Conformational Analysis of Three NK1 Tripeptide Antagonists: A Proton Nuclear Magnetic Resonance Study

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Two new peptides, tailored after Ac-Thr-D-Trp(CHO)-Phe-NMeBzl (TRI), namely, Ac-Thr-D-Trp(CHO)-Phe-NMeαMeBzl (TRA) and Ac-Thr-D-Trp(CHO)-Oic-NMeBzl (TOI), in which Phe is replaced by (3a*S*,7a*S*)-octahydroindole-2-carboxylic acid, proved more potent and selective NK1 antagonists. The conformational properties of all three compounds were investigated in solution by NMR spectroscopy and those of TRI analyzed in greater detail by means of systematic computer-assisted modeling. All conformers whose energy differs by less than 9 kcal/mol from the absolute minimum are different from the conformer proposed in a previous molecular modeling study by the discoverers of TRI. Parallel calculations for TRA and TOI yield low-energy conformers similar to those of TRI but in a slightly different order. Comparison of the shapes of low-energy conformers of all three peptides with those of four typical rigid NK1 antagonists shows that putative bioactive conformations are indeed present in solution.

Introduction

Tachykinins represent a class of important neuropeptides that exert several biological functions.[1] The three most well-known agonists, namely, Substance P (SP), neurokinin A (NKA), and neurokinin B (NKB), interact with distinct receptor subtypes named NK1, NK2, and NK3, respectively. NK1 agonists and antagonists[2−4] are implicated in crucial biological functions. The most relevant are probably those connected with the onset of asthma, notably with the so-called neurogenic inflammation mechanism.[5]

In this context, the design of new NK1 antagonists is always of considerable theoretical and practical interest. A fairly recent series of papers[6−8] described a new, potent NK1 antagonist, N^{α}-[N^{α}-(N^{α}-acetyl-L-threonyl)-N^{1}-formyl-D-tryptophyl]-*N*-methyl-*N*-(phenylmethyl)-L-phenylalaninamide, i.e., Ac-Thr-D-Trp(CHO)-Phe-NMeBzl (henceforth called TRI), and its congeners together with structure−activity relationship studies based on computer-assisted molecular modeling.[8] This study made no use of experimental data, either in solution or in the solid state, but started (deliberately) from an "arbitrarily selected" low-energy conformation.[8] Such a procedure may be questionable since it is difficult to avoid getting trapped in local minima, i.e., the most dangerous of the pitfalls in all search procedures.

In our experience, the search for potential bioactive conformations in solutions of small, flexible peptides is best performed by a combination of NMR spectroscopic investigations and MM (or MD) calculations.

We have examined the NMR spectra of TRI and two more potent analogs recently synthesized by some of us, Ac-Thr-D-Trp(CHO)-Phe-NMeαMeBzl (TRA), in which the C-terminal benzyl group is substituted by an α-methylbenzyl group,[9] and Ac-Thr-D-Trp(CHO)-Oic-NMeBzl (henceforth called TOI), in which Phe is replaced by (3a*S*,7a*S*)-octahydroindole-2-carboxylic acid (Oic),[10] and used these data as the starting point for a computer-assisted molecular modeling. A detailed conformational analysis was deemed necessary in our case also to try to explain the large increase in antagonist activity observed in going from the parent peptide (TRI, characterized by a pA_2 of 8.0) to TRA (with a pA_2 of 9.1) and TOI (with a pA_2 of 9.3).

Here we present an NMR study of all three compounds (TRI, TRA, and TOI) together with the detailed conformational analysis of TRI and the comparison of the resulting structures with the shape of rigid NK1 antagonists.

Chemistry

Oic was prepared according to the procedure of Vincent et al.[11] as described in the literature. The peptides TOI and TRA were synthesized by the conventional method in solution using the combination of *tert*-butyloxycarbonyl and fluorenylmethyloxycarbonyl protecting groups. The starting Fmoc- or Boc-X_{aa}-R compounds were produced from Fmoc- or Boc-X_{aa}-OH and the corresponding amine by the TBTU−HOBT method. The Boc-protected tripeptides were synthesized from Fmoc- or Boc-X_{aa}-OH derivatives according to the conventional way which comprises two cycles of deprotection of the Fmoc or Boc group with diethylamine in tetrahydrofuran (33%) and 4 N hydrochloric acid in dioxane and the subsequent coupling process with a Boc-protected amino acid. The introduction of a nonnatural amino acid (Oic) and D-residue (Trp) did not require exceptional coupling procedures, although activation with the WSCD−HOBT and TBTU method[7,12] was preferred to DCC−HOBT. The final compounds were obtained from the corresponding Boc-protected tripeptides by deprotection of the Boc group with 4 N hydrochloric acid in dioxane and subsequent acetylation with acetic anhydride. Crude peptide products were purified by reverse-phase HPLC to greater than 96%

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Table 1. pA_2 (±SEM) Values of the Analogs TRA and TOI and the Reference Peptide TRI Measured against the Standard Agonist SP in the GPI (NK-1), RC (NK-2), and RPV (NK-3) Bioassays

peptide	NK-1	NK-2	NK-3	n[a]
TRI[b]	8.0 ± 0.29	7.0 ± 0.40	5.8 ± 0.40	6
TRA[b]	9.1 ± 0.45	6.7 ± 0.31	5.0 ± 0.42	6
TOI[c]	9.3 ± 0.18	8.4 ± 0.35		6

[a] n = number of independent experiments. [b] Taken from ref 9. [c] Taken from ref 10.

purity. The reference compound was synthesized according to Hagiwara et al.[7]

All final compounds were characterized by FAB mass spectroscopy, reverse-phase HPLC, and amino acid analysis. The resulting compounds were then tested in three bioassay preparations: the guinea pig ileum longitudinal smooth muscle preparation (GPI), the rat colon muscolaris mucosae (RC), and the rat everted portal vein (RPV), representative of the NK-1, NK-2, and NK-3 receptor types, respectively.

