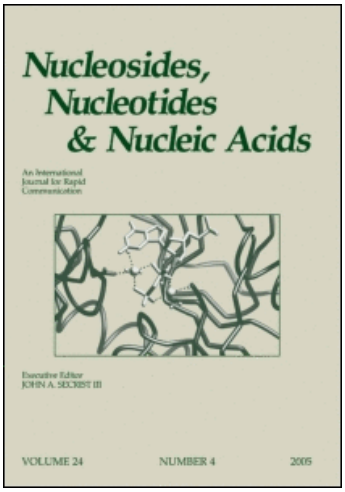

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ALKALINE DEGRADATION OF 1,3-DI-(2-HYDROXYETHYL)ADENOSINE 3',5'-CYCLIC PHOSPHATE. STUDIES ON THE REACTION PRODUCTS

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Abstract

1,3-Di-(2-hydroxyethyl)adenosine 3',5'-cyclic phosphate (1) in 1 M NaOH failed to undergo the expected Dimroth rearrangement. Rather, pyrimidine ring opening followed by loss of ethylene oxide and formate yielded 5-amino-1-(β-D-ribofuranosyl)imidazole-4-(N-(2-hydroxyethyl)-carboxamidine) 3',5'-cyclic phosphate (2) as the major product. The 3'-monophosphate (3) and 2'-O-hydroxyethyl (4) derivatives of 2 were also isolated and characterized.

INTRODUCTION

Adenosine 3',5'-cyclic phosphate (cAMP) is well documented as an important effector molecule which participates in the regulation of many biochemical reactions (1). It seems possible that alkylating agents such as ethylene oxide, which are known mutagens, (2) may target cAMP. We have therefore been involved with elucidating the characteristics of cAMP alkylation.

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We have recently pointed out (3) that cAMP triethylammonium salt reacts with ethylene oxide at ∿5°C to afford 1-(2-hydroxyethyl)-cAMP instead of cAMP-P-O-(2-hydroxyethyl) ester as claimed earlier in the literature (4). Besides experimental NMR evidence (proton coupled ^{13}C spectra), the structure of the monoalkylated cAMP was also confirmed by noting its behavior upon exposure to mild alkaline conditions (NH_4HCO_3 in D_2O, pD 10, 50°C, 24 h). N^6-(2-hydroxyethyl)-cAMP (isolated as the NH_4^+ salt) was obtained via the Dimroth rearrangement (5). At ambient temperature the triethylammonium salt of cAMP also afforded 1,3-di-(2-hydroxyethyl)-cAMP (1) as the second major product in the reaction with ethylene oxide. The structure of 1, sole representative of the potential 1,3-disubstituted-cAMP's, was also verified by its proton coupled ^{13}C NMR spectrum (3). In this communication we describe the unique behavior of 1 towards treatment with hydroxide ion, leading to pyrimidine ring opened imidazole derivatives 2 - 4 (Reaction Scheme).

RESULTS AND DISCUSSION

In mild alkali (aq. NaOH, pH 10, room temperature) compound 1 remained unchanged even after 24 h. But when dissolved in 1 M sodium hydroxide at ambient temperature all the starting material disappeared within about 2 days and several new compounds were present in the reaction mixture as evidenced by silica gel and cellulose TLC. A partial separation of the anionic mixture was achieved on a DEAE Sephadex A-25 anion exchange column using salt gradient elution. Finally four samples were isolated by means of cellulose TLC. The Dimroth rearrangement product, 3,N^6-di-(2-hydroxyethyl)cAMP was not detected.

By way of comparison in 1 M NaOH at room temperature 1-(2-hydroxyethyl)cAMP rearranged fast (1 day) to N^6-(2-hydroxyethyl)cAMP, but no further transformation was observed even after a prolonged (8 days) reaction time. cAMP was actually stable in 1 M NaOH (3 days, ambient temperature). However, after 7-8 days definite decomposition, very likely to the different mononucleotides, could be observed by TLC.

