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Structure–Activity Relationships for Potent Phthalane and Thiophthalane Inhibitors of Norepinephrine Uptake

A Comparison with Desipramine and Related Compounds

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SUMMARY

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The bicyclic phthalane and thiophthalane compounds, Lu 3-010 and Lu 5-003, although containing a ring system which is distinct from the ring system of tricyclic antidepressants, nonetheless, are approximately equipotent with tricyclic compounds in inhibiting the amine pump in adrenergic neurons. The Lu compounds require the presence of a phenyl ring on the bicyclic nucleus for full potency to be expressed. Molecular models of both of the enantiomers of the Lu compounds, when superimposed on a model of the ring system of desipramine, have their phenyl rings coincide closely with the phenyl rings of desipramine. With the enantiomers in these conformations the orientation of their propylamine side chains with respect to the phenyl rings is very similar to the orientation seen in desipramine. In addition, the Lu compounds have methyl groups on their bicyclic ring system which are involved in binding to the receptor. There are no analogous groups in the tricyclic system. The data suggest that the receptor for the amine pump in peripheral and central adrenergic nerve membranes, in addition to having primary sites for binding phenethylamine substrates, also has additional ancillary binding sites to which inhibitors attach themselves. Furthermore, the data also suggest that the number of ancillary sites is greater than is utilized by any one class of inhibitors. Although desipramine and the Lu compounds have binding sites in common, it appears that the Lu compounds are equipotent with desipramine only because they bind to an additional site.

INTRODUCTION

Maxwell *et al.* (1–7) have developed a hypothesis which accounts, in molecular terms, for the high potency of the tricyclic antidepressants as inhibitors of the amine pump in adrenergic neuronal membranes and for the weaker activity of analogous diphenyl and monophenyl compounds. In brief, the receptor for the amine pump was surmised to have one primary site for binding the phenyl ring and a second primary site for binding the protonated nitrogen atom of phenelthylamine substrates (e.g., norepinephrine). In addition, it was concluded that the receptor possessed an ancillary site for binding a second phenyl ring (a constituent of many potent inhibitors). The data indicated further that these two sites for binding the phenyl rings, one primary and one ancillary, were arranged spatially so that optimal binding occurred when the rings of an inhibitor were held in a conformational relationship like the one in the inflexible, noncoplanar tricyclic ring system. The lower affinity of dicyclic compounds (diphenyls) was attributed in part to the fact that their phenyl rings, unlike those of the tricyclic compounds, were relatively free to rotate and therefore to have considerable differences in their orientation to one another. Hence, they were not optimally disposed for binding (Fig. 1). Finally, the data indicated that a second ancillary binding site was present that accepted the single methyl group attached to the exocyclic nitrogen atom in the tricyclic secondary methylamine derivatives.

Carlsson *et al.* (8) and Petersen *et al.* (9) reported on a series of phthalane and thiophthalane inhibitors. These compounds had a ring system which was quite distinct from that of tricyclic antidepressants, nonetheless, some members of the series were very potent (Fig. 1). Two

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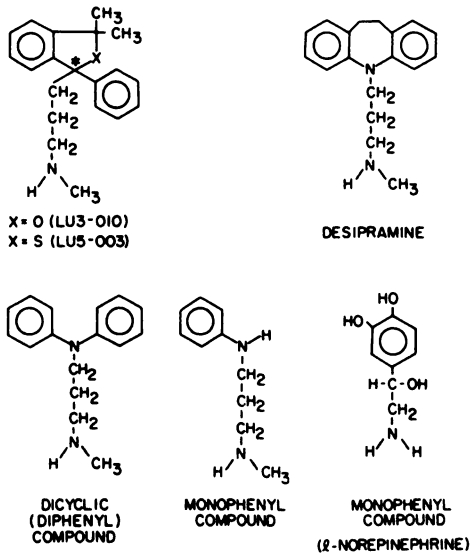


FIG. 1. *Structural formulas for Lu compounds, desipramine, and related diphenyl and monophenyl compounds including norepinephrine*

* = Asymmetric carbon atom.

compounds, Lu 5-003 and Lu 3-010, were as potent *in vitro* as the most potent tricyclic antidepressants, e.g., desipramine or protriptyline. We have studied the properties of several compounds in the Lu series in an attempt to analyze why they were so potent despite their obvious structural differences from tricyclic antidepressants and for what they might tell us about the way that the amine pump receptor site accommodated inhibitors.

