Engineering of the Spinosyn PKS: Directing Starter Unit Incorporation

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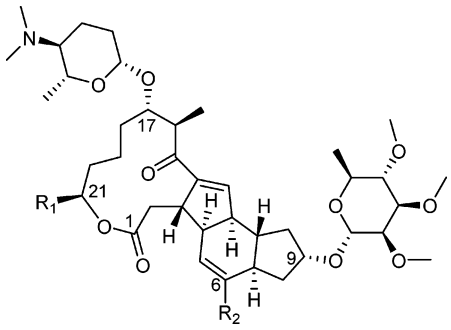
The spinosyns are a family of potent and highly selective insect control agents that display a favorable environmental profile. As some regions of the spinosyn molecule are recalcitrant to chemical modification, a targeted genetic approach was carried out to generate new analogues. The polyketide synthase (PKS) loading modules from the avermectin PKS of *Streptomyces avermitilis* and the erythromcyin PKS of *Saccharopolyspora erythraea* were each used to replace the spinosyn PKS loading module. Both of the resulting strains containing hybrid PKS pathways produced the anticipated spinosyn analogues. Supplementation of the culture media with a range of exogenous carboxylic acids led to the successful incorporation of these novel elements to yield further novel spinosyn molecules, some of which demonstrated potent and new insecticidal activities. Furthermore, it has been demonstrated that semisynthesis of such novel metabolites can then be used to generate active analogues, demonstrating the effectiveness of utilizing these complementary methods to search the chemical space around this template.

The spinosyns are a family of tetracyclic macrolide polyketides first identified in the soil organism *Saccharopolyspora spinosa*. Spinosyns are the active ingredients in Dow AgroSciences' (DAS) Naturalyte family of potent agricultural insect control agents. Products such as Tracer are highly effective against target insects and have an excellent environmental and mammalian toxicological profile.[1] Fermentation of *S. spinosa* produces two major active components, spinosyn A (**1**) and spinosyn D (**2**), which differ from each other by a single methyl group at C-6 (Figure 1). Other members of the spinosyn family, produced as minor metabolites in *S. spinosa*, exhibit different methylation patterns and are significantly less active.[2]

The spinosyn biosynthetic gene cluster has previously been cloned from *S. spinosa* and sequenced.[3] Biosynthesis is proposed to occur by processive polyketide chain extension on a modular type I polyketide synthase (PKS) to form a decaketide product. Formation of the decaketide is followed by, or accompanied by, an intramolecular cyclization in a (presumably enzyme-mediated) process resembling a Diels−Alder cycloaddition, as discussed by Martin and co-workers,[4] to yield a tetracyclic aglycone. Biosynthesis then proceeds with the addition of L-rhamnose to the hydroxyl group at C-9 of the aglycone, which is subsequently 2,3,4-tri-*O*-methylated to yield compounds described as pseudoaglycones. This is followed by addition of D-forosamine to the hydroxyl group at C-17 to yield the final spinosyn molecule. Addition of the two deoxysugars L-rhamnose and D-forosamine is essential for biological activity.[5,6]

The search for novel spinosyns with a different spectrum of activity has led to the generation of many semisynthetic analogues by derivatization of the available chemical handles of the spinosyn molecules.[7,8] The "western shore" of **1**, which is biosynthesized by the first three modules of the PKS, is not readily amenable to semisynthetic modification. Manipulation of the pathway governing the biosynthesis of spinosyns is therefore a preferred route for modifying this region of the molecule.

Compounds produced by type I PKSs exhibit a wide range of structural diversity, which is reflected in the range of bioactivities they display, including antibiotic, insecticidal, antifungal, anticancer, antiparasitic, and immunosuppressive properties. Type I PKSs consist of giant multifunctional proteins that contain discrete modules of active domains. Each module catalyzes a single specific cycle of polyketide chain extension and processing of the nascent β-keto functionality. The modular nature of these systems as first described for the erythromycin (*ery*) PKS in *Saccharopolyspora erythraea*[9,10] lends itself to engineering approaches for rationally altering the structures of their products in a targeted manner. Hybrid PKSs have been used to generate targeted novel compounds in a number of ways including loading module swaps,[11] module insertions,[12] and acyl transferase (AT) domain swaps.[13] These reports demonstrate the effectiveness of biosynthetic engineering approaches in the production of libraries of novel compounds. Here, we investigate loading module swaps in the spinosyn (*spn*) PKS with the aim of constructing hybrid PKS pathways that generate spinosyns with novel C-21 groups.



R_1 = Et, R_2 = H, spinosyn A (**1**)
R_1 = Et, R_2 = Me, spinosyn D (**2**)
R_1 = Me, R_2 = H, spinosyn E (**3**)

Figure 1. Chemical structures of natural spinosyns.

In the erythromycin PKS, the loading module consists of an acyltransferase domain (AT0) and an acyl carrier protein domain (ACP0). AT0 is responsible for recruiting a propionyl-CoA starter unit, which is then extended by the action of six extension modules and macrocyclization to produce a heptaketide polyketide molecule. The avermectin (*ave*) PKS from *Streptomyces avermitilis* also has an AT0ACP0 type loading module, which has been demonstrated, using a precursor-directed approach, to accept numerous C2 branched carboxylic acids as alternative starter units and thus

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generate novel avermectin analogues.[14] Replacing the native loading module of the erythromycin PKS with the *ave* loading module, by genetic engineering methods, transferred the specificity for the recruitment of C2 branched carboxylic acids to the resulting hybrid PKS, allowing the production of novel erythromycin analogues.[11] Erythromycin analogues produced following recruitment of branched starter units, equivalent to those of avermectin biosynthesis (isobutyryl-CoA and (*2S*)-*sec*-butyryl-CoA), were observed as products from this hybrid PKS. Moreover, further novel erythromycin analogues with branched starter units were produced when the culture medium was enriched with the appropriate carboxylic acids.[15] Interestingly, it has been demonstrated that the *ery* PKS loading module can also accept a range of branched chain or cyclic carboxylic acids, albeit a more limited range and with lower efficiency than the *ave* loading module.[15]

As further PKS sequences were published, it became apparent that AT0ACP0 didomain-type loading modules are rather uncommon. The majority of modular PKSs identified so far possess loading modules that contain an additional N-terminal domain, named KSQ. Each KSQ closely resembles a KS domain, but the active site cysteine residue is replaced by a glutamine (Q) to give the conserved amino acid sequence VDxx**Q**SSS. It has been demonstrated that polyketide chain initiation on these PKS clusters occurs via KSQ-catalyzed decarboxylation of the CoA thioesters of dicarboyxlic acids that are recruited by the AT0 domain.[16] In general the KSQ-containing tridomain loading modules appear to have a stricter substrate specificity than the AT0ACP0 didomain loading modules.[17]

The first open reading frame of the spinosyn PKS (*spnA*) encodes the loading and first extension modules.[3] The loading module is of the tridomain-type, comprising KSQ, AT0, and ACP0. Unusually for a KSQ-containing loading module, it has flexible substrate specificity and recruits both methylmalonyl-CoA and malonyl-CoA. Incorporation of malonyl-CoA results in production of spinosyn E (**3**), which differs from **1** in that it has a methyl at position C-21 instead of an ethyl moiety (Figure 1). This alteration also results in a reduction in insecticidal activity.[18]

We describe engineering of the portion of *spnA* encoding the loading module, in order to produce novel spinosyn analogues. The specificities of the *ery* and *ave* loading modules were transferred to generate hybrid PKSs, resulting in the production of novel compounds containing modifications at C-21. This is the first report of successful genetic engineering of the spinosyn biosynthetic gene cluster and demonstrates the potential of this approach for the generation of multiple novel compounds. The compounds produced in this study were found to exhibit new and potent insecticidal activity.

