Pyrones. VIII.[1] Biosynthetic investigations of the fungal metabolite phacidin

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Biosynthetic feeding experiments with singly and doubly ^{13}C-labelled acetate show that phacidin (4-methoxy-6-nonanoyl-2*H*-pyran-2-one-3-carboxaldehyde) is formed from a heptaketide precursor. A similar feeding with sodium ^{13}C-formate has shown that the methoxyl and aldehyde carbons are derived from the one-carbon pool.

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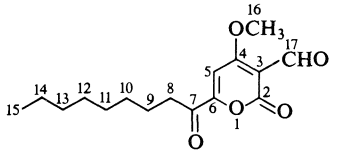
Des expériences d'alimentation biosynthétique avec un acétate marqué avec un ou deux ^{13}C révèlent que la phacidine (méthoxy-4 nonanoyl-6 2*H*-pyranone-2 carbonaldéhyde-3) se forme à partir d'un précurseur heptacétide. Une alimentation analogue avec le formate ^{13}C de sodium montre que les carbones du méthoxy et de l'aldehyde proviennent de sources contenant un seul carbone.
[Traduit par le journal]

Introduction

Phacidin, 4-methoxy-6-nonanoyl-2*H*-pyran-2-one-3-carboxaldehyde **1**, is an antibiotic (1, 2)



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metabolite isolated (2) from cultures of the fungus *Potebniamyces balsamicola* Smerlis var. *boycei* Funk, whose synthesis we have recently reported (3). During our investigations, we were desirous of obtaining further supportive evidence of the structure of phacidin and information relating to its possible mode of formation by the fungus, and thus undertook to perform some simple biosynthetic experiments. The biogenetic origin of the carbon skeleton of a natural product can now be determined routinely in favourable cases by the use of ^{13}C-enriched precursors (4). Since phacidin possessed many of the characteristics of an acetogenin (polyoxygenated ring, linear carbon skeleton, long saturated side-chain, etc.), we commenced our investigations with the feeding of singly- and doubly-labelled sodium acetate.

Results and discussion

In order to test the predictions concerning the biosynthesis of a metabolite, the position of the labelled atom(s) in the product must be determined. The use of carbon-13 magnetic resonance, combined with the use of carbon-13 enriched precursors, has greatly simplified biosynthetic studies of secondary metabolites since chemical degradation of the product becomes unnecessary. Accurate assignment of the ^{13}Cmr spectrum is, however, a necessity, since any misassignment may lead to false assumptions and/or conclusions about the biosynthesis.

(A) Assignment of ^{13}Cmr spectrum of phacidin

Many 4-oxy-2-pyrones are known, and the difficulties encountered in accurately assigning resonances due to C-2, C-4, and C-6 have been discussed (5). Indeed, one of the original ^{13}C-labelled biosynthetic investigations of an α-pyrone (radicinin, **2** (6)) had to be repeated and the resonances reassigned (7). Similar studies such as the assignment (8) and reassignment (9) of the ^{13}Cmr spectrum of Aurovertin B **3** have further emphasized the need for caution when analyzing the spectra of 2-pyrones. Considerable care was therefore taken in the assignment of the ^{13}Cmr spectra of phacidin and its derivatives.[4]

An examination of the chemical shift values of a variety of 4-oxypyrones (see Table 1) indicates that the assignment of C-4 is straightforward, since it is consistently the most downfield signal as compared to the other ring carbons. This same trend also holds for most hydroxycoumarins (e.g. compound **12**). It has also been shown that for 2-pyrones the magnitude of the two-bond spin–spin coupling constant between C-2 and a proton at C-3 (H-3) is less than that of C-6 to H-5 or to protons on a side-chain at C-6 (12), resulting in C-6 normally

[1]For Part VII, see ref. 16.

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[4]Copies of ^{13}Cmr spectra of phacidin (natural abundance, sodium 1-^{13}C and 1,2-$^{13}C_2$-acetate derived, and sodium 1-^{13}C-formate derived) and of the ^{1}Hmr spectrum of phacidin (^{13}C-formate derived) have been placed with the Depository of Unpublished Data, CISTI, National Research Council of Canada, Ottawa, Ont., Canada K1A 0S2.

