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

Synthesis of a Tritium Labelled Phospholipase A_2 Inhibitor: A Ligand for Macromolecular ^3H NMR Spectroscopy

A.S. Culf[1¶], H. Morimoto[2], P.G. Williams[2], W.J.S. Lockley[3], W.U. Primrose[4] and J.R. Jones[1]

1. Department of Chemistry, University of Surrey, Guildford, Surrey, GU2 5XH, UK.
2. The National Tritium Labelling Facility, Structural Biology Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA.
3. Department of Medicinal Chemistry, Astra Charnwood, Bakewell Road, Loughborough, Leics., LE11 ORH, UK.
4. Department of Biochemistry, University of Leicester, Leicester, Leics., LE1 7RH, UK.

Summary

A tritium labelled phospholipase A_2 (PLA_2) amide analogue inhibitor was prepared by the reduction of an alkene precursor. Diimide, heterogeneous and homogeneous metal catalyzed reduction methods were assessed for their suitability for the preparation of a tritiated ligand for ^3H NMR spectroscopic studies. The chosen homogeneous metal-catalyzed method gave a product of specific activity 57 Ci $mmol^{-1}$, which was isolated by flash chromatography. The binding of this tritiated substrate to bovine pancreatic PLA_2 was observed by ^3H NMR spectroscopy in the presence of calcium ions. Chemical shift changes suggest that the tritium atoms are located within the hydrophobic pocket of the protein, close to two phenylalanine residues.

Keywords: Diimide, Wilkinson's Catalyst, Tritium, Tritium NMR, PLA_2, Inhibitor

Introduction

Phospholipase A_2 (PLA_2) is a 14 kDa enzyme which stereospecifically hydrolyses the *sn*-2 acyl chains of triglyceride phospholipids.[1] The enzyme occupies a central role in the mediation of inflammatory disease.[2] Although the mechanism of action[3,4] and the location of the active site[5-7] in PLA_2 is now well defined, the role of the long alkyl and alkenyl chains of the natural substrate and the nature and extent of their interaction with amino acid side chains at the active site are less well understood.[3-7] Examples of solid-state X-ray crystallographic PLA_2/inhibitor complexes that

¶ ASC is currently in the Department of Chemistry, University of California, Santa Barbara, CA 93106, USA.

CCC 0362–4803/96/040373–12
©1996 by John Wiley & Sons, Ltd.
Received 1 November 1995
Revised 17 November 1995

have appeared in the literature[3-7] indicate that the *sn-2* alkyl chain has a sharp kink at the α-carbon and is accommodated by the side chains of hydrophobic amino acid residues.

The large number and coincidence of ^1H signals makes the NMR investigation of interactions between the alkyl chains of the substrate and the PLA_2 enzyme very difficult. One option is the design of an inhibitor such that some of its ^1H NMR signals occur in regions of the spectrum relatively free of overlapping protein signals.[8] A second approach is the specific labelling of either the inhibitor or protein molecules and selective "filtered" observation of the desired interactions without the overwhelming background of protein signals. Tritium benefits from having the highest sensitivity to NMR detection and zero background,[9] making tritium labelling combined with ^3H NMR spectroscopy a useful approach in biological NMR studies. Hence, in the current context, the use of ^3H NMR spectroscopy should allow the direct, detailed investigation of the binding characteristics of inhibitor alkyl chains. Similar applications of ^3H NMR spectroscopy have been demonstrated in several biomolecular structural[10-13] and dynamical[14] studies for obtaining selective information.

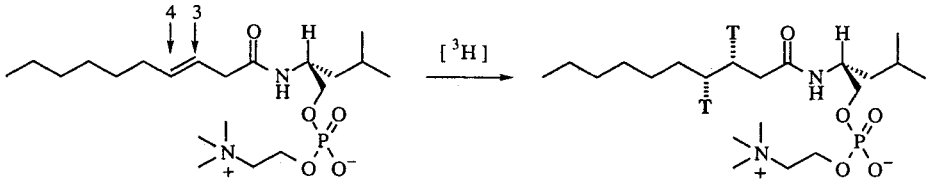
Crucial to these studies is the ability to prepare ligands or receptors at very high specific activities. One of the most widely used methods is that of catalytic tritiation of a suitable precursor, which can be achieved in a number of ways, *e.g.* through the use of a [^3H]diimide reagent,[15] or by application of either heterogeneous or homogeneous metal-catalyzed reduction with tritium gas. Ideally, high specific activity should be associated with high regiospecificity, and one of the objectives of the present investigation was to see which of the three approaches proposed above best satisfied these criteria.

