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Design and Synthesis of a Bicyclic Non-Peptide ß-Bend Mimetic of Enkephalin

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Abstract: *If the important functional groups of a biologically active peptide can be arrayed on a non-peptide framework in the "biologically active conformation" it may be possible to maximize the receptor interaction and eliminate or reduce the number of amide bonds that are available for proteolytic enzyme cleavage. Thus, we have designed and synthesized the 2,5,7-trisubstituted 2,3,4,4a,7,7a-hexahydro-pyrano[2,3-b]pyrrole ring system as the basis for non-peptide mimetic analogs for peptides that prefer the β-bend conformation. Computer assisted molecular modeling studies have indicated that the resulting analogs closely resemble the functional group positioning particularly of type I and I' peptide β-bends. Completed analogs of Leu-enkephalin have been synthesized and shown to be potent naloxone-reversible agonists in the electrically stimulated guinea pig ileum assay.*

The β-bend, also known as a β-turn, β-loop or reversed turn, is a 10-membered intramolecularly H-bonded ring and is a conformational feature existing in many biologically active peptides and proteins. The main feature of a β-bend is the reversal of the direction of the peptide chain by approximately 180°. Several types of β-bend conformations were originally described by Venkatachalam in 1968.[1] By varying ϕ_2, ψ_2, ϕ_3, ψ_3, there are at least 14 types of β-bend structure which have been tabulated by Chou and Fasman.[2] Among

them, types I (42%), II (15%), III (18%) are prevalent; their mirror images {I' (3%), II' (5%) and III' (3%)} are much less common and the other β-bend types are distinctly rare (types V, V', VI and VII total 5% and type IV 8%).[3] Several biologically active peptides have been proposed to have a β-bend structure in their active conformation, for example, somatostatin,[4] LHRH,[5] oxytocin,[6] bradykinin,[7] and enkephalin.[8]

The approach taken in our laboratory for the design of mimetic analogs of bioactive peptides is to take advantage of the β-bend structural feature in order to synthesize compounds that are forced into this desired conformation when this is the biologically active conformation, i.e., the conformation of the peptide ligand at the receptor site, and leave the moieties which are responsible for intrinsic activity unchanged. Leucine-enkephalin has been used as a model system in which to implement our design and to demonstrate the effectiveness of the non-peptide mimetic.

Enkephalins are pentapeptides, with the sequence Tyr-Gly-Gly-Phe-Leu (Leu-enkephalin) or Tyr-Gly-Gly-Phe-Met (Met-enkephalin), that are the endogenous ligands for at least some of the opioid receptors.[9] Studies of structure-activity relationships (SAR) for enkephalin analogs provided the minimal requirements, i.e., the essential amino acid residues or functional groups of enkephalin, needed for intrinsic activity which are as follows: 1) the tyrosyl phenolic group is essential for opiate activity;[10-13] 2) the tyrosyl amino group should be present and unalkylated for good activity;[14-16] 3) the glycine unit at the two position of the peptide can be replaced with a number of D-amino acids or 2-amino-isobutyric acid (Aib), but replacement with an L-amino acid results in loss of activity;[10,17,18] 4) the glycine unit at the third position should be retained for good activity;[19] 5) the phenylalanine at the fourth position can not be replaced by D-Phe, Tyr, or (O-Me)-Tyr, but (N-Me)Phe, (6H)Phe, (p-NO_2)Phe, or (p-Cl)Phe can replace the phenylalanine without loss of activity;[18,20,21] 6) the residue at the fifth position can be replaced by a variety of D- or L-amino acids without loss of activity;[16,22-24] and 7) the terminal carboxyl group can be eliminated, esterified, converted to a primary or secondary amide, or coupled to other amino acids without considerable loss of activity.[22]

However, just knowing the active amino acid residues of enkephalin is not sufficient in order to design a non-peptide mimetic enkephalin analog. Knowing the active conformations of enkephalin is much more helpful in order to include the proper architecture in the design. Various approaches have been used in order to predict the active conformation of enkephalin, such as, x-ray crystallography, NMR spectroscopy, and energy calculations.

