86. Alkylation of Adenine, Adenosine, and NAD^+ with 1,3-Propanesultone. Synthesis of N^6-(3-Sulfonatopropyl)-NAD^+, a New NAD^+ Derivative with Substantial Coenzyme Activity

by **Giacomo Carrea**, **Gianluca Ottolina**, and **Sergio Riva***

Istituto di Chimica degli Ormoni, CNR, Via Mario Bianco, 9, I-20131 Milano

and **Bruno Danieli**, **Giordano Lesma**, and **Giovanni Palmisano**

Dipartimento di Chimica Organica ed Industriale, Facoltà di Scienze, Università degli Studi di Milano, Via Venezian, 21, I-20133 Milano

(26. II. 88)

The reactivity of 1,3-propanesultone with adenine, adenosine, and NAD^+ was studied in order to prepare N^6-(3-sulfonatopropyl)-NAD^+ (**3b**), a new NAD^+ derivative substituted at the purine moiety with substantial coenzyme activity for several dehydrogenases. The regiochemistry of the alkylation at the purine nucleus was investigated by UV, ^{1}H-NMR, and FAB-MS. FAB-MS proved to be a powerful tool for determining the molecular weight of these polar and poorly volatile compounds. In addition, regular fragmentation of **3b** and other NAD^+ derivatives was observed.

Introduction. – Large-scale transformations that use enzymes not dependent on coenzymes, such as hydrolases and isomerases, are now well established in organic synthesis and biotechnological processes [1]. NAD(P)-dependent enzymes have also been successfully employed in a variety of cases for laboratory-scale synthesis of chiral synthons [1a][1b][2], resolutions of racemates [1a][1b], oxidoreduction of steroids [3] and bile acids [4], and synthesis of labelled compounds [5], carnitine [6], and amino acids [7].

Use of these enzymes was made possible by the development of effective enzymatic methods for *in situ* regeneration of the expensive nicotinamide cofactors [8]. For large-scale applications of NAD(P)-dependent enzymes, continuous-flow reactors with ultrafiltration membranes which only retain high molecular weight compounds have been proposed [9]. To prevent their escape from the reactor, cofactors have been linked to several H_2O-soluble polymers through a proper functionalization of $NAD(P)^+$ with iodoacetic acid, propionolactone, and ethyleneimine [10]. Changes in the activity of these 'macromolecular' coenzymes and in their stability are current problems of the methodology. Recently, a significant and attractive contribution to the solution of the problem of coenzyme retention was proposed by *Kitpreechavanich et al.* [11] and *Howaldt et al.* [12]. They suggested using a sulfonated-polysulfone membrane which would prevent the passage of the anionic coenzyme, taking advantage of the electrostatic repulsion of the negatively charged functional groups of the membrane.

We reasoned that the residence time of $NAD(P)^+$ inside this kind of membrane reactor could be greatly enhanced by introducing a sulfonic group into the cofactor molecule. For this purpose, we reacted NAD^+ (**3**) with the bifunctional reagent 1,3-propanesultone, and in this paper, we report the synthesis and the physico-chemical charac-

terization of the new NAD^+ derivatives 1-(3-sulfonatopropyl)-NAD^{++} (**3a**) and N^6-(3-sulfonatopropyl)-NAD^+ (**3b**) and the coenzymatic properties of **3b**. Before carrying out this study, the reactivity of 1,3-propanesultone with adenosine (**1**) and adenine (**2**) was investigated, since the purine moiety is the reactive site of the coenzyme towards alkylating agents. From this investigation, we obtained information about the regiochemistry of the reaction, and we verified the usefulness of ^{1}H-NMR and FAB mass spectrometry for probing the alkylation sites.

Results and Discussion. – The great biological importance of the ubiquitous adenine derivatives in living systems has led to a large variety of studies and to continually expanding research in the synthesis of alkyl-substituted adenine and adenosine analogues. From these studies, a complex and somewhat intriguing picture emerges, indicating that the purine moiety is a multident nucleophilic system that can react at different N-sites, with variable yields depending on solvent composition and type of alkylating reagent [13]. In dipolar aprotic solvents, adenosine (**1**) furnishes mainly products of N(1) alkylation accompanied to a lesser extent by N(7) alkylation. There is no reaction at N(3) because of hindrance by the ribose unit [14]. In aqueous solvents, products involving alkylation at the ring atoms N(1) and N(7) as well as at the exocyclic N^6 have been obtained [14]. On the other hand, in neutral media (aqueous solution or dipolar aprotic solvents), adenine (**2**) furnishes N(3)-alkyl derivatives as the dominant products, accompanied by N(9)- and N(1)-alkylated by-products [15]. The alkaline salts of adenine are alkylated at N(9) in dipolar aprotic solvents and at N(9) and N(3) under phase-transfer-catalysis conditions [16].

