Investigations of the biosynthesis of the phytotoxin coronatine[1]

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This paper is dedicated to Professors Ian Spenser and David B. MacLean

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The biosynthesis of the phytotoxin coronatine has been investigated by administration of isotopically labeled precursors to *Pseudomonas syringae* pv. *glycinea*. The structure of coronatine contains two moieties of distinct biosynthetic origin, a bicyclic, hydrindanone carboxylic acid (coronafacic acid) and a cyclopropyl α-amino acid (coronamic acid). Investigations of coronafacic acid biosynthesis have shown that this compound is a polyketide derived from three acetate units, one butyrate unit, and one pyruvate unit. The two carbonyl oxygen atoms of coronafacic acid were found to be derived from the oxygen atoms of acetate. Additional experiments are described that rule out some possible modes for assembly of the polyketide chain. Coronamic acid is shown to be derived from L-isoleucine via the intermediacy of L-alloisoleucine. Examination of the mechanism of the cyclization of L-alloisoleucine to coronamic acid revealed that the formation of the cyclopropane ring takes place with the removal of only two hydrogen atoms from the amino acid, one at C-2 and the other at C-6. The nitrogen atom at C-2 of L-alloisoleucine is shown to be retained. On the basis of these observations, a mechanism is postulated for the cyclization reaction that involves the diversion of an enzymatic hydroxylation reaction into an oxidative cyclization. Finally, a precursor incorporation experiment with deuterium-labeled coronamic acid demonstrated that free coronamic acid can be efficiently incorporated into coronatine. This observation indicates that the cyclization of L-alloisoleucine to coronamic acid can occur before formation of the amide bond between coronafacic acid and coronamic acid.

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On a étudié la biosynthèse de la phytotoxine coronatine en administrant des précurseurs marqués au *Pseudomonas syringae* pv. *glycinea*. La structure de la coronatine contient deux portions d'origines biosynthétiques différentes : un acide hydrindanone carboxylique bicyclique (l'acide coronafacique) et un acide cyclopropylique α-aminé (l'acide coronamique). Des études sur la biosynthèse de l'acide coronafacique ont montré que ce composé est un polycétide dérivé de trois unités acétique, d'une unité butyrique et d'une unité pyruvique. On a trouvé que les deux atomes d'oxygène carbonyles de l'acide coronafacique sont dérivés des atomes d'oxygène de l'acide acétique. On décrit des expériences additionnelles qui permettent d'éliminer un certain nombre de modes d'assemblage de la chaîne polycétide. On démontre que l'acide coronamique est dérivé de la L-isoleucine par le biais de la L-alloisoleucine comme intermédiaire. L'examen du mécanisme de cyclisation de la L-alloisoleucine en acide coronamique a révélé que la formation du cyclopropane se produit avec l'enlèvement de seulement deux atomes d'hydrogène à partir de l'acide aminé, l'un en C-2 et l'autre en C-6. On a démontré que l'atome d'azote en C-2 de la L-alloisoleucine est conservé. Sur la base de ces observations, on propose un mécanisme pour la réaction de cyclisation qui implique la transformation d'une réaction enzymatique d'hydroxylation en une cyclisation oxydante. Finalement, une expérience d'incorporation d'un précurseur avec de l'acide coronamique marqué au deutérium a permis de démontrer que l'acide coronamique libre peut être incorporé efficacement dans la coronatine. Cette observation indique que la cyclisation de la L-alloisoleucine en acide coronamique peut se produire avant la formation de la liaison amide entre l'acide coronafacique et l'acide coronamique.

[Traduit par la rédaction]

Introduction

Coronatine (**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), pv. *tomato* (4), and pv. *morsprunorum* (2). Infection of the host plants by these bacteria induces chlorosis on the leaves due to the production of coronatine(1,2). In addition, coronatine distorts leaf growth, inhibits root elongation, and causes hypertrophy when applied to the cut surface of potato tubers (5,6). Treatment of plant tissues with coronatine has been shown to stimulate the production of ethylene, and it has been hypothesized that the biological effects of coronatine on plant tissues are due to this stimulative effect (7).

Coronatine can be hydrolyzed to yield two components, the bicyclic acid (**2**), which is called (+)-coronafacic acid, and the cyclopropyl amino acid **3**, which has been dubbed (+)-coronamic acid (Fig. 1). The structure and absolute stereochemistry of coronafacic acid were elucidated by X-ray crystallographic analysis of its 7a epimer (1). The absolute stereochemistry of coronamic acid has been determined by X-ray analysis of its *N*-acetyl derivative (8). The structures of **2** and **3** suggest they should have distinct biosynthetic origins. The results of detailed

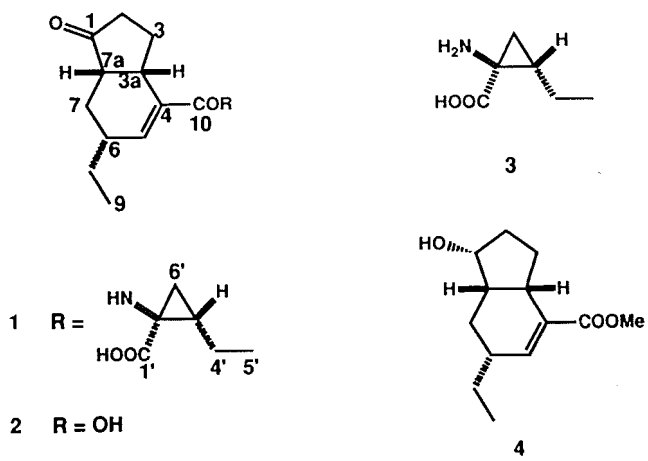

FIG. 1. Structures of coronatine, coronafacic acid, and coronamic acid.

[1]The author is pleased to dedicate this article to Professors Ian Spenser and David MacLean for their friendship, counsel, and many contributions to natural products chemistry.

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TABLE 1. Carbon-13 chemical shifts for methyl ester of coronafacic acid (**2**)

Carbon atom	Shift (ppm)[a]	Carbon atom	Shift (ppm)[a]
1	220.5	7	25.8
2	38.1	7a	46.6
3	28.1	8	27.8
3a	36.2	9	11.2
4	131.3	10	167.3
5	144.2	11	51.7
6	37.7		

[a]Shifts were measured in deuterochloroform at 75.47 MHz.

investigations of the biosynthesis of coronafacic acid and coronamic acid will now be described.[3]

Results and discussion

Investigations of coronafacic acid (**2**) and coronamic acid (**3**) biosynthesis were carried out by administration of labeled precursors to liquid cultures of *Pseudomonas syringae* pv. *glycinea* strains PDDCC 4182 and 4180. These strains produce both coronafacic acid and coronatine when grown in Hoitink–Sinden medium (2). Compounds **1** and **2** were isolated from the culture broth as their methyl esters, after treatment with diazomethane.

Coronafacic acid

To elucidate the biosynthesis of coronafacic acid, it was necessary to assign the resonances present in its ^{13}C NMR spectrum. The complete assignment (Table 1) required the use of several techniques. Treatment of methyl coronafacate with D_2O/DCl led to the disappearance of the ^{13}C signal for C-2 from the noise-decoupled spectrum due to exchange of the hydrogens at C-2 for deuterium. Reduction of methyl coronafacate with lithium tri-*tert*-butoxyaluminum hydride yielded a single alcohol (**4**) (Fig. 1) in which the ^{13}C NMR signals for C-2 and C-7a were shifted upfield to 31.05 and 42.28 ppm, respectively. The hydroxyl group in **4** was assigned the α configuration on the basis of the assumption that the reagent will approach from the less-hindered face of the coronafacate skeleton.

Precursor incorporation experiments also provided a very powerful tool for assignment. The structure of coronafacic acid suggested that the compound was likely to be a polyketide. Therefore, sodium (1-^{13}C)-, (2-^{13}C)-, and (1,2-$^{13}C_2$)acetate were administered to *P. syringae* cultures. The results of these experiments (Table 2, expts. 1–3) clearly indicated that five molecules of acetate were incorporated into **2**. The positions of the labels and their connectivities were unequivocally established by a ^{13}C COSY analysis (10,11) of the methyl coronafacate derived from doubly ^{13}C-labeled acetate.

Since the incorporation experiments with ^{13}C-labeled sodium acetate indicated that C-1 and C-10 of the coronafacate moiety are derived from C-1 of an acetate unit, an experiment was carried out to determine whether or not the carbonyl oxygen atoms present at C-1 and C-10 are derived from the oxygen atoms of acetate. Administration of sodium (1-^{13}C, $^{18}O_2$)acetate yielded a sample of methyl coronatine in which the ^{13}C NMR signals for C-1 and C-10 exhibited upfield ^{18}O isotope-induced shifts of ca. 0.05 and 0.03 ppm, respectively (Table 2, expt. 4). One can therefore conclude that the carbonyl oxygens present at C-1 and C-10 of coronatine are derived from the oxygens atoms of an acetate unit (12).

The presence of an ethyl side chain in coronafacic acid suggested that butyrate might also serve as a specific precursor of this compound, since butyrate has been found to be the source of the ethyl side chains that are present in a number of polyketide antibiotics (13). This expectation was confirmed by administration of sodium (1-^{13}C)butyrate to *P. syringae* and isolation of a sample of methyl coronafacate that exhibited a 38% enrichment at C-5 (Table 2, expt. 5).[4]

The incorporation experiments with labeled acetate demonstrated that two carbon atoms of the coronafacate acid skeleton, C-3 and C-3a, are not derived from acetate. Glycine, glycerol, and pyruvate were therefore evaluated as sources of these carbon atoms. Of these compounds, only pyruvic acid appeared to be specifically incorporated. Administration of sodium (1-^{13}C)-, (2-^{13}C)-, and (3-^{13}C)pyruvate revealed the somewhat surprising fact that C-3 and C-3a of coronafacate are derived from C-2 and C-3 of pyruvate, respectively (Table 2, expts. 6–8). In these experiments, the remaining carbon atoms of coronafacate exhibited a labeling pattern consistent with the partial degradation of pyruvate to acetate. The intact incorporation of C-2 and C-3 of pyruvate into coronafacate was confirmed by administration of (2,3-$^{13}C_2$)pyruvate followed by analysis of the resulting labeled coronafacate by a ^{13}C COSY experiment (Table 2, expt. 9). The COSY experiment revealed the presence of ^{13}C–^{13}C connectivity between C-3 and C-3a, as well as the connectivities expected from degradation of the doubly labeled pyruvate to doubly labeled acetate.

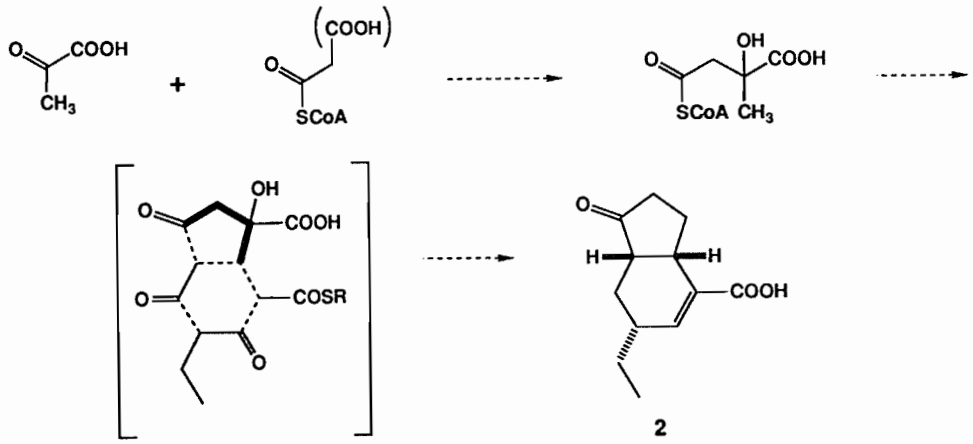
Although the results of preceding experiments reveal the origin of all of the carbon atoms of the skeleton of coronafacic acid, they do not define the starting point for assembly of the polyketide chain, nor do they provide any insight into the mechanism for incorporation of pyruvate into the coronafacate skeleton. A potential solution to both these uncertainties is provided by the hypothesis shown in Scheme 1. This hypothesis postulates that pyruvate serves as the starter unit and that the first step in the assembly of the polyketide chain involves the condensation of acetyl or malonyl CoA with pyruvate to yield the mono CoA ester of citramalic acid (**5**). The plausibility of this hypothesis is enhanced by the fact that both (+)-(*S*) and (–)-(*R*) forms of citramalic acid have been reported to occur naturally (14,15), although the biosynthetic origins of citramalate have apparently never been examined. Initially, we decided to prepare the appropriate mono *N*-acetylcysteamine (NAC) ester of (^{13}C)-labeled (*S*)-citramalate for evaluation as a precursor. The NAC ester was utilized because the NAC esters of a number of polyketide intermediates have been successfully incorporated intact into polyketide natural products (16). The NAC ester of (5-^{13}C)-(*S*)-citramalate (**6**) was synthesized from l-malic acid as shown in Scheme 2*a*. The key steps in the synthesis make use of chemistry reported by Seebach et al. (17) and Schwab and Klassen (18). Unfortunately, administration of **6** to the coronatine fermentation yielded methyl coronafacate that exhibited no

[3]Portions of this work have been the subject of preliminary communications (9).