X-ray crystallographic studies of Leu-enkephalin crystallized from aqueous methanol showed a type I β-turn with hydrogen-bonds between the NH of the Phe residue and the C=O of the Tyr residue, and also one between the C=O of the Phe residue and the NH of the Tyr residue.[25] Other studies on Leu-enkephalin by the Karle[26] and Camerman[27] research groups revealed that the unit cell contains four molecules and that all four chains have an extended backbone conformation. However, in all four molecules, the side chain orientations are different.[27] The conformation of Met[5]-enkephalin has been determined by Ishida et al. and

also shown to have an extended backbone structure.[28]

Conformational preferences in solution for enkephalins as determined by NMR spectroscopic methods have been investigated by several research groups since 1976. They can be briefly summarized as follows: 1) a type I β-turn with a hydrogen-bond between the amide proton of Met^5 and the carbonyl oxygen of Gly^2 was suggested by Roques et al.[29] and Jones et al.;[30] 2) three hydrogen bonds were proposed for methionine-enkephalin based on ^{1}H-NMR, ^{13}C-NMR, UV, and CD methods in DMSO-d_6 by Khaled et al.[31,32]: (a) an intramolecular hydrogen-bond between the Met^5 amide hydrogen and the Gly^2 carbonyl oxygen (i.e., a 5-2 β-bend); (b) an intramolecular hydrogen-bond between the Gly^3 amide hydrogen and the Tyr^1 carbonyl oxygen; and (c) an intramolecular hydrogen-bond between the Tyr^1 phenolic group and the Gly^3 carbonyl oxygen, which are also consistent with the results obtained by Niccolai et al.[33]; 3) an extended conformation for Met^5-enkephalin in equilibrium with a folded conformation was proposed by Higashijima et al.;[34] and 4) Garbay-Jaureguiberry et al.[35] suggested that a type II' β-bend is present in the active conformation of enkephalin.

One of the earliest classical energy calculations was reported by Isogai et al.,[36] which predicted a type II β-bend structure for Met-enkephalin with a hydrogen-bond between the phenolic hydrogen of Tyr^1 and carbonyl oxygen of Gly^3. These findings are also consistent with the NMR data of Roques et al.,[29] and Jones et al.[30]

Based on traditional methods, restriction of backbone or side chain rotation of enkephalin can be achieved by replacing an amino acid residue which is responsible for holding the enkephalin in its active conformation, with a more sterically bulky or rigid amino acid residue. 2-Aminoisobutyric acid (Aib) is frequently used for this purpose. [Aib^2], [Aib^2, Aib^3], and [Aib^3]-Met^5-enkephalinamide have been synthesized by Nagaraj et al.[37,38] The first two analogues have greater in vivo activity in mice than [Met^5]-enkephalin, while the [Aib^3] analogue has slightly less activity.

Cyclization through side chain moieties not involving the tyrosine amino group has also proven to be very fruitful. Schiller et al.[38] have found that the [D-Orn^2, Glu^5]-enkephalinamide with the Orn and Glu side chains cyclized as an amide was a potent μ selective agonist. Schiller et al.[39] and Hruby et al.[40] have found that [D-Pen^2, D-Pen^5]-enkephalin analogs cyclized by a disulfide bridge were potent δ selective agonists. DiMaio et al. used D-Orn to replace the glycine in position 2 and cyclized the side-chain amino group with the leucine carboxyl group which resulted in analogs with μ receptor selectivity.[41]

Nonpeptide mimetics were recently reviewed by Ball and Alewood and classified into two groups, external and internal.[42] External β-bend mimetics are molecules which reduce the conformational flexibility of a peptide with a rigidifying structure whose skeleton lies outside the pseudo 10-membered ring formed by a typical β-bend. Alternatively, in the internal β-bend mimetics, the rigidifying skeleton lies inside the β-bend. Freidinger et al.[43] reported the first conformationally constrained biologically active peptide which

utilized a nonpeptide β-bend mimetic strategy. A substituted lactam was used very successfully as a replacement for Gly[6]-Leu[7] in LHRH to produce an agonist with 8.9 times the in vitro potency of LHRH. Freidinger et al. subsequently reported the replacement of Gly[2]-Gly[3] with a γ-lactam to produce a [Met[5]]-enkephalinamide analog.[44] In a different study by Douglas et al.[45], a similar lactam was used to replace Tyr[12]-Gly[13] in gastrin which produced potent agonists. These results support the work of Peggion et al.[46] who proposed a β-bend involving residues Ala[11] to Trp[14] based on the circular dichroism spectra of gastrin.

