Phytochemistry, 1978, Vol 17, pp 1743–1745 © Pergamon Press Ltd. Printed in England. 0031-9422/78/1001-1743 $02.00/0

A ^{13}C NMR STUDY OF THE BIOSYNTHESIS OF THE ANTHRAQUINONE DOTHISTROMIN BY *DOTHISTROMA PINI*

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(*Received* 21 *October* 1977)

Key Word Index—*Dothistroma pini*; Sphaeropsidales; fungi imperfecti; biosynthesis; ^{13}C labelling; acetate–polymalonate; dothistromin; bistetrahydrofuranoanthraquinone.

Abstract—A ^{13}C-NMR study of the biosynthesis of dothistromin by *Dothistroma pini* was undertaken. The biosynthetic labelling pattern in this bistetrahydrofuranoanthraquinone was consistent with the incorporation of 9 intact acetate units.

INTRODUCTION

The phytotoxin, dothistromin (1) is the major metabolite elaborated by cultures of *Dothistroma pini* [1–4] and it has been shown, using chemical, spectroscopic, [2, 3] and crystallographic evidence [5, 6], to have a bistetrahydrofuranoanthraquinone structure. More recently dothistromin has been isolated from cultures of *Cercospora smilacis* [7].

Several biosynthetic schemes [8–10] leading to the dothistromin carbon skeleton have been proposed by virtue of its structural similarity to a possible intermediate in the biosynthesis of sterigmatocystin [9] and the aflatoxins, e.g. aflatoxin B_1 [9]. In the absence of any biosynthetic studies leading to bistetrahydrofuranoanthraquinones [8] and because of their possible involvement as intermediates in these other pathways, we have applied the ^{13}C labelling technique to investigate the biosynthesis of this metabolite.

RESULTS AND DISCUSSION

To elucidate the most favourable culture conditions for ^{13}C enriched precursor incorporation, a preliminary study using sodium acetate-[1-^{13}C] and -[2-^{13}C] was undertaken.

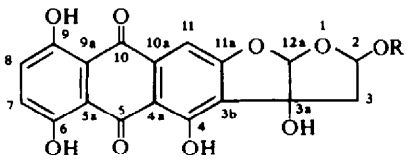
In 100 ml submerged malt cultures of *D. pini* (see Experimental), dothistromin production, as determined spectrophotometrically, was initiated about 76 hr after spore inoculation. The rate of synthesis was observed to increase most rapidly between 110 and 170 hr after spore inoculation.

From the addition of sodium acetate-[1-^{14}C] and sodium acetate-[2-^{14}C] at various times after the start of dothistromin synthesis and at a concentration of 12 mM (1 mg/ml), the lowest isotopic dilution values were observed when these precursors were added to the cultures 86 hr after spore inoculation. Results are summarized in Table 1.

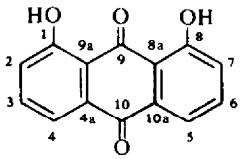
Table 1. Incorporation of radioactivity from sodium acetate-[1-^{14}C] and -[2-^{14}C] into dothistromin by *Dothistroma pini*

Labelled precursor	Hr after spore inoculation	Isotopic dilution value*	Concentration of dothistromin (mg/flask)
NaOAc-[1-^{14}C] (12 mM)	86	—	0.5
	118	34.0	5
	144	27.4	9
	164	23.2	10.7
	174.5	30.9	11.3
	198.5	32.6	11.6
	222.5	34.6	12.1
NaOAc-[2-^{14}C] (12 mM)	86	—	0.4
	118	17.7	4.8
	144	15.2	8.8
	164	13.1	10.6
	174.5	16.5	11.2
	198.5	18.4	11.5
	222.5	19.5	12.0

* Isotopic dilution value = $\frac{\text{Molar specific radioactivity of precursor}}{\text{Molar specific radioactivity of product}}$



(1) R = H
(2) R = Et



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Based on these experiments, ^{13}C enriched dothistromin was prepared, in separate experiments, by supplementing 100 ml submerged cultures of *D. pini* with 100 mg sodium acetate-[1-^{13}C] (90 atoms %^{13}C) and sodium acetate-[2-^{13}C] (90 atoms %^{13}C) per flask, 86 hr after spore inoculation of the culture medium. Following a further 80 hr incubation, isotopically enriched dothistromin was exhaustively extracted with ethyl acetate, purified by TLC and converted to the ethyl acetal (2). Under these conditions, yields of purified dothistromin were consistently 10–12 mg per flask.

