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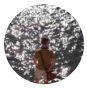

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Combining UHPLC-High Resolution MS and Feeding of Stable Isotope Labeled Polyketide Intermediates for Linking Precursors to End Products

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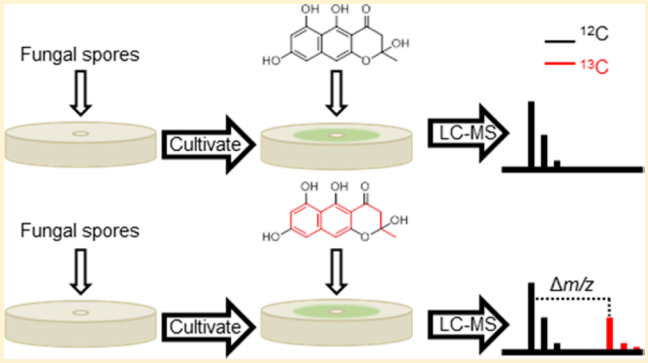
Supporting Information

ABSTRACT: We present the results from stable isotope labeled precursor feeding studies combined with ultrahigh performance liquid chromatography-high resolution mass spectrometry for the identification of labeled polyketide (PK) end-products. Feeding experiments were performed with $^{13}C_8$-6-methylsalicylic acid (6-MSA) and $^{13}C_{14}$-YWA1, both produced in-house, as well as commercial $^{13}C_7$-benzoic acid and $^{2}H_7$-cinnamic acid, in species of *Fusarium*, *Byssochlamys*, *Aspergillus*, and *Penicillium*. Incorporation of 6-MSA into terreic acid or patulin was not observed in any of six evaluated species covering three genera, because the 6-MSA was shunted into (2*Z*,4*E*)-2-methyl-2,4-hexadienedioic acid. This indicates that patulin and terreic acid may be produced in a closed compartment of the cell and that (2*Z*,4*E*)-2-methyl-2,4-hexadienedioic acid is a detoxification product toward terreic acid and patulin. In *Fusarium* spp., YWA1 was shown to be incorporated into aurofusarin, rubrofusarin, and antibiotic Y. In *A. niger*, benzoic acid was shown to be incorporated into asperrubrol. Incorporation levels of 0.7−20% into the end-products were detected in wild-type strains. Thus, stable isotope labeling is a promising technique for investigation of polyketide biosynthesis and possible compartmentalization of toxic metabolites.



Filamentous fungi are a rich source of bioactive metabolites, including the polyketides (PKs), which constitute one of the largest groups of natural products. PKs include important pharmaceutics such as lovastatin, mycophenolic acid, and griseofulvin.[1] Three of the five major economically important mycotoxins are also of PK origin: aflatoxins, zearalenones, and fumonisins, aflatoxins being the most carcinogenic natural compounds currently known and zearalenones being highly estrogenic.[2]

With the rapid decrease in the cost of fungal genome sequencing, a much more efficient foundation for elucidation of biosynthetic pathways is now available.[3] This can be used for direct studies of biosyntheses, for improving cell factories via metabolic engineering, or for product yield optimization.[4] Alternatively, biosynthetic clusters can be transferred to a heterologous host for higher yields, which is often vital for producing sufficient amounts of a new drug candidate for toxicological and pharmacological evaluation. However, linking of fungal biosynthetic genes to their products by genetic engineering approaches is still very time-consuming. This is mainly due to the difficulties with bioinformatic prediction of the products being synthesized by iterative polyketide synthases (PKSs).[5] In a recent study we have used feeding experiments and ultrahigh performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) to show that $^{13}C_8$-labeled 6-methylsalicylic acid (6-MSA, **1**; Chart 1) and not the previously hypothesized precursor shikimic acid was a central building block in formation of yanuthone D in *Aspergillus niger*.[6]

The earliest biosynthetic studies using labeled precursors were based on ^{14}C and other radioactive isotopes to enable detection.[7] However, this has been overtaken by NMR spectroscopy using stable isotope labeled (SIL) compounds, where a (usually) ^{13}C-, ^{15}N-, ^{2}H-, or ^{34}S-labeled precursor is used. NMR data can also reveal labeling positions in the final products.[8] The downside of NMR spectroscopy is the poorer sensitivity compared with liquid chromatography mass spectrometry (LC-MS), requiring time-consuming isolation of SIL-labeled product(s) as well as much higher consumption of SIL precursors. However, MS may not yield information on the position of the labeling unless MS/MS can be used to form assignable labeled fragments of the compound of interest.

SIL precursor feeding has been used in several studies of the aflatoxin pathway,[9] the asticolorin pathway (both NMR based),[10] and as noted above the yanuthone D pathway in *A. niger* (MS based).[6,11]

