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Further Studies on the Biosynthesis of the Manumycin-Type Antibiotic, Asukamycin, and the Chemical Synthesis of Protoasukamycin

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Abstract: Asukamycin (**2**), a metabolite of *Streptomyces nodosus* ssp. *asukaensis* ATCC 29757 and a member of the manumycin family of antibiotics, is assembled from three components, an "upper" polyketide chain initiated by cyclohexanecarboxylic acid, a "lower" polyketide chain initiated by the novel starter unit, 3-amino-4-hydroxybenzoic acid (3,4-AHBA), and a cyclized 5-aminolevulinic acid moiety, 2-amino-3-hydroxycyclopent-2-enone (C_5N unit). To shed light on the order in which these components are assembled, we synthesized in labeled form various potential intermediates and evaluated their incorporation into **2**. The assembly of the molecular framework of **2** from 3,4-AHBA and cyclohexanecarboxylic acid apparently does not involve free, unactivated intermediates. However, protoasukamycin (**12**), the total synthesis of which is reported, was efficiently converted into **2**, demonstrating that the modification of the aromatic ring to the epoxyquinol structure is the terminal step in the biosynthesis. The results suggest that the two polyketide chains are synthesized separately and that the "upper" chain must be connected to the "lower" polyketide chain before the C_5N unit.

Introduction

Manumycin A (**1a**) and asukamycin (**2**) were the first two members of the manumycin family of antibiotics[1] discovered. Manumycin A was isolated in 1963 from the bacterium *Streptomyces parvulus* Tü 64 by Zähner and co-workers,[2] its structure and stereochemistry were reported by the Zeeck group,[3] and its stereochemistry was revised by Taylor and co-workers.[4] The isolation[5] and structure[6] of asukamycin from *S. nodosus* subsp. *asukaensis* was reported by the group of Omura, with a subsequent revision of its stereochemistry.[7] Both compounds share the structural elements of a central 2-amino-4-hydroxy-5,6-epoxycyclohex-2-enone moiety (the epoxyquinol moiety or mC_7N unit) and a "lower" chain extending from it, an all-trans triene terminating in a carboxyl group amide-linked to a 2-amino-3-hydroxycyclopent-2-enone moiety (the C_5N unit). They only differ in the structure of the "upper" chain which is amide-linked to the nitrogen of the mC_7N unit. In manumycin A, the "upper" chain consists of a methyl branched unsaturated fatty acid, whereas in asukamycin it is a linear trienoic acid terminating in a cyclohexane ring. Since the initial discovery of **1a** and **2**, some 30 structurally closely related members of the manumycin family have been isolated and structurally characterized from natural sources,[1] in addition to numerous unnatural analogues generated by precursor-directed biosynthesis.[8,9] Compounds of the manumycin family exhibit a broad range of biological activities. Most of these, such as their antibacterial (gram-positive), anticoccidial, antifungal, and insecticidal activities, are modest and probably not of clinical interest.[1] However, the discoveries that manumycin A is a potent and selective inhibitor of RAS farnesyltransferase in vitro and in vivo,[10] and that several manumycins act as inhibitors of interleukin-1β converting enzyme,[11] making them potential lead structures for the development of anticancer and antiinflamma-

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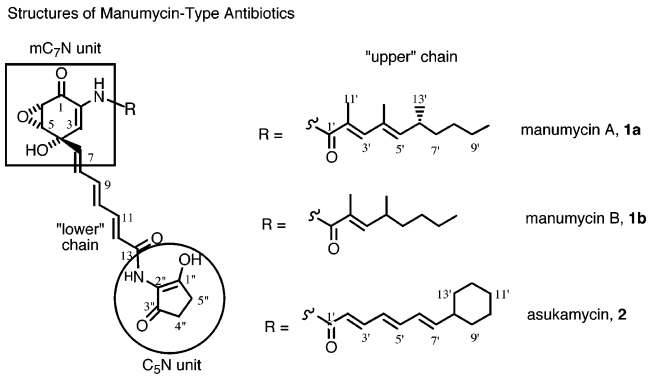
(1) Sattler, I.; Thiericke, R.; Zeeck, A. *Nat. Prod. Rep.* **1998**, *15*, 221−240.
(2) Buzzetti, F.; Gäumann, E.; Hütter, R.; Keller-Schierlein, W.; Neipp, L.; Prelog, V.; Zähner, H. *Pharm. Acta Helv.* **1963**, *38*, 871−874.
(3) (a) Schröder, K.; Zeeck, A. *Tetrahedron Lett.* **1973**, *14*, 4995−4998. (b) Zeeck, A.; Schröder, K.; Frobel, K.; Grote, R.; Thiericke, R. *J. Antibiot.* **1987**, *40*, 1530−1540. (c) Thiericke, R.; Stellwaag, M.; Zeeck, A.; Snatzke, G. *J. Antibiot.* **1987**, *40*, 1549−1554.
(4) Alcarz, L.; Macdonald, G.; Ragot, J. P.; Lewis, N.; Taylor, R. J. K. *J. Org. Chem.* **1998**, *63*, 3526−3527
(5) Omura, S.; Kitao, C.; Tanaka, H.; Oiwa, R.; Takahashi, Y.; Nakagawa, A.; Shimida, M. *J. Antibiot.* **1976**, *29*, 876−881.
(6) Kakinuma, K.; Ikegawa, N.; Nakagawa, A.; Omura, S. *J. Am. Chem. Soc.* **1979**, *101*, 3402−3404.
(7) Cho, H.; Sattler, I.; Beale, J. M.; Zeeck, A.; Floss, H. G. *J. Org. Chem.* **1993**, *58*, 7925−7928.
(8) Thiericke, R.; Zeeck, A. *J. Chem. Soc., Perkin Trans. I* **1988**, 2123−2127.
(9) Thiericke, R.; Langer, H. J.; Zeeck, A. *J. Chem. Soc., Perkin Trans. I* **1989**, 851−855.
(10) (a) Hara, M.; Akasaka, K.; Akinaga, S.; Okabe, M.; Nakano, H.; Gomez, R.; Wood, D.; Uh, M.; Tamanoi, F. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 2281−2285. (b) Hara, M.; Han, M. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 3333−3337.
(11) Tanaka, T.; Tsukuda, E.; Uosaki, Y.; Matsuda, Y. *J. Antibiot.* **1996**, *49*, 1085−1090.

tory agents, respectively, has generated considerable interest in this class of compounds.



Biosynthetic studies of the manumycins have mainly focused on asukamycin and manumycin A and have involved feeding experiments with radioactive and stable isotope-labeled precursors to identify the biosynthetic building blocks. These compounds appear to be assembled from several different components, each of which has its own biosynthetic origin. Both the "upper" and the "lower" chain are of polyketide origin. In **1a**, the "upper" chain is built up from acetyl-CoA as the starter unit through chain elongation by one malonyl-CoA and three methylmalonyl-CoA extender units. The "upper" chain in **2** arises from cyclohexanecarboxylic acid as the starter unit by chain extension with three molecules of malonyl-CoA.[12] The cyclohexanecarboxylic acid in turn is derived from shikimic acid through a series of dehydrations and reductions.[13] The assembly of the "lower" chain in both compounds is initiated by the mC_7N unit, with chain extension by three malonyl-CoA. The C_5N unit terminating the "lower" chain, as in other antibiotics,[14] arises by an intramolecular cyclization from δ-aminolevulinic acid,[12] the common precursor of porphyrins and corrins. The mode of formation of the mC_7N unit is not clear yet. Contrary to an earlier suggestion,[15] its origin is different from that of the mC_7N units found in ansamycin and mitomycin antibiotics, which arise from 3-amino-5-hydroxybenzoic acid formed by the aminoshikimate pathway.[16] Rather, its seven carbon atoms originate from one molecule of a 4-carbon dicarboxylic acid, such as succinate or oxalacetate, and one molecule of a glycerol-derived metabolite, such as a triose phosphate.[12] The epoxide and the hydroxy, but not the carbonyl oxygen of the epoxyquinol moiety, were shown to be derived from molecular oxygen,[17] and some features of the mode of incorporation of glycerol have been established,[7,12] but the mechanism of the assembly of the mC_7N unit from its building blocks is not known.

