^{17}O NMR in Biosynthetic Studies: Aspyrone, Asperlactone and Isoasperlactone, Metabolites of *Aspergillus melleus*

James Staunton* and Andrew C. Sutkowski

University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, UK

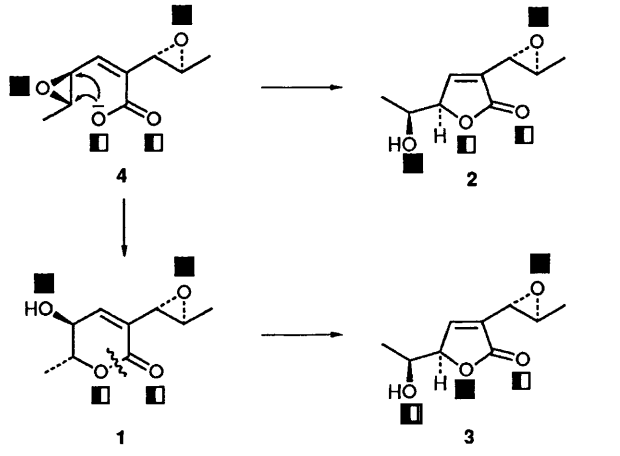
Incorporation studies with *Aspergillus melleus*, using ^{17}O labelled acetate and ^{17}O labelled gas as precursors, and ^{17}O NMR spectroscopy as the analytical tool, have established that all three isomeric metabolites, aspyrone, asperlactone and isoasperlactone, arise from a common diepoxide intermediate.

In comparison with ^{18}O, ^{17}O has been relatively little used as a biosynthetic tracer despite its advantage of being observable directly by ^{17}O NMR spectroscopy, rather than being limited to indirect observation through an isotopic effect[1] on a neighbouring NMR active nucleus (^{13}C). The principal reason for this neglect is not expense but the difficulty of acquiring good quality ^{17}O NMR spectra.[2] The nucleus is quadrupolar and relaxes very rapidly. Signals are therefore broad. Also the free-induction decay (FID) can be badly corrupted by pulse breakthrough from the radio frequency used to excite the nucleus, which can lead to serious distortion of the baseline of the transformed spectrum. The latter effect can be partially compensated for by setting the early points in the FID to zero, a technique used in the present work. On the positive side, the low natural abundance of ^{17}O (0.04%) compared with ^{18}O (0.20%) and ^{13}C (1.1%) can be an important advantage in biosynthetic studies because the natural abundance level of the tracer isotope, or of the reporter nucleus in an isotopic shift experiment, sets a limit on the degree of dilution allowed in the biosynthetic processes under study.

The three isomeric metabolites aspyrone **1**, asperlactone **2** and isoasperlactone **3**, are polyketides produced by the fungus *Aspergillus melleus*. It has been suggested that they derive from a common biosynthetic precursor, the diepoxide **4**, as indicated in Scheme 1.[3] Alternative modes of nucleophilic attack by the carboxy group on one of the two epoxide

residues leads to the five-membered ring of asperlactone or the six-membered ring of aspyrone as indicated. The absolute configurations of all the chiral centres in these two metabolites are satisfactorily explained by this scheme. The difference in stereochemistry between asperlactone **2** and isoasperlactone **3** can be explained on the assumption that the latter compound is formed from aspyrone **1** by nucleophilic cleavage of the acyl–oxygen bond of the lactone ring, followed by recyclisation to give the five-membered ring lactone. An alternative origin for **3**, paralleling the direct conversion of the diepoxide **4** to **2**, is shown in Scheme 2, where the equivalent epoxide ring of a diastereoisomeric diepoxide **5** is opened by nucleophilic attack by the carboxy group.

