



SYNTHESIS AND STRUCTURAL CHARACTERIZATION OF CONJUGATES OF ADENOSINE AND TETRA-ASPARTATE, NOVEL ANALOGS OF ATP

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Abstract: Solid phase synthesis of conjugates of adenosine and tetra-aspartate, potential ligands of P₂ (adenosine triphosphate) receptors, is described. Different spatial arrangement of the peptide chain relative to the adenosine moiety in these highly charged compounds is shown by ¹H and ¹³C NMR spectroscopy. pK_a values for the three internal aspartates and adenine base were determined. © 1997 Elsevier Science Ltd.

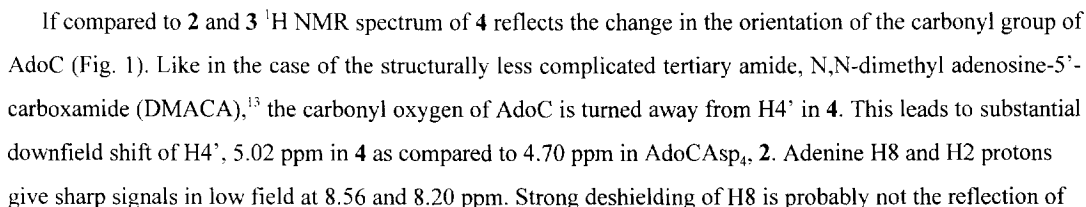
Intracellular nucleotides play fundamental and ubiquitous roles in energy metabolism, nucleic acid synthesis, and enzyme regulation. Extracellular nucleotides also exert significant biological actions in many tissues and cells through cell membrane adenosine triphosphate (ATP) P₂ receptors.¹ Up to the present the development of ligands for P₂ receptors has given few active compounds, at least in part due to the difficulties in chemical synthesis and purification of nucleoside triphosphates.² Most of the ATP analogs tested have been commercially available compounds, although some reports on specially designed and synthesized ATP analogs and derivatives as ligands for P₂ receptors have now been published.²⁻⁶

Triphosphate chain of ATP has great flexibility, so it is difficult to predict the spatial arrangement of the charges in its complex with P₂ receptors. However, in the case of globular proteins (enzymes) it has been found that protein bound nucleotides adopt structures corresponding to low-energy conformations of free nucleotides and phosphate binding to the main-chain NH groups is the usual way for phosphate/protein interactions.⁷ Modeling of the P_{2y} purinoceptor using rhodopsin as the template has been described⁸ where positively charged side chains of certain amino acid residues have been shown to be essential for ATP binding.

In 1994 Uri et al.³ introduced peptidyl derivatives of adenosine-5'-carboxylic acid⁹ (AdoC) as possible ligands for P₂ purinoceptors. The simple and productive procedure of the synthesis of new ATP analogs was based on the conventional solid phase peptide synthesis method, where the peptide chain was assembled and AdoC attached to it on a Wang-type polystyrene resin. Two of the new conjugates, AdoCAsp₃ and AdoCAsp₄, derivatives of AdoC with three and four aspartate residues, inhibited ATP response on P₂ receptors of C6 cells.³ AdoCAsp₄ has also shown activity in other systems carrying P₂ receptors.¹⁰ Although these two conjugates resemble ATP by their high negative charge concentrated in a small volume close to the adenosine part of the molecule, the spatial arrangement of these charges and distances between them should be quite different.

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Scheme



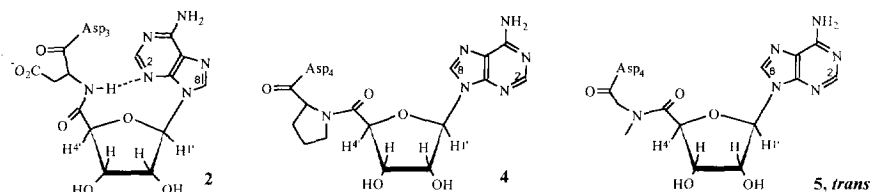


Figure 1. Orientation of the C(O) group and adenine base of AdoC relative the sugar ring in **2**, **4** and the *trans* form of **5**.

the close proximity of negative charges of aspartates to H8, since DMACA also exhibits signals for these hydrogens at 8.67 and 8.20 ppm.¹³ The great downfield shift of H8 may be attributed to the proximity of the carboxyl oxygen of AdoC to H8 or even a weak hydrogen bond between these two atoms.¹⁴ The *cis* - *trans* isomerization equilibrium about AdoC-Pro in **4** is shifted far toward the *trans* form (>95% by ¹³C NMR).

