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Conformational study of eight-membered diazocine turn mimics by two-dimensional NMR spectroscopy

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Abstract The eight-membered ring conformations of two diazocine turn mimics, methyl-[2,5-dioxo-3-(*S*)-(3-ω-tosyl-guanidino-propyl)-4-methyl-octahydro-1,4-diazocin-1-yl]acetate (**I**) and methyl-[2,5-dioxo-3-(*S*)-(3-ω-tosyl-guanidino-propyl)-octahydro-1,4-diazocin-1-yl]acetate (**II**), were determined using torsion angle constraints derived from $^3J(C,H)$ coupling constants extracted from ^{13}C-filtered TOCSY spectra with ^{13}C in natural abundance. For **I**, the torsion angle constraints derived from $^3J(C,H)$ coupling constants were in agreement with torsion angle constraints derived from $^3J(H,H)$ coupling constants extracted from a P.E.COSY spectrum. Similar $^3J(C,H)$ coupling constants were found for **I** and **II**, and they shared an identical eight-membered ring conformation characterized by two *cis*-amide bonds and a staggered conformation of the trimethylene group in which the H3 proton is proximal to both the H6 and H8 protons.

Key words: NMR; Coupling constant; Conformational mimetics; Arg-Gly-Asp (RGD) peptide; Molecular modeling

1. Introduction

Synthetic peptides containing the Arg-Gly-Asp (RGD) sequence inhibit the binding of fibrinogen to its receptor, GP IIb/IIIa, resulting in inhibition of platelet aggregation and thrombus formation. The introduction of local and regional constraints, such as cyclic amides and disulfides, have resulted in potent inhibitors of platelet aggregation [1–3]. Extensive analysis of a series of cyclic RGD-containing peptides by high resolution NMR, X-ray crystallography and molecular modeling, has led to the development of a pharmacophore model of the peptide receptor interaction [4]. This model consists of a turn around Arg, an extended Gly and a γ-turn around Asp. Our initial efforts to confirm this model involved the replacement of the C-terminal region of the peptide with a γ-turn mimetic [5]. This mimetic-containing analog retained both affinity and anti-aggregatory activity. With this information in hand we shifted our focus to the conformation about the Arg residue. One mimetic of the proposed turn about the Arg residue involved an eight-membered diazocine ring that bridged the *N*-acyl carbonyl of the Arg to the N–H of the Gly with a three carbon bridge. The two compounds, methyl-[2,5-dioxo-3-(*S*)-(3-ω-tosylguanidino-propyl)-4-methyl-octahydro-1,4-diazocin-1-yl]acetate (**I**) and methyl-[2,5-dioxo-3-(*S*)-(3-ω-tosylguanidinopropyl)-octahydro-1,4-diazocin-1-yl]acetate (**II**) (Fig. 1), differ only in their substituent at the diazocine position 4 nitrogen, which is an important substitution in the cyclic peptide antagonists [3]. Here, using high resolution NMR techniques, we have investigated the conformations of the two diazocine turn mimics which were later incorporated into GPIIb/IIIa peptide antagonists. Of particular interest was the single substitution of a methyl group for a proton that resulted in a tenfold higher affinity of the resulting 4-Me- compared to 4-H-containing mimetic analogs for the GPI/Iii receptor (unpublished results). It was necessary to determine if the difference observed in the antagonistic potency of these analogs was related to constitutional (Me for H) or, perhaps, conformational differences.

The eight-membered ring conformation of these two molecules can be determined by measuring vicinal homonuclear and heteronuclear coupling constants along the trimethylene portion of that ring and by measuring interproton NOE intensities between the diazocine methine proton and the protons of the trimethylene group. $^3J(H,H)$ coupling constants were measured using the P.E.COSY experiment, and interproton distances were calculated from ROESY buildup curves. For compound **II**, unfortunately, partial overlap of the central H7 and H7′ protons made it impossible to measure $^3J(H,H)$ coupling constants from a P.E.COSY spectrum. We therefore used a ^{13}C-filtered TOCSY experiment, capitalizing on the chemical shift dispersion among the H6, H6′, H8, and H8′ protons, to measure heteronuclear $^3J(C,H)$ coupling constants with ^{13}C in natural abundance in both compounds **I** and **II**.