The structure of the three major alkaline degradation products of 1 were determined basically by ^1H and ^{13}C NMR spectroscopy. Molecular weight by FAB MS and uv characteristics were also determined. Subsequent to MS analysis the sample in lowest amount proved to be a mixture of 2-3 compounds by TLC and NMR spectroscopy. Presumably this was an unstable

compound and therefore the structures of the components were not determined.

Discussion of NMR Results

(Compound 2; Reaction Scheme, Table 1 and Figs. 1 and 2). Note that numbering of the product heterocycle conforms to that of the purine ring of the starting material. The 270 MHz ribose proton spectrum (Fig. 1) illustrates that the cyclic phosphate is intact with the 3'-carbon endo such that $^{3}J_{1'2'}$ is very small (6). Moreover, a grouping of adjacent methylenes of a hydroxyethyl substituent is evident from the two triplets at δ 3.44 and 3.68 ppm, each integrating for two protons. The β methylene is assigned to lower field due to the attached hydroxyl. That the adenine ring structure is broken is indicated by the fact that only one low field resonance is seen at δ 7.40 ppm.

The proton broad band decoupled 67.8 MHz carbon-13 spectrum (Fig. 2) confirms the suggestions from the proton spectra that 1) the cyclic phosphate exists, 2) two aliphatic carbons are present and 3) only four low field base carbons are found, illustrating that the adenine ring is broken, most likely in the pyrimidine portion. Ribose carbon assignments were those of Uesugi, et al. (7). Also note in Fig. 2 the two and three bond PC couplings at C2', C3' and C4'.

Specific proton decoupling during ^{13}C-observe was used to assign the position of the hydroxyethyl substituent (to N1 or N3). In the proton coupled spectrum , C8 and C5 are readily assigned from $^{1}J_{C8H8}$ (216 Hz) and $^{3}J_{C5H8}$ (9 Hz) couplings. C6 and C4 were distinguishable by specific irradiation of H-1'. Here the higher field resonance is simplified, therefore C4, whereas the lowest field peak is unchanged, therefore C6. When the α-methylene protons were irradiated, the C6 triplet at 169 ppm was collapsed to a singlet, thus the hydroxyethyl group is attached to N1. We write 2 as the N^{6}-imino tautomer since in DMSO a triplet (J = 12 Hz) one proton exchangeable resonance (presumably N1H) is observed $\sim$0.2 ppm downfield from H-8.

Compound 3 (Table 2):

Comparison of the proton spectrum of compound 3 with that of compound 2 illustrates that in the former, H-3' shifts downfield about 0.2 ppm, H-4' shifts upfield 0.2 ppm, H-5', 5'' shift upfield 0.45 ppm and there is a

