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Bis(isopropylidene adenosine)—A Novel Base-Stacked Dinucleoside with Two Deamination Sites for Adenosine Deaminase

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Two adenosine molecules are connected via their ribose moieties by transacetalation with 2,2,5,5-tetraethoxyhexane, yielding diastereoisomeric bis(isopropylidene adenosine) compounds with *S,S*- (**1a**) or *R,S*-configurated (**1b**) acetal carbons. The *S,S* isomer shows high hypochromicity and a pronounced positive Cotton effect, which implies strong stacking interactions. The stacking of **1b** is less pronounced. Both isomers are substrates for mammalian adenosine deaminase (EC 3.5.4.4). Whereas compound **1a** is slowly deaminated due to steric hindrance and stacking interactions, the diastereoisomer **1b** is a much better substrate for the enzyme. Because of the difference in configuration in **1b** the adenosine moieties are processed stepwise. Moreover, isomer **1b** is a strong competitive inhibitor for the deamination of adenosine by the enzyme.

INTRODUCTION

Hydrogen bonding and base stacking are the main interactions which stabilize the secondary structure of nucleic acids. Even solutions of nucleosides, e.g., adenosine in high concentration, show strong interactions, which can be observed by changes in their uv, CD, or NMR spectra (*1*). For a more detailed examination of these interactions in uncharged molecules two adenine or adenosine molecules have been connected via their exocyclic amino groups by hydrocarbon spacers (*2, 3*). In these molecules the nucleobases are forced into close proximity. However, in contrast to polynucleotides where the sugar moieties are connected in those compounds the aglycons are linked.

We have now been able to connect two adenosine molecules at their ribose 2′,3′-hydroxyl groups. These ribose linked dinucleosides are good models for the study of intramolecular base stacking, since they preserve the flexibility of the nucleobase moiety. Furthermore these molecules can be used as novel bis substrates for nucleobase converting enzymes (*4–6*).

DISCUSSION

Recently we demonstrated that adenosine can be acetalized with nonsymmetri-

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cal ketones yielding diastereoisomeric acetals, which were employed as stereochemical probes for the active site of adenosine deaminase (*6*). Through the use of 2,5-hexanedione for acetalation of adenosine the formation of the three (bis)adenosine derivatives, **1a–c** was expected. However, since this reaction is unfavored we used 2,2,5,5-tetraethoxyhexane, prepared from 2,5-hexanedione and triethyl orthoformate/ethanol as colorless leaflets. Transacetalation of this acetal with adenosine was accomplished by acidic catalysis in anhydrous *N,N*-dimethylformamide. From the reaction mixture only two of the three possible diastereoisomers could be detected and isolated. Their chromatographic separation was achieved on Dowex 1 × 2 ion exchange resin (*7*), yielding the faster migrating material **1a** with a melting point of 275°C, and a slower migrating compound **1b** (mp 166°C) in a ratio of 1 : 3.

Since the absolute configuration at the acetal carbon of *O*-2′,3′-[1-(2-carboxyethyl)ethylidene]adenosine (*8*) was determined by X-ray analysis and corroborated by proton NMR spectroscopy (*9*), the configuration of the new chiral centers of **1a** and **1b** was confirmed on the basis of these data. Using the methyl signals as probes for this assignment the structure of the compound with one signal at 1.50 ppm was established as the *S,S*-isomer **1a**, and the compound with two signals at 1.31 and 1.56 ppm as the *R,S*-isomer **1b**.

Ultraviolet and CD spectra of **1a** and **1b** provide a considerable body of information about the interaction of the adenosine residues (*10*). The measurements were carried out at 30–50 μM, thereby excluding intermolecular interaction (self-association). Compared to isopropylidene adenosine both compounds demonstrate a hypochromicity of 19% (**1a**) and 13% (**1b**) in water and a hypochromic shift of the long-wavelength maximum of 2 nm. The hypochromicities agree well with values observed with concentrated solutions of adenosine (22%) (*1*) or adenosine dinucleotides (11.9–22.1%) (*11*). As the CD spectra of *O*-2′,3′-alkyli-

