Oligonucleotide synthesis reagents catalogue

3rd edition

BIOSEARCH™ TECHNOLOGIES

GENOMIC ANALYSIS BY LGC

重松貿易株式会社 化学品部
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Integrated tools.
Accelerated science.

Contents

Your partner of choice for all your oligonucleotide synthesis reagent and instrument needs.

LGC, Biosearch Technologies™

A global leader in the life sciences sector, serving customers in healthcare, applied markets (including food, agricultural biotechnology and the environment), research and government institutions.

Offering a comprehensive range of products for genomics reagents and instrumentation, research and measurement services, our scientific tools and services form an essential part of your experience of quality and compliance, allowing you to provide safer products, develop new solutions and advance research - we are with you, every step of the way.

In recent years, Biosearch Technologies has paved the way forward with its complete end-to-end nucleic acid chemistry solution and now offers you a truly unrivaled product portfolio of specialised phosphoramidites, solid supports and synthesizers to build your oligonucleotides.

Nucleic acid chemistry (NAC)

You will find our NAC portfolio offers you a complete and convenient point for all your oligonucleotide needs; everything from an extensive range of solid supports and common phosphoramidite modifications such as 2’-O-methyl and 2’-fluoro, through more specialist modifications and peptide nucleic acid monomers, to nucleosides, carbohydrates and fluorescent markers.

This extensive range of over 1000 specialist products, combined with our range of synthesizers, offers you the complete comprehensive portfolio for all your oligonucleotide synthesis needs.

Quality at Biosearch Technologies

We understand product quality is important to you and so have been manufacturing reagents for oligonucleotide synthesis to an unrivalled level of quality. We control and check the whole manufacturing process, repeatedly, at every stage.

We put more than 35 years' experience in chemistry - in particular with respect to oligo synthesis reagents - at your disposal, and whether you want 1 g or 1 kg, Biosearch Technologies is your partner of choice. We combine a comprehensive catalogue of off-the-shelf materials together with a full bespoke custom service so you can order precisely what you need, every time.

As the biggest specialist company in this field, we ensure great value for money.

Our team is made up of passionate, committed professionals who deliver the most efficient, helpful service you could imagine.

Biosearch Technologies is the brand you can trust

At Biosearch Technologies, we are very customer focused and strive to not only meet but exceed the requirements and expectations of our customers. As a measure of this, our Quality Management System (QMS) ISO 9001 certified.

While continuing to provide robust and reliable services and products, we are committed to continual improvement and as such once an improvement project has been determined, we put together Continual Improvement Groups consisting of key people to work on the project. Many of these project ideas come from the customer. In conjunction with our QMS, our Customer Relationship Management (CRM) System allows all customer contact (sales, enquiries, technical support) to be monitored as a means of identifying trends which enables us to act faster in terms of any changes that may be required as a result of a trend, e.g. scale up of a particular product. This then becomes a continual improvement project. Biosearch Technologies is committed to providing high quality products, therefore, our QC processes allow for testing of all key raw materials and all intermediates in addition to our final products. After QC, all batches undergo QA review prior to release.

Our QC operation, and associated QA quarantine areas, are housed in defined areas and separate from process development and routine manufacturing.

Not only do we have a wide variety of in-house analytical techniques such as HPLC, LCMS-TOF and UV/vis, we also provide NMR and fluorescence measurement, enabling us to fully characterise and analyse all our products, intermediates and raw materials.

All our oligonucleotide synthesizer reagents products are functionally tested on one of our in-house oligo synthesizers and the resulting oligo subsequently analysed.

Enhanced specification

Although at Biosearch Technologies our QMS is ISO 9001:2015 certified, we understand that many of our customers are certified to other quality standards (e.g. ISO 13485) and therefore have different requirements. For these customers, we have implemented processes which will allow us to meet these specific needs for our NAC customers.

For instance, all our products undergo QC testing and QA review to ensure they meet a defined specification and a Certificate of Analysis (CoA) is issued and shipped to the customer along with the product. For many customers this is sufficient. However, for others there is a benefit for Biosearch Technologies' NAC services to align our QC analysis for a product (or set of products) to that of the client's own testing and copies of the analytical data along with the CoA are sent along with the product. This is in place for many of our customers.

For other clients, we go one step further and carry out impurity profiling based on LCMS, NMR and HPLC data. Where applicable, we can provide the analytical data and analysis report prior to shipping the material for pre-approval by the customer.

Custom synthesis and bulk packaging

We take care to provide our off-the-shelf products in popular pack sizes and packing formats. As your needs grow, so do the options available to you. We can supply most manufactured products in bulk quantities.

Biosearch Technologies' NAC portfolio can either ship in single containers or aliquot material; the unit size and type of packaging is entirely at your discretion – tell us what you need. For any bulk order, the packaging will be agreed at the outset of manufacturing.

At Biosearch Technologies, our catalogue range of oligo reagents is exceptional but it may not be enough for our more specialist clients. You know who you are – CMOs, GMP manufacturers, diagnostic oligo manufacturers and others. You may work to ISO 13485, but you're certainly a customer with highly specialised, possibly unique, requirements. You need a partner you can trust to deliver the highest quality products, to your exacting standard, via a managed and resilient supply chain, with multiple manufacturing sites in UK and US.

Have a look through our catalogue and find what you are looking for. But if you don't see it, it does not mean that we do not supply this reagent or support. Our product offering is consistently evolving to meet all your needs. So give our Customer Service a call on +44 (0) 1698 849911 and we may be able to help you find what you are looking for.

Corporate social responsibility

At Biosearch Technologies, we are very much aware of our responsibility to the environment and consider the impact of our operations on society very seriously. We strive to minimise our waste production and maximise recycling at all times - whether in our laboratories, canteen, or paper for our marketing materials.

Of course, chemical production will always produce waste but we always ensure that the hazardous material element of what waste we do produce is properly contained and dealt with responsibly.

Every year, we support a charity chosen by our employees. We raise funds for charities that demonstrate links to our business activities and have an international reach to reflect our global footprint. Donations to our chosen corporate charity are being boosted by Biosearch Technologies matching any funds raised through team-building events.

For more information on this, see our website for further details.

Chemistry of oligonucleotide synthesis

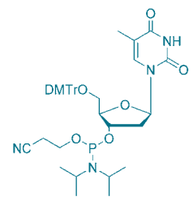
Nearly 40 years since its introduction, the use of phosphoramidite chemistry remains the method of choice for the automated synthesis of oligonucleotides.

Oligonucleotide synthesis cycle

Most techniques used in molecular biology today rely on synthetic oligonucleotides, including PCR, DNA sequencing, and Single-Nucleotide Polymorphism (SNP) assays. The vast majority of oligonucleotides are synthesised on automated synthesizers using phosphoramidite methodology.

Oligonucleotide phosphoramidite chemistry was first introduced 38 years ago.[1] The method is based on the use of DNA phosphoramidite nucleosides which are modified with a 4,4'-dimethoxytrityl (DMTr or DMT) protecting group on the 5'-OH, a ß-cyanoethyl-protected 3'-phosphite, and appropriate conventional protecting groups on the reactive primary amines in the heterocyclic nucleobase. The four classic protected DNA nucleoside phosphoramidites are benzoyl-dA, benzoyl-dC, iso-butyryl-dG and dT (which requires no base protection), products **LK2003**, **LK2004**, **LK2002** and **LK2001** respectively. As discussed in the following pages, both acetyl-dC (**LK2034**) and dimethylformamidine-dG (**LK2030**) are now also routinely used.

1 **An investigation of several deoxynucleoside phosphoramidites useful for synthesising deoxyoligonucleotides**, *L.J. McBride and M.H. Caruthers, Tetrahedron Lett., 24, 245-248, 1983.*



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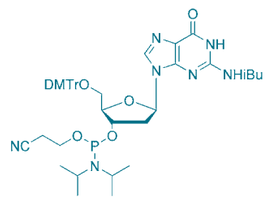

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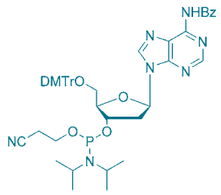

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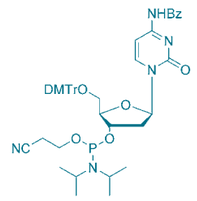


LK2004

The phosphoramidite approach is today carried out almost exclusively on automated synthesizers using controlled-pore glass (CPG) or polystyrene solid supports.[2] These supports are held in small synthesis 'columns' or wells within plate (e.g. 96 well plates) that act as the reaction vessel. These columns or plates are loaded onto the synthesizer and phosphoramidite and ancillary reagents are passed through the column in cycles of four distinct reactions thus extending the oligonucleotide chain.

The synthesis cycle consists of four steps: deblocking (detritylation); activation/coupling; capping; and oxidation (or sulphurisation). These steps are shown in Figure 1. Synthesis occurs in the 3' to 5' direction; which is opposite to enzymatic synthesis by DNA polymerases.

Conventionally, the 3' base in the sequence is incorporated by use of a base-functionalised CPG or polystyrene support (1), although 'universal' supports are available (see below). Synthesis initiates with removal ('deblocking' or 'detritylation') of the 5'-dimethoxytrityl group by treatment with acid (classically 3% trichloroacetic acid in DCM (**LK4140**)[3] to afford the reactive 5'-OH group (2). The phosphoramidite corresponding to the second base in the sequence (3) is activated[4] (using a tetrazole-like product such as ETT (**LK0237** or **LK3140**/**LK3142**/**LK3145**/**LK3146**) or BTT (**LK0234** or **LK3160**/**LK3162**), then coupled to the first nucleoside via the 5'-OH to form a phosphite linkage (4).

Solid phase phosphoramidite coupling usually proceeds to around 99% efficiency. If the 1% of molecules remaining with reactive 5'-OH groups are left untreated, unwanted side-products will result. To prevent this, a 'capping' step is introduced typically prior to the oxidation to acetylate the unreacted 5'-OH (5). Where sulphurisation is performed, capping must come after this step. This is typically achieved using a solution containing acetic anhydride (Cap Mix A – **LK4010**/**LK4110**/**LK4012**) and the catalyst N-methylimidazole (Cap Mix B – **LK4120**/**LK4122**). Unless blocked these truncated oligonucleotides can continue to react in subsequent cycles giving near full- length oligonucleotide with internal deletions (species referred to as (N-1)mers).

The unstable trivalent phosphite triester linkage is oxidised, via an iodine-phosphorous adduct, to the stable pentavalent phosphotriester (6) using iodine in a THF/(pyridine or lutidine)/water solution (**LK4230**/**LK4330**/**LK4132**). After oxidation, the cycle is repeated, starting with detritylation of the second molecule and so on.

The synthesis cycle is repeated until the desired length of oligonucleotide is achieved. At this point the synthesis is complete.

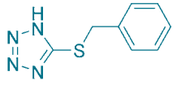
At this point, there are two choices: either the final 5'-DMTr group can be left in place as a purification 'handle' (DMT ON option on the synthesizer; see below) or it can be removed by a final acid treatment (DMT OFF). The oligonucleotide can then be cleaved from the solid support using a suitable deprotection solution, e.g. ammonium hydroxide solution at room temperature.

If desired, cleavage and deprotection can be carried out simultaneously. In addition to cleaving the support, the cyanoethyl groups are removed from the sugar-phosphate backbone. Nucleobase protection is also removed at this time. The specific cleavage and deprotection conditions will vary from oligo to oligo depending on the nucleobase protection employed and any modifiers present. This is typically done by heating the resin in the deprotection solution or in gaseous ammonia.

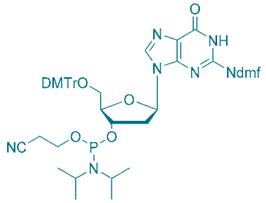
2 For a recent review see: **A brief review of DNA and RNA chemical synthesis**, M.H. Caruthers, Biochem. Soc. Trans., 39, 575-580, 2011.

3 *In larger production environments 2-5% dichloroacetic acid in toluene is commonly used; we can provide this by request - for 5% quote item* **LK4500**. *3% DCA in DCM (***LK4040***) is also available.*

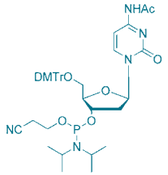
4 **A description of the mechanism of activation via the phosphorotetrazolide intermediate can be found in Studies on the role of tetrazole in the activation of phosphoramidites**, *S. Berner, K. Mühlegger and H. Seliger, Nucleic Acids Research, 17, 853-864, 1989.*



LK0234



LK2030



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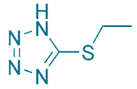


LK0237

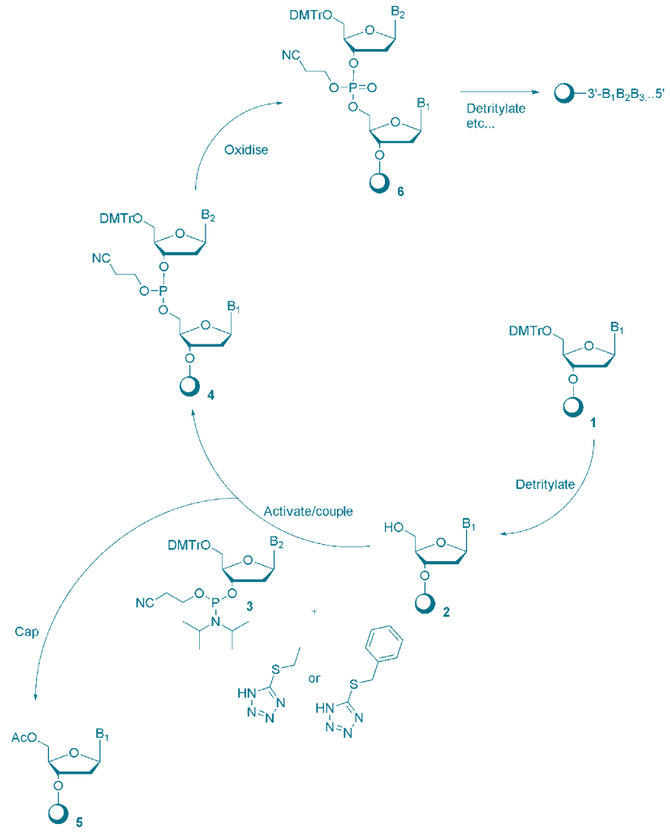


Figure 1. The oligonucleotide synthesis cycle using phosphoramidite chemistry.

After deprotection, oligos are typically de-salted (also removes small-molecule side products) or purified by methods such as Polyacrylamide Gel Electrophoresis (PAGE), reverse-phase (RP) HPLC, cartridge methods or ion-exchange (IE) HPLC.

Leaving the DMTr protecting group in place aids the purification of full-length sequences, since the hydrophobic DMTr group is retained by reverse phase chromatographic media. In contrast, non-DMTr containing failure sequences are much less retained on chromatography. This is the basis on which oligonucleotides are purified by reverse-phase (RP) HPLC. Note that if the capping or detritylation steps are inefficient, N-1, N-2, ... species can occur but the 5'-end of the failures is still protected with DMTr.

Classically, after preparative chromatography the 5'-DMTr group would be removed by acetic acid treatment to give the biologically active oligonucleotide. Whilst this is still a valid method, it is not now commonly carried out in this manner. There are many available preparative columns (e.g. Hamilton PRP-3, ABI POROS, Waters X-Bridge) that will allow DMT ON purification and the detritylation to be carried out on the column, thus allowing the product to be collected already detritylated. This is fast and less likely to lead to depurination than solution- phase acetic acid treatment.

Ordering unmodified DNA phosphoramidites

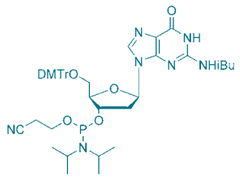
As well as the above, Biosearch Technologies is able to offer the following DNA phosphoramidites from its legacy product portfolios. For further information on these products, see our website or contact Customer Service.

Many cartridge purification systems based on this principle are available. In this case, the crude oligonucleotide is adsorbed on to the cartridge and failure sequences are washed out by elution with water, leaving the pure full-length product on the solid medium. The cartridge is then treated with acid to remove the DMTr group and the pure oligonucleotide is eluted with acetonitrile/water.

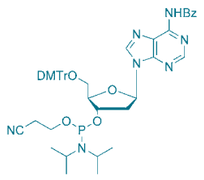
The synthesis of RNA (where the chemistry is complicated by the presence of an additional 2'-OH functional group) is discussed on page 20.

Ordering ancillary reagents

Biosearch Technologies now offer a range of ancillary reagents, from activators, oxidisers to deblok, as well as Cap Mix A and B., to complement our MerMade™ DNA/RNA synthesizers. To view our full product for this range, have a look at our website.



LK2002



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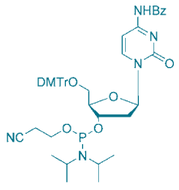
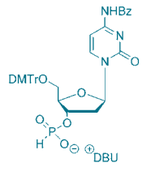
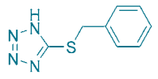


LK2004



LK2035



LK0234

Choosing a solid support

Controlled Pore Glass (CPG) has been widely used as a solid support for oligo synthesis for several decades. Biosearch Technologies' has perfected CPG manufacture for maximum oligo purity and yield. Our advanced CPG production techniques were developed to improve control of particle size and shape, pore size, pore volume, and specific surface area. These physical parameters influence solution exchange behaviour, ligand loading and distribution, and reaction kinetics to increase the efficiency, purity, and reproducibility of syntheses.

Proprietary chemical attachment procedures were developed to further optimise ligand distributions, providing increased accessibility to synthesis reagents and washing solutions and facilitating even better oligo yields and purities. Furthermore, process refinements and proprietary assays were developed to minimise the troublesome "N-1" impurity levels in an oligo synthesis.

Biosearch Technologies' CPG is considered to be the gold-standard solid support used in all sectors of the market. Our collaborative process has resulted in solid supports which are optimised for the synthesis of the latest therapeutic oligo classes including LNA, delivery enhancing lipid ligands, SiRNA and Spiegelmers. Biosearch Technologies' CPG solid supports are available in a variety of pore sizes and functionalised nucleoside loadings. Six pore sizes are offered from 500 Å to 3000 Å to enable the synthesis of oligonucleotides for all applications. Which pore size required is dependent on the length, complexity and application of the oligo. Some guidelines are below for the most widely used pore sizes.

- 500 Å CPG;
  - ≤30mers medium to large scale oligo synthesis
  - High yields of product are required such as therapeutic oligos
  - High loaded support is required. 500 Å can load up to ~100 µmol/g
- 1000 Å CPG;
  - >20mers or highly modified oligonucleotides.
  - The loading is typically 25-40 µmol/g and most of our modifiers are functionalised onto this pore size as standard.
- 3000 Å CPG;
  - >80mers
  - The loading is typically 10-20 µmol/g.
  - With a few exceptions, it is possible to have any of our 1000 Å products with a 3000 Å pore size.

In general large scale oligo synthesis for therapeutic applications requires high loaded 500-600 Å and small to medium scale synthesis for diagnostic or research use require higher pores sizes.

CPG products

Many research use CPG products are available which originated from the legacy LINK, Biosearch and Berry portfolios. These include a number of unmodified and modified products, noted throughout the catalogue. Many more bulk and column-packed products are available from our Prime business; a summary of which can be seen in Table 1.

Table 1. Summary of typical CPGs available.

Type	Modification
CPG 500 Å, 600 Å, 1000 Å	Native, AMP, CNA/LCAA
CPG 2000 Å, 3000 Å	Native, AMP, CNA/LCAA
DNA CPG 500 Å, 600 Å, 1000 Å	dA, dC (Ac), dC (Bz), dG (iBu), dG (dmf), dT
DNA CPG 2000 Å, 3000 Å	dA, dC (Ac), dC (Bz), dG (iBu), dG (dmf), dT
RNA CPG 1000 Å	rA (Bz), rC (Ac), rC (Bz), rG (iBu), rG (dmf), rU, rA (Bz), 2'-OAc, rA, (Ac), 2'-OAc, rC (Ac), 2'-OAc, rG (iBu), 2'-OAc, rU, 2'-OAc
RNA CPG 2000 Å, 3000 Å	rA (Bz), rC (Ac), rC (Bz), rG (iBu), rG (dmf), rU, rA (Bz), 2'-OAc, rA (Ac), 2'-OAc, rC (Ac), 2'-OAc, rG (iBu), 2'-OAc, rU, 2'-OAc
RNA CPG (Pac); all pore sizes	rA (Pac), rG (iPr-Pac), rG (iPr-Pac), 2'-OAc
2'-OME CPG 1000 Å	2' OMe rA (Bz), 2' OMe rC (Ac), 2' OMe rC (Bz), 2' OMe rG (iBu) 2' OMe rG (dmf), 2' OMe rU
2'-OME CPG 2000 Å, 3000 Å	2' OMe rA (Bz), 2' OMe rC (Ac), 2' OMe rC (Bz), 2' OMe rG (iBu), 2' OMe rG (dmf), 2' OMe rU
Reverse CPG	3' DMT dT, 3' DMT dA (Bz), 3' DMT dC (Bz)
LNA CPG	LNA A (Bz), LNA C (Bz), LNA G (dmf), LNA T
2'-F CPG	2' Fluoro A (Bz), 2' Fluoro C (Ac), 2' Fluoro G (iBu), 2' Fluoro U

Employing universal supports

As outlined above, in oligo synthesis the 3'-end is generally determined by the base or modifier functionalisation of the solid support. Whilst the synthesis of any oligo is efficient, given the choice of functionalised supports available, there are advantages in using a 'universal' support where there is no nucleobase or modification already present (e.g. when using plate synthesizer). In this case, the first base at the 3'-end is determined by the first phosphoramidite addition in the synthesis cycle.

When preparing wells in plate synthesizers this eliminates the possibility of the incorrect resin being placed in a well. It also allows automated preparation of the plates to stock ready for synthesis. There is an added benefit in large scale syntheses, where supply chain is simplified as only the one support is required.

A universal support can also be applied in situations where a 3'-modification support is not available, using a phosphoramidite modifier as the first addition in the cycle (this will only work with phosphoramidites capable of extending the oligo chain, i.e. not 5'-modifiers and are compatible with the universal support cleavage and deprotection conditions).

Although we still offer the original McLean 'universal' support, and a Q-linker version (**LK2300**/**LK2410**/**LK2411**), our most effective product for universal synthesis is the Unprotected Universal Solid Support with a labile N-iPr linker*[5] (**B1-3500**, **B2-3500** and **B5-3500**). It is similar in structure to the N-Ph product developed at Isis Pharmaceuticals as UnyLinker™. The advantage of the N-iPr version is that the removal of the linker from the 3'-terminus of synthetic oligonucleotides occurs about 5 times more rapidly.

The advantage of the unprotected ('DMT-off') universal supports over the respective DMT-protected supports is that no linker is cleaved from the solid support during the initial detritylation step. With DMT-protected supports, 10 to 25% of the linker is cleaved off during this step thus reducing the loading and the yield of oligonucleotides.

Further, this eliminates the need for an initial detritylation step. The DMT group in Universal supports is more stable than in nucleosidic supports by a factor of 5 to 7. When using DMT-protected supports, therefore, the user is required to create a custom detritylation protocol for the initial detritylation. This requirement is removed by using our DMT-off product.

The methyl version of the support (preferable over the phenyl version since methylamine rather than aniline is formed on deprotection) is available. Contact our Customer Service group for more information.

See our website for our full selection on offer.

5 This product is distributed on behalf of AM Chemicals LLC.

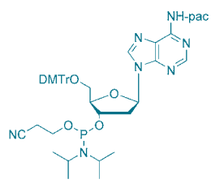
Protection group strategies

UltraMILD deprotection

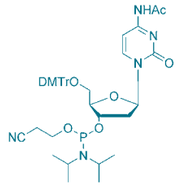
In oligonucleotide synthesis, the classic heterocyclic base protection groups (Bz-dA, Bz- dC and iBu-dG) are routinely removed using ammonium hydroxide solution with heating. Unfortunately, many modifiers and labels used in oligonucleotide synthesis will not withstand prolonged exposure to such strongly alkaline conditions. The UltraMILD monomers - phenoxyacetyl (Pac)-dA (**LK2059**), acetyl (Ac)-dC (**LK2034**), and iso-propyl-phenoxyacetyl (iPr-Pac)-dG (**LK2060**) - were developed to alleviate this. This alternative protection allows milder deprotection conditions to be used where sensitive labels and tags have been incorporated into the oligonucleotide. This strategy allows the use of very mild deprotection conditions such as 0.05 M potassium carbonate in methanol at room temperature. The UltraMILD monomers can also be deprotected using ammonium hydroxide solution, and, in fact, acetyl- is currently the protecting-group of choice for dC since this is compatible with all deprotection conditions.

The corresponding Pac-dA, Ac-dC, and iPr-Pac-dG functionalised supports are also available for UltraMILD compatibility of the first 3' base, as are UltraMILD capping reagents.

It should be noted that using the alternative capping solution containing Pac-anhydride (**LK4210**) avoids the possibility of formation of acetyl-dG by exchange in regular capping solutions. N2-acetyl-dG would not be deprotected under UltraMILD conditions.



LK2059



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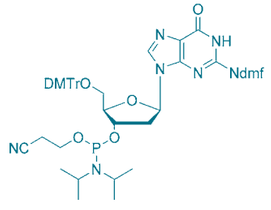
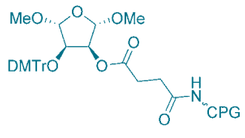
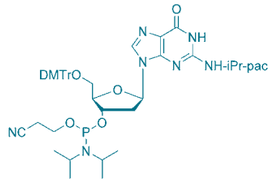


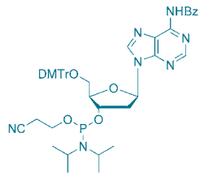
LK2030



LK2304



LK2060



LK2003

FAST deprotection

The use of dimethylformamidine-(dmf)-dG (**LK2030**) has over the years gained favour over iBu-dG (**LK2003**), originally due to its ability to deprotect with ammonium hydroxide in 1h at 65 °C (or 2h at 55 °C). This, together with the availability of the Ac-dC phosphoramidite developed for UltraMILD protocols, led to the creation of a new monomer set allowing rapid deprotection by the FAST method. By using Ac-dC, Bz-dA and dmf-dG monomers, FAST cleavage and deprotection can be effected by a 1:1 mixture of aqueous ammonium hydroxide and aqueous methylamine (known as AMA) in 10 minutes. Cleavage takes place over 5 minutes at room temperature, then deprotection follows by heating to 65 °C for a further 5 minutes.[6] Deprotection also takes place at room temperature if left for 120 minutes. AMA deprotection is not recommended for use in the presence of sensitive labels such as cyanine or rhodamine (TAMRA) dyes, or where there are Bz-protected C nucleosides as this will result in transamidation with methylamine.

dmf-dG works particularly well with tbutylamine/methanol/water (1:1:2) as used for rhodamine containing modifiers (e.g. TAMRA).

The Ac-dC and dmf-dG protected supports are also available.

Other methods

While all common deprotection methods require purification to remove the residual protective groups (e.g. benzamide) and insoluble silicates, an ammonia-free reagent mixture that allows avoidance of additional purification has been reported.[7] The method, which uses a mixture of lithium hydroxide and triethylamine, can be applied to deprotect oligos synthesised using the classical or Pac-protected phosphoramidites.

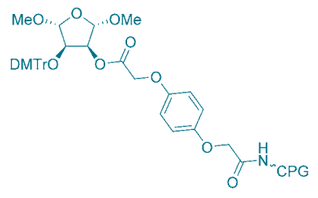
A modified "ultra-mild" protocol has also been reported that is compatible with the known UltraMILD monomers.[8] This method, utilising 10% diisopropylamine in methanol with 0.25 M of ß-mercaptoethanol, was developed for studies incorporating the base-sensitive dG-AAF into DNA; it could well be of more general use for the incorporation of base-labile functionalities into DNA.

See our website for a complete view of our unmodified UltraMILD phosphoramidites and solid supports.

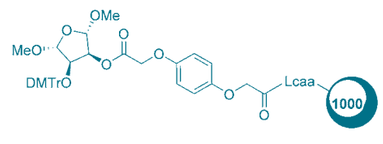
6 Using AMA, the order of hydrolysis of the base protecting groups is the acetyl group on dC, followed by the benzoyl group on dA, and then the dmf groups from dG. The hydrolysis of Ac-dC is almost instantaneous, thereby precluding the unwanted transamidation reaction to the side-product N-Me-dC possible with alkylamine deprotection.

7 **Advanced method for oligonucleotide deprotection**, *S.A. Surzhikov, E.N. Timofeev, B.K. Chernov, J.B. Golova and A.D. Mirzabekov, Nucleic Acids Research, 28, e29, 2000.*

8 **Site-specific incorporation of N-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-AAF) into oligonucleotides using modified 'ultra-mild' DNA synthesis**, *L.C.J. Gillet, J. Alzeer and O.D. Schärer, Nucleic Acids Research, 33, 1961-1969, 2005.*



LK2300



LK2410/LK2411

Oligoribonucleotide synthesis

There are many uses of RNA oligonucleotides such as understanding the role of ribozymes (catalytic RNA) and cellular RNA as a target for antisense therapeutics. However, the need for chemically synthesised RNA oligonucleotides has become increasingly important since the advent of synthetic siRNA for use in antisense technologies such as gene silencing, and the therapeutic application of RNAi.[9]

Chemistry and protection strategies

DNA and RNA have very similar structures and differ only in the presence of the 2'-OH moiety in the latter, and that in RNA thymidine (T) is replaced with uridine (U). As such, the chemistries in terms of synthesis and deprotection of the oligonucleotides differ.[10]

There are many 2'-OH protection chemistries available: tert-butyldimethylsilylester (TBDMS)[11], Xeragon's 2'-O-triisopropylsilyoxymethyl, (TOM™)[12], Dharmacon's 2'-O-bis(2-acetoxyethoxy) methyl (ACE™)[13] and very recently Agilent's 2'-O-thiomorpholine-4-carbothioate (TC).[14] However, to date, TBDMS chemistry remains the most widely accepted and utilised in RNA synthesis, particularly where the RNA is used in therapeutic applications (**LK2033**, **LK2036**, **LK2037**, **LK2038**, **LK2039**, **LK2040**).

9 **RNA interference in the clinic: challenges and future directions**, *C.V. Pecot, G.A. Calin, R.L. Coleman, G. Lopez- Berestein and A.K. Sood, Nat. Rev. Cancer, 11, 59-67, 2011.*

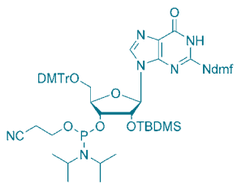
10 **For a recent overview of this area see: Current Strategies for the Synthesis of RNA**, *S. Muller, J. Wolf and S.A. Ivanov, Current Organic Synthesis, 1, 293-307, 2004.*

11 **Chemical synthesis of biologically active oligoribonucleotides using beta-cyanoethyl protected ribonucleoside phosphoramidites**, *S.A. Scaringe, C. Francklyn and N. Usman, Nucleic Acids Research, 18, 5433- 5441, 1990.*

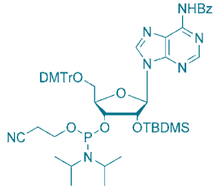
12 **Synthesis and pairing properties of oligoribonucleotide analogues containing a metal-binding site attached to beta-D-allofuranosyl cytosine**, *X. Wu and S. Pitsch, Nucleic Acids Research, 26, 4315-4323, 1998.*

13 **Novel RNA synthesis method using 5'-O-silyl-2'-O-orthoester protecting groups**, *S.A. Scaringe, F.E. Wincott and M.H. Caruthers, J. Amer. Chem. Soc., 120, 11820-11821, 1998. The ACE™ method also requires that the 5'-OH is protected with a silyl ether rather than the common DMTr group. Moreover, at present the phosphoramidites are not commercially available.*

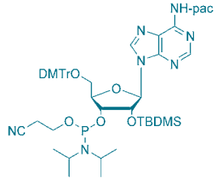
14 **The Development of a Cost-Effective Large Scale Synthesis Process for RNA Therapeutics**, *D. Dellinger, Presentation at TIDEs®, May 18, 2009.*



LK2033



LK2036



LK2037

The coupling reaction for RNA synthesis is much longer in comparison with DNA synthesis. Using TBDMS chemistry coupling times of up to 12 minutes are required depending on the choice of activator, although with 0.25 M BTT, 3-5 minute couplings are possible.

Until recently, the synthesis of longer RNA oligos (~80mers) in reasonable yields was met with some difficulty due to the premature partial deprotection of the TBDMS group during the step to remove the nucleobase protection with ammonium hydroxide solution with heating. Reaction between the now free 2'-OH group and the 3'-phosphate resulted in either cleavage of the oligo at this point or rearrangement to 2'-phosphate and 3'-OH. The desilylation can be suppressed by the use of anhydrous ethanolic ammonia or ethanolic ammonium hydroxide.[15] However, the most significant improvement is with the use of AMA[16] (aqueous ammonium hydroxide/ methylamine 1:1) or ethanolic AMA[17] in conjunction with the use of fast deprotection amidites which allows the nucleobases to be deprotected in 10 minutes at 65˚C. The use of DMSO/ethanolic methylamine (1:1) has also been reported.[18] It must be noted that the use of Bz-dC or Bz-C with AMA leads to transamidation with methylamine and these monomers are therefore not suited to this deprotection method.

The most critical step in obtaining high quality RNA is removing the 2'-TBDMS group. Before starting on this part of the deprotection process, it is important that the sample is completely dry and all the nucleobase deprotection solutions have been removed. Removal of the TBDMS groups is most commonly achieved using N-methylpyrrolidone/triethylamine/ triethylamine trihydrofluoride (NMP/Et^{3}N/Et^{3}N.3HF) or with either DMSO or DMF to replace the NMP.

While to date this has been the most widely used RNA chemistry, the two step deprotection is time consuming and detrimental to more sensitive modifications (e.g. cyanine dyes). A one-pot deprotection method for TBDMS chemistry which uses anhydrous methylamine and neat Et^{3}N/Et^{3}N.3HF has been described.[19] The deprotection time is still 2-3 hours and is still incompatible with sensitive modifications.

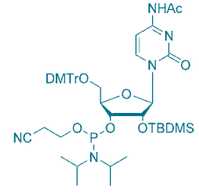
15 **Prevention of chain cleavage in the chemical synthesis of 2'-silylated oligoribonucleotides**, *T. Wu, K.K. Ogilvie and R.T. Pon, Nucleic Acids Research, 17, 3501-3517, 1989.*

16 **Methylamine deprotection provides increased yield of oligoribonucleotides**, *M.P. Reddy, F. Farooqui and N.B. Hanna, Tetrahedron Lett., 36, 8929-8932, 1995. For an account of the use of this protection approach in DNA synthesis see: Fast cleavage and deprotection of oligonucleotides, M.P. Reddy, N.B. Hanna and F. Farooqui, Tetrahedron Lett., 35, 4311-4314, 1994.*

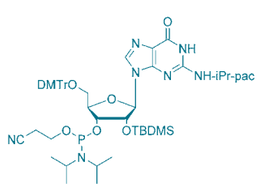
17 **Synthesis, deprotection, analysis and purification of RNA and ribozymes**, *F. Wincott, A. DiRenzo, S. Scaringe and N. Usman, Nucleic Acids Research, 23, 2677-2684, 1995.*

18 **Comparison of coupling and deprotection protocols for RNA synthesis**, *R.T. Pon and S. Yu, Poster Presentation at ABRF 2004, Integrating Technologies in Proteomics & Genomics, Portland, Oregon, Feb. 28 – Mar. 2, 2004.*

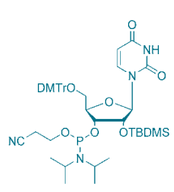
19 **"One-pot" oligoribonucleotide deprotection with anhydrous methylamine and neat triethylamine trihydrofluoride**, *L. Bellon, in Current Protocols in Nucleic Acid Chemistry, Unit 3.6, Eds. S.L. Beaucage, D.E. Bergstrom, G.D. Glick and R.A. Jones, John Wiley & Sons, 2000.*



LK2038



LK2039



LK2040

Recently, TC-RNA chemistry has been introduced. This has a one-step deprotection method that removes both the nucleobase and 2'-OH protection simultaneously; this technology however is not widely available for commercial use.

See our website for a complete view of our unmodified RNA CPG supports.

Alternative activators

Activators containing tetrazole (traditionally as a 0.45 M solution in anhydrous acetonitrile) have classically been the reagents of choice in routine automated DNA and RNA synthesis. There are, however, two main disadvantages to using this product. Firstly, at lower laboratory temperatures (typically 18 °C), solid tetrazole can crystallise from the near-saturated solution causing blockage of delivery lines.

Secondly, the product has become more difficult to obtain because of shipping restrictions due to its classification as an explosive (current UK law permits shipping of tetrazole only as a solution; even this cannot be shipped by air). As a consequence, we now no longer offer this product.

An alternative activator, 5-Ethylthio-1H-tetrazole (ETT, **LK3140**/**LK3142**/**LK3145**/**LK3146**), can offer more effective activation than tetrazole without crystallisation problems. In particular it has been shown to decrease the coupling times in both RNA synthesis[20] and DNA synthesis.

20 (a) **Synthesis, deprotection, analysis and purification of RNA and ribozymes**, *F. Wincott, A. DiRenzo, C. Shaffer, S. Grimm, D. Tracz, C. Workman, D. Sweedler, C. Gonzalez, S. Scaringe and N. Usman, Nucleic Acids Research, 23, 2677-2684, 1995;* (b) **An efficient method for the isolation and purification of oligoribonucleotides**, *B. Sproat, F. Colonna, B. Mullah, D. Tsou, A. Andrus, A. Hampel and R. Vinayak, Nucleosides & Nucleotides, 14, 255-273, 1995;* (c) **Large-scale synthesis of oligoribonucleotides on high-loaded polystyrene (HLP) support**, *D. Tsou, A. Hampel, A. Andrus and R. Vinayak, Nucleosides & Nucleotides, 14, 1481-1492, 1995.*

However, it is believed that shipping restrictions will also be imposed on this product in the future. It has also been demonstrated that the acidity of tetrazole based activators is sufficient to deprotect the trityl group in monomer solution - to a small extent - leading to some dimer (n+1) formation.[21]

The less acidic 4,5-dicyanoimidazole (DCI) has been used as an alternative activator to avoid this side-reaction.[22] The increased nucleophilicity of this molecule also increases the rate of activation. We offer 0.25 M dicyanoimidazole in anhydrous acetonitrile (**LK3150**). This is available in a 450 mL (16 oz), 28-405 screw neck, amber bottle, suitable for Expedite 8909, upgraded ABI 392/394, ABI 3400 and 3900, and all MerMade synthesizers.

Despite these advances, a need remains for additional activators, particularly in RNA synthesis in which the longer coupling times, due to the steric effects of protecting the 2'-OH, ideally could still be reduced. We provide 5-Benzylthio-1H-tetrazole (BTT) activator (**LK3160**/**LK3162**) to specifically meet this need.

BTT has been classed as a non-explosive material, and therefore, availability of the product is not restricted. Furthermore, this product is available at a cost equivalent to other tetrazole activators. In DNA synthesis, coupling efficiency is routinely at least as good as with tetrazole, and often better. In RNA synthesis, the coupling of TBDMS or TOM monomers with 1H-tetrazole activation conditions can require 12-15 min. Using BTT 3 min coupling times are recommended, although 90s has been used effectively using 6.5eq of 0.25 M BTT and 6eq of 0.1 M phosphoramidite.[23] BTT is, in fact, very slightly more acidic than ETT, however, it has been shown that N+1 peaks are no more significant using BTT with shorter coupling times than ETT with a 6 min coupling time or 1H-tetrazole for 12 min.

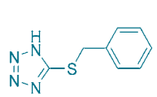
Note that we now also provide crystalline BTT (**LK0234**) and ETT (**LK0237**) - these can ship as non-hazardous products thereby reducing costs.

Although we still find BTT to be the most widely effective activator, some customers still prefer to use DCI or ETT in certain situations, therefore, we are happy to support this.

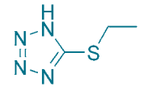
21 **On the formation of longmers in phosphorothioate oligodeoxyribonucleotide synthesis**, *A.H. Krotz, P.G. Klopchin, K.L. Walker, G.S. Srivatsa, D.L. Cole and V.T. Ravikumar, Tetrahedron Lett., 38, 3875-3878, 1997.*

22 **Efficient activation of nucleoside phosphoramidites with 4,5-dicyanoimidazole during oligonucleotide synthesis**, *C. Vargeese, J. Carter, J. Yegge, S. Krivjansky, A. Settle, E. Kropp, K. Petersen and W. Pieken, Nucleic Acids Research, 26, 1046-1050, 1998.*

23 **The synthesis of 2'-O-[(triisopropylsilyl)oxy]methyl (TOM) phosphoramidites of methylated ribonucleosides for the use in automated RNA solid-phase synthesis**, *C. Höbartner, C. Kreutz, E. Flecker, E. Ottenschläger, W. Pils, K. Grubmayr and R. Micura, Monatchefte für Chemie, 134, 851-873, 2003.*



LK0234



LK0237

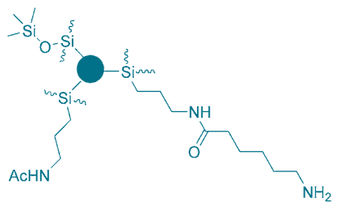
Bespoke 3’-in-corporation: employing unfunctionalised Amino-CPG

The use of our Amino-CPG products (**LK1308**/**LK1383**/**LK1385**/**LK1397**) allows the user to directly incorporate a bespoke nucleobase or modifying unit of their own choosing at the 3’-end.

Synthetically, this is done by reacting the Amino-CPG with the succinate (or other suitable derivative) of the nucleoside (or modifier) in the presence of e.g. a carbodiimide and base in a suitable solvent. The modifying unit of course requires a protected alcohol available for further chain extension in the synthesis cycle (i.e. in nucleosides the 5’-OH is protected by a DMTr group). This 3’-modified CPG can then be used to synthesise an oligonucleotide.

Our Amino-CPG products are fully activated and are used without further treatment.

See our website for a complete view of our unfunctionalised CPG supports



LK1308/**LK1383**/**LK1385**/**LK1397**

Applications of modified oligonucleotides

Modified oligonucleotides are now being used in many applications, most notably, diagnostics and therapeutics.

Introduction

As we will see in the following sections, modifying an oligonucleotide enables the development of diagnostic tests, therapeutics, detection methods and genetic analysis tools. Whilst there is a multitude of applications such as gene synthesis, genetic profiling, biosensors, cosmetics and agriculture, the two main sectors utilising modified oligonucleotides are diagnostics and therapeutics.

Diagnostics

The diagnostics sector is the fastest growing sector in the oligonucleotide market. This is primarily driven by the advances in terms of detection methods, particularly with respect to qPCR and sequencing techniques. These improvements in turn demand the need for new and improved detection labels such as fluorophores or electrochemical labels to fine tune these techniques to give higher sensitivity and selectivity.

There is a vast array of oligonucleotide related diagnostic tests but, with the exception of one or two techniques, they fall into one of three categories: fluorescence (e.g. probe based qPCR); electrochemical (e.g. CombiMatrix microarrays); or colourimetric detection (e.g. ELISA assay), see pages 78, 91 and 89, respectively, for further information on each topic..

