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SYNTHESIS AND ^{13}C NMR SPECTRA OF 3'-AMIDO ANALOGS OF ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE

M. MORR, M.-R. KULA* and L. ERNST
Gesellschaft für Molekularbiologische Forschung mbH, D-3300 Braunschweig-Stöckheim, Mascheroder Weg 1, West Germany

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Abstract—A new method is described for the synthesis of P–N bonds under mild conditions and in high yields: water-soluble carbodiimides are used to cyclize 3'-amino-3'-deoxyadenosine 5'-monophosphate and 3'-N-benzylamino-3'-deoxyadenosine 5'-monophosphate in aqueous soln at pH 7·5 and 37°. When 3'-amino-3'-deoxyadenosine 2',5'-diphosphate is treated with carbodiimide the 3',5'-cyclic derivative is formed rather than the 2',3'-cyclic derivative. ^{13}C NMR spectra are used for structure determination of the products. The chemical shifts and ^{13}C–^{31}P spin–spin coupling constants are discussed.

Water-soluble carbodiimides such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl) or 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (CMC) have been used almost exclusively to synthesize peptide or amide bonds –NH–CO– in peptide chemistry[1,2] or for the preparation of suitable carriers for affinity chromatography.[3] The water-insoluble dicyclohexylcarbodiimide (DCC) is widely employed as a condensing agent for the synthesis of esters and anhydrides in organic solvents. DCC was also used by Chambers and Moffat[4] to prepare adenosine 5'-phosphoramidate from adenosine 5'-monophosphate and ammonia in a solvent mixture composed of formamide, t-butanol, and water. In a similar reaction mixture we cyclized 3'-amino-3'-deoxyadenosine 5'-monophosphate (**1a**) and obtained the desired 3'-amido-3'-deoxyadenosine 3',5'-cyclophosphate (**3a**) after 8 hr at 100° in 40% yield.[5] To improve the yield of this cyclisation reaction in order to get sufficient quantities of material for biological studies of the cAMP-analogs, water-soluble carbodiimides were also tested and found to react under very mild conditions. **1a** and 3'-N-benzylamino-3'-deoxyadenosine 5'-monophosphate (**1b**) were cyclized in aqueous soln at pH 7·2–7·5 and 37° within 2–5 hr in yields up to 95% after purification. Nucleophilic attack of the 3'-amino group on the intermediate O-acylurea may proceed via a cyclic structure as formulated (2) and this probably accounts for the rather high reaction rates and for the fact that intramolecular condensation occurs almost exclusively. The reaction rate varies with the carbodiimide used. EDC·HCl reacts faster than CMC, presumably for steric reasons. The final yield appears also to depend to some extent on the counterion of the carbodiimide. Cyclization of **1a** with EDC·HCl gave **3a** in 92% yield and no side reactions were observed, while EDC·HCO_3 gave **3a** in 80% yield only and some other products could be detected in the mixture. The effect was opposite for the cyclization of **1b**, a secondary amine, where one of the hydrogens of the 3'-amino group is substituted by a benzyl group. Using EDC·HCl, 65% of 3'-N-benzylamido-3'-deoxyadenosine 3',5'-cyclophosphate (**3b**) was obtained, while reaction with EDC·HCO_3 gave **3b** in 95% yield. The complete removal of salts proved difficult when chloride was present in the mixture. Therefore, EDC·HCO_3 was used to prepare **3a** and **3b** for spectroscopic studies.

Substitution of hydrogen by the benzyl group in the 3'-amido position of the newly prepared **3b** makes this compound more susceptible to acidic hydrolysis of the P–N bond than **3a**. Hydrolysis is pronounced below pH 7. After 5 hr incubation at 37° and pH 7·0, 10% of **3b** were hydrolysed. Complete hydrolysis was found at pH 5·0 whereas no hydrolysis was noted at pH 9·0. In this respect, **3b** resembles the isomeric 5'-N-benzylamido-5'-deoxyadenosine 3',5'-cyclophosphate.[6] As starting material for the preparation of **3b**, 3'-N-benzylamino-3'-deoxyadenosine 5'-monophosphate was prepared by a procedure commonly used for the preparation of N-benzylaminoacids.[7] **6** was reacted with benzaldehyde and the resulting Schiff base was reduced with sodium borohydride. The free acid **8** crystallized readily from water and is comparable to N-benzylamino acids.

