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Design, synthesis and solution structure of a renin inhibitor

Structural constraints from NOE, and homonuclear and heteronuclear coupling constants combined with distance geometry calculations

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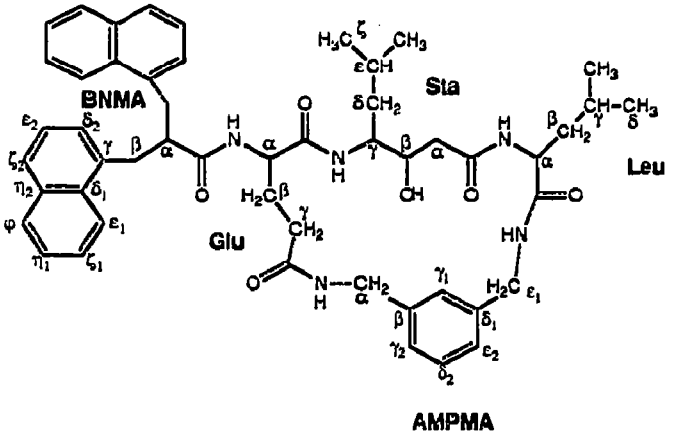
A macrocyclic renin inhibitor was designed using molecular modeling and a model of human renin. The synthesized molecule displayed poor binding affinity. To investigate the reasons for the observed inactivity, the structure of the compound has been studied by NMR spectroscopy and distance geometry. Structural constraints for distance geometry calculations were derived from nuclear Overhauser effects and homonuclear and heteronuclear three bond coupling constants. Homonuclear coupling constants were measured directly from the resolution-enhanced proton spectra and heteronuclear coupling constants were measured from the natural abundance ^{15}N- and ^{13}C-edited TOCSY experiments. One ϕ angle was determined uniquely by this method and two were reduced to two possible values each. By using a statistical analysis of 400 structures generated with distance geometry, two families of structures were found to be consistent with the NMR data. The solution structures so derived were different from the originally designed structure, including an internal hydrogen bond. This provides a possible explanation for the lack of effectiveness of this compound.

Renin inhibitor; Solution structure; Heteronuclear coupling constant; Distance geometry; Molecular modeling; Drug design

1. INTRODUCTION

Rational, de novo design of drugs is an on-going objective in many pharmaceutical and academic laboratories [1]. In pursuit of this goal, effective use of computer-aided drug design can guide synthetic efforts, resulting in more efficient discovery of therapeutic candidates. Recent technological advances in macromolecular crystallography, NMR spectroscopy and computational efficiency have begun to provide the tools necessary to apply this methodology to pharmaceutically relevant problems. Experimental verification of modeled structures is an important feedback mechanism in the development of this approach. In this report, we describe molecular modeling studies involving a human renin enzyme model [2] leading to the design of a conformationally constrained renin inhibitor (Scheme 1), its synthesis, biological evaluation and subsequent structural analysis. In the structural analysis, a method for measuring heteronuclear coupling constants at natural abundance [3] is applied to this compound. The combined use of homonuclear $^3J_{NH,H\alpha}$ and heteronuclear $^3J_{NH,C\beta}$ and $^3J_{H\beta,N}$ coupling constants has minimized the ambiguities that arise from the periodic nature of the Karplus relationship [4] and has provided valuable constraints that are used in conjunction with NOE distances in distance geometry calculations.



Scheme 1.

Abbreviations: ROESY, rotating frame nuclear Overhauser spectroscopy; COSY, correlated spectroscopy; TOCSY, total correlated spectroscopy; AMPMA, *meta*-di(aminomethyl)benzene; BNMA, bis-[(1-naphthyl)methyl]acetic acid; BOC, *N*-*t*-butyloxycarbonyl; DCC, 1,3-dicyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole hydrate.

