Sirodesmin PL Biosynthesis in *Phoma lingam* Tode

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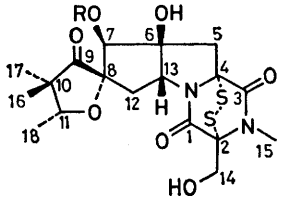
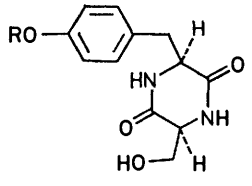
The biosynthesis of sirodesmin PL (1) in the fungus *Phoma lingam* Tode has been investigated using different labelled precursors: [1-^{14}C]-, [1-^{13}C]-, [1,2-$^{13}C_2$]-acetates, L-[*U*-^{14}C]serine, L-[*U*-^{14}C]tyrosine, [^{14}C]phomamide (3) or [^{14}C]cyclo-L-tyrosyl-L-serine (4). A scheme is proposed in agreement with the demonstration of the mevalonic origin of the tetrahydrofuranone ring, the incorporation of radioactivity from serine and tyrosine, and the easy conversion of the two cyclodipeptides (3) and (4) into sirodesmin PL (1).

SIRODESMIN PL (1) and deacetylsirodesmin PL (2) are two phytotoxins produced by the fungus *Phoma lingam* Tode,[1] and related to a family of epidithiopiperazinediones with antiviral properties precedingly isolated from *Sirodesmium diversum*.[2] These molecules present an unique skeleton, different from already known epidithiopiperazinediones,[3,4] due to the tetrahydrofuranone ring spiro-fused to a cyclopentylpyrrolidine moiety. The origin of this part of the molecule is an interesting problem and this paper deals with the biogenetic formation of (1) in cultures, using [1-^{14}C]-, [1-^{13}C]-, [1,2-$^{13}C_2$]-acetates, L-[*U*-^{14}C]serine, and L-[*U*-^{14}C]tyrosine. These labelled precursors have been selected in relation to the proposed biosynthetic pathways for such compounds [5] and with a previous hypothesis concerning sirodesmin biosynthesis.[2] Further investigations on the metabolites produced by *Phoma lingam* lead recently [6] to the isolation of phomamide (3) from the culture filtrate. According to the



(1) Sirodesmin PL: R = $-\overset{19}{\underset{}{C}}(=O)-\overset{20}{C}H_3$

(2) Deacetylsirodesmin PL: R = H



(3) Phomamide: R = γ,γ-dimethylallyl

(4) Cyclo-L-tyrosyl-L-serine: R = H

former incorporation results, this metabolite seemed to be the expected intermediate on the biosynthetic pathay to sirodesmin PL (1). The formation of phomamide (3) from L-[U-^{14}C]serine and L-[U-^{14}C]tyrosine has been investigated as well as the conversion of [^{14}C]phomamide {cyclo-*O*-(γγ-dimethylallyl)-L-[*U*-^{14}C]tyrosyl-L-serine} and its presumed direct precursor, cyclo-L-[*U*-^{14}C]-tyrosyl-L-serine (4), into sirodesmin PL (1).

In order to rationalize the result from the incorporation of ^{13}C precursors, preliminary experiments have

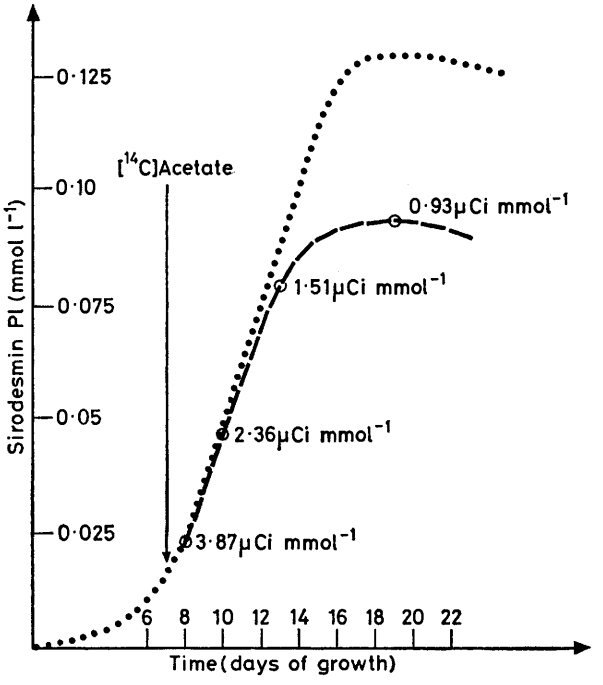


FIGURE 1 · · · · [1-^{14}C]acetate, 0.44 μmol l^{-1} — — [1-^{14}C]acetate, 1.52 mmol l^{-1}. Incorporations in sirodesmin PL (1)

been performed using [1-^{14}C]acetate in tracer and non-tracer amounts, and the results are represented in Figures 1 and 2. It appears in both cases that the maximum production phase of sirodesmins begins at the 7th day after inoculation with a maximum yield occuring between the 16th and 18th day of culture. From preliminary studies and to get optimal incorporations, all precursors were added 7 days after inoculation. Using non-tracer amounts of acetate (1.52 mmol l^{-1}) induced a 30% decrease of the maximum production of sirodesmins (Figure 1). Moreover, the maximum incorporation of acetate is reached about the 6th day after addition, this

corresponding to an average dilution per labelled site (Figure 2) of 26 which agrees [7] with the ^{13}C enrichment required for n.m.r. spectroscopy.