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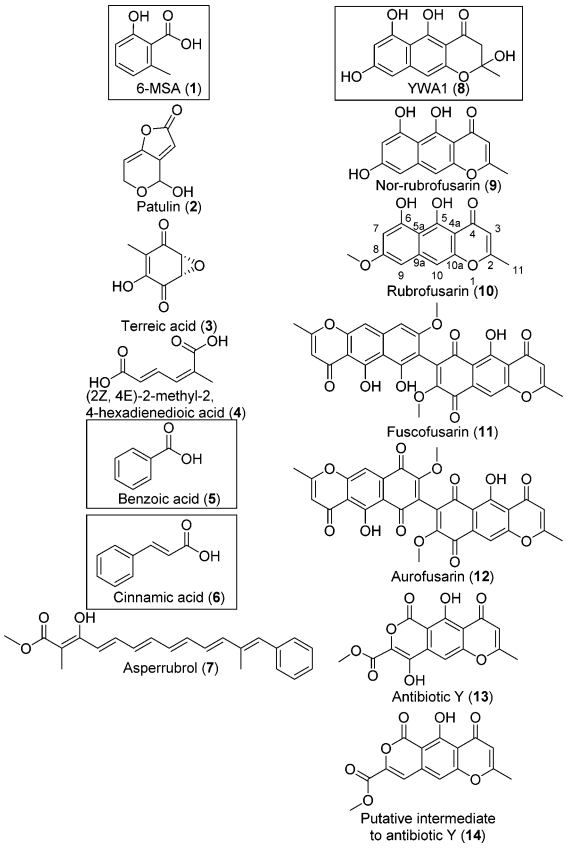
Chart 1. Chemical Structures of Compounds Investigated[a]



[a]Compounds are arranged according to biosynthetic origin. The boxed compounds correspond to the SIL compounds used in the study.

To ease interpretation of LC-MS results from these labeling experiments, it is advantageous to use a 100% labeled precursor that will result in formation of one distinct product isotopomer. Furthermore, the mass shift induced should preferably be large enough to be free of interference from the natural isotopomers of the target.

For MS investigation of pathways where SIL precursors are not available, the organism could be cultivated using fully isotope labeled media leading to nearly complete isotope enrichment in a so-called reciprocal or inverse labeling experiment.[12−14] This approach requires a minimal medium where all C, N, H, or S sources can be labeled, which is not available for complex media containing components that are often required to induce expression of fungal secondary metabolite pathways.

In recent studies, we were able to achieve close to 20% labeling of PK end-products in *A. niger* using $^{13}C_8$-6-MSA produced by heterologous expression of a 6-MSAS gene (*yanA*) in *Aspergillus nidulans*.[6] Based on these results, we speculated that it would be of scientific value to produce numerous SIL precursors this way and use them for examination of various biosynthetic pathways. To test the applicability of this strategy, we used two commercially available precursors [benzoic acid (**5**) and cinnamic acid (**6**)] and two in-house produced precursors [6-MSA and YWA1 (**8**)] to investigate a number of pathways where these four compounds are known or suspected to be precursors to other compounds.

Since labeled 6-MSA was already available, it seemed obvious to examine other known compounds biosynthesized using 6-MSA as precursor. A well-known compound is the mycotoxin patulin (**2**), for which the biosynthesis has already been elucidated.[15] Patulin is found in many species throughout three different genera (*Byssochlamys, Penicillium*, and *Aspergillus*), making it an excellent case for testing for broad versatility of labeling across organisms. The compound terreic acid (**3**,[16] Figure 1), produced by *A. terreus* ATCC 20542 (the original mevinolin producer)[17,18] is related to patulin and is also biosynthesized from a 6-MSA precursor.[19] Thus, terreic acid was also selected for investigation.

A. niger is a producer of numerous PKs including asperrubrol (**7**).[20] It has previously been hypothesized that cinnamic acid is a precursor to asperrubrol.[21] Cinnamic acid is a known precursor of benzoic acid in *Phanerochaete chrysosporium*,[22] which means that benzoic acid might also be used to investigate the biosynthesis of cinnamic acid. Because both cinnamic acid and benzoic acid were commercially available as SIL compounds, feeding experiments were performed using both.

The PK YWA1 (**8**)[23] is a key precursor to several different compounds in a variety of different fungal species; in *A. nidulans* YWA1 (produced by WA, encoded by *wA*) is the precursor to the green melanin responsible for pigmentation of conidia.[23] In *A. niger*, YWA1 (produced by AlbA, encoded by *albA*) is also the precursor to conidial pigment; however here the YWA1 is converted into 1,8-dihydroxynaphthalene (1,8-DHN) by chain shortening, after which the 1,8-DHN is polymerized into black melanin. YWA1 is also the precursor to the naphtho-γ-pyrones, of which the predominant compounds are the aurasperones.[24,25]

In *Fusarium graminearum*, YWA1 is the first stable intermediate formed during biosynthesis of the red pigment aurofusarin (**12**).[26−28] In *F. graminearum*, YWA1 is biosynthesized by PKS12,[29] an orthologue of the WA PKS in *A. nidulans*,[24] resulting in the formation of a nonreduced heptaketide. Folding of the heptaketide can result in the formation of either YWA1 or isocoumarins.[27] After release from the PKS, YWA1 is converted into nor-rubrofusarin (**9**), rubrofusarin (**10**), 9-hydroxyrubrofusarin, and finally the dimers fuscofusarin (**11**) and aurofusarin.[29]

Antibiotic Y (**13**) (avenacein Y) was first isolated from *F. avenaceum* in 1986, and although its biosynthetic pathway is unknown,[30] it displays several structural features in common with YWA1 and rubrofusarin. This suggest that it may also be formed via the nonreducing polyketide biosynthetic pathway.[5] The carbon backbone of antibiotic Y includes a lactone, which is atypical for nonreduced polyketides, and in this study, we hypothesize that it is formed either by the fusion of a tri- and tetraketide or by a previously undescribed carbon backbone cleavage of YWA1 followed by recondensation into a lactone.

In this study, we have used LC-MS to investigate the biosynthetic pathways of different filamentous fungi using SIL precursors. Both well-known metabolites such as patulin and terreic acid and metabolites biosynthesized from undescribed pathways (antibiotic Y and asperrubrol) were investigated to explore advantages and limitations of the approach.

