spectively. The relative mobilities of these two peptides are consistent with the structures of the expected histidine-containing peptides from a chymotryptic digestion—H-Ava-Ser-Met-Arg-His-Phe-OH and H-Ava-Ser-Met-Glu-His-Phe-OH from peptides III and II, respectively.

Biological Assay. In vitro steroidogenesis in isolated rat adrenal cells was measured by the method of Moyle et al.[14] In vitro lipolytic activity in isolated rat and rabbit fat cells was measured by the method of Ramachandran and Lee.[23] In vitro melanotropic activity was determined by the method of Shizume et al.[24] Potency was measured against highly purified sheep ACTH[25] standard. In vivo melanotropic activity was determined by the method of Hogben and Slome.[26]

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References and Notes

(1) For part 48, see J. Blake and C. H. Li, *Int. J. Pept. Protein Res.*, in press. All asymmetric amino acids occurring in the peptides mentioned in this paper are of the L configuration. Abbreviations used are ACTH, adrenocorticotropin; Boc, *tert*-butyloxycarbonyl; Nps, *o*-nitrophenylsulfenyl; Ava, δ-aminovaleric acid; Tos, *p*-toluenesulfonyl; Z, benzyloxycarbonyl.
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Pharmacological Activity of Nitroxide Analogues of Dichloroisoproterenol and Propranolol

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Spin-labeled analogues of dichloroisoproterenol and propranolol were synthesized. It was found that the K_D's of both probes for the β-adrenergic receptors of frog erythrocytes were about 30-fold higher than the K_D's previously reported for the parent antagonists. Thus the introduction of a bulky nitroxide moiety in place of the isopropyl group on the amino nitrogen is associated with a decrease in affinity for the β-adrenergic receptors. Nonetheless, the affinity of the spin-labeled propranolol would appear to be within a range compatible with EPR measurements.

In recent years, the use of nitroxides as free-radical probes in the study of drug interactions has received considerable attention.[1-3] In particular, spin-labeled probes of acetylcholine[4,5] have been developed to study the topography of the muscarinic cholinergic receptor[6] and acetylcholinesterase.[7,8] Furthermore, we have observed that in order to "map" the surface of the receptor, the dissociation constant of a spin-labeled probe must be no greater than 1×10^{-5} M. Recently, Sinha and Chignell[9] reported the synthesis of a spin-labeled analogue of dichloroisoproterenol (2) and propranolol (4). In their article, they reported that 2 and 4 inhibit, at 6.65×10^{-5} M, the isoproterenol-stimulated adenylate cyclase of fat cell membranes by 19 and 41%, respectively. Unfortunately, the authors[9] did not publish the dissociation constant for either agent. With this in mind, we felt that it might be advantageous to repeat their experiments to determine the feasibility of using these probes to study the topography of the β-adrenergic receptors.

In our experience with sodium cyanoborohydride reductive aminations,[10] the use of high-molecular-weight amines or hindered amines has given unsatisfactory yields of the desired products. For example, Sinha and Chignell[9] have reported a 36% yield of 4-[[2-hydroxy-3-(1-naphthalenyloxyl)propyl]amino]-2,2,6,6-tetramethyl-1-piperidinyloxyl (4) and presumably a low yield of 4-[[2-(3,4-dichlorophenyl)-2-hydroxyethyl]amino]-2,2,6,6-tetramethyl-1-piperidinyloxyl (2) following the method discussed in the literature.[10,11] However, we observed that reaction of 3,4-dichlorophenyloxirane (1) with 4-amino-2,2,6,6-tetramethyl-1-piperidinyloxy gave better than an 80% yield of the desired product 2. In addition, we were able to obtain 85% of 4 by reacting (1-naphthalenyloxyl)methyloxirane with the precursor spin-labeled probe,

