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Biosynthetic Origin of BE-10988 in *Streptomyces* sp. BA10988

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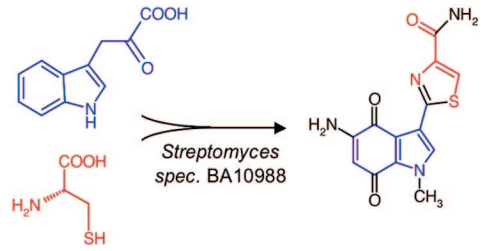

The biosynthetic origin of the tumor-inhibitory derivative, BE-10988, was studied in *Streptomyces* sp. BA10988 by retrobiosynthetic NMR analysis using [U-$^{13}C_6$]glucose as a precursor. The isotopologue compositions of the indole moieties of BE-10988 and tryptophan were virtually identical. This indicates that tryptophan or a closely related metabolite served as a biosynthetic precursor of BE-10988 in analogy to the biosynthetic pathway of camalexin, a structurally related phytoalexin in *Arabidopsis thaliana*. Labeling experiments with [U-$^{13}C_8{}^{15}N$]indole, L-[*ring*-$^2H_5$]tryptophan, or L-[U-$^{13}C_3{}^{15}N$]cysteine confirmed this hypothesis. However, transfer of the label from [*ring*-$^2H_5$]indole pyruvic acid, but not from the known camalexin precursor, [*ring*-$^2H_5$]indole-3-acetaldoxime, showed that plants and bacteria have evolved independent mechanisms of tryptophan modification in the biosynthesis of thiazolylindole derivatives.

Introduction

Plants, as well as actinomycetes, contribute substantially to the enormous structural variety of natural products. However, structurally related secondary metabolites are rather rarely found among these two evolutionarily very distantly related groups of organisms.[1] In order to understand the mechanisms of ecologically driven convergent evolution of metabolism between organisms from different kingdoms, the biosynthetic origins of additional structural metabolite classes have to be comparatively analyzed.

3-Thiazol-2′-ylindole (camalexin, **10**) is the characteristic phytoalexin of the model plant *Arabidopsis thaliana*. Its biosynthesis is induced by a great variety of plant pathogens. While some fungi developed resistance by degradation of camalexin, other pathogens, as well as a human breast cancer cell line, are growth inhibited by the compound.[2] The biosynthesis of camalexin (**10**) has been studied in some detail. The nitrogen and sulfur atoms, as well as C-4′ and C-5′ of the thiazole ring, are derived from cysteine (**7**), while the remaining atoms are introduced from tryptophan (**2**). A number of cytochrome P450-catalyzed steps are involved in the biosynthesis of camalexin. Tryptophan (**2**) is converted to indole-3-acetaldoxime (IAOx, **3**) by CYP79B2 and CYP79B3[3–5] and then dehydrated to indole-3-acetonitrile (IAN, **8**) by CYP71A13.[6] Dihydrocamalexic acid (**9**), synthesized from IAN (**8**) and

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(1) Moore, B. S.; Hertweck, C.; Hopke, J. N.; Izumikawa, M.; Kalaitzis, J. A.; Nilsen, G.; O'Hare, T.; Piel, J.; Shipley, P. R.; Xiang, L.; Austin, M. B.; Noel, J. P. *J. Nat. Prod.* **2002**, *65*, 1956–62.
(2) Glawischnig, E. *Phytochemistry* **2007**, *68*, 401–6.
(3) Glawischnig, E.; Hansen, B. G.; Olsen, C. E.; Halkier, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 8245–50.
(4) Hull, A. K.; Vij, R.; Celenza, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 2379–84.
(5) Mikkelsen, M. D.; Hansen, C. H.; Wittstock, U.; Halkier, B. A. *J. Biol. Chem.* **2000**, *275*, 33712–7.
(6) Nafisi, M.; Goregaoker, S.; Botanga, C. J.; Glawischnig, E.; Olsen, C. E.; Halkier, B. A.; Glazebrook, J. *Plant Cell* **2007**, *19*, 2039–52.

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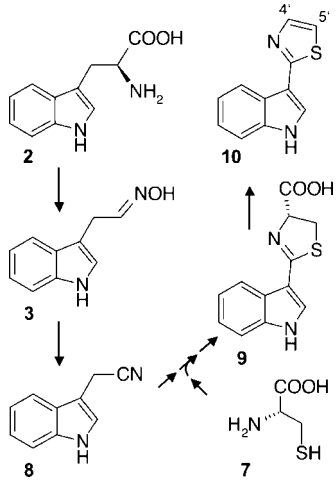
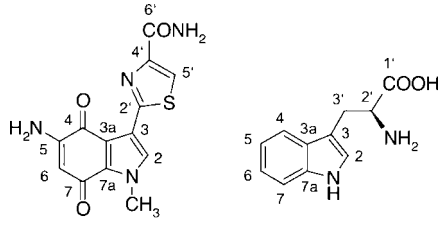
SCHEME 1. Biosynthesis of Camalexin (10) [3,6–8]



SCHEME 2. BE-10988 (1) and Tryptophan (2): Designation of Carbon Atoms



cysteine (**7**) by a yet unidentified mechanism, is finally decarboxylated to camalexin (**10**) by CYP71B15 (PAD3)[7] (Scheme 1).

Streptomyces species synthesize a wide range of secondary metabolites including compounds with thiazole moieties.[9,10] A bioactive thiazolylindole derivative has been identified from the culture broth of the *Streptomyces* strain BA 10988 and termed BE-10988 (**1**) (Scheme 2).[11,12] It exhibited increased DNA−topoisomerase complex formation and inhibited the growth of murine leukemia cell lines. A number of examples show that in bacteria thiazole rings can be formed from cysteine (**7**) by a nonribosomal peptide synthetase.[13] By analogy, an origin of the thiazole ring of BE-10988 (**1**) from cysteine (**7**) appeared plausible. For the biosynthetic origin of the indoloquinone moiety of BE-10988 two possible pathways can be envisaged (i) from tryptophan, similarly to camalexin (**10**) (Scheme 3C), or (ii) via a pathway analogous to that of the mitomycin antibiotics.[14] In such a "mitomycin-like" pathway, the secondary metabolite synthesis would branch off from early shikimate pathway intermediates (Scheme 3B).

Retrobiosynthetic NMR analysis using common carbon sources in ^{13}C-labeled form, such as [U-$^{13}C_6$]glucose, has been shown to be a relatively fail-safe method for the determination of the biosynthetic history of a target metabolite (for a recent review, see Eisenreich and Bacher[15]). It allows elimination of predicted pathways by comparison of isotopologue profiles in the target compound and other metabolic products (typically amino acids) from the same experiment. In this study, we show by labeling experiments that BE-10988 (**1**), like camalexin (**10**), is synthesized from cysteine (**7**) and tryptophan (**2**). However, different mechanisms of tryptophan modification have evolved in the plant and the bacterial system, as BE-10988 (**1**) is not synthesized via IAOx (**3**).