The choice of PLA_2 inhibitor culminated in the selection of a synthetic amide analogue[8] bearing an amide function in place of the natural substrates' carboxylate ester. This structural change abolishes PLA_2 catalytic activity[5,16] by displacing an essential water molecule at the catalytically active Asp-99/His-48 couple and by forming a hydrogen bond with His-48.[5,16]

Results

As noted above, to ensure the most effective ^3H NMR study, a tritium labelling procedure was sought that would give both high specific activity (S.A.) and good label regiospecificity. The high S.A. was necessary to overcome the limitations of low enzyme concentrations in solution (*ca.* 1 mmolar[16]) and regiospecific labelling minimizes NMR assignment problems caused by general labelling of the alkyl chain.[14,17] Reduction by [^3H]diimide,[15] 10% palladium-on-carbon and

Wilkinson catalyzed reduction by tritium gas were selected as likely methods to fulfil the requirements for tritium labelling of the inhibitor (Scheme 1). An alkene precursor of the inhibitor was synthesized in 18% overall yield using the method of Bennion *et al.*[8]



Scheme 1

Tritium labelling using high specific radioactivity [^{3}H]diimide is selective for non-polarized multiple bonds, giving specific and symmetrical *cis*-addition of the label across the multiple bond.[15] In the current case, [^{3}H]diimide labelling of the PLA_2 inhibitor was incomplete even after 16 hours, and both the S.A. and radiochemical yield were very low, with 74 mCi of the labelled inhibitor resulting from 10.9 Ci of [$^3H_2$]water. On a positive note, the labelling of the double bond was specific and symmetrical (Table 1), and no degradation occurred. The ^{1}H-decoupled ^{3}H NMR spectrum of the product (Figure 1a) revealed an extraneous signal at 2.05 ppm, a result of

TABLE 1: ^{3}H NMR Data for [^{3}H]Phospholipids in ^2H_2O at 25 °C

Method of Reduction	Positions Labelled	Chemical Shift[a] (ppm)	Coupling Constant $^3J_{TT}$ (Hz)	Integral[a] (%)
N_2T_2, dioxane	H3	1.65, 1.57	—	18.1[b]
	H4	1.30, 1.27	—	18.2[b]
10% Pd/C, T_2 CH_3OH	H2	2.24	—	1.9
	H3	1.62 - 1.56	—	29.7
	H4 and H5-H9	1.30 - 1.24	—	67.0
	H10	0.78	—	1.4
$RhCl(PPh_3)_3$, T_2, 3:2 ratio of benzene: CH_3CH_2OH	H3	1.64, 1.62[c]	5.4	49.0
		1.58, 1.56[d]	6.1	
	H4	1.31, 1.29[c]	6.5	51.0
		1.29, 1.27[d]	6.4	

a. ^{1}H decoupled
b. The remainder (63.7%) of the ^{3}H NMR signal was $CH_2TCO_2^-$ at 2.05 ppm.
c. These signals of the diastereotopic H3 and H4 doublets are J-coupled and hence belong to one of the two diastereomers.
d. As footnote c above.

acid-catalyzed exchange of [O-^{3}H]acetic acid reagent to yield [methyl-^{3}H]acetic acid which then formed a double salt with the phosphocholine headgroup of the inhibitor. In summary, use of [^{3}H]diimide was able to satisfy only some of our stringent labelling requirements.

