Synthesis, Biological Evaluation, and Quantitative Receptor Docking Simulations of 2-[(Acylamino)ethyl]-1,4-benzodiazepines as Novel Tifluadom-like Ligands with High Affinity and Selectivity for κ-Opioid Receptors[1]

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The synthesis and biological evaluation of a series of 2-substitued 5-phenyl-1,4-benzodiazepines, structurally related to tifluadom (**5**), the only benzodiazepine that acts simultaneously as a κ-opioid agonist and a cholecystokinin-A (CCK-A) antagonist, are reported. The radioligand binding models used in these studies were [^{125}I](BH)-CCK-8 in rat pancreas (CCK-A), [^{3}H]-(MeNLE^{28,31})-CCK-8 in guinea pig cerebral cortex (CCK-B), and [^{3}H]U-69593 (κ_1), [^{3}H]DAMGO (μ), and [^{3}H]DADLE (δ) in guinea pig brain. All the title compounds were devoid of significant affinity for both CCK-A and CCK-B receptors, while some of them bound with nanomolar affinity and high selectivity for κ-opioid receptors. In particular, the 2-thienyl derivative **7a** (X = H) with a K_i = 0.50 nM represents a clear improvement with respect to tifluadom, showing a comparable potency but higher selectivity. The application of computational simulations and linear regression analysis techniques to the complexes between guinea pig κ (κ_1)-receptor and the title compounds allowed the identification of the structural determinants for recognition and quantitative elucidation of the structure−affinity relationships in this class of receptors.

Introduction

For more than a decade, the search has continued for a highly selective κ-opioid agonist with clinical potential as a safe and effective analgesic.[2,3] Most of the κ-agonists synthesized during this period were derived from the prototype selective κ-agonist U-50,488 (**1**),[4] which is an arylacetamide compound. Successive modifications of this structure led to U-62,066 (spiradoline) (**2a**),[5] CI 977 (enadoline) (**2c**),[6] GR 89696 (**3**),[7] and BRL 52656 (**4**).[8] All these compounds are selective for the κ-opioid receptor and are potent in a range of antinociceptive models in animals;[9] where tested, these compounds have also produced analgesia in man.[10]

Administration of these compounds, however, is also associated with the appearance of a spectrum of side effects including diuresis, sedation/locomotor incoordination, and an array of central nervous system (CNS) effects.[11,12] The side-effect profile of the above compounds is almost certainly related to their selective interaction with κ-receptors. All feature, however, the same pharmacophore sequence [N-C-C-N (sp^2)][13] which may be important in this respect, since it has been hypothesized that, even at a single receptor, the chemical structure of an agonist may determine which second-messenger response pathways are activated and hence which biological response is apparent (see review by Kenakin).[14] Another possibility is that the diverse biological activities of existing κ-opioid agonists represent the functional correlates of activities at the various

subtypes of the κ-receptor (κ_1, κ_2, and κ_3) described in the literature.[15] However, of these receptor subtypes, only one, with the characteristics of a κ_1-receptor [specific ligand, the arylacetamide U-69,593 (**2b**)[16]], has been isolated, cloned, and expressed from a variety of species[17−19] including man[20] and, like the other opioid receptors belongs to the G-protein coupled receptors (GPCRs) superfamily, appears to be a classical seven-transmembrane receptor.[21]

Tifluadom (**5**)[22] is a 2-[(acylamino)methyl]-1,4-benzodiazepine with negligible effects upon classical central benzodiazepine receptors,[23] which also shows high affinity for the κ-opioid receptor[24] and causes antinociception in several animal models.[25] Additional activities noted with tifluadom include locomotor disturbances,[26] diuresis,[27] anti-inflammatory activity,[28] and effects on food consumption:[29] all of these effects are thought to be associated with activity at κ-opioid receptor. Tifluadom, however, also has significant affinity for the μ- and δ-opioid receptors,[30] and activities at these sites may serve to modulate the κ-related effects *in vivo*.[31] Furthermore, it has been shown that tifluadom is also a cholecystokinin (CCK) antagonist,[32] and several studies have indicated that agonists and antagonists at the CCK receptors can interact with the actions of opioids.[33]

There are several reports in the literature of medicinal chemistry effort to separate the various opioid and CCK components in tifluadom analogues.[34] Bock et al.[35] and Meurisse et al.[36] prepared a series of tifluadom derivatives to try to clarify the relationship between affinity for the CCK receptors and chemical structure. In the former study the influence of the molecular flexibility of the compounds on the κ-opioid and CCK receptors was also examined. The present study was
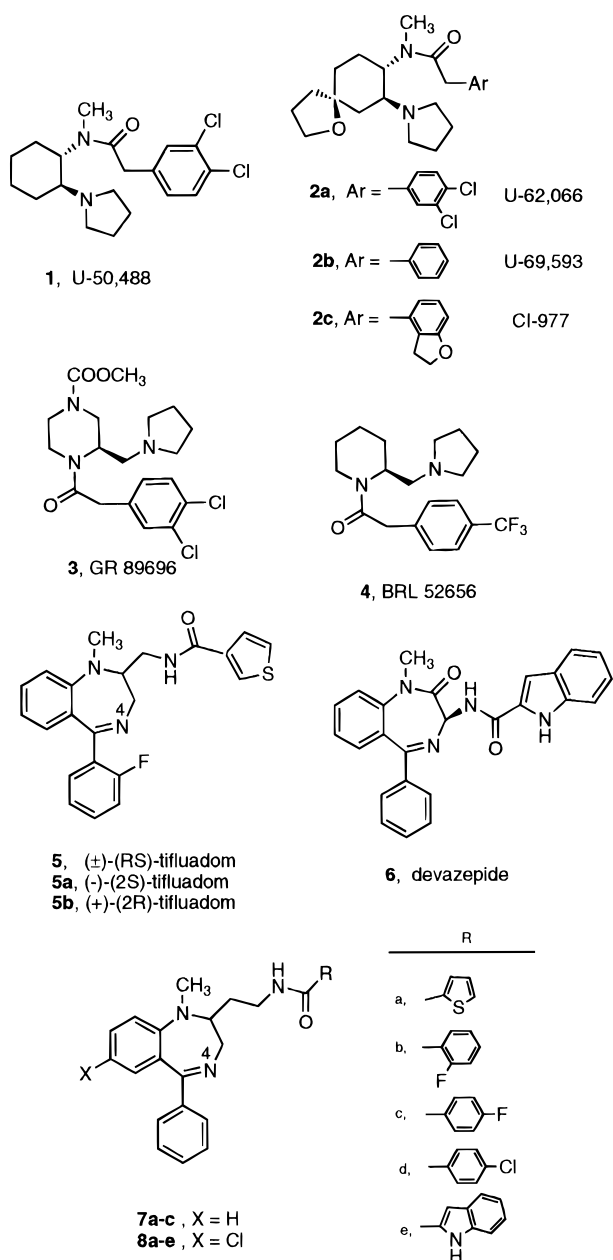
Chart 1

1, U-50,488

2a, Ar = [structure] U-62,066

2b, Ar = [structure] U-69,593

2c, Ar = [structure] CI-977

3, GR 89696

4, BRL 52656

5, (±)-(RS)-tifluadom
5a, (−)-(2S)-tifluadom
5b, (+)-(2R)-tifluadom

6, devazepide

7a-c, X = H
8a-e, X = Cl

R
a, [thiophene]
b, [phenyl-F]
c, [phenyl-F]
d, [phenyl-Cl]
e, [indole]

Scheme 1[a]

9 a,b[b]

10 a,b

11 a-b, R = NPhth
12 a-b, R = NH₂

7 a-c
8 a-e

[a] (i) SOCl₂, CH₂Cl₂; (ii) potassium phthalimide, NaI, DMF; (iii) NH₂NH₂, MeOH; (iv) RCOCl, NEt₃, THF. [b] **a**, X = H; **b**, X = Cl.
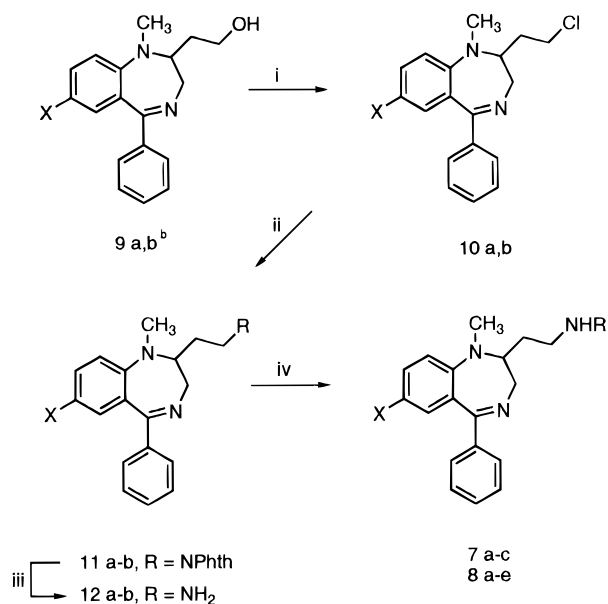
initiated to ascertain whether, using tifluadom as a starting point, it would be possible to prepare selective κ_1-agonists which do not feature the [N-C-C-N (sp²)] pharmacophore of existing compounds: such compounds might ultimately gain insight into the role of the chemical structure of a selective agonist on its biological effects *in vitro* and *in vivo*.

The interpretation of the structure−affinity relationship (SAFIR) data generated (for details, see Results and Discussion section) prompted the design of compounds **7** and **8** as receptor probes able to discriminate κ-opioid from CCK receptors. Although it is generally accepted that the constraint of molecules of various chemical structures having a nonselective binding profile confers selectivity for a particular receptor,[37] the title compounds which possess further degrees of conformational freedom represented more flexible tifluadom analogues showing higher selectivity with respect to the parent compound.

In the present study, the influence of the increasing degree of molecular flexibility on binding affinities for

μ- and δ-opioid receptors was also evaluated. The SAFIR in the very limited series of the title compounds are briefly discussed. Furthermore, in this study the heuristic direct approach to quantitative structure−activity relationship (QSAR) analysis[38,39] has been applied to the three-dimensional models of the complexes between the κ_1-receptors and the newly synthesized ligands in order to rationalize, quantitatively, through the combination of molecular modeling, computational simulations, and correlation analysis, their structure−affinity features.