RESULTS AND DISCUSSION

$^{13}C_8$-6-MSA Was Not Incorporated into Patulin or Terreic Acid. Feeding experiments were performed using several organisms that were known to produce patulin (*P.*

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Table 1. Results from the Labeling Experiments, Where the Highest Determined Degree of Incorporation Is Listed

target compound	producer organism	precursor	time of precursor addition (d)	degree of incorporation (%, average of duplicates)
patulin (**2**)	*P. griseofulvum, P. paneum, P. carneum, A. clavatus, B. nivea*	6-MSA (**1**)	3	ND[a]
(2*Z*,4*E*)-2-methyl, 4-hexadienoic acid (**4**)				45[b]
terreic acid (**3**)	*A. hortai, A. floccosus*	6-MSA (**1**)	3	ND[a]
(2*Z*,4*E*)-2-methyl, 4-hexadienoic acid (**4**)			6	ND[a]
			3	76[c]
			6	58[c]
asperrubrol (**7**)	*A. niger*	Cinnamic acid (**6**)	3	ND[a]
			6	ND[a]
		Benzoic acid (**5**)	3	1.3[d]
			6	ND[a]
aurofusarin (**12**)	*F. avanaceum, F. graminearum*	YWA1 (**8**)	3	1.2[f]
			7	0.3[g]
			10	0.4[g]
antibiotic Y (**13**)			3	ND[a]
			7	0.7[e]
			10	0.4[e]
rubrofusarin (**10**)			3	0.4[g]
			7	10[g]
			10	17[g]
putative intermediate to antibiotic Y (**14**)			3	ND[a]
			7	2.2[e]
			10	2.2[e]

[a]No incorporation detected. [b]*A. clavatus*. [c]*A. floccosus*. [d]*F. avanaceum* cultivated on DFM. [e]*F. avanaceum* cultivated on Bell's medium. [f]*F. graminearum* cultivated on DFM. [g]*F. graminearum* cultivated on Bell's medium.

griseofulvum, P. paneum, P. carneum, A. clavatus, B. nivea) or to produce terreic acid (*A. hortai* and *A. floccosus*).

No changes in morphologies or chemical profiles (acquired base peak chromatograms, BPC) were observed for any of the fungi fed with SIL precursors. Chemical analysis showed no signs of incorporated $^{13}C_8$-6-MSA into either patulin or terreic acid. The analysis was conducted by examining extracted ion chromatograms (EIC, ±0.02 Da) corresponding to both the labeled and unlabeled forms of the compounds (Table 2) and comparing these to reference standards of the compounds.

This was a surprise because 6-MSA is a known precursor to both compounds.[15,19] Since chemical analysis showed that the $^{13}C_8$-6-MSA was removed from the medium, we hypothesize that this result could be due to the fungi degrading the 6-MSA as a source of nutrient. Another explanation could be that the enzymatic activities involved in biosynthesis are linked in a manner that does not allow entry of an advanced precursor. A recent paper by Guo et al.[19] showed that (2*Z*,4*E*)-2-methyl-2,4-hexadienedioic acid is a shunt product in the terreic acid pathway, and we subsequently detected a peak corresponding to the correct accurate mass of this compound in an extract from *A. floccosus*. Investigation of the mass spectrum also revealed the presence of an ion corresponding to one incorporating $^{13}C_7$ (Supporting Information, Figure S1). We define the degree of labeling as

$$\frac{\text{Signal}_{\text{labeled form}}}{\text{Signal}_{\text{labeled form}} + \text{Signal}_{\text{unlabeled form}}}$$

For (2*Z*,4*E*)-2-methyl-2,4-hexadienedioic acid, the degree of labeling was thus 76% in *A. floccosus* fed after 3 days (Table 1). Interestingly (2*Z*,4*E*)-2-methyl-2,4-hexadienedioic acid was also found in the extracts from the patulin producers (Table 1), in both labeled and unlabeled form, showing that it is also a shunt product in the patulin biosynthesis. This strongly indicates that it is a result of a detoxification reaction in the cytoplasm and that patulin and terreic acid are produced in defined compartments. This would make sense, since patulin is an antifungal compound. The need for a detoxification process also seems to be important because (2*Z*,4*E*)-2-methyl-2,4-hexadienedioic acid was detected in amounts corresponding to 10−20% of the produced patulin as determined using UV. To test for compartmentalization, the peptide sequence of the proteins involved in the terreic acid pathway[19] were analyzed in order to predict any membrane bound proteins, using a range of different prediction tools,[31] including TargetP 1.1,[32] PSORT II,[33] and MultiLoc2.[34] However, no conclusive results were returned on whether the proteins are membrane bound.

Benzoic Acid Is a Precursor to Asperrubrol in *A. niger*. Asperrubrol biosynthesis in *A. niger* was investigated by addition of the two proposed precursors, cinnamic acid and benzoic acid. After feeding with $^2H_7$-cinnamic acid, no changes in morphologies or the BPCs were observed (data not shown).

