Nonactin Biosynthesis: Unexpected Patterns of Label Incorporation from 4,6-Dioxoheptanoate Show Evidence of a Degradation Pathway for Levulinate through Propionate in *Streptomyces griseus*

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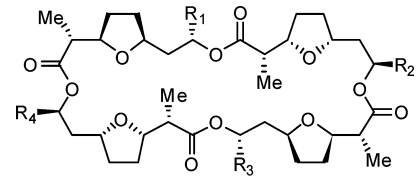
The polyketide nonactin, a polyketide possessing antitumor and antibacterial activity, is produced by an unusual biosynthesis pathway in *Streptomyces griseus* that uses both enantiomers of the nonactin precursor, nonactic acid. Despite many studies with labeled precursors, much of the biosynthesis pathway remains unconfirmed, particularly the identity of the last achiral intermediate in the pathway, which is believed to be 4,6-diketoheptanoyl-CoA. We set out to confirm the latter hypothesis with feeding studies employing [4,5-$^{13}C_2$]-, [5,6-$^{13}C_2$]-, and [6,7-$^{13}C_2$]-4,6-diketoheptanoate thioester derivatives. In each case the isotopic label was incorporated efficiently into nonactin; however, at positions inconsistent with the currently accepted biosynthesis pathway. To resolve the discrepancy, we conducted additional feeding studies with a [3,4-$^{13}C_2$]levulinate thioester derivative and again observed efficient label incorporation. The latter result was intriguing, as levulinate is not an obvious precursor to nonactin. Levulinate, however, is known to be efficiently degraded into propionate even though the pathway for the conversion is not known. On the basis of both our levulinate and diketoheptanoate isotope incorporation data we can now postulate a pathway from levulinate to propionate that can also account for the conversion of 4,6-diketoheptanoate into levulinate in *S. griseus*.

Streptomyces griseus subsp. g*riseus* ETH A7796 (DSM40695) makes a series of ionophore antibiotics known as the macrotetrolides (Figure 1).[1] The most prevalent homologue, nonactin, has been shown to possess antitumor activity and to be an effective inhibitor of the P170-glycoprotein-mediated drug resistance in cancer cells.[2] The 32-membered ring of a macrotetrolide is composed of four monomers, the latter being either nonactic acid or homononactic acid. In the case of nonactin, two monomers of (+)-nonactic acid and two monomers of (−)-nonactic acid are linked such that nonactin has S_4 symmetry and is achiral. Recently, we were able to demonstrate that diastereoisomers of nonactin, derived from only one enantiomer of nonactic acid, were inactive.[3] The production of both enantiomers of the precursor acids, and that such acids originate from succinic acid,[4] show that nonactin is an unusual polyketide.

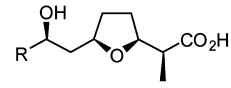
The broad outlines of the nonactin biosynthesis pathway were originally established by stable isotope feeding experiments with primary metabolites[5−9] and more advanced precursors.[4,10] The latter data showed that after an initial condensation of malonyl-CoA and succinyl-CoA (Scheme 1) an adipate derivative **8** was generated. Acylation of **8** with acetyl-CoA led to the generation of both enantiomers of nonactic acid; acylation with propionyl-CoA led to both enantiomers of homononactic acid. The conversion of **9a/b** into **10a/b** can be accomplished by transposition of the CoASH thioester followed by spontaneous decarboxylation. Such a *trans*-thioesterification reaction is supported by data from the sequencing and annotation of the nonactin biosynthesis gene cluster.[11,12] The deduced product of *orf8* has high sequence identity to *E*-glutaconate: acetyl-CoA CoASH transferase[13−15] as well as a number of other enzymes that catalyze the transfer of CoASH.[16−19] The *E*-glutaconate transferase acts in a ping-pong mechanism to first transfer CoASH from the donor thioester to a glutamate residue. In a second step, the CoASH moiety is transferred from the glutamate to the acceptor carboxylate.[15] The thioester transposition proposed in nonactin biosynthesis (**9** to **10**) is the intramolecular analogue of the succinyl-CoA:3-ketoacid CoASH transferase-catalyzed reaction. As intermediates **9** and **10** are achiral or readily epimerizable, they are common to both enantiocomplementary pathways that generate nonactic acid, and the divergence in stereochemistry must occur later in the pathway. The divergence presumably arises through the stereoselectivity of dehydrogenase enzymes acting on such substrates. In this case **10** would not be an intermediate in nonactin biosynthesis. Consequently, we would have strong evidence that our notion of a thioester translocation was valid if we were able to show that **10** was indeed a biosynthesis intermediate, a hypothesis testable using a traditional feeding experiment.

