The Synthesis of Oligonucleotide–Polyamide Conjugate Molecules Suitable as PCR Primers

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A new class of oligonucleotide–polyamide conjugates having free 3′-termini has been developed. These molecules have the potential to act as multiply-labeled nonradioactive polymerase chain reaction (PCR) primers. The linkage between the oligonucleotide and the polyamide is via a C5-modified deoxyuridine, synthesized in three steps from 5-iodo-2′-deoxyuridine (**6**). Subsequent palladium-catalyzed coupling of the 5′-O-protected deoxyuridine derivative **7** and the N-protected propargylamines **11** and **12** gave the alkynyl nucleosides **3** and **4**, respectively. Both of these were then incorporated onto succinyl-CPG resin by diisopropylcarbodiimide/DMAP-mediated esterifications to give **18** and **19**, respectively. Acidolytic removal of all protecting groups, selective acylation at the C5 pendant amine by an activated N^{α}-(9-fluorenylmethoxycarbonyl) (Fmoc)-protected amino acid followed by reprotection of the 5′-hydroxyl by tritylation with 4,4′-dimethoxytrityl chloride gave the solid support **22**. Standard Fmoc solid-phase peptide synthesis on the C5 pendant arm of **22** followed by oligonucleotide synthesis using standard phosphoramidite chemistry on the 5′-hydroxyl gave the desired oligonucleotide–polyamide conjugate **5**. A single biotin label was incorporated into the polyamide using the biotinylated lysine synthon **29**; this method is expected to be readily applicable for the attachment of multiple biotin residues in a facile and highly-controlled manner. The conjugate **5** was characterized by UV spectroscopy, amino acid analysis, 5′-end labeling with ^{32}P and reversed-phase C_{18} HPLC analysis of enzymatic digests. Preliminary experiments with **5** have shown that it is an efficient PCR primer.

Introduction

The polymerase chain reaction (PCR)[1a-c] is a recent advance in molecular genetics which has wide-ranging implications for any technique requiring the production or detection of specific DNA sequences. PCR is a gene amplification technique which allows many copies of a specific DNA to be generated from only a few target molecules within a period of 1–2 h and consequently has found applications in nucleic acid detection techniques previously limited by low sensitivity.[2-5] For a comprehensive description of the practical applications of PCR, there is a review article by Eisenstein.[6]

A PCR cycle has three steps (a typical PCR experiment has 30 cycles): (i) heat denaturation of the double-stranded native DNA, (ii) annealing of two synthetic oligonucleotide primers to complementary sequences on opposite strands of the DNA at a lower temperature, and (iii) extension in the 3′ direction by a thermostable DNA polymerase (usually Taq polymerase). The majority of PCR-based experiments use agarose gel electrophoresis followed by UV visualization for the characterization of the PCR products. A number of procedures have been introduced recently that do not require an electrophoretic step,[7] a condition that has to be met if a test is to have application in mass screening, e.g., in the detection of HIV genetic material. These procedures use oligonucleotides modified to contain a single nonradioactive label.

We have previously described the synthesis of multiply-labeled oligonucleotide–polyamide conjugates.[8] Specifically, we were able to show that, using chemiluminescent detection of multiply-biotinylated probes, a sensitivity could be achieved that was considerably higher than that of monobiotinylated probes and was comparable to that of ^{32}P-labeled probes.[8c] These nonradioactive probes have significant advantages over isotopically-labeled probes in being stable for extended periods of time and much safer to handle. The combination of an assay which is amenable to mass screening of samples with the high sensitivity and convenience afforded by nonradioactive, multiply-labeled PCR primers would provide a powerful tool for DNA detection. The multiply-labeled conjugates we have prepared previously[8a,b] had oligonucleotide moieties with blocked 3′-termini and were thus unsuitable for use as PCR primers. The aim of this work was to develop

† School of Chemistry.

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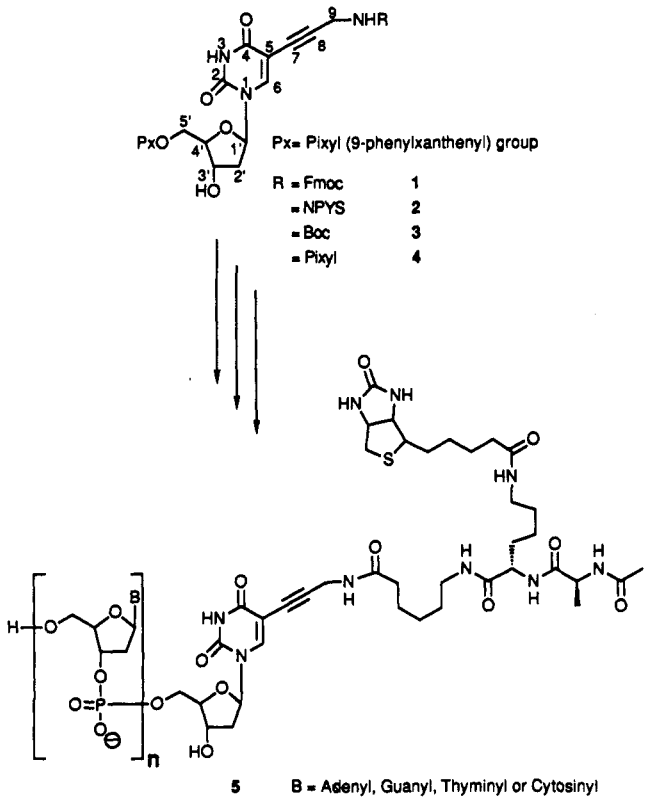
Scheme I



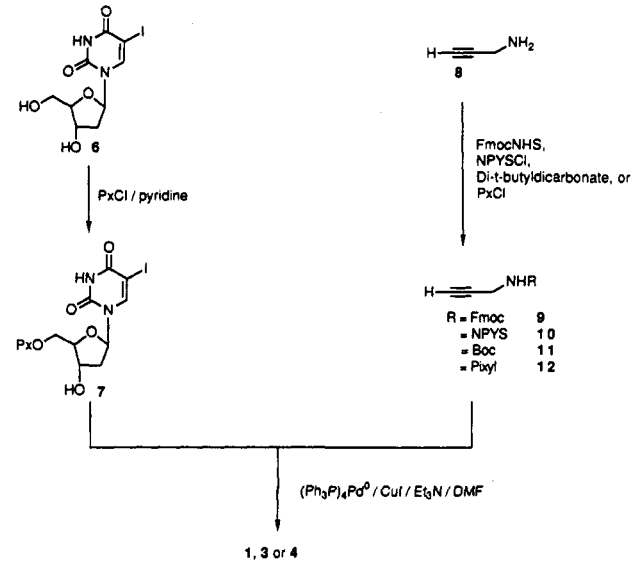
methodology suitable for the synthesis of oligonucleotide–polyamide conjugates possessing free 3′-termini.

One type of oligonucleotide–polyamide conjugate that retains a free 3′-hydroxyl is **5**, in which the linkage is through a modified nucleoside (Scheme I). The oligonucleotide can act as a PCR primer, while the polyamide can carry multiple labels such as biotin residues or fluorophores. The salient features of this conjugate are that the rigid alkyne linker arm on C5 of the deoxyuridine decreases the likelihood of the polyamide interfering in the interaction between the DNA polymerase and the 3′-terminus of the oligonucleotide, there is a free 3′-hydroxyl to allow chain elongation to proceed, and the conjugate can be synthesized by assembly of the polyamide on the modified-nucleoside derivatized solid support, followed by assembly of the oligonucleotide. The polyamide moiety of this conjugate contains a 6-aminohexanoic acid (ϵAhx) residue as a spacer, an ϵ-biotinylated lysine residue as a label for the detection of the PCR amplification products, and an alanine residue with an acetylated α-amino group (to avoid any interference during oligonucleotide synthesis) as a reference amino acid (Scheme I). The incorporation of the labels can be achieved in a highly controlled manner, using Fmoc-L-Lys(Biotinyl)-OPfp (**29**).

The synthesis of this type of oligonucleotide–polyamide conjugates could be attempted in two ways. In the first, a nucleoside with orthogonal protection, such as **1** or **2** (Scheme I), is attached to a solid support via the 3′-hydroxyl. Removal of the base labile 9-fluorenylmethoxycarbonyl (Fmoc) group of **1** or the reduction-labile 3-nitropyridine-2-sulfenyl (NPYS) group of **2** provides an amino group for the synthesis of the polyamide. Then, acidolytic removal of the pixyl group at C5′ allows oligonucleotide synthesis to take place.

The second route is based on the large difference in nucleophilicity between primary amines and primary alcohols. Thus, if a mildly activated acylating agent is employed, such as the pentafluorophenyl ester of an Fmoc

Scheme II



amino acid, selective acylation of the amine should be possible. In this route, the protecting group R in Scheme I could be one of the acid-labile protecting groups Boc (as in **3**) or 9-phenylxanthen-9-yl (pixyl) (as in **4**). We now describe the synthesis and characterization of a simple model conjugate which allows both the synthetic methodology and the efficacy of these conjugates as PCR primers to be assessed.