Results and Discussion

The loading module of the *ery* PKS controls the selection of propionyl-CoA, but also allows "non-natural" starters to be incorporated when exogenous carboxylic acids are fed to the fermentation medium.[15] In order to transfer this relaxed specificity to the spinosyn PKS, the *ery* load was spliced upstream of, and in-frame with, the KS1 domain of *spnA* as described in the Experimental Section. An *Nhe*I restriction enzyme sequence was introduced at the junction between the two PKS fragments, which were spliced together at the start of the spinosyn KS1 domain. The introduction of this site resulted in a conservative amino acid change, isoleucine to leucine, close to the beginning of the *spn*KS1 (Figure 2). The strategy then involves splicing the *ery*AT0ACP0 didomain loading module as an *Nde*I-*Nhe*I fragment to the spinosyn KS1 at the *Nhe*I site in the equivalent position. The hybrid gene construct was cloned into the apramycin resistant plasmid pLSB3, to give plasmid pLSB62, placing it under the control of the promoter for resistance to pristinamycin (P_{ptr}).[19] Upon introduction into *S. spinosa*, homologous recombination occurred within the region of

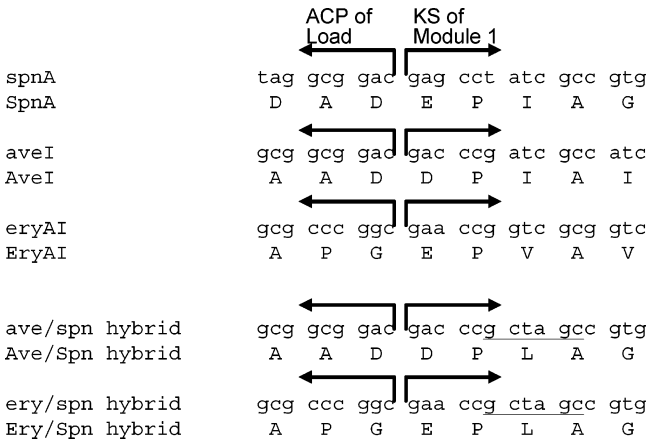


Figure 2. Introduction of conservative amino acid changes by PCR amplification to facilitate generation of the hybrid PKS gene fragments.

the *spnA* gene contained on pLSB62, to yield a strain containing a hybrid *ery/spn* PKS under the control of P_{ptr} (Figure 3). Integration within *spnA* results in a truncated *spnA* gene under the control of the natural spinosyn promoter. However, only the full-length *ery*/*spn* hybrid PKS protein encodes the natural C-terminus of the protein, which includes a "docking region" for association with SpnB and therefore will be able to dock successfully with SpnB to form an active PKS.[20] This means there is no competition from the truncated form of SpnA and it is not necessary to resolve the plasmid. This strategy also addresses the problem of chromosomal instability inherent to this organism; the spinosyn gene cluster of *S. spinosa* lies in an unstable region of the chromosome and is prone to deletion, which leads to nonproductive isolates.[21] As the engineered strains reported here were each generated by a single integration event, they retain the resistance marker of the introduced plasmid, which is used for selection and thus to maintain strain integrity during fermentation.

S. spinosa NRRL 18537 was transformed by conjugation with plasmid pLSB62 using standard techniques.[21] A number of exconjugants were screened by PCR amplification for the presence of integrated plasmid. One correct colony was demonstrated to carry the plasmid integrated within *spnA* and, more specifically, to contain the *ery/spn* hybrid gene under the control of P_{ptr} (Figure 3). This strain was designated strain *S. spinosa* BIOT-1066.

Cultures of *S. spinosa* BIOT-1066 were grown in production medium as described in the Experimental Section. The major compounds produced were identified as spinosyns A (**1**) and E (**3**) along with spinosyn D (**2**) as a minor product. These products are fully "processed", indicating that the timing of production of the PKS proteins from the heterologous promoter is compatible with production of the enzymes catalyzing the later steps of biosynthesis. The titer of this strain was 15−25 mg/L of total spinosyns, with approximately equal amounts of **1** and **3**. Most significantly the ratio of products was different from the wild-type strain, with the relative production of **3** being significantly increased. *S. spinosa* NRRL 18537 produces predominantly **1** and **2** at a ratio of 85:15 and accounting for 90% of total spinosyn production, with **3** being produced at 2−5% of total spinosyns. The altered ratio of products most probably reflects the different substrate specificity of the *ery* loading module, relative to that of the *spn*PKS, as well as the substrate supply available within *S. spinosa*. Recruitment of acetyl-CoA by the *ery* loading module occurs naturally in *S. erythraea*, most notably when the PKS pathway is upregulated.[22]

This result shows that the heterologous promoter, the promoter for resistance to pristinamycin, can be successfully used in *S. spinosa*. This is important to the success of these experiments due to the lack of knowledge regarding the regulation of the spinosyn biosynthetic gene cluster. The decoupling of regulation from

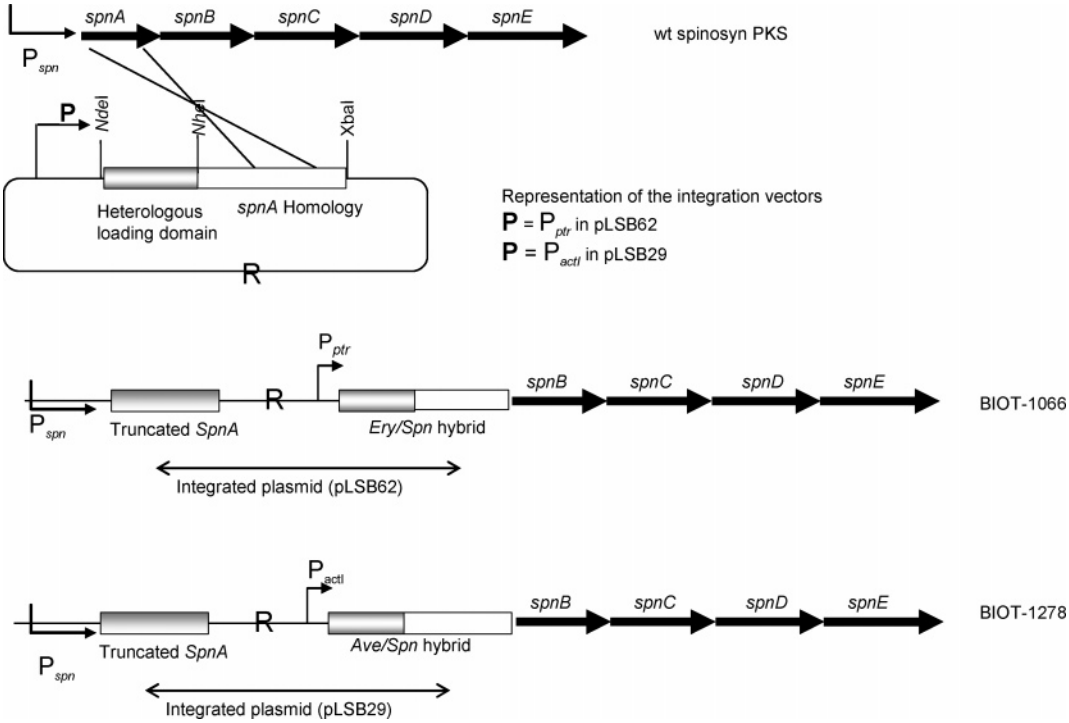


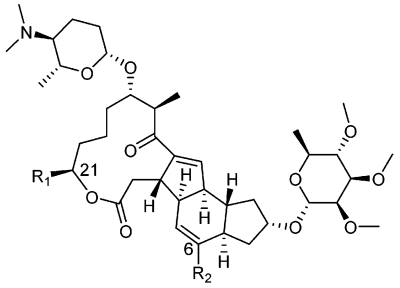
Figure 3. Diagrammatic representation of the organization of the spinosyn biosynthetic gene cluster and the hybrid strains BIOT-1066 and BIOT-1278.

compound production should avoid any regulation issues, particularly as the post-PKS processing steps are complex and the enzymes involved are challenged to accept novel substrates. The additional observation that not only does this promoter function in *S. spinosa*, but also the timing of expression of the PKS is compatible with expression of the late genes from their native promoters was crucial to our success.