Results and Discussion

Incorporation of [U-$^{13}C_6$]Glucose into BE-10988 and Tryptophan. *Streptomyces* sp. BA10988 was grown in medium containing 0.475% glucose and 0.025% [U-$^{13}C_6$]glucose (w/v). BE-10988 (**1**) (5.5 mg) were isolated from 10 L of culture medium. Tryptophan (**2**) was isolated after alkaline hydrolysis of protein. Both compounds were analyzed by quantitative NMR spectroscopy. The ^{13}C NMR signals displayed intense ^{13}C-coupling satellites indicating the presence of multiply labeled isotopologues at significant abundance (Table 1, Table 2, Figure 1, Scheme 3).

As a prerequisite for biosynthetic studies by ^{13}C NMR spectroscopy, all ^{13}C NMR signals of the target molecules must be unequivocally assigned. The published assignments of BE-10988 (**1**)[12] were confirmed by a detailed analysis of the $^{13}C^{13}C$ coupling constants that were observed in the ^{13}C NMR spectrum of the ^{13}C-enriched sample (Table 1).

The isotopologue composition of tryptophan (**2**) shows the same qualitative pattern as in tryptophan from plants after incorporation of [U-$^{13}C_6$]glucose.[16] Characteristic abundant isotopologues include [3a,7a-$^{13}C_2$]-, [4,5,6-$^{13}C_3$]-, and [2,3-$^{13}C_2$]Trp (Scheme 3C, Table 2). The profile is consistent with biosynthesis via the shikimic acid pathway, which is also active in *Streptomyces* strains.[17] Accordingly, the indole ring of tryptophan (**2**) is derived from erythrose 4-phosphate (C-4, C-5, C-6, C-7), phosphoenolpyruvate (C-3a, C-7a), and ribose 5-phosphate (C-2, C-3).

The isotopologue composition of BE-10988 (**1**) labeled with 5% [U-$^{13}C_6$]glucose was also determined by NMR spectroscopy (Table 1, Scheme 3A, Figure 1) and was compared with predictions of the pattern via two hypothetical pathways (Scheme 3B,C). The structural and possible evolutionary relation of BE-10988 (**1**) and mitomycins (Scheme 3D), antibiotics of actinomycete origin, suggested a similar biosynthetic origin for BE-10988 (**1**) (Scheme 3B). In this hypothetical pathway, 3-amino-5-hydroxybenzoic acid (**12**), which is derived from the shikimate pathway, serves as a precursor. A qualitative pattern for this intermediate can be predicted according to the origin of the shikimate intermediate from erythrose 4-phosphate and phosphoenolpyruvate,[17] confirmed by the observed isotopologue pattern of tryptophan (Scheme 3C, Table 2). The side chain at C-3, as well as C-2/C-3 of the indoloquinone ring in **1**, could be derived from a sugar or amino sugar.[14] Following this hypothetical route, the isotopologue composition of BE-10988 can be predicted, as shown in Scheme 3B. Alternatively, the isotopologue profile of BE-10988 was predicted from tryptophan

(7) Schuhegger, R.; Nafisi, M.; Mansourova, M.; Petersen, B. L.; Olsen, C. E.; Svatos, A.; Halkier, B. A.; Glawischnig, E. *Plant Physiol.* **2006**, *141*, 1248–54.
(8) Zook, M.; Hammerschmidt, R. *Plant Physiol.* **1997**, *113*, 463–8.
(9) Chang, Z.; Flatt, P.; Gerwick, W. H.; Nguyen, V. A.; Willis, C. L.; Sherman, D. H. *Gene* **2002**, *296*, 235–47.
(10) Cheng, Y. Q.; Tang, G. L.; Shen, B. *J. Bacteriol.* **2002**, *184*, 7013–24.
(11) Oka, H.; Yoshinari, T.; Murai, T.; Kawamura, K.; Satoh, F.; Funaishi, K.; Okura, A.; Suda, H.; Okanishi, M.; Shizuri, Y. *J. Antibiot. (Tokyo)* **1991**, *44*, 486–91.
(12) Suda, H.; Matsunaga, K.; Yamamura, S.; Shizuri, Y. *Tetrahedron Lett.* **1991**, *32*, 2791–2.
(13) Crosa, J. H.; Walsh, C. T. *Microbiol. Mol. Biol. Rev.* **2002**, *66*, 223–49.
(14) Mao, Y.; Varoglu, M.; Sherman, D. H. *Chem. Biol.* **1999**, *6*, 251–63.
(15) Eisenreich, W.; Bacher, A. *Phytochemistry* **2007**, *68*, 2799–815.
(16) Glawischnig, E.; Tomas, A.; Eisenreich, W.; Spiteller, P.; Bacher, A.; Gierl, A. *Plant Physiol.* **2000**, *123*, 1109–19.
(17) Smithers, C. M.; Engel, P. P. *Genetics* **1974**, *78*, 799–808.

SCHEME 3. Retrobiosynthetic Analysis of BE-10988 Biosynthesis

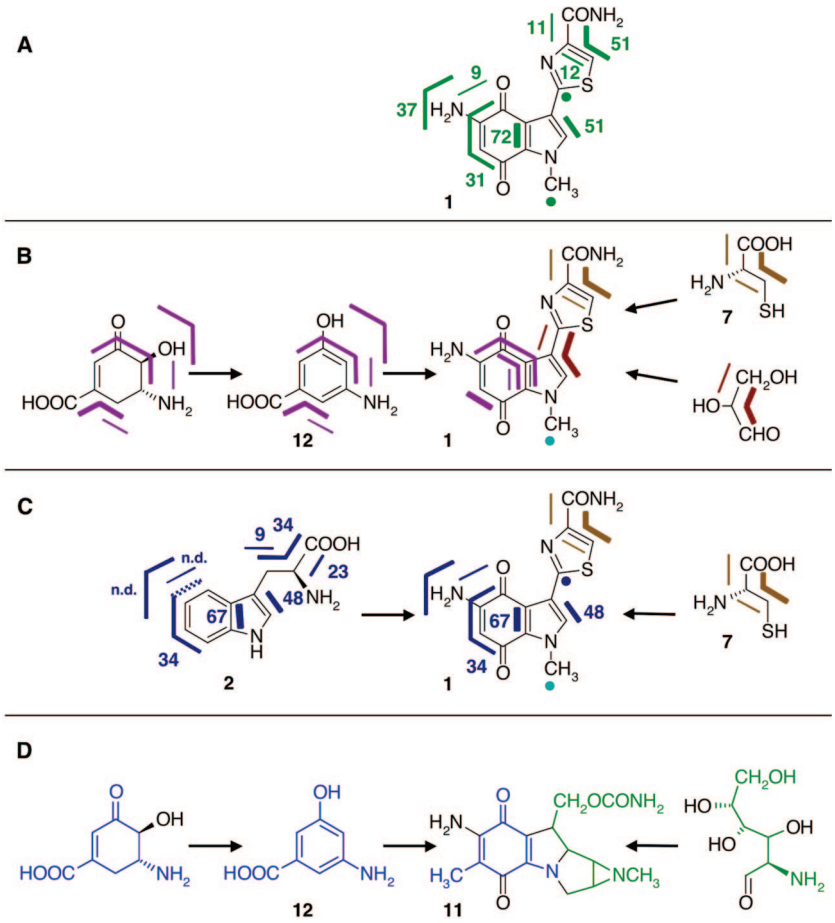


TABLE 1. ^{13}C NMR Data of BE-10988 from the Experiment with [U-$^{13}C_6$]Glucose

