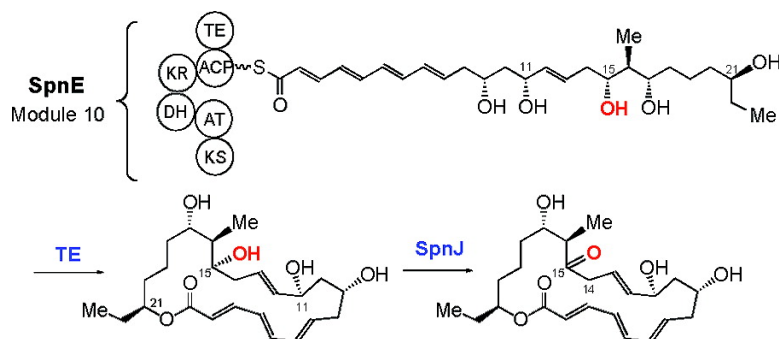## Communication

The Biosynthesis of Spinosyn in *Saccharopolyspora spinosa*: Synthesis of the Cross-Bridging Precursor and Identification of the Function of SpnJ

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The Biosynthesis of Spinosyn in *Saccharopolyspora spinosa*: Synthesis of the Cross-Bridging Precursor and Identification of the Function of SpnJ

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Spinosyns A (**1**) and D (**2**) are polyketide-derived macrolides produced by *Saccharopolyspora spinosa*.[1] The combination of **1** and **2** serves as the active ingredient in a commercial insecticide, Naturalyte. This mixture exhibits excellent insecticidal activity with low mammalian toxicity and little detrimental environmental effects.[2] Structurally, the spinosyns consist of a 22-membered macrolactone ring fused to a perhydro-*as*-indacene core scaffold. In addition, they are glycosylated with tri-*O*-methylrhamnose and forosamine at C-9 and C-17, respectively. The aglycone portion of spinosyns is unusual among polyketide-derived secondary metabolites due to the presence of three intramolecular carbon–carbon bonds that constitute the *as*-indacene skeleton. The unusual nature of the spinosyn aglycone suggests an intriguing biosynthetic pathway which has generated much recent attention.[3,4]

The spinosyn (*spn*) biosynthetic gene cluster was cloned from *S. spinosa*.[3] Sequence analysis coupled with gene disruption experiments suggested that macrolactone **4**, which is derived from the linear polyketide precursor **3**, is a likely intermediate to form the *as*-indacene aglycone (**9**),[3,4] and the proteins encoded by *spnF*, *spnJ*, *spnL*, and *spnM* could be involved in the cross-bridging modifications. SpnJ exhibits sequence homology to a flavin-dependent dehydrogenase (55% identity, 68% similarity) from *Streptomyces steffisburgensis*,[5] and the SpnF, SpnL, and SpnM proteins show resemblance to methyltransferase (SpnF and SpnL) and lipase (SpnM),[3] but lack part of the feature residues/motifs conserved among members of each enzyme family. Hence, their biological functions are not apparent and their roles in the cross-bridging reactions cannot be established with certainty.

If **4** is indeed a precursor of the polycyclic aglycone and the cross-bridging reactions are all post-cyclization events, oxidation of the 15-hydroxyl group of **4** to a keto group (**4 → 5**) and deprotonation at C-14 to form the C-3/C-14 linkage (**5 → 6 → 7**) likely occur first (Scheme 1, route A). Subsequent deprotonation at C-14 of **7** and elimination of the 11-hydroxyl group to give **8** is expected to trigger the second cross-coupling reaction (**8 → 9**) to produce the *as*-indacene core. However, it is also possible that the initial oxidation of the 15-hydroxyl group takes place on the linear precursor **3** when it is still linked to the acyl carrier protein (ACP) associated with the polyketide synthase SpnE (Scheme 1, route B). The keto product **10** is next cleaved from ACP catalyzed by a thioesterase (TE) to give **5**, which then undergoes sequential cyclizations in a similar manner as described above. In this pathway, compound **4** is bypassed.

