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Structural and Conformational Analogues of L-Methionine as Inhibitors of the Enzymatic Synthesis of S-Adenosyl-L-methionine

I. Saturated and Unsaturated Aliphatic Amino Acids

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SUMMARY

COULTER, A. W., LOMBARDINI, J. B., AND TALALAY, PAUL: Structural and conformational analogues of L-methionine as inhibitors of the enzymatic synthesis of *S*-adenosyl-L-methionine. I. Saturated and unsaturated aliphatic amino acids. *Mol. Pharmacol.* **10,** 293-304 (1974).

Aliphatic amino acid analogues of L-methionine that inhibit the enzymatic synthesis of *S*-adenosyl-L-methionine have been designed on the basis of structural, conformational, and electronic considerations. The inhibitory activity of these compounds has been evaluated with partially purified preparations of ATP:L-methionine *S*-adenosyltransferase (EC 2.5.1.6) obtained from bakers' yeast, *Escherichia coli*, and rat liver. The effects of variation in length and branching of carbon chain, steric configuration, degree or position of unsaturation, and the introduction of chloro groups have been analyzed in an effort to deduce the most favorable features for inhibition. Within this class of compounds, 2-amino-4-hexynoic acid, (*E*)-2-amino-*trans*-4-hexenoic acid, and (*Z*)-2-amino-5-chloro-*trans*-4-hexenoic acid are among the most powerful inhibitors synthesized. In contrast, (*Z*)-2-amino-*cis*-4-hexenoic acid and (*E*)-2-amino-5-chloro-*cis*-4-hexenoic acid are weak inhibitors or are inactive. The activity of the more powerful inhibitors appears to reside exclusively in the L isomers. (*Z*)-L-2-Amino-5-chloro-*trans*-4-hexenoic acid displays considerably greater specificity for the inhibition of rat liver enzyme (I_{50} = 0.55 mM) than for the yeast (I_{50} = 3.0 mM) or *E. coli* (I_{50} = 4.2 mM) adenosyltransferase. Examination of molecular models reveals a close similarity in the size, shape, and molecular contour between an extended conformation of L-methionine and L-2-amino-4-hexynoic acid and (*Z*)-L-2-amino-5-chloro-*trans*-4-hexenoic acid. Another compound with significant inhibitory activity is (2*S*, 4*S*)-2-amino-4,5-methylene-5-hexenoic acid (hypoglycin A).

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INTRODUCTION

The central role of *S*-adenosyl-L-methionine in transmethylation reactions (1, 2), in

the synthesis of polyamines (3), and in a number of regulatory metabolic roles (4) has prompted a detailed study of the control of the synthesis of *S*-adenosyl-L-methionine by ATP:L-methionine *S*-adenosyltransferase (EC 2.5.1.6) in various species and tissues, and a search for inhibitors of this reaction. Earlier studies from this laboratory (5) have delineated the relatively narrow substrate specificity of this enzyme, and have uncovered three basic types of amino acids which inhibit the adenosyltransferase[2] reaction in competition with L-methionine but differ with respect to their steric, electronic, and conformational relationships to L-methionine. These inhibitory compounds include (a) saturated and unsaturated aliphatic amino acids endowed with appropriate conformational rigidity and critically placed regions of unsaturation, such as 2-amino-*trans*-4-hexenoic acid (IV) and L-2-amino-4-hexynoic acid (XIVb); (b) derivatives of serine and cysteine, such as *O*-acetyl-L-serine, *O*-carbamyl-L-serine, and *S*-carbamyl-L-cysteine; and (c) carbocyclic amino acids, such as 1-aminocyclopentane-1-carboxylic acid. These findings have led to deductions concerning the probable conformation of L-methionine at the active site of the adenosyltransferase. In the present group of papers, the topography of the active site has been mapped in further detail by means of a series of additional inhibitors that fall into three structural classes: aliphatic amino acids, aromatic amino acids (6), and carbocyclic and heterocyclic amino acids (7). The effects of the administration of representative inhibitors of these types on tissue levels of *S*-adenosyl-L-methionine and L-methionine in rats and mice have been the subject of a detailed recent study (8).

[2] The abbreviations used are: adenosyltransferase, ATP:L-methionine *S*-adenosyltransferase (EC 2.5.1.6); *cis*- and *trans*-crotylglycine, (*Z*)-2-amino-*cis*-4-hexenoic acid and (*E*)-2-amino-*trans*-4-hexenoic acid, respectively. The expression pI_{50} is used to denote the value of $\log_{10}(1/I_{50})$ for a given inhibitor, where the I_{50} value refers to the molar concentration of inhibitor required to produce 50% inhibition of enzyme activity under conditions specified in the text. It should be noted that most I_{50} values in the text are expressed in millimolar concentrations, whereas the pI_{50} values refer to *molar* concentrations.

