Bioorganic & Medicinal Chemistry 17 (2009) 4185–4196

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Discovery of the butenyl-spinosyn insecticides: Novel macrolides from the new bacterial strain *Saccharopolyspora pogona*

Paul Lewer, Donald R. Hahn, Laura L. Karr, Dennis O. Duebelbeis, Jeffrey R. Gilbert, Gary D. Crouse, Thomas Worden, Thomas C. Sparks, Pat McKamey Rex Edwards, Paul R. Graupner *

Dow AgroSciences, 9330 Zionsville Road, Indianapolis, IN 46033, USA

ARTICLE INFO

Article history:
Received 26 September 2008
Revised 11 February 2009
Accepted 18 February 2009
Available online 23 February 2009

Keywords:
Spinosyn
Saccharopolyspora pogona
Natural products

ABSTRACT

A new bacterium, *Saccharopolyspora pogona* (NRRL30141) was discovered which produced a series of very potent insecticidal compounds structurally related to the 'classical' (i.e., C-21-ethyl) spinosyns. A series of fermentations gave sufficient extract to allow the isolation and characterization of a total of 31 new metabolites. The majority of these compounds contained a but-1-enyl group at C-21 of the macrolide in place of the ethyl group in the 'classical' spinosyn series, corresponding to an additional acetate group incorporated during their biosynthesis. Additionally a variety of other new functionality was seen including hydroxylations, several novel forosamine sugar replacements, and a novel 14-membered macrolide ring analog.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

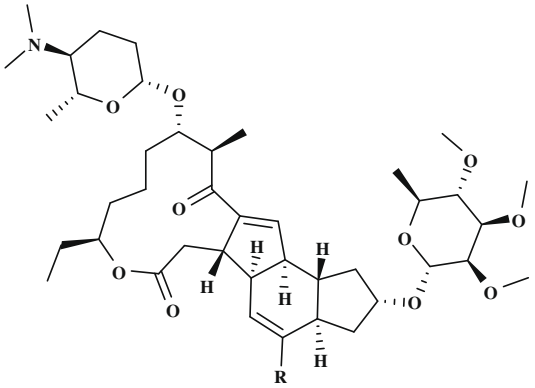
The spinosyns are a family of bacterial natural products with a unique cross-bridged macrocyclic structure.[1] Fermentation of the actinomycete *Saccharopolyspora spinosa* produces mixtures of several analogs with two, known as spinosyn A and D predominating. Extracts of the fermentation broth containing this mixture, known as Spinosad, are extremely potent insect control agents. Consequently, several products containing Spinosad as the active ingredient have been commercialized, including Tracer®, Success®, SpinTor® and Conserve®.[2,3]

S. spinosa was discovered in the early 1980s from a soil sample collected in the Caribbean.[4] The spinosyns it and subsequent derived strains produced were given trivial names based on letters of the alphabet and are referred to herein as the 'classical' spinosyns. Quantitatively, spinosyn A was the major compound produced, with spinosyn D the predominant structural variant, being typically produced at 25% of the level of factor A. Many other structural variants were present at much lower levels.

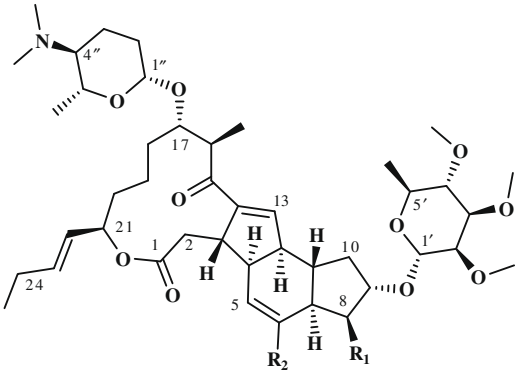
In the search for a second-generation commercial product a significant effort to enhance the potency and expand the insecticidal spectrum in this series was made through the preparation of semi-synthetic analogs. Well over 1000 synthetic analogs were made based on the various natural factors isolated from the fermentation broth.[2] These primarily focused on altering substituents on the rhamnose and forosamine moieties and/or substitution/reduction of the macrolide double bonds. While this effort was limited by the amount of starting material(s) available and particularly by the functional groups present in those molecules, a second-generation product candidate known as Spinetoram was discovered through this approach.[5,6] Two recent reviews discuss this work in much greater detail so this area will not be expanded upon here.[2,3] However, certain areas of the molecule still proved to be synthetically inaccessible, in particular the region of the macrocycle spanning carbon atoms 18 through 21 and the C21 side chain termed the 'western shore'. In an effort to address this, two alternative strategies were adopted. In one approach, use of targeted genetically-modified bacteria allowed for production of differently-C17-sugar substituted analogs, as well as alternatively C21-substituted analogs.[7,8] In a second approach, spinosyn A and its aglycone were reported to be selectively oxidized at C-22 by a *Streptomyces* strain, thus introducing a potential synthetic handle into this region.[9] While those efforts were in progress, a new actinomycete strain, known as *Saccharopolyspora pogona* was discovered.[10,11] This proved to be the source of many new spinosyn analogs with a much wider degree of structural variation than was seen with *S. spinosa* and, in particular, containing functionality allowing synthetic access to the 'western shore'. The isolation of these analogs and their structural elucidation are described herein. We also describe a systematic 'modular' nomenclature for the new spinosyn series which accommodates the wider degree of structural types, while retaining clear links to the alphabetically-named 'classical' spinosyns.