The assignments of ^{13}C-NMR chemical shifts in dothistromin ethyl acetal (Table 2) were based on results from substituent calculations [11], off-resonance proton decoupling experiments, specific single frequency proton decoupling experiments and from a comparison of carbon chemical shift data measured for 1,4-dihydroxy-, 1,8-dihydroxy- and 1,3-dimethoxy-2-methylanthraquinone (Table 3) and from comparable naturally occurring metabolites.

The aliphatic side-chain C-3 (43.7 ppm) and C-3a (85.5 ppm) were readily assigned from off-resonance proton decoupling experiments. C-12a (117.5 ppm) was shifted to higher field because of the shielding effect of the two furan oxygen substituents and was assigned from off-resonance proton decoupling results and by comparison with the analogous carbon in the bistetrahydrofurano side-chain of sterigmatocystin (113.1–113.3 ppm) [12, 13], 6-methoxysterigmatocystin (113.3 ppm) [12] and aflatoxin B_1 (113.6 ppm) [14]. The hemiacetal C-2 (106.2 ppm) was distinguished from C-11 (103.4 ppm) by specific single frequency proton decoupling experiments. The aromatic C-3b (123.3 ppm), C-4 (159.9 ppm), C-10a (136.1 ppm) and C-11a (165.6 ppm) were all identified by comparison of similar carbon chemical shifts in 1,3-dimethoxyanthraquinone and substituent shift calculations. The two high field resonances at 185.1 ppm and 188.6 ppm were assigned to C-10 and C-5, respectively, based on results for analogous carbons in 1,8-dihydroxyanthraquinone (3).

The remaining 7 carbons, namely 6,9; 7,8 and 4a, 5a, 9a were grouped according to the carbon chemical shifts observed in 1,4-dihydroxy- and 1,8-dihydroanthraquinone. Unfortunately unequivocal assignment of these carbons was not possible.

The ^{13}C labelling pattern found for dothistromin (Table 2) was consistent with the condensation of 9 intact acetate units. Interestingly, the unique labelling pattern revealed in the bistetrahydrofurano side-chain (Scheme 1) is identical to that found in both sterimatocystin [13] and aflatoxin B_1 [14]. Consistent with these findings, the ^{13}C signals due to isotopic enrichment of

Table 2. ^{13}C labelling and chemical shift data for dothistromin ethyl acetal (2)*

Carbon number	Chemical shift(δ_c) sodium acetate-[1-^{13}C]	sodium acetate-[2-^{13}C]
2		106.2
3	43.7	
3a		85.5
3b		123.3
4a, 5a, 9a	112.0	111.6, 111.1
4	159.9	
5	188.6	
6, 9	156.4	157.3
7, 8	129.1	129.6
10	185.1	
10a	136.1	
11		103.4
11a	165.6	
12a	117.5	
13 (62.6)		
14 (15.2)		

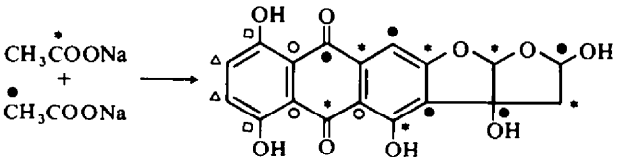
* Proton noise decoupled F.T. ^{13}C NMR spectra were obtained from 0.2 mmol DMSO-d_6 solutions. ^{13}C enrichments were *ca* 5 times natural abundance. Chemical shifts were measured relative to internal DMSO-d_6 but are reported relative to TMS using the realationship $\delta_{ext\,TMS} = \delta_{DMSO} - 39.5$ ppm.

Table 3. ^{13}C chemical shifts of some anthraquinones*

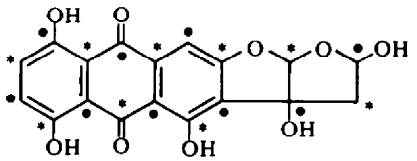
Compound	Carbon number† 1	2	3	4	4a	5	6	7	8	8a	9	9a	10	10a
Anthraquinone	127.0	133.9			133.3						182.2			
1,4-Dihydroxy-	156.7	129.4	129.4	156.7	112.7	126.7	135.1	135.1	126.7	132.9	186.7	112.7	186.7	132.9
1,8-Dihydroxy-	161.4	119.4	137.5	124.4	133.3	124.4	137.5	119.4	161.4	116.0	181.3	116.0	192.1	133.3
1,3-Dimethoxy-2-methyl-	160.6	120.4	163.0	105.1	135.3[a]	126.9[b]	133.3[c]	133.4[c]	127.5[b]	134.8[a]	185.4[d]	129.1	181.3[d]	133.0[a]

* ^{13}C NMR spectra were obtained from DMSO-d_6 solutions; shifts reported relative to TMS; a, b, c, d: assignments may be reversed.