The enzymatic synthesis of *S*-adenosyl-L-methionine involves the transfer of the adenosyl moiety of ATP to the sulfur atom of L-methionine with the formation of a positively charged, high-energy sulfonium derivative and the release of inorganic phosphate and pyrophosphate, according to the following stoichiometry (9):

$$\text{L-Methionine} + \text{ATP} \xrightarrow{K^+,\ Mg^{2+}} S\text{-adenosyl-L-methionine} + PP_i + P_i$$

The adenosyltransferase also displays an intrinsic hydrolytic activity that promotes the cleavage of enzyme-bound or exogenous tripolyphosphate to inorganic phosphate and pyrophosphate (9). The interesting mechanism of this reaction has been the object of intensive studies, which have been reviewed by Mudd (9), and to which more recent information has been added by Greene (10), by Chou and Talalay (11), and by Lombardini, Chou, and Talalay (12). Unusual kinetic complexities have been encountered in these studies. Double-reciprocal plots of the reaction velocity of yeast, rat liver, and *Escherichia coli* adenosyltransferases as a function of L-methionine concentrations (under saturating ATP conditions) demonstrate downward deflections from linearity for yeast and *E. coli* adenosyltransferases and an upward deflection for the rat liver enzyme (11, 12). Moreover, the liver enzyme differs in a unique manner from other mammalian and microbial enzymes examined, in that the kinetics is sigmoidal with respect to L-methionine, and nonsubstrate analogues of L-methionine at low concentration stimulate the synthesis of *S*-adenosyl-L-methionine by obliterating these sigmoidal relations (12). These kinetic anomalies preclude accurate determinations of Michaelis constant (K_m) values for the substrates or of K_i values for inhibitors which are independent of substrate concentrations. The potencies of inhibitors have consequently been expressed as concentrations required to reduce the velocity to 50% (I_{50} values) at a specified concentration of L-methionine (37.5 μM) under precisely defined reaction conditions (5). When Michaelis-Menten kinetics is obeyed, the relation between fractional inhibition values (such as I_{50}) and the inhibition constant (K_i) depends upon the reaction

mechanism, as recently shown by Chou (13). In the present case an analysis of this type is not possible, and kinetic interpretations of inhibitory potencies are precluded. Nevertheless, the I_{50} values obtained under standard conditions are highly reproducible, and apparent Michaelis-Menten kinetics is observed over even quite extended concentration ranges (7) (see Fig. 1).

In addition, it has been convenient to quantitate inhibitory potency in terms of an inhibition index, pI_{50}, which is the negative logarithm of the I_{50} value expressed in *molar* concentrations in order to obtain values of convenient magnitude and avoid negative numbers. Thus I_{50} values of 100, 10, 1, and 0.1 mM correspond to pI_{50} values of 1, 2, 3, and 4, respectively. The advantages of the use of pI_{50} values rather than I_{50} measurements is that the former relate directly (rather than reciprocally) to the inhibitory potencies, and in some instances relatively constant increments in pI_{50} values can be ascribed to specific structural changes.

EXPERIMENTAL PROCEDURE

Materials

All solutions were prepared in deionized, glass-distilled water from chemicals of the best commercial grades available.

Cellulose phosphate ion exchange paper discs (23-mm diameter, type P 81) were purchased from Reeve Angel, Clifton, N. J. Adenosine 5′-triphosphate (disodium) was purchased from P-L Biochemicals.

The primary and secondary scintillators PPO (2,5-diphenyloxazole) and POPOP {*p*-bis[2-(5-phenyloxazolyl)]benzene} were purchased from New England Nuclear. Spectroscopic quality *p*-dioxane and glycerol and reagent grade naphthalene were purchased from Matheson, Coleman, and Bell. L-[*methyl*-^{14}C]Methionine (53.6 mCi/mmole) and [8-^{14}C]adenosine 5′-triphosphate tetralithium (47 mCi/mmole) were the products of Amersham/Searle Corporation and Schwarz/Mann Research Laboratories, respectively. Crystallized bovine plasma albumin was purchased from Armour Pharmaceutical Company.

Amino Acid Analogues

The structures of all amino acid analogues are represented in Table 1. Roman numerals refer to the structures shown in this table. The geometric configurations of olefinic compounds are designated as *cis* or *trans* in accordance with the orientation of the longest carbon chain. If the double bond is terminal and carries a halogen substituent, *cis* and *trans* designations are used to relate the orientation of the substituent to the longest carbon chain. However, in order to avoid any ambiguity, the compounds are further designated as *E* (*entgegen*) and *Z* (*zusammen*) according to more recent proposals for nomenclature (14).

Commercial. L-Methionine, L-norleucine (I), and D- and L-norvaline (VII) were supplied by Schwarz/Mann Research Laboratories. D-Norleucine (I) was purchased from Cyclo Chemical Corporation.

Gifts. L-2-Amino-3-methylenehexanoic acid (II) (15) was a gift of Dr. G. Dardenne, Faculté des Sciences Agronomiques de l'Etat, Gembloux, Belgium. DL-2-Amino-5-methylhexanoic acid (III) (16) and (*E*)-DL-2-amino-4-methyl-*trans*-4-hexenoic acid (VI) (16) were gifts of Dr. W. Shive, University of Texas, Austin. The latter compound was derived from tiglaldehyde, and is presumably the *E* isomer. Synthetic DL-2-amino-4,5-hexadienoic acid (XVI) (17, 18) was a gift of Dr. W. S. Chilton, University of Washington, Seattle. Hypoglycin A (2*S*,4*S*-2-amino-4,5-methylene-5-hexenoic acid, XVIII) (17), (2*S*,3*R*-2-amino-3,4-methylene-5-pentenoic acid, XVII) (19, 20), and 2-amino-4-methyl-5-hexynoic acid (XIX, tentatively assigned the 2*S*,4*R* configuration) (21) were natural products and were gifts of Professor L. Fowden, University College, London.

