Articles

Isozyme-Specific Enzyme Inhibitors. 10.[1] Adenosine 5'-Triphosphate Derivatives as Substrates or Inhibitors of Methionine Adenosyltransferases of Rat Normal and Hepatoma Tissues

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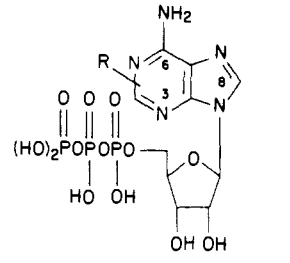
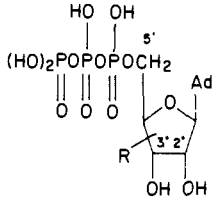
Monosubstituted adenosine 5'-triphosphate (ATP) derivatives with a substituent of up to four atoms at any of eight positions in the adenosine moiety, or with an isosteric group replacement at O5' or in the triphosphate moiety, have been evaluated kinetically as substrates and inhibitors of liver (I), kidney (II), and Novikoff hepatoma (T) variants of rat methionine adenosyltransferase. Inhibitory potencies were expressed as $K_M(ATP)/K_i$ (for competitive inhibition vs. ATP) or as $K_M(ATP)/K_M$ when no K_i value was available. Variant I was inhibited more powerfully than II or T by all of four ATP derivatives for which comparative data were obtained. Among 15 ATP derivatives, four were substrates of II or T and the remainder inhibited II and T competitively with respect to ATP; most derivatives exhibited at least moderate (>0.5) inhibitory potency. Differential inhibition of II and T was shown by 11 of 14 ATP derivatives; relative inhibitory potencies (T:II) ranged from 5.5 with 2-SCH_3-ATP [$K_M(ATP)/K_i$ = 1.3 with T] to 0.24 with the ATP isostere with a C5'-CH_2-P^α system [$K_M(ATP)/K_i$ = 1.9 with II]. The most effective inhibitor was the P^β,P^γ imido isostere of ATP with inhibitory potencies of 25 and 35 for II and T, respectively. The findings provide further evidence that substrate derivatives with single short groups attached at various positions, or with single isosteric group replacements, are frequently useful probes in the design of isozyme-selective inhibitors.

Data on the isozyme composition of mammalian normal and neoplastic tissues suggests, on grounds outlined previously,[2] that isozyme-specific enzyme inhibitors might, if available, be useful in the design of new types of antineoplastic agents. Studies of the interactions of substrate derivatives at four substrate sites located on isozymic variants of three enzymes have indicated that monosubstituted substrate derivatives that bear short substituents at various positions are potentially useful probes in the design of isozyme-specific inhibitors.[1] Each of the enzymes that were so studied occurs as a variant that is of interest as a possible target in cancer chemotherapy. In an extension of this approach, the present report describes interactions of monosubstituted substrate derivatives at a substrate site of a fourth such enzyme, ATP:L-methionine *S*-adenosyltransferase (EC 2.5.1.6) (MAT), which catalyzes bond formation between C5' of adenosine 5'-triphosphate (ATP) and the sulfur of L-methionine to form *S*-adenosylmethionine. The study employed ATP derivatives monosubstituted at six positions in the purine and ribofuranose rings (**1**–**8b**), and analogues of ATP resulting from single isosteric group replacements at four positions in the tripolyphosphate region (**9**–**13**). 2'-Deoxy-ATP (**14**) was also studied.

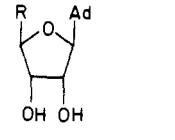
There is evidence for four variants of MAT in normal rat tissues: MAT-I comprises 15% of the total MAT activity in the liver;[3] MAT-II comprises 5% of the liver activity[3] and is reported as the sole form in 11 other tissues;[4,5] MAT-III comprises 85% of MAT activity in liver[6-8] and could be the major form in lenticular tissue also;[9] a MAT-IV, together with MAT-II, is reportedly present in

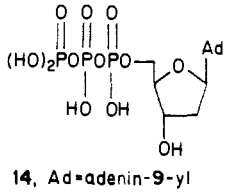


1, R = 2-SMe
2, R = N^6-*n*-Bu
3, R = 6-desamino-6-S(*n*-Bu)
4a, R = 8-NHMe
4b, R = 8-SMe
4c, R = 8-S$(CH_2)_2$CH$(NH_2)CO_2H$