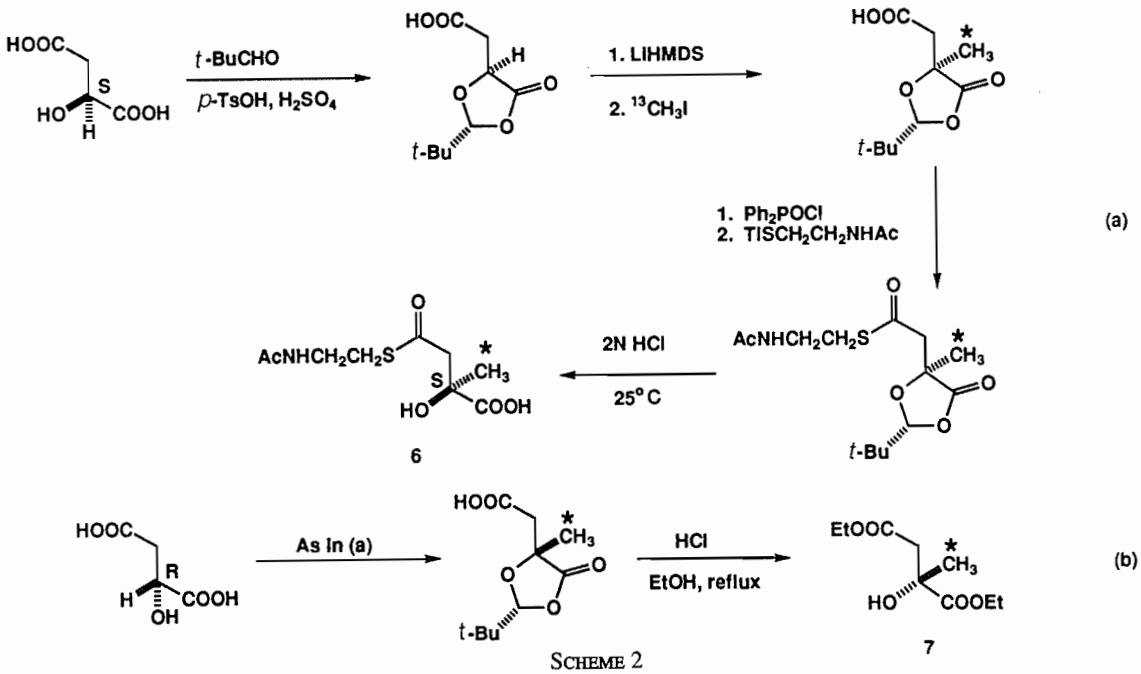
[4]In initial studies of coronafacate biosynthesis (ref. 9), an incorporation experiment with (1-^{13}C)butyrate failed to show enrichment. The reasons for this behavior are unclear.

TABLE 2. Incorporation of precursors into the coronafacic acid moiety (**2**) of coronatine

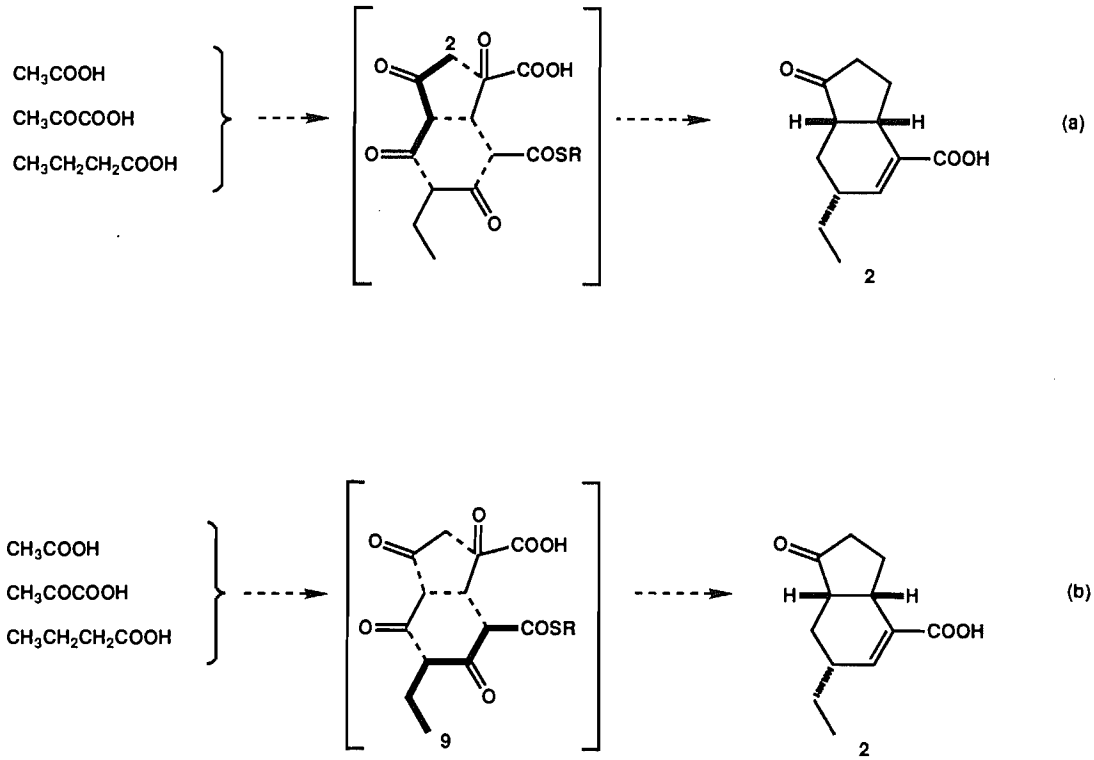
Expt.	Precursor	Labeling pattern
1	Sodium (1-^{13}C)acetate	C-1, C-5, C-7, C-8, C-10
2	Sodium (2-^{13}C)acetate	C-2, C-4, C-6, C-7a, C-9
3	Sodium (1,2-$^{13}C_2$)acetate	Connectivities between C-1, C2; C-4, C-10; C-5, C-6; C-7, C-7a; C-8, C-9
4	Sodium (1-^{13}C, $^{18}O_2$)acetate	Upfield isotope shifts of 0.05 ppm at C-1, 0.03 ppm at C-10
5	Sodium (1-^{13}C)butyrate	C-5
6	Sodium (1-^{13}C)pyruvate	No enrichment
7	Sodium (2-^{13}C)pyruvate	C-3; C-1, C-5, C-7, C-8, C-10
8	Sodium (3-^{13}C)pyruvate	C-3a; C-2, C-4, C-6, C-7a, C-9
9	Sodium (2,3-$^{13}C_2$)pyruvate	Connectivities between C-3, C-3a; C-1, C-2;C-4, C-10; C-5, C-6; C-7, C-7a; C-8, C-9
10	Ethyl (3-^{13}C)acetoacetate	C-8
11	Ethyl (2,3-$^{13}C_2$)acetoacetate	Connectivity between C-6, C-8 ($^1J_{CC}$ = 33.9 Hz)
12	Ethyl (2,3-$^{13}C_2$)-3-ketohexanoate	C-5
13	*N*-Acetylcysteamine ester of (2,3-$^{13}C_2$)-3-ketohexanoate	C-5



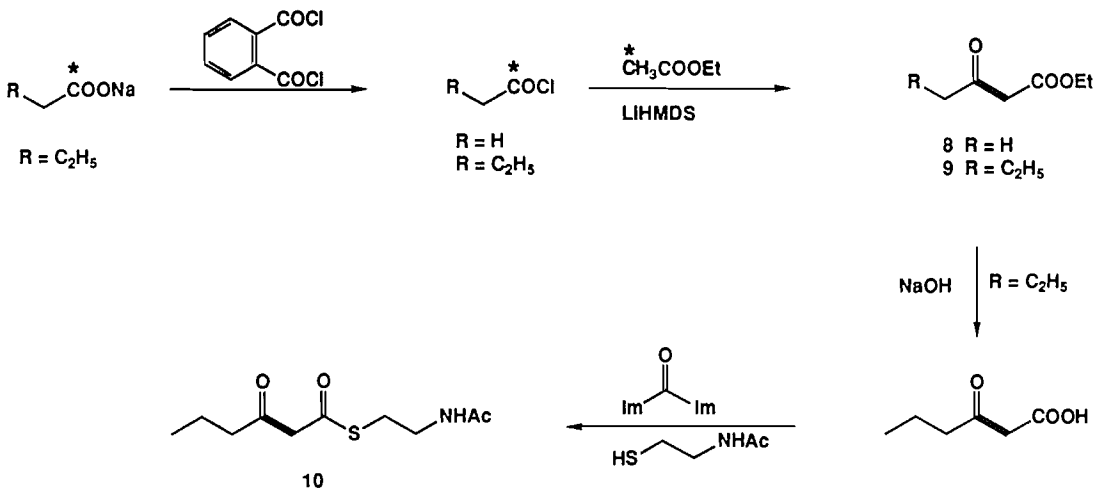
SCHEME 1



SCHEME 2



SCHEME 3



SCHEME 4

evidence of enrichment, not even that resulting from catabolism of the precursor to yield (2-^{13}C)acetate (data not shown). It therefore appeared necessary to examine the (*R*) form of citramalic acid as a precursor. For this purpose, the diethyl ester of (5-^{13}C)-(*R*)-citramalate (**7**) was synthesized (Scheme 2*b*) and evaluated. The diethyl ester was employed since no evidence of catabolism had been observed with the (*S*) precursor **6**. In the event, compound **7** also yielded methyl coronafacate that showed no signs of ^{13}C enrichment (data not shown). The results of these two experiments suggest that the hypothesis for polyketide assembly shown in Scheme 1 is incorrect. Although these observations indicate that pyruvate is not incorporated into coronafacate via the intermediacy of citramalate, they do not rule out the possibility that some metabolite of pyruvate serves as a starter unit. For example, pyruvate could be converted to phosphoenolpyruvate, which could then condense with malonyl CoA to yield a mono CoA ester of itaconic acid. The potential role of itaconic acid in coronafacate biosynthesis remains to be evaluated.

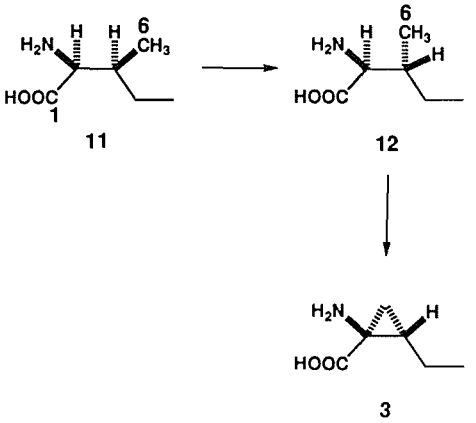
An alternative mode for assembly of the polyketide skeleton of coronafacic acid that can be envisioned proceeds from C-2 of the skeleton, with acetate serving as the starter unit and pyruvate serving as the terminating unit (Scheme 3*a*). If this hypothesis were correct, then a thiol ester of acetoacetic acid should be the intermediate resulting from the first polyketide condensation step. Investigations of this hypothesis were initiated by administering commercial ethyl (3-^{13}C)acetoacetate to *P. syringae*. The results of this experiment were quite unexpected: the isolated methyl coronafacate exhibited 27% enrichment at C-8, with no other detectable enrichments (Table 2, expt. 10)! This

observation was confirmed by the synthesis (Scheme 4) and administration of ethyl (2,3-$^{13}C_2$)acetoacetate (**8**). In this instance, the resulting methyl coronafacate exhibited ^{13}C–^{13}C coupling between C-6 and C-8, but no trace of ^{13}C–^{13}C coupling was apparent between C-1 and C-7a (Table 2, expt. 11). Three conclusions can be drawn from these two precursor incorporation experiments. First, ethyl acetoacetate does not appear to be significantly catabolized to acetate by *P. syringae*. Second, ethyl acetoacetate is incorporated intact into the butyrate-derived portion of the coronafacate skeleton. Finally, since no coupling appears between C-1 and C-7a in the coronafacate derived from doubly labeled acetoacetate, the polyketide assembly process does not appear to begin at C-2 of the coronafacate skeleton. Consequently, the polyketide assembly mode hypothesized in Scheme 3*a* is probably not correct. The efficient and intact incorporation of ethyl acetoacetate observed in experiments 10 and 11 is quite remarkable since it is usually necessary to administer thiol esters of polyketide intermediates in order to observe intact incorporation (16). Furthermore, Vederas and co-workers (19) have reported that even the thiol esters of *acetate*-derived polyketide intermediates tend to be completely degraded unless special measures are taken.

A third mode of polyketide assembly that can be considered for coronafacate is one in which the assembly proceeds from C-9 of the coronafacate skeleton, with acetate serving as the starter unit (Scheme 3*b*). This hypothesis is consistent with the results obtained from the ethyl acetoacetate experiments, but it requires the rather unorthodox insertion of a pyruvate unit into the middle of the growing polyketide chain. If polyketide assembly indeed begins at C-9, then a thiol ester of 3-ketohexanoate should be an intermediate in the assembly process. Emboldened by the results with ethyl acetoacetate, we therefore synthesized ethyl (2,3-$^{13}C_2$)-3-ketohexanoate (**9**) (Scheme 4) and administered it to the coronatine fermentation. The results from this experiment provided a sharp contrast to those observed with ethyl acetoacetate, since the methyl coronafacate exhibited a 35% enrichment at C-5, but no ^{13}C coupling to C-4 was apparent (Table 2, expt. 12). Curiously, no significant enrichments were present at those carbons expected to be derived from C-2 of acetate. This may reflect the fact that the amount of acetate generated by catabolism of this precursor would be significantly less than the amount that was administered when acetate was used as a precursor. In any case, the labeling pattern indicates that the ethyl (2,3-$^{13}C_2$)-3-ketohexanoate had been catabolized to give (1-^{13}C)butyrate, which was then incorporated into coronafacate. Much the same result was observed when the NAC ester of (2,3-$^{13}C_2$)-3-ketohexanoate (**10**) was synthesized (Scheme 4) and evaluated as a precursor (Table 2, expt. 13). These observations can be interpreted in one of two ways: either 3-ketohexanoate is not an intermediate in coronafacate biosynthesis and the polyketide assembly does not proceed from C-9 (Scheme 3*b*) or 3-ketohexanoate is so readily catabolized by *P. syringae* that its intact incorporation into methyl coronafacate cannot be observed under the usual fermentation conditions.

