dithiothreitol. Aliquots of the dialyzed solution were frozen at -20 °C and used as the source of soluble phosphodiesterase. The 105000g pellet was resuspended in the original volume of sucrose buffer and rehomogenized. After a second 105000g centrifugation, the washed pellet was resuspended in a minimal volume of 20 mM Tris-HCl, pH 7.5, buffer containing 1 mM $MgCl_2$ and 0.1 mM dithiothreitol and dialyzed overnight against the same buffer. This preparation was kept frozen at -20 °C and used as the source of particulate enzyme.

Enzyme Assay. Cyclic nucleotide phosphodiesterase activity was assayed as previously described,[27] following a modified method based on the original procedure of Thompson et al.[28] Substrate cAMP and cGMP concentration was 0.25 μM. All the test compounds were solubilized in dimethyl sulfoxide (Me_2SO) and brought to the adequate concentration with the incubation buffer. Final Me_2SO concentration did not exceed 5%. This Me_2SO

amount slightly inhibited cyclic nucleotide phosphodiesterase activity but did not modify the percent inhibition found with water-soluble drugs. Test compounds and Me_2SO were examined to ensure that they did not interfere either with the nucleotidase step of the assay or with adenosine and guanosine recoveries. I_{50}'s were calculated by interpolating three to four values of inhibition, ranging from 30 to 80%, against the logarithm of inhibitor concentrations. All the assays were carried out at 30 °C and performed in triplicate at enzyme dilutions that gave 10-15% hydrolysis of substrate in the absence of inhibitor.

"Low K_m's" of the cytosolic and the particulate phosphodiesterase preparations were determined with cAMP and cGMP concentrations ranging from 0.25 to 5 μM. With the cytosolic preparation, the apparent low K_m's were 2-4 μM for cAMP and 3-5 μM for cGMP. With the particulate preparation, low K_m's values were 3-4 and 20-22 μM for cAMP and cGMP as substrate, respectively.

Penta-*O*-ethylquercetin (**2b**), one of the more selective quercetin analogues, was found to be a competitive inhibitor of both the cytosolic and the particulate cAMP phosphodiesterase with K_i of 200 and 15 μM, respectively.

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Importance of the Aromatic Ring in Adrenergic Amines. 7. Comparison of the Stereoselectivity of Norepinephrine *N*-Methyltransferase for Aromatic vs. Nonaromatic Substrates and Inhibitors[1a,b]

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Some nonaromatic analogues of amphetamine and α-methylbenzylamine were prepared and evaluated as competitive inhibitors of norepinephrine *N*-methyltransferase (NMT). All of the nonaromatic analogues were significantly more active than their aromatic counterparts [K_i for amphetamine = 740 μM; K_i for 1-cyclooctyl-2-aminopropane = 86 μM]. In order to determine if the aliphatic ring of these analogues bound to the same binding site as the phenyl ring of amphetamine and α-methylbenzylamine, the stereoselectivity of NMT toward the different compounds was determined. Stereochemical requirements for aromatic and nonaromatic inhibitors were similar (in all cases the *S* isomer was more potent at inhibiting NMT). The stereochemical preference expressed for phenylethanolamine substrates and corresponding nonaromatic analogues was also found to be the same; however, as the lipophilicity of the nonaromatic ethanolamine analogues was increased, a loss in both stereoselectivity and substrate activity occurred. The results presented here are consistent with an aromatic ring binding site that is part of, or bordered by, a large hydrophobic area. The larger, more hydrophobic nonaromatic phenylethanolamine derivatives are drawn into the hydrophobic area, which reduces side-chain hydroxy interactions necessary for substrate activity.
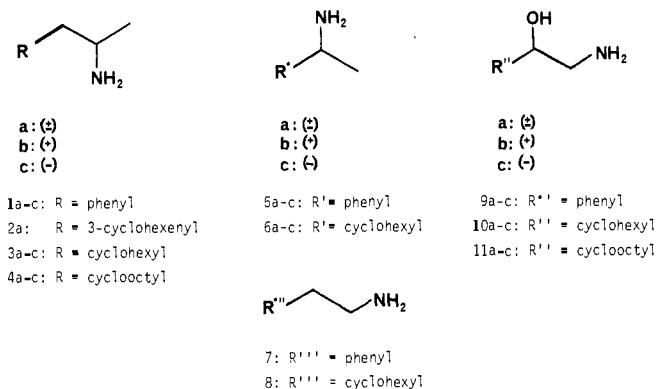
The last step in the biosynthetic pathway of epinephrine is the N-methylation of norepinephrine, which is catalyzed by the enzyme norepinephrine *N*-methyltransferase (EC 2.1.1.28), usually abbreviated as NMT (also called

phenylethanolamine *N*-methyltransferase, PNMT). *S*-Adenosyl-L-methionine (AdoMet) serves as the methyl donor in the reaction. Although NMT is primarily localized in the adrenal medulla, in 1974 Hokfelt et al.[2] employed immunohistochemical techniques to demonstrate the presence of NMT-containing cell bodies within the brainstem and hypothalamus; this observation led to the conclusion that epinephrine was utilized as a neurotransmitter. The fact that some of the NMT-containing nuclei within the brainstem had previously been thought to participate in the central control of blood pressure led several investigators to compare the NMT activity in the brainstem of spontaneously hypertensive rats (SHR) with normotensive controls; it was found that NMT activity was

(1) (a) Contents of this paper were presented at the Second Chemical Congress of the North American Continent and 180th National Meeting of the American Chemical Society, Law Vegas, NV, Aug. 25-29, 1980; see "Abstracts of Papers"; American Chemical Society: Washington, DC, 1980; Abstr MEDI 72. They were also presented at the 10th National Meeting of the Society of Neuroscience, Cincinnati, OH, Nov. 14, 1980, Abstr 286:5; *Soc. Neurosci. Abstr.* 1980, 6, 849. (b) Taken, in part, from the Ph.D. Dissertation submitted to the Graduate School of the University of Kansas by M.F.R., 1982. (c) Support provided by NIH predoctoral training Grant GM07775. (d) NSF undergraduate research participant, 1979 (Grant SP178-26939). (e) University of Kansas undergraduate research participant, 1980 (Grant KU-3944).