Chemistry

Scheme 1 summarizes the synthetic procedures used to obtain the 2-[(acylamino)ethyl]-1,4-benzodiazepines considered in this study. The title compounds were prepared starting from the appropriate 2-hydroxyethyl derivative **9a,b**. By treatment with thionyl chloride in methylene chloride at room temperature, compounds **9a,b** were converted into the respective chloroethyl derivatives **10a,b** which, via the classical Gabriel synthesis, were transformed into the 2-(aminoethyl)-1,4-benzodiazepines **12a,b**.[40] These compounds were, in turn, acylated by the suitable acyl chloride to afford the expected 1,4-benzodiazepines **7a−c** and **8a−e**. While the synthesis of compound **9b** has been already reported,[41] compound **9a** was prepared starting from the 1,4-benzodiazepin-2-ylidene acetic acid ethyl ester 4-oxide (**13**) which we previously described[42] (Scheme 2). Briefly, by reduction with phosphorus trichloride, compound **13** gave **14**, which was in turn transformed into its N_1-methyl derivative **15** using methyl *p*-toluenesulfonate in a two-phase system (benzene/50% aqueous sodium hydroxide), in the presence of tetrabutylammonium bromide as a phase-transfer catalyst. The *Z*-geometry of **13** was assigned on the basis of its ¹H-NMR spectrum[42] only, while the *E/Z*-geometry at the exocyclic double bond of compounds **14** and **15** was assigned on the basis of their ¹H-NMR spectra and 2D-NOESY experiments. The 2D-NOESY spectrum of **14** showed a nuclear Overhauser effect (NOE) between the olefinic

Scheme 2[a]
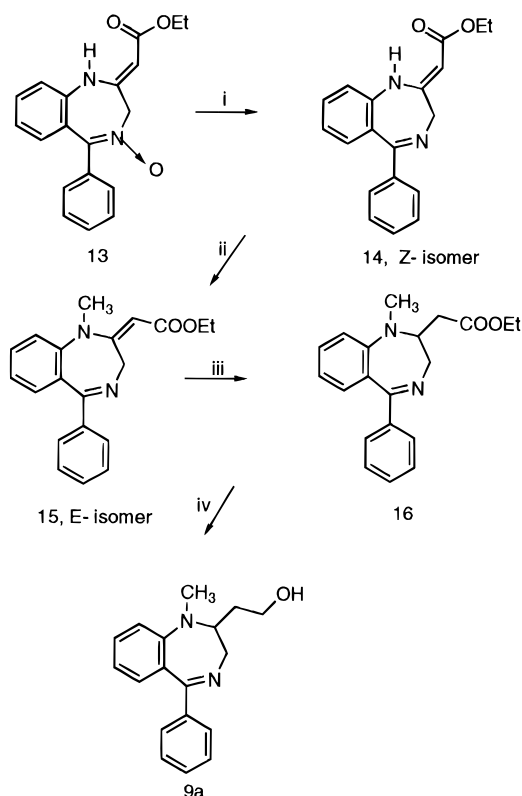
[a] (i) PCl$_3$, CHCl$_3$; (ii) TsOCH$_3$, (*t*-Bu)$_4$N$^+$Br$^-$, NaOH (50%), C$_6$H$_6$; (iii) Et$_3$SiH, TFA; (iv) LAH, ether.

proton (δ 4.89) and the C-3 methylene protons (δ 4.30), supporting *Z*-geometry at the exocyclic olefin. In the case of compound **15**, a detectable NOE, between the olefinic proton and the protons of the N_1-methyl group, allowed to assign the *E*-geometry to this compound. Compounds **14** and **15** were isolated as single isomers. Selective reduction of **15** with triethylsilane in trifluoroacetic acid yielded the saturated ester **16**, which was reduced with lithium aluminum hydride in dry ether at room temperature to afford the expected 2-(hydroxyethyl)-1,4-benzodiazepine **9a**.

Biology

In vitro receptor binding assays were used to measure affinities at κ-, μ-, and δ-opioid and CCK receptors. The radioligand binding models used in these studies were [^{125}I](BH)-CCK-8 in rat pancreas (CCK-A),[43] [^{3}H](MeN-LE^{28,31})-CCK-8 in guinea pig cerebral cortex (CCK-B),[44,45] and [^{3}H]U-69,593 (κ_1),[16] [^{3}H]DAMGO (μ), and [^{3}H]DADLE (δ) in guinea pig brain tissues,[46] respectively. The results are reported in Table 2.

Results and Discussion

A careful study of the SAFIR performed on tifluadom,[35] devazepide (MK-329, formerly L-364,718) (**6**),[47] and related 1,4-benzodiazepines[48] revealed that the insertion of a methylene bridge between the 1,4-benzodiazepine nucleus and the exocyclic amidic nitrogen in devazepide structure led to an increase of κ-opioid affinity while dramatically decreasing the affinity for CCK-A receptors. Further speculation about the different steric tolerance in the receptor areas corresponding to position 2 or 3 of the 1,4-benzodiazepine core of tifluadom-like compounds is possible. The replacement

Table 1. Physicochemical Data of Compounds **7−16**

compd	formula	yield,[a] %	mp, °C	MS,[b] molecular ion	anal.[c]
7a	C$_{23}$H$_{23}$N$_3$OS	52	*d*	389	C, H, N
7b	C$_{25}$H$_{24}$FN$_3$O	40	*d*	401	C, H, N
7c	C$_{25}$H$_{24}$FN$_3$O	42	*d*	401	C, H, N
8a	C$_{23}$H$_{22}$ClN$_3$OS	65	85−86	423	C, H, N
8b	C$_{25}$H$_{23}$ClFN$_3$O	74	62−63	435	C, H, N
8c	C$_{25}$H$_{23}$ClFN$_3$O	78	79−80	435	C, H, N
8d	C$_{25}$H$_{23}$Cl$_2$N$_3$O	66	82−83	452	C, H, N
8e	C$_{27}$H$_{25}$ClN$_4$O	62	138−139	456	C, H, N
9a	C$_{18}$H$_{20}$N$_2$O	80	*d*	280	C, H, N
10a	C$_{18}$H$_{19}$ClN$_2$	83	*d*	298	C, H, N
10b	C$_{18}$H$_{18}$Cl$_2$N$_2$	76	*d*	333	C, H, N
11a	C$_{26}$H$_{23}$N$_3$O$_2$	74	*d*	409	C, H, N
11b	C$_{26}$H$_{22}$ClN$_3$O$_2$	83	65−66	443	C, H, N
12a	C$_{18}$H$_{21}$N$_3$	70	*d*	279	C, H, N
12b	C$_{18}$H$_{20}$ClN$_3$	85	*d*	313	C, H, N
14	C$_{19}$H$_{18}$N$_2$O$_2$	75	122−123	306	C, H, N
15	C$_{20}$H$_{20}$N$_2$O$_2$	68	*d*	320	C, H, N
16	C$_{20}$H$_{22}$N$_2$O$_2$	84	*d*	322	C, H, N

[a] Yields were not optimized. [b] Determined by low-resolution mass spectrometry; fragmentation patterns were consistent with the expected structure. [c] Combustion analyses are within ±0.4% of the theoretical value. [d] Oil.

of the 3-thienyl group of tifluadom with a 2-indolyl or 4-chlorophenyl moiety led to subnanomolar κ-ligands provided with a CCK-A affinity (expressed as IC$_{50}$) ranging from 29 to 800 nM. The critical inspection of the literature data about the influence of the 7-substitution of the benzodiazepine ring revealed that the presence of a chlorine atom depresses CCK-A affinity, while up to now, no data concerning its importance on κ-opioid receptors affinity were available. So, the designed 7-chloro substitution in the compounds of the present series could lead to a better κ/CCK-A selectivity. On the other hand, in a series of devazepide-like compounds,[47] 2′-fluoro substitution appears in some instances to have a positive effect on CCK-A affinity. Thus, all these considerations allowed us to predict high κ-selectivity versus CCK-A receptors for compounds **7** and **8**. The results of receptor binding studies performed on compounds **7** and **8**, and summarized in Table 2, confirmed that our working hypothesis proved to be correct. All the title compounds were completely devoid of affinity for CCK receptors, while most of them bound with high affinity and selectivity to the κ-opioid site. In particular, 2-thienyl derivative **7a** represents a clear improvement with respect to tifluadom, showing a comparable potency but higher κ/CCK and κ/μ selectivity.

It should be noted that all the compounds in this series are racemic, and therefore no conclusions can be drawn concerning the relative contributions of the corresponding enantiomers to the inhibition of [^{3}H]U-69,593 guinea pig brain binding. From the data reported in Table 2, some SAFIR can be obtained about the influence of the acyl substituent. In the series of the acyl groups examined, negligible difference in the κ-opioid affinities could be found, suggesting that, most probably, the amidic functionality of the acylamino moiety gives the main contribution to the binding process. However, additional steric hindrance in the zone corresponding to the *para*-position of the phenyl moiety of compounds **8** is not well tolerated, and both *p*-chlorophenyl and 2-indolyl derivatives **8d**,**e** (Chart 1) resulted less active at κ-opioid receptors. The designed

Table 2. Receptor Binding Affinities for the 2-[(Acylamino)ethyl]-1,4-benzodiazepines **7** and **8**

	K_i, nM[a]				
compd[b]	κ_1, [³H]U-65,693	μ, [³H]DAMGO	δ, [³H]DADLE	CCK-A, [¹²⁵I](BH)-CCK-8	CCK-B, [³H](MeNLE²⁸,³¹)-CCK-8
5, tifluadom	0.78 ± 0.19	1.93 ± 0.08	153 ± 1.71	47 ± 16[c]	>100 000[c]
7a	0.50 ± 0.10	6.42 ± 0.96	105 ± 48	NA[d]	13 000
7b	1.03 ± 0.42	4.69 ± 0.46	>300	NA[d]	NA[e]
7c	0.56 ± 0.10	2.26 ± 0.03	275 ± 43	NA[e]	NA[e]
8a	8.84 ± 1.47	69.4 ± 15	>1000 (2)	NA[d]	17 000
8b	17.9 ± 6.44	62.1 ± 16	>1000 (2)	NA[f]	NA[d]
8c	4.74 ± 1.33	53.2 ± 1.33	>1000 (2)	NA[f]	NA[d]
8d	35.0 ± 5.1	≥100 (2)	>1000 (2)	NA[f]	NA[d]
8e	296 ± 37	>100 (2)	>1000 (2)	NA[f]	NA[d]
lorglumide				129 ± 19	
6, devazepide					3.69 ± 0.84

[a] K_i values followed by SEM derived from three independent experiments (except where otherwise indicated). [b] All compounds were tested as racemates. [c] Data drawn from ref 32. [d–f] NA: compound which did not reach the half-maximal displacement at the maximal concentration tested, [d]30 μM, [e]3 μM, and [f]10 μM.