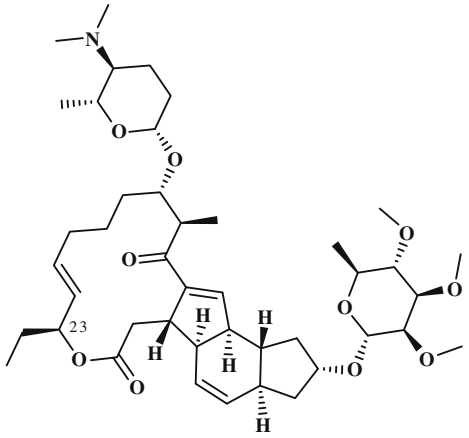
* Corresponding author. Tel.: +1 317 337 3496; fax: +1 317 337 3546.
E-mail address: prgraupner@dow.com (P.R. Graupner).

0968-0896/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2009.02.035



Spinosyn A (R = H), and D (R = Me)





	R^1	R^2	Spinosyn
1	H	H	α1
2	H	Me	δ1
3	OH	H	β1
4	OH	Me	6-Methyl β1

5 Spinosyn γ1

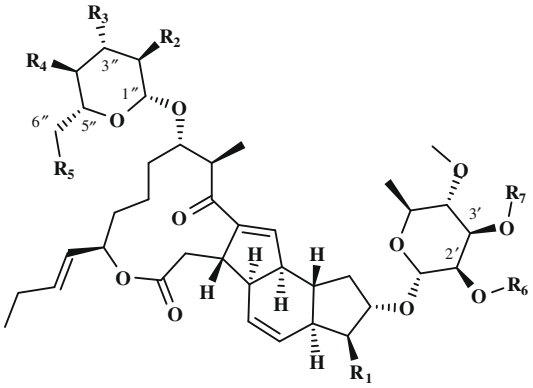
2. Results and discussion

2.1. Nomenclature

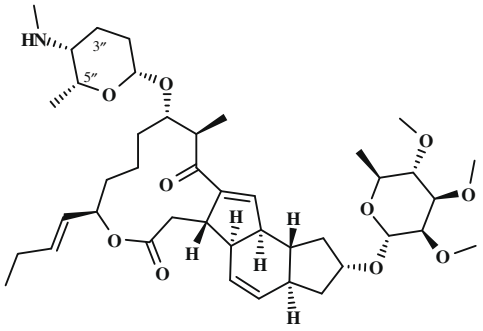
Although the new compounds were produced by a strain distinct from *S. spinosa*, the structural similarity to the 'classical' spinosyns ultimately led us to keep the name 'spinosyn' for the new compounds. In order to distinguish them from the 'classical' molecules while introducing a systematic approach, a new naming scheme was devised.[12] The three main structural elements in these molecules in which variations were seen are: (i) the macrocyclic ring system, (ii) the sugar attached to C17 and (iii) the side chain attached to C21, as exemplified by compounds **1**–**5**, **6**–**16** and **17**–**22**, respectively. Therefore, the new naming system is a composite of these three elements. First, the macrocycle type is designated by a Greek letter, for example α represents the macrocycle as it appears in spinosyn A, while β represents this macrocycle bearing an 8-OH group, δ represents the base macrocycle bearing a 6-methyl group and γ represents a 14-membered ring-expanded macrocycle. Second, the C17-sugar type is designated by a number, for example 1 represents β-D-forosamine and 2 represents 3-*O*-methyl-glucose. Third, a lower case Roman letter represents the side chain (unless it is but-1-enyl, the parent type, in which case it is not used), for example a = buta-1,3-dienyl, b = prop-1-enyl and c = 3-hydroxy-but-1-enyl. Finally, more minor changes such as O- or N-demethylation are added as prefixes to the systematic name. Examples illustrating this naming system include the major factor, named α1 (**1**), its 6-methyl analog (**2**) named δ1, the 3′-*O*-demethyl analog of **1**, named 3′-*O*-demethyl-α1 (**10**) and the 1,3-butadienyl side chain analog of **1**, named α1a (**19**). In this naming system the 21-butenyl analogs of spinosyns A and D are named α1 and δ1, thereby keeping a link to the 'classical' series, while being able to accommodate potential findings of new analogs in the future.

