The Use of Topographical Constraints In Receptor Mapping: Investigation of the Topographical Requirements of the Tryptophan 30 Residue for Receptor Binding of Asp-Tyr-D-Phe-Gly-Trp-(*N*-Me)Nle-Asp-Phe-NH_2 (SNF 9007), a Cholecystokinin (26−33) Analogue That Binds to both CCK-B and δ-Opioid Receptors†

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The cholecystokinin (26−33) [CCK (26−33)] octapeptide analog Asp-Tyr-D-Phe-Gly-Trp(*N*-Me)-Nle-Asp-Phe-NH_2 (SNF 9007) is a potent and selective ligand for both the CCK-B and δ-opioid receptors. Pharmacological studies of SNF 9007 suggest a relationship between the ligand requirements of CCK-B and δ-opioid receptors, which further implies a possible structural relationship between these receptors. We have utilized topographical constrainment of the important Trp^{30} residue to investigate structural features of SNF 9007 that would distinguish between binding requirements in this region for the CCK-B and δ-opioid receptors. Thus, the four optically pure isomers of β-MeTrp were substituted for L-Trp^{30} of SNF 9007. Receptor binding results suggest that the preferred topography of the Trp^{30} residue for CCK-B receptor binding may be the 2*S*,3*S* (*erythro*-L) configuration whereas for the δ-opioid receptor it may be the 2*S*,3*R* (*threo*-L) configuration. Molecular modeling studies of these ligands further support the recently revised receptor-bound model for CCK-B octapeptide ligands (Kolodziej et al. *J. Med. Chem.* **1995**, *38*, 137−149) and are in good agreement with the DPDPE-δ opioid receptor "template" model (Nikiforovich et al. *Biopolymers* **1991**, *31*, 941−955).

Introduction

Cholecystokinin 8 (CCK 8) is a linear octapeptide found in the periphery as a hormone and in the central nervous system as a neurotransmitter/neuromodulator.[1,2] The main subtypes of receptors for CCK are CCK-A, found predominantly in the peripheral tissues, and CCK-B, localized in the central nervous system.[3,4] Previous efforts in our laboratory have led to the design of highly potent and selective ligands for these receptor subtypes.[5,6] Of these ligands, one octapeptide, Asp-Tyr-D-Phe-Gly-Trp-(*N*-Me)Nle-Asp-Phe-NH_2 (SNF 9007, **1**), was found to be unique. It is a highly potent and extremely selective peptide for the CCK-B receptor.[7] Its uniqueness arises from the fact that it is also a potent ligand for the δ-opioid receptor.[8] It also elicits an analgesic response *in vivo*, a property associated with the opioid receptors.

Pharmacological results of SNF 9007 (**1**) suggest a relationship between the ligand requirements of CCK-B and δ-opioid receptors, which further implies a possible structural relationship between these receptors.[8,9] In a case of a peptidic ligand like **1** which interacts with two types of vastly different biological receptors such as the CCK-B and the δ-opioid receptors, the topography[10] of the side chain groups can be expected to play an important role in distinguishing between the binding sites of different receptor types.[8,9] Previous reports indicate that the tryptophan residue of CCK is a critical pharmacophore for CCK receptor recognition and binding[11−13] with very limited tolerance for modification of the residue. We describe herein an investigation of the topographical surfaces of the tryptophan side chain in SNF 9007 (**1**) that may be involved in receptor recognition and potent binding and which help distinguish binding requirements for the CCK-B and δ-opioid receptors.

Results

The four isomers of *N*-indolyl(2-mesitylenesulfonyl)-β-methyltryptophan were synthesized in optically pure form as described previously[14] for preparing the β-Me-Trp^{30}-containing analogs of SNF 9007 (**3**−**6**; Table 1). They were synthesized using an N^{α}-*t*-Boc protection strategy on *p*-methylbenzhydrylamine (MBHA) resin. Cleavage of the assembled peptides was achieved using a modified HF cleavage procedure in order to deprotect the *N*-indolyl(2-mesitylenesulfonyl) group simultaneously.[15] All peptides were purified to homogeneity (>98%) by semipreparative reversed-phase HPLC as described in the Experimental Section.

