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Biosynthesis of Structurally Unique Fungal Metabolite GKK1032A$_2$: Indication of Novel Carbocyclic Formation Mechanism in Polyketide Biosynthesis

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The biosynthesis of the antitumor agent GKK1032A$_2$ (**1**) has been investigated by administration of isotopically labeled (^{13}C and ^{2}H) precursors to *Penicillium* sp. GKK1032. These studies showed that the backbone of **1** is constructed from L-tyrosine and a nonaketide chain flanked with five methyl groups probably by a polyketide synthase and a nonribosomal peptide synthetase hybrid. On the basis of the oxidation level of the starter unit and unusual 13-membered macroether formation between the tyrosine hydroxy group and the polyketide chain, novel cyclization mechanisms on the formation of a tricarbocyclic system and a macroether have been proposed. Involvement of a similar type of cyclization in the biosynthesis of structurally related metabolites is discussed.

Introduction

GKK1032 A$_2$ (**1**), A$_1$ (**2**), and B (**3**) are fungal metabolites produced by *Penicillium* sp. GKK1032 (Figure 1).[1] These metabolites exhibit antimicrobial and antitumor activities against epithelioid cell HeLa S3 originated from human uterine-cervix carcinoma.[1a] The structures of **2** and **3** were determined by extensive spectral analysis including various 1D- and 2D-NMR techniques. The proposed structure of **3** was further confirmed by X-ray crystallographic analysis.[1] The differences in the structures among these compounds are located only in the nitrogen-containing ring: (1) the γ-lactam moiety in **1** and **2** is replaced to succimide in **3**, and (2) the relative stereochemistry of two substituents is changed from cis in **1** and **2** to trans in **3**. Very recently, He et al. have reported isolation of pyrrocidines A (**4**) and B (**5**), 3,6-bisepi-3-desmethyl analogues of GKK1032s, from unidentified filamentous fungus LL-Cyan426 as an antimicrobial agent against Gram-positive bacteria including drug-resistant strains.[2] Members of the GKK1032 family show very unique structural features such as (1) 12 or 13-membered macrocyclic ether-containing 1,4-disubstituted phenyl and γ-lactam or succimide moieties and (2) a reduced tricarbocyclic system which is biosynthetically rare in polyketide metabolites. Herein, this paper describes the biosynthetic origins of **1** and proposes a biosynthetic pathway constructing its unique molecular skeleton.

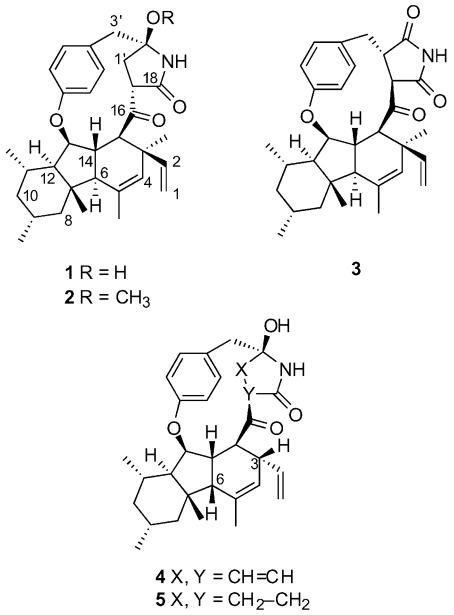

FIGURE 1. Structures of GKK1032 members.

Results and Discussion

Assignments of the NMR signals of **1** have not been carried out since its structure was deduced by comparison of the spectra of **2** to those of **1**. Thus, complete assignments of all signals in the ^{13}C and ^{1}H NMR spectra of **1** were established by extensive 2D-NMR techniques used

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TABLE 1. Results of Incorporation Experiments in *Penicillium* sp. GKK1032. ^{13}C Chemical Shifts of GKK1032A$_2$ (1) in ^{13}C NMR (125 MHz, CDCl$_3$)

