of tetrahydrofuran was then added, and the reaction was left overnight at room temperature. Dicyclohexylurea was removed by filtration, and the filtrate was evaporated under vacuum. The residue was purified on a silica gel column (15 cm × 2.5 cm) with chloroform. Those fractions containing the ester were evaporated, and the residue was crystallized from methanol–water: yield 1.05 g (85%); mp 236–238 °C; mass spectrum m/e 413 (6.0, parent, M), 55 (base).

Amides of 4-Androsten-3-one-17β-carboxylic Acid (16a–d). A solution of *N*-hydroxysuccinimide ester of 4-androsten-3-one-17β-carboxylic acid (**15**; (316 mg, 1 mmol) in 10 mL of tetrahydrofuran was added to a mixture of the appropriate amine (1 mmol) and $NaHCO_3$ (84 mg, 1 mmol) dissolved in 10 mL of tetrahydrofuran–water (1:1). The reaction was left at room temperature for 16 h and then acidified with 1 N HCl. The organic solvent was removed under vacuum, and the precipitate in the resulting aqueous media was collected by filtration, dried, and crystallized from aqueous ethanol. The yields ranged from 70 to 75%. These crystalline products were homogeneous by TLC and analytical HPLC on a 25 cm × 4.6 mm LiChrosorb-Diol column with methylene chloride for **16c** and methylene chloride–isooctane (7:3) for **16a,b,d**. All of these amides, **16a–d**, gave satisfactory (±0.4%) combustion analysis. The steroidal amides were readily characterized by their mass spectra. **16a**: mp 194–196 °C; mass spectrum m/e 405 (29, parent, M), 91 (base, $CH_2C_6H_5$). Anal. ($C_{27}H_{35}NO_2$) C, H, N. **16b**: mp 182–184 °C; mass spectrum, m/e 435 (7, parent, M), 121 (base, $CH_2C_6H_4OCH_3$). Anal. ($C_{28}H_{37}NO_3$) C, H, N. **16c**: mp 242–245 °C; mass spectrum m/e 435 (0.6, parent, M), 120 (base, $CH_2CHC_6H_4OH$). Anal. ($C_{28}H_{37}NO_3$) C, H, N. **16d**: mp 183–184 °C; mass spectrum m/e 449 (1.0, parent, M), 134 (base, $CH_2CHC_6H_4OCH_3$). Anal. ($C_{29}H_{39}NO_3$) C, H. N.

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Alkylating β-Blockers: Activity of Isomeric Bromoacetyl Alprenolol Menthanes

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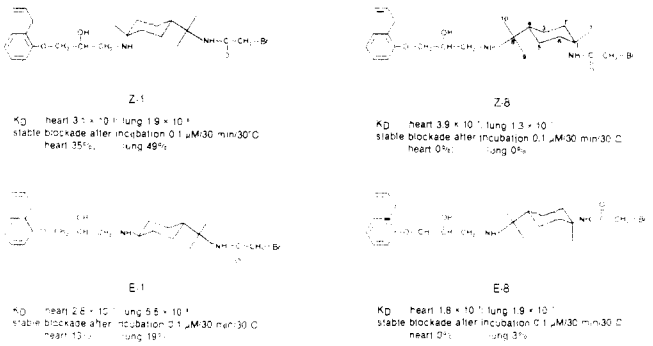
An affinity label for β-adrenoceptors, *N*-(bromoacetyl)-*N'*-[3-(*o*-allylphenoxy)-2-hydroxypropyl]-1,8-diamino-*p*-menthane, has been extensively used in the form of a mixture of four isomers. In the present study, all four isomers were isolated, their structures elucidated, and their interactions with β-adrenoceptors characterized. The isomer with the aromatic (pharmacophore) group on carbon 1 of *p*-menthane and with the *Z* configuration (*Z*-1) predominates in the mixture and has the highest affinity for β-adrenoceptors of rat heart ($K_D = 3 \times 10^{-8}$ M) and lungs ($K_D = 2 \times 10^{-8}$ M). This isomer acts as a ligand that binds irreversibly at the drug binding site of the receptor (i.e., after treatment and extensive washing of the membrane preparation, the concentration of the receptors is decreased in a dose-dependent manner), while binding characteristics of the remaining receptors are not changed. The corresponding *E* diastereomer (*E*-1) also binds irreversibly to the drug binding site of the receptor. The isomer with the aromatic group on carbon 8 and the *Z* configuration (*Z*-8) modifies the receptor noticeably only at higher concentrations and then on a site apparently different from the drug-binding site, i.e., affinity of receptors after the treatment and washing is changed. The corresponding *E* diastereomer (*E*-8) modified both the drug-binding and alternative binding site. The results suggest that there is some flexibility in the conformation of the β-adrenoceptor that enables pairs of ligands, differing by axial or equatorial positions of critical groups, to alkylate the receptor in an analogous manner.