Binding affinities to CCK-A receptors were measured with guinea pig pancreatic membranes, and binding affinities to CCK-B receptors were determined with membranes prepared from the cortex of guinea pigs as described previously.[8] Opioid receptor binding affinities

† Abbreviations: CCK, cholecystokinin; (NMe)Nle, *N*-methylnorleucine; DPDPE, cyclo[D-Pen^2,D-Pen^5]enkephalin; FAB-MS, fast atom bombardment mass spectrometry; β-MeTrp, β-methyltryptophan; Pen, penicillamine; DMF, dimethylformamide; DIEA, diisopropylethylamine; BOP reagent, (benzotriazol-1-yloxy)tris(diethylamino)phosphonium hexafluorophosphate; Boc, *tert*-butyloxycarbonyl; HF, hydrofluoric acid.

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Table 1. Binding Data for Peptides **1**−**6** for CCK-A and -B Receptors and μ- and δ-Opioid Receptors[a]

		IC_{50} (nM)			
		CCK receptors		opioid receptors	
compd	structure	CCK-A	CCK-B	μ	δ
1	H-Asp-Tyr-D-Phe-Gly-**Trp**-N-MeNle-Asp-Phe-NH_2 (SNF 9007)	>12000	0.79 ± 0.48	700 ± 140	29 ± 10
2	H-Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH (DPDPE)	inactive[b]	inactive[b]	7300 ± 1700	4.1 ± 0.46
3	H-Asp-Tyr-D-Phe-Gly-(**2*S*,3*S***)-β-MeTrp-*N*-MeNle-Asp-Phe-NH_2	>12000	88 ± 22	6800 ± 520	140 ± 62
4	H-Asp-Tyr-D-Phe-Gly-(**2*R*,3*R***)-β-MeTrp-*N*-MeNle-Asp-Phe-NH_2	3700 ± 1100	1540 ± 510	>30000	820 ± 180
5	H-Asp-Tyr-D-Phe-Gly-(**2*R*,3*S***)-β-MeTrp-*N*-MeNle-Asp-Phe-NH_2	8600 ± 1240	6500 ± 1600	>30000	1050 ± 210
6	H-Asp-Tyr-D-Phe-Gly-(**2*S*,3*R***)-β-MeTrp-*N*-MeNle-Asp-Phe-NH_2	9000 ± 1000	1900 ± 710	3050 ± 1500	65 ± 12

[a] Affinities are described for μ- and δ-opioid receptors and CCK-B receptors in guinea pig brain and for CCK-A type of receptors in guinea pig pancreas as described in the Experimental Section. The results are expressed with their SEM to the nearest whole number obtained from the average of at least three experiments done in duplicate. [b] DPDPE (**2**) does not show any appreciable binding to CCK receptors at high concentrations.

were measured using guinea pig whole brain membranes using [^{3}H]CTOP, [^{3}H][4′-Cl-Phe^4]DPDPE, and [^{3}H] U-69,593 for labeling of μ-, δ-, and κ-opioid receptors, respectively. The results are summarized in Table 1.

It is known that central and peripheral administration of cholecystokinin (26−33) (CCK 8) can cause analgesia.[16] As mentioned earlier, the highly potent CCK-B ligand SNF 9007 (**1**, Table 1) binds to δ-opioid receptors and can produce analgesia.[8] Evidence suggesting that CCK-B and δ-opioid receptors may have overlapping topographical structural requirements for their agonist ligands was presented recently,[9] and the Trp^{30} residue appears to play a critical role.

Since we have previously shown that topographic constraints alone can greatly affect receptor potency and selectivity (e.g. refs 17−20) like in the case of the four diastereoisomers of cyclo[D-Pen^2,β-Me-*p*-NO_2Phe^4,D-Pen^5]enkephalin,[17] the use of the four β-MeTrp isomers was chosen for further examination. As can be seen in Table 1, SNF 9007 (**1**) and its β-MeTrp-substituted analogs (**3**−**6**) generally do not interact well with CCK-A and μ-opioid receptors, although several binding interactions occur with IC_{50} values in the 3−9 μM range. (κ-Opioid receptor binding potencies for all compounds were weaker than 15 000 nM and are not reported). The (2*R*,3*R*)-β-MeTrp- (**4**), and (2*R*,3*S*)-β-MeTrp-containing (**5**) analogs which have the D-configuration at the α-carbon of β-MeTrp also show greatly reduced binding potencies for the CCK-B receptor when compared to the parent peptide, **1**. This was not surprising, since substitution of L-Trp with a D-Trp residue has been shown to disrupt the binding of peptide ligands to CCK receptors, presumably by altering the bioactive conformation of the backbone.[13] Compound **4** and **5** also do not bind well to CCK-B and δ-opioid receptors. Compounds **3** and **6** which contain the L-isomers of β-MeTrp also showed reduced binding potencies for the CCK-B receptor when compared to **1**, though the (2*S*,3*S*)-β-MePhe^{30}-containing analog **3** has an IC_{50} of 88 nM at the CCK-B receptor. However compounds **3** and **6** do interact quite well with the δ opioid receptor relative to **1** (Table 1).

