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CORONAMIC ACID, AN INTERMEDIATE IN CORONATINE BIOSYNTHESIS BY *PSEUDOMONAS SYRINGAE*

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Key Word Index—*Pseudomonas syringae* pv. *glycinea*; coronatine; biosynthesis; coronamic acid; 2-ethyl-1-aminocyclopropane-1-carboxylic acid.

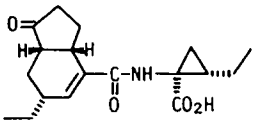
Abstract—Experiments were undertaken on the biosynthetic pathway to coronatine in *Pseudomonas syringae* pv. *glycinea*, using mutants blocked in coronatine synthesis, wild type strains and radioactive labelling with L-[U-^{14}C] isoleucine and ^{14}C-coronamic acid. Evaluation of the kinetics of coronatine appearance in the medium revealed an initial exponential phase with a maximum rate of 0.86 μmol hr^{-1} culture^{-1} after 3.1 days growth. Beyond this the rate declined to a steady rate of 0.20 μmol hr^{-1} culture^{-1}. With *P. syringae* 4185, 8.6% of L-[U-^{14}C] isoleucine added at three days growth was incorporated into coronatine during a 90 min exposure period. At the same time there was negligible incorporation (0.04%) to coronamic acid. Two mutants blocked in coronatine synthesis were shown to produce coronamic acid by isolation and purification of this product from culture supernatants. L-[U-^{14}C]Isoleucine was added to one of these mutants at four days growth and 1.9% of the radioactivity was incorporated into coronamic acid, these data showed that coronamic acid is a distinct biosynthetic entity. However, the coronatine-producing strain from which these mutants were derived, *P. syringae* 4180, showed only 0.08% incorporation into a coronamic acid fraction, although culture supernatants after five days growth did reveal a trace of coronamic acid as detected by ninhydrin after electrophoresis and chromatography on thin layer cellulose plates. When a three-day culture of *P. syringae* 4185 was fed coronamic acid prior to ^{14}C-isoleucine, a 3.7-fold reduction in the incorporation of label into coronatine occurred; instead the radioactivity was diverted to extracelluar coronamic acid which showed 7% incorporation. This result demonstrates that coronamic acid is a biosynthetic intermediate of coronatine. *Pseudomonas syringae* 4185 very efficiently utilized ^{14}C-coronamic acid, giving 94% incorporation into coronatine after 90 min exposure in a four-day culture, showing the specific utilization of this compound. Other experiments were undertaken to evaluate the possible involvement of coronafacoylisoleucines in the biosynthesis of coronatine, by use of a cor$^-$ mutant blocked in coronafacic acid synthesis, with the conclusion that this was not an operative pathway to coronatine.

INTRODUCTION

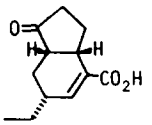
Several pathovars of *Pseudomonas syringae*, pathogenic on a range of host plants, produce the chlorosis-inducing phytotoxin coronatine (**1**) [1, 2]. The general importance of coronatine to organisms capable of producing it is clearly indicated by a report of enhanced pathogenicity it imparts [3]. Our recent work has thus been directed towards determining the molecular and biochemical basis for coronatine production. The present paper reports more detail on the biosynthesis of coronatine specifically relating to the amino acid pathway and the point where coupling to coronafacic acid (**2**) occurs.

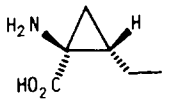
Previous work has shown that the two structural entities of coronatine are derived from different biosynthetic pathways, the cyclopropane structure being of amino acid (isoleucine) origin [4, 5] and the coronafacoyl moiety being of polyketide origin [5]. The end-product from the polyketide pathway, coronafacic acid (**2**), is often excreted as a co-metabolite with coronatine [4, 6]. In contrast, there is no evidence for the free existence of an end-product of the amino acid pathway, e.g. 2-ethyl-1-aminocyclopropane-1-carboxylic acid (**3**, coronamic acid). An accessible source of this compound to date has been from the chemical hydrolysis of coronatine.

