# Approaches to Isozyme-Specific Inhibitors. 16. A Novel Methyl-C5′ Covalent Adduct of L-Ethionine and β,γ-Imido-ATP as a Potent Multisubstrate Inhibitor of Rat Methionine Adenosyltransferases[1]

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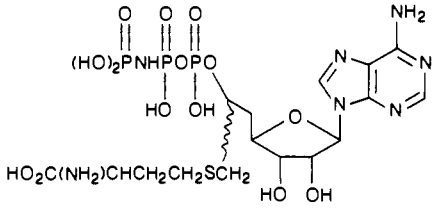
$N^6$,$N^6$-Dibenzoyl-2′,3′-*O*-isopropylideneadenosine, which is readily synthesized by one-pot 5′-O-trimethylsilylation, $N^6$-benzoylation, and desilylation, was converted to the corresponding 5′-aldehyde. This was treated with $CH_2$=CHMgBr to afford, after debenzoylation, a 1:3 mixture of the 5′*S* and 5′*R* epimers, respectively, of 5′-*C*-vinyl-2′,3′-*O*-isopropylideneadenosine. The configurations were established by single-crystal X-ray diffraction analysis of the 5′*R* epimer. Hydroboration of the 5′-*O*-tetrahydropyranyl derivative of the mixed epimeric 5′-*C*-vinyl nucleosides readily furnished 5′(*S*,*R*)-*C*-(2-hydroxyethyl)-2′,3′-*O*-isopropylideneadenosine. Treatment of the 5′(*S*,*R*)-*C*-(2-*O*-tosyl) derivative of this with disodium L-homocysteinate permitted facile introduction of the L-ethionine system. By means of methods developed earlier in the synthesis of homologous methionine–ATP adducts, the α-amino acid group was protected, a β,γ-imidotriphosphoryl group was introduced at O5′, and blocking groups were removed to give the title adduct as a 2:3 mixture of its two 5′ epimers. It was a powerful inhibitor [$K_M$(ATP)/$K_i$ = 520 and 340] of the M-2 (normal tissue) and M-T (hepatoma tissue) forms, respectively, of the title enzyme and displayed predominantly competitive kinetics with the two substrates L-methionine and MgATP. It inhibited M-2 and M-T slightly less effectively than its homologue possessing one less $CH_2$ between sulfur and C5′ and gave kinetic evidence of an increased tendency to form L-methionine–enzyme–adduct and MgATP–enzyme–adduct complexes.

Evidence indicates that the M-T variant of methionine adenosyltransferase (predominant in rat ascitic hepatoma cells) is of interest as a model mammalian target in anticancer drug design.[2,3] The enzyme catalyzes attack of the sulfur of L-methionine (Met) on C5′ of ATP with expulsion of inorganic tripolyphosphate. The isozyme predominant in most nonhepatic rat normal tissues is a second variant, M-2. In the course of the present series of studies on possibly useful approaches to the design of isozyme-specific inhibitors, we found that inhibitors that are both potent and significantly isozyme-selective can be derived systematically and relatively readily by attaching a short substituent to an appropriate atom of a multisubstrate adduct which itself is a potent but not an isozyme-selective inhibitor.[4,5] To help determine the scope and limitations of this approach we synthesized five types of covalent Met–ATP adducts (e.g. 1 and 12).[1,6-8] Four of these



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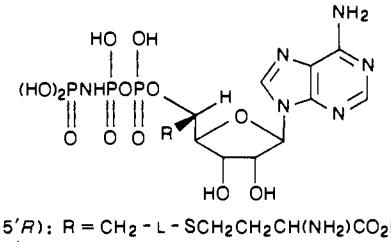
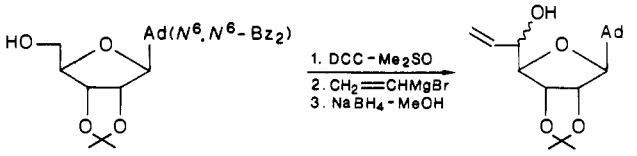


1 (5′*R*): R = $CH_2$-L-$SCH_2CH_2CH(NH_2)CO_2H$
2a (5′*S*): R = $CH_2CH_2$-L-$SCH_2CH_2CH(NH_2)CO_2H$

proved to be potent, virtually nonselective inhibitors of M-T and M-2, and the kinetic data supported a dual substrate site mode of interaction with the two isozymes. The most effective inhibitor was 1, the 5′*R* epimer of a

Scheme I




3 4a,b

$CH_3$-C5′ adduct of L-Met and β,γ-imido-ATP.[8] An important determinant of inhibitory potency in a multisubstrate inhibitor is the number of spacer atoms that link its component substrate moieties.[9,10] As a step toward delineating the number of spacer atoms required in Met–ATP adducts for maximal inhibition of M-T and M-2, we recently introduced a $CH_2$ group between C4′ and C5′ of 1 (giving 12) and reported that this caused a small loss of potency.[1] Because the reaction catalyzed by M-2 and M-T involves bond formation between the sulfur of Met and C5′ of ATP, the S–C5′ distance in Met–ATP adducts can be expected to exert a major influence on inhibitory potency. Described here is the effect of lengthening the S–C5′ distance in 1 by insertion of a second $CH_2$ carbon, a modification that produces a novel $CH_3$-C5′ covalent adduct of L-ethionine and β,γ-imido-ATP. This adduct was secured in the present work as a 2:3 mixture of its 5′ epimers **2a,b**, an approach that was more direct, synthetically, than preparation of the individual 5′ epimers,

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yet allowed us to determine, as planned, the effect of the extra $CH_2$ on inhibitory potency. In addition, the C5′ configurations of the two epimeric 5′ alcohols **8a,b** from which **2a,b** were derived have been established by single-crystal X-ray diffraction analysis of a precursor in the synthetic sequence. Findings from this analysis are included here.