Results and Discussion

Our first approach to testing our hypotheses was to attempt the obvious feeding experiment with a ^{13}C-labeled derivative of **10**. We chose a dual-^{13}C labeling pattern for our probe **10a** (Scheme 2, compound **15**), as the dual label offers greater sensitivity and allows us to determine if degradation of the intermediate occurs prior to incorporation into nonactin. Benzyl acetate was prepared



	R_1	R_2	R_3	R_4
Nonactin (**1**)	Me	Me	Me	Me
Monactin (**2**)	Me	Me	Et	Me
Dinactin (**3**)	Et	Me	Et	Me
Trinactin (**4**)	Et	Et	Et	Me
Tetranactin (**5**)	Et	Et	Et	Et



6 R = CH_3 (+)-nonactic acid
7 R = C_2H_5 (+)-homononactic acid

Figure 1. Structures of the naturally occurring macrotetrolides and their precursors.

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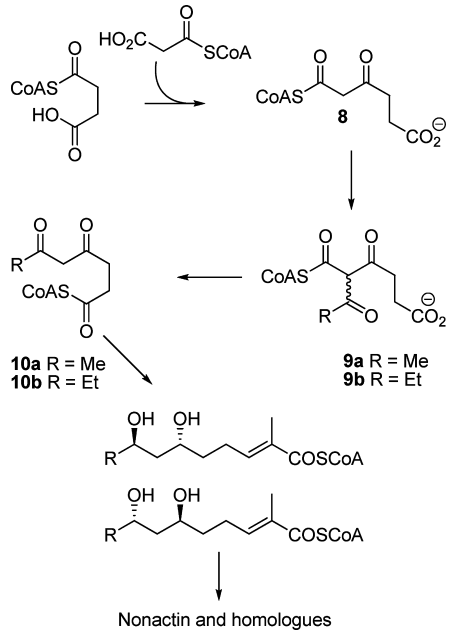
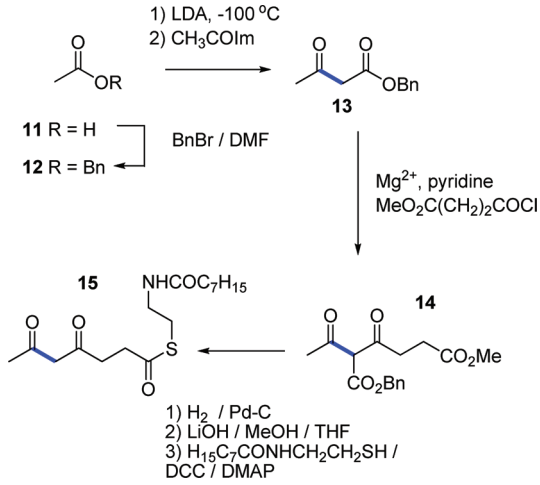
Scheme 1. Early Steps of the Nonactin Biosynthesis Pathway