	δ_C	[1-^{13}C]-acetate[a] enrich-ment	[1,2-^{13}C]-acetate J_{C-C} (Hz)	[1-^{13}C,^{2}H$_3$]-acetate[b] Δ (ppm)	[1-^{13}C]-L-Tyr[a] enrich-ment	[*S*-^{13}CH$_3$]-L-Met[a] enrich-ment
C-1	112.0		69			
C-2	146.3	3.6	70	0.182		
C-3	41.6		42			
C-4	130.7	3.2	43			
C-5	138.5		41			
C-6	53.0	2.9	41			
C-7	41.5		35			
C-8	49.0	3.5	34			
C-9	28.0		33			
C-10	45.5	3.5	33			
C-11	27.3		35			
C-12	60.9	3.9	35			
C-13	90.7		36			
C-14	50.6	2.1	36			
C-15	56.7		39			
C-16	200.2	4.0	40			
C-17	56.7		45			
C-18	171.8	3.2	44			
C-1′	33.4				26.2	
C-2′	88.8					
C-3′	47.0					
C-4′	127.9					
C-5′a	133.3					
C-5′b	131.5					
C-6′a	118.8					
C-6′b	124.4					
C-7′	159.8					
3-Me	25.7					18.1
5-Me	20.9					15.9
7-Me	16.0					13.3
9-Me	22.8					14.8
11-Me	19.8					14.6

[a] The values of enrichments were determined by comparison of the relative peak intensities of the corresponding carbons in labeled and unlabeled spectra. [b] The isotope shift is given as Δ (ppm) upfield from the natural abundance signal.

in structure determination of **2** and **3** (Table 1). These results were supported by incorporation experiments described below.

Feeding experiments with [1-^{13}C]acetate resulted in enrichment that established the biosynthesis of **1** via a polyketide pathway. The enrichments of the carbons 2, 4, 6, 8, 10, 12, 14, 16, and 18 were consistent with the initial prediction that a linear polyketide chain from C1 to C18 was constructed by nonaketides (Table 1). This result was further confirmed by incorporation with [1, 2-^{13}C$_2$]acetate. In the ^{13}C NMR spectrum of enriched **1**, nine pairs of coupled signals were observed as shown in Table 1. The results indicate that these carbon pairs were originated from intact acetate units.

The results of incorporation shown above suggest that the starter unit of the polyketide chain of **1** is C1−C2, although the terminal methyl group is oxidized to an olefin. To obtain information of the starter unit of the polyketide, [1-^{13}C,^{2}H$_3$]acetate was incorporated. In the ^{13}C NMR spectrum of enriched **1**, an upfield-shifted signal was observed at C2, and the magnitude of the shift (0.182 ppm) indicates that the signal carried two deuteriums at the β-position (C1) (Table 1). This was supported by the HSQC-TOCSY spectrum[3] of the enriched **1** (Figure 2); the shifted signal showed no correlation with protons

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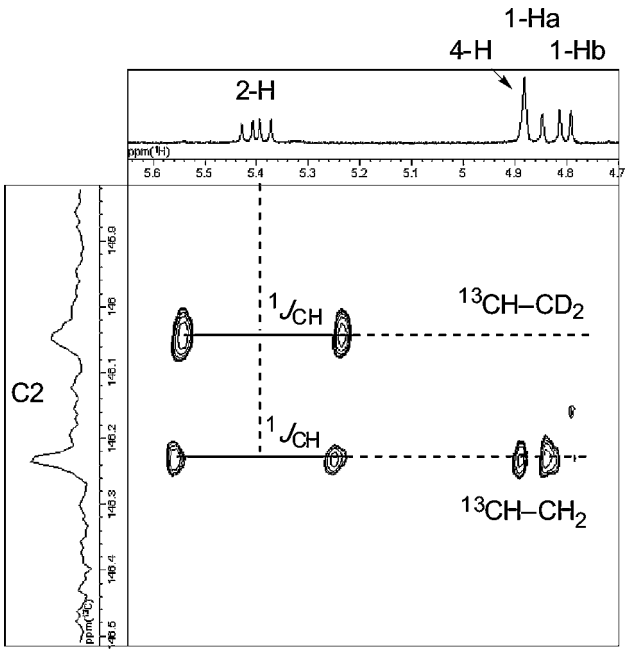