**Synthesis of 2a,b.** Initially, it was planned to obtain the key 1,3-diol **2a,b** by means of a Grignard reaction between an O-protected 2-haloethanol and a suitably blocked adenosine-5′-aldehyde derivative. To this end, 2-chloroethanol was converted to its *O*-(2-tetrahydropyranyl) derivative; attempts to convert this to a Grignard reagent were unsuccessful, however. In an alternative approach, the readily available $N^6$-benzoyl-2′,3′-*O*-isopropylideneadenosine-5′-aldehyde[11] was treated in THF with a 10-fold excess of vinylmagnesium bromide. Much solid separated in the mixture and the yield of the desired $N^6$-benzoyl derivative of 5′(*S*,*R*)-*C*-vinyl-2′,3′-*O*-isopropylideneadenosine (**4a,b**) was only 2.5%. It was decided to study as starting material the 5′-aldehyde derived from the alcohol $N^6$,$N^6$-dibenzoyl-2′,3′,-*O*-isopropylideneadenosine (**3**, Scheme I) because this was expected to be more THF-soluble and, furthermore, it lacked the reactive $N^6$ proton of the above $N^6$-monobenzoyl aldehyde. Alcohol **3** is reportedly obtainable in 74% overall yield by benzoylation of 2′,3′-*O*-isopropylidene-5′-*O*-trityladenosine and subsequent detritylation; no experimental details were given.[12] In the present studies, **3** was found to be easily accessible by one-pot 5′-O-silylation of 2′,3′-*O*-isopropylideneadenosine in pyridine with trimethylsilyl chloride, followed by $N^6$,$N^6$-dibenzoylation with benzoyl chloride, and finally by removal of the trimethylsilyl group by addition of water. The conditions found satisfactory in the first two of these conversions were similar to those reported to give efficient conversion of adenosine to its $N^6$-monobenzoyl derivative.[13] Silica gel chromatography furnished homogeneous **3** in 68% yield. Pfitzner-Moffatt oxidation[11] smoothly converted **3** to the corresponding nucleoside-5′-aldehyde. This was converted to the nonhydrated aldehydic form shown to be advantageous in the case of Grignard reactions carried out with $N^6$-monobenzoyl-2′,3′-*O*-isopropylideneadenosine-5′-aldehyde.[11] In THF at −78 °C a mixture of the anhydrous 5′-aldehyde derived from **3** and vinylmagnesium bromide yielded a homogeneous solution. The aldehyde **3** was rapidly converted to four products that had TLC properties corresponding to the $N^6$-mono- and $N^6$,$N^6$-dibenzoyl derivatives of the two 5′ epimeric forms **4a,b** of 5′-*C*-vinyl-2′,3′-*O*-isopropylideneadenosine. All benzoyl groups were conveniently and cleanly removed by addition of $NaBH_4$ to a solution of the products in MeOH. Column chromatography on silica gel then gave homogeneous **4a,b** with an **a**:**b** ratio of 1:3 from HPLC analysis and from the $^1H$ NMR spectrum. The two epimers were separated by reversed-phase semipreparative HPLC, following which the major component (**4b**) was crystallized from MeOH. Single-crystal X-ray diffraction analysis revealed that **4b** possessed the 5′*R* configuration (Figure 1).

Hydroboration of unprotected **4a,b** with 9-BBN in THF produced the 1,3-diol **5a,b** in no more than 10% yield. By acid-catalyzed condensation with 2,3-dihydropyran in dioxane under conditions described for conversion of

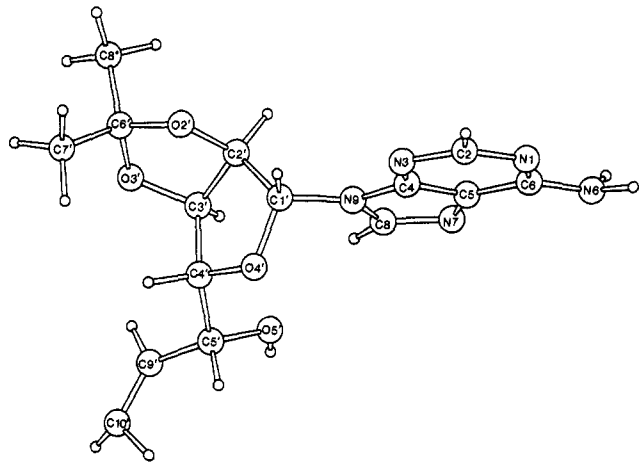


**Figure 1.** 5′(*R*)-*C*-Vinyl-2′,3′-*O*-isopropylideneadenosine, **4b**, molecular structure and atomic numbering scheme.

3′,5′-di-*O*-acetyladenosine to its 2′-*O*-tetrahydropyranyl derivative,[14] **4a,b** was converted quantitatively to material with the TLC and HPLC properties expected for its 5′-*O*-(2-tetrahydropyranyl) derivative. Without purification this was treated in THF with 9-BBN, leading, after removal of the tetrahydropyranyl group, to the hydroborated product **5a,b** (52% yield from **4a,b**). Epimers **5a** and **5b** were separable by preparative TLC on silica gel. The **a**:**b** ratio (1:2) in **5a,b** as determined by HPLC was the same as in the sample of **4a,b** that served as starting material. Assignment of **5a** as the 5′*S* epimer was made through its preparation from epimerically homogeneous **4a**.

Diols **5a,b** could be converted to their 7′-*O*-tosylates **6a,b** almost quantitatively. Without purification, the isolated tosylates were treated in EtOH with disodium L-homocysteinate to give, after reversed-phase chromatography, the homogeneous 7′-thioethers **7a,b** (**a**:**b** = 1:2) in 62% yield from **5a,b**. The α-amino acid residue in **7a,b** was then converted to an *N*-Boc derivative of its methyl ester by procedures found in previous work to be suitable with structurally related compounds.[6] The protected intermediate (**8a,b**) was obtainable in homogeneous form in 80% yield with an unaltered **a**:**b** ratio when the final reaction mixture was worked up immediately. When product isolation was delayed, significant amounts formed of a more polar compound identified as the sulfoxide **9a,b** from its $^1H$ NMR spectrum and from its quantitative formation when **8a,b** was treated with $H_2O_2$ in MeOH. Formation of **9a,b** as a byproduct presumably arises from the action on **8a,b** of traces of peroxides present in THF and $Et_2O$ used in the final $CH_2N_2$-mediated methylation step.

Phosphorylation of **8a,b** with β-cyanoethyl phosphate–dicyclohexylcarbodiimide in pyridine[15] furnished the β-cyanoethyl ester of the nucleoside 5′-phosphate **10a,b**. Removal of the cyanoethyl group is usually accomplished with aqueous base under conditions[16] that would cause hydrolysis of the carbomethoxy group of **10a,b**. When the present phosphodiester was treated at room temperature in pyridine with 2 equiv of *t*-BuOK, rapid elimination of the cyanoethyl group occurred, and concomitant attack on the carbomethoxy group could not be detected. The resulting nucleoside 5′-phosphate **10a,b** was isolated in 34% yield from **8a,b**. By means of procedures worked out earlier for synthesis of another adduct,[6] **10a,b** was con-