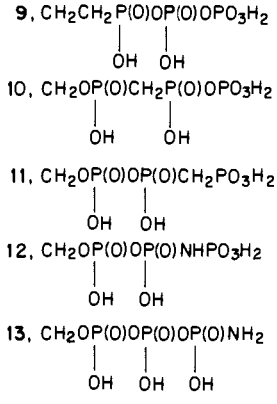


5, R = 2'-*O*-Me
6, R = 3'-*O*-Me
7a, R = 5'-*R*-Me
7b, R = 5'-*R*-*n*-Pr
8a, R = 5'-*S*-Me
8b, R = 5'-*S*-*n*-Pr





14, Ad = adenin-**9**-yl



9, $CH_2CH_2P(O)OP(O)OPO_3H_2$
10, $CH_2OP(O)CH_2P(O)OPO_3H_2$
11, $CH_2OP(O)OP(O)CH_2PO_3H_2$
12, $CH_2OP(O)OP(O)NHPO_3H_2$
13, $CH_2OP(O)OP(O)OP(O)NH_2$

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Table I. Physical Properties of Adenosine 5′-Triphosphate (ATP) Derivatives

compd	yield,[a] %	UV: λ_{max}, nm ($\epsilon \times 10^{-3}$) pH 1	UV: λ_{max}, nm ($\epsilon \times 10^{-3}$) pH 6.8	electrophoresis, pH 3.6, rel mobility[b]	R_f (syst B)[b]	HPLC retn time,[b] min	formula	anal.
ATP				1.0	0.16	15		
1	72	270 (15.9)	273 (15.6)	1.0	0.2	20	$C_{11}H_{14}N_5O_{13}SP_3Na_4 \cdot 2H_2O$	C, H, N, P, S
4a	70	277 (14.7)	279 (17.3)	0.98	0.17	17	$C_{11}H_{15}N_6O_{13}P_3Na_4 \cdot 2H_2O$	C, H, N, P
4c	37	281 (18.9)	281 (18.6)	0.94	0.12[c]	13	$C_{14}H_{19}N_6O_{15}SP_3Na_4 \cdot 5.5H_2O \cdot CH_3OH$	C, H, N, P, S
5	40		260 (15.1)	0.98	0.24	17	$C_{11}H_{14}N_5O_{13}P_3Na_4 \cdot 2H_2O$	C, H, N, P
6	45		260 (14.9)	0.97	0.24	16	$C_{11}H_{14}N_5O_{13}P_3Na_4 \cdot 3H_2O$	C, H, N, P
13	94	258 (14.9)	260 (15.1		*d*		$C_{10}H_{14}N_6O_{12}P_3Na_3 \cdot 4H_2O \cdot 0.25CH_3OH$	C, H, N, P

[a] Yields are of purified sodium salts. [b] For conditions, see the Experimental Section. [c] R_f 0.30 in system C. [d] R_f 0.40 (ATP, 0.33), 0.17 (ATP, 0.06), and 0.71 (AMP, 0.67; ATP, 0.31) in systems C–D–E, respectively.

lactating mammary gland.[4] During chemically induced hepatocarcinogenesis in rats, MAT-I and MAT-III become replaced by an additional form, designated here as MAT-T.[10] In the rat Novikoff hepatoma and several other transplantable rat hepatomas, MAT-T was the major form detected,[6,10] prompting the proposal that this form might be a potential target for chemotherapy.[6] A metabolic disorder in children in which hepatic (but not extrahepatic) MAT levels are ca. 10% of normal has been reported to be attended by apparent clinical fitness during observation periods extending over several years.[11,12] Possibly, therefore, an effective antitumor agent need not inhibit MAT-T selectively with respect to MAT-III, but with respect only to MAT-II and possibly also MAT-I. In the present work, the ATP derivatives **1**–**14** have been evaluated kinetically as substrates and inhibitors of MAT-T of rat Novikoff hepatoma cells and MAT-II of rat kidney in a search for differential effects. Also reported are substrate and inhibitor properties of some of these ATP derivatives with MAT-I of rat liver.