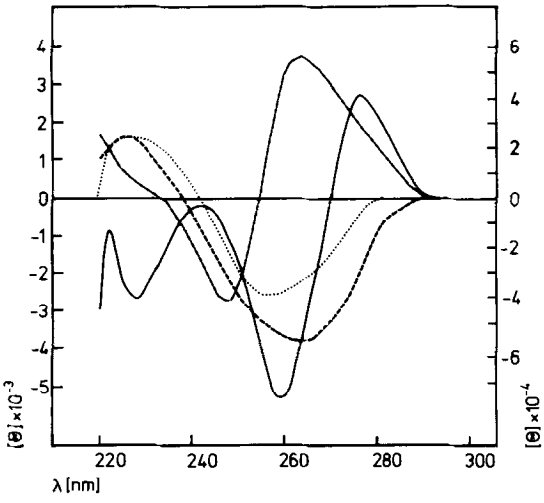


FIG. 1. CD spectra of adenosine (– – –), **1a** (——), **1b** (· · ·) (all left scale), and poly(A) (– · –) (right scale) in 0.07 *M* phosphate buffer (pH 7.0); [Θ] values per mole residue (base).

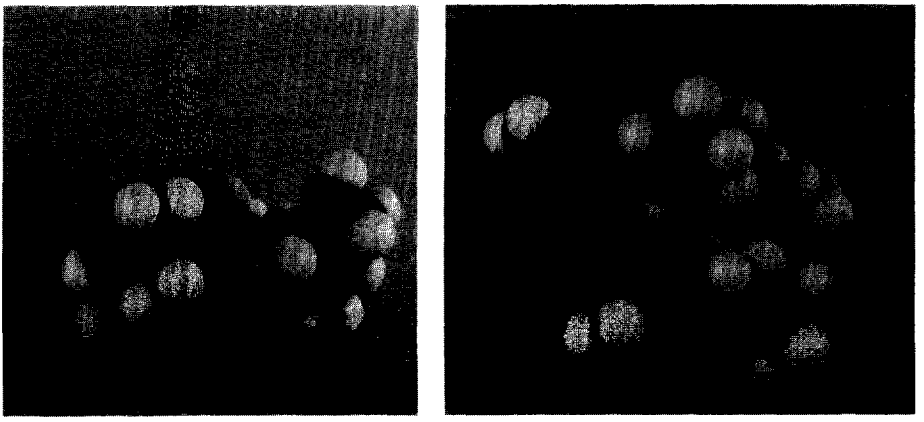


FIG. 2. CPK models of the diastereoisomers **1a** (a) and **1b** (b).

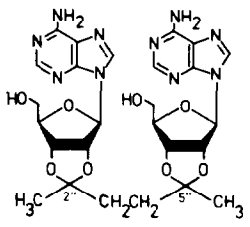
dene adenosines (*6*) do not differ from that of adenosine at the above concentration, the exciton splitting of compound **1a** (Fig. 1) or the positive absorption band at 275 nm, respectively, is not caused by the nonsymmetric carbon chain and so it indicates strong base stacking (*12*). Whereas the CD spectrum of the *R,S*-isomer **1b** is similar to that of adenosine, the spectrum of the *S,S* isomer resembles that of poly(A), an effect not observed with base-linked adenosine molecules (*3*). CPK models show that the bases of **1a** can be arranged parallel to each other and oriented in a manner similar to helical poly(A); the bases of **1b**, however, are always at an angle to each other (Fig. 2). These data confirm the strong base stacking in the *S,S*-isomer **1a** and suggest a poly(A)-like orientation of the nucleobases.

Both bis(isopropylidene adenosine) compounds **1a** and **1b** are deaminated by adenosine deaminase from calf intestine mucosa (EC 3.5.4.4). This behavior is in contrast to that of (3′ → 5′)-phosphate linked adenosine oligomers, such as (3′ → 5′)ApA, which cannot be deaminated by this enzyme (*13*). The successful deamination of **1a** and **1b** proves for the first time that not only monofunctional but also bifunctional substrates are accepted by the mammalian enzyme.