REACTION SCHEME

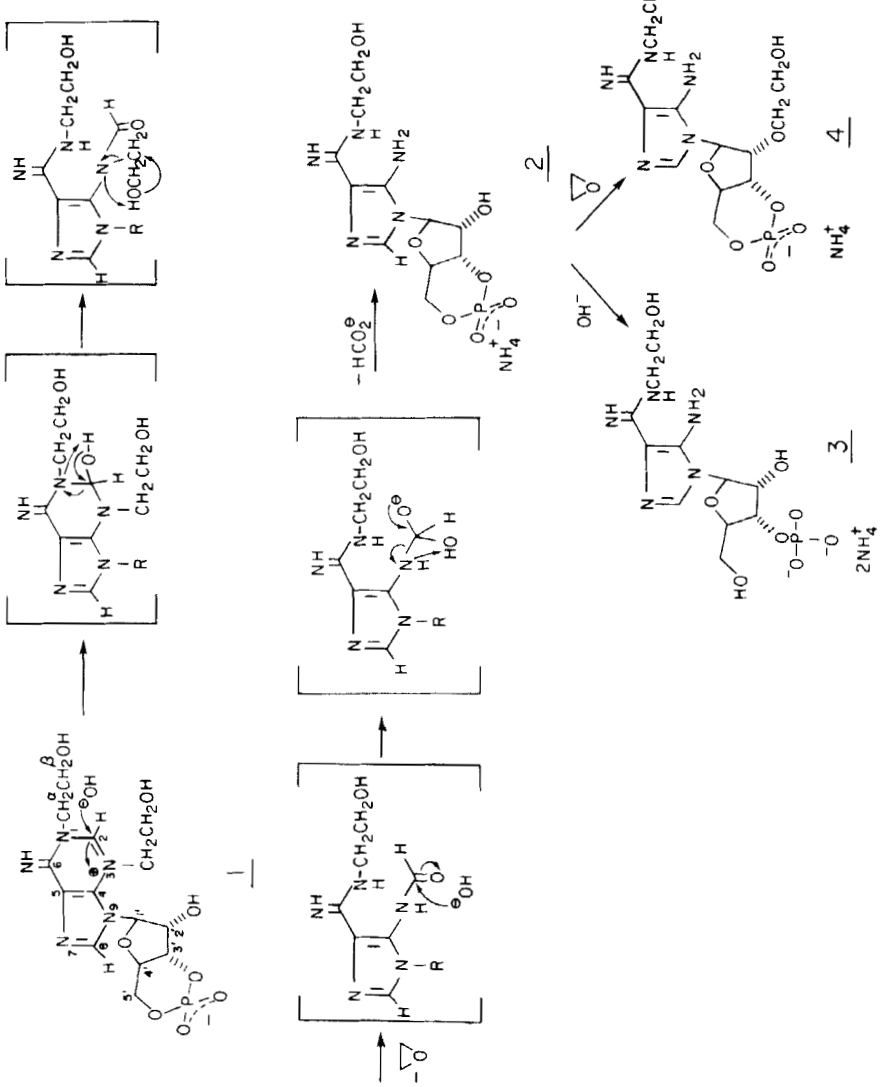

Table 1. Chemical shifts and coupling constants of 2.

C[a,b]	δ (ppm)[c]	JCH, JPC (Hz)	H[d,b]	δ (ppm)[c]
α	43.60	^{1}JCH=139	α	3.44
β	63.44	^{1}JCH=143	β	3.68
5'	70.18	^{1}JCH=152, ^{2}JPC=7	5',5''	4.24-4.21[e]
4'	74.60	^{1}JCH=155, ^{3}JPC=4	4'	4.48-4.27[e]
2'	75.02	^{1}JCH=159, ^{3}JPC=7	3'	
3'	80.26	^{1}JCH=147, ^{2}JPC=4	2'	4.63-4.61[e]
1'	94.04	^{1}JCH=168	1'	5.75
5	115.87	^{3}JCH8=9	8	7.40
8	132.59	^{1}JCH=216, ^{3}JCH1'=4		
4	145.14	^{3}JCH8=4, ^{3}JCH1'=2		
6	169.02	^{3}JCHα=4		

[a] 67.8 MHz. [b] The numbering refers to structure 1 (Reaction Scheme).
[c] Chemical shifts measured from external DSS in D_2O (separate sample).
[d] 270 MHz. [e] Width of resonance; 3' and 4' overlapped.