Since the absence of interaction with specific receptors might be caused by conformational features of these analogs, and by other reasons as well, for the modeling work described in this paper, we have selected only the compounds with high to moderate affinity toward CCK-B and/or δ-opioid receptors, i.e. compounds **1**, **3**, and **6**. For each of these compounds, the buildup procedures have found the sets of low-energy three-dimensional structures differing in backbone conformations, or in backbone conformations and rotamers of Tyr and Trp residues, namely, 178 structures for **1** (SNF 9007), 211 for the (*S*,*S*)-β-MeTrp-containing analog **3**, and 45 for the (*S*,*R*)-β-MeTrp-containing analog **6** ($E_i - E_{min} \leq 10$ kcal/mol) (see the Experimental Section and Supporting Information).

Discussion

SNF 9007 (**1**), and one of the best known δ-selective opioid peptides, DPDPE (**2**), have certain resemblances in amino acid sequences. Namely, both peptides contain two aromatic residues (Tyr and Trp/Phe) separated by a D-Xxx-Gly fragment, where Xxx = Phe for SNF 9007 and Xxx = DPen for DPDPE. Since SNF 9007 (**1**), compound **3**, and compound **6** possess high to moderate specific binding to the δ-opioid receptor, it is reasonable to suggest for them some resemblance to the δ-opioid receptor bound DPDPE conformer as well. The model of the DPDPE receptor-bound conformer proposed by us earlier[21] was strongly supported by synthesis and biological testing of conformationally constrained analogs of opioid peptides.[22,23] This model suggests the placement of the Phe side chain in a definite position in space corresponding to the *gauche* (−) rotamer. We have used this model as a "template" for geometrical comparison with low-energy conformers of the SNF 9007 related compounds, which were found by energy calculations. The DPDPE model[21] was assumed to adapt a certain conformation for the peptide backbone and a certain χ_1 rotamer for the Phe side chain, whereas possible χ_1 rotamers of the Tyr side chain were still uncertain.[21] Accordingly, geometrical comparison of the two fragments, i.e. the Tyr-D-Pen-Gly-Phe in DPDPE (**2**) and the Tyr-D-Phe-Gly-Trp in SNF 9007 (**1**) was performed by overlapping all $C^{\beta,\gamma}$-atoms for Tyr and Phe/Trp residues as well as the C^{α}-atoms of the D-Pen/D-Phe residues.

Geometrical comparison showed that there are several low-energy conformers similar to the DPDPE template at the level of rms ≤ 1 Å for the three compounds **1**, **3**, and **6**. The levels of similarity are depicted in Figure 1. The values of dihedral angles, which influence the spatial arrangement of the aromatic residues in Figure 1, are listed in the upper part of Table 2 (only one example for each compound, that displaying the lowest rms value, is listed). These values fluctuate within the sets of similar conformers for each particular peptide, but not more than by a few degrees. Interestingly, the rotamers of χ_1 angle for the Trp residues are not those of the g(−) type in all cases. However, the