Coronamic acid

The structure and absolute configuration of coronamic acid (**3**) suggest that the compound could be derived from L-isoleucine (**11**) via the intermediacy of L-alloisoleucine (**12**) (Scheme 5). This hypothesis was evaluated by administration of commercial (1-^{13}C)-L-isoleucine and (1-^{13}C)-L-alloisoleucine to *P. syringae*, followed by isolation of coronatine methyl ester. The results of these experiments (Table 3, expts. 1,2) demonstrated



SCHEME 5

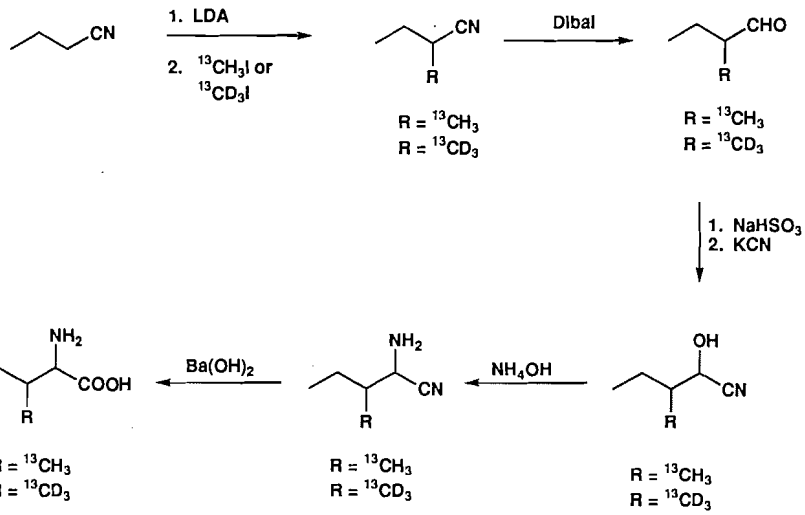
that both of these amino acids are specific precursors, and, furthermore, they indicated that L-alloisoleucine is more efficiently incorporated than L-isoleucine. Since the formation of the cyclopropane ring of coronamic acid by cyclization of L-alloisoleucine would constitute a highly unusual process, it appeared important to verify that the methyl group of the amino acid (C-6) is indeed the source of the cyclopropane bridge. This was accomplished by administration of a mixture of (6-^{13}C)-DL-isoleucine and (6-^{13}C)-DL-alloisoleucine synthesized according to Scheme 6 to the coronatine fermentation. The ^{13}C NMR spectrum of the resulting coronatine methyl ester clearly showed that the cyclopropane bridge is derived from the methyl group of isoleucine (Table 3, expt. 3). These observations set the stage for more detailed investigations of the mechanism of the cyclization process.

The first mechanistic probe utilized a mixture of (6-^{13}C, 6-$^2H_3$)-DL-isoleucine and (6-^{13}C, 6-$^2H_3$)-DL-alloisoleucine prepared from (^{13}C, $^2H_3$)methyl iodide (Scheme 6) as a precursor. Examination of the ^{13}C NMR spectrum of the coronatine methyl ester obtained in this experiment while carrying out simultaneous ^1H and ^2H decoupling revealed the presence of a new ^{13}C resonance shifted upfield by 0.59 ppm relative to the signal for C-6′ of coronatine. The magnitude of the upfield isotopic shift indicates that two deuterium atoms are located at C-6′ (20). The conversion of L-alloisoleucine to coronamic acid therefore proceeds with the removal of one hydrogen atom from C-6 of the amino acid. It is also noteworthy that the level of ^{13}C incorporation observed in this experiment (Table 3, expt. 4) was dramatically lower than that observed when no deuterium was present at C-6 (Table 3, expt. 3). This difference in incorporation efficiency is presumably due to the operation of a substantial deuterium isotope effect.

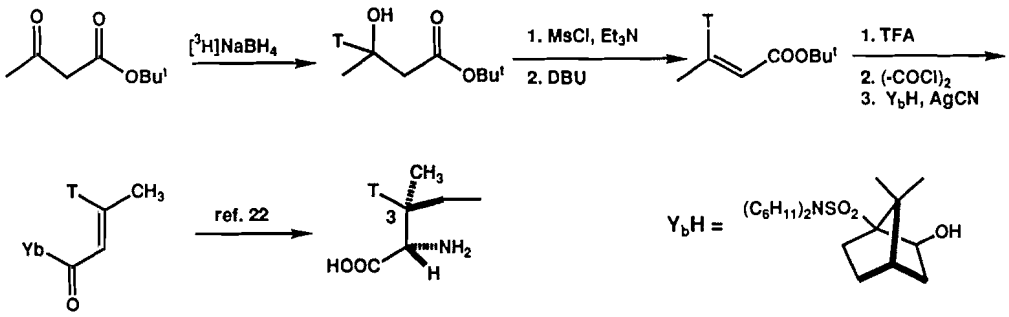
The loss of only one hydrogen atom from C-6 of L-alloisoleucine during coronamic acid formation rules out the intermediacy of species with a C-6 oxidation level higher than that of an alcohol. Putative intermediates with the appropriate oxidation level would therefore include 3-methylene-L-norvaline and 6-hydroxy-L-alloisoleucine. The possible intermediacy of the former amino acid was evaluated by means of a precursor incorporation experiment with [L-^{14}C, 3-^3H]-L-alloisoleucine. The synthesis of L-alloisoleucine carrying the appropriate isotopic labels was accomplished using modifications of chemistry devised by Oppolzer et al. (21) (Scheme 7). Administration of a mixture of the labeled amino acids to *P. syringae* generated a sample of coronatine methyl ester that was purified chromato-

TABLE 3. Incorporation of precursors into the coronamic acid moiety (**3**) of coronatine

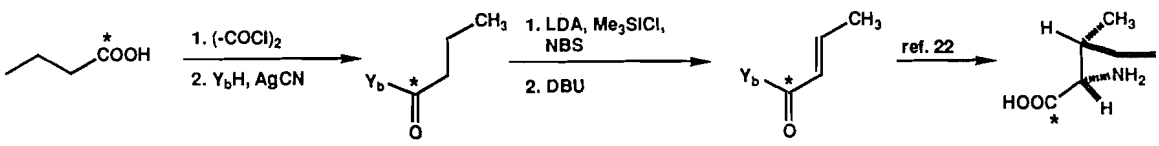
Expt.	Precursor ($^3H/^{14}C$)	Percent enrichment or incorporation	Labeling pattern
1	(1-^{13}C)-L-Isoleucine	9	C-1′
2	(1-^{13}C)-L-Alloisoleucine	60	C-1′
3	(6-^{13}C)-DL-Isoleucine + (6-^{13}C)-DL-alloisoleucine	31	C-6′
4	(6-^{13}C, 6-$^{2}H_3$)-DL-Isoleucine + (6-^{13}C, 6-$^{2}H_3$)-DL-alloisoleucine	0.5	^{13}C and 2 D at C-6′
5	[1-^{14}C, 3-^{3}H]-L-Alloisoleucine (3.89)	1.8	$^3H/^{14}C$ = 3.67 (94% ^{3}H retention)
6	(2-^{13}C, ^{15}N)-DL-Isoleucine + (2-^{13}C, ^{15}N)-DL-alloisoleucine	7.8% ^{13}C 2.4% ^{15}N	Connectivity between C-2′ and ^{15}N ($^1J_{CN}$ = 12 Hz)
7	(4-$^{2}H_1$, 5-$^{2}H_1$)-DL-Coronamic acid	ca. 32%	1 D at C-4′, 1 D at C-5′



SCHEME 6

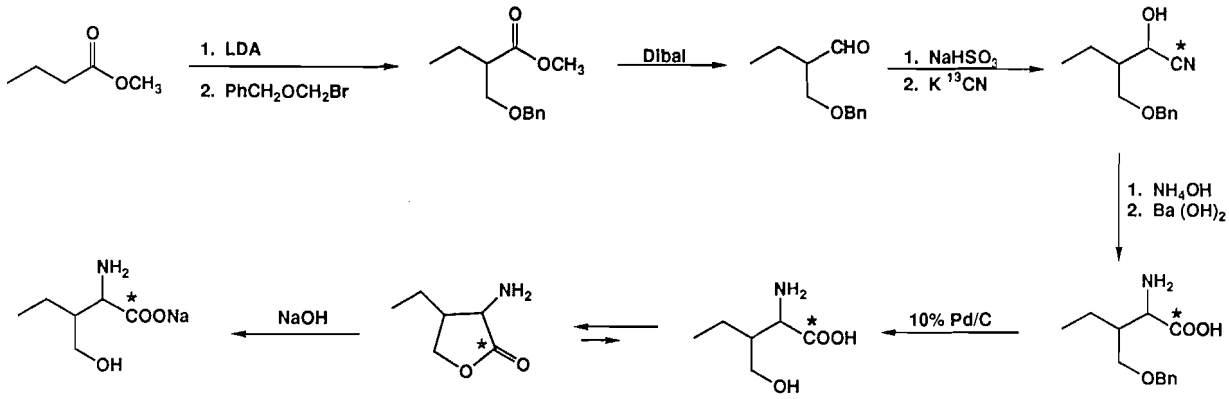


(a)



(b)

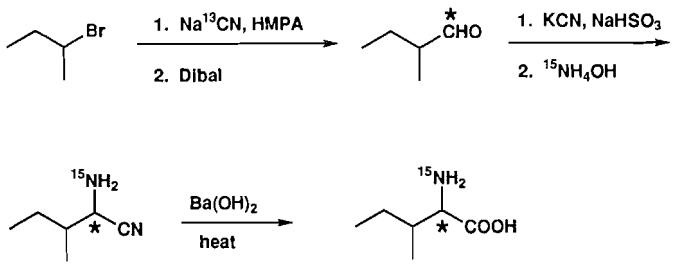
SCHEME 7



SCHEME 8

graphically and then reduced to the corresponding alcohol with lithium tri-*tert*-butoxyaluminum hydride. The radioactive alcohol was subjected to additional chromatography in order to remove any lingering radiochemical impurities. Comparison of the $^{3}H/^{14}C$ ratio in the doubly labeled alcohol with that in the doubly labeled alloisoleucine (Table 3, expt. 5) clearly shows that alloisoleucine is cyclized to coronamic acid without significant loss of the hydrogen atom present at C-3. The intermediacy of 3-methylene-L-norvaline in the cyclization process is thereby ruled out. To evaluate the potential intermediacy of 6-hydroxy-L-alloisoleucine in coronamate biosynthesis, a mixture of (1-^{13}C)-6-hydroxy-DL-isoleucine and (1-^{13}C)-6-hydroxy-DL-alloisoleucine was synthesized as shown in Scheme 8. Since the NMR spectrum of this mixture of amino acids indicated that they exist as the γ-lactones, the compounds were administered both in the lactone form and as the ring-opened sodium salts prepared by treatment of the lactones with one equivalent of sodium hydroxide. Both forms of the hydroxy amino acids failed to exhibit incorporation into coronatine (data not shown).

Another question to be addressed with respect to the mechanism of cyclization of L-alloisoleucine to coronamic acid concerns the fate of the nitrogen atom present at C-2 of the amino acid, i.e., one may inquire whether the cyclization process requires the loss of the α-amino group. This question was answered by means of an incorporation experiment with a mixture of (2-^{13}C, ^{15}N)-DL-isoleucine and (2-^{13}C, ^{15}N)-DL-alloisoleucine synthesized according to Scheme 9. When this mixture of amino acids was supplied to *P. syringae*, the resulting coronatine displayed a total ^{13}C enrichment at C-2′ of about 7.8%, of which 2.4% exhibited coupling to ^{15}N (Table 3, expt. 6). One can therefore conclude that the cyclization reaction proceeds without obligatory loss of the amino group of L-alloisoleucine.