3'-Amino-3'-deoxyadenosine 2',5'-diphosphate was treated with EDC·HCO_3 to measure the competition of the 2'- and 5'-phosphate groups in the cyclization reaction. Products were separated by column chromatography on DEAE-Sephadex A 25 (Fig. 1) and the main peak treated with alkaline phosphatase to remove the residual free phosphate group. From the NMR spectra it follows unequivocally that the final product isolated is **3a**. Cyclization therefore takes place primarily between the 3'-amino and the 5'-phosphate groups rather than between the vicinal 3'-amino and the 2'-phosphate groups. The 6-membered ring in **3a** containing three heteroatoms is favoured over the 5-membered ring resulting from a condensation between the 3'-amino and the 2'-phosphate groups. ^{13}C NMR was found to be the most useful method of differentiating between the various species and is discussed below in detail. The biological properties of the newly synthesized cAMP analogs will be reported elsewhere.[8,9] Water-soluble carbodiimides valuable in the synthesis of cyclic phosphoramidates should also be useful in the synthesis of linear compounds.

^{13}C NMR spectra

Proton noise-decoupled and single frequency off-resonance decoupled (SFORD) ^{13}C NMR spectra were obtained for **1a**, **1b**, **3a**, **3b**, **4** and **5**. The results, chemical shifts and ^{13}C–^{31}P spin–spin coupling constants, are given in Table 1.

Assignment of the signals for the base moiety in all compounds was straightforward as the chemical shifts are

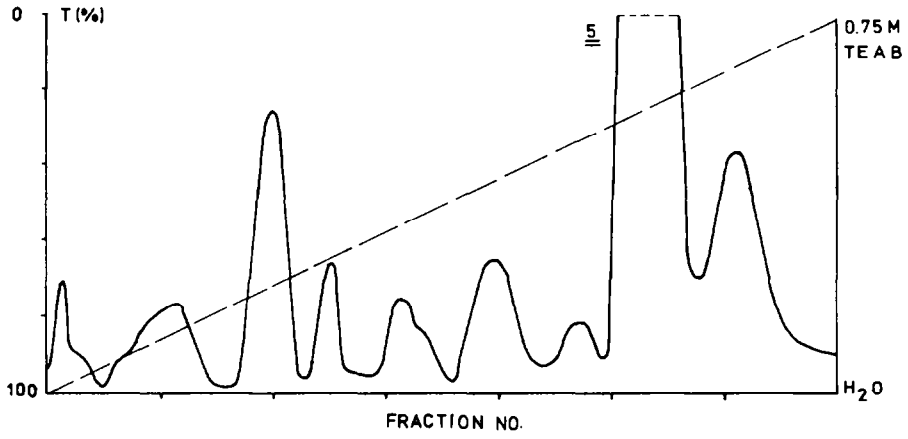


Fig. 1. Chromatography of the reaction products on DEAE-Sephadex A 25 after condensation of 3'-amino-3'-deoxyadenosine 2',5'-diphosphate with EDC·HCO_3. For details see text.

very similar to those of adenosine 5'-monophosphate[10] and adenosine 3',5'-cyclophosphate.[11] For the sugar moieties, C-1' could be assigned because of its low-field shift and C-5' because of its triplet structure in the SFORD spectra. Assignment of C-3' was possible due to its very high-field shift (18 ppm in the open, 12 ppm in the cyclic forms) compared to the 3'-oxygen analog. These high-field shifts are rationalized in terms of the lower electronegativity of the amino versus the hydroxy substituent. Distinction of the two remaining signals due to C-2' and C-4' was facilitated by inspection of the ^{13}C–^{31}P coupling constants. In the open compounds, C-4' experiences a 3-bond coupling to phosphorus of the order of 9 Hz. ^{31}P spin–spin interaction with C-2' would involve a 5-bond path which, of course, does not give rise to any observable coupling constants. In the cyclic forms, however, ^{31}P coupling over three bonds is observed for both C-2' and C-4'. While the coupling path to the C-2' nucleus involves a P–N–C–3'–C–2' dihedral angle of *ca.* 180°, the corresponding angle for the P–N–C–3'–C–4' path is about 60°. One therefore would expect a large coupling constant ($^3J_{trans}$) of *ca.* 9 Hz[11] to C-2' and a much smaller one ($^3J_{gauche}$) of *ca.* 2 Hz[11] to C-4'. Yet one has to take into account that coupling between ^{31}P and C-4' can also be transmitted over a second path, viz P–O–C–5'–C–4' which also involves a dihedral angle of *ca.* 60°. $^3J_{gauche}$ is therefore expected to be near 4 Hz. The observed values are consistent with these expectations: the $^3J(^{31}P–^{13}C–2')$ are 8·9–10·6 Hz, the $^3J(^{31}P–^{13}C–4')$ 3·1–4·7 Hz. Thus unequivocal assignment of all C atoms is achieved. In **4** and **5** additional splittings of the C–1'–, C–2'– and C–3'-signals are caused by ^{31}P–2'.

