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R- and *S*-Alkylidene Acetals of Adenosine: Stereochemical Probes for the Active Site of Adenosine Deaminase

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Condensation of adenosine with unsymmetrical ketones leads to 2′,3′-*O*-alkylidene acetals with a new chiral center. The diastereoisomers were separated chromatographically, and the ratio of products was found to be 3 : 1. The configuration of the new chiral centers was determined by nmr spectroscopy. The diastereoisomers were used as stereochemical probes for the active site of adenosine deaminase. By determination of the K_m values it was shown that the binding of the *S*-diastereoisomers is strongly decreased in comparison with the *R*-compounds. The data imply a close proximity of the 2′,3′-site of the ribose moiety to the active site of adenosine deaminase.

INTRODUCTION

Adenosine deaminase (*1–3*) is of special interest since its absence, which can be genetically determined, causes severe immunodeficiency diseases (*4*). On the other hand it inactivates potent antiviral nucleosides like β-D-arabinofuranosyl adenine (*5, 6*). A thorough understanding of deamination mechanism and the effect of modified substrates on enzymatic activity could afford the know-how necessary to design effective chemotherapeutic agents that would either be resistant to, or inhibitors of, adenosine deaminase.

The enzyme shows high stereoselectivity during complex formation with substrates and inhibitors, which can be substantiated by chiral N-9 glycoside analogs (*7*). Recently we have reported that alkylidene residues attached to the 2′,3′-hydroxyl groups of adenosine influence binding of the altered substrate to the enzyme (*8*). Besides that, the alkylidene acetals have been used as ligands for the immobilization of adenosine to polymers (*8, 9*). These polymers bind adenosine deaminase specifically, whereby the methylene groups between the acetal carbon and the carbonyl residue function as spacers (*10*). All of the acetals examined possessed the *R* configuration at the acetal carbon (*8*). We now report on the synthesis of diastereoisomeric adenosine acetals (*R* and *S*) and their use as stereochemical probes for the active site of adenosine deaminase.

DISCUSSION

The adenosine derivatives **3a/3b**, **4a/4b**, and **5a/5b** were prepared by condensa-

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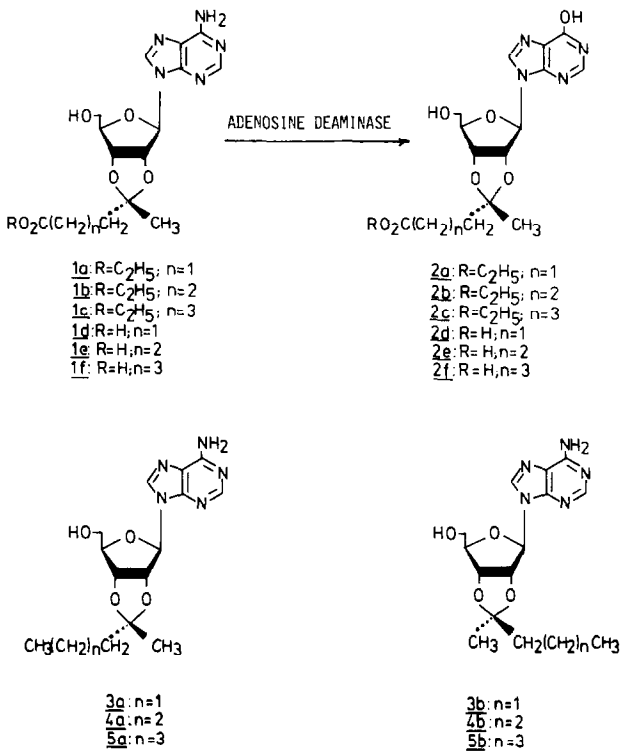

tion of the corresponding methyl alkyl ketones with adenosine, following the pattern of a previously published method (*11*). The newly synthesized compounds possess a further chiral center, the acetal carbon. In contrast to the adenosine acetals with keto acids (*8*), both diastereoisomers are found when using simple ketones (*R*/*S* ration 3/1) and can be separated by preparative thick-layer chromatography on silica gel plates. Because of the very small difference in the R_f values, separation is achieved by repeated development in ethyl acetate/benzene. The zones were eluted from silica gel with methanol, and after evaporation the yellowish amorphous residues were recrystallized twice from ethyl acetate/*n*-pentane as colorless needles.