An earlier study of the origins of the oxygen atoms using ^{18}O in association with ^{13}C as a reporter nucleus (isotopic shifts in the ^{13}C NMR spectra) established that in the major metabolite, aspyrone, all four oxygens are labelled by molecular oxygen, but not uniformly: the level of enrichment in the two oxygens of the lactone group is half that in the remaining two sites.[4] Therefore, in individual aspyrone molecules three of the four oxygens derive from molecular oxygen: the hydroxy and epoxide oxygens together with one or the other of the oxygen atoms of the lactone group. No oxygen isotope was incorporated from the carboxy group of acetate,[4] so it can be assumed that the fourth oxygen comes from water. This labelling pattern is consistent with the proposed derivation of **1** from **4**, assuming that the two epoxide residues of **4** are derived from molecular oxygen in the standard way, and that one of the two carboxylate oxygens is introduced at some stage by an oxidation using molecular oxygen.



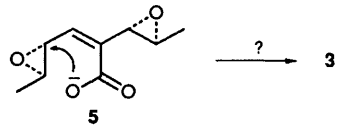
Scheme 1

The very small quantities of **2** and **3** produced in these experiments, combined with the low incorporation of ^{18}O, precluded the detection of isotopically shifted peaks in the ^{13}C NMR. We now show that this limitation of sensitivity can be overcome by using ^{17}O as the tracer isotope and ^{17}O NMR spectroscopy as the method of detection. In practical terms these new experiments were equivalent to the earlier ones with ^{18}O, except that the enrichment of ^{17}O in the precursors was lower (10–20% instead of 100%). The precursors, $^{17}O_2$ and $MeC^{17}O_2H$, were administered to growing cultures of *A. melleus* on day seven after inoculation, when the rate of metabolite production reached a maximum, and metabolites were extracted four days later.

The ^{17}O NMR spectra were run at 54.2 MHz on a Bruker instrument. For all spectra the FID was recorded over 256 data points and the first 8–10 points were set to zero prior to transformation to reduce baseline drift. Chemical shifts were measured relative to an internal capillary reference of water, and the peaks assigned by analogy with published data.[5] In the aspyrone spectrum the signals for the hydroxy group and epoxide are superimposed (δ 9), but the two key oxygens of the lactone group give rise to well resolved signals at δ 160 (ether oxygen) and δ 360 (carbonyl oxygen). The spectra of the two minor metabolites **2** and **3** were identical to each other with three peaks at δ 15 (epoxide and hydroxy oxygens signals superimposed), δ 188 (lactone ether) and δ 321 (lactone carbonyl).

The relative intensities of the peaks in the natural abundance spectrum of aspyrone (spectrum 1, Table 1) differed significantly from the values (1 : 1 : 2) expected for a uniform distribution of the isotope because the available excitation pulse, which was placed near the centre of the displayed spectrum, was not sufficiently strong to give uniform excitement of nuclei over the whole of the spectra width. Measurements of relative intensity were nevertheless satisfactorily reproducible (±20%) as long as standard conditions were used to acquire spectra. The natural abundance spectrum for each metabolite can therefore serve as a control in assessing relative degrees of enrichment at different sites in enriched biosynthetic samples.

Spectrum 2 gives the results for aspyrone of an experiment in which [^{17}O]acetate was used as the precursor. The resulting spectrum is not significantly different from the natural abundance spectrum, showing that no oxygen label was incorporated. Similarly, no evidence was found for incorpora-



Scheme 2

Table 1 Relative intensities of peaks in ^{17}O NMR spectra of metabolites[a]

Spectrum	Metabolite	Source of ^{17}O label	Intensities of resonances relative to lactone carbonyl[b]		
			Lactone carbonyl	Lactone ether	Hydroxy + epoxide
1	Aspyrone	Natural abundance	1	1.2	1.6
2	Aspyrone	[^{17}O]Acetate	1	1.1	1.6
3	Aspyrone	$^{17}O_2$	1(1)	1.3(1)	4.3(4)
4	Asperlactone	$^{17}O_2$	1(1)	1.2(1)	4.8(4)
5	Isoasperlactone	$^{17}O_2$	1(1)	2.0(2)	3.1(3)