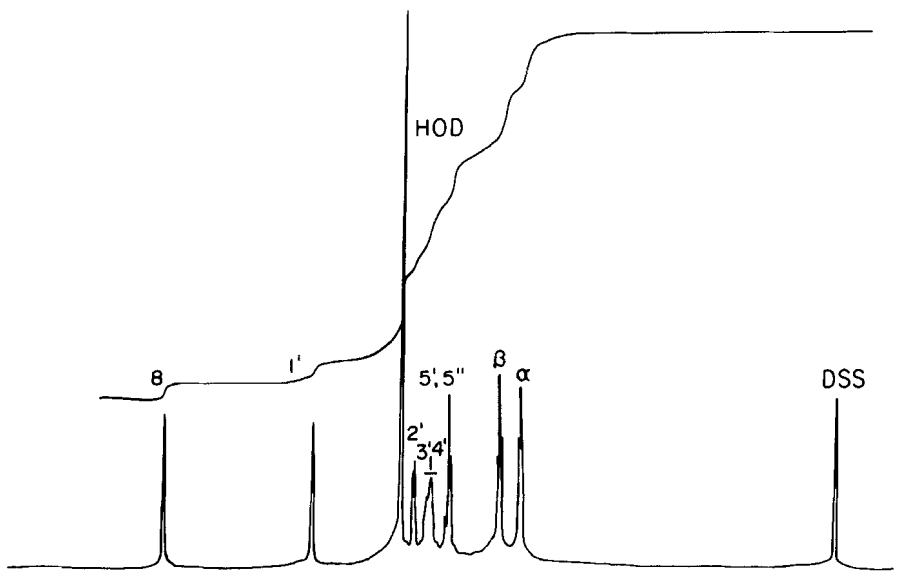

Figure 1. 270 MHz proton spectrum of 2. See text for details.

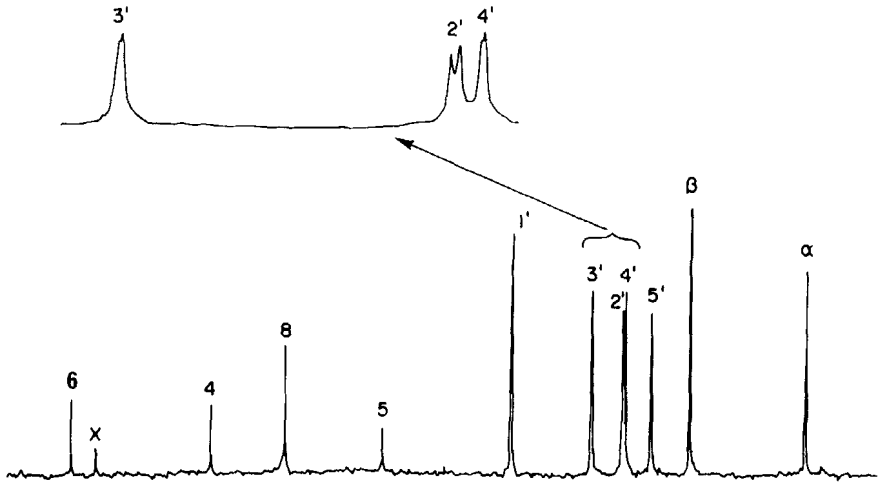

Figure 2. 67.8 MHz proton decoupled carbon-13 spectrum of 2. See text for details. "X" is unidentified.

Table 2. Chemical shifts and coupling constants of 3

C[a,b]	δ(ppm)[c]	JCH, JPC (Hz)	H[d,b]	δ(ppm)[c]
α	43.19	^{1}JCH=139	α	3.44
β	63.08	^{1}JCH=143	β	3.69
5'	63.66	^{1}JCH=143	5',5''	3.78-3.82[e]
4'	75.40f	^{1}JCH=152	4'	4.30
2'	75.46f	^{1}JCH=153	2',3'	4.55-4.62[e]
3'	87.44	^{1}JCH=151, ^{2}JPC=5	1'	5.67
1'	90.15	^{1}JCH=165	8	7.47
5	115.28	^{3}JCH8=8		
8	133.12	^{1}JCH=215, ^{3}JCH1'=3		
4	145.52	^{3}JCH8=3, ^{3}JCH1'=2		
6	168.82	^{3}JCHα=4		

a, b, c, d, e, See notes of Table 1.

f Due to spectral overlap, assignment of C2' and C4' is not possible.

measureable $^{3}J_{1'2'}$ (5.5 Hz) seen in the H-1' resonance at δ5.67 ppm. These results suggest that the cyclic phosphate ring has opened, yielding a 3'-monophosphate. Again, a single downfield proton and two, two-proton methylene triplets point to a ring opened base with one hydroxyethyl substituent, as in 2.