7-chloro substitution has proved to be inappropriate because it depresses κ-opioid affinity to a higher extent than CCK-A affinity. Although, as pointed out above, the high κ/CCK-A selectivity could be easily predicted on the basis of available SAFIR, it was rather difficult to rationalize them in terms of ligand–receptor interaction in absence of three-dimensional models of the receptors.

Computational Simulations. 1. 3D Model of the κ₁-Receptor. A comparison of the primary sequences of the κ-, δ-, and μ-opioid receptors and of their hydrophobic plots[50] was carried out, in order to identify the seven putative transmembrane (TM) regions. The alignment was facilitated by the occurrence of several residues of the TM domains which are highly conserved across the GPCR superfamily. Figure 1 shows the zones selected as the TM portions of guinea pig κ (κ₁)-, mouse κ-, rat κ-, mouse δ-, rat δ-, and rat μ-receptors.[19] Residues conserved in most of the GCPR superfamily are in bold, while residues mainly conserved in the peptide receptor subclass are boxed. The putative TM regions of the CCK-A receptor sequence[50] are also reported in Figure 1 for comparison.

The seven TM domains were constructed as right-handed α-helices with ϕ = −59° and ψ = −44°. The backbone coordinates of bacteriorhodopsin (Brookhaven Protein Data Bank, code 1BRD)[51] were used as initial templates for the packing of the bundle. The helices were rotated around their principal axes in order to orient the maximum number of charged, polar, and conserved residues toward the inside of the receptor, according to the helical wheel projection model proposed by Baldwin.[52] Uncertainty in the relative vertical position of the helices was partially overcome by taking into account data from site-directed mutagenesis experiments on bovine rhodopsine[53] and cationic neurotransmitter or peptide receptors[54,55] together with the need for an efficient side-chain packing.[56,57]

The structure of the receptor model obtained was energy minimized to relieve steric conflicts of the amino acid side chains. The minimized coordinates of the receptor structure were then used as starting point for a simulation which combines a sequence of quenched molecular dynamics ($T = 0 \rightarrow 300$ K) and a 80 ps molecular dynamics (MD) run at 300 K. In this way the search for conformational space can be expanded in a limited fashion and the conformational behavior of the conserved amino acids, which are invoked to play a

Table 3. Energies and Geometric Description of the Hydrogen-Bonding Network Established in the Minimized Average Structure of the κ₁-Receptor

residue	atom type	residue	atom type	IE (kcal/mol)	IE(HB) (kcal/mol)	D−H−A (deg)	d(H−A) (Å)
Thr104	HG1	**Pro219**	O	−7.06	−3.82	169.64	2.00
Ser108	O	Thr216	HG1	−6.78	−3.98	171.50	1.93
Asn118	HD22	Phe205	O	−6.61	−3.22	171.29	1.97
Tyr107	HH	Ala709	O	−6.53	−3.97	167.19	1.92
Leu207	O	Thr314	HG1	−7.15	−3.37	162.00	1.99
Asp211	OD1	Asn311	HD22	−4.72	−2.37	155.60	2.06
Asp211	OD2	Ser716	HG	−7.59	−4.54	165.34	2.04
Cys301	HT1	Ile421	O	−5.06	−2.64	175.70	2.49
Tyr309	OH	Ser414	HG	−7.35	−4.15	176.92	1.91
Tyr309	HH	Ser418	OG	−5.32	−3.59	164.78	1.97
Thr320	HG1	Asn405	OD1	−7.05	−4.11	176.06	1.93
Asp308	OD1	**Tyr712**	HH	−8.12	−3.92	174.21	2.03
Asn311	HD21	**Ser715**	OG	−6.34	−3.32	173.47	2.05
Ser315	O	**Asn718**	HD22	−5.80	−4.16	153.56	2.19
Leu319	O	Tyr722	HH	−7.14	−3.91	173.24	1.97
Ser412	HG	Phe506	O	−7.75	−3.88	172.34	1.97
Phe504	O	His617	HE2	−7.83	−3.15	167.63	2.03
Ala507	O	**Trp613**	HE1	−5.62	−3.26	169.29	1.93
Tyr519	HH	Leu603	O	−9.37	−4.10	174.89	1.92
Val 622	HCT	Ser703	OG	−6.09	−4.12	168.15	1.93
Phe609	O	**Asn714**	HD22	−7.22	−3.26	169.29	1.94
Phe619	O	Ser702	HG	−7.82	−4.06	178.23	1.98

[a] The residues which are conserved in most of the GPCRs are in bold.

role in the receptor folding and function, can be investigated during the simulation period of the dynamics run. Table 3 lists the amino acids that participate in the complex network of interhelix interactions established during dynamics. The energy contributions of the interacting amino acids (*IE*), the hydrogen-bonding components of the *IE* [*IE*(HB)], the hydrogen–acceptor (H−A) distances, and the donor–acceptor angles (D−H−A) of each hydrogen bond are computed over the minimized average structure of κ₁-receptor.

Most of the residues present in virtually all the GPCRs are involved in this network, as expected. They are reported in bold in Table 3. Among the other residues, the dynamics behavior of the ones specifically conserved in the subclass of opioid receptors or in the κ-receptor subtypes is particularly important as they might play a crucial role in ligand recognition and binding, in a direct or indirect manner. Thr104, Thr320, and Ser716 (the first digit corresponds to the helix, the next two digits indicate the position of the residue in the helix), which have the function of hydrogen bond donors with respect to Pro219, Asn405, and Asp211, respectively, are conserved only in the κ- and δ-receptor subtypes. The μ-receptor subtype presents Met, Cys,

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κ₁ (guinea pig)   V I I T A V[Y]S V V F V V G L V G N S L V M F V I
κ₁ (mouse)        - - - - - - - - - - - - - - - - - - - - - - - - -
κ₁ (rat)          - - - - - - - - - - - - - - - - - - - - - - - - -
δ (mouse)         I A _ _ _ L _ _ A _ C A _ _ _ L _ _ V _ _ _ _ _ G  H1
δ (rat)           I A _ _ _ L _ _ A _ C A _ _ _ L _ _ V _ _ _ _ _ G
μ (rat)           I T _ M _ L _ _ I _ C _ _ _ _ _ F _ _ F _ _ _ _ Y _
CCK-A (human)     A V Q I L L Y S L I F L L S V L G N T L V I T V L


κ₁ (guinea pig)   N I Y I F[N]L A[L]A D A L V T T T M P F Q S T V Y
κ₁ (mouse)        - - - - - - - - - - - - - - - - - - - - - A _ _
κ₁ (rat)          - - - - - - - - - - - - - - - - - - - - _ A _ _
δ (mouse)         - - - - - - - - - - - - A _ S _ L _ _ _ _ A K _  H2
δ (rat)           - - - - - - - - - - - - A _ S _ L _ _ _ _ A K _
μ (rat)           - - - - - - - - - - - - A _ S _ L _ _ _ _ V N _
CCK-A (human)     N I F L L S L A V S D L M L C L F C M P F N L I P


κ₁ (guinea pig)   C[K]I V I S I D Y Y N M F T S I[F]T L T M M S V
κ₁ (mouse)        - - - - - - - - - - - - - - - - - - - - - - - -
κ₁ (rat)          - - - - - - - - - - - - - - - - - - - - - - - -
δ (mouse)         _ _ A _ L _ - - - - - - - - - - - - - - - - - -  H3
δ (rat)           _ _ A _ L _ - - - - - - - - - - - - - - - - - -
μ (rat)           - - - - - - - - - - - - - - - - - - C T _ _ _
CCK-A (human)     C K T T T Y F M G T S V S V S T F N L V A I S L


κ₁ (guinea pig)   A K I I N I C[T]W L L S S S V G I S A I I L G
κ₁ (mouse)        - - - - - - - - - - - - A _ - - - - - _ V _ _
κ₁ (rat)          - - - - - - - - - - - - A _ - - - - - _ V _ _
δ (mouse)         _ _ L _ _ _ _ _ _ V _ A _ G _ _ V P I M V M A  H4
δ (rat)           _ _ L _ _ _ _ _ _ V _ A _ G _ _ V P I M V M A
μ (rat)           _ _ _ V _ V _ N _ I _ _ _ A I _ L P V M F M A
CCK-A (human)     A L K V I A A T W C L S F T I M T P Y P I Y S


κ₁ (guinea pig)   I C V F V F A F V I P V L I I I V C Y T L M
κ₁ (mouse)        - - - - - - - - - - - - - - - - - - - - - -
κ₁ (rat)          - - - - - - - - - - - - - - - - - - - - - -
δ (mouse)         _ _ _ _ L _ _ _ _ V _ I _ _ _ T _ _ _ G _ _  H5
δ (rat)           _ _ _ _ L _ _ _ _ V _ I _ _ _ T _ _ _ G _ _
μ (rat)           _ _ _ _ I _ _ _ I M _ _ _ _ _ T _ _ _ G _ _
CCK-A (human)     T F L L L I L F L I P G I V M M V A Y G L I


κ₁ (guinea pig)   L V L[V V V]A V F I I C W T P I[H]I F I L V
κ₁ (mouse)        - - - - - - - - - - - - - - - - - - - - - -
κ₁ (rat)          - - - - - - - - - - - - - - - - - - - - - -
δ (mouse)         M _ _ _ _ _ G A _ V V _ _ A _ _ _ _ V I _  H6
δ (rat)           M _ _ _ _ _ G A _ V V _ _ A _ _ _ _ V I _
μ (rat)           M _ _ _ _ _ _ _ _ _ V _ A _ _ _ _ Y V I I
CCK-A (human)     M L I V I V V L F F L C W M P I F S A N A W


κ₁ (guinea pig)   L S S Y Y F C I A[L]G Y T N S S L N P I L Y A F L
κ₁ (mouse)        - - - - - - - - - - - - - - - - - - _ V _ _ _ _ _
κ₁ (rat)          - - - - - - - - - - - - - - - - - - _ V _ _ _ _ _
δ (mouse)         V A A L H L _ _ _ _ _ _ A _ _ _ _ _ V _ _ _ _ _
δ (rat)           V A A L H L _ _ _ _ _ _ A _ _ _ _ _ V _ _ _ _ _  H7
μ (rat)           T V _ W H _ _ _ _ _ _ _ _ _ _ C _ _ _ V _ _ _ _ _
CCK-A (human)     G T P I S F I L L L S Y T S S C V N P I I Y C F M
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Figure 1. Comparison of the transmembrane amino acid sequences of opioid and CCK-A receptors. Residues conserved in most of the GPCRs are in bold, and residues conserved in the peptide receptor subclass are boxed.