2.2. Microbiology of producing organism

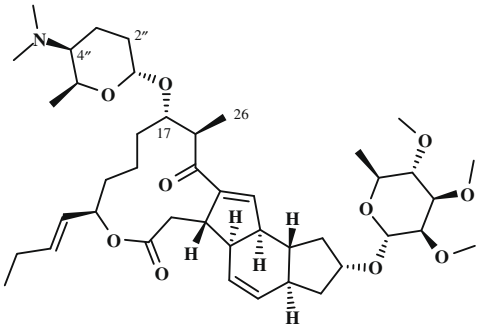
The actinomycete isolate NRRL 30141, which was used in this work was found to be a member of the actinomycete genus *Saccharopolyspora* by BLAST analysis of the 16S rRNA gene sequence.[13] The designation as *Saccharopolyspora* was supported by its sensitivity to *Saccharopolyspora* specific bacteriophage, biochemical



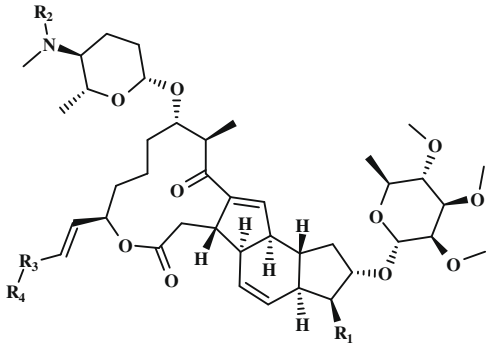
	R^1	R^2	R^3	R^4	R^5	R^6	R^7	Spinosyn
6	H	H	H	$N(O)Me_2$	H	Me	Me	α1 *N*-oxide
7	H	H	H	NHMe	H	Me	Me	*N*-Demethyl α1
8	H	OH	H	NMe_2	H	Me	Me	2-OH α1
9	H	H	H	NMe_2	H	H	Me	2`-*O*-Demethyl α1
10	H	H	H	NMe_2	H	Me	H	3`-*O*-Demethyl α1
11	H	H	H	OH	H	Me	Me	α4
12	H	OH	OMe	OH	OH	Me	Me	α2
13	OH	H	H	OH	H	Me	Me	β4
14	OH	H	OMe	OMe	H	Me	Me	β3



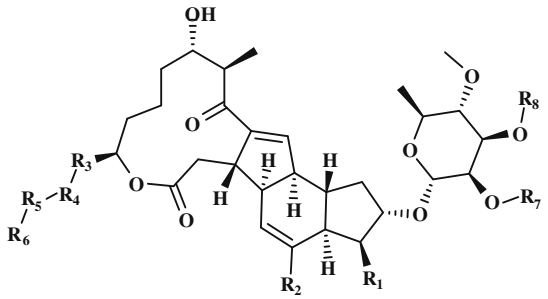
15 *N*-Demethyl spinosyn α6



16 Spinosyn α5



	R^1	R^2	R^3	R^4	Spinosyn
17	H	Me	CH(OH)	Me	α1c
18	H	H	CH(OH)	Me	*N*-Demethyl α1c
19	H	Me	CH=	$=CH_2$	α1a
20	H	Me	Me		α1b
21	OH	Me	CH=	$=CH_2$	β1a
22	OH	Me	Me		β1b



	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	Spinosyn
23	H	H	CH=	=CH	CH_2	Me	Me	Me	α
24	H	Me	CH=	=CH	CH_2	Me	Me	Me	δ
25	OH	Me	CH=	=CH	CH_2	Me	Me	Me	6-Methyl β
26	H	H	CH=	=CH	CH(OH)	Me	Me	Me	αc
27	OH	H	CH=	=CH	CH(OH)	Me	Me	Me	βc
28	H	H	CH=	=CH	CH=	$=CH_2$	Me	Me	αa
29	H	H	CH_2	CH_2	CH_2	Me	Me	Me	αd
30	H	H	CH=	=CH	CH_2	Me	H	Me	2`-O-Demethyl α
31	H	H	CH=	=CH	CH_2	Me	Me	H	3`-*O*-Demethyl α

testing, antibiotic resistance and morphological observations. Strain NRRL 30141was highly similar to *S. spinosa* based on 16S rRNA phylogeny, growth characteristics and physiological testing. However, the spore morphology, 16S rRNA sequence and bacteriophage sensitivity indicated that strain NRRL 30141 was a new species. Based on its 'bearded' morphology when viewed by electron microscopy, the strain was named *S. pogona*.

2.3. Isolation and identification of active metabolites

2.3.1. Isolation

Following the initial discovery that extracts from the fermentation broth of *S. pogona* had potent lepidopteran activity, an aliquot was analyzed using LC–MS and the eluent was collected in fractions for bioassay (LC–MS-bio). The biologically-active region of the chromatogram showed only very low-level UV-detectable peaks. While there were several +ESI-MS-detectable components in this region their mass spectra did not match any compounds in our mass spectral library, so they were isolated to allow structure elucidation. To facilitate this, several batches of fermentation broth were made on increasingly large scale, ranging from 4 to 400 L. For brevity, we describe the isolation work from only three of those batches here, which resulted in the identification of the 31 compounds we report. All of these compounds were novel and many contained structural features unprecedented in the 'classical' spinosyn series, as discussed below. The three fermentation batches were subjected to different work-up conditions so that the same compounds were not always isolated from each batch (see Supplementary data). In particular, the 4-L batch was processed using a 'total extract' approach where the whole extract was chromatographed to separate the components. In contrast, the 400-L batch was processed using an acid partitioning step which isolated only those analogs having a basic nitrogen. The 140-L batch was processed using a combination of the two methods.