Comparison of the chemical shifts between the open and cyclic forms (Fig. 2 and Table 1), i.e. the differences δ(**3a**) − δ(**1a**), δ(**3b**) − δ(**1b**) and δ(**5**) − δ(**4**), reveals drastic changes for all of the ribose carbons except for C–2' in **1b/3b**. Moreover, these changes have similar magnitudes within the series. Cyclization shifts $\Delta\delta$ are (+)3·0 − (+)3·2 ppm for C–1', (+)5·0 − (+)7·1 ppm for C–3', (−)8·5 − (−)10·0 ppm for C–4' and (+)2·6 − (+)4·0 ppm for C–5' (positive signs denoting downfield shifts on cylization). The positive value of $\Delta\delta$ for C–3' can be explained in that

Table 1. ^{13}C Chemical shifts (δ) and ^{13}C–^{31}P coupling constants (J) of aqueous solns of 3'-amino analogs of 5'-AMP, 3',5'-cAMP and their 2'-phosphorylated derivatives[a]

δ	1a	3a	1b[b]	3b[c]	4	5
C-2	153.81	154.08	153.71	153.84	154.10	154.28
C-4	149.59	149.38	149.5	149.04	150.21	150.11
C-5	119.80	119.82	119.8	119.75	119.92	119.9
C-6	156.61	156.66	156.59	156.51	156.86	156.84
C-8	141.29	140.74	141.19	140.27	141.95	142.08
C-1'	89.93	93.09	90.38	93.37	89.04	92.26
C-2'	76.66	74.88	74.43	74.58	78.39	76.67
C-3'	53.31	60.43	59.35	64.30	53.70	60.44
C-4'	85.71	75.76	84.28	75.81	85.29	75.97
C-5'	64.49	68.50	65.18	67.80	65.06	68.49
$J(^{13}C-^{31}P)$:						
C-1'	-	-	-	-	5.0 (2'-P)	1.8 (2'-P)
C-2'	-	10.6	-	8.9	4.4 (2'-P)	4.7 (2'-P), 10.3 (5'-P)
C-3'	-	3.0	-	< 1	3.2 (2'-P)	2.5 (2'-P)[d], 6.2 (5'-P)[d]
C-4'	8.7	5.7	9.2	3.1	8.8 (5'-P)	6.2 (5'-P)
C-5'	4.9	6.8	4.3	6.3	4.9 (5'-P)	6.3 (5'-P)

a) Chemical shifts in ppm (±0.03 ppm) to low field of external TMS. Coupling constants in Hz (±0.6 Hz).

b) Benzyl substituent: CH_2 δ 52.39, J(C,P) 0; $C_{quart.}$ δ 139.89, J(C,P) 0; C_o, C_m δ 129.83; C_p δ 128.69.

c) Benzyl substituent: CH_2 δ 51.56, J(C,P)<1; $C_{quart.}$ δ 139.58, J(C,P)4.0; C_o and C_m δ 130.01 and 129.52; C_p δ 128.55.

d) These coupling constants could be interchanged.

C-3' obtains another β-substituent on cyclization. Similarly, ring formation acts as introduction of a γ-substituent for C-2' and C-4' thereby giving rise to negative $\Delta\delta$-values. The large magnitudes of the C-4' shifts are, however, somewhat surprising and for the time being we refrain from a more detailed discussion.

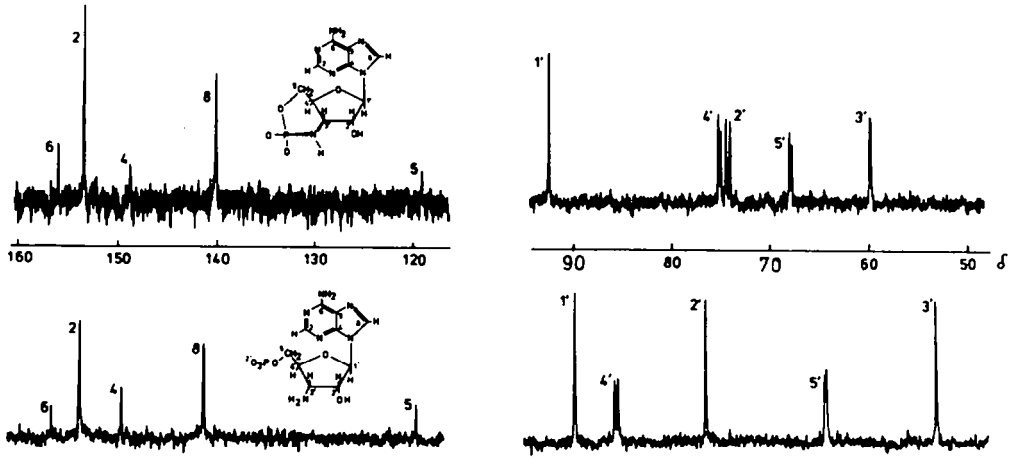


Fig. 2. ^{13}C NMR spectra of **1a** (bottom) and **3a** (top) in D_2O solution. Chemical shifts are relative to external TMS.