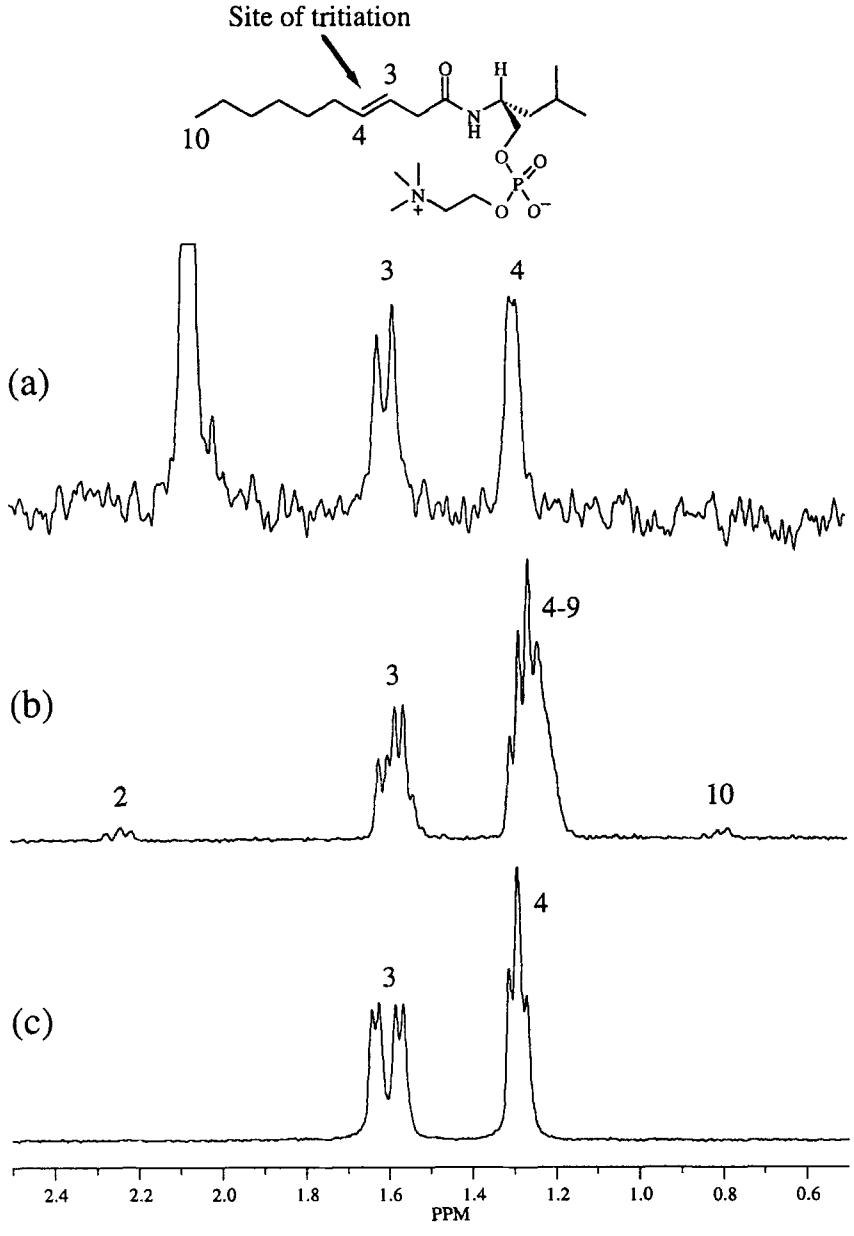


Figure 1. 320 MHz ^{3}H NMR spectra of the tritiated PLA_2 inhibitor in ^2H_2O. (a) ^{1}H-decoupled; [^{3}H]diimide reduction. (b) ^{1}H-decoupled; Heterogeneous catalyzed reduction. (c) ^{1}H-decoupled; Homogeneous catalyzed reduction.

Heterogeneous catalytic reduction is a widely used method for hydrogen isotope labelling as it is experimentally simple, quick and highly efficient.[18] However, non-specific exchange reactions and double bond migration processes can yield products of uncertain tritium distribution,[19] and ^3H NMR analysis of the labelled products of heterogeneous tritiation reactions may be complex due to the numerous isotopomers present.[17] In this example, a saturated product with a specific radioactivity of approximately 85 Ci $mmol^{-1}$ was obtained. Mass spectral analysis of products from model studies of this reaction, using the appropriate decenoic acid and deuterium gas, showed that more than three deuterium atoms were incorporated per molecule, consistent with the high tritium content observed here. As Table 1 shows and Figure 1b illustrates, tritium was distributed along the *sn-2* alkyl chain, indicating that a combination of double bond migration, saturation and non-specific exchange processes had taken place. The ^3H NMR spectrum (Figure 1b) could not be fully assigned as both the chemical shift and coupling constants for all the tritons were very similar. Hence, heterogeneous catalytic reduction failed to satisfy the regiospecificity criterion of our labelling requirements.