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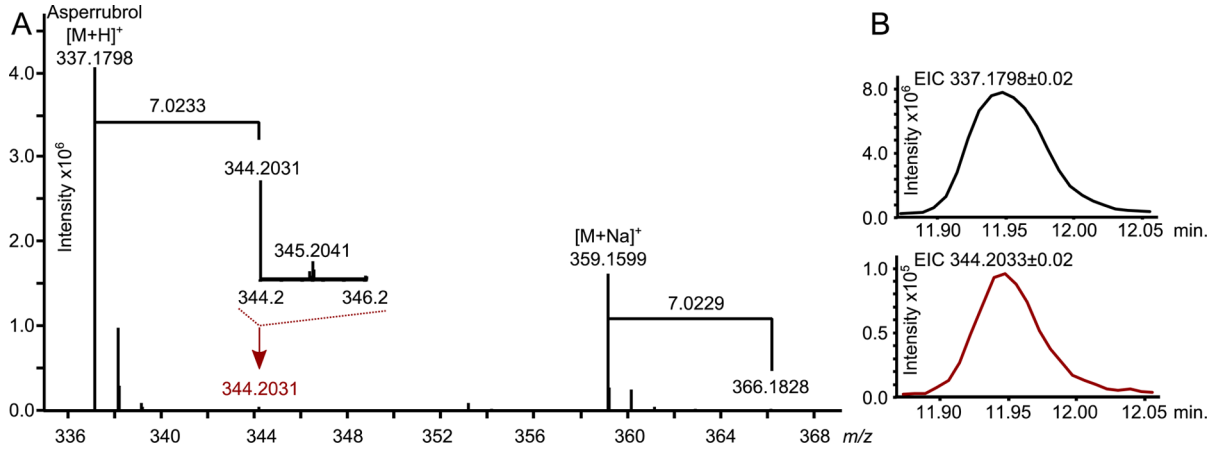

Figure 1. (A) Mass spectrum extracted at RT 12.0 min contained the $[M + H]^+$ (m/z 337.1798, mass deviation m/z 0.06 ppm) and $[M + Na]^+$ (m/z 359.1599). Mass shift of 7.0233 Da (m/z 344.2031, mass deviation 0.60 ppm) suggests incorporation of $^{13}C_7$ (red arrow). (B) EICs corresponding to asperrubrol (**7**, top) and asperrubrol with $^{13}C_7$ incorporated (bottom).

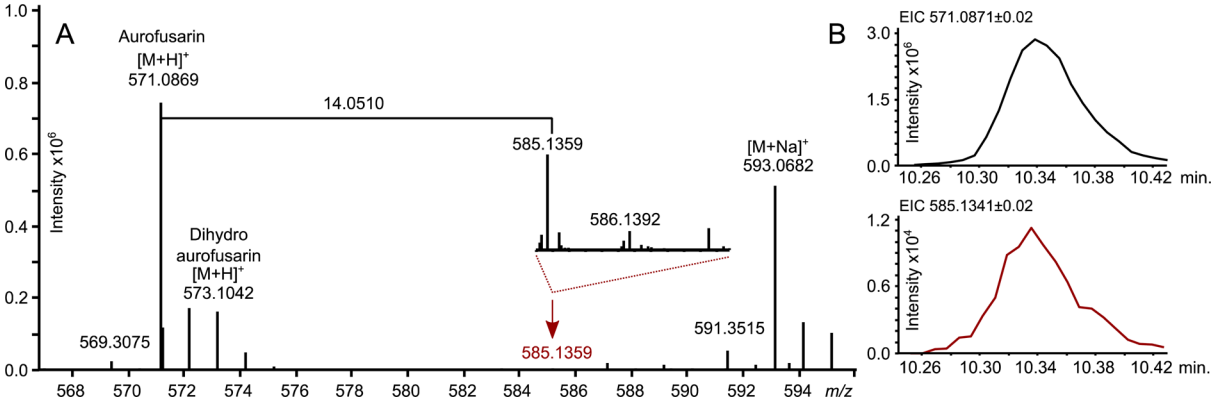

Figure 2. (A) Mass spectrum extracted at RT 10.3 min showing $[M + H]^+$ (m/z 571.0869, mass deviation −0.35 ppm) and $[M + Na]^+$ (m/z 593.0682) pseudomolecular ions. A mass shift of 14.0510 Da (m/z 585.1359 mass deviation, 3.1 ppm) suggests incorporation of $^{13}C_{14}$ (red arrow). (B) EICs corresponding to aurofusarin (**12**, top) and aurofusarin with $^{13}C_{14}$ incorporated (bottom).

Mass spectra of asperrubrol from samples fed with $^2H_7$-cinnamic acid exhibited no changes compared with the control samples. If cinnamic acid was converted into benzoic acid or another advanced precursor prior to incorporation into asperrubrol, extracted ion chromatograms corresponding to asperrubrol labeled with five, six, or seven ^2H atoms should be detectable; our experiments showed this was not the case.

Cultures of samples fed with $^{13}C_7$-benzoic acid also did not exhibit any changes in morphologies nor any peaks appearing or disappearing in the BPCs (Supporting Information, Figure S2), but investigation of the peak corresponding to asperrubrol revealed an ion with m/z 344.2031, corresponding to a difference of m/z 7.0233 compared with the $[M + H]^+$ ion of asperrubrol (Figure 1A). The ion corresponding to the $[M + Na]^+$ pseudomolecular ion of asperrubrol, as well as its labeled form, was also detected. This corresponded to incorporation of $^{13}C_7$ into the asperrubrol molecule. EICs of asperrubrol and its labeled form (Figure 1B) exhibited similar peak shapes and retention time (RT) and had a degree of incorporation of around 1.3% (Table 1).

