triphenylphosphonium chloride in 15 ml of MeOH. After 2 hr of stirring at ambient temp, the mixt was poured into 300 ml of H_2O. H_2O was decanted from the yellow solid which was digested in 100 ml of hot H_2O. After 2 digests, the yellow solid was collected by filtration and recrystd from EtOH–H_2O to give 1.75 g (91%) of light yellow needles, mp 115–116°; lit.[26] mp 116°, prepd by an alternate route.

4-(3-Methylphenethyl)pyridine (45) *p*-Toluenesulfonate.—A mixt of 2.0 g (10.3 mmoles) of **12** as the free base and 50 mg of 10% Pd/C was reduced as described for **14**. After filtration of the catalyst and evapn of the solvent, 2.0 g (10.4 mmoles) of TsOH in 100 ml of Et_2O was added to the residue. The salt was collected and recrystd twice from EtOAc; yield, 3.46 g (92%), mp 112–113°. *Anal.* ($C_{21}H_{23}NO_3S$) C, H, N.

4-(3-Methylphenethyl)pyridinium Methiodide (46).—A mixt of 6.14 g (31 mmoles) of **45** and 10 ml of MeI was heated on a steam bath for 30 sec when it solidified. The solid was heated 5 min more, than recrystd from Me_2CO–petr ether (bp 65–110°)–MeOH; yield, 8.37 g (81%), mp 166–167°. *Anal.* ($C_{15}H_{18}IN$) C, H, N.

Preparation of Enzyme and Assay Methods.—The enzyme prepn was a modification of the method of Potter, *et al.*,[14] used for rat brain. A mixt of 2.0 g of rabbit brain Me_2CO powder and 40 ml of ice-cold 0.1 m*M* Versene was homogenized in a pre-cooled head of a Waring blender for 2 min. After the addition of 50 μl of 1 *M* NH_4OH and 0.45 ml of *n*-BuOH, the mixt was blended an additional 30 sec. It was centrifuged at 20,000 rpm for 20 min in a No. 40 rotor of a Spinco L centrifuge. The supernatant was sepd and adjusted to pH 5 with 1 *M* HOAc (about 0.2 ml). The mixt was centrifuged at 20,000 rpm for 15 min and the supernatant rejected. The pellet was carefully rinsed with 5 ml of ice-cold H_2O, then stirred for 20 min with 0.2 *M* KCl at 0° for 15 min. The mixt was centrifuged at 20,000 rpm for 20 min. The supernatant (18 ml) was stored at 3°. Choline acetyltransferase activity gradually decreased, but was sufficiently active up to storage for 1 month. The AChE activity was stable over several months.

Choline acetyltransferase activity was measured by modification of the method of McCaman and Hunt;[15] the assay was run in the presence of 10% DMSO, and inhibitors were added in this solvent. The assay mixt contained 1 m*M* choline, 0.1 m*M* eserine, 0.1 *M* KCl, 0.025 *M* Tris buffer, and 0.1 m*M* acetyl CoA (5 mCi/mmole). The final Reinecke salt in Me_2CO was spotted on a glass filter paper, dried, and counted in PhMe containing 0.4% PPO and 0.01% dimethyl POPOP.

AChE activity was measured by modification of the method of Potter;[16] the assay was run in the presence of 2.5% MeOEtOH and inhibitors were dissolved in 25% MeOEtOH since 10% DMSO completely inhibited the reaction. The assay contained 1 m*M* ACh·Cl^- (0.9 mCi/mmole), 0.05 *M* Tris buffer, and 0.02 *M* $MgCl_2$.