2. Materials and methods

The preparation of **I** and **II** has been described [6,7]. Chloroform-d (99.96%) was obtained from Cambridge Isotope Laboratories, Woburn, MA. The sample concentrations for **I** and **II** were 30 mM in $CDCl_3$ for the P.E.COSY experiments and 90 mM in $CDCl_3$ for the ^{13}C-filtered TOCSY experiments.

The NMR data were collected on a Bruker AMX-500 spectrometer and processed using the FELIX software package (BIOSYM, Inc.). All one-dimensional (1D) ^1H NMR spectra collected at various temperatures were recorded with a sweep width of 3816 Hz and 32K complex points. 1,024 × 4,096 complex data matrices were recorded for P.E.COSY spectra [8,9] at 293 K and 220 K. These matrices were zero filled and Fourier transformed to yield 4K × 4K matrices with digital resolution of 0.93 Hz along both frequency dimensions. 256 × 2,048 complex data matrices were recorded at 293 K for ROESY spectra [10] using mixing times varying from 15 ms up to 120 ms, and with a spin locking field of 2,500 Hz. The data matrices were processed to yield 2K × 2K matrices with digital resolution of 1.96 Hz along both

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Abbreviations: NMR, nuclear magnetic resonance spectroscopy; TOCSY, total correlation spectroscopy; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy; P.E.COSY, primitive exclusive correlation spectroscopy.

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frequency dimensions. 384 × 2,048 complex data matrices were recorded at 293 K for the ^{13}C-filtered TOCSY experiments [11–13] with a mixing time of 44 ms. An in-house TOCSY mixing pulse sequence with an improved resonance offset efficiency compared to the MLEV-17 [14] and DIPSI-2 [15] pulse sequences (to be published) was used. The ^{3}J(C,H) coupling constants were extracted from strip-Fourier-transformed spectra that had a digital resolution of 0.5 Hz in both frequency dimensions. For distinguishing negative and positive frequencies along the ω_1 frequency dimension the States method was used for the P.E.COSY and ROESY experiments, and the TPPI method was used for the ^{13}C-filtered TOCSY experiments.

The P.E.COSY crosspeak multiplet pattern for a particular pair of spins belonging to K mutually coupled spins consists of the superposition of 2^{K-2} displaced two-spin antiphase square patterns. The peak separation of the two-spin antiphase square pattern along ω_1 and ω_2 is determined by the active coupling of that particular crosspeak. The coupling constants between the two spins and their mutually coupling partners can be directly extracted from the K-2 displacements vectors [16]. For the six-spin system of the trimethylene group of the eight-membered ring of **I**, the multiplet pattern consists of up to 16 square patterns with, in principle, four displacement vectors. ^{2}J(H,H) and ^{3}J(H,H) coupling constants were extracted from the eight vicinal crosspeaks. The extraction was done in combination with simulation of these crosspeaks using the MATLAB software package (The MathWorks, Inc.) and the extracted coupling constants.

Torsion angles were calculated from the ^{3}J(C,H) and ^{3}J(H,H) coupling constants using appropriate Karplus relationships [17,18]: the Karplus relation for the heteronuclear vicinal coupling constants were based on the ^{3}J(C,H) of propane [18]. Torsion angle constraints were set to allow a rotation of ± 30° about the calculated value. Interproton distances were calculated from initial buildup rates of interproton crosspeak volumes in the ROESY spectra, as has been done previously [19]. The buildup rates of the geminal proton crosspeak volumes of the trimethylene group of the eight-membered diazocine ring were taken as a rate corresponding to a fixed distance of 1.75 Å.

The conformational search was performed by MacroModel (Version 4) [20]. In both constrained and unconstrained searches, 1,000 conformers were generated by Monte Carlo simulation and then minimized within a $CHCl_3$ matrix using the MM2 force field. Conformers within 50 kJ/mol of the global minimum were saved. For the unconstrained search, side chains were simplified to methyl groups to focus on the eight-membered ring conformation. The constrained search was run on both the simplified and complete molecules.