FIGURE 2. Part of the HMQC-TOCSY spectrum of **1** from feeding with [1-^{13}C,^{2}H$_3$]-acetate.

at C1 although the same signal gave directly coupled correlation with 2-H. The observation that no shifted signal was observed at C14, C16, and C18 indicates that significant washout occurred during chain elongation. These data suggest that the starter unit is acetate but not another possible unit such as malonate.[4]

To examine the origin of the branched methyl groups, a feeding experiment with [*S*-^{13}CH$_3$]-L-methionine was conducted. High enrichments (13.3−18.1) were observed at the carbons 3-Me, 5-Me, 7-Me, 9-Me, and 11-Me (Table 1). In the biosynthesis of fungal polyketides, pendant methyl groups on the polyketide chain are commonly derived from L-methionine although the corresponding methyl group is originated from propionate in that of Actinomycetes polyketides.[5] Recently, methyl transferase domains have been found in iterative fungal polyketide synthetase (PKS) genes of lovastatin[6a] and fumonisin[6b] and also in bacterial modular PKS genes which are responsible for the biosynthesis of epothilone.[7] Thus, all branched methyl groups in **1** are most likely introduced by a similar type of PKS.

On the basis of the structure of **1**, the remaining C$_9$-unit is assumed to be derived from L-tyrosine. This was confirmed by incorporation of [1-^{13}C]-L-tyrosine (Table 1). Significant enrichment (26.2) was observed at C1′, indicating that the carboxyl group of tyrosine is condensed with the polyketide chain. A recent finding that hybrid

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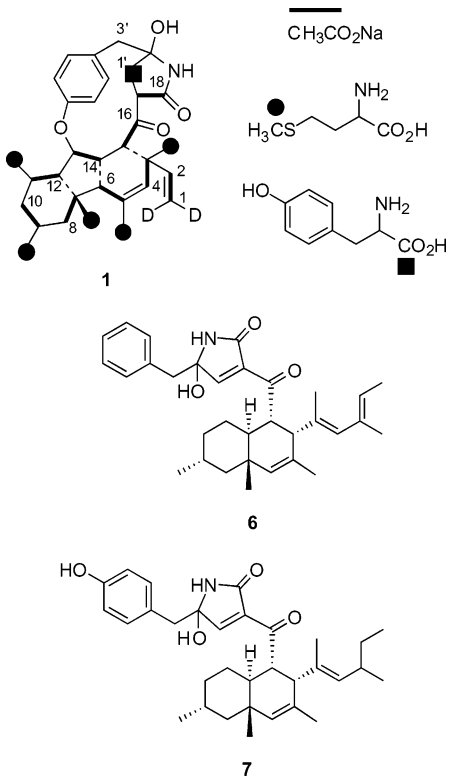

FIGURE 3. Labeling patterns of GKK1032A_2 (**1**) from incorporation experiments and biosynthetically related fungal metabolites.

genes of PKS and nonribosomal peptide synthetase (NRPS) are responsible for the biosynthesis of various nitrogen-containing polyketides[8] suggests that the backbone of **1** is constructed by PKS-NRPS hybrid enzyme.

Occurrence of oteromycin (**6**)[9] and ZG-1494a (**7**)[10] (Figure 3) having an acyldesoxytetramate moiety with the nonaketide derived backbone strongly indicated that GKK1032 family members and **6** and **7** are derived from linear polyketide intermediates such as **8** (Scheme 1). An iterative fungal PKS-NRPS hybrid carrying methyltransferase domains may be involved in construction of methyl branched nonaketide which is then condensed with the amino group of enzyme-bound tyrosine, followed by intramolecular condensation to yield free acyltetramate **8**. Subsequent keto-reduction, dehydration, and C2′-hydroxylation furnish a plausible key intermediate **9**. Occurrence of a number of polyketides with an acyltetramate moiety[11] gives further circumstantial evidence for this biosynthetic route. In the biosynthesis of **6**[12] and **7**, intramolecular Diels−Alder reaction of intermediates similar to **8** may take place. Involvement of Diels−Alder reaction in the biosynthesis of polyketides with a decalin moiety was proved in the biosynthesis of solanapyrone[13] and lovastatin[14] and was proposed in a number of polyketides.[12,15] On the other hand, the carbon skeleton of **1** cannot be constructed by simple Diels−Alder reaction.

