Synthesis and biophysical studies of short oligodeoxynucleotides with novel modifications: a possible approach to the problem of mixed base oligodeoxynucleotide synthesis

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ABSTRACT

The syntheses of 1,2-dideoxy-D-ribofuranose and 1,2-dideoxy-1-phenyl-β-D-ribofuranose are described. Oligodeoxynucleotides containing these analogues have been synthesised and hybridized to their complementary strands. Hypochromicity studies have shown that these duplices are less stable than either the totally complementary duplex or those containing A.C and G.T mismatches.

INTRODUCTION

The identification and isolation of genes often involves the use of specific hybridisation probes which, historically, were derived from natural sources such as mRNA, or prepared from cDNA. More recently it has been demonstrated that synthetic oligonucleotides predicted from the amino acid sequence of a protein can be used for the isolation and sequencing of genes from both mRNA and DNA sources. However, owing to redundancies in the genetic code for most amino acids it is often necessary to synthesise a large number of oligonucleotide sequences to represent all the possible combinations of triplet codon usage. These oligonucleotides may either be synthesised separately or, more commonly, as a mixture. To identify a single target sequence in a DNA population a mixed probe must then be used under stringent hybridisation conditions. [1]

During the chemical synthesis of a mixed probe using solid phase methodology there are three major stages where problems can occur; differential rates during the coupling step [2], the purification step and the sequencing step. One solution to the first problem has been demonstrated by Caruthers et al [3] who has employed different triarylmethyl protecting groups for the 5' hydroxyl function of each of the four common monomer units. The major difficulty that occurs during the purification of complex mixed probes arises from the fact that the individual oligonucleotides of the mixture are rarely present in equal proportions and the isolation of minor

components cannot be guaranteed.

Finally, whilst the sequencing of simple mixtures by the Maxam and Gilbert method is possible [4,5] the detailed analysis of complex mixtures is not possible by any method.

Our approach to the solution of these problems is to design an analogue base which can be incorporated into a synthetic oligonucleotide at points of redundancy. This universal base may also give some insight into the important factors involved in DNA helix stability. As part of a continuing study we wish to report the syntheses of 1,2-dideoxy-D-ribofuranose and 1,2-dideoxy-1-phenyl-β-D-ribofuranose and their incorporation into synthetic oligonucleotides using automated solid phase phosphotriester methodology.

In order to investigate the effect of the introduction of base analogues on duplex stability it was decided to use sequences from the chymosin gene as a model system. The chymosin A and B genes differ by only one base, an A to G change at Asp-286 and oligonucleotides of 15 residues (sequences A and B) containing this site were synthesised. The complementary strand (sequences E and F) to each of these had been synthesised previously [6] and in order to test the effect of all base pair combinations it was decided to synthesise sequences C and D. The no-base and phenyl analogues were incorporated into the modified complementary strand at position 5 as shown below and the effects on helix stability were investigated by observing changes in the "melting temperature" (T_m).

5' AACCAGTACGATGAG 3' - Sequence A
5' AACCAGTACGGTGAG 3' - Sequence B
5' AACCAGTACGCTGAG 3' - Sequence C
5' AACCAGTACGTTGAG 3' - Sequence D
3' TTGGTCATGCTACTC 5' - Sequence E (complementary strand to Sequence A)
3' TTGGTCATGCCACTC 5' - Sequence F (complementary strand to Sequence B)
3' TTGGTCATGC*ACTC 5' - Sequence G (complementary strand modified at *)

Extensive physical studies have been reported on oligonucleotides with either non-Watson-Crick mis-matched base pairs, or with extra impaired bases [7-9]. However, these systems have only limited analogy with the base-deleted oligonucleotides discussed here.

RESULTS

The basic synthetic strategies for no-base and phenyl analogues are outlined in Schemes I and II respectively.

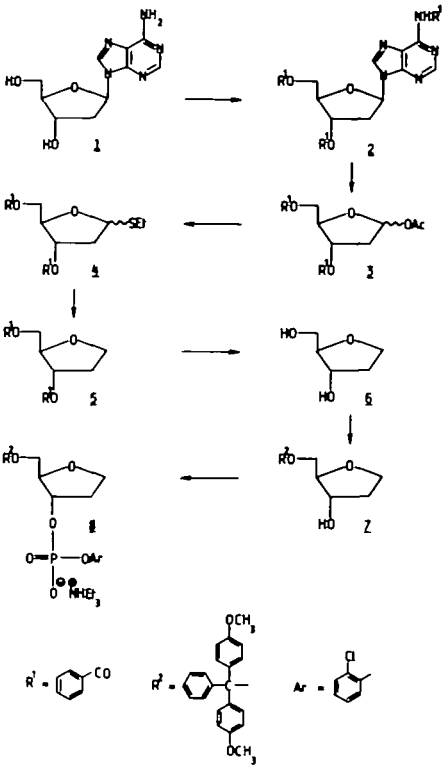

Figure 1 (Scheme I)

Scheme I (Figure 1)

The nucleotide starting material is the readily available 2'-deoxyadenosine monohydrate (1). Acylation and subsequent depurination was carried out as previously described [10] except that a mixture of benzoyl chloride and 4-dimethylaminopyridine was used as the acylating agent. The more polar anomer of the acetal (3) was predominant in the ratio of 3:1 and the overall yield of (3) based on 2'-deoxyadenosine was 72%. Reaction of the acetal (3) with ethanethiol in dry dichloromethane using one equivalent of anhydrous zinc bromide gave an anomeric mixture of the thioacetal (4) in excellent yield (90%). Raney nickel desulphurization of (4) dissolved in ethanol gave none of the expected product (5) using the method of Tomoya et al [11] and only very low yields were obtained when ethyl acetate [12] or acetone [13] were used as solvents. However, when the reaction was carried out in dioxan as solvent and in the presence of powdered calcium