Homogeneous hydrogenation in the presence of Wilkinson's catalyst is selective for carbon-carbon multiple bonds and shows specific *cis*-addition across the multiple bond.[20,21] The rate of reduction is dependent upon multiple bond stereochemistry and substitution pattern.[22] The experimental procedure is complicated by the need to isolate the labelled product free from the homogeneous catalyst[23] and this is usually achieved by filtration, distillation[23] or chromatography.

In the current work, the radiochemically pure tritium labelled PLA_2 inhibitor was isolated from the homogeneous reaction mixture by flash chromatography on silica gel using a chloroform:methanol:water solvent system (Figure 2). Tritium incorporation was specific to the original double bond giving a symmetrical distribution across the two carbons (Table 1). A $^3H/^3H$ NMR COSY spectrum (Figure 3) confirmed that the signals present in the ^1H-decoupled ^3H NMR spectrum of the tritium labelled inhibitor (Figure 1c) arose from two diastereomers. The extent of tritium incorporation (*ca.* 57 Ci $mmol^{-1}$) was determined by a gravimetric method, but the accuracy of this measurement was limited by the hygroscopic nature of the inhibitor. All our labelling requirements were satisified by this homogeneous catalytic reduction method, which yielded a pure, high specific activity, regiospecifically tritiated PLA_2 inhibitor molecule.

533 MHz ^3H NMR spectra, shown in Figures 4a-4c, were obtained in Teflon-lined 5mm tubes at 57 °C in deuterium oxide. The Teflon liners containing the sample were sonicated

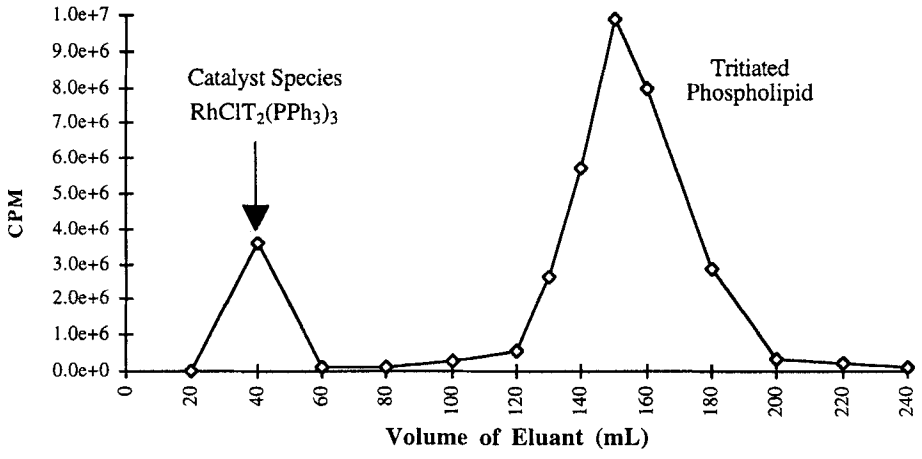


Figure 2. Radiochromatogram of the chromatographic separation of Wilkinson's catalyst and the tritiated PLA_2 inhibitor using 65:25:4 (v/v/v) chloroform:methanol:water as the solvent system on a column of silica gel.