Although a [4 + 2] cyclization reaction has been speculated to be a key step in the biosynthesis of many natural products, few have been investigated at the molecular level.[6] In the case of the polyketide-derived lovastatin (**14**, Scheme 2), which contains an octahydronaphthalene core, in vitro study with purified polyketide synthase (PKS) showed that it is capable of converting a linear *N*-acetylcysteamine mimic of the appropriate enzyme-bound
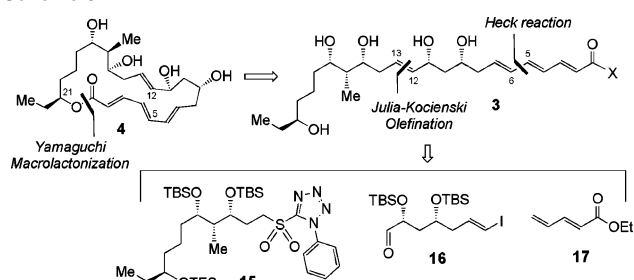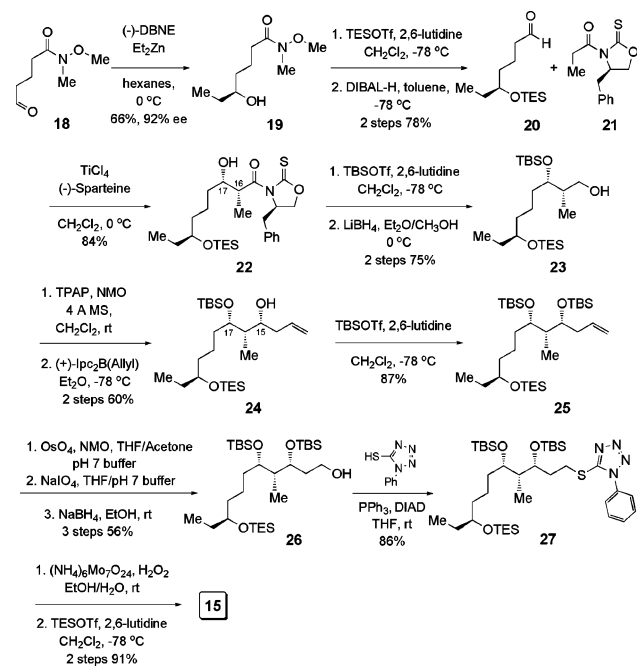
Scheme 1
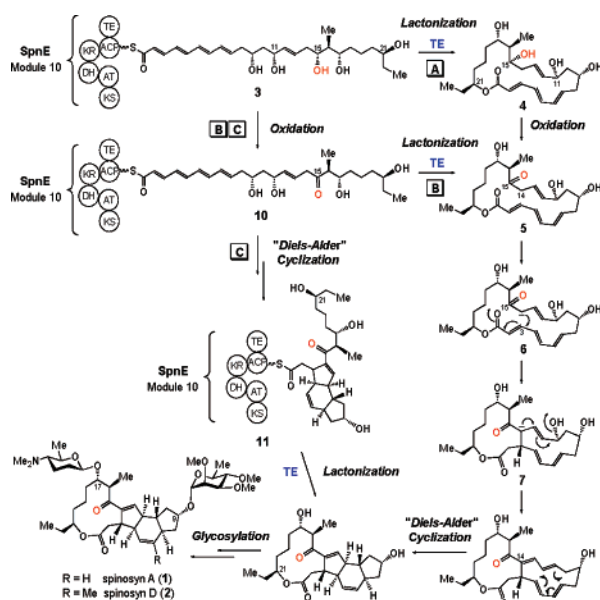
Scheme 2
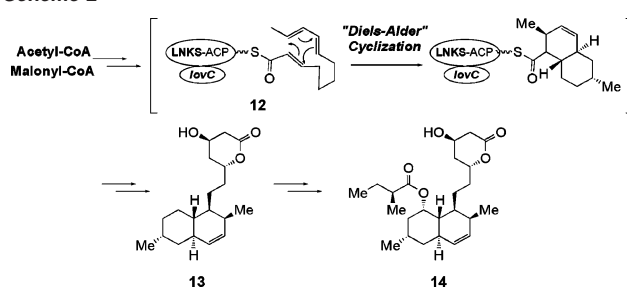
Scheme 3

polyketide intermediate (**12**) to the correct isomer of the octahydronaphthalene moiety (**13**).[6b,f,g] By analogy to **14**, the cross-bridging reactions in the spinosyn case could also take place when the polyketide intermediate **10** is still tethered to ACP. In this scenario, C-15 oxidation should occur on the polyketide intermedi-

Scheme 4

Scheme 5

ate **3** to give **10**, which would then undergo cyclization to form the tricyclic polyketide **11** (Scheme 1, route C). In this route, the intermediacies of **4**−**8** are no longer necessary.