Figure 2. General view of the κ₁-receptor model. The amino acids which constitute the binding site are highlighted.

and Cys in these positions. A conservative mutation is found for the hydrogen bond donor Thr216 which is exchanged with a Ser in the δ- and μ-receptor subtypes. Ser414 and Ser702 act as hydrogen bond donors with respect to Tyr309 and Phe619, while Ser418 and Ser703 accept hydrogen bonds from Tyr309 and Val622. These serines are the most variable residues among the opioid receptor subtypes. In fact, Ser414 is exchanged with a Gly in the δ-receptor subtype and with an Ala in the μ-receptor subtype; Ser418 substitutes a Pro residue, which is widely conserved among the GPCR superfamily. Ala is found in positions 702 and 703 in the δ-subtype, while Ser702 is exchanged with a Val in the μ-receptor subtype.

Three charged amino acid residues are predicted to lie within the TM region of the opioid receptors. They are Asp211 and Asp308, highly conserved within the GCPR superfamily, and His617, which is conserved
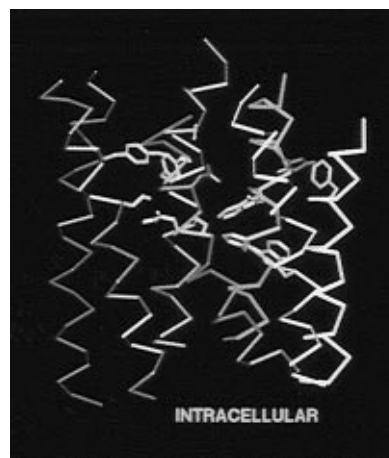
among a number of peptide receptors (e.g., cannabinol, adenosine, bombesin, endothelin, and tachykinin). Their role in the ligand binding and receptor function of the δ- and μ-receptor subtypes has been investigated by site-directed mutagenesis studies.[58,59] In agreement with mutagenesis studies on other GPCRs,[54,55] the data show the fundamental importance of a negatively charged residue at position 211 for efficient G-protein coupling, the implication of Asp308 in direct ligand recognition, and the contribution of His617 to the binding of both agonist and antagonist ligands, although its implication in the intrinsic activity of the receptor is not clear.

Chemical modifications of the κ-opioid receptor expressed on the mouse lymphoma cell line R1.1 indicate that a sulfhydryl group or groups, but not a disulfide bond, are present at or near the binding site.[60] Moreover, the existence of a Cys residue in the ligand-binding site is emphasised by the finding that a sulfhydryl affinity labeling ligand for opioid receptor is able to modify the receptor covalently.[61] In our 3D-minimized model of κ₁-receptor subtype, this sulfhydryl group may be identified as the Cys707 which points toward the inside of the receptor pore at a minimal side-chain distance to Asp308 of 3.6 Å. The same residue has been proposed to participate in the binding of S-activated (−)-6β-sulfhydryldihydromorphine with the μ-receptor in a very recently published model building study of the opioid receptor.[63]

2. κ₁-Tentative Binding Domain and Docking Experiments. On the basis of this information, a tentative binding domain can be identified for the nonpeptide κ₁-ligands considered in this study. It consists of the Asp308, His617, and Cys707 amino acid residues and a large hydrophobic pocket containing widely conserved residues: Trp409, Phe508, Pro511, Phe609, and Trp613. The distance between the center of the cavity and the Asp308 side chain is 5.5 Å. On the contrary, several polar residues are clustered in a pocket delimited by H1, H2, H3, and H7. Among these, Tyr712 and Ser715 are conserved in the GPCR superfamily, and Asn311, Thr215, and Tyr107 are present only in the opioid receptor subtypes. A general view of the κ₁-receptor model obtained is given in Figure 2, where the amino acids which constitute the binding site are highlighted.

Compounds **7a–c** and **8a–c** were used for docking purposes together with tifluadom (**5**) and the higly

Table 4. Experimental Binding Constants and Computed Energy Descriptors (kcal/mol) for the Ligand−κ_1-Receptor Interactions

compd	pK_i	BE	$IE_{\text{k-L}}$	$E_L{}^d$	$E_k{}^d$
5a	9.10	−49.62	−73.06	6.42	20.09
1	9.04	−49.63	−71.59	5.46	16.50
2b	8.75	−44.82	−74.94	8.93	21.19
7a	9.30	−51.72	−73.27	4.84	16.71
7b	8.99	−51.25	−74.94	5.71	17.98
7c	9.25	−50.57	−76.16	6.53	19.02
8a	8.05	−31.24	−70.86	5.66	33.96
8b	7.75	−21.73	−75.74	8.62	45.43
8c	8.32	−35.56	−80.95	6.90	38.49

selective κ_1-agonists U-50,488 (**1**) and U-69,593 (**2b**) whose experimental binding affinity constants were determined in our laboratories. The amine nitrogen atom in compounds **1** and **2b** and the imine nitrogen atom in compounds **5**, **7a**−**c**, and **8a**−**c** were considered as protonated for docking purposes.

In order to test the ability of the receptor model thus obtained to reproduce the stereoselectivity observed for the (−)-2S-enantiomer of tifluadom [(−)-(2S)-tifluadom (**5a**), IC$_{50}$ = 1.1 nM; (+)-(2R)-tifluadom (**5b**), IC$_{50}$ = 50 nM],[36] the docking of the two enantiomers was carried out, whereas 2S was assumed to be the most active configuration of ligands **7a**−**c** and **8a**−**c**, as in the case of tifluadom. The ligands were manually docked in the minimized average structure of the κ_1-receptor subtype using as a main criterium the formation of a charge-reinforced hydrogen bond between the protonated nitrogen N$_4$ atom of the ligands **5**, **7a**−**c**, and **8a**−**c** (Chart 1) and the Asp308. This can only be achieved at the expense of disrupting the charge-reinforced hydrogen-bonding interaction with the Tyr712 residue which occurs in the free receptor. The rest of the ligands were accommodated in the adjacent hydrophobic pocket in such a way that the carbonylic oxygen of the substituent and the HD1 atom of the His617 residue were found at hydrogen-bonding distance, subject to the removal of the interhelix hydrogen bond which stabilized the isolated receptor.

3. Energy Description of the κ_1-Binding Site Model and QSAR Analysis. The energies for the selected κ_1-ligand complexes are reported in Table 4, together with their experimental binding affinities (pK_i). The variation in the measured binding constants is very well explained by the computed binding energies (*BE*) which take into account the ligand−receptor interaction energy ($IE_{\text{k-L}}$) and the mutual conformational adjustment of the ligand (E^d_L) and of the receptor (E^d_k) upon binding. The quantitative relationship obtained between the binding affinity data values and the computed binding energies for the ligands considered is shown in Figure 3.

From the analysis of the data reported in Table 4 clearly emerges that the changes in the potential energy of the receptor upon formation of the complexes are mainly responsible for the correlation obtained, being the changes in the interaction energies disorganized and ranging in a smaller interval. This implies that repulsion forces play the most important role in discriminating between the compounds considered. Thus, from a dynamic point of view, a reorganization of the receptor architecture is needed in order to accommodate the ligands.
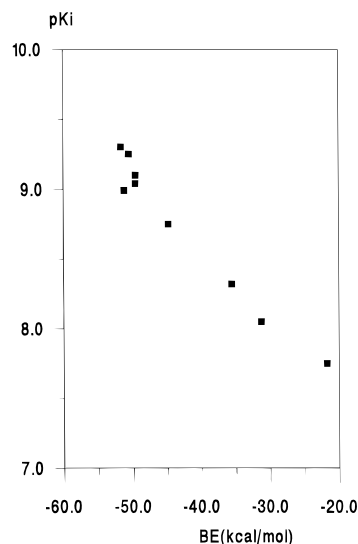

Figure 3. Correlation between the binding affinity values and the calculated binding energies for the minimized ligand−receptor complexes. The linear regression is pK_i = 6.56($\pm$0.16) − 0.05($\pm$0.003)*BE*, n = 9, r = 0.98, s = 0.11, where n is the number of compounds, r is the correlation coefficient, s is the standard deviation, and the values in parentheses give the 95% confidence intervals.

This is mainly achieved by means of Pro15 in helix 6, positioned just below the putative active site, which allows the kinking of the C-terminal part of the helix away from the core of the receptor. The extent of this structural change depends on the structural features of the ligands and influences the overall packing of the bundle. This is also shown by the data of Table 5, where the amino acid residues of the κ_1-receptor mainly involved in the interactions with the ligands are listed and the energy contributions of each residues are given. A few important observations arise from a detailed analysis of the output structures of the complexes. (a) (−)-(2S)-Tifluadom performs the best interaction with Asp308. It is the only ligand able to achieve a good geometry for hydrogen-bonding interaction with both the protonated nitrogen and amidic nitrogen atoms simultaneously. In fact, the hydrogen bond contributors to the Asp308 total *IE* is 6.8 kcal/mol, while it is only 3.0 and 3.89 kcal/mol for compounds **1** and **7a**, respectively. (b) In order to interact with the ligands, residues Asn311, Trp613, His617, Tyr712, Asn714, and Ser715 induce slight modifications in the interhelix hydrogen-bonding network observed in the isolated receptor. Tyr712 gives dispersion interactions with the ligand while it establishes new interhelix hydrogen-bonding interactions with the Thr215 and Asn311 or Asp211 residues. The protonated state of His617 is able to maintain the hydrogen bond with the backbone of H5 present in the isolated receptor and establishes at the same time a hydrogen bond with the carbonyl oxygen of the substituent side chain of the ligands **7a**−**c** and **8a**−**c**. Only dispersive forces are involved in the interaction of this residue with the other ligands. (c) The stereoselectivity of the κ_1-receptor for the (−)-(2S)-tifluadom (**5a**) can be partially explained, in this model, by the inability of the carbonylic oxygen of the 2-substituent in the R-enantiomer to achieve a correct spatial orientation for the formation of a good hydrogen bond with His617. Moreover, the lack of this specific interaction cannot be compensated by dispersion interaction

