Tritiated Peptides. Part 13.[1] Synthesis of [4,5-^{3}H-Leu^2]- and [3,4-^{3}H-Pro^6]-Locust Adipokinetic Hormone

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The synthesis of [4,5-^{3}H-Leu^2]- and [3,4-^{3}H-Pro^6]-locust adipokinetic hormones by the catalytic tritiation of [4,5-dehydroLeu^2]-(ΔLeu^2-) and [3,4-dehydroPro^6]-(ΔPro^6-) precursors is described.† The products had specific radioactivities of 115 and 12.1 Ci mmol^{-1} respectively. The distribution of isotope in the former was investigated by ^{3}H n.m.r. spectroscopy which confirmed the conclusions from ^{2}H n.m.r. spectroscopy of catalytically deuteriated *N*-acetyl-4,5-dehydroleucine. It was concluded that the δ-methyl groups and the γ-methinyl group of leucine are the only tritiated positions in the molecule.

In order to explore the precise site of action and mode of degradation of locust adipokinetic hormone (1; LAKH),[2] radioisotopically labelled peptide was required. Material of a high specific activity, 50—100 Ci mmol^{-1}, is desirable to ensure that useful results can be obtained at dose levels of the order of those found naturally in the insect (10 pg). Unnaturally high dose levels may lead to non-specific binding and degradation by other routes. Specific tritiation of peptides has in previously reported work predominantly been carried out by catalytic tritiation of protected peptides containing iodine substituted in aromatic amino-acids.[3] Although the LAKH sequence contains phenylalanine, we have chosen to extend our studies and examine non-aromatic amino-acid side-chains as sites of tritiation. Accordingly, we describe the catalytic tritiation of ΔLeu^2- and ΔPro^6-LAKH, two analogues themselves of interest in correlating structure-activity relationships, whose synthesis and biological activity has been earlier reported.[4]

The tritiation of 3,4-dehydroproline residues in thyroliberin and bradykinin analogues has been explored by Felix *et al.*[5] Theoretical labelling of the former tripeptide was obtained (58.4 Ci mmol^{-1}) but no specific activity was given for the latter nonapeptide, and it was stated that ' complete reduction of the 3,4-double bond of the prolyl residue in longer peptides may require more forcing conditions than those described ' (PdO, MeOH, $^3H_2$, 3.5 h). There are two reported cases of the specific tritiation of peptides by catalytic reduction of alkenes. A protected gastrin C-terminal tetrapeptide amide analogue containing a 4,5-dehydronorleucyl residue gave, on reduction with $^3H_2$ gas in a large volume of methanol, material of low specific activity (0.14 Ci mmol^{-1}),[6] while protected [allylglycine^4]-β-corticotropin (1—24)-tetracosapeptide gave the 4,5-[^{3}H]norvaline analogue with specific activity 7.42 Ci mmol^{-1}.[7] Prior to our work no peptide longer than a tripeptide had been reported as completely tritiated by reduction of an alkene linkage to give material with a specific activity of the order of 50 Ci mmol^{-1}.

The catalytic hydrogenation of ΔLeu^2- and ΔPro^6-LAKH was initially examined on a small scale using high-pressure liquid chromatography (h.p.l.c.) to follow the progress of the reaction. Using the conditions reported by Felix *et al.*, palladium oxide in methanol, reduction was slow in both cases. Substitution of palladium on charcoal did not improve the rate; only *ca.* 20% reduction occurred during 1 h. However, replacement of the solvent by *N*,*N*-dimethylformamide (DMF) effected a dramatic improvement. Reduction was now largely complete after 1 h in both cases. The occurrence of side reactions under these conditions was investigated by reduction of ΔLeu^2-LAKH in DMF for 24 h. We were unable to show the presence of products other than LAKH by h.p.l.c.

Tritiation of the decapeptides containing dehydro-residues was therefore carried out (2—4 μmolar scale) in DMF with 10% Pd/C as catalyst. The reductions were for 4 h although tritium gas uptake was no longer measurable after 1 h. The products were purified by h.p.l.c. and then passed down an anion exchange column to remove phosphate ions (phosphoric acid was a component of the h.p.l.c. solvent mixture). The products on analytical h.p.l.c. were indistinguishable from authentic LAKH. Amino-acid analysis and counting after both acidic and enzymic hydrolysis gave results that were consistent with (3,4-[^{3}H]-Pro^6)- and (4,5-[^{3}H]-Leu^2)-LAKH ‡ (Table 1). Yields of *ca.* 50% were obtained, and the specific activities were 12.1 and 115 Ci mmol^{-1} respectively; 100% incorporation of two tritons per molecule should give a specific activity of 58.25 Ci mmol^{-1}.