In the present paper, we report on the synthesis of a number of labeled potential biosynthetic intermediates, including the total synthesis of protoasukamycin, and their evaluation for incorporation into **2**. On the basis of these results, a hypothetical pathway for the biosynthesis of the manumycin-type antibiotics is proposed. Some of the results have been published in preliminary form.[18]

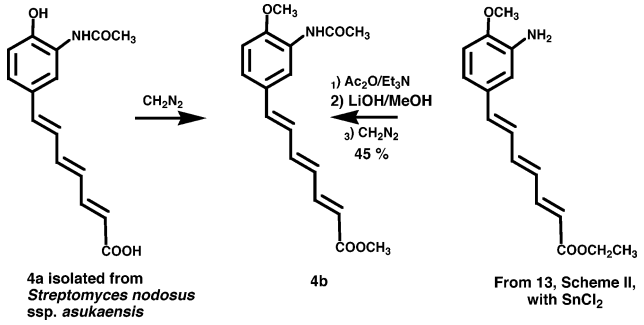
Results

Nature of the mC_7N Unit. The labeling pattern of the mC_7N unit of **1** and **2** from acetate, succinate, and glycerol or glucose is duplicated in a metabolite, 4-hydroxy-3-nitrosobenzamide, an iron chelator isolated from mutant strains of *S. murayamaensis*.[19] This compound represents a mC_7N unit, and 3-amino-4-hydroxybenzoic acid (3,4-AHBA, **3**) was shown to be its specific precursor.[19a] These findings led to the suggestion[19b] that 3,4-AHBA may also be the precursor of the mC_7N unit in manumycin-type compounds; that is, it serves as the polyketide starter unit for the synthesis of the "lower" chain. A preliminary feeding experiment with [2-^{2}H]-3,4-AHBA supported the proposal by showing 16.5% incorporation of deuterium into **2**.[18] To test this hypothesis further, we synthesized [7-^{13}C]-3,4-AHBA (99 atom % ^{13}C) from 4-hydroxy-[7-^{13}C]benzoic acid[14] by nitration and subsequent reduction using procedures developed by Kondo et al.[20] and Balcom and Fürst,[21] respectively. Feeding of this compound (10 mg/100 mL) to liquid cultures of *S. nodosus* ssp. *asukaensis* ATCC 29757 and *S. parvulus* Tü 64 under previously described conditions[12] gave samples of **2** and **1a**, respectively, which were analyzed by ^{13}C NMR spectroscopy. Both compounds showed enriched signals at δ 136 ppm (in $CDCl_3$, C-7), with16.5 atom % excess ^{13}C in **2** and 5.4 atom % excess ^{13}C in **1a**. The fermentation of *S. parvulus* also yielded manumycin B (**1b**) which was also enriched (5.7 atom % excess ^{13}C) at C-7. This result demonstrates the intact incorporation of **3** into the manumycins and shows that 3,4-AHBA indeed serves as the polyketide starter unit for the formation of the "lower" chain.

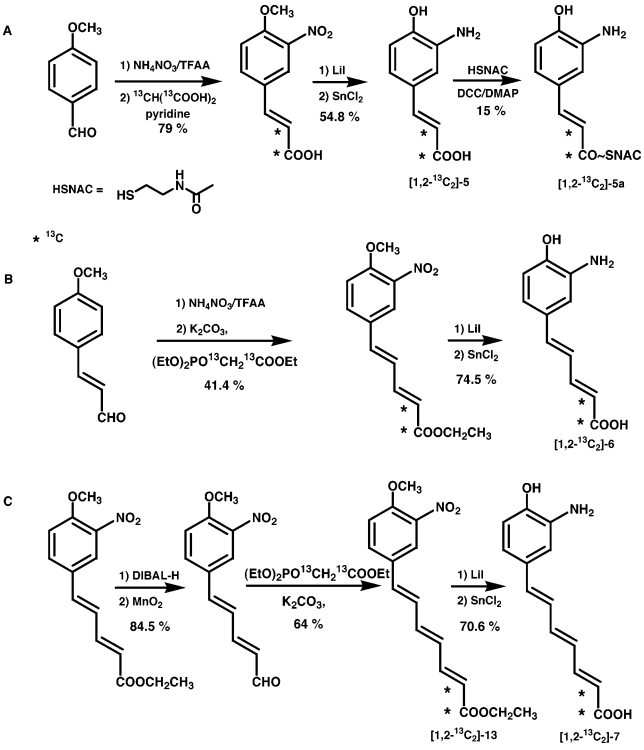
Isolation of a Shunt Metabolite. The ^{13}C NMR spectra of crude extracts from early stage (day 2 after feeding) fermentations of *S. nodosus* ssp. *asukaensis* with [7-^{13}C]-3,4-AHBA showed two enriched signals in the olefinic region, one at δ 137 ppm and the other at δ 139 ppm (in acetone-d_6). The former belongs to C-7 of **2**, but the latter represents a new compound formed from **3**. Because the chemical shift suggests that this new compound is formed from **3** by polyketide chain extension at C-7, its structure might shed light on the biosynthetic pathway. The new compound (**4a**, Scheme 1) was therefore isolated from the fermentation and purified by flash column chromatography on RP18 silica gel followed by HPLC. FAB-MS indicated a molecular weight of 273 Da; methylation with CH_2N_2 gave a dimethyl derivative **4b** of molecular formula $C_{17}H_{19}NO_4$ (by HR-MS), establishing the molecular formula of the parent compound as $C_{15}H_{15}NO_4$. The general similarity of the NMR spectra of **4a** and **4b** to those of the synthetic 7-(3-amino-4-