EXPERIMENTAL

Compounds **1a** and **4** were synthesized as described.[12] EDC was prepared according to Sheehan[13] by reacting ethyl isocyanate with N,N-dimethyl-1,3-propanediamine and dehydrating the urea derivative with *p*-toluenesulfonyl chloride. The free base was converted just prior to use into the bicarbonate by bubbling CO_2 through the aqueous soln until a pH of 7·8 was reached. EDC·HCl was purchased from Ega-Chemie (Steinheim, Germany), CMC from Aldrich-Europe (Düsseldorf, Germany) and DEAE-Sephadex A 25 from Pharmacia (Uppsala, Sweden). Alkaline phosphatase (EC 3.1.3.1) from calf intestine was a product of Boehringer (Mannheim, Germany).

TLC was carried out on silicagel SiF plates from Riedel de Haën (Seelze, Germany) developed with MeOH unless otherwise noted.

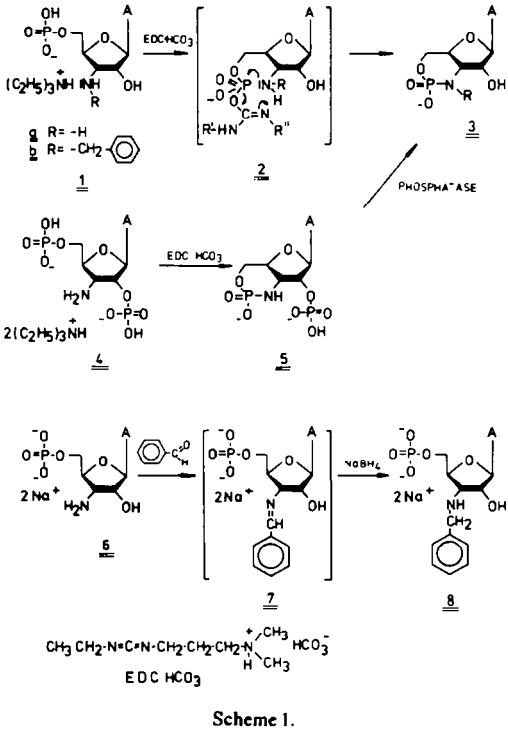
^{13}C NMR spectra were run on a Varian XL-100-12 spectrometer at 25·16 MHz in the pulse Fourier transform mode. Accumulation of 12 K and transformation of 16 K data points for 5 kHz spectral widths gave an accuracy of the chemical shifts of ±0·03 ppm and of ±0·6 Hz for the coupling constants. The compounds were studied as 0·1 to 0·2 M solns in D_2O which served as lock. Capillaries of neat TMS were used as external standard.

3'-Amido-3'-deoxyadenosine 3'5'-cyclophosphate (**3a**)

(a) (Compound **1a** (120 mg, 0·27 mmoles) was dissolved in 50 ml water and the pH of the soln adjusted to 7·5 by addition of triethylamine. 1·5 mmoles of EDC·HCO_3 were added with stirring. The reaction proceeded at 37° and was monitored by TLC. The pH was adjusted to 7·5 if necessary by bubbling CO_2 through the soln. After completion of the reaction (2·5 hr) the mixture was directly applied to a column packed with DEAE-Sephadex A 25 (HCO_3^- form, 2·5 × 40 cm). **3a** was eluted with a linear gradient between 1000 ml water and 1000 ml 0·2 M triethylammonium bicarbonate. The effluent was monitored at 254 nm and collected in fractions. Fractions containing **3a** were combined and concentrated by rotary evaporation. The product was finally freeze-dried several times from water; yield: 80% by UV-absorbance. **3a** was characterized by its chromatographic and electrophoretic properties and by chemical and enzymatic degradation.

