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DETERMINATION OF SPECIFIC RADIOACTIVITY OF [^{14}C]-COMPOUNDS BY MASS SPECTROSCOPY

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Abstract—A method is presented by which the specific radioactivity of [^{14}C]-labelled compounds can be determined using MS data from GLC–MS. The specific activity of [^{14}C]-labelled *ent*-kaurene, *ent*-7α-hydroxykaurenoic acid (as the Me ester and Me TMSi ether), *ent*-kaurenoic acid (as the Me ester) and GA$_{12}$-aldehyde (as the Me ester) have been determined by this method. These compounds were thus shown to be derived from 2-[^{14}C]MVA without significant dilution of the label in a cell-free enzyme system from *Cucurbita pepo*. The accuracy and sensitivity of the method is discussed.

INTRODUCTION

THE IDENTIFICATION of μg quantities of enzymatic conversion products and the demonstration that they are derived from a radioactive precursor are problems frequently encountered in biochemical studies. We have previously shown[1] that the first of these problems can be solved by GLC–MS. This paper shows how the same data may be used to detect the [^{14}C]-label and to calculate specific activities, thus solving the latter problem. The method takes advantage of the high degree of labelling often obtained with cell-free enzyme systems in which dilution of the label by endogenous precursors and intermediates can be avoided.

Using a cell-free enzyme system from the endosperm of *Cucurbita pepo* we have previously shown that mevalonic acid (MVA) is converted into *ent*-kaurene (I),[2] *ent*-kaurenoic acid (II),[1] *ent*-7α-hydroxykaurenoic acid (IV),[1] and *ent*-7-oxo-gibberell-16-en-19-oic acid (GA$_{12}$-aldehyde, VII).[1] These four compounds serve as intermediates in the fungal bio synthesis of the gibberellins (for reviews, see Cross[3] and MacMillan[4]). Although the last three compounds were identified by GLC–MS, and the incorporation of label from 2-[^{14}C]MVA of specific activity 0·5 μCi/μmol was demonstrated, the specific activities were not determined.

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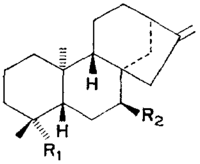
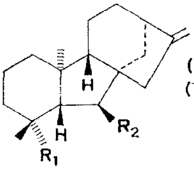


(I) $R_1 = CH_3$; $R_2 = H$
(II) $R_1 = COOH$; $R_2 = H$
(III) $R_1 = COOMe$; $R_2 = H$
(IV) $R_1 = COOH$; $R_2 = OH$
(V) $R_1 = COOMe$; $R_2 = OH$
(VI) $R_1 = COOMe$; $R_2 = OSiMe_3$



(VII) $R_1 = COOH$; $R_2 = CHO$
(VIII) $R_1 = COOMe$; $R_2 = CHO$

When the same four compounds (I), (II), (IV) and (VII) were obtained by incubation of the enzyme system containing 2-[^{14}C]MVA with a specific activity of 10·3 μCi/μmol the GLC–MS scans of *ent*-kaurene (I) and the derivatized acids (II), (IV) and (VII) showed strong [^{14}C]-isotopic peaks. Thus the MS of *ent*-kaurene (I), Me *ent*-kaurenoate (III), Me *ent*-7α-hydroxykaurenoate TMSi ether (VI) and GA$_{12}$-aldehyde Me ester (VIII) showed $M^+ + 2$, $M^+ + 4$, $M^+ + 6$ peaks while that of Me *ent*-7α-hydroxykaurenoate (V) showed $M^+ + 2$ and $M^+ + 4$ peaks. Mass matching of the $M^+ + 2$ ion in the MS of *ent*-kaurene (I) confirmed the formula $^{12}C_{19}{}^{14}C_1H_{32}$. From the intensities of these peaks, specific activities can be calculated from the general expression

$$\sum_{j/2=1}^{j/2=n} j/2\ h_j\ a \Bigg/ \sum_{j/2=0}^{j/2=n} h_j$$

where n = number of [^{14}C]-atoms per molecule;
$j/2 = 0, 1, 2 \ldots\ldots n$; a = specific activity of a[^{14}C]-atom;
h_j = intensity of the $M^+ + j$ ion.