Other workers have also reported procedures for the introduction of multiple labels at the 5′-end, the 3′-end, or internal positions.[9] However, the methods described in this report are the only ones to our knowledge in which the label is included as part of the solid support and an oligonucleotide is synthesized by standard methods and is still able to act as a primer for a polymerase enzyme.

Results

Synthesis of the Modified Nucleoside. The modified nucleosides **1**, **3**, and **4** which act as the linker between the oligonucleotide and the polyamide were synthesized in three steps starting from commercially-available 5-iodo-2′-deoxyuridine (IdU) **6** (Scheme II). The first step involved the preparation of the four different N-protected propargylamines **9**–**12** by reaction of 3-aminopropyne with *N*-[(fluoren-9-ylmethoxycarbonyl)oxy]succinimide, 3-nitropyridine-2-sulfenyl chloride (NPYSCl), di-*tert*-butyl dicarbonate, and 9-chloro-9-phenylxanthene, respectively. In the case of the NPYS derivative **10**, the presence of a base was necessary for efficient reaction. Triethylamine was found to readily effect nucleophilic substitution of the NPYSCl to form the quaternary ammonium salt $[(Et_3N)S(C_5H_3NO_2)]^+Cl^-$, so a 2.5 molar excess of 3-aminopropyne was used to act as base as well. The Pd(0)-catalyzed oxidative coupling of a protected aminoalkyne to the unprotected nucleoside resulted in complex mixtures inseparable by silica gel chromatography. Protection of the 5′-hydroxyl of IdU via DMAP-catalyzed alkylation with 9-chloro-9-phenylxanthene gave the 5′-protected nucleoside **7** in high yield and purity, and this was reacted

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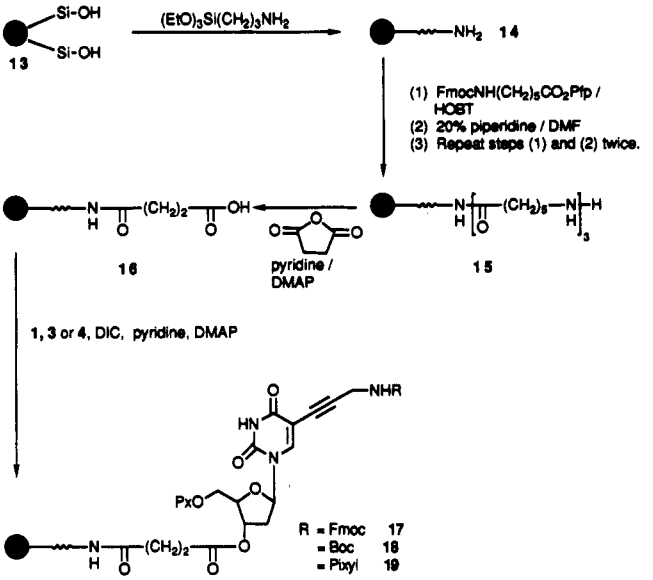
with protected propargylamines **9–12**, according to the method of Hobbs.[10] The reaction of the protected nucleoside **7** with the Fmoc-protected propargylamine **9** produced the desired product **1** in quite low yields (30%), probably due to partial deprotection of **9** by triethylamine. The free primary amino groups generated by the deprotection may also coordinate with the palladium catalyst and hinder the coupling reaction.[10] The product **1** coeluted with the starting material in silica gel chromatography under a wide range of eluting conditions, and both the ^1H and ^{13}C NMR spectra showed that it contained a small amount of **7**. The coupling reaction between **7** and the NPYS-protected propargylamine **10** produced a complex mixture which could only be resolved by silica gel chromatography followed by C_{18} reversed-phase HPLC. Analysis of the HPLC fractions by ^1H and ^{13}C NMR spectroscopy and FABMS showed that none contained the desired product. In contrast, the coupling of the Boc and pixyl-protected propargylamines **11** and **12** to **7** were straightforward. The Boc-protected nucleoside **3** also coelutes with the starting nucleoside **7** but the product **3** could be isolated in high yield nevertheless due to the absence of significant contaminating amounts of **7**. The preparation of the dipixylated nucleoside **4** could be monitored by TLC (5% MeOH/1% Et_3N/CH_2Cl_2), and the reaction was judged to be complete after 5 h; this reaction also had a high yield.

Adequate characterization of the alkynyl nucleosides **3** and **4** could be achieved by one-dimensional ^1H and ^{13}C NMR spectroscopy, since the multiplets in the former compound are well separated and most of the latter's resonances correspond quite closely to those of analogous compounds we have previously reported.[11] Some salient features of the ^{13}C NMR spectrum of these alkynyl nucleosides are the two resonances in the regions δ 73.8–74.6 and δ 90.1–91.2 corresponding to the alkynyl quaternary carbons C8 and C7, respectively (see Scheme I for numbering system). The downfield shift of C5 from δ 69.9 in IdU to δ 98.4–98.7 is consistent with the substitution of a quaternary alkynyl carbon for the iodine atom. All other resonances were consistent with the proposed structures of **3** and **4**.

Derivatization of a Solid Support with the Modified Nucleoside. The next stage in the synthesis of the conjugate was derivatization of a solid support. In our experience, controlled-pore glass (CPG) resin allows both the polyamide and the oligonucleotide to be synthesized efficiently. Conventional peptide synthesis resins such as Pepsyn K[12] have a higher loading than CPG but efficient DNA synthesis by the phosphoramidite approach is not possible, so CPG is the solid support of choice.

Derivatization of CPG resin for DNA synthesis is conventionally effected by amination of the CPG, followed by coupling with a nucleoside succinate active ester[13] or a carbodiimide coupling, typically with DCC.[13,14] Damha et al.[15] have recently described a method for the attachment

Scheme III



of nucleosides to succinylated CPG resin via a 1-[3′-(dimethylamino)propyl]-3-ethylcarbodiimide (DEC)-mediated condensation. This procedure is more convenient since it obviates additional solution-phase manipulation of the nucleoside derivatives. The effectiveness of DEC compared to that of DCC has been attributed to its smaller steric requirement.[14] With this in mind, we anticipated that diisopropylcarbodiimide (DIC) might also be better than DCC. Hence, a comparison of DIC with DCC and DEC was undertaken using a standard nucleoside, 5′-*O*-(dimethoxytrityl)-N^4-benzoyl-2′-deoxycytidine (dimethoxytrityl = bis(*p*-methoxyphenyl)phenylmethyl). Succinylated-CPG resin was treated with the nucleoside, DMAP (0.5 molar equiv), and the appropriate condensation agent in dry pyridine. After 24 h, the degree of nucleoside coupling was assessed by trityl assay. The nucleoside loadings were determined to be 22, 18, and 30 μmol/g for DCC, DEC, and DIC, respectively, indicating that DIC is the condensation reagent of choice.

The solid support for the synthesis of the conjugate was prepared in four steps starting from CPG resin **13** (Scheme III). The CPG was aminated with (3-aminopropyl)-triethoxysilane,[16] and then three 6-aminohexanoic acid spacer residues were attached using standard Fmoc solid-phase peptide synthesis techniques[12] to give resin **15**. It was anticipated that incorporation of the three spacer units would allow more efficient oligonucleotide synthesis due to the increased accessibility of the terminal resin-bound nucleoside to reagents, in an analogous manner to long-chain alkylamine (LCAA) CPG.[17] The aminated resin **15** was subjected to DMAP-catalyzed succinylation with succinic anhydride to give resin **16**. Attachment of the appropriate nucleoside to the succinyl resin **16** was achieved by DIC/DMAP-mediated condensation, and any free carboxylic acid and amine groups remaining were blocked[15] by DCC/4-nitrophenol/piperidine and acetic anhydride/DMAP treatments, respectively.

The attachment of the Fmoc-nucleoside derivative **1** to the succinyl resin **16** to give **17** was problematic. The use of DMAP is necessary to achieve adequate nucleoside

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loadings of approximately 30 μmol/g, but we have found[18] that the concentration used effects 45% cleavage of the Fmoc group in 24 h. After two successive 24-h coupling reactions between the Fmoc-nucleoside 1 and the succinyl resin 16, comparison of the Fmoc and pixyl assays suggested that about half of the nucleoside had undergone amine deprotection and was probably attached to the resin by an amide bond. This side product was not expected to interfere in the preparation of the desired conjugate since it would be expected to be stable to the final cleavage conditions and thus stay on the solid support. However, resin derivatized with the Fmoc-nucleoside 1 consistently gave very low yields of conjugate and was not further investigated.

