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A NEW CHEMICAL SYNTHESIS OF 2-AMINO-(*N*-D-RIBOFURANOSYL)ACETAMIDE 5′-PHOSPHATE

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ABSTRACT

2-Amino-(*N*-D-ribosyl)acetamide 5′-phosphate (GAR, **10**) has been prepared in a readily scaled-up synthesis from 2,3-*O*-isopropylidene-D-ribofuranosylamine *p*-toluenesulfonate (**3**) by condensation with the mixed anhydride of *N*-(benzyloxycarbonyl)glycine, followed by phosphorylation with 2-cyanoethyl phosphate and removal of the protecting groups. By varying the conditions, the α-**4** and the β-**5** anomers could be obtained and separated from each other. Anomerization occurs upon removing the *O*-isopropylidene group from either **7** or **8**, so that the final compound (**10**) is an equimolar mixture of both anomers, of which only one is active toward GAR-transformylase.

INTRODUCTION

2-Amino-(*N*-D-ribofuranosyl)acetamide 5′-phosphate (GAR, **10**) is an intermediate that occurs early in the purine biosynthetic pathway[1–4]. The 2-amino group of this compound is formylated by the enzyme, 5,10-methenyltetrahydrofolate: 2-amino-(*N*-D-ribofuranosyl)acetamide 5′-phosphate transformylase (EC 2.1.2.2), hereafter referred to as GAR-transformylase, to give 2-formamido-(*N*-D-ribofuranosyl)acetamide 5′-phosphate (FGAR, **11**) and 5,6,7,8-tetrahydrofolic acid (tetrahydrofolate)[5–8].

$$\text{GAR} + \text{5,10-methenyltetrahydrofolate} + H_2O \rightarrow \text{FGAR} + \text{tetrahydrofolate} + H^+ \quad (1)$$

Our interest in this enzyme arises from studies on model compounds for tetrahydrofolic acid derivatives[9–13]. In this paper, we report a readily scaled-up synthesis of GAR (**10**) that offers distinct improvements in yield and product purity over earlier nonenzymic and enzymic syntheses.

Earlier preparations of **10** were conducted enzymically from D-ribose 5-phosphate. Two chemical syntheses of FGAR (**11**) have been reported[14,15]. However,

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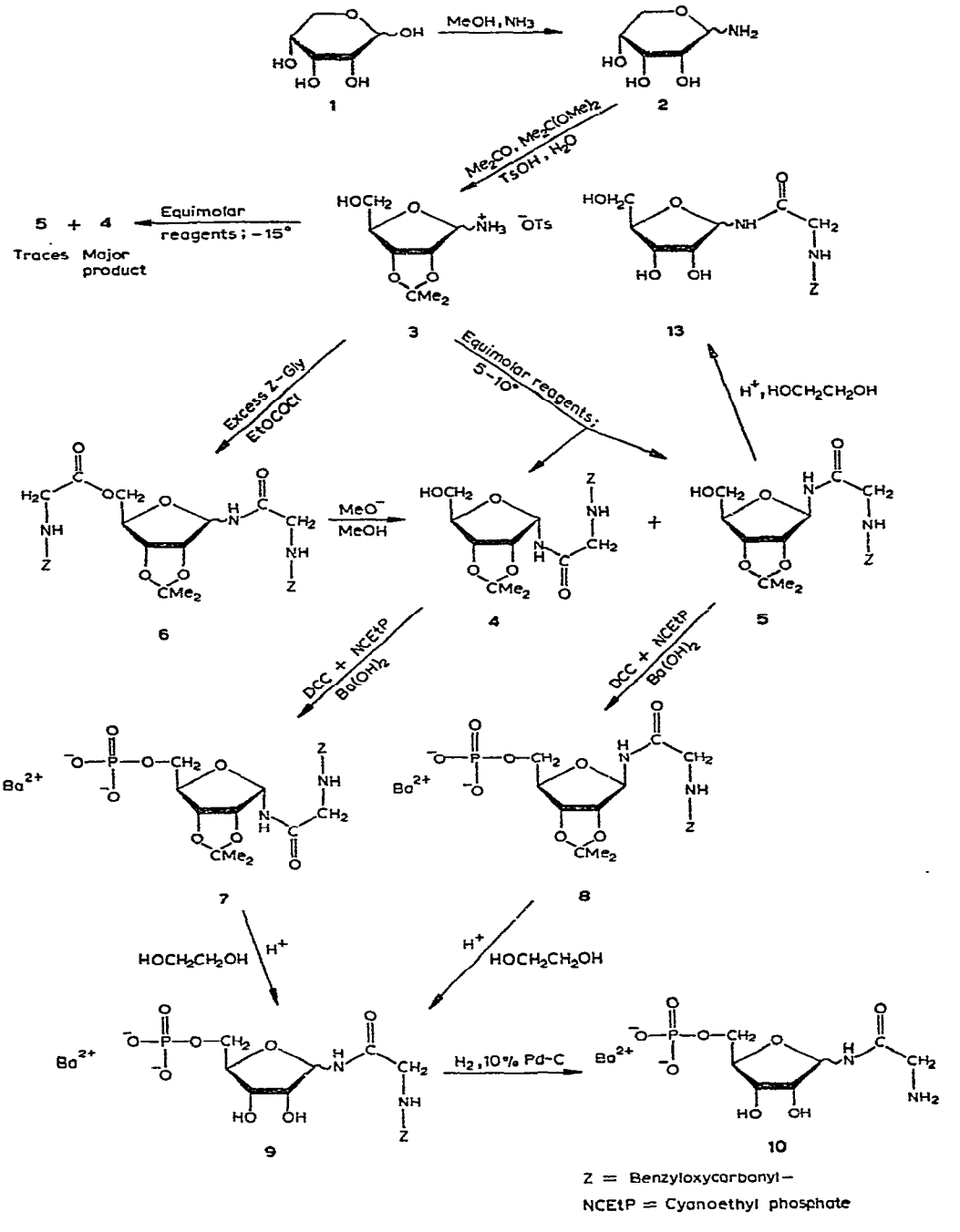
the key intermediate in both routes is 2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl azide, which is prepared from the corresponding chloride, and the latter is very reactive and difficult to synthesize[16,17]. The first reported procedure introduced the *O*-phosphoryl moiety before reduction of the azide and condensation with glycine; the second, in an attempt to improve the yield, condensed the ribosylamine with glycine before phosphorylation. Difficulties with the latter azide route include (1) rapid anomerization on reduction of the azide, (2) migration of the 2-benzoyl residue to the 1-position during hydrogenation[18,19], and (3) formation of various amounts of 2′- and 3′-phosphates in addition to the 5′-phosphate, despite use of the phosphorus oxychloride–triethyl phosphate[20,21] method.

