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Conformational Preferences of the κ-Selective Opioid Agonist U50488. A Combined Molecular Mechanics and Nuclear Magnetic Resonance Study

Mark Froimowitz,* Christine M. DiMeglio, and Alexandros Makriyannis

Alcohol and Drug Abuse Research Center, McLean Hospital, Harvard Medical School, 115 Mill Street, Belmont, Massachusetts 02178, and Section of Medicinal Chemistry and Pharmacognosy, School of Pharmacy, University of Connecticut, Storrs, Connecticut 06268. *Received April 20, 1992*

The conformational preferences of the κ-selective opioid agonist U50488 have been studied using MM2-87 calculations and nuclear magnetic resonance (NMR) spectroscopy. The calculations were performed for the protonated form with a dielectric constant of 80 and the unprotonated form with dielectric constants of 1.5 and 80. A systematic search found 72 stable conformers with certain consistent conformational preferences for some of the important dihedral angles. The preferred conformers proved to be compact structures stabilized by intramolecular attractive van der Waals interactions, though at least some of these appear to be electrostatically unfavorable. The conformation of U50488 was also examined in aqueous solution using one-dimensional (1D) and two-dimensional (2D) high-resolution ^{1}H NMR techniques such as the interpretation of ^{1}H–^{1}H vicinal coupling constants, 1D and 2D nuclear Overhauser effect (NOE) experiments, and 2D correlated spectroscopy (COSY) experiments. Five crystallographic conformations were examined as well. There was generally good agreement between all three methods of conformational analysis. There appeared to be a reasonable geometrical agreement between the relatively rigid κ-agonist (–)-ketazocine and a gauche conformer of U50488. The proposed pharmacophore is also consistent with other κ-selective analogs of U50488 including one in which the peptide bond is incorporated into a lactam ring. The low affinity of U50488 for μ-receptors was attributed to its cyclohexane ring which occupies space not present in the nonselective (–)-ketazocine.

The opioid U50488 (*trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide) (Figure 1) is the prototype for selective κ-receptor agonists.[1-4] Unlike previously described opioids that interact with κ-receptors, U50488 has little affinity for μ-receptors and there is little cross tolerance between it and μ-agonists such as morphine. Despite interacting with a different opioid receptor subtype, U50488 has been shown to have potent analgesic properties but does not appear to induce physical dependency as do morphine-like opioids.

There have been systematic structure–activity studies reported for analogs of U50488.[5-8] Among the structural features noted were the following: (1) Electron-rich aromatic systems provided optimal κ-receptor activity and selectivity. (2) (Amine) N-substituents requirements were different for μ- and κ-receptors since opening the pyrrolidine ring, as in the *N*,*N*-diethyl analog, reduced affinity for κ-receptors but not for μ-receptors. Also, N-substituents that normally convert μ-agonists into antagonists enhanced affinity for μ-receptors but not for κ-receptors. (3) A methoxy group in the equatorial 4-position of the cyclohexane ring produced the greatest κ-selectivity. The absolute configuration of the active enantiomer of U50488 and some of its analogs has been reported.[5,9]

This work was initiated to provide information on the conformational properties of U50488. A combined study

* To whom correspondence should be addressed at the Alcohol and Drug Abuse Research Center, McLean Hospital, Harvard Medical School, 115 Mill Street, Belmont, MA 02178.

(1) Szmuszkovicz, J.; VonVoigtlander, P. F. Benzeneacetamide Amines: Structurally Novel Non-μ Opioids. *J. Med. Chem.* **1982**, *25*, 1125–1126.
(2) Piercey, M. F.; Lahti, R. A.; Schroeder, L. A.; Einspahr, F. J.; Barsuhn, C. U50488H, a Pure κ Agonist with Spinal Analgesic Loci in the Mouse. *Life Sci.* **1982**, *31*, 1197–1200.
(3) Lahti, R. A.; VonVoigtlander, P. F.; Barsuhn, C. Properties of a Selective κ Agonist, U50,488H. *Life Sci.* **1982**, *31*, 2257–2260.
(4) VonVoigtlander, P. F.; Lahti, R. A.; Ludens, J. H. U-50,488: A Selective and Structurally Novel Non-μ (κ) Opioid Agonist. *J. Pharm. Exp. Ther.* **1983**, *224*, 7–12.
(5) Clark, C. R.; Halfpenny, P. R.; Hill, R. G.; Horwell, D. C.; Hughes, J.; Jarvis, T. C.; Rees, D. C.; Schofield, D. Highly Selective κ Opioid Analgesics. Synthesis and Structure-Activity Relationships of Novel N-[(2-aminocyclohexyl)aryl]acetamide and N-[(2-aminocyclohexyl)aryloxy]acetamide Derivatives. *J. Med. Chem.* **1988**, *31*, 831–836.
(6) Halfpenny, P. R.; Hill, R. G.; Horwell, D. C.; Hughes, J.; Hunter, J. C.; Johnson, S.; Rees, D. C. Highly Selective κ-Opioid Analgesics. 2. Synthesis and Structure–Activity Relationships of Novel N-[(2-aminocyclohexyl)aryl]acetamide Derivatives. *J. Med. Chem.* **1989**, *32*, 1620–1626.
(7) Halfpenny, P. R.; Horwell, D. C.; Hughes, J.; Hunter, J. C.; Rees, D. C. Highly Selective κ-Opioid Analgesics. 3. Synthesis and Structure–Activity Relationships of Novel N-[2-(1-pyrrolidinyl)-4 or -5-substituted-cyclohexyl]arylacetamide Derivatives. *J. Med. Chem.* **1990**, *33*, 286–291.

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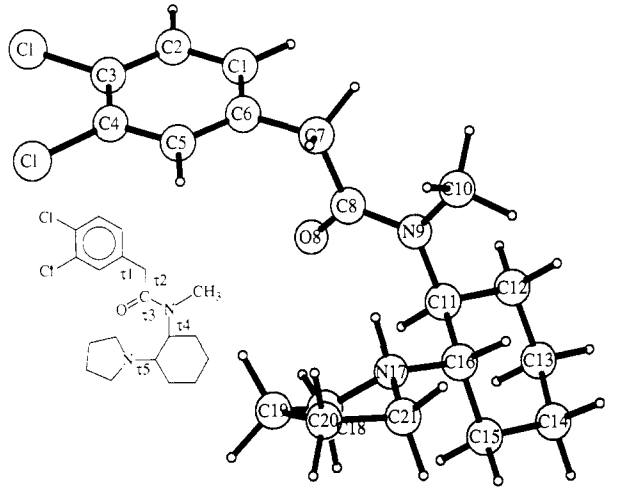

Figure 1. The [80,-176,176,116,-52] conformer of the active enantiomer of protonated U50488.

was performed using molecular mechanics calculations with the MM2-87 computer program and ^{1}H NMR spectroscopy. For the MM2-87 studies, systematic conformational searches were performed for U50488, a sterically bulkier 4-benzo[*b*]thiophene analog, and the *N*,*N*-dimethyl analog. Both of these analogs retain good affinity and selectivity for κ-receptors.[1,5] Other analogs that were considered but not examined include those like spiradoline[10] in which additional groups are attached to the cyclohexane ring. However, these are attached to the side of the cyclohexane ring away from the flexible parts of the molecules and the compounds are, therefore, unlikely to have significantly different conformational preferences from those of U50488.

Results

MM2 Calculations. The three-dimensional structure of U50488 is primarily a function of the rotation about five single bonds. These are τ1(C8–C7–C6–C5), τ2(N9–C8–C7–C6), τ3(C11–N9–C8–C7), τ4(C12–C11–N9–C8) and τ5(C18–N17–C16–C15) (Figure 1). Preliminary MM2-87 calculations and the conformational preferences of simpler molecules were used to determine the local minima for each of the five dihedral angles. With respect to τ1, which specifies the orientation of the phenyl ring, the two minima are in the vicinity of ±90° and correspond to flipping the ring over. For τ2, which corresponds to rotation about the C7–C8 bond, there are two gauche values and one trans value. τ3 specifies the conformation about the amide bond with the two minima in the vicinity of 0° and 180°. τ4, which corresponds to rotation about the amide nitrogen–cyclohexane bond, appears to have only two minima in the vicinity of 110° and −70°. Finally, τ5, which specifies the conformation of the pyrrolidine ring relative to the cyclohexane ring, has two gauche values and one trans value. Permuting all of the possibilities, there are 72 different combinations of these five dihedral angles. The conformer that corresponds to each of these was energy minimized and the results are shown in Table I. The conformers will be referred to as [τ1,τ2,τ3,τ4,τ5] according to the actual value of these five dihedral angles for the energy-minimized structure. The conformers shown in the figures will also be referred to by the figure number in which they appear.

