Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXVIII, No. 4

A TRITIATED PHOTOLABEL SPECIFIC FOR THE ACTIVE SITE OF MONOAMINE OXIDASE

F.R.C. Backwell & C.H. Williams

Division of Biochemistry,

School of Biology and Biochemistry,

Medical Biology Centre,

The Queen's University of Belfast, Belfast BT9 7BL.

SUMMARY

We report the synthesis of a tritiated inhibitor of monoamine oxidase, containing a photolabile azido group. The compound, N-(4-azido-2-nitrophenyl)-N'-3-[^{3}H]-methyl-N'-(3-prop-1-ynyl)-1,3-diamino propane, is a novel, specific photolabel designed to interact covalently with the flavin of the enzyme in absence of light and to form a second covalent linkage with the polypeptide on photoirradiation. The compound is shown to label a sample of monoamine oxidase isolated from bovine liver, and may be useful in helping to map the active site of the enzyme.

Key words: monoamine oxidase, inhibitor, photoaffinity label.

INTRODUCTION

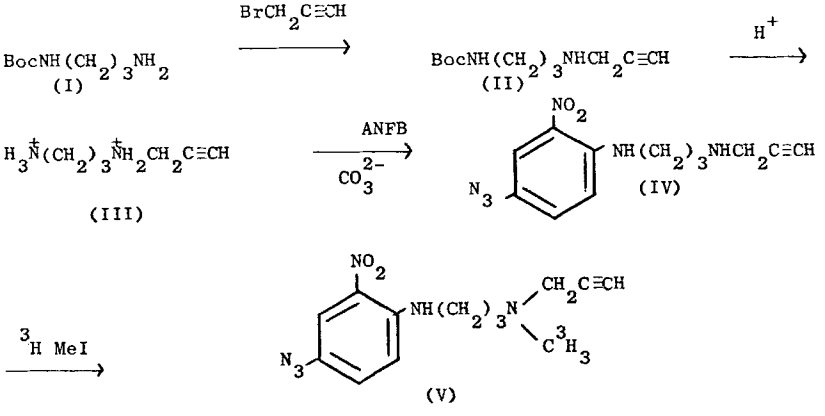
The enzyme monoamine oxidase (MAO: EC 1.4.3.4) occurs in two forms, designated A and B, which differ in substrate specificity and in their sensitivity to some inhibitors. It has recently been shown that the two forms

0362–4803/90/040421–06$05.00

Received September 4, 1989
Revised October 2, 1989

of MAO differ in amino acid sequence, as deduced from their cDNA sequences (1), though the region around the covalently bound FAD is totally conserved. Consequently, specificity differences between the two enzymes are likely to be due to differences in amino acid sequence in less highly conserved regions of their respective polypeptide chains. We have shown that azidonitrophenyl propylamines inactivate and photolabel the enzyme (2) and we now report the synthesis of a tritiated form of one of these which may be useful as a specific probe for the active site sequences in the two forms of MAO. The compound, N-(4-azido-2-nitrophenyl)-N'-[^{3}H]methyl-N'-(3-prop-1-ynyl)-1,3-diamino propane belongs to the class of propynylamines which are k_{cat} inhibitors of MAO and which are known to label the flavin (3,4). The azido group becomes activated on photoirradiation and is designed to photolabel the polypeptide chain in the active site region after an initial dark reaction has anchored the compound to the FAD.

The synthetic route to the tritiated photolabel is shown in Scheme I.



Boc = t-butyloxycarbonyl
ANFB = 4-azido-2-nitrofluorobenzene

Scheme I

EXPERIMENTAL

Unless stated otherwise all steps involving azido compounds were performed in the dark or under subdued room lighting. 4-azido-2-nitrofluorobenzene (ANFB) was prepared by the method of Fleet et al. (5). Compound (I) (Scheme I) was prepared by conventional means from 1-amino-3-bromopropane and

di-t-butyldicarbonate. It was obtained as a mobile oil in 75% yield and was used without further purification.