Intrigued by the complexity of the tetracyclic nucleus of the spinosyns, especially the possible involvement of a Diels−Alder-type [4 + 2] cyclization (**7** → **8** or **10** → **11**), we decided to study the proposed cross-bridging reactions by first focusing on the initial oxidation step to distinguish the three possible pathways. Herein, we report the expression and purification of SpnJ, the chemical synthesis of **3** and **4**, and the results showing that **4**, but not **3**, is a substrate for SpnJ. Our results clearly demonstrate that SpnJ is the predicted oxidase and is specific for C-15 oxidation of mac-rolactone **4**. These findings provide compelling evidence establishing the early sequence of events in the cross-bridging reactions.

Outlined in Scheme 3 is our retrosynthetic analysis for the preparation of **3** and **4**, in which two main couplings were envisioned: a Julia-Kocienski olefination of sulfone **15** with aldehyde **16** to establish the *E*-alkene geometry at C-12/C-13, and a palladium-mediated Heck reaction to connect the C-5/C-6 carbon−carbon bond with **17**. Once the linear polyketide **3** is constructed, a Yamaguchi macrolactonization to form the ester linkage at C-1 will afford **4**.[7]

Our synthesis of the aryl sulfone **15**, shown in Scheme 4, began with the Weinreb amide **18** derived from δ-valerolactone.[8] Asymmetric addition[9] of diethyl zinc catalyzed by (−)-(1*S*,2*R*)-*N*,*N*-dibutylnorephedrine [(−)-DBNE] provided **19**, whose enantiomeric purity (92% ee) and absolute configuration were determined by NMR analysis of the corresponding (*R*)- and (*S*)-Mosher esters. Subsequent silylation followed by reduction furnished **20**. Aldol reaction[10] between **20** and Crimmins' oxazolidinethione auxiliary **21** gave the *syn*-aldol adduct **22**. The configurations at C-16/C-17 were determined by application of the Zimmerman−Traxler transition state model as well as analysis of the $J_{16,17}$ values, which are in accord with literature precedent.[11] Compound **23**, derived from **22** via silylation and reduction, was subjected to Ley oxidation and then Brown's (+)-Ipc$_2$B(allyl) reagent[12] to yield **24** as a single stereoisomer. The relative configurations at C-15/C-17 were determined by conversion of **24** to the corresponding 1,3-acetonide followed by application of Rychnovsky's ^{13}C NMR method,[13]
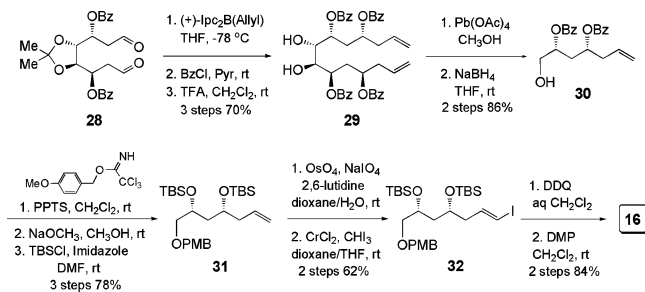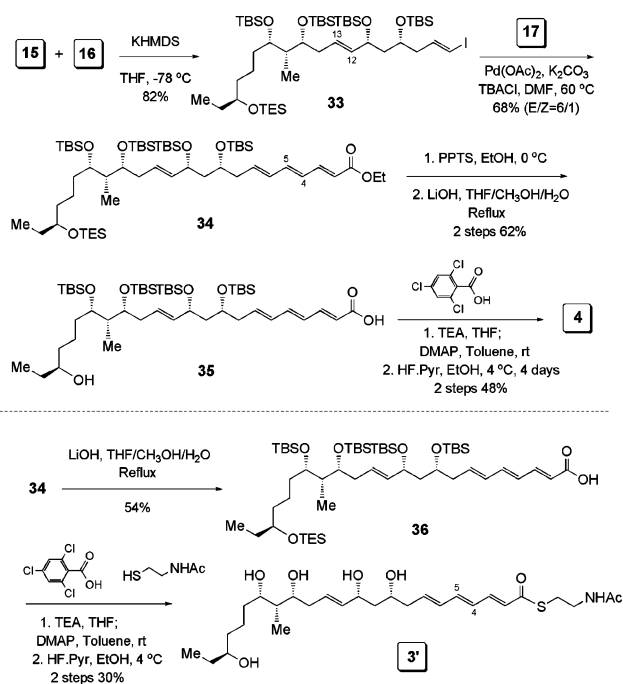
providing proof for a 1,3-*syn* relationship. Silyl ether protection followed by a two-step Lemieux−Johnson oxidation of the terminal olefin and reduction of the resulting aldehyde gave **26**. It was crucial to conduct the oxidation of the terminal alkene under neutral pH as a nonbuffered reaction medium resulted in unwanted deprotection of the triethylsilyl ether at C-15 which required reprotection. Completion of the requisite sulfone **15** was achieved by conversion of **26** to aryl sulfide **27** under Mitsunobu conditions followed by oxidation employing hydrogen peroxide and ammonium molyb-date.[14]

