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A Plant-like Biosynthesis of Benzoyl-CoA in the Marine Bacterium '*Streptomyces maritimus*'

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Dedicated to Professor Paul J. Scheuer, B. S. M.'s scientific grandfather, in celebration of his 50-year tenure at the University of Hawaii and his inspiration to the field of marine natural products

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Abstract—The first plant-like biosynthesis of benzoic acid in a prokaryote, the marine actinomycete '*Streptomyces maritimus*', has been characterized. Feeding experiments with ^{2}H- and ^{13}C-labeled intermediates revealed that phenylalanine is metabolized to benzoyl-coenzyme A (CoA) by means of a phenylalanine ammonia lyase and subsequent β-oxidation of cinnamoyl-CoA. Benzoyl-CoA serves as the rare starter unit of a type II polyketide synthase producing the structurally novel bacteriostatic polyketides enterocin and the wailupemycins. The results from the feeding study are consistent with the proposed biosynthetic model deduced from the cloned enterocin biosynthesis gene cluster.

Introduction

The bacteriostatic agents enterocin (vulgamycin) (**2**) and wailupemycins A–D (**3**–**6**) are members of a series of structurally diverse polyketides produced by the marine bacterium '*Streptomyces maritimus*' (Scheme 1).[1–3] Classical feeding experiments demonstrated that enterocin is derived from benzoic acid (**1**), seven acetate units, and the methyl group of methionine and undergoes a novel Favorskii-like carbon rearrangement.[4] We recently cloned, sequenced, and heterologously expressed the enterocin biosynthesis gene cluster (*enc*) and demonstrated that the 21.3 kb cluster contains an iterative type II polyketide synthase (PKS) and is extremely versatile in encoding the biosynthesis of the diverse set of polyketides.[3,5] Feeding studies with [ring-d_5]benzoic acid to the transformant *S. lividans* K4-114/pJP15F11-1 that carries the *enc* cluster established the breadth of polyketide biosynthesis by this single gene set as nine uncharacterized structural analogs were additionally produced.[3]

Benzoic acid is a rare PKS starter unit that has been implicated in only one other bacterial polyketide biosynthetic pathway. The myxobacterial antifungal macrolide soraphen A is derived from a modular type I PKS that is primed with benzoyl-CoA.[6] Other rare examples of bacterial natural products possessing a probable benzoate-derived residue include thiangazole[7] and benzoyl α-L-rhamno-pyranoside.[8] In eukaryotic systems, benzoic acid is conversely a common metabolite and is a component of many important natural products, including salicylic acid,[9] cocaine,[10] taxol,[11] and the zaragozic acids.[12]

In spite of its wide occurrence in plants and fungi, the biosynthesis of benzoic acid has not been firmly established.[9] Two pathways have been characterized which similarly involve conversion of phenylalanine (**7**) to cinnamic acid (**8**) by phenylalanine ammonia lyase (PAL) (Scheme 2). The routes diverge at this intermediate, one involving a β-oxidation pathway (route *a*) and the second a retro-aldol path through benzaldehyde (route *b*). The only known bacterial benzoate pathway is anaerobic and involves transamination of phenylalanine to phenylpyruvate followed by two successive α-oxidative decarboxylations (route *c*).[13] Recently, a direct conversion of shikimic acid to benzoic acid was postulated in a streptomycete (route *d*).[8]

Analysis of the *enc* gene cluster revealed seven open reading frames (ORFs) arranged on four transcripts neighboring the minimal *enc* PKS that are putatively involved in the biosynthesis and attachment of the benzoyl-CoA starter unit.[5] The nucleotide sequence of these ORFs is very suggestive for a eukaryotic-like benzoic acid biosynthetic pathway in this prokaryote. Feeding experiments with labeled intermediates were conducted in '*S. maritimus*' and verified that the enterocin benzoic acid starter unit is biosynthesized via the plant-like pathway.

