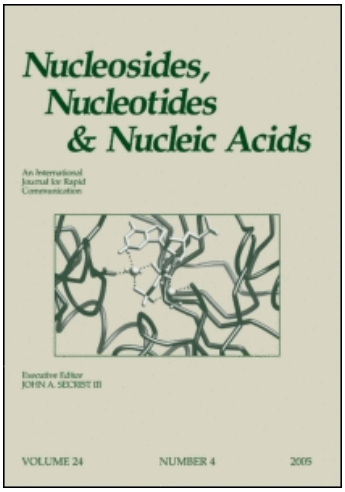

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Synthesis and Properties of 7-Acetyl-1, N^6-etheno-AMP and 7-Acetyl-1, N^6-etheno-NAD

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SYNTHESIS AND PROPERTIES OF 7-ACETYL-1,N^6-ETHENO-AMP AND 7-ACETYL-1,N^6-ETHENO-NAD^+.

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ABSTRACT

Synthesis, PMR- and UV/Vis-Spectroscopic data of 7-Acetyl-εAMP and 7-Acetyl-εNAD^+ are described. Due to their unique optical properties (strong absorption and fluorescence well above 300 nm) these nucleotide analogs appear well suited as fluorescent probes in protein-ligand studies.

INTRODUCTION

1,N^6-ethenoadenosine-nucleotides[1],first described by Secrist et al. in 1972[2,3],have been used as fluorescent coenzyme analogs in several protein-ligand interaction studies[4-12]. Subsequently,several fluorescent etheno-analogs of other nucleotides were designed for specific kinetic and equilibrium fluorescence studies[13-15]. Quantitative evaluation of fluorescence data obtained with ethenoadenosine nucleotides and proteins, however,was complicated by the broad overlap of the respective fluorescence and absorption spectra. To avoid this difficulty,we prolonged the conjugated double bond system of the etheno-ring by introducing an acetyl side chain in position 7 of two common ethenonucleotides,1,N^6-etheno-AMP and 1,N^6-etheno-NAD^+. The new compounds have UV/Vis-optical properties, which make them especially suited for fluorescence studies with proteins.

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RESULTS AND DISCUSSION

PMR-Spectroscopic Data

Table 1 summarizes the chemical shifts of the newly synthesized compounds 1 and 2,of εAMP and εNAD$^+$ and of AMP and NAD$^+$. To have all the data under exactly the same conditions,the resonance values of these latter nucleotides were redetermined,although their pmr-data are already published[2,16]. Comparison of the chemical shifts of compound 1 with those of εAMP and AMP leads by the following considerations to the proposed structure of compound 1 (Figure 1):

1) The resonance at δ = 2.67 ppm (three identically bound protons) must originate from the acetylgroup of the α-BKB used for the reaction. This group must be present now as a side chain in aromatic linkage[17],because the aliphatic acetylgroup of ß-KBDA shows resonance only at 2.20 ppm (own data,not shown).

2) Instead of the two doublet-protons εH7 and εH8,which are characteristic for the ethenobridge,the isolated compound 1,as compared

TABLE 1: PMR-Data (chemical shifts in ppm) of Different Nucleotides. Numbering of the protons follows the nomenclature for ethenoadenine-nucleotides[2,3] (see fig.1). Solvent: 10% D_2O in d_6-DMSO. Each resonance corresponds to 1 proton,except for the acetylgroup,were 3 protons were detected*)

	Pyridinium protons in position no.:				Ethenoadenine protons in position no.:(see fig.1)				Acetyl side chain
Compound	2(s)	6(d)	4(d)	5(t)	8	2(s)	5(s)	7(d)	
AMP	-	-	-	-	-	8.59	8.19	-	-
εAMP	-	-	-	-	7.64 (d)	8.61	9.35	8.19	-
Compound 1	-	-	-	-	10.0 (s)	8.60	8.70	-	2.67
NAD$^+$	9.53	9.39	9.08	8.37	-	8.77	8.55	-	-
εNAD$^+$	9.76	9.56	9.10	8.44	7.84 (d)	8.80	9.54	8.32	-
Compound 2	9.68	9.50	9.10	8.40	10.2 (s)	8.72	8.90	-	2.68

*) Abbreviations used in the table are: s = singlet-proton; d = doublet-proton; t = triplet-proton.