MATERIALS AND METHODS

Rabbit Aortic Strips

Uptake studies. Aortic strips were prepared according to the method of Furchgott (10) from male rabbits weighing approximately 2.5 kg.

The uptake of tritiated norepinephrine into the strips was studied by procedures already described elsewhere (1–4). In brief, tissues suspended in 10 ml of Krebs–bicarbonate solution at pH 7.4 were exposed to 0.1 μM ^{3}H-(−)-norepinephrine for 10 min, washed for 10 min, removed from the bath, blotted, and homogenized in 10% trichloroacetic acid, and an aliquot was counted. In some instances the tissues were removed from the bath, blotted, solubilized with NCS tissue solubilizer, neutralized, and counted. Control studies demonstrated that these procedures allowed quantitative recovery of radioactivity that had been added to tissue homogenates. The counting efficiency was 25%. All samples were kept in the liquid scintillation spectrometer for sufficient time to register at least 2000 counts. Inhibitors were preincubated with strips for 15 min prior to the addition of the labeled substrate. The weight of aortic strips cut to 2 mm × 2-cm size was approximately 25 mg, with only an occasional extreme variation of 15%, and therefore, values have been expressed per strip, rather than per milligram of strip weight.

A minimum of three and in some cases four to five different concentrations of drugs producing from 20 to 80% inhibition of uptake of norepinephrine was used to determine the IC_{50} values. The results were plotted on semilogarithmic paper and the line was determined by connecting the points on the graph.

For plotting kinetic data regarding norepinephrine uptake, calculations were performed by computer analysis using enzyme kinetic equations 1, 8, and 10 presented by Cleland (11).

Release studies. In order to study the release of norepinephrine, tissues were exposed to 0.1 μM ^{3}H-(−)-norepinephrine for 10 min and washed for 10 min. The strips were then incubated with the inhibitors for a period of 15 min and washed again for 10 min. Strips were then removed, blotted, extracted, and counted as before. The efficacy of inhibitors in releasing norepinephrine was assessed by comparing the counts from the inhibitor-treated tissues with those from strips that had been run simultaneously and subjected to all manipulations except that they were not exposed to inhibitor.

Synaptosomal Preparations of Rat Hypothalamus

Uptake studies. Male albino rats of the Sprague–Dawley strain weighing from 200 to 250 g were sacrificed by decapitation, their brains were rapidly removed, and the hypothalamus was dissected from the rest of the tissue on an ice-filled petri dish. The hypothalamus was immediately weighed and homogenized in 20 vol of 0.32 M sucrose containing 25 mM Tris–HCl buffer, pH 7.4. All homogenizations were performed with 10 up-and-down strokes with a pestle revolution of 840 rpm. The Teflon pestle (A. H. Thomas Company, Philadelphia, Pa.) was ground to obtain a clearance of 0.025 cm between it and the glass vessel. The nuclei and cell debris were separated by centrifugation at 1000*g* for 10 min. The supernatant was gently separated from the 1000*g* pellet and was thoroughly mixed to yield a uniform suspension. This preparation will be referred to as the crude synaptosomal preparation. A 0.5-ml aliquot of the crude synaptosomal preparation was suspended in 2.5 ml of a standard incubation medium consisting of the following substances: NaCl, 145 mM; KCl, 3 mM; $CaCl_2$, 1.28 mM; $MgCl_2$, 1.19 mM; glucose, 11 mM; Tris–HCl buffer (pH 7.4), 25 mM; ascorbate, 1 μM; iproniazid, 10 μM; and ^{3}H-(−)-norepinephrine, 0.2 μM. All incubations were conducted for a period of 5 min at 37°C in an atmosphere of 95% O_2–5% CO_2. The uptake at 0°C was also studied for each experiment in order to determine the amount of tritiated amines accumulated by the temperature-insensitive uptake process. The amount of ^{3}H-amine accumulated by the temperature-insensitive process (uptake at 0°C) was subtracted from the total uptake at 37°C to yield the uptake by the temperature-sensitive process only. All drugs were dissolved in incubation medium and preincubated with the crude synaptosomal preparation for 5 min at 37°C in an atmosphere of 95% O_2–5% CO_2 before the addition of the labeled substrate. The reaction was stopped by the addition of 2.0 ml of ice-cold 0.32 M sucrose containing 25 mM Tris–HCl buffer, pH 7.4, and the tubes were centrifuged at 49,600*g* for 10 min. The supernatant was discarded and the pellet was washed by