The oxidation level of the starter unit and unusual ether formation at C13 indicate the cationic cyclization of polyolefin. Several plausible mechanisms for construction of the tricyclic system in **1** are shown in Scheme 2. The mechanisms can be divided into two groups: (1) C1- or C2−C3-oxidation/cyclization and (2) C12−C13-epoxidation/cyclization. Route Ia involves two-electron oxidation at C1 in the plausible intermediate **9** with an enzyme such as FAD dependent oxidase[16] and isomerization of C4−C5 double bond (from *E* to *Z*) in the resultant conjugated allylic cation. The cation **10** triggers sequential cyclization and the penultimate cation at C13 is trapped with phenoxy oxygen derived from tyrosine to give cyclization product **11**. In this case, a single enzyme is responsible for this complex cyclization. The stereochemistry on the carbocyclic systems newly formed can be defined by isomerization of polyene precursor **9** in allylic conjugated carbocation **10**. Though all *E*-olefin was selected as the intermediate in this report, an olefin carrying *E*- and *Z*-double bonds can be an intermediate of similar stereospecific cyclization to explain the configuration of product **11**.

Routes Ib and Ic require more than two enzymes which catalyze the conversion of **9** to **11**. In route Ib, hydroxylation at C1 in **9**, followed by elimination of a hydroxy group gives the same allylic cation **10** as the one in route Ia. Route Ic is initiated by epoxidation at C2-olefin. The subsequent ring-opening of the epoxide triggers cyclization in a similar way to that of route Ia, followed by dehydration to furnish **11**.

Alternative modes of the cyclization can take place from C12−C13-oxidation. In route IIa, epoxidation and acid-mediated ring-opening provide α-hydroxy aryl ether **12** which is converted to **11** by acid-catalyzed cyclization and isomerization of the C4 olefin. Similar macroether formation is proposed by Moore in the biosynthesis of bicyclic depsipeptide salinamide.[17] He also made an interesting biogenetic proposal on Fe(II)-dependent oxygenase-mediated [2 + 2] cycloaddition.[17] Currently, no data is available to determine the actual cyclization mode. However, route Ia is attractive to explain the biosynthetic pathways of structurally similar polyketides described below.

Isolation of a series of GKK1032 derivatives (Figure 1) indicates that the final transformations from **11** to **1** and **3** proceed as shown in Scheme 3. Thus, acid-