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Inhibition of Phenethanolamine *N*-Methyl Transferase by Ring-Substituted α-Methylphenethylamines (Amphetamines)

RAY W. FULLER,* JACK MILLS, AND MAX M. MARSH

The Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana

Received September 28, 1970

Phenethanolamine *N*-methyl transferase (PNMT) transfers a Me group from *S*-adenosylmethionine to phenethylamines with an OH group β to the N. Phenethylamines (including α-methylphenethylamines) without such a β substitution combine with and inhibit the enzyme. Amphetamines with various aromatic substituents were studied as inhibitors; there was greater than a 1000-fold range in their inhibitor potency. The inhibitor activity of these compounds showed a correlation with the Hammett σ and π (a lipophilic parameter derived from the partition coefficient) associated with the aromatic substituent. The *d* isomers were more active as inhibitors than *l* isomers of amphetamines. *d*-3,4-Dichloroamphetamine was the most active inhibitor in the series and is the most potent inhibitor of PNMT reported to date; its inhibition was reversible and competitive. Inhibitors of PNMT that are effective *in vivo* should be of pharmacological importance.

Phenethanolamine *N*-methyl transferase (PNMT) transfers a Me group from *S*-adenosylmethionine to a Me acceptor, which apparently has to be either a phenethanolamine or a phenylethylenediamine.[1-3] The physiological role of PNMT is to convert norepinephrine into epinephrine, primarily in the adrenal medulla.[4] Although the physiological effects of norepinephrine and epinephrine are qualitatively similar in general, there are numerous differences in the responses of various target organs to these two catecholamines.[5-7] Thus an inhibitor of PNMT which altered the ratio of epinephrine:norepinephrine in the adrenal gland ought to be an interesting pharmacological tool and at least potentially useful as a drug. However, few studies on PNMT inhibitors have been published. PNMT is inhibited by sulfhydryl binding agents.[1,8,9] Fuller and Hunt[2] reported that some phenethylamines structurally related to substrates but lacking the β-OH essential for substrate activity were inhibitory to PNMT. Krakoff and Axelrod[10] reported the inhibition of PNMT by several amines, among which the monoamine oxidase inhibitor tranylcypromine was one of the most potent inhibitors.

Both the reports by Fuller and Hunt and by Krakoff and Axelrod showed that amphetamine was a relatively weak PNMT inhibitor. We describe here the inhibition by a series of substituted amphetamines, the most active of which represent the most potent PNMT inhibitors reported up to this time.

Experimental Section

The enzyme prepn and the method of enzyme assay were as reported previously.[11] An $(NH_4)_2SO_4$ fraction of the high-speed

(1) J. Axelrod, *J. Biol. Chem.*, **237**, 1657 (1962).
(2) R. W. Fuller and J. M. Hunt, *Biochem. Pharmacol.*, **14**, 1896 (1965).
(3) R. W. Fuller, B. J. Warren, and B. B. Molloy, *Biochim. Biophys. Acta*, **222**, 210 (1970).
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(8) A. E. Kitabchi and R. H. Williams, *Biochim. Biophys. Acta*, **178**, 181 (1969).
(9) R. J. Connett and N. Kirshner, *J. Biol. Chem.*, **245**, 329 (1970).
(10) L. R. Krakoff and J. Axelrod, *Biochem. Pharmacol.*, **16**, 1384 (1967).
(11) R. W. Fuller and J. M. Hunt, *Anal. Biochem.*, **16**, 349 (1966).

centrifugal supernatant from a homogenate of whole rabbit adrenals was used as enzyme.[11] Norepinephrine (40 μM) was the substrate. Enzyme activity was calcd as $\mu\mu$moles of epinephrine formed per 30 min of incubation.

The compds tested as inhibitors were synthesized in the Lilly Research Laboratories, and their chemical structures were verified by physicochemical methods. The compds in most cases were used as soluble salts, mainly as hydrochlorides. Several concns of each inhibitor were studied; the concns were spaced on a logarithmic basis, *e.g.* 1, 3, 10, 32, 100, 317 μM, and were selected to achieve a range of inhibition from <10% at the lowest to >90% at the highest concn. The results were plotted as per cent inhibition *vs.* concn on a logarithmic scale. From this plot, the inhibitor concn required for 50% inhibition (pI_{50}) was detd by interpolation. Inhibitors with pI_{50} differences of 0.1 or more were readily distinguishable from one another.