BIOT-1066 was also used as a platform to generate novel spinosyn compounds by feeding exogenous carboxylic acids to fermentation cultures. A variety of acids were examined. LC-MS analysis of culture broths showed new components consistent with the successful incorporation of cyclobutanecarboxylic acid, cyclopropanecarboxylic acid, methylthioacetic acid, and cyanoacetic acid (Figure 4) to generate 21-desethyl-21-cyclobutylspinosyns A (**4**) and D (**5**), 21-desethyl-21-cyclopropylspinosyns A (**6**) and D (**7**), 21-desethyl-21-methylthiomethylspinosyns A (**8**) and D (**9**), and 21-desethyl-21-cyanomethylspinosyns A (**10**) and D (**11**), respectively (Figure 4). These novel compounds all displayed UV chromophores and MS fragmentation patterns characteristic of the spinosyn class of compounds and could not be observed in fermentations to which the exogenous acids were not fed (see Table 1).

To isolate and confirm the identities of **4**−**7**, larger-scale fermentations were carried out. *S. spinosa* BIOT-1066 was grown in production medium in a bioreactor for 7 days. Cyclobutanecarboxylic acid was fed 24 h post-inoculation to a final concentration of 5 mM. The Experimental Section describes the isolation of **4** and **5** from this fermentation. Cyclopropanecarboxylic acid was fed in similar fashion to *S. spinosa* BIOT-1066 to yield **6** and **7**. Each of the compounds **4**−**7** was structurally characterized using a combination of multidimensional NMR techniques, MS/MS analysis, UV analysis, and comparison to related compounds as described in the Experimental Section. Briefly, for compound **4** the majority of assignments were straightforward, especially when compared to other standard compounds (i.e., spinosyn A). Key COSY, HMQC, and HMBC correlations were all observed, allowing clear assignment of the cyclobutyl moiety attached to C-21. The limited amount of **5** precluded full assignment, but LCMS/MS and the identification of spin systems for the C-6 and C-21 were consistent with the anticipated structure. For **6** the majority of assignments were again straightforward when compared to other compounds. However, assignment of the cyclopropyl moiety was difficult, as no HMQC correlations could be observed; the assignment presented here was made on the basis of strong COSY correlations and other observations. The H-21 resonance was shifted upfield from δ_H 4.67 (spinosyn A) to δ_H 4.18. The H-23 methyl group resonance characteristic of an ethyl group attached to C-21 was not observed. The chemical shifts of three new resonances (δ_H 0.89, 0.44, and 0.14) were consistent with the introduction of a cyclopropyl moiety attached to C-21. A COSY correlation from the H-21 resonance (δ_H 4.18) to a resonance at δ_H 0.89 allowed assignment of H-22. The H-22 resonance was correlated to protons resonating at δ_H 0.44 and 0.14 (H-23 and H-24). Compound **7** was not isolated, but analysis of the crude extract by LCMS/MS and UV data was consistent with the anticipated structure.

The avermectin loading module AT0ACP0 is responsible for incorporation of the branched chain isopropionic and *sec*-butanoic acids into the avermectin molecule. Previous work has shown that placing the avermectin loading module upstream of the erythromycin PKS in place of its natural loading module leads to erythromycin compounds with branched chain starters.[11] The avermectin loading module has also been shown to incorporate CoA esters of a broad range of free acids fed to the production medium, both in its native environment[14] and as part of the genetically engineered *ave/ery* hybrid pathway.[15] With the aim of transferring this broad specificity to the *spn* PKS, splicing the avermectin loading module adjacent to the spinosyn KS1 should lead to branched units at C-21 of the spinosyn molecule. Using a strategy analogous to that for construction of the *ery/spn* hybrid PKS, the avermectin AT0ACP0 was cloned upstream of and in-frame with *spnA* using the same *Nhe*I restriction site at the start of the spinosyn KS1 as the junction between the two PKS fragments (Figure 2), as described in the Experimental Section, to give plasmid pLSB29. Plasmid pLSB29 is an apramycin-resistant conjugative vector that contains the avermectin loading module spliced in-frame with the spinosyn KS1 under the control of P_{actI}, with sufficient *spnA* homology to allow homologous recombination into the spinosyn PKS. Plasmid pLSB29 was transformed by conjugation into *S.*



R_1	R_2	Compound	
	H	21-desethyl-21-(cyclobutyl)spinosyn A	**4**
	Me	21-desethyl-21-(cyclobutyl)spinosyn D	**5**
	H	21-desethyl-21-(cyclopropyl)spinosyn A	**6**
	Me	21-desethyl-21-(cyclopropyl)spinosyn D	**7**
	H	21-desethyl-21-(methylthiomethyl)spinosyn A	**8**
	Me	21-desethyl-21-(methylthiomethyl)spinosyn D	**9**
	H	21-desethyl-21-(cyanomethyl)spinosyn A	**10**
	Me	21-desethyl-21-(cyanomethyl)spinosyn D	**11**
	H	21-desethyl-21-(isopropyl)spinosyn A	**12**
	Me	21-desethyl-21-(isopropyl)spinosyn D	**13**
	H	21-desethyl-21-(*sec*-butyl)spinosyn A	**14**
	Me	21-desethyl-21-(*sec*-butyl)spinosyn D	**15**
	H	21-desethyl-21-(*n*-propyl)spinosyn A	**19**
	H	21-desethyl-21-(2-furanyl)spinosyn A	**17**
	H	21-desethyl-21-(2-methylcyclopropyl)spinosyn A	**18**

Figure 4. Compounds derived from feeding carboxylic acids to production cultures of engineered *S. spinosa* strains.

Table 1. LCMS/MS Data for Novel Compounds Produced by Strain BIOT-1066 When Fermentation is Supplemented by Exogenous Acids

carboxylic acid fed	retention time (min)[a]	key mass spectral data (*m*/*z*)	novel product
cyclopropanecarboxylic acid	23.5	744.4 $[M + H]^+$; 142.4 [forosamine − OH]$^+$	21-desethyl-21-cyclopropyl-spinosyn A (**6**)
cyclopropanecarboxylic acid	25.0	758.5 $[M + H]^+$; 142.3 [forosamine − OH]$^+$	21-desethyl-21-cyclopropyl-spinosyn D (**7**)
cyclobutane carboxylic acid	25.7	758.5 $[M + H]^+$; 142.3 [forosamine − OH]$^+$	21-desethyl-21-cyclobutyl-spinosyn A (**4**)
cyclobutanecarboxylic acid	27.3	772.5 $[M + H]^+$; 142.2 [forosamine − OH]$^+$	21-desethyl-21-cyclobutyl-spinosyn D (**5**)
methylthioacetic acid	22.9	764.4 $[M + H]^+$; 142.3 [forosamine − OH]$^+$	21-desethyl-21-methylthiomethyl-spinosyn A (**8**)
methylthioacetic acid	24.3	778.5 $[M + H]^+$; 142.3 [forosamine − OH]$^+$	21-desethyl-21-methylthiomethyl-spinosyn D (**9**)

[a] Chromatographic conditions are described in the Experimental Section.

spinosa NRRL 18538. Apramycin-resistant exconjugants were confirmed to contain the *ave/spn* hybrid genes by PCR analysis and Southern blot (Figure 3). One such strain was designated *S. spinosa* BIOT-1278.