The low specific radioactivity (*ca.* 21% of theory) of (3,4-[^{3}H]-Pro^6)-LAKH is probably a reflection of the rate of reduction of the precursor. Protons present as traces of water and readily exchangeable protons in the peptide itself can clearly compete effectively with tritium in the presence of the catalyst.

The extremely high specific activity of the (4,5-[^{3}H]-

Table 1. The percentage of the total tritium content associated with amino-acids after hydrolysis

	Amino-acids	Acidic hydrolysis	Enzymic hydrolysis
3,4-[$^3H_2$]-Pro^6-LAKH(1—10)	Pro	97.5	91.4
	Phe	1.2	1.2
	Trp	1.3	5.6
4,5-[^3H_n]-Leu^2-LAKH(1—10)	Asp	0.14	—
	Leu	98.3	43.3
	Phe	0.17	0.13
	Trp	0.23	0.65
	Glp-Leu	—	55.9

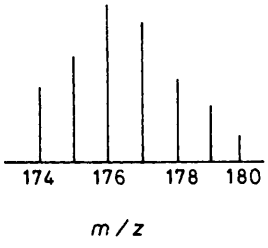
† In this paper, all amino-acid residues are of the L-configuration unless otherwise stated. Abbreviations for amino-acids and their use in the formulation of derivatives follow the revised recommendations of the IUPAC-IUB Commission on Biochemical nomenclature entitled ' Symbols for Amino-acid Derivatives and Peptides. Recommendations (1971).'

‡ The origin of the slightly low Asp values has previously been discussed.[4]

Table 2. Mass spectra of *N*-acetyl-4,5-dehydro-DL-leucine and reduced derivatives

	Catalyst	M^+ (*m/z* range)	Most abundant M^+ peak
N-Acetyl-4,5-dehydro-DL-leucine	—	—	171
N-Acetyl-DL-leucine	10% Pd/C	—	173
N-Acetyl-[^2H_n]-DL-leucine	10% Pd/C	174—180 †	176
	5% $Rh/CaCO_3$	174—180 †	176
	PtO	174—180 †	176
	Wilkinson's	174—176 *	175

* *m/z* 174 and 176 were of such low intensity as to be almost negligible. † The relative intensities were as shown below.



Leu^2]-LAKH, which corresponds to the presence of *ca.* four tritons per molecule, was useful for our purposes but demanded further investigation. It has been established that all the tritium was located in the leucine residue. Catalytic deuteriation of *N*-acetyl 4,5-dehydro-DL-leucine was carried out, and the product subjected to n.m.r. and mass spectroscopy (m.s.) to explore the deuteriation pattern. The m.s. results obtained using four different catalysts in DMF are shown in Table 2. The heterogeneous catalysts all gave similar results, the product being mixtures of compounds containing from one to seven deuterons, the triply deuteriated species being the most abundant. Wilkinson's homogeneous catalyst [8] by contrast gave almost exclusively the dideuteriated species. These results confirmed the occurrence of substantial isotopic exchange during saturation of the alkene linkage of 4,5-dehydroleucine using a heterogeneous catalyst.

The ^1H n.m.r. spectrum of dehydroleucine and its simple *N*-acyl or *N*-alkoxycarbonyl derivatives shows two interesting features (Table 3). The protons on the β-carbon atom are non-equivalent, although the chemical shifts are smaller than those observed in the cyclic derivative α-amino-γ-methylvalerolactone, where almost totally separate signals are seen for each proton. These results are indicative of a favoured conformation for the amino-acid. The signal for the terminal methylene protons is solvent dependent. In $CDCl_3$ the protons are non-equivalent, giving a complex 'doublet', but in deuterioacetone only a singlet is observed. If the preferred conformation of the amino-acid involves the close proximity of the alkene link to the carbonyl of the carboxy-group (3), then approach of the carbonyl group of an acetone molecule on the other side of the alkene link could effectively restore the symmetry of the magnetic field. Such a conformation, of course, should facilitate lactonisation.

