Synthesis of a New N^1-Pentyl Analogue of Cyclic Inosine Diphosphate Ribose (cIDPR) as a Stable Potential Mimic of Cyclic ADP Ribose (cADPR)

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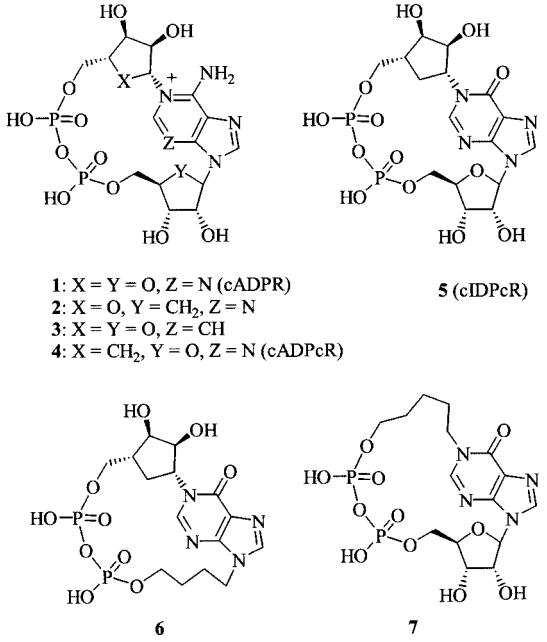
Keywords: cADPR analogues / cIDPR / Nucleotides

The new analogue **7** of cADPR (**1**), a cyclic nucleotide bis(phosphate) involved in Ca^{2+} metabolism, was prepared starting from 2′,3′-isopropylideneinosine (**8**) which was alkylated at N-1, leading to the intermediate **11**. Bis(phosphorylation) of **11** through two alternative procedures, followed by phosphate deprotection steps, afforded derivatives **15** and **16**, the substrates for the intramolecular pyrophosphate bond formation. Both **15** and **16** were converted into derivative **17** in high yields, which was finally deprotected to give the target compound **7**.

Introduction

Cyclic-ADP ribose (cADPR, **1**, Scheme 1), a naturally occurring compound related to NAD^+, has been shown to be a general mediator involved in Ca^{2+} signalling and intracellular mobilization in various cells.[1] This cyclic nucleotide is characterized by a very labile glycosidic bond to N-1 which is rapidly hydrolyzed both enzymatically, by cADP hydrolase, and nonenzymatically, to give ADP-ribose even in a neutral aqueous solution.[2,3] This biological and chemical instability deeply hinders further studies on cADPR aimed at elucidating its physiological role, particularly as far as the regulation of Ca^{2+} mobilization in the cells is concerned. Hence, stable, yet active, cADPR analogues give rise to great interest in this field.

Several enzymes involved in the metabolism of cADPR have been described, among which is the ubiquitous ADP-ribosyl cyclase, first discovered in sea urchin eggs and particularly abundant in *Aplysia californa*.[4] The *Aplysia* cyclase shows a significant catalytic activity in the cyclization of NAD^+ and $NADP^+$ to cADPR and has been utilized to produce new analogues of the natural metabolite from their linear counterparts.[5−8] However, the enzyme specificity severely limits the applicability of enzymatic or chemo-enzymatic procedures. Further analogues have been synthesized and their biological properties investigated.[9−12] Some of these analogues are by far more stable to hydrolysis than the natural metabolite and exhibit interesting biological activity. This is the case, for example, in compounds **2**−**5** where the structural changes are limited to the substitution of one or two atoms of the original metabolite, thus preventing substantial conformational modifications. Particularly in compound **5** (cIDPcR), a stable mimic of cADPR synthesized by Matsuda and co-workers,[10] the adenine base is replaced by the structurally related hypoxanthine whereas a carbocyclic moiety is linked at N-1. In the same paper, a general method for the chemical synthesis of new cADPR analogues is supplied through an extensive study



Scheme 1

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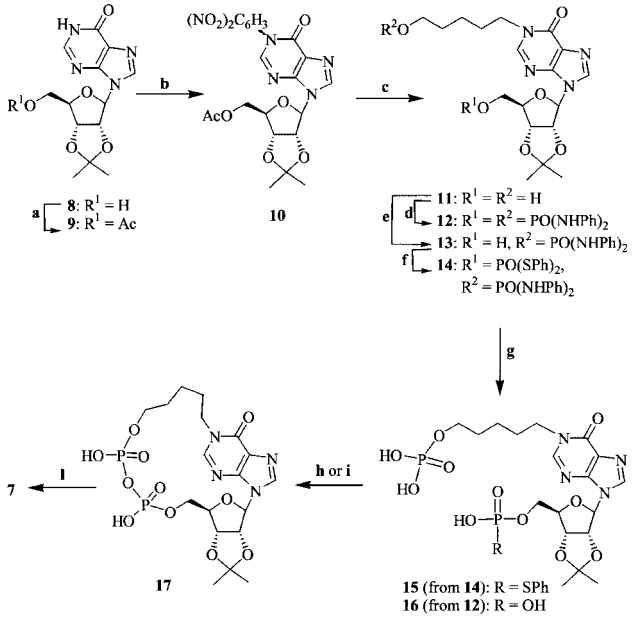
of the pyrophosphate bond formation involved in the cyclization step.