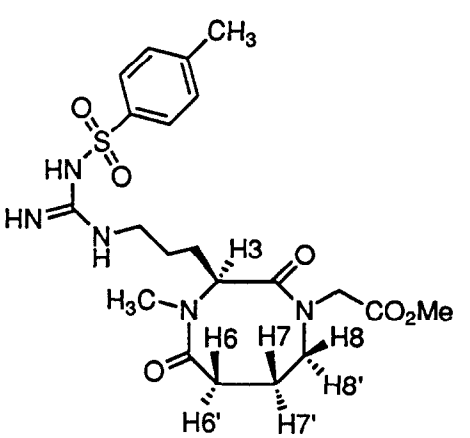
3. Results and discussion

The 1D ^{1}H NMR spectra of **I** at different temperatures are shown in Fig. 2. By reducing the temperature, it is sometimes possible [21] to detect additional conformations which are slowly exchanging on a time scale or with energy barriers that are potentially visible with the temperature range that we used. As the temperature is lowered from 293 K to 220 K, one observes the progressive appearance of two sets of resonances which is general and not restricted to any particular region of the molecule. The ^{1}H NMR assignments of **I** at 293 K and 220 K and **II** at 293 K are listed in Table 1. The two sets of resonances observed in the spectra of **I** at 220 K are not the result of conformational averaging within the eight-membered ring backbone; simulation of the crosspeak multiplets of **I** at 220 K using the same coupling constants measured at 293 K was in good agreement with the experimental crosspeaks (Fig. 3). Furthermore, as shown in Fig. 3b, the trimethylene protons of the eight-membered ring of the major and minor conformers of **I** at 220 K, within experimental error, have similar multiplet patterns. It is likely that geometrical isomerism at a C–N bond, possibly originating from resonance hybridization at the guanidino group, is responsible for the observed duplicity of resonances.

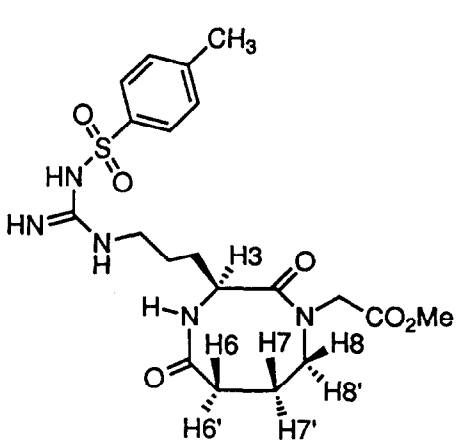
Table 1
Proton chemical shifts of **I** and **II**

Protons	**I**, δ in ppm 293 K	**I**, δ in ppm 220 K major	**I**, δ in ppm 220 K minor	**II**, δ in ppm 293 K
H8	2.88	3.01	2.94	2.78
H8′	2.58	2.59	2.59	2.52
H7	1.89	1.94	1.94	1.94
H7′	1.78	1.78	1.77	1.91
H6	3.98	4.12	4.01	4.04
H6′	3.18	3.22	3.21	3.29
NMe/NH	2.74	2.73	2.70	6.61
H3	4.73	4.80	4.74	4.46
Hβ1/Hβ2	2.00/1.84	1.89/1.89	1.84/1.84	1.86/1.80
Hγ1/Hγ2	1.69/1.61	1.68/1.59	1.54/1.47	1.63/1.58
Hδ1/Hδ2	3.30/3.30	3.33/3.16	3.33/3.24	3.23/3.23
tosyl H2/H6	7.80	7.79	7.70	7.72
tosyl H3/H5	7.30	7.34	7.22	7.21
tosyl CH_3	2.41	2.42	2.36	2.36
ester CH_3	3.69	3.67	3.67	3.67
acetyl CH_2	4.18/3.87	4.16/3.86	4.21/3.83	4.11/3.94

The chemical shift dispersion of the protons along the



I



II

Fig. 1. Chemical structures of methyl-[2,5-dioxo-3-(*S*)-(3-ω-tosyl-guanidino-propyl)-4-methyl-octahydro-1,4-diazocin-1-yl]acetate (**I**) and methyl-[2,5-dioxo-3-(*S*)-(3-ω-tosyl-guanidino-propyl)-octahydro-1,4-diazocin-1-yl]acetate (**II**).

