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Trinucleotide phosphoramidites: ideal reagents for the synthesis of mixed oligonucleotides for random mutagenesis

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ABSTRACT

Trinucleotide phosphoramidites representing codons for all 20 amino acids have been prepared and used in automated, solid-phase DNA synthesis. In contrast to an earlier report, we show that these substances can be used to introduce entire codons into oligonucleotides in excess of 98% yield, and are ideal reagents for the synthesis of mixed oligonucleotides for random mutagenesis.

INTRODUCTION

Oligonucleotide-directed mutagenesis is the most commonly-used method for preparing variants of a polypeptide or protein of interest. Increasingly, oligonucleotides of mixed composition are being used to generate libraries of variants for the study of biomolecular function, and in the search for peptides and proteins with improved properties (1). In preparing the peptide or protein library, the goal is usually to introduce subsets of the 20 amino acids at pre-determined positions in the molecule. Although schemes have been designed to improve the efficiency of this process (2) there is a limit to the precision with which mononucleotides can be mixed, and because of the degeneracy of the code, only limited control over the distribution of amino acids is possible. Furthermore, undesired amino acids and stop codons often cannot be avoided. The most direct route to controlled mutagenesis is to use trinucleotide synthons which correspond directly to the amino acid codons needed. Until now, the necessary trinucleotide phosphoramidites have not been available. By splitting the solid support prior to coupling each mononucleotide, and then recombining after coupling, it is possible to achieve DNA synthesis codon by codon (3). However this is a laborious method which becomes prohibitively time-consuming as the complexity of the mutagenesis scheme increases. Furthermore, the limit of complexity is determined by the number of particles in the solid support, which is generally of the order of 10^6. We report here the synthesis of the 20 trinucleotides required for general use of the method, and show that, in contrast to an earlier report (4), these substances couple in excellent yield during conventional solid-phase synthesis on a commercial DNA synthesizer.

EXPERIMENTAL

General materials and methods

Base-protected dimethoxytrityl nucleosides (DMT-dA^{Bz}, DMT-dC^{Bz}, DMT-dG^{iBu} DMT-T, **1a–d**) were obtained from Pharma Waldhof (Düsseldorf, Germany). Methyl *N,N*-diisopropylphosphoramidochloridite was synthesized from methyl phosphorodichloridite (Lancaster) and diisopropylamine (Fluka) and used to prepare the DMT-nucleoside phosphoramidites **3a–d** according to the literature (5–7). Dry acetonitrile, dichloromethane, methanol and pyridine were bought over molecular sieves (Fluka). Phenoxyacetylchloride (PAC-Cl, Fluka), *N,N*-diisopropylethylamine (Fluka) and tetrazole (Aldrich) were used without further purification. Amberlyst® 15 was purchased from Fluka. *tert*-Butylhydroperoxide was used as an 80% solution in di-*tert*-butylhydroperoxide (Fluka). Methanolic ammonia (7.5 M) was prepared by passing ammonia gas through cooled methanol until saturation. Solvents and buffers for HPLC were bought from Fluka or Roth (Karlsruhe, Germany). All operations with compounds sensitive to oxygen or humidity were carried out under a nitrogen atmosphere.

TLC analysis was performed on aluminium-backed silica gel plates (Merck, Kieselgel 60 F_{254}, Art. 5554) using CH_2Cl_2/MeOH (90/10, v/v) as the solvent system. Column chromatography was performed on silica gel 60 (Fluka) and on LiChroprep® DIOL, 40–63 μm (Merck). LiChroprep® DIOL was washed with triethylamine-containing solvent prior to chromatography to prevent detritylation.

^{1}H NMR spectra were measured at 300 MHz, ^{13}C NMR spectra at 75 MHz using a Bruker ARX-300 spectrometer. ^{31}P NMR spectra were measured at 120 MHz using a Bruker A-C-300 spectrometer. Chemical shifts for ^{1}H and ^{13}C NMR are given in p.p.m. (δ) relative to tetramethylsilane and for ^{31}P as

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p.p.m. (δ) relative to 85% H_3PO_4 as external standard. FAB ionization mass spectrometry was performed on a Finnigan MAT 900 spectrometer, using m-nitrobenzylalcohol (m-NBA) as matrix, unless otherwise stated. Electrospray ionization (ESI) mass spectrometry was performed on a Finnigan TSQ 700 spectrometer, using solutions in methanol containing sodium iodide.

Automated oligonucleotide synthesis was performed on an Applied Biosystems DNA Synthesizer 380B, using the standard synthesis protocols, unless otherwise stated. The synthesis reagents were obtained from Applied Biosystems and Roth. Trinucleotide phosphoramidite solutions were prepared by drying the compound or the mixture of compounds for 24 h over phosphorus pentoxide under vacuum, dissolving in acetonitrile (water ≤ 0.0009%, Roth), filtering the solution through a Millipore filter (type GV, 0.22 μ) and adding acetonitrile to yield a 0.1 M solution. When trinucleotide phosphoramidites were incorporated, the methyl phosphate ester was cleaved by thiophenol treatment (thiophenol/dioxane/triethylamine, 1/2/2, v/v, 2×30 min) prior to ammonia treatment (60°C, 16 h).

HPLC-analysis was carried out on a Merck-Hitachi L-6220 pump, equipped with a variable wavelength UV detector, type 655A, and a Chromato-Integrator, type D-2500A. Oligonucleotides carrying the DMT group were analysed on an RP8 (Vydac 228TP1010) column [0−45% acetonitrile in 0.1 M triethylammonium acetate buffer (pH 6.5) within 15 min], completely deprotected oligonucleotides on an RP18 (Vydac 218TP1010) column [0−15% acetonitrile in 0.1 M triethylammonium acetate buffer (pH 6.5) within 25 min].

Preparation of monomers[8]

3'-Phenoxyacetylthymidine (**2a**). 5'-O-(4,4'-Dimethoxytrityl)-thymidine (**1d**, 10.0 g, 18.4 mmol, dried by repeated evaporation with dry pyridine), was dissolved in 50 ml of pyridine at 0°C, and phenoxyacetylchloride (2.80 ml, 20.0 mmol) was slowly added. The reaction mixture was warmed to room temperature and stirred for 1 h. When TLC showed completion, 1 ml of methanol was added and the mixture was concentrated to dryness. The residue was repeatedly dissolved in toluene and evaporated to remove pyridine. The remaining oil was dissolved in 75 ml CH_2Cl_2/MeOH (8/1, v/v), and Amberlyst® 15 was added in portions until the surface of the resin remained orange coloured (~4 g). The suspension was stirred for 24 h, the resin was filtered off, the solution concentrated under vacuum, and the product precipitated twice from petroleum ether (40−60°C, 500 ml) to yield 6.1 g (88%) of **2a** as a colourless powder; R_f 0.55; ^1H NMR see lit. (8); ^{13}C NMR ($CDCl_3$): δ 12.56 (CH_3 of T), 37.08 (2'-C), 62.46 (5'-C), 65.23 (CH_2 of PAC), 75.88 (3'-C), 84.96 and 86.26 (1'-C, 4'-C), 111.46 (5-C of T), 114.66 (2,6-C of PAC), 122.06 (4-C of PAC), 129.70 (3,5-C of PAC), 136.45 (6-C of T), 150.49 (2-C of T), 157.59 (1-C of PAC), 163.75 (4-C of T), 168.81 (CO of PAC); FAB MS (m-NBA, m/z): 377.1 (M + H^+).