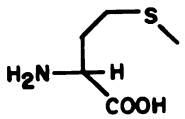
Synthetic. DL- and L-2-Amino-4-hexynoic acids were synthesized according to procedures developed in this laboratory (22), and by A. W. Coulter and J. Salt.[3] The *cis*-(*Z*) and *trans*-(*E*) isomers of DL-2-amino-4-hexenoic acid have been previously described (22, 23).

The *cis* isomer (V) was prepared by Lindlar catalyst reduction of ethyl 2-acetamido-2-carbethoxy-4-hexynoate, followed by hydrolysis and decarboxylation (22). The *trans* isomer (IV) was prepared by selective chromous reduction of DL-2-amino-4-hexy-

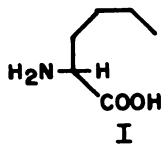
[3] A. W. Coulter and J. Salt, unpublished procedure.

noic acid, under solvent conditions favoring *trans* reduction.[3] The geometrical isomers (IV and V) are obtained in pure form by high-resolution ion-exchange chromatography. When chromatographed under the specified conditions (22) with appropriate standards, the times of elution of these amino acids were as follows: cysteine, 500

TABLE 1



L-METHIONINE



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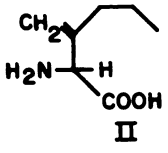

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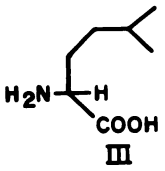

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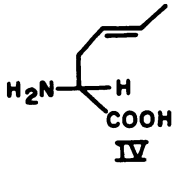

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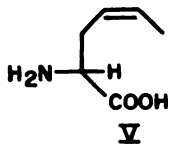

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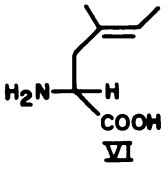

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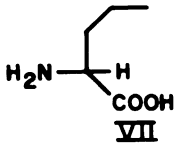

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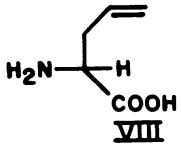

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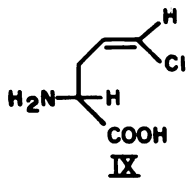

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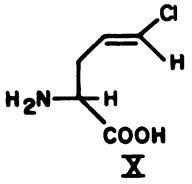

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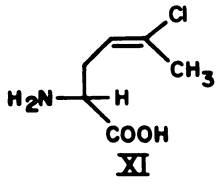

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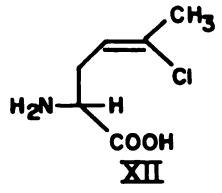

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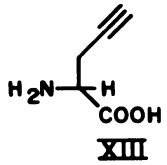

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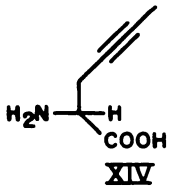

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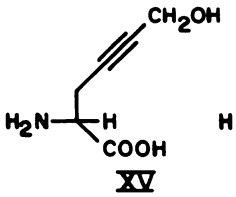

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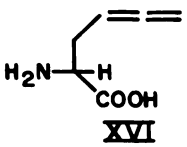
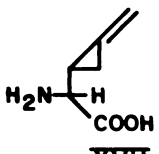


XVI



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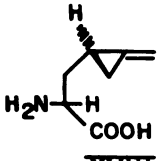
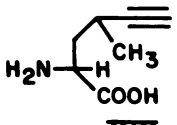


XVIII



XIX

min; methionine, 522 min; V, 549 min; IV, 563 min; isoleucine, 594 min; leucine, 617 min.

DL-2-Amino-4-pentenoic acid (VIII) was prepared according to Goering *et al.* (24). The geometrical isomers of DL-2-amino-5-chloro-4-pentenoic acid (IX and X) were prepared by a modification of the procedures described (25–27). Condensation of (*Z*)-1,3-dichloropropene and (*E*)-1,3-dichloropropene with diethyl acetamidomalonate furnished (*Z*)-ethyl 2-acetamido-2-carbethoxy-5-chloro-4-pentenoate (mp 55–56°) and the (*E*)-isomer (mp 69–71°), respectively. Hydrolysis was accomplished by refluxing for 4 hr in 6 N HCl. The amino acids were isolated by large-scale ion-exchange chromatography, characterized by NMR and mass spectral data, and analyzed with a single column (0.6 × 140 cm) automatic amino acid analyzer, using Bio-Rad Aminex Q-15 S resin. The column was operated at 60° at a flow rate of 39 ml/hr. Elution of the amino acids was carried out with a nine-chamber variable-gradient device, using citrate buffers of pH 2.88–5.00 (22). Under these operating conditions IX and X were chromatographed with appropriate standards and the times of their elution were as follows: valine, 363 min; cysteine, 400 min; X, 406 min; IX, 413 min; methionine, 426 min.