This product (5 g; 21 mmoles) was allowed to react with propargylamine (2.3 g; 42 mmoles) in acetonitrile as described by MacGregor et al. (6) for the analogous synthesis of clorgyline. The solvent was evaporated and the residue was partitioned betweeen ether and ice-cold 10% $NaHSO_4$ solution. The aqueous phase was immediately run into an excess of 2 M NaOH and extracted with ether. The extract was washed (H_2O), dried ($MgSO_4$) and evaporated to yield 1.0 g (22%) of (II). This was deprotected by stirring for 2 h at room temperature in 2 M methanolic HCl (10 ml). The solvent was evaporated and the solid residue (III) was crystallised from ethanol/petroleum ether (b.p. 60-80°). Yield 0.62 g (71%). Found C, 39.1; H, 7.6; N, 14.38. $C_6H_{14}N_2Cl_2$ requires C, 38.9; H, 7.6; N, 15.1%.

The product III (0.5 g, 2.7 mmoles) was taken up in 0.1 M $NaHCO_3/Na_2CO_3$, pH 9.5 (5 ml) and added to ANFB (164 mg, 0.9 mmoles) in ethanol (5 ml). Sufficient further ethanol was added to give a solution. The apparent pH was adjusted to 9.5 with NaOH and the solution was heated at 60°C for 16 h in the dark. The solvents were evaporated, the residue was taken up into 0.1 M HCl and washed with ether. The pH of the aqueous phase was adjusted to 8.6 and the product was extracted into ether, which was then washed (H_2O), dried ($MgSO_4$) and evaporated. Yield 69.1 mg (29%). ^1H n.m.r. δ (ppm) in [^{2}H]dimethyl sulphoxide; 1.95[2H, m, $-CH_2\underline{CH}_2CH_2-$], 2.24[1H, m, $\equiv C\underline{H}$], 2.88[2H, m, $C\underline{H}_2-N-CH_2C\equiv$], 3.4[2H, m, $-C\underline{H}_2(CH_2)-N-CH_2-$], 3.5[2H, m, $N-C\underline{H}_2C\equiv$], 6.9[H, d, ring C-H, position 6], 7.12[H, m, ring C-H, position 5], 7,85[H, d, ring C-H, position 2], 8.3[H, broad, aryl NH].

A sample was converted to an HCl salt by dissolving in ether and adding an excess of HCl gas in ether. Found C, 46.1; H 4.8; $C_{12}H_{15}N_6Cl\ O_2$ requires C, 46.4; H, 4.8%. The i.r. spectrum showed absorbance bands at 2140-2120 cm^{-1} (N_3 str.) and 3240 cm^{-1} ($\equiv$C-H str.) FAB Mass Spectroscopy showed a molecular ion peak with M/z 275 ($M+H^+$).

N-[^{3}H]methylation of (IV). The procedure used is based on those described by MacGregor et al. (6) and Halldin et al. (7) for the N-methylation of other propargylamines. The desmethyl amine (32 mg, 0.12 mmoles) in a mixture of dimethyl sulphoxide (0.15 ml) and dimethyl formamide (0.85 ml) was added to a glass ampoule containing [^{3}H]methyl iodide (10 mCi; 120 nmoles) in toluene (86 µls). The ampoule was sealed and then heated at 60^{o}C for 5 min. After it had cooled to room temperature the contents of the ampoule were injected, in portions, on to the Spherisorb C18 semi-preparative HPLC column. Gradient elution with methanol (A) and 0.05 M ammonium formate, pH 3.5 (B), was used to separate the products under the following conditions:- flow rate 3 ml/min; 0-7 min, 100% A; 7-32 min, from 100% A to 50% A/50% B; 32-35 min, from 50% A/50% B to 100% A; 35-45 min, 100% A. Fractions of 3 ml were collected and aliquots (2 x 20 µl) of each were taken for liquid scintillation counting. The tritiated amine was eluted with the same retention time as an authentic unlabelled reference sample (16.4 min) and the unchanged starting material eluted at 20.8 min. Fractions containing radioactivity were pooled and evaporated to dryness after adding an excess of 2 M HCl. The residue was dissolved in 0.1 M sodium phosphate buffer, pH 7.5. Radioactivity in the recovered product totalled 6.97 x 10^{9} d.p.m. (3.17 mCi). This is equivalent to 37.3 nmoles (31%) of labelled amine, based on the sp. act. of the [^{3}H]methyl iodide.