N^2-Isobutyryl-3'-phenoxyacetyl-2'-deoxyguanosine (**2b**). This was prepared using the same method as described above, from 5'-O-(4,4'-dimethoxytrityl)-N^2-isobutyryl-2'-deoxyguanosine (**1c**, 9.90 g, 15.5 mmol). Yield 6.3 g (86%), colourless powder; R_f 0.51; ^1H NMR see lit. (8); ^{13}C NMR ($CDCl_3$): δ 18.96 (CH_3 of ^iBu), 36.00 (CH of ^iBu), 36.92 (2'-C), 62.28 (5'-C), 65.19 (CH_2 of PAC), 76.51 (3'-C), 85.52 and 85.81 (1'-C, 4'-C), 114.59 (2,6-C of PAC), 121.49 (5-C of G), 121.94 (4-C of PAC), 129.65 (3,5-C of PAC), 138.92 (5-C of G), 148.02 and 148.13 (2,4-C of G), 155.52 (6-C of G), 157.57 (1-C of PAC), 168.74 (CO of PAC), 180.10 (CO of ^iBu); FAB MS (m-NBA, m/z): 472.2 (M + H^+).

Preparation of dimers

P-Methylthymidylyl-(3'→5')-3'-phenoxyacetylthymidine (**4d**). To a solution of **2a** (5.50 g, 14.6 mmol, dried by repeated coevaporation with acetonitrile/toluene) and tetrazole (1.70 g, 24.3 mmol, dried by repeated coevaporation with acetonitrile/toluene) in 50 ml dry acetonitrile was added a solution of 3'-*O*-[(*N*,*N*-diisopropylamino)-methoxyphosphino]-5'-*O*-(4,4'-dimethoxytrityl)-thymidine (**3d**, 10.6 g, 15.0 mmol, dried by repeated coevaporation with toluene) in 30 ml dry acetonitrile. The reaction was followed by TLC and more phosphoramidite added if necessary. The mixture was cooled in an ice bath, *tert*-butylhydroperoxide (5.0 ml, 40 mmol) was added, and after 15 min the solution was evaporated to dryness under reduced pressure. The remaining oil was dissolved in ethyl acetate (100 ml), the solution successively washed with phosphate buffer (0.2 M, pH = 6.8) and water, dried over anhydrous sodium sulphate, filtered, and evaporated to dryness. The crude, fully protected dinucleotide was dissolved in 75 ml CH_2Cl_2/MeOH (8/1, v/v), and deprotected with Amberlyst® 15 as described above, to give 8.5 g (84%) of **4d** as a colourless powder. For the acquisition of analytical data a small sample was purified by column chromatography on silica gel using 8% methanol in dichloromethane as eluent.; R_f 0.44; ^1H NMR ($CDCl_3$): δ 1.75 (3H, s, CH_3 of T), 1.94 (3H, s, CH_3 of T), 2.49 (4H, m_c, 2'-H), 3.25 (1H, bs, OH), 3.7−3.9 (2H, m, 5'-H), 3.80 and 3.84 (3H, 2s, $POCH_3$), 4.20 (2H, m, 5'-H), 4.34 (2H, m, 4'-H), 4.70 (2H, s, CH_2 of PAC), 5.15 (1H, m, 3'-H), 5.45 (1H, m, 3'-H), 6.15 (1H, m_c, 1'-H), 6.27 (1H, m_c, 1'-H), 6.90 (2H, d, J = 7.9 Hz, 2,6-H of PAC), 7.01 (1H, m, 4-H of PAC), 7.26−7.34 (2H, m, 3,5-H of PAC), 7.38 (1H, m, 6-H of T), 7.45 (1H, m, 6-H of T), 9.22 (1H, bs, NH), 9.40 (1H, bs, NH); ^{13}C NMR ($CDCl_3$): δ 12.52 (2×CH_3 of T), 36.78 (2'-C), 38.48 (2'-C), 54.96 ($POCH_3$), 62.10 (5'-C), 65.13 (CH_2 of PAC), 67.21 (5'-C), 74.80 and 75.04 (3'-C), 79.0 and 79.1 (3'-C), 82.64, 85.24 and 86.31 (2×1'-C, 2×4'-C), 111.29 (5-C of T), 111.38 (5-C of T), 114.64 (2,6-C of PAC), 122.14 (4-C of PAC), 129.74 (3,5-C of PAC), 135.40 (6-C of T), 136.56 (6-C of T), 150.47 (2×2-C of T), 157.51 (1-C of PAC), 163.84 (2×4-C of T), 168.91 (CO of PAC); ^{31}P NMR ($CDCl_3$): δ −0.40 (P(V) diast. a), −0.76 (P(V) diast. b); FAB MS (m-NBA, m/z): 695.2 (M + H^+).

P-Methylthymidylyl-(3'→5')-N^2-isobutyryl-3'-phenoxyacetyl-2'-deoxyguanosine (**4g**). This was prepared using the same method as described above, from **2b** (6.10 g, 12.9 mmol), tetrazole (1.70 g, 24.3 mmol), **3d** (9.39 g, 13.3 mmol) and *tert*-butylhydroperoxide (5.0 ml, 40 mmol). Yield: 8.25 g (81%), colourless powder; R_f 0.36; ^1H NMR ($CDCl_3$): δ 1.21 (6H, m, CH_3 of ^iBu), 1.85 (3H, s, CH_3 of T), 2.2−2.6 (4H, m, 2'-H), 2.76 (1H, m, CH of ^iBu), 3.34 (1H, bs, OH), 3.81 and 3.84 (3H, 2s, $POCH_3$), 3.89 (2H, m, 5'-H), 4.15 and 4.27 (1H, 2m, 4'-H), 4.33 (2H, m, 5'-H), 4.40 and 4.54 (1H, 2m, 4'-H), 4.72 and 4.73 (2H, 2s, CH_2 of PAC), 5.15 and 5.26 (1H, 2m, 3'-H), 5.57 and 5.68 (1H, 2m, 3'-H), 6.02 (1H, m_c, 1'-H), 6.17 and 6.25 (1H, 2m, 1'-H), 6.91 (2H, d, J = 8.1 Hz, 2,6-H of PAC), 7.01 (1H, m, 4-H of PAC), 7.28−7.33 (2H, m, 3,5-H of PAC), 7.52 and 7.58 (1H, 2m, 6-H of T), 7.66 (1H, m, 8-H of

G), 9.28 and 9.31 (1H, 2s, NH), 10.68 (1H, bs, NH), 12.18 (1H, bs, NH); ^{13}C NMR ($CDCl_3$): δ 12.45 (CH_3 of T), 18.61, 18.99 and 19.39 (CH_3 of ^{i}Bu), 35.10 and 35.49 (2′-C), 35.86 (CH of ^{i}Bu), 38.53 (2′-C), 54.77, 54.85 and 54.99 ($POCH_3$), 61.78 (5′-C), 65.21 (CH_2 of PAC), 66.50 and 66.99 (5′-C), 75.74 and 75.92 (3′-C), 79.04 and 79.20 (3′-C), 82.88, 85.75, 86.00, 86.09, 86.40 and 86.62 (2×1′-C, 2×4′-C), 111.13 and 111.20 (5-C of T), 114.63 (2,6-C of PAC), 122.10 (4-C of PA-C), 122.47 (5-C of G), 129.75 (3,5-C of PAC), 136.57 and 136.76 (6-C of T), 139.33 (5-C of G), 147.98 and 148.17 (2,4-C of G), 150.50 and 150.56 (2-C of T), 155.59 and 155.74 (6-C of G), 157.58 (1-C of PAC), 163.96 (4-C of T), 168.74 and 168.97 (CO of PAC), 180.27 (CO of ^{i}Bu); ^{31}P NMR ($CDCl_3$): δ −1.44 (P(V) diast. a, −1.22 (P(V) diast. b; FAB MS (m-NBA, m/z): 790 (M + H^+).