The geometrical isomers of DL-2-amino-5-chloro-4-hexenoic acid (XI and XII) were prepared by condensing diethyl acetamidomalonate with 1,3-dichloro-2-butene to give a mixture of (*E*)- and (*Z*)-ethyl 2-acetamido-2-carbethoxy-5-chloro-4-hexenoate (27). These isomers were separated by chromatography on a dry silica gel column to afford pure *E* (*cis*) (mp 62–62.5°) and *Z* (*trans*) (mp 75–76°) adducts, the stereochemical configurations being assigned after the determination of the structure of XI and XII by NMR experiments. The diesters were hydrolyzed and decarboxylated to (*Z*)-2-acetamido-5-chloro-*trans*-4-hexenoic acid (mp 113–114°; when sublimed it melted at 140°) and (*E*)-2-acetamido-5-chloro-*cis*-4-hexenoic acid (mp 124–125°). Base hydrolysis afforded XIa and XIIa, while acylase I hydrolysis yielded XIb and XIIb. All intermediates and products were characterized by infrared, NMR, and mass spectrometry, and by elemental analyses. Under the above operating conditions for the amino acid analyzer, compounds XI and XII were chromatographed with appropriate standards, and the times of their elution were as follows: methionine, 405 min; isoleucine, 472 min; XI, 482 min; leucine, 490 min; XII, 505 min; tyrosine, 545 min. The assumed geometric stereochemistry of the pure amino acids was unequivocally confirmed by observation of a clear-cut nuclear Overhauser effect in the case of XII and its absence in the case of XI in the 60-MHz NMR spectrometer. Compound XII has also been isolated and synthesized by Chilton and Tsou (28) from *Amanita solaria*, but not rigorously characterized with respect to stereochemistry.

L-2-Amino-6-hydroxy-4-hexynoic acid was prepared by alkylation of diethyl acetamidomalonate with 1,4-dichloro-2-butyne according to Jansen *et al.* (29). Base hydrolysis and decarboxylation resulted in an oil (the *N*-acetyl derivative of DL-XV), which failed to crystallize. The oil was hydrolyzed with hog kidney acylase I in the usual manner (30) to afford L-XV, decomposing above 250°; infrared spectrum (KBr) 3415, 3060–2400, 2250, 2000, 1658, 1587, 1505, 1031 cm^{-1}; NMR (D_2O, external trimethylsilane) δ 2.88 (d of t, 2, J = 5.5, 2 Hz C≡C—$C\underline{H}_2$—), 3.90 (t, 1, J = 5.5 Hz —$C\underline{H}$—COO^-), 4.22 (t, 2, J = 2 Hz O—$C\underline{H}_2$—C≡C—); mass spectrum (70 eV) *m/e* (relative intensity) 143 (0.15), 126 (2.5), 99 (23), 98 (37), 80 (19), 75 (60), 74 (100), 53 (31), 52 (35).

$C_6H_9NO_3$

Calculated: C 50.34, H 6.34, N 9.79, O 33.53
Found: C 50.34, H 6.27, N 9.97, O 33.36

Under the designated operating conditions for the amino acid analyzer, the elution times of XV when chromatographed with appropriate standards were: aspartic acid, 96 min; XV, 105 min; serine, 116 min; glutamic acid, 133 min.

Methods

Preparation of methionine adenosyltransferase. Procedures for purification of the

yeast, *E. coli*, and rat liver ATP:L-methionine *S*-adenosyltransferases have been described (5). The specific activities [micromoles of *S*-adenosyl-L-methionine synthesized per milligram of protein per 30 min of incubation at 37° under specified conditions (5)] of the enzyme preparations used in these studies were as follows: yeast, 4.7; *E. coli*, 4.2; rat liver, 6.2. The reaction system for determination of specific activities of the enzymes was the same as used for inhibitor studies (below) except that the L-methionine concentration was 2 mM.

Enzymatic assays and determination of inhibitory potency. The assay conditions used for inhibition studies were described by Mudd *et al.* (31) and modified by Lombardini *et al.* (5). The reaction system contained the following in a final volume of 0.1 ml (in micromoles): KCl, 20; $MgCl_2$, 30; glutathione, 0.8; Tris-HCl, pH 7.6, 16; L-[*methyl*-^{14}C]methionine (350,000 cpm), 0.00375; ATP, 2; inhibitor in varied amounts; and enzyme which was used to initiate the reaction. Linearity between velocity of reaction (formation of *S*-adenosyl-L-methionine) as a function of time or protein concentration was established, and optimal conditions for the three isofunctional enzymes were determined (5): yeast, 10 min, 2.4 μg of protein; *E. coli*, 30 min, 8.4 μg of protein; rat liver, 6 min, 9.4 μg of protein. By utilizing very low levels of L-methionine in the incubation system (37.5 μM final concentration) the ratio of inhibitor to substrate (L-methionine) could be made very large, thereby magnifying the inhibitory potency of weak inhibitors. In this system, saturating concentrations of L-methionine produce maximum velocities. The potencies of the analogue inhibitors were calculated by the method of Dixon (32), which involves a graphical representation of the reciprocal of the percentages of control velocity as a function of the inhibitor concentration. In this manner the I_{50} value (the concentration of inhibitor at which the control activity is reduced by 50%) could be readily determined. Linearity of the graphical representation of the data was observed over a wide range of inhibitor concentrations (e.g., Fig. 1, ref. 7), despite the deviations from Michaelis-Menten kinetics discussed above. When the experimentally attainable concentrations of inhibitors did not produce 50% inhibition, I_{50} values for very weak inhibitors were determined by graphical extrapolation. In order to determine whether a methionine analogue was a substrate for the adenosyltransferase reaction, 1 μmole of the amino acid analogue was added to the above assay system in place of L-methionine and the quantity of ATP was reduced to 0.5 μmole of [8-^{14}C]-ATP (containing 300,000 cpm). Velocities were compared with those observed with 1 μmole of L-methionine.