Table I. Conformers of Protonated U50488 ([τ1,τ2,τ3,τ4,τ5]) and Their Computed Steric Energies (kcal/mol) after Minimization with a Dielectric Constant of 80

τ1(C8–C7–C6–C5) ≈ 90°			
τ3(C11–N9–C8–C7) ≈ 180°		τ3(C11–N9–C8–C7) ≈ 0°	
τ4(C12–C11–N9–C8) ≈ 110°			
[44,67,−178,140,56]	23.3	[62,67,1,112,48]	17.1
[45,66,−179,111,166]	24.0	[44,77,2,95,176]	23.0
[44,67,180,114,−53][a]	22.6	[48,78,−2,95,−45]	21.6
[88,180,−179,102,57]	23.7	[86,−178,3,101,57]	24.2
[82,−178,179,112,166]	24.3	[87,−179,1,105,172]	25.3
[80,−176,176,116,−52][b]	22.6	[94,−178,−3,105,−50]	23.8
[119,−76,175,99,56]	22.2	[128,−69,1,106,55]	22.3
[123,−76,174,106,162][c]	22.3	[127,−67,−1,107,172]	23.3
[126,−66,173,111,−56][d]	19.9	[128,−63,−2,108,−48]	21.8
τ4(C12–C11–N9–C8) ≈ −70°			
[62,75,180,−76,56]	23.9	[45,68,−1,−87,62]	29.7
[62,74,178,−69,164]	24.5	[43,73,−1,−80,162]	30.9
[62,75,−164,−72,−59]	20.5	[40,65,7,−92,−60]	27.9
[94,175,−178,−78,57]	25.4	[92,168,−6,−80,60]	29.3
[95,177,−179,−71,163]	26.1	[90,174,−7,−75,162]	30.3
[88,−179,−179,−71,−57]	24.7	[80,−168,11,−63,−50]	29.0
[133,−68,180,−75,57]	25.2	[143,−90,−8,−75,66]	28.9
[133,−67,179,−70,164]	25.9	[140,−94,−9,−70,160]	30.7
[134,−68,180,−70,−55]	24.4	[137,−75,5,−60,−55]	28.2
τ1(C8–C7–C6–C5) ≈ −90°			
τ4(C12–C11–N9–C8) ≈ 110°			
[−133,68,−179,103,56]	23.4	[−122,65,0,113,48][e]	16.6
[−133,66,180,111,166]	24.1	[−135,77,1,95,176]	23.2
[−134,67,180,114,−53]	22.7	[−132,77,−1,95,−45]	21.8
[−90,180,−179,102,57]	23.8	[−91,77,−1,95,−45]	24.2
[−91,−178,179,112,166]	24.4	[−90,−179,1,105,172]	25.3
[−92,−178,177,117,−52]	22.7	[−86,−178,−4,106,−50]	23.8
[−59,−75,176,99,56]	21.9	[−49,−68,1,106,55]	22.4
[−59,77,176,107,163]	22.0	[−50,−66,−1,107,172]	23.5
[−59,−69,177,114,−53]	20.1	[−49,−62,−2,108,−47]	22.0
τ4(C12–C11–N9–C8) ≈ −70°			
[−118,74,180,−76,56]	24.3	[−135,68,−1,−87,62]	29.5
[−118,74,178,−69,164]	24.9	[−135,72,−4,−81,162]	30.6
[−123,72,−164,−71,−59]	20.3	[−140,65,5,−92,−60]	27.7
[−82,174,−178,−78,57]	25.3	[−84,167,−5,−80,60]	29.3
[−82,176,−179,−71,163]	26.0	[−86,172,−7,−75,162]	30.2
[−68,162,−172,−95,−60]	23.3	[−96,−169,11,−63,−50]	29.1
[−45,−68,179,−75,57]	25.1	[−34,−90,−7,−75,68]	28.8
[−44,−67,179,−70,164]	25.8	[−40,−93,−11,−70,160]	30.7
[−43,−67,180,−70,−56]	24.4	[−27,−84,−13,−75,−71]	23.8

[a] Figure 2c. [b] Figure 1. [c] Figure 6b. [d] Figure 2b. [e] Figure 2a.

It should be noted that Table I has been organized to allow the comparison of the conformational trends associated with the different dihedral angles. For example, the first two columns differ from the last two only with respect to τ1. Similarly, if one compares the first column with the second and the third with the fourth, one can examine the effect of the two possible values of τ3. Conformers which vary only in τ5 are grouped together in the same column. Thus, the first three entries in the first column are for the same conformer in which only τ5 has been varied. Similarly, the first, fourth, and seventh entries in each column, the second, fifth, and eighth entries, etc. are for the same conformer in which only τ2 has been varied.

Using the above procedure on Table I, it is possible to discern consistent conformational trends for the 72 conformers. For the two possible values of τ1, the difference in energy is consistently small (tenths of a kcal/mol) and the 72 conformers can be considered to be 36 pairs in which the phenyl ring is flipped over. In view of this, only the

(8) Halfpenny, P. R.; Horwell, D. C.; Hughes, J.; Humblet, C.; Hunter, J. C.; Nerhaus, D.; Rees, D. C. Highly Selective κ Opioid Analgesics. 4. Synthesis of Some Conformationally Restricted Naphthalene Derivatives with High Receptor Affinity and Selectivity. *J. Med. Chem.* **1991**, *34*, 190–194.
(9) DeCosta, B.; George, C.; Rothman, R. B.; Jacobson, A. E.; Rice, K. C. Synthesis and Absolute Configuration of Optically Pure Enantiomers of a κ-Opioid Receptor Selective Agonist. *FEBS Lett.* **1987**, *223*, 335–339.
(10) VonVoigtlander, P. F.; Lewis, R. A. Analgesic and Mechanistic Evaluation of Spiradoline, a Potent *Kappa* Opioid. *J. Pharm. Exp. Ther.* **1988**, *246*, 259–262.

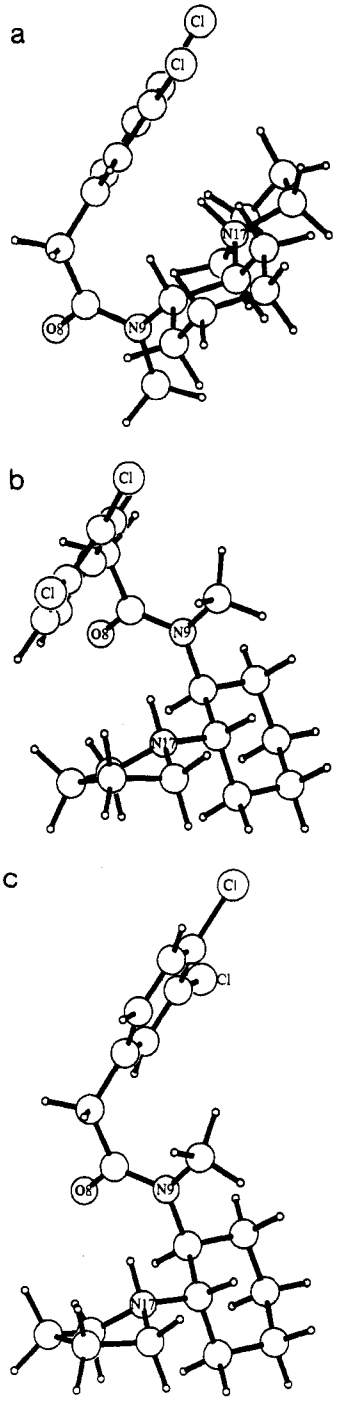

Figure 2. (a) The [−122,65,0,113,48] conformer for protonated U50488 that is the computed global minimum with a dielectric constant of 80. This conformer, however, appears to be electrostatically unfavorable. (b) The [126,−66,173,111,−56] conformer that is also stabilized by attractive van der Waals interactions and is consistent with the NMR data. (c) The [44,67,180,114,−53] conformer that is also consistent with the NMR data.

lower energy member of each pair will be discussed below. With respect to $\tau 4$, conformers with a torsional angle of 110° are almost always preferred. This is especially true for conformers with $\tau 3 \approx 0°$ which are preferred by substantial amounts (5–12 kcal/mol). With respect to $\tau 5$, conformers with dihedral angles in the vicinity of −60° are preferred by small amounts in almost every case.

Dihedral angles for the computed global minimum [−122,65,0,113,48] and several other conformers of interest are listed in Table II and their structures are shown in Figures 1 and 2(a–c). Also included in Table II are the

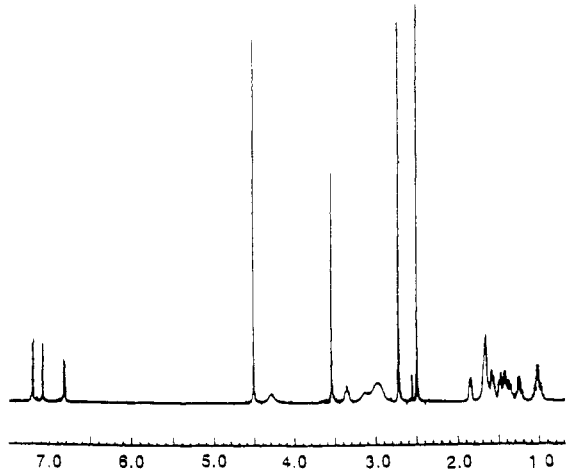

Figure 3. 500-MHz ^{1}H NMR spectra of U50488 (0.09 M in D_2O) recorded at 21.7 °C.