Therapeutics

Oligonucleotide therapeutics[29] is a broad term which actually covers a number of modes of action of similarly structured molecules. Therefore, whilst the design and construction of the oligos is often similar, there are several ways in which therapeutic effects can be induced. These include antisense, anti-miRs, aptamers,[30] DNAzymes and ribozymes, exon skipping, siRNA, transcription factor decoys (TFD) and immunostimulatory effects. Currently, the main focus in terms of oligonucleotide therapeutics is on antisense and siRNA technologies.

29 **Therapeutic Oligonucleotides**, RSC Biomolecular Sciences, Ed. J. Kurreck, 2008, ISBN 978-0-85404-116-9.
30 **Aptamers as therapeutics**, *A.D. Keefe, S.* Pai and A. Ellington, Nature Reviews Drug Discovery, *9, 537-550, 2010.*

Antisense therapy

The concept underlying antisense technology is relatively straightforward: the use of a sequence, complementary by virtue of base pair hybridisation, to a specific mRNA can inhibit its expression and then induce a blockade in the transfer of genetic information from DNA to protein.[31] The development of antisense oligonucleotide technologies as therapeutics agents in recent years led to the first FDA approval for the commercialisation of an antisense oligonucleotide, Vitravene (for cytomegalovirus retinitis[32]), and to numerous clinical trials of therapeutic oligonucleotides.[33]

Phosphorothioate oligonucleotides (see page 62) were the first modified oligos to be used in antisense applications (e.g. Vitravene). Their endonuclease resistance and their RNase H activity make them suitable candidates for this purpose, for both RNase H directed and steric block antisense applications.

However, where the oligonucleotide has a high degree of phosphorothiolation, non-specific binding is known to occur.[34] There is also the issue where binding efficiency to RNA is much lower than that of DNA. In spite of this, full phosphorothioate and part-phosphorothioate oligos are still used in the development of antisense oligonucleotide therapeutics.

In order to resolve these issues base, sugar and other phosphate modifications have been developed. These "second-generation" oligonucleotides are resistant to degradation by cellular nucleases (see page 71) and hybridise specifically to their target mRNA with higher affinity than the isosequential phosphodiester or phosphorothioate. However, such antisense effects result from RNase H-independent mechanisms.

In this respect, the most common oligonucleotide modification involves use of 2-O-methyl groups (see page 72). These oligonucleotides form high melting heteroduplexes with targeted mRNA[35] and induce an antisense effect by a non-RNase H-dependent mechanism[36], i.e. via a steric blocking mechanism.

Stable oligos have also been produced that do not possess the natural phosphate-ribose backbone. PNAs (see page 54) have an uncharged, flexible, polyamide backbone comprised of repeating N-(2-aminoethyl) glycine units to which the bases are attached. These oligomers can form very stable duplexes or triplexes with nucleic acids: single or double-strand DNA or RNA.[37] The property of high-affinity nucleic acid binding can be explained by the lack of electrostatic repulsion because of the absence of negative charges on the PNA oligomers. Because PNAs are not substrates for the RNase H or other RNases, the antisense mechanism of PNAs depends on steric hindrance. PNAs can also bind to DNA and inhibit RNA polymerase initiation and elongation,[38] as well as the binding and action of transcription factors.[39] PNAs can also bind mRNA and inhibit splicing[40] or translation initiation and elongation.[41]

Although such modifications in their own right have proved efficient in terms of antisense applications, it is the combination of these modifiers, including the use of phosphorothioate linkages, which have given the most dramatic improvements. Specificity, as well as efficacy, can be increased by using a chimeric oligonucleotide, in which the RNase H-competent segment, usually a phosphorothioate moiety, is flanked on one or both termini by a higher- affinity region of modified RNA,[42] frequently 2'-O-alkyloligoribonucleotides. This substitution not only increases the affinity of the oligonucleotide for its target but reduces the cleavage of nontargeted mRNAs by RNase H.[43]

Other examples of "second-generation" antisense oligonucleotides include phosphorodiamidate morpholino oligomers,[44] and N3'-P5' PN, which result from the replacement of the oxygen at the 3' position on ribose by an amine group.[45]

siRNA

Unlike antisense oligonucleotides, siRNA is a duplex made up of a sense (passenger) and antisense (guide) strands. In this case, the mechanism of gene silencing is more complex. First the duplex loads onto the RNA-Induced Silencing Complex (RISC) where the strands separate. The antisense strand then guides sequence specific cleavage of the target mRNA with the protein Agronaute, the latter being the catalytic component of RISC.

Just like antisense oligonucleotides, it is important to build in nuclease resistance to the therapeutic. Similarly, high binding efficiency between the antisense strand and the target mRNA is highly desirable. As a consequence, the aforementioned modifiers developed to improve antisense technology are equally applicable to siRNA. Typical modifications are combinations of 2'- or sugar modified nucleosides such as 2'-OMe, 2'-F, LNA and phosphorothioate. The first FDA-approved siRNA drug; Patisiran for the treatment of hATTR is an siRNA oligo developed by Alnylam.

31 **Antisense Oligonucleotides: Basic Concepts and Mechanisms**, *N. Dias and C. A. Stein, Mol. Cancer Ther., 1, 347, 2002.*

32 *(a)* **Technology evaluation: fomivirsen. Isis Pharmaceuticals Inc/CIBA vision**, *R.M. Orr, Curr. Opin. Mol. Ther., 3, 288 –294, 2001; (b)* **Fomivirsen approved for CMV retinitis**, *B. Roehr, J. Int. Assoc. Physicians AIDS Care, 4, 14 –16, 1998.*

33 **Antisense therapeutics: is it as simple as complementary base recognition?**, *S. Agrawal and E.R. Kandimalla, Mol. Med. Today, 6, 72 –81, 2000.*

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Aptamers

Nucleic acid 'aptamers' are single stranded (ss) oligonucleotides (DNA or RNA), which fold into well-defined three-dimensional structures, forming shapes with complementary interactions with a desired target (e.g. small molecules, proteins, cells and even whole organisms).[46,47] As a consequence of this 'lock and key' binding mechanism, they generally have very high affinity (nano- and pico-molar dissociation constants) and specificity (>1000-fold) equivalent to that of antibodies (Figure 2). Importantly they offer several advantages over antibodies. Firstly, from a synthetic perspective, aptamers can be cost-effectively produced on a large scale and site-specifically modified by chemical methods.[48,49]

In addition, they are intrinsically stable to heat and, unlike antibodies, can undergo multiple heat denaturation steps and still refold. Secondly, for therapeutic applications, they penetrate tissue faster due to their smaller size (8–25 kDa aptamers versus ~150 kDa of antibodies)[47] and generate lower toxicity and immunogenicity as they are not normally recognised by the host immune system.

Aptamers are generally derived from a random library of 10^{58} – 10^{61} ssDNA or ssRNA molecules through an *in vitro* selection technique called SELEX (systematic evolution of ligands by exponential enrichment), developed in the 1990s.[51,52] The process is depicted in Figure 3. ssDNA libraries are often prepared by the strand separation of double-stranded PCR products.[53] In contrast, ssRNA aptamer libraries are prepared by *in vitro* transcription of double stranded (ds) DNA using recombinant T7 RNA polymerase[51] SELEX involves the incubation of random oligonucleotide libraries with the target molecule, separation of bound from unbound nucleic acids, elution of the bound nucleic acids from the target and amplification of enriched population to use as the 'new' starting libraries in the next round of selection. Subsequently, cloning and sequence analysis are carried out. Conventional SELEX carried out manually can take up to several weeks to be completed. Several types of *in vitro* selection methods have been studied including nitrocellulose membrane filtration-based SELEX,[7,54,55] affinity chromatography[56,57,58] and magnetic bead-based SELEX,[59,60,61] capillary electrophoresis-based SELEX,[62,63,64] microfluidic-based SELEX,[65,66] cell-SELEX[67,68,69] and other less common techniques.[70,71] Recently developed technologies have shown a significant improvement in SELEXbased discovery of aptamers.[72]

From a clinical point of view there have been a small number of important advances. An anti- VEGF aptamer, (Pegaptanib or Macugen) has been approved for the treatment of neovascular (wet) age-related degeneration disease (AMD).[73,74] In addition, numerous aptamers are now at preclinical or clinical trial stages for gene

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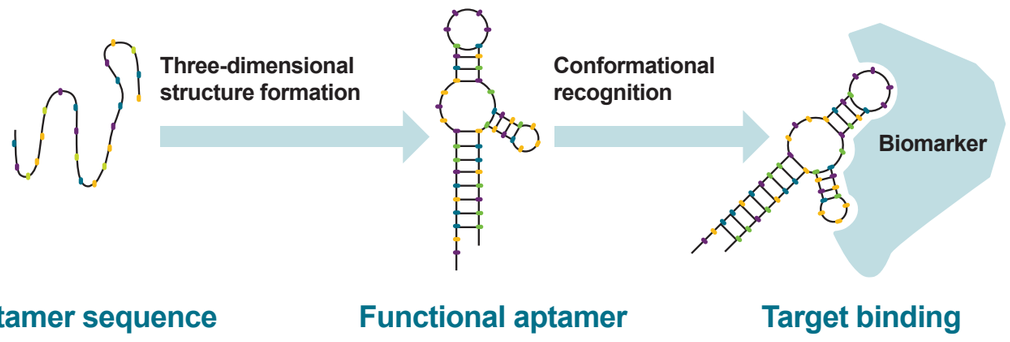

Figure 2. Schematic presentation of aptamer conformational interaction with its target to form an aptamer-target complex.[22]

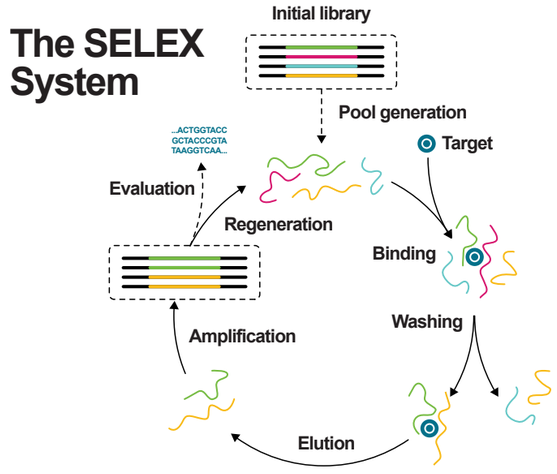

Figure 3. Overview of a typical SELEX procedure

therapy,[75,76,77,78] immunotherapy,[79,80] cancer therapy[81,82,83] and as molecular imaging agents.[84,85,86]

For therapeutic applications, a major limitation of natural nucleic acid-based aptamers is their poor stability in biological media. They are susceptible to nuclease degradation and are sensitive to the composition of the local environment (e.g. acidic or basic media, metal ions).[70,87] To address the former point, the 2'-position of the ribose sugar is often functionalised with fluoro (-F), amino (-NH2), O-alkyl (e.g. -OMe) or thiol (-SH) groups (Figure 4).[70,88,89,90,91]

Recently, Tolle et al. reported a 'click-SELEX' procedure that greatly enhances the structural diversity of aptamers by introducing bulky modifications during post-PCR step by click chemistry (CuAAC reaction).[92] This eliminates problems caused by the limitations of DNA polymerases in incorporating modified dNTPs that must otherwise be overcome during the PCR amplification steps in SELEX.[93] Furthermore, Kimoto et al. discovered DNA aptamers that contain two hydrophobic artificial (unnatural) nucleotides in addition to four natural bases, enhance aptamer affinity and target specificity.[94]

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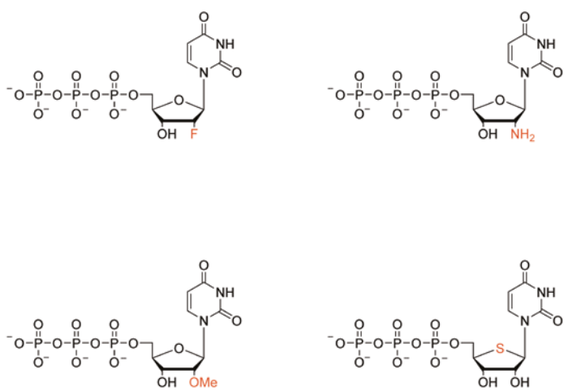

Figure 4 Chemical structures of sugar modified nucleotides used in the aptamer generation resulting in increased nuclease resistance : 2'-fluorouridine-5'-triphosphate, 2'-aminouridine-5'-triphosphate, 2'-methoxyuridine-5'-triphosphate and 4'-thiouridine-5'-triphosphate, These are currently not available from Biosearch Technologies as stock items. However, contact our Customer Service for a custom order (terms apply).

CRISPR/Cas gene editing

The ability to make targeted changes to genes within living organisms has undergone a revolution in recent years, largely due to the rapid development of CRISPR/Cas technology. This technology has its origins in a bacterial immune defence mechanism that is defined by its use of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) nucleases to recognise and destroy foreign DNA from invading viruses.[95]

Several types of CRISPR/Cas nucleases have been identified, of which Cas9 and Cas12a (also known as Cpf1) are the most commonly used. Both nucleases require an RNA molecule, referred to as guide RNA, to specifically target a genetic locus. Together, the guide RNA (gRNA) and nuclease target and cut both strands of double-stranded DNA. When this is performed *in vivo*, the DNA is repaired by an error-prone mechanism that can result in a genetic change. As an RNA molecule, gRNAs are susceptible to hydrolysis and this liability can hinder utilisation of CRISPR/Cas systems in environments where cellular nucleases are present.

Chemical modifications that protect gRNAs from degradation have been shown to improve gene editing by CRISPR/CAS systems.[96,97,98,99] These modifications include the addition of phosphorothioate bonds in the backbone between the first and last three nucleotides of gRNA, as well as modifications to the 2'-hydroxyl, such as replacement with 2'-O-methyl or 2'-fluoro groups. While these modifications protect gRNAs from cellular RNA exonuclease activity, they can only be placed in certain locations within the gRNA sequence without affecting Cas activity.

Fluorescently labelled gRNAs have been used in various applications. Fluorescent guides enable monitoring of Cas-gRNA delivery into cells following transfection. Additionally, cells that have received a fluorescent gRNA can be sorted by fluorescent-activated cell sorting (FACS), which allows for enrichment of cells containing Cas-gRNA complexes and therefore improves the likelihood of obtaining edited cells. Inactive Cas nucleases complexed with gRNA can be used to tag loci without cleaving them. In an example of this approach, a gRNA was engineered to include an annealing site for a molecular beacon, which is a structured oligonucleotide appended with a fluorophore and quencher.[100] CRISPR/Cas has also been used in synthetic biology applications. One such approach uses fluorescent guides to study CRISPR/Cas synthetic DNA circuits.[101]

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Modifications for epigenetics

The study of DNA damage resulting from metabolic processes and environmental factors, along with their associated repair mechanisms has led to a better understanding of the occurrence of genetic mutations, neurodegenerative diseases, cancers and the aging process.

Ordering epigenetic reagents

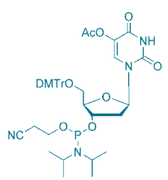
At Biosearch Technologies, we offer a range of modifiers for this purpose. In particular we have amidites of 5-hydroxy-dC (**LK2543**), 5-hydroxy-dU (**LK2541**), 5-hydroxymethyl-dU (**LK2542**), 5-hydroxymethyl-dC (**LK2544**), 5-carboxy-dC (**LK2545**), 5-formyl-dC (**LK2546/ BA0367**), 5-hydroxymethyl-dC II (**LK2547/ BA0371**) and 5-formyl-dC III (**LK2548**) for use in the study of oxidative damage and repair, methylation and epigenetics.

Oxidised pyrimidines such as 5-hydroxy dU and 5-hydroxy dC are derived from dC via oxidative metabolic processes, UV or ionising radiation to form 5-HO-dC which spontaneously undergoes deamination to form 5-HO-dU (see Figure 5).

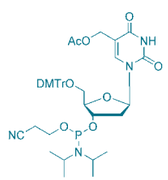
Although there are repair mechanisms to convert 5-HO-pyrimidines back to dC,[102] the fact that they are observed in cellular DNA at consistent levels suggests that these repair mechanisms are inefficient,[103] at least in certain cell types. Oligonucleotides modified with **LK2541** or **LK2543** are useful in understanding such processes.

The presence of either 5-HO-dU or 5-HO-dC can both lead to mutations resulting from their ability to mismatch with A and A/C respectively hence where the repair mechanism fails, such mutations can be permanently incorporated into the resulting gene.

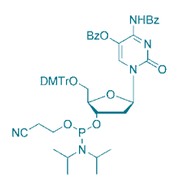
102 **Base excision repair in a network of defence and tolerance**, *H. Nilsen and H.E. Krokan, Carcinogenesis, 22, 987-998, 2001.*
103 **Endogenous oxidative damage of deoxycytidine in DNA**, *J.R. Wagner, H. Chia-Chieh and B.N. Ames, Proc. Nat. Acad. Sci., 89, 3380-3384, 1992.*



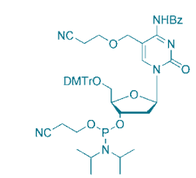
LK2541



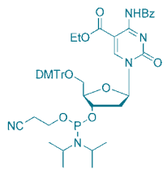
LK2542



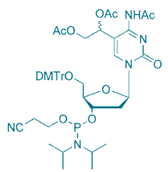
LK2543



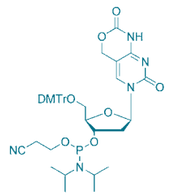
LK2544



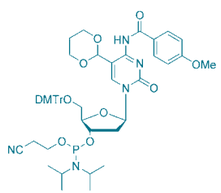
LK2545



LK2546



LK2547



LK2548

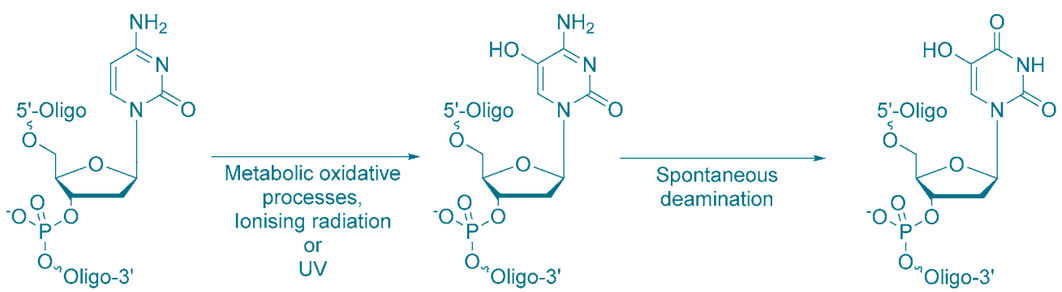


Figure 5 Formation of 5-HO-dC and 5-HO-dU from dC.

5-Hydroxymethyl-dU (5-hmdU, **LK2542**) is also a result of oxidative process or ionizing radiation but in this case dT is modified.[104] It is also possible that 5-hmdU is formed by deamination of 5-hmdC but Müller and Carell recently showed that this does not contribute to the steady state levels of hmdU in mouse embryonic stem cells, but that dT is a substrate for ten eleven translocation enzymes (Tet) leading to the formation of 5-hmdU.[105] Hence, **LK2542** is an important reagent for the study of both oxidative processes and epigenetics.

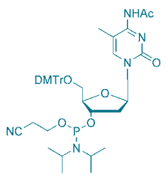
Epigenetics is the study of heritable silencing of genes where there is no change to the coding sequence. Interest in this area has grown significantly over the past few years particularly looking at changes induced and sustained by non-coding RNA gene silencing, histone modification and DNA methylation of cytidine in CpG islands.[106] Phosphoramidites **LK2544** - **LK2548** are applicable to the latter.

Once incorporated into an oligonucleotide, these modifiers represent the various products in the biochemical pathway of the modification of dC (see Figure 6).

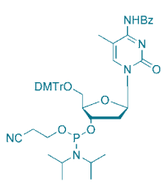
In DNA, cytidine is methylated by a DNA methyl transferase catalysed reaction with S-adenosylmethionine to form 5-mdC. This is oxidised by Tet enzymes to 5-hydroxymethyl-dC which is further oxidised to 5-formyl-dC, which in turn is further oxidised to 5-carboxy-dC. Both 5-carboxy-dC and 5-formyl-dC can be converted back to dC via thymidine DNA glycosylase mediated base excision repair.[107]

Until now our range of products in this area of research has been limited to 5-methyl-dC (**LK2017** [N-Bz] and **LK2529** [N-Ac]) therefore the addition of these modifiers to our catalogue provides our customers working in this area the tools required to progress our understanding of these important pathways.

104 **Oxidative damage to DNA: formation, measurement, and biological significance**, *J. Cadet, M. Berger, T. Douki and J.-L. Ravanat, Rev. Physiol. Biochem. Pharmacol., 131, 1-87, 1997.*
105 **Tet oxidizes thymine to 5-hydroxymethyluracil in mouse embryonic stem cell DNA**, *T. Pfaffeneder, F. Spada, M. Wagner, C. Brandmayr, S.K. Laube, D. Eisen, M. Truss, J. Steinbacher, B. Hackner, O. Kotljarova, D. Schuermann, S. Michalakis, O. Kosmatchev, S. Schiesser, B. Steigenberger, N. Raddaoui, G. Kashiwazaki, U. Müller, C.G. Spruijt, M. Vermeulen, H. Leonhardt, P. Schär, M. Müller and T. Carell, Nat. Chem. Biol., 10 (7), 574-81, 2014.*
106 **Epigenetics in human disease and prospects for epigenetic therapy**, *G. Egger, G. Liang, A. Aparicio and P.A. Jones. Nature, 429, 457-463, 2004.*
107 **Tet enzymes, TDG and the dynamics of DNA methylation**, *R.M. Kholi and Y. Zhang, Nature, 502, 472-479, 2013.*



LK2529



LK2017

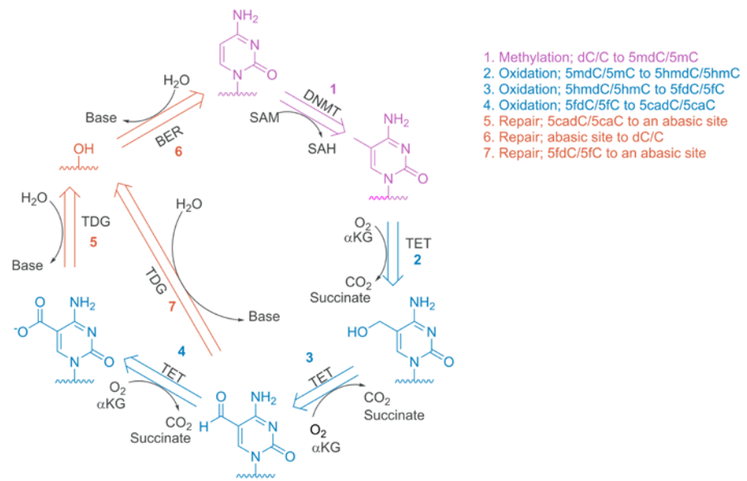


Figure 6. A complete pathway for dynamic modifications of C (adapted from reference 100).

Modifiers and their use in oligonucleotide synthesis

Modifying oligonucleotide structure is becoming an increasingly important tool in a vast range of applications, as the breadth of modifications continues to grow.

Introduction

Although only ~10% of oligonucleotides manufactured are modified, this is an extremely important sector of the oligonucleotide market. Modifying an oligonucleotide enables the development of diagnostic tests, therapeutics, detection methods and genetic analysis tools. As these develop, the need for new improved modifiers grows. For instance, the first examples of phosphorylated oligonucleotides introduced 5'-phosphate enzymatically. Today there are many examples of phosphorylating reagents (e.g. **LK2101/BNS-5010, LK2127**) available for direct incorporation during oligonucleotide synthesis. When used in combination with a sulphurisation reagent, the resulting thiophosphate was once a common means of conjugating biomolecules (e.g. HRP) to oligonucleotides. This was often inefficient due to the proximity of the biomolecule to the oligonucleotide. Since then, a range of amino and thiol linkers have been developed to allow more efficient coupling and, when used in conjunction with a spacer, can not only improve the conjugation efficiency further, depending on the nature and length of the spacer, can improve the efficiency of the oligonucleotide in its intended application.

While post-synthetic labelling is still important, many phosphoramidites have been developed to eliminate the need for this. 5'-FAM (**LK2134/BA0054**) phosphoramidite for instance now allows the incorporation of fluorescein to the 5'-end of an oligonucleotide without the need for a linker and an active form of the dye and the additional downstream processing associated with post labelling.

The need to improve hybridisation properties to obtain higher specificity in terms of detection or a more stable duplex for therapeutic use has driven the development of modified bases and modified backbones. PNA for instance forms a very strong duplex as a result of the lack of charge on the backbone. It is now possible to fine tune the T_m of a duplex with the use of modifiers. For instance, 2'-OMe nucleosides will increase the T_m of a duplex by 1-4 ºC per addition whereas the incorporation of UNA nucleosides will decrease the T_m of a duplex by 5-10 ºC. Therefore, it is possible that a precise T_m can be dictated by the use of such modifications. Where the sugar is modified, this provides a means of protecting the oligonucleotide against nucleases.

In short, although the majority of oligonucleotides manufactured are unmodified there is an important and continually evolving need for modifiers.

Spacers

In general terms, a spacer is introduced into an oligonucleotide to add distance between the oligonucleotide and a modifier. This reduces the possibility of any adverse interaction between the modifier and the sequence. For instance, G-rich sequences are known to quench fluorescein therefore the use of a suitable spacer will remove the dye label from the proximity of the oligonucleotide minimising the quenching effect. In a similar fashion, spacers are often used to distance between multiple additions of self-quenching dyes e.g. fluorescein.[108]

The application of the modified oligonucleotide will dictate whether a hydrophilic (Spacer 18; **LK2129**/**BNS-5036**, Spacer 9; **LK2128**/**BNS-5035**) or hydrophobic spacer (Spacer C3; **LK2113**/**BNS-5041**, Spacer C12; **LK2147**) is required. Multiple incorporations of varying lengths of these spacers allow the precise length of the spacer arm to be controlled. This can be important in hairpin loop[109] and duplex studies[110] of DNA.

Several spacers have specific uses. Spacer C3 phosphoramidite (**LK2113**/**BNS-5041**), when incorporated into an oligonucleotide, mimics the three carbon spacing between the 3' and 5' hydroxyls of sugar unit.[111] Although useful where the base at a specific site is unknown, the flexibility of the alkyl chain distorts the sugar-phosphate backbone. This can be alleviated with the use of dSpacer (**LK2146**) since incorporation of this modifier sits directly into the natural sugar-phosphate backbone with no adverse effect. This modifier mimics abasic sites[112] and is useful in the study of mutations resulting from depurination.

In some cases, it is advantageous to remove the modifier from the oligonucleotide e.g., if using biotin as a means of capture, the target is bound to the biotin labelled probe. This is then captured using a streptavidin affinity column and the target-probe duplex can be eluted if a cleavable spacer is used (e.g. our photocleavable spacer **LK2131**; see page 67 for more information).

Although less common than terminal spacing, but equally important, spacers have been incorporated within an oligonucleotide. This adds distance between sections of the sequence. For instance, Cytocell's SMART detection assay[113] uses spacer 18 or HEG (**LK2129**/**BNS-5036**) in the template probe where one section acts as an anchor in binding to the target leaving the other section free for hybridisation to the extension probe to allow amplification during PCR. In this case, the spacer gives flexibility to the template probe to enable hybridisation to both the target and the extension probe.

In similar way, HEG is used in Scorpions™ Primers to separate the probe and primer section. However, in this case, this not only provides the flexibility to allow the probe to flip back to hybridise to the amplicon but also acts as a PCR blocker to prevent read through to the probe.[114]

3'-Spacers such as **LK2245**/**BG1-5011** and **LK2395** are often used as an alternative to 3'-phosphate as blockers since, when incorporated at the 3'-end, the resulting oligonucleotide shows nuclease and polymerase resistance. In fact, spacer C3 is often incorporated at the 3'-end of an oligonucleotide for use with restriction enzymes rather than phosphate since the latter is thought to partially cleave during the assay.

Ordering spacer modifiers

Biosearch Technologies offers a wide range of spacer phosphoramidites and solid supports as part of our NAC product offering. See our website for a complete view of our spacers.

108 *See for example:* **Design of multidye systems for FRET-based applications**, *M.S. Shchepinov and V.A. Korshun, Nucleosides, Nucleotides & Nucleic Acids, 20, 369-374, 2001.*

109 **Circular dichroism studies of an oligodeoxyribonucleotide containing a hairpin loop made of a hexaethylene glycol chain: conformation and stability**, *M. Durand, K. Chevrie, M. Chassignol, N.T. Thuong and J.C. Maurizot, Nucleic Acids Research, 18, 6353-6359, 1990.*

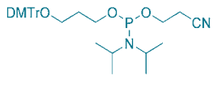
110 **A nicked duplex decamer DNA with a PEG6 tether**, *L. Kozerski, A.P. Mazurek, R. Kawecki, W. Bocian, P. Krajewski, E. Bednarek, J. Sitkowski, M. P. Williamson, A.J.G. Moir and P.E. Hansen, Nucleic Acids Research, 29, 1132-1143, 2001.*

111 **Enhancing sequence-specific cleavage of RNA within a duplex region: Incorporation of 1,3-propanediol linkers into oligonucleotide conjugates of serinol-terpyridine**, *B.N. Trawick, T.A. Osiek and J.K. Bashkin, Bioconjugate Chem., 12, 900-905, 2001.*

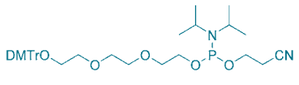
112 *(a)* **Oligodeoxynucleotides containing synthetic abasic sites model substrates for DNA-polymerases and apurinic apyrimidinic endonucleases**, *M. Takeshita, C.N. Chang, F. Johnson, S. Will and A.P. Grollman, J. Biol. Chem., 262, 10171-10179, 1987; (b)* **NMR-studies of abasic sites in DNA duplexes deoxyadenosine stacks into the helix opposite the cyclic analog of 2-deoxyribose**, *M.W. Kalnik, C.N. Chang, A.P. Grollman and D.J. Patel, Biochemistry, 27, 924-931, 1988.*

113 **Detection of virus mRNA within infected host cells using an isothermal nucleic acid amplification assay: marine cyanophage gene expression within *Synechococcus sp***, *S.D. Wharam, M.J. Hall and W.H. Wilson, Virology Journal, 4, 52-59, 2007.*

114 **Duplex Scorpion primers in SNP analysis and FRET applications**, *A. Solinas, L.J. Brown, C. McKeen, J.M. Mellor, J.T.G. Nicol, N. Thelwell and T. Brown, Nucleic Acids Research, 29 (20), e96, 2001.*



LK2113



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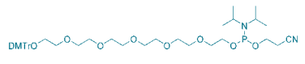

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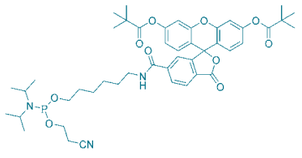

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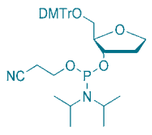

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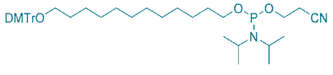

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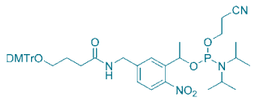
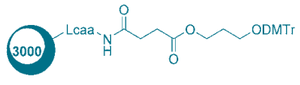
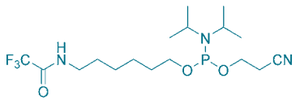


LK2131

LK2245



LK2395



LK2124

Modifiers and their use in oligonucleotide synthesis

Conjugation reagents

Incorporation of reactive functional groups, particularly primary amine, thiol, or carboxylate, at specific sites within an oligonucleotide allows for subsequent post-synthesis conjugation of the oligo with a number of different affinity, reporter or protein labels, depending on the application. Such labels need to be reactive towards the incorporated functional group, for example, NHS esters or isothiocyanates will react with primary amines, and iodoacetamides or maleimides will conjugate with primary thiols. This approach is often necessary where the desired label or tag is either not available as a phosphoramidite, or is sensitive or unstable to the conditions of oligonucleotide synthesis or deprotection. A common example is the attachment of a rhodamine dye using the TAMRA NHS ester. Functionally-derivitised oligos can also be covalently attached to surfaces such as glass slides or gold microspheres for use in various microarray or nanoelectronic applications.

Amino modification

5’-Amino Linkers

One of the most common modifications is the incorporation of a primary amine at the 5’-terminus of the oligonucleotide using an ‘amino-linker’ phosphoramidite, protected with either the base labile trifluoroacetate[115] (TFA) (e.g. 5’-TFA-Amino-Modifier C6-CE Phosphoramidite, **LK2124**/**BNS-5017**) or the acid-labile monomethoxytrityl[116] (MMT) (e.g. 5’-MMT-Amino- Modifier C6-CE Phosphoramidite, **LK2123**/**BNS-5015**, or 5’-MMT-Amino-Modifier C12-CE Phosphoramidite, **LK2133**/**BNS-5039**) groups.

The choice between the MMT and TFA-protected C6 amino modifiers is dependent on the purification strategy used on the oligo, or whether on-column or solution-phase conjugation is required. If this is purified, the MMT protection is preferable since the trityl group, stable to the basic cleavage and deprotection conditions, can be used as a ‘handle’ in e.g. cartridge purification where the MMT group is removed during the purification process. Otherwise, the TFA protection is perfectly suitable. A variety of molecules can be attached to the liberated 5’-amine such as fluorescent dyes or haptens such as biotin.

The MMT group can also be removed by extended deblocking on the synthesizer, allowing a solid-phase conjugation of a label containing e.g. an activated carboxylic acid. However, in this case it is important to remember that the conjugate must be stable to the subsequent cleavage and deprotection conditions.

The shorter C5 or C6 carbon chain linkers may be used to attach compounds where proximity to the oligonucleotide causes no problem. The longer C12 analogue has specific applications in e.g. affinity chromatography, where the oligo must be sufficiently distanced from the surface, and in some cases labelling with fluorescent tags, where close interaction may lead to partially quenched fluorescence.

Our hydrophilic Amino-Modifier 11 CE-Phosphoramidite (**LK2182**) is particularly useful for solution-phase couplings of labels to oligos. It is often found that when using hydrophobic amino-linkers, e.g. **LK2123**/**BNS-5015**, an additional hydrophilic spacer is required. This extends the distance of the label from the oligo. Our product has this hydrophilicity “built-in” and can therefore be used where a hydrophilic linker is required. Once incorporated into an oligo this linker is equivalent to ~2 base units. It is available in both TFA (**LK2182**) and MMT (**LK2193**) protected forms, the latter allowing oligo purification based on exploiting the trityl group, or on-column conjugations as described above.

Internal amino linkers

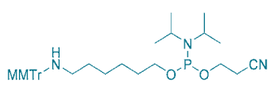
Internal amino-functions, ready for further post-synthetic modification, can be introduced to oligonucleotides by a number of products. Amino-Modifier C6-dC-CE Phosphoramidite (**LK2141**/**BA0163**) and both the Amino-Modifier C2-dT (**LK2149**) and C6-dT (**LK2135**/**BNS-5040**) products can be added in place of a 2’-deoxycytidine and a thymidine residue, respectively, during oligonucleotide synthesis.

In the case of the C6 analogues, after deprotection, the primary amine is distanced from the oligonucleotide by a total of 10 atoms and can be labelled or attached to a biomolecule such as an enzyme. The C2 analogue is more appropriate for applications where the attached label is designed to interact with the oligonucleotide. It has been shown that duplexes containing a modified T base have no adverse effect on melting behaviour.[117]

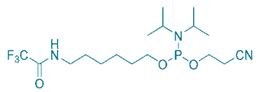
115 *This is a standard amino-protecting group in organic synthesis. See for example: Greene’s Protective Groups in Organic Synthesis, 5th Edition, P.G.M. Wuts (Ed.), Wiley-Blackwell, 2013.*

116 **The synthesis of oligonucleotides containing a primary amino group at the 5’-terminus**, *B.A. Connolly, Nucleic Acids Research, 15, 3131-3139, 1987.*

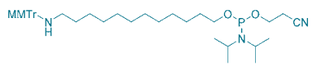
117 **Synthesis and characterization of DNA oligomers and duplexes containing covalently attached molecular labels: comparison of biotin, fluorescein, and pyrene labels by thermodynamic and optical spectroscopic measurements**, *J. Telser, K.A. Cruickshank, L.E. Morrison and T.L. Netzel, J. Amer. Chem. Soc., 111, 6966-6976, 1989.*



LK2123

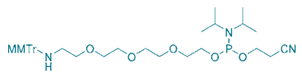


LK2124



LK2133

LK2182



LK2193

However, there are times when it is advantageous to have an interaction between the duplex and the label. For instance, incorporating dansyl directly onto the 5-position of dU allows the study of the interaction of antibiotics with the minor groove by measuring the change in fluorescence signal.[118]

The related Amino-Modifier C6-dA-CE Phosphoramidite (**LK2071**) is useful for introducing an amino function at a dA site, although the linker on the 8-position does cause some destabilisation of the duplex pairing to T (approximately 2 ºC per insertion).

Applications of the internal modification technique are varied. For example, an internally amino-modified oligo is ideal for incorporating dyes not normally available as phosphoramidites, such as ROX, via an NHS ester. For example, when synthesising wavelength shifting FRET probes using FAM/ROX, combine the ROX-modified amino-dT with a FAM modification at the 5’-end.

3’-Amino-linkers

The most commonly used product for introducing a 3’-amino functionality is Fmoc-protected 3’-Amino-Modifier C7 CPG 1000 (**LK2350**). Use of an Fmoc-protected amine has both advantages and disadvantages. This is quite stable to oligo synthesis conditions however, if not handled correctly, some loss of Fmoc may occur. This leads to capping of the free amine with acetic anhydride and hence loss of functionality. The main advantage of Fmoc is that it can be removed selectively without cleavage from the support allowing solid-phase conjugation of the desired label. This can be done prior to or subsequent to oligonucleotide synthesis. It should be noted that, due to the 1,3-diol configuration, **LK2350** contains a chiral centre and will generate a pair of diastereomers in oligo synthesis, although this is rarely observed in HPLC.

Alternatives to Fmoc protection have been investigated. Phthalimide (PT) chemistry has been used in the development of 3’-PT-Amino-Modifier C6 CPG[119] (**LK2365**), where the nitrogen which will ultimately provide the 3’-amino function is part of the PT group attached to the support through an imide group attached to the aromatic ring. This linkage is stable to all conditions of oligo synthesis and the resulting amino functionality does not add any additional chiral centres/diastereomers to the oligo. Cleavage and deprotection is achieved using an extended ammonium hydroxide treatment. A completely analogous C3 product (**LK2371**) is also available.

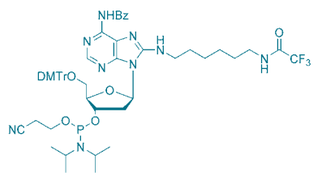
Two additional products are available for introducing 3’-amino functionality without blocking the terminus from any desired enzymatic activity. These are 3’-Amino-Modifier C6-dC CPG (**LK2369**) and the equivalent dT analogue (**LK2367**).

Ordering amino-linkers

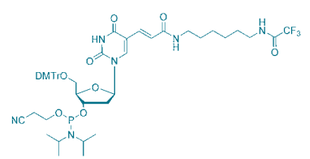
See our website for a complete view of our amino linkers.

118 **Fluorescent d(CGCGAATTCGCG): characterization of major groove polarity and study of minor groove interactions through a major groove semantophore conjugate**, *D.A. Barawkar and K.N. Ganesh, Nucleic Acids Research, 23, 159-164, 1995.*

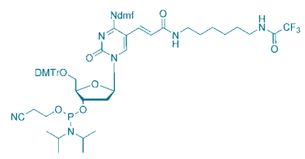
119 **An improved CPG support for the synthesis of 3’-amine-tailed oligonucleotides**, *C.R. Petrie, M.W. Reed, A.D. Adams and R.B. Meyer, Jr., Bioconjugate Chem., 3, 85-87, 1992.*



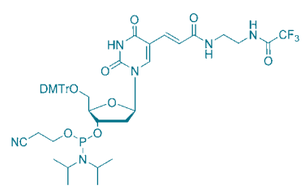
LK2071



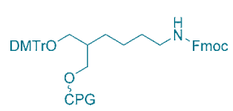
LK2135



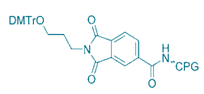
LK2141



LK2149



LK2350



LK2371

Thiol modification

5’-thiol-linkers

5’-thiol-modifiers, phosphoramidites used to introduce a 5’-thio functionality[120] to an oligo, have very similar applications to amino-modifiers. The thiol group is used to attach labels such as fluorescent tags[121] and biotin.[122] Conjugation to fluorescent markers is possible, for example, via reactions of the thiol with iodoacetate and maleimide derivatives to form thioether linkages.

Since conjugation to a thiol is orthogonal to that of an amino functionality, it is not uncommon to have both an amino (e.g. 3’) and a thiol (e.g. 5’) in the same oligo.

In general, thiol modification at the 5’-end of the oligonucleotide is achieved with 5’-thiol-modifier C6-CE Phosphoramidite (**LK2125**/**BNS-5019**) or, more commonly, the thiol-modifier C6 S-S CE Phosphoramidite (**LK2126**/**BNS-5042**). As with the MMT protected amino-modifiers, the trityl group on **LK2125**/**BNS-5019** is usually retained after cleavage of the oligonucleotide to assist purification. However, because the S-trityl group is not acid labile, it must be removed by treatment with silver nitrate. Although, this procedure is commonly used it must be very carefully carried out. Use of **LK2126**/**BNS-5042** offers an alternative and more robust protocol, whereby the thiol is liberated by use of tris(2-carboxyethyl)phosphine (TCEP). This disulphide product can also be used to modify the 3’-position by using the phosphoramidite as the first adduct in the oligo sequence. Incorporation of **LK2126**/**BNS-5042** at the 5’-end allows the possibility of DMT-ON purification prior to reduction of the disulphide bridge.

Following on from the hydrophilic amino modifiers described above, we have prepared a hydrophilic thiol product, S-Bz TEG-CE Phosphoramidite (**LK2187**), which offers all the same advantages of the analogous amino products, but for use in applications where conjugation to a thiol is preferred, or necessary.

We have shown that the post-labelling efficiency of the hydrophilic products is comparable with the current most commonly used amino and thiol linkers, used on their own or in combination with a HEG spacer.[123] **LK2187** is not compatible with thiotetrazoles as activators therefore DCI is recommended for this modification.

An alternative route to thiol modification can be used with our thioctic acid product (**LK2166**, see below).