For quantitative studies of the enzymatic reaction Michaelis–Menten kinetics (*14*) were employed giving a first approximation of the apparent K_M and V_{max} values. A more detailed examination will be required in order to determine the absolute values. Compared to isopropylidene adenosine the deamination kinetics of the *S,S*-isomer **1a** (Table 1) showed significantly higher K_m and smaller V_{max} values per residue. This behavior may be caused by intramolecular stacking and steric hindrance of the *S*-oriented residues at the active site of the enzyme, which has already been substantiated with (*S*)-*O*-2′,3′-(2-pentylidene)adenosine (K_m, 304 μM; V_{max}, 7%) (*6*). In contrast the *R,S* compound, which is stacked to a smaller extent and contains one residue in the less hindered *R* configuration, is more readily deaminated. Furthermore the time-dependent reaction rate exhibits a biphasic response (Fig. 3) allowing a separate determination of the apparent K_m value of the *R*-oriented residue (Table 1). The calculation of the K_m of the *S*

TABLE 1

KINETIC DATA FOR DEAMINATION OF ADENOSINE AND DERIVATIVES BY ADENOSINE DEAMINASE[a]



1a: 2''S; 5''S
1b: 2''R; 5''S
1c: 2''R; 5''R

Compound	K_m (μM)	Relative V_{max} (%)
Adenosine	30	100
Isopropylidene adenosine	48	49
(Bis)adenosine derivatives		
1a *SS* isomer	300	0.5
1b *R* moiety	9	2
S moiety	400	0.03

[a] Values refer to one adenosine residue.

moiety in **1b** according to Michaelis and Menten can only lead to an apparent value, since the initial velocities cannot be exactly determined and the use of this model leads only to an approximation. Because of the distinct differences in the

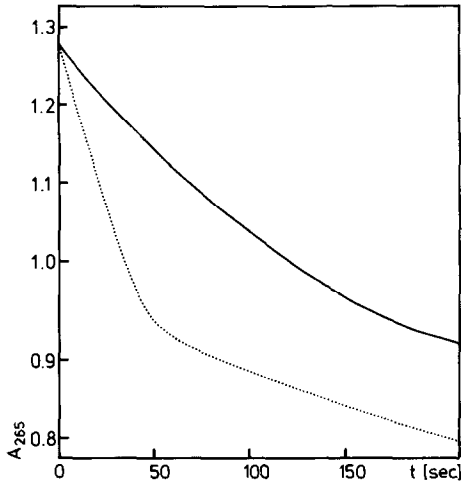


FIG. 3. Deamination observed at 265 nm of **1a** (——) and **1b** (· · ·) in 0.07 *M* phosphate buffer (pH 7.0) at 25°C; 5.0 or 1.25 units adenosine deaminase, respectively.

reaction rates of the *R* and *S* moieties the deamination kinetics of the slower part may still be considered to be in the initial phase with the additional supposition that the enzymatically generated inosine residue does not inhibit the reaction. This interpretation is underlined by reasonable agreement of the K_m of **1b** (*S* moiety, K_m, 400 μM) with that of (*S*)-*O*-2′,3′-(2-pentylidene)adenosine (K_m, 304 μM). In the case of the *R* residue evidence for negligible inhibition by the *S* residue was obtained by investigating a mixture of (*R*)- and (*S*)-*O*-2′,3′-(2-pentylidene)adenosine. The K_m value for the *R* compound (K_m, 26 μM) in the mixture was virtually identical to that of the pure *R* isomer (K_m, 31 μM) (*6*). Surprisingly, the *R* moiety of **1b** possesses a lower K_m than isopropylidene adenosine or even adenosine itself (Table 1). From previous observations it can be concluded that this result is not due to a hydrophobic effect, since the increased alkyl chain length in the endo position of (*R*)-*O*-2′,3′-(2-heptylidene)adenosine does not decrease the K_m value (K_m, 31 μM) (*6*) significantly.

However, the kinetic examination according to Michaelis and Menten encounters two problems. If a close proximity exists between two substrate moieties for a monofunctional enzyme, the status of quasiequilibrium between enzyme, substrate, and Michaelis complex is questionable and this may conflict with the Michaelis–Menten equation. A second restriction is caused by an intermediate product of the reaction containing both an adenosine and an inosine residue, in which the latter is a competitive inhibitor of the enzyme (inosine, K_i, 160 μM).