(12) Thiericke, R.; Zeeck, A.; Nakagawa, A.; Omura, S.; Herrold, R. E.; Wu, S. T. S.; Beale, J. M.; Floss, H. G. *J. Am. Chem. Soc.* **1990**, *112*, 3979−3987.
(13) (a) Moore, B. S.; Cho, H.; Casati, R.; Kennedy, E.; Reynolds, K. A.; Mocek, U.; Beale, J. M.; Floss, H. G. *J. Am. Chem. Soc.* **1993**, *115*, 5254−5266. (b) Moore, B. S.; Poralla, K.; Floss, H. G. *J. Am. Chem. Soc.* **1993**, *115*, 5267−5274.
(14) Cho, H.; Beale, J. M.; Graff, C.; Mocek, U.; Nakagawa, A.; Omura, S.; Floss, H. G. *J. Am. Chem. Soc.* **1993**, *115*, 12296−12304.
(15) Becker, A, M.; Rickards, R. W.; Brown, R. F. C. *Tetrahedron* **1983**, *39*, 4189−4192.
(16) (a) Kim, C.-G.; Kirschning, A.; Bergon, P.; Zhou, P.; Su, E.; Sauerbrei, B.; Ning, S.; Ahn, Y.; Breuer, M.; Leistner, E.; Floss, H. G. *J. Am. Chem. Soc.* **1996**, *118*, 7486−7491. (b) Yu, T.-W.; Müller, R.; Müller, M.; Zhang, H.; Draeger, G.; Kim, C.-G.; Leistner, E.; Floss, H. G. *J. Biol. Chem.* **2001**, *276*, 12546−12555.
(17) Thiericke, R.; Zeeck, A.; Robinson, J. A.; Beale, J. M.; Floss, H. G. *J. Chem. Soc., Chem. Commun.* **1989**, 402−403.
(18) Hu, Y.; Melville, C. R.; Gould, S. J.; Floss, H. G. *J. Am. Chem. Soc.* **1997**, *119*, 4301−4302.
(19) (a) Cone, M. C.; Melville, C. R.; Carney, J. R.; Gore, M. P.; Gould, S. J. *Tetrahedron* **1995**, *51*, 3095−3102. (b) Gould, S. J.; Melville, C.; Cone, M. C. *J. Am. Chem. Soc.* **1996**, *118*, 9228−9232.
(20) Kondo, S.; Murase, K.; Kuzuya, M. *Chem. Pharm. Bull.* **1994**, *42*, 768−773.
(21) Balcom, D.; Fürst, A. *J. Am. Chem. Soc.* **1953**, *75*, 4334−4335.

Scheme 1



Scheme 2



hydroxyphenyl)hepta-2,4,6-trienoic acid (**7**, Scheme 2), except for the presence of signals corresponding to an acetyl group and, in **4b**, two additional *O*-methyl groups, suggested that **4a** might be the *N*-acetyl derivative of **7**. This was confirmed by synthesizing an authentic sample of **4b** by acetylation of the ethyl ester of **7** followed by ester hydrolysis with LiOH and methylation with diazomethane (Scheme 1), which was in all respects identical to the material obtained from the isolated **4a**. The specific incorporation of [7-^{13}C]-**3** into **4a** was determined by mass spectrometry as 19.5%. The yield of **4a** depended very much on the culture conditions and ranged up to 4−5 mg/L.

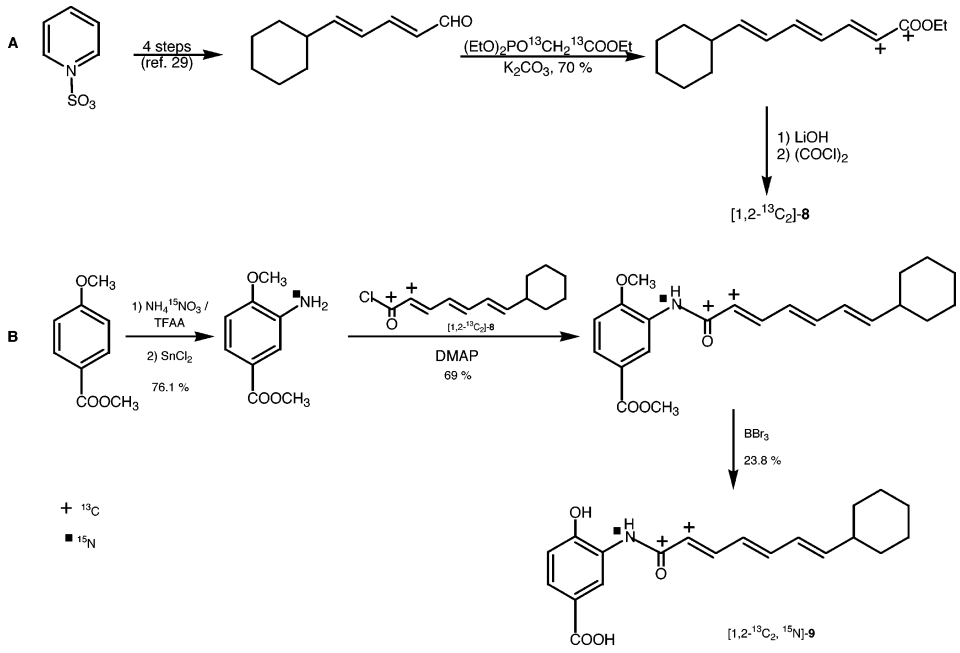
To define the role of **4a** in asukamycin biosynthesis, we prepared a sample of [1,2-$^{13}C_2$]-**4a** by acetylation of [1,2-$^{13}C_2$]-**7** (synthesis: Scheme 2) and subsequent mild base hydrolysis of the resulting diacetate. Feeding of this compound to *S. nodosus* ssp. *asukaensis* and analysis by MS and ^{13}C NMR showed no incorporation into **2**. To determine whether polyketide chain extension or *N*-acetylation occurs first in the formation of **4a**, a sample of [7-^{13}C, acetyl-$^{2}H_3$]-*N*-acetyl-3,4-AHBA was prepared by acetylation of [7-^{13}C]-**3** with [$^{2}H_3$]-acetyl chloride and hydrolysis of the *N*,*O*-diacetate to the *N*-acetate. No incorporation of either ^{13}C or deuterium into **4a** was observed upon feeding of this material to *S. nodosus* ssp. *asukaensis*. These results indicate that **4a** is a shunt metabolite of the asukamycin pathway, which cannot be channeled back into the biosynthetic manifold. The formation of **4a** may be a reflection of the fact that formation of the cyclohexanecarboxylic acid starter unit, and hence of the "upper chain", appears to be rate limiting for the biosynthesis of **2**. Supplementation experiments with *S. nodosus* ssp. *asukaensis* showed that whereas feeding of 0.65 mM 3,4-AHBA increased the ratio of **4a** to **2** by a modest 70%, feeding of 4 mM cyclohexanecarboxylic acid increased the ratio of **2**:**4a** 10-fold, that is, almost completely suppressed **4a** formation.

Synthesis of Potential Biosynthetic Intermediates. The architecture of the manumycins embodies several different building blocks. It was logical to expect that these are synthesized separately from their respective precursors and are then assembled into the complete molecular framework. To try to elucidate in which order these building blocks are assembled, we synthesized, in labeled form, the various components and partial assemblies of components and determined which ones are incorporated into the final product, asukamycin. In choosing the targets, we made the assumption, borne out by the subsequent experiments, that the modification of the aromatic ring to the epoxyquinol structure is probably a late step in the biosynthesis. Consequently, we targeted the synthesis of 3,4-AHBA chain-extended by one (**5**), two (**6**), and three (**7**) acetate units, 3,4-AHBA chain-extended to carry the complete "lower" chain including the C_5N unit (**10**), the complete "upper" chain (**8**), 3,4-AHBA *N*-acylated with the complete "upper" chain (**9**), 3,4-AHBA with both the "upper" and the "lower" chain, but without the C_5N unit (**11**), and protoasukamycin (**12**), which consists of the entire framework of asukamycin but lacks the modification of the aromatic ring to the epoxyquinol structure.

