New Substituted Piperazines as Ligands for Melanocortin Receptors. Correlation to the X-ray Structure of "THIQ"

Felikss Mutulis,[†] Sviatlana Yahorava,[†] Ilze Mutule,[†] Aleh Yahorau,[†] Edvards Liepinsh,[‡] Sergei Kopantshuk,[†,§] Santa Veiksina,[†] Kaspars Tars,[#] Sergey Belyakov,[||] Anatoly Mishnev,[||] Ago Rinken,[§] and Jarl E. S. Wikberg*[,†]

Department of Pharmaceutical Biosciences, Division of Pharmacology, Uppsala University, Box 591, Biomedicum, SE-751 24 Uppsala, Sweden, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden, Institute of Organic and Bioorganic Chemistry, Tartu University, Jakobi 2, 51014 Tartu, Estonia, Department of Cell and Molecular Biology, Uppsala University, Box 596, SE-751 24 Uppsala, Sweden, and Latvian Institute of Organic Synthesis, LV-1006, Riga, Latvia

Received December 8, 2003

A series of piperazine analogues of the melanocortin 4 receptor (MC_4R) specific small-molecule agonist "THIQ" was synthesized and characterized structurally and pharmacologically. First, several THIQ imitations lacking the triazole moiety were prepared. Syntheses included acylation of 4-phenylpiperazine or 4-cyclohexylpiperazine. In two cases the tertiary amine function was replaced by the corresponding *N*-oxide. To obtain more complex structures, a 4-substituted piperazine ring was formed by alkylation of the primary amino group of cyclohexane-derived amino alcohols with *N*,*N*-bis(2-chloroethyl)benzylamine. The hydroxylic group of the intermediate was first activated with methanesulfonyl chloride, and the sulfonic ester formed in situ was introduced into the reaction with the sodium salt of 1,2,4-triazole. In one case (i.e., preparation of **23c**) introduction of the 1,2,4-triazole moiety was performed at a carbon of the cyclohexane ring. In addition, this intermediate contained a piperazine moiety connected via its nitrogen atom to a cyclohexane ring carbon neighboring the reaction center. As established in NMR and X-ray investigations herein, this substitution proceeded with retention of the initial trans configuration of 1,2-disubstituted cyclohexane. To obtain pure enantiomers of **23c**, its precursor **21c** was subjected to chiral chromatography on a Chirobiotic V column. The derivatives (*R*,*R*)-**21c**and (*S*,*S*)-**21c** obtained were introduced into further syntheses steps, giving (*R*,*R*)-**23c** and (*S*,*S*)-**23c**, respectively. Melanocortin $MC_{1,3-5}$ receptor binding studies showed that all tested piperazine derivatives were active. Several compounds showed clear selectivity for MC_4R, with submicromolar affinities being obtained. Among them, one substance, (*R*,*R*)-**23c**, displayed a biphasic curve in displacement of [^{125}I]NDP-MSH on MC_4R [$K(i)_{high} = 1$ nM and $K(i)_{low} = 260$ nM]. This biphasic competition curve was similarly biphasic to the competition curve obtained herein using THIQ. An X-ray study performed on crystals of the THIQ sulfate salt revealed two closely related conformations, which resemble the shape of the letter "Y", where piperidine and 4-chlorophenyl groups are situated close to each other, but the 1,2,3,4-tetrahydroisoquinoline residue is remote, the triazole function being highly exposed to the environment. The crystals of the dinitrate salt of (*R*,*R*)-**23c** showed a different conformation, where parts of the molecule are spread out almost symmetrically around the central section. Molecular modeling, based on the THIQ crystal structure and the functional similarity of THIQ and (*R*,*R*)-**23c**, allowed us to suggest a possible "bioactive" conformation of (*R*,*R*)-**23c** that is similar to the crystal conformation of THIQ.

Introduction

Melanocortin receptors belong to the family of heptahelical transmembrane G-protein-coupled receptors.[1] Up to now five subtypes of melanocortin receptors ($MC_{1-5}R$) have been cloned.[2] These receptors are involved in multiple physiological processes. For example, the MC_1R is expressed in the skin where it plays a major role in skin and hair pigmentation, and it has a role in controlling the immune system.[3] The MC_2R is found in the adrenal gland where it is responsible for the ACTH mediated control of steroid production.[4] The MC_3R is present in the hypothalamus and is there involved in energy homeostasis regulation.[5] The MC_4R is expressed in the brain and other tissues, and it is known to control feeding. In addition, this receptor (as well as presumably the MC_3R receptor) regulates sexual behavior.[3,6] MC_5R is found in the skin among other places and appears to regulate functions of exocrine glands.[7]

Agonists and antagonists of melanocortin receptors have a potential for treatment of several diseases and health disorders such as overweight, sexual dysfunctions, and conditions related to inflammation.[8] MC_4R agonists reduce appetite and decrease body weight, while antagonists of the receptor has the opposite effect, where long-term treatment causes a dramatic increase in body weight.[3,9] The natural agonists for the melano-

* To whom correspondence should be addressed. Phone: +46-18-4714238. Fax: +46-18-559718. E-mail: Jarl.Wikberg@farmbio.uu.se.
[†] Division of Pharmacology, Uppsala University.
[‡] Karolinska Institutet.
[§] Tartu University.
[#] Department of Cell and Molecular Biology, Uppsala University.
[||] Latvian Institute of Organic Synthesis.

10.1021/jm0311285 CCC: $27.50
Published on Web 08/03/2004

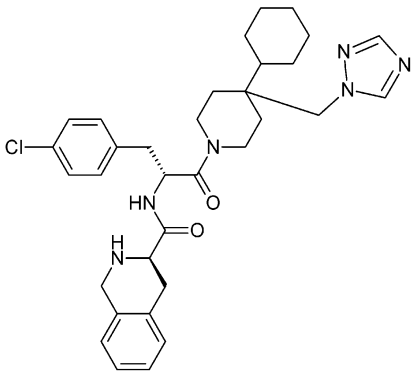


Figure 1. THIQ.

cortin receptors are the melanocortins. These peptides include adrenocorticotropic (ACTH) and α-, *β*-, and *γ*-melanocyte stimulating hormones (α-, *β*-, and *γ*-MSH), all which are formed by post-translational cleavages of the precursor protein proopiomelanocortin (POMC).[10] In addition, the activity of the melanocortin receptors is regulated by endogenous antagonists, namely, the agouti protein and agouti-related peptide (AgRP).[11]

Investigations of melanocortins include preparation of many peptide analogues.[3] Among them, substances displaying valuable properties such as antagonism, high activity, and selectivity were discovered.[12−16] However, in most cases peptides are not suited for therapeutic application because they usually do not pass the blood−brain barrier and are rapidly metabolized in blood and tissues.[17] Accordingly there is a demand for non-peptide ligands with low molecular weight showing high affinity and selectivity for the melanocortin receptors. Many drug development companies exert considerable efforts to create MC_4R-directed drugs for treatment of obesity or anorexia (for reviews see refs 8, 18, and 19). A large number of low molecular weight compounds were reported to show a binding activity on melanocortin receptors.[8,18−21] Among them, "THIQ" (Figure 1) exhibits outstanding specificity for the MC_4 receptor and a high agonist activity.[22] Publishing of its structural formula and activity data in the year 2000 was followed by an extensive exploration of related structures by Merck and several other drug development companies, which is reflected in patents (see refs 8, 18, and 19). However, up to now only few academic studies were devoted to this theme.[23−26]

In the present study we have synthesized some new piperazine derivatives related to THIQ and investigated their molecular structures and receptor binding properties. For comparison, we resynthesized also THIQ[22] for use in parallel investigations.

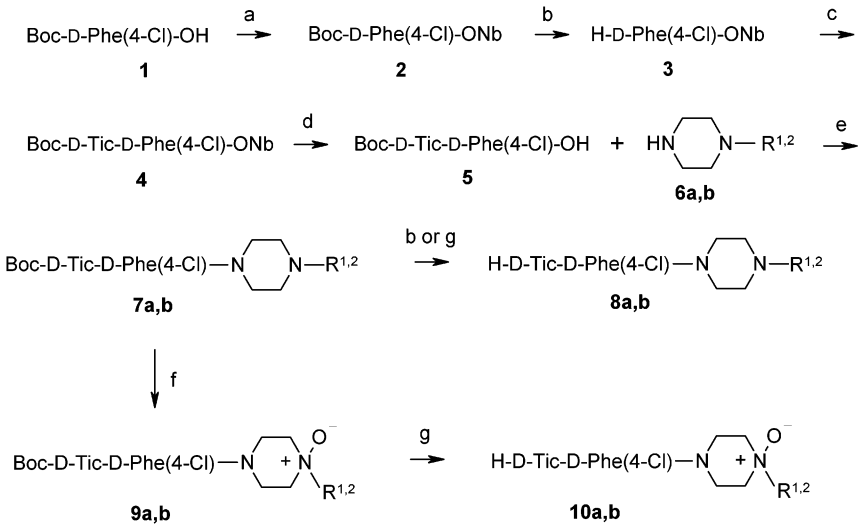
Chemistry

To prepare some piperazine derivatives related to THIQ that lacked the triazole function, we started with Boc-4-chloro-D-phenylalanine **1** (Scheme 1). Reaction with 4-nitrobenzyl bromide[27] gave the corresponding 4-nitrobenzyl ester **2**. Elimination of the Boc group then followed. The amino acid derivative **3** formed was coupled[28] with Boc-D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid to obtain the protected dipeptide **4**. Further, alkaline hydrolysis[27] of the 4-nitrobenzyl ester function led to dipeptide acid **5**, which was reacted with 1-phenylpiperazine **6a** or 1-cyclohexylpiperazine **6b**, giving amides **7a** and **7b**. Elimination of the Boc group converted them to compounds **8a** and **8b**, bearing a free amino group.

To prepare *N*-oxides, Boc-protected amide **7a** or **7b** were oxidized by hydrogen peroxide.[29] Protected *N*-oxide **9a** or **9b** formed. Deprotection with trifluoroacetic acid provided the desired *N*-oxides **10a** and **10b**.

For preparation of more complex end products, diethanolamine **11** was treated with benzyl bromide (Scheme 2), as described in the literature.[30] The N-

Scheme 1[a]

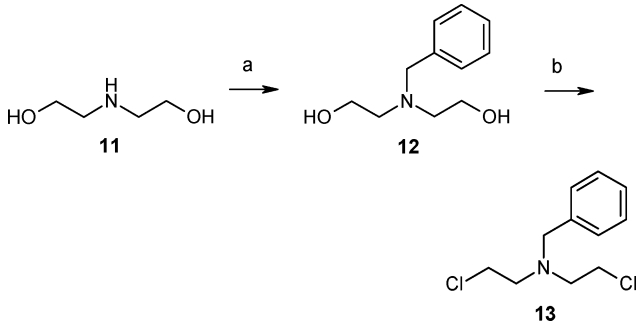


R^1 = phenyl (**6-10a**),

R^2 = cyclohexyl (**6-10b**).

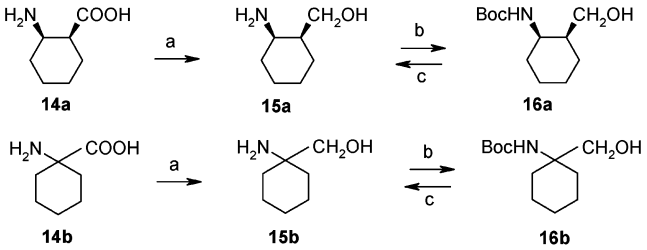
[a] Reagents and conditions: (a) 4-nitrobenzylbromide, TEA, EtOAc, reflux, 7 h; (b) 4 M HCl/dioxane, AcOH, room temp, 1 h; (c) Boc-D-Tic-OH, HATU, DIEA, DMF, room temp, 2 h; (d) 2 N aqueous NaOH, MeOH, room temp, 1.5 h; (e) HATU, DIEA, DMF, room temp, 2 h; (f) 30% aqueous H_2O_2, room temp, 1 h, then 60 °C, 2 h; (g) TFA/CH_2Cl_2 (2:1), room temp, 1 h.

Scheme 2[a]



[a] Reagents and conditions: (a) benzyl bromide, K_2CO_3, toluene, 60−70 °C, 1 h; (b) $SOCl_2$, $CHCl_3$, reflux, 4 h.

