53023-56-6; **33**, 58222-69-8; **34**, 58222-70-1; **35**, 58222-71-2; **36**, 58222-72-3; **37**, 58222-73-4; **38**, 41096-52-0; **39**, 58222-74-5; **40**, 58239-26-2; 41, 91384-64-4; **42**, 58222-77-8; **43**, 58222-67-6; **44**, 91384-65-5; **45**, 35529-97-6; **46**, 58222-75-6; **47**, 58222-80-3; **48**, 58222-81-4; **49**, 58222-82-5; **50**, 58222-83-6; **51**, 58222-84-7; **52**, 58222-85-8; **53**, 58222-86-9; **54**, 58222-87-0; **55**, 58222-88-1; **56**, 58222-89-2; **57**, 58222-90-5; **58**, 58222-91-6; **59**, 58222-78-9; **60**, 58222-79-0; **61**, 58222-13-2; **62**, 58222-14-3; **63**, 58222-15-4; **64**, 58222-16-5; **65**, 58222-17-6; **66**, 58222-18-7; **67**, 53023-54-4; **68**, 58222-19-8; **69**, 58222-20-1; **70**, 58222-21-2; **71**, 58222-25-6; **72**, 58222-26-7; **73**, 58222-27-8; **74**, 58222-28-9; **75**, 58222-29-0; **76**, 58222-30-3; **77**, 58222-32-5; **78**, 58222-33-6; **79**, 58222-34-7; **80**, 58222-35-8; **81**, 58222-36-9; **82**, 58222-31-4; **83**, 58222-42-7; **84**, 58222-43-8; **85**, 58222-44-9; **86**, 58222-45-0; **87**, 58222-47-2; **88**, 58222-48-3; **89**, 58222-49-4; **90**, 58222-50-7; **91**, 58222-51-8; **92**, 58222-52-9; **93**, 58222-22-3; **94**, 58222-23-4; **95**, 58222-24-5; **96**, 58222-37-0; **97**, 58222-38-1; **98**, 58222-39-2; **99**, 58222-40-5; **100**, 58222-41-6; **101**, 91423-74-4; **102**, 58222-54-1.

Potentiation of the Tolerogenicity of Benzylpenicilloylated Eicosa-L-lysine by Conjugation with 4-(Hydroxymethyl)benzyl 3β-Cholestanyl Succinate

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It was previously found that amino acid polymers such as oligolysines bearing haptenic groups in high densities efficiently suppress anti-hapten IgE antibody formation. Such conjugates are strong elicitors of anaphylaxis and therefore may not be used for desensitization of drug allergic patients. Here we report on the synthesis and immunological evaluation of benzylpenicilloylated (BPO) eicosa-L-lysines containing none, one, or two lipophilic *p*-(hydroxymethyl)benzyl cholestan-3β-yl succinate (OSuco) groups. The lipophilic derivatives suppress primary as well as ongoing anti-BPO IgE antibody formation in mice much more efficiently than their hydrophilic counterpart. The lipophilic but not the hydrophilic derivatives form stable micelles in water and suppress the antibody formation according to different cellular mechanisms. The relationship between structure, hydrophobicity, and mode of action is discussed.

Drug allergies of the immediate or anaphylactic type, such as, for example, the penicillin allergy, still constitute an unsolved, though more and more urgent problem in medical therapy. It is well established that these allergies are mediated by specific IgE antibodies that are formed against, and recognize the drug or its metabolites in the form of a conjugate with autologous protein(s). Thus, in the case of the penicillin allergy the benzylpenicilloyl (BPO)[1] moiety was shown to be the major antigenic determinant.[2a] One approach to the treatment of allergies would consist in the abrogation of the allergen-specific IgE antibody formation, and several such attempts have been made. Chiorazzi et al.[2b] have reported that primary as well as ongoing anti-BPO IgE antibody formation in mice can be suppressed by injecting BPO-poly(D-Glu,D-Lys). Similarly carbohydrates,[3,4] isologous γ-globulins,[5] or poly(D- or L-lysines)[6] bearing haptenic groups in high densities were found to suppress antibody formation with hapten but not isotype specificity. These compounds were shown to exert their action by interfering with the function of the antibody-forming cells.[2,3,5] It must be noted, however, that these B-cell tolerogens also strongly elicit anaphylaxis in sensitized individuals, thereby limiting their potential therapeutic usefulness.

In previous studies on BPO-oligolysines[6-8] it was found that BPO_{21}-eicosalysine is the smallest homomer that still displays a high tolerogenicity. Having developed a synthesis in solution for BPO_{21}-eicosa-L-lysine, we were interested to study whether the introduction of hydrophobic auxillary groups into this conjugate potentiates its tolerogenicity, such that the required dosage and/or epitope density could be lowered correspondingly.

Several literature reports provide evidence that lipid modification of antigens tends to lower their humoral immunogenicity and often even leads to immune tolerance. Some years ago Dailey and Hunter[9,10] observed that the introduction of dodecanoyl residues into bovine serum albumin (BSA) completely abrogates its humoral *but increases* its cellular immunogenicity. Furthermore, Machida et al.[11,12] demonstrated that the suppressed humoral immunogenicity of dodecanoylated BSA is a consequence of the action of BSA-specific suppressor T cells. A similar effect of lipid modification was reported by Benacerraf and co-workers, who found that palmitoyl conjugated poly(L-Glu,L-Lys,L-Phe) peptides[13] or fowl γ-globulin[14] bind

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(1) Symbols and abbreviations are in accordance with the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem.*, **74**, 1 (1977)). In addition, the following abbreviations are used: Adip, adipinoyl; BPO, benzylpenicilloyl; DCC, dicyclohexylcarbodiimide; DNP, dinitrophenyl; EDAC, *N*-ethyl-*N'*-[3-(dimethylamino)propyl]-carbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; HONSu, *N*-hydroxysuccinimide; DCHA·MIA, dicyclohexylammonium (2-methyl-1-indolyl)acetate; OSuco, 4-(hydroxymethyl)benzyl 3β-cholestanyl succinate; PBS, phosphate balanced saline; PCA, passive cutaneous anaphylasix; PVm/BPO, molar penamaldate value per BPO group; BSA, bovine serum albumin; Asc, *Ascaris suum* protein(s); HVE, high-voltage electrophoresis.

