A Synthesis of Pseudouridine and of 5-β-D-Ribofuranosyluridine

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5-Bromo-2,4-di-t-butoxypyrimidine is converted into the pyrimidin-5-yl-lithium and coupled to 2,4:3,5-di-*O*-benzylideneribose. Mild acid treatment of the intermediate protected 1-pyrimidin-5-ylpentitol affords 5-β-D-ribofuranosyluracil, pseudouridine, with some α-anomer and only traces of the pyranose isomers. Likewise 5-bromo-2′,3′-*O*-isopropylideneuridine leads to 5-β-D-ribofuranosyluridine (1,5-diribosyluracil).

AMONG the many nucleoside components of transfer ribonucleic acid (tRNA) there is one, pseudouridine, unique in having a C-glycosidic linkage.[1,2] Its structure has been determined as 5-β-D-ribofuranosyluracil (III).[3,4] Acid treatment leads to an equilibrium mixture of isomers including the α-anomer and the pyranose isomers, all of which have been characterised.[3] The chemistry of these, and of their phosphorylated derivatives, has been reviewed recently.[5] The biosynthesis of pseudouridine, still unresolved, presents an interesting problem.[6] One proposed solution, among others,[6] involves the transfer of a ribosyl residue to uridine to form an intermediate 1,5-diribosyluracil followed by loss of the ribose residue attached to N-1.[7] The isolation of a compound with the diribosyl structure from natural sources has been claimed,[8,9] although more recent evidence does not support this.[10] The present paper describes the synthesis of the α- and β-anomers of pseudouridine. The synthetic route is also applied to 1,5-di-β-D-ribofuranosyluracil. These experiments have already been described briefly.[11] More recently, syntheses of 5-xylo- and 5-arabino-furanosyluracil by a similar route, but using 2,4-dibenzyloxy-5-bromopyrimidine, have been described.[12]

5-Bromo-2,4-dimethoxypyrimidine can be converted into the 5-lithio-derivative.[13] Our attempts to couple this with acylated 1-ribosyl halides were unsuccessful, although Shapiro and Chambers obtained a very low yield of pseudouridine by this means.[14] Dehalogenation and deacylation of the ribosyl halide appeared to be a cause of the low yields. Moreover, removal of both methyl protecting groups required acidic conditions vigorous enough for a furanose–pyranose interconversion to take place and become a serious disadvantage. The 2,4-dimethoxypyrimidin-5-yl-lithium, too, undergoes self-condensation reactions.[15] To surmount these difficulties, 5-bromo-2,4-di-t-butoxypyrimidine (I) was synthesised from 5-bromo-2,4-dichloropyrimidine [16] and sodium t-butoxide. It is hydrolysed very rapidly by acid to give 5-bromouracil. In practice, the 5-lithio-derivative was more stable, possibly because dimerisation reactions involving addition to a C=N bond [15] were sterically hindered. This was coupled with 2,4:3,5-di-*O*-benzylideneribose [17] at −70° in dry tetrahydrofuran. Lower yields attended the use of 2,3:4,5-di-*O*-isopropylideneribose.[18] The product (II) was formed as a mixture of the C-1′-isomers, although in the present experiments it was not isolated. However, its characterisation, separation into two isomers, and some further transformations that have been effected will be described elsewhere.[19]

The C-1′-position in pseudouridine shows considerable benzylic character,[3] as does 5-hydroxymethyluracil.[20] On these grounds it was expected that mild acid treatment, sufficient to remove the t-butyl and benzylidene