Construction of the final coronatine structure requires coupling (amide bond formation) between coronafacic acid and an amino acid component. Two routes are possible (Fig. 1), the first yielding coronatine from coupling of coronafacic and coronamic acids in the final step (Fig. 1, route A), the second involving coupling of coronafacic acid and alloisoleucine at an earlier stage to form coronafacoylalloisoleucine, followed by cyclopropane ring formation resulting in coronatine (Fig. 1, route B). Although there has been no definitive evidence for either of these two routes, the latter was suggested on the basis of the natural occurrence of *N*-coronafacoyl-L-isoleucine



1 coronatine





2 coronafacic acid 3 coronamic acid

and *N*-coronafacoyl-L-alloisoleucine in the bacterial fermentation [7], while the former process has sometimes been implied to be operative [8]. The genes for coronatine biosynthesis have been found to reside in a 31 kb region of plasmid DNA in *Pseudomonas syringae* pv. *glycinea* 4180 [9–11]. Several mutants that were blocked in coronatine biosynthesis were isolated and their phenotype characterized, initially by co-cultivation experiments, and then by substrate feeding experiments. Two mutants in particular were regarded to be important for biosynthetic studies; the phenotypes of these were coronafacate$^+$, cyclization$^-$ and coupling$^+$ (PG4180.CO), and coronafacate$^-$, cyclization$^+$ and coupling$^+$ (PG4180.E9) [10]. Preliminary evidence from experiments with these mutants favoured the involvement of coronamic acid rather than coronafacoylisoleucine in the biosynthetic route [10]. However, more extensive proof was not available from this work. We herewith report on results of experiments which clearly prove the pathway operating in the biosynthesis of coronatine.

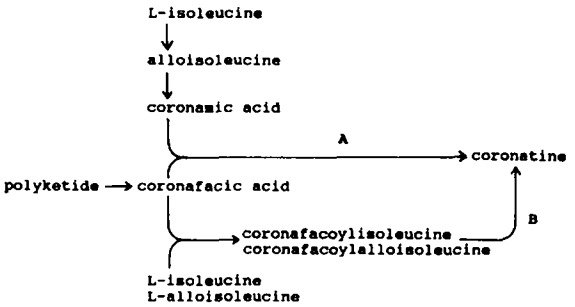


Fig. 1. Partial biosynthetic routes to coronatine.

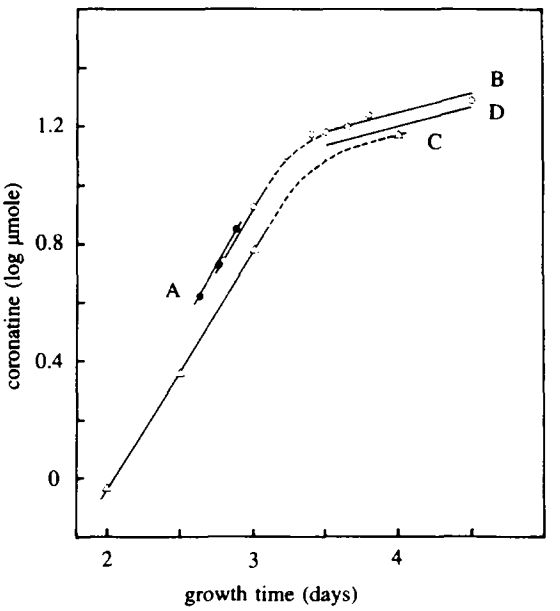


Fig. 2. Coronatine production in *Pseudomonas syringae* pv. *glycinea* 4185. A–C are different data sets, while D is the calculated slope for steady coronatine production of 0.20 µmol hr^{-1}.

RESULTS AND DISCUSSION

Aspects of coronatine production by P. syringae 4185

Coronatine was detected and measured in *P. syringae* 4185 supernatants as early as two days, when the culture was at an early stage of its growth. Between 2.0 and 3.1 days of growth coronatine was produced at an exponential rate (Fig. 2), and thus the rate of coronatine synthesis during this period was increasing with time. At *ca* 3.1 days, coronatine synthesis reached a maximum rate of 0.86 µmol hr^{-1} culture^{-1} (Fig. 2, data set B). However, between 3.1 and 3.4 days the rate declined to a level that approximately matched a calculated rate of *ca* 0.20 µmol hr^{-1} culture^{-1} during the next 24 hr–4.5 days (Fig. 2).