Recently, we reported the synthesis of a new product, **6**,[13] with more severe structural changes than the previously reported cADPR analogues. This compound, designed to investigate the role of the N-9-attached ribosyl moiety in the mechanism of Ca^{+2} intracellular modulation, displays a carbaribosyl and a butyl moiety at N-1 and N-9 of a hypoxanthine base, respectively. We wish to report here the synthesis of a new cIDPR congener **7** having a pentyl chain at N-1 of a hypoxanthine base, which is expected to be resistant to both enzymatic and chemical hydrolysis as similar N^1-alkylated analogues.[14]

Results and Discussion

The synthetic route adopted for **7** (Scheme 2) shows three main steps: i) introduction of the 5-hydroxypentyl chain on N-1 of the protected inosine **9** which leads to **11**; ii) bis(phosphorylation) of derivative **11** through two alternative procedures to give **12** or **14**, the direct precursors of **15** and **16**, respectively; iii) cyclization of derivatives **15** or **16** by pyrophosphate bond formation. For the N^1-alkylation of inosine we utilized the already described procedure based on the reaction of N^1-(2,4-dinitrophenyl)inosine with alkylamine.[15] Thus, acetylation of 2′,3′-isopropylideneinosine (**8**) with Ac_2O in pyridine led to the 5′-*O*-acetyl derivative **9**[16] which was, in turn, converted into the N^1-(2,4-dinitrophenyl) derivative **10** by reaction with 2,4-dinitrochlorobenzene and K_2CO_3 in DMF (90% yields). Compound **10** was obtained as a 1:1 mixture of atropisomers at the N-1 position.[15] Treatment of **10** with 5-aminopentan-1-ol afforded the N^1-(5-hydroxypentyl)inosine derivative **11** (85% yield), with the concomitant aminolysis of the 5′-*O*-acetyl protecting group. The formation of the N^1-alkylated derivative **11** proceeds through a rearrangement of the pyrimidine ring, induced by nucleophilic attack of the amine at C-2, whose electrophilicity was enhanced by the presence of the 2,4-dinitrophenyl group at N-1. Compound **11**, after purification, underwent two distinct phosphorylation procedures leading to the bis(phosphate) derivatives **12** and **14**. Thus, reaction of **11** with a 1.2 molar excess of *N*,*N*′-diphenylphosphorodiamidic chloride[17] afforded a mixture of monophosphate and bis(phosphate) derivatives **13** and **12** in a 9:1 ratio, which were separated by silica gel chromatography. Treatment of **13** with *S*,*S*′-diphenyl dithiophosphate as a cyclohexylammonium salt,[18] in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) and 1*H*-tetrazole[19] gave the protected bis(phosphate) **14** (80% yield). Alternatively, **11** treated with a 3.5 molar excess of $(PhNH)_2POCl$ furnished the bis(phosphate) **12** (85% yield).

Deprotection of the phosphate groups in **14** was achieved by sequential treatments with isoamyl nitrite in pyridine/AcOH/Ac_2O (2:1:1, v/v)[20] and H_3PO_2 [19] in pyridine affording compound **15** (75% yield). The complete deprotection of both phosphate groups in **12**, achieved by treatment with isoamyl nitrite in pyridine/AcOH/Ac_2O, led to **16** (85% yield). Both **15** and **16**, the substrates for the intramolecular condensation, were purified by HPLC on a C_{18} reversed-phase column and characterized as triethylammonium salts by NMR and MS data. Particularly, the proton-decoupled ^{31}P NMR spectrum of **15** showed two singlets at $\delta = 2.3$ and 18.9 ppm attributed to the phosphate and phenylthiophosphate groups, respectively. As expected, in the ^{31}P NMR spectrum of **16** two close signals at $\delta = 0.4$ and -0.1 ppm were present, pertinent to the phosphomonoester functions.



Scheme 2. Reagents and conditions: a) Ac_2O, pyridine, room temp., 6 h; b) 1-chloro-2,4-dinitrobenzene, K_2CO_3, DMF, 80 °C, 2.5 h; c) 5-aminopentan-1-ol, 80 °C, 6 h; d) $(PhNH)_2POCl$ (excess), pyridine, room temp., ca. 12 h; e) $(PhNH)_2POCl$, pyridine, room temp., ca. 12 h; f) *S*,*S*′-diphenyl dithiophosphate, 1*H*-tetrazole, TPSCl, pyridine, N_2, room temp., 8 h; g) isoamyl nitrite, pyridine/AcOH/Ac_2O, room temp., 8 h; h) **15**, I_2, pyridine, 3-A molecular sieves, room temp., 15 h; i) **16**, EDC, MPD, room temp.; 60 h; l) aq. HCO_2H, room temp., 3.5 h

Two alternative intramolecular condensation reactions were performed to cyclize derivatives **15** and **16**. In the former method **15**, dissolved in pyridine, was added over 15 h to I_2, dissolved in the same solvent, in the presence of activated molecular sieves (3 Å).[21] HPLC purification on a C_{18} reversed-phase column furnished the cyclic product **17** (60% isolated yield). In the latter case the intramolecular condensation reaction of the two phosphate groups of **16** was performed by addition of a slight excess of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) in *N*-methylpyrrolidone (MPD) and allowing the mixture to stand at 50 °C for 60 h. The desired cyclic product **17** was obtained in 80% yield after HPLC purification.