The results, reported as pA_2 values,[9,10] are summarized in Table 1 and compared to the corresponding values of TRI, i.e., the antagonist Ac-Thr-D-Trp(CHO)-Phe-CONMeBzl (FR113680) taken as reference drug.

NMR

All peptides were examined both in DMSO-d_6 at room temperature and in a 90:10 (v:v) DMSO-d_6/water cryomixture at low temperature. Sequential assignment of all protons was straightforward and was achieved by standard methods[13] via the usual systematic application of DQF-COSY,[14] TOCSY,[15] and NOESY[16] experiments. The use of cryomixtures at low temperature often results in a great improvement of the quality of the NOESY spectra even with respect to ROESY[17] experiments at room temperature, with a selective enhancement of diagnostically valuable effects, mainly among backbone protons.[18]

In spite of the simplicity of the chemical constitution, the spectra of all three peptides are rather complex. Figure 1 shows the low-field part of a typical NOESY spectrum of TRI in the 90:10 (v:v) DMSO-d_6/water cryomixture at 273 K. In the NOESY spectrum of TRI in the 90:10 (v:v) DMSO-d_6/water cryomixture at 273 K, the number of resonances is twice-doubled with respect to those expected from the number of protons of the structural formula: The observed signals stem from the presence of cis−trans isomers around the formyl bond and around the terminal amide bond. The four isomers have different populations, slightly influenced by the isomerism due to the formyl group and with the two isomers containing either a trans or cis terminal amide bond in the ratio of 7:3, respectively. We have concentrated our efforts in the study of the trans isomer only since the NOEs of the other isomers are very similar. The spectra of TRA are virtually identical with those of TRI, except of course for the resonance of the additional methyl group and its interactions with other signals.

The complexities introduced by cis−trans isomerism around amide bonds become nearly impossible to cope with in the case of TOI, for which the spectra do show the expected eight isomers due to the additional isomerism afforded by Oic (Figure 1). We decided to study the two main isomers of TRI in greater detail, both experimentally and by computer analysis, and then test the consistency of the results with the data of the other two peptides.

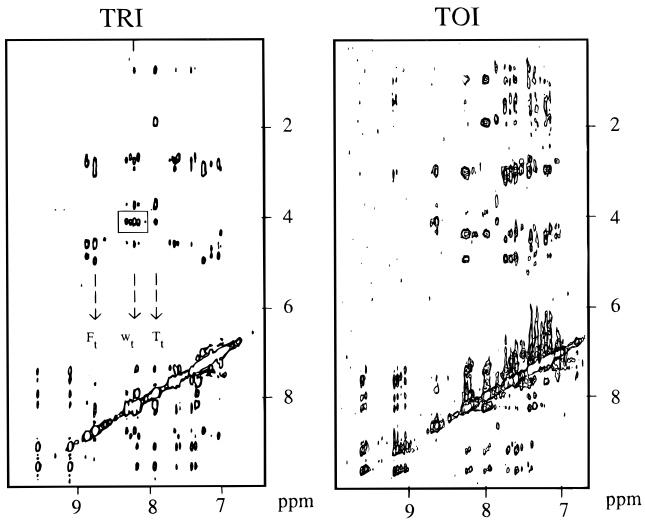

Figure 1. Comparison of the low-field portion of the 500 MHz ^{1}H NOESY spectra of Ac-Thr-D-Trp(CHO)-Phe-NMeBzl (TRI) and Ac-Thr-D-Trp(CHO)-Oic-NMeBzl (TOI) in a 90:10 (v:v) DMSO-d_6/water cryomixture at 273 K, mixing time 300 ms. The boxed resonances in the spectrum of TRI correspond to the cross-peaks of Thr CH_α with the D-Trp NH's of the four isomers. The positions corresponding to the NH resonances of Thr_t, D-Trp_t, and Phe_t are indicated by arrows.

Table 2. Relevant Chemical Shift Data[a] of the Two Families of TRI Isomers Generated by Cis−Trans Isomerism around the Terminal Amide Bond

	Thr_t	Thr_c	D-Trp_t	D-Trp_c	Phe_t	Phe_c
NH	7.90	7.90	8.18	8.12	8.73	8.84
α	4.05	4.01	4.60	4.60	4.90	4.82
β	3.70	3.60	2.80/2.60	2.70/2.60	2.99/2.77	2.80/2.75
γ	0.58	0.54				
others			H_{ald} 9.1	H_{ald} 9.5		

[a] Referred to residual DMSO at 2.5 ppm.

Table 2 summarizes chemical shift data of the two main isomers of TRI. An analysis of the intensities of the main cross-peaks in the NOESY spectrum of TRI shows that the pattern is identical for the two isomers, indicating that these isomers do not differ substantially in their conformational preferences, i.e., the cis−trans difference has but marginal influence on the conformation of the whole molecule.

The number of observable NOEs, however, is too small to justify an interpretation based on a single, rigid structure. Nonetheless we tested the hypothesis of a single ordered structure by means of MD calculations that included all backbone NOEs (both observed and not observed) as distance constraints. Not observed NOEs were used to generate lower limit distances of the order of 4 Å. The results of the MD calculations show that there are several different structures consistent with experimental NOEs. However, unconstrained minimization does not discriminate among them and, worst of all, reveals that a few of the constraints used in the MD procedure are not entirely conserved. Accordingly, we pursued an analysis that can afford a reasonable number of low-energy structures to be compared, a posteriori, to the NMR data.