position	δ, ppm	J_{cc}[a] (Hz)	% $^{13}C^{13}C$[a]
4	179.9	48.0 (5), 3.3 (6 or 7)	74.5 (5, 5 and 6, 5 and 6 and 7)
7	178.1	63.9 (6)	36.9 (4 and 5 and 6)
5′	162.8	74.1 (4′), 7.8 (6′)	49.4 (4′ and 6′), 12.8 (4′)
2′	159.3	61.5 (3)	<3 (3)
5	151.3	67.3 (6), 48.3 (4),3.3 (7)	67.0 (6 and 4), 8.9 (6)
4′	150.1	73.9 (5′), 64.1 (6′)	48.3 (5′and 6′), 10.2 (6′),9.3 (5′)
7a	131.9	57.5 (3a)	70.6 (3a)
2	130.4	67.7 (3)	51.2 (3)
6′	124.0	63.7 (4′), 8.6 (5′)	53.7 (4′and 5′), 12.9 (4′)
3a	117.4	57.7 (7a)	73.8 (7a)
3	116.7	67.5 (2)	51.7 (2)
6	98.7	67.0 (5), 4.3 (4)	37.3 (5 and 4), 30.4 (4 and 5 and 7)

[a] Fractions of ^{13}C-coupled satellite signals in the overall signal intensity of the indexed atom. Coupled atoms are given in parentheses.

TABLE 2. ^{13}C NMR Data of Tryptophan from the Experiment with [U-$^{13}C_6$]Glucose

position	δ, ppm	J_{cc}[a] (Hz)	% $^{13}C^{13}C$[a]
1′	182.8	53.3 (2′)	55.6 (2′)
7a	136.0	53.7 (3a)	69.5 (3a)
3a	127.1	53.7 (7a)	65.1 (7a)
2	124.2	69.8(3)	46.4 (3)
6	121.6	nd (multiplet)	nd (multiplet)
4	118.9	nd (higher order)	nd (higher order)
5	118.7	nd (higher order)	nd (higher order)
7	111.6	58 (6), 2 (4 or 5)	33.9 (4 and 5 and 6)
3	110.5	69.9 (2)	50.2 (2)
2′	56.4	53.5 (1′), 33.6 (3′)	33.9 (1′ and 3′), 23.8 (1′), 8.8 (3′)
3′	30.2	33.4 (2′)	39.6 (2′)

[a] Fractions of ^{13}C-coupled satellite signals in the overall signal intensity of the indexed atom. Coupled atoms are given in parentheses.

as a precursor via a pathway resembling camalexin (**10**) biosynthesis (Scheme 3C). Tryptophan is also synthesized via the shikimic acid pathway,[17] but the topology of the shikimic acid precursor relative to the product **1** differs, resulting in highly specific isotopologue patterns of **1** by the two predictions.

Scheme 3 shows a comparison of the observed labeling pattern (**A**) of BE-10988 (**1**), isolated from [U-$^{13}C_6$]glucose-containing cultures, with predicted labeling patterns (**B**, **C**) via two different hypothetical pathways: **A**, observed pattern; **B**, prediction via a pathway similar to mitomycin[14] biosynthesis; **C**, prediction via a pathway similar to camalexin biosynthesis from tryptophan (**2**) and cysteine (**7**). The colored bars indicate multiple ^{13}C-labeled isotopologues connecting ^{13}C atoms in a given molecule and the thickness of the bars is scaled to the relative abundances of the indexed isotopologues. The numbers indicate relative abundances of multiple ^{13}C-labeled isotopologues as determined from the intensities of the ^{13}C-coupled satellite pairs in the ^{13}C NMR spectra of BE-10988 (**1**) (Table 1, Figure 1) and tryptophan (**2**) (Table 2). Single ^{13}C-labeled isotopologues in excess (as determined from the overall NMR intensities) are indicated by closed circles. The predictions are based on the observed patterns in tryptophan (Table 2) and established metabolic pathways.[17,18] For comparison, the biosynthetic pathway of mitomycin[14] (**11**) is shown schematically under **D**. For the biosynthesis of camalexin, see Scheme 1.

The observed labeling pattern of BE-10988 (Scheme 3A) was in good agreement with the prediction via tryptophan (**2**) but