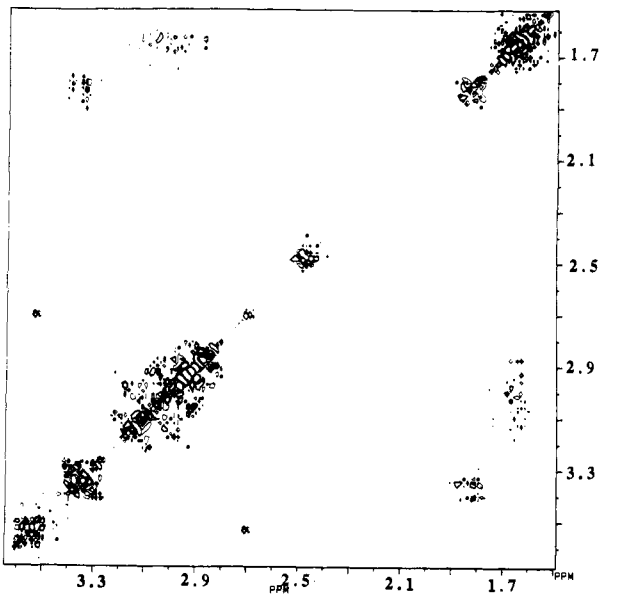

Figure 4. Expanded-scale 200-MHz 2D ^{1}H–^{1}H DQF-contour map of U50488 (0.05 M in D_2O) recorded at 21.7 °C. The contour map shows coupling between the pyrrolidinyl protons H19 and H20 (1.65 ppm) and the pyrrolidinyl protons H18 and H21 (3.25–2.70 ppm).

two recently determined crystal structures of U50488,[11,12] a 4-benzo[*b*]thiophene analog,[5] and a spiro (amide) *N*-demethyl analog in which two nonequivalent conformers appear.[13] A breakdown of the components of the computed steric energy for the energy-minimized conformers is shown in Table III. The factor that appears to be responsible for the favorability of the lowest energy conformers is their compactness which maximizes the attractive van der Waals interaction term. A gauche value for $\tau 2$ is necessary to achieve a compact structure. For the

(11) Doi, M.; Ishida, T.; Inoue, M. Structure of κ-Agonist, U-50488. *Acta Crystallogr.* **1990**, *C46*, 676–678.
(12) Costello, G. F.; James, R.; Shaw, J. S.; Slater, A. M.; Stuchbury, N. C. J. 2-(3,4-Dichlorophenyl)-N-methyl-N-[2-(1-pyrrolidinyl)-1-substituted-ethyl]acetamides: The Use of Conformational Analysis in the Development of a Novel Series of Potent Opioid κ Agonists. *J. Med. Chem.* **1991**, *34*, 181–189.
(13) Fujimoto, R. A.; Boxer, J.; Jackson, R. H.; Simke, J. P.; Neale, R. F.; Snowhill, E. W.; Barbaz, B. J.; Williams, M.; Sills, M. A. Synthesis, Opioid Receptor Binding Profile, and Antinociceptive Activity of 1-Azaspiro[4.5]dec-10-yl Amides. *J. Med. Chem.* **1989**, *32*, 1259–1265.

Table II. Dihedral Angle Values (deg) for Important Conformers of U50488 and the Five X-ray Conformers

	minimized conformers					X-ray conformers				
	2a	2b	1	2c	6b	X-ray[a]	X-ray[b]	X-ray[c]	X-ray[d]	X-ray[d]
C8–C7–C6–C5 (τ1)	−122	126	80	44	123	−93	−104	−90	−60	26
N9–C8–C7–C6 (τ2)	65	−66	−176	67	−76	−177	173	−176	109	−106
C11–N9–C8–C7 (τ3)	0	173	176	180	174	173	−174	−172	−177	−179
C12–C11–N9–C8 (τ4)	113	111	116	114	106	117	110	106	103	130
C16–C11–N9–C8	−120	−125	−120	−123	−130	−119	−125	−130	−127	−108
C13–C12–C11–N9	−171	180	−179	−178	−177	−179	179	−179	−173	−179
cyclohexane										
C14–C13–C12–C11	−57	−56	−56	−56	−56	−57	−59	−58	−53	−57
C15–C14–C13–C12	56	57	56	56	57	58	63	62	49	57
C16–C15–C14–C13	−57	−57	−57	−57	−58	−59	−64	−62	−47	−59
C11–C16–C15–C14	57	57	58	58	58	57	59	59	47	58
C12–C11–C16–C15	−57	−56	−58	−58	−56	−56	−54	−54	−53	−58
C13–C12–C11–C16	58	55	56	56	56	56	54	55	57	58
pyrrolidine										
N17–C16–C15–C14	178	179	179	180	−177	180	−178	180	177	180
C18–N17–C16–C15 (τ5)	48	−56	−52	−53	162	−55	−62	−59	−60	−46
C21–N17–C16–C15	165	67	71	70	−74	69	63	63	70	82
C19–C18–C17–C16	166	167	177	177	−179	115	140	150	e	e
C20–C19–C18–N17	−33	−14	−34	−32	−36	32	−25	−43	e	e
C21–C20–C19–C18	10	−15	7	6	11	−41	30	45	e	e
N17–phenyl center, Å	3.2	4.5	6.5	6.9	5.0	6.6	7.1	6.9	7.2	5.1
N17–phenyl plane, Å	3.2	3.7	2.3	3.5	4.5	2.9	2.0	3.0	1.7	0.3
energy (kcal/mol)	16.6	19.9	22.6	22.6	22.3					

[a] U50488 methanesulfonic acid, computed from fractional coordinates in ref 11. [b] 4-Benzo[b]thiophene analog, computed from fractional coordinates in ref 5. [c] U50488 maleate, computed from fractional coordinates in ref 12. [d] Spiro (amide) N-demethyl analog, free base, two nonequivalent molecules, ref 13. [e] No exact equivalent.

Table III. Contribution to the Computed Steric Energy (kcal/mol) for Conformers Discussed in the Text

	conformer				
	2a	2b	1	2c	6b
bond stretching	2.2	1.9	2.0	2.0	2.4
bond angle bending	6.9	7.6	7.2	7.7	8.8
stretch-bond	0.4	0.3	0.2	0.2	0.2
van der Waals					
1,4 interactions	16.9	16.1	16.6	16.2	16.1
other	−13.1	−8.2	−5.2	−5.2	−7.7
torsional	3.1	2.1	1.9	1.7	2.7
charge–dipole D = 80	0.2	0.1	0.1	0.1	0.1
D = 1.5	10.8	5.6	3.7	3.9	5.3
dipole–dipole D = 80	−0.1	−0.1	−0.1	−0.1	−0.1
D = 1.5	−5.3	−5.1	−5.4	−5.0	−5.2

global minimum, the sum of the van der Waals interaction terms for atoms separated by four or more bonds is −13.1 kcal/mol. This compares with −5.1 kcal/mol for conformers in which τ2 has a trans value.

Nuclear Magnetic Resonance

Chemical Shifts. The 500-MHz ^{1}H NMR spectrum of U50488 is shown in Figure 3. The chemical shifts of the aromatic, benzylic methylene, and *N*-methyl protons were assigned on the basis of integrated chemical shifts and analysis of the ^{1}H–^{1}H 2D COSY cross-peak pattern. Assignment of the protons of the pyrrolidine ring system was accomplished by analyzing the results of 2D phase sensitive double quantum filtered (DQF) COSY and ^{1}H homonuclear decoupling experiments. Examination of the DQF COSY contour map (Figure 4) show scalar coupling between protons centered at 1.65 ppm and protons in the chemical shift region 3.25–2.70 ppm. Homonuclear decoupling with the ^{1}H irradiation frequency centered at 1.65 ppm greatly simplifies the overlapping multiplets from 3.25 to 3.10 ppm. The ^{1}H decoupled spectrum (not shown) shows three doublets at 3.12 ppm (1 H), 2.96 ppm (2 H), and 2.83 ppm (1 H) which are assigned to the four pyrrolidine protons α to the pyrrolidinyl N$^+$ (H18a,b and H21a,b). The four pyrrolidine protons β to N$^+$ (H20a,b and H19a,b) are then assigned to the broad peak at 1.65 ppm.

Table IV. ^{1}H Chemical Shifts for U50488H (0.09 M in D_2O) at 21.7 °C[a]

		chemical shift (ppm)
aromatic	H1	6.82
	H2	7.20
	H5	7.08
methylene	H7	3.52
NCH_3	H10	2.71
cyclohexyl	H11a	4.29
	H12a,e	1.41, 1.49
	H13a,e	1.01, 1.36
	H14a,e	1.01, 1.56
	H15a,e	1.24, 1.84
	H16a	3.35
pyrrolidinyl	H18a,b, 21a,b	3.12 (1 H), 2.96 (2 H), 2.83 (1 H)
	H19a,b, 20a,b	1.65
$CH_3SO_3^-$		2.48

[a] Spectra were recorded at 500.133 MHz and chemical shifts measured relative to internal D_2O at 4.5 ppm. Numbering refers to the structure in Figure 1.