The incubations were carried out in glass-stoppered tubes at 37° with agitation. For some of the measurements the reaction was terminated as previously described (5) and the radioactive *S*-adenosyl-L-methionine was separated from L-methionine on ion-exchange columns. For the majority of the measurements reported in this paper, a new and simplified method has been developed (33) for the separation of *S*-adenosyl-L-methionine from L-methionine on cellulose phosphate cation-exchange discs, on which an aliquot (50 μl) of the incubation mixture is dried, washed with a minimum of 200 ml of distilled water on a filtration apparatus (to remove L-[*methyl*-^{14}C]methionine), and then directly placed in a scintillation vial containing 7 ml of Bray's (34) scintillation fluid. Radioactivity was determined with a liquid scintillation spectrometer with an efficiency for ^{14}C of about 70%. This assay system is extremely sensitive and capable of measuring 0.05 nmole of *S*-adenosyl-L-methionine.

For the inhibition studies the enzyme preparations were diluted with bovine serum albumin (2 mg/ml, pH 7.0) containing 20% glycerol in order to stabilize the dilute enzyme preparations.

RESULTS AND DISCUSSION

Quantitation of inhibitory activities. The structures of the aliphatic amino acid analogues involved in these studies are shown in Table 1, and their inhibitory potencies for partially purified isofunctional adenosyltransferases of yeast, *E. coli*, and rat liver are presented in Table 2 in terms of both I_{50} and pI_{50} values. A few of the I_{50} values

TABLE 2

Inhibitory potencies of aliphatic analogues of L-*methionine on ATP:*L-*methionine adenosyltransferases of yeast, E. coli, and rat liver*

The enzyme activity was measured at 37.5 μM L-methionine, and the inhibitory potency was determined as described under EXPERIMENTAL PROCEDURE.

Compound No.	Compound	Concentration required for 50% inhibition			Inhibition index (pI_{50})		
		Yeast	*E. coli*	Liver	Yeast	*E. coli*	Liver
		mM	mM	mM			
Ia	L-2-Aminohexanoic acid (L-norleucine)	56[a]	38[a]	23[a]	1.25	1.42	1.64
Ib	D-2-Aminohexanoic acid (D-norleucine)	170[a]	118[a]	310[a]	0.77	0.93	0.51
II	L-2-Amino-3-methylenehexanoic acid	146[a]	26	63[a]	0.84	1.59	1.20
III	DL-2-Amino-5-methylhexanoic acid	210[a]	190[a]	I[b]	0.68	0.72	
IV	(*E*)-DL-2-Amino-*trans*-4-hexenoic acid (*trans*-crotylglycine)	11	12.5	6.5	1.96	1.90	2.19
V	(*Z*)-DL-2-Amino-*cis*-4-hexenoic acid (*cis*-crotylglycine)	170[a]	55[a]	80[a]	0.77	1.26	1.10
VI	(*E*)-DL-2-Amino-4-methyl-*trans*-4-hexenoic acid	275[a]	80[a]	165[a]	0.56	1.10	0.78
VIIa	L-2-Aminopentanoic acid (L-norvaline)	144[a]	45[a]	124[a]	0.84	1.35	0.91
VIIb	D-2-Aminopentanoic acid (D-norvaline)	122[a]	45[a]	76[a]	0.91	1.35	1.21
VIII	DL-2-Amino-4-pentenoic acid	110[a]	27[a]	65[a]	0.96	1.57	1.19
IX	(*Z*)-DL-2-Amino-5-chloro-*cis*-4-pentenoic acid	113[a]	55[a]	48[a]	0.95	1.26	1.32
X	(*E*)-DL-2-Amino-5-chloro-*trans*-4-pentenoic acid	37	40	13	1.43	1.40	1.89
XIa	(*E*)-DL-2-Amino-5-chloro-*cis*-4-hexenoic acid	I[b]	I[b]	I[b]			
XIb	(*E*)-L-2-Amino-5-chloro-*cis*-4-hexenoic acid	I[b]	I[b]	I[b]			
XIIa	(*Z*)-DL-2-Amino-5-chloro-*trans*-4-hexenoic acid	5.15	8.0	1.0	2.28	2.09	3.00
XIIb	(*Z*)-L-2-Amino-5-chloro-*trans*-4-hexenoic acid	3.0	4.2	0.55	2.52	2.38	3.26
XIII	L-2-Amino-4-pentynoic acid	26	9.0	20.5	1.59	2.05	1.69
XIVa	DL-2-Amino-4-hexynoic acid	4.7	6.6	3.7	2.33	2.18	2.43
XIVb	L-2-Amino-4-hexynoic acid	2.2	3.1	1.5	2.66	2.51	2.82
XV	L-2-Amino-6-hydroxy-4-hexynoic acid	77[a]	I[b]	87[a]	1.11		1.06
XVI	DL-2-Amino-4,5-hexadienoic acid	33[a]	23	12.5	1.48	1.64	1.90
XVII	(2*S*,3*R*)-2-Amino-3,4-methylene-4-pentenoic acid	75[a]	25	45[a]	1.12	1.60	1.35
XVIII	(2*S*,4*S*)-2-Amino-4,5-methylene-5-hexenoic acid (hypoglycin A)	6.5	9.6	7.1	2.19	2.02	2.15
XIX	(?2*S*,?4*R*)-2-Amino-4-methyl-5-hexynoic acid	I[b]	90[a]	I[b]		1.04	

[a] These values were obtained by graphical extrapolation, and are not bracketed by experimental observations, since the range of concentrations employed was limited by solubility.