Scheme 2. Synthesis of Labeled 4,6-Diketoheptanoate Derivatives



from [2-$^{13}C_1$]acetate and then used to generate an enolate that, at low temperature, was quenched with an imidazolide generated from [1-$^{13}C_1$]acetic acid. The benzyl ester was chosen so as to add molecular weight to the synthetic intermediates and as a useful chromophore to aid in purification. Acylation of **13** using Mg^{2+}/pyridine[20] to generate the required enolate worked well. Hydrogenolysis of **14** occurred with spontaneous decarboxylation. The latter process is analogous to the conversion of **9** to **10** in nonactin biosynthesis, where we postulate that the *trans*-thioesterification reaction also occurs with concomitant, spontaneous decarboxylation. Straightforward replacement of the methyl ester with *N*-caprylcysteamine under standard conditions[21] gave our first required, labeled probe. The probe was administered to a fermentative culture of *S. griseus* at the onset of nonactin production, and after 48 h macrotetrolides were recovered.[4] A preliminary ^{13}C NMR survey showed significant incorporation of ^{13}C label into the macrotetrolide mixture. To simplify the analysis, the macrotetrolides were subjected to acid methanolysis to afford methyl nonactate (**6**-OMe) and methyl homononactate (**7**-OMe), which were separated by chromatography. Reduction of methyl nonactate with $LiAlH_4$ gave the expected, known diol[10] as a mixture of enantiomers. Derivatization of the diol with Mosher's chloride was followed by ^{13}C NMR analysis. It was evident that the contiguous $^{13}C-^{13}C$ labeling of the precursor

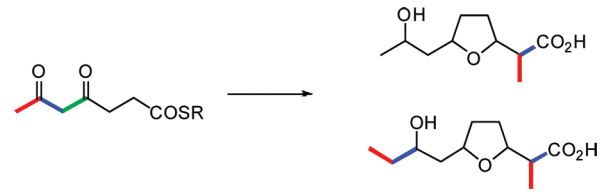
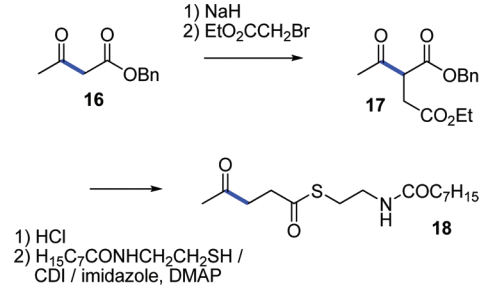


Figure 2. Summary of feeding results obtained with [4,5-$^{13}C_2$]- (green), [5,6-$^{13}C_2$]- (blue), and [6,7-$^{13}C_2$]-4,6-diketoheptanoate (red) derivatives. The ^{13}C label is efficiently incorporated into nonactin, via nonactic acid, at positions derived from propionate.

Scheme 3. Synthesis of a Thioester Derivative of [3,4-$^{13}C_2$]Levulinate

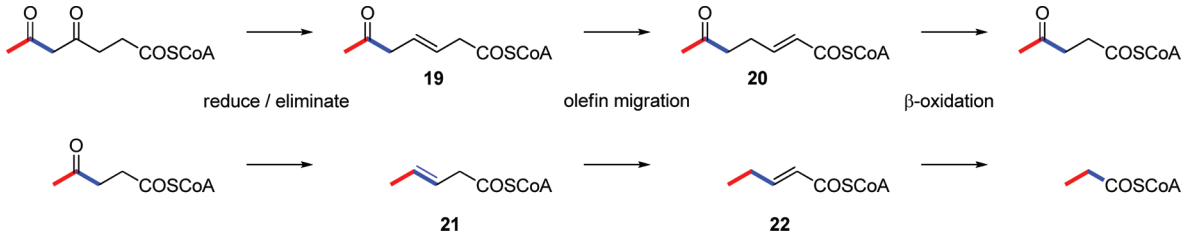


remained intact, although the incorporation of the label at the C1−C2 (Figure 2, blue highlight) positions rather than the expected C7−C8 positions was a surprise. In repetitions of the experiment, label incorporations of 1% to 10% were obtained, with higher incorporations being observed at lower overall levels of macrotetrolide synthesis. The levels of ^{13}C incorporation were comparable to those observed in feeding experiments with labeled propionate. No significant enrichments by single ^{13}C labels were observed. Furthermore, the intact $^{13}C-^{13}C$ label was found at C1−C2 and C8−C9 in methyl homononactate. Analysis of diastereoisomeric derivatives made from both methyl nonactate and methyl homononactate showed that there was no stereoselectivity associated with incorporation. Both enantiomers of each acid were labeled to the same extent and at the same positions. The observed labeling pattern is inconsistent with the proposed biosynthesis pathway but does suggest that **15** is degraded to propionate, as the observed labeling patterns match those observed from feeding [2,3-$^{13}C_2$]propionate to *S. griseus*.