In the light of the literature data, the above results are rather surprising. Although a number of well-established procedures have been described for the formation of a pyrophosphate bridge, most of the available data refer to inter-

molecular reactions with only a few papers dealing with cyclization processes involving an intramolecular condensation of two phosphate groups. From these data, it was apparent that the following factors play a major role in regulating the course of the reaction: i) the size of the cycle; ii) the conformation adopted by the linear precursor; iii) the electrostatic repulsion between the two phosphate species to be condensed. Studies on the synthesis of compounds **4** and **5** seem to indicate the electrostatic repulsion as the dominant factor in determining the cyclization yields. In fact, very low (23%) or not detectable yields are reported for the preparation of **5** through the cyclization with EDC of the linear precursors possessing favorable or nonfavorable conformational requisites (*syn* or *anti* conformation around the glycosidic bond, respectively). On the other hand, very high yields were observed if the cyclization was performed minimizing the charge repulsion between the two phosphates, independently from the conformation. On the contrary, our finding suggests that the electrostatic repulsion is not so crucial, at least in the cyclization of linear precursors characterized by high conformational flexibility, as in the case of **15** and **16**. In fact, in the procedure adopted for compound **15**, neutral, reactive metaphosphate species are generated, whereas cyclization of **16** involves two charged phosphomonoester functions, one of them activated by EDC. Nevertheless, a higher yield for the cyclization is observed for **16** than for **15**.

The structure of **17** was confirmed by ^{1}H, ^{13}C, ^{31}P NMR and ESI-MS data. Particularly, the ^{31}P NMR spectrum showed two broad singlets at $\delta = -10.4$ and -11.5 ppm, which are diagnostic for a pyrophosphate moiety.

Final removal of the isopropylidene protecting group, performed by treating **17** with aqueous HCO_2H at room temperature for 3.5 h, furnished **7** (85%, after purification). Product **7** was purified by HPLC on a C_{18} reversed-phase column and its structure was confirmed by ^{1}H and ^{31}P NMR and MS data.

In conclusion, we have reported the high-yield synthesis of a new stable analogue **7** of the cIDPR carrying a pentyl chain at N-1 of inosine, which substitutes the ribosyl moiety. In the synthesis of this compound, characterized by high conformational flexibility, we tested two condensation procedures on the linear bis(phosphate) derivatives **15** and **16** to obtain the closure of a 19-membered ring, by a pyrophosphate bond formation. The surprisingly and unprecedented high cyclization yields observed using EDC as a condensing agent of the two phosphomonoester functions of the linear precursor **16**, indicates this reaction as an efficient and easy method to obtain large rings characterized by a high conformational mobility.

Experimental Section

General Methods: ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ with a Bruker WM 500 spectrometer. Residual proton and carbon signals of the solvent ($CDCl_3$: $\delta = 7.24$ ppm and 77.5; CD_3OD: $\delta =$ and 77.5; D_2O: $\delta = 4.80$ ppm) were used as internal references. ^{31}P NMR spectra were recorded with a Bruker WM 400 spectrometer (85% H_3PO_4 as an external standard). NMR signals were assigned to the pertinent nuclei through two-dimensional ^{1}H-^{1}H and ^{1}H-^{13}C COSY experiments. Mass spectra were registered with a Finnigan MAT instrument. General reagents and solvents were purchased from Sigma-Aldrich-Fluka. UV measurements were carried out with a Jasco V-530 UV spectrophotometer. HPLC purifications were performed on a Nucleosil C18 column (Macherey−Nagel, 250/10, 7 μm). The following abbreviations were used throughout the text: TPSCl = 2,4,6-triisopropylbenzenesulfonyl chloride, EDC = 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, MPD = *N*-methylpyrrolidone, DMF = *N*,*N*-dimethylformamide, TEAA = triethylammonium acetate, TEAB = triethylammonium bicarbonate, DNP = 2,4-dinitrophenyl, s's = singlets, d's = doublets, m's = multiplets, q's = quadruplets.

5′-*O*-Acetyl-2′,3′-*O*-isopropylideneinosine (9): Commercially available 2′,3′-*O*-isopropylideneinosine (**8**) (1.0 g, 3.25 mmol) was treated with Ac_2O/pyridine solution (6:4 v/v, 10 mL). After 6 h at room temperature, the dried mixture was dissolved in $CHCl_3$ and washed with water. The dried organic layer gave **9**[16] (1.08 g, 95%) as a white solid.

