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The Remarkable Substrate Activity for Phenylethanolamine *N*-Methyltransferase of Some Conformationally Defined Phenylethylamines Lacking a Side-Chain Hydroxyl Group

Conformationally Defined Adrenergic Agents. 6

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SUMMARY

A number of conformationally defined phenylethylamines were found to be active as substrates for the enzyme phenylethanolamine *N*-methyltransferase (PNMT). These compounds have the ethylamine side chain locked into a fully extended conformation and represent the first known examples of PNMT substrates which are not ethanolamines or closely related structures. The most potent of these substrates are trifluoromethyl-substituted derivatives of *exo*-2-aminobenzobicyclo[2.2.1]heptene, with the substituent attached to position 5 or 6 on the aromatic ring. For example, the 6-substituted compound was found to have a K_m of 92 ± 16 μM with a V_{max} of 3.07 nmoles of product per milligram of protein per minute; the values for phenylethanolamine are K_m = 108 ± 23 μM and V_{max} = 1.75 nmoles of product per milligram of protein per minute. The 7- and 8-substituted derivatives displayed negligible substrate activity and instead were found to be competitive inhibitors of the enzyme. Trifluoromethyl-substituted *endo*-2-aminobenzobicyclo[2.2.1]heptenes, which are analogues of phenylethylamine in a *gauche* conformation, were found to be uncompetitive inhibitors, with the 7-substituted derivative being most potent. Examination of some other fully extended side-chain analogues of phenylethylamine revealed that substrate activity depended on the inflexibility of the carbon skeleton; conformationally mobile compounds such as 2-aminotetralin were devoid of activity. The conclusions drawn from this study are that PNMT substrates are required to assume a fully extended conformation within the active site in order for methyl transfer to occur, and that the side-chain hydroxyl of ethanolamine apparently serves to restrict the movement of the acceptor amino group during the approach of the methyl donor. Since these conclusions do not account for the ability of some folded phenylethylamine analogues such as tetrahydroisoquinolines and the related benzylamines to inhibit PNMT competitively, it is suggested that the active site exists in two different conformational states. The lower-energy state is not catalytically active and accommodates benzylamine-type inhibitors, while a higher-energy state is able to bind substrates in a fully extended conformation and this state is capable of effecting the methyl transfer.

INTRODUCTION

The fact that phenylethanolamines are considerably more active as substrates for PNMT[1] (EC 2.1.1.28) than the corresponding phenylethylamines was first reported by Axelrod (1) and Hoffman *et al.* (2) and later by Fuller *et al.* (3). Among the compounds previously evaluated for substrate activity which are not ethanolamines, only arterenone and α-(aminomethyl)-3,4-dichlorobenzylamine showed more than just a trace of activity (4). The nature of the contribution of the side-chain hydroxyl group to substrate activity is unknown, although it appears to be more than just a net contributor to the overall affinity of the substrate, since a number of phenylethylamine and amphetamine derivatives are very potent competitive dead-end inhibitors of the enzyme (5). Recently, we reported the inhibitory activity of a series of conformationally defined analogues of phenylethylamine (6) in an effort to determine the conformational requirements for optimal interaction with the active site. The results of this study indicated that phenylethylamine

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[1] The abbreviations used are: PNMT, phenylethanolamine *N*-methyltransferase; AdoMet, *S*-adenosyl-L-methionine.

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inhibitors were required to assume a fully extended side-chain conformation in order to act as competitive inhibitors of the enzyme. We now have on hand a series of trifluoromethyl-substituted derivatives of two of the compounds used in this study (*exo*- and *endo*-2-aminobenzobicyclo[2.2.1]heptene) and, since hydrophobic, electron-withdrawing aromatic substituents (such as trifluoromethyl) are known to increase inhibitory potency of phenylethylamines toward PNMT (5), we examined these compounds (**1** through **7**, Table 1) as inhibitors of the enzyme.