The data of Fig. 2 allow the rate of utilization of precursors at particular times to be calculated on the basis of unrestricted access (uptake). Thus, at 3.0 days growth, production of coronatine is 0.48 µmol hr^{-1} culture^{-1} (data set C, Fig. 2). If the per cent incorporation into coronatine from 2 µmol isoleucine is an arbitrary 10% (therefore yielding 0.2 µmol coronatine), the utilization of precursor by the cell could be complete in 25 min. We chose to use an exposure period of 90 min, which was 3.6-fold in excess of that required for 10% incorporations. This was found to be a satisfactory protocol, since in our analyses of the products, the precursors were not present in the amino acid fractions isolated from the supernatants after a 90 min exposure period. Also the exposure period adopted was very adequate for incorporations of $< 10\%$ that were recorded.

These data for coronatine production have application in experiments aimed at preparation of radioactive coronatine. For example, if the precursor is administered in the late exponential phase of coronatine synthesis (near

the maximum rate of synthesis), then maximum incorporation of radioactivity into the product may well be achieved. However, it is likely that coronatine producers will vary in the time period for the maximum rate of production to be achieved. In our experience we found difficulty in precise replication of these experiments with respect to the time scale, where variation was presumably arising in the inoculum density and the lag time prior to commencement of coronatine synthesis.

Incorporations into coronatine and carrier coronamic acid from ^{14}C-L-*isoleucine*

The relatively high incorporations of ^{14}C-L-isoleucine into coronatine with both *P. syringae* 4180 and 4185 (Table 1) were consistent with this amino acid being a precursor to the cyclopropyl moiety of coronatine as determined in earlier work [4, 5]. Incorporation data were measured on the acid fraction as obtained from the extraction procedure. To ensure the validity of these data, the coronatine extracted in one experiment was purified by silica CC, and a comparison of specific activities (Table 2) showed a close correspondence (5–6%) between the samples. For this reason, radioactivity data for coronatine in all remaining experiments were recorded on the acid extracts without further purification.

Radioactivity levels were also determined in coronamic acid that may have remained in the aqueous phase, in the event that this was a secreted product, with the use of carrier coronamic acid (unlabelled) added during the work-up of the product. The carrier coronamic acid was subsequently isolated and purified, and its radioactivity determined (Table 1). Although these data (0.08% incorporation) gave no conclusive evidence that coronamic acid was an excreted product, they did suggest that very low levels of coronamic acid may transfer from the cell to the external medium during the synthesis of coronatine.

Coronamic acid production in wild type and mutant P. syringae *strains*

Mutant strains blocked in coronatine synthesis may produce end-products which function as intermediates in the coronatine pathway, depending on the nature of the genetic block. The two mutants PG4180.E9 and PG4180.G70 were obtained from previous work by Tn5 mutagenesis, and their phenotypes were evaluated to be, respectively, coronafacate$^-$, coupling$^+$ and cyclization$^+$ [10], and coronafacate$^+$, coupling$^-$ and cyclization$^+$ [11]. In previous work the coronafacate phenotype was determined directly by the chemical presence or absence of this compound, while the cyclization phenotype was determined by either coronatine synthesis (E9 fed with coronafacic acid) or by co-cultivation experiments [10, 11]. In the present study, we examined these mutants directly for coronamic acid production. For each mutant, the supernatant obtained from a four-day culture yielded an amino acid fraction and from this a purified product was obtained (2.3 mg in each case) that was identical with coronamic acid in comparisons by TLC and TLE. For each of these products, FAB MS gave an $[M+1]^+$ ion at m/z 130, as did authentic coronamic acid.

Table 1. Incorporation of ^{14}C from ^{14}C-L-isoleucine into coronatine and coronamic acid by wild-type and mutant strains of *P. syringae*. Cultures after three or four days growth were fed with 2.0 μmol and 5 μCi of ^{14}C-L-isoleucine, and after 90 min were harvested and the products isolated

		^{14}C Incorporation into	
Strain	Growth stage days	Coronatine %	Coronamic acid %
4185	3	8.6	0.04
4180	4	8.7	0.08*
4180.G70†	4	(0.4)‡	1.9

*In this experiment unlabelled coronamic acid was used as a radioactive carrier in the isolation and purification of product.

†Cor$^-$ Tn5 mutant derived from *P. syringae* 4180, with the phenotype coronafacate$^+$, cyclization$^+$, coupling$^-$.

‡Value determined for crude carboxylic acid fraction. GC analysis did not show the presence of coronatine.