Cultures of *S. spinosa* BIOT-1278 produced mainly **1**, **2**, and **3** through the recruitment of propionyl- and acetyl-CoA. Analysis by LC-MS also showed components consistent with production of low levels of the expected novel products 21-desethyl-21-isopropylspinosyns A (**12**) and D (**13**) and 21-desethyl-21-*sec*-butylspinosyns A (**14**) and D (**15**) (Table 2 and Figure 4). Feeding the branched chain acids isopropyl and 2-methylbutyl carboxylic acid did not alter the yield of branched starter products made by fermentation of BIOT-1278. Low amounts of these compounds were present in all fermentations presumably due to the intracellular concentration of precursors. As the *ave/ery* hybrid pathway had been shown previously to incorporate a wide range of novel starter acids, it was anticipated that the strain containing the *ave/spn* pathway could be similarly used to generate an array of novel compounds. A broad range of free acids were thus fed to *S. spinosa* BIOT-1278. New compounds were identified on the basis of retention time, characteristic UV chromophore, and mass spectral fragmentation.

Table 2. LCMS/MS Data for Branched Starter Compounds Produced by Strain BIOT-1278

carboxylic acid starter unit	retention time (min)[a]	key mass spectral data (m/z)	novel product
isopropylcarboxylic acid	25.1	746.5 $[M + H]^+$; 142.3 [forosamine − OH]$^+$	21-desethyl-21-isopropylspinosyn A (**12**)
isopropylcarboxylic acid	26.3	760.5 $[M + H]^+$; 142.3 [forosamine − OH]$^+$	21-desethyl-21-isopropylspinosyn D (**13**)
2-methylbutylcarboxylic acid	26.7	760.4 $[M + H]^+$; 142.3 [forosamine − OH]$^+$	21-desethyl-21-*sec*-butylspinosyn A (**14**)
2-methylbutylcarboxylic acid	27.5	774.5 $[M + H]^+$; 142.3 [forosamine − OH]$^+$	21-desethyl-21-*sec*-butylspinosyn D (**15**)

[a] Chromatographic conditions are described in the experimental section

Feeding cyclobutanecarboxylic acid led to production of **4** and **5** along with a significant amount of a new compound (**16**), the C-17-pseudoaglycone of **4** (Figure 4). In this strain **4** and **5** were detected at higher titers than from BIOT-1066, with **4** being produced at ~5−10 mg/L. Feeding cyclopropanecarboxylic acid led to the production of **6** and **7** as anticipated. These analogues were again identical to those isolated from the engineered strain *S. spinosa* BIOT-1066 but were produced at higher titer. Methylthioacetic acid was also recruited by the PKS and again led to increased yields of **8** compared to that of the equivalent *ery/spn* loading experiment. In further experiments, 2-furoic acid and methylcyclopropanecarboxylic acid were also accepted by the PKS to give detectable, but low yields of the novel products 21-desethyl-21-(2-furanyl)spinosyn A (**17**) and 21-desethyl-21-(2-methylcyclopropyl)spinosyn A (**18**).

Isolation of some of these novel spinosyns was carried out in order to confirm their identities and to obtain an assessment of insecticidal activities. *S. spinosa* BIOT-1278 was grown in production medium in a 20 L stirred bioreactor as described in the Experimental Section. The culture was fed with 2-methylbutyric acid at 26 and 37.5 h post-inoculation to an overall final concentration of 4 mM with the aim of isolating the branched chain starter unit derived spinosyns. However, feeding of 2-methylbutylcarboxylic acid had no effect on the titer of 21-desethyl-21-*sec*-butylspinosyn A (**14**), and this was not isolated due to insufficient material being present. However, 21-desethyl-21-isopropylspinosyn A (**12**) was produced at a low but sufficient level to allow its isolation after protracted HPLC. Similarly, 21-desethyl-21-*n*-propylspinosyn A (**19**) was isolated, and details are presented in the Experimental Section. Although a full NMR assignment of **12** could not be completed, a clear A-type H-5 resonance was observed. The pair of doublets resonating at δ_H 0.85 was consistent with the presence of an isopropyl group; these correlated to C-21 at δ_C 80 and importantly to one another in a HMBC experiment. For **19** the majority of assignments were straightforward when compared to other compounds. However, assignment of the *n*-propyl moiety itself was problematic due to weak signals in the correlation experiments. Differentiation from the potential isopropyl moiety of alternative compounds was straightforward, as the doublet signal, which would be required for any prochiral methyl groups, was not present. In the COSY spectrum methyl protons resonating at δ_H 0.86 ($J = 7.4$ and 7.3 Hz) were correlated to a pair of protons resonating at δ_H 1.25; these signals were assigned as H-24 and H-23, respectively. The H-21 resonance was correlated to a proton resonance at δ_H 1.50 (H-22). A very faint correlation could be observed between H-22 and H-23 in the COSY spectrum.

Semisynthesis of spinosyn has previously been used to generate libraries of compounds.[7,8] To illustrate the utility of these methods for templates generated by genetic engineering, **4** was hydrogenated as described in the Experimental Section to yield 5,6-dihydro-21-desethyl-21-cyclobutylspinosyn A (**20**).

In the experiments described above, the titers of compounds produced by both of the engineered strains were much lower than the wild-type strain. In the following discussion, productivity is compared to the production of total spinosyns by the wild-type strain, which is consistently in the region 300(±30) mg/L. For BIOT-1066 (erythromycin loading module) the major products were **1** and **3**, which were produced at a total of 15−25 mg/L in approximately equal amounts, representing 5−8% of the total spinosyn production from the wild-type. Interestingly, the titer of spinosyn E from BIOT-1066 and the wild-type are roughly the same, but when produced from BIOT-1066, there is significantly less production of other spinosyns. BIOT-1066 would therefore represent a preferred production strain for spinosyn E. BIOT-1278 (avermectin loading module) produced predominantly **1**−**3**, and these together represent 2−5% of the wild-type production titer (6−15 mg/L), with the branched starter spinosyns **12**−**15** also being produced in all fermentations of BIOT-1278 but equivalent only to 1% of the wild-type titers. When exogenous starter acids were added to the fermentations of either of these engineered strains, **1**−**3** were always observed, and this had no impact on the titers of these products. Titers of the compounds generated by addition to BIOT-1066 were approximately 5 mg/L for cyclobutanecarboxylic acid (production of **4** and **5**) and 4 mg/L for cyclopropyanecarboxylic acid (production of **6** and **7**). Titers of the compounds generated by feeding to BIOT-1278 were better with the mixture of **4** and **5** (and the equivalent C-17-pseudoagalycone), being approximately 10−15 mg/L, very similar to the production of **1**−**3** by this strain; **6** and **7** were produced at similar levels when cyclopropancarboxylic acid was added to the fermentation. The novel products generated by the addition of all other exogenous starter acids were produced at titers too low to allow quantification.

While no attempt was made to optimize the production of these compounds, it is clear that our PKS engineering had a detrimental effect on productivity. This reduction in efficiency may be caused by nonoptimal protein structure in the hybrid PKS with poor cooperation between the heterologous loading module and the native module 1. Alternatively an issue may reside in the substrate selectivity of the AT domain of the loading module or in the substrate tolerance of the downstream domains. Finally, a problem may reside in the cellular machinery required to convert the exogenous carboxylic acids to their CoA esters for use as substrates. However, the similar production levels for the branched chain analogues **12**−**15** in comparison to **1**−**3** would argue against this. In short, there are multiple starting points for an investigation to improve the productivity of this system.