Preparation of trimers

*N^2-Isobutyryl-P-methyl-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyguanylyl-(3′→5′)-P-methylthymidylyl-(3′→5′)-3′-phenoxyacetylthymidine (**5n**).* The synthesis of the fully protected trinucleotide proceeded as described above for the fully protected dinucleotide intermediate. Thus **4d** (7.90 g, 10.0 mmol) was reacted with N^2-isobutyryl-3′-*O*-[(*N*,*N*-diisopropylamino)-methoxyphosphino]-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyguanosine (**3c**, 8.41 g, 10.5 mmol), tetrazole (1.12 g, 16.0 mmol) and *tert*-butylhydroperoxide (3.1 ml, 25 mmol). After the aqueous work up, the crude product was dissolved in dichloromethane (35 ml) and purified by precipitation from ether (1000 ml). Yield: 13.0 g (92%), off-white powder; R_f 0.54; ^{1}H NMR ($CDCl_3$): δ 1.21−1.26 (6H, m, CH_3 of ^{i}Bu), 1.83−1.95 (6H, m, 2×CH_3 of T), 2.37−3.12 (7H, m, 2′-H and CH of ^{i}Bu), 3.70−3.90 (14H, m, 2×5′-H, 2×OCH_3 of DMT and 2×$POCH_3$), 4.20−4.34 (7H, m, 4×5′-H and 3×4′-H), 4.69 (2H, bs, CH_2 of PAC), 5.20 (2H, m_c, 3′-H), 5.44 (1H, m_c, 3′-H), 5.99−6.25 (3H, m, 1′-H), 6.83 (4H, δ, *J* = 8.8 Hz, 3,3′,5,5′-H of DMT), 6.89 (2H, d, *J* = 8.1 Hz, 2,6-H of PAC), 6.98−7.02 (1H, m, 4-H of PAC), 7.17 (4H, d, *J* = 8.8 Hz, 2,2′,6,6′-H of DMT), 7.22−7.57 (9H, m, 3,5-H of PAC, 2′,3′,4′,5′,6′-H of DMT, 2×6-H of T), 7.77−8.08 (1H, m, 8-H of G), 9.28−10.13 (2H, m, NH), 10.30−10.47 (1H, m, NH), 12.19−12.25 (1H, m, NH); ^{13}C NMR ($CDCl_3$): δ 12.36 (2×CH_3 of T), 18.97 and 19.12 (CH_3 of ^{i}Bu), 35.98 and 36.17 (CH of ^{i}Bu), 36.82 (2′-C), 38.75 (2×2′-C), 54.86 (2×$POCH_3$), 55.26 (OCH_3 of DMT), 61.90 (5′-C), 65.14 (CH_2 of PAC), 67 (2×5′-C), 74.81, 77.24 and 80.0 (3×3′-C), 81.44 (*tert*-C of DMT), 82.54, 85.08, 85.52 and 85.90 (3×1′-C, 3×4′-C), 111.11 (5-C of T), 111.73 (5-C of T), 113.17 (3,3′,5,5′-C of DMT), 114.63 (2,6-C of PAC), 121.8 (5-C of G), 122.10 (4-C of PAC), 127.07 (4″-C of DMT), 127.79 and 127.84 (2″,3″,5″,6″-C of DMT), 129.14 (2,2′,6,6′-C of DMT), 129.71 (3,5-C of PAC), 136.0 (6-C of T), 137.2 (6-C of T), 138.65 (5-C of G), 139.50 (1,1′-C of DMT), 147.36 (1′-C of DMT), 148.02 (2,4-C of G), 150.44 (2×2-C of T), 155.27 (6-C of G), 157.52 (1-C of PAC), 158.64 (4,4′-C of DMT), 163.85 (2×4-C of T), 168.82 (CO of PAC), 180.0 (CO of ^{i}Bu); ^{31}P NMR ($CDCl_3$): δ −0.90 to −0.51 (2×P(V)); FAB MS (m-NBA, m/z): 1431.9 (M + Na^+), 1409.9 (M + H^+).

*N^6-Benzoyl-P-methyl-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyadenylyl-(3′→5′)-P-methylthymidylyl-(3′→5′)-N^2-isobutyryl-3′-phenoxyacetyl-2′-deoxyguanosine (**5t**).* This was prepared using the same method as described above, from **4g** (7.90 g, 10.0 mmol), N^6-benzoyl-3′-*O*-[(*N*,*N*-diisopropylamino)-methoxyphosphino]-2′-deoxyadenosine (**3a**, 8.60 g, 10.5 mmol), tetrazole (1.12 g, 16.0 mmol) and *tert*-butylhydroperoxide (3.1 ml, 25 mmol). Yield: 12.9 g (85%), off-white powder; R_f 0.58; ^{1}H NMR ($CDCl_3$): δ 1.15−1.28 (6H, m, CH_3 of ^{i}Bu), 1.78−1.90 (3H, m, CH_3 of T), 2.05−2.85 (6H, m, 5×2′-H and CH of ^{i}Bu), 3.0−3.15 (1H, m, 2′-H), 3.40−3.52 (2H, m, 5′-H), 3.69−3.90 (12H, m, 2×$POCH_3$, 2×OCH_3 of DMT), 4.10−4.55 (7H, m, 4×5′-H and 3×4′-H), 4.69−4.70 (2H, m, CH_2 of PAC), 4.90−5.15 (1H, m, 3′-H), 5.20−5.35 (1H, m, 3′-H), 5.50−5.62 (1H, m, 3′-H), 5.99−6.22 (2H, m, 1′-H), 6.49−6.54 (1H, m, 1′-H), 6.76−6.90 (6H, m, 2,6-H of PAC and 3,3′,5,5′-H of DMT), 6.96−7.03 (1H, m, 4-H of PAC), 7.15−7.36 (11H, m, 3,5-H of PAC, 2,2′,6,6′-H and 2′,3′,4′,5′,6′-H of DMT), 7.46−7.71 (5H, m, 3,4,5-H of Bzl, 6-H of T, 8-H of G), 8.03 (2H, d, *J* = 7.6 Hz, 2,6-H of Bzl), 8.16−8.23 (1H, m_c, 2-H of A), 8.67−8.77 (1H, m, 8-H of A), 9.0−9.8 (2H, m, NH), 10.42−10.60 (1H, m, NH), 12.19−12.23 (1H, m, NH); ^{13}C NMR ($CDCl_3$): δ 12.34 (CH_3 of T), 18.78−19.21 (CH_3 of ^{i}Bu), 35.50 (2′-C), 35.93 (CH of ^{i}Bu), 38.04 (2×2′-C), 54.88 (2×$POCH_3$), 55.26 (OCH_3 of DMT), 63.0 (5′-C), 65.18 (CH_2 of PAC), 66.83 (2×5′-C), 75.50, 77.73 and 79.28 (3×3′-C), 82.82−87.61 (3×1′-C, 3×4′-C), 86.85 (*tert*-C of DMT), 111.61 (5-C of T), 113.17 and 113.25 (3,3′,5,5′-C of DMT), 114.60 (2,6-C of PAC), 122.12 (4-C of PAC), 122.8 (5-C of G), 123.8 (5-C of A), 127.06, 127.80, 127.83, 127.95, 128.11, 128.77, 129.16, 129.74 and 130.01 (2,2′,6,6′-C and 2″,3″,4″,5″,6″-C of DMT, 2,3,5,6-C of Bzl, 3,5-C of PAC), 132.79 (4-C of Bzl), 133.5 (1-C of Bzl), 135.36 (1,1′-C of DMT), 135.67 (6-C of T), 139 (5-C of G), 142.2 (2-C of A), 144.5 (1′-C of DMT), 147.90 (2,4-C of G), 149.78 (4-C of A), 150.29 (2-C of T), 151.7 (6-C of A), 152.6 (8-C of A), 155.54 (6-C of G), 157.54 (1-C of PA-C), 158.65 (4,4′-C of DMT), 163.7 (4-C of T), 165 (CO of Bzl), 168.76 (CO of PAC), 179.82 (CO of ^{i}Bu); ^{31}P NMR ($CDCl_3$): δ −1.65 to −0.5 (2×P(V)); FAB MS (m-NBA, m/z): 1545.0 (M + Na^+), 1522.9 (M + H^+).