Table 2. Comparison of radioactive purity of coronatine in ethyl acetate extracts and after purification. Coronatine extracts from cultures of *P. syringae* 4185 were purified by CC on silica gel and their specific activities compared

	Specific activity of coronatine in		
Growth stage days	Acid extract cpm μmol^{-1} $\times 10^{-4}$	SiCC Prod., cpm μmol^{-1} $\times 10^{-4}$	% Increase
2.9	10.60	11.25	6.1
3.4	5.85	6.17	5.5

When PG4180.G70 was fed with ^{14}C-L-isoleucine for 90 min after four days growth, the radioactivity distribution into coronamic acid was 1.9% (Table 1) and 0.4% into the organic acid fraction, which was shown to be void of coronatine by GLC examination. These data (Table 1) demonstrate that when coronatine synthesis is genetically blocked outside of the amino acid pathway, coronamic acid indeed accumulates as a metabolic product of the organism and provides the first evidence that coronamic acid is a discrete biosynthetic entity.

In contrast to the mutant strains, the parent strain *P. syringae* 4180 did not produce comparable levels of coronamic acid. The amino acids isolated from the supernatant were fractionated on a column of LH20 Sephadex and then fractions spanning the elution range for coronamic acid were closely examined for the presence of coronamic acid on both TLE and TLC by spotting 5 and 10% of each fraction collected. In this way, coronamic acid was positively detected, although not unequivocally established, and from the colour intensity the total level present in a 600-ml culture was estimated to

be *ca* 5 μg. This equates to a very low concentration of coronamic acid, and is probably the main reason why this compound has not previously been reported as a naturally occurring product.

Effects of exogenous coronamic acid on ^{14}C-isoleucine incorporation into coronatine

The preceding evidence utilizing mutants was consistent with the requirement for coronamic acid in a coupling process with coronafacic acid to form coronatine. However, for the wild-type parent strain, *P. syringae* 4180, the data obtained above for the existence of free coronamic acid was equivocal due to the low level isolated. Rather than undertake an enormous scale-up of the experiment to produce sufficient purified material for more rigorous evidence to be obtained, we sought alternative evidence for the involvement of coronamic acid in coronatine biosynthesis by wild-type strains of *P. syringae*. If coronamic acid truly lay on the pathway to coronatine, then an exogenous source of coronamic acid would probably interfere with the flow of radioactivity into coronatine when cultures were supplied with a labelled earlier precursor in the pathway, on the proviso that the exogenous pool of coronamic acid led to an effective concentration within the cell. We therefore compared the effects of a few selected amino acids, including coronamic acid, on the incorporation of ^{14}C from ^{14}C-L-isoleucine into coronatine (Table 3). Exogenous coronamic acid caused a substantial reduction to the flow of ^{14}C through to coronatine, which was not the case with threonine, or with 1-aminocyclopropane-1-carboxylic acid (ACC). Two repeats of this experiment gave comparable reductions in the case of coronamic acid (3.2-fold, 3.3-fold), but not with the other two amino acids. These results imply that ^{14}C-coronamic acid was a biosynthetic intermediate and show that intracellular ^{14}C-coronamic acid has been substantially influenced (diluted) by the extracellular pool of (added) coronamic acid.

Effect of exogenous coronamic acid on biosynthetic activity

Because of the reduced radioactivity incorporated from ^{14}C-L-isoleucine into coronatine in the presence of exogenous coronamic acid we sought more data on the fate of the ^{14}C-isoleucine supplied, since this was still taken up and utilized as in the control experiment where no coronamic acid was added. The aqueous phase remaining after extraction of the organic acids, and the amino acid fraction isolated from this, both showed elevated radioactivity. Fractionation of the amino acids by chromatography on LH20 Sephadex gave a radioactive peak coincident with unlabelled coronamic acid (as originally added for the experiment), and further purification by ion exchange chromatography on QAE Sephadex yielded ^{14}C-coronamic acid. The incorporation from ^{14}C-L-isoleucine into coronamic acid was 7% (Table 4), and was compared with the incorporation into coronamic acid in the control experiment (0.04% incorporation, Table 4) by isolation and purification of the products after addition of unlabelled coronamic acid to act as radioactive carrier. Thus, these data show that the exogenous coronamic acid has not affected biosynthetic activity in the pathway, since the total incorporation from ^{14}C-L-isoleucine (%coronamate + %coronatine) was maintained (Table 4). The data also demonstrate that there is a free and rapid interchange between the intra- and extracellular pools of coronamic acid, where the exogenous coronamic acid effectively has intercepted and diverted the intracellular ^{14}C-coronamic acid as it has formed. The data provide strong evidence that coronamic acid is the direct amino acid intermediate of coronatine. On repeat of this experiment, similar results were obtained.