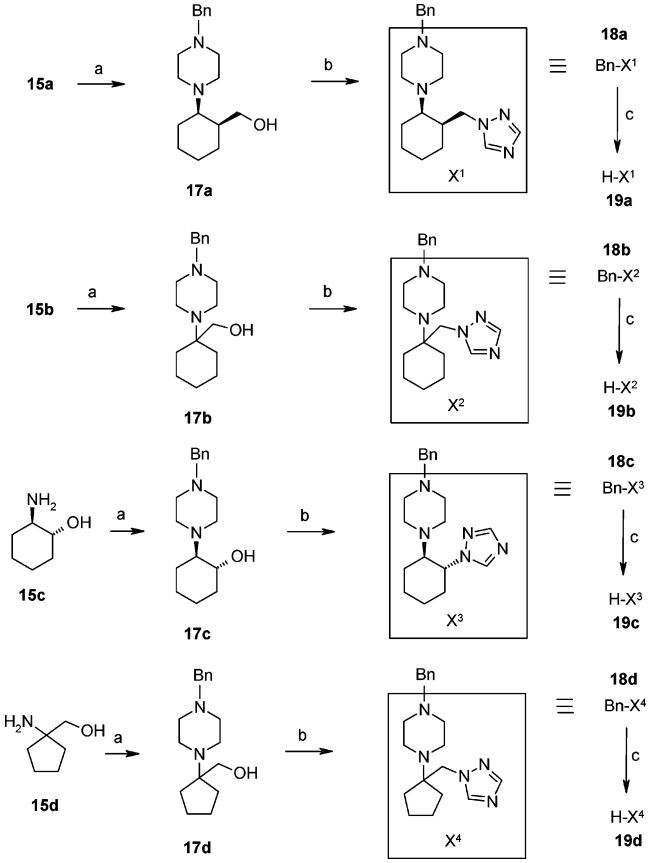
Scheme 3. Relative Stereochemistry[a]



[a] Reagents and conditions: (a) $LiAlH_4$, THF under Ar atmosphere, 70−80 °C, 28 h; (b) Boc_2O, TEA/MeOH (1:9), room temp, 4 h; (c) 4 M HCl/dioxane, room temp, 1 h.

protected diol **12** formed. Reaction with $SOCl_2$ converted it to the dichloride **13**.[30] To obtain the two reactants **15a** and **15b** for reaction with **13**, *cis*-2-aminocyclohexanecarboxylic acid (**14a)** or 1-aminocyclohexanecarboxylic acid (**14b)** was reduced with $LiAlH_4$ (Scheme 3).[31,32] However, it was difficult to isolate alcohols **15a**,**b** from the mixture formed. To enable chromatography on silica gel, the reaction products were converted to Boc derivatives **16a**,**b**.[33] After purification by flash chromatography the Boc group was removed to afford pure alcohol **15a** or **15b**. Two other alcohols **15c** and **15d** were commercial available. In the next step (Scheme 4), the cyclic alcohols **15a**−**d** were introduced into reaction with dichloride **13** (preparation detailed above). This led to the creation of piperazine derivatives **17a**−**d**.[34,35] The next stage was the introduction of the triazole moiety. The hydroxyl group was first activated with methanesulfonyl chloride, and the sulfonic acid derivative formed in situ was reacted with the sodium salt of 1,2,4-triazole.[22,36] Triazole derivatives **18a**−**d** were obtained. In one case (preparation of **18c**), the introduction of the triazole moiety proceeded at an asymmetrical carbon atom included in the cyclohexane ring. We found only one example in the literature where a similar reaction had been investigated earlier. There it was found that upon reaction of 2,3-disubstituted oxirane (substituents: halogen and tertiary butyl group) with sodium triazolide only the trans triazole introduction product was obtained.[37] In the present study the orientation of substituents was determined by a more insightful NMR analysis of the end product of the whole synthesis of **23c** (trans configuration was established; a full account of this study is given below). Further, it could be inferred that already in the 1,2-disubstituted cyclohexane **18c** and its further synthesis products the piperazine and triazole moieties were in trans relation to each other.

Scheme 4. Relative Stereochemistry[a]



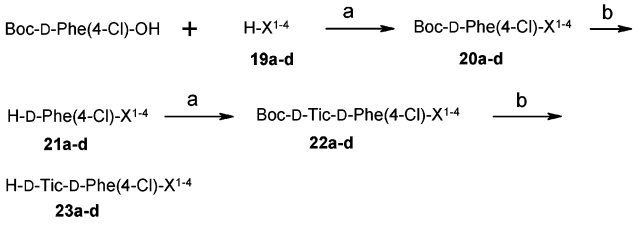
[a] Reagents and conditions: (a) **13**, $NaHCO_3$, EtOH, reflux, 3−12 h; (b) (1) $MeSO_2Cl$, CH_2Cl_2, TEA, 0 °C to room temp, 2 h; (2) sodium salt of 1,2,4 triazole, DMF, under Ar atmosphere, 80−100 °C, 12 h; (c) $HCOONH_4$, 10% Pd/C, 1 N HCl, MeOH, reflux, 1.5 h.

Thus, the reaction where the triazole moiety was introduced using the secondary alcohol **17c** proceeded with retention of the initial relative configuration (trans for the starting material 2-aminocyclohexanol, **15c)**. We assume that the reaction mechanism includes two successive S_N2 steps. First, the adjacent piperazine nitrogen forms a transient aziridinium ion by back-side attack on the mesylate. The *meso*-aziridinium ion is then attacked by the triazolide from the far side, leading to overall retention of stereochemistry.[38]

Removal of the *N*-benzyl group by transfer hydrogenation[39] afforded diamines **19a**−**d**. They were subjected to stepwise peptide synthesis in solution (Scheme 5). First, the Boc derivative of 4-chloro-D-phenylalanine was attached, giving intermediates **20a**−**d**. After deprotection (products **21a**−**d**), coupling with Boc-D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid followed. Protected derivatives **22a**−**d** were obtained. Removal of the Boc group furnished raw end products **23a**−**d**, which were purified by HPLC.

Several other compounds obtained in the present study included an asymmetrical center in their cyclohexane ring, namely, **23a** and its precursors (**15a**−**22a**). However, there is no doubt that the relative cis configuration, which was present in the starting amino acid (**14a**), was retained in the end product **23a** because its multistep preparation process did not touch the asymmetrical carbon atoms (Schemes 3−5).

Scheme 5[a]



X^1 = 4-[*cis*-2-(1,2,4-triazol-1-yl-methyl)cyclohexyl]-piperazine (**19-23a**),

X^2 = 4-[1-(1,2,4-triazol-1-yl-methyl)cyclohexyl]-piperazine (**19-23b**),

X^3 = 4-[*trans*-2-(1,2,4-triazol-1-yl)cyclohexyl]-piperazine (**19-23c**),

X^4 = 4-[1-(1,2,4-triazol-1-yl-methyl)cyclopentyl]-piperazine (**19-23d**).

[a] Reagents and conditions: (a) Boc amino acid, HATU, DIEA, DMF, room temp, 2 h; (b) TFA/CH_2Cl_2 (2:1), room temp, 1 h.

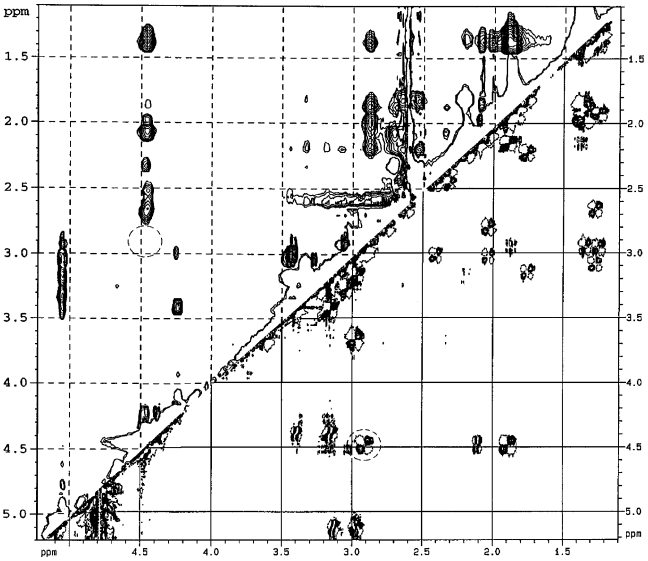


Figure 2. Combined picture of a ROESY (above the diagonal) and double-quantum filtered COSY (below the diagonal) NMR spectra for **23c** in DMSO-d_6. Presence (in COSY spectrum) or absence (in ROESY spectrum) of a cross-peak reflecting interaction between protons at the cyclohexane asymmetrical carbon atoms is shown by circles drawn with a broken line.

NMR Investigations

The aim of the investigations was to establish the relative configuration of the carbon atom in cyclohexane position 2, which could be affected by the nucleophilic reaction introducing the triazole moiety. **23c** was investigated by NMR at 600 MHz in DMSO, 28 °C: proton spectra, 60 ms mixing time TOCSY,[40] 250 ms mixing time ROESY,[41] HSQC and HMBC ($^{13}C-^{1}H$ correlation).[42,43] Protons, which are attached to carbon atoms of the cyclohexane ring (positions 1 and 2) were detected at resonances 2.77 and 4.36 ppm, respectively. They showed a cross-peak in a double-quantum-filtered COSY spectrum (500 MHz, in deuterium oxide, pH 5.5) but did not exhibit any cross-peak in the ROESY spectrum (Figure 2). In other words, the COSY spectrum showed the presence of a spin−spin coupling between protons at the cyclohexane asymmetrical centers, but from the ROESY data there was no sign of a nuclear Overhauser effect between them. This reflects the diaxial location of these protons. Other spectra, which were recorded in deuterium oxide (500 MHz, pH 4.4, 25 °C), allowed us to estimate the coupling constant between the above protons. $^3J_{HH}$ was found to be about 11.3 Hz, something which again corresponds to the interaction between protons in axial−axial positions.[44] Consequently, both substituents at cyclohexane are equatorial. Such a relation between cyclohexane 1,2-substituents presumes that they are in a trans relative configuration. Analysis was problematic, however, because signal doubling was observed in most cases. This could be explained as the presence of equal amounts of two trans isomers at cyclohexane asymmetric centers: *R*,*R* and *S*,*S*.

Other NMR observations for the **23c** molecule (DMSO-d_6 solution) indicated that the aliphatic ring of the tetrahydroisoquinoline ring system mainly exists in a half-chair conformation, where the carbon atoms of the CH_2 groups are situated in the same plane as the benzene ring, whereas the asymmetrical carbon atom and charged nitrogen atom are each located in opposite sides of this plane. (This could be reasoned from data concerning the spin−spin coupling constants and the nuclear Overhauser effect in the aliphatic cycle, including those for the disubstituted ammonium ion.) Besides, the ROESY spectra showed several types of exchange peaks for piperazine protons and the whole analysis was complicated because of broadness of the signals. This can be explained both by a conversion process between several chair conformations of this ring and by a slow rotation around the amide bond attached to the piperazine cycle occurring at the rates intermediate to the NMR time scale.

After separation of isomers (described below), an additional NMR study, which again involved a wide application of two-dimensional approaches, allowed us to make a full assignment of most ^1H and ^{13}C NMR spectral signals for both forms of **23c** [(*R*,*R*)-**23c**and (*S*,*S*)-**23c**] and their intermediates (*R*,*R*)-**21c** and (*S*,*S*)-**21c**. Data for this are provided in Supporting Information.

Separation of Isomers

According to our NMR data, substance **23c** had been initially prepared as an equimolar mixture of *R*,*R* and *S*,*S* isomers in relation to its trans 1,2-substituents at the cyclohexane ring. In our binding experiments on the MC_4R, this mixture gave a strongly biphasic competition binding curve, showing one apparent high-affinity binding component with about 1 nM affinity and one apparent low-affinity component with more than 200-fold lower affinity (similarly as did THIQ), and we decided that **23c** should deserve further studies using its individual isomers.

We investigated both **23c** and those intermediates of its synthesis that included disubstituted cyclohexane, trying to separate the isomers. Because **23c** and some of its precursors (**20c**, **21c**, **22c**) contained one or two fixed asymmetric centers (included in the residues of 4-chloro-D-phenylalanine and D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) in addition to the above cyclohexane centers, their separation by conventional chromatography was in principle possible. However, no double peaks were observed in our experiments on reversed-phase HPLC columns. Crystallization of salts to obtain individual diastereomers was unsuccessful too (we prepared salts of **19c** with L-tartaric acid, dibenzoyl-

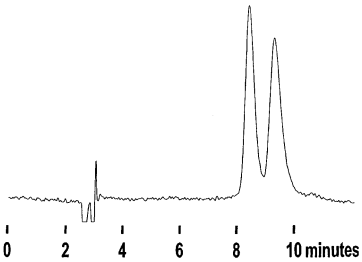


Figure 3. Analytical separation of **21c** isomers on Chirobiotic V 4.6 mm × 250 mm column: (eluent) dioxane−MeOH−AcOH−NEt_3−H_2O (50:50:0.04:0.01:0.03); detection at 220 nm; flow rate of 1 mL/min.

D-tartaric acid, and di-*p*-toluoyl-D-tartaric acid and tried their fractional crystallization, but no separation of isomers was observed).

The problem was solved using chiral HPLC (analytical and preparative) on a Chirobiotic V (Advanced Separation Technologies Inc.) stationary phase. A somewhat less satisfactory separation was obtained also on a Chirobiotic T column. These phases are obtained covalently bonding the natural glycopeptides vancomycin (18 chiral centers) or teicoplanin (20 chiral centers) to silica gel.[45] Data on our trials with Chirobiotic V and Chirobiotic T columns are included in Supporting Information. After some optimization in analytical scale experiments with intermediate **21c**, a division close to baseline separation was achieved (Figure 3). On a semipreparative Chirobiotic V column using a mobile phase containing dioxane−methanol−acetic acid−triethylamine (50:50:0.02:0.01), we thereafter succeeded with a multimilligram scale separation. Both products obtained, (*R*,*R*)-**21c** (faster moving on chiral HPLC) and (*S*,*S*)-**21c** (a slower moving isomer), were introduced into the two remaining synthesis steps (Scheme 6) to give individual diastereomers (end products (*R*,*R*)-**23c** and (*S*,*S*)-**23c**) as ditrifluoroacetates. The absolute stereochemistry of them was established later when we obtained the X-ray structure for (*R*,*R*)-**23c** dinitrate (described below).

Receptor Binding Studies

The synthesized compounds were investigated for their ability to displace the specific binding of [^{125}I]NDP-MSH to recombinant human melanocortin receptors $MC_{1,3-5}Rs$ in Sf9 cell membranes.[46,47] All substances displayed some binding affinity (Table 1). By comparison of data for **8a** and **8b**, which differ only by the presence of an aromatic (benzene) or aliphatic (cyclohexane) ring, it can be seen that the benzene-related **8a** displays an approximately equal affinity for all four MC receptors, the K_i values in the range 13−30 μM with about a 2-fold lower affinity for MC_4R than that for the three other receptors. The cyclohexane derivative **8b** on the other hand showed the same or higher affinity for all receptors compared with **8a**, on the MC_1R the affinities being roughly the same as **8a**, on the MC_3 and MC_5Rs being twice as high, and on the MC_4R being 20-fold as high as **8a**. Thus, **8b** displayed some selectivity for the MC_4R. When **8a** was converted into the *N*-oxide (substance **10a**), the affinities decreased about 2- to 5-fold. As in **8a**, some loss of affinity was seen for the MC_4R. **10b**, which is the *N*-oxide derived from **8b**, behaved like **10a**, showing a 12- to 41-fold lower affinity when compared with the parent compound, but still it retained about a 2-fold selectivity for the MC_4R. From these data, we can make the conclusion that the benzene derivatives of our THIQ-related piperazines, and particularly the *N*-oxides, are less compatible with efficient MC_4R binding. Therefore, in the subsequent studies we concentrated on alicyclic cyclohexane and cyclopentane derivatives introducing additionally a 1,2,4-triazole moiety (which is present also in THIQ, Figure 1).