5′-*O*-Acetyl-1-(2,4-dinitrophenyl)-2′,3′-*O*-isopropylideneinosine (10): A mixture of **9** (1.0 g, 2.86 mmol), 1-chloro-2,4-dinitrobenzene (1.15 g, 5.72 mmol) and K_2CO_3 (790 mg, 5.72 mmol) was stirred in anhydrous DMF (8.0 mL) at 80 °C for 2.5 h. After cooling, the mixture was filtered and the solid was washed with $CHCl_3$. The filtrate and washings, concentrated to dryness under reduced pressure, were purified on a silica gel column eluted with an increasing amount of CH_3OH in $CHCl_3$ (from 0 to 5%) to give **10** (1.33 g, 90%) as a 1:1 mixture of atropisomers at the N-1−phenyl bond as a pale yellow amorphous solid. M.p. 204−207 °C (from CH_3OH). ^{1}H NMR ($CDCl_3$): $\delta = 9.07$ (s, 1 H, 3-H DNP), 8.65 (d, $J =$ 9.0 Hz, 1 H, 5-H DNP), 8.04, 8.03, 7.98, 7.95 (s's, 0.5 H each, 2-H and 8-H), 7.72 and 7.70 (d's, 0.5 H, each $J = 9.0$ Hz, 6-H DNP), 6.12 and 6.14 (s's, 0.5 H each, 1′-H), 4.95 (m, 1 H, 3′-H), 4.56 and 4.51 (m's, 0.5 H each, 4′-H), 4.34 and 4.26 (m's, 1 H each, 5′-H_2), 2.27 (m, 1 H, 2′-H), 2.05 (s, 3 H, CH_3CO) 1.63 and 1.40 (s's, 3 H each, isopropylidene) ppm. ^{13}C NMR ($CDCl_3$): $\delta = 170.1$, 158.0, 152.8, 151.4, 149.9, 144.9, 143.0, 142.2, 129.1, 126.7, 121.9, 121.8, 114.7, 91.3, 84.9, 84.2, 81.3, 63.8, 27.0, 25.1, 20.4 ppm. ESI-MS: calcd. for $C_{21}H_{20}N_6O_{10}$ 516.12, found 517 $[M + H]^+$. UV ($CHCl_3$): $\lambda_{max} = 249$ nm.

1-(5-Hydroxypentyl)-Substituted Compound 11: A solution of **10** (1.0 g, 1.94 mmol) in dry DMF (8.0 mL) was treated with 5-aminopentan-1-ol (2.0 g, 19.4 mmol) at 80 °C for 6 h whilst stirring. The resulting solution, dried under reduced pressure, was purified on a silica gel column eluted with an increasing amount of CH_3OH in $CHCl_3$. The fractions eluted with 15% CH_3OH furnished **11** (0.65 g, 85%) as a white amorphous solid which could not be induced to crystallize. ^{1}H NMR ($CDCl_3$): $\delta = 7.99$ and 7.87 (s, 1 H each, 2-H and 8-H), 5.85 (d, $J = 4.5$ Hz, 1 H, 1′-H), 5.07 and 5.04 (m's, 1 H each, 2′-H and 3′-H), 4.49 (br. s, 1 H, 4′-H), 4.05 (t, $J =$ 7.2 Hz, 2 H, CH_2N), 3.94 (d, $J = 12.4$ Hz, 1 H, 5′-H_a), 3.77 (m, 1 H, 5′-H_b), 3.62 (t, $J = 6.3$ Hz, 2 H, CH_2O), 1.80 (m, 2 H, pentyl chain methylene), 1.61 (s, 3 H, isopropylidene), 1.57 and 1.43 (m's, 2 H each, pentyl chain methylene groups), 1.35 (s, 3 H, isopropylidene) ppm.^{13}C NMR ($CDCl_3$): $\delta = 158.2$, 149.7, 148.6, 141.3, 125.3, 115.3, 92.3, 88.5, 85.9, 82.9, 63.4, 62.9, 48.0, 33.2, 30.5, 27.6, 25.6, 24.3 ppm. ESI-MS: calcd. for $C_{18}H_{26}N_4O_6$ 394.18, found 395 $[M + H]^+$. UV (CH_3OH): $\lambda_{max} = 247$, 251, shoulder 268 nm.