The ^1H n.m.r. spectrum of catalytically deuteriated *N*-acetyl-4,5-dehydro-DL-leucine is complicated by the mixture of compounds present. However, the non-equivalence of the

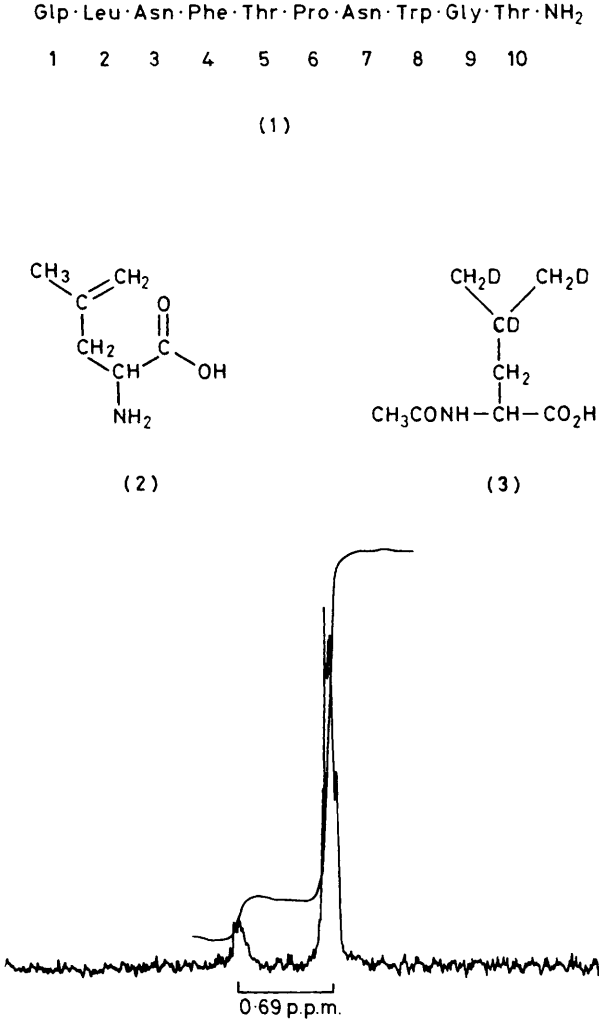


Figure Proton decoupled ^3H n.m.r. spectrum of (4,5-[^3H_n]-Leu^2)-LAKH(1—10)

δ-methyl groups aids in the determination of deuteriation sites. The relative integration values of the signals shows that the two δ-methyl groups are equally isotopically substituted. While the γ-carbon atom is totally deuteriated, the β-methylene group contains no isotope. The most abundant species is confirmed to be triply substituted, and its structure is defined (3). ^{13}C N.m.r. spectra support these conclusions. The product of deuteriation in the presence of Wilkinson's catalyst was shown by ^1H n.m.r. spectroscopy to be the expected γδ-dideuteriated compound.

The sensitivity of ^3H n.m.r. enabled us also to examine (4,5-[^3H]-Leu^2)-LAKH itself. The sample concentration was 300 mCi cm^{-3}. The proton decoupled spectrum showed two signals only, both multiplets, separated by 0.69 p.p.m. (Figure). Although tritium attached to the β-carbon atom would give a signal overlapping that at the γ-carbon atom, the ratio of the integrals observed for γ-C-^3H and γ-C-^3H (6.5 to 1) is higher in fact than that seen in the deuteriation experiments, indicating that hydrogen can compete more effectively with tritium than with deuterium at the γ-position rather than that there is any tritiation of the β-position. The splitting pattern of the signal for the tritium atoms on the δ-carbon atoms supports the other results indicative of equal isotopic substitution in the two δ-methyl groups. Bioassay of the (4,5-[^3H]-Leu^2)-LAKH showed its activity to be the same as that of LAKH itself.

Table 3. ^{1}H N.m.r. spectra of *N*-acetyl-4,5-dehydroleucine and reduced derivatives

Derivative	p.p.m.	Signal	Integration	Assignment
N-Acetyl-leucine	0.9	Complex	6 H	Leu CH_3's
	1.7	Complex	3 H	CH_2 and γ-CH
	2.0	Singlet	3 H	Acetyl CH_3
	4.3	Complex	1 H	α-CH
N-Acetyl-4,5-dehydro-DL-leucine	1.7	Singlet	3 H	Leu CH_3
	1.8	Singlet	3 H	Acetyl CH_3
	2.4	Complex	2 H	CH_2
	4.4	Complex	1 H	α-CH
	4.8	Singlet	2 H	$=CH_2$
N-Acetyl[^{2}H]leucine (10% Pd/C)	0.9	Complex	4 H	Leu CH_3's
	1.5	Complex	2 H	CH_2
	1.9	Singlet	3 H	Acetyl CH_3
	4.2	Complex	1 H	α-CH
N-Acetyl[^{2}H]leucine (5% Rh–$CaCO_3$)	0.9	Complex	4 H	Leu CH_3's
	1.5	Complex	2 H	CH_2
	2.0	Singlet	3 H	Acetyl CH_3
	4.2	Complex	1 H	α-CH
N-Acetyl[^{2}H]leucine (PtO)	0.9	Complex	4 H	Leu CH_3's
	1.6	Complex	2 H	CH_2
	2.0	Singlet	3 H	Acetyl CH_3
	4.2	Complex	1 H	α-CH
N-Acetyl[^{2}H]leucine (Wilkinson's catalyst)	0.9	Complex	5 H	Leu CH_3's
	1'6	Complex	2 H	CH_2
	2.0	Singlet	3 H	Acetyl CH_3
	4.3	Complex	1 H	α-CH

