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^{13}C AND ^{2}H LABELLING STUDIES ON THE BIOSYNTHESIS OF SCYTALONE IN *PHIALAPHORA LAGERBERGII*

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Abstract—The regio- and stereospecificity of incorporation of label from [$^{2}H_3$] acetate into scytalone, a dihydronaphthalene metabolite of *Phialaphora lagerbergii*, has been determined by high field ^{1}H and ^{2}H NMR studies. Incorporation studies with [2-^{13}C] malonate have failed to reveal an acetate "starter" effect suggesting that scytalone may be derived from a hexaketide precursor rather than a pentaketide as previously proposed.

Scytalone (**1**) is a dihydronaphthalene metabolite produced by a number of fungi including a *Scytalidium sp.*,[1] *Phialaphora lagerbergii*,[2] and *Verticillium dahliae*[3] in which it has been shown to be an intermediate on the pathway to fungal melanins.[4] A number of biosynthetic studies using ^{13}C and ^{2}H labelled precursors have been reported. Incorporation of singly labelled ^{13}C-acetates suggested a pentaketide origin for scytalone,[2] and studies with [1, 2-$^{13}C_2$]acetate[5,6] showed a randomisation of ^{13}C–^{13}C couplings consistent with scytalone being biosynthesised *via* a symmetrical intermediate, 1,3,6,8-tetrahydroxynaphthalene (**2**), as indicated in the scheme, and this compound can indeed be converted *in vitro* to scytalone by sodium borohydride reduction.[7] Incorporation of [2-^{13}C, $^{2}H_3$]acetate and examination of both proton noise-decoupled and ^{2}H noise-decoupled spectra indicated that ^{2}H was incorporated at C-4 and C-5 only.[8] Surprisingly no ^{2}H incorporation could be observed at C-2 or C-7. In order to obtain more information on the stereospecificity of labelling at C-4 and to study further the question of labelling at C-2 and C-7 we have used the more sensitive approach of direct ^{2}H NMR. We have also studied the incorporation of ^{13}C-malonate to try to obtain more information on the nature of the assembly pattern of the precursor polyketide chain.

A necessary prerequisite of any stable isotope labelling study is an unambiguous assignment of the NMR spectrum. The ^{1}H NMR spectrum of scytalone had not been rigorously assigned and indeed at normal field strengths the signals due to the C-2 and C-4 methylenes appear as complex overlapping, non-first order multiplets. However at 360.13 MHz a full analysis of the spectrum and assignment of all the signals was possible and revealed a wealth of long-range coupling data. The ^{1}H chemical shifts and couplings are summarised in the Table. These have all been confirmed by the appropriate decoupling experiments. H-7 appears as a doublet of doublets (J, 2.2 and 0.6 Hz) due to coupling to H-5 and a 6-bond coupling to the 4-axial hydrogen;[9] H-6 as an overlapping doublet of triplets (J, 2.2 and 1.1 Hz) the triplet splitting being due to 4-bond coupling to the 4-methylene hydrogens. The 3-OH substituent clearly occupies an equatorial orientation as H-3 appears as a symmetrical septet due to equal trans diaxial couplings (7.8 Hz) and equal axial-equatorial couplings (3.8 Hz) to the 2- and 4-methylene hydrogens, which in turn show geminal couplings of 17.1 and 16.1 Hz respectively. In addition the equatorial hydrogens on C-2 and C-4 show a mutual 4-bond "W" coupling of 1.0 Hz, and the axial hydrogens also show a mutual coupling of 1.1 Hz.