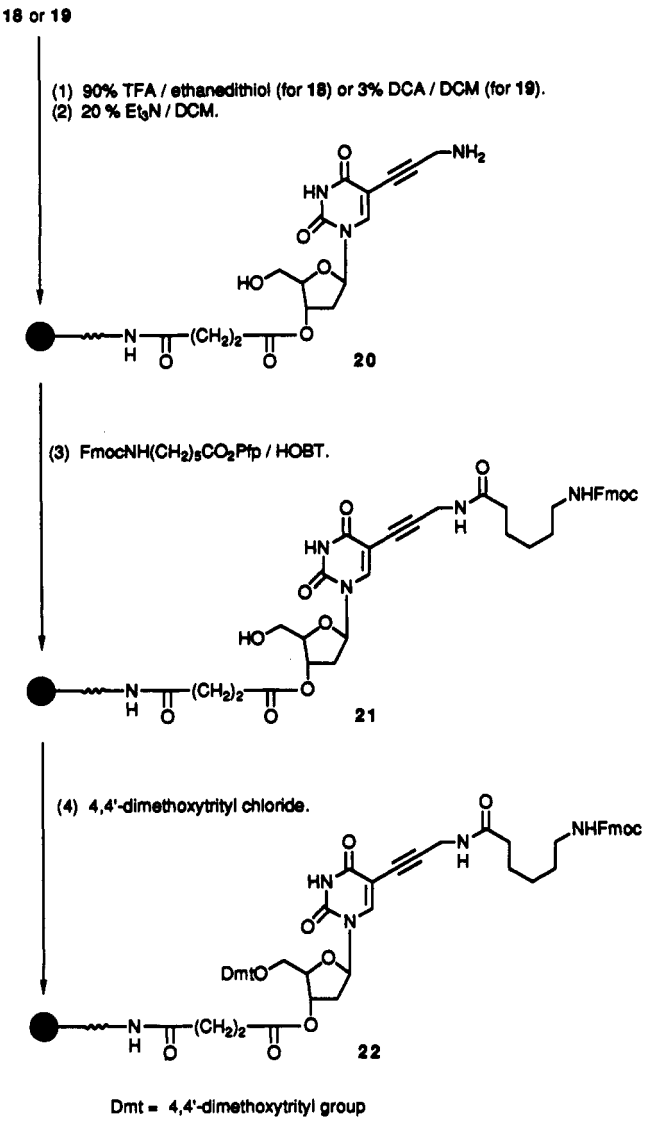
The Boc and pixyl nucleosides 3 and 4 were readily coupled to the solid support 16 under the above conditions, resulting in loadings of 22 μmol/g (for 17) and 40 μmol/g (for 18), respectively, as assessed by pixyl assay. Both the Boc and the pixyl derivatives 3 and 4 gave sufficient nucleoside loadings for efficient oligonucleotide synthesis, but the latter is preferred due to its facile preparation and the milder deprotection conditions required.

Synthesis of the Oligonucleotide–Polyamide Conjugate. The model compound 5 was initially synthesized to test the efficacy of these types of conjugates as PCR primers. The oligonucleotide moiety was a 23mer (see Experimental Section) which, together with another 23mer d(GGTTATCGAAATCAGCCACAGCG), amplifies a 500 base-pair region (7131–7630) of the DNA of λ phage; the template is provided with the standard Cetus PCR kit for control reactions.

As with the synthesis of conjugates we have previously described,[8] the polyamide part of the present conjugate was synthesized first on the derivatized solid support described above, by the conventional Fmoc strategy,[12] since peptide synthesis conditions are harsher than those of DNA synthesis. The pendant amino and 5′-hydroxyl groups of the derivatized solid supports 18 and 19 were deprotected (Scheme IV) by treatment with 90% TFA/ethanedithiol and 3% DCA/CH_2Cl_2, respectively, and then neutralized with 20% triethylamine/CH_2Cl_2 to give the solid support 20. They were then subjected to a double (45-min each) coupling with a 1:1 mixture of *N*-Fmoc-6-aminohexanoic acid pentafluorophenyl ester (Fmoc-ϵAhx-OPfp) and 1-hydroxybenzotriazole (HOBT) (2.5 molar equiv of each) to give 21. A qualitative trinitrobenzenesulfonic acid test[19] showed only trace amounts of free amino groups at the end of the first coupling and no free amino groups after the repeat coupling. Reprotection of the 5′-hydroxyl was achieved by tritylation with 4,4′-dimethoxytrityl chloride in pyridine (without DMAP as catalyst because of the presence of the Fmoc group) to give resin 22. The trityl content of the resin after two successive 24-h treatments was comparable to the nucleoside content determined prior to the acylation reaction, confirming that the acylation had occurred with a high degree of selectivity at the pendant amine. Any remaining free hydroxyl and amino groups were blocked as already described.

The biotinylated lysine synthon 29 (see below and Scheme VII) and Fmoc-Ala-OPfp were coupled to solid support 22 (Scheme V) using standard Fmoc chemistry with a 5-fold excess of amino acid active ester and HOBT to give the solid support 23. The coupling efficiency of 29

Scheme IV



Dmt = 4,4′-dimethoxytrityl group

was similar to that of the standard alanine derivative, the reaction being complete in 1 h. Amino acid analysis of the solid support at this stage gave the expected ratio of lysine to alanine, with loadings of 26 and 28 μmol/g for lysine and alanine, respectively. The Fmoc group of alanine was removed with 20% piperidine/DMF and the resulting free amino group acetylated by treatment with Ac_2O/DMAP to give the intermediate 24.

Following the synthesis of the polyamide, an oligonucleotide was synthesized at the 5′-position of 24 using standard β-cyanoethyl phosphoramidite chemistry.[8a,13a,20] In short, one synthetic cycle consists of the following steps (Scheme VI): (1) detritylation with 3% DCA/DCM (2) tetrazole-catalyzed phosphitylation with a β-cyanoethyl-protected nucleoside phosphoramidite to give an intermediate with a phosphite linkage (not shown), (3) capping of any remaining free hydroxyl groups with acetic anhydride/DMAP, and finally (4) oxidation by iodine to form the more stable phosphotriester linkage in 25. By repeating these steps "n – 1" times, ending with a detritylation step, the base- and phosphate-protected, resin-bound conjugate 26 was synthesized. The repetitive coupling yields of nucleotide addition as assessed by trityl

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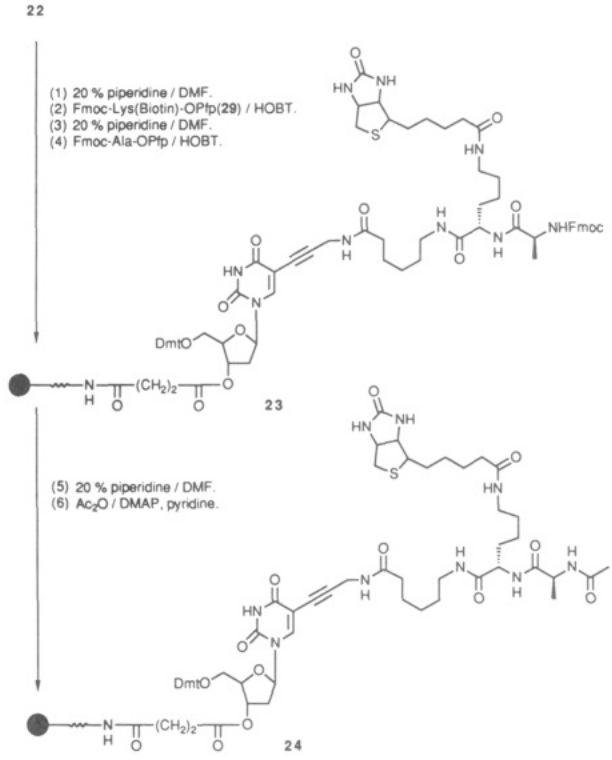
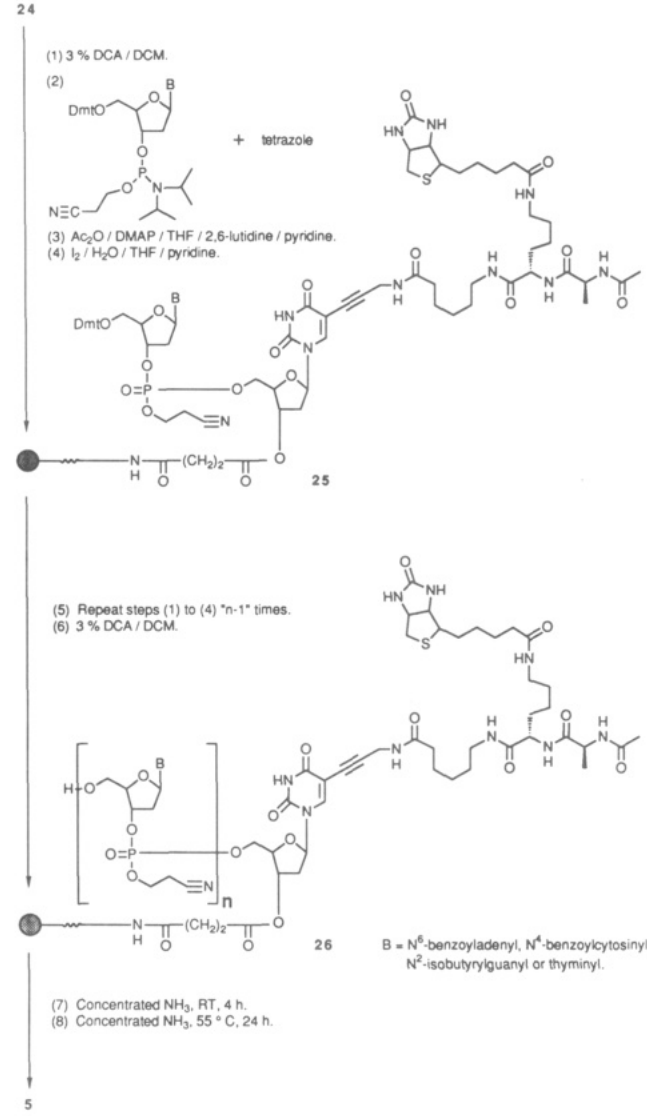
Scheme V



Scheme VI



assays were comparable with that of DNA synthesis using normal solid supports. Cleavage from the solid support, phosphate, and base deprotection by ammonia treatment[8a,13a,16a] gave a material which was shown to be composed of two major products by polyacrylamide gel electrophoresis (PAGE). The product with the higher electrophoretic mobility contained no peptidic material (as assessed by amino acid analysis) while the slower moving one contained the desired composition of amino acids (the relative molar ratio of these products being 1.30:1.00). Purification by preparative PAGE resulted in high yields of the desired conjugate.