Evidence for the labelling of MAO by (V). A detailed account of the labelling of MAO by the photolabel will be reported elsewhere. Briefly, a sample of the enzyme (1.2 mg of protein), prepared as previously reported (8), was incubated for 2 h at 30^{o}C with 37 nmoles of (V) in 1.65 ml of 0.1 M phosphate buffer, pH 7.5. The sample was then centrifuged at 260000 x g for 1.5 h to sediment the MAO. The pellet was resuspended in buffer and again sedimented by centrifugation. It was taken up into PAGE loading buffer and an aliquot was electrophoresed in 10% polyacrylamide gel. Proteins were then electroblotted onto a nitrocellulose membrane and stained with Ponceau S. Channels containing MAO were cut into strips about 3 mm wide and these were assayed for radioactivity by liquid scintillation counting. Radioactivity was

detected in only 3 adjacent strips, the middle one staining for a protein of apparent mol. wt. 60000 which corresponds to MAO.

DISCUSSION

We have previously shown that in the absence of light an unlabelled form of (V) produces irreversible inhibition of MAO. After irradiation and electrophoresis of the enzyme, previously treated with the tritiated photolabel, the enzyme retains radioactivity. On the other hand the analogue containing Me instead of $-CH_2C{\equiv}CH$, a competitive inhibitor of MAO, was found to irreversibly inactivate the enzyme only after photoirradiation, showing that these compounds can photolabel the enzyme (2). Hence compound (V) may prove to be useful for labelling the polypeptide at the active site. The advantage of this particular inhibitor is that because it can be anchored to the active site FAD prior to photoactivation, little if any non-specific photolabelling should occur, provided that excess inhibitor is first removed.

It is noteworthy that (V) eluted earlier than its desmethyl analogue from the reverse-phase HPLC column, since the latter may be expected to be less hydrophobic. At pH 3.5 (the pH of the eluting solvent) both amines will carry a single positive charge which might in any case override small differences in hydrophobicity. The simplest explanation of their elution behaviour is that steric factors are important determinants of affinity for the column under the conditions used..

ACKNOWLEDGEMENTS

We wish to thank the Wellcome Trust for financial support.

REFERENCES

1. Bach, A.W., Lan, N.C., Johnson, D.L., Abell, C.W., Bembener, M.E., Kwan, S-W., Seeburg, P.H. and Shih, J.C. - Proc. Natl. Acad. Sci. USA. 85: 4934 (1988).

2. Backwell, F.R.C. and Williams, C.H. - Biochem. Soc. Trans. 16: 214 (1988).

3. Maycock, A.L., Abeles, R.H., Salach, J.I. and Singer, T.P. - Biochemistry, 15: 114 (1976).

4. Singer, T.P. and Salach, J.I. - in Monoamine Oxidase Inhibitors - The State of the Art (Eds. Youdim, M.B.H. and Paykel, E.S.) pp. 17-29. John Wiley, New York (1981).

5. Fleet, G.W.J., Knowles, J.R. and Porter, R.R. - Biochem. J. 128: 499 (1972).

6. MacGregor, R.R., Fowler, J.S., Wolf, A.B., Halldin, C. and Langstrom, B. - J. Labelled Compounds & Radiopharmaceuticals 25: 1 (1988).

7. Halldin, C., Bjurling, P., Langstrom, B., MacGregor, R.R. and Fowler, J.S. - J. Labelled Compounds & Radiopharmaceuticals 25: 545 (1988).

8. Williams, C.H., Lawson, J. and Backwell, F.R.C. - Biochem. J. 256: 911 (1988).