An additional reason for our interest in compounds **1–7** as PNMT inhibitors is the fact that the benzobicyclo[2.2.1]heptene skeleton locks the phenyl ring in a single orientation such that there are two distinguishable positions which correspond to the *m*-position of phenylethylamine (positions 5 and 7). Since a 3,4-disubstituent pattern is reported to be optimal for enhancement of PNMT affinity of phenylethylamines (4, 5), the compounds employed in this study are potential probes of the substituent orientation of bound phenylethylamines and could contribute to our understanding of the 3-dimensional requirements of the active site. In order to correlate the activities of the compounds used in this study with similarly substituted substrates and inhibitors, we prepared *m*- and *p*-trifluoromethylphenylethanolamine (**9** and **10**) and the analogous amphetamines (**11** and **12**) and included these compounds in this study. Only one of these compounds, *p*-trifluoromethylamphetamine, has been previously examined for PNMT activity (5). The results found for these and other compounds are presented in this paper.

MATERIALS AND METHODS

The hydrochloride salts of all of the compounds in this study were synthesized in our laboratories, with the exception of 2-aminoindan·HCl (**16**), which was purchased from Aldrich Chemical Company (Milwaukee, Wisc.). [^{3}H]AdoMet, which was used in the radiochemical enzyme assay, was purchased from New England Nuclear Corporation (Boston, Mass.). The details of the synthesis of the trifluoromethyl-substituted derivatives **1–7** will be published elsewhere. References to the synthesis of compounds **13–15** can be found in our earlier report (6). The syntheses of ethanolamines **9** and **10** have been described by Poos *et al.* (7), and the amphetamines **11** and **12** were prepared according to the method of Holland *et al.* (8). All of the compounds used in this study were fully characterized by spectroscopic methods and gave satisfactory combustion analyses. Bovine adrenal glands required for the purification of the enzyme used in this study were purchased from Pel-Freez Biologicals (Rogers, Ark.).

Radiochemical assay for PNMT activity. The assay used in this investigation has been previously described (6, 9). Bovine adrenal PNMT which had been purified according to the method of Connett and Kirshner (10) through the isoelectric precipitation step was used. For the determination of substrate kinetic constants, at least six concentrations of the variable substrate were assayed. Inhibition constants in this study for the compounds which did not display significant substrate activity were determined using at least three different inhibitor concentrations, as previously described (6), with phenylethanolamine as the variable substrate. Briefly stated, a typical assay mixture consisted of 50 μl of 0.5 M phosphate (pH 8.0); 25 μl of a 10 mM solution of unlabeled AdoMet; 5 μl of [*methyl*-^{3}H]AdoMet, containing approximately 2 × 10^6 dpm (specific activity 15 mCi/mmole; New England Nuclear Corporation); 25 μl of substrate solution; 25 μl of inhibitor solution (if added); 25 μl of the enzyme preparation; and sufficient water to achieve a final volume of 250 μl. After incubation for 30 min at 37° the reaction was quenched by the addition of 250 μl of 0.5 M borate (pH 10) and extracted with toluene-isoamyl alcohol (7:3). The organic layer was removed and transferred to a scintillation vial and diluted with cocktail for counting. The mode of inhibition was ascertained by inspection of the $1/V$ versus $1/S$ plot of the data.

Gas chromatographic analysis of the enzymatic product. A preparative scale enzymatic reaction mixture suitable for product identification studies consisted of 500 μl of the partially purified enzyme preparation, 250 μl of 10 mM AdoMet, 250 μl of a 20 mM solution of **14**, 500 μl of 0.5 M phosphate buffer (pH 8.0), and 1.0 ml of water for a total volume of 2.5 ml. A small amount of [^{3}H]AdoMet was also added in order to be able to detect the formation of product and to allow for calculation of the amount of product formed. After incubation at 37° for 22 hr, the mixture was made alkaline by the addition of 0.5 ml of 1 N NaOH and extracted three times with toluene (2 ml each time). The pooled organic layers were dried (K_2CO_3), and a 100-μl aliquot was transferred to a counting vial, along with 0.9 ml of toluene and 5.0 ml of scintillation cocktail (3a-70; Research Products International Corporation, Mt. Prospect, Ill.). The rest of the toluene extract was taken to dryness, and the residue (1.7 mg) was redissolved in 300 μl of ethyl acetate prior to analysis.

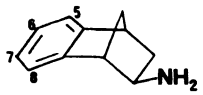
The instruments used in the product identification study were a Hewlett-Packard 5880A gas chromatograph equipped with a Model 7672A automatic sampler, containing a ¼ inch × 6 foot glass column of 10% Apiezon L, 2% KOH on 80–120 mesh Chromosorb W-AW; and a Varian Model 3700 gas chromatograph containing a ¼ inch × 10 foot glass column of 3% SE-30 on 80–120 mesh Chromosorb W-AW. Conditions were varied from 185° to 205° (isothermal) and temperature-programmed analyses of 140° to 250° at 20°/min. In each case, peak identity was confirmed by coinjection with an aliquot of a solution of authentic material.

