Tritiated Peptides. Part 16[1]. Synthesis of [^{3}H-Tyr^{8}]-Physalaemin

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SUMMARY

The title compound was synthesised with a specific activity of 30 Ci mmol^{-1} and with radiochemical purity of 96% by iodination of the sulphoxide followed by catalytic tritiation of an isolated fraction. Iodination and reduction studies on model peptides are reported. Alternative philosophies for preparing tritiated peptides are critically discussed.

Key words: ^{3}H-Physalaemin, Tritiation, ^{3}H-Peptides, Iodination

INTRODUCTION, RESULTS AND DISCUSSION

Peptides labelled with tritium are available by two distinctly different approaches: (i) by rigorous chemical synthesis of a protected precursor molecule for tritiation, and (ii) by tritiation procedures applied to free peptides, often isolated natural products.

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The first approach allows freedom in the choice of labelling site and complete confidence in the final precursor for labelling to be obtained from cumulative chemical analyses throughout the synthetic procedure and by the provision of a sufficient quantity of the precursor to allow powerful purification techniques to be employed (e.g. ref. 2). Synthesis of a stock of the precursor also allows repeat preparations of tritiated peptide to be carried out with little effort whenever material is needed. We feel these are significant advantages over a recently reported total 'hot-synthesis' approach (3). Repetition of such a synthesis is excessively wasteful of time. Also, such protracted syntheses using large quantities of radioactive material could be potentially dangerous both to the environment and to operators, and to the compound by radiolysis. The advantages claimed for this approach are total specificity of labelling and maximised specific activity. However, non-specific labelling is generally minor (1 and previous papers in the series) and, in any case, total specificity is not absolutely essential as it is obvious that in all subsequent biological applications the identity of label with compound must be rigorously verified. Lower specific activities than those maximally achievable (3) will be generally acceptable. Biological investigations might indeed not be feasible if levels of labelling achieved were an order of magnitude lower than maximal but as materials of specific activity of 30-100% maximal are readily prepared (cf. ref. 1 etc, and 3) such investigations are not likely to be disallowed.

Although the approach (i) has been applied to quite large peptides, for example human corticotrophin (molecular weight 4541) (4), the approach is obviously limited, when applied to very large peptides, by the labour involved in synthesis. Additionally, if the target peptide contains a number of disulphide bridges, e.g. insulin, synthesis of the precursor would be a daunting challenge and catalytic tritiation would probably be accompanied by extensive chemical degradation and by catalyst poisoning. Tritiated insulin has been prepared by semi-synthesis (5), but the options open for the choice of labelling site with this method are limited in such complex molecules.

The second approach, starting with the unlabelled target peptide, consists of iodine chloride-mediated iodination followed by catalytic reduction (exemplified in ref. 6). This approach circumvents the labour of peptide synthesis for the radiochemist if the peptide is available and the method is also applicable to peptides isolated from biological sources. This is an especially valuable feature when it is wished to

label very large peptides (7). However, in such cases, the side reactions associated with iodination procedures (8) may not be avoidable and the final tritiated products may be significantly impure as a result (7) even after extensive and laborious purification procedures. No choice of labelling site is available by this method. The outcome is dependent on the structure of the peptide (primary for small peptides, tertiary for large peptides) and is limited to the tritiation of tyrosine and histidine residues. Nevertheless, it should be strongly emphasised that large labelled peptides, even if not absolutely pure, are only readily obtainable by this approach. The non-expert should, however, beware of the pitfalls of assuming that chromatographic purity represents chemical purity in the absence of sufficient material to perform a rigorous chemical characterisation and of the limited value of bioassay which is too imprecise to be a criterion of purity.

A request from Prof. J.M. Musacchio of New York University Medical Center for the supply of [^{3}H]-physalaemin afforded us an opportunity to study this second approach using a relatively small peptide (11 residues, molecular weight 1264) where carboxyl-terminal methionine amide presented the complication of an interfering oxidisable function during iodination and a potential catalyst poison during catalytic tritiation.

Preliminary investigations of the competitive behaviour of the model peptides Ser-Tyr-Ser-Met and Synacthen (Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro) towards the reagent iodine monochloride will be described but are not detailed in the experimental section.