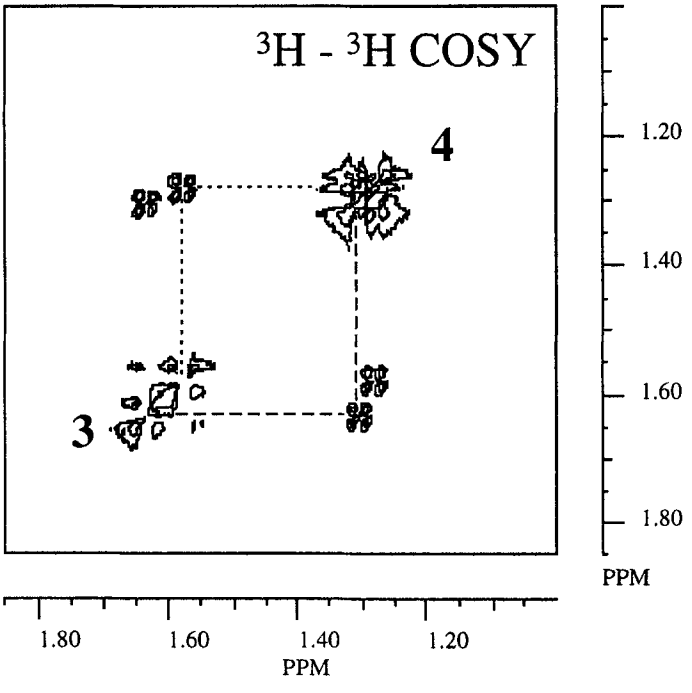


Figure 3. Phase sensitive 320 MHz $^3H/^3H$ NMR COSY of the homogeneous catalyzed reduction product. The signal structure in Figure 1(c) is due to two J-coupled diastereomers.

immediately before acquiring the spectra in order to remove dissolved air. ^3H NMR spectra of 1:1 PLA_2:[$^3H_2$]inhibitor complexes were obtained without (Figure 4a) and with (Figure 4b) calcium ions,[24,25] an essential co-factor for extracellular PLA_2's. An excess of the [$^3H_2$]inhibitor was used to obtain Figure 4c. The stoichiometry of PLA_2:[$^3H_2$]inhibitor complexes prepared as NMR samples were determined by liquid scintillation counting.

Discussion

The ^3H NMR signals observed in the sample containing PLA_2 enzyme and tritiated inhibitor (Figure 4a) arise from the non-complexed inhibitor. In the absence of calcium, the inhibitor does not bind to the active site of the enzyme. The signals are broadened relative to those in Figure 1c as the inhibitor undergoes a weak, non-specific association with the surface of the enzyme. The two signals each represent the sum of the resonances from equivalent tritons from each diastereomer.

On the addition of calcium (Figure 4b), there are considerable chemical shift changes as the inhibitor binds to the enzyme. This interaction was also followed by ^1H NMR (data not shown) and identical changes to the spectrum of the protein were seen as had previously been observed for other amide substrate analogues.[8,16] In the ^3H NMR spectrum of the complex (Figure 4b), all of the tritium signals are displaced downfield from their positions in the free inhibitor spectrum. Assuming that the two diastereomers bind identically to the enzyme, the appearance of the spectrum suggests that enantiomeric tritons experience quite different chemical environments within the binding pocket. Integration of the peaks shows that the three peaks correspond to 2 (2.1 ppm), 1 (1.7 ppm) and 1 (1.5 ppm) tritons respectively. By analogy with the binding of the parent olefin compound,[16] it is likely that the broad signal at 2.1 ppm corresponds to tritons at positions 3 and 4 of one of the diastereomers bound with the two tritons pointing towards the edges of the aromatic rings of Phe-5 and Phe-106 whilst the signals at 1.7 ppm and 1.5 ppm arise from the other diastereomer with the two tritons pointing away from these residues.

Addition of an excess of the inhibitor (Figure 4c) gives rise to a sum of the spectra of the free inhibitor and the complex. This can be seen in the peak at 1.4-1.5 ppm, which is clearly a combination of the resonances in the other two spectra with no sign of any exchange broadening. This suggests that the inhibitor is in slow exchange on the NMR time-scale and is therefore comparatively tightly bound.