Results

Inhibition by Phenethylamines.—Our study of amphetamines as inhibitors was based on results with phenethylamines. The data in Table I illustrate the pertinent findings with respect to inhibition by phenethylamines. Table Ia shows that aromatic substituents markedly influenced the inhibitor potency of phenethylamines. We have previously reported that, among phenethanolamines studied as PNMT substrates, the 3,4-dichloro compd had a greater affinity for the enzyme than any other phenethanolamine studied.[3] 3,4-Dichlorophenethylamine was thus expectedly a potent inhibitor. The data in Table Ib show that a Me branch on either C of the side chain had little effect on inhibition of PNMT by phenethylamines. The data in Table Ic show that alkyl substitutions on the amino group decreased the inhibitory activity directly proportionate to the size of the substituent. These data in Table I led us to study amphetamines rather than phenethylamines as PNMT inhibitors, since (a) the α-Me branch did not decrease inhibitor activity, (b) a wider variety of compds with aromatic substituents were available to us in the amphetamine series, and (c) the differences in inhibitor activity among the few phenethylamines with aromatic substituents indicated further exploration of ring substitution would be fruitful.

TABLE I

PNMT INHIBITION BY PHENETHYLAMINES. STRUCTURE–ACTIVITY RELATIONSHIPS

Inhibitor	pI_{50}
a. Phenethylamines with Aromatic Substituent	
3,4-Cl_2	4.75
3-Cl-4-OH	4.15
3,4-$(OH)_2$	3.19
None	3.26
b. Phenethylamines with Side Chain Substituent	
α-Me	3.34
β-Me	3.28
None	3.26
c. 3,4-Dihydroxyphenethylamines with N Substituents	
None	3.19
Me	2.93
Et	2.37
n-Pr	2.12

Inhibition by 3,4-Disubstituted Amphetamines.—Figure 1 illustrates the inhibition of PNMT by 5 compds in the amphetamine series. The most active inhibitor, 3,4-dichloroamphetamine, was about 600 times as effective as amphetamine. Replacement of 4-Cl with OH reduced the inhibitor activity, and replacement of both Cl with OH reduced the potency still more. The 3,4-difluoro compd was intermediate in potency between 3,4-dichloro and 3,4-dihydroxy.

Correlation of PNMT Inhibitor Potency with Physicochemical Properties of Amphetamines.—We examined a total of 33 amphetamines as PNMT inhibitors and calcd pI_{50} values from graphs like Figure 1. The pI_{50} value was the neg log of the molar concn of inhibitor needed for 50% inhibition of enzyme activity. We attempted to correlate the activity of these compounds with the Hammett constant (σ) and a hydrophobic parameter (π) in a manner successfully used by us with monoamine oxidase inhibitors[12] and used by Hansch and his associates extensively.[13]

Table II lists the observed pI_{50} values for a group of 3- and 4-substituted amphetamines. By linear regression analysis the following equation was derived to fit the obsd data.

$$pI_{50} = 0.455\pi + 2.18\sigma + 2.747 \quad (1)$$

The level of significance for this equation by the f test was $P = 0.01$, and the square of the correlation coeff was 0.71. The fit of the equation was not improved by the addn of a π^2 term or by use of the Taft steric constant, E_s.[14] The calcd pI_{50} values shown in Table II were derived from the preceding equation. There was reasonable agreement between obsd and calcd pI_{50}