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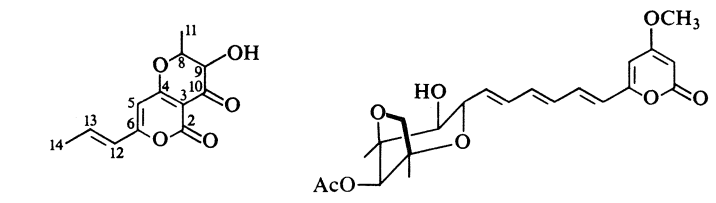

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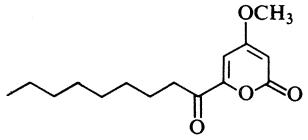

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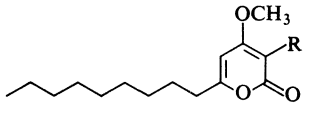
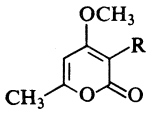
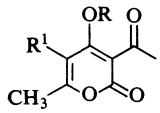


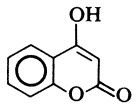
5, R = CHO
6, R = H



7, R = H
11, R = Br



8, R = R¹ = H
9, R = CH₃, R¹ = H
10, R = CH₃, R¹ = Br



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appearing as a multiplet, while C-2 remains as a singlet or doublet.

The signals in the ^{13}Cmr spectrum due to the ring carbons of phacidin were thus assigned as follows:

(*i*) δ 172.9 (m), C-4, being the most deshielded ring carbon.

(*ii*) δ 160.2 (d, $^{3}J_{C-H}$ = 2.5 Hz), C-2. The chemical shift range of C-2's of similar compounds was approximately δ 160–165 ppm, and the analogous compound **5** showed a similar three-bond coupling constant of 3.3 Hz.

(*iii*) δ 158.5 (d, $^{2}J_{C-H}$ = 1.4 Hz), C-6. The chemical shifts for C-6 of a variety of analogous compounds had a relatively wide range of δ 154–175. The two extremes of this range can be represented by compounds **5** (174.4) and **4** (155.2), consistent with the donor/acceptor nature of the carbonyl substituents and its transmission via the conjugated system (13). The carbon α to the carbonyl experiences an increase in electron density (shielding), while those β or δ to it show a decrease (deshielding). In phacidin, the C-6 carbon is influenced by both the aldehyde substituent at C-3 (deshielding on C-6) and the directly attached nonanoyl group (shielding at C-6); the latter is expected to dominate, and phacidin therefore shows the C-6 resonance at δ 158.5, shielded with respect to its analogue **6** which is lacking an aldehyde substituent at C-3 (i.e. no C-17), and which has the resonance at δ 165.8 ppm.

TABLE 1. ^{13}Cmr spectra of 4-oxy-2-pyrones

Compound	C-2	C-3	C-4	C-5	C-6	Reference
1	160.2	105.9	172.9	97.0	158.5	3
2	156	97.8	174.7	98.2	162.5	7
3	163.4	88.6	170.4	107.4	154.1	9
4	162.5	93.7	170.2	103.8	155.2	3
5	162.2	102.1	174.6	94.3	174.4	3
6	165.0	87.5	171.3	99.6	165.8	3
7	164.6	87.3	171.4	100.3	162.1	10
8	161.2	99.9	181.1	101.4	169.2	10
9	161.8	105.1	170.4	95.2	166.9	10
10	159.5	100.0	178.2	98.3	166.9	10
11	160.8	88.4	166.9	95.2	162.9	10
12	161.8	91.0	165.5	115.7	153.4	11

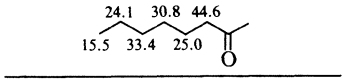
(*iv*) δ 105.9 (dd, $^{2}J_{C-H}$ = 24.5 Hz, $^{3}J_{C-H}$ = 4.2 Hz), C-3. In the series of analogous compounds, C-3 resonated at δ 88–105 ppm. The derivative **5** likewise showed C-3 at δ 102.1 (dd, $^{2}J_{C-H}$ = 23.9 Hz, $^{3}J_{C-H}$ = 4.2 Hz), consistent with this assignment.

(*v*) δ 97.0 (d, $^{1}J_{C-H}$ = 175.3 Hz), C-5. The assignment of C-5 was trivial, since it was the only carbon directly bonded to proton. Normal chemical shift ranges and coupling constants are δ 95–108 and $^{1}J_{C-H}$ = 170–176 Hz.

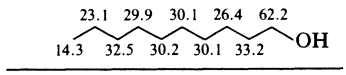
In addition, it was a trivial matter to assign the nonanoyl ketone C-7 (δ 193.5, m), the 3-carboxaldehyde C-17 (δ 186.3, d, $^{1}J_{C-H}$ = 186.2 Hz), the methoxyl C-16 (δ 58.4, q, $^{1}J_{C-H}$ = 148.4 Hz), and the methylene α to the ketone, C-8 (δ 38.3, t, $^{1}J_{C-H}$ = 123.6 Hz). The remaining carbons of the nonanoyl side chain were assigned by comparison with the assignments of 2-octanone and 1-decanol (14) in Table 2.