[a] Measured by cutting and weighing peaks. [b] Numbers in parentheses give calculated values based on the labelling patterns shown in Scheme 1.

tion of label in either of the other two metabolites, **2** or **3**, isolated from this experiment. It should be stressed that these results provide an extremely sensitive test, because even a very low incorporation of isotopic label into the oxygens of the lactone group would have resulted in a major relative increase in the intensities of their signals relative to the epoxide and hydroxy signals. In marked contrast, the co-metabolite hydroxymellein isolated from this experiment gave very strong signals in the ^{17}O NMR.[6]

Spectra 3, 4 and 5 listed in Table 1 give the results of an experiment in which the organism was grown in an atmosphere enriched with $^{17}O_2$ gas. For each metabolite the measured relative intensities in the ^{17}O NMR spectrum are consistent with the values expected on the basis of the transformations shown in Scheme 1. Thus the distribution in **2** parallels that found for **1**, but the distribution in isoasperlactone **3** is significantly different in a way consistent with its proposed derivation *via* rearrangement of aspyrone, rather than by the direct opening of an epoxide as shown in Scheme 2. These results therefore support the proposed common biosynthetic origin of all three metabolites from the single diepoxide intermediate **4**.

The experiments described here demonstrate the relative merits of ^{17}O *vs.* ^{18}O as a biosynthetic tracer. The principal weakness of the ^{17}O NMR method lies in the relatively limited resolution. Thus in the present work the uniform distribution of labels in the hydroxy and epoxide residues of aspyrone could not be demonstrated because the relevant resonances were very broad and therefore were superimposed. This limitation could probably have been overcome by preparing a suitable acyl derivative of the hydroxy group so as to alter the chemical shift of this oxygen, but this possibility was not explored because the uniformity of labelling had already been established in the earlier study using ^{18}O as the isotopic tracer.[4] A second disadvantage can be the distortion of relative intensities of widely dispersed signals because of inadequate power in the excitation pulse, but this limitation will be less of a problem as more powerful instruments are developed.

Two compensating advantages of ^{17}O as a tracer are also evident. Because of the low natural abundance of ^{17}O, we were able to demonstrate more convincingly than was possible in our earlier work with ^{18}O that no oxygen label was retained from the carboxy group of acetate in the lactone residue. Also, because of the greater sensitivity of the ^{17}O NMR technique, we were able to make useful measurements of the isotopic distributions for the minor metabolites **3** and **4**, as well as for aspyrone. The optimum choice of isotope in a particular biosynthetic study therefore depends on the nature of the problem to be solved and the obstacles encountered. In our earlier experiments using ^{18}O and in the present ones using ^{17}O, both the scale of the work and the expense of materials were comparable. In future biosynthetic tracer studies using labelled oxygen, therefore, ^{17}O deserves serious consideration as the isotope of choice.

We thank Dr A. Bassindale and Dr D. Wilson who acted as internal supervisors to A. C. S. while he was an external research student at the Open University, Milton Keynes, UK.

Received, 15th March 1991; Com 1/01252E

References

1 J. C. Vederas, *Can. J. Chem.*, 1982, **60**, 1637.
2 I. P. Gerothanassis and J. Lauterwein, *J. Magn. Res.*, 1985, **66**, 32.
3 M. J. Garson, P. G. Jones and J. Staunton, *J. Chem. Soc., Perkin Trans. 1*, 1984, 1021.
4 S. A. Ahmed, T. J. Simpson, J. Staunton, A. C. Sutowski, L. A. Trimble and J. C. Vederas, *J. Chem. Soc., Chem. Commun.*, 1985, 1685.
5 J. P. Kitzinger, *Oxygen -17 and Silicon-29 N.M.R., Basic Principles and Progress*, eds. P. Diehl, E. Fluck and R. Kosfield, Springer Verlag, Berlin, 1981.
6 C. Abell, A. C. Sutkowski and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1987, 586.