5′-*O*-(Dianilinophosphoryl)-1-[5-*O*-(dianilinophosphoryl)pentyl]-2′,3′-*O*-isopropylideneinosine (12): $(PhNH)_2POCl$ (1.18 g, 4.43 mmol) was added to **11** (500 mg, 1.27 mmol), dissolved in dry pyridine (4.0 mL), and the solution was stirred at room temperature overnight. The resulting mixture, dried under reduced pressure, was chromatographed on a silica gel column eluted with an increasing amount of CH_3OH in $CHCl_3$. The fractions eluted with 5% CH_3OH furnished **12** (0.92 g, 85%) as a pale yellow amorphous solid which could not be induced to crystallize. ^1H NMR ($CDCl_3$): δ = 7.81 and 7.51 (s's, 1 H each, 2-H and 8-H), 7.15−6.25 (complex signals, 20 H, phenyl groups), 6.97 (br. s, 1 H, 1′-H), 6.66 and 6.53 (d's, 2 H each, *J* = 7.3, 7.3 Hz, NH), 5.21 (m, 1 H, 2′-H), 5.24 (m, 1 H, 3′-H), 4.51 (m, 1 H, 4′-H), 4.06 (complex signals 4 H, CH_2N and 5′-H_2), 1.66, 1.55 and 1.29 (m's, 2 H each, pentyl chain methylene groups), 1.60 and 1.31 (s's, 3 H each, isopropylidene) ppm. ^{13}C NMR ($CDCl_3$): δ = 156.6, 147.7, 146.7, 139.8, 140.2, 140.0, 129.5, 129.3, 125.6, 122.1, 121.9, 118.2, 118.1, 114.6, 91.9, 86.1, 84.8, 82.4, 65.7, 64.8, 46.9, 29.5, 28.7, 27.3, 25.4, 22.8 ppm. ^{31}P NMR ($CDCl_3$): δ = 6.9, 6.4 ppm. ESI-MS: calcd. for $C_{42}H_{48}N_8O_8P_2$ 854.31, found 855 $[M + H]^+$. UV (CH_3OH): λ_{max} = 276, 250 nm.

1-[5-*O*-(Dianilinophosphoryl)pentyl]-2′,3′-*O*-isopropylideneinosine (13): $(PhNH)_2POCl$ (405 mg, 1.52 mmol) was added to **11** (500 mg, 1.27 mmol), dissolved in dry pyridine (5.0 mL), and the solution was stirred at room temperature overnight. The resulting mixture was dried under reduced pressure and chromatographed on a silica gel column eluted with an increasing amount of CH_3OH in $CHCl_3$. The fraction eluted with 15% of CH_3OH furnished **13** (0.55 g, 70%) as a yellow oil and **12** (0.08 g, 8%) was also recovered from the column. ^1H NMR ($CDCl_3$): δ = 8.00 and 7.88 (s's, 1 H each, 2-H and 8-H), 7.15−6.87 (complex signals, 10 H, phenyl groups), 6.18 (br. s, 2 H, NH), 5.88 (d, *J* = 4.0 Hz, 1 H, 1′-H), 5.08 (dd, *J* = 4.0 and = 5.1 Hz, 1 H, 2′-H) 5.03 (dd, *J* = 5.1, 5.1 Hz, 1 H, 3′-H), 4.48 (br. s, 1 H, 4′-H), 4.09 (m, 2 H, CH_2O), 3.90 (complex signal, 3 H, 5′-H_a and CH_2N), 3.76 (d, *J* = 12.0 Hz, 1 H, 5′-H_b), 1.68 (m, 4 H, pentyl chain methylene groups), 1.61 (s, 3 H, isopropylidene), 1.39 (m, 2 H, pentyl chain methylene), 1.35 (s, 3 H, isopropylidene) ppm. ^{13}C NMR ($CDCl_3$): δ = 155.5, 147.5, 146.2, 140.1, 139.8, 129.5, 126.4, 122.1, 118.5, 114.2, 93.7, 86.3, 84.0, 81.6, 66.1, 63.3, 47.0, 29.8, 29.1, 27.8, 25.5, 22.8 ppm. ^{31}P NMR ($CDCl_3$): δ = 6.2 ppm. ESI-MS: calcd. for $C_{30}H_{37}N_6O_7P$ 624.25, found 625 $[M + H]^+$. UV (CH_3OH): λ_{max} = 275, 250 nm.

1-[5-*O*-(Dianilinophosphoryl)pentyl]-2′,3′-*O*-isopropylidene-5′-*O*-[bis(phenylthio)phosphoryl]inosine (14): *S*,*S*′-Diphenyl dithiophosphate (cyclohexylammonium salt, 350 mg, 0.92 mmol) and TPSCl (695 mg, 2.3 mmol) were added to a solution of **13** (500 mg, 0.81 mmol) in dry pyridine (5.0 mL) and the mixture was stirred for 8 h at room temperature under N_2. The mixture was dried under reduced pressure and the residue was purified on a silica gel column by eluting with an increasing amount of CH_3OH in $CHCl_3$. The fraction eluted with 5% of CH_3OH furnished **14** (0.57 g, 80%) a yellow oil. ^1H NMR (CD_3OD): δ = 7.87 and 7.70 (s's, 1 H each, 2-H and 8-H), 7.50−7.18 (complex signals, 10 H, PhN), 7.16−6.79 (complex signals, 10 H, PhS), 6.10 (m, 2 H, 2NH), 6.01 (br. s, 1 H, 1′-H), 5.09 (m, 1 H, 2′-H), 4.89 (m, 1 H, 3′-H), 4.38 (m, 3 H, 4′-H and CH_2O), 4.08 (m, 2 H, 5′-H_2), 3.90 (m, 2 H, CH_2N), 1.68 (m, 4 H, pentyl chain methylene groups), 1.58 (s, 3 H, isopropylidene), 1.35 (m, 2 H, pentyl chain methylene), 1.30 (s, 3 H, isopropylidene) ppm. ^{13}C NMR (CD_3OD): δ = 156.5, 147.5, 147.0, 140.1, 144.1, 136.2, 129.2, 128.1, 127.9, 125.8, 124.4, 122.0, 118.0, 114.9, 91.4, 85.2, 84.9, 81.9, 66.5, 65.5, 46.8, 29.8, 29.0, 27.6, 25.2, 22.5 ppm. ^{31}P NMR (CD_3OD): δ = 49.8, 5.9 ppm. ESI-MS: calcd. for $C_{42}H_{46}N_6O_8P_2S_2$ 888.23, found 889 $[M + H]^+$. UV (CH_3OH): λ_{max} = 270, 256, 235 nm.

