C-5 PROPYNES, CYTOFECTIN AND OTHER PRODUCT UPDATES

C-5 Propynes, Cytofectin GSV

In *The Glen Report*, 1993, **6(2)**, 1, we introduced the C-5 Propynyl Pyrimidine derivatives to the research community, under license from Gilead Sciences, Inc. Oligos containing these modifications have proved to be attractive for antisense experiments because of their enhanced binding to their purine partners. At that time, we noted that these properties would also prove to be useful in primers and probes and, in recent years, the majority of uses seem to have been in more routine biological experiments. In *The Glen Report*, 1996, **9(1)**, 1, we introduced Cytofectin GSV, a transfection reagent, designed at and licensed by Gilead, to be used to transport antisense oligonucleotides into cells even in the presence of serum. Although optimal for the transfection of antisense oligonucleotides, Cytofectin GSV has also been found to be suitable for plasmid transfection.

In December 1998, Gilead's antisense technology, including the patents covering the C-5 Propynes and Cytofectin GSV, was purchased by Isis Pharmaceuticals, Inc. In March, 2000, Isis terminated our license to continue to manufacture and supply these products, effective July 9, 2000. Glen Research and Isis have been unable to reach a new agreement to allow Glen to continue supplying these compounds in the long term. Further supply of these products is covered by our original contract with Gilead and we may continue to supply inventory existing on July 9, 2000 for a further 12 months, or until the supply is depleted.

We sincerely regret that our association with these products will be ending and, especially, we regret any inconvenience that our customers may incur as a result. Please check our web site for up-to-date information about supplies and for the name and contact information of the responsible person at Isis.

The Glen Report 13.1

In this issue, we fortunately have lots of good news to go with the preceding announcement. We have another fine article from Misha Shchepinov from his work in Oxford. This time, he discusses a resurrection of the trityl group with applications in fluorescence, combinatorial chemistry and mass spectroscopy. We would appreciate your input as to which of the products discussed should be made commercially available. In addition, beginning on Page 2, Scott Strobel has kindly updated us on the state of play with Nucleotide Analog Interference Mapping (NAIM) and the newer, more advanced Interference Suppression. And it would not be *The Glen Report* if there were no new products to discuss, so here we go.

3'-Amino-Modifier C7 CPG 1000

Over the last 18 months, we have been changing our support for minor bases and modifiers to 1000Å CPG. This change has been made to allow longer oligos to be routinely prepared. The analytical information has been amended to include a test to determine where the drop-off point for each specific lot occurs. We think this is more relevant than the pore size of the

(Continued on Page 13)

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A Rapid Method for Atomic Mutagenesis of Nucleic Acids

Interference Mapping

Nucleotide Analog Interference Mapping (NAIM) is a powerful new approach to perform atomic mutagenesis simultaneously, yet individually, at every position in an RNA using materials easily prepared by *in vitro* transcription.[1] Through the use of a collection of nucleotide analogs, now available from Glen Research, it is possible to assess the importance of every 2'-OH, exocyclic amine, or imino group throughout the length of an RNA. By comparing the results of multiple nucleotide analogs, it is possible to determine which ribose rings adopt unusual sugar puckers, which 2'-OH groups serve as hydrogen bond donors and/or acceptors, which nucleotides have perturbed pK_as for potential use in folding or catalysis. Furthermore, this approach can reveal which nucleotides make tertiary contacts, on which nucleotide face the contacts are made, and where metal ions are coordinated within an RNA fold.

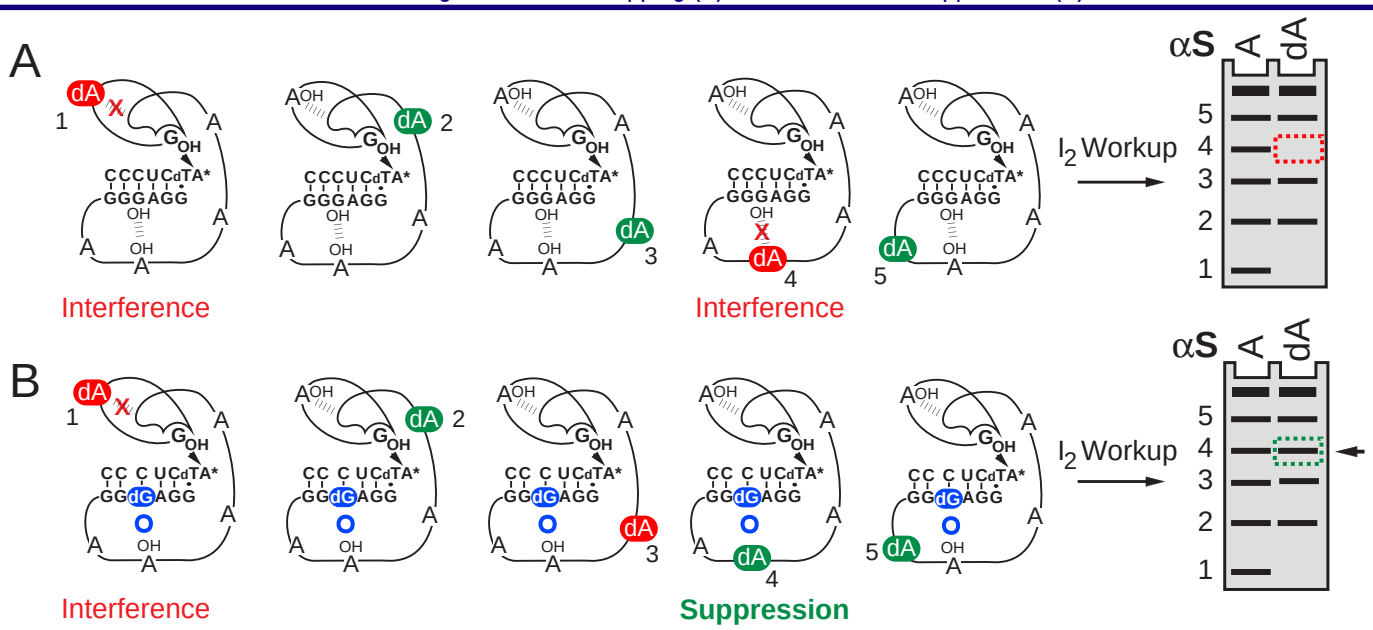
This extraordinary level of chemical detail is available quite rapidly using a collection of nucleotide analogs that modify individual functional groups on the RNA base or ribose sugar. The analogs are all α-thiotriphosphates which mark the sites of their incorporation within the transcript with an α-phosphorothioate linkage[2]. This linkage allows simple iodine cleavage and makes it possible to identify the sites where incorporation of the analog interfered with or enhanced the activity of the RNA molecule. The magnitude of an interference effect at each position in the molecule can be quantified by simply viewing a sequencing gel of the reaction products (Figure 1A).

A complete description of this approach was the subject of *Glen Report*, 1998, **11(2)**, 6-8, and can be accessed at: http://www.glenres.com/ProductFiles/Information/Strobel.HTML

An up-to-date listing of the available α-thiotriphosphates is maintained at: http://www.glenres.com/catalog/NAIM.html

This rapid and inexpensive alternative to single site atomic mutagenesis has been implemented for the study of several systems, including RNA folding,[3] ribozyme catalysis,[4-6] RNA-protein interactions,[7,8] and even *in vivo* RNA modification. The method can be applied to the study of any nucleic acid that can be functionally selected from among a larger pool of less active variants. Selection schemes have included native gel mobility shifts, nitrocellulose filter binding, selective radiolabeling by RNA ligation, and size selection in a denaturing polyacrylamide gel.[3-8] It has been applied toward the study of catalytic RNA reactivity, tRNA-synthetase interactions, mRNA processing, rRNA modification and snoRNA assembly. RNAs successfully studied by NAIM range from less than 50 to almost 1000 nucleotides in size, yet in all cases the data defines the contribution of a specific functional group at the individual nucleotide level.

FIGURE 1: Schemes of Nucleotide Analog Interference Mapping (A) and Interference Suppression (B).



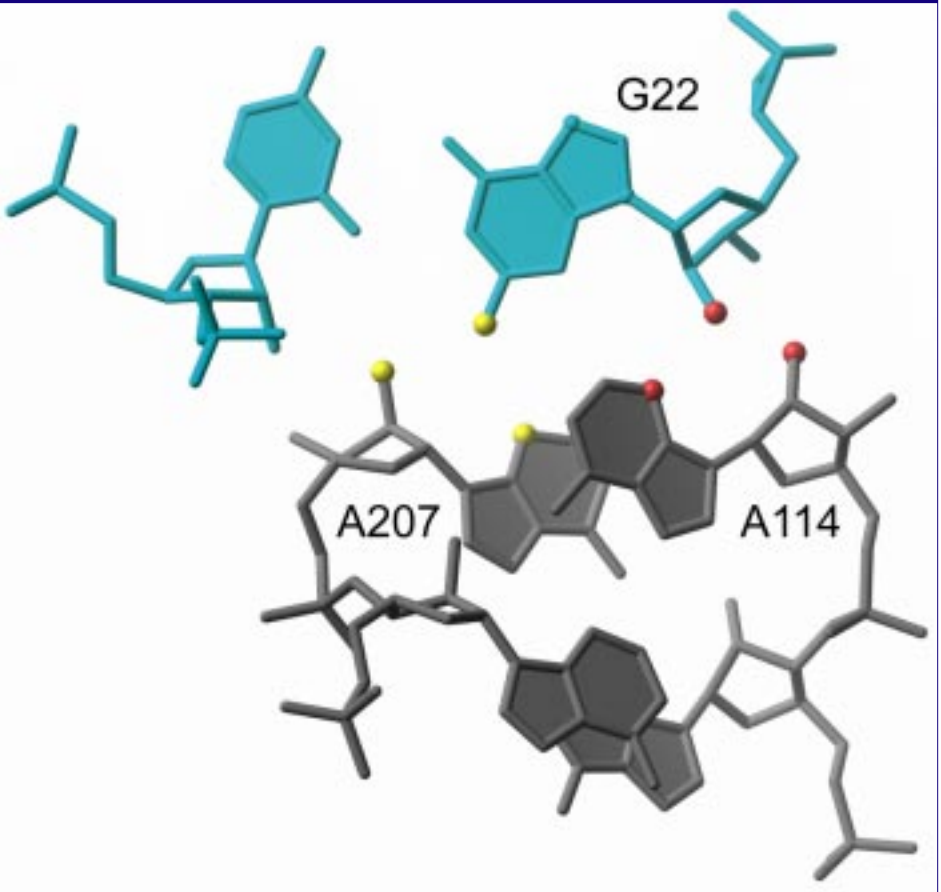
In this particular example, the RNA has five A's and the 2'-OH groups of two of them (1 and 4 shown in red) are important for activity based upon the NAIM experiment with dAαS (A). Interference suppression is performed in the same manner as NAIM, but in this case a 2'-OH known to be important for activity is substituted with a 2'-deoxy G nucleotide within all the molecules in the population. This eliminates one of the critical hydrogen bonds (blue circle) and causes all of the molecules in the population to be less active. However, deletion of the second hydrogen bond in the pair has no additional deleterious effect on activity, so it can be removed without significant penalty. In this example, specific suppression of dAαS interference is observed at A4, which is evidence that the 2'-OH of A4 and the 2'-OH of the substituted G participate in a hydrogen-bonding interaction.