RESULTS AND DISCUSSION

The route we have adopted is shown in Scheme I. The key intermediate, the *p*-toluenesulfonate salt (3) of 2,3-*O*-isopropylidene-D-ribofuranosylamine was readily prepared via the ribosylamine in two steps starting from D-ribose[19,22]. D-Ribopyranosylamine (2) crystallized from the medium in 86% yield during a two-week period. As this product exhibited no change in m.p. after preliminary purification[23], it was used directly in the next step. Introduction of the 2,3-*O*-isopropylidene group was accompanied by pyranose → furanose isomerization. The yield of 2,3-isopropylidene acetal (3) was 79–82%.

The next step envisaged acylation of the amino group at C-1 in preference to the hydroxyl group at C-5. Tipson[23] had earlier effected *N*-acetylation of D-ribopyranosylamine with acetic anhydride in 1:1 acetic acid–water. The free base of 2-amino-2-deoxy-D-glucose is readily acylated in supersaturated methanolic solution by reaction at ambient temperature with 1.2–1.5 equivalents of acid anhydride or acid chloride[24]. Bodanszky[25] has demonstrated selective *N*-acylation of serine and tyrosine, employing the activated *p*-nitrophenyl esters, and the reaction has been extended to the *N*-acylation of unprotected 2-amino-2-deoxy-D-glucose[26]. The *N*-acylation of 3 was patterned on these precedents, utilizing *N*-benzyloxycarbonylglycine, as this protecting group is more readily removed by hydrogenolysis than the *N*-formyl group. Coupling of *N*-benzyloxycarbonylglycine to the ribosylamine, employing the *p*-nitrophenyl ester of the amino acid, gave only poor yields of the desired mono-*N*-acylated material, even when various solvents, such as ethyl acetate, chloroform, and dimethyl sulfoxide in the presence of triethylamine were tried. Additional products, including the *O*-acylated and *N*,*O*-diacylated product (6) were also obtained. Similar low yields resulted when *N*,*N*′-dicyclohexylcarbodiimide was employed as the coupling reagent. The desired compound was synthesized in good yield (≥ 71%) as a mixture of anomers by generating the mixed anhydride from the *N*-protected amino acid and ethyl chloroformate in the presence of triethylamine, with ethyl acetate as solvent.