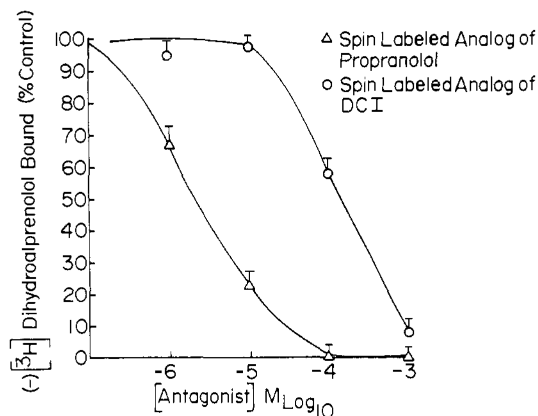
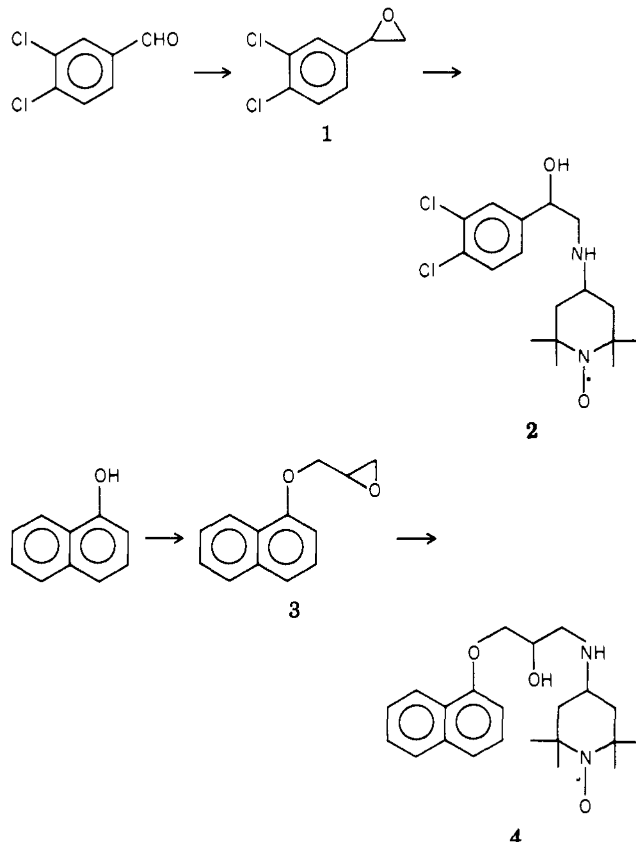


Figure 1. Inhibition of (−)-[^{3}H]dihydroalprenolol binding to frog erythrocyte membranes by spin-labeled dichloroisoproterenol and spin-labeled propranolol. Values shown are mean ± SEM of duplicate determinations from two experiments.

4-amino-2,2,6,6-tetramethyl-1-piperidinyloxy.



In this communication we report the dissociation constants of spin-labeled dichloroisoproterenol (2) and spin-labeled propranolol (4) for the β-adrenergic receptors in a model system, the frog erythrocyte membrane.

Discussion

Elsewhere it has been documented that dissociation constants of β-adrenergic agents may be simply determined by assessing their ability to compete with (−)-[^{3}H]dihydroalprenolol for occupancy of the receptors in membrane fractions.[12,13] Figure 1 depicts the results obtained when the two spin-labeled analogues were tested in the (−)-[^{3}H]dihydroalprenolol binding assay. Since the concentration of (−)-[^{3}H]dihydroalprenolol in the assay and the dissociation constant of (−)-[^{3}H]dihydroalprenolol for the receptors are known, the concentration of unlabeled ligand causing 50% displacement (EC_{50}) may be used to calculate its dissociation constant (see Experimental Section). The calculated dissociation constants are shown in Table I.

Table I. Dissociation Constants of Spin-Labeled β-Adrenergic Antagonists Determined by (−)-[^{3}H]Dihydroalprenolol Binding and Adenylate Cyclase Assays

Compd	(−)-[^{3}H]Dihydroalprenolol binding, K_D, μM	Adenylate cyclase, K_D, μM
(±) spin-labeled DCI (2)	16.0 ± 2.0	8.2 ± 1.1
(±) spin-labeled propranolol (4)	0.27 ± 0.03	0.14 ± 0.02
(−)-Propranolol	0.0046 ± 0.0003[a]	0.004 ± 0.001[a]
(±)-DCI	0.57 ± 0.03[a]	0.4 ± 0.01[a]

[a] See ref 13.