Insect control assays were performed, as described in the Experimental Section, with those novel compounds from this study that could be isolated in sufficient yield (Table 3). All of the new compounds tested exhibited activity against one or more agronomically important pests. However, the species clearly differed in their susceptibility to both the C-21 derivatives from this study and spinosyn A. With the exception of some enhanced activity against beet armyworm and cabbage looper larvae, simple extension of the C-21 substituent from ethyl (spinosyn A, **1**) to *n*-propyl (**19**) provided only a marginal improvement in activity. Cyclization of the C-21-*n*-propyl (**19**) to C-21-cyclopropyl (**6**) resulted in lower activity. While only limited data are available, replacement with a C-21-isopropyl (**12**) appeared to result in activity that is at best equivalent to, or less than, the C-21-*n*-propyl (**19**). Compared to the C-21-*n*-propyl derivative (**19**) and **1**, the C-21-cyclobutyl derivative (**4**) exhibited improved activity against some of the sucking insect pests, but declined against some of lepidopteran insects. Interestingly, the largest consistent improvement in activity among these new derivatives is observed for the C-21-cyclobutyl-5,6-dihydro derivative (**20**). With the exception of green peach

Table 3. Bioassay (LC_{50}) Data for Selected Novel Spinosyn Derivatives

		cotton aphid	green peach aphid	whitefly	tobacco budworm	beet armyworm	cabbage looper	two-spotted spider mite
cpd #	compound	LC_{50} ppm	LC_{50} ppm	LC_{50} ppm	LC_{50} μg/larva	LC_{50} μg/larva	LC_{50} μg/larva	LC_{50} ppm
1	spinosyn A	65	50	20	0.03	0.63	0.03	5.30
2	spinosyn D	~50	50	1.7				19.0
3	spinosyn E	>50	>50	3.9				10−100
4	21-cyclobutyl A	~3	>50	55	0.013	>1	0.124	>50
5	21-cyclobutyl D	15	>50	13.5	0.016	0.385	0.01	
20	5,6-dihydro-21-cyclobutyl A	1.7	>50	0.7	0.02	0.134	0.013	
6	21-cyclopropyl A	~46	>50	>50	0.125	~1	0.157	>50
12	21-isopropyl A				0.02	0.123	0.022	
19	21-*n*-propyl A	27.3	>50	>50	0.025	0.031	0.004	<50

aphid, it is at least as active as spinosyn A against the target panel and in most cases is more active, especially against cotton aphids and whiteflies. Thus, these data demonstrate that modifications to the C-21 position of the spinosyn can lead to improvements in activity, depending on the substitution and the species. This conclusion is further supported by the discovery of the C-21-butenyl series of spinosyns in *Saccharopolyspora pogona*.[23,24] These natural analogues, characterized by a but-2-enyl group at C-21, also exhibit strong activity against lepidopteron pests and improved performance against aphids.

The desire to generate novel spinosyns with alterations at C-21 was driven by the need to identify new insecticides. While a semisynthetic approach has previously been successful in making alterations to much of the spinosyn molecule,[5−8] it did not allow access to the types of compounds described in this study. The production of spinosyn analogues correctly processed by post-PKS enzymes was encouraging and allowed a direct comparison of the insecticidal activity of the novel analogues with spinosyns A and D. The novel compounds showed similar efficacies to spinosyn A and, for certain species, a significant increase in activity. This work demonstrates the value of biosynthetic engineering to complement chemical approaches in creating analogues of a molecule with a validated biological activity.

Experimental Section

Media, Growth Conditions, and Fermentations. *Escherichia coli* DH10B cells (Gibco BRL/Invitrogen) were grown at 37 °C in 2TY media. Ampicillin or apramycin were added when appropriate to a final concentration of 100 and 50 mg/L, respectively. *S. spinosa* strains NRRL 18537 and NRRL 18538 were routinely cultured from frozen vegetative stocks (1:1 CSM culture:[25] cryopreservative (20% (w/v) glycerol, 10% lactose in water)). Strains were grown either in volumes of 6 mL in 25 mL Erlenmeyer flasks or in 30 mL in 250 mL Erlenmeyer flasks. Seed cultures were grown in CSM medium in an Erlenmeyer flask with a steel spring and shaken at 250 rpm with a 2 in. throw at 30 °C and 75% relative humidity for 3 days. Vegetative cultures were cultured in vegetative medium[26] from a 5% v/v inoculum and grown in the same manner for 2 days. A vegetative culture was used to inoculate production medium[26] at 5% v/v. Small-scale production cultures in 6 mL or 30 mL volumes in Erlenmeyer flasks (25 or 250 mL) were fermented under the same conditions for 7−10 days.

Larger-scale fermentations were performed at 12−14 L volumes in a 20 L stirred bioreactor. Airflow was set at 0.75 vvm, overpressure was set at 0.5 barg or below, and impeller tip speed was controlled between 0.39 and 1.57 ms^{-1} in order to maintain dissolved oxygen tension at or above 30% of air saturation. Pluronic acid L0101 (BASF) was used as required to control foaming.

Feeding of Substrates to Fermentations. Cultures were routinely grown in 6 mL, 30 mL, or 12−14 L production medium as described above. After 24 h cultures were fed with the carboxylic acid to a final concentration of 2−6 mM. Where appropriate, cultures were additionally fed 37.5 and 45 h post-inoculation. The fermentations typically lasted between 6 and 9 days.

DNA Manipulation, Sequencing, and Analysis. Standard methods for DNA isolation and manipulation were used.[27,28] All restriction enzymes were purchased from New England Biolabs. Pwo polymerase (Roche Molecular Biochemicals) was used for PCR reactions. Oligos used for PCR were purchased from MWG Biosciences. Sequencing was performed using standard techniques. Southern blots were carried out using Roche DIG labeling and detection kit. Plasmids were conjugated from *E. coli* S17-1 into *S. spinosa* strains NRRL 18537 and NRRL 18538 as previously reported.[21]

Construction of Plasmids. To introduce the *Nhe*I site at the start of spinosyn KS1 (Figure 2), the region of *spnA* from the beginning of the KS1 was amplified by PCR using pRHB3E11[3] as the template and oligos SP14 and SP15. SP14 (aagctagccgtgatcgggatgggctgtcggtt) introduces an *Nhe*I site at bases 24107−24112 (numbers refer to the deposited sequence in GenBank under the accession number AY007564). SP15 (atagcggccgccccccagcccccacagatccggtcaccaa) binds across a naturally occurring *BstE*II site approximately 1500 bp downstream of the junction site (restriction enzyme recognition sites are underlined and the bases altered from the natural sequence to introduce the *Nhe*I site are shown in bold). A *Not*I site was also incorporated into SP15 to facilitate subsequent cloning steps. The resulting PCR product was cloned into pUC19 and sequenced and this plasmid designated pLSB5. To increase the region of *spnA* homology for recombination, a further 2.6 kbp of *spnA* (from the naturally occurring *BstE*II to *Not*I sites) was cloned from pRHB3E11 into pLSB5 to yield pLSB8.

The *ery* loading module was amplified by PCR using pCJR26[20] as the template and oligos SP28 and SP29. SP28 (aggacacatatggcggacctgtcaaagctctc) incorporates a *Nde*I site overlapping the start codon of *eryAI* and changing the GTG start codon to an ATG. SP29 (cccgctagcggttcgccgggcgccgcttcgttgg) incorporates an *Nhe*I site at the beginning of the KS1 domain (as above, restriction enzyme recognition sites are underlined and bases altered from the natural sequence shown in bold). The resulting PCR product was cloned into pUC19 and sequenced and the plasmid designated pLSB44. The *Nde*I/*Nhe*I fragment from pLSB44, containing the erythromycin loading module, was cloned into pLSB8 that had been previously digested with *Nde*I and *Nhe*I to give pLSB56. The *Nde*I/*Xba*I fragment of pLSB56 was cloned into *Nde*I/*Xba*I cut pLSB3 to give pLSB62. Plasmid pLSB3 is derived from pKC1132;[29] it harbors the apramycin-resistance gene *oriT* for conjugal transfer of DNA into actinomycetes and contains P_{ptr} cloned from pCJR81.