The occurrence of deuterium exchange during catalytic deuteriation of alkenes under mild conditions has previously been reported,[9] notably in the steroids.[10] For example, in a Pt-catalysed reaction, reduction of cholesteryl acetate gave a product containing 2.44D, 0.40 of which were located at C-7. The presence of a tertiary carbon atom is not a controlling factor since under similar conditions cholest-2-ene yielded cholestane containing 2.42D.[10] du Vigneaud *et al.* have described the tritiation of *N*-trityl-4,5-dehydroleucine by catalytic reduction, but the resulting free 4,5-[^{3}H]leucine was not purified, so no specific activity could be given.[11] Although both in steroids and our dehydroleucine compounds exchange during reduction occurs only at allylic positions, in each case one allylic position is resistant. This position is always clearly the more sterically hindered one.

We conclude that 4,5-dehydroleucine residues might be generally useful in the preparation of specifically tritiated peptides of high activity.

Experimental

For general remarks, see the preceding paper, except that the following t.l.c. solvent systems were used (all v/v): (A) $CHCl_3$–MeOH–H_2O, 65 : 25 : 4; (B) butan-1-ol–AcOH–H_2O, 10 : 1 : 3; (C) EtOAc–pyridine–AcOH–H_2O, 5 : 5 : 1 : 3; (D) propan-2-ol–H_2O–AcOH, 25 : 10 : 1; (E) $CHCl_3$–MeOH–H_2O, 60 : 30 : 10. In addition ^{1}H n.m.r. spectra were recorded on a JEOL MH 100 spectrometer, the proton decoupled ^{3}H n.m.r. spectrum on a 300 Hz Brucker PFT spectrometer, and ^{13}C n.m.r. spectra on a JEOL PFT-100 spectrometer at 25 MHz, while mass spectra were obtained with a Micromass 16F single focusing magnetic vector mass spectrometer using isobutane as the ionising gas with a source temperature of 200 °C when chemical ionisation was required.

(3,4-[$^3H_2$]-*Pro*)*LAKH*(1—10).—Pyroglutamyl-leucyl-asparaginyl-phenylalanyl-threonyl-3,4-dehydroprolyl-asparaginyl-tryptophanylglycyl-threonine amide[4] (400 mg, 3.44 μmol) was dissolved in freshly distilled dimethylformamide (DMF: 0.5 ml) and reduced using 98% tritium gas (3.2 ml; 8 Ci) in the presence of 10% palladium on charcoal catalyst (5.60 mg) for 4 h at 20 °C. The catalyst was removed by filtration through a pad of cellulose (Macherey-Nagel M 300) and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in water (0.53 ml) and the bulk (0.5 ml) of the solution was applied to a column (50 × 0.7 cm) of Nucleosil 10C_{18}. The column was eluted at 2.4 ml min^{-1} with solvent system 2. The eluate was monitored at 210 nm and fractions (1.0 min) were collected automatically. Fractions 34—40 were combined and evaporated under reduced pressure to *ca.* 1 ml. The solution was passed through a column (0.5 ml) of Dowex 1 (acetate form) resin and the eluate was evaporated to dryness. The residue (1.52 μmol, 48% 12.1 Ci mmol^{-1}) was dissolved in water (10 ml) and stored in liquid nitrogen. This decapeptide solution gave the following amino-acid analysis after acidic hydrolysis: Glu, 1.00; Leu, 1.00; Asp, 1.56; Phe, 1.00; Thr, 2.04; Pro, 1.00; Trp, 0.33; Gly, 1.02; and after hydrolysis with a mixture of carrier-bound enzymes;[12] Leu, 0.27 *; Asp, 1.54; Phe, 1.00; Thr 1.53; Thr-NH_2, 0.57; Pro, 0.99; Trp, 0.98; Gly, 0.98.

The material was homogeneous on analytical h.p.l.c. (using a Nucleosil 10C_{18} column (25 × 0.45 cm) and solvent system 2) with a retention time identical with that of authentic LAKH.