The oligonucleotide–polyamide conjugate **5** was characterized by UV spectroscopy, amino acid analysis, nuclease digestion to its component nucleotides, and PAGE. Quantitation of the oligonucleotide moiety by its UV absorbance and quantitation of the polyamide moiety by amino acid analysis gave a 1.2:1 ratio of oligonucleotide to polyamide. In addition, the ratios of the amino acids were as expected, suggesting that the polyamide moiety was intact and was stable to oligonucleotide synthesis conditions. The conjugate was also 5′-end labeled with a radioactive phosphate by γ-[^{32}P]-ATP and T_4 polynucleotide kinase and analyzed by PAGE with the resulting autoradiogram (Figure 1) confirming the conjugate's homogeneity. Preliminary experiments using this conjugate as a PCR primer gave a product of the expected length. Subsequent chemiluminescent detection of the biotin label in this product showed unequivocally that the conjugate was incorporated into the PCR product as expected (data will be reported elsewhere). These types of conjugates are thus viable PCR primers.

Preparation of the Biotinylated Lysine Synthon 29. In the past, we have incorporated biotin residues into conjugates via global biotinylation of the polyamide moiety after deprotection of the ϵ-amino group of the lysine residues.[8b] This batchwise approach gave limited control of the placement of the biotins, and in larger polyamides containing up to 10 lysine residues, where the biotinylation reaction does not go to completion, an uneven distribution of biotins may result. The synthon **29** (Scheme VII) allows the incorporation of biotins in a highly controlled manner using conventional Fmoc peptide synthesis. It was prepared in a two-step procedure by biotinylation of N^{α}-Fmoc-L-Lys-OH (**27**) with the *N*-hydroxysuccinimidyl ester of biotin to give the free acid **28**, followed by DCC-mediated condensation with pentafluorophenol to give the desired active ester **29**. A synthesis of N^{α}-Fmoc-D-Lys(Biotin)-OH has been reported by Jacobson et al.[21] but this method is not practical for large-scale syntheses because the product is only sparingly soluble in the solvents used for the extraction step and has a yield of 47% starting from N^{α}-Fmoc-D-Lys-OH compared to our overall yield of 54% for the active ester **29**.

Characterization of the pentafluorophenyl ester **29** was made difficult by its highly complex ^{1}H NMR spectrum and the similarity of its ^{13}C NMR spectrum to that of the starting free acid **28**. The only significant difference in the ^{13}C NMR spectrum of the active ester was the upfield shift of 5 ppm of the lysine α-carbonyl and the presence of some unresolved multiplets in the aromatic region due to the pentafluorophenyl group. To provide more conclusive evidence for the structures of both the free acid **28**

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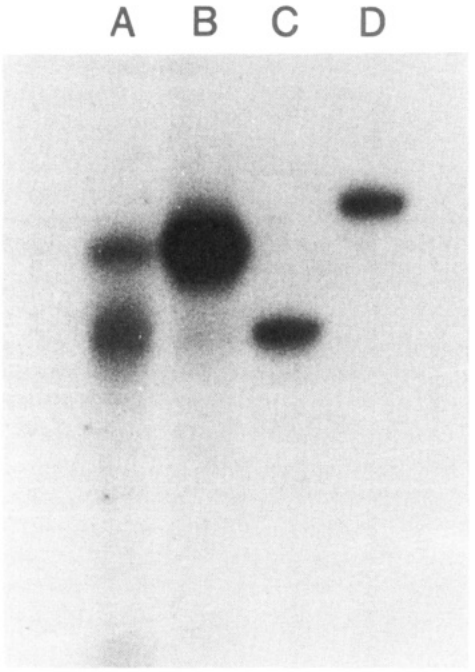
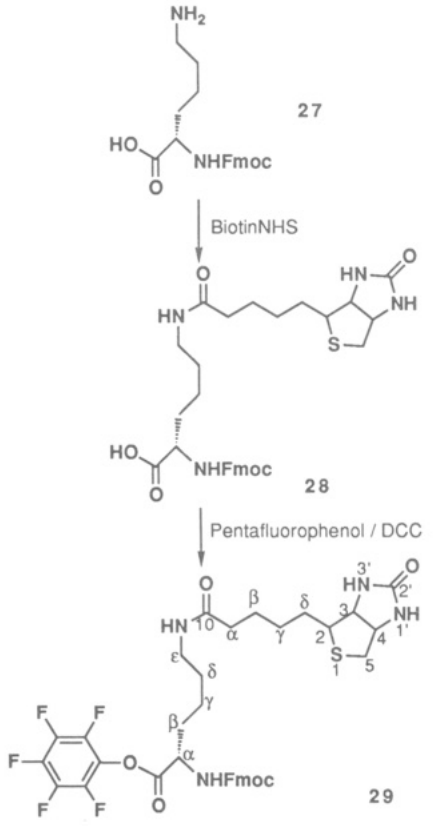


Figure 1. Autoradiogram of ^{32}P end-labeled oligonucleotides electrophoresed on a 16% polyacrylamide gel: lane A, crude conjugate **5**; lane B, purified **5**; lane C, the normal 23mer oligonucleotide d(GATGAGTTCGTGTCCGTACAACT)—same sequence as **5**; lane D, the 30mer d(GTAGTCGGCGTTGAAACCCGAGAACATCAT).

Scheme VII



and the active ester **29**, a double quantum filtered homonuclear shift correlation experiment in the phase-sensitive mode of data accumulation (DQFPh COSY) was performed on **28** and a heteronuclear multiple bond connectivity (HMBC) experiment was undertaken on **29**. The cross-peaks in the COSY spectrum of biotinylated lysine **28** were sufficiently well-resolved to provide unequivocal assignments of all multiplets in the one-dimensional ^1H NMR spectrum. The HMBC spectrum of the active ester **29** showed strong connectivities between all the carbonyls and their adjacent protons except for the resonance at δ 169.2, which was tentatively assigned to the α-carbonyl. This had no correlation to any protons under these particular experimental conditions. However, by providing unambiguous assignments for three out of the four resonances in the carbonyl region, the HMBC experiment indirectly confirmed that the resonance at δ 169.2 was due to the lysine α-carbonyl and also confirmed the assignment of the remaining resonances which were essentially the addition of the spectra of N^{α}-Fmoc-L-Lys-OH and biotin.[22] Finally, the IR spectrum of **29** has a band at 1787 cm^{-1} corresponding to the ester carbonyl, a significant shift from the carboxylic acid band of **28** at 1702 cm^{-1}.