Table 5. Energy Contribution (kcal/mol) to the Total *IE* of κ_1-Receptor Residues Interacting with the Ligand Considered

compd	Ile307	Asp308	Asn311	Met312	Ile417	Ile420	Trp613	Ile616	His617	Ile620	Cys707	Tyr712	Asn714	Ser715
5a	−2.34	−20.25	−2.16	−3.11	−1.66	−1.59	−2.33	−2.58	−6.34	−3.13	−2.07	−3.64	−3.28	−3.94
5b	−2.29	−16.07	−0.56	−1.77	−2.79	−1.29	−1.15	−3.15	−1.24	−1.64	−1.50	2.52	−2.55	−1.19
1	−1.67	−19.18	−2.82	−3.31	−3.57	−2.87	−1.60	−2.49	−6.11	−2.03	−2.67	−2.60		−2.62
2b		−16.18	−3.59	−3.05	−3.65	−2.05	−6.58	−2.81	−7.65	−2.92	−2.37	−2.09		−3.15
7a	−2.77	−14.80	−1.83	−4.12	−2.41	−2.06	−4.77	−3.09	−7.07	−2.32	−2.08	−3.87	−1.82	−3.37
7b	−2.75	−15.21	−2.00	−4.53	−3.08	−2.63	−2.22	−2.68	−7.35	−1.97	−1.84	−3.49	−3.01	−3.02
7c	−2.75	−15.56	−2.05	−4.69	−2.81	−2.35	−2.63	−2.74	−7.54	−2.07	−1.82	−3.61	−3.29	−2.82
8a	−2.88	−15.07	−1.52	−5.33	−2.12	−1.89	−2.95	−3.84	−4.70	−2.57	−1.66	−3.09	−2.01	−3.75
8b	−2.31	−14.10	−1.56	−4.38	−1.03	−3.61	−5.70	−4.47	−5.56		−1.53	−3.76	−2.01	−3.74
8c	−2.53	−15.12	−1.93	−4.54	−2.52	−4.04	−5.75	−2.10	−7.27	−2.02	−1.97	−3.14	−1.13	−3.90
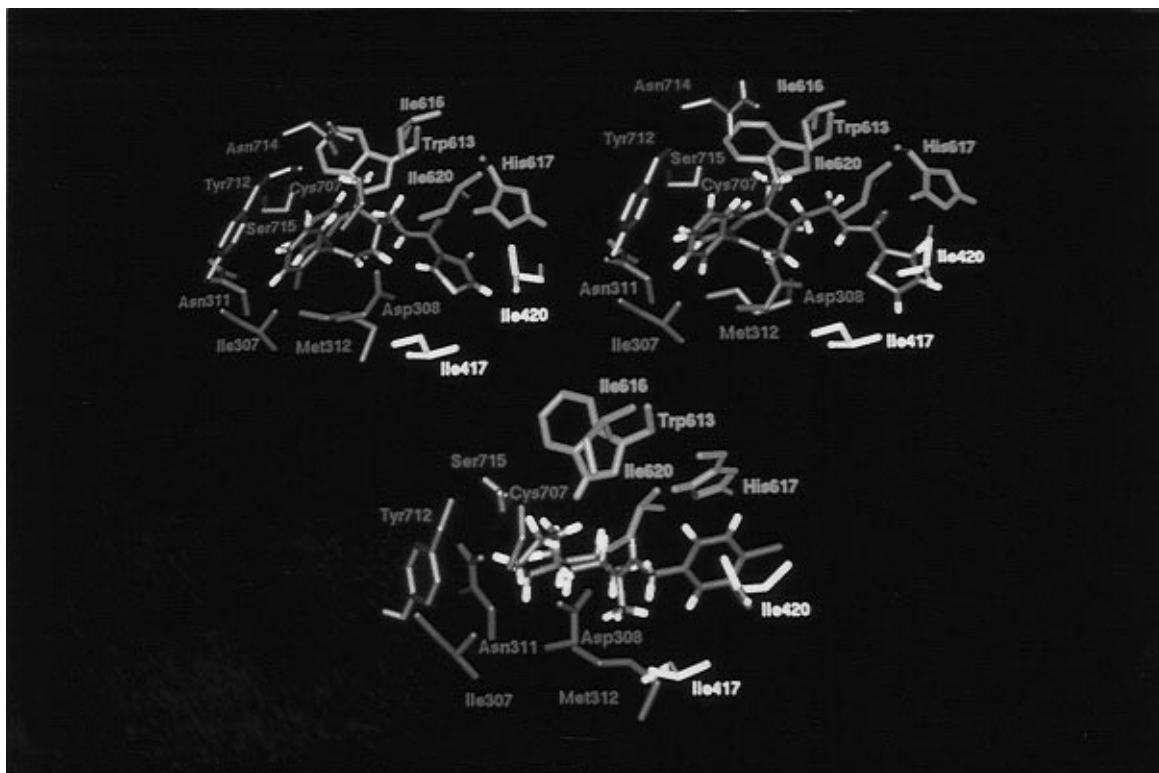
Figure 4. Details of the interaction preferences between the κ_1-binding site residues and the most relevant ligands: tifluadom, **7a**, and U-50,488 (clockwise from top left).

between this residue and the thienyl ring. However, the substantial difference in the total interaction energies of the two enantiomers (ΔIE=15 kcal/mol) indicates that a large reorganization of the receptor architecture is needed in order to accommodate the *R*-enantiomer. (d) Ile417, Ile420, and Ile620 are only present in the κ_1-subtypes. Although highly conservative substitutions occur in these positions among the opioid receptor subtypes (see Figure 1), they might contribute to receptor selectivity affecting the local conformation of the binding pocket. In fact, variability in the aliphatic side chain of a single amino acid residue in the H6 domain of the cholecystokinin receptor (from Val to Leu) was proved to be entirely responsible for ligand selectivity.[63] (e) The amino acids showed to be involved in the interaction with these ligands are not conserved in the CCK-A receptor (Figure 1). On this basis, different loci for ligand−receptor interaction can be invoked in order to explain the observed selectivity. Figure 4 shows a detailed picture of the interaction preferences of the **1**, **5**, and **7a** ligands with the κ_1-binding site residues.

Common κ-recognition requirements for nonpeptide and peptide ligands can be identified by comparing the three-dimensional structure of the compounds considered with a low-energy conformation of the endogenous

peptide agonist. Better clues are provided when the interacting conformations are considered.

Structural analysis of the endogenous opioid peptides indicates that the N-terminal tetrapeptide sequence, shared by dynorphins and enkephalins, is required for agonist activity and binds to the message subsite of the receptor which is located in the TM region. The divergent C-terminal residues define the subtype specificity by binding to the address domain located on the extracellular loops of the receptors.[64,65] This view has, very recently, been supported by the results obtained from studies on chimeric μ/κ receptor.[66] On this basis, the N-terminal "message" fragment of dynorphin [(COOCH$_3$)-Leu-Phe-Gly-Gly-Tyr-NH$_3^+$] was modeled in the κ_1-receptor, and the conformation assumed in the minimized complex is compared in Figure 5 with those assumed by the most relevant compounds studied, tifluadom (**1**), U-50,488 (**2**), and compound **7a**. The overlay of these ligands (Figure 6) helps to formulate a peptidomimetic hypothesis which may be used to guide future synthesis of 1,4-benzodiazepine derivatives with improved characteristics.

The results obtained show the ability of the heuristic direct QSAR approach to handle the available heterogeneous experimental information on receptors and
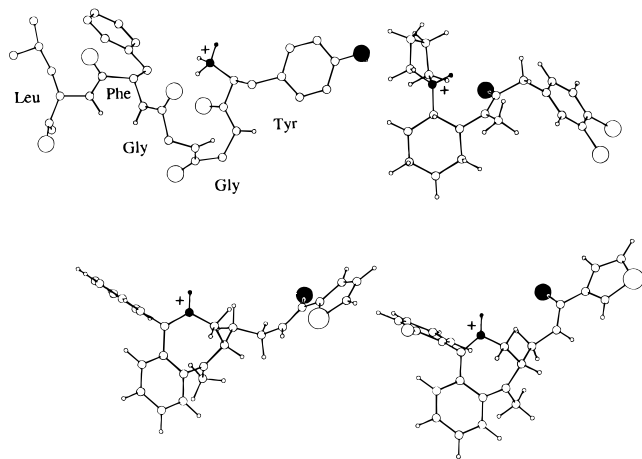
Figure 5. Conformation assumed in the minimized complexes by the N-terminal pentapeptide sequence of dynorphin, U-50,488, tifluadom, and **7a** (clockwise from top left).
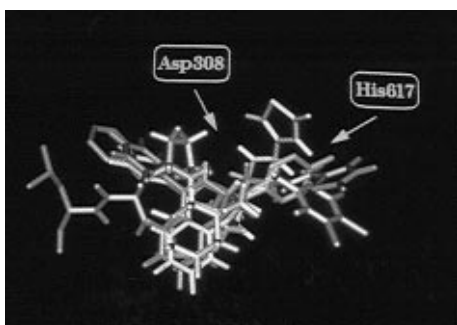

Figure 6. Superposition of the N-terminal pentapeptide sequence of dynorphin (green), tifluadom (violet), U-50,488 (pink), and **7a** (blue).

ligands and to summarize and translate them into quantitative models. Moreover, this procedure constitutes a powerful tool for the design of new leads based on intermolecular interactions and for the suggestion of site-directed mutagenesis studies, in order to give, interactively, further support and improvement to the predictive and interpretive aspects of the model.

Summary and Conclusions

The present study describes the synthesis of a series of tifluadom analogues which feature the introduction of a methylenic bridge between the 1,4-benzodiazepine nucleus and the (acylamino)methyl group of the parent compound.

Affinity for the κ_1-receptor was maintained with close analogues of tifluadom which had the 2-thienyl, 2-fluorophenyl, 4-fluorophenyl, and 2-chlorophenyl substituents. Strict derivatives of these compounds which had a chlorine atom in position 7 of the benzodiazepine ring showed, however, a marked loss of activity at the κ_1-receptor. Compared to tifluadom, however, all the compounds synthesized in this study had lower affinity for the CCK receptors; thus, they exhibited a greater CCK-A/κ selectivity ratio. This was particularly evident in compound **7a**, the 2-[(2-thienylcarbonyl)amino]ethyl derivative, which had a ratio of 60 000 compared to 60 for tifluadom [each value is the ratio of K_i value for the CCK-A site over that for κ_1-receptor (from data reported in Table 2)].

The heuristic direct approach to QSAR analysis applied to the three-dimensional models of the com-

plexes between the κ_1-receptors and the newly synthesized ligands allowed us to rationalize, quantitatively, through the combination of molecular modeling, computational simulations, and correlation analysis, their structure−affinity features. Notwithstanding the limited set of compounds studied, the detailed picture of the ligand−receptor interactions emerging from this approach constitutes an important working hypothesis to direct the design and synthesis of new congeneric ligands with improved characteristics, which, in turn, will be used to evaluate the capability and the limitations of the model, enlarging, in this way, its local reliability.

