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NONPEPTIDE β-TURN MIMETICS OF ENKEPHALIN

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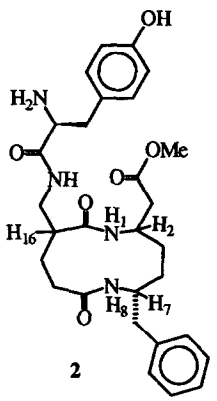
Abstract: The design and synthesis of a conformationally restricted peptide mimetic of the endogenous opiate peptide leucine enkephalin is described. This mimetic incorporates a nonpeptide β-turn prosthetic unit; conformational analysis indicates that the active isomer exhibits a conformation closely corresponding to that of a highly potent, δ-selective enkephalin analog, Tyr-D-Pen-Gly-Phe-D-Pen (DPDPE, Pen=Penicillamine). Preliminary pharmacological investigation indicated that one isomer of the mimetic species exhibits significant *in vivo* analgesic activity, despite relatively low *in vitro* binding affinity.

The intriguing relationship between morphine and the endogenous opiate peptide leucine enkephalin, **1**, discovered in 1975 by Hughes and Kosterlitz,[1] has stimulated numerous efforts to delineate the structural basis for their common analgesic effects.[2] The inherent mobility of the enkephalin framework, its rapid degradation *in vivo*,[3] and the presence of multiple receptors[4] has hampered the assessment of its bioactive conformations. Several turn conformations have been proposed based upon computational models,[5,6] X-ray crystallography,[7] and spectroscopic studies.[8] To examine these hypotheses and explore the role of potential bioactive turn conformations of enkephalin, we have designed, synthesized and evaluated the 2 → 5 β-turn mimetic **2**,[3,9] utilizing our previously described conformationally constrained turn mimetic templates.[10,11]

Tyr-Gly-Gly-Phe-Leu
1



2

Synthesis. Wittig reaction of aldehyde **3**[12] with ylid **4** provided the (E,Z) diene **5** in 75% yield after chromatography. Saponification and deconjugation[13] afforded the (E,E) diene **6** in 80% yield. Coupling of diene **6** to the previously described diacyl hydrazide **7** proceeded smoothly using Bop reagent.[14] Cycloaddition was smoothly effected at room temperature upon treatment with iodobenzene diacetate,[10] to provide the tricyclic system **9** which was readily cleaved with