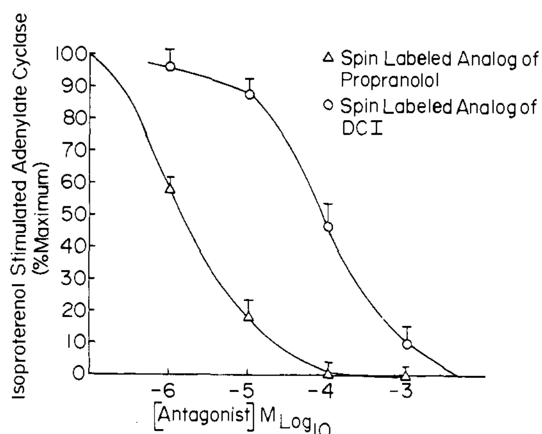


Figure 2. Inhibition of isoproterenol-stimulated adenylate cyclase in frog erythrocyte membranes by spin-labeled dichloroisoproterenol and spin-labeled propranolol. Isoproterenol was present at 1×10^{-5} M. Values shown are mean ± SEM of duplicate determinations from two experiments.

In separate experiments the ability of the two spin-labeled agents to antagonize isoproterenol-stimulated adenylate cyclase in the frog erythrocyte membranes was tested. These data are shown in Figure 2. The concentrations of agents causing 50% inhibition of isoproterenol-stimulated enzyme activity can be used to calculate dissociation constants for these agents (see Experimental Section). These dissociation constants are also listed in Table I and are in reasonable agreement with those determined independently by the direct binding studies.

It was of interest that both spin-labeled analogues had considerably lower affinities for the β-adrenergic receptors than did the parent compounds. The ratio of K_D (2)/K_D [(±)-DCI] was 28. The ratio of K_D (4)/K_D [(−)-propranolol] was 59. However, if we make the reasonable assumption that (−)-propranolol has a twofold better affinity for the receptors than (±)-propanolol,[13] than the ratio K_D (4)/K_D [(±)-propranolol] is 29. Since all the other agents were racemates this would appear to be a better value for comparative purposes. Thus the substitution of a nitroxide for an isopropyl moiety on the amino nitrogen is associated with about 30-fold loss of affinity of both analogues for the β-adrenergic receptors. Nonetheless, the affinity of the spin-labeled analogue of propranolol (4) would appear to be within a range compatible with its utility as an EPR probe for these receptors.

Experimental Section

General Comments. Melting points were obtained on a Thomas-Hoover melting point apparatus and are corrected.

Infrared spectra were recorded on a Perkin-Elmer Model 727 spectrophotometer. Electron paramagnetic resonance spectra were obtained from a Varian E-9 spectrometer.

3,4-Dichlorophenyloxirane (1). Oil-free NaH (0.35 g, 15 mmol) was added to a solution of 1.75 g (10 mmol) of 3,4-dichlorobenzaldehyde in 25 ml of Me_2SO at 20°. After stirring this mixture for 10 min, 3.0 g (15 mmol) of trimethylsulfonium iodide was added in several portions over 10 min. The temperature of the reaction was slowly raised to 50° and allowed to stir for 1 h. The mixture was then cooled and poured into 400 ml of cold phosphate buffer (1 M at pH 6.0). This mixture was extracted with Et_2O, dried over anhydrous $MgSO_4$, and evaporated to dryness giving 1.5 g of a pale yellow oil. Identification was based on the absence of ir absorption in the region of the precursor aldehyde (1720 cm^{-1}) and observation of a single spot on silica gel TLC plates using $CHCl_3$ as the solvent.