Discussion

The critical factor in the synthesis of this class of conjugates was the choice of protecting groups for the amino and the 5′-hydroxyl functions of the nucleosides **1**–**4** that would act as a linkage between the peptide and the oligonucleotide parts of the molecule. As the widely used acid-sensitive pixyl and dimethoxytrityl ethers were judged to be suitable for the protection of the 5′-hydroxyl group, appropriate protection had to be found for the pendant amine. The use of the NPYS-protected propargylamine **10** did not give any of the desired nucleoside product **2**, although the Fmoc-protected homologue **9** gave **1** in low yield. The use of **1** to derivatize succinylated-CPG resin **16** gave rise to appreciable (~50%) Fmoc cleavage, due to the basic conditions of the coupling reaction. In contrast, the nucleosides **3** and **4**, in which the 5′-hydroxyl and the pendant amino groups are both protected by acid-sensitive protecting groups, were prepared in high yields and gave the derivatized solid supports **18** and **19**. On removal of both protecting groups, the amino group was selectively acylated with the pentafluorophenyl ester of Fmoc-ϵAhx and the 5′-hydroxyl group then reprotected by treatment with 4,4′-dimethoxytrityl chloride to give **22**. From this substrate, any desired oligonucleotide–polyamide conjugate can be synthesized using an automated DNA Synthesizer. The use of the solid support **22** gave a good yield of the conjugate **5**, which contains one biotinylated lysine residue. The proximity of the labeled peptide to the 3′-hydroxyl position did not decrease its efficiency as a PCR primer. Other studies[23] have shown that oligonucleotides containing a 3′-thymidine residue are efficient as PCR primers irrespective of the pairing residue on the template; i.e., a match to a deoxyadenosine residue on the opposite strand is not necessary. As the 3′-nucleoside in **5** is essentially a derivatized thymidine residue, it appears that it would act as a PCR primer even if this nucleoside did not form a base pair due to the presence of a large interfering C-5 substituent. It should be emphasized, however, that this nucleoside was designed with the label-carrying substituent on the side of the molecule that is not involved in Watson–Crick hydrogen bonding. The substituent on C-5 would be expected to point out of the major groove of double-stranded DNA. Incorporation of other simpler C-5

(22) Ikura, M.; Kunio, H. *Org. Magn. Reson.* **1982**, *20*, 266–273.
(23) Kwok, S.; Kellogg, D. E.; McKinney, N.; Spasic, D.; Goda, L.; Levenson, C.; Sninsky, J. J. *Nucleic Acids Res.* **1990**, *18*, 999–1005.

substituents through an alkyne linker and inclusion of the resulting nucleotide in an internal position on an oligonucleotide have been shown to give oligonucleotide probes that retain their ability to hybridize efficiently.[11] Furthermore, the 2′,3′-dideoxy derivatives of pyrimidine nucleoside triphosphates in which a fluorophore is bound to the C-5 position through an alkyne linker are used as chain terminators in the dideoxy DNA sequencing protocol;[24] in this procedure the modified nucleoside must recognize its deoxyadenosine pairing base on the template strand with high efficiency and specificity.

The fact that the conjugate **5** can act as an efficient PCR primer also means that the interaction of the Taq polymerase with the double-stranded priming region and the 3′-hydroxyl group are not greatly affected. In further experiments, we will synthesize conjugates containing multiply-biotinylated lysine residues and test them as nonradioactively labeled PCR primers. In summary, the use of a modified 3′-nucleoside has resulted in the synthesis of oligonucleotides that are labeled at the 3′-end and retain the ability to act as PCR primers. These oligonucleotide–polyamide conjugate molecules have the potential to carry multiple reporter groups. The reporter groups are introduced prior to oligonucleotide synthesis, and they form part of the solid phase on which the oligonucleotide is synthesized. This prelabeled solid support can then be used for the synthesis of any desired oligonucleotide.

Experimental Section

3-Aminopropyne, 5-iodo-2′-deoxyuridine, biotin, and (3-aminopropyl)triethoxysilane were purchased from Sigma. *N*-[(Fluoren-9-ylmethoxycarbonyl)oxy]succinimide, N^{α}-Fmoc-L-lysine, pentafluorophenol, DCC, and Fmoc-Ala-OPfp were obtained from Auspep, Melbourne. 3-Nitropyridine-2-sulfenyl chloride was obtained from Kokusan, Tokyo. 9-Chloro-9-phenylxanthene and tetrakis(triphenylphosphine)palladium(0) were supplied by Aldrich. *N*-Hydroxysuccinimide was obtained from Pierce. Triethylamine (puriss grade), controlled pore glass (200–400 mesh, 500-Å pore size, cat. no. 27720) and diisopropylcarbodiimide (DIC) were purchased from Fluka. Reagents for the ninhydrin assay were supplied by Applied Biosystems. DMF was distilled under reduced pressure and used within 14 days. Pyridine was distilled over CaH_2 at atmospheric pressure and stored over 5-Å molecular sieves. All other reagents were used without further purification. Thin-layer chromatography was performed on Merck SG-60 precoated plastic plates, and flash chromatography was performed on Merck silica gel (SG-60, 230–240 mesh). Biotin *N*-hydroxysuccinimidyl ester was prepared as described previously[25] except that 95% ethanol/1% acetic acid/4% H_2O was substituted as recrystallization solvent. *N*-Fmoc-ϵAhx-OPfp[8a] and *N*-Boc-3-aminopropyne[11] were prepared according to literature methods.

NMR spectra were recorded at 399.9 MHz (^{1}H) and at 99.98 MHz (^{13}C). DEPT experiments were performed with a 135° ^{1}H selection pulse. The DQFPh COSY experiment was run in the phase-sensitive mode of data accumulation with a 256 × 2K data matrix and 16 transients per increment. The spectrum was obtained after zero-filling to a 1K × 1K final data matrix and application of an exponential weighting function in both directions. The HMBC experiment was run in the absolute absorption mode with a 128 × 2K raw data matrix and 128 transients per increment. The spectrum was obtained after zero-filling four times in the t_1 dimension and application of a sine-bell weighting function in both directions. The experiment was run assuming a $^2J_{C-H}$ value of 8 Hz. The numbering system used for nucleosides **1**, **3**, and **4** is outlined in Scheme I while the numbering system for compounds **28** and **29** is as shown in Scheme VII. IR spectra were recorded using KBr discs. UV spectra were recorded in 0.1 mM EDTA solutions. HPLC analyses were performed on a Hewlett-Packard HP 1090 liquid chromatograh, using a Spherisorb reversed-phase C_{18} column (ODS2, 5 μm, 4.6 × 250 mm) at 40 °C with buffer A being 0.1 M triethylammonium acetate, pH 7.0, and buffer B acetonitrile, running at 0.5 mL/min.

Elemental analyses were obtained from CMAS Pty Ltd, Melbourne. Samples for high-resolution FAB-MS measurements were suspended in a polyethylene glycol (600)/thiolglycerol/glycerol/DMSO matrix and low-resolution samples in a thioglycerol matrix. The ionization gas in both cases was Xe.

3-[(Fluoren-9-ylmethoxycarbonyl)amino]propyne (9). 3-Aminopropyne (359 μL, 5.25 mmol) was added dropwise to a solution of *N*-[(fluoren-9-ylmethoxycarbonyl)oxy]succinimide (FmocNHS) (1.69 g, 5.00 mmol) in THF (8 mL) at 0 °C and stirred for 2 h. The solution was allowed to reach room temperature, and the solvent was removed under vacuum. The crude residue was dissolved in ethyl acetate (100 mL) and the solution washed with H_2O (3 × 30 mL) and then dried (Na_2SO_4). Recrystallization from ethyl acetate gave **9** as colorless needles (1.11 g, 80%), mp 129–130 °C. ^{1}H NMR (DMSO-d_6): δ 3.13 (t, 1 H, H1, J = 2.3 Hz), 3.80 (dd, 2 H, H3, J = 5.9, 2.5 Hz), 4.23 (t, 1 H, Fmoc CH, J = 6.8 Hz), 4.33 (d, 2 H, Fmoc CH_2, J = 7.1 Hz), 7.33 (ddd, 2 H, Fmoc H2 and H7, J = 7.6, 7.5, 1.2 Hz), 7.41 (ddd, 2 H, Fmoc H3 and H6, J = 7.6, 7.5, 0.9 Hz), 7.71 (d, 2 H, Fmoc H2 and H8, J = 7.6 Hz), 7.81 (t, 1 H, NH, J = 5.9 Hz), 7.90 (d, 2 H, Fmoc H4 and H5, J = 7.6 Hz). ^{13}C NMR (DMSO-d_6): δ 29.8 (C3), 46.6 (Fmoc CH), 65.7 (Fmoc CH_2), 73.1 (C1), 81.4 (C2), 120.1 (Fmoc C3 and C6), 125.2 (Fmoc C2 and C7), 127.1 (Fmoc C4 and C5), 127.6 (Fmoc C1 and C8), 140.7 (C4a and C4b), 143.8 (Fmoc C8a and C9a), 155.9 (Fmoc CO). FABMS: m/z 300 (M + Na), 278 (M + H). Anal. Calcd for $C_{18}H_{15}NO_2$: C, 78.0; H, 5.45; N, 5.05. Found: C, 78.1; H, 5.45; N, 5.01.

3-[(3-Nitro-2-sulfenylpyridinyl)amino]propyne (10). To a stirred solution of 3-aminopropyne (855 μL, 12.5 mmol) in DMF (20 mL) was added 3-nitropyridine-2-sulfenyl chloride (0.94 g, 5.0 mmol) in two equal portions over 0.5 h. After 1.5 h, the reaction mixture was poured into ethyl acetate (500 mL), washed with H_2O (3 × 200 mL), and dried (Na_2SO_4) and the solvent removed under vacuum. Recrystallization from MeOH/H_2O gave **10** as red crystals (0.653 g, 62%), mp 107–9 °C. ^{1}H NMR (DMSO-d_6): δ 3.18 (t, 1 H, H1, J = 2.6 Hz), 3.77 (dd, 2 H, H3, J = 4.4, 2.6 Hz), 5.34 (t, 1 H, NH, J = 4.4 Hz), 7.47 (dd, 1 H, NPYS H5, J = 8.3, 4.6 Hz), 8.61 (dd, 1 H, NPYS H6, J = 8.4, 1.5 Hz), 8.90 (dd, 1 H, NPYS H4, J = 4.4, 1.5 Hz). ^{13}C NMR (DMSO-d_6): δ 30.7 (C3), 74.8 (C1), 81.7 (C2), 120.4 (NPYS C5), 134.3 (NPYS C4), 139.8 (NPYS C3), 154.1 (NPYS C6), 163.1 (NPYS C2). FABMS: m/z 210 (M + H). Anal. Calcd for $C_8H_7N_3O_2S$: C, 45.9; H, 3.37; N, 20.1. Found: C, 45.7; H, 3.33; N, 20.1.