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Chart I

a: (±)
b: (+)
c: (-)

1a–c: R = phenyl
2a: R = 3-cyclohexenyl
3a–c: R = cyclohexyl
4a–c: R = cyclooctyl

a: (±)
b: (+)
c: (-)

5a–c: R' = phenyl
6a–c: R' = cyclohexyl

a: (±)
b: (+)
c: (-)

9a–c: R'' = phenyl
10a–c: R'' = cyclohexyl
11a–c: R'' = cyclooctyl

7: R''' = phenyl
8: R''' = cyclohexyl

significantly elevated in the SHR.[3-6] Similar changes in NMT activity have since been reported to occur in phenylethanolamine hypertensive rat models.[7,8] In an attempt to uncover the role of epinephrine and NMT in hypertension and other pathological conditions, several laboratories have developed potent in vitro and in vivo inhibitors of NMT.[9,10] Recently, some of these inhibitors have been reported to lower brainstem epinephrine levels in vivo and also to reduce blood pressure to normal in hypertensive rat models;[4,11-14] however, reports of adrenergic side effects (e.g., α-adrenoceptor blockade) for some of these compounds have made it impossible to attribute the hypotensive activity solely to the inhibition of brainstem NMT.[15-17]

The fact that questions concerning the role of epinephrine in the CNS could potentially be answered by more selective inhibitors of NMT prompted us to undertake an investigation of the binding requirements of substrates and inhibitors to the active site of the enzyme. The object of the present study has been to develop a conceptual model of the active site that would guide the design of novel and selective inhibitors of the enzyme. The initial focus of this investigation was to determine the nature of

the binding forces that involve the aromatic ring of bound substrates. We have reported that these binding forces appear to be primarily hydrophobic, by virtue of our finding that replacement of the phenyl ring of phenylethanolamine with more hydrophobic saturated structures, such as a cyclooctyl ring, yielded a significant increase in active-site affinity.[18] Extending this investigation to include a wide variety of hydrophobic analogues of phenylethanolamine (9a)[19-21] has allowed us to draw the following conclusions about the ring-binding region of the active site: (1) the region is primarily hydrophobic in character; (2) the minimum dimensions of this region are roughly 2-3 × 6-7 Å; and (3) charge-transfer, π-complex, or other binding forces which are more specific for aromatic systems are apparently not an important contributor to binding in this region.

Some of the saturated ethanolamines prepared earlier, such as cyclooctylethanolamine (11a, Chart I) have been found to be potent alternate substrate inhibitors for NMT.[19] Since compounds such as 11a are less related structurally to the catecholamines than many of the presently used NMT inhibitors, it occurred to us that these compounds might be more selective in their action in vivo. Compound 11a has been found to be a weaker α₂-receptor antagonist than some other widely studied NMT inhibitors, lending support to this hypothesis.[16,22] However, alternate substrate inhibitors have drawbacks, including the obvious fact that they are substrates for NMT and, therefore, subject to conversion by the enzyme to N-methyl derivatives of unknown activity, as well as other possible metabolic conversions following in vivo administration. The potential utility of 11a and similar inhibitors for animal studies must, therefore, be approached with caution.

With this in mind, we have sought to apply our previous findings to the development of some novel nonaromatic dead-end inhibitors of NMT that might be more suitable for in vivo studies. The prototype competitive inhibitors of this enzyme are the phenylethylamines and the benzylamines, and as reported by Fuller and co-workers,[23,24] the α-methyl derivatives of these compounds retain the ability to inhibit NMT and yet are resistant to metabolic inactivation by monoamine oxidase (MAO). In order to determine if nonaromatic derivatives of these structural classes could be effective inhibitors of NMT, we have prepared some fully saturated analogues of amphetamine (α-methylphenylethylamine, 1a) and α-methylbenzylamine (5a) for in vitro evaluation. We have also considered the possibility that the hydrophobic region that we have been characterizing in NMT might not, in fact, represent the true ring-binding site. The fact that the catechol ring of the natural substrate norepinephrine is a relatively hydrophilic structure would argue for this possibility. Although NMT is not highly stereoselective in comparison to the dramatic selectivity displayed by some other en-

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Scheme I
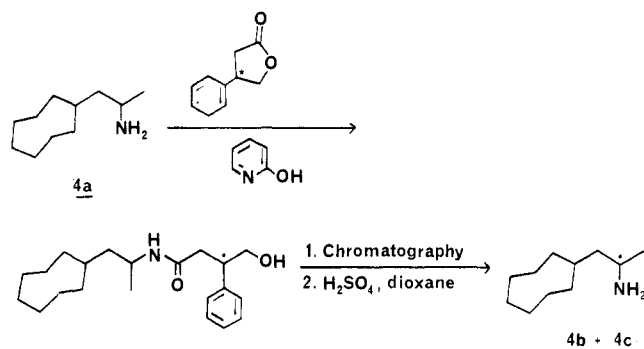
zymes, significant differences in activity have been reported for the enantiomers of both substrates and inhibitors. Since this stereoselectivity is dependent upon the composition of the active site in the vicinity of the side chain of bound ligands, we considered that interaction of the hydrophobic ring of nonaromatic compounds with an area of the active site that is distinct from the aromatic ring-binding site would change the orientation of the side chain. This could result in a difference in the stereochemical behavior of NMT toward nonaromatic substrates and inhibitors, relative to the corresponding aromatic compounds. We report here the synthesis, resolution, and activities of the stereoisomers of some nonaromatic substrates and inhibitors, along with the enantiomers of phenylethanolamine, amphetamine, and α-methylbenzylamine, as a means of probing for different active-site orientations arising from different ring-binding sites.