Affinity labeling of receptors is a useful technique for biochemical and physiological studies of these proteins. Photoaffinity probes have been extensively used for biochemical studies of β-adrenoceptors.[1-4] Chemically reactive probes were also prepared and used.[5-10] One of these probes, called bromoacetyl alprenolol menthane or BAAM, has proven to be very well suited for in vivo studies and its use has made possible the selective destruction of β-adrenoceptors in living cells or in animals. This procedure enabled us (a) to study the rates of de novo synthesis of β-adrenoceptors in rat heart and lungs and to measure the decrease in these rates in senescent animals,[11,12] (b) to study the effects of a decrease in β-adrenoceptor number on the adenylate cyclase activity in rat heart,[13] (c) to study the release of amylase from cells of rat salivary glands activated by catecholamines,[14] (d) to study de novo synthesis of β-adrenoceptors in guinea pig lungs and to show that regeneration of physiological functionality is slower than regeneration of antagonist binding capacity of re-

(1) Lavin, T. H.; Heald, S. L.; Jeffs, R. W.; Shorr, R. G. L.; Lefkowitz, R. J.; Caron, M. G. *J. Biol. Chem.* **1981**, *256*, 11944.
(2) Wrenn, S. M.; Homcy, C. J. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 4449.
(3) Ruoho, A. E.; Kiefer, H.; Singer, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 2567.
(4) Darfler, F. J.; Marinetti, G. V. *Biochem. Biophys. Res. Commun.* **1977**, *79*, 1.
(5) Atlas, D.; Steer, M.; Levitzki, A. *Proc. Natl. Acad. Sci U.S.A.* **1976**, *73*, 1921.
(6) Fraser, C. M.; Venter, J. C. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 390.
(7) Ramkin, A.; Broadley, K. J. *Biochem. Pharmacol.* **1982**, *31*, 1325.
(8) Pitha, J.; Zjawiony, J.; Nasrin, N.; Lefkowtiz, R. J.; Caron, M. G. *Life Sci.* **1980**, *27*, 1791.
(9) Homburger, V.; Gozlan, H.; Bouhelal, R.; Lucas, M.; Bockaert, J. *Arch. Pharmacol.* **1985**, *328*, 279.
(10) Chorev, M.; Feigenbaum, A.; Keenan, A. K.; Gilon, C.; Levitzki, A. *Eur. J. Biochem.* **1985**, *146*, 9.
(11) Baker, S. P.; Pitha, J. *J. Pharmacol. Exp. Ther.* **1982**, *220*, 247.
(12) Pitha, J.; Hughes, B. A.; Kusiak, J. W.; Dax, E. M.; Baker, S. P. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 4424.
(13) Kusiak, J. W.; Pitha, J. *Biochem. Pharmacol.* **1984**, *33*, 483.
(14) Kousvelari, E. E.; Kusiak, J. W.; Hand, A. R.; Pitha, J.; Baum, B. J. *J. Pharmacol. Exp. Ther.* **1983**, *227*, 238.

Chart I. Structures of the Studied Alkylating β-Blockers and the Corresponding Abbreviations (Data on Interactions of These Compounds with β-Adrenoceptors and Characteristics of Persistent Blockades of Membrane Preparations Also Given)



ceptors,[15] (e) to study de novo synthesis of β-adrenoceptors in S-49 cells in culture and to show that regeneration of antagonist binding capacity of receptor is faster than capacity to produce cyclic adenosine monophosphate upon isoproterenol stimulation,[16] and (f) to study the regulation of the cardiovascular systems of hypertensive turkeys and show that pharmacologically important β-adrenoceptors are located peripherally rather than centrally in the nervous system.[17] This useful label is easy to prepare, but unfortunately, its synthesis yields a mixture of two diastereomeric pairs. This complication arises from the use of the commercial chemical 1,8-diamino-*p*-menthane, which contains both *Z* and *E* diastereomers, each of which has two nonequivalent amino groups of similar reactivity.

In the present report we describe the synthesis, purification, and interactions with β-adrenoceptors of the four compounds that comprise the mixture known as BAAM.

Results

Chemistry. 1,8-Diamino-*p*-menthane, used in the synthesis of BAAM, is prepared by the Ritter reaction and hydrolysis from α-pinene in the form of a mixture of *Z* and *E* diastereomers.[18] Previously, this mixture was oxidized to the corresponding 1,8-dinitro-*p*-menthanes that were then separated by chromatography and recrystallization.[18] We repeated this procedure, and in addition we also reduced catalytically the dinitro derivatives to pure *Z* and *E* diastereomers of 1,8-diamino-*p*-menthane. These were also prepared by direct separation of the diamine by chromatography on silica gel. The previously suggested attribution of structures to dinitro derivatives[18] was confirmed by ^{13}C NMR spectroscopy of the diamines (see the Experimental Section).

Reaction of *o*-allylphenyl glycidyl ether with either *E* or *Z* diastereomers of 1,8-diamino-*p*-menthane in a 1:1 ratio of the reactants yielded mainly disubstituted derivatives; at a 1:5 ratio mainly monosubstituted derivatives were obtained whereas the disubstituted derivatives did not exceed 5%. The degree of substitution was determined from integrated ^1H NMR spectra; furthermore, these spectra enabled us to confirm the structures of the products. That confirmation was necessary since we observed that the direction of the ring cleavage of the above epoxide depends on the amine used; thus, with (*Z*)- or (*E*)-1,8-diamino-*p*-menthane the epoxide was opened by an attack on the primary carbon, whereas with tris(hydroxymethyl)aminomethane both carbons were attacked. Confirmation of the structures was based on the difference of CH_2NH signals that appear at 2.90–2.70 ppm, compared to those of CH_2OH at 3.20–3.40 ppm. Only the former band was observed in our products.

