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DETERMINATION OF LOW LEVELS OF INCORPORATION OF ^{13}C-LABELLED PRECURSORS. BIOSYNTHESIS OF THE 2,5-DIHYDROXY*CYCLO*HEXADIENE-1,4-DIONE SYSTEM OF COCHLIODINOL

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Key Word Index—*Chaetomium globosum*; cochliodinol; biosynthesis; ^{13}C NMR; mass spectra.

Abstract—A method for the determination of small enrichments of ^{13}C is described. The method is illustrated by measurements on a synthetic sample of DL-tryptophan-[2-^{13}C] and a metabolite of this amino acid, cochliodinol, found in cultures of *Chaetomium globosum*

INTRODUCTION

Recently biosynthetic studies have benefited from the use of ^{13}C-labelled precursors [1]. Generally speaking, comparison of broadband [^{1}H]-decoupled ^{13}C NMR spectra of the labelled and unlabelled metabolite allows estimation of the levels and sites of ^{13}C enrichment. However, accurate measurement of low levels of incorporation ($<0.5\%$) is difficult [1]. This limitation of the ^{13}C NMR method has been circumvented to some extent by the use of multiple labels, the use of relaxation agents [2] or by growing organisms on media containing only ^{12}C and subsequently introducing the ^{13}C labelled precursor [1, 3]. Another approach is to establish incorporation of a ^{13}C precursor by determining the $(M + 1)^+/(M)^+$ ratio of the metabolite by MS. The ^{13}C NMR data may then be manipulated statistically to arrive at the most probable location for ^{13}C enrichment in the metabolite and a $(M + 1)^+/(M)^+$ ratio comparable to the MS results. Such a procedure is illustrated in this paper in connection with the biosynthesis of the 2,5-dihydroxy*cyclo*hexadiene-1,4-dione ring of cochliodinol (**1**, R = 5-prenylindolyl-3). [4].

RESULTS

Measurement of enrichment and $(M + 1)^+/(M)^+$ ratios from NMR data

The measurement of ^{13}C enrichment at individual carbon positions from ^{13}C NMR spectra of enriched and unenriched material has been discussed previously [5]. An estimate of the $(M + 1)^+/(M)^+$ ratio can be obtained from these measurements. Let p_i, p_j be the probabilities that the ith, jth atom in a molecule containing n atoms, has an atomic weight 1 atomic mass unit (a.m.u.) more than its isotope of lower mass. Then the probability of an M^+ ion is

$$(M)^+ = \prod_{j=1}^{n} (1 - p_j).$$

Similarly

$$(M + 1)^+ = \sum_{j=1}^{n} \left[p_i \prod_{\substack{j \neq i \\ j=i}}^{n} (1 - p_j) \right]$$

assuming that only the isotopes of lowest atomic weight, or lowest atomic weight plus 1 a.m.u., are present. This is true for C, H, N and O, if ^{18}O is neglected. Thus

$$(M + 1)^+/(M)^+ = \sum_{j=1}^{n} [p_j/(1 - p_j)]. \qquad (1)$$

MS and NMR data were used to determine the position and degree of ^{13}C enrichment in both DL-tryptophan-[2-^{13}C] (henceforth try-[2-^{13}C] and cochliodinol (**1**, R = 5-prenylindolyl-3) a metabolite of *Cheatomium globosum*. Try-[2-^{13}C] (a precursor of cochliodinol) was synthesized from malonic-[2-^{13}C] acid [6]; the procedure given in the Experimental contains several modifications which improved the overall yield.

In the MS of try-[2-^{13}C] the most abundant ion in the group of ions of greatest mass had m/e 205.0936 ($^{12}C_{10}{}^{13}CH_{12}N_2O_2$ requires 205.0932). The m/e (204/205) ratio of ion currents for try-[2-^{13}C] was 1.178 with standard deviation ($\sigma =$) 0.047, whereas the value 0.1315 ($\sigma = 0.0087$) was obtained for this ratio for a sample of DL-tryptophan synthesized in the same way from natural malonic acid. The value calculated for this ratio from the natural isotopic abundances [7] of ^{2}H, ^{13}C, ^{15}N, ^{17}O and ^{18}O for a molecule $C_{11}H_{12}N_2O_2$ is 0.1332.