P. lagerbergii was grown on a medium supplemented with [$^{2}H_3$]acetate and the 55.28 MHz ^{2}H NMR spectrum of the isolated scytalone was determined. This showed, Fig. 1(a), signals at δ_H 2.85, 3.10 and 6.32 ppm with relative intensities of 0.7, 1.6 and 1.0 respectively. The latter signals are readily assigned to the 4-equatorial and 5-hydrogens. However the remaining signal at 2.85 ppm could be assigned to either of the 4-axial or 2-equatorial hydrogens which are not resolved in the ^{2}H NMR spectrum. This was demonstrated by determining the spectrum of universally-labelled scytalone, prepared by the simple expedient of growing *P. lagerbergii* on a medium supplemented with 5% $^{2}H_2O$. The spectrum, Fig. 1(b), showed an equal degree of labelling at all the possible positions in scytalone. However the problem was resolved by carrying out a series of exchange experiments in deuteriated methanol using sodium methoxide as base. It was found, see Fig. 1(c), that the 2-equatorial H exchanged most rapidly, at approximately twice the rate of the 2-axial H. In addition the 7-H exchanged about three times more slowly than the 2-equatorial H and on prolonged treatment complete exchange of the 5-H was also obtained. However no exchange of the 4-H's occurred even under forcing conditions. On subjecting the sample of [2-$^{2}H_3$]acetate enriched scytalone to a reverse exchange process using sodium methoxide and [^{1}H]methanol and redetermining the ^{2}H NMR spectrum it was apparent that no loss of ^{2}H label had occurred, and so the signal at 2.85 ppm in the spectrum of [2-$^{2}H_3$]acetate-enriched scytalone must be due to incorporation of ^{2}H at the 4-axial and not the 2-equatorial position. As previous work[8] has shown that only one ^{2}H can be incorporated at C-4 the presence of label at both the equatorial and axial positions indicates that reduction to the dihydronaphthalene occurs in a non-stereospecific manner. This contrasts with recent results for O-methyl aspar-

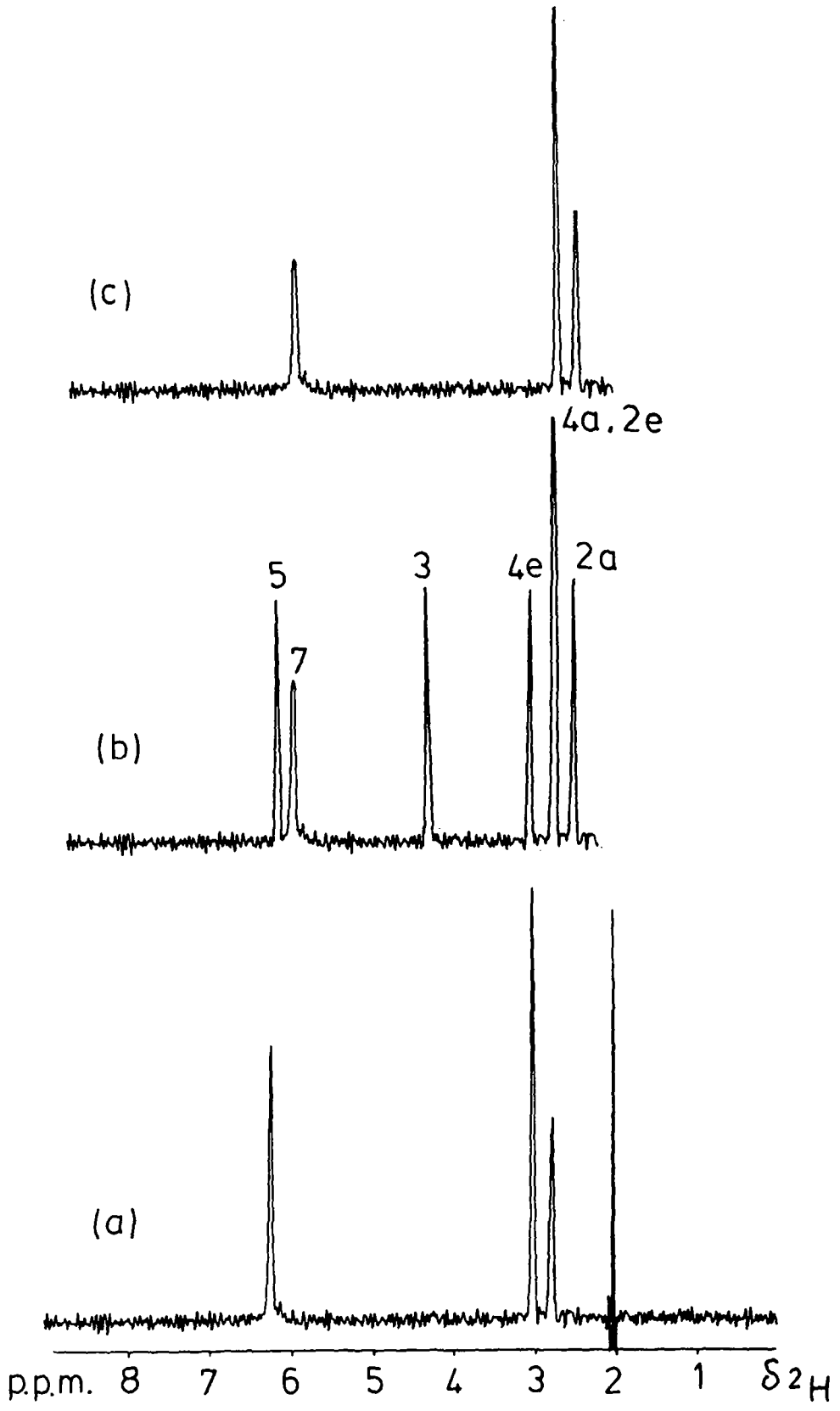
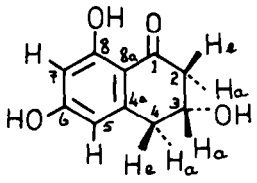