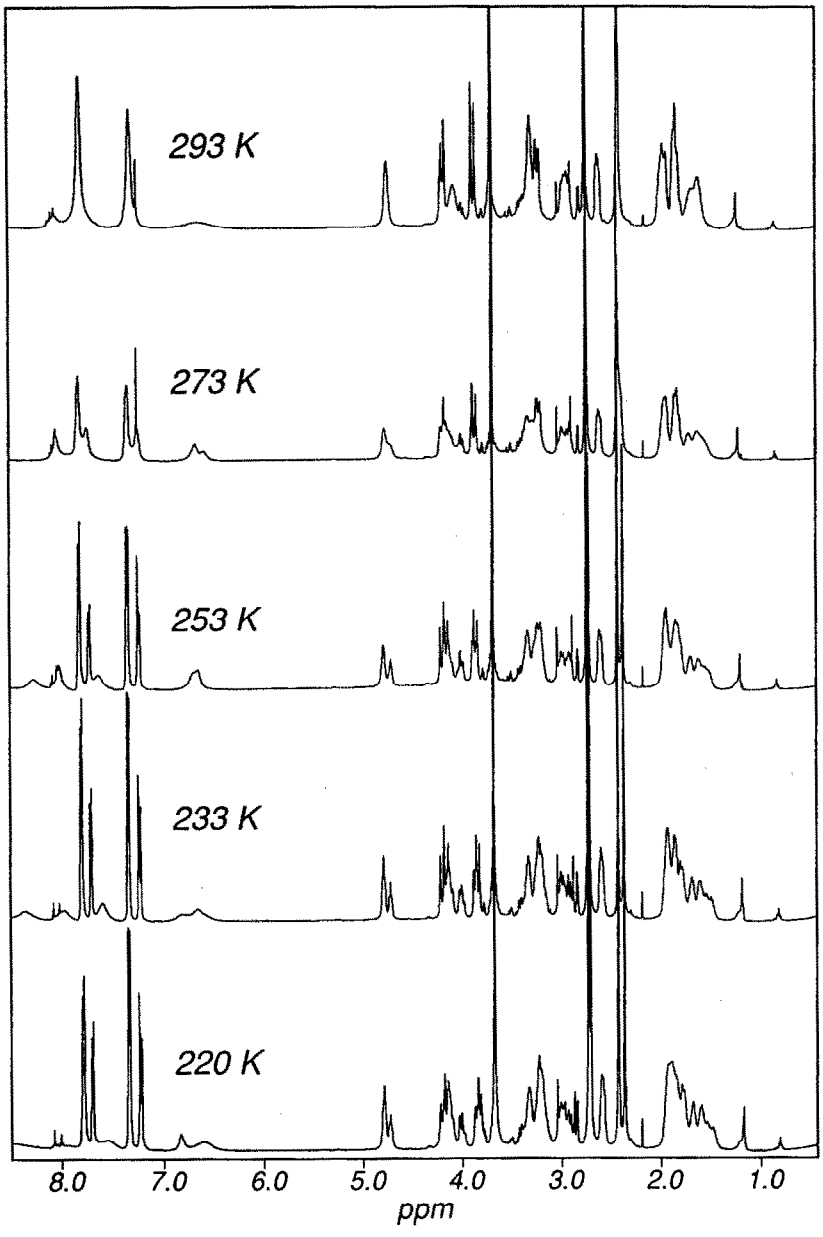


Fig. 2. One dimensional ^{1}H NMR spectra of I in CD_3OH at 293 K, 273 K, 253 K, 233 K, and 220 K. Two sets of resonances are visible at lower temperatures, and are most likely due to resonance hybridization at the guanidino group.