(4,5-[^3H_n]-*Leu*^{2})*LAKH*(1—10).—(4,5-Dhl^2)LAKH(1—10) was tritiated in a manner identical to the above on a 2.16 μmolar scale. The h.p.l.c. purification was carried out as before but with a flow rate of 5.2 ml min^{-1} and fractions were collected at 30 s intervals and after ion-exchange chromatography and evaporation gave decapeptide (1.14 μmol; 53%, 115 Ci mmol^{-1}) which was dissolved in water (14 ml) and stored in liquid nitrogen. This material gave the following amino-acid analysis after acidic hydrolysis: Glu, 0.99; Leu, 1.00; Asp, 1.53; Phe, 1.00; Thr, 2.00; Pro, 1.00; Trp, 0.37;

* This low value is ascribed to incomplete hydrolysis of the pyroglutamyl-leucyl peptide bond.

Gly, 1.03; and after enzyme hydrolysis:[12] Leu, 0.44; Asp, 1.93; Phe, 1.00; Thr, 1.80; Thr-NH_2, 0.20; Pro, 0.97; Trp, 0.96; Gly, 0.97. On analytical h.p.l.c. (conditions as above) the product gave a symmetrical peak with an elution time identical with that of authentic LAKH. The product was homogeneous on t.l.c. in systems A—E when examined using the Panax scanning system. The proton decoupled H^3 n.m.r. spectrum of this decapeptide in water is shown in the Figure (sample concentration 300 mCi cm^{-3}).

Analysis of Distribution of Tritium.—Samples (2 nmol) of the acidic and enzymic hydrolysates from the above two decapeptides were separately chromatographed in the normal manner on the Beckman 120C amino-acid analyser and the eluates were collected automatically (0.5 min fractions) from the end of the column without addition of ninhydrin or passage through the colorimeter. After suitable dilution, the fractions were assayed for radioactivity. The percentage of the total tritium content associated with the amino-acids are given in Table 1.

Deuteriation of N-*Acetyl*-4,5-*dehydro*-DL-*leucine using Heterogeneous Catalysis.*—*N*-Acetyl-4,5-dehydro-DL-leucine (0.50 g, 2.9 mmol)[11] was dissolved in freshly distilled DMF (20 ml) and reduced using 99.99% deuterium in the presence of 10% Pd/C catalyst (0.50 g) for 4 h at 20 °C. (T.l.c. showed the reduction to be complete.) The catalyst was filtered off and the filtrate evaporated under reduced pressure to afford a white crystalline solid. This experiment was repeated in an identical manner using platinum oxide (0.10 g) or 5% rhodium on calcium carbonate (0.50 g) as the catalyst. In each case the ^1H n.m.r. and mass spectra were examined and the results are summarised in Tables 3 and 2 respectively.

Deuteriation of N-*Acetyl*-4,5-*dehydro*-DL-*leucine using Homogeneous Catalysis.*—*N*-Acetyl-4,5-dehydro-DL-leucine (0.50 g, 2.9 mmol) was dissolved in freshly distilled DMF (20 ml) and reduced using 99.99% deuterium in the presence of tris(triphenylphosphine)rhodium(I) chloride (0.27 g, 2.9 mmol) for 4 h at 20 °C. (T.l.c. showed reduction to be complete.) The solvent was removed under reduced pressure and the residue dissolved in water (20 ml). After extraction with CH_2Cl_2 (3 × 15 ml) the aqueous phase was evaporated to dryness, to afford the product as a white crystalline solid. This material was used for spectroscopic studies (see Tables 2 and 3) without further purification.

α-Amino-γ-methyl-γ-valerolactone Hydrochloride.—4,5-Dehydro-DL-leucine (0.20 g, 1.6 mmol) was dissolved in a 1 : 1 (v/v) mixture of 6M-HCl and propionic acids (10 ml). The solution was heated under reflux for 5 h and cooled to 20 °C before evaporation under reduced pressure (<30 °C). The residual solid was recrystallised from ethanol–ether (2 : 5, v/v) to give the pure lactone (0.22 g, 86%), m.p. 213—215 °C (lit.,[13] 210—211 °C); ^1H n.m.r. (D_2O): δ 1.44 (3 H, s, CH_3), 1.53 (3 H, s, CH_3), 2.00—3.00 (2 H, m, CH_2), and 4.60 (1 H, q, CH) (Found: C, 43.7; H, 7.4; N, 8.3%. Calc. for $C_6H_{11}NO_2 \cdot HCl$: C, 43.5; H, 7.3; N, 8.5%).

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