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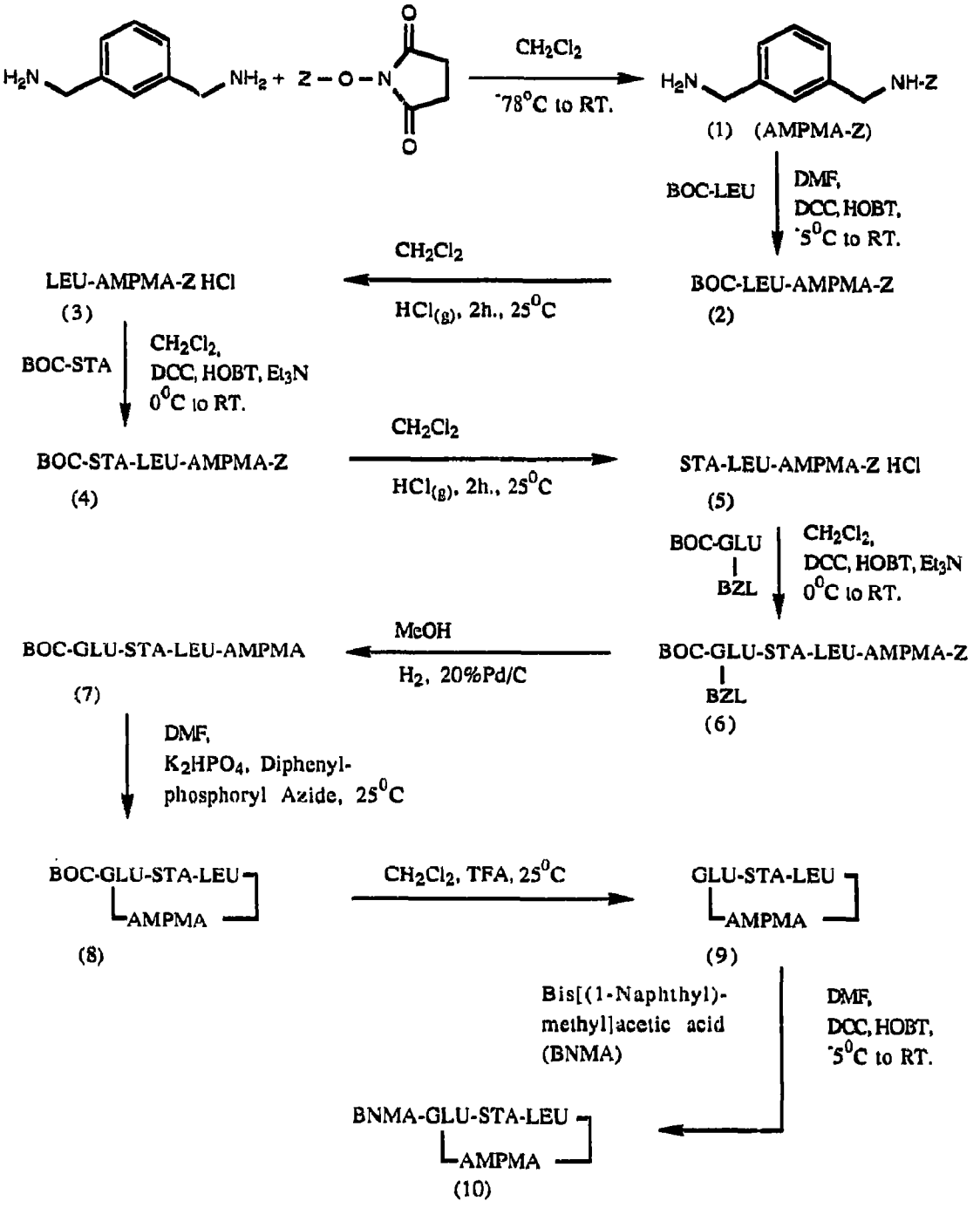


Scheme 2.

2. EXPERIMENTAL

2.1. *Peptidomimetic synthesis*

The synthesis of the cyclic peptidomimetic is outlined in Scheme 2. Compound **1**, mono-carbobenzyloxy-*m*-xylenediamine, AMPMA-Z, [5] was isolated from the reaction of *m*-xylenediamine with benzyloxycarbonyl-(oxy)succinimide. This material was coupled to BOC-Leu using DCC-HOBT, followed by deprotection with anhydrous HCl gas in dichloromethane, and the isolation of **3** as the hydrochloride. Following neutralization of **3** with triethylamine in dichloromethane, BOC-Sta [6] was coupled to **3** using DCC-HOBT to give **4**. The deprotection of **4** gave **5**, which was coupled to BOC-(γ-benzyl)Glu, giving product **6**. The γ-benzyl ester and carbobenzyloxy protecting groups were then simultaneously removed by catalytic hydrogenation which gave **7**. A 1% solution of **7** in DMF was cyclized to **8** in 71% yield with diphenylphosphorylazide by the application of a procedure by Brady et al. [7]. Removal of the BOC group from **8** with (50:50) TFA/dichloromethane, followed by the DCC-HOBT coupling of BNMA [8] gave the product **10**. Further purification was accomplished by chromatography on silica gel, eluted with chloroform/methanol (95:5).