To further understand the unusual incorporation of **15**, we prepared the isotopomers [4,5-$^{13}C_2$]- and [6,7-$^{13}C_2$]-4,6-diketoheptanoate through analogous synthetic chemistry. Incorporation of the latter labels was again evident in the macrotetrolides but at positions, and in patterns, consistent with the degradation of 4,6-diketoheptanoate into propionyl-CoA. The efficient incorporation of ^{13}C label shows that the probe compounds are capable of entering the cell and participating in metabolism. The lack of incorporation of label at the expected positions is suggestive that **15** is not an intermediate in nonactin biosynthesis. However, to discount **15** as an intermediate, we need to understand how **15** is efficiently converted into propionate. Such a metabolic pathway is not immediately obvious, as the 6-keto group of **15** must become a methylene and the 5-position of **15** must become the carboxyl group of propionate without scrambling or breaking of the C5−C6 and C6−C7 bonds of **15**.

At this point we decided to regroup and evaluate some less likely pathways to nonactic acid. One such pathway involved a key reaction in which levulinate could be acylated to generate 4,6-diketoheptanoate. We prepared, therefore, a thioester derivative of [3,4-$^{13}C_2$]levulinate as a probe (Scheme 3). Benzyl [2,3-$^{13}C_2$]acetoacetate was generated from benzyl [2-$^{13}C_1$]acetate and [1-$^{13}C_1$]acetic acid as described above for **13**. Deprotonation of **16** with NaH, followed by reaction with ethyl 2-bromoacetate, led to

Scheme 4. Primary Metabolic Pathways That Account for the Conversion of 4,6-Diketoheptanoyl-CoA into Levulinoyl-CoA and Levulinoyl-CoA into Propionyl-CoA[a]



[a] The pathways account for the labeling patterns observed in the feeding experiments and for the use of levulinate as a propionate precursor in fermentation.

17, which, upon saponification, underwent spontaneous decarboxylation to afford [3,4-$^{13}C_2$]levulinic acid. The latter compound was converted to activated *N*-caprylcysteamine thioester **18** using standard procedures.[21]

We used both levulinic acid and **18** in feeding experiments in fermentative cultures of *S. griseus*. The macrotetrolides recovered from these experiments were converted into methyl nonactate and methyl homononactate and analyzed by ^{13}C NMR. The dual label was incorporated intact at C1−C2 of nonactate and both C1−C2 and C8−C9 of homononactate, a pattern identical to that observed in the feeding experiment of the [5,6-$^{13}C_2$]-4,6-diketoheptanoate thioester. Resolution of the enantiomers of nonactic acid was achieved this time using a *Rhodococcus*-mediated biotransformation, a stereoselective oxidation of methyl (−)-nonactate to methyl (−)-8-ketononactate.[22] Dual ^{13}C-labeling was observed in both methyl (+)-nonactate and methyl (−)-8-ketononactate, confirming our expectation that no stereoselection is observed in the conversion of levulinate.

The conversion of levulinate to propionyl-CoA, such that levulinate is a precursor of nonactin, is, at best, unlikely. The levulinate feeding experiment, however, was of significance, as it drew our attention to pathways for levulinate degradation and provided the final clue needed to make a rational hypothesis for the unusual degradation of 4,6-diketoheptanoate into propionate that was observed in our original set of feeding experiments. It is known that levulinate can act as a source of propionate and is often used as such in fermentation media, although the details of the conversion are not clear.[23] Our initial feeding experiments can be rationalized on the basis of the degradation of 4,6-diketoheptanoyl-CoA into levulinoyl-CoA (Scheme 4).