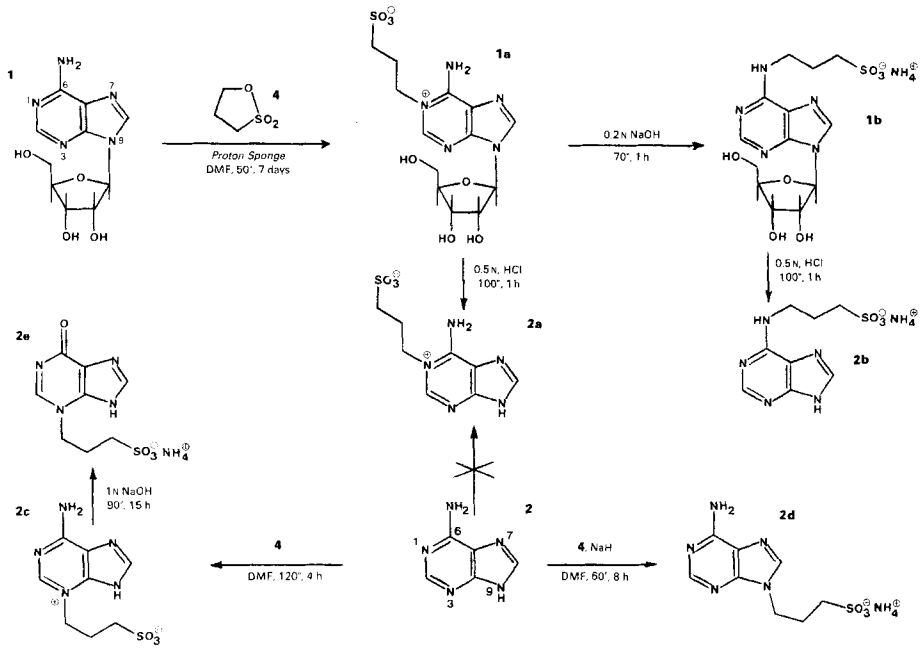
The 1,3-propanesultone (**4**) is a versatile intermediate which can react with a large variety of substrates [17]. Unlike the corresponding γ-lactone which behaves as an acylating agent in the reaction with nucleophyles, **4** behaves as a sulfoalkylating agent, which undergoes alkyl–O bond cleavage rather than sulfonyl–O bond fission: in this respect, its reactivity is similar to that of β-propionolactone. The zwitterionic sulfopropylated derivatives of many N-compounds, including pyridine, are among the most interesting products that can be obtained from **4** [17b]. These chemical properties strongly point to 1,3-propanesultone as the ideal reagent for the functionlization of NAD^+ with a moiety carrying a SO_3^- group.

The reactions of adenosine (**1**) and adenine (**2**) with 1,3-propanesultone (**4**) are shown in *Scheme 1*. When **1** was reacted at 50° with 5 equiv. of **4** in DMF in the presence of the proton scavenger 1,8-bis(dimethylamino)naphthalene (= *Proton Sponge*®), there was conversion of as much as 77% into a single product (direct HPLC analysis of the reaction mixture)[1]), 3-(1-adenosinio)propanesulfonate (**1a**), which was isolated after ion-exchange chromatography in 51% yield. The ammonium 3-(adenosin-N^6-yl)propanesulfonate (**1b**) could be obtained from **1a** by amidine rearrangement (*Dimroth* rearrangement) [18]: when **1a** was heated at 70° in alkaline aqueous solution (pH 11.5), it was rapidly and completely converted into **1b** and then isolated as the ammonium salt after ion-exchange chromatography.

Adenine (**2**) reacted with **4** in hot DMF solution to give a product recognized as 3-(3-adeninio)propanesulfonate (**2c**), which crystallized from the cold reaction mixture

[1]) Minor yields were observed in the absence of the proton scavenger.

Scheme 1



[19]; other minor products remained in the mother liquor and were not isolated. Nucleosides **1a** and **1b** were easily hydrolyzed in acid solution (0.5M HCl) providing the corresponding adenine derivatives 3-(1-adeninio)propanesulfonate (**2a**) and ammonium 3-(adenin-N^6-yl)propanesulfonate (**2b**), not accessible by direct reaction [20]. Finally, when **2** was reacted with **4** in the presence of NaH, ammonium 3-(adenin-9-yl)propanesulfonate (**2d**) was the major product (accompanied by a small amount of **2c**) [19] and was isolated as ammonium salt by ion-exchange chromatography.

UV analysis is established as a convenient and reliable routine method for determining the alkylation site of the purine moiety of **1** and **2** [21]. Accordingly, **1a** and **1b** had UV spectra with λ_{max} at 258.5 and 267 nm, while in the adenine series, the λ_{max} for **2a–d** were, respectively, 264, 267, 274, and 260 nm. Although these spectral differences are characteristic, they are not very large, and attribution could be troublesome, particularly when there is only one derivative. Therefore, we investigated other spectroscopic properties of our alkyladenosines and -adenines, hoping to find other sound criteria for differentiation. We focused first on the ^{1}H-NMR spectra. It has been reported [22a] that the separation between H–C(2) and H–C(8) signals could be of diagnostic value for simple alkylated purines. In a subsequent study [22b] on N^6-acyl-7 (or 9)-alkyladenine, this criterion was not of general validity, but the relative chemical shift of the introduced N-CH_2 group proved to be more reliable. The chemical shifts of purine H-atoms and propanesulfonate methylene groups of compounds **1**, **1a**, **1b**, **2**, and **2a–d** are reported in *Table 1*, with the data for N^6- and 1-methyladenine and 1-methyladenosine for comparison.

Table 1. *Chemical Shifts of Purine H-Atoms and Propanesulfonate Methylene Groups of Adenosine and Adenine Derivatives*[a])

Compound	Chemical shift [ppm]	
	purine H-atoms	propanesulfonate methylene groups
Adenosine (**1**)	8.35, 8.15	
1a	8.75	4.45, 2.60, 2.10
1b	8.35, 8.20	3.55, 2.55, 1.90
Adenine (**2**)	8.13, 8.12	
2a	8.62, 8.45	4.40, 2.55, 2.10
2b	8.16, 8.06	3.55, 2.50, 1.90
2c	8.76, 8.59	4.52, 2.50, 2.20
2d	8.23	4.27, 2.46, 2.12
1-Methyladenosine	8.12, 8.08	
1-Methyladenosine/CF_3COOH	8.77, 8.72	
1-Methyladenine	8.20, 8.10	
1-Methyladenine/CF_3COOH	8.55, 8.37	
N^6-Methyladenine	8.20, 7.85	

[a]) All the spectra were recorded in (D_6)DMSO with TMS as internal standard.

