Biosynthesis of Carolic Acid in *Penicillium charlesii*: The Intermediate Precursors

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Summary A number of intermediate precursors of carolic acid in *P. charlesii* has been studied, the most notable being γ-methyltetronic acid and carlosic acid which appear to be incorporated *via* separate metabolic pathways.

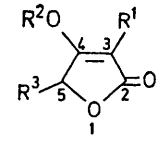
THE manner of biosynthesis of the tetronic acid nucleus is of considerable interest in that it occurs in ascorbic acid (**1**) as well as a family of eight different metabolites of *Penicillium spp.* Whereas (**1**) is derived without skeletal modification from glucose,[1] Bentley[2] has demonstrated that carolic acid (**2**) [which exists as (**2a**) in aqueous solution but which is isolated as (**2b**) in crystalline form from non-aqueous solvents] from *P. charlesii* arises *via* a more subtle mechanism, where C(4), C(5), and R^3 represent a subunit intimately related to succinate, and C(2), C(3), and R^1 are formed from a subunit bearing the normal polyketide labelling pattern but, intriguingly, considerably less label in the 'methyl terminal' end as contrasted to the 'carboxy terminal' end, in diametric opposition to the order usually observed in such processes.

We thus considered the possibility that (**3**) or a closely related metabolite might be involved in the primary stage of the biosynthetic process, in spite of prior negative evidence obtained by replacement culture techniques on unlabelled substrates.[2]

Since most of the required precursors were not available in labelled form, our initial experiments used competition techniques, whereby unlabelled precursors were fed simultaneously with [*U*-^{14}C]glucose and the resultant activity of (**2**) isolated was compared with a series of blanks with no competitors present.

To test for the intermediacy of (**3**), initially (5*RS*)-(**3**)[3] was used in competition experiments, where an excellent incorporation into (**2**) was observed (see Table). We then prepared (5*R*)-(**3**) from (5*R*)-(**2**)[4] and fed it at the same level, with very comparable results. Finally, a more precise confirmation of these studies was obtained when [*U*-^{14}C]-(5*R*)-(**2**) obtained biologically from [*U*-^{14}C]glucose was converted into [*U*-^{14}C]-(5*R*)-(**3**) which was again fed at the same level as in the foregoing experiments. The agreement between both series of experiments using (5*R*)-(**3**) was excellent.

Since (**3**) might be degraded prior to incorporation into (**2**), we subjected (**2**) obtained from the [*U*-^{14}C]-(5*R*)-(**3**) feeding experiment to degradation as previously described.[2,4] Over 98% of the total radioactivity was observed in the tetronic acid nucleus, clearly indicating incorporation of (**3**) as a unit.



(**1**) R^1 = OH, R^2 = H, R^3 = HO·CH_2CH(OH)
(**2a**) R^1 = HO·CH_2·CH_2·CH_2·CO, R^2 = H, R^3 = Me
(**2b**) R^1R^2 = –CO·CH_2·CH_2·CH_2, R^3 = Me
(**3**) R^1 = R^2 = H, R^3 = Me
(**4**) R^1 = Pr^nCO, R^2 = H, R^3 = HO_2C·CH_2
(**5**) R^1 = R^2 = H, R^3 = HO_2C·CH_2

Previous work had not determined whether or not carlosic acid (**4**), the co-metabolite of (**2**), could act as its precursor. This was of particular importance to our studies, since acylation of (**3**), if it were the sole pathway,

would preclude (**4**) as a precursor, and a very low incorporation of activity from (**4**) into (**2**) would be expected. In practice, when (**4**) was used as a competitor *vs.* [*U*-^{14}C]-glucose, the estimated relative incorporation was excellent. As in our other experiments, this was cross-checked by the feeding of labelled precursor. Since the amount of [*U*-^{14}C]-(—)-(**4**) available from culture filtrates was very limited, much less of this material was administered in the radioactive feeding experiments than in the competition experiment.

Since (**3**) was detected in culture filtrates of the organism by Bentley[2] as well as ourselves, we believe its incorporation into (**2**) may represent more than a mere aberrance, though this has not been strictly proven.

It appeared that (**5**) could represent a pivotal intermediate from which either (**3**) or (**4**) might be derived. The estimated relative incorporation of (**5**) into (**2**) by the competitive technique was good; however, it has not been possible to cross-check this value by direct feeding experiments [owing to lack of both amount and specific activity of (**4**) currently available].

TABLE

Competition experiments

Competitor added[a]	Competitor added per flask[a] (μmol)	(**2**) isolated per flask (μ mol)	% Decrease in radioactivity of (**2**) with competitor present[c]	Estimated relative incorporation[d]
(5*RS*)-(**3**)[b]	446	1689	5·71	21·7
(5*R*)-(**3**)[b]	443, 441	1689,1969	6·35, 5·56	24·3, 24·9
(—)-(**4**)[b]	215	1447	7·95	53·5
(5*RS*)-(**5**)[b]	323	941	2·85	8·3

Radioactive feeding experiments

Precursor added[e]	Precursor added per flask[e] (μ mol)	(**2**) isolated per flask (μmol)	% incorporation (%)
[*U*-^{14}C]-(5*R*)-(**3**)	441	1474	24·7
	441	1375	24·9
[*U*-^{14}C]-(—)-(**4**)	57	2200	87[f]
	28	2629	91·6

[a] Fed to 100-ml cultures in 500-ml Erlenmeyer flasks on the 3rd to 5th day after inoculation, using the medium of Bentley[2] and *P. charlesii* NRRL 1887. [b] Run simultaneously. [c] Compared to the average of three control experiments with no competitor added, also run simultaneously. [d] Defined as: estimated relative incorporation = 100 × [μmol non-glucose derived (**2**)/(μmol competitor added), where: μmol non-glucose derived (**2**) = [μmol (**2**) isolated per flask] × (% decrease in specific activity)/100. Since it was not known *a priori* which carbon atoms of (**2**) isolated were derived from the competitor, this calculation was carried out as if all were, so that "μmol non-glucose derived (**2**)" above is an estimate. The estimated relative incorporation is therefore *not* a true percentage incorporation. [e] Culture conditions were the same as for the competition experiments. [f] While this experiment supports a high incorporation, the amount of precursor added was only measured to ± 3%.

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