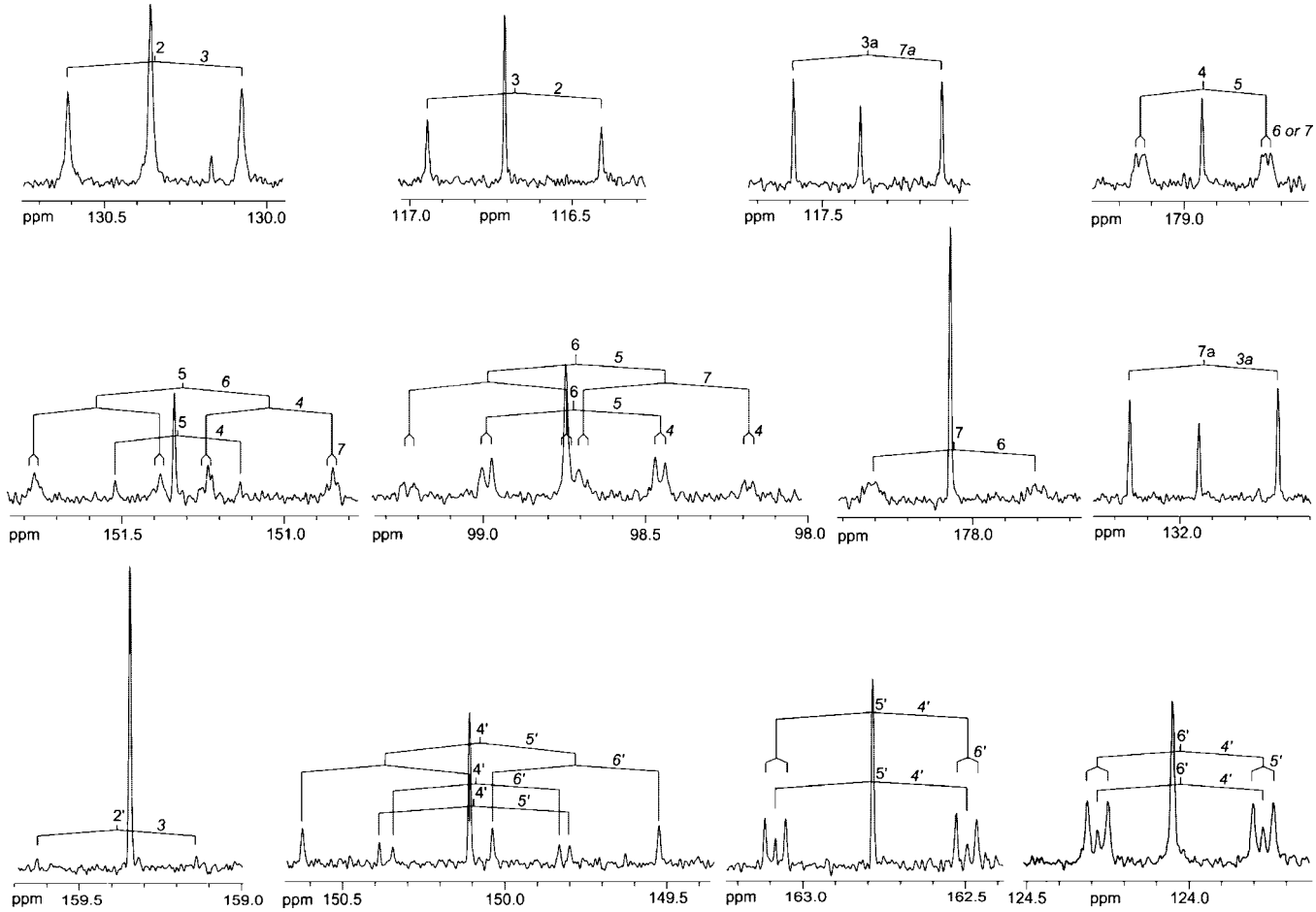
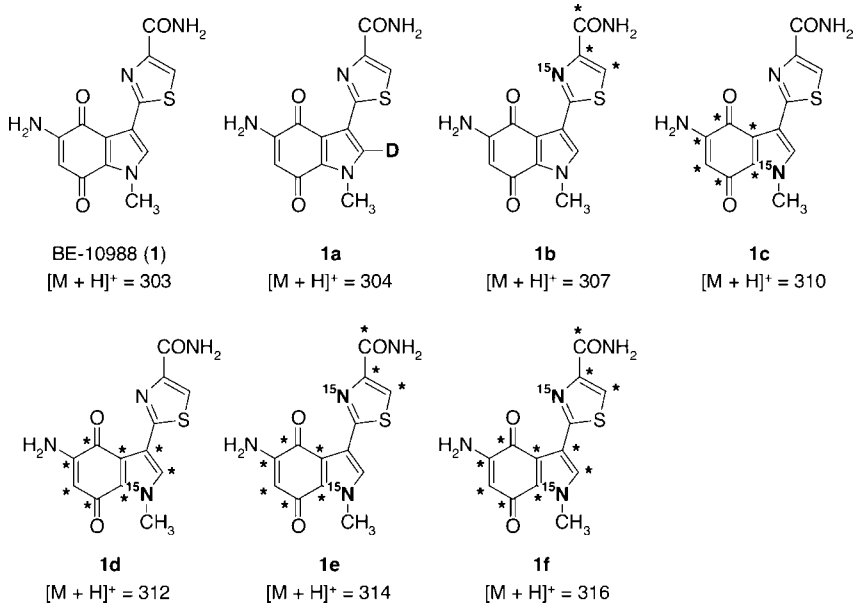


FIGURE 1. ^{13}C NMR Signals of BE-10988 Isolated from Cultures Grown with [U-$^{13}C_6$]Glucose. Coupling patterns are indicated.

SCHEME 4. BE-10988 (1) and Labeled Analogues (* = ^{13}C)



clearly at odds with the prediction via the mitomycin (**11**) pathway (Scheme 3B). Specifically, [3,2′-$^{13}C_2$]-, [2,3,2′-$^{13}C_3$]-, and [3a,4,7a-$^{13}C_3$]-isotopologues were not detected in BE-10988, while a [4,5,6-$^{13}C_3$]-isotopologue was observed at high abundance.

Biosynthesis from Tryptophan and Cysteine: Incorporation of Specific Precursors. In order to confirm that BE-10988 (**1**) (Scheme 4) is synthesized from L-cysteine (**7**) and L-tryptophan (**2**), L-[*ring*-$^2H_5$]tryptophan (**2a**), [U-$^{13}C_8{}^{15}N$]indole (**4a**), and L-[U-$^{13}C_3{}^{15}N$]cysteine (**7a**) were applied as specific biosynthetic precursors in labeling experiments (Schemes 4 and 5). For this purpose, 30 mL of minimal medium, supplemented with a specific precursor, was inoculated with 0.75 mL of a

TABLE 3. Calculated Incorporation Rates of Specific Precursors into BE-10988 According to the LC-ESIMS Spectra; Labeled Product (1a−f) According to Scheme *4*

	relative abundance (%)						
precursor (50 μM, if not otherwise stated)	**1**	**1a**	**1b**	**1c**	**1d**	**1e**	**1f**
[$^2H_5$]Trp (**2a**)[a]	36.7	63.3	0	0	0	0	0
[$^2H_5$]Trp (**2a**)[a] + [U-^{13}C,^{15}N]ind (**4a**)[a]	9.7	50.5	0	1.8	38.0	0	0
[$^2H_5$]IAOx (**3a**)	100	0	0	0	0	0	0
[$^2H_5$]IAOx (**3a**) + [U-^{13}C,^{15}N]ind (**4a**)	57.6	(3.3)	0	28.5	10.6	0	0
[^{15}N]anthranilate (**5a**)	58.9	41.1[b]	0	0	0	0	0
5a + **4a**	42.7	32.5[b]	0	11.9	12.9	0	0
[$^2H_5$]IPyr (**6a**)	71.4	28.6	0	0	0	0	0
[$^2H_5$]IPyr (**6a**) + [U-^{13}C,^{15}N]ind (**4a**)	32.0	14.4	0	11.2	42.4	0	0
[U-^{13}C,^{15}N]Cys (**7a**)	67.3	0	32.7	0	0	0	0
7a + **4a**	29.4	0	8.1	10.5	35.8	4.7	11.5
[U-^{13}C,^{15}N]Cys (**7a**)[a]	48.7	0	51.3	0	0	0	0

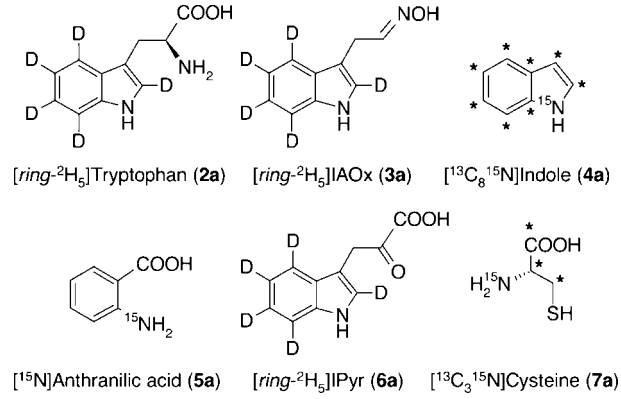
[a] 200 μM instead of 50 μM. [b] [1-^{15}N-BE-10988 + H]$^+$; ind: indole.