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tightly to lymphocytes and macrophages, thereby converting these cells into inducers of immune tolerance.

For the murine IgE system Segawa et al.[15] found that administration of ovalbumin conjugated with various fatty acids effectively abrogate primary and ongoing IgE antibody formation. This suppression was T cell mediated and isotype specific. Similarly Kishimoto et al.[16] observed that preadministration of antigen conjugated *N*-acetyl-6-*O*-mycoloylmuramyl peptides into mice suppressed IgE antibody production upon subsequent immunization; this T cell mediated suppression was greatly dependent on the presence of the hydrophobic mycoloyl residues in these derivatives. In this paper we report on the syntheses and immunological testing of fully benzylpenicilloylated eicosa-L-lysines containing none, one, or two hydrophobic *p*-(hydroxymethyl)benzyl cholestan-3β-yl succinate (OSuco) groups. The compounds were tested for IgE suppression in BALB/c mice. It was found that the hydrophobic derivatives suppressed the primary as well as the ongoing anti-BPO IgE antibody formation much more efficiently than the hydrophilic counterpart. The effect of OSuco modification on the tolerogenicity of BPO-eicosalysine is discussed in the light of different molecular interactions with macrophages and lymphocytes, thus activating different cellular mechanisms of unresponsiveness.

Syntheses of BPO-eicosa-L-lysines. Two previously described oligolysine syntheses by the solid-phase method showed that this technique yields quite heterogeneous products.[17,18] Therefore we decided to use a synthesis in solution based on the liquid–liquid extraction technique as described by Schneider et al.[19] Hereby a better control of the reaction is possible. If required, the purification of intermediates is also possible by chromatography. In a first approach it was attempted to synthesize N^{ϵ}-benzyloxycarbonyl (N^{ϵ}-Z) protected eicosalysine by stepwise condensation of *tert*-butyloxycarbonyl (Boc)-Lys(Z)-OH to Boc-Lys(Z)-OCH_2PhCH_2-O-Gly-COPh. However, already at the pentapeptide level, gel formation and limited solubility were encountered. By use of the hydrophobic OSuco group for C-terminal protection, solubility properties were improved; however, at the octapeptide level, again limiting solubility occurred. Since this strategy did not allow fragment condensation (the OSuco ester could not be saponified), the synthesis using Boc for N^{ϵ} and 2-nitrophenylsulfenyl (Nps) for N^{α}-protection was tried. The Nps deprotection was rapidly and selectively achieved by NH_4SCN in the presence of the extractable Nps scavenger (2-methyl-1-indolyl)acetic acid (MIA).[20]

Although using Boc instead of Z groups for side-chain protection improved solubility, it appeared advisable to reach the eicosalysine **10** by fragment condensation of the decalysines **8** and **9** or the eicosalysine **11** by dimerization of the decalysine **9** via adipic acid, respectively (see Scheme I). Whereas the synthesis of the small homomeres was

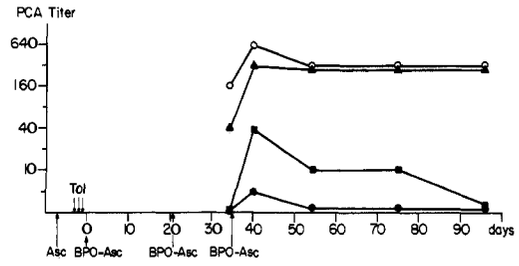

Figure 1. Groups of mice were preimmunized with Asc (10 μg) absorbed on $Al(OH)_3$ (2 mg). Seven days later at day 0 and later at days 21 and 35, all animals were immunized with BPO_9-Asc (10 μg) absorbed on $Al(OH)_3$ (2 mg). At days −3, −2, and −1, 1 mg of the tolerogens was injected intravenously. At days 34, 40, 54, 75, and 96, bleedings and measurements of the anti-BPO IgE titers were performed. The titers of the groups treated with compound **12** (▲), **13** (■), **14** (●), and saline (○) are shown.

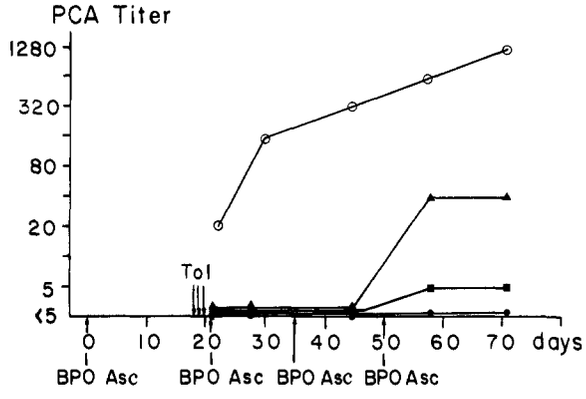

Figure 2. Groups of mice were immunized on days 0, 21, 35, and 50 with BPO_9-Asc (6 μg) absorbed on $Al(OH)_3$ (2 mg). The tolerogens were injected at days 18, 19, and 20. At days 21, 30, 44, 57, and 71, bleedings and measurements of the anti-BPO IgE titers were performed. The titers of the groups treated with compound **12** (▲), **13** (■), **14** (●), and saline (○) are shown.

readily achieved by condensing the protected amino acids by means of *N*-ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide (EDAC) in the presence of 1-hydroxybenzotriazole (HOBt),[21] this procedure was inefficient in the case of the higher homomeres.

In these instances coupling was achieved by reacting Nps-Lys(Boc)-ONSu with the amino peptide in dimethyl sulfoxide with use of HOBt as catalyst. It should be noted that HOBt is acidic enough to cleave the Nps protection and therefore has to be neutralized prior to use.

Each reaction mixture was dissolved in CH_2Cl_2 and extracted in an automatized extractor with 0.1 M HCl, 0.2 M K_2CO_3, and H_2O.[19] Additional purification steps by chromatography on silica gel using various $CHCl_3$–CH_3OH mixtures were followed as indicated below.