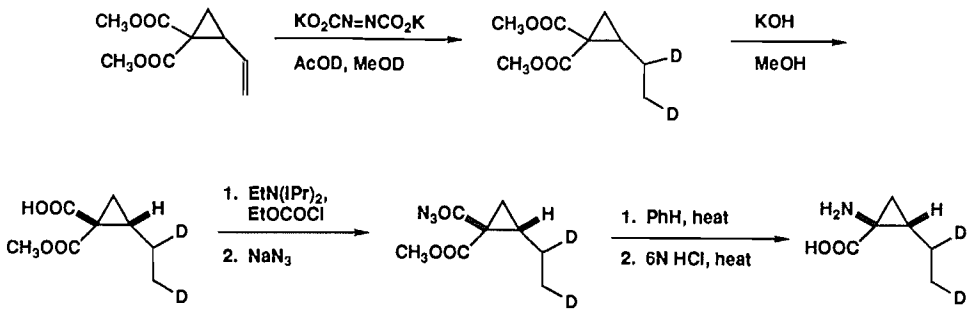


SCHEME 9

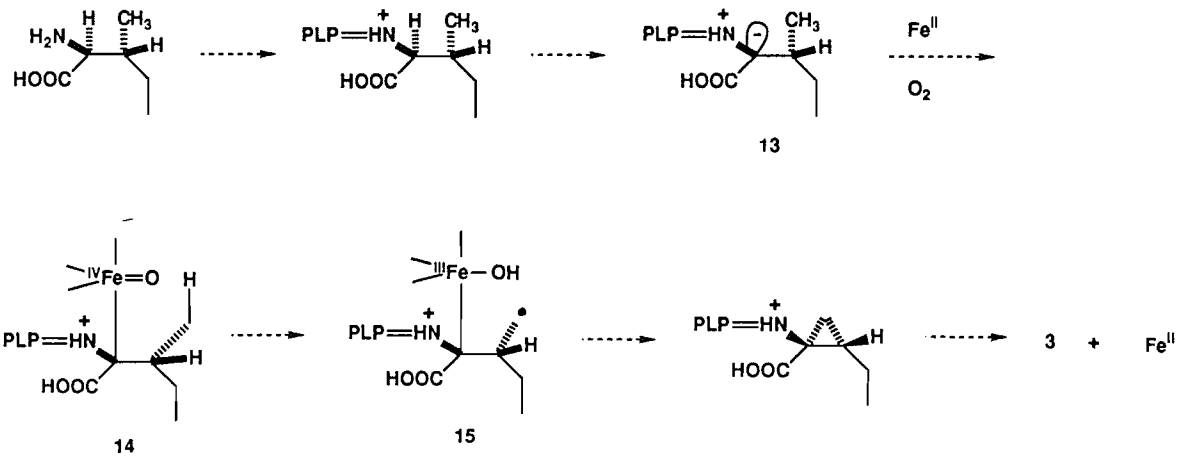
The fact that the total ^{13}C enrichment exceeds the ^{15}N enrichment indicates that some loss of ^{15}N from the mixture of doubly labeled amino acids occurs in vivo. This loss might be associated with the conversion of L-isoleucine to L-alloisoleucine since one mechanism for this epimerization reaction could involve the intermediacy of an α-keto acid.

Coronatine is accompanied in the fermentation broth of *P. syringae* by a number of congeners, including *N*-coronafacoyl-L-valine (22), *N*-coronafacoyl-L-isoleucine (23), and *N*-coronafacoyl-L-alloisoleucine (23). The co-occurrence of these congeners has led to the suggestion that the formation of the cyclopropane ring of coronamic acid may take place after formation of the amide bond between L-isoleucine or L-alloisoleucine and coronafacic acid (22). If this hypothesis were correct, then *P. syringae* might not possess the capability of utilizing free coronamic acid for the biosynthesis of coronatine. Information bearing on this aspect of coronatine biosynthesis was obtained by carrying out an incorporation experiment with (4-$^{2}H_1$, 5-$^{2}H_1$)-DL-coronamic acid, which was synthesized as outlined in Scheme 10. The synthesis makes use of a coronamic acid synthesis reported by Baldwin et al. (24). Analysis of the resulting coronatine methyl ester by ^{13}C NMR revealed the presence of two triplets at 13.0 and 20.0 ppm, which could be assigned to C-5′ and C-4′ of coronatine methyl ester, respectively. The center of each triplet was shifted upfield by ca. 0.25 ppm relative to the resonance positions of C-4′ and C-5′ in unlabeled coronatine methyl ester, thereby indicating that one deuterium atom was present at C-4′ and at C-5′. Analysis of the same sample of coronatine methyl ester by ^{2}H NMR revealed the presence of deuterium resonances at 1.00 and 1.65 ppm. Integration of these two absorptions with respect to an internal benzene standard indicated a deuterium incorporation of ca. 32% at both C-4′ and C-5′ (Table 3, expt. 7). Consequently, it appears that coronamic acid is a highly efficient precursor of coronatine.

The results of the experiments summarized in Table 2 limit the number of mechanisms that can be envisioned for the oxidative cyclization of L-alloisoleucine to coronamic acid. The available evidence is consistent with the possibility that this cyclization is related to the oxidative cyclizations observed in the biosynthesis of isopenicillin N (25), deacetoxycephalosporin C (25), and clavaminic acid (26). Based upon the analogy provided by these processes, one could postulate that the formation of coronamic acid proceeds by the mechanism outlined in Scheme 11. The cyclization could be initiated by reac-



SCHEME 10



SCHEME 11

tion of L-alloisoleucine with pyridoxal phosphate (PLP) to yield a Schiff base, which could then be deprotonated to give the α-carbanion (**13**).[5] This carbanion could then react with a ferryl species formed from ferrous iron and molecular oxygen to yield the intermediate **14**. In this step, the carbanion would mimic the role of the thiol group or hydroxyl group in the isopenicillin N and clavaminate cyclizations, respectively. The next stage of the cyclization would involve abstraction of a hydrogen atom from the C-6 methyl group by the iron-oxo species to yield a carbon radical (**15**), which then collapses to give the PLP adduct of coronamic acid. Hydrolysis of the PLP adduct would finally yield free coronamic acid.

In conclusion, investigations of the biosynthesis of coronatine by means of precursor incorporation experiments have revealed that the biosynthesis of this phytotoxin possesses some novel features. The derivation of the coronafacic acid moiety of coronatine from C-2 and C-3 of pyruvate, as well as acetate and butyrate, appears to be unprecedented. Likewise, the formation of the coronamic acid moiety of coronatine by cyclization of L-alloisoleucine appears to involve a mechanism for cyclopropane ring formation that has not been previously encountered in Nature. A more detailed understanding of the biochemical processes underlying coronatine biosynthesis is likely to require the application of both chemistry and molecular biology to the solution of this problem.

[5]Since some amino acid epimerases do not require a cofactor to generate a carbanion at the α position of an amino acid, it is conceivable that the formation of a carbanion at the α position of L-alloisoleucine may be cofactor independent. See ref. 27.

Experimental

General procedures

Proton nuclear magnetic resonance spectra were taken on either a JEOL FX-90Q (90 MHz), an IBM AF300 (300 MHz), or a Bruker AC250 (250 MHz) spectrometer. Chemical shifts are given in parts per million downfield from tetramethylsilane (0.0 ppm) for spectra taken in $CDCl_3$. In other organic solvents, the solvent itself was used as the reference. In D_2O the water signal (4.80 ppm) was used as reference. Carbon-13 NMR spectra were taken on the same three instruments at 22.5, 75.45, and 62.89 MHz, respectively. Methanol (49.0 ppm) or *p*-dioxane (66.5 ppm) were used as an internal reference when D_2O was the solvent. Deuterium NMR spectra were obtained with the IBM AF300 instrument at 46.07 MHz. Deuterium-depleted water was used both as solvent and as reference. Benzene was added as a standard for the calculation of ^2H enrichment. Mass spectra were run on Finnigan 3300 and CEC 111021-110B mass spectrometers. Infrared spectra were recorded on a Nicolet 205 FT-IR spectrophotometer. All melting points were taken on a Fisher–Johns melting point apparatus and are uncorrected. Preparative thin-layer chromatography was accomplished using 0.75-mm layers of Merck silica gel, type PF-254. Analytical TLC was carried out using glass plates precoated with Cellulose F or Merck silica gel, type 60, F-254. Cellulose plates were visualized with short-wavelength ultraviolet light or by spraying with a 2% solution of ninhydrin in ethanol and heating. Silica gel plates were visualized with short-wavelength ultraviolet light, iodine vapor, or phosphomolybdic acid. Flash chromatography was carried out using silica gel, type 60A, 230–400 mesh as the stationary phase. Cellulose column chromatography utilized Merck microcrystalline cellulose. Coronatine and coronafacic acid production were monitored by high-pressure liquid chromatography (HPLC) on a Whatman octadecylsilane reversed-phase column (4.6 mm i.d. × 25 cm, 5 μm) using a Spectraphysics SP model 8700 solvent delivery system. Microbiological media for slants, agar plates, and fermentation broths were either pur-

chased from Difco or prepared from the appropriate reagents. Fermentations were performed using a New Brunswick G-25R rotary shaker. The shaker was connected to a Forma Scientific CH/P 2067 circulating water bath in order to carry out fermentations of *P. syringae* at 18°C. Slants were either grown at room temperature or incubated at 30°C. Measurement of radioactivity was carried out with a Beckmann LS-3801 liquid scintillation counter.

Materials

Authentic samples of coronafacic acid and of coronatine were obtained as a gift from Dr. R.E. Mitchell. All reagents and compounds were purchased from Aldrich Chemical Co., Fluka, ICN, and sigma Chemical Co. and were purified, where necessary, by standard techniques. Radiolabeled compounds were purchased from Amersham Life Sciences. Stable isotopically labeled compounds were generally purchased from Cambridge Isotope Laboratories. (1-^{13}C)-l-Isoleucine and (1-^{13}C)-l-alloisoleucine were purchased from Tracer Technologies, Inc. (Somerville, Mass.). Unless otherwise stated, all reactions were performed under a dry nitrogen atmosphere.

Organism and fermentation

Pseudomonas syringae pv. *glycinae* (PDDCC no. 4182) was obtained from the Culture Collection of Plant Diseases Division of the New Zealand Department of Scientific and Industrial Research; *P. syringae* pv. *glycinae* (PDDCC no. 4180) was a gift from Professor Carol Bender of Oklahoma State University. The organism was maintained on mannitol–glutamate yeast agar slants which consisted of mannitol 1%, sodium L-glutamate 0.2%, KH_2PO_4 0.05%, NaCl 0.02%, $MgSO_4 \cdot 7H_2O$ 0.02%, yeast extract 0.025%, agar 1.5%, pH adjusted to 7.0 with dilute sodium hydroxide. For long-term storage, the organism was preserved by lyophilization in mist desiccans (28). For fermentation, *P. syringae* growing on one mannitol slant was suspended in 1.0 mL of sterile water and the suspension used to inoculate 100 mL of seed medium, which consisted of yeast extract (500 mg), glucose (500 mg), $MgSO_4 \cdot 7H_2O$ (20 mg), KH_2PO_4 (410 mg), K_2HPO_4 (360 mg), dissolved in 100 mL water with the pH adjusted to 6.8. The inoculated seed medium was incubated at 25°C, 250 rpm, for 24 h. After 24 h, the seed medium was used to inoculate 10 1-L flasks each containing 200 mL of production medium (10 mL of inoculum per flask). Production medium consisted of two solutions: (A) glucose (20.0 g), $MgSO_4 \cdot 7H_2O$ (0.4 g), KH_2PO_4 (8.2 g), K_2HPO_4 (7.2 g), dissolved in 2.0 L of water, and adjusted to pH 6.8 with 1 N NaOH. This solution was sterilized by autoclaving at 121°C for 20 min. After this solution had cooled to room temperature, 1 mL of solution (B) consisting of NH_4Cl (2.0 g) and biotin (2.0 mg) dissolved in 10 mL water was added to each production medium flask through a sterile filter. The inoculated fermentation flasks were incubated at 18°C, 200 rpm, and monitored for coronatine and coronafacic acid production by HPLC using 28% acetonitrile in H_2O containing 0.05% trifluoroacetic acid (flow rate = 1.5 mL/min, R_T = ca. 7 min for coronafacic acid, ca. 12 min for coronatine). The HPLC analysis indicated that coronatine production usually begins on day 1 and reaches a maximum after 5 days.

Isolation of methyl coronafacate and methyl coronatine

After 5 days the cells were removed by centrifugation at 8000 rpm for 40 min. The supernatant broth was filtered through glass wool, and stirred with a mixture of activated charcoal (20 g) and Celite (20 g) for 30 min before being loaded into a column and washed with 400 mL of distilled water. The column was then eluted with ca. 300 mL of acetone. The acetone elutant was concentrated fivefold, adjusted to pH 2.5 with 2.5 M H_2SO_4, and vigorously extracted three times with ethyl acetate. The combined ethyl acetate extracts were dried over anhydrous $MgSO_4$, filtered through Celite, and concentrated in vacuo. The acidic residue was dissolved in 2.0 mL methanol and treated with excess diazomethane. The excess diazomethane was blown off in the hood under a stream of nitrogen and the methanol removed in vacuo. The residue was further purified by preparative thin-layer chromatography (0.75 mm noncommercial silica gel plates, eluant 10% isopropanol in hexane). To obtain an adequate purification, it was necessary to develop the plate two or three times. This yielded, on average, 10 mg of coronatine as its methyl ester derivative and 4–6 mg of the methyl ester of coronafacic acid.

Administration of labeled precursors to P. syringae *pv.* glycinea

Precursors were pulse fed to the producing organism 24, 48, and 72 h after initiation of the fermentation. Both stable and radiolabeled precursors were dissolved in a suitable volume of Milli-Q water or ethanol and administrated to the fermentation flasks through a sterile Millipore filter (pore size 0.22 μm). In experiments employing precursors labeled with stable isotopes, about 100 mg were fed each day.

Synthesis of labeled precursors

*Synthesis of (5-^{13}C)-*N*-acetylcysteamine ester of* (S)*-citramalic acid (**6**)*

*A. Synthesis of (methyl-^{13}C)-(2S, 5S)-2-(*tert*-butyl)-5-(methoxycarbonyl)-5-(methyl)-1,3-dioxolan-4-one.* The ^{13}C-labeled dioxolane acid was prepared from L-malic acid and (^{13}C)methyl iodide using the procedure of Seebach et al. (17).

