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New Type II Manumycins Produced by *Streptomyces nodosus* ssp. *asukaensis* and Their Biosynthesis

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Five new type II manumycins, containing the hydroxyquinol mC_7N unit, asukamycins A-II, B-II, C-II, D-II, E-II, were discovered in cultures of *Streptomyces nodosus* ssp. *asukaensis*. The biosynthetic origin of the type II manumycins from the type I compounds, containing an epoxyquinol mC_7N unit, was deduced from the time course of production and proven by preparing [7′-^{13}C]asukamycin A and demonstrating its incorporation into asukamycin A-II.

The antibiotics of the manumycin family,[1] exemplified by asukamycin A (**3a**)[2] are produced by a variety of Streptomycetes and are characterized by two linear polyketide chains, the "lower" chain extending from a six-membered carbocyle, the mC_7N starter unit, and terminating in an amide linkage to a 2-aminocyclopentenolone moiety, and a more variable "upper" chain amide-linked to the nitrogen of the mC_7N unit. They are defined as belonging to type I or type II based on the structure of their central mC_7N unit.[1] The compounds with an oxirane at C-5/C-6 (epoxyquinol structure) are named type I manumycins. Those with a hydroxyethylene group at C-5/C-6 (hydroxyquinol structure) are referred to as type II manumycins. So far, 17 type I manumycins have been isolated from 10 Streptomycetes and 6 type II manumycins from 3 Streptomycetes in which both type I and type II compounds are produced. In some cases, the corresponding type I and type II manumycins that have identical carbon skeletons are produced by the same strain, *e.g.*, manumycin A (type I) and D (type II) are produced by *Streptomyces parvulus* and *Streptomyces* sp. A-230,[3~5] manumycin C and TMC-1C by *Streptomyces* sp. A-230.[5] In some respects, type I and type II manumycins share the same pharmacological properties, such as activity against various cancers, including pancreatic cancer, colon cancer and ovarian cancer.[5~12] On the other hand, significant differences in biological activities have also been observed. For example, the type I manumycins have activity against various Gram-positive and some Gram-negative bacteria but the type II manumycins do not.[5] These findings revealed the biological significance of the structure of the mC_7N unit and highlight the importance of understanding the mode of formation of type II manumycins. In this paper, we report the discovery a series of new type II manumycins from the asukamycin producer, *S. nodosus* ssp. *asukaensis*, and the results of experiments which reveal their biosynthetic origin.

Results

New Type II Manumycins Discovered in *S. nodosus* ssp. *asukaensis* and Their Structure Elucidation

In our earlier study, 3-amino-4-hydroxybenzoic acid (3,4-AHBA) **1** was identified as the key precursor of the mC_7N unit in the biosynthesis of asukamycin and manumycin A[13]. In the present work, feeding of 3,4-[7-^{13}C]-AHBA[13] as a probe was therefore used to follow the course of antibiotic production and to discover new pathway-related metabolites by virtue of their ^{13}C enrichment. A series of type I manumycins, asukamycin A **3a**, B **3b**, C **3c**, D **3d** and E **3e** (identical with the known compounds asukamycin, manumycin G, U-56,407,