Thus, in the feeding experiments, all cultures were harvested 6—7 days after addition of the precursor, that

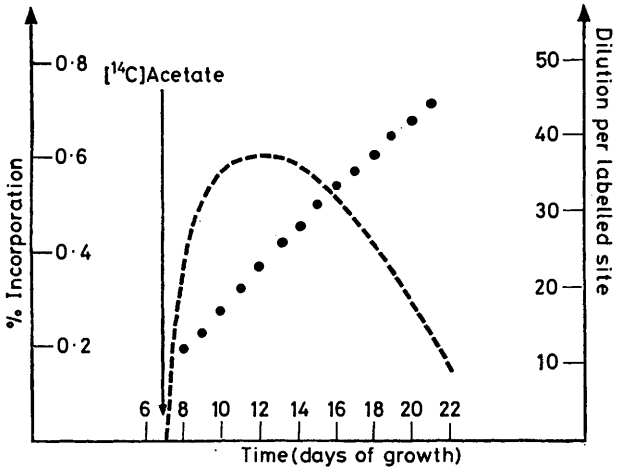


FIGURE 2 Absolute incorporation (— — —) and dilutions per site (· · · ·) assuming 3 sites to be labelled at C-10, C-18, and C-19 of the sirodesmin PL (1) molecule with [1-^{14}C]acetate (1.52 mmol l^{-1})

is, 13—14 days after inoculation. For ^{14}C incorporation experiments the chloroform–ethyl acetate-soluble metabolites were successively subjected to t.l.c. and LH-20 Sephadex column chromatography. In order to confirm the labelling results and due to crystallisation difficulties of (1), each purified sirodesmin fraction was decoupled (s.f.o.r.d.) natural ^{13}C abundance spectra of (1) and of four derivatives.[1] The unequivocal assignments for the C-16, C-17, and C-18 methyl resonances in sirodesmin PL (1) had to be made before any interpretation of the biosynthetic results and this has been established by selective decoupling experiments ($CDCl_3$) at δ 1.03 (16-H_3 or 17-H_3), 1.11 (16-H_3 or 17-H_3), and 1.27 (18-H_3) corresponding to the three methyl groups of the tetrahydrofuranone moiety. The ^{13}C high field methyl signal at δ_C 14.2 corresponds to the C-18 resonance, the 1.03 and 1.11 ^1H n.m.r. signals being respectively related to the δ_C 17.4 and 20.4 resonances of the geminal methyl groups at C-16 and C-17. The C-18 resonance undergoes two γ shielding effects [8] from the neighbouring methyl groups at C-16 and C-17. These two last signals undergo reciprocal β effects similar to the 8.6 p.p.m. deshielding effect described [9] for 1,1-dimethylcyclopentane although these effects are balanced by γ shielding occurring from the vicinal oxygen of the tetrahydrofuranone [8,10] moiety. The respective experimental assignments of the two geminal C-16 and C-17 methyl signals have not been possible from the n.m.r. spectra. However, assuming that the major contributions to their chemical-shift differences are the *cis*- and *trans*-shielding effects from the vicinal C-18 methyl as already shown [9] for 1,2-dimethylcyclopentane, the higher field signal at δ_C 17.4 may be assigned to the *cis* C-17 methyl and the most deshielded signal at δ_C 20.4 to the *trans*-C-16 methyl, these two assignments being unfortunately inverted in a preceding ^1H n.m.r. study.[1]

In a preliminary feeding experiment with [1-^{13}C]-

TABLE 1

Results of incorporations of [^{14}C]acetate, [^{14}C]serine, and [^{14}C]tyrosine into sirodesmin PL (1) by cultures of *Phoma lingam* Tode.

Precursor	Total activity (μCi)	Specific activity (mCi $mmol^{-1}$)	Concentration μmmol/100 ml of medium	μmol/100 ml at the 13th day	Sirodesmin PL (1) Total activity d.p.m.	Sirodesmin PL (1) Specific activity (d.p.m. $mmol^{-1}$)	% Incorporation Absolute	% Incorporation Specific
Sodium [1-^{14}C]acetate	2	45	4.4×10^{-2}	8.8	2.7×10^{4}	3.06×10^{6}	0.61	3×10^{-3}
Sodium [1-^{14}C]acetate diluted with cold material	4	1.3×10^{-2}	152	7.8	2.6×10^{4}	3.35×10^{6}	0.59	11.6
L-[*U*-^{14}C]serine	10	150	6.6×10^{-2}	32	4.88×10^{5}	1.52×10^{7}	2.20	4.56×10^{-3}
L-[*U*-^{14}C]tyrosine	10	150	6.6×10^{-2}	15.6	3.80×10^{5}	2.24×10^{7}	1.71	6.72×10^{-3}

then acetylated and the 6,14-diacetylsirodesmin PL so afforded was subjected to a further t.l.c. purification step.

