Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXIV, No. 10

SYNTHESIS OF DEUTERIUM LABELLED PENICILLAMINE AND ITS USE FOR THE ASSIGNMENT OF THE ^{1}H NMR SPECTRA OF TWO CYCLIC ENKEPHALIN ANALOGS

Henry I. Mosberg*, John R. Omnaas,
Kondareddiar Ramalingam, and Ronald W. Woodard*
Department of Medicinal Chemistry
College of Pharmacy
The University of Michigan
Ann Arbor, Michigan 48109-1065

SUMMARY

The synthesis of D,L-[4,4'-$^2H_6$]penicillamine (D,L-[$^2H_6$]Pen) and its incorporation into the cyclic, disulfide-containing peptides, [D-Pen^2,D-[$^2H_6$]Pen^5]enkephalin (DPDPE) and [D-Pen^2,L-[$^2H_6$]Pen^5]enkephalin (DPLPE), both highly δ opioid receptor-selective analogs, are described. The deuterium-labelled peptides allowed the assignment of the four Pen methyl group resonances in each of the corresponding unlabelled peptides to specific Pen residues.

Key Words : D,L-[4,4'-$^2H_6$]penicillamine, ^{1}H-NMR, deuterium labelling, enkephalin analogs, DPDPE, DPLPE

INTRODUCTION

Many endogenous peptide hormones and neurotransmitters are relatively small, flexible molecules which can utilize their inherent flexibility to interact with different subclasses of receptors which mediate different physiological events and which presumably place different conformational requirements upon the ligand. In order to elucidate the molecular mechanism of action of a particular peptide hormone or neurotransmitter it is necessary to unravel the distinct actions mediated by the individual receptor subclasses and to determine the bioactive conformation of the peptide ligand at each of these receptors. A particularly useful approach toward these ends is the design and synthesis of analogs of the native peptide into which conformational restrictions are incorporated. One benefit of this approach is that the proper choice of conformational restriction can result in an analog capable of assuming the conformational requirements for interaction

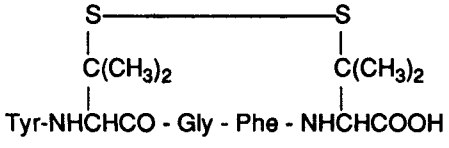
*To whom correspondence should be addressed.

0362-4803/87/101265-07$05.00

Received September 2, 1986
Revised December 17, 1987

with one subclass of receptor but not other subclasses. Such highly receptor-selective analogs can then be used to determine the physiological actions mediated by distinct receptor subclasses. An additional benefit of conformationally restricted analogs is that while the conformational analysis of flexible peptides is hampered by dynamic averaging of conformation-dependent spectroscopic parameters leading to the determination of an average solution conformation of dubious physical or biological significance, more rigid analogs do not share this liability. Thus, a conformationally restricted analog can be expected to assume a more well-defined solution conformation and allow a more reliable extrapolation to the active, receptor-bound conformation. For conformationally restricted, receptor-selective analogs this allows the determination of the bioactive conformation at **specific** receptor subclasses.

Analogs of the enkephalins, one class of endogenous opioid peptides, provide an example of the benefits of the approach outlined above. Opioid receptor heterogeneity has been amply demonstrated and it is now clear that three distinct opioid receptor subtypes, designated μ, δ, κ, exist (1,2). A number of enkephalin analogs have been reported which display enhanced receptor selectivity relative to the native ligands. Among these highly selective analogs are the cyclic, disulfide-containing compounds, [D-Pen^2,D-Pen^5]enkephalin (DPDPE) and [D-Pen^2,L-Pen^5]enkephalin (DPLPE), in which Pen, penicillamine is β,β-dimethylcysteine, which display the highest selectivity reported to date for the δ opioid receptor (3-5). These diastereomeric enkephalin analogs which have the structure:



are conformationally restricted due to the imposed cyclization *via* a disulfide bond and are further conformationally restricted due to the presence of the gem-dimethyl substituents within the 14-membered cyclic moiety (3). Thus DPDPE and DPLPE represent valuable tools for elucidating the active conformation at a specific receptor subtype, the δ opioid receptor.