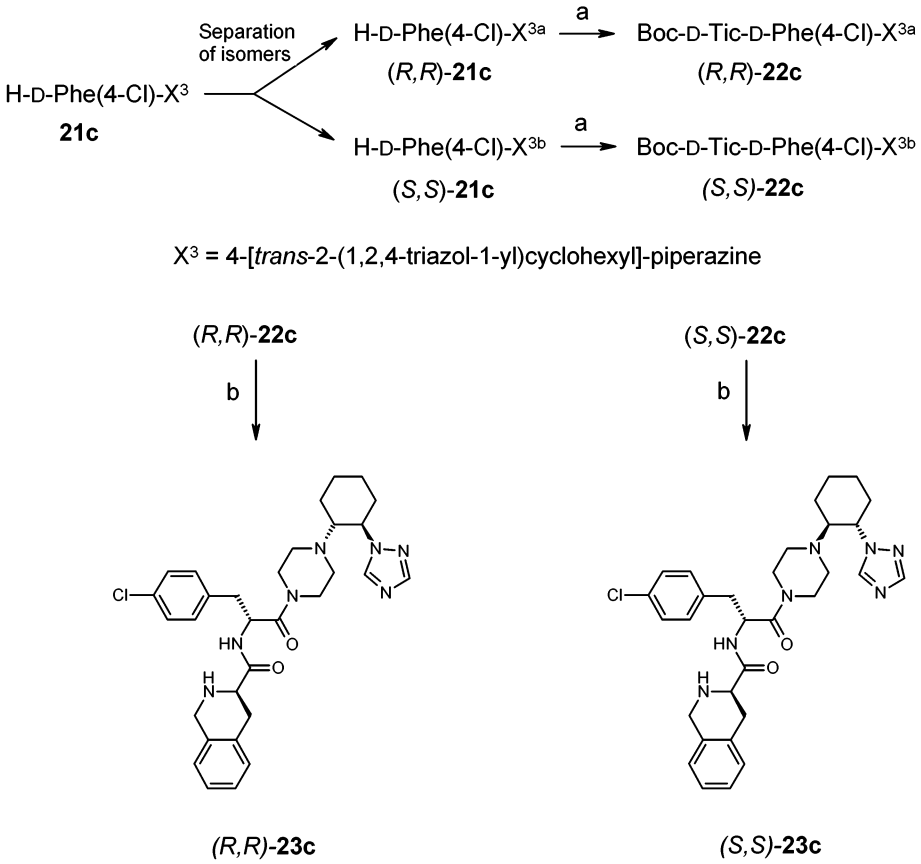
The first substance of the more complex type, **23a**, contains two substituents at neighboring carbon atoms in the cyclohexane ring (Schemes 4 and 5). **23a** showed a submicromolar affinity on the MC_4R and low micromolar affinities on MC_1R and MC_3R. Thus, the affinities for **23a** on these receptors were improved when compared with the more simple cyclohexane derivative **8b** described above. In contrast, the affinity of **23a** for the MC_5R turned out to be 2-fold lower compared with the simpler compound **8b**.

23b, similar to THIQ (Figure 1), contains a carbon atom with no hydrogens attached to it, which is connected to the 1,2,4-triazol-1-ylmethyl group. Two substituents are attached to the same carbon atom, which is also part of the cyclohexane ring, and there are no asymmetric centers in this part of the molecule (Schemes 4, 5). **23b** displayed an affinity pattern that was very similar to that of **23a** except that the affinity to MC_4R was diminished by a factor of 2.5.

23c, which has a rigid trans triazole substitution at the carbon atom neighboring the carbon where the piperazine is attached to the cyclohexane ring, showed obvious MC_4R selectivity. Moreover, the curve for displacement of [^{125}I]NDP-MSH binding was heterogeneous, which can be interpreted as the interaction with two apparent binding sites (e.g., representing stabilized receptor conformations). When the heterogeneous binding curves were analyzed using law-of-mass-action modeling under the assumption that two discrete MC_4R binding sites were present, the apparent low-affinity site appeared to show a submicromolar affinity, while the high affinity binding site appeared to show a low nanomolar affinity for **23c**. In parallel experiments using THIQ, we obtained a similar heterogeneous curve (Figure 4). Thus, the binding patterns of THIQ and **23c** were closely similar, indicating that their "biologically active" structures should be similar. However, **23c** was a mixture of two isomers. These isomers were obtained as individual substances, (*R*,*R*)-**23c** and (*S*,*S*)-**23c** (see "Separation of Isomers"). Binding experiments showed that these isomers exhibit different properties; (*R*,*R*)-**23c** was more active than (*S*,*S*)-**23c** on the MC_3, MC_4, and MC_5Rs, but the activity toward MC_1R was the same for both isomers. However, the most striking difference between the isomers was the absence of any apparent high- and low-affinity binding sites for (*S*,*S*)-**23c** on the MC_4R. On the MC_4R, (*S*,*S*)-**23c** exhibited a classical monophasic competition binding curve. The heterogeneity of binding to the MC_4R, which was observed for the mixture of isomers **23c**, showed up again in the binding experiments using (*R*,*R*)-**23c** (Figure 4).

The next synthesis product, **23d**, contains a cyclopentane moiety and a triazole group attached to the rest of the molecule through a methylene group. We found that the MC_4R selectivity, which had been observed for the

Scheme 6. Absolute Stereochemistry[a]



[a] Reagents and conditions: (a) Boc amino acid, HATU, DIEA, DMF, room temp, 2 h; (b) TFA/CH_2Cl_2 (2:1), room temp, 1 h.

cyclohexane derivatives, had disappeared for this compound. However, **23d** was more active on the MC_1R compared to all other compounds tested in the present study because it showed a submicromolar affinity for this receptor. For the MC_4R, its affinity was 6-fold lower compared to that for (*R*,*R*)-**23c** (low affinity site), and the affinities for the MC_3 and MC_5Rs were 2-fold lower.

X-ray Analysis

THIQ was synthesized according to the patent description.[22] Its trifluoroacetate salt was dissolved in methanol and passed through a small column filled with Dowex 1 × 4 in OH^- form. The solution of the base obtained was diluted with water (20% of volume), sulfuric acid (0.6 molar equiv) was added, and the mixture was evaporated to dryness in vacuo. The residue was dissolved in a small volume of hot water. When the mixture was slowly cooled to room temperature, crystals formed. The largest of these crystals was sampled together with the mother liquid, using a glass capillary. The mother liquor was then removed with a small piece of filter paper. The crystal in the capillary was subjected to X-ray diffraction. However, it turned out that the crystals were unstable when exposed to air. An initial trial experiment resulted in a model for the structure that could not be refined to a reliable *R* factor. To resolve this, different glues were tried for coating the crystals. It turned out that the best results were obtained using nitrocellulose glue, which resulted in practically no intensity decay during the course of 12 h of X-ray exposure. The final successful measurements were obtained from a crystal in the form of a thin plate with linear dimensions 0.05 mm × 0.17 mm × 0.45 mm. (Further experimental details, fractional atomic coordinates, and crystal data are presented in Supporting Information).

From the X-ray study it was found that the structure of the crystals corresponded to the formula $2C_{33}H_{41}ClN_6O_2 \cdot H_2SO_4 \cdot 6H_2O$; i.e., two protonated THIQ molecules form a complex with one sulfate ion and six water molecules. Both the crystal conformations of THIQ differ insignificantly from each other; some difference could be seen for the location of the triazole rings. These structures resemble the shape of the letter Y, where the 4-chlorophenyl group and cyclohexylpiperidine are close to each other and the D-Tic residue is remote. The triazole function turns away from the rest of the molecule and is highly exposed to the environment (Figure 5).

(*R*,*R*)-**23c** was synthesized as described above. Its ditrifluoroacetate salt was dissolved in methanol and passed through a small column filled with Dowex 1 × 4 in OH^- form. The solution of the base obtained was evaporated. To the oily residue formed, a solution of nitric acid (2 molar equiv) was added in a small volume of water. After shaking the mixture on a Vortex, a homogeneous solution formed, from which after a short standing crystals precipitated.

Crystals of (*R*,*R*)-**23c** showed sufficient stability in air, and therefore, no special measures to protect them were required. To perform X-ray structure analysis, essentially the same instrumentation and experimental

Table 1. Affinity of Synthesized Substances to Recombinant Human Melanocortin Receptors in Membrane Preparations, K_i (in μM Unless Otherwise Noted)

Compound	Structure[a]	MC_1R	MC_3R	MC_4R	MC_5R
8a		13±2	15±2	30±4	17±2
8b		11±1.5	6.8±0.9	1.4±0.3	8.2±1.3
10a		36±5	35±10	169±23	94±23
10b		152±38	114±20	57±7	102±16
23a		2.0±0.1	5.2±0.2	0.39±0.04	16±2
23b		3.2±0.6	5.6±0.7	1.0±0.15	14±2
(R,R)-**23c**		2.0±0.2	4.4±0.3	0.26±0.02	9.8±0.7
		High affinity MC_4R binding site: K_i = 1.0±0.9 nM			
(S,S)-**23c**		2.2±0.2	6.5±1.3	0.86±0.08	17±2
23d		0.53±0.14	8.8±0.3	1.5±0.18	18±6
THIQ		0.76±0.18	4.5±3.0	0.08±0.02	5.0±0.15
		High affinity MC_4R binding site: K_i = 0.059±0.027 nM			

[a] R = H-D-Tic-D-Phe(4-Cl)-; **23a** structure is shown in terms of relative stereochemistry.

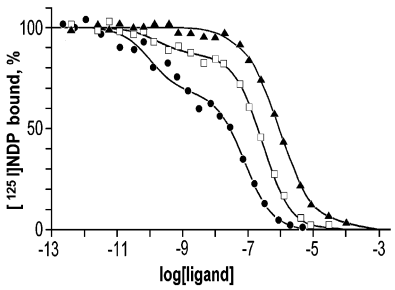


Figure 4. Displacement of receptor-bound [^{125}I]NDP-MSH by THIQ (experimental points shown as circles), (*R,R*)-**23c** (squares), and (*S,S*)-**23c** (triangles). Experiments were made on MC_4R containing membranes.

methods as described for THIQ were used. The crystal structure was solved by use of direct methods and refined by full-matrix least squares on F^2. All non-hydrogen atoms were refined anisotropically. All hydrogen atoms were located by difference Fourier synthesis. The X-ray study showed that the structure of the crystals corresponded to the formula $C_{31}H_{38}ClN_7O_2 \cdot 2HNO_3 \cdot 3H_2O$. The single conformation of (*R,R*)-**23c**, which was found in the crystals, is shown in Figure 6. These data allowed us to establish the absolute stereochemistry of the molecule, which was *R,R* in relation to the substituents at the cyclohexane ring. Accordingly, the corresponding configuration of the other isomer (*S,S*)-**23c** is *S,S* (Scheme 6). More experimental facts are presented in Supporting Information.

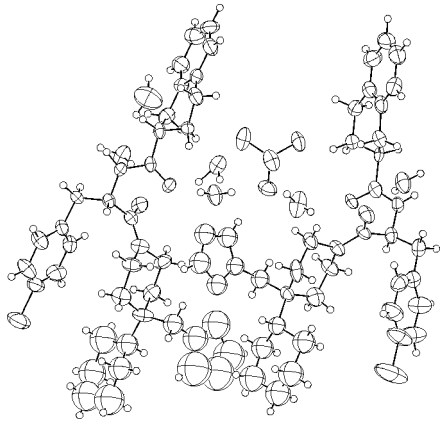


Figure 5. ORTEP image of two molecules of THIQ in elementary crystallographic unit.

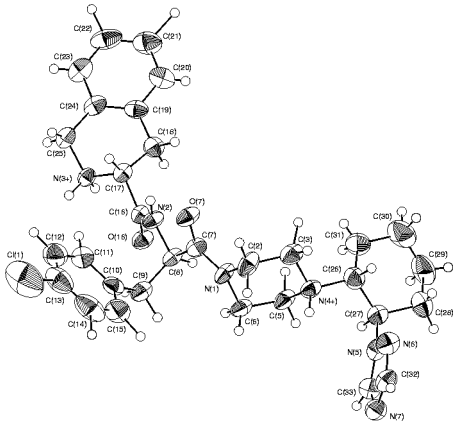


Figure 6. ORTEP image of (*R,R*)-**23c** conformation in crystals.

The crystallographic data for the structure analysis of THIQ and (*R,R*)-**23c** have been deposited at the Cambridge Crystallographic Data Centre.

Molecular Modeling

A THIQ tertiary structure, which is very similar (if not identical) to those that we found in crystals, was presented in a previous publication[25] without any information about its origin. According to the authors of this study, this conformation correlated with a calculated low-energy structure of the highly active cyclic melanocortins related peptide MT-II. It was proposed that it represents a "biologically active" conformation interacting with the MC_4R. We have utilized the same assumption in our present work.

Taking into account that both isomers of compound **23c** (Scheme 6) showed an increased affinity and selectivity for the MC_4R and that these are characteristic features of THIQ, we suggested that their "biologically active" structure is in general similar to that of THIQ and the differences should be located in the differing part. To investigate this further, we first introduced fractional atomic coordinates obtained from the X-ray structure of THIQ (form 1 from two forms found in crystals; see Supporting Information) into the CRYSIN program, which is included in molecular modeling software package SYBYL.[48] Application of the "Connect" command led to an image of the tertiary structure of the molecule. This was then saved as a mol2 file to enable its modification following molecular mod-

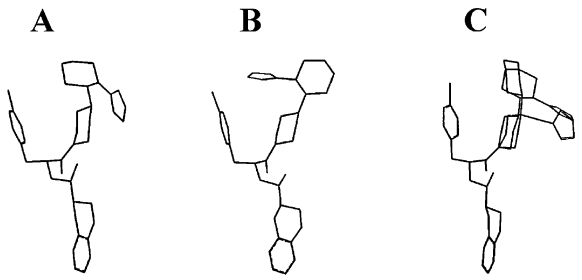


Figure 7. Modeling on SYBYL: (A) Proposed "bioactive" conformation of (*R*,*R*)-**23c**; (B) proposed "bioactive" conformation of (*S*,*S*)-**23c**; (C) overlaid proposed "bioactive" conformations of THIQ and (*R*,*R*)-**23c**.

eling in SYBYL. Further, the part of the THIQ molecule that is identical to that of (*R*,*R*)-**23c** and (*S*,*S*)-**23c** molecules (residues of 4-chloro-D-phenylalanine and D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid and nitrogen atom of piperidine (THIQ) or piperazine (**23c**) residues) was defined as an "aggregate" to exclude change of its tertiary structure during the following modeling. The image of THIQ was then modified using the "Sketch molecule" option, changing its differing part in relation to the isomers of **23c** in order to obtain their approximate tertiary structure. To suggest a minimum energy conformer for the investigated molecules, the imaginary structures of (*R*,*R*)-**23c** and (*S*,*S*)-**23c**, whose conformational freedom was limited by the "aggregates", were introduced into the SYBYL program MAXIMIN.