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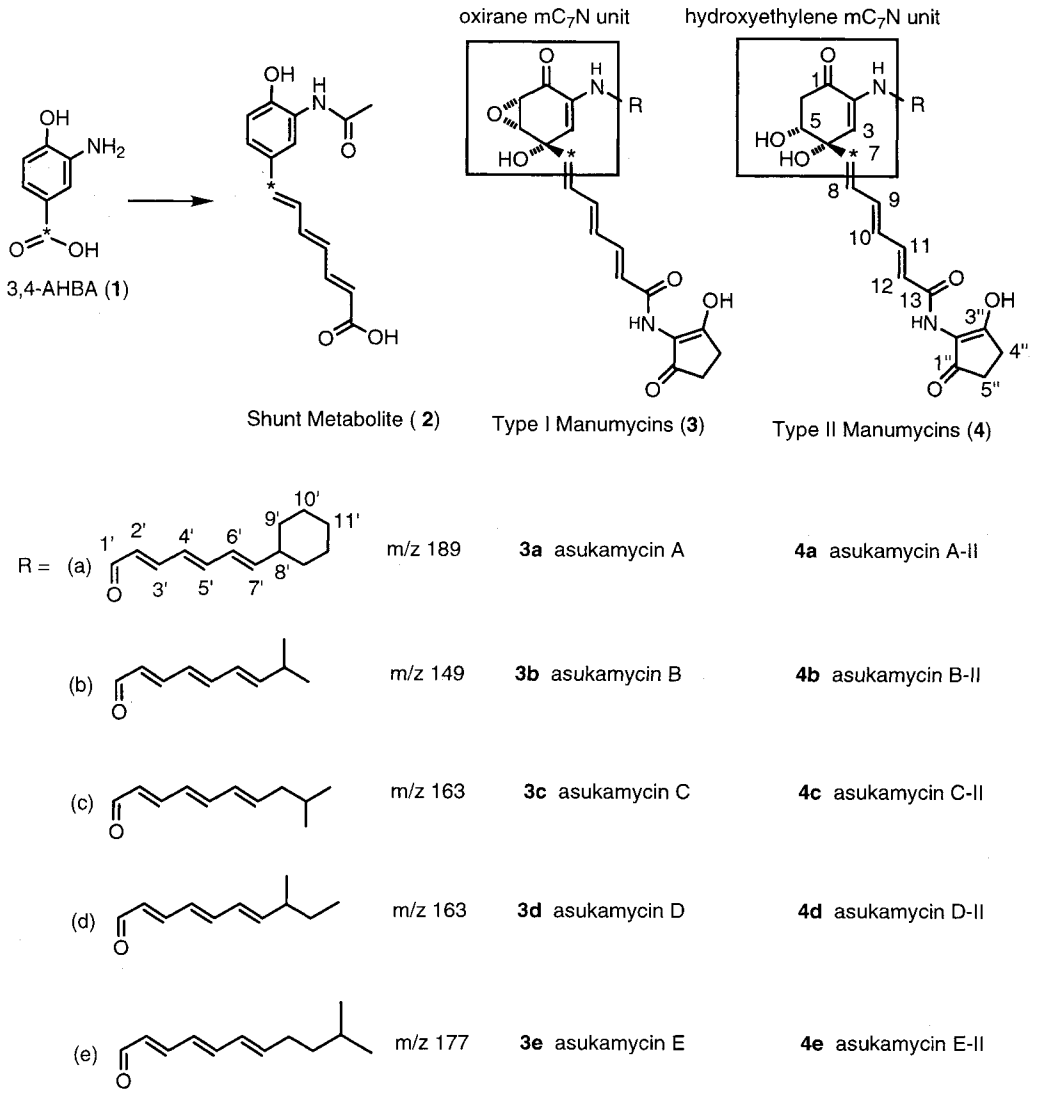
Fig. 1. Metabolites from *Streptomyces nodosus* ssp. *asukaensis*.



* Denotes the position of a biosynthetic ^{13}C label from 3,4-[7-^{13}C]-AHBA.

EI-1511-5 and manumycin E, respectively) and a shunt metabolite, 7-(3-*N*-acetylamino-4-hydroxyphenyl)-(2*E*,4*E*,6*E*)-hepta-2,4,6-trienoic acid **2**, were detected (Fig. 1).[14] Subsequently, additional new pathway-related compounds were isolated and purified as four HPLC fractions. ^{1}H-NMR, LC-MS, LC-MS/MS and HR-MS techniques were used to elucidate the structures of these new compounds.

First, the molecular mass of the new compounds was determined by LC-MS as 508 (fraction 1), 522 (fraction 2), 536 (fraction 3) and 548 (fraction 4), two mass units higher than the type I compounds **3b**, **3c** and **3d**, **3e**, and **3a**, respectively, produced by *S. nodosus* ssp. *asukaensis*. MS/MS spectra gave significant fragments of the "upper" chain at *m/z* 149, 163, 177 and 189, respectively, in fractions 1 to 4. These fragments are identical to those from the "upper" chains of asukamycins B, C and D, E, and A, respectively (Fig. 1). Compared with the type I manumycins, this new series of compounds gives relatively more intense fragments resulting from the loss of water $[M-18]^+$. This suggests that the new metabolites contain a hydroxyethylene mC_7N unit, which is more easily dehydrated than an oxirane mC_7N unit. The ^{1}H-NMR spectra of each fraction display almost identical signal

Table 1. Time course of relative production of shunt metabolite **2**, type I and type II manumycins[a].

Time (hr) [b]	Shunt Metabolite	Type I Manumycins	Type II Manumycins
24	0.13	0.87	0
30	0.20	0.80	0
48	0.15	0.77	0.08
54	0.14	0.72	0.14
66	0.19	0.68	0.13
68	0.13	0.70	0.17
72	0.22	0.60	0.18
90	0.13	0.52	0.35

a) Relative amounts were determined by integration of the enhanced ^{13}C signals after feeding [7-^{13}C]3,4-AHBA b) Hours of fermentation in production medium.