Proton coupled carbon-13 spectra of the base carbons shows the same splitting patterns as in 2. Furthermore, the nearly identical ^{13}C chemical shifts (Tables 1 and 2) for base carbons four, five, six and eight and the two methylene carbons lend credence for the postulate that the aglycone in 3 is the same as in 2.

Further evidence for the 3'-monophosphate comes from the ribose carbon shifts; C5' occurs ∿6 ppm upfield in 3, whereas C3' resonates ∿7 ppm downfield. Comparative ^{1}H and ^{13}C data on the ribose portion with 3'-AMP show close correspondence. (Some conformational difference is indicated compared with 3'-AMP since in 3, C2' is 1 ppm to higher field and the $^{3}J_{H-1', H-2'}$ is smaller.) Complete overlap of C2' and C4' resonances prevents detection of $^{3}J_{PC4'}$.

Compound 4 (Table 3):

The proton spectrum of 4 shows the single downfield resonance typical for the ring opened base and a singlet anomeric H-1' characteristic of a cyclic phosphate. The high field part of the spectrum is quite congested and indicates the possibility of two alkyl substituents (the FAB MS shows a MW with 44 mass units greater than compound 3, consistent with the addition of a second hydroxyethyl group).

Two additional high field carbon peaks are seen in the ^{13}C-spectra; both are methylene carbons. One of these peaks falls in the ribose carbon region.

The four carbon base region again is about the same as for compounds 2 and 3. The chemical shifts for the base carbons (Table 3) are nearly identical with those of 2 and 3 (Tables 1 and 2).

The second hydroxyethyl substituent was placed on the 2'-oxygen based upon comparison of ^{13}C and ^{1}H chemical shift differences between 2 and 4 and with reported 2'-O-alkyl substituted nucleosides (8,9). As can be seen in Tables 1 and 3, the ribose carbon chemical shifts are fairly close, with the exception of C2', which is 7.2 ppm downfield in 4. 2'-O-alkylation has been shown to result in downfield shifts at the 2'-carbon

Table 3. Chemical shifts and coupling constants of 4

C[a,b]	δ (ppm)[c]	JCH, JPC (Hz)	H[d,b]	δ (ppm)[c]
α1	43.54	^{1}JCH=141	α 1	3.45
β1	63.36	^{1}JCH=144	β 1	3.71
α2'	75.20	^{1}JCH=145	α 2'	3.76
β2'	63.55	^{1}JCH=144	β 2'	3.82
5'	70.08	^{1}JCH=150, ^{2}JPC=7	5',5''	4.25-4.21[e]
4'	74.76	^{1}JCH=148, ^{3}JPC=4	4',3',2'	4.40-4.55[e]
2'	82.62	^{1}JCH=150, ^{3}JPC=8	1'	5.87
3'	80.47	^{1}JCH=143, ^{3}JPC=4	8	7.42
1'	92.32	^{1}JCH=168		
5	115.97	^{3}JCH8=10		
8	132.68	^{1}JCH=215, ^{3}JCH1'=4		
4	145.14	^{3}JCH8=4, ^{3}JCH1'=2		
6	169.03	^{3}JCHα=3		

a, b, c, d e See notes of Table 1.

of this magnitude (8). Furthermore, 2'-O-alkylation causes upfield shifts in the corresponding 2'-proton (9) as seen in comparison of 2 and 4, where H-2' moves upfield to merge with H-3' and H-4'.