[1,2-$^{13}C_2$]-**5** was synthesized in 43.3% overall yield from 4-methoxybenzaldehyde by nitration with NH_4NO_3/trifluoroacetic anhydride (TFAA)[22] to 3-nitro-4-methoxybenzaldehyde, followed by a Knoevenagel condensation with [U-$^{13}C_3$]malonic acid,[23] demethylation with LiI,[24]and reduction of the nitro group with $SnCl_2$[25] (Scheme 2A). Preparation of the corresponding *N*-acetylcysteamine (SNAC) thioester **5a** proved problematic because of interference of the phenolic hydroxy group and failure of common protecting groups (e.g., TBDMS, Boc), but was finally accomplished in poor yield (15%) by direct esterification of **5** with *N*-acetylcysteamine catalyzed by DCC and DMAP.[26] The 5-(3-amino-4-hydroxyphenyl)-(2*E*,4*E*)-penta-2,4-dienoic acid **6** was synthesized analogously from 4-methoxycinnamaldehyde, but using a Wadsworth−Emmons reaction with triethyl phosphonoacetate[27] for the two-carbon chain extension. For the preparation of [1,2-$^{13}C_2$]-**6**, triethyl phosphono-[1,2-$^{13}C_2$]acetate was used in this step. The subsequent cleavage of the methyl ether with LiI also removed the ethyl ester function and, after $SnCl_2$ reduction of the nitro group, gave **6** in 40.9% overall yield (Scheme 2B). For the synthesis of 7-(3-amino-4-hydroxyphenyl)-(2*E*,4*E*,6*E*)-hepta-2,4,6-trienoic acid **7**, a second

(22) Crivello, J. V. *J. Org. Chem.* **1981**, *46*, 3056−3060.
(23) Ressler, C.; Goodman, F. J.; Tsutsui, R.; Tsutsumi, M. *J. Org. Chem.* **1979**, *44*, 2027−2029.
(24) Harrison, I. T. *J. Chem. Soc., Chem. Commun.* **1969**, 616.
(25) Bellamy, F. D.; Ou, K. *Tetrahedron Lett.* **1984**, *25*, 839−842
(26) Witter, D. J.; Vederas, J. C. *J. Org. Chem.* **1996**, *61*, 2613−2623
(27) (a) Williams, D. R.; White, F. H. *J. Org. Chem.* **1987**, *52*, 5067−5079. (b) Seguineau, P.; Villieras, J. *Tetrahedron Lett.* **1988**, *29*, 477−480.

Scheme 3



chain extension with triethyl phosphonoacetate was carried out on 5-(3-nitro-4-methoxyphenyl)-(2*E*,4*E*)-penta-2,4-dienal, which was obtained from ethyl 5-(3-nitro-4-methoxyphenyl)-(2*E*,4*E*)-penta-2,4-dienoate, the intermediate in the synthesis of **6**, by DIBAL reduction and MnO_2 oxidation[28] of the resulting alcohol. Following dealkylation with LiI and reduction with $SnCl_2$, the overall yield of **7** starting from 3-nitro-4-methoxycinnamaldehyde was 33.2%. [1,2-$^{13}C_2$]-**7** was prepared by using triethyl phosphono-[1,2-$^{13}C_2$]acetate in the second Wadsworth−Emmons reaction (Scheme 2C). All attempts to prepare the SNAC thioesters of **6** or **7**, either by direct esterification or by use of protecting groups, were unsuccessful.

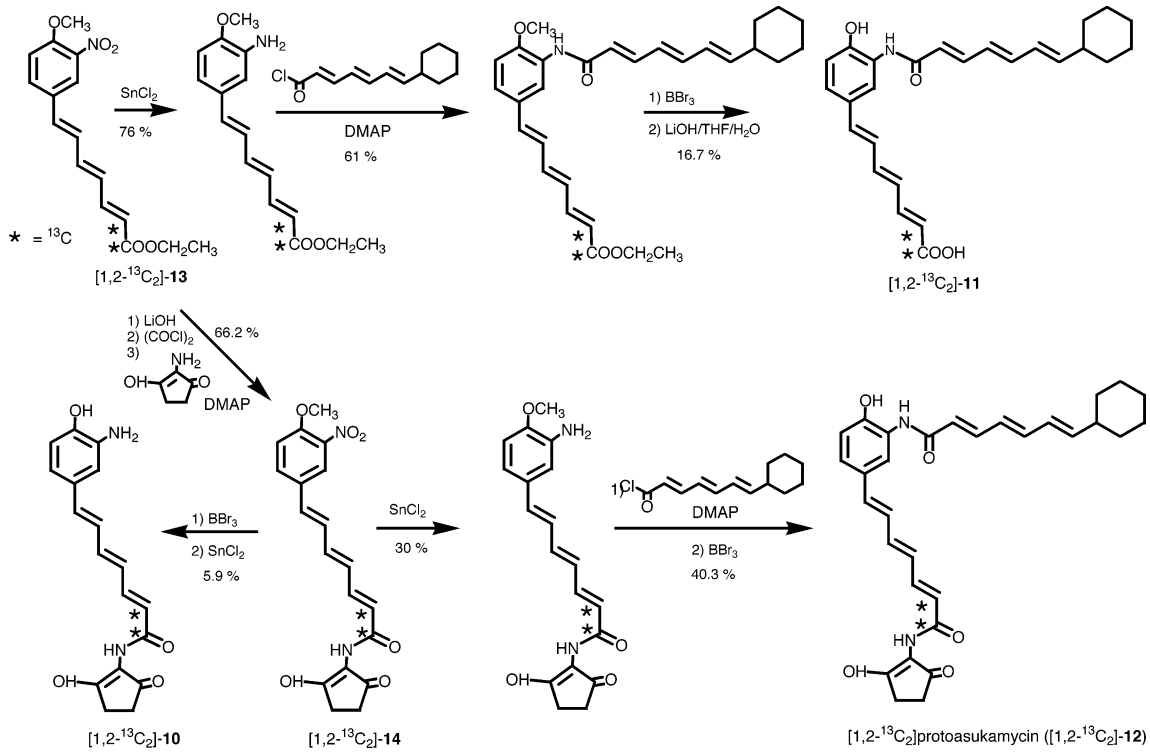
The synthesis of the "upper" chain, 7-cyclohexyl-(2*E*,4*E*,6*E*)-hepta-2,4,6-trienoic acid **8**, proceeded from pyridinium-1-sulfonate via glutaconaldehyde using chemistry developed by the Taylor and Wipf groups.[29] Reaction of the TBDMS-protected enol of glutaconaldehyde with cyclohexylmagnesium bromide and acid hydrolysis gave 5-cyclohexylpenta-2,4-dienal (32.4% from pyridinium-1-sulfonate), which was subjected to Wadsworth−Emmons chain extension with triethyl phosphonoacetate followed by ester hydrolysis with LiOH to give **8** (22.5% from dienal). Again, [1,2-$^{13}C_2$]-**8** was prepared by using triethyl phosphono-[1,2-$^{13}C_2$]acetate in the Wadsworth−Emmons reaction (Scheme 3A). A third building block, 2-amino-3-hydroxycyclopent-2-enone, was prepared from cyclopentane-1,3-dione as described by Ebenezer.[30]