In the ^{13}C nmr spectrum each of the new acetals shows an upfield shift of approx 10 ppm for the 2′,3′-carbons, indicating that acetalation took place at the 2′,3′-hydroxyl groups (Table 1). The problem of the configuration at the acetal carbon was solved by proton nmr spectra. Since the absolute configuration of the acetal carbon of **1d** has been determined as *R* by X-ray analysis (*12*) and the ^1H nmr signals of the acetal methyl groups of the main products **3a**, **4a**, and **5a** coincide with that of **1d** (δ_{CH_3} 1.3 ppm), the configuration of these compounds must also be *R*; this means that the alkyl chains of these compounds are endo, whereas in the isomers of the *S* configuration (compounds **3b**, **4b**, and **5b**) the alkyl chains are exo (δ_{CH_3} 1.5 ppm).

The adenosine derivatives were then used to investigate the active site of adenosine deaminase with respect to hydrophobic regions of the enzyme and the

TABLE 1

^{13}C nmr SHIFTS OF ADENOSINE AND ISOPROPYLIDENE ADENOSINE IN $(CD_3)_2SO$, ALL OTHER DERIVATIVES IN $CDCL_3$, δ VALUES RELATIVE TO TMS

Compound	Nucleobase					Glycon				
	C-2	C-4	C-5	C-6	C-8	C-1′	C-2′	C-3′	C-4′	C-5′
Adenosine	152.5	149.1	119.4	156.2	140.1	88.1	73.6	70.8	86.0	61.8
Isopropylidene adenosine	152.7	148.9	119.2	156.2	139.6	89.8	83.3	81.4	86.4	61.7
3a	152.8	148.6	120.9	156.3	140.1	93.7	83.2	81.4	86.5	63.3
3b	152.8	148.6	120.9	156.3	140.1	93.9	83.5	81.9	86.7	63.3
4a	152.8	148.7	121.0	156.3	140.2	93.8	83.2	81.5	86.5	63.3
4b	152.8	148.7	121.0	156.3	140.2	94.1	83.6	81.9	86.7	63.3
5a	152.8	148.6	121.0	156.3	140.1	93.7	83.2	81.5	86.5	63.3
5b	152.8	148.6	121.0	156.3	140.1	94.0	83.6	81.9	86.6	63.3

	Alkylidene residue						
	CH_3-acetal	C-acetal	α-C	β-C	γ-C	δ-C	ε-C
Adenosine	—	—	—	—	—	—	—
Isopropylidene adenosine	25.1	113.1	27.1	—	—	—	—
3a	23.4	115.9	42.4	17.4	14.3	—	—
3b	25.4	116.2	40.9	17.8	14.3	—	—
4a	23.4	116.1	40.0	26.3	22.9	14.0	—
4b	25.5	116.4	38.5	26.4	22.9	14.0	—
5a	23.4	116.0	40.2	23.8	31.9	22.6	14.0
5b	25.2	116.3	38.7	23.9	31.9	22.6	14.0

geometry at the active center. Information about the nature of the active site results from affinity labeling with active-site-directed inhibitors (*13*) and altered substrates. This mechanistic investigation showed that the amino group at C-6 of the aglycon is not necessary for binding. 6-Chloro or 6-mercapto derivatives are bound almost as well as the regular substrate (*14*). An increase in binding can be achieved with C-6 derivatives having a tetrahedral carbon at this position (*15, 16*). No binding is observed with substrates lacking the nitrogen at position 7, so, for example, tubercidin is neither a substrate nor an inhibitor (*17*). Whereas the 5′-hydroxyl group of the ribose moiety is a possible binding site (5′-AMP cannot be deaminated), the 2′- or 3′-hydroxyl groups are not of major importance. The latter conclusion results from the kinetic data of 2′-deoxy and 3′-deoxy derivatives (*6*), as well as the fact that isopropylidene adenosine is bound almost as strongly as adenosine (*14*).

Our kinetic data (Table 2) show that there also is no significant difference in the K_m values when the endo chain of the *R*-alkylidene adenosine acetals is lengthened (compounds **3a**, **4a**, and **5a**). In the case where polarizable (**1a–c**) or anionic functional groups (**1d–f**) are attached to the endo alkylidene chain, the V_{max} value decreases if the chain is shortened. The latter may be explained either by

TABLE 2

KINETIC DATA FOR SUBSTRATES OF ADENOSINE DEAMINASE

Compound	K_m (μM)	Relative V_{max} (%)
Adenosine	30	100
Isopropylidene adenosine[a]	48	49
Pentylidene derivatives		
1a: *R*	31	24
1d: *R*	37	1
3a: *R*	43	59
3b: *S*	304	7
Hexylidene derivatives		
1b: *R*	17	34
1e: *R*	68	21
4a: *R*	29	39
4b: *S*	875	12
Heptylidene derivatives		
1c: *R*	20	31
1f: *R*	60	25
5a: *R*	31	34
5b: *S*[b]	199	5

[a] See Ref. (*14*).
[b] Only slight solubility.

hydrophobic sites surrounding the active center (*18*) or by electrostatic interactions between the enzyme and the altered substrates.

