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Biosynthesis of Penitrems and Roquefortine by *Penicillium crustosum*

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Roquefortine and the penitrems were biosynthesised concurrently at an approximately equimolar rate by *Penicillium crustosum* after growth and sporulation. [^{14}C]mevalonic acid was incorporated (15% efficiency) into the isoprenoid regions of the penitrem and roquefortine molecules to an extent consistent with their 6:1 molar ratio of isoprenoid components. [^{14}C]penitrem A (specific activity, 3.4×10^2 μCi mmol^{-1}) and ^{14}C-penitrems B, C, and E readministered to young cultures were metabolically interconverted, indicating considerable metabolic flux, though generally directed towards penitrem A as the end product and suggesting a metabolic grid for the penitrem metabolites. Addition of bromide to the medium preferentially favored the production of bromo-analogs rather than the usual chloropenitrems.

Penicillium spp., yielding penitrem A and related metabolites B through F (Fig. 1), frequently also produce roquefortine (6–8). However, only a general indication of the relative dynamics of the biosynthetic processes leading to these partially isoprenoid metabolites has been given (8); the assay for roquefortine was fairly crude, values being estimated by visual comparison of spots on thin-layer chromatograms (TLC), and the course of fermentation was not clearly monitored through the earlier part of the toxin production phase. The present paper describes a more precise quantitative assay for roquefortine, thus facilitating demonstration of the coincidence of penitrem and roquefortine biosynthesis. The coincidence was confirmed experimentally through the biosynthesis of ^{14}C-labeled metabolites of high specific activity, ^{14}C-penitrems being used subsequently to explore the extent of interconversions of penitrems during the early phase of penitrem biosynthesis. The brief report (9) that bromopenitrem A could be produced by substituting the chloride in the medium with bromide was also explored further.

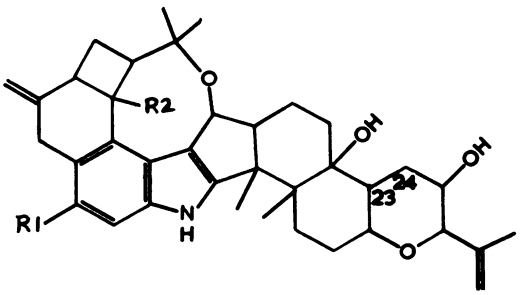
MATERIALS AND METHODS

Fungal culture. *Penicillium crustosum* (6) cultures were grown in Erlenmeyer flasks (250 or 500 ml) on Czapek Dox broth (50 or 100 ml, respectively) supplemented with yeast extract (0.5%). Spores from potato glucose agar cultures were seeded onto the liquid surface, and the flasks were incubated statically at 27°C for up to 12 days.

Extraction, purification, and estimation of penitrems and roquefortine. Since 99% of total penitrems were to be found in the mycelium, the broth was routinely discarded when penitrems only were to be considered. Otherwise, some additional roquefortine was obtained from a chloroform extract (two equal volumes) of the broth. The mycelium from each flask was homogenized in water, freeze-dried, extracted for 3 h with 200 ml of chloroform-acetone (1:1, vol/vol), filtered, and then treated with acetone (50 ml) for 24 h. Combined extracts were taken to dryness in vacuo. A small portion of each extract, dissolved in methanol, was taken for quantitative determination of total penitrems (5); 3 ml of extract, suitably diluted, and 0.18 ml of H_2SO_4 were incubated at 70°C for 20 min, and the resulting blue color was measured spectrophotometrically at 630 nm and compared with a standard curve for penitrem A.