With reference to assignment of the α and β carbons of the two hydroxyethyl groups, it was assumed that for the substituent on N1, the carbons would appear at δ 43 ppm (α) and δ 63 ppm (β) as per the single substituted derivatives 2 and 3. In 4, the two β carbons should resonate at about the same field, thus α 2' is assigned downfield at δ 75.2 ppm. Perhaps this carbon experiences deshielding anisotropy from the cyclic phosphate as well as electron withdrawing from the attached oxygen. It is worth noting that a change of N to OR substitution results in a 25 ppm downfield shift of C2' in adenosine derivatives (8).

The structures elucidated by NMR were further confirmed by electron impact MS (for 2) and UV spectroscopy. Since the UV spectra (Table 4) of 2-4 are essentially identical, it can also be concluded that 2-4 have the same base chromophore as was shown by NMR spectroscopy.

Table 4.

Compd.	Molecular weight[a]	UV Data at Different pH Values						Rf	
		pH 2		pH 7		pH 11		cAMP[b]	(1)[c]
		λ max	λ min	λ max	λ min	λ max	λ min		
cAMP[f]		256	227	257	225	258	225		
2	364	265 243	250 212	264	221	264	220	0.45	0.11
3	382	268 245	253 218	265	221	265	220	0.37	0.08
4	347[d], 408	265 247	250 215	263	220	263	220	0.45	0.12
X[e]	346	291	248	283	231	264	226	0.37	0.04
1[f]		262	235	262	234	264	232		
1-(2-hydroxyethyl)cAMP[f]		257	230	257	229	259	229		
N^6-(2-hydroxyethyl)cAMP[f]		262	229	265	229	266	228		

[a] Determined by fast atom bombardment (FAB) ionization mass spectrometry in positive ion mode.

[b] R_f values on silica gel TLC sheets in solvent system (v/v): isobutyric acid /28% NH_4OH/H_2O (66/1/33:).

[c] R_f values on cellulose TLC plates in solvent system: n-butanol/ethanol/H_2O (16/2/5).

[d] Due to loss of OCH_2CH_2OH from the 2'-carbon.

[e] A mixture of 2-3 components according to NMR spectra.

[f] Compounds for comparison under identical conditions.

Mechanism of the novel pyrimidine ring opening and hydroxy ethyl elimination reactions.

In the Reaction Scheme a plausible mechanistic explanation is depicted for the transformation 1 2-4 in alkali. The first step in the degradation process of the base ring should be an OH^- attack on C2 followed by ring opening to give the hydroxyethyl formamide intermediate, which subsequently loses ethylene oxide to yield the formamide species. One should note that this is the same intermediate formed prior to the Dimroth rearrangement, which does not occur. The only difference in reaction conditions is the use of 1 M NaOH as opposed to $(NH_4)_2CO_3$. It is therefore assumed that in the presence of strong base, loss of the formate ion to yield major product 2 occurs before rearrangement and cyclization can take place.

Ring-opening of the 3',5'-cyclic phosphate moiety of 2 produces a 3'-monophosphate 3, similar to alkaline hydrolysis of natural cyclic nucleotides (10). Finally, 4 is formed presumably via alkylation at the 2'-oxygen with ethylene oxide, the latter coming from the elimination step described above. It is worth noting here that under very alkaline conditions the ribose moiety of a given nucleoside is preferentially alkylated (2).

The base of 2 - 4 is a 5-aminoimidazole-4-carboxamidine derivative. 5-amino-1-(β-D-ribofuranosyl)imidazole-4-carboxamidine 3',5'-cyclic phosphate is a key intermediate for the synthesis of 2-substituted cAMP analogs and is produced by alkaline ring-opening starting from cAMP N1-oxide or N1-alkoxides (10).

EXPERIMENTAL

Materials. cAMP was a gift from Prof. R. K. Robins, Department of Chemistry, Brigham Young University, Provo, Utah. Precoated silica gel ($60F_{254}$, 0.02 x 20 x 20 cm, Merck, Darmstadt, FRG) and cellulose (F_{254}, 0.01 x 20 x 20 cm, Merck) TLC plates were used to follow the reaction, separate the products and check the purity. Solvent systems used were (v/v): isobutyric acid /28% aq. NH_4OH/ H_2O (66/1/33) and n-butanol/ethanol/ H_2O (16/2/5). DEAE Sephadex A-25 was the product of Pharmacia Fine Chemicals, Sweden and was purchased from Sigma Chemical Co, St. Louis, Missouri.