Compounds **1a**, **2a**, and **2c** showed pronounced downfield shift of their aromatic purine protons to values above 8.45 ppm (from 8.13, 8.12 for adenine (**2**) and 8.35, 8.15 for adenonsine (**1**)). On the other hand, in the N^6-sulfonatopropyl derivatives **1b** and **2b** and in the N(9) derivative **2d**, the same protons resonated close to those of the parent compounds. In addition, the resonances of **1b** and **2b** were in fair agreement with those of N^6-methyladenine. The same correspondence was not observed between the 1-sulfonatopropyl derivatives **1a** and **2a** and 1-methyladenosine or -adenine, because in the latter, H–C(2) and H–C(8) resonated in the same region as in the non-substituted compounds. However, if 1 equiv. of CF_3COOH was added to the solution of 1-methyladenosine or -adenine in DMSO, the corresponding cationic purine was formed and H–C(2) and H–C(8) experienced downfield shifts comparable to those of **1a** and **2a**. The different behaviour of **1a** and **2a** has been attributed to their delocalized alkyladeninium structure, internally stabilized by the sulfonate group (sulfobetaine). Analysis of the shifts of the 3 CH_2 groups of the propanesulfonate moiety in **1a–b** and **2a–d** gave additional support to this interpretation. Whereas the shift of the central CH_2 and of CH_2SO_3 were practically the same in all our products, the CH_2 group linked to the purine skeleton was found at different values, being downfield at 4.4–4.5 ppm in **1a**, **2a**, and **2c** (deshielded by positively charged N) and upfield at 3.5 ppm in **1b** and **2b**, where it is connected to the exocyclic N-atom. The same CH_2 group was found at 4.2 ppm in **2d**, as expected for an alkylbenzimidazole, allowing differentiation between N(9) and N^6 derivatives.

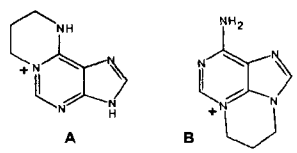
The above NMR data were useful for distinguishing propanesulfonates at N(1) and N(3) of adenine and adenosine from the corresponding N^6 and N(9) compounds. However, they were not very useful for differentiating **2a** from **2c**. This became possible through their chemical behaviours: **2a** underwent the amidine rearrangement on heating in mild alkaline solution (pH 11.5) at 70°, whereas **2c** was stable under the same conditions and was transformed in ammonium 3-(6,9-dihydro-6-oxo-3*H*-purin-3-yl)propanesulfonate (**2e**) under much more harsh conditions (1N NaOH, 90°, 15 h).

The MS behaviour of our products was investigated with the FAB technique, a recent but well settled methodology for direct analysis of thermally sensitive, highly polar, non volatile compounds [23]. The sulfobetaines **1a**, **2a**, and **2c**, which have a positive charge on the base and a negative charge on the sulfonate group, might be analyzed in either positive- or negative-ion modes, because of the ability of the compounds to undergo both protonation and deprotonation. Compounds **1b**, **2b**, and **2d**, which lack the positive charge on the purine nucleus, appeared to be amenable only to negative-ion detection.

The negative-ion FAB spectra of the sulfonatopropylated compounds were examined first, but the analysis was soon abandoned. In fact, intense $[M-H]^-$ and M^- ions were displayed at *m/z* 388 by **1a** and **1b** and at *m/z* 256 by **2a–d**; however, the unique fragment ions were at *m/z* 82, 95, and 109, due to the cleavage of the sulfonatopropyl chain with charge retention. We next turned our attention to the positive FAB, in the hope that, in the energized condensed phase, the purine skeleton would have undergone protonation in the presence of a proton-donating matrix. The usual first-choice matrix, glycerol (Gly), satisfied this requirement and was found to give good spectra containing structurally significant ions and limited background interference.

The adenosine derivatives and **1a–1b** displayed a strong $[M + H]^+$ ion at *m/z* 390 accompanied by weak cationized molecular ion $[M + Na]^+$ and glycerol adduct $[M + H + Gly]^+$. The unique route of fragmentation of $[M + H]^+$ was glycosidic bond cleavage, accompanied by proton transfer from ribose to purine, to form the base peak at *m/z* 258 corresponding to the sulfonatopropylated adenines. The two other important fragments resulting from cleavage across the ribose moiety [24] which should have appeared at $[M + H - 90]^+$ and $[M + H - 104]^+$ were missing. In addition, there was no evidence of the sugar ion at *m/z* 132 resulting from glycosidic-bond cleavage and retention of charge on the carbohydrate, thus reflecting the greater stability of alkyladeninium ions than of the other O-stabilized ions.

The four adenine derivatives **2a–d** gave the same protonated molecular ions at *m/z* 258; compounds **2b** and **2d** also gave peaks at *m/z* 276 due to the protonated ammonium salt. Fragmentation occurred on the propanesulfonate chain, generating the ions *m/z* 176 ($[M - HSO_3]^+$), 149, and 136 (protonated adenine) in a unimolecular way, as indicated by daughter-ion analysis carried out by B/E linked-scan methodology. The *m/z* 176 can be represented by a tricyclic alkyladeninium structure **A** and **B** for **2a–b** and **2c–d**, respectively. The *m/z* 149 ion has an obscure mechanistic origin as well as a puzzling and not easily drawable structure. In fact, HR-MS measurements of all compounds (relative to *m/z* 136, $C_5H_6N_5$) revealed a common $C_6H_7N_5$ composition, which calls for a radical ion. The appearance of radical ions in a FAB spectrum is rare and unfavorable compared with the kinetically and energetically facile loss of even-electron neutral residues. Also, under EI conditions, the fragmentation of even-electron ions into odd-electron ions with loss of radicals is a disfavoured high-energy decomposition. Probably, the generation of the *m/z* 149 ion is triggered by formation of a stable species due to the delocalization of both ionic and radical sites on the purine nucleus. The four isomeric adenine-derived propanesulfonates could not be differentiated by the described fragmentation ions because quantitative changes were small. The B/E linked-scan spectra of ion 258 revealed a fragmentation pathway leading not only to ions 176, 149, and 136, but also to an ion at *m/z* 162 with a structure analogous to **A** and **B** but with one CH_2 fewer.



A **B**

In conclusion, reliable structural characterization of sulfonatopropylated adenosines and adenines could be obtained by combined use of UV and ^{1}H-NMR spectra and chemical reactivity. On the other hand, FAB-MS data although crucial in providing molecular-ion information, were not suitable for differentiating isomers[2]).