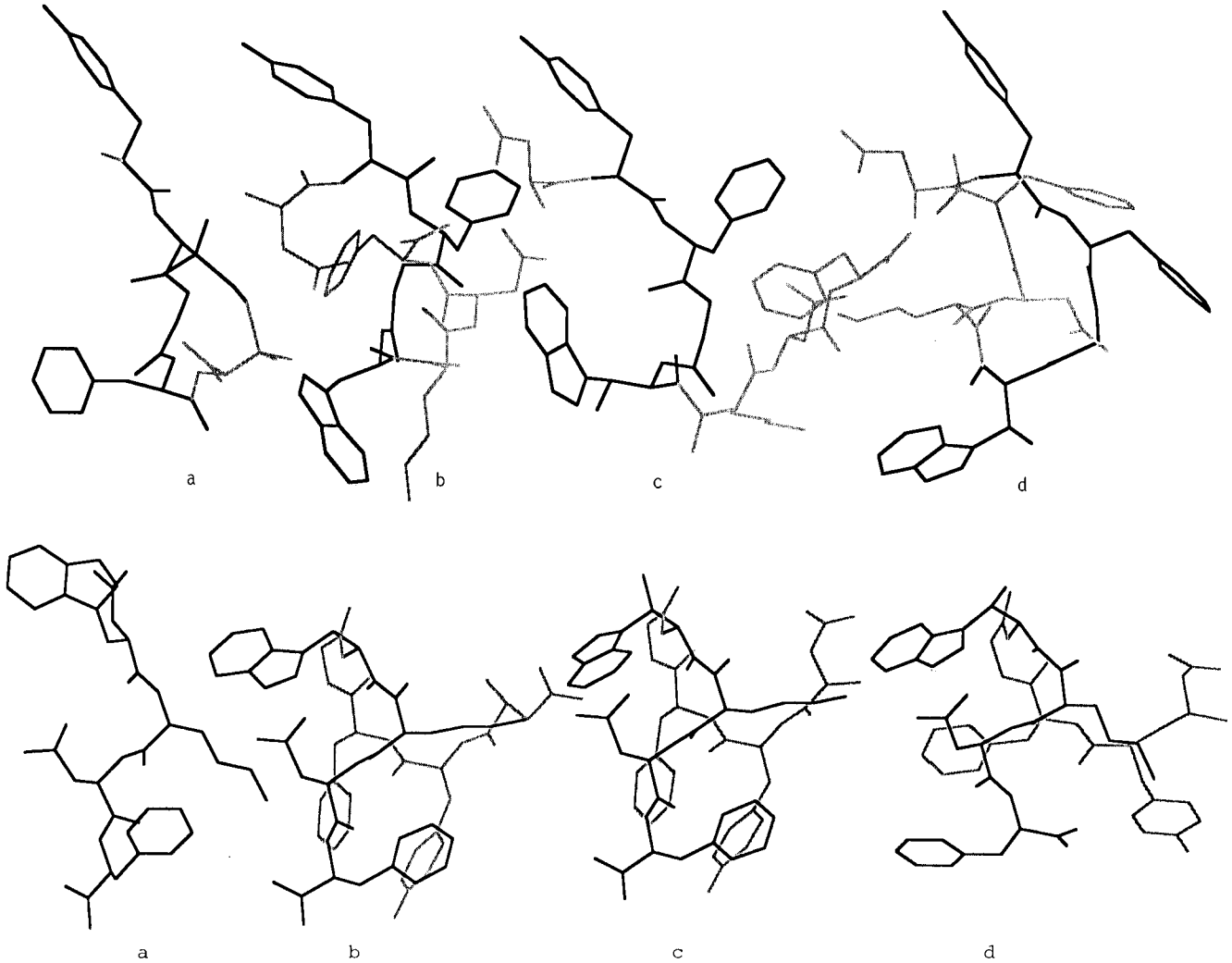

Figure 1. (Top) The DPDPE template conformer (a) and compatible low-energy conformers of **1** (b), **3** (c), and **6** (d). All hydrogens are omitted. The compared tetrapeptides are shown in bold. (Bottom) The CCK-B template conformer (a) and compatible low-energy conformers of **1** (b), **3** (c), and **6** (d). All hydrogens are omitted. The compared tetrapeptides are shown in bold.

Table 2. Low-Energy Conformers of SNF 9007 (**1**) and Its Analogs **3** and **6** Compatible to the DPDPE Template Conformers[21] and the CCK-B Template[26]

residue	Tyr	D-Phe/D-Pen		Gly		Trp/Phe	
angle	ψ	ϕ	ψ	ϕ	ψ	ϕ	χ_1
SNF 9007 (**1**)	49	124	−8	−69	−28	−144	−67
compound **3**	137	83	−169	77	−66	−69	−48
compound **6**	137	134	−148	−94	−49	−137	174
DPDPE (**2**)	142	81	−145	66	28	−157	−74

residue	Trp	N-MeNle			Asp		Phe
angle	ψ	ω	ϕ	ψ	ϕ	ψ	ϕ
SNF 9007 (**1**)	52	−164	47	81	−98	135	−134
compound **3**	52	−164	45	81	−93	134	−138
compound **6**	45	−148	33	78	−66	−43	−141
Ac-CCK-4	169	180	−83	93	−71	149	−118

overall spatial arrangements of these side chains are topologically similar to the Phe side chain in our DPDPE template (see Figure 1).