Chemistry. The cyclohexyl analogues 10a–c, 3a–c, and 6a–c were obtained by reduction of phenylethanolamine (9a–c), amphetamine (1a–c), and α-methylbenzylamine (5a–c) using PtO_2 as the catalyst. Optically active phenylethanolamine was prepared according to Meyers and Slade[25] starting with the appropriate isomer of mandelic acid. Resolution of cyclooctylethanolamine (11a) was accomplished by repeated crystallization of the (+)- or (−)-*O*,*O*-dibenzoyltartrate salts from methanol. The cyclooctyl analogue of amphetamine, 1-cyclooctyl-2-aminopropane (4a), was prepared by treatment of cyclooctylacetic acid with excess methyllithium, followed by conversion of the resulting methyl ketone to the oxime and reduction. This procedure is a modification of the method described by McCarthy and Kahl.[26] A similar approach, starting with 3-cyclohexenylacetonitrile, was adopted for the synthesis of 2a. Attempts to resolve 4a by fractional crystallization of the salts of several optically active acids were not successful; however, clean separation of the enantiomers of 4a was achieved by employing the method of Helmchen et al.,[27] which is illustrated in Scheme I. Optically active 3-phenylbutyrolactone, which had been resolved chromatographically as the (+)-α-methylbenzylamine derivative, was condensed with racemic 4a to afford a mixture of diastereomeric amides. These amides were conveniently separated by preparative medium-pressure chromatography on silica gel and, after hydrolysis, gave (+)- and (−)-4a (4b and 4c). Gas chromatographic analysis of the *N*-(trifluoroacetyl)-*S*-prolinamide (TPC) derivatives showed the separated enantiomers to be greater than 90% optically pure and allowed tentative assignment of the *S* absolute stereochemistry to the (+) isomer 4b by

Table I. NMT Inhibition Constants for Aromatic and Nonaromatic Amphetamines and α-Methylbenzylamines

no.	absolute config	$K_i \pm$ SEM, μM
1a	(±)	740 ± 68
1b	S(+)	422 ± 43
1c	R(−)	1381 ± 147
2a	(±)	110 ± 13
3a	(±)	109 ± 11
3b	S(+)	22 ± 6
3c	R(−)	290 ± 64
4a	(±)	86 ± 34
4b	S(+)	34 ± 6
4c	R(−)	157 ± 7
5a	(±)	460 ± 52
5b	R(+)	1679 ± 209
5c	S(−)	149 ± 13
6b	R(+)	412 ± 41
6c	S(−)	44 ± 7
7		467 ± 75
8		41 ± 9

^{a} Indicated direction of rotation is of the free base at 589 nm.

Table II. Substrate Constants for Aromatic and Nonaromatic Ethanolamines

no.	absolute config	$K_m \pm$ SEM, μM	$V_{max}{}^a$	$V_{max}/K_m \times 100$
9a	(±)	108 ± 23	1.75	1.62
9b	S(+)	96 ± 15	0.92	0.93
9c	R(−)	286 ± 130	4.85	1.69
10a	(±)	40 ± 10	0.99	2.47
10b	S(+)	34 ± 5	0.64	1.88
10c	R(−)	76 ± 15	1.85	2.43
11a	(±)	43 ± 4	0.27	0.62
11b	(+)	40 ± 15	0.27	0.67
11c	(−)	43 ± 6	0.38	0.88

^{a} Units of V_{max} are nanomoles of product per milligram of protein per minute.

virtue of the order of the retention times for the TPC-derivatized enantiomers.[28]

Biochemistry. For this study, we employed a radiochemical assay that has been detailed elsewhere.[29] A partially purified preparation of NMT derived from bovine adrenal glands was used for this study. The K_m and K_i values presented here are all expressed in micromolar units, unless otherwise stated, and V_{max} values were calculated in terms of nmoles of product formed per milligram of protein per minute. Since the object of this investigation was to determine the relative activities of the enantiomers of the substrates in Table II and not the absolute values for K_m and V_{max} in each case, we did not correct the values presented for the extraction efficiency of each compound in the toluene/isoamyl alcohol step of the radiochemical assay.

Results and Discussion

In Table I the K_i values for the fully saturated analogues of amphetamine and α-methylbenzylamine are presented. In all cases, competitive inhibition (Lineweaver–Burk analysis) with respect to the substrate (±)-phenylethanolamine was observed. Comparison of the activities found for nonaromatic derivatives 2a, 3a, and 4a with amphetamine (1a) reveals that, as was the case with the

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saturated phenylethanolamine analogues, replacement of the aromatic ring with more hydrophobic structures results in increased binding affinity for the active site. The most hydrophobic of these derivatives, **4a**, was also the most active, being approximately 10 times as potent as **1a** at inhibiting NMT. Likewise, a consideration of the values found for **5a–c** and the cyclohexyl analogues **6b,c** shows that the same holds true for α-methylbenzylamines and indicates that the preparation of nonaromatic inhibitors of NMT is a valid approach toward potentially more specific agents.