2′,3′-*O*-Isopropylidene-5′-*O*-[(phenylthio)phosphoryl]-1-[5-*O*-(phosphoryl)pentyl]inosine (15): A mixture of **14** (60 mg, 0.068 mmol) and isoamyl nitrite (200 μL, 3.3 mmol) in pyridine/AcOH/Ac_2O (2:1:1, v/v, 1.5 mL) was stirred at room temperature for 8 h. The mixture, dried under reduced pressure (at < 50 °C), was dissolved in a mixture of H_3PO_2 (70 μL, 4.3 mmol), Et_3N (292 μL, 2.1 mmol) and pyridine (5.5 mL) and the resulting solution was stirred at room temperature for 11 h. The mixture was dried under reduced pressure (at < 50 °C) and the residue, dissolved in H_2O, was partitioned between $CHCl_3$ and H_2O. Pyridine (11.0 mL) was added to the aqueous layer and the resulting solution was dried under reduced pressure (at < 50 °C). The resulting solid residue was dissolved in a TEAA buffer (0.1 M, pH = 7.0) and chromatographed on a C_{18} reversed-phase HPLC column, eluted with a linear gradient from 0 to 30% of CH_3CN in a TEAA buffer (0.1 M, pH = 7.0) in 30 min, flow 2.0 mL/min. The product with retention time 12.2 min was concentrated under reduced pressure and excess of TEAA was removed by C_{18} reversed-phase HPLC column eluted with 30% of aqueous CH_3CN to give **15** (0.04 g, 75%) as a mono(-triethylammonium) salt as a white amorphous solid. M.p. > 230 °C (dec., from CH_3OH). ^1H NMR (D_2O): δ = 8.32 and 8.22 (s, 1 H each, 2-H and 8-H), 7.25−7.05 (complex signals, 5 H, PhS), 6.31 (br. s, 1 H, 1′-H), 5.48 (m, 1 H, 2′-H), 5.11 (m, 1 H, 3′-H), 4.69 (m, 1 H, 4′-H), 4.25 and 4.13 (m's, 1 H each, CH_2O), 4.05 (m, 2 H, 5′-H_2), 3.85 (m, 2 H, CH_2N), 3.21 (q, *J* = 7.2 Hz, 6 H, CH_2 triethylammonium), 1.74−1.63 (m's, 4 H, pentyl chain methylene groups), 1.44 and 1.41 (s's, 3 H each, isopropylidene), 1.35 (m, 2 H, pentyl chain methylene), 1.17 (t, 9 H, *J* = 7.2 Hz, CH_3 triethylammonium) ppm. ^{13}C NMR (D_2O): δ = 158.0, 149.7, 147.5, 140.2, 134.4, 129.7, 127.2, 126.1, 125.0, 115.1, 92.3, 86.4, 85.5, 82.6, 66.9, 65.7, 59.8, 47.7, 31.4, 30.4, 27.5, 25.8, 24.0, 8.8 ppm. ^{31}P NMR (D_2O): δ = 18.9, 2.3 ppm. ESI-MS: calcd. for $C_{24}H_{32}N_4O_{11}P_2S$ 646.13, found 647 $[M + H]^+$. UV (H_2O): λ_{max} = 244, shoulder 268 nm.

2′,3′-*O*-Isopropylidene-5′-*O*-phosphoryl-1-[5-*O*-(phosphoryl)-pentyl]inosine (16): A mixture of **12** (800 mg, 0.94 mmol) and isoamyl nitrite (1.2 mL, 6.1 mmol) in pyridine/AcOH/Ac_2O (2:1:1, v/v, 15 mL) was stirred at room temperature for 8 h. The mixture was dried under reduced pressure and the residue, dissolved in a TEAA buffer (0.1 M, pH = 7.0), was purified by a C_{18} reversed-phase HPLC column, eluted with a linear gradient from 0 to 50% of CH_3CN in a TEAA buffer (0.1 M, pH = 7.0) in 45 min. The product with retention time 22.9 min was concentrated and excess of TEAA was removed by C_{18} reversed-phase HPLC column eluted with 30% aqueous CH_3CN to give **16** (0.52 g, 85%) as a mono(triethylammonium) salt as a white amorphous solid. M.p. > 230 °C (dec., from CH_3CH_2OH). ^1H NMR (D_2O): δ = 8.37 and 8.36 (s, 1 H each, 2-H and 8-H), 6.27 (br. s, 1 H, 1′-H), 5.40 (m, 1 H, 2′-H), 5.17 (m, 1 H, 3′-H), 4.61 (m, 1 H, 4′-H), 4.13 and 4.09 (m's, 2 H each, 5′-H_2 and CH_2O), 3.83 (m, 2 H, CH_2N), 3.19 (q, *J* = 7.1 Hz, 6 H, CH_2 triethylammonium), 1.80 and 1.68 (m's, 4 H, pentyl chain methylene groups), 1.65 and 1.43 (s's, 3 H each, isopropylidene), 1.40 (m, 2 H, pentyl chain methylene), 1.22 (t, *J* = 7.1 Hz, 9 H, CH_3 triethylammonium) ppm. ^{13}C NMR (D_2O): δ = 158.7, 149.4, 148.1, 140.8, 124.0, 115.4, 94.7, 91.3, 85.8, 81.8, 65.8, 65.0, 47.5, 42.6, 29.8, 28.7, 26.1, 24.3, 22.1, 9.2 ppm. ^{31}P NMR (D_2O): δ = 0.4, −0.1 ppm. ESI-MS: calcd. for $C_{18}H_{28}N_4O_{12}P_2$ 554.12, found 553 $[M - H]^-$. UV (H_2O): λ_{max} = 250 nm.