Sodium [1-^{14}C]acetate, L-[*U*-^{14}C]serine, and L-[*U*-^{14}C]-tyrosine incorporation results are summarized in Table 1 and show a *ca.* 3-fold higher incorporation of amino-acids than acetate. The acetate incorporation pattern was further investigated using sodium [1-^{13}C]- and [1,2-^{13}C]-acetates and the position of the labelling determined by ^{13}C n.m.r. spectroscopy.

The ^{13}C n.m.r. determinations have been made on the basis of pulsed Fourier transform (p.F.t.) ^1H noise decoupled (p.n.d.) and single frequency off resonance

acetate (90.2% isotopic purity) the (1) isolated has been monoacetylated under already reported [1] conditions, in order to standardise the line intensities of the labelled and unlabelled products to the n.m.r. carbonyl signal of the introduced acetate.

The direct comparison of the ^{13}C natural abundance and ^{13}C enriched p.n.d. n.m.r. spectra of 14-acetylsirodesmin PL samples, recorded under the same conditions, showed the introduction of the labelling in the carbon atoms C-10, C-18, and C-19. The average ^{13}C incorporation calculated from n.m.r. data is 3.2% per labelled site, corresponding to a ^{13}C precursor dilution of 28 per

site, in agreement with the result observed in the pilot experiment. The 0.22% absolute incorporation value well fits with the experimental result [11] of 0.21% from mass spectrometry.

order to get more unambiguous results on skeleton carbon atom enrichments, the hydrolysis (mild conditions) of the acetyl group in position 7 of (1) has been performed. The ^{13}C n.m.r. spectra [$(CD_3)_2SO$] of the labelled deacetyl-

TABLE 2

Incorporations of sodium [1-^{13}C]acetate and [1,2-$^{13}C_2$]acetate into sirodesmin PL (1); results from p.n.d. ^{13}C n.m.r. spectra [$CDCl_3$, δ (p.p.m.) from $SiMe_4$], enrichment per labelled site

Carbon atoms	Chemical shifts [a]	[1-^{13}C]acetate enrichment/site [b] (%)	[1,2-^{13}C]Acetate enrichment/site [c] (%)	[1,2-^{13}C]Acetate $^1J(^{13}C-^{13}C)$ [d]/Hz
1	162.7 (s)			
2	77.2 (s)			
3	165.1 (s)			
4	75.3 (s)			
5	43.6 (t)			
6	82.3 (s)			
7	79.0 (d)			
8	89.5 (s)			
9	223.2 (s)			
10	47.6 (s)	1.7	2.6	38.2
11	80.1 (d)		3.9	41.9
12	33.7 (t)			
13	67.1 (d)			
14	60.7 (t)			
15	27.4 (q)			
16	20.4 (q)		2.9	36.8
17	17.4 (q)		3.6	
18	14.2 (q)	4.0	3.4	41.2
–CO–19 } OAc	169.8 (s)	3.8	3.3	58.9
–CH_3–20 } OAc	20.4 (q)		3.3	58.8

[a] Bracketted multiplicities are from s.f.o.r.d. spectra. [b] Enrichment per site: $\frac{1.1 \text{ peak height of enriched sample}}{\text{peak height natural abundance}} - 1$. [c] Calculated from modified McInnes calculation. For complex split signals (C-10 and C-11) all satellites contributions were included in calculation. [d] Values deduced from the main satellites pairs of complex signal splitting at C-10 and C-11.

The acetate incorporation pattern was further investigated using [1,2-$^{13}C_2$]acetate (87% isotopic purity). The p.n.d. ^{13}C n.m.r. spectrum of the corresponding labelled sirodesmin PL (1) shows an enrichment of seven carbon atom signals in spite of a coalescence of the C-16 and acetate methyl group C-20 signals (Table 2). In

sirodesmin (2) compared with the spectra of the naturally labelled product recorded under the same conditions, showed the centres C-10 (δ_C 46.9) and C-11 (78.4) to be enriched, as well as the methyl group carbon atoms at C-16 (δ_C 19.5), C-17 (16.2), and C-18 (14). As shown on the spectrum (Figure 3) the expected satellites for intact