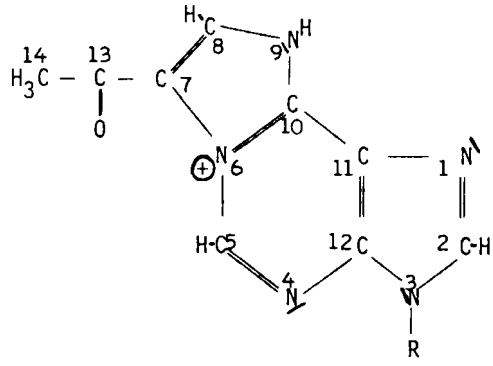

FIGURE 1: Proposed structures for compound 1 (R = Ribose-5'-phosphate; 7-Acetyl-εAMP) and compound 2 (R = Ribose-5'-phosphate-NMN$^+$; 7-Acetyl-εNAD$^+$).

to AMP,contains only one new proton in the aromatic region (δ = 10.0). In analogy to the formation of the ethenonucleotides,its appearance indicates the formation of an acetylated heterocyclic ring between aN1 and the aminogroup at aC6. With δ = 10.0 ppm,this proton is shielded very weakly as compared to the other protons of this compound. The reason for this is the close neighbourhood of the acetylgroup at εC7, whose carbonyl exerts a strong attractive force upon the electrons along the system of conjugated double bonds in the heterocyclic etheno-ring. The electronic environment of εH2 seems to be influenced only weakly by this force,whereas the resonance of εH5 is lowered from δ =9.35 ppm to δ = 8.70 ppmby the shielding effect of the acetylgroup at εC7.

The chemical shifts of the reaction product of NAD^+ with α-BKB (compound 2) reveal that the protons of the pyridinium-ring[4] have been completely preserved; hence this ring has not been attacked by the aldehyde. The protons εH2 and εH5 exhibit similar chemical shifts in compound 1 as well as in compound 2. The weakly shielded proton with δ = 10.2 ppm is the same as in compound 1,likewise the 3-proton resonance at δ = 2.68 ppm of the acetyl side chain.

UV/Vis-Spectroscopic Data of compounds 1 and 2

To confirm the chemical structure of these two new nucleotides and to establish their suitability for protein-ligand studies,we examined the pH-characteristics of their UV/Vis-spectra.