Results of the performed geometrical comparison are in agreement with the obtained binding data, thus further supporting the DPDPE template model proposed by us earlier.[21] Moreover, inspection of the structures depicted in Figure 1 suggest that an even better resemblance of SNF 9007 (**1**) and its conformers to the DPDPE template would be achieved by elimination of the N-terminal Asp residue and establishing a covalent link between positions occupied by the Tyr and *N*-MeNle residues. Following this line of reasoning, the Tyr-cyclo-[D-Pen-Gly-Trp-3-*trans*-mercaptoproline]-Asp-Phe-NH_2 analog was designed and tested, showing low nanomolar affinity and high selectivity toward δ-opioid receptors.[24] This analog, however, did not interact with both CCK-A and CCK-B receptors.[24]

The template model for CCK-B receptor bound conformer is not currently developed to such extent as the δ-opioid receptor bound template. The model, proposed by us earlier,[25] very recently was refined according to

new data obtained with tetrapeptides, containing alkyl substituents of various mercaptoprolines in position 31.[26] The model suggests a certain backbone conformation of the C-terminal tetrapeptide of CCK (CCK-4); accordingly, geometrical comparison in this case was performed for spatial arrangement of all C^{α}- and C^{β}-atoms in the C-terminal tetrapeptides of SNF 9007 (**1**) and the (*S*,*S*)-*β*-MeTrp- and (*S*,*R*)-*β*-Me-Trp-containing analogs **3** and **6**, respectively.

Only two compounds, SNF 9007 (**1**) and **3** possess several low-energy conformers compatible to the CCK-B template. The examples of these conformers are depicted in Figure 1 and are described in the lower part of Table 2. It is noteworthy that these conformers are not the same as those compatible with the DPDPE template. [(*S*,*R*)-Trp]-SNF 9007 has no low-energy conformers similar to the CCK-B template at the level of rms ≤ Å; the conformer with a minimal rms value is also depicted in Figure 1. It is clearly seen that in the case of this compound the spatial orientation of the crucial Phe side chain is different from that in the template (see also Table 2). Taken together with the drastical loss of affinity of **6** toward CCK-B receptors, this finding could be interpreted as indirect evidence in favor of the CCK-B receptor bound model suggested in ref 26. However, as we have pointed out above, the reasons for the loss of binding might be not only in conformational differences between 6 and the CCK-B template. Again it is appropriate to mention that the potency of CCK related peptides is extremely sensitive to chemical modifications in the Trp residue as discussed in the Introduction.

Experimental Section

TLC was performed on silica gel 60 F254 (Merck) with detection of the peptides by ninhydrin spray. Optical rotations were measured on a Perkin-Elmer 141 polarimeter. Mass spectra were obtained at the University of Arizona Microanalysis Center using a Finnegan fast atom bombardment mass spectrometer. Amino acid analysis was performed on an Applied Biosystems Model 420A instrument with automatic hydrolysis (vapor phase at 160 °C for 1 h 40 min in 6 N HCl) and precolumn phenylthiocarbamyl amino acid (PTC-AA) analysis. No corrections were made for amino acid decomposition.

The optically pure analogs of *N*-indolyl(2-mesitylenesulfonyl)-*β*-methyltryptophan were synthesized as described previously.[14] Peptides were synthesized using the N^{α}-Boc/benzyl strategy on *p*-methylbenzhydrylamine resin (approximate substitution of 0.5 mequiv/g). The protected amino acids N^{α}-Boc-PheOH, N^{α}-Boc-Asp(O-Bzl)OH, N^{α}-Boc-Trp(N^{in})OH, N^{α}-Boc-GlyOH, N^{α}-Boc-Tyr(O-Bzl)OH, N^{α}-Boc-D-PheOH, and N^{α}-Boc-NleOH were obtained from Bachem (Torrance, CA). N^{α}-Boc-NleOH was N-methylated according to the procedure of Cheung and Benoiton.[27] Peptides were assembled on the solid-phase resin using N^{α}-Boc amino acid (4 equiv), BOP reagent[28] (4.2 equiv), and diisopropylethylamine (DIEA) (7.8 equiv) in dimethylformamide (DMF). Coupling of the *β*-methyltryptophans to the *N*-MeNle residues was monitored with 2% bromophenol blue in toluene. Trifluoroacetic acid (50% in dichloromethane) was used to deprotect the N^{α}-Boc group of the amino acids followed by neutralization with 10% DIEA in DMF prior to coupling the next amino acid. Cleavage of the peptides with complete deprotection of the side chain protecting groups was achieved with a modified HF cleavage procedure.[15] This modified HF procedure was necessary to completely remove the *N*-indolyl(mesitylenesulfonyl) protecting groups of the *β*-MeTrp residues. Crude peptides were purified by semipreparative HPLC with detection at 280 nm using a reversed phase C_{18}-bonded silica column (Vydac, 218TP1010, 1.9 × 25 cm). The analytical data for **3**−**6** are listed in the Supporting Information.