As a result, conformations of both **23c** forms were generated (Figure 7A,B). If we look at the parts of the molecules not included in the "aggregate", it can be seen that only the conformation of (*R*,*R*)-**23c** (Figure 7A) is very similar to that of the X-ray structure of THIQ (Figure 5). The generated conformation of (*S*,*S*)-**23c** (Figure 7B) differs substantially because the triazole moiety is here turned to the rest of the molecule. To obtain a better comparison with the X-ray structure of the THIQ form 1 found in crystals, its structure was overlaid with the conformation of (*R*,*R*)-**23c** created by MAXIMIN using the SYBYL "Fit atoms" function. From the overlaid image (Figure 7C), the following features could be noted. The position of the piperazine of (*R*,*R*)-**23c** differs insignificantly from the position of the piperidine of THIQ. However, the cyclohexane ring of (*R*,*R*)-**23c** is turned away from its place in THIQ and the same can be said about the triazole residue. Both in THIQ and (*R*,*R*)-**23c** the triazole is exposed to the environment, i.e., turned away from the rest of the molecule. Upon measurement of the distances between centers of the corresponding cycles in both molecules (SYBYL options "Centroid" and "Measure"), the following results were obtained: distance piperidine−piperazine 0.54 Å, distance cyclohexane−cyclohexane 3.82 Å, distance triazole−triazole 3.27 Å. Such deviations could, at least partly, explain the reduced activity of (*R*,*R*)-**23c** compared with THIQ.

Performing similar modeling for **23b** and comparing the structure obtained with that of THIQ gave very similar data as above in terms of positions of rings and distances between them. However, **23b** displayed uniform and considerably lower affinity on the MC_4R compared with **23c** (i.e., no high- and low-affinity sites involved). This could be explained if we assume that the protonated charged nitrogen atom, which is present in the piperazine ring, is not favorable for interactions with the receptor. This disubstituted ammonium ion is shielded in **23c** by the rigidly connected triazole so that the ion is not able to contact the receptor. In **23b** the triazole is connected through a methylene group and has more conformational freedom. Therefore, upon interaction with the receptor the disubstituted ammonium ion might interfere negatively with the binding process. This explanation could be expanded also to **23d** and **23a**, which have structures similar to **23c** but reduced activity and selectivity for the MC_4R.

The importance for separation of the piperazine substituted ammonium ion from the receptor is supported by the fact that other recently disclosed MC_4R agonists related to THIQ contain piperazine with a bulky substituent close to the ammonium nitrogen.[18,49] A steric hindrance here could be an important factor to maintain high MC_4R activity.

Conclusion

Comparison of the binding data for THIQ-related piperazine derivatives, which do not include a triazole moiety, indicated the importance of the cyclohexane ring for attaining MC_4R selectivity over other MC receptors. *N*-Oxides were considerably less active than the corresponding amines. The cyclopentane derivative **23d** exhibited preference for the MC_1R and not for the MC_4R.

Introduction of a triazole moiety improved the affinity of all compounds for all melanocortin receptors evaluated, the affinities reaching a low micromolar to submicromolar level. Among the compounds studied, only THIQ and (*R*,*R*)-**23c** displayed an MC_4R-selective, heterogeneous binding profile in competition with [^{125}I]-NDP-MSH. According to the two binding sites interpretation, they showed submicromolar affinity for the apparent low-affinity site and a nanomolar or subnanomolar affinity for the apparent high-affinity site.

In line with a previous study by others,[25] the crystal conformation of THIQ may be looked upon as a "bioactive" one. By adoption of this assumption, a model for explaining the different activity patterns of the **23c** isomers was developed. According to this model, an important feature for a "bioactive" form of (*R*,*R*)-**23c** is the location of its triazole function so that it is turned away from the rest of the molecule, similarly as in THIQ, whereas for (*S*,*S*)-**23c** it is turned toward the rest of the molecule, thus not being exposed to the receptor. According to our interpretation, this is the reason (*S*,*S*)-**23c** does not delineate the apparent high- and low-affinity binding sites of THIQ and (*R*,*R*)-**23c**.

Experimental Section

Materials and Methods. Reagents were used without purification. Unless otherwise noted, they were obtained from Aldrich or Fluka. DMF, DIEA, and HATU were supplied from Applied Biosystems. Boc-D-Tic-OH was a product of Neosystem. Most of ^{1}H NMR spectra were recorded on a JEOL JNM-EX270 spectrometer, using DMSO-d_6 (2.50 ppm downfield from TMS) as an internal standard. In a particular study the Bruker DMX-600 and Bruker DRX-500 instruments were used. Exact molecular masses were determined on Micromass Q-Tof2 mass spectrometer equipped with an electrospray ion source. LC/MS was performed on a Perkin-Elmer instrument PE SCIEX API 150EX with a Turboionspray ion source and a Dr. Maisch Reprosil-Pur C18-AQ, 5 μm, 150 mm × 3 mm

HPLC column using a gradient formed from water and acetonitrile with 5 mM ammonium acetate additive. Small-scale preparative HPLC was carried out on an LKB system consisting of a 2150 HPLC pump, 2152 LC controller, a 2151 variable-wavelength monitor, and a Vydac RP C_{18} column (10 mm × 250 mm, 90 Å, 201HS1010), with the eluent being an appropriate concentration of MeCN in water + 0.1% TFA, a flow rate of 5 mL/min, and detection at 280 nm. TLC was performed using Merck silica gel 60 F 254 glass plates; flash chromatography was performed using Merck Silica gel (70−230 mesh). Evaporations of solvents were made on a vacuum rotary evaporator at 30 °C and 20 mbar. Freeze-drying was carried out at 0.01 bar on a Lyovac GT2 freeze−dryer (Finn-Aqua) equipped with a Trivac D4B (Leybold Vacuum) vacuum pump and a liquid nitrogen trap.

***N*-(*tert*-Butoxycarbonyl)-4-chloro-D-phenylalanine 4-Nitrobenzyl Ester (2).** To a solution of Boc-D-Phe(4-Cl)-OH (0.99 g, 3.30 mmol) in EtOAc (50 mL), 4-nitrobenzyl bromide (0.75 g, 3.47 mmol) and TEA (0.69 mL, 4.95 mmol) were added. The resulting mixture was refluxed with stirring for 7 h. After the mixture was cooled to room temperature, the precipitate formed was filtered off. The filtrate was diluted with EtOAc to 200 mL and washed successively with aqueous 10% $KHSO_4$ (3 × 50 mL), saturated aqueous $NaHCO_3$ (2 × 20 mL), and water (2 × 50 mL). The organic layer was dried over $MgSO_4$ and filtered, and the filtrate was evaporated. Product **1** was obtained as a white solid (yield 1.28 g, 89%). ^{1}H NMR (270 MHz, DMSO-d_6): δ 8.20 (2H, m), 7.54 (2H, m), 7.47 (1H, d, J = 7.6 Hz), 7.29 (4H, m), 5.24 (2H, s), 4.25 (1H, m), 2.95 (2H, m), 1.31 (9H, s). MS calculated for $C_{21}H_{23}ClN_2O_6$: 434.1, found 435.1 $(M + H)^+$. R_f = 0.90 ($CHCl_3$−MeOH, 7:1).

4-Chloro-D-phenylalanine 4-Nitrobenzyl Ester Hydrochloride (3). To a solution of **2** (1.00 g, 2.30 mmol) in AcOH (2 mL) 4 M HCl was added in dioxane (4 mL), and the mixture was stirred for 1 h at room temperature. The resulting solution was evaporated and the residue was triturated with dry ether to give product **3** as a white solid (yield 0.85 g, 96%). ^{1}H NMR (270 MHz, DMSO-d_6): δ 8.70 (3H, br s), 8.19 (2H, m), 8.49 (2H, m), 7.29 (4H, m), 5.28 (2H, s), 4.42 (1H, m), 3.15 (2H, m). HRMS ($M + H^+$): 335.0784, $C_{16}H_{16}ClN_2O_4$ requires 335.0789. R_f = 0.54 ($CHCl_3$−MeOH, 7:1).

(*N*-*tert*-Butoxycarbonyl-D-1,2,3,4-tetrahydroisoquinoline-3-carbonyl)-4-chloro-D-phenylalanine 4-Nitrobenzyl Ester (4). To a solution of Boc-D-Tic-OH (0.46 g, 1.65 mmol) in DMF (3 mL) were added **3** (0.65 g, 1.65 mmol), HATU (0.69 g, 1.81 mmol), and DIEA (0.85 mL, 4.94 mmol). The reaction mixture was stirred for 2 h at room temperature, diluted with CH_2Cl_2 to 100 mL, and washed with aqueous 10% $KHSO_4$ (2 × 25 mL), saturated aqueous $NaHCO_3$ (2 × 25 mL), and water (2 × 25 mL), dried over $MgSO_4$, filtered, and evaporated to afford product **4** (yield 0.97 g, 99%) as a fine white power. ^{1}H NMR (270 MHz, DMSO-d_6): δ 8.39 (1H, d, J = 7.6 Hz), 8.17 (2H, m), 7.48 (2H, m), 7.15 (8H, m), 5.19, 5.12 (2H, 2s), 4.50 (4H, m), 3.00 (4H, m), 1.20 (9H, m). MS calculated for $C_{31}H_{32}ClN_3O_7$: 593.2, found 594.2 ($M + H^+$). R_f = 0.80 ($CHCl_3$−MeOH, 7:1).

(*N*-*tert*-Butoxycarbonyl-D-1,2,3,4-tetrahydroisoquinoline-3-carbonyl)-4-chloro-D-phenylalanine (5). To a stirred suspension of **4** (0.15 g, 0.25 mmol) in MeOH (2 mL), aqueous 2 N NaOH (131 μL, 0.26 mmol) was added, and the mixture was stirred for 1 h at room temperature. Reaction progress was monitored using TLC and LC/MS. After 1 h, 31 μL (0.06 mmol) of aqueous 2 N NaOH was additionally added, and the reaction mixture was stirred for 30 min more and then evaporated. The residue was partitioned between EtOAc (50 mL) and aqueous 10% $KHSO_4$ (25 mL), and the aqueous layer was extracted with EtOAc (2 × 25 mL). The combined organic extracts were washed with water (2 × 25 mL), dried over $MgSO_4$, filtered, and evaporated to give **5** (0.11 g, quantitative yield) as a somewhat yellow oil. ^{1}H NMR (270 MHz, DMSO-d_6): δ 12.80 (1H, br s), 8.00 (1H, m), 7.15 (8H, m), 4.40 (4H, m), 2.90 (4H, m), 1.30 (9H, m). MS calculated for $C_{24}H_{27}ClN_2O_5$: 458.2, found 459.2 ($M + H^+$). R_f = 0.22 ($CHCl_3$−MeOH, 7:1).

1-[(*N*-*tert*-Butoxycarbonyl-D-1,2,3,4-tetrahydroisoquinoline-3-carbonyl)-4-chloro-D-phenylalanyl]-4-phenylpiperazine (7a). To a solution of **5** (0.15 g, 0.33 mmol) in DMF (1.5 mL) were added 1-phenylpiperazine (50 μL, 0.33 mmol), HATU (0.14 g, 0.36 mmol), and DIEA (0.17 mL, 0.99 mmol). The reaction mixture was stirred for 2 h at room temperature, diluted with CH_2Cl_2 to 50 mL, and washed with aqueous 10% $KHSO_4$ (2 × 10 mL), saturated aqueous $NaHCO_3$ (2 × 10 mL), and water (2 × 5 mL), dried over $MgSO_4$, and evaporated to afford **7a** as a somewhat yellow oil (0.10 g, quantitative yield). MS calculated for $C_{34}H_{39}ClN_4O_4$: 602.3, found 603.3 ($M + H^+$).

1-[(*N*-*tert*-Butoxycarbonyl-D-1,2,3,4-tetrahydroisoquinoline-3-carbonyl)-4-chloro-D-phenylalanyl]-4-cyclohexylpiperazine (7b) was prepared from **5** (0.114 g, 0.25 mmol), 1-cyclohexylpiperazine (42 mg, 0.25 mmol), HATU (0.104 g, 0.27 mmol), and DIEA (0.13 mL, 0.75 mmol) as described above for **7a**. **7b** was obtained as a clear oil (0.15 g, quantitative yield). MS calculated for $C_{34}H_{45}ClN_4O_4$: 608.3, found 609.3 ($M + H^+$). R_f = 0.56 ($CHCl_3$−MeOH, 7:1).

1-[(D-1,2,3,4-Tetrahydroisoquinoline-3-carbonyl)-4-chloro-D-phenylalanyl]-4-phenylpiperazine Dihydrochloride (8a). To a solution of **7a** (90 mg, 0.15 mmol) in MeOH (1 mL) was added 4 M HCl in dioxane (0.38 mL), and the reaction mixture was stirred for 1.5 h at room temperature and evaporated. The residue was dissolved in MeOH (1 mL) and treated with dry ether. The crude **8a** was precipitated as a white-yellow powder (80 mg), which was purified by HPLC (eluent, 19% MeCN; k' = 0.47) followed by freeze-drying. A light powder formed. Yield 30 mg (36%). ^{1}H NMR (270 MHz, DMSO-d_6): δ 9.87 (1H, m), 9.50 (1H, m), 9.13 (1H, d, J = 7.6 Hz), 8.18 (2H, m), 7.46 (2H, m), 7.25 (9H, m), 5.60 (1H, br), 5.02 (1H, m), 4.20 (3H, m), 3.65 (4H, m), 3.00 (8H, m). HRMS ($M + H^+$): 503.2236, $C_{29}H_{32}ClN_4O_2$ requires 503.2214. R_f = 0.70 (*n*-BuOH−AcOH−H_2O, 4:1:1).