The above results also indicated that 1,3-propanesultone has a simplified behaviour toward adenine and adenosine in dipolar aprotic solvents, affording regioselective attack at one of the pyrimidine N-atoms, with exclusion of the exocyclic N^6 [3]).

---

[2]) CAD-MIKE spectroscopy and measurements of kinetic-energy release were utilized to distinguish positional isomers in a series of benzylated guanosines [25].

[3]) Other alkylating reagents like diethyl sulfate and ethyl methanesulfonate are much less selective and afford a mixture of products [14a][14b].

This preliminary information encouraged us to study the reactivity of NAD^+ (**3**) with **4**, hoping to reproduce the high specificity of the reaction in a different solvent. In fact, since NAD^+ is insoluble even in the most polar organic solvent, functionalization had to be carried out in H_2O as the reaction medium. The 1-(3-sulfonatopropyl)-NAD^{++} (**3a**) and N^6-(3-sulfonatopropyl)-NAD^+ (**3b**) were synthesized by standard procedures, as shown in *Scheme 2*. A ten-fold excess of **4** was added stepwise to a H_2O solution of **3**, because of spontaneous hydrolysis of the reagent. The reaction was allowed to continue for 24 h and the sulfonatopropylated NAD^+ **3a** isolated in low yield by ion-exchange chromatography. Since all the oxidized nicotinamide cofactors are particularly unstable under alkaline conditions [8], **3b** was obtained from **3a** in a three-step process involving chemical reduction of the nicotinamide moiety with sodium dithionite, heating at 70° at pH 11.5, and enzymatic reoxidation with yeast alcohol dehydrogenase. The process was

Scheme 2

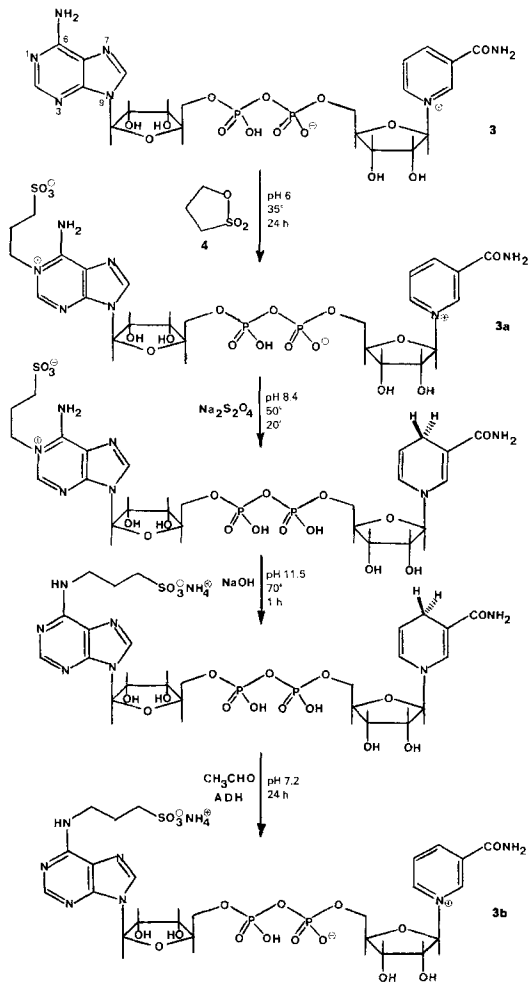


Table 2. *Chemical Shifts of Purine H-Atoms in NAD$^+$ Derivatives*

Compound	Solvent	Chemical shift [ppm]
NAD^+ (**3**)	$(D_6)DMSO$	8.37, 8.20
3a	$(D_6)DMSO$	8.80, 8.69
3b		8.30, 8.12
NAD^+ (**3**)	D_2O, pH 6.5	8,46, 8.15
1-(Carboxylatomethyl)-NAD^{++}		8.68, 8.51
N^6-(Carboxylatomethyl)-NAD^+	D_2O, pH 6.5	8.47, 8.20
1-(2-Aminoethyl)-NAD^+		8.43, 8.38
N^6-(2-Aminoethyl)-NAD^+		8.44, 8.27

carried out without isolation of the intermediates, and the final product **3b** was purified by ion-exchange chromatography.

In addition to their different UV absorptions (λ_{max} 259 and 265.5 nm), compounds **3a** and **3b** had the expected markedly different ^{1}H-NMR spectra in DMSO *(Table 2)*. In **3a**, H–C(2) and H–C(8) appeared to be deshielded at 8.68 and 8.51 ppm, while in **3b** they were similar to those of the parent NAD^+, thus paralleling the data obtained with N^6- and 1-sulfonatopropyl-substituted adenines and adenosines. Moreover, we found that the same behaviour was shared by another pair of NAD^+ derivatives, 1-(carboxylatomethyl)-NAD^{++} and N^6-(carboxylatomethyl)-NAD^+, prepared from NAD^+ and iodoacetic acid as described in the literature [10a][10d]. On the other hand, 1-(2-aminoethyl)-NAD^+ and N^6-(2-aminoethyl)-NAD^+ [10c] had chemical-shift values close to the ones of NAD^+ (see *Table 2*), a situation similar to the one observed above for methyladenine and methyladenosine *(cf. Table 1)*.

NAD^+ was one of the first compounds used to demonstrate the efficiency of the FAB-MS technique [26]. In the positive mode, significant molecular-ion species were obtained because of protonated and cationized molecules and adducts with the matrix; these ions were accompanied by important fragments providing sequence information. Excellent FAB-MS positive spectra were obtained for **3a** and **3b** in glycerol matrix, supporting unequivocally the localization of the sulfonatopropyl group on the adenine moiety.

