Investigations of Coronatine Biosynthesis. Elucidation of the Mode of Incorporation of Pyruvate into Coronafacic Acid

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Coronatine (**1**) (Scheme 1) is a phytotoxin of novel structure that is produced by many pathovars of *Pseudomonas syringae*, including pv. *atropurpurea*,[1,2] pv. *glycinea*,[2,3] and pv. *tomato*.[4] Infection of the host plants by these bacteria induces chlorosis on the leaves due to coronatine production.[1,2] Coronatine also possesses other interesting biological activities including distortion of leaf growth, inhibition of root elongation, and induction of hypertrophy when applied to the cut surface of potato tubers.[5,6] Although coronatine's mode of action and target site remain elusive, structural and functional homologies have been reported between coronatine, methyl jasmonate, and 12-oxophytodienoic acid, suggesting that coronatine may partially function as a molecular mimic of the octadecanoid signaling molecules of higher plants.[7,8]

Coronatine can be hydrolyzed to yield two components, the bicyclic acid **2**, which is called (+)-coronafacic acid, and a cyclopropyl amino acid which has been named (+)-coronamic acid (Scheme 1). Previous biosynthetic investigations in our laboratory have established that coronamic acid is derived by the cyclization of L-alloisoleucine, while coronafacic acid was shown to be a novel polyketide derived from three acetate units, one butyrate unit, and one unit of pyruvate connected in the manner outlined in Scheme 1.[9] The discovery that the hydrindane ring system of coronafacic acid is assembled in this manner provided an interesting mechanistic puzzle, since it appeared that creation of the ring system would require C−C bond formation between three nucleophilic carbon atoms (C-3a, C-4, C-7a). While previous efforts to clarify the mechanism of incorporation of pyruvate into coronafacate were unsuccessful,[9] we now report the results of experiments that provide the solution to this problem.

It occurred to us that pyruvate could be incorporated into coronafacate via the operation of the TCA cycle. This hypothesis, which is outlined in Scheme 2, postulates that, first, pyruvate is carboxylated to oxaloacetate and that the oxaloacetate is then converted into α-ketoglutarate through the usual stages of the TCA cycle. The α-ketoglutarate (or its nonsymmetrical decarboxylation product, succinic semialdehyde) could then serve as the starter unit for assembly of coronafacic acid. This hypothesis possesses several attractive features. It accounts for the labeling pattern produced by administration of pyruvate, it accounts for the link between C-2 of pyruvate and C-2 of acetate found in coronafacic acid, it provides a rationale for the loss of the pyruvate carboxyl group, and it provides a plausible mechanism for the formation of two C−C bonds to C-3 of pyruvate.

Since α-ketoglutarate should be interconvertible with glutamic acid *in vivo*, the hypothesis has been evaluated by utilizing labeled forms of glutamic acid for precursor incorporation experiments. A preliminary evaluation of the hypothesis was carried out by administration of commercial [1,2-$^{13}C_2$]-L-glutamate to *Pseudomonas syringae* pv. *glycinea* P4180 and isolation of coronafacic acid as its methyl ester. This led to the formation of coronafacic acid that exhibited ca. 1.1% enrichment at C-3a, a result that is consistent with the hypothesis shown in Scheme 2 (Table 1, experiment 1). The hypothesis was further evaluated by administration of commercial [U-^{13}C]-L-glutamate. The resulting methyl coronafacate exhibited a complex labeling pattern, which was analyzed at 150.8 MHz. Couplings were observed that appeared to be consistent with the presence of ^{13}C−^{13}C connectivity between C-1, C-2, C-3, and C-3a, but the interpretation was not entirely unambiguous due to the presence of additional couplings. Some of these additional couplings could be attributed to catabolism of the uniformly labeled glutamate to give both doubly-labeled acetate and doubly- or triply-labeled pyruvate which were then reincorporated (data not shown).