Methods.

NMR spectroscopy. All samples were treated with Biorad Chelex 100 resin to remove paramagnetic impurities. Aqueous solutions were stirred several hours at ambient temperature with the solid resin. The resin was filtered off, rinsed and the total solutions lyophilized to dryness. The dried materials were dissoved in Stohler 99.8% D_2O, relyophilized and redissolved. In the case of sample 2, the total material was dissolved in 0.5 ml D_2O and transferred to a Wilmad #528 mm tube. For samples 3 and 4, the solids were dissolved in 0.25 ml D_2O and transferred to Wilmad 5 mm microcells (0.25 ml volume).

All samples were examined on a JEOL FX-270 spectrometer at 23 °C in a 5 mm C/H fixed tuned probe. ^1H spectral conditions were commonly: 20-24 transients accummulated; flip angle 90°, pulse delay 0.2 sec., sweep width 10 ppm. ^{13}C spectral conditions were generally: for total carbon spectra, 149 ppm sweep width, flip angle, 75°; pulse delay 6 sec; 12-26 x 10^3 transients collected. For examination of the low field carbon region, sweep widths of 62 ppm were used and 8-24 x 10^3 transients collected under the conditions listed above. During complete broad band proton noise decoupling, 4 watts decoupler power was used; no sample heating was detected. For selective proton decoupling, 0.1 watt decoupler power was used. Proton coupled ^{13}C-spectra were obtained with full NOE (decoupler gated on during pulse delay).

All chemical shifts were measured from an external reference of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) in D_2O.

FAB mass spectra were acquired using a Varian MAT 731 instrument. An Ion Tech FAB 11N ion source was used with a neutral 6 keV xenon beam for ion desorption.

EI mass spectrometric measurements were carried out on a Varian MAT 112S instrument with ionizing energy of 80eV and an ion source temperature of 175 °C. The sample was introduced by direct probe. Trimethylsilylation was carried out following the addition of N,O-bis(trimethylsilyl)trifluoroacetamide with 1% of trimethylchlorosilane added (30 µL) and pyridine (10 µL) to ca. 0.2 mg of sample which was heated at 110 °C for 1 h in a glass capillary tube.

Transformation of 1,3-Di-(2-hydroxyethyl)-cAMP (1) in Alkali

Compound 1 (0.242 g, 0.5 mmol) was dissolved in 1 M sodium hydroxide (5 mL) at ambient temperature. After 2-4 days the reaction mixture was put on a column (2.5 x 50 cm) of DEAE Sephadex A-25 (HCO_3).

The column was first washed with distilled water (1 L), then eluted using a linear gradient of water (1.5 L) and 1 M ammonium bicarbonate (1.5 L). Fractions (18 mL/9 min.) 62-66 contained mainly compound 2 (0.043 g, 22%). Compound 3 appeared almost solely in fractions 76-84 (0.099 g, 47%). Mixed fractions (67-75) contained 2-4 and X(0.056 g). Final purification of 2 (37 mg) was performed on four cellulose plates using n-BuOH/EtOH/H_2O (16/2/5) developing system (two developments). Compound 2 (26 mg) was extracted from cellulose with water. The same procedure was applied for compound 3 (14 mg, one plate, one development, 8 mg of pure 3) and for X (39 mg, 14 plates, one development, 6 mg of pure 4 and 2 mg of X).

EI-MS data of 2, m/e (rel. intensity %): 580, M^+ + 3TMS(2); 565, M^+ + 3TMS-15 (1); 652, M^+ + 4TMS (2); 637, M^+ + 4TMS-15 (13).

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