*N^2-Isobutyryl-P-methyl-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyguanylyl-(3′→5′)-P-methylthymidylyl-(3′→5′)-thymidine (**6n**).* To a solution of **5n** (4.00 g, 2.84 mmol) in methanol (20 ml) and acetonitrile, was added methanolic ammonia (5 ml, 38 mmol). The reaction was followed by TLC, and more methanolic ammonia was added if necessary. After completion of the deprotection (~20−30 min) the solution was rapidly evaporated to dryness without heating. The off-white foam was dissolved in dichloromethane and chromatographed on 250 g LiChroprep® DIOL with dichloromethane as eluent, applying a stepwise gradient of methanol (0−10%). The fractions containing **5n** were combined and concentrated to give a colourless foam, which was dried under vacuum. Yield: 1.9 g (52%); R_f 0.25; ^{1}H NMR ($CDCl_3$): δ 1.09−1.33 (6H, m, CH_3 of ^{i}Bu), 1.84−2.00 (3H, m, 2×CH_3 of T), 2.10−3.00 (6H, m, 5×2′-H and CH of ^{i}Bu), 3.20−3.45 (2H, m, 2′-H and OH), 3.68−3.85 (14H, m, 2×5′-H, 2×OCH_3 of DMT and 2×$POCH_3$), 4.10 (1H, m, 4′-H), 4.34 (5H, m_c, 4×5′-H and 1×4′-H), 4.51 (1H, m, 4′-H), 5.00−5.28 (3H, m, 3′-H), 6.04−6.28 (3H, m, 1′-H), 6.78 (4H, d, *J* = 8.3 Hz, 3,3′,5,5′-H of DMT), 7.15−7.45 (11H, m, 2,2′,6,6′-H and 2′,3′,4′,5′,6′-H of DMT, 2×6-H of T), 7.74 and 7.77 (1H, 2m, 8-H of G), 9.7−10.4 (3H, m, NH), 12.10−12.25 (1H, m, NH); ^{13}C NMR ($CDCl_3$): δ 12.34

(2×CH_3 of T), 18.83 and 19.02 (CH_3 of ^iBu), 35.92 (CH of ^iBu), 38.23 (2×2′-C), 39.80 (2′-C), 54.97 (2×$POCH_3$), 55.26 (OCH_3 of DMT), 63.30 (5′-C), 66.78 and 67.65 (2×5′-C), 70.44, 77.27 and 79.20 (3×3′-C), 83.6–86.75 (3×1′-C, 3×4′-C), 86.82 (*tert*-C of DMT), 111.22 (5-C of T), 111.90 (5-C of T), 113.27 (3,3′,5,5′-C of DMT), 121.20 (5-C of G), 127.09 (4″-C of DMT), 127.96 and 128.07 (2″,3″,5″,6″-C of DMT), 130.02 (2,2′,6,6′-C of DMT), 135.36 (1,1′-C of DMT), 136.28 (2×6-C of T), 136.5 and 137.0 (5-C of G), 144.32 (1′-C of DMT), 148.37 (2,4-C of G), 150.55 (2×2-C of T), 155.55 (6-C of G), 158.67 (4,4′-C of DMT), 164.27 (4-C of T), 164.56 (4-C of T), 180.08 (CO of ^iBu); ^{31}P NMR ($CDCl_3$): δ −0.90 to −0.34 (2×P(V)); FAB MS (m-NBA, m/z): 1276.1 (M + H^+).

N^6-Benzoyl-P-methyl-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyadenylyl-(3′→5′)-P-methylthymidylyl-(3′→5′)-N^2-isobutyryl-2′-deoxyguanosine (**6t**). This was prepared using the same method as described above, from **5t** (4.00 g, 2.63 mmol). Yield: 1.8 g (49%), colourless foam; R_f 0.29; ^1H NMR ($CDCl_3$): δ 1.17–1.35 (6H, m, CH_3 of ^iBu), 1.81 (3H, m_c, CH_3 of T), 2.10–2.85 (7H, m, 6×2′-H and CH of ^iBu), 3.42 (2H, m, 5′-H), 3.74 (12H, m_c, 2×$POCH_3$, 2×OCH_3 of DMT), 3.95–4.55 (6H, m, 4×5′-H and 2×4′-H), 4.65 (1H, m, 4′-H), 4.95 (1H, m_c, 3′-H), 5.29 (1H, m_c, 3′-H), 5.55 (1H, m_c, 3′-H), 6.0–6.3 (2H, m, 1′-H), 6.53 (1H, m_c, 1′-H), 6.78 (4H, m_c, 3,3′,5,5′-H of DMT), 7.15–7.95 (14H, m, 2,2′,6,6′-H and 2′,3′,4′,5′,6′-H of DMT, 3,4,5-H of Bzl, 6-H of T, 8-H of G), 8.02 (2H, m, 2,6-H of Bzl), 8.28 (1H, m_c, 2-H of A), 8.64 (1H, m_c, 8-H of A), 9.2–10.0 (2H, m, NH), 10.4–11.1 (1H, m, NH); ^{13}C NMR ($CDCl_3$): δ 12.5 (CH_3 of T), 19.03 (CH_3 of ^iBu), 36.0 (CH of ^iBu), 38.1–39.5 (3×2′-C), 55.1 (2×$POCH_3$), 55.19 (OCH_3 of DMT), 63.13 (5′-C), 67.0–68.0 (2×5′-C), 74.5–80.5 (3×3′-C), 82.8–86.8 (3×1′-C, 3×4′-C), 86.67 (*tert*-C of DMT), 111.5 (5-C of T), 113.14 (3,3′,5,5′-C of DMT), 122.0 (5-C of G), 123.8 (5-C of A), 126.9 (4″-C of DMT), 127.0–130.2 (2,2′,6,6′-C and 2″,3″,5″,6″-C of DMT, 2,3,5,6-C of Bzl), 132.7 (4-C of Bzl), 133.5 (1-C of Bzl), 135.34 (1,1′-C of DMT), 135.6 (6-C of T), 138.5 (5-C of G), 142.3 (2-C of A), 144.27 (1′-C of DMT), 148.22 (2,4-C of G), 149.9 (4-C of A), 150.41 (2-C of T), 151.53 (6-C of A), 152.6 (8-C of A), 155.6 (6-C of G), 158.53 (4,4′-C of DMT), 163.7 (4-C of T), 165.35 (CO of Bzl), 180.25 (CO of ^iBu); ^{31}P NMR ($CDCl_3$): δ −1.18 to −0.54 (2×P(V)); FAB MS (m-NBA, m/z): 1411.1 (M + Na^+), 1389.1 (M + H^+).