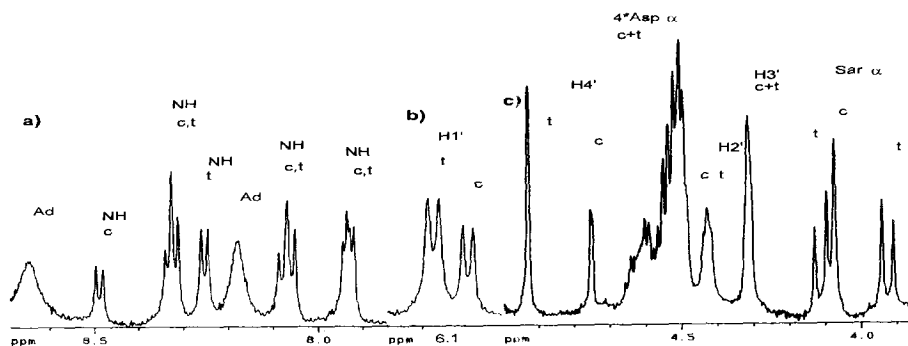


Figure 2. Fragments from the ¹H NMR spectrum of AdoCSarAsp4, **5** (500 MHz; DMSO-d₆; r.t.).

In AdoCSarAsp₄, **5**, two conformers with *trans* and *cis* peptide groups between AdoC and Sar are represented respectively in ratio 2:1 (by ¹H NMR in DMSO-d₆). ¹H (Fig. 2) and ¹³C NMR spectra indicate substantial differences in the 3-D structures of the rotamers. ¹H signals of sarcosine N-CH₃ appear for the *trans* and *cis* forms at 3.01 and 2.80 ppm. Sarcosine CH₂ protons of the *cis* conformer show a single peak at 4.07 ppm, whereas two methylene protons of the *trans* conformer appear in a different field (anisochronism), at 4.10 and 3.92 ppm (doublets, *J*_{gem}=16.2Hz). This may indicate the presence of an extra hydrogen bond in the *trans* conformation that makes the form less flexible. Isomerization of the amide group is also reflected in the spectrum of protons of the sugar and adenine parts of the conjugate. Signals of H4' appear at 4.93 and 4.75 ppm, the large (0.18 ppm) upfield chemical shift of the *cis* conformer may indicate the location of strongly anisotropic carbonyl group of sarcosine near H4'. Signals of H1' (Fig. 2, b) for the *trans* and *cis* conformers at 6.08 and 6.04 ppm are also separated. Similar coupling constants (*J*_{1',2'}=6.8 Hz for *trans* and 6.2 Hz for *cis*; *J*_{3',4'}=1.5 Hz for *trans* and 2.4 Hz for *cis*) point to the minute changes in the puckering mode of the sugar as the result of the *trans*-*cis* isomerization of AdoCSar amide group. All 4 pairs of the NH signals between 7.9 and 8.5 ppm (Fig. 2, a) are separated and thus confirm the differences in the structure of the peptide chain in the two

conformers. Adenine H2 and H8 both give broad signals. In the ^{13}C NMR spectrum of **5** double peaks in ca 2/1 ratio appear at 86.43 and 86.86 (C1'), 81.13 and 80.74 (C4'), 74.81 and 74.63 (C2'). C3' gives a single line at 72.65 ppm. Also large (if compared to <0.5 ppm for AdoCAsp₄) chemical shift difference of C2' and C3' should be noted. ^{13}C NMR signals from 10 carboxyl groups are clearly grouped into amide CO (167.7–170.6 ppm) and acid (171.7–172.1 ppm) signals. The amide CO resonances are characterized with pairs of signals from *trans* and *cis* conformers: 167.82/167.68, 170.64/170.50, 169.90/170.00, 170.31/170.33, 170.13 (single peak).

The pattern of chemical shifts in the ^{13}C NMR spectrum of the adenosine part of **6** is similar to that of ATP.¹⁴ The ^{13}C chemical shift difference of C2' and C3' bigger than 2.8 ppm (3.6 ppm in **6**) is characteristic for the *anti* conformation of nucleosides.¹⁵ Close proton-proton $J_{1,2}$ coupling constants (4.8 Hz for **6** and 5.6 Hz for adenosine phosphates¹⁶) indicate similar sugar puckering modes in these compounds. Therefore, it is possible to prepare ATP analogs, conjugates of Ado and highly charged peptides, with retained 3-D structure of the adenosine part of the molecule by introducing a C(O) group between the adenosine O5' and peptide chain.