By way of overview, 14 compounds were isolated and identified from the 4-L fermentation, with recovered weights in the 1–27 mg range (corresponding to approx. 0.25–7 ppm in the broth). They included the parent compound α1 (**1**) as well as seven new C-17-glycosylated metabolites. Of these, three were based on the macrolide-α structure, with structural variation being due only to forosamine-substitution in *N*-demethyl-spinosyn α1 (**7**), or replacement in spinosyn α4 (**11**) and spinosyn α2 (**12**). A further three were based on the macrolide-β (i.e., 8-hydroxylated) structure as exemplified by spinosyn β1 (**3**), with structural variation being due to forosamine replacement in spinosyns β4 (**13**) and β3 (**14**). The final C-17 glycoside, spinosyn δ1 (**2**) was the sole C17-glycosylated representative of the macrolide-δ (i.e., 6-methyl) structural class from this batch. Additionally, six C-17 *pseudo*aglycones (those with no sugar attached to C17) were identified. Of these, two were based on the macrolide-α structure, either not further elaborated, as in spinosyn α (**23**) or 24-hydroxylated in spinosyn αc (**26**): three were based on the macrolide-β (8-hydroxylated) structure, either not further elaborated in spinosyn β (**25**) or 24-hydroxylated in spinosyn βc (**27**) (two epimers at C-24) and again one, spinosyn δ (**24**) which was the sole representative of the macrolide-δ structural class.

The early identification of these metabolites, all based on the novel spinosyn α1 core, suggested the new organism to have considerable biosynthetic prowess. This organism clearly possessed biosynthetic capabilities similar to *S. spinosa*, in terms of assembling the core macrolide, but also some critically different ones, particularly in the biosynthesis and attachment of a variety of C17-sugars such as D-amicetose (e.g., **11** and **13**); 4-*O*-methyl-oleandrose (**14**) and 3-*O*-methyl-glucose (**12**). Additionally, the presence of two new (8- and 24-) hydroxylation positions was intriguing from the standpoint of the biological activity of the resulting metabolites, the biosynthetic route of introduction of these oxygen atoms and their utility as semi-synthetic starting materials, which is discussed in the following paper.

The larger-scale isolations subsequently performed further substantiated the biosynthetic abilities of *S. pogona*. From the 140-L fermentation, we identified a further 12 family members. Incompletely methylated rhamnose analogs had been previously observed in the classical spinosyn series, and this defect in the biosynthetic pathway was demonstrated in *S. pogona* by the presence of 2′- and 3′-*O*-demethyl-α1 (**9** and **10**) and their C17-pseudoaglycones (**30** and **31**). The presence of three new C21-side chains in molecules also fully glycosylated was first demonstrated by the four compounds **17**, **18**, **19** and **20**. Thus, spinosyn α1c (**17**) and *N*-

demethyl-spinosyn α1c (**18**) incorporated a 3-hydroxybut-1-enyl side chain, spinosyn α1a (**19**) incorporated a 1,3-butadienyl side chain and spinosyn α1b (**20**) incorporated a prop-1-enyl side chain in an otherwise complete spinosyn α1 base structure. An additional new C21 side chain, the fully saturated *n*-butyl group, was present in the C17-pseudoaglycone spinosyn αd (**29**). Further structural variations were observed in the very low abundance spinosyns α1 N-oxide (**6**), 2″-hydroxy-α1 (**8**) and the C17-pseudoaglycone spinosyn αa (**28**). Finally, isolation of the major components from the 400-L batch revealed further new analogs. Of these, spinosyns α5 (**16**) and *N*-demethyl-spinosyn-α6 (**15**) resulted from epimerization of selected substituents on the glycoside (making them osamine glycosides). Thus the spinosyn α6 osamine moiety was N-demethylated and C-4″-epimerized relative to spinosyn α1 while that in spinosyn α5 was epimerized at C-5″, thereby being equivalent of spinosyn G.[14] Three of the remaining new compounds belonged to the (8-hydroxy-) macrolide-β series. Thus 6-methyl spinosyn β1 (**4**) combined both 6-methylation and 8-hydroxylation with the C21-butenyl side chain, whereas spinosyns β1a (**21**) and -β1b (**22**) incorporated 1,3-butadienyl and 1-propenyl C21-side chains, respectively, in fully glycosylated 8-hydroxy-macrocycles. Finally, spinosyn γ1 (**5**) was found to be a macrolide ring-expanded product without precedent in the spinosyn field.

While small quantities of spinosyn A were isolated from the larger fermentations, its source was not clear. These batches were fermented in tanks used to occasionally ferment *S. spinosa* at the production plant, so contamination seemed a possible explanation. However, the other major component from *S. spinosa* fermentations, spinosyn D, was never isolated from the *S. pogona* fermentations, nor was there evidence for its presence. Considering that the spinosyn A:D ratio is typically approx. 75:25, it would be surprising not to detect spinosyn D if the presence of spinosyn A resulted from contamination.

