Tetrahedron Vol. 36, pp 3305 to 3307
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0040-4020/80/1115-3305/$02.00/0

SELECTIVE INHIBITION OF THE BIOSYNTHESIS OF PENICILLIC ACID

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(*Received in the UK* 10 *February* 1980)

Abstract—5-Chloroorsellinic acid inhibits the formation of penicillic acid by *Penicillium cyclopium* at sub-lethal concentrations.

Existing knowledge of the terminal stages in biosynthetic sequences leading to secondary metabolites suggests the possibility of a rational approach to the selective inhibition of specific pathways, using antimetabolites consisting of strategically designed analogues of advanced intermediates.[1]

As an initial feasibility study, it was decided to examine the influence of appropriately modified precursors on the formation of penicillic acid **1**. The biosynthesis of this mycotoxin requires a C(4)–C(5) ring cleavage of a metabolite of the phenolic tetraketide orsellinic acid **2**,[2] probably involving its prior conversion to 3-methoxytoluquinol **3**[3] as outlined in the simplified scheme.

In the course of a study of the inhibitory effects of a number of orsellinic acid analogues, it has been observed that the 5-chloro derivative **4**, inhibits the formation of **1** by *Penicillium cyclopium* at sub-lethal concentrations, while stimulating accumulation of the intermediates **2** and **3**.

MATERIALS AND METHODS

Culture and fermentation conditions

Penicillium cyclopium CMI 89372 was grown in Raulin–Thom medium containing 5% glucose (60 ml/250 ml Erlenmeyer flask) on a Gallenkamp incubator orbital shaker (240 rpm) at 24°, under which conditions it accumulates penicillic acid as the principal $CHCl_3$-extractable acidic constituent of the metabolism solution[2] (optimum yield after 5 days growth *ca* 1·5 mg/ml). Penicillic acid production in the presence of orsellinic acid and its derivatives was monitored by UV absorption at 225 nm of ethanolic solutions of $CHCl_3$ extracts of acidified culture filtrates (Table 1).

Chromatographic fractionation of P. cyclopium *metabolites*

In addition to penicillic acid, two minor constituents of $CHCl_3$ extracts of acidified filtrates of 3 day old cultures were fractionated by preparative thin layer chromatography (tlc) on silica plates (GF 254), using a four-component solvent mixture ($CHCl_3$:CH_3OH:H_2O:HCO_2H = 250:25:24:1 v/v).[3] Following elution with MeOH, these metabolites were characterised as 3-methoxy-2,5-toluquinol **3**. R_f 0.4 to 0.5, colourless crystals (CCl_4), m.p. 131–132°, and the

