The potential role of fatty acid initiation in the biosynthesis of the fungal aromatic polyketide aflatoxin B_1[1]

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This paper is dedicated to Professor Ian D. Spenser

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Earlier work in this laboratory has shown the intact incorporation of [1-^{13}C]hexanoate into averufin (**1**), a key intermediate in aflatoxin B_1 biosynthesis. Parallel experiments with equimolar amounts of [1-^{13}C]butyrate, [1-^{13}C]-3-oxo-octanoate, and [1-^{13}C]-5-oxo-hexanoate gave no detectable specific incorporation of heavy isotope but low and equivalent background incorporation comparable to [1-^{13}C]acetate. Three of these potential intermediates in polyketide formation were reexamined as their corresponding *N*-acetylcysteamine (NAC) thioesters. The NAC thioester of [1-^{13}C]hexanoic acid gave a remarkably high 22% intact incorporation while the NAC thioester of [1-^{13}C]-3-oxo-octanoic acid afforded nearly 5% when an equimolar amount was administered to the producing organism *Aspergillus parasiticus* (ATCC 24551). In contrast, the NAC thioester of [1-^{13}C]butyric acid showed no selective enrichment of averufin. This negative result was tested further in a more sensitive experiment with the NAC thioester of [2,3-$^{13}C_2$]butyric acid. No $^1J_{CC}$ coupling was detectable, indicating an incorporation efficiency of <0.1%. [1-^{13}C,$^{18}O_2$]Hexanoate was prepared and gave a 53% retention of ^{18}O relative to the ^{13}C internal standard in keeping with previous experiments with [1-^{13}C,$^{18}O_2$]acetate. It is concluded from these data that the initial C_6 segment of polyketide biosynthesis is unlikely to arise by β-oxidation of a higher fatty acid but more probably is generated by a specialized fatty acid synthase (FAS) that provides this unit either separately to the polyketide synthase (PKS) or as part of a larger FAS/PKS fusion. While these two physical arrangements cannot be distinguished by these experiments, both must accommodate comparatively efficient exchange of the NAC thioesters of both hexanoic and 3-oxo-octanoic acid, but not the NAC thioester of butyric acid.

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Des travaux antérieurs effectués dans notre laboratoire ont démontré que le [1-^{13}C]hexanoate s'incorpore d'une façon intacte dans l'avérufine (**1**), un intermédiaire clé dans la biosynthèse de l'aflatoxine B_1. Des expériences parallèles avec des quantités équimoléculaires de [1-^{13}C]butyrate, de [1-^{13}C]-oxo-octanoate et de [1-^{13}C]-5-oxo-hexanoate n'ont pas permis de détecter d'incorporation spécifique de l'isotope lourd; seulement des incorporations faibles, équivalentes au bruit de fond, comparables à celle du [1-^{13}C]acétate. On a réexaminé trois de ces intermédiaires potentiels dans la formation du polycétides sous la forme de thioesters de la *N*-acétylcystéamine (NAC). Lorsqu'on administre des quantités équimoléculaires à l'organisme *Aspergillus parasiticus* (ATCC 24551) qui les produit, le thioester de la NAC de l'acide [1-^{13}C]hexanoïque conduit à une incorporation intacte étonnante de 22% alors que le thioester de la NAC de l'acide [1-^{13}C]-3-oxo-octanoïque en fournit environ 5%. Par opposition, le thioester de la NAC de l'acide [1-^{13}C]butyrique ne présente pas d'enrichissement sélectif de l'avérufine. On a évalué ce résultat négatif plus à fond à l'aide d'une expérience sensible impliquant le thioester de la NAC de l'acide [2,3-$^{13}C_2$]butyrique. On n'a pas observé de couplage $^1J_{CC}$; ce résultat indique que l'efficacité de l'incorporation est inférieure à 0,1%. On a préparé du [1-^{13}C,$^{18}O_2$]hexanoate et il conduit à une rétention de 53% du ^{18}O par rapport au ^{13}C agissant comme référence interne; ce résultat est en accord avec les expériences antérieures avec du [1-^{13}C,$^{18}O_2$]acétate. Sur la base de ces données on conclut que le segment initial en C_6 de la synthèse du polycétide ne provient probablement pas d'une oxydation β d'un acide gras de poids moléculaire plus élevé, mais qu'il est vraisemblablement généré par une synthase d'acide gras spécialisée (FAS) qui fournit cette unité soit d'une façon séparée par rapport à la synthase de polycétide (PKS) ou dans le cadre d'une fusion plus large FAS/PKS. Alors que l'on ne peut pas distinguer entre ces deux arrangements physiques sur la base des expériences réalisées, les deux doivent toutefois accommoder un échange efficace des thioesters de la NAC des acides tant hexanoïque que 3-oxo-octanoïque, mais pas de l'acide butyrique.

[Traduit par la rédaction]

In 1982 we made the unexpected and intriguing observation that [1-^{13}C]hexanoate incorporated label intact into averufin (**1**), a central intermediate in the biosynthesis of the potent mycotoxin aflatoxin B_1 (**2**). The efficiency of linear fatty acid utilization in these whole-cell experiments was 3–4% at C-1′. The specific incorporation was seen in a background of ca. 0.5% at all carboxyl-derived carbons throughout the molecule. This nonspecific labeling is due to β-oxidation of the hexanoate to [1-^{13}C]acetyl CoA and secondary incorporation of the latter in accord with the conventional view of polyketide biosynthesis (1). This experiment was followed by a series under the same experimental conditions in which *equimolar* amounts of [1-^{13}C]butyrate, [1-^{13}C]-5-oxo-hexanoate, and [1-^{13}C]-3-oxo-octanoate were tested. In each instance no intact incorporation of these potential substrates could be detected by ^{13}C{^{1}H}NMR spectroscopy. Only degradation to [1-^{13}C]acetyl CoA was seen and secondary incorporation of heavy isotope as above, comparable to that observed from [1-^{13}C]acetate itself (2).

While in bacteria and plants polyketide starter units have been demonstrated from low molecular weight branched acids (derived from transamination and oxidative decarboxylation of the homologous amino acids (see for example ref. 3)) and propionate (4), exceptions are few to the general observation that "extra" carbon atoms in fungal polyketides are derived from methionine (5). The classic exception to this general rule is the perturbation of orsellinic acid biosynthesis in *Penicillin baarnense* reported by Mosbach in which added propionate gave rise to the appearance of homoorsellinate (6). Similarly, Steyn's group determined that the C_3 primer of aurovertin B (7) and

[1] This paper is dedicated to Professor Ian Spenser and a career of accomplishment in natural products chemistry.