B. Synthesis of N*-acetylcysteamine ester of (methyl-^{13}C)-(2S, 5S)-2-(*tert*-butyl)-5-(methoxycarbonyl)-5-(methyl)-1,3-dioxolan-4-one.* The labeled dioxolane acid prepared in step A (375.0 mg, 1.74 mmol) was dissolved in 7.5 mL of dry CH_2Cl_2. *N*-Methylmorpholine (194.4 mg, 1.92 mmol) was then added at room temperature. The reaction mixture was cooled to –20°C for 15 min and diphenylphosphinic chloride (438 mg, 1.92 mmol) was then added. The reaction mixture was then stirred at room temperature for 20 min. Ether (10 mL) was added to the mixture and the precipitated *N*-methylmorpholine hydrochloride was removed by filtration. The salt was then washed with ether (3 × 30 mL) and the combined ether portions were washed with 15 mL of 10% $NaHCO_3$ and then with brine. After drying of the organic phase over anhydrous $MgSO_4$, the solvent was removed to furnish a thick oil, which was dissolved in dry THF (57 mL). The resulting solution was cooled in an ice bath to –3 to –4°C. A suspension of the thallium salt of *N*-acetylcysteamine in THF (19.8 mL, 1.92 mmol), prepared by the method of Schwab et al. (18), was added quickly, with vigorous stirring. The bright yellow reaction mixture was then stirred at –2 to –4°C for 18 h under an inert atmosphere. The solvent was removed from the reaction mixture and the residue redissolved in ether and filtered through Celite. The filtrate was taken to dryness in vacuo to give a thick, yellowish oil (835 mg). This oil was chromatographed on Florisil. Gradient elution with an ethyl acetate – hexane mixture furnished the product (401 mg) in 73% yield: ^{1}H NMR (250 MHz, $CDCl_3$) δ: 0.94 (s, 9H), 1.47 (d, $^1J_{CH}$ = 127.5 Hz, 3H), 1.97 (s, 3H), 2.9–3.5 (m, 6H), 5.17 (s, 1H), 5.8–6.0 (bs, 1H); ^{13}C NMR (62.89 MHz, $CDCl_3$) δ: 19.7 (enriched) ppm.

*C. (5-^{13}C)-*N*-acetylcysteamine ester of* (S)*-citramalic acid (**6**).* The 1,3 dioxolane NAC ester prepared in step B (254 mg, 0.8 mmol) was hydrolyzed with 2 N HCl (5.4 mL) at room temperature for 48 h under an argon atmosphere. The aqueous acid was then removed under reduced pressure, and remaining traces of HCl removed by twice adding water, which was then removed in vacuo. This procedure afforded the product as a colorless viscous oil (168 mg) in 84% yield: ^{1}H NMR (250 MHz, $CDCl_3$) δ: 1.45 (d, $^1J_{CH}$ = 125 Hz, 3H), 1.94 (s, 3H), 2.9–3.5 (m, 4H); ^{13}C NMR (62.89 MHz, $CDCl_3$) δ: 21.7 (enriched), 25.6, 28.1, 38.5, 52.3 ppm.

Synthesis of (5-^{13}C)-(R)*-citramalic acid diethyl ester (**7**)*

(*methyl*-^{13}C)-(2*R*, 5*R*)-2-(*tert*-Butyl)-5-(methoxycarbonyl)-5-(methyl)-1, 3-dioxolan-4-one (202 mg, 1 mmol), prepared (17) from D-malic acid using $^{13}CH_3I$, was dissolved in 30.0 mL of dry ethanol. Dry HCl gas was bubbled through the solution for 5 h. An exothermic reaction ensued. The reaction mixture was thenrefluxed for 18 h, after which time the ethanol was removed in vacuo. Column chromatography on silica gel furnished 161 mg of the diethyl ester of (*methyl*-^{13}C)citramalic acid in 79% yield: ^{1}H NMR (250 MHz, $CDCl_3$) δ: 1.24 (t, 3H), 1.29 (t, 3H), 1.42 (d, $^1J_{C-H}$ = 130 Hz, 3H), 2.67 (d, *J* = 15.6 Hz, 1H), 2.97 (d, *J* = 15.6 Hz, 1H), 4–4.35 (m, 4H); ^{13}C NMR (62.89 MHz, $CDCl_3$) δ: 14.09, 26.29 (enriched), 44.20, 60.83, 61.94, 103.5, 170.5, 175.5 ppm.

Synthesis of (2,3-$^{13}C_2$)ethyl acetoacetate (8)

A solution of LiHMDS in THF (10.5 mL, 10.5 mmol) was cooled to –78°C under dry nitrogen, and (2-^{13}C)ethyl acetate (440 mg, 5.0 mmol) was added to it. The solution was then stirred for 30 min to allow generation of the ester anion. To this mixture was then added (1-^{13}C)acetyl chloride (354 mg, 4.5 mmol) dissolved in dry THF (0.4 mL). The mixture was stirred at –78°C for 30 min. To this mixture 24 mL of 2 N HCl was added at the same temperature. The reaction was brought to room temperature over 2 h. It was then further acidified to pH 3 with 2 N HCl. The acidic solution was extracted with ether (3 × 4 mL). The combined ethereal extracts was dried over $MgSO_4$. Solvent removal and Kugelrohr distillation furnished 351 mg of (2,3-$^{13}C_2$)ethyl acetoacetate in 60% yield: ^{1}H NMR (250 MHz) δ: 1.28 (t, 3H), 2.27 (d, J_{CH} = 7.5 Hz, 3H), 3.45 (dd, J_{CH} = 130 Hz, $^2J_{CH}$ = 7.5 Hz, 2H), 4.19 (q, 2H); ^{13}C NMR (62.89 MHz, $CDCl_3$) δ: 14.01, 30.01, 50.14 (d, J = 38.4 Hz, enriched), 61.30, 167.5, 200.6 (d, J = 38.4 Hz, enriched) ppm.

Synthesis of (2,3-$^{13}C_2$)ethyl 3-ketohexanoate (9)

The compound was prepared from (2-^{13}C)ethyl acetate and (1-^{13}C)butyryl chloride following the procedure employed to synthesize doubly labeled ethyl acetoacetate. The yield was 69%: ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.92 (t, 3H), 1.28 (t, 3H), 1.5–1.75 (m, 2H), 2.45–2.6 (q, 2H), 3.63 (dd, $^1J_{CH}$ = 131.3 Hz, $^2J_{CH}$ = 6.3 Hz, 2H), 4.1–4.25 (q, 2H); ^{13}C NMR (75.45 MHz, $CDCl_3$) δ: 49.28 (d, J = 37.7 Hz, enriched), 202.83 (d, J = 37.7 Hz, enriched) ppm.

Synthesis of the N*-acetylcysteamine ester of (2,3-$^{13}C_2$)-3-ketohexanoate (10)*

A. Synthesis of (2-3-$^{13}C_2$)-3-ketohexanoic acid. Hydrolysis of ethyl (2,3-$^{13}C_2$)-3-ketohexanoate (**9**) was carried out at room temperature by stirring the ester (1.29 g, 8.2 mmol) with aqueous sodium hydroxide (0.82 g in 10 mL H_2O) for 20 h. The reaction mixture was doubly diluted with water and the pH adjusted to 12. The alkaline solution was then extracted with ether (3 × 30 mL) to remove neutral compounds. The aqueous phase was then acidified with 1 N HCl to pH 2. The free ß-keto acid was then extracted with $CHCl_3$ (3 × 80 mL) and the chloroform extracts dried over anhydrous Na_2SO_4. Removal of the $CHCl_3$ at room temperature furnished colorless crystals of the ß-keto acid (669 mg, 61% yield): ^{1}H NMR (250 MHz, $CDCl_3$) δ: 0.95 (t, 3H), 1.5–1.8 (m, 2H), 2.5–2.65 (q, 2H), 3.51 (dd, J = 13 Hz, J = 2.5 Hz, 2H).

B. Synthesis of the N*-acetylcysteamine ester of (2,3-$^{13}C_2$)-3-ketohexanoate (10).* 1,1′-Carbonyldiimidazole (488 mg, 3 mmol) was added to a solution of (2,3-$^{13}C_2$)-3-ketohexanoic acid (325 mg, 2.5 mmol) in dry acetonitrile (7.2 mL) at –5°C. The mixture was stirred for 1.5 h below 0°C, and a solution of freshly prepared *N*-acetylcysteamine (353 mg, 2.7 mmol) in dry acetonitrile (0.5 mL) was then added. The resulting mixture was stirred for another 1 h at the same temperature. The solvent was then removed in vacuo and residue dissolved in 15 mL water. This aqueous solution was extracted with ethyl acetate (3 × 50 mL), the combined extracts dried over anhydrous sodium sulfate, and the solvent was removed in vacuo to give 200 mg of residue. This residue was purified by flash chromatography on silica gel. Gradient elution with a mixture of ethyl acetate and hexane furnished nearly pure fractions of the ester. Traces of *N*-acetylcysteamine were removed from these fractions by preparative TLC to give 161 mg of the product in 28% yield: ^{1}H NMR (250 MHz, $CDCl_3$) δ: 0.93 (t, 3H), 1.5–1.75 (m, 2H), 1.98 (s, 3H), 2.4–2.6 (q, 2H), 2.9–3.15 (t, 2H), 3.35–3.55 (t, 2H), 3.71 (dd, $^1J_{CH}$ = 119.8 Hz, $^2J_{CH}$ = 5.2 Hz, 2H); ^{13}C NMR (62.89 MHz, $CDCl_3$) δ: 57.18 (d, J = 36.5 Hz, enriched), 202.15 (d, J = 36.5 Hz, enriched) ppm; HRMS, calcd. for $^{13}C_2C_8H_{17}NO_3S$: 233.0996; found (M^+): 233.0996; calcd. for $^{13}C_3C_7H_{17}NO_3S$: 234.1030; found (M + 1): 234.1028.

Synthesis of a mixture of (6-^{13}C)-DL-isoleucine and (6-^{13}C)-DL-alloisoleucine

The first synthesis developed for the preparation of these labeled amino acids involved the alkylation of the anion of *n*-butyl butyrate with (^{13}C)methyl iodide followed by diisobutyl aluminum hydride (DIBAL) reduction of the resulting ester to yield the corresponding aldehyde. Since difficulties were encountered with overreduction in the DIBAL step, an alternative synthetic route was devised. This is the one outlined below.

A. (5-^{13}C)-2-Methylbutyronitrile. *n*-Butyronitrile (2.0 g, 28.9 mmol) was added dropwise to a stirred solution of commercially available lithium diisopropylamide (3.71 g, 34.7 mmol) in 50 mL of dry THF at –78°C.The resulting yellow solution was stirred at –78°C for 30 min before (^{13}C)methyl iodide (4.13 g, 28.9 mmol) was added. The reaction mixture was stirred at –78°C for a further 1 h and then allowed to warm to 0°C over 3.5 h. The reaction mixture was quenched at 0°C by the addition of saturated aqueous NH_4Cl (30 mL). The THF was removed in vacuo and the residue extracted with CH_2Cl_2. The combined organic extracts were washed with water, saturated NaCl, dried over $MgSO_4$, filtered, and concentrated at atmospheric pressure. This gave 2.5 g of crude (5-^{13}C)-2-methylbutyronitrile as a pale yellow liquid, ^{1}H NMR (250 MHz, $CDCl_3$) δ: 1.0–1.1 (m, 3H), 1.35 (dd, $^1J_{CH}$ = 125 Hz, $^3J_{HH}$ = 7.9 Hz, 3H), 1.65–1.8 (m, 2H), 3.5–3.65 (m, 1H); ^{13}C NMR (62.89 MHz, $CDCl_3$) δ: 11.35, 17.58 (enriched), 26.58, 27.56, 77.0 ($CDCl_3$), 122.91.

B. (5-^{13}C)-2-Methylbutyraldehyde. Diisobutyl aluminum hydride (DIBAL) (37.2 mL, 37.2 mmol) was added dropwise to a stirred solution of (5-^{13}C)-2-methylbutyronitrile (2.5 g, 29.8 mmol) in dry CH_2Cl_2 (50 mL) at room temperature. The reaction mixture was stirred at room temperature for 1 h and quenched by cautiously adding 5% aqueous H_2SO_4. The aqueous and organic layers were separated and the aqueous layer extracted with CH_2Cl_2. The combined organic extracts were dried over $MgSO_4$, filtered through Celite, and concentrated at atmospheric pressure. This gave 2 g of crude (5-^{13}C)-2-methyl butyraldehyde.

C. (6-^{13}C)-2-Hydroxy-3-methylvaleronitrile. Ethanol (50 mL) was added to a solution of (5-^{13}C)-2-methylbutyraldehyde and sodium bisulfite (3.53 g, 34.6 mmol) in water (50 mL) until the solution became homogeneous and clear. The solution was stirred at room temperature for 1 h before potassium cyanide (1.99 g, 30.6 mmol) was added and the reaction mixture stirred at room temperature for 3 h. The ethanol was then removed in vacuo and the aqueous residue extracted with ether. The combined ether extracts were washed with water, saturated NaCl, dried over $MgSO_4$, filtered, and concentrated in vacuo. This gave 2.3 g of crude (6-^{13}C)-2-hydroxy-3-methylvaleronitrile.

D. (6-^{13}C)-2-Amino-3-methylvaleronitrile. The crude cyanohydrin from the previous step was dissolved in concentrated NH_4OH (34.6 mL) and ethanol (30 mL) was added until the solution became homogeneous and clear. The reaction mixture was then stirred at room temperature for 18 h. The ethanol was removed in vacuo and the aqueous residue extracted with ether. The combined ether extracts were washed with water, saturated NaCl, dried over $MgSO_4$, filtered, and concentrated in vacuo. This gave 1.7 g of the crude aminonitrile.