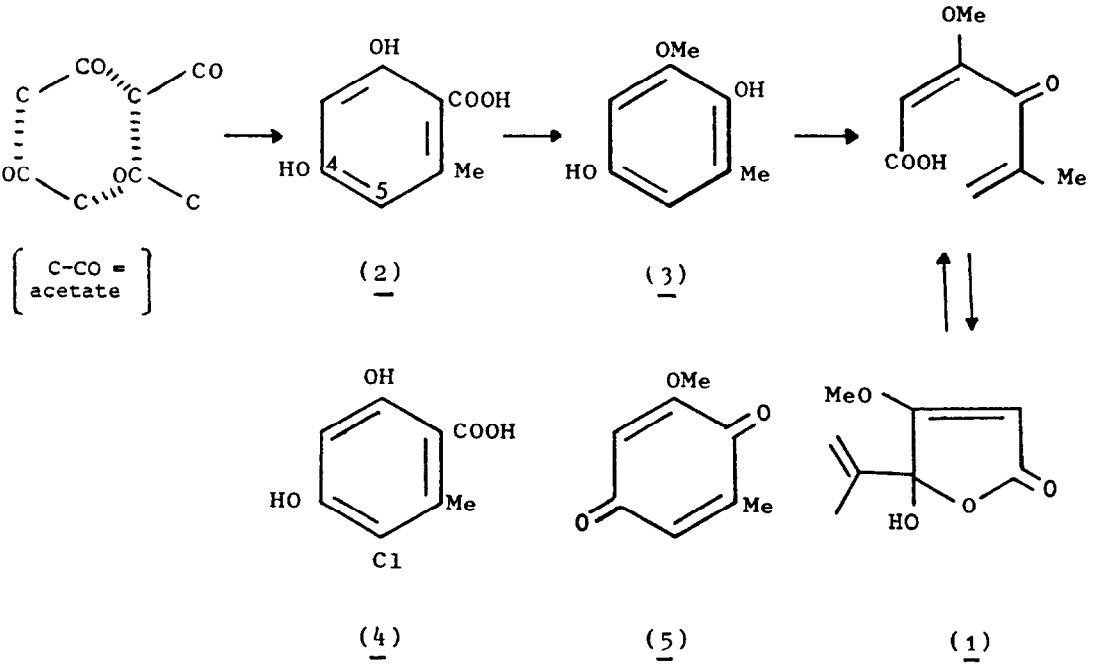

Table 1. Influence of orsellinic acid analogues on mycelial growth and penicillic acid production by *P. cyclopium*

Orsellinic acid derivative[a]	Concentration[b] ($M \times 10^{-4}$)	Mycelial wt.[c] (mg/flask)	(% control)	Penicillic acid yield[d] (mg/flask)	(% control)
Orsellinic acid (OA)	0	542	100	34.0	100
	2.5	563	104	35.5	104
	5.0	560	103	35.0	102
	9.9	561	104	28.5	84
5-Chloro OA	0	475	100	36.0	100
	2.1	462	97	22.0	61
	4.1	396	83	11.0	31
	8.2	320	67	1.5	4
3-Chloro OA	0	470	100	30.5	100
	2.1	438	93	30.5	100
	4.1	465	99	31.5	103
	8.2	370	79	12.5	41
OA-4-methyl	0	369	100	25.5	100
	0.9	339	92	19.0	75
	1.8	287	78	12.5	49
	2.7	191	52	0	0

[a]Addition to culture after 30 hr growth at 24° on 5% glucose/Raulin-Thom medium (60 ml/250 ml flask).
[b]Initial concentration of orsellinic acid and its analogues.
[c]Mycelial weight 6 days after inoculation (average of duplicate flasks).
[d]Penicillic acid recovered by $CHCl_3$ extraction of filtrate at pH 2.5.

corresponding quinone **5**, R_f 0.8 to 0.9, yellow needles (CCl_4) m.p. 155–156°. Their identity was confirmed by comparison with authentic samples provided by Professor S. Gatenbeck.

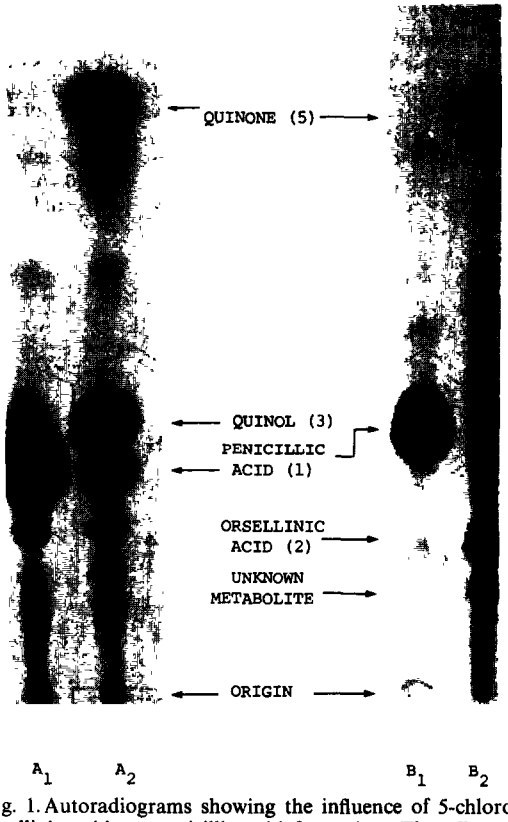

Fig. 1. Autoradiograms showing the influence of 5-chloro-orsellinic acid on penicillic acid formation. The effect of incubating *P. cyclopium* mycelium with (1-^{14}C)-acetate in the presence of 5-chloroorsellinic acid at 2.47×10^{-4}M (A_2) and 4.90×10^{-4}M (B_2) is shown relative to controls obtained with H_2O (A_1 and B_1).