Incorporation of ^{14}C-coronamic acid into coronatine

^{14}C-coronamic acid (0.5 μmol) was fed to four-day-old cultures of *P. syringae* 4180 and 4185, and after a 90 min exposure the cultures were harvested and the fate of the radioactivity in the various products was determined (Table 5). High incorporation levels into coronatine (81 and 94% for 4180 and 4185, respectively) were calculated from the data, and almost complete recovery of radioactivity was obtained (Table 5). The utilization of 0.5 μmol of coronamic acid within 90 min, and most of this appearing in the end-product coronatine, indicates that *P. syringae* uses coronamic acid very efficiently, and that its involvement in coronatine biosynthesis is very specific.

Table 3. Effects of exogenous amino acids on ^{14}C-incorporation from ^{14}C-L-isoleucine into coronatine by *P. syringae* 4185. After three days growth, the medium was supplemented with 20 μmol of amino acids coronamic acid, or ACC, or threonine, then after 5 min 5 μCi (0.2 μmol) of ^{14}C-L-isoleucine was added, the culture was harvested after a further 90 min growth

Added amino acid	Radioactivity isolated in organic acids, cpm × 10^{-6}	Incorporation %	Change (over control)
Nil (^{14}C-Ile only)	0.842	8.6	
Coronamic	0.230	2.3	−3.7-fold
Threonine	1.038	10.5	+1.2-fold
ACC*	0.655	6.7	−1.3-fold

*1-Aminocyclopropane-1-carboxylic acid.

Experiments with cor⁻ mutant PG4180.E9 using unlabelled substrates

The phenotype of PG4180.E9, (coronafacate⁻, cyclization⁺, coupling⁺) made it ideally suited for testing the second pathway outlined in Fig. 1, which requires cyclization of coronafacoylalloisoleucine in the final step. Previous work has shown that this mutant is cor⁻ because it is defective in coronafacic acid synthesis, based on the resulting coronatine production on supplying cultures of the mutant with coronafacic acid [10]. This result demonstrated that the organism maintains the mechanism to couple amino acids to coronafacic acid, and also to complete the cyclization step to form the cyclopropane ring. It also showed that coronafacic acid was freely taken up by the mutant. When 4180.E9 was supplied with coronafacic and coronamic acids and harvested after a further 1.7 days growth, the coronatine level was sevenfold higher than a culture supplied with coronafacic acid alone. This indicated that the mutant indeed did have an affinity for coronamic acid as a substrate. In marked contrast to these results, when either coronafacoyl-L-isoleucine or coronafacoyl-L-alloisoleucine were supplied to the mutant under the same conditions, GC analyses of the products showed predominantly the presence of unaltered coronafacoylisoleucine or coronafacoylalloisoleucine, and an absence of a component that could be assigned as coronatine. This result indicated that either these compounds could not enter the cell, or they were not substrates for cyclopropane ring formation. However, since free coronafacic acid is taken up, it seems likely that the coronafacoyl compounds would also be assimilated by the cells. On this basis we favour the latter explanation and conclude that the pathway involving coronafacoylisoleucines is not operative in coronatine biosynthesis. For this reason further extension to this line of experimentation (such as with synthetically derived labelled precursors) was not pursued.

Table 4. Effect of exogenous coronamic acid on the incorporation of ^{14}C-L-isoleucine into coronatine by *P. syringae* 4185. Additions were made after three days growth and cultures harvested following exposure to ^{14}C for 90 min

	^{14}C Incorporation into		
Additive	Coronamate %	Coronatine %	Total pathway* %
+ ^{14}C-L-Ile only			
Expt 1	0.04	8.6	8.6
Expt 2	0.04	6.7	6.7
+ Unlabelled coronamate then + ^{14}C-L-Ile			
Expt 1	7.0	2.3	9.3
Expt 2	6.8	2.0	8.8

*% Into coronamate + % into coronatine.

Biosynthesis of norcoronatine

The results reported here invite comment on the likely variation in the coronatine pathway that would give rise to the coronatine analogue, norcoronatine, which is a phytotoxin produced in minor quantity by *P. syringae* pv. *glycinea* [2]. Norcoronatine has a 2-methyl cyclopropane ring substituent in place of the 2-ethyl substituent of coronatine. Therefore, by analogy with coronatine, it is probable that the cyclopropane amino acid norcoronamic acid is the biosynthetic intermediate which is coupled to coronafacic acid to form norcoronatine. Norcoronamic acid itself may be derived by cyclopropane ring formation in L-valine by the same mechanism that yields coronamic acid.