The ^{13}C NMR spectrum of try-[2-^{13}C] was not determined because of its low solubility, but the spectrum of its derivative (**4**, R = R′ = H, R″ = CO$_2$Me, R‴ = CHO, R⁗ = Me), an intermediate in its synthesis, confirmed the location of the ^{13}C label in try-[2-^{13}C]. The broadband-[^{1}H]-decoupled ^{13}C NMR spectrum contained 12 signals: δ_C 168.5 (ester CO), 160.7 (amide CO), 129.3, 124.6 (2-indole), 121.8, 119.4, 119.1, 111.8 (aromatic carbons), 109.2 (3-indole), 67.6 (side-chain quaternary carbon), 53.0 (OMe), 29.4 (CH$_2$), assigned from their chemical shifts and multiplicities in high-resolution and off-resonance decoupled spectra. Intensities for each resonance of the compound at natural abundance and ^{13}C enriched were normalized to the line at δ_C 109.2, and the ratios, r, of the normalized intensities (enriched/unenriched) were determined for each resonance [5]. Thus $r > 1$ indicated enrichment.

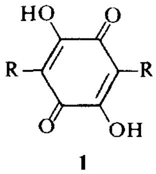
A comparison of 4 determinations of the 'natural abundance' spectrum gave the standard deviation of normalized intensities for each signal, from which the error in r was derived. For the enriched material only the signal at δ_C 67.6 showed a significant ($> \pm 2\sigma$) intensity difference. It was increased to 34 ± 10 times the natural abundance intensity. The mean r value for the remaining carbons was 1.10 ($\sigma = 0.34$). Hence the probability (p_α) that the side-chain quaternary carbon is ^{13}C is $34 \times 0.011 = 0.38 \pm 0.12$. However, because of the high ^{13}C enrichment, the resonances of carbons directly bonded to the side-chain quaternary carbon showed ^{13}C–^{13}C spin–spin coupling effects. As the $\underline{C}H_2$ is a well-relaxed carbon, the total intensities of the satellites (I_s) and the central peak (I_c) of this resonance determine the probability that the side-chain carbon atom is ^{13}C, i.e. $p_\alpha = I_s/(I_s + I_c) = 0.44 \pm 0.05$. This value is more accurate than, but concordant with, that obtained from the intensity ratios. The value derived from the intensity ratios for p_α, the known [7] values for the ^{13}C probabilities of the ten other carbons that persist in try-[2-^{13}C] and the probabilities for ^2H, ^{15}N and ^{17}O, when inserted into equation 1 gives a $(M + 1)^+/(M)^+$ value of 0.779 ± 0.29 which is compatible with the value found by MS (1.178 ± 0.047).

Biosynthesis of the quinone ring of cochliodinol

The try-[2-^{13}C] was mixed with try-[1-^{14}C] (1.6 μCi/mmol) and the mixture added to a culture of *C. globosum*. The cochliodinol produced (**1**, R = 5-prenylindolyl-3, 0.033 μCi/mmol, 2% recovery) was converted [4] into its diacetate (0.04 μCi/mmol) to establish its radioactive purity. It was also oxidized [4] to the *cyclo*pentadieneone (**3**, R = 5-prenylindolyl-3, 0.028 μCi/mmol) thus indicating that the radioactive label resided in the oxygenated carbon atoms of the quinone ring (since all of the oxygenated carbon atoms are equivalent). The cochliodinol diacetate was hydrolysed [4] and the cochliodinol obtained oxidized [4] to cochliodinone (**2**, R = 5-prenylindolyl-3, 0.032 μCi/mmol). This procedure was necessary because 2,5-dihydroxy*cyclo*hexadiene-1,4-diones have abnormal MS [8] and ^{13}C NMR spectra [4].