Experimental Section

Melting points were determined in open capillaries on a Büchi 510 apparatus and are uncorrected. Microanalyses were carried out using a Perkin-Elmer 240C elemental analyzer. Merck silica gel 60, 70−230 mesh, was used for column chromatography, and Riedel-de Haen DC-Mikrokarten SI F 37341 were used as TLC. Infrared spectra (IR) were recorded in Nujol mulls with a Perkin-Elmer model 397 spectrophotometer. ^{1}H-NMR spectra were recorded with a Bruker AC 200 spectrometer in the indicated solvents (TMS as internal standard); the values of chemical shifts are expressed in ppm and coupling constants (J) in Hz. Mass spectra (EI, 70 eV) were recorded on a VG 70-250S spectrometer. IR, NMR spectra, and elemental analyses were performed in the Dipartimento Farmaco Chimico Tecnologico, Università di Siena. Mass spectra were performed by Centro di Analisi e Determinazioni Strutturali, Università di Siena. Commercial chemicals were used as obtained without further purification, except for solvents, which were purified and dried, where appropriate, before use by standard methods.

2,3-Dihydro-2-(2-chloroethyl)-1-methyl-5-phenyl-1H-1,4-benzodiazepine (10a). To an ice-cooled solution of 2,3-dihydro-2-(2-hydroxyethyl)-1-methyl-5-phenyl-1H-1,4-benzodiazepine, **9a** (1.8 g, 6.2 mmol), in dry CH$_2$Cl$_2$ (40 mL) was added thionyl chloride (2.2 mL, 30 mmol) dropwise. The ice bath was removed, and the resulting mixture was stirred at room temperature for 4 h. The solvent was evaporated under reduced pressure, and thionyl chloride excess was removed by azeotropic distillation with benzene. The residue was partitioned between methylene chloride and saturated NaHCO$_3$ solution. The organic phase was washed with a saturated NaHCO$_3$ solution (2 × 20 mL) and brine, dried (Na$_2$SO$_4$), and concentrated under reduced pressure. The crude product was purified by column chromatography (CH$_2$Cl$_2$−EtOAc, 95:5, v/v) to give 1.54 g (83%) of **10a** as a light yellow oil: ^{1}H NMR (CDCl$_3$) δ 2.12−2.22 (m, 2 H), 2.88 (s, 3 H), 3.64 (t, 2 H, J = 6.9), 3.69−3.76 (m, 2 H), 3.88−3.99 (m, 1 H), 6.81−7.10 (m, 3 H), 7.30−7.48 (m, 4 H), 7.54−7.58 (m, 2 H). Anal. Calcd for C$_{18}$H$_{19}$ClN$_2$: C, H, N.

7-Chloro-2,3-dihydro-2-(2-chloroethyl)-1-methyl-5-phenyl-1H-1,4-benzodiazepine (10b). This compound was prepared by the identical procedure described for **10a**, starting from **9b**,[41] and 1.57 g (76%) of **10b** was isolated as an oil. An analytical sample was obtained by column chromatography (CH$_2$Cl$_2$−EtOAc, 95:5, v/v) as a light yellow oil: ^{1}H NMR (CDCl$_3$) δ 2.11−2.22 (m, 2 H), 2.85 (s, 3 H), 3.63 (t, 2 H, J = 7.0), 3.69−3.74 (m, 2 H), 3.86−3.94 (m, 1 H), 6.93−7.0 (m, 2 H), 7.26−7.44 (m, 4 H), 7.52−7.57 (m, 2 H). Anal. Calcd for C$_{18}$H$_{18}$Cl$_2$N$_2$: C, H, N.

2,3-Dihydro-2-(2-phthalimidoethyl)-1-methyl-5-phenyl-1H-1,4-benzodiazepine (11a). To a solution of **10a** (1.19 g, 4 mmol) in dry DMF (30 mL) was added sodium iodide (0.6 g, 4 mmol), and the resulting mixture was stirred at room temperature for 0.5 h. Potassium phthalimide (0.83 g, 4.5 mmol) was then added, and the reaction mixture was heated at 70 °C for 14 h. After cooling the mixture was poured onto crushed ice and extracted with CH$_2$Cl$_2$. The organic phase was thoroughly washed with water to remove the DMF excess, dried (Na$_2$SO$_4$) and concentrated under reduced pressure to

give 1.21 g (74%) of nearly pure **11a** as a yellow oil which was used as such in the next step. An analytical sample was obtained by column chromatography (CH$_2$Cl$_2$−EtOAc, 90:10, v/v) as a light yellow oil: ^{1}H NMR (CDCl$_3$) δ 1.90−2.18 (m, 2 H), 2.88 (s, 3 H), 3.65−3.98 (m, 5 H), 6.88−7.12 (m, 3 H), 7.25−7.45 (m, 4 H), 7.52−7.63 (m, 2 H), 7.68−7.75 (m, 2 H), 7.78−7.89 (m, 2 H). Anal. Calcd for C$_{26}$H$_{23}$N$_3$O$_2$: C, H, N.

7-Chloro-2,3-dihydro-2-(2-phthalimidoethyl)-1-methyl-5-phenyl-1H-1,4-benzodiazepine (11b). This compound was prepared with the same procedure used for **11a**, starting from **10b,** and allowed to react for 20 h. When the reaction mixture was poured onto crushed ice, nearly pure **11b** precipitated as a yellow solid (1.47 g, 83%) which was filtered, dried, and used with no further purification in the next step. An analytical sample melting at 65−66 °C was obtained after recrystallization from EtOAc−n-hexane as light yellow needles: ^{1}H NMR (CDCl$_3$) δ 1.97−2.12 (m, 2 H), 2.88 (s, 3 H), 3.76−3.94 (m, 5 H), 6.97−7.02 (m, 2 H), 7.28−7.45 (m, 4 H), 7.53−7.58 (m, 2 H), 7.69 −7.75 (m, 2 H), 7.81−7.86 (m, 2 H). Anal. Calcd for C$_{26}$H$_{22}$ClN$_3$O$_2$: C, H, N.

2,3-Dihydro-2-(2-aminoethyl)-1-methyl-5-phenyl-1H-1,4-benzodiazepine (12a). A suspension of the phthalimido derivative **11a** (1.0 g, 2.5 mmol) in MeOH (30 mL) treated with 98% hydrazine (1.21 mL, 25 mmol) was stirred at room temperature for 4 h, diluted with MeOH (20 mL), and filtered. The precipitate was discarded, and the filtrate was concentrated under reduced pressure. The residue was partitioned between CH$_2$Cl$_2$ (50 mL) and ice-cold aqueous dilute hydrochloric acid (10 mL). The organic layer was discarded, and the acidic aqueous solution was made alkaline with ice-cold 3 M sodium hydroxide solution. The reaction product was then extracted with CH$_2$Cl$_2$ and the organic phase washed to neutrality with brine and dried over MgSO$_4$. Evaporation under reduced pressure gave pure **12a** (0.56 g, 80%) as a colorless oil, which was used as such in the next reaction. An analytical sample was obtained by column chromatography (EtOAc−MeOH−Et$_3$N, 90:10:10, v/v): ^{1}H NMR (CDCl$_3$) δ 1.69−2.10 (m, 4 H, two of which disappeared with D$_2$O), 2.57−3.0 (m, 5 H), 3.52−3.85 (m, 3 H) 6.84−7.0 (m, 3 H), 7.26−7.41 (m, 4 H), 7.52−7.57 (m, 2 H). Anal. Calcd for C$_{18}$H$_{21}$N$_3$: C, H, N.

7-Chloro-2,3-dihydro-2-(2-aminoethyl)-1-methyl-5-phenyl-1H-1,4-benzodiazepine (12b). Starting from compound **11b** and following the same procedure as described for **12a**, amine **12b** (0.66 g, 85%) was obtained as a yellowish oil and used with no further purification in the next step. An analytical sample was obtained by column chromatography (EtOAc−MeOH−Et$_3$N, 90:10:10, v/v) as a light yellow oil: ^{1}H NMR (CDCl$_3$) δ 1.75−2.10 (m, 4 H, two of which disappeared with D$_2$O), 2.72−2.95 (m, 5 H), 3.54−3.85 (m, 3 H) 6.91−7.0 (m, 2 H), 7.28−7.56 (m, 6 H). Anal. Calcd for C$_{18}$H$_{20}$ClN$_3$: C, H, N.

General Procedure for the Preparation of 2-[(Acylamino)ethyl]-1,4-benzodiazepines 7 and 8. The 2-(aminoethyl)-1,4-benzodiazepine derivatives **12a,b** were acylated as previously described.[35] Briefly, a magnetically stirred solution of the suitable amine (1.76 mmol) in dry THF (12 mL) cooled at 0 °C was treated in succession with Et$_3$N (245 μL) and the appropriate acyl chloride (1.76 mmol) dissolved in dry THF (3 mL). The ice bath was removed, and the reaction mixture was warmed at 23 °C on overnight stirring and then diluted with ethyl acetate (100 mL). The organic layer was washed with a saturated NaHCO$_3$ solution (2 × 30 mL) and brine, dried (MgSO$_4$), and concentrated under reduced pressure to give the crude product, which after purification by column chromatography (EtOAc−n-hexane, 70:30, v/v) afforded the corresponding acylamino derivative.

2,3-Dihydro-2-[[(2-thienylcarbonyl)amino]ethyl]-1-methyl-5-phenyl-1H-1,4-benzodiazepine (7a) and 7-Chloro-2,3-dihydro-2-[[(2-thienylcarbonyl)amino]ethyl]-1-methyl-5-phenyl-1H-1,4-benzodiazepine (8a). These compounds were prepared according to the general procedure starting from **12a,b** and using 2-thiophenecarbonyl chloride as acylating agent. Compound **7a** was obtained in 52% yield. The purification by column chromatography (EtOAc−n-hexane, 70:30, v/v) gave an analytical sample as an orange oil:

^{1}H NMR (CDCl$_3$) δ 2.02−2.14 (m, 2 H), 2.90 (s, 3 H), 3.35−3.54 (m, 2 H), 3.57−3.91 (m, 3 H), 6.82 (d, 1 H, J = 7.5), 6.95−7.12 (m, 3 H), 7.19−7.70 (m, 6 H), 7.73−7.78 (m, 2 H), 7.97 (t, 1 H, J = 5.4, disappeared with D$_2$O). Anal. Calcd for C$_{23}$H$_{23}$N$_3$OS: C, H, N.

Compound **8a** was obtained as a solid in 65% yield. Recrystallization from cyclohexane−EtOAc afforded an analytical sample as light orange fluffy flakes melting at 85−86 °C: ^{1}H NMR (DMSO-d_6) δ 1.72−2.07 (m, 2 H), 2.84 (s, 3 H), 3.30−3.40 (m, 2 H), 3.61−3.82 (m, 3 H), 6.93 (d, 1 H, J = 2.7), 7.11−7.20 (m, 2 H), 7.45−7.54 (m, 6 H), 7.73−7.78 (m, 2 H), 8.54 (t, 1 H, J = 5.4, disappeared with D$_2$O). Anal. Calcd for C$_{23}$H$_{22}$ClN$_3$OS: C, H, N.