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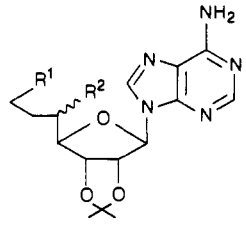

a series: 5′S
b series: 5′R

| no. | $R^1$ | $R^2$ |
|---|---|---|
| **5a,b** | OH | OH |
| **6a,b** | OTs | OH |
| **7a,b** | L-$SCH_2CH_2CH(NH_2)CO_2H$ | OH |
| **8a,b** | L-$SCH_2CH_2CH(NH\text{-}Boc)CO_2Me$ | OH |
| **9a,b** | L-$S(O)CH_2CH_2CH(NH\text{-}Boc)CO_2Me$ | OH |
| **10a,b** | L-$SCH_2CH_2CH(NH\text{-}Boc)CO_2Me$ | $OPO_3H_2$ |
| **11a,b** | L-$SCH_2CH_2CH(NH\text{-}Boc)CO_2Me$ | $OP(OH)(O)OP(OH)(O)NHPO_3H_2$ |

verted into its imidopyrophosphoroxy derivative **11a,b**. Removal of blocking groups then furnished adduct **2a,b** in homogeneous form in 17% yield from **10a,b**. HPLC analysis indicated that the ratio of 5′ epimers in **2a,b** was the same, within experimental error, as in its parent alcohol **8a,b**. The yield of **2a,b** from **8a,b** was not high enough to permit unequivocal assignment of the C5′ configuration in the major and minor components, respectively, of **2a,b**.

**Studies of Isozyme Inhibition.** Inhibition constants of 1 and the above **2a,b** mixture with the M-2 and M-T variants of methionine adenosyltransferase are set out in Table I. Adduct **2a,b** was a potent inhibitor and gave predominantly competitive kinetics vs MgATP [$K_M$(ATP)/$K_i$ = 520 and 340 for M-2, M-T respectively] and vs L-methionine [$K_M$(Met)/$K_i$ = 0.93] in the case of M-2. With M-T and variable [Met], **2a,b** furnished competitive–noncompetitive kinetics [$K_M$(Met)/$K_i$ = 5.9] with more noncompetitive character than seen in the other three kinetically analyzed systems. The high potency and competitive or partly competitive character of the inhibitions is consistent with a dual substrate site mode of interaction of **2a,b** with M-2 and M-T.

$K_i$ values of **2a,b** determined with variable [Met] were higher than with variable [ATP] by factors of 6.6 and 20 with M-T and M-2, respectively. Similar effects with M-2 and M-T are produced by all four of the markedly inhibitory adducts studied earlier.[1,7,8] Along lines set out previously,[8] these differences in $K_i$ values are taken to indicate that **2a,b** may have higher affinity for each isozyme–Met complex than for the corresponding isozyme–ATP complex, and/or that enzyme–ATP may form more often than enzyme–Met during the catalytic cycle of one or both isozymes.

Inhibition of both isozymes by **2a,b** was predominantly competitive with respect to ATP, but a significant contribution from noncompetitive inhibition was noted also. Inasmuch as under the same conditions 1 did not exhibit noncompetitive inhibition with respect to ATP, it is possible that the extra $CH_2$ in **2a,b** might facilitate reversible conversion of the respective enzyme–ATP and/or **2a,b**–enzyme complexes to ternary **2a,b**–enzyme–ATP complexes by lessening interference from the bulky $\beta,\gamma$-imido-ATP residue of **2a,b**. In the case of M-T, **2a,b** is seen to exhibit more noncompetitive inhibition vs Met than did 1, suggesting that the extra $CH_2$ might also promote conversion of (M-T)–Met and/or **2a,b**–(M-T) complexes to **2a,b**–(M-T)–Met complexes.

Earlier studies with another Met–ATP adduct revealed a large (74-fold) influence of C5′ chirality upon inhibitory potency toward M-T.[7] In light of this, minimum possible $K_i$ values for either **2a** or **2b** were calculated (Table I) from

**Table I.** Inhibition Constants of Adenine Nucleotide Derivatives with Kidney (M-2) and Novikoff Ascitic Hepatoma (M-T) Forms of Rat Methionine Adenosyltransferase[a]

| | M-2: $K_i$, μM (type of inhibn)[b] | | M-T: $K_i$, μM (type of inhibn) | |
|---|---|---|---|---|
| compd | ATP varied | Met varied | ATP varied | Met varied |
| 1[c] | 0.13 (C) | 0.65 (C) | 0.21 (C) | 0.67[d] (M) |
| **2a,b** | 0.27[e] (M) | 5.4 (C) | 0.41[f] (M) | 2.7[g] (M) |
| **2a** or **2b**[h] | (0.11) | (2.1) | (0.16) | (1.1) |

[a] When methionine (Met) was the variable substrate, [MgATP] was 2 mM with both MAT forms; with variable MgATP, [Met] was 60 μM with MAT-2 and 120 μM with MAT-T. Remaining conditions are given in ref 1. The Michaelis constant ($K_M$) of MgATP was 0.14 mM with both M-2 and M-T. The $K_M$ of L-Met was 5 μM with M-2 and 16 μM with M-T under the conditions of the $K_i$ determinations. [b] C = competitive; NC = simple noncompetitive (the inhibitor reduces $V_{max}$ and does not change $K_M$); M = mixed C and NC. $K_i$ values for M type inhibitions were derived from double-reciprocal plots (ref 1) as $K_i$ (slope) and provide a measure of the C component; $K_i$ values calculated from intercepts on the vertical ($1/V$) axis (a measure of the NC component) are listed in footnotes. [c] Data from ref 8. [d] $K_i$ (intercept), 1.75 μM. [e] $K_i$ (intercept), 1.6 μM. [f] $K_i$ (intercept), 2.6 μM. [g] $K_i$ (intercept), 3.4 μM (inhibition was predominantly NC). [h] The figures in parentheses are $K_i$ values calculated from the assumption that all the observed inhibitions by **2a,b** are mediated solely by the minor component (39% of the total), be that **2a** or **2b**.

the assumption that the minor component of the **2a,b** 5′ epimeric mixture was responsible for all of the observed inhibition. With the M-2 variant, comparison of these values with those of 1 shows that the extra of $CH_2$ of **2a** or **2b** could not have significantly enhanced inhibitory potency as judged by experiments with variable [MgATP], and actually decreased potency as judged by varying [Met]. In the case of M–T and variable [MgATP] the extra $CH_2$ culd not have increased inhibitory potency by more than 1.3-fold, while with variable [Met] it decreased potency at least 1.6-fold. The findings thus indicate that **2a** and/or **2b** are, overall, slightly less effective inhibitors of M-2 and M-T than **1**. In addition, 1 is seen to inhibit M-2 significantly more effectively than M-T, whereas **2a** and/or **2b** show only small, offsetting selective effects with variable [MgATP] and [Met], respectively, and no significant net isozyme selectivity.