Fig. 1. 55.28 MHz NMR spectra of scytalone (a) [$^2H_3$]acetate enriched; (b) [*U*-^{2}H]-labelled; (c) after reaction for 72 hr at room temperature in NaOME, MeO^{2}H. All spectra were determined with proton noise-decoupling using 1000 Hz sweep widths, 2 K data points acquired and transformed into 16 K data points, pulse width 23 μs, acquisition time 1.0 s, line broadening -2.5 Hz, gaussian multiplier 0.40.

Table 1. ^{1}H NMR data for scytalone (1)

Hydrogen	δ_H (ppm)	Multiplicity	J (Hz)
7	6.15	dd	2.2, 0.6
5	6.28	dt	2.2, 1.1
3	4.31	septet	3.9
4-equat	3.20	dddd	16.1, 3.8, 1.1, 1.1
4-axial	2.86	ddddd	16.1, 7.8, 1.1, 1.0, 0.6
2-equat	2.84	ddd	17.1, 3.9, 1.0
2-axial	2.62	ddd	17.1, 7.8, 1.1



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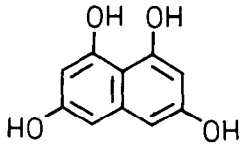

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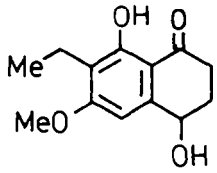
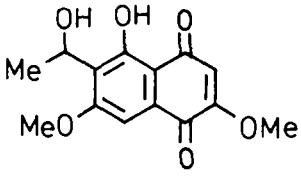


(3)



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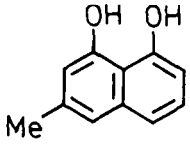

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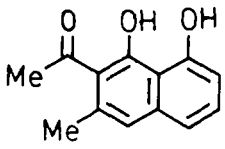

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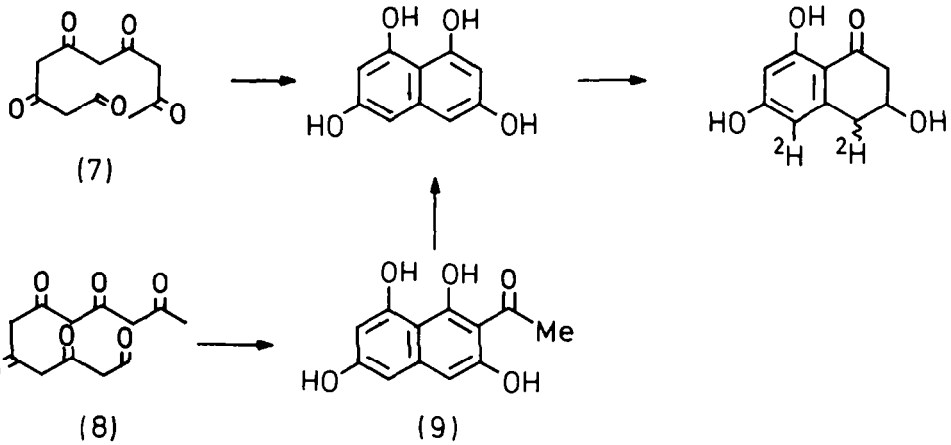

Scheme 1. Alternative pentaketide and hexaketide biosynthetic pathways to scytalone.

venone (**3**) where the corresponding reduction is clearly stereospecific.[10] Thus there is no incorporation of ^2H, even at a low level on C-2 or C-7 of scytalone.

