Phytochemistry, Vol. 24, No. 2, pp. 247–249, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00

CORONATINE BIOSYNTHESIS: INCORPORATION OF L-[U-^{14}C]ISOLEUCINE AND L-[U-^{14}C]THREONINE INTO THE 1-AMIDO-1-CARBOXY-2-ETHYLCYCLOPROPYL MOIETY

ROBIN E. MITCHELL

Division of Horticulture and Processing, DSIR, Mt Albert Research Centre, Private Bag, Auckland, New Zealand

(*Revised received* 2 *July* 1984)

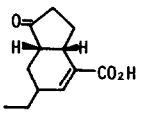
Key Word Index—*Pseudomonas syringae* pv. *atropurpurea*; toxin biosynthesis; coronatine; coronafacoylvaline; coronafacoylisoleucine; isoleucine incorporation; threonine incorporation.

Abstract—Addition of either L-[U-^{14}C]threonine or L-[U-^{14}C]isoleucine to 2.7-day-old shaking liquid cultures of *Pseudomonas syringae* pv. *atropurpurea* resulted in incorporation of radioactivity into coronatine, but not into *N*-coronafacoylvaline, another phytotoxin excreted by *P.s. atropurpurea*. In contrast, addition of L-[U-^{14}C]valine did not lead to incorporation of radioactivity into coronatine, but instead into coronafacoylvaline. Acid hydrolysis of the purified [^{14}C]coronatine obtained after incorporation of either [^{14}C]isoleucine or [^{14}C]threonine demonstrated that $> 94\%$ of the radioactivity was present in the 1-amido-1-carboxy-2-ethylcyclopropyl moiety of coronatine, and $< 6\%$ was in the coronafacoyl moiety. These findings are used to propose a biosynthetic pathway for coronatine.

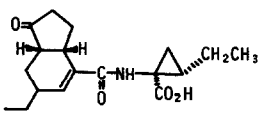
INTRODUCTION

Coronatine (**2**) is a phytotoxin produced in liquid cultures by the plant pathogenic bacteria *Pseudomonas syringae* pv. *atropurpurea* (Reddy & Godkin 1923) Young, Dye & Wilkie 1978 [1], and *Pseudomonas syringae* pv. *glycinea* (Coerper 1919) Young, Dye & Wilkie 1978 [2], pathogens of Italian ryegrass (*Lolium multiflorum* Lam.) and soybean (*Glycine max*), respectively. Other chemically related compounds produced by certain strains of these organisms are coronafacic acid (**1**) [1] and coronafacoylvaline (**3**) [3]. The co-production of the three compounds **1**, **2** and **3** by a strain of *P.s. atropurpurea* 4328 [3] suggests that one of the steps on a biosynthetic pathway to coronatine could be a coupling of coronafacic acid and isoleucine to form coronafacoylisoleucine (**4**), a homologue of **3** which, however, does not accumulate.

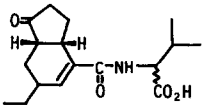
Experiments reported here were conducted to measure the incorporation of amino acids into coronatine. The results obtained allow a biosynthetic pathway to coronatine to be formulated.



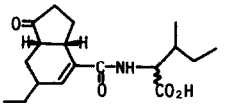
1 coronafacic acid



2 coronatine



3 N-coronafacoylvaline



4 N-coronafacoylisoleucine

RESULTS AND DISCUSSION

Interpretation of the results of experiments to test possible precursors to coronatine should be strengthened if some assessment can be made of the degree of randomization of the label from the precursor. With this objective, *P.s. atropurpurea* 4328 was chosen for the study. Since this organism has previously been found [3] to produce approximately equal amounts of coronatine and coronafacoylvaline in liquid culture, it is eminently suitable for studies to evaluate the specificity of incorporation of ^{14}C-labelled precursors into either of these two products. Furthermore, with the assumption that coronafacoylvaline does not lie on the biosynthetic pathway to coronatine, then at least one of these two products in any labelling experiment should be an indicator of randomization of the ^{14}C-label.

When L-[U-^{14}C]valine was added to a growing liquid culture of *P.s. atropurpurea* 4328, there was a 16-fold greater incorporation of ^{14}C into coronafacoylvaline compared with coronatine (Table 1). This result demonstrates that valine was not a direct precursor of either the cyclopropyl or the coronafacoyl moieties of coronatine, and that under the experimental conditions used there was little metabolic degradation then re-utilization of exogenous [^{14}C]valine. It also suggests that the valine moiety of coronafacoylvaline is incorporated directly as valine.