In summary, the Met–ATP adduct 1 was previously established as the most effective of four Met–ATP adducts that act as potent dual substrate site inhibitors of the rat M-T and M-2 variants of methionine adenosyltransferase. Described here is a practicable synthesis of the mixed 5′ epimers **2a,b** of a higher homologue of 1 in which the number of methylene groups between S and C5′ has been increased from one to two. This modification produced a slight diminution in inhibitory potency and evidence for an increased tendency for formation of ternary enzyme–Met–adduct and enzyme–ATP–adduct complexes.

## Experimental Studies

**Chemical Synthesis. General Procedures.** $(CH_3)_3SiCl$, PhCOCl, and 2,3-dihydropyran were distilled prior to use. Disodium L-homocysteinate was prepared as previously.[1] 9-Borabicyclononane (9-BBN) and vinylmagnesium bromide were purchased from Aldrich Chemical Co. Dimethyl sulfoxide (DMSO), pyridine, and dimethylformamide (DMF) were distilled from $CaH_2$ and stored over molecular sieves. DEAE Trisacryl M was purchased from LKB Instruments, Inc. Thin-layer chromatography (TLC) was run with Merck silica gel 60 F-254 plates in (A) EtOAc–$CH_2Cl_2$ (3:2), (B) $CH_3COCH_3$–$CH_2Cl_2$ (1:9), (C) $CHCl_3$–MeOH (95:5), (D) $CHCl_3$–MeOH (9:1), (E) $CHCl_3$–MeOH–$H_2O$ (60:30:4), and (F) $CHCl_3$–MeOH–4% $CH_3COOH$ (lower layer (3:2:1). Paper chromatography was done by the ascending technique on Whatman No. 1 paper in (G) 1-propanol–$NH_4OH$–$H_2O$ (55:10:35) and (H) isobutyric acid–

$NH_4OH$–$H_2O$ (66:1:33). $C_{18}$ silica gel in column chromatography was flash grade from J. T. Baker. HPLC was performed on a Waters Model 204 chromatograph equipped with a dual solvent delivery system, a Waters 740 data module, and a Waters RCM-100 radial compression unit containing a 4 μm particle size Nova-Pak $C_{18}$ cartridge, 8 mm × 10 cm. Gradients were linear and were run for 10 min at 2 mL/min in 40–100% MeOH in $H_2O$ unless stated otherwise. Semipreparative HPLC was performed on a Dynamax $C_{18}$ silica column (21.4 mm × 25 cm) supplied by the Rainin Instrument Co., Inc. (catalog no. 83-221-C). Electrophoresis was carried out on Whatman No. 1 paper at pH 7.5 (0.05 M $Et_3NH{\cdot}HCO_3$) for 0.5 h at 4 kV. UV spectra were obtained with a Cary Model 15 spectrophotometer and $^1H$ NMR spectra with a Nicolet NT-300 spectrometer. Chemical shifts are given as ppm (δ) downfield from $SiMe_4$. Melting points are uncorrected. Elemental analyses were by Galbraith Laboratories Inc., Knoxville, TN, and were within ±0.4% of the theoretical values. Compounds for analysis were dried at 25 °C. Evaporations were performed under reduced pressure.

**$N^6$,$N^6$-Dibenzoyl-2′,3′-*O*-isopropylideneadenosine (3).** A suspension of 2′,3′-*O*-isopropylideneadenosine (30.7 g, 100 mmol) in pyridine (200 mL) was evaporated to dryness. A solution of the residue in pyridine (450 mL) was cooled in an ice bath under argon and $Me_3SiCl$ (51 mL, 4 equiv) was added over 10 min. The suspension was stirred at 22 °C for 2 h and then cooled in an ice bath. PhCOCl (26 mL, 2.2 equiv) was added slowly. The suspension was stirred at 22 °C for 2 h. Water (10 mL) was added, and volatiles were evaporated. A solution of the residual syrup in $CHCl_3$ (800 mL) was washed in succession with cold 2 N $H_2SO_4$ (3 × 200 mL), saturated $NaHCO_3$ (2 × 200 mL), and $H_2O$ (2 × 200 mL), then dried ($Na_2SO_4$), and evaporated. A solution of the gummy residue in $CH_2Cl_2$ (200 mL) was applied to a column (12.5 cm diameter) of 625 g of silica gel (flash chromatography grade) that had been preeluted with $CH_2Cl_2$ (1.2 L). The column was eluted under aspirator vacuum with $CH_2Cl_2$ (700 mL), EtOAc–$CH_2Cl_2$ (3:7, 1 L), EtOAc–$CH_2Cl_2$ (2:3, 1 L), EtOAc–$CH_2Cl_2$ (1:1, 1 L), and finally EtOAc–$CH_2Cl_2$ (3:2, 1 L), and 500-mL fractions were collected. This gave homogeneous **3** (35 g, 68%; $R_f$ 0.44, solvent A) as a white foam with the properties reported.[17] Anal. ($C_{27}H_{25}N_5O_6$) C, H, N.