2,3-Dihydro-2-[[(2-fluorobenzoyl)amino]ethyl]-1-methyl-5-phenyl-1H-1,4-benzodiazepine (7b) and 7-Chloro-2,3-dihydro-2-[[(2-fluorobenzoyl)amino]ethyl]-1-methyl-5-phenyl-1H-1,4-benzodiazepine (8b). Compounds **12a,b** were acylated with 2-fluorobenzoyl chloride as reported above to give **7b** (yield 40%) and **8b** (yield 74%), respectively. An analytical sample of compound **7b** was obtained by column chromatography (EtOAc−n-hexane, 70:30, v/v) as an orange oil: ^{1}H NMR (CDCl$_3$) δ 1.96−2.15 (m, 2 H), 2.86 (s, 3 H), 3.44−3.58 (m, 2 H), 3.62−3.85 (m, 3 H), 6.84 (d, 1 H, J = 3.6), 6.90−7.65 (m, 12 H), 7.91 (t, 1H, J = 6.2, disappeared with D$_2$O). Anal. Calcd for C$_{25}$H$_{24}$FN$_3$O: C, H, N.

Compound **8b** was obtained as a solid and recrystallization from cyclohexane−CH$_2$Cl$_2$ afforded an analytical sample melting at 62−63 °C as yellowish flakes: ^{1}H NMR (DMSO-d_6) δ 1.71−2.12 (m, 2 H), 2.83 (s, 3 H), 3.29−3.42 (m, 2 H), 3.64−3.83 (m, 3 H), 6.93 (d, 1 H, J = 2.2), 7.14 (d, 1 H), 7.25−7.66 (m, 10 H), 8.39 (t, 1 H, J = 6.1, disappeared with D$_2$O). Anal. Calcd for C$_{25}$H$_{23}$ClFN$_3$O: C, H, N.

2,3-Dihydro-2-[[(4-fluorobenzoyl)amino]ethyl]-1-methyl-5-phenyl-1H-1,4-benzodiazepine (7c) and 7-Chloro-2,3-dihydro-2-[[(4-fluorobenzoyl)amino]ethyl]-1-methyl-5-phenyl-1H-1,4-benzodiazepine (8c). Compounds **12a,b** were acylated with 4-fluorobenzoyl chloride as reported above to give **7c** (yield 42%) and **8c** (yield 78%), respectively. An analytical sample of compound **7c** was obtained by column chromatography (EtOAc−n-hexane, 70:30, v/v) as an orange oil: ^{1}H NMR (CDCl$_3$) δ 1.78−2.13 (m, 2 H), 2.86 (s, 3 H), 3.05−3.49 (m, 2 H), 3.52−3.81 (m, 2 H), 399−4.07 (m, 1 H), 6.81 (t, 1 H, J = 7.4), 7.0−7.11 (m, 4 H), 7.31−7.58 (m, 6 H), 7.75 −7.88 (m, 2 H), 8.15 (t, 1H, J = 5.4, disappeared with D$_2$O). Anal. Calcd for C$_{25}$H$_{24}$FN$_3$O: C, H, N.

Compound **8c** was obtained as a solid, and recrystallization from cyclohexane−CH$_2$Cl$_2$ afforded an analytical sample melting at 79−80 °C as yellow flakes: ^{1}H NMR (DMSO-d_6) δ 1.74−2.10 (m, 2 H), 2.83 (s, 3H), 3.32−3.42 (m, 2H), 3.63−3.80 (m, 3H), 6.93 (d, 1 H, J = 2.1), 7.14 (d, 1 H, J = 8.8), 7.32 (t, 2 H, J = 8.8), 7.40−7.54 (m, 6 H), 7.95 (q, 2 H), 8.56 (t, 1H, J = 5.3, disappeared with D$_2$O). Anal. Calcd for C$_{25}$H$_{23}$ClFN$_3$O: C, H, N.

7-Chloro-2,3-dihydro-2-[[(4-chlorobenzoyl)amino]ethyl]-1-methyl-5-phenyl-1H-1,4-benzodiazepine (8d). This compound, prepared by acylation of **12b** with 4-chlorobenzoyl chloride according to the reported procedure was obtained as a solid in 66% yield. Recrystallization from cyclohexane−CH$_2$Cl$_2$ gave an analytical sample melting at 82−83 °C as yellow flakes: ^{1}H NMR (DMSO-d_6) δ 1.74−2.06 (m, 2 H), 2.83 (s, 3 H), 3.32−3.46 (m, 2 H), 3.63−3.80 (m, 3 H), 6.93 (d, 1 H, J = 2.5), 7.13 (d, 1 H, J = 8.7), 7.39−7.58 (m, 8 H), 7.89 (d, 2 H, J = 8.7), 8.62 (t, 1 H, J = 5.2, disappeared with D$_2$O). Anal. Calcd for C$_{25}$H$_{23}$Cl$_2$N$_3$O: C, H, N.

7-Chloro-2,3-dihydro-2-[(1H-indol-2-ylamino)ethyl]-1-methyl-5-phenyl-1H-1,4-benzodiazepine (8e). By acylation of compound **12b** with 2-indolylcarbonyl chloride,[67] compound **8e** was obtained as a solid in 62% yield. Recrystallization from EtOAc−n-hexane gave an analytical sample melting at 138−139 °C as light yellow needles: ^{1}H NMR (DMSO-d_6) δ 1.76−2.12 (m, 2 H), 2.84 (s, 3 H), 3.35−3.42 (m, 2 H), 3.70−3.83 (m, 3 H), 6.93 (d, 1 H, J = 2.8), 7.05−7.24 (m, 4 H), 7.39−7.65 (m, 8 H), 8.53 (t, 1 H, J = 5.3, disappeared with D$_2$O), 11.56 (s, 1H, disappeared with D$_2$O). Anal. Calcd for C$_{27}$H$_{25}$ClN$_4$O: C, H, N.

(*Z*)-2,3-Dihydro-2-[(ethoxycarbonyl)methylene]-5-phenyl-1*H*-1,4-benzodiazepine (14). A solution of 2,3-dihydro-2-[(ethoxycarbonyl)methylene]-5-phenyl-1*H*-1,4-benzodiazepine 4-oxide, **13**[42] (2.25 g, 7.0 mmol), in CHCl$_3$ (70 mL) containing phosphorus trichloride (6.72 g, 49.0 mmol) was refluxed for 45 min while stirring. The solution was cooled, the reaction quenched with ice, and the mixture basified with a saturated Na$_2$CO$_3$ solution and then extracted with CH$_2$Cl$_2$. The organic phase was washed to neutrality with brine, dried (Na$_2$SO$_4$), and evaporated under reduced pressure to give a gummy residue. The crude product was purified by column chromatography (CHCl$_3$) affording an oil (75%) which crystallized on standing. After recrystallization from cyclohexane an analytical sample melting at 122−123 °C was obtained as colorless prisms: IR 3200 (NH), 1635 (CO) cm^{-1}; ^{1}H NMR (CDCl$_3$) δ 1.27 (t, 3 H, $J = 7.3$), 4.15 (q, 2 H), 4.30 (bs, 2 H), 4.89 (s, 1 H), 6.98−7.51 (m, 9 H), 10.5 (s, 1 H, disappeared with D$_2$O). Anal. Calcd for C$_{19}$H$_{18}$N$_2$O$_2$: C, H, N.

(*E*)-2,3-Dihydro-2-[(ethoxycarbonyl)methylene]-1-methyl-5-phenyl-1*H*-1,4-benzodiazepine (15). To a well-stirred solution of compound **14** (1.78 g, 5.8 mmol) in benzene (30 mL 50%) were added aqueous sodium hydroxide (30 mL.), tetrabutylammoniun bromide (0.386 g, 1.2 mmol), and methyl *p*-toluenesulfonate (2.45 g,13.2 mmol) in sequence. The two-phase system was refluxed under vigorous stirring for 90 min, cooled, diluted with water (100 mL), and extracted with CH$_2$Cl$_2$ (3 × 50 mL). The organic layer, washed to neutrality with brine, was dried over Na$_2$SO$_4$ and evaporated to dryness *in vacuo*. The residue, purified by column chromatography (CH$_2$Cl$_2$−EtOAc, 95:5, v/v), gave an orange oil in 68% yield: IR 1640 cm $^{-1}$ (CO); ^{1}H NMR (CDCl$_3$) δ 1.27 (t, 3 H, $J = 7.1$), 4.15 (q, 2 H), 4.30 (bs, 2 H), 4.89 (s, 1 H), 6.98−7.51 (m, 9 H), 10.5 (s, 1 H, disappeared with D$_2$O). Anal. Calcd for C$_{20}$H$_{20}$N$_2$O$_2$: C, H, N.

2,3-Dihydro-1-methyl-5-phenyl-1*H*-1,4-benzodiazepine-2-acetic Acid Ethyl Ester (16). A solution of compound **15** (1.0 g, 3.1 mmol) in TFA (15 mL) was stirred under N$_2$ atmosphere and cooled in an ice bath. The solution was treated with triethylsilane (0.72 g, 6.2 mmol) added in a period of 5 min. The ice bath was removed, and the stirring was continued for 30 min. The mixture was then poured onto crushed ice, made alkaline by addition of ammonia, and extracted with CH$_2$Cl$_2$. The extracts were combined, dried (Na$_2$SO$_4$), and evaporated under reduced pressure. The residue was purified by column chromatography (CH$_2$Cl$_2$−EtOAc, 95:5, v/v), and **16** was obtained in 84% yield as a thick yellow oil. Further purification by chromatography afforded an analytical sample as a colorless oil: ^{1}H NMR (CDCl$_3$) δ 1.25 (t, 3 H, $J = 7.4$), 2.46 (dd, 1 H, $J = 14.8$, 7.7), 2.70 (dd, 1 H, $J = 15$, 4.3), 2.86 (s, 3 H), 3.51 (dd, 1 H, $J = 11$, 9.4), 3.87 (dd, 1 H, $J = 11$, 4.2), 4.11 (q, 2 H), 4.29 (m, 1 H), 6.92−7.05 (m, 3 H), 7.26−7.40 (m, 4 H), 7.53−7.57 (m, 2 H). Anal. Calcd for C$_{20}$H$_{22}$N$_2$O$_2$: C, H, N.