The conversion of 4,6-diketoheptanoyl-CoA into levulinoyl-CoA would presumably occur via reduction of the ketone at C-4 of the probe, followed by elimination of the intermediate hydroxy group to give **19**. Migration of the olefin into conjugation with the thioester would afford intermediate **20**, which can undergo one round of standard β-oxidation to be converted into a levulinoyl-CoA. Degradation of levulinoyl-CoA to propionyl-CoA would use the same sequence of reduction−elimination (gives **21**), olefin migration (gives **22**), and β-oxidation that then accounts fully for both the conversion of 4,6-diketoheptanoate into levulinate and levulinate into propionate and that agrees with the disposition of the ^{13}C-labeling patterns observed in our feeding experiments.

Our original plan to confirm that 4,6-diketoheptanoate was an intermediate in nonactin biosynthesis through the use of labeling experiments was foiled, as there is likely a very active pathway for the degradation of levulinoyl-CoA to propionyl-CoA operating in *S. griseus*. The latter pathway would account for the conversion of 4,6-diketoheptanoyl-CoA into levulinoyl-CoA and the overall conversion of our labeled probes into propionyl-CoA. The overall result is that our initial feeding experiments can neither confirm nor rule out 4,6-diketoheptanoyl-CoA as a nonactin biosynthesis intermediate. Our labeling studies do shed light, however, on the known but little understood conversion of levulinate into propionate and allow us to propose a pathway for the latter conversion that accounts for all our feeding results.

Experimental Section

General Experimental Procedures. NMR spectra were acquired at 400 MHz (^1H) and 100 MHz (^{13}C), were referenced to the residual solvent, and are reported as chemical shift (δ/ppm), intensity, splitting pattern, and coupling constant (*J*/Hz). Solvents were obtained from Fisher Scientific. All other reagents, unless noted otherwise, were obtained from Aldrich. The solvents CH_2Cl_2 (over CaH_2) and THF (over Na/K-benzophonone) were dried and distilled prior to use. Solutions were concentrated by evaporation *in vacuo*. All synthesis procedures, unless noted otherwise, were carried out under a slight positive pressure of dry argon gas.

Microorganism. *Streptomyces griseus* subsp. griseus ETH A7796 was obtained from the Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (DSM 40695). Fermentation of *S. griseus* and feeding studies were performed according to our standard procedures.[4]

Benzyl [2-$^{13}C_1$]Acetate (12). Benzyl bromide (2.40 mL, 20.0 mmol) was added to a solution of sodium [2-$^{13}C_1$]acetate (0.836 g, 10.0 mmol) in anhydrous DMF (20 mL). The solution was heated at 96 °C for three days. Water (80 mL) and Et_2O (120 mL) were added to the cooled mixture, which was then stirred. The organic layer was recovered, and the aqueous layer then extracted with Et_2O (2 × 120 mL). The Et_2O fractions were combined, dried over $MgSO_4$, filtered, and concentrated. Chromatography on silica gel, eluting with EtOAc−hexanes (15:85), gave the product as a liquid (1.36 g, 89.8%): ^1H NMR (400 MHz, $CDCl_3$) δ 7.30 (5H, m), 5.10 (2H, s), and 2.08 (3H, d, $J_{^1H-^{13}C}$ = 134.5 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.7 (d, $J_{^{13}C-^{13}C}$ = 59.4 Hz), 135.8, 128.1, 66.1, and 20.9.