TABLE 4. Incorporation Rates into BE-10988 According to the HR-LC-ESIMS Spectra

	relative abundance (%)						
precursor (50 μM, if not otherwise stated)	**1**	**1a**	**1b**	**1c**	**1d**	**1e**	**1f**
[$^2H_5$]Trp (**2a**)[a]	47.5	52.5	0	0	0	0	0
[$^2H_5$]Trp (**2a**)[a] + [U-^{13}C,^{15}N]ind (**4a**)[a]	9.8	57.3	0	1.0	31.9	0	0
[$^2H_5$]Trp (**2a**)[a] + [U-^{13}C,^{15}N]ind (**4a**)	19.0	79.0	0	0.8	1.2	0	0
[$^2H_5$]IPyr (**6a**) + [U-^{13}C,^{15}N]ind (**4a**)	33.3	12.4	0	10.4	43.9	0	0

[a] 200 μM instead of 50 μM; ind: indole.

SCHEME 5. Compounds Fed to BE-10988 (1) for MS Analysis (* = ^{13}C)



stationary culture of *Streptomyces* sp. BA 10988 and cultured under aerobic conditions for 2 days. BE-10988 (**1**) was isolated from the broth, and the isotopic composition was analyzed by LC-MS. Efficient incorporation of the label from L-cysteine, indole, and L-tryptophan was observed (Table 3).

After application of [*ring*-$^2H_5$]tryptophan (**2a**), [(m + 1) + H]$^+$ (**1a**), but not [(m + 2) + H]$^+$ was significantly enriched (Table 3, Table 4). In accordance with these data, we propose that deuterium at C-6 is exchanged when oxygen at C-7 is introduced or as a result of an intermediate quinol tautomerisation. Generally, tryptophan (**2**) is expected to be synthesized from indole-3-glycerol phosphate by a tryptophan synthase complex without release of intermediately formed indole (**4**).[19] However, [U-$^{13}C_8{}^{15}$N]indole (**4a**) is efficiently incorporated into BE-10988 [(m + 9) + H]$^+$ (**1d**). Similarly, high incorporation rates of indole were observed into camalexin (**10**).[3] It is therefore suggested that the concerned tryptophan synthase complexes are either highly accessible for indole (**4**) or tryptophan synthase β functions efficiently independently of alpha subunits. In addition, detection of the [(m + 7) + H]$^+$ (**1c**), which was observed when [U-$^{13}C_8{}^{15}$N]indole (**4a**) was applied suggests that externally applied indole is partially degraded and an intermediate, such as anthranilic acid (**5**), re-enters the tryptophan biosynthetic pathway.

[U-$^{13}C_3{}^{15}$N]cysteine (**7a**) incorporation is in accordance with an origin of the thiazole ring from cysteine (**7**), thus excluding C-2, as it has been demonstrated for other thiazole compounds from bacteria.[13] Enrichment of [(m + 13) + H]$^+$ (**1f**) after double labeling with **4a** and **7a** confirmed that both precursors give rise to different parts of the heterocycle, i.e., the indoloquinone and the thiazole moiety (excluded C-2, included the amide carbon, Figure 2).

Evidence for the incorporation of the side chain C-3′ of tryptophan into BE-10988 (**1**) was obtained by labeling a 10 mL culture for 2 days with [3′-^{14}C]tryptophan. BE-10988 was isolated, purified by HPLC, and subsequently separated by TLC. Co-chromatography of an abundant radioactive metabolite with BE-10988 was observed (Figure 2D). These data confirm that tryptophan (**2**) is a precursor of **1** and suggest that C-2′ of **1** derives from **2**.

Indole 3-Acetaldoxime Is Not a Biosynthetic Intermediate. During the biosynthesis of camalexin (**10**), tryptophan (**2**) is converted to indole-3-acetaldoxime (IAOx, **3**) by a cytochrome P450 reaction. In addition, it was shown for *Nocardia uniformis* that oxime synthesizing P450 enzymes can be present in actinomycetes.[20] Therefore, IAOx (**3**) was tested as a potential precursor of BE-10988 (**1**). As indolocarbazoles from actinomycetes are synthesized via indole-3-pyruvic acid (IPyr, **6**) or 7-chloroindole-3-pyruvic acid,[21,22] IPyr (**6**) was also analyzed as an alternative precursor.

BA 10988 cultures were grown in the presence of [$^2H_5$]IAOx (**3a**) or [$^2H_5$]IPyr (**6a**), and isolated BE-10988 was analyzed

(18) Borodina, I.; Krabben, P.; Nielsen, J. *Genome Res.* **2005**, *15*, 820–9.
(19) Miles, E. W. *Chem. Rec.* **2001**, *1*, 140–51.
(20) Kelly, W. L.; Townsend, C. A. *J. Am. Chem. Soc.* **2002**, *124*, 8186–7.
(21) Howard-Jones, A. R.; Walsh, C. T. *Biochemistry* **2005**, *44*, 15652–63.
(22) Nishizawa, T.; Aldrich, C. C.; Sherman, D. H. *J. Bacteriol.* **2005**, *187*, 2084–92.