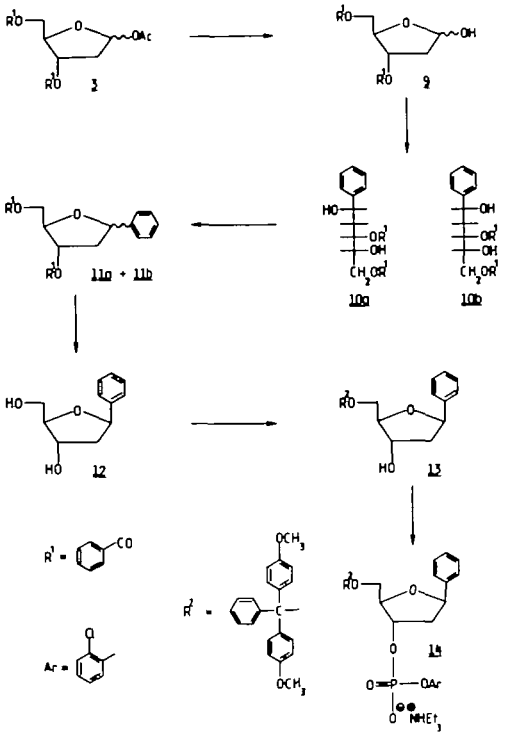

Figure 2 (Scheme II)

hydride the ether (5) was obtained in 64% yield.

Quantitative removal of the benzoyl groups was accomplished using freshly prepared sodium methoxide in dry methanol. Protection of the C_5-hydroxyl group of the ether (6) was achieved using a slight excess of 4,4'-dimethoxytrityl chloride in anhydrous pyridine giving a 90% yield of the 1,2-dideoxy-D-ribofuranose derivative (7). The synthesis of the phosphodiester (8) was completed by phosphorylation of the alcohol (7) with a mixture of 2-chlorophenylphosphorodichloridate and triazole [14] in anhydrous pyridine in 90% yield. The phosphodiester (8) was incorporated into the oligonucleotide sequence $^{5'}$CTCA-CGTACTGGTT$^{3'}$ using automated solid phase phosphotriester methodology [15]. (- = no base analogue).

Scheme II (Figure 2)

The synthetic strategy for the synthesis of the phosphodiester (14) is modelled on the C-nucleoside studies of Buchanan et al [16]. When the hemiacetal (9), prepared by acid hydrolysis of the acetal (3), was treated

with a two fold excess of phenylmagnesium bromide in dry tetrahydrofuran at -23°C, a mixture of the diols (10a) and (10b) was obtained in 64% yield. By conducting the reaction at -23°C, selective attack of the Grignard reagent at C_1 was achieved and debenzoylation minimized.

Ring closure of the diols (10a) and (10b) using a catalytic amount of benzenesulphonic acid in refluxing toluene gave an anomeric mixture of the ethers (11a) and (11b). The pure β-anomer [17] (11b) was obtained in 33% yield after three recrystallizations from diethyl ether. Due to the poor solubility of (11b) in methanolic sodium methoxide at ambient temperature, debenzoylation was carried out at 40°C to give the diol (12) in 98% yield. The synthesis of the phosphodiester (14) was then completed by protection of the C_5-hydroxyl with the 4,4'-dimethoxytrityl group followed by phosphorylation as previously described. The phosphodiester (14) was then incorporated into the oligonucleotide sequence $^{5'}$CTCAΦCGTACTGGTT$^{3'}$ (Φ = phenyl analogue).

Purification and Sequencing

The oligonucleotides were deprotected by published methods [18] and purified using ionexchange chromatography. The purified oligonucleotides were labelled using (γ-^{32}P) ATP and T4 polynucleotide kinase and their sequences determined using the mobility shift method of Jay et al [19]. In the case of the no-base analogue, it was found that under the snake venom phosphodiesterase conditions employed, the digestion stopped at the point of incorporation of the no-base analogue. The same was generally true for the aromatic analogue although complete digestion could be seen faintly with the hydrolysis of the phenyl analogue giving a "jump" similar to that observed on losing 5'-thymidine monophosphate.

Melting Curves

The melting curves are plotted in Figures 3 to 6 and the derived "melting temperatures" are shown in Tables I and II. The duplex formed by sequence A and its complementary strand has a T_m of 42.5°C compared with a T_m of 45.0°C for the duplex containing sequence B and its complementary strand. The duplex formed by sequence A and the complementary strand to sequence B (i.e. an A.C mismatch) and that formed by sequence B and the complementary strand to sequence A (i.e. a G.T mismatch) each show a reduction in T_m of 6.5°C. However, the duplices formed by sequences A, B, C and D with a complementary strand containing either a no-base or a phenyl analogue at position 5 all show considerable destabilization with reductions in T_m >16°C. The difference between the "melting temperature" of

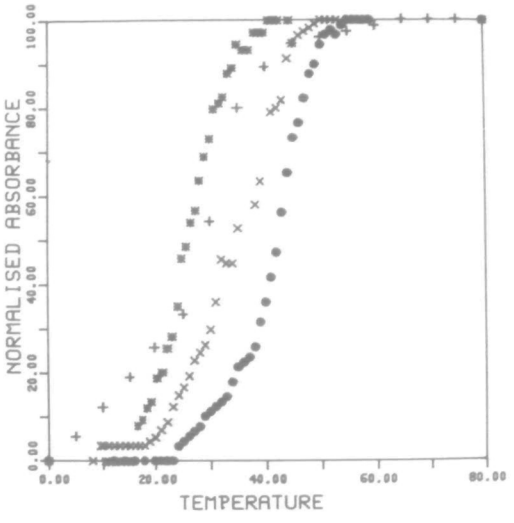

Figure 3. Melting curves for sequence A
● Native; x A-C mismatch; * no-base analogue; + Phe analogue

the duplices in which the complementary strand contains a no-base or a phenyl analogue is within experimental error and is therefore not significant except in the case where the duplex containing the A.Phe mismatch has a