E. (6-^{13}C)-DL-Isoleucine/(6-^{13}C)-DL-alloisoleucine. The crude aminonitrile was dissolved in water (100 mL) and barium hydroxide (19.3 g, 61 mmol) added. The reaction mixture was then heated at reflux for 18 h. After cooling to room temperature, the solution was filtered and the filtrate taken to dryness. The solid white residue was redissolved in water (50 mL). This solution was treated twice with solid $(NH_4)_2CO_3$ to remove any remaining barium as insoluble barium carbonate. The filtrate was stirred in a water bath at 60–65°C for 1 h before being finally concentrated in vacuo. This gave 0.5 g of the crude amino acid mixture as a white solid. The remaining impurities were removed by repeated cellulose column chromatography (eluant: *n*-butanol:HOAc:H_2O (BAW), 4:1:2). This gave 169 mg (4.5% from *n*-butyronitrile) of the pure amino acid mixture as a flaky white solid: ^{1}H NMR (250 MHz, D_2O) (ca. 1:1 mixture of isoleucine and alloisoleucine) δ: 0.85 (dd, $^3J_{HH}$ = 6.9 Hz, $^1J_{CH}$ = 125.3 Hz, 3H, one diastereomer), 1.00 (t, $^3J_{HH}$ = 6.25 Hz, 3H), 1.25 (dd, $^3J_{HH}$ = 7.5 Hz, $^1J_{CH}$ = 125.9 Hz, 3H, other diastereomer), 1.36–1.50 (m, 2H), 2.04–2.12 (m, 1H), 3.69 (dd, $^3J_{HH}$ = 3.95 Hz, $^3J_{CH}$ = 3.92 Hz, 1H, one diastereomer), 3.76 (dd, $^3J_{HH}$ = 5.92 Hz, $^3J_{CH}$ = 5.90 Hz, 1H, other diastereomer); ^{13}C NMR (62.89 MHz, D_2O) δ: 11.14, 11.21, 13.42 (enriched), 14.79 (enriched), 24.56, 25.61, 35.70, 35.99, 58.58, 59.66, 174.3, 175.2 ppm.

Synthesis of a mixture of (6-^{13}C,$^{2}H_3$)-DL-isoleucine and 6-^{13}C,$^{2}H_3$)-DL-alloisoleucine

The synthesis of this mixture of labeled amino acids was carried out in the same manner as described for the preparation of the (6-^{13}C)-labeled compounds, except that (6-^{13}C, 6-$^{2}H_3$)methyl iodide was substituted for (6-^{13}C)methyl iodide. From 2.4 g of *n*-butyronitrile (34.3 mmol), 185 mg (4.1% yield) of a mixture of (6-^{13}C,$^{2}H_3$)-DL-isoleucine and (6-^{13}C,$^{2}H_3$)-DL-alloisoleucine was obtained as a flaky white solid: ^{1}H NMR (250 MHz, D_2O) (ca. 1:1 mixture of isoleucine and alloisoleucine) δ: 0.9–1.1 (m, 3H), 1.3–1.6 (m, 2H), 3.69 (dd, $^3J_{HH}$ = 3.95 Hz, $^3J_{CH}$ = 3.92 Hz, 1H), 3.76 (dd, $^3J_{HH}$ = 5.92 Hz, $^3J_{CH}$ = 5.90 Hz, 1H); ^{13}C NMR (62.89 MHz, D_2O) δ: 11.14, 11.21, 13.42 (enriched septet), 14.79 (enriched septet), 24.56, 25.61, 35.70, 35.99, 58.58, 59.66, 174.3, 175.2 ppm.

Synthesis of [3-^{3}H]-L-alloisoleucine

A. [3-^{3}H]-tert-Butyl 3-hydroxybutyrate. Unlabeled sodium borohydride (9 mg) was added to a stirred solution of *tert*-butyl acetoacetate (600 mg, 3.8 mmol) in dry ethanol (2.7 mL) at 0°C. The solution was stirredat 0°C for 15 m in and [^{3}H]sodium borohydride (100 mCi, specific activity 500 mCi/mmol) added. The reaction mixture was then stirred at 0°C for 30 min. The ice bath was removed and the mixture stirred at room temperature overnight. The reaction mixture was then cooled at 0°C, a further 65 mg of unlabeled sodium borohydride was added, and the reaction mixture was stirred at 0°C for 15 min and then at room temperature for 2 h. The ethanol was removed in vacuo, water was added, and the pH of the solution was adjusted to 5 with glacial acetic acid. The aqueous layer was extracted with ethyl acetate and the combined organic extracts were dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The clear residual oil was further purified by Kugelrohr distillation in vacuo (0.1 Torr). This gave 0.439 g (72%) of [3-^{3}H]-*tert*-butyl 3-hydroxybutyrate: bp 60–65°C at 0.1 Torr (1 Torr = 133.3 Pa); radiochemical yield, 11.1%; specific activity, 0.025 m Ci/mg: ^{1}H NMR (250 MHz, $CDCl_3$) δ: 1.2 (d, 3H), 1.4 (s, 9H), 2.4 (s, 3H), 3.1 (s, -OH), 3.9–4.2 (m, 2H); HRMS, calcd. for $C_8H_{16}O_3$: 160.1099; found (M^+): 160.1095; calcd. for $^{13}C_1C_7H_{16}O_3$: 161.1132; found (M + 1): 161.1129.

B. [3-^{3}H]-tert-Butyl 3-mesyloxybutyrate. Triethylamine (338 mg, 3.34 mmol) was added to a stirred solution of [3-^{3}H]-*tert*-butyl 3-hydroxybutyrate (0.435 g, 271 mmol) in 16 mL dry CH_2Cl_2 at room temperature. The solution was stirred at room temperature for 10 min, cooled in an ice bath to 0°C, and methanesulfonyl chloride (0.382 g, 3.34 mmol) added dropwise. After stirring at room temperature for a further 1 h, the reaction mixture was carefully poured into 1 M $NaHCO_3$ and the aqueous and organic layers separated. The aqueous layer was further extracted with CH_2Cl_2 and the combined organic extracts washed once with water, dried over anhydrous $MgSO_4$, filtered, and concentrated. The residual oil was further purified by Kugelrohr distillation. This gave [3-^{3}H]-*tert*-butyl 3-mesyloxybutyrate, 0.622 g (96%): ^{1}H NMR (250 MHz, $CDCl_3$) δ: 1.4 (s, 9H), 1.5 (s, 3H), 2.5–2.7 (m, 2H), 3.0 (s. 3H), 5.0–5.2 (m, 1H); HRMS, calcd. for $C_8H_{15}O_2$: 143.0690; found (M^+ – MsO): 143.0694; calcd. for $^{13}C_1C_7H_{15}O_2$: 144.0724; found (M + 1 – MsO): 144.0718.

C. [3-^{3}H]-trans-Crotonic acid. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) (796 mg, 5.23 mmol) was added dropwise to a stirred solution of [3-^{3}H]-*tert*-butyl 3-mesyloxybutyrate (622 mg, 2.6 mmol) in 25 mL dry CH_2Cl_2. The reaction mixture was stirred at room temperature for 24 h before being quenched by the addition of 20 mL of 1 M acetic acid. The aqueous layer was extracted with CH_2Cl_2 and the combined organic extracts washed with water, dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residual oil was purified by Kugelrohr distillation. This gave 0.37 g (2.6 mmol) of [3-^{3}H]-*tert*-butyl crotonate: ^{1}H NMR (90 MHz, $CDCl_3$) δ: 1.5 (s, 9H), 1.82 (dd, $^3J_{HH}$ = 7.7 Hz, $^4J_{HH}$ = 2.5 Hz, 3H), 5.74 (dd, $^3J_{HH}$ = 16.7, $^4J_{HH}$ = 2.5 Hz, 1H), 6.7–7.0 (m, 1H); HRMS, calcd. for $C_8H_{14}O_2$: 142.0993; found (M^+): 142.0994; calcd. for $^{13}C_1C_7H_{14}O_2$: 143.1027; found (M + 1): 143.1028.

The tritiated *tert*-butyl crotonate was dissolved in 25 mL of freshly distilled benzene and *p*-toluenesulfonic acid (84 mg, 0.44 mmol) added. The flask was fitted with a Dean–Stark trap and condenser and the whole heated at reflux for 1 h. The reaction mixture was cooled to room temperature and the benzene removed in vacuo. The white, crystalline solid was further purified by passage through a CC-4 silica gel column (15 g, eluant 5%–20% ethyl acetate – hexane). This gave 0.189 g (84%) of [3-^{3}H]-*trans*-crotonic acid as a white solid: ^{1}H NMR (90 MHz, $CDCl_3$) δ: 1.90 (dd, $^3J_{HH}$ = 7.7 Hz, $^4J_{HH}$ = 2.6 Hz, 3H), 5.82 (dd, $^3J_{HH}$ = 16.7 Hz, $^4J_{HH}$ = 2.6 Hz, 1H), 6.7–7.1 (m, 1H).

D. [3-^{3}H]-(+)-10′-Dicyclohexylsulfamoyl-L-isobornyl crotonate. Oxalyl chloride (290 mg, 2.29 mmol) was added dropwise to a stirred solution of [3-^{3}H]crotonic acid (190 mg, 2.20 mmol) in 10 mL dry benzene at room temperature. The solution was stirred at room temperature for 24 h and then at 40°C for 1 h. Silver cyanide (294 mg, 2.20 mmol) and (+)-10-dicyclohexylsulfamoyl-L-isoborneol (665 mg, 1.67 mmol) were added, the flask fitted with a reflux condenser, and the mixture heated at reflux for 72 h. After 72 h no chiral auxillary remained as shown by TLC analysis (SiO_2, 15% ethyl acetate – hexane). The reaction mixture was cooled to room temperature, filtered through Celite, and the benzene removed in vacuo. The residue was purified by flash chromatography (eluant 5%–20% ethyl acetate – hexane). This gave 232 mg (29.8%) of [3-^{3}H]-(+)-10-dicyclohexylsulfamoyl-L-isobornyl crotonate: ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.85 (s, 3H), 1.0 (s, 3H), 1.05–2.15 (m, 30H), 2.68 (d, *J* = 12.9 Hz, 1H), 3.1–3.35 (m, 3H), 5.05 (d, *J* = 8.6 Hz, 1H) 5.83 (d, *J* = 17.2 Hz, 1H), 6.8–7.03 (m, 1H); ^{13}C NMR (75.47, $CDCl_3$) δ: 17.86, 19.98, 20.39, 25.06, 26.34, 26.95, 29.77, 32.68, 39.33, 44.45, 49.03, 49.38, 53.56, 57.29, 77.87, 123.27, 143.69, 164.78, 205.5 ppm.

E. [3-^{3}H]-(3R)-(+)-10′-Dicyclohexylsulfamoyl-L-isobornyl-3-methylpentanoate. Ethyllithium (1.09 mmol) was added dropwise to a stirred solution of tetra-(tri-*n*-butylphosphine) copper(I) iodide (29) (424 mg, 0.27 mmol) in 7 mL dry ether at –78°C. The solution was stirred at –78°C for 30 min before boron trifluoride etherate (154 mg, 1.09 mmol) was added. After stirring at –78°C for a further 30 min, the tritiated isobornyl crotonate (225 mg, 0.49 mmol) was added as a solution in 2 mL ether/1 mL THF and the reaction mixture stirred for 2 h at –78°C. After 2 h the reaction mixture was quenched by the addition of 7 mL of saturated aqueous NH_4Cl and the aqueous layer extracted with ether. *m*-Chloroperoxybenzoic acid (245 mg, 1.42 mmol) was added to the combined ether extracts and the solution stirred for 10 min, filtered through Celite, and washed with 1 M NaOH until colorless. The ether extracts were further dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash chromatography (SiO_2, elutant 5%–20% ethyl acetate –hexane). This gave [3-^{3} H]-(+)-10-dicyclohexylsulfamoyl-L-isobornyl-3-methylpentanoate, 173 mg (71.5%) as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.85 (s, 6H), 0.93 (d, 3H), 1.00 (s, 3H), 1.05–2.4 (m, 32H), 2.55–2.7 (d, *J* = 14 Hz, 1H), 3.1–3.4 (m, 3H), 4.85–4.95 (dd, *J* = 5.1 Hz, *J* = 3.8 Hz, 1H); ^{13}C NMR (75.45 MHz, $CDCl_3$) δ: 11.25, 19.23, 19.93, 20.42, 25.13, 26.45, 26.97, 29.37, 30.09, 31.81, 32.71, 39.71, 41.85, 44.42, 49.06, 49.22, 53.63, 57.39, 78.34 ppm.