Table I. Affinity of Alkylating β-Blockers at Rat Heart and Lung β-Adrenoceptors

compd	tissue	
	heart	lung
BAAM mixture	$(4.24 \pm 0.39) \times 10^{-8}$ (4)[a]	$(2.93 \pm 0.48) \times 10^{-8}$ (4)
Z-1	$(3.12 \pm 0.42) \times 10^{-8}$ (4)	$(1.98 \pm 0.39) \times 10^{-8}$ (4)
Z-8	$(3.91 \pm 1.16) \times 10^{-7}$ (3)	$(1.26 \pm 0.61) \times 10^{-7}$ (3)
E-1	$(2.80 \pm 1.00) \times 10^{-7}$ (3)	$(5.45 \pm 0.88) \times 10^{-8}$ (3)
E-8	$(1.81 \pm 0.11) \times 10^{-7}$ (3)	$(1.88 \pm 0.14) \times 10^{-7}$ (3)

[a] The results are expressed as K_D values (M) ±1 SEM. The numbers in parentheses are the number of times the experiment was performed for each compound.

Efforts to separate the monosubstituted product (i.e., 1- or 8-isomer) failed, but after the mixture was converted to monobromoacetyl derivatives these compounds could be separated by column chromatography. Structures of the compounds are depicted in Chart I where the abbreviations used are also indicated: steric configuration (*Z* or *E*), followed by position of aromatic group (1 or 8).

The structures of BAAM isomers were ascertained by mass spectrometry. Isomers *Z*-1 and *E*-1 had characteristic *m/e* 248 ions, which are ions originating from the fragmentation between carbon 4 and carbon 8 and containing the aromatic part of the molecule. These two isomers also had strong molecular ions; these were very weak in compounds *Z*-8 and *E*-8.

Inspection of the ^1H NMR spectra of isomers *Z*-1 and *Z*-8 showed that the anisotropic effect of the carbonyl group can also aid in the structure determination. In the case of isomer *Z*-1 the methyl protons of carbon 7s are shielded and resonate at 1.33 ppm, whereas the signal of the two methyl groups of carbon 9s and carbon 10s appears at lower field (1.40 ppm). In isomer *Z*-8 the shifts are reversed: the methyl protons of carbon 9s and carbon 10s are shielded and resonate at 1.30 ppm, whereas the protons of the methyl group carbon 7s appear at 1.40 ppm.

Pharmacology. The equilibrium dissociation constant (K_D) of the BAAM mixture at rat heart and lung membranes was about (3–4) × 10^{-8} M at both tissues. This result is very similar to previously reported values for a number of different preparations of the BAAM mixture. The BAAM mixture contains about 50–55% *Z*-1 and 35–40% *Z*-8 isomers; in the remaining portion *E*-1 and *E*-8 isomers are about equally distributed. When the individual isomers were isolated and tested separately, the *Z*-1 isomer, which predominates in the above mixture, had slightly higher affinity, whereas the *Z*-8 isomer had about 10-fold lower affinity at these same sites (Table I). The two *E* isomers, which are very minor components, were of intermediate affinity (Table I). The affinity of no single isomer was more than 10-fold greater or smaller than the affinity of the mixture.

The irreversible inhibition exerted by the BAAM mixture or its components was evaluated by preincubation of membranes with the compounds at 0.1 μM concentration for 30 min at 30 °C, extensive washing, and subsequent assay for specific binding of [^3H]dihydroalprenolol. Such treatment of membranes with the BAAM mixture led to a loss of 36% of the binding in heart and 23% in lung preparations; the corresponding results for the components of the BAAM mixture are summarized in Chart I. These data show that the *Z*-1 isomer is the main active compo-

(15) Baker, S. P. et al., manuscript in preparation.
(16) Insel, P. A.; Mahan, L. C. *Fed. Proc.* **1984**, *43*, 876.
(17) Kuenzel, W. J.; Kusiak, J. W.; Augustine, P. C.; Pitha, J. *Comp. Biochem. Physiol.* **1983**, *76C*, 371.
(18) Morat, C.; Rassat, A.; Rey, P. *Tetrahedron* **1975**, *31*, 2927.

Table II. Binding Parameters of Dihydroalprenolol to Membrane Preparations Pretreated with Alkylating β-Blockers[a]

			heart		lung	
alkylating blocker	concn, M	n	B_{max}, fmol/mg protein	K_D, nM	B_{max}, fmol/mg protein	K_D, nM
none (control)		16	32 ± 2	2.4 ± 0.4	397 ± 26	1.5 ± 0.2
BAAM mixture	1×10^{-7}	3	20 ± 2	3.2 ± 1.0	280 ± 41	2.5 ± 1.0
Z-1	1×10^{-7}	3	16 ± 1	2.1 ± 0.4	165 ± 17	1.6 ± 0.6
Z-8	1×10^{-5}	3	28 ± 3	3.0 ± 0.4	367 ± 21	4.8 ± 0.4
E-1	1×10^{-7}	3	26 ± 3	1.5 ± 0.2	305 ± 34	1.3 ± 0.2
E-8	1×10^{-6}	3	25 ± 2	2.8 ± 0.1	289 ± 11	2.8 ± 0.6

[a] Membranes were preincubated with alkylating β-blockers at given concentrations for 30 min at 30 °C, washed, and assayed as described in the Experimental Section. The parameters given were then calculated from Scatchard analysis of binding data and are the means ±1 SEM.