The advanced potential precursors **9**−**12** were prepared from **7** and/or **8** or intermediates from their synthesis. For the synthesis of [1,2-$^{13}C_2$,^{15}N]-**9**, methyl 4-methoxybenzoate was nitrated with $NH_4{}^{15}NO_3$/TFAA and the nitro group was reduced with $SnCl_2$ to give methyl 4-methoxy-3-[^{15}N]aminobenzoate. The latter was coupled with 7-cyclohexyl-(2*E*,4*E*,6*E*)-[1,2-$^{13}C_2$]-hepta-2,4,6-trienoyl chloride, obtained from [1,2-$^{13}C_2$]-**8** with oxalyl chloride. Initial demethylation with BBr_3, surprisingly, removed only the ester function, but a second BBr_3 treatment of the methyl ether gave 3-{7-cyclohexyl-(2*E*,4*E*,6*E*)-[1,2-$^{13}C_2$]-hepta-2,4,6-trienoyl}-[^{15}N]amino-4-hydroxybenzoic acid **9** in a modest 12.5% overall yield (Scheme 3B). Labeled **10**, **11**, and **12** were all prepared from ethyl 7-(4-methoxy-3-nitrophenyl)-(2*E*,4*E*,6*E*)-[1,2-$^{13}C_2$]hepta-2,4,6-trienoate ([1,2-$^{13}C_2$]-**13**), the intermediate in the synthesis of [1,2-$^{13}C_2$]-**7 (**Scheme 2C), using more or less the same standard reactions but in different orders, as shown in Scheme 4. Thus, ester hydrolysis of **13**, conversion to the acid chloride with oxalyl chloride, and condensation with 2-amino-3-hydroxycyclopent-2-enone hydrochloride gave N_1-(2-hydroxy-5-oxo-cyclopent-1-enyl)-7-(4-methoxy-3-nitrophenyl)-(2*E*,4*E*,6*E*)-[1,2-$^{13}C_2$]hepta-2,4,6-trienamide ([1,2-$^{13}C_2$]-**14**) in 66% yield. Demethylation of **14** with BBr_3 and nitro group reduction with $SnCl_2$ proceeded in very poor yield (6%) to give [1,2-$^{13}C_2$]-**10**. On the other hand, when the nitro group of **14** was reduced with $SnCl_2$ first, and then the product was coupled with 7-cyclohexyl-(2*E*,4*E*,6*E*)-hepta-2,4,6-trienoyl chloride and demethylated with BBr_3, [1,2-$^{13}C_2$]protoasukamycin ([1,2-$^{13}C_2$]-**12**) was obtained in 12% yield from **14**. Similarly, $SnCl_2$ reduction of [1,2-$^{13}C_2$]-**13**, coupling with 7-cyclohexyl-(2*E*,4*E*,6*E*)-hepta-2,4,6-trienoyl chloride, followed by demethylation with BBr_3 and ester hydrolysis with LiOH gave [1,2-$^{13}C_2$]-**11** in 8% yield. The last two steps in particular accounted for the low overall yield.

Feeding of Potential Biosynthetic Intermediates. The labeled compounds **5** and its SNAC derivative **5a**, **6**, **7**, and **9**−**12** were each fed to a 100 mL (**5**, **5a**, **6**, **7**, **9**) or 60 mL (**10**−**12**) fermentation culture of *S. nodosus* ssp. *asukaensis* in 500 or 250 mL baffled Erlenmeyer flasks 24 h after inoculation. After incubation for 48 h with shaking, the cultures were harvested, and asukamycin and any other relevant compounds, such as **4a** or metabolites of the administered precursors, were extracted from the culture broth and from the mycelium and purified by flash column chromatography and reverse-phase

(28) (a) Rao, A. V. R.; Reddy, S. P.; Reddy, E. R. *J. Org. Chem.* **1986**, *51*, 4158−4159. (b) Hoeger, C. A.; Johnston, A. D.; Okamura, W. H. *J. Am. Chem. Soc.* **1987**, *109*, 4690−4698.
(29) (a) Lewis, N.; McKen, P. W.; Taylor, R. J. K. *Synlett* **1991**, 898−900. (b) Wipf, P.; Coish, D. G. *Tetrahedron Lett.* **1997**, *38*, 5073−5076.
(30) Ebenezer, W. *Synth. Commun.* **1991**, *21*, 351−358.

Scheme 4



HPLC. The degree of isotope enrichment was measured by electrospray mass spectrometry (ES-MS), using selective ion monitoring, and the location of the isotope was determined by ^{13}C NMR spectroscopy. In the feeding experiment with [1,2-$^{13}C_2$,^{15}N]-**9**, no production of either asukamycin or the shunt metabolite **4a** was observed. Feeding of [1,2-$^{13}C_2$]-**5**, [1,2-$^{13}C_2$]-**5a**, [1,2-$^{13}C_2$]-**6**, or [1,2-$^{13}C_2$]-**7** allowed the production of **2** at normal yields, but no incorporation of isotope into the product was detected. Likewise, no incorporation of ^{13}C from [1,2-$^{13}C_2$]-**10** and [1,2-$^{13}C_2$]-**11** into **2** was seen, and neither compound underwent modification of the aromatic ring to the epoxyquinol structure. However, the amount of **2** produced in the fermentation with [1,2-$^{13}C_2$]-**11** was greatly reduced relative to that of unfed cultures. In contrast, feeding of [1,2-$^{13}C_2$]-**12** resulted in an unambiguous incorporation of ^{13}C into **2**, with ^{13}C NMR showing labeling of the expected positions C-12 and C-13 (δ 122.9 and 167.1 ppm, d, J = 65.9 Hz) and coupling between the two labeled carbon atoms. The specific incorporation was determined by ES-MS as 30.5%, showing that protoasukamycin is a very efficient biosynthetic precursor of asukamycin.

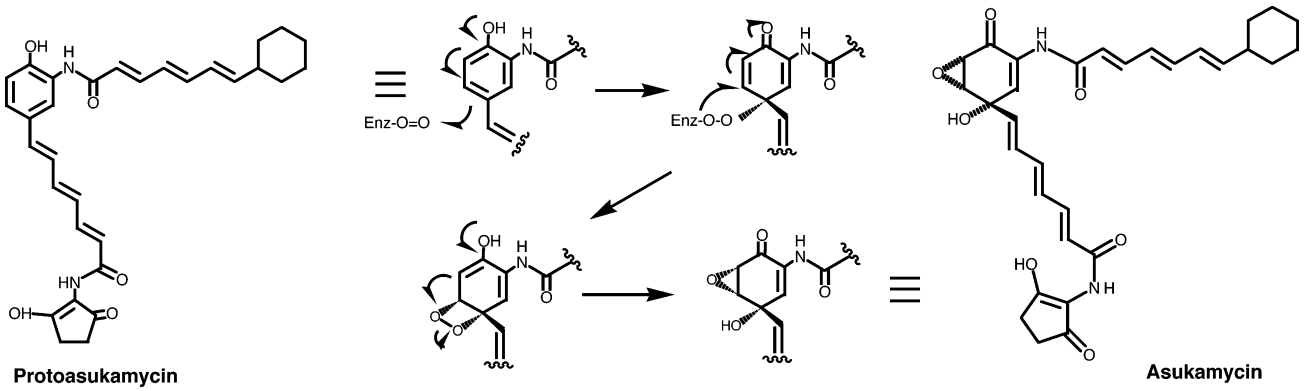
Discussion

Previous work[12] had shown that the mC_7N unit of the manumycins is not derived from 3-amino-5-hydroxybenzoic acid, as is that of the ansamycin and mitomycin antibiotics.[31] Instead, the feeding experiments presented here and elsewhere[18] show that it comes from a very similar compound, the regioisomeric 3-amino-4-hydroxybenzoic acid (3,4-AHBA). 3,4-AHBA, following conversion either to the CoA thioester or as the free acid which is activated on the loading domain of a polyketide synthase,[32,33] evidently serves as the starter unit for the assembly of the lower polyketide chain. However, the mode of its formation from the established building blocks, a glycerol-derived three-carbon fragment and a four-carbon dicarboxylic acid, is still unclear. A plausible pathway has been proposed by Gould et al.,[19b] but conclusive evidence to support or disprove this hypothetical sequence of reactions is still lacking.