N^2-Isobutyryl-P-methyl-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyguanylyl-(3′→5′)-P-methylthymidylyl-(3′→5′)-3′-O-[(N,N-diisopropylamino)-methoxyphosphino]-thymidine (**7n**). Methyl *N,N*-diisopropylphosphoramidochloridite (0.52 ml, 2.66 mmol) was added to a solution of **6n** (1.70 g, 1.33 mmol, dried for 2 h under vacuum) in dry dichloromethane, containing *N,N*-diisopropylethylamine (0.92 ml, 5.32 mmol). After 30 min at ambient temperature, methanol (1 ml) was added, the solution diluted with ethyl acetate (saturated with nitrogen) and washed with phosphate buffer (0.4 M, pH = 7.8, saturated with nitrogen) and water. The aqueous layer was back extracted with ethyl acetate. The combined organic fractions were dried over sodium sulphate, filtered and concentrated to dryness. The residue was dissolved in dichloromethane (5 ml) and precipitated from ether (500 ml) to yield 1.64 g (86%) of **7n** as a colourless powder; R_f 0.47; ^1H NMR ($CDCl_3$): δ 1.10–1.25 (18H, m, CH_3 of ^iBu and ^iPr), 1.85–2.05 (6H, m, 2×CH_3 of T), 2.15–2.85 (7H, m, 6×2′-H and CH of ^iBu), 3.35 and 3.40 (3H, m, $NPOCH_3$), 3.45–3.70 (2H, m, CH of ^iPr), 3.70–3.90 (14H, m, 2×5′-H, 2×OCH_3 of DMT and 2×$POCH_3$), 4.05–4.55 (7H, m, 4×5′-H and 3×4′-H), 5.00–5.35 (3H, m, 3′-H), 6.10–6.30 (3H, m, 1′-H), 6.75–6.85 (4H, m, 3,3′,5,5′-H of DMT), 7.1–7.5 (11H, m, 2,2′,6,6′-H and 2′,3′,4′,5′,6′-H of DMT, 2×6-H of T), 7.67–7.75 (1H, m, 8-H of G), 9.9–10.5 (3H, m, NH); ^{13}C NMR ($CDCl_3$): δ 12.37 (CH_3 of T), 12.55 (CH_3 of T), 19.10 and 19.28 (CH_3 of ^iBu), 24.80 and 24.88 (CH_3 of ^iPr), 36.14 (CH of ^iBu), 38.29 (2′-C), 38.88 (2′-C), 39.26 (2′-C), 43.22 and 43.38 (CH of ^iPr), 50.48 and 50.70 ($NPOCH_3$), 55.14 (2×$POCH_3$), 55.49 (OCH_3 of DMT), 63.91 and 63.56 (5′-C), 66.99 and 67.31 (5′-C), 67.74 (5′-C), 72.40 and 72.63 (3′-C), 77.59 and 77.90 (3′-C), 79.65 and 79.89 (3′-C), 83.59–86.85 (3×1′-C, 3×4′-C), 87.05 and 87.13 (*tert*-C of DMT), 111.37 and 111.53 (5-C of T), 111.80 (5-C of T), 113.51 (3,3′,5,5′-C of DMT), 121.58 and 121.75 (5-C of G), 127.34 (4″-C of DMT), 128.20 and 128.29 (2″,3″,5″,6″-C of DMT), 130.26 (2,2′,6,6′-C of DMT), 135.54 (1,1′-C of DMT), 136.29 and 136.47 (6-C of T), 136.72, 137.00 and 137.14 (6-C of T, 8-C of G), 144.46 (1′-C of DMT), 148.32, 148.78, 148.84 and 149.38 (2,4-C of G), 150.62 (2-C of T), 150.83 (2-C of T), 155.68 (6-C of G), 158.92 (4,4′-C of DMT), 164.17 (4-C of T), 164.63 and 164.82 (4-C of T), 179.97 and 180.08 (CO of ^iBu); ^{31}P NMR ($CDCl_3$): δ −0.43 to +0.34 (2×P(V)), 150.08–150.75 (P(III)); FAB MS (m-NBA, m/z): 1510.9 (M−HN^iPr_2 + m-NBA + Na^+), 1488.9 (M−HN^iPr_2 + m-NBA + H^+), 1474.8 (M + O + Na^+), 1452.9 (M + O + H^+), 1353.9 (M−HN^iPr_2 + H_2O + H^+); FAB MS (o-nitrophenyloctylether, m/z): 1453 (M + O + H^+), 1437 (M + H^+); ESI MS (MeOH/NaI, m/z): 1481.8 (M + Na_2+),1460.9 (M + Na^+).