2,3-Dihydro-2-(2-hydroxyethyl)-1-methyl-5-phenyl-1*H*-1,4-benzodiazepine (9a). A solution of compound **16** (2.7 mmol) in dry ether (25 mL) was added dropwise to a stirred suspension of lithium aluminum hydride (5.7 mmol) in dry ether (25 mL). After stirring under N$_2$ atmosphere for 15 min, the excess of reducing agent was decomposed by careful addition of H$_2$O (1 mL). The inorganic material was filtered off and washed with ether. The filtrate was dried (Na$_2$SO$_4$) and evaporated under reduced pressure. A light yellow oil was obtained in 80% yield and used in the next step without further purification. An analytical sample was obtained by column chromatography using EtOAc as eluant: ^{1}H NMR (DMSO-*d*$_6$) δ 1.54−1.68 (m, 1 H), 1.80−1.91 (m, 1 H), 2.76 (s, 3H), 3.44−3.62 (m, 3 H), 3.64−3.77 (m, 2 H), 4.48 (bs, 1 H, disappeared with D$_2$O), 6.91 (d, 2 H, $J = 4.5$), 7.05 (d, 1 H, $J = 8.1$), 7.32−7.50 (m, 6 H). Anal. Calcd for C$_{18}$H$_{20}$N$_2$O: C, H, N.

***In Vitro* Binding Assays. 1. CCK Receptor Binding Studies.** CCK-A receptor binding assays were carried out on rat isolated pancreatic acini, prepared by enzymatic digestion of pancreas obtained from male Sprague−Dawley (SD) rats (Charles River, Calco, Italy) weighing 250−300 g and fasted 24 h before sacrifice.[43] Briefly, tissue was quickly removed, extensively minced, and dispersed into 30 vol of Krebs−Henseleit buffer (KHB) (118 mM NaCI, 25 mM NaHCO$_3$, 4.7 mM KCI, 1.2 mM NaH$_2$PO$_4$, 0.1 mM CaCI$_2$, 14 mM glucose, 0.1 mg/mL SBTI, 1% BSA) adjusted to pH 7.4. To this medium, continuously shaken and gassed with 95% O$_2$−5% CO$_2$, was added 0.5 mg/mL crude collagenase. The resulting suspension was filtered with a nylon mesh (320 μm), layered over KHB containing 4% BSA, 0.5 mM CaCI$_2$, and 0.1 mg/mL SBTI, and centrifuged for 5 min at 13200*g*. The final resulting pellet was suspended in 10 vol of Hepes-Ringer buffer, pH 7.4 (118 mM NaCI, 10 mM Hepes, 1.13 mM MgCI$_2$, 1.28 mM CaCI$_2$, 1% BSA, 0.2 mg/mL SBTI). This preparation gave a final concentration of $(1-3) \times 10^7$ cells/mL (assessed by light microscope with the aid of a Neubauer chamber), which was diluted in Hepes-Ringer buffer to about 5×10^6 cells/mL. Acini (400 μL), [^{125}I](BH)-CCK-8 (25 pM), and displacing agents were incubated in 0.5 mL total volume in polypropylene tubes for 30 min at 37 °C. At the end of incubation 1 mL of ice-cold assay buffer was added, and the tubes were centrifuged for 5 min at 12500*g*. The supernatant was aspirated and the radioactivity associated to the pellet measured in a Canberra Packard B-5002 γ-counter (80% efficiency). Specific binding was estimated as the difference between the [^{125}I](BH)-CCK-8 binding values found in the absence of 1 μM CCK-8 and those in the presence.

CCK-B receptor binding assays were carried out on membranes obtained from male albino guinea pig (Bettinardi, Momo, Italy) cerebral cortex as previously described[44] with modifications. Tissue (about 1.5 mg) was homogenized in ice-cold 10 mM Hepes (pH 7.4) and centrifuged twice at 4 °C for 15 min at 48000*g*. The final membrane pellet was suspended in tissue buffer (10 mM Hepes, 118 mM NaCl, 4.7 mM KCI, 5.0 mM MgCl$_2$, 1.0 mM EGTA, pH 7.4) to a concentration of 100 mg/mL (original wet weight). Protein concentration was determined according to the method of Bradford.[45] Cortical membranes (0.35−0.4 mg of protein/tube), [^{3}H](N-Me,NLe^{28,31})-CCK-8 (0.5 nM), and displacing agents were incubated for 150 min at 25 °C. All the components, other than cortical membrane suspension, were prepared in an assay buffer consisting of 1 mg/mL BSA, 50 μM bestatin, and 0.1 mg/mL bacitracin dissolved in tissue buffer. Specific binding was estimated as the difference between the [^{3}H](N-Me,NLe^{28,31})-CCK-8 binding values in absence of 1 μM CCK-8 and those in the presence. Bound radioligand was separated by rapid filtration on glass fiber filters (GFB; Whatman), pretreated for at least 1 h with 0.1% BSA solution, and washed three times with 4.0 mL of ice-cold normal saline. Filters were counted in a liquid scintillation spectrophotometer (55% efficiency), after adding 8 mL of Ultima Gold fluid (Canberra Packard).

BSA (fraction V), soybean trypsin inhibitor (SBTI), and crude collagenase A (from *Clostridium hystoliticum*) were purchased from Boehringer Mannheim GmbH (West Germany). [^{125}I](BH)-CCK-8 (2000 Ci/mmol) was purchased from Amersham Int. plc (Buchinghamshire, U.K.). [^{3}H](MeNle^{28,31}) CCK-8 (2000 Ci/mmol) was purchased from New England Nuclear (Boston, MA); bacitracin was purchased from Sigma (St. Louis, MO).

2. κ_1-, μ-, and δ-Opioid Receptor Binding Studies. Male guinea pigs (Dunkin-Hartley; 300−350 g) were killed by decapitation and the brains (without cerebella) rapidly removed. The tissues were homogenized in 10 vol of TRIS-HCl (50 mM, pH 7.4), using a PBI polytron (setting 5 for 15 s), and centrifuged at 49000*g* for 10 min at 4 °C. The pellets obtained were resuspended in 10 vol of TRIS buffer, incubated at 37 °C for 45 min, and centrifuged at 49000*g* for 10 min. The resultant pellets were finally resuspended in 100 vol of TRIS buffer (0.5−0.6 mg of protein/mL), and 1.9 mL aliquots were used for the assay. The tubes containing the homogenate and tritiated and unlabeled compounds were incubated at 25 °C at the final volume of 2 mL.

The following radioligands were used, [^{3}H]U-69,593 (65 Ci/mmol; Amersham, Italy) to bind κ_1-sites,[16] [^{3}H]-[DAla^2,MePhe^4, Gly-ol^5]-enkephalin ([^{3}H]DAMGO) (50.5 Ci/mmol; New England Nuclear, Italy) to bind μ-sites, and [^{3}H]-[DAla^2, DLeu^5]-enkephalin ([^{3}H]DADLE) (33.5 Ci/mmol; New England Nuclear,

Italy) to label δ-sites, in the presence of 40 nM of unlabeled DAMGO to prevent cross-reactivity with μ-receptors.[46] The nonspecific binding was determined in the presence of naloxone, 10 μM. The time necessary to reach equilibrium conditions, previously determined with specific kinetic studies, was 40 min for DAMGO and DADLE and 50 min for U-69,593. The bound ligand was separated from the free ligand by filtration through Whatman GF/B filters using a Brandell cell harvester; the radioactivity on the disc was measured by liquid scintillation counting using a Camberra Packard 2500TR β-counter.

3. Analysis of Binding Data. The binding parameters deriving from competition experiments (IC_{50} values) were calculated by nonlinear regression analysis using the RS/1 software.[68] The inhibition constants (K_i) were calculated from IC_{50} values using the Cheng and Prusoff equation.[69]

Computational Methodology. Model building, docking experiments, energy minimization, and molecular dynamics studies were carried out using the software package QUANTA, version 3.3 (Molecular Simulations, Inc., 200 Fifth Ave., Waltham, MA 02154), implemented on a Hewlett-Packard 720 workstation.

The minimization procedure consisted of 50 steps of steepest descent followed by a conjugate gradient minimization until the rms gradient of the potential energy was <0.001 kcal/mol/Å. The united atom force field parameters, a distance-dependent dielectric term ($\epsilon = 4r$), and a 12 Å nonbonded cutoff were employed. During dynamics the lengths of the bonds involving hydrogen atoms were constrained according to the SHAKE algorithm, allowing an integration time step of 0.001 ps. Moreover, weak harmonic constraints (30 kJ/mol/Å) were applied between the backbone oxygen atoms of residue i and backbone nitrogen atom of residue $i+4$ by using the NOE facility in the CHARMM program[70] in order to maintain the helical structure.

Crystallographic coordinates are available for tifluadom,[71] U-69,593,[72] and U-50,488.[73] The other ligands were obtained by modification of the atomic coordinates of (+)-(2S)-tifluadom. Standard geometric parameters were used. The fully optimized geometries of the ligands were obtained in the AM1 framework,[74] by applying the molecular mechanics correction to the amidic bonds. The N-terminal pentapeptide portion of dynorphin was constructed in the sequence builder module of QUANTA, and the minimized average conformation obtained from a 50 ps dynamics run was used for docking purposes. The ligands were manually docked in the minimized average structure of the κ_1-receptor subtype using as a main criterium the formation of a charge-reinforced hydrogen bond between the protonated nitrogen atom of the ligand and the Asp308. In order to obtain optimal complementarity between each ligand and the receptor cavity, several AM1[74] energetically permitted conformations of the protonated ligands were tested and, as a consequence, rotation of the side-chain torsional angles of different residues forming the binding site were necessary to remove bad steric contacts from each input structure of the complexes. Several complexes were, therefore, obtained for each ligand which were energy minimized by means of molecular mechanics calculation. The choice of the "best complex" among those obtained was performed on the basis of the following criteria: (a) the most favorable energy state for the complex achieved by favorable energy states of the individual components (CHARMm energy[70]), (b) a reasonable distorsion of the ligand upon binding, judged by comparing the AM1 energy of the bound conformation of the ligand to that of the global mimimun (a range of 6 kcal/mol was allowed), and (c) consistency in the binding preferences among the ligands considered.

The binding energies (*BE*) of the ligand–receptor-minimized complexes were computed according to the following equation:[75] $BE = IE_{k-L} + E^d_L + E^d_k$, where IE_{k-L} is the total interaction energy between the receptor and the ligand, E^d_L is the distorsion energy of the ligand calculated with respect to the optimized energy of the free molecule, and E^d_k is a measure of the conformational energy change in the receptor induced by ligand binding. Ligand and receptor distorsion energies were obtained by minimizing the ligand and the

receptor separately, giving as geometrical input the same geometry they assume in each minimized complex.

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