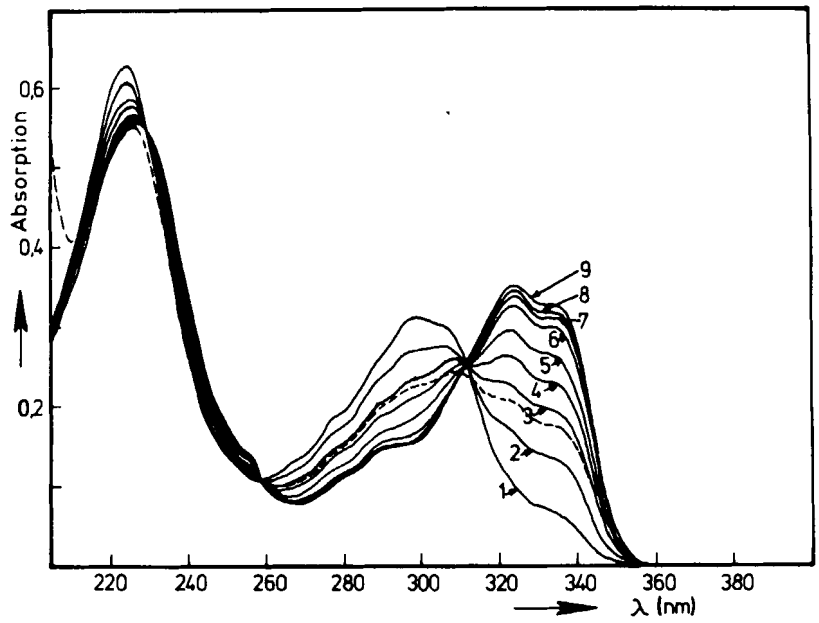

FIGURE 2: UV/Vis-absorption of 7-Acetyl-εAMP at different pH-values.
A 30 μM solution of the nucleotide in 0.10 N HCl at 20°C was titrated with increasing amounts of 3N NaOH. The numbers indicate the following pH-values: 1=pH 1.2; 2=pH 1.6; 3=pH 1.95; 4=pH 2.20; 5=pH 2.50; 6=pH 3.00; 7=pH 3.60; 8=pH 5.60; 9=pH 11.00. Broken line: Readjustment from pH 11 to pH 2.0.

The absorbance spectrum of 7-Acetyl-εAMP at different pH-values is picked up in figure 2. The spectrum contains 3 isosbestic points at 311, 259 and 229 nm,which persist over the whole pH-range,proving the purity of the compound or at least the absence of chromophores with other spectral properties. The pH-dependence of the absorption at 324 nm corresponds to a pK_a of 1.90,a value being almost 2.5 units lower than the pK_a of the unsubstituted etheno-chromophore[16]. This difference can easily be explained with the proposed structure for compound 1,where the C=O - double bond of the 7-acetyl group stands in resonance with the conjugated double bond system of the etheno-heterocycle. Therefore,the free π-electronpairs of the two neighbouring nitrogen atoms are included more strongly in limiting mesomeric structures than in the unsubstituted etheno-compounds. This view is supported by the extremely low electron density at εC8.

Contrary to the etheno-chromophore,whose maximal absorption is pH-independent,the absorption maximum of 7-Acetyl-εAMP is shifted by ca.30 nm to higher wavelenths with raising pH-value. As already stated for the lower pK_a of 7-Acetyl-εAMP,this effect as well is possibly due to the

contribution of additional mesomeric structures in the unprotonated state of 7-Acetyl-εAMP. The absorption maxima of the unprotonated state of 7-Acetyl-εAMP and εAMP,respectively,differ by ca. 60 nm. This shift is similar to the one observed when benzene and acetophenone are compared[18] and may serve as an additional proof for the proposed structure of compound 1.

The absorption spectrum of 7-Acetyl-εNAD^+ above 290 nm was identical with that found for 7-Acetyl-εAMP. The additional nicotinamide chromophore (λ_{max} = 260 nm) in the former,however,changes its absorption at lower wavelenths. The position of the isosbestic points was exactly the same as already described for 7-Acetyl-εAMP,likewise the pK_a of 1.90 (no figs.). This indicates that in both cases the same chromophore is titrated and supports the proposed structures (see fig.1).

Fluorescence Properties of 7-Acetyl-εAMP and 7-Acetyl-εNAD^+

Excitation and emission spectra of 7-Acetyl-εAMP at different pH-values are shown in figure 3. The excitation and emission maxima are centered around 330 and 374 nm,respectively,and are pH-dependent. Their intensity, however,is - very similar to the absorption (figure 2) - ca. 4-times higher at pH 7.6 than at pH 1.3. This contrasts with the behaviour of εAMP, whose fluorescence in neutral solution is ca. 50-times higher than at pH 1.0,although its absorption is lower by a factor of 0.5 at pH 7.6 than under acidic conditions[16]. The Stoke's shift of 7-Acetyl-εAMP is only 44 nm (9.5 kcal/mol) as compared to 110 nm (26 kcal/mol) observed with εA·HCl[2]. From this it may be concluded that the polarities of the ground and the excited state of 7-Acetyl-εAMP are similar and that reorientation of the solvent molecules does not occur after excitation of the chromophore.

The fluorescence behaviour of 7-Acetyl-εNAD^+ was qualitatively the same as that observed for 7-Acetyl-εAMP,the quantum yield,however,was ca. 10-times less. Similarly,a reduced quantum yield by a factor of about 10 of εNAD^+ as compared to εAMP was observed[10] and was ascribed to a "closed" conformation of free εNAD^+ in aqueous solution. The same consideration seems to apply for 7-Acetyl-εNAD^+.