These results suggest that asperrubrol is indeed biosynthesized from benzoic acid, which may in turn be synthesized from cinnamic acid in a different compartment. These results support the structure of asperrubrol reported by Rabache et al.[20]

Labeling in *Fusarium* spp. The compound YWA1 is known to be a biosynthetic precursor of several compounds including nor-rubrofusarin, rubrofusarin, fuscofusarin, and aurofusarin in fusaria. To investigate the biosynthesis of these, ^{13}C-labeled YWA1 (**8**) was used in labeling studies with two wild-type *Fusarium* strains, as well as two PKS12 deletion strains, deficient in the production of YWA1, grown under conditions that induce production of the compounds of interest. The two wild-type *Fusaria* did not exhibit any changes in morphologies or BPCs as a result of adding labeled substrate (data not shown). The mass spectrum extracted at the RT of the peak corresponding to aurofusarin showed ions corresponding to both unlabeled aurofusarin (**12**) and aurofusarin labeled with $^{13}C_{14}$ (Figure 2A).

EICs corresponding to labeled and unlabeled aurofusarin (Figure 2B) exhibited similar peak shapes and RTs with an incorporation degree of 0.4% (Table 1). No ions corresponding to aurofusarin with incorporation of two labeled YWA1 units were detected. This result was not surprising due to the low frequency of incorporation, that is, the frequency of incorporation of two units into aurofusarin would be $(0.4\%)^2 \approx 0.0016\%$, which is below the limit of detection.

Based on the previously established biosynthetic pathway of aurofusarin,[26,28,29] intermediates of the biosynthesis were investigated to determine if labeling of these could be detected.

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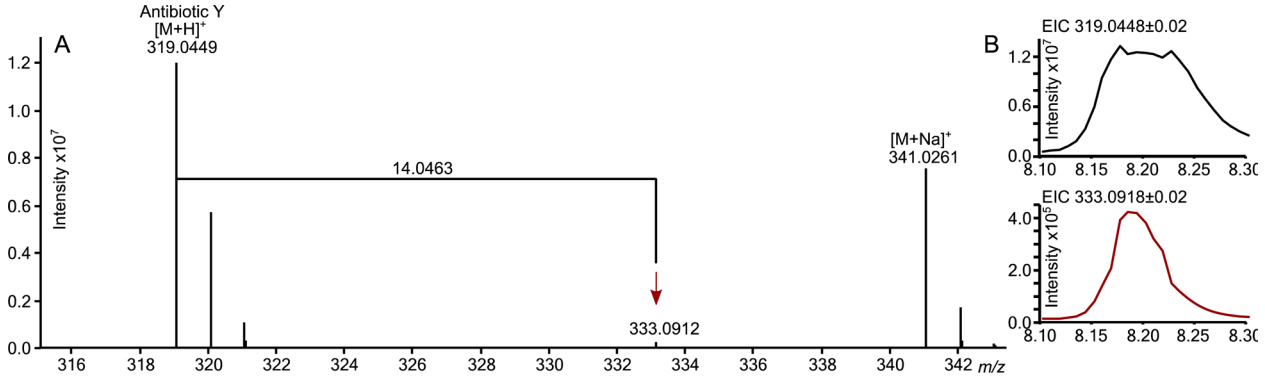

Figure 3. (A) Mass spectrum extracted at RT 8.2 min with $[M + H]^+$ (m/z 319.0449, mass deviation 0.18 ppm) and $[M + Na]^+$ (m/z 341.0261) pseudomolecular ions corresponding to antibiotic Y. Mass shift of $^{13}C_{14}$ suggest incorporation of labeled YWA1 (red arrow). (B) EICs corresponding to antibiotic Y (**13**; m/z 333.0912, mass deviation −1.8 ppm; top) and antibiotic Y with $^{13}C_{14}$ (bottom).

Only one precursor to aurofusarin, rubrofusarin (See Supporting Information, Figure S3), was detected in its labeled form and exhibited an incorporation degree of 20% (Table 1).

The two PKS12 deletion strains, *F. graminearum* Δ*PKS12* P1b and *F. graminearum* PH-1 HUEA (Δ*PKS12*), were also investigated by feeding with $^{13}C_{14}$-YWA1. These should not be able to produce YWA1 or aurofusarin. The PH-1 HUEA strain is thus pale white, while the wild-type *F. graminearum* is deep red. For one of these strains, PH-1 HUEA, addition of YWA1 resulted in visual changes: addition of $^{13}C_{14}$-YWA1 on day three resulted in bright red coloring around the reservoir, and addition after 7 days resulted in brownish coloring (Supporting Information, Figure S4). Addition of $^{13}C_{14}$-YWA1 after 10 days did not result in any color change. The colors of the control samples were unchanged throughout all 14 days. BPCs from the analysis did not reveal any changes in the chemical profiles (Supporting Information, Figure S5). Chemical analysis showed that the samples fed on days three and seven contained a compound with the same RT as aurofusarin. The mass spectrum (Supporting Information, Figure S6) contained an ion (m/z 599.1807) corresponding to aurofusarin with two YWA1 units ($^{13}C_{28}$) incorporated. Because this strain is not able to biosynthesize YWA1 on its own, all aurofusarin produced must be a product of the added $^{13}C_{14}$-YWA1, thus allowing detection of aurofusarin with two YWA1 units incorporated. This demonstrated that the fungus is indeed able to take up YWA1 from the medium and that YWA1, as expected, is a precursor to aurofusarin.

To test the hypothesis that antibiotic Y in *F. avanaceum* was also formed from YWA1, a wild-type *F. avenaceum* was fed with $^{13}C_{14}$-YWA1 under conditions that were known to induce production of antibiotic Y. As expected, feeding did not affect the metabolite profile (Supporting Information, Figure S7). However, closer investigation of the mass spectrum from the peak corresponding to antibiotic Y (Figure 3) revealed an ion (m/z 333.0912) corresponding to antibiotic Y with $^{13}C_{14}$ incorporated.