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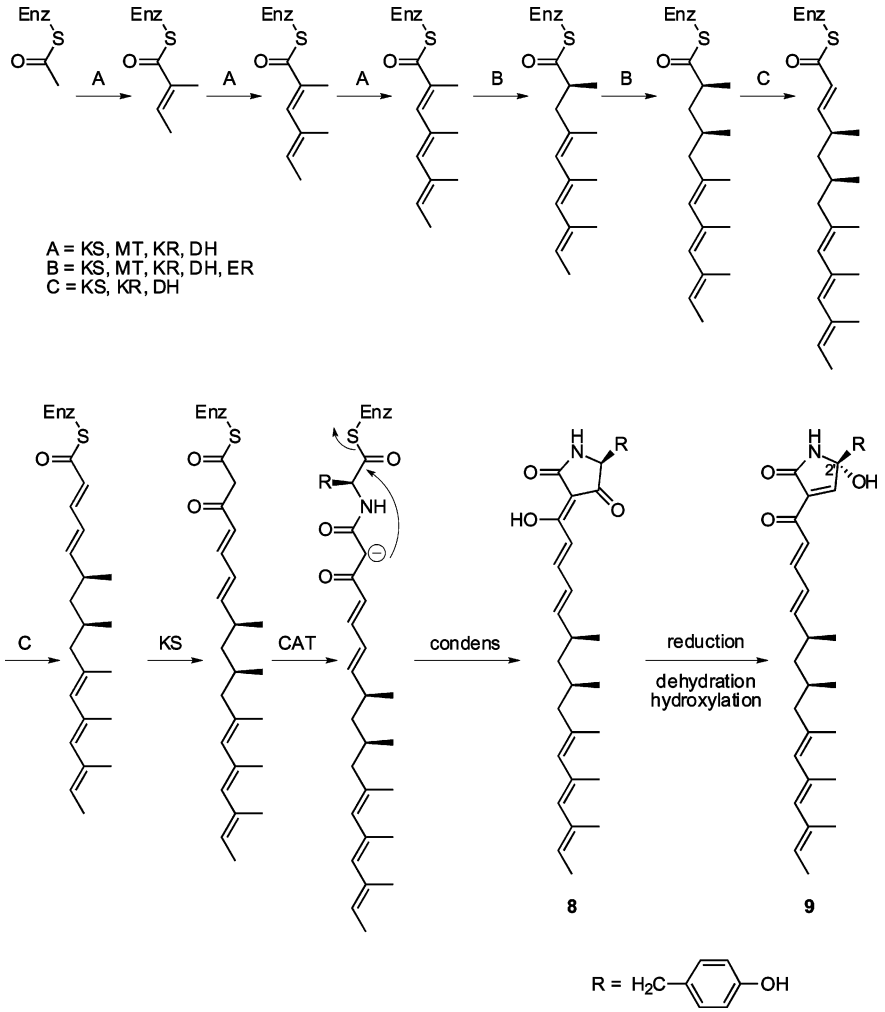
SCHEME 1. Proposed Biosynthetic Pathway for the Construction of Backbone of 1[a]



[a] Catalytic domains: KS, ketosynthase; MT, methyl transferase; KR, ketoreductase; DH, dehydratase; ER, enoyl reductase; CAT, NRPS condensation−adenylation−thiolation.

catalyzed 1,2-carbon shift from C2′ to C1′ affords imide **3**. This ring contraction caused significant ring strain on the macroether, forcing C17 configuration to be *R*. On the other hand, hydrogenation of **11** provides **1** which has the *S*-configuration at C17.

A similar tricyclic system is found in the commercially available insecticide spinosyn A (**13**, A83543A)[18] shown in Figure 4. In the case of **13**, the biosynthetic gene cluster spanning ~80 kb has been identified.[19] Based on the modular arrangement of the PKS and several gene disruption experiments, Waldron et al. suggests involvement of the biosynthetic intermediate macrolide **14**. In this cluster, the genes possibly encoding two methyltransferases (*spnF*, *spnL*) and FAD-dependent oxidase (*spnJ*) are found. Since these genes are not involved in the biosynthesis of deoxysugar and the plausible intermediate **14**, the authors speculated that they encode the enzymes responsible for polyketide bridging reaction from **14** to aglycon **15**. Thus, it can be proposed that similar oxidative cyclizations are involved in the biosynthesis of **1** and **13**.

The ikarugamycin group of natural products has characteristic structural features such as an ornithine-derived acyltetramate moiety and fused bi- and tricyclic systems which derived from two polyketide chains[20] (*N*-acyl chain and the acyl chain with tetramate) (Figure 5). This group consists of ikarugamycin,[21] capsimycin,[20] xanthobaccins A[22a] and D[22b] discodermide,[23] cylindramide,[24] and alteramide A.[25] In the case of ikarugamycin, the Diels−Alder reaction has been proposed for the construction of the cyclohexene ring, but this cannot fully explain the formation of the tricyclic system. In analogy