gentle resuspension in 5.0 ml of 0.9% NaCl by means of a thin glass rod. The suspension was again centrifuged at 49,600*g* for 10 min. The supernatant was discarded and the pellet lysed by homogenization with 2.0 ml of 0.4 N perchloric acid. The precipitated protein was removed by centrifugation at 49,600*g* for 15 min and a 1.0-ml aliquot of supernatant was counted for radioactivity as previously described (12).

Release studies. A crude synaptosomal fraction was obtained as previously described in uptake studies. A 5.0-ml aliquot was incubated in the presence of 0.2 μM ^{3}H-(−)-norepinephrine for 5 min at 37°C in an atmosphere of 95% O_2–5% CO_2. The reaction was stopped by the addition of 2.0 ml of ice-cold 0.32 M sucrose containing 25 mM Tris–HCl buffer, pH 7.4, and the tubes were centrifuged at 49,600*g* for 15 min. After centrifugation, the pellet was washed with 5.0 ml of 0.9% NaCl and recentrifuged at 49,600*g* for 10 min and the resulting pellet was resuspended with incubation mixture to its original volume. A 0.5-ml aliquot of this preparation was added to 2.5 ml of fresh incubation medium and the release of ^{3}H-(−)-norepinephrine was studied at 37°C in the presence and absence of the drug. The rest of the procedure was identical to that already described for uptake studies.

Kinetic plots of norepinephrine uptake were determined with the aid of a computer program as mentioned previously for rabbit aortic strips.

7-^{3}H-(−)-Norepinephrine (5 to 10 Ci/mmol; radiochemical purity, >99%) was obtained from New England Nuclear Corp. (Boston, Mass.).

The Lu compounds were kindly supplied by Dr. I. Møller Nielsen of H. Lundbeck & Co. A/S, Denmark.

RESULTS

A Comparison of the Potencies of Lu 3-010, Lu 5-003, Desipramine, and their Primary Amine and Tertiary Dimethylamine Derivatives as Inhibitors of the Uptake of Norepinephrine

In rabbit aortic strips and in crude synaptosomal preparations from rat hypothalamus, the secondary methylamine derivatives, Lu 3-010 and Lu 5-003, were as potent as desipramine. They were more potent than their respective primary amine and tertiary dimethylamine derivatives (Fig. 2). In this respect they clearly resembled desipramine, protriptyline, and nortriptyline (4), which are also more potent than their primary amine and tertiary dimethylamine derivatives. The IC_{50} values for desipramine and derivatives are plotted in Fig. 2 for purposes of comparison.

The Influence of Several Substituents in Lu 3-010 and Lu 5-003 on Potency

An inspection of Fig. 3 shows that Lu 3-071, the derivative of Lu 3-010 which lacks the phenyl ring, is 60- to 400-fold weaker as an inhibitor of norepinephrine uptake than either Lu 3-010 or Lu 5-003 in both the aortic and the hypothalamic synaptosomal preparations.

It can also be seen in Fig. 3 that the removal of one (Lu 5-001) or both (Lu 4-070) of the methyl groups attached to the carbon atom adjacent to the sulfur atom in Lu 5-003 leads to a 6- to 10-fold loss in potency.