Table 1
Summary of mass spectrometry data for isolated spinosyns

Compound	Molecular ion species[a]	Fragment ion data[b]
1	$[M+H]^+$ = 758.4	142.1 (forosamine)
2	$[M+H]^+$ = 772.4	142.1 (forosamine)
3	$[M+H]^+$ = 774.4	142.1 (forosamine)
4	$[M+H]^+$ = 788.7	ND
5	$[M+H]^+$ = 758.4	142.1 (forosamine)
6	$[M+H]^+$ = 774.5	158.0 (forosamine + O)
7	$[M+H]^+$ = 744.4	128.1 (*N*-demethyl-forosamine)
8	$[M+H]^+$ = 774.4	158.1 (forosamine + O)
9	$[M+H]^+$ = 744.4	142.1 (forosamine)
10	$[M+H]^+$ = 744.4	142.1 (forosamine)
11	$[M+NH_4]^+$ = 748.0	115 (C-17 sugar), 189 (trimethylrhamnose)[d]
12	$[M+NH_4]^+$ = 810.5	617.4 (M-C17 sugar + 2H)
13	$[M+NH_4]^+$ = 764.5	633.4 (M-C17 sugar + 2H)
14	$[M+NH_4]^+$ = 808.5	633.4 (M-C17 sugar + 2H)
15	$[M+H]^+$ = 744.4	128.1 (*N*-demethyl-osamine)
16	$[M+H]^+$ = 758.4	142.1 (osamine)
17	$[M+H]^+$ = 774.4	142.1 (forosamine)
18	$[M+H]^+$ = 760.5	128.1 (*N*-demethyl-forosamine)
19	$[M+H]^+$ = 756.4	142.1 (forosamine)
20	$[M+H]^+$ = 744.3	142.1 (forosamine)
21	$[M+H]^+$ = 772.4	142.1 (forosamine)
22	$[M+H]^+$ = 760.4	142.1 (forosamine)
23	$[M+ NH_4]^+$ = 634	189 (trimethylrhamnose)[d]
24	$[M+H]^+$ = 631.4	189 (trimethylrhamnose)[d]
25	$[M+H]^+$ = 633.4	189 (trimethylrhamnose)
26	$[M+NH_4]^+$ = 650.4	189.1 (trimethylrhamnose)
27[c]	$[M+NH_4]^+$ = 666.4	189.1 (trimethylrhamnose)
28	$[M+H]^+$ = 615.4	189.1 (trimethylrhamnose)
29	$[M+NH_4]^+$ = 636.4	617.4 ($M+H^+$), 189.1 (trimethylrhamnose)
30	$[M+NH_4]^+$ = 620.3	175.1 (3,4-dimethyl-rhamnose)
31	$[M+NH_4]^+$ = 620.3	175.1 (2,4-dimethyl-rhamnose)

[a] MS ion from which MW was determined.
[b] MS/MS ion which determined nature of C-17 or C-9 sugars.
[c] Both C-24 epimers.
[d] EI probe MS conditions.

2.3.2. Structures of the new spinosyns

The first compound in this new family (spinosyn α2; **12**; discussed in more detail below) to be analyzed by NMR did not contain an amino sugar, but still contained 2 glycoside units—a *per*-methylated rhamnose and a 3-O-methylated glucose. The similarity to the spinosyns—which have been well studied in these laboratories—was noted from an HSQC experiment, where key differences from the 'classical' spinosyns were identified. The main spectral differences apart from the glucose signals and lack of the dimethylamine in forosamine were caused by the addition of two new olefinic carbons and protons. While the LC–MS data did not contain sufficient information to solve the structures, it was clear that many of the new metabolites contained nitrogen atoms with evidence of forosamine sugars (e.g., the characteristic fragment ion at *m/z* 142). Full characterization was completed on the more abundant compounds, as discussed below. This discussion focuses primarily on NMR spectral data; however in each case, the MS parent ion and fragment ion data (Table 1) supported these assignments.

2.3.3. Spinosyn α1

Comparing the NMR spectra of spinosyn α1 (**1**) with spinosyn A revealed few differences (Fig. 1). Two additional olefinic proton signals were present in the spectrum of spinosyn α1. These showed a coupling constant of 16 Hz, indicating a *trans* orientation. Further, H-21 was subject to a significant downfield shift. A 2D gCOSY experiment showed that H-21 was coupled to this olefin, which in turn was coupled to an ethyl group, for which the methylene was more first order than that found in spinosyn A. Thus it was determined that **1** contained a but-1-enyl group at C-21 rather than the ethyl group of spinosyn A. This was consistent with the MS data, which indicated that spinosyn α1 contained an extra C_2H_2 unit compared with spinosyn A. All other signals from the spectra of spinosyn α1 correlated with those from spinosyn A (Table 2).

2.3.4. Macrocycle variants

Three variations on the central ring system were found in these samples (Fig. 2 and Table 3).