[b] The designation I, for inactive, indicates that at the concentrations of compounds that were attainable less than 10% inhibition was observed. The highest concentrations tested were: III, 20 mM; XIa, 35 mM; XIb, 17.5 mM; XV, 35 mM; XIX, 35 mM.

have been reported earlier (5) and are included in Table 2 for purposes of comparison and completeness of discussion. In order to obtain some indication of the utility of the derived inhibition index (pI_{50}), we have examined its consistency in the three enzymatic systems (Fig. 1). In this illustration the successive effects on the inhibition index of converting DL-2-aminopentanoic acid (VII) to DL-2-amino-4-pentenoic acid (VIII) and to DL-2-amino-4-pentynoic acid (XIII) have been compared. The first change produces a mean increase in pI_{50} of 0.15 unit (range, 0.08–0.22 unit in the three systems), and the second (from DL-VIII to DL-XIII), a mean increase in pI_{50} of 0.23 unit (range, 0.17–0.32 unit) (Fig. 1). Thus, although the absolute magnitudes of the pI_{50} values vary considerably, the reasonably parallel lines obtained suggest that the same structural alterations tend to produce similar changes in pI_{50} values in each enzyme system.

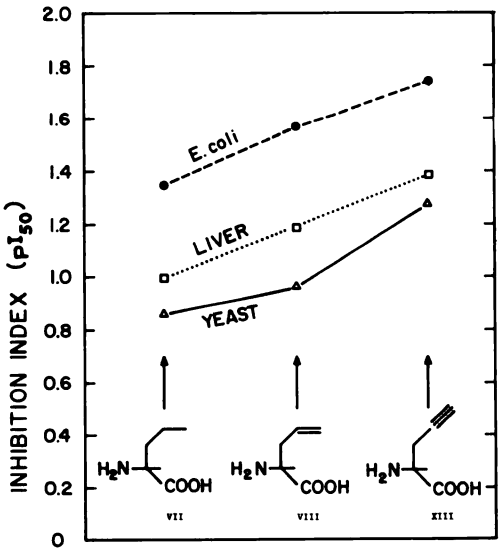


FIG. 1. *Effects of progressive introduction of unsaturation in aliphatic amino acids on inhibition index (pI_{50}) for ATP:L-methionine S-adenosyltransferase of yeast, E. coli, and rat liver*

The graph shows the effect of conversion of DL-norvaline (VII) to DL-2-amino-4-pentenoic acid (VIII) and DL-2-amino-4-pentynoic acid (XIII). The inhibition index for DL-XIII was calculated from twice the I_{50} values for L-XIII, and that for DL-VII, from those of L-VII and D-VII by the formula (DL-I_{50}) = 2 (D-I_{50}) (L-I_{50})/(D-I_{50} + L-I_{50}) (35).

Length of carbon chain. Norvaline (VIIa and VIIb) is an extremely weak inhibitor of the adenosyltransferases of yeast and liver, but considerably more active for the *E. coli* enzyme. If we consider L-norvaline (VIIa) specifically, the addition of a terminal methyl group to give L-norleucine (Ia) markedly enhances the inhibitory activity for the yeast and liver enzymes (ΔpI_{50} = 0.4–0.7), but this change has little effect in the *E. coli* system. The positive effect of the terminal methyl group on inhibitory potency is also observed among the olefinic (compare IV and VIII) and the acetylenic (compare XIII and XIVa) analogues. For the yeast and liver enzymes, the addition of a terminal methyl group to the C_5 olefin to give the *E* configuration enhances the pI_{50} value by 1.0 unit, and the analogous methyl group addition to the C_5 acetylene (compare XIII and XIVa) augments the inhibitory strength by 0.7 unit. The effects of these terminal methyl group additions appear to be far less marked for the *E. coli* enzyme. Moreover, it should be noted that all the C_5 compounds are considerably more powerful inhibitors for the *E. coli* enzyme than for the other two enzyme systems.

Configuration of amino group. Among the saturated compounds, there is little difference in the C_5 series between the weak inhibitory powers of the D and L isomers (cf. VIIa and VIIb). However, in the saturated C_6 series, there is a clear-cut preference for the L configuration (cf. Ib and Ia) and the pI_{50} values differ by 0.5–1.1 units, in favor of the L compound. Furthermore, a comparison of the inhibitory activity of the DL- and L-acetylenic C_6 compounds XIVa and XIVb leads to the conclusion that virtually all of the inhibitory power of DL-2-amino-4-hexynoic acid (XIVa) resides in the L isomer (XIVb). This preference for the L configuration at C-2 is consistent with the fact that the enzymes are unable to activate D-methionine and that this isomer has little inhibitory activity (5). In the case of the DL- and L-2-amino-4-chloro-*trans*-4-hexenoic acids (XIIa and XIIb), the entire inhibitory activity of the racemic mixture can also be ascribed to the L isomer. Thus, in all reason-

ably potent inhibitors, the inhibition activity is due to the L isomer.