The cyclohexyl proton H16 was assigned by analogy with related compounds[14] and from the analysis of 2D COSY and homonuclear decoupling experiments. The remaining cyclohexyl resonances were assigned on the basis of coupling networks identified by the difference decoupling and 2D COSY experiments. For example, H11 couples through a vicinal mechanism to H16 and the two H12 protons. Chemical shift assignments for U50488 are summarized in Table IV.

Coupling Constants. Determination of ^{1}H–^{1}H vicinal coupling constants (^3J) can provide important structural information through the calculation of dihedral angles from the well-known Karplus equation.[15] The 1D spectrum of U50488 (Figure 3) shows that many resonances of the

(14) Aldrich Library of NMR Spectra, Charles J. Pouchert and John R. Campbell, Aldrich Chemical Company, 1974.
(15) Karplus, M. Contact Electron-Spin Coupling of Nuclear Magnetic Moments. *J. Chem. Phys.* **1959**, *30*, 11–15.

Table V. ^2J, ^3J, and ^4J Coupling Constants Determined from Spectral Simulation of the 500.133 MHz ^{1}H Spectrum and the Corresponding Dihedral Angles

type of coupling	Ha–Hb[a]	coupling constant, Hz	dihedral angle, deg
geminal (^2J)	12a–12e	−12.2	*b*
	13a–13e	−11.8	*b*
	14a–14e	−12.0	*b*
	15a–15e	−11.5	*b*
vicinal (^3J)	11a–16a	12.3	164
	11a–12a	11.8	160
	11a–12e	3.0	63
	13a–14a	12.7	168
	13a–14e	2.5	65
	13e–14e	2.3	64
	14a–15a	13.0	171
	14a–15e	2.6	64
	14e–15e	2.3	62
	15a–16a	12.2	163
	15e–16a	3.6	59
^4J	H7–H1	1.3	*c*
	H7–H5	1.2	*c*

[a] a and e refer to axial and equatorial protons, respectively. [b] These dihedral angles cannot be determined quantitatively because of the myriad of electronic and geometric factors which contribute to ^2J. [c] See text.

cyclohexyl ring overlap, precluding the accurate determination of ^2J and ^3J values directly from the spectra. Thus, ^2J and ^3J values were first estimated from analyses of the 1D and phase-sensitive 2D COSY spectra,[16] and then used along with chemical shift values given in Table IV as the starting point for an iterative simulation of subspectra containing the aliphatic resonances of the cyclohexyl ring. This procedure allowed for the determination of all vicinal and geminal coupling constants between hydrogens of the cyclohexyl ring, and these data are compiled in Table V. The values of the dihedral angles (θ), which also appear in the table, were calculated from the Karplus equation ($^3J = K \cos^2 \theta$) using K values (K_{aa} = 13.31 Hz, $K_{ae} = K_{ea}$ = 14.36 Hz, K_{ee} = 12.24 Hz) calculated from ^3J values measured in 1,1′,4,4′-tetradeuteriocyclohexane[17] and dihedral angles independently determined for cyclohexane.[18] The ^{1}H–^{1}H vicinal coupling constants for the cyclohexyl ring indicate a chair conformation similar to cyclohexane. This is consistent with the conformations of the crystal structures (Table II) and was assumed for the MM2-87 calculations.

Also included in Table V are the $^4J_{HH}$ scalar coupling constants between aromatic protons H1 and H5 and the benzylic protons H7. The approximate coupling constants were determined using the method of line width difference in the absence and presence of decoupling because of the inability to separate contributions to line widths from long-range coupling, natural line width, and magnetic field inhomogeneities.[19]

^{1}H–^{1}H NOE. NOE interactions can be used to determine spatial relationships of protons within a molecule, and thus, the NOE effect is one of the most important

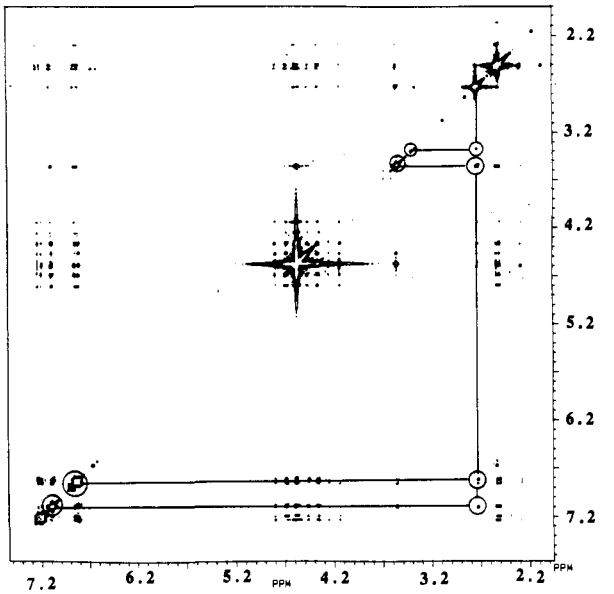

Figure 5. 200-MHz expanded-scale ^{1}H–^{1}H 2D NOESY spectrum of U50488 (0.05 M in D_2O). NOE cross-peaks between the *N*-methyl protons H10 and aromatic protons H1 and H5, the benzylic methylene protons H7, and the cyclohexyl proton H16 are indicated.

NMR parameters used in conformational analysis. 1D and 2D NOE data show through-space dipolar coupling between the *N*-methyl protons H10 on the one hand and the aromatic protons H1 and H5, the benzylic methylene protons H7, and cyclohexyl proton H16 (Figure 5). These are also listed in Table VI along with the interproton distances as determined by the MM2-87 calculations.

Discussion

The MM2-87 calculations indicate that compact structures of U50488 that maximize the attractive van der Waals interactions are favored (Tables I, II). However, there is no evidence for the existence of the global minimum conformer 2a from the NMR data and it is also quite different from the five crystal conformations (Table II). Upon closer examination, it appears that the global minimum is an artifact of using a dielectric constant of 80 in the calculations to approximate an aqueous solution of the compound. While using this model is reasonable under most circumstances, this approach breaks down when electrostatically unfavorable groups are placed sufficiently close to each other as to exclude intervening water molecules. This appears to be the case for the global minimum since computing the energy of the conformer (without further minimization which would cause the structure to converge to another minimum) with a dielectric constant of 1.5 (the default) showed that the charge–dipole interaction is an unfavorable 10.8 kcal/mol. This compares with an unfavorable 5.6 kcal/mol for the conformer 2b which is also stabilized by attractive van der Waals forces. For the closely related conformers 1 and 2c, which are not stabilized in this way, the charge–dipole interactions are an unfavorable 3.7 and 3.9 kcal/mol. Based on this, it appears that the global minimum is an artifact due to unrealistic damping of highly unfavorable electrostatic interactions. With regard to conformer 2b, the situation is less clear since it is only 1.7 kcal/mol less than conformer 2c and it appears likely that one of these two is observed by NMR (see below).

As discussed in the Results section, there are five dihedral angles that have a significant effect on the three-dimensional structure of U50488. For τ1, the MM2-87

(16) Marion, D.; Wüthrich, K. Application of Phase Sensitive Two-Dimensional Correlated Spectroscopy (COSY) for Measurements of ^{1}H–^{1}H Spin–Spin Coupling Constants in Proteins. *Biochem. Biophys. Res. Commun.* **1985**, *115*, 967–974.
(17) Booth, H. Applications of Hydrogen Nuclear Magnetic Resonance Spectroscopy to the Conformational Analysis of Cyclic Compounds. *Prog. NMR Spectrosc.* **1969**, *5*, 149–381.
(18) Garbisch, E. W.; Griffen, M. G. Proton Coupling Constants in Cyclohexane. *J. Am. Chem. Soc.* **1968**, *90*, 6543–6544.
(19) Kriwacki, R. W.; Makriyannis, A. The Conformational Analysis of Δ^9- and $\Delta^{9,11}$-Tetrahydrocannabinols in Solution Using High Resolution Nuclear Magnetic Resonance Spectroscopy. *Mol. Pharmacol.* **1989**, *35*, 495–503.

Table VI. Closest Interatomic Distances (Å) for the Indicated Protons and a List of the NOEs That Are Important for Determining Conformation

comformer	H1–H10[a]	H5–H10	H7–H10	H10–H16	H1–H18[a]	H1–H19[a]	H5–H18	H5–H19
2a	4.4	6.0	4.4	2.4	5.4	4.2	4.8	5.4
2b	3.9	2.9	2.2	2.3	2.8	2.5	6.2	6.3
1	4.6	4.4	2.3	2.3	6.3	6.7	4.0	2.9
2c	3.2	3.5	2.3	2.2	6.8	6.4	5.6	6.7
[134,–68,180,–70,–55]	3.5	2.9	2.3	4.6	6.6	7.5	6.2	6.0
NOE observed?	yes	yes	yes	yes	no	no	no	no

[a] While this distance involving the phenyl proton H1 may be outside the NOE range for the indicated conformer, it will be similar to that for the phenyl proton H5 for the conformer in which the phenyl ring is flipped over.

calculations suggest that there is little energy difference associated with the phenyl ring being flipped over (Table I). This result is supported by the NMR data in that NOEs are observed between the *N*-methyl protons H10 and both of the ortho phenyl protons H1 and H5 (Table VI).