6, like all other synthesized conjugates, showed five separated carboxylate ^{13}C signals (178–179 ppm) for four aspartates (Fig. 3, a; separation of the two high field signals is not seen in this compressed Figure) at higher (>6) and five separated carboxylic acid signals (173–174 ppm) for four protonated acids (Fig. 3, c) at lower (<2) pH values, and also substantial protonation shifts (PS) of C α and C β ^{13}C signals of the aspartates. These PS and their behavior during the titration should be a convenient source for the determination of pK_a values of the adenine and different Asp residues. Actually, in weakly acidic medium (2<pH<6) strong broadening of many signals, including the most sensitive positions to the protonation process - carboxylate and C β carbons, was

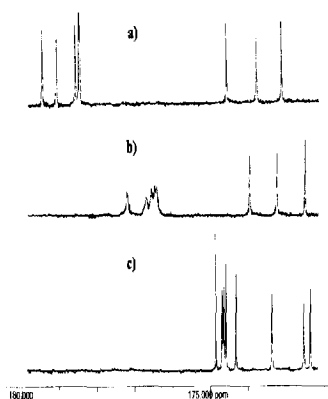


Figure 3. Carboxyl carbon region (without urethane CO) from the ^{13}C NMR spectra of AdoOC(O)Asp₄, **6** (125 MHz; D₂O; r.t.); a: pH=9.0, b: pH=4.1, c: pH=1.1.

observed (Fig. 3, b). This may indicate the inter- or intramolecular association of aspartate groups, like it has been established for oligo- and polyglutamic acids,¹⁷ although other explanations for the phenomenon are possible (e.g., traces of paramagnetic metal ions). Regardless of the broadening of some C α signals their positions could be determined in the course of titration. C α of the terminal δ -Asp is unambiguously assigned due to its large PS (-4.11 ppm) and the largest broadening of the signal during the titration process. The next residue (γ -Asp) with the PS of α -carbon -1.44 ppm was assigned on the basis of some broadening of the signal. The remaining α - (PS -1.77 ppm) and β - (PS -1.51 ppm) Asp residues were assigned on the basis of their chemical shift and PS values. It is reasonable to assume that in β - and γ -Asp residues α -carbons have close values of chemical shifts (50.69 and 50.82 ppm at pH=1; C α of α -Asp gives a signal at

52.04 ppm). Direct plotting of the signals from α -, β - and γ -Asp C α carbons against pH meter readings in D₂O solution (MI-412 microelectrode from Microelectrodes Inc., 8 pH values from 1.1 to 12) and the curve fitting (PC Origin 4.10 from Microcal Software, Inc.) gave the following pK_a values: α -Asp, pK_a=4.06 \pm 0.06; β -Asp,

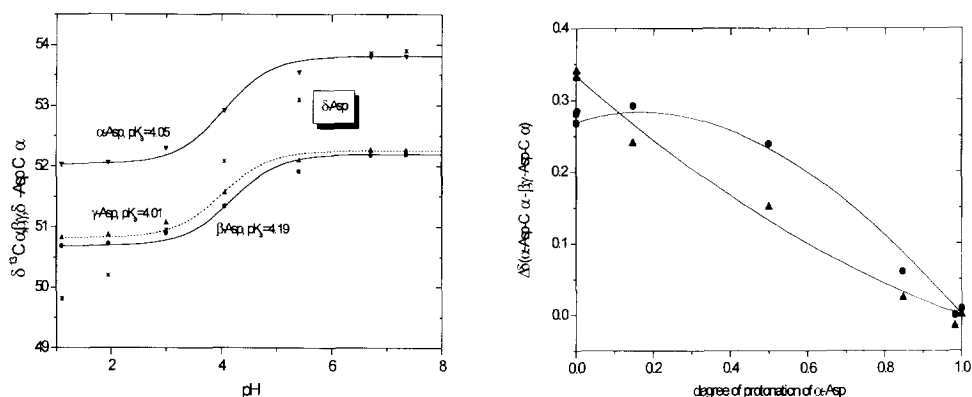


Figure 4. Comparison of dissociation constants of different carboxylates of **6** on the basis of the peptide chain $C\alpha$ chemical shifts; left graph: traditional pH - δ plots; right graph: comparison of dissociation constants of β - (●) and γ - (▲) Asp residues to α -Asp residue according to the method of pK_a difference ^{13}C NMR spectroscopy.¹⁸

$pK_a=4.20\pm0.08$, and γ -Asp, $pK_a=4.01\pm0.06$; as shown on the left side of Fig. 4. Relatively large errors result from the line broadenings observed, small PS of $C\alpha$ carbons remote from the protonation site and small number of measurements in the protonation region. δ -Asp was not included into the curve fitting as PS of its $C\alpha$ was caused by the protonation of both acid groups.