When either L-[U-^{14}C]isoleucine or L-[U-^{14}C]threonine was added to growing liquid cultures of *P.s. atropurpurea* 4328, ^{14}C was substantially incorporated into coronatine (1.81, 1.11%, respectively), but only slightly into coronafacoylvaline (0.024, 0.023%, respectively). Since the coronafacoyl moiety is common to both coronatine and coronafacoylvaline, the selective incorporation of radioactivity from [^{14}C]isoleucine or [^{14}C]threonine into coronatine but not into coronafacoylvaline suggested that the coronafacoyl moiety of the radioactive

Table 1. Incorporation of ^{14}C into coronatine and coronafacoylvaline by *Pseudomonas syringae* pv. *atropurpurea*

Assayed metabolite	^{14}C (cpm × 10^{-3}) incorporated* from 10 μCi amino acid: Ile	Thr	Val
Coronatine	390.5	238.6	1.7
Coronafacoylvaline	5.2	5.0	27.5

Ten μCi of L-[U-^{14}C]Ile, or L-[U-^{14}C]Thr, or L-[U-^{14}C]Val was added to liquid cultures of *P.s. atropurpurea* 4328 2.7 days after inoculation, then the products were isolated after a further 18 hr.

*Percentage incorporations into coronatine were 1.81% from Ile and 1.11% from Thr. Percentage incorporation into coronafacoylvaline was 0.13% from Val.

coronatine would be unlabelled, and that the radioactivity was specifically incorporated into the 1-amido-1-carboxy-2-ethylcyclopropyl moiety. This interpretation was tested by measuring the radioactivity of the products from acid hydrolysis of the [^{14}C]coronatine derived from [^{14}C]isoleucine and from [^{14}C]threonine (Table 2). In each experiment, coronafacic acid, the major hydrolysis product* in the ethyl acetate-soluble carboxylic acid fraction was found to have 5–6% of the total radioactivity recovered in the experiment, while coronamic acid† (1-amino-1-carboxy-2-ethylcyclopropane), obtained in the water-soluble fraction, had 94–95% of the recovered radioactivity.

These results demonstrate that there are two distinctly separate biosynthetic origins for the two structural moieties of coronatine; the coronamic acid moiety is derived via threonine–isoleucine amino acid metabolism whereas the origin of the coronafacoyl moiety remains unknown. The latter may, however, be derived from the metabolism of glucose via the shikimic acid pathway, with a branching point prior to the usual aromatization sequences leading to the characteristic end-products of the shikimate pathway.

The separate biosynthetic origins for the two structural moieties of coronatine, together with the knowledge that one structural component, coronafacic acid, is excreted into the medium, suggest that coronafacic acid is an intermediate in the pathway to coronatine and that this is coupled to an amino acid, probably isoleucine, by amide-bond formation. Indeed, the incorporation of both threonine and isoleucine into coronatine, and the higher incorporation rate recorded for isoleucine suggest that the amino acid that is coupled to a coronafacoyl compound is biosynthetically at or beyond isoleucine, i.e. threonine *per se* is not coupled but rather is metabolized, at least to isoleucine, prior to the coupling step. Furthermore, a compound having the chemical characteristics of coronafacoylisoleucine (**4**) has recently been isolated from a coronatine-producing pseudomonad (Mitchell, R. E., unpublished data), consistent with the proposal that **4** is a biosynthetic intermediate to coronatine which precedes the steps involving ring-closure to the cyclopropane compound. However, there is, as yet, no experimental validation of this proposal. Alternative possibilities are coupling with an isoleucine derivative, intermediate to the cyclopropyl ring formation, or coupling in the final biosynthetic step with the cyclopropyl amino acid, coronamic acid. Further studies will be directed towards testing these various alternatives.

Collectively, the present results indicate a possible sequence to the biosynthesis of coronatine, summarized as follows: coronafacic acid (**1**) + isoleucine → coronafacoylisoleucine (**4**) → unknown intermediate → coronatine (**2**). This points to the possibility of a parallel cyclopropyl ring formation in the compound coronafacoylvaline (**3**) to form a methyl-substituted cyclopropane (norcoronatine) analogous to the ethylcyclopropane of coronatine. Recent studies (Mitchell, R. E., unpublished data) have been directed towards examination of the minor components of coronatine-producing pseudomonads and indicate that a low concentration of norcoronatine does exist in nature.

EXPERIMENTAL

General procedures have been described previously [3]. Radioactivity was measured without quench correction at 97%

*By GLC analysis, which also showed that the hydrolysis of coronatine was essentially complete (> 98%).

†In each hydrolysis experiment, this product contained *ca* 87% of the radioactivity of the parent [^{14}C]coronatine and consisted of a single homogeneous ^{14}C-labelled component by two-dimensional (2D) thin-layer electrophoresis (TLE)/TLC, identical to coronamic acid obtained from coronatine.