Additional evidence that there is little energy difference associated with flipping the phenyl ring over is provided by the observation that both aromatic protons H1 and H5 have similar $^4J_{HH}$ scalar coupling constant values with the benzylic protons H7. The magnitude of the benzylic coupling constant is greatest (–1.5 Hz) when the aliphatic C–H bond is perpendicular to the aromatic ring and at a minimum (–0.4 Hz) when the C–H bond lies in the plane of the ring.[20,21] The absolute values of the ^4J coupling constants in the spectra were found to be 1.3 Hz (±0.2 Hz) for coupling between H1 and H7 and 1.2 Hz for coupling between H5 and H7 (Table V). Although these coupling constants have not been determined with sufficient accuracy to allow an exact calculation of the benzylic dihedral angle, they do give approximate information about the conformation about the C6–C7 bond. The data are consistent with two conformations of the phenyl ring which interconvert by a 180° rotation about the C6–C7 bond. For a given conformation, one benzylic hydrogen is perpendicular to the aromatic ring while the other forms an angle of approximately 30° with the aromatic ring.

With regard to $\tau 2$, the MM2-87 calculations indicate that there are two gauche conformers and one trans conformer possible (Table I). Three of the crystal conformers have a trans dihedral angle for $\tau 2$ while two have gauche values (Table II). The MM2-87 calculations suggest that gauche conformers are required to achieve the compact structures that are stabilized by attractive van der Waals interactions. The presence of a gauche conformer is supported by the 1D and 2D NOE experiments which show NOEs between the phenyl protons H1 and H5 and the *N*-methyl protons H10 (Table VI). For conformer 1, which has a trans value of the dihedral angle, the closest approach for the *N*-methyl protons H10 and phenyl protons H1 and H5 is 4.4 Å whereas the distance is 2.9–3.2 Å for conformers 2b and 2c which have gauche values. Observation of NOEs between phenyl H1 and the pyrrolidinyl H18, H19 protons could also be used to discriminate between the two gauche forms since conformer 2b has interproton distances of 2.5–2.8 Å while conformer 2c has distances >6.4 Å (Table VI). However, 1D and 2D NOE experiments did not reveal the presence of dipolar coupling between these protons. The lack of an observable NOE, however, does not rule out the presence of conformer 2b or of an interpretation which involves dynamic averaging of the two gauche conformers.

For $\tau 3$, the carbonyl group is trans either to the *N*-methyl group or the cyclohexane ring. The MM2-87 calculations suggest a slight preference for the former (Table I) and this is also observed in all five crystal conformers (Table II). The NMR data (Table VI) agrees with this since there are NOEs between the methylene protons H7 and the *N*-methyl protons H10 where the distance of closest approach is 2.2–2.3 Å for conformers 1 and 2b–2c with $\tau 3 \approx 180°$ as opposed to 4.4 Å for conformers such as 2a where $\tau 3 \approx 0°$.

The MM2-87 calculations (Table I) suggest that, of the two possibilities for $\tau 4$, a value in the vicinity of 110° is consistently preferred over the value in the vicinity of –70°. This value of the dihedral angle is also observed by NMR since there are NOEs between the *N*-methyl protons H10 and the cyclohexyl proton H16. For conformers 1 and 2a–2c where $\tau 4 \approx 110°$, the distance of closest approach for H10 to H16 is 2.2–2.4 Å, while the distance is 4.6 Å for [134,–68,180,–70,–55] where $\tau 4 = -70°$ (Table VI). An additional NOE interaction between H10 and H12a, which would confirm this value of $\tau 4$, could not be unambiguously assigned due to spectral overlap at 200 MHz. All five crystal conformers have values in the vicinity of 110° (Table II). A similar conclusion with regard to $\tau 4$ was made on the basis of more limited conformational energy calculations on model compounds with varying patterns of substitution.[12]

With regard to $\tau 5$, the MM2-87 calculations (Table I) suggest a slight preference for a dihedral angle of –60° which is also the value observed in the three crystal conformers in which the pyrrolidine ring can rotate (Table II). The NMR experiments did not provide information regarding this dihedral angle.

The two crystal structures for U50488 and its 4-benzo[*b*]thiophene analog appear to be quite similar to conformer 1 with the major difference being the conformation of the pyrrolidine ring (Table II). However, it is well known that the pseudorotation of 5-membered rings such as cyclopentane and pyrrolidine result in only small differences in energy. As can be seen from Table II, the three crystal structures also vary among themselves with regard to the conformation of the pyrrolidine ring. The major difference between the three crystal structures and the conformer observed by NMR is that the $\tau 2$ dihedral angle is trans in the three crystal structures while it is gauche in solution. This can be explained by the observation that the trans conformer in at least two of the three crystal structures appears to be stabilized by intermolecular stacking interactions between adjacent aromatic rings.

In summary, the NOE data (Table VI) is most consistent with conformer 2c (and its equivalent with the phenyl ring flipped over). However, conformer 2b (and its equivalent) should also be considered since the lack of observed NOEs between the phenyl and pyrrolidine protons is not conclusive. The observed NOEs can be used to rule out low

(20) Wasylishen, R.; Shaefer, T. INDO Molecular Orbital Calculations on the Conformational Dependence of Long-Range Spin–Spin Coupling of Methyl Protons in Toluene, *p*-Fluorotoluene, and the Xylenes. *Can. J. Chem.* **1972**, *50*, 1852–1862.

(21) Griessner-Prettre, C.; Pullman, B. Molecular-Orbital Study of the Ortho-Benzylic Long-Range Proton–Proton Coupling Constants $^4J_{HH}$ in Biological Phenethyl Amines. *J. Magn. Reson.* **1975**, *18*, 564–568.

concentrations of conformers in which $\tau 3 \approx 180°$ and $\tau 4 \approx 110°$. The presence of observed NOEs between H1-H10 and H5-H10 suggest that there is little difference in energy between conformers in which $\tau 1 \approx 90°$ and $-90°$.

Superposition Study

It is becoming increasingly clear that there may be more than one way for a drug molecule to satisfy the requirements of a receptor. For example, opioid activity can be achieved with compounds that are conformationally constrained with the phenyl ring in either an axial or equatorial position of the piperidine ring.[22-24] Recently, it has been shown that constrained phenyl-equatorial phenylmorphans also have high affinity for the same μ_1 and μ_2 receptors as constrained phenyl-axial opioids such as morphine.[25] The apparent explanation for this is that the phenyl ring of both phenyl-axial and phenyl-equatorial opioids appears to bind to the same molecular component of the receptor site. Although this results in the different placement of the ammonium nitrogen, the ammonium hydrogen appears to be pointing toward the same region of space in both classes of opioids and could, therefore, interact with a common negative charge in the receptor site.[22-24] An analogous example occurs for κ-agonists which are characterized as having an additional oxygen-containing group.[26] For example, the carbonyl oxygen in ketazocine is clearly placed differently from the alcohol or ether oxygen in MR2034 and bremazocine. Nevertheless, a κ-receptor which binds to all of these compounds can be envisioned.[26]

Given the above caveat, one would nevertheless like to determine the common molecular features responsible for the κ-receptor activity of U50488 and other κ-agonists. In general, κ-agonists contain either a carbonyl group, as in ketazocine (Figure 6a), or an ether or alcohol group in the N-substituents of benzomorphans which are located in different portions of the molecules.[26] U50488 contains a carbonyl group and can best be compared to ketazocine.

