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The Synthesis, Bioactivity and Enzyme Stability of D-Ala^2, ∇^EPhe^4,Leu^5-Enkephalins

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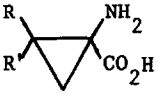
Summary. We have synthesized the first enkephalin analog containing a "cyclopropyl" phenylalanine (∇Phe) residue. The E-configuration of this residue is apparently responsible for its low activity in the MVD and GPI muscle assays. The enkephalin is very stable to cleavage by carboxypeptidase Y.

1-Aminocyclopropane carboxylic acid analogs (1) having substituents which are structurally equivalent to the α-substituents of natural protein amino acids are of interest due to their inherent steric properties. The



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quaternary α-carbon atom sterically inhibits nucleophilic attack at the α-substituents,[1] which may confer resistance to enzymatic degradation on peptides containing these amino acid residues. Also, rigid positioning of the β-substituent constrains the χ_1 angle to only two possible values (0° or 120°) depending on whether the Z-(1, R'=H) or E-isomer (1, R=H) is in hand. Models show that incorporation of these rigid amino acid moieties into a peptide chain will have a profound effect on its shape, since the ϕ and ψ angles of cyclopropyl amino acids (∇AA^2) will adopt small values at conformational energy minima, possibly leading to helical and/or β-turn conformations.[3] Thus, the incorporation of a single ∇AA into a peptide will fix several of its conformational parameters and restrict its shape severely. We have previously[4] incorporated the Δ^ZPhe (Z-dehydrophenylalanine) moiety into the 4-position of D-Ala^2,Leu^5-enkephalin and we now report the synthesis and biological properties of a ∇^EPhe (1, R=H,R'=Ph)

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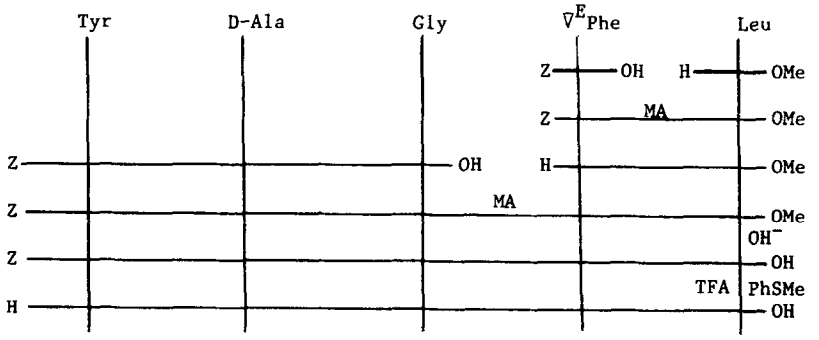
enkephalin in which the phenyl group is constrained to the opposite side of the rigid functionality.[5]

Materials and Methods

The overall synthesis of D-Ala^2,∇^EPhe^4,Leu^5-enkephalin is summarized in the Scheme. The ∇^EPhe was resolved using the brucine salt of its N-



MA = mixed anhydride; TFA = CF_3CO_2H; PhSMe = thioanisole

Scheme 1

benzyloxycarbonyl derivative, which was prepared using previously reported[6] methods. The separated Z-∇^EPhe enantiomers were deblocked and the CD spectra of their hydrochlorides were determined.[7] By analogy with the absolute configurations of α-methylphenylalanine,[8] it was decided that the (-)-isomer [$[\alpha]_D^{25}$-74.6° (c, 1.0 H_2O)] had the 2S,3R configuration corresponding to the L-form and the (+)-isomer [$[\alpha]_D^{25}$74.4° (c, 1.0 H_2O)] had the 2R,3S configuration equivalent to D-∇^EPhe. A sample of the 2S,3R enantiomer was then coupled with L-leucine methyl ester and the retention time of the peptide was determined on RPLC (C_{18} Lichrosorb, 20 cm x 0.46 cm, CH_3CN-H_2O (55:45), 2 ml/min). Racemic Z-∇^EPhe was then coupled with L-leucine methyl ester by the mixed anhydride procedure and the diastereomeric mixture was separated by RPLC using the above system; (S-isomer, 6.2 min; R-isomer, 8.1 min). One of those isomers was identical in RPLC retention time, IR and NMR spectra, to that peptide containing the 2S,3R amino acid. Each diastereomer of the dipeptide was then deblocked and coupled with the tripeptide, Z-Tyr-D-Ala-Gly·OH (prepared by standard methods), and the product was converted to the free pentapeptide by standard methods.