patterns in the polyene region (5.8~8.1 ppm), which integrate to thirteen protons. These signals correspond to the "upper" and "lower" polyketide chains plus H-3 from the mC_7N unit. The major differences in the ^1H-NMR spectra of these compounds compared to those of the corresponding type I manumycins are the absence of two signals at 3.8 and 3.7 ppm for the two oxirane protons, H-5 dd and H-6 d, and the appearance of one new proton signal at 4.04 ppm corresponding to H-5 in type II manumycins.[4,5] Another new signal at 2.74~2.77 ppm from the two methylene protons at C-6 is largely obscured by the water signal. The only differences among the spectra of the different compounds of this new series are found in the saturated hydrocarbon region from 0.8 to 2.4 ppm, which is attributed to the "upper" chain terminus. Analysis of this region indicates that fraction 1 contains an "upper" chain terminus with an isopropyl group, fraction 2 a mixture of an isobutyl and an anteisobutyl group, fraction 3 contains an "upper" chain terminus with two *geminal* methyl groups, and fraction 4 has a cyclohexane ring as chain terminus. Therefore, the four new metabolites were established as the new type II manumycins, asukamycin A-II **4a**, B-II **4b**, C-II **4c**, D-II **4d** and E-II **4e**, corresponding to their type I manumycin counterparts already isolated from the same fermentation (Fig. 1).

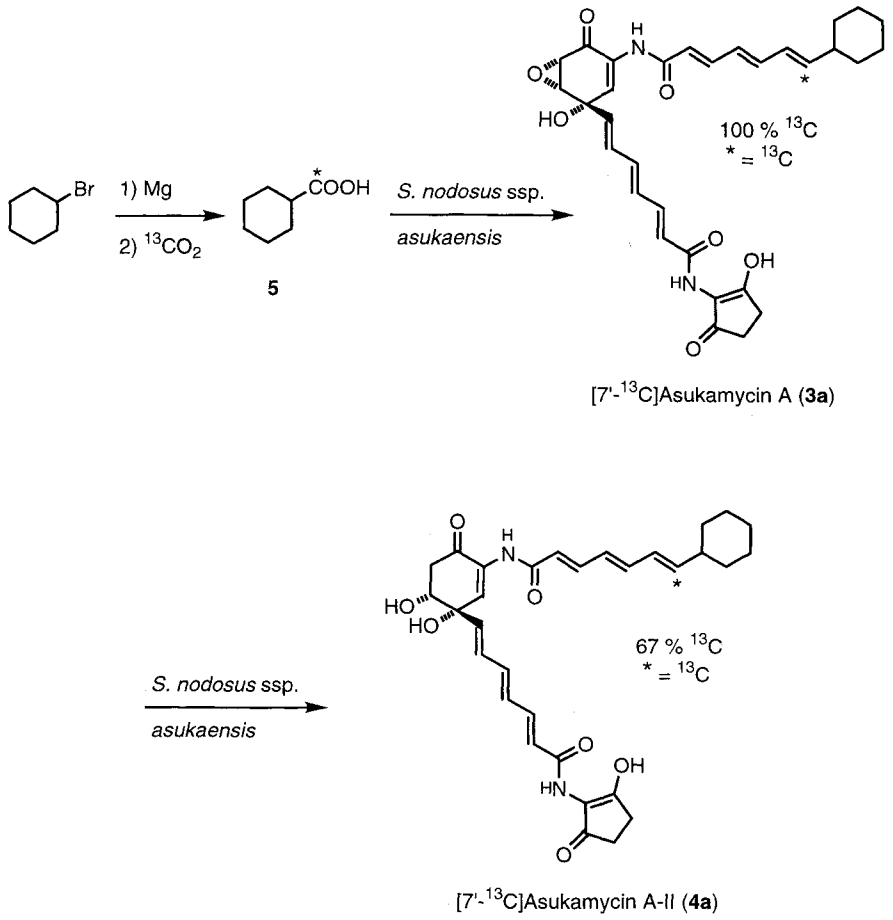
Time Course of the Relative Production of Different Fermentation Metabolites

The incorporation of 3,4-[7-^{13}C]-AHBA gave enhanced ^{13}C-NMR signals at 137 ppm, 139 ppm and 141 ppm (in acetone-d_6), corresponding to shunt metabolite **2**, the type I and the type II manumycins, respectively. The integration of these enhanced signals was used to follow the time course of the product pattern in the fermentation (Table 1). This study revealed that in the early stage of the fermentation (one to two days in production medium), only shunt metabolite **2** and type I manumycins were present. The type II manumycins were first detected in the middle stage (after two days in production medium). The levels of the type I manumycins declined and those of the type II manumycins increased with the progress of the fermentation. The late appearance of the type II manumycins suggests that the type II compounds may be formed from the type I manumycins by reduction of the epoxide group.