Comparing the conformers of U50488 in Figures 1 and 2 with ketazocine, conformer 2b appears to be the best fit since the distance between the ammonium nitrogen and the center of the phenyl ring is 4.5 Å (Table II) which matches a distance of 4.5 Å in ketazocine (as computed from the crystallographic fractional coordinates).[27] For conformer 2a, which is electrostatically unfavorable (Table III), this distance is 3.2 Å while the distances are 6.5–6.9 Å for conformers 1 and 2c. However, the orientation of the ammonium hydrogen in conformer 2b, which appears

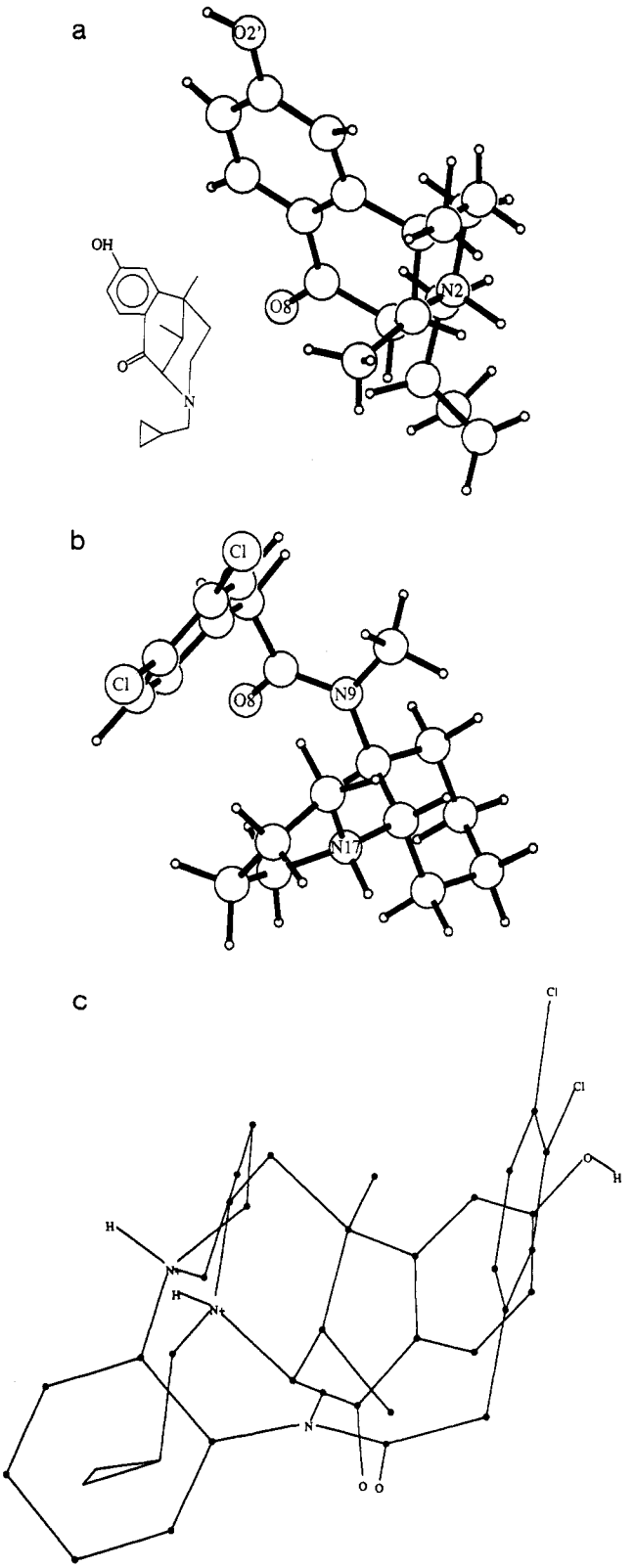

Figure 6. (a) Crystal conformation of the active enantiomer of ketazocine.[27] (b) The [123,-76,174,106,162] conformer of protonated U50488 that appears to be the best match to the structure of ketazocine. (c) Least-squares overlap of conformer **6b** with ketazocine using the two atoms of the carbonyl group and the ammonium nitrogen. This was performed with the PCMODEL program from Serena Software, Box 3076, Bloomington, IN 47402-3076.

(22) Tecle, H.; Hite, G. Narcotic Antagonists—The Azabicyclanes. I. New Insights into the Molecular Modes of Receptor Interactions of Narcotic Analgetics and Narcotic Antagonists. In *Problems of Drug Dependence 1976*; National Academy of Sciences: Washington, D.C., 1976; pp 464–470.

(23) Fries, D. S.; Portoghese, P. S. Stereochemical Studies on Medicinal Agents. 20. Absolute Configuration and Analgetic Potency of α-Promedol Enatiomers. The Role of the C-4 Chiral Center in Conferring Stereoselectivity in Axial- and Equatorial-Phenyl Prodine Congeners. *J. Med. Chem.* **1976**, *19*, 1155–1158.

(24) Froimowitz, M.; Salva, P.; Hite, G. J.; Gianutsos, G.; Suzdak, P.; Heyman, R. Conformational Properties of α- and β-Azabicyclane Opiates. The Effect of Conformation on Pharmacological Activity. *J. Comput. Chem.* **1984**, *5*, 291–298.

(25) Froimowitz, M.; Pick, C. G.; Pasternak, G. W. Phenylmorphans and Analogs: Opioid Receptor Subtype Selectivity and the Effect of Conformation on Activity. *J. Med. Chem.* **1992**, *35*, 1521–1525.

(26) De Ranter, C. J.; Verlinde, C. L.; Blaton, N. M.; Peeters, O. M. The kappa-Active Oxygen in the Opioid Pharmacophore of Some Benzomorphans: Charges, Proton Affinities and Binding Modelling. *Neuropeptides* **1984**, *5*, 209–212.

(27) Verlinde, C. L.; De Ranter, C. J. (1S,5R,9R)-2-Cyclopropylmethyl-2'-hydroxy-5,9-dimethyl-8-oxo-6,7-benzomorphan Hydrochloride Monohydrate (Ketazocine), $C_{18}H_{23}NO_2 \cdot HCl \cdot H_2O$. *Acta Crystallogr.* **1983**, *C39*, 1703–1706.

to be an important factor,[22-24] is clearly different (Figures 2b and 6a). Rotating the pyrrolidine ring to match ketazocine, the conformer in Figure 6b appears to be the best match and the most likely pharmacophore for U50488. This can also be seen in Figure 6c which shows the least-squares overlap (using the two atoms of the carbonyl group and the ammonium nitrogen) between this U50488 conformer and ketazocine. It should be noted that while the phenyl ring in ketazocine is relatively rigid, the barrier for rotation of the phenyl ring in U50488 is quite low. The energy of conformer 6b is 2.4 kcal/mol higher in energy than conformer 2b due to more unfavorable bond-stretching, bond angle bending, and torsional terms and less favorable attractive van der Waals interactions (Table III).

Superimposing conformer 6b with ketazocine suggests that the cyclohexane ring in U50488 is responsible for its lack of affinity at μ-receptors since this portion of the molecule occupies space that is not occupied in ketazocine and other benzomorphans which have activity at both μ- and κ-receptors. The κ-receptor appears to have considerable tolerance for steric bulk in this portion of the molecule.[6-8,10,27] Additional evidence for this proposal is that the introduction of a benzo or naphtho group to the cyclohexane ring of a μ-agonist analog of U50488 (in which the dichlorophenyl group is directly attached to the carbonyl carbon) results in compounds with decreased affinity for μ-receptors and increased affinity for κ-receptors.[28] Since these bulky groups are placed on the side of the cyclohexane ring away from the flexible parts of the molecules, they are unlikely to affect conformation. Rather, their effect appears to be due to a direct steric interaction with the receptor. Interestingly, the cyclohexane ring in U50488 also appears to occupy the same region of space as *N*-alkyl groups that convert μ agonists into antagonists. There is some evidence that μ-antagonism may be associated with κ-agonists. For example, β-FNA is a κ-agonist but an irreversible μ-antagonist.[29] Similarly, U50488 and other κ-agonists have been found to antagonize the actions of some but not all μ-agonists.[30,31] More recently, it has been shown that the μ-antagonist nalorphine, the *N*-allyl analog of morphine, exerts its antinociceptive effects through κ-receptors.[32] Thus, steric bulk in the region of the cyclohexane ring appears to be associated with decreased affinity for μ-receptors and increased affinity for κ-receptors including κ-agonist activity.

An additional series of U50488 analogs consists of compounds in which the pyrrolidine ring has been incorporated into a spiro structure with the cyclohexane ring (Figure 7).[13] This results in a fixed orientation of the ammonium group similar to conformer 2a. The effect of this molecular

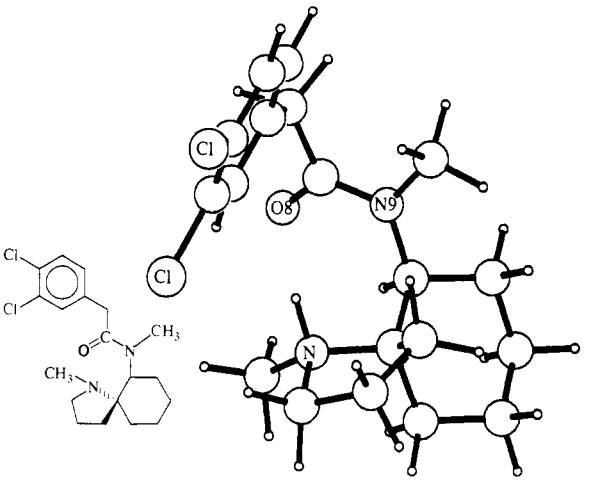

Figure 7. Spiro analog of U50488 which is inactive at κ-receptors. This has been attributed to either an incorrect orientation of the ammonium hydrogen or steric factors due to the non-rotating pyrrolidine ring.[13]

change on the closest analog to U50488 is to abolish all affinity for κ-receptors while affinity for μ-receptors is relatively unaffected. Two explanations were provided for this dramatic difference in receptor affinity.[13] One was that the orientation of the ammonium hydrogen was incorrect for κ-receptors and this may be taken as evidence for conformer 6b as the biologically active form. The second explanation was a steric one in that the rigid pyrrolidine ring is forced to be perpendicular to the cyclohexane ring and, thus, occupies space that a freely rotating pyrrolidine ring cannot occupy. The κ-receptor does appear to be sensitive to steric bulk in this part of the molecule since opening the pyrrolidine ring as in the *N,N*-diethyl derivative of a 4-benzo[*b*]thiophene analog results in a 500-fold decrease in affinity for κ-receptors while having little effect on μ-receptor affinity.[5] Based on our calculations on the *N,N*-dimethyl analog, opening the ring should not have a significant conformational effect on the orientation of the ammonium hydrogen and a steric explanation does appear likely for the loss of κ-receptor affinity in the *N,N*-diethyl analog.