Both **3a** and **3b** showed the same pseudomolecular ion at *m/z* 786, corresponding to protonated molecules. Cationized species were weak, and there were only a few structurally significant fragments (see *Table 3* and **C**). The first was at *m/z* 664, corresponding to the loss of either the sulfonatopropyl group or the nicotinamide moiety: the other peaks in the spectra showed that this first fragmentation was due to the fission of the nicotinamide-ribofuranosyl bond (see **a** in **C**). The ions *m/z* 550, 470, 390, and 258 were generated by fission along the molecule and were assigned to the sulfonatopropylated adenosine diphosphate (ion **b**), monophosphate (ion **c**), adenosine (ion **d**), and adenine (ion **e**), respectively. The cascade of the fragmentations and the gase-phase decomposition of the single ions were analyzed by the B/E linked-scan technique. Important metastable transitions were found to correlate the protonated molecular ion with the ions *m/z* 664, 646 (664 – H_2O), 622 (due to the cleavage across the ribofuranosyl

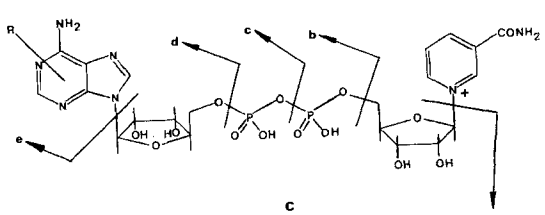


Table 3. *FAB-MS Fragmentation Ions of NAD$^+$ and NADH Derivatives*

Compound	Ion [m/z][a])					
	$[M+H]^+$	**a**	**b**	**c**	**d**	**e**
NAD^+	664	542	428	348		136
3a	786	664	550	470	390	258
3b	786	664	550	470		258
1- or N^6-(Carboxylatomethyl)-NAD^+	722	600	486	406		194
1- or N^6-(2-Aminoethyl)-NAD^+	707	585	471	391		179
NADH	666	542	428	348		136
8-[(6-Aminohexyl)amino]-NADH	780	656	542	462		250

[a]) Ion structures are depicted in **C**.

ring linked to the nicotinamide), 550, 470, and 452 (470 – H_2O). In the same way, the m/z 664 ion was correlated with the ions m/z 550, 470, 452, and 258. In turn, the m/z 550 ion decomposed to m/z 470, 452, 258, and finally, the m/z 470 ion gave origin to m/z 452, 390, 348, and 258. These data indicate that the formation of the ions in the spectra could take place not only in the energized condensed phase under atom bombardment but also outside the source, through spontaneous decomposition. A subsequent systematic investigation of the FAB-MS of the above mentioned 1- and N^6-(carboxylatomethyl)-NAD^+, 1- and N^6-(2-aminomethyl)-NAD^+, and of 8-[(6-aminohexyl)amino]-NADH [27] [10d] showed the same ordered fragmentation pathway (*Table 3*).

Enzymatic Assays. It is well known that NAD^+ substituted at N(1) of the adenine moiety no longer functions as a coenzyme [28]. For this reason we limited our enzymatic studies to compound **3b**. *Table 4* shows the K_m and V_{max} values for several enzymes of practical interest. Formate dehydrogenase, glucose dehydrogenase, glutamate dehydrogenase, and lactate dehydrogenase are currently used for the enzymatic regeneration of cofactor in preparative-scale synthesis [8]. The 3α- and 7α-hydroxysteroid dehydrogenase have been used for the regio- and stereoselective oxidoreduction of steroids [4a] [4b].

Table 4. *Kinetic Constants of NAD$^+$* (**3**) *and Ammonium* N^6*-(3-Sulfonatopropyl)-NAD$^+$* (**3b**) *for Different Dehydrogenases* (DH)

Enzyme	K_m [μm]		V_{max} (rel) NAD = 100
	3	**3b**	
Formate DH	10	5	36
Glucose DH	93	996	8
3α-Hydroxysteroid DH	34	102	43
7α-Hydroxysteroid DH	177	1614	13
Glutamate DH	124	52	20
Alcohol DH	192	118	27
Lactate DH	148	19	21

Alcohol dehydrogenase has found wide application in the preparation of chiral synthons [29]. Our derivative **3b** was active as a coenzyme with most of the enzymes tested, and its kinetic constants were comparable with data in the literature for carboxylated analogues of NAD^+ [30].

Conclusions. – We have demonstrated that 1,3-propanesultone can be successfully employed to obtain new sulfonatopropylated adenosines and adenines and to synthesize a new NAD^+ derivative (N^6-(3-sulfonatopropyl)-NAD^+ (**3b**)) which retains substantial

coenzyme activity with most of the enzymes tested. The N^6-(3-sulfonatopropyl)-NAD^+ (**3b**) is properly functionalized to be retained inside continuous-flow anionic membrane reactors.

Identification of the alkylation site in NAD^+ is a troublesome problem. Our systematic investigation of the spectroscopic properties of numerous NAD(H) derivatives showed that FAB mass spectroscopy is a powerful tool for characterization of these compounds, even though it can not distinguish between different isomers alkylated on the adenine moiety. ^{1}H-NMR analysis of the chemical shifts of the aromatic protons of adenine and adenosine derivatives proved to be a reliable method to solve this problem, even for more complex molecules such as NAD^+. The 1,3-propanesultone has been reported to be mutagenic and a potent carcinogen [31]. Its facile reactivity with the purine bases adenine and adenosine, together with its reported reactions with guanosine and deoxyguanosine [14b] could account for these properties.