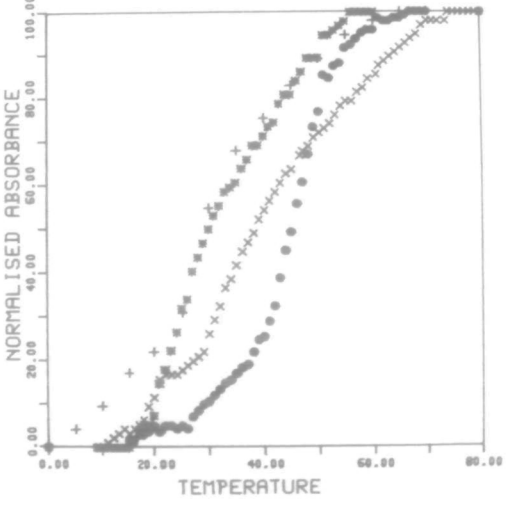

Figure 4. Melting curves for sequence B
● Native; x G-T mismatch; * no-base analogue; + Phe analogue

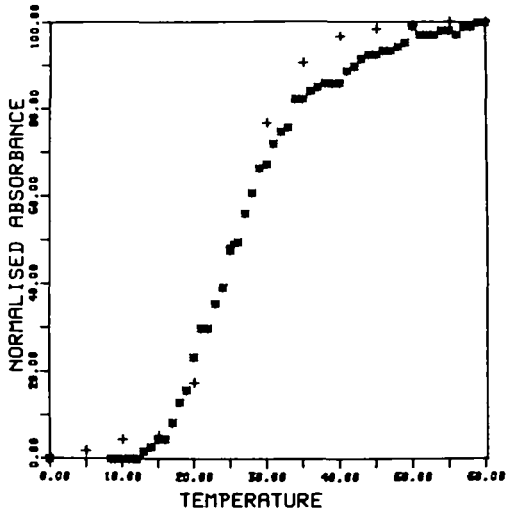

Figure 5. Melting curves for sequence C
* no-base analogue; + Phe analogue.

T_m of 32.0°C, whereas that containing the A.no-base mismatch has a T_m of 26.0°C. None of the melting curves appear to be monphasic but each series of curves is sigmoidal with the exception of duplices containing sequence B.

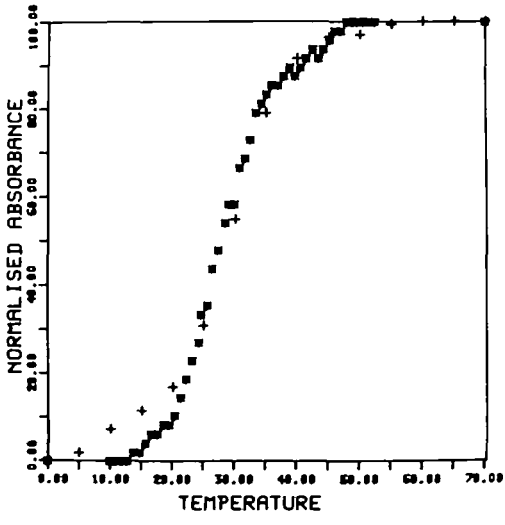

Figure 6. Melting curves for sequence D
* no-base analogue; + Phe analogue

Table I: T_m arranged according to modification type

Sample Composition	T_m °C
Sequence A + Sequence E (A.T)	42.5
Sequence B + Sequence F (G.C)	45.0
Sequence A + Sequence F (A.C)	36.0
Sequence B + Sequence E (G.T)	38.5
Sequence A + Sequence G (A.no-base)	26.0
Sequence B + Sequence G (G.no-base)	30.0
Sequence C + Sequence G (C.no-base)	26.0
Sequence D + Sequence G (T.no-base)	28.0
Sequence A + Sequence G (A.Phe)	32.0
Sequence B + Sequence G (G.Phe)	29.0
Sequence C + Sequence G (C.Phe)	25.5
Sequence D + Sequence G (T.Phe)	29.0

Only the native duplex containing sequence B has a sigmoidal curve and all other duplices in this series deviate from this form indicating some change in co-operativity.

DISCUSSION

It is known that the hypochromicity of the U.V. absorption spectra of nucleic acids is due to base stacking effects. The increase in optical density at 260nm on heating DNA results from the disruption of base stacking in the transition from double helix to random coil. Thus the T_m of the

Table II: T_m arranged according to sequence type

Sample Composition	T_m °C
Sequence A + Sequence E (A.T)	42.5
Sequence A + Sequence F (A.C)	36.0
Sequence A + Sequence G (A.no-base)	26.0
Sequence A + Sequence G (A.Phe)	32.0
Sequence B + Sequence F (G.C)	45.0
Sequence B + Sequence E (G.T)	38.5
Sequence B + Sequence G (G.no-base)	30.0
Sequence B + Sequence G (G.Phe)	29.0
Sequence C + Sequence G (C.no-base)	26.0
Sequence C + Sequence G (C.Phe)	25.5
Sequence D + Sequence G (T.no-base)	28.0
Sequence D + Sequence G (T.Phe)	29.0

Table III: Calculated energies for native trinucleotides and no-base analogues

DNA Composition	Sequence	E Kcal mole^{-1}	ΔE Kcal mole^{-1}
GGT.ACC	B	-150.8	7.5
GGT.A*C		-143.3	
GCT.AGC	C	-150.6	27.1
GCT.A*C		-133.5	
GAT.ATC	A	-161.2	20.1
GAT.A*C		-141.1	
GTT.AAC	D	-160.8	26.6
GTT.A*C		-134.2	

duplex is not merely a measure of the thermal stability of the molecule but its inherent stability in terms of base stacking interactions.

The replacement of a complementary base by a no-base analogue in the chymosin sequences leads to a considerable destabilisation of the duplices, as shown by a reduction in T_m of approximately 40%. This modification leads to a change in both base stacking and base pairing energies but the destabilisation of these duplices is much greater than one might have expected from the calculated ΔE of 20.1 Kcal/mol for sequence A and 7.5 Kcal/mol for sequence B. The calculated energies (Table III) are only providing information on the destabilisations caused by loss of stacking in the no-base analogues, and are based on a model that preserves B-DNA conformation and stacking. Differences in ΔE values reflect differences in nearest neighbour stacking stabilities [20]. The fact that ΔT_m values for duplices containing sequence A and B are very similar, wherease their ΔE values are not, suggests that this model is not fully adequate. Further theoretical studies are in progress that involve systematic relaxation of a B-DNA model. This may in part be due to the position of the modification, which is towards one end of the duplex and so may combine in its action with the instabilities of end effects.