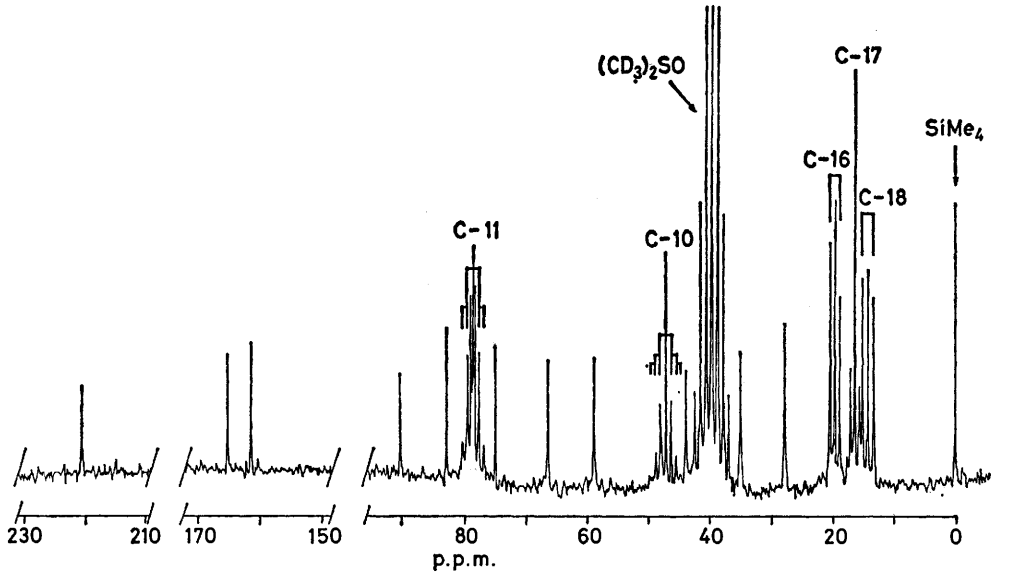


FIGURE 3 Proton noise decoupled ^{13}C n.m.r. spectrum of deacetylsirodesmin (2) [$(CD_3)_2SO$, δ (p.p.m.) from $SiMe_4$] labelled with [1,2-$^{13}C_2$]acetate

two-carbon ^{13}C-^{13}C unit incorporation are observed for the signals due to the methyl group carbon atoms at C-16 and C-18. However, a more complex pattern than either of these satellite pairs or than the singlet enhancement expected for a cleaved ^{13}C acetate unit incorporation [12] is observed for the resonances from centres C-10, C-11, and C-17. The complex signal due to the C-10 and C-11 centres exhibits a multiplet pattern and the coupling constants between the main satellite pairs are in agreement with the incorporation of the C-10, C-16 [$^1J(^{13}C\text{-}^{13}C) \sim 37$ Hz] and C-11, C-18 [$^1J(^{13}C\text{-}^{13}C) \sim 41$ Hz] pairs of carbon atoms issued from [1,2-$^{13}C_2$]acetate. An important singlet enhancement ($\times$ 2.3) is observed, associated with symmetrical satellite pairs, for the resonance due to the C-17 methyl carbon atom. A similar complex pattern has been already reported for islandicin [13] and griseofulvin.[14]

These results are indeed consistent with an incorporation of more than one labelled acetate at adjacent positions, and hence, non-negligible coupling occurs between the C-10, C-11 and C-10, C-17 adjacent carbon atoms with, in the latter case, a ^{13}C split into the unexpected satellites reaching *ca.* 40%.

Mass spectrometry gives a 0.45% absolute ^{13}C incorporation (3.6% average enrichment with a dilution of 24 per labelled site) and this is consistent with the calculated value of 3.3% from the ^{13}C n.m.r. analysis performed on the less perturbated signals of the carbon atoms at C-16 and C-18. The total enrichment per site, including incorporation of doubly-labelled ^{13}C-^{13}C (76%) and singly-labelled ^{13}C-^{12}C (22.5%) from acetate units, has been obtained from the ^{13}C n.m.r. spectra, using a modified McInnes calculation [15] with 1.1 $(I_S + fI_S)/(I_c - fI_S)$ where I_S is the sum of the intensities of the two satellite resonances, I_c the intensity of the central peak and fI_c the ratio mono/double labelled acetate introduced in culture.

The observed incorporations and the pilot experiments are in agreement. However, the complex interacetate unit ^{13}C coupling occuring from incorporations of labelled acetate units at adjacent positions could not be anticipated from the average overall enrichment (3.5%, average dilution of *ca.* 26 per site). Although the quantitative approach of the problem remains rather complex,[16] two factors at least can be taken into account for this unexpected result, in agreement with Simpson and Holker explanations in the case of griseofulvin.[14]

(1) The pilot experiments (Figure 1) show a substantial pool (20%) of (1) to be already present when the ^{13}C labelled precursor is added, this resulting in an artificially lowered average enrichment.

(2) The average dilution per site monitored as a function of time (Figure 2) shows an increase from 12.5 one day after addition of the precursor to 26, when the culture is harvested. This denotes a high decrease of the labelling probabilities with time. During a short period after its addition, the large amount of ^{13}C acetate probably shunts the endogenous acetate metabolism and is rapidly consumed by the fungus to give highly labelled sirodesmin molecules exhibiting a high probability of having two adjacent ^{13}C units incorporated. Since unenriched sirodesmin is subsequently produced when endogenous acetate metabolism starts again, these first enriched molecules make an important contribution to the final average incorporations and give the unexpected ^{13}C n.m.r. splittings for the carbon atoms C-10, C-11, and C-17.

Assuming that the observed ^{13}C n.m.r. splitting can arise from such phenomena, thus eliminating alternative biosynthetic pathways, unambiguous conclusions can be drawn from the feeding experiments using labelled acetate.

From the ^{14}C and ^{13}C acetate incorporation experiments, the mevalonic origin of the carbon atoms C-10, C-11, C-16, C-17, and C-18 of the tetrahydrofuranone moiety of (1) is demonstrated (see Scheme), the C-17 methyl group resulting from the decarboxylated acetate unit of mevalonic acid as represented.