Reaction of Ser-Tyr-Ser-Met with two molar proportions of iodine monochloride in acetic acid produced the sulphoxide without any iodination of tyrosine. In contrast, similar treatment of Synacthen resulted in partial sulphoxidation, modest levels of tyrosine iodination and side-reactions, as suggested by observation of asymmetric peak profiles on column chromatography, and poor amino acid analyses on recovered fractions (for details of enzymic hydrolysis procedures, see ref. 9). These observations suggest that methionine oxidation is easier than iodination of tyrosine but that steric effects may modify the outcome in favour of iodination in larger molecules. It is known that the carboxyl-terminal methionine residue in substance P (Arg-Pro-Lys-Pro-Gln-Phe-Phe-Gly-Leu-Met-NH_2) is exceptionally susceptible to oxidation in the unlabelled peptide, for example by repeatedly freezing and thawing a dilute aqueous solution. Thawing by heating frozen tritiated aqueous substance P solution can effect complete conversion to the sulphoxide, presumably by the action of radiation-induced radicals (10). As

physalaemin (Glp-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2) shows considerable structural homology to substance P, it was considered that sulphoxidation of physalaemin by iodine monochloride was likely to be a troublesome and complicating side-reaction to the desired iodination of the 8-tyrosine residue and that controlled iodination of tyrosine would only be achievable if physalaemin sulphoxide was used as the substrate.

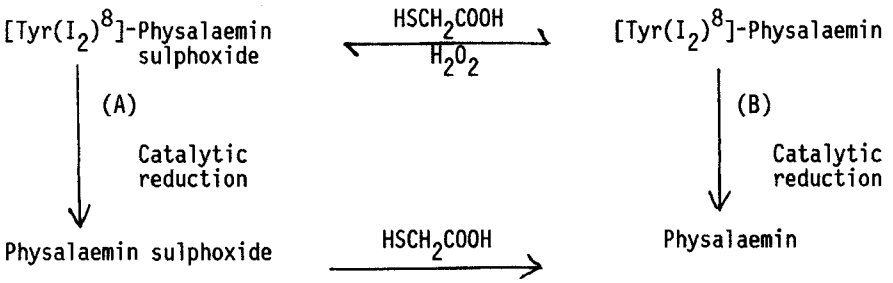
This strategy was also attractive for considerations concerning the tritiation step. Methionine has been observed to undergo reductive desulphurisation to yield labelled α-aminobutyric acid (11). As it is not possible to predict whether peptides containing this amino acid will be separable from the methionine-containing peptides from which they are derived, it was thought wise to take steps to avoid this side-reaction. Conversion of the thioether to the sulphoxide should reduce the poisoning of the catalyst (10). Sulphoxidation has been suggested as a protection strategy for methionine in peptide synthesis to avoid unwanted side-reactions (13). In this latter study, it was noted that catalytic reduction of methionine sulphoxide itself was slow using palladium on charcoal and that Raney nickel caused some degradation to α-aminobutyric acid.

We have observed (10), using the deprotected di-iodotyrosine--containing free peptides [$Tyr(I_2)^2$]- and [$Tyr(I_2)^{23}$]-Synacthen (14,15) that no deiodination can be achieved in aqueous solution using conditions published for smaller iodinated peptides (6,16). This may be due to conformational effects in these larger molecules. This was demonstrated for protected calcitonin substances in non-aqueous solutions where a sulphur-protected dotriacontapeptide containing iodine was resistant to catalytic hydrogenation while a mixture of the decapeptide and docosa-peptide fragments from which it was assembled underwent easy dehalo-genation under identical reaction conditions (17) thus confirming that sulphur protection had successfully averted catalyst poisoning.

Although some attempt has been made to extract rational concepts in the foregoing discussion by considerations of sulphur protection (and how that bears on side-reactions and on catalyst poisoning) and of steric effects, it should be remembered that other variables such as solvent composition and pH, and catalyst nature will have profound influences on the course of reductive dehalogenations. Insufficient information is available in the literature to allow any evaluation of these points.

Based on the arguments presented, we decided to attempt the preparation of tritiated physalaemin from synthetic physalaemin using sulphur protection by oxidation in order to simplify the iodination step and reduce catalyst poisoning in the reduction step.

Physalaemin was oxidised using hydrogen peroxide in glacial acetic acid (13). The product was one component as judged by high pressure liquid chromatography (h.p.l.c.). Iodination of the oxidised peptide was effected using an excess of iodine monochloride in aqueous sodium acetate solution. The mixture was separated by preparative reverse-phase h.p.l.c. and contained two major peptidic products. The more polar (A) of these two compounds was converted into the other (B) by treatment with thioglycollic acid solution and B was converted into A by oxidation with hydrogen peroxide. The reduced form B gave physalaemin directly on catalytic dehalogenation whereas the oxidised form A afforded physalaemin sulphoxide. This was confirmed by conversion to physalaemin using thioglycollic acid. Ultraviolet spectroscopy indicated that compound A contained di-iodotyrosine. It was concluded that treatment of physaelaemin sulphoxide with an excess of iodine monochloride had yielded roughly equal amounts (as judged by h.p.l.c. and by the weights of recovered materials) of [8-di-iodotyrosine]-physalaemin (B) and its sulphoxide (A) (Scheme).