Interference Suppression

Although NAIM provides a great deal of chemical information about the functional importance of each nucleotide in an RNA sequence, it can be difficult to reach specific conclusions about the tertiary structure from primary interference data alone. While a single NAIM experiment can identify all of the important 2'-OH groups and exocyclic amines within an RNA, it does not define how these groups interact within the overall RNA fold. However, such information can be obtained using a variation of NAIM, termed Interference Suppression.[9] This method, which combines site-specific analog substitution and interference modification approaches, makes it possible to identify specific tertiary hydrogen-bonding partners within an RNA structure.[10] The principle behind the approach is that, if an interaction is disrupted by deletion or alteration of one functional group in an interacting pair, then no additional energetic penalty will result from deletion or alteration of the second functional group in the pair. Interference suppression is scored by the reappearance of a specific band on the sequencing gel which showed interference in the context of the initial NAIM experiment (Figure 1B).

For example, if every RNA in the population lacks a 2'-OH group that is known to be important for activity, interference will be suppressed at the specific site with the specific functional group that makes a hydrogen bonding interaction with the deleted group. This type of experiment has provided a series of hydrogen bonding structural restraints that have made it possible to construct a detailed model of the group I intron active site (Figure 2).[10] Within this catalytic RNA, deletion of the 2'-OH at G22, resulted in suppression of 2'-deoxy and 3-deaza interference at A114 (red atoms in Figure 2), while similar suppressions were observed exclusively at A207 if the exocyclic amine of G22 was deleted (yellow atoms in Figure 2).[9] Suppression experiments do not necessarily require single atom substitution, as mutation of a specific amino acid residue in a bound protein or mutation of a nucleotide base involved in a tertiary contact can also accomplish a similar effect.

FIGURE 2: Tertiary interactions in the group I ribozyme active site.



The sets of interacting functional groups identified by interference suppression are shown in yellow and red spheres of larger radius. The sheared A·A pairs in the J4/5 segment of the active site are shown in gray and the G·U wobble pair in the P1 helix is shown in cyan. The nucleotide numbers within the Tetrahymena group I intron are also indicated. See text for discussion of specific interactions.

Nucleic acid biochemistry is about to enter a new era. With the soon to be revealed high resolution structure of the ribosome, the vast majority of the structural motifs available to RNA will be known, as will many of the rules by which proteins interact with RNA molecules. The next challenge will be to identify high resolution biochemical signatures for these folding motifs and to assign biological function to the amazingly complex structures that nucleic acids are capable of adopting. NAIM provides a particularly valuable tool in this effort and Glen Research is pleased to make this collection of reagents available to the scientific community for the multitude of applications that can be envisioned for them.

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Trityl Group in the 3rd Millenium: New Perspectives for Oligonucleotide Chemistry and Beyond

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ABSTRACT*(or what can now be done with trityl-based compounds)*

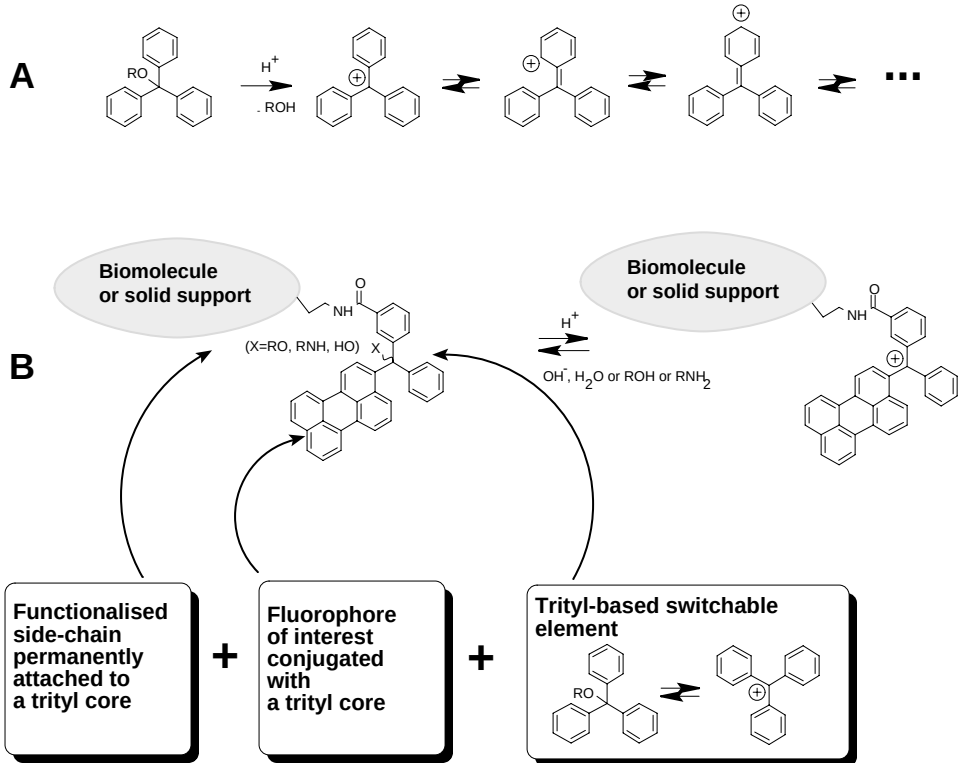
Fluorescence

- Novel trityl-based fluorescent labels incorporating polycyclic aromatic hydrocarbons (PAHs), such as pyrene and perylene, allow controlled activation/deactivation of fluorescence (Section 2.1).
- Trityl-based labels are useful tools for surface chemistry applications, such as oligonucleotide array/DNA chip technology (Section 2.1).
- The use of these tritylized fluorescent tags in fluorescence resonance energy transfer (FRET) allows an additional degree of control of the energy transfer process, which could be beneficial in studying phenomena like DNA charge transfer and electroluminescence (Section 2.2).

Combinatorial chemistry and mass-spectrometry

- Extremely high desorption properties of trityl-based compounds in mass-spectrometry, partially due to the high stability of the cations, make them valuable as encoding tags for combinatorial synthesis (Section 3.1).
- Mass-tags forming a monolayer on the surface can still be unambiguously detected by mass-spectrometry, suggesting numerous applications in surface sciences, including oligonucleotide array/DNA chip technology (Section 3.1).
- Unique desorption efficiency of trityl mass-tags even in a mixture makes them extremely useful for calibrating mass-spectrometers with a very high degree of precision, consistent with modern demands for 1-5 ppm range measurements (Section 3.2).

SCHEME 1: Structure of Trityl-based Fluorophores.



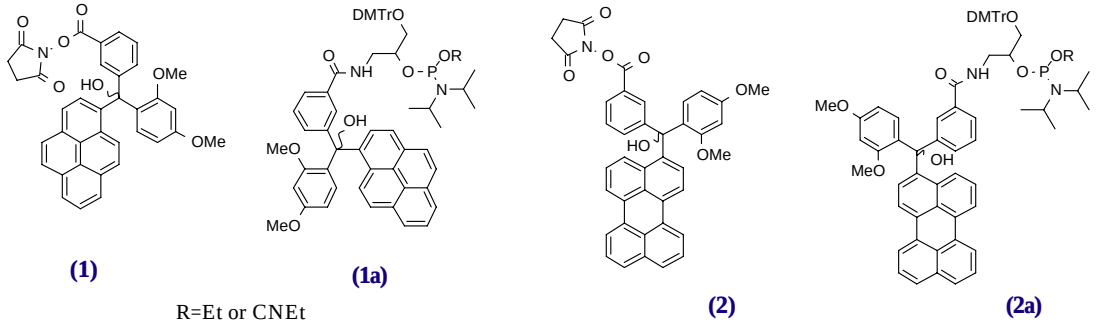
1. Introduction

That the trityl group is full of surprises was first demonstrated one hundred years ago this year, when it became the first stable free radical to be discovered[1]. The positive charge on the α–carbon atom is stabilized by the resonance effect of three aromatic rings (Scheme 1A). This particular property, which makes trityl ethers acid-labile, turned out to be useful, and the next few decades saw the trityl being developed into a major class of protective groups widely used in nucleoside[2], oligonucleotide[3], peptide, carbohydrate, and indeed in almost all other fields of organic and bioorganic chemistry[4]. Trityl-based compounds also occupy an important niche in organic dye chemistry. More recent applications in the life sciences include multi-color monitoring of oligonucleotide synthesis yields[5] and the use of a modified trityl group as a cleavable cross-linking moiety, for example, to purify oligos by immobilizing on to a solid support after synthesis either through a Diels-Alder[6] process or an amide bond formation[7]. Modified trityl groups have also been used to accelerate the formation of internucleotide bonds in the phosphotriester approach[8], to quantify the amount of amino groups on a solid support[9], and to controllably activate pro-drug antibody conjugates[10]. A modified trityl group bearing a pyrenyl residue in place of one of the aryl groups has been used for more precise fluorescent detection (down to 10^{-10} M) of detritylation[11], and a ^{14}C-labelled 4,4'-dimethoxytrityl (DMTr) group was used for more sensitive monitoring of coupling reactions on an aminated polypropylene support[12]. Some new derivatives of the trityl group and their applications are discussed below.