Modification of oligonucleotides with thioctic acid for gold and silver bioconjugation

Immobilisation of DNA and other biopolymers on solid surfaces has wide application in microarrays, biosensors and related technologies. Recently, the detection of specific DNA sequences—a central theme of molecular diagnostics—has been achieved using oligonucleotide probes conjugated to metallic nanoparticle substrates.[124]

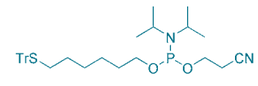
120 **Chemical synthesis of oligonucleotides containing a free sulphydryl group and subsequent attachment of thiol specific probes**, *B.A. Connolly and P. Rider, Nucleic Acids Research, 13, 4485-4502, 1985.*

121 **Efficient methods for attachment of thiol specific probes to the 3’- ends of synthetic oligodeoxyribonucleotides**, *R. Zuckermann, D. Corey and P. Schultz, Nucleic Acids Research, 15, 5305-5321, 1987.*

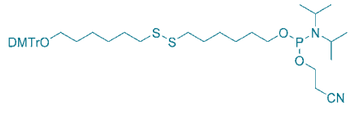
122 **The synthesis of protected 5’-mercapto-2’,5’-dideoxyribonucleoside-3’-O-phosphoramidites**; *uses of 5’-mercapto-oligodeoxyribonucleotides, B.S. Sproat, B. Beijer, P. Rider and P. Neuner, Nucleic Acids Research, 15, 4837-4848, 1987.*

123 **A comparison of hydrophilic and hydrophobic amino and thiol linkers for use in post-synthetic labelling of oligonucleotides**, *S. Aitken, U. Ixkes, C. McKeen and D. Picken, poster presented at IS3NA XIX IRT, Lyon, 2010. Available online: https://linktechsupport.zendesk.com/hc/en-us/articles/200143418-A-Comparison-of-Hydrophilic- and-Hydrophobic-Amino-and-Thiol-Linkers-for-use-in-Post-Synthetic-Labelling-of-Oligonucleotides.*

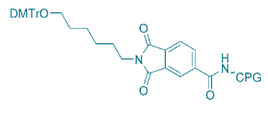
124 **For reviews of this area see: (a) Nanostructures in biodiagnostics**, *N.L. Rosi and C.A. Mirkin, Chem. Rev., 105, 1547-1562, 2005; (b) Surface recognition of bio-macromolecules using nanoparticle receptors, A. Verma and V. Rotello, Chem. Comm., 303-312, 2005; and (c) Nanoparticles, proteins and nucleic acids: Biotechnology meets materials science, C.M. Niemeyer, Angew. Chem. , Int. Ed., 40, 4128-4156, 2001.*



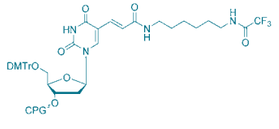
LK2125



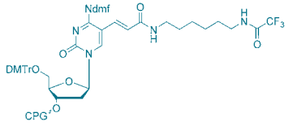
LK2126



LK2365



LK2367



LK2369

Attachment of molecules to gold surfaces (planar or nanoparticle) can be achieved via thiol-based linkers that have a natural affinity for the metal.[125]

Recognised 5’-thiol-modifiers such as the aforementioned 5’-thiol-modifier C6 CE Phosphoramidite (**LK2125**/**BNS-5019**) have been used in such applications.[126] Alternatively, Yoo et al[127] have demonstrated the modification of a planar gold surface using the dithiol-containing thioctic acid, which was then activated as an NHS ester to allow attachment of an anti-DNA antibody. Taira and Yokoyama have also reported DNA- conjugated polyallylamines employing thioctic acid-based amides as side-chains.[128]

Researchers at the University of Strathclyde have further investigated the use of Thioctic Acid NHS Ester (**LK2166**) in oligonucleotide immobilisation[129] and, in particular, demonstrate the superior conjugate stability afforded by the dithiol modification when compared to mono- thiols. Furthermore, they extend the use of this modification to silver conjugation, until now difficult to achieve successfully.[130] Sharma et al have also recently used this molecule in gold nanoparticle patterning on self-assembled DNA.[131]

LK2166 can be attached after cleavage to the 3’-end of an oligonucleotide using a 3’-amino- modified solid support, or to the 5’-end post-synthetically to an amino-modified oligo in the same way as, e.g., TAMRA NHS ester. Availability of this product therefore allows the simple synthesis of dithiol-modified oligos for attachment to gold and silver surfaces.

Internal thiol-linkers

Although thiol-modified oligonucleotides are routinely used to introduce labels such as dyes, haptens and enzymes via reaction with maleimides or haloacetamides, this method has been traditionally limited to the 5’ or 3’ end of the oligonucleotide.

The use of thiol reactive labels for internal modification until now required the conversion of an amino functionality, e.g. amino-dT (**LK2135**/**BNS-5040** or **LK2149**) with thioctic acid (**LK2166**). To overcome this we have developed a thiol-dT modification (**LK2191**) that can be incorporated within an oligonucleotide and reacts directly with maleimides and haloacetamides.

3’-thiol-linkers

Using different strategies, both the thiol-modifier C6 S-S CE-Phosphoramidite (**LK2126**/**BNS-5042**, see above) and the 3’-thiol-modifier C3 S-S CPG (**LK2361**) can be used to introduce a 3’-thio functionality. The latter is simply used as any other support, with subsequent cleavage of the disulphide linkage affording the free thiol. This functionality can be exploited in much the same way as 5’-thiol modification. In addition, 3’-conjugation of phosphorothioates to peptides via a disulphide linkage has been reported.[132]

125 **Some recent advances in nanostructure preparation from gold and silver particles: a short topical review**, *M. Brust and C.J. Kiely, Colloids Surf., A: Physicochemical and Engineering Aspects, 202, 175-186, 2002. For a recent RNA application see: Enzyme-free interrogation of RNA sites via primers and oligonucleotides 3’-linked to gold surfaces, U. Plutowski, S.R. Vogel, M. Bauer, C. Deck, M.J. Pankratz and C. Richert, Org. Lett., 9, 2187-2190, 2007 and references therein.*

126 **See for example: (a) A multi-step chemical modification procedure to create DNA arrays on gold surfaces for the study of protein-DNA interactions with surface plasmon resonance imaging**, *J.M. Brockman, A.G. Frutos and R.M. Corn, J. Amer. Chem. Soc., 121, 8044-8051, 1999; (b) Formation, spectroscopic characterization and application of sulfhydryl-terminated alkanethiol monolayers for the chemical attachment of DNA onto gold surfaces, E.A. Smith, M.J. Wanat, Y. Cheng, S.V.P. Barreira, A.G. Frutos and R.M. Corn, Langmuir, 17, 2502-2507, 2001; and (c) The effect of surface probe density on DNA hybridisation, A.W. Peterson, R.J. Heaton and R.M. Georgiadis, Nucleic Acids Research, 29, 5163-5168, 2001.*

127 **A radioimmunoassay method for detection of DNA based on chemical immobilization of anti-DNA antibody**, *S.-K. Yoo, M. Yoon, U.J. Park, H.S. Han, J.H. Kim, and H.J. Hwang, Exp. Mol. Medicine, 31, 122-125, 1999.*

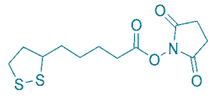
128 **DNA-conjugated polymers for self-assembled DNA chip fabrication**, *S. Taira and K. Yokoyama, Analytical Sciences, 20, 267-271, 2004.*

129 *(a)* **Enhanced oligonucleotide-nanoparticle conjugate stability using thioctic acid modified oligonucleotides**, *J.A. Dougan, C. Karlsson, W.E. Smith and D. Graham, Nucleic Acids Research, 35, 3668-3675, 2007; (b)* **Highly sensitive detection of dye-labelled DNA using nanostructured gold surfaces**, *R.J. Stokes, A. Macaskill, J.A. Dougan, P.G. Hargreaves, H.M. Stanford, W.E. Smith, K. Faulds and D. Graham, Chem. Commun., 2811-2813, 2007.*

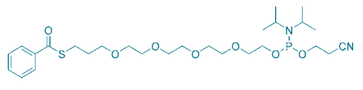
130 **Ultrasensitive DNA detection using oligonucleotide-silver nanoparticle conjugates**, *D.G. Thompson, A. Enright, K. Faulds, W.E. Smith and D. Graham, Anal. Chem., 80, 2805-2810, 2008.*

131 **Ultrasensitive DNA detection using oligonucleotide-silver nanoparticle conjugates**, *D.G. Thompson, A. Enright, K. Faulds, W.E. Smith and D. Graham, Anal. Chem., 80, 2805-2810, 2008.*

132 *(a)* **Synthesis of peptide-oligonucleotide phosphorothioate conjugates by convergent or step-wise solid- phase strategies**, *M. Antopolsky and A. Azhayev, Nucleosides, Nucleotides & Nucleic Acids, 20, 539-550, 2001; (b)* **Efficient synthesis of oligonucleotide-peptide conjugates on large scale**, *S.O. Doronina, A.P. Guzaev and M. Manoharan,*



LK2166



LK2187

The 3’-phosphorothioate (thiophosphate) is generated by the addition of the first base to the 3’-phosphate resin (e.g. **LK2279/BG1-5000**), but one linkage is sulphurised with e.g. EDITH (**LK2171**). Cleavage and deprotection releases the 3’-thiophosphate modified oligo. In a similar way, thiol-modified oligos are conjugated to maleimide-modified enzymes or peptides.

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Carboxylate modification

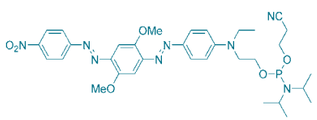
The use of the 5’-carboxylate modifier (**LK2057**), first described by Kachalova et al,[133] allows the introduction of a carboxylic acid function at the 5’-end of an oligonucleotide that is available for conjugation to amines whilst still on the solid support. This strategy avoids the problems of low yields, long reaction times and the need for excess reactants often encountered by other post-cleavage solution methods.

This non-nucleosidic building block is incorporated into the final step of automated DNA synthesis using phosphoramidite chemistry. The 2’-chlorotrityl protecting group is stable during coupling, capping and oxidation or sulphurisation but is easily removed during the deblock step; typically 3% TCA in DCM. At this stage the 5’-carboxylate oligonucleotide can be cleaved and deprotected or further modified by on-column conjugation. Alternatively the 2-chlorotrityl protection can be retained until after cleavage and deprotection.

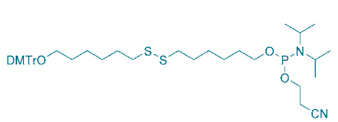
If the conjugation step has already been completed, provided the label is stable, most deprotection conditions are applicable. Otherwise it is best to use 0.4 M NaOH in methanol/water (4:1) overnight at room temperature. This will avoid the formation of an amide as would be the case using ammonium hydroxide or AMA deprotection conditions.

Nucleosides, Nucleotides & Nucleic Acids, 20, 1007-1010, 2001.

133 **A new and efficient method for the synthesis of 5’-conjugates of oligonucleotides through amide-bond formation on solid phase**, *A.V. Kachalova, D.A. Stetsenko, E.A. Romanova, V.N. Tashlitsky, M.J. Gait and T.S. Oretskaya, Helvetica Chimica Acta, 85, 2409-2416, 2002.*



LK2155



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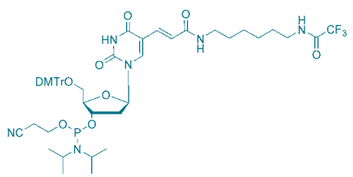

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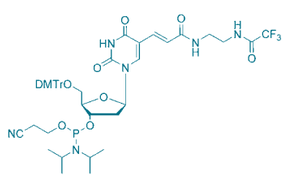

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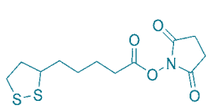

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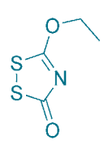
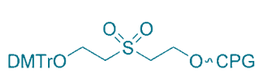


LK2171



LK2279

Subsequent conjugation of the 5’-carboxylic acid function to a range of primary and secondary aliphatic amines can be achieved through amide bond formation on the solid support. We have demonstrated the flexibility of this modifier through reactions with the diene furfurylamine, an aminocaproic ester spacer and ß-casomorphin-5-amide, the latter providing a simple and expedient synthesis of an oligonucleotide-peptide conjugate. Coupling yields up to 93% have been attained after cleavage from the solid support.

Internal carboxylate functions can be achieved using Carboxy-dT-CE Phosphoramidite (**LK2142**). The methyl ester is hydrolysed during deprotection and can be coupled directly to a molecule containing a primary amino group via a peptide coupling reaction.

Ordering carboxylate modifiers

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Aldehyde modification

The aldehyde function is often used to conjugate biopolymers to other molecules by processes such as reductive amination or adduct formation with hydroxylamines, hydrazines and semicarbazides. Aldehydes have also been used as a means of immobilising oligonucleotides onto solid surfaces.[134]

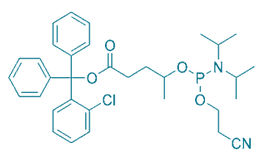
The use of this functionality has been hampered by the complexity of existing routes such as post synthetic periodate oxidation of a diol to produce the aldehyde, and the lack of conveniently available ready-made phosphoramidites or supports to incorporate an aldehyde functionality into an oligonucleotide.

Researchers at Kyoto University in Japan described the first facile incorporation of an aldehyde function into DNA without any protection/deprotection of the aldehyde by using 3-formylindole 2’-deoxynucleoside (**LK2056**).[135] This formylindole modifier can be placed either in the centre of or at the 5’-end of an oligonucleotide, but an extended coupling time of 15min for this modifier is recommended to provide a coupling efficiency of >95%.

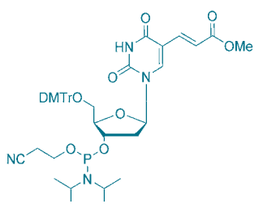
Since the sugar unit of the pseudo nucleoside is unmodified, multiple incorporations of dR- formylindole are possible. This not only provides multiple conjugation sites but formylindole is known to act as a universal base resulting in destabilisation of the duplex by 7-10 ºC per

134 **For a review of this area see: Use of carbonyl group addition-elimination reactions for synthesis of nucleic acid conjugates**, *T.S. Zatsepin, D.A. Stetsenko, M.J. Gait and T.S. Oretskaya, Bioconjugate Chemistry, 16, 471-489, 2005.*

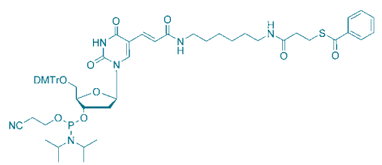
135 **A facile incorporation of the aldehyde function into DNA: 3-formylindole nucleoside as an aldehyde containing universal nucleoside**, *A. Okamoto, K.Tainaka and I. Saito, Tetrahedron Lett., 43, 4581-4583, 2002.*



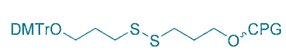
LK2057



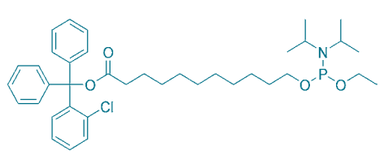
LK2142



LK2191



LK2361



LK2531

addition when compared with the natural duplex. This modification is stable to most cleavage and deprotection conditions.

Post-synthetic modification of oligonucleotides bearing this moiety, and still bound to the solid support, has also been achieved. In essence, the options for post-synthetic modification of the aldehyde functionalised oligonucleotide are limited only by the reactive nature of aldehydes and the conditions to which the conjugate is stable.

We have used the aldehyde function to conveniently attach molecules such as O-benzylhydroxylamine and diphenylhydrazine. The use of DMT ON oligonucleotides produced extremely hydrophobic material with these substituents, however, DMT OFF oligonucleotides reacted in a mixture of acetate buffer (pH 4.7) and DMSO (1:1) at 37 °C overnight to give conjugation yields in excess of 70% when modified in the centre and in excess of 80% when modified at the 5'-end of the oligonucleotide. Alternative examples of aldehyde conjugations are available in the literature.[136] Aldehyde functionalised oligos have also successfully been coupled with 6-hydrazine nicotinamide (HyNic) modified labels.[137]

Ordering aldehyde modifiers

For more details on aldehyde modifiers offered by Biosearch Technologies, see our website.

On-column oligonucleotide conjugations

Modified oligonucleotides where labels (e.g. reporter, carrier, biomolecule) have been conjugated to the oligonucleotide have a vast array of applications such as diagnostics, capture and therapeutics.[138]

While the preferred method of conjugation is via solid phase synthesis using phosphoramidite chemistry, there remain many examples where this method is not feasible, either because the label does not exist as a phosphoramidite or solid support, or that the label is not compatible with oligonucleotide synthesis and/or deprotection. In these cases, there are two choices:

1. The label is conjugated on solid phase after oligonucleotide synthesis but prior to cleavage and deprotection.

In this case, the active functional group in the oligonucleotide must be easily deprotected without cleaving the oligonucleotide from the resin and the label must be compatible with the required deprotection conditions.

or

2. The label is conjugated in solution phase after cleavage and deprotection.

In this case, the label must have some degree of solubility and must be stable in aqueous solution even if mixed with a co-solvent such as DMSO or DMF.

In either scenario, the oligonucleotide is most commonly functionalised with one reactive group and the label functionalised with a complementary reactive group. The most commonly used pairings are amines/NHS esters and thiols/maleimides, although many others are available e.g. alkynes/azides (click chemistry) and furan/maleimides (Diels-Alder chemistry).

Of these scenarios, solution phase conjugation is the most common, but on-column conjugations are particularly useful where the label and the conjugation product are difficult to separate or where the label is not soluble in aqueous phase - hence solution phase coupling is not feasible. On completion of the coupling reaction, excess label is washed from the column prior to cleavage and deprotection leaving only separation of the conjugate, unlabelled oligonucleotide and failure sequences from the reaction mixture. Typical labels include dyes such as ROX and TMR, lipophilic compounds that are not available as synthesis reagents, and amino acids or small peptides.

A number of Biosearch Technologies' phosphoramidites and solid supports allow on-column conjugations with many of the amino, thiol, carboxy and aldehyde modified oligonucleotides discussed previously in this section.

Amino modified oligonucleotides

The Fmoc group of 3'-Amino-C7 CPG (**LK2350**) is easily removed with 20% piperidine in MeCN with no cleavage of the oligonucleotide from the support.[139] The MMT group of 5'-MMT-Amino- Modifier C6-CE Phosphoramidite (**LK2123/BNS-5015**), 5'-MMT-Amino-Modifier C12-CE Phosphoramidite (**LK2133/BNS-5039**) and 5'-MMT-Amino-Modifier-11-CE Phosphoramidite (**LK2193**) is removed using an elongated detritylation step. In this case it is recommended that the resin is washed with 20% diethylamine in acetonitrile to ensure the free amine is not in the protonated form. Additionally, this removes the cyanoethyl groups preventing acrylamide formation during cleavage and deprotection.

A label, typically an active ester such as an NHS ester, can then be conjugated to the free amine. For small molecules such as fluorescent dyes, this is often carried out in DMF with up to six equivalents of the NHS ester. Short peptides and amino acid residues are generally added via a typical peptide coupling using a coupling agent (e.g. HATU or DCC) or a crosslinker (such as DSS) where the C-terminus of the peptide is coupled to the amino functionality of the oligonucleotide. Figure 7 depicts on-column labelling of an oligonucleotide modified with **LK2350**.

Thiol modified oligonucleotides

Thioctic Acid NHS Ester (**LK2166**) must be used in conjunction with one of the amino-modifiers mentioned previously, but thereafter has the ability to be used as a thiol reactive site. However, this is generally used as a means of conjugating oligonucleotides to silver or gold nanoparticles.[140]

Thiol-modifier C6 S-S CE Phosphoramidite (**LK2126/BNS-5042**), once incorporated into an oligonucleotide, introduces the possibility of reducing the disulphide bridge with e.g. TCEP in water or mercaptoethanol followed by conjugation to a maleimide or acetamide active label. See Figure 8.

Carboxylate modified oligonucleotides

In the case of 5'-Carboxylate Modifier-CE Phosphoramidite (**LK2057**), the oligonucleotide is synthesised 'DMT OFF' to remove the chlorotrityl group and conjugation of the label carried out prior to cleavage and deprotection. Typically, the coupling reaction is carried out using a peptide coupling reagent such as HATU to an amino functionalised label to form a stable amide linkage. This is indicated in Figure 9. Labels in this case are generally amino functionalised dyes such as the near infrared dye Cyanine 7 amine, or amino acids and small

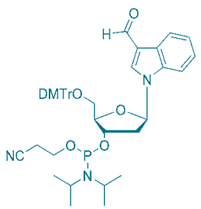
136 *(a)* **Synthesis of peptide-oligonucleotide conjugates with single and multiple peptides attached to 2'-aldehydes through thiazolidine**, *oxime, and hydrazine linkages, T.S. Zatsepin, D.A. Stetsenko, A.A. Arzumanov, E.A. Romanova, M.J. Gait and T.S. Oretskaya, Bioconjugate Chemistry, 13, 822-830, 2002; (b)* **Hydrazine oligonucleotides: new chemical modification for chip array attachment and conjugation**, *S. Raddatz, J. Mueller- Ibeler, J. Kluge, L. Wäß, G. Burdinski, J.R. Havens, T.J. Onofrey, D. Wang and M. Schweitzer, Nucleic Acids Research, 30, 4793-4802, 2002.*

137 **Biomolecule/polymer conjugates**, *D.A. Schwartz, US Patent No. 6,911,535 B2, 2005.*

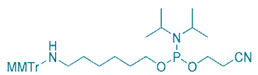
138 **Bioconjugate Techniques, 3rd Edition**, *G.T. Hermanson, 2013.*

139 *US Patent no. 5736626, 1998; Solid Support Reagents for the Direct Synthesis of 3'-Labelled Polynucleotides.*

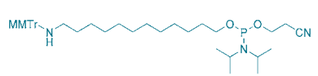
140 *(a)* **Enhanced oligonucleotide-nanoparticle conjugate stability using thioctic acid modified oligonucleotides**, *J.A. Dougan, C. Karlsson, W.E. Smith and D. Graham, Nucleic Acids Research, 35, 3668-3675, 2007;(b)* **Highly sensitive detection of dye-labelled DNA using nanostructured gold surfaces**, *R.J. Stokes, A. Macaskill,J.A. Dougan, P.G. Hargreaves, H.M. Stanford, W.E. Smith, K. Faulds and D. Graham, Chem. Commun., 2811-2813, 2007.*



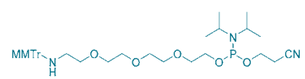
LK2056



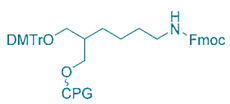
LK2123



LK2133



LK2193



LK2350

Modifiers and their use in oligonucleotide synthesis

peptides where coupling occurs between the N-terminus of the peptide and the 5'-end of the oligonucleotide.

Aldehyde modified oligonucleotides

Among other functional groups, aldehydes, such as that in Formylindole Modifier-CE Phosphoramidite (**LK2056**), will react with amines to form an imine (Schiff's base) which is generally followed by a borohydride reduction due to the instability of the imine bond.[141] These will also react with a hydrazine to form a hydrazone.[142] Solulink HyNic™ conjugation technology[143] is derived from this type of coupling. Although semi-carbizide couplings are commonly used to attach oligonucleotides to glass slides, it is feasible the reaction of an aldehyde with a semi-carbizide to form a semi-carbizone[144] can be applied to on-column coupling. These couplings are shown in Figure 10.

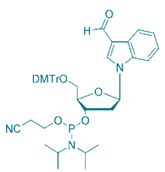
By combining both options, i.e. on-column and solution phase conjugations, it is possible to incorporate the same functional group with orthogonal protection into an oligonucleotide where each position can be labelled in turn. This is illustrated in Figure 11; an oligonucleotide modified at the 3'-end with **LK2350**, the 5'-end with **LK2193**, and internally with Amino-Modifier C6-dT-CE Phosphoramidite (**LK2135/BNS-5040**), allows stepwise conjugation in three positions. The oligonucleotide is synthesised 'DMT-ON' and the Fmoc group at the 3'-end is removed and labelled at this position followed by a capping step as per solid phase oligonucleotide synthesis. Detritylation to remove the MMT group from the 5'-end is then carried out which is in turn labelled followed by a capping step. The resin is then treated with 20% DEA in MeCN then cleaved, deprotected and - if necessary -

141 **Use of carbonyl group addition-elimination reactions for the synthesis of nucleic acid conjugates**, *T.S. Zatsepin, D.A. Stetsenko, M.J. Gait and T.S. Oretskaya, Bioconjugate Chemistry, 16, 471-489, 2005.*

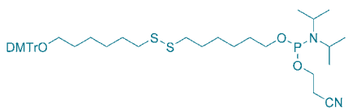
142 **Rapid oxime and hydrazone ligations with aromatic aldehydes for biomolecular labelling**, *A. Dirksen and P.E. Dawson, Bioconjugate Chemistry, 19, 2543-2548, 2008.*

143 **Technetium 99m human polyclonal IgG radiolabeled via the hydrazino nicotinamide**, *M.J. Abrams, M. Juweid, C.I. TenKate, D.A. Schwartz, M.M. Hauser, F.E. Gaul, J. Fuccello, R.H. Rubin, H.W. Strauss and A.J. Fischman, J. Nuclear Med., 31, 2022-2028, 1990.*

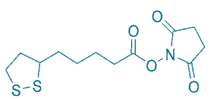
144 **Attachment of benzaldehyde-modified oligonucleotide probes to semi-carbazide coated glass**, *M.A. Podyminogin, E.A. Lukhtanov and M.W. Reed, Nucleic Acids Research, 29, 5090-5098, 2001.*



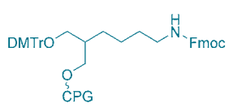
LK2057



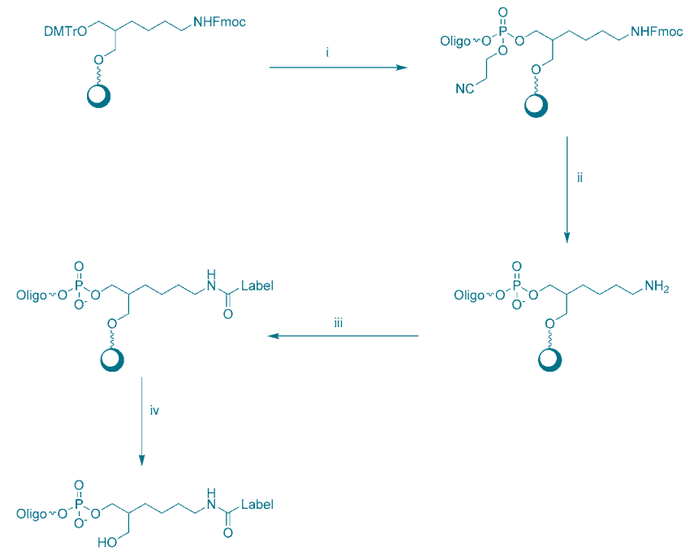
LK2126



LK2166

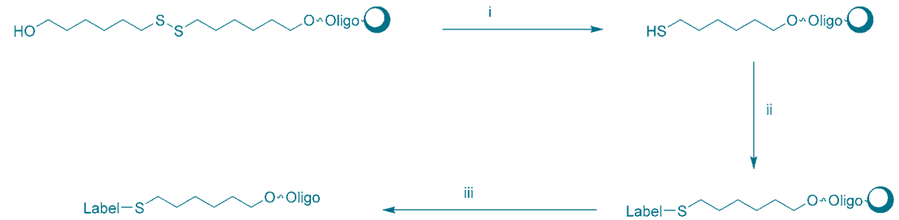


LK2350



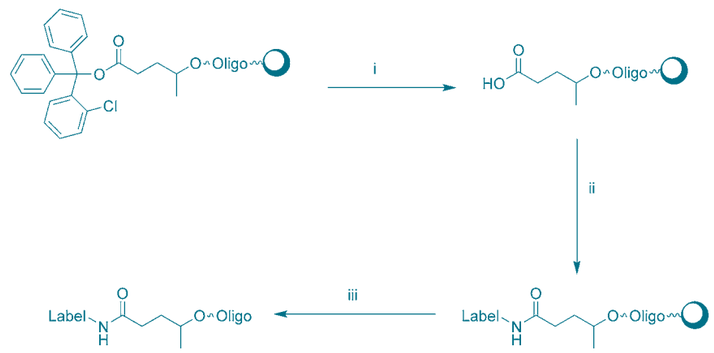
i. Oligo Synthesis, ii. 20% Piperidine/MeCN, iii. Label-NHS Ester, iv. Oligo Deprotection

Figure 7. On-column conjugation to an amino-modified oligonucleotide.



i. Disulphide Reduction, ii. Label Coupling, iii. Cleavage and Deprotection

Figure 8. On-column conjugation to a thiol-modified oligonucleotide.



i. Detritylation, ii. Label Coupling, iii. Cleavage and Deprotection

Figure 9. On-column conjugation to a carboxylate-modified oligonucleotide.

purified. The final conjugation step can now be carried out resulting in an oligonucleotide modified in three positions.

Alternatively, more than one functional group can be incorporated into the oligonucleotide, e.g. **LK2135**/**BNS-5040** can be replaced with Bz-S-C6-dT-CE Phosphoramidite (**LK2191**) or **LK2193** replaced with **LK2126**/**BNS-5042**. Here one of the conjugation reactions would become thiol/maleimide or thiol/acetamide.

While on-column post synthetic conjugations are not the most widely used method of labelling an oligonucleotide, this method opens up the ability to improve on solution phase couplings where the label is either unstable or has poor solubility in aqueous buffers. This also opens up the possibility of carrying out multiple post synthetic coupling reactions on the same oligonucleotide.

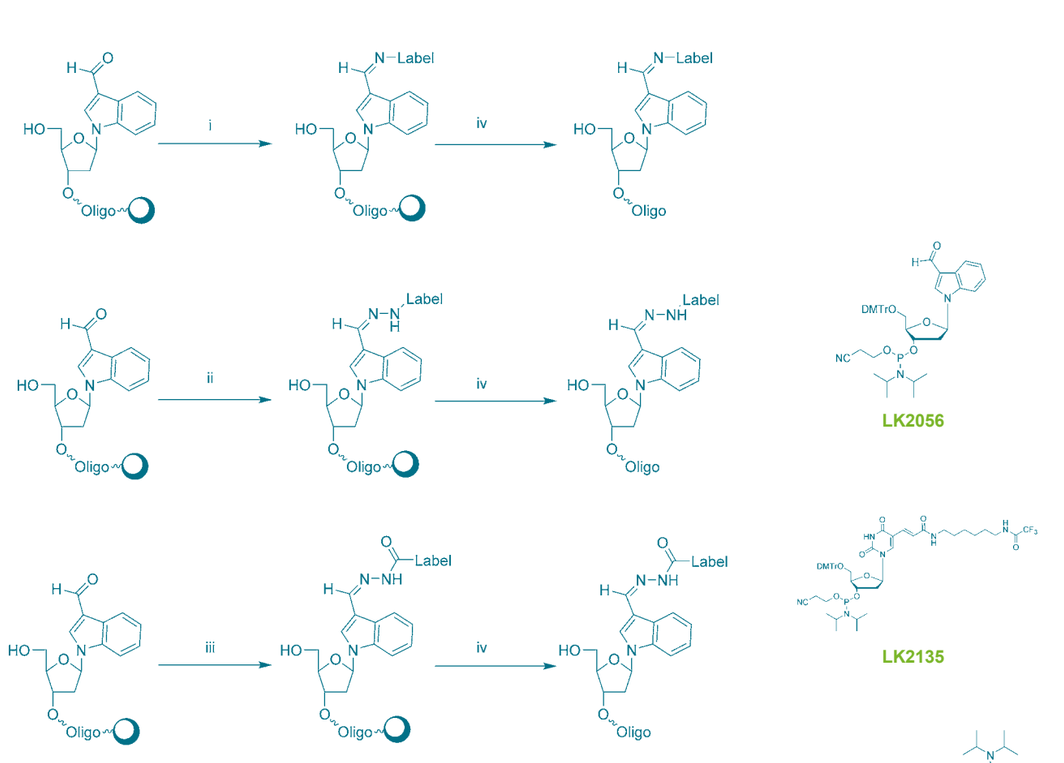
Click chemistry within oligonucleotide synthesis

Since its introduction in 2002, the click reaction has become a valuable tool spanning many fields of research from surface science to biomolecules. Originally introduced as a copper(I) catalyzed alkyne–azide cycloaddition (CuAAC), the click reaction is clean, efficient, and compatible with a wide range of solvents and functional groups. However, the required copper can degrade oligonucleotides[145] and can compromise cell function,[146] thereby limiting the utility of the Cu-catalyzed click reaction for these purposes. Since Bertozzi's report in 2004,[147] many cyclooctynes have been developed to capitalise upon the unique nature of a ring-bound alkyne for strain-promoted alkyne-azide cycloaddition (SPAAC). Biosearch Technologies provides bicyclo[6.1.0]nonyne

145 **Click chemistry as a reliable method for the high-density postsynthetic functionalization of alkyne-modified DNA,Gierlich,** *J.; Burley, G. A.; Gramlich, P. M. E.; Hammond, D. M.; Carell, T. Org. Lett. 2006, 8, 3639-3642.*

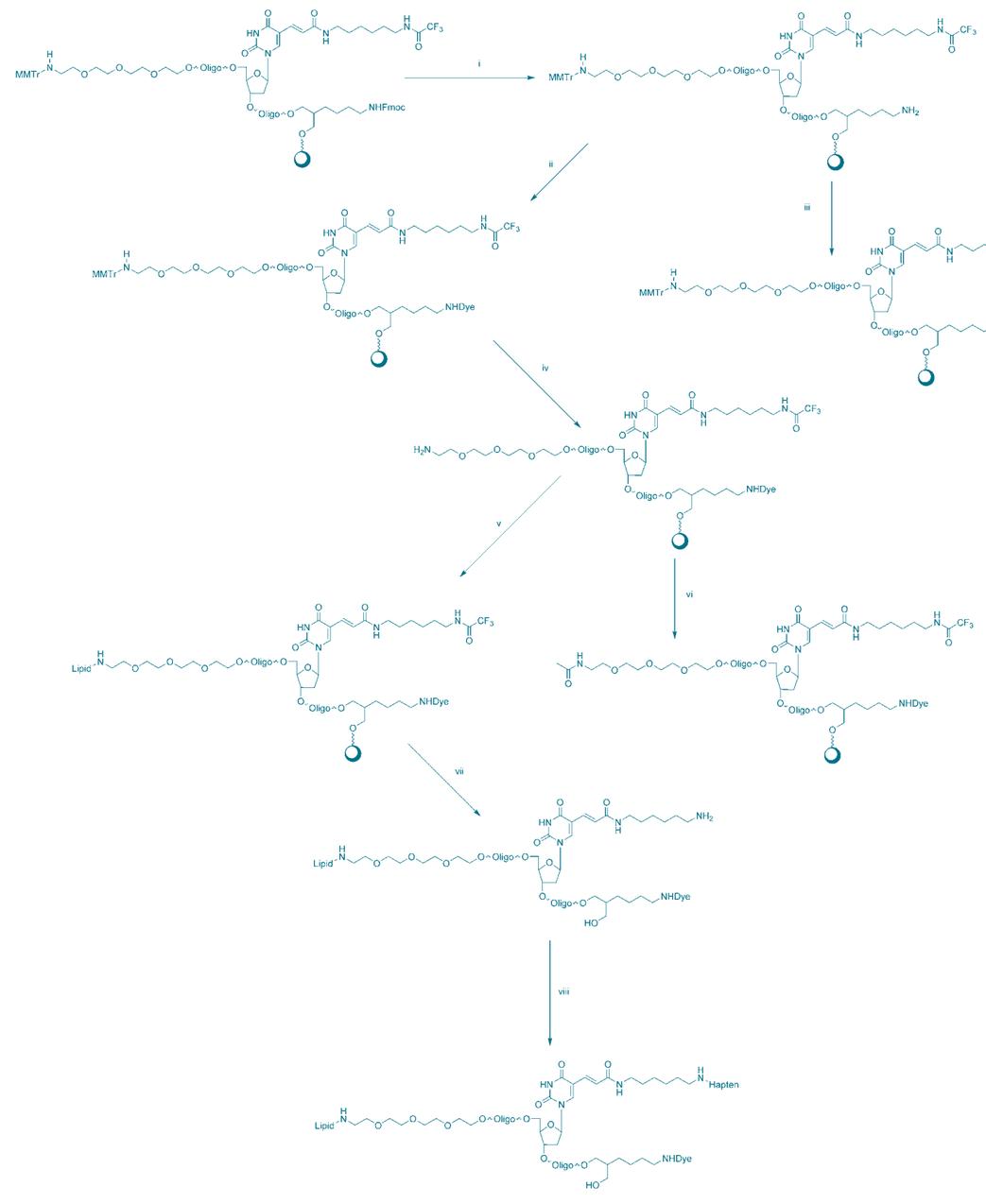
146 **Presentation and detection of azide functionality in bacterial cell surface proteins**, *Link, A.J.; Vink, M.K.S.; Tirrell, D.A. J. Am. Chem. Soc. 2004, 126, 10598-10602.*

147 **A strain-promoted [3 + 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems**, *Agard, N.J.; Prescher, J.A.; Bertozzi, C.R. J. Am. Chem. Soc. 2004, 126, 15046-15047.*



i. Label-NH_2, ii. Label-$NHNH_2$, iii. Label-$NHCONHNH_2$, iv. Cleavage and Deprotection

Figure 10. On-column conjugation to an aldehyde-modified oligonucleotide.



i. 20% Piperidine/MeCN, ii. Dye Coupling, iii. Capping, iv. 3% TCA/DCM, v. Lipid Coupling, vi. Capping, vii. Cleavage and Deprotection, viii. Solution-Phase Coupling of Hapten

Figure 11. Conjugation at three amino-functionalised positions within an oligonucleotide.

(BCN) alkyne scaffold for catalyst free clicking (Figure 12).[148]

This substituted cyclooctyne balances between reactivity and lipophilicity of all the substituted cyclooctynes reported to date.[149] Since a Cu(I) catalyst is not required for click reactions with azides, BCN modification provides a quick and easy solution to many oligo conjugation reactions.

The Click-easy™ BCNs (**BA0373**, **LKD4320**, and **LKD4330**) can be used to efficiently prepare oligonucleotides and other biomolecules labelled with the BCN motif. 5'-BCN-oligonucleotides react cleanly with a variety of azide reagents. Even PQQ-TEG azide (**FC8170**), which contains a highly reactive quinone functionality is smoothly ligated to a BCN-oligo.

In one illustrative example, 5'-Click-easy BCN CEP II (**BA0373**)[150] is added at the 5'-terminus of an oligonucleotide with >99% efficiency. Following a diethylamine wash, standard cleavage, and salt switch, a triethylamine salt of the T6 oligo was taken up in a buffer/acetonitrile solution and treated with PQQ-TEG azide (Figure 13).

The reactivity of the quinone functionality in PQQ presents a challenging barrier to successful oligonucleotide conjugation via Cu-catalyzed click and Staudinger ligation strategies. The clean SPAAC conjugation of PQQ-TEG azide to the BCN-oligo is attainable using approximately a three-fold molar excess of the azide.

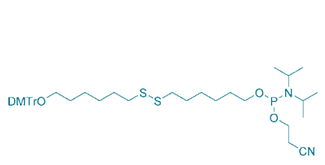
Although the SPAAC ligation reaction shown in Figure 13 requires >26 hours, it is possible to increase the reaction rate by increasing the concentration of azide, i.e, when the same BCN-oligo was treated with Biotin-TEG azide (**BT1085**) in approximately 100-fold molar excess in the same reaction volume, the reaction was nearly complete in just 30 minutes (Figure 14).

Biosearch Technologies' Click-easy lineup for catalyst-free ligation are the MFCO.[151] We offer the analogous Click-easy MFCO-N-hydroxysuccinimide ester (**LKD4300**) for post synthetic incorporation.

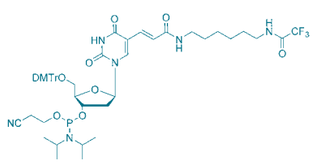
Ordering BCN alkynes for Copper-Free Clicking

Biosearch Technologies' current offering of Click Reagents is available for view on our website for our full selection on offer.

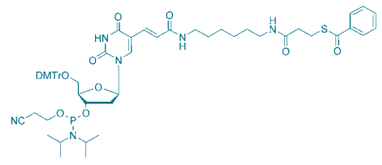
148 *For more information regarding SynAffix, see* **www.synaffix.com**.
149 **Bioconjugation with strained alkenes and alkynes**, *Debets, M.F.; van Berkel, S.S.; Dommerholt, J.; Dirks, A.J.; Rutjes, F.P.J.T.; van Delft, F.L. Accounts of Chem. Res. 2011, 44, 805-815.*
150 *Patent pending.*
151 *a)* **Synthesis of a DOTA–Biotin Conjugate for Radionuclide Chelation via Cu-Free Click Chemistry**, Schultz, M.K.; Parameswarappa, S.G.; Pigge, F. C. Organic Lett. 2010, 12, 2398- 2401. b) **A DOTA-peptide conjugate by copper-free click chemistry**, *Martin, M.E.; Parameswarappa, S.M.; O'Dorisio, M.S.; Pigge, F.C.; Schultz, M.K. Bioorg. & Med. Chem. Lett. 2010, 20, 4805-4807.*



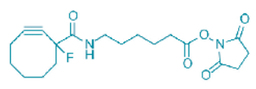
LK2126



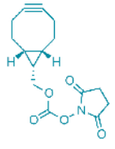
LK2135



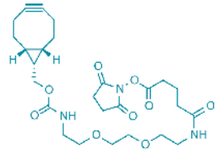
LK2191



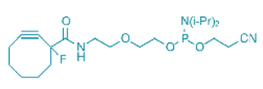
LK4300



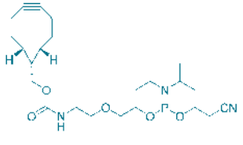
LK4320



LK4330



BA0368



BA0373

Double and triple clicking protected alkynes

Although the click reaction with standard alkynes and cyclooctynes is straightforward and efficient, it does not lend itself well to the sequential labelled of oligonucleotides. Fortunately, the baseclick platform provides tools for introduction of up to three different labels using variably protected diynes.[152] To affect the triple click, the three orthogonally protected diyne substituted bases could be incorporated into an oligonucleotide, followed by on column click reaction of the free alkyne, deprotection of the TMS alkyne during cleavage of the oligonucleotide from the solid support with ammonia, a second click, TBAF deprotection of the TIPS and then the final click.

Biosearch Technologies offers the two alkynes in the dU family: 5-Octadiynyl-dU CEP (**BA0308**) and 5-Octadiynyl-TMS-dU CEP (**BA0364**). For oligonucleotides requiring dC, the unprotected (**BA0366**, 5-Octadiynyl-dC CEP) alkyne is also available.

Ordering protected alkynes

See our website for all Click Chemistry reagents from Biosearch Technologies' NAC product portfolio.

Click-Mate™ azides

Due to the efficiency and simplicity of the click reaction, biologically significant azides are in high demand. For those who prefer the reliability of cholesterol for its lipophilicity and ability to improve efficiency of delivery of oligonucleotides to targeted cells, we now offer Cholesteryl-TEG azide (**FC8180**).

Since cholesteryl labelling does have some limitations, folate TEG azide (**FC8150**), a vitamin E analogue Tocopherol-TEG azide (**FC8160**) and the cofactor PQQ-TEG azide (**FC8170**) for use as a colourimetric probe for biomolecules,[153]are available from Biosearch Technologies. For the convenient ligation of 6-tetrachloro fluorescein either via click chemistry or Staudinger ligation, 6-TET-TEG azide (**FF6130**) is used. Tetrachloro fluorescein, Psoralen TEG azide (**PS5030**), has been widely used for labelling a variety of biomolecules and has the advantage of being fluorescent at physiological pH.

Ordering Click-Mates Azides

Biosearch Technologies offers a wide range of Click Chemistry reagents. See our website for full product offering.

