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Note

High-performance liquid chromatographic separation of the isomeric 5-fluorocytidine 2′, 3′- and 3′, 5′-cyclic monophosphates

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The key role in the bioregulation of purine cyclic nucleotides, adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP) is fairly well known. Recently 3′,5′-cyclic monophosphates of pyrimidine nucleosides (cytidine and uridine) have also been isolated from different living sources[1,2], but knowledge of their biological function is limited[3–5]. Moreover, some controversy has recently emerged as to the natural occurrence of cytidine 3′,5′-cyclic phosphate (cCMP)[6,7]. Several hundred analogues of cAMP and cGMP have been synthesized and evaluated biologically[8–10], but the number of analogues of natural pyrimidine cyclic nucleotides is small[8,11].

We have recently prepared some 5-substituted analogues of 3′,5′-cyclic monophosphates of pyrimidine nucleosides[12]. When ring-closure of 5-fluorocytidine 5′-monophosphate (the purity of which was checked by HPLC and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy) was performed according to a published procedure[13], a mixture was obtained. This mixture could not be resolved by chromatography on a DEAE Sephadex A-25 (HCO_3^-) anion-exchange column or by silica gel thin-layer chromatography.

The components of this mixture were determined to be isomeric 3′,5′- and 2′,3′-cyclic phosphates of 5-fluorocytidine by UV and NMR spectroscopy and mass spectrometry. This paper describes an HPLC system used to separate these isomeric cyclic phosphates and the spectroscopic results used to identify them in the mixture. Numerous papers in the literature deal with HPLC separation of various cyclic nucleotides from each other[14–16]. After completion of this work, a paper appeared on reversed-phase HPLC separation of nucleosides and nucleotides, which presented the separation of isomeric nucleoside 2′,3′- and 3′,5′-cyclic monophosphates[17]. Resolution of 2′,3′- and 3′,5′-cyclic ribonucleotides by Amberlite XAD-4 column chromatography has also been reported[18].

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EXPERIMENTAL

The HPLC analyses were performed on a Perkin-Elmer Series 3 liquid chromatograph equipped with a Perkin-Elmer LC-55 variable-wavelength detector set at 260 nm, and a Rheodyne Model 7120 injector with a 20-μl loop. Separations were carried out on a stainless-steel column (25 cm × 4.6 mm I.D.), packed with APS-Hypersil, (5 μm, Shandon Southern, U.K.), at ambient temperature. A Sigma 10 data system (Perkin-Elmer) was used for peak integration and identification. The mobile phase was 0.05 *M* potassium dihydrogen orthophosphate, adjusted to pH 3.5 with phosphoric acid, flow-rate 1 ml/min. Double distilled water purified on a Lobar RP-8 column (Merck, Darmstadt, F.R.G.)[19] was used. The compounds were dissolved in the mobile phase.

Spectroscopy

^{1}H NMR spectra were recorded with a Varian XL-100 Fourier transform (FT) NMR system (100.1 MHz) in deuterium oxide. Tetramethylsilane served as internal reference (0.00 ppm). ^{13}C spectra were recorded using a Varian XL-100/15 disk-augmented FTNMR system (25.2 MHz) in deuterium oxide. Dioxane served as internal reference (67.71 ppm). ^{31}P spectra were acquired with a Varian SC-300 FTNMR system (121.5 MHz) in deuterium oxide. Phosphoric acid served as external reference (0.00 ppm). UV spectra were recorded with a Varian Cary 17D UV-VIS spectrophotometer system at three different pH levels (pH 2, 10^{-2} *M* hydrochloric acid; pH 7, 0.02 *M* potassium dihydrogen phosphate–0.02 *M* dipotassium hydrogen phosphate; pH 11, 10^{-3} *M* sodium hydroxide).

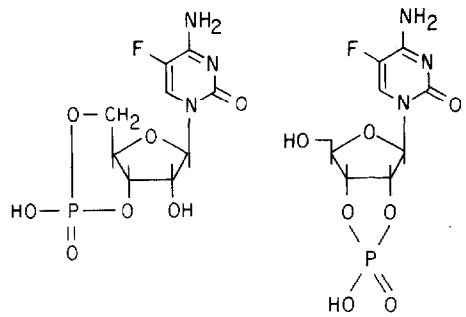
Mass spectrometry

Mass spectra were acquired using a Varian MAT 731 instrument. An Ion Tech FAB 11N ion source was used, modified as described by Martin *et al.*[20]. A neutral 6-keV xenon beam was used for ion desorption.

Trimethylsilylation was carried out following the addition of N,O-bis(trimethylsilyl)acetamide, trimethylchlorosilane and pyridine (100:1:10) to *ca.* 20 μg of the isomeric mixture which was heated at 100°C for 1 h in a glass capillary tube. Electron impact spectra were acquired under the following conditions: ion source temperature, 250°C; ionizing energy, 70 eV. The sample was introduced by direct probe.