2. Fluorescence

2.1 Tritylization of Polycyclic Aromatic Hydrocarbons (PAHs): a New Family of Switchable Fluorescent Labels

SCHEME 2: New Trityl-based PAH labels.



Both single fluorophore[13] and energy transfer[14] based fluorescence detection methods find wide applications in the analysis of nucleic acids. Some PAHs have certain advantages over the fluorophores, such as fluorescein, currently used to label biomolecules: they are less prone to photobleaching and have high molar absorbance and quantum yields. They are also chemically more stable and do not degrade in the conditions of oligonucleotide and peptide synthesis. Furthermore, molecules are available with a range of excitation and emission maxima and large Stokes shifts. Another potentially useful feature is sensitivity to chemical environment. This is especially so for pyrene and perylene, which make them PAHs with very useful fluorescent properties. The introduction of a trityl-based carbinol-carbocation switchable element to these PAHs would allow their fluorescence to be controllably turned on and off by changing pH. In addition, the conversion of these PAHs into trityl-type structures would be advantageous due to the non-planar conformation of triarylmethanols (the angle of twist is *ca* 35° for a cation[15] and is even bigger for tritanol), which would prevent the π-π stacking interactions with a resulting increase in the solubility.

A modified trityl group bearing a pyrenyl residue in place of one of the phenyls has fluorescent properties similar to non-modified pyrene[11]. The cation derived from the triarylmethyl group on acid treatment has substantially red-shifted spectral characteristics due to the conjugation of all aromatic rings through the cationic central α–atom. The cation will still remain covalently linked to a probe molecule if attached to it through a side-chain, as shown in Scheme 1B[16]. These features are combined in compounds 1 and 2 (Scheme 2), which are designed to label amino group(s).

The UV spectra of model (butyl) amides based on 1 and 2 (Figure 1A) are the same shape but slightly red-shifted (3-10 nm) relative to the starting PAHs (Figure 1A). Their fluorescence spectra are shown in Figure 1B. The pH-threshold for the formation of trityl carbocations (Scheme 1) from corresponding tritanols at low pH can be controlled by electron withdrawing or donating groups in the aromatic rings[3]. Two methoxy groups and one carboxyl group give 1 and 2 an acidic stability similar to that of the 4-monomethoxytrityl (MMTr) group.

FIGURE 1A: Absorption spectra of butylamides **1** (solid line) and **2** (dotted line) (10^{-5} M in DCM).

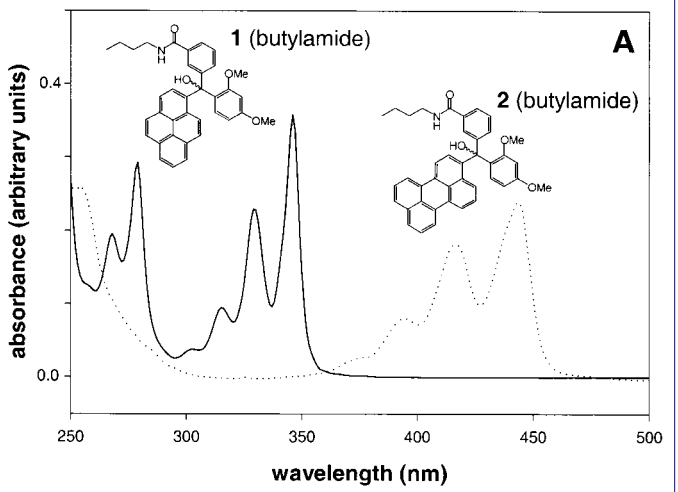


FIGURE 1B: Fluorescence spectra of butylamides **1**, **2** (10^{-6}M in DCM); excitation wavelengths: 330 nm (**1**), 420 nm (**2**).

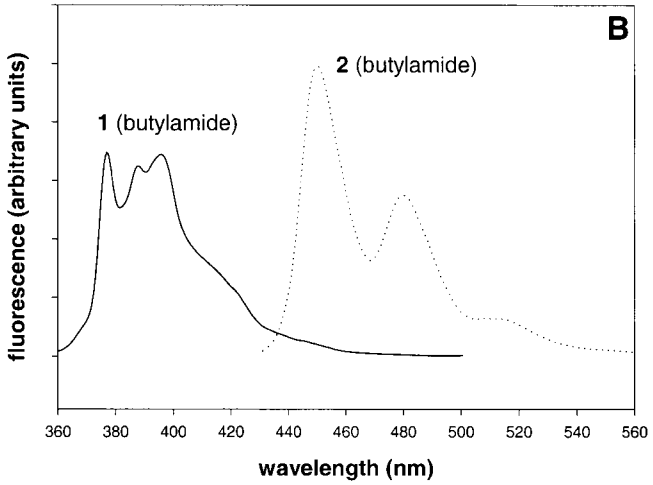


FIGURE 1C: Absorption spectra of cations of butylamides **1**, **2** (10^{-5} M in 1% TFA/DCM).

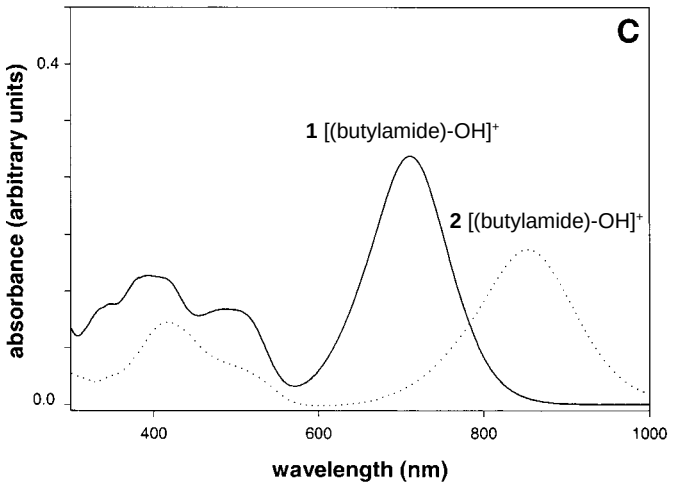
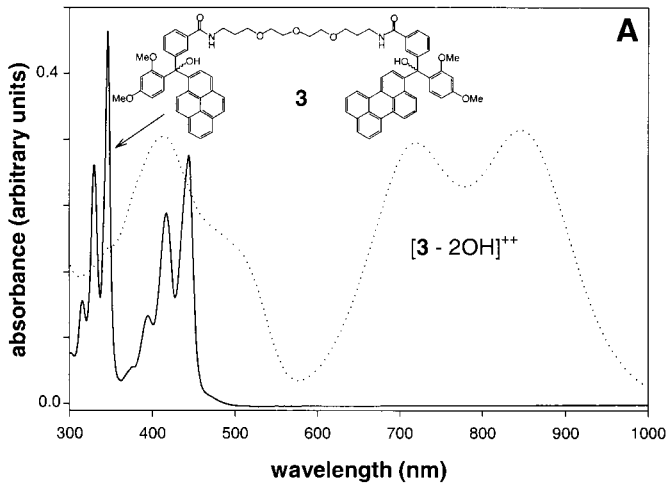


FIGURE 2A: Absorption spectra of **3** (10^{-5} M in DCM) in non-ionized (solid line) and bis-cationic (dotted line) forms (in 1% TFA/ DCM).



Compounds like 1 and 2 (Figure 1A and 1B) can be used to expand the palette of fluorophores for multicolor DNA detection on DNA chips. Multicolor detection (use of more than one fluorophore in one reaction) is a useful feature of fluorescent dyes; it enables different sequences to be detected simultaneously and was employed in DNA sequencing[13], FISH[17], and gene expression analysis on DNA chips[18]. The size of the palette is limited by the overlap of the excitation and emission spectra and it has proved difficult to use more than four colors in FISH, two colors being more normal in expression analysis. An advantage of trityl-based fluorescent tags is the potential to 'switch' the spectra on and off by simply changing the pH. The magnitude of the shifts is very large. For example, moving from neutral or alkaline to acidic pH shifts the excitation maximum of the pyrene-based compound 1 from 346 to 711 nm (Figure 1C).

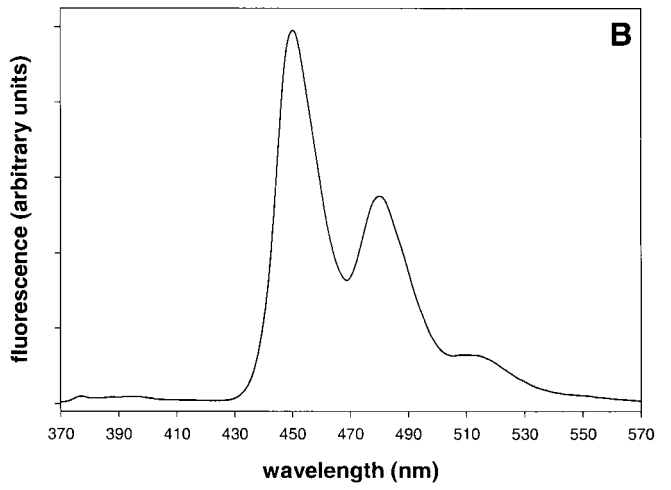
Trityl carbocations do not fluoresce in the range detected for the corresponding tritanols. This property can be used to improve the discrimination of labels: first by increasing the accuracy of intensity measurements; and, second, increasing the potential number of colors in the palette. For example, targets can be labelled with two fluorophores having similar excitation and emission spectra, but only one of which is switchable by pH change. After hybridization, measurements are taken at two pH values: under ambient conditions, and after exposing the array to acidic vapor, which is enough to switch the emission of fluorescent trityls off immediately but reversibly. Using a single excitation source, both fluorophores emit at neutral pH but only one will emit in acid. These two measurements alone would be enough to distinguish the two patterns of hybridization. But a third measurement, using a source which excites the second fluorophore in acid, allows even more analysis. In this way it may be possible to double the number of labels that can be used together.