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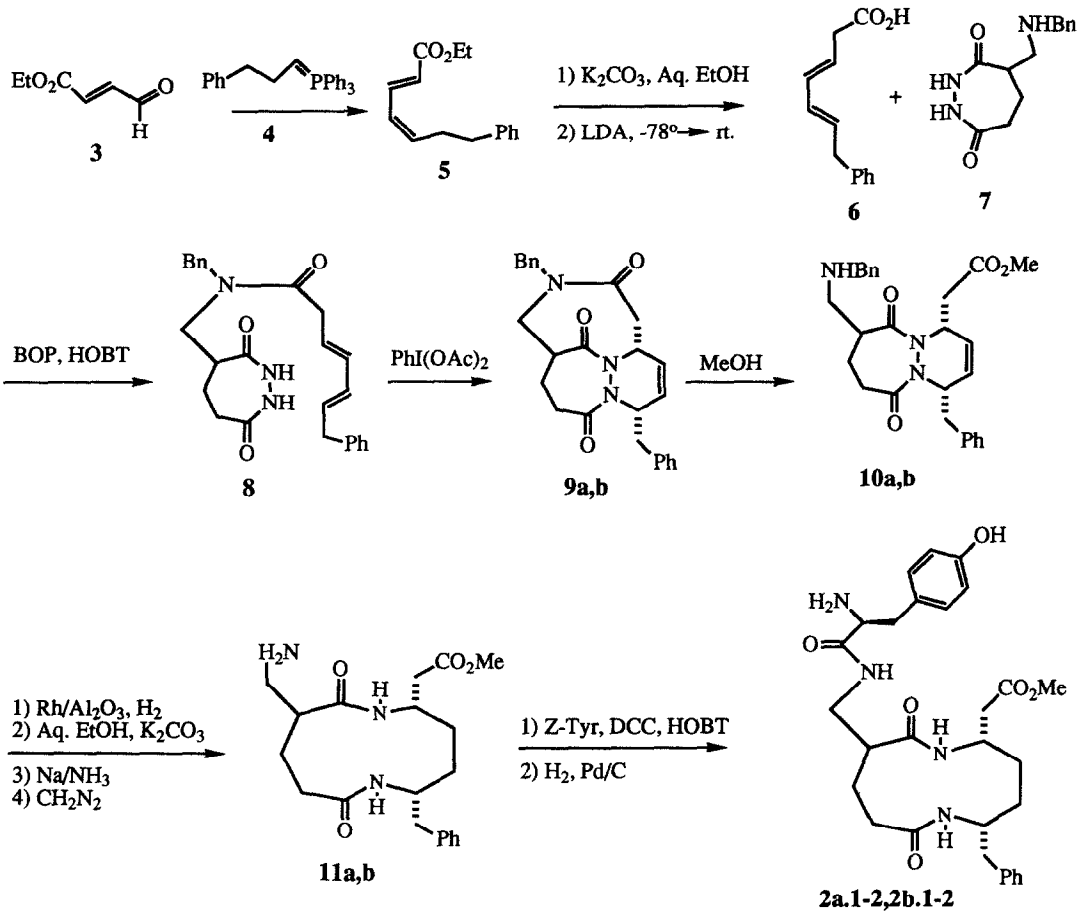
methanol to afford **10** in 36% yield after chromatography. The *syn* and *anti* diastereomers **10a,b** were separated



at this stage. Hydrogenation, saponification, reductive cleavage of the diacylhydrazide[15] with concomitant removal of the N-benzyl protecting group, and the subsequent esterification afforded the 11-membered ring bislactams **11a** and **11b**. The synthesis of chimeric peptide **2**, which perforce generates an additional pair of diastereomers was readily accomplished via a standard protocol. The newly generated pair were separated by reverse phase HPLC.[16]

Conformational Analysis. Phase sensitive 2D-DQFCOSY and 2D-ROESY nmr spectroscopy was used to analyze the ring conformations of **2a.2** and **2b.2**, the more abundant isomers.[17] (The ring structures of **2a.1** and **2b.1** are simply the mirror images of **2a.2** and **2b.2**, respectively.) Chemical shifts for both structures are nearly identical, suggesting similar conformations.[16] Both ring amide protons H_1 and H_8 are well resolved at 25 °C, suggesting the presence of hydrogen bonding that protects the amide protons from undergoing fast exchange with residual water. Selective broadening of H_8 at higher temperature indicates that it is less

protected than H_1. The coupling constants (J = 8.2-9.0 Hz) indicate that the H_1-H_2 and H_7-H_8 dihedral angles are about 0 or 180°. Weak NOESY cross-peaks between H_1 and H_2, coupled with strong cross-peaks between H_7 and H_8, and the absence of cross-peaks between H_1 and H_8 indicate that H_1 and H_2 are *anti* (180° dihedral angle), while H_7 and H_8 are *syn* (0°). Additionally, a strong H_1-H_{16} cross-peak for **2a.2**, and the absence of the same cross-peak for **2b.2** further confirm the H_1-H_2 *anti* orientation, since the H_2 and H_{16} protons are *anti* for **2a.2**, and *syn* for **2b.2**. Thus the two peptide bonds must be nearly antiparallel with each other.

The modeled structure of **2b.2**,[18] minimized after 50 ps of molecular dynamics simulation at 300 K was compared with that of the cyclic, δ-selective enkephalin analog, Tyr-D-Pen-Gly-Phe-D-Pen, as determined by NMR measurements and 250 ps molecular dynamics simulation in explicit water.[6] Fig. 1 compares the two structures, showing a remarkable similarity in the positioning of the Tyr ring, and a close correspondence of the type II' β-turn region. The low energy calculated mimetic ring structure is fully consistent with the NMR analysis. Details of the NMR and modeling analyses will be reported elsewhere.