Molecular Mechanics Calculations

In the case of a tripeptide it is generally possible to perform a molecular mechanics (MM) study, consisting

of a full-grid search followed by energy minimization (e.g., see ref 19). Alternatively, one can use statistical methods such as the Monte Carlo method. Both approaches yield a satisfactory description of the global conformational state of flexible molecules. The average conformational state, however, may be peculiarly uninteresting when one is searching for specific bioactive conformers. Besides, in our case, the number of conformers is augmented by the two quoted cis−trans isomerisms so that we have 15 torsional angles to deal with in the search, a fairly high number for a full search. In fact, our attempts led to unwieldy numbers of conformers of comparable energy.

On the other hand, in a combined study of NMR spectroscopy in solution and model building based on semiempirical energy calculations, it is always preferable, even on a purely speculative basis, to tailor the search on the available NMR data. Thus, we made the maximum possible use of both observed and absent NOEs. When using NOE-derived constraints corresponding to short backbone distances, we must include, in all possible averages, also conformers containing long distances since, by the very nature of the dependence of NOEs from interproton distances, they will contribute negligibly to the observed intensity, that is, they are nearly invisible. Typically, a short sequential d_{NH-NH} hints the presence, in the conformational mixture, of folded conformers but, at the same time, does not preclude the presence of extended conformers characterized by a long sequential d_{NH-NH} since such a conformer would be "invisible" by NMR. On the other hand, when "absent NOEs" are concerned, that is, nonobserved effects, it is safe to exclude from all possible averages conformers containing short distances since, owing to the inverse sixth power dependence, even small populations would contribute significantly to the observed intensity. In spite of these limitations the number of conformers allowed for a tripeptide is very large and can only be dealt with by specialized search programs.

It is possible to show that, by an appropriate combination of the above-described criteria on absent and observed NOEs with a peculiar choice of torsion angles, it is possible to reduce the number of conformers to numbers manageable by direct procedures. In other words, it is possible to find a collection of structures representative enough of the whole conformational space and use them as starting structures for minimization procedures in a MM or MD calculation.

Minisearch. The choice of low-energy conformers based on a complete grid search, even for a mere tripeptide, is a very time-consuming procedure. On the other hand, if one needs starting structures for extensive unrestrained minimizations or for MD calculations, the choice of full-grid search may be unduly redundant and, in the case of a small sequence, can be profitably substituted by an ad hoc choice of internal rotation angles.

In all MD calculations performed in our laboratory on small peptides, we have consistently observed a regularity in the pairs of accessible ϕ,ψ angles for a given residue, that is, the pairs are invariably close to those of canonical turns or extended conformations. Thus one can take, for ϕ angles, all values foreseen in canonical turns (−60°, −90°, 80°, 77°, and −77°) plus a value typical of extended structures (−120°). Correspondingly, we have for ψ the values: −30°, 0°, −65°, 65°, and 120°. At first sight there is not a great improvement with respect to a normal search procedure: The six ϕ angles and the five ψ values are equivalent to a grid with a fairly coarse mesh. However, it is possible to further simplify these choices.

The first simplification, considering that we are simply looking for starting structures, is to unify several slightly different values using averages. Thus, we chose a single value of ±70° in lieu of −60°, ±65°, ±77°, 80°, and −90° and a value of 0° for both 0° and −30°. If we consider that these values originate from canonical turns, that is, the most significant structures for short peptides, we can easily impose a further limitation by choosing ϕ,ψ pairs instead of single independent ϕ or ψ values. With these rather drastic but sensible limitations, we have the simple menu of the following pairs for ϕ,ψ values: −70°,0° (henceforth dubbed ζ), −70°,-120° (η), 70°,0° ($-\zeta$), 70°,−70° (γ), −70°,70° ($-\gamma$), and −120°,120° (ϵ). In this simple shorthand notation the sequence ζ,ζ corresponds to both type I and type III β-turns, and the sequence $\eta,-\zeta$ corresponds to a type II β-turn, whereas γ, $-\gamma$, and ϵ correspond to a γ-turn, an inverse γ-turn, and an extended conformation, respectively. In the case of an R residue the corresponding six pairs of ϕ,ψ values are $-\zeta$, $-\eta$, ζ, γ, $-\gamma$, and $-\epsilon$. In a case like that of our tripeptide, these considerations limit enormously the number of starting conformations, but we still have rather large numbers for a direct investigation: 729 conformations if we ignore the contributions from cis−trans isomerisms and side chain conformations.

In order to pursue the main goal of our work, that is, to find conformers of potential biological significance (e.g., those that may bear resemblance with known antagonists) among those present in the conformational mixture in solution, we can make use of the experimental NMR data. Thus, we can restrict our investigation to conformers whose terminal amide is trans (since cis isomers are far less stable) and to either a cis or trans formyl conformer (since they are essentially isoenergetic), provided a suitable a posteriori check tells us that this isomerism does not significantly alter the global conformations. It must be noted that all these approximations would be too rough if our goal were the accurate description of the conformational mixture in solution. However, it is necessary to emphasize that we are simply looking for the most relevant conformations consistent with experimental data.

Furthermore, we can restrict our sample of starting conformations to those that have at least one short NH−NH distance. This choice reduces the number of structures to a mere 120. Since this number is quite reasonable for any MM or MD approach, we chose not to introduce any further limitation. It is better to make a preliminary selection on the basis of energy criteria and then discriminate among the final conformers on the basis of their consistency with experimental data.