This mixture of 4 and 5 chromatographed as two u.v.-active spots, having R_F values of 0.17 and 0.23, respectively. The spots were inactive toward ninhydrin,


MeOH, NH3
1
2
Me2CO, Me2C(OMe)2
TsOH, H2O
5 + 4
Traces
Major product
Equimolar reagents; −15°
3
13
Excess Z-Gly
EtOCOCl
Equimolar reagents; 5-10°
H+, HOCH2CH2OH
MeO−
MeOH
6
4
5
DCC + NCEtP
Ba(OH)2
7
8
HOCH2CH2OH
H+
H2, 10% Pd-C
9
10
Z = Benzyloxycarbonyl−
NCEtP = Cyanoethyl phosphate

Scheme I.

whereas both ribopyranosylamine and the 2,3-*O*-isopropylideneribofuranosylamine salt were developed by this reagent. Hydrolysis of the amide linkage by pretreatment with concentrated hydrochloric acid, followed by ninhydrin treatment, permitted reaction with the latter reagent at areas coincident with those found under u.v. light. The requirement of prior hydrolysis before ninhydrin development eliminated the possible presence of the mono-*O*-acyl derivative. Various lines of evidence further support the structure (**4**+**5**) assigned to the mixture. The mass-spectral fragmentation pattern of the anomers showed prominent fragments at *m/e* 237, 209, and 208 that were readily assigned to (base+30), (base+2H), and (base+H), where the base is the *N*-benzyloxycarbonylglycineamide ion-radical. Thus the *O*-acylated compound was excluded. In addition, fragments arising from loss of a methyl group from the *O*-isopropylidene moiety (M−15), the 5′-CH_2OH group (M−31), acetone and a $(CH_3)_2CO_2$ group (+H)[27] (M−58 and M−75, respectively), were detected, as well as the parent ion (M^+).

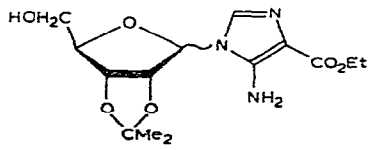
By conducting the reaction at −15°, it was possible to obtain the material having R_F 0.17 as the major product; at 5–10° equal amounts of both the anomers (R_F 0.17 and 0.23) were formed. The latter (R_F 0.23) was recrystallized from ethyl acetate, methanol, or ethanol–water mixtures, the former was purified chromatographically. The mass spectra of the purified compounds and the anomeric mixture were identical, both in fragmentation and relative intensity. I.r. spectra of the separated isomers were not particularly informative, owing to the presence of the benzyloxycarbonyl group, which absorbs at frequencies anticipated for the amide of glycine. However, both spectra were totally superposable, suggesting the presence of two anomers rather than a mixture of *O*- and *N*-acyl derivatives.

Examination of the proton n.m.r. spectra permitted conclusive identification of **4** and **5**. For **5** in $CDCl_3$, the 1′-NH proton resonated as a doublet centered at δ 8.07 ($J_{1'\text{-NH},1'\text{-H}}$ 8.4 Hz) and the proton at C-1′ gave a doublet, δ 5.73 ($J_{1'\text{-NH},1'\text{-H}}$ 8.4 Hz). For **4** in $CDCl_3$, the 1′-NH proton resonated at δ 8.02 ($J_{1'\text{-NH},1'\text{-H}}$ 8.4 Hz) and the proton at C-1′ gave a quartet centered at δ 5.74 ($J_{1'\text{-NH},1'\text{-H}}$ 8.4 Hz, $J_{1'\text{-H},2'\text{-H}}$ 3.0 Hz). In CD_3OD, the proton at C-1′ gave a doublet, δ 5.76 (J 3.0 Hz). Although the anomeric configuration of simple aldofuranosyl derivatives cannot be unequivocally determined from J values, a choice is possible in five-membered rings that cannot deviate substantially from planarity, in which case $J_{cis} > J_{trans}$. This situation occurs in **4** and **5**, where the C-2′ and C-3′ are incorporated into a 1,3-dioxolane ring. On this basis, compound **4** is assigned the α configuration and **5** the β form.

Phosphorylation of **4** and **5** by the 2-cyanoethyl phosphate procedure gave the respective monoesters in 40% yield. Although the 5′-OH was the only group available for phosphorylation, methods employing phosphorus oxychloride, phosphorus oxychloride in the presence of trialkyl phosphate, or dibenzyl phosphorochloridate gave very low yields of the anticipated products. The phosphate moncester gave a single spot in the solvent employed, having R_F values 0.68 for **8** and 0.62 for **7**. No inorganic phosphate could be detected by paper chromatography or spectrophoto-

metric analysis. The compound was unreactive toward periodate. Both of the barium salts were soluble in methanol and could readily be separated from excess cyanoethyl phosphate and inorganic phosphate. Proton n.m.r. spectroscopy in CD_3OD confirmed the presence of protons from both the *O*-isopropylidene and the *N*-benzyloxycarbonyl protecting groups in the ratio of 6:5.