nent of that mixture, but there is also some contribution from *E*-1. Furthermore, the character of irreversible inhibition was investigated at different concentrations of [^{3}H]dihydroalprenolol, and data were analyzed by Scatchard plots (Figure 1). Membranes treated with isomers *Z*-1 and *E*-1 at 0.1 μM concentration had reduced concentrations of binding sites with essentially no change in affinity for [^{3}H]dihydroalprenolol (Table II). Compound *Z*-8 had practically no activity at 0.1 μM, but preincubation with a higher concentration (100 μM) yielded Scatchard plots of [^{3}H]-DHA binding that were not parallel to control, indicating an apparent reduction in affinity with no change in the concentration of binding sites (Table II; Figure 1). This suggests a modification of receptor away from the site to which [^{3}H]dihydroalprenolol was bound. Membrane preparations treated with the *E*-8 isomer at 1 μM concentration had both a small reduction in binding site concentration and a small increase in K_D value, suggesting a dual type of inhibition for this isomer.

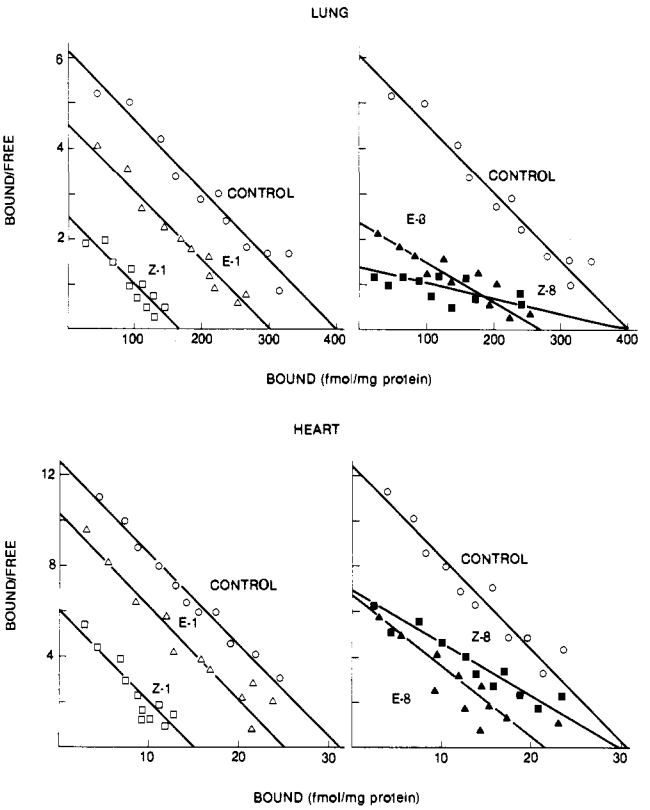


Figure 1. Scatchard plots of saturation experiments performed on membrane preparations after preincubation with the studied alkylating β-blockers. Membrane preparations were preincubated without (control, O) or with the studied compounds at the concentrations given below, washed, and then assayed in saturation experiments for specific [^{3}H]dihydroalprenolol binding as described in the text. Data points are from representative experiments performed at least three times. Preincubation conditions: *Z*-1, 1×10^{-7} M, □; *E*-1, 1×10^{-7} M, △; *Z*-8, 1×10^{-5} M, ■; *E*-8, 1×10^{-6} M, ▲.

Discussion

An effective affinity label has to be recognized by two sites of the receptor, by the hormone binding site that mediates the specific binding, and by the nucleophilic site that is covalently alkylated in the process.[19,20] BAAM is a derivative of alprenolol, an antagonist that is believed to bind to the hormone binding site of the receptor. The reactive group of BAAM is the bromoacetamido group, and the corresponding nucleophilic site of the receptor is probably a thiol group of a cysteine residue. At least in qualitative tests, BAAM was very reactive toward thiols (thiourea or methyl mercaptide) and quite inert toward compounds containing amino groups (phosphatidylethanolamine); the same reactivity toward thiols and lack of reactivity toward amino groups were previously detected for another reactive group, mesylate of α-hydroxy ketones, which is similar to the present bromoacetamide group.[21] The linking of the hormone binding and the nucleophilic sites of a receptor by an affinity label is a rather selective property, as documented by compounds that contain all the necessary groups but do not act as affinity labels. In this laboratory numerous additional candidates were synthesized; three of those related to alprenolol and two of those related to bunitrolol had acceptable affinity at β-adrenoceptors, but their binding was fully reversible. Similar results were obtained for other receptors, for example, opiates.[20]

BAAM, in the form of a mixture, fulfills a number of criteria for an affinity label of β-adrenoceptors: (a) Treatment of preparations of receptors in vitro and subsequent extensive washing lead to a decrease of receptor concentration while the binding affinity of the remaining receptors is not changed.[8,11-17] (b) The alkylation of the receptor by BAAM may be prevented by β-adrenergic agonists or antagonists.[8] (c) Radioactive dihydro–BAAM preparations label a protein that upon electrophoresis migrates as a subunit of the β-adrenoceptor.[8] (d) Effects of BAAM on β-adrenoceptors in living animals persist for days or weeks while those of alprenolol last only hours.[11-13] The principal component of the BAAM mixture, the *Z*-1 isomer, indeed has been found to have high affinity at β-adrenoceptors and to decrease the concentration of receptors without any change in affinity of the remaining receptors; this was an expected finding. Furthermore, the data in Chart I indicate that the presence of the other three isomers in the BAAM mixture is of no great consequence for its effects as an affinity label. Nevertheless, in light

