Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 15, pp. 1679-1684, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00280-4

ENZYMATIC SYNTHESIS OF 1-(2-DEOXY-β-D-RIBOFURANOSYL) IMIDAZOLE-4-CARBOXAMIDE, A SIMPLIFIED DNA BUILDING BLOCK

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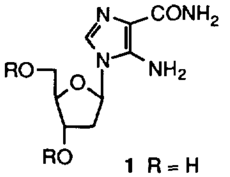
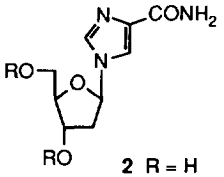
Abstract: Coupling of silylated 4-(5)-imidazolecarboxamide (**5**) with 1-chloro-2-deoxy-3,5-di-O-*p*-toluoyl-α-D-erythro-pentofuranose (**6**) in the presence of $SnCl_4$ in CH_3CN or condensation of the sodium salt of **5** with the halogenose **6**, afforded a mixture of 1-β-glycosylated 4-(5)-imidazolecarboxamide (**11**) and the corresponding α anomer (**12**). Using a crude extract of nucleoside N-deoxyribosyltransferases from *Lactobacillus leichmannii* only one product was formed, the N-1-β-stereoisomer (**2**), in a good yield.

Introduction

Deoxynucleotides bearing simplified bases hold great promises for altering the recognition and replication of nucleic acids. We previously reported the synthesis of 5-amino-1-(2-deoxy-β-D-ribofuranosyl)imidazole-4-carboxamide (**1**, dZ) from 2'-deoxyinosine by an alkaline ring opening reaction[1]. The triphosphate derivative dZTP is a good substrate for terminal deoxynucleotidyl transferase and homooligomers of dZMP can be obtained in this way[1]. It is also a substrate for certain DNA polymerases which incorporate it in place of dATP and dCTP. However, polymerases fail to further elongate the lagging strand after dZMP incorporation[2]. To diversify base-pairing in DNA, we focussed on 2-deoxy-β-D-ribofuranosyl)imidazole-4-carboxamide (dICAR, **2**), in which the 5-amino group from the imidazole moiety of dZ is deleted. Indeed, steric clashes by the 5-amino group with sugar and carboxamide atoms likely restrict the pairing potential of dZ by hindering rotation of the imidazole and carboxamide groups and thus limiting the number of hydrogen-bond donor and acceptor patterns that can be alternatively displayed. The entity lacking the 5-amino group, dICAR (**2**), whose rotamer distribution should be wider than that of dZ, is expected to form stable pairs with the four canonical bases as well as with itself (Fig. 1). This self-pairing capability deserves scrutiny, as its deployment during replication reactions would lead to a new mode of information transfer by nucleic acids.



1 R = H
3 R = Ac



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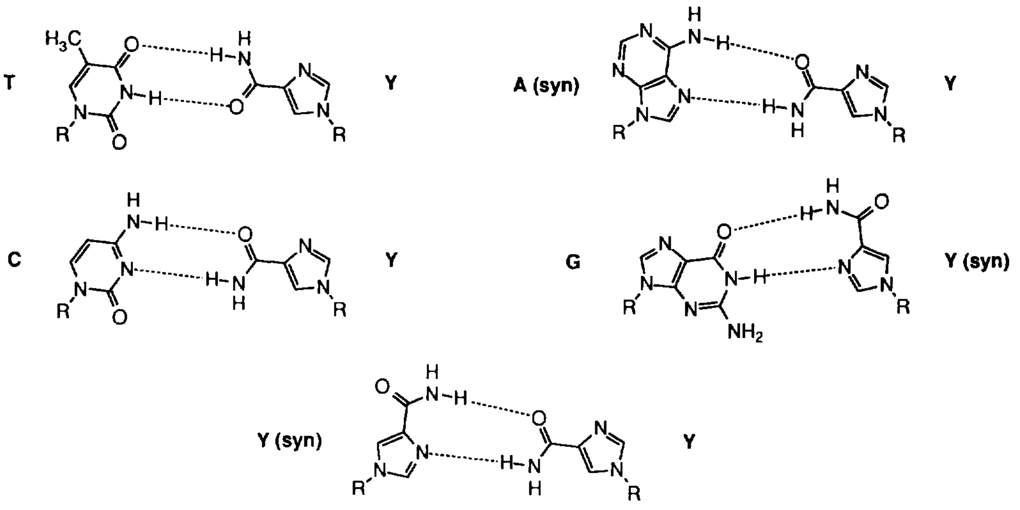