3-[(9-Phenylxanthen-9-yl)amino]propyne (12). 9-Chloro-9-phenylxanthene (14.7 g, 50 mmol) was added in two equal portions over 0.5 h to a stirred solution of 3-aminopropyne (8.6 mL, 125 mmol) in DMF (100 mL). After 3 h, MeOH (20 mL) was added, and the reaction mixture was stirred for 15 min. The mixture was then extracted into diethyl ether (500 mL), washed with H_2O (3 × 300 mL), and dried (Na_2SO_4) and the solvent removed under vacuum. The resulting yellow oil was dried under high vacuum (4 h) and then redissolved in hot diethyl ether (70 mL). The solution was kept at −20 °C for 24 h and filtered. The solid was washed with ice-cold hexane (2 × 50 mL) followed by ice-cold diethyl ether (2 × 50 mL), giving **12** as a colorless powder (12.7 g, 82%), mp 116–8 °C. ^{1}H NMR (DMSO-d_6): δ 2.86 (dd, 2 H, H3, J = 7.3, 4.4 Hz), 2.99 (t, 1 H, H1, J = 2.4 Hz), 4.01 (t, 1 H, NH, J = 7.2 Hz), 6.98–7.50 (m, 13 H, Px CH). ^{13}C NMR (DMSO-d_6): δ 32.8 (C3), 59.7 (Px C9), 73.4 (C1), 82.7 (C2), 116.0 and 123.5 (Px CH), 125.0 (Px C8a and C9a), 126.3, 126.4, 128.0, 128.6, 128.7 (Px CH), 149.5 (Px C1′), 150.7 (Px C4a and C10a). FABMS: m/z 311 (M^+). Anal. Calcd for $C_{22}H_{17}NO$: C, 84.8; H, 5.51; N, 4.50. Found: C, 84.4; H, 5.73; N, 4.30.

5-Iodo-5′-*O*-(9-phenylxanthen-9-yl)-2′-deoxyuridine (7). 5-Iodo-2′-deoxyuridine (IdU) (3.54 g, 10 mmol) was coevaporated with dry pyridine (3 × 20 mL). The IdU was redissolved in dry pyridine (15 mL), and 9-phenyl-9-chloroxanthene (3.82 g, 13 mmol) was added to the stirring solution in two equal portions over 0.5 h. After 1 h, MeOH (5 mL) was added and the solution stirred for a further 0.5 h. The solvent was then removed under

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(25) Becker, J. M.; Wilchek, M.; Katchaliski, E. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 2604–7.

vacuum and the residue recrystallized from a 1% Et_3N/ethyl acetate solution giving **7** as colorless crystals (4.27 g, 70%), mp 200–2 °C. ^1H NMR (DMSO-d_6): δ 2.20 (m, 2 H, H2′), 2.99 (dd, 1 H, H5′, J = 10.5, 4.2 Hz), 3.10 (dd, 1 H, H5″, J = 10.5, 2.7 Hz), 3.85 (m, 1 H, H4′), 4.15 (m, 1 H, H3′), 5.29 (d, 1 H, 3′OH, J = 4.2 Hz), 6.08 (t, 1 H, H1′, J = 7.0 Hz), 7.10–7.42 (m, 13 H, Px CH), 8.08 (s, 1 H, H6). ^{13}C NMR (DMSO-d_6): δ 40.4 (C2′), 63.8 (C5′), 69.9 (C5), 71.0 (C3′), 75.6 (Px C9), 85.2 (C1′), 85.9 (C4′), 116.3, 116.4 (Px CH), 122.3, 122.4 (Px C8a and C9a), 123.8, 124.0, 125.8, 126.8, 128.2, 129.3, 129.69, 129.7, 129.73 (Px CH), 143.9 (C6), 148.4 (Px C1′), 150.0 (C2), 150.51, 150.58 (Px C4a and C10a), 160.6 (C4). FABMS: m/z 633 (M + Na^+). Anal. Calcd for $C_{28}H_{23}N_2O_6I$: C, 55.1; H, 3.81; N, 4.59. Found: C, 55.0; H, 3.81; N, 4.54.

5-[3-[(*tert*-Butyloxycarbonyl)amino]prop-1-yn-1-yl]-5′-*O*-(9-phenylxanthen-9-yl)-2′-deoxyuridine (3). To a degassed (Ar) solution of **11** (1.22 g, 2.00 mmol) in DMF (6 mL) was added CuI (0.076 g, 0.40 mmol), Et_3N (558 μL, 4.00 mmol), 3-[(*tert*-butyloxycarbonyl)amino]propyne[11] (0.932 g, 6.00 mmol) and $(Ph_3P)_4Pd^0$ (0.232 g, 0.20 mmol) successively and the solution was stirred for 5 h. AG1X8(HCO_3^-) ion-exchange resin (6 molar equiv) was added with MeOH (10 mL) and CH_2Cl_2 (10 mL) and the mixture stirred for 30 min. The resin was removed by filtration and the solvent removed under reduced pressure. The crude residue was dissolved in ethyl acetate (200 mL), and the solution was washed with H_2O (3 × 100 mL), dried (Na_2SO_4), and filtered. After removal of the solvent, flash silica gel chromatography (70 g silica, 0–10% MeOH/CH_2Cl_2) followed by recrystallization from chloroform/diethyl ether gave **3** (0.602 g, 47%) as colorless crystals, mp 157–160 °C: ^1H NMR (DMSO-d_6): δ 1.35 (s, 9 H, 3 × Boc CH_3), 2.25 (m, 2 H, H2′), 3.00 (dd, 1 H, H5′, J = 10.5, 4.4 Hz), 3.12 (dd, 1 H, H5″, J = 10.4, 2.6 Hz), 3.69 (m, 2 H, H9), 3.91 (m, 1 H, H4′), 4.15 (m, 1 H, H3′), 5.30 (d, 1 H, 3′OH, J = 4.2 Hz), 6.09 (t, 1 H, H1′, J = 6.7 Hz), 7.10–7.45 (m, 13 H, Px CH), 7.96 (s, 1 H, H6). ^{13}C NMR (DMSO-d_6): δ 28.2 (3 ×Boc CH_3), 30.0 (C9), 40.3 (C2′), 63.8 (C5′), 70.8 (C3′), 73.8 (C8), 75.5 (Px C9), 78.2 (Boc C), 85.3 (C1′), 85.9 (C4′), 90.1 (C7), 98.4 (C5), 116.1, 116.3 (Px CH), 122.0 (Px C8a and C9a), 123.9 (Px CH), 124.0 (Px CH), 125.8 (2 × Px CH), 126.7 (Px CH), 128.1 (2 × Px CH), 129.0 (Px CH), 129.1 (Px CH), 129.7 (2 × Px CH), 142.9 (C6), 148.3 (Px C2), 149.3 (Px C1′), 150.5, 150.7 (Px C4a and C10a), 155.1 (Boc CO), 161.6 (C4). FABMS: m/z 660 (M + Na). Anal. Calcd for $C_{36}H_{35}N_3O_8$: C, 67.8; H, 5.54; N, 6.59. Found: C, 67.7; H, 5.68; N, 6.54.

The Fmoc-nucleoside **1** was prepared in an analogous manner, but it could not be purified due to persistent contaminating starting nucleoside **7**. ^{13}C NMR ($CDCl_3$): δ 31.2 (C9), 41.8 (C2′), 46.9 (Fmoc C9), 60.3 (C5′), 63.4 (C8), 66.4 (Fmoc CH_2), 72.4 (C3′), 74.3 (Px C9), 86.1 (C1′), 86.7 (C4′), 89.5 (C7), 99.4 (C5), 116.4 (Px CH), 119.8 (Fmoc C3 and C6), 122.1, 122.3 (Px C8a and C9a), 123.7, 123.8 (Px CH), 124.8 (Fmoc C2 and C7), 126.2 (Px CH), 126.85, 126.95 (Fmoc C4 and C5), 127.8 (Fmoc C1 and C8), 129.4, 129.6 (Px CH), 141.1 (Fmoc C4a and C4b), 143.2, 143.6 (Fmoc C8a and C9a), 143.7 (C6), 148.1 (C2), 149.4 (Px C1′), 151.06, 151.12 (Px C4a and C10a), 155.6 (Fmoc CO), 162.25 (C4).