According to Scheme I, the purified, fully protected peptides **10** and **11** were either completely deprotected by liquid HF or partially deprotected by trifluoroacetic acid (TFA). The deprotected peptides were penicilloylated with potassium penicillinate in K_2CO_3 solutions at pH 10.5–11. Under these conditions the OSuco esters were completely stable. The penicilloylated peptides were obtained in pure form after gel permeation chromatography and lyophilization. As recorded in Table III, the products were assayed for homogenicity by thin-layer chromatography (TLC), by high-voltage electrophoresis (HVE), and by gel permeation chromatography. As shown in Table IV, the compounds were characterized by elemental analysis, by penamaldate

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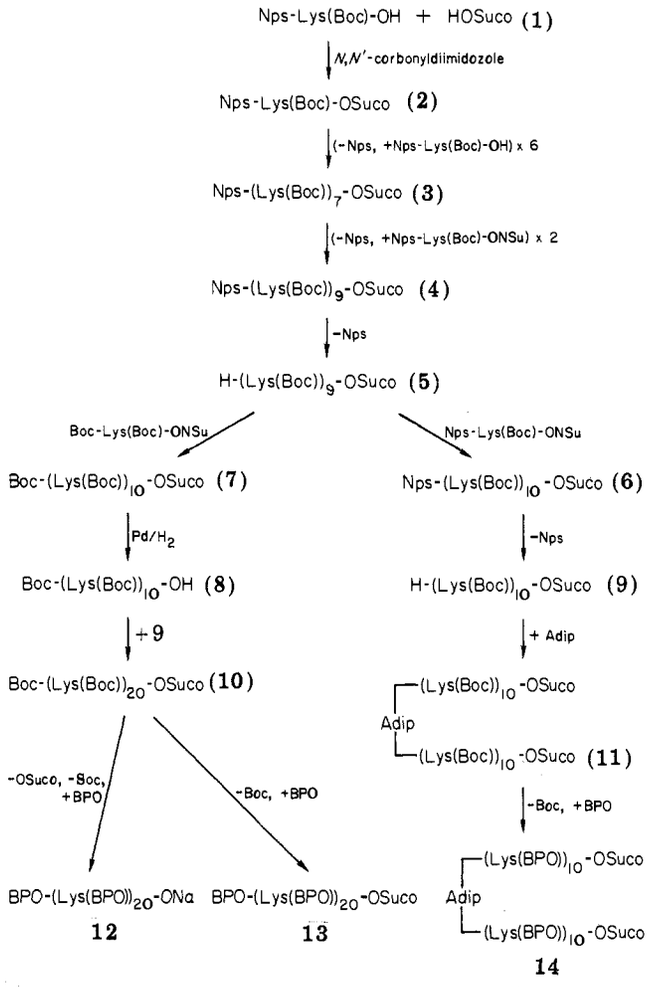
Scheme I. Syntheses of the BPO-eicosalysines 12–14



analysis,[22,23] and by the TNBS assay.[24]

Suppression of the Murine Anti-BPO IgE Antibody Formation by Compounds 12–14. Groups of mice were immunized with BPO-Ascaris suum proteins (BPO_9-Asc) absorbed on $Al(OH)_3$. As shown in the protocols in Figures 1 and 2, the compounds **12–14** were injected once before primary immunization (Figure 1) and once before secondary immunization (Figure 2). The sera were assayed for anti-BPO and anti-Asc IgE antibodies as described in the Experimental Section.

As shown in Figure 1, compound **12** mediates only a moderate IgE suppression when administered prior to primary immunization. Furthermore, this suppression was not stable on further immunizations and is completely abrogated after the second booster injection. Quite a different situation is observed with substances **13** and **14**. Here a strong suppression was noticed, which was barely affected by two subsequent booster immunizations. Compound **14** caused an almost complete suppression, which was also not affected by a fourth immunization at day 99; at day 112 again a titer below 10 was measured (data not shown).

In comparison and as shown in Figure 2, the hydrophilic compound **12** shows a higher tolerogenicity when administered after primary immunization. Also the hydrophobic compounds **13** and **14** induce a stronger suppression than in the previous experiment. Again upon subsequent booster immunizations, the immune suppressions brought about by compounds **13** and **14** are more persistent. In both experiments compound **14** caused a complete suppression of the anti-BPO IgE response, which was not affected by three subsequent booster immunizations.

Measurements of the corresponding anti-Asc IgE titer showed that the tolerogen injections showed no effect on the anticarrier response,[25] thus indicating that their effectiveness is hapten specific and not due to nonspecific immune suppression.

Discussion

Mechanism of Tolerance Induction. The pronounced differences in tolerance induction as observed for compound **12** vs. its hydrophobic analogues suggests that there are different cellular mechanisms involved. For tolerance induction by polymerized amino acids bearing haptenic groups in high densities, such as compound **12**, it has been shown that these compounds directly interfere with the antibody production of B lymphocytes, i.e., they establish a central B cell tolerance.[2b,5]

For the amphipatic derivatives **13** and **14** an additional involvement of T suppressor cells may be expected from related reports from the literature. Thus it has been found that BSA,[11,12] poly(L-Glu,L-Lys,L-Phe),[13] fowl γ-globulin,[14] or ovalbumin[15] strongly induce T suppressor cells upon conjugation with fatty acids. That compound **14** induces and acts via T suppressor cells was shown in two adoptive cell transfer experiments.[26] In one experiment spleen cells from BALB/c mice that were previously immunized with BPO_9-Asc in $Al(OH)_3$ were transferred into syngeneic irradiated (650 rad) recipients. The secondary anti-BPO IgE response in these animals was strongly suppressed when spleen cells from tolerized animals were cotransferred. This suppression was reversed when the tolerized spleen cells were treated with anti-Lyt-2.2 antibody plus complement prior to cell transfer. Since this treatment deletes T suppressor cells that bear the Lyt-2.2 marker,[27] the observed immune suppression was indeed mediated by T suppressor cells.

In a similar experiment, spleen cells from immunized or tolerized mice were transferred into syngeneic, nonirradiated recipients. A secondary anti-BPO IgE response was obtained only in the case where primed spleen cells were transferred. However, when the recipients were treated with cyclophosphamide 2 h prior to cell transfer, both groups gave the same secondary IgE response. Since cyclophosphamide treatment inhibits T cell mediated suppression of the murine IgE response,[28] this experiment again demonstrates that compound **14** induces and acts via T suppressor cells.

Relationship between Hydrophobicity, Antigen Structure, and the Mechanism of Tolerance Induction. In the previous paragraph evidence was presented that lipid-modified antigens induce suppressor T cells in mice. In particular it was shown that the hydrophobic compound **14** induces and acts via T cells.