trimethylene group of I was such, and in particular those of the H7 and H7′ protons (Fig. 4a), that all eight ^{3}J(H,H) coupling constants were measurable from the P.E.COSY spectrum at 293 K. They are listed in Table 2. Unfortunately, it was not possible to extract a complete set of ^{3}J(H,H) coupling constants at 220 K because of chemical shift degeneracies among the major and minor conformers (Table 1). For compound II, the small chemical shift dispersion between the H7 and H7′ protons prevented the measurement of the ^{3}J(H,H) coupling constants from the P.E.COSY spectrum. The crosspeak multiplets between the H8, H8′, H6, or H6′ protons and the H7 or H7′ protons were overlapped, as illustrated in Fig. 4c. This overlap problem was obviated in the ^{13}C-filtered TOCSY experiment since the ^{3}J(C,H) coupling constants of interest were visible as displacements along ω_2 at the H8, H8′, H6, and H6′ resonances, and these four resonances were well separated along ω_2 (Fig. 5). The ^{3}J(C,H) coupling constants measured at 293 K are listed in Table 3. These were used to calculate dihedral angles along the trimethylene group of the eight-membered rings of both I and II. The calculated dihedral angles for I were in close agreement with those calculated with the ^{3}J(H,H) coupling constants.

Table 2
^{3}J(H,H) coupling constants for the trimethylene group of the eight-membered-ring backbone of compound I

J(H8,H7)	2.0 Hz	J(H7,H6)	1.3 Hz	J(H8,H8′)	−14.5 Hz
J(H8,H7′)	12.6 Hz	J(H7,H6′)	5.3 Hz	J(H7,H7′)	−15.3 Hz
J(H8′,H7)	7.2 Hz	J(H7′,H6)	12.1 Hz	J(H6,H6′)	−16.5 Hz
J(H8′,H7′)	2.0 Hz	J(H7′,H6′)	2.0 Hz		

Table 3
Heteronuclear $^3J(C,H)$ coupling constants for the trimethylene group of the eight-membered-ring backbone of compounds I and II

Compound I		Compound II	
J(C6,H8)	3.8 Hz	J(C6,H8)	2.7 Hz
J(C6,H8′)	9.5 Hz	J(C6,H8′)	10.0 Hz
J(C8,H6)	3.9 Hz	J(C8,H6)	3.5 Hz
J(C8,H6′)	8.8 Hz	J(C8,H6′)	9.3 Hz

Analyses of interproton NOE buildup rates in the ROESY spectra of I and II at 293 K yielded identical interproton distances between the H3 methine proton and the H8 and H6 methylene protons of 2.2 Å and 2.3 Å, respectively.

Monte Carlo simulations (1,000 structures, MM2 force field, $CHCl_3$ solvent matrix) of simplified compounds I and II yielded, respectively, eight and seven conformations which were within 50 kJ/mol of their respective lowest energy structure. Comparison of these two relatively small sets of conformations showed that only one conformer from each set was