1-[(D-1,2,3,4-Tetrahydroisoquinoline-3-carbonyl)-4-chloro-D-phenylalanyl]-4-cyclohexylpiperazine Ditrifluoroacetate (8b). To a solution of **7b** (75 mg, 0.125 mmol) in CH_2Cl_2 (1 mL) was added TFA (2 mL), and the resulting solution was allowed to stand for 1 h at room temperature. The reaction mixture was evaporated to dryness, and the residue was triturated with a dry ether and hexane mixture to furnish crude **8b** as a fine white powder (70 mg), which was purified by HPLC (eluent, 13% MeCN; k' = 0.67) followed by freeze-drying. A light powder formed. Yield 37 mg (40%). ^{1}H NMR (270 MHz, DMSO-d_6): δ 10.10 and 9.93 (1H, 2 br s), 9.58 (1H, br s), 9.34 (1H, br s), 9.01 (1H, m), 7.30 (8H, m), 5.00 (1H, m), 4.30 (5H, m), 3.30 (6H, m), 2.90 (4H, m), 2.20 (1H, m), 1.85 (4H, m), 1.60 (1H, m), 1.20 (5H, m). HRMS ($M + H^+$): 509.2697, $C_{29}H_{38}ClN_4O_2$ requires 509.2683. R_f = 0.28 (*n*-BuOH−AcOH−H_2O, 4:1:1).

1-[(*N*-*tert*-Butoxycarbonyl-D-1,2,3,4-tetrahydroisoquinoline-3-carbonyl)-4-chloro-D-phenylalanyl]-4-oxy-4-phenylpiperazine (9a). A solution of **7a** (90 mg, 0.15 mmol) in 30% H_2O_2−AcOH (2 mL) was allowed to stand for 1 h at room temperature and for 2 h at 60 °C. The reaction mixture was evaporated to afford **9a** (82 mg, 88%). MS calculated for $C_{34}H_{39}ClN_4O_5$: 618.3, found 619.3 ($M + H^+$).

1-[(*N*-*tert*-Butoxycarbonyl-D-1,2,3,4-tetrahydroisoquinoline-3-carbonyl)-4-chloro-D-phenylalanyl]-4-oxy-4-cyclohexylpiperazine (9b) was synthesized from **7b** as described for the preparation of **9a.** Yield 92%. MS calculated for $C_{34}H_{43}ClN_4O_5$: 624.3, found 625.3 ($M + H^+$). R_f = 0.41 ($CHCl_3$−MeOH, 7:1).

1-[(D-1,2,3,4-Tetrahydroisoquinoline-3-carbonyl)-4-chloro-D-phenylalanyl]-4-oxy-4-phenylpiperazine Ditrifluoroacetate (10a). 9a (82 mg, 0.13 mmol) was dissolved in CH_2Cl_2 (1 mL) and treated with TFA (2 mL). The resulting solution was allowed to stand for 1 h at room temperature. The reaction mixture was evaporated, and the residue was triturated with a dry ether and hexane mixture to furnish crude **10a** (76 mg), which was purified by HPLC (eluent, 14% MeCN; k' = 0.67) followed by freeze-drying. A light powder formed. Yield 36 mg (36%). ^{1}H NMR (270 MHz, DMSO-d_6): δ 9.50 (1H, br s), 9.31 (1H, br s), 9.17 and 9.08 (1H, 2d, J = 8.6

and 8.3 Hz), 8.10 (2H, m), 7.67 (2H, m), 7.30 (9H, m), 5.20 (1H, m), 4.70−3.80 (9H, m), 3.50 (3H, m), 3.00 (3H, m). HRMS (M + H$^+$): 519.2143, $C_{29}H_{32}ClN_4O_3$ requires 519.2163. R_f = 0.30 (*n*-BuOH−AcOH−H_2O, 4:1:1).

1-[(D-1,2,3,4-Tetrahydroisoquinoline-3-carbonyl)-4-chloro-D-phenylalanyl]-4-oxy-4-cyclohexylpiperazine Ditrifluoroacetate (10b). 10b was synthesized from **9b** as described for the preparation of **10a**. HPLC eluent, 16% MeCN; k' = 2.47. Yield 52%. ^{1}H NMR (270 MHz, DMSO-d_6): δ 9.62 (1H, br, s), 9.37 (1H, br, s), 9.04 and 9.05 (1H, 2d, J = 6.9 and 7.6 Hz), 7.30 (8H, m), 5.05 (1H, m), 4.30 (5H, m), 3.65 (5H, m), 3.30 (3H, m), 2.96 (3H, m), 2.17 (2H, m), 1.87 (2H, m), 1.70−1.00 (6H, m). HRMS (M + H$^+$): 525.2617, $C_{29}H_{38}ClN_4O_3$ requires 525.2632. R_f = 0.33 (*n*-BuOH−AcOH−H_2O, 4:1:1).

1-[*cis*-2-(*tert*-Bytoxycarbonylamino)cyclohexyl]-methanol (16a). To a stirred suspension of lithium aluminum hydride (0.40 g, 10.50 mmol) in anhydrous THF (25 mL) was added *cis*-2-amino-1-cyclohexanecarboxylic acid (0.50 g, 3.49 mmol). The reaction mixture was stirred under Ar atmosphere for 28 h at 70−80 °C, then cooled to 0 °C and quenched with saturated aqueous K_2CO_3 (25 mL). The resulting white emulsion was diluted with EtOAc (50 mL) and filtered through a short pad of Celite, which was afterward rinsed with EtOAc (25 mL). The filtrate was evaporated, 1-butanol (50 mL) was added, and the organic layer formed was washed with brine (3 × 10 mL), dried over Na_2SO_4, and evaporated to afford a white solid. MS analysis of the product showed that it contained alcohol **15a**. MS calculated for $C_7H_{15}NO$: 129.1, found 130.1 (M + H$^+$).

The above product was dissolved in 10% TEA solution in MeOH (30 mL), di-*tert*-butyl dicarbonate (0.76 g, 3.49 mmol) was added, and the reaction mixture was stirred for 4 h at room temperature. The mixture was evaporated, the residue was dissolved in EtOAc (70 mL) and successively washed with saturated aqueous $NaHCO_3$ (2 × 10 mL), water (2 × 10 mL), and brine (1 × 10 mL), dried over Na_2SO_4, and filtered, and the filtrate was evaporated to afford a clear colorless oil (0.53 g). The product was purified on a silica gel column (2 cm × 20 cm) eluting it with a petroleum ether and ether mixture (gradually changing their volume proportions from 4:1 to 5:3). Eluate fractions containing the desired product were pooled and evaporated to furnish **16a** as a clear colorless oil (0.254 g, 32% for two steps). ^{1}H NMR (270 MHz, DMSO-d_6): δ 6.67 (1H, d, J = 8.6 Hz), 3.69 (1H, m), 3.32−3.17 (2H, m), 1.50 (4H, m), 1.37 (9H, s), 1.40−1.15 (6H, m). MS calculated for $C_{12}H_{23}NO_3$: 229.2, found 230.2 (M + H$^+$). R_f = 0.67 ($CHCl_3$−MeOH, 7:1).

1-(*cis*-2-Aminocyclohexyl)methanol Hydrochloride (15a). 16a (0.218 g, 0.952 mmol) was dissolved in a 4 M solution of HCl in dioxane (4.76 mL, 19.04 mmol), allowed to stand for 1 h at room temperature, and evaporated. The residue was dried in vacuo in the presence of KOH and treated with dry ether to furnish the product **15a** as white oil (0.157 g, quantitative yield). ^{1}H NMR (270 MHz, DMSO-d_6): δ 7.86 (3H, br s), 4.86 (1H, br s), 3.40 (2H, m), 1.90−1.20 (10H, m). HRMS (M + H$^+$): 130.1237, $C_7H_{16}NO$ requires 130.1232. R_f = 0.57 (*n*-BuOH−AcOH−H_2O, 4:1:1).

1-[1-(*tert*-Bytoxycarbonylamino)cyclohexyl]-methanol (16b) was synthesized from 1-aminocyclohexanecarboxylic acid as described for the preparation of **16a.** Yield 68%. ^{1}H NMR (270 MHz, DMSO-d_6): δ 6.03 (1H, br s), 4.54 (1H, br s), 3.34 (2H, s), 1.89 (2H, dm, J = 12.2 Hz), 1.50−1.10 (8H, m), 1.35 (9H, s). MS calculated for $C_{12}H_{23}NO_3$: 229.2, found 230.2 (M + H$^+$). R_f = 0.69 ($CHCl_3$−MeOH, 7:1).

1-(1-Aminocyclohexyl)methanol Hydrochloride (15b). 15b was synthesized from **16b** as described for the preparation of **15a**. Yield 80%. ^{1}H NMR (270 MHz, DMSO-d_6): δ 7.80 (3H, br s), 5.41 (1H, br s), 3.45 (2H, s), 1.80−1.10 (10H, m). HRMS (M + H$^+$): 130.1235, $C_7H_{16}NO$ requires 130.1232. R_f = 0.45 (*n*-BuOH−AcOH−H_2O, 4:1:1).

1-Benzyl-4-(*cis*-2-hydroxymethylcyclohexyl)piperazine (17a). To a solution of **13**[30] (0.251 g, 0.937 mmol) in EtOH (25 mL) were added **15a** (0.155 g, 0.937 mmol) and $NaHCO_3$ (0.283 g, 3.373 mmol). The reaction mixture was refluxed for 5 h, then cooled to room temperature and evaporated. EtOAc (50 mL) was added to the residue, and the mixture was extracted with saturated aqueous $NaHCO_3$ (30 mL). The organic layer was washed with brine (2 × 15 mL), dried over $MgSO_4$, filtered, and evaporated to give the crude product as a white oil. It was purified by crystallization from petroleum ether to afford **17a** (yield 0.70 g, 69%). ^{1}H NMR (270 MHz, DMSO-d_6): δ 7.42 (5H, m), 4.42 (1H, m), 4.00 (2H, m), 3.75 (1H, m), 3.70−2.80 (8H, m), 2.40 (1H, m), 2.00−1.00 (10H, m). MS calculated for $C_{18}H_{28}N_2O$: 288.2, found 289.2 (M + H$^+$). R_f = 0.43 ($CHCl_3$−MeOH, 7:1).

1-Benzyl-4-(1-hydroxymethylcyclohexyl)piperazine (17b) was synthesized from **15b** as described for the preparation of **17a**. Yield 25%. ^{1}H NMR (270 MHz, DMSO-d_6): δ 7.26 (5H, m), 4.23 (1H, m), 3.65−3.1 (5H, m), 2.60 (4H, m), 2.28 (3H, m), 1.70−1.00 (10H, m). MS calculated for $C_{18}H_{28}N_2O$: 288.2, found 289.2 (M + H$^+$). R_f = 0.47 ($CHCl_3$−MeOH, 7:1).

1-Benzyl-4-(*trans*-2-hydroxycyclohexyl)piperazine (17c) was synthesized from *trans*-2-aminocyclohexanol hydrochloride as described for the preparation of **17a**. Yield 69%. ^{1}H NMR (270 MHz, DMSO-d_6): δ 7.40 (5H, m), 3.97 (1H, m), 3.80−2.60 (11H, m), 1.90 (2H, m), 1.65 (2H, m), 1.20 (5H, m). MS calculated for $C_{17}H_{26}N_2O$: 274.2, found 275.2 (M + H$^+$). R_f = 0.43 ($CHCl_3$−MeOH, 7:1).

1-Benzyl-4-(1-hydroxymethylcyclopentyl)piperazine (17d) was synthesized from 1-amino-1-cyclopentanemethanol as described for the preparation of **17a**. Yield 74%. ^{1}H NMR (270 MHz, DMSO-d_6): δ 7.27 (5H, m), 4.44 (1H, br s), 4.07 (1H, m), 3.61 (1H, m), 3.35 (2H, m), 2.66 (4H, m), 2.32 (4H, m), 1.50 (8H, m). MS calculated for $C_{17}H_{25}N_2O$: 274.2, found 275.2 (M + H$^+$). R_f = 0.27 ($CHCl_3$−MeOH, 7:1).

1-Benzyl-4-[*cis*-2-(1,2,4-triazol-1-ylmethyl)cyclohexyl]-piperazine (18a). To a cooled solution of **17a** (0.27 g, 0.937 mmol) and TEA (0.26 mL, 1.87 mmol) in CH_2Cl_2 (10 mL) at 0 °C was added dropwise with stirring a solution of methanesulfonyl chloride (0.145 mL, 1.87 mmol) in CH_2Cl_2 (5 mL). The reaction mixture was allowed to warm to room temperature and was stirred for 2 h more. The volatiles were removed under reduced pressure, and the residue containing 1-benzyl-4-[*cis*-(2- methanesulfonyloxymethyl)cyclohexyl]piperazine was used in the next step without any purification as clear-yellow oil. MS calculated for $C_{19}H_{29}N_2O_3S$: 366.2, found 367.2 (M + H$^+$).