A bicyclic dipeptide mimetic, which is based on a thiazolidine ring system, was reported to mimic the type II' β-bend conformation[47-49] and was used as a replacement of D-Phe[4]-Pro[5] and D-Phe[4']-Pro[5'] in gramicidin S to give a potent analog, which provided strong support that the solution conformation of gramicidin S is also the bioactive one.[50] This bicyclic dipeptide mimetic was also used as a replacement for Gly[6]-Leu[7] to give an LHRH agonist which retained 10% of the biological activity of LHRH.[51] This was used as further support for a Tyr[5]-Arg[8] type II' β-bend in the bioactive conformation of LHRH. However, replacement of Gly[2]-Gly[3] in enkephalin[47] and Phe[6]-Pro[7] in somatostatin[52] by this same dipeptide mimetic resulted in weakly active analogs.

Kemp and Stites[53] have reported a compound as an internal mimic of a type II β-bend but without supporting biological data. Kahn and colleagues have designed internal ß-bend mimetics for several peptides, including jaspamide,[54] type I ß-bends,[55] an immunosuppressing tripeptide (Lys-Pro-Arg),[56] erabutoxin b,[57] enkephalin,[58] and fibrinopeptide A.[59] The work of Kahn and colleagues will be discussed elsewhere in this volume. Belanger et al. also reported a design for a bicyclic ß-bend mimetic analog of enkephalin, but the resulting analogs showed only weak biological activity.[60,61]

ß-Bend mimetic design. As reported earlier by Krstenansky et al.,[62] a non-peptide replacement for a peptide ß-bend was designed which replaced the usual hydrogen-bond between the anti-parallel backbone N-H and C=O with a covalent linkage. The concept was to produce a relatively conformationally rigid replacement for a ß-bend which would produce the correct disposition of the peptide functional groups attached to it and also would be more stable toward proteolytic digestion. Thus, attention was paid to the angle of attachment of the substituents at what became the 2 and 7 positions and the dihedral angle between the substituents during the design of the ß-bend mimetic. The resulting mimetic was the 2,5,7-trisubstituted 2(S)-5-H-6-oxo-2,3,4,4a,7,7a-hexahydropyrano[2,3-b]pyrrole shown in Figure 1. This design was initially based on the x-ray crystal structure of Leu-enkephalin reported by Smith and Griffin[25] which was in a 4→1 ß-bend. However, it was recognized that with appropriate substitution of the ring system either the 4→1 or the 5→2 ß-bend of enkephalin could be represented, as well as ß-bends in other peptides.

The synthetic approach that was initially used for this ß-bend mimetic has been reported by Krstenansky et al.[63] as well as an improvement in the synthesis of the ring system.[64] Synthesis of this

substituted bicyclic ring system produced two isomers; the syn-, where the 2-sustituent and the lactam ring are on the same side relative to the tetrahydopyran ring, and the anti-, where the 2-substituent and the lactam ring are on opposite sides relative to the tetrahydropyran ring. The assignment of structure to the syn- and anti-isomers using NMR spectroscopy and a comparison of the bicyclic system to a type I' ß-bend using molecular modeling are reported herein.

EXPERIMENTAL METHODS

NMR. The experimental parameters for the NOE difference experiments were optimized as discussed by Derome.[65] A sample of the lactone in $CDCl_3$ was prepared. A gated decoupling sequence in which the proton resonance of interest was presaturated for a duration equal to at least three times the longest T_1 of the sample was used. A control experiment to give the "unperturbed" spectrum was performed by setting the irradiation frequency well away from the resonances at the same decoupler strength used for the "saturated" experiments and an equivalent number of scans was taken. Instrumental instabilities due to random error and systematic long-term drift were addressed by interleaving the acquisition of saturated and non-saturated experiments over the duration of the entire experiment. The difference NOE spectra were obtained by computer subtraction of the "unperturbed" FIDS from the "perturbed" FIDS. The difference FIDS were exponentially multiplied with a line broadening of 0.1 and Fourier transformed to give the difference spectra.