Our synthesis of aldehyde **16** is depicted in Scheme 5. Asymmetric Brown allylation of dialdehyde **28**, obtained from D-mannitol,[15] followed by benzoylation of the newly generated secondary alcohols and removal of the isopropylidene protecting group under acidic hydrolysis afforded diol **29** in excellent overall yield. Oxidative cleavage of the vicinal diol in **29** facilitated by lead tetraacetate followed by sodium borohydride reduction of the ensuing aldehyde afforded primary alcohol **30**. Next, protection of the alcohol functionality in **30** as its PMB ether followed by exchange of the benzoate protecting groups for silyl ethers provided **31**. Following an oxidation of the terminal olefin in **31** using Jin's protocol,[16] the resulting aldehyde was subjected to Takai olefina-tion,[17] affording the desired (*E*)-vinyliodide **32** in good overall yield as a single stereoisomer. Oxidative removal of the PMB ether employing DDQ in aqueous dichloromethane and oxidation of the liberated hydroxy group with Dess−Martin periodinane then provided aldehyde **16**.

With fragments **15** and **16** in hand, we assembled the presumed biosynthetic precursors **3** and **4** (Scheme 6). The use of KHMDS in THF provided the best yield and stereoselectivity in forming the C(12)−C(13) olefin by the Julia-Kocienski olefination[18] pro-tocol. Completion of the triene moiety was accomplished by a Heck reaction involving **33** with ethyl dienoate **17**[19] under ligand-free conditions,[20] affording **34** as a mixture of isomers (*E*/*Z* = 6/1 at C(4)−C(5)) that were separated by flash column chromatography. Chemoselective deprotection of the TES ether utilizing PPTS in ethanol followed by saponification furnished *seco*-acid **35**. Acid **35** was then subject to Yamaguchi macrolactonization[21] followed by global deprotection of the silicon-based protecting groups employing HF•Pyr in cold ethanol for 4 days to give **4** in moderate yields.[22] The preparation of the linear substrate **3** was achieved in a similar manner. Due to the difficulty of preparing the ACP-bound linear polyketide chain, the intended linear substrate **3** was synthesized as the *N*-acetylcysteamine (NAC) thioester **3′**. Since the corresponding *N*-acetylcysteamine (NAC)-linked thioesters have been commonly used in the biosynthetic studies as surrogates for ACP-bound acyl esters, **3′** is expected to be an effective substitute for **3**. As shown in Scheme 6, saponification of **34** afforded acid **36**, which was then coupled with *N*-acetylcysteamine under Yamaguchi esterification conditions. Final deprotection using HF•Pyr complex led to the desired linear substrate **3′**.[22]