On another hand, L-[*U*-^{14}C]serine and L-[*U*-^{14}C]-tyrosine are incorporated into sirodesmin PL (1). If the C-1, C-2, C-14, and N (N-CH_3) centres of (1) are assumed to derive from serine, the origin of the cyclopentylpyrrolidine moiety as well as the C-8 and C-9 centres in (1) is less obvious and implies a complex molecular modification of the tyrosyl aromatic ring. Since natural epithiopiperazinediones have been shown to occur from condensation of two amino-acids [4,5] *via* cyclodipeptides as intermediates, a precursor resulting from the condensation of serine and tyrosine could be postulated on the biosynthetic route to sirodesmin PL. According to Curtis's hypothesis [2] such an intermediate might be an *O*-isoprenyl derivative of cyclotyrosylserine. The recent isolation of phomamide (3) from the culture filtrate of *Phoma lingam* supported the idea that this metabolite, shown to be cyclo-*O*-(γγ-dimethylallyl)-L-tyrosyl-L-serine, was the intermediate expected in the biosynthetic pathway from serine and tyrosine to sirodesmins. We decided therefore to test whether (3) was a precursor of (1). This hypothesis was supported by the pertinent results already achieved in the gliotoxin field, where the role of cyclo-L-phenylalanyl-L-serine has been demonstrated.[17,18]

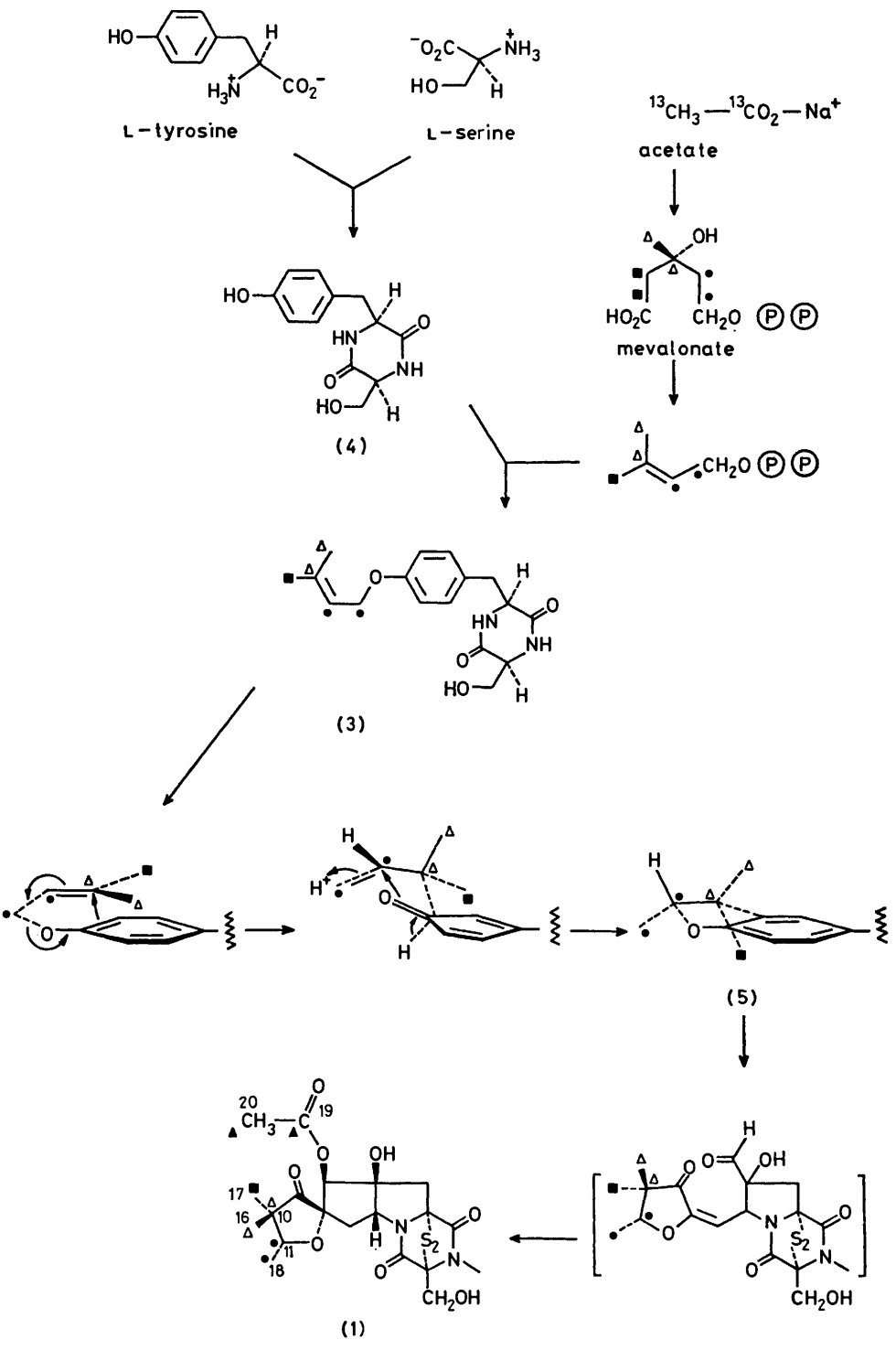
Incorporations of L-[*U*-^{14}C]serine and L-[*U*-^{14}C]tyrosine into phomamide (3) have been investigated as well as the conversion of radiolabelled (3) and its presumed direct precursor cyclo-L-tyrosyl-L-serine into sirodesmin PL (1). Feeding experiments were carried out as described above, all cultures being harvested 6 days after introduction of the labelled material.