Keywords: benzoic acid; biosynthesis; phenylalanine ammonia lyase; polyketide synthase; *Streptomyces*.
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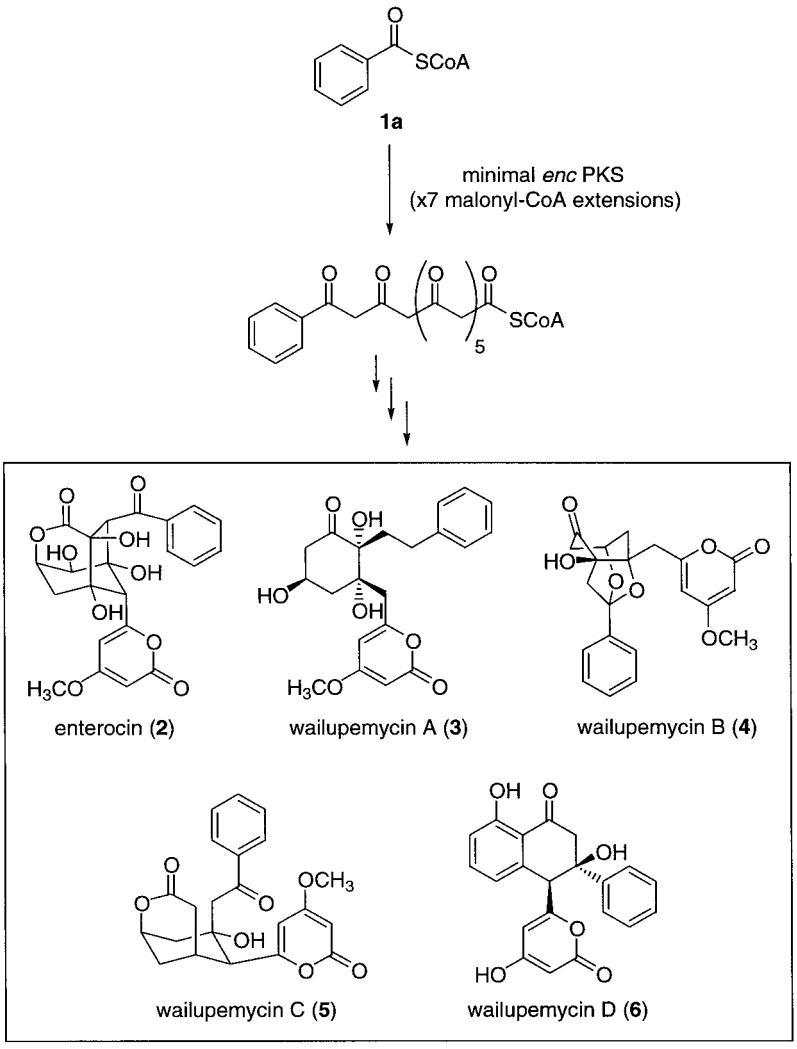
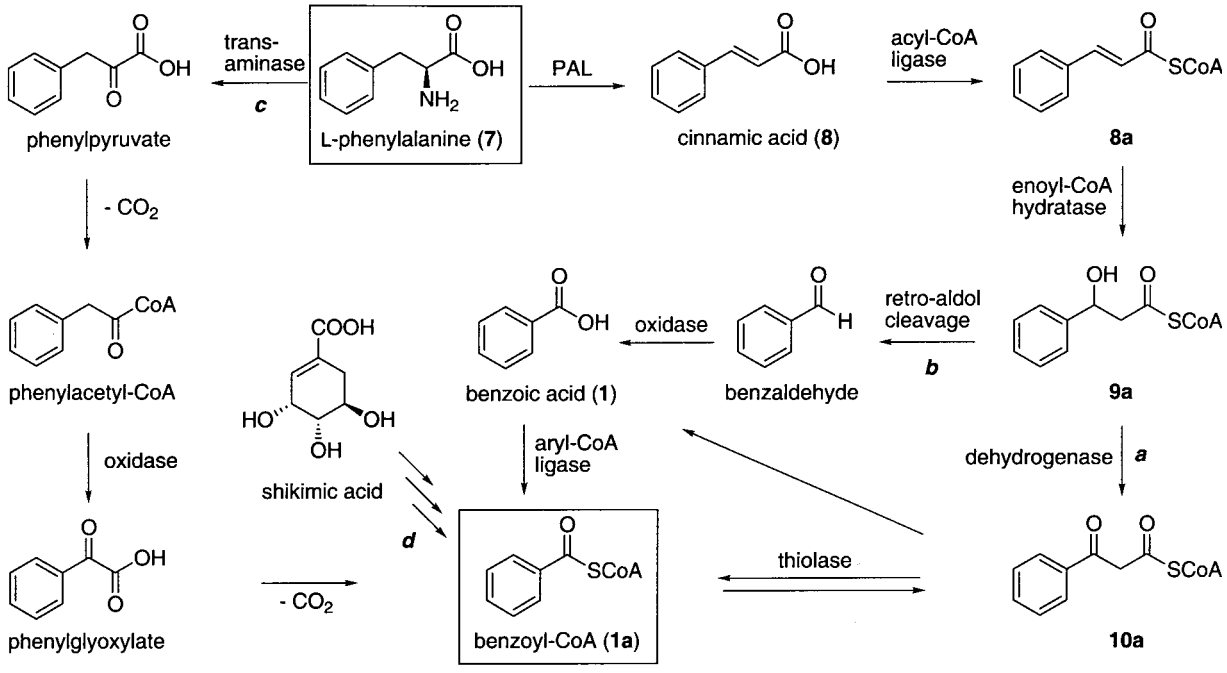
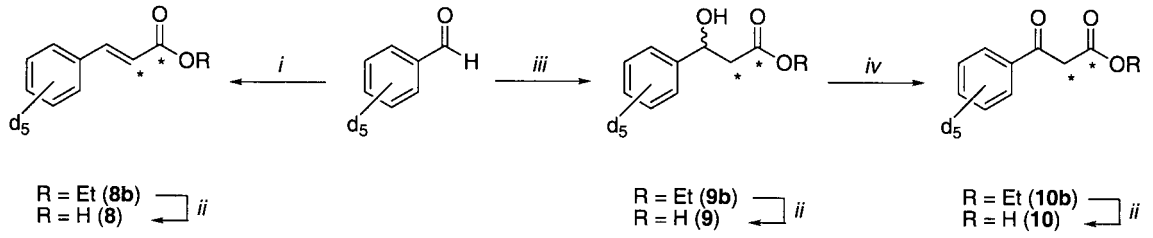