Proton NMR is an extremely powerful method for determining the solution conformation of relatively small peptides such as DPDPE and DPLPE with the obvious first requirement being the assignment of all the resonances within the spectra. In this regard, DPDPE and DPLPE present difficulties. While the α-carbon proton resonances arising from Pen^2 and Pen^5 can be differentiated by observing chemical shift changes upon pH titration of the Pen^5 carboxylic acid function, the β methyl proton shifts are insensitive to this pH variation. Thus, while four distinct resonances are observed for the penicillamine methyl groups, unequivocal assignment to the individual Pen residues is not possible. In order to remedy this uncertainty, we report here the synthesis of D,L-[4,4'-$^2H_6$]penicillamine (D,L-[$^2H_6$]Pen), its incorporation into DPDPE and DPLPE as the carboxy terminal (Pen^5) residue, and the subsequent unequivocal assignment of the Pen methyl ^{1}H-NMR resonances.

EXPERIMENTAL

Melting points were determined on a Mel-Temp apparatus and are uncorrected. All chemicals were obtained from Aldrich Chemical Co., Vega Biochemicals, and Pierce Co. and were used without further purification. Percent deuterium incorporations were determined from ^{1}H-NMR spectral data. ^{1}H-NMR spectra were recorded at 270 MHz on an IBM WP-270-SY NMR spectrometer. Peptide samples for NMR were prepared by first dissolving in D_2O and lyophilizing to replace exchangeable protons with deuterons and then dissolving in 100.0% D_2O and adjusting to pH 3.0 (uncorrected meter reading) with CD_3CO_2D. Chemical shift values are reported in parts per million (ppm) downfield from internal 3-(trimethylsilyl)tetradeuteriopropionate (TSP).

S- Benzyl-D,L-[4,4'-$^2H_6$]penicillamine.

A 50-mL three-necked flask, equipped with a magnetic stirring bar, a pressure-equalizing addition funnel, and a nitrogen inlet valve was charged with 1.13 g (0.01 mol) ethyl isocyanoacetate (6) in 10 mL of freshly distilled tetrahydrofuran and cooled to -78°C while 4.3 mL of a 2.5 M solution of *n*- butyllithium (0.011 mol) in THF was added dropwise. After addition of the *n*- butyllithium, the mixture was stirred for 15 min at -78°C, a solution of [$^2H_6$]acetone (1.28 g, 0.02 mol, 99.5 atom %) in 5 mL of THF was added dropwise during a period of 5 min and the solution was then stirred for an additional 4 h at -78°C. The reaction mixture was allowed to warm to 25°C and 1.25 g (0.01 mol) of benzyl

mercaptan was added all at once. After the solution had stirred for an additional 30 min at 25°C, the solvent was removed and 15 mL of a 1 N KOH solution was added to the residue. The mixture was stirred until a homogeneous solution was obtained (ca. 30 min). The basic solution was neutralized with concentrated hydrochloric acid and allowed to stand at 10°C overnight. The precipitated solid was filtered and recrystallized from aqueous ethanol to give 1.51 g (55.7% yield) *N*- formyl-*S* -benzyl[4,4'-$^2H_6$]-D,L-penicillamine, m.p. 156-157°C (Lit. (7) m.p. 157°C for the nondeuterated analog). ^{1}H-NMR (DMSO-d_6) δ 3.28 (s, 1H, α-C*H*-), 3.79 (s, 2H, $C_6H_5CH_2$), 7.2-7.42 (m, 6H, C_6H_5- and C*H*O merged), 7.52 (bs, 1H, N*H*).

A solution containing 0.5 g of the above *N*- formyl-*S* -benzyl[4,4'-$^2H_6$]-D,L-penicillamine in 10 mL of 6N HCl was heated under reflux for 12 h. The solvent was removed on a rotary evaporator (45°C, water aspirator pressure) and the residue was recrystallized from ethanol-diethyl ether to yield 450 mg (87.5%) of the title compound hydrochloride salt, m.p. 192-194°C. The atom percent of deuterium in the product was estimated to be 99% from the integrated ^{1}H-NMR spectra: ^{1}H-NMR (DMSO-d_6) δ 3.2 (s, 1H, α-C*H*-), 3.8 (s, 2H, $C_6H_5CH_2$), 7.2-7.4 (m, 5H, C_6H_5-).