The following protocol was used both in the quantitative determination of roquefortine and in the separation of ^{14}C-labeled penitrems and roquefortine before further purification by preparative high-pressure liquid chromatography. Mycelial extract, dissolved in chloroform-methanol (3:1, vol/vol), was loaded in subdued light on Silica Gel GF_{254} (Merck type 60) layered (1 mm) on glass (40 by 20 cm). Chromatograms were developed across the short axis of the plate in chloroform-acetone (93:7, vol/vol), and the position of the most polar penitrems (C plus D) was noted by quenching of the fluorescent dye under UV (254 nm) light. The chromatograms were redeveloped in chloroform-acetone (80:20, vol/vol) until the solvent front approached closely to the rear of the region of penitrems C plus D. The position of roquefortine was then seen as a prominent fluorescence-quenching region clearly resolved from the origin. Silica regions corresponding to roquefortine and to penitrems C plus D, A plus E, and B plus F were removed from the chromatogram and eluted with methanol or acetone, and the eluate was taken to dryness in vacuo.

Partially purified penitrems and roquefortine were resolved further by repetitive high-pressure liquid chromatography in a column (25 by 1 cm) of Ultra-



Penitrem	R1	R2	C-23–C-24
A	Cl	OH	Epoxide
B	H	H	Epoxide
C	Cl	H	Δ
D	H	H	Δ
E	H	OH	Epoxide
F	Cl	H	Epoxide

FIG. 1. Structure of penitrems.

sphere ODS reversed-phase silica, using methanol-water (5:1, vol/vol, for resolving penitrems; 4:1, vol/vol, for roquefortine). The flow rate was 2.5 ml min^{-1}, and products were monitored spectrophotometrically in the column eluate passing through a flow cell (335 nm for penitrems, 325 nm for roquefortine). The process was followed on a chart recorder. Measurement of the area under the trace corresponding to roquefortine allowed a reliable and reproducible quantitative determination of roquefortine in the linear range of 5 to 30 μg, which was entirely adequate for the present investigation and could, no doubt, be refined further by using an analytical column. Collected fractions, pooled where necessary from repetitive processing, were freeze-dried, and the quality of the products was monitored by TLC in the usual solvent systems or by electron impact mass spectrometry (4) or by both methods.

Where nondestructive quantitative assay of penitrems was required, their absorption at 233 nm was used for spectrophotometric estimation.

Radioactivity measurement. Radiolabeled products of metabolism of ^{14}C-penitrems were measured on planchet disks in a low-background counter (Nuclear-Chicago). Other measurements were made by standard liquid scintillation techniques.

RESULTS

Course of production of penitrems. Pairs of 250-ml flasks were harvested at intervals over 12 days (Fig. 2). Slight variations in the efficiency of establishing a complete mat in the early stages of fermentation were reflected in the course of total penitrem and roquefortine production, but it is clear that these metabolites are only biosynthesized after growth and concomitant sporulation and that the syntheses are closely concurrent. The relative yields, when adjusted to allow for their relative molecular mass, corresponded approximately to an equimolar ratio. TLC of penitrem extracts showed a similar relative composition of components throughout the metabolite production phase.

Production of ^{14}C-labeled penitrems and roquefortine. DL-[2-^{14}C]mevalonic acid sodium salt (125 μCi) was divided equally between two cultures in 500-ml flasks. The precursor in 2.5 ml of water was injected through the mycelial mat and into the culture medium 4 days after inoculation. The procedure was repeated 3 days later in the same flasks, after which the two together had received a total of 250 μCi of precursor. The flasks were harvested on day 12, each yielding 0.76 g (dry weight) of mycelial material. A flask inoculated at the same time as these was analyzed at the stage at which the others were first fed with ^{14}C-labeled precursor and was found to have produced 3 mg of penitrem. The fed flasks at harvest were assayed at 21.5 mg of penitrem each. The overall efficiency of purification (Ta-