EICs corresponding to unlabeled antibiotic Y and antibiotic Y with $^{13}C_{14}$ incorporated (Figure 3B) exhibited similar RT, confirming that the labeled YWA1 precursor is incorporated into antibiotic Y. The unlabeled form was present in high enough amounts to saturate the detector, which accounts for the differences observed for the peak shapes. To calculate the degree of incorporation, the intensity of the $[^{13}C_1M + H]^+$ ion, which was not saturated, was then used to estimate the nonsaturated intensity of $[M + H]^+$, calculated using the theoretical ratio between these two. This showed that the degree of incorporation of YWA1 into antibiotic Y was 0.4% (Table 1). These results confirmed the hypothesis that YWA1 is a precursor to antibiotic Y and that its biosynthesis must depend on a yet undescribed structural rearrangement. To further investigate the biosynthesis of antibiotic Y, several putative intermediates were proposed and their chemical formulas formed the basis for a targeted analysis. One of these putative intermediates to antibiotic Y exhibited a mass spectrum indicative of YWA1 incorporation (Supporting Information, Figure S8), with an incorporation degree of 2.3%.

Comparison of the aurofusarin gene clusters in the genome-sequenced aurofusarin-producing fusaria revealed that the three antibiotic Y producing *F. avenaceum* strains contained an additional gene (*aurE*, FAVG1_08663) located centrally in the gene cluster.[35] AurE is predicted to encode a soluble epoxide hydrolase (EC: 3.3.2.3) based on its enzymatic domains. It is possible that the product of this unique gene is responsible for cleavage of YWA1 (**8**), and molecular genetics studies have been initiated to test this hypothesis.

Degrees of Incorporation. Overall the feeding experiments showed that the degrees of incorporation of the labeled precursors obtained by direct addition to wild-type strains varied significantly from 0.3% to 76%, with two further cases of incorporation into a presumed detoxification product. As expected, strains deficient in production of the precursor showed 100% incorporation. The degree of incorporation seemed to correlate inversely with the quantity of end product biosynthesized, with the signal of (2*Z*,4*E*)-2-methyl-2,4-hexadienedioic acid being very low in the patulin producers that have a 100-fold higher production of the compound than the terreic acid producing strains. In other published labeling studies, the degrees of incorporation of precursor have also varied. In a study of the mycotoxin terretonin by McIntyre et al., incorporation of several different differentially labeled precursors was investigated.[36] They found incorporation degrees of 0.3−2.5% depending on the precursor and cultivation conditions used. A study by Yoshizaws et al. investigated the incorporation of acetate in the biosynthesis of dehydrocurvalarin and found that these were incorporated at approximately 2%.[37] Finally, Yue et al. reported a 6% incorporation of ethyl (2*R*,3*R*)-2-methyl-3-hydroxy pentanoate into tylactone for an investigation of macrolide biosynthesis.[38]

The results revealed several important parameters for successful labeling of a compound through the use of an advanced labeled precursor. The organism must be able to take

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up the labeled precursor and, if necessary, transport it to a specific biosynthetic compartment in the cell. Second, the labeled compound must be included in the biosynthesis of a compound to act as a precursor. Finally, the precursor must be recognized by the tailoring enzymes as a substrate, and it is dependent on tailoring enzymes that are not physically coupled to the PKS synthesis, for example, as a protein complex. One hypothesis could be that synthesis of the PKs takes place in a so-called metabolon, where the SIL precursor cannot be inserted, as described for the tricarboxylic acid cycle.[39]

Examination of the data showed that the highest degree of incorporation of the labeled precursors was obtained at different time points, which is not surprising because biosynthesis also occurs at different time points during growth. For antibiotic Y, the highest degree of incorporation was obtained by addition after 7 days, but for aurofusarin, the highest incorporation was obtained with addition on day three. Presumably, the best strategy is to add the labeled compound at the onset of biosynthesis for the compound(s) to be studied. Another complication is that produced compounds may be recycled as part of the primary metabolism, as described for the nonribosomal peptide roquefortine C.[40]

Due to the low incorporation degrees observed for wild-type strains, a targeted analysis approach was required for determination of the incorporation levels. This could be combined with more systematic feeding studies, where fungi of interest could be cultivated using a whole panel of SIL precursors to investigate the biosynthesis of more complex compounds, since it is well suited for confirming hypotheses concerning biosynthetic pathways.

EXPERIMENTAL SECTION

General Experimental Procedures. All LC-MS analysis was performed using ultrahigh-performance liquid chromatography (UPHLC) UV/vis diode array detector (DAD) high-resolution MS (HRMS). The equipment used was an Agilent 6550 iFunnel Q-TOF LC/MS system (Torrance, CA) with an electrospray ionization (ESI) source operating in positive polarity, connected to an Agilent 1290 infinity UHPLC. The column used was an Agilent Poroshell 120 phenyl hexyl 2.7 μm, 250 mm × 2.1 mm column.

Chemicals. Solvents were LC-MS grade, and all other chemicals were analytical grade. All were from Sigma-Aldrich (Steinheim, Germany) unless otherwise stated. Water was purified using a Milli-Q system (Millipore, Bedford, MA). Electrospray ionization time-of flight (ESI-TOF) tune mix was purchased from Agilent.