It remained to establish conditions for protection that would minimize anomeric interconversion. The rate of removal of the isopropylidene group from **12** varies with



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anomeric configuration, requiring 1.5 h for the β-anomer and 3 h for the α-anomer (100°, 10% acetic acid) and apparently proceeds without anomerization[19]. Buchanan *et al.*[8] have reported that best yields of enzymically active GAR (**10**) can be obtained from FGAR (**11**) in 0.1M hydrobromic acid after 15 min at 100°. When **4** and **5** were heated for 20 min at 70° at pH ~2, the isopropylidene group was removed, as detected by paper chromatography followed by the benzidine–metaperiodate test. However, during the same period, **7** and **8** were only partially deprotected and, in addition, inorganic phosphate, D-ribose 5-phosphate, and starting material were detected. At pH 2 and 25°, no appreciable removal of the isopropylidene group occurred after 96 h. Hampton *et al.*[28] have shown that the removal of the isopropylidene group is promoted by an order of magnitude by an alcohol-exchange reaction. In 1:1 (v/v) ethylene glycol–water at pH 1.5 and 100°, complete removal of the *O*-isopropylidene group was effected in 15 min. After removal of the isopropylidene group, the product could be precipitated free of inorganic phosphate. This compound (**9**) was reactive toward periodate and the molar ratio of periodate consumption to organic phosphate was 1:1. It did not react with ninhydrin.The foregoing procedure for removing the *O*-isopropylidene group was also attempted with the non-phosphorylated isomer (**5**), but the α isomer of **13** was obtained, indicating extensive anomerization. Apparently anomerization likewise occurs with the phosphorylated compounds (**7** and **8**), as evidenced by the comparable enzymic activity of the final products obtained from either the α or β anomer, respectively.

The second deprotecting step involved the removal of the benzyloxycarbonyl moiety by hydrogenolysis over palladium on charcoal. It was not necessary to observe this sequence; hydrogenolysis followed by hydrolysis produced **10** of comparable enzymic activity. However, it is important for the isopropylidene group to be present during the base-catalyzed removal of the cyanoethyl group. Prior removal of the 2′,3′ protecting group apparently caused cleavage of the furanose ring, as evidenced

by a change in the ratio of organic phosphate to periodate consumption from 1:1 to 1:2 and the lack of enzymic activity toward the isolated material.

After filtering off the catalyst, the barium salt of GAR (**10**) precipitated from the solution when the pH was brought to 8.5. The mole ratio of organic phosphate to periodate consumption was 1:1 and the material was ninhydrin active. The compound gave a single spot (R_F 0.15) in the solvent system employed. Both the ninhydrin and organic phosphate tests coincided on the paper chromatogram. The ^{13}C n.m.r. spectrum of the sodium salt in D_2O at pH 7.5 was consistent with the assigned structure. The observed resonances in **10** for C-1′–C-5′ were assigned on the basis of the established order C-1′, C-4′, C-3′, C-2′, and C-5′ in the direction of increasing field, utilizing the separate anomers **4** and **5** and uridine 5′-monophosphate as reference materials[29,30]. Resonances for glycine agreed with literature values[31] and were unaffected by the stereochemistry at C-1′. From the integration data, the two anomers were present in equal amounts in GAR (**10**).

Enzymic activity toward GAR (**10**) was measured by a modification of the transformylase assay[8], in which 5,10-methenyltetrahydrofolate was in excess and the amount of GAR was limiting. The enzyme used was a partially purified transformylase from chicken liver. Enzymic acitivity was based on the tetrahydrofolate produced, and was found to be 45–49% of the chemically found organic phosphate or periodate-active material. As the enzyme is specific for the β anomer, the results of the enzymic assay compare favorably with the anomeric distribution found by ^{13}C n.m.r. spectroscopy.

In conclusion, the foregoing synthesis, which is readily scaled up, gives **10** in an overall yield of 23%, commencing with **3**. The preparation furnishes GAR (**10**) of high purity on the basis of enzymic and chromatographic analysis. The Ba^{2+} ion is readily exchanged for other cations, such as Li^+ or Na^+, by ion-exchange chromatography. However, it is recommended that the compound be stored as the less hygroscopic Ba^{2+} salt.