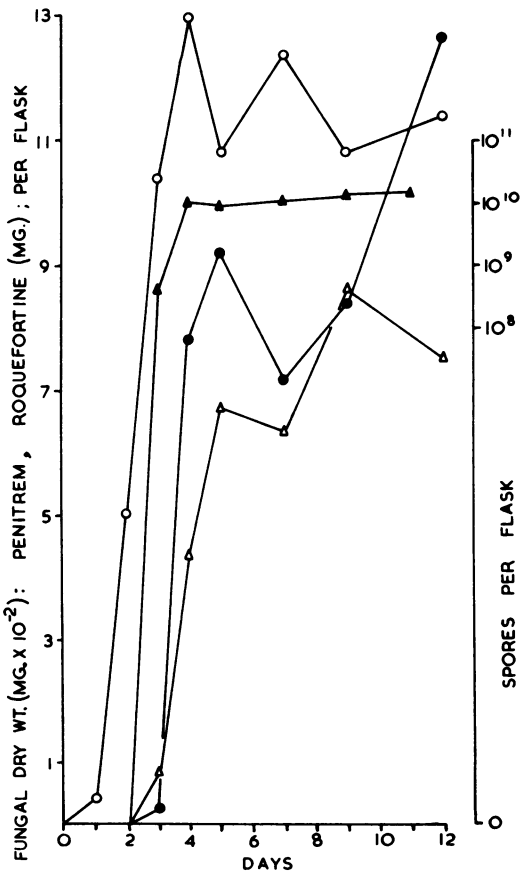


FIG. 2. Course of 12-day fermentation of *P. crustosum* in surface culture for the production of penitrems and roquefortine. Symbols: ○, fungal dry weight; ▲, sporulation; △, total penitrems expressed as penitrem A; ●, roquefortine.

ble 1) was therefore 76%, and the overall efficiency of incorporation of ^{14}C-labeled precursor into recovered metabolites was 15.2%. The molar ratio of total penitrems to roquefortine (Table 1) is approximately 1:1, and the approximately fivefold difference found between the specific activities of penitrem A and roquefortine is consistent with closely coincident biosynthetic rates in metabolites having a molar ratio of isoprene units of 6:1. Although the penitrem products appeared to be chromatographically pure on TLC, autoradiography of this chromatogram revealed small amounts of contaminating penitrems with close chromatographic mobilities. These were not considered to detract significantly from the value of the penitrem products, labeled to relatively high specific activity, with respect to their subsequent use for probing their own metabolism in *P. crustosum*.

Interconversions of penitrems in *P. crustosum*. Penitrems are particularly hydrophobic metabolites and thus require an acceptable organic solvent in which to readminister them to cultures of the fungus. Ethanol was chosen, having been shown in a pilot experiment at 0.3% to have little or no effect on penitrem production.

Samples of ^{14}C-penitrems A, B, C, and E (0.5 to 1.2 mg) were dissolved in ethanol (200 μl), 1 μl was taken for scintillation counting, and the remainder was distributed equally to each of two 500-ml flask cultures on day 3, when biomass production was virtually complete and as penitrem biosynthesis was commencing. Controls with and without ethanol alone were also provided. The flasks were harvested 4 days later (day 7), and total penitrems were assayed and found to be in the range of 4.16 to 8.42 mg per flask. The extract from the two flasks was combined, individual penitrems from each treatment were separated, the radioactivity was counted, and the weight of each penitrem was estimated by the direct spectrophotometric method. The results were adjusted to take account of the usual relative amounts of the various penitrem products of the fungus (Table 1), since a constant efficiency of processing could not be expected for all component penitrems on a small scale. It is apparent (Table 2) that considerable metabolism of the fed penitrems occurred. Thus, the traces of contaminating penitrems in the ^{14}C-penitrem fractions fed would also have been metabolized and therefore dispersed to constitute insignificant amounts.

TABLE 1. Yields and specific activities of ^{14}C-labeled penitrems and roquefortine biosynthesised from [2-^{14}C]mevalonate in two flasks of *P. crustosum*[a]

Material	Mol wt	Wt (mg) of product purified	Sp act (μCi $mmol^{-1}$) ($\times 10^2$)
Penitrem A	633	19.4	3.4
Penitrem B	583	5.1	0.7
Penitrem C	601	3.0	2.4
Penitrem E	599	5.3	2.7
Roquefortine	389	24.1[b]	0.7

[a] Specific activity of [^{14}C]mevalonate, 53 mCi $mmol^{-1}$.