An *Nde*I/*Nhe*I fragment containing the avermectin loading module was cloned from pIG1[11] into pLSB8 that had been previously digested with *Nde*I and *Nhe*I to give pLSB14. The fragment of pLSB14 was cloned into *Nde*I/*Xba*I cut pLSB2 to give pLSB29. Plasmid pLSB2 is derived from pKC1132;[29] it harbors the apramycin-resistance gene *oriT* for conjugal transfer of DNA into actinomycetes and contains P_{actI} and its cognate activator a*ctII*-ORF4 cloned from pCJR24.[22]

Identification of Metabolites from *S. spinosa* Fermentations. An aliquot of the fermentation broth (1 mL) was taken and adjusted to pH ~10 by the addition of 20% ammonia solution, shaken vigorously with EtOAc (1 mL, 20 min), and then centrifuged. The upper phase was removed to a clean tube and the solvent removed by evaporation. Residues were dissolved in MeOH (0.25 mL) and clarified by centrifugation. Samples were analyzed by HPLC and LCMS.

Analytical HPLC was performed on a Hewlett-Packard 1100 System. Chromatography was achieved over a Hypersil C_{18} BDS column (150 × 4.6 mm, 3 μm) eluting with a gradient of mobile phases A and B; flow rate, 1 mL/min; mobile phase A: 10% CH_3CN:90% H_2O, containing 10 mM NH_4OAc and 0.15% HCO_2H; mobile phase B: 90% CH_3CN:10% H_2O, containing 10 mM NH_4OAc and 0.15% HCO_2H;

gradient: $t = 0$ min, 10% B; $t = 1$, 10% B; $t = 25$, 95% B; $t = 29$, 95% B; $t = 29.5$, 10% B. LCMS was performed on a Waters-Micromass ZQ system using the chromatographic conditions described above; data were collected over the range $m/z = 100-1000$. Authentic standards of spinosyns A, D, and E and pseudoaglycones A and D were kindly provided by Dow AgroSciences.

General Method for Compound Isolation. Fermentation broths were clarified by centrifugation to provide supernatant and cell fractions. The cell fraction was extracted by mixing thoroughly with an equal volume of MeOH and then allowed to stand for 30 min. The resulting slurry was centrifuged and the supernatant retained. The residue was similarly extracted once more, and the MeOH supernatants were combined. The fermentation supernatant was adjusted to pH ~10 by addition of 5 N NaOH and then stirred gently with 0.75 volume of ethyl acetate for 8 h. The organic phase was removed by aspiration, and the extraction was repeated. The EtOAc and MeOH fractions were combined and the solvents removed in vacuo to yield a yellow-brown oil−aqueous mixture (typically 1 L). This mixture was mixed with ethyl acetate (2 L) and repeatedly extracted with a solution of 50 mM tartaric acid (3 × 1.5 L). The tartaric acid extracts were combined, adjusted to pH ~10 with 5 N NaOH, and repeatedly extracted with ethyl acetate (3 × 1.5 L). The EtOAc extracts were combined and the solvent was removed to leave a brown, oily residue (typically 5−10 g). This residue was dissolved into EtOAc (500 mL) and extracted again with 50 mM tartaric acid (3 × 350 mL). The tartaric acid fractions were combined, adjusted to pH ~10, and re-extracted with EtOAc (3 × 500 mL). The EtOAc fractions were combined and the solvent removed in vacuo to yield a brown, oily residue (typically 0.5−1 g).

The resulting oily solid was dissolved into MeOH, and chromatography was achieved over a Hypersil C_{18} BDS column (21 × 250 mm, 5 μm) eluting with a gradient of 35% to 95% mobile phase B over 30 min with a flow rate of 21 mL/min. Fractions were collected every 30 s. The mobile phases are described above. Fractions containing spinosyns were combined, and the acetonitrile was removed in vacuo. The residual aqueous was applied to a C_{18}-BondElute cartridge (200 mg) and washed with H_2O (10 mL) to remove buffer, and the product was eluted with MeOH (2 × 10 mL).

Characterization of Compounds. NMR spectra were recorded on a Bruker Advance 500 spectrometer at 298 K operating at 500 and 125 MHz for ^{1}H and ^{13}C, respectively. Standard Bruker pulse programs were used to acquire the ^{1}H−^{1}H COSY, APT, HMQC, and HMBC spectra. All compounds were dissolved in $CDCl_3$, and spectra were referenced to $CHCl_3$ (δ_H 7.26) and $CDCl_3$ (δ_C 77.0). Where material was limited, ^{13}C chemical shifts were assigned from correlations observed in the HMQC and HMBC spectra. High-resolution mass spectra were recorded on a Bruker Daltonics BioApexII 4.7 T Fourier transform ion cyclotron resonance MS instrument.

Isolation and Characterization of 21-Desethyl-21-cyclobutylspinosyn A (4). Compound **4** was isolated as an amorphous solid (3.1 mg) using the general method described above; UV (DAD) λ_{max} = 245 nm; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.76 (1H, s, H-13), 5.88 (1H, d, J = 9.8 Hz, H-6), 5.80 (1H, ddd, J = 9.8, 3.0, 3.0 Hz, H-5), 4.85 (1H, d, J = 1.7 Hz, H-1′), 4.80 (1H, ddd, J = 10.0, 7.7, 2.6 Hz, H-21), 4.42 (1H, dd, J = 9.2, 2.1 Hz, H-1″), 4.31 (1H, m, H-9), 3.61 (1H, m, H-17), 3.56 (3H, s, OCH_3-4′), 3.54 (1H, m, H-5′), 3.54 (1H, m, H-4), 3.50 (3H, s, OCH_3-3′), 3.49 (3H, s, OCH_3-2′), 3.49 (1H, m, H-2′), 3.48 (1H, m, H-5″), 3.46 (1H, dd, J = 9.4, 3.4 Hz, H-3′), 3.26 (1H, dq, J = 10.0, 6.8 Hz, H-16), 3.12 (1H, dd, J = 13.9, 5.1, H-2b), 3.12 (1H, dd, J = 9.4, 9.4, H-4′), 3.01 (1H, m, H-3), 2.88 (1H, m, H-12), 2.43 (1H, dd, J = 13.9, 3.0 Hz, H-2a), 2.34 (1H, m, H-22), 2.26 (1H, m, H-10b), 2.26 (1H, m, H-4″), 2.26 (6H, s, H−N(CH_3)$_2$), 2.17 (1H, m, H-7), 1.98 (1H, m, H-2″b), 1.98 (1H, m, H-3″b), 1.92 (1H, dd, J = 13.4, 7.0, H-8b), 1.86 (1H, m, H-25a), 1.86 (1H, m, H-25b), 1.76 (1H, m, H-24a), 1.76 (1H, m, H-24b), 1.65 (1H, m, H-23a), 1.65 (1H, m, H-23b), 1.49 (1H, m, H-18a), 1.49 (1H, m, H-18b), 1.47 (1H, m, H-2″a), 1.47 (1H, m, H-2″b), 1.44 (1H, m, H-20b), 1.37 (1H, m, H-8a), 1.34 (1H, m, H-20a), 1.33 (1H, m, H-10a), 1.28 (3H, d, J = 6.2 Hz, H-6′), 1.26 (3H, d, J = 6.2 Hz, H-6″), 1.17 (3H, d, J = 6.8 Hz, H-26), 0.91 (1H, dddd, J = 11.7, 11.7, 11.7, 6.4 Hz, H-11); ^{13}C NMR ($CDCl_3$, 125 MHz) 202.9 (C-15), 172.8 (C-1), 147.5 (C-13), 144.2 (C-14), 129.3 (C-6), 128.8 (C-5), 103.4 (C-1′′), 95.4 (C-1′), 82.2 (C-4′), 81.0 (C-3′), 80.6 (C-17), 78.0 (C-21), 77.7 (C-2′), 76.0 (C-9), 73.5 (C-5″), 67.9 (C-5′), 64.8 (C-4″), 60.9 (O-CH_3, C-4′), 59.0 (O-CH_3, C-2′), 57.7 (O-CH_3, C-3′), 49.4 (C-12), 47.8 (C-16), 47.5 (C-3), 46.0 (C-11), 41.7 (C-4), 41.1 (C-7), 40.6 (N-CH_3)$_2$, C-4″), 40.5 (C-22), 37.3 (C-10a), 36.2 (C-8a), 34.4 (C-18a), 33.8 (C-2a), 30.8 (C-2″a), 28.2 (C-20a), 24.6 (C-25a), 24.5 (C-23a), 21.7 (C-19a), 19.0 (C-6″), 18.5 (C-3′′a), 17.8 (C-24a), 17.8 (C-6′), 16.1 (C-26); ESIMS m/z 758.5 $[M + H]^+$, 142.4 [forosamine − OH]$^+$; HRFTICRESIMS m/z 758.4830 (calcd for $C_{43}H_{68}NO_{10}$, 758.4838).