Scheme 1. Structures of enterocin (**2**) and wailupemycins A–D (**3**–**6**) from '*Streptomyces maritimus*' and their biosynthesis from benzoyl-CoA (**1a**).



Scheme 2. Biosynthetic routes to benzoyl-CoA in plants and fungi (paths *a* and *b*), in anaerobic bacteria (path *c*) and from shikimic acid (path *d*).



Scheme 3. *Reagents*: (i) DBU, LiCl, $(EtO)_2P(O)$ $^{13}CH_2{}^{13}CO_2Et$, MeCN, rt; (ii) 1 M NaOH, rt; (iii) $CrCl_2$, LiI, $Br^{13}CH_2{}^{13}CO_2Et$, THF, rt −55°C; (iv) PDC, DCM, rt. The asterisks denote ^{13}C atoms.

Results and Discussion

Although analysis of the *enc* gene cluster suggested a plant-like benzoic acid biosynthetic pathway,[5] it did not unequivocally differentiate between the β-oxidative and the retro-aldol routes. Furthermore, while benzoyl-CoA is the accepted enterocin PKS starter unit, β-ketophenylpropionyl-CoA **10a** may be an alternative starter unit that requires one fewer malonyl-CoA condensations. Thus, doubly labeled phenylpropanoid biosynthetic intermediates were synthesized with label in both the phenyl ring and the propyl side chain to determine the metabolic fate of the precursor in enterocin biosynthesis.

Synthesis of precursors

Synthesis of the doubly labeled putative phenylpropionyl intermediates **8**–**10** was straightforward and highly efficient as outlined in Scheme 3. Horner–Emmons reaction of commercially available [ring-d_5]benzaldehyde and triethyl [$^{13}C_2$]phosphonoacetate using DBU/LiCl as base[14] provided the ethyl (*E*)-cinnamate **8b** in high yield. GC and NMR analyses corroborated that the cinnamic ester was free from the (*Z*)-isomer and displayed a high degree of isotope labeling. Labeled ethyl 3-hydroxy-3-phenylpropionate **9b** was conveniently prepared as the racemate from [ring-d_5]benzaldehyde and ethyl [1,2-$^{13}C_2$]bromoacetate by a novel chromium Reformatsky reaction.[15] While the classic zinc variant of this reaction is known to be sluggish and unreliable on a small scale, the chromium variation offered an excellent yield (78%) even on a microscale (0.5 mmol). Subsequent PDC oxidation[16] of the β-hydroxy ester almost quantitatively furnished the corresponding β-keto propionate **10b** under mild conditions. Basic hydrolysis of all three ethyl esters with 1 M NaOH at ambient temperature yielded the pure free acids **8**–**10** without side reactions. All labeled compounds exhibited a very high (>98%) degree of isotopic labeling.