Figure 1 : Potential hydrogen bonding of the base ICAR (noted Y) with A, C, G, T and itself.

We report here the preparation of dICAR (**2**) following two pathways, via chemical glycosylation of imidazole-4-carboxamide on one hand, and via enzymatic transglycosylation with a crude extract of deoxyribosyl transferases from *Lactobacillus leichmannii* on the other hand. In the course of this study, Bergstrom *et al.* reported the synthesis of a C-nucleoside isomer of dICAR designed to function as a universal base, 2-carbamoyl-4-(2-deoxy-β-D-ribofuranosyl)-imidazole[3].

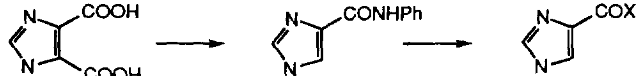
Results and Discussion

Previously reported syntheses of 2-deoxy-D-ribofuranosyl-imidazole nucleosides included degradation or ring opening of purine nucleosides,[1] condensation of heavy metal salts of preformed imidazoles with acylglycosyl halides,[4,5] direct condensation or acid-catalyzed fusion,[6,7] or condensation in the presence of a Lewis acid.[7] In contrast to ribofuranoses which possess an acyloxy substituent at position C-2 that directs base attack on the β face of the sugar, glycosylation with 2-deoxyribofuranoses results in the formation of a mixture of α and β anomeric products. Various factors including solvent, temperature and ratio of Lewis acid, seem to influence the anomeric ratio of the products as well as the regioselectivity during the silylation-glycosylation.[8] Selectivity was observed during the sodium salt glycosylation of 4(5)-amino-imidazole-5(4)-carbonitrile with 1-chloro-2-deoxy-3,5-di-O-*p*-toluoyl-α-D-erythropentofuranose; the N-1 and N-3 isomers, both in β-configuration, were obtained in a good yield.[9] Some 2-deoxy-β–D-ribofuranosyl-imidazole nucleosides were synthesized by bioconversion.[10-12]

In a preliminary step, an authentic sample of dICAR (**2**) was obtained by deamination of 5-amino-1-(3,5-di-O-acetyl-2-deoxy-β-D-erythropentofuranosyl)imidazole-4-carboxamide (**3**) as described for the corresponding ribonucleoside.[13] The reductive deamination of **3** was achieved at low temperature (-20°C) using 50% hypophosphorous acid and sodium nitrite. 1-(3,5-di-O-acetyl-2-deoxy-β-D-erythropentofuranosyl) imidazole-4-carboxamide (**4**) was isolated by silica gel chromatography as a crystalline product in a low yield (15%). Treatment of **4** with 1% sodium methoxide in methanol provided dICAR (**2**) in quantitative yield.

The low yield of the reductive deamination in the deoxy series did not permit the use of this route on a large scale. Therefore, the synthesis of dICAR was accomplished coupling 4-(5)-imidazolecarboxamide (5)[14] with 1-chloro-2-deoxy-3,5-di-O-*p*-toluoyl-α-D-erythropento-furanose (6)[15] according to the following approaches : the modified silyl Hilbert-Johnson procedure and the sodium salt procedure.