Preparation of [7′-^{13}C]Asukamycin A and Its Incorporation into Asukamycin A-II

To further probe whether the type I manumycins were

Fig. 2. Preparation of [7′-^{13}C]asukamycin A and its incorporation into asukamycin A-II.



indeed the biosynthetic precursors of the type II compounds, we wanted to prepare and feed a labeled sample of asukamycin A **3a**. In a parallel study[14] we had observed that the spectrum of compounds formed could be greatly influenced by feeding *S. nodosus* ssp. *asukaensis* different acids, particularly cycloalkylcarboxylic acids, as exogenous "upper" chain starter units. Consequently, [7-^{13}C]cyclohexanecarboxylic acid **5** was synthesized from cyclohexyl bromide *via* a Grignard reaction with $^{13}CO_2$ and fed to *S. nodosus* ssp. *asukaensis*. The resulting type I manumycins were isolated and shown by mass spectrometric analysis to consist of 78 % asukamycin A. Surprisingly, the asukamycin A obtained was derived from [7-^{13}C]cyclohexanecarboxylic acid without any isotopic dilution, *i.e.*, it was enriched with ^{13}C to the extent of over 99 atom%. The purified [7′-^{13}C]asukamycin A was fed to a new culture of *S. nodosus* ssp. *asukaensis* and the type II manumycins were isolated. The incorporation of [7′-^{13}C]asukamycin A into asukamycin A-II **4a** was determined by SIM-MS to be 67%, demonstrating that the type I manumycins are efficient precursors of the type II manumycins.

The Relative Production of Type I and Type II Manumycins

To determine whether there is any substrate preference in the conversion of type I into type II manumycins, we analyzed the relative production of the different type I and type II manumycins by ion-trap mass spectroscopy. The results are listed in Table 2. Asukamycin A is the major type I component making up 41% of the total. Asukamycin C+asukamycin D combined are next at 35%, followed by asukamycin B (15.8%) and E (8.8%). In contrast, for the type II manumycins, asukamycin C-II+asukamycin D-II combined are the major components at 45% of the total,

Table 2. Relative production of different type I and type II manumycins.

	Type I	Type II
Asukamycin B	15.8 %[a)]	17.1 %
Asukamycin C + D	34.7 %	44.9 %
Asukamycin E	8.8 %	4.5 %
Asukamycin A	40.7 %	33.5 %

a) Determined by ^{13}C NMR after feeding [7-^{13}C]3,4-AHBA

while asukamycin A-II is second at 33.5%, followed by asukamycin B-II (17.1%) and E-II (4.5%). The production of type I and type II manumycins is thus not strictly correlated but rather, some substrate selectivity in the conversion of type I into the type II compounds is evident.

