Biotinyl and phosphotyrosinyl phosphoramidite derivatives useful in the incorporation of multiple reporter groups on synthetic oligonucleotides

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ABSTRACT

Non-nucleosidic phosphoramidite linker units suitable for use on commercial DNA synthesis machines have been designed for the direct incorporation of biotin and a new reporter group, phosphotyrosine, at multiple sites on synthetic oligonucleotides. The units are based on a 3-carbon glyceryl backbone where the reporter group is attached to the 2-O-position through a 3-aminopropyl spacer. 17-mer oligonucleotides were synthesized carrying at the 5'-end 1, 2, 4 or 8 biotinyl units or 1, 2, 4 or 8 phosphotyrosinyl units respectively and used for the detection of DNA on nitrocellulose filters by hybridization. Subsequent incubation of the filters with a monoclonal antibody to the reporter group followed by secondary detection using enhanced chemiluminescence (ECL) resulted in amplification of signal strengths as the number of reporter groups was increased. The results were quantitated by use of a charge couple device (CCD) camera. Spacing of biotin moieties by thymidyl residues resulted in further improvements in signal strengths, whereas similar spacing of phosphotyrosinyl units did not.

INTRODUCTION

There are now numerous methods for the attachment of a single biotin moiety or other single reporter groups to the 5'-end of a synthetic oligodeoxyribonucleotide. Most of these involve the use of a linker phosphoramidite or H-phosphonate as the final coupling step in machine-aided assembly of the oligonucleotide (1–4). After deprotection, an amino, thiol, or other functional group is generated at the 5'-end of the oligonucleotide and this group must then be reacted with an activated biotin derivative in a separate step.

For the attachment of multiple biotins and other labels, the most common procedures involve the preparation of a nucleoside derivative specially functionalized on the heterocyclic base to give a reactive functional group upon deprotection. The functionalized nucleoside is incorporated either enzymatically (5,6) or chemically as a phosphoramidite derivative (7,8). Once again an extra step is necessary in order to convert the functional groups into the appropriate polybiotinylated species. More recently, Roget et al. (9) have shown that it is possible to use 4-N-(6-N-biotinylaminohexyl)-2'-O-deoxycytidine (or -5-methyl-2'-deoxycytidine) derivatised as a phosphoramidite in machine-aided assembly of oligonucleotides to generate upon deprotection biotinylated nucleotide tails on the 5'-end of oligonucleotides. A 45-mer oligonucleotide tailed in this way was claimed to be more sensitive in *in situ* hybridisation using a streptavidin-alkaline phosphatase detection system than the same 45-mer tailed at the 3'-end with biotin dUTP by an enzymatic method (10) although no quantitation was reported.

Cytidine derivatives functionalized on the heterocycle with biotin are not particularly conveniently prepared in that the 4-thiodeoxynucleoside starting materials are expensive. Moreover, the use of oligonucleotide tails may limit the stereochemical accessibility of the biotin moieties or alter the hybridisation properties of the oligonucleotide probe to which it is attached. Thus the use of a much simpler, non-nucleosidic linker phosphoramidite reagent capable of allowing the incorporation of multiple biotins or other reporter groups would seem worth exploring.

Recently Nelson et al. (11) have reported that a 3-amino-1,2-propanediol unit can be functionalized to provide a phosphoramidite that can be used in oligonucleotide assembly. After deprotection, the oligonucleotide contains a 5'-tail of aliphatic primary amino groups on a repeating branched 3-carbon backbone. Whereas five such units could be efficiently assembled at the 5'-end of an oligonucleotide, only 65% of the amino groups could be functionalized subsequently with biotin, however. Very recently Haralambidis et al. (12) have reported the incorporation of up to 10 biotin residues on the 3'-end of an oligonucleotide by means of a combination of synthetic peptide and oligonucleotide chemistry on solid-phase. One disadvantage of this approach is that two different machines are required for assembly of the peptide-oligonucleotide composite. Moreover, it was necessary for biotin to be conjugated after assembly of the polyamide chain.

In the case of biotin and other chemically stable reporter

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groups, it would seem preferable to incorporate the reporter group directly into the phosphoramidite derivative rather than to have to rely on post-assembly functionalization. Although two reports have recently appeared of biotinyl linker phosphoramidites having been used to attach single biotin moieties to the 5'-end of synthetic oligonucleotides (13,14), to our knowledge no biotinyl linker phosphoramidite has been described which is capable of allowing incorporation of multiple biotins into a synthetic oligonucleotide. Here we describe the convenient synthesis of a biotinyl linker phosphoramidite and also a linker phosphoramidite containing the alternative reporter group, phosphotyrosine, which has not hitherto been used in connection with nucleic acid probes. We also show that multibiotinylated and multiphosphotyrosinylated oligonucleotides have significant advantages over singly functionalised probes in sensitivity and signal strength in detection of DNA immobilized on filters.

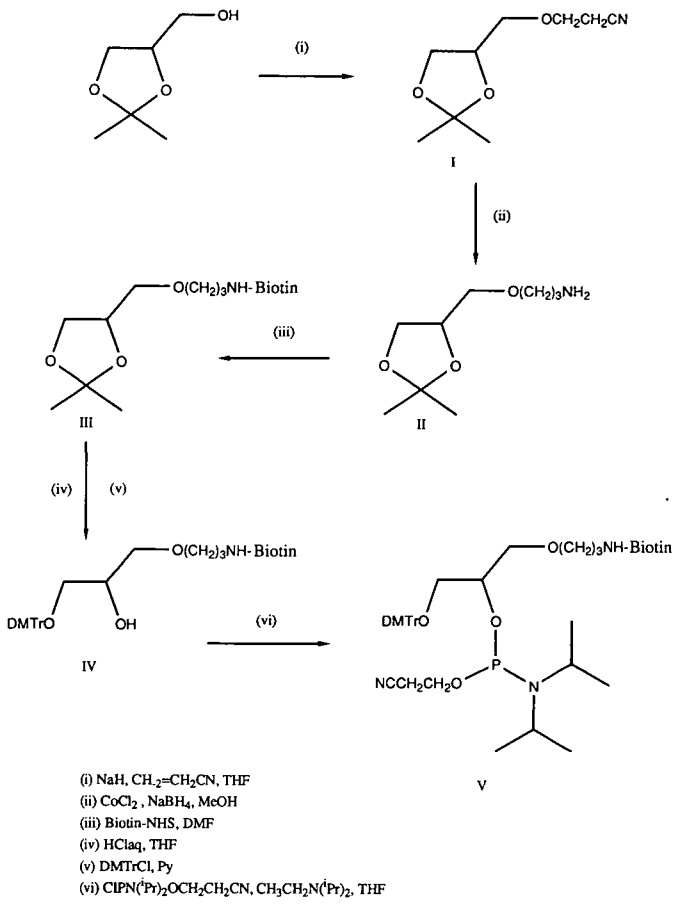
RESULTS

For the design of a repeating linker unit, we have chosen a simple 3-carbon glyceryl backbone to give maximum flexibility as well as good aqueous solubility properties. Thus, reaction of readily available solketal with acrylonitrile in the presence of sodium hydride in tetrahydrofuran afforded the addition product, 2-cyanoethyl solketal (I), in 79% yield (Scheme 1). Reduction of nitrile I required carefully controlled conditions since it was found that the use of strong reductants (such as lithium aluminium hydride) caused preferential elimination. Best results were found using sodium borohydride in the presence of cobalt (II) chloride in methanolic solution (15) to afford 3-aminopropyl solketal which was purified by distillation in 43% yield. Reaction of amine II with biotin N-hydroxysuccinimide ester in DMF solution gave N-biotinyl-3-aminopropyl solketal (III) in 89% yield.

Biotin derivative III was treated with a mixture of 1M hydrochloric acid and tetrahydrofuran (1:1) to remove the isopropylidine group and, without isolation, the product was reacted with 4,4'-dimethoxytrityl chloride in anhydrous pyridine to give 1-O-(4,4'-dimethoxytrityl)-3-O-(N-biotinyl-3-aminopropyl)glycerol (IV) which was purified by silica column chromatography in 63%yield. Phosphitylation of compound IV was carried out using an equimolar proportion of 2-cyanoethyl N,N-diisopropylaminochlorophosphite (16) in tetrahydrofuran in the presence of N,N-diisopropylethylamine. Under these conditions, the predominant product was the desired singly phosphitylated product, 1-O-(4,4'-dimethoxytrityl)-3-O-(N-biotinyl-3-aminopropyl)glyceryl 2-O-(N,N-diisopropylamino) (2-cyanoethyl)phosphite (V), which was readily separated by silica column chromatography in 58% yield. ^{31}P nmr of V showed just four peaks of approximately equal intensity corresponding to the four possible diastereomers. Analytical reversed-phase h.p.l.c. showed more than 90% of the UV absorption in two closely eluting peaks each presumably corresponding to a pair of diastereoisomers.