First, the 4,5-imidazolecarboxamide (5) was obtained from imidazole-4,5-dicarboxylic acid (7) in four steps. Reflux of 7 in aniline afforded 4-(5)-imidazolecarboxanilide (8),[16] which was submitted to acid hydrolysis followed by esterification of the resulting 4-(5)-imidazolecarboxylic acid (9) to give ethyl 4-(5)-imidazolecarboxylate (10).[17] Treatment of 10 with ammonia afforded compound 5[14] in a 20% overall yield.

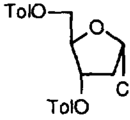


7

8

9 X = OH
10 X = OEt
5 X = NH_2

The carboxamide 5 was then converted into its trimethylsilyl derivative by refluxing with hexamethyldisilazane (HMDS) and chlorotrimethylsilane (TMSCl) in pyridine. After removal of the volatiles, crude silylated 5 was obtained as a moisture sensitive oil which was used without further purification. The silylated base was reacted with the halogenose 6 (1 eq) in dry acetonitrile in the presence of $SnCl_4$ (1.3 eq). The crude product was chromatographed on silica gel to afford as the major fraction, a mixture of β-N-1 (11) and α-N-1 (12) protected nucleosides (ratio : 60/40) in 52% yield. After deprotection with 1% sodium methoxide, the mixture was separated by preparative reverse-phase HPLC to provide pure α and β-anomers (2 and 13) in 75% yield. The site of glycosylation (N-1) and the anomeric configuration at carbon 1' were established by ^{1}H- and ^{13}C-NMR spectroscopy[18,19] and by comparison with the spectra of the authentic sample previously obtained by reductive deamination of dZ. The regio- and stereochemistry of the reaction was established from NOESY experiments which showed proximity in space of H-2, H-5 and H-1' for both nucleosides 2 and 13.



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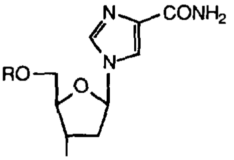
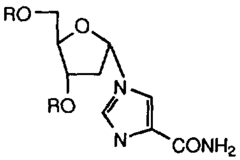


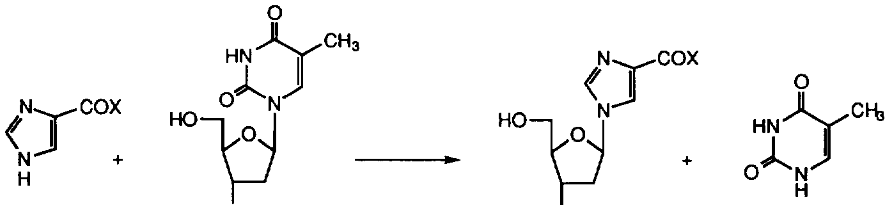
11 R = Tol
2 R = H



12 R = Tol
13 R = H

In a second set of experiments, the carboxamide 5 was subjected to nucleobase anion glycosylation.[20] Thus, the sodium salt of 5, produced *in situ* by 60% NaH in anhydrous acetonitrile, was treated with the halogenose 6 at room temperature. The major product isolated in 36% yield by chromatography on silica gel was in fact a mixture of α and β anomers (ratio 50/50). Removal of the toluoyl groups with 1% sodium methoxide in methanol provided the corresponding deprotected nucleosides 2 and 13 (87% yield). The mixture was further resolved by reverse phase HPLC to obtain pure anomers in approximately the same ratio.

We also investigated the biosynthesis of dICAR through enzymatic transglycosylation. The nucleoside N-deoxyribosyltransferases are known to catalyze the exchange of the deoxyribosyl moiety between a 2'-deoxy-β-D-ribonucleoside (donor) and a purine or pyrimidine base (acceptor).[21] In particular the known dZ[10,12] and some related imidazole nucleosides[12] have been synthesized through this route. The regioselectivity and the yield of the resulting transfer products were dependent upon the type of substituents on the imidazole ring. Recently, Hutchinson et al.[22] have reported the beneficial effect of organic solvents on the enzymatic synthesis of deoxynucleoside analogs using a crude extract of N-deoxyribosyltransferases from *Lactobacillus leichmannii*. We have studied the possible transfer of our 4-substituted imidazole bases (**8**, **10** and **5**) on a 0.5 mmol scale using a crude preparation of N-deoxyribosyltransferases from *L. leichmannii* in the presence of ethanol (10%) as a cosolvent and thymidine (3 eq.) as the donor. The three substituted imidazoles were all substrates for the bacterial extract. The corresponding β-N-1 nucleosides (**14**, **15** and **2**) were isolated as the sole products (12-40% yield) as established by ^{1}H and ^{13}C-NMR spectroscopy.