TABLE 2

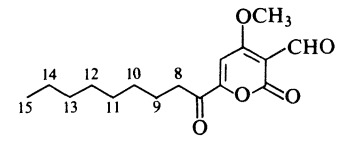
(*a*) Assignment of 2-octanone



(*b*) Assignment of 1-decanol



(*c*) Assignment of nonanoyl ketone*



Carbon	δ (ppm)	$^1J_{C-H}$ (Hz)	Multiplicity
15	14.1	124.6	q
14	22.7	127.5	t
13	31.8	125.5	t
12	29.3	125.1	t
11	29.1	125.1	t
10	29.0	125.1	t
9	23.1	127.5	t
8	38.3	123.6	t

*We were unable to distinguish C-10, C-11, and C-12 since their chemical shift values were only very slightly different from each other.

(B) Sodium acetate-1-^{13}C labelling experiment

Labelled sodium acetate was fed to growing cultures of *P. balsamicola* and the distribution of the ^{13}C label in the resulting phacidin determined according to the method of McInnes *et al.* (15), by comparing the ^{13}Cmr spectra of natural abundance and enriched compounds recorded under identical instrument parameters. In order to make the comparisons more valid, the carbons to be compared should have similar substituents. Since it was known, *vide infra*, part D, that the aldehyde and methoxyl carbons were not acetate-derived, they were used as reference signals to which the unsaturated (C-2 to C-7) and saturated (C-8 to C-15) were compared.

The results (Table 3) indicate that C-2, C-4, C-6, C-8, and C-14 have been enriched by the feeding of sodium acetate-1-^{13}C to *P. balsamicola*; it is obvious also that C-10 and C-12 are similarly labelled.

(C) Sodium acetate-1,2-$^{13}C_2$ labelling experiment

P. balsamicola was fed sodium acetate-1,2-$^{13}C_2$ in the same manner and for the same duration as described in the above labelling experiment. The ^{13}Cmr spectrum of labelled phacidin clearly showed the presence of coupled ^{13}C–^{13}C units (see Table 4) demonstrating the utilization of intact acetate units in the biosynthesis. In addition, the labelling pattern corroborated the chemical shift assignments discussed above.

Intact incorporation of acetate units into the phacidin skeleton was demonstrated for C-2—C-3, C-4—C-5, C-6—C-7, C-8—C-9, C-12—C-13, C-14—C-15, and was assumed for C-10—C-11, thereby indicating its probable biogenesis from an hepta-acetate precursor as indicated below.

(D) Sodium formate-1-^{13}C labelling experiment

P. balsamicola cultures were fed labelled sodium formate as described below, with the incorporation of ^{13}C in the methoxy (C-16) and aldehyde (C-17) groups clearly being indicated by spectral examination. The extent of incorporation was so large in these two positions that it was readily detectable in

TABLE 3. Calculation of incorporation of sodium acetate-1-^{13}C into phacidin

Carbon	$R = I_N^*/I_S$	$R^1 = I_L/I_S$	$r = R^1/R$	$\%^{13}C = rf$†	Error‡
2	0.388	0.534	1.38	2.47	0.23
3	0.351	0.176	.501		
4	0.360	0.608	1.69	3.03	0.19
5	1.10	0.929	0.876		
6	0.238	0.273	1.15	2.06	0.28
7	0.435	0.208	0.478		
8	1.06	2.54	2.40	2.17	0.031
9	0.923	1.17	1.27		
10, 11, 12	1.48	4.88	3.30	2.99	0.022
13	0.752	0.918	1.22		
14	0.775	2.64	3.41	3.09	0.022
15	0.521	0.617	1.18		

*I_N = peak height in spectrum of unlabelled compound; I_L = peak height in spectrum of labelled compound; I_S = peak height of standard carbon; C-16 used as standard for aliphatic (C-8 to C-15) carbons, C-17 for unsaturated (C-2 to C-7) ones.

†f = factor required to scale average r for unlabelled carbons to natural abundance.

‡Error = standard deviation (r) × average enrichment (r) ÷ r.

TABLE 4. The $^{13}C-^{13}C$ spin–spin coupling constants of phacidin and radicinin (7)

Phacidin (1)*			Radicinin (2)	
Carbon	δ	$^1J_{C-C}$	Carbon	$^1J_{C-C}$
2	160.2	78.7	2	80
3	105.9	78.2	3	80
4	172.9	58.9	4	62
5	97.0	58.8	5	62
6	158.5	59.4	6	64
7	193.5	59.4		
8	38.3	34.8	8	40
9	23.1	34.8	9	42
10	29.2	†	10	43
11	29.3	†	11	†
12	29.1	34.7	12	64
13	31.8	34.7	13	42
14	22.7	34.6	14	†
15	14.1	34.7		

*Kindly recorded by Dr. A. G. McInnes and co-workers at the Atlantic Regional Laboratory, National Research Council of Canada, Halifax.
†Values not obtained due to overlapping of signals.