SCHEME: Interconversion and proof of identity of compounds A and B (for details see the text and experimental).

Reductions of methionine sulphoxide to the thioether amino acid residue have been reported using various relatively harsh reducing conditions, but this is the first report of a reduction effected under such mild (and indeed oxidising) conditions.

Catalytic reduction of compound B using tritium gas followed by h.p.l.c. isolation gave [^{3}H]-physalaemin which was substantially radiochemically pure as judged by thin layer chromatography (t.l.c.) in three solvent systems, analytical h.p.l.c., amino acid analysis and by bioassay and a receptor cross-desensitisation assay with the unlabelled hormone (18).

It was concluded from the specific activity achieved that the presence of methionine (containing divalent sulphur) in the substrate for

tritiation did not lead to significant catalyst poisoning. This may be dependent on the choice of solvent and catalyst (6) used in this instance. In addition, there was no evidence of conversion of methionine to α-aminobutyric acid despite use of a large excess of catalyst. This is again probably a function of the solvent and catalyst employed. Reports exist of a trouble-free deiodination in the presence of sulphur (16) and of an instance in which complete desulphurisation occurred (19).

We conclude that the iodination/catalytic tritiation approach applied to free peptides has, in this instance, afforded material which is demonstrably pure by a sufficient range of independent criteria, notwithstanding that both the iodination and the reduction steps were accompanied by significant side-reactions.

Variable recoveries of aspartic acid were observed after acidic hydrolysis of the peptide followed by amino acid analysis. Similar problems with this amino acid have also been observed in the analysis of locust adipokinetic hormone (20). Analytical values for methionine and proline were low. This is sometimes observed when only small amounts of material are available for analysis.

EXPERIMENTAL

Physalaemin was purchased from Cambridge Research Biochemicals Ltd., Harston, Cambridgeshire, CB2 5NX, England. [^{3}H]-Physalaemin was examined with a Panax E.0111/XPD-05 radiochromatogram scanner system after t.l.c. on thin layers of silica gel developed in the following solvent systems: (A) n-butanol:pyridine:acetic acid:water (30:20:6:24, by vol.); (B) ethyl acetate:pyridine:acetic acid:water (5:5:1:3, by vol.); (C) chloroform:-methanol:ammonia solution sp. gr. 0.88 (65:45:20, by vol.).

Physalaemin Sulphoxide

Physalaemin (10 mg) was dissolved in acetic acid (0.4 ml), hydrogen peroxide (30 µl of 6%) was added and the mixture was kept at room temperature for 45 min. After addition of water (2 ml), the mixture was freeze-dried to yield a white solid. A portion (20 µl) of an aqueous solution (1 mg ml^{-1}) of the product was examined by analytical h.p.l.c. on a column (25 x 0.46 cm) of Nucleosil 10C$_{18}$ ODS-silica which was eluted at a flow rate of 2 ml min^{-1} with acetonitrile:water:phosphoric acid (250:750:1, by vol.). Detection was at 200 nm (Cecil CE2012). The product chromatographed as a single peak (k = 1.0). Under the same conditions, physalaemin chromatographed as a single peak (k = 3.5).

Iodination of Physalaemin Sulphoxide

The sulphoxide was dissolved in water (2.0 ml), the solution was cooled to 0°C and sodium acetate (3.3 mg) in water (0.1 ml) was added. A solution of iodine monochloride (4.88 mg, 3 equivalents) in methanol (100 µl) was added to the stirred solution in five equal portions at intervals of 3 min. Acetic acid was then added dropwise until the precipitate was dissolved. After stirring for a further 15 min, SO_2-water was added dropwise until the colour of iodine was discharged. The solution was freeze-dried to give a light yellow powder. This was dissolved in aqueous acetic acid (2 ml of approx. 20%) and applied using a Rheodyne injection valve to a column (50 x 0.7 cm) of Nucleosil $10C_{18}$ ODS-silica which was eluted at a flow rate of 5.0 ml min^{-1} with a constant volume (100 ml) gradient of acetonitrile:water:phosphoric acid (from 250:750:1 to 500:500:1, by vol.). Detection was at 210 nm and fractions (30 sec) were collected automatically. Two major peaks were eluted, in fractions 18-21 (compound A) and fractions 27-34 (compound B). The two solutions were separately reduced in volume (to 1 ml) by rotary evaporation and passed through columns (1 ml) of Dowex 1 (acetate form) resin, the eluates were then freeze-dried to give A (3.4 mg) and B (3.3 mg) as white powders.