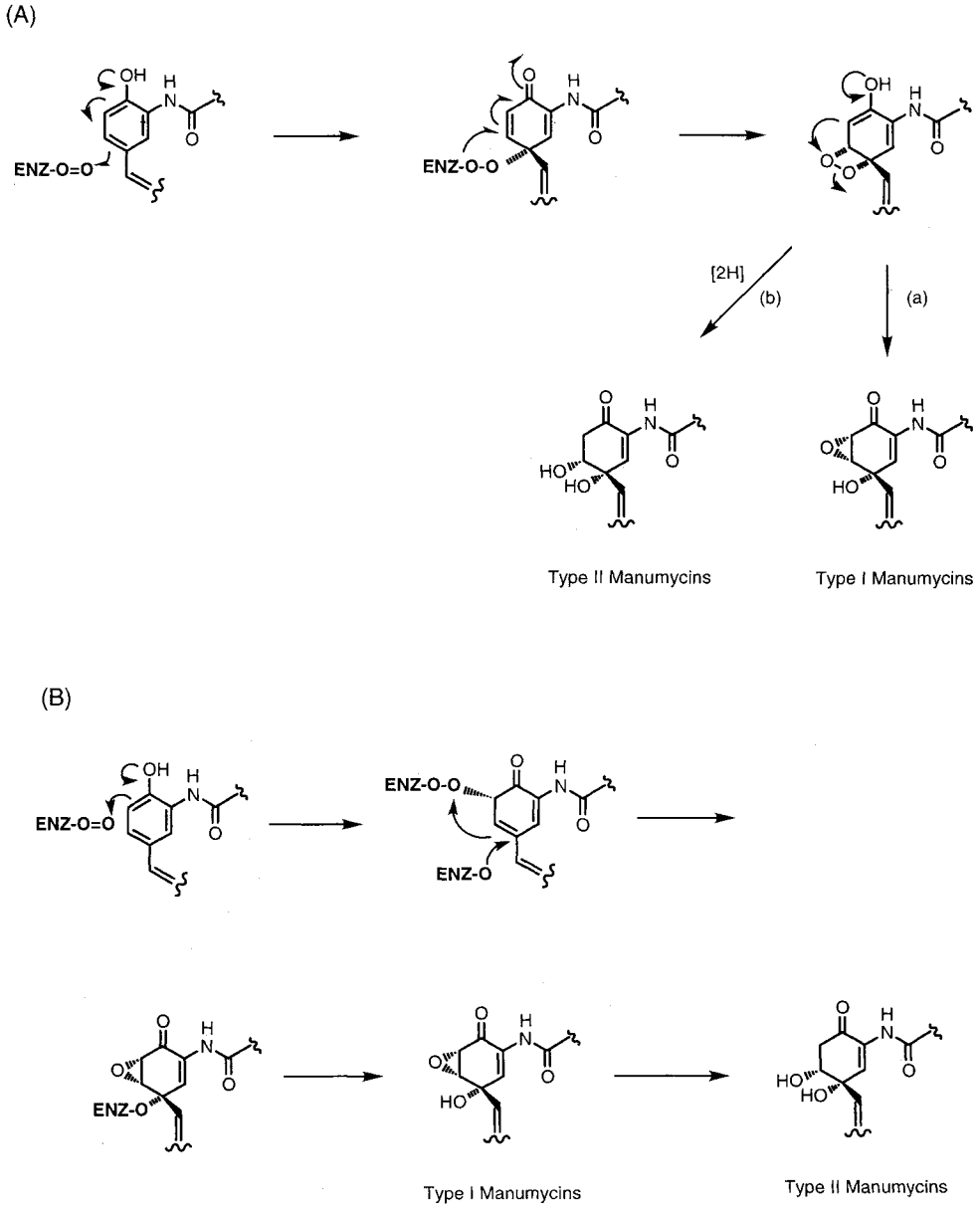
Discussion

The present work demonstrates that the asukamycin fermentation produces not only the parent compound **3a** and various "upper" chain variants of it, all type I manumycins, but also smaller amounts of the type II manumycins **4a**~**4e**. The latter compounds all correspond in the structure of their "upper" chain to type I compounds present in the same fermentation, indicating that the formation of the type I and type II compounds is correlated. This raises the question whether the two types of compounds are formed independently from a common intermediate or whether one class is the precursor of the other.

Two mechanisms have been considered for the formation of the epoxyquinol structure in the manumycins and other compounds from an aromatic precursor. GOULD and coworkers[13,15)] invoked a dioxygenase mechanism, similar to the one originally proposed by DOWD for the oxidation of dihydrovitamin K[16)] and also demonstrated for the formation of the antibiotics LL-C10037 and MPP3051[17)] (Fig. 3A). In this mechanism both oxygens of the epoxyquinol moiety are derived from the same molecule of dioxygen and their stereochemical relationship of necessity has to be *cis*. A second mechanism in which the two oxygens are introduced sequentially by two separate oxygenations has also been considered by ZEECK and collegues[1)] (Fig. 3B). With the revision of the stereochemistry of manumycin A from a *trans* to a *cis* arrangement of the epoxy and quinol oxygens by TAYLOR and coworkers[18)] a major contradiction to the dioxygenase mechanism has been removed, and although unproven in the case of the manumycins, this mechanism now seems by far the most plausible. In this mechanism, the type II manumycins may arise by reductive opening of the epoxide ring of the type I compounds or, as shown in Fig. 3A, they could arise independently by a reductive cleavage (path b) of the same cyclic peroxide that gives rise to the type I compounds (path a).[1)] The experiments reported here, particularly the time-course of their formation and the biosynthetic conversion of ^{13}C-labeled asukamycin into asukamycin A-II, demonstrate clearly that the type II manumycins are not synthesized independently but are derived from the corresponding type I compounds.

The remaining unknown is whether the conversion of the type I to the type II manumycins is catalyzed by an enzyme or represents a non-enzymatic chemical process. It has been reported that in the total synthesis of colabomycin D[19)] the epoxide could be reduced chemically by Na[PhSeB(OEt)$_3$], and we have observed that in aqueous solution the epoxide function of asukamycin was easily reduced to the hydroxyethylene group by 1,4-dithioerythritol.[14a)] However, this facile chemical conversion does not exclude the involvement of an enzyme in the biochemical reaction, possibly even a sulfhydryl enzyme. If the type I to type II transformation is a spontaneous chemical reaction, structural variations in the "upper" chain terminus should

Fig. 3. Dioxygenase (A) and monooxygenase (B) mechanisms for the formation of the manumycins, and two modes of formation of the type II manumycins.



not affect the reaction rate, since the oxirane reaction site is far away. Therefore, the percentage distribution of the different type I congeners should be the same as that of the corresponding type II series. However, in an enzymatic process there are likely to be some differences in the efficiency of different type I congeners as substrates of the reductase, and hence one might see some difference in the percentage distribution of the type I and type II congeners. The latter is indeed what the experiments showed, providing substantial evidence for an enzyme-mediated conversion of the type I into the type II manumycins. The enzyme slightly favors a branched "upper" chain terminus in its substrate over the cyclohexane ring. However, some contribution of the non-enzymatic reaction to the overall conversion can not be excluded.