2.3.5. Spinosyn δ1 (2)

Three compounds (**2**, **4**, **24**) containing a 6-methyl group were isolated during this work. In the case of the direct spinosyn D equivalent, spinosyn δ1 (**2**), the two olefinic signals for H-5 and H-6 of spinosyn α1 at 5.8 and 5.9 ppm were exchanged for a broad singlet integrating for one proton at 5.5 ppm, plus a broad methyl singlet at 1.72 ppm (Fig. 2). The latter was consistent with an olefinic methyl group—for which a gCOSY experiment indicated allylic coupling to H-5 and homo-allylic coupling to H-4, equivalent to that seen in spinosyn D. Interestingly, in the *S. pogona* series, the relative abundance of 6-methyl analogs was much lower than in the *S. spinosa* series. For example, in the *S. pogona* batches discussed here, the spinosyn α1:δ1 ratio averaged approx. 28:1, versus the more common ratio of 3:1 encountered for spinosyn A:D from *S. spinosa*.

2.3.6. Spinosyn β1 (3)

Eight-hydroxylation was the most commonly-encountered derivative of the macrocycle, with eight examples (**3**, **4**, **13**, **14**, **21**, **22**, **25**, **27**) being isolated. Compound **3**, for example, was characterized by MS as having a molecular weight 16 amu (Table 1) higher than spinosyn α1. Analysis of the proton spectrum and

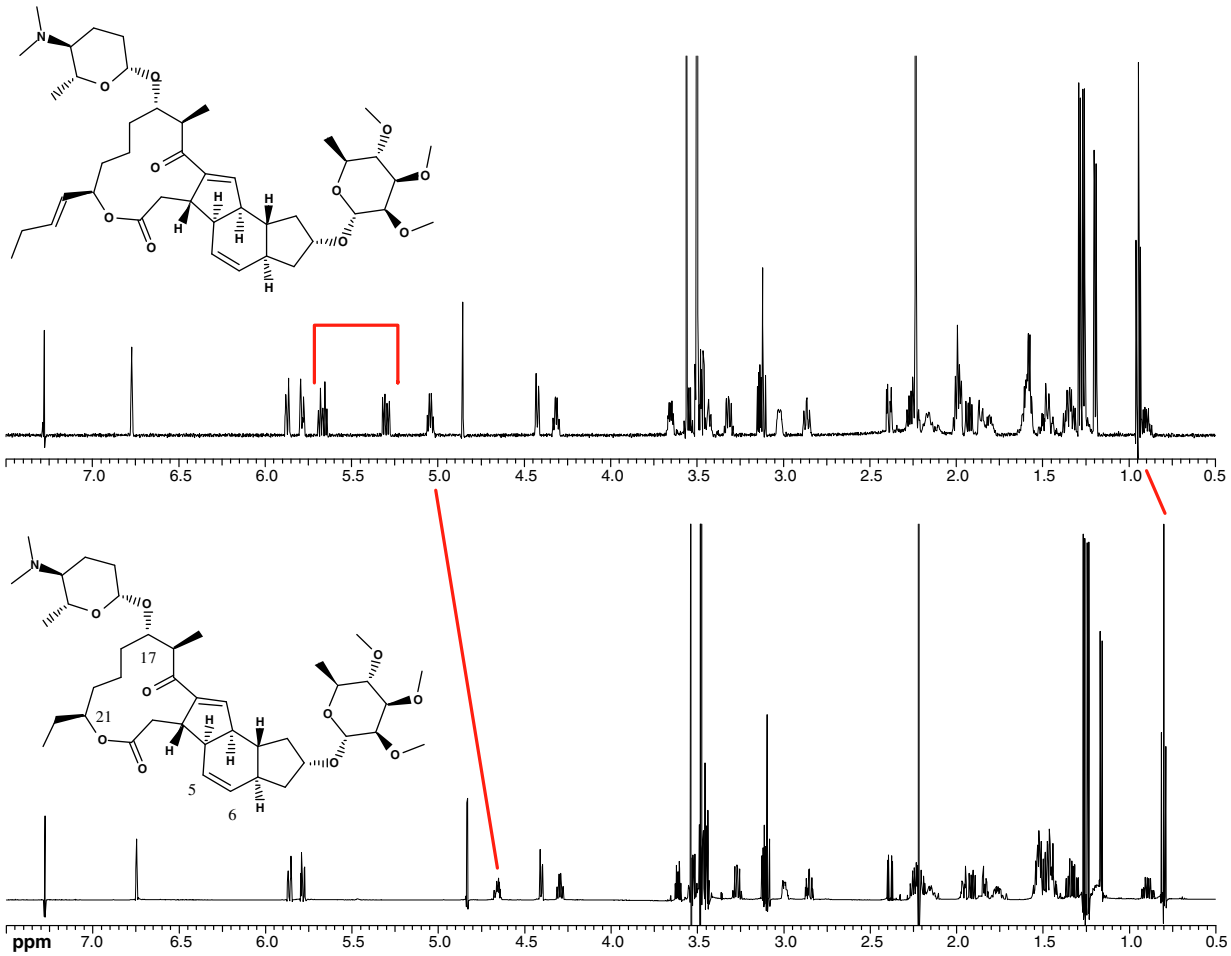


Figure 1. Comparison of the ^{1}H NMR spectra of spinosyn α1 (**1**, upper) and 'classical' spinosyn A (lower). In the upper spectrum, the two side chain olefinic protons are bracketed. The most significant differences that are shown are H-21 and the terminal methyl resonance H-25 (corresponding to H-21 in spinosyn A).