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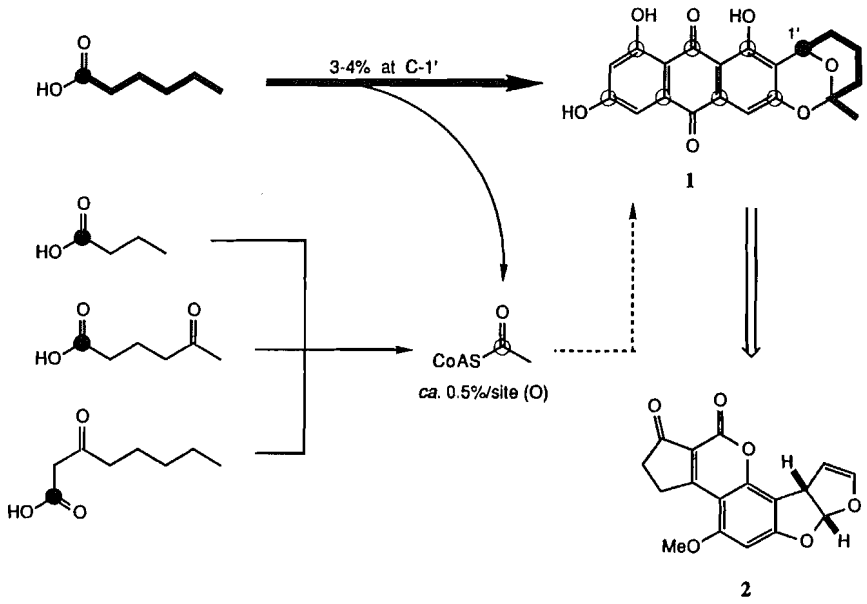

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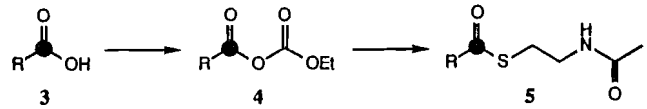
SCHEME 1

asteltoxin (8) can arise either by direct incorporation of propionate or by the net utilization of acetate and a C_1 unit from methionine.

The suggestion that fatty acids could initiate polyketide biosynthesis was made by Birch more than 30 years ago (9). Yet, apart from two examples that can be recalled from plants (butyrate into margaspidin (10) and octanoate into coniine (11)), failed incorporations of fatty acids, when reported, have been the general rule (see for example ref. 12). The utilization of hexanoate in the biosynthesis of the fungal metabolite averufin, therefore, was an important experimental step forward in the understanding of polyketide biosynthesis. Report of these results (1, 2) was soon followed by the incorporation of acetoacetate into nonactin (13) and octanoate into the chain-terminating unit of fungichromin (14). Successes, however, were limited. Attempts to demonstrate the intact incorporation of free acids derived from linking together of two or three ketide units into macrolides, polyethers, and other secondary metabolites were completely unsuccessful (15, 16). The earlier, crucial finding of Lynen (17) that *N*-acetylcysteamine (NAC) thioesters could substitute for the corresponding CoA thioesters to load biosynthetic intermediates onto fatty acid synthase (FAS) was recalled at this time by the NMR studies of Schwab et al. (18) examining the inhibition of β-hydroxydecanoylthioester dehydrase by the NAC thioester of 3-decynoic acid (19). Testing of potential polyketide intermediates as their NAC thioesters (15, 16) came as a methodological breakthrough that has since been successfully applied in a number of systems and has experimentally secured the central idea of processivity in polyketide chain elongation (see for example ref. 20).

Questions remained, however, from our first experiments. Why was butyrate not detectably incorporated if hexanoate gave a readily observable enrichment? Should one expect that 3-oxo-octanoate, derived by the addition of the first clearly polyketide C_2 unit to a saturated primer, might be successfully incorporated? It could be the case that both of these putative intermediates were utilized but at such low levels that they could not be experimentally detected above natural abundance by single-site ^{13}C-labeling methods. In the hope of achieving greater sensitivity in these experiments, a series of NAC thioesters were prepared and examined for their incorporation into averufin.

The mixed anhydride method of Kass and Brock (21) using freshly prepared *N*-acetylcysteamine (22) gave the NAC thioesters of [1-^{13}]butyric acid and [1-^{13}C]hexanoic acid in 70–80% yields. This approach is summarized in Scheme 2. [2,3-$^{13}C_2$]Butyric acid was prepared by reaction of the dianion of [2-^{13}C]acetic acid and [1-^{13}C]iodoethane in the presence of added HMPA (23). [1-^{13}C]Hexanoic acid was synthesized according to the protocol of Cane et al. from 1-iodopentane and K^{13}CN (24). The yields of the latter two acids were greater than 80%.



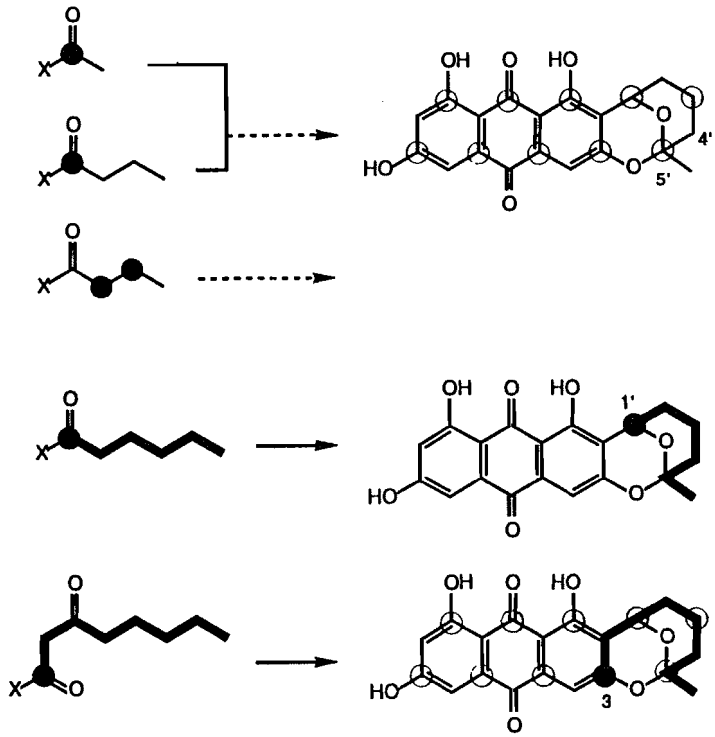
3 4 5

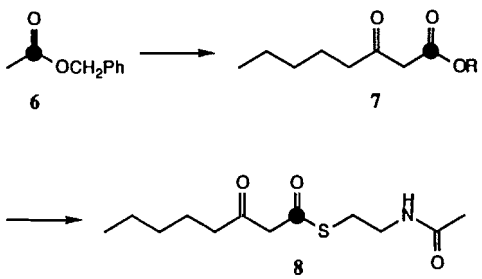
SCHEME 2

Benzyl [1-^{13}C]acetate (25) was treated with lithium hexamethyldisilazide followed by hexanoyl chloride to give the β-ketoester **7** (R = CH_2Ph) in 77% yield. Hydrogenolysis proceeded smoothly to afford the known acid **7** (R = H) (26). Not unexpectedly, thioesterification using the method of Kass and Brock (21) did not take place owing presumably to the self-condensation of the mixed anhydride **4** under the reaction conditions. Literature precedent (27) notwithstanding, attention was turned toward DCC-activated coupling in the presence of 4-dimethylaminopyridine (28). The NAC thioester **8** of 3-oxo-octanoic acid was obtained in about 70% yield as a crystalline solid.