[b] Including 7.8 mg recovered from culture filtrate.

Penitrem A remained mainly as A, but substantial catabolism, possibly through both pathways of the proposed metabolic grid (Fig. 3), had occurred. Very little of the added penitrem B remained as such; most followed the anabolic route to penitrem A, but some was also catabolized to penitrems D and C. Penitrems C and E were also substantially metabolized to other penitrems, the most activity eventually residing in penitrem A.

The three samples which were unfortunately lost (Table 2) may have significantly improved the overall recoveries of added radiolabel, two being samples metabolically adjacent to the labeled penitrem fed. Nevertheless, these experimental results are consistent with a considerable metabolic flux during the early stages of penitrem biosynthesis, although the tendency is clearly for accumulation of the dominant end product, penitrem A.

Biosynthesis of bromo-penitrem A. Potassium bromide (50 mg in 1 ml of sterile water) was added to one 500-ml flask before inoculation, resulting in a 2:1 molar ratio of Cl^- to Br^-. The flask, together with a control, was harvested on day 7, and the mycelium was found to contain 8.8 mg of total penitrem (10.6 mg in the control). A TLC analysis of the Br^--treated product suggested a relative reduction in the deshalo-penitrems (E and B) and showed a slight increase in the R_f values of penitrems A and F, consistent with apparently decreased polarity. High-pressure liquid chromatography showed a double peak in the penitrem A region, the fraction with the longer retention time representing the larger mass of product. The mass spectra of these two adjacent components showed clearly that the less polar component was bromo-penitrem A, the other being penitrem A. The preferential production of the bromo-analog in spite of an unfavorable halide molar ratio of 1:2 suggests a metabolic preference for Br in penitrem biosynthesis. The sequence Br- > Cl- > deshalo seems to operate.

DISCUSSION

Although penitrems are particularly hydrophobic molecules, they were obviously administered to the culture broth quite efficiently in ethanol and were taken up by the mycelium.

TABLE 2. Interconversions of penitrems A, B, C, and E during the early phase of penitrem biosynthesis by *P. crustosum*

^{14}C-penitrem		Wt (μg) isolated by HPLC[a] and counted	Calculated[b] total radioactivity (dpm × 10^3) in penitrems[c]	Recovery (%) of label fed
Fed	Found			
A (0.5 mg)	A	584	144.4	34
	B	—[d]	—	
	C	322	5.8	
	D	237	3.9	
	E	5	39.3	
	F	15	20.2	
B (1.2 mg)	A	128	62.2	32
	B	139	5.4	
	C	565	10.6	
	D	5	32.4	
	E	—	—	
	F	63	1.2	
C (1.2 mg)	A	307	66.6	16
	B	81	15.2	
	C	184	37.8	
	D	538	24.5	
	E	200	31.1	
	F	—	—	
E (0.6 mg)	A	18	251.4	68
	B	10	76.7	
	C	449	30.4	
	D	66	10.5	
	E	290	28.7	
	F	5	2.4	

[a] HPLC, High-pressure liquid chromatography.
[b] Calculated to allow for the usual relative abundance of the various penitrems produced by this fungus (Table 1). This can be expected only to emphasize gross trends.
[c] In a 7-day mycelium in a 500-ml flask.
[d] —, Sample lost.

However, the extent of translocation through the mycelial mat cannot be predicted with confidence in a surface culture, particularly where a significant proportion of the penitrem biosynthetic activity appears to reside in the spores which were, at the time of radiolabel administration, fully formed. Thus, we have been cautious in interpreting the results of the interconversion experiment other than recognizing the evident metabolic flux and the dominant biosynthetic trend of putative intermediates toward penitrem A, the most complex of all the penitrems.