152 *Click-click-click: single to triple modification of DNA, Gramlich, P.M.E.; Warncke, S.; Gierlich, J.; Carell. T. Angew. Chem. Int. Ed. 2008, 47, 3442 –3444.*
153 Pyrroloquinoline Quinone-Doped Polymeric Nanospheres as Sensitive Tracer for Binding Assays, Shen, D.; Meyerhoff, M. E. Anal. Chem. 2009, 81, 1564-1569.

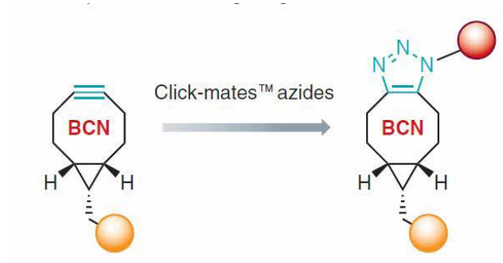


Figure 12. BCNs for copper-free clicking

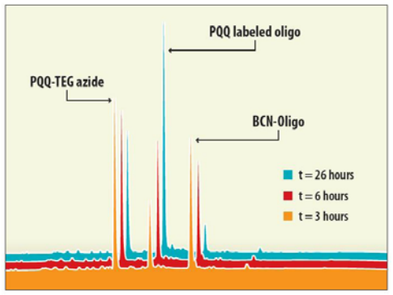


Figure 13. BCN-Oligo with PQQ-TEG azide (FC 8170)

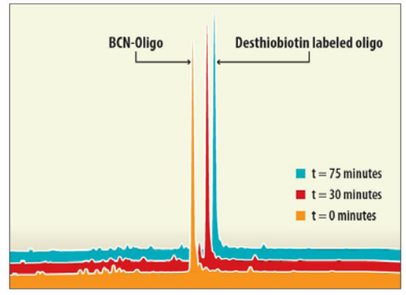


Figure 14. BCN-Oligo with excess Desthiobiotin-TEG azide (BT 1075).

Backbone modification

PNA

Introduction

Peptide Nucleic Acid (PNA) was originally conceived as a ligand for the recognition of double- stranded DNA.[154] The concept was to mimic an oligonucleotide binding to double stranded DNA via Hoogsteen base pairing. However, it is the favourable properties of PNA when mimicking and/ or binding to single strands of DNA that have seen PNA gather interest in many areas of modern chemical biology.

Structure and properties of PNA

The structure of PNA is quite simple (see Figure 15), consisting of repeating N-(2-aminoethyl)- glycine units linked by amide bonds. The purine (A, G) and pyrimidine (C, T) bases are attached to the backbone by methylene carbonyl linkages. Unlike DNA or its analogues, PNAs do not contain any sugar moieties or phosphate groups. Again, unlike DNA, the backbone is acyclic, achiral and neutral.

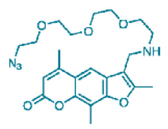
It is tempting to regard PNA as a DNA analogue, however its chemical structure shows that it is in fact more similar to a protein or peptide molecule. Nevertheless, for applications using PNA the basis of analysis is using sequence information just like with DNA etc. By convention, PNAs are represented like peptides, with the N-terminus (or pseudo 5') at the left hand side position and the C-terminus (pseudo 3') at the right.

PNA oligomers are less soluble in water than DNA, and in some aqueous buffers (especially phosphate) poor solubility can be an issue. This is particularly true with increasing length (>12 units) and purine content (especially G above 60%). Often the inclusion of one or two lysine residues can alleviate this problem, as can use of the AEEA spacer (**LK5005**).

The neutrality of the PNA backbone is a significant feature that has several consequences. One of the most important is the stronger binding between complementary PNA/DNA strands than between DNA/DNA strands at low to medium ionic strength. This can be attributed to the lack of charge repulsion between PNA and DNA. This is also thought to be the reason that the sequence specificity of PNA to DNA is also higher than in native DNA/DNA strands.[155]

154 (a) **Sequence selective recognition of DNA by strand displacement with a thymine-substituted polyamide**, *P.E. Nielsen, M. Egholm, R.H. Berg and O. Buchardt, Science, 254, 1497-1500, 1991; (b)* **Peptide nucleic acids (PNA). Oligonucleotide analogues with an achiral peptide backbone**, *M. Egholm, O. Buchardt, P.E. Nielsen and R.H. Berg, J. Amer. Chem. Soc., 114, 1895-1897, 1992; (c)* **Peptide nucleic acids (PNA). DNA analogues with a polyamide backbone, P.E. Nielsen**, *M. Egholm, R.H. Berg and O. Buchardt, In "Antisense Research and Application", S. Crook and B. Lebleu (eds.), CRC Press, Boca Raton, pp. 363-373.*

155 **PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen bonding rules**, *M. Egholm, O. Buchardt, L. Christensen, C. Behrens, S.M. Freier, D.A. Driver, R.H. Berg, S.K. Kim, B. NordJn and P.E. Nielsen, Nature, 365, 556-568, 1993.*



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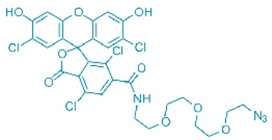

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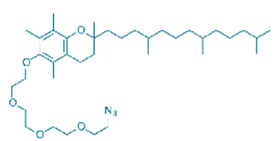

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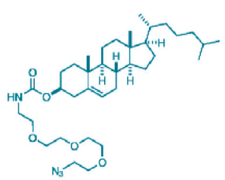

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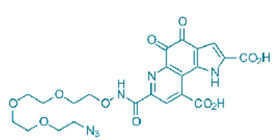


FC8170

In general, homopyrimidine PNAs form extremely stable triplexes that have sufficient stability to invade intact double stranded DNA. Studies have also shown that 2PNA/DNA triplex formation follows the rules of homopyrimidine DNA triplex formation, i.e. with an antiparallel Watson-Crick duplex and a parallel bound Hoogsteen strand. Even more stable triplexes can be formed when the Watson-Crick PNA strand is connected by continuous synthesis via ethylene glycol type linkers (e.g. AEAA, **LK5005**) to the Hoogsteen strand. Such constructs are called bis-PNAs.[156]

Applications of PNA

PNA may be used in many of the same applications as synthetic DNA, but with the additional benefits gained from tighter binding and greater specificity. It has therefore become a versatile tool in genetic diagnostics and a variety of molecular biology techniques; particularly *in situ* hybridisation and PCR clamping, but also nucleic acid capture, plasmid vector tagging, duplex DNA targeting, and solution-phase hybridisation detection.[157] bis-PNAs, particularly, provide a tool for selectively targeting any short homopurine sequence in intact double stranded DNA with very high specificity and efficacy.

The ability to bind to both DNA and RNA is a key feature of PNA, as compared to other analogues that favour RNA. In a typical *in situ* hybridisation probing application of mRNA, PNA probes offer faster hybridisation, higher signal, and better specificity. In this application, a set of longer DNA probes with multiple labels can be substituted by a single PNA 15mer with one label.[158] Using one shorter probe improves sequence discrimination, and the PNA has added advantages of increasing overall specificity of the assay, lower background signal and long-term stability of the probes.

The lack of a sugar-phosphate backbone makes PNA resistant to nucleases and polymerases. As a result, unmodified PNAs cannot be used as primers in PCR (or other amplification techniques). However, the improved hybridisation properties of PNA are utilised in PCR clamping assays. This technique involves PNA blocking extension of a DNA primer by competing for binding at, or around, the primer site. It has been shown that the superior specificity of the competing PNA results in an

156 **Single and bis peptide nucleic acids as triplexing agents: binding and stoichiometry**, *M.C. Griffith, L.M. Risen, M.J. Greig, E.A. Lesnik, K.G. Sprangle, R.H. Griffey, J.S. Kiely and S.M. Freier, J. Amer. Chem. Soc., 117, 831-832, 1995.*

157 **For a review see: Peptide nucleic acid: a versatile tool in genetic diagnostics and molecular biology**, *P.E. Nielsen, Curr. Opin. Biotechnol., 12, 16-20, 2001 and references therein.*

158 **Cellular uptake and intracellular fate of antisense oligonucleotides**, *A.R. Thierry, E. Vives, J.P. Richard, P. Prevot, C. Martinand-Mari, I. Robbins and B. Lebleu, Curr. Opin. Mol. Ther., 1, 226-243, 1999.*

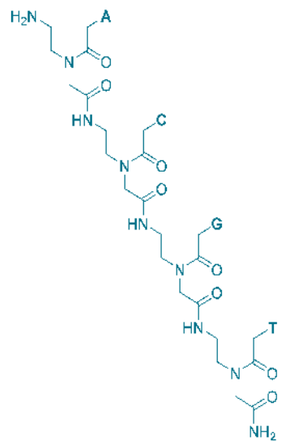


Figure 15. General structure of PNA, where A, C, G and T are the standard purine and pyrimidine nucleobases.

assay that allows for discrimination of single base pair differences.[159] This technique has recently been extended to using PNA as both a PCR clamp and probe.[160]

In a similar way PNA-DNA chimeras have been used to enhance DNA amplification.[161] The PNA part binds to the target with greater specificity and the DNA part is amplified.

More recently attention has also turned to the chemical modification of PNA to improve cellular uptake and binding to double-stranded DNA and RNA.[162]

PNA vs LNA

Locked Nucleic Acid (LNA)[163], like PNA, is a DNA analogue of much interest. Structurally these analogues are very different, however their application is in many respects very similar. Each technology has its own advantages and choice between them principally depends upon the experimental conditions and specifics of the application.[164]

In diagnostics, LNA has found particular use in single nucleotide polymorphism (SNP) assay analysis, owing to its excellent thermal stability and mismatch discrimination.[165] LNA-DNA chimera also exhibit RNase H activity (PNA does not) and this can be exploited in therapeutic applications.[166] A significant advantage of PNA is its neutral backbone. This greatly assists in cell delivery when combined with cell-penetrating peptides in antisense therapeutics.[167]

The synthesis of LNA homo-oligomers is less common than with PNAs. DNA (or RNA) is usually "modified" with LNA by incorporation of LNA units into a DNA oligomer to form a chimera. This is easily done as LNAs are synthesised using conventional phosphoramidite chemistry. PNA synthesis, on the other hand, more closely resembles peptide chemistry but it is possible to synthesise PNA-DNA chimera using modified PNA monomers.[131,168]

159 *(a)* **Single base pair mutation analysis by PNA directed PCR clamping**, *H. Ørum, P.E. Nielsen, M. Egholm, R.H. Berg, O. Buchardt and C. Stanley, Nucleic Acid Research, 21, 5332-5336, 1993; (b)* **Simple and sensitive detection of mutations in ras proto-oncogenes using PNA-mediated PCR clamping**, *C. Thiede, E. Bayerdörffer, R. Blasczyk, B. Wittig and A. Neubauer, Nucleic Acids Research, 24, 983-984, 1996; (c)* **Facilitated detection of oncogene mutations from exfoliated tissue material by a PNA-mediated 'enriched PCR' protocol**, *M. Behn, C. Thiede, A. Neubauer, W. Pankow and M. Schuermann, J. Pathol., 190, 69-75, 2000; (d)* **Peptide nucleic acid-mediated PCR clamping as a useful supplement in the determination of microbial diversity**, *F. Von Wintzingerode, O. Landt, A. Ehrlich and U.B. Göbel, Appl. Environ. Microbiol., 66, 549-557, 2000; (e)* **The age-related accumulation of a mitochondrial DNA control region mutation in muscle, but not brain, detected by a sensitive PNA-directed PCR clamping based method**, *D.G. Murdock, N.C. Christacos and D.C. Wallace, Nucleic Acids Research, 28, 4350- 4355, 2000.*

160 **Single-tube reaction using peptide nucleic acid as both PCR clamp and sensor probe for the detection of rare mutations**, *C.-C. Chiou, J.-D. Luo and T.-L. Chen, Nature Protocols, 1, 2604-2612, 2006.*

161 **PNA-DNA oligomers and methods of use thereof**, *L.T. Bortolin, C.M. Rudzinski and A.L. Stephens, US Patent No. 2008/0131880 A1.*

162 **Recent advances in chemical modification of peptide nucleic acids**, *E. Rozners, J. Nucleic Acids, 2012, Article ID 518162, 8pp, 2012.*

163 *LNA is locked by means of a methylene bridge that connects the 2'-oxygen atom to the 4' carbon atom. This bridge 'locks' the structure conferring a RNA-like C3'-endo conformation to the sugar part of the molecule. LNA products are available exclusively from Exiqon A/S. Also see:* **LNA (Locked Nucleic Acids): Synthesis of the adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil bicyclonucleoside monomers, oligomerisation, and unprecedented nucleic acid recognition**, *A.A. Koshkin, S.K. Singh, P. Nielsen, V.K. Rajwanshi, R. Kumar, M. Meldgaard, C.E. Olsen, and J. Wengel, Tetrahedron, 54, 3607-3630, 1998.*

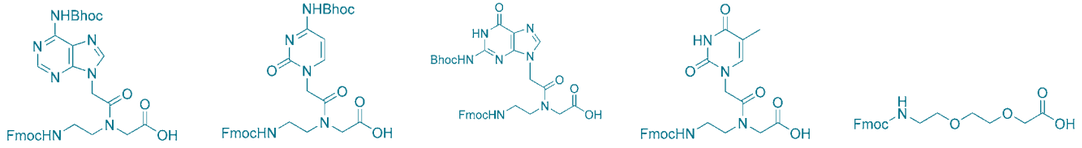
164 *For a comparative review of the two technologies see:* **Promising nucleic acid analogs and mimics: characteristic features and applications of PNA, LNA, and morpholino**, *S. Karkare and D. Bhatnagar, Appl. Microbiol. Biotechnol., 71, 575-586, 2006.*

165 **Detection of the Factor V Leiden mutation by direct allele-specific hybridization of PCR amplicons to photo immobilized locked nucleic acids**, *H. Ørum, M.H. Jakobsev, T. Koch, J. Vuust and M.B. Borre, Clin. Chem., 45, 1898-1905, 1999.*

166 **Design of antisense oligonucleotides stabilized by locked nucleic acids**, *J. Kurreck, E. Wyszko, C. Gillenand and V.A. Erdmann, Nucleic Acids Research, 30, 1911-1918, 2002.*

167 **Effects in live cells of a c-myc anti-gene PNA linked to a nuclear localization signal**, *G. Cutrona, E.M. Carpaneto, M. Ulivi, S. Roncella, O. Landt, M. Ferrarini and L.C. Boffa, Nature Biotechnol., 18, 300-303, 2000.*

168 *(a)* **Novel synthetic routes to PNA monomers and PNA-DNA linker molecules**, *G. Breipohl, D.W. Will, A. Peyman and E. Uhlmann, Tetrahedron, 53, 14671-14686, 1997; (b)* **New synthesis of PNA–3' DNA linker monomers, useful building blocks to obtain PNA/DNA chimeras**, *D. Musumeci, G.N. Roviello, M. Valente, R. Sapio, C. Pedone and E.M. Bucci, Peptide Science, 76, 535–542, 2004.*



PNA synthesis by Fmoc-chemistry

Although PNA was first synthesised using tBoc/Z chemistry[169], the milder chemistry of the Fmoc/Bhoc protection allows the synthesis of PNA with e.g. sensitive reporter groups. The simplified final cleavage and deprotection can also be achieved in minutes, provided a suitable resin is used.

After extensive screening, the benzhydryloxycarbonyl (Bhoc) group was selected as the best choice for protecting the exocyclic amino groups of the nucleobases. This group provides sufficient protection during synthesis, is readily removed under the cleavage conditions, and renders solubility to the monomers. For PNA synthesis, therefore, we provide the four Fmoc/ Bhoc monomers (items **LK5001** - **LK5004**) and a hydrophilic spacer molecule, AEEA (**LK5005**).

The latter is used in bis PNA and can be added to PNA to aid solubility. It is also useful to add to the N-terminus (pseudo 5') when labelling PNA with e.g. biotin, ROX, TAMRA etc.

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Locked nucleic acid (LNA™) oligonucleotides

In the cell, double-stranded DNA exists as a B-form helix, while double-stranded RNA adopts an A-form helical structure. This arises from the differences in the preferred conformations of the sugar ring of deoxyribose and ribose. In DNA, the furanose ring of deoxyribose predominately exists in the C2'-endo conformation resulting in the B-form helix. In RNA, the furanose ring exists in C3'-endo confirmation due to the presence of the 2'-OH resulting in the A-form helix.

In 1998, laboratories in Japan and Denmark first described the synthesis and properties of a novel series of nucleic acid analogues called Locked Nucleic Acids (LNA)[170], which have subsequently been developed by Exiqon A/S, Denmark. These are locked in the C3'-endo conformation by means of a methylene bridge that connects the 2' oxygen atom to the 4' carbon atom (see Figure 16). This bridging restricts the conformational flexibility and results in the pre-organisation of the sugar in an RNA-like form.

Physical studies of LNA-containing oligonucleotides hybridised to DNA or RNA revealed some remarkable properties. The melting temperatures (T_m) were dramatically increased and, most importantly, not at the expense of mismatch discrimination. The specificity of LNA for its perfectly matched complement is significantly higher. An increase in T_m of as much as 41 °C for a full-LNA:DNA duplex relative to the corresponding DNA:DNA duplex has been reported. In general, an increase in T_m of about 3-8 °C per LNA modification is observed. The structures of LNA containing oligonucleotides hybridised to both DNA and RNA have been determined in solution by NMR. These show that the LNA residues induce a conformational change in the surrounding DNA causing it to adopt a similar C3'-endo conformation. This leads to an increased local organisation of the phosphate backbone, enhancing the strength of base stacking interactions, leading to increased duplex stability observed as increased T_m values.

169 **Synthesis of peptide nucleic acid monomers containing the four natural nucleobases: thymine, cytosine, adenine and guanine, and their oligomerization**, *K.L. Dueholm, M. Egholm, C. Behrens, L. Christensen, H.F. Hansen, T. Vulpius, K. Petersen, R.H. Berg, P.E. Nielsen and O. Buchardt, J. Org. Chem., 59, 5767-5773, 1994.*

170 *(a)* **Stability and structural features of the duplexes containing nucleoside analogues with a fixed N-type conformation, 2 '-O,4 '-C-methyleneribonucleosides, S. Obika, D. Nanbu, Y. Hari, J. Andoh, K. Morio, T. Doi, and T. Imanishi, Tetrahedron Lett., 39, 5401-5404, 1998. (b) LNA (Locked Nucleic Acids): Synthesis of the adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil bicyclonucleoside monomers, oligomerisation, and unprecedented nucleic acid recognition, A.A. Koshkin, S.K. Singh, P. Nielsen, V.K. Rajwanshi, R. Kumar, M. Meldgaard, C.E. Olsen, and J. Wengel, Tetrahedron, 54, 3607-3630, 1998.**

The binding of LNA to double-stranded DNA under physiological conditions has also been observed. This can occur by triplex formation or by strand invasion mechanisms and raises the prospect of being able to use LNA to recognise double-stranded DNA within living cells.

Full-LNA is nuclease resistant, this can also be achieved in chimeras by the incorporation of phosphorothioate linkages in the DNA sections of the molecule.

The commercial availability of LNA monomers (products **LK2061-63** and **LK2065**, plus a variety of 3'-LNA CPGs) now enables individual researchers to synthesise LNA containing oligonucleotides.

Unlike some other modifications, such as morpholino oligonucleotides or PNA, oligonucleotides can be made routinely by automated synthesis by phosphoramidite chemistry with no additional reagents with only minor modifications to synthesis cycles. Longer coupling time compared to DNA additions are required in addition to a prolonged oxidation step.

The use of standard nucleobase protection and the stability of LNA to base results in compatibility to the most common cleavage and deprotection strategies. Although there is an increased hydrophobicity, LNA has similar solubility and handling properties established purification methods can be utilised. This simplicity of preparation combined with its outstanding hybridisation properties makes LNA an extremely attractive choice in DNA modification (see Table 3).

In addition to offering the LNA phosphoramidites, Biosearch Technologies also manufacturers a vast range of LNA CPGs in different pore sizes, in bulk and in columns. See our website for the full range, or contact us for custom options.

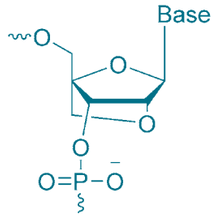

Figure 16. General Structure of ß-D-LNA.

Applications of LNA

The favorable ΔT_m of LNA:DNA or LNA/DNA:DNA duplexes of a perfect match versus a mismatched makes LNA a very powerful tool in diagnostic applications.[171]

In a later study,[172] LNA probes were compared in a 5'-nuclease PCR assay with minor groove binder (MGB) probes. In this real-time PCR assay, a fluorogenic probe hybridises with single-stranded DNA target within the region bound by the PCR primers. The fluorophore is quenched because of the close proximity to quencher. During PCR, the 5'-nuclease activity of the polymerase removes any bound nucleic acid on the target strand one base at a time cleaning the target for amplification. This includes the probe releasing the fluorophore resulting in a fluorescent signal which is directly related to the quantity of specific PCR product. In the study, the MGB probes hybridise to single-stranded target with increased specificity compared to unmodified probes enabling the use of shorter oligonucleotides. The authors showed the same effect is achieved by LNA containing probes and observed specificity and sensitivity of both approaches were equivalent.

Several groups have reported on the use of LNA probes for SNP genotyping. Methods have been developed using microtitre plates with covalently attached LNA probes to capture PCR products and detect hybridisation events using an ELISA format. In homogeneous assays, it has been shown that a 3' LNA residue improves allelic discrimination in allele-specific PCR.[173] This assay proved to be robust and to function under a wide variety of PCR conditions. Due to the high T_m values, and excellent mismatch discrimination of even short LNA probes, it has been proposed that a complete set of genotyping probes could be generated.[174]

The use of oligonucleotides as therapeutic drugs has proved successful in the last few years with 13 drugs being approved since 2016. One such class of therapeutic are antisense oligonucleotides, (see page 28 for more details). The proposed mechanism of action of these molecules generally involve RNase H, an enzyme that cleaves the RNA strand of an RNA:DNA heteroduplex. The DNA oligonucleotides bind to mRNA and cause it to degrade, after which the oligonucleotide is free to bind to further mRNA resulting in a catalytic inhibition of gene expression. Oligonucleotides that contain only LNA have been shown to activate RNase H less well than unmodified DNA. This disadvantage is overcome by using a gapmer strategy, constructing oligonucleotides with LNA residues at each end and a stretch of DNA residues in the middle. Such constructs efficiently support RNase H cleavage, have increased T_m and increased resistance to nuclease degradation. Their properties are reported[175] to be superior in many respects to those of many other DNA analogue constructs. Since full-LNA oligonucleotides bind so tightly with RNA, these are feasible antisense agents by a non-RNase mechanism, either by targeting the 5'-untranslated region to prevent translation or at other sites where they may cause premature termination.

A similar hybrid LNA-DNA-LNA approach has been taken in antigene studies [176] for the construction of decoy oligonucleotides. These are double-stranded oligonucleotides that contain the consensus binding sequence for a specific transcription factor. Once in the cell these constructs bind the target factor resulting in the

171 **Design of LNA probes that improve mismatch discrimination**, *Y. You, B.G. Moreira, M.A. Behlke and R. Owczarzy, Nucleic Acids Res., 34, e60, 2006.*
172 **Evaluation of the performance of LNA and MGB probes in 5 '-nuclease PCR assays**, *C. Letertre, S. Perelle, F. Dilasser, K. Arar, and P. Fach, Molecular Cellular Probes, 17, 307-311, 2003.*
173 **Enhanced allele-specific PCR discrimination in SNP genotyping using 3 ' locked nucleic acid (LNA) primers**, *D. Latorra, K. Campbell, A. Wolter, and J.M. Hurley, Human Mutation, 22, 79-85, 2003.*
174 **Single nucleotide polymorphism genotyping using short, fluorescently labeled locked nucleic acid (LNA) probes and fluorescence polarization detection**, *A. Simeonov, T.T. Nikiforov, Nucleic Acids Research, 30, art. no. e91, 2002.*
175 **Design of antisense oligonucleotides stabilized by locked nucleic acids**, *J. Kurreck, E. Wyszko, C. Gillen, and V.A. Erdmann, Nucleic Acids Research, 30, 1911-1918, 2002.*
176 **Design and characterization of decoy oligonucleotides containing locked nucleic acids**, *R. Crinelli, M. Bianchi, L. Gentilini, and M. Magnani, Nucleic Acids Research, 30, 2435-2443, 2002.*

Table 3. Properties of Modified Oligonucleotides.

Chemistry	Increased affinity	RNase H activity	Nuclease resistance
LNA	Yes	Yes (as chimera)	Yes
DNA	No	Yes	No
RNA	No	No	No
Phosphorothioate DNA	No	Yes	Yes
PNA	Yes	No	Yes
Morpholino	Yes	No	Yes

reduction or even blockade of transcriptional activation. As indicated above, LNA can also be useful to form stabilised triple helical structures.[177] Along with other advances in this area, such as the development of new bases that recognise pyrimidine: purine inversion sites, LNA raises the possibility of being able to modify and control the cellular function of genomic DNA. LNA oligonucleotides have been bound to supercoiled double- stranded plasmid DNA and shown to remain associated with plasmid after transfection.[178] In this case it was demonstrated that the main mechanism was strand displacement.

Specific cleavage of RNA may be catalysed by short oligonucleotides termed DNAzymes. Taking advantage of the improved affinity of LNA for its complementary sequence, LNAzymes have been developed that have significantly improved cleavage kinetics.[179]

Recently, the study and application of LNA has widened considerably. For example, LNA-Molecular Beacons[180] have been developed with improved properties. RNA experiments, in particular, have become more common; LNA has been utilised in the thermodynamic study of 2'-OMe RNA/RNA heteroduplexes, [181] *in situ* detection of miRNAs in animal embryos,[182] and quantification of miRNA gene expression.[183] The therapeutic potential of LNA has also been reviewed.[184]

Alpha-L-LNA

Initial LNA research focused on the ß-D form of LNA (shown in Figure 2.4.1), however positive results, particularly in antisense studies, have prompted the study of various stereoisomers – most notably α-L-LNA (see Figure 17. Compared to ß-D- LNA, α-L-LNA shows superior stability against 3'-exonuclease activity and has thus proved to be a useful tool in enabling the construction of different gapmers and chimeras that present potent antisense activity. As a result, the incorporation of α-L-LNA into modified oligos for potential use in antisense drug development has come under investigation.[185]

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177 *(a)* **The potential for gene repair via triple helix formation**, *M.M. Seidman and P.M. Glazer, J. Clinical Investigation, 112, 487-494, 2003. (b)* **Triplex formation with α-L-LNA (α-L-ribo-configured locked nucleic acid),** *N. Kumar, K.E. Nielsen, S. Maiti and M. Petersen, J. Am. Chem. Soc., 128, 14-15, 2006.*

178 **Use of locked nucleic acid oligonucleotides to add functionality to plasmid DNA**, *K.M.L. Hertoghs, J.H. Ellis, and I.R. Catchpole, Nucleic Acids Research, 31, 5817-5830, 2003.*

179 **Improved RNA cleavage by LNAzyme derivatives of DNAzymes**, *B. Vester, L.B. Lundberg, M.D. Sorensen, B.R. Babu, S. Douthwaite, and J. Wengel, Bicochem. Soc. Trans., 32, 37-40 Part 1, 2004.*

180 **Locked nucleic acid molecular beacons**, *L. Wang, C.J. Yang, C.D. Medley, S.A. Benner and W. Tan, J. Amer. Chem. Soc., 127, 15664-15665, 2005.*

181 **The influence of locked nucleic acid residues on the thermodynamic properties of 2'-O-methyl RNA/RNA heteroduplexes**, *E. Kierzek, A. Ciesielska, K. Pasternak, D.H. Mathews, D.H. Turner and R. Kierzek, Nucleic Acids Res., 33, 5082-5093, 2005.*

182 ***In situ* detection of miRNAs in animal embryos using LNA-modified oligonucleotide probes**, *W.P. Kloosterman, E. Wienholds, E. de Bruijn, S. Kauppinen and R.H.A. Plasterk, Nature Methods, 3, 27-29, 2006.*

183 **A single-molecule method for the quantitation of microRNA gene expression**, *L.A. Neeley, S. Patel, J. Garver, M. Gallo, M. Hackett, S. McLaughlin, M. Nadel, J. Harris, S. Gullans and J. Rooke, Nature Methods, 3, 41-46, 2006.*

184 *(a)* **LNA: a versatile tool for therapeutics and genomics**, *M. Petersen and J. Wengel, Trends in Biotechnology, 21, 74-81, 2003; (b)* **Novel antisense and peptide nucleic acid strategies for controlling gene expression**, *D.A. Braasch, D.R. Corey, Biochemistry, 41, 4503-4510, 2002.*

185 *(a)* **NMR Structure of an α-L-LNA:RNA hybrid: structural implications for RNase H recognition**, *J.T. Nielsen, P.C. Stein, and M. Petersen, Nucleic Acids Res., 31, 5858-5867, 2003; (b)* **Expanding the design horizon of antisense oligonucleotides with alpha-L-LNA**, *M. Frieden, S.M. Christensen, N.D. Mikkelsen, C. Rosenbohm, C.A. Thrue, M. Westergaard, H.F. Hansen, H. Ørum, and T. Koch, Nucleic Acids Res., 31, 6365-6372, 2003.*

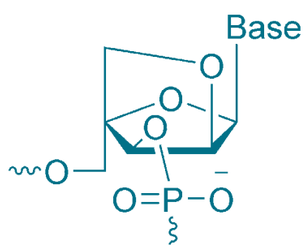


Figure 17. General Structure of α-L-LNA.

H-Phosphonates

H-phosphonate monomers (**LK2005-7**) and (**LK2035**) are useful for the preparation of internucleotide linkages that are not attainable by phosphoramidite chemistry.[186] The advantage of this chemistry over phosphoramidite chemistry is that in one reaction the backbone of the entire oligonucleotide is converted to the required form, (see Figure 18). This is typically oxidation to give a sugar-phosphate backbone or sulphurised to give a thiophosphate-sugar backbone, or conversion to the silyl phosphite triester which provides a useful means of generating a variety of phosphorus analogues.[187]

The H-phosphonate moiety renders phosphate protection unnecessary and the nucleobases are deprotected using ammonium hydroxide conditions applicable to any unmodified or phosphorothioate oligonucleotide. A popular application of H-phosphonate method is the synthesis of radiolabelled phosphorothioates.[188]

Ordering H-Phosphonates

Several H-Phosphonates linkers are available from Biosearch Technologies. Select the product you need from our website.

186 **Nucleoside H-phosphonates. Chemical synthesis of oligodeoxyribonucleotides by the hydrogenphosphonate approach**, *P.J. Garegg, I. Lidh, T. Regberg, J. Stawinski and R. Strömberg, Tetrahedron Lett., 27, 4051-4054, 1986.*

187 **Synthesis of DNA/RNA and their analogs via phosphoramidite and H-phosphonate chemistries**, *S. Roy and M. Caruthers, Molecules, 18, 14268-14284, 2013.*

188 **Preparation of 35S-labelled polyphosphorothioate oligodeoxyribonucleotides by the use of H-phosphonate chemistry**, *C.A.Stein, C.A. Iversen, C. Subashinge, J.S. Cohen, W.J. Stec and G. Zon, Analytical Biochem., 188, 11-16, 1990.*

Phosphorothioates

Introduction

Phosphorothioate-containing oligonucleotides (PS-Oligos), containing one sulphur atom in place of an oxygen atom (see Figure 19), have found widespread use in molecular biology.

The increased resistance to nuclease digestion that is exhibited by sulphur-containing backbone analogues has prompted consideration of these molecules for medical applications. Phosphorothioate-containing antisense oligos have been used *in vitro* and *in vivo* as inhibitors of gene expression.[189]

Site-specific attachment of reporter groups onto the DNA or RNA backbone is facilitated by the introduction of single phosphorothioate sites.[190] Phosphorothioates have also been incorporated into oligos for mechanistic studies on DNA-protein[191] and RNA-protein[192] interactions. Backbone modifications, including phosphorothioate substitutions, are also being explored as an approach for increasing the nuclease resistance, and therefore enhancing the therapeutic potential, of ribozymes.[193]

Using solid-phase oligonucleotide assembly, phosphorothioates can be prepared in two ways: by use of H-phosphonates (see above) or by using a sulphurising reagent in conjunction with phosphoramidite chemistry, discussed below.

Sulphurisation

During synthesis using the phosphoramidite approach, the backbone of either DNA or RNA can be modified by sulphurisation (or sulphur-transfer) reagents to replace one non-bridging oxygen atom in the phosphodiester, thus creating a phosphorothioate (PS) linkage.

This makes this method more suitable than H-phosphonate chemistry for controlling the state of each linkage [P=O versus P=S] in a site-specific manner. Compatibility with automated protocols is what gives this technique widest appeal.

Classically, elemental sulphur has been used as a sulphurising reagent,[194] however it is not an efficient process due to poor solubility and slow kinetics. It is imperative that an efficient sulphurisation reagent is used in phosphorothioate synthesis, particularly as synthesis scale and cost increase during commercial oligo production.

A number of sulphurising reagents have been described. These include phenylacetyl disulphide (PADS),[195] tetraethylthiuram disulphide (TETD),[196] 3H-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage Reagent),[197] 3-ethoxy-1,2,4-dithiazolidine-5-one (EDITH),[198]

189 *See for example:* **Improved biological activity of antisense oligonucleotides conjugated to a fusogenic peptide**, *J.-P. Bongartz, A.-M. Aubertin, P.G. Milhaud and B. Lebleu, Nucleic Acids Research, 22, 4681-4688, 1994.*

190 **Acceptor helix interactions in a Class II tRNA synthetase: Photoaffinity crosslinking of an RNA miniduplex substrate**, *K. Musier-Forsyth and P. Schimmel, Biochemistry, 33, 773-779, 1994.*

191 **Application of phosphate-backbone-modified oligonucleotides in the studies on EcoRI endonuclease mechanism of action**, *M. Koziolkiewicz and W.J. Stec, Biochemistry, 31, 9460-9466, 1992.*

192 **Determination of RNA-protein contacts using thiophosphate substitutions**, *J.F. Milligan and O.C. Uhlenbeck, Biochemistry, 28, 2849-2855, 1989.*

193 **Ribozymes as human therapeutic agents**, *R.E. Christoffersen and J.J. Marr, J. Med. Chem., 38, 2023-2037, 1995.*

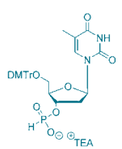
194 **Synthesis of dinucleoside monophosphorothioates via addition of sulphur to phosphite triesters**, *P.M. Burgers and F. Eckstein, Tetrahedron Lett., 40, 3835-3838, 1978.*

195 *(a)* **An efficient approach toward the synthesis of phosphorothioate diesters via the Schönberg reaction**, *P.C.J. Kamer, H.C.P.F. Roelen, H. van den Elst, G.A. van der Marel and J.H. van Boom, Tetrahedron Lett., 30, 6757-6760, 1989; (b)* **A study on the use of phenylacetyl disulfide in the solid-phase synthesis of oligodeoxynucleoside phosphorothioates**, *H.C.P.F. Roelen, P.C.J. Kamer, H. van den Elst, G.A. van der Marel and J.H. van Boom, Recl. Trav. Chim. Pays-Bas., 110, 325-331, 1991.*

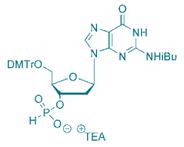
196 **Internucleotide phosphite sulfurization with tetraethylthiuram disulfide. Phosphorothioate oligonucleotide synthesis via phosphoramidite chemistry**, *H. Vu and B.L. Hirschbein, Terahedron Lett., 32, 3005- 3008, 1991.*

197 *(a)* **3H-1,2-benzodithiole-3-one 1,1-dioxide as an improved sulfurizing reagent in the solid- phase synthesis of oligodeoxyribonucleoside phosphorothioates**, *R.P. Iyer, W. Egan, J.B. Regan and S.L. Beaucage, J. Amer. Chem. Soc., 112, 1253-1254, 1990; (b) The automated synthesis of sulfur-containing oligodeoxyribonucleotides using 3H-1,2-benzodithiole-3-one 1,1-dioxide, R.P. Iyer, L.R. Phillips, W. Egan, J.B. Regan and S.L. Beaucage, J. Org. Chem., 55, 4693-4699, 1990.*

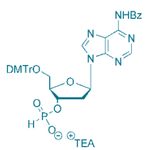
198 *(a)* **Use of 1,2,4-dithiazoline-3,5-dione (DtsNH) and 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH) for synthesis of phosphorothioate-containing oligodeoxyribonucleotides**, *Q. Xu, K. Musier-Forsyth, R.P. Hammer and G. Barany, Nucleic Acids Research, 24, 1602-1607, 1996; (b)* **Efficient introduction of phosphorothioates into RNA oligonucleotides by 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH),** *Q. Xu, G. Barany, R.P. Hammer and K. Musier- Forsyth, Nucleic Acids Research, 24, 3643-3644, 1996; (c)* **Synthetic, mechanistic,**



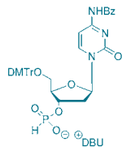
LK2005



LK2006



LK2007



LK2035

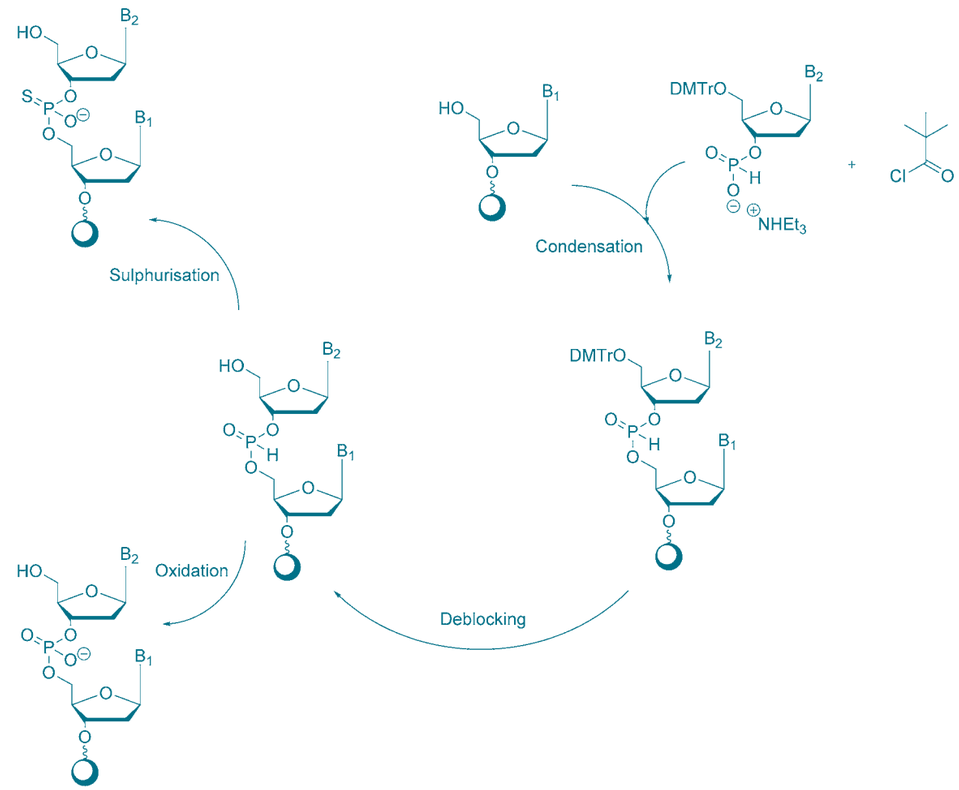


Figure 18. H-phosphonate synthesis cycle.

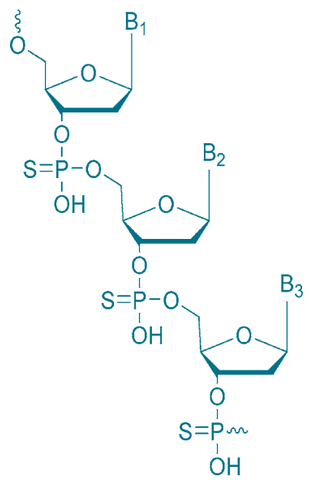


Figure 19. General structure of phosphorothioates.

1,2,4-dithiazolidine-3,5-dione (DtsNH),[199] 3-methyl-1,2,4-dithiazolin-5-one (MEDITH),[200] dibenzoyl tetrasulphide,[201] bis(O,O-diisopropoxyphosphinothioyl) disulphide (S-Tetra),[202] benzyltriethylammonium tetrathiomolybdate (BTTM),[203] bis(p-toluenesulphonyl) disulphide[204] and 3-amino-1,2,4-dithiazole-5-thione (ADTT).[205]

Of these, Beaucage Reagent and TETD were the first commercially available, although the former has been most widely used until now, principally due to its better performance and better stability under comparable conditions.

Beaucage Reagent is a relatively efficient sulphurising agent, however it is inherently unstable and has a tendency to precipitate from solution and therefore clog the delivery lines of an automated DNA synthesizer. Furthermore, the by-product formed in the sulphurisation reaction (3H-2,1-benzoxanthiolan-3-one-1-oxide) is a potent oxidising agent, leading to side-products, e.g. phosphodiesters, which are difficult to separate from the desired product. TETD's sulphurisation rate is slow and therefore a significant molar excess of this reagent is required. Even with this excess, the sulphurisation yields are low.