The stereochemical factors for binding of these nonaromatic inhibitors may be assessed by an examination of the inhibition constants in Table I for the enantiomers of amphetamine analogues **3a** and **4a** and α-methylbenzylamine analogue **6a**. Fuller et al.[23] found the $S(+)$ isomer of amphetamine to be approximately three times more potent at inhibiting the enzyme than the $R(-)$ isomer. The K_i values for **1b** and **1c** in Table I agree with this finding. For the fully saturated derivatives, we found the S isomer was again more potent, by 7- to 10-fold over the R isomer, which represents a slightly greater display of stereoselectivity for the nonaromatic analogues. Fuller[30] also reported that for α-methylbenzylamines the S isomer was the better inhibitor by approximately 10-fold; the values that we found for the cyclohexyl derivatives **6b** and **6c** agree very closely with these findings. Taken together, the results presented in Table I for the fully saturated inhibitors suggest that the orientation of the hydrophobic analogues within the active site is similar to that assumed by the aromatic compounds, since the absolute stereoselectivity and relative stereoselectivity of NMT toward these two different types of compounds are essentially the same. This would argue in favor of the hydrophobic and aromatic ring binding sites being either the same site or being two different sites located very near one another, such that the features of the active site which result in one isomer being preferentially bound are the same for both aromatic and nonaromatic inhibitors.

In Table II are presented the kinetic data found for some fully saturated ethanolamine substrates. Axelrod[31] and Fuller et al.[32] have both observed that NMT displays a slight preference for the natural $R(-)$ isomer of norepinephrine, which is methylated approximately twice as fast as the $S(+)$ isomer. We found for phenylethanolamine that, although the S isomer **9b** has a lower value for K_m, consideration of V_{max} values reveals that the R isomer **9c** is the preferred substrate by approximately 1.8-fold (ratio of the V_{max}/K_m terms for **9b** and **9c**). For the cyclohexylethanolamines **10b** and **10c**, similar results were obtained in that the S isomer **10b** possessed a K_m value that was approximately one-half that found for the R isomer; however, when the V_{max} terms for **10b–c** were considered again, it became apparent that the R isomer was the better substrate, but the magnitude of stereoselectivity was found to be smaller (1.3-fold). Surprisingly, however, the results for the cyclooctyl derivative **11a** were significantly different in that no difference could be detected in the K_m values for the resolved isomers. The V_{max} was also found to be significantly lower for **11a** than for the closely related **10a** despite the fact that the K_m values for **10a** and **11a** were very similar, suggesting that **11a** was a much poorer substrate for NMT than **10a**. We had previously found that

11a was a very potent inhibitor of NMT with an IC_{50} of 7 μM,[19] which indicated that the diminished substrate activity of **11a** did not result from a decreased capacity to bind to the active site. The dropoff in substrate activity might be accounted for if the binding of the side-chain hydroxy group in **11a** was being interfered with, since it is well established for NMT that the ethanolamine side chain is a virtual requirement for substrates.[31] Since the hydroxy group is also directly attached to the asymmetric center of **11a**, decreased interaction between this group and its binding site could also account for the loss of stereoselectivity by NMT toward **11a**. In an attempt to assess the importance of the contribution of the side-chain hydroxy group toward binding of nonaromatic substrates to NMT, relative to phenylethanolamine, we compared the K_i values found for phenylethylamine (**7**) and cyclohexylethylamine (**8**) with the K_m values of **9a** and **10a**. Compounds **7** and **8** are included in Table I. Phenylethylamine is a relatively weak inhibitor of NMT, with a K_i of 467 μM; in contrast, **8** is nearly 12 times more potent, with a K_i of 41 μM. This dramatic difference in active-site affinities is not reflected in the K_m values for the substrates **9a** and **10a**, in which the difference is only 2.5-fold. While K_m is not a true affinity constant, there is a dependence of K_m upon the substrate affinity constant. The above data, therefore, suggest that the hydroxy group is a more important contributor toward binding of aromatic substrates, such as **9a**, than it is for more hydrophobic derivatives, since omitting the side-chain hydroxy from **9a** yields **7**, which is a poor ligand for the active site. The nonhydroxylated derivative of **10a** is still tightly bound, as indicated by the low K_i value for **8**.

The loss of binding interactions between the hydroxy group and its binding site for hydrophobic ethanolamines is consistent with a change in orientation of the ethanolamine side chain relative to aromatic substrates. It is apparent that the fully saturated derivatives, such as **10a** and **11a**, are bound such that the hydroxy group is held farther away from its binding site than is the case for **9a**, and the distance that the hydroxy group is removed seems to increase with increasing hydrophobic character of the ring portion of the substrate (going from **10a** to **11a**). For the cyclooctyl derivative **11a**, this effect reaches the point at which no significant interaction can occur between the hydroxy group and the binding site, resulting in a loss of stereoselectivity for binding and greatly diminished substrate activity. A reasonable explanation to account for this effect is that the hydrophobic and aromatic rings are bound to slightly different regions of the active site, which would occur if the ring binding site was comprised of a large hydrophobic area adjacent to the "true" ring binding site. Since our earlier investigations[19,20] failed to detect any binding interactions other than hydrophobic forces in this region, it is suggested that the aromatic ring of substrates such as **9a** is normally bound at one end of the hydrophobic region. As the lipophilicity of the ring portion of the substrate is increased, the substrate is attracted deeper into the hydrophobic region, which affects the orientation of the side chain within the active site. As discussed earlier, no significant difference could be detected in the stereochemical behavior of NMT toward aromatic and nonaromatic inhibitors; in this case, substrate activity was a more sensitive indicator of changes within the active site that were not of sufficient magnitude to be observed with inhibitors alone. Our ring-binding hypothesis is depicted diagrammatically in Figure 1.

In summary, we have presented evidence that nonaromatic analogues of dead-end inhibitors of NMT are

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are continuing to refine and extend the observations presented in this report.