F. [3-^{3}H]-(2R, 3R)-2-Bromo-(+)-10′-dicyclohexylsulfamoyl-L-isobornyl-3-methylpentanoate. Butyllithium (0.595 mmol) was added dropwise to a stirred solution of diisopropylamine (60 mg, 0.595 mmol) in 6 mL dry THF between 0°C and –10°C. The solution was stirred for 30 min before being cooled to –78°C. A solution of the ester prepared under *E* was added in 4 mL of dry THF and the whole stirred at –78°C for 1 h. Trimethylsilyl chloride (105.8 mg, 0.974 mmol) was added and the reaction mixture stirred for 45 min before carefully recrystallized and dried *N*-bromosuccinimide (105.9 mg, 0.595 mmol) was added. The reaction mixture was stirred at –78°C for 5 h. After 5 h the reaction was quenched by the addition of aqueous NH_4Cl. The aqueous layer was extracted with ether; the combined extracts were washed with water and brine, dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash chromatography (SiO_2, eluant 4%–20% ethyl acetate – hexane). This gave 118 mg of the tritiated bromide as a white solid: ^{1}H NMR (90 MHz, $CDCl_3$) δ: 0.85 (s, 6H), 1.0 (s, 6H), 1.1–2.2 (m, 30H), 2.5–2.8 (d, *J* = 14 Hz, 1H), 3.0–3.6 (m, 3H), 4.0–4.22 (d, *J* = 7.6 Hz, 1H), 4.8–5.1 (m, 1H).

G. [3-^{3}H]-(2S, 3R)-2-Azido-(+)-10′-dicyclohexylsulfamoyl-L-isobornyl-3-methylpentanoate. DMF (3 mL) was added to a mixture of sodium azide (30.7 mg, 0.472 mmol) and the tritiated bromo ester (90.3 mg, 0.157 mmol) prepared under *F*, and the reaction was stirred at room temperature for 3 days with periodic monitoring by TLC (SiO_2, 10% ethyl acetate – hexane). After 3 days the reaction mixture was quenched with water and extracted with ether. The combined ether extracts were washed with water and saturated sodium chloride. They were then dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash chromatography (SiO_2, eluant 5%–15% ethyl acetate – hexane). This gave 72 mg (85%) of the tritiated azide: ^{1}H NMR (90 MHz, $CDCl_3$) δ: 0.75–1.00 (m, 12H), 0.95 (s, 3H), 1.0–2.2 (m, 31H), 2.4–2.7 (d, J = 14 Hz, 1H), 2.9–3.4 (m, 3H), 4.8–5.1 (m, 1H).

H. [3-^{3}H]-(2S, 3R)-2-Amino-(+)-10′-dicyclohexylsulfamoyl-L-isobornyl-3-methylpentanoate. A mixture of the tritiated azide from procedure *G* (72.1 mg, 0.133 mmol) and 5% palladium – barium sulfate catalyst (50.4 mg) in 5 mL absolute ethanol was stirred at room temperature for 24 h under hydrogen at atmospheric pressure. After 24 h the reaction mixture was filtered through Celite to remove the catalyst and the residue washed with $CHCl_3$. The filtrate was concentrated in vacuo and the residue purified by flash chromatography (SiO_2, 5%–50% ethyl acetate – hexane). This gave 53 mg (78%) of [3-^{3}H]-2-amino-(+)-dicyclohexylsulfamoyl-L-isobornyl-3-methylpentanoate: ^{1}H NMR (90 MHz, $CDCl_3$) δ: 0.75–1.2 (m, 12H), 1.2–2.2 (m, 30H), 2.5–2.9 (dd, J = 14 Hz, 1H), 2.95–3.5 (m, 3H), 3.8–4.1 (d, J = 3.8 Hz, 1H), 4.8–5.1 (m, 1H).

I. [3-^{3}H]-L-Alloisoleucine. A mixture of the tritiated amino ester prepared under *H* (53.3 mg, 0.103 mmol) and barium hydroxide (57.5 mg, 0.18 mmol) in 4 mL of water and 2 mL of ethanol was heated at reflux for 24 h. After 24 h the reaction mixture was cooled to room temperature, filtered through Celite, and concentrated in vacuo. The residue was redissolved in 10 mL water and treated with 8 mg $(NH_4)_2CO_3$. The milky white solution was filtered and concentrated before being redissolved in water and treated with a second batch of $(NH_4)_2CO_3$. The solution was filtered one more time to remove any remaining $BaCO_3$ and then stirred in a water bath at 60–65°C for 1 h. The water was then removed in vacuo and the white residue purified by flash chromatography (cellulose, eluant butanol:acetic acid:water (BAW) 12:3:5). This gave 13 mg (95%) of [3-^{3}H]-L-alloisoleucine whose specific activity was 11 μCi/mg: ^{1}H NMR (250 MHz, D_2O) δ: 1.0 (m, 6H), 1.3–1.5 (m, 2H), 1.9–2.1 (m, 1H), 3.6 (d, J = 0.36 Hz, 1H), 4.9 (s, D_2O); ^{13}C NMR (75.46 MHz, D_2O) δ: 11.17, 13.43, 25.65, 35.78, 58.58, 174.89 ppm.

Synthesis of [1-^{14}C]-L-alloisoleucine

A. [1-^{14}C]-(+)-10′-Dicyclohexylsulfamoyl-L-isobornyl butyrate. [1-^{14}C]Butyric acid (44 mg, 0.5 mmol) and oxalyl chloride (46 μL, 0.52 mmol) were allowed to react in 1 mL of dry benzene for 18 h at room temperature. The reaction mixture was then heated to 42°C for 30 min. After cooling to room temperature, (+)-10-dicyclohexylsulfamoyl-l-isoborneol (150 mg, 0.38 mmol) and AgCN (134 mg, 1.0 mmol) were added to the crude acid chloride along with 0.5 mL of dry benzene. The mixture was heated to gentle reflux for 48 h. The silver salts were removed by filtration through Celite and the filtrate evaporated in vacuo to give a residue that was chromatographed on silica gel to furnish the coupling product (131 mg) in 56% yield with a specific activity of 8.1 μCi/mg: ^{1}H NMR (90 MHz, $CDCl_3$) δ: 0.85 (s, 3H), 0.95 (t, 6H), 1.04–2.4 (m, 31H), 2.64 (d, J = 14 Hz, 1H), 2.95–3.4 (m, 3H), 4.8–5.04 (m, 1H). HRMS, calcd. for $C_{26}H_{45}NO_4S$: 467.3069; found (M^+): 467.3076; calcd. for $^{13}C_1C_{25}H_{45}NO_4S$: 468.3103; found (M + 1): 468.3110.

B. [1-^{14}C]-(+)-10′-Dicyclohexylsulfamoyl-L-isobornyl-2-bromobutyrate. Under a nitrogen atmosphere, diisopropylamine (290 mg, 2.9 mmol) in dry THF (45 mL) was cooled to 0°C and *n*-BuLi (1.1 mL, 2.6 M in hexane, 2.9 mmol) was added. After stirring at 0°C for 30 min, the reaction mixture was cooled to –78°C before a solution of the radioactive ester prepared in step *A* (808 mg, 1.7 mmol) in dry THF (15 mL) was added. The colorless reaction mixture was stirred at –78°C for 1 h before trimethylsilyl chloride (0.565 mL, 4.4 mmol) was added. After stirring for an additional 45 min at –78°C, recrystallized *N*-bromosuccinimide (518.7 mg, 2.82 mmol) in dry THF (22 mL) was added. The resulting cloudy yellowish reaction mixture was stirred at –78°C for 5 h and then quenched with water. After the usual aqueous work-up, the crude product was purified by flash chromatography on silica gel to furnish pure bromide (762 mg) in 81% yield: ^{1}H NMR (90 MHz, $CDCl_3$) δ: 0.85 (s, 3H), 1.0 (s, 3H), 1.04–2.4 (m, 32H), 2.8 (d, J = 14 Hz, 1H), 2.9–3.5 (m, 3H), 4.15 (dd, J = 5.8 Hz, 1H), 4.8–5.1 (m, 1H); ^{13}C NMR (75.47 Hz, $CDCl_3$) δ: 11.83, 19.80, 20.24, 24.91, 26.19, 26.76, 28.55, 30.20, 32.03, 33.03, 39.04, 44.06, 49.08, 49.27, 49.59, 53.36, 57.12, 79.91, 167.5 ppm; HRMS, calcd. for $C_{26}H_{44}NO_4BrS$: 545. 2174; found (M^+): 545.2171; calcd. for $^{13}C_1C_{25}H_{44}NO_4BrS$: 546.2208; found (M + 1): 546.2216.

C. [1-^{14}C]-(+)-10′-Dicyclohexylsulfamoyl-L-isobornyl crotonate. The radioactive bromide prepared in part *B* (762 mg, 1.37 mmol) was allowed to react with DBU (823 mg, 5.49 mmol) in 46 mL of dry benzene at reflux under nitrogen for 18 h. The resulting yellowish brown reaction mixture was worked up by addition of 1 M $NaHCO_3$ and water in succession. After drying the organic phase, the solvent was removed in vacuo and the residue purified by flash chromatography on silica gel to afford the pure crotonyl ester (468 mg) in 72% yield: ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.85 (s, 3H), 1.0 (s, 3H), 1.05–2.15 (m, 30H), 2.68 (d, J = 12.9 Hz, 1H), 3.1–3.35 (m, 3H), 5.05 (d, J = 8.6 Hz, 1H) 5.83 (d, J = 17.2 Hz, 1H), 6.8–7.03 (m, 1H); ^{13}C NMR (75.47 MHz, $CDCl_3$) δ: 17.86, 19.98, 20.39, 25.06, 26.34, 26.95, 29.77, 32.68, 39.33, 44.45, 49.03, 49.38, 53.56, 57.29, 77.87, 123.27, 143.69, 164.78, 205.5 ppm.

D. [1-^{14}C]-L-Alloisoleucine. The [^{14}C]-labeled crotonate ester prepared in part *C* was converted into [1-^{14}C]-L-alloisoleucine using the same sequence of reactions that was employed to convert [3-^{3}H]-(+)-10-dicyclohexylsulfamoyl-L-isobornyl crotonate into [3-^{3}H]-L-alloisoleucine. The specific activity of the resulting [1-^{14}C]-L-alloisoleucine was 7.5 μCi/mg.

Synthesis of a mixture of (2-^{13}C,^{15}N)-DL-isoleucine and (2-^{13}C,^{15}N)-DL-alloisoleucine

A. (1-^{13}C)-2-Methylbutyronitrile. (^{13}C)Sodium cyanide (2.0 g, 40.8 mmol) was added to a solution of 2-bromobutane (3.72 g, 27.2 mmol) in 50 mL HMPA at room temperature. The reaction mixture was stirred at room temperature for 48 h and then connected via a U-tube, cooled in liquid nitrogen, to a vacuum pump and distilled at room temperature. This gave 0.913 g (40%) of (1-^{13}C)-2-methylbutyronitrile as a colorless liquid: ^{1}H NMR (250 MHz, $CDCl_3$) δ: 1.05 (s, 3H), 1.3 (s, 3H), 1.55 (m, 2H), 2.5–2.65 (m, 1H); ^{13}C NMR (62.89 MHz, $CDCl_3$) δ: 11.347, 11.392, 26.585, 27.237, 26.266, 27.464, 122.914 (enriched) ppm.

B. (1-^{13}C)-2-Methylbutyraldehyde. DIBAL (13.4 mL, 13.4 mmol) was added dropwise to a stirred solution of (1-^{13}C)-2-methylbutyronitrile (0.913 g, 11 mmol) in dry CH_2Cl_2 (25 mL) at room temperature. After 1.5 h the reaction mixture was cautiously poured into 5% H_2SO_4 (50 mL) and the aqueous and organic layers separated. The aqueous layer was further extracted with dichloromethane and the combined organic extracts were dried over anhydrous $MgSO_4$, filtered, and concentrated by distillation at atmospheric pressure. The crude (1-^{13}C)-2-methylbutyraldehyde distilled at 90–92°C: ^{1}H NMR (250 MHz, $CDCl_3$) δ: 0.9–1.0 (t, 3H), 1.1–1.15 (t, 3H), 1.3–1.8 (m, 2H), 2.2–2.35 (m, 1H), 9.25–9.95 (d, $^1J_{CH}$, J = 40.9 Hz, 1H).

C. (2-^{13}C)-2-Hydroxy-3-methylvaleronitrile. Ethanol was added to a stirred solution of (1-^{13}C)-2-methylbutyraldehyde (0.95 g, 11 mmol) and sodium bisulfite (1.3 g, 12.5 mmol) in water (10 mL) until the solution became homogenous and clear. After stirring at room temperature for 1 h, potassium cyanide (0.73 g, 11.2 mmol) was added and the reaction mixture stirred at room temperature for 3 h. The ethanol was removed in vacuo and the aqueous layer extracted with ether. The combined ether extracts were washed with water, and with saturated NaCl. They were then dried over $MgSO_4$, filtered, and concentrated in vacuo. This gave 0.76 g (61%) of crude (2-^{13}C)-2-hydroxy-3-methyl valeronitrile.

D. (2-^{13}C, ^{15}N)-2-Amino-3-methylvaleronitrile. Ethanol was added to a solution of (2-^{13}C)-2-hydroxy-3-methylvaleronitrile (0.76 g, 6.72 mmol) in (^{15}N)ammonium hydroxide (4.1 mL of a 3.3 N solution) until the solution became homogenous and clear. The reaction mixture was stirred at room temperature for 18 h, the ethanol removed in vacuo, and the aqueous layer extracted with ether. The combined ether extracts were washed with water, saturated NaCl, dried over $MgSO_4$, filtered, and concentrated. This gave 0.72 g of crude (2-^{13}C, ^{15}N)-2-amino-3-methylvaleronitrile.