**5′(*S*,*R*)-*C*-Vinyl-2′,3′-*O*-isopropylideneadenosines (4a,b).** A solution of **3** (10.3 g, 20 mmol) in toluene (100 mL) was evaporated to dryness. The residue was treated in DMSO with DCC and $Cl_2CHCO_2H$ as described for the oxidation of $N^6$-benzoyl-2′,3′-*O*-isopropylideneadenosine.[11] TLC (solvent B) showed that conversion of the alcohol ($R_f$ 0.50) to the aldehyde ($R_f$ 0.28) was complete in 1.5 h. A solution of oxalic acid monohydrate (2.75 g, 1.1 equiv) in MeOH (20 mL) was added to the reaction mixture. Dicyclohexylurea was filtered off and the filtrate was diluted with EtOAc (500 mL) and washed with water (2 × 150 mL). The aqueous portion was extracted with EtOAc (2 × 100 mL). The combined EtOAc extract was dried ($Na_2SO_4$) and evaporated. From a solution of the resulting gum in benzene (200 mL) $H_2O$ was removed azeotropically for 1 h with a Dean–Stark trap. The benzene was evaporated. To a solution of the residue in THF (100 mL) at −78 °C under argon $CH_2CHMgBr$ in THF (1.0 M, 30 mL) was added over 10 min. The dark solution was stored at −78 °C for 1 h. TLC (solvent B) of the EtOAc-soluble fraction obtained as below showed disappearance of aldehyde and formation of compounds of $R_f$ 0.74, 0.66, 0.59, and 0.50 respectively. The mixture was stirred vigorously for 20 min with saturated aqueous $NH_4Cl$ (400 mL) containing HOAc (5 mL). The aqueous phase was extracted with EtOAc (3 × 100 mL). The combined THF–EtOAc solutions were dried ($Na_2SO_4$) and evaporated. Toluene was evaporated from the residue to remove AcOH. The residue was dissolved in MeOH (50 mL) and $NaBH_4$ (430 mg) was added at 0 °C. The solution was refluxed for 1 h. The apparent pH was adjusted to 7 with HOAc. The MeOH was evaporated and the residue was partitioned between $CHCl_3$ (300 mL) and $H_2O$ (150 mL). The aqueous layer was extracted with $CHCl_3$ (2 × 100 mL). The combined $CHCl_3$ extracts were dried ($Na_2SO_4$) and evaporated. A solution of the gum in EtOAc (50 mL) was applied to a column of silica gel (500 g) prewashed with EtOAc (500 mL). Elution under suction was carried out with 1 L each of EtOAc, $CH_3COCH_3$–EtOAc (3:7), $CH_3COCH_3$–EtOAc (2:3), $CH_3COCH_3$–EtOAc (1:1), and finally $CH_3COCH_3$; 0.5-L fractions were collected. Fractions 7, 8, and 9 yielded upon evaporation a pale yellow foam which from MeOH gave white crystalline **4a + 4b** (1:3 ratio by HPLC; 1.5 g, 23% yield) homogeneous by HPLC ($t_R$ 7.3, 7.8 min, respectively) and TLC ($R_f$ 0.33 in D). The yield was 15% upon 3.5-fold scale-up; $UV_{max}$ (EtOH) 260 nm (ε 14 600); $^1H$ NMR ($CDCl_3$) δ 8.34, 7.83, 7.80 (2 H, 3 s, H-2 and H-8), 5.97–5.81 (2 H, m, H-1′ and H-6′), 5.72 (2 H, br s, $NH_2$), 5.59, 5.53 (1 H, m, H-2′), 5.36, 5.33 (1 H, m, H-3′), 5.16–4.95 (3 H, m, H-5′ and H-7′), 4.54, 4.50 (1 H, m, H-4′), 4.43 (1 H, OH), 1.62, 1.61, 1.36, 1.34 (6 H, 4 s, $2CH_3$). Anal. ($C_{15}H_{19}N_5O_4$) C, H, N.

**Crystalline 5′(*R*)-*C*-Vinyl-2′,3′-*O*-isopropylideneadenosine (4b).** A solution in MeOH (12.5 mL) of 100 mg of a 3:7 mixture of the *S* and *R* forms, respectively, was injected in 0.5-mL portions onto a Rainin (catalog no. 83-221-C) axially compressed HPLC column (2.14 cm × 25 cm) of $C_{18}$-bonded silica equilibrated with MeOH–$H_2O$ (1:1). Elution with this solvent was carried out at a rate of 10 mL/min. The *S* and *R* forms had $t_R$ values of 18.3 min and 23.3 min, respectively. The homogeneous *R* epimer so obtained was crystallized from MeOH (5 mL) to give transparent crystals, mp 246–247 °C (uncorr), that were used in the X-ray analysis detailed below.

**5′(*S*)- and 5′(*R*)-*C*-(2-Hydroxyethyl)-2′,3′-*O*-isopropylideneadenosines (5a and 5b).** To a solution of **4a + 4b** (1:2 ratio) (2.06 g, 6.19 mmol) and *p*-toluenesulfonic acid monohydrate (1.17 g, 6.16 mmol) in dioxane (19.5 mL) was added 2,3-dihydropyran (3.1 mL). After 20 min at 20 °C, TLC (solvent D, two developments, apparent $R_f$(**4a,b**) 0.54, product 0.58) and HPLC ($t_R$(**4a,b**) 7.3, 7.8 min, $t_R$ product 9.68, 10.00, 1028 min) indicated disappearance of **4a,b**. Volatiles were evaporated. The residue was partitioned between saturated aqueous $NaHCO_3$ (60 mL) and $CHCl_3$ (40 mL). The aqueous layer was extracted with $CHCl_3$ (2 × 40 mL). The combined $CHCl_3$ solutions were dried ($Na_2SO_4$) and evaporated to give a pale yellow gum. This was dried by evaporation of toluene (30 mL) from it, after which 9-BBN in THF (0.5 M, 48.8 mL, 4 equiv) was added under argon. The solution was refluxed for 2 h and then cooled in ice, and NaOH (3 M, 13 mL) and 30% $H_2O_2$ (5.3 mL) were added. The mixture was heated at 50 °C for 1 h. Solvents were evaporated, and the residue was partitioned between EtOAc (100 mL) and $H_2O$ (30 mL). The aqueous phase was extracted with EtOAc (2 × 50 mL). The EtOAc solutions were dried ($Na_2SO_4$) and evaporated. The residue contained UV-absorbing components of $t_R$ 8.1, 8.3, and 8.6 min. It was dissolved in MeOH (25 mL) and refluxed with pyridinium *p*-toluenesulfonate (1.55 mmol) for 3 h to remove the THP group. The MeOH was evaporated and the residue was partitioned between $CHCl_3$ (100 mL) and saturated aqueous $NaHCO_3$ (50 mL). The aqueous phase was extracted with $CHCl_3$ (2 × 50 mL). The $CHCl_3$ solutions were dried ($Na_2SO_4$) and evaporated. The residue was dissolved in $CHCl_3$ (30 mL) and subjected to flash chromatography on silica gel with $CHCl_3$–MeOH (19:1, 1.75 L); 50-mL fractions were collected. Fractions containing pure **5a** or pure **5b** were used for NMR spectral characterizations. Fractions containing **5a**, **5b**, or mixtures of **5a** and **5b** were combined to give a white foam (3.23 mmol by $OD_{260}$ units, 52% yield, 1:2 ratio of **5a**:**5b** from integration of H-1′ in the NMR spectrum) that was homogeneous by TLC (solvent D, two developments, apparent $R_f$ of **5a** 0.30, of **5b** 0.34) and by HPLC, $t_R$ 4.9 min. **5a**: $^1H$ NMR ($CDCl_3$) δ 8.28, 7.87 (2 H, 2 s, H-2 or H-8), 5.88 (1 H, d, H-1′, $J_{1',2'}$ = 4.7 Hz), 5.86 (2 H, br s, $NH_2$), 5.16–5.00 (2 H, m, H-2′ and H-3′), 4.42 (1 H, m, H-4′), 4.02 (1 H, m, H-5′), 3.85–3.81 (2 H, m H-7′), 1.70 (2 H, m, H-6′), 1.62, 1.36 (6 H, 2 s, $2CH_3$). **5b**: $^1H$ NMR ($CDCl_3$) δ 8.28, 7.83 (2 H, 2 s, H-2 or H-8), 5.91 (2 H, br s, $NH_2$), 5.82 (1 H, d, H-1′, $J_{1',2'}$ = 4.6 Hz), 5.13–5.05 (2 H, m, H-2′ and H-3′), 4.31 (1 H, m, H-4′), 4.19–4.16 (1 H, m, H-5′), 3.89 (2 H, m, H-7′), 1.76 (2 H, m, H-6′), 1.61, 1.36 (6 H, 2 s, $2CH_3$). A portion of **5a,b** (10 mg) was dissolved in $CHCl_3$ (0.5 mL) and pentane (2 mL) was added, giving a white solid. Anal. ($C_{15}H_{21}N_5O_5{\cdot}0.2CHCl_3{\cdot}0.1C_5H_{12}$) C, H, N.