Molecular modeling. Molecular models of the ß-bend mimetic analogs were built using the MacroModel molecular modeling program.[66] Monte Carlo conformational searches for low-energy ring conformers utilized the Batchmin program, retaining only ß carbons in the side chains. Each conformation was minimized by the MacroModel MM2 force field by the Conjugate Gradient method. Modeling was done both in vacuum and using a volume-based continuum solvation model.[67] Conformers within @50 kj/mol energy window, starting from the lowest energy conformer, were stored and compared with the standard ß-turns.

RESULTS AND DISCUSSION

Isomer assignment by NMR methods. In the synthesis of the ß-bend analogs developed by Krstenansky et al.,[62,63] cis fusion of the bicyclic ring renders isomers in which the 5-membered ring is either syn- (in the same plane) or anti- (in the opposite plane) to the 2S-phthalimidomethyl substituent. The substituted γ-lactam isomers were previously separated by HPLC,[62] and in each NMR spectra, different chemical shifts for the 7a proton doublets were observed. Previously, the doublets lying downfield and

upfield were assigned as the syn- and anti-isomers respectively. These assignments were based on the theoretical assumption that in the anti-isomer, the 7a proton would lie in the shielding region of the phthalimide ring and result in an upfield shift of this proton. This assignment was not supported by experimental evidence such as NMR spectroscopy and necessitated further investigation to differentiate the isomers.

Nuclear Overhauser enhancement difference spectroscopy. Stereochemical assignment of the syn- and anti-isomers of the bicyclic γ-lactone by determination of the presence or absence of NOE enhancements arising from the dipolar interaction between the 7a-bridge and 2- protons was investigated.[68] As shown in Table 1, there are two doublets in the region between 5 and 6 ppm in each spectrum. These represent the isomeric 7a-bridge protons of the syn- and anti-isomers of each γ-lactone. Presaturation of the downfield doublet at 6.06 ppm resulted in an 8% NOE of the isomeric 4a-bridge multiplet, and no NOE for the 2-proton. Pre-saturation of the upfield doublet at 5.49 ppm showed a 13% enhancement of the other 4a-multiplet and a 3% NOE for the 2-proton. The reverse experiment, pre-saturation of the 2-multiplet, resulted in a 7% NOE for the upfield 7a-doublet. These experiments support the assignment of the upfield doublet as that of the syn-isomer, opposite to the assignment which had been previously proposed. The measured T_1 times for the downfield and upfield doublets were 1.82 and 1.16 s respectively. The shorter T_1 (1.16 sec) for the upfield doublet can be attributed to intramolecular relaxation between the 7a-bridge proton and the 6-proton in the syn-isomer, also supporting the assignment made from the NOE difference experiments.

Carbon-carbon anisotropy. Assignment of isomers based on theoretical carbon-carbon anisotropy in 6-membered rings[69] was also studied. The bulky sidechain at the 2-position of the 6-membered ring will exist predominantly in the equatorial position. Thus, in the bicyclic anti-isomer, the 7a-proton will be equatorial and lie in the deshielding cone of the carbon-carbon bond one bond removed. In contrast, the 7a-bridge proton of the bicyclic syn-isomer will be axial and lie outside this deshielding cone, supporting the assignment to the upfield doublet. This effect is known to produce chemical shift differences between such protons in the range of 0.2 to 0.7 ppm. Tabulation of the chemical shifts and coupling constants for the isomeric 7a-proton doublets for compounds 1, 2, and 3 (Table 1) shows a chemical shift difference range of 0.3 to 0.57 ppm. The downfield doublets also have significantly greater coupling constants which are consistent with the different conformations represented by the syn- and anti-isomers.

Taken together these NMR studies clearly establish that the upfield doublet arises from the syn-isomer and the downfield doublet from the anti-isomer.

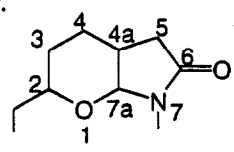

Table 1. Chemical Shift and Coupling Constant Data for Syn- and Anti-Isomers of Bicyclic Lactones.