(19) Jakoby, W. B., Wilchek, M., Eds. *Methods Enzymol.* **1977**, 46.
(20) Sayre, L. M.; Larson, D. L.; Fries, D. S.; Takemori, A. E.; Portoghese, P. S. *J. Med. Chem.* **1983**, *26*, 1229.
(21) Simons, S. S.; Pons, M.; Johnson, D. F. *J. Org. Chem.* **1980**, *45*, 3084.

of the mentioned high selectivity of affinity labeling, it was expected that other components of the mixture would be inactive; that expectation has been found incorrect. The *E*-1 isomer differs from the principal component *Z*-1 by an equatorial conformation of the aromatic substituent (Chart I) and was found also to be an affinity label, albeit of lesser affinity. The *Z*-8 and *E*-8 isomers also labeled the adrenoceptors, but both of these compounds changed the binding affinity of the remaining receptors, a sign that the receptors were modified on sites that are outside of the hormone binding area. Thus, there is more similarity within diastereomeric pairs that have the same substituents on carbons 1 and 4 (e.g., *Z*-1 and *E*-1 pair) than in pairs of compounds having the same steric configuration (e.g., *Z*-1 and *Z*-8 pair). These findings suggest that there is a certain amount of steric flexibility in the receptor since only some steric accommodations by the receptor may explain why the probes with *Z* and *E* configuration may both interact with the receptor in a similar manner. Such steric accommodation by receptors has not often been detected but is not unique. Stereochemical effects on affinity labeling were carefully evaluated with morphine derivatives and found to depend greatly on the chirality of the critical substituent.[20,22] Nevertheless, in one instance (murine vas deferens receptors and a probe containing the isothiocyano group) a change in chirality of the reactive group made little difference.[20] Those and the present findings indicate that there may be as much serendipity in the preparation of affinity probes as there is in drug design. Hopefully, the variability in the receptor–probe reaction may be used to obtain a probe recognizing subtypes of receptors. This was realized in the field of opiate receptors[20] but not in the present case; it should be noted that the affinity of compounds studied presently at receptors in rat lungs (approximately 15% β_1 adrenoceptor subtype) and rat heart (approximately 80% β_1) do not differ significantly.

Experimental Section

Melting points were determined on hot-stage (Fisher-Johns Melting Point) apparatus and were not corrected. ^{1}H NMR spectra were measured on a JEOL PMX-60SI (60-MHz) instrument, and ^{13}C NMR spectra were measured on a JEOL 90Q (Fourier transform, 90-MHz) instrument; internal tetramethylsilane standard was used. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN, and by the National Institutes of Health, Bethesda, MD, where mass spectra were also measured.[8] Precoated 0.2-mm sheets, EM reagent silica gel 60F-254, were used for TLC, and silica gel of grade 60 and 230–400 mesh was used for flash column chromatography and silica gel H for short-column chromatography.

[^{3}H]Dihydroalprenolol, 90 Ci/mmol, was purchased from Amersham Corp. (±)-Alprenolol was a generous gift from Hässle. All other chemicals were of analytical grade and were purchased from various sources.

(*Z*)- and (*E*)-1,8-Dinitro-*p*-menthane. A commercial sample of 1,8-diamino-*p*-menthane (10.2 g) was oxidized as previously described,[18] yielding 5.35 g (39%) of a mixture of (*Z*)- and (*E*)-1,8-dinitro-*p*-menthane from which 1.43 g (10%) of pure *Z* diastereomer was obtained by recrystallization (six times) from *n*-hexane; mp 112 °C (lit.[18] mp 112 °C); R_f 0.33 (*n*-hexane–ethyl acetate (9:1)). The mother liquor was processed by flash chromatography (120 g of silica gel) with an *n*-hexane–ethyl acetate (9:1) solvent system to obtain 670 mg (15% total yield) more of the *Z* diastereomer and 150 mg (1%) of pure (*E*)-1,8-dinitro-*p*-menthane: mp 130–132 °C (lit.[18] mp 132 °C); R_f 0.24 (*n*-hexane–ethyl acetate (9:1)).

(*Z*)-1,8-Diamino-*p*-menthane. (a) (*Z*)-1,8-Dinitro-*p*-menthane (1.3 g) was hydrogenated (atmospheric pressure, room temperature) in 200 mL of ethanol on 0.3 g of palladium-on-charcoal (10%) for 48 h; the diamine was isolated in the form of a colorless oil: 0.813 g (84%); R_f 0.34 (ethanol–NH_4OH (9:1)), 0.60 (ethanol–NH_4OH (8:2)); hydrochloride, crystallized from methanol–ether, mp 269–270 °C. Anal. ($C_{10}H_{24}Cl_2N_2$) C, H, N. ^{13}C NMR chemical shifts (δ) of the compound in base form ($CDCl_3$ solution): C-1 (47.67), C-2,6 (40.14), C-3,5 (22.69), C-4 (49.08), C-7 (33.10), C-8 (51.14), C-9,10 (28.44). The assignment of signals was made on the basis of (a) multiplet formation in the proton-decoupled off-resonance spectra, (b) relative intensity of the signals, (c) signals of C-3,5, assumed to be at higher fields than that of C-2,6 due to the gauche interaction between these carbons and that of the axial substituent on C-1,[23] (d) axial methyl groups that resonate at higher fields than the equatorial ones.[24]

(b) (*Z*)/(*E*)-1,8-Diamino-*p*-menthane mixture (5.0 g) was processed by flash chromatography on 300 g of silica gel using ethanol–concentrated NH_4OH (8:2) as the eluent. Yield of the *Z* diastereomer was 2.15 g (43%), identical with the compound obtained by the reduction of (*Z*)-1,8-dinitro-*p*-menthane. It is also possible to achieve some separation of *Z* and *E* diastereomers by distillation; the *Z* diastereomer is more volatile.