Syntheses. Syntheses of ATP derivatives **2** and **3**,[13] **4b**,[14] and **7a**, **7b**, **8a**, **8b**, and **9**[15] were described previously. ATP derivatives **1**, **5**, and **6** were prepared from the corresponding derivatives of adenosine 5′-phosphate (AMP),[16] first by converting these to their 5′-phosphoroimidazolidates by the action of *N,N′*-carbonyldiimidazole.[17] This intermediate was obtained as its 2′,3′-*O*-carbonyl derivative[18] in the case of the AMP derivative corresponding to **1**, in which the 2′,3′-*cis*-diol system is unsubstituted. The phosphoroimidazolidates were brought into reaction with tri-*n*-butylammonium pyrophosphate under conditions described by Hoard and Ott[17] for the conversion of nucleoside 5′-phosphates to nucleoside 5′-triphosphates. The 2′,3′-*O*-carbonyl group attached to **1** was readily removed by mild basic hydrolysis.[18] The ATP derivatives **1**, **5**, and **6** were obtained in 40–70% yield as tetrasodium salt that were homogeneous in the paper chromatographic, electrophoretic, and HPLC systems of Table I.

8-(Methylamino)adenosine 5′-diphosphate (8-MeNH-ADP) is readily converted to 8-MeNH-ATP (**4a**) by rat pyruvate kinases.[1] The present studies showed that **4a** could be obtained in homogeneous form in 70% yield by this reaction (catalyzed by commercially available rabbit muscle pyruvate kinase), in which the phosphate reside of phosphoenolpyruvate becomes transferred to 8-MeNH-ADP. 8-[(L-1-amino-1-carboxypropyl)thio]adenosine 5′-triphosphate (**4c**) was obtained by condensation of disodium L-homocysteine with tetralithium 8-Br-ATP in MeOH–H_2O under conditions used previously for the synthesis of other 8-(alkylthio)-ATP derivatives.[14] Finally, the tributylammonium salt of adenosine 5′-trimetaphosphate,[19,20] prepared as described previously,[13] was treated in DMF solution with an excess of ammonia, following which ATP γ-amidate (**13**) was isolated in 94% yield as a homogeneous trisodium salt. The preparation of **13** by amination of γ-*O*-mesitoyl-ATP[21] has been reported.[22]

Enzyme Studies. Catalysis by methionine adenosyltransferase (MAT) requires the presence of K^+ and a divalent metal such as Mg^{2+}. Substrate and inhibitor constants of the present series of ATP derivatives with MAT-I, MAT-II, and MAT-T were determined with levels of K^+ and Mg^{2+} and at pH values that were similar or identical for all three forms and have been shown to be near-optimal for their activity.[3,6,23] Methionine was employed at levels that produced near-maximal reaction velocities but were not inhibitory. ATP and its derivatives were added as 1:1 Mg complexes in order to minimize variations in the level of uncomplexed Mg^{2+} in reaction mixtures. The isolation and purification procedures used for MAT-II and MAT-T were identical.

Substrate activity with MAT-II or MAT-T (Table II) was shown only by the 8-substitutued ATP derivatives **4a,b** and by P^{γ}-NH_2-ATP (**13**) and 2′-deoxy-ATP (**14**). The relatively high K_m values of **13** and **14** suggest that the 2′-hydroxyl of ATP and the dianionic form of the ATP γ-phosphate residue may play roles in reversible binding of ATP to these enzymes. The low V_{max} of 2′-deoxy-ATP with MAT-II suggests possible involvement of the 2′-

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Table II. Substrate and Inhibitor Constants of ATP Derivatives with Liver (MAT-I), Kidney (MAT-II), and Novikoff Hepatoma (MAT-T) Forms of Rat Methionine Adenosyltransferase