The shortcomings of these reagents, particularly evident in large-scale synthesis, has seen increased interest in the alternatives to Beaucage and TETD. PADS, for example, has found favour in some quarters for the synthesis of antisense oligonucleotides[206] and siRNA,[207] although there is a requirement to "age" the solution prior to synthesis to achieve optimum results.[208] More recently, effective sulphurisation using 3-((N,N-dimethyl-aminomethylidene) amino)-3H-1,2,4-dithiazole-5-thione (DDTT) has been described commercially, however the use of this reagent is dependent on dilution with a mixture of anhydrous pyridine in acetonitrile or THF.

and structural studies related to 1,2,4-dithiazolidine-3,5-dione, *L. Chen, T.R. Thompson, R.P. Hammer and G. Barany, J. Org. Chem., 61, 6639-6645, 1996; (d)* **Evaluation of 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH) as a new sulfurizing reagent in combination with labile exocyclic amino protecting groups for solid-phase oligonucleotide synthesis**, *M.Y.-X. Ma, J.C. Dignam, G.W. Fong, L. Li, S.H. Gray, B. Jacob-Samuel and S.T. George, Nucleic Acids Research, 25, 3590-3593, 1997.*

199 **Stepping towards highly flexible aptamers: enzymatic recognition studies of unlocked nucleic acid nucleotides**, *C. Dubois, M.A. Campbell, S.L. Edwards, J. Wengel and R.N. Veedu, Chem. Commun., 48, 5503-5505, 2012.*

200 **Solid phase synthesis of oligonucleotide phosphorothioate analogues using 3-methyl-1,2,4-dithiazolin- 5-one (MEDITH) as a new sulfur-transfer reagent**, *Z. Zhang, A. Nichols, J.X. Tang, Y. Han and J.J. Tang, Tetrahedron Lett., 40, 2095-2098, 1999.*

201 **Dibenzoyl tetrasulphide—A rapid sulphur transfer agent in the synthesis of phosphorothioate analogues of oligonucleotides**, *M.V. Rao, C.B. Reese and Z. Zhengyun, Tetrahedron Lett., 33, 4839-4842, 1992.*

202 **Bis(O,O-diisopropoxy phosphinothioyl) disulfide—a highly efficient sulfurizing reagent for cost- effective synthesis of oligo(nucleoside phosphorothioate)s**, *W.J. Stec, B. Uznanski and A. Wilk, Tetrahedron Lett., 34, 5317-5320, 1993.*

203 **Solid phase synthesis of phosphorothioate oligonucleotides using benzyltriethylammonium tetrathiomolybdate as a rapid sulfur transfer reagent**, *M.V. Rao and K. Macfarlane, Tetrahedron Lett., 36, 6741- 6744, 1994.*

204 **New efficient sulfurizing reagents for the preparation of oligodeoxyribonucleotide phosphorothioate analogues**, *V.A. Efimov, A.L. Kalinkina, O.G. Chakhmakhcheva, T.S. Hill and K. Jayaraman, Nucleic Acids Research, 23, 4029-4033, 1995.*

205 **Large-scale synthesis of oligonucleotide phosphorothioates using 3-amino-1,2,4-dithiazole-5-thione as an efficient sulfur-transfer reagent**, *J.-Y. Tang, Y. Han, J.X. Tang and Z. Zhang, Org. Proc. Res. Dev., 4, 194-198, 2000.*

206 **Synthesis of antisense oligonucleotides: Replacement of 3H-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage Reagent) with phenylacetyl disulfide (PADS) as efficient sulfurization reagent: From bench to bulk manufacture of active pharmaceutical ingredient**, *Z.S. Cheruvallath, R.L. Carty, M.N. Moore, D.C. Capaldi, A.H. Krotz, P.D. Wheeler, B.J. Turney, S.R. Craig, H.J. Gaus, A.N. Scozzari, D.L. Cole and V.T. Ravikumar, Org. Proc. Res. Dev., 4, 199- 204, 2000.*

207 **Development of siRNA for therapeutics: Efficient synthesis of phosphorothioate RNA utilizing phenylacetyl disulfide (PADS),** *V.T. Ravikumar, M. Andrade, R.L. Carty, A. Dan and S. Barone, Bioorganic & Medicinal Chem. Lett., 16, 2513-2517, 2006.*

208 *(a)* **Phosphorothioate oligonucleotides with low phosphate diester content: Greater than 99.9% sulfurization efficiency with "aged" solutions of phenylacetyl disulfide (PADS),** *A.H. Krotz, D. Gorman, P. Mataruse, C. Foster, J.D. Godbout, C.C. Coffin and A.N. Scozzari, Org. Proc. Res. Dev., 8, 852-858, 2004; (b) An alternative advantageous protocol for efficient synthesis of phosphorothioate oligonucleotides utilizing phenylacetyl disulfide (PADS), R.K. Kumar, P. Olsen and V.T. Ravikumar, Nucleosides, Nucleotides and Nucleic Acids, 26, 181-188, 2007.*

It is EDITH (**LK2171**), however, that is now emerging as the reagent of choice amongst users due to its all-round capability and ease of use. This product is soluble in acetonitrile (other Beaucage alternatives require either pyridine or picoline), reportedly stable in solution for several months (a silanised bottle is not required), and exhibits high sulphurisation efficiency with both DNA and RNA. Its high efficiency in RNA synthesis, often unobtainable with other reagents, is of particular benefit, giving a reported >99% sulphurisation efficiency.[167]

EDITH's efficiency in sulphurising DNA in comparison to TETD has been demonstrated by Xu et al,[167] who reported a 0.5 M solution of TETD and contact time of 15 min giving 96% PS, whilst EDITH at 0.05 M and contact time 30s gave >98% PS.

The compatibility of EDITH with labile (fast) deprotection chemistry has also been noted.[167] Some deleterious G modification has been observed, however this can be eliminated by using a modified coupling-cap-thio-cap cycle. This allows the preparation of certain phosphorothioates that may be sensitive to prolonged ammonium hydroxide solution treatment. However, it should be noted that capping prior to sulphurisation can lead to some oxidation of the PIII species.

Ordering sulphurising reagents

Sulphurising reagents, such as EDITH, are available from Biosearch Technologies. See our website for full details.

Creating methylated and ethylated backbones

Methyl phosphonamidites

Since methyl (Me) phosphonate linkages are uncharged and nuclease resistant, oligonucleotides containing these have many applications, particularly in developing novel strategies for targeted cellular delivery of antisense therapeutic agents.[209] These were among the first modified oligonucleotides shown to inhibit protein synthesis via an antisense mechanism.

Synthesis using these monomers (**LK2073**/**LK2074**/**LK2075**/**LK2077**) requires a low water content oxidiser and changes are necessary from commonly used deprotection procedures because the linkages are more base-labile. EDA in 95% EtOH (1:1) is typically used, but other methods have been reported.[210] To help in purification and isolation of these oligos, it is best to incorporate as many phosphodiester linkages (prepared from ß-cyanoethyl phosphoramidites) into each oligo as possible.

Methyl phosphoramidites

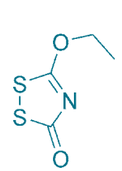
The UltraMILD set of methyl phosphoramidites (**LK2050** - **LK2052**, **LK2078**), in conjunction with UltraMILD deprotection conditions, can be used to prepare the interesting, nuclease resistant methyl phosphotriester linkage. Again, these have potential therapeutic applications.

Ethyl phosphoramidites

As a result of several customer requests, we have introduced a range of ethyl phosphoramidites with classical nucleobase protection (iBu-dG, Bz-dA, Bz-dC) (**LK2516** - **LK2519**).

209 *See for example: (a)* **Comparative hybrid arrest by tandem antisense oligodeoxyribonucleotides or oligodeoxyribonucleoside methylphosphonates in a cell-free system**, *L.J. Maher, III and B.J. Dolnick, Nucleic Acids Research, 16, 3341-3358, 1988; (b)* **Solid-phase synthesis of oligo-2-pyrimidinone-2'-deoxyribonucleotides and oligo-2-pyrimidinone-2'-deoxyribose methylphosphonates**, *Y. Zhou and P.O.P. Ts'o, Nucleic Acids Research, 24, 2652-2659, 1996; and (c)* **Nuclear antisense effects of neutral, anionic and cationic oligonucleotide analogs**, *P. Sazani, S.-H. Kang, M.A. Maier, C. Wei, J. Dillman, J. Summerton, M. Manoharan and R. Kole, Nucleic Acids Research, 19, 3965-3974, 2001.*

210 **Deprotection of methyiphosphonate oligonucleotides using a novel one-pot procedure**, *R.I. Hogrefe, M.M. Vaghefi, M.A. Reynolds, K.M. Young and L. Arnold Jr, Nucleic Acids Research, 21, 2031-2038, 1993.*



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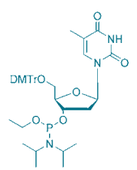

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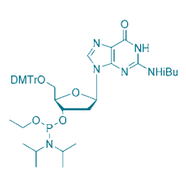

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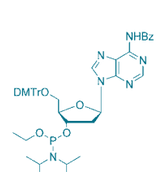

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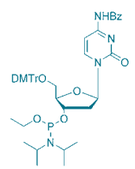


LK2519

Nuclease resistant P-ethoxy oligonucleotides (a hydrophobic analogue of phosphodiesters) have been shown, through incorporation into liposomes, to be effective in the inhibition of protein expression and cell growth in therapeutic applications.[211,212] The neutral charge and slight lipophilic character appears to improve the delivery of the oligonucleotide into a cell.

Ordering methyl phosphonamidites

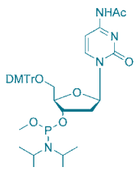
Biosearch Technologies have expanded its offering of methyl phosphoramidites to now include Pac-dA-Me. To see the full range of products, see our website.

Ordering ethyl phosphoramidites

Popular demand for ethyl phosphoramidites means LGC Biosearch Technologies now offer an expanded range of these products. Visit our website for full details.

211 Cellular pharmacology of P-ethoxy antisense oligonucleotides targeted to Bcl-2 in a follicular lymphoma cell line, Y. Gutiérrez-Puente, A.M. Tari, R.J. Ford, R. Tamez-Guerra, R. Mercado-Hernandez, M. Santoyo-Stephano, and G. Lopez-Berestein, Leuk. Lymphoma., 44, 1979-85, 2003.

212 Safety, pharmacokinetics, and tissue distribution of liposomal P-ethoxy antisense oligonucleotides targeted to Bcl-2, Y. Gutiérrez-Puente, A.M. Tari, C. Stephens, M. Rosenblum, R.T. Guerra, G. Lopez-Berestein, J. Pharmacol. Exp. Ther., 291, 865-9, 1999.



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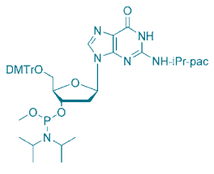

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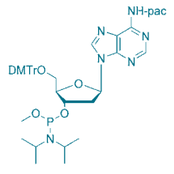

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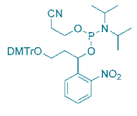

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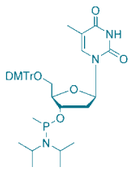

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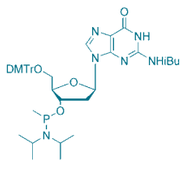

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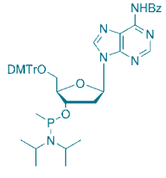

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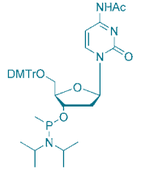
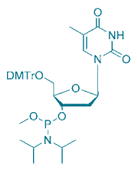


LK2077



LK2078

Using photocleavable (PC) modifiers

Introduction

The versatility of common modifiers and labels has been extended by the introduction of several photocleavable analogues of the Amino- and Spacer-Modifiers and the Biotin label. This range is complemented by a PC linker molecule, suited to a wide variety of applications.

Design of photocleavable modifiers

The general design of the PC monomers is based on an α-substituted 2-nitrobenzyl group.[213] The photo-reactive group is derivatised as a cyanoethyl phosphoramidite for use in automated DNA synthesis.[214]

The PC 5'-Biotin-CE Phosphoramidite (**LK2122**) contains a biotinyl moiety that bears a trityl group on the N-1 nitrogen atom. This is primarily for N-protection (cf. **LK2109/BNS-5021**) rather than to facilitate coupling efficiency monitoring by trityl cation assay. However, as with **LK2109/BNS-5021**, the N-DMTr group enables cartridge purification of the oligo.

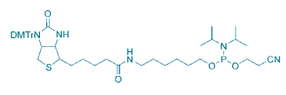
5' Addition of PC Amino-Modifier-CE Phosphoramidite (**LK2130**) to an oligonucleotide, followed by cleavage from the support and deprotection, results in an amino-linker separated from the oligo by a photocleavable linker. The amino group is then used in post-synthetic modification with amine reactive reagents or to attach synthetic oligonucleotides to activated solid supports. This is particularly useful for capturing DNA or RNA where the oligonucleotide/DNA duplex is cleaved from the surface by photolysis of the PC linker.

While the biotin and amino modifiers are both 5'-terminus modifiers, both the PC Spacer (**LK2131**) and PC Linker (**LK2066**) Phosphoramidites can be used as mid-sequence modifiers (for example for use with mass markers).

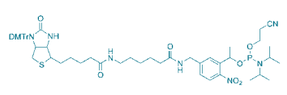
Upon irradiating a PC-modified oligo with near-UV light, the phosphodiester bond between the linker and the phosphate is cleaved, resulting in the formation of a 5'-monophosphate on the released oligonucleotide. **LK2066** has the added advantage in that photocleavage results in monophosphate fragments at both the 3'- and 5'-termini (see Figure 20).

213 *(a)* **Photocleavage of a 2-nitrobenzyl linker bridging a fluorophore to the 5' end of DNA**, *X. Bai, Z. Li, S. Jockusch, N. J. Turro, and J. Ju, PNAS, 100, 409–413, 2003; (b)* **Model studies for new o-nitrobenzyl photolabile linkers: substituent effects on the rates of photochemical cleavage**, *C.P. Holmes, J. Org. Chem., 62, 2370-2380, 1997.*

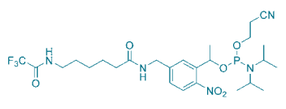
214 *For examples of applications of related, non-phosphoramidite, molecules see: (a)* **Photochemical control of the infectivity of adenoviral vectors using a novel photocleavable biotinylation reagent**, *M.W. Pandori, D.A. Hobson, J. Olejnik, S. Sonar, E. Krzymańska-Olejnik, K.J. Rothschild, A.A. Palmer, T.J. Phillips and T. Sano, Chemistry & Biology, 9, 567-573, 2002; and (b)* **Design and synthesis of a photocleavable biotinylated nucleotide for DNA analysis by mass spectrometry**, *X. Bai, S. Kim, Z. Li, N. J. Turro, and J. Ju, Nucleic Acids Research, 32, 535-541, 2004.*



LK2109



LK2122



LK2130

Application of photocleavable modifiers

Affinity conjugation and purification

Photocleavable amino-tag phosphoramidites represent a more general approach compared to PC biotin.[215] 5'-PC amino oligonucleotides can be reacted with a wide variety of activated molecules and surfaces, facilitating the formation of a variety of photocleavable conjugates.[216] Upon exposure to near UV light the unmodified oligonucleotide and/or marker molecule can be released and recovered.

The 5'-PC amino group can be used as an affinity tag for photo-cleavage-mediated affinity purification and phosphorylation of synthetic oligonucleotides in conjunction with activated supports. 5'-PC amino labelled oligos suggest applications including multiple non-radioactive probing of DNA/RNA blots, affinity isolation and purification of nucleic acids binding proteins, diagnostic assays requiring release of probe-target complex or specific marker, cassette mutagenesis and PCR.

Oligonucleotide isolation and purification

PC Biotin-labelled DNA can be captured with streptavidin beads in a similar fashion to oligonucleotides modified with conventional 5'-biotin phosphoramidite (see Figure 21)[217] and therefore biotin PC linkers are particularly useful in capture probes. The PC biotin is rapidly and quantitatively cleaved from the 5'-terminus, releasing the DNA into solution, by simply illuminating with a hand-held UV light source at 300- 350 nm. After photo-cleavage the DNA is suitable for further biological manipulations like gene construction and cloning after ligation. However, more commonly this technique is used to isolate DNA by first hybridisation to the biotin labelled probe, then release of the probe/DNA duplex after photolysis.

Avidin-biotin technology has found applications as diverse as detection of proteins by non- radioactive immunoassays, cytochemical staining, cell separation, isolation of nucleic acids, detection of specific DNA/RNA sequences by hybridisation, and probing conformational changes in ion channels.

The use of PC biotin facilitates these applications with the additional benefit of providing an easily removable label. More advanced applications now envisaged include the selective release of biomolecules from 2-dimensional arrays and the assembly of biomolecular constructs at the nanometer scale. PC 5'-Biotin labelled oligonucleotides are useful in a variety of applications in molecular biology including cassette mutagenesis and PCR, where the biotin is used as a means of capture. PC 5'-Biotin Phosphoramidite has been used for the synthesis, purification and phosphorylation of 50mer and 60mer oligonucleotides.

See page 90 for other biotin products.

Photo-triggered strand cleavage

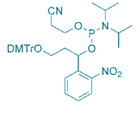
Photo-triggered DNA cleavage is a major tool used for studying conformational changes and strand breaks, as well as for studying activation of nucleic-acid-targeted drugs, such as antisense oligonucleotides.[218] The PC Linker Phosphoramidite (**LK2066**), first described for use

215 **Photocleavable aminotag phosphoramidites for 5'-termini DNA/RNA labelling**, *J. Olejnik, E. Krzymańska- Olejnik, and K.J. Rothschild, Nucleic Acids Research, 26, 3572-3576, 1998.*

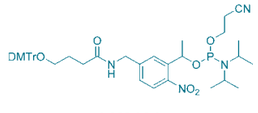
216 **Photocleavable peptide-DNA conjugates: synthesis and applications to DNA analysis using MALDI-MS**, *J. Olejnik, H.-C. Lüdemann, E. Krzymańska-Olejnik, S. Berkenkamp, F. Hillenkamp and K.J. Rothschild, Nucleic Acids Research, 27, 4626-4631, 1999.*

217 *(a)* **Photocleavable affinity tags for isolation and detection of biomolecules**, *J. Olejnik, E. Krzymańska- Olejnik, and K.J. Rothschild, Methods in Enzymology, 291, 135-154, 1998; (b)* **Photocleavable biotin phosphoramidite for 5'-end-labelling, affinity purification and phosphorylation of synthetic oligonucleotides**, *J. Olejnik, E. Krzymańska-Olejnik, and K.J. Rothschild, Nucleic Acids Research, 24, 361-366, 1996; (c)* **Photocleavable biotin derivatives: A versatile approach for the isolation of biomolecules**, *J. Olejnik, S. Sonar, E. Krzymańska- Olejnik, and K.J. Rothschild, Proc. Natl. Acad. Sci. USA, 92, 7590-7594, 1995.*

218 **Using photolabile ligands in drug discovery and development**, *G. Dormán and G.D. Prestwich, Trends in Biotechnology, 18, 64-77, 2000.*



LK2066



LK2131

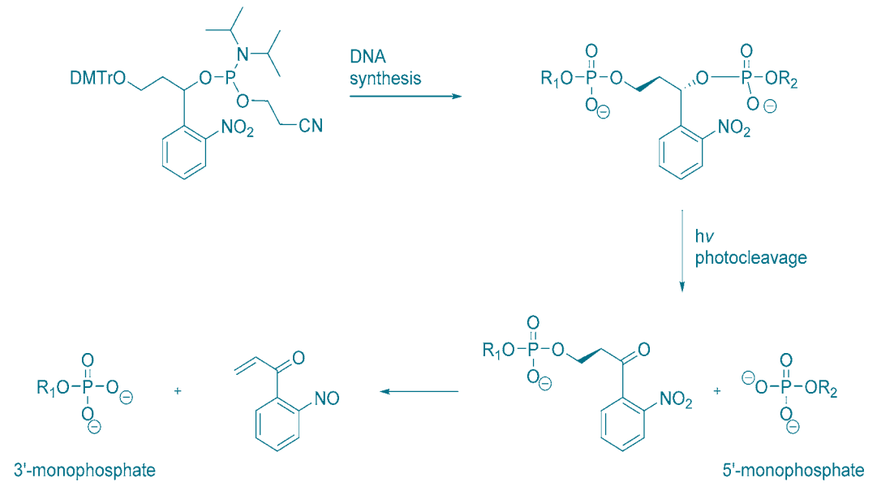


Figure 20. Photocleavage using PC Linker Phosphoramidite.

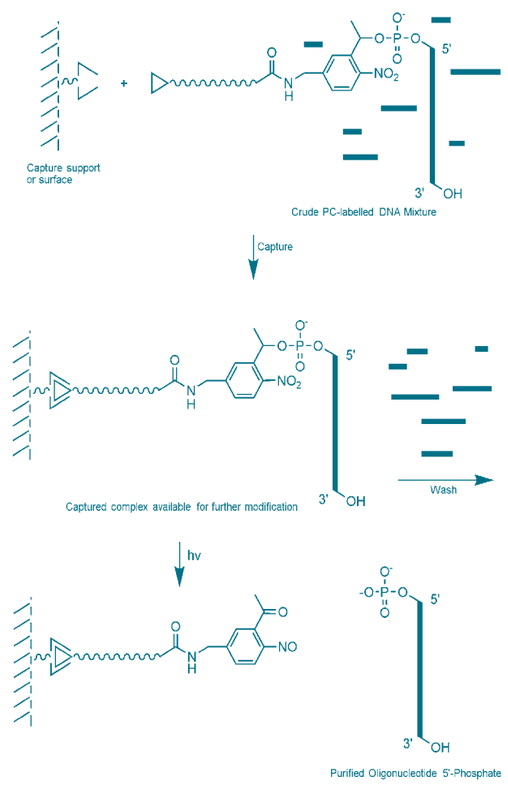


Figure 21. DNA purification using PC-5'-Biotin Phosphoramidite

in phototriggered hybridisation,[219] has also been used in the design of multifunctional DNA and RNA conjugates for the *in vivo* selection of new molecules catalysing biomolecular reactions.[220]

The genoSNIP method[221] for single-nucleoside polymorphism (SNP) genotyping by MALDI- TOF mass spectrometry utilises this modification; the method uses size reduction of primer extension products by incorporation of the PC Linker for photo-triggering strand breaks near the 3'-end of the extension primer.

In addition, the PC Spacer Phosphoramidite, can be used as an intermediary to attach any modified phosphoramidite to the terminus of the oligonucleotide. This allows the modification to be removed by photolysis if required.

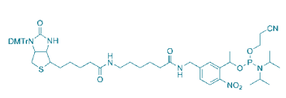
Ordering photocleavable modifiers

PC 5'-Biotin and PC 5'-Amino are now part of the photocleavable modifiers offered from Biosearch Technologies. See our website for our full offering of these modifiers.

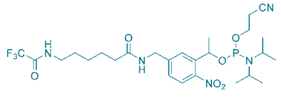
219 **Design and synthesis of a versatile photocleavable DNA building block. Application to phototriggered hybridization**, *P. Ordoukhanian and J-S. Taylor, J. Amer. Chem. Soc., 117, 9570-9571, 1995.*

220 *(a)* **Libraries of multifunctional RNA conjugates for the selection of new RNA catalysts**, *F. Hausch and A. Jäschke, Bioconjugate Chem., 8, 885-890, 1997; (b)* **A novel carboxy-functionalized photocleavable dinucleotide analog for the selection of RNA catalysts**, *F. Hausch and A. Jäschke, Tetrahedron Lett., 39, 6157-6158, 1998; (c)* **Multifunctional DNA conjugates for the in vitro selection of new catalysts**, *F. Hausch and A. Jäschke, Nucleic Acids Research, 28, e35, 2000; (d)* **Multifunctional dinucleotide analogs for the generation of complex RNA conjugates**, *F. Hausch and A. Jäschke, Tetrahedron, 57, 1261-1268, 2001.*

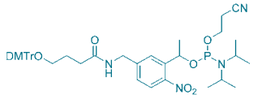
221 **genoSNIP: SNP genotyping by MALDI-TOF MS using photocleavable oligonucleotides**, *T. Wenzel, T. Elssner, K.Fahr, J. Bimmler, S. Richter, I. Thomas, and M. Kostrzewa, Nucleosides, Nucleotides and Nucleic Acids, 22, 1579- 1581, 2003.*



LK2122



LK2130



LK2131

Modifications for nuclease resistance

Synthetic oligonucleotides, just like their natural counterparts, are prone to degradation once introduced into a cell. This degradation is due to the presence of exo- and endonuclease enzymes, as well as inherent chemical instability (particularly for RNA). Under cellular conditions, this leads to fast *in vivo* degradation of oligos and a short half-life.[222] To reduce or eliminate this susceptibility, nuclease-resistant modifications can be introduced into oligonucleotides. For antisense or RNAi applications, incorporation of modifications conferring nuclease resistance is essential and such modifications are used routinely. There are a number of ways to introduce nuclease resistance into a synthetic oligonucleotide.

When considering such an oligo, one must also try to minimise potential deleterious side- effects (such as reduced duplex stability, increased toxicity, or induction of off-target biological effects). One method of achieving this is by creating a 'gapmer', in which the linkages of the three terminal 5'- and 3'-bases are phosphorothiolated (see phosphorothioates on page 62), with the remaining bases in the middle having phosphorodiester linkages. Such oligos are highly resistant to both 5'- and 3'-exonuclease degradation. In addition, because phosphorothiolation lowers the binding affinity of the oligo for its target (T_m of the oligo-target duplex is lowered between 0.5 ºC and 1.5 ºC per linkage), use of as few as six such linkages can give an acceptable balance between nuclease resistance and binding affinity[223] If increased binding affinity is required, other modifications can also be incorporated into the oligo, such as 2'-fluoro pyrimidines or 2'-OMe bases. The downside of using phosphorothiolation is that sulphur-containing linkages can be toxic, limiting the applicability of this approach.

Alternatively, methylphosphonates (see page 62) can be used for the 5'- and 3'-end positions of the 'gapmer'. Methylphosphonates lower an oligo's binding affinity more than phosphorothiolation, therefore the use of additional modifications, such as 2'-fluoro nucleosides, is used to counteract this effect. Most commonly, the substitution of 2'-OMe bases at some or all positions of an oligo is used as the preferred route to inducing nuclease resistance[224] Since the nuclease resistance conferred by 2'-OMe lies between that of unmodified nucleosides (no resistance) and phosphorothiolation (highly resistant), extensive/complete 2'-O-methylation is frequently chosen when a high level of nuclease resistance is required. 2'-O-methylation also confers the desirable property of higher binding affinity (that is, higher duplex T_m) to the oligo for its target. For these reasons, 2'-OMe nucleosides are extensively used in siRNA and aptamer applications.

222 **Rate of degradation of [alpha] and [beta]-oligodeoxynucleotides in Xenopus oocytes. Implications for anti-messenger strategies**, *C. Cazenave, M. Chevrier, T.T. Nguyen and C. Helene, Nucleic Acids Research, 15, 10507- 10521, 1987.*

223 **Evaluation of different types of end-capping modifications on the stability of oligonucleotides toward 3'-and 5'-exonucleases**, *D. Pandolfi, F. Rauzi and M.L. Capobianco, Nucleosides & Nucleotides, 18, 2051-2069, 1999.*

224 *(a)* **Evaluation of 2'-Modified Oligonucleotides Containing 2'-Deoxy Gaps as Antisense Inhibitors of Gene Expression**, *B.P. Monia, E.A. Lesnik, C. Gonzalez, W.F. Lima, D. McGee, C.J. Guinosso, A.M. Kawasaki, P.D. Cook and S.M. Frier, J. Biol. Chem., 268, 14514-14522, 1993; (b)* **Nuclease Resistance and Antisense Activity of Modified Oligonucleotides Targeted to Ha-ras**, *B.P. Monia, J.F. Johnston, H. Sasmor and L.L. Cummins, J. Biol. Chem., 271, 14533-14540, 1996.*

2’-O-Methyl modifications

2’-O-Methyloligoribonucleotides[225] are extremely useful reagents for a variety of molecular biology applications. The 2’-OMe RNA-RNA duplex is more thermally stable than the corresponding DNA-RNA one.[226] In addition, 2’-OMe-RNA is chemically more stable than either DNA or RNA and is resistant to degradation by RNA- or DNA-specific nucleases.[227] It is worth noting though that duplexes formed between oligos having 2’-OMe bases at all positions and RNA are incapable of RNase H activity, thus making them ineffective in RNaseH dependent antisense applications[228], although they can suppress gene expression by blocking the mRNA translation process via steric hindrance.[229]

Ordering modified/unmodified 2’-OMe RNA phosphoramidites and CPG supports

An expanded range of both modified and unmodified 2’-OMe RNA phosphoramidites and associated CPG supports are offered. Details on product specifics are available to view on our website.

The enhanced RNase and DNase resistance, and the increased thermal stability of their duplexes and triplexes, have been examined in a number of ways.[230,231] Applications range from simple antigene type experiments to the correction of aberrant splicing. Researchers have also made use of biotinylated 2’-OMe RNA for the affinity selection or affinity depletion of ribonucleoprotein complexes, most notably in the field of RNA processing.[232]

We provide a range of 2’-OMe phosphoramidites (**LK2041**, **LK2042**, **LK2043**, **LK2044**, **LK2045**, **LK2083**/**LK2084**) and CPG supports (**LK2310**/**BG5-1300MR**, **LK2311**/**BG5-1200MR**, **LK2312**/**BG5-1000MR**, **LK2313**/**BG5-1100MR**) with a variety of protecting groups. Whilst we have for some time offered these, increasingly researchers are looking for nucleobase modifications to RNA-type molecules to extend the experimentation available. In fact, many of these modifiers are increasingly being used in larger scale oligonucleotide manufacture. To this end we have introduced a number of other 2’-OMe RNA products.

Oligonucleotides containing 2’-OMe-5-Me-U (2’-OMe-T) (**LK2099**), 2’-OMe-N-Ac-5-Me-C (**LK2192**) or 2’-OMe-I (**LK2098**) are particularly applicable to triplex and antisense studies using 2’-OMe-RNA. For example, the immune stimulatory activity of CpG containing oligonucleotides in which C or G was substituted with 2’-OMe ribonucleotides, 5-Me-dC, or 2’-OMe-5-Me-C has been studied alone and in combination with TLR agonists.[233]

When 2’-OMe residues are incorporated into triplex forming oligonucleotides[234] (TFOs), similar trends in nuclease resistance and triplex stability are seen as with duplexes.

Hence they have been used to develop TFOs for use as gene targeting reagents.[235] Triplex stability can be increased further with the incorporation of 2’-OMe-5-Me-U residues into the oligonucleotide[236] using **LK2099**. Interestingly, 2’-OMe-5-Me C (using **LK2192**) can have the opposite effect and destabilise the triplex, yet is still more stable than a DNA and/or RNA triplex. Incorporation of both 2’-OMe-5-Me U and 2’-OMe-5-Me C enables fine-tuning of the T_m of the resulting triplex.

Conveniently, the deprotection of 2’-OMe oligoribonucleotides are exactly the same as for unmodified oligodeoxynucleotides. However, due to the higher degree of hydrophobicity, some alterations may be required in terms of purification. Because the 2’-OMe oligos are nuclease resistant, unless the oligo contains RNA residues, the need to decontaminate equipment and glassware with e.g. RNase away is not an absolute requirement.

225 *Note this is not a 2’-OH protecting group strategy; the 2’-OMe group cannot be cleaved under RNA synthesis and deprotection conditions.*

226 **Synthesis and hybridization studies on two complementary nona(2’-O-methyl)ribonucleotides**, *H. Inoue, Y. Hayase, A. Imura, S. Iwai, K. Miura, and E. Ohtsuka, Nucleic Acids Research, 15, 6131-6148, 1987.*

227 **Highly efficient chemical synthesis of 2’-O-methyloligoribonucleotides and tetrabiotinylated derivatives; novel probes that are resistant to degradation by RNA or DNA specific nucleases**, *B.S. Sproat, A.I. Lamond, B. Beijer, P. Neuner and U. Ryder, Nucleic Acids Research, 17, 3373-3386, 1989.*

228 **Sequence-dependent hydrolysis of RNA using modified oligonucleotide splints and RNase** *H, H. Inoue, Y. Hayase, S. Iwai and E. Ohtsuka, FEBS Lett., 215, 327-330, 1987.*

229 **Antisense technologies. Improvement through novel chemical modifications**, *J. Kurreck, Eur. J. Biochem., 270, 1628-1644, 2003.*

230 **Effective incorporation of 2’-O-methyl-oligoribonucleotides into liposomes and enhanced cell association through modification with thiocholesterol**, *B. Oberhauser and E. Wagner, Nucleic Acids Research, 20, 533-538, 1992.*

231 **Enhancement of ribozyme catalytic activity by a contiguous oligodeoxynucleotide (facilitator) and by 2’-O-methylation**, *J. Goodchild, Nucleic Acids Research, 20, 4607-4612, 1992.*

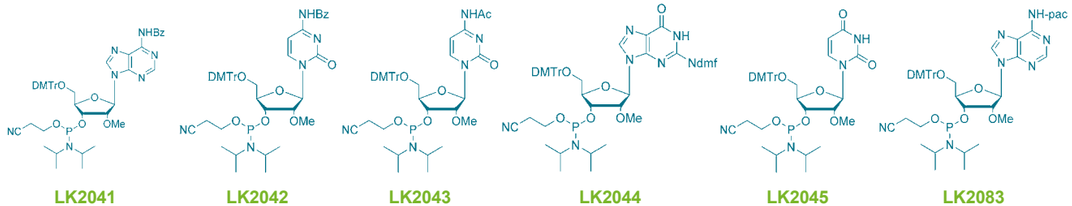
232 *See for example:* **Mapping U2 snRNP – premRNA interactions using biotinylated oligonucleotides made of 2’-OMe RNA**, *S.M.L. Barabino, B.S. Sproat, U. Ryder, B.J. Blencowe, A.I. Lamond, The EMBO Journal, 8, 4171-4178, 1989.*

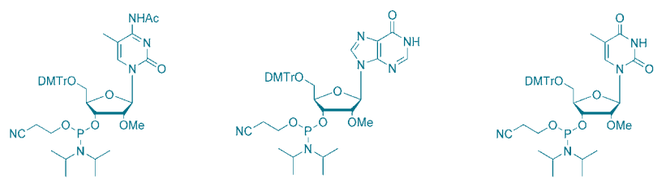
233 **Modifications incorporated in CpG motifs of oligodeoxynucleotides lead to antagonist activity of toll-like receptors 7 and 9**, *D. Yu, D. Wang, F.-G. Zhu, L. Bhagat, M. Dai, E. R. Kandimalla and S. Agrawal, J. Med. Chem., 52, 5108-5114, 2009.*

234 **Pyrimidine motif triplexes containing polypurine RNA or DNA with oligo 2’-O-Methyl or DNA triplex forming oligonucleotides**, *M. Behan and P.S. Miller, Biochim. Biophys. Acta., 1492, 155-162, 2000.*

235 **The development of bioactive triple helix-forming oligonucleotides**, *M.M. Seidman, N. Puri, A. Majumdar, B. Cuenoud, P.S. Miller and R. Alam, in volume 1058, Therapeutic Oligonucleotides: Transcriptional and Translational Strategies for Silencing Gene Expression, 119–127, November 2005, Wiley-Blackwell.*

236 **Effects of 5-methyl substitution in 2’-O-methyl oligo(pyrimidine)nucleotides on triple-helix formation**, *M. Shimizu, T. Koizumi., H. Inoue and E. Ohstuka, Bioorg. Med. Chem. Letts., 4, 1029-1032, 1994.*





2’-Fluoro modifications

2’-F-RNA oligonucleotides (synthesised using **LK2079 - LK2082**) adopt an A-form helix on hybridisation to a target. Whereas a hydroxyl group of RNA is a hydrogen bond donor, fluorine appears to be a weak acceptor. These features of 2’-F-RNA oligonucleotides lead to certain interesting properties. For example, it was demonstrated that oligonucleotides hybridise to a RNA oligonucleotide in the following order of increasing stability: DNA < RNA < 2’-OMe-RNA < 2’-F-RNA.[237]

Aptamers composed of 2’-F–RNA bind targets with higher affinities and are more resistant to nucleases, compared to RNA aptamers.[238] In addition, 2’-F-RNA can be effectively used in siRNA applications. It has been shown that siRNA synthesised with 2’-F pyrimidine nucleosides are more inhibitory, and show considerably increased stability in human plasma, compared to siRNA.[239] 2’-F-RNA is now finding a number of applications, especially in RNA interference for the specific silencing of genes in cells and in vivo.[240]

Ordering 2’-F products

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Reverse (5’ to 3’) oligonucleotides

The chemical synthesis of DNA using the phosphoramidite method proceeds in a 3’ to 5’ direction principally as a consequence of the use of building blocks activated as 3’-O-phosphoramidites. The primary 5’-OH group is significantly more reactive than the secondary 3’-OH (or 2’-OH) group, making it straightforward to protect with the DMT group leaving the 3’-OH available to form the phosphoramidite. In contrast, ‘reverse’ oligonucleotide synthesis (i.e. in a 5’ to 3’ direction) has not been utilised to nearly the same extent.

Nevertheless, there are several applications of this chemistry, most notably in nuclease resistance.

An interesting addition to the protection of antisense oligonucleotides is to modify the terminal linkages from the natural 3’-5’ to 3’-3’ and/or 5’-5’ linkages. In this way, the oligonucleotides are protected against exonuclease activity, especially 3’-exonuclease activity which is by far the most significant enzymatic degradation route, resulting in nucleosides with no toxicity concerns. This strategy has been applied by Beaucage and co-workers who have used 5’-O-phosphoramidites in the formation of oligonucleotides having alternating 3’-3’ and 5’-5’ linkages to maintain effective hybridisation.[241] A simpler approach is in fact to modify only the linkage at the 3’ terminus.[242] This is conveniently carried out and results in effective resistance with minimal disruption to hybridisation.

In addition to the established applications in nuclease resistance and hairpin loops, other technologies exploit the flexibility of reverse oligo synthesis. For example, with the increasing use of DNA chip technology, interest has focused upon the synthesis of support-bound, fully deprotected oligonucleotides.[243] Such molecules are accessible through the use of 2-(4-nitrophenyl)-ethyl/[2-(4-nitrophenyl)ethoxy]carbonyl (npe/npeoc) protecting groups[244] on the nucleobase.

The use of 5’-O-phosphoramidites has not generally been used for the elaboration of oligonucleotides, even though this approach offers a facile route to 3’-modified oligodeoxynucleotides. The potential for this approach has recently been demonstrated by Hecht and co-workers using a phosphoramidite derived from tyrosine.[245] The derived oligonucleotide was shown to have chromatographic and electrophoretic properties identical with the modified oligo resulting from the proteinase K digestion of a topoisomerase-DNA complex.

Ordering reverse phosphoramidites and support

We offer reverse phosphoramidites (**LK2020 - LK2023, LK2093**) and solid supports (**LK2294/BG1-1300i, LK2298/BG1-1200i, LK2355/BG1-1000i, LK2356**), with classical heterocyclic base protection groups.

See our website for Biosearch Technologies full offering of reverse phosphoramidites.

237 **Uniformly modified 2’-deoxy-2’-fluoro phosphorothioate oligonucleotides as nuclease-resistant antisense compounds with high affinity and specificity for RNA targets**, *A.M. Kawasaki, M.D. Casper, S.M. Freier, E.A. Lesnik, M.C. Zounes, L.L. Cummins, C. Gonzalez and P.D. Cook, J. Med. Chem., 36, 831-841, 1993.*

238 **Neutralization of infectivity of diverse R5 clinical isolates of human immunodeficiency virus type 1 by gp120-binding 2’-F-RNA aptamers**, *M. Khati, M. Schüman, J. Ibrahim, Q. Sattentau, S. Gordon and W. James, J. Virology, 77, 12692-12698, 2003.*

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240 **Molecular requirements for degradation of a modified sense RNA strand by *Escherichia coli* ribonuclease H1**, *D.R. Yazbeck, K.-L. Min and M.J. Damha, Nucleic Acids Research, 30, 3015-3025, 2002.*

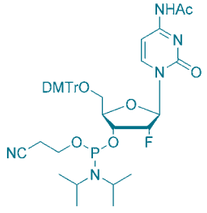
241 *(a)* **Alternating α,β-oligothymidylates with alternating (3’-3’)- and (5’-5’)-internucleotidic phosphodiester linkages as models for antisense oligodeoxyribonucleotides**, *M. Koga, M.F. Moore and S.L. Beaucage, J. Org. Chem., 56, 3757-3759, 1991; (b)* **Synthesis and physicochemical properties of alternating α,β- oligodeoxyribonucleotides with alternating (3’-3’)- and (5’-5’)-internucleotidic phosphodiester linkages**, *M. Koga, A. Wilk, M.F. Moore, C.L. Scremin, L. Zhou and S.L. Beaucage, J. Org. Chem., 60, 1520-1530, 1995.*

242 *(a)* **Antisense effect of oligodeoxynucleotides with inverted terminal internucleotidic linkages: a minimal modification protecting against nucleolytic degradation**, *J.F.R. Ortigao, H. Rosch, H. Selter, A. Frohlich, A. Lorenz, M. Montenarh and H. Seliger, Antisense Res. & Dev., 2, 129-146, 1992; (b)* **Oligonucleotide analogs with terminal 3’-3’- internucleotidic and 5’-5’-internucleotidic linkages as antisense inhibitors of viral gene-expression**, *H. Seliger, A. Frohlich, M. Montenarh, J.F.R. Ortigao and H. Rosch, Nucleosides & Nucleotides, 10, 469-477, 1991.*

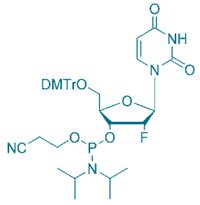
243 **Synthesis of 2’-deoxyribonucleoside 5’-phosphoramidites: New building blocks for the inverse (5’-3’)-oligonucleotide approach**, *T. Wagner and W. Pfleiderer, Helv. Chim. Acta., 83, 2023-2035, 2000.*

244 **Improved synthesis of oligodeoxyribonucleotides**, *K.P. Stengele and W. Pfleiderer, Tetrahedron Lett., 31, 2549-2552, 1990.*

245 **3’-Modified oligonucleotides by reverse DNA synthesis**, *C.D. Claeboe, R. Gao and S. M. Hecht, Nucleic Acids Research, 31, 5685-5691, 2003.*



LK2079



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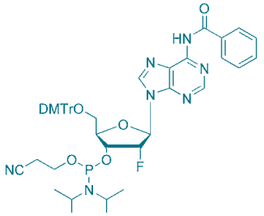

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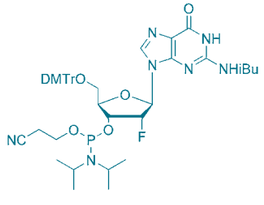

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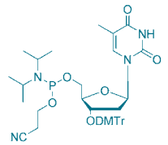
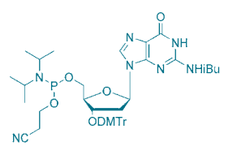


LK2020



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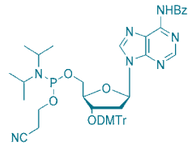

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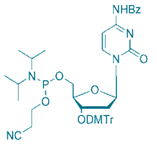
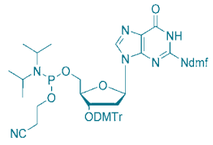
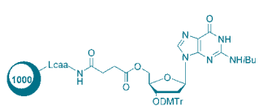


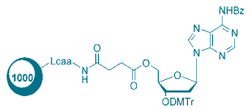
LK2023



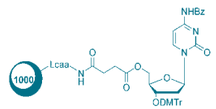
LK2093



LK2298



LK2355



LK2356

Chemical phosphorylation

Chemical phosphorylation is a cost-effective alternative to enzymatic methods (using T4 polynucleotide kinase and ATP), allowing efficient introduction of terminal phosphate groups. Oligonucleotides containing a 5'-phosphate group have various applications, being most widely used as a means of ligating one oligo to another, e.g. as linkers and adapters, in cloning, gene construction, and ligation in general. This is still the most common method of gene synthesis. 3'-Phosphorylations, however, are used to block enzyme activity. For example, this is an efficient and commonly used PCR blocking technique.

Phosphorylation of the 5'-terminus on oligonucleotides is routinely achieved, with higher yields than using kinase, using Phosphate-ON (**LK2101**/**BNS-5010**) (also known as Chemical Phosphorylation Reagent (CPR))[246]. Aside from its inherent convenience, CPR also has the advantage over enzymatic methods in allowing determination of the phosphorylation efficiency due to the presence of the DMTr protecting group. However, the trityl group cannot be used as a purification handle. It is eliminated along with the sulphonyl ethyl group to produce the 5'-phosphate during the ammonium hydroxide deprotection.

LK2101/**BNS-5010** can also be used at the 3'-end to incorporate a 3'-phosphate by addition to any support (e.g. a dT column). This is particularly useful for labelling long oligos where higher pore sized resins for modification are not available. It is for this reason we introduced the 3000 Å phosphate support (see below).

This technique is not only limited to phosphate modification, since any modifying phosphoramidite can be added to the phosphate-ON-T. In this case the oligo will be terminated at the 3'-end with "modifier-phosphate-3'".