RESULTS AND DISCUSSION

A good chromatographic separation of the 3',5'-cyclic phosphate (I) and the isomeric 2',3'-cyclic phosphate (II) of 5-fluorocytidine was achieved using the HPLC



I II

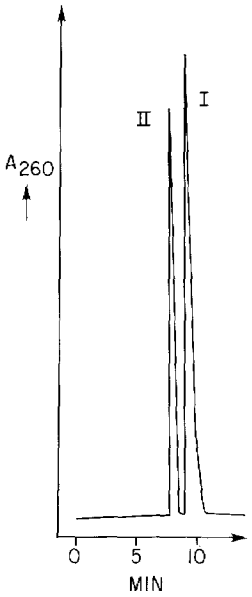


Fig. 1. HPLC separation of isomeric 5-fluorocytidine 2′,3′- and 3′,5′-cyclic monophosphates. For conditions see Experimental section.

system described in the Experimental section (Fig. 1). It is interesting to note that there was no separation using methanol or mixtures of methanol and potassium dihydrogen orthophosphate buffer. On a Hypersil CPS column no separation could be obtained with various mixtures of methanol and ammonium formate or acetonitrile–ammonium formate buffer as an eluent. The identity of I was confirmed by its coelution with authentic 5-fluorocytidine 3′,5′-cyclic monophosphate. This authentic sample was obtained by a new synthetic route[12] and its identity was confirmed spectroscopically as discussed below. Under the HPLC conditions used, the starting material, 5-fluorocytidine 5′-monophosphate, elutes at 7.68 min and thus is easily separated from I and II.

The identity of II was established by the following techniques.

UV spectroscopy

The UV data of authentic I and the isomeric mixture are shown in Table I.

TABLE I

UV DATA AT DIFFERENT pH VALUES

	pH 2		*pH 7*		*pH 11*	
	λ_{max}	λ_{min}	λ_{max}	λ_{min}	λ_{max}	λ_{min}
Authentic I	286	249	279	259	279	258
	211	–	237	–	237	–
Mixture of I + II	285	249	278	259	278	259
	212	–	238	–	238	–

TABLE II

^{13}C NMR DATA

Chemical shifts in ppm (coupling constants in Hz).

Carbon atom	*Authentic I*	*Isomeric mixture*	*2',3'-cCMP**
2	**	**	158.23
4	**	**	167.77
5	Not observed	Not observed	97.30
6	126.2 (32.9)	126.46 (33.0), 127.94 (33.0)	144.91
1'	94.9	95.24, 93.70 (5.9)	94.97 (6.3)
2'	72.6 (7.8)	72.85 (7.8), 81.71 (2.0)	82.11 (2.5)
3'	77.3 (4.3)	77.52 (4.4), 77.79 (0.5)	78.54 (0.5)
4'	72.1 (4.1)	72.35 (4.3), 85.90 (2.3)	86.37 (2.2)
5'	67.5 (6.8)	67.76 (6.8), 61.66 (<0.2)	62.32 (<0.2)

* Data from ref. 22.
** Could not be analyzed.

Since the UV spectra are essentially identical, it is concluded that both I and II have the same chromophore in the base.

NMR spectroscopy

In the ^1H NMR spectrum of authentic I, a doublet at 7.88 ppm (J_{HF} = 5.8 Hz) for H-6 of the base and a singlet at 5.93 ppm for anomeric H-1' were observed. The isomeric mixture showed two doublets at 7.86 and 7.98 ppm, both with splitting of *ca.* 5.8 Hz, and two singlets at 5.92 and 6.01 ppm. This indicates that the mixture contains I and that the other component (*i.e.*, II) is isomeric.

The ^{31}P NMR spectrum showed two resonances for the mixture, one at −0.84 ppm due to the phosphorus in a six-membered ring as in I and the other at +20.91 ppm, which corresponds to a phosphorus in a five-membered ring as in II[21].

The ^{13}C NMR data (Table II) also indicate that this mixture contains I and II. ^{13}C NMR data for cytidine 2',3'-cyclic monophosphate from ref. 22 are shown for comparison.

Mass spectrometry

The recently developed technique of fast atom bombardment (FAB) mass spectrometry, which has shown great potential in the analysis of polar molecules[23], was applied for the analysis of this isomeric mixture. In the positive ion mode, this mixture gave MH^+ (*m/z* 324) and base $(B)H_2^+$ (*m/z* 130); in the negative ion mode complementary information was obtained: $[M-H]^-$ (*m/z* = 322) and B^- (128). No other sample-related peaks were detected. This information establishes the molecular weight of the components in the mixture to be 323 and of the (free) base 129. This is consistent with the structures I and II.

On trimethylsilylation the mixture produced M^+ (*m/z* 539) and $[M-15]^+$ (*m/z* 524) in the electron impact mass spectrum. On subtraction of the three trimethylsilyl groups from the molecular ion at 539 the molecular weight of the components of the mixture is again established to be 323. Mass spectrometry was not used to distinguish

I and II since the analysis was done on a mixture of the two, but to verify the structure of the cyclic nucleotides and to show lack of any contamination.

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