Isolation and Characterization of 21-Desethyl-21-cyclobutylspinosyn D (5). Compound **5** was isolated as an amorphous solid (0.5 mg) using the general method described above. The low yield for this compound precluded full structural characterization, but the data accumulated were consistent with the proposed structure; UV (DAD) λ_{max} = 245 nm; ESIMS m/z 772.5 $[M + H]^+$, 142.4 [forosamine − OH]$^+$; HRFTICRESIMS m/z 772.4990 (calcd for $C_{44}H_{70}NO_{10}$, 772.4994).

Isolation and Characterization of 21-Desethyl-21-cyclopropylspinosyn A (6). Compound **6** was isolated as an amorphous solid (1 mg) using the general method described above; UV (DAD) λ_{max} = 245 nm; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.74 (1H, s(br), H-13), 5.87 (1H, b, J = 9.9 Hz, H-5), 5.79 (1H, ddd, J = 9.9, 2.7, 2.7, H-6), 4.84 (1H, s(br), H-1′), 4.41 (1H, d, J = 8.2 Hz, H-1″), 4.30 (1H, m, H-9), 4.18 (1H, m, H-21), 3.62 (1H, m, H-17), 3.55 (3H, s, OCH_3-4′), 3.54 (1H, m, H-5′), 3.49 (3H, s, OCH_3-2′), 3.49 (1H, m, H-2″), 3.49 (1H, m, H-4), 3.48 (1H, m, H-5″), 3.48 (3H, s, OCH_3-3′), 3.45 (1H, dd, J = 9.3, 3.2 Hz, H-3′), 3.26 (1H, m, H-16), 3.13 (1H, dd, J = 13.5, 5.1 Hz, H-2b), 3.10 (1H, dd, J = 9.3, 9.3 Hz, H-4′), 3.00 (1H, m, H-3), 2.85 (1H, m, H-12), 2.43 (1H, dd, J = 13.5, 3.0 Hz, H-2a), 2.24 (6H, s, N(CH_3)$_2$-4″), 2.24 (1H, m, H-4″), 2.24 (1H, m, H-10b), 2.15 (1H, m, H-7), 1.98 (1H, m, H-2″b), 1.91 (1H, dd, J = 13.2, 7.0 Hz, H-8b), 1.85 (1H, m, H-3″b), 1.74 (1H, m, H-19b), 1.62 (1H, m, H-20a), 1.62 (1H, m, H-20b), 1.51 (1H, m, H-18a), 1.51 (1H, m, H-18b), 1.47 (1H, m, H-2″a), 1.45 (1H, m, H-3″a), 1.34 (1H, m, H-8a), 1.32 (1H, m, H-10a), 1.27 (3H, d, J = 6.4 Hz, H-6″), 1.27 (3H, d, J = 6.4 Hz, H-6′), 1.18 (1H, m, H-19a), 1.17 (3H, J = 6.8 Hz, H-25), 0.89 (1H, m, H-11), 0.89 (1H, m, H-22), 0.44 (1H, m, H-23a), 0.44 (1H, m, H-24a), 0.14 (1H, m, H-23b), 0.14 (1H, m, H-24b); ^{13}C NMR ($CDCl_3$, 125 MHz) 147.4 (C-13), 129.1 (C-5), 128.6 (C-6), 103.4 (C-1″), 95.2 (C-1′), 82.1 (C-4′), 80.9 (C-3′), 80.6 (C-17), 79.3 (C-21), 77.5 (C-2′), 75.9 (C-9), 73.4 (C-5″), 67.7 (C-5′), 64.7 (C-4″), 60.7 (O−CH_3, C-4′), 58.8 (O-CH_3, C-2′), 57.5 (O-CH_3, C-3′), 47.5 (C-16), 47.3 (C-3), 45.9 (C-11), 41.4 (C-4), 40.6 (N(CH_3)2, C-4″), 37.0 (C-10), 36.0 (C-8), 34.0 (C-2), 34.2 (C-18), 34.0 (C-12), 30.9 (C-20), 30.8 (C-2″), 22.0 (C-19), 18.7 (C-6″), 18.2 (C-3″), 17.6 (C-6′), 16.5 (C-22), 16.0 (C-25), 3.7 (C-24), 2.3 (C-23); ESIMS m/z 744.5 $[M + H]^+$, 142.2 [forosamine − OH]$^+$; HRFTICRESIMS m/z 744.7680 (calcd for $C_{42}H_{66}NO_{10}$, 744.4681).

Isolation and Characterization of 21-Desethyl-21-*n*-propylspinosyn A (19). Compound **19** was isolated as an amorphous solid (1 mg) using the general method described above; UV (DAD) λ_{max} = 244 nm; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.75 (1H, 1H, m, H-13), 5.87 (1H, m, H-5), 5.78 (1H, ddd, J = 9.6, 2.7, 2.7, H-6), 4.84 (1H, d, J = 1.6 Hz, H-1′), 4.73 (1H, m, H-21), 4.41 (1H, d(br), J = 8.2 Hz, H-1″), 4.30 (1H, m, H-9), 3.62 (1H, m, H-17), 3.55 (3H, s, OCH_3-4′), 3.54 (1H, m, H-5′), 3.50 (1H, m, H-2′), 3.49 (3H, s, OCH_3-2′), 3.48 (3H, s, OCH_3-3′), 3.48 (1H, m, H-4), 3.48 (1H, m, H-5″), 3.46 (1H, dd, J = 9.4, 3.2 Hz, H-3′), 3.28 (1H, dq, J = 9.8, 6.8 Hz, H-16), 3.10 (1H, dd, J = 13.5, 5.3 Hz, H-2b), 3.10 (1H, dd, J = 9.3, 9.3 Hz, H-4′), 3.00 (1H, m, H-3), 2.86 (1H, m, H-12), 2.40 (1H, dd, J = 13.5, 3.1 Hz, H-2a), 2.26 (1H, m, H-10b), 2.23 (6H, s, N(CH_3)$_2$-4″), 2.23 (1H, m, H-4″), 2.16 (1H, m, H-7), 1.97 (1H, m, H-2″b), 1.91 (1H, dd, J = 13.2, 7.1 Hz, H-8b), 1.85 (1H, m, H-3″b), 1.75 (1H, m, H-19b), 1.57 (1H, m, H-20a), 1.57 (1H, m, H-20b), 1.53 (1H, m, H-18a), 1.53 (1H, m, H-18b), 1.50 (1H, m, H-22b), 1.47 (1H, m, H-2″a), 1.46 (1H, m, H-3″a), 1.37 (1H, m, H-22a), 1.34 (1H, m, H-8a), 1.32 (1H, m, H-10a), 1.27 (3H, d, J = 6.2 Hz, H-6′), 1.25 (3H, d, J = 6.2 Hz, H-6″), 1.25 (2H, m, H-23), 1.19 (1H, m, H-19a), 1.17 (3H, d, J = 6.8 Hz, H-25), 0.91 (1H, m, H-11), 0.86 (3H, dd, J = 7.4, 7.3 Hz, H-24); ^{13}C NMR ($CDCl_3$, 125 MHz) 202.9 (C-15), 172.6 (C-1), 147.5 (C-13), 144.1 (C-14), 129.3 (C-5), 128.8 (C-6), 103.5 (C-1″), 95.4 (C-1′), 82.3 (C-4′), 81.0 (C-3′), 80.7 (C-17), 77.7 (C-2′), 76.1 (C-9), 75.4 (C-21), 73.6 (C-5″), 67.9 (C-5′), 64.8 (C-4″), 61.0 (OCH_3-4′), 59.0 (OCH_3-2′), 57.7 (OCH_3-3′), 49.4 (C-12), 47.7 (C-16), 47.6 (C-3), 46.0 (C-11), 41.5 (C-4), 41.2 (N(CH_3)$_2$-4″), 41.2 (C-7), 37.9 (C-22), 37.4 (C-10), 36.3 (C-8), 34.4 (C-18), 34.1 (C-2), 30.9 (C-2″), 30.7 (C-20), 21.6 (C-19), 19.0 (C-6″), 18.4 (C-3″), 18.4 (C-23), 17.8 (C-6′), 16.2 (C-25), 14.0 (C-24); ESIMS m/z 746.5 $[M + H]^+$, 142.2 [forosamine − OH]$^+$.