Cyanoethyl phosphoramidites 1a and 2a (Scheme 2, R=CNEt) are suitable for standard oligonucleotide synthesis and deprotection. However, if it is desirable to have the labels attached through a non-charged linker, then the ethyl phosphoramidites (R=Et) should be employed, since they can yield non-charged phosphotriesters after ammonolysis.

2.2 Energy Transfer Applications: No Fret with FRET

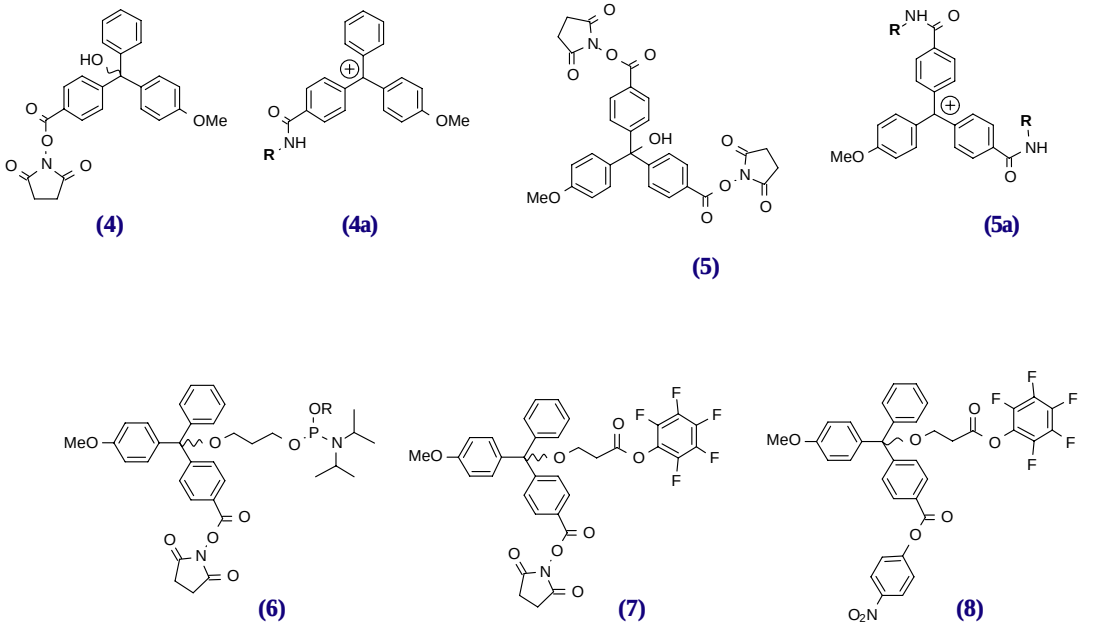
To evaluate the suitability of compounds 1 and 2 as components for FRET, a model compound 3 (Figure 2A) was prepared. While the absorption spectra for both non-ionized and bis-cationic forms (Figure 2A) essentially represent a superimposition of 1 and 2, compound 3 fluoresces only at 450, 480 and 515 nm when excited at the pyrene absorption

FIGURE 2B: Fluorescence spectrum of bis-chromophore **3** (10^{-6} M in DCM); excitation wavelength: 330 nm.



maximum of 330 nm (Figure 2B), with no detectable fluorescence of pyrene (at 377, 388 or 396 nm). When mixed in equimolar amounts, model amides of 1 and 2 (Scheme 2) retain their own fluorescence properties. This suggests a possibility of designing fluorescent labels having increased Stokes shifts by arranging the necessary fluorophores (perhaps even more than two) in the vicinity of each other[19]. Furthermore, an additional control can be achieved by making some of these parts more acid-labile than the others, so that some selected components of the chain may be reversibly switched off by decreasing the pH.

SCHEME 3: Structures of Mass-tags for Combinatorial Synthesis.



Interestingly, MALDI-TOF MS of 3 shows fragments lacking one (1193.46) and two (1176.47) hydroxyl groups. The fact that the latter flies in mono-charged (mono-cationic) and not in bis-cationic form (the signal for 1176.49/2 = 588.25 was not detected) suggests some unusual interactions, perhaps FRET during the LDI-TOF process, initiated by laser irradiation at 340 nm, which is almost a perfect match with the absorption maximum for 3.

Applications may be possible for such trityl tags (probably in more acid-labile form, with three or more methoxyls) in the burgeoning field of DNA charge transport[20], where methods are needed for monitoring carrier mobility (or lack of it) through the stacked aromatic 'core' of a double helix.

The fluorophore-containing trityls attached to solid surfaces, such as glass, silicon oxide, polypropylene, can be used to monitor local environmental changes, such as pH, as previously described[16]. The ability of a trityl group to ionize under the influence of light[25] can be utilized to reversibly produce monolayers of these species on the surfaces. The field of electroluminescence[21] can also benefit from using the compounds which combine the hole-transporting properties (trityl) with the presence of moieties routinely used as dopants for polyphenylenevinylene (PPV) (perylene for blue color) and are easy to attach (side-chain).

3. Applications in Mass-Spectrometry

3.1 Trityl Mass-Tags: Encoding of Combinatorial Libraries[27]

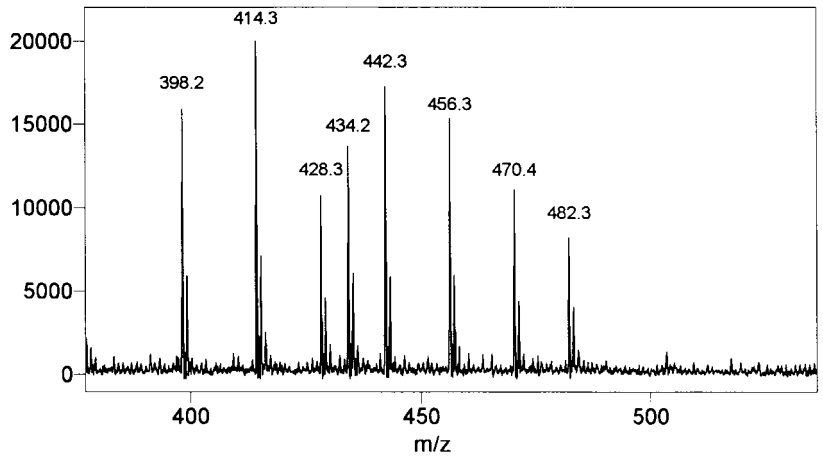
The combinatorial approach to the simultaneous synthesis of large numbers of compounds on solid supports has been an important recent development in biological and medicinal chemistry[22]. Two methods predominate: spatially addressable arrays, in which synthesis steps are performed simultaneously on sets of physically separated starting materials or areas[23]; and bead libraries, consisting of mixtures of microscopic resin beads each of which carries a single compound, usually synthesized using the 'split-and-mix' method[24]. Although resin libraries are quickly screened, their application is limited if the compound on a selected 'hit' bead cannot be readily identified.

One way around this is to attach one or more tags to the bead, which can be cleaved and identified even at very low concentration, and which will encode the synthesis steps that the bead has undergone. An encoding tag must survive the synthesis and assay, be cleaved specifically and orthogonally from the tethered compound, and be readily identifiable in pmol or fmol quantities. The problem of encoding during 'one-bead-one-compound' combinatorial synthesis has been addressed by several groups (the methods of identification of the tags include HPLC, GC, MS, IR, and NMR) and recently reviewed[22]. However, none of these methods has been particularly successful for encoding large libraries, such as those formed by a complete set of all possible oligonucleotides of a defined length. The sheer numbers involved point to the use of bead libraries for their synthesis and screening, but the limited sensitivity of gel-based

sequencing methods rules out direct identification.

The characteristic signal of the DMTr cation (monoisotopic peak, exact mass 303.139 Da) is frequently present in mass-spectra of DMTr-containing compounds, suggesting that derivatives of trityl groups with different masses could serve as unique markers in combinatorial synthesis[27]. This unique application is based on the high desorption rate of trityl cation-based tags in the conditions of LDI-TOF MS, which makes detection simpler than in previously described encoding systems[22]. Trityl cations can be released by acidic treatment and detected by LDI-TOF analysis with or without a matrix. Alternatively, the cations can be generated directly by laser irradiation[25], which permits direct detection of tagged DNA on surfaces, for example, when hybridized at different positions of a DNA chip.

FIGURE 3: LDI-TOF of a set of MMTr(NHS)-based tags (**4a**) encoding for an 8-mer oligonucleotide on a single Rapp bead[27].



Trityl-based synthons 4 and 5 (Scheme 3) are suitable for use as mass-markers. To produce the mass-tags 4a and 5a, these synthons are treated with different amines.

The masses of the majority of cheap commercially available primary amines which would withstand the conditions of oligonucleotide synthesis and deprotection (thus excluding, for example, all aromatic amines unless 'fast' phosphoramidites are used for the combinatorial synthesis) lie mainly in the range of 50-300 Da. For some applications it is desirable to have several hundred mass-tags available. The resolution of the tags in TOF mass-spectrometry is satisfactory with ≥2 Da difference between the masses of tags. Secondary amines are not useful because they are less reactive and usually there are primary amines with the same masses. Amines bearing other reactive groups cannot be used as tags in the conditions of oligonucleotide synthesis unless they are introduced at the very last step or an additional capping step is employed. Therefore, the above range of amines can only yield a limited number of tags.

The compound 5 allows attachment of two amines to the same trityl moiety, thus extending the series of mass-tags into the higher mass range (Scheme 3). NHS-activated trityls react with amines in THF or dioxane to give well-flying mass-tags. A typical LDI-TOF spectrum is shown in Figure 3. The acidic stability of the corresponding ethers is as follows: MMTrOR < MMTr(NHS)OR < MMTr(2NHS)OR ~ Tr and the MMTr(NHS) group is more than 50 times more stable to acid than the DMTr group. However, no difference was detected in the signal intensity of tritanols as compared to the corresponding trityl ethers when using laser ionization instead of acidic treatment, suggesting photocleavage by the laser irradiation[25] as a good alternative to acidic cleavage.