The replacement of a complementary base by a non-polar aromatic ring in the form of a phenyl group is as destabilising as having no base present. This indicates that the phenyl derivative makes little or no contribution to base stacking and that hydrophobicity alone is not a sufficient prerequisite for base stacking. This is in agreement with the findings of Bugg et al [21] that, in the solid state, bases which have polar substituents stack whereas purely aromatic molecules such as benzene and anthracene do not.

The substitution of a complementary base by a mismatch is intermediate in effect. In this case, although stable Watson-Crick type base pairs are unable to form, the mismatched pyrimidines are still able to contribute to the free energy of the molecule in terms of base stacking.

From these experiments it is apparent that the stability of a duplex is dependent upon the base-stacking contribution of its composite bases. The substitution of an aromatic ring for a base is not sufficient to maintain stability if it has no dipole moment indicating that dipole-induced dipole interactions are of prime importance in maintaining stability by base-stacking. An effective gene probe has to be capable of forming a stable duplex. Therefore in the design of any universal base the effects of polar substituents on base-stacking as well as base-pairing must be taken into consideration.

EXPERIMENTAL

General Methods

^{1}H-nuclear magnetic resonance (N.M.R.) spectra were obtained using Varian HA-100 (Reading University) and Perkin Elmer R34 (P.C.M.U., Harwell) spectrometers. ^{13}C-N.M.R. spectra were obtained using Bruker WH-180 (P.C.M.U., Harwell) and Jeol FX-90 (C.L.P. N.M.R. Service) spectrometers. In all cases, chemical shifts are reported in ppm downfield of an internal standard of tetramethylsilane. ^{31}P-N.M.R. spectra were obtained using a Jeol FX-60 (Leicester University) spectrometer and chemical shifts are reported in ppm relative to an external standard of phosphoric acid. Mass spectral data were supplied by M-Scan Ltd., Ascot. Elemental analysis was carried out by Butterworths Laboratories, Middlesex. Melting points (uncorrected) were determined on a Baird and Tatlock electrothermal melting point apparatus. Ultraviolet (U.V.) spectra were obtained using a Beckman DU-8 spectrophotometer. Analytical thin layer chromatography (t.l.c.) was carried out on Merck plates pre-coated with silica gel $60F_{254}$. Visualization was by absorption of ultraviolet light or alkaline permanganate staining followed by heat. Synthetic oligonucleotides were prepared on Celltech's automated synthesiser using modified phosphotriester chemistry. High pressure liquid chromatography (h.p.l.c.) was carried out on a Varian Vista 5040 liquid chromatograph fitted with a U.V. 100 detector. All solvents and reagents were purified in accordance with general laboratory procedures. The exception was tetrahydrofuran which was purified by standing over calcium hydride for at least 2 days, then heated under reflux

over lithium aluminium hydride for a period greater than 4 hours and finally distilled under nitrogen and used immediately.

Melting Curves

Samples consisting of approximately 1 O.D. unit of single-stranded oligonucleotide were dissolved in 1.0 ml of 0.01M SHE buffer, (9.5mM NaCl, 2mM HEPES, 20μm EDTA pH=7.0). Equal volumes of parent and complementary strand, native or modified, were then mixed and annealed at 50°C to give approximately 1 O.D. unit of duplex in each case.

Profiles of absorbance vs. temperature were measured at 260nm on a Cary-14 spectrophotometer. The temperature was controlled using circulating water from a Haake bath, the rate of temperature increase being approximately 2°C/min.

For the oligonucleotide containing the phenyl analogue, melting curves were measured using a Gilford 250 spectrophotometer with a temperature increase of 1°C/min.

Molecular graphics and semi-empirical energy calculations were performed on an interactive raster graphics system interfaced to a PDP11/34 computer, using computer programs written by S.A. Islam at King's College. The relative energy, (E) and hence stability of a particular structural model was approximated by the non-bonded interaction energy

$$\frac{A}{r^6} - \frac{B}{r^{12}}$$

with parameters A and B as used previously [22]. Models with a base deleted, or changed in one instance to a benzene ring, were not altered from standard B-DNA nucleotide backbone conformations, since no structural information is available on such modifications. Thus, the calculations of E estimated only the changes in stabilisation due to different base-base stacking interactions.

The representative models used in these calculations were of double-stranded trinucleotides with Watson-Crick base pairs and exact B-DNA helical geometry, as found from fibre diffraction studies [23]. Changes were made in the central residue of this model, and it was assumed that their effects on stacking properties would not be propagated beyond the flanking bases.

Synthesis

6-Benzamido-9-(3',5'-di-O-benzoyl-2'-deoxy-β-D-ribofuranosyl) purine (2)

2'-Deoxyadenosine monohydrate 1 (27.17g, 100mmol) was dried by coevaporation with anhydrous pyridine (3x50ml) and suspended in anhydrous pyridine (500ml). The suspension was cooled in an ice bath, 4-dimethylamino-

pyridine (4.02g, 33mmol) and benzoyl chloride (208.7g, 450mmol) were added and the solution allowed to warm up to room temperature. After stirring overnight, the orange coloured solution was poured into ice cold sodium bicarbonate (1M, 1l) and extracted with dichlormethane (4x400ml). The separate organic extracts were sequentially washed with sodium bicarbonate (1M, 500ml), combined and dried (Na_2SO_4). Evaporation of the solvent *in vacuo* gave a yellow oil. Coevaporation with toluene gave 2 (68g) as a buff solid.

t.l.c. : Rf = 0.86 in dichloromethane/methanol (9:1).