8 X = NHPh
10 X = OEt
5 X = NH$_2$

14 X = NHPh
15 X = OEt
2 X = NH$_2$

The same reaction was carried out on a preparative scale for the synthesis of dICAR; thus, 4-(5)-imidazolecarboxamide (6 mmol) and thymidine (2 eq.) were incubated at 40°C for five days in 20 mM citrate buffer (180 mL, pH 6.0) with the crude enzyme extract (5 mL) in the presence of ethanol (18 mL). The nucleoside dICAR (**2**) was isolated in 56% yield after silica gel chromatography with a purity of 95% (estimated by reverse-phase HPLC).

In summary, we have proposed a simple and efficient route to 1-(2-deoxy-β-D-ribofuranosyl) imidazole-4-carboxamide (dICAR) and related imidazole nucleosides based on the use of a crude extract of N-deoxyribosyl-transferases from *L. leichmannii*. The synthesis of oligodeoxynucleotides containing this new building-block as well as of the triphosphate derivative of dICAR are underway.

Experimental

NMR spectra were recorded on a Bruker 300 at 300 MHz for ^{1}H and at 75 MHz for ^{13}C. Column chromatography was performed on silica gel Merck 60 (70-230 mesh). Acetonitrile, pyridine were distilled from CaH_2. $SnCl_4$ (Aldrich) was used without distillation. Analysis by HPLC was carried out on a Perkin Elmer system using a Nucleosil 5C18 column and a linear gradient of acetonitrile (A) in 10 mM triethylammonium acetate buffer (pH 7.3) (B) over 20 min at a flow rate of 1 mL/min.

1-(3,5-di-O-acetyl-2-deoxy-β-D-ribofuranosyl)imidazole-4-carboxamide (4). To a solution of **3** (0.25 g, 0.77 mmol) in 50% H_3PO_2 (5.2 mL) at -20°C was added dropwise a solution of $NaNO_2$ (60 mg) in water (0.6 mL). After 2 h, TLC showed the reaction to be complete and that two products were formed. The reaction mixture was adjusted to pH 6.2 by careful addition of 30% aqueous ammonia and extracted with ethyl acetate (4 x 70 mL). The organic layers were dried and concentrated to dryness. Chromatography on silica gel (CH_2Cl_2/MeOH) gave compound **4** (35 mg, 15%): δ_H (DMSO-d_6) 2.04 (3H, s, CH_3), 2.07 (3H, s, CH_3), 2.50 (1H, m, 2'-H), 2.70 (1H, m, 2"-H), 4.20 (3H, m, 4'-H, 5'-H and 5"-H), 5.25 (1H, m, 3'-H), 6.15 (1H, dd, *J* 5.9

and 8.1, 1'-H), 7.15 (1H, broad s, $CONH_2$), 7.35 (1H, broad s, $CONH_2$), 7.90 and 7.95 (2H, each d, *J* 0.9, 2-H and 5-H); δ_C (DMSO-d6) 20.43 and 20.70 (CH_3), 37.08 (C-2'), 63.52 (C-5'), 74.24 (C-3'), 81.63 (C-1'), 85.70 (C-4'), 119.87 (C-5), 136.49 (C-2), 137.34 (C-4), 163.62 ($CONH_2$), 169.87 and 170.02 ($COCH_3$).