To a solution of the above-mentioned oil in DMF (2 mL) was added the sodium salt of 1,2,4-triazole (0.257 g, 2.81 mmol). The resulting suspension was heated for 12 h at 80−100 °C and cooled to room temperature. The reaction mixture was poured into water (15 mL) and extracted with EtOAc (2 × 20 mL). The combined organic extracts were washed with brine (2 × 10 mL), dried over $NaSO_4$, and filtered, and the filtrate was evaporated, affording a yellow oil (0.444 g). The product was purified on a silica gel chromatography column (2 cm × 25 cm) and eluted with a hexanes−EtOAc−MeOH mixture (gradually changing their volume proportions from 1:1:0 to 1:6:1). Eluate fractions containing the desired product were pooled and evaporated to furnish **18a** as a clear colorless oil. Yield 36 mg (11% for two steps). ^{1}H NMR (270 MHz, DMSO-d_6): δ 8.50 (1H, s), 7.92 (1H, s), 7.28 (5H, m), 4.23 (2H, d, J = 7.3 Hz), 3.42 (2H, s), 3.33 (8H, m), 2.36 (2H, m), 2.07 (1H, m), 1.78 (2H, m), 1.50−1.00 (5H, m). MS calculated for $C_{20}H_{29}N_5$: 339.2, found 340.2 (M + H$^+$). R_f = 0.36 ($CHCl_3$−MeOH, 7:1).

1-Benzyl-4-[1-(1,2,4-triazol-1-ylmethyl)cyclohexyl]piperazine (18b) was synthesized from **17b** as described for the preparation of **18a**. Yield 19% (oil). ^{1}H NMR (270 MHz, DMSO-d_6): δ 8.49 (1H, s), 7.97 (1H, s), 7.46 (5H, m), 4.29 (2H, s), 4.16 (2H, s), 3.21 (2H, m), 2.96 (6H, m), 1.74 (2H, m), 1.60−1.00 (8H, m). MS calculated for $C_{20}H_{29}N_5$: 339.2, found 340.2 (M + H$^+$). R_f = 0.41 ($CHCl_3$−MeOH, 7:1).

1-Benzyl-4-[*trans*-2-(1,2,4-triazol-1-yl)cyclohexyl]piperazine (18c) was synthesized from **17c** as described for the preparation of **18a**. Yield 23% (oil). ^{1}H NMR (270 MHz, DMSO-d_6): δ 8.41 (1H, s), 7.88 (1H, s), 7.25 (5H, m), 4.32 (1H, m), 3.34 (3H, m), 2.74 (1H, m), 2.62 (2H, m), 2.50 (1H, m), 2.13 (4H, m), 1.89 (2H, m), 1.74 (2H, m), 1.30 (4H, m). MS calculated for $C_{19}H_{26}N_5$: 325.2, found 326.2 (M + H$^+$). R_f = 0.65 ($CHCl_3$−MeOH, 7:1).

1-Benzyl-4-[1-(1,2,4-triazol-1-ylmethyl)cyclopentyl]piperazine (18d) was synthesized from **17d** as described for the preparation of **18a**. Yield 21%. ^{1}H NMR (270 MHz, DMSO-d_6): δ 8.61 (1H, s), 7.97 (1H, s), 7.46 (5H, m), 4.24 (2H, s), 3.40 (2H, m), 3.33 (4H, m), 2.28 (4H, m), 1.70−1.30 (8H, m). MS calculated for $C_{19}H_{27}N_5$: 325.2, found 326.2 (M + H$^+$). R_f = 0.45 ($CHCl_3$−MeOH, 7:1).

4-[*cis*-2-(1,2,4-Triazol-1-ylmethyl)cyclohexyl]piperazine Dihydrochloride (19a). To a solution of **18a** (32 mg, 0.094 mmol) in MeOH (3 mL) were added 10% Pd/C (55 mg) and ammonium formate (0.16 g, 2.51 mmol). The mixture was heated under reflux for 1.5 h. After cooling to room temperature, the mixture was filtered and the filtrate was evaporated. The residue was dissolved in water (1.0 mL) and purified by HPLC (detection at 205 nm; eluent, water + 0.1% CF_3COOH). Eluate fractions containing the presumed product of interest were pooled and freeze-dried. The oil formed was dissolved in MeOH (1.0 mL), and the solution slowly passed through a column (5 mm × 10 mm) packed with Dowex 1 × 4 in Cl$^-$ form. The column was rinsed with methanol, and the united eluate evaporated. Yield 20 mg (66%, amorphous solid). ^{1}H NMR (270 MHz, DMSO-d_6): δ 11.91 (1H, br s), 9.70 and 9.46 (2H, 2br s), 8.72 (1H, m), 8.06 (1H, m), 4.02 (2H, m), 3.69−2.68 (8H, m), 1.95−1.50 (10H, m). HRMS (M + H$^+$): 250.2030, $C_{13}H_{24}N_5$ requires 250.2031. R_f = 0.06 (*n*-BuOH−AcOH−H_2O, 4:1:1).

4-[1-(1,2,4-Triazol-1-ylmethyl)cyclohexyl]piperazine Dihydrochloride (19b). 19b was synthesized from **18b** as described for the preparation of **19a.** The HPLC eluent contained 1.6% MeCN. Yield 72% (amorphous solid). ^{1}H NMR (270 MHz, DMSO-d_6): δ 9.30 (1H, m), 8.50 (1H, s), 8.51 (2H, m), 7.98 (H, s), 4.16 (2H, m), 3.00 (4H, m), 2.71 (4H, m), 1.77 (2H, m), 1.45 (5H, m), 1.18 (3H, m). HRMS (M + H$^+$): 250.2028, $C_{13}H_{24}N_5$ requires 250.2031. R_f = 0.16 (*n*-BuOH−AcOH−H_2O, 4:1:1).

4-[*trans*-2-(1,2,4-Triazol-1-yl)cyclohexyl]piperazine Dihydrochloride (19c). 19c was synthesized from **18c** as described for the preparation of **19a**. HPLC eluent, water + 0.1% CF_3COOH. Yield 35% (amorphous solid). ^{1}H NMR (270 MHz, DMSO-d_6): δ 9.45 (1H, m), 8.67 (1H, s), 8.62 (2H, m), 8.03 (1H, s), 4.42 (1H, m), 3.08 (3H, m), 2.82 (5H, m), 2.40 (2H, m), 2.03−1.68 (4H, m), 1.30 (3H, m). HRMS (M + H$^+$): 236.1876, $C_{12}H_{22}N_5$ requires 236.1875. R_f = 0.13 (*n*-BuOH−AcOH−H_2O, 4:1:1).

4-[1-(1,2,4-Triazol-1-ylmethyl)cyclopentyl]piperazine Dihydrochloride (19d). 19d was synthesized from **18d** as described for the preparation of **19a**. The HPLC eluent contained 1.6% MeCN. Yield 47% (amorphous solid). ^{1}H NMR (270 MHz, DMSO-d_6): δ 9.31 (1H, m), 8.94 (1H, s), 8.41 (2H, m), 8.19 (1H, s), 4.50 (2H, m), 2.88 (4H, m), 2.53 (4H, m), 1.92 (3H, m), 1.75−1.44 (5H, m). HRMS (M + H$^+$): 236.1876, $C_{12}H_{22}N_5$ requires 236.1875. R_f = 0.14 (*n*-BuOH−AcOH−H_2O, 4:1:1).

1-(*N*-*tert*-Butoxycarbonyl-4-chloro-D-phenylalanyl)-4-[*cis*-2-(1,2,4-triazol-1-yl-methyl)cyclohexyl]piperazine (20a). To a solution of **19a** (17.0 mg, 0.0528 mmol) in DMF (200 μL) were added Boc-D-Phe(4-Cl)-OH (15.8 mg, 0.0528 mmol), HATU (22.1 mg, 0.0581 mmol), and DIEA (27.1 μL, 0.158 mmol). The reaction mixture was stirred for 2 h at room temperature, then diluted with EtOAc to 20 mL and washed with aqueous 10% $KHSO_4$ (2 × 5 mL), saturated aqueous $NaHCO_3$ (2 × 5 mL), water (2 × 5 mL), and brine (1 × 5 mL), dried over Na_2SO_4, filtered, and evaporated to afford **20a** as a clear oil (21.0 mg, 75%). MS calculated for $C_{27}H_{39}ClN_6O_3$: 530.3, found 531.3 (M + H$^+$). R_f = 0.44 ($CHCl_3$−MeOH, 7:1).

1-[*N*-(*tert*-Butoxycarbonyl)-4-chloro-D-phenylalanyl]-4-[1-(1,2,4-triazol-1-ylmethyl)cyclohexyl]piperazine (20b) was synthesized from **19b** as described for the preparation of **20a**. Yield, quantitative (oil). MS calculated for $C_{27}H_{39}ClN_6O_3$: 530.3, found 531.3 (M + H$^+$). R_f = 0.40 ($CHCl_3$−MeOH, 7:1).

1-[*N*-(*tert*-Butoxycarbonyl)-4-chloro-D-phenylalanyl]-4-[*trans*-2-(1,2,4-triazol-1-yl)cyclohexyl]piperazine (20c) was synthesized from **19c** as described for the preparation of **20a**. Yield 82% (oil). MS calculated for $C_{26}H_{37}ClN_6O_3$: 516.3, found 517.3 (M + H$^+$). R_f = 0.63 ($CHCl_3$−MeOH, 7:1).

1-[*N*-(*tert*-Butoxycarbonyl)-4-chloro-D-phenylalanyl]-4-[1-(1,2,4-triazol-1-ylmethyl)cyclopentyl]piperazine (20d) was synthesized from **19d** as described for the preparation of **20a**. Yield 81% (oil). MS calculated for $C_{26}H_{37}ClN_6O_3$: 516.3, found 517.3 (M + H$^+$). R_f = 0.51 ($CHCl_3$−MeOH, 7:1).

1-(4-Chloro-D-phenylalanyl-4-[*cis*-2-(1,2,4-triazol-1-ylmethyl)cyclohexyl]piperazine Ditrifluoroacetate (21a). To a solution of **20a** (21.0 mg, 0.0396 mmol) in CH_2Cl_2 (1 mL), TFA (2 mL) was added, and the resulting mixture was stirred for 1 h at room temperature. The volatiles were removed in vacuo, and the residue was triturated with a mixture of dry ether and hexane to furnish the product **21a** as a fine white powder (19.0 mg, 73%). ^{1}H NMR (270 MHz, DMSO-d_6): δ 8.53 (1H, m), 8.39 (1H, m), 8.21 (3H, m), 8.01 (1H, s), 7.42 (2H, m), 7.26 (2H, m), 4.67 (1H, m), 4.25 (2H, m), 3.44 (2H, m), 3.44−2.85 (8H, m), 1.95−1.65 (2H, m), 1.40 (8H, m). HRMS (M + H$^+$): 431.2318, $C_{22}H_{32}ClN_6O$ requires 431.2326. R_f = 0.24 (*n*-BuOH−AcOH−H_2O, 4:1:1).

1-(4-Chloro-D-phenylalanyl)-4-[1-(1,2,4-triazol-1-ylmethyl)cyclohexyl]piperazine Ditrifluoroacetate (21b). 21b was synthesized from **20b** as described for the preparation of **21a**. Yield 79% (white powder). ^{1}H NMR (270 MHz, DMSO-d_6): δ 8.57 (1H, br s), 8.23 (4H, m), 8.04 (1H, br s), 7.42 (2H, m), 7.25 (2H, m), 4.66 (1H, m), 4.15 (4H, m), 3.08−2.75 (8H, m), 1.78−1.16 (10H, m). HRMS (M + H$^+$): 431.2301, $C_{22}H_{32}ClN_6O$ requires 431.2326. R_f = 0.30 (*n*-BuOH−AcOH−H_2O, 4:1:1).

1-(4-Chloro-D-phenylalanyl)-4-[*trans*-2-(1,2,4-triazol-1-yl)cyclohexyl]piperazine Ditrifluoroacetate (21c). 21c was synthesized from **20c** as described for the preparation of **21a.** Yield 72% (white powder). ^{1}H NMR (270 MHz, DMSO-d_6): δ 8.48 (1H, s), 8.14 (4H, m), 7.92 (1H, s), 7.39 (2H, m), 7.19 (2H, m), 4.59 (1H, m), 4.38 (1H, m), 3.51 (2H, m), 3.45−3.15 (4H, m), 3.15−2.76 (4H, m), 2.26−1.08 (9H, m). HRMS (M + H$^+$): 417.2173, $C_{21}H_{30}ClN_6O$ requires 417.2169. R_f = 0.22 (*n*-BuOH−AcOH−H_2O, 4:1:1).

1-(4-Chloro-D-phenylalanyl)-4-[*R*,*R*-2-(1,2,4-triazol-1-yl-)cyclohexyl]piperazine Dihydrochloride [(*R*,*R*)-21c] and 1-(4-Chloro-D-phenylalanyl)-4-[*S*,*S*-2-(1,2,4-triazol-1-yl)-cyclohexyl]piperazine Dihydrochloride [(*S*,*S*)-21c]. 21c (50.6 mg) was dissolved in 8.5 mL of methanol. This solution was introduced in 150 μL portions into a 11034 Chirobiotic V 250 mm × 10 mm HPLC column (Advanced Separation Technologies Inc.). Eluent, dioxane−methanol−acetic acid−triethylamine (50:50:0.02:0.01); flow rate 5 mL/min; UV monitoring at 220 nm. At each run, three eluate fractions were collected. The first fraction contained pure (*R*,*R*)-**21c**, the second contained a mixture of (*R*,*R*)-**21c** and (*S*,*S*)-**21c**, and the third contained pure (*S*,*S*)-**21c**. When all of the initial mixture was passed through the column, the corresponding eluate fractions were separately pooled in three flasks and evaporated. The residues were dried at 0.01 bar for 24 h, and then each of them was dissolved in 50 mL of water and freeze-dried for 2 days. Each of the products obtained were dissolved in water (2.0 mL) and the solutions separately and slowly passed through one of three columns (5 mm × 10 mm) with Dowex 1 × 4 in Cl$^-$ form. Columns were rinsed with water, and the correspondingly united eluates were freeze-dried. Each of three residues was dissolved in 1.5 mL of water and freeze-dried again. Three samples of fluffy white powders were obtained. The yield of (*R*,*R*)-**21c** was 12.4 mg (32%), ee = 95.5%, and the yield of (*S*,*S*)-**21c** was 12.7 mg (33%), ee = 92.6%. (Optical purities, ee, were determined from data of analytical chiral HPLC on Chirobiotic V.) The intermediate fraction gave 9.8 mg (25%) of a regenerated isomer mixture.