RESULTS

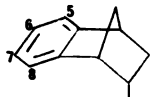
Unless otherwise stated, the K_m and K_i values presented here are in micromolar units, and the V_{max} values in units of nanomoles of product formed per milligram of protein per minute. Table 1 lists the kinetic constants found for the trifluoromethyl-substituted compounds **1–7**. Compounds **1** and **2** were found to be substrates for PNMT, with K_m and V_{max} values equivalent to those of phenylethanolamine (**8**) as measured in our laboratory. Compounds **3** and **4**, which like **1** and **2** are fully extended analogues of phenylethylamine, displayed very weak substrate activity and were instead competitive inhibitors of the enzyme. In keeping with the results found by us for similar unsubstituted *endo*-compounds (6), the substi-

TABLE 1

PNMT activity of some trifluoromethyl-substituted derivatives of exo- and endo-2-aminobenzobicyclo[2.2.1]heptene



1: 5 - CF_3
2: 6 - CF_3
3: 7 - CF_3
4: 8 - CF_3



5: 5 - CF_3
6: 6 - CF_3
7: 7 - CF_3

	K_i ± SEM (uM)	K_m ± SEM (uM)	V_{max}[a]
1	----	36 ± 13	0.99
2	----	92 ± 16	3.07
3	204 ± 65	----	---
4	350 ± 59	----	---
5[b]	>2 mM	----	---
6[b]	893 ± 154	----	---
7[b]	69 ± 15	----	---
8[c]	----	108 ± 23	1.75

[a] Units of V_{max}: nanomoles of product formed per milligram of protein per minute.

[b] These compounds displayed uncompetitive inhibition.

[c] Phenylethanolamine.

tuted derivatives **5–7** were uncompetitive inhibitors (showed parallel Lineweaver-Burk plots for varying inhibitor concentrations), although the potency varied dramatically depending on the position of substituent attachment, with the 7-substituted compound **7** being the most potent of the series (the 8-substituted *endo*-derivative was not available for this study).

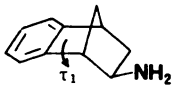
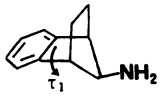
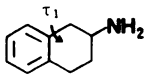
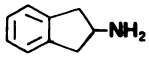
The trifluoromethyl-substituted ethanolamines **9** and **10** (Table 2) proved to be excellent substrates for PNMT, as expected. The *p*-isomer **9** appeared to be slightly better, as evidenced by the lower K_m as compared with **10**, but the difference was only about 2-fold. The amphetamine derivatives **11** and **12** were found to be much more potent than amphetamine (K_i for amphetamine, 740 μM; ref. 6), but in this case the *m*-substituted isomer was slightly more potent, although the difference was very small. The K_i reported in Table 2 for **11** is in good agreement with the pI_{50} value reported by Fuller *et al.* (5) for the same compound.

Table 3 lists the K_m and V_{max} values for some fully

TABLE 3

PNMT substrate activity and τ_1 angle of some conformationally restricted phenylethylamine analogues

The τ_1 angle represents the torsional angle of the phenyl ring with respect to the ethylamine side chain

		K_m ± SEM (uM)	V_{max}[a]	τ_1
13		393 ± 27	0.038	69°
14		150 ± 15	0.173	34°
15		inactive		18°
16		inactive		

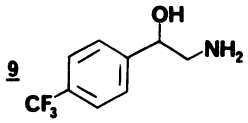
[a] For units see Table 1.

extended side-chain analogues which were found in our earlier study to be competitive inhibitors of PNMT (6). Compound **13**, which is the unsubstituted precursor of compounds **1–4** of Table 1, was an extremely poor substrate with a V_{max} of only 0.038, which is 1.2% of the V_{max} observed for **2**. However, compound **14**, which like **12** is a fully extended side-chain analogue of phenylethylamine, was a much better substrate, although still much poorer than either **1** or **2**. The main conformational difference between compounds **13** and **14** is the torsional angle of the phenyl ring (τ_1, Table 3), which in the case of **13** is 69° as compared with 34° for **14**. Neither 2-aminotetralin (**15**) nor 2-aminoindan (**16**) possessed any detectable substrate activity at concentrations up to 1 mM, even though both are bound considerably tighter by PNMT than either **13** or **14** as evidenced by their lower K_i values (15 μM for **15**; 12 μM for **16**; ref. 6).