1-O-Acetyl-3,5-di-O-benzoyl-2-deoxy-D-ribofuranose (3)

Acetic anhydride (23ml, 250mmol) was added to crude compound 2 (68g) suspended in glacial acetic acid (200ml) and the mixture heated to 100°C (internal temperature) for 2 hours. The reaction mixture was cooled in an ice bath, dichloromethane (200ml) added and the solution concentrated *in vacuo*. The resulting brown solid was resuspended in dichloromethane (500ml), washed with ice cold sulphuric acid (1M, 300ml), saturated sodium bicarbonate (500ml), ice cold water (300ml) and dried (Na_2SO_4). Evaporation of the solvent *in vacuo* gave a brown oil. The product was purified by column chromatography on silica gel (Merck 9385, 1kg). Elution with dichloromethane/hexane (1:1) and evaporation of the solvent *in vacuo* gave an anomeric mixture of 3 (28.7g, 75% based on 1) as a pale yellow oil. The anomeric mixture was separated by crystallization from ethanol to give a white solid (7.2g, mp=87.5-89°C) of the less polar anomer and a colourless oil (20g) of the more polar anomer, t.l.c.: Rf=0.45 in dichloromethane/acetone (97:3). ^{1}H-NMR ($CDCl_3$) of less polar anomer, δ 8.00-8.30 (4H,m) 7.40-7.70 (6H,m), 6.54-6.64 (1H,m), 5.68-5.84 (1H,m), 4.50-4.80 (3H,m), 2.48-2.80 (2H,m), 1.93 (3H,s); ^{1}H-NMR ($CDCl_3$) of more polar anomer, δ 8.02-8.20 (4H,m), 7.42-7.70 (6H,m), 6.57 (1H,d,J=5Hz), 5.58-5.66 (1H,m), 4.75-4.82 (1H,m), 4.60 (2H,d,J=4.5Hz), 2.60-2.76 (2H,m), 2.09 (3H,s); ^{13}C-NMR ($CDCl_3$) of less polar anomer, δ 170.04, 166.09 (C=O), 128.41-133.51 (aromatics), 98.25 (C_1), 82.91 (C_3), 74.58 (C_4), 64.43 (C_5), 38.48 (C_2), 21.13 (CH_3); ^{13}C-NMR ($CDCl_3$) of more polar anomer, δ 170.17, 166.10, 166.01 (C=O), 128.48-133.41 (aromatics), 98.56 (C_1), 83.77 (C_3), 74.50 (C_4), 64.24 (C_5), 38.66 (C_2), 21.30 (CH_3); mass spectrum m/z 384 (M^+, 3.5%), 341 (M^+-CH_3CO, 35), 325 ($M^+-CH_3CO_2$, 86), 262 ($M^+-C_6H_5CO_2H$,35), 249 ($M^+-C_6H_5CO_2CH$,100), 202 ($M^+-C_6H_5CO_2H-CH_3CO_2H$), 100). Anal. Calcd. for $C_{21}H_{20}O_7$ (384.39): C, 65.62; H, 5.24. Found: C, 65.85; H, 5.27.

Ethyl-3,5-di-O-benzoyl-1,2-dideoxy-1-mercapto-D-ribofuranose (4)

To the acetal 3 (5.6g, 14.5 mmol) dissolved in dry dichloromethane (20ml) was added ethanethiol (1.08g, 17.4 mmol) and the solution stirred under an atmosphere of dry nitrogen gas at room temperature. After 5 minutes anhydrous zinc bromide (3.26g, 14.5 mmol) was added and the reaction mixture stirred at room temperature for a further 30 minutes. The orange coloured solution was poured into ice cold sodium hydroxide (5% w/v, 250ml) and extracted with dichloromethane (3x100ml). The separate organic extracts were washed with ice cold water (100ml), combined and dried (Na_2SO_4). Evaporation of the solvent in vacuo gave a colourless oil which was purified by column chromatography on silica gel (Merck 9385, 200g). Elution with dichloromethane/hexane (9:1) and evaporation of the solvent in vacuo gave an anomeric mixture of 4 (5.0g, 89%) as a colourless oil.
t.l.c. : Rf = 0.6 in dichloromethane/acetone (97:3). ^{1}H-NMR ($CDCl_3$), δ 8.10-8.25 (4H,m), 7.40-7.65 (6H,m), 5.0-5.75 (2H,m), 4.50-4.75 (3H,m), 2.20-3.00 (4H,m), 1.28-1.40 (3H,m); ^{13}C-NMR ($CDCl_3$), δ 166.25 (C=O), 128.42-133.35 (aromatics), 84.62 and 83.77 (C_1), 82.98 and 80.61 (C_3), 74.83 (C_4), 64.70 and 64.18 (C_5) 39.58 (-S$\underline{C}H_2CH_3$), 38.99 (C_2), 15.12 (CH_3); mass spectrum m/z 325 (M^+-SCH_2CH_3, 30%), 105 (100), 77 (100). Anal. Calcd. for $C_{21}H_{22}O_5S$ (386.47): C, 65.27; H, 5.74; S, 8.29. Found: C, 66.15; H, 5.84; S, 7.95.

3,5-Di-O-benzoyl-1,2-dideoxy-D-ribofuranose (5)

Raney nickel (10g wet) was washed with dry dioxan (10x15ml) and suspended in dry dioxan (10ml). The suspension was refluxed for 1.5 hours under nitrogen and in the presence of powdered calcium hydride. The thioacetal 4 (1.0g, 2.59mmol) was dissolved in dry dioxan (5ml), added to the Raney nickel suspension and the reaction continued under reflux for a further 10 minutes. The reaction mixture was cooled, filtered through Hyflo supercel and the catalyst washed with dioxan (4x20ml). The combined filtrate was concentrated in vacuo to give a clear oil which was purified by column chromatography on silica gel (Merck 9385, 50g). Elution with dichloromethane/hexane (9:1) and evaporation of the solvent in vacuo gave 5 (0.537g, 64%) as a colourless oil.
t.l.c. : Rf=0.52 in dichloromethane/acetone (97:3). ^{1}H-NMR ($CDCl_3$) δ 8.10-8.20 (4H,m), 7.45-7.70 (6H,m), 5.52-5.60 (1H,m), 4.57 (2H,d,J=5.7Hz), 4.40-4.47 (1H,m), 4.02-4.28 (2H,m), 2.33-2.50 (1H,m), 2.16-2.30 (1H,m); ^{13}C-NMR ($CDCl_3$) δ 166.22 (C=O), 128.42-133.28 (aromatics), 82.12 (C_3), 76.73 (C_4), 67.73 (C_1), 64.70 (C_5), 32.88 (C_2); mass spectrum m/z 325

(M^+-1, 18%), 191 (M^+-$C_6H_5CO_2CH_2$, 37), 105 (100), 77 (100). Anal. Found: M^+, 326.1149. $C_{19}H_{18}O_5$ requires M, 326.1152.