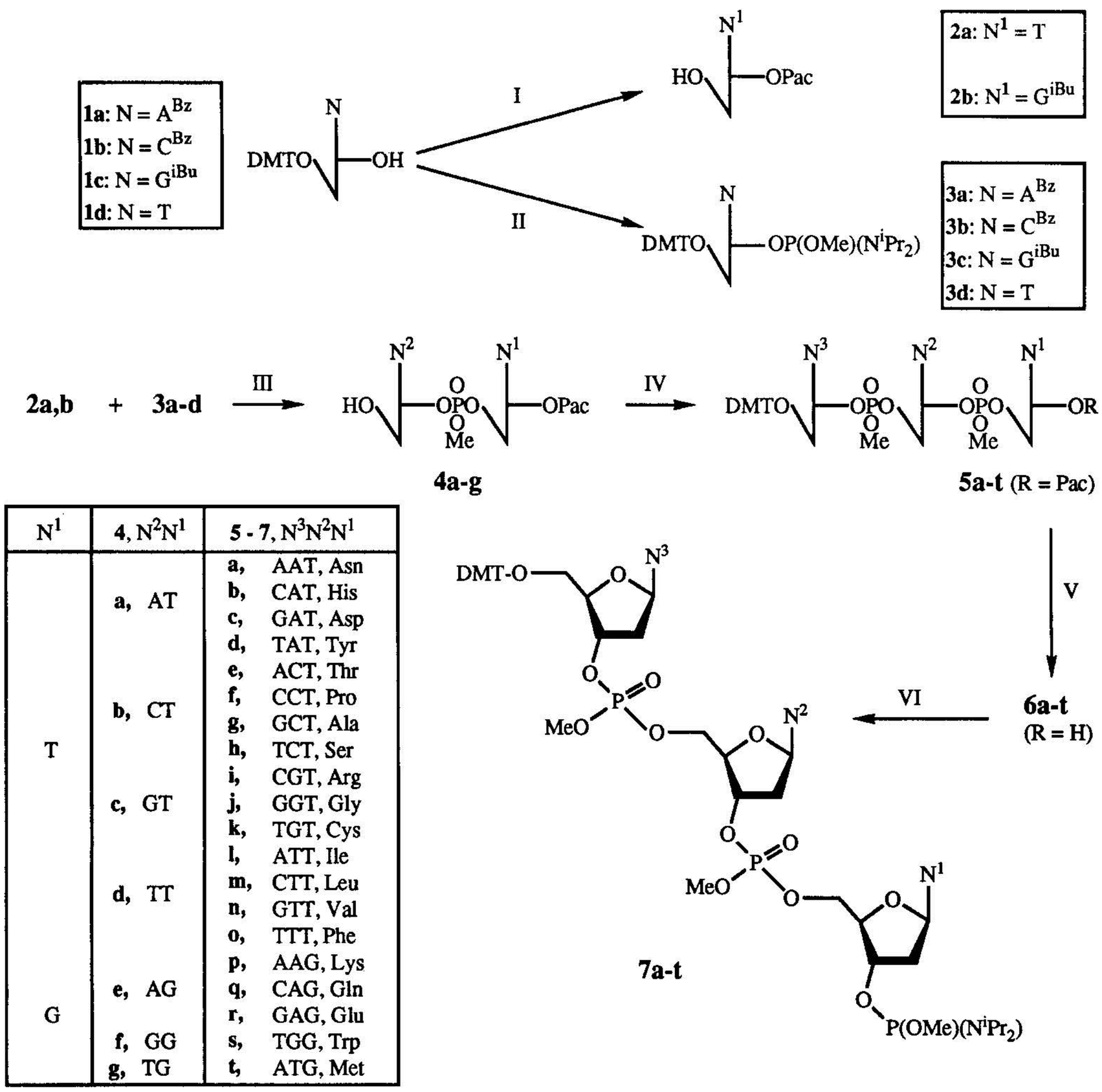
N^6-Benzoyl-P-methyl-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyadenylyl-(3′→5′)-P-methylthymidylyl-(3′→5′)-N^2-isobutyryl-3′-O-[(N,N-diisopropylamino)-methoxyphosphino]-2′-deoxyguanosine (**7t**). This was prepared using the same method as described above, from **6t** (1.6 g, 1.15 mmol), methyl *N,N*-diisopropylphosphoramidochloridite (0.45 ml, 2.30 mmol) and *N,N*-diisopropylethylamine (0.79 ml, 4.60 mmol). Yield: 1.62 g (91%), colourless powder; R_f 0.47; ^1H NMR ($CDCl_3$): δ 1.15–1.25 (18H, m, CH_3 of ^iBu and ^iPr), 1.80–1.90 (3H, m, CH_3 of T), 2.0–3.25 (7H, m, 6×2′-H and CH of ^iBu), 3.30–3.80 (19H, m, 2×5′-H, 2×OCH_3 of DMT, 3×OCH_3 of $POCH_3$ and 2×CH of ^iPr), 4.00–4.70 (7H, m, 4×5′-H and 3×4′-H), 4.85–5.65 (3H, m, 3′-H), 6.0–6.3 (2H, m, 1′-H), 6.4–6.6 (1H, m, 1′-H), 6.75–6.85 (4H, m, 3,3′,5,5′-H of DMT), 7.17–7.65 (13H, m, 2,2′,6,6′-H and 2′,3′,4′,5′,6′-H of DMT, 3,4,5-H of Bzl, 6-H of T), 7.65–7.75 (1H, m, 8-H of G), 8.04 (2H, m, 2,6-H of Bzl), 8.10–8.28 (1H, m, 2-H of A), 8.68 (1H, m_c, 8-H of A), 10.4–11.0 (1H, m, NH); ^{13}C NMR ($CDCl_3$): δ 12.47 (CH_3 of T), 18.98–19.38 (CH_3 of ^iBu), 24.79 and 24.88 (CH_3 of ^iPr), 36.16 (CH of ^iBu), 38.35 (3×2′-C), 43.17 and 43.33 (CH of ^iPr), 50.25 and 50.80 ($NPOCH_3$), 55.11 (2×$POCH_3$), 55.43 (OCH_3 of DMT), 63.34, 67.04 and 67.85 (3×5′-C), 73.27 and 73.89 (3′-C), 77.43 and 77.98 (3′-C), 79.58 (3′-C), 83.38–86.9 (3×1′-C, 3×4′-C), 86.97 (*tert*-C of DMT), 111.78 (5-C of T), 113.42 (3,3′,5,5′-C of DMT), 122.85 and 123.07 (5-C of G), 123.84 (5-C of A), 127.24 (4″-C of DMT), 128.10 (2,6-C of Bzl), 128.29 (2,2′,6,6′-C of DMT), 128.95 (3,5-C of Bzl), 130.20

(2″,3″,5″,6″-C of DMT), 132.96 (4-C of Bzl), 133.81 (1-C of Bzl), 135.56 (1,1′-C of DMT), 135.79 (6-C of T), 138.89 (8-C of G), 142.12 (2-C of A), 144.46 (1′-C of DMT), 148.02 (2,4-C of G), 149.95 (4-C of A), 150.46 (2-C of T), 151.75 (6-C of A), 152.62 (8-C of A), 155.86 (6-C of G), 158.82 (4,4′-C of DMT), 163.77 (4-C of T), 165.17 (CO of Bzl), 179.84 (CO of ^{i}Bu); ^{31}P NMR ($CDCl_3$): δ −0.94 to −0.09 (2×P(V)), 149.89−150.04 (P(III)); FAB MS (m-NBA, m/z): 1623.8 (M−HN^{i}Pr$_2$ + m-NBA + Na$^+$), 1601.8 (M−HN^{i}Pr$_2$ + m-NBA + H$^+$), 1566.0 (M + O + H$^+$), 1466.7 (M−HN^{i}Pr$_2$ + H_2O + H$^+$); FAB MS (o-nitrophenyloctylether, m/z): 1565.9 (M + O + H$^+$), 1550.2 (M + H$^+$); ESI MS (MeOH/NaI, m/z): 1596.2 (M + Na$_2$+),1574.9 (M + Na$^+$).

Cloning and sequencing

The single-chain Fv fragment (scFv) of a benzoylampicillin-binding antibody was obtained from hybridoma 2H10 and was cloned into the phage-display vector of pIG vector series (9). The phagemid was used as template for the PCR. PCR for generating individual segments (Figure 2A) were carried out in 50 μl total volume containing 0.2 μg of phagemid template, 25 pmol of each primer (Figure 2B) and *Taq* polymerase (Boehringer Mannheim, 5 U). The polymerase was added after the first cycle of denaturation and annealing. 30 cycles of PCR were performed (92°C, 1 min; 50°C, 1 min; 72°C, 1 min). The fragments of correct size were isolated using low-melting (LMP) agarose and phenol−chloroform extracted. The fragments were 'end-polished' by 30 cycles of PCR (92°C, 1 min; 50°C, 1 min; 72°C, 1 min) using Vent polymerase (New England Biolabs, 2 U), and gel purified as above. The ethanol-precipitated fragments were dissolved in 20 μl of TE, pH 7.6. Assembly PCR (10) was performed in 50 μl total volume containing all three purified PCR segments (A2, A3 and A4, 50 ng each), with 2 U of Vent polymerase. Touch-down PCR (10) was applied (92°C, 1 min; 56−50°C, ΔT = 2°C, 2 min; 72°C, 1 min). Subsequently, the mixture was denatured and the temperature was lowered to 48°C. At this point terminal primers (A-1.1 and A-1.6, 25 pmol each) were added and annealing was allowed to continue for another 2 min, followed by extension at 72°C, 1 min. 28 cycles of PCR were used to generate the full-length products (92°C, 1 min; 48°C, 1 min; 72°C, 1 min). As control, the phagemid (0.2 μg) was similarly amplified.