$^{13}C_8$-Labeled 6-MSA (Table 2), 98.7%, had been produced by fermentation of a genetically modified *A. nidulans* by cultivation on labeled media, as described by Holm et al.[6] $^{13}C_7$-Benzoic acid, 99% labeled, and $^{2}H_7$-cinnamic acid, 98%, were purchased from Sigma-Aldrich (Steinheim, Germany).

Table 2. SIL Compounds Used in the Study

compound	elemental composition[a]	monoisotopic mass [Da]	mass difference[b] [Da]
6-MSA	$^{13}C_8H_8O_3$	152.0473	8.0268 (7.0235)[c]
cinnamic acid	$C_9{}^{2}H_7HO_2$	148.0524	7.0439
benzoic acid	$^{13}C_7H_6O_2$	122.0368	7.0235 (6.0201)[c]
YWA1	$^{13}C_{14}H_{12}O_6$	276.0634	14.0450

[a]Elemental composition denotes the formula of the compound and indicates the presence of labeled atoms. [b]Mass difference denotes the mass difference between the SIL compound and the natural predominant isotype. [c]Mass difference of compound following potential decarboxylation.

Construction of YWA1 Producing Strain. Protoplasting and gene targeting procedures were performed as described previously for *A. nidulans*.[41,42] The *wA* ORF (AN8209) was amplified with primers wA-fw (5′-GAGCGAUATGGAGGACCCATACCGTGT-3′) and wA-rv (5′-TCTGCGAUTATTAGAACCAGAGGATTATTATTGTT-3′) and inserted into the expression vector pDH57 via USER cloning, as described by Holm et al.[6] The gene targeting substrate for insertion of the YWA1 synthase gene was excised from pDH57-wA by *Not*I digestion and transformed into IBT 29539, as previously described.[6] Transformants with *wA* integrated into IS1 were verified by diagnostic PCR as described by Hansen and co-workers.[43]

Production and Purification of $^{13}C_{14}$-Labeled YWA1. The constructed YWA1 producing strain was propagated on solid MM medium prepared as described by Cove[44] and supplemented with 4 mM arginine. Spores were harvested after 14 days incubation at 30 °C with 10 mL of saline (0.9% NaCl in water) with 0.01% Tween 80 and filtered through Miracloth (Merck Millipore, Billerica, MA, USA). The spores were washed twice with saline prior to application. The batch fermentation was initiated by inoculation of 5×10^9 spores/L. A 1 L bioreactor (Sartorius, Goettingen, Germany) with a working volume of 0.8 L equipped with two Rushton six-blade disc turbines was used. The pH electrode (Mettler, Greifensee, Switzerland) was calibrated according to manufacturer standard procedures. For batch cultivation, the following media composition was applied: 20 g/L D-glucose-$^{13}C_6$ (99 atom % ^{13}C, Sigma-Aldrich) or D-glucose, 7.5 g/L $(NH_4)_2SO_4$, 1.5 g/L KH_2PO_4, 1.0 g/L $MgSO_4{\cdot}7H_2O$, 1.0 g/L NaCl, 0.1 g/L $CaCl_2$, 0.1 mL of Antifoam 204 (Sigma-Aldrich), 1 mL/L trace element solution (0.4 g/L $CuSO_4{\cdot}5\ H_2O$, 0.04 g/L $Na_2B_2O_7{\cdot}10H_2O$, 0.8 g/L $FeSO_4{\cdot}7H_2O$, 0.8 g/L $MnSO_4{\cdot}H_2O$, 0.8 g/L $Na_2MoO_4{\cdot}2H_2O$, 8.0 g/L $ZnSO_4{\cdot}7H_2O$.

The bioreactor was sparged with sterile atmospheric air, and off-gas concentrations of oxygen and carbon dioxide were measured with a Prima Pro Process mass spectrometer (Thermo-Fischer Scientific, Waltham, MA, USA). Temperature was maintained at 30 °C, and pH was controlled by addition of 2 M NaOH and H_2SO_4. Start conditions were as follows: pH 3.0, stir rate 100 rpm, and air flow 0.1 volume of air per volume of liquid per minute (vvm). These conditions were changed linearly in 720 min to pH 5.0, stir rate 800 rpm, and air flow 1 vvm. The cultivation was ended at glucose depletion, as measured by glucose test strips (Macherey-Nagel, Düren, Germany), and the culture had entered stationary phase as monitored by off-gas CO_2 concentration. The entire volume of the reactor was harvested, and the biomass was removed by filtration through a Whatman No. 1 qualitative paper filter followed by centrifugation at 8000*g* for 20 min to remove fine sediments. The YWA was then recovered from the supernatant by repetitive liquid−liquid extraction using ethyl acetate with 0.5% formic acid. The organic extract was completely dried in vacuo resulting in a crude extract that was redissolved in 20 mL of ethyl acetate and dry loaded onto 3 g of Sepra ZT C_{18} (Phenomenex, Torrence, CA, USA) resin prior to packing into a 25 g SNAP column (Biotage, Uppsala, Sweden) with 22 g of pure resin in the base. The crude extract was fractionated on an Isolera flask purification system (Biotage) using a water−acetonitrile gradient starting at 15:85 going to 100% acetonitrile in 23 min at a flow rate of 25 mL min^{-1} and kept at that level for 4 min. Fractions were collected using UV detection at 210 and 254 nm, resulting in a total of 20 fractions, of which two were pooled and analyzed. The total yield of 0.6 g of $^{13}C_{14}$−YWA1 was estimated to be 90% pure by UHPLC-UV/vis-TOFMS analysis and have a labeling degree of 98.2% based on the $^{13}C_{13}{}^{12}C/^{13}C_{14}$ ratio.