Starting compound IV was also recovered as a later eluting fraction from the silica column in 32% yield. A small amount of doubly phosphitylated product was observed in the crude reaction product, the formation of which was considerably exacerbated by the use of excess phosphitylating agent. ^{31}P nmr evidence (not shown) suggested that this contaminant contained one phosphite moeity attached to the biotin ring at N-3, which is in line with the findings of Alves et al (13).

The phosphoramidite V was used in the final coupling steps



Scheme 1. Synthesis of biotinyl phosphoramidite.

in oligonucleotide assembly by the phosphoramidite procedure (17) using an Applied Biosystems 380B 3-column DNA synthesiser. Three parallel assemblies were carried out of the 17-mer d(GTAAAACGACGGCCAGT) (corresponding to the sequence of the universal M13 sequencing primer (18)) with respectively one, two, and four extra cycles of coupling with phosphoramidite V after the assemblies of the core oligonucleotide 17-mers. The efficiencies of addition of phosphoramidite V averaged 99% as judged by release of dimethoxytrityl cation before subsequent coupling steps. An oligonucleotide containing a 5'-tail of eight biotins was also prepared by assembly of the same 17-mer followed by 8 sequential additions of the biotinyl linker V. In each of these assemblies, the final terminal dimethoxytrityl group was not removed. This was to maintain the terminal primary hydroxyl group in a masked configuration during subsequent ammonia treatment in order to prevent attack of the terminal hydroxyl group of the glyceryl moiety on the nearest phosphate linkage giving rise to elimination of the terminal glyceryl unit.

After complete deprotection, the four 17-mers were purified by reversed phase chromatography and in each case a major component corresponding to the desired product was seen (Figure 1). The singly biotinylated 17-mer bio-17 was retarded in mobility compared to an unbiotinylated control. The multiply biotinylated 17-mers $(bio)_2$-17, $(bio)_4$-17 and $(bio)_8$-17 were each retarded in turn according to their biotin content. Thus, reversed phase chromatography is a convenient system for both purification and

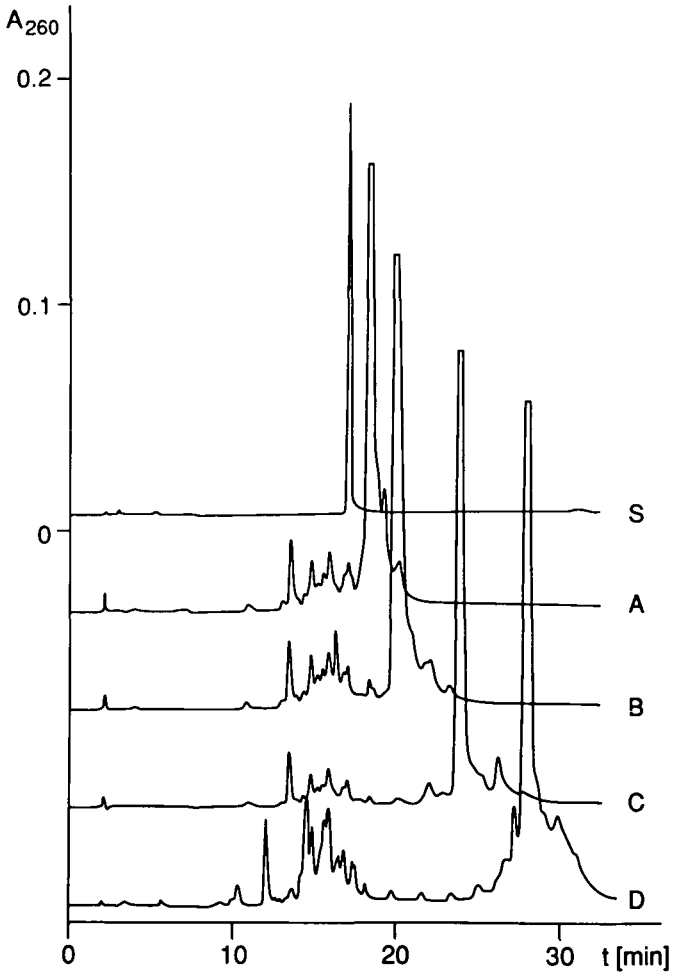
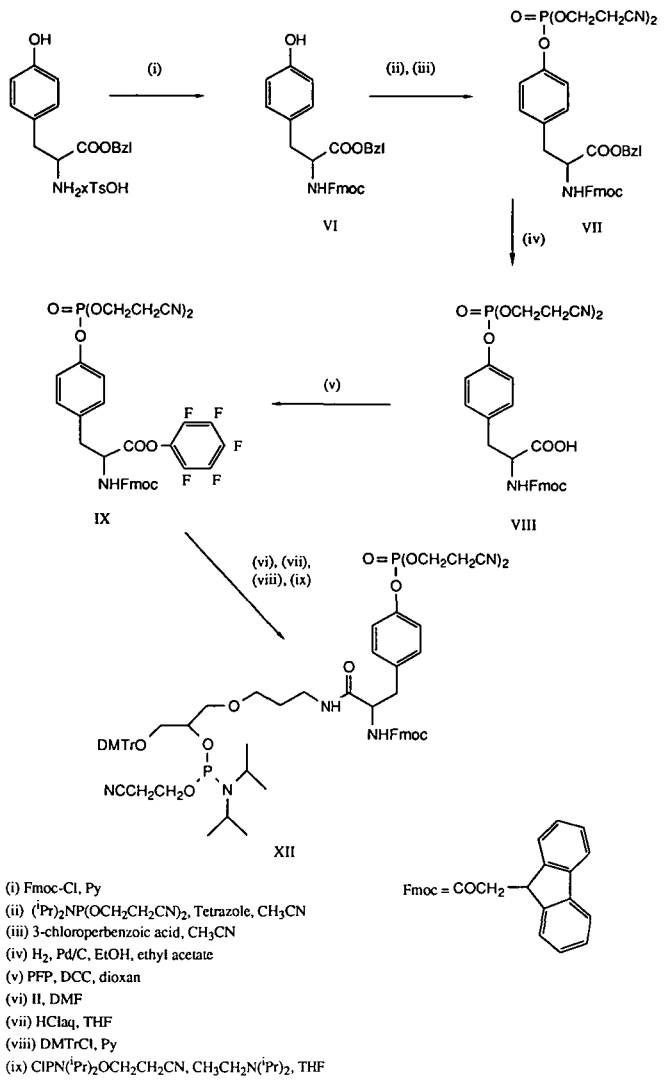


Figure 1. Reversed phase h.p.l.c. purification of crude reaction products in synthesis of multibiotinylated 17-mers. A: bio-17, B: $(bio)_2$-17, C: $(bio)_4$-17, D: $(bio)_8$-17, S: control unmodified 17-mer oligonucleotide (purified sample). Conditions: 1', 0%B then 30', 0–30%B (see experimental section)



Scheme 2. Synthesis of phosphotyrosinyl phosphoramidite.

for assessment of the homogeneity of multiply biotinylated oligonucleotides. Overall isolated yields after assembly and purification were 26, 25, 19% and 19% for bio-17, $(bio)_2$-17, $(bio)_4$-17 and $(bio)_8$-17 respectively based on the amount of first nucleoside attached to the support.

In order to determine the effect of the spacing of biotin residues on the detection ability, another assembly of the 17-mer was carried out followed by four additions of biotin linker interspersed with three thymidyl residues. The overall isolated yield of $(bio\text{-}dT)_3$-bio-17 was 19% after reversed-phase purification.

For the preparation of a suitable phosphoramidite derivative containing phosphotyrosine, it was necessary to consider the question of protection of the phosphate group of tyrosine. By analogy with nucleoside phosphate derivatives, it was thought that bis(2-cyanoethyl) protection should afford sufficient stability under acidic conditions yet the cyanoethyl groups should be removable with aqueous ammonia under conditions needed to remove base protecting groups. For N-protection, the 9-fluorenylmethoxycarbonyl group was chosen (19).