Experimental Section

All melting points were determined on a Thomas-Hoover capillary melting point apparatus calibrated with known compounds. Combustion analyses were performed on a Hewlett-Packard 185B CHN Analyzer at the University of Kansas. NMR spectra were recorded in CCl_4 or $CDCl_3$ with Me_4Si as the internal standard on either a Varian EM-360 or T-60 spectrometer. IR spectra were taken on either a Beckmann IR-33 or a Perkin-Elmer 727 spectrophotometer. Mass spectra were obtained on a Varian Atlas CH-5 electron-impact mass spectrometer. Gas chromatographic analyses were performed on a Varian 3700 gas chromatograph. Optical rotations were determined on a Perkin-Elmer Model 141 polarimeter at 589 nm.

Compounds 5a,b and 7 were purchased from Aldrich Chemical Co. and converted to the HCl salts where necessary before use. S-Adenosyl-L-methionine was purchased from Sigma Chemical Co. Methyllithium was obtained from Alfa Products, Inc., and N-(trifluoroacetyl)-S-prolinamide (TPC) was purchased from Regis Chemical Co. [^{3}H]-S-Adenosyl-L-methionine (CH_3 labeled) was purchased from New England Nuclear Corp. Analytical thin-layer plates (silica gel, 0.1-mm precoated plastic plates) were purchased from Brinkmann. All other starting materials, unless specified, were purchased from Aldrich Chemical Co. Solvents were routinely distilled just prior to use; anhydrous ether and THF were obtained by distillation from sodium-benzophenone ketyl, and toluene was dried by distillation from CaH_2. Compounds 3a-c and 6b-c were prepared by catalytic reduction of 1a-c and 5b-c according to published methods.[33] Unless specified as absolute, ethanol and EtOH refer to 95% ethanol.

Synthesis of 1-Cyclooctyl-2-aminopropane (4a). To a solution of 1.0 g of cyclooctylacetic acid[26] (5.88 mmol) in 75 mL of freshly distilled dry THF was added 6.34 mL of methyllithium (2.04 M solution; 12.9 mmol) under N_2 at room temperature. The milky solution was then brought to reflux and maintained for 18 h. The clear reaction mixture was allowed to cool to room temperature and was then treated with 25 mL of 2 N HCl over a period of several minutes. The mixture was evaporated in vacuo to approximately 20 mL, at which point it was extracted with ether (3 × 25 mL). The pooled ether layers were dried over $MgSO_4$ and evaporated to yield 823 mg of crude 1-cyclooctyl-2-propanone. The product was purified by chromatography on silica gel [2.5 × 10 cm bed of 70–230 mesh eluting with hexane/ethyl acetate (11:1)] to yield 565 mg (57%) of pure ketone: NMR (CCl_4) δ 2.18 (2 H, br s, CH_2CO), 1.97 (3 H, s, CH_3); IR (film) 1718 (C=O) cm^{-1}.

The above ketone was dissolved in 15 mL of pyridine/ethanol (1:1) to which 0.7 g (3 equiv) of $NH_2OH·HCl$ was added .n one portion. The mixture was heated at reflux for 3 h. Most of the solvent was removed by evaporation in vacuo, after which the residue was extracted several times with small portions of ether. The pooled ether layers were then washed with 1 N HCl (2 × 20 mL) and dried over Na_2SO_4. Evaporation left 611 mg (99%) of pale yellow oxime, with no detectable ketone by TLC or by IR: IR (film) 3550–3000 (N–OH stretch), 1660, 1460, 1440 cm^{-1}. The oxime was not purified further but was transferred to a Parr shaker bottle in approximately 50 mL of 95% EtOH. Concentrated HCl (0.5 mL) was added, followed by 60 mg of PtO_2 as a slurry in 10 mL of 95% EtOH. Catalytic reduction of the mixture was performed on a Parr shaker apparatus at an initial hydrogen pressure of 40 psi. The reaction was complete after 24 h, at which time the reaction was stopped and the suspension was filtered to remove the catalyst. Evaporation left a white residue, which was treated with 1 N NaOH and extracted with ether (3 × 50 mL). After drying (K_2CO_3), the ether layers were evaporated to leave a pale yellow oil, which was purified by bulb to bulb distillation [bp 62–66 °C (0.05 mm)] to yield 380 mg (67%) of purified 4a: NMR (CCl_4) δ 4.83 (2 H, NH_2, D_2O exchangeable), 2.93 [1 H, m, CH(NH_2)], 1.8–0.9 ppm (20.6 H, br m); IR (film) 3380, 3290 cm^{-1}; MS (70 eV), m/e (relative intensity) 170 ($M^+ + 1$, 3.8), 169 (M^+, 7), 154 ($M^+ - 15$, 26), 152 (31), 124 (27), 111 (22), 110 (27), 109 (26), 97 (11), 96 (19), 95 (27), 83 (15), 82 (51), 81 (42), 72 (20), 71 (15), 70
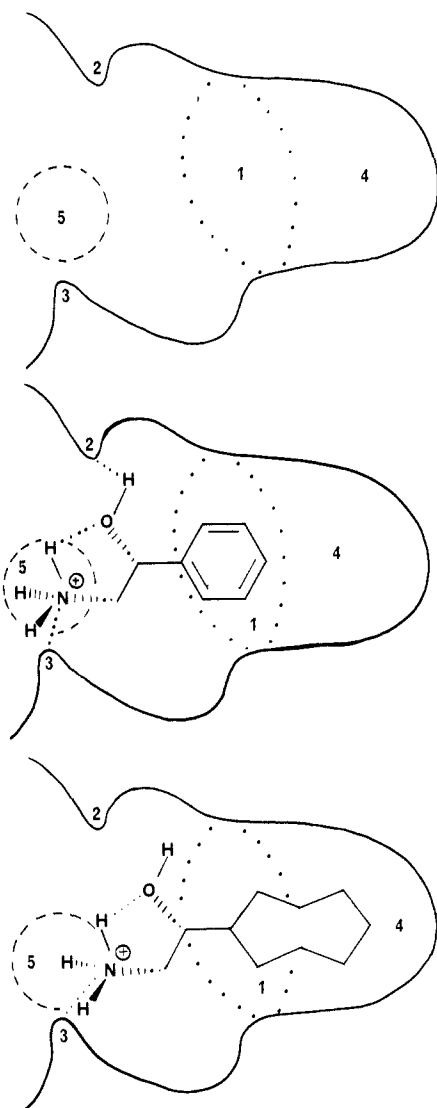