The diastereoisomeric *S*-acetals show a significant difference in their Michaelis–Menten constants, compared to the *R*-compounds. While all *R*-acetals with endo alkyl chains, including isopropylidene adenosine, have approximately the same K_m values, an increase of 5- to 10-fold is observed on changing the configuration at the acetal carbon to give the *S*-compounds **3b**, **4b**, and **5b**.

Thus, on binding of the ribose moiety to the enzyme, a small group like methyl can be accommodated at the exo position (Fig. 1a), as indicated by the fact that all analogs with an exo methyl group have a K_m similar to adenosine. However, a larger group at this position (Fig. 1b) impairs binding, as shown by an increase in K_m. This behavior can be interpreted as evidence of close proximity of the 2′,3′-sites of the ribose moiety to the enzyme, in agreement with the finding that the size of substituents in the endo position is not crucial to the binding.

EXPERIMENTAL

Melting points were determined on a Berl apparatus (Wagner & Munz, Munich, FRG) and were not corrected. Thin-layer chromatography was performed on thin-

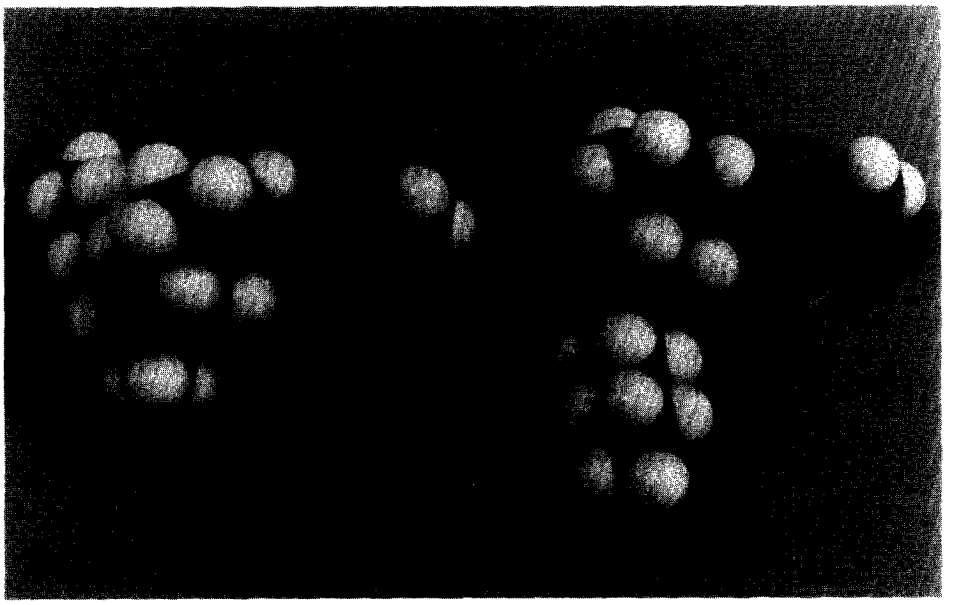

FIG. 1. CPK models of the diastereoisomers **5a** (a) and **5b** (b).

layer silica gel plates F_{254} (Woelm, Eschwege, FRG) with (A) chloroform–methanol (8:2, v/v), and (B) ethyl acetate–benzene (9:1, v/v). For preparative thick-layer chromatography silica gel PSC plates F_{254} (Merck, Darmstadt, FRG) were used with (B). Ultraviolet absorption spectra and enzyme kinetics were measured on Zeiss PMQ 3 or Varian SuperScan 3 spectrophotometer. ^1H nmr spectra and ^{13}C nmr spectra were measured on Varian EM-390 and Bruker HX-60 spectrometers, respectively; δ values are reported in parts per million relative to tetramethylsilane (TMS) as internal standard. Microanalyses were performed by Mikroanalytisches Labor Beller (Göttingen, FRG). Adenosine deaminase from calf intestine mucosa (EC 3.5.4.; 5 mg/ml glycerol; 1 mg $\cong$ 200 units) was purchased from Boehringer (Mannheim, FRG), and nucleosides were delivered by Pharma Waldhof (Dusseldorf, FRG).