The chemical nature of the compounds **12–14** is defined (see Tables III and IV) and their structure has been studied. Conformational studies on compound **12** in

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aqueous medium at neutral pH indicated 30% α-helix structure.[29] Furthermore, conformational analysis of N^{ϵ}-Boc-oligolysines showed that the C-terminal OSuco group does not interfere with the conformation of the peptide chain.[30] Taken collectively and as suggested by the molecular weights determined by gel permeation chromatography (see Table III), the major structural implication of OSuco conjugation seems to be the formation of micelles.

Thus the question arises why and how hydrophobic moieties, in this study the OSuco group, promote the activation of T suppressor cells. A definitive answer can not be given at present; however, there is growing evidence that lipid modification of antigens alters their interaction with macrophages and that this altered antigen processing and presentation is directly responsible for the activation of suppressor T cells.

It has been found that antigen uptake is greatly increased for lipid modified antigens. For dodecanoylated BSA, a 25–50-fold increased uptake has been observed for macrophages but not for lymphocytes.[10] Similarly a 20–30-fold higher uptake by BALB/c macrophages was noted for compound **14** than for compound **12** (Lüscher and Gitler, unpublished results).

It is tempting to speculate that there is a direct relationship between the micellar structure of such antigens and their uptake by macrophages. Antigen uptake is a metabolically active process[10] that is initiated by binding to membrane receptors.[31] It is conceivable than a micellar antigen due to its increased particle size and potential to undergo additional hydrophobic interaction with membrane components still further exerts a stimulatory effect on the uptake.

Furthermore, there are data suggesting that lipid modification of antigens leads to their association with plasma membranes. Janeway et al. detected dodecanoylated dinitrophenyl (DNP) BSA on macrophage surfaces in high quantities and over long periods of time.[32] Low amounts of fatty acid conjugated antigens are directly absorbed on cell surfaces, presumably due to spontaneous incorporation into the plasma membrane.[10,14,33,34]

For the antigens **12** and **14**, preliminary studies in subcellular antigen tracing, as assessed by fractionating antigen pulsed homogenized macrophages on self-generating percol gradients, showed differences in antigen distribution; in particular it was found that compound **14** largely resides in the plasma membrane fraction (Lüscher and Gitler, unpublished results). Thus, lipid modification of antigens may have further implications. First, due to their hydrophobic sites they may become intimately membrane associated or membrane integrated. Second, as a consequence of the unnatural lipid conjugation, the high hydrophobicity, and micellar structure, these antigens may be rather stable toward lysosomal enzymes. Immunologically such alterations in antigen processing and presentation may be decisive since the efficiency of antigen handling by macrophages directly influences T suppressor vs. T helper cell activation.[35] In fact, evidence was provided that macrophages, but no other cell type, when pulsed with palmitoyl γ-globulin, strongly induce suppressor T cells.[14] Similary Yokomuro et al.[36] observed that glass-adherent spleen cells after pulsing with heavily dinitrophenylated keyhole limpet hemocyanin strongly induced suppression of the anti-DNP IgE response in mice. Dailey et al. found that macrophages pulsed with dodecanoylated BSA efficiently induced delayed-type hypersensitivity[10] and suppression of antibody formation,[9] which was shown to be mediated by suppressor T cells.[11,12]

The role and significance of macrophages for the activation of the suppressor pathway is not clear and often controversial. In contrast to the above-mentioned findings, there exist several reports according to which macrophages are not required for the induction of suppressor T cells.[37-39] It is difficult to explain this contradiction; however, it may be mentioned that Morimoto et al.[40] recently reported that complete removal of macrophages is difficult to achieve and leads to the abrogation of T suppressor cell formation.

It also must be mentioned that antigen processing and presentation by macrophages is a well-known requirement for the activation of T helper cells.[35,39,41] Thus if macrophages were required for the induction of T helper and T suppressor cells, the question arises as to what is the difference in these macrophage functions. In the light of the feedback suppression concept proposed by Gershon et al.,[42-44] one could visualize that differences in antigen processing and/or presentation by macrophages lead to the preferential stimulation of the $Ly1^+,Qa^-$ or the $Ly1^+,Qa^+$ T helper cell subset, thus leading either to the activation of B lymphocytes or to the induction of T suppressor cells.

Experimental Section

Reagents and Methods. Amino acid derivatives and other chemicals were purchased from Fluka AG., Buchs, Switzerland. Solvents, silica gel plates 60 F-254 for preparative and analytical TLC, and silica gel 60 for column chromatography (particle size 0.063–0.2 mm) were obtained from Merck AG., Darmstadt, Germany. For gel permeation chromatography Sephadex G-10, G-25m, G-150, or LH-20 from Pharmacia, Uppsala, Sweden, were used. HVE was performed on cellulose plates from Merck or cellulose acetate foils from Camag AG., Muttenz, Switzerland. Potassium benzylpeinicillinate was from Glaxo Ltd., London, England. DCHA·MIA was synthesized and used as previously described.[20] $HOCH_2C_6H_4CH_2OH$ and mono-3β-cholestanyl succinate were prepared as described by Rolli.[45] Workup I designates dissolving the reaction mixture in CH_2Cl_2 (400 mL) and extracting

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Table I. Reaction Parameters for the Synthesesis of Smaller N^{ϵ}-Boc Oligolysines