Autoradiographic procedure

Based on a previously established procedure,[4] freshly harvested 72 hr *P. cyclopium* mycelium (1 g wet wt.) was resuspended in an aqueous solution of (1:^{14}C)-acetate (5 μCi/2 ml) for 8 hr at 24°, followed by autoradiographic detection of tlc-resolved ^{14}C-labelled components of $CHCl_3$ extracts of the filtrate, with appropriate modification as indicated in Fig. 1. Sodium (1-^{14}C)-acetate was purchased from the Radiochemical Centre, Amersham and autoradiograms of thin layer silica chromatograms prepared using Kodak X-ray film (cat. no. 305 6207).

Synthesis of orsellinic acid derivatives

These were prepared by previously described procedures: orsellinic acid **2**, m.p. 175–176°, (aq. acetic acid), 3-chloroorsellinic acid, m.p. 173–174° (benzene), and 5-chloroorsellinic acid **4**, m.p. 196–197° (H_2O) were synthesised by the method of Santesson,[5] while orsellinic acid 4-methyl ether,[6] m.p. 174° (aq. EtOH) was obtained by methylation of **2** with CH_2N_2 followed by hydrolysis of the resulting partially methylated ester with 2M-NaOH (reflux/2 hr).

Satisfactory spectroscopic data were obtained for all the products described above.

RESULTS AND DISCUSSION

The results shown in Table 1 demonstrate the relative inhibitory effects of low concentrations of selected orsellinic acid derivatives on the metabolism of *P. cyclopium*. Thus 5-chloro-orsellinic acid was observed to block penicillic acid formation effectively at levels which only partially reduce mycelial growth, while orsellinic acid 4-methyl ether markedly inhibits both growth and penicillic acid formation at appreciably lower concentrations. By contrast 3-chloro-orsellinic acid exhibits only slight inhibitory activity.

The selective nature of the inhibitory effect of low concentrations of 5-chloroorsellinic acid is clearly apparent from the results of autoradiographic experiments (Fig. 1), which also demonstrated the concomitant accumulation of other metabolites, the

yields of which were dependent upon the concentration of the inhibitor. Two of these were isolated by preparative tlc and identified as 3-methoxytoluquinol **3** and the corresponding quinone **5**, while a third product was chromatographically indistinguishable from orsellinic acid. All three compounds have been shown to serve as precursors of penicillic acid ($C_8H_{10}O_4$),[3] although the quinone ($C_8H_8O_3$), which is readily formed from the quinol on exposure to air, is not necessarily a direct intermediate. 3-Methoxytoluquinol **3** has been previously isolated from *Penicillium baarnense* along with orsellinic acid **2**, from which it is presumably derived.[7] Evidence for its occurrence in *P. cyclopium* was reported by the same group,[3] based on a chromatographic comparison with the synthetic quinol and also aerial oxidation of the ($^{14}CH_3$) methionine-derived culture filtrate extract followed by isotopic dilution with the unlabelled quinone **5**.

This inhibitory effect is superficially analogous to that frequently observed in studies with auxotrophic mutants, where however the accumulation of an intermediate in a particular pathway is typically a consequence of the deletion of a gene responsible for the synthesis of a specific enzyme in the normal metabolic sequence. In the present instance, the biosynthetic block appears to result from the action of a selective inhibitor on an existing enzyme. Although orsellinic acid 4-methyl ether is a more potent inhibitor of penicillic acid formation than is 5-chloroorsellinic acid, in view of its pronounced inhibitory effect on mycelial growth (Table 1), it may conceivably act through a different mechanism. The 4-methyl ether is known to occur naturally in an esterified form in lichens as the depside evernic acid.[8]

The finding that penicillic acid biosynthesis can be inhibited by sub-lethal concentrations of 5-chloroorsellinic acid, supports the view that it is a secondary metabolite which is not directly essential to cell growth and reproduction. In addition, it demonstrates the feasibility of this strategy for designing selective inhibitors of secondary metabolism based on a knowledge of obligatory steps in advanced stages of biosynthetic sequences, which in this instance requires cleavage of the C(4)-C(5) bond of orsellinic acid or a subsequent metabolite.

Further studies are in progress to examine the mode of action of these inhibitors and the wider application of this antimetabolite approach to the specific inhibition of individual steps in the biosynthesis of other fungal metabolites, including mycotoxins.

Acknowledgements—This investigation was supported by a grant from Ferdowsi University and a studentship (to J.L.) from the University of Kerman, Iran. Samples of 3-methoxytoluquinol and the corresponding quinone were kindly supplied by Professor S. Gatenbeck.

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