EXPERIMENTAL

General methods. — Solutions were evaporated with a Büchi rotary evaporator at flask temperatures below 50°. Melting points were determined with a Fisher–Johns apparatus and are uncorrected. T.l.c. was performed on silica gel PF-254 and GF-254 (Merck) activated at 110°, with ethyl acetate as the eluant (unless otherwise indicated). Ascending paper chromatograms were run on Whatman No. 1 paper, which was used without prior acid washing. The solvent front was allowed to travel at least 15 cm. Butanol–17.6M acetic acid–water (2:1:1) was used as solvent. Phosphates were detected on paper with the Isherwood and Hanes[32] reagent and visualized by u.v. irradiation[33]. Amino groups were detected on paper by ninhydrin (0.3 g ninhydrin per 100 ml of butanol, containing 3% by volume of acetic acid) and heating for 20 min at 100°. On t.l.c. plates the *N*-protected derivatives were detected by spraying first with concentrated hydrochloric acid, followed by ninhydrin, and heating on a hot

plate. The D-ribose phosphate derivatives unprotected at C-2′ and C-3′ were detected on paper chromatograms by the benzidine–metaperiodate spray[34]. For cation-exchange procedures, Dowex-50W (25–50 mesh, Na^+ form) was used. Inorganic phosphate was determined by the method of Chen *et al.*[35]. Total phosphate was determined, after wet ashing with 0.2 ml of concentrated sulfuric acid, by treatment with 0.1 ml of 70% perchloric acid to decompose the carbon. The amount of organic phosphate is expressed as the difference between total phosphate and the amount of inorganic phosphate found before hydrolysis. The pentose concentration was determined by measuring the consumption of periodate spectrophotometrically, by the method of Dixon and Lipkin[36]. The periodate method was calibrated by using adenosine 5′-monophosphate, inosine 5′-monophosphate, and D-ribose 5-phosphate as primary standards. All u.v.-absorption measurements were recorded on a Gilford 240 spectrophotometer. A Perkin–Elmer Model 735 or Model 257 spectrophotoment. was used for recording i.r. spectra. Optical rotations were measured on a Rudolph Model 80 high-precision polarimeter with a Model 200 photoelectric attachment. Mass spectra were recorded with an AEI-MS902 spectrometer at an ionizing potential of 70 eV and a direct-introduction source operating at 250°. N.m.r. spectra at 60 MHz were recorded with a Varian A-60A spectrometer, with tetramethylsilane as the internal standard. Natural abundance ^{13}C n.m.r. spectra were recorded on a J.E.O.L. Model JNM-PS-100 instrument equipped with noise decoupling, using $CDCl_3$ or D_2O as the internal lock signal, and tetramethylsilane in $CDCl_3$ or sodium 4,4-dimethyl-4-silapentane-1-sulfonate in D_2O as the external standard. Microanalyses were performed by the Midwest Microlab Ltd., (Indianapolis, Indiana).

The amount of enzymically active GAR (**10**) in the preparation was determined by taking limiting amounts of the substrate in the presence of the enzyme, GAR-transformylase, isolated from chicken liver, with an excess of 5,10-methenyltetrahydrofolate. The quantity of tetrahydrofolate formed was measured as *p*-aminobenzoylglutamate by the Bratton–Marshall procedure[37]. 5,10-Methenyltetrahydrofolate was prepared according to the method of Rowe[38]. The assay contained the following materials in a final volume of 0.9 ml: 0.1 ml of 0.003M ethylenedinitrilo-(tetraacetic acid) (tetrasodium salt), 12 units (1 unit = 0.01 μmol/30 min) of GAR-transformylase, 0.1 μmole of 5,10-methenyltetrahydrofolate (0.1 ml of a freshly prepared solution in 0.05M maleate buffer, pH 6.8) and 20 μl of a solution of GAR in 0.05M maleate buffer, pH 6.8, containing approximately 0.01 μmol on the basis of chemical assay. The assay containing all materials except the 5,10-methenyltetrahydrofolate was incubated for 10 min at 37°, the methenyl solution added and the resulting solution incubated for 30 min at 37°. The reaction was then quenched with 0.1 ml of 30% trichloroacetic acid, the mixture centrifuged, and 0.75 ml of supernatant analyzed by the Bratton–Marshall procedure. An extinction coefficient of 40,500 at 540 nm was employed for the product dye. In an alternative assay, tetrahydrofolate was converted quantitatively into 5,10-methenyltetrahydrofolate and the latter measured by the procedure of Blakley and Ramasastri[39]. The concentration of tetrahydrofolate determined by the two assays was in satisfactory agreement.