S- Benzyl-*N-t*- Boc-D,L-[4,4'-$^2H_6$]penicillamine.

The above *S*- protected amino acid hydrochloride salt (280 mg, 1 mmol) was dissolved in a solution of dioxane/H_2O (2:1, 7 mL) and 212 mg (2 mmol) of sodium carbonate and cooled to 0°C with an ice-water bath. When the evolution of carbon dioxide had ceased, 240 mg of di-*tert*- butyl dicarbonate (1.1 mmol) was added in portions with stirring. An additional 110 mg (1 mmol) of sodium carbonate in 3 mL of water was added and the reaction mixture was stirred for 1 h at 0°C and then 12 h at room temperature. The dioxane was removed on a rotary evaporator. The remaining aqueous solution was acidified (pH 6) by the dropwise addition of 2N HCl and extracted with ethyl acetate. The ethyl acetate was washed with water, dried over sodium sulfate and evaporated *in vacuo* to afford 310 mg (93%) of the title compound as a clear oil. ^{1}H-NMR (DMSO-d_6) δ 1.42 (s, 9H, *t*- C_4H_9), 3.25 (s, 1H, α–C*H*), 3.82 (d, 2H, $C_6H_5CH_2$-), 7.00 (bs, 1H, N*H*-), 7.24-7.42 (m, 5H, C_6H_5-).

[D-Pen^2,D-[$^2H_6$]Pen^5]enkephalin and [D-Pen^2,L-[$^2H_6$]Pen^5]enkephalin.

The *S* - Benzyl-*N* -*t* -Boc-D,L-[4,4'-$^2H_6$]penicillamine was coupled to chloromethylated (1.16 mmol Cl^-/g resin) polystyrene resin 1% cross-linked with divinylbenzene using the procedure of Gisin (8) and the resin-bound hexadeuterated pentapeptides were synthesized using solid phase methods as previously described (3). Cleavage of the peptides from the resin and simultaneous deprotection of the Pen side chain sulfurs were effected by treatment with anhydrous HF in the presence of 10% anisole (0°C, 60 min). Extraction of the resin with 80% acetic acid followed by lyophilization yielded the deuterium labelled, free sulfhydryl-containing peptides which were then oxidized to the cyclic, disulfide-containing forms by treatment with $K_3Fe(CN)_6$ as previously described (3). The deuterium-labelled diastereomeric peptides [D-Pen^2,D-[$^2H_6$]Pen^5]enkephalin and [D-Pen^2,L-[$^2H_6$]Pen^5]enkephalin were separated by high performance liquid chromatography (HPLC) on a Vydac 218TP C-18 column (2.5 cm x 22 cm) using the solvent system 0.1% trifluoroacetic acid in H_2O/ 0.1% trifluoroacetic acid in acetonitrile (75/25). Analytical HPLC (Vydac 218TP, 0.46 cm x 25 cm) of the products showed them to be > 98% pure and each deuterium-labelled peptide co-eluted with the corresponding unlabelled analog.

RESULTS AND DISCUSSION

Several chiral glycine-type synthons (9,10) are known that should allow for the asymmetric synthesis of L-[$^2H_6$]Pen and/or D-[$^2H_6$]Pen in high optical enrichment (>98%). However, since the two diastereomers, DPDPE and DPLPE, are readily separable by HPLC, it was more logical to synthesize D,L-[$^2H_6$]Pen, carry out one solid-phase peptide synthesis and then separate the isomers. The method chosen for the synthesis of D,L-[$^2H_6$]Pen involved the use of the glycine synthon, ethyl isocyanoacetate. Perdeuterated acetone was added to the lithiated ethyl isocyanoacetate to give first ethyl 3,3-dimethyl-2-hydroxy-2-[3,3-$^2H_6$]isocyanoacetate (a β-hydroxyvaline analog) which under the reaction conditions used, dehydrated to ethyl 3,3-dimethyl-2-[3,3-$^2H_6$]isocyanoacrylate, a dehydro amino acid derivative. The *in situ* dehydration was attributed to the acidity of the remaining α-hydrogen atom of the monoalkylated ethyl isocyanoacetate. Benzyl mercaptan was added across the double bond giving, after base and acid hydrolysis, the *S*- benzyl-D,L-[$^2H_6$]Pen in 50% overall yield. Treatment of the *S*- benzyl-D,L-[$^2H_6$]Pen with