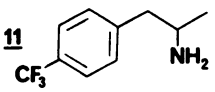
Gas chromatographic analysis of the enzymatic product mixture for compound **14** clearly demonstrated the presence of the expected *N*-methylated product. Injection of a sample of *N*-methyl **14** onto the Apiezon column at a column temperature of 185° produced a single peak with a retention time of 3.7 min. Compound **14** itself had a retention time of 3.2 min under the same conditions. Analysis of the toluene-extractable material from the preparative scale enzyme reaction mixture of **14** pro-

TABLE 2

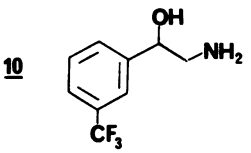
PNMT activity of some trifluoromethyl-substituted derivatives of phenylethanolamine and amphetamine



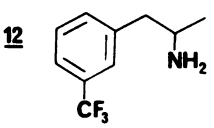
9 K_m = 6 ± 2.7 uM
V_{max} = 0.51[a]



11 K_i = 92 ± 6.7 uM



10 K_m = 13 ± 6 uM
V_{max} = 0.56[a]



12 K_i = 79 ± 4.7 uM

[a] For units see Table 1.

duced a series of peaks, among which a peak corresponding to **14** and a smaller peak with a retention time of 3.7 min were clearly identifiable. Coinjection of the product mixture with a small amount of *N*-methyl **14** increased the size of the peak at 3.7 min and confirmed its identity as *N*-methyl **14**. The ratio of the integrated areas of these peaks was approximately 3:1, suggesting 25% conversion of the substrate to product. Calculation of the amount of product formed from the amount of radiolabel incorporated into the toluene-extractable material indicated a total conversion of 29% of the substrate to product, in good agreement with the gas chromatographic results. Several experiments employing different temperatures were conducted and were repeated on a different column as described in the previous section, and in every case the retention time of the product peak corresponded to that of authentic *N*-methyl **14**.

A similar experiment was conducted using compound **2** as the substrate. Although we did not have an authentic sample of the expected product on hand, gas-liquid partition chromatography of the product mixture revealed the presence of a sharp peak with a retention time of 2.1 min on the Apiezon column at 200° (**2** has a retention time of 1.8 min under the same conditions). The peak areas indicated that 54% of the substrate had been converted to product; calculation from the amount of radiolabel incorporated into the product mixture indicated a 52% conversion.

DISCUSSION

The potent substrate activity of compounds **1** and **2** clearly differs from the previously accepted structural requirement of an ethanolamine side-chain or related structure for PNMT substrates. All of the bicyclic compounds employed in this study are highly inflexible structures, which greatly restrict the mobility of the amino group. Compounds **15** (2-aminotetralin) and **16** (2-aminoindan) are considerably more flexible and would allow the amino group a reasonable amount of freedom of movement. The fact that the more rigid structures displayed substrate activity, whereas the flexible analogues did not, suggests a possible role of the side-chain hydroxyl of ethanolamine substrates in immobilizing the amino group during methyl transfer. The hydroxyl group could, for instance, be bound both to the active site and to the side-chain amino group simultaneously by electrostatic or hydrogen bond interactions; intramolecular amino and hydroxyl group interactions have been demonstrated by a variety of techniques, such as nuclear magnetic resonance spectroscopic evidence obtained for phenylethanolamine (11). Such an arrangement would require a fully extended side-chain conformation, which is in fact the conformation required by the enzyme for catalysis, as indicated by the results of this study. Phenylethylamines lacking this added stabilization of the amino group fail to act as substrates because the amino group is free to swing away at the approach of the AdoMet methyl within the active site. In the case of the compounds presented in this study, the rigid bicyclic skeleton prevents the amino group from moving away at the approach of the methyl donor; however, the loss of additional binding interactions normally contributed by the side-chain hydroxyl must be made up in order to achieve significant substrate activity, and this extra affinity is provided by the attachment of a trifluoromethyl group to the aromatic ring in the case of compounds **1** and **2**.