	MAT-I			MAT-II			MAT-T			inhib potency $[K_M(ATP)/K_i$ or $K_M]$			rel inhib potency,
compd	K_i,[a] mM	K_m,[b] mM	rel V_{max}	K_i, mM	K_M, mM	rel V_{max}	K_i, mM	K_M, mM	rel V_{max}	I	II	T	T/II
ATP		0.14	100		0.14	100		0.14	100				
1				0.60			0.11				0.23	1.27	5.5
2				0.53			0.21				0.26	0.66	2.5
3				0.31			0.10				0.45	1.40	3.1
4a					0.16	37		0.23	19		0.88	0.61	0.69
4b					0.10	3.1	0.53	0.20	7.3		1.40	0.70	0.50
4c	3.3[c]			0.52[d]							0.27		
5				0.14			0.17				1.00	0.82	0.82
6				0.17			0.18				0.82	0.78	0.95
7a	0.0090			0.19			0.080			15.6	0.74	1.75	2.4
7b				0.048			0.037				2.92	3.78	1.3
8a	0.014			0.41			0.75			10.0	0.34	0.19	0.56
8b				0.27			0.14				0.52	1.00	1.9
9	0.015			0.075			0.31			9.3	1.87	0.45	0.24
10				0.30			0.34				0.46	0.41	0.89
11				[e]			[e]						
12				0.0055			0.0040				25.5	35.0	1.4
13					3.3	97			[f]		0.042		
14		1.54	26		4.0	6.5			[f]	0.091	0.035		

[a] Inhibitions were competitive with respect to Mg-ATP unless indicated otherwise. [b] K_M = concentration of Mg-ATP for half-maximal velocity (V_{max}). [c] Inhibition by **4c** was noncompetitive with respect to Mg–ATP and also with respect to methionine, when a K_i of 2.3 mM was obtained. [d] **4c** showed competitive inhibition, K_i = 1.1 mM, with respect to methionine. [e] Compound 11 (0.5 mM) produced no inhibition in the presence of 0.15 mM Mg-ATP. [f] Very weak substrate activity was observed that could have originated from traces of ATP which HPLC analysis (see the Experimental Section) indicated could be present in 13 and 14.

hydroxyl of ATP in catalyic events. 2′-Deoxy-ATP had 10-fold higher substrate efficiency (V_{max}/K_m) with MAT-I than with MAT-II.

Inhibition constants of the ATP derivatives are given in Table II together with inhibitory potencies expressed as $K_m(ATP)/K_i$ or, to a first approximation, as $K_m(ATP)/K_m$ in the few cases when a K_i value was unavailable. Competitive inhibition with respect to MgATP was observed with all MAT–inhibitor combinations tested except the S-substituted homocysteine derivative **4c** and MAT-1. Most of the ATP derivatives exhibited at least moderate inhibitory potency [$K_m(ATP)/K_i$ or $K_m > 0.5$] with MAT-II and MAT-T. The β,γ-imido analogue of ATP (**12**) was a relatively strong inhibitor whereas its methylene counterpart **11** was a much weaker inhibitor, conceivably because imidodiphosphate is sterically and electronically more similar to pyrophosphate than is methylene diphosphonate.[24] MAT-I was markedly inhibited by the epimeric 5′-*C*-methyl derivatives of ATP (**7a** and **8a**) and by the phosphonate isostere **9** of ATP.

A majority of the ATP derivatives produced differential effects with MAT-II and MAT-T, as shown by their relative inhibitory potencies listed in Table II. Substituents at the C2-, C6-, N^6-, or C5′-positions or substitution of P^β-NH-P^γ for P^β-O-P^γ tended to produce selective inhibitors of MAT-T, whereas substituents at C8 or substitution of CH_2 for O5′ gave selective inhibitors of MAT-II. In addition, the inhibitory potencies of **7a**, **8a**, **9**, and **14** were significantly higher with MAT-I than with MAT-II or MAT-T.

Previous studies have shown that a high proportion of AMP or ATP derivatives that bear a single short substituent behave as species- or isozyme-selective inhibitors of adenylate kinases by reason of differential affinities for the AMP and ATP substrate sites, respectively, of those enzymes.[13-16] Likewise, a large proportion of monosubstituted thymidine derivatives were species- or isozyme-selective inhibitors of thymidine kinases,[25,26] and a large proportion of monosubstituted ADP derivatives were isozyme-selective inhibitors of pyruvate kinases (for which ADP is a substrate).[1] The present findings with methionine adenosyltransferases follow the same pattern and thus afford additional evidence that substrate derivatives that bear short substituents (usually one to four atoms exclusive of H) are potentially useful probes in early stages of the design of isozyme-selective inhibitors. It is not yet possible to assess the general utility of an approach along these lines. One limitation is that a series of monosubstituted substrate derivatives can, on occasion, exhibit isozyme-selective effects that are almost exclusively in a therapeutically undesirable direction, as was found, for example, in a study of AMP derivatives and AMP kinases.[16] A more general problem is that substrate substituents have tended to produce inhibitors of low or moderate potency. We have noted, however, that the selectivity and/or inhibitory potency of these compounds can sometimes be significantly improved by modifying the substituent[25] or by retaining the substituent and elaborating the substrate moiety so that it can bind simultaneously and powerfully to two adjoining substrate sites.[13,15]