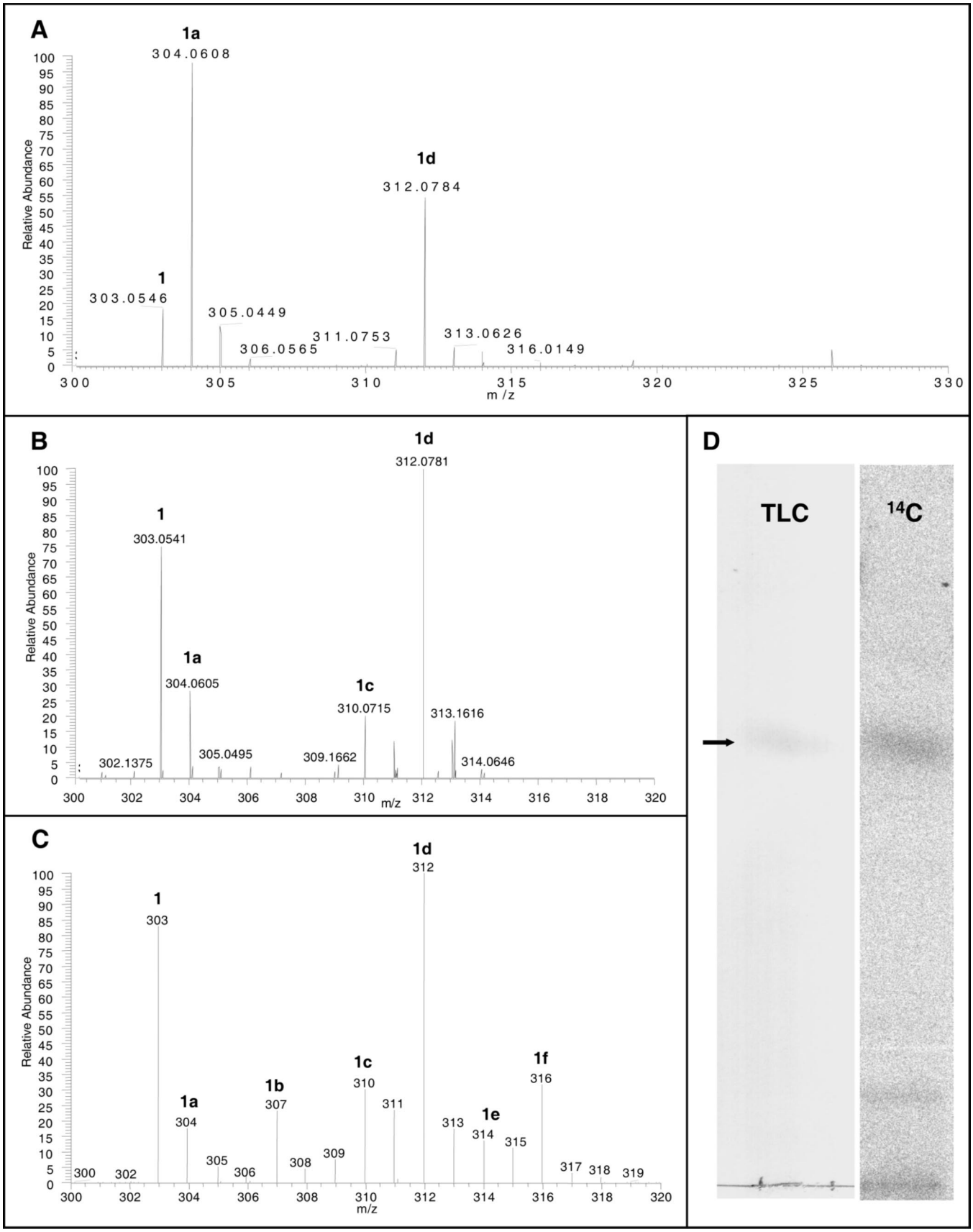


FIGURE 2. Incorporation of specific precursors. **A**, **B**: HR-LC-ESIMS spectrum of BE-10988 isolated after feeding (**A**) 200 μM [$^2H_5$]Trp (**2a**)/200 μM [U-$^{13}C_8{}^{15}N$]indole (**4a**) or (**B**) 50 μM [U-$^{13}C_8{}^{15}N$]indole (**4a**)/50 μM [$^2H_5$]IPyr (**6a**). At the displayed magnification, **1a** is not separated from the signal derived from naturally abundant $^{13}C_1$-BE-10988 isotopologues. **C**: ESIMS spectra of BE-10988 isolated after feeding 50 μM [U-$^{13}C_8{}^{15}N$]indole (**4a**)/50 μM [U-$^{13}C_3{}^{15}N$]cysteine (**7a**). Here: **1a** signal derived from natural abundance $^{13}C_1$-BE-10988 isotopologues. **D**: TLC separation (left) of HPLC-purified BE-10988 labeled with [3′-^{14}C]tryptophan (marked with arrow) and autoradiogram thereof (right).

TABLE 5. Incorporation Rates of Specific Precursors into BE-10988 (1) and Tryptophan (2) According to HR-LC-ESIMS Data[a]

precursor	product	labeled product (rel abundance) (%)
50 μM [$^2H_5$]IPyr (**6a**)	BE-10988 (**1**)	26.0
50 μM [$^2H_5$]IPyr (**6a**)	tryptophan (**2**)	27.8
50 μM [$^2H_5$]Trp (**2a**)	BE-10988 (**1**)	29.8
50 μM [$^2H_5$]Trp (**2a**)	tryptophan (**2**)	66.4

[a] The percentage of total labeled isotopologues is given, taking into account natural abundance isotopologues.

for enrichment of **1a** (Table 3). No incorporation was observed after feeding of 50 μM **3a**. In contrast, 50 μM [$^2H_5$]IPyr (**6a**) labeling resulted in approximately 29% incorporation (Table 3). After feeding of **6a** and **4a**, 12% deuterium incorporation (**1a**) was detected by HR-LC-ESIMS (Table 4). These data indicate that, unlike in camalexin (**10**) biosynthesis, not IAOx (**3**), but rather IPyr (**6**), can serve as a biosynthetic intermediate in the BE-10988 pathway.

However, the results could also reflect that the applied labeled IPyr (**6a**) was transaminated to labeled tryptophan (**2a**) prior to conversion into **1a** via unknown mechanisms. To further analyze the role of IPyr in the pathway, we have grown the culture in the presence of 50 μM ^2H-labeled tryptophan (**2a**) or IPyr (**6a**), respectively. BE-10988 (**1**) was isolated from the culture medium and tryptophan (**2**) was isolated from the protein hydrolysate. Incorporation of label into **1** and **2** was determined by HR-LC-ESIMS. Both **2a** and **6a** yielded labeled BE-10988 (**1**) and tryptophan (**2**) (Table 5). The incorporation values show that label from IPyr (**6a**) is transferred to BE-10988 (**1**) and tryptophan (**2**) at similar rates, whereas label from exogeneous tryptophan (**2a**) was transferred more efficiently (by a factor of 2.4) to protein-derived tryptophan than to BE-10988. The incorporation experiment with **6a** demonstrates that **6** can be converted into BE-10988 (**1**) as well as into tryptophan (**2**) yielding apparent isotopic equilibrium between **1**, **2**, and **6**. On the other hand, the higher incorporation rate of labeled tryptophan into protein as compared to BE-10988 suggests that IPyr (**6**) is an intermediate in the biosynthetic pathway of BE-10988.