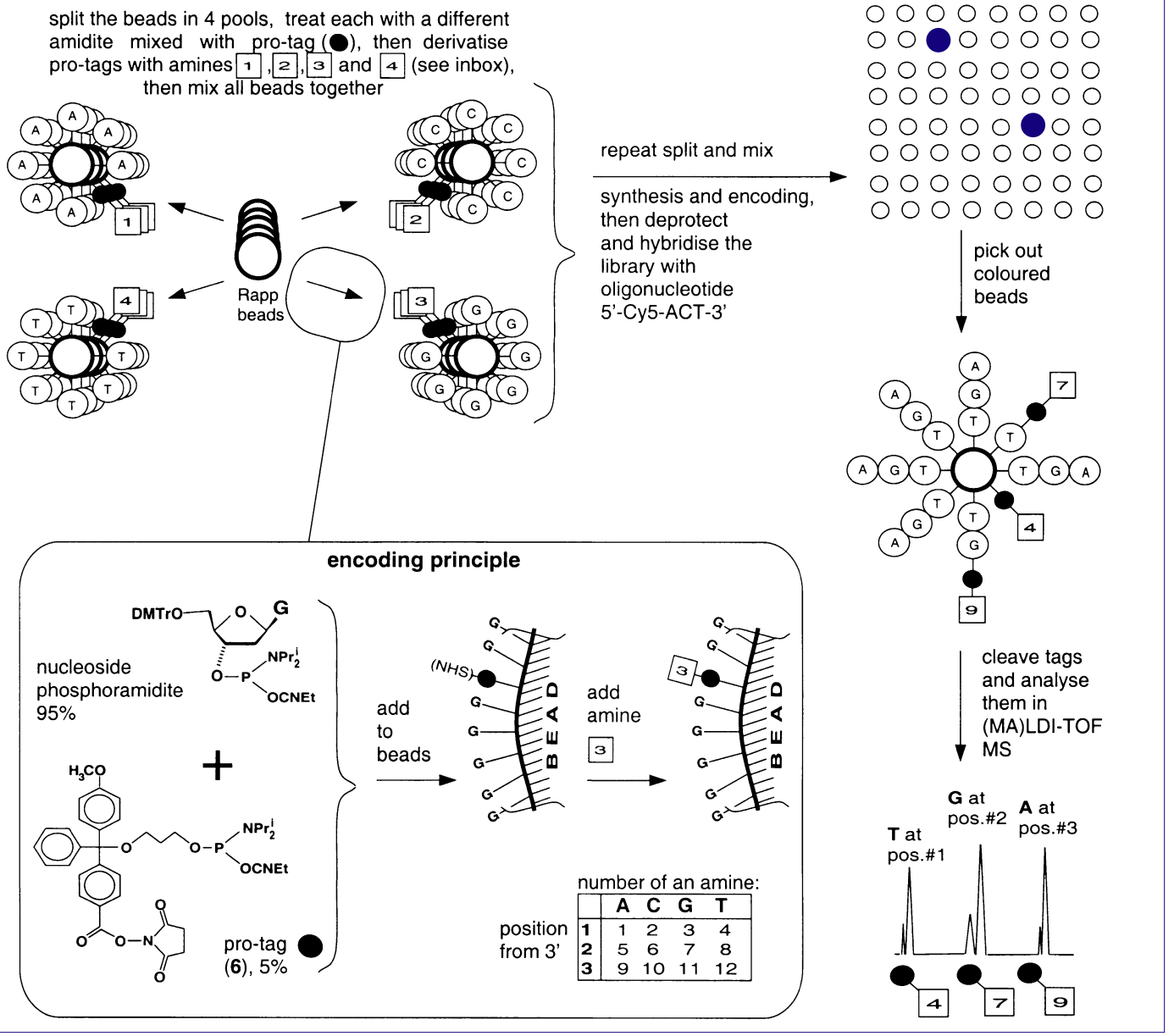
Dilution experiments showed that the lower limit of (MA)LDI-TOF (either with or without matrix) detection of trityl-based tags is around 10^{-13} M concentration level. The diameter of the spot on the sample well that is covered by the laser beam is about 100-300 micron, which means that the actual amount of sample analyzed is in the fmol range. About 5% of sites are occupied by tags (Scheme 4), which is more than enough for detection.

To be used as a tag in oligonucleotide synthesis, the trityl group should give clean, high-intensity signal in (MA)LDI-TOF analysis. It should also survive several steps of the acidic treatment used to remove the 5'-DMTr group in oligonucleotide synthesis, that is, be orthogonal to the groups involved. The MMTr(NHS) group 4 (Scheme 3) remains attached to a primary hydroxyl group after at least 8-9 cycles of acidic deprotection in oligo synthesis under conditions described below, and is easily released as 4a using 1-3% TFA.

To introduce a tagging moiety during oligonucleotide synthesis (Scheme 4), the phosphoramidite synthon 6 (Scheme 3) was prepared. Based on a propanediol structure, compound 6 provides reactivity similar to that of standard 2'-deoxynucleoside phosphoramidites. The molar ratio between 6 and a standard amidite, with which it is premixed in solution, will therefore be maintained on the solid support after condensation. The phosphoramidite 6 is stable in acetonitrile solution at room temperature for at least 2 days.

Standard A, C, G and T phosphoramidites (either 3' or 5') are premixed with *ca* 3-6 mol% of 6 prior to oligonucleotide synthesis. Assuming the stepwise yield of oligonucleotide synthesis to be about 99%, for an 8-mer library synthesized using 6 as a 5% additive to all bases, *ca* 60% of all sites of the beads are occupied by full length oligonucleotides in the final product. The concentration of the first tag (5% of all initial sites) would be about two-fold greater than that of the last tag (5% of the remaining 60% of the sites), which still makes it possible to detect all of them in the same mixture. Split-and-mix synthesis of oligonucleotides can be carried out in an Applied Biosystems 394 DNA/RNA four column synthesizer. The solid support used was Tenta Gel Macrobeads OH, 280-320 mm, polystyrene beads grafted with polyethyleneglycol chains (Rapp Polymere), but other supports can also be employed. The natural loading of the beads can be dramatically increased by employing the Trebler

SCHEME 4: Encoding and Decoding of Combinatorial Libraries.



position from 3'	A	C	G	T
1	1	2	3	4
2	5	6	7	8
3	9	10	11	12

phosphoramidite[26] (Glen Research), each addition of which triples the amount of reactive groups on the beads. The beads were placed in four reusable polypropylene DNA synthesis columns (Glen Research). The oligonucleotide synthesis was carried out on a 1 mmol scale but the supply of deblocking reagent (diluted to 50% of its original concentration with CH_2Cl_2) to the columns was reduced to 10-15 sec, with a subsequent waiting step (10 sec). Thorough CH_3CN washing ensured that all $DMTr^+$ is desorbed. Before each detritylation step, the columns were washed with CH_3CN in Manual Control mode, and then treated with corresponding amines (0.5 ml of 0.5-1 M solutions in dioxan/THF) for 1 min using 1 ml syringes, again washed with CH_3CN and dried *in vacuo* for 15 min. The beads from all columns were then combined, mixed, and split again. The procedure was repeated until the end of the synthesis. The beads were then deprotected in concentrated ammonia and washed with distilled water.

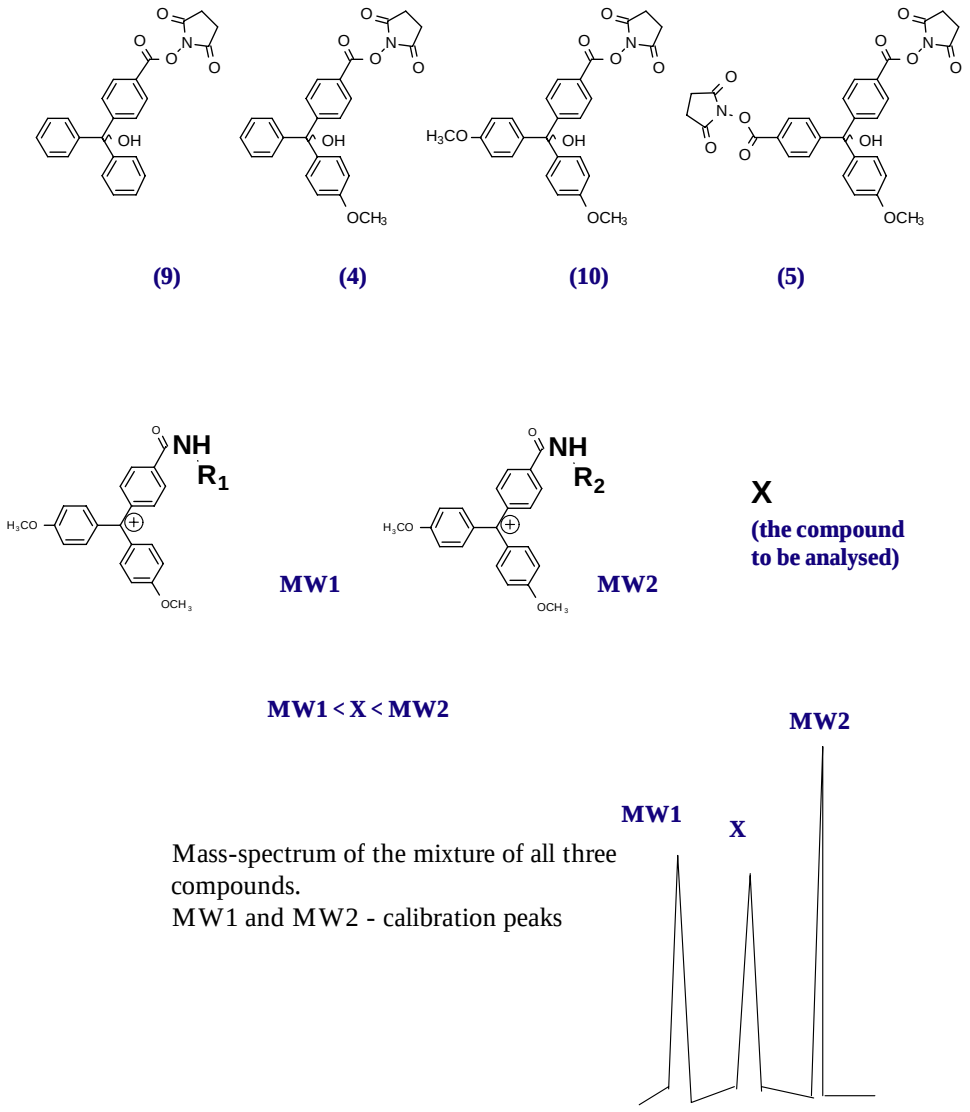
At the end of the synthesis, each bead would contain a unique oligonucleotide sequence covalently attached to it. Beads can be selected by hybridization with labelled (for example, Cy5-labelled) oligonucleotide. After washing in the same buffer, the beads are transferred on to the surface of a microscope slide and the excess of the buffer removed by blotting with tissue paper. Colored (or otherwise identified beads) are then removed, washed with water at elevated temperatures, acetone and dried. The trityl tags can be cleaved with a few mL of 1-2% solution of trifluoroacetic acid in standard Deblock Solution (Glen Research; TCA/CH_2Cl_2) for 3-4 min. The supernatant is evaporated several times with acetone and methanol and the residue analyzed by (MA)LDI-TOF HRMS. The size of Rapp-beads (~0.3 mm) allows for manual removal of positively identified beads from the pool. For smaller beads, automated