Experimental Part

1. *General.* The 3α-hydroxysteroid dehydrogenase (EC 1.1.1. 50, 15 U·mg^{-1} protein), 7α-hydroxysteroid dehydrogenase (EC 1.1.1.159, 7 U·mg^{-1} protein), glutamate dehydrogenase from beef liver (EC 1.4.1.3, 300 U·mg^{-1} protein), glucose dehydrogenase (EC 1.1.1.47, 200 U·mg^{-1} protein), lactic dehydrogenase (1.1.1.27, 800 U·mg^{-1} lyophilized powder), 1-methyladenosine, 1-methyladenine, and N^6-methyladenine were purchased from *Sigma*. Formate dehydrogenase (EC 1.2.1.2, 3 U·mg^{-1} protein), alcohol dehydrogenase (EC 1.1.1.1, 400 U·mg^{-1} protein), adenine, adenosine, and NAD^+ were obtained from *Boehringer*, the 1,3-propanesultone and 1,8-bis(dimethylamino)naphthalene form *Aldrich*. All other reagents and compounds were of anal. grade. HPLC: *Jasco Trirotar* pump equipped with a *GP-A30* solvent delivery system, a *Uvidec 100 III* detector and a 5-μm-*Finepak Sil* C_{18} column (250 mm × 4.6 mm i.d.); a 32-min linear gradient from 20 mM Na_3PO_4, pH 7, to 20% MeCN in 20 mM Na_3PO_4, pH 7, (eluent *C*) was employed for adenosine and NAD^+ derivatives; a 32-min linear gradient from 20 mM Na_3PO_4, pH 7, to 12% MeCN in 20 mM Na_3PO_4, pH 7, (eluent *D*) was used for adenine products. TLC: precoated silica gel *60* F_{254} plates from *Merck;* eluent *A* = 2-BuOH/AcOEt/H_2O/MeOH/AcOH 3.5:2:2:1.5:1, *B* = i-PrCOOH/H_2O/NH_4OH 66:33:1. UV spectra (λ_{max} in nm): in 20 mM sodium phosphate, pH 7; *Jasco UVIDEC 420*. ^{1}H-NMR spectra: *Bruker WP-80* (80 MHz) and *VARIAN XL-200* (200 MHz). FAB-MS: *VG Analytical 70–70 EQ-HF*.

2. *Enzyme Assays.* Assays were carried out at 25° in 1-ml cuvettes (1-cm light path) with spectrophotometric monitoring of the reduced cofactor at 340 nm. The conditions for the various enzymes were: formate dehydrogenase in 0.1M K_3PO_4, pH 7.3, containing 0.1M formate; glutamate dehydrogenase in 0.1M K_3PO_4, pH 8, containing 0.1M glutamate and 30 mM ADP; glucose dehydrogenase in 0.1M K_3PO_4, pH 7, containing 20 mM glucose; 3α-hydroxysteroid dehydrogenase and 7α-hydroxysteroid dehydrogenase in 0.1M K_3PO_4, pH 9, containing 50 mM sodium cholate; lactate dehydrogenase in 0.1M K_3PO_4, pH 9, containing 50 mM L-lactic acid; alcohol dehydrogenase in 0.1M K_3PO_4, pH 9, containing 4% EtOH. The concentrations of nicotinamide cofactors were: NAD^+, 5–300 μM; N^6-(3-sulfonatopropyl)-NAD^+, 4–220 μM.

3. *3-(1-Adenosinio)propanesulfonate* (**1a**). To a soln. of 200 mg of **1** (0.75 mmol) in 3 ml of DMF at 50°, 1,8-bis(dimethylamino)naphthalene (333 mg, 1.5 mmol) and 1,3-propanesultone (**4**; 500 mg, 4.1 mmol) were added, and the soln. was incubated at 50° in the dark for a week. The soln. was poured into 30 ml of cold acetone and the precipitate collected by centrifugation and purified by ion-exchange chromatography on a *50W-X8* column (acid form, 1.2 × 47 cm) with a linear MeOH gradient in 10 mM NH_4OOCH, pH 4.5 (0–20%, 0.6 l). After prolonged lyophilization to remove NH_3 and HCOOH as well as H_2O, the yield of **1a** was 149 mg (51%). TLC (*A*): R_f 0.36. HPLC (*C*): t_R 10 min. UV: 258.5. FAB-MS: 390 (*M* + H, 50), 258 (100), 176 (11.5), 149 (21.2), 136 (23.1), 121 (21.2), 115 (46.2).

4. *Ammonium 3-(Adenosin-*N^6*-yl)propanesulfonate* (**1b**). Crude **1a**, obtained as described above from adenosine (1 g), was dissolved in 10 ml of H_2O, the pH was adjusted to 11.5 and the soln. heated for 1 h at 70°, monitoring the degree of conversion by TLC and UV. The pH was lowered to 7.0 and the product purified by ion-exchange chromatography on a *DE-32* column (carbonate form, 2.5 × 40 cm) with a linear $(NH_4)_2CO_3$

gradient (0–0.5M, 1.6 l). After prolonged lyophilization, 664 mg (46% overall yield) of pure **1b** were obtained. TLC (*A*): R_f 0.56. HPLC (*C*): t_R 20.6 min. UV: 267. FAB-MS: 390 (M + H, 56), 258 (100), 176 (32), 149 (28), 148 (7.4), 136 (48), 121 (32).

5. *3-(1-Adeninio)propanesulfonate* (**2a**). A soln. of 80 mg of **1a** in 2 ml of 0.5N HCl was heated at 100° for 1 h. A white solid was filtered off, washed with H_2O and dried to give 11 mg (0.04 mmol, 20%) of pure **2a**. TLC (*A*): R_f 0.33. HPLC (*D*): t_R 4.80 min. UV: 264. FAB-MS: 258 (M + H, 100), 176 (13.8), 149 (7.4), 136 (21.3).

6. *Ammonium 3-(Adenine-N^6-yl)propanesulfonate* (**2b**). As described above, 80 mg of **1b** were hydrolyzed. The soln. was diluted to 50 ml with H_2O and adjusted to pH 8. Ion-exchange chromatography on a *DE 32* column (carbonate form, 1.2 × 24 cm) with a linear $(NH_4)_2CO_3$ gradient (0–0.4 M, 0.4 l) provided, after prolonged lyophilization, 7.2 mg (0.03 mmol, 14%) of pure **2b**. TLC (*A*): R_f 0.50. HPLC (*D*): t_R 12 min. UV: 267. FAB-MS: 258 (M + H, 100), 167 (17), 149 (18), 147 (14), 136 (11), 131 (11).