The NAC thioesters of [1-^{13}C]acetic acid, [1-^{13}C]butyric acid, [1-^{13}C]hexanoic acid, and [1-^{13}C]-3-oxo-octanoic acid were administered in equimolar amounts to cell suspensions of *Aspergillus parasiticus* under carefully controlled conditions as was done earlier with the carboxylic acids (1, 2). Freshly prepared mycelial pellets were weighed into 12 250-mL Erlenmeyer flasks (10 g per flask) containing 100 mL of a replacement medium (29). The ^{13}C-labeled thioesters were

TABLE 1. Incorporation of ^{13}C-labeled carboxylic acids (X = OH) and their corresponding *N*-acetylcysteamine thioesters (X = NAC) into averufin by mycelial suspensions of *A. parasiticus* (ATCC 24551)





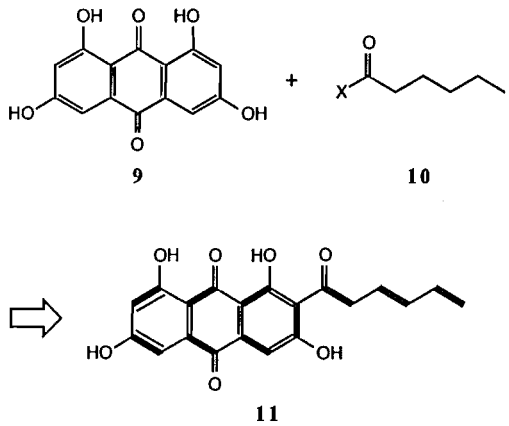
SCHEME 3

apportioned equally among the shaken cultures in acetone solution and, after 48 h, the averufin produced was isolated and purified for $^{13}C\{^1H\}$NMR analysis. The results from the free acids and corresponding NAC thioesters are shown in Table 1.

The sites and levels of isotopic enrichment were determined by comparison to averaged signal intensities at natural abundance. The results were striking in their clarity. [1-^{13}C]-Hexanoate, which as its free acid had given a 3–4% incorporation, as its NAC thioester gave a remarkably high 22% specific incorporation superimposed once again on a ca. 0.5% secondary incorporation from [1-^{13}C]acetyl CoA. [1-^{13}C]-3-Oxo-octanoate, which previously had given no detectable incorporation as its free acid, now showed a 4.7 ± 0.7% enhancement of the resonance for C-3 (30) within the anthraquinone nucleus of averufin when incorporated as its NAC thioester. While substantially lower in efficiency than the NAC thioester of [1-^{13}C]hexanoic acid, this result suggested successful linking of the apparent aliphatic starter with the first C_2 unit at the keto oxidation state. In contrast, the NAC thioester of [1-^{13}C]butyric acid showed no convincing enrichment of the averufin side chain. More sensitive analysis using the NAC thioester of [2,3-$^{13}C_2$]butyric acid revealed no intact incorporation of a C_4 unit measurable as $^1J_{CC}$ coupling between C-4′ and C-5′. The detection limit of this analysis correlated to an incorporation rate of <0.1% given the high signal/noise ratio of the NMR spectrum obtained. Therefore, apart from the intact incorporations shown by the labeled NAC thioesters of hexanoic acid and 3-oxo-octanoic acid, *all* of the tested substrates gave low extents of enrichment comparable to those from [1-^{13}C]acetate or its NAC thioester at carboxyl-derived centers throughout averufin.

Based on these observations, several conclusions can be drawn about the first steps of aflatoxin biosynthesis. First, it could be supposed that a 1,3,6,8-tetrahydroxyanthraquinone **9**, a known natural product from a related *Aspergillus* (31), and a C_6 fatty acid/CoA ester **10** are separately generated and joined to form norsolorinic acid (**11**, Scheme 4), the first anthraquinone intermediate of aflatoxin biosynthesis (32). This possibility can be eliminated on symmetry grounds. Thus, while **9** is symmetrical, its labeling pattern from [1,2-$^{13}C_2$]acetate in **11** is not (30, 33). A Friedel–Crafts-like reaction of this sort is unprecedented in natural product biosynthesis and, if it were to occur, it would require condensation in a non-random manner on the synthase-bound anthraquinone prior to its release from the protein. Owing as well to the inherently poor reactivity of anthraquinones to electrophilic addition, this possibility seems highly unlikely. Moreover, it would not accommodate the specific incorporation of the NAC thioester of [1-^{13}C]-3-oxo-octanoic acid.

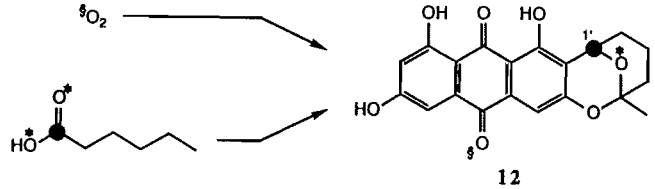
Second, it could be visualized that a polyketide synthase (PKS) responsible for assembling norsolorinic acid (**11**) may selectively and with high affinity bind hexanoyl CoA arising



SCHEME 4

from normal fatty acid degradation (2). This possibility is probably not likely. [1-^{13}C,$^{18}O_2$]Acetate was incorporated into averufin and shown to label every oxygen except that at C-10, which was derived from molecular oxygen ($O^{\S}$, Scheme 5) (34). Some exchange of acetate-bound oxygen isotope was observed, as is commonly the case in experiments of this sort, to give an incorporation of 40% at C-1′, that carbon corresponding to C-1 of a potential hexanoyl starter. While 60% of the oxygen at this ^{13}C-labeled site was not accounted for in this experiment, the comparable levels of heavy isotope uptake at this and other sites in averufin suggest that β-oxidation cannot be the source of the first six carbons. Oxygen incorporation through this latter mechanism would be from the aqueous medium, not from acetate. Moreover, given the efficiency with which the NAC thioester of hexanoic acid is incorporated when supplied exogenously, it seems unlikely that hexanoyl CoA ester produced intracellularly could not compete with the already poor incorporation of administered labeled acetate (0.5–1% per site) and, thus, reduce the observed labeling of both isotopes at C-1′. Such a reduction is not evident.