methods such as FACS might be used. An example of a decoding spectrum is shown in Figure 3.

To eliminate the problem of gradual loss of encoding MMTr-based tags 4 or 8 during the detritylation step in oligonucleotide synthesis, the use of Fmoc as a 5'-protecting group,[28] (thus omitting an acidic treatment altogether) was also investigated. After each oxidation step, the columns were removed from the synthesizer, and the beads were treated with the corresponding amines, washed with CH_3CN and then treated with 0.1 M DBU in CH_3CN to remove Fmoc-protection. The tags encoding for up to 9-mer oligos synthesized using this strategy were detected using (MA)LDI-TOF analysis. Any other method employing non-acidically removed 5'-protective groups could also be used. For longer sequences, the 3'-ethyl or 3'-methyl phosphoramidites of 5'-Fmoc- (or other) protected nucleosides should preferably be used instead of cyanoethyl phosphoramidites, to prevent the loss of the CNEt protecting group due to the treatment with amines and DBU. Similarly, for longer sequences it is better to use the methyl derivative of 6 (Scheme 3).

The trityl mass-tags can also be used for encoding in combinatorial peptide synthesis. For that, the reagents 7 and 8 (Scheme 3) can be used, which have carboxyl groups activated to different extents: first, the more active pfp-activated group reacts with the amino-groups of amino-acids on the solid support in a way similar to that depicted on Scheme 4. The excess of the tagging amine then converts less reactive NHS-activated or pNP-activated carboxyl group into an amide, thus completing the encoding procedure.

SCHEME 5: Mass-tags for High Precision Mass-spectrometer Calibrations.

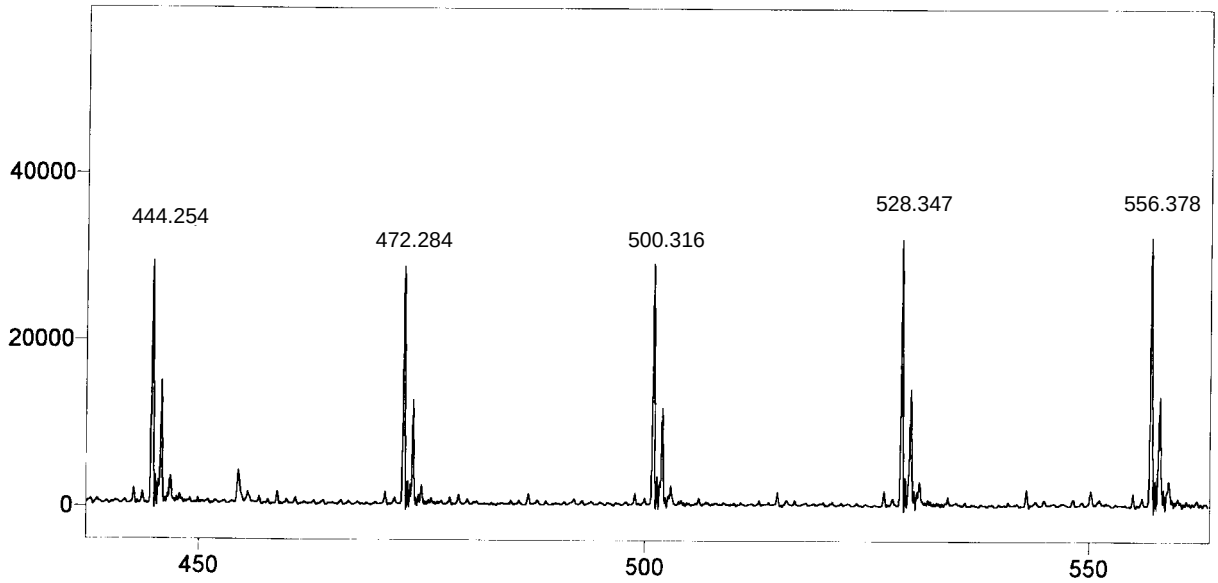


3.2 Trityl Mass-Tags as 'Trityl Ladders': Calibration of Mass-Spectrometers and High Precision Mass Measurements

It has become standard practice in modern organic chemistry to characterize new compounds by mass-spectrometry with a precision of at least 5 parts per million. For a compound with the mass of a few hundred Da, this would mean a complete match between the theoretically calculated exact mass and that found for at least two digits after a decimal point. To achieve that degree of precision, one needs mass-markers which possess a very high desorption rate (in other words, fly well), and can potentially cover a long range of masses. Peptide-based mass-markers or the dextran derivatives presently used possess neither of these two properties to the desired extent.

Trityl mass-markers are easy to design and make just by treating activated trityl blocks like Tr(NHS) (9), MMTr(NHS) (4), DMTr(NHS) (10) and MMTr(2NHS) (5) with appropriate amines (Scheme 5). The pool of amines is not limited by the demands of stability to the conditions of oligonucleotide synthesis and deprotection, as is the case for the mass-tags, so aromatic amines and amines with functional groups can also be used. The exact masses for compounds with molecular weights of 350-800 Da were routinely measured with a precision of 0.5-1 ppm using trityl mass-tags as markers, whereas with standard peptides it was usually 5-7 ppm. For heavier samples, where the availability of amines becomes a limiting factor, the methods of synthesizing these amines can be developed based on solid-support synthesis.

FIGURE 4: An example of Trityl Ladder produced by reacting DMTr(NHS) group **10** with amines containing odd numbers of carbon atoms (heptylamine (444), nonylamine (472), undecylamine (500), tridecylamine (528) and pentadecylamine (556)).



The calibration curve for the majority of modern mass-spectrometers is not linear. It is therefore highly desirable to have more than two mass-markers in the same experiment. At the same time, the markers should not interfere with the analyte by decreasing its desorption rate. Mixtures of trityl-based tags ('trityl ladders') seem to be an ideal choice, since they possess an almost uniform desorption rate regardless of the amine used and can be detected at a really low concentration (Figure 4). With these properties, it looks like trityl-based mass-tags have a potential to become a mass-spec equivalent of the DNA/RNA ladders routinely used in gel electrophoresis!

Another potential application of the activated trityl blocks can be to activate compounds which are usually difficult to analyze, like carbohydrates, which will increase their desorption rate and will make it easier to identify them mass-spectrometrically.

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POLYPLEX SYNTHESIZER FROM GENEMACHINES

PolyPlex™ is a fast, cost-effective 96-channel oligonucleotide synthesizer capable of generating a full plate of different 20-mers in less than 3 hours. Synthesis is carried out in a 96-well format, which is amenable to downstream high-throughput processing and handling. Parallel dispensing of reagents reduces both synthesis time and reagent consumption, while also eliminating the flushing of reagent lines. The synthesis chamber provides a unique, inert-gas environment where synthesis progress can be monitored by using full-plate trityl collection after any base addition.

Synthesis scales are in the range 20 - 50 nmole and two additional amidite ports are available for incorporating modified bases. There is flexibility in being able to run partial plates and, if the the trityls are not looking good for a particular well, then that well can be stopped while the others continue. The control software is available in both Apple and Windows formats. For more information about this synthesizer, see: http://genemachines.com/PolyPlex.html

In parallel synthesizers, there is a good possibility that tetrazole will crystallize on tips. This situation would lead to termination of flow which is bad in itself but, even worse, the blockage may be temporary. A solution to this problem is to use 4,5-dicyanoimidazole (DCI) as activator. DCI is typically used at a concentration of 0.25M in acetonitrile which is far below its saturation level at greater than 1.1M.