1,2-Dideoxy-D-ribofuranose (6)

A solution of freshly prepared sodium methoxide in dry methanol (0.4M, 10ml) was added to the ether 5 (0.537g, 1.64 mmol) dissolved in dry methanol (10ml) and the solution stirred at room temperature for 20 minutes. The reaction mixture was neutralised by the addition of Dowex-50 (pyr^+ form) ion exchange resin. The resin was filtered, washed with methanol (4x20ml) and the combined filtrate concentrated in vacuo to give a colourless oil. The product was purified by column chromatography on silica gel (Merck 9385, 20g). Elution with dichloromethane/methanol (95:5) and evaporation of the solvent in vacuo gave 6 (0.184g,95%) as a colourless oil.
t.l.c. : Rf = 0.15 in dichloromethane/methanol (9:1).
^{1}H-NMR (C_5D_5N), δ 5.06 (2H, b.s.), 4.78-4.88 (1H,m), 4.40-4.50 (1H,m), 3.86-4.28 (4H,m), 2.05-2.30 (2H,m); ^{13}C-NMR (C_5D_5N), δ 88.53 (C_3), 73.22 (C_4), 67.40 (C_1), 63.66 (C_5), 36.09 (C_2); mass spectrum m/z 100 (M^+-H_2O,10%), 87 (M^+-CH_2OH, 100), 69 (37).

1,2-Dideoxy-5-O-(4,4'-dimethoxytrityl)-D-ribofurnaose (7)

The diol 6 (0.184g, 1.56 mmol) was coevaporated with anhydrous pyridine (3x10ml) and dissolved in anhydrous pyridine (10ml). 4,4'-Dimethoxytrityl chloride (0.63g, 1.87 mmol) was added and the mixture stirred at room temperature for 4 hours. The reaction mixture was poured into saturated sodium bicarbonate (100ml) and extracted with dichloromethane (4x75ml). The separate organic extracts were washed with sodium bicarbonate (100ml), combined, washed with ice cold water (100ml) and dried (Na_2SO_4). Evaporation of the solvent in vacuo gave a pale yellow foam which was purified by column chromatography on silica gel (Merck 9385, 10g). Elution with dichloromethane/hexane (8:2) and evaporation of the solvent in vacuo gave 7 (0.589g, 90%) as a white foam.
t.l.c. : Rf = 0.5 in dichloromethane/methanol (9:1).
^{1}H-NMR ($CDCl_3$) δ 7.20-7.55 (9H,m), 6.84-6.90 (4H,m), 4.25-4.36 (1H,m), 3.90-4.04 (3H,m), 3.76 (6H,s), 3.24 (1H,dd,J=9.7 and 5.0Hz), 3.12 (1H,dd,J=9.7 and 5.0Hz), 2.42 (1H,b.s.), 2.05-2.20 (1H,m), 1.80-1.94 (1H,m); ^{13}C-NMR ($CDCl_3$) δ 126.77-158.47(aromatics), 113.16 ($\underline{C}$-Ar_2Ph), 84.95 (C_3), 74.56 (C_4), 67.07 (C_1), 64.57 (C_5), 55.23 (O-$\underline{C}H_3$), 34.58 (C_2); mass spectrum m/z 420 (M^+, 45%), 303 (100). Anal. Found: M^+, 420.1952. $C_{26}H_{28}O_5$ requires M, 420.1934.

1,2-Dideoxy-5-O-(4,4'-dimethoxytrityl)-3-(2-chlorophenyl) - phosphate-D-ribofuranose, triethylammonium salt (8)

1,2,4-triazole (0.77g, 11.2 mmol) was coevaporated with anhydrous pyridine (3x10ml) and dissolved in anhydrous pyridine (15ml). The solution was cooled on an ice bath and stirred under an atmosphere of dry nitrogen gas. 2-chlorophenylphosphorodichloridate (0.631g, 2.56 mmol) was added dropwise and the suspension stirred for 20 minutes. A solution of the alcohol 7 (0.589g, 1.40 mmol) dissolved in anhydrous pyridine (10ml) was added dropwise and the pale yellow solution stirred for 20 minutes. Following the addition of triethylamine (2ml) and water (1.2ml) the emulsion was stirred at room temperature for a further 30 minutes. The reaction mixture was poured into saturated sodium bicarbonate (100ml) and extracted with dichloromethane (6x40ml). The separate organic extracts were sequentially washed with saturated sodium bicarbonate (100ml), combined and dried (Na_2SO_4). Evaporation of the solvent in vacuo gave a pale yellow oil. The product was purified by column chromatography on silica gel (Merck 9385, 60g). Elution with dichloromethane/methanol/triethylamine (94:5:1) and evaporation of the solvent in vacuo gave 8 (0.92g, 90%) as a white foam.
t.l.c. : Rf = 0.25 in dichloromethane/methanol (9:1);
^{1}H-NMR ($CDCl_3$) δ 12.1 (1H,b.s.), 6.80-7.70 (17H,m), 4.93-5.02 (1H,m), 4.24-4.32 (1H,m), 3.96-4.16 (2H,m), 3.80 (6H,s), 3.20 (1H,dd,J=8.8 and 5Hz), 3.10 (1H,dd,J=8.8 and 5.0Hz), 2.96 (6H,q,J=6.6Hz), 2.16-2.30 (2H,m), 1.23 (9H,t,J=8.8Hz); ^{13}C-NMR ($CDCl_3$) δ 121.50-158.66 (aromatics), 113.16 (C-(Ar)$_2$ Ph), 85.15 (C_3), 81.34 (C_4), 66.87 (C_1), 63.19 (C_5), 55.23 (OCH_3), 45.76 ($-CH_2CH_3$), 33.94 (C_2), 8.57 ($-CH_2CH_3$); ^{31}P-NMR (CH_2Cl_2) δ -6.23. mass spectrum m/z 609 ($(M-H)^-$, 50%), 309 (25), 307 (100). Anal. Found: $(M-H)^-$, 609.1439, $C_{32}H_{31}O_8P^{35}Cl$ requires M, 609.1445. Found: $(M-H)^-$ 611.1418. $C_{32}H_{31}O_8P^{37}Cl$ requires 611.1416.