Evaluation of Iodination Fractions

Samples (0.1 mg) of compounds A and B were separately reduced as described below but using protium gas. Compound A yielded physalaemin sulphoxide, as judged by analytical h.p.l.c. (see conditions above). On heating this product in H_2O (1 ml) with thioglycollic acid (50 µl) for 16 h at 50°C, physalaemin was produced (~50% conversion, h.p.l.c.).

Compound A (3 mg) in H_2O (1 ml) was heated under N_2 at 50°C for 24 h with thioglycollic acid (50 µl). The reducing agent was removed by repeated evaporation to dryness with addition of portions (3 x 1 ml) of H_2O and the residue was dissolved in H_2O (3 ml) for analysis by h.p.l.c. (conditions and results are given below).

Compound B in H_2O (1 mg ml^{-1}, 200 µl) was kept for 1 h at 20°C with glacial acetic acid (100 µl) and 6% aqueous H_2O_2 (100 µl) and the mixture was analysed directly by h.p.l.c.

Samples (20 µl) of each of the two above preparations were examined on a column (30 x 0.46 cm) of Nucleosil $10C_{18}$ which was eluted at a flow rate of 1.5 ml min^{-1} with acetonitrile:water:phosphoric acid (300:700:1,

by vol.) with detection at 210 nm. The results are given below (Table 1).

Table 1

H.p.l.c. analysis of treated iodinated physalaemin sulphoxide fractions. Details are given in the text.

Compound	Treatment	k
A	-	1.08
B	-	4.08
A	$HSCH_2COOH$	1.08 (63%); 4.08 (37%)
B	H_2O_2	1.08 (100%)

Compound A (3 mg) was dissolved in tris buffer (0.05M, pH 7.5, 4 ml) and the solution was adjusted to pH 8.0 (NaOH). Examination by UV showed λ_{max} = 304 nm and λ_{min} = 282 nm.

[^{3}H-Tyr^8]-Physalaemin

Compound B (1.6 mg) was dissolved in water (0.45 ml) and 0.5M-sodium phosphate buffer pH 7.0 (5 µl) was added. Catalytic reduction using 8 Ci (3.2 ml) of tritium gas in the presence of 5% $Pd/CaCO_3$ catalyst (35 mg) was carried out for 40 min at room temperature. The catalyst was removed by centrifugation and washed with water (0.8 ml, then 1.3 ml). The combined supernatants were dried by rotary evaporation and ethanol (2 x 3 ml) was evaporated from the residue to give a white solid. This was dissolved in water (0.5 ml) and the solution was applied using a Rheodyne injection valve to a column (50 x 0.7 cm) of Nucleosil $10C_{18}$ ODS-silica which was eluted at a flow rate of 5.0 ml min^{-1} with acetonitrile:water:-phosphoric acid (250:750:1, by vol.). Detection was at 210 nm and fractions (30 sec) were collected automatically. Fractions 17-20 were combined, evaporated to a volume of approx. 1 ml and the solution was passed through a column (0.5 ml) of Dowex 1 (acetate form) resin. The eluate was evaporated to dryness and the residue was redissolved in water (1.5 ml).

Analysis

A portion (0.2 ml) of the product was hydrolysed in a sealed, evacuated tube in the presence of phenol (3 mg) using 6.7M-HCl (1 ml) at 110° for 16 h. On opening the tube, a portion (10 µl) of the hydrolysate was diluted to 5 ml (H_2O) and portions (10 µl) of this solution were counted in BBOT scintillation fluid using [^{3}H]-hexadecane as internal standard. The remaining hydrolysate was used for amino acid analysis and for estimation of the distribution of tritium amongst the amino acid residues (15). As the label in tyrosine is completely removed by acidic hydrolysis (21), the quantity present in this residue was estimated by calculation. Analyses are given in Table 2 together with a reference analysis for the 'cold' physalaemin taken as starting material.