In order to provide clearer evidence for the hypothesis, it was decided that glutamate carrying a ^{13}C label at both C-3 and C-4 should be utilized as a precursor. If α-ketoglutarate is indeed the starter unit, then the coronafacic acid derived from this form of labeled glutamate should exhibit ^{13}C−^{13}C connectivity between C-2 and C-3. Since these carbons are known to be derived from C-2 of acetate and C-2 of pyruvate,[9] respectively, a display of ^{13}C−^{13}C connectivity between these carbons would provide particularly strong evidence for the incorporation of pyruvate in the manner outlined in Scheme 2. Therefore [3,4-$^{13}C_2$]-DL-glutamate (**3**) was synthesized (Scheme 3) and administered to *P. syringae*. The outcome of this experiment was very gratifying in that the ^{13}C NMR spectrum of the resulting methyl coronafacate clearly exhibited coupling between C-2 and C-3 ($^1J_{CC}$ = 34 Hz) with the enrichment at each carbon atom corresponding to ca. 1.1%. In addition, couplings were observed between those carbon atoms that are known to be derived from acetate and pyruvate, but the enrichments at these coupled carbon atoms were significantly lower than at C-2 and C-3 (Table 1, experiment 2). This indicates that the observed coupling between C-2 and C-3 is unlikely to be due to the incorporation of labeled acetate and labeled pyruvate into the *same* molecule of methyl coronafacate.

Mitchell has recently described[10] the isolation of the cyclopentenone derivative **4** (Scheme 2) from coronatine-producing species of *Pseudomonas*. Some additional insight into the biosynthesis of coronafacic acid was obtained by examining the ^{13}C-labeling pattern in the methyl ester of **4** isolated from the [3,4-$^{13}C_2$]-DL-glutamate feeding. This compound exhibited ^{13}C−^{13}C coupling between C-2 and C-3 ($^1J_{CC}$ = 35 Hz) with an enrichment at each carbon of ca. 0.89%. The only other coupling that could be observed in the methyl ester of **4** was at C-4 (Table 1, experiment 2). The presence of this coupling suggests that doubly-labeled pyruvate formed by catabolism of the doubly-labeled glutamate has been incorporated into C-3 and C-4 of **4**. Unlike the methyl coronafacate, the methyl ester of **4** did not exhibit couplings attributable to the incorporation

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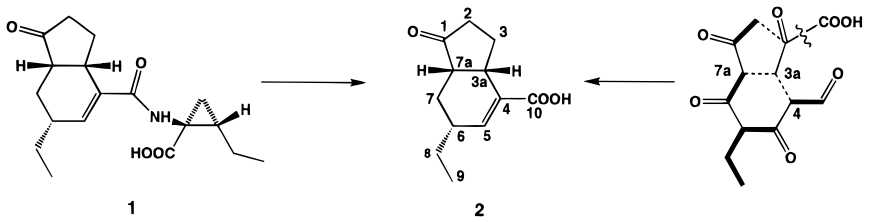
Scheme 1



Scheme 2

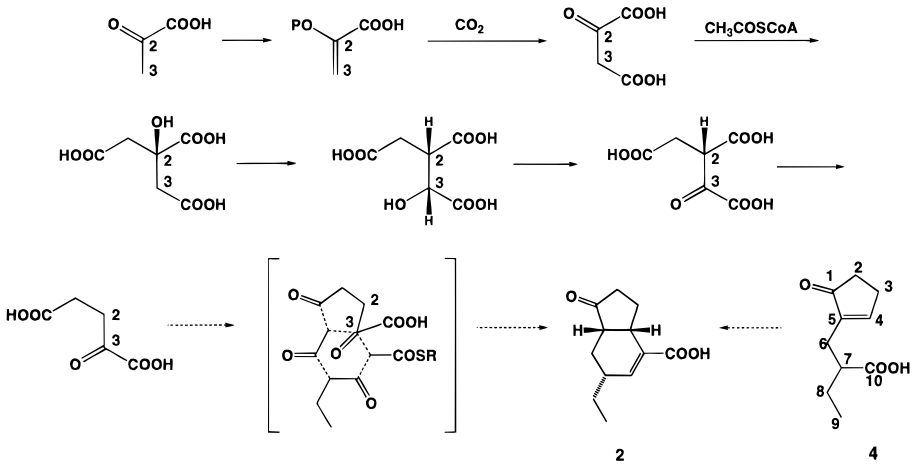
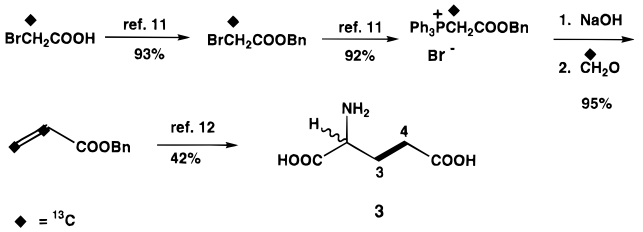