[^{14}C]Serine and [^{14}C]tyrosine incorporation results in (3) are summarized in Table 3. Phomamide (3) was subjected to two different t.l.c. purification steps followed by acetylation and a further t.l.c. of the provided monoacetyl phomamide to check the radioactivity. As expected, phomamide (3) has been formed from both serine and tyrosine.

The ^{14}C-labelled cyclodipeptide (4) has been prepared [6,19] from *N*-(t-butoxycarbonyl)-L-serine and L-

[*U*-^{14}C]tyrosine used as its methyl ester hydrochloride as described. Cyclo-L-[*U*-^{14}C]tyrosyl-L-serine (4) was further purified by preparative t.l.c. until constant specific activity was achieved. In order to check the tracer conditions of the feeding experiment, labelled (4) was added to the growth at two different concentrations (Table 4, experiments 1 and 2).

The labelled phomamide (3) was prepared from (4) by treatment of its sodium salt with 1-bromo-3-methylbut-2-ene in dimethylformamide. After t.l.c. purification the cyclo-*O*-(γγ-dimethylallyl)-L-[*U*-^{14}C]tyrosyl-L-serine afforded was likewise added to cultures at two different concentrations (Table 4, experiments 3 and 4). Sirodesmin PL was purified as reported. Radioscanning of



thin-layer chromatograms showed radioactivity mainly at R_F values of (1) and, to a lesser extent, of the precursors as expected.

The highest absolute incorporations were observed when labelled (3) and (4) were fed to cultures at the lowest concentration (experiments 1 and 3), this agreeing with an already reported [17] observation. However, higher specific incorporations were found using higher concentrations of the precursors (experiments 2 and 4), this probably denoting a stronger shunt of the endogenous biosynthesis of each intermediate. Finally, the highest incorporation was observed with phomamide (3) (25.5%, experiment 3) which is, of course, a closer intermediate than (4) in the biosynthetic route to sirodesmin PL (1). The cyclodipeptide (4) is probably the true precursor of phomamide (3) although it has not been found in *Phoma lingam* until now.

These last results are in agreement with the sequence (4) → (3) → (1) for the biosynthesis of sirodesmin PL as was also suggested from the acetate, serine, and tyrosine feeding experiments reported above. The question still remains as to why phomamide (3) is excreted by the mycelium in the culture filtrate, and a deficiency in the enzymatic system involving, for example, the sulphur requirement of the fungus may be the answer.

TABLE 3

Results of incorporations of [^{14}C]serine and [^{14}C]tyrosine into phomamide (3) by cultures of *Phoma lingam* Tode.

					Phomamide (3)			
	Total activity	Specific activity	Concentration	μmol/ 100 ml at the	Total activity	Specific activity	% Incorporation	
Precursors	(μCi)	(mCi mmol^{-1})	μmol/100 ml of medium	13th day	(d.p.m.)	(d.p.m. mmol^{-1})	Absolute	Specific
L-[*U*-^{14}C]serine	10	150	6.6×10^{-2}	6.3	6.47×10^{4}	1.03×10^{7}	0.29	3.09×10^{-3}
L-[*U*-^{14}C]tyrosine	10	150	6.6×10^{-2}	5	1.08×10^{5}	2.16×10^{7}	0.49	6.48×10^{-3}

EXPERIMENTAL

General.—The ^{13}C and ^{14}C precursors used in this work have been synthesised by the C.E.A. Saclay, France, details of the labelling being reported under.

All ^{13}C n.m.r. spectra were measured in $CDCl_3$ or $(CD_3)_2SO$ at 22.63 MHz (pulse length 4.5 μs) on a Bruker HX90FT spectrometer. Selective proton-decoupling experiments were performed on a 62.8 MHz Cameca apparatus and all chemical shifts are given in p.p.m. downfield from an internal $SiMe_4$ standard.

The ^{14}C radioactivity determinations were carried out in toluene with an Intertechnique SL30 scintillator. A Berthold LB 2720 Dunnschicht-Scanner was used for the radioscanning of t.l.c.

Mass spectral measurements for ^{13}C content were performed on an electron impact AEI MS 50 instrument and the ions corresponding to the loss of S_2 ($M^+ - 64$) have been monitored for calculation.