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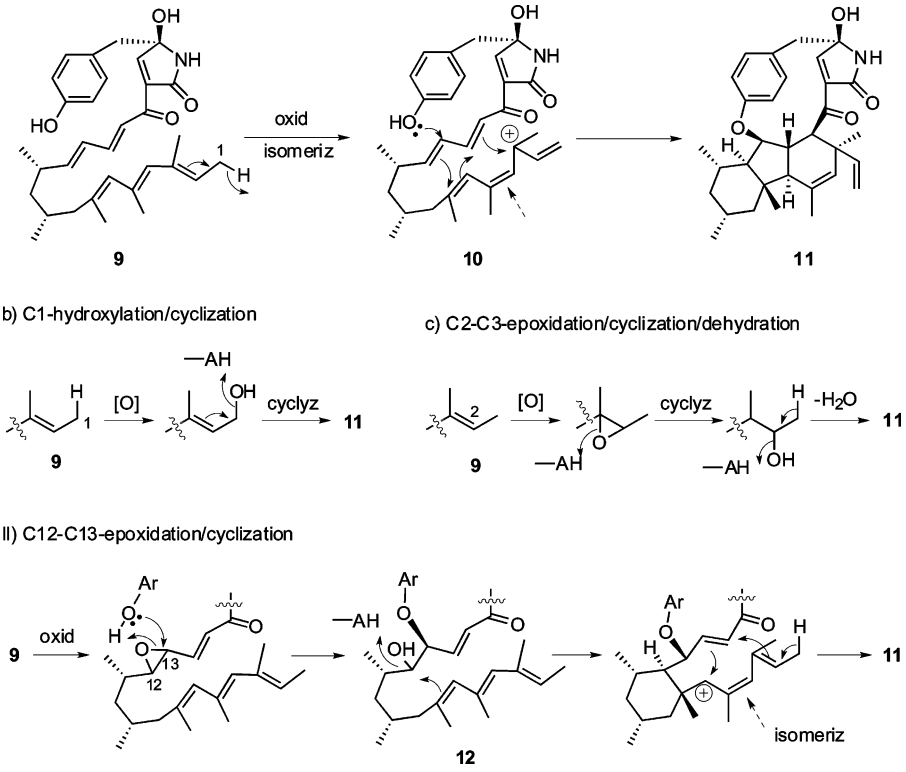
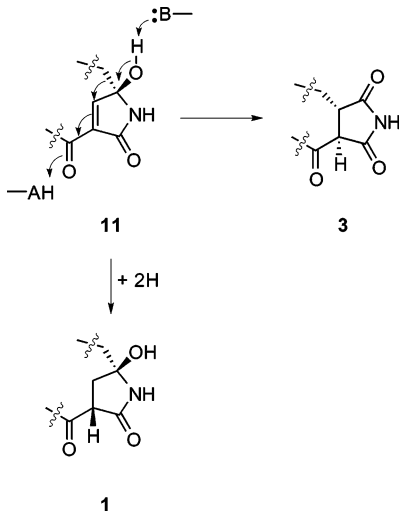
SCHEME 2. Proposed Mechanisms for Formation of the Unusual GKK1032 Skeleton



SCHEME 3. Proposed Mechanism of Conversion of the Plausible Late Intermediate 11 to 1 and 3



to the proposed biosynthetic pathways for **1** and **13**, one can reasonably assume that the carbocyclic systems in these metabolites are also constructed by oxidative cyclizations of the corresponding polyene intermediates similar to **9** and **14**. The presence of extra olefins in bicarbocyclic metabolites and distinct carbocyclic systems (5−5, 5−5−6, or 5−6−5) provide circumstantial evidence for this hypothesis. Various stereochemical types on the fused rings can be explained by reaction of polyolefins carrying *E*- and *Z*-olefins or olefin isomerization during conjugated carbocation intermediates. Similar cationic cyclization of polyolefin is known in the biosynthesis of terpenoids. The cyclization of terpenoids utilizes polyolefin with double bonds located regularly while the one in polyketides described above utilizes conjugated polyolefins with double bonds in varied positions. The flexibility of this tailoring enzyme might add further structural diversity to polyketide metabolites.