Table 1. Incorporation of Precursors into Methyl Coronafacate (**2**) and Cyclopentenone **4**

expt no.	precursor	labeling pattern in **2** (% enrichment)	$^{13}C-^{13}C$ couplings in **2**	labeling pattern in **4** (% enrichment) $^{13}C-^{13}C$ couplings
1	[1,2-$^{13}C_2$]-L-glutamate	C-3a (1.1)	none	ND[a]
2	[3,4-$^{13}C_2$]-DL-glutamate	C-2 (1.1, 0.20), C-3 (1.1, 0.47); C-1 (0.20); C-3a (0.47); C-4 (0.23), C-10 (0.23); C-5 (0.39), C-6 (0.39); C-7 (0.23), C-7a (0.23); C-8 (0.21), C-9 (0.21)	$J_{2,3} = 34$ Hz; $J_{1,2} = 36$ Hz, $J_{3,3a} = 30$ Hz, $J_{4,10} = 73$ Hz, $J_{5,6} = 41$ Hz, $J_{7,7a} = 30$ Hz, $J_{8,9} = 35$ Hz	C-2 (0.89), C-3 (0.89), $J_{2,3} = 35$ Hz; C-4 (0.44), $J_{3,4} = 35$ Hz
3	[2,3-$^{13}C_2$]succinic acid	C-1 (0.41), C-2 (0.41); C-3 (0.44), C-3a (0.44); C-4 (0.43), C-10 (0.43); C-5 (0.70), C-6 (0.70); C-7 (0.37), C-7a (0.37); C-8 (0.41), C-9 (0.41)	$J_{1,2} = 36$ Hz, $J_{3,3a} = 30$ Hz, $J_{4,10} = 73$ Hz, $J_{5,6} = 41$ Hz, $J_{7,7a} = 30$ Hz, $J_{8,9} = 35$ Hz	ND[a]

[a] Not determined.

Scheme 3



of doubly-labeled acetate. This is probably due to lower levels of acetate incorporation combined with the fact that only a very small amount of **4** (0.5 mg) was available for NMR analysis. In any case, the observed coupling pattern for the methyl ester of **4** derived from [3,4-$^{13}C_2$]-DL-glutamate is completely consistent with that observed in methyl coronafacate. This suggests that **4** is biosynthesized in the same manner as coronafacic acid and that it may be an intermediate in coronafacic acid biosynthesis.

The results of the preceding experiments provide strong support for the hypothesis for coronafacic acid biosynthesis shown in Scheme 2. The evidence also suggests that succinic acid cannot be the starter unit for coronafacate assembly, since [1,2-$^{13}C_2$]-L-glutamate was incorporated nonsymmetrically. Additional proof that succinic acid does not serve as the starter unit was obtained by administration of [2,3-$^{13}C_2$]succinic acid to *P. syringae*. The methyl coronafacate derived from this precursor exhibited a $^{13}C-^{13}C$ coupling pattern that was consistent with catabolism of the precursor to give doubly-labeled acetate and doubly-labeled pyruvate. However, *no coupling was observed between C-2 and C-3 of the methyl coronafacate* (Table 1, experiment 3). This observation indicates that the succinic acid was not incorporated intact into coronafacic acid. The most likely starter unit for coronafacic acid assembly therefore appears to be either α-ketoglutarate or its decarboxylation product, succinic semialdehyde, although the possibility that L-glutamate serves as the starter unit cannot be ruled out at this time.

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