2.2. *NMR measurements*

Homonuclear and heteronuclear NMR experiments were carried

Table 1

Selected NMR data for the cyclic peptidomimetic in DMSO-d6,[a]

Residue	Chemical shifts	$^3J_{HN,H}$	$J_{H,C}$[b]	$J_{H,N}$[c]	$\Delta\delta/\Delta T$[d]
Glu	α-4.22 β-1.64, 1.64 γ-2.03, 2.03 NH-8.32	7.8	Nβ 1.0, Nα 2.3 βα −2.5, γα 3.0 αβ − 3.0, γβ − 4.0 Nγ 1.0, αγ 2.0 βγ −4.0	α 2.5 β −1.0	−7.4
Sta	α-2.18, 21.8 β-3.88 γ-3.85 δ-1.37, 1.27 ε-1.58 ζ-0.92, 0.83 OH-4.90 NH-7.77	9.5	Nδ 0.7, Nα 2.0 δγ −3.0, δ'γ 3.0 γδ −1.5, HOα 3.0 βα −1.0	δ −5.0[e] δ' −5.0[e] γ 2.5	−7.1
Leu	α-4.18 β-1.47, 1.47 γ-1.67 δ-0.89, 0.85 NH-8.05	6.9	Nβ 1.5, Nα 2.0 βα −3.0, αβ −4.0 γβ −2.5	β −5.8 α 0.0	−6.1
AMPMA	γ-7.08 δ-7.20 ε-4.55, 3.99 ε2-7.09 α-4.46, 4.46 αNH-7.92 eNH-8.46	α 7.6 α' 4.6 ε 7.6 ε' 4.6	Nα 2.5 Nε1 2.0	α 1.5 α' 1.3 ε1 2.0 ε' 0.5	−2.8 −6.1

[a] Chemical shifts relative to DMSO-d5 = 2.50 ppm, coupling constants in Hz.
[b] Pairs of Greek characters refer to proton and carbon, respectively, nomenclature as in Scheme 1. N indicates the amide proton.
[c] Greek character refers to a proton as in the Scheme. Coupling is to the amide nitrogen 2 to 4 bonds away.
[d] Amide proton dependence in ppb/K between the 280 and 300K.
[e] The −5.0 Hz coupling constants observed for both the β protons possibly due to the nonpeptide structure of the Sta residue.

out with 15 mg and 50 mg peptidomimetic, respectively, in 0.5 ml of dimethylsulfoxide-d6 at 300K. Two-dimensional double quantum filtered COSY [9] and ROESY [10] data (512 t1 blocks of 2048 t2 data points) were acquired on a Bruker AMX500. For the ROESY data, a 4 kHz continuous wave spin-lock field was applied during the 250 ms mixing time at the transmitter frequency. The temperature dependence of the proton spectrum was determined by acquiring spectra at 280K, 290K and 300K. Homonuclear coupling constants were measured directly from spectra that were resolution enhanced by application of a gaussian function (GB=0.5, LB=−5) to the free induction decay prior to Fourier transformation. Heteronuclear coupling constants were measured from ^{15}N-edited and ^{13}C-edited TOCSY spectra as described previously for peptides [3] and isotopically enriched proteins [11]. To allow measurement at natural isotopic abundance, a BIRD type heteronuclear editing pulse was incorporated to select protons attached to ^{13}C or ^{15}N nuclei followed by a conventional TOCSY pulse sequence (see Fig. 1).