E. Mixture of (2-^{13}C, ^{15}N)-DL-isoleucine and (2-^{13}C, ^{15}N)-DL-alloisoleucine. Barium hydroxide (8.2 g, 26 mmol) was added to a solution of (2-^{13}C, ^{15}N)-2-amino-3-methylvaleronitrile (0.721 g, 6.32 mmol) in water (50 mL). The flask was fitted with a reflux condenser and the mixture refluxed for 18 h. The reaction mixture was then cooled to room temperature, filtered through Celite, and the filtrate treated twice with $(NH_4)_2CO_3$ to remove barium as insoluble barium carbonate. The resulting clear filtrate was stirred at 60–65°C in a water bath for 1 h and finally concentrated in vacuo. Chromatography of the residue on a cellulose column (eluant BAW 12:3:5) gave 0.35 g (42%) of doubly labeled amino acid as a white solid: ^{1}H NMR (250 MHz, $CDCl_3$) δ: 0.6–0.75 (m, 6H), 1.0–1.25 (m, 2H), 1.65–1.85 (m, 1H), 3.4 (ddd, $^1J_{CH}$ = 162 Hz, $^3J_{HH}$ = 25 Hz, $^2J_{NH}$ = 6.4 Hz, 1H); ^{13}C NMR (62.89 MHz, $CDCl_3$) δ: 11.23, 11.29, 13.46, 14.82, 22.83, 24.61, 25.63, 35.99, 58.50, 58.59, 59.58, 59.67 (doublet, $^1J_{CN}$ = 68 Hz), 180.62 ppm.

Synthesis of (5-$^2H_1$, 6-$^2H_1$)-DL-coronamic acid

A. (6-$^2H_1$, 7-$^2H_1$)-1,1-Dicarbomethoxy-2-ethylcyclopropane. A flame-dried and cooled 1000-mL three-necked round-bottomed flask, fitted with a reflux condenser and dropping funnel, was charged with 1,1-dicarbomethoxy-2-vinylcyclopropane (1.0 g, 5.43 mmol) (30) and dipotassium azodicarboxylate (31) (68.9 g, 355 mmol) in 100 mL methanol-O-*d*. Acetic acid-O-*d* (30.1 mL) was added dropwise. The original deep yellow solution turned pale yellow and the methanol began to reflux. The acetic acid was added at such a rate as to maintain reflux. Once the addition was complete, the reaction mixture was stirred at room temperature. After 2.5 h the pale yellow solution had become white. After stirring for 5.5 h the reaction mixture was transferred to a 500-mL round-bottomed flask and the methanol removed in vacuo. The residue was dissolved in water (50 mL) and extracted with ether. The combined ether extracts were washed with 1 N $NaHCO_3$, water, and brine, dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash chromatography (SiO_2, elutant 5%–15% ethyl acetate – hexane). This gave 0.8 g (80%) of (6-$^2H_1$, 7-$^2H_1$)-1,1-dicarbomethyoxy-2-ethylcyclopropane as a clear liquid: ^{1}H NMR (250 MHz, $CDCl_3$) δ: 0.9 (m, 2H), 1.2–1.3 (m, 1H), 1.3–1.5 (m, 2H), 1.8–2.0 (m, 1H), 3.7 (s, 3H), 3.8 (s, 3H).

B. (6-$^2H_1$, 7-$^2H_1$)-1-Carboxy-1-carbomethoxy-2-ethylcyclopropane. Potassium hydroxide (0.25 g, 4.4 mmol) was added to a stirred solution of (6-$^2H_1$,7-$^2H_1$)-1,1-dicarbomethoxy-2-ethylcyclopropane in methanol (18 mL) at room temperature. The reaction mixture was stirred at room temperature for 4 days. The methanol was removed in vacuo and the residue dissolved in water (20 mL). The aqueous layer was extracted with ethyl acetate and the combined ethyl acetate extracts washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. This gave 0.41 g (85%) of (6-$^2H_1$, 7-$^2H_1$)-2-carboxy-1-carbomethoxy-2-ethylcyclopropane as a clear liquid, ^{1}H NMR (250 MHz, $CDCl_3$) δ: 0.95 (m, 2H), 1.4–1.7 (m, 1H), 1.7–1.8 (m, 1H), 1.9–2.15 (m, 2H), 3.7 (s, 3H).

C. (5-$^2H_1$, 6-$^2H_1$)-DL-Coronamic acid. Ethyl chloroformate (0.68 mL, 7.1 mmol) was added dropwise to a stirred solution of (6-$^2H_1$,7-$^2H_1$)-1-carboxy-1-carboxymethyl-2-ethylcyclopropane (0.41 g, 2.35 mmol) and *N*-ethyldiisopropylamine (0.82 mL, 4.72 mmol) in acetone (5 mL) at 0°C. The solution was stirred at 0°C for 30 min, a solution of sodium azide (0.76 g, 11.82 mmol) in water (5 mL) was added, and the reaction mixture was stirred at 0°C for 2 h. The acetone was removed in vacuo and the aqueous layer extracted with ethyl acetate. The combined ethyl acetate extracts were washed with water and saturated NaCl, dried over $MgSo_4$, filtered, and concentrated in vacuo. This gave 0.45 g of the crude acyl azide as a pale yellow oil. The crude acyl azide was dissolved in toluene (6 mL) and heated at reflux for 30 min. The reaction mixture was cooled to roomtemperature, and the toluene removed in vacuo. The brown residual oil was dissolved in 6 N HCl (6 mL) and the solution refluxed for 6 h. The solution was cooled to room temperature and the acid removed in vacuo. The residue was redissolved in water (7 mL) and loaded onto a strongly acidic ion-exchange resin (Dowex 50W-X8, H^+-form, 15 mL). After washing the column with water, the amino acid fraction was eluted with 2 N NH_4OH (60 mL) and concentrated in vacuo. This gave 0.250 g (40%) of (5-$^2H_1$, 6-$^2H_1$)-DL-coronamic acid as a pale yellow solid: TLC (cellulose, BAW, 4:1:2, R_f = 0.69); ^{1}H NMR (300 MHz, D_2O) δ: 0.75 (m, 2H), 1.15 (d, $^3J_{HH}$ = 1.2 Hz, 2H), 1.3–1.4 (m, 1H), 1.4–1.5 (m, 1H); ^{13}C NMR (62.89 MHz, D_2O) δ: 12.44 (t, $^1J_{CD}$ = 19 Hz), 17.05, 19.57 (t, $^1J_{CD}$ = 20 Hz), 27.61, 39.85, 174.11 ppm.

Synthesis of a mixture of (1-^{13}C)-6-hydroxy-DL-isoleucine and (1-^{13}C)-6-hydroxy-DL-alloisoleucine

A. Methyl 2-benzyloxymethylbutanoate. A solution of *n*-BuLi (2.04 M in hexane, 5.5 mL) was added with stirring to a solution of diisopropylamine (1.11 g, 11.0 mmol) in dry THF (20 mL) at 0°C. The resulting solution was stirred at 0°C for 30 min, then cooled to –78°C before adding methyl butanoate (1.05 g, 10.3 mmol). After the ester addition, the reaction mixture was stirred at –78°C for 1 h, and bromomethyl benzyl ether[6] (2.27 g, 11.3 mmol) was then added. Stirring at –78°C was continued for 30 min, after which time the reaction was allowed to warm to room temperature and then quenched with excess saturated aqueous NH_4Cl solution. The resulting mixture was concentrated in vacuo and ether (10 mL) was added. The organic phase was washed with 1 N HCl (3 × 20 mL), separated, and dried over $MgSO_4$. Removal of the ether in vacuo gave the crude product, which was purified by flash chromatography (SiO_2, 15% EtOAc in hexane) to yield 1.39 g (61%) of a yellowish oil: ^{1}H NMR (90 MHz, $CDCl_3$) δ: 0.90 (t, *J* = 7 Hz, 3H), 1.45–1.76 (m, 2H), 2.50–2.88 (m, 1H), 3.53–3.59 (m, 2H), 3.69 (s, 3H), 4.50 (s, 2H), 7.30 (s, 5H); ^{13}C NMR (22.5 MHz, $CDCl_3$) δ: 11.49, 21.96, 47.53, 51.43, 70.56, 73.03, 127.55, 128.27, 138.16, 174.85 ppm.

B. 2-Benzyloxymethylbutanal. A solution of DIBAL (5.3 mL, 1.0 M in CH_2Cl_2) was added with stirring to a solution of methyl 2-benzyloxymethylbutanoate (1.16 g, 5.22 mmol) in dry CH_2Cl_2 (20 mL) chilled to –78°C. The resulting solution was stirred at –78°C for 1 h and then quenched with excess saturated aqueous NH_4Cl. The reaction mixture was then warmed to room temperature and the aluminum salts solubilized by addition of 1 N HCl (30 mL). The organic layer was separated, washed with brine (10 mL), dried ($MgSO_4$), and concentrated in vacuo. The crude aldehyde was purified by flash chromatography (SiO_2, 15% EtOAc in hexane) to yield 845 mg (84%) of a colorless oil: ^{1}H NMR (90 MHz, $CDCl_3$) δ: 0.93 (t, *J* = 7 Hz, 3H), 1.45–1.90 (m, 2H), 2.32–2.65 (m, 1H), 3.64–3.71 (m, 2H), 4.50 (s, 2H), 7.31 (s, 5H), 9.69 (d, *J* = 2 Hz, 1H); ^{13}C NMR (22.5 MHz, $CDCl_3$) δ: 11.29, 18.97, 53.58, 68.22, 73.23, 127.55, 127.68, 128.40, 137.96, 203.93 ppm; MS [EI], *m/z*: 121, 116, 107 ($C_7H_7O^+$, base peak), 106, 105, 91.

C. Mixture of (1-^{13}C)-6-benzyloxy-L-isoleucine and (1-^{13}C)-6-benzyloxy-DL-alloisoleucine. 2-Benzyloxymethylbutanal (530 mg, 2.76 mmol) was added to a solution of sodium bisulfite (370 mg) in water (10 mL). Ethanol was then added with stirring until the mixture became homogeneous. The resulting solution was stirred at room temperature for 1 h, at which time (^{13}C)-KCN (184 mg, 2.83 mmol) was added. Stirring of the reaction mixture was then continued for 3 h. The solution was then concentrated in vacuo and the concentrated mixture extracted with ether. After removal of the ether, the residue was suspended in concentrated NH_4OH (10 mL) and ethanol added until the mixture was homogeneous. The resulting solution was stirred overnight at room temperature. The solution was again concentrated in vacuo and the concentrated mixture extracted with ether. Removal of the ether yielded a residue, which was added to a solution of $Ba(OH)_2 \cdot 8H_2O$ (2.25g, 7.14 mmol) in water (10 mL) and the mixture

[6]Prepared using gaseous HBr instead of gaseous HCl following the procedure of Conner et al. (32).

refluxed overnight. The white precipitate was filtered off and washed twice with hot water. The filtrate was concentrated in vacuo and then treated with solid ammonium carbonate (400 mg). The solution was boiled for 10 min and the precipitated $BaCO_3$ was removed by filtration. The filtrate was then treated again with solid ammonium carbonate. This process was repeated until no white precipitate formed. Finally, the clear solution was boiled to destroy excess ammonium carbonate and then taken to dryness in vacuo. The residue was purified by flash chromatography (SiO_2, 25% MeOH in $CHCl_3$) to afford 160 mg (25%) of a mixture of (1-^{13}C)-6-benzyloxy-DL-isoleucine and (1-^{13}C)-6-benzyloxy-DL-alloisoleucine as a yellowish oil: ^{1}H NMR (90 MHz, D_2O) δ: 0.64–0.94 (m, 3H), 1.16–1.56 (m, 2H), 2.04–2.40 (m, 1H), 3.42–3.56 (m, 2H), 4.09 (d, J = 3.6 Hz, 1H), 4.40 (s, 2H), 7.28 (s, 5H); ^{13}C NMR (22.5 MHz, D_2O, *p*-dioxane ref.) δ: 10.94, 19.66, 20.44, 40.87, 55.18, 69.49, 73.07, 128.44, 128.76, 175.1 (enriched) ppm.

D. Mixture of (1-^{13}C)-6-hydroxy-DL-isoleucine and (1-^{13}C)-6-hydroxy-DL-alloisoleucine. A mixture of (1-^{13}C)-6-benzyloxy-DL-isoleucine and (1-^{13}C)-6-benzyloxy-DL-alloisoleucine 332 mg, 1.40 mmol) was dissolved inmethanol (35 mL), 10% Pd/C (143 mg) and concentrated HCl (3 drops) were added, and the suspension was stirred under H_2 at atmospheric pressure for 24 h. The catalyst was then removed by filtration and the solution taken to dryness. The residue was purified by column chromatography (cellulose, BAW 6:1:1) to yield a mixture of the free amino acids weighing 70 mg (39%). On standing in aqueous solution at room temperature, this mixture was converted into a mixture of the corresponding γ-lactones: TLC (cellulose, BAW 6:1:1), R_f = 0.49 for free amino acids, R_f = 0.33 for lactonized amino acids; spectral data for the lactones: ^{1}H NMR (90 MHz, D_2O) δ: 0.96 (t, J = 7.5 Hz, 3H), 1.20–1.96 (m, 2H), 2.08–2.72 (m, 1H), 3.49 (d, J = 11 Hz, 1H), 3.89–4.10 (m, 1H), 4.33–4.65 (m, 1H); ^{13}C NMR (22.5 MHz, D_2O, *p*-dioxane ref.) δ: 11.13, 18.55, 42.17, 57.26, 61,95, 180.25 (enriched) ppm; HRMS, calcd. for $C_6H_{11}NO_2$: 129.07897; found (M^+): 129.07895; calcd. for $^{13}C_1C_5H_{11}NO_2$: 130.08232; found (M + 1): 130.08205.

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