7. *3-(3-Adeninio)propanesulfonate* (**2c**). Adenine (1 g, 3.75 mmol) was reacted with **4** as described by *Kondo et al.* [19]. The white precipitate was filtered off and recrystallized from H_2O (417 mg, 1.07 mmol). TLC (*A*): R_f 0.34. HPLC (*D*): t_R 8.40 min. UV: 274. FAB-MS: 258 (M + H, 100), 181 (53), 179 (23), 176 (8), 149 (56), 136 (14).

8. *Ammonium 3-(Adenin-9-yl)propanesulfonate* (**2d**). As described in [19], **2d** was synthesized starting from 200 mg (1.48 mmol) of **2**. Purification on a *DE 32* column (carbonate form, 1.2 × 24 cm) with a linear $(NH_4)_2CO_3$ gradient (0–0.5M, 0.4 l) yielded 100 mg (0.39 mmol) of pure **1d**. TLC (*A*): R_f 0.48. HPLC (*D*): t_R 12 min. UV: 260. FAB-MS: 258 (M + H, 100), 176 (10), 149 (12), 147 (9), 136 (15).

9. *Ammonium 3-(6,9-Dihydro-6-oxo-3H-purin-3-yl)propanesulfonate* (**2e**). A soln. of 300 mg (1.2 mmol) of **2c** in 5 ml of 1N NaOH was heated at 90° for 15 h. The soln. was diluted with H_2O, adjusted to pH 7, and the product purified by ion-exchange chromatography (*DE 32* column, carbonate form, 2.5 × 40 cm, linear $(NH_4)_2CO_3$ gradient 0–0.5M, 1.2 l). After prolonged lyophilization, 150 mg (0.6 mmol) of pure **2e** were obtained. TLC (*A*): R_f 0.24. UV: 264. ^{1}H-NMR (80 MHz, (D_6)DMSO): 8.30 (br. *s*, H–C(8)); 8.15 (br. *s*, H–C(2)); 4.30 (*t*); 2.50 (*m*); 2.15 (*m*). ^{13}C-NMR ((D_6)DMSO): 162.1 (*s*, C(6)); 148.0 (*d*, C(2)); 146.3 (*s*, C(4)); 140.6 (*d*, C(8)); 114.8 (*s*, C(5)); 47.9, 47.2, 25.2 (3 *t*, $(CH_2)_3$. FAB-MS: 259 (M + H, 100), 177 (10), 166 (7), 149 (13), 137 (26).

10. *1-(3-Sulfonatopropyl)-NAD^{++}* (**3a**). NAD$^+$ (500 mg, 0.75 mmol) was dissolved in 7 ml of distilled H_2O, and the pH was adjusted to 6. The 1,3-propanesultone (185 mg, 1.5 mmol) was added slowly and the mixture gently stirred for 24 h at 35°. During this time, the pH was kept at 6.0 with an automatic titrator filled with 1N LiOH, and additional reagent was dissolved in the reaction mixture (total 7.5 mmol). The soln. was poured into 20 volumes of cold EtOH and the mixture kept overnight at –20°. The precipitate was collected by centrifugation and redissolved in H_2O (500 ml). The soln. was adjusted to pH 7.5 and chromatographed over *DE 32* (carbonate form, 2.5 × 40 cm) with a linear NH_4HCO_3 gradient (0–0.4M, 1.2 l). After prolonged lyophilization, 55 mg (0.07 mmol) of pure **3a** were obtained. TLC (*B*): R_f 0.20. HPLC (*C*): t_R 10.0 min. UV: 259. FAB-MS: 786 (M + H, 23), 664 (5), 550 (25), 470 (25), 390 (10), 294 (96), 258 (100).

11. *Ammonium N^6-(3-Sulfonatopropyl)-NAD$^+$* (**3b**). A soln. of 55 mg of **3a** (0.07 mmol) in 3 ml of 0.2M $NaHCO_3$ was adjusted to pH 8. Sodium dithionite (84 mg, 0.42 mmol) was added and the soln. heated at 50° for 20 min under N_2. In this way, **3a** was converted to the alkali-stable reduced form. The excess of dithionite was oxidized by bubbling air through the soln. for 15 min at 25°. Addition of 1N NaOH to pH 11.5 and heating at 70° for 1 h gave the rearranged **3b**. The soln. was cooled to r.t. the pH adjusted to 7.2, and the nucleotide enzymatically oxidized by addition of acetaldehyde (10 µl) and yeast alcohol dehydrogenase. The soln. was then diluted with 30 ml of H_2O and purified by ion-exchange chromatography (*DE 32* column, carbonate form, 1.2 × 24 cm; linear NH_4HCO_3 gradient, 0–0.5M, 300 ml) to give, after prolonged lyophilization, 14 mg (0.02 mmol) of pure **3b**. TLC (*B*): R_f 0.25. HPLC (*C*): t_R 12.9 min. UV: 265.5. FAB-MS: 786 (M + H, 17), 664 (3), 550 (12), 470 (12), 386 (22), 294 (100), 258 (70).

We thank the *Biotechnology Action Programme of the Commission of the European Communities* for financial support of this work and Dr. *A. F. Bückmann* for providing us 1-(2-aminoethyl)-NAD$^+$ and *N*^{6}-(2-aminoethyl)-NAD$^+$.

REFERENCES

[1] For reviews, see: a) G.M. Whitesides, C.H. Wong, *Angew. Chem. Int. Ed.* **1985**, *24*, 617; b) J.B. Jones, *Tetrahedron* **1986**, *42*, 3351; c) A.M. Klibanov, *Chemtech* **1986**, *16*, 354; d) 'Methods in Enzymology', Ed. K. Mosbach, Academic Press, Orlando-London, 1987, Vol. 136.

[2] a) J.B. Jones, in 'Enzymes in Organic Synthesis', Ciba Found. Symp. III, Pitman, London, 1985, pp. 3–14; b) E. Keiman, K.K. Seth, R. Lamed, *J. Am. Chem. Soc.* **1986**, *108*, 3474.

[3] a) G. Carrea, *Trends Biotechnol.* **1984**, *2*, 102; b) G. Carrea, P. Cremonesi, in 'Methods in Enzymology', Ed. K. Mosbach, Academic Press, Orlando-London, 1987, Vol. 136, pp. 150–157.