Thus reaction of L-tyrosine benzyl ester with 9-fluorenylmethyl-chloroformate in pyridine at 0°C (Scheme 2) afforded after crystallisation an 84% yield of N-fluorenylmethoxycarbonyl-L-tyrosine benzyl ester (VI). Phosphitylation of VI with bis(2-cyanoethyl)-N,N-diisopropylaminophosphine in acetonitrile in the presence of tetrazole followed by oxidation with 3-chloroperbenzoic acid gave an 86% yield of crystalline N-fluorenylmethoxycarbonyl-O-[bis(2-cyanoethyl)phosphate]-L-tyrosine benzyl ester (VII). Debenzylation of VII was accomplished with hydrogen (Pd/C). Concomitant loss of the fluorenylmethoxycarbonyl group was minimised by use of ethyl acetate as a co-solvent with ethanol. Small amounts of liberated dibenzofulvene were removed by diethyl ether extraction and the desired N-fluorenylmethoxycarbonyl-O-[bis(2-cyanoethyl)phosphate]-L-tyrosine (VIII) was purified by extraction from acidic solution into ethyl acetate and isolated in 65% yield.

The phosphotyrosine derivative VIII when treated with a mixture of 1M hydrochloric acid and tetrahydrofuran (1:1) at room temperature for 3 hours gave less than 5% loss of the phosphate group. Treatment of VIII with concentrated ammonia in a sealed tube for 5 hours at 60°C gave rise to complete removal of both 2-cyanoethyl groups with only a trace of loss of phosphate (data not shown).

Reaction of VIII with pentafluorophenol in the presence of dicyclohexylcarbodiimide in dioxane solution gave the corresponding pentafluorophenyl derivative IX in 82% yield. IX was coupled to 3-aminopropyl solketal (II) in DMF solution to yield after silica column chromatography an 86% yield of N-fluorenylmethoxycarbonyl-O-[bis(2-cyanoethyl)phosphate]-L-tyrosinyl-3-aminopropyl solketal (X). The isopropylidene group of the solketal derivative X was removed using 1M hydrochloric acid/tetrahydrofuran and without isolation, the product was reacted in pyridine solution with 4,4'-dimethoxytrityl chloride to give after silica column chromatography 1-O-(4,4'-dimethoxytrityl)-3-O-(N-{N-fluorenylmethoxycarbonyl-O-[bis(2-cyanoethyl)phosphate]-L-tyrosinyl}-3-aminopropyl)glycerol (XI) in 70% yield. Phosphitylation of XI by 2-cyanoethyl N,N-diisopropylaminochlorophosphite in the presence of N,N-diisopropylethylamine gave after silica column chromatography the corresponding 2-O-(N,N-diisopropylamino)(2-cyanoethyl)phosphite derivative (XII) as a solid foam in 44% yield. The ^{31}P nmr spectrum of XII showed a doublet at δ −148.65 and −148.64 corresponding to the tyrosinyl phosphate and three peaks at δ 7.41, 7.68 and 7.96 in the ratio of 1:1:2. These latter 3 signals are presumably accounted for by only partial resolution of the expected 4 diasteroisomers due to chirality at the C-2 of the glyceryl moiety and the P of the phosphoramidite group.

Phosphotyrosinyl linker XII was used in oligonucleotide assembly of the following derivatives of the 17-long M13 primer: PTyr-17, $(PTyr)_2$-17, $(PTyr)_4$-17, $(PTyr)_8$-17 and the thymidyl spaced derivative $(PTyr\text{-}dT)_3$-PTyr-17. Average coupling yields for the phosphotyrosinyl linker were 96% as judged by analysis of liberated dimethoxytrityl groups. In the cases of the oligonucleotides with short phosphotyrosine tails, purification could be effected by ion exchange h.p.l.c. (Figure 2) making use of the extra formal negative charges on the phosphotyrosine moieties to aid separation (overall isolated yields of 25, 18, 18 and 14% respectively). In the case of the oligomer $(PTyr)_8$-17, the retention on ion exchange h.p.l.c. was too great and the oligomer could not be eluted. In this case, preparative polyacrylamide gel electrophoresis was used for purification (overall isolated yield: 14%). It can be seen (Figure 3) that oligonucleotides with different phosphotyrosine contents were resolved well.

Purified biotinylated and phophotyrosinylated oligonucleotides were used as probes for the detection of M13 DNA immobilized on nitrocellulose filters. Standard biotin detection systems frequently involve primary interaction with streptavidin directly coupled to alkaline phosphatase or with streptavidin followed by biotinylated alkaline phosphatase (methods a and b, Experimental section). In each case, there is a colorimetric endpoint based on dye precipitation on the filter. Using either detection system, there was no difference in sensitivity of detection of M13 DNA irrespective of the number of biotin residues (from 1 to 8) or with the probe where four biotins were spaced with three

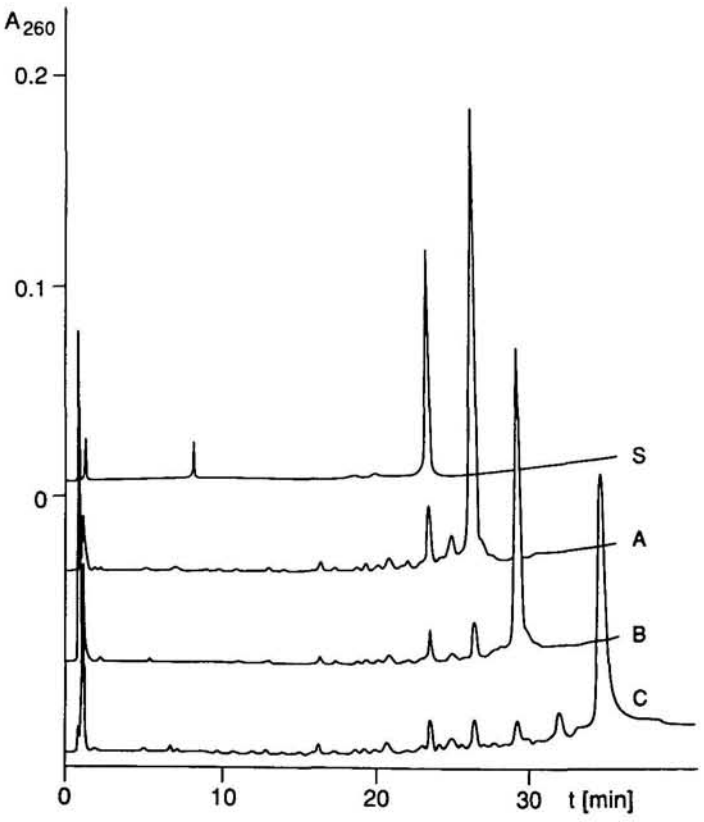


Figure 2. Ion exchange h.p.l.c. purification of crude reaction products in synthesis of 17-mers containing multiple phosphotyrosine residues. A: PTyr-17, B: $(PTyr)_2$-17, C: $(PTyr)_4$-17, S: control unmodified 17-mer oligonucleotide (purified sample). Conditions: 1', 0%B then 33', 0−100%B (see experimental section)

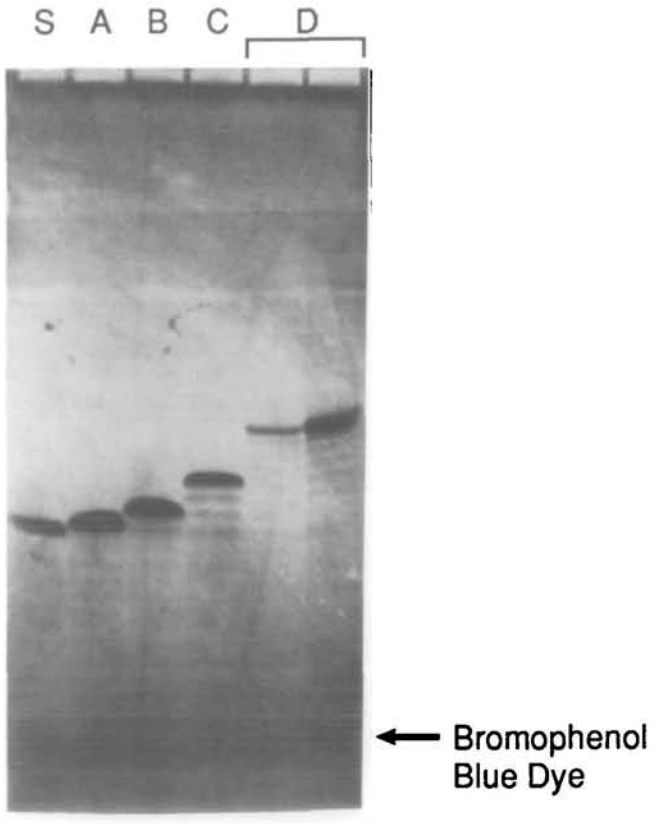


Figure 3. Polyacrylamide gel electrophoresis of crude reaction products in synthesis of 17-mers containing multiple phosphotyrosine residues. S: control unmodified 17-mer oligonucleotide (purified sample) A: PTyr-17, B: $(PTyr)_2$-17, C: $(PTyr)_4$-17, D: $(PTyr)_8$-17 (two lanes with different loadings). Visualization by UV shadowing.

thymidine residues. The detection limit was 0.5 ng of M13 DNA which corresponds to ca. 1 pg of detected sequence.