If adenosine is deaminated in the presence of the *S,S*-isomer **1a**, no significant inhibition is observed. In contrast the *R,S*-isomer **1b** is a strong competitive inhibitor. Because of the low V_{max} value of **1b** (Table 1) the inhibitory constant can be measured and is found to be $K_i = 9\ \mu M$. This value is comparable with that of the purine ribonucleoside (K_i, 9.3 μM). Therefore the linking of two nucleoside molecules by acetalation seems to be a very promising approach in designing novel powerful inhibitors for adenosine deaminase.

Since the problem of linked substrates is also of interest in regard to enzyme reactions on polymers or polymer-bound substrates (*15*), further investigations on the effect on enzyme binding to di substrates (*9*) and di-inhibitors will be necessary.

EXPERIMENTAL PROCEDURES

Melting points were determined on a Berl apparatus (Wagner & Munz, Munich, F.R.G.) and were not corrected.

Silica gel 60, 230–400 mesh ASTM (Merck, Darmstadt, F.R.G.), was used with chloroform–methanol (9:1, v/v) for silica gel chromatography. Ion exchange chromatography was performed on Dowex 1×2, OH^- form (Serva, Heidelberg, F.R.G.).

Ultraviolet absorption spectra and enzyme kinetics were measured on Zeiss PMQ 3 or Varian SuperScan 3 spectrophotometers. CD spectra were obtained with a Mark V UV-VIS Auto-Dichrograph (Instruments S.A., France) with quartz cuvettes of 1-cm lightpath length.

^{1}H-NMR spectra were measured on a Varian EM 390 spectrometer; ^{13}C-NMR

spectra, on a Bruker HX-60 spectrometer; δ values are in parts per million relative to tetramethylsilane (TMS) as internal standard.

Microanalyses were performed by Mikroanalytisches Labor Beller (Göttingen, F.R.G.).

Adenosine deaminase from calf intestine mucosa (EC 3.5.4.4; 5 mg/ml glycerol; 1 mg protein ≙ 200 units) was purchased from Boehringer (Mannheim, F.R.G.), and nucleosides were delivered by Pharma-Waldhof (Düsseldorf, F.R.G.).

2,2,5,5-Tetraethoxyhexane. A solution of 2,5-hexanedione (9.0 ml, 76 mmol), triethyl orthoformate (25.0 ml, 150 mmol), absolute ethanol (9.0 ml, 154 mmol), and sulfuric acid (50 μl) was stirred on an ice bath for 1 hr. After addition of triethylamine (150 μl) the precipitate was sucked off and washed with a small amount of ice-cold ethanol yielding colorless leaflets (12.9 g, 65%) with mp 55°C; ^{1}H-NMR ($CDCl_3$, 90 MHz) δ 1.15 (t, J = 7.5 Hz, 4 × ethyl-CH_3), 1.28 (s, 2 × acetal-CH_3), 2.66 (s, 2 × CH_2), 3.45 (q, J = 7.5 Hz, 4 × ethyl-CH_2); ^{13}C-NMR ($CDCl_3$) δ 15.54 (4 × CH_3-ethyl), 22.08 (2 × CH_3-acetal), 32.38 (2 × CH_2), 55.63 (4 × CH_2-ethyl), 101.48 (2 × C-acetal).

Anal. Calcd for $C_{14}H_{30}O_4$ (262.39): C, 64.08; H, 11.52. Found: C, 64.28; H, 11.49.

Condensation of adenosine with 2,2,5,5-tetraethoxyhexane. To a suspension of adenosine (4.0 g, 14.9 mmol) in anhydrous *N,N*-dimethylformamide (70 ml) 2,2,5,5-tetraethoxyhexane (2 g, 7.6 mmol) and a solution of 7 *M* hydrogen chloride in dry *p*-dioxane (6 ml) were added. The mixture was stirred 24 hr at room temperature and then poured in anhydrous diethyl ether (1000 ml). After decantation the precipitate was washed with ether and then dissolved in chloroform by addition of 2% aqueous sodium hydrogencarbonate. The organic layer was washed with water, dried, and then the solvent was evaporated *in vacuo*. The glassy residue dissolved in chloroform–methanol (9:1, v/v) was chromatographed on a silica gel column (70 × 3.5 cm) with the same solvent. The slow migrating main zone was pooled, the solvent evaporated, and solid material precipitated (ethanol/*n*-hexane) yielding a colorless, amorphous mixture of isomers (437 mg, 10%) with mp 150–180°C; uv (CH_3OH) λ_{max} 260 (ε 29,800).