Experimental

General

S. nodosus ssp. *asukaensis* (ATCC 29757) was obtained from the American Type Culture Collection. Fermentation ingredients were purchased from Difco and Sigma, chemicals from Aldrich and Lancaster. 3-Amino-4-hydroxy-[7-^{13}C]benzoic acid (3,4-AHBA) was material which had been synthesized previously.[13]

^{1}H- and ^{13}C-NMR spectra were recorded on Bruker AF 300 and AM 500 spectrometers in acetone-d_6 as solvent. Chemical shifts are given in parts per million (ppm) and are adjusted to the TMS scale by reference to the solvent signal. Electrospray mass spectra and tandem mass spectra were recorded on a Bruker Esquire ion trap mass spectrometer. LC-MS was carried out with Shimadzu LC-10AD pumps (2), a SPD-10AV UV-Vis variable detector and a Micromass Quattro II Tandem Quadrupole mass spectrometer. High resolution mass spectra were obtained on a Micromass 70SEQ tandem hydrid mass spectrometer. The isotopic distribution in asukamycin A-II was 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) from EM Science. Mobilities are quoted relative to the solvent front (Rf). Preparative TLC was carried out on precoated silica gel plates (glass backing, 2.0 mm layer, UV-254 fluorescence) from EM Separations Technology. Flash column chromatography was performed on 230~400 mesh silica gel from Aldrich. HPLC was conducted with a Beckman model 116 isocratic pump and Beckman model 166 absorbance detector using C_{18} reverse-phase analytical or semi-preparative columns.

Synthesis

[7-^{13}C]Cyclohexanecarboxylic acid

Cyclohexyl bromide (3.6 g, 22.3 mmol) was added to a mixture of magnesium shavings (1.6 g, 67 mmol) in anhydrous THF (20 ml) under argon at 50°C. Three drops of 1,2-dibromoethane were added to initiate the reaction. After 2 hours, the mixture was cooled to 0°C and 480 ml of $^{13}CO_2$ (99+ atom% ^{13}C, 22.3 mmol) was bubbled through the solution *via* syringe. The reaction mixture was stirred for 0.5 hour and poured into water, acidified and extracted with diethyl ether. The ether layer was washed with brine. The crude mixture resulting from evaporation of the ether was dissolved in 1 M NaOH and extracted with Et_2O. The aqueous layer was acidified and extracted with Et_2O. The ether layer was dried over $MgSO_4$, and ether removed *in vacuo* to give [7-^{13}C]cyclohexanecarboxylic acid (1.4 g, 11.02 mmol, 49.4%): ^{1}H-NMR (300 MHz, acetone-d_6) δ_H 2.28 (m, 1H, H-1), 1.91~1.85 (m, 2H, H-2$_{(eq)}$), 1.73~1.55 (m, 4H, H-3), 1.47~1.35 (m, 2H, H-2$_{(ax)}$), 1.32~1.22 (m, 2H, H-4). ^{13}C-NMR (75 MHz, acetone-d_6) δ_C 177.7 (enriched, C-7), 43.2 (d, J=56.2 Hz, C-1), 29.7, 26.5, 26.0 (d, J=3.7 Hz, C-2). EI-MS *m/z* (relative intensity) 115 (M^+, 42), 87 (71), 74 (94), 69 (82), 43 (100).

[7′-^{13}C]Asukamycin A ([7′-^{13}C]-**3a**)

[7-^{13}C]Cyclohexanecarboxylic acid was fed to twenty 100 ml production cultures of *S. nodosus* ssp. *asukaensis* at a concentration of 4 mM at the time of inoculation, and the cultures were harvested after 3 days. After extraction of the supernatant with ethyl acetate and the mycelium with acetone, the combined crude extracts were subjected to silica gel column chromatography, eluting with methylene chloride/methanol 50 : 1. Type I manumycins (53 mg) were obtained and shown by ion-trap MS to consist of 77.9% asukamycin.

[7′-^{13}C]Asukamycin A: Rf 0.42 [silica gel, MeOH/$CHCl_3$ (10 : 1)]. $[M-H]^-$ 546, daughter ions 528, 510, 415, 357, 340, 216, 178. Enrichment: 99+ atom% ^{13}C, incorporation of [7-^{13}C]cyclohexanecarboxylic acid: 100%.