(b) Compound **1a** (56 mg; 0·125 mmoles) was dissolved in 20 ml water and the pH of the soln adjusted to 7·5 by addition of triethylamine. Solid EDC·HCl (115 mg = 0·60 mmoles) was added to the soln with stirring. The pH was readjusted and kept at 7·5. After 2 hr the reaction products were separated as described above. **3a** was isolated in 92% yield as judged by UV absorbance at 259 nm. The product contained some triethyl-ammonium chloride. The cyclic phosphoramidates can be adsorbed from water onto the resin Amberlite XAD-2. The resin is washed with water to remove the remaining salt. The cyclic phosphoramidates are eluted with a mixture of water and t-BuOH and finally freeze-dried.[14]

(c) Compound **1a** (2·2 mg; 5 μmoles) was dissolved in 0·2 ml water and mixed with 0·3 ml of a soln containing 35 mg CMC per ml of water. The pH of the soln was raised to 7·3 by adding triethylamine. The mixture was kept at 37°. After 10 hr, the products were separated by TLC using cellulose-coated plates developed with n-propanol:ammonia:water, 20:20:3, v:v:v. Material containing **3a** (R_f 0·67) was scraped off the plates, **3a**



Scheme 1.

eluted with 0·1 M ammonia and the yield estimated by UV absorbance at 259 nm. 50% of **3a** were isolated under the conditions described. In a similar experiment, 65% of **3a** were obtained after 1·5 hr at 80°.

3'-N-Benzylamino-3'-deoxyadenosine 5'-monophosphate (**1b**)

Compound **1a** (346 mg; 1 mmol) was dissolved in 2 ml water and titrated with NaOH to give the disodium salt **6**. Excess benzaldehyde (0·5 ml) was added and the emulsion shaken vigorously at room temp. After 6 hr, residual benzaldehyde was extracted with ether, the aqueous phase cooled to 0°, and the Schiff base **7** reduced by adding $NaBH_4$. Reaction with benzaldehyde and reduction with $NaBH_4$ were repeated twice to ensure high yields of **8**. The products were purified by column chromatography using DEAE-cellulose (HCO_3^- form, 2·5 × 50 cm) and a linear gradient between 1500 ml of water and 1500 ml of 0·5 M triethylammonium bicarbonate, pH 7·5. The effluent was monitored at 254 nm and collected in fractions. The main peak was concentrated, adjusted to pH 12 and applied to a column of Dowex 1 × 8 (acetate form, 2·5 × 40 cm). **8** was eluted as the free acid with 1M AcOH, yield 70%. 10 mg (23 μmoles) of **8** were incubated with 7 units of alkaline phosphatase at 37° in 1 ml of 50 mM tris buffer, pH 7·5, containing 0·1 μmoles of $ZnCl_2$. Upon cooling of the mixture, fine white needles (7 mg = 82%) of 3'-N-benzylamino-3'-deoxyadenosine appeared which were recrystallized from water (m.p. 154–156°) and characterized by mass spectra. The latter showed the molecular ion *m/e* 356, the fragments of the base (B + 2H) *m/e* 136, (B + H) *m/e* 135, and the fragment *m/e* 91 derived from the benzyl group.

3'-N-Benzylamido-3'-deoxyadenosine 3',5'-cyclophosphate (**3b**)

Compound **1b** (110 mg; 0·2 mmoles) was cyclized with EDC·HCO_3 and purified essentially as described for **1a**. **3b** was isolated in 95% yield. It is resistant to the action of alkaline phosphatase. The structure of **3b** also follows from its electrophoretic and chromatographic properties and its NMR spectra.

Reaction of 3'-amino-3'-deoxyadenosine 2',5'-diphosphate (**4**) *with* EDC·HCO_3

Compound **4** (305 mg; 0·5 mmoles) was dissolved in 100 ml water and treated with EDC·HCO_3 as described above. Purification of the products was achieved by column chromatography on DEAE-Sephadex A 25 (HCO_3^- form, 2·5 × 60 cm). Elution was carried out with a linear gradient between 1500 ml of water and 1500 ml of 0·75 M triethylammonium bicarbonate. The effluent was monitored at 254 nm (Fig. 1) and collected in fractions. The main fraction was lyophilized from water to give 180 mg (60%) of **5**. **5** was dissolved in 30 ml of 50 mM tris buffer, 0·1 mM $ZnCl_2$, pH 7·5, and incubated at 37 with 15 units of alkaline phosphatase. After 5 hr the mixture was diluted to 60 ml and applied to a column of DEAE-Sphadex A 25 (HCO_3^- form, 2·5 × 60 cm). Elution was carried out using a linear gradient between 1000 ml of water and 1000 ml of 0·2 M triethylammonium bicarbonate. **3a** was obtained in 90% yield with respect to **5**. The product gave a positive reaction with periodate–benzidine after treatment with acid and was also shown by ^{13}C NMR spectra to have structure **3a**.

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