By contrast, primary interaction of biotin with a mouse monoclonal antibody against biotin followed by secondary detection using an anti-mouse IgG conjugated to alkaline phosphatase (method c), or using an anti-mouse IgG coupled to biotin followed by streptavidin conjugated to alkaline phosphatase (method d) gave in each case an increased sensitivity of detection as the numbers of biotin residues attached to the 17-mer increased. For example, whereas the detection limit of the singly biotinylated 17-mer was about 1 ng, the detection limit of the 17-mer functionalized with 8 biotins was 0.1 ng. Moreover, the spacing of four biotin residues with thymidine residues improved the detection limit to 0.1 ng compared to 0.5 ng obtained with the unspaced probe.

Unfortunately, accurate quantitation of colorimetric endpoints on nitrocellulose filters is difficult. However, we now report excellent quantitave results obtained by use of Enhanced Chemiluminescence (ECL) (20) and a Charge Couple Device (CCD) camera, which very recently has been shown to give good quantitation of images produced on membranes following

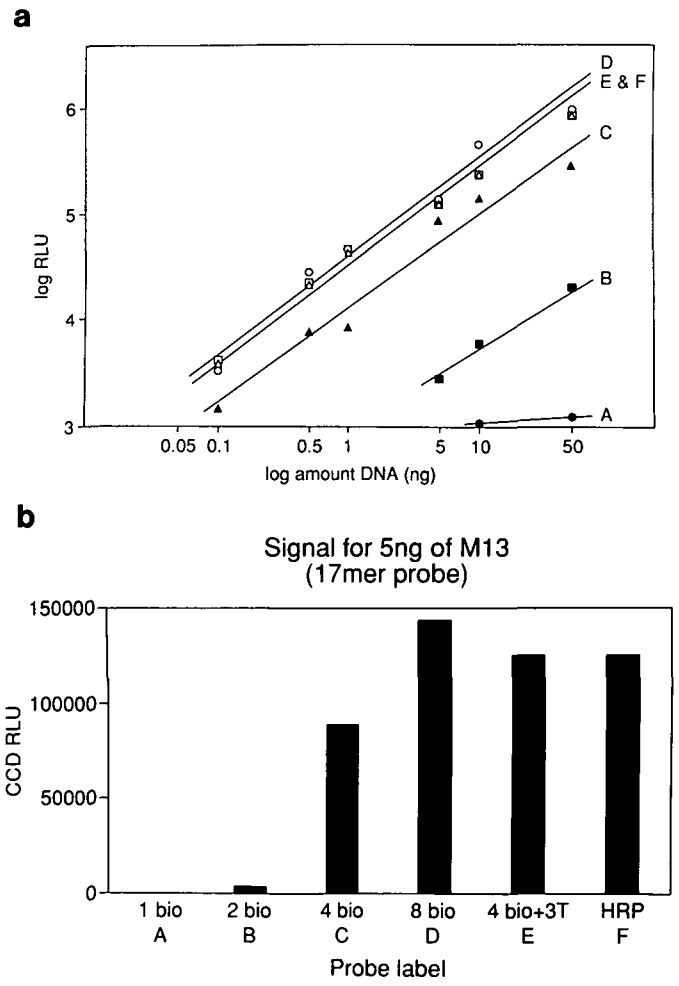


Figure 4. a) Double logarithmic plot of light emission in Relative light units against the amount of M13 DNA spotted on the filter. A (filled circles): bio-17, B (filled boxes): (bio)2-17, C (filled triangles): (bio)4-17, D (open circles): (bio)8-17, E (open boxes): (bio-dT)3-bio-17, F (open triangles): 17-mer linked directly to horseradish peroxidase (23). b) Signal strength of detection of 5 ng of M13 DNA for each of the five biotinylated probes A-E and probe F (17-mer linked to horseradish peroxidase).

electrophoretic separation of DNA fragments (21). Thus, nitrocellulose filters hybridized with biotinylated 17-mers were incubated with a mouse monoclonal anti-biotin antibody followed by an anti-mouse IgG coupled to horseradish peroxidase. This enzyme catalyses the oxidation of luminol and light is emitted which can be quantitated using the CCD camera.

Using the ECL system and the singly biotinylated probe, a linear logarithmic response was observed between the amount of light produced and the amount of DNA spotted on the filter (Figure 4a). Moreover, as the number of biotin residues in the probe was increased, there was a substantial increase in signal strength in detection of M13 DNA (Figure 4b). Spacing of biotin with thymidinyl residues resulted in a 50% increase in signal strength compared to the unspaced probe at this M13 DNA concentration. In addition, the sensitivity of detection using either the $(bio)_8$-17 probe or the spaced $(bio\text{-}dT)_3$-bio-17 probe was very similar to that obtained with the same 17-mer directly conjugated to horseradish peroxidase (23).

Phosphotyrosine-containing oligonucleotides were also used as probes for the detection of M13 DNA on filters. In qualitative studies, phosphotyrosine was detected using a mouse monoclonal anti-phosphotyrosine antibody followed by an alkaline phosphatase secondary antibody system (methods c and d, experimental section). Once again there was an increase in the detection limit obtained with increasing numbers of phosphotyrosine residues. Thus the detection limit of PTyr-17 was about 10 ng whereas that for $(PTyr)_8$-17 was 1 ng. When the alternative enzyme-linked detection system, horseradish peroxidase coupled to anti-mouse IgG, was used, the sensitivity of detection was several fold lower. This result suggests that the decreased sensitivity of the multiphosphotyrosinylated probes compared to the multobiotinylated probes was not due to partial removal by alkaline phosphatase of phosphate groups from phosphotyrosine residues but instead due to the nature of the primary antibody itself.

Quantitative detection of phosphotyrosine was obtained using the ECL system and CCD detection. Once again a linear logarithmic response was observed between the amount of light produced and the amount of DNA spotted on the filter (Figure 5a). There was also a significant increase in signal strength obtained as the number of phosphotyrosinyl residues was increased (Figure 5b), although the effect was less pronounced than in the case of biotinylated oligonucleotides. Spacing of phosphotyrosine with thymidinyl residues resulted in a slight decrease in signal strength compared to the unspaced probe.

DISCUSSION

We have shown that chemiluminescent detection of DNA on nitrocellulose filters by hybridization of oligonucleotide probes is a powerful quantitative technique that benefits considerably by the incorporation of multiple haptens. Signal strengths increased particularly dramatically as a function of the number of biotin moieties, but much more modestly in the case of multiply phosphotyrosinylated probes. Similarly, the overall sensitivity of chemiluminescent detection was approximately 10-fold higher in the case of biotin detection. It therefore seems likely that the most critical parameter in determining the utility of this oligonucleotide-multihapten technique is the nature of the particular monoclonal antibody itself. In the case of the anti-biotin antibody when biotin residues on the probe were spaced by thymidinyl residues, further improvements in signal strengths

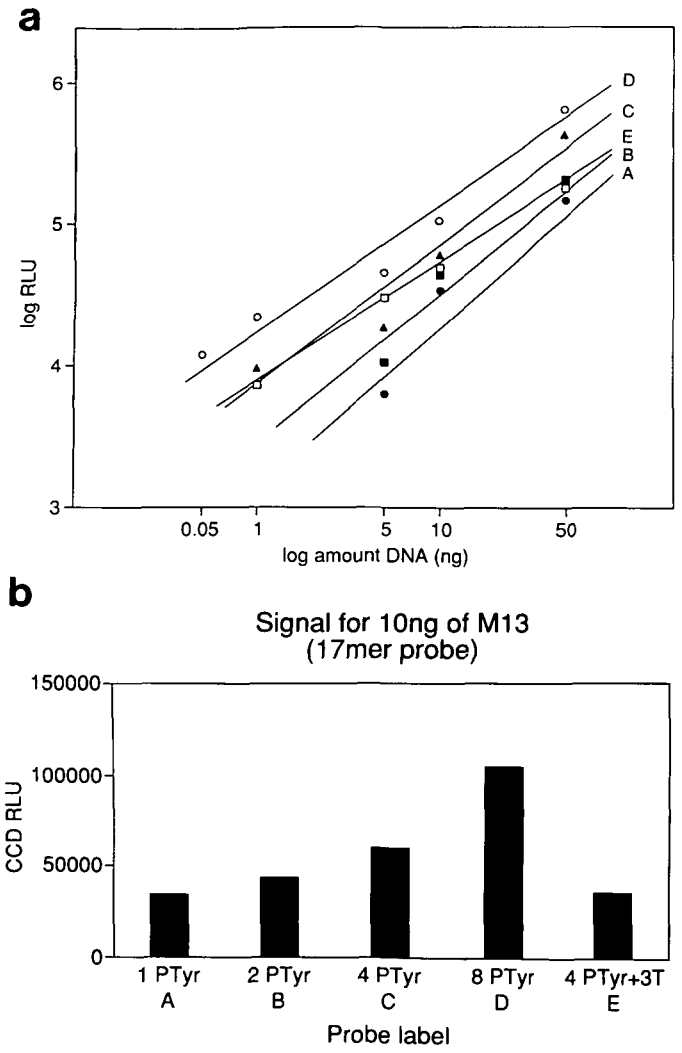


Figure 5. a) Double logarithmic plot of light emission in Relative light units against the amount of DNA spotted on the filter. A (filled circles): PTyr-17, B (filled boxes): $(PTyr)_2$-17, C (filled triangles): $(PTyr)_4$-17, D (open circles): $(PTyr)_8$-17, E (open boxes): $(PTyr\text{-}dT)_3$-PTyr-17. b) Signal strength of detection of 10 ng of M13 DNA for each of the 5 phosphotyrosinylated probes A–E.