A Comparison of the Relative Potencies of the Lu Inhibitors with those of Desipramine and Related Inhibitors

While it is clear from Figs. 2 and 3 that the inhibitors in this study are generally more potent in the hypothalamic synaptosomes than in aortic strips, the relative potencies are very similar (Table 1). In Table 1 the potencies are expressed relative to a standard that we have used in the past and which we continue to use in order to make comparisons with this earlier literature easier. The standard compound is the tertiary dimethylamine derivative of the diphenylmethylidine series (1, 2, 5).

The Capacity of Lu 3-010 and Lu 5-003 to Release Tritiated 1-Norepinephrine

Lu 3-010 and Lu 5-003 at concentrations which produced approximately 80% inhibition of uptake did not produce a significant loss of label from aortic strips which had been pretreated with tritiated norepinephrine (Table 2).

Likewise, Lu 3-010 and Lu 5-003 at concentrations which inhibited uptake by approximately 80% did not release radioactive label from a crude preparation of rat hypothalamic synaptosomes which had been pretreated with tritiated norepinephrine (Table 3).

Kinetic Studies

Double-reciprocal plots for the uptake of norepinephrine into aorta in the absence and presence of Lu 3-010 and in the absence and presence of the weak derivative lacking the phenyl ring (Lu 3-071) are presented in Fig. 4. Similar plots from crude synaptosomal preparations from rat hypothalamus in the absence and presence of Lu 5-003 are also presented in Fig. 4. As can be seen, all three compounds were competitive inhibitors of the uptake of tritiated norepinephrine.

DISCUSSION

Since it was demonstrated that the potent inhibitors used in this study did not release norepinephrine, the data in Figs. 2 and 3 and Table 1 were taken to reflect uncomplicated estimates of relative capacity to inhibit uptake. Additionally, since representative compounds in the Lu series have been demonstrated to be competitive inhibitors of the uptake of norepinephrine, we considered it reasonable to conclude that the inhibitors as a group and the substrate, norepinephrine, fit the same receptor. Desipramine as well as other tricyclic antidepressants have been shown to be competitive inhibitors of norepinephrine uptake (2).

The greater potency of secondary methylamines (Lu 3-010 and Lu 5-003) relative to primary amine and tertiary dimethylamine derivatives is also very characteristic of tricyclic inhibitors (1, 13, 14). Such selective potency for secondary methylamines is not observed with the analogous diphenyl and monophenyl compounds (5). These facts further strengthen the notion that the Lu compounds and the tricyclic inhibitors act at the same receptor and have binding sites in common.

The phenyl ring substitution on the bicyclic ring sys-

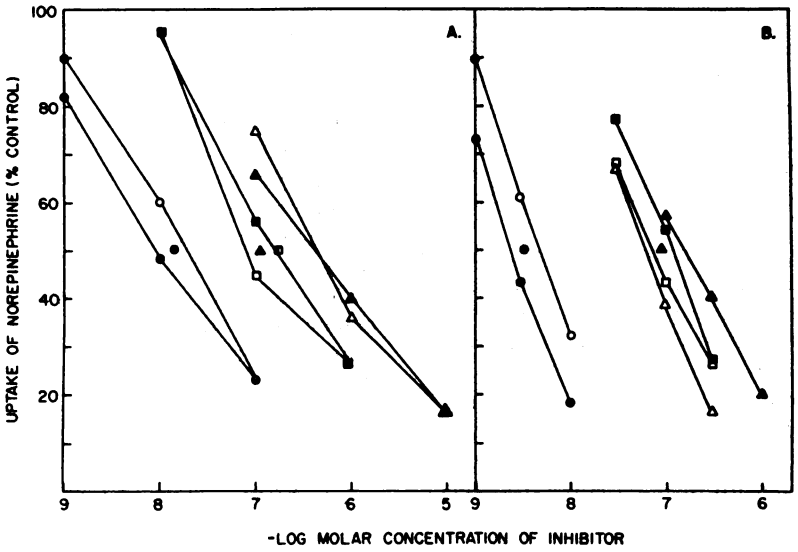


FIG. 2. *The effect of Lu 3-010 and Lu 5-003 and their primary amine and tertiary dimethylamine derivatives on the uptake of tritiated 1-norepinephrine*