compd	reagents (molar excess in %)	concn (mL/mmol) in	rctn time, h	yield, %
H-(Lys(Boc))$_2$-OSuco	NH_4SCN (100), DCHA·MIA (100)	38 *a*	1	99
Nps-(Lys(Boc))$_3$-OSuco	Nps-Lys(Boc)-OH (10), HOBt (5), EDAC (10)	3 CH_2Cl_2	12	96
H-(Lys(Boc))$_3$-OSuco	NH_4SCN (100), DCHA·MIA (100)	38 *a*	1	98
Nps-(Lys(Boc))$_4$-OSuco	Nps-Lys(Boc)-OH (10), HOBt (5), EDAC (10)	3 CH_2Cl_2	12	95
H-(Lys(Boc))$_4$-OSuco	NH_4SCN (100), DCHA·MIA (100)	38 *a*	1	98
Nps-(Lys(Boc))$_5$-OSuco	Nps-Lys(Boc)-OH (10), HOBt (5), EDAC (10)	3 CH_2Cl_2	12	95
H-(Lys(Boc))$_5$-OSuco	NH_4SCN (100), DCHA-MIA (100)	38 *a*	1	98
Nps-(Lys(Boc))$_6$-OSuco	Nps-Lys(Boc)-OH (15), HOBt (10), EDAC (15)	3 CH_2Cl_2	12	94
H-(Lys(Boc))$_6$-OSuco	NH_4SCN (100), DCHA·MIA (100)	38 *a*	1	97
Nps-(Lys(Boc))$_7$-OSuco	Nps-Lys(Boc)-OH (15), HOBt (10), EDAC (15)	4 CH_2Cl_2	20	96
H-(Lys(Boc))$_7$-OSuco	NH_4SCN (100), DCHA·MIA (100)	38 *a*	1	97
H-(Lys(Boc))$_8$-OSuco	NH_4SCN (100), DCHA·MIA (100)	38 *a*	1.5	89[b]
Nps-(Lys(Boc))$_9$-OSuco	Nps-Lys(Boc)-ONSu (15), HOBt (0.2)	3 $(CH_3)_2SO$	24	87[b]
H-(Lys(Boc))$_9$-OSuco	NH_4SCN (100), DCHA·MIA (100)	38 *a*	1.5	
H-(Lys(Boc))$_{10}$-OSuco	NH_4SCN (100), DCHA·MIA (100)	38 *a*	1.5	95

[a] CH_2Cl_2–CH_3OH–CH_3CO_2H (15:2:2, v/v/v). [b] Overall yield of the coupling and deprotection reaction.

in an automatic extractor[19] with 0.1 M HCl (2 L), H_2O (300 mL), 0.2 M K_2CO_3 (2 L), and H_2O (500 mL). Alternatively and in order to improve solubility and phase separation, 10–15% CH_3OH (v/v) was added to the extractants (workup II). After drying over Na_2SO_4, the CH_2Cl_2 solutions were evaporized and dried in vacuo. Unless mentioned otherwise, reactions were performed by stirring at room temperature in the dark and under argon. TLC was performed in the following systems: A, $CHCl_3$–CH_3OH (95:5, v/v); B, $CHCl_3$–CH_3OH (92.5:7.5, v/v); $CHCl_3$–CH_3OH (9:1, v/v); D, $CHCl_3$–CH_3OH (85:15, v/v); E, $CH_3CO_2C_2H_5$–CH_3CO_2H–H_2O (3:1:1, v/v); F, 1-butanol–CH_3CO_2H–H_2O (4:1:1, v/v/v). The substances were detected by UV light at 254 nm (UV) and by treatment with fluorescamine (fluram), ninhydrin, ninhydrin and heating to 130 °C (a.h.), H_2SO_4 and heating (a.h.), Cl_2 vapor followed by KI/o-tolidine[46] 2,6-dichlorophenolindophenol, $AgNO_3$ in aqueous NH_3 ($[Ag(NH_3)_2]NO_3$) and I_2 in aqueous NaN_3 (I_2/NaN_3). HVE was performed at 40 V/cm in 0.05 M phosphate buffer of pH 7.4 by using a horizontal electrophorese apparatus from Camag. HVE(I) refers to electrophoresis on cellulose acetate for 20 min, HVE(II) to electrophoresis on cellulose for 40 min. Penamaldate analysis was performed as described by Schneider and de Weck,[22,23] using a Pye-Unicam SP8-100 spectrophotometer. The molar penamaldate values (PVm) all showed a penamaldate stability of $PS_{10} \geq 95\%$.

$HOCH_2C_6H_4CH_2OSuc$-3β-cholestane (1). To a solution of mono-3β-cholestanyl succinate (5 g, 10.2 mmol) in dry tetrahydrofuran (8 mL) was added *N,N'*-carbonyldiimidazole (1.74 g, 10.7 mmol) and the mixture was stirred for 1 h. This solution was slowly added to a solution of 1,4-bis(dihydroxymethyl)benzene (6.0 g, 44.1 mmol) in dry dimethylformamide (10 mL). After 36 h and workup I, a colorless resin (5.25 g; 85%) was obtained: TLC R_f(A) 0.56 (UV, H_2SO_4) main spot, 0.74 (UV, H_2SO_4) little (symmetric diester).

Nps-Lys(Boc)-OSuco (2). To a solution of Nps-Lys(Boc)-OH (5.05 g, 12.64 mmol) in dry tetrahydrofuran (25 mL) was added *N,N'*-carbonyldiimidazole (2.08 g, 12.8 mmol). After 1 h, a solution of 1 (6.93 g, 11.37 mmol) in dry tetrahydrofuran (12 mL) was added. After stirring for 36 h and workup I, a yellow solidified resin was obtained (9.80 g). This material was chromatographed on a silica gel column (200 g, 5.7 × 16 cm). After eluting with $CHCl_3$ (600 mL) and 1% CH_3OH in $CHCl_3$ (520 mL), the main zone was isolated: yield 8.0 g (71%); TLC R_f(A) 0.73 (UV, ninhydrin (a.h.) and H_2SO_4 (a.h.)) one spot.

H-Lys(Boc)-OSuco (2a). Compound **2** (3.68 g, 3.71 mmol) was dissolved in CH_2Cl_2–CH_3OH–CH_3CO_2H (15:2:2, v/v/v) (141 mL) and stirred with NH_4SCN (565 mg, 7.43 mmol) and DCHA·MIA(2.75 g, 7.43 mmol) for 1 h. After workup I, 3.05 g (98%) of **2a** was obtained as a colorless, solidified foam: TLC R_f(A) 0.23 (UV, ninhydrin, fluram, H_2SO_4 (a.h.)) one spot.

Nps-(Lys(Boc))$_2$-OSuco (2b). Compound **2a** (3.05 g, 3.64 mmol) and HOBt (577 mg, 3.82 mmol) were dissolved in CH_2Cl_2 (7.2 mL) and *N*-methylmorpholine (1 mL), and the mixture was stirred with Nps-Lys(Boc)-OH (1.59 g, 4.00 mmol) and EDAC (767 mg, 4.00 mmol) for 1 h at 0 °C and overnight at room temperature. After workup I, 4.30 g (97%) of a yellow solidified foam was obtained: TLC R_f(A) 0.73 (UV, ninhydrin (a.h.) and H_2SO_4 (a.h.)) main spot, 0.70 and 0.80 (UV and ninhydrin (a.h.)) traces.