4-(5)-imidazolecarboxamide (5). A suspension of 4,5-imidazoledicarboxylic acid (**7**, 10 g, 64 mmol) in aniline (100 mL) was refluxed overnight at which time the acid had passed into solution. Water (500 mL) was added and the reaction mixture was concentrated to a small volume. The procedure was repeated several times to remove aniline. The residual aqueous solution was filtered and the precipitate was washed with Et_2O. The filtrate was concentrated and triturated with Et_2O to furnished another crop of 4-(5)-imidazolecarboxanilide (**8**[16], 6.4 g, 53%): δ_H (DMSO-d6) 7.00 (t, 1H, Ar), 7.30 (t, 2H, Ar), 7.80 (d and s, 4H, Ar, 5-H and 2-H), 9.80 (s, 1H, *NH*Ar), 12.65 (broad s, 1H, NH Im); Anal. Calcd for $C_{10}H_9N_3O$ (187.19): C, 64.15; H, 4.84; N, 22.45. Found: C, 64.20; H, 4.76; N, 22.29.

Compound **8** (2.8 g, 14.9 mmol) was heated with 10% HCl (30 mL) at 150°C (oil bath) overnight. The reaction mixture was concentrated to dryness, the residue was redissolved in water (30 mL) and Na_2CO_3 was added until a white precipitate appeared. It was filtered and recrystallized in water to give 4-(5)-imidazolecarboxylic acid as white needles (**9**[16], 1.5 g, 13.3 mmol, 89%).

A suspension of **9** (3.2 g, 28.6 mmol) in absolute ethanol saturated with HCl (50 mL) was refluxed. At the end of the reaction (4-6 h), the solution was concentrated and residual HCl was removed by coevaporation with toluene. The crude ester could be used without purification for the next step. For the transfer reaction, the residue was dissolved in a small quantity of water and precipitated with a saturated K_2CO_3 solution. Chromatography on silica gel (CH_2Cl_2/MeOH) gave ethyl 4-(5)-imidazolecarboxylate (**10**[17], 1.8 g, 45%): δ_H (DMSO-d6) 1.30 (t, 3H, CH_3), 4.20 (q 2H, CH_2), 7.80 (s, 2H, 5-H and 2-H).

To a solution of **10** (1.7 g, 12.1 mmol) in MeOH (20 mL) was added 30% aqueous ammonia (10 mL). The reaction mixture was stirred for 60 h at room temperature. After evaporation to dryness, the residue was purified by chromatography on silica gel (CH_2Cl_2/MeOH) to give 4-(5)-imidazolecarboxamide (**5**[14], 1.3 g, 96%): δ_H (DMSO-d6) 6.95 (1H, broad s, $CONH_2$), 7.25 (1H, broad s, $CONH_2$), 7.50 and 7.55 (each s, 2H, 5-H and 2-H), 12.50 (broad s, 1H, NH Im).

Glycosylation with silylated carboxamide 5. To a solution of **5** (0.6 g, 5.4 mmol) in pyridine (10 mL) was added HMDS (14.5 mL) and TMSCl (0.6 mL) at room temperature. The resulting mixture was refluxed for 5 h under anhydrous conditions, then cooled to room temperature. Excess of solvent and reagents was removed under reduced pressure to provide the silylated base as a brownish oil, which was used for coupling reactions without purification. To silylated **5** (0.6 g, 5.4 mmol) in CH_3CN (50 mL) was added halogenose **6**[15] (2.0 g, 5.4 mmol), followed by $SnCl_4$ (0.82 mL 6.9 mmol) dropwise at 4°C. The reaction mixture was stirred at room temperature for 1 h, then dissolved in CH_2Cl_2 and treated with a cold saturated $NaHCO_3$ solution. The mixture was filtered through a pad of Celite, the residue was washed with CH_2Cl_2. The combined organic layers were washed with H_2O, dried over Na_2SO_4, and concentrated under reduced pressure. Chromatography on silica gel (CH_2Cl_2/MeOH) afforded the protected nucleosides as a mixture of α and β anomers (1.3 g, 52%).