To examine this implication more closely, a sample of [1^{13}C,$^{18}O_2$]hexanoate was prepared by extension of Cane's method (24) and administered to mycelial suspensions of *A. parasiticus*. ^{13}C{^{1}H}NMR analysis of the isolated averufin (**12**, Scheme 5) showed a 53% incorporation of ^{18}O relative to the ^{13}C internal standard at C-1′, as evidenced by the relative proportion of a 0.027 ppm upfield-shifted resonance (for an overview of this method see ref. 35). Therefore, both [1-^{13}C,$^{18}O_2$]-acetate and [1-^{13}C, $^{18}O_2$]hexanoate label C-1′ and its attached oxygen with high and comparable efficiencies.



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SCHEME 5

In view of these arguments we are led to the following interpretation of the data. The hexanoyl unit is likely prepared by a specialized FAS that releases, for example, hexanoyl CoA, hexanoyl ACP, or channels this unit in an enzyme complex with the PKS to lead on to norsolorinic acid. Alternatively, but indistinguishable by the information presently available, the FAS and PKS are part of one large polyprotein such that the saturated C_6 unit is never released, for example, as a CoA ester, yet can fairly readily exchange with exogenously supplied thioester to load the C_6 site for further PKS elaboration with malonyl CoA. Recent disclosures of the organization and proposed function of the erythronolide genes (36) could be cited to favor this latter view. Nonetheless, the role of a specialized FAS, whether a separately expressed enzyme or part of a larger fusion to give a multifunctional protein, accommodates the "starter effect" noted by Chandler and Simpson on incorporation of diethyl [2-^{13}C]malonate into averufin (37), and, indeed, the slightly increased levels of secondary acetate incorporation detected at C-5′ in the present experiments (ca. 0.7%). Moreover, stereochemical experiments of the enoyl thioester reductase steps resulting in formation of both the averufin side chain and the fatty acids of *A. parasiticus* show a common configurational outcome (38) in contrast to polyketide natural products from other fungi (39). Purification of the protein or proteins responsible for norsolorinic acid synthesis and determination of the genetic organization and expression of the gene or genes that encode them are of considerable interest. As experiments advance to this stage, it may be possible to answer why the NAC thioesters of hexanoic acid and 3-oxo-octanoic acid can be utilized so efficiently in the biosynthesis while the corresponding thioester of butyric acid cannot.

Experimental

General synthetic methods

Melting points were determined in open capillaries using a Thomas–Hoover apparatus and are uncorrected. IR spectra were recorded on a Perkin–Elmer model 599B or a Series 1600 FTIR spectrometer. Proton NMR spectra were obtained using a Varian CFT-20 spectrometer fitted with a probe to operate at 80 MHz or, as indicated, a Varian XL-400 spectrometer operating at 400 MHz. Unless otherwise noted, chemical shifts (δ) are reported downfield of tetramethylsilane as an internal standard. ^{13}C NMR spectra were recorded using a Varian XL-400 spectrometer operating at 100 MHz with the chemical shifts given from deuteriochloroform (δ_C 76.89) or d_6-DMSO (δ_C 39.69) as internal reference. Mass spectra were obtained through the services of mass spectrometry facilities in the Department of Chemistry at Johns Hopkins University (VG Micromass 70-S). Elemental combustion analyses were carried out by Galbraith Laboratories, Inc. (Knoxville, Tenn.).

Analytical thin-layer chromatography was performed on Analtech GHLF Uniplate glass plates coated with silica gel (0.25 mm) containing a fluorescent indicator, and using the mobile phase noted. Column chromatography was conducted on Merck Silica Gel 60 (230–400 mesh), using the column size and mobile phase indicated. Flash column chromatography was conducted under 60 Torr (1 Torr = 133.3 Pa) pressure with silica gel 60, size finer than 0.063 mm (E. Merck).

Dry tetrahydrofuran (THF) and dry ether were distilled from sodium benzophenone ketyl prior to use. Hexamethylphosphoric triamide (HMPA) was distilled under reduced pressure from calcium hydride and stored over molecular sieves (4 Å) under nitrogen. All other reagents were of reagent grade or purified further as indicated.

Biological procedures: strains and culture conditions

The averufin-accumulating mutant *Aspergillus parasiticus* ATCC 24551 was maintained on slants of potato dextrose agar (Difco) plus 0.5% yeast extract. Plates subcultured from these slants were grown for 7 days in the dark before use.

The growth medium used for cultivation of the organisms in submerged culture in shaken flasks was the minimum mineral medium (A&M) of Adye and Mateles (40). The replacement medium (RM) was the nitrogen-free resting cell medium of Hsieh and Mateles (29) containing various amounts of glucose.

Shaken cultures were incubated in cotton-stoppered Erlenmeyer flasks in the dark at 28°C and the indicated revolutions/min in a New Brunswick Scientific model G-25-K gyrotary incubator shaker; static cultures were grown in cotton-stoppered Erlenmeyer flasks or Fernbach flasks at 28°C in the dark. For inoculation of liquid cultures, a 7-day-old plate culture was flooded with a 0.85% saline solution (9 mL) containing 0.5% Tween (1 mL) and a 5-mm inoculating loop was used to generate a suspension of spores; the suspension was transferred to a tube containing the saline solution (9 mL) and mixed well to produce a suspension of ca. 10^6 conidiospores per 1 mL.

All broths, slants, and equipment were sterilized at 125°C/20 psi (1 psi = 6.9 kPa) for 20 min prior to inoculation.

N-(2-Mercaptoethyl)acetamide (N-acetylcysteamine)

The procedure of Kass and Brock was used (21). A three-necked 250-mL round-bottomed flask equipped with a pH electrode and argon inlet was charged with *N,S*-diacetylcysteamine (2.09 g, 13.0 mmol; prepared by the method of Schwab and Klassen (22)) and 70 mL of water. The solution was cooled to 0°C and solid potassium hydroxide (2.43 g, 43.3 mmol) was added. The reaction mixture was stirred 1 h at room temperature, and the solution was adjusted to pH 7, saturated with NaCl, and extracted three times with dichloromethane. The combined organic extracts were dried over anhydrous sodium sulfate and concentrated in vacuo to give *N*-acetylcysteamine (1.42 g, 91% yield) as a clear, colorless oil that was prepared just prior to use. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.2–5.8 (m, 1H, NH), 3.43 (dt, J = 6, 6 Hz, 2H, -CH_2NH-), 2.71 (dt, J = 8, 6 Hz, 2H, -CH_2SH), 2.00 (s, 3H, CH_3CO), 1.34 (t, J = 8 Hz, SH).