2′,3′-Isopropylidene-Substituted Cyclopyrophosphate Compound 17 from 15: A solution of **15** (20 mg, 0.027 mmol) in pyridine (20.0 mL) was added slowly over 15 h, using a syringe pump, to a mixture of I_2 (125 mg, 0.475 mmol) and dried 3-Å molecular sieves

(1.2 g) in pyridine (20 mL) at room temperature in the dark. After filtering and washing, the combined filtrate and washings were concentrated to dryness. The residue was dissolved in a TEAA buffer (0.1 M, pH = 7.0) and purified by a C_{18} reversed-phase HPLC column eluted with a linear gradient (from 0 to 70% in 70 min) of CH_3CN in a TEAA buffer (0.1 M, pH = 7.0) flow 2.0 mL/min. The fractions with retention time 28.8 min were concentrated to dryness and excess of TEAA was removed by C_{18} reversed-phase HPLC column eluted with 40% of aqueous CH_3CN to give **17** (0.01 g, 60%) as a bis(triethylammonium) salt as a white amorphous solid which could not be induced to crystallize. ^{1}H NMR (D_2O): δ = 8.43 and 8.22 (s, 1 H each, 2-H and 8-H), 6.37 (br. s, 1 H, 1′-H), 5.82 (m, 1 H, 2′-H), 5.46 (m, 1 H, 3′-H), 4.56 (m, 1 H, 4′-H), 4.08 and 3.93 (m's, 2 H each, CH_2O and CH_2N), 3.77 (m, 2 H, 5′-H_2), 3.20 (q's, 12 H, J = 7.2 Hz, CH_2 triethylammonium), 1.91 (m, 2 H, pentyl chain methylene), 1.65 (s, 3 H, isopropylidene), 1.58 (m, 2 H, pentyl chain methylene), 1.47 (s, 3 H, isopropylidene), 1.35 (m, 2 H, pentyl chain methylene), 1.23 (t's, 18 H, J = 7.2 Hz, CH_3 triethylammonium) ppm. ^{13}C NMR (D_2O): δ = 158.0, 148.8, 147.3, 142.2, 124.2, 114.4, 91.2, 87.3, 83.8, 82.1, 65.9, 65.3, 47.3, 46.7, 29.0, 26.5, 25.8, 24.2, 21.6, 8.1 ppm. ^{31}P NMR (D_2O): δ = 10.4, −11.5 ppm. ESI-MS: calcd. for $C_{18}H_{26}N_4O_{11}P_2$ 536.11, found 537 $[M + H]^+$. UV (H_2O): λ_{max} = 249 nm.

Product 17 from 16: EDC (151 mg, 0.79 mmol) was added to a solution of **16** (100 mg, 0.15 mmol) in MDP (20 mL) and the mixture stirred at room temperature for 60 h. The solvents were evaporated under reduced pressure and the residue was dissolved in 20 mL of a TEAA buffer (0.1 M, pH = 7.0). The solution was purified and desalted by a C_{18} reversed-phase HPLC column, as described before for the same compound to give **17** (0.09 g, 80%) as a bis(triethylammonium) salt. Spectroscopic data are identical to product **17** obtained from **15**.