3,5-Di-O-benzoyl-2-deoxy-D-ribofuranose (9)

To the acetal 3 (10g, 26.04 mmol) dissolved in dioxan (100ml) was added hydrochloric acid (1M in dioxan, 40ml) and the solution stirred at room temperature for 4hr. The reaction mixture was poured into saturated sodium bicarbonate (300ml) and extract with dichloromethane (3x150ml). The separate organic extracts were washed with saturated sodium chloride (200ml), combined and dried (Na_2SO_4). Evaporation of the solvent in vacuo gave a clear oil which was purified by column chromatography on silica gel (Merck 9385, 300g). Elution with dichloromethane to dichloromethane/acetone (97:3) and evaporation of the solvent in vacuo gave an anomeric mixture of 9

(7.5g, 86%) as a colourless oil.

t.l.c. : Rf = 0.22 in dichloromethane/acetone (97:3);
^{1}H-NMR ($CDCl_3$)δ 7.90-8.2 (4H,m), 7.20-7.70 (6H,m), 5.40-5.85 (2H,m), 4.35-4.80 (3H,m), 3.70 (1H,b.s.), 2.15-2.70 (2H,m); ^{13}C-NMR ($CDCl_3$)δ 166.3, 166.1 (C=O), 120.4-133.4 (aromatics), 99.3 and 98.9 (C1), 81.9 and 81.6 (C3), 75.7 and 75.3 (C4), 65.6 and 64.5 (C5), 39.9 and 39.7 (C2); mass spectrum m/z 325 (M^+-H_2O, 5%), 220 ($M^+-C_6H_5CO_2H$, 15), 202 ($M^+-C_6H_5CO_2H-H_2O$, 25), 122 (15), 105 (100), 77 (30). Anal. Found: (M^+-H_2O), 325.1076, $C_{19}H_{17}O_5$ requires M, 325.1076.

3,5-Di-O-benzoyl-2-deoxy -1-phenyl-D-altro and D-allo-ribitol (10a and 10b)

To the hemiacetal 9 (7.5g, 21.93 mmol) dissolved in dry tetrahydrofuran (100ml) at -23°C (CCl_4/CO_2) under an atmosphere of dry nitrogen was added phenylmagnesium bromide (3M in diethyl ether, 15ml, 43.86 mmol) in portions over a 12 minutes period. After a total time of 40 minutes, the reaction mixture was poured into ice cold ammonium chloride (10% w/v, 500ml) and extracted with dichloromethane (4x400ml). The separate organic extracts were washed with saturated sodium chloride (500ml), combined and dried (Na_2SO_4). Evaporation of the solvent in vacuo gave a yellow oil which was purified by column chromatography on silica gel (Merck 9385, 300g). Elution with dichloromethane to dichloromethane/acetone (97:3) and evaporation of the solvent in vacuo gave a mixture of 10a + 10b (5.77g, 63%) as an oily foam.

t.l.c. : Rf = 0.21 and 0.18 in hexane/ethyl acetate (6:4);
^{1}H-NMR ($CDCl_3$)δ 7.80 - 8.10 (4H,m), 7.00 - 7.60 (11H,m), 5.20 - 5.70 (1H,m), 4.60 - 5.10 (1H,m), 4.00 - 4.55 (3H,m), 3.80 (2H,b.s.), 2.00 - 2.60 (2H,m); ^{13}C-NMR ($CDCl_3$)δ 166.8, 166.0 (C=O), 129.7 - 143.7 (aromatics), 73.0 and 72.8 (C3), 71.7 and 70.8 (C1), 70.6 and 70.3 (C4), 65.7 and 65.6 (C5), 41.1 and 39.4 (C2); mass spectrum m/z 421 ($M + H^+$, 2%), 403 ($M + H^+$) - H_2O, 43), 299 (($M+H^+$) - $C_6H_5CO_2H$, 2) 281 (($M+H^+$) - $C_6H_5CO_2H$ - H_2O, 24), 159 (($M+H^+$) - $2C_6H_5CO_2H$ - H_2O, 43), 105 (100). Anal. Found: ($M+H^+$) - H_2O, 403.1542. $C_{25}H_{23}O_5$ requires M, 403.1545.

3,5-Di-O-benzoyl-1,2-dideoxy-1-phenyl-α (and β)-D-ribofuranose (11a and 11b)

Benzenesulphonic acid (100mg, 0.56 mmol) was added to dry toluene (60ml) and the solution refluxed through molecular sieves (3A, 100g) for 1.5 hours. A mixture of the diols 10a + 10b (5.0g, 11.9 mmol) dissolved in dry toluene (50ml) was added dropwise over 1 hour. After a further 20 minutes, the mixture was cooled, poured into ammonium acetate (10% w/v, 200ml) and extracted with dichloromethane (4x100ml). The separate organic extracts

were washed with saturated sodium chloride (200ml), combined and dried (Na_2SO_4). Evaporation of the solvent in vacuo gave a brown solid. Recrystallization (3 times) from diethyl ether gave the pure β-anomer 11b (1.6g, 33%) as white needles (m.p. = 124-126°C).
t.l.c. : Rf = 0.28 in hexane/diethyl ether (7:3);
^{1}H-NMR ($CDCl_3$)δ 7.90 - 8.20 (4H,m), 7.15 - 7.70 (11H,m), 5.5 - 5.7 (1H,m), 5.51 - 5.30 (1H,dd,J=9.0 and 5Hz), 4.50 - 4.8 (3H,m), 2.05 - 2.70 (2H,m); ^{13}C-NMR ($CDCl_3$)δ 125.8 - 140.6 (aromatics), 82.8 (C3), 80.8 (C1), 77.4 (C4), 64.9 (C5), 41.6 (C2). Anal. Calcd. for $C_{25}H_{22}O_5$ (402.45): C, 74.61; H, 5.51. Found: C, 74.54; H, 5.61.