Experimental Section

Chemical Synthesis. General Procedures. *N,N*-Dimethylformamide (DMF) was distilled from CaH_2 and stored over molecular sieves. Paper chromatography was carried out by the ascending technique on Whatman No. 1 paper in (A) 1-butanol–acetic acid–water (5:2:3), (B) isobutyric acid–concentrated NH_4OH–water (66:1:33), (C) 1-propanol–concentrated NH_4OH–water (55:10:35), (D) 2-propanol–concentrated NH_4OH–water (7:1:2), and (E) polyethyleneimine cellulose with 1.5 M LiCl. Electrophoresis was carried out on Whatman No. 1 paper at pH 3.5 (0.05 M citrate). UV spectra were obtained with a Cary Model 15 spectrophotometer. Elemental analyses were performed by Galbraith Laboratories Inc., Knoxville, TN. and were within ±0.4% of the theoretical values unless noted otherwise. Compounds were dried over P_2O_5 at 25 °C. HPLC was performed with

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a Waters Model 204 chromatograph equipped with a Model M-6000A dual solvent delivery system and a Model 660 programmer. Analyses employed a Waters RCM-100 unit containing a μ-Bondapak C_{18} column eluted at 1.2 mL/min with 0.1 M KH_2PO_4–0.025 M Bu_4NHSO_4 (pH 5) with a linear gradient of 10–40% MeOH over 10 min.

The ATP derivatives **10–12** and **14** were purchased from the Sigma Chemical Co.; **13** contained 1% of ATP, and **14** contained 0.06% of ATP as indicated by HPLC using 0.5 M aqueous $Et_3NH^+HCO_3^-$ as eluant. ATP derivatives synthesized as described below were purified by one of the two following procedures. The products so obtained were homogeneous in the systems of Table I.

Method I. The crude product (0.4 mmol) was chromatographed at 5 °C on a DEAE cellulose column (4 × 20 cm). Elution with a linear gradient of 0–0.35 M $Et_3NH^+HCO_3^-$ (4 L), vacuum evaporation of appropriate fractions, and several evaporations of EtOH from the residue gave the triethylammonium salt of the product which was dissolved in sufficient MeOH so that addition of 1 M NaI in MeOH (7.5 equiv) gave no precipitate. The methanol solution was evaporated to a minimum volume and diluted with acetone (ca. 10 volumes) to precipitate the tetrasodium salt.

Method II. When byproducts were present that coeluted with the desired product from columns of DEAE-cellulose, the crude product was chromatographed in solvent A on Whatman No. 17 paper (width 46 cm, 0.2–0.3 mmol/sheet). The band containing the product was eluted with water at 5 °C, the eluate was applied to a column of DEAE cellulose, and purification was completed by method I.

General Method for Synthesis of the Nucleoside 5′-Triphosphates 1, 5, and 6. The requisite nucleoside 5′-monophosphate (0.4 mmol)[16] was converted into the corresponding 5′-phosphoroimidazolidate and this was reacted with tri-*n*-butylammonium pyrophosphate according to the Hoard–Ott procedures.[17] The mixture was filtered, and the DMF used as solvent was evaporated in vacuo. In the synthesis of **1**, the residue was dissolved in EtOH–H_2O (1:1, 100 mL) and the solution was brought to pH 11 with Et_3N to remove 2′,3′ cyclic carbonate residues.[18] After 4 h at 23 °C, volatiles were removed in vacuo and EtOH was evaporated from the residue to remove residual Et_3N. Crude **1** was purified by method I. Crude **5** and **6** both contained an unidentified byproduct of higher R_f in solvent A and were purified by method II. Physical properties are listed in Table I.