The 120 structures underwent totally unrestrained energy minimization up to a rms gradient of at least 10^{-4} to yield a wide spread of energies. The torsion angles and relative energies of the best structures within a range of 15 kcal/mol are reported in Table 3. None of them can account for all experimental NMR data, in particular for all observed and absent NOEs, but there are several possible mixtures of the lower

Table 3. Relevant Torsion Angles of the Minimum Energy Conformers of TRI

conf	E	ϕ_1	ψ_1	χ_1^1	ϕ_2	ψ_2	χ_1^2	ϕ_3	ψ_3	χ_1^3
XIV	0	−84.8	38.5	−54.6	81.5	−64.0	173.7	−132.6	61.1	−57.4
IV	0.5	−75.1	−21.5	−61.9	87.3	−40.0	50.1	−116.4	99.8	175.3
XV	0.8	−70.8	−8.9	−62.1	−71.8	−2.0	165.3	−128.1	61.0	−56.7
VI	1.9	80.4	−50.6	−164.7	85.7	−26.3	−49.2	−103.4	116.6	174.9
XIII	2.7	−65.9	−11.3	−66.2	−65.7	−7.8	47.4	−121.6	80.2	178.6
I	2.9	−84.2	3.47	−59.5	82.7	−18.1	42.9	−71.2	−39.8	177.4
X	5.7	−80.1	−7.0	−167.2	86.8	−65.1	55.4	−74.3	−47.9	178.1
XI	6.1	−71.3	118.7	180.0	105.8	−33.7	57.1	−134.9	60.7	−177.3
II	6.3	−78.4	76.6	−169.8	88.0	−1.1	51.88	−69.8	−37.6	−179.2
V	7.0	−79.6	69.2	180.0	132.5	−24.4	54.4	−131.9	65.2	−176.0
III	7.2	79.5	−61.1	−166.8	74.7	−5.0	54.2	−69.9	−37.5	−173.8
VII	9.4	−77.5	2.6	−57.6	80.5	5.4	54.8	77.8	−100.6	−53.2
VIII	11.8	−80.4	68.6	−169.1	93.8	−1.1	53.2	72.3	−103.7	−56.1
XII	13.0	−80.9	−6.7	−60.0	87.2	−55.9	60.2	60.8	−103.1	−172.9
IX	13.2	78.0	−64.1	−166.0	82.6	−11.4	52.2	80.6	−101.8	−53.1

Table 4. Comparison of Representative NOEs with Corresponding Distances in Relevant Conformers of TRI

Thr	D-Trp	Phe	intensity	XIV	IV	XV	VI	XIII	I
	NH	NH	m	3.66	3.10	2.63	2.87	2.70	2.80
NH	NH		m	3.14	2.44	2.56	3.49	2.67	2.52
	a	NH	m	2.45	2.77	3.18	2.98	3.12	3.10
α	NH		m	2.84	3.50	3.39	3.60	3.42	3.29
β	NH		m-w	4.58	4.00	4.25	2.59	4.16	4.41
CH_3	NH		w	5.0/5.2/6.0	4.5/5.2/5.3	4.7/5.3/5.6	4.0/4.8/4.9	4.7/5.3/5.6	4.7/5.3/5.7

energy structures that can account for the whole set of experimental data. First of all we have discarded all conformers that contain short distances corresponding to "absent NOEs"; subsequently we have simply restricted the choice of a significant sample to the smallest possible number of low-energy structures: They can be combined to yield a reasonable agreement with experimental data. The qualitative comparison between a representative sample of calculated distances of the six best conformers and corresponding NOE-derived ranges is reported in Table 4. It can be appreciated that several pairs of low-energy conformers have crucial interproton distances that are nicely complementary in comparison with experimental NOEs.

Discussion

Figure 2 shows the molecular models of conformers XIV, IV, and XV. It is interesting to note that none of the low-energy conformers consistent with the NMR data bears any resemblance with the conformers proposed in the quoted molecular modeling study by Hagiwara et al.[8] Actually, only the less stable conformers of Table 3 (VII−IX) are similar to the reference structure of TRI.[8] On the other hand, the four lowest energy conformers (XIV, IV, XV, and VI) resemble the shape of well-known rigid antagonists[4,20−22] very closely. We chose three different molecules in order to increase the probability of finding putative bioactive conformations among our minimum energy structures: L-732,-244[4] and CP-96,345[20] that are very rigid molecules and L-732,138[22] that is only partially rigid but can be compared to our peptides in the conformation proposed by the authors.[17] Figure 3 shows the molecular models[23] of CP-96,345, L-732,244, and L-732,138. These nonpeptide Substance P antagonists were then compared to the three best conformers of TRI. Figure 4 shows the overlays of conformers XV and IV with L-732,244 and of conformer XIV with both CP-96,345 and L-732,138. These molecular superpositions were performed using the minimum possible number of atoms, not to force the fit; typically only three atoms per aromatic ring were employed in the calculation. It is easy to see that the superposition of the aromatic rings of nonpeptidic antagonists with corresponding rings of TRI is good in all cases. It is also interesting to note that in the comparisons XV/L-732,244, XIV/CP-96,345, and XIV/L-732,138 the aromatic rings of TRI involved in the overlays are those of Trp and Phe, whereas in the other, i.e., IV/L-732,244, the rings involved are those of Phe and the N-terminal benzyl group.