Analytical and preparative t.l.c. were carried out on Schleicher Schüll LS 254 plates (SiO_2)

Cyclo-L-[U-^{14}C]*tyrosyl*-L-*Serine* (4).—L-[*U*-^{14}C]tyrosine (21 mg, 0.116 mmol, 95 μCi mmol^{-1}) was esterified in anhydrous hydrochloric acid–methanol (1 ml) and condensed to *N*-(t-butoxycarbonyl)-L-serine (23 mg, 0.116 mmol) using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodi-imide hydrochloride (24.5 mg, 0.128 mmol) in dichloromethane (2 ml) containing triethylamine (0.128 mmol) at 0 °C overnight. After removal of dichloromethane the residue was dissolved in ethyl acetate and successively washed with water, citric acid (1N), sodium hydrogen carbonate, and water, and then dried (Na_2SO_4) and ethyl acetate evaporated off. Treat-

TABLE 4

Results of incorporations of [^{14}C]cyclo-L-tyrosyl-L-serine (4) and of [^{14}C]phomamide (3) into sirodesmin PL (1) by cultures of *Phoma lingam* Tode.

			Specific		μmol/		Specific		
		Total	activity	Concentration	100 ml	Total	activity	% Incorporation	
No.	Precursors	activity (μCi)	(μCi mmol^{-1})	μmol/100 ml of medium	at the 13th day	activity (d.p.m)	(d.p.m. mmol^{-1})	Absolute	Specific
1	Cyclo-L-[*U*-^{14}C]-Tyr-L-Ser (4)	0.66	94.2	1.4	7.2	1.83×10^{5}	5.07×10^{6}	12.5	2.4
2	Cyclo-L-[*U*-^{14}C]Tyr-L-Ser (4)	0.66	13.1	10	8.1	6.36×10^{4}	1.57×10^{6}	4.35	5.4
3	Phomamide (3) cyclo-*O*-(γγ-dimethylallyl)-L-[*U*-^{14}C]-Tyr-L-Ser	0.80	94.5	1.7	9.1	4.53×10^{5}	9.6×10^{6}	25.5	4.75
4	Phomamide (3) cyclo-*O*-(γγ-dimethylallyl)-L-[*U*-^{14}C]-Tyr-L-Ser	0.80	16.2	10	5.8	1.32×10^{5}	4.55×10^{6}	7.4	12.6

The spiro-linked tetrahydrofuranone part of (1) is thus derived from (3) through the isoprenyl chain of the *O*-substituted tyrosyl rest. A mechanism involving the formation of a bicycloderivative (5) (Scheme) can be postulated which accounts for the stereospecificity of the [^{13}C]acetate incorporation *via* the mevalonate intermediate, as noted from the ^{13}C n.m.r. of enriched sirodesmin PL. The complex mechanism leading to sirodesmin PL (1) from (5) and involving the sulphur addition,

ment of the protected dipeptide according to standard procedure [6,19] afforded cyclo-L-[*U*-^{14}C]tyrosyl-L-serine (4) which was crystallised from water–methanol (14 mg, 0.056 mmol, 94.2 μCi mmol^{-1}). Radiochemical and stereochemical purity of (4) was checked by radioscanning of analytical thin layer chromatograms [chloroform–methanol–ethyl acetate (14 : 2 : 1), R_F 0.30] and by crystallisations of an aliquot from water–methanol after dilution with authentic unlabelled material.

Cyclo-P-(*γγ-dimethylallyl*-L-[U-^{14}C]*tyrosyl*-L-*serine* (*Phommide*) (3).—Cyclo-L-[*U*-^{14}C]tyrosyl-L-serine (4) (8 mg, 0.032 mmol, 94.2 μCi mmol^{-1}) was treated for 2 h (5 °C) with 0.3 ml of potassium hydroxide (1 equiv.) After lyophylisation, the potassium salt was dissolved in dimethylformamide (0.5 ml) and converted into phomamide by the action of 1-bromo-3-methylbut-2-ene (5.3 mg, 0.035 mmol) overnight at room temperature. Preparative t.l.c. of the product after the removal of the solvent [chloroform–methanol–ethyl acetate (14 : 2 : 1), R_F 0.62] yielded pure cyclo-*O*-(γγ-dimethylallyl)-L-[*U*-^{14}C]tyrosyl-L-serine (6.4 mg, 0.02 mmol, 94.5 μCi mmol^{-1}). Further recrystallisations from methanol of an aliquot, after addition of unlabelled (3), showed the specific radioactivity to be constant.

Culture Conditions and Purification of Labelled (1) and (2). —The strain of *Phoma lingam* used in this work has been kindly supplied by Dr. Boudart, Lille, France and was grown at room temperature and in the dark in static Roux flasks, each containing 100 ml of medium under the already reported conditions.[1] For each labelling experiment, the precursors were supplied as sterile aqueous solutions [except for (3) and (4) supplied as $(CD_3)_2SO$–H_2O solutions] to the culture broth 7 days after inoculation and, except for pilot assays, the harvest occurred after growing a further week. The collected culture filtrate was then successively extracted with chloroform (×3) and ethyl acetate (×3), the extracts being then evaporated under reduced pressure and subjected to a first preparative t.l.c. development with chloroform–ethyl acetate (1 : 1).