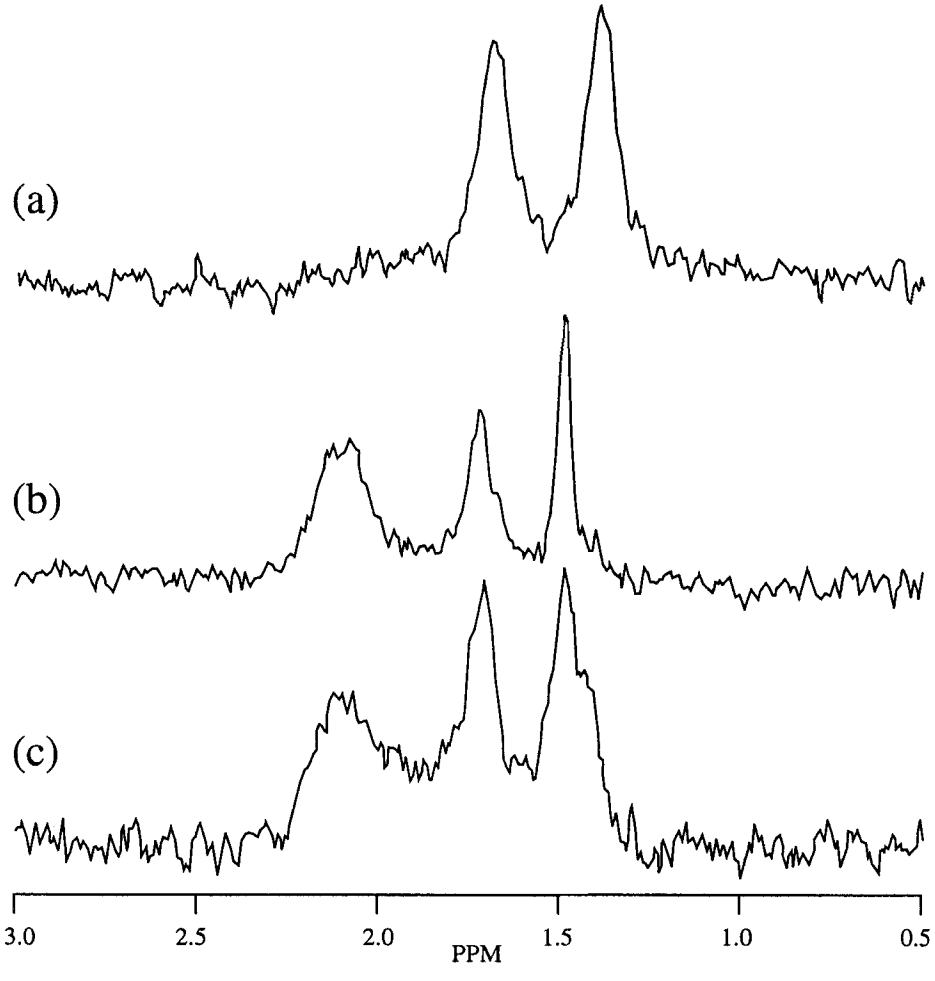


Figure 4. 533 MHz ^{3}H NMR spectra of the tritiated inhibitor binding to bovine pancreatic PLA_2. (a) ^{1}H-decoupled ^{3}H NMR spectrum of the non-complexed tritiated inhibitor in the presence of PLA_2; 1:1 molar ratio. (b) As in (a), but with calcium ions present at 50 mM, showing the effects of binding at the active site; 1:1 molar ratio. (c) As for (b) but with a molar excess of tritiated inhibitor.

We have demonstrated that tritiation of an alkene precursor using homogeneous catalysis yields a substrate suitable for ^3H NMR studies of inhibition and binding interactions with PLA_2 enzyme. This substrate can probe the inhibitor—enzyme interactions in a region inaccessible to ^1H NMR. More extensive ^3H NMR studies are in progress.

Experimental

Tritium gas was purchased from Oak Ridge National Laboratory. Silica gel (60Å, 230-400 mesh), platinum dioxide, 10% palladium-on-carbon and Wilkinson's catalyst were purchased from Aldrich Chemical Co. Potassium azodicarboxylate was a gift from Dr. M. Saljoughian, of the National Tritium Labelling Facility (NTLF). All the tritiation reactions were conducted inside a glove box at the NTLF, using a custom-built micro-hydrogenation apparatus.