2.3. *Molecular modeling*

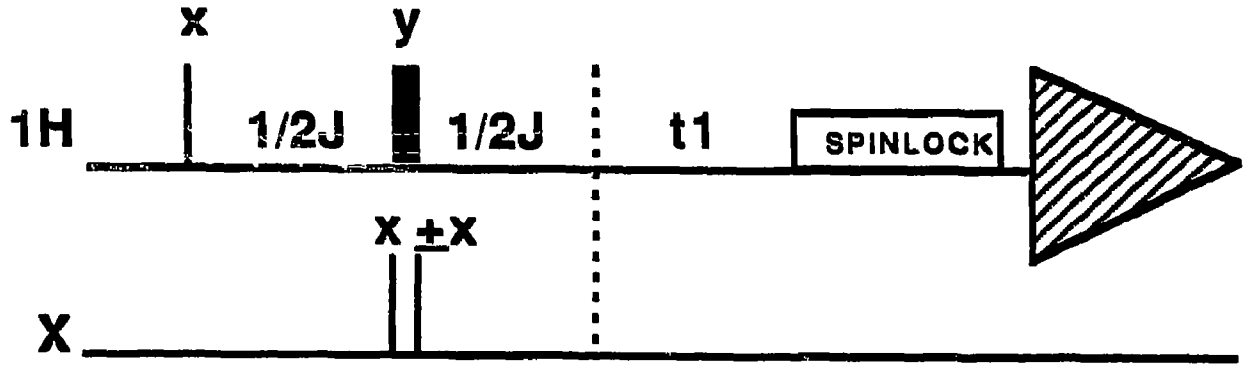
The peptidomimetic was designed using Sybyl molecular modeling software [12] and a previously described model for human renin [2]. Distance geometry calculations were carried out using DGEOM [13] and the cluster analysis software, Compare, as previously described [14]. Statistical Analysis Software (SAS) [15] was used to analyze the distance geometry structures.

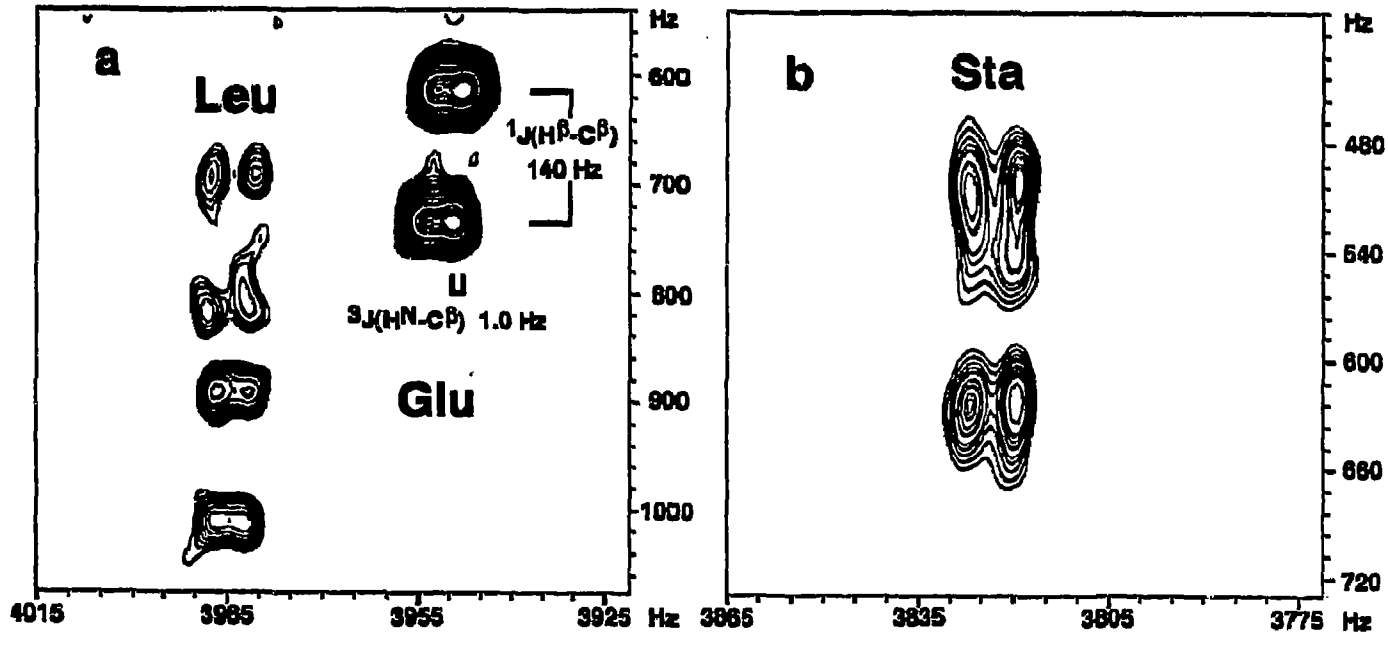
Energy minimizations and molecular dynamics calculations were carried out without electrostatic potentials using the Tripos force field in Sybyl [12]. Molecular dynamics calculations were run in the gas phase using a Boltzmann weighted starting velocity with gradual heating for 4.5 ps followed by 20 ps at 300 K. In the dynamics runs the φ angles were restrained to a designated value with a force constant of 0.01 and interproton distances restrained with a force constant of 10 and a functional power of 2.