We had assumed as our working hypothesis that the modification of the aromatic ring to the epoxyquinol structure of the mC_7N unit would be a late step in the biosynthesis of the manumycins. We therefore synthesized for the first time a putative late intermediate in asukamycin biosynthesis, protoasukamycin (**12**), which carries the unmodified aromatic ring in place of the epoxyquinol structure of **2**. The efficient and specific incorporation of this compound into **2** leaves no doubt that the entire molecular framework of the manumycins is assembled first before the aromatic ring is modified to the final epoxyquinol structure of the type I[34] manumycins. The latter transformation is presumably catalyzed by a dioxygenase, and a likely mechanism[36] is shown in Scheme 5. Such a mechanism has been demonstrated for the oxidation of dihydrovitamin K[37−39] and for the oxidation of dihydroxyacetanilid in the formation of antibiotics LL-C10037 and MPP3051.[40] Consistent with this mechanism, both the epoxide and the hydroxy oxygen

(31) Floss, H. G. *Nat. Prod. Rep.* **1997**, *14*, 433−452.
(32) Admiraal, S. J.; Walsh, C. T.; Khosla, C. *Biochemistry* **2001**, *40*, 6116−6123.
(33) Lowden, P. A. S.; Wilkinson, B.; Böhm, G. A.; Handa, S.; Floss, H. G.; Leadlay, P. F.; Staunton, J. *Angew. Chem., Int. Ed.* **2001**, *40*, 777−779.
(34) The type I manumycins with the epoxyquinol moiety in turn are the precursors of the type II manumycins which carry a diol structure instead and are derived from the former by a transformation late in the fermentation.[35]
(35) Hu, Y.; Floss, H. G. *J. Antibiot.* **2001**, *54*, 340−348.
(36) Ham, S. W.; Dowd, P. *J. Am. Chem. Soc.* **1990**, *112*, 1660−1661.
(37) Kuliopulos, A.; Hubbard, B. R.; Lam, Z.; Koski, I. J.; Furie, B.; Furie, B. C.; Walsh, C. T. *Biochemistry* **1992**, *31*, 7722−7728.
(38) Naganathan, S.; Hershline, R.; Ham, S. W.; Dowd, P. *J. Am. Chem. Soc.* **1994**, *116*, 9831−9839.
(39) Dowd, P.; Hershline, R.; Ham, S. W.; Naganathan, S. *Science* **1995**, *269*, 1684−1691.
(40) Gould, S. J.; Kirchmeier, M. J.; LaFever, R. E. *J. Am. Chem. Soc.* **1996**, *118*, 7663−7666.

Scheme 5



of the epoxyquinol are derived from molecular oxygen,[17] and these two oxygens in all naturally occurring manumycins are syn oriented.[1,4] The availability of protoasukamycin as substrate opens the way for the discovery and characterization of the enzyme(s) catalyzing this interesting reaction.

Surprisingly, none of the potential intermediates in the assembly of protoasukamycin from 3,4-AHBA tested were incorporated into **2**. The nonincorporation of the two partially chain-extended versions of 3,4-AHBA, the carboxylic acids 5 and 6, which should be enzyme-bound intermediates in the assembly of the "lower" polyketide chain, is not unexpected. In general, the free acids corresponding to such intermediates cannot be activated and can therefore not be loaded onto the PKS. More surprising, however, is the failure of compound **5a**, the SNAC thioester derivative of the diketide **5**, to label **2**. It has been shown in a number of systems that polyketide synthases, particularly modular type I enzymes, can accept intermediates presented to them in the form of their SNAC thioesters, load them onto the cognitive module, and process them through the remainder of the assembly steps to generate the expected endproducts.[26,41] Possibly, this paradigm does not hold for the enzyme assembling the asukamycin "lower" chain, which therefore cannot utilize compound **5a** in this way. The nonincorporation into **2** of [1,2-$^{13}C_2$]-**7**, representing the fully assembled "lower" chain polyketide, suggests that **7** is not released from the PKS as the free acid. It may be released as an activated species, such as the CoA thioester, or may be transferred directly from the PKS to the nitrogen of the C_5N unit. Yet, the product of this reaction, compound **10** representing the starter unit with the entire "lower" chain, is also not incorporated. Another possible explanation for nonincorporation of **5**, **5a**, **6**, **7**, and **10** would be a scenario in which 3,4-AHBA is first acylated on the nitrogen by the complete "upper" chain to give compound **9** before the "lower" chain is built up. ^{15}N- and ^{13}C-double labeled **9** was, however, not converted into **2**, ruling out this version of a biosynthetic pathway. Interestingly, this compound completely shuts off the production of both **2** and the shunt metabolite **4a** in the fermentation. Therefore, **9**, based on its similarity to an intermediate in the biosynthesis of **2**, probably acts as an inhibitor of one of the biosynthetic enzymes. As another scenario that would explain the nonincorporation of **7** as well as **10**, the PKS-bound compound **7** may have to be acylated by the complete "upper" chain before it can be released from the enzyme and coupled to the C_5N unit. However, if this is the case, the product cannot be released from the enzyme as the free acid, because compound **11**, representing protoasukamycin without the C_5N unit, is also not incorporated into **2**.