Effects of unsaturation. Since the *trans*- and *cis*-crotylglycines (IV and V) were only available to us as racemic mixtures, direct comparisons with the L- and D-norleucines (Ia and Ib) are somewhat uncertain. However, if we estimate I_{50} values for DL-I from the I_{50} values for the D and L isomers by the procedure of Schaeffer *et al.* (35), it may be seen that *trans*-crotylglycine (IV) is more powerful as an inhibitor (ΔpI_{50} = +0.66–0.88) than the saturated DL-norleucine (I), whereas DL-*cis*-crotylglycine (V) is probably an equally weak or somewhat poorer inhibitor than DL-norleucine. Thus any advantages imposed by the presence of the olefinic group are counteracted by the presence of the terminal methyl group in the *cis* configuration. The conclusion that L-methionine therefore probably exists in the extended conformation on the surface of the enzyme has been drawn earlier and illustrated with photographs of molecular models (5). The superiority of IV over V as an inhibitor of the yeast adenosyltransferase was first reported by Mudd (9).

The beneficial effect of an olefinic linkage in the C-4 position is also inferred from a comparison of the inhibitory potencies of the C_5 compounds: 2-aminopentanoic acid (VII) and 2-amino-4-pentenoic acid (VIII). If we estimate the inhibitory activity of DL-VII and compare it with DL-VIII, the olefinic C_5 compound has a pI_{50} 0.08–0.22 unit greater than its saturated analogue (Fig. 1). It may be noted that the effect of a C-4 double bond is far greater in the C_6 series than in the C_5 series.

An acetylenic linkage joining C-4 and C-5 introduces even greater inhibitory power than that conferred by a double bond in this position. Thus in the C_6 series L-2-amino-4-hexynoic acid (XIVb) is one of the best inhibitors examined. The acetylenic linkage results in an increase in pI_{50} values of 1.1–1.4 units over the saturated compounds. As already mentioned, the terminal methyl group is important for inhibition (cf. XIVb and L-2-amino-4-pentynoic acid, XIII). If this methyl function is hydroxylated, as in L-2-amino-6-hydroxy-4-hexynoic acid (XV), the inhibitory potency is dramatically lowered. The relative greater effectiveness of the terminal acetylenic linkage, in contrast to the terminal olefin, may also be seen by comparing the inhibitory potencies in the C_5 series (cf. VII and VIII with XIII).

The presence and orientation of the terminal methyl group among C_6 compounds appears to be of considerable importance. The DL-2-amino-4,5-hexadienoic acid (XVI) is a somewhat poorer inhibitor than DL-*trans*-crotylglycine (IV), and XVI is a much better inhibitor (ΔpI_{50} = 0.4–0.9 unit) than DL-*cis*-crotylglycine (V). The linear allenic system places the terminal carbon atom in a position intermediate to that assumed by the corresponding group in IV and V. These differences are most marked for the yeast and liver enzymes and less prominent for the *E. coli* enzyme.

Branching of carbon skeleton. The introduction of a methylene group (II) at C-3 of L-2-aminohexanoic acid (Ia) reduces the inhibitory potency (ΔpI_{50} = 0.4 unit) for the yeast and rat liver enzymes, and slightly improves the inhibitory potency for the *E. coli* enzyme. The insertion of a terminal branching methyl group (III) into L-2-aminohexanoic acid (Ia) is a further impediment to the already poor inhibitory potency of Ia, even if adjustment is made for the fact that III was only available as the DL mixture.

Effects of chlorine substitution. Chlorine atoms and methyl groups have quite similar van der Waals radii (i.e., 1.8 A and 2.0 A, respectively), but differ in the electronic influences that they exert. Consequently we have examined the effects of the introduction of chloro and methyl groups (alone or in combination) on the inhibitory powers of various amino acid analogues.

The two isomeric (*Z*)- and (*E*)-DL-2-amino-5-chloro-4-pentenoic acids (IX and X) may be viewed as structural derivatives of DL-2-amino-4-pentenoic acid (VIII). For the *E. coli* enzyme, both IX and X are somewhat less inhibitory than the parent compound. However, in the yeast and liver systems, the (*E*)-chloro derivative (X), which carries the chloro substituent in the same spatial configuration as the methyl group of *trans*-crotylglycine (IV), is a more powerful inhibitor (by pI_{50} = 0.4–0.7 unit) than the unsubstituted aminopentenoic acid (VIII), whereas the (*Z*)-chloro compound

(IX) has the same relatively low inhibitory potency as the unsubstituted parent compound (VIII) and is an inhibitor comparable to *cis*-crotylglycine (V). Moreover, it can be concluded for the yeast and liver systems that in the steric position favorable for inhibition, the effect of the methyl group (ΔpI_{50} = 1.0 unit) is considerably larger than that of a chloro group (ΔpI_{50} = 0.47–0.70 unit).