Experimental Section

Sodium [1-^{13}C]- (99 atom % ^{13}C), [1-^{13}C, $^{2}H_3$]- (99 atom % ^{13}C, 98 atom % ^{2}H), and [1, 2-$^{13}C_2$]acetates (99 atom % ^{13}C), [1-^{13}C]-L-tyrosine (90 atom % ^{13}C), and [*S*-^{13}C H_3]-L-methionine (99 atom % ^{13}C) were purchased from Cambridge Isotope Laboratory. ^{13}C NMR spectra were obtained on a Bruker AM-500 spectrometer for solutions of $CDCl_3$ and referenced to the solvent signal ($CDCl_3$, 77.0 ppm). Resolution of the spectra is 1.01 Hz/point. *J* values are reported in Hz.

NMR spectra of GKK1032A$_2$ (1): ^{13}C NMR see Table 1; ^{1}H NMR (500 MHz, $CDCl_3$) δ [multiplicity, coupling constant *J* (Hz), assignment] 7.14 (1H, dd, *J* = 8.1, 2.0, 5′-Ha), 6.96 (1H, dd, *J* = 8.8, 2.2, 6′-Hb) and 6.92 (1H, dd, *J* = 8.8, 2.0, 5′-Hb), 6.81 (1H, dd, *J* = 8.1, 2.2, 6′-Ha) and 5.92 (1H, br s, NH), 5.43 (1H, dd, *J* = 17.6, 10.7, 2-H) and 4.91 (1H, br s, 4-H), 4.89 (1H, dd, *J* = 17.6, 1.0, 1-Ha) and 4.82 (1H, dd, *J* = 10.7, 1.0, 1-Hb), 4.25 (1H, dd, *J* = 7.7, 4.4, 13-H) and 3.52 (1H, d, *J* = 10.0, 15-H), 3.13 (1H, dd, *J* = 12.0, 4.6, 17-H) and 2.95 (1H, d, *J* = 13, 3′-Ha), 2.92 (1H, d, *J* = 13, 3′-Hb) and 2.90 (1H, m, 1′-Ha), 2.62 (1H, ddd, *J* = 13.1, 10.0, 4.4, 14-H), 1.99−1.86 (3H, m, 8-Ha, 11-H, 6-H) and 1.86 (3H, s, 5-Me), 1.86−1.76 (3H, m, 9-H, 10-Ha, 1′-H) and 1.19 (3H, s, 3-Me), 1.17 (3H, s, 7-Me) and 1.10 (3H, d, *J* = 6.3, 11-Me), 1.07 (1H, dd, *J* = 11.0, 7.7, 12-H) and 0.91 (3H, d, *J* = 6.3, 9-Me), 0.80 (1H, dd, *J* = 12, 12, 8-Hb), 0.62 (1H, ddd, *J* = 12, 12, 12, 10-Hb).

Growth of *Penicillium* sp. GKK1032. *Penicillium* sp. GKK1032 was inoculated to the seed medium (100 mL, pH 6.5) in a 500 mL Erlenmeyer flask containing mashed potato 30 g/L, glucose 100 g/L, and yeast extracts 5 g/L. After inoculation, the organism was grown at 25 °C and 180 rpm for 2 days. Two mililiters of seed culture was used to inoculate production medium (100 mL, pH 6.5) in a 500 mL Erlenmeyer flask containing sucrose 30 g/L, starch 20 g/L, dry yeast 5 g/L,