Cyclopyrophosphate Compound 7: A solution of **17** (56 mg, 0.076 mmol) in aqueous HCO_2H (60%, 2.0 mL) was stirred at room temperature for 3.5 h. After the solvent was evaporated under reduced pressure, the residue, dissolved in H_2O, was purified by a C_{18} reversed-phase HPLC column, eluted with a linear gradient (from 0 to 40% in 80 min) of CH_3CN in a TEAB buffer (0.1 M, pH = 7.0), flow 2.0 mL/min. The fractions with retention time 24.4 min were concentrated under reduced pressure and excess of TEAB was coevaporated with H_2O. The residue was freeze-dried to give **7** (0.05 g, 85%) as a bis(triethylammonium) salt as a white amorphous solid. M.p. > 230 °C (dec., from CH_3CH_2OH). ^{1}H NMR (D_2O): δ = 8.39 and 8.21 (s, 1 H each, 2-H and 8-H), 6.04 (br. s, 1 H, 1′-H), 5.38 (m, 1 H, 2′-H), 4.50 (m, 1 H, 3′-H), 4.31 (m, 1 H, 4′-H), 3.96 and 3.84 (m's, 2 H each, CH_2O and CH_2N), 3.76 (m, 2 H, 5′-H_2), 3.20 (q's, J = 7.0 Hz, 12 H, CH_2 triethylammonium), 1.85, 1.58 and 1.32 (m's, 2 H each, methylene groups), 1.21 (t's, J = 7.0 Hz, 18 H, CH_3 triethylammonium) ppm. ^{13}C NMR (D_2O): δ = 158.4, 148.6, 147.5, 142.1, 125.2, 90.4, 84.1, 72.1, 70.5, 66.1, 65.4, 59.0, 47.0, 29.1, 26.3, 21.5, 8.7 ppm. ^{31}P NMR (D_2O): δ = −10.2, −11.1 ppm. ESI-MS: calcd. for $C_{15}H_{22}N_4O_{11}P_2$ 496.08, found 495 $[M - H]^-$. UV (H_2O): λ_{max} = 249 nm (10100), shoulder 266 (5400).

Acknowledgments

This work was supported by the Italian M.U.R.S.T. (PRIN 2001) and Regione Campania (L. 41). The authors are grateful to "Centro Ricerche Interdipartimentale di Analisi Strumentale", C.R.I.A.S., for supplying NMR and MS facilities.

[1] H. C. Lee, *Annu. Rev. Pharmacol. Toxicol.* **2001**, *41*, 317−345.
[2] A. Galione, *Science* **1993**, *259*, 325−326.
[3] A. H. Guse, *Cell Signalling* **1999**, *11*, 309−316.
[4] R. Aarhus, M. R. Graeff, D. M Dickey, T. F. Walseth, H. C. Lee, *J. Biol. Chem.* **1995**, *270*, 30327−30333.
[5] R. Zocchi, L. Franco, L. Guida, U. Benfatti, A. Bargellesi, E. Malavasi, H. C. Lee, A. De Flora, *Biochem. Biophys. Res. Commun.* **1993**, *196*, 1459−1465.
[6] K. Inageda, K. Takahashi, K. Tokita, H. Nishina, Y. Kanaho, I. Kukimoto, K. Kontani, S. Hoshino, T. J. Katada, *Biochemistry (Tokio)* **1995**, *117*, 125−131.
[7] L. Guida, L. Franco, R. Zocchi, S. Buzzone, U. Benfatti, G. Damonte, H. C. Lee, *J. Biol. Chem.* **1997**, *272*, 12945−12951.
[8] Y. Fukushi, I. Kato, S. Takasawa, T. Sasaki, B. H. Ong, M. Sato, A. Ohsaga, K. Sato, H. Okamoto, Y. Maruyama, *J. Biol. Chem.* **2001**, *276*, 649−655.
[9] S. Shuto, M. Shirato, Y. Sumita, Y. Ueno, A. Matsuda, *J. Org. Chem.* **1998**, *52*, 1986−1994.
[10] M. Fukuoka, S. Shuto, N. Minakawa, Y. Ueno, A. Matsuda, *J. Org. Chem.* **2000**, *65*, 5238−5248.
[11] S. Shuto, M. Fukuoka, A. Manikowsky, Y. Ueno, T. Nakano, R. Kuroda, H. Kuroda, A. Matsuda, *J. Am. Chem. Soc.* **2001**, *123*, 8750−8759.
[12] T. F. Walseth, H. C. Lee, *Biochim. Biophys. Acta* **1993**, *1178*, 235−242.
[13] A. Galeone, L. Mayol, G. Oliviero, G. Piccialli, M. Varra, *Tetrahedron* **2002**, *58*, 363−368.
[14] F. Zhang, Q. Gu, C. J. Sih, *Bioorg. Med. Chem.* **1999**, *7*, 653−664.
[15] L. De Napoli, A. Messere, D. Montesarchio, G. Piccialli, M. Varra, *J. Chem. Soc., Perkin Trans. 1* **1997**, 2079−2082.
[16] K. H. Scheit, *Angew. Chem. Internat. Edit.* **1967**, *6*, 180−181.
[17] J. Smrt, *Tetrahedron Lett.* **1973**, *47*, 4727−4728.
[18] T. Hata, K. Yamaguchi, S. Honda, I. Nakagawa, *Chem. Lett.* **1978**, 507−508.
[19] M. Sekine, J. Matsuzaki, T. Hata, *Tetrahedron Lett.* **1981**, *22*, 3209−3212.
[20] M. Sekine, T. Hata, *Tetrahedron Lett.* **1983**, *24*, 5741−5744.
[21] Y. Nakagawa, S. Koni, S. Ohtani, T. Hata, *Synthesis* **1980**, 556−557.

Received July 9, 2002
[O02372]