Benzyl [2,3-$^{13}C_2$]-3-Oxobutanoate (13). Diisopropylamine (1.3 mL, 9.2 mmol) was added to dry THF (10 mL) in a 50 mL three-neck round-bottom flask, cooled in a dry ice-acetone bath at −60 °C. *n*-BuLi (5.1 mmol) in hexanes (17.4 mL) was added via syringe. Compound **12** (1.16 g, 7.7 mmol) was dissolved in dry THF (5 mL), and the solution added dropwise via syringe. The temperature was maintained below −60 °C for 45 min. [1-$^{13}C_1$]Acetic acid (0.27 mL, 4.6 mmol) was added via syringe to a stirred suspension of recrystallized 1,1′-carbonyldiimidazole (0.845 g, 5.20 mmol) in dry THF (5 mL) in a 10 mL round-bottom flask. Stirring was maintained for 1 h at rt. The acetylimidazole solution was then transferred to the enolate solution via syringe over 5 min, maintaining the internal temperature below −20 °C. The reaction was stirred for 45 min, quenched by addition of water (25 mL), acidified to pH 4 with 2 M HCl, and then extracted with $CHCl_3$ (3 × 50 mL). The organic layers were combined and dried over $MgSO_4$, filtered, and concentrated. The product was purified by chromatography on silica gel, eluting with EtOAc−hexanes (15:85), as an oil (0.46 g, 51.5%): ^1H NMR (400 MHz, $CDCl_3$) δ 7.35 (5H, m), 5.16 (2H, s), 3.48 (2H, dd, $J_{^1H-^{13}C}$ = 130.4 Hz, 6.59 Hz), and 2.23 (3H, d, $J_{^{13}H-^{13}C}$ = 6.59 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 200.3 (keto, enriched, d, $J_{^{13}C-^{13}C}$ = 36.6 Hz), 175.8 (enol, enriched, d, $J_{^{13}C-^{13}C}$ = 71.7 Hz), 135.1, 128.5, 128.4, 128.3, 89.5 (enol, enriched, d, $J_{^{13}C-^{13}C}$ = 73.2 Hz), 67.0, 49.9 (keto, enriched, d, $J_{^{13}C-^{13}C}$ = 38.1 Hz), and 30.0.

1-Benzyl 6-Methyl [2-^{13}C]-([1-^{13}C]-2-Acetyl)-3-Oxohexanedioate (14). 13 (1.31 g, 6.8 mmol) was added to a suspension of anhydrous $MgCl_2$ (0.65 g, 6.9 mmol) in dry CH_2Cl_2 (7 mL), which was then cooled to 0 °C. Dry pyridine (1.1 mL, 13.7 mmol) was added via syringe to the stirred suspension. After 15 min at 0 °C, carbomethoxypropionyl chloride (0.85 mL, 6.9 mmol) was added via syringe. After a further

15 min at 0 °C the suspension was allowed to warm to rt, and stirring continued for 2 h. Aqueous HCl solution (6 M, 5 mL) was added carefully, and the mixture extracted with Et_2O (3 × 25 mL). The organic extracts were combined, dried, and concentrated. Chromatography on silica gel, eluting with EtOAc−hexanes (2:3), gave the product as a faint yellow oil (1.54 g, 73.3%): ^{1}H NMR (250 MHz, $CDCl_3$) δ 17.8 (1H, dd, $J_{^1H-^{13}C}$ = 6.5, 4.5 Hz), 7.3 (5H, m), 5.2 (2H, s), 3.6 (3H, s), 3.0 (2H, t, $J_{^1H-^1H}$ = 6.8 Hz), 2.6 (2H, tt, $J_{^1H-^1H}$ = 6.8 Hz) and 2.3 (3H, dd, $J_{^1H-^{13}C}$ = 6.1, 2.2 Hz); ^{13}C NMR (67 MHz, $CDCl_3$) δ 199.3, 193.9 (enriched, d, $J_{^{13}C-^{13}C}$ = 63.6 Hz) 183.5 (d, $J_{^{13}C-^{13}C}$ = 70.1 Hz), 172.6, 135.5, 128.5, 128.4, 128.3, 72.7 (enriched, d, $J_{^{13}C-^{13}C}$ = 36.2 Hz), 66.6, 51.5, 33.6, 28.4, and 24.7.