The similarity of the shape of the C-terminal moiety of the peptide with the shape of different rigid antagonists of somewhat different constitution points to the fact that the exact nature of the rings is less important than the global U-shaped pharmacophore. This observation is in line with the remark by Hagiwara et al.[8] that "... the amino acid residue adjacent to the Phe-NMeBzl structure can accept a wide variety of modifications irrespective of the nature of the substituent". Thus, it is of secondary importance that the indole rings of TRI and L-732,244 are not superposed in the overlay IV/L-732,244 of Figure 4, as long as both aromatic rings of L-732,244 are superposed to the two C-terminal aromatic rings of TRI.

Owing to the similarity of the NMR data of the other two peptides, we assumed that it was possible to use the 120 basic conformers of TRI as a meaningful starting point for a MM analysis. Therefore, we used the same backbone angles for the starting conformers of TRA and TOI in energy minimizations and checked whether the final structures were in the same relative energy order and consistent or not with the NMR data. Table 5 summarizes the results for TRA and TOI.

It can be seen that the lowest energy conformers are similar to those of TRI, although not in the same relative order. Besides, in the case of TOI the energy difference between the absolute minimum (XV_{TOI}) and the conformer (IV_{TOI}) nearest to it energywise is considerably higher than for the other two peptides: 3.2 vs 0.5 and 0.7 kcal/mol for TRI and TRA, respectively.

The overlay of the minimum energy conformer of TRA with L-732,244 is very similar to the corresponding one of Figure 4, consistent with the fact that the additional

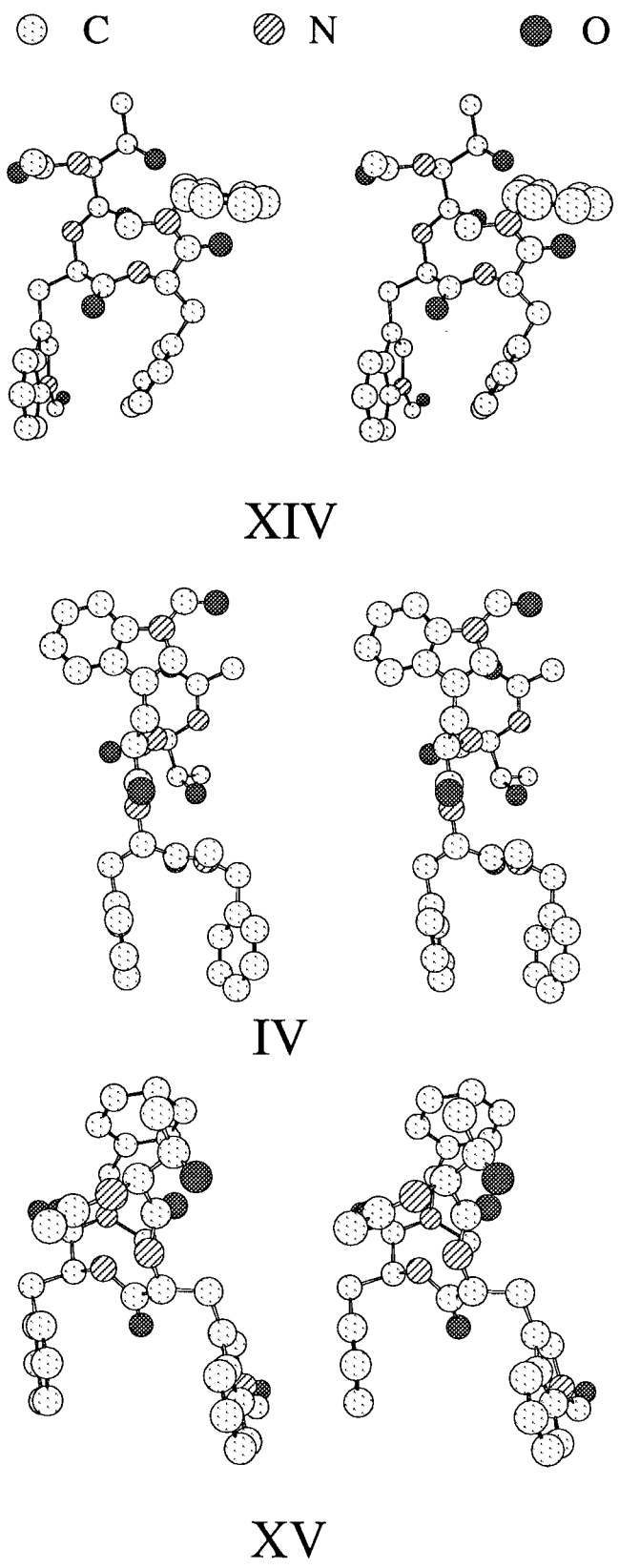

Figure 2. Stereoviews of the molecular models of the three conformers of TRI of lower energy (XIV, IV, XV).

methyl group seems not to interfere significantly with the stable conformations of TRI. The example shown in Figure 5 (IV_{tra}/L-732,244) is almost superimposable to the corresponding one of TRI reported in Figure 4. Thus, although it is reassuring to note that similar pharmacological profiles (Table 1) are paralleled by very similar conformational preferences, it is difficult to explain the increased activity of this peptide with respect to TRI (a pA_2 of 9.1 vs 8.0) on a conformational