HSQC revealed the following differences from the spectrum of **1**. Protons 5 and 6 had collapsed to a highly second order doublet of doublets (Fig. 2). H-9 was not obviously assignable: whereas in **1** it appeared as a broad ddd multiplet at 4.32 ppm, in **3** there was a sharp doublet at 4.15 ppm and a sharp double doublet at 4.10 ppm. These two signals were consistent with oxygen substitution (HSQC revealed δ_C 72 and 85 ppm, respectively). Further, H-7 had become a sharp multiplet rather than the broad unresolved multiplet that was seen in other spinosyn examples. Finally H-11 had moved downfield by nearly 0.5 ppm. In a gCOSY experiment, cross peaks were identified from H-7 to H-5 and/or H-6, and for homo-allylic coupling to H-4 as expected, but also to the doublet signal at 4.32 ppm, suggesting that this be assigned as H-8 with a hydroxy group replacing the other proton. The double doublet at 4.10 ppm was coupled to a pair of methylene signals at 2.35 and 1.5 ppm which were assigned as H-10, assigning this multiplet as H-9. This assignment was confirmed in an HMBC experiment, with a cross peak to C-9 from H-1′ confirming attachment of the trimethylrhamnose unit. There was no cross peak found, however, between H-8 and H-9. Examination of the X-ray structure for spinosyn A[15] showed that the vicinal angles between H-9 and the two H-8 protons were 4° (*syn*) and 118° (*anti*) thus in **3** it follows that H-8 and H-9 are *anti* subtending a vicinal angle close to 90° leading to a coupling constant $^{3}J_{HH}$ approaching zero. Such 8-hydroxy substitution has not been seen in previous naturally-occurring spinosyns, but recently in a paper describing a synthetic approach to spinosyn A, substitution of a steric directing group on C-8 was employed in order to direct Diels–Alder ring closure to the correct stereochemistry.[16] In that work, the oxygen substituent was synthesized such that the produced spinosyn model had the opposite stereochemistry at C-8 (protons H-8 and H-9 *syn*) to that determined in **3**, and in that case coupling was seen between H-9 and H-8 ($^{3}J_{HH}$ = 6.3 Hz for a measured torsion angle of 146°) as well as a significantly different coupling constant between H-7 and H-8 (8.8 Hz vs 4.6 Hz measured in **3**).

2.3.7. Spinosyn γ1 (5)

The proton spectrum of this 14-membered ring macrocycle variant showed significant differences to the other compounds (Fig. 2). An additional olefin was present relative to spinosyn A, although the multiplet structures were different than those in **1**. The proton adjacent to the lactone oxygen atom (H-21 in **1**) had shifted upfield to 4.3 ppm, and was more simple; and the alkyl side-chain 'tail' had reverted to an ethyl group as found in spinosyn A. A gCOSY experiment indicated that the olefinic protons were still coupled to the lactone proton, indicating that the olefinic bond was integrated into the macrocyclic ring, while TOCSY and HSQC-TOCSY experiments revealed that there were still three methylene carbon atoms in this ring, with expected movements in their chemical shifts due to the presence of the olefin. Thus the macrocycle in **5** was 2 carbon atoms larger than in the other spinosyns. NOESY experiments, careful assessment of coupling constants and molecular modeling experiments were used to deduce the 3D solution structure of this molecule. The results indicated that the stereo-

Table 2
^{1}H NMR data for spinosyn α1 (**1**, 600 MHz, $CDCl_3$)