General procedure for preparing NAC thioesters (21)

To a flame-dried three-necked round-bottomed 250-mL flask equipped with magnetic stirring bar and argon inlet was added 50 mL of dry THF and the carboxylic acid **3** (2.5 mmol). The solution was cooled to 0°C and triethylamine (0.35 mmol, 0.25 g, 2.5 mmol) followed by ethyl chloroformate (0.24 mL, 0.27 g, 2.5 mmol) was added and stirred 1 h at 0°C under argon. To a second three-necked 100-mL round-bottomed flask equipped with magnetic stirring bar, potassium hydroxide (1.4 g, 25 mmol) and 40 mL of water were added and stirred until the KOH had dissolved. Diacetylcysteamine (1.2 g, 7.5 mmol) was dissolved in the alkaline solution and allowed to stir at room temperature for 1 h. The pH was then adjusted to 7.8 with 6 N HCl and the solution was added via syringe to the mixed anhydride **4** at 0°C. If the mixture was cloudy, sufficient water was added to maintain a homogeneous solution. The pH was adjusted to 8.0 with 1 N NaOH and maintained at that pH throughout the reaction. The solution was allowed to stir for 1 h at room temperature, was acidified to pH 3.0 with 6 N HCl, and then was saturated with NaCl. The mixture was extracted three times with ether. The combined organic extracts were washed with 5% $NaHCO_3$ and brine and were dried over magnesium sulfate. Filtration and concentration in vacuo gave a clear colorless semisolid. TLC visualization under shortwave UV showed one spot (6:1 dichloromethane:acetone). Spray I followed by Spray II showed magenta spots indicating thioesters and (or) disulfides.

Spray I (sodium nitroprusside (41): 1.5 g of sodium nitroferricyanide, 5 mL of 2 N HCl, 95 mL of absolute methanol, and 10 mL of concentrated ammonium hydroxide (28%). Filter after mixing the above reagents together.

Spray II (methanolic sodium hydroxide): 2 g of NaOH, 5 mL of distilled water, and 95 mL of absolute methanol.

Dipping (spraying) a TLC plate with Spray I showed free thiols (here any residual *N*-acetylcysteamine). Taking the same TLC plate and dipping it in Spray II showed thioesters and disulfides as magenta spots on a yellow background. Dipping in ether intensifies the spots if weak (41). Purification by flash chromatography (20 g, 230–400 mesh, 6:1 dichloromethane:acetone) afforded the *N*-acetyl-*S*-acylcysteamines **5** as white solids. Other data are listed separately for each compound below.

[1-^{13}C]Butanethioic acid S-*[2-(acetylamino)ethyl]ester*

The compound was prepared from [1-^{13}C]butyric acid by acidification of the sodium salt (0.29 g, 2.6 mmol. 90 at% ^{13}C). The NAC thioester was obtained in 73% yield as a white solid, mp 21–23°C (lit. (42) mp 21–23°C). R_f = 0.25 (6:1 dichloromethane:acetone, visualized with Sprays I and II). IR($CHCl_3$): 3448, 3330, 3008, 2969, 2936, 2875, 1673, 1521, 1463, 1440, 1408, 1373, 1360, 1266, 1236, 1221, 1174, 1150, 1114, 1040 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.9–5.8 (m, 1H, NH), 3.44 (dt, J = 6, 6 Hz, 2H, -CH_2NH-), 3.03 (t, J = 6.6 Hz, 2H, -CH_2S-), 2.55 (dt, J = 7.5, 5.8 Hz, 2H, -C$H_2{}^{13}$CO-), 1.96 (s, 3H, CH_3CO-), 1.73–1.6 (m, 2H, -CH_2CH_3), 0.96 (t, J = 7.3 Hz, 3H, -CH_3); EIMS, *m/z* (relative intensity) (unlabeled material): 189 (M^+, 2), 161 (4), 130 (18), 119 (75), 118 (30), 102 (14), 86 (27), 71 (70), 60 (98), 43 (100). Exact Mass (unlabeled material): 189.0825; calcd. for $C_8H_{15}NO_2S$: 189.0824.

[1-^{13}C]Hexanethioic acid S-*[2-(acetylamino)ethyl]ester*

[1-^{13}C]Hexanoic acid (0.29 g, 2.5 mmol) gave the corresponding NAC thioester in 79% yield as a white solid, mp 43–45°C. R_f = 0.56 (6:1 dichloromethane:acetone, visualized by Sprays I and II). IR ($CHCl_3$): 3450, 3330, 3007, 2959, 2930, 2870, 1672, 1520, 1463, 1440, 1407, 1373, 1360, 1266, 1234, 1221, 1210, 1174, 1147, 1120, 1040 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.0–5.9 (m, 1H, NH), 3.43 (dt, J = 6, 6 Hz, 2H, -CH_2NH-), 3.02 (t, J = 6.6 Hz, 2H, -CH_2S-), 2.57 (dt, J = 7.3, 5.8 Hz, 2H, -$CH_2{}^{13}$CO-), 1.97 (s, 3H, CH_3CO-), 1.71–1.63 (m, 2H, -CH_2CH_2CO-), 1.33–1.28 (m, 4H, -C$H_2CH_2CH_3$), 0.90 (t, J = 7 Hz, 3H, -CH_3); EIMS, *m/z* (relative intensity) (unlabeled material): 217 (M^+, 1), 161 (1), 158 (8), 132 (7), 119 (78), 118 (23), 102 (11), 99 (40), 86 (17), 76 (15), 72 (26), 71 (39), 60 (85), 55 (12), 43 (100). Exact Mass (unlabeled material): 217.1139; calcd. for $C_{12}H_{21}NO_2S$: 217.1137.

Dicyclohexylcarbodiimide-activated esterification of carboxylic acids (28)

To a flame-dried 10-mL three-necked round-bottomed flask equipped with magnetic stirring bar and argon inlet was added the carboxylic acid (2.5 mmol), 3 mL dichloromethane, 10 mol% dimethylaminopyridine, and *N*-acetylcysteamine (8.5 mmol) in 2 mL of dichloromethane. The mixture was cooled to 0°C and DCC (2.5 mmol) was added. The reaction mixture was stirred at 0°C for 5 min and then warmed to room temperature, at which time dicyclohexylurea (DCU) precipitated. After stirring for 3.5 h at room temperature, the reaction mixture was filtered through Celite to remove DCU and was concentrated in vacuo, providing a yellow semisolid. Purification by flash chromatography (19 g silica, 230–400 mesh, 6:1 dichloromethane: acetone) gave the *N*-acetyl-*S*-cysteamine thioesters as white solids. Other data are listed below for each compound.