The PCR products were purified (Qiagen PCR spin purification kit, Diagen GmbH) and eluted in 50 μl of H_2O. They were



N^1	4, N^{2}N^1	5 - 7, N^{3}N^{2}N^1
T	a, AT	a, AAT, Asn b, CAT, His c, GAT, Asp d, TAT, Tyr
	b, CT	e, ACT, Thr f, CCT, Pro g, GCT, Ala h, TCT, Ser
	c, GT	i, CGT, Arg j, GGT, Gly k, TGT, Cys l, ATT, Ile
	d, TT	m, CTT, Leu n, GTT, Val o, TTT, Phe
G	e, AG	p, AAG, Lys q, CAG, Gln r, GAG, Glu
	f, GG	s, TGG, Trp
	g, TG	t, ATG, Met

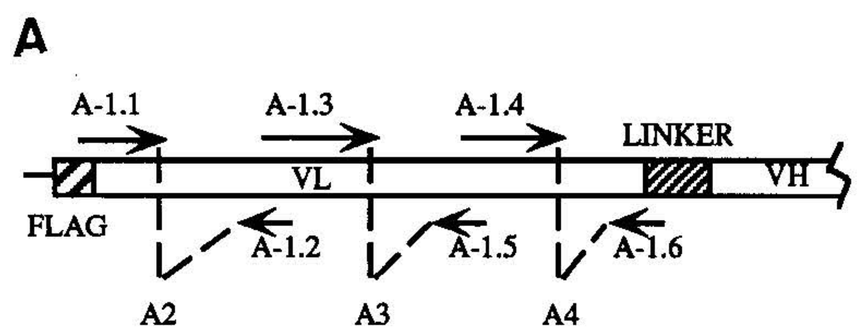
Figure 1. Synthetic route to and identity of the 20 trinucleotides prepared: I, Pyridin, Pac-Cl; then Amberlyst® 15; II, EtN^{i}Pr$_2$, MeOP(N^{i}Pr$_2$)Cl; III, Tetrazole; ^{t}BuOOH; then Amberlyst® 15; IV, **3a−d**, Tetrazole; ^{t}BuOOH; V, NH_3, MeOH; VI, EtN^{i}Pr$_2$, MeOP(N^{i}Pr$_2$)Cl.

digested with 40 U of *Eco*RV and 40 U of *Xho*I (New England Biolabs) overnight. The fragments were gel purified (Qiagen) and ligated into pre-digested, purified pSL301·*Eco*RV−*Xho*I vector. The ligated products were used to transform competent *E.coli* JM-83 cells. Transformants containing the V_L inserts were screened and sequenced (Pharmacia[T7] SequencingTM kit).

RESULTS AND DISCUSSION

Our synthetic strategy took account of two factors: we wanted to make the route to all 20 trinucleotides as convergent as possible, and since we intended to use the resulting oligonucleotides for expression of proteins in *E.coli*, it was necessary to accommodate codon usage requirements in the host organism. Since there is no evidence that the most abundant codons in well-expressed genes of *E.coli* offer any advantage in expression yield over codons of medium abundance (11), we set out only to avoid rare codons.

Accordingly, we devised a scheme which would enable synthesis of all 20 fully protected trinucleotides via two protected mononucleosides, and only seven dinucleotides (see Figure 1). This scheme provides access to the 20 trinucleotides using the fewest possible coupling reactions. As with standard solid-phase DNA synthesis using mononucleotides, the use of trinucleotides requires that the bases, 5′-hydroxyl group, and phosphate esters are protected. The choice of protecting groups took into account the need to liberate the hydroxyl group at the 3′-terminus prior to formation of the phosphoramidite. For this purpose we employed phenoxyacetyl (PAC) as the protecting group at the 3′-position. PAC was chosen after problems were encountered with our original choice of 3′-protecting group, 9-fluorenylmethoxycarbonyl (FMOC). Although reaction of 5′-DMT-thymidine (**1d**) with FMOC-chloride (1.1 eq.) in pyridine (0°C, 30 min) proceeded smoothly to provide the 5′-DMT-3′-FMOC derivative, treatment of 5′-DMT-N^2-isobutyryldeoxyguanosine (**1c**) under the same conditions led to depurination and almost quantitative formation of an FMOC−guanine derivative (identified by mass spectrometry). The lability of PAC under basic conditions meant that we were forced to use the more stable methyl- rather than cyanoethyl-protected phosphate esters. As a result, it was necessary to use thiophenol during deprotection of oligonucleotides which incorporated trinucleotides.



B

A-1.1 5'- TACAAAGATA TCGTGZACCZ TCTCCAGCA

A-1.2 5'- *GGTCATGGTG ACCCG*

A-1.3 5'- *CGGGTCACCA TGACCZ*ACTG CCAGTTCAAG TGTAAGTTCC TCTTACZCAC ZZCAGCAGAA G

A-1.4 5'- *TCTCTCACAA TCAGCZ*ATGZ GCTGAGGATG CTGCCACTTA TTACZCACCA GTAT

A-1.5 5'- *GCTGATTGTG AGAGA*

A-1.6 5'- CCGTTTTATC TCGAGCTTGG TCCCCCC(A/T)(G/C)C GAACGTGGG

C

Trinucleotide	Occurrence	Trinucleotide	Occurrence
TTT (Phe)	13/63	ACT (Thr)	8/63
ATT (Ile)	15/63	GCT (Ala)	7/63
CTT (Leu)	4/63	ATG (Met)	6/63
GTT (Val)	9/63	TGG (Trp)	1/63

Figure 2. (**A**) Schematic arrangement of the PCR primers relative to the V_L template. Primers A-1.1, A-1.3 and A-1.4 contain the trinucleotide mixture as shown in Figure 2B. Separate PCR was carried out with each primer pair indicated and the resulting fragments (A2, A3 and A4) were used in subsequent assembly PCR (see Experimental). FLAG, three amino acid immuno detection tag; LINKER, peptidal linker sequence (9). (**B**) Sequences of the primers used in assembling the 2H10 scFv light chain gene. Underlined sequences are *Eco*RV (A-1.1) and *Xho*I (A-1.6) restriction sites used in cloning the PCR product. Sequences in italics represent regions of overlap of the initial PCR products during subsequent assembly PCR. **Z** denotes an approximately uniform mixture of the trinucleotides TTT, ATT, CTT, GTT, ACT, GCT, ATG and TGG. (**C**) Table showing the distribution of the eight trinucleotides determined by sequencing of seven clones each containing nine trinucleotide sites (designated **Z** in Figure 2B) resulting from insertion of the PCR product into sequencing vector pSL301.