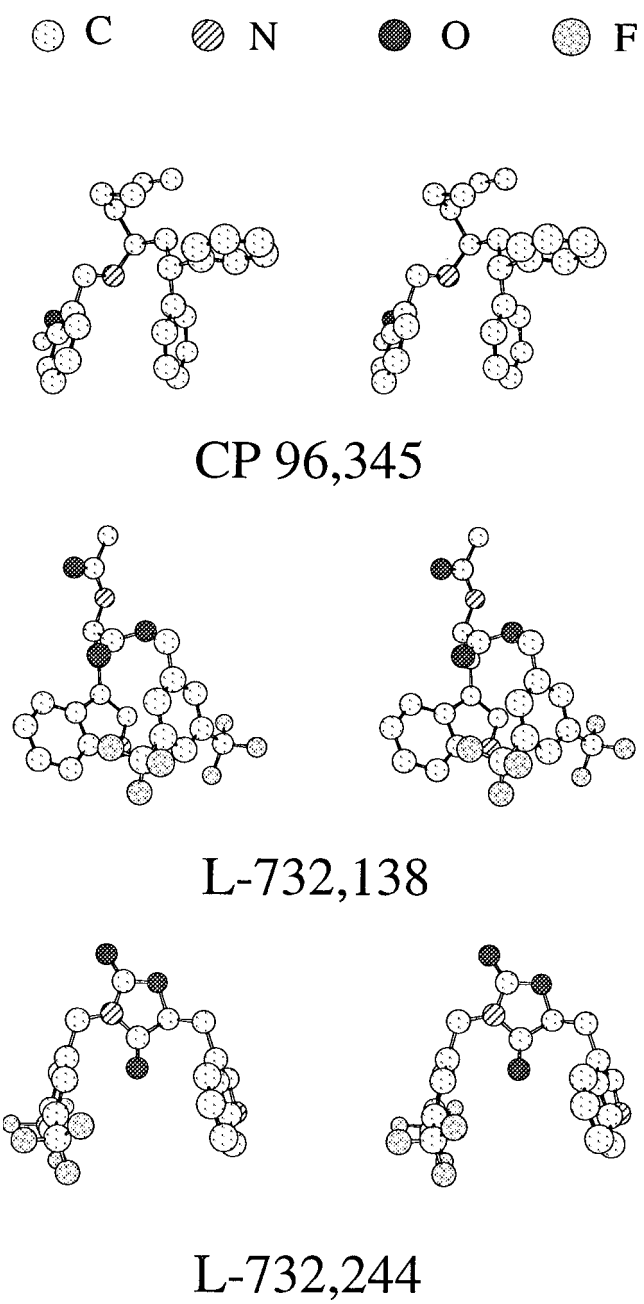

Figure 3. Stereoviews of the molecular models of three nonpeptide Substance P antagonists: CP-96,345, L-732,244, and L-732,138.

basis. It seems more likely to assume that the extra methyl group interacts favorably with a complementary niche of the receptor affording the additional interaction energy measured by the pA_2.

In the case of TOI, on the other hand, a good overlay could be obtained only in the case of the absolute minimum (XV_{TOI}). This finding, coupled with the fact that the energy differences between XV_{TOI} and any of the other conformers are higher than for TRI and TRA, hints that the requirements for the bioactive conformation of TOI are more specific.

Taking into account also the increased rigidity with respect to the other two peptides, the conformer XV_{TOI} appears to be a promising candidate for further antagonist design.

Summary

The biological activity of the tripeptide NK1 antagonist Ac-Thr-D-Trp(CHO)-Phe-NMeBzl (TRI, $pA_2 = 8.0$)