***S*-[2-(Octanoylamino)ethyl] [5,6-$^{13}C_2$]-4,6-Dioxoheptanethioate (15).** Pd on C (5%, 1.077 g) was added to a solution of **14** (1.54 g, 5.0 mmol) in MeOH (40 mL), and the mixture was stirred under a H_2 atmosphere (balloon) for 3 h. Celite was added, and the suspension filtered. The filtrate was concentrated, and the product, methyl [5,6-$^{13}C_2$]-4,6-dioxoheptanoate, was obtained as an oil (0.64 g, 74.4%) that was carried through to the next reaction without further purification. Aqueous LiOH solution (2.5 M, 4.5 mL) was added to a stirred solution of methyl [5,6-$^{13}C_2$]-4,6-dioxoheptanoate (0.64 g, 3.7 mmol) in THF (13.5 mL) and methanol (9 mL). The single phase solution was stirred at rt overnight. The reaction was extracted with EtOAc (5 × 150 mL), and the organic extracts were combined, dried, filtered, and concentrated to give a yellow solid (0.46 g, 73%). The material was carried through without further purification. Dicyclohexylcarbodiimide (0.47 g, 2.3 mmol) was added to a stirred solution of 4,6-dioxoheptanoic acid (0.18 g, 1.1 mmol), *N*-caprylcysteamine (0.36 g, 1.8 mmol), and DMAP (0.037 g, 0.03 mmol) in CH_2Cl_2 (12 mL). After two days the copious white precipitate was removed by filtration. The filtrate was concentrated and then fractionated on silica gel, eluting with EtOAc−EtOH−hexanes (38:2:60), to give the title compound (0.31 g, 78.3%): ^{1}H NMR (250 MHz, $CDCl_3$) δ 15 (0.5H, br, s), 5.87 (0.9H, br, s), 5.5 (0.5H, dd, $J_{^1H-^1C}$ = 4.8, 164.8 Hz), 3.65 (0.5H, dd, $J_{^1H-^{13}C}$ = 6.4, 89.2 Hz), 3.4 (2H, q, $J_{^1H-^1H}$ = 6.0 Hz), 3.02 (2H, t, $J_{^1H-^1H}$ = 6.4 Hz), 2.87 (2H, t, $J_{^1H-^1H}$ = 6.8 Hz), 2.7 (2H, t, $J_{^1H-^1H}$ = 6.8 Hz), 2.2 (1H, dd, $J_{^1H-^{13}C}$ = 1.2, 6.4 Hz), 2.1 (2H, m), 2.00 (2H, dd, $J_{^1H-^{13}C}$ = 3.6, 6.4 Hz), 1.57 (2H, t, $J_{^1H-^1H}$ = 6.8 Hz), 1.25 (9H, br, m), and 0.84 (3H, t, $J_{^1H-^1H}$ = 6.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 208.3 (d, $J_{^{13}C-^{13}C}$ = 38.2 Hz), 201.7 (d, $J_{^{13}C-^{13}C}$ = 36.6 Hz), 199.6, 198.4, 194.8, 187.1 (d, $J_{^{13}C-^{13}C}$ = 62.6 Hz), 173.4, 99.7 (d, $J_{^{13}C-^{13}C}$ = 64.1 Hz), 57.7 (d, $J_{^{13}C-^{13}C}$ = 36.6 Hz), 43.0 (d, $J_{^{13}C-^{13}C}$ = 39.7 Hz), 39.3, 38.3, 36.6, 33.9 (dd, $J_{^{13}C-^{13}C}$ = 10.7, 31.1 Hz), 31.6, 29.2, 28.9, 28.6, 25.6, 23.6, 22.5, and 14.0.

1-Benzyl 4-Ethyl [2-$^{13}C_1$]-([1-$^{13}C_1$]-2-Acetyl)succinate (17). To a solution of **13** (0.36 g, 1.86 mmol) in benzene (10 mL) was added in small portions 60% NaH in mineral oil (0.078 g, 1.95 mmol). The mixture was stirred at rt for 1 h. Ethyl bromoacetate (0.25 mL, 2.23 mmol) was added via syringe. The solution was heated at 90 °C overnight, after which the reaction was quenched by careful addition of H_2O (10 mL), followed by the addition of EtOAc (10 mL). The mixture was shaken, and the organic layer recovered. The aqueous layer was extracted with EtOAc (2 × 20 mL). The organic layers were combined and dried over $MgSO_4$, filtered, and concentrated. Purification via silica gel chromatography, eluting with EtOAc−hexanes (40:60), gave 1-benzyl 4-methyl [2-$^{13}C_1$]-([1-$^{13}C_1$]-2-acetyl)succinate (0.405 g, 77.8%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 (5H, m), 5.15 (2H, d), 4.17 (0.5H, m), 4.08 (2H, q, $J_{^1H-^1H}$ = 6.6 Hz), 3.85 (0.5H, m), 2.90 (2H, m), 2.30 (3H, d, $J_{^1H-^{13}C}$ = 6.6 Hz), and 1.20 (3H, t, $J_{^1H-^1H}$ = 6.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.5 (keto, enriched, d, $J_{^{13}C-^{13}C}$ = 35.1 Hz), 201.7 (keto, enriched, d, $J_{^{13}C-^{13}C}$ = 36.6 Hz), 175.0 (enol, enriched, d, $J_{^{13}C-^{13}C}$ = 76.3 Hz), 128.8, 128.7, 128.5, 94.8 (enol, enriched, d, $J_{^{13}C-^{13}C}$ = 76.3 Hz), 67.7, 61.2, 59.5 (keto, enriched, d, $J_{^{13}C-^{13}C}$ = 35.1 Hz), 54.8 (keto, enriched, d, $J_{^{13}C-^{13}C}$ = 36.6 Hz), 32.5 (d), 30.2 (m), and 14.3.