(*E*)-1,8-Diamino-*p*-menthane. (a) (*E*)-1,8-Diamino-*p*-menthane was hydrogenated as described for the *Z* isomer, yielding 238 mg (80.4%) of a highly viscous oil: R_f 0.20 (ethanol–NH_4OH (9:1)), 0.39 (ethanol–NH_4OH (8:2)); hydrochloride mp 263 °C. Anal. ($C_{10}H_{24}Cl_2N_2$) C, H, N. ^{13}C NMR chemical shifts (δ): C-1 (48.86), C-2,6 (41.87), C-3,5 (24.43), C-4 (49.08), C-7 (22.57), C-8 (51.03), C-9,10 (28.38). For the conditions of measurements and assignment of the signals see above.

(b) From 5.0 g of a 1,8-diamino-*p*-menthane mixture, 0.76 g (15%) of the *E* diastereomer was obtained (see above).

N^1- and N^8-[3-(*o*-Allylphenoxy)-2-hydroxypropyl]-(*Z*)-1,8-diamino-*p*-menthane. (*Z*)-1,8-Diamino-*p*-menthane (2.55 g, 15 mmol) and *o*-allylphenyl glycidyl ether (0.57 g, 3 mmol) were reacted for 16 h at 70 °C. The excess amine was then removed by distillation in vacuo, and the residue (1.30 g) was purified by flash chromatography on 150 g of silica gel with ethanol–NH_4OH (95:5) as the eluent. The yield of the monosubstituted products was 815 mg (75.4%): R_f 0.56 (ethanol–NH_4OH (95:5)), 0.26 (ethanol–NH_4OH (97.5:2.5)); NMR ($CDCl_3$) δ 7.40–6.90 (m, 4 H, aromatic protons), 6.35–5.80 (m, 1 H, CH_2=C*H*CH_2), 5.10 (d, 2 H, C*H*$_2$=CHCH_2), 4.05 (br s, 3 H, C*H*$_2$O + >C*H*OH), 3.55 (d, 2 H, CH_2=CHC*H*$_2$), 2.95 (br s, 2 H, C*H*$_2$NH), 2.15 (br s, 3 H, NH and NH_2), 1.80–0.95 (m, 18 H, 3-C*H*$_3$ and nine protons of the cyclohexane ring).

The disubstituted products (N^1,N^8-bis(3-(*o*-allylphenoxy)-2-hydroxypropyl)-(*Z*)-1,8-diamino-*p*-menthane) had a higher chromatographic mobility than the monosubstituted compounds: R_f 0.72 (ethanol–NH_4OH (95:5)); NMR ($CDCl_3$) δ 7.35–6.70 (m, 8 H, aromatic protons), 6.05–5.70 (m, 2 H, 2 CH_2=C*H*CH_2), 5.05 (d, 4 H, 2 *H*$_2$C=CHCH_2), 3.85 (m, 6 H, 2 CH_2O + 2 >C*H*OH), 3.35 (d, 4 H, 2 CH_2=CHC*H*$_2$), 2.70 (br s, 4 H, 2 C*H*$_2$NH), 2.30 (br s, 2 H, 2-OH), 2.00–0.90 (m, 20 H, 3-CH_3, nine protons of the cyclohexane ring and 2-NH).

N^1- and N^8-[3-(*o*-Allylphenoxy)-2-hydroxypropyl]-(*E*)-1,8-diamino-*p*-menthane. The reaction was performed as described for the *Z* diastereomer, yielding an oily monosubstituted compound (85%): R_f 0.49 (ethanol–NH_4OH (95:5)); NMR ($CDCl_3$) δ 7.35–6.60 (m, 8 H, aromatic protons), 6.33–5.65 (m, 2 H, 2 CH_2=C*H*CH_2), 5.00 (d, 4 H, 2 C*H*$_2$=CHCH_2), 4.00 (br s, 6 H, 2 CH_2O + 2 >C*H*OH), 3.52 (d, 4 H, 2 CH_2=CHC*H*$_2$), 2.86 (br s, 4 H, 2 C*H*$_2$NH), 2.43 (br s, 4 H, 2 OH + 2 NH), 1.95–1.00 (m, 18 H, 3-CH_3 and nine protons of the cyclohexane ring).