(A) Uptake into adrenergic nerves of rabbit aortic strips. Each point represents the mean of at least six measurements. Norepinephrine concentration in the bath was 10^{-7} M. ●, Lu 5-003; ■, 1° amine derivative; ▲, tertiary dimethylamine; ○, Lu 3-010; □, 1° amine derivative; △, tertiary derivative. (B) Uptake into crude synaptosomal preparations of rat hypothalamus. Each point represents the mean of four or five measurements. In A and B the SE did not exceed ±8%. Norepinephrine concentration in the incubation mixture was 2×10^{-7} M. Symbols the same as in A. In A the IC_{50} values for desipramine (◐), desmethyldesipramine (◧), and imipramine (◭) are also presented. In B data for desmethyldesipramine were not available (data taken from Ref. 5).

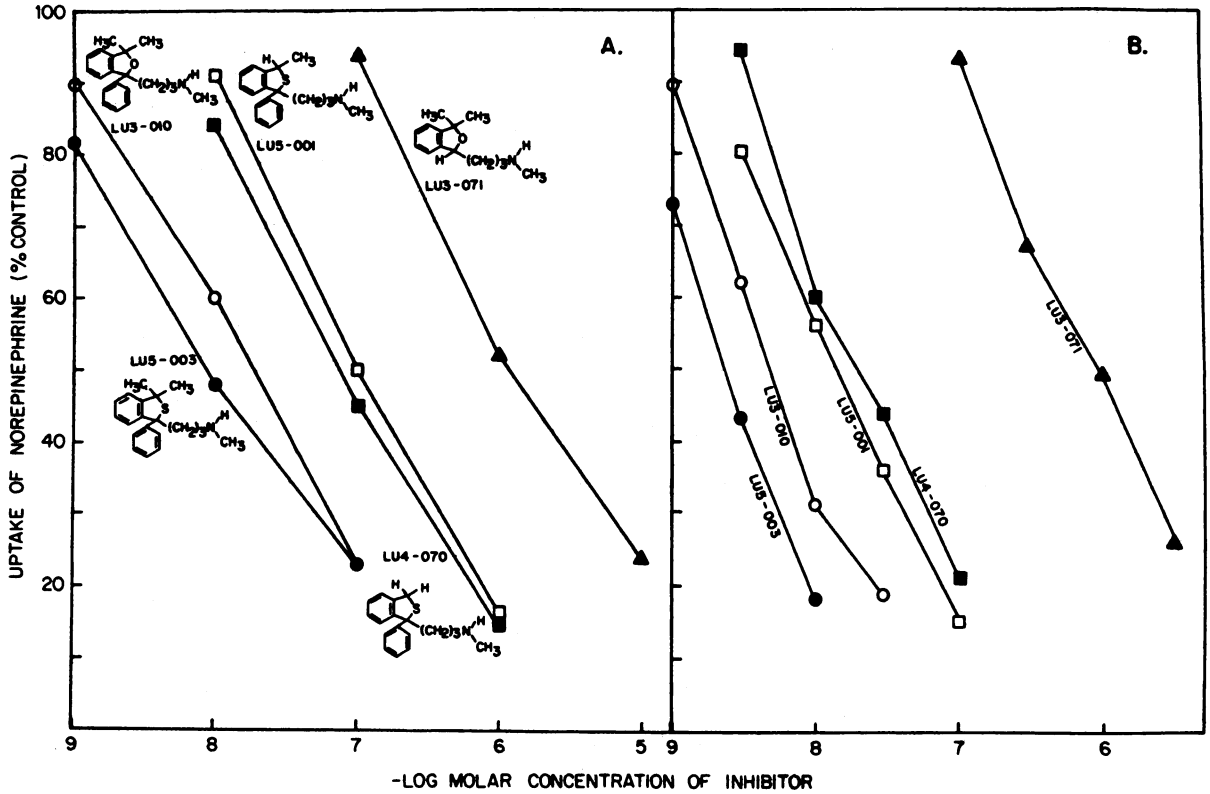


FIG. 3. *The effect of Lu 3-010 and Lu 5-003 and related compounds on the uptake of tritiated 1-norepinephrine*