† See structure 3 for carbon numbering.



Scheme 1. The experimental ^{13}C labelling pattern for dothistromin. △, ○, □—absolute assignments in doubt.



Scheme 2. The proposed labelling pattern based on biosynthetic schemes for sterigmatocystin and aflatoxin B_1.

C-3a and C-3b satellite resonance showed ^{13}C-^{13}C spin-spin coupling. The coupling constant of 50 Hz was similar to that observed previously and confirmed the assignment of these two carbons.

The discernible labelling pattern in the anthraquinone moiety strongly suggests a biosynthetic pathway involving an acetate–polymalonate intermediate. If this deduction is correct, then the labelling pattern is consistent with that predicted (Scheme 2) for a possible substituted bistetrahydrofuranoanthraquinone intermediate leading to sterigmatocystin or aflatoxin B_1. These proposed biosynthetic schemes have recently been reviewed [15].

EXPERIMENTAL

Mps were taken on a Kofler hostage and are uncorr. PFT-^{13}C-NMR spectra were recorded on either a Varian XL100 (25.2 MHz) or JOEL HNM FX60 (15.04 MHz) spectrometers.

Culture. The fungus, *Dothistroma pini* Hulbary (ATCC No. 26810), was obtained from cultures held at the Forestry Research Institute, Rotorua, New Zealand. The culture was maintained at 18° on solid growth media consisting of 5% dried malt extract and 2% agar in H_2O. A spore inoculum for submerged culture was obtained by flooding a Petri dish culture with sterile H_2O and transferring the spore suspension to 250 ml Erlenmeyer flasks containing 100 ml of the same medium without agar. The cultures were shaken at 18° on a shaker platform (*ca* 200 rpm).

Isotopic incorporation studies. (a) Sodium acetate-[^{14}C]. For radiochemical analysis of ^{14}C-dothistromin, 3 ml culture medium was extracted with EtOAc (2 × 6 ml) and purified by TLC on Si gel developed with EtOAc–$CHCl_3$ (6:4) (R_f, 0.4). The product was made up to 3 ml in EtOH and the concn calculated from the visible absorption maximum at 490 nm. λ_{max}^{EtOH} nm (log ε): 230 (4.28), 255 (4.04), 268 (4.08), 280 (4.14), 478 (3.91) 490 (3.92), 508 (3.83) and 523 (3.76).

The specific activity was determined by scintillation counting using a 1,4-dioxan based scintillation solvent (7 g PPO, 0.05 g POPOP, 50 mg naphthalene per l. of 1,4-dioxan). Counting efficiencies were measured with hexadecane-[^{14}C] as internal standard.

(b) Sodium acetate-[^{13}C]. To each of 10 submerged cultures was added 100 mg of NaOAc-[1-^{13}C] or -[2-^{13}C] (90 atoms $\%^{13}C$), *ca* 86 hr after spore inoculation. After a further 80 hr incubation, the culture medium was exhaustively extracted with EtOAc (3 × 150 ml) and the combined extracts were dried over Na_2SO_4, filtered and evapd *in vacuo*. Labelled dothistromin was purified as above. Yield 10–12 mg per flask.

Dothistromin ethyl acetal (2). To a soln of dothistromin (50 mg) in dry EtOH (50 ml) was added a catalytic amount of $SOCl_2$ and stirred at room temp. for 3 hr. The residue, after evapn of the solvent, was chromatographed as above (R_f, 0.75). Yield 15 mg. The sample was crystallized from EtOH, mp 203–205°. Lit. [3] 197–210°. Found: MS, 400.0793. $C_{20}H_{16}O_9$ requires 400.0794. MS, *m/e* (rel. int.): 400 (22) (M^+), 371 (22), 355 (6), 325 (10), 309 (6), 299 (100), 283 (10), 272 (10).

Acknowledgements—The authors with to thank Dr. K. W. Jolley (Massey University) and Mr. E. Sokoloski (N.I.H.) for the ^{13}C-NMR spectra.

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