The apparently concurrent biosynthesis of penitrems and roquefortine theoretically offers a potential system for exploring the neglected and difficult area of the control of secondary metabolism. The consistent failure to induce the fungus to biosynthesize these metabolites in submerged culture in spores, however, imposes a constraint on time course experiments which are most conveniently performed by sampling one homogeneous fermentation. Therefore, *P. crustosum* stands in contrast to *Penicillium simplicissimum (P. estinogenum)* which produces another potent tremorgenic mycotoxin, verruculogen, the fungus being induced to sporulate and produce the metabolite in submerged culture under the influence of calcium ions (2).

The structures of penitrems A through F have been described (3), and the nomenclature used in that report has been followed in the present paper, although there is some discrepancy between it and that used in other previous publications on penitrems. The penitrems differ as indicated in Fig. 3, the hydroxylation step forming a substituent of the eight-membered ring (Fig. 4). Part of the penitrem carbon skeleton has been shown (3) to be acetate derived, and the measured carbon-carbon coupling constants in the ^{13}C nuclear magnetic resonance spectrum of [1,2-^{13}C]acetate-derived penitrem A are in accordance with the expected derivation of this part of the molecule from isoprenoid precursors. In concurrent studies (P. G. Mantle, J. B. Day, P. A. Fellows, and E. S. Waight, unpublished data) penitrem A from *P. crustosum* fed with [2-^{13}C]mevalonic acid showed a specific several-fold enhancement of the naturally abundant ^{13}C of the carbon atoms theoretically derived in the terpenoid region from the 2-position of mevalonic acid. Therefore, we infer that the ^{14}C label in the radiolabeled penitrems of high specific activity, herein described, was as indicated in Fig. 4. Penitrem molecules with multiple car-

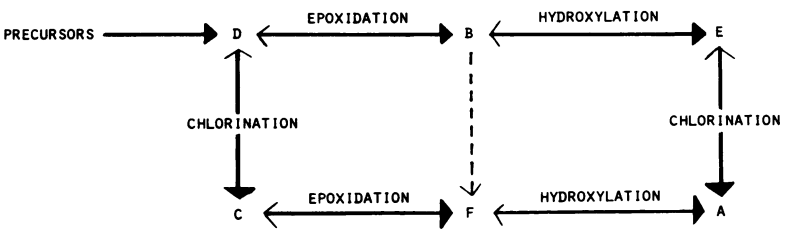
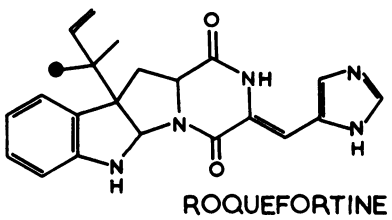
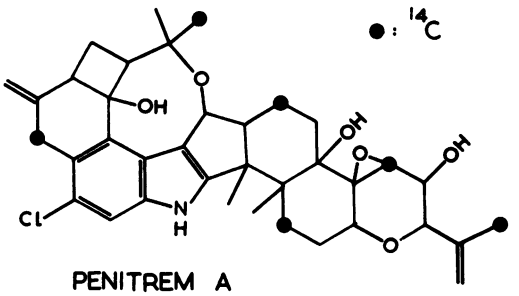


FIG. 3. Putative metabolic grid for biosynthetic interrelationships among penitrems A through F.



ROQUEFORTINE



PENITREM A

FIG. 4. Positions of radiolabel in [^{14}C]penitrem A and [^{14}C]roquefortine biosynthesised from [2-^{14}C]mevalonic acid. In penitrem A some scrambling of label into the adjacent methylene carbon derived from the methyl carbon of mevalonic acid may be expected, at least at the extreme ends of the molecule.

bons labeled are particularly suitable for metabolism experiments, not only those concerning biosynthetic interrelationships among the penitrems, but also those used in exploring distribution and metabolism in sheep livers and elimination in sheep bile (P. G. Mantle and B. L. Smith, unpublished data).

It may also be deduced from a biosynthetic study of roquefortine (1) that the roquefortine produced concurrently with the radiolabeled penitrems was radiolabeled as indicated in Fig. 4.

ACKNOWLEDGMENTS

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