7-Acetyl-εNAD^+ as a Coenzyme of Glutamate Dehydrogenase

Using 7-Acetyl-εNAD^+ as coenzyme,glutamate dehydrogenase from bovine liver exhibited ca. 70% of its catalytic activity as compared to standard assay mixtures[19] containing NAD^+.

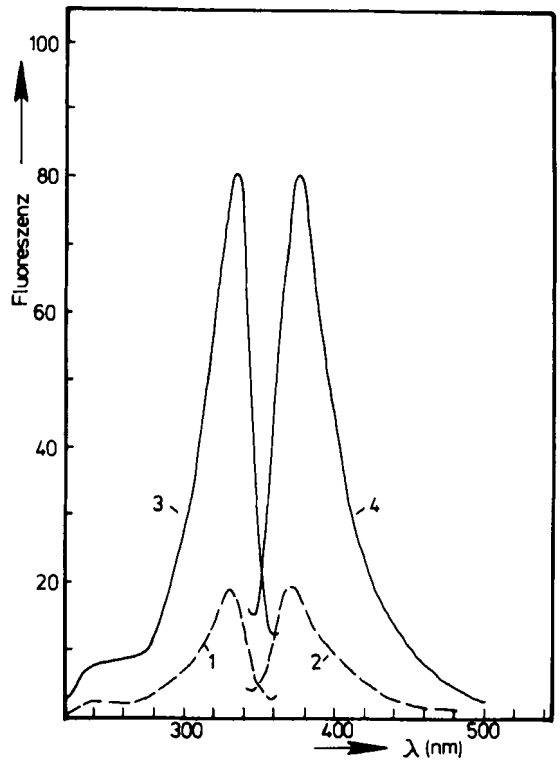

FIGURE 3: Fluorescence of 7-Acetyl-εAMP at pH 1.3 (curves 1 and 2) and pH 7.0 (curves 3 and 4).

Measurment in 0.10 N HCl or PP (pH 7.0),respectively. Nucleotide concentration was 16.6 μM.

Wavelengths (uncorrected): Excitation at 330 nm (curves 2 and 4)
Emission at 374 nm (curves 1 and 3).

METHODS AND MATERIALS

Methods

1) Synthesis of α-Bromo-ß-ketobutanal (α-BKB): α-BKB was synthesized as described in20) with the following modifications: To a strongly stirred mixture of 15 g (110 mmoles) of ß-KBDA and 11 g (55 mmoles) $BaCO_3$ in 30 ml H_2O are added dropwise 6.20 ml Br_2 (120 mmoles). During this,the temperature is raised slowly to 50°C under irradiation by a reaction lamp (white light). The reaction is initiated at 50°C by addition of ca. 10 mg solid benzoylperoxide. Initially after decoloration the mixture is cooled in an ice bath,organic and aqueous phase are separated and the latter is extracted with 3x200 ml diethylether. The extracts are combined with the oily organic phase and dried over Na_2SO_4. The solvent is evaporated under reduced pressure below 10°C,leaving behind a light yellow mixture of oily and crystalline material,which is used immediately or stored at -20°C. Yield: 9.5 g or 52%.

2) Synthesis and purification of 7-Acetyl-εAMP: 1 g (2.5 mmoles) AMP,free acid,is dissolved in 20 ml 0.25 N LiOH,the pH is adjusted to