8-(Methylamino)adenosine 5′-Triphosphate (4a). A solution of trisodium 8-MeNH-ADP[1] (0.06 mmol) and cyclohexylammonium phosphoenolpyruvate (Sigma Chemical Co.; 0.12 mmol) in 0.1 M $MgCl_2$ (0.6 mL) was added to a mixture of 0.05 M triethanolamine hydrochloride (2 mL, pH 7.0), 0.1 M $MgCl_2$ (1.5 mL), and 4 M KCl (0.5 mL). Crystalline pyruvate kinase (Boehringer Co.; 1 mg) was added. The solution was stored at 22 °C for 16 h and then heated in a steam bath for 3 min. Precipitated protein was removed by centrifugation and extracted once with water. The combined aqueous solutions were diluted to 200 mL with water, after which **4a** was purified by method I. Properties of **4a** are given in Table I.

8-[(L-1-Amino-1-carboxypropyl)thio]adenosine 5′-Triphosphate (4c). Tetralithium 8-bromoadenosine 5′-triphosphate[27] (0.2 mmol) and disodium L-homocysteine[28] (1 mmol) were condensed under conditions previously used in the synthesis of other 8-(alkylthio)-ATP derivatives[14] to give tetrasodium **4c** as a white powder after purification by method I. The product gave a positive ninhydrin test. Other properties are listed in Table I.

Adenosine 5′-Triphosphate γ-Amidate (13). Dry NH_3 was bubbled into a solution of tri-*n*-butylammonium adenosine 5′-trimetaphosphate[13] (1 mmol) in DMF (5 mL) at −20 °C. After 15 min, addition of NH_3 was stopped, and the mixture was kept 15 min at room temperature. The residue obtained upon evaporation of volatiles in vacuo at 35 °C was homogeneous in paper chromatographic systems C–E. Purification of the product by method I gave the trisodium salt of **13** as a white powder. Properties of **13** are given in Table I.

Enzyme Kinetic Studies. Dextran (industrial grade, mol wt 15000–20000) was obtained from the Sigma Chemical Co. Dialysis tubing (Spectrapor, mol wt cutoff 12000) and a collodion bag concentrator–dialyzer apparatus (mol wt cutoff 25000) were purchased from A. H. Thomas Co. Novikoff ascites rat hepatoma cells were provided by Dr. Sidney Weinhouse, Fels Research Institute, Temple University, Philadelphia, PA, and were propagated in female Sprague–Dawley rats (150–175 g) purchased from the Holtzman Co., Madison, WI. After 6–7 days, host rats were sacrificed by diethyl ether inhalation. The peritoneal fluid was transferred to a receiver cooled in ice and diluted with 3 volumes of cold (2 °C) Dulbecco's phosphate buffered saline (PBS) (0.90 mM $CaCl_2$, 2.68 mM KCl, 0.49 mM $MgCl_2$, 0.137 M NaCl, 1.47 mM KH_2PO_4, 8.06 mM Na_2HPO_4). Subsequent operations were conducted at 2–4 °C. Novikoff hepatoma cells were separated from erythrocytes by low-speed centrifugation. The supernatent, which contained the erythrocytes, was decanted off. To the residual slurry of hepatoma cells was added an equal volume of cold PBS solution. The cell line was propagated by intraperitoneal injection of 2 mL of this supension into each of several recipient rats. The remaining suspension was sedimented by low-speed centrifugation, and the tumor cells were washed again with PBS solution by centrifugation to remove final traces of erythrocytes. A cytosol extract of the packed tumor cells (1–10 mL) was prepared as described by Liau et al.[6] and was chromatographed on a column of DEAE-cellulose as described by Liau et al.[7] Fractions containing MAT activity derived from 1.5–2.0 mL of cells were placed in a bag of dialysis tubing. All such bags were immersed in dextran powder until the volume was reduced to ca. 0.5 mL/mL of cells extracted. The above operations were carried out in a single day. The MAT-T preparations were combined and dialyzed overnight against 0.05 M Tris·HCl (pH 7.8)–50 mM KCl–5 mM $MgCl_2$–5 mM $HS(CH_2)_2OH$ and then concentrated (with simultaneous dialysis against the same mixture) with a Selectron collodion bag apparatus to a final volume of ca. 0.3 mL/mL of cells extracted. This stock solution was stored at −20 °C. Portions were diluted 10-fold in the above buffer to provide a working solution; the activity of this preparation remained constant for 1 day at 0 °C. Under the conditions given below, K_M^{Met} was 15 μM (lit.[6] 21 μM), and K_M^{ATP} was 0.14 mM (lit.[6] 0.14 mM).