MS of two samples of cochliodinone (**2**, R = 5-prenylindolyl-3) at natural isotopic abundance showed a $(M + 1)^+/(M)^+$ ratio of 0.365 ± 0.013 (calc. value for $C_{32}H_{28}N_2O_4 = 0.372$). The value of this ratio was 0.394 ± 0.014 as measured from the MS of ^{13}C-enriched cochliodinone. A Student's t test of the results showed that these ratios were different at the 99% confidence level, establishing the cochliodinol derived from try-[2-^{13}C] had a greater than natural abundance of ^{13}C ($2.7 \pm 1.7\%$). This result is consistent with the ^{14}C measurements. A similar result was obtained by comparison of the ions of m/e 27.9949 (^{12}CO requires 27.9949) and m/e 28.9983 (^{13}CO requires 28.9983) in the MS of the ^{13}C enriched sample of cochliodinone and the sample of natural abundance.

Resonance intensities in the [^1H] broadband decoupled ^{13}C NMR spectra of cochliodinone were normalized to the signal at δ_C 98.6, assigned [4] to the non-oxygenated carbon atoms of the lactone rings. When the normalized intensities of the signals in the spectrum of cochliodinone of natural abundance were compared (like signal with like) with those of the signals in the spectrum of this compound derived from try-[2-^{13}C] values of $r = 1$ were obtained for 9 of the 15 signals; those at δ_C 168 (C=O), 152 (=C—O—), 136, 132, 127 and 26 were > 1, (for other assignments see ref. [4]). However the intensity of the signal at δ_C 26 was uncertain because it was not completely resolved from one of the signals of the solvent; it was not therefore included in the analysis. The problem concerning which of these 5 remaining absorptions were significantly increased in intensity in the cochliodinone biosynthesized from try-[2-^{13}C] was solved by taking the 5 ratios and comparing them to all of the others. The calculations were then repeated with all combinations of 4 of the 5 signals, then 3 and so on. All comparisons that showed no significant differences were rejected. The only combination retained at the end of the analysis was that of the signals resonating at δ_C 168 (C=O) and 152 (=C—O—) compared to all the others. The statistics were: mean of the ratios r for the signals at δ_C 136, 135, 132, 128, 125, 124.5, 121, 112, 105, 99, 35, 18 = 1.04 ($\sigma = 0.28$); for the signals at δ_C 168 and 152 in the sample of cochliodinone enriched in ^{13}C, $r = 2.34$ ($\sigma = 0.98$). Thus there is a 90% probability that the oxygenated carbon atoms in cochliodinone (and hence cochliodinol) biosynthesized from try-[2-^{13}C] have been enriched. The $(M + 1)^+/(M)^+$ ratio calculated from the NMR data using equation 1 was 0.43 ± 0.05, which includes, within error, the value reported above from the MS measurements (0.394).



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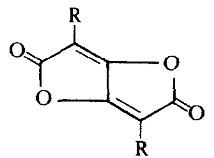

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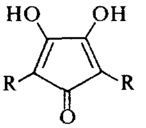

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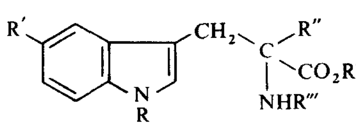

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DISCUSSION

Grove *et al.* [9] reported the incorporation of $Me^{13}CO_2^-$ into asperentin dimethyl ether by *Aspergillus flavus*. The spectra were normalized with respect to the mean intensities of the methoxyl carbon atoms and 'enrichment ratios' were calculated, by comparing the ratios of the normalized intensities. Tamm *et al.* [10] have studied the biosynthesis of the cytochalasins by a *Phoma* sp. from $^{13}CH_3CO_2^-$ and used the MS of the metabolites to determine the likely increase in intensity of the ^{13}C signals of the labelled carbon nuclei in their NMR spectra. The method used in this paper combines the ideas of earlier workers [9, 10] and reduces the NMR and MS results to ratios that are comparable. In addition, a statistical treatment of the comparison of measurements of ^{13}C abundance in normal compounds and those thought to be enriched in this isotope is introduced into the calculations. It is likely that the detection of small enrichments of ^{13}C will require considerable refinement of this statistical treatment.