Fermentation and Feeding Experiments

S. nodosus ssp. *asukaensis* was grown on yeast-malt extract agar plates incubated at 28°C for 4 days, which were 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 grown on a rotary shaker at 300 rpm for 2 days at 28°C. For production cultures, 10 ml of seed culture was used to inoculate 100 ml of culture medium which contained glucose 20 g, Bacto Peptone 5 g, K_2HPO_4 0.25 g, $MgSO_4$ 0.25 g, trace elements $(NH_4)_6Mo_7O_{24}\times4H_2O$ 5 mg, $FeSO_4\times7H_2O$ 50 mg, $CuSO_4\times5H_2O$ 5 mg, $ZnSO_4\times7H_2O$ 5 mg, $MnCl_2\times4H_2O$ 10 mg, deionized water 1000 ml, pH 7.0. Media were sterilized for 20 minutes at 121°C in an autoclave.

In general, single doses of labeled compounds were administered to the production cultures 24 hours after inoculation. The labeled compounds were added in the amounts indicated per culture volume: 3,4-[7-^{13}C]-AHBA dissolved in 5% K_2CO_3, 10 mg/100 ml (0.649 mM), [7′-^{13}C]asukamycin A dissolved in 5% K_2CO_3, 20 mg/100 ml (0.356 mM). In the fermentation time course study, 3,4-[7-

^{13}C]AHBA was administered to the production cultures at the time of inoculation.

Isolation and Purification of Type II Manumycins

The production cultures were harvested after 3 1/2 days. After extraction of the supernatant with ethyl acetate and of the mycelium with acetone, the combined crude extracts were fractionated on a silica gel column by elution with methylene chloride/methanol 50:1. The fractions containing type II manumycins with an enhanced ^{13}C-NMR signal at 141 ppm were further purified by HPLC eluting with a gradient of acetonitrile/water 57.5:42.5 to 62.5:37.5 over 15 minutes at a flow rate of 4 ml/minute. Four fractions were collected and assigned as the following type II manumycins.

Asukamycin B-II (**4b**) (HPLC fraction 1, t_{ret} 8.5 minutes): Rf 0.30 [silica gel, MeOH/$CHCl_3$ (10:1)]. ^{1}H-NMR (500 MHz, acetone-d_6) δ_H 7.53 (d, 1H, J=1.9 Hz, H-3), 7.34 (dd, 1H, J=14.8, 11.4 Hz, H-11), 7.25 (dd, 1H, J=14.8, 11.1 Hz, H-3′), 6.79 (dd, 1H, J=14.8, 11.1 Hz, H-9), 6.64 (dd, 1H, J=15.1, 11.1 Hz, H-8), 6.62 (dd, 1H, J=14.8, 10.8 Hz, H-5′), 6.58 (d, 1H, J=14.8 Hz, H-12), 6.50 (dd, 1H, J=14.8, 11.4 Hz, H-10), 6.38 (d, 1H, J=14.8 Hz, H-2′), 6.30 (dd, 1H, J=14.8, 11.2 Hz, H-4′), 6.22 (d, 1H, J=14.8 Hz, H-7), 6.18 (dd, 1H, J=15.5, 10.8 Hz, H-6′), 5.93 (dd, 1H, J=15.5, 7.4 Hz, H-7′), 4.04 (m, 1H, H-5), 2.77 (m, 2H, H-6_{eq}, H-6_{ax}, obscured by H_2O peak), 2.46 (br s, 4H, H-4″, H-5″), 2.40 (m, 1H, H-8′), 1.01 (d, 6H, J=6.8 Hz, H-9′). ES-MS/MS $[M+H]^+$ 509, daughter ions 491, 149. $[M-H]^-$ 507, daughter ions 489, 376, 341, 216.

Asukamycin C-II (**4c**) (minor) and asukamycin D-II (**4d**) (major) (HPLC fraction 2, t_{ret} 11.1 minutes), data from the major component (**4d**): ^{1}H-NMR (500 MHz, acetone-d_6) δ_H 7.54 (d, 1H, J=1.9 Hz, H-3), 7.36 (dd, 1H, J=14.8, 11.7 Hz, H-11), 7.26 (dd, 1H, J=14.8, 11.4 Hz, H-3′), 6.79 (dd, 1H, J=14.8, 11.4 Hz, H-9), 6.65 (dd, 1H, J=14.8, 11.1 Hz, H-8), 6.65 (dd, 1H, J=14.8, 11.1 Hz, H-5′), 6.58 (d, 1H, J=14.8 Hz, H-12), 6.51 (dd, 1H, J=14.8, 11.4 Hz, H-10), 6.39 (d, 1H, J=14.8 Hz, H-2′), 6.33 (dd, 1H, J=14.8, 11.4 Hz, H-4′), 6.23 (d, 1H, J=14.8 Hz, H-7), 6.19 (dd, 1H, J=15.5, 11.1 Hz, H-6′), 5.85 (dd, 1H, J=15.5, 7.4 Hz, H-7′), 4.04 (m, 1H, H-5), 2.74 (m, 2H, H-6_{eq}, H-6_{ax}, obscured by H_2O peak), 2.47 (br s, 4H, H-4″, H-5″), 2.10 (m, 1H, H-8′, beneath acetone peak), 1.35 (m, 2H, H-9′), 1.00 (d, 3H, J=6.8 Hz, H-11′), 0.85 (t, 3H, J=7.4 Hz, H-10′), for minor component (**4c**), 2.04 (m, 2H, H-8′, beneath acetone peak), 1.71 (m, 1H, H-9′), 0.89 (d, 6H, J=6.8 Hz, H-10′), HR-MS [M+Na] for $C_{29}H_{34}N_2O_7Na$, calculated 545.2264, found 545.2286; 24.0% ^{13}C incorporation (not calibrated against unlabeled sample). ES-MS/MS $[M+H]^+$ 523, daughter ions 505, 163. $[M-H]^-$ 521, daughter ions 503, 390, 341, 216.