di*tert*- butyl dicarbonate gave the *S*-benzyl-*N*-*tert*- boc-D,L-[4,4'-$^2H_6$]penicillamine which was then used to synthesize [D-Pen^2,D-[$^2H_6$]Pen^5] enkephalin and [D-Pen^2,L-[$^2H_6$]Pen^5]-enkephalin via standard solid-phase peptide methodology.

The ^{1}H-NMR chemical shift values, δ, for the four penicillamine methyl resonances (all singlets) of DPDPE and of DPLPE are shown in Table 1 along with the corresponding chemical shifts observed for [D-Pen^2,D-[$^2H_6$]Pen^5]enkephalin and [D-Pen^2,L-[$^2H_6$]Pen^5]enkephalin.

Table 1.

Compound	δ-values (Pen methyl signals)
DPDPE	1.48; 1.34; 1.29; 0.84
[D-Pen^2,D-[$^2H_6$]Pen^5]enkephalin	1.48; 0.84
DPLPE	1.44; 1.29; 1.27; 0.86
[D-Pen^2,L-[$^2H_6$]Pen^5]enkephalin	1.44; 0.86

The results compiled in Table 1 clearly demonstrate that in each of the unlabelled enkephalin analogs the central pair of penicillamine methyl resonances arise from the Pen^5 residue while the downfield-most and upfield-most penicillamine methyl resonances are due to Pen^2. It should be noted that more sophisticated NMR techniques, such as two-dimensional COSY optimized to detect long-range coupling constants (11), when applied to the unlabelled peptides were unable to provide this unequivocal assignment (unpublished observation) and thus the judicious use of deuterium-labelled amino acids remains an important tool for the elucidation of ^{1}H NMR spectra of peptides.

ACKNOWLEDGMENT

This work was supported by U. S. Public Health Service Grant DA 03910 (H. I. M.) and the Program in Protein Structure and Design at the University of Michigan (H. I. M. and R. W. W.). We are grateful to the U. S. P. H. S. and the College of Pharmacy for their contribution to the purchase of the IBM 270 MHz NMR.

REFERENCES

1. Lord, J. A. H., Waterfield, A. A., Hughes, J. and Kosterlitz, H. W. - *Nature* **267**: 495 (1977).

2. Paterson, S. J., Robson, L. E., and Kosterlitz, H. W. - *Br. Med. Bull.* **39**: 31 (1983).
3. Mosberg, H. I., Hurst, R., Hruby, V. J., Gee, K., Yamamura, H. I., Galligan, J. J. and Burks, T. F. - *Proc. Natl. Acad. Sci. USA* **80**: 5871 (1983).
4. James, I. F. and Goldstein, A. - *Mol. Pharmacol.* **25**: 337 (1984).
5. Corbett, A. D., Gillan, M. G. C., Kosterlitz, H. W., McKnight, A. T., Paterson. S. J., and Robson, L. E. - *Br. J. Pharmacol.* **83**: 271 (1984).
6. Ramalingam, K., Kalvin, D. and Woodard, R. W. - *J. Labelled Compounds,* **XXI**: 833 (1984).
7. Schöllkopf, U. and Hoppe, D. - *Angew. Chem. Internat. Edit.,* **9**: 236 (1970).
8. Gisin, B. F. - *Helv. Chim. Acta,* **56**: 1476 (1973).
9. Schöllkopf, U. - *Tetrahedron,* **39**: 2085 (1983).
10. Seebach, D., Boes, M., Naef, R. and Schweizer, W. B. - *J. Am. Chem. Soc.,* **105**: 5390 (1983).
11. Bax, A. and Freeman, R. - *J. Magn. Resonance* **44**: 542 (1981).