The specific activity of the product (0.25 µmole) was 30.4 ± 2.3 Ci $mmol^{-1}$. The majority (94.0 ± 0.01%) of the tritium was present in the tyrosine residue.

Radiochemical purity was estimated by t.l.c. using cold physalaemin (1 mg ml^{-1}) as carrier. The results are given in Table 3. Radiochemical purity was estimated as 95.5 ± 0.6% by analytical h.p.l.c. followed by counting of the column effluent, exclusive of 3.2 ± 0.4% of the sulphoxide (total notional purity = 98.7 ± 0.7%).

Table 2

Amino acid analysis of [^{3}H]-physalaemin. Details are given in the text.

Residue	[^{3}H]-Physalaemin	[^{1}H]-Physalaemin
Ala	0.98	1.02
Asp	1.97	2.10
Glu	1.00	1.04
Gly	1.02	0.93
Leu	0.95	1.02
Lys	1.07	1.00
Met	0.72	0.87
Phe	1.02	0.98
Pro	0.72	1.04
Tyr	0.96	0.73
NH_3	8.0	1.98

Table 3

Radiochemical purity (%) of [^{3}H]-physalaemin as judged by t.l.c. Details are given in the text.

Solvent System	% as Physalaemin (x)	% as Physalaemin Sulphoxide (y)	x + y
A	98.4 ± 0.1	1.1 ± 0.1	99.5
B	96.6 ± 0.1	2.5 ± 0.1	99.1
C	95.1 ± 0.4	Not resolved	95.1

The tritiated physalaemin was equipotent with reference peptide in a bioassay based on the contraction produced in a guinea-pig ileum myenteric plexus longitudinal muscle preparation. Additionally, the labelled peptide desensitised the muscle preparation towards unlabelled physalaemin and vice versa (experiments by Prof. J.M. Musacchio).

The product was stored in water at a concentration of 5.2 mCi ml^{-1} at the temperature of liquid nitrogen (-196°C).

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REFERENCES

1. Part 15. Allen M.C., Brundish D.E., Fullerton J.D. and Wade R. - (in preparation)
2. Allen M.C., Brundish D.E., Wade R., Sandberg B.E.B., Hanley M.R. and Iversen L.L. - J. Med. Chem. 25: 1209 (1982)
3. Parnes H. and Shelton E.J. - J. Labelled Compounds Radiopharm. 21: 263 (1984).
4. Brundish D.E. and Wade R. - J. Chem. Soc. Perkin I: 462 (1980)
5. Hablan P.A. and Offord R.E. - Biochem. J. 151: 219 (1975)
6. Morgat J.L., Hung L.T. and Fromageot P. - Biochem. Biophys. Acta. 207: 374 (1970)

7. Menez A., Morgat J.L., Fromageot P., Ronseray A.M., Boquet P., and Changeux J.P. - FEBS Lett. 17: 333 (1971)

8. Ramachandran L.K. - Chem. Rev. 56: 199 (1956)

9. Bennett H.P.J., Elliott D.F., Evans B.E., Lowry P.J. and McMartin C. - Biochem. J. 129: 695 (1972)

10. Brundish D.E. (unpublished observations)

11. Brundish D.E. and Wade R. - J. Labelled Compounds Radiopharm. 18: 1123 (1981)

12. Rylander E. in 'Catalytic Hydrogenation over Platinum Metals', Academic Press, N.Y.: p. 21 (1967)

13. Iselin B. - Helv. Chim. Acta 44: 61 (1961)

14. Brundish D.E. and Wade R. - J. Chem. Soc. Perkin I: 2875 (1973)

15. Brundish D.E., Martin J.L. and Wade R. - J. Chem. Soc. Perkin I: 2182 (1976)

16. Morgat J.L., Hung L.T., Cardinaud R., Fromageot P., Bockaert J., Imbert M. and Morel F. - J. Labelled Compounds 6: 276 (1970)

17. Brundish D.E. and Wade R. - J. Chem. Soc. Perkin I: 318 (1981)

18. Bahouth S.W., Lazaro D.M., Brundish D.E. and Musacchio J.M. - Mol. Pharmacol. (in the press)

19. Fluoret B., Terada S., Yang F., Nakagawa S.H., Nakagawa T. and Hechter O. - Biochemistry 16: 2119 (1977)

20. Hardy P.M., Sheppard P.W., Brundish D.E. and Wade R. - J. Chem. Soc. Perkin I: 731 (1983)

21. Hempl K. in 'Proceedings of Conference on Methods of Preparing and Storing Marked Molecules', Euratom, p. 1009 (1964)