From the practical point of view the relative order of acidities of different ionization sites in the molecule is often more important than the absolute pK_a values. For the comparative analysis of pK_a values the method based on ^{13}C chemical shift differences gives more reliable results.¹⁸ One of its main advantages is the use of the degree of protonation of a reference compound and no need for pH measurements. In the right side of Fig. 4 the plots for the calculation K_a values of β - and γ -Asp related to α -Asp are given. Concave curve for γ -Asp (▲) shows that it is stronger acid than α -Asp. The opposite is true for β -Asp (●) with resulting convex curve. Curve fitting [$K\alpha/K\beta = 1.30\pm0.04$ ($\Delta pK_a=-0.11$) and $K\alpha/K\gamma = 0.91\pm0.03$ ($\Delta pK_a=0.04$)] gives pK_a differences along the Asp_4 chain. Observed ΔpK_a values of Asp residues originate from the effect of the connected neighbors. Thus γ -Asp must have the lowest pK_a due to strong influence of the terminal δ -Asp with two free carboxylate groups, and the proximity of the AdoOC(O) moiety to the acid group of α -Asp is going to enhance somewhat its acidity, leaving β -Asp with the highest pK_a value. The adenine base of **6** showed large PS for all carbon atoms. Curve fitting from C6 (PS -5.60 ppm) gives $pK_a=3.74\pm0.10$, from C2 (PS -8.19 ppm) $pK_a=3.71\pm0.10$ and from C8 (PS +2.81 ppm) $pK_a=3.78\pm0.11$. These values point to the site of protonation (N1) and are in good agreement with the pK_a values reported for the protonation of N1 sites of the adenine residue of AMP ($pK_a=3.61$) and adenosine 5'-O-thiomonophosphate ($pK_a=3.72$).²⁰ The amide group in the carbamate moiety of **6** probably occurs in more usual *trans* conformation,¹⁹ however closer inspection of the ^{13}C NMR

spectra revealed several smaller lines (<10% intensity by ^{13}C NMR) close to the main ones in the sugar part of the molecule at medium and higher pH values, which may indicate the presence of *cis*-orientated structures.

Taken together, the synthetic methods outlined in the Scheme provide highly charged (-5 at pH=7.4, as compared to -3.8 of ATP) ATP analogs with different arrangement of negative charges. Consequently, it is possible to generate great numbers of novel ATP analogues as pure compounds or in the form of their equimolar mixtures ("chemical libraries") that can be further tested for biological (pharmacological) activity. Due to the peptide nature of the new compounds it is possible to take advantage of all drug development methods for peptide-type hormones. Taking into account the importance of ATP in many enzymatic systems novel potential ligands of purinoceptors should also be tested as inhibitors of ATP consuming enzymes.

Acknowledgment: We thank the Estonian Science Foundation for financial support (grants 1754 and 2293).

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12. **AdoOC(O)Asp**, **6**. The synthesis of 5'-(4-nitrophenyl carbonate) of 2',3'-IpAdo, **10**, followed the published procedure.²¹ Chromatographically (silica; acetone - ethyl acetate 1:1. v/v; R_f =0.54) purified product showed correct ^1H NMR spectrum: (DMSO, d_6 , TMS, δ : 1.36 and 1.57 (2 x s, 6H, 2 x CH₃ of isopropylidene); 4.3 - 4.6 (m, 3H, H4' and 2xH5'); 5.17 (dd, 1H, 6.2 and 2.4 Hz, H3'); 5.50 (dd, 1H, 6.2 and 2.2 Hz, H2'); 6.25 (d, 1H, 2.2 Hz, H1'); 7.37 (s, 2H, 6-NH₂); 7.49 (d, 2H, 9.4 Hz, 2 x o-H); 8.17 (s, 1H, H2); 8.29 (d, 2H, 9.4 Hz, 2 x m-H), 8.34(s, 1H, H8). **10** (33 mg, 0.077 mmol) was dissolved in 1.5 mL DMF containing DIEA (90 μL) and the solution added to **1** (0.061 mmol NH₂). After 40 h reaction **6** was cleaved from the resin with a 2 h treatment in 90% trifluoroacetic acid and purified on a preparative C₁₈ HPLC column. MS: M/z = 772(M+H), 794(M+Na), calculated M =771; NMR in D₂O, discussed in the text.
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