Asukamycin E-II (**4e**) (HPLC fraction 3, t_{ret} 14.6 minutes): ^{1}H-NMR (500 MHz, acetone-d_6) δ_H 7.54 (d, 1H, J=1.9 Hz, H-3), 7.35 (dd, 1H, J=14.8, 11.1 Hz, H-11), 7.25 (dd, 1H, J=14.8, 11.7 Hz, H-3′), 6.78 (dd, 1H, J=14.8, 10.8 Hz, H-9), 6.63 (dd, 1H, J=14.8, 10.8 Hz, H-8), 6.61 (dd, 1H, J=15.1, 10.5 Hz, H-5′), 6.57 (d, 1H, J=14.8 Hz, H-12), 6.51 (dd, 1H, J=14.8, 11.1 Hz, H-10), 6.39 (d, 1H, J=14.8 Hz, H-2′), 6.31 (dd, 1H, J=15.1, 11.7 Hz, H-4′), 6.23 (d, 1H, J=14.8 Hz, H-7), 6.19 (m, 1H, H-6′), 5.97 (dd, 1H, J=15.5, 8.0 Hz, H-7′), 4.04 (m, 1H, H-5), 2.77 (m, 2H, H-6_{eq}, H-6_{ax}, obscured by H_2O peak), 2.47 (br s, 4H, H-4″, H-5″), 2.16 (m, 2H, H-8′, beneath acetone peak), 1.57 (m, 1H, H-10′), 1.30 (m, 2H, H-9′), 0.88 (d, 6H, J=6.8 Hz, H-11′). ES-MS/MS $[M+H]^+$ 537, daughter ion 521, 177. $[M-H]^-$ 535, daughter ions 517, 404, 341, 216.

Asukamycin A-II (**4a**) (HPLC fraction 4, t_{ret} 16.0 minutes): Rf 0.30 [silica gel, MeOH/$CHCl_3$ (10:1)]. ^{1}H-NMR (500 MHz, acetone-d_6) δ_H 7.54 (d, 1H, J=1.9 Hz, H-3), 7.35 (dd, 1H, J=14.8, 11.7 Hz, H-11), 7.25 (dd, 1H, J=14.8, 11.1 Hz, H-3′), 6.78 (dd, 1H, J=14.8, 10.5 Hz, H-9), 6.63 (dd, 1H, J=14.8, 10.5 Hz, H-8), 6.61 (dd, 1H, J=14.8, 10.5 Hz, H-5′), 6.57 (d, 1H, J=14.8 Hz, H-12), 6.50 (dd, 1H, J=14.8, 11.1 Hz, H-10), 6.38 (d, 1H, J=14.8 Hz, H-2′), 6.32 (dd, 1H, J=14.8, 11.2 Hz, H-4′), 6.23 (d, 1H, J=14.8 Hz, H-7), 6.18 (dd, 1H, J=15.5, 10.5 Hz, H-6′), 5.91 (dd, 1H, J=15.5, 7.4 Hz, H-7′), 4.04 (m, 1H, H-5), 2.77 (m, 2H, H-6_{eq}, H-6_{ax}, obscured by H_2O peak), 2.47 (br s, 4H, H-4″, H-5″), 2.08 (m, 1H, H-8′, beneath acetone peak), 1.71 (m, 4H, H-10′), 1.28 (m, 2H, H-11′), 1.20~1.10 (m, 4H, H-9′). ES-MS/MS $[M+H]^+$ 549, daughter ions 531, 189. $[M-H]^-$ 547, daughter ions 529, 416, 341, 216. HR-MS [M+Na] for $C_{31}H_{36}N_2O_7Na$, calculated 571.2420, found 571.2424. 21.2% incorporation of 3,4-[7-^{13}C]-AHBA (not calibrated against unlabeled sample). ^{13}C-Asukamycin A-II (67 atom% ^{13}C) from incorporation of ^{13}C-asukamycin A, $[M-H]^-$ 548, daughter ions 530, 417, 341, 216.

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