3. RESULTS AND DISCUSSION

Molecular modeling studies led to the design of an inhibitor which was sterically and electrostatically compatible with the enzyme binding site. After synthesis and biological testing, the inhibitor proved to have very low binding affinity for renin (26% inhibition of renin at 1 μM) [16]. In an attempt to explain the failure of the modeling studies, heteronuclear and proton NMR studies, coupled with distance geometry and structural analyses were undertaken to explore the conformational properties of the cyclic peptidomimetic in solution.

The site-specific proton assignments were obtained using standard two-dimensional techniques; the AMPMA methylene protons were assigned stereospecifically based on $^3J_{NH,H\alpha}$ and NOE data. The chemical


x
y
1H
1/2J
1/2J
t1
SPINLOCK
x ±x
X


a
Leu
1J(Hβ-Cβ)
140 Hz
3J(HN-Cβ) 1.0 Hz
Glu
Hz
600
700
800
900
1000
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3955
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b
Sta
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540
600
660
720
3865
3835
3805
3775 Hz

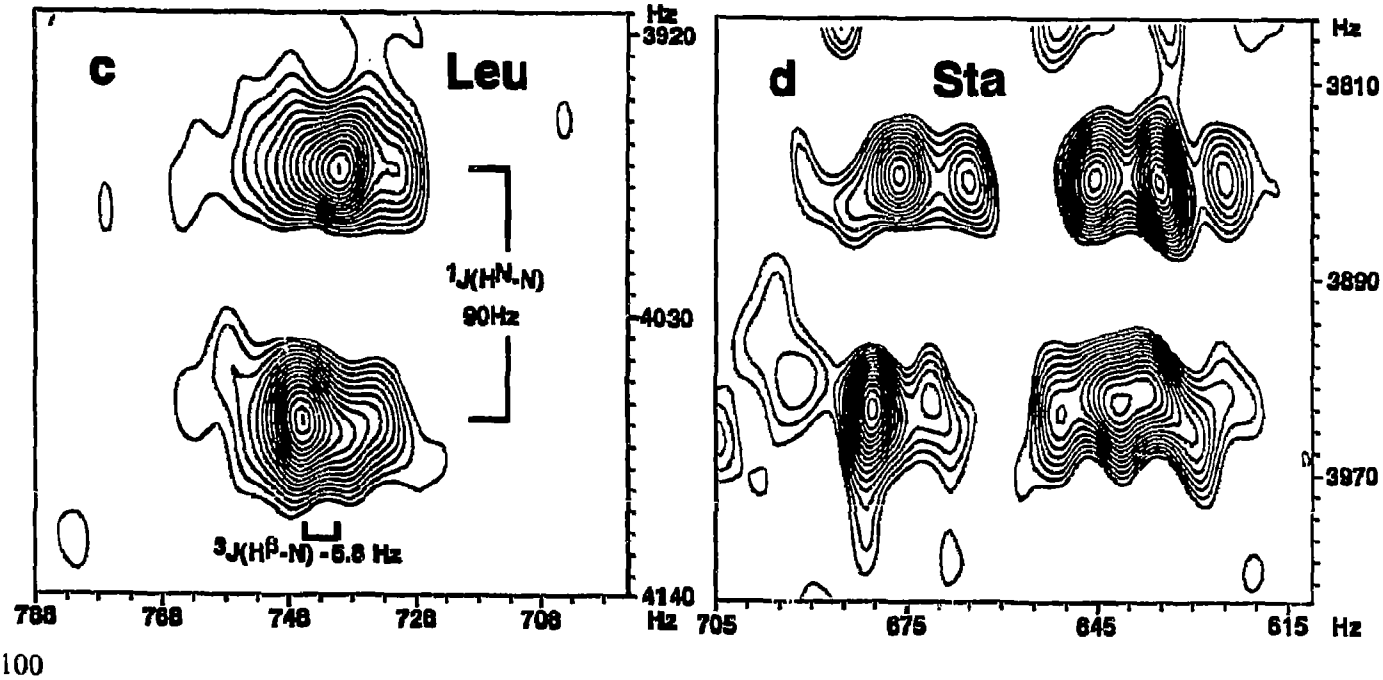
c
Leu
1J(HN-N)
90Hz
3J(Hβ-N) ~5.8 Hz
Hz
3920
4030
4140
768
748
728
708 Hz
d
Sta
3810
3890
3970
705
675
645
615 Hz

Table II

Interproton upper bound distances derived from ROESY data for the Peptidomimetic in DMSO-d6[a].