(A) Uptake into adrenergic nerves of rabbit aortic strips. Each point represents the mean of at least six measurements. Norepinephrine concentration in the bath was 10^{-7} M. (B) Uptake into crude synaptosomal preparations of rat hypothalamus. Each point represents the mean of four or five measurements. In A and B the SE did not exceed ±8%. Norepinephrine concentration in the incubation mixture was 2×10^{-7} M. Structures as in A.

tem in the Lu compounds is important for binding as evidenced by the 60- to 400-fold drop in potency in aorta and hypothalamus, respectively, when the phenyl ring is not present (Table 1).

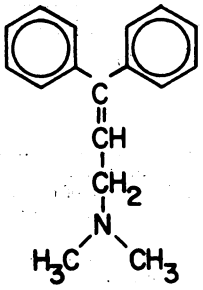
From Fig. 1 it is clear that there are considerable structural differences between the "ring end" of the Lu compounds and that of the tricyclic compounds. Unlike desipramine, and other tricyclic antidepressants, the Lu compounds have an asymmetric carbon atom. This might be expected to lead to reduced potency since the stereochemistry of the two enantiomers is distinctly different and only one enantiomer might be expected to fit the receptor very well. However, as is apparent in Fig. 5, the ring systems of both enantiomers of Lu 3-010 (or Lu 5-003) can readily superimpose over desipramine. In so doing the phenyl ring contained in the phthalane, or thiophthalane, nucleus (which is maximally restricted in its rotatability) and the "unrestricted" phenyl ring exchange places, but the primary orientation of the side chain with respect to the two phenyl rings remains very similar in both enantiomers and very similar to the orientation in the tricyclic compounds.

Maxwell *et al.* (1, 5) have presented evidence which supports the idea that the relatively inflexible, noncoplanar positioning into which the two phenyl rings are forced by the 2-carbon bridge in tricyclic ring systems is one which is favorable for alignment of these rings with their hydrophobic binding sites in the receptor. Since, as already mentioned, one of the phenyl rings in the Lu compounds is not fixed as in desipramine but is free to rotate, it is surprising that Lu 3-010 and Lu 5-003 are as potent as desipramine. It might be expected that these phthalane and thiophthalane derivatives have potencies of the same order as those of diphenyl compounds. In fact, however, the primary amine and the tertiary dimethylamine derivatives in the Lu series have relative potencies which range between 8 and 54 (calculated from the ED_{50} values in Fig. 2 and the ED_{50} values for the standard given in Table 1), whereas the analogous diphenyl compounds have potencies in the range of 1 to 12 (5). However, the picture is more complex than it appears. Lu 3-010 and Lu 5-003 have two methyl groups on the carbon atom adjacent to the oxygen or sulfur atoms in the phthalane and thiophthalane ring systems. There are no analogous substitutions in the tricyclic ring system of desipramine. In Fig. 5 it can be seen that one methyl group in each of the enantiomers of the Lu compounds projects into the same region. As pointed out in the Results, the removal of one or both methyl groups leads to a 6- to 11-fold drop in potency. These results suggest that one of the methyl groups is involved in hydrophobic bonding with an ancillary site not used by tricyclic inhibitors. If we use an average figure of 9-fold for the influence of the methyl group on relative potencies and divide by this number, the relative potencies for the primary amine and tertiary dimethylamine derivatives drop from 8–54 to 1–6. This adjusted range of values is very similar to that of the diphenyl compounds, i.e., 1–12. This then indicates that except for the extra binding by a methyl