R,S-2',3'-O-(2"-Pentylidene)adenosine (3a/3b). To a suspension of adenosine (3.0 g, 11.3 mmol) in dry *N,N*-dimethyl formamide (35 ml), 2-pentanone (2.4 ml, 22.6 mmol) and triethyl orthoformate were added, and insoluble material was dissolved by adding a solution of 7 *M* hydrogen chloride in dry *p*-dioxane (5 ml). The mixture was kept 24 hr at room temperature and then poured into dry diethyl ether (500 ml). The upper layer was decanted, and the oily residue 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 concentrated, and the residue was crystallized and recrystallized from ethyl acetate/*n*-pentane to give colorless needles of **3a/3b** (3.23 g, 85%): mp 145°C; tlc (A), R_f 0.93; uv (CH_3OH), λ_{max} 259.5 (ϵ15,400).

Anal. Calcd for $C_{15}H_{21}N_5O_4$ (335.37): C, 53.72; H, 6.31; N, 20.88. Found: C, 53.70; H, 6.46; N, 20.82.

R-2',3'-O-(2"-Pentylidene)adenosine (*3a*). The diastereoisomers **3a**/**3b** were separated on silica gel thick-layer plates with (B). The plates were repeatedly developed and the slower migrating zone was scratched out and eluted by methanol from silica gel. Recrystallization of the evaporated amorphous residue yielded colorless needles from ethyl acetate/*n*-pentane: mp 156°C; tlc (B), R_f 0.10; ^{1}H nmr [$(CD_3)_2SO$, 90 MHz] δ0.97 (t, J = 6 Hz, γ-CH_3), 1.28 (s, acetal-CH_3), 1.4–1.5 (m, β-CH_2), 1.6–1.8 (m, α-CH_2), 3.53 (d, J = 4.5 Hz, 5'-H), 4.1–4.3 (m, 4'-H), 5.02 (dd, J = 3 Hz, 3'-H), 5.40 (dd, J = 3 Hz, 2'-H), 6.17 (d, J = 3 Hz, 1'-H), 7.33 (s, NH_2), 8.19 (s, 8-H), 8.38 (s, 2-H). ^{13}C nmr, see Table 1.

S-2',3'-O-(2"-Pentylidene)adenosine (*3b*). The faster migrating zone from the thick-layer plate was treated as described above yielding colorless needles of **3b** with: mp 191°C; tlc (B), R_f 0.11; ^{1}H nmr [$(CD_3)_2SO$, 90 MHz] δ0.87 (t, J = 6 Hz, γ-CH_3), 1.4–1.5 (m, β-CH_2), 1.51 (s, acetal-CH_3), 1.5–1.7 (m, α-CH_2), 3.53 (d, J = 4.5 Hz, 5'-H), 4.1–4.3 (m, 4'-H), 4.95 (dd, J = 3 Hz, 3'-H), 5.33 (dd, J = 3 Hz. 2'-H), 6.16 (d, J = 3 Hz, 1'-H), 7.30 (s, NH_2), 8.14 (s, 8-H), 8.36 (s, 2-H). ^{13}C nmr, see Table 1.

R,S-2',3'-O-(2"-Hexylidene)adenosine (*4a*/*4b*). Adenosine (3.0 g, 11.3 mmol) was treated in the manner described above with 2-hexanone (2.8 ml, 22.7 mmol) yielding colorless needles from ethyl acetate/*n*-pentane (3.47 g, 88.6%): mp 169°C; tlc (A), R_f 0.94; uv (CH_3OH), λ_{max} 259.5 (ε15,700).

Anal. Calcd for $C_{16}H_{23}N_5O_4$ (349.40): C, 55.00; H, 6.63; N, 20.05. Found: C, 55.11; H, 6.62; N, 20.13.

R-2',3'-O-(2"-Hexylidene)adenosine (*4a*). For separation and isolation of the diastereoisomers the technique developed above was used. The slower migrating zone contains **4a**, crystallizing in colorless needles (ethyl acetate/*n*-pentane) with: mp 180°C; tlc (B), R_f 0.12; ^{1}H nmr [$(CD_3)_2SO$, 90 MHz] δ0.92 (t, J = 6 Hz, δ-CH_3), 1.30 (s, acetal-CH_3), 1.2–1.5 (m, 2 × CH_2), 1.6–1.8 (m, α-CH_2), 3.52 (d, J = 4.5 Hz, 5'-H), 4.1–4.3 (m, 4'-H), 4.99 (dd, J = 3 Hz, 3'-H), 5.38 (dd, J = 3 Hz, 2'-H), 6.14 (d, J = 3 Hz, 1'-H), 7.30 (s, NH_2), 8.17 (s, 8-H), 8.37 (s, 2-H). ^{13}C nmr, see Table 1.