[^{3}H]Diimide Reduction.[15] The alkene precursor (16.0 mg, 37 μmol) and potassium azodicarboxylate (50 mg, 257 μmol) were added to a 5 mL flame-dried flask equipped with a side-arm and a stirrer bar. The materials were thoroughly mixed and the flask was rigorously evacuated. In a separate flask, [$^3H_2$]water (190 μmol, 10.9 Ci) was synthesized by the combustion of $^3H_2$ with PtO_2. [O-^{3}H]Acetic acid (382 μmol) was prepared by adding acetic anhydride to the tritiated water and heating at 60 °C for 30 minutes. The acid was dissolved in anhydrous dioxane (0.5 mL), and the solution was transferred through a double-ended needle to the reaction flask. The flask was isolated and the pressure maintained at 270 mmHg with dry nitrogen gas whilst stirring the reaction mixture at room temperature for 16 hours. The solvent and excess [O-^{3}H]acetic acid were then removed under vacuum and methanol (1 mL) was added and evaporated. The reaction products were then dissolved in acetonitrile, filtered through glass wool, lyophilized, and analyzed by ^3H NMR spectroscopy (320 MHz, Figure 1a) as a solution in ^2H_2O (250 μL). Liquid scintillation counting showed that the product contained 74 mCi, and the ^3H NMR analysis showed that *ca.* 25 mCi of that radioactivity was in the desired product.

Heterogeneous Metal Catalyzed Reduction. A solution of the alkene precursor (8.0 mg, 18.5 μmol) in methanol (2 mL) was prepared in a flame-dried flask containing a stirrer bar and catalyst spoon charged with 10% palladium-on-carbon (8.3 mg). The solution was subjected to three freeze-thaw cycles under N_2 gas. After evacuation, tritium gas was admitted to 723 mmHg, the solution allowed to thaw and the catalyst added. Uptake of $^3H_2$ gas was complete after two hours. The solution was then frozen, the tritium gas evacuated, the flask flushed with nitrogen and

the suspension was thawed. Methanol (1 mL) was added and evaporated. After a further addition of methanol (1 mL) the suspension was passed through a PTFE filter and the filtrate lyophilized. The yield was 1.6 Ci and the specific radioactivity was determined by gravimetric analysis to be approximately 85 Ci $mmol^{-1}$. A 50 mCi portion was analyzed by ^3H NMR spectroscopy (320 MHz, Figure 1b) as a solution in ^2H_2O (250 μL).

Homogeneous Metal Catalyzed Reduction. A solution of the alkene precursor (10.0 mg, 23 μmol) in 3:2 (v/v) benzene:ethanol (1 mL) was prepared in a flame-dried flask equipped with a stirrer bar and catalyst spoon charged with tris(triphenylphosphine)chlororhodium (I) (20.5 mg, 22.2 μmol). The solution was subjected to three freeze-thaw cycles under N_2. The homogeneous catalyst was then added to the stirred solution, and tritium gas was admitted to one atmosphere pressure. The reaction mixture colour changed to yellow after approximately 20 minutes and the solution was stirred vigorously for 3.5 hours. The solution was then frozen in liquid N_2, tritium gas was evacuated and, after flushing with N_2 gas, the solution was thawed and methanol (1 mL) was added. The mixture was stirred, lyophilized and the resultant solid was dissolved in 65:25:4 (v/v/v) chloroform:methanol:water (1 mL) and applied to a flash chromatography column (silica, 250 x 20 mm). Elution with the same solvent system (Figure 2) yielded fractions containing the tritiated inhibitor which were pooled and lyophilized. The yield was 1.3 Ci and the specific radioactivity was approximately 57 Ci $mmol^{-1}$. An aliquot (50 mCi) was analyzed by ^3H NMR spectroscopy (Figures 1c, 3) as a solution in ^2H_2O (250 μL).

^3H NMR Spectra of the PLA_2-Inhibitor Complex. For samples devoid of calcium ions, the solution components were: bovine pancreatic PLA_2 (1 mM); NaCl (300 mM); EDTA (250 μM); $^2H_{11}$-TRIS (10 mM); $^2H_4$-succinate (10 mM) and [$^3H_2$]inhibitor (10 mCi, 1 mM) in ^2H_2O (200 μL, Figure 4a). For samples with calcium ions (Figure 4b), the differences in solution components were: NaCl (200 mM); $CaCl_2$ (50 mM). All buffers and salts were present as sodium salts and pH^*=7.3, uncorrected for the deuterium isotope effect. PLA_2 was isolated from bovine pancreas following literature procedures, with slight amendments as reported previously.[8]

Acknowledgements

ASC would like to thank Mr. D. Hunter, Mr. C. Bennion, Dr. S. Connolly and Dr. D.H. Robinson, all of Astra Charnwood, for their invaluable assistance. This work was supported by the SERC (a CASE award to ASC) and the Biomedical Research Technology Program, National

Center for Research Resources, U.S. National Institutes of Health, under Grant P41 RR01237, through Contract DE-AC03-76SF00098 with the U.S. Department of Energy.