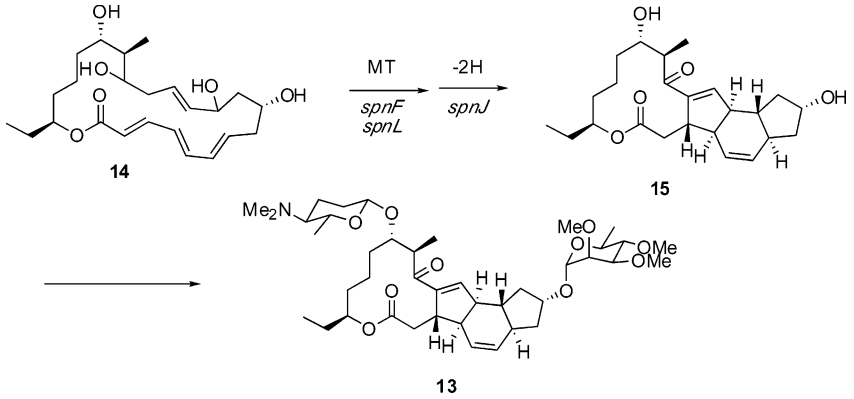


FIGURE 4. Proposed pathway for carbocyclic formation in the biosynthesis of spinosyn A (**13**).

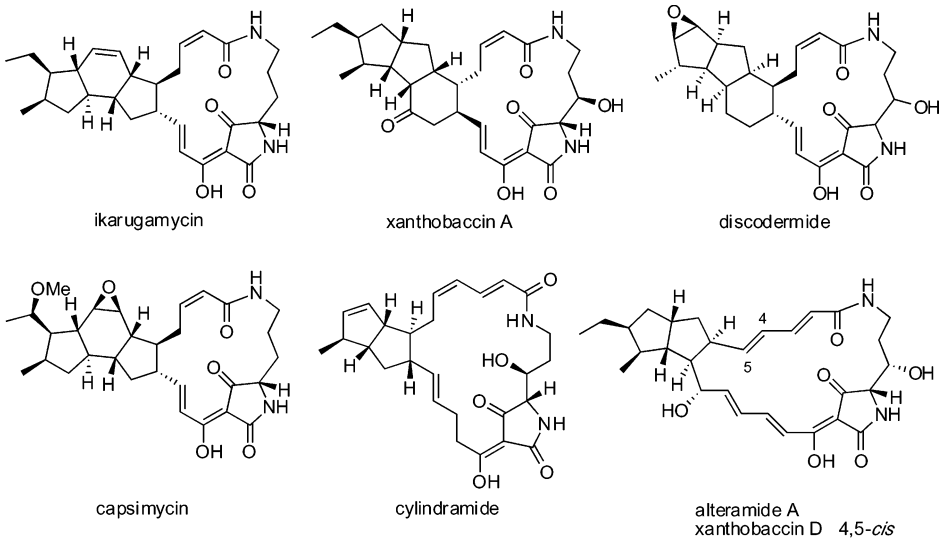


FIGURE 5. Ikarugamycin group of natural products.

malt extracts 10 g/L, corn steep liquor 5 g/L, V8 vegetable juice 200 mL/L. After inoculation, the organism was grown at 25 °C and 180 rpm for a week.

General Procedure for Feeding Experiments with Isotopically Labeled Compounds. On the second day after inoculation, the aqueous solution (8.8 mL) of 400 mg of isotopically labeled compounds filtered through sterilized microfilter (0.2 μm) was equally distributed into four 500 mL flasks. In the case of [1-^{13}C]-L-tyrosine, autoclaved solution was used. After further incubation for 5 days, mycelium was collected by filtration and extracted with acetone. This extract was concentrated in vacuo, and the resultant aqueous layer was extracted with EtOAc. Drying over Na_2SO_4, the EtOAc extract was concentrated in vacuo to afford the oily residue which was chromatographed with SiO_2 column (hexane/EtOAc, 2/3) to collect fractions containing **1**. These fractions were concentrated and the residue was separated by HPLC with the GL Science reversed-phase column (Inertsil ODS-3, 5 μm, ϕ 10 × 250 mm; flow rate 2.5 mL/min; UV 205 nm) with solvent systems (CH_3CN/H_2O, 85/15) to give **1**. The yield of **1** was 1−3 mg in each experiment.

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Supporting Information Available: ^{13}C NMR spectra for all labeled GKK1032A$_2$ (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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