In order to obtain information on the disposition of the "starter" acetate group of the polyketide precursor the incorporation of [2-^{13}C]malonate into scytalone was also examined. This resulted in high enrichment (*ca* 5 at.%) at each of the five carbons enriched from [2-^{13}C]acetate,[2] *viz* C-2, C-4, C-5, C-7 and C-8a but there was no discernible difference in enrichment levels. On repeating the experiment with simultaneous addition of unlabelled sodium acetate, a technique commonly used to facilitate observation of malonate "starter" effects,[11] the overall enrichments were lower (*ca* 3 at.%) but again no significant differences in enrichment levels were apparent.

These ^2H and ^{13}C labelling results are open to two possible interpretations. Although studies have been reported in which ^2H from acetate has been incorporated only into the acetyl-CoA derived "starter" unit of a polyketide chain and not into the malonyl CoA derived chain extending units,[12] we ourselves, in a reasonably large number of studies to date have always observed incorporation into both positions.[13] However, this could imply that C-4 and C-5 were partially derived from the acetate "starter" unit of a pentaketide precursor and so make assembly pattern (**7**) likely. However the absence of ^2H on C-2 and C-7 can be partially explained along with the failure to observe a "starter" effect on incorporation of [2-^{13}C]malonate by proposing that the 1,3,6,8-tetrahydroxynaphthalene (**2**) is formed by loss of an acetyl moiety from the corresponding 2-acetyl-derivative (**9**), which itself would be formed from a *hexaketide* precursor e.g. **8**. Such hexaketide-derived naphthalene metabolites e.g. **3** and **4** have been isolated from a number of fungi, including *Scytalidium* species.[14] There is also evidence that the naphthol (**5**) is derived by loss of the acetyl moiety from nepodin (**6**) in *Rumex alpinus*.[15]

Studies with potential advanced intermediates to further test these proposals are in progress.

EXPERIMENTAL

NMR spectra were determined on a Bruker WH 360 spectrometer: ^1H NMR spectra at 360·13 MHz in hexadeuterioacetone solns; ^2H NMR spectra at 55.28 MHz in acetone solns; and ^{13}C NMR spectra at 90.56 MHz in hexadeuterioacetone solns.

Incorporation of labelled precursors. Phialaphora lagerbergii (CMI 96745) was grown at 25° in shaken culture in 500 ml conical flasks each containing 200 ml Czapek-Dox medium containing 0.1% yeast extract and 5% sucrose. Preliminary experiments indicated that scytalone production commenced after 4 days' growth and reached a maximum after 12–15 days' growth. Further experiments were carried out with [^{14}C]acetate and [^{14}C]malonate to determine the optimum regime for feeding ^2H- and ^{13}C-labelled precursors.

(a) *Incorporation of* [$^2H_3$]*acetate.* Sodium [$^2H_3$]acetate (2 g) was dissolved in distilled water (4 ml) and the sterilised soln was distributed among eight shake flask cultures of *P. lagerbergii* after 5 days' growth. After a further 4 days' growth the mycelium was separated from the culture liquors by filtration and the acidified filtrate was extracted with ethyl acetate (4 × 100 ml). Evaporation of the extract gave a brown solid (1.011 g) which was purified by preparative thin layer chromatography on 20 × 20 × 0.05 cm silica GF_{254} plates eluted with 50% acetone in light petroleum (b.p. 60–80°) to give pure scytalone (600 mg).

(b) *Incorporation of* [2-^{13}C]*malonate.* Diethyl [2-^{13}C]malonate (0.25 g) dissolved in EtOH (3 ml) was added in equal portions to one shake flask culture of *P. lagerbergii* after one, two and three days' growth. After a further 3 days' growth, the culture was worked up as above to give scytalone (109 mg).