The synthetic route and the identity of the trinucleotides and the necessary intermediates is illustrated in Figure 1. The synthesis used standard conditions (5−7) with the exception of cleavage of the 5′-dimethoxytrityl group where we used a strongly acidic ion exchange resin (Amberlyst® 15) in methanol, which we found superior to trichloroacetic acid in furnishing the intermediate 5′-deprotected mononucleosides **2** and dinucleotides **4**. Synthesis and ^{1}H NMR data of **2a** and **2b** were recently published (8).

All trinucleotides were prepared in the same way, as described in the Experimental section for the phosphoramidites **7n** and **7t**. Typical overall yields of trinucleotide phosphoramidites **7** were 25−40%, based on the precursors **2**. The least efficient step in the synthesis was cleavage of the PAC group, which proceeded in yields ranging from 75% (**6o**, TTT), in which only phosphate ester deprotection occurred, down to 47% (**6r**, GAG), in which both phosphate ester and base deprotection occurred, as shown by TLC. We are currently optimizing this step. The final products **7a** to **7t** were obtained as white powders which were, in all cases, at least 85% pure. We found that the phosphoramidites **7** were sufficiently stable that they could be stored dry at −20°C for at least one year without significant decomposition. Importantly for their use in DNA synthesis, all 20 trinucleotide phosphoramidites showed good solubility in acetonitrile, being soluble to at least 0.1 M concentration.

NMR spectra (^{1}H, ^{13}C and ^{31}P) for intermediates and final products were largely as expected, although interpretation was complicated by the increasing number of diastereomers present. The trinucleotide phosphoramidites showed remarkably different behaviours on mass spectrometry compared to the monomer phosphoramidites **3**. In the positive FAB mass spectra of **7n** and **7t** using m-NBA as matrix, the molecular ions could not be detected. Instead, reaction of the protonated species with the matrix alcohol (giving the phosphotriester), and water (giving the phosphonate), as well as oxidation (giving the phosphoramidate) occurred and the corresponding ions were observed. In contrast, the FAB spectra of the phosphoramidites **3a** and **3b** showed the molecular ions and only traces of the decomposition products, suggesting a lower reactivity of the monomer phosphoramidites. However, changing the matrix to o-nitrophenyloctylether yielded the M+H$^+$-ions for all 20

trinucleotides **7**. Apart from an intense signal for the oxidation products, almost no decomposition products could be detected. Alternatively, in the electrospray ionization mass spectra, solutions of **7n** and **7t** in methanol containing sodium iodide gave the corresponding $M+Na^+$-ions. No decomposition products were detected in this case. The absence of ions corresponding to the oxidized phosphoramidites suggested that in the FAB spectra partial oxidation occurred under the influence of the nitroaromatic matrices.

We used each of the 20 trinucleotide phosphoramidites separately in automated DNA synthesis, and found that they coupled equally well under conditions generally used for mononucleotide coupling. In the first experiment, we measured coupling yields (determined by measuring released dimethoxytrityl cation) of 80–85% for trinucleotides at 0.1 M concentration in acetonitrile, using a coupling time of 15 s, conditions under which mononucleotide coupling yields of 99% were observed. The discrepancy between trinucleotide and mononucleotide coupling efficiencies may reflect either intrinsic differences in reactivity, or the effect of hydrolysis of trinucleotides by adventitious water. Hydrolysis clearly plays a role as we consistently observed that coupling yields were improved as the scale on which phosphoramidite solutions were prepared was increased. However we cannot rule out the possibility that the trinucleotides are unable to couple as efficiently as mononucleotides.

By increasing the time allowed for coupling, and performing 'double-couple' cycles (in which the trinucleotide coupling step was performed twice in succession) the yield could be improved. For example, we observed that by allowing 1 min per coupling, in a double-couple cycle, coupling yields of 96–98.5% were achieved for the trinucleotides. As each coupling of a trinucleotide adds three bases to the growing oligonucleotide chain, these figures correspond to stepwise couplings of mononucleotides of 98–99.5%.

To verify that the DNA generated by incorporation of trinucleotide phosphoramidites was as expected, we synthesized the tetranucleotides ACTT, GCTT, ATTT, CTTT, GTTT, TTTT, GAGT, TGGT and ATGT in duplicate, using either exclusively conventional cyanoethyl mononucleoside phosphoramidites under standard conditions, or coupling the corresponding trinucleotide phosphoramidites onto a support bearing T. In each case the deprotected products behaved identically on HPLC (DMT on and DMT off) and gel electrophoresis.

The most important application of the trinucleotide phosphoramidites will be in preparing mixed oligonucleotides for peptide and protein mutagenesis. We therefore sought to ascertain whether coupling of a mixture of trinucleotide phosphoramidites during DNA synthesis would result in the desired mixture of oligonucleotides. Several oligonucleotides were synthesized, incorporating a mixture of trinucleotides corresponding to the eight hydrophobic amino acids alanine, phenylalanine, isoleucine, leucine, methionine, threonine, valine, and tryptophan at several positions (see Figure 2b). The trinucleotide mixture was made equimolar by weighing appropriate amounts of the phosphoramidites. As a result, the relative amounts of the trinucleotides may vary by, at most, 15% as a result of varying levels of impurities in the individual phosphoramidite preparations. In each case the trinucleotide phosphoramidite mixture coupled in very high yield. For example, the 69mer A-1.3 (Figure 2b) which contains the trinucleotide mixture at four sites, was prepared in an overall yield of 27%, corresponding to a stepwise yield of over 98%.

The oligonucleotides listed in Figure 2b were used pairwise as primers in PCR amplification of the light chain gene for the single chain Fv version of the antibody 2H-10 (9), as shown in Figure 2a. The resulting double-stranded fragments were used to form the full-length gene segment by assembly PCR (10), the product was digested with *Eco*RV and *Xho*I, and cloned into the sequencing vector pSL301. Sanger sequencing covering a total of 63 trinucleotide-containing sites confirmed that all trinucleotides had been faithfully incorporated into the primers at the expected sites. As shown in Figure 2c, although all trinucleotides were seen, they were present in differing amounts. As the original mixture of trinucleotide phosphoramidites used in synthesizing the primers was approximately uniform, these differences probably reflect variations in relative coupling rates during synthesis. We are currently investigating this feature of the trinucleotides in detail.

In the early days of DNA synthesis, trinucleotide phosphotriester were prepared in order to reduce the number of steps required in assembling oligonucleotides, and the use of these reagents either singly (12) or in mixtures (13) during DNA synthesis has been reported. However the replacement of phosphotriesters by the advent of superior phosphoramidite chemistry has meant that these reagents have not found general use.

It has been claimed that trinucleotides of the type described here can be used in solid-phase DNA synthesis, although the coupling yields obtained were less than 5% even when extended reaction times were used (4). It was suggested (4) that the poor coupling yields were most probably attributable to the steric bulk of the trinucleotides. We have shown that each of the 20 trinucleotide phosphoramidites can be coupled in very good yields using a standard DNA synthesizer, under the same conditions as are used for mononucleotide coupling. By using slightly longer reaction times, and double couple cycles, excellent yields can be achieved, corresponding to mononucleotide coupling yields in excess of 98%. Our results are consistent with what is seen with dinucleotides (13), and show that steric bulk is not a major factor affecting the coupling of these substances. With the synthesis of the 20 trinucleotide phosphoramidites and the demonstration that they perform well in standard DNA synthesis, the way is now clear for their use in random mutagenesis.

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