Table 1. Relative incorporation of labeled precursors into enterocin (**2**)

Precursor[a]	% Enrichment in **2**
[ring-$^2H_5$]Benzoic acid (**1**)	31
[ring-$^2H_5$]Phenylalanine (**7**)	24
(2*E*)-[ring-$^2H_5$,1,2-$^{13}C_2$]Cinnamic acid (**8**)	36[b]
[ring-$^2H_5$,1,2-$^{13}C_2$]-3-Hydroxy-3-phenylpropionic acid (**9**)	16[b]
[ring-$^2H_5$,1,2-$^{13}C_2$]-3-Oxo-3-phenylpropionic acid (**10**)	10[b]
[ring-$^2H_5$]Phenylacetic acid (**11**)	n.d.[c]

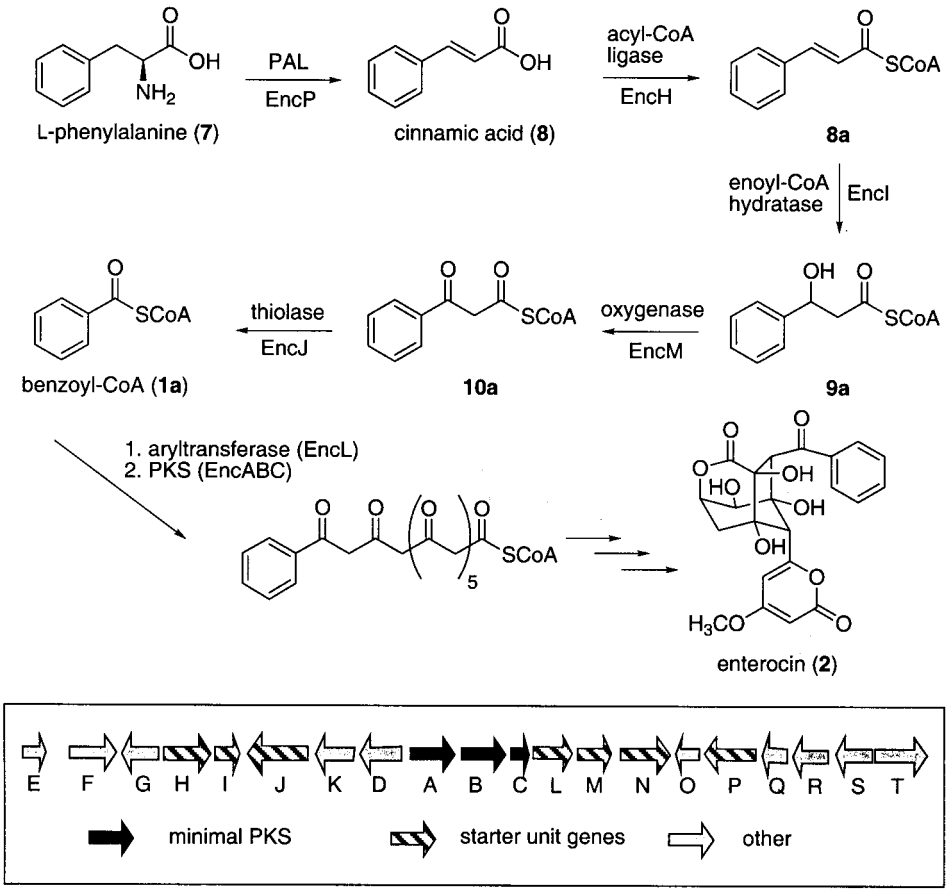
[a] 40 μmol precursor/100 mL '*S. maritimus*' culture.
[b] Enrichment based on the incorporation of the d_5-benzyl group with loss of the side chain $^{13}C_2$-label.
[c] Not detectable.