The disubstituted compound (N^1,N^8-bis[3-(*o*-allylphenoxy)-2-hydroxypropyl]-(*E*)-1,8-diamino-*p*-menthane) had R_f 0.80 in the same system: NMR ($CDCl_3$) δ 7.28–6.70 (m, 8 H, aromatic protons), 6.30–5.60 (m, 2 H, 2 CH_2=C*H*CH_2), 5.04 (d, 4 H, 2 C*H*$_2$=CHCH_2), 4.00 (m, 6 H, 2 CH_2O + 2 >C*H*OH), 3.50 (d, 4 H, 2 CH_2=CHC*H*$_2$), 2.85 (br s, 4 H, 2 C*H*$_2$NH), 2.36 (br s, 2 H, 2-OH), 1.90–1.00 (m, 20 H, 3-CH_3, nine protons of the cyclohexane ring and 2 N*H*).

(22) Crouch, R. C.; Bhatia, A. V.; Lever, O. W. *Tetrahedron Lett.* **1983**, *24*, 4801.
(23) Gorenstein, D. G. *J. Am. Chem. Soc.* **1977**, *99*, 2254.
(24) Liptak, A.; Fügedi, P. *Angew. Chem., Int. Ed. Engl.* **1983**, *22*, 255.

N^1-[3-(*o*-Allylphenoxy)-2-hydroxypropyl]-N^8-(bromoacetyl)-(*Z*)-1,8-diamino-*p*-menthane (*Z*-1) and N^8-[3-(*o*-Allylphenoxy)-2-hydroxypropyl]-N^1-(bromoacetyl)-(*Z*)-1,8-diamino-*p*-menthane (*Z*-8). The mixture of the isomeric *N*-[3-(*o*-allylphenoxy)-2-hydroxypropyl]-(*Z*)-1,8-diamino-*p*-menthane (620 mg, 1.72 mmol) was treated in 30 mL of ethyl acetate with bromoacetyl bromide (391 mg, 1.94 mmol) for 20 min at 0 °C. The reaction mixture was diluted with 50 mL of ethyl acetate; the precipitated salts were dissolved by washing with 2 × 10 mL of saturated $NaHCO_3$ solution and water (3 × 15 mL). The organic layer was dried ($MgSO_4$) and evaporated to a solid foam (770 mg). TLC examination of the crude product showed the presence of two minor components with very high R_f values and two main spots. For the separation of the two main isomers short-column chromatography (40 g of silica gel) was used with chloroform–methanol (88:12) as the eluent.

The faster moving main component proved to be compound *Z*-1: 300 mg (35%); mp 156–158 °C; R_f 0.45 (chloroform–methanol (88:12)), 0.80 (ethyl acetate–methanol–NH_4OH (75:25.1)). Anal. ($C_{24}H_{37}BrN_2O_3$) H, Br, N; C: calcd, 59.86; found, 58.52. NMR ($CDCl_3$) δ 7.50–6.85 (m, 4 H, aromatic protons), 6.30–5.75 (m, 1 H, CH_2=C*H*CH_2), 5.00 (d, 2 H, CH_2=CHCH_2), 4.55 (br s, 1 H, NHCO), 4.05 (d, 3 H, CH_2O + >C*H*OH), 4.00 (s, 2 H, CH_2Br), 3.45 (d, 2 H, CH_2=CHCH_2), 3.15 (d, 2 H, CH_2NH), 2.00–1.05 (m, 20 H, 3-CH_3, nine protons of the cyclohexane ring, OH and NH).

The second isomer, *Z*-8, having R_f values of 0.27 and 0.60, respectively in the systems above, was isolated as a thick syrup (279 mg, 32%) powdered under *n*-hexane; mp 114 °C (sintered at 98 °C). Anal. ($C_{24}H_{37}BrN_2O_3$) C, H, Br, N. NMR ($CDCl_3$) δ 7.40–6.70 (m, 4 H, aromatic protons), 6.35–5.60 (m, 1 H, CH_2=C*H*CH_2), 5.00 (d, 2 H, CH_2=CHCH_2), 4.50 (br s, 1 H, N*H*CO), 4.03 (d, 3 H, CH_2O + >C*H*OH), 3.90 (s, 2 H, CH_2Br), 3.40 (d, 2 H, CH_2=CHCH_2), 3.10 (d, 2 H, CH_2NH), 2.10 (s, 2 H, OH and NH), 1.95–0.98 (m, 18 H, 3-CH_3 and nine protons of the cyclohexane ring).

N^1-[3-(*o*-Allylphenoxy)-2-hydroxypropyl]-N^8-(bromoacetyl)-(*E*)-1,8-diamine-*p*-menthane (*E*-1) and N^8-[3-(*o*-Allylphenoxy)-2-hydroxypropyl]-N^1-(bromoacetyl)-(*E*)-1,8-diamino-*p*-menthane (*E*-8). The mixture of isomeric *N*-[3-(*o*-allylphenoxy)-2-hydroxypropyl]-(*E*)-1,8-diamino-*p*-menthanes (464 mg, 1.28 mmol) was treated in 25 mL of ethyl acetate with bromoacetyl bromide (259 mg, 1.28 mmol) as described for the *Z* isomers.

TLC showed the presence of two main components with R_f 0.52 and 0.46 values, using an ethyl acetate–methanol–NH_4OH (75:25:1) solvent system. The two isomers were separated with the same solvent system by the short-column technique.

The first component proved to be *E*-1 (167 mg, 27%), mp 85 °C. Anal. ($C_{24}H_{37}BrN_2O_3$) C, H, N, Br.