Figure 1. (a) Schematic representation of the NMT phenylethanolamine binding site. Area 1 corresponds to the proposed aromatic ring binding site; 2 is the side-chain hydroxy binding site; 3 is the amino group binding site; and 4 represents the hydrophobic component of the ring binding area. Area 5 is a proposed "methylation zone" within which the amino group must be located in order for methyl transfer to occur. (b) This figure depicts the postulated binding mode for (R)-(-)-phenylethanolamine. The ethanolamine side chain is in a fully extended conformation in order to allow the amino group to interact with site 3 and lie within methylation zone 5. This conformation is possibly promoted and stabilized by intramolecular hydrogen bonding as illustrated. Hydroxy binding site 2 is pictured as being an H-bond acceptor. (c) This figure represents the proposed binding mode for 11a. If the cyclooctyl ring is attracted into hydrophobic area 4, then the side-chain amino and hydroxy groups will be pulled away from the optimal locations for substrate activity. The amino group can still bind to site 3 as indicated by the potent competitive inhibition shown by 11a; however, the loss of stereoselectivity is consistent with diminished interaction with site 2. The reduced substrate activity for nonaromatic ethanolamines is likely due to the amino group being outside the "methylation zone" 5.

themselves significantly more potent than their aromatic parent. The stereochemical requirements for both inhibitor and substrate activity are similar for aromatic and nonaromatic compounds; however, increasing hydrophobic character of the ring portion of substrates produces a gradual loss in substrate activity and in stereoselectivity. These findings are consistent with a ring binding site that is composed of a large hydrophobic area that contains or is adjacent to the aromatic ring binding site. Investigations

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(71), 69 (23), 68 (48), 44 (100). The HCl salt was recrystallized from ethanol/ethyl acetate, mp 187–190 °C (lit.[34] mp 182–184 °C). Anal. ($C_{11}H_{23}N\cdot HCl$) C, H, N.

Resolution of 4a. Optically active 3-phenylbutyrolactone was prepared by the method outlined by Helmchen et al.[27] [mp 57–58 °C; [α]^{25}$_D$ 46.6° (c 1.95, CH_2Cl_2)]. To a solution of 578 mg (3.57 mmol) of the lactone was added 339 mg (3.57 mmol) 2-hydroxypyridine and 402 mg (2.38 mmol) of 4a in 25 mL of dry toluene. The mixture was brought to reflux under N_2 and maintained for 26 h. After addition of 50 mL of $CHCl_3$, the mixture was extracted with 1 N HCl (3 × 10 mL), followed by H_2O (2 × 10 mL). The organic layer was dried over Na_2SO_4 and evaporated, leaving 785 mg of a brownish residue. This residue was applied to a 2.5 × 30 cm medium-pressure liquid chromatography column of silica gel 60 (Merck, 270–400 mesh) which had been equilibrated with $CHCl_3$/THF (9:1) and eluted with the same solvent at 10 mL/min. Two amides were easily separated (TLC R_f values of 0.24 and 0.14, same solvent) to yield 202 mg of amide I ([α]^{27}$_D$ +10.7° (c 0.22, CH_2Cl_2); R_f 0.24) and 182 mg of amide II ([α]^{24}$_D$ +20.3° (c 0.30, CH_2Cl_2); R_f 0.14). The amides were hydrolyzed by refluxing in 10 mL of a 1:1 mixture of dioxane/1 M H_2SO_4 for 24 h to yield 64.4 mg of amine I and 64.8 mg of amine II (as the free base). Both amines were converted to the HCl salts and recrystallized from ethanol/ethyl acetate.

For amine I (4c): rotation of the free base in $CHCl_3$, [α]^{22}$_D$ −5.26° (c 0.19); rotation of the HCl salt in absolute EtOH, [α]^{22}$_D$ +7.8° (c 0.47); mp 194.5–195.5 °C. Anal. ($C_{11}H_{23}N\cdot HCl$) C, H, N.

For amine II (4b): rotation of the free base in $CHCl_3$, [α]^{22}$_D$ +5.38° (c 0.14); rotation of the HCl salt in absolute EtOH, [α]^{22}$_D$ −8.4° (c 0.47); mp 195 °C.

We evaluated the optical purity of both amines by converting the free base to the TPC derivatives, using the procedure provided by Regis Chemical Co. Both samples were applied to a 10 ft, 0.25 in. glass column of 3% SE-30 on Chromosorb W-AW, 80–120 mesh, at an oven temperature of 250 °C (isothermal analyses). The derivatized amine I (4c) had a retention time of 11.9 min and that of amine II (4b) had a retention time of 12.7 min. The calculated optical purities of the two amines based on the relative peak areas were 90 and 92%, respectively. The amine I (4c) with the shorter retention time was assigned the R configuration (see text).