4-[[2-(3,4-Dichlorophenyl)-2-hydroxyethyl]amino]-2,2,-6,6-tetramethyl-1-piperidinyloxyl (2). A mixture of 1.5 g (7.9 mmol) of 3,4-dichlorophenyloxirane (1) and 1.21 g (7 mmol) of 4-amino-2,2,6,6-tetramethyl-1-piperidinyloxyl[10] was dissolved in 50 ml of CH_3OH and the solution was refluxed overnight. The reaction was then poured into 400 ml of H_2O which was then extracted exhaustively with Et_2O. The Et_2O extracts were combined and washed with three portions of an acetate buffer (0.5 M at pH 3.8). The aqueous extracts were combined, made basic with NaOH, and extracted with $CHCl_2$. The organic solution was dried over anhydrous $MgSO_4$ and evaporated to dryness, giving 2.1 g (83%) of a red oil which crystallized upon standing. The compound was recrystallized from hexane–C_6H_6 to give red crystals, mp 100–101.5° (lit.[9] mp 100–102°).

(1-Naphthalenyloxy)methyloxirane (3). A mixture of 1-naphthol (4.3 g, 30 mmol), epichlorohydrin (8.5 g, 92 mmol), Et_3N (9.1 g, 90 mmol), and 50 ml of C_6H_6 was refluxed overnight and then the solution was poured into 300 ml of H_2O. The mixture was twice extracted with 100-ml portions of Et_2O. The Et_2O solution was washed with 2% HCl and then washed with 2% NaOH, dried over anhydrous $MgSO_4$, and evaporated to dryness giving 4.0 g (66%) of a clear oil.[14,15] Identification was based on the absence of ir absorption in the region of the precursor, 1-naphthol, and the observation of a single spot on silica gel TLC plates using $CHCl_3$ as the solvent.

4-[[2-Hydroxy-3-(1-naphthalenyloxy)propyl]amino]-2,-2,6,6-tetramethyl-1-piperidinyloxy (4). A mixture of the oxirane 3 (300 mg, 15 mmol) and 4-amino-2,2,6,6-tetramethyl-1-piperidinyloxyl[10] (258 mg, 1.5 mmol) was dissolved in 30 ml of CH_3OH and refluxed overnight. The mixture was poured into dilute HCl and extracted twice with 50-ml portions of Et_2O. The aqueous mixture was then made basic with NaOH and extracted with Et_2O. This solution was dried over anhydrous $MgSO_4$ and evaporated to dryness giving 474 mg (85%) of a red oil. The oxylate salt of this red oil was prepared and recrystallized from absolute EtOH: mp 139–141° (lit.[9] mp 140–142°).

Pharmacological Evaluation. Frog (*Rana pipiens*) erythrocyte membranes were prepared as previously described.[12,13] (–)-[^{3}H]Dihydroalprenolol binding assays were performed with radioligand present at 25 nM as described previously using a 10-min incubation at 37° in a 150-μl vol in 50 mM Tris–HCl buffer, pH 7.4. Incubations were terminated by vacuum filtration over Whatman glass fiber filters (GFC) followed by two 5-ml buffer washes.[13,16,17] Filters were counted in a Triton–toluene based flour. Nonspecific binding was determined in the presence of 10^{-5} M (±)-propranolol and was only 5–10% of total binding. Dissociation constants were calculated from the binding EC_{50} values using the equation[18]

$$K_D = EC_{50}/(1 + S/K_m)$$

where S = the concentration of (–)-[^{3}H]dihydroalprenolol in the binding assay and K_m = the dissociation constant of (–)-[^{3}H]-dihydroalprenolol for the receptors, 3 nM.[12,13]

Adenylate cyclase assays were performed exactly as previously described.[12,13,19,20] Isoproterenol-stimulated activity refers to the increment in enzyme activity caused by 10^{-5} M (–)-isoproterenol. Complete inhibition of this activity refers to enzyme activity which is at basal level. Basal adenylate cyclase was 12 pmol of cAMP generated per minute per milligram of protein and was stimulated 10–15-fold by 10^{-5} M (–)-isoproterenol. Dissociation constants were calculated from the 50% inhibitory concentrations of the agents using the equation[18]

$$K_D = EC_{50}/(1 + S/K_m)$$

where S = the concentration of (–)-isoproterenol in the assay (10^{-5} M) and K_m = the apparent dissociation constant of (–)-isoproterenol for enzyme stimulation (10^{-6} M).

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