AMPHA NHε	Leu Hα	2.5	Sta Hβ	Sta Hα	4.0
	AMP HεS	3.0		Sta Hδ	4.0
	AMP HεR	3.5		Glu Hγ	4.0
	Leu Hβ	4.0			
	AMP Hγ	3.0	Sta OH	Sta Hα	4.5
	Leu NH	3.5		Sta Hβ	2.5
	Sta Hγ	4.0		Sta Hδ'	4.0
				Sta Hδ	4.0
AMPHA NHα	AMP HαR	3.0			
	AMP HαS	3.5	Sta Hγ	Sta Hα	4.0
	Glu Hγ	2.5		Sta Hε	3.0
	AMP Hγ	2.5		Sta Hδ	3.0
	Sta Hβ	4.0		Sta Hζ	4.2
	Glu Hβ	4.0			
	Sta Hα	4.0	Sta Hα	Sta Hδ	4.0
AMPMA Hγ	Glu Hβ	4.0	Leu NH	Leu Hα	3.0
	Glu Hγ	4.0		Sta Hβ	4.0
	AMP HαR	3.0		Sta Hα	2.5
	AMP HαS	3.5		Leu Hγ	3.0
	AMP HεS	3.0		Leu Hβ	3.0
	AMP HεR	3.5			
	Leu Hα	3.5	Leu Hα	Leu Hγ	3.5
				Leu Hβ	3.5
AMPMA Hε2	AMP HεS	3.5		Leu Hδ	4.0
	AMP HεR	2.5	Leu NH	Sta OH	4.0
AMPMA Hγ2	AMP HαR	3.5			
	AMP HαS	2.5	Glu NH	Glu Hγ	4.0
				Glu Hβ	3.0
Sta NH	Sta OH	3.5		Glu Hα	3.5
	Glu Hα	2.5		BNA Hα	3.0
	Sta Hγ	3.0			
	Sta Hα	3.0	Glu Hγ	Sta Hα	4.0
	Glu Hγ	3.5		Sta Hβ	4.0
	Sta Hε	4.0			
	Sta Hδ	3.0	Glu Hα	Glu Hγ	4.0
	Sta Hδ'	4.0		Glu Hβ	4.0
	Sta Hβ	3.5			
	Glu Hβ	3.5			

[a] Approximate upper distance bounds were estimated by classifying crosspeak intensity as strong, medium or weak relative to the strongest and weakest crosspeaks in the spectrum.

Table III

Evaluation of conformational families of the cyclic peptidomimetic

	Criteria[a]			Starting Φ angle (degrees)		
Run No.	1	2	3	Glu	Sta	Leu
CPOH17	N	Y	Y	-170	-120	-160
CPOH113	N	Y	Y	-170	-120	-160
CPOH236	N	Y	Y	-170	-120	60
CPOH28	N	Y	Y	-170	-120	60
CPOH329	N	Y	Y	60	-120	60
CPOH317	N	Y	N	60	-120	60
CPOH424	N	Y	Y	60	-120	-160
CPOH425	N	N	Y	60	-120	-160
CPOS113	N	N	N	-170	-120	-160
CPOS135	N	Y	N	-120	-120	-160
CPOS147	N	N	N	-170	-120	-160
CPOS236	Y	Y	Y	-170	-120	60
CPOS27	N	Y	Y	-170	-120	60
CPOS29	Y	Y	Y	-170	-120	60
CPOS328	N	Y	Y	60	-120	60
CPOS333	N	Y	N	60	-120	60
CPOS415	N	Y	N	60	-120	-160
CPOS438	N	Y	N	60	-120	-160
CPOS444	N	N	Y	60	-120	-160
PREDICTED	N	N	N	-	-	-

[a] Criteria for compatibility with NMR data:
1, interproton distances < 5 Å for all observed NOEs;
2, torsion angle values within 30° of the applied constraint for each Φ angle;
3, hydrogen bond involving the α NH of AMP and either the hydroxyl or the carbonyl oxygen of statine;
Y, meets criterion;
N, does not meet criterion.

shifts are listed in Table I. Sixty-four interproton distances were obtained from ROESY spectra and are listed in Table II. Approximate upper bound constraints were determined based on the relative intensity of the ROESY crosspeak. Additional constraints were provided by three bond homonuclear and heteronuclear coupling constants. Portions of the ^{13}C- and ^{15}N-edited TOCSY spectra are shown in Fig. 1. Analysis of the $^3J_{HN,H\alpha}$ coupling constants alone, using published empirical relationships [4], gave four possible ϕ angles for the Leu, Sta and Glu residues. This resulted in a total of 64 possible combinations. Incorporating the heteronuclear coupling constants into the analysis reduced the total number of possible combinations to four, making their use as constraints in a distance geometry program tractable (see Table I and the following discussion).