Another phosphorylation reagent, known as CPR II (Figure 22, **LK2110**/**BNS-5009**), has been described by researchers at the University of Turku in Finland.[247]

With this product, conventional ammonium hydroxide cleavage gives rise to an oligonucleotide protected at the 5'-phosphate with a DMTr-ether. At this stage, the oligo may be easily separated from truncated impurities by e.g. RP-HPLC or cartridge-purification. The DMTr-group is then removed by aqueous acid and brief ammonium hydroxide treatment yields the 5'-phosphate. Alternatively, the yield of the last coupling may be quantified by detritylation of the oligo whilst still on the support. Deprotection then leads to the 5'-phosphorylated oligo.

This CPR II reagent has been further refined (by substituting the ethyl esters for methyl amides) to provide a product (**LK2127**, also known as CPR IIa),[248] that offers all the benefits of CPR II whilst also being a stable solid that permits easy weighing, handling and dissolution

246 **A chemical 5'-phosphorylation of oligodeoxyribonucleotides that can be monitored by trityl cation release**, *T. Horn and M. Urdea, Tetrahedron Lett., 27, 4705-4708, 1986.*
247 **A new approach for chemical phosphorylation of oligonucleotides at the 5'-terminus**, *A. Guzaev, H. Salo, A. Azhayev and H. Lonnberg, Tetrahedron, 51, 9375-9384, 1995.*
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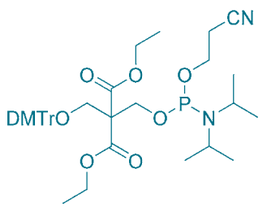
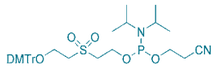
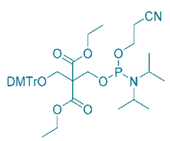


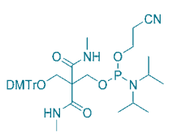
Figure 22. CPR II (LK2110/ BNS-5009).



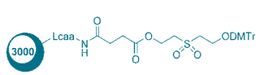
LK2101



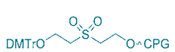
LK2110



LK2127



LK2398



LK2279

(**LK2101**/**BNS-5010** and **LK2110**/**BNS-5009** are both viscous glasses). This product also allows the option of DMT ON purification (see Figure 23).

Although **LK2101**/**BNS-5010** can be used in 3'-phosphorylations, 3'-Phosphate CPG 1000/110 and 3000/110 (**LK2279**/**BG1-5000** and **LK2398**) allow direct preparation of oligonucleotides with a 3'-phosphate group. **LK2127** or **LK2110**/**BNS-5009** cannot be used for 3'-phosphorylation since the DMTr- protected OH is required to release the phosphate group.

The presence of the methylamides in **LK2127** protects the modification from ß-elimination reactions until the base hydrolysis during deprotection. This can therefore be used in conjunction with Fmoc or levulinyl protected branching monomers (e.g. **LK2150**, see page 93) without forming the phosphate moiety until the deprotection step. **LK2101**/**BNS-5010** is not protected against ß-elimination and would form the phosphate moiety during deprotection of the branching point. Hence if this is used at the 3' end it would cleave the oligo from the support (as would **LK2279**/**BG1-5000**, **LK2398**).

Ordering chemical phosphorylation reagents

A growing range of Chemical Phosphorylation Reagents are available from Biosearch Technologies' NAC product catalogue. See our website for full product offering.

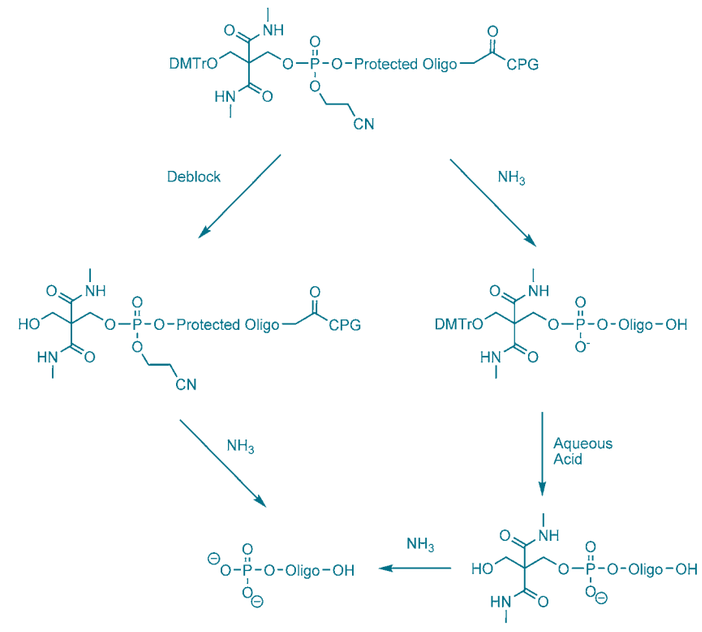
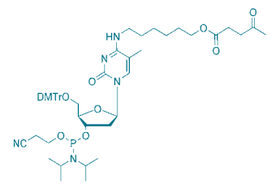


Figure 23. 5'-Phosphorylation using LK2127.



LK2150

Fluorescence detection

Introduction

Dye-labelled oligonucleotides have many important biochemical and analytical uses. For certain applications, such as DNA Sanger sequencing and *in situ* hybridisation (e.g. FISH), oligos are required to be singly labelled. Subsequent detection and analysis rely on the fluorescent properties of the dye, most of which emit light in the visible spectrum.

Other types of oligonucleotides - e.g. probes for Real-time quantification of DNA and RNA[249] (Fluorophore-Quencher (FQ) probes) and allele discrimination[250] (Molecular Beacons™) - are doubly labelled, one dye acting as a fluorophore, the other as a quencher.

Where a fluorophore/quencher pair is used in such applications dynamic quenching occurs via either FRET (Fluorescence Resonance Energy Transfer) or by collisional quenching. For the most part, the mechanism is dependent on the quencher. For example, BHQ™s are FRET quenchers, whereas dabcyl works via collision. The latter is thought to work independently of the extinction coefficient, whereas in FRET a high extinction coefficient is important. This is thought to be the reason for the high quenching efficiency of BHQs.

Consideration must also be given to the overlap of the donor emission and acceptor absorption spectra. It is crucial that this is effective to generate efficient quenching.

Regardless of the quenching mechanism, the result is essentially the same. A dual-labelled probe (Taqman® Probes, Molecular Beacons, Scorpion Primers etc) hybridise to the amplicon formed during PCR and the fluorophore becomes separated from the quencher. This mechanism is dependent on the probe type but in general either the probe opens, increasing the distance between the fluorophore and quencher such that quenching no longer occurs (Molecular Beacons), or the fluorophore (or quencher) is cleaved from the probe (Taqman Probes) (most common).

The fluorophore/quencher pair of choice is dependent on the emission wavelength of the fluorophore, i.e. the detection signal required. However, there are cases where there is a requirement to modify the emission wavelength of the fluorophore. Typically a combination of FAM/ROX/quencher (e.g. DDQ-I) is used. In this case there are two donor-acceptor interactions. FAM/ROX shifts the wavelength of the signal and the quencher acts as the acceptor for the FAM/ROX emission.

While the use of a quencher in this type of application is the most widely used, FRET studies are known where two fluorophores are used.[251] This is often applied in structural studies, e.g. RNA/proteins[252], although it has been shown that FRET signal is dependent on the position of the dyes in the oligo.[253]

This section gives an overview of the dye labelled products available from Biosearch Technologies that can be used in such applications.

249 **Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems**, *S.A. Bustin, J. Molecular Endocrinology, 29, 23-39, 2002.*
250 **Multiplex detection of single-nucleotide variations using molecular beacons**, *S.A.E. Marras, F.R. Kramer and S. Tyagi, Genetic Analysis, 14, 151-156, 1999.*
251 **Spectroscopic investigation of a FRET molecular beacon containing two fluorophores for probing DNA/RNA sequences**, *S. Jockusch, A.A. Marti, N.J. Turro, Z. Li, X. Li, J. Ju, N. Stevens and D.L. Akins, Photochemical & Photobiological Sciences, 5, 493-498, 2006.*
252 **Single-Molecule Observation of the Induction of k-Turn RNA Structure on Binding L7Ae Protein**, *J. Wang, T. Fessl, K.T. Schroeder, J. Ouellet, Y. Liu, A.D.J. Freeman and D.M.J. Lilley, Biophysical Journal, 103, 2541–2548, 2012.*
253 **Orientation dependence in fluorescent energy transfer between Cy3 and Cy5 terminally attached to double-stranded nucleic acids**, *A. Iqbal, S. Arslan, B. Okumus, T.J. Wilson, G. Giraud, D.G. Norman, T. Ha and D.M. J. Lilley, PNAS, 105, 11176-11181, 2008.*

Fluorophores

TAMRA labelling

This is the most commonly used rhodamine dye in oligonucleotide-based applications. The fluorescent properties of TAMRA are sometimes used in oligonucleotide labelling, however TAMRA is more often used as a quencher (see page 83).

Fluorescein labelling

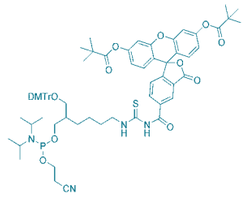
There are several ways of labelling an oligonucleotide with fluorescein-type dyes. The choice of label is diverse, depending on the degree of chlorination of the aromatic rings. This determines the fluorescence emission of the dye. 5'-Fluorescein-CE Phosphoramidite (6-FAM) (**LK2134**/**BA0054**), derived from the single isomer 6-carboxyfluorescein, 5'-Hexachloro-fluorescein-CE Phosphoramidite (HEX) (**LK2136**/**BNS-5032**) and 5'-Tetrachlorofluorescein-CE Phosphoramidite (TET) (**LK2137**/**BA0377**), can all be used to efficiently label an oligonucleotide at the 5'-end.

There are two other phosphoramidites available from LINK that can be used for labelling an oligonucleotide with fluorescein. While both 6-Fluorescein-CE Phosphoramidite (**LK2139**) and Fluorescein-CE Phosphoramidite (**LK2148**) incorporate the same fluorescent dye as **LK2134**/**BA0054**, the linking backbone differs. **LK2139** has a 1,3-diol structure, where the additional OH is protected with DMTr. This not only allows coupling efficiency monitoring by DMTr release, but it also allows the possibility of multiple additions within the oligo for use in, e.g. chromosome painting. However, this often requires a linker (e.g. spacer-18 **LK2129**/**BNS-5036**) to be incorporated between each addition to prevent self-quenching of fluorescein. In the same way, spacer-C3 (**LK2113**/**BNS-5041**) is used to mimic the distance between the 3' and 5'-O of dR, the 1,3-diol arrangement of **LK2139** provides the same scenario. It must be noted that, as with spacer-C3, a distortion of the backbone occurs, particularly with multiple incorporations. As with all 5'-DMTr protected (or pseudo 5' species), the DMTr group can be used to aid purification.

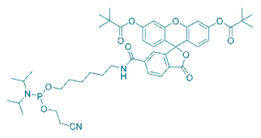
Fluorescein-CE Phosphoramidite (**LK2148**) offers the same possibilities as **LK2139** but in this case the linker is attached to the fluorescein via a thiourea linkage. This mimics the original method of incorporating fluorescein to an amino-modified oligo. It must be noted however that the linkage is attached via the 5 position of the ring system in this case.

Internal sequence additions of Fluorescein are achieved using Fluorescein-dT-CE Phosphoramidite (**LK2068**/**BNS-5047**), by substituting any suitable dT residue. Again, multiple additions can be carried out but the spacing between each fluorescein-dT is crucial to prevent self-quenching.

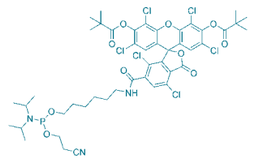
Labelling the 3'-end of an oligo with fluorescein can be achieved using one of four available supports. 3'-Fluorescein CPG (**LK2359**), based on the 5-isomer of the substituted fluorescein, and 3'-(6-Fluorescein) CPG (**LK2368**), prepared from 6-FAM, are commonly chosen for this purpose. In addition, we offer 3'-(6-FAM) CPG (**LK2366**) and Fluorescein-dT CPG (**LK2370**), which are also derived from 6-carboxy fluorescein. **LK2366** also allows the effective blockage of the 3'-terminus from polymerase extension, as well as exonuclease activity. **LK2370** allows both of these activities to proceed. Cleavage and deprotection, typically with ammonium



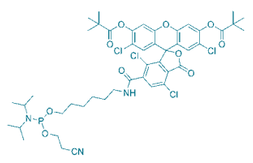
LK2148



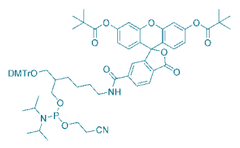
LK2134



LK2136



LK2137



LK2139

hydroxide, liberates the fluorescein-labelled oligo when using any of these supports.

Ordering Fluorescein labelling reagents

A wide range of fluorescein labelling reagents are available from Biosearch Technologies' NAC product portfolio. See our website for our full selection on offer.

Cyanine dyes (including Quasar™ dyes)

Cyanine-based dyes have been used for many years in areas such as textiles. Their application in nucleic acid labelling[254] increased firstly with their commercial availability as succinimidyl esters, then latterly as phosphoramidites. Today they are a central part of many diagnostic platforms and assays based on fluorophore labelling and detection.

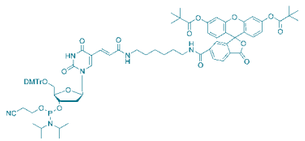
Cyanine dyes are used as fluorescent markers in oligonucleotide synthesis,[255] primarily for molecular diagnostics such as the preparation of probes used in monitoring real-time PCR, fluorescence *in situ* hybridisation (FISH) and in Surface-Enhanced Resonance Raman Spectroscopy (SERRS) based DNA detection assays. Their emission spectra can be tuned by altering the length of the polymethine chain and solubility in organic or aqueous solvents can be altered via the substituents on the aromatic ring.

The most commonly used phosphoramidite dyes are Cyanine-3 (**LK2520**/**BA0407**) and Cyanine-5 (**LK2521**/**BA0404**). These are generally attached to the 5'-end of an oligonucleotide during synthesis, however are easily incorporated within the sequence. The MMT-protected hydroxyl group is removed in the same way as DMTr protection. Internal incorporation is not common due to the lack of heterocyclic base in their structure and as such they do not have the ability to participate in base pairing. This destabilises any duplexes formed.

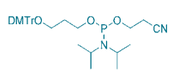
For 3'-attachment, we have introduced the equivalent 3'-modified 1000 Å CPG supports, 3'-Cyanine-3 (**LK2412**/**BA0408**) and 3'-Cyanine-5 (**LK2413**/**BA0406**). Previously this was done by adding the dye post- synthetically onto an amino-modified oligonucleotide or by adding the amidite to a support functionalised with a modification that will not interfere with the use of the oligonucleotide (e.g. phosphate, spacer). 3'-Labelling is particularly useful in

254 **Molecular Probes Based on Cyanine Dyes for Nucleic Acid Research**, *T.G. Deligeorgiev, p125, in Near Infrared Dyes for High Technology Applications, Ed. S. Daehne, U. Resch-Genger and O. Wolfbeis, NATO ASI Series Publ., Kluwer Academic, 1998.*

255 **Fluorescence based strategies for genetic analysis**, *R.T. Ranasinghe and T. Brown, Chem. Commun., 5487- 5502, 2005.*

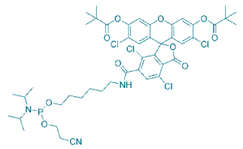


LK2068



LK2113

LK2129



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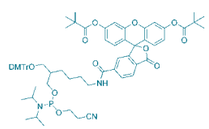

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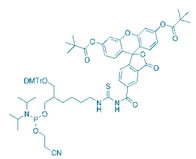

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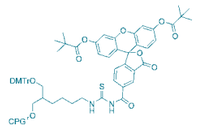

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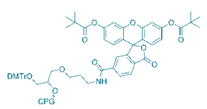
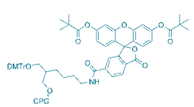


LK2366



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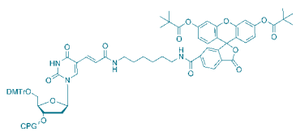


LK2370

FRET where the FRET partner is incorporated either at the 5'-end or within the oligonucleotide sequence.[256]

Quasar dyes 570 and 670 (available as phosphoramidites **LK2158**/**BNS-5063** and **LK2159**/**BNS-5065** respectively) are fluorescent indocarbocyanines, which fluoresce in the yellow-orange (**LK2158**/**BNS-5063**) and red (**LK2159**/**BNS-5065**) regions of the visible spectrum. Both dyes are directly analogous in application to the common cyanine (Cy™) dyes, Quasar 570 for cyanine-3/Cy3 and Quasar 670 for cyanine-5/Cy5 in the labelling of fluorescent probes. The dye phosphoramidites are used directly in automated oligo synthesis. Note, unlike **LK2520**/**BA0407** and **LK2521**/**BA0404**, Quasar dyes do not have the ability to be added internally within an oligo sequence. Both Quasar 570 and 670 are quenched by BHQ-2 (see page 84).

CAL Fluor™ dyes

CAL Fluor Dyes are a set of fluorescent dyes specifically designed for qPCR instruments. These novel xanthene fluorophores can replace previous dyes as a lower-cost alternative. The dyes can be efficiently manufactured, and remain stable to the conditions of oligo synthesis and work up. The attachment chemistry linking the CAL Fluor Dyes to biomolecules eliminates the problem of multiple isomers. This results in dye labels that are easier to manufacture, have a single RP-HPLC peak and have well-defined emission spectra.

CAL Fluor Dyes are available as CPGs and phosphoramidites, allowing facile incorporation during oligo synthesis, which, as we have noted with other products, results in more efficient label incorporation and fewer purification steps than post-synthesis labelling. The CAL Fluor Dyes have emission maxima from 520 nm to 635 nm and can be paired with BHQ-1 or BHQ- 2 for efficient quenching in a variety of probe formats.

Ordering cyanine dyes (including Quasar dyes)

Cyanine dyes are available from Biosearch Technologies. A full offering can be viewed on our website. Please note, all cyanine products require to be shipped on ice.

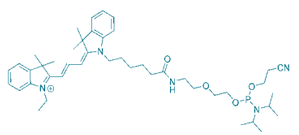
Ordering CAL Fluor dyes

Biosearch Technologies are the proud manufacturers of CAL Fluor reagents and our full offering can be viewed on our website. All CAL Fluor products require to be shipped on ice.

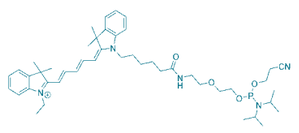
ROX (6-carboxy-X-rhodamine) labelling

ROC fluorophore dye (carboxy-X-rhodamine) is routinely used as a passive reference dye to provide a stable baseline for the fluorescent reporter dyes, i.e SYBR Green I or TaqMan probes, in multiplex quantitative polymerase chain reaction (qPCR) or real time (real-time PCR). The baseline also allows the correction of pipetting errors, fluorescence fluctuations, and instrumental drift such as change of lamp intensity output over time. As the dye does

256 **Fluorescence resonance energy transfer in near-infrared fluorescent oligonucleotide probes for detecting protein-DNA interactions**, *S. Zhang, V. Metelev, D. Tabatadze, P.C. Zamecnik, A. Bogdanov Jr, Proc. Natl. Acad. Sci. USA, 105, 4156-4161, 2008.*



LK2158



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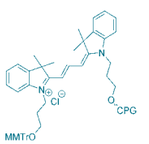
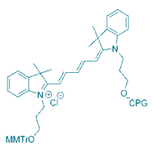
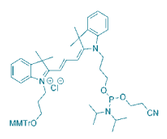


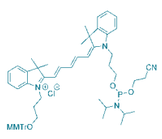
LK2412



LK2413



LK2520



LK2521

not interfere qPCR amplification, a constant amount is added to all samples as a control.

Dependent on the filter and real-time PCR instrument used during experiments, concentration of ROX may need be to be adjusted. Earlier real-time PCR instruments did not have filter matching ROX and thus high concentrations were needed to establish the baseline. Later instruments addressed this short fall with internal standard to correct for light intensity. With the ROX dye being compatible with all PCR instruments, the later instruments are now calibrated for ROX spectral settings and thus removes the need to re-calibrate the thermal cycler.

With an excitation/emission maxima of 586 and 610, the dye has a different emission spectrum compared against the SYBR Green I or TaqMan probes, making it ideal to be used with our BHQ-2 quencher.

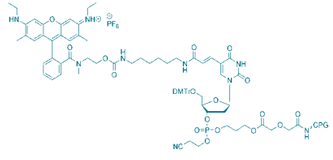
Ordering ROX

ROX conjugated support is available from Biosearch Technologies. Have a look at our website for more details.

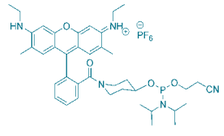
Table 4. Dye Selection Chart. Note the dyes are listed in order of absorbance maxima; the colour scale is used only as a pictorial representation.

Fluorophore	Abs. max. (nm)	Em. max. (nm)
Cy5.5™	675	694
Alexa 647	650	668
Quasar 670/Cyanine-5 (Cy5™)	647	667
BODIPY™ 650/665-X	646	660
BODIPY 630/650-X	625	640
CAL Fluor Red 635	618	637
BODIPY TR-X/Alexa 594	590	617
CAL Fluor Red 610	590	610
Cy3.5™	581	596
BODIPY 581/591	581	591
Redmond Red™	580	594
Texas Red X/Alexa 578	578	603
ROX	575	602
CAL Fluor Red 590	569	591
BODIPY 564/570	563	569
Alexa 546	556	573
TAMRA/Rhodamine Red-X	555	580
Alexa 555	555	565
Quasar 570	548	566
BODIPY TMR/ Cyanine-3 (Cy3)	544	570
CAL Fluor Orange 560	538	559
HEX	535	556

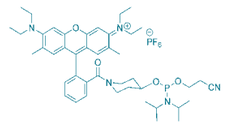
Fluorophore	Abs. max. (nm)	Em. max. (nm)
Alexa 532/VIC/BOD-IPY 530/550	532	554
Yakima Yellow™	531	549
Rhodamine 6G	528	550
CAL Fluor Gold 540	522	544
TET	521	536
JOE	520	548
Oregon Green™ 514	506	526
Rhodamine Green-X	503	528
BODIPY FL	502	513
Alexa 488	495	519
6-FAM	494	525
BODIPY 493/503	493	503
Cy2™	489	506
Alexa 430	433	539
Coumarin	432	472
Pacific Blue	416	451
Acridine	362	462
Marina Blue	362	459
Alexa 350	346	442
Edans	336	468
Fluorescein/DANSYL	335	518



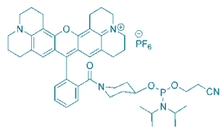
LK2423



LK2538



LK2539



LK2540

Quenchers

TAMRA labelling

The light-absorbing properties of TAMRA, and spectral overlap with several commonly used fluorophores - including FAM, HEX, TET and JOE, make it useful as a quencher for the design of dual-labelled probes. The usefulness of TAMRA is, however, limited because of its broad emission spectrum, which reduces its capabilities in multiplexing. Its intrinsic fluorescence contributes to the background signal, potentially reducing the sensitivity of assays based on TAMRA. Despite these limitations, TAMRA has been used extensively in the design of probe-based assays, perhaps most notably in Taqman probes for Real-Time PCR.

Oligonucleotides can be labelled with TAMRA using two distinct methodologies. TAMRA is not sufficiently stable to strong bases; the molecule degrades in the presence of ammonium hydroxide. If this deprotection is required, the oligonucleotide is synthesised with an amino group at either the 3'- (most common), or 5'-end and labelled with TAMRA post-synthetically using TAMRA-NHS Ester. Oligonucleotides synthesised using mild deprotection monomers can be labelled directly with TAMRA, either internally by substituting any suitable dT residue with TAMRA-dT-CE Phosphoramidite (**LK2143**), or at the 3'-end using 3'-TAMRA CPG support (**LK2434**). Subsequent deprotection of the oligo is achieved with tbutylamine/ methanol/ water (1:1:2) for 2.5h at 70 ˚C. Although there is still a small amount of TAMRA degradation, this is easily removed during purification. As previously mentioned, TAMRA is also a fluorophore and, although one of the most widely used quenchers, applications often require the use of a dark quencher.

Ordering TAMRA labelling reagents

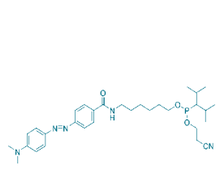
TAMRA reagents are available for view on our website.

Non-fluorescent (Dark) quenchers

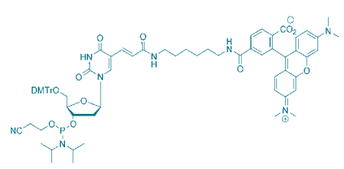
Dabcyl labelling

Dabcyl, because of its light absorbance properties and lack of residual fluorescence, has been widely used as a quencher in diagnostic probes such as Molecular Beacons.[257] A Molecular Beacon is a hybridisation probe consisting of a fluorophore, a quencher and a defined section of the oligonucleotide sequence complementary to that of the target nucleic acid. In the inactive state, when the probe is not hybridised to its target sequence, the fluorescence energy of the fluorophore is transferred to the quencher by a process of collisional quenching. For light energy transfer to take place efficiently, both fluorophore and quencher have to be in close proximity. This requirement is accounted for in the design of Molecular Beacons in that the two parts of the stem hybridise to hold the F/Q pair in close proximity. Hybridisation of the Molecular Beacon probe to its target sequence results in the separation of the stem and hence the F/Q pair, resulting in fluorescence.

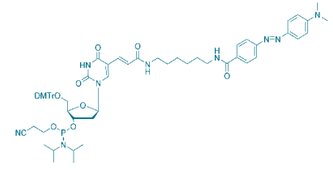
257 **Molecular beacons: probes that fluoresce upon hybridisation**, *S. Tyagi and F.R. Kramer, Nature Biotechnology, 14, 303-308, 1996.*



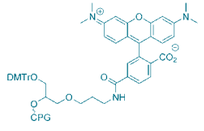
LK2085



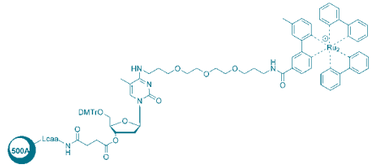
LK2143



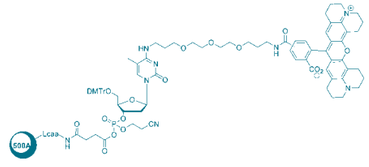
LK2144



LK2372



BG5-5070



BG5-5021

Since dabcyl is stable to oligo synthesis it can be incorporated at any point in the sequence via one of our modifiers: at the 5' end using 5'-Dabcyl-CE Phosphoramidite (**LK2085**/**BNS-5023**) - e.g. for use in TwistAmp fpg probes; at the 3'-end using 3'-Dabcyl CPG (**LK2374**) - e.g. for Taqman probes, duplex Scorpions and Molecular Beacons); or internally using Dabcyl-dT-CE Phosphoramidite (**LK2144**) - e.g. in Scorpion Primers. Deprotection of the oligo is dependent on the F/Q pair but in general is as per unmodified oligos.

Dabcyl's absorption properties limit the range of dyes it can quench to those emitting at 400-550 nm (absorption maximum, 471 nm). However, when used in Molecular Beacons, the fluorophore and dabcyl are brought close enough to allow a slightly broader spectrum of dyes to be quenched, thereby increasing the versatility of the dabcyl molecule.

Although dabcyl was originally the quencher of choice, for the most part this has been superceded by BHQ dyes.

Black Hole Quencher reagents

The demands of modern genomic and diagnostic applications, which are typically centred around an ever increasing need for greater assay sensitivity, has led to the development of a series of new non-fluorescent quenchers. Some of the best known of these are the Black Hole Quencher™ reagents (BHQ reagents) that have been specifically optimised for FRET-based quenching.

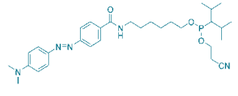
Due to the high extinction co-efficiency and the broad spectral overlap covered by each BHQ dye, the efficiency of quenching is increased, when compared to molecules like dabcyl. This in turn means that BHQ dyes provide access to a much larger range of wavelengths for detection purposes, covering visible into near IR regions of the spectrum (480-730 nm). Coupling this with the fact that these molecules have no residual background fluorescence (they are true dark quenchers) makes BHQ dyes a favourable choice for real-time PCR applications. All four original BHQ quenchers are available from Biosearch Technologies, and while all are used readily within the industry, BHQ-1 and BHQ-2 are the more popular, either as the 5'-Phosphoramidites (items **LK2154**/**BNS-5051N** and **LK2155**/**BNS-5052N** respectively), the dT-Phosphoramidites (**LK2156**/**BNS-5051T** and **LK2157**/**BNS-5052T**) or the 3'-CPGs (**LK2379**/**BG1-5041G** and **LK2380**/**BG1-5042G**). Only considering the excitation and emission values suggests Cy5, Cyanine-5 and Quasar 670 require BHQ-3 for efficient quenching, however BHQ-2 is recommended because it is less susceptible to degradation. BHQ-1 is typically used to quench in the range 480-580 nm and can be used in conjunction with the commonly used fluorophores; e.g. FAM, TET, JOE and HEX. BHQ-2 is used to quench in the range 550- 650 nm and is most effective in quenching fluorophores such as TAMRA, ROX, Cyanine-3, Cy3, Cy3.5™ and Red 640. Each of the available BHQ phosphoramidites and CPGs are used directly in automated synthesis.

Ordering dabcyl labelling reagents

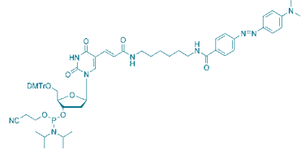
Our website contains all of Biosearch Technologies' offering of dabcyl labelled reagents for sale. Please review and if you have any questions, contact Customer Service.

Ordering Black Hole Quencher reagents

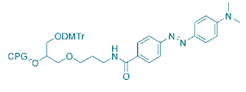
Black Hole Quencher reagents are the most referenced quenchers in peer reviewed scientific journals. To see which BHQ reagents you need, see our website. Note, all BHQ products require to be shipped on ice.



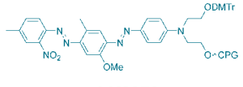
LK2085



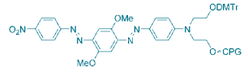
LK2144



LK2374



LK2379



LK2380

Deep Dark Quencher 1

Deep Dark Quencher 1 is a non-fluorescent molecule quenching the shorter wavelength dyes such as FAM. As such, its quenching properties are very similar to that of dabcyl (see Figure 24 below). This modification is available as a 3'-modifier CPG 1000 Å support (**LK2349**).

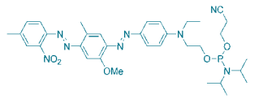
In this case the quencher is attached to the anomeric position of dRibose. This removes the possibility of losing the label during deprotection, a problem often associated with dabcyl due to the 1,2-diol configuration. Additionally, incorporation of DDQ-1 results in preservation of the natural sugar-phosphate backbone meaning there is no adverse effect on the structure of the oligo.

Ordering Deep Dark quencher 1

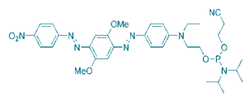
A range of Deep Dark Quenchers are available from Biosearch Technologies. See our website for our full selection on offer.

BlackBerry™ Quencher

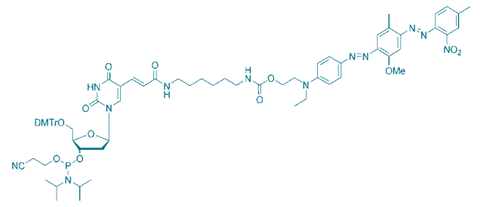
While in the excited state, fluorophores are sensitive to their environment and may lose excitation energy by several processes besides emission of a fluorescence photon. Such fluorescence quenching can occur by collision or molecular motion (dynamic quenching), excited state reaction with other molecules (photobleaching), contact quenching (the formation of a nonfluorescent ground state complex, also known as static quenching), or energy transfer to another molecule via fluorescence resonance energy transfer, (FRET). Many nucleic acid fluorescence detection techniques use probes that bear both a fluorophore and a quencher, relying on FRET- or contact-mode quenching to diminish fluorescence until a hybridisation event occurs. Upon hybridisation, the fluorophore and quencher are separated in space, resulting in an increase in fluorescence. The efficiency of FRET from the fluorophore to the quencher (i.e., the magnitude of quenching) depends on the relative orientation of their transition dipoles, the distance between them, and how well the absorption spectrum of the quencher overlaps the emission spectrum of the



LK2154



LK2155



LK2156

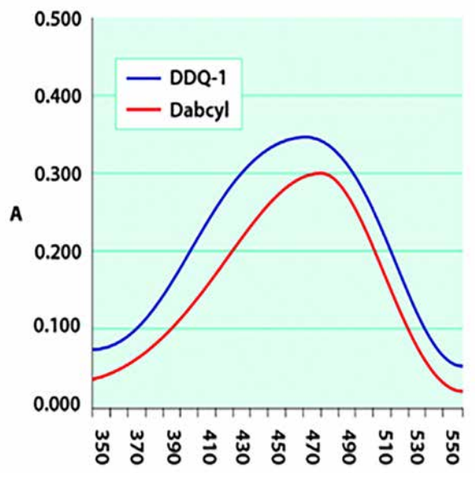
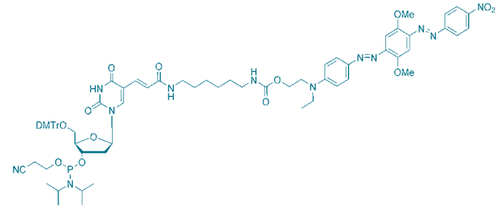
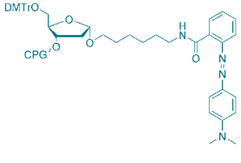


Figure 24. Comparative UV absorption spectra of 3'-Dabcyl– and 3'-DDQ-1- labelled oligonucleotides (DDQ-1 λmax 471nm, Dabcyl λmax473nm).



LK2157



LK2349

fluorophore. The efficiency of contact quenching depends on the ability of the fluorophore and quencher to form a ground-state complex and correlates with the mutual affinity of the two species and the distance between them. Quenchers may themselves be fluorescent, emitting a photon at a longer wavelength than the acceptor fluorophore.

More conveniently, dark quenchers can be used,(see above) which are non-fluorescent chromophores that, in FRET mode, can absorb energy from the excited state of the fluorophore, preventing emission of a fluorescence photon. The resultant excited state of a dark quencher relaxes to the ground state by radiationless decay (heat). In contact mode, the dark quencher forms a non-fluorescent ground-state complex with the fluorophore, masking its fluorescence until the complex is disrupted by a hybridisation event.

The archetypal dark quencher is dabcyl (page 83). A desirable characteristic of such quenchers is a long-wavelength absorption maximum. Unfortunately, the chemical stability of such compounds is often diminished. Extended π systems and/or the use of stronger donor-acceptor functional group pairings can lead to sensitivity to oligonucleotide synthesis reagents, e.g., oxidants such as iodine and deblocking agents such as ammonia and AMA.

Biosearch Technologies offers our BlackBerry Quencher 650 (BBQ-650) as a synthesis-stable dark quencher of long wavelength fluorescence (Figure 25). An 8-alkoxyjulolidine moiety was found to be a powerful π -electron donor, affording a surprising bathochromic shift when compared to related compounds. The absorption spectrum of a BBQ-650-tagged oligonucleotide is shown in Figure 26. The broad absorbance centred around 650 nm effectively overlaps the emission maxima of popular

Table 5. Dye/Quencher Selection Chart. Note the dyes are listed in order of emission maxima; the colour scale is used only as a pictorial representation.

Fluorophore	Abs. max. (nm)	Em. max. (nm)	Quencher	
Cy5.5	675	694		
Alexa 647	650	668	BHQ-2 559-670 Abs. Max. 579nm	
Quasar™ 670/Cyanine-5 (Cy5)	647	667		
BODIPY 650/665-X	646	660		
BODIPY™ 630/650-X	625	640		
CAL Fluor Red 635	618	637		
BODIPY TR-X/Alexa 594	590	617		
CAL Fluor Red 610	590	610		
Texas Red X/Alexa 568	578	603		
ROX	575	602		
Cy3.5	581	596		
Redmond Red	580	594		
BODIPY 581/591	581	591		
CAL Fluor Red 590	569	591		
TAMRA/Rhodamine Red-X	555	580		BHQ-1 480-580 Abs. Max. 534nm
Alexa 546	556	573		
BODIPY TMR/Cyanine-3 (Cy3)	544	570		
BODIPY 564/570	563	569		
Quasar 570	548	566		
Alexa 555	555	565		
CAL Fluor Orange 560	538	559		
HEX	535	556	BHQ-1 480-580 Abs. Max. 534nm	
Alexa 532/VIC/BODIPY 530/550	532	554		
Rhodamine 6G	528	550		
Yakima Yellow	531	549		
JOE	520	548		
CAL Fluor Gold 540	522	544		
Alexa 430	433	539		
TET	521	536		
Rhodamine Green-X	503	528		
Oregon Green 514	506	526		
6-FAM	494	525		
Alexa 488	495	519		
Fluorescein/DANSYL	335	518		
BODIPY FL	502	513		
Cy2™	489	506		
BODIPY 493/503	493	503		
Coumarin	432	472		
Edans	336	468		
Acridine	362	462		
Marina Blue	362	459		
Pacific Blue	416	451		
Alexa 350	346	442		

long-wavelength fluorophores such as Cy3, TAMRA, Texas Red, ROX, Cy5, and Cy5.5, allowing efficient quenching.

BlackBerry Quenchers may be installed at the 3' terminus, internally, or at the 5' position using the reagents shown in Figure 27. To evaluate BlackBerry Quencher 650 in contact quenching mode, molecular beacon probes bearing various 5'-fluorophores (FAM, Cy3™, Texas Red, Cy5™, Cy5.5™) were synthesised using 3'-BBQ-650 CPG. Signal-to background ratios upon binding to fully-complementary target were noted to be excellent, e.g., >90 with Cy5 and >88 with Cy5.5. The probes are known to be successful in typing C to T transitions at positions 627 and 630 of the human chemokine receptor 5 gene,[258] and produced excellent results in real-time PCR studies.

BlackBerry Quencher 650 are known to be excellent FRET-mode quenchers. Pairs of complementary strands were designed that would bring a 5'-Cy5.5 fluorophore to within 5 or 10 base pairs (20-40 Å) of a 3'-BBQ-650 upon hybridisation,[259] where quenching efficiencies of ≥98.3 and ≥98.9%, respectively, were observed. Melting temperatures of these hybrids were unchanged from non-labelled hybrids, showing that these quenching efficiencies were due to FRET quenching and not contact quenching.

Ordering BlackBerry Quencher products

See our website for all BlackBerry Quenchers on offer from Biosearch Technologies' NAC product catalogue.

258 **Genotyping SNPs With Molecular Beacons**, *Marras, S. A. E.; Kramer, F. R.; Tyagi, S. Methods in Molecular Biology 2003, 212, 111-128.*

259 **Efficiencies of fluorescence resonance energy transfer and contact-mediated quenching in oligonucleotide probes.** , *Marras, S. A. E.; Kramer, F. R.; Tyagi, S. Nucleic Acids Res. 2002, 30, e122.*

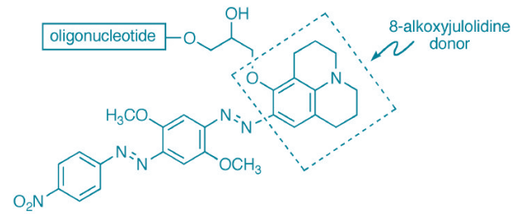


Figure 25. BlackBerry Quencher 650 (BBQ-650).

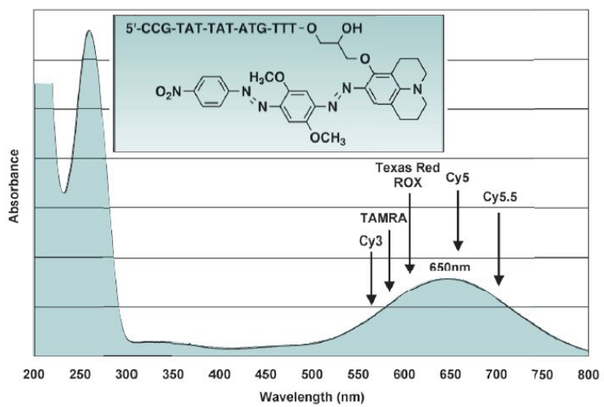


Figure 26. Absorption spectrum of a 15-mer bearing a 3' BlackBerry Quencher 650.

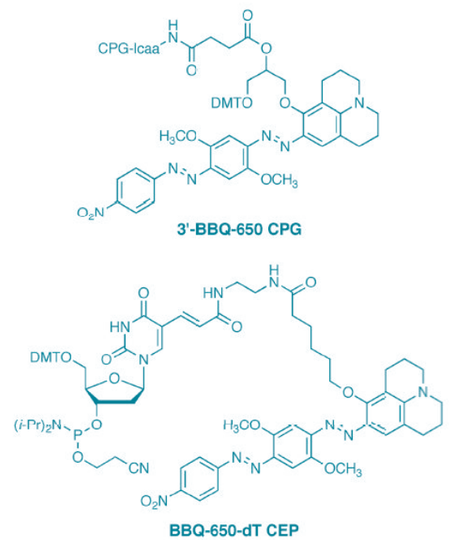


Figure 27. Reagents for the incorporation of BlackBerry Quencher 650 into oligonucleotides.

Blueberry Quenchers

With our legacy partnerships, Biosearch Technologies have developed a novel line of pH sensitive quenchers. The ability to tune absorbance as a function of pH sets these quenchers apart from others on the market.

Imaging ions in a cellular environment is an ongoing challenge for advancing research in clinical diagnostics, biochemical research, and environmental science among other specialties. The field has expanded from the invention of fluorescent calcium imaging indicators[260] to include a variety of sensing tools one of which is nanoscale ion-selective optodes.[261] These nanoscale optodes are the equivalent of ion-selective electrodes and offer an advantage over traditional molecular indicators in that the recognition moiety and the optical reporter are separate components. This modular construction enables tuning the sensor for dynamic range, sensitivity, wavelength and selectivity.

Efforts are underway to overcome the optode limitations arising from the use of fluorescent indicators from the Nile red series.[262] A key step in this work has been the successful use of the quencher dye Blueberry-C6-ester-652 (**BLU00652**) in place of the more traditional chromoionophore III (CH III) in the potassium specific nanosensor formulation.[264] Figure 28 shows the sensor mechanism utilizing the pH sensitive Blueberry-C6-ester-652 along with a static fluorophore in ion-selective optodes. The change in FRET between quencher and fluorophore results from the change in absorbance of the quencher as a function of potassium ion extraction. Figure 29 shows both the extinction coefficient characterisation (A) and the pH titration (B).

In addition to Blueberry-C6-ester-652 (**BLU00652**), Biosearch Technologies now provides all Blueberry Quenchers including the Blueberry pyridyl C6 ester with a maximum absorption of 556 nm, pKa of 5.6 and e = 36,750 M-1 cm-1, and Blueberry-cyano-C6-ester (**BLU00655**) which has a maximum absorption of 623 nm, pKa of 8.0 and e = 24,500 M-1 cm-1. These quenchers exhibit broad absorption ranges from 450 nm–700 nm, and cover a wide range of physiologically relevant pKa's.