[1-^{13}C]Ethanethioic acid S-*[2-(acetylamino)ethyl]ester*

[1-^{13}C]Acetic acid (0.25 g, 4.09 mmol, 99 at% ^{13}C) and one equivalent of *N*-acetylcysteamine were combined to give the NAC thioester (0.56 g, 85% yield) as a clear colorless oil. R_f = 0.36 (6:1 dichloromethane:acetone, visualized by Spray I followed by Spray II). ^{1}H NMR (80 MHz, $CDCl_3$) δ: 6.5–6.25 (m, 1H, NH), 3.40 (dt, J = 6, 6 Hz, -CH_2NH-), 3.06 (t, J = 6.7 Hz, 2H, -CH_2S-), 2.35 (d, J = 6.2 Hz, 3H, $CH_3{}^{13}$CO-), 1.96 (s, 3H, CH_3CONH).

[2,3-$^{13}C_2$]Butanethioic acid S-*[2-(acetylamino)ethyl]ester*

[2,3-$^{13}C_2$]Butyric acid (2.66 mmol) yielded the NAC thioester (250 mg, 50% yield) as a white solid, mp 21–23°C (lit. (42) mp 21–23°C). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.5–6.27 (m, 1H, NH), 3.42 (dt, J = 6, 6 Hz, 2H, -CH_2NH-), 3.03 (t, J = 6.5 Hz, 2H, -CH_2S-), 2.56 ($^1J_{CH}$ = 124 Hz, $^2J_{CH}$ = 4.7 Hz, $^3J_{HH}$ = 7.4 Hz, 2H, -^{13}CH_2CO-), 1.98 (s, 3H, CH_3CO-), 1.68 ($^1J_{CH}$ = 123 Hz, $^2J_{CH}$ = 4.2 Hz, $^3J_{HH}$ = 7.4 Hz, 2H, -^{13}CH_2CH_3), 0.95 ($^2J_{CH}$ = 4.7 Hz, $^3J_{CH}$ = 4.4 Hz, $^3J_{HH}$ = 7.4 Hz, 3H, -CH_3).

[1-^{13}C]-3-Oxo-octanoic acid S-*[2-(acetylamino)ethyl]ester*

[1-^{13}C]-3-Oxo-octanoic acid (0.398 g, 2.5 mmol) afforded the NAC thioester in 69% yield as a white solid, mp 86–89°C. IR ($CHCl_3$): 3450, 3330, 3004, 2960, 2933, 2870, 1721, 1672, 1613, 1524, 1464,

1440, 1407, 1365, 1266, 1233, 1217, 1177, 1101, 1087, 1040 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.75–6.25 (m, 1H, NH), 3.70 (d, J = 6.2 Hz, -^{13}CH_2CO-), 3.42 (dt, J = 6, 6 Hz, 2H, -CH_2NH-), 3.13 (t, J = 6.4 Hz, -CH_2S-), 2.53 (t, J = 7.3 Hz, 2H, -CH_2CO-), 1.99 (s, 3H, CH_3CO-), 1.75–1.25 (m, 6H, -$CH_2CH_2CH_2$-), 0.90 (t, J = 6.8 Hz, CH_3CH_2-); EIMS *m/z* (relative intensity) (unlabeled material): 259 (1), 200 (1), 141 (25), 119 (80), 118 (19), 102 (2), 99 (32), 86 (12), 84 (50), 76 (10), 72 (23), 71 (19), 69 (31), 60 (94), 56 (15), 44 (20), 43 (64), 41 (24), 30 (100). Exact Mass (unlabeled material): 259.1248; calcd. for $C_{12}H_{21}NO_3S$: 259.1242.

[1-^{13}C]Hexanoic acid (24)

To a 25-mL three-necked round-bottomed flask equipped with nitrogen inlet, magnetic stirring bar, and reflux condenser were added MeOH (6.3 mL), H_2O (0.24 mL), 1-iodopentane (3.27 mL, 4.97 g, 25.1 mmol), and potassium [^{13}C]cyanide (1.63 g, 24.6 mmol, 99% ^{13}C). The mixture was allowed to heat to reflux for 48 h. The hexanenitrile (assume 24.6 mmol) was distilled directly into a 50-mL round-bottomed flask at 55°C (15 Torr) as a clear colorless liquid. Water (0.95 mL, 53 mmol) and 29.5 mL of 0.85 M potassium *tert*-butoxide (25 mmol) in *tert*-butanol were added; the solution was heated to reflux for 48 h under nitrogen and was then cooled to room temperature, affording a white precipate. The residue obtained upon evaporation of the solvent was redissolved in water and acidified with 10% aqueous HCl to pH 2. The aqueous solution was extracted with ether three times. The organic layers were combined and washed with water, brine, and were dried over anhydrous magnesium sulfate. Filtration and concentration in vacuo gave a clear yellow liquid. The liquid was purified by Kugelrohr distillation at 85°C to give [1^{13}C]hexanoic acid as a clear colorless liquid (2.35 g, 80% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.33 (dt, J = 7.3, 5.8 Hz, 2H, CH_2^{13}CO-), 1.80–1.50 (m, 2H, -C$H_2$$CH_2$^{13}CO-), 1.50–1.25 (m, 4H, CH_2C$H_2$$CH_3$), 0.90 (t, J = 7 Hz, 3H, -CH_3).

[1-^{13}C,$^{18}O_2$]Hexanoic acid, sodium salt

The method used was similar to that for preparing analogously labeled sodium propionate by Cane et al. (24). To a flame-dried 25-mL round-bottomed flask equipped with nitrogen inlet, magnetic stirring bar, and reflux condenser were added methanol (3.8 mL), H_2^{18}O (98% ^{18}O, 0.15 mL), 1-iodopentane (1.98 mL, 15.19 mmol), and potassium [^{13}C]cyanide (99% ^{13}C, 0.99 g, 15.03 mmol). The mixture was heated to reflux for 48 h. After cooling, the mixture was distilled into a 50-mL round-bottomed flask for direct hydrolysis of the nitrile. To the latter flask, equipped with magnetic stirring bar, reflux condenser, and nitrogen inlet, were added 0.85 M potassium *tert*-butoxide in *tert*-butanol (17.8 mL, 15.13 mmol) and H_2^{18}O (98% ^{18}O, 0.64 mL, 32 mmol). The product was obtained as described above to give [1-^{13}C,$^{18}O_2$]hexanoic acid as a colorless liquid (0.78 g, 45% yield). Water (10 mL) and 1 N NaOH (6.7 mL) were added to the carboxylic acid. The mixture was stirred for 30 min at room temperature and then concentrated in vacuo to give a white solid, mp 260°C (dec.). ^{1}H NMR (400 MHz, D_2O) δ: 2.19 (dt, J = 7.3, 5.9 Hz, 2H, -CH_2 ^{13}C$^{18}O_2$), 1.61–1.54 (m, 2H, -C$H_2$$CH_2^{13}C^{18}O_2$), 1.37–1.26 (m, 4H, -C$H_2CH_2$$CH_3$), 0.89 (t, J = 7.1 Hz, 3H, -CH_3); ^{13}C NMR (100 MHz, D_2O, partial) δ: 183.07 (^{13}C$^{16}O_2$), 183.04 (^{13}C^{16}O^{18}O), 183.01 (^{13}C$^{18}O_2$). Estimation of isotope content of the acid by ^{13}C NMR was 17.9% (^{13}C,$^{16}O_2$), 45.6 (^{13}C,^{16}O,^{18}O), and 36.5 (^{13}C,$^{18}O_2$). The mass spectrum gave the isotopic cluster (M + Na^+) as follows: EIMS *m/z* (unlabeled): 161 (100), 162 (7.03), 163 (1.03), 164 (0.02), 165 (0.32) and 166 (0.03); (labeled): 161 (6.96), 162 (39.99), 163 (8.3), 164 (100), 165 (10.73), 166 (81.4), and 167 (4.54). From these data it can be shown that the contributions of labeled materials are as follows: 18.1% (^{13}C$^{16}O_2$), 45.4% (^{13}C^{16}O^{18}O), and 36.5% (^{13}C$^{18}O_2$).