As shown in Scheme I, further chain elongation was achieved by successive repeating of Nps removal and condensation of Nps-Lys(Boc)-OH as described in the two preceding paragraphs. In order to remove accumulated side products, the N^{α}-protected hexapeptide (3.85 g) was applied to a silica gel column (120 g, 5.7 × 16 cm) and eluted with 1% CH_3OH in $CHCl_3$ (720 mL) and 2% CH_3OH in $CHCl_3$ (600 mL). The main zone isolated gave the pure hexapeptide (3.02 g, 78%): TLC R_f(B) 0.47, R_f(C) 0.63 one spot.

Due to decreasing reactivities, the higher homomeres were obtained by reacting the amino peptides with Nps-Lys(Boc)-ONSu in dimethyl sulfoxide and in the presence of HOBt.

Nps-(Lys(Boc))$_8$-OSuco (3a). H-(Lys(Boc))$_7$-OSuco (5.19 g, 2.35 mmol) and HOBt (71 mg, 0.47 mmol) were dissolved in dimethyl sulfoxide (7.0 mL) and *N*-methylmorpholine (600 μL) and stirred with Nps-Lys(Boc)-ONSu (1.34 g, 2.70 mmol) for 24 h. After workup II the product still contained excess Nps-Lys-(Boc)-ONSu. This material was removed by Nps cleavage followed

Table II. R_f values of N^{ϵ}-Boc Oligolysines

compd	no.	R_f values in solvent system: A	B	C	D
Nps-Lys(Boc)-OSuco	**2**	0.73	0.82		
H-Lys(Boc)-OSuco	**2a**	0.23	0.32		
Nps-(Lys(Boc))$_2$-OSuco	**2b**	0.60	0.68		
H-(Lys(Boc))$_2$-OSuco		0.14	0.19		
Nps-(Lys(Boc))$_3$-OSuco		0.46	0.59		
H-(Lys(Boc))$_3$-OSuco		0.10	0.18		
Nps-(Lys(Boc))$_4$-OSuco		0.41	0.58		
H-(Lys(Boc))$_4$-OSuco		0.09	0.15		
Nps-(Lys(Boc))$_5$-OSuco		0.39	0.50		
H-(Lys(Boc))$_5$-OSuco			0.14	0.35	
Nps-(Lys(Boc))$_6$-OSuco			0.47	0.63	
H-(Lys(Boc))$_6$-OSuco			0.15	0.33	
Nps-(Lys(Boc))$_7$-OSuco	**3**		0.41	0.62	
H-(Lys(Boc))$_7$-OSuco			0.17	0.26	
Nps-(Lys(Boc))$_8$-OSuco	**3a**		0.47	0.60	
H-(Lys(Boc))$_8$-OSuco			0.18	0.30	
Nps-(Lys(Boc))$_9$-OSuco	**4**		0.48	0.61	
H-(Lys(Boc))$_9$-OSuco	**5**		0.19	0.32	
Nps-(Lys(Boc))$_{10}$-OSuco	**6**		0.48	0.64	
H-(Lys(Boc))$_{10}$-OSuco	**9**		0.18	0.30	0.60
Boc-(Lys(Boc))$_{10}$-OSuco	**7**		0.53	0.65	0.81
Boc-(Lys(Boc))$_{20}$-OSuco	**10**			0.54	0.70

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Table III. Homogeneity Criteria for the Products 12–14

no.	TLC[a] R_f(E)	TLC[a] R_f(F)	HVE(II):[b] $s_{cat.}$, cm	gel filtration[c] V_e, mL	gel filtration[c] M_r calcd	gel filtration[c] M_r found
12	0.48	0.49	4.6	53.3	9747.6	7943
13	0.43	0.39	2.2	25.3[d]	10410.6	131826
14	0.42	0.37	1.8	24.7	10735.3	138038

[a] The chromatograms showed one spot in UV and following treatment with Cl_2/tolidine,[46] $[Ag(NH_3)_2]NO_3$, and I_2/NaN_3. [b] The electrophoreses showed one band after treatment with Cl_2/tolidine or I_2/NaN_3. [c] The products (6 mg) were run in PBS (flow rate 12.6 mL/h) on a Sephadex G-150 column (1.3 × 47.5 cm). The optical densities of the fractions (1.3 mL) were read at 257 nm. Likewise the following proteins were run: cytochrome c (V_e = 49.4 mL, M_r 12384), BSA (V_e = 32.5 mL, M_e 68750), and bovine γ-globulin (V_e = 23.4 mL, M_r 160000). The enlisted M_r values were obtained by extrapolation from a graph (migration coefficient vs. log M_r) of these molecular weight markers. [d] Compound 13 was eluted in a broader peak (V = 13 mL) than compound 14 (9 mL).

Table IV. Characterization of the Compounds 12–14

no.	formula[a]	M_r[a]	elemental anal.[b] N calcd	N found	S calcd	S found	NH_2,[c] %	PVm/BPO,[d] mol^{-1} L cm^{-1}
12	$C_{440}H_{562}Cl_1N_{80}O_{101}S_{20}Na_{21}$	9747.6	11.50	11.31	6.58	7.04	4.8	16866
13	$C_{484}H_{625}Cl_1N_{81}O_{106}S_{20}Na_{20}$	10410.6	10.90	10.48	6.16	6.69	3.4	16720
14	$C_{511}H_{674}Cl_1N_{78}O_{109}S_{10}Na_{19}$	10735.3	10.18	10.55	5.67	5.40	4.1	15515

[a] These values were calculated for the free Na/Cl salts at pH 7.4 assuming that (i) the thiazolidine carboxyl group occurs as sodium salt, while (ii) the thiazolidine nitrogen is not protonated (Woodward et al.[47]), and (iii) the free amino groups are present as hydrochlorides (Schnabel[48]). [b] Performed by H. Frohofer, Institute for Organic Chemistry, University of Zürich. The combustion data for C were too low, presumably due to incomplete conversion of $NaHCO_3/Na_2CO_3$ into NaO and CO_2. The values for H were correct. [c] Free amino groups in percent of theoretically substitutable amino groups remaining in the products as determined by the TNBS assay.[24] The incompleteness of the penicilloylation of oligolysines is described in ref 29 and 48. [d] PVm/BPO is the molar penamaldate value[22] divided by the theoretical number of BPO groups. It is of the order of 16000–18000 for disubstituted and multisubstituted BPO derivatives.[23,29] The corresponding penamaldate stability values PS_{10}[23] were ≥95%, thus indicating the absence of benzylpenicillinate.

by workup II (see Table I). The overall yield of the coupling and deprotection reaction was 5.09 g (89%). R_f values are given in Table II.