Recently, the opioid activity and receptor affinity of a novel series of 1-(arylacetyl)-2-(aminomethyl)piperidines were reported.[33] It had been noted that the dihedral angle between the amide and ammonium nitrogens in U50488 is constrained to be 60° and the novel compounds were designed to reproduce this dihedral angle. Some of the resultant compounds proved to be extremely potent and selective κ-agonists. The conformational properties of some of the compounds were also investigated with the MM2 and AMPAC computer programs and with NMR spectroscopy. The conclusions of that work appear to be consistent with the present study on a number of points. Using NMR, it was found that the carbonyl oxygen was oriented exclusively toward the chain containing the ammonium group as is also the case for U50488. For some of the compounds, NOEs were noted between both ortho phenyl protons and a methyl group, suggesting that there is little energy difference associated with flipping the ring over. Finally, while these novel κ-agonists do not contain a cyclohexane ring, the amide-containing six-membered ring is placed in the same general space as the cyclohexane ring of U50488.

(28) Freeman, J. P.; Michalson, E. T.; D'Andrea, S. V.; Baczynskyj, L.; VonVoigtlander, P. F.; Lahti, R. A.; Smith, M. W.; Lawson, C. F.; Scahill, T. A.; Mizsak, S. A.; Szmuszkovicz, J. Naphtho and Benzo Analogues of the κ Opioid Agonist *trans*-(±)-3,4-Dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide. *J. Med. Chem.* **1991**, *34*, 1891–1896.

(29) Ward, S. J.; Portoghese, P. S.; Takemori, A. E. Pharmacological Profiles of β-Funaltrexamine (β-FNA) and β-chlornaltrexamine (β-CNA) on the Mouse Deferens Preparation. *Eur. J. Pharmacol.* **1982**, *80*, 377–384.

(30) Porreca, F.; Tortella, F. C. Differential Antagonism of Mu Agonists by U50,488H in the Rat. *Life Sci.* **1987**, *41*, 2511–2516.

(31) Sheldon, R. J.; Nunan, L.; Porreca, F. Mu Antagonist Properties of Kappa Agonists in a Model of Rat Urinary Bladder Motility *in Vivo*. *J. Pharm. Exp. Ther.* **1987**, *243*, 234–240.

(32) Paul, D.; Pick, C. G.; Tive, L. A.; Pasternak, G. W. Pharmacological Characterization of Nalorphine, a Kappa$_3$ Analgesic. *J. Pharm. Exp. Ther.* **1991**, *257*, 1–7.

(33) Vecchietti, V.; Giodani, A.; Giardina, G.; Colle, R.; Clarke, G. D. (2S)-1-(Arylacetyl)-2-(aminomethyl)piperidine Derivatives: Novel, Highly Selective κ Opioid Analgesics. *J. Med. Chem.* **1991**, *34*, 397–403.

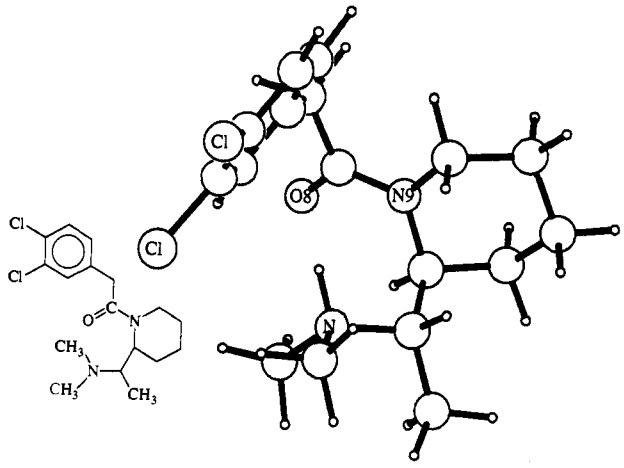

Figure 8. Analog of U50488 which maintains good affinity and selectivity for κ-receptors. The compound has been placed in a conformation consistent with previous results[33] and the results obtained here.

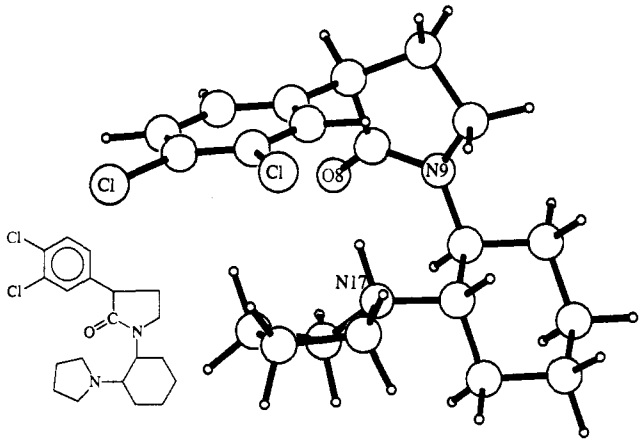

Figure 9. The [94,−13,165,134,−49] conformer and global minimum for the more active and κ-selective diastereomer of U50488 analogs in which the peptide bond has been incorporated into a lactam ring.[34]

While the conformation of the arylacetyl group was not determined in that study, it appears that this moiety can assume the same general conformation as U50488 (Figure 8). Thus, it appears that κ-agonists such as these are compatible with the pharmacophore proposed for U50488.

After the completion of this work, we became aware of a recent and relevant paper in which novel analogs of U50488 were synthesized and assayed for affinity at opioid μ, κ, and δ receptors.[34] In these analogs, the amide group of U50488 has been incorporated into a lactam ring resulting in additional conformational rigidity (Figure 9). Despite this structural change, one diastereomer maintains good affinity and selectivity for κ receptors. The major conformational effect of the lactam ring is to fix $\tau 3 \approx 180°$ in agreement with the conclusions of this work. The lactam ring also limits $\tau 2$ to negative values for the more active and κ-selective diastereomer while the less active diastereomer is limited to positive values. MM2-87 calculations were performed on the more active diastereomer to determine the effect of the lactam ring on conformational preferences within the molecule. It was found that most of these were the same as for U50488. For example, there was once again only small energy differences associated with flipping the ring over and the preferences for $\tau 4 \approx 110°$ and $\tau 5 \approx -60°$ were maintained. The global minimum was found to be the gauche conformer [94, −103,165,134,−49] (Figure 9) which was again stabilized by attractive van der Waals forces. This was found to be 0.6 kcal/mol more favorable than the trans conformer [50, −143,176,136,−46]. It should be noted that while the lactam ring does have some constraining effect on $\tau 2$ for both of these conformers, the affinity and κ-selectivity of the more active diastereomer (Figure 9) is consistent with the conclusions of this paper that the biologically active conformer of U50488 has values of $\tau 2 \approx -60°$ and $\tau 3 \approx 180°$. Moreover, the greatly reduced affinity of the other diastereomer would appear to rule out the gauche conformer in which $\tau 2 \approx +60°$.

Experimental Section

Molecular Mechanics. The calculations were performed with the MM2-87 program and parameter set.[35,36] Two torsional parameters involving the amide nitrogen were not provided by the program. These were for C_{sp2}–C_{sp3}–$C_{carbonyl}$–N_{amide} (atom types 2-1-3-9) and N_{amide}–C_{sp3}–C_{sp3}–$N_{amine(ammonium)}$ (atom types 9–1–1–8(39)). These were approximated by the parameters for C_{sp3}–C_{sp3}–$C_{carbonyl}$–N_{amide} (atom types 1–1–3–9) and N_{amide}–C_{sp3}–C_{sp3}–C_{sp3} (atom types 9–1–1–1), respectively. The only nonzero term for both parameters is the V2 term which is 0.4 kcal/mol, which translates into a maximum contribution of 0.2 kcal/mol. This suggests that this approximation will have only a small effect on the conformational energy. This is especially true for the second torsional parameter which is for a dihedral angle within the cyclohexane ring which does not change significantly between conformers. A chair conformation of the cyclohexane ring with the two substituents in the equatorial position was assumed. The numbering system used for U50488 is shown in Figure 1. The initial Cartesian coordinates for the energy minimizations were generated by a previously described program.[37] The most comprehensive calculations were performed on U50488. Since results for the 4-benzo[*b*]thiophene and *N*,*N*-dimethyl analogs were essentially the same as those for U50488, only the latter are reported.