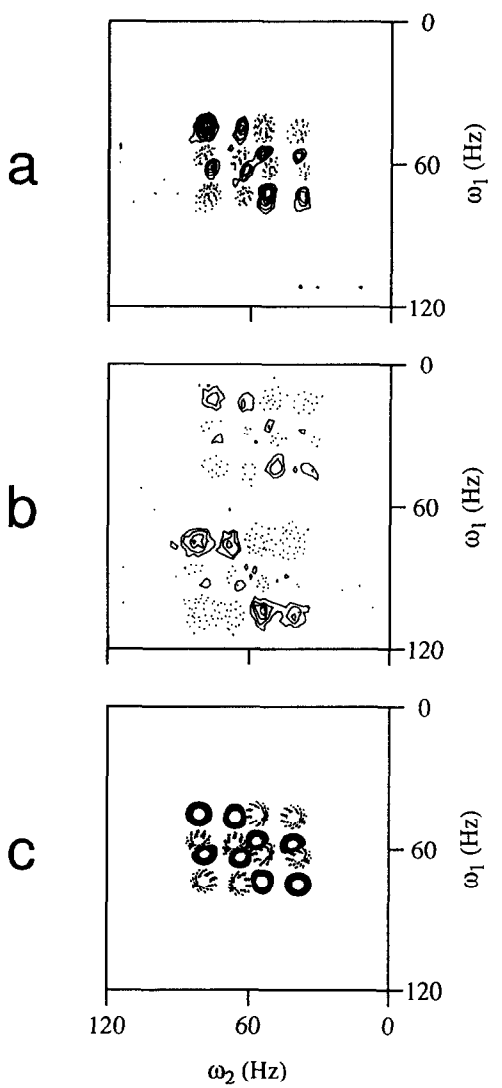


Fig. 3. Experimental H6→H7′ crosspeak of I at 293 K (a), at 220 K (b), and simulated at 293 K (c). The coupling constants extracted from the P.E.COSY spectrum of I at 293 K were used for the simulation.

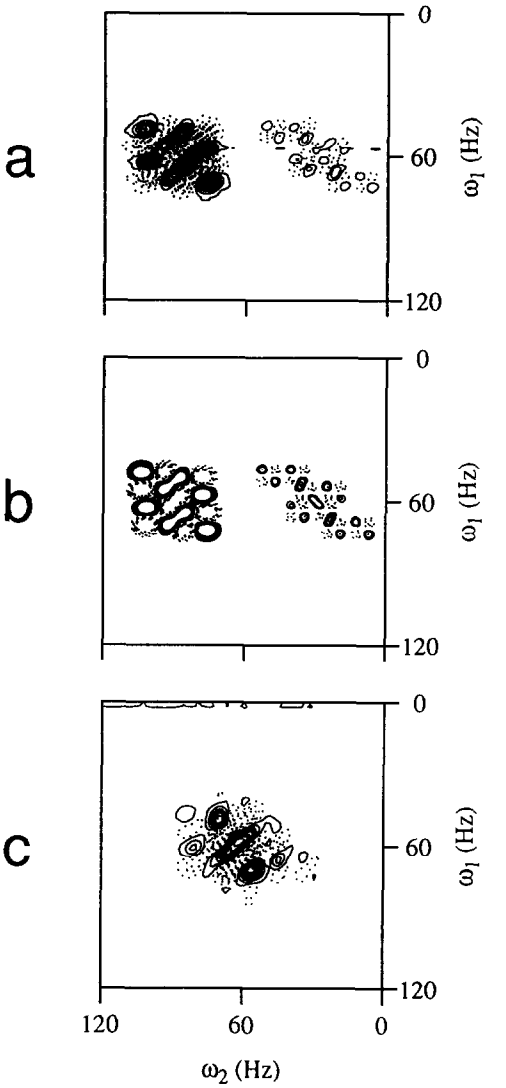


Fig. 4. Experimental H8′→H7′ and H8′→H7 PECOSY crosspeaks of I at 293 K (a); simulated (b); and experimental H8′→H7′ and H8′→H7 P.E.COSY crosspeaks of II, (c). The coupling constants extracted from the P.E.COSY spectrum (Table 2) were used to simulate the experimental crosspeak multiplets. Due to the small chemical shift separation between the H7 protons of II, all the crosspeak multiplets of interest were overlapped. It was not possible, therefore, to extract vicinal homonuclear coupling constants from these crosspeaks.

consistent with all of the measured NMR parameters. This observation was confirmed by performing a constrained Monte Carlo simulation on each compound incorporating two torsion angle constraints (∠C6-H8′ = 180° ± 30°, ∠C8-H6′ = 180° ± 30°) obtained from the ^{13}C-filtered TOCSY experiment. Only one common conformation was found for both compounds that met the torsion angle constraints and the experimentally determined NOE distances. Repeating the constrained search on the complete molecules I and II resulted in a set of conformations with a single eight-membered ring conformation identical to the one found using simplified molecules. The only variation was seen in the C-3 side chain. Both I and II adopt an identical eight-membered ring conformation characterized by two *cis*-amide bonds and a stag-