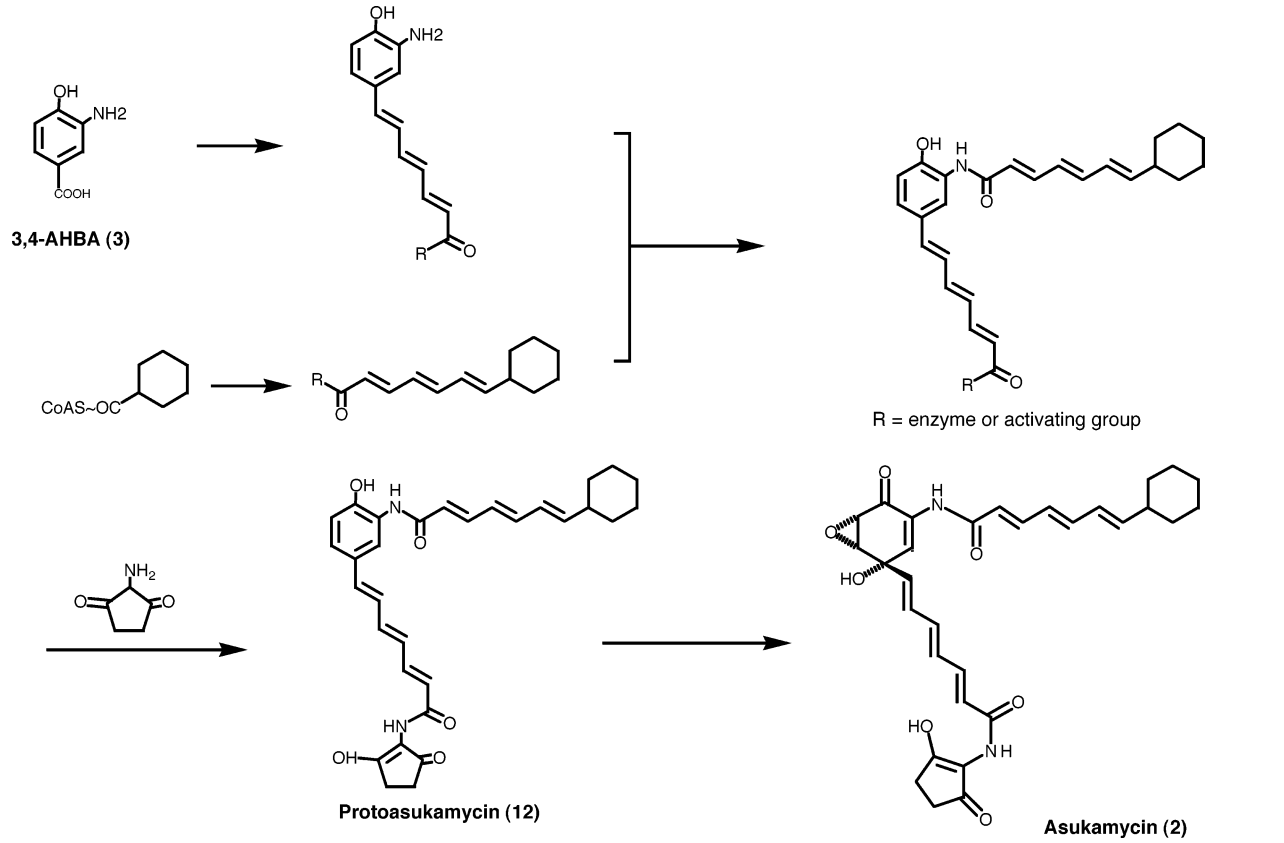
Negative results from feeding experiments always have to be interpreted with caution, as permeability barriers may prevent the administered labeled precursors from entering the cells and reaching the site of synthesis. In the present case, the efficient incorporation of **12**, a molecule similar in structure to and larger in size than the other compounds fed, argues against the possibility that the nonincorporation of the potential intermediates fed is due to permeability problems. However, it must be kept in mind that **12** is the only molecule among the compounds fed that does not contain an easily ionizable (at neutral pH) group. Nevertheless, the results most likely indicate that the assembly of **12** from 3,4-AHBA involves no free, unactivated intermediates. It may involve some free activated intermediates, such as CoA thioesters of the acids **7** and/or **8**, but it is also possible that all intermediates between the starter unit, 3,4-AHBA, and the first identified product, protoasukamycin, remain enzyme-bound during the entire assembly process.

The above results leave open the question in which order the different building blocks of **2** are attached to each other. Because compound **9** is not incorporated, attachment of the "upper" chain to the nitrogen of the mC_7N unit is probably not the first step. It follows that the assembly of the "lower" chain must be the first reaction which 3,4-AHBA undergoes. However, because neither **10** nor **11** are incorporated, it is not clear whether the C_5N unit or the "upper" chain is attached first to an activated version of **7**. Several lines of evidence, however, point toward the latter scenario, that is, transfer of the "upper" chain to the nitrogen of an activated **7** before formation of the amide bond to the aminocyclopentenolone moiety. (i) The fact that **9** and **11**, but not **10**, inhibit asukamycin formation suggests that **9** and **11**, but not **10**, resemble intermediates in the biosynthesis and thus act as competitive inhibitors. (ii) The shunt metabolite **4a** must arise by acetylation of free, activated, or enzyme-bound **7**. The *N*-acetylation of arylamines is a commonly observed reaction in Actinomycetes.[42] The fact that no formation of an acetylated shunt metabolite equivalent to **10**

(41) (a) Yue, S.; Duncan, J. S.; Yamamoto, Y.; Hutchinson, C. R. *J. Am. Chem. Soc.* **1987**, *109*, 1253−1255. (b) Cane, D. E.; Yang, C. *J. Am. Chem. Soc.* **1987**, *109*, 1255−1257. (c) Cane, D. E.; Lambalot, R. H.; Prabhakaran, P. C.; Ott, W. R. *J. Am. Chem. Soc.* **1993**, *115*, 522−526. (d) Cane, D. E.; Tan, W.; Ott, W. R. *J. Am. Chem. Soc.* **1993**, *115*, 527−535. (e) Tsantrizos, Y. S.; Zhou, F.; Famili, P.; Yang, X. *J. Org. Chem.* **1995**, *60*, 6922−6929.

(42) Yu, T.-W.; Shen, Y.; Doi-Katayama, Y.; Tang, L.; Park, C.; Moore, B. S.; Hutchinson, C. R.; Floss, H. G. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 9051−9056.

Scheme 6



was observed suggests that acylation of the arylamine function with the "upper" chain occurs before attachment of the C_5N unit. In the absence of a sufficient supply of the limiting "upper" chain precursor, the biosynthesis stalls, leading to aberrant release and acetylation of **7**. On the basis of these considerations, we propose a sequence for the assembly of the components of protasukamycin as shown in Scheme 6, involving first chain extension of 3,4-AHBA to the activated "lower" polyketide chain, in parallel chain extension of cyclohexanecarboxylic to the activated "upper" chain, followed by transfer of the "upper" chain to the arylamine nitrogen, and finally amide bond formation between the activated carboxyl group of the resulting assembly and the nitrogen of the aminocyclopentenolone moiety to give protoasukamycin. The genetic analysis of the asukamycin biosynthetic gene cluster, currently underway in this and a collaborating laboratory, will hopefully show whether this proposed sequence and other aspects of the biosynthesis of **2** deduced from isotopic tracer experiments are indeed valid.

Experimental Section

Materials and General Methods. *Streptomyces nodosus* ssp. *asukaensis* ATCC 29757 was obtained from the American Type Culture Collection, and *Streptomyces parvulus* Tü 64 was obtained from Professor Axel Zeeck, University of Göttingen, Germany. Fermentation ingredients were purchased from Difco and Sigma, chemicals were from Aldrich and Lancaster, and nitrogen dioxide was from Sigma. 4-Hydroxy-[7-^{13}C]benzoic acid was purchased from Cambridge Isotope Laboratories, triethyl phosphono-[1,2-$^{13}C_2$]acetate was from Sigma, and [U-$^{13}C_3$]malonic acid had been synthesized previously in this laboratory. All chemicals and solvents were of reagent or HPLC grade and were used without further purification unless otherwise noted.

NMR spectra were obtained on Bruker AF 300 and AM 500 spectrometers. Electron-impact mass spectrometry (EI-MS) was carried out on a Kratos Profile HV-3 mass spectrometer, and electrospray mass spectra (ES-MS) and tandem mass spectra (ES-MS/MS) were recorded on a Bruker Esquire ion trap mass spectrometer. High-resolution mass spectra (HR-MS) were obtained on a Micromass 70SEQ tandem hydrid mass spectrometer. The isotope distributions in **2**, **1a**, **1b**, and **4a** were determined by selected ion monitoring (SIM) on a Micromass Quattro II tandem quadrupole mass spectrometer. Fermentations were carried out in a New Brunswick G25 controlled environment incubator shaker or in an Adolf Kühner ISF-4-V rotary shaker cabinet. Analytical TLC was performed on precoated silica gel plates (aluminum backing, 0.25 mm layer, UV-254 fluorescence), and preparative TLC was performed on precoated silica gel plates (glass backing, 2.0 mm layer, UV-254 fluorescence), both from EM Science. Flash column chromatography was performed on 230−400 mesh silica gel from Aldrich, and reverse-phase chromatography was performed on C_{18} silica gel from Whatman. HPLC was conducted with a Beckman model 116 isocratic pump and a model 166 absorbance detector using C_{18} reverse-phase analytical or semipreparative columns.