Despite the facts that cochliodinone was unstable under the conditions of the NMR experiment, that the incorporation of the ^{13}C label was 2%, that the nuclei enriched were poorly relaxed and that only about 20 mg of material was available, the NMR, MS and degradation chemistry results, all indicated that DL-tryptophan-[1-^{14}C] and try-[2-^{13}C] were incorporated into the oxygenated carbon atoms of the benzoquinone ring of cochliodinol, and to about the same extent. Mosbach [11] has suggested, on indirect evidence that this reaction also occurs in *Erwinia* spp. where the side-chain of phenylalanine is incorporated into the lactone rings of pulvinodilactone (**2**, R = C_6H_5); polyporic acid (**1**, R = C_6H_5) being regarded as an intermediate. Recently, an isomer of cochliodinol, asterriquinone (**1**, R = 1-prenylindolyl-3, 12) has been reported to be produced by *Aspergillus terreus*. It appears, therefore, that the enzyme systems capable of catalysing this remarkable reaction are common in fungi, and that they might not be fastidious with respect to the substrates they accept. However, we could obtain no evidence that the tryptophan analogues (**4**, R′ = R″ = R‴ = R⁗ = H, R = Me; **4**, R = R″ = R‴ = R⁗ = H, R′ = $C_6H_5CH_2OCO$) were used by *C. globosum* for the production of 2,5-dihydroxy-*cyclo*hexadiene-1,4-diones.

EXPERIMENTAL

All MS were measured on samples introduced as solids into the source. Isotope ratios were determined by repeatedly scanning the appropriate part of the spectrum under conditions (determined on a previously run sample) such that the relative abundance of ions remained steady during the scans and least affected by slight variations in ionization conditions. $(M + 1)^+/(M)^+$ ratios of carbon monoxide were measured at an estimated resolution of 1:2500. Exact mass measurements (±3 ppm) were obtained by the peak matching method using an appropriate ion in the MS of perfluorokerosene as a standard. ^{13}C NMR spectra were obtained using a Varian XL-100 pulse Fourier transform spectrometer equipped with a Varian 620-L computer. Conditions were: frequency 25.16 MHz, spectral band width 5120 Hz, aquisition time 0.8 sec (tryptophan derivative), 3.2 sec (cochliodinone), flip angle 40°, temp. 30°, proton noise decoupling field strength ($\gamma H_2/2\pi$) *ca* 3800 Hz, noise band width 3 KHz, solvent tetrahydrofuran-[$^2H_8$], concns *ca* 60 mg/ml (tryptophan derivative in 12 mm diameter microcell), 40 mg/ml (cochliodinone in 5 mm diameter tube), low field signal (^2H) of the solvent used for pulse lock, time constant for weighting the free induction decay 0.8 sec. All chemical shifts refer to TMS as an int. stand. The radioactivity of tryptophan samples was obtained by converting the amino acid into its acetyl derivative [13]. All determinations were done using samples dissolved in toluene (15 ml) containing 2,5-diphenyloxazole (60 mg). Identities were established by comparison of IR spectra and NMR spectra. Mps are not corr. Statistical calculations were performed on a Control Data 6400 computer, using programmes written in the beginners all-purpose symbolic instruction code (BASIC).