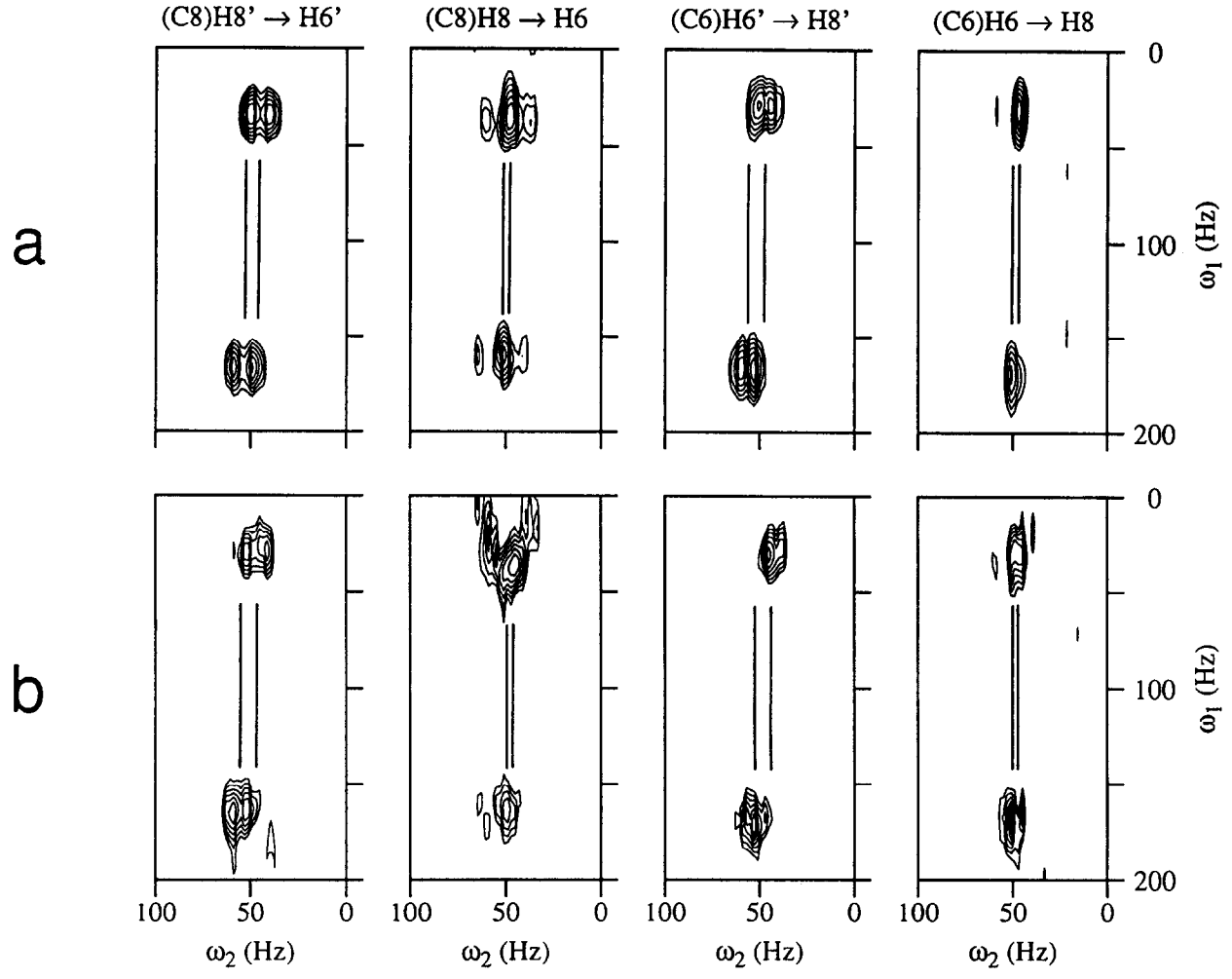


Fig. 5. Contour plot of the ^{13}C-filtered TOCSY spectra of compounds **I** and **II** showing the relevant crosspeaks for determining heteronuclear vicinal coupling constants, ^{3}J(C,H), along the trimethylene bridge. The ^{3}J(C,H) coupling constants are visible as displacements along ω_2. The multiplet components are separated along ω_1 by the large ^{1}J(C,H) coupling.

gered conformation of the trimethylene group in which the H3 proton is within 2.3 Å of both the H6 and H8 protons (Fig. 6).