The temperature dependence of the amide NH protons (Table I) indicated that only the AMPMA α NH had a low temperature coefficient and was therefore a good candidate for involvement in hydrogen bonding [17]. An NOE between the AMPMA α NH and the α methylene protons of statine suggested that the best hydrogen bond acceptors were probably either the hydroxyl oxygen or the carbonyl oxygen of statine.

The torsion angle and NOE distance constraints from the NMR data were entered into the distance geometry program, DGEOM, to generate acceptable conformations. Distance geometry runs were initiated with eight different sets of constraints. Each set had a hydrogen bond constraint between the α NH of AMPMA and

←

Fig. 1. Expansions of the ^{13}C-edited (a and b) and ^{15}N-edited (c and d) TOCSY spectra of 100 mM peptidomimetic in 0.5 ml of dimethylsulfoxide-d6 at 298K. Shown are selected NH, Hβ crosspeaks. Horizontal displacements of the indicated crosspeaks arise from the one bond Cβ-Hβ or N-H coupling and vertical displacement corresponds to the three bond NH-Cβ or Hβ-N coupling. The pulse sequence used in this study is shown; see text for experimental details.

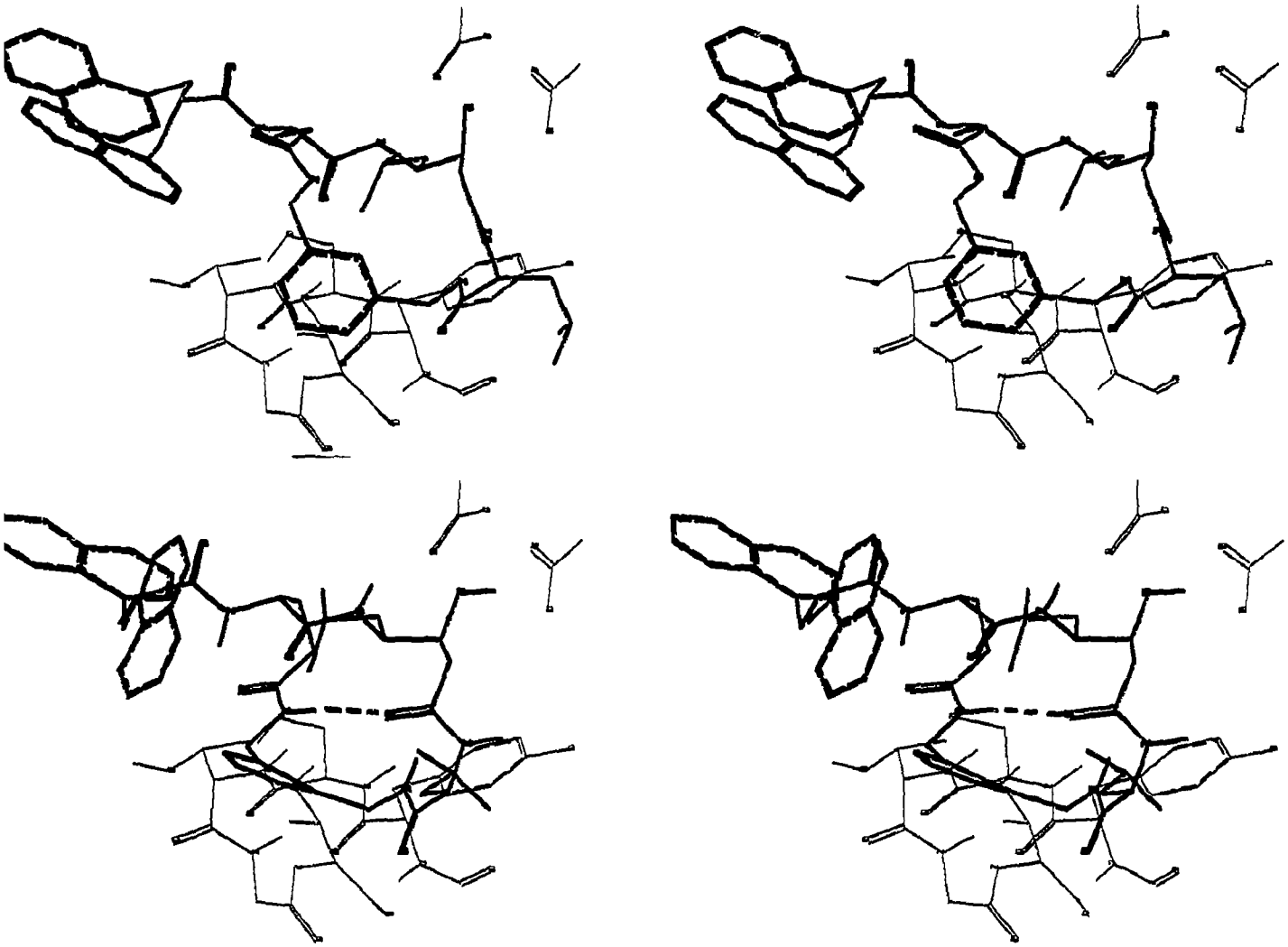

Fig. 2. Stereo views of the (a) predicted and (b) experimental cyclic peptidomimetic conformations (heavy lines) in the renin binding pocket (light lines). The dotted line in (b) indicates a possible hydrogen bond.

either the hydroxyl oxygen (cpoh series) or the carbonyl oxygen (cpos series) of statine and one of the four possible combinations of the three constrained ϕ angles (designated 1 through 4). Fifty structures were obtained from each run. Using the program Compare [13], the rms fit of each of the fifty conformers to every other conformer was determined. Based on the rms fits, the conformations were classified into families of structures which differed by less than 1.0 Å average RMSD (all atoms excluding the two methylenenapthyl groups) using a hierarchical cluster analysis [15]. A total of 19 families were found using this approach.

A representative conformer for each of the 19 families was minimized using thirty-four maximal NOE distance constraints and three torsion constraints determined from the coupling constant analysis. The structure was then subjected to constrained molecular dynamics. In order to obtain an energetically reasonable structure, one conformer extracted from the last 5 ps of each dynamics run was energy minimized without any constraints. The constraint violations of these final structures as well as the originally modeled conformation are listed in Table III. A structure was considered compatible with NMR results if it met all three criteria listed in Table III.