*Benzyl [1-^{13}C]acetate (**6**) (25)*

To a flame-dried 10-mL round-bottomed flask equipped with reflux condenser, argon inlet, and magnetic stirring bar were added sodium [1-^{13}C]acetate (99% ^{13}C, 0.80 g, 9.64 mmol), methyl ethyl ketone (3 mL), benzylchloride (1.02 mL, 1.12 g, 8.87 mmol), and triethylamine (0.04 mL, catalytic). The heterogeneous mixture was heated to a vigorous reflux for 8.5 h. At this time water was added to dissolve the salt and the aqueous and organic layers were separated. The aqueous phase was extracted with dichloromethane and the organic layers were combined, washed with brine, and dried over anhydrous magnesium sulfate. Filtration and concentration in vacuo provided 1.32 g of **6** as a clear colorless liquid (98% yield). ^{1}H NMR (80 MHz, $CDCl_3$) δ: 7.35 (s, 5H, phenyl), 5.11 (d, J = 3.2 Hz, 2H, $C_6H_5C$$H_2$-), 2.10 (d, J = 6.9 Hz, 3H, CH_3^{13}CO-).

Benzyl [1-^{13}C]-3-oxo-octanoate (7, R = CH_2Ph)

To a 50-mL flame-dried three-necked round-bottomed flask containing a nitrogen inlet and magnetic stirring bar were added 13 mL of dry THF and 18.3 mL (18.3 mmol) of lithium hexamethyldisilazide (1.0 M in THF). The stirred solution was cooled to −78°C and benzyl [1-^{13}C]acetate **6** (1.26 mL, 8.7 mmol) was added, followed in 30 min by hexanoyl chloride (1.2 mL, 8 mmol). After 35 min, 2 N hydrochloric acid (13 mL) was added at −78°C and the reaction was allowed to warm to room temperature and stir 35 min more. The mixture was poured into water and extracted with ethyl acetate three times. Concentration of the dried organic extract in vacuo afforded a yellow oil. Purification by flash chromatography (80 g silica, 230–400 mesh, 10:1 hexane:ethyl acetate) provided 1.54 g of product **7** (R = CH_2Ph) as a clear colorless oil (77% yield). R_f = 0.55 (10:1 hexane:ethyl acetate). ^{1}H NMR (80 MHz, $CDCl_3$) δ: 7.35 (s, 5H, phenyl), 5.17 (d, J = 3.3 Hz, 2H, $C_6H_5CH_2$-), 3.47 (d, J = 7.4 Hz, 2H, -^{13}COCH_2CO-), 2.49 (t, J = 7.2 Hz, 2H, -CH_2CO-), 1.75–1.25 (m, 6H, -$CH_2CH_2CH_2$-), 0.87 (virtually coupled triplet, 3H, -CH_3).

[1-^{13}C]-3-Oxo-octanoic acid (7, R = H)

To a 250-mL Parr bottle were added benzyl [1-^{13}C]-3-oxo-octanoate (1.54 g, 9.67 mmol), ethyl acetate (10 mL), and 10% palladium on carbon (100 mg). Debenzylation was carried out under hydrogen at 40 psi for 12 h. The catalyst was removed by filtration through Celite and washed with 1:1 ethyl acetate: ethanol. Concentration in vacuo provided [1-^{13}C]-3-oxooctanoic acid as a while solid (0.89 g, 90% yield), mp 73–74°C (lit. (26) mp 73–74°C). R_f = 0.07 (10:1 hexane: ethyl acetate, visualized with bromocresol green.). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.47 (d, J = 7.4 Hz, 2H, -^{13}COCH_2CO-), 2.49 (t, J = 7.2 Hz, 2H, CH_2CO-), 1.75–1.25 (m, 6H, -$CH_2CH_2CH_2$-), 0.87 (t, J = 7 Hz, 3H, CH_3).

[2,3-$^{13}C_2$]Butanoic acid (23)

To a flame-dried 50-mL round-bottomed flask equipped with reflux condenser, magnetic stirring bar, and argon inlet were added THF (9.5 mL) and diisopropylamine (1.99 mL, 1.44 g, 14.2 mmol). After cooling to 0°C, [2-^{13}C]acetic acid (99% ^{13}C, 0.37 mL, 6.37 mmol) was added dropwise giving a milky white suspension, which became homogeneous after the addition of three equivalents of HMPA (3.3 mL, 19.11 mmol). The resulting solution was heated for 2 h at 50°C to facilitate dianion formation. After cooling to room temperature, [1-^{13}C]-1-iodoethane (99% ^{13}C, 500 mg, 3.18 mmol) was added slowly via syringe and the alkylation was allowed to proceed for 12 h. The product was isolated by acidification with ice-cold 10% HCl and extraction with ether. The combined organic layers were washed three times with 10% HCl, water, and brine. The organic extract was dried over anhydrous sodium sulfate, filtered, and concentrated to obtain the crude product as an orange liquid. Any ethyl esters formed in the reaction were saponified with 1 N NaOH and extracted with ether. The aqueous layer was acidified to pH 2 with 2 N HCl and was extracted back into ether. The ether extract was washed with water, brine, and was dried over anhydrous sodium sulfate. Filtration and concentration in vacuo gave a pale yellow liquid, which was Kugelrohr distilled (50°C, 15 Torr) to give a colorless liquid (0.24 g, 85% yield based on 1-iodoethane). ^{1}HNMR (400 MHz, $CDCl_3$) δ: 2.32 ($^1J_{CH}$ = 124 Hz, $^2J_{CH}$ = 4.7 Hz, $^3J_{HH}$ = 7.4 Hz, 2H, -^{13}CH_2CO-), 1.65 ($^1J_{CH}$ = 123 Hz, $^2J_{CH}$ = 4.2 Hz, $^3J_{HH}$ = 7.4 Hz, 2H, -^{13}C$H_2$$CH_3$), 0.95 ($^2J_{CH}$ = 4.7 Hz, $^3J_{CH}$ = 4.4 Hz, $^3J_{HH}$ = 7.4 Hz, 3H, -CH_3).