Feeding Experiments. *Streptomyces nodosus* ssp. *asukaensis* was grown on yeast extract-malt (YM) agar plates at 28 °C for 4 days, and was then stored at 0 °C. A loop of mycelium was transferred into 100 mL of culture medium in a 500 mL baffled Erlenmeyer flask and was grown on a rotary shaker at 300 rpm for 2 days at 28 °C (seed culture). Ten (or six) milliliters of seed culture was used to inoculate each 100 (or 60) mL of culture medium in 500 (or 250) mL baffled Erlenmeyer flasks, which were incubated on a rotary shaker at 300 rpm for 3 days at 28 °C. Both culture media consisted of glucose, 20 g; Bacto Peptone, 5 g; K_2HPO_4, 0.25 g; $MgSO_4$, 0.25 g; trace elements $(NH_4)_6Mo_7O_{24} \times 4H_2O$, 5 mg; $FeSO_4 \times 7H_2O$, 50 mg; $CuSO_4 \times 5H_2O$, 5 mg; $ZnSO_4 \times 7H_2O$, 5 mg; $MnCl_2 \times 4H_2O$, 10 mg; deionized water, 1000 mL; pH

7.0, and were sterilized for 20 min at 121 °C in an autoclave. Fermentations with *S. parvulus* Tü 64 were carried out as described earlier.[12]

Labeled compounds were administered to the production cultures 24 h after inoculation as single doses of filter-sterilized solutions in the amounts indicated per culture volume: 3,4-[7-^{13}C]-AHBA ([7-^{13}C]-**3**) dissolved in 5% K_2CO_3, 10 mg/100 mL (0.649 mM); 3-(7-cyclohexyl-(2*E*,4*E*,6*E*)-[1,2-$^{13}C_2$]hepta-2,4,6-trienoyl)-[^{15}N]amino-4-methoxybenzoic acid ([1,2-$^{13}C_2$,^{15}N]-**9**) dissolved in 5% K_2CO_3, 12 mg/100 mL (0.348 mM); 3-(3-amino-4-hydroxyphenyl)-(*E*)-[1,2-$^{13}C_2$]prop-2-enoic acid ([1,2-$^{13}C_2$]-**5**) dissolved in 5% K_2CO_3, 15 mg/100 mL (0.829 mM); 5-(3-amino-4-hydroxyphenyl)-(2*E*,4*E*)-[1,2-$^{13}C_2$]penta-2,4-dienoic acid ([1,2-$^{13}C_2$]-**6**) dissolved in 5% K_2CO_3, 15 mg/100 mL (0.725 mM); 7-(3-amino-4-hydroxyphenyl)-(2*E*,4*E*,6*E*)-[1,2-$^{13}C_2$]hepta-2,4,6-trienoic acid ([1,2-$^{13}C_2$]-**7**) dissolved in 5% K_2CO_3, 20 mg/100 mL (0.858 mM); 3-(3-amino-4-hydroxyphenyl)-(*E*)-[1,2-$^{13}C_2$]prop-2-enoic acid *N*-acetylcysteamine thioester ([1,2-$^{13}C_2$]-**5a**) dissolved in DMSO, 20 mg/100 mL (0.707 mM); N_1-(2-hydroxy-5-oxocyclopent-1-enyl)-7-(3-amino-4-hydroxyphenyl)-(2*E*,4*E*,6*E*)-[1,2-$^{13}C_2$]hepta-2,4,6-trienamide ([1,2-$^{13}C_2$]-**10**) dissolved in 5% K_2CO_3, 7 mg/60 mL (0.356 mM); 7-[3-{7-cyclohexyl-(2*E*,4*E*,6*E*)-hepta-2,4,6-trienoyl}-amino-4-hydroxyphenyl]-(2*E*,4*E*,6*E*)-[1,2-$^{13}C_2$]hepta-2,4,6-trienoic acid (**11**) dissolved in 5% K_2CO_3, 9 mg/60 mL (0.356 mM); proto-asukamycin ([1,2-$^{13}C_2$]-**12**) dissolved in 5% K_2CO_3, 11 mg/60 mL (0.356 mM).

After fermentation for 72 h, cultures were centrifuged at 9000 rpm for 25 min. The supernatant was saturated with NaCl and extracted three times with ethyl acetate. The mycelium was extracted with acetone, and the acetone extracts were concentrated. The residue was extracted with ethyl acetate. The combined ethyl acetate extracts were dried, and the solvent was evaporated in vacuo. The crude product was purified on 2.0 mm preparative TLC plates developed three times with chloroform/methanol 9:1, or on a silica gel column eluting with methylene chloride/methanol 100:3. The asukamycin was further purified by semipreparative C_{18} reverse-phase HPLC eluting with methanol/water or acetonitrile/water. Yields of asukamycins averaged about 20−25 mg/L.

Isolation and Identification of Shunt Metabolite 4a. *S. nodosus* ssp. *asukaensis* cultures fed with [7-^{13}C]-**3** were harvested after 2 days of fermentation. The supernatant extracts were fractionated on an RP18 silica gel column eluted with acetone/water 20:80, and the compound responsible for an enriched NMR signal at 137 ppm was further purified on HPLC with water/2-propanol 72.5/27.5 as eluent. The structure of this unknown compound was determined as 7-(3-*N*-acetylamino-4-hydroxyphenyl)-(2*E*,4*E*,6*E*)-hepta-2,4,6-trienoic acid (**4a**) from its ^{1}H, ^{13}C NMR and FAB-MS spectra. The compound was methylated with diazomethane, and the molecular formula of the resulting derivative (**4b**) was determined by HR-MS. NMR comparison of the derivative to an authentic sample prepared from synthetic **13** showed them to be identical. **4a**: R_f 0.21 (silica gel, CH_2Cl_2/MeOH 10:1); UV absorption maximum 368 nm (MeOH); ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 7.90 (d, 1H, J = 2.0 Hz, H-2′), 7.23 (dd, 1H, J = 15.1, 11.2 Hz, H-3), 7.12 (dd, 1H, J = 8.3, 2.0 Hz, H-6′), 6.84 (d, 1H, J = 8.3 Hz, H-5′), 6.80−6.68 (m, 3H, H-5, H-6, H-7), 6.49 (dd, 1H, J = 14.2, 11.2 Hz, H-4), 5.86 (d, 1H, J = 15.1 Hz, H-2), 2.09 (s, 3H, CH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C 169.0, 167.7, 148.6, 144.3, 141.2, 137.1 (enriched, C-7), 129.0, 127.6, 126.7, 126.6, 125.6, 123.6, 120.8, 115.9, 23.7; FAB-MS m/z 274 $[M + H]^+$. HR-MS for **4b**: $[M]^+$ $C_{17}H_{19}NO_4$, calculated 301.1322, found 301.1314.

Supporting Information Available: Synthesis of labeled compounds (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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