Isolation and Characterization of 21-Desethyl-21-isopropylspinosyn A (12). A mixture of spinosyn D (**2**), **12**, and **19** (~5 mg) was

isolated as described above. This was further separated analytically by HPLC using isocratic elution. The conditions used were as follows: Hypersil C_{18} BDS column (250 × 4.6 mm, 5 μm) eluting with a mixture of 10 mM NH_4OAc/MeOH/THF (40:45:15) at a flow rate of 1 mL/min. Although full NMR assignment was not possible, the accumulated data were sufficient to verify the structure as an A-type spinosyn (6-H) with a novel isopropyl group at position C-21 (see text); UV (DAD) λ_{max} = 244 nm; ESIMS *m*/*z* 746.5 $[M + H]^+$, 142.2 $[forosamine]^+$.

Preparation of 5,6-Dihydro-21-desethyl-21-cyclobutylspinosyn A (20). A solution of 21-desethyl-21-cyclobutylspinosyn A (3.1 mg, 0.004 mmol) in 2.0 mL of toluene and 0.5 mL of EtOH was purged with a slow stream of nitrogen for 20 min, then 2.0 mg of chlorotris-(triphenylphosphine)rhodium was added and the solution hydrogenated at 60 °C for 16 h. After cooling and removal of solvent, the residue was chromatographed using a 10 cm × 2 cm Si gel column, eluting with 5 × 25 mL fractions of CH_2Cl_2 containing 0%, 1%, 2%, 3%, 4%, and 5% MeOH, respectively. The product-containing fractions were combined and concentrated to give 2.1 mg of 5,6-dihydro-21-desethyl-21-cyclobutylspinosyn A: ^{1}H NMR ($CDCl_3$, 600 MHz) δ 6.88 (1H, m, H-13); 0.88 (3H, dd, J = 7.4, 7.3 Hz, H-24); ESIMS *m*/*z* 760.4. Small olefinic proton signals at 5.87 and 5.78 (C5 and C6) indicate that ca. 20% of the material was unreduced starting material.

Insect Bioassays. Topical toxicity was examined for three lepidopteran species: fourth instar beet armyworm (*Spodoptera exigua*), third instar corn earworm (*Helicoverpa zea*), and third instar cabbage looper (*Trichoplusia ni*). Stock solutions were prepared by dissolving materials in acetone at 1 μg/μL. Topical treatments were 1 μL aliquots deposited from a 50 μL Hamilton syringe along the dorsa of each larva. Each dilution was applied to six insects, and six control insects of each species were treated with blank solvent. Following treatment, insects were held individually in wells of six-well tissue culture plates (Falcon 3046) containing an appropriate food substrate. Treated insects were held under ambient laboratory conditions (21 °C), and mortality was noted at intervals for 5 days post-treatment. LD_{50} values were calculated from the corrected dose−response data using probit analysis.[30]

Representative sucking insects were cotton aphid (*Aphis gossypii*, CA) and green peach aphid (*Myzus persicae*, GPA). For CA, summer crookneck squash seedlings (*Cucurbita pepo*), approximately 1 week old, were pruned to a single cotyledon. A population of cotton aphids was transferred to the cotyledons 16−24 h prior to the application of test materials. For GPA, head cabbage seedlings (*Brassica oleracea capitat*), approximately 12 days old, were thinned and selected for uniformity. Four days prior to the application of the test materials these untreated plants were infested with all stages of GPA. Test compounds were dissolved in 90:10 acetone/EtOH to form a stock solution, from which an appropriate amount was diluted in H_2O containing 0.05% Tween 20 to form the spray solutions (ranging from 100 to 0.024 ppm). For both species, compound applications were made with a handheld DeVilbiss airbrush sprayer. Aphid-infested plants were sprayed on both the upper and lower surfaces of the leaves until runoff. For each rate/pest there were four replicate plants. Controls consisted of eight replicates treated with diluent prepared with a blank stock solution only (solvent blank). Treated plants were held for 72 h at 23 °C and 40% RH with a 24 h photoperiod prior to grading. Counts of live aphid (all nonwinged stages) were compared to the populations on the solvent blank controls. The dose−response data were analyzed in the same way as the lepidopteran bioassays.

Efficacy against immature sweet potato whitefly (*Bemisia tabaci*) was measured on preinfested greenhouse-grown cotton plants (about 5 weeks old) that had been defoliated except the first pair of true leaves. Spray solutions of experimental compounds were prepared in 90:10 acetone/EtOH solvent mixture. To this stock was added H_2O containing 0.05% v/v Tween 20 surfactant, to produce serial solutions ranging from 240 to 0.938 ppm. Plants were sprayed (both upper and lower leaf surfaces) using a handheld syringe fitted with a hollow cone nozzle (Teejet TN-3, Spraying Systems Co.). One milliliter of spray solution was applied to each leaf surface for a total of 4 mL per cotton plant. Two cotton plants for each treatment were used, for a total of four leaf replicates per treatment. Plants were allowed to air-dry and then placed in a holding room at 30 °C and 60% RH. Compound efficacy was determined by larval survival 13 days after application. At this time whitefly nymphs that had successfully developed to the third and fourth nymphal stadia on the underside of the leaves were viewed with the aid of an illuminated magnifier. Percent mortality was calculated as the reduction of the mean number of living nymphs relative to that observed in the solvent check. LC_{50} values were calculated as above.

Activity against mites was determined using two-spotted spider mites (*Tetranychus urticae*). Test compounds were dissolved in a formulation blank comprised of acetone, Tween 20, M-pyrol, and Exxon 200 to concentrations of 0.195, 0.78, 3.12, and 12.5 ppm. The preinfested squash plants (trimmed to one cotyledon) were sprayed with compound solutions using a handheld syringe equipped with a TX-3 nozzle. Each treatment was applied to the upper and lower surface of eight cotyledons. Following application, the treated, infested plants were kept under low-light conditions in the laboratory for 4 days prior to grading. Live mites were counts on the underside of each cotyledon. The dose−response data were analyzed as described above.

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