[4] a) S. Riva, R. Bovara, P. Pasta, G. Carrea, *J. Org. Chem.* **1986**, *51*, 2902; b) S. Riva, R. Bovara, L. Zetta, P. Pasta, G. Ottolina, G. Carrea, *ibid.* **1988**, *53*, 88.

[5] a) A.R. Battersby, J. Staunton, H.R. Wiltshire, *J. Chem. Soc., Perkin Trans. 1* **1975**, 1156; b) C.H. Wong, G.M. Whitesides, *J. Am. Chem. Soc.* **1983**, *105*, 5012.

[6] J.P. Vandecasteele, *Appl. Environ. Microbiol.* **1980**, *39*, 327.

[7] R. Wichmann, C. Wandrey, A.F. Bückmann, M.R. Kula, *Biotechnol. Bioeng.* **1981**, *23*, 2789.

[8] For a review, see: H.K. Chenault, G.M. Whitesides, *Appl. Biochem. Biotechnol.* **1987**, *14*, 147.

[9] For a review, see: C. Wandrey, R. Wichmann, 'Coenzyme Regeneration in Membrane Reactors', in 'Application of Isolated Enzymes and Immobilized Cells to Biotechnology', Ed. A. Laskin, Addison Wesley, 1985, 177–208.

[10] a) M. Lindberg, P.O. Larsson, K. Mosbach, *Eur. J. Biochem.* **1973**, *40*, 187; b) S. Furukawa, N. Katayama, T. Iizuka, I. Urabe, H. Okada, *FEBS Lett.* **1980**, *121*, 239; c) A.F. Bückmann, M.R. Kula, R. Wichmann, C. Wandrey, *J. Appl. Biochem.* **1981**, *3*, 301; d) S. Riva, G. Carrea, F.M. Veronese, A.F. Bückmann, *Enzyme Microb. Technol.* **1986**, *9*, 556.

[11] V. Kitpreechavanich, N. Nishio, M. Hayashi, S. Nagai, *Biotechnol. Lett.* **1985**, *7*, 657.

[12] M. Howaldt, A. Gottlob, K.D. Kulbe, H. Chmiel, *Ann. N.Y. Acad. Sci.*, in press.

[13] For a review, see: J.H. Lister, in 'The Chemistry of Heterocyclic Compounds. Fused Pyrimidines, Part II, Purines', Ed. D.J. Brown, John Whiley & Sons, New York, 1971, pp. 342–50.

[14] a) B. Singer, L. Sun, H. Fraenkel-Conrat, *Biochemistry* **1974**, *13*, 1913; b) R.C. Moschel, W.R. Hudgins, A. Dipple, *J. Org. Chem.* **1979**, *44*, 3324.

[15] A.E. Beasley, M. Rasmussen, *Aust. J. Chem.* **1981**, *34*, 1107.

[16] I. Shinkai, M.C. Van der Zwan, F.W. Hartner, R.A. Reamer, R.J. Tull, I.M. Weinstock, *J. Heterocycl. Chem.* **1981**, *18*, 197.

[17] a) R.F. Fischer, *Ind. Eng. Chem.* **1964**, *56*, 41; b) D.W. Roberts, D.L. Williams, *Tetrahedron* **1987**, *43*, 1027.

[18] a) J.D. Engel, *Biochem. Biophys. Res. Commun.* **1975**, *64*, 581; b) T. Itaya, F. Tanaka, T. Fujii, *Tetrahedron* **1972**, *28*, 535.

[19] K. Kondo, H. Aoi, K. Takemoto, *Synth. Commun.* **1980**, *10*, 267.

[20] J.W. Jones, R.K. Robins, *J. Am. Chem. Soc.* **1963**, *85*, 193.

[21] N.J. Leonard, J.A. Deyrup, *J. Am. Chem. Soc.* **1962**, *84*, 2148.

[22] a) L.B. Townsend, R.K. Robins, R.N. Loeppky, N.J. Leonard, *J. Am. Chem. Soc.* **1964**, *86*, 5320; b) M. Rasmussen, J.H.S. Chan, *Aust. J. Chem.* **1975**, *28*, 1031.

[23] a) M. Barber, R.S. Bordoli, R.D. Sedgwick, A.N. Tyler, B.N. Green, *J. Chem. Soc., Chem. Commun.* **1981**, 325; b) D.J. Surman, J.C. Vickerman, *ibid.* **1981**, 324; c) D.L. Showikowski, K.H. Schram, *Nucleoside Nucleotide* **1985**, *4*, 309.

[24] S.J. Shaw, D.M. Desiderio, K. Tsuboyama, J.A. McCloskey, *J. Am. Chem. Soc.* **1970**, *92*, 2510.

[25] Y. Tondeour, R.C. Moschel, A. Dipple, S.R. Koepke, *Anal. Chem.* **1986**, *58*, 1316.

[26] For a review, see: H.R. Schulten, H.M. Schiebel, 'Mass Spectrometry of Pyridine Nucleotides', in 'Pyridine Nucleotides Coenzymes, Part A', Eds. D. Dolphin, R. Poulson, and O. Avramovic, John Wiley & Sons, New York, 1987, pp. 231–253.

[27] C.J. Lee, N.O. Kaplan, *Arch. Biochem. Biophys.* **1974**, *161*, 561.

[28] G. Grenner, H.L. Schmidt, W. Völkl, *Hoppe-Seyler's Z. Physiol. Chem.* **1976**, 887.

[29] R. MacLeod, H. Prosser, L. Fikentsher, J. Lanyi, H.S. Mosher, *Biochemistry* **1964**, *3*, 838.

[30] N. Katayama, K. Hayakawa, I. Urabe, H. Okada, *Enzyme Microb. Technol.* **1984**, *6*, 538.

[31] P.D. Lawley, in 'Chemical Carcinogens, Second Edition', Ed. C.E. Searle, American Chemical Society, Washington, D.C., 1984, Vol. 1, p. 325–484.