were obtained, suggesting that each biotin moiety is recognised independently and that steric factors predominantly determine the number of antibody molecules that can be simultaneously bound to the probe. By contrast, the anti-phosphotyrosine antibody was adversely affected when the phosphotyrosine moieties were spaced apart, suggesting that a single phosphotyrosinyl moiety, presented in the context of this glyceryl backbone, does not represent a complete hapten to the antibody and that at least part of the increased signal strength must have been due to a co-operative effect of more than one phosphotyrosinyl residue.

It is interesting to note also that the amplification effect for multibiotinylated probes was not obtained when streptavidin was used as the primary detection system, even when the biotin residues were spaced apart by one thymidinyl residue. Presumably the large size of streptavidin prevents more than a single streptavidin molecule from interacting with the probe under these circumstances.

The synthetic glyceryl backbone used for presentation of haptens is convenient in terms of the suitability for detection using monoclonal antibodies and also in terms of the ease of preparation of phosphoramidites in solid-form appropriate for use in DNA synthesis machines. Other chemically stable haptens (such as dinitrophenyl) could potentially be presented in this way as well as fluorescent groups (eg dansyl). Moreover, there are numerous possibilities for development of this technique to suit particular diagnostic formats by adjustment of the numbers of individual reporter groups, their locations at either end of or within an oligonucleotide, and by spacing with nucleotidyl or other linkers. In addition, these adjustments can be carried out within the context of mechanised DNA synthesis without the need for post-assembly labelling technology.

EXPERIMENTAL SECTION

Pyridine, acetonitrile and N,N-diisopropylethylamine were dried by distillation from calcium hydride. Tetrahydrofuran and dioxane were dried by distillation from sodium/benzophenone. N,N-dimethylformamide (DMF) was dried by distillation under reduced pressure (18 mm Hg). Biotin N-hydroxysuccinimide ester was prepared from biotin by the method of Becker et al (23). L-Tyrosine benzyl ester p-toluenesulphonate salt was obtained from Sigma and 9-fluorenylmethyl-chloroformate from Fluka. Bis(2-cyanoethyl)-N,N-diisopropylaminophosphine was obtained from dichloro-N,N-diisopropylaminophosphine (Aldrich) by the method of Uhlmann and Engels (24). Organic solutions were dried over anhydrous sodium sulphate. Column chromatography was carried out by the short column method using Kieselgel 60H (Merck).

Melting points were measured on a Koefler hot stage apparatus and are uncorrected. Thin layer chromatography (t.l.c.) was carried out using Kieselgel 60 F254 plates (Merck) with aluminium backing and devolpment with the following solvents: A, chloroform/absolute ethanol (19:1); B, chlorofom/ethanol (9:1); C, chloroform/ethanol (4:1); D, acetonitrile/methanol (4:1); E, methylene chloride/methanol (9:1) containing 1% pyridine; F, chloroform/ethanol (39:1); G, chloroform/ethanol (9:1) containing 2% acetic acid; H, methylene chloride/methanol (19:1); I, methylene chloride/ethyl acetate (1:1) containing 1% 2,6-lutidine. Plates were visualised under shortwave ultraviolet light, with iodine vapour, or by spraying with 2% ethanolic molybdophosphoric acid. Dimethoxytrityl-containing compounds were visualised by exposing the t.l.c. plate to vapour of concentrated hydrochloric acid. Biotin-containing derivatives were visualised by spraying with a reagent containing p-dimethylamino- cinnamaldehyde (25).

Proton nuclear magnetic resonance (nmr) spectra were recorded on a Bruker WM-250 spectrometer (250MHz) with chemical shifts given relative to tetramethylsilane and ^{31}P-nmr were recorded on a Bruker AM-400 spectrometer (162MHz) with chemical shifts given relative to trimethyl phosphite. All spectra were taken with compounds as deuterochloroform solutions unless otherwise stated. Mass spectra were recorded on a Kratos model MS 890 spectrometer for fast atom bombardment (FAB) ionisation using 3-nitrobenzylalcohol as matrix and on a Kratos MS 902 spectrometer for electron impact (EI) ionisation.

Quantitative chemiluminescent endpoint detection was carried out using a Charge-coupled Device (CCD) imaging system. This consisted of a Wright Instruments (Milton Keynes, UK) model AT1 liquid nitrogen-cooled CCD camera fitted with an Olympus f1.2/50 mm objective lens. The distance of the lens from the sample was 275 mm, giving an approximate 40×75 mm field

of view. Hard copy images were collected on a Mitsubishi P60B video copy processor. To create a light-tight environment, an enclosure was designed to surround the complete assembly.

2-Cyanoethyl solketal (I)

Solketal (2,2-dimethyl-1,3-dioxolane-4-methanol) (26.4g, 200 mmole) and acrylonitrile (26.4 ml, 400 mmole) were dissolved in dry tetrahydrofuran (500 ml). To the stirred and cooled (waterbath) solution, sodium hydride (0.96g, 40 mmole) was added in two portions and stirring was continued for 1 hour. Then water (100 ml) was added dropwise and the resultant suspension was concentrated to remove tetrahydrofuran. Water (200 ml) was again added and the mixture was extracted with methylene chloride (2×300 ml). The extracts were dried and concentrated to give an oil (44.06g) which was distilled under reduced pressure to give the title compound (23.42g, 79% yield) as an oil (bp. 96−97°C at 0.5 mm Hg.).T.l.c. in Solvent A, R_f 0.76. ^{1}H nmr, δ :1.35(s,3H), 1.41(s,3H), 2.61(t,J=6.3Hz, 2H), 3.51−3.58 (m, 2H), 3,69−3.76 (m, 3H), 4.05 (dd, J = 8.2Hz, J=6.4Hz, 1H), 4.23 (quintet, J = 5.5Hz, 1H). Mass Spectrum (EI), m/z 186 ($M^{+\cdot}$ + 1).

3-Aminopropyl solketal (II)

2-Cyanoethyl solketal (I)(27.75g, 150 mmole) was dissolved in methanol (900 ml) and cobalt(II) chloride.$6H_20$ (71.37g, 0.3 mole) was added. To this stirred and cooled (waterbath) solution was added sodium borohydride (56.76g, 1.5 mole) in two portions (caution, foaming). Stirring was continued for 1 hour and then concentrated ammonia solution (300 ml) was added. The resultant suspension was filtered and concentrated to remove methanol. The mixture was extracted with chloroform (2×300 ml) and the extracts dried and evaporated to give an oil (20.82g) which was distilled under reduced pressure to yield the title compound (12.12g, 43% yield) as an oil (bp. 78−9°C at 0.5 mm Hg). T.l.c. in Solvent B, R_f 0.10. ^{1}H-nmr, δ: 1.34 (s, 5H), 1.40 (s, 3H), 1.70 (quintet, J = 6.5Hz, 2H), 2.77 (t, J = 6.8Hz, 2H), 3.38−3.57 (m, 4H), 3.70 (dd, J = 8.2Hz, J = 6.3Hz, 1H), 4.03 (dd, J = 8.2Hz, J = 6.3Hz, 1H), 4.24 (quintet, J = 5.8Hz, 1H). Mass Spectrum (EI), m/z 190 ($M^{+\cdot}$ + 1).