TABLE 1

Relative potencies (IC_{50} standard/IC_{50} test compound) of Lu compounds and desipramine and related compounds as inhibitors of the uptake of norepinephrine into the adrenergic nerves of rabbit aorta and into crude rat hypothalamic synaptosomal preparations

Compound	Relative potencies	
	Aorta	Hypothalamus
Lu-5-003	480	522
Lu-3-010	210	255
Lu-4-070	56	60
Lu-5-001	42	86
Lu-3-071	3.5	1.3
Desipramine	300	363
Imipramine	38	13
Desmethyldesipramine	24	—



Standard compound:
IC_{50} for aorta = 4×10^{-6} M
IC_{50} for hypothalamus = 1.2×10^{-6} M

TABLE 2

The capacity of Lu 3-010 and Lu 5-003 to release tritiated norepinephrine from adrenergic nerves of rabbit aorta

Tissues were exposed to 0.1 μM ^{3}H-(−)-norepinephrine for 10 min and washed for 10 min. The strips were then incubated with the inhibitors for a period of 15 min and washed again for 10 min. Strips were then removed, blotted, extracted, and counted. Counts from the inhibitor-treated tissues are compared with those from strips run simultaneously and subjected to all manipulations except that they were not exposed to inhibitor.

Compound	Conc.	No. of strips	Norepinephrine in tissue
	M		*cpm/strip* × 10^{-2} ± *SE*
Untreated control		10	107 ± 13
Lu-3-010	10^{-7}	6	113 ± 8*
Lu-5-003	10^{-7}	8	115 ± 9*

* Not significantly different from control value, $P > 0.05$ as determined by Student's *t* test.

TABLE 3

The capacity of Lu 3-010 and Lu 5-003 to release tritiated norepinephrine from crude synaptosomal preparations of rat hypothalamus

A 5.0-ml aliquot of a crude synaptosomal preparation was incubated with a 0.2 μM solution of ^{3}H-(−)-norepinephrine for 5 min, washed, and resuspended in incubation medium, and a 0.5-ml aliquot was added to 2.5 ml of fresh medium at 37°C. At this time some tissues were exposed to drugs and others were untreated. At the end of the 5 min, the ^{3}H-amine present in control and drug-treated pellets was extracted and compared.

Compound	Conc.	No. of expts.	Norepinephrine released
	M		% ± *SE*
Untreated control		6	13.3 ± 1.8
Lu-3-010	3×10^{-6}	6	13.8 ± 0.8
Lu-5-003	3×10^{-6}	6	10.8 ± 0.8

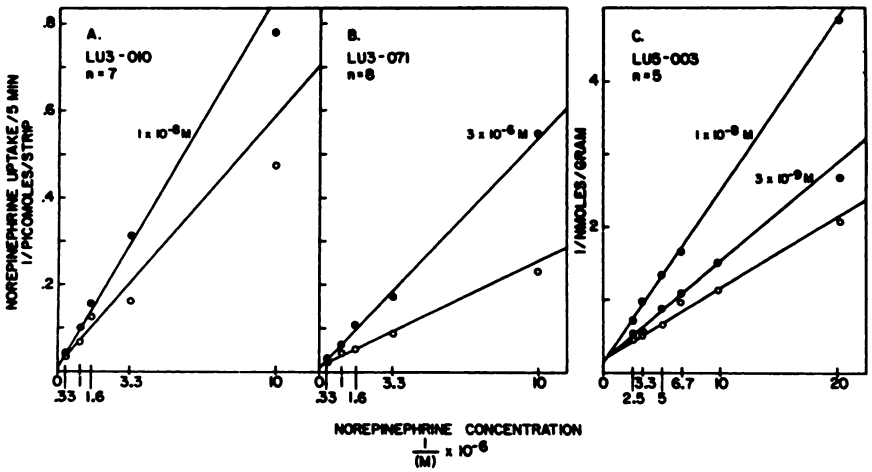


FIG. 4. *Reciprocal plots of the relationship between the rate of uptake of norepinephrine and the concentration of tritiated l-norepinephrine in the absence and presence of Lu compounds*

(A, B) Uptake into adrenergic nerves of rabbit aorta. (C) Uptake into crude synaptosomal preparation of rat hypothalamus. ○, Untreated; ●, treated with inhibitor.

group, the Lu compounds would indeed be equivalent in potency to the diphenyl compounds.