***S*-[2-(Octanoylamino)ethyl] [3,4-$^{13}C_2$]-4-Oxopentanethioate (18).** **17** (0.405 g, 1.45 mmol) was mixed with HCl (2 M, 8 mL) and heated in an oil bath at 105 °C for 3.5 h, then stirred gently overnight at rt. The reaction was extracted with EtOAc (3 × 20 mL). The organic phases were combined, dried over $MgSO_4$, and concentrated; the product was purified by silica gel chromatography, eluting with EtOAc−hexanes−AcOH (50:49:1), as a white solid (0.104 g, 61.1%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.9 (br), 2.88 (1H, q), 2.58 (4H, m), and 2.16 (3H, dd, $J_{^1H-^{13}C}$ = 5.86 Hz, $J_{^1H-^{13}C}$ = 1.47 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.7 (d, $J_{^{13}C-^{13}C}$ = 38.15 Hz), 178.7, 37.5 (d, $J_{^{13}C-^{13}C}$ = 39.7 Hz), 30.0 (m), and 27.7 (d, $J_{^{13}C-^{13}C}$ = 38.1 Hz). 1,1-Carbonyldiimidazole (0.0855 g, 0.527 mmol) was added in one portion to a stirred solution of [3,4-$^{13}C_2$]-4-oxopentanoic acid (0.051 g, 0.44 mmol) in CH_2Cl_2 (1 mL). The solution was stirred for a further 30 min. 4-Dimethylaminopyridine (0.016 g, 0.13 mmol) was then added, and the mixture was stirred for another 30 min, followed by addition of *N*-caprylcysteamine (0.177 g, 1.10 mmol) in one portion. The reaction mixture was stirred overnight and then quenched by addition of H_2O (10 mL). The mixture was extracted with EtOAc (3 × 10 mL), and the organic layers were combined, dried over $MgSO_4$, and concentrated. The thioester product was recovered by chromatography on silica gel eluting with EtOAc−hexanes (6:4) as a white solid (0.126 g, 93.8%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.90 (1H, br), 3.40 (2H, q, $J_{^1H-^1H}$ = 6.6 Hz), 3.00 (2H, q), 2.82 (2H, p), 2.61 (1H, q), 2.16 (3H, dd, $J_{^1H-^{13}C}$ = 5.86 Hz, $J_{^1H-^{13}C}$ = 1.47 Hz), 2.10 (2H, $J_{^1H-^1H}$ = 7.32 Hz), 1.58 (2H, p, $J_{^1H-^1H}$ = 7.3 Hz), 1.25 (9H, m), 0.75 (3H, t, $J_{^1H-^1H}$ = 6.59 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.0 (d, $J_{^{13}C-^{13}C}$ = 38.1 Hz) 198.6, 173.3, 39.2, 38.0 (d, $J_{^{13}C-^{13}C}$ = 38.1 Hz), 36.5, 31.6, 29.7 (m), 29.2, 28.9, 28.6, 25.6, 22.5, and 14.0.

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Supporting Information Available: ^{13}C NMR spectra of methyl nonactate and methyl homononactate prepared from nonactin isolated in isotope feeding experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

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