Feeding studies

The intermediacy of phenylalanine (**7**) in the '*S. maritimus*' benzoic acid biosynthetic pathway was initially confirmed in a feeding experiment with [ring-d_5]phenylalanine. A 24% incorporation was measured by mass spectrometry on the enriched enterocin (Table 1). The MS fragmentation pattern of the pentadeuterated enterocin was consistent with the label confined to the benzyl ring. This high incorporation was consistent with that of enterocin derived from [d_5]benzoic acid which in a parallel culture was likewise enriched at 31%.

Similarly, high incorporation of the phenylpropanoids **8**–**10** (10–36%) was detected by MS analysis (Table 1), yet only after loss of the $^{13}C_2$-label in the side chain. No intact incorporation was detected, clearly indicating that the propionate side chain is cleaved before incorporation of the benzoyl unit. Thus, benzoyl-CoA (**1a**), and not β-ketopropionyl-CoA **10a**, is the *enc* starter unit. Relative to cinnamate, approximately half of racemic 3-hydroxypropionate **9** was incorporated, leading to the assumption that only one enantiomer is accepted by the dehydrogenase. In fact, similar observations have been made during biosynthetic studies of cocaine, where a dramatic preference for the *R*-enantiomer was exhibited.[10] Whether or not a stereospecific dehydrogenase is involved in the '*S. maritimus*' benzoic acid pathway, as well as its stereospecificity, will be addressed at a later time by asymmetric synthesis and feeding of labeled (*R*)- and (*S*)- hydroxypropionates. Furthermore, the relative low incorporation of the β-ketoacid **10** is probably due to its instability in culture, which results in spontaneous decarboxylation. Its incorporation, nonetheless, provides strong support for the plant-like β-oxidative pathway. The bacterial α-oxidative pathway (route *c*) does not appear to be operative in '*S. maritimus*', as in addition to the genetic analysis of the cluster, the α-oxidative intermediate phenylacetate (**11**) was not incorporated into the benzoate-derived moiety of enterocin (Table 1).

These data are consistent with the genetic analysis of the *enc* biosynthetic gene set. Conversion of L-phenylalanine to *trans*-cinnamic acid by the putative *encP* gene product PAL constitutes the initial reaction in the pathway (Scheme 4). This is only the second report of a PAL catalyzed reaction in a bacterium[17] and the first associated with a bacterial biosynthesis of benzoic acid. Activation by the putative



Scheme 4. Proposed biosynthesis of benzoyl-CoA in '*S. maritimus*' and arrangement of the putative biosynthetic genes in the *enc* gene cluster (GenBank accession number AF254925). EncH: acyl-CoA ligase; EncI: Enoyl-CoA hydratase; EncJ: β-ketothiolase; EncABC: minimal PKS; EncL: aryl transferase; EncM: oxygenase; EncN: aryl-CoA ligase; EncP: phenylalanine ammonia lyase (PAL).

acyl-CoA ligase EncH to cinnamoyl-CoA (**8a**) followed by a β-oxidative pathway involving hydration (putatively catalyzed by EncI), dehydrogenation (putatively catalyzed by EncM), and thiolysis (putatively catalyzed by EncJ) yields the benzoyl-CoA starter unit. Thiolysis may alternatively generate the free acid, which would then require conversion to the CoA thioester **1a** by the putative EncN aryl-CoA ligase. Correlation of the biochemical reactions to the putative functional assignments of the *enc* gene products will be resolved at the biochemical and genetic levels with recombinant proteins.

In conclusion, the first plant-like benzoate pathway in a bacterium has been characterized in '*S. maritimus*'. Feeding experiments with labeled intermediates support the biochemical pathway suggested by the sequence analysis of the *enc* gene cluster. Due to the clustering of the benzoate biosynthesis genes in this prokaryote, we regard '*S. maritimus*' as an excellent model to fully characterize the eukaryotic-like benzoic acid biosynthetic pathway at the enzymatic and genetic levels.