References

1. Lapetina, E.G. — Ann. Reports Med. Chem. 19: 213 (1984)
2. Pruzanski, W: and Vadas, P. — Immunology Today 12: 143 (1981)
3. Dijkstra, B.W., Drenth, J. and Kalk, K.H. — Nature 289: 604 (1981)
4. Renetseder, R., Brunie, S., Dijkstra, B.W., Drenth, J. and Sigler, P.B. — J. Biol. Chem. 260: 11627 (1985)
5. Thunnissen, M.M.G.M., Ab, E., Kalk, K.H., Drenth, J., Dijkstra, B.W., Kuipers, O.P., Dijkman, R., de Haas, G.H. and Verheij, H.M. — Nature 347: 689 (1990)
6. White, S.P., Scott, D.L., Otwinowski, Z., Gelb, M.H. and Sigler, P.B. — Science 250: 1560 (1990)
7. Scott, D.L., Otwinowski, Z., Gelb, M.H. and Sigler, P.B. — Science 250: 1563 (1990)
8. Bennion, C., Connolly, S., Gensmantel, N.P., Hallam, C., Jackson, C.G., Primrose, W.U., Roberts, G.C.K., Robinson, D.H. and Slaich, P.K. — J. Med. Chem. 35: 2939 (1992)
9. Evans, E.A., Warrell, D.C., Elvidge, J.A. and Jones, J.R. — "Handbook of Tritium NMR Spectroscopy and Applications", Wiley (1985)
10. O'Connell, T.M., Gerig, J.T. and Williams, P.G. — J. Chem. Soc., Chem. Commun. 1502 (1990)
11. Gehring, K., Williams, P.G., Pelton, J.G., Morimoto, H. and Wemmer, D.E. — Biochemistry 30: 5524 (1991)
12. Highsmith, S., Kubinec, M.G., Jaiswal, D.K., Morimoto, H., Williams, P.G. and Wemmer, D.E. — J. Biomolecular NMR 3: 325 (1993)
13. Curtis, N., Moore, S., Birdsall, B., Bloxsidge, J., Gibson, C.L., Jones, J.R. and Feeney, J. — Biochem. J. 303: 401 (1994)
14. O'Connell, T.M., Gerig, J.T. and Williams, P.G. — J. Am. Chem. Soc. 115: 3048 (1993)

15. Saljoughian, M., Williams, P.G., Morimoto, H., Goodlett, D.R. and van Breemen, R.B. — J. Chem. Soc., Chem. Commun. 414 (1993)

16. Slaich, P.K., Primrose, W.U., Robinson, D.H., Wharton, C.W., White, A.J., Drabble, K. and Roberts, G.C.K. — Biochem. J. 288: 167 (1992)

17. Williams, P.G., Morimoto, H. and Wemmer, D.E. — J. Am. Chem. Soc. 110: 8038 (1988)

18. Rylander, P.N. — "Catalytic Hydrogenation in Organic Synthesis", Academic Press (1979)

19. Dinh-Nguyen, N. and Ryhage, R. — Acta Chem. Scand. 13: 1032 (1959)

20. Osborn, J.A., Jardine, F.H., Young, J.F. and Wilkinson, G. — J. Chem. Soc.(A) 1711 (1966)

21. James, B.R. — Adv. Organometal. Chem. 17: 319 (1979)

22. Masters, C. — "Homogeneous Transition Metal Catalysis", Chapman and Hall (1981)

23. Birch, A.J. and Walker, K.A.M. — J. Chem. Soc.(C) 1894 (1966)

24. Aguiar, A., de Haas, G.H., Jansen, E.H.J.M., Slotboom, A.J. and Williams, R.J.P. — Eur. J. Biochem. 100: 511 (1979)

25. de Haas, G.H., Bonsen, P.P.M., Pieterson, W.A. and van Deenen, L.L.M. — Biochim. Biophys. Acta 239: 252 (1971)