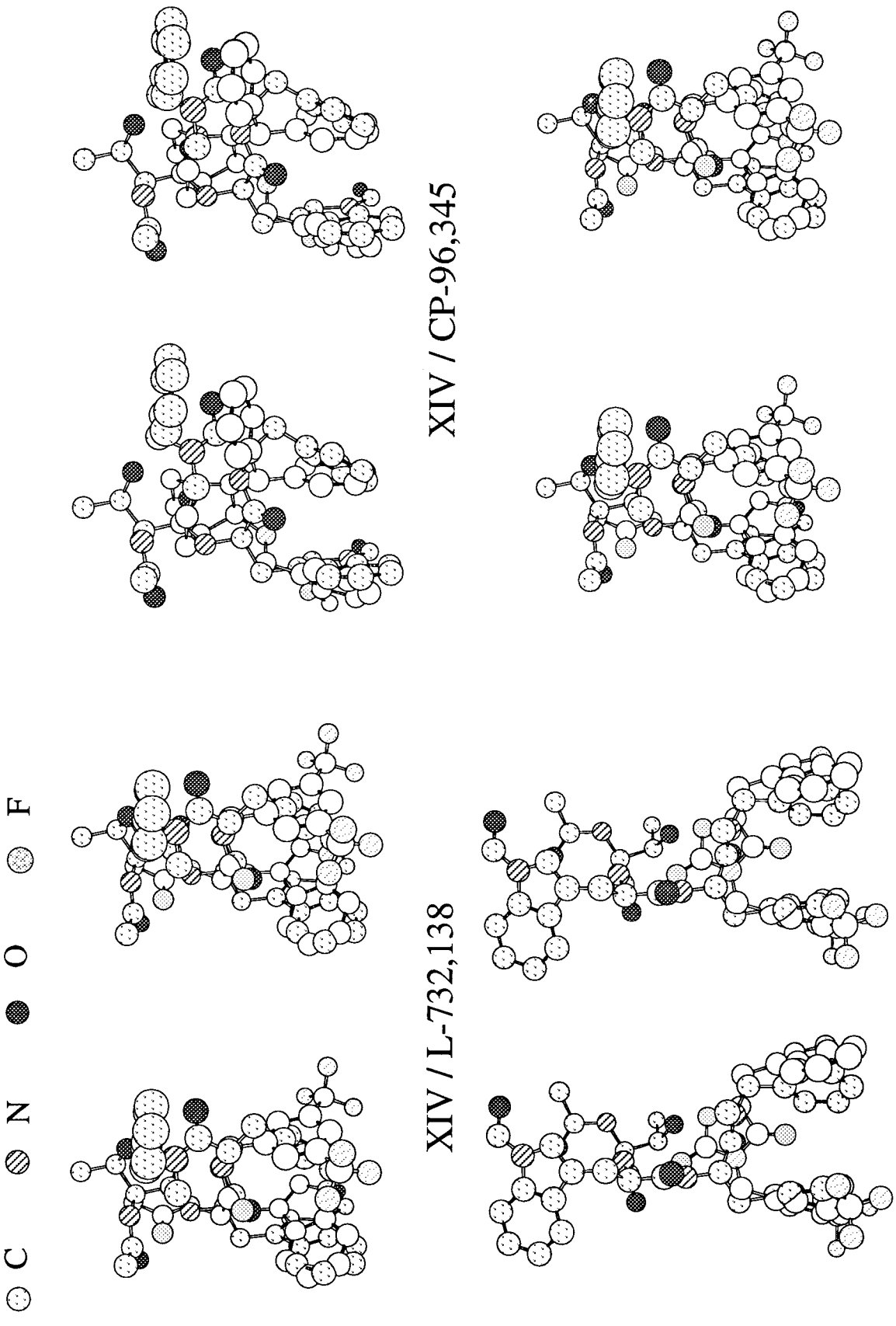

Figure 4. Stereoviews of the overlays of the molecular models of conformers XIV, XV, and IV with nonpeptide Substance P antagonists CP-96,345, L-732,244, and L-732,138. The atoms of the nonpeptide antagonists are represented as blank balls.

Table 5. Relevant Torsion Angles of the Minimum Energy Conformers of TRA and TOI

conf	E	ϕ_1	ψ_1	χ_1^1	ϕ_2	ψ_2	χ_1^2	ϕ_3	ψ_3	χ_1^3
IV_{TRA}	0.0	−72.7	−27.3	−64.3	87.4	−50.7	49.7	−101.0	115.5	177.2
XV_{TRA}	0.7	−70.0	−10.9	−63.4	−73.9	−1.3	166.8	−129.9	56.1	−55.2
VI_{TRA}	0.9	78.7	−41.0	−164.7	86.0	−29.1	−49.5	−111.3	102.7	172.0
XIV_{TRA}	1.3	−85.1	45.2	−55.4	84.5	−65.0	171.9	−135.7	50.7	−57.2
XV_{TOI}	0.0	−91.2	−38.3	−160.8	131.5	−66.4	−54.0	−77.8	141.2	
IV_{TOI}	3.2	−85.9	38.3	−159.3	70.2	37.2	51.1	−74.1	146.7	
$XIII_{TOI}$	3.8	−84.1	51.5	−163.5	−62.2	−37.8	40.0	−104.5	80.9	
VI_{TOI}	4.1	60.1	−76.6	−52.2	77.7	40.3	180.1	−74.1	141.6	

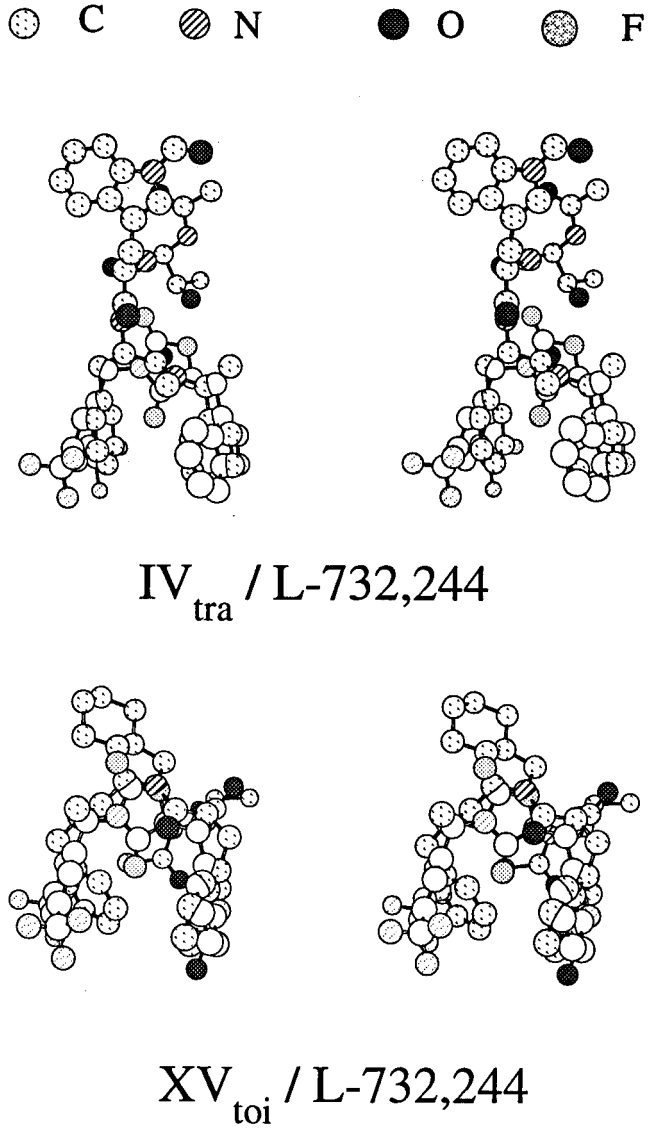

Figure 5. Stereoviews of the overlays of the molecular models of conformers IV_{TRA} and XV_{TOI} with nonpeptide Substance P antagonist L-732,244. The atoms of the nonpeptide antagonists are represented as blank balls.

can be considerably increased by inserting a methyl group in the terminal benzyl group (TRA, pA_2 = 9.1) or by substituting Phe with Oic, a cyclic residue (TOI, pA_2 = 9.3).