5-[3-[(9-Phenylxanthen-9-yl)amino]prop-1-yn-1-yl]-5′-*O*-(9-phenylxanthen-9-yl)-2′-deoxyuridine (4). The procedure was identical to that of **3** except that the crude residue was redissolved in CH_2Cl_2 (200 mL). The solution was washed with 10% $NaHCO_3$ (2 × 100 mL) and H_2O (1 × 100 mL). After the solution had been dried (Na_2SO_4) and filtered and the solvent evaporated, flash silica gel chromatography (0–5% MeOH/CH_2Cl_2, 1% Et_3N) gave **4** as a fawn solid (0.608 g, 76%). A portion was recrystallized from MeOH to analytical purity, mp 156–8 °C dec. ^1H NMR (DMSO-d_6): δ 2.25 (m, 2 H, H2′), 2.55 (dd, 1 H, H9a, J = 16.3, 7.15 Hz), 2.66 (dd, 1 H, H9b, J = 16.3, 7.14 Hz), 2.95 (dd, 1 H, H5′, J = 10.6, 3.67 Hz), 3.07 (dd, 1 H, H5″, J = 10.4, 2.38 Hz), 3.51 (t, 1 H, N9H, J = 7.20 Hz), 3.9 (m, 1 H, H4′), 4.21 (m, 1 H, H3′), 5.31 (d, 1 H, 3′OH, J = 4.03 Hz), 6.11 (t, 1 H, H1′, J = 6.78 Hz), 6.85–7.40 (m, 26 H, NPx and OPx CH), 8.05 (s, 1 H, H6), 11.6 (br s, 1 H, H3). ^{13}C NMR (DMSO-d_6): δ 33.4 (C9), 40.8 (C2′), 59.6 (NPx C9), 63.6 (C5′), 70.9 (C3′), 74.6 (C8), 75.7 (OPx C9), 85.2 (C1′), 86.0 (C4′), 91.2 (C7), 98.7 (C5), 115.86, 115.91, 116.21, 116.30 (NPx and OPx CH), 122.17 and 122.24 (OPx C8a and C9a), 123.50, 123.54, 123.92, 124.05 (NPx and OPx CH), 124.65, 124.68 (NPx C8a and C9a), 125.8 (2 × Px CH), 126.3 (2 × Px CH), 126.5 (2 × Px CH), 127.8 (2 × Px CH), 127.9 (2 × Px CH), 128.46 (Px CH), 128.58 (Px CH), 128.68 (Px CH), 128.74 (Px CH), 128.98 (Px CH), 129.25 (Px CH), 129.64 (Px CH), 129.76 (Px CH), 142.7 (C6), 148.1 (C2), 149.3 (NPx and OPx C1′), 150.4, 150.61, 150.66, 150.75 (NPx and OPx C4a and C10a), 161.6 (C4). FABMS: m/z 816 (M + Na), 794 (M + H). HR-FABMS: exact mass found 794.2871, calcd for ($C_{50}H_{39}N_3O_7$) + H 794.2868.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-N^{ϵ}-biotinyllysine, Fmoc-Lys(Biotin)-OH (28). A solution of Et_3N (698 μL, 5.00 mmol) in DMF (70 mL) was added to a mixture of the *N*-hydroxysuccinimidyl ester of biotin (3.41 g, 10.0 mmol) and N^{α}-Fmoc-lysine (**27**) (1.84 g, 5.00 mmol), and the resulting mixture was stirred for 6 h and then filtered. Cold aqueous HCl (pH 2, 500 mL) was then added and the precipitate filtered and washed with aqueous HCl (pH 2, 3 × 200 mL) and H_2O (3 × 200 mL). The residue was found to contain a large amount of water at this stage and was consequently lyophilized (48 h) to give a fluffy colorless solid (2.41 g, 81%), mp 181–2 °C dec. ^1H NMR (DMSO-d_6): δ 1.20–1.40 (m, 4 H, Btn Hγ and Lys Hδ), 1.40–1.52 (m, 6 H, Btn Hβ and Btn Hδ and Lys Hγ), 1.52–1.62 (m, 2 H, Lys Hβ), 2.03 (t, 2 H, Btn Hα, J = 7.3 Hz), 2.56 (d, 1 H, Btn H5b, J = 12.5 Hz), 2.79 (dd, 1 H, Btn H5a, J = 12.4, 5.1 Hz), 3.00 (m, 2 H, Lys Hε), 3.06 (m, 1 H, Btn H2), 3.9 (m, 1 H, Lys Hα), 4.1 (m, 1 H, Btn H3), 4.18–4.30 (m, 4 H, Btn H4 and Fmoc CH_2 and Fmoc H9), 6.36 (s, 1 H, Btn H1′), 6.42 (s, 1 H, Btn H3′), 7.32 (ddd, 2 H, Fmoc H2 and H7, J = 7.4, 7.4, 1.1 Hz), 7.41 (dd, 2 H, Fmoc H3 and H6, J = 7.2, 7.2 Hz), 7.61 (d, 1 H, Lys $N^{\alpha}H$, J = 8.1 Hz), 7.72 (d, 2 H, Fmoc H1 and H8, J = 7.4 Hz), 7.76 (t, 1 H, Lys $N^{\epsilon}H$, J = 5.6 Hz), 7.88 (d, 2 H, Fmoc H4 and H5, J = 7.4 Hz). ^{13}C NMR (DMSO-d_6): δ 23.1 (Lys Cγ), 25.3 (Btn Cβ), 28.0 (Btn Cδ), 28.2 (Btn Cγ), 28.8 (Lys Cδ), 30.5 (Lys Cβ), 35.2 (Btn Cα), 38.2 (Lys Cε), 39.9 (Btn C5), 46.7 (Fmoc C9), 53.8 (Lys Cα), 55.2 (Btn C2), 59.2 (Btn C4), 61.0 (Btn C3), 65.6 (Fmoc CH_2), 120.1 (Fmoc C3 and C6), 125.3 (Fmoc C2 and C7), 127.1 (Fmoc C4 and C5), 127.7 (Fmoc C1 and C8), 140.7 (Fmoc C4a and C4b), 143.79, 143.84 (Fmoc C8a and C9a), 156.2 (Fmoc CO), 162.7 (Btn C2′), 171.9 (Btn C10), 174.0 (Lys CO_2H). HR-FABMS: exact mass found 595.2566, calcd for ($C_{31}H_{38}N_4O_6S$) + H 595.2590. IR: 1702 (Fmoc CO and lysine α CO), 1638 cm^{-1} (amide CO).

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-N^{ϵ}-biotinyllysine Pentafluorophenyl Ester, Fmoc-Lys(Biotin)-OPfp (29). A solution of pentafluorophenol (1.75 g, 9.29 mmol) and DCC (1.27 g, 6.32 mmol) in DMF (5 mL) was added to a solution of **28** (2.21 g, 3.72 mmol) in DMF (40 mL) and the mixture stirred for 16 h. The colorless precipitate was filtered off and discarded. The filtrate was kept and after removal of the solvent under vacuum followed by trituration with diethyl ether (4 × 100 mL) afforded a colorless solid. Recrystallization from ethyl acetate/ethanol/acetic acid (80:19:1) gave **29** as fine colorless crystals (1.90 g, 67%), mp 162–5 °C dec. ^1H NMR (DMSO-d_6): δ 1.23–1.32 (m, 2 H, Btn Hγ), 1.38–1.53 (m, 6 H, Btn Hβ and Btn Hδ and Lys Hδ), 1.54–1.63 (m, 2 H, Lys Hβ), 2.04 (t, 2 H, Btn Hα, J = 7.3 Hz), 2.55 (d, 1 H, Btn H5b, J = 12.5 Hz), 2.79 (dd, 1 H, Btn H5a, J = 12.5, 5.1 Hz), 2.98–3.04 (m, 2 H, Lys Hε), 3.05–3.10 (m, 1 H, Btn H2), 4.09 (m, 1 H, Lys αH), 4.20–4.30 (m, 2 H, Btn H3 and Fmoc H9), 4.32–4.42 (m, 3 H, Btn H4 and Fmoc CH_2), 6.35 (s, 1 H, Btn H1′), 6.42 (s, 1 H, Btn H3′), 7.28–7.34 (m, 2 H, Fmoc H2 and H7), 7.40 (dd, 2 H, Fmoc H3 and H6, J = 7.5, 7.5 Hz), 7.70 (d, 2 H, Fmoc H1 and H8, J = 7.3 Hz), 7.77 (t, 1 H, Lys $N^{\epsilon}H$, J = 5.7 Hz), 7.88 (d, 2 H, Fmoc H4 and H5, J = 7.7 Hz), 8.12 (d, 1 H, Lys $N^{\alpha}H$, J = 7.3 Hz). ^{13}C NMR (DMSO-d_6): δ 27.7 (Lys Cγ), 25.3 (Btn Cβ), 28.1 (Btn Cδ), 28.3 (Btn Cγ), 28.7 (Lys Cδ), 29.9 (Lys Cβ), 35.3 (Btn Cα), 38.1 (Lys Cε), 39.9 (Btn C5), 46.7 (Fmoc C9), 53.9 (Lys Cα), 55.5 (Btn C2), 59.2 (Btn C4), 61.1 (Btn C3), 65.9 (Fmoc CH_2), 120.2 (Fmoc C3 and C6), 125.2 (Fmoc C2 and C7), 127.1 (Fmoc C4 and C5), 127.7 (Fmoc C1 and C8), 136.5, 138.5, 139.0 (3 × m, C – CF), 140.8 (Fmoc C4a and C4b), 142.0 (m, C – CF), 143.68, 143.71 (Fmoc C8a and C9a), 156.2 (Fmoc CO), 162.7 (Btn C2′), 169.2 (Lys CO), 171.9 (Btn C10). HR-FABMS: exact mass found 761.2413, calcd for ($C_{37}H_{37}N_4O_6SF_5$) + H 761.2432. IR: 1787 (ester CO), 1702 (Fmoc CO), 1641 cm^{-1} (amide CO).