Lactone[a]	Downfield Doublet	Upfield Doublet	Difference Chemical Shift	Difference Coupling Constant
1	5.76 ppm 4.50 Hz	5.46 ppm 3.42 Hz	0.30 ppm	1.08 Hz
2	6.06 ppm 4.29 Hz	5.49 ppm 3.91 Hz	0.57 ppm	0.38 Hz
3	5.98 ppm 4.40 Hz	5.47 ppm 3.90 Hz	0.51 ppm	0.50 Hz

[a] 1 = (2S)-2-phthalimidomethyl-; 2 = (2S)-2-phthalimidomethyl-5-carbomethoxy-; and 3 = 2-N-Boc-aminomethyl-5-carbomethoxy-6-oxo-2,3,4,4a,7,7a-hexahydro[2,3-b]furan[64]

Molecular modeling. Monte Carlo searches produced minimum energy conformations for the syn- and anti-isomers. These calculations were done for the analogs with and without a substitutent at the 5-position. Superposition with ideal ß-bends produced a close resemblance of the syn-isomer to a type I' ß-bend with a clear difference in the fit between the syn- and anti-isomers with the syn-isomer having a four atom RMS of 0.133Å while the anti-isomer had a four atom RMS of 0.425Å. The minimum energy conformation of the syn-isomer is shown in Figure 1 and the superposition of the syn-isomer on a type I' ß-bend is shown in Figure 2.

The addition of a substituent at the 5-position, which would correspond to the position of the benzyl side-chain of phenylalanine in enkephalin, produced little change in the conformation when the substituent was in the **R** configuration with a four center RMS of 0.146Å. The comparison is of the C^{α}_{i}, C_i, N_{i+3}, and C^{α}_{i+3} of the peptide with the carbon attached to C-2, C-2, N-7, and the carbon attached to N-7. When the substituent was in the S configuration, two lower energy conformers were obtained. One has almost identical conformation to that of the **R** configuration while the other resulted in a change to an alternate chair conformation of the tetrahydopyran ring which was less favorable in comparison to the type I' ß-bend with an RMS of 0.741Å.

The difference between the syn- and anti-isomers was also borne out in the biological assay. The syn-isomer without a substituent at the 5-position was a naloxone reversible agonist in the electrically stimulated guinea pig ileum assay with an IC_{50} of 3×10^{-7} M compared to 25×10^{-7} M for the anti-isomer and 1×10^{-7} M for morphine. Thus, the syn-isomer was an agonist of the same order of magnitude as morphine and one order of magnitude more potent than the anti-isomer. This agonist effect was quite selective since neither isomer was an agonist in vitro in a κ-receptor assay[70] or for the release of growth hormone or prolactin from

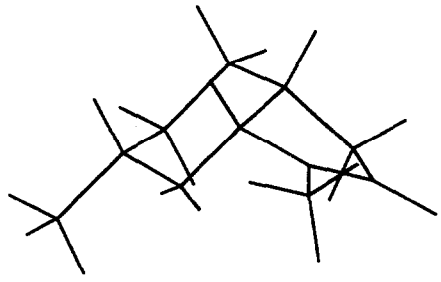

Figure 1. B-Bend mimetic analog (cis-syn) in the energy minimized conformation.

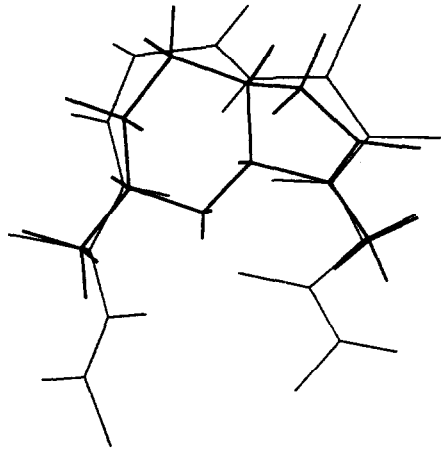

Figure 2. B-Bend mimetic analog of figure 1 superimposed on a standard type I' B-turn.

rat pituitaries.[71] These results support the 5→2 ß-bend as the biologically active conformation of Leu-enkephalin on the μ receptor.

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