Incorporation of sodium [1-^{13}C,$^{18}O_2$]hexanoate into averufin

Four 500-mL portions of A&M medium (40) contained in 2-L Erlenmeyer flasks were inoculated and allowed to shake at 175 rpm at 28°C in the dark for 48 h. The mycelial pellets were filtered on cheesecloth and rinsed with RM (1.62 g glucose/L) (29). Ten grams each (wet weight) was resuspended in 100 mL of RM contained in each of 12 250-mL Erlenmeyer flasks. Sodium [1^{13}C,$^{18}O_2$]hexanoate (178 mg, 1.25 mmol, 14.9 mg per flask) was dissolved in 24 mL of H_2O and filtered through a sterile 0.2-μm membrane and 2 mL of the filtrate was pipetted into each flask. The flasks were allowed to shake 48 h at 28°C in the dark. The mycelial pellets were filtered on cheesecloth and rinsed with water. The collected mycelial pellets were steeped in acetone and filtered. The acetone extract was concentrated to ca. 300 mL and was extracted four times with ether until the ether layer was nearly colorless. The combined organic extracts were washed with water and brine, and were dried over anhydrous sodium sulfate. Filtration and concentration in vacuo gave an orange solid. Purification by flash chromatography (18 g silica, 230–400 mesh, 97:3 $CHCl_3$:MeOH) gave 59 mg of averufin as an orange solid. ^{13}C NMR (100 MHz, $CDCl_3$ with five drops d_6-DMSO) δ: 188.81 (C-9), 180.99 (C-10), 164.67 (C-6), 164.30 (C-8), 159.71 (C-3), 158.34 (C-1), 134.53 (C-11), 132.86 (C-14), 115.63 (C-2), 109.06 (C-5), 108.57 (C-12), 108.15 (C-13), 107.99 (C-7), 107.5 (C-4), 100.53 (C-5′), 66.32 (C-1′), 35.33 (C-4′), 27.33 (C-6′), 26.9 (C-2′), 15.43 (C-3′); δ: 66.324 (C-1′–^{16}O), 66.297 (C-1′–^{18}O).

Enrichment of averufin labeled with both ^{13}C and ^{18}O was determined to be 31.5% based on peak areas in the NMR spectrum. EIMS gave *m/z* (unlabeled) 368 (100), 369 (22.4), 370 (5.1), 371 (1.2), and 372 (0.02); and for the labeled compound 369 (100), 369 (25.2), 370 (5.8), 371 (2.7), and 372 (1.3). From these data it can be shown that the contributions of labeled species are as follows: 67.7% (^{13}C,^{16}O) and 32.3% (^{13}C,^{18}O). To determine the incorporation of doubly labeled hexanoate into averufin, an assumption was made about the utilization of the hexanoate fed. Of the three peaks integrated in the ^{13}C NMR spectrum of [1-^{13}C,$^{18}O_2$]hexanoate, half of the area of the peak corresponding to ^{13}C,^{16}O,^{18}O-labeled material was used since statistically only half of the oxygens could be retained in the conversion to averufin. With this in mind a corrected figure of 59.3% was obtained for the maximum ^{13}C,^{18}O-enrichment that could be observed in averufin. Dividing the 31.5% enrichment actually obtained for the [^{13}C,^{18}O]-averufin by 59.3% gave 53% retention of ^{18}O in averufin relative to the ^{13}C internal standard.

Administration of NAC thioesters to ATCC 24551

A common procedure was used to test the incorporation of all the NAC thioesters. Mycelial pellets were prepared as above and resuspended in 12 250-mL Erlenmeyer flasks containing 100 mL each of RM. The ^{13}C-labeled NAC thioester was dissolved in 24 mL of acetone and 2 mL was pipetted into each flask. The flasks were allowed to shake 48 h at 28°C in the dark at 175 rpm. The mycelial pellets were then filtered on cheesecloth and rinsed with distilled water. The averufin produced was isolated as above.

Administration of [1-^{13}C]ethanethioic acid S-*[2(acetylamino)-ethyl]ester*

The yield of averufin was 8 mg. The estimation of incorporation by ^{13}C NMR was 0.3% at C-5′. EIMS *m/z* (unlabeled): 368 (100), 369 (22.4), 370 (5.1), and 371 (1.2); (labeled): 368 (100), 369 (26.6), 370 (7.9), and 371 (3.5). The mass spectrum (EI) indicated that the overall level (for 10 sites) of carbon-13 in the molecule was 3.8% above natural abundance.

Administration of [1-^{13}C]butanethioic acid S-*[2(acetylamino)-ethyl]ester*

The yield of averufin was 11.6 mg. Estimation of incorporation by ^{13}C NMR was 0.36% at C-3′ (however, see below). EIMS *m/z* (unlabeled): 368 (100), 369 (22.4), and 370 (5.1); and for the labeled compound: 368 (100), 369 (27.0), and 370 (6.8). The mass spectrum indicated that the level of carbon-13 was 4.4% above natural abundance (for 10 sites).

Administration of [2,3-$^{13}C_2$]butanethioic acid S-*[2(acetylamino)-ethyl]ester*

The yield of averufin was 25 mg. A ^{13}C NMR spectrum showed no intact incorporation of the thioester, as evidenced by the lack of coupling at a detection limit of <0.1% between C-4′ (δ 35.78) and C-5′ (δ 100.9).

Administration of [1-^{13}C]hexanethioic acid S-*[2(acetylamino)-ethyl]ester*

The yield of averufin was 18 mg. Estimation of incorporation by ^{13}C NMR spectroscopy was 22% at C-1′ by peak height analysis compared to natural abundance. EIMS *m/z* (unlabeled): 368 (100), 369 (22.4), 370 (5.1), and 371 (1.2); and for the labeled compound: 368 (100), 369 (62.8), 370 (25.7), and 371 (7.5). From these data it can be shown that the level of enrichment (M + 1) by mass spectrometry was 26%.

Administration of [1-^{13}C]-3-oxo-octanoic acid S-*[2(acetylamino)-ethyl]ester*

Estimation of incorporation by ^{13}C NMR spectroscopy gave 4.7 ± 0.7% at C-3 compared to natural abundance. Incorporation was ca 0.7% per site at alternating carbons, giving a total of 7% secondary incorporation due to acetate. EIMS gave *m/z* (unlabeled): 368 (100), 369 (22.4), 370 (5.1), and 371 (1.2); (labeled): 368 (100), 369 (35.1), 370 (10.5), and 371 (2.8). The level of enrichment (M + 1) estimated by mass spectrometry was 10.9% in the entire molecule.

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