In another experiment, **4a,b** (2.5 mmol) was converted to **5a,b** by the above procedure, and **5a,b** were separated by flash chro-

(17) Vrudhula, V. M.; Hampton, A. *Synthetic Procedures in Nucleic Acid Chemistry*; Townsend, L. B., Tipson, R. S., Eds.; John Wiley and Sons: New York; Vol. 4, in press.

matography as described above to give **5b** (450 μmol by $OD_{260}$ units) and **5a+b** (500 μmol by $OD_{260}$ units). Portion (280 μmol) of **5a+b** was chromatographed on three preparative silica gel plates (20 × 20 × 0.2 cm) in solvent D (six developments) to give **5a** (175 μmol by $OD_{260}$ units) as a white crystalline solid.

**5′(*S*,*R*)-*C*-[2-(L-Homocystein-*S*-yl)ethyl]-2′,3′-*O*-isopropylideneadenosines (7a,b).** A solution of **5a,b** (3.11 mmol; **a**:**b** = 1:2) in anhydrous pyridine (20 mL) was evaporated to dryness. The residue was dissolved in anhydrous pyridine and treated with *p*-$CH_3$-$C_6H_4SO_2Cl$ (1.6 equiv) as described for tosylation of isopropylideneadenosine.[18] TLC (solvent D, $R_f$ of **6a,b** = 0.39 and 0.34; $R_f$ of **5a** = 0.14, **5b** = 0.19) indicated that the reaction was complete after 18 h. Pyridine was evaporated off and crude **6a + 6b** was isolated by a described method.[18] To a solution of **6a + 6b** in EtOH (15 mL) was added 6 mL of freshly prepared 0.78 M disodium L-homocysteinate (1.5 equiv) in EtOH. TLC (solvent D, $R_f$ of **7a,b** = 0.0; solvent E, $R_f$ of **6a,b** 0.84, of **7a,b** 0.18) showed that reaction was complete after 1 h at 22 °C. The EtOH was evaporated and the pH of a solution of the residue in $H_2O$ was adjusted to 6.5 with 1 N HCl. The solution was extracted with $Et_2O$ (50 mL). The $Et_2O$ was washed with $H_2O$ (20 mL). The combined aqueous solutions were concentrated to a small volume and applied to a column of $C_{18}$ silica gel (50 g, flash chromatography grade) equilibrated with $H_2O$ in a funnel under aspirator vacuum. Elution was done under vacuum with $H_2O$ (150 mL), MeOH–$H_2O$ (3:7, 600 mL), and finally MeOH (100 mL); 50-mL fractions were collected. Fractions 4–10 contained **7a,b** (HPLC, 20–100% MeOH–$H_2O$, $t_R$ 7.6 min) and were evaporated to dryness. EtOH (3 × 30 mL) was evaporated from the residue to give a white solid (900 mg, 62% yield) homogeneous by TLC and HPLC in the solvents given above. Integration of the H-1′ proton in the NMR spectrum showed **7a**:**7b** to be 7:15. $UV_{max}$ (MeOH) 260 nm (ε = 14 000); $^1H$ NMR ($D_2O$) for **7a**: δ 8.06, 8.00 (2 H, 2 s, H-2 and H-8), 5.98 (1 H, d, H-1′, $J_{1',2'}$ = 3.8 Hz), 5.16 (1 H, dd, H-2′, $J_{2',3'}$ = 6.0 Hz), 4.94 (1 H, dd, H-3′, $J_{3',4'}$ = 3.3 Hz), 4.16 (1 H, t, H-4′, $J_{4',5'}$ = 3.3 Hz), 3.79–3.46 (2 H, m, H-5′ and H-2″), 2.58–2.23 (4 H, m, H-7′, H-4″), 2.1–1.85 (2 H, m, H-3″), 1.75–1.41 (2 H, m, H-6′), 1.48, 1.24 (6 H, 2 s, $2CH_3$). **7b**: 8.05, 8.00 (2 H, 2 s, H-2 and H-8), 6.04 (1 H, d, H-1′, $J_{1',2'}$ = 2.1 Hz), 5.35 (1 H, dd, H-2′, $J_{2',3'}$ = 6.2 Hz), 5.02 (1 H, dd, H-3′, $J_{3',4'}$ = 1.9 Hz), 4.00 (1 H, dd, H-4′, $J_{4',5'}$ = 6.3 Hz), 3.79–3.46 (2 H, m, H-5′ and H-2″), 2.58–2.23 (4 H, m, H-7′, H-4″), 2.1–1.85 (2 H, m, H-3″), 1.75–1.41 (2 H, m, H-6′), 1.46, 1.27 (6 H, 2 s, $2CH_3$). Anal. ($C_{19}H_{28}N_6O_6S·H_2O$) C, H, N; S: calcd, 6.58; found, 7.30.