(c) *Incorporation of* [^{13}C]*malonate in the presence of unlabelled sodium acetate.* Diethyl [2-^{13}C]malonate (0.2 g) dissolved in ethanol (2 ml) was added in two equal portions to one shake flask culture of *P. lagerbergii* after one and two days' growth along with sodium acetate (0.25 g) in water (2.5 ml) each day. Work up as above yielded scytalone (90 mg).

(d) *Fermentation in* D_2O *supplemented medium.* Two shake flask cultures of *P. lagerbergii* were grown for one day, 10 ml of culture medium was then removed from each flask and replaced by 10 ml ^2H_2O. After a further 7 days' growth the cultures were worked up to give [*U*-^2H]scytalone (150 mg).

^2H *exchange experiments.* Scytalone (100 mg) was dissolved in d_4-methanol (1.5 ml) and NaOMe (10 mg) was added. The mixture was left at room temp and the rate of exchange monitored by ^1H NMR. After 3 days complete exchange of the 2-equatorial H and partial exchange of the 2-axial and 7-H's had occurred. After 5 days, exchange of the 2-axial H was complete and leaving for 5 weeks resulted in almost complete exchange of the 5- and 7-H's. After acidification and preparative TLC, pure scytalone (62 mg) was isolated.

Exchange of [$^2H_3$]*acetate-enriched scytalone in* [^1H]*-methanol.* The labelled scytalone (254 mg) was dissolved in MeOH (5 ml) and NaOMe (30 mg) was added and the mixture was left to stand for 5 days at room temp. The mixture was then poured into water (30 ml) and the soln acidified to pH 2 with dil HCl. Extraction with EtOAc (4 × 100 ml) followed by evaporation of the solvent gave the crude product (200 mg) which was purified by preparative TLC to give pure scytalone (154 mg).

REFERENCES

[1]J. A. Findlay and D. Kwan, *Can. J. Chem.* **51**, 1617 (1973).
[2]D. C. Aldridge, A. B. Davis, M. R. Jackson and W. B. Turner, *J. Chem. Soc.* Perkin 1, 1540 (1974).
[3]A. A. Bell, R. D. Stipanovic and J. E. Puhalla, *Tetrahedron* **32**, 1353 (1976).
[4]R. D. Stipanovic and A. A. Bell, *J. Org. Chem.* **41**, 2468 (1976).
[5]U. Sankawa, H. Shimada, T. Sato, T. Kinoshita and K. Yamasaki, *Tetrahedron Letters* 483 (1977).
[6]H. Seto and H. Yonehara, *Ibid.* 487 (1977).
[7]B. W. Bycroft, M. M. Cashyap and T. K. Leung, *J. Chem. Soc.* Chem. Commun. 443 (1974).
[8]U. Sankawa, H. Shimada and Y. Yamasaki, *Tetrahedron Letters* 3375 (1978).
[9]R. E. Wasylishen and T. Shaefer, *Can. J. Chem.* **80**, 1852 (1972).
[10]T. J. Simpson and D. J. Stenzel, *J. Chem. Soc.* Chem. Commun. 239 (1981).
[11]A. J. Birch, A. Cassera and R. W. Rickards, *Ibid.*, 654 (1961).
[12]R. Wyss, C. Tamm, and J. C. Vederas, *Helv. Chim. Acta* **63**, 1538 (1980); I. Kurobane, L. C. Vining, A. G. McInnes and J. A. Walter, *Can. J. Chem.* **58**, 1380 (1980).
[13]E. Bardshiri and T. J. Simpson, *J. Chem. Soc.* Chem. Commun. 195 (1981); Ref. 10; T. J. Simpson, A. E. de Jesus, P. S. Steyn and R. Vleggaar, *Ibid.* Chem. Commun. 632 (1982); T. J. Simpson and D. J. Stenzel, *Ibid.* 890 (1982).
[14]G. W. van Eijk and J. J. Roeymans, *Experientia* **34**, 1257 (1978).
[15]H. J. Bauch, R. P. Labadie and E. Leistner, *J. Chem. Soc.* Perkin 1. 689 (1975).