Radioligand binding assays were conducted as previously described.[5,7,8] Compounds **1**−**6** were tested against [^{125}I]BH-CCK_8 (for CCK-A binding), [^{3}H] SNF 8702 (for CCK-B binding), [^{3}H]CTOP (*μ*-opioid receptor binding), [^{3}H][4′-Cl-Phe^4]DPDPE (*δ*-opioid receptor binding), and [^{3}H]U-69,593 (*κ*-opioid receptor binding).[8]

Conformational Search and Energy Calculations. Conformational search and energy calculations were conducted as described previously.[21] The ECEPP potential field[29,30] was used for conformational energy calculations assuming rigid valence geometry with planar *trans* peptide bonds (both *trans* and *cis* peptide bonds were examined for the NMeNle residue; in this case, the *ω* angle also was allowed to rotate). The valence geometries of (2*S*,3*S*)- and (2*S*,3*R*)-*β*-MeTrp were derived by an analogy with those for (2*S*,3*S*) and (2*S*,3*R*)-*β*-MePhe residues.[31] Aliphatic and aromatic hydrogens were generally included in united atomic centers of CH_n type; H^{α} atoms, amide hydrogens, and aromatic hydrogens in Trp and (2*S*,3*S*)- and (2*S*,3*R*)-*β*-MeTrp residues were described explicitly. The buildup calculation scheme was the same for all compounds in question, i.e. from the D-Phe-Gly-Xxx-*N*-MeNle fragments to Tyr-D-Phe-Gly-Xxx-*N*-MeNle-Asp-Phe-NH_2 to the entire molecules (Xxx = Trp, (2*S*,3*S*)-*β*-MeTrp, or (2*S*,3*R*)-*β*-MeTrp). At the first step of the calculations, all possible combinations of local energy minima for the peptide backbone of each amino acid residue were considered. According to the notation method described previously[32] there were minima of *E*, *F*, *C*, *A*, and *A** for each residue of the L-configuration, minima of *E**, *F**, *C**, *A**, and *A* types for the D-Phe residue, and of *E*, *F*, *C*, *A* and *E**, *F**, *C**, and *A** types for the Gly residue. Minima of *E*, *C*, and *A* types were considered for the N-terminal Asp residue, and the minima of *E*, *C*, and *A** types were considered for the C-terminal Phe-NH_2 residue. Two filters were used to eliminate conformers from further consideration. First, only the backbone structures selected at the previous step by $E - E_{min} < \Delta E = 10$ kcal/mol were considered at subsequent steps. Second, from the set of low-energy structures obtained at the previous step, only those differing by more than 60° in at least one value of any backbone dihedral angle were selected for the next step. The dihedral angle values of side chain groups and of the terminal groups of the backbone were optimized at every step before energy minimization to achieve their most favorable spatial arrangements employing the algorithm described earlier.[21] However, at the last step of calculations, three possible rotamers of the χ_1 dehedral angle (*g*+, *t*, and *g*−) were separately considered for Tyr, Trp, (2*S*,3*S*)-*β*-MeTrp, and (2*S*,3*R*)-*β*-MeTrp residues.

Geometric comparison of low-energy conformers, which belong to **1** and its analogs **3** and **6**, and the "template" models of bioactive conformers for *δ*-opioid and CCK-B receptors included an assessment of the best fit of the spatial arrangement for the atomic centers chosen to represent a fragment bearing the pharmacophoric groups presumably interacting with a particular receptor type. The conformers were regarded as geometrically consistent with the templates when the corresponding rms values were less than 1.0 Å.

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Supporting Information Available: Three figures showing (1) interaction of the indole ring with the amide backbone with different χ_1 values, (2) structures of the four isomers of *β*-methyltryptophan, and (3) Newman projections of about χ_1 of the (2*S*,3*R*)- and (2*S*,3*S*)-*β*-methyltryptophan, and three tables of (1) buildup procedures for determining low-energy conformers of **1**, **3**, and **6**, (2) amino acid and MS analyses of **3**, **4**, **5**, and **6**, and (3) HPLC, TLC, and $[\alpha]_D$ for **3**, **4**, **5,** and **6** (7 pages). Ordering information is given on any current masthead page.

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