The constant a, calculated from the half-life of [^{14}C] is 63·0 μCi/μmol. The $M^+ + j$ ions were corrected for their [^{13}C], [^{29}Si], and [^{30}Si] content. For *ent*-kaurene (I) and the Me esters (III), (V) and (VIII), the [^{13}C]-contribution to the $M^+ + 2$, $M^+ + 4$, and $M^+ + 6$ peaks were 2·4, 2·2 and 1·9% of their respective parent ions, M^+, $M^+ + 2$, and $M^+ + 4$. For the MeTMSi ether (VI), $C_{24}H_{40}O_3Si$, the intensity of the [^{14}C]-component of the $M^+ + 2$ peak was obtained by subtracting the contributions of the $^{12}C_{24}H_{40}O_3{}^{30}Si$, $^{12}C_{23}{}^{13}C_1H_{40}O_3{}^{29}Si$, and $^{12}C_{22}{}^{13}C_2H_{40}O_3{}^{28}Si$ which were respectively 3·4, 1·4, and 3·5% of the M^+ peak. Similarly the $M^+ + 4$ peak of the MeTMSi ether (VI) was corrected for $^{12}C_{23}{}^{14}C_1H_{40}O_3{}^{30}Si$, $^{12}C_{22}{}^{13}C_1{}^{14}C_1H_{40}O_3{}^{29}Si$, and $^{12}C_{21}{}^{13}C_2{}^{14}C_1H_{40}O_3{}^{28}Si$ contributions of 3·4, 1·3, and 3·2% respectively of the $M^+ + 2$ peak. The $M^+ + 6$ peak was also corrected for $^{12}C_{22}{}^{14}C_2H_{40}O_3{}^{30}Si$, $^{12}C_{21}{}^{13}C_1{}^{14}C_2H_{40}O_3{}^{29}Si$, and $^{12}C_{20}{}^{13}C_2{}^{14}C_2H_{40}O_3{}^{28}Si$ which were respectively 3·4, 1·3, and 2·9% of the $M^+ + 4$ peak. These corrections were calculated from the expression $(x + y)^m$ where x is the natural abundance of [^{12}C] or [^{30}Si], y is the abundance of [^{13}C], [^{29}Si], or [^{30}Si], and m is the number of carbon or silicon atoms in the molecule. The specific activities determined in this way are shown in Table 1.

TABLE 1. SPECIFIC ACTIVITIES OF METABOLIC PRODUCTS FROM 2-[^{14}C]MVA (10·3 μCi/μmol)

Compound	Measured specific activity (μCi/μmol) (theoretical 41·2 μCi/μmol)
I	41·3, 40·6, 41·1
III	43·5
V	37·0, 41·4
VI	38·4, 45·2, 39·4
VIII	34·2, 35·2, 35·4

The relative heights of M^+ and its [^{14}C]-peaks can also provide information on the number of [^{14}C]-atoms incorporated from the precursor in the following convenient way. The theoretical relative peak heights are readily calculated from the expression $(b + c)^n$ where b and c are the frequencies of precursor molecules without and with [^{14}C]-atoms, and n is the number of labelled positions in the ion. The frequency b for MVA is given by the specific activity, in μCi/μmol, divided by the previously calculated constant a (= 63 μCi/μmol). Since $b = 1 - c$, the relative intensities of the isotopic peaks can be calculated from the ratios of the successive terms in the expansion of $(b + c)^n$. The theoretical values for 2-[^{14}C]MVA of specific activity 10·3 μCi/μmol are shown in Table 2 and the measured ratios are shown in Table 3. The theoretical relative intensities in Table 2 can be used for di-, sesqui- and mono-terpenes. They also apply to any ion in the MS, subject to the provisos noted below, and could be used to determine the fragmentation pathway if the position of the labelled atoms is known or vice versa. Tables, analogous to Table 1, can be readily constructed for MVA, or other precursors, of any specific activity.