N-Biotinyl-3-aminopropyl solketal (III)

Biotin N-hydroxysuccinimide ester (3.41g, 10 mmole) was dissolved in hot dry DMF (40 ml). After cooling, a solution of 3-aminopropyl solketal (II) (2.27g, 12 mmole) in dry DMF (20 ml) was added dropwise with stirring. The solution was left for 1 hour and then concentrated. The residue was dissolved in chloroform (100 ml) and washed with saturated sodium bicarbonate solution (50 ml). The aqueous layer was washed with chloroform (50 ml) and the chloroform extracts were combined, dried and concentrated. The resultant solid was washed with pentane (40 ml), filtered off and dried to give the title compound (3.71g, 89% yield) as crystals (mp. 126−7°C). T.l.c. in Solvent C, R_f 0.38. ^{1}H-nmr, δ : 1.35 (s, 3H), 1.42 (s, 3H), 1.42 (quintet, J = 7.2Hz, 2H), 1.61−1.82 (m, 6H), 2.19 (t, J = 7.5Hz, 2H), 2.81 (d, J = 12.8Hz, 1H), 2.90 (dd, J = 12.8Hz, J = 4.8Hz, 1H), 3.10−3.16 (m, 1H), 3.31−3.36 (m, 2H), 3.48 (d, J = 5.2Hz, 2H), 3.57 (t, J = 5.4Hz, 2H), 3.71 (dd, J = 8.2Hz, J = 6.2Hz, 1H), 4.05 (dd, J = 8.1Hz, J = 6.4Hz, 1H), 4.25−4.33 (m, 2H), 4.48−4.53 (m, 1H), 5.46 (br. s, 1H), 6.28 (br. s, 1H), 6.53 (br. s, 1H). Mass spectrum (+FAB), m/z 416.3 ($M^{+\cdot}$ +1).

1-O-(4,4'-dimethoxytrityl)-3-O-(N-Biotinyl-3-aminopropyl) glycerol (IV)

N-biotinyl-3-aminopropyl solketal (III) (2.50g, 6 mmole) was disolved in a mixture of tetrahydrofuran (12 ml) and 1 M hydrochloric acid (12 ml). The solution was left for 0.5 hours and then absolute ethanol (12 ml) was added. The solution was concentrated, the residue was dissolved in absolute ethanol (12 ml) and concentrated again. The resultant product was dried by co-evaporation with pyridine (2×12 ml) to give an oil (2.46g) which was redissolved in dry pyridine (24 ml) and 4,4'-dimethoxytrityl chloride (2.03g, 6 mmole) added in a two portions with stirring. Stirring was continued for 15 min and the resultant solution was left for 1 hour. Absolute ethanol (12ml) was added and the solution was concentrated. The residue was dissolved in chloroform (60 ml) and washed with saturated sodium bicarbonate solution (30 ml). The aqueous layer was washed with chloroform (30 ml) and the chloroform extracts were combined, dried and evaporated to an oil (5.28g). The product was chromatographed on a silica column (120g) eluting with acetonitrile/methanol (9:1) and then acetronitrile/methanol (4:1). Fractions containing a single component were collected and evaporated to dryness to yield the title compound (2.55g, 63% yield) as a foam. T.l.c. in Solvent D, R_f 0.39. ^{1}H-nmr, δ : 1.36−1.42 (m, 2H), 1.61−1.73 (m, 6H), 2.12−2.20 (m, 2H), 2.62 (d, J = 12.8Hz), 2.79−2.86 (m, 1H), 3.03−3.21 (m, 3H), 3.28−3.34 (m, 2H), 3.46−3.58 (m, 4H), 3.77 (s, 6H), 3.93−3.96 (m, 1H), 4.15−4.24 (m, 1H), 4.35−4.41 (m, 1H), 5.43 (br. s, 1H), 6.61 (br. s, 1H), 6.78 (br. s, 1H), 6.78−6.82 (m, 4H), 7.18−7.43 (m, 9H). Mass Spectrum (+ FAB), m/z 678.4 ($M^{+\cdot}$ +1).

1-O-(4,4'-dimethoxytrityl)-3-O-(N-biotinyl-3-aminopropyl) glyceryl 2-O-(N,N-diisopropylamino)(2-cyanoethyl) phosphite (V).

1-O-(4,4'-dimethoxytrityl)-3-O-(N-biotinyl-3-aminopropyl)-glycerol (IV) (1.36g, 2 mmole) was dissolved in dry tetrahydrofuran (4 ml) and N,N-diisopropylethylamine (0.52 ml, 3 mmole) was added. Then a solution of 2-cyanoethyl N,N-diiso-propylaminochlorophosphite (0.47g, 2 mmole) in dry tetrahydrofuran (1 ml) was added dropwise with stirring. The reaction mixture was left for 1 hour, filtered, and the filtrate was diluted with ethyl acetate (100 ml). The resultant solution was washed with 0.5M phosphate buffer pH 7.0 (2×20 ml), dried and concentrated. The residue (1.92g) was chromatographed on a silica column (60g) eluting with methylene chloride/methanol (39:1) and then methylene chloride/methanol (19:1) both containing 1% triethylamine. Two fractions were collected. The faster eluting product was evaporated to gave a foam (1.10g) which was dissolved in toluene (10 ml) and precipitated into pentane (200 ml). The precipitate was washed with pentane (200 ml), collected by centrifugation, and dried. The title compound (1.02g, 58% yield) was obtained as a fine powder. T.l.c. in Solvent E, R_f 0.33. ^{1}H-nmr, δ : 1.01−1.18 (m, 12H), 1.39 (quintet, J = 7.2Hz, 2H), 1.62−1.70 (m, 6H), 2.07−2.14 (m, 2H), 2.45 (t, J = 6.5H2, 1H), 2.63 (t, J = 6.5Hz, 1H), 2.65 (d, J = 13.0Hz, 1H), 2.87 (dd, J = 12.8Hz, J = 4.8Hz, 1H), 3.07−3.34 (m, 5H), 3.47−3.75 (m, 8H), 3.77 (s, 3H), 3.88 (s, 3H), 4.07−4.13 (m, 1H), 4.24−4.29 (m, 1H), 4.43−4.48 (m, 1H), 5.04 (br.s, 1H), 5.74 (br.s, 1H), 6.22 (br.d, J = 18.4Hz, 1H), 6.78−6.83 (m, 4H), 7.18−7.45 (m, 9H). ^{31}P-nmr, δ : 6.09, 6.12, 7.61, 7.63. Mass Spectrum (+FAB) 876.4 ($M^{+\cdot}$ −1). Elemental analysis, found: C, 63.10; H, 7.62; N,

7.84; calculated for $C_{46}H_{64}N_5O_8P$: C, 62.92; H, 7.35; N, 7.98. Reversed phase h.p.l.c. using isocratic elution at 90% buffer B showed two closely eluting peaks corresponding two two pairs of diastereoisomers (R_t 4.82 and 5.16 min).

Fractions containing the slower eluting product were evaporated to dryness to give unreacted starting compound IV (0.44g, 32% recovery).

N-Fluorenylmethoxycarbonyl-L-tyrosine benzyl ester (VI)

L-Tyrosine benzyl ester p-toluenesulphonate salt (11.09g, 25mmole) was dissolved in dry pyridine (125ml). The solution was cooled in an ice/water bath and then 9-fluorenylmethyl-chloroformate (6.47g, 25mmole) was added with stirring. Stirring was continued for 1 hour at 0°C and for 1 hour at room temperature. The reaction mixture was concentrated, dissolved in toluene (50ml) and concentrated once more to give an oil (24.0g). Crystallization from acetonitrile (50ml) gave an initial crop of 6.85g. The filtrate was concentrated and the resultant oil was dissolved in chloroform (200ml) and washed with 0.5M hydrochloric acid (50ml). The aqueous layer was extracted with chloroform (2×50ml) and the chloroform extracts were combined, dried and evaporated to dryness. The resultant crystalline solid (6.77g) was combined with the first crop and recrystallized from acetonitrile (60ml) to yield the title compound (10.38g, 84% yield): mp. 150−1°C. T.l.c. in Solvent F, R_f 0.48. ^{1}H nmr (d_6 DMSO), δ: 2.85−2.93 (m, 2H), 3.32 (d, J = 12.2Hz, 1H), 4.18−4.25 (m, 4H), 5.09 (s, 2H), 6.25 (d, J = 8.4Hz, 2H), 7.03 (d, J = 8.4Hz, 2H), 7.26−7.44 (m, 8H), 7.63 −7.67 (m, 2H), 7.87−7.93 (m, 3H), 9.25 (m, 1H). Mass spectrum (+FAB), m/z 494.2 ($M^{+\cdot}$ + 1).