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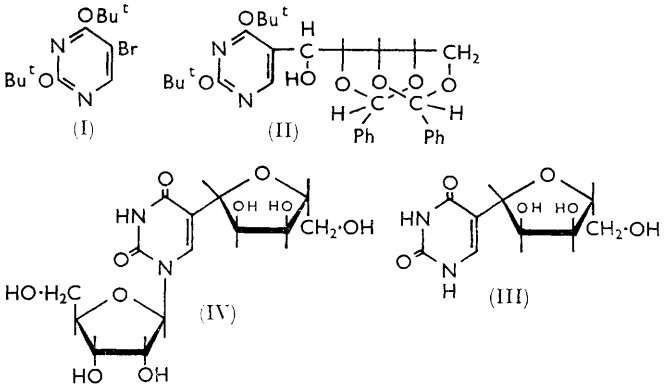
protecting groups, would also cause ring-closure of the sugar residue and that furthermore, if the conditions were mild enough, the kinetically controlled furanoside products would predominate.[21] Treatment with methanolic hydrogen chloride at 60° for 2 min. was sufficient to effect ring-closure and the products were then separated by anion-exchange chromatography using a borate gradient elution system. This allows the separation of all four pseudouridine isomers.[3,22] Traces (0·3% each) of the pyranosides were present but not isolated. The α- and β-furanosides were isolated in 8% and 18% yields respectively [based on (I)]. The latter was identical in all respects with naturally occurring pseudouridine (III).



It has been shown [19] that both C-1′-isomers of (II) give on acid treatment the same mixture of α-(30%) and β-(70%) furanose products. Thus the ring closure must involve an S_N1 process, the intermediate carbonium ion being possibly stabilised in part by electron accession from N-1. Ring formation is clearly kinetically controlled since only traces of the pyranose isomers were formed; after acid-catalysed equilibration the pyranose isomers predominate.[3]

An extension of this synthetic route led to 5-β-D-ribofuranosyluridine (IV). 5-Bromo-2′,3′-*O*-isopropylideneuridine was treated with three equivalents of n-butyllithium to form the 3,5,$O^{5'}$-trilithio-derivative (cf. ref. 15), and di-*O*-benzylideneribose was then added. Mild acid treatment of the intermediate gave two products which were separable by chromatography on a cellulose column. One of these proved to be a diribosyluracil and the other, the structure of which is unclear, was converted completely into the former by further treatment with acid. The product crystallised from ethanol and was shown to be 5-β-D-ribofuranosyluridine. The n.m.r. spectrum in D_2O showed a one-proton singlet at τ 2·4 characteristic of the 6-proton of uracil and pseudouridine,[3] thus indicating that the 5-position is substituted. Periodate titration gave an uptake of 1·9 moles of oxidant per mole, showing that both ribose residues are furanose.

Definitive evidence on this point was obtained by treatment of the diribosyluracil with a yeast nucleosidase [23] which hydrolyses uridine to uracil. It gave pseudouridine, identical with the natural material. Thus the structure (IV) is confirmed.

The o.r.d. spectrum showed a very small positive Cotton effect. Since uridine shows a large positive Cotton effect and pseudouridine a small negative one this supports the view that both ribose residues are in the β-configuration.[24,25]

Recently Dlugajczyk and Eiler [10] have claimed the synthesis of (IV) which, although not isolated in substance, was shown by Dr. J. J. Eiler to be chromatographically and electrophoretically identical to our product. A comparison of their product with that isolated from natural sources by Lis and Lis [8] showed them to be dissimilar. The question of the existence of 5-ribosyluridine in living systems must, then, remain an open one, although the compound detected by Kuriki [26] in a study of pseudouridine biosynthesis in yeast has not been compared with the synthetic material.

EXPERIMENTAL

Paper chromatography was carried out by the descending technique using the solvent system n-butanol–acetic acid–water (5 : 2 : 3 v/v). Paper electrophoresis was performed in 0·1M-sodium tetraborate solution at 40 v/cm.