TABLE 2. THEORETICAL RELATIVE INTENSITIES FROM 2-[^{14}C]MVA (10·3 μCi/μmol)

Ionic species	Relative intensities 4[^{14}C] per ion	3[^{14}C] per ion	2[^{14}C] per ion	1[^{14}C] per ion
M^+	100	100	100	100
$M^+ + 2$	78·5	58·9	39·2	19·6
$M^+ + 4$	23·1	11·5	3·8	0·0
$M^+ + 6$	3·0	0·8	0·0	0·0
$M^+ + 8$	0·15	0·0	0·0	0·0

There is reasonable agreement between the theoretical specific activity of 41·2 μCi/μmol for the incorporation of [2-^{14}C]MVA without dilution and the determined specific activities (Table 1) and also between the theoretical (Table 2) and measured (Table 3) relative intensities of the $M^+ + j$ ions. Nevertheless there is a considerable scatter in the determined values and in the case of GA_{12}-aldehyde the three values are low. However, these values were obtained from single, fast GLC–MS scans which, as discussed below, are subject to considerable statistical variation. Agreement between the determined and theoretical values was very much better when the TIC peaks of compounds (I), (VI) and (VIII) were cyclically scanned at the same scan speed over the M^+ isotopic peaks; the average specific activities for (I, six scans), (VI, ten scans), and (VIII, five scans) were respectively 40·7, 40·7, and

TABLE 3. MEASURED RELATIVE INTENSITIES FROM 2-[^{14}C]MVA (10·3 μCi/μmol)

Ionic species	Relative intensities in compounds I		III	V		VI	VIII
M^+	100·0	100·0	100·0	100·0	100·0	100·0	100·0
$M^+ + 2$	72·1	70·1	75·6	68·9	64·4	68·2	57·9
$M^+ + 4$	21·0	21·9	27·7	20·7	32·4	31·0	16·0
$M^+ + 6$	3·8	4·8	4·5	0·0	0·0	0·0	2·8

TABLE 4. MEASURED RELATIVE PEAK INTENSITIES FOR CYCLIC SCANS OF (VI) FROM 2-[^{14}C]MVA (10·3 μCi/μmol)

m/e	Relative intensities*									
404	100·0	100·0	100·0	100·0	100·0	100·0	100·0	100·0	100·0	100·0
406	66·2	67·7	78·7	73·4	66·7	72·2	71·7	79·7	64·7	77·7
408	25·5	20·0	25·8	21·2	23·7	25·7	20·0	26·5	22·6	15·4
410	2·7	5·0	2·9	2·9	3·4	3·8	5·1	3·4	4·0	4·2

* The mean values give specific activity of 40·7 μCi/μmol.

40·0 μCi/μmol. The relative peak intensities for the ten scans of the TMSi ether (VI), corrected for [^{13}C], [^{29}Si], and [^{30}Si] as described above, are shown in Table 4. The peak heights, measured directly from the chart paper and from which the data in Table 4 were derived, are shown in Table 5 since the accuracy of the determined specific activities and relative peak heights depends upon these actual peak heights in the following two ways. First, the precision of measurement above the base line is about 3 % for a peak 1 cm in height, increasing to 0·3 % for a 10 cm peak. Secondly, the statistical variation in peak height depends upon the number of ions collected at that *m/e* value. It was calculated that, under the conditions used to obtain the data in Table 5, a peak of 1 cm in height is obtained from only 180 ions at the photomultiplier. The mean deviation for a 1 cm peak is therefore 7·5 %. Thus the statistical error of many of the peak heights in Table 5 is considerable. Indeed there is a high probability that some peaks may not be detected in a single fast scan. For example no M$^+$ + 6 peak was observed in the MS of compounds (V) and (VI) (see Table 3) which show M$^+$ ions of weak intensity. These considerations apply in general to MS obtained from fast scans of GLC peaks where small numbers of ions at particular *m/e* values are detected. Thus a weak but significant peak can be present or absent from such MS obtained under the same conditions.