Anal. Calcd for $C_{26}H_{32}N_{10}O_8$ (612.62): C, 50.98; H, 5.27; N, 22.87. Found: C, 51.05; H, 5.40; N, 22.71.

S,S-Bis(isopropylidene adenosine) (**1a**). The mixture of diastereoisomers **1a/1b** (100 mg) was applied on an ion exchange column (Dowex 1×2, OH^- form, 40 × 2.0 cm) and eluted with water : methanol (8 : 2, 500 ml)/water : methanol (3 : 2, 500 ml), linear gradient. Compound **1a** was eluted first. The fractions were pooled, the solvent was evaporated, and the remaining material was crystallized from ethanol/*n*-hexane yielding colorless crystals (25 mg, 25%) with mp 275°C decomp.; uv (H_2O) λ_{max} 258 (ε 24,400), (CH_3OH) λ_{max} 260 (ε 28,800); ^{1}H-NMR [$(CH_3)_2SO$, 90 MHz] δ 1.50 (s, 2 × (*S*)-methyl), 1.64 (s, 2 × CH_2), 3.48 (d, J = 5 Hz, 2 × 5′-H), 4.1–4.3 (m, 2 × 4′-H), 4.91 (dd, J = 3 Hz, 2 × 3′-H), 5.31 (dd, J = 3 Hz, 2 × 2′-H), 6.12 (d, J = 3 Hz, 2 × 1′-H), 7.26 (s, 2 × NH_2), 8.13 (s, 2 × 2-H), 8.30 (s, 2 × 8-H).

Anal. Calcd for $C_{26}H_{32}N_{10}O_8$ (612.62): C, 50.98; H, 5.27; N, 22.87. Found: C, 50.55; H, 5.19; N, 22.77.

R,S-Bis(isopropylidene adenosine) (**1b**). Compound **1b** was isolated from the slower migrating zone and crystallized as described above. The colorless crystals (75 mg, 75%) showed a mp of 166°C; uv (H_2O) λ_{max} 258 (ϵ 26,100), (CH_3OH) λ_{max} 260 (ϵ 29,300); ^{1}H-NMR [$(CH_3)_2SO$, 90 MHz] δ 1.31 (s, (*R*)-methyl), 1.56 (s, (*S*)-methyl), 1.83 (s, 2 × CH_2), 3.4–3.6 (m, 2 × 5′-H), 4.1–4.3 (m, 2 × 4′-H), 4.9–5.2 (m, 2 × 3′-H), 5.3–5.5 (m, 2 × 2′-H), 6.12 (d, J = 3 Hz, 1′-H), 6.18 (d, J = 3 Hz, 1′-H), 7.31 (s, 2 × NH_2), 8.17 (s, 2 × 2-H), 8.31 (s, 8-H), 8.35 (s, 8-H).

Anal. Calcd for $C_{26}H_{32}N_{10}O_8$ (612.62): C, 50.98; H, 5.27; N, 22.87. Found: C, 50.81; H, 5.47; N, 22.66.

Deamination assay. The assay based on a method of Kalckar (*16*) and was performed at 25°C in a Varian SuperScan 3 spectrophotometer with quartz cuvettes of 1-cm lightpath length. Compound **1a** or **1b** varying in 10 different concentrations from 4 to 80 μM in 0.07 *M* Sørensen phosphate buffer (pH 7.0) were treated with varying amounts (0.50, 0.63, or 1.25 units) of adenosine deaminase. Deamination was followed at 265 nm and rates were recalculated in terms of concentration using the molar extinction coefficients at that wavelength. K_m and V_{max} values were obtained from double-reciprocal substrate concentration–initial velocity plots (*17*) with data weighted by v versus $v/[S]$ plots (*18, 19*).

The K_i value was calculated according to Dixon (*20*) based on studies with constant adenosine concentrations and varying the concentration of **1b** between 4 and 40 μM.

The lines of best fit were calculated by the method of least squares. K_m and V_{max} for adenosine were determined as 30 μM and 200 m*M*/min mg protein, respectively.

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