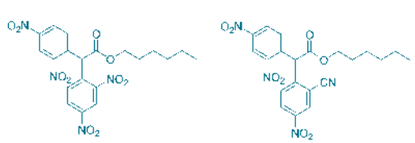
Ordering Blueberry Quenchers

The full range of Blueberry Quenchers are available from Biosearch Technologies. See our website for a full review of products.

260 **A new generation of Ca2+ indicators with greatly improved fluorescence properties**, *Grynkiewicz, G.; Poenie, M.; Tsien, R.Y. J. Biol. Chem. 1985, 260, 3440-3450.*

261 *a)* **Ion-Selective Optodes Measure Extracellular Potassium Flux in Excitable Cells**, *Harjes, D.I.; Dubach, J.M.; Rosenzweig, A.; Das, S.; Clark, H.A. Macromolecular Rapid Commun. 2010, 31, 217-221. b)* **Visualizing sodium dynamics in isolated cardiomyocytes using fluorescent nanosensors**, *Dubach, J.M.; Das, S.; Rosenzweig, A.; Clark, H.A. Proc. Nat. Academy of Sci. 2009, 106, 16145-16150. c)* **Ion selective optodes: from the bulk to the nanoscale**, *Dubach, J.M.; Integrative Biol. 2011, 3, 142-148. d) Xie, X.; Bakker, E. Anal. Bioanal. Chem. 2015, 407, 3899-3910.*

262 **Development of an Optical Nanosensor Incorporating a Novel Quencher Dye for Potassium Imaging**, *Sahari, A.; Ruckh, T.; Hutchings, R.; Clark, H. submitted to Anal. Chem. 2015.*



Colourimetric detection and capture

Colourimetric detection is one of the oldest diagnostic techniques. This is based on the interaction of an enzyme e.g. HRP interacting with a substrate. This also requires some form of capture of the target with a hapten labelled probe and the duplex captured using an affinity column or matrix loaded with a suitable protein or antibody. Examples of haptens are biotin, DNP and DIG, the most commonly used being biotin in conjunction with streptavidin or avidin. The enzyme labelled oligo then hybridises to another part of the immobilised target and treatment with the substrate produces a distinctive colour.

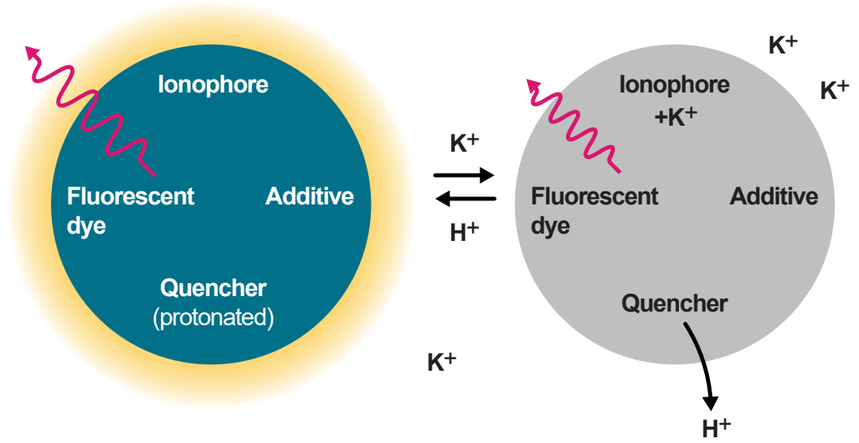


Figure 28. The pH-based mechanism of potassium nanosensors.

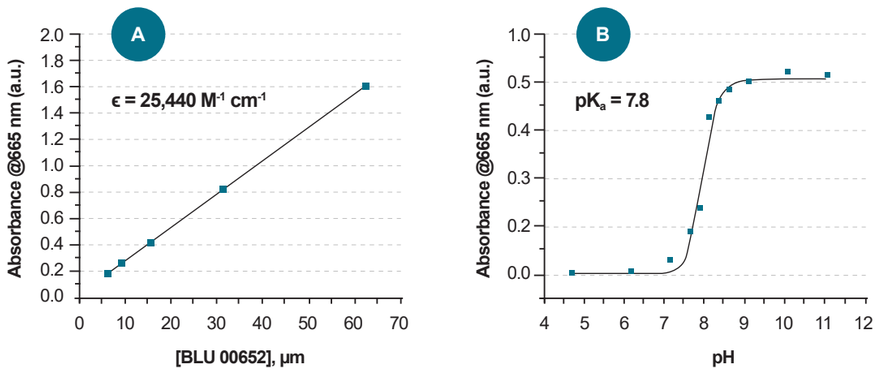


Figure 29. Blueberry-C6-ester-652 quencher dye: (A) extinction coefficient characterisation, and (B) pH titration in CH3CN/ Buffer (1:1).

Biotin labelling

The uses of avidin-biotin technology are diverse.[263] Applications include the detection of proteins by non-radioactive immunoassays, cytochemical staining, cell separation, isolation of nucleic acids, detection of specific DNA/RNA sequences by hybridisation, and probing of conformational changes in ion channels.

Many of these applications require the use of oligos containing biotin at one or more positions. The availability of functional biotin, in turn, provides the opportunity for immobilisation on pre-coated solid surfaces.[264] An extension of this technology using the photocleavable biotin product (**LK2122**) is described on page 67.

Several different reagents are available for labelling nucleic acids with biotin. Choosing the right one will depend largely on the position within the oligonucleotide requiring to be labelled. Biotin-CE Phosphoramidite (**LK2140**) is based on a 1,3-diol structure where one hydroxyl is protected with DMTr and the other is the phosphoramidite, hence it can be used for adding multiple biotins to either the 3', or 5' end of an oligonucleotide. It has been suggested that this property could be exploited in the development of diagnostic probes, in applications such as ELISA, in which signal amplification is often beneficial. This has been shown using *in situ* hybridisation studies where three biotins at either end of the oligo gives the optimal signal.[265]

Biotin-TEG-CE Phosphoramidite (**LK2132**) can be used in a similar way to **LK2140** for adding biotin to the 3'- and 5'- ends of an oligo. This phosphoramidite also has an extended 15 atom mixed polarity spacer arm based on a triethylene glycol linker. The benefits of an extended spacer arm separating the biotin function from the rest of the oligo may be seen in applications where possible steric hindrance effects could be reduced as a result, e.g. when dual-labelling with bulky reporter molecules, such as haptens, dyes, or enzymes. Note the 1,2-diol arrangement makes cleavage during deprotection possible therefore it is advisable to keep the 5'-DMTr group on until after deprotection.

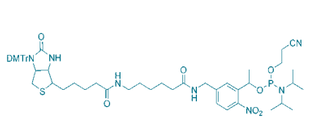
5'-Biotin-CE Phosphoramidite (**LK2109/BNS-5021**) can also be used for adding biotin to an oligo, but only to the 5'-end.[266] The DMTr protection on the N1 of biotin prevents branching during coupling. The DMTr group can, however, be used to assist in reverse-phase cartridge and HPLC purification although biotin is hydrophobic enough to obtain good separation of biotin labelled oligos (DMT OFF) and unlabelled oligos.

263 *See for example: (a)* **Avidin-Biotin Technology**, *M. Wilchek and E.A. Bayer (Eds.), in Methods in Enzymology, J.N. Abelson and M.I. Simon (Series Eds.), Volume 184, 671pp, Academic Press, 1990; (b)* **The biotin-(strept)avidin system: Principles and applications in biotechnology**, *E.P. Diamandis and T.K. Christopoulos, Clinical Chem., 37, 625-636, 1991.*

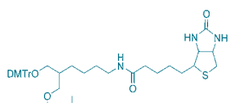
264 *See for example:* **Electrochemical detection of non-labelled oligonucleotide DNA using biotin-modified DNA(ss) on a streptavidin-modified gold electrode**, *J.W. Park, H.-Y. Lee, J.M. Kim, R. Yamasaki, T. Kanno, H. Tanaka, H. Tanaka and T. Kawai, J. Bioscience and Bioengineering, 97, 29-32, 2004.*

265 **A comparative study of digoxigenin, 2,4-dinitrophenyl, and alkaline phosphatase as deoxyoligonucleotide labels in non-radioisotopic in situ hybridisation**, *S.J. Harper, E. Bailey, C.M. McKeen, A.S. Stewart, J.H. Pringle, J. Feeholly and T. Brown, J. Clinical Pathology, 50, 686-690, 1997.*

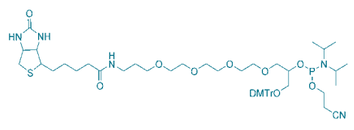
266 *For a recent diagnostic application see:* **Detection and differentiation of Plasmodium species by polymerase chain reaction and colorimetric detection in blood sample of patients with suspected malaria**, *D.M. Whiley, G.M. LeCornec, A. Baddeley, J. Savill, M.W. Syrmis, I.M. Mackay, D.J. Siebert, D. Burns, M. Nissen and T.P. Sloots, Diagnostic Microbiology and Infectious Disease, 49, 25-29, 2004.*



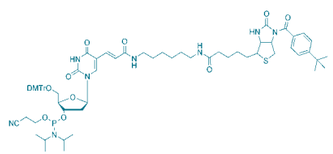
LK2122



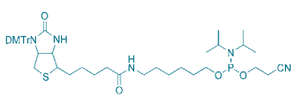
LK2140



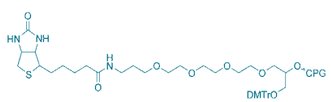
LK2132



LK2067



LK2109



LK2353

The addition of biotin internally within an oligonucleotide sequence is achieved using Biotin-dT-CE Phosphoramidite (**LK2067/BNS-5022**), where any suitable dT position within the sequence can be replaced with biotin-dT. The tert-butylbenzoyl group, used to increase solubility and to protect the biotin, is removed in the ammonium hydroxide deprotection step.

Finally, the direct labelling of the 3'-end of an oligonucleotide sequence with biotin is also possible and is routinely achieved using 3'-Biotin-TEG CPG (**LK2353**), which incorporates biotin at the first step in the synthesis process.

Ordering biotin labelling reagents

The expanded NAC products now offers Biotin labelled products. See our website for our full selection on offer.

Electrochemical detection

Ferrocene labelling

Ferrocene (Fc) and its derivatives are attractive electrochemical probes for nucleic acid analysis because of their stability and convenient synthetic chemistry. Early examples of Fc labelling have utilised the conjugation of carboxy-Fc to 5'-amino-modified oligos.[267] Internal post-synthetic labelling of DNA probes has been obtained by reaction with ferrocenecarboxaldehyde or aminoferrocene.[268] For direct incorporation into oligonucleotides, Fc phosphoramidites[269] and monomers with a ferrocenyl moiety linked to position 5 of 2'- dU[270] and dC[237] or the 2' sugar position of dA and dC[271] have been described, as has on column derivatisation of I-dU with ferrocenyl propargylamide.[272] Methods using redox tagging have also been employed.[273]

Recently Brisset and co-workers[274] have described the synthesis and use of abasic Fc-modified phosphoramidites, including the preparation of Fc-

267 *(a) Electrochemically active DNA probes: Detection of target DNA sequences at femtomole level by high-performance liquid chromatography with electrochemical detection, S. Takenaka, Y. Uto, H. Kondo, T. Ihara and M. Takagi, Anal. Biochem., 218, 436-443, 1994; (b) Ferrocene-oligonucleotide conjugates for electrochemical probing of DNA, T. Ihara, Y. Maruo, S. Takenaka and M. Takagi, Nucleic Acids Research, 24, 4273-4280, 1996; (c) Electrochemical analysis of DNA amplified by the polymerase chain reaction with a ferrocenylated oligonucleotide, Y. Uto, H. Kondo, M. Abe, T. Suzuki and S. Takenaka, Anal. Biochem., 250, 122-124, 1997.*

268 *Electrochemical detection of sequence-specific DNA using a DNA probe labelled with aminoferrocene and chitosan modified electrode immobilized with ssDNA, C. Xu, H. Cai, P. He and Y. Fang, Analyst, 126, 62-65, 2001.*

269 *(a) M. Wiessler and D. Schutte, European Patent WO9709337 (1997); (b) T.Chunlin, US Patent Application US2009/0155795 A1 (2009).*

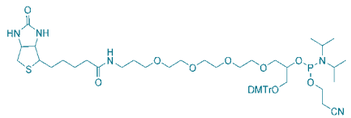
270 *(a)* **Uridine-conjugated ferrocene DNA oligonucleotides: Unexpected cyclization reaction of the uridine base**, *C.J. Yu, H. Yowanto, Y. Wan, T.J. Meade, Y. Chong, M. Strong, L.H. Donilon, J.F. Kayyem, M. Gozin and G.F. Blackburn, J. Amer, Chem. Soc., 122, 6767-6768, 2000; (b)* **Ferrocene-modified pyrimidine nucleosides: synthesis, structure and electrochemistry**, *H. Song, X. Li, Y. Long, G. Schatte and H.-B. Kraatz, Dalton Trans., 4696-4701, 2006.*

271 **2'-Ribose-ferrocene oligonucleotides for electronic detection of nucleic acids**, *C.J. Yu, H. Wang, Y. Wan, H. Yowanto, J.C. Kim, L.H. Donilon, C. Tao, M. Strong and Y. Chong, J. Org. Chem., 66, 2937-2942, 2001.*

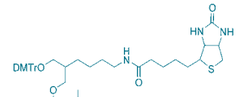
272 **On-column derivatization of oligodeoxynucleotides with ferrocene**, *A.E. Beilstein and M.W. Grinstaff, Chem. Commun., 509-510, 2000.*

273 *(a)* **Synthesis of the first ferrocene-labelled dideoxynucleotide and its use for the 3'-redox end-labelling of 5'-modified single-stranded oligonucleotides**, *A. Anne, B. Blanc and J. Moiroux, Bioconjug. Chem., 12, 396-405, 2001; (b)* **Ferrocene conjugates of dUTP for enzymatic redox labelling of DNA**, *W.A Wlassoff and G.C. King, Nucleic Acids Research, 30, e58, 2002.*

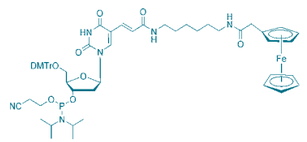
274 *(a)* **Automated synthesis of new ferrocenyl-modified oligonucleotides: study of their properties in solution**, *A.E. Navarro, N. Spinelli, C. Moustrou, C. Chaix, B. Mandrand and H. Brisset, Nucleic Acids Research, 32, 5310- 5319, 2004; (b)* **Supported synthesis of ferrocene modified oligonucleotides as new electroactive DNA probes**, *A.-E. Navarro, N. Spinelli, C. Chaix, C. Moustrou, B. Mandrand and H. Brisset, Bioorg. Med. Chem. Lett., 14, 2439-2441, 2004; (c) C. Chaix-Bauvais et al, US Patent Application US2005/0038234*



LK2132



LK2140



LK2167

modified phosphorothioates[275] however, to our knowledge, these are not commercially available. In any case, reported coupling efficiencies and oligo synthesis yields are relatively low.

To provide a robust phosphoramidite for direct incorporation into oligos we chose a structure (item **LK2167**) analogous to our current dT products (amino, dabcyl, biotin, fluorescein etc). This both simplifies its synthesis and imparts the benefits of having a nucleobasic structure consistent with natural DNA-sugar-phosphate backbone. Further, as the Fc-modification is on the 5-position of the pyrimidine, natural base-pairing to dA will still occur.

Ordering electrochemical labelling reagent

Electrochemical reagents are manufactured by Biosearch Technologies and now available on our website.

Branching modification

Branched DNA (bDNA) has become a significant tool in diagnostics research and, in particular, gene expression analysis.[276] For example, branching possibilities can be exploited to achieve multiplicity of labelled probe hybridisation to target sequences leading to enhanced signals. 5-Me-dC-Brancher CE Phosphoramidite (**LK2150**)[277] has been designed to provide a facile route to incorporate branching capability into an oligonucleotide.

The Me-dC Brancher is a 5'-trityl-protected, 3'-phosphoramidite dT analogue, that can be incorporated into an oligonucleotide during synthesis. The levulinyl group on the branching chain is removed with buffered hydrazine at neutral pH—conditions that do not affect any other groups (e.g. it does not cleave from the support)—yet it does not degrade during storage and synthesis, unlike the Fmoc protection used on other commercially available branching phosphoramidites. This product has the advantage of being nucleosidic, thereby preserving internucleotide distance, therefore perturbs DNA structure less than, for example, a non-nucleosidic doubler or trebler molecule.

A1 (2005).

275 **The first automated synthesis of ferrocene-labelled phosphorothioate DNA probe: A new potential tool for the fabrication of microarrays**, *H. Brisset, A.-E. Navarro, N. Spinelli, C. Chaix and B. Mandrand, Biotechnol. J., 1, 95-98, 2006.*

276 *(a)* **Nucleic Acid Detection Technologies—Labels, Strategies, and Formats**, *L.J. Kricka, Clinical Chemistry, 45, 453-458, 1999; (b)* **Signal amplification through nucleotide extension and excision on a dendritic DNA platform**, *S. Capaldi, R.C. Getts, and S.D. Jayasena, Nucleic Acids Research, 28, e21, 2000.*

277 *(a)* **Forks and combs and DNA: The synthesis of branched oligodeoxyribonucleotides**, *T. Horn and M.S. Urdea, Nucleic Acids Research, 17, 6959-6967, 1989; (b)* **An improved divergent synthesis of comb-type branched oligodeoxyribonucleotides (bDNA) containing multiple secondary sequences**, *T. Horn, C-A. Chang and M.S. Urdea, Nucleic Acids Research, 25, 4835-4841, 1997; (c)* **Chemical synthesis and characterization of branched oligodeoxynucleotides (bDNA) for use as signal amplifiers in nucleic acid quantification assays**, *T. Horn, C-A. Chang and M.S. Urdea, Nucleic Acids Research, 25, 4842-4849, 1997.*

Ordering branching modifier

Our website highlights all Branching Modifiers from Biosearch Technologies.

It must be noted that, although visually this structure resembles Me-dC, the linker on the N-4 position results in hybridisation akin to dT. Therefore, if hybridisation is required at the branching point, this modifier must replace a T base within the natural DNA sequence.

Use of branching Me-dC CE phosphoramidites to incorporate site specific modifiers

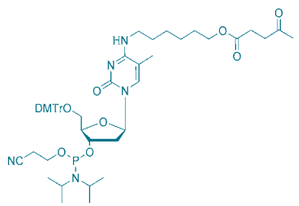
It is widely known that branching monomers are used in the preparation of oligonucleotide dendrimers[278] and **LK2150** has been used to generate comb and fork like oligonucleotide structures for use in nucleic acid hybridisation assay as a means of signal amplification.[265]

However, it is also possible to incorporate reporter groups into specific sites within an oligonucleotide sequence using this monomer in an analogous manner to the method reported by Brown et al.[279] Here they used a branching dT phosphoramidite (1) to incorporate dyes such as Cyanine-5 (**LK2521**/**BA0404**) within the sequence for use in real-time probes such as HyBeacon or Angler probes.

In this case 1, (Figure 30) was incorporated within an oligonucleotide where the 5'-end is blocked either by retaining the DMT group, capping with acetyl protection or by the incorporation of a terminal modifier e.g. 6-FAM CE Phosphoramidite (**LK2134**/**BA0054**). While keeping the oligonucleotide on the column, the Fmoc group is removed with 20% piperidine in MeCN or DMF and the cyanine dye phosphoramidite is added to the branching point under the same conditions as incorporation at the 5'-end. This is outlined in Figure 31.

278 *(a)* **Oligonucleotide dendrimers: Synthesis and use as polylabelled DNA probes**, *M. S. Shchepinov, I. A. Udalova, A. J. Bridgman and E. M. Southern, Nucleic Acids Research, 25, 4447-4454, 1997; (b)* **Branched oligonucleotides induce in vivo gene conversion of a mutated EGFP reporter**, *P. A. Olsen, C. McKeen and S. Krauss, Gene Therapy, 10, 1830-1840, 2003.*

279 **Synthesis of a modified thymidine monomer for site-specific incorporation of reporter groups into oligonucleotides**, *L. J. Brown, J. P. May, T. Brown, Tetrahedron Letters, 42, 2587-2591, 2001.*



LK2150

Although it is possible to incorporate **LK2521/BA0404** within an oligonucleotide sequence, this results in a destabilised duplex whereas the use of the modified dT has no adverse effect. This is also true when **LK2150** is used in the same way. Although a Me-dC anaolgue, the presence of the branching chain on the N4 position of the pyrimidine results in this modifier having hybridisation properties akin to dT rather than dC hence is incorporated as a 'dT' position of the oligonucleotide sequence. In this case (see Figure 32), the levulinyl protection is removed using 0.5 M hydrazine hydrate in pyridine/acetic acid 1:1.

The use of such branching monomers opens up the possibility of incorporating modifiers only available as 5'-addition amidites internally within the sequence. For instance this gives a means of generating HEX-dT (2) using **LK2150** and **LK2136/BNS-5032** or cholesteryl dT (3) using **LK2150** and **LK2170** as shown in Figure 33 on page 96. Neither of these dT modifiers are commercially available as amidites.

This can be particularly useful in evaluating which marker works best in a given application without the expense of synthesising a range of modified dT amidites to get the same result. Using this information, the preferred modified dT amidite can be synthesised with the peace of mind of knowing that the resulting oligonucleotide will give the desired result when used in an assay.

Although originally **LK2150** was designed for the preparation of highly branched oligonucleotides at Biosearch Technologies we see the potential of this product to allow the incorporation of modified dT bases not commercially available within the sequence.

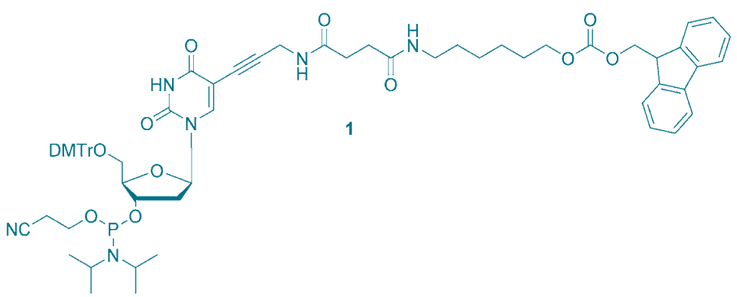


Figure 30. Branching dT

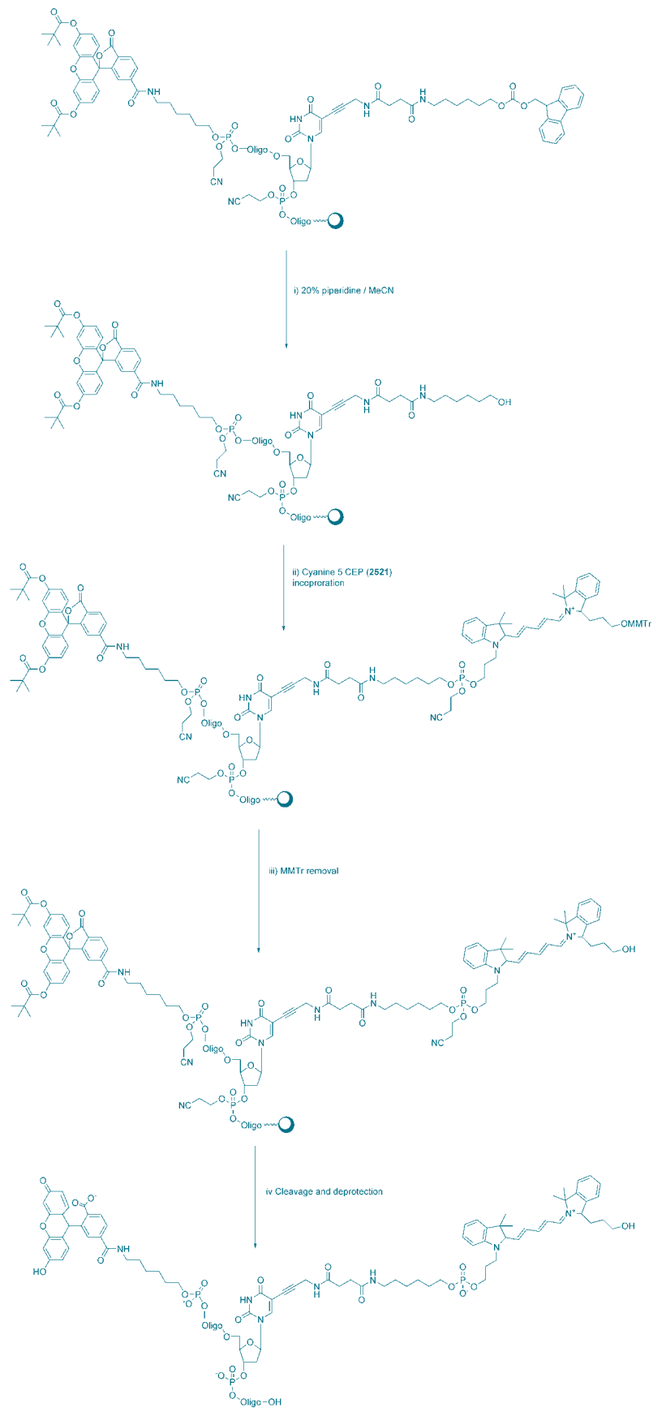
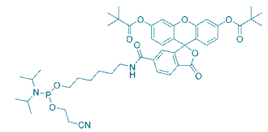
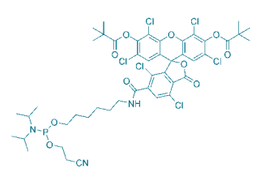


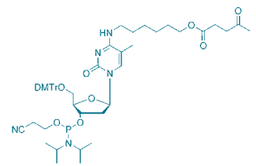
Figure 31. Use of Fmoc protected branching dT amidite for incorporation of cyanine-5.



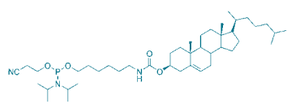
LK2134



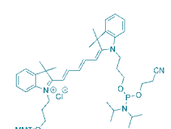
LK2136



LK2150



LK2170



LK2521

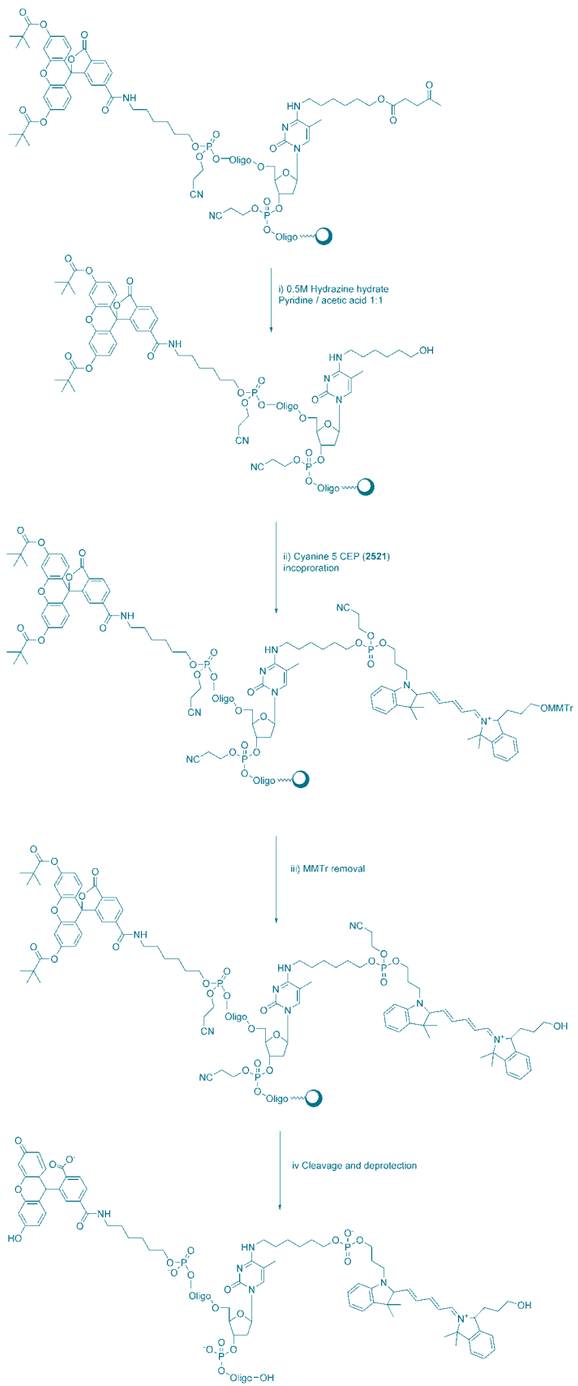


Figure 32. Use of Levulinyl protected branching Me-dC amidite for incorporation of cyanine-5.

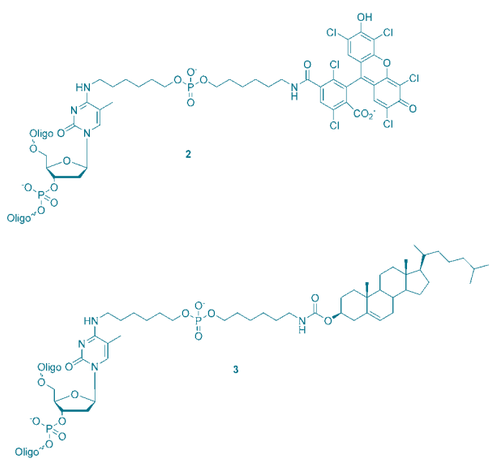


Figure 33. Generation of HEX dT (2) and cholesteryl dT (3) within an oligonucleotide sequence using 2150.

Cell delivery and uptake

Despite advances in oligonucleotide therapeutics, the main issues remain cell delivery and cellular uptake. A number of strategies have been developed to combat this, the most widely used being the conjugation of a 'delivery' reagent to the oligonucleotide. In general, the reagent is hydrophobic in nature, e.g. cholesterol, and is often attached via a cleavable linker.

This is typically incorporated at the 5'-end of the oligo, and for siRNA is incorporated on the sense (passenger) strand.

Lipophilic modification

The introduction of hydrophobic (lipophilic) residues into oligonucleotides with a view to improving their penetration into cells has recently met with some success.[280] Cholesteryl- conjugated oligonucleotides have in particular been the subject of substantial interest in antisense and other studies due to the lipophilicity and good availability of cholesterol. One such study[281] has shown the use of cholesteryl-modified siRNA in therapeutic gene silencing.

Historically this has been attached by post-synthetic conjugation of an amino-modified oligo to cholesterol chloroformate,[282] however direct attachment during synthesis is much more convenient. 5'-Attachment is possible via a modified phosphoramidite.[283,284,285] By comparison to other cholesterol amidites available we have found 5'-Cholesterol-CE Phosphoramidite (**LK2170**) to offer specific advantages in oligo synthesis.[286]

Since the cholesterol is attached directly to aminohexanol, it is not susceptible to 1,2-diol elimination as observed in some other products. Lack of a trityl group simplifies purification; some cholesterol products must be used in trityl-on mode (to prevent 1,2-diol elimination during deprotection), then detritylated, and can subsequently be very difficult to purify.

280 *See for example:* **Cholesterol conjugated oligonucleotide and LNA: A comparison of cellular and nuclear uptake by Hep2 cells enhanced by Streptolysin-O**, *Š. Holasová, M. Mojžíšek, M. Bunček, D. Vokurková, H. Radilová, M. Šafářová, M. Červinka and R. Haluza, Molecular and Cellular Biochem., 276, 61-69, 2005.*

281 **Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs**, *J. Soutschek, A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, M. John, V. Kesavan, G. Lavine, R.K. Pandey, T. Racie, K.G. Rajeev, I. Röhl, I. Toudjarska, G. Wang, S. Wuschko, D. Bumcrot, V. Koteliansky, S. Limmer, M. Manoharan and H.-P. Vornlocher, Nature, 432, 173-178, 2004.*

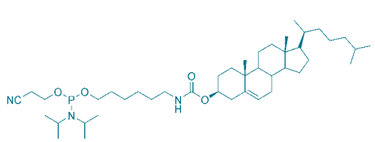
282 *See for example: (a)* **Cholesteryl-conjugated oligonucleotides: synthesis, properties, and activity as inhibitors of replication of human immunodeficiency virus in cell culture**, *R.L. Letsinger, G. Zhang, D.K. Sun, T. Ikeuchi and P.S. Sarin, Proc. Natl. Acad. Sci., 86, 6553-6556, 1989; and (b)* **A simplified synthesis of acridine and/or lipid containing oligodeoxynucleotides**, *C.J. Marasco, Jnr., N.J. Angelino, B. Paul and B.J. Dolnick, Tetrahedron Lett., 35, 3029-3032, 1994. Other methods, such as conjugation via a disulphide bond to terminal phosphate groups, have also been used. See for example:* **Antisense effects of cholesterol-oligodeoxynucleotide conjugates associated with poly(alkylcyanoacrylate) nanoparticles**, *G. Godard, A.S. Boutorine, E. Saison-Behmoaras and C. Hélène, Eur. J. Biochem., 232, 404-410, 1995.*

283 **Assembling liposomes by means of an oligonucleotide tagged with a lipophilic unit**, *N. Maru, K. Shohda and T. Sugawara, Nucleic Acids Symposium Series No. 48, 95-96, 2004.*

284 **Synthesis and physical properties of anti-HIV antisense oligonucleotides bearing terminal lipophilic groups**, *C. MacKellar, D. Graham, D.W. Will, S. Burgess and T. Brown, Nucleic Acids Research, 20, 3411-3417, 1992.*

285 **Mode of action of 5'-linked cholesteryl phosphorothioate oligodeoxynucleotides in inhibiting syncytia formation and infection by HIV-1 and HIV-2 in vitro**, *C. A. Stein, Ranajit Pal, A. L. DeVico, G. Hoke, S. Mumbauer, O. Kinstler, M. G. Sarngadharan and R. L. Letsinger, Biochemistry, 30, 2439 - 2444, 1991.*

286 *For a detailed assessment of our cholesterol modifications see:* **Plant derived cholesterol modifications: Comparative use in oligonucleotide synthesis**, *S. Aitken, D. Hannah, U. Ixkes, C. McKeen and D. Picken, available online: https://linktechsupport.zendesk.com/hc/en-us/articles/200143398-Plant-Derived-Cholesterol-Modifications-A-Comparison-of-Commercially-Available-Cholesterol-Phosphoramidites-and-Solid-Supports-for-use-in-Oligonucleotide-Synthesis-of-DNA-and-RNA-TC-and-TBDMS-chemistries-.*



LK2170

Significantly, its coupling efficiency (final modification step) is routinely >90% giving a high yield of modified product. This compares favourably to final (5') modification efficiencies we have observed with other commercial products. This product can readily be used in automated synthesis. Although, like most cholesterol products, it is not soluble in acetonitrile, it is easily dissolved using dichloromethane as the diluent. Unlike competing products, there is no requirement for solvent mixtures that include THF (this solvent can cause problems in some large-scale automated instruments).

Aside from other oligonucleotide design criteria, 3'-modification can offer the added benefit of, at least partially, protecting the oligo from exonucleases in the cell. For this purpose we offer 3'-Cholesterol CPG 1000/110 (**LK2394**).

This product has a couple of notable advantages over competing products. Like the phosphoramidite (**LK2170**), it is not susceptible to the 1,2-diol elimination observed in some other supports. Furthermore, since the modification is based on the natural sugar-phosphate backbone, there are no adverse structural effects on the oligo. The product can also be used without IP restriction.

At the request of several customers, we have extended our cholesterol-modification range to include a TEG-based product, 5'-Cholesterol-TEG-CE Phosphoramidite (**LK2189**). This, too, is a simple 5'-modifier without the complications of a 1,2-diol and trityl protection. This product has the added benefit of solubility in acetonitrile.[288]

The strict guidelines imposed by regulatory authorities now make it essential to use nonanimal-based products in pharmaceutical drug development for humans. With increasing frequency, therefore, our customers are requesting that we supply products with BSE/TSE statements. We have now developed an alternative route to these products that uses entirely plant-derived cholesterol, making them even better choices for modification of oligos.

Of similar application, but comparatively less studied to date, is the incorporation of the palmitoyl moiety into oligonucleotides. One such use employs an oligonucleotide conjugate with a 5'-palmitoyl group attached through an amide bond.[287] This has been used to modify GRN163, a thio-phosphoramidate oligonucleotide, to enhance the potency of telomerase inhibition. We offer both 5'-Palmitate-C6-CE Phosphoramidite (**LK2199**) and 3'-Palmitate CPG 1000/110 (**LK2393**) for direct incorporation of a palmitoyl group during oligo synthesis, at the 5' and 3' end respectively.

See our website for ordering lipophilic modifiers from Biosearch Technologies.

As with cholesterol modifications, other lipophiles such as tocopherol (vitamin E) have been shown to have potential use in the delivery of oligonucleotides into cells. Vitamins such as tocopherol are not produced by the target cells, but are used by the latter and therefore vitamins are recognised. They are thought to be internalised by cells only after interaction with a binding protein and therefore have the potential for specific targeting of a cell type.[288'289,290,291]

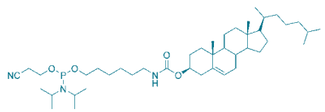
287 **Lipid modification of GNR163, an N3' P5' thio-phosphoramidate oligonucleotide, enhances the potency of telomerase inhibition**, *B.-S. Herbert, G.C. Gellert, A. Hochreiter, K. Pongracz, W.E. Wright, D. Zielinska, A.C. Chin, C.B. Harley, J.W. Shay and S.M. Gryaznov, Oncogene, 24, 5262-5268, 2005.*

288 **Delivery of oligonucleotides and analogues: The oligonucleotide conjugate-based approach**, *F. Marlin, P. Simon, T. Saison-Behmoaras and C. Giovannangeli, ChemBioChem., 11, 1493-1500, 2010.*

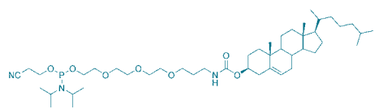
289 **Efficient in vivo delivery of siRNA to the liver by conjugation to alpha-tocopherol**, *K. Nishina, T. Unno, Y. Uno, T. Kubodera, T. Kanouchi, H. Mizusawa and T. Yokota, Mol. Ther., 16, 734-740, 2008.*

290 **Resolution of liver cirrhosis using vitamin-A coupled liposomes to deliver siRNA against a collagen- specific chaperone**, *Y. Sato, K. Murase, J. Kato, M. Kobune, T. Sato, Y. Kawano, R. Takimoto, K. Takada, K. Miyanishi, T. Matsunaga, T. Takayama and Y. Niitsu, Nat. Biotechnol., 26, 431-442, 2008.*

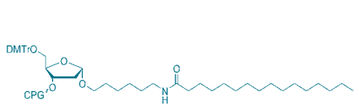
291 **Attachment of vitamin E derivatives to oligonucleotides during solid-phase synthesis**, *D. Will and T. Brown, Tet. Letts ., 33, 2729-2732, 1992.*



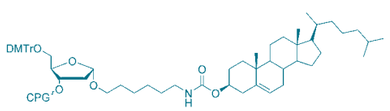
LK2170



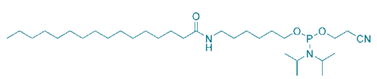
LK2189



LK2393



LK2394



LK2199

We have extended our line of lipophilic modifiers to include two products, namely 5'-Tocopherol-CE Phosphoramidite (**LK2163**) and the analogous 5'-Octyltocopherol-CE Phosphoramidite (**LK2194**). These can be used to introduce tocopherol at the 5' end, either directly on the 5'-OH of the final base or in conjunction with a linker such as C6 S-S thiol (**LK2126/BNS-5042**). This latter approach enables the tocopherol to be cleaved via the disulphide bridge, for example once the oligo has been delivered to the cell. As a spacer arm is often required for label distancing, **LK2194** was developed with a "built in" C8 spacer.[292]

As an aside, the hydrophobic nature of tocopherol has also been utilised as a means of improving the purification of ribozymes.[293] We have also demonstrated the use of tocopherol products as a means of allowing an initial purification of thiol-modified oligos with a view to improving the efficiency of a second, e.g. ion-exchange, purification.[294,295]

More recently, the conjugation of therapeutic oligos to a trivalent N-acetylgalactosamine (GalNAc) ligand has become the technology of choice to deliver nucleic acid-based therapeutics to the liver. GalNAc avidly binds to the Asialoglycoprotein receptor (ASGPR) that is predominantly and abundantly expressed on liver hepatocytes, resulting in rapid and efficient endocytosis.[296] The first GalNAc-siRNA conjugate therapeutics have already received regulatory approval and many more are in clinical development. GalNAc conjugates offer a simpler approach than LNP formulations and has become the method of choice for hepatic delivery.

For that reason, we have developed our own GalNAc ligands which offer the flexibility to construct individualised GalNAc clusters. For insertion at the 3' end, we offer the dR-GalNAc (Alpha) CPG (**LK2440**, **LK2489** and **LK2568**), while for internal and 5'end addition, the dR-GalNAc (Beta) Phosphoramidite (**LK2568**) would be the product of choice. Our GalNAc monomers can be used to build up flexible GalNAc clusters, varying in the number of GalNAc monomers and in their position within the oligo. Additionally, it is possible to add various spacers between each GalNAc monomer and to incorporate cleavable linkers between the oligo and the GalNAc cluster. Additional advantages include the fact that these GalNAc units can be easily inserted into the oligo during the synthesis and they have a dR moiety that wits directly into the natural sugar-phosphate backbone with little disruption of the duplex, meaning there is minimal effect on the Tm.

See our website for our full list of products and pack sizes.

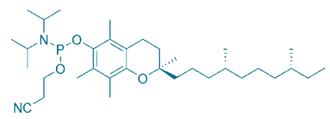
292 **Tocopherol (Vitamin E) modified oligonucleotides**, *S. Aitken, R. Archer, G. McGeoch, C. McKeen and D. Picken, poster presented at TIDES 2011. Available online: https://linktechsupport.zendesk.com/hc/en-us/ articles/200143388-Tocopherol-Vitamin-E-Modified-Oligonucleotides.*

293 **Fast and simple purification of chemically modified hammerhead ribozymes using a lipophilic capture tag**, *B.S. Sproat, T. Rupp, N. Menhardt, D. Keane, and B. Beijer, Nucleic Acids Research, 27, 1950-1955, 1999.*

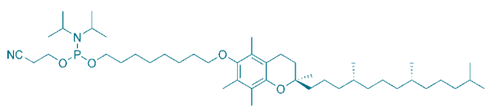
294 **Tocopherol (Vitamin E) modified oligonucleotides II: Utilising hydrophobicity to aid purification**, *S. Aitken, R. Archer, G. McGeoch, C. McKeen and D. Picken, poster presented at the 6th Cambridge Symposium on Nucleic Acids Chemistry and Biology, 2011. Available online: https://linktechsupport.zendesk.com/hc/en-us/articles/200143428- Tocopherol-Vitamin-E-Modified-Oligonucleotides-II-Utilising-Hydrophobicity-to-Aid-Purification.*

295 **Oligonucleotide delivery and purification: Tocopherol modification improves product purification and aids delivery into cells**, *C. McKeen, Gen. Eng. News, 32(3), 22-23, February 1, 2012. Available online: www. genengnews.com/gen-articles/oligonucleotide-delivery-and-purification/3991/.*

296 **Multivalent N-acetylgalactosamine-conjugated siRNA localizes in hepatocytes and elicits robust RNAi-mediated gene silencing.** *Nair JK, Willoughby JL, Chan A, Charisse K, Alam MR, Wang Q, Hoekstra M, Kandasamy P, Kel'in AV, et al. (2014). J Am Chem Soc 136:16958–16961. Crossref, Medline, Google Scholar.*



LK2163



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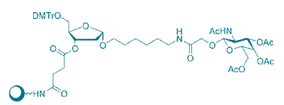
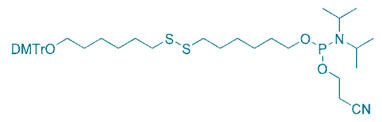
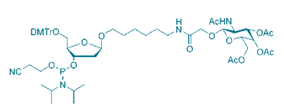


LK2440/LK2489



LK2126



LK2568

Structural studies

Duplex stability/instability

The hybridisation properties of synthetic oligonucleotides are crucial for almost all applications. Optimisation of base pairing, and subsequent duplex stabilisation, is therefore desirable.