We have tried to explain this increase by finding likely bioactive conformations for these peptides. The low-energy conformers resulting from a detailed conformational analysis are indeed consistent with all known rigid nonpeptide NK1 antagonists. The conformational differences can be the main cause of the different activity between TOI and TRI, but in the case of TRA the differences are negligible; thus, it is more likely that the different activity can be ascribed to a specific interaction of the extra methyl group with the receptor.

Experimental Section

Materials and Bioassays. The peptides Ac-Thr-D-Trp-(CHO)-X_{aa}-N(CH_3)CH(R)-Ph (R = H, X_{aa} = Phe, TRI; R = H, X_{aa} = Oic, TOI; R = CH_3, X_{aa} = Phe, TRA) were synthesized by conventional solution methods using the combination of *tert*-butyloxycarbonyl and fluorenylmethyloxycarbonyl protecting groups as previously reported.[7,9−10]

Final compounds were tested in three bioassay preparations:[9] the guinea pig ileum longitudinal smooth muscle preparation (GPI), the rat colon muscolaris mucosae (RC), and the rat everted portal vein (RPV), representative of the NK-1, NK-2, and NK-3 receptors.

^{1}H NMR. Samples were prepared by dissolving appropriate amounts of each peptide in DMSO-d_6 and diluting to a final concentration of 1 mM for both the DMSO-d_6 and the DMSO-d_6/water (90:10, v:v) cryomixture solutions. NMR spectra were run at 500 MHz on a Bruker AMX-500 instrument. One-dimensional (1D) NMR spectra were recorded in the Fourier mode, with quadrature detection, and the water signal was suppressed by a low-power selective irradiation in the homogated mode. DQF-COSY,[14] TOCSY,[15] NOESY,[16] and ROESY[17] experiments were run in the phase-sensitive mode using quadrature detection in ω_1 by time-proportional phase incrementation of the initial pulse.[24] Data block sizes were 2048 addresses in t_2 and 512 equidistant t_1 values. Before Fourier transformation, the time domain data matrices were multiplied by Lorentz−Gauss functions in the F_2 dimension and by a shifted sine-bell in the F_1 dimension. Mixing times of 70 and 120 ms were employed for TOCSY and ROESY experiments, respectively. NOESY experiments were run at mixing times in the range 100−300 ms. NOEs of potential diagnostic value were translated into interatomic distances by the method of Esposito and Pastore[25] using the distances between the CH_2 protons of terminal benzyl (0.180 nm) for calibration, but only intervals were used for comparison with calculated structures. The full list of observed NOEs and absent NOEs employed in model building is reported in Table 6. The intervals corresponding to medium, medium-weak, and weak NOEs of Tables 4 and 6 are 2.75−3.50, 3.0−4.0, and 3.5−4.5 Å, respectively.

Molecular Mechanics and Molecular Dynamics. Energy calculations were based on the all-atom parametrization of the AMBER force field[26,27] (as implemented in the SYBYL package). Tentative fits of experimental constraints to a single low-energy conformer were performed through a combination of restrained simulated annealing (SA) MD calculations, combined with local conformational searches (CS) and final restrained and unrestrained energy minimizations (EM). Both SA and EM calculations have been performed with a distance-dependent dielectric constant ($\epsilon = r$) and no distance cutoff for nonbonded interactions. Distance restraints from NOESY spectra have been applied by using a harmonic potential outside the fixed distance ranges, with a force constant value of 100 kcal mol^{-1} Å^{-2}, while a null-restraining potential is applied inside that range. The harmonic term is switched to a linear one when the absolute value of a calculated violation exceeds 0.5 Å at either range limits. The computational procedure for energy minimizations was divided into two steps: a preliminary calculation, using a quasi-Newton method, stopping when the gradient norm is 10^{-3} or less and a final refinement obtained using a full Newton−Raphson minimization with a convergence criterium on the gradient norm comprised between 10^{-4} and 10^{-6}.

Table 6. List of Observed and Absent NOEs of TRI Employed in Model Building[a]

				NOEs	
Thr	D-Trp	Phe	benzyl	obsd	absent
NH	NH			m	
NH	α				ADC[b]
NH	*β*, *β*′				ADC
NH		NH			ADC
NH		α			ADC
NH		*β*, *β*′			ADC
α	NH			m	
α	α				ADC
α	*β*, *β*′				ADC
α		NH			ADC
α		α			ADC
α		*β*, *β*′			ADC
α			*β*, *β*′		ADC
β	NH			m-w	
CH_3	NH			m-w	
NH-α				s-m	
NH-*β*				s-m	
NH-CH_3				m	
α-CH_3				m	
α-*β*				m	
	NH	NH		m	
	α	NH		m	
	NH	α			ADC
	NH	*β*, *β*′			ADC

[a] Observed values are derived from spectra of 1 mM solution in a DMSO-d_6/water (90:10, v:v) cryomixture. [b] ADC stands for absent distance constraint.

During SA runs, in addition to the molecular temperature, also the value of the restraining potential force constant was varied, using a time-dependent weight function. The system was coupled to a temperature bath at 300 K with a coupling constant $\tau_T = 0.1$ ps. The equations of motion were integrated using the Verlet algorithm[28] with an integration step of 1 fs and with starting random velocities. The bond lengths of bonds with hydrogen were kept rigid using the SHAKE method.

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