Although the data presented in Tables 1, 3–5 were obtained on the M$^+$ peaks, peaks arising from simple fragmentations can also be used. For example, the M$^+$ −90 ions of the TMSi ether (VI) give a specific activity of 40·0 μCi/μmol. However, values based upon fragment ions are normally not reliable unless the fragmentation is known with certainty and the MS of the unlabelled compound, obtained under identical conditions, is available for correction.

Several incubations were carried out using 2-[^{14}C]MVA of specific activity between 5·0 and 5·85 μCi/μmol. The determined specific activities of the products (I), (II), (IV), and (VII) were in the range 20–23·4 μCi/μmol. The lower limits of detection of [^{14}C]-label

TABLE 5. MEASURED PEAK HEIGHTS (cm) FOR CYCLIC SCANS OF (VI) FROM 2-[^{14}C]MVA (10·3 μCi/μmol)

					Scan No.					
m/e	1	2	3	4	5	6	7	8	9	10
404	4·3	7·7	11·5	16·5	21·0	18·0	15·0	10·02	7·0	3·70
405	1·25	3·1	4·5	5·8	7·5	6·10	4·5	4·05	1·98	1·15
406	3·20	5·85	10·0	13·5	15·8	14·5	12·0	9·00	5·10	3·15
407	1·32	2·35	3·5	4·85	5·00	4·85	4·13	2·95	1·70	0·85
408	1·25	1·95	3·68	4·45	6·10	5·65	3·85	3·35	1·95	0·80
409	0·50	0·80	1·3	1·65	1·75	1·65	1·22	0·85	0·53	0·40
410	0·20	0·50	0·55	0·75	1·10	1·05	1·00	0·55	0·40	0·20

depend upon the relative intensities of the molecular ion. In the case of *ent*-kaurene (I), with an intense M^+ ion, it is estimated that the lower limit is approximately 2·0 μCi/μmol. In compounds such as (V) with M^+ ions of low relative intensity, the lower limit is about 4·0 μCi/μmol.

When metal columns were used for GLC of methyl *ent*-7α-hydroxykaurenoate (V), the MS were complicated by $M^+ - 2$ peaks. Although the MS can be corrected for these $M^+ - 2$ peaks, this complication can be avoided by using glass columns.

EXPERIMENTAL

2-[^{14}C]-MVA (specific activity 10·3 μCi/mol) was obtained from the Radiochemical Centre, Amersham. Cell-free extracts were prepared from unripe seeds of *Cucurbita pepo* as previously described[4] except that the extracts were dialysed at pH 8·0 and that centrifugation after dialysis was omitted. The acidic products were generated in a 50 ml incubation mixture. Incubation, extraction and chromatography were as previously reported.[2] [^{14}C]*ent*-Kaurene was generated in the same way but omitting NADPH from the incubation mixture. It was purified by TLC on silic gel G developed with light petroleum (40–60°). The incubation products of specific activity 20·0–23·4 μCi/μmol were obtained from several incubations using 2-[^{14}C]MVA of specific activity 5·0–5·85 μCi/μmol.

GLC–MS. Derivatized fractions were chromatographed on a glass or stainless steel column (243 cm × 1·7 nm i.d.) packed with 2% SE.33 on Gaschrome Q (100–120 mesh) fitted to a Varian MAT CH7 mass spectrometer. The helium carrier gas flow of 5 ml/min was removed by a single stage Biemann–Watson separator. The column temperature was programmed from 190° at 2°/min. The single and cyclic scans were carried out at a scan rate of approximately 1·2 sec/100 m.u. Cyclic scans were carried out over a mass range of 50 m.u. above and below the parent ion.

High-resolution MS. Mass matching of the $M^+ + 2$ ion in (I) was performed on an MS9 (Found: 274·255. $^{12}C_{19}{}^{14}C_1H_{32}$ requires 274·253; $^{12}C_{18}{}^{13}C_2H_{32}$ requires 274·257).

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