Resolution of Cyclooctylethanolamine (11a). To a solution of (+)-dibenzoyltartaric acid monohydrate (994 mg, 2.64 mmol) in 18 mL of hot anhydrous methanol was added 452 mg (2.60 mmol) cyclooctylethanolamine (11a),[19] and the solution was heated to a boil until the total volume was reduced to approximately 12 mL by evaporation. The clear solution was allowed to cool slowly to room temperature, and the flask was capped to prevent further evaporation. Large, clear crystals slowly formed, which were recovered and recrystallized from methanol. The initial crystals had a melting point of 178–187 °C dec; further recrystallization eventually yielded crystals with mp 204–205 °C dec. This level was generally achieved after three recrystallizations; however, in one instance five recrystallizations were necessary in order to reach this melting point. Further recrystallization did not produce any change in melting point. Parallel to the changes observed in the melting point was the gradual change in the optical rotation of the free base recovered from these crystals, which also leveled off when the melting point was maximized. The rotation of the free base of the isomer of 11 that was resolved by (+)-dibenzoyltartrate (11b): [α]^{22}$_D$ +5.37° (c 1.75, absolute EtOH); for the isomer resolved by (−)-dibenzoyltartrate (11c): [α]^{22}$_D$ −5.21° (c 2.46, absolute EtOH). The amines were converted to their HCl salts, mp 232–234 °C [lit.[19] mp for (±), 228–229 °C].

(+)- and (−)-2-Cyclohexyl-2-hydroxyethylamine (10b,c). Phenylethanolamine [9b; 50 mg; [α]^{21}$_D$ +32.3° (c 0.44, absolute EtOH)][35] was dissolved in 20 mL of EtOH containing 1 mL of

1 N HCl. PtO$_2$ (10 mg) was added, and the mixture was hydrogenated at 50 psi initial pressure over a period of 7 h. Filtration of the catalyst and evaporation left 10b as the HCl salt, which was recrystallized from EtOH/ethyl acetate, mp 205–206.5 °C [lit.[18] mp for (±), 204–206 °C]. The free base (34.5 mg) was recovered as a waxy solid: [α]^{22}$_D$ +10.35° (c 0.61, absolute EtOH); NMR ($CDCl_3$) showed no evidence of aromatic protons. Anal. ($C_8H_{17}NO\cdot HCl$) C, H, N.

From 59.2 mg of 9c was obtained, in the same manner, 43 mg of 10c free base: [α]^{22}$_D$ −10.22° (c 1.875, absolute EtOH). Anal. ($C_8H_{17}NO\cdot HCl$) C, H, N.

2-Cyclohexylethylamine (8). Compound 7 (12.45 g, 0.102 mol) was catalytically reduced as described for 10b,c to yield, after recrystallization (EtOH/ethyl acetate), 11.8 g (68%) of 8·HCl, mp 255–262 °C (lit.[36] mp 252–253 °C). Anal. ($C_8H_{17}N\cdot HCl$) C, H, N.

1-(3-Cyclohexenyl)-2-aminopropane (2a). (3-Cyclohexenyl)acetonitrile[37] (2.5 g, 21 mmol) was dissolved in 125 mL of dry ether under N_2 and cooled to 3 °C in a flask equipped with reflux condenser and a septum-capped addition funnel. Into the addition funnel was transferred 1.36 g of methyllithium (62 mmol; 47 mL of a 1.32 M solution). The methyllithium was added dropwise, and the mixture was maintained at 3 °C for 2 h with stirring, followed by 2 h at room temperature. The reaction was quenched by the addition of 20 mL of saturated NH_4Cl and extracted with ether (3 × 100 mL), and the pooled ether layers were dried ($MgSO_4$) and evaporated to leave 2.42 g of an oily residue. The oil was applied to a 2.5 × 100 cm medium-pressure liquid chromatography column packed with silica gel 60 (270–400 mesh, Merck) which was equilibrated in hexanes/benzene (1:1). Elution with the same solvent mixture yielded 425 mg of starting nitrile and 1.16 g (39% yield) of 1-(3-cyclohexenyl)-2-propanone: IR (film) 3022, 1708 (C=O) cm^{-1}; NMR (CCl_4) δ 5.57 (br s, 2 H, olefin), 2.08 (s, 3 H, CH_3), 2.2–0.95 (br m, 9 H).

The oxime was prepared as follows: 1.64 g (12 mmol) of the above ketone was heated at reflux in 15 mL of pyridine/ethanol (1:1) containing 2.48 g $NH_2OH\cdot HCl$ (35.7 mmol) for a period of 2 h. Evaporation and extraction of the residue with ether yielded 2.44 g of oxime as an oil (95%): IR (film) 3250 (br, OH) cm^{-1}. No further purification was necessary prior to the next step. Oxime (693 mg, 4.53 mmol) was taken up in 3 mL of dry benzene and added via syringe to a solution of Red-Al (5.0 mL of a 70% solution) in 30 mL of dry benzene at reflux. Heating was continued for 2 h, after which the reaction was quenched by pouring the mixture onto 100 g of ice containing 5 mL of 10% NaOH. The resulting mixture was filtered through a 2-cm pad of Celite which was washed extensively with small portions of benzene. The benzene layer was dried (KOH pellets) and evaporated to leave a reddish oil, which was distilled [bp 81–83 °C (22 mm)] to leave amine 2a (462 mg, 73% yield): IR (film) 3400, 3280 (NH_2) cm^{-1}; NMR ($CDCl_3$) δ 5.50 (br s, 2 H, olefin), 2.96 (doublet of triplets, J = 6 Hz, 1 H, $CHNH_2$), 1.26 (br s, 2 H, D_2O exchangeable, NH_2), 1.05 (d, J = 6 Hz, 3 H, CH_3), 2.2–0.95 (br m, 10 H); MS (70 eV), m/e (relative intensity) 139 (M$^+$, 7.3), 124 (3), 81 (8.3), 80 (11), 79 (8), 57 (9.7), 45 (5.3), 44 (100).