1-(4-Chloro-D-phenylalanyl)-4-[1-(1,2,4-triazol-1-ylmethyl)cyclopentyl]piperazine Ditrifluoroacetate (21d). 21d was synthesized from **20d** as described for the preparation of **21a.** Yield 59% (white powder). ^{1}H NMR (270 MHz, DMSO-d_6): δ 8.62 (1H, s), 8.38 (1H, m), 8.13 (3H, m), 7.98 (1H, s), 7.39 (2H, m), 7.21 (2H, m), 4.57 (1H, m), 4.35 (2H, m), 3.69 (2H, m), 3.15−2.85 (4H, m), 2.30 (4H, m), 2.02−1.45 (m, 8H).

HRMS (M + H$^+$): 417.2184, $C_{21}H_{30}ClN_6O$ requires 417.2169. R_f = 0.25 (*n*-BuOH−AcOH−H_2O, 4:1:1).

1-[*N*-(*tert*-Butoxycarbonyl)-D-1,2,3,4-tetrahydroisoquinoline-3-carbonyl-4-chloro-D-phenylalanyl]-4-[*cis*-2-(1,2,4-triazol-1-ylmethyl)cyclohexyl]piperazine (22a) was synthesized from **21a** (17.0 mg, 0.0258 mmol), Boc-D-Tic-OH (7.2 mg, 0.0258 mmol), HATU (10.8 mg, 0.0284 mmol), and DIEA (13.2 mL, 0.0774 mmol) as described for the preparation of **20a**. The title compound was obtained as a clear oil (17.8 mg, quantitative yield). MS calculated for $C_{37}H_{48}ClN_7O_4$: 689.3, found 690.3 (M + H$^+$). R_f = 0.40 ($CHCl_3$−MeOH, 7:1).

1-[*N*-(*tert*-Butoxycarbonyl)-D-1,2,3,4-tetrahydroisoquinoline-3-carbonyl-4-chloro-D-phenylalanyl]-4-[1-(1,2,4-triazol-1-ylmethyl)cyclohexyl]piperazine (22b) was synthesized from **21b** as described for the preparation of **22a**. Yield, quantitative (oil). MS calculated for $C_{37}H_{48}ClN_7O_4$: 689.3, found 690.3 (M + H$^+$). R_f = 0.39 ($CHCl_3$−MeOH, 7:1).

1-[*N*-(*tert*-Butoxycarbonyl)-D-1,2,3,4-tetrahydroisoquinoline-3-carbonyl-4-chloro-D-phenylalanyl]-4-[*trans*-2-(1,2,4-triazol-1-yl)cyclohexyl]piperazine (22c) was synthesized from **21c** as described for the preparation of **22a**. Yield, quantitative (oil). MS calculated for $C_{36}H_{46}ClN_7O_4$: 675.3, found 676.3 (M + H$^+$). R_f = 0.60 ($CHCl_3$−MeOH, 7:1).

1-[*N*-(*tert*-Butoxycarbonyl)-D-1,2,3,4-tetrahydroisoquinoline-3-carbonyl-4-chloro-D-phenylalanyl]-4-[*R*,*R*-2-(1,2,4-triazol-1-yl)cyclohexyl]piperazine [(*R*,*R*)-22c] was synthesized from (*R*,*R*)-**21c** as described for the preparation of **22a**. Yield, quantitative (oil). MS calculated for $C_{36}H_{46}ClN_7O_4$: 675.3, found 676.3 (M + H$^+$). R_f = 0.60 ($CHCl_3$−MeOH, 7:1).

1-[*N*-(*tert*-Butoxycarbonyl)-D-1,2,3,4-tetrahydroisoquinoline-3-carbonyl-4-chloro-D-phenylalanyl]-4-[*S*,*S*-2-(1,2,4-triazol-1-yl)cyclohexyl]piperazine [(*S*,*S*)-22c] was synthesized from (*S*,*S*)-**21c** as described for the preparation of **22a**. Yield, quantitative (oil). MS calculated for $C_{36}H_{46}ClN_7O_4$: 675.3, found 676.3 (M + H$^+$). R_f = 0.60 ($CHCl_3$−MeOH, 7:1).

1-[*N*-(*tert*-Butoxycarbonyl)-D-1,2,3,4-tetrahydroisoquinoline-3-carbonyl-4-chloro-D-phenylalanyl]-4-[1-(1,2,4-triazol-1-ylmethyl)cyclopentyl]piperazine (22d) was synthesized from **21d** as described for the preparation of **22a.** Yield 92% (oil). MS calculated for $C_{36}H_{46}ClN_7O_4$: 675.3, found 676.3 (M + H$^+$). R_f = 0.34 ($CHCl_3$−MeOH, 7:1).

1-(D-1,2,3,4-Tetrahydroisoquinoline-3-carbonyl-4-chloro-D-phenylalanyl)-4-[*cis*-2-(1,2,4-triazol-1-ylmethyl)cyclohexyl]piperazine Ditrifluoroacetate (23a). 23a was synthesized from **22a** as described for the preparation of **21a**. The crystalline crude product obtained was purified by HPLC (the eluent contained 8% MeCN, k' = 4.00). Freeze-drying of eluate fractions afforded the pure product as a white powder. Yield 10%. ^{1}H NMR (270 MHz, DMSO-d_6): δ 9.47 (2H, 2m), 9.32 (1H, m), 8.95 (1H, d, J = 7.6 Hz), 8.57 (1H, s), 7.98 (1H, s), 7.40−7.12 (8H, m), 5.00 (1H, m), 4.60−4.03 (5H, m), 3.85−3.16 (8H, m), 3.00−2.65 (4H, m), 1.90−1.05 (10H, m). HRMS (M + H$^+$): 590.3034, $C_{32}H_{41}ClN_7O_2$ requires 590.3010. R_f = 0.17 (*n*-BuOH−AcOH−H_2O, 4:1:1).

1-(D-1,2,3,4-Tetrahydroisoquinoline-3-carbonyl-4-chloro-D-phenylalanyl)-4-[1-(1,2,4-triazol-1-ylmethyl)cyclohexyl]-piperazine Ditrifluoroacetate (23b). 23b was synthesized from **22b** as described for the preparation of **21a**. HPLC eluent contained 9.6% MeCN, k' = 1.87. Yield 18% (white powder). ^{1}H NMR (270 MHz, DMSO-d_6): δ 9.50 (2H, H), 9.38 (1H, m), 8.98 (1H, d, J = 7.6 Hz), 8.48 (1H, br s), 7.95 (1H, br s), 7.45−7.20 (8H, m), 4.99 (1H, m), 4.48−3.20 (13H, m), 3.10−2.75 (4H, m), 1.85−0.98 (10H, m). HRMS (M + H$^+$): 590.2999, $C_{32}H_{41}ClN_7O_2$ requires 590.3010. R_f = 0.37 (*n*-BuOH−AcOH−H_2O, 4:1:1).

1-(D-1,2,3,4-Tetrahydroisoquinoline-3-carbonyl-4-chloro-D-phenylalanyl)-4-[*trans*-2-(1,2,4-triazol-1-yl)cyclohexyl]-piperazine Ditrifluoroacetate (23c). 23c was synthesized from **22c** as described for the preparation of **21a**. The HPLC eluent contained 9.6% MeCN, k' = 0.87. Yield 41% (white powder). ^{1}H NMR (270 MHz, DMSO-d_6): δ 9.48 (2H, m), 9.37 (1H, m), 8.94 (1H, m), 8.47 (1H, s), 7.90 (1H, s), 7.39−7.15 (8H, m), 4.93 (1H, m), 4.44−4.05 (6H, m), 3.30 (2H, m), 3.20−2.70 (8H, m), 2.20−1.60 (6H, m), 1.26 (3H, m). HRMS (M + H$^+$): 576.2830, $C_{31}H_{39}ClN_7O_2$ requires 576.2854. R_f = 0.52 (*n*-BuOH−AcOH−H_2O, 4:1:1).

1-(D-1,2,3,4-Tetrahydroisoquinoline-3-carbonyl-4-chloro-D-phenylalanyl)-4-[*R*,*R*-2-(1,2,4-triazol-1-yl)cyclohexyl]-piperazine Ditrifluoroacetate [(*R*,*R*)-23c]. (*R*,*R*)-**23c** was synthesized from (*R*,*R*)-**22c** as described for the preparation of **21a**. The HPLC eluent contained 9.6% MeCN, k' = 0.87. Yield 67% (white powder). HRMS (M + H$^+$): 576.2834, $C_{31}H_{39}ClN_7O_2$ requires 576.2854. R_f = 0.52 (*n*-BuOH−AcOH−H_2O, 4:1:1).

1-(D-1,2,3,4-Tetrahydroisoquinoline-3-carbonyl-4-chloro-D-phenylalanyl)-4-[*S*,*S*-2-(1,2,4-triazol-1-yl)cyclohexyl]-piperazine Ditrifluoroacetate [(*S*,*S*)-23c]. (*S*,*S*)-**23c** was synthesized from (*S*,*S*)-**22c** as described for the preparation of **21a**. The HPLC eluent contained 9.6% MeCN, k' = 0.87. Yield 43% (white powder). HRMS (M + H$^+$): 576.2838, $C_{31}H_{39}ClN_7O_2$ requires 576.2854. R_f = 0.52 (*n*-BuOH−AcOH−H_2O, 4:1:1).

1-(D-1,2,3,4-Tetrahydroisoquinoline-3-carbonyl-4-chloro-D-phenylalanyl)-4-[1-(1,2,4-triazol-1-ylmethyl)cyclopentyl]piperazine Ditrifluoroacetate (23d). 23d was synthesized from **22d** as described for the preparation of **21a**. The HPLC eluent contained 9.6% MeCN, k' = 1.00. Yield 17% (white powder). ^{1}H NMR (270 MHz, DMSO-d_6): δ 9.42 (2H, m), 9.33 (1H, m), 8.92 (1H, d, J = 7.9), 8.62 (1H, s), 7.97 (1H, s), 7.26 (8H, m), 4.92 (1H, m), 4.42−4.02 (5H, m), 3.70−3.15 (4H, m), 3.00−2.69 (4H, m), 2.30 (4H, m), 2.03−1.47 (8H, m). HRMS (M + H$^+$): 576.2865, $C_{31}H_{39}ClN_7O_2$ requires 576.2854. R_f = 0.41 (*n*-BuOH−AcOH−H_2O, 4:1:1).

Melanocortin Receptor Binding Studies. Cell Cultures. Sf9 cells were grown in 50−100 mL Sf-900 II medium (Gibco-BRL) at 27 °C in small spinner bottles (250 mL) as described.[47] Recombinant viruses for human MC_1, MC_3, MC_4, and MC_5R were added to the cell culture ((2−3) × 10^6 cells/mL), and the incubation continued for an additional 72 h before harvest.

Membrane Preparations. Cells were centrifuged at 800*g* for 5 min and Dounce homogenized (5 times by 10 stokes with 30 s intervals) in ice-cold 50 mL/(5 × 10^8 cells) in 20 mM Na-HEPES, 0.1 mM phenylmethylsulfonyl fluoride, 0.25 mM benzamidine, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL soybean trypsin inhibitor, pH 7.4. The homogenate was centrifuged at 700*g* for 5 min at 4 °C, and the pellet was then homogenized and centrifuged again. The combined supernatants were collected, sedimented at 70000*g* for 60 min at 4 °C, washed once in new buffer, and recentrifuged. The final pellet was resuspended in homogenization buffer at a protein concentration of 1−3 mg of protein/mL, and aliquots were stored at −80 °C. Protein was determined using the Bradford method[46] with bovine serum albumin as a standard.

Radioligand Binding Assays. Assays were performed by incubating membranes (10 μg of protein/100 μL) in 20 mM K-Hepes (pH 7.4), 5 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.5 mg/mL BSA with appropriate concentrations of [^{125}I]-NDP-MSH ([^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH) and competitive nonlabeled ligands. Incubations were for 3 h at 25 °C and were terminated by rapid filtration through 0.3% polyethyleneimine and 1 mg/mL BSA pretreated GF/B glass-fiber filters (Whatman International, Ltd., Madistone, U.K.) using a Brandell cell harvester and three washes of 5 mL of ice-cold 50 mM Tris-HCl, pH 7.4. Nonspecific binding was determined by using 3 μM NDP-MSH ([Nle^4,D-Phe^7]α-MSH). All binding data were analyzed by nonlinear least-squares regression analysis using the commercial program GraphPad PRISM 3.0 (GraphPad Software, San Diego, CA), and the statistical analysis was carried out with the StatView 4.5 package (Abacus Concepts, Berkeley, CA) for Macintosh.

Methods and Conditions for Molecular Modelng. By use of the subprogram MAXIMIN, default parameters were introduced with following exceptions: charges, Gasteiger−Marsili;[50] PBC, minimum. Some other parameters were the following: method, Powell; initial optimization, simplex; termination gradient, 0.05 kcal/mol; max iterations, 2000; force

field, Tripos;[51] NB cutoff, 12.0; dielectric function, constant; dielectric constant, 80.

Acknowledgment. This work was supported by the Swedish VR (Grant 04X-05957) and Estonian SF (Grant 4603).

Supporting Information Available: ^{1}H NMR spectra and assignments of ^{1}H and ^{13}C signals for (*R*,*R*)-**21c**, (*S*,*S*)-**21c**, (*R*,*R*)-**23c**, and (*S*,*S*)-**23c**, chiral HPLC data, experimental methods for X-ray structure analysis, crystal data and structure refinements for THIQ sulfate and (*R*,*R*)-**23c** dinitrate, fractional atomic coordinates and *U*(iso) for THIQ sulfate, and fractional atomic coordinates and *U*(eq) for (*R*,*R*)-**23c** dinitrate. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Haskell-Luevano, C.; Hadley, M. E. The Melanocortin Receptors. *Drug News Perspect.* **1999**, *12*, 197−205.

(2) Wikberg, J. E. S. Melanocortin Receptors: Perspectives for Novel Drugs. *Eur. J. Pharmacol.* **1999**, *375*, 295−310.