The fact that compound **14** displays substrate activity which is considerably higher than that found for **13** suggests that the phenylethylamine conformation mimicked by **13** and compounds **1–4** may not be optimal for active-site interaction, and an over-all more planar conformation for phenylethylamine such as that approximated by **14** is closer to the true active-site conformation of phenylethanolamine substrates. The half-chair conformer of compound **15** has a τ_1 angle of 18° for its most stable conformer, which translates to a much more planar conformation than compound **14**, and would, therefore, suggest that **15** would be an even better substrate than either **13** or **14**. The fact that no substrate activity was observed suggests that the compound is sufficiently flexible to allow the amino group to swing away at the approach of the AdoMet methyl group as described earlier; thus, methyl transfer cannot occur.

The reported existence of nonspecific methyltransferases in mammalian tissues which are capable of *N*-methylating a wide variety of simple amines (12, 13) raises the possibility that PNMT is, in fact, not responsible for the substrate activity of **1**, **2**, **13**, and **14**. However, to our knowledge this enzyme has not been detected in the adrenal medulla. A report that several benzylamine derivatives, which are potent dead-end inhibitors of PNMT, are excellent substrates for this nonspecific methyltransferase (14) would make detection of this enzyme quite feasible even in the presence of large amounts of PNMT. No such activity has been reported, nor have we observed any such activity with our enzyme preparation.

One of the most striking features of this study is the profound influence of the position of substituent attachment on the activity of both the *exo*- and *endo*-compounds. It is clear from the results for Compounds **1–4** that positions 5 and 6 allow interaction with the ring-binding region to a much greater extent than positions 7 and 8. Compound **1**, therefore, appears to be a better model for the active site conformation of the *m*-substituted compounds **10** and **12** than does compound **3**. The extent of the contribution of the trifluoromethyl group attached to positions 7 and 8 is considerably less than the nearly 10-fold increase in affinity observed for **11** and **12** as compared with amphetamine; this suggests that these positions are away from the ring-binding site and substituents on these positions are unable to bind to any significant extent.

For the *endo*-compounds **5–7**, a considerably different positional requirement for substituents with respect to uncompetitive inhibition was revealed. In contrast to the results for **1–4**, moving the trifluoromethyl group from position 5 to position 6 and on to position 7 produced a parallel increase in inhibitory activity, with the result that compound **7** was found to be a remarkably potent uncompetitive inhibitor of PNMT. The contrasting positional influences found for **5–7** as compared with the *exo*-compounds **1–4** indicate that the binding site at which the *endo*-compounds exert their uncompetitive inhibition is separate and distinct from the active site, and could be a target for future inhibitor design.

One of the principal conclusions of this study is that substrates for PNMT assume a fully extended conformation in order for catalysis to occur. If this is true, then the mechanism by which benzylamine derivatives can exert such potent competitive inhibition against the enzyme remains unclear. One possible explanation is that the active site exists in two different conformational states, one which can accommodate benzylamine-like structures and a second (catalytically active) state which binds fully extended phenylethylamines. Evidence that PNMT does undergo conformational changes is found in the substrate protection studies of Masover *et al.* (15) in which it was demonstrated that PNMT is considerably more resistant to thermal and proteolytic degradation in the presence of substrates (norepinephrine and/or AdoMet). Phenylethylamine was also reported to protect PNMT from tryptic degradation, but to a lesser extent than either phenylethanolamine or norepinephrine. That these changes occur only in the presence of phenylethylamine inhibitors and substrates suggests that the conformationally adapted state of the enzyme is a higher-energy state, with the energy required to assume this state being supplied, in effect, by the ligands. One would assume, therefore, that phenylethylamines would exhibit a lower observed binding energy due to the energy lost in adapting the active site as compared with ligands, i.e., benzylamines, which can bind to the enzyme without having to induce a conformational change in the active site. Although we have no direct evidence for differences in the conformational response of PNMT toward benzylamines and phenylethylamines which would support our proposed two-state model of the active site, ample literature precedence for this mechanism exists (16, 17). Furthermore, an examination of both the data presented in the literature and findings in our laboratory shows that, in comparing similarly substituted benzylamines and phenylethylamines, the benzylamines are invariably more potent inhibitors, indicative of a higher binding energy. Calculations from K_i values indicate that the ΔG for benzylamine binding is 0.5–1 kcal/mole higher in energy than for phenylethylamines, suggesting that this amount of energy is required to adapt the active site for phenylethylamines; this is less than the calculated energy difference between a fully extended phenylethylamine conformation and a tightly folded conformation corresponding to tetrahydroisoquinoline or benzylamine (18). The fact that there are observable differences in the extent to which phenylethylamine inhibitors and phenylethanolamine substrates stabilize PNMT could indicate that, while this small amount of energy is sufficient to induce a conformational change in the active site, it is not enough to activate fully the enzyme for catalysis. Alternatively, the difference in binding energy could represent the energy needed by phenylethylamine to assume a folded "benzylamine-like" conformation which then binds to the inactive form of the active site, if the calculated conformational energy differences actually overestimate the true values. This would also account for the lack of substrate activity for phenylethylamine. This mode of phenylethylamine binding would seem to be contradicted by the observed tryptic stability of PNMT which is imparted by phenylethylamine; however, the fact that phenylethylamine does not provide thermal stability as does phenylethanolamine suggests that there is a difference in conformational response of the active site to the two compounds, which might be reflected in the binding of phenylethylamine in a different conformation from that of substrates. In either case, activation of the active site for catalysis requires either the added binding contribution and/or conformational influences of the side-chain hydroxyl, or the added support of a conformationally defined skeleton coupled with enhanced binding affinity of the aromatic ring. This hypothesis is illustrated in Fig. 1.