Sirodesmin PL (1) (R_F 0.65) was further purified by a Sephadex LH20 column chromatography eluted by chloroform–ethyl acetate (3 : 2). Deacetylsirodesmin PL (2) also collected from the t.l.c. purification step (R_F 0.30) represented less than 10% of (1). For [^{14}C]serine and [^{14}C]-tyrosine experiments, sirodesmin PL was subjected to a further t.l.c. using toluene–methanol (4 : 1; R_F 0.35). Finally all ^{14}C-radiolabelled sirodesmin pools were treated with pyridine–acetic anhydride for 72 h at room temperature and the 6,14-diacetylsirodesmin PL so obtained was purified on t.l.c. plates [chloroform–ethyl acetate (3 : 2)].

Feeding Experiments with Cultures of Phoma lingam *Tode.* —[1-^{14}C]*Acetate*. Series of parallel experiments were carried out supplying the cultures with sodium [1-^{14}C]-acetate either in tracer amounts (45 mCi mmol^{-1}, 0.44 μmol l^{-1}, 14 × 100 ml) or in non-tracer amounts (0.013 mCi mmol^{-1}, 1.52 mmol l^{-1}, 36 × 100 ml); 100 ml of the former and 2 × 100 ml of the last culture were harvested each day after addition of the precursor, from the 7th to the 22nd day of culture.

[1-^{13}C]*Acetate*. Sodium [1-^{13}C]acetate (500 mg) of 90.2% isotopic purity was added to 30 × 100 ml cultures (2 mmol l^{-1}) and after work up of the filtrate as described (on the 13th day of culture), 65 mg of (1) were obtained with a 0.21% absolute incorporation from mass spectrometry. Sirodesmin PL was then treated overnight at 5 °C with pyridine–acetic anhydride to give 61 mg of the ^{13}C-labelled 14-acetyl derivative needed for n.m.r. spectroscopy.

[1,2-$^{13}C_2$]*Acetate*. Culture flasks (40 × 100 ml) were fed with 500 mg (1.52 mmol l^{-1}) of sodium [1,2-^{13}C]acetate of 87% isotopic purity, leading after purification to 93 mg of (1) (0.45% absolute incorporation from mass spectrometry). ^{13}C Enriched sirodesmin PL was then hydrolysed overnight at room temperature in 1N-hydrochloric acid methanol, affording 63 mg of [^{13}C]deacetylsirodesmin PL (2) used for the ^{13}C n.m.r. study.

L-[U-^{14}C]*Serine and* L-[U-^{14}C]*Tyrosine*. L-[*U*-^{14}C]serine (150 mCi mmol^{-1}, 0.66 μmol l^{-1}, 100 ml) and L-[*U*-^{14}C]-tyrosine (150 mCi mmol^{-1}, 0.66 μmol l^{-1}, 100 ml) feeding experiments were conducted in parallel affording respectively 15.5 mg (4.88×10^5 d.p.m.) and 7.6 mg (3.80×10^5 d.p.m.) of sirodesmin PL (1) after purification.

^{14}C-Labelled phomamide (3) was analysed as follows: from the first t.l.c.-purification step (chloroform–ethyl acetate; 1 : 1), the most-polar fraction (R_F 0) was collected and subjected to two subsequent t.l.c. separations (chloroform–methanol–ethyl acetate 14 : 2 : 1, R_F 0.45; ethyl acetate–methanol 4 : 1, R_F 0.50) to yield pure phomamide (2 mg, 6.47×10^4 d.p.m. from [^{14}C]serine and 1.6 mg, 1.08×10^5 d.p.m. from [^{14}C]tyrosine). After acetylation (pyridine–acetic anhydride, overnight at room temperature) the specific activity of the monoacetylphomamide obtained was checked after t.l.c. development in chloroform–methanol–ethyl acetate (28 : 3 : 2; R_F 0.50).

Cyclo-L-[U-^{14}C]*tyrosyl*-L-*serine* (4) *and Cyclo*-O-(*γγ-dimethylallyl*)-L-[U-^{14}C]*tyrosyl*-L-*serine* (3).—Four parallel experiments were carried out: ^{14}C-labelled (4) was supplied at two different concentrations (1.75 mg, 14 μmol l^{-1}, 94.2 μCi mmol^{-1}, 5 × 100 ml and 12.5 mg, 100 μmol l^{-1}, 13.1 μCi mmol^{-1}, 500 ml) to 8 day-old *Phoma lingam* cultures (experiments 1 and 2). ^{14}C-Labelled phomamide (3) was likewise added at two concentrations to the broth (2.69 mg, 17 μmol l^{-1}, 94.5 μCi mmol^{-1} 5 × 100 ml and 15.7 mg, 100 μmol l^{-1}, 16.2 μCi mmol^{-1}, 5 × 100 ml) (experiments 3 and 4). The culture filtrate was worked up as usual on the 13th day, affording respectively 17.5 mg (experiment 1, 1.83×10^5 d.p.m.), 19.7 mg (experiment 2, 6.36×10^4 d.p.m.), 22.1 mg (experiment 3, 4.53×10^5 d.p.m.), and 14 mg (experiment 4, 1.32×10^5 d.p.m.) of ^{14}C-labelled sirodesmin PL (1). Purity and labelling specificity of (1) were checked as above, including t.l.c. of the diacetylsirodesmin derivatives, until the level of radioactivity was constant.

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