(3) Wikberg, J. E. S.; Muceniece, R.; Mandrika, I.; Prusis, P.; Post, C.; Skottner, A. New aspects on the melanocortins and their receptors. *Pharmacol. Res.* **2000**, *42*, 393−420.

(4) Penhoat, A.; Naville, D.; Begeot, M. The Adrenocorticotropic Hormone Receptor. *Curr. Opin. Endocrinol. Diabetes* **2001**, *8*, 112−117.

(5) Kesterson, R. A. The Melanocortin-3 Receptor. In *Melanocortin Receptors*; Cone, R. D., Ed.; Humana Press Inc.: Totowa, NJ, 2000; pp 385−403.

(6) Cone, R. D. The Melanocortin-4 Receptor. In *Melanocortin Receptors*; Cone, R. D., Ed.; Humana Press Inc.: Totowa, NJ, 2000; pp 405−447.

(7) Chen, W. The Melanocortin-5 Receptor. In *Melanocortin Receptors*; Cone, R. D., Ed.; Humana Press Inc.: Totowa, NJ, 2000; pp 449−472.

(8) Wikberg, J. E. S. Melanocortin Receptors: New Opportunities in Drug Discovery. *Expert Opin. Ther. Pat.* **2001**, *11*, 61−76.

(9) MacNeil, D. J.; Howard, A. D.; Guan, X.; Fong, T. M.; Nargund, R. P.; Bednarek, M. A.; Goulet, M. T.; Weinberg, D. H.; Strack, A. M.; Marsh, D. J.; Chen, H. Y.; Shen, C.-P.; Chen, A. S.; Rosenblum, C. I.; MacNeil, T.; Tota, M.; MacIntyre, E. D.; Van der Ploeg, L. H. T. The Role of Melanocortins in Body Weight Regulation: Opportunities for the Treatment of Obesity. *Eur. J. Pharmacol.* **2002**, *440*, 141−157.

(10) Tatro, J. B. Receptor Biology of the Melanocortins, a Family of Neuroimmunomodulatory Peptides. *NeuroImmunoModulation* **1996**, *3*, 259−284.

(11) Wilkison, W. O. Regulation of the Melanocortin Receptors by Agouti. In *Melanocortin Receptors*; Cone, R. D., Ed.; Humana Press Inc.: Totowa, NJ, 2000; pp 475−490.

(12) Szardenings, M.; Muceniece, R.; Mutule, I.; Mutulis, F.; Wikberg, J. E. S. New Highly Specific Agonistic Peptides for Human Melanocortin MC1 Receptor. *Peptides* **2000**, *21*, 239−243.

(13) Schioth, H. B.; Kask, A.; Mutulis, F.; Muceniece, R.; Mutule, I.; Mutule, I.; Mandrika, I.; Wikberg, J. E. S. Novel Selective Melanocortin 4 Receptor Antagonist Induces Food Intake after Peripheral Administration. *Biochem. Biophys. Res. Commun.* **2003**, *301*, 399−405.

(14) Grieco, P.; Lavecchia, A.; Cai, M.; Trivedi, D.; Weinberg, D.; MacNeil, T.; Van der Ploeg, L. H. T.; Hruby, V. J. Structure−Activity Studies of the Melanocortin Peptides: Discovery of Potent and Selective Affinity Antagonists for the hMC3 and hMC4 Receptors. *J. Med. Chem.* **2002**, *45*, 5287−5294.

(15) Bednarek, M. A.; MacNeil, T.; Kalyani, R. N.; Tang, R.; Van der Ploeg, L. H. T.; Weinberg, D. H. Selective, High Affinity Peptide Antagonists of α-Melanotropin Action at Human Melanocortin Receptor 4: Their Synthesis and Biological Evaluation in Vitro. *J. Med. Chem.* **2001**, *44*, 3665−3672.

(16) Haskell-Luevano, C.; Holder, J. R.; Monck, E. K.; Bauzo, R. M. Characterization of Melanocortin NDP-MSH Agonist Peptide Fragments at the Mouse Central and Peripheral Melanocortin Receptors. *J. Med. Chem.* **2001**, *44*, 2247−2252.

(17) Rocchi, R.; Gobbo, M. Peptide Science in the Years to Come**.** *Chim. Oggi* **2002**, *20*, 26−29.

(18) Speake, J. D.; Bishop, M. J. Recent Advances in the Development of Melanocortin-4 Receptor Agonists. *Expert Opin. Ther. Pat.* **2002**, *12*, 1631−1638.

(19) Andersson, P. M.; Boman, A.; Seifert, E.; Skottner, A.; Lundstedt, T. Ligands to the Melanocortin Receptors. *Expert Opin. Ther. Pat.* **2001**, *11*, 1583−1592.

(20) Mutulis, F.; Mutule, I.; Wikberg, J. E. S. *N*-Alkylaminoacids and Their Derivatives Interact with Melanocortin Receptors. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1039−1042.

(21) Mutulis, F.; Mutule, I.; Lapins, M.; Wikberg, J. E. S. Reductive Amination Products Containing Naphthalene and Indole Moieties Bind to Melanocortin Receptors. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1035−1038.

(22) Bakshi, R. K.; Barakat, K. J.; Nargund, R. P.; Palucki, B. L.; Patchett, A. A.; Sebhat, I.; Ye, Z.; Van Der Ploeg, L. H. T. World Patent Application WO 0074679 A1, December 14, 2000; *Chem. Abstr.* **2001**, *134*, 42445.

(23) Martin, W. J.; McGowan, E.; Cashen, D. E.; Gantert, L. T.; Drisko, J. E.; Hom, G. J.; Nargund, R.; Sebhat, I.; Howard, A. D.; Van der Ploeg, L. H. T.; MacIntyre, D. E. Activation of Melanocortin MC4 Receptors Increases Erectile Activity in Rats Ex Copula. *Eur. J. Pharmacol.* **2002**, *454*, 71−79.

(24) Van der Ploeg, L. H. T.; Martin, W. J.; Howard, A. D.; Nargund, R. P.; Austin, C. P.; Guan, X.; Drisko, J.; Cashen, D.; Sebhat, I.; Patchett, A. A.; Figueroa, D. J.; DiLella, A. G.; Connolly, B. M.; Weinberg, D. H.; Tan, C. P.; Palyha, O. C.; Pong, S.-S.; MacNeil, T.; Rosenblum, C.; Vongs, A.; Tang, R.; Yu, H.; Sailer, A. W.; Fong, T. M.; Huang, C.; Tota, M. R.; Chang, R. S.; Stearns, R.; Tamvakopoulos, C.; Christ, G.; Drazen, D. L.; Spar, B. D.; Nelson, R. J.; MacIntyre, D. E. A Role for the Melanocortin 4 Receptor in Sexual Function. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11381−11386.

(25) Sebhat, I. K.; Martin, W. J.; Ye, Z.; Barakat, K.; Mosley, R. T.; Johnston, D. B. R.; Bakshi, R.; Palucki, B.; Weinberg, D. H.; MacNeil, T.; Kalyani, R. N.; Tang, R.; Stearns, R. A.; Miller, R. R.; Tamvakopoulos, C.; Strack, A. M.; McGowan, E.; Cashen, D. E.; Drisko, J. E.; Hom, G. J.; Howard, A. D.; MacIntyre, D. E.; van der Ploeg, L. H. T.; Patchett, A. A.; Nargund, R. P. Design and Pharmacology of *N*-[(3*R*)-1,2,3,4-Tetrahydroisoquinolinium-3-ylcarbonyl]-(1*R*)-1-(4-chlorobenzyl)-2-[4-cyclohexyl-4-(1*H*-1,2,4-triazol-1-ylmethyl)piperidin-1-yl]-2-oxoethylamine (1), a Potent, Selective, Melanocortin Subtype-4 Receptor Agonist. *J. Med. Chem.* **2002**, *45*, 4589−4593.

(26) Mutulis, F.; Yahorava, S.; Mutule, I.; Yahorau, A.; Kopanchuk, S.; Veiksina, S.; Rinken, A.; Wikberg, J. E. S. A Non-Peptide Radioiodinated High Affinity Melanocortin-4 Receptor Ligand. *J. Labelled Compd. Radiopharm.* **2003**, *46*, 1007−1017.

(27) Schwarz, H.; Arakawa, K. The Use of *p*-Nitrobenzyl Esters in Peptide Synthesis. *J. Am. Chem. Soc.* **1959**, *81*, 5691−5695.

(28) Albericio, F.; Bofill, J. M.; El-Faham, A.; Kates, S. A. Use of Onium Salt-Based Coupling Reagents in Peptide Synthesis. *J. Org. Chem.* **1998**, *63*, 9678−9683.

(29) Pruesse, W.; Amschler, H.; Schoetensack, W. German Patent 2638184, March 10, 1977; *Chem. Abstr.* **1977**, *87*, 6022.

(30) Szarvasi, E.; Festal, D.; Grand, M.; Depin, J. C.; Chabert, J. Spiro[piperidine-4,6′-thiazolo[3,2-*a*]pyrimidines]. Thymoanaleptics and Blood Platelet Aggregation Inhibitors. *Eur. J. Med. Chem.* **1976**, *11*, 115−24.

(31) Hsiao, Y.; Hegedus, L. S. Synthesis of Optically Active Imidazolines, Azapenams, Dioxocyclams, and Bis-dioxocyclams. *J. Org. Chem.* **1997**, *62*, 3586−3591.

(32) Veeresa, G.; Datta, A. Stereoselective Synthesis of the Antifungal Antibiotic (+)-Preussin. *Tetrahedron* **1998**, *54*, 15673−15678.

(33) Pozdnev, V. F. Acylation of Amino Acids by *tert*-Alkyl Pyrocarbonates. *Khim. Prir. Soedin.* **1971**, *7*, 384−385; *Chem. Abstr.* **1971**, *75*, 110568.

(34) Natsuka, K.; Nakamura, H.; Uno, H.; Umemoto, S. 1-Substituted 4-(1,2-diphenylethyl)piperazine Derivatives and Their Analgesic Activities. *J. Med. Chem.* **1975**, *18*, 1240−1244.

(35) Natsuka, K.; Nakamura, H.; Nishikawa, Y.; Negoro, T.; Uno, H.; Nishimura, H. Synthesis and Structure−Activity Relationships of 1-Substituted 4-(1,2-diphenylethyl)piperazine Derivatives Having Narcotic Agonist and Antagonist Activity. *J. Med. Chem.* **1987**, *30*, 1779−1787.

(36) Kaulen, J. The Enantiomers of the Azole Fungicide HWG-1608. Asymmetric Synthesis of a 2-Hydroxyethylazole Fungicide. *Angew. Chem.* **1989**, *101*, 507−508.

(37) Gasteiger, J.; Kaufmann, K. Diastereospecific Synthesis of Fungicidal Threo- and Erythro-α-hydroxy Aminals. *Tetrahedron Lett.* **1985**, *26*, 4341−4344.

(38) Sekar, G.; Nishiyama, H. Nonenzymatic kinetic resolution of *β*-amino alcohols: chiral BINAP mediated SN2 displacement of hydroxy groups by halogens through formation of an aziridinium ion intermediate. *Chem. Commun.* **2001**, 1314−1315.

(39) Diouf, O.; Depreux, P.; Chavatte, P.; Poupaert, J. H. Synthesis and Preliminary Pharmacological Results on New Naphthalene Derivatives as 5-HT4 Receptor Ligands. *Eur. J. Med. Chem.* **2000**, *35*, 699−706.

(40) Otting, G.; Wuethrich, K. Pre-TOCSY, a New Experiment for Obtaining Complete Two-Dimensional Proton NMR Spectra of Proteins in Aqueous Solution. *J. Magn. Reson.***1987**, *75*, 546−549.

(41) Bax, A.; Davis, D. G. Practical Aspects of Two-Dimensional Transverse NOE Spectroscopy. *J. Magn. Reson.* **1985**, *63*, 207−213.

(42) Bodenhausen, G.; Ruben, D. J. Natural Abundance Nitrogen-15 NMR by Enhanced Heteronuclear Spectroscopy. *Chem. Phys. Lett.* **1980**, *69*, 185−189.
(43) Kessler, H.; Mronga, S.; Mueller, G.; Moroder, L.; Huber, R. Conformational Analysis of a IgG1 Hinge Peptide Derivative in Solution Determined by NMR Spectroscopy and Refined by Restrained Molecular Dynamics Simulations. *Biopolymers* **1991**, *31*, 1189−1204.
(44) Brownstein, S.; Miller, R. Conformational Analysis of Disubstituted Cyclohexanes by Proton Resonance Spectroscopy. *J. Org. Chem.* **1959**, *24*, 1886−1887.
(45) Armstrong, D. W.; Tang, Y.; Chen, S.; Zhou, Y.; Bagwill, C.; Chen, J. R. Macrocyclic Antibiotics as a New Class of Chiral Selectors for Liquid Chromatography. *Anal. Chem.* **1994**, *66*, 1473−1484.
(46) Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein−Dye Binding. *Anal. Biochem.* **1976**, *72*, 248−354.
(47) O'Reilly, D. R.; Miller, L. K.; Luckow, V. A. *Baculovirus Expression Vectors: A Laboratory Manual*; Freeman: New York, 1992.
(48) *SYBYL Molecular Modeling Package*, version 6.5; Tripos: St. Louis, MO, 1998.
(49) Backer, R. T.; Briner, K.; Collado, C. I.; De Frutos-Garica, O.; Doecke, C. W.; Fisher, M. J.; Garcia-Paredes, C.; Kuklish, S. L.; Mancoso, V.; Martinelli, M. J.; Mateo, H. A. I.; Mullaney, J. T.; Ornstein, P. L.; Wu, Z.; Xie, C. World Patent Application WO 0259095 A1, August 1, 2002; *Chem. Abstr.* **2002**, *137*, 140775.
(50) Gasteiger, J.; Marsili, M. Iterative Partial Equalization of Orbital Electronegativity: A Rapid Access to Atomic Charges. *Tetrahedron* **1980**, *36*, 3219−3222.
(51) Clark, M.; Cramer, R. D., III.; Van Opdenbosch, N. Validation of the General Purpose Tripos 5.2 Force Field. *J. Comput. Chem.* **1989**, *10*, 982−1012.

JM0311285