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AN APPROACH TO VECTORISATION OF PHARMACOLOGICALLY ACTIVE MOLECULES : THE COVALENT BINDING OF LEU-ENKEPHALIN TO A MODIFIED β- CYCLODEXTRIN.

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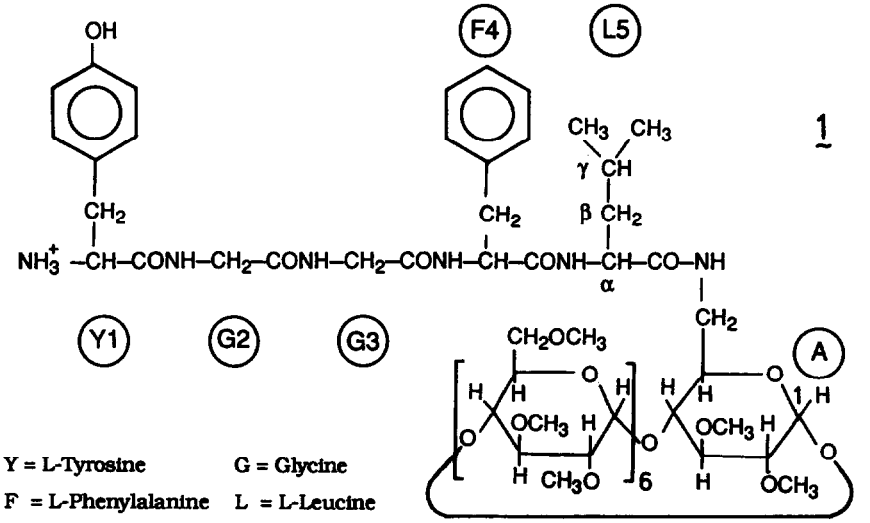
Summary : The neurotropic peptide Leu-enkephalin has been coupled to a mono-6-amino permethyl β - cyclodextrin at the C-terminal residue. The resulting compound has been fully characterized by proton NMR in D2O and d6-DMSO evidencing complete reduction of the molecular symmetry of the cyclodextrin.

Coupling of a bio-active marker onto the carrier cyclodextrin may provide a site-specific transport and delivery system, the ability of these cyclic oligosaccharides to form inclusion complexes with hydrophobic organic molecules being well established[1]. The first example of this newly designed series is provided by grafting the neuropeptide Leu-enkephalin onto mono-6-amino permethyl β-cyclodextrin. Besides the potential uses of this new class of transporter, coupling of bio-active peptides to cyclodextrins should also be regarded as providing routes to increased solubility and bioavailability of hydrophobic peptides and as ways to protect them against fast degradation by proteases. Coupling of enkephalin to monosaccharides has recently been demonstrated[2] and the biological activity of the enkephalin moiety is retained[3] .
We report here on the synthesis and NMR characterization of the first compound in this series.