Regular 500Å supports may be used to fill the wells. However, a universal support clearly removes the need for four specific supports and makes preparing plates straightforward. The conditions to eliminate the terminal phosphodiester linkage entirely to 3'-OH are very forcing in our current universal support. However, the use of ammonium hydroxide for 6 hours at 55°C gives around 50% 3'-OH and oligos prepared this way are perfectly usable sequencing primers. For more stringent applications, the use of 0.4M sodium hydroxide in aqueous methanol gives 100% 3'-OH in 0.5 hours at 80°C. The universal Q support allows cleavage of the oligos from the plate in 2 minutes.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
Monomers			
dA-CE Phosphoramidite	10-1000-20	2.0g	100.00
dC-CE Phosphoramidite	10-1010-20	2.0g	100.00
Ac-dC-CE Phosphoramidite	10-1015-20	2.0g	100.00
dG-CE Phosphoramidite	10-1020-20	2.0g	100.00
dmf-dG-CE Phosphoramidite	10-1029-20	2.0g	100.00
dT-CE Phosphoramidite	10-1030-20	2.0g	100.00
Solvents/Reagents			
Activator			
0.45M Tetrazole, sublimed in Acetonitrile	30-3100-52	200mL	100.00
0.25M DCI in Acetonitrile	30-3150-52	200mL	100.00
Diluent			
Acetonitrile, anhydrous	40-4050-45	45mL	12.00
Cap Mix A			
THF/Lutidine/Ac_2O	40-4010-52	200mL	30.00
Cap Mix B			
16% MeIm in THF	40-4220-52	200mL	40.00
Oxidizing Solution			
0.1M I_2 in THF/Pyridine/H_2O	40-4230-52	200mL	30.00
0.02M I_2 in THF/Pyridine/H_2O	40-4330-52	200mL	30.00
Deblocking Mix			
3% DCA/DCM	40-4040-62	2L	144.00
3% TCA/DCM	40-4140-62	2L	144.00
Wash Solvent			
DCM	40-4100-62	2L	120.00
Supports			
dA-CPG 500	20-2000-10	1.0g	75.00
dC-CPG 500	20-2010-10	1.0g	75.00
Ac-dC-CPG 500	20-2015-10	1.0g	75.00
dG-CPG 500	20-2020-10	1.0g	75.00
dmf-dG-CPG 500	20-2029-10	1.0g	75.00
dT-CPG 500	20-2030-10	1.0g	75.00
Universal Support 500	20-5000-10	1.0g	95.00
Universal Q Support 500	21-5000-10	1.0g	95.00

(Continued from Front Page)

support in determining the length of oligos that can be made on the support. The drop-off point is now reported for every lot of support. Some products like 3'-Amino-Modifier C7 CPG (20-2957) are in use in the manufacturing of oligos and we will continue to supply the 500Å support. When we add the 1000Å version of such a support, it is given a new catalog number and 3'-Amino-Modifier C7 CPG 1000 (1) is 20-2958.

5-Hydroxy-2'-deoxyCytidine

We cannot correctly claim that we are introducing 5-Hydroxy-2'-deoxyCytidine (5-OH-dC) (2) in this issue. Regular readers will, of course, remember that we originally introduced 5-OH-dC in 1998 as a damaged nucleoside analogue ideal for studying free-radically induced DNA damage and enzymatic repair. Unfortunately, the synthesis of the phosphoramidite proved to be "capricious" and we were unable to make any reasonable quantities for quite some time. However, our chemists' perseverance has paid off and we can now offer it again with some confidence.

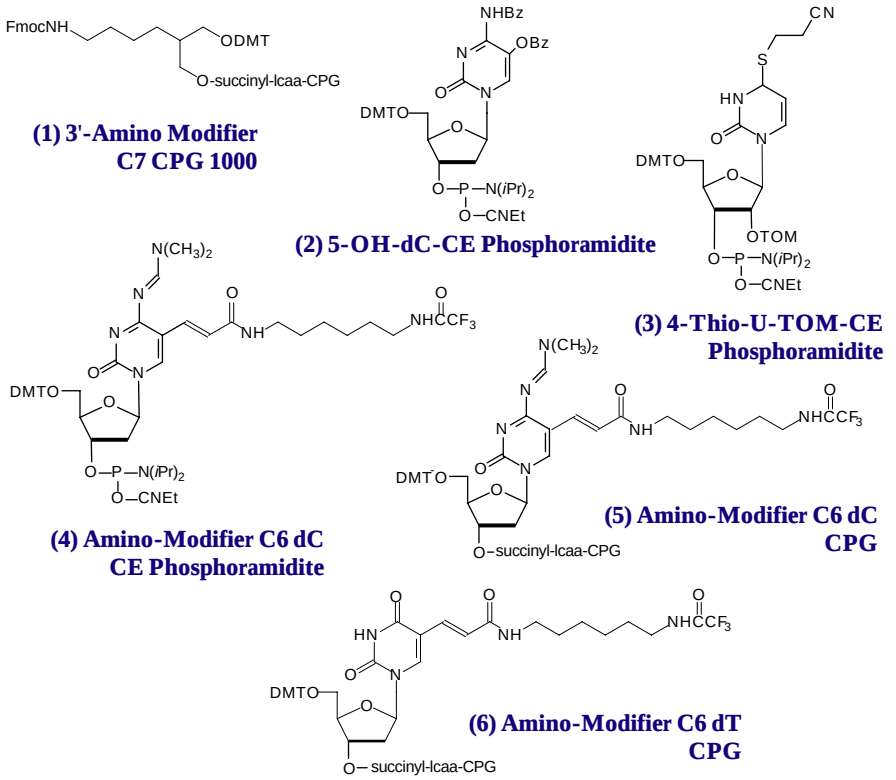
4-Thio-Uridine

We have been asked frequently in the past to prepare 4-thio-U for RNA synthesis. We were not brave enough to face the difficulties of selecting cleavage and deprotection conditions compatible with this labile base. Now we have made the TOM-protected 4-thio-U phosphoramidite (3) and we have fortunately been able to develop conditions for the cleavage and deprotection steps.

New Amino-Modifiers

The success of Amino-Modifier C6 dT has prompted us to consider the possibility of supplying the other three standard nucleosides in an amino-modified form. The jury is still out on the two purines but we have been able to produce Amino-Modifier C6 dC as both the phosphoramidite (4) and CPG (5). The side chain is attached at the 5-position of the base and this point of attachment guarantees that there is little or no disruption of hybridization relative to the unmodified dC. We also have taken the opportunity at this time to add the Amino-Modifier C6 dT CPG (6) to our product line.

FIGURE 1: STRUCTURES OF NEW PRODUCTS



ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
3'-Amino-Modifier C7 CPG 1000	20-2958-01	0.1g	95.00
	20-2958-10	1.0g	675.00
1 µmole columns	20-2958-41	Pack of 4	140.00
0.2 µmole columns	20-2958-42	Pack of 4	85.00
10 µmole column (ABI)	20-2958-13	Pack of 1	250.00
15 µmole column (Expedite)	20-2958-14	Pack of 1	375.00
5-OH-dC-CE Phosphoramidite	10-1063-90	100 µmole	225.00
	10-1063-02	0.25g	675.00
4-Thio-U-TOM-CE Phosphoramidite	10-3052-95	50 µmole	212.50
	10-3052-90	100 µmole	425.00
	10-3052-02	0.25g	975.00
Amino-Modifier C6 dC	10-1019-90	100 µmole	225.00
CE Phosphoramidite	10-1019-02	0.25g	450.00
	10-1019-05	0.5g	900.00
3'-Amino-Modifier C6 dC CPG	20-2019-01	0.1g	120.00
	20-2019-10	1.0g	995.00
1 µmole columns	20-2019-41	Pack of 4	200.00
0.2 µmole columns	20-2019-42	Pack of 4	120.00
10 µmole column (ABI)	20-2019-13	Pack of 1	300.00
15 µmole column (Expedite)	20-2019-14	Pack of 1	450.00
3'-Amino-Modifier C6 dT CPG	20-2039-01	0.1g	96.00
	20-2039-10	1.0g	800.00
1 µmole columns	20-2039-41	Pack of 4	160.00
0.2 µmole columns	20-2039-42	Pack of 4	96.00
10 µmole column (ABI)	20-2039-13	Pack of 1	240.00
15 µmole column (Expedite)	20-2039-14	Pack of 1	360.00

NEW MONOMERS: 2,4-DIFLUOROTOLUENE, FLUORESCEIN PHOSPHORAMIDITE

2,4-Difluorotoluene

Hydrogen bonding between the DNA bases as well as to the amino acid side chains in proteins has been considered to be largely responsible for the specificity of DNA-DNA and DNA-protein interactions. These electrostatic effects are considered to be the primary force behind the exquisite specificity of many polymerases and some nucleases. A wide variety of nucleoside analogues have been examined for their utility in modifying or confusing normal enzymatic functions in medicine and molecular biology. Over the last several years, Eric Kool's group at the University of Rochester has been working on a base pair which seems to confound conventional logic – there is virtually no hydrogen bonding between the bases.

Among several non-polar, aromatic nucleoside analogs that have been studied, 2,4-difluorotoluene (F)[1, 2] has proved to be remarkable in its ability to act as a non-polar, indeed hydrophobic, mimic of thymidine. F is an identical shape to that of T (Figure 1) and, when substituted for T, is specifically and efficiently replicated[3] by the Klenow fragment of *E. coli* DNA polymerase I, a polymerase known to make an error in nucleotide insertion only once in 10^3 - 10^5 bases. This is in spite of F's very low capacity for hydrogen bonding. And the corollary works equally well in that F (as triphosphate) is inserted efficiently[4] by the polymerase opposite A. Interestingly, melting studies of oligos with F substituting for T indicate[4] that the duplex is significantly destabilized. In these studies, F shows little interest in preferring to associate with A over the other bases.

These results would lead to the conclusion that shape selection may be the key factor in base selection by DNA polymerases. Therefore, complementarity of shape may be the salient feature in the design of nucleoside analogues destined to probe and modify enzyme interactions. The use of this analogue should allow researchers to probe the level of significance of Watson-Crick hydrogen bonding in any given protein-DNA interaction.

We are delighted to offer the 2,4-difluorotoluene 2'-deoxynucleoside analogue (F) as a phosphoramidite, (1) Figure 3. F is offered under license from the University of Rochester.

References:

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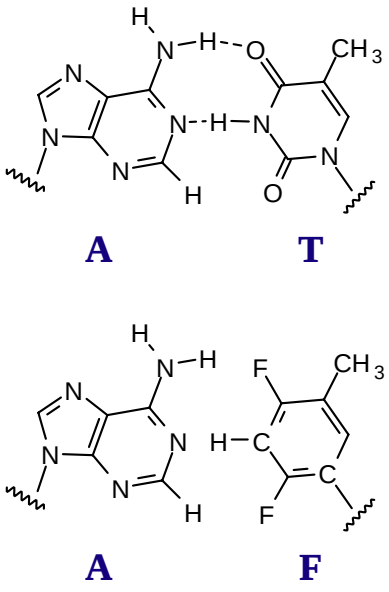
6-Fluorescein

For many years, our most popular fluorescein reagents were Fluorescein Phosphoramidite, (1) Figure 2, and the corresponding Fluorescein CPG. Both of these products are presented on the popular 1,3-diol C7 spacer.[1] The fluorescein section of the molecules was derived from fluorescein isothiocyanate (FITC), which, at that time, was far and away the most popular reagent for post-synthesis labelling. While these reagents have performed well over the years, recent developments in the design of oligonucleotide probes have made us revisit these products.