Preparation of Succinyl-CPG Resin (16). A solution of (3-aminopropyl)triethoxysilane (3 g, 13.6 mmol) in ethanol (60 mL) was added to CPG (200–400 mesh, 500-Å pore size, cat. no. 27720) **13** (6 g), and the mixture was shaken gently at regular intervals over a period of 6 h. The resin was collected by gravity filtration (*without* any washing), air dried (24 h), and kept at 110

°C (24 h) to give the aminated resin **14**. The amino loading was determined to be 129 μmol/g by ninhydrin assay. *N*-Fmoc-εAhx-OPfp (2.5 molar equiv) and HOBT (2.5 molar equiv) in DMF were coupled to **14** (double coupling, 1.5 h each reaction) in a sintered-glass column. Two more aminohexanoic acid residues were attached using 1.5-h single couplings, giving resin **15**. Residual amino groups were acetylated by reaction with Ac_2O (250 μL) and DMAP (50 mg) in dry pyridine for 5 min. The Fmoc group was cleaved by treatment with 20% piperidine/DMF (5 min), the resin washed with DMF, and then a solution of succinic anhydride (50 molar equiv) and DMAP (10 molar equiv) in a minimum volume of dry pyridine was added. After the suspension was shaken for 1 h, the resin was rinsed with pyridine, DMF, and CH_2Cl_2, and dried under vacuum. The reaction was monitored by ninhydrin assay, and the loading of carboxylic acid groups as calculated from the disappearance of amino groups was 46 μmol/g.

Coupling of Modified Nucleosides 3 and 4 to Succinyl-CPG Resin 16. Resin **16** was treated twice with a solution of **3** or **4** (5 molar equiv), diisopropylcarbodiimide (5 molar equiv), and DMAP (0.5 molar equiv) in a minimum volume of dry pyridine in two separate 16-h couplings with only a washing step in between (double coupling). After the second coupling, the resin was rinsed with pyridine, and pixyl assay gave a nucleoside loading of 39 μmol/g. The remaining carboxylic acid and amino groups were capped with piperidine and Ac_2O/DMAP according to the method of Damha,[15] giving resins **18** or **19**.

Derivatization of 18 and 19 To Give 22. In the case of **18**, the resin was treated with 90% TFA/ethanedithiol for 10 min, rinsed with CH_2Cl_2, and neutralized with 20% Et_3N/CH_2Cl_2. The resin was then rinsed with CH_2Cl_2, dried, and treated with a 1:1 mixture of Fmoc-εAhx-OPfp/HOBT in DMF (2.5 molar equiv, 45 min, twice). After rinsing with DMF, the resin was subjected to two successive 16-h reactions with 4,4′-dimethoxytrityl chloride (50 molar equiv each time) in a minimum volume of dry pyridine to give resin **22**. Trityl assay showed 30 μmol/g of dimethoxytrityl group present. Any remaining free hydroxyl and amino groups were acetylated by reaction with Ac_2O (250 μL) and DMAP (50 mg) in dry pyridine for 5 min. The procedure for the derivatization of **19** to give **22** is identical to the procedure for **18** except that 3% DCA/CH_2Cl_2 was substituted for 90% TFA/ethanedithiol.

Polyamide and Oligonucleotide Synthesis on Resin 22. A biotinylated lysine residue and an alanine residue were attached to **22** by Fmoc solid-phase peptide synthesis, using **29** and N^{α}-Fmoc-Ala-OPfp respectively, in a manual glass-sinter peptide synthesis cell. A 5-fold excess of amino acid pentafluorophenyl ester and HOBT were used, with a coupling time of 1 h and 20% piperidine/DMF as deprotection agent. The α-amino group of alanine was deprotected and capped with Ac_2O (250 μL) and DMAP (0.050 g) in dry pyridine (0.5 h). After rinsing with DMF, CH_2Cl_2, and drying under vacuum, a portion of the resin was used in oligonucleotide synthesis on an Applied Biosystems 380A DNA synthesizer[11] using standard β-(cyanoethyl)-protected phosphoramidites (with a 60-s Ac_2O/DMAP capping step[8a]), on a 1 μmol scale. The sequence of the oligonucleotide synthesized was d(GATGAGTTCGTGTCCGTACAACT*) (T* being the modified nucleoside linker). The resulting conjugate was cleaved from the solid support by treatment with concentrated ammonia (22 °C, 6 h). The resulting solution of the conjugate was heated at 50 °C for 24 h to effect base deprotection. The ammonia was removed under reduced pressure and the conjugate redissolved in 0.1 mM EDTA (2 mL). Separation by preparative PAGE (16% polyacrylamide gel)[26] gave two products, the one with the lowest electrophoretic mobility was isolated to give **5** in an overall yield of 2.1% from **24**.

Characterization of the Oligonucleotide–Polyamide Conjugate. A sample of the conjugate **5** was 5′-end labeled[26] with γ-[^{32}P]-ATP and T_4 polynucleotide kinase and was shown to be homogeneous by PAGE (16% polyacrylamide gel). The UV spectrum showed a maximum of absorption at 260 nm. A 3.0 nmol aliquot of the conjugate (amount calculated from UV absorption at 260 nm) was analyzed for amino acid content and showed a ratio of 1.01 mol Ala to 0.99 mol Lys (expected: 1 Ala to 1 Lys); the amount of peptide found in the sample was 2.6 nmol. A 10-μg aliquot of **5** (in 100 μL of 0.1 mM EDTA) was digested with P_1 nuclease (50 μg, in 50 μL of 0.05 M NaOAc, pH 6.0) and 0.5 M NaOAc pH 6.0 (25 μL) at 37 °C for 30 min. The pH was then increased by the addition of 135 μL of Tris buffer (0.5 M tris(hydroxymethyl)aminomethane, 1 mM EDTA, pH 9.0) followed by 40 U of calf intestinal alkaline phosphatase (Boehringer Mannheim enzyme immunoassay grade cat. no. 567-774) and allowed to incubate at 37 °C for 27 h. The dephosphorylation of the resulting nucleotides in this system produced complete deamination of dA to dI, as determined on a sample of authentic dA. The digest was lyophilized, the residue redissolved in 60 μL of H_2O, and 15 μL of this was analyzed by reversed-phase HPLC isocratically at 3% B. Typical elution times using this system were dC 7.5 min, dI, 10.6 min, dG, 11.6 min, dT, 13.6 min, dA, 21.0 min. The response factor of each nucleoside was determined by the injection of a mixture containing 1.5 nmol of each nucleoside and manual integration of the resulting HPLC profile, taking the average of three runs. The molar ratios of the nucleosides from digested **5** were determined to be dC, 4.8; dI, 5.1; dG, 5.9; dT, 6.3. Expected ratios: dC, 5; dI, 5; dG, 6 dT, 6.

Ninhydrin Assay. This is a modified version of the original assay[27] which specified an incubation time of 7 min at 100 °C. Accurately weighed aliquots of resin were treated with 76% w/w phenol/ethanol (4 drops from a Pasteur pipette), 0.0002 M potassium cyanide/pyridine (8 drops), and 0.28 M ninhydrin/ethanol (4 drops) at 110 °C for 10 min. After dilution with 60% ethanol (3.8 mL), the absorptions were measured at 570 nm (ϵ = 15 000 M^{-1} cm^{-1}).

Pixyl and Trityl Assays. Accurately weighed aliquots of resin were treated with 10% toluenesulfonic acid/acetonitrile (3 mL) for 10 min at room temperature. Absorptions were measured at 445 nm (ϵ = 4400 M^{-1} cm^{-1}) for pixyl and 507 nm (ϵ = 66 500 M^{-1} cm^{-1}) for trityl.

Fmoc Assay.[28] Accurately weighed aliquots of resin were treated with piperidine (200 μL) and CH_2Cl_2 (200 μL) for 30 min at room temperature. After dilution with CH_2Cl_2 (3.6 mL), the absorptions were measured at 301 nm (ϵ = 7800 M^{-1} cm^{-1}).

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Supplementary Material Available: ^{13}C NMR spectra for compounds **4**, **28**, and **29** (3 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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