**5′(*S*,*R*)-*C*-[2-[L-Methyl *N*-(*tert*-butyloxycarbonyl)-homocysteinat-*S*-yl]ethyl]-2′,3′-*O*-isopropylideneadenosines (8a,b).** To a solution of **7a,b** (7:15 by $^1H$ NMR, 859 mg, 1.84 mmol) in DMF (72 mL) was added $Et_3N$ (0.27 mL) and ($t$-BuO-$CO_2$)O (0.53 g, 1.3 equiv). After 1.5 h at 20 °C, TLC (solvent E) revealed complete conversion of **7a,b** ($R_f$ 0.17) to a product ($R_f$ 0.47) negative to ninhydrin spray. DMF was evaporated and a solution of the colorless gum in THF was treated with ethereal $CH_2N_2$ until the color of the $CH_2N_2$ persisted. HPLC showed **8a,b** as a single peak of $t_R$ 9.9 min. Volatiles were evaporated. Storage of the gum at −15 °C for 18 h led to conversion of 31% of **8a,b** to **9a,b** ($t_R$ 8.3 min). TLC, solvent D: $R_f$ of **8a,b** 0.42, of **9a,b** 0.26). Flash chromatography on silica gel with $CHCl_3$–MeOH (97:3) gave **8a,b** as a while foam (843 μmol by $OD_{260}$ units homogeneous by TLC (solvent D) and HPLC and stable to storage at −15 °C for several weeks. The **a**:**b** ratio was 1:2 by $^1H$ NMR. $^1H$ NMR ($CDCl_3$) δ 8.30, 8.29, 7.83 (2 H, 3 s, H-2 and H-8), 6.00–5.81 (3 H, m, $NH_2$ and H-1′), 5.13–5.02 (2 H, m, H-2′ and H-3′), 4.45–4.28 (2 H, m, H-4′ and H-5′), 4.02 (1 H, m, H-2″), 3.73 (3 H, s, $CH_3$), 2.88–2.46 (4 H, m, H-7′ and H-4″), 2.2–1.64 (4 H, m, H-3″ and H-6′), 1.61 (3 H, s, $CH_3$), 1.42 (9 H, s, $C_4H_9$), 1.41, 1.35 (6 H, 2 s, $2CH_3$). Anal. ($C_{25}H_{38}N_6O_8S·0.1CHCl_3$) C, H, N; S: calcd, 5.39; found, 6.31.

**Sulfoxide Derivatives of 8a,b (9a,b).** The foregoing fractions containing **9a,b** gave a white foam (548 μmol by $OD_{260}$ units) that appeared to be homogeneous by TLC (solvent D) and HPLC analysis and appeared from the $^1H$ NMR spectrum to contain one minor and two major components. $^1H$ NMR ($CDCl_3$) δ 8.30, 8.28, 7.88, 7.87 (2 H, 4 s, H-2 and H-8), 6.00–5.81 (3 H, m, $NH_2$, H-1′), 5.11–5.03 (2 H, m, H-2′ and H-3′), 4.48–4.30 (2 H, m, H-5′ and H-4′), 3.99 (1 H, m, H-2″), 3.76, 3.75 (3 H, 2 s, $CH_3$), 3.05–2.63 (4 H, m, H-7′, H-4″), 2.46–1.84 (4 H, m, H-3″ and H-6′), 1.62, 1.61 (3 H, 2 s, $CH_3$), 1.42, 1.41, 1.40 (9 H, 3 s, $C_4H_9$), 1.35 (3 H, s, $CH_3$). Anal. $C_{25}H_{38}N_6O_9S·H_2O$) C, H, N; S: calcd 5.19; found, 5.65.

**Conversion of 8a,b to 9a,b.** To **8a,b** (2 mg) in MeOH (100 μL) was added aqueous $H_2O_2$ (30%, 20 μL). TLC and HPLC analysis (including coinjection with **9a,b**) indicated that after 1 h at 22 °C complete conversion had occurred to a compound indistinguishable from **9a,b**.

**5′(*S*,*R*)-*C*-[2-(L-Homocystein-*S*-yl)ethyl]adenosine 5′-(β,γ-Imidotriphosphates) (2a,b).** To **8a + 8b** (**a**:**b** = 1:2 by $^1H$ NMR; 450 μmol determined by $OD_{260}$ units) was added pyridinium cyanoethyl phosphate (900 μmol) in pyridine (900 μL).[15] The resulting solution was evaporated to dryness. Pyridine (6 mL) was added and evaporated three times. The gum was dissolved in anhydrous pyridine (4.5 mL) and DCC (750 mg) was added. The pale yellow suspension was stored under argon for 3 days. TLC (solvent D, $R_f$ of the cyanoethyl ester of **10a,b** 0.0; solvent F, $R_f$ of **8a,b** 0.9, $R_f$ of the cyanoethyl ester 0.40) showed complete conversion of starting material to the cyanoethyl ester and other products. HPLC confirmed that the cyanoethyl ester ($t_R$ 5.6 min) was the major UV-absorbing product (43% of the total) and that a number of less polar components were present. To the mixture was added water (4.5 mL). After 1 h volatiles were evaporated. The residue was suspended in MeOH (10 mL). Dicyclohexylurea (DCU) was filtered off. From the MeOH solution a second crop of DCU was filtered off. To the filtrate was added water dropwise to incipient cloudiness. The mixture was loaded onto a column of $C_{18}$ silica gel and this was eluted with $H_2O$ (150 mL), MeOH–$H_2O$ (1:1, 150 mL), MeOH–$H_2O$ (7:3, 100 mL), and MeOH (100 mL). Fractions containing the cyanoethyl ester were evaporated to give a yellow gum (185 μmol by $OD_{260}$ units). The electrophoretic mobility relative to AMP (1.00) was 0.40. The gum was dissolved in pyridine (2 mL) and $t$-BuOK (41 mg, 360 μmol) was added. Electrophoresis showed the reaction to be complete after 0.5 h at 22 °C. The solution was evaporated. A solution of the residue in MeOH–$H_2O$ (1:4, 5 mL) was applied to a column (2 × 15 cm) of $C_{18}$ silica gel. The column was washed with MeOH–$H_2O$ (1:4, 200 mL) and then with MeOH–$H_2O$ (3:1, 200 mL) to elute **10a,b** (152 μmol by $OD_{260}$ units). After removal of volatiles in vacuo, **10a,b** was converted to its phosphoroimidazolidate and this in turn to **2a,b** via **11a,b** by procedures detailed previously[6] except that MeOH–$H_2O$ (1:1) was required in the step involving elution of product from a $C_{18}$ silica gel column. Chromatography of **2a,b** was performed at 4 °C on a DEAE Trisacryl M ($HCO_3^-$) column (2.5 × 10 cm) with a linear gradient of aqueous $Et_3NH$–$HCO_3$ (0–0.4 M, 2 L). Fractions containing **2a,b** (800 $OD_{260}$ units, 11.8% from **8a,b**) were combined and evaporated in vacuo. The residue was dissolved in MeOH (1 mL) and 1 M NaI (270 μL, 5 equiv) in acetone was added. Acetone (50 mL) was added. The precipitate was washed with acetone and dried in vacuo over $P_2O_5$ to give tetrasodium **2a,b** (41 mg) as an off-white powder. This material did not yield satisfactory elemental analyses. Purification by means of paper chromatography (system G) and a DEAE Trisacryl M ($HCO_3^-$) column gave tetrasodium **2a,b** (22 mg) as a white powder which had given a smooth symmetrical peak on the DEAE Trisacryl elution diagram and which also appeared homogeneous as judged by paper chromatography in G ($R_f$ 0.38; ATP, 0.48) and H ($R_f$ 0.40, ATP 0.33) and HPLC with 0–30% MeOH in 0.1 M $K_2HPO_4$–0.025 M ($n$-Bu)$_4$$NHSO_4$ (pH 7.6) [$t_R$ 10.7 and 12.1 min (39:61 ratio), ATP 7.8 min, 1 11.8 min]. $UV_{max}$ pH 2, 258 nm (ε 14 800), pH 11, 260 nm (ε 15 300). Anal. ($C_{16}H_{24}N_7O_{14}P_3SNa_4·3H_2O·2MeOH$) C, H, N, P; S: calcd, 3.67; found, 4.52.