Table 2. Radioactivity of the products of hydrolysis of [^{14}C]coronatine derived from [^{14}C]Ile and [^{14}C]Thr, and of [^{14}C]coronafacoylvaline derived from [^{14}C]Val

^{14}C-Labelled metabolite (from ^{14}C-labelled precursor)	Radioactivity (cpm × 10^{-3}) hydrolysed	Radioactivity (cpm × 10^{-3}) of hydrolysis products in: EtOAc extract*	aq. phase remaining†
Coronatine (Ile)	195.3	8.4	171.2
Coronatine (Thr)	119.3	6.2	103.4
Coronafacoylvaline (Val)	12.7	0.76	10.4

*Coronafacic acid by GC.

†Coronamic acid from coronatine samples, and valine from coronafacoylvaline sample, by 2D TLE/TLC.

efficiency using a Beckman LS2800 liquid scintillation counter.

Culture. The culture used was *P.s. atropurpurea* 4328, as in ref. [3]. The method of liquid culture is described in refs. [2, 4, 5] for *P.s. glycinea.* In the present experiments, 300 ml medium for each ^{14}C-labelling experiment (in 1 l. flasks) was inoculated with 1 ml of a 24 hr yeast inoculum culture of the organism, and allowed to grow at 18° for 64 hr; this brought the culture to early log-phase of growth. In each experiment, 0.35 μmol of unlabelled L-amino acid (either Ile, Thr, or Val) was then added, followed after 3.5 hr by the same amino acid ^{14}C-labelled (10 μCi; *ca* 0.035 μmol). After 2 hr, a further 0.35 μmol of the unlabelled amino acid was added and the culture was harvested after a further 18 hr.

Isolation of carboxylic acids. The culture was centrifuged at 16000 *g* for 30 min and the supernatant concd (rotary evapn, bath temp. 40°). The carboxylic acid fraction was isolated from the concentrate by a reduced scale of the method described in ref. [3]. Yields of the acids obtained were 7.4, 3.7 and 2.5 mg, respectively, from Ile, Val and Thr.

Purification of coronatine and coronafacoylvaline. Coronatine and coronafacoylvaline were purified by prep. TLC (0.5 mm, then 0.25 mm) [3]. Autoradiography (of the TLC plates) revealed that either coronatine or coronafacoylvaline only was radioactive in a given feeding experiment. The radioactivities of the purified products were measured (Table 1) and the identity of each was confirmed by GLC analysis of its methyl ester.

Acid hydrolysis of [^{14}C]coronatine. Half of the coronatine derived from [^{14}C]Ile or [^{14}C]Thr (1.953×10^5 cpm or 1.193×10^5 cpm, respectively) was sealed in a glass tube with 0.8 ml 6 M HCl and heated in steam for 6 hr. The contents of the tube were then partitioned between 1 ml H_2O and 5 ml EtOAc, and the aq. phase was extracted with a further 3×5 ml EtOAc. Combined EtOAc extracts were dried (Na_2SO_4) and evapd, and the residue was dissolved in 0.5 ml EtOAc; aliquots were taken for radioactivity measurement (Table 2), and for methylation and GC, which showed the presence of mainly coronafacic acid.

The aq. phase remaining from the acid hydrolysis was evapd to dryness and the residue dissolved in 0.5 ml 1:1 MeOH–H_2O; aliquots were taken for radioactivity measurement (Table 2), and for 2D TLE/TLC, which showed a single ^{14}C-labelled product with R_E 0.95 and R_C 1.55 (relative to alanine) [6] that gave an orange colour after reaction with ninhydrin, properties characteristic of coronamic acid.

Acid hydrolysis of [^{14}C]coronafacoylvaline. This was hydrolysed as described above for [^{14}C]coronatine (Table 2).

Acknowledgement—Excellent technical assistance from Edith J. Frey is much appreciated.

REFERENCES

1. Ichihara, A., Shiraishi, K., Sato, H. and Sakamura, S. (1977) *J. Am. Chem. Soc.* **99**, 636.
2. Mitchell, R. E. and Young, H. (1978) *Phytochemistry* **17**, 2028.
3. Mitchell, R. E. (1984) *Phytochemistry* **23**, 791.
4. Mitchell, R. E. (1982) *Physiol. Plant Pathol.* **20**, 83.
5. Hoitink, H. A. J. and Sinden, S. L. (1970) *Phytopathology* **60**, 1236.
6. Mitchell, R. E. (1976) *Phytochemistry* **15**, 1941.