Sodium salt procedure. To a solution of **5** (0.5 g, 4.5 mmol) in dry acetonitrile (20 mL) was added NaH (60% in oil) (0.20 g, 5 mmol) and the mixture was stirred at room temperature under a nitrogen atmosphere for 1 h. After addition of the halogenose **6** (1.7 g, 4.5 mmol) in one portion, the mixture was stirred at room temperature until the reaction was judged complete by TLC (CH_2Cl_2/MeOH:90/10) (approx. 1 h). Then the insoluble material was filtered. Evaporation of the solvent followed by chromatography on silica gel (CH_2Cl_2/MeOH) afforded the protected nucleosides as a mixture of α and β anomers (0.75 g, 36%).

General procedure for deacylation. To a solution of protected nucleoside in anhydrous methanol was added 1% sodium methoxide (3 eq) at room temperature and the solution was stirred until TLC showed completion of the reaction. The mixture was then neutralized by addition of dry ion exchange resin (Dowex 50WX8, H^+ form), filtered, rinsed with MeOH. After evaporation of the solvent in vacuo, H_2O was added and the mixture was extracted several times with Et_2O. The aqueous layer was purified by gel filtration on Sephadex G-10 (H_2O) to give two fractions which were further purified to homogeneity by reverse phase HPLC on a C18 column using a 0-15% gradient of acetonitrile in H_2O over 20 min at 5.5 mL/min. The yields in purified nucleosides varied from 75 to 87%.

1-(2-deoxy-β-D-ribofuranosyl)imidazole-4-carboxamide (2): as an oil. Rt 9.91 min; δ_H (D_2O, pH 7.5) 2.23 (2H, m, 2'-H and 2"-H), 3.60 (2H, m, 5'-H and 5"-H), 3.90 (1H, m, 4'-H), 4.38 (1H, m, 3'-H), 5.93 (1H, t, *J* 7, 1'-H), 7.70 (2H, s, 2-H and 5-H); δ_C (D_2O) 39.41 (C-2'), 62.76 (C-5'), 72.41 (C-3'), 81.00 (C-1'), 86.84 (C-4'), 122.70 (C-5), 134.12 (C-4), 138.06 (C-2), 165.78 ($CONH_2$); MS (CI, NH_3), 228; Anal. Calcd for $C_9H_{13}N_3O_4 + 0.25\ H_2O$ (231.5): C, 46.65; H, 5.61; N, 18.20. Found: C, 46.87; H, 5.66; N, 18.68.

1-(2-deoxy-α-D-ribofuranosyl)imidazole-4-carboxamide (13): as a white powder. Rt 11.61 min; δ_H (D_2O, pH 7.8) 2.05 (1H, m, 2'-H), 2.50 (1H, m, 2"-H), 3.55 (2H, m, 5'-H and 5"-H), 4.05 (1H, m, 4'-H), 4.33 (1H, m, 3'-H), 5.88 (1H, dd, *J* 6.8 and 3.3, 1'-H), 7.70 and 7.71 (2H, each s, 2-H and 5-H); δ_C (D_2O) 39.41 (C-2'), 62.26 (C-5'), 72.16 (C-3'), 80.96 (C-1'), 86.58 (C-4'), 122.70 (C-5), 134.12 (C-4), 138.06 (C-2), 165.32 ($CONH_2$); Anal. Calcd for $C_9H_{13}N_3O_4$ + 0.25 H_2O (231.5): C, 46.65; H, 5.61; N, 18.20. Found: C, 46.85; H, 5.60; N, 18.51.