TABLE II

INHIBITION OF PNMT BY 3- AND 4-SUBSTITUTED AMPHETAMINES

Aromatic substituents	pI_{50} Obsd	pI_{50} Calcd[a]	Difference
3,4-Cl_2	5.10	4.68	−0.42
3-Cl	4.23	3.87	−0.36
4-CF_3	4.00	4.43	+0.43
3,4-F_2	3.85	3.77	−0.08
3-F	3.75	3.54	−0.21
4-Cl	3.60	3.55	−0.05
4-*i*-Pr	3.30	3.05	−0.25
3-Me	3.17	2.83	−0.34
4-Me	3.14	2.58	−0.56
4-F	3.01	2.95	−0.06
None	2.89	2.75	−0.14
3,4-Me_2	2.85	2.65	−0.20
4-PhO	2.76	3.37	+0.61
4-MeO	2.57	2.14	−0.43
3-MeO	2.07	3.05	+0.98
3-MeO-, 4-EtO	2.06	2.69	+0.63
3,4-$(MeO)_2$	2.00	2.45	+0.45
Hydroxylated Compounds			
3-Br-4-OH	4.15	2.96	−1.19
3-Cl-4-OH	4.15	2.81	−1.34
3,4-$(OH)_2$	3.30	1.45	−1.85
4-$(OH)_2$	3.12	1.69	−1.43
3-$(OH)_2$	2.77	2.52	−0.25

[a] Calcd pI_{50} values were calcd from eq 1.

(12) R. W. Fuller, M. M. Marsh, and J. Mills, *J. Med. Chem.*, **11**, 397 (1968).
(13) C. Hansch, *Annu. Rep. Med. Chem.*, **1967**, 348 (1968).
(14) E. Kutter and C. Hansch, *J. Med. Chem.*, **12**, 647 (1969).

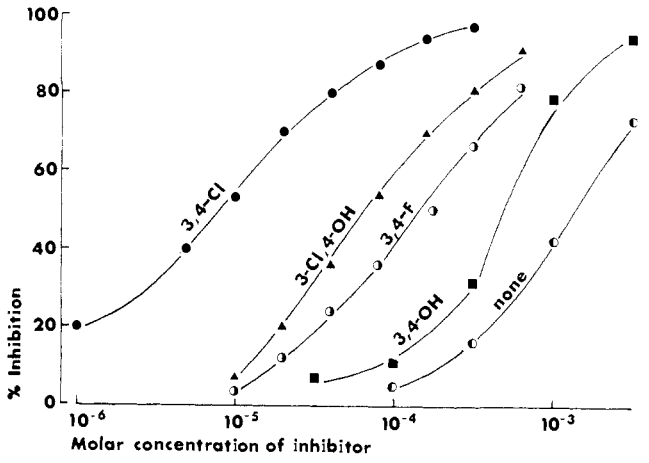

Figure 1.—Inhibition of PNMT by 3,4-disubstituted amphetamines.

values except with the weaker inhibitors (which were less active than the equation predicted) and for the hydroxylated derivs. The compds with an OH substituent were not included in the derivation of the equation. The pI_{50} values for the compds with a 4-OH substituent were poorly predicted by the equation; all of those compds were more than 10 times as active as the equation predicted. The 3-OH compd was well predicted by the equation. These results suggest that the 4-OH confers a special affinity for the enzyme, possibly by interacting in some way such as H bonding with a group near or at the active site of the enzyme.

Table III lists obsd and similarly calcd pI_{50} values for

TABLE III
INHIBITION OF PNMT BY 2-SUBSTITUTED AMPHETAMINES

Aromatic substituent	pI_{50} Obsd	pI_{50} Calcd[a]	Difference
2,4-Cl_2	4.02	3.73	−0.29
2,5-F_2	3.48	3.51	+0.03
2,6-Cl_2	3.47	3.67	+0.20
2-Me	3.25	2.92	−0.33
2-Cl	3.24	3.27	−0.03
2-F	3.17	3.12	−0.05
2,4-F_2	3.08	3.23	+0.15
None	2.89	2.87	−0.02
2,4-Me_2	2.85	2.93	+0.08
2,5-Me_2	2.83	3.02	+0.19
2,3-$(MeO)_2$	1.65	2.52	+0.87
2,4-$(MeO)_2$	1.51	2.08	+0.57

[a] Calcd pI_{50} values were calcd from eq 2.