1,2-Dideoxy-1-phenyl-β-D-ribofuranose (12)

To the β-anomer 11b (1.5g, 3.73 mmol) in dry methanol (60ml) was added sodium methoxide (0.4M, 40ml) and the mixture warmed in a water bath (40°C). After 20 minutes the solution was neutralized by the addition of Dowex-50 (pyr^+ form) ion exchange resin. The resin was filtered, washed with methanol (6x20ml) and the combined filtrate concentrated in vacuo to give an off white solid. The product was purified by column chromatography on silica gel (Merck 9385, 30g). Elution with dichloromethane/methanol (95:5) to dichloromethane/methanol (9:1) and evaporation of the solvent in vacuo gave 12 (0.71g, 98%) as a white solid. An analytical sample of 12 was obtained by recrystallization from diethyl ether (m.p. = 93-94°C).
t.l.c. : Rf = 0.3 in dichloromethane/methanol (9:1);
^{1}H-NMR ($CDCl_3/CD_3OD$)δ7.15 - 7.45 (5H,m), 5.05 - 5.27 (1H, dd, J = 9.0 and 6Hz), 4.26 - 4.45 (1H,m), 4.20 (2H, b.s.), 3.90 - 4.10 (1H,m), 3.60 - 3.80 (2H,m), 1.80 - 2.38 (2H,m). ^{13}C-NMR($CDCl_3$)δ126.0 - 141.0 (aromatics), 87.2 (C3), 80.1 (C1), 73.67 (C4), 63.4 (C5), 43.8 (C2). Anal. Calcd. for $C_{11}H_{14}O_3$ (194.23): C, 68.02; H, 7.26. Found: C, 68.18; H, 7.13.

1,2-Dideoxy-5-O-(4,4'-dimethoxytrityl)-1-phenyl-β-D-ribofuranose (13)

The diol 12 (0.58g, 2.99 mmol) was coevaporated with dry pyridine (4x10ml) and dissolved in dry pyridine (25ml). 4,4'-Dimethoxytrityl chloride (1.2g, 3.587 mmol) was added and the brown solution stirred for 3 hours at room temperature. The reaction mixture was poured into saturated sodium bicarbonate (200ml) and extracted with dichloromethane (4x100ml). The separate organic extracts were washed with saturated sodium chloride (200ml), combined and dried (Na_2SO_4). Evaporation of the solvent in vacuo gave a brown foam which was purified by column chromatography on silica gel (Merck 9385, 60g). Elution with dichloromethane/methanol (98:2)

and evaporation of the solvent in vacuo gave 13 (1.4g, 95%) as a yellow foam.

t.l.c. : Rf = 0.48 in dichloromethane/methanol (95:5);
^{1}H-NMR ($CDCl_3$)δ 7.10 - 7.60 (14H,m), 6.70 - 6.90 (4H, d, J=8Hz), 5.05 - 5.26 (1H, dd, J=5Hz), 4.30 - 4.50 (1H,m), 3.98 - 4.14 (1H,m), 3.75 (6H,s), 3.15 - 3.45 (2H,m), 1.85 - 2.40 (3H,m); ^{13}C-NMR ($CDCl_3$)δ 125.9 - 158.4 (aromatics) 113.1 (C-AR_2Ph), 86.3 (C3), 79.9 (C1), 74.6 (C4), 64.5 (C5), 55.1 (-OCH_3) 43.8 (C2); mass spectrum m/z 496 (M^+, 10%), 303 (100).

1,2-Dideoxy-5-O-(4,4'-dimethoxytrityl)-3-(2-chlorophenyl)-phosphate-1-phenyl-β-D-ribofuranose, triethylammonium salt (14)

1,2,4-triazole (1.18g, 17.096 mmol) was coevaporated with pyridine (4x10ml) and dissolved in pyridine (20ml). The solution was cooled in an ice bath and stirred under an atmosphere of nitrogen gas. 2-chlorophenyl phosphorodichloridate (0.963g, 0.65ml, 3.925 mmol) was added dropwise to give a cloudy suspension which was stirred for a further 10 minutes. The alcohol 13 (1.06g, 2.137 mmol) dissolved in anhydrous pyridine (10ml) was added dropwise and the yellow solution stirred at room temperature for a further 30 minutes after which triethylamine (3.3ml) and water (2ml) were added. After 20 minutes, the reaction mixture was poured into saturated sodium bicarbonate (100ml) and extracted with dichloromethane (6x70ml). The separate organic extracts were washed with sodium bicarbonate (100ml), combined and dried (Na_2SO_4). Evaporation of the solvent in vacuo gave a pale yellow foam. The product was purified using column chromatography on silica gel (Merck 9385, 60g). Elution with dichloromethane/methanol/triethylamine (94:5:1) and evaporation of the solvent in vacuo gave 14 (1.6g, 95%) as a white foam.

t.l.c. : Rf = 0.3 in dichloromethane/methanol (9:1);
H^1-NMR ($CDCl_3$)δ 12.00 (1H, b.s.), 7.00 - 7.80 (16H,m), 6.75 - 7.02 (6H,m), 5.10 - 5.25 (1H, dd, 4.8Hz), 4.98 - 5.10 (1H,m), 4.32 - 4.45 (1H,m), 3.80 (6H, s), 3.14 - 3.40 (2H,m), 2.98 (6H,q, J = 9.0 and 6.6Hz), 2.50 - 2.62 (1H,m), 1.92 - 2.12 (1H,m), 1.22 (9H,t, J = 8.8Hz); ^{13}C-NMR ($CDCl_3$)δ 121.5 - 158.3 (aromatics), 112.9 (C-Ar_2Ph) 85.4 and 85.2 (C3), 80.25 (C1), 79.3 and 79.0 (C4), 64.8 (C5), 55.1 (-OCH_3), 45.53 (-NCH_2CH_3), 42.7 (C2), 8.47 (NCH_2CH_3); ^{31}P-NMR (CH_2Cl_2)δ - 7.86; mass spectrum m/z 383. $(M-H)^-$ - $C_{21}H_{19}O_2$). Anal. Found: $(M-H)^-$ $C_{21}H_{19}O_2$, 383.0451. $C_{17}H_{17}O_6P^{35}Cl$ requires 383.0452.

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