The amine was converted to the sulfate salt by careful dropwise addition of 5% H_2SO_4 in ether to an ethereal solution of 2a, filtering after the addition of each 6 drops of acid solution. The precipitated salt was recrystallized from EtOH and ethyl acetate, mp 274–278 °C dec. Anal. [($C_9H_{17}N)_2SO_4\cdot H_2O$] C, H, N.

Biochemistry. The assay employed in this study has been described elsewhere.[29] Briefly, a typical assay mixture consisted of 25 μL of 15 mM AdoMet, 25 μL of substrate solution, 5 μL of [^{3}H]AdoMet (C^{3}H$_3$, 15 Ci/mmol; diluted so that a 5-μL aliquot contained approximately 2 × 10^6 dpm), 25 μL of inhibitor solution, 50 μL of 0.5 M phosphate buffer (pH 8.0), 25 μL of enzyme preparation (from bovine adrenal glands; purified through the isoelectric step according to Connett and Kirshner[38] and stored frozen prior to use), and H_2O sufficient to bring the final volume to 250 μL. The mixture was incubated for 30 min at 37 °C, after which the reaction was quenched by the addition of 250 μL 0.5

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M borate, pH 10. To this was added 2 mL of toluene/isoamyl alcohol (7:3), and the phases were mixed and centrifuged to facilitate separation. An aliquot (1 mL) was transferred to a counting vial, which contained 5 mL of scintillation cocktail (3a-70; Research Products International Corp.) for counting. Substrate constants were calculated by a hyperbolic curve-fitting program and also by linear regression of the reciprocal plots of the data; numbers calculated by the two different methods were in good agreement. Racemic phenylethanolamine was used as the variable substrate for the determination of inhibitor constants, with at least three different concentrations of inhibitor used to calculate each K_i value.

Directional Probes of the Hydrophobic Component of the Aromatic Ring Binding Site of Norepinephrine N-Methyltransferase[1a]

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We investigated the directional nature of the bulk tolerance and hydrophobic binding in the aromatic ring binding region of the active site of norepinephrine N-methyltransferase (NMT) by comparing the substrate and inhibitor activities of m- and p-phenyl-substituted derivatives of amphetamine, phenylethanolamine, and α-methylbenzylamine. The para isomers of amphetamine and phenylethanolamine displayed significantly greater activities as inhibitor and substrate, respectively, than the meta isomers, which indicated that the bulk tolerance was near the para position. For benzylamines, the greatest inhibitory activity was observed for the meta isomer, demonstrating a significant difference in the binding requirements for phenylethylamines and benzylamines. These findings are consistent with a two-state model for the NMT active site that has been proposed elsewhere to account for its ability to bind both benzylamines and phenylethylamines in a fully extended side-chain conformation.

There now exists a considerable body of evidence that relates the increased activity of certain brainstem and hypothalamic nuclei, which appear to utilize epinephrine as their neurotransmitter, to the development of hypertension.[2-5] As a result, development of a means of selective modulation of epinephrine levels in these regions has become an attractive target for drug design. A possible approach to reduction of epinephrine levels without directly affecting other catecholamine levels is by the inhibition of norepinephrine N-methyltransferase (NMT, EC 2.1.1.28; also known as phenylethanolamine N-methyltransferase, PNMT), which is the only enzyme unique to the biosynthesis of epinephrine. In order to establish an experimental basis for the rational design of inhibitors of this enzyme, we have been attempting to characterize the active site of NMT by determining the optimum structural requirements for binding of substrates and inhibitors.[6-8] One aspect of this investigation has led to the conclusion that the region of the active site that secures the aromatic ring of substrates such as phenylethanolamine is primarily hydrophobic in character and can accommodate structures much larger than the phenyl ring.[8] The region appears to be roughly rectangular, with dimensions of 2-3 × 6-7 Å.

Aromatic substrates and inhibitors are apparently bound to one end of this region, while more hydrophobic nonaromatic analogues tend to be attracted toward the center of the hydrophobic area.[9] Since the ring binding region promises to be an important factor in the design of novel and selective inhibitors for NMT, we have continued our investigation of this binding region.

Directionality in hydrophobic binding by the NMT active site has been suggested by Hansch and Glave as an interpretation of a QSAR correlation of the inhibitory activity of some ring-substituted amphetamines.[10] Their conclusion was that hydrophobic substituents contributed toward affinity only when attached to the ortho and/or the meta position but not the para position, which was taken as evidence of a hydrophobic "cleft" near the ortho and meta positions of the bound phenyl ring. A drawback in this earlier investigation, however, is that the size of the substituents employed, particularly in the meta position, was not large enough to allow unambiguous determination of the directionality of the bulk tolerance of the active site.

The phenyl substituent appeared to be well suited for probing the bulk tolerance and the directional nature of the hydrophobic region, since it is considerably larger and more hydrophobic than the substituents previously examined. In addition, the phenyl ring is planar, and we know from previous studies that the hydrophobic region best accommodates planar structures and has a severely limited "height" tolerance.[7,11] While the phenyl substituent extends the conjugated system and could enhance binding interactions involving the π electrons (π complex, charge transfer), our previous studies[7] have suggested this binding interaction to be minimal within the NMT active site. The phenyl substituent has little σ character, so that any

(1) (a) This paper has been taken, in part, from the Ph.D. Dissertation submitted to the Graduate School of the University of Kansas by M.F.R., 1982. (b) Supported by NIH Predoctoral Training Grant GM 07775.

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