The MVD and GPI assays were carried out using standard methods.[9] The CPase assay was carried out essentially as previously described[10]. At intervals of 2,4 and 24 hrs, an aliquot (100 µl) was removed and lyophilized. The residue was dissolved in citrate-buffer (pH 2.2, 200 µl), and the solution was centrifugated for 10 min and injected into an amino acid analyzer. The amino acids were calculated by a computing integrator as actual nanomoles.

Results and Discussion

The bioactivities of the two peptides, as tested against the electrically stimulated mouse vas deferens and guinea pig ileum, are reported in Table 1. These results show that the ∇^EPhe moiety has a very deleterious effect on the ability of the peptide to inhibit muscle contraction. Since it is known that the D-Phe^4 enkephalin is essentially in-

Table 1

Enkephalin	Muscle Assays (IC_{50},μM) Mouse vas Deferens	Guinea Pig Ileum
D-Ala^2,(2R,3S)∇^{E}Phe^4,Leu^5	34	64
D-Ala^2,(2S,3R)∇^{E}Phe^4,Leu^5	96	260
D-Ala^2,Leu^5	0.011	---
D-Ala^2,D-Leu^5	0.0026	---

active, it is notable that the 2R,3S isomer is the more active of the pair.

Very significantly, it was also shown that absolutely no leucine (using an amino acid analyzer as detector) was liberated from either the 2S,3R or 2R,3S-isomers of ∇^{E}Phe^4-enkephalin when they were treated with CPase Y for 24 hours (substrate: enzyme; 274:1). D-Ala^2,Leu^5-enkephalin was hydrolyzed to the extent of 17% in 2 hrs and 100% in 24 hrs under the conditions used. The dehydro analog, D-Ala^2, Δ^{Z}Phe^4,Leu^5-enkephalin was also very resistant to CPase Y, but leucine was slowly liberated.[10]

Since the dehydro peptide, D-Ala^2, Δ^{Z}Phe,Leu^5-enkephalin (χ_1=0 for ∇^{Z}Phe) was strongly active in the muscle assays it is clear that the E-configuration of the ∇Phe moiety, which has a χ_1 angle of ~120°, is "wrong" for these muscle receptors. Whether this change in χ_1 or changes in the ϕ,ψ angles are responsible for the gross loss in activity would be difficult to establish. Synthesis of the ∇^{Z}Phe^4 enkephalins is underway with the hope that further clarification of these steric effects can be developed.

Acknowledgment

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References

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2. The inverted triangle, ∇, is used to designate "cyclopropyl" with superscripts, ∇^{E} or ∇^{Z}, to designate the configurations about the cyclopropane ring. This is not to be confused with the symbol, Δ, meaning "dehydro", as in Δ^{Z}Phe, etc.

3. The similarity between α-aminoisobutyric acid (Aib) and ∇Ala is evident. For a recent description of the conformational effects of the former, see Nagaraj, R. and Balaram, P. (1981) Acc. Chem. Res. **14**, 356-362.

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5. The thermodynamic instability of Δ^{E}Phe (see Nitz, T. J., Holt, E. M., Rubin, B. and Stammer, C. H. (1981) J. Org. Chem., **46**, 2667) makes incorporation of a dehydro moiety of opposite configuration problematical.

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7. We are grateful to Dr. Frank Robinson of Merck & Co. Rahway, N.J., for these determinations.

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