The ring structure of the tricyclic compounds, in contrast to the diphenyl and monophenyl ring structures, aids in bringing about good binding of the single *N*-methyl group of the secondary methylamine derivatives. They do this by fixing the orientation of the atom between the two rings (either carbon or nitrogen) as well as the first carbon of the propylamine chain attached to this atom (Fig. 5). Thus, considerably more orientation is given to the chain of the tricyclic compounds than is given in either diphenyl or monophenyl compounds. This orientation is highly desirable because it permits a "lock and key" binding between the single *N*-methyl and the receptor (2, 5). In the Lu compounds the phenyl ring which is incorporated in the phthalane and thiophthalane

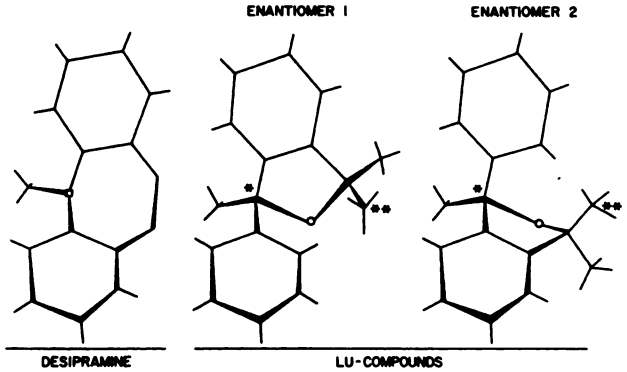


FIG. 5. *Drawings of Dreiding stereomodels of the ring systems of desipramine and the two enantiomers of potent Lu compounds*

The phenyl rings of the enantiomers have been made to take the conformation of the phenyl rings of desipramine. □ = Nitrogen; ○ = oxygen or sulfur; * = asymmetric carbon; ** methyl groups sharing common space. As an aid to visualizing the stereochemistry, wedge-shaped lines are used to indicate a projection of the line outward from the page. The thickened end is closest to the viewer. For clarity, only the first methylene group of the propylamine side chains is shown. The remainder of the side chain, —$CH_2CH_2NHCH_3$, is identical in the three compounds.

ring systems and one of the attached methyl groups presumably act in conjunction with the "free" phenyl ring, so that when they are all bound to the receptor they apply an orientation to the side chain which is similar to if not identical to that in desipramine. This permits binding of the *N*-methyl group of the secondary methylamine compounds, Lu 3-010 and Lu 5-003.

From a more general point of view the data suggest that there are more sites available on the receptor for binding than are used by any one series of compounds. Thus one can conceive of a restricted region in the center of the amine pump receptor (consisting minimally of a site to receive the phenyl ring and one to receive the nitrogen) to which norepinephrine and other substrates bind reversibly and as a consequence of which they are passed through the membrane. Surrounding this "substrate receptor site" is an additional array of molecular species, e.g., groups in the amino acid residues of proteins, phospholipid constituents, and other molecules found in membranes. This surrounding "foliage" provides potential ancillary sites for the binding of potent inhibitors. This view is consonant with the fact that potent inhibitors are generally "bulkier" molecules than are substrates (e.g., compare desipramine and cocaine with norepinephrine). The array of potential ancillary binding sites is greater than is used by any one class of inhibitors, e.g., tricyclic compounds, and may be the reason why other classes of compounds, e.g., Lu compounds, can be equipotent with desipramine as inhibitors of norepinephrine uptake despite certain structural differences which should make them weaker inhibitors. In addition to binding to sites in common, they may also bind to groups which are not utilized by the tricyclic agents.

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