4.5 (1 N HCl) and the solution is kept at 40°C in a water bath. Under strong stirring (hood!),4 g (25 mmoles) α-BKB,containing some MgO for stabilization are added quickly. The pH is kept constant at 4.5 with 2 N LiOH. After one hour,again 4 g of α-BKB are added. The proceeding of the reaction is monitored by precipitating 0.10 ml with 2 ml of cold acetone and measuring the extinction of the redissolved precipitate at 324 nm in PP of pH 7.6. A_{324} is constant after ca. 12 hrs. After extraction with diethylether (3x200 ml),the aqueous phase is concentrated under reduced pressure at 40°C to ca. 10 ml and then poored into 500 ml of cold acetone. The precipitate is collected by centrifugation and is almost salt-free because of the good solubility of LiBr in acetone. The redissolved (H_2O) precipitate is adjusted to pH 4.5 and applied to a Dowex 1x2-column (40x1.5 cm,formate-form in H_2O). After washing with 400 ml H_2O a linear gradient (250 ml H_2O ------> 250 ml 2 M HCOOH) is applied,eluting ca. 50% of starting material(AMP) and 5-7 minor unidentified products. The following gradient (250 ml H_2O ------> 250 ml 4 M HCOOH) removes some more unknown products and ends at the beginning of the 7-Acetyl-εAMP - peak. The compound is eluted with ca. 300 ml 4 M HCOOH; fractions exhibiting identical absorption spectra at pH 6-9 (λ_{max} = 324 nm;shoulders at 335 and 290 nm; λ_{min} = 265 nm,see fig.2) are combined and lyophilized several times for complete removal of formic acid. Yield: 400 mg or 32% of pale yellow 7-Acetyl-εAMP.

3) Synthesis and purification of 7-Acetyl-εNAD$^+$:The reaction mixture contains NAD^+ and α-BKB in the same molar ratios and quantities as already described for the synthesis of 7-Acetyl-εAMP. After ca. 20 hrs, A_{324} is constant. The product mixture is prepared for chromatography as already described for the AMP-derivative. After washing the column with ca. 400 ml H_2O,a linear gradient (250 ml H_2O ------>250 ml 3 M HCOOH) is applied which elutes mainly unreacted NAD^+. 7-Acetyl-εNAD$^+$ appears at the end of the following gradient (250 ml 3 M HCOOH ------>250 ml 5 M HCOOH) and is eluted with some more 5 M HCOOH. Fractions exhibiting identical absorption spectra (λ_{max} = 324nm; shoulders at 335,290,275 and 255 nm;λ_{min} = 280 nm in PP at pH 7.6) are combined and lyophilized several times. Yield: 500 mg or 28% pale yellow 7-Acetyl-εNAD$^+$.

4) Photometric determination of nucleotide concentrations: The extinction coefficient at 324 nm of 7-Acetyl-εAMP and 7-Acetyl-εNAD (pH 7.60) was determined gravimetrically and by estimation of phosphate according to Chen[21]. For both nucleotides ε_{324} was 12 $mM^{-1}.cm^{-1}$.

5) PMR-Spectra: The purified and formate-free nucleotides were dissolved at 15-25 mg/ml (AMP and derivatives) or 30-50 mg/ml (NAD^+ and derivatives). Solvents were D_2O and d_6-DMSO. The spectra were calibrated internally by comparison of experimental resonance values with the resonance of TMS (δ = 0 in d_6-DMSO) or the sodium salt of TriMPS (δ = 0 in D_2O).

6) Fluorescence spectra were measures on a Perkin-Elmer spectrofluorimeter without correction for spectral characteristics of lamp,monochromator and detector.

7) Absorption spectra were recorded on a CARY 18 spectrophotometer.

8) Oxidation of glutamate by 7-Acetyl-εNAD$^+$ in presence of glutamate dehydrogenase: This assay was performed as described by Dieter et al. for NAD^+ as coenzyme[19].

Materials

ß-KBDA from Fluka-AG,CH-9470 Buchs,was distilled under reduced pressure before use. Dowex 1x2,200-400 mesh and AMP (free acid) were from Serva,Heidelberg. ß-NAD^+·Na_2 as well as bovine liver glutamate dehydrogenase were from Boehringer,Mannheim. D_2O (Uvasol quality),TMS and TriMPS·Na were from Merck,Darmstadt,d_6-DMSO from the "Eidgenössische Institut für Reaktorfor-

schung" in CH-5303 Würenlingen. All other chemicals were of biochemical reagent grade from commercially available sources.