The second isomer *E*-8 (79 mg, 13%) was isolated as a solid foam, was powderable under *n*-hexane, and melted at 73 °C. Anal. ($C_{24}H_{37}BrN_2O_3$) C, H, N, Br.

Reactivity of Bromoacetyl Alprenolol Menthanes with Thiols and Amines. All of the above bromoacetamido derivatives reacted (20 °C, methanolic solutions) rapidly and to completion with thiourea (1 h) or with sodium methyl mercaptide (30 min); no reaction was observed with phosphatidylethanolamine (pH 13.0, 15 h).

Membrane Preparation. Rat heart and lung membranes were prepared as described previously.[13] Briefly, rat heart or lung tissue was homogenized by use of a Polytron PT-10 homogenizer in 20 vol of 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM EDTA and 150 mM sodium perchlorate. Homogenates were centrifuged at 48000*g* for 10 min at 4 °C. The pellets were resuspended by a Polytron in 30 vol of 50 mM Tris-HCl, pH 7.4, containing 10 mM $MgCl_2$ (binding buffer) and recentrifuged as before. The pellets were resuspended in 30 vol of binding buffer by a hand-held glass homogenizer and were centrifuged. The final pellets were resuspended in either 10 vol (heart) or 15 vol (lung) of the binding buffer.

Measurement of Affinity. The affinity of the BAAM mixture and isomers was determined as follows. Solutions of alkylating β-blockers were prepared to give final concentrations in the assay between 1×10^{-9} and 1×10^{-4} M (16 different concentrations). The assay tubes contained binding buffer, 5 nM [^{3}H]dihydroalprenolol, and either no further additions (total binding), or 10 μM (±)-alprenolol (nonspecific binding), or aliquots of the solutions of alkylating β-blockers in a total volume of 150 μL. Reactions were initiated by the addition of membranes (100 μL, containing ~400 μg of protein for heart, ~100 μg of protein for lung). Incubations were carried out for 20 min at 30 °C and were terminated by dilution with 4 mL of ice-cold binding buffer and rapid filtration under vacuum on GF/C filters. The reaction tubes and filters were washed an additional three times with 4 mL of binding buffer. Dried filters were placed in scintillation vials containing Ready-Solv MP (Beckman) and were counted by liquid scintillation spectrometry (Beckman LS-5800) at approximately 47% efficiency. Duplicate samples were used throughout, and each experiment was performed at least three times. In all cases specific binding is reported and is defined as the difference between total binding and nonspecific binding (i.e., binding in the presence of 10 μM (±)-alprenolol).

Drug concentrations causing 50% inhibition of specific [^{3}H]-dihydroalprenolol binding were estimated by computer-fitted logistic curves, and inhibitor dissociation constants were calculated from the equation[25]

$$K_1 = IC_{50}/[1 + (S/K_D)]$$

Measurement of Persistent Blockade. The irreversible potency of the alkylating β-blockers was assessed by preincubating heart or lung membranes at 30 °C for 30 min with various concentrations of these compounds. The membranes were then diluted with ice-cold binding buffer and incubated on ice for 10 min before centrifugation at 48000*g* for 10 min at 4 °C. The pellets were resuspended in 5 mL of binding buffer with a hand-held glass Teflon homogenizer and then diluted to 30 mL with more binding buffer and recentrifuged. This washing procedure was repeated twice, and the final pellets were resuspended in their original volumes. These membrane preparations either were assayed for specific [^{3}H]dihydroalprenolol binding at a fixed concentration of [^{3}H]dihydroalprenolol (5 nM) in dose–response experiments (performed twice) or were assayed at various concentrations of [^{3}H]dihydroalprenolol (0.3–6.0 nM) in saturation experiments performed three times. In the latter type of experiment, the data were analyzed by Scatchard plots.

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Registry No. (*Z*)-1, 97879-26-0; (*E*)-1, 97879-28-2; (*Z*)-8, 97879-27-1; (*E*)-8, 97879-29-3; 1,8-diamino-*p*-menthane, 80-52-4; (*Z*)-1,8-dinitro-*p*-menthane, 54166-26-6; (*E*)-1,8-dinitro-*p*-menthane, 58940-69-5; (*Z*)-1,8-diamino-*p*-menthane, 54166-24-4; (*E*)-1,8-diamino-*p*-methane, 54166-25-5; *o*-allylphenyl glycidyl ether, 4638-04-4; (N^1,N^8-bis[3-(*o*-allylphenoxy)-2-hydroxypropyl]-(*Z*)-1,8-diamino-*p*-menthane, 97879-20-4; (N^1,N^8-bis[3-(*o*-allylphenoxy)-2-hydroxypropyl]-(*E*)-1,8–diamino-*p*-menthane, 97879-23-7; N^8-[3-(*o*-allylphenoxy)-2-hydroxypropyl]-(*Z*)-1,8-diamino-*p*-menthane, 97879-21-5; N^8-[3-(*o*-allylphenoxy)-2-hydroxypropyl]-(*E*)-1,8-diamino-*p*-menthane, 97879-24-8; N^1-[3-(*o*-allylphenoxy)-2-hydroxypropyl]-(*Z*)-1,8-diamino-*p*-menthane, 97879-22-6; N^1-[3-(*o*-allylphenoxy)-2-hydroxypropyl]-(*E*)-1,8-diamino-*p*-menthane, 97879-25-9.

(25) Cheng, Y. C.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099.