The 5-chlorohexenoic acids (XI and XII) may be regarded either as additions of methyl groups to X and IX, respectively, or as additions of chloro groups to V and IV, respectively. The addition of a 5-chloro group to *trans*-crotylglycine enhances the inhibitory power considerably, especially for the liver enzyme (cf. IV and XIIa). On the other hand, the *E* isomer is quite inactive as an inhibitor, although the sparing solubility of this compound precluded its evaluation at concentrations that might have detected a low order of inhibitory potency. It is therefore not possible to ascertain whether XIa is in fact significantly different in inhibitory potency from DL-*cis*-crotylglycine (V), and consequently the quantitative effect of the chloro substitution in XI cannot be rigorously evaluated. We may now consider the combined addition of methyl and chloro groups at C-5 of 2-amino-4-pentenoic acid, in the optimal steric configuration for inhibition. In the series of conversions of VIII to XII via IV or IX, it can be seen that the inhibition index increments are not strictly additive, and that the effects of the methyl group and the chloro group are powerfully synergistic. An examination of the photographs of molecular models of L-methionine and (*Z*)-L-2-amino-5-chloro-*trans*-4-hexenoic acid in the appropriate conformation shows striking similarities in molecular size and shape (Fig. 2). The photograph shows that the chloro group precisely overlies the sulfur atom, and that the same conformation is also similar spatially to L-2-amino-4-hexynoic acid (XIVb).

The van der Waals radii of chlorine and sulfur atoms are almost identical (1.80 and 1.85 A, respectively), and do not differ greatly from the radius of the average methyl group (2.0 A). In analyzing the effects of chloro and methyl groups in a

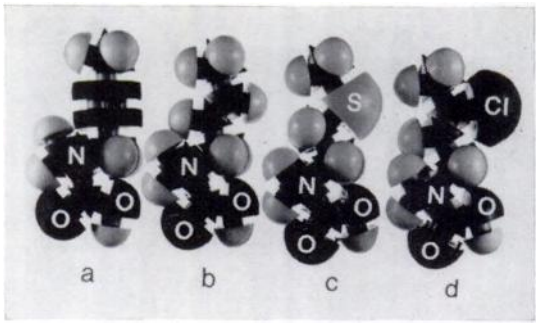


FIG. 2. *Photographs of Courtauld molecular models of* L-*2-amino-4-hexynoic acid* (*a, compound XIVb*), (*E*)-L-*2-amino-4-trans-hexenoic acid* (*b, compound IV*), L-*methionine* (*c*), *and* (*Z*)-L-*2-amino-5-chloro-trans-4-hexenoic acid* (*d, compound XIIb*)

Note the close similarities in conformation and size of the molecules. The chloro group in *d* is closely superimposable on the sulfur atom of *c*, which also corresponds to the acetylenic and olefinic groups of *a* and *b*, respectively.

group of inhibitory analogues, consideration should be also given to the electronic influences of these groups and to their "deformability." It may be relevant to point out that while chloro substituents frequently act as electron-withdrawing groups, they may, when attached to a doubly bonded carbon atom, tend to share their electrons with olefinic linkage, thereby becoming less electronegative. There are some reasons for believing that a partial positive charge in the region analogous to the sulfur atom of L-methionine may more closely simulate the transition state of the enzymatic reaction and consequently contribute to more favorable binding to the enzyme surface (6).

The topographical similarities between the assumed conformation of L-methionine and (*Z*)-L-2-amino-5-chloro-*trans*-4-hexenoic acid shown in Fig. 2 seem quite convincing. In examining molecular models, certain features do not become evident; among these we have already discussed the electronic influences of certain substituents, but further consideration should also be given to the ease with which certain groups or atoms are subject to "compression" or "deformation." Various lines of evidence support the view that chlorine and sulfur are far more "compressible" atoms than a methyl group. One

manifestation of this property is the mean free energy difference between axial and equatorial conformations of substituents on a cyclohexane skeleton. Typical values for such conformational energy differences (in kilocalories per mole) are −1.70 for —CH_3, −0.43 for —Cl, and −0.7 for —SCH_3 (36). Thus the effects of chloro and methyl groups on the inhibitory properties of this series of compounds depend in all likelihood on a combination of van der Waals radii, electronic properties, and deformability of the relevant groups.

Dienes and cyclopropyl derivatives. DL-2-Amino-4,5-hexadienoic acid (XVI) is a considerably better inhibitor than DL-2-amino-4-pentenoic acid (VIII) for the yeast and liver enzymes, and equipotent for the *E. coli* adenosyltransferase. This observation further supports several observations that the *E. coli* enzyme has a far more elastic requirement for a terminal carbon atom than the eukaryotic enzymes. The inhibitory potency of XVI is not as great as that of DL-*trans*-crotylglycine (IV), and considerably greater than DL-*cis*-crotylglycine (V). It would seem that the spreading out of the electronegative double-bond system or the improper steric orientation of the terminal methylene group accounts for the differences between IV and XVI. If the diene (XVI) is further modified by a 4,5-cyclopropyl bridge, as in compound XVIII, the inhibitory potency is somewhat improved for the yeast system and probably is unchanged for the *E. coli* and liver enzymes (on the assumption that only the L isomer of XVI is active). Hypoglycin A (XVIII) has the 4*S* configuration, and it would therefore be of great interest to examine the 4*R* compound. Transposition of the cyclopropyl group to the 3,4-position and displacement of the double bond to the 4,5-position, as in methylene cyclopropylglycine (XVII), destroys the inhibitory activity.

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