The nonapeptide **4** was obtained as described for the octapeptide **3a**. The reaction parameters used to prepare the precursors of the decalysines **6** and **7** are collected in Table I; Table II shows the R_f values of these compounds.

Nps-(Lys(Boc))$_{10}$-OSuco (6). Compound **5** (1.78 g, 0.67 mmol) was dissolved in dimethyl sulfoxide (2 mL) and *N*-methylmorpholine (200 μL) and stirred with Nps-Lys(Boc)-ONSu (381 mg, 0.77 mmol) and HOBt (20 mg, 0.133 mmol) overnight. The reaction mixture was subjected to workup II, followed by chromatography on nine preparative silica gel plates, using solvent system C. The main zone isolated gave **6** as a yellow solidified foam: yield 1.77 g (87%); TLC R_f(B) 0.48, R_f(C) 0.64 (UV, ninhydrin (a.h.) and H_2SO_4 (a.h.)) one spot.

Boc-(Lys(Boc))$_{10}$-OSuco (7). Compound **5** (3.55 g, 1.33 mmol) was reacted with Boc-Lys(Boc)-ONSu as described in the previous paragraph. After workup II the material was chromatographed on silica gel plates, using solvent system C. Compound **7** was obtained as a solidified, colorless resin: yield 2.95 g (75%); TLC R_f(B) 0.53, R_f(C) 0.65, R_f(D) 0.81 (UV, ninhydrin (a.h.) and H_2SO_4 (a.h.)) one spot.

Boc-(Lys(Boc))$_{10}$-OH (8). Compound **7** (0.50 g, 0.17 mmol) was dissolved in CH_3CO_2H–CH_3OH (4:1, v/v) (10 mL) and hydrogenated at room temperature and normal pressure in the presence of active Pd-black (150 mg) for 14 h. The catalyst was filtered off and the solvent evaporized in vacuo. After chromatography on three silica gel plates using solvent system C, **8** was obtained as a colorless solidified resin: yield 338 mg (84%); TLC R_f(C) 0.09, R_f(D) 0.20, one spot with 2,6-dichlorphenolindophenol, ninhydrin (a.h.); UV and H_2SO_4 (a.h.) negative.

Boc-(Lys(Boc))$_{20}$-OSuco (10). Compounds **8** (320 mg, 0.137 mmol) and **9** (397 mg, 0.137 mmol) were dissolved in dimethyl sulfoxide (550 μL) and triethylamine (150 μL), and the solution was stirred with HOBt (23 mg, 0.17 mmol) and DCC (31 mg, 0.15 mmol). After 12 h again DCC (4.2 mg, 0.02 mmol) was added and after 24 h the reaction mixture was dried in vacuo and chromatographed on six silica gel plates, using solvent system C. The main zone isolated gave **10** as a colorless solidified resin: yield 506 mg (69%); TLC R_f(C) 0.54, R_f(D) 0.70 (UV, ninhydrin (a.h.) and H_2SO_4) one spot.

Adip[(Lys(Boc))$_{10}$-OSuco]$_2$ (11). Compound **9** (600 mg, 0.207 mmol) and adipinic acid (15.2 mg, 0.104 mmol) were dissolved in dimethyl sulfoxide (700 μL) and triethylamine (180 μL), and the solution was stirred with HOBt (31 mg, 0.104 mmol) and DCC (49 mg, 0.238 mmol) for 36 h. The reaction mixture was worked up as described in the previous paragraph. Compound **11**: yield 360 mg (64%); TLC R_f(C) 0.58 (UV, ninhydrin (a.h.) and H_2SO_4 (a.h.)) one spot.

CH_3CO_2H·H-(Lys(H·CH_3CO_2H))$_{20}$-OH (10a). Compound **10** (175 mg, 33.2 μmol) was dissolved in anisole (300 μL) and liquid HF (8–10 mL), and the solution was stirred for 45 min at 0 °C. The solvents were evaporated in vacuo, and the residue was washed with ether (4 × 20 mL) and dried in vacuo. The colorless amorphous powder was dissolved in 0.5 M CH_3CO_2H (2 mL) and applied on a Sephadex G-25m column (1.8 × 34 cm) and eluted with 0.5 M CH_3CO_2H (flow rate 13 mL/h) to give **10a** (V_e = 39.6 mL); yield following lyophilization, 111 mg (97%); HVE(I), s_{an} = 6.3 cm.

BPO-(Lys(BPO))$_{20}$-ONa (12). Compound **10a** (96 mg, 25 μmol) and potassium benzylpenicillinate (195 mg, 0.525 mmol) were dissolved in H_2O (1.2 mL), and the pH was adjusted to 11 by adding 3 M K_2CO_3. After 12 and 24 h again potassium benzylpenicillinate (195 mg, 0.525 mmol) was added and the pH adjusted to 11. After 36 h the reaction mixture was applied to a Sephadex G-25m column (1.8 × 34 cm) and eluted with PBS (flow rate 13 mL/h). The fractions of the first peak (V_e = 38 mL) were collected and demineralized by filtrating over Sephadex G-10 with H_2O. Upon lyophilization **12** was obtained as a colorless amorphous powder: yield 229 mg (92%). For homogenicity criteria and characterization, see Tables III and IV.

CH_3CO_2H·H-(Lys(H·CH_3CO_2H))$_{20}$-OSuco (10b). Compound **10** (92 mg, 17.4 μmol) was dissolved in CF_3CO_2H–H_2O (4:1, v/v) (6 mL) and stirred at room temperature for 90 min. After evaporization and drying in vacuo, the colorless viscous oil was dissolved in CH_3CO_2H–H_2O (4:1, v/v) (2 mL) and applied to a Sephadex LH-20 column (1.8 × 34 cm). The column was eluted with CH_3CO_2H–H_2O (4:1, v/v) (flow rate 13 mL/h). The main peak (V_e = 40.5 mL) gave upon lyophilization 74 mg (89%) of **10b**: HVE(I), s_{an} = 4.6 cm.