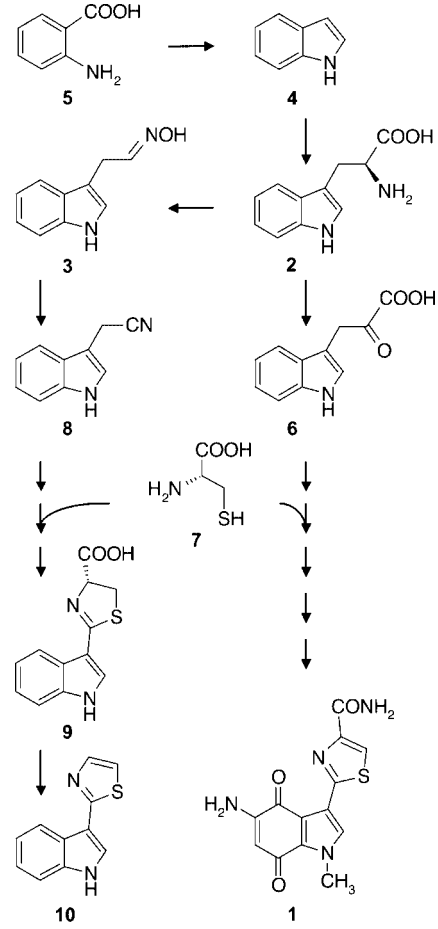
In the biosynthesis of the indolic antibiotic rebeccamycin, synthesized by the actinomycete *Lechevalieria aerocolonigenes*, tryptophan (**2**) is oxidized to IPyr (**6**) by the amino acid oxidase RebO.[21,22] A RebO homologue might also be involved in the biosynthesis of BE-10988 (**1**). Identification and functional analysis of this proposed specific amino acid oxidase in *Streptomyces* sp. BA 10988 will finally prove a functional role of IPyr (**6**) as intermediate of BE-10988 (**1**).

Conclusions

Both thiazolylindoles, the *Arabidopsis* defense compound camalexin (**10**) and the bacterial derivative BE-10988 (**1**), are synthesized from cysteine (**7**) and tryptophan (**2**) (Scheme 6). Our data show that in each case, the side chain of the tryptophan precursor is shortened to one carbon prior to the assembly of the complex heterocycles in **1** and **10**. However, the early steps in the biosynthesis of **1** and **10** are different. In camalexin (**10**) biosynthesis, the carboxylic group of tryptophan is eliminated in the first step yielding IAOx (**3**). Our data exclude IAOx as a precursor of BE-10988 (**1**) but rather suggest that IPyr (**6**) serves as an early intermediate in the bacterial pathway.

In the biosynthesis of camalexin (**10**), tryptophan (**2**) modification is carried out by cytochrome P450 enzymes of the plant-specific families 71 and 79. Possibly also in the *Streptomyces*

SCHEME 6. Biosynthetic Pathway of BE-10988 and Camalexin: Comparison of the Biosynthetic Pathway of Camalexin (10) (Left,[3,6–8]) and the Proposed Pathway for BE-10988 (1) (Right, This Study)



system, cytochrome P450s have been recruited to modify the indolic precursors and might have convergently evolved structural similarities with the *Arabidopsis* enzymes. For the formation of the thiazolylindole heterocycle in *Streptomyces*, we propose the contribution of a nonribosomal peptide synthetase (NRPS). No apparent NRPS genes are present in the *Arabidopsis thaliana* genome and the plant enzyme catalyzing the reaction of the indolic precursor with cysteine (**7**) or a cysteine derivative remains to be identified. It will be interesting to learn whether analogous reactions have evolved in the two kingdoms. As demonstrated for the biosynthesis of vitamin B_6,[23] corresponding enzymes, although unrelated concerning their primary sequence, could share similarities in their 3D-structures and mechanisms as a consequence of their common biological function.

Experimental Section

Chemicals. Labeled precursors, commercially available: L-[*ring*-$^2H_5$]tryptophan (**2a**), [U-$^{13}C_8{}^{15}N$]indole (**4a**), [^{15}N]anthranilate (**5a**), L-[U-$^{13}C_3{}^{15}N$]cysteine (**7a**), [U-$^{13}C_6$]glucose, and [3′-^{14}C]tryptophan (58.1 mCi mmol^{-1}).

[*ring*-$^2H_5$]IAOx (**3a**) was synthesized from (**2a**) essentially as described by Hofmann et al.[24] with the following modifications: 10 mg of L-[*ring*-$^2H_5$]tryptophan (**2a**) as educt, reaction volume 2 mL. [$^2H_5$]IAOx was purified by TLC (chloroform/methanol, 9:1). Total yield: 25% [$^2H_5$]IAOx (**3a**). UV_{max}: 280 nm. ESI-MS [M + H]$^+$: 180.

[*ring*-$^2H_5$]Indole-3-pyruvate (**6a**) was synthesized enzymatically from (**2a**) by modifying the protocol of Cooney and Nonhebel:[25] 10 mg of L-[*ring*-$^2H_5$]tryptophan (**2a**) was oxidized with 2.5 U of amino acid oxidase and 2.5 U of catalase in 5 mL of 0.25 mM KP_i, pH 7 for 2 h at 25 °C, acidified with 1.15 mL of 2 M citric acid, and extracted 3 times with 2.4 mL of ethyl acetate. [$^2H_5$]IPyr (**6a**) was purified by preparative HPLC: 250-10, RP-18, 5 μm; 7 mL min^{-1}; 0.3% HCOOH in H_2O/acetonitrile (4:1) for 2 min, followed by a 14 min linear gradient to 0.3% HCOOH in H_2O/acetonitrile (2:3). Fractions containing **6a** were collected, acetonitrile was removed under reduced pressure, and the remaining H_2O phase was extracted twice with 1 volume of ethyl acetate. Yield: 20%. Product identity and purity (>95%) were confirmed by HPLC (250-4, RP-18, 5 μm; 1 mL min^{-1}; 0.3% HCOOH in H_2O/acetonitrile (9:1) for 2 min, followed by a 11.5 min linear gradient to 0.3% HCOOH in H_2O/acetonitrile (2:3), t_R = 13.2 min, OD_{max}=327 nm, both identical to IPyr (**6**) standard. ESI-MS: $[M + H]^+$ 209.

Culture Conditions and Product Isolation. *Streptomyces* strain BA 10988[11] was kindly provided by Dr. Saeki, Banyu Inc., Japan, and grown in liquid dYT medium or on solid mannitol soya flour medium.[26] For production of BE-10988, the minimal medium described by Hopwood[27] was modified as follows: $(NH_4)_2SO_4$ (0.1%), K_2HPO_4 (0.05%), $MgSO_4 \times 7H_2O$ (0.02%), $FeSO_4 \times 7H_2O$ (0.001%) in H_2O at pH 7; addition of 1% glucose in labeling experiments with specific precursors or alternatively 0.475% glucose and 0.025% [U-$^{13}C_6$]glucose (retrobiosynthetic analysis).