2-substituted amphetamines. The equation derived from the obsd data for these compds was

$$pI_{50} = 0.460\pi + 1.151\sigma + 2.732 \quad (2)$$

The fit of this equation was significant (f test) at $P = 0.01$, and the square of the correlation coeff was 0.80. Again, the correlation was not improved by adding a π^2 term. None of the 2-substituted amphetamines was as potent as 3,4-dichloroamphetamine in inhibiting PNMT. 2,4-Dimethoxyamphetamine was the weakest inhibitor of the entire series.

Influence of Stereoisomerism on Inhibition by Amphetamines.—Figure 2 shows a comparison of stereoisomers of amphetamine and two substituted amphetamines. In all cases, the d isomer was more active than the l isomer. The differences in pI_{50} values were 0.4 for the most potent compd (3,4-dichloro-*N*-methyl), 0.3 for the 2,4-dichloro compd, and about 0.7 for amphetamine itself.

Studies on the Type of Inhibition.—The most active inhibitor of PNMT in this series was d-3,4-dichloroamphetamine. Further studies were made with this compd; Table IV shows the effect of dialysis on the inhibition. A concn of inhibitor sufficient to give 60–70% inhibition when not subjected to dialysis was used. Dialysis for 24 hr completely restored the enzyme activity. Thus the inhibition by 3,4-dichloroamphetamine was reversible by dialysis. Another experiment for which data are not shown revealed that incubation of 3,4-dichloroamphetamine with enzyme prior to substrate addn did not increase the inhibitory potency. These are characteristics of a reversible inhibitor.

TABLE IV
REVERSAL BY DIALYSIS OF THE INHIBITION BY d-3,4-DICHLOROAMPHETAMINE[a]

Conditions	PNMT activity	% inhibition
(a) No inhibitor	37 ± 1	
(b) Inhibitor added at end of dialysis	14 ± 1	62
(c) Inhibitor added at beginning but not dialyzed	12 ± 1	68
(d) Inhibitor added at beginning and dialyzed	36 ± 1	3

[a] Enzyme prepns in 0.05 M, pH 7.4, sodium phosphate buffer were kept at 4° for 24 hr and then used as the enzyme in the PNMT assay. Mixts a, b, and d were dialyzed during this time against frequent changes of the same buffer. Inhibitor was added before (d) or after (b) dialysis. In c, inhibitor was added at the beginning of the 24-hr period, but the mixt was not dialyzed. Inhibitor concn was 212 μM in the dialysis mixts and 32 μM in the final assay mixt. PNMT activity is in $\mu\mu$moles of epinephrine formed per 30 min of incubation.

The competitive nature of the inhibition by 3,4-dichloroamphetamine is shown in Figure 3. A concn of 4 μM of the inhibitor increased the apparent K_m without altering V_{max}. The 16 μM concn further increased the apparent K_m and only slightly lowered V_{max}. These results are characteristic of a competitive inhibitor. The average K_i value calcd from these data was 2.6 μM, which may be compared to the K_m of 8.8 μM for norepinephrine.

Discussion

Varying the aromatic substitution on amphetamines led to large changes in the ability of the compds to inhibit PNMT. The most active compds in the series all had 3-halogen substituents. 3-Chloro-, 3,4-dichloro-, 3-chloro-4-hydroxy-, and 3-bromo-4-hydroxyamphetamines were all more potent than previously described PNMT inhibitors. Krakoff and Axelrod[10] reported tranylcypromine to be the most active of a group of arylalkylamines, which they studied as PNMT inhibitors. Tranylcypromine in our assay with norepinephrine as substrate had a pI_{50} of 4.05. Tryptamine, another inhibitor reported by Krakoff and Axelrod,[10] had a pI_{50} of 3.46 in our system.