Conclusions

Coronamic acid, 2-ethyl-1-aminocyclopropane-1-carboxylic acid, is a biosynthetic amino acid product of *P. syringae* pv. *glycinea* secreted in only minute amounts and therefore not usually detectable. Instead, coronamic acid is the functional/active intermediate in the biosynthesis of the phytotoxin coronatine, and *in vivo*, it apparently very efficiently couples to the polyketide coronafacic acid to form coronatine which is excreted from the cell. In mutants which retain the amino acid pathway but where coronatine synthesis has been blocked, either by interruption of coronafacic acid synthesis, or by a block in the coupling function, coronamic acid synthesis still proceeds. In the absence of its further utilization, the compound is secreted from the cell in measurable quantities. We conclude that an alternative pathway involving coronafacoylisoleucine is not operative.

Table 5. Incorporation of ^{14}C into coronatine from ^{14}C-coronamic acid in *P. syringae* 4180 and 4185. Four-day-old cultures were supplied with 0.032 μCi and 0.5 μmol of ^{14}C-coronamic acid for 90 min, then harvested and the products isolated

	^{14}C Incorporation into			
Strain	Coronatine %	Neutrals %	Radioactivity in ion exchange fraction %	Total radioactivity recovered %
4180	80.8	0.7	11.3	92.8
4185	93.7	0.7	4.3	98.7

EXPERIMENTAL

Bacterial strains and culture. Two strains of *P. syringae* pv. *glycinea* used were 4180 and 4185 from the International Collection of Microorganisms from Plants (formerly PDDCC). Strain 4185 was recovered from a lyophilized state by plating on to King's medium B, and maintained on slants of King's medium B held at 4°. Mutant strains were obtained by Tn5 mutagenesis and designated PG4180 with suffixes; information concerning them is reported in refs [10 and 11]. Liquid cultures consisted of 600 ml of the medium of ref. [12], in 2-l conical flasks, inoculated with a 24-hr yeast inoculum culture as described in ref. [12] and grown at 18°.

Labelling experiments. Cultures of *P. syringae* were grown for 3.0 days and then L-[U-^{14}C]isoleucine (2 μmol, 4–5 μCi) or ^{14}C-coronamic acid (as in Table 5) were added. Cultures were harvested 90 min after addition of ^{14}C labelled compounds. In some experiments, unlabelled amino acids (20 μmol) were added to cultures 5 min before addition of a reduced level (0.2 μmol) of L-[U-^{14}C]isoleucine.

Isolation of organic acids. Culture supernatant was concd 25-fold and then extracted with EtOAc as described in ref. [13].

Isolation of amino acids. Culture supernatant, or reconstituted supernatant following extraction of organic acids from its concentrate, was percolated through Amberlite CG 120 $[H]^+$, and the amino acid fr. collected in 15 ml, starting from the point of pH change in elution of the column with 5% NH_3.

Chromatographic methods. GC analysis of Me ester derivatives of organic acids and coronatine is described in ref. [14], using a temp. programme 120° 5 min^{-1}, 10° min^{-1} to 220°. Ion exchange columns were conducted as used previously for the isolation of rhizobitoxine in ref. [15]. Purification of coronatine was by CC on silica gel using freshly distilled EtOH–free $CHCl_3$. Purification of coronamic acid was by chromatography on Sephadex LH20 using 1:1 MeOH–H_2O as solvent, followed by ion exchange chromatography on a column (14 × 2.6 cm) of QAE Sephadex (12.5 g) and elution with a gradient of NH_4HCO_3 (2 × 300 ml, 0.02 – 0.3 M); amino acid composition of frs was determined by either thin layer electrophoresis or TLC on cellulose plates as described in ref. [15].

Coronatine levels for kinetics, or specific activity determinations, were quantitated by GC as above.

Coronamic acid, for use as a carrier for radioactive biosynthetic product, or radioactive material, was prepared by the acid hydrolysis of coronatine or ^{14}C-coronatine, respectively, and purified as detailed above. ^{14}C-Coronamic acid was quantitated by hydrolysis of coronatine of known specific activity.

Radioactivity of samples was counted in emulsions of 0.5 ml H_2O and 3.1 ml of ACS II aq. counting scintillant (Amersham).

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