N-Fluorenylmethoxycarbonyl-O-[bis (2-cyanoethyl)phosphate]-L-tyrosine benzyl ester (VII)

N-Fluorenylmethoxycarbonyl-L-tyrosine benzyl ester (VI) (7.41g, 15 mmole) and 1H-tetrazole (1.58g, 22.5 mmole) were dissolved in dry acetonitrile (225ml) and then a solution of bis(2-cyanoethyl)-N,N-diisopropylaminophosphine (6.10g, 22.5mmole) in dry acetonitrile (22.5ml) was added dropwise with stirring. The reaction mixture was left for 1 hour and then 50% 3-chloroperbenzoic acid (5.16g, 15 mmole) was added with stirring and cooling by use of a water bath. Stirring was continued at room temperature for 0.5 hour and the solution was concentrated to an oil (13.50g). The oil was dissolved in chloroform (450 ml) and washed with saturated sodium bicarbonate solution (225 ml). The chloroform solution was dried, evaporated to dryness to give an oil (14.80g). Crystallization from a mixture of methylene chloride (60 ml) and diethyl ether (120 ml) gave the title compound (8.80g, 86% yield): mp. 103−4°C. T.l.c. in Solvent A, R_f 0.44. ^{1}H nmr, δ: 2.65−2.79 (m, 2H), 4.19 (t, J = 6.8Hz, 1H), 4.28−4.47 (m, 6H), 4.68 (dt, J =12.1Hz and J=5.7Hz, 1H), 5.12 (d, J = 12.1Hz, 1H), 5.19 (d, J = 12.1Hz, 1H), 5.36 (d, J = 12.1Hz, 1H), 6.97 (d, J = 8.3Hz, 2H), 7.07 (d, J = 8.3Hz, 2H), 7.27−7.43 (m, 9H), 7.57 (d, J = 7.0Hz, 2H), 7.76 (d, J = 7.4Hz, 2H). ^{31}P nmr, δ: −148.60. Mass spectrum (+ FAB), m/z 680.3 ($M^{+\cdot}$ + 1).

N-Fluorenylmethoxycarbonyl-O-[bis(2-cyanoethyl)phosphate]-L-tyrosine (VIII)

N-Fluorenylmethoxycarbonyl-O-[bis(2-cyanoethyl)phosphate]-L-tyrosine benzyl ester (VII) (6.80g, 10 mmole) was dissolved in a mixture of 95% ethanol (200ml) and ethyl acetate (200ml) and 10% palladium on charcoal (1.0g) was added. The suspension was stirred under hydrogen until nearly all the substrate was gone (T.l.c. assay). The reaction mixture was filtered and the filtrate was concentrated. The resultant oil (6.20g) was dissolved in a 1% solution of sodium carbonate (200ml) and the solution was shaken with diethyl ether (100ml). The aqueous solution was acidified with citric acid to pH4 and the resultant suspension was extracted with ethyl acetate (200ml). The organic phase was dried and evaporated to give the title compound (3.83g, 65% yield) as a solid foam. T.l.c. in Solvent G, R_f 0.38. ^{1}H nmr, δ: 2.71 (t, J = 6.0Hz, 4H), 3.13 (d, J = 5.3Hz, 2H), 4.20 (t, J = 6.7Hz, 1H), 4.30−4.51 (m, 6H), 4.65 (dt, J = 12.0Hz and J = 5.4Hz, 1H), 5.48 (d, J = 12.1Hz, 1H), 7.13, (s,4H), 7.28−7.43 (m, 4H), 7.58 (d, J = 7.2Hz, 2H), 7.77 (d, J = 7.4Hz, 2H). ^{31}P nmr, δ: −148.85. Mass spectrum (+FAB), m/z 590.2 ($M^{+\cdot}$ + 1).

N-Fluorenylmethoxycarbonyl-O-[bis(2-cyanoethyl)phosphate]-L-tyrosine pentafluorophenyl ester (IX)

N-Fluorenylmethoxycarbonyl-O-[bis(2-cyanoethyl)phosphate]-L-tyrosine (VIII) (2.95g, 5mmole) was dissolved in dry dioxane (20ml) and a solution of pentafluorophenol (1.02g, 5.5mmole) in dry dioxane (5ml) was added. Then dicyclohexylcarbodiimide (1.13g, 5.5mmole) was added with stirring. Stirring was continued for 1 hour and the resultant suspension was filtered. The filtrate was concentrated to an oil (4.18g) which was dissolved in chloroform (100ml) and washed with saturated sodium bicarborate solution (50ml). The organic phase was dried and concentrated to an oil (3.52g). The product was chromatographed on a silica column (60g) eluting with chloroform/ ethanol (39:1) and then chloroform/ethanol (19:1). Fractions containing a single component were collected and evaporated to dryness to yield the title compound (3.10g, 82% yield) as a solid foam. T.l.c. in Solvent A, R_f 0.33. ^{1}H nmr, δ: 2.74 (t, J = 6.0Hz, 4H), 3.27 (d, J = 5.8Hz, 2H), 4.21 (t, J = 6.6Hz, 1H), 4.32−4.48 (m, 6H), 4.97−5.02 (m, 1H), 5.45 (d, J = 8.5Hz, 1H), 7.20 (s, 4H), 7.28−7.43 (m, 4H), 7.56−7.59 (m, 2H), 7.77 (d, J = 7.4Hz, 2H). ^{31}P nmr, δ: −148.67. Mass spectrum (+FAB), m/z 756.1 ($M^{+\cdot}$ + 1).

N-{N-Fluorenylmethoxycarbonyl-O-[bis(2-cyanoethyl)phosphate]-L-tyrosinyl}-3-aminopropyl solketal (X)

N-Fluorenylmethoxycarbonyl-O-[bis(2-cyanoethyl)phosphate]-L-tyrosine pentafluorophenyl ester (IX) (3.02g, 4 mmole) was dissolved in dry DMF (20ml). Then a solution of 3-aminopropyl solketal (II) (0.91g, 4.8 mmole) in dry DMF (8ml) was added dropwise with stirring. The reaction mixture was left for 0.5 hours and then concentrated to an oil (5.61g) which was dissolved in chloroform (80ml) and washed with saturated sodium bicarbonate solution (40ml). The organic phase was dried and concentrated to an oil (4.10g). The product was chromatographed on a silica column (100g) eluting with chloroform/ethanol (39:1) and then chloroform/ethanol (19:1). Fractions containing a single component were evaporated to dryness to give the title compound (2.60g, 86% yield) as a thick oil. T.l.c. in Solvent A, R_f 0.26. ^{1}H nmr, δ: 1.40 (s, 3H), 1.65 (s, 3H), 1.87−1.90 (m, 2H), 2.76 (t, J = 6.1Hz, 4H), 2.95−3.11 (m, 2H), 3.32−3.56 (m, 9H), 4.16−4.20 (m, 1H), 4.32−4.40 (m, 7H), 5.63−5.82 (m, 1H), 6.56−6.81 (m, 1H), 7.11−7.16 (m, 4H), 7.28−7.43 (m, 4H), 7.56 (d, J = 7.4Hz, 2H), 7.76 (d, J = 7.4Hz, 2H). ^{31}P nmr, δ: −148.65, −148.49.

1-O-(4,4'-dimethoxytrityl)-3-O-(N-{N-fluorenylmethoxycarbonyl-O-[bis(2-cyanoethyl) phosphate]-L-tyrosinyl}-3-aminopropyl)glycerol (XI)

N-{N-Fluorenylmethoxycarbonyl-O-[bis(2-cyanoethyl)phosphate]-L-tyrosinyl}-3-aminopropyl solketal (X) (2.28g, 3 mmole) was dissolved in a mixture of tetrahydrofuran (12ml) and 1M hydrochloric acid (6ml). The solution was left for 1 hour and then absolute ethanol (12ml) was added. The solution was concentrated, the residue was dissolved in absolute ethanol (12ml) and concentrated again. The resultant product was dried by co-evaporation with pyridine (2×6ml) to give an oil (2.20g) which was redissolved in dry pyridine (12ml) and 4,4'-dimethoxytritylchloride (1.02g, 3 mmole) was added with stirring. Stirring was continued for 15 mins and the resultant solution was left for 1 hour. Absolute ethanol (6ml) was added and the solution was concentrated. The residue was dissolved in chloroform (60ml) and washed with saturated sodium bicarbonate solution (30ml). The organic phase was dried and evaporated to an oil (4.32g). The product was chromatographed on a silica column (90g) eluting with methylene chloride/methanol (39:1) and then methylene chloride/methanol (19:1). Fractions containing a single component were collected and evaporated to dryness to yield the title compound (2.15g, 70% yield) as a solid foam. T.l.c. in Solvent H, R_f 0.28. ^{1}H nmr, δ: 1.61−1.67 (m, 2H), 2.66 (q, J = 5.7Hz, 2H), 2.76 (t, J = 6.0Hz, 2H), 3.03−3.14 (m, 4H), 3.29−3.52 (m, 7H), 3.74 (s, 3H), 3.79 (s, 3H), 3.88 (br s, 1H), 4.23−4.40 (m, 8H), 5.56−5.82 (m, 1H), 6.56−6.68 (m, 1H), 6.78 (d, J = 8.9Hz, 2H), 6.82 (d, J = 9.0Hz, 2H), 7.13−7.54 (m, 19H), 7.72−7.78 (m, 2H). ^{31}P nmr, δ: −148.59, −148.57. Mass spectrum (+FAB), m/z 1022.6 ($M^{+\cdot}$).