Experimental

General methods

All reactions were carried out under argon in flame-dried glassware using standard gas tight syringes, cannulas and septa. Solvents and reagents were dried prior to use according to standard procedures. For column chromatographic separations 230–400 mesh silica gel (Aldrich) was used. NMR spectra were recorded on a Bruker AM-500 spectrometer. Chemical shifts are given in ppm and are adjusted to the TMS scale by reference to the solvent signal. Coupling constants (*J*) are given in Hertz (Hz). GC-MS was carried out on a Hewlett–Packard 5890 GC connected to a 5971A Mass Spectrometer. LC-MS analyses were performed using a Hewlett–Packard HP1100 LC pump with a photo diode-array detector linked to a Bruker HP Esquire Ion Trap Mass Spectrometer operating in the negative ion mode. A Beckman Ultrasphere C18 column (4.6 mm×15 cm) was used at a flow rate of 1 mL/min with a linear solvent gradient of 5% MeOH in 0.15% TFA/H_2O to 100% MeOH over a period of 45 min.

Materials

Triethyl [$^{13}C_2$]phosphonoacetate, chromium (II) chloride (anhydrous), and LiI (anhydrous) were purchased from Aldrich Co., [ring-$^2H_5$]benzoic acid, [ring-$^2H_5$]phenylalanine, and ethyl [$^{13}C_2$]bromoacetate were obtained from Cambridge Isotope Labs, and [ring-$^2H_5$]benzaldehyde and [ring-$^2H_5$]phenylacetic acid were acquired from CDN Isotopes.

Fermentation

'*S. maritimus*' spores from a plate culture were inoculated into 100 mL of A1 medium in a 500 mL Erlenmeyer flask. The medium contained 10 g soluble potato starch, 4 g yeast extract, 2 g peptone, and 28 g Instant Ocean® per liter H_2O and buffered at pH 8 with 10 mL of 1 M Tris buffer. The inoculated culture was incubated at 28°C for 2 days on a rotary shaker at 200 rpm. The inoculum (10 mL) was then transferred to 90 mL of A1 and incubated at 28°C for 5 days with shaking at 200 rpm.

Feeding experiments with labeled precursors

Single doses of precursors were administered to the fermentation at the time of inoculation, and the cultures were harvested 5 days later. Precursors were added as sterile solutions (40 μmol in 100 μl DMSO) in the amounts indicated per 100 mL culture: [ring-$^2H_5$]phenylalanine (7.9 mg), (2′*E*)-[ring-$^2H_5$,1,2-$^{13}C_2$]cinnamic acid (7.3 mg), [ring-$^2H_5$,1,2-$^{13}C_2$]-3-hyroxy-3-phenylpropionic acid (8.1 mg), [ring-$^2H_5$,1,2-$^{13}C_2$]-3-oxo-3-phenylpropionic acid (8.0 mg), [ring-$^2H_5$]phenylacetic acid (5.6 mg), and [ring-$^2H_5$]benzoic acid (5.1 mg). The cultures were centrifuged at 6000×*g* for 8 min, and the supernatants were extracted with EtOAc (3×50 mL). The crude extracts were dried (Na_2SO_4) and evaporated in vacuo. The residue was dissolved in 0.5 mL MeOH and analyzed by LC-ESMS (5 μl in 500 μl MeOH). Enterocin (**2**): ESMS (*m*/*z*) 443 (M−H), 425 (M−H_3O), 399, 381, 355, 337, 275, 257. Enterocin-d_5: ESMS (*m*/*z*) 448 (M−H), 430 (M−H_3O), 404, 386, 360, 342, 280, 262.