BPO-(Lys(BPO))$_{20}$-OSuco (13). Compound **10b** (118 mg, 24.4 μmol) and potassium benzylpenicillinate (191 mg, 0.512 mmol) were dissolved in H_2O (1.4 mL), and the pH was adjusted to 11 by adding 3 M K_2CO_3. After 12 and 24 h again potassium benzylpenicillinate (191 mg, 0.512 mmol) was added and the pH adjusted to 11. After 36 h the reaction mixture was applied to a Sephadex G-25m column (1.8 × 34 cm) and eluted with PBS (flow rate 13 mL/h). The fist peak (V_e = 40 mL) was collected, demineralized by passing over Sephadex G-10, and lyophilized.

Compound 13 was obtained as a colorless amorphous powder: yield 230 mg (89%). For homogenicity criteria and characterization, see Tables III and IV.

Adip[(Lys(H·CH_3CO_2H))$_{10}$-OSuco]$_2$ (11a). Compound 11 (360 mg, 66.78 μmol) was deprotected and worked up as described for **10b**: yield 300 mg (98%).

Adip[(Lys(BPO))$_{10}$-OSuco]$_2$ (14). Compound **11a** (115 mg, 25.1 μmol) and potassium benzylpenicillinate (186 mg, 0.501 mmol) were dissolved in H_2O (1.5 mL), and the pH was brought to 11 by adding 3 M K_2CO_3. After 12 and 24 h again potassium benzylpenicillinate (186 mg, 0.501 mmol) was added and the pH adjusted to 11. After 36 h the reaction mixture was worked up as described for **13**. After demineralization and lyophilization, 14 was obtained as a colorless, amorphous powder: yield 306 mg (90%). For homogenicity criteria and characterization, see Tables III and IV.

Immunological Methods. Immunizations. Six to eight weeks old BALB/c mice of either sex were obtained from Blomholtgård, Ry, Denmark, and assembled in groups of four, mice were immunized by intraperitoneal injections of 6 or 10 μg BPO_9-Asc absorbed on 2 mg $Al(OH)_3$. BPO_9-Asc was prepared as described elsewhere.[49] Each mouse was bled from the retical orbital sinus as indicated in Figures 1 and 2, and pooled sera were assayed for anti-BPO and anti-Asc IgE titers.

Determination of IgE Titers. IgE titers were evaluated using rat passive cutaneous anaphylaxis (PCA) as described by Watanabe and Ovary.[50] In brief, Wistar rats of either sex, weighing 200–280 g, were obtained from the Institute's own animal facilities and were intradermally injected (100 μL) with the diluted mouse sera (1/5, 1/10, ..., 1/1280). After 24 h, 2 μmol of $(BPO)_{20}$-polylysine or 4 mg of Asc, dissolved in 1 mL 0.5% Evans blue, was injected intravenously. After 20 min the BPO or Asc specific anaphylactic reaction was read. All reactions were set up in duplicate, and the titer was expressed as the reciprocal of the antiserum dilution yielding a reaction of 5-mm diameter (endpoint). The maximal variation is one doubling dilution step.

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Registry No. 1, 91177-68-3; 2, 47551-11-1; **2a**, 91110-85-9; **2b**, 91110-86-0; **3a**, 91110-88-2; 4, 91110-83-7; **5**, 91110-84-8; **6**, 91129-62-3; **7**, 91129-63-4; **8**, 90522-20-6; **9**, 91129-64-5; **10**, 91239-82-6; **10a**, 91110-89-3; **10b**, 91110-91-7; 11, 91239-83-7; **11a**, 91110-93-9; **12**, 91239-84-8; **12**·Na, 87713-97-1; **13**, 91239-86-0; 14, 91239-85-9; Nps-(Lys(Boc))-OSuco, 91110-87-1; Nps-Lys(Boc)-OH, 47551-11-1; H-(Lys(Boc))$_7$-OSuco, 91110-94-0; Nps-Lys(Boc)-ONSu, 60654-30-0; Boc-Lys(Boc)-ONSu, 30189-36-7; mono-3β-cholestanyl succinate, 91177-69-4; 1,4-bis(hydroxymethyl)benzene, 589-29-7; adipinic acid, 124-04-9; potassium benzylpenicillinate, 113-98-4.

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Nontricyclic Antidepressant Agents Derived from *cis*- and *trans*-1-Amino-4-aryltetralins

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The need for drugs that lack the obtrusive and limiting side effects of the tricyclic antidepressants has prompted the search for agents with greatly enhanced selectivity for specific mechanisms believed to be essential for antidepressant efficacy. The potential role of derangements of 5-HT pathways in the etiology of depression has long been suspected and has given impetus to the development of newer compounds that accentuate inhibition of serotonin reuptake. This paper presents structure–activity relationships for a series of *cis*-1-amino-4-(substituted-aryl)tetralins, which are surprisingly potent and selective inhibitors of serotonin uptake in in vitro models. These compounds are pharmacologically distinct from corresponding members of the trans series, which also potently block uptake of dopamine and norepinephrine. The activity in both cis and trans series is stereospecific, being restricted to the cis-(1*S*,4*S*) and the trans-(1*R*,4*S*) enantiomers.

Research directed toward establishing an etiology of endogenous depression on a molecular basis has continued over two decades. The early "catecholamine hypothesis" of Schildkraut,[1] suggesting a deficiency of monoamine neurotransmitters at their postsynaptic receptors, led to the discovery of maprotiline, desmethylimipramine, and nortriptyline, compounds that are relatively specific inhibitors of norepinephrine (NE) uptake.[2,3] The slow onset of clinical improvement with these antidepressants has led to recent proposals that desensitization of norepinephrine transmission (down regulation of β-adrenoreceptors) is involved in the therapeutic activity of antidepressants.[4] Other investigators have suggested a role for serotonin in depression.[5,6] Supporting the latter view are observations that certain subgroups of depressed patients responded better to an antidepressant with more serotonin uptake blocking activity[7,8] and other reports suggesting that the serotonin synthesis inhibitor *p*-chlorophenylalanine caused rapid, reversible relapse of depressed patients stabilized with imipramine[9] or tranylcypromine.[10] The search for more selective serotonin uptake blockers with reduced anticholinergic and cardiovascular liabilities of the tricyclic antidepressants has resulted in newer agents like zimelidine, fluvoxamine, and fluoxetine, which are reported to exert antidepressant activity in man.[11]

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