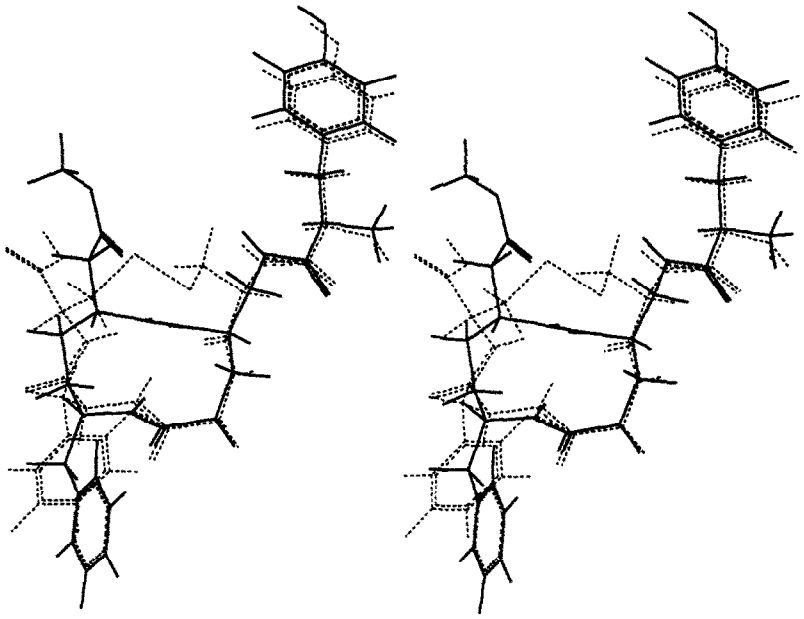


Fig. 1. Low energy conformation of **2b.2** (bold) overlaid with that of Tyr-D-Pen-Gly-Phe-D-Pen[6] (dashed, light tracing). Conformational comparison is excellent, except at the Phe position where the D-Phe of our mimetic is positioned away from that of the L-Phe of DPDPE, and may explain the relatively poor binding of the mimetics. The structural comparison suggests that inversion of the H_7 stereochemistry would enhance activity.

Biological Activity. The separated diastereomers of **2** were evaluated for their ability to bind to both μ and δ opioid receptors. Enkephalin mimetic **2b.2** displaced 53% at 10μM at the μ receptor and 50% at 6μM

at the δ receptor. The other diastereomers displayed no significant displacement at either the μ or δ receptor at the 10 μM level.[19] Despite the relatively weak *in vitro* binding activity, this analog exhibited relatively potent (ED_{50} < 10 μg/mL icv) analgesic activity in a mouse writhing assay.[20] The relatively low binding activity could be reconciled with the aid of molecular modeling, which indicates significant correspondence between DPDPE and **2b.2** in most respects. However, the D-Phe configuration in the mimetic, rather than the L-Phe in DPDPE is probably important, and the degree of conformational flexibility of these two backbones differs significantly and may play a critical role in allowing for productive binding. Efforts to further investigate the role of reverse turns in the bioactive conformations of enkephalin are in progress utilizing a recently developed more flexible framework,[21] and will be reported in due course.