A preparation of MAT-I free of MAT-II and MAT-III was obtained from freshly obtained rat liver as described by Hoffman[23] and was concentrated in a collodion bag apparatus to a volume of 0.17 mL/g of liver extracted and stored at −20 °C in 100-μL portions. A working solution was prepared by diluting the stock solution 5-fold with 150 mM KCl–5 mM $MgSO_4$–2 mM dithiothreitol–25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) adjusted to pH 7.5 with KOH. K_M^{Met} was 55 μM (lit.[23] 42 μM); K_M^{ATP} was 0.14 mM (lit.[6] 0.14 mM; lit.[29] 0.15 mM).

Preparations of MAT-II were obtained from rat kidney. Frozen kidneys (Rockland Inc., Gilbertsville, PA) were thawed at 4 °C for 30 min in the appropriate buffer, and a tissue extract was prepared[4] and subjected to DEAE chromatography[7] according to Liau et al. Enzymatically active fractions were combined and dialyzed in a collodion bag apparatus against 0.05 M Tris·HCl (pH 7.8)–50 mM KCl–5mM $MgCl_2$–5mM $HS(CH_2)_2OH$ with simultaneous concentration to a final volume of 0.35 mL/g of kidney extracted. This stock solution was stored at −20 °C in 50-μL portions. A working solution was prepared daily by diluting the stock solution 10-fold with the above buffer. K_M^{Met} was 5 μM (lit.[7] 3.6 μM; lit.[4] 3.1 μM); K_M^{ATP} was 0.14 mM (lit.[5] 0.07 mM at pH 9.0 in the presence of 250 μM L-methionine; lit.[6] 0.14 mM (mixture of MAT-I and MAT-II)).

Enzyme assays were conducted at 37 °C for 30 min in a final volume of 0.1 mL. Each mixture was made in duplicate. MAT-I was studied in 150 mM KCl–7.5 mM $MgSO_4$–2 mM dithiothreitol–50 mM HEPES buffer, pH 7.5;[23] and MAT-II and MAT-T were studied in 150 mM KCl–15 mM $MgCl_2$–5 mM dithiothreitol–50 mM Tris·HCl, pH 8.2.[6] (The activities of MAT-I and MAT-II are relatively constant in the range pH 7.5–9.3) L-[*methyl*-^{14}C]Methionine (New England Nuclear Co.; 54 Ci/mol)

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and Mg-ATP were included at the levels specified below. Reactions were started by addition of 10 μL of working enzyme solution and terminated by addition of 10 μL of 4 N $HClO_4$–10 mM-L-methionine with cooling in an ice bath. Each suspension was centrifuged, and 50 μL of supernatent was applied to a 2.3-cm disk of phosphocellulose paper. Disks were washed as described[23] and then immersed in a toluene solution of phosphors and counted in a Packard liquid scintillation spectrometer (Model 2425). Blanks were provided by incubations carried out in the absence of ATP. Reaction velocities were linear for at least 30 min and were proportional to the amount of enzyme added at the levels of enzyme activity employed.

Substrate constants were determined from double-reciprocal plots of velocity vs. substrate concentration; all plots were linear. K_M values reported for methionine were determined at 5 mM ATP for MAT-I (18.8–112.8 μM [^{14}C]methionine) and 2 mM ATP for MAT II (2.1–21 μM [^{14}C]methionine) and MAT-T (10.5–63 μM [^{14}C]methionine). Substrate constants for ATP as well as substrate and inhibition constants for ATP analogues were determined in the presence of 380 μM [^{14}C]methionine for MAT-I and 60 μM [^{14}C]methionine for MAT-II and MAT-T. These methionine levels were noninhibitory. All ATP analogues were tested for substrate activity at a level of 1 mM, using a higher level of MAT (10 μL of stock solution) per assay than in the remaining studies.