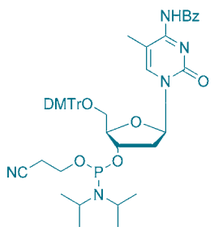
C-5 Methyl pyrimidine nucleosides are known to stabilise duplexes relative to the non- methylated bases. Therefore, the use of 5-Me-dC-CE Phosphoramidite (available either as N-Bz (**LK2017**) or N-Ac (**LK2529**))[297] rather than dC results in enhanced binding (a similar comparison can be made between thymidine and 2'-deoxyuridine). This increase in duplex stabilisation is attributed to the hydrophobic nature of the methyl groups that helps eliminate water molecules from the duplex. We also offer a 5-Me-dC CPG (**LK2323**) for modification at the 3'-end. The stabilisation properties of Me-dC make this a suitable modification for stabilisation of triplex strands, where its presence raises the melting temperature of the third strand.

During duplex hybridisation of unmodified oligos, A-T base pairs have two hydrogen bonds, whereas G-C base pairs have three. One of the simplest methods of improving duplex stabilisation is the use of 2-Amino-dA-CE Phosphoramidite (**LK2145**) (2,6-diaminopurine) in place of dA.[298] This forms an additional hydrogen bond with thymidine (see Figure 34). However, **LK2145** also destabilises A-G wobble mismatches, thus increasing specificity.

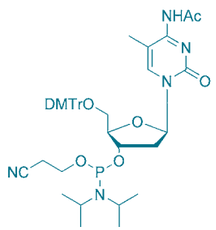
It is also worth noting that 2'-OMe modifications, primarily used to confer nuclease resistance, have the complementary property of duplex stabilisation (see page 72 for more details).

297 **Effect of 5-methylcytosine on the stability of triple-stranded DNA-a thermodynamic study**, *L.E. Xodo, G. Manzini, F. Quadrifoglio, G.A. van der Marel and J.H. van Boom, Nucleic Acids Research, 19, 5625-5631, 1991.*

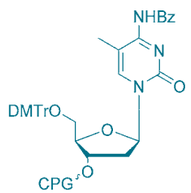
298 **Oligonucleotides containing 2-aminoadenine and 5-methylcytosine are more effective as primers for PCR amplification than their non-modified counterparts**, *Y. Lebedev, N. Akopyants, T. Azhikina, Y. Shevchenko, V. Potapov, D. Stecenko, D. Berg and E. Sverdlov, Genetic Analysis – Biomolecular Engineering, 13, 15-21, 1996.*



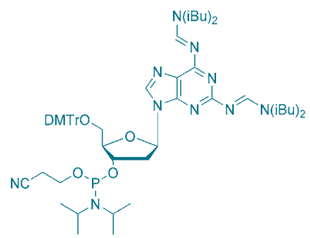
LK2017



LK2529



LK2323



LK2145

Other duplex effects

In sequencing applications, the design of primers can be complicated by the degeneracy of the genetic code (there are 64 possible 3-base codon configurations and only 21 amino acids, and therefore the third base in a sequence codon is often unknown). The problem of degeneracy can also be tackled by the use of universal bases.[299] Deoxyinosine is often used as a degenerate base in an oligonucleotide to alleviate this problem.[300] This is possible since its structure allows it to base pair with all four bases in various 'wobble' structures. However, the base-pairing is not equivalent with each of the 4 naturally occurring bases. The overall preferential order of base-pairing is: dI-dC > dI-dA > dI-dG = dI-dT. We provide both dI-CE Phosphoramidite (**LK2016**/**BNS-5030**) and dI CPG 1000/110 (**LK2293**/**BG1-5015**). 2'-Deoxynebularine[301] is another example. Incorporation of a deoxyuridine base within a DNA sequence can be used to induce mutagenic effects. The enzyme uracil-N-glycosylase (UNG) can specifically remove uracil to create abasic sites at the deoxyuridine positions. This property is used to generate site-specific strand breaks in the oligonucleotide. We provide both dU-CE Phosphoramidite (**LK2013**/**BNS-5031**) and dU CPG 1000/110 (**LK2287**/**BG1-5016**).

Duplex stability/instability modifiers

Deoxyxanthosine

Xanthosine (Figure 35) is a naturally occurring nucleoside containing a purine heterocycle that presents an H-bonding pattern to a complementary strand distinct from that presented by unmodified purines found in encoded oligonucleotides.

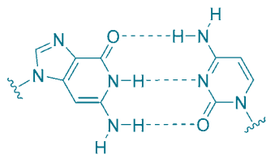
Xanthosine has been proposed as a 'universal base', i.e. a heterocycle that can pair equally well with all four natural nucleosides.[302] As such, several studies have been carried out (as far back as the mid-1980s), incorporating deoxyxanthosine (dX) into oligonucleotides. However, the expected base-pairing properties were not observed.

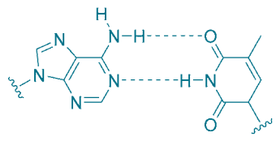
299 **The applications of universal DNA base analogues**, *D. Loakes, Nucleic Acids Research, 29, 2437-2447, 2001.*

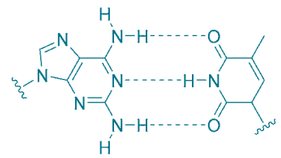
300 *(a)* **Base pairing involving deoxyinosine: implications for probe design**, *F.H. Martin, M.M. Castro, F. Aboul- ela and I. Tinoco, Jr, Nucleic Acids Research, 13, 8927-8938, 1985; (b)* **Studies on the base pairing properties of deoxyinosine by solid phase hybridisation to oligonucleotides**, *S.C. Case-Green, E.M. Southern, Nucleic Acids Research, 22, 131-136, 1994.*

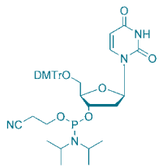
301 *(a)* **Synthesis and properties of oligonucleotides containing 2'-deoxynebularine and 2'-deoxyxanthosine**, *R. Eritja, D.M. Horowitz, P.A. Walker, J.P. Ziehler-Martin, M.S. Boosalis, M.F. Goodman, K. Itakura and B.E. Kaplan, Nucleic Acids Research, 14, 8135-8153, 1986; (b) As a custom item we have prepared the phosphoramidite (***LK2024/BA0016***), see:* **A convenient synthesis of deoxynebularine phosphoramidite**, *D. Picken and V. Gault, Nucleosides, Nucleotides and Nucleic Acids, 16, 937-939, 1997. Please enquire regarding availability.*

302 *(a)* **Double protection of the heterocyclic base of xanthosine and 2'-deoxyxanthosine**, *A. van Aerschot, M. Mag, P. Herdewijn and H. Vanderhaeghe, Nucleosides & Nucleotides, 8, 159-178, 1989; (b)* **Synthesis and properties of oligonucleotides containing 2'-deoxynebularine and 2'-deoxyxanthosine**, *R. Eritja, D.M. Horowitz, P.A. Walker, J.P. Ziehler-Martin, M.S. Boosalis, M.F. Goodman, K. Itakura and B.E. Kaplan, Nucleic Acids Research, 14, 8135-8153, 1986.*

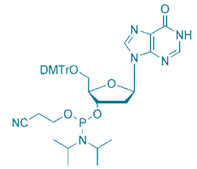




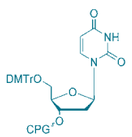




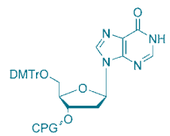
LK2013



LK2016



LK2287



LK2293

Figure 34. Hydrogen bonding patterns.

Other duplex effect modifiers

Other notable properties have been reported however. Benner and co-workers have described the extension of the 'genetic alphabet' by purine partnering dX with 5-(ß-D- ribofuranosyl)pyrimidine-2,4-diamine, a pyrimidine analogue presenting an H-bonding pattern complementary to dX.[303] Recently dX has been used in the study of the physiologically important nitrosative deamination of DNA which is one of the main causes of genomic mutations.[304,305]

Although a number of monomers for the incorporation of dX have been reported (using phosphotriester or phosphoramidite chemistry), the most effective of these is the 2-(4-nitrophenyl)ethyl (NPE) O^2/O^6 doubly-protected monomer, our product 2'-Deoxyxanthosine-CE Phosphoramidite (**LK2164**/**BA0313**)[294] **LK2164**/**BA0313** is used as per standard protocols, with an extra deprotection reagent to remove the NPE groups.

Photocrosslinking

Halogenated nucleosides are versatile reagents in oligo applications. We provide a wide range of halogenated nucleoside phosphoramidites and CPG supports.

Photocrosslinking is a useful technique for the partial definition of the nucleic acid-protein interface of nucleoprotein complexes.[306] Photoactive bases may also be used to probe the crystal structure of the protein-DNA complexes.[307] Photoactive analogues of dC (5-Iodo- and 5-Bromo-dC (**LK2009** and **LK2011**/**BA0124**)) and dT analogues (5-Iodo- and 5-Bromo-dU (**LK2014**/**BA0376** and **LK2012**) are available as phosphoramidites. The Br-dU support (**LK2325**) is also available.

8-Br-dA (**LK2054**/**BA0004**)[308] and 8-Br-dG (**LK2055**) phosphoramidites have been proposed to complete the set of the four photoactive bases required to examine base to amino acid contact pairs, although work in this regard has been limited. 8-Br-dG is also useful in promoting the formation of Z-form DNA structures and for

303 **Differential discrimination of DNA polymerases for variants of the non-standard nucleobase pair between xanthosine and 2,4-diaminopyridine, two components of an expanded genetic alphabet**, *M.J. Lutz, H.A. Held, M. Hottiger, U. Hübscher and S.A. Benner, Nucleic Acids Research, 24, 1308-1313, 1996.*

304 *(a)* **Stability of 2'-deoxyxanthosine in DNA**, *V. Vongchampa, M. Dong, L. Gingipalli and P. Dedon, Nucleic Acids Research, 31, 1045-1051, 2003 ; (b)* **A bifunctional DNA repair protein from Ferroplasma acidarmanus exhibits O6-alkylguanine-DNA alkyltransferase and endonuclease V activities**, *S. Kanugula, G.T. Pauly, R.C. Moschel and A.E. Pegg, PNAS, 102, 3617-3622, 2005.*

305 *(a)* **Synthesis and characterisation of oligonucleotides containing 2'-deoxyxanthosine using phosphoramidite chemistry**, *S.C. Jurczyk, J. Horlacher, K.G. Devined, S.A. Benner and T.R. Battersby, Helv. Chim. Acta., 83, 1517-1524, 2000; (b)* **Stability, miscoding potential and repair of 2'-deoxyxanthosine in DNA: Implications for nitric oxide-induced mutagenesis**, *G.E. Weunschell, T.R. O'Connor and J. Termini, Biochemistry, 42, 3608-3616, 2003*

306 **Photocross-linking of nucleic acids to associated proteins**, *K.M. Meisenheimer and T.H. Koch, Critical Reviews in Biochemistry and Molecular Biology, 32, 101-140, 1997.*

307 **Crystal structure of chromomycin-DNA complex**, *C.M. Ogata, W.A. Hendrickson, X. Gao and D. Patel, J. Abstr. Amer. Cryst. Assoc. Mtg. Ser., 2, 1753, 1989.*

308 **Synthesis of photoactive DNA: Incorporation of 8-bromo-2'-deoxyadenosine into synthetic oligonucleotides**, *J. Liu and G.L. Verdine, Tetrahedron Lett., 33, 4265-4268, 1992.*

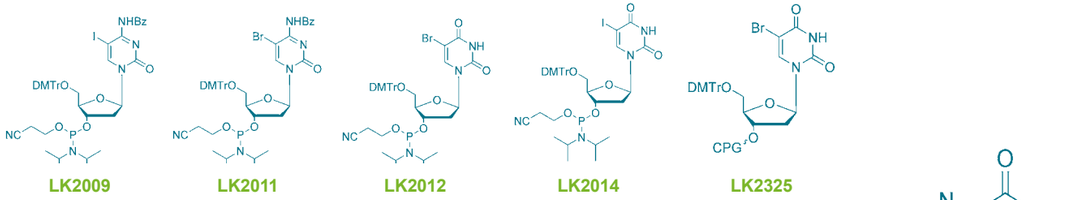


Figure 35. Xanthosine.

locating subtle differences in DNA polymerases and repair enzymes.[309]

Sulphur modified bases are of particular use for cross-linking. 4-Thio-dT-CE Phosphoramidite (**LK2070**) provides a convenient modification for photo cross-linking and photo-affinity labelling applications.

Other structural studies

The three-dimensional structure of DNA can be probed by x-ray crystallography using several halogenated nucleoside phosphoramidites.[310] In addition, antibodies exist which are specific for Br-dU so that oligonucleotides containing Br-dU can be used as probes.

5-Fluoro-deoxyuridine (**LK2010**) is a base analogue that has the potential to bind to A and G. It does not destabilise duplex formation and is an alternative to using mixed bases A/G for degeneracy.[311]

In addition to halogenated nucleosides, Biosearch Technologies offers several other phosphoramidites that have uses in various structural studies.

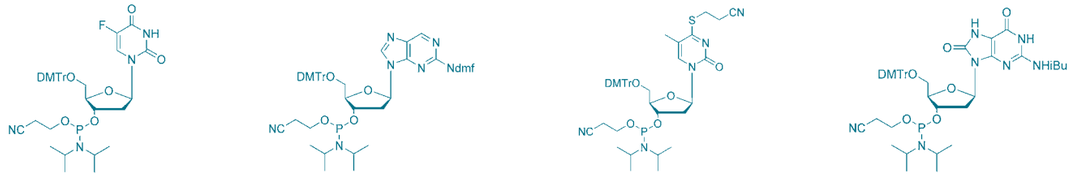
2-Aminopurine-CE Phosphoramidite (**LK2069**) is useful for investigating structural changes, as the base is deficient in hydrogen bonding sites. It is also mildly fluorescent.

8-oxo-dG-CE Phosphoramidite (**LK2072**) allows investigation of the structure and activity of oligonucleotides containing an 8-oxo mutation. This is formed naturally when DNA is subjected to oxidative conditions or ionising radiation. The resulting 8-oxo modification is significant in mutagenesis and ultimately carcinogenesis.

309 **Synthesis and properties of oligonucleotides containing 8-bromo-2'-deoxyguanosine**, *C. Fàbrega, M.J. Macías and R. Eritja, Nucleosides, Nucleotides & Nucleic Acids, 20, 251-260, 2001.*

310 **Effects of cationic charge on three-dimensional structures of intercalative complexes: structure of a bis- intercalated DNA complex solved by MAD phasing**, *X. Shui, M.E. Peek, L.A. Lipscomb, Q. Gao, C. Ogata, B.P. Roques, C. Garbay-Jaureguiberry, A.P. Wilkinson and L.D. Williams, Curr. Med. Chem., 7, 5971, 2000.*

311 *The less studied halogenated minor base phosphoramidites 5-I-U (2031) and 5-Br-U (2032) (structures not drawn) can be useful in cross-linking and x-ray studies. Please contact us regarding their availability.*

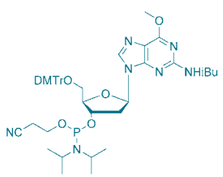


Methylating agents are common carcinogens which function by methylation of nucleobases in DNA. To examine the resulting mutagenic effects, the methylated products O6-Me-dG- CE Phosphoramidite (**LK2018**), N6-Me-dA-CE Phosphoramidite (**LK2019**/**BA0002**), and O4-Me-dT-CE Phosphoramidite (**LK2025**) can be incorporated in oligonucleotides.

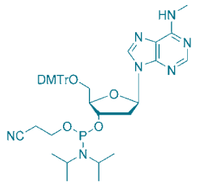
Oligonucleotides containing a hairpin loop are used routinely for structural studies of duplex formation. The hairpin loop allows the oligonucleotide to bend back on itself thereby forming a duplex in an anti-parallel formation. The hairpin may be nucleosidic or it may consist of a polyethylene glycol spacer.[312] By using “reverse” 5’-O-phosphoramidites (see page 74) for part of the synthesis, oligos with hairpin loops can be formed in which the strands are parallel.[313] These parallel stranded oligos can be readily prepared with 5’-5’ or 3’-3’ sense. Parallel stranded oligos are now also used in triplex formation studies.

Ordering structural study modifiers
Several structural modifiers are available from Biosearch Technologies and can be viewed on our website.

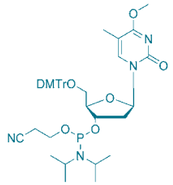
312 **Triple-helix formation by an oligonucleotide containing one (dA)12 and two (dT)12 sequences bridged by two hexaethylene glycol chains**, *M. Durand, S. Peloille, N.T. Thuong and J.C. Maurizot, Biochemistry, 31, 9197-9204, 1992.*
313 **Parallel Stranded DNA**, *J.H. van der Sande, N.B. Ramsing, M.W. Germann, W. Elhorst, B.W. Kalisch, E. van Kitzing, R.T. Pon, R.C. Clegg and T.M. Jovin, Science, 241, 551-557, 1988.*



LK2018



LK2019



LK2025

Nucleosides

With modified and unmodified nucleosides now part of our catalogue, you now have access to an unparalleled product range.

Introduction

Since their chemical syntheses being published in 1948, adenosine and guanosine has paved way for a steady increase in the understanding of synthesis of nucleosides and nucleic acids in general. Precursors for nucleic acid synthesis and essential for metabolism and control of growth within cells, nucleosides are glycosylamines, sugar molecule linked to a nitrogen-containing organic ring compound. In this instance, they can be thought of as nucleotides without a phosphate group. Much like nucleotides, the sugar within nucleosides is either ribose or deoxyribose and thus glycosylamines is either a purine or a pyrimidine.

Modified and unmodified

Biosearch Technologies features a wide range of modified nucleosides. This unique collection of reagents contains popular nucleoside building blocks such as a protected amino modified deoxyuridine analogue (**PY7530**), 5-Bromo-2'-deoxyuridine (**PY7117**), 5-Iodo-2'-O-methyluridine, a useful cyclic cytodine analogue (**PY7270**) and a 8-aza adenosine (**PRA10007**) – a substrate for adenosine kinase, 8-azaadenosine inhibits cells lacking adenine phoshporibosyltransferase (APRTase). 8-azaadenosine is also good substrate for adenosine deaminase, and as the neutral species is moderately fluorescent.

We also offer a large selection of nucleosides that facilitate epigenetics research (see page 34 for this topic within the catalogue). Among others, this collection contains 5-Hydroxymethyl-2'-deocymethylcytidine (**PY7588**), 5-Formyl-2'-deoxycytidine (**PY7589**), and 5-Formylcytidine (**PY7599**). In addition, we also offer a family of Pseudouridine analogues including Pseudouridine (**PYA11080**) and N1-Methylpseudouridine (**PYA11052**).

Ordering modified and unmodified nucleosides

With over 300 specialised products for nucleosides, Biosearch Technologies offers the largest range of on the market. Moreover, several fluorescent nucleosides are also available from the NAC portfolio. See our website for our full selection on offer.

Miscellaneous products

We also provide nucleoside synthesis reagents, Molecular Traps, plus empty synthesizer bottles and columns.

Nucleoside synthesis reagents

Nucleosides are prepared for oligonucleotide synthesis using 4,4'-dimethoxytrityl chloride (**LK0021**) for DMTr-protection of the 5'-OH. The 3'-phosphoramidite functionality is achieved using a phosphitylating reagent (**LK1002**) in the presence of an activator, DIHT (**LK1001**). For ribonucleosides, or other cases where the 2' position is hindered - or where rapid reaction is required, a more reactive chlorophosphitylating reagent (**LK1028**) is used instead of **LK1002**.

Ordering nucleoside synthesis reagents

Our full range of product offering can be found on our website.

Molecular Traps

Molecular Traps™ are highly activated molecular sieve packets designed to provide and maintain very low water levels in solvents and solutions. Originally designed for oligonucleotide synthesis, Molecular Traps are a convenient, dust and lint free, way of adding molecular sieves to many solvents and organic solution. They can generally be used in any application where molecular sieves are utilised such as to dehydrate or maintain anhydrous solvents through repeated air exposures, or to remove small molecule contaminants and known breakdown products.

Tested on selected DNA synthesizers, Molecular Traps will maintain sub-50 ppm water levels in the acetonitrile and activator bottles directly on the instrument without issues of clogging valves or restrictors with sieve dust or pouch 'fuzz'. Used directly in 4 liter solvent bottles, they provide a ready source of dry solvent for amidite dilution or other bench work, without the cost of discarding half-used bottles or buying many separate, small bottles.

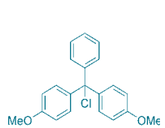
Molecular Traps are available in three sizes for bottles from 50 mL through 4 liters. Figure 36 illustrates the dynamic water scavenging effect of these traps from acetonitrile samples "spiked" with large amounts of water.

Applications

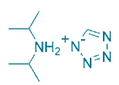
Molecular Traps have been functionally tested for use on a wide range of DNA synthesis including Biosearch Technologies' MerMade synthesizer range. Molecular Traps are designed to be used directly on the instrument in the main acetonitrile bottle, the activator bottle and in the amidite bottles.

Ordering Molecular Traps

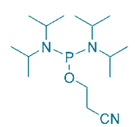
Biosearch Technologies is able to offer you a wide range of traps to cover you specific needs. See our website for further details.



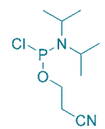
LK0021



LK1001



LK1002



LK1028

Empty synthesis bottles and columns

Our unmodified DNA phosphoramidites are packaged ready for use on either ABI, MerMade, or Expedite synthesizers. All other products are packaged by default in ABI compatible bottles, however other bottles can be provided on request (see detailed information on product packaging on page 113.

Similarly, synthesis supports are routinely packed in ALL-FIT luer columns, compatible with most ABI and Expedite instruments (not ABI 3900), and many are available as pipette-tip (e.g. MerMade or ABI3900) columns. Customers buying bulk supports may wish to source empty columns.

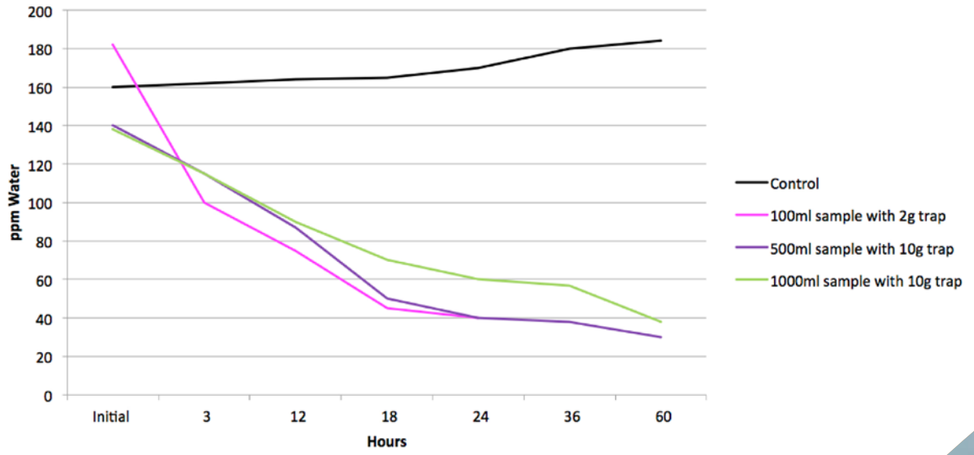
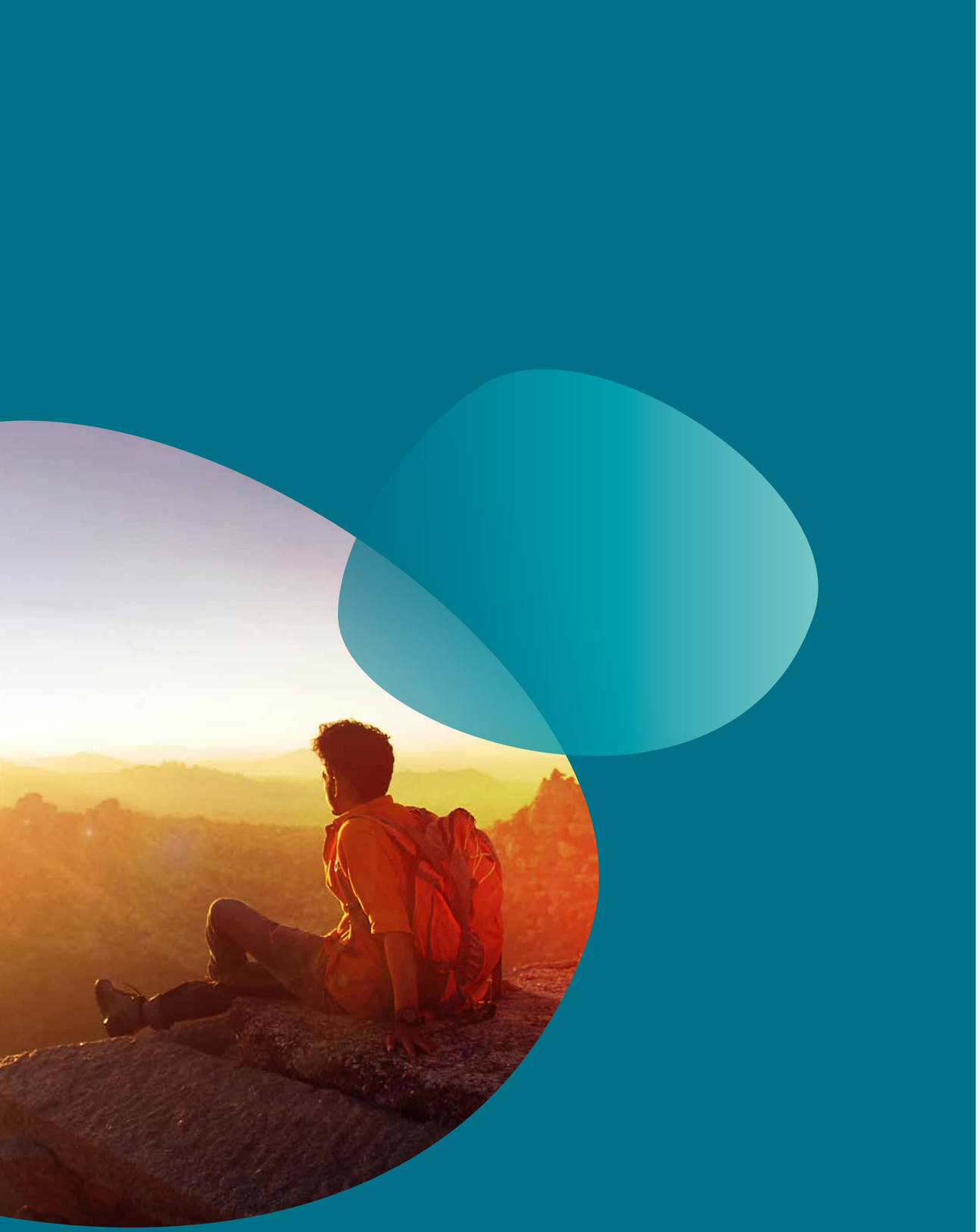


Figure 36. Colourimetric determination of water in Acetonitrile when using Molecular Traps



Ordering information

All you need to know about getting your oligonucleotide reagents from us

Product packaging

There are a number of different automated DNA/RNA synthesizers in current use. Our standard product packaging is designed to be compatible with the original configuration of as many of the popular small to medium scale models as possible, however, our recommendation is to use the MerMade range available from Biosearch Technologies.

Table 6 on page 114 shows the standard configuration for the majority of Biosearch Technologies' MerMade synthesizers available.

Given the variety of instruments available, we do not list every possible bottle size or column type. In many instances, however, where we do not list a type of packaging or bottle type we may still be able to assist you with what you require. Therefore, please ask and we'll do our best to help.

Where we supply bulk quantities of a product this will be supplied either in HDPE Nalgene™ bottles (powders) or Schott/Duran bottles (oils) unless otherwise specified.

Ordering

Online

Orders can be placed online on our website. Please look to navigate through the appropriate portal to find your product of interest.

If you are having any difficulties with this, please contact our Customer Service for advice and assistance.

By telephone or email

When using any of these methods, please ensure that you provide us with the following information:

- *Customer number (if known)*
- *Contact name and telephone number*
- *Email address for order confirmation, shipping notification and invoice*
- *Purchase order number*
- *Catalogue numbers and product descriptions*
- *Order quantity and unit size of products*

Orders can be placed by:

Telephone: +44 (0) 1698 849911
or +1 415 883 8400

Email: genomics.emea@lgcgroup.com
genomics.americas@lgcgroup.com
genomics.apac@lgcgroup.com
genomics.china@lgcgroup.com

Terms and conditions of sale

A full description of our terms and conditions is available on our website.

Technical support

In the first instance, technical support is available via our online Help Centre. If the information you require is not already detailed on the website, then a facility is provided to submit a technical support ticket.

Whilst we think this is an excellent online resource, please contact our support team by telephone on +44 (0) 1698 849911 with any query you might have, or email us as techsupport@lgcgroup.com.

Table 6. Summary of automated DNA/RNA MerMade synthesizer rage from Biosearch Technologies. This table is for reference purposes only and should not be used for instrument buying decisions. Please check with the Customer Service for detailed and current specification information as this may change.

Model	Scale	Format	Trityl monitor	Amidite ports (A, G, C, T/U) ports	Amidite ports (modifiers)	Ancillary reagent ports	Standard column type
MerMade 4	50 nmole to 5 µmol	Column	Yes	10		7	Pipette-tip
MerMade 6	50 nmol - 200 µmol	Column	Yes	10 to 20		7	Pipette-tip
MerMade 12	50 nmol - 200 µmol	Column	Yes	10 to 20		7	Pipette-tip
MerMade 48X	50 nmol - 1 µmol	Column	No	2 x 4	6	7	Pipette-tip
MerMade 192X	5 nmol - 1 µmol	Plate or Column	No	10 to 20		7	Pipette-tip (if column)
MerMade 192E	50 nmol - 1 µmol	Column	No	4		7	Pipette-tip

Synthesizers

If you are interested in purchasing a synthesizer, please visit our range of offerings for both small to medium range platforms on our website.

If at any point you need assistance, please contact our Customer Service.

Distributors

Although we are happy to ship worldwide, you may find the convenience of a local distributor beneficial. For this reason, we are appointing partners to offer this service. Please check our web site for an updated list of distributors.

Please note that some catalogue products may not be available in some territories due to licencing restrictions.

Standard bottle type (amidites)	Standard bottle type (ancillary reagents)	Synthesis positions	Notes and availability
45 mm (GL45) or 28-405 screw	Activator/Caps/Oxidiser: 28-405 screw; Wash/Deblock: 45 mm (GL45) screw	4	For availability contact Customer Service
45 mm (GL45) or 28-405 screw	Activator/Caps/Oxidiser: 28-405 screw; Wash/Deblock: 45 mm (GL45) screw	6 expandable to 12	Dedicated sulphurisation port. For availability contact Customer Service
45 mm (GL45) or 28-405 screw	Activator/Caps/Oxidiser: 28-405 screw; Wash/Deblock: 45 mm (GL45) screw	12	Dedicated sulphurisation port. For availability contact Customer Service
20 mm slider	Activator/Caps/Oxidiser: 28-405 screw; Wash/Deblock: 45 mm (GL45) screw	48	For availability contact Customer Service
45 mm (GL45) or 28-405 screw	Activator/Caps/Oxidiser: 28-405 screw; Wash/Deblock: 45 mm (GL45) screw	2 x 96	Dedicated sulphurisation port. For availability contact Customer Service
45 mm (GL45) or 28-405 screw	Activator/Caps/Oxidiser: 28-405 screw; Wash/ Deblock: 45 mm (GL45) screw	192	For availability contact Customer Service

Legal statements

The small print

Black Hole Quencher, CAL Fluor and Quasar dyes

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The CAL Fluor technology is covered by U.S. patent numbers 7,344,701 and 9,228,225, and European patent 1740100. The Quasar technology is covered by U.S. patent numbers 7,705,150, 9,435,796 and 8,436,153.

EDITH

The use of EDITH in the US is protected under US Patent No. 5,852,168 and licence for such use must be sought from The University of Minneapolis.

2’-Fluoro

A licence may be required from Ionis Pharmaceuticals, Inc. to incorporate 2’-fluoro modified nucleosides into oligonucleotides as claimed in US Patent Numbers 5,670,633, 6,005,087, 6,531,584 and foreign equivalents.

PNA

PNA monomers are manufactured and sold pursuant to licence under one or more of US Patents Nos. 5,773,571, 6,133,444, 6,172,226, 6,395,474, 6,414,112, 6,613,873, 6,710,163 and 6,713,602, or corresponding patent claims outside the US. PNA Monomers are sold to be used for internal research use only, and are not to be resold unless by separate licence.

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LK4300 is protected by US 7,807,619, the rights of which are assigned to The Regents of the University of California. Additional patents covering methods for their use in the modification of biomolecules are pending. They may be used for research purposes only. They are not licensed for resale and may only be used by the buyer. These products may not be used and are not licensed for clinical assays, where the results of such assays are provided as a diagnostic service. If a diagnostic or therapeutic use is anticipated, then a license must be requested from the University of California. The availability of such diagnostic and therapeutic use license(s) cannot be guaranteed from the University of California.

Photocleavable (PC) modifiers

The Photocleavable (PC) Modifiers were developed by Ambergen Inc., Massachusetts, US, LCG Limited, England and Glen Research Corp., Virginia, US and are made available under licence from Ambergen Inc. These products or the use of these products may be covered by one or more patents including: US Patent Nos. 6,218,530; 7,195,874; 8,906,700; 8,932,879 and International Patent Nos. EP1086251; JP,4058704,B; JP,4147230,B.

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The GalNAc reagents [dR-GalNAc (beta) phosphoramidite (**LK2568**) and dR-GalNAc (alpha) CPG (**LK2440** and **LK2489**)] are sold exclusively under joint license from MiNA Therapeutics Limited and LGC Genomics Ltd., and for which patent protection is being sought (under WO2021/032777A1). GalNAc reagents are sold under the condition that they be used for research use only and are prohibited from use in commercial applications (including, but not limited to, clinical, diagnostic, therapeutic or other applications) unless explicitly authorized by separate written agreement with LGC Genomics Ltd. Please enquire via licensing@lgcgroup.com. A licence is required from MiNA Therapeutics Ltd. to incorporate GalNAc-dR (beta) phosphoramidite or GalNAc-dR (alpha) CPG into small activating RNA (saRNA) for up-regulation. Please inquire via info@minatx.com.

Legal statements

Glossary

Anti-parallel
Hybridised strands of oligonucleotides that are directionally opposed.

Antisense
Single stranded oligonucleotide complementary to a specific DNA or RNA sequence which upon binding prevents any further action by the sequence (e.g. prevention of protein translations of mRNA).

Amplicon
The product of an amplification reaction of a nucleic acid either naturally or by PCR.

Aptamer
Oligonucleotides selected from a random pool as a result of their binding properties to a specific target.

Carcinogenesis
The process by which healthy cells are transformed into cancerous cells.

Cassette mutagenesis
Insertion of an oligonucleotide carrying a gene mutation into a plasmid where the insertion site is cleaved by a restriction enzyme followed by ligation of the oligonucleotide into the plasmid.

Cell penetrating peptide
A short peptide generally chemically linked to a nucleic acid (e.g. DNA fragment or oligonucleotide) which aids cellular uptake via endocytosis.

Collisional quenching
Collisional quenching occurs when a fluorophore and quencher are in close enough proximity to enable molecular interactions (e.g. p-π orbital overlapping) allowing non-radiative transitions to the ground state resulting in quenching. In this case quenching is not highly dependent on the wavelength overlap of the fluorophore/quencher pair.

CpG motif
Unmethylated C-phosphate-G dinucleotides within a nucleic acid sequence.

Degeneracy/Wobble
A defined position or positions within a mixture of oligonucleotides where two or more different bases are possible. For instance the defined position may contain either A or G.

Duplex
The result of the hybridisation of two single complementary single strands of nucleic acids.

ELISA
Enzyme-Linked ImmunoSorbent Assay is a detection assay to determine the presence and quantify of a substance (e.g. protein). This involves the generation of an antigen-antibody complex where the antibody is linked to an enzyme. Detection is achieved by measuring the product of the enzyme acting on a specific substrate.

FISH
Fluorescence *In Situ* Hybridisation employs the hybridisation of a fluorescent probe complementary to a specific sequence on a chromosome which is then visualised by fluorescent microscopy.

FRET
Fluorescence Resonance Energy Transfer is the transfer of energy from a high energy donor (fluorophore) to an acceptor. The latter can be a quencher (non-radiative transfer) or a second fluorophore (radiative transfer). Efficient FRET is achieved when there is good overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor. Where the acceptor acts as a quencher, a high extinction co-efficient is thought to be an important factor is terms of quenching efficiency.

Gapmer
Antisense oligonucleotides where the central region is recognised by RNase H but the flanking 5’ and 3’ sections are chemically modified to be RNase H resistant.

Gene silencing
The prevention of gene expression (‘switching off’ the gene) by interruption or suppression of transcription or translation.

In situ hybridisation
The hybridisation of a single stranded probe to denatured cellular DNA or RNA in order to detect a specific sequence. Visualisation is achieved with microscopy.

mRNA
Messenger RNA is transcribed from genes then involved in the transcription of proteins.

miRNA
MicroRNAs are highly conserved small RNA molecules which regulate gene expression by binding to 3'-untranslated regions of specific mRNA molecules.

Mass marker
A modifier attached to an oligonucleotide whereby after hybridisation to a specific target the marker can be released (usually by photolysis) and detected by mass spectroscopy.

Molecular beacons
This is a hybridisation probe whereby in the absence of target the fluorophore is quenched but as the target is amplified during PCR the probe hybridises to the amplicon separating the fluorophore from the quencher generating a fluorescent signal. In this case the fluorophore and quencher are incorporated at the 5' and 3' ends of the probe which are held in close proximity by the stem (short complementary sequences at the 5' and 3' ends which are hybridised together in the absence of a target). The probe (complementary to the target) is found in the centre of the oligonucleotide.

Mutagenesis
The process by which a stable genetic mutation is generated from a healthy gene.

Parallel
Hybridised strands of oligonucleotides that are directionally identical.

PCR
The Polymerase Chain Reaction (PCR) is a technique widely used to amplify a section of target DNA that is flanked by two known genetic sequences. Two short primers are prepared and are designed such that each is complementary to sections of the known sequences. The latter are typically 18-30 bases in length, with similar (% G+C) content to ensure similar annealing temperatures. The amplification is achieved by thermal cycling using nucleotide triphosphates and a thermally stable enzyme e.g. Taq Polymerase.

PCR blocker
A modification incorporated into an oligonucleotide generally at the 3'-end but in some specific cases internally (e.g.

Scorpions Primers) resulting in a DNA polymerase resistant oligonucleotide at the site of the blocker.

PCR clamping
Allows selective amplification of target DNA where sequences differ by a single base pair. This generally involves the use of PNA oligos to block one or more sequence due to the highly stable PNA-DNA duplex formed on hybridisation which is resistant to DNA polymerase. This leaves only the desired target available for amplification.

Photolysis
Chemical reaction induced by light (or photons) resulting in either a rearrangement or division of the molecule.

pKa
Acid dissociation constant, used to define the strength of an acid. A strong acid will have a low pKa value whereas a weak acid will have a high value.

Quadruplex
G-rich nucleic acid sequence with the ability to form a highly stable four sided square planar structure (guanine tetrad) via Hoogsteen hydrogen bonding. Where two or more guanine tetrads stack, a G-quadruplex is formed.

RNAi
RNA interference (RNAi) is a biological process in which RNA molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules.

RT-PCR
Reverse Transcription Polymerase Chain Reaction is a means of quantitatively detecting the level of RNA expression by generating complementary DNA transcripts from RNA using reverse transcriptase followed by PCR.

Real-time PCR/qPCR
Real-Time Polymerase Chain Reaction/Quantitative Polymerase Chain Reaction is a means of measuring the amplification of specific DNA sequences using PCR where the formation of the amplicon(s) is measured by the generation of a fluorescent signal by use of a fluorescent probe with a sequence complementary to the target DNA sequence.

Scorpions Primers
This is a hybridisation probe whereby in the absence of target the fluorophore is quenched but as the target is amplified during PCR the probe hybridises to the amplicon separating the fluorophore from the quencher generating a fluorescent signal. This is similar to a molecular beacon in that the fluorophore and quencher are held in close proximity via a stem. However, it differs from a molecular beacon in that the quencher is placed internally within the sequence connecting the probe to a primer via a PCR blocker. In this case when the probe hybridises to the target this is an intramolecular process since after hybridisation the probe is chemically linked to the amplicon.

Sense
Single stranded oligonucleotide complementary to a corresponding antisense strand (i.e. has the same sequence as the target e.g. mRNA).

SERRS
Surface Enhanced Resonance Raman Scattering is a detection method by which a Raman signal is generated from a Raman- active molecule absorbed onto a metal surface. The Raman-active molecule can be a labelled (e.g. TAMRA) oligonucleotide attached to a metal surface (e.g. gold) via a suitable linkage (e.g. thiol-gold).

siRNA
Small interfering RNA (siRNA), sometimes known as short interfering RNA or silencing RNA, is a class of double-stranded RNA molecules, 20-25 base pairs in length. siRNA plays many roles, but its most notable is in the RNA interference (RNAi) pathway, where it interferes with the expression of specific genes with complementary nucleotide sequence.

SNP
Single Nucleotide Polymorphism is a variation between two or more nucleic acid sequences (generally genes) by one nucleotide base. This can also be an insertion or deletion.

Splicing
The process by which nucleic acid fragments are combined to form larger fragments. For example the formation of recombinant DNA or chimeric genes.

Taqman
This is a hybridisation probe whereby in the absence of target the fluorophore is quenched. During PCR in addition to amplification, Taq polymerase cleaves the probe from the target by way of its 5'-3' exonuclease activity releasing the fluorophore into solution generating a fluorescent signal.

Triplex
A structure in which three oligonucleotides are hybridised together to form a triple helix where the third strand is bound to the duplex by Hoogsteen hydrogen bonding.

Universal base
A base with the ability to base pair with any of the four natural bases with minimal detriment to the stability or functionality of the resulting duplex.

Wobble/Degeneracy
A defined position or positions within a mixture of oligonucleotides where two or more different bases are possible. For instance the defined position may contain either A or G.

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