1-O-(4,4'-dimethoxytrityl)-3-O-(N-{N-fluorenylmethoxycarbonyl-O-[bis(2-cyanoethyl) phosphate]-L-tyrosinyl}-3-aminopropyl)glyceryl 2-O-(N,N-diisopropylamino)(2-cyanoethyl)-phosphite (XII)

1-O-(4,4'-dimethoxytrityl)-3-O-(N-{N-fluorenylmethoxycarbonyl-O-[bis(2-cyanoethyl)phosphate]-L-tyrosinyl}-3-aminopropyl)-glycerol (XI) (2.04g, 2mmole) was dissolved in dry tetrahydrofuran (4ml) and N,N-diisopropylethylamine (0.70ml, 4 mmole) was added. Than a solution of 2-cyanoethyl N,N-diisopropylamino- chlorophosphite (0.71g, 3mmole) in dry tetrahydrofuran (2ml) was added dropwise with stirring. The reaction mixture was left for 1 hour, filtered and the filtrate was diluted with ethyl acetate (100ml). The resultant solution was washed with 0.5M phosphate buffer pH 7.0 (2×20ml), dried and concentrated. The residue (2.52g) was chromatographed on a silica column (80g) eluting with methylene chloride/ethyl acetate (3:1) and then methylene chloride/ethyl acetate (1:1), both containing 1% of 2,6-lutidine. Fractions containing a single component were collected and evaporated to dryness. The resultant oil (1.62g) was dissolved in toluene (16ml) and product precipitated with pentane (320ml). The precipitate was washed with pentane (2×320ml), collected by centrifugation, and dried. The title compound (1.08g, 44% yield) was obtained as a fine powder. T.l.c. in Solvent I, R_f 0.26. ^{1}H nmr, δ: 1.14−1.33 (m, 12H), 1.56−1.75 (m, 2H), 2.54−2.79 (m, 6H), 2.93−3.26 (m, 6H), 3.38−3.61 (m, 7H), 3.76 (s, 6H), 6.21−6.33 (m,1H), 6.79−6.83 (m, 4H), 7.27−7.57 (m, 19H), 7.76 (d, J=7.6Hz, 2H). ^{31}P nmr, δ: −148.65, −148.64, 7.41, 7.68, 7.96. Mass spectrum (+FAB), m/z 1223.9 ($M^{+\cdot}$ + 1). Elemental analysis; calculated for $C_{66}H_{76}N_6O_{13}P_2$: C 64.81,H 6.26, N 6.87; found: C 64.70, H 6.34, N 6.92. Reversed phase h.p.l.c. using isocratic elution at 90% buffer B showed two closely eluting peaks, each peak corresponding to a pair of diasteroisomers (R_t 6.14 and 6.86 min).

Oligonucleotide Assembly

Oligonucleotides were assembled using an Applied Biosystems 380B 3-column DNA Synthesiser following manufacturers recommendations with the cyanoethyl phosphoramidite procedure. 0.2 μmole scale columns were used throughout. For couplings with biotinyl phosphoramidite V or phosphotyrosinyl phosphoramidite XII a 0.2M concentration in anhydrous acetonitrile was used and the coupling wait time was increased to 300 secs (compared to 30 secs for normal nucleotide coupling). Both these modifications were necessary to obtain high coupling yields for phosphoramidites V and XII. In each final coupling cycle, the Trityl ON configuration was used. After assembly, the oligonucleotides were cleaved from the support using concentrated ammonia at room temperature using the manufacturer's end procedure cycle. The ammoniacal solution was then heated to 60°C in a sealed tube for 5 h and evaporated to dryness. The residue was dissolved in 0.3 ml acetic acid/ water (8:2) and after 20 minutes at room temperature the mixture was evaporated to dryness. To the residue was added water (0.5 ml) and the resultant suspension filtered. The aqueous solution now contained the deprotected oligonucleotide ready for purification.

Oligonucleotide Purification

Reversed-phase h.p.l.c. was carried out on an analytical or a semi-preparative μ Bondapak C18 reversed phase column (Waters) using gradients of buffer A (0.1M ammonium acetate solution) and buffer B (20% buffer A/80% acetonitrile) at flow rates of 1.5 ml/min (analytical runs) or 3 ml/min (purification runs). Desired product was obtained by evaporation to dryness of the material in the product peak.

Ion exchange h.p.l.c. was carried out on an analytical Partisphere 5-SAX cartridge (Whatman) using gradients of potassium phosphate buffer (pH 6.3) (buffer A, 1mM; buffer B, 0.3M) containing 60% formamide and a flow rate of 2 ml/min. Material in the product peak was dialysed exhaustively against deionised water to remove formamide and salts.

Polyacrylamide gel electrophoresis was carried out on 20% gels (200×400×1.5 mm) at 37 watts using a marker dye of bromophenol blue and product bands were visualized by UV shadowing at 254 nm. Oligomers were eluted with 0.5M ammonium acetate, 0.01M magnesium acetate and desalted using OPC cartridges (Applied Biosystems) following manufacturers protocols.

Oligonucleotide hybridization to M13 DNA

M13mp19 ssDNA (Gibco BRL) was spotted on pre-wet (water, then 1M ammonium acetate solution) nitrocellulose filters (Schleicher and Schuell) in serial dilutions in 10mM Tris HCl (pH 7.4), 5mM NaCl, 0.1mM EDTA (amounts 0.05−50 ng). The filters were baked at 80°C for 1 hour and washed in 6×SSC, 0.2%BSA, 0.2% PVP 40, 0.2% Ficoll 400, 0.2% SDS at 60°C for 10 mins. After brief rinsing in 6×SSC, hybridization was carried out in solutions of biotinylated or phosphotyrosinylated oligomers (10 nM) at 37°C for 2 hours. Filters were washed

in 2×SSC, 0.1% SDS twice for 1 minute and then for 1 minute in 2×SSC.

Detection of biotin and phosphotyrosine

Biotin was detected qualitatively on filters by the following methods: a) streptavidin-alkaline phosphatase kit (bluGENE, BRL); b) streptavidin-biotinylated alkaline phosphatase kit (DNA Detection System, BRL); c) mouse monoclonal against biotin (DAKO) (10μg/ml in Tris buffered saline plus 0.05% Tween 20 (TBST)) followed by alkaline phosphatase conjugated rabbit-anti mouse IgG (DAKO) (10 μg/ml in TBST) with colorimetric endpoint detection; d) mouse monoclonal against biotin as in c) followed by biotinylated goat-anti mouse IgG (BRL)(5μg/ml in TBST) and then streptavidin-alkaline phosphatase conjugate (BRL)(0.2 μg/ml in TBST)(Immunoselect system kit, BRL, 9590SA) with colorimetric endpoint detection.

Quantitative detection of biotin involved blocking of the filter with 5% w/v reconstituted milk powder in TBST for 1 hour, incubation with a mouse monoclonal antibody against biotin (0.1 μg/ml in TBST), washing with TBST (6×5 mins) and then incubation with sheep anti-mouse IgG-horseradish peroxidase conjugate (Amersham, NA 931) at a 1/1000 dilution in TBST. After washes with TBST (6×5 mins) and TBS (1×5 min), detection was carried out using the enhanced chemiluminescent (ECL) detection reagents (Amersham, RPN 2105). The still moist filters were photographed and quantitated using a CCD camera (600 second exposures). Control 17-mer oligonucleotide directly linked to horseradish peroxidase was prepared using the ECL oligonucleotide labelling and detection system (22) (Amersham, RPN 2111/2113).

Phosphotyrosine was detected qualitatively by methods c) and d) except that in each case the primary detection antibody was a mouse monoclonal antibody against phosphotyrosine (Amersham, RPN 138) used at 10μg/ml in TBST buffer, and also by method e) where the secondary antibody was goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma, 10μg/ml in phosphate buffered saline plus 0.05% Tween 20). Phosphotyrosine was detected quantitatively using the ECL method. Primary detection was by a mouse monoclonal antibody against phosphotyrosine (0.5μg /ml) and the secondary antibody was sheep anti-mouse IgG conjugated to horseradish peroxidase (Amersham, NA931) used at 1/1000 dilution.

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