the 90 MHz ^{1}Hmr spectrum: the methoxy protons appeared as a singlet at 4.14 ppm (^{12}C—H) and a doublet centred at 4.15 ppm (^{13}C—H, ^1J = 148 Hz), the ratio of intensities indicating 29% ^{13}C incorporation, while the aldehyde proton appeared as a singlet (10.85 ppm, ^{12}C—H) overlapped by a doublet centred at 10.84 (^{13}C—H, ^1J = 188 Hz), the ratio of intensities indicating 36% ^{13}C incorporation. This was substantiated further by the 62.8 MHz ^{13}Cmr spectrum[5] which showed dramatically increased intensities of the 186 (aldehyde) and 58 ppm (methoxyl) carbons as compared to the remaining signals. The methoxy and aldehyde carbons thus do not originate from an acetate-derived precursor, but from the C-1 metabolic pool.

(E) Possible biogenesis

The above feeding experiments indicate that phacidin can be formed from seven acetate units with two one-carbon insertions. This may occur via an elaboration of an enzyme-bound caprate (e.g. **13**) produced by normal fatty acid routes to a fourteen-carbon unit (**14**) which may then undergo *O*-cyclization to give a pyrone derivative. The sequence of the other necessary stages, that is, one-carbon introduction and oxidation at C-7, cannot, of course, be determined without much more extensive investigation (see Fig. 1).

Experimental

Carbon magnetic resonance spectra were obtained on Nicolet TT-14 (15.1 MHz, Victoria), Bruker WP250 (62.8 MHz, Bruker Canada Ltd., Mississauga) and Varian XL100/15 (25.2 MHz, Atlantic Regional Laboratory, Halifax) instruments in $CDCl_3$, and are referenced to internal TMS.

[5]Kindly run by Dr. C. Roger, Bruker Spectrospin Canada, Mississauga, Ont., Canada L5L 1J9.

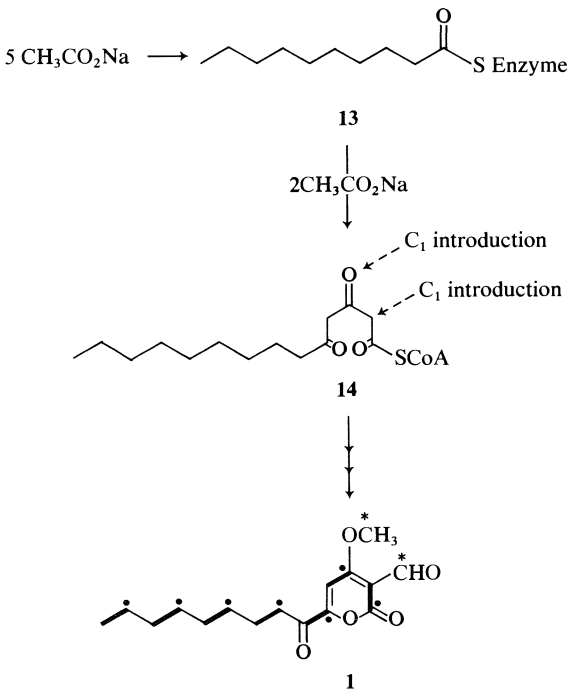


FIG. 1. Feeding of $CH_3{}^{13}\dot{C}O_2Na$, $^{13}CH_3$—$^{13}CO_2Na$, and H—$^{13}CO_2Na$ to *P. balsamicola*; possible biosynthetic route.

Sodium acetate-1-^{13}C feeding

Cultures of *P. balsamicola* were grown at the Pacific Forest Research Centre by A. Funk. They were allowed to grow for two weeks, unshaken, at 15°C in 75 mL of 5% malt extract in water. Sodium acetate-1-^{13}C (100 mg, 90% enriched, Stohler Isotope Chemicals) in water (5 mL) was added and the cultures allowed to grow for a further six weeks. Labelled phacidin was then isolated by benzene extraction, and purified by recrystallization from cyclohexane.

Sodium acetate-1,2-$^{13}C_2$ feeding

Cultures were grown in a manner similar to that detailed above, and sodium acetate-1,2-$^{13}C_2$ (100 mg, 90% ^{13}C-enriched, Stohler Isotope Chemicals) added in a similar fashion. Phacidin was isolated by benzene extraction and purified by recrystallization.

Sodium formate-1-^{13}C feeding

Cultures were grown in a manner similar to that detailed above. After 10 weeks, labelled sodium formate (90% enriched, Stohler Isotope Chemicals) was added (304 mg in four portions) over a period of two weeks. Blank feedings using unlabelled sodium formate were also carried out. After two further weeks, phacidin was isolated by hot 1,2-dichloroethane extraction and purified by recrystallization.

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