Molecular mechanics calculations are usually performed on an isolated molecule. In an effort to make the calculations more realistic and to examine the conformational effect of different molecular environments, the calculations were performed under a variety of conditions on the protonated and unprotonated forms of the compounds. For the protonated forms, a dielectric constant of 80 was used to approximate a high dielectric environment, such as water, which would tend to damp out intramolecular electrostatic interactions which would otherwise dominate in a strongly polar molecule. For the unprotonated forms, dielectric constants of 1.5 (the default) and 80 were used. However, with some notable exceptions that are discussed, there was little difference in the results among the different ways of performing the calculations. It should be noted that this is the first version of the MM2 program to explicitly calculate electrostatic interactions between a charged ammonium group and the remaining dipoles within a molecule.

Nuclear Magnetic Resonance. The 500-MHz 1D ^{1}H NMR spectrum was recorded at 500.133 MHz with a Bruker MSL-500 FT spectrometer equipped with an Aspect 3000 computer. Acquisition parameters were as follows: spectral width, 4310.345 Hz (8.6 ppm); data size 32K; recycle delay, 5.8 s; pulse width, 6.5 μs (90° tip angle); 128 transients.

The 200-MHz 1D ^{1}H homonuclear decoupling and ^{1}H difference NOE spectra were recorded at 200.13 MHz with a Bruker WP-200

(34) Cheng, C. Y.; Lu, H.-Y.; Lee, F.-M.; Tam, S. W. Synthesis of (1′,2′-Trans)-3-phenyl-1-[2′-(N-pyrrolidinyl)cyclohexyl]-pyrrolid-2-ones as κ-Selective Opiates. *J. Pharm. Sci.* **1990**, *79*, 758–762.

(35) Allinger, N. L.; Yuh, Y. H. *Quantum Chem. Progr. Exch. 1980*, 12, program 395.

(36) Quantum Chemistry Program Exchange, Department of Chemistry, Indiana University, Bloomington, IN 47405.

(37) Froimowitz, M.; Gans, P. J. Comparison of Semiempirical Classical and Semiempirical Quantum Mechanical Calculations on Acetylcholine. *J. Am. Chem. Soc.* **1972**, *94*, 8020–8025.

SY FT spectrometer equipped with an Aspect 3000 computer. Acquisition parameters were as follows: spectral width, 1785 Hz (8.9 ppm); data size, 16K; recycle delay, 5 s; pulse width, 2 μs (40° tip angle); 128 transients. The ^1H decoupled spectra were examined with respect to a control spectrum in which the irradiated frequency corresponded to a blank region of the spectrum. The ^1H difference NOE spectra were recorded with a pulse program available in the Bruker library of programs. The spectra were recorded by sequential acquisition and storage of eight transients with irradiation at each of several frequencies. The list of frequencies included one corresponding to a blank spectral region, and the resulting spectrum was used as a control. A subsaturating irradiation pulse (0.8 s) was used before acquisition. The cycle was repeated ×32 to give a total of 256 transients. The stored free induction decays (FIDs) were processed with identical normalization and phase constants. The control spectrum was subtracted from each decoupled spectrum to give a difference spectrum.

Spectral regions of the 1D 500.133 MHz ^1H NMR spectrum of U50488 corresponding to the cyclohexyl ring were simulated with the LAOCOON-based PANIC software, available in the Bruker library of computer programs, using an Aspect 3000 computer. The computer program accepts a maximum of nine nuclear spins and requires the input of coupling constant and chemical shift data. Line width, spectral width, and data size are adjustable parameters. Chemical shifts and ^2J and ^3J coupling constant values estimated from analysis of the 1D and phase-sensitive 2D COSY spectra were used as starting points for an iterative simulation of subspectra.

The 2D ^1H-^1H NOE, COSY, and DQF COSY spectra were recorded on the Bruker WP-200 SY FT spectrometer. The pulse sequences for data acquisition and phase cycling routines were those available in the Bruker library of programs. Typical acquisition parameters for the experiments were as follows: 90° pulse width; 3.4 μs; initial t_1 value, 3 μs; 64 transients per t_1 value; sweep width in f_1 and f_2, 1638.2 Hz; recycle delay, 3.5 s. The 2D NOE spectra were recorded with a mixing time of 0.8 s with random variation for suppression of cross-peaks due to zero order scalar (J) coupling. The 2D DQF COSY spectra required a presaturation pulse. Data processing included application of a sine bell window function in f_1 and f_2, 2D Fourier transformation, and symmetrization of the data about the diagonal.

Materials. U50488H (the $CH_3SO_3^-$ salt) was obtained from the Drug Supply Program of the National Institute on Drug Abuse (Bethesda, MD). D_2O (99.5%) was obtained from the Aldrich Chemical Co. (Milwaukee, WI). U50488H was dissolved in D_2O, thoroughly degassed, and sealed in high-quality 5-mm NMR tubes (728-pp; Wilmad Glass Co., Buena, NJ). All spectra were recorded at 21.7 °C.

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Mapping the Binding Site of Tissue Kallikrein: Preparation and Testing of All Possible Substrate Analog Inhibitors Homologous with the Sequence of Kininogen between Ser^{386} and Gln^{392} †

Milind S. Deshpande‡ and James Burton*

Unit for Rational Drug Design, Department of Medicine, The University Hospital, Boston, Massachusetts 02118.
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Programs aimed at converting peptide inhibitors of proteolytic enzymes into more traditional drug structures require an understanding of the role played by the individual amino acid residues in the inhibitor. To this end, all possible substrate analogues occurring within the sequence Ser^{386}-Pro-Phe-Arg-Ser-Val-Gln^{392} from bovine kininogen were synthesized and tested as inhibitors of tissue kallikrein (EC 3.4.21.35, β-PPK). Of the 21 sequences which can be formed from the heptapeptide, 11 have inhibitory constants which could be measured in the chromogenic assay employed in these studies. No dipeptide and only one tripeptide, Ac-Phe-Arg-Ser-NH_2 (K_i = 718 μM), measurably inhibits the enzyme. All longer peptides inhibit β-PPK. The heptapeptide Ac-Ser-Pro-Phe-Arg-Ser-Val-Gln-NH_2 is the most effective inhibitor in this series (K_i = 101 μM). Each amino acid residue in the sequence appears to alter binding in a relatively independent manner. The N-terminal seryl residue (P_4) and the prolyl residue (P_3) slightly improve the K_i of the various inhibitors. The phenylalanyl residue at P_2 appears to have a more pronounced effect on K_i. The arginyl residue at P_1 and the seryl residue at P_1' appear to be the most important residues in the inhibitory sequence. They contribute approximately one-third and one-fourth of the binding energy to the interaction between the substrate analogues and β-PPK, respectively. The valyl residue at P_2', and the C-terminal glutaminyl residue improve K_i of each of the peptides tested. Almost 80% of the binding energy of the substrate analogue inhibitors comes from the core sequence Phe-Arg-Ser which occurs between P_2 and P_1'. Molecular models developed from the Chen-Bode coordinates of the aprotinin-β-PPK complex have been used to interpret the results of these studies.

Introduction

Tissue kallikrein (EC 3.4.21.35) is a serine protease which releases kinins such as kallidin (lysylbradykinin) from low molecular weight kininogen.[1] Numerous functions have been assigned to kinins including regulation of blood flow in some organs,[2] the enhancement of pain,[3] and vascular leakage resulting from rhinitis.[4] Clinical evidence

† Abbreviations used: ACE, angiotensin I converting enzyme; BAEE, benzoyl-L-arginine ethyl ester; HUK, human urinary kallikrein (α-form); KKI, kallikrein inhibitor (Rational Drug Design Number); β-PPK, porcine pancreatic kallikrein (β-form); RUK, rat urinary kallikrein (α-form); S-2266, D-valylleucyl-arginyl-p-nitroanilide; PNA, 4-nitroaniline; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

* Unit for Rational Drug Design, E301, The University Hospital, 88 East Newton Street, Boston, MA 02118-2393.

‡ Current address: Bristol-Myers Squibb Pharmaceutical Research Institute, 5 Research Parkway, Wallingford, CT 06492.

(1) Bhoola, K. D.; Figuero, C. D.; Worthy, K. Bioregulation of Kinins: Kallikreins, kininogens, and Kininases. *Pharmacol. Rev.* **1992**, *44*, 1-80.
(2) Levinsky, N. G. The renal kallikrein-kinin system. *Circ. Res.* **1979**, *44*, 441-451.
(3) Steranka, L. R.; Manning, D. C.; DeHaas, C. J.; Ferkany, J. W.; Borosky, S. A.; Connor, J. R.; Vavrek, R. J.; Stewart, J. M.; Snyder, S. H. Bradykinin as a pain mediator: receptors are localized to sensory neurons, and antagonists have analgesic actions. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 3245-3249.
(4) Proud, D.; Reynolds, C. J.; Lacapra, S.; Kagey-Sobotka, A.; Lichtenstein, L. M.; Naclerio, R. M. Nasal provocation with bradykinin induces symptoms of rhinitis and a sore throat. *Am. Rev. Respir. Dis.* **1988**, *137*, 613-616.

0022-2623/92/1835-3094$03.00/0