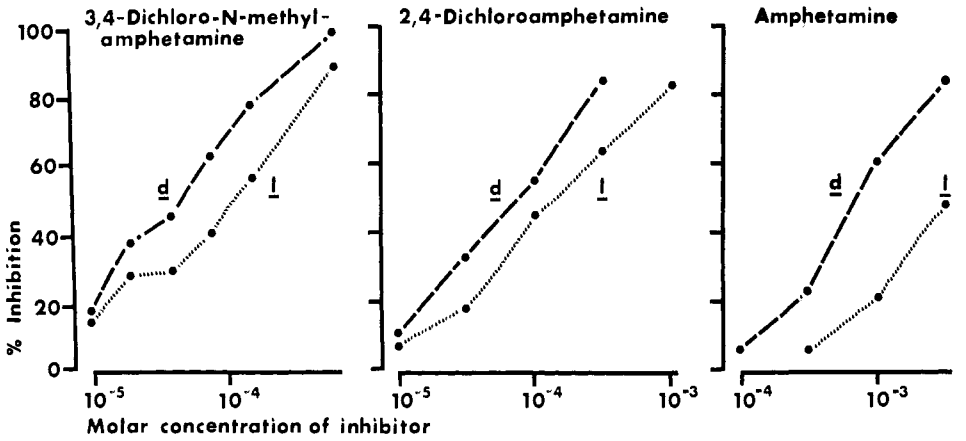

Figure 2.—Inhibition by *d* and *l* isomers of amphetamines.

The pharmacol activity of 3,4-dichloroamphetamine[14a] and other compds in this series in animals is qualitatively similar to that of amphetamine, with CNS stimulation being the dominant feature. The observed pharmacological activity is probably unrelated to PNMT inhibition and would be a side effect complicating the study of these compds as PNMT inhibitors *in vivo*. We have begun some preliminary studies of *d*-3,4-dichloroamphetamine as a PNMT inhibitor in rats. The compound is distributed in tissues similar to the way in which 4-chloroamphetamine is distributed,[15] and several organs have higher concns than do the adrenal glands after a single dose of the compd. Repeated injections led to an increase in the concn of drug found in the adrenals, but we do not yet know if *in vivo* inhibition of PNMT by this compd can be achieved at nontoxic doses.

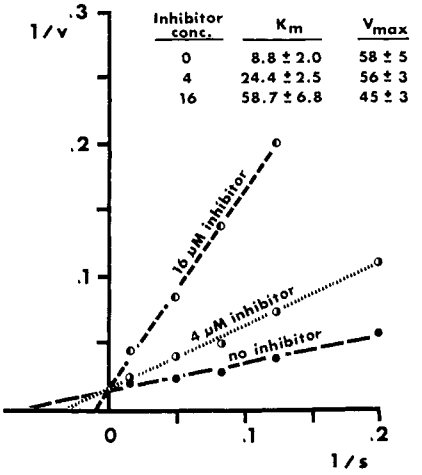

Figure 3.—Competitive inhibition by *d*-3,4-dichloroamphetamine. The reciprocals of velocity (v) in $\mu\mu$moles of epinephrine formed per 30 min of incubation and of μM substrate concn (s) are plotted. K_m (μM) and V_{max} ($\mu\mu$moles/30 min) values were calcd by the method of Wilkinson with the use of an Olivettti Programma 101 desk-top computer.

Acknowledgments.—We are grateful to Betty J. Warren and Mary Jo Brandt for technical assistance.

(14a) NOTE ADDED IN PROOF.—Mandel, *et al* [L. R. Mandel, C. C. Porter, F. A. Kuehl, Jr., N. P. Jensen, S. M. Schmitt, T. B. Windholz, T. R. Beattie, J. A. Carty, B. G. Christensen, and T. Y. Shen, *J. Med. Chem.*, **13**, 1043 (1970)], recently reported that substituted benzimidazoles inhibited bovine PNMT. Their most active compound *in vivo* was 5, 6-dimethyl-2-aminobenzimidazole, which in our system had a pI_{50} of 5.05 and was not distinguishable in potency from 3,4-dichloroamphetamine.

(15) R. W. Fuller and C. W. Hines, *J. Pharm. Sci.*, **56**, 302 (1967).