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REFERENCES AND FOOTNOTES

1) Abbreviations used in the text are:

aC2,aC5,...: carbon atom in position 2,5,...of the adenine-chromophore.
aH2,aH5,...:proton in position 2,5,...of the adenine-chromophore.
α-BKB: α-Bromo-β-ketobutanal
β-KBDA: β-Ketobutanal-dimethylacetal
d_6-DMSO: completely deuterated dimethylsulfoxide
εC7,εC8,...: carbon atom 7,8,...of the ethenoadenine-chromophore.
εH7,εH8,...: proton in position 7,8,...of the ethenoadenine-chromophore.
εAMP,εNAD$^+$: compounds with an etheno-bridge between aC1 and the nitrogen atom at aC6. The correct designation of this chromophore is imidazo(2,1 - i)-purine.
ethenonucleotide,etheno-chromophore,...: see εAMP,εNAD$^+$.
NMN$^+$: Nicotinamide-mononucleotide (oxidized form)
PMR: Proton magnetic resonance
PP: 0.067 M potassium phosphate buffer,pH-values are indicated in the text.
TMS: Tetramethylsilane; TriMPS: Trimethylsilyl-phosphosulfonic acid

2) J.A.Secrist III,J.R.Barrio,N.J.Leonard and G.Weber,Biochemistry 11, 3499 (1972).

3) J.R.Barrio,J.A.Secrist III and N.J.Leonard , Proc.Nat.Acad.Sci.U.S.A. 69,2039 (1972).

4) C.Y.Lee and J.Eversee,Arch.Biochem.Biophys.157,83 (1973).

5) P.B.Chock,C.Y.Huang,R.B.Timmons and E.R.Stadtman,Proc.Nat.Acad.Sci. 70,3134 (1973).

6) M.Deluca,N.J.Leonard,B.J.Gates and W.B.McElroy,Proc.Nat.Acad.Sci. U.S.A.70,1664 (1973).

7) G.E.Willick,K.Oikawa,W.D.McCubbin and C.M.Kay,Biochem.Biophys.Res. Commun.53,923 (1973).

8) J.R.Barrio,J.A.Secrist III,Y.Chien,P.J.Taylor,J.L.Robinson and N.J. Leonard,FEBS Lett.29,215 (1973).

9) H.H.Dieter,R.Koberstein and H.Sund,FEBS Lett.47,90 (1974).

10) P.L.Luisi,A.Baici,F.J.Bonner and A.A.Aboderin,Biochemistry 14,362 (1975).

11) R.Koberstein,H.H.Dieter and H.Sund,in "Protein-Ligand Interactions" (H.Sund and G.Blauer,eds.),Walter de Gruyter,Berlin-New York, p.97 (1975).

12) L.D.Burtnick and P.L.Schaar,FEBS Lett.97,166 (1979)

13) R.Gregory,D.Recktenwald,B.Hess,H.J.Schäfer,P.Scheurich and K.Dose, FEBS Lett.108,253 (1979).

14) J.P.Miller,T.S.Yagura,R.B.Meyer jr.,R.K.Robbins and H.Uno,J.Carbohydr. Nucl.Nucl.7,167 (1980).

15) G.A.Bhat,K.H.Scram and L.B.Townsend,J.Carbohydr.Nucl.Nucl.7,333 (1980).

16) G.R.Penzer,Europ.J.Biochem.34,297 (1973).

17) D.H.Williams and L.Fleming,in "Spektroskopische Methoden in der Organischen Chemie",Georg Thieme Verlag,Stutlgart (1968).

18) L.Doub and J.M.Vandenbelt,J.Am.Chem.Soc.69,2714 (1947).

19) H.H.Dieter,R.Koberstein and H.Sund,Europ.J.Biochem.115,217 (1981).

20) N.N.Kotchetkov and E.E.Nifantev,Ber.Akadem.Wiss.USSR (engl.translation) 121,553 (1958).

21) P.S.Chen,T.Y.Toribara and H.Warner,Anal.Chem.28,1756 (1956).

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