Production of cochliodinol. Inocula were prepared from slants of *Chaetomium globosum* (HLX 707)* on 2% malt agar. Cultures were grown in 2 l. Erlenmeyer flasks, each flask containing 1 l. of the following medium (g/l.): vitamin-free amino acids (Difco), 4; K_2HPO_4, 1; $MgSO_4 . 7H_2O$, 0.5; KCl, 0.5; sucrose, 30; $FeSO_4 . 7H_2O$, 0.01; thiamin hydrochloride, 0.0001; niacin, 0.0001; calcium pantothenate, 0.0001; riboflavin, 0.0001; pyridoxin, 0.0001; folic acid, 0.0001; *p*-aminobenzoic acid, 5×10^{-5}; inositol, 0.003; vitamin B_{12}, 4×10^{-6}. All cultures were grown for 7 days at 25° on a shaker. Each flask described a circle in a horizontal plane of diameter 5.6 cm at 220 rpm. Aq. solns of hydrochlorides of the precursors were sterilized by filtration and added to the cultures aseptically after 3.5 days growth. Cochliodinol (**1**, R = 5-prenylindolyl-3, 130 mg/l.) and cochliodinone (**2**, R = 5-prenylindolyl-3, 1 mg/l.) were isolated from the lyophilized mycelium as previously described [14].

Preparation of precursors. DL-5-Benzyloxytryptophan (**4**, R = R″ = R‴ = R⁗ = H, R′ = $C_6H_5CH_2OCO$) was synthesized in 70% yield by the method of ref. [15] and DL-1-methyltryptophan in 80% yield as described in ref. [16].

DL-*Tryptophan-*[*2-*^{13}C]. A soln of CH_2N_2 (0.8 g) in Et_2O (100 ml) was added slowly, beneath the surface of a soln (20 ml) of malonic-[2-^{13}C] acid (58 atom %, Merck, Sharpe and Dohme, 1 g) in Et_2O. After 10 min the soln was evapd through a short Fenski column. The residue was dissolved in a soln (2 ml) of dry NaAc (0.2 g) in HOAc, and a soln (5 ml) of $NaNO_2$ (1.65 g) in H_2O added. The reaction mixture was kept for 4 hr at 30–40°, extracted with Et_2O, the extract washed with satd NaCl soln, dried (Na_2SO_4) and evapd. The residue (1.35 g, mp 55–65°) in formic acid (98%, 15 ml) was heated to 80° and Zn dust (2.5 g) added, keeping the exothermic reaction mixture at 90–100°. After 2 hr at 100°, the mixture was filtered hot, the Zn salts washed with hot formic acid and the filtrate and washings evapd. The residue (1.4 g, mp 80–82°) was dissolved in toluene (50 ml) and 20 ml distilled, when a soln (50 ml) of gramine (1.6 g) in toluene was added. The soln, concd to 30 ml, was treated with a suspension of NaOH (0.1 g) in toluene and the solvent allowed to distil slowly while the vol. of the reaction mixture was kept at about 40 ml by continuous addition of toluene. When dimethylamine could not be detected in the distillate (4 hr), the reaction mixture was concd to 20 ml, cooled, EtOAc (100 ml) added, the soln extracted with dil. HCl, washed with H_2O, dried and evapd. The residue (**4**, R = R′ = H, R″ = CO_2Me, R‴ = CHO, R⁗ = Me, 0.96 g, mp 185–189°) suspended in 10% NaOH soln (5 ml) was heated under reflux in N_2 for 18 hr. HOAc (4 ml) was added, the mixture heated under reflux for 2 hr, cooled to 20°, filtered and kept at 4° for 24 hr. The DL-tryptophan-[2-^{13}C] (0.38 g, 19% overall, mp 278–280°) was collected and recrystallized from H_2O (0.35 g, mp 280–281°). Cochliodinone (**2**, R = 5-prenylindolyl-3, 39 mg) and the *cyclo*pentadieneone (**3**, R = 5-prenylindolyl-3, 9 mg) were prepared as described [4] from cochliodinol (**1**, R = 5-prenylindolyl-3, 130 mg).

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