1'-N-(Benzyloxycarbonylglycyl)-2',3'-O-isopropylidene-α-D-ribofuranosylamine- (**4**). — Compound[19] **3** (19.2 g, 53.2 mmol) was stirred in 200 ml of dry ethyl acetate and cooled to −15°. To a mixture of *N*-benzyloxycarbonylglycine (11.1 g, 53.1 mmol) suspended in 200 ml of dry ethyl acetate was added 7.5 ml of freshly distilled triethylamine. The mixture was cooled to −20° and 5.3 ml of distilled ethyl chloroformate was added. After 15 min, another 7.5 ml of triethylamine was added to this flask, and its contents were quickly transferred to the flask containing compound **3**. After the transfer, the vessel was closed with a Drierite guard-tube and the solution stirred at −20°. Over a period of 10 h, the mixture was allowed to warm to ~25°, at which time the precipitate was filtered off and washed several times with small quantities of ethyl acetate. The filtrate and washings were transferred to a separatory funnel in which the ethyl acetate layer was washed with equal volumes of the following solutions: 20% sodium chloride (twice), M potassium carbonate (four times), 20% sodium chloride (once), ice-cold M hydrochloric acid (once) and finally with 20% sodium chloride (twice). After the final wash, the organic layer was dried (magnesium sulfate) and evaporated under vacuum to a thick syrup. The latter was kept overnight at 10 mtorr to remove the last traces of solvent and gave 14.4 g (38 mmol, 71%) of **4**; as the α-anomer and a trace of the β-anomer; $[\alpha]_D^{27}$ +21.8° (*c* 12.4, chloroform); R_F 0.17 (ethyl acetate), R_F 0.23; $\lambda_{max}^{CHCl_3}$ 2.96, 3.05 (NH), 3.4 (CH), 5.8–5.95 (CONH), 6.7 (NH), 7.3 (CH_3), 7.9–8.5 (aromatic CH), and 9.5 μm (C–O); n.m.r. (60 MHz, $CDCl_3$): δ 1.29, 1.44 (CMe_2), 3.5–4.7 (furanosyl protons, and $NHCH_2CO$ multiplet), 5.08 (singlet, CH_2 of benzyl), 5.76 (quartet, $J_{1',2'}$ 3.0 Hz, $J_{1'-NH}$ 9.0 Hz, H-1 of α-anomer; in CD_3OD, doublet, $J_{1',2'}$ 3.2 Hz), 6.24 (NH of *Z*-protecting group; exchanged by CD_3OD), 7.27 (s, Ph), 8.02 (doublet, $J_{1'-NH}$ 9.0 Hz, exchanged by CD_3OD, 1'-NH); *m/e* 381 (2, $[M+1]^+$), 380 (4, M^+), 365 (24, $[M-CH_3]^+$), 349 (2, $[M-CH_2OH]^+$), 322 (4, [M − acetone]$^+$), 305 (75, $[M-CH_3]^+ - CH_3CO_2(+H)$, 173 (75, [M − base]$^+$), 208 (49, [base + H]$^+$), 209 (32, [base + 2H]$^+$), and 237 (30, [base + 30]$^+$).

Anal. Calc. for $C_{18}H_{24}N_2O_7$: C, 56.84; H, 6.32; N, 7.37; O, 29.47. Found: C, 56.59; H, 6.35; N, 7.05; O, 29.49.

2',3'-O-Isopropylidene-1'-N-(benzyloxycarbonylglycyl)-β-D-ribofuranosylamine (**5**). — When the foregoing condensation with mixed anhydride was performed at 0–5°, the two anomers were formed in equal amounts. The anomer having R_F 0.23 crystallized from the ethyl acetate layer after final processing, when the solvent was partially removed under vacuum. The white, fibrous crystals (yield, 50% of the mixture of anomers) were filtered off and recrystallized from 1:1 ethanol–water; m.p. 128–130°, $[\alpha]_D^{27}$ −22.0° (*c* 4.57, chloroform); R_F 0.23 (ethyl acetate); $\lambda_{max}^{CHCl_3}$ 2.94, 3.05 (NH), 3.4 (CH), 5.75–5.95 (CONH), 6.7 (NH), 7.3 (CH_3-geminal), 7.9–8.5 (aromatic CH), and 9.5 μm (C–O); n.m.r. (60 MHz, $CDCl_3$): δ 1.29, 1.48 (CMe_2), 3.5–4.8 (furanosyl protons and $NHCH_2CO$ multiplet), 5.09 (CH_2 of benzyl), 5.73 (doublet $J_{1'-NH}$ 9.0 Hz, H-1 of β-anomer; in CD_3OD, 5.63, singlet), 6.11 (triplet, *J* 6 Hz, exchanged by CD_3OD, NH of Z-glycine) 7.31 (s, Ph), and 8.07 (doublet, $J_{1'-NH}$ 8.0 Hz, exchanged by CD_3OD, 1'-NH); *m/e* 381 (1.5, $[M+1]^+$), 380 (2.5,

$M^{\ddagger}$), 365 (49, $[M-CH_3]^{\ddagger}$), 349 (1.5, $[M-CH_2OH]^{\ddagger}$), 322 (2.5, [M−acetone]‡), 305 (93, $[M-CH_3]^{\ddagger}-CH_3CO_2(+H)$), 173 (84, [M-base]‡), 208 (59.5 [base+H]‡), 209 (49, [base+2H]‡), 237 (37.5, [base+30]‡).