Feeding of [U-$^{13}C_6$]Glucose and Isolation of BE-10988 and Tryptophan. Streptomyces BA 10988 was precultured for 48 h in liquid dYT medium (5 mL) at 28 °C. The preculture was used to inoculate 50 mL of production medium, incubated for 48 h on a rotary shaker at 28 °C and 200 rpm. This second preculture was used to inoculate 1 L of production medium. A total of 10 flasks (total culture volume: 10 L) were incubated on a rotary shaker at 28 °C and 200 rpm for 100 h. BE-10988 (**1**) was isolated by extracting each culture (1 L) three times with ethyl acetate (300 mL). The extract was then dried over sodium sulfate. After evaporation of the ethyl acetate, BE-10988 (**1**) was purified by preparative HPLC (250-10 column, RP-18e, 10 μm) at a flow rate of 5 mL min^{-1} with 25% acetonitrile for 2 min, followed by a 7 min linear gradient from 25% to 100% acetonitrile. BE-10988 (**1**) was eluted after 6.6 min. After removal of the acetonitrile under reduced pressure, the remaining aqueous phase was extracted three times with 1/3 of its volume of ethyl acetate. The solvent was evaporated, and the combined residues were dissolved in deuterated DMSO. Tryptophan (**2**) was isolated after alkaline hydrolysis of bacterial pellet as described by Eisenreich and Bacher.[28]

Labeling Experiments with Specific Precursors. Thirty milliliters of production medium containing the specific precursor (Scheme 5) was inoculated with 0.75 mL of preculture and incubated for 48 h on a rotary shaker at 28 °C and 200 rpm. Then the culture was extracted with ethyl acetate (2 × 15 mL). After evaporation of the ethyl acetate, BE-10988 (**1**) was isolated by preparative TLC (chloroform/methanol, 9:1).

Instrumentation and Labeling Analysis. LC-ESIMS. For LC-MS analysis, 10 μL of sample was separated on a RP18 column (150 × 2 mm, 4 μm particle size). The mobile phase consisted of water containing 1 mM ammonium acetate and 0.1% HCOOH (A) and acetonitrile + 0.1% HCOOH (B). Samples were separated using a gradient program as follows: (flow rate of 300 μL min^{-1}) 90% A isocratic for 3 min, linear gradient to 50% A over 12 min, and to 100% B for another 1 min. After 100% B isocratic for 6 min, the system was returned to its initial conditions (100% A) within 1 min and was equilibrated for 6 min before the next run was started. The HPLC system was coupled with a TSQ Quantum Ultra AM equipped with an APCI ion source (Ion Max) operating in positive mode (mass range of 150−650 mu). Nitrogen was employed as both the sheath (40 arbitrary units) and auxiliary (5 arbitrary units) gas and argon served as the collision gas with a pressure of 1.5 mTorr. The capillary temperature for the TSQ was set to 200 °C. The vaporizer temperature was set to 450 °C.

HR-LC-ESIMS. The ESI-FT-MS spectra were obtained with an LTQ-Orbitrap spectrometer. The spectrometer was operated in positive mode (1 spectrum s^{-1}; mass range: 50−1000) with nominal mass resolving power of 60000 at *m/z* 400 with a scan rate of 1 Hz) with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation using one internal standard; polydimethylcyclosiloxane- $[(CH_3)_2SiO]_6$: *m/z* = 445.120025.

The spectrometer was equipped with a UV detector at 254 nm, flow manager, and autosampler (injection volume 1 μL). Nitrogen was used as sheath gas (5 arbitrary units), and helium served as the collision gas. The separations were performed by using a C18 column (3 μm, 0.3 × 150 mm) with a H_2O (+ 0.1% HCOOH) (A)/acetonitrile (+0.1% HCOOH) (B) gradient (flow rate 4 μL min^{-1}). Samples were analyzed by using a gradient program as follows: 90% A isocratic for 2 min, linear gradient to 100% B over 8 min; after 100% B isocratic for 10 min, the system returned to its initial condition (90% A) within 1 min and was equilibrated for 6 min.

Calculation of Incorporation Rates. The peak ratios of all significant isotope $[M + H]^+$ ions were determined from the area of the corresponding ion trace at *m/z* = 303, 304, 307, 310, 312, 314, and 316. In the case of labeling experiments except with *ring*-deuterated samples, the ion at *m/z* = 304 was not included, since it occurs due to the natural abundance of ^{13}C and does not represent a species arising from the labeled precursor. In the case of feeding experiments with *ring*-deuterated samples, the area of the ion at *m/z* = 304 was diminished by a value that represents 16.6% of the corresponding area of the ion at *m/z* = 303 in order to take into account the natural abundance of isotopes in BE-10988. Finally, the ratios of the remaining areas were recalculated to yield 100% for all unlabeled and labeled species together. BE-10988 (**1**): LC-HR-ESIMS: *m/z* = 303.0540 $[M + H]^+$, calcd 303.0546 for $C_{13}H_{11}N_4O_3S$. **1a**: LC-HR-ESIMS: *m/z* = 304.0605 $[M + H]^+$, calcd 304.0609 for $C_{13}H_{10}{}^2HN_4O_3S$. **1c**: LC-HR-ESIMS: *m/z* = 310.0721 $[M + H]^+$, calcd 310.0718 for $C_7{}^{13}C_6H_{11}N_3{}^{15}NO_3S$. **1d**: LC-HR-ESIMS: *m/z* = 312.0778 $[M + H]^+$, calcd 312.0785 for $C_5{}^{13}C_8H_{11}N_3{}^{15}NO_3S$.

Incorporation of Radioactivity. ^{14}C incorporation into BE-10988 was analyzed after TLC (chloroform/methanol, 9:1) separation on a phosphoimager.

NMR spectroscopy. NMR spectra were recorded at 27 °C using a DRX 500 spectrometer. BE-10988 was measured in deuterated DMSO as solvent; tryptophan was measured in 0.1 M NaOD.

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(23) Fitzpatrick, T. B.; Amrhein, N.; Kappes, B.; Macheroux, P.; Tews, I.; Raschle, T. *Biochem. J.* **2007**, *407*, 1–13.
(24) Hofmann, F.; Rausch, T.; Hilgenberg, W. *J. Labelled Compd. Radiopharm.* **1981**, *18*, 1491–5.
(25) Cooney, T. P.; Nonhebel, H. M. *Biochem. Biophys. Res. Commun.* **1989**, *162*, 761–6.
(26) Hobbs, G.; Frazer, C. M.; Gardner, D. C. J.; Cullum, J. A.; Oliver, S. G. *Appl. Microbiol. Biotechnol.* **1989**, *31*, 272–7.
(27) Hopwood, D. A. *Microbiol. Mol. Biol. Rev.* **1967**, *31*, 373–403.
(28) Eisenreich, W.; Bacher, A. *J. Biol. Chem.* **1991**, *266*, 23840–9.