Notably, the structure of the peptidomimetic predicted in the design phase of the project does not meet any of the criteria listed above. Furthermore, none of the final structures from the cpoh series are consistent with the NOE data. Of the eleven structures minimized from the cpos series, two structures (cpos29 and cpos236) were compatible with the NMR data. Forty-seven of the 50 total structures which resulted from the distance geometry starting set cpos2 were in families represented by these two structures. The average RMSD of the macrocyclic backbone heavy atoms be-

tween cpos29 and cpos236 is 0.35 Å Stereoviews of the originally predicted conformation and the cpos29 structure are shown in Fig. 2a and b, respectively.

4. CONCLUSION

A solution conformation derived from the above NMR and distance geometry analyses supports the formation of an internal hydrogen bond involving the AMPMA α NH and the carbonyl oxygen of statine. The phi angles predicted for this structure are −170 (Glu), −120 (Sta) and 60 (Leu), consistent with homo and heteronuclear coupling constants. In the model of the inhibitor–enzyme complex, the peptidomimetic binds to the enzyme with the Sta hydroxyl group located directly between the active site aspartic acid residues while the macrocyclic system extends out over the enzyme flap region (Fig. 2a). The occurrence of the hydrogen bond across the peptidomimetic ring in the two structures that satisfy the NMR constraints causes a significant conformational change relative to that predicted by the modeling studies. This apparently hinders the desired interactions in the renin active site.

One explanation for the observed weak binding is that the molecule may transiently exist in the originally predicted bound conformation and there is a considerable free energy cost in achieving this conformation. It is thus demonstrated that inhibitor design based on the three dimensional structure of an enzyme warrants additional conformational studies of the uncomplexed inhibitor.

We have demonstrated the use of both ^{15}N- and ^{13}C-edited TOCSY to measure, at natural abundance, heteronuclear three bond coupling constants that can greatly reduce the number of possible torsional angles that result from homonuclear analysis alone. These constraints are necessary supplements in these small, partially constrained systems where NOE information alone is not sufficient to define a structure.

To gain additional insight into this system, we are currently pursuing crystallographic verification of the structure of the peptidomimetic bound to human renin and kinetic analysis of the binding.

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