Anal. Calc. for $C_{18}H_{24}N_2O_7$: C, 56.84; H, 6.32; N, 7.37; O, 29.47. Found: C, 57.04; H, 6.40; N, 7.08; O, 29.69.

1'-N-(*Benzyloxycarbonylglycyl*)-α-D-*ribofuranosylamine* (**13**). — To compound **5** (380 mg, 1 mmol), in 5 ml of ethylene glycol was added *p*-toluenesulfonic acid monohydrate (20 mg) and the solution was stirred for 15 h at room temperature. The mixture was then poured into 150 ml of ether and enough methanol was added to form a single homogeneous layer. This mixture was kept in a freezer overnight, whereupon crystallization began. After another two days the crystals were filtered off, washed with ether and dried; yield, 160 mg (47%); m.p. 165–167° (lit. m.p. 169–170°), $[\alpha]_D^{27}$ +68.5° (*c* 1.00, methanol); *m/e* 322 (10, $[M-H_2O]^{\ddagger}$), 133 (7, [M−base]‡), 208 (10, [base+H]‡), 209 (25, [base+2H]‡), 237 (7, [base+30]‡).

Anal. Calc. for $C_{15}H_{20}N_2O_7$: C, 52.94; H, 5.88; N, 8.24; O, 32.94. Found: C, 52.83; H, 5.87; N, 8.14; O, 33.21.

2',3'-O-*isopropylidene-1'*-N-(*benzyloxycarbonylglycyl*)-α- *or* -β-D-*ribofuranosylamine* 5'-*phosphate* (**7** *or* **8**). — The β-nucleoside (2.1 g, 5.5 mmoles), was dissolved in 10 ml of dry pyridine (freshly distilled and stored over molecular sieves). 2-Cyanoethyl phosphate barium salt dihydrate (8.06 g) (Aldrich) was converted into its pyridinium salt and a standard M solution (based on the weight of barium salt) was prepared according to the procedure of Tener[40]. The reagent was found on analysis to contain 0.65 mmol of organic phosphorus per ml. This reagent (16.9 ml, 11 mmol) was added to the nucleoside solution in pyridine and the mixture evaporated to a thick syrup. Another 15 ml of pyridine was added, and the mixture again evaporated to a thick syrup. The foregoing procedure was repeated three more times, with 20 ml of dry pyridine each time, and at the end the resulting syrup was kept at 1 torr for 15 min at 45°. *N,N'*-Dicyclohexylcarbodiimide (6.8 g, 33 mmol) was dissolved in 35 ml of pyridine and quickly filtered through glass wool into the reaction vessel. The flask was stoppered and stirred for 4 days at room temperature. The reaction was then quenched by adding 25 ml of distilled water and stirring for 7 h. The urea derivative was filtered off and washed with small quantities of distilled water. The filtrate and washings were evaporated to low volume, and 80 ml of distilled water was added, and the mixture stirred overnight. The precipitated urea was filtered off and washed with distilled water. Paper chromatography indicated that all of the organic phosphates were in the water extract. The volume of filtrate and washings was decreased by half and two volumes of abs. ethanol were added. The apparent pH was 3.2 (glass electrode). The pH was brought to 8.5 with 5% ammonia, and 6 ml of M barium acetate was added. The mixture was stored overnight in a freezer and then filtered. The filtrate was evaporated to low volume, 300 ml of 9M ammonia was added, and the solution was then heated for 90 min at 60° under reflux. The flask was then cooled rapidly to room temperature in an ice bath and the mixture filtered. The filtrate was evaporated to one quarter of its volume and any more precipitate formed