Nucleoside N-deoxyribosyltranferase. The enzyme extract was obtained from *Lactobacillus leichmannii* ATCC 4797 (strain n° 54.3 from CIP) as described by Hutchinson et al.[23]

Transfer reaction. To the imidazole derivative (**5**, **8** or **10**, 0.5 mmol) and thymidine (0.36 g, 1.48 mmol) dissolved in 20 mM citrate buffer (25 mL, pH 6 containing 0.2% sodium azide) was added ethanol (2.5 mL) followed by crude transferase extract (0.75 mL). The mixture was incubated at 40°C for 5 to 8 days until the reaction had reached equilibrium (TLC). The solution was concentrated to dryness and the residue was purified by chromatography on silica gel (CH_2Cl_2/MeOH) followed by reverse phase HPLC (C18, H_2O-CH_3CN).

1-(2-deoxy-β-D-ribofuranosyl)imidazole-4-carboxanilide (14): yield 59 mg (39%) as a white powder. Rt 12.64 min [5-50% A in B over 20 min at 1.0 mL/min]; δ_H (DMSO-d6) 2.25 (m, 1H, 2'-H), 2.45 (m, 1H, 2"-H), 3.55 (m, 2H, 5'-H and 5"-H), 3.85 (m, 1H, 4'-H), 4.35 (m, 1H, 3'-H), 5.00 (t, 1H, OH-5'); 5.30 (d, 1H, OH-3'), 6.20 (t, 1H, *J* 7, 1'-H), 7.05 (t, 1H, Ar), 7.30 (t, 2H, Ar), 7.90 (d, 2H, Ar), 8.05 (s, 2H, 5-H and 2-H), 9.95 (s, 1H, *NH*Ar); δ_C (DMSO-d6) 41.24 (C-2'), 61.76 (C-5'), 70.89 (C-3'), 86.23 (C-1'), 88.11 (C-4'), 120.07 (Ar), 121.20 (CH), 123.39 (CH), 128.72 (Ar), 136.74 (CH), 136.96 (Cq); 139.04 (Cq), 160.65 (CONHAr); MS (CI, NH_3), 304, 188.

Ethyl 1-(2-deoxy-β-D-ribofuranosyl)imidazole-4-carboxylate (15): yield 15 mg (12%) as an oil. Rt 9.60 min [5-50% A in B over 20 min at 1.0 mL/min]; δ_H (D_2O) 1.30 (t, 3H, CH_3), 2.50 (m, 2H, 2'-H and 2"-H), 3.75 (m, 2H, 5'-H and 5"-H), 4.05 (m, 1H, 4'-H), 4.30 (q, 2H, CH_2), 4.45 (m, 1H, 3'-H), 6.65 (dd, 1H, *J* 6 and 8, 1'-H), 7.75 and 8.15 (each s, 2H, 5-H and 2-H); MS (CI, NH_3), 257, 141.

1-(2-deoxy-β-D-ribofuranosyl)imidazole-4-carboxamide (2): yield 44 mg (40%) as a white foam. Rt 7.03 min (7.35 min for dZ) [0-15% A in B over 20 min at 1.0 mL/min]; δ_H (D_2O) same data as those obtained by chemical synthesis; δ_H (DMSO-d6) 2.10 (m, 1H, 2'-H), 2.20 (m, 1H, 2"-H), 3.35 (m, 2H, 5'-H and 5"-H), 3.65 (m, 1H, 4'-H), 4.10 (m, 1H, 3'-H), 4.90 (t, 1H, OH-5'), 5.20 (d, 1H, OH-3'), 5.90 (t, 1H, *J* 7, 1'-H), 6.95 and 7.20 (each s, 2H, $CONH_2$), 7.70 and 7.75 (each s, 2H, 2-H and 5-H); δ_C (DMSO-d6) 41.27 (C-2'), 61.86 (C-5'), 71.00 (C-3'), 86.15 (C-1'), 88.10 (C-4'), 120.30 (C-5), 136.67 (C-4),137.00 (C-2), 164.01 ($CONH_2$); MS (CI, NH_3), 228.

Acknowledgements: We are grateful to Dr Jean-Michel Neumann (Laboratoire de Biophysique, CEN Saclay) for performing the NOESY experiments.

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(*Received in Belgium* 6 *March* 1995; *accepted* 24 *June* 1995)