Scheme 6

15 + **16** →(KHMDS, THF, -78 °C, 82%)→ **33** →(**17**, Pd(OAc)$_2$, K$_2$CO$_3$, TBACl, DMF, 60 °C, 68% (E/Z=6/1))→

34 →(1. PPTS, EtOH, 0 °C; 2. LiOH, THF/CH$_3$OH/H$_2$O, Reflux, 2 steps 62%)→ **35** →(1. TEA, THF; DMAP, Toluene, rt; 2. HF·Pyr, EtOH, 4 °C, 4 days, 2 steps 48%)→ **4**

34 →(LiOH, THF/CH$_3$OH/H$_2$O, Reflux, 54%)→ **36** →(1. TEA, THF; DMAP, Toluene, rt; 2. HF·Pyr, EtOH, 4 °C, 2 steps 30%)→ **3′**

Figure 1. (1) TLC and (2) HPLC analysis of incubation mixture without SpnJ (A) or with SpnJ (B)[21] (TLC: silica gel, CH$_2$Cl$_2$/MeOH = 93:7; HPLC: C-18, 2% NH$_4$OAc/CH$_3$CN = 70:30 to 20:80 over 120 min).

remaining enzymes (SpnF, SpnL, and SpnM) believed to be involved in the cross-bridging event as well as their biological roles leading to an understanding of the mechanism of formation for the spinosyn aglycone.

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

With sufficient quantities of **3′** and **4** now available, we evaluated whether the linear polyketide thioester **3′** or the macrolactone **4** is a substrate for SpnJ, which is predicted to be an oxidase. Since the functions of SpnF, SpnL, and SpnM are not obvious, it would be interesting to examine whether SpnJ is a multifunctional enzyme capable of catalyzing not only oxidation at C-15 but also deprotonation at C-14 to initiate the Michael addition to form a C–C bond between C-3 and C-14, or the 1,4-elimination of the 11-OH group setting the stage for a possible Diels–Alder-type [4 + 2] cycloaddition.

To characterize SpnJ, the *spnJ* gene was cloned into a pET28b-(+) vector and the resulting construct was expressed in *E. coli* BL21(DE3) cells.[22] After induction under 25 mM isopropyl β-D-thiogalactopyranoside (IPTG), the N-terminal His$_6$-tagged SpnJ protein (60.6 kDa) was purified to near homogeneity using Ni-NTA resin.[22] The absorption spectrum of the purified protein is typical for that of a flavoprotein. To determine the function of SpnJ, SpnJ (20 μM) was incubated with 2 mM **3′** and 40 μM FAD in Tris-HCl buffer (pH 8.0) at 29 °C. No new product was detected after overnight incubation. Because **3′** cannot be processed by SpnJ, it is reasonable to conclude that **3** is not a substrate for SpnJ.

In contrast, TLC and reverse-phase HPLC analysis of the incubation mixture containing SpnJ (20 μM), 2 mM **4**, and 40 μM FAD in Tris-HCl buffer (pH 8.0) revealed the time-dependent formation of a new product (nearly quantitative conversion after 4 h at 29 °C, see Figure 1), which was identified by ^{1}H and ^{13}C NMR spectroscopy as well as HR-CIMS analysis as the C-15 ketone derivative **5**.[22] These results clearly established that SpnJ is a flavin oxidase and **4** is the substrate for SpnJ. The fact that no trace of cross-bridging products was detected indicated that SpnJ is only a monofunctional oxidase. These results provide strong support for the conversion of **4** to **5** as the first step in the post-cyclization reactions (vide supra) and set the stage for the study of the putative Diels–Alderase activity.

In summary, the experiments described herein detail a convergent synthesis of **4** postulated to be involved in spinosyn biosynthesis. More importantly, we have validated the catalytic function of SpnJ and established **4** as the precursor of the tricyclic nucleus of spinosyns. Future work will encompass characterization of the
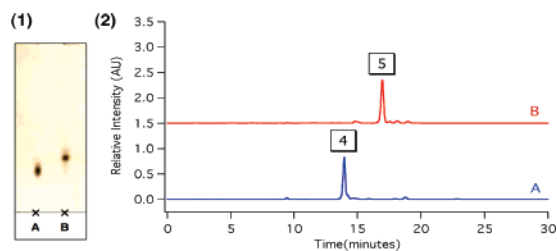
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