Synthesis of labeled precursors

(2*E*)-[ring-$^2H_5$,1,2-$^{13}C_2$]Cinnamic acid (8). To a supension of LiCl (32.2 mg, 0.6 mmol) in dry MeCN (1.0 mL) at 0°C were subsequently added triethyl [$^{13}C_2$]phosphonoacetate (120 μl, 0.6 mmol), 1,8-diazabicyclo-[5.4.0]-undec-7-ene (75 μl, 0.5 mmol) and [ring-$^2H_5$]benzaldehyde (51 μl, 0.5 mmol). After stirring for 10 min at ambient temperature, H_2O (1 mL) was added and immediately the pH was adjusted to 7.0 by quick titration with dilute HCl. The aqueous phase was extracted with Et_2O (3×5 mL) and the combined organic phases were washed with brine and dried over $MgSO_4$. After evaporation of the solvent in vacuo, the residue was purified on silica with 1:1 pentane/Et_2O, providing 80.2 mg ethyl ester **8b** as a colorless oil in 87% yield. EIMS [IP 70 eV; *m*/*z* (% rel int)] 183 ($M^{+\cdot}$, 28), 155 (17), 138 (100), 109 (42), 81 (15), 54 (7). Ester **8b** (0.3 mmol, 55 mg) was added to 1 M aqueous NaOH (2 mL, excess) and the emulsion was stirred overnight at ambient temperature, at which time no ester was detectable by TLC analysis. The reaction mixture was extracted with Et_2O (2×2 mL), and the ethereal solution was discarded. The aqueous phase was acidified to pH 1 with 1N HCl and extracted with Et_2O (5×3 mL). The combined organic extracts were dried (Na_2SO_4) and concentrated to afford 38 mg (81%) of pure acid **8**. ^{1}H NMR ($CDCl_3$) δ 6.41 (1H, ddd, J=163, 16, 2.8 Hz, H-2), 7.75 (1H, ddd, J=16, 6.75, 2.6 Hz, H-3), (no aromatic H detectable).[18]

[ring-$^2H_5$,1,2-$^{13}C_2$]-3-Hydroxy-3-phenylpropionic acid (9). To a gray-green suspension of anhydrous $CrCl_2$ (251 mg, 2.05 mmol) and anhydrous LiI (11 mg, 0.08 mmol) in dry THF (3 mL) were added [ring-$^2H_5$]benzaldehyde (75 μl, 0.74 mmol) and ethyl [$^{13}C_2$]bromoacetate (91 μl, 0.82 mmol). The mixture was stirred at ambient temperature for 15 min and then warmed to 55°C for 1 h. The maroon suspension was cooled to ambient temperature, quenched with brine (3 mL) and vigorously stirred for 15 min. The organic layer was separated, and the aqueous phase was extracted with Et_2O (3×5 mL). The combined organic extracts were washed with H_2O, dried (Na_2SO_4), and concentrated under reduced pressure. The residue was purified on silica with 2:1 hexane/Et_2O and gave the ethyl ester **9b** (116 mg) as a colorless oil in 78% yield. EIMS [IP 70 eV; *m*/*z* (% rel int)] 201 ($M^{+\cdot}$, 31), 172 (4), 156 (6), 126 (12), 112 (100), 110 (62), 90 (21), 84 (62), 72 (10), 62 (19), 52 (11). Ester **9b** (40 mg, 0.2 mmol) was subsequently hydrolyzed as described for **8b** to the colorless oil **9** (28 mg) in 81% yield.[19] ^{1}H NMR ($CDCl_3$) δ 2.78 (2H, dddd, J=129, 17.8, 6.2, 3.7 Hz, H-2), 5.15 (1H, m, H-3), (no aromatic H detectable).[20]

[ring-$^2H_5$,1,2-$^{13}C_2$]-3-Oxo-3-phenylpropionic acid (10). β-Hydroxyester **9b** (50 mg, 0.25 mmol) was added to an ice-cold suspension of pyridinium chlorochromate (0.4 mmol) in CH_2Cl_2 (1.5 mL) and stirred at ambient temperature for 15 h. The suspension was filtered, the filtrate dried in vacuo and the residue chromatographed on silica with 3:1 hexane/ Et_2O providing 45 mg ethyl ester **10b** as a colorless oil in 91% yield.[16] EIMS [IP 70 eV; *m*/*z* (% rel int)]: 200 ($M^{+\cdot}$+1, 35), 154 (9) 138 (17), 126 (24), 110 (100), 82 (41), 54 (17), 45 (57). Ester **10b** (40 mg, 0.2 mmol) was subsequently hydrolyzed as described for **8b** to give the free acid **10** (29 mg, 84% yield) as a colorless oil.[21] ^{1}H NMR ($CDCl_3$) δ (mixture of 76% keto and 24% enol tautomers[22]) 3.98 (1.52H, dd, J=131, 18 Hz, H-2), 5.69 (0.24H, d, J=176 Hz, enol H-2), (no aromatic H detectable).[23]

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