In summary, we have found that some trifluoromethyl-substituted derivatives of a conformationally defined analogue of phenylethylamine are excellent substrates for PNMT. These compounds constitute the first known examples of potent substrates for this enzyme which do not possess a polar, hydrogen-bonding functional group

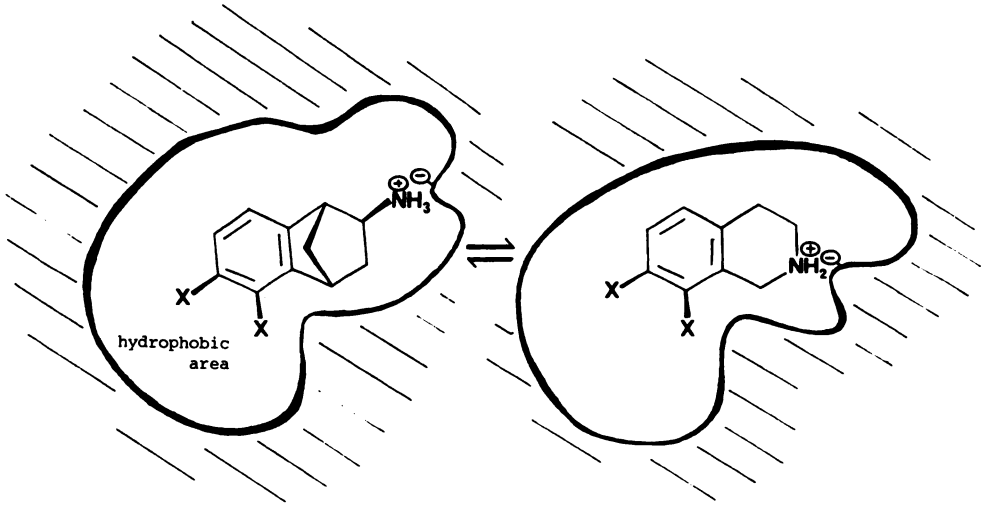


FIG. 1. *Schematic illustration of the proposed two-state model of the PNMT active site*

Left, the active site in a catalytically active conformation to which a fully extended conformationally defined analogue of phenylethylamine is bound. Right, the active site in a catalytically inactive state, which is able to accommodate structures such as tetrahydroisoquinolines (shown) and benzylamines. The two states are in equilibrium, with the right-hand conformational state favored.

(usually hydroxyl) on the α, or benzylic, position of the ethylamine side-chain. The results indicate that substrates assume a fully extended side-chain conformation for catalytic activity. In order to account for the potent inhibitory activity of benzylamines, we have proposed a two-state model for the PNMT active site. The positional requirements for the aromatic ring substituent with respect to activity have contributed to our understanding of the orientation of aromatic *m*-substituents of bound substrates. Finally, the substrate activity of some other conformationally restricted analogues of phenylethylamine suggests that the actual active-site conformation of substrates is slightly different than the conformation approximated by compounds 1–4, and further investigation with additional conformationally defined analogues is warranted.

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