5-Bromo-2,4-di-t-butoxypyrimidine.—Dry t-butanol (350 ml.) was added slowly to a stirred suspension of a 50% dispersion of sodium hydride in oil (18·6 g.) in light petroleum (100 ml., b.p. 80—100°) under nitrogen. When the initially vigorous reaction had subsided, the mixture was heated under reflux for 10 min., by which time the white precipitate had almost entirely dissolved. After cooling, 5-bromo-2,4-dichloropyrimidine [16] was added dropwise during 10 min. so that refluxing was just maintained. The mixture was heated for 2 hr. and cooled, and solvents were removed under reduced pressure. The residues from two such preparations were combined, water (250 ml.) was added, and the solution was extracted rapidly with ether (4 × 250 ml.). The extract was washed with sodium chloride solution, dried ($MgSO_4$) and evaporated. The residue was distilled through a 15 cm. Vigreux column and the fraction having b.p. 94—102°/0·7 mm. (29·3 g., 61%) was collected and crystallised. Refractionation gave the pure *product*, m.p. 63—64° (Found: C, 47·3; H, 6·6; N, 8·9. $C_{12}H_{19}N_2Br$ requires C, 47·5; H, 6·3; N, 9·2%). Purification may also be conveniently carried out by sublimation at 60°/0·2 mm.

5-α- and 5-β-D-Ribofuranosyluracil.—Freshly sublimed 5-bromo-2,4-di-t-butoxypyrimidine (0·91 g., 3 mmoles) was dissolved in dry tetrahydrofuran (20 ml., distilled from $LiAlH_4$) and the solution was cooled to −70° and stirred in an atmosphere of dry, oxygen free nitrogen. A solution

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of n-butyl-lithium (3 mmoles) [27] in ether was added and the solution stirred for 10 min. 2,4:3,5-Di-*O*-benzylideneribose (0·97 g., 3 mmoles, dried at 100°/1 mm. for 3 hr. until the band at 3500 $cm.^{-1}$ in the i.r. spectrum was absent) in dry tetrahydrofuran (20 ml.) was then added dropwise during 0·5 hr. After strring for 2 hr. at 70°, the solution was allowed to warm up to room temperature overnight.

Water (100 ml.) and ether (100 ml.) were added and the water layer was further extracted with ether (2 × 30 ml.). The combined ether extracts were dried ($MgSO_4$) and evaporated. The residual gum was heated at 80°/10^{-2} mm. for 3 hr. to remove all unchanged (I). The residue was taken up in methanol (10 ml.), concentrated hydrochloric acid (1 ml.) was added and the solution was warmed at 60° for 2 min. before removing the solvents and acid at 20°/10^{-2} mm. The residue was dissolved in water (20 ml.), the solution was ether-extracted, and the aqueous phase was made approximately 0·5M in ammonia. This solution was applied to a Dowex-1 column (18 × 2 cm., chloride form) and elution was carried out using a linear borate gradient in which 5 l. of a solution 0·005M with respect to ammonium chloride, ammonium hydroxide, and sodium tetraborate was replaced by 5 l. of ammonium chloride solution (0·02M).[3] Five peaks were obtained containing (a) residual uracil, (b) and (c) the two pyranose isomers (A_f and A_s in Cohn's nomenclature,[3] 0·3% of each), (d) the α-furanose isomer (pseudouridine B), and (e) the β-furanose isomer (pseudouridine C). These were characterised by comparison with the mixture of isomers derived from the acid-catalysed isomerisation of naturally occurring pseudouridine by means of the elution volume on an analytical ion-exchange column,[3] and by paper electrophoresis.

The last two peaks were each made 0·5M in ammonium hydroxide and absorbed on a Dowex-1 column as above. This was then washed to neutrality with water and the product was eluted with 0·1M-acetic acid. The eluate was taken to dryness and the residue was evaporated thrice with methanol and crystallised from methanol. 5-β-D-Ribofuranosyluracil formed crystals (131 mg., 18%), m.p. 222—224° (lit.,[3] 220—221°) (Found: C, 44·0; H, 5·2; N, 11·7. Calc. for $C_9H_{12}N_2O_6$: C, 44·3; H, 4·95; N, 11·5%); $\lambda_{max.}$ (H_2O) 262 mμ (ε 7900) (pH 7); $\lambda_{max.}$ 587 mμ (ε 7800) (pH 12). The o.r.d. spectrum (*a* — 22 in water) was identical to that of natural material.[28]

On periodate oxidation it showed an uptake of 0·9 mol. oxidant. The n.m.r. spectrum in D_2O was identical with that of the natural substance.