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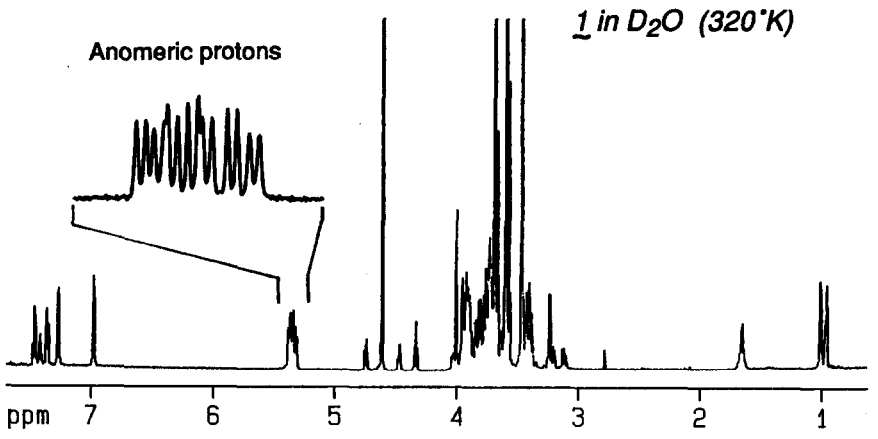
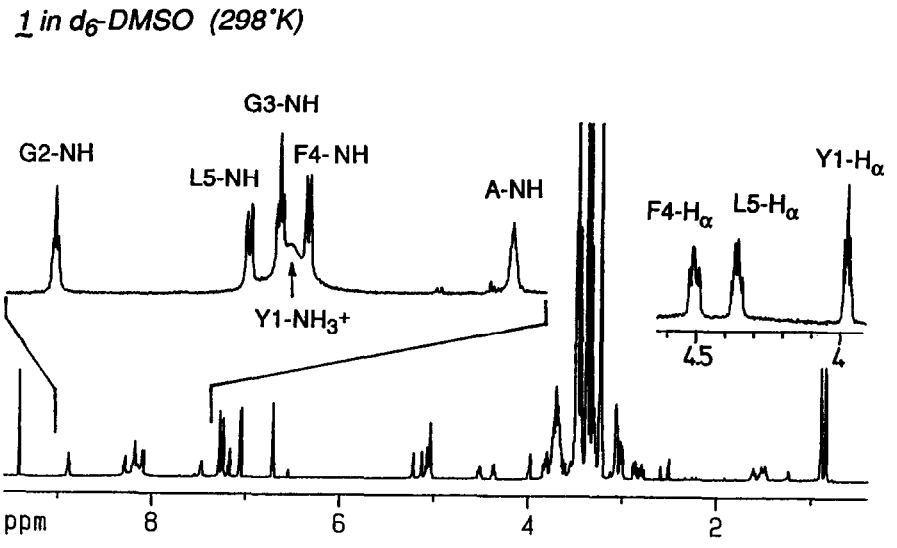
The NMR analysis of 1 was performed in D_2O and d_6-DMSO and demonstrated that the purified sample was free of any included by-products or reagents. Digital integration of selected NMR signals arising from the cyclodextrin and peptide moieties unambiguously showed the monosubstitution of the cyclodextrin.