Inhibition studies were made with six to eight levels of Mg-ATP in the range 0.5–4.0 × K_M of Mg-ATP for each of two inhibitor levels that were in the range 1–5 × K_i and for control mixtures lacking inhibitor. Inhibitors were tested as their 1:1 complexes formed by admixture of stock solutions with equimolar amounts of $MgCl_2$. Inhibition constants (K_i values) were obtained from replots of inhibitor concentrations vs. slopes of double-reciprocal plots of velocity vs. substrate level. All of the latter plots were linear.

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Influence of Alkyl-Chain Fluorination on the Action of Mammary Tumor Inhibiting 2,3-Bis(hydroxyphenyl)butanes and 2,3-Bis(hydroxyphenyl)but-2-enes

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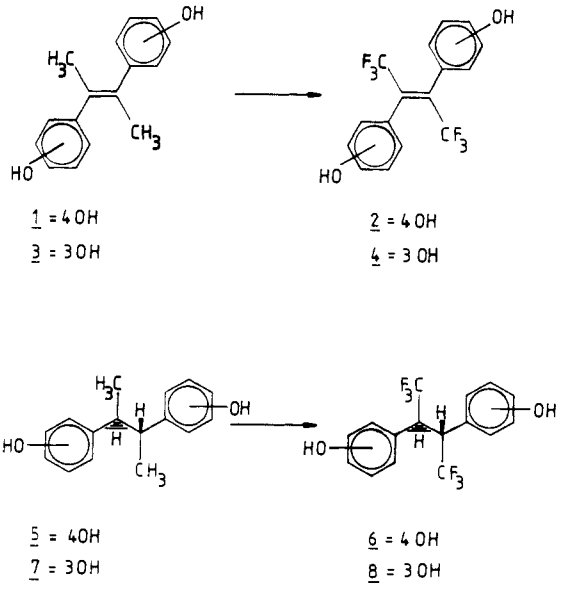
trans-1,2-Bis(trifluoromethyl)-1,2-bis(4- and 3-hydroxyphenyl)ethenes **2** and **4** were prepared by reductive coupling ($TiCl_4$/Zn/pyridine) of the methoxy-substituted α,α,α-trifluoroacetophenones, separation of the resulting *cis*- and *trans*-stilbene derivatives, and ether cleavage with BBr_3. The *cis*-stilbenes were catalytically hydrogenated to give *meso*-1,1,1,4,4,4-hexafluoro-2,3-bis(4- and 3-hydroxyphenyl)butanes **6** and **8**. Compounds **2**, **4**, **6**, and **8** showed 2- to 10-fold increased binding affinities for the estradiol receptor (E_2R) and enhanced estrogenicity in the uterine weight test of the immature mouse compared to their unfluorinated analogues. Compound **8** exhibited a 46% inhibition of the estrone-stimulated uterine growth. Antitumor activity was evaluated with use of the transplantable, hormone-dependent MXT mammary tumor of the $BD2F_1$ mouse. All compounds showed tumor growth inhibitory activity corresponding to their RBA values. The most interesting compound **8** led to a significant inhibition of the tumor growth on the DMBA-induced hormone-dependent mammary carcinoma of the Sprague–Dawley rat.

Searching for new mammary tumor inhibiting antiestrogens, we recently found that modification of the synthetic estrogens hexestrol and diethylstilbestrol by displacement of the phenolic hydroxy groups into the 3,3′-positions provided compounds with strongly decreased estrogenicity, marked antiestrogenicity, and strong inhibitory activity on the established 9,10-dimethyl-1,2-benzanthracene (DMBA) induced mammary carcinoma of the Sprague–Dawley (SD) rat.[1-3] Replacement of the ethyl groups by methyl substituents resulted in two compounds totally lacking estrogenic activity (compounds **3** and **7**, Chart I).[1,3,4] Shortening of the alkyl chain, however, led to a reduction of the estradiol receptor (E_2R) affinity[1,3,4] and thus to a decrease of mammary tumor inhibiting activity.[1,3]

In a structure–activity study dealing with the influence of an aromatical disubstitution of 1,2-diphenylethane estrogens and antiestrogens on their biological properties, we have shown that it is possible to raise the binding affinity and the antitumor activity of 1,2-diphenylethanes by placing substituents ortho to the ethane bridge.[4-7] All

Chart I



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