5-α-D-*Ribofuranosyluracil* formed crystals (58 mg., 8%), m.p. 207—210° (Found: C, 44·2; H, 5·2; N, 11·5. $C_9H_{12}N_2O_6$ requires C, 44·3; H, 4·95; N, 11·5%); $\lambda_{max.}$ (H_2O) 263 mμ (ε 7900) (pH 7); $\lambda_{max.}$ 287 mμ (ε 6300) (pH 12). The o.r.d. curve showed a positive Cotton effect (*a* + 10 in water).

As an alternative to the above purification the residue from the treatment with methanolic hydrochloric acid was taken up in methanol (5 ml.) and cooled. The β-furanose isomer crystallised directly and was generally obtained in about 10% yield after two recrystallisations from methanol.

5-β-D-*Ribofuranosyluridine*.— 5-Bromo-2′,3′-*O*-isopropylideneuridine (2·5 g., 7 mmoles) [29,30] was dried under vacuum for 2 hr. at 150°. It was dissolved in dry tetrahydrofuran (60 ml.) and cooled to −70° under oxygen-free nitrogen. A solution of n-butyl-lithium (approximately N) (21 mmoles) was added and the solution was stirred for 10 min. at −70°. A light precipitate occasionally formed at this point. A solution of well dried 2,4:3,5-di-*O*-benzylideneribose (2·3 g., 7 mmoles) in dry tetrahydrofuran (40 ml.) was added dropwise during 0·5 hr. After 2 hr. at −70° the solution was allowed to warm up to room temperature overnight.

Ether (200 ml.) and water (200 ml.) were added and, after separation, the aqueous layer was neutralised with dilute hydrochloric acid and thoroughly extracted with ether. The combined ether extracts were dried (Na_2SO_4) and evaporated to a yellow syrup. This was dissolved in methanol (30 ml.), water (5 ml.), and concentrated hydrochloric acid (1 ml.) and the mixture was heated at 60° for 2 min. It was then dissolved in the minimum quantity of n-butanol–water (86 : 14), applied to a cellulose column (25 × 6 cm.), and eluted with the same solvent. In addition to some uridine and 5-bromouridine, two products were eluted separately and isolated by removal of the solvent *in vacuo*. The first, 5-β-D-*ribofuranosyluridine*, crystallised from ethanol, m.p. 241—244° (130 mg., 5%) (Found: C, 44·7; H, 5·4; N, 7·3. $C_{14}H_{20}N_2O_{10}$ requires C, 44·75; H, 5·3; N, 7·4%); $\lambda_{max.}$ (H_2O) 265 mμ (ε 9800) (pH 2—12). The n.m.r. spectrum in D_2O showed a resonance at τ 2·4 (singlet) due to the 6 proton. Periodate uptake: 1·9 mol. oxidant. The o.r.d. spectrum showed a positive Cotton effect (*a* +8 in water), but with an amplitude smaller than that of uridine. Its electrophoretic mobility (in 0·1M-sodium tetraborate) was 1·2 times that of uridine and its R_F on paper chromatography was 0·6 times that of uridine.

The second substance was obtained as an amorphous, hygroscopic solid (260 mg., 10%). Its u.v. spectrum was identical to that of uridine at all pH values. Its n.m.r. spectrum in D_2O showed singlets at τ 3·7 and 4·6 and a doublet at τ 4·2. Its electrophoretic mobility (in 0·1M-sodium tetraborate) was 1·4 times that of uridine and its R_F on paper chromatography was 0·4 times that of uridine. Periodate oxidation continued over an extended period and reached an indefinite end-point at 3 mol. On heating with N-hydrochloric acid at 100° for 0·5 hr. the substance was converted quantitatively into 5-ribosyluridine, which was isolated as a crystalline material identical with that isolated above.

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