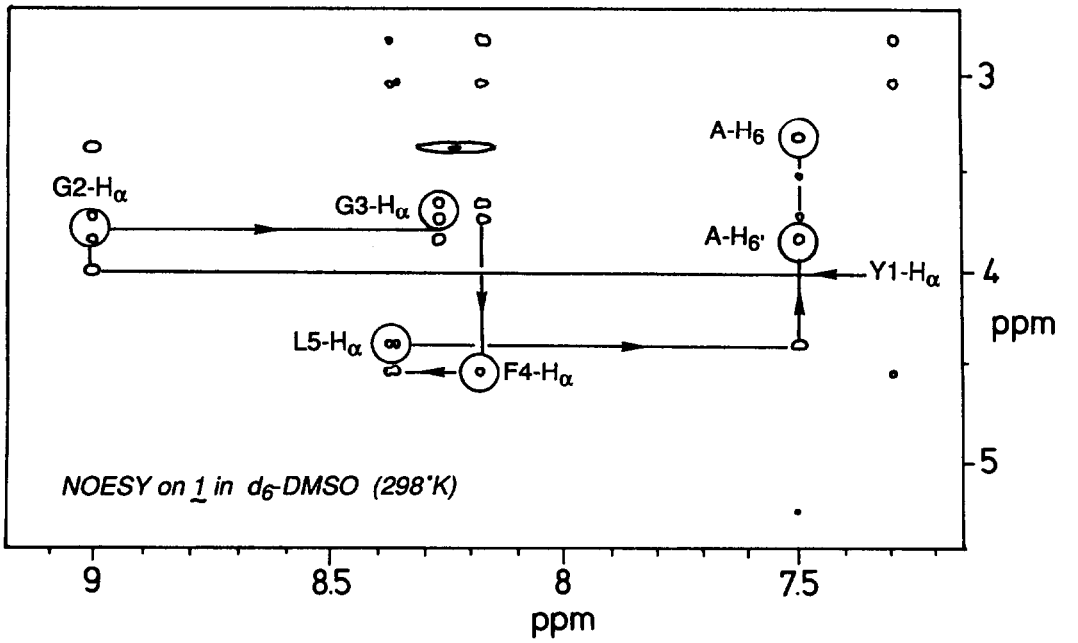


The ^1H NMR spectrum of 1 in D_2O reveals several interesting features. The total reduction of molecular symmetry expected for single substitution is clearly evidenced, all 7 doublets from the anomeric protons being visualized although, at the present time, they have not been assigned to given glucopyranosyl units of the cyclodextrin. This complete non-equivalence does not arise from partial methylation of the osidic hydroxyls as the 20 expected O-CH_3 groups can be observed.



This is further evidenced by the absence of residual lines from the cyclodextrin hydroxyl groups in the proton NMR spectrum of 1 in d6-DMSO where all signals in the 4-9 ppm region have been assigned by a sequence specific approach using of a combination of scalar and dipolar bidimensional correlation experiments.

A phase-sensitive double-quantum filtered COSY experiment[4] has allowed the identification of all amino-acids spin systems and assignment of Y1-Hα at 3.98 ppm due to lack of coupling to amide protons.



Sequential assignment of all protons of the peptide backbone was then derived from a phase sensitive NOESY experiment[5] performed in d6-DMSO at 298°K with 500 msec mixing time. Cross-peaks already present on the COSY contour plot are circled and correspond to correlations between amide and α protons within the same residue. Starting from Y1-Hα , all Hα and amide signals were assigned unambiguously as well as the 6,6' methylene protons of the substituted glucopyranosyl unit A. It is noteworthy that the NOESY contour plot shows many other cross-peaks which arise from spatial proximity between protons as well as from some spin diffusion effects. They will be used for a subsequent conformational analysis of 1 in solution. NMR experiments dedicated to afford a complete sequential assignment of protons from the cyclodextrin moiety are presently on the way.

Further work in progress indeed deals with the determination of the three-dimensional conformation in solution using quantitative NOESY data in comparison with the structure of natural enkephalins in terms of affinity for opiate receptors and susceptibility to enzymatic degradation.

Experimental :

Mono- 6-tosyl-β-cyclodextrin[6] (12g, 9.3 mmol.) was converted to the mono-azido derivative by action of lithium azide (6g, 0.122 mol.) in water (82 ml) at 90 °C for 4 hours (84% overall yield after recrystallization in water). This compound was permethylated under solid-liquid phase transfer catalysis conditions. A mixture of mono-6-azido-β-cyclodextrin (5.0g, 4.31 mmol.), powdered potassium hydroxide (7.25g, 0.13 mol.) and Aliquat, 0.2g (Aldrich) was treated with dimethylsulfate (12.5 ml) at 20 °C for 4 hours affording mono-6-azido-permethyl-β-cyclodextrin in 32% yield after chromatography on silicagel using ethyl acetate-methanol (95:5, v:v). Reduction of the latter compound with hydrogen in the presence of platinum oxide in methanol-aqueous hydrochloric acid gives the hydrochloride of mono-amino-permethyl-β-cyclodextrin in 84.5% yield. 1 was prepared in 75% yield by coupling Boc-Leu-enkephalin (Bachem, Switzerland) to the mono-amino permethyl derivative using the dicyclohexylcarbodiimide/hydroxybenzotriazole procedure[7] in methylene chloride at 0 °C. The crude material was purified by chromatography on silicagel using ethyl acetate - methanol (98:2, v:v) as eluant. Quantitative removal of the Boc protecting group was achieved in neat trifluoroacetic acid. The final compound was isolated as the trifluoroacetate. All intermediate compounds were checked for purity by NMR.

Proton NMR experiments were performed at 600 MHz on a Bruker AM600 spectrometer using 10 mM solutions of 1. Phase sensitive COSY and NOESY experiments were obtained at 298K in d6-DMSO. 400 time increments have been used to generate the second dimension. After zero-filling and Fourier transformation, a 1024x1024 real data points matrix is obtained. Only negative contours are plotted.

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