UHPLC-DAD-Quadrupole Time-of-Flight (qTOF) MS. Analysis was performed using UPHLC-DAD-HRMS. The equipment used was an Agilent 6550 iFunnel Q-TOF LC/MS system (Agilent Technologies, Torrence, CA, USA), connected to an Agilent 1290 infinity UHPLC. The column used was an Agilent Poroshell 120 phenyl hexyl 2.7 μm, 250 mm × 2.1 mm, and the column was maintained at 60 °C. The UV was used to measure at 280 nm. A linear water−acetonitrile (LC-MS-grade) gradient was used (both solvents were buffered with 20 mM formic acid) starting from 10% (v/v) acetonitrile and increased to 100% in 15 min, maintaining this rate for 2.5 min before returning to starting conditions in 0.1 min and staying

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there for 2.4 min before the following run. A flow rate of 0.35 mL/min was used. MS was performed in both ESI^+ and ESI^- in the mass range m/z 30−1700. Additional parameters and settings are published in Kildgaard et al.[45]

Cultivation of Fungi. Attempted labeling of patulin and terreic acid was carried out using the following fungi: *Penicillium griseofulvum* (IBT 18169), *P. paneum* (IBT 24722), *P. carneum* (IBT 26356), *Byssochlamys nivea* (CBS 546.75), *Aspergillus clavatus* (IBT 27903), *A. hortai* (IBT 26384 = NRRL 274, formerly identified as *A. terreus*), and *A. floccosus* (IBT 22556 = WB 4872 = NRRL 4872, formerly identified *A. terreus* var. *floccosus*). The IBT strains are available from the IBT culture collection at authors' address, NRRL strains from National Center for Agricultural Utilization Research (Peoria, IL, USA), and the CBS strain from Centraalbureau voor Schimmelcultures (Utrecht, Netherlands).

With a 5 mm plug drill, a reservoir was cut in the middle of a solid YES 9 cm media plate (Figure 4), prepared as described Frisvad and Samson.[46] Into this reservoir was added 65 μL of spore suspension, and the fungi were incubated for 7 days at 30 °C in darkness. On day three, 100 μg of ^{13}C-labeled 6-MSA dissolved in 100 μL of EtOH−H_2O (1:4) was added to the reservoir. Control samples without addition and with addition of 100 μL of EtOH−H_2O (1:4) were also prepared. On day seven, five plugs were excised from across the fungus using a 5 mm plug drill, and the plugs were extracted using acidic ethyl acetate−dichloromethane−methanol (3:2:1 vol/vol/vol) as described by Smedsgaard,[47] followed by analysis using LC-MS. All experiments were performed in duplicate.

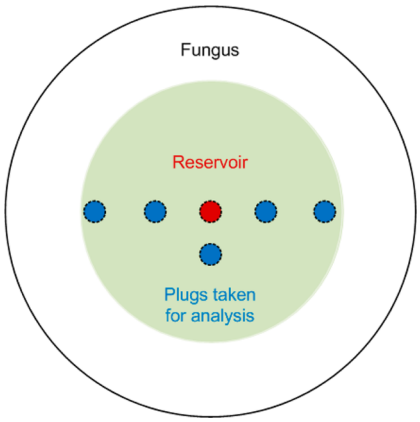

Figure 4. Diagram depicting the experimental setup. A reservoir (red) was cut in the middle of the media in the 9 cm Petri dish, and the fungus was then inoculated therein. At a specific time point, the labeled compound was added to the reservoir. At the end of the experiment plugs (blue) were removed from the fungal colony (green) and extracted as described in the text.

A. niger experiments, for the labeling of asperrubrol, were carried out following the described procedure, with addition of 100 μg of $^{13}C_7$-labeled benzoic acid or $^{2}H_7$-cinnamic acid dissolved in 100 μL of Milli-Q water on day 3 or 6, respectively. Separate control samples without labeled compounds were also fed to the strains. $^{2}H_7$-Cinnamic acid was only fed to *A. niger* KB1001. All experiments were prepared in duplicate. Sampling and extraction was performed as described above.

For the *Fusarium* labeling experiments four strains were used: *F. avanaceum* (IBT 41708), *F. graminearum* PH-1 (NRRL 31084) , *F. graminearum* ΔPKS12 P1b,[48] and *F. graminearum* PH-1 HUEA.[49] Fungi were inoculated on both Bells medium[50] and defined *Fusarium* medium (DFM)[51] and cultivated for 14 days at 30 °C in darkness to produce spores for the feeding experiment.

For the feeding experiments, solid Bells and DFM plates were prepared using a plug 5 mm drill to make a reservoir in the middle of the plate. Into this plate was added 65 μL of spore suspension, and the fungi were then cultivated for 14 days at 30 °C in darkness. After 3, 7, and 10 days, respectively, 100 μg of labeled YWA1, dissolved in 55 μL of ACN, was added to the reservoirs in the plates. Separate controls without labeled compounds and controls with 100 μL of ACN were also prepared. All experiments were prepared in duplicate. Sampling and extraction was performed as described above.

■ ASSOCIATED CONTENT

Supporting Information

Photographs of *F. graminearum* HUEA strain. BPCs from analysis of *A. niger*, *F. avanaceum*, and *F. graminearum* HUEA. Mass spectra of rubrofusarin, putative intermediate to antibiotic Y, and (2Z,4E)-2-methyl-2,4-hexadienedioic acid indicting labeling. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/np500979d.

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Notes

The authors declare no competing financial interest.

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