S-2',3'-O-(2"-Hexylidene)adenosine (*4b*). The faster migrating zone gave **4b** as colorless needles from ethyl acetate/*n*-pentane with: mp 187°C; tlc (B), R_f 0.14; ^{1}H nmr [$(CD_3)_2SO$, 90 MHz] δ0.88 (t, J = 6 Hz, δ-CH_3), 1.2–1.4 (m, 2 × CH_2), 1.52 (s, acetal-CH_3), 1.5–1.7 (m, α-CH_2), 3.52 (d, J = 4.5 Hz, 5'-H) 4.2–4.3 (m, 4'-H), 4.98 (dd, J = 3 Hz, 3'-H), 5.35 (dd, J = 3 Hz, 2'-H), 6.17 (d, J = 3 Hz, 1'-H), 7.33 (s, NH_2), 8.18 (s, 8-H), 8.37 (s, 2-H). ^{13}C nmr, see Table 1.

R,S-2',3'-O-(2"-Heptylidene)adenosine (*5a*/*5b*). The synthesis of **5a**/**5b** was carried out in the usual way described for **3a**/**3b**, starting with adenosine (3.0 g, 11.3 mmol) and 2-heptanone (3.2 ml, 23.6 mmol) yielding colorless needles from ethyl acetate/*n*-pentane (3.62 g, 88.9%): mp 157°C; tlc (A), R_f 0.96; uv (CH_3OH), λ_{max} 259.5 (ε15,200).

Anal. Calcd for $C_{17}H_{25}N_5O_4$ (363.43): C, 56.18; H, 6.93; N, 19.27. Found: C, 56.32; H, 6.77; N, 19.37.

R-2',3'-O-(2"-Heptylidene)adenosine (*5a*). The diastereoisomers **5a**/**5b** were separated in the usual manner and recrystallized as colorless needles from ethyl

acetate/*n*-pentane. The material of the slower migrating zone is identified as **5a** with: mp 170°C; tlc (B), R_f 0.14; ^{1}H nmr [$(CD_3)_2SO$, 90 MHz] δ0.89 (t, J = 6 Hz, ϵ-CH_3), 1.30 (s, acetal-CH_3), 1.2–1.5 (m, 3 × CH_2), 1.5–1.9 (m, α-CH_2), 3.52 (d, J = 4.5 Hz, 5′-H), 4.1–4.3 (m, 4′-H), 4.99 (dd, J = 3 Hz, 3′-H), 5.37 (dd, J = 3 Hz, 2′-H), 6.13 (d, J = 3 Hz, 1′-H), 7.32 (s, NH_2), 8.17 (s, 8-H), 8.37 (s, 2-H). ^{13}C nmr, see Table 1.

S-2′,3′-O-(2″-Heptylidene)adenosine (*5b*). The faster migrating compound could be characterized as **5b** with: mp 194°C; tlc (B), R_f 0.16; ^{1}H nmr [$(CD_3)_2SO$, 90 MHz] δ0.85 (t, J = 6 Hz, ϵ-CH_3), 1.2–1.4 (m, 3 × CH_2), 1.51 (s, acetal-CH_3), 1.5–1.7 (m, α-CH_2), 3.53 (d, J = 4.5 Hz, 5′-H), 4.1–4.3 (m, 4′-H), 4.97 (dd, J = 3 Hz, 3′-H), 5.33 (dd, J = 3 Hz, 2′-H), 6.16 (d, J = 3 Hz, 1′-H), 7.32 (s, NH_2), 8.17 (s, 8-H), 8.36 (s, 2-H). ^{13}C nmr, see Table 1.

Deamination of the acetals with adenosine deaminase. The assay was based on differential spectrophotometry, and measurements were performed at 25°C in a Varian SuperScan 3 spectrophotometer with cuvettes of 1-cm light path length. The reaction mixture contained per milliliter of 0.06 M Sørensen phosphate buffer (pH 7.0) 0.36, 0.72, or 1.2 units adenosine deaminase, and the compounds indicated in 10 different concentrations varying from 10 to 170 μM. Deamination was followed at 265 nm according to the method of Kalckar (19), and rates were recalculated in terms of concentration using the molar extinction coefficients at that wavelength. K_m and V_{max} values (20) were obtained from double reciprocal substrate–initial velocity plots (21) with data weighted by v versus v/[S] plots (22, 23), and the line of best fit was calculated by the method of least squares. K_m and V_{max} for adenosine were determined as 30 μM and 206 μM/min/mg protein, respectively.

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