The chemical shift dispersion of the protons of a given geminal proton pair along the trimethylene portion of the eight-membered ring is greater for **I** than for **II** (Table 1). This could be explained by either greater conformational averaging in **II** relative to **I** or by a constitutional effect, i.e. the substitution of the amide for the *N*-methyl group. However, ^{3}J(C,H) coupling constants measured along the trimethylene bridges of these two molecules are very similar (Table 3), and the constrained conformational search yielded a single identical eight-membered ring conformation for **I** and **II** (Fig. 6). The differences observed in the chemical shift dispersion of the trimethylene protons of these two compounds and their different affinities for the GP IIb/IIIa receptor are most likely due to constitutional and not conformational differences.

The ^{13}C-filtered TOCSY experiment has proven to be a valuable tool for extracting vicinal heteronuclear coupling constants which were then used for calculating torsion angle constraints along the trimethylene bridge of the eight-membered ring. Additionally, the ^{3}J(C,H) coupling constants were measured with ^{13}C in natural abundance. This is an important practical consideration since ^{13}C labeling is not always available and can be costly and time consuming. For **I**, independent determination of torsion angles using ^{3}J(H,H) coupling constants helped to verify the applicability of the heteronuclear Karplus relation for determining torsion angle constraints for **II** where homonuclear vicinal coupling constants were not available. Finally, interproton distances calculated from the unique conformation found with the constrained search were in good agreement with those derived from NOE buildup curves extracted from ROESY spectra.

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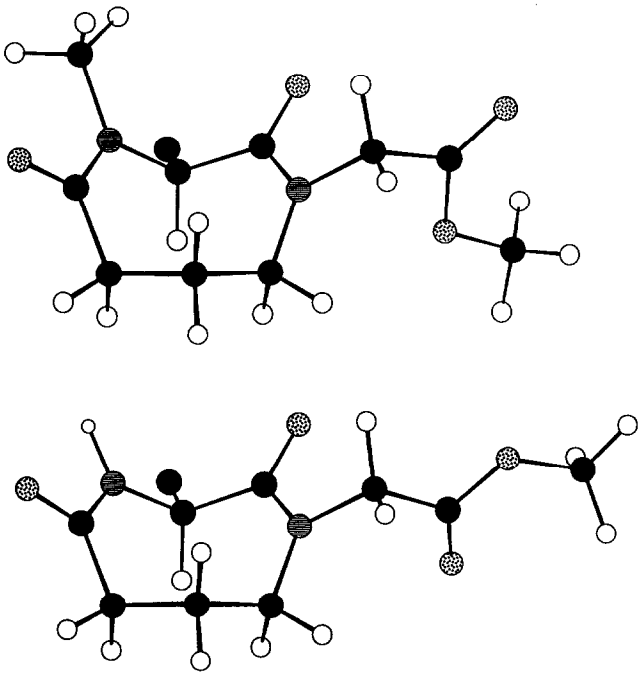

Fig. 6. Eight-membered ring backbone conformation of I and II. The constrained search performed on each compound resulted into a single common conformation for the eight-membered-ring backbone. The constrained search yielded the following torsion angles and interproton distances: (I) ∠ C6-H8′ = −153°, ∠ C8-H6′ = 156°, d_{H3-H8} = 2.15 Å, d_{H3-H6} = 2.16 Å; (II) ∠ C6-H8′ = −152°, ∠ C8-H6′ = 158°, d_{H3-H8} = 2.16 Å, d_{H3-H6} = 2.28 Å.

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