was filtered off and washed with small quantities of distilled water. The filtrate and washings (pH 7.1) were brought to pH 8.5 with 5% ammonia and an additional 6 ml of M barium acetate was added. The mixture was stored for 3 h in a freezer and then filtered. The precipitate was washed with 10-ml quantities of methanol and the filtrate and washings were made up to 200 ml with methanol (ratio of methanol to water in this solution was ~3:1). This solution was evaporated until turbidity developed, and then kept overnight in a freezer and the mixture filtered. The filtrate was further concentrated and two more crops of the organic phosphate precipitated. The precipitates were combined, air dried, and desiccated at <0.01 torr for 6 h at 25°. This precipitate (1.77 g, 32% for **8** based on the anhydrous barium salt) chromatographed on paper as a single spot having R_F 0.68. An analogous procedure starting with the α-nucleoside (1.0 g, 2.7 mmoles) yielded 0.79 g (39% for **7** based on the anhydrous Ba salt), which chromatographed as a single spot, R_F 0.62. No inorganic phosphate could be detected. The n.m.r. (60 MHz, CD_3OD) spectrum showed δ 1.3, 1.5 (CMe_2), 3.5–4.7 (furanosyl protons and $NHCH_2CO$ multiplet), 7.23 (phenyl protons of Z group). The ratio of the phenyl protons to isopropylidene protons were 5:6. The compound did not react with ninhydrin nor with benzidene–metaperiodate reagent on paper. Phosphate analysis showed this material to contain 1 μmol of organic phosphate/mg (for the β-anomer), and 1.34 μmol of organic phosphate/mg (for the α-anomer), as compared with 1.68 μmol calculated for the anhydrous Ba salt.

1′-N-(Glycyl)-α- and -β-D-ribofuranosylamine 5′-phosphate (**10**). — Compound **8** (Ba salt, 959 mg, 0.96 mmol), was suspended in 10 ml of 1:1 ethylene glycol–distilled water, and the pH was adjusted to 1.6 (glass electrode) with M hydrochloric acid. The salt dissolved to give a clear solution that was heated in a boiling water-bath for 15 min at 100° and then chilled in ice to 25°. The pH was brought to 8.5 with M potassium hydroxide. The barium salt precipitated and the precipitation was made complete by adding 50 ml of methanol and cooling the mixture for 3 h in a freezer before centrifuging. The precipitate was washed repeatedly with 2:1 methanol–water, and air-dried overnight. The air-dried residue was powdered and kept for 6 h at 0.05 torr at 60°; yield 756 mg (100% based on the anhydrous barium salt). An analogous procedure starting with **7** (760 mg, 1.0 mmol) gave 620 mg (78% based on the anhydrous Ba salt). On paper the compound showed as a single, major spot for organic phosphate (R_F 0.52), with traces of inorganic phosphate at R_F 0.31. Phosphate assay showed this product to contain 1.27 μmol of organic phosphorus/mg (theoretical 1.80 μmol/mg for the anhydrous barium salt). Periodate assay showed this product to contain 1.32 μmol of periodate-reactive material/mg (theoretical 1.80 μmol/mg). The ratio of phosphate to periodate was 1:1.04. The paper chromatogram did not develop color when sprayed with ninhydrin, and only a single, active spot (R_F 0.52) could be detected with benzidine–metaperiodate spray. This material, (740 mg, 0.94 mmol) was suspended in 10 ml of distilled water and stirred with Dowex-50W (Na^+) until dissolution was complete. The resin was filtered off, washed several times with small quantities of distilled water, and the filtrate and washings combined with

an equal volume of methanol. The solution was hydrogenated at atmospheric pressure with 100 mg of 10% Pd on charcoal. After the absorption of hydrogen had ceased, the catalyst was filtered off, washed with small quantities of 1:1 methanol–water solution and finally washed with 10 ml of 1:1 solution of 50% methanol–water and 5% ammonia. The filtrate and washings were concentrated, the pH was adjusted to 8.5 if necessary, and 2 ml of M barium acetate was added. The mixture was stirred for 10 min, cooled for 30 min at 0°, and then centrifuged. The supernatant was mixed with five times its volume of methanol, cooled at 0° overnight, and centrifuged. The precipitate was washed repeatedly with 80% methanol, methanol and ether, and then air dried; yield 539 mg (84% based on the anhydrous barium salt). Phosphate assay showed this product to contain 1.46 μmol of organic phosphorus/mg (theoretical 2.37 for the anhydrous barium salt). The air-dried residue chromatographed on paper as a single spot having R_F 0.15. Both the organic phosphate and ninhydrin spots coincided on paper. The ^{13}C n.m.r. spectrum (D_2O, pH 8.5) showed δ 85.83 (C-1′ and C-4′ of the β-anomer), 83.77 (C-1′ of the α-anomer), 82.31 (C-4′ of the α-anomer), 75.87 (C-3′ of the β-anomer), 73.05 (C-2′ of the β- and C-3′ of the α-anomers), 72.17 (C-2′ of the α-anomer), 66.22 (C-5′ of the α- and β-anomers), 174.91 (C=O of $-CH_2CO-$), and 44.62 (CH_2 of $-CH_2CO-$). The ratio of organic phosphate to periodate-reactive material was 1:1.01. Enzymic activity toward **10** was measured by the tetrahydrofolate produced in the transformylase assay[8].

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