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16. The two diastereometric pairs **2a** and **2b** were separated via reverse phase HPLC on a Hitachi instrument (Model L 6299 with a preparative column Hibar-preparative LiChrosorb Particles C-18, 10x250 mm from E. Merck) and with a mixture of 20% CH_3CN in H_2O containing 0.05% CF_3COOH. Retention times, monitored at 214 nm, for the two diastereomers of **2a** were 28.8 min (**2a.1**) and 31.0 min (**2a.2**) correspondingly, with a flow rate of 3 ml/min. For those of **2b**, the corresponding retention times were 29.8 min (**2b.1**) and 32.2 min (**2b.2**). Compounds **9**, **10** and **11** are racemic. Compound **2** is a mixture of 4 diastereomers all of which have the L-tyrosine absolute configuration.
17. Compounds were dissolved in 0.5 ml of deuterated DMSO and all experiments were performed on a GE 500 MHz instrument, and referenced to the DMSO peak at 2.49 ppm. Chemical shift assignments were based on 2D-DQFCOSY and ROESY experiments at 25 °C. The presence of water residue caused fast exchange between amide and water protons that resulted in broadening or disappearance of amide proton peaks under certain conditions. In temperature dependent experiments, the H_8 peak of **2b.2** started broadening at about 30 °C, and disappeared at about 35 °C, while H_1 showed no broadening under comparable conditions. In ROESY experiments, a 300 ms spin lock and a 5 kHZ field strength were applied with spectral widths of 6-8 kHz. Several carrier frequencies were used to identify and to avoid false ROESY peaks. Typical ROESY experiments were run at 25 °C, with 2K data points and 516 FIDs and a repetition time of 2 sec. Qualitative results were obtained by setting ROESY peaks between the vinyl protons of Tyr as very strong (vs) and the merely detectable ones as weak (w). Peak intensities falling in between were labeled as strong (s) and medium (m).

18. The 11-member ring moieties for **2a.2** and **2b.2,** with three methyl groups attached at the positions of chain attachment, were built in a computer using the MACROMODEL program [Still, W.C.; Tempczyk, A; Hawley, R.C.; Hendrickson, T. *J. Am. Chem. Soc.* **1990** *112*, 6127, and references therein]. Monte Carlo conformational searches were carried out to find the global minimum and the distribution of the conformers by using BATCHMIN with the MM2/MACROMODEL force field by randomly rotating torsional angles in the ring systems. The solvation effect was taken into account using a volume-based continuum model for the electrostatic component using an option in BATCHMIN. Molecular dynamics calculations were carried out using the BATCHMIN program for compounds **2a.2** and **2b.2** with 1 fs time steps. Solvation effects were again included through the volume based continuum solvation model in MACROMODEL/ BATCHMIN.
19. Binding at μ and δ receptors was measured in a twice-washed P2 membrane fraction obtained from whole rat brain (minus cerebellum) using a 50 mM Tris-HCl buffer (pH 7.4 at 37 °C). Assay tubes contained 0.8 mL of membrane homogenate (0.5 mg of protein), 0.1 mL of ^{3}H-labeled ligand (1.0 nM DTLET or 1.0 nM DSLET for δ, 2.0 nM DAMGO for μ), and 0.1 mL of the test compound in replicates of three. After 60 min incubation at 37 °C, reactions were terminated by rapid filtration on Whatman GF/B glass-fiber filters and subsequent 10 mL wash of ice-cold buffer. Filters were prepared for liquid scintillation counting. Specific binding was calculated as the difference in radioactivity bound in the absence and presence of 10 μL of levorphanol. IC_{50} values, the concentration of test compound that inhibited ^{3}H-labeled ligand binding by 50%, were obtained by regression analysis of a log-logit transformation of binding data.
20. Male albino mice (Charles River Laboratory; CD-1HAM/1LRE, 20-30 g) were used for the writhing assay. The compound was injected i.c.v. in a volume of 5 μl into 10 animals. Ten minutes later, 0.025% phenylbenzoquinone (PBQ) was injected i.p., and, following a five minute delay, each animal was then placed into a large glass beaker. The number of writhes that occurred in the subsequent 10 minutes was counted. A writhe consisted of dorsoflexion of the back, extension of the hindlimbs and strong contraction of the abdominal musculature. The test compound was considered to have significantly inhibited writhing (produced antinociception in a mouse) if the number of writhes elicited by PBQ was less than equal to one-half the median number of writhes recorded for the control group of mice that day. The data were expressed as the number of mice (out of a possible ten) in which the test compound produced antinociception. The ED_{50} for DPDPE in the mouse hot plate assay was 1.4 μg/mL, and was 2.0 μg/mL in the mouse tail flick assay i.c.v.
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