**Crystal data for 4b**: $C_{15}H_{19}N_5O_4$, $M_4$ = 333.349 daltons, orthorhombic, $P2_12_12_1$, $a$ = 7.974 (1) Å, $b$ = 9.482 (1) Å, c = 21.213 (1) Å, $V$ = 1603.89 Å$^3$, $Z$ = 4, $D_x$ = 1.380 (3) g/cm$^3$, $D_m$ = 1.384 (5) g/cm$^3$, λ(Cu Kα) = 1.541 78 Å, μ 7.64 cm$^{-1}$, $T$ = 21 °C (figures in parentheses indicate estimated standard deviations); crystal dimensions 0.5 × 0.4 × 0.15 mm. Density was measured by the flotation method in $C_6H_5Cl$–$CCl_4$.

**Crystallographic Measurements.** Preliminary characterization of the **4b** crystal was obtained from precession photographs. Intensity data were collected on an Enraf Nonius CAD-4 diffractometer with graphite monochromated Cu Kα radiation using

(18) Kuhn, R.; Jahn, W. *Chem. Ber.* **1965**, *98*, 1699.

$\omega/2\theta$ scans. Unit cell parameters were obtained from least-squares fitting of the diffractometer angles of 25 centered reflections ($40° < 2\theta < 50°$). No decay was observed in the four standard reflections or in the crystal itself during data collection. Intensities were corrected for Lorentz and polarization effects, and an empirical absorption correction was applied, with $\Psi$ scan intensity data. A total of 1742 reflections were measured, of which 1671 were considered observed [$I > 2\sigma(I)$].

**X-ray Structure Analysis of 4b.** The structure was solved by direct methods using the 246 highest $E$ values with the program system MULTAN 80.[19] Refinement by full-matrix least squares was based on $|F_o|$ with 1671 data [$I > 2\sigma(I)$] with all the non-H atoms assigned anisotropic thermal parameters. Hydrogen atoms bonded to the C atoms on the adenine ring system were given fixed geometry (C–H 1.08 Å), and only their thermal parameters were refined. The remaining hydrogen atoms were assigned isotropic thermal parameters according to the type of atom to which they are bonded; their positions and thermal parameters were refined. In the final refinement cycle the two $CH_3$, the vinyl $=CH_2$, the OH, and the $NH_2$ groups were allowed to move as rigid bodies, to obtain a well-defined structure. The weighting scheme used was $w^{-1} = \sigma^2|F_o| + 0.0004|F_o|^2$. Scattering factors for C, N, and O were those of Cromer and Mann.[20] The scattering factor used for H was that for a spherical bonded H atom.[21] The structure converged to a final residuals $R = 0.052$ and $wR = 0.051$ for 231 parameters. Least-squares refinement and geometric calculations were performed with SHELX76.[22] The final difference Fourier map showed no distinct features (maximum +0.28 e/Å$^3$, and minimum −0.41 e/Å$^3$). The molecular structure and atomic numbering scheme of the molecule is shown in Figure 1. The bond lengths and angles are comparable to those in an X-ray structure of the parent compound, 2',3'-*O*-isopropylideneadenosine.[23] Structure factor data, coordinates, and thermal parameters have been deposited with the British Library.

**Enzyme Studies.** Assay of M-2 and M-T activity and determination of inhibition constants were carried out as detailed previously.[1] The type of double-reciprocal plots so obtained have been illustrated previously.[7]

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**Supplementary Material Available:** Table I listing final fractional atomic coordinates and temperature factors, Table II listing interatomic distances and angles (2 pages); Table III listing structure factor data (7 pages). Ordering information is given on any current masthead page.

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# [(1*H*-Imidazol-1-yl)methyl]- and [(3-Pyridinyl)methyl]pyrroles as Thromboxane Synthetase Inhibitors[1]

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Several [(1*H*-imidazol-1-yl)methyl]- and [(3-pyridinyl)methyl]pyrroles were prepared and evaluated in vitro as thromboxane synthetase inhibitors in human platelet aggregation studies. A number of structures, e.g. **10b,f,g,i** (respective $IC_{50}$ values: 1 μM, 50 nM, 42 nM, 44 nM) showed superior in vitro inhibition of $TXA_2$ synthetase when compared to the standard dazoxiben (1). However, it was found that in vitro potency did not translate into nor correlate with in vivo activity when these compounds were evaluated in mice in a collagen–epinephrine-induced pulmonary thromboembolism model. (*E*)-1-Methyl-2-[(1*H*-imidazol-1-yl)methyl]-5-(2-carboxyprop-1-enyl)pyrrole (**10b**) was found to offer protection against collagen–epinephrine-induced mortality in mice, thereby demonstrating that oral administration is an effective route for absorption of this drug. Additional evidence for the oral effectiveness of **10b** in lowering serum $TXB_2$ levels was obtained by performing ex vivo radioimmunoassay experiments with rats. A 13-week study of **10b** in rats with reduced renal mass was conducted in order to evaluate the role of $TXA_2$ production in hypertension and renal dysfunction. Although serum and urinary $TXB_2$ levels in rats were found to be lowered during this study by **10b**, the levels of urinary protein excretion remained comparable to that of the control group.

Thromboxane $A_2$ ($TXA_2$), a potent and labile mediator of platelet aggregation and vasoconstriction, is endogenously generated mainly by blood platelets by the enzyme $TXA_2$ synthetase from the endoperoxide $PGH_2$. Prostacyclin ($PGI_2$) is similarly produced from this endoperoxide by an enzyme located primarily in endothelial cells of the arteries and blood vessels, and is a potent inhibitor of platelet aggregation and a vasodilator. The symbiotic relationship between the effects of $PGI_2$ and $TXA_2$ has been suggested as one of the natural balancing mechanisms for physiological hemodynamics.[2] The imbalanced overproduction of $TXA_2$ has been suggested in numerous pathological events.[3-7]

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