So, what's the problem? The isothiocyanate group in FITC, when reacted with an amino compound, forms a thiourea linkage, (2) Figure 2. Unfortunately, this linkage is labile during ammonium hydroxide cleavage and deprotection. According to mass spectroscopic evidence from some of our customers, a new species with mass 16 daltons less than expected is also formed after deprotection. It seems clear that this is formed by hydrolysis of the thiourea group to a urea group, (3) Figure 2, (substitution of sulfur by oxygen). A recent publication[2] also describes the modification of the thiourea linkage to a guanidinium linkage, (4) Figure 2, by ammonolysis. These results help explain the presence of two or three fluorescein components in the reverse phase HPLC traces of oligos prepared from Fluorescein

FIGURE 1: A-T AND A-F BASE PAIRS



Phosphoramidite. (The same species are likely present in equivalent 3'-labelled oligos but they do not readily resolve.) It was difficult to explain to a customer that all three peaks in the HPLC represent "the fluorescein oligo". Now add the presence of another fluorophore in doubly-labelled probes and the situation gets really messy.

The solution is fairly simple; use 6-carboxyfluorescein to prepare the fluorescein portion of two new products. 6-Fluorescein Phosphoramidite, (2) Figure 3, and 6-Fluorescein CPG, (3) Figure 3, are designed to supplement our existing fluorescein product range and we would envisage that the original two products will be eclipsed by the new versions.

References:

(1) P.S. Nelson, M. Kent, and S. Muthini, *Nucleic Acids Res.*, 1992, **20**, 6253-6259.
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2',3'-Dideoxynucleosides

Many of our customers have expressed an interest in preparing oligos with a 2',3'-dideoxynucleoside at the 3'-terminus. Unfortunately, the 2',3'-

dideoxynucleoside supports are very difficult to prepare and we currently only offer ddC.

An alternative approach to the preparation of these oligos is to synthesize in the 5'-3' direction, using 5'-supports and phosphoramidites, with the 2',3'-dideoxynucleoside 5'-phosphoramidite being added to the 3'-terminus in the last cycle. Unfortunately, since the 2',3'-dideoxynucleoside 5'-phosphoramidite has no DMT group, this approach is incompatible with DMT-on purification techniques. Also, since failure sequences contain a 3'-OH group, it is imperative that they be removed from the product by ion-exchange HPLC or gel electrophoresis. The structures of the 2',3'-dideoxynucleoside monomers are shown, (4) - (7), Figure 3.

FIGURE 2: HYDROLYSIS OF C=S

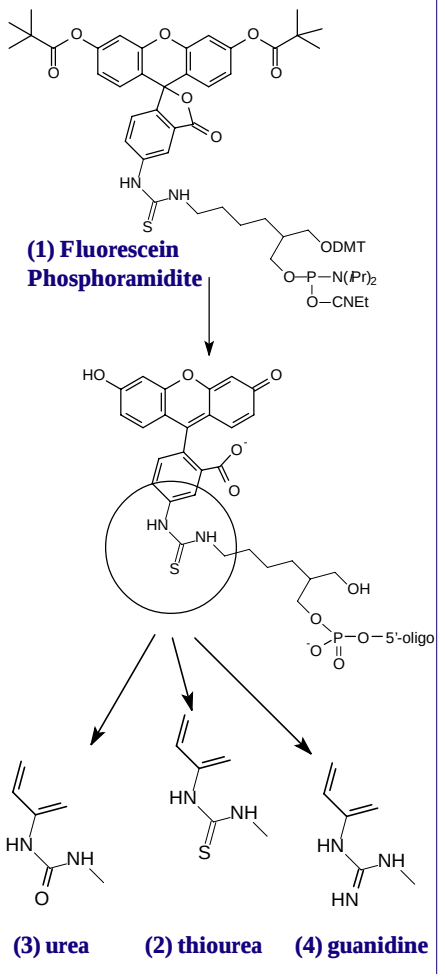
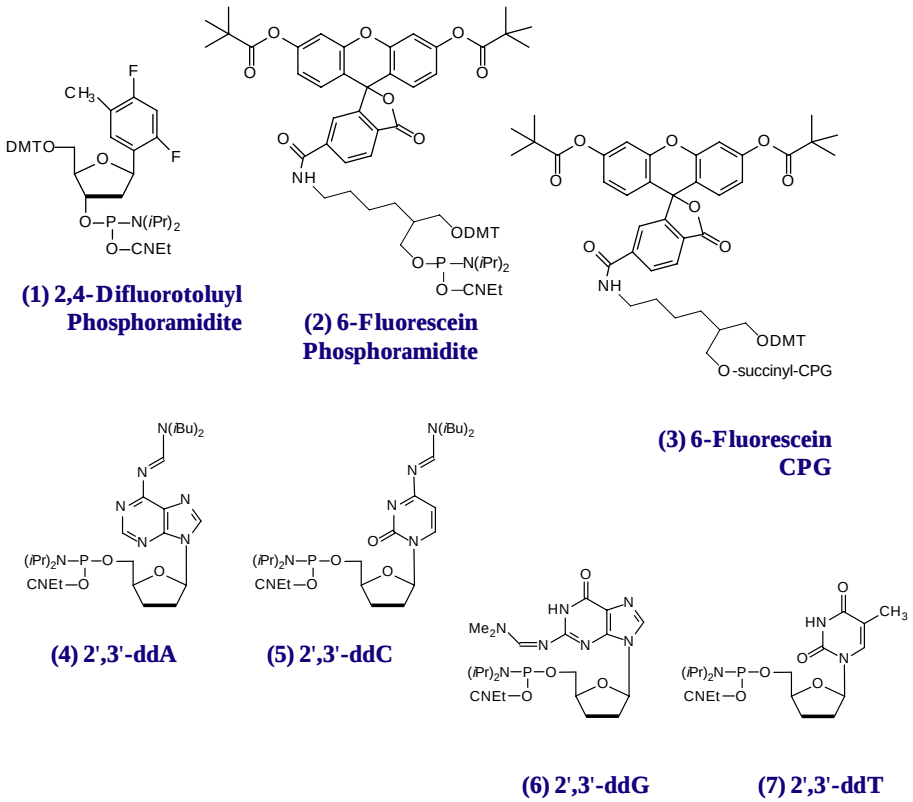


FIGURE 3: STRUCTURES OF NEW PRODUCTS



ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
2,4-Difluorotoluyl Phosphoramidite	10-1095-95	50 µmole	245.00
	10-1095-90	100 µmole	495.00
	10-1095-02	0.25g	1495.00
6-Fluorescein Phosphoramidite	10-1964-95	50 µmole	165.00
	10-1964-90	100 µmole	295.00
	10-1964-02	0.25g	595.00
3'-Fluorescein CPG	20-2964-01	0.1g	120.00
	20-2964-10	1.0g	995.00
1 µmole columns	20-2964-41	Pack of 4	200.00
0.2 µmole columns	20-2964-42	Pack of 4	120.00
10 µmole column (ABI)	20-2964-13	Pack of 1	300.00
15 µmole column (Expedite)	20-2964-14	Pack of 1	450.00
2',3'-ddA-CE Phosphoramidite	10-7001-90	100 µmole	130.00
	10-7001-02	0.25g	545.00
2',3'-ddC-CE Phosphoramidite	10-7101-90	100 µmole	130.00
	10-7101-02	0.25g	545.00
2',3'-ddG-CE Phosphoramidite	10-7201-90	100 µmole	130.00
	10-7201-02	0.25g	545.00
2',3'-ddT-CE Phosphoramidite	10-7301-90	100 µmole	100.00
	10-7301-02	0.25g	495.00

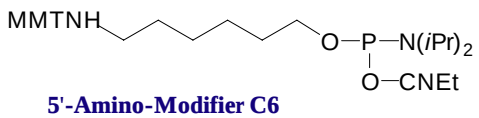
PRODUCT UPDATE - WHICH 5'-AMINO-MODIFIER?

Our most popular 5'-amino-modifier is the C6 version, available with a monomethoxytrityl (MMT) or a trifluoroacetyl (TFA) protecting group. Which is appropriate for what set of circumstances?

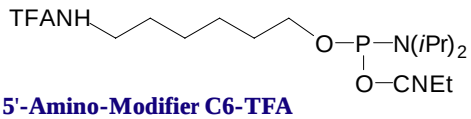
If you wish to purify the 5'-amino-modified oligonucleotide, the MMT group is preferable since the oligo can be easily purified by reverse phase techniques. The MMT group is then removed with aqueous acid. Also, the MMT group can be removed by extended deblocking on the synthesizer, allowing a solid-phase conjugation of a tag containing an activated carboxylic acid. However, the conjugate must be stable to the subsequent conditions of cleavage and deprotection.

The base-labile TFA group is preferred if the amino-modified oligo is not going to be purified prior to the conjugation reaction. It is logical to assume that only the amino-modified oligo is full-length and, therefore, that the conjugation reaction will select for only the full-length oligo.

FIGURE 1: STRUCTURES OF 5'-AMINO-MODIFIER C6 REAGENTS



5'-Amino-Modifier C6



5'-Amino-Modifier C6-TFA

ORDERING INFORMATION

Item	Catalog No.	Pack	Price($)
5'-Amino-Modifier C6	10-1906-90	100 µmole	60.00
	10-1906-02	0.25g	200.00
5'-Amino-Modifier C6-TFA	10-1916-90	100 µmole	30.00
	10-1916-02	0.25g	100.00

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