Position	^{1}H	Multiplicity	^{13}C	HMBC correlations
1	—	—	172.1	2, 2, 21
2	3.14	dd, 13.3, 5.1	34.5	
	2.39	dd, 13.3, 3.1		
3	3.02	m, br	47.6	2, 2, 4, 13
4	3.42	m	41.3	2, 5, 13
5	5.79	dt, 9.7, 2.6	128.7	8, 11
6	5.87	d (br), 9.7	129.3	5
7	2.16	m	41.1	6, 8, 8, 9, 11
8	1.92	dd, 13.7, 7.4	36.2	6, 10
	1.35	m		
9	4.30	ddd, 7.1, 6.1	76.0	1′, 8, 8
10	2.26	dd, 13.3, 6.7	37.3	7, 8, 11
	1.34	m		
11	0.90	ddd, 11.8, 6.7	46.0	6, 8, 9 (wk), 10
12	2.86	m	49.4	5, 13
13	6.77	s (br)	147.5	11
14	—	—	144.1	2, 2, 12, 13
15	—	—	202.8	13, 16, 17 (wk), 26
16	3.31	dq, 9.7, 7.2	47.4	13, 17
17	3.66	dt, 9.7, 4.6	80.8	1″, 16, 19 (wk), 26
18	1.60	m	34.2	
19	1.80	m	21.4	16, 17
	1.23	m		
20	1.60	m	31.6	21, 22
21	5.04	ddd, 6.7, 6.7, 6.7	76.3	19, 19, 22, 22, 23
22	5.30	dd, 15.4, 7.2	128.0	21, 24
23	5.66	dt, 15.4, 6.1	134.9	21, 24, 25
24	1.99	dq, 7.2, 6.1	25.0	22, 23, 25
25	0.95	t, 7.2	13.1	23, 24
26	1.20	d, 7.2	16.3	16, 17
1′	4.86	s (br)	95.4	9
2′	3.50	dd, 3.1, 1.5	77.7	1′, 2′-OMe
2′-OMe	3.48	s	57.6	
3′	3.46	dd, 9.2, 3.1	81.0	1′, 4′, 3′-OMe
3′-OMe	3.48	s	59.0	
4′	3.11	dd, 9.2, 9.2	82.2	3′, 6′, 4′-OMe
4′-OMe	3.56	s	60.9	
5′	3.55	dq, 9.2, 6.1	67.9	1′, 4′, 6′
6′	1.28	d, 6.1	7.8	
1″	4.42	d (br), 7.7	103.6	2″, 2″, 3″, 3″, 5″, 17
2″	1.98	m	30.9	1″, 3″
	1.46	m		
3″	1.86	m	18.3	1″, 2″
	1.50	m		
4″	2.22	m	64.8	2″, 3″, 4″-NMe_2
4″-NMe_2	2.23	s	40.7	4″, NMe_2, 6″
5″	3.50	m	73.7	3″, 4″, 6″
6″	1.26	d, 6.1	18.9	

chemistry at C-23 (where the lactone OH attaches to the carbon backbone) is consistent with those found in the 'classical' spinosyns.[17] This was the first example of a spinosyn with a different number of atoms in the macrocyclic lactone that has been identified to date.

2.3.8. Glycosylation variants

The trimethylrhamnose sugar on C-9 was conserved in the majority of compounds discovered in this series. As with the 'classical' spinosyns, the only variations found were due to the degree of O-methylation, for example in compounds **9**, **10**, **30** and **31**, in which methoxy groups were substituted with hydroxy groups at either C-2′ and C-3′ (Table 4). More interesting was the group of compounds where the nature of the sugar substituted at C-17 differed. In the 'classical' spinosyn series, the only variations found on this sugar were due either to the degree of methylation on the amino function (spinosyns B and C), or to the presence of an epimer at the 5″ position (spinosyn G).[14] *S. pogona* produced two distinct classes of carbohydrates linked to the macrolide at C17: those with an amino function at C-4″, and those with an alcohol at C-4″, as discussed below (Table 5).

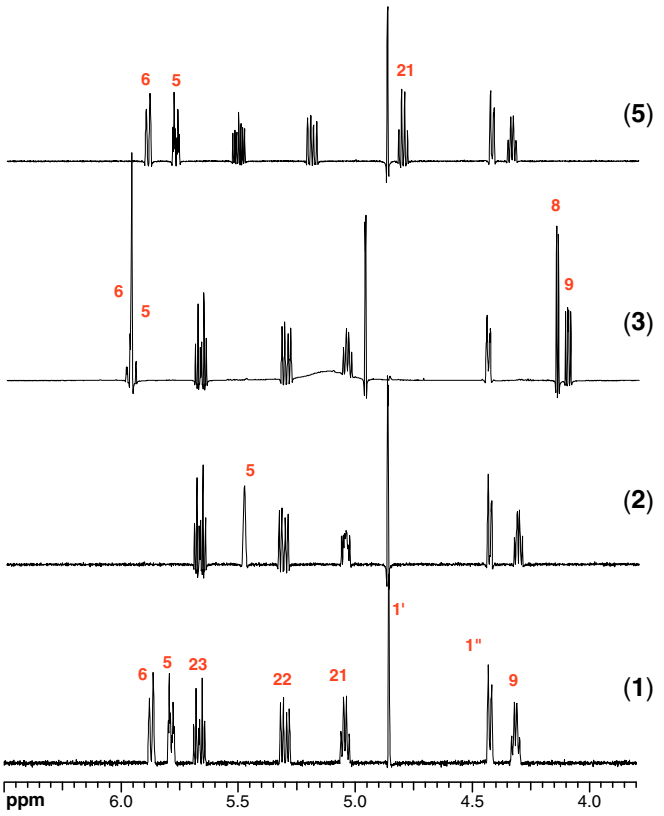


Figure 2. Expansion of the downfield portion of the ^{1}H spectra for **1** (lowest), **2**, **3** and **5** (top) with key assignments shown as discussed in the text.

Table 3
Comparison of ^{1}H spectra for macrocyclic variants (600 MHz, $CDCl_3$)

	α1 (**1**)	β1 (**3**)	γ1 (**5**)	δ1 (**2**)
2	3.14	3.13	3.29	3.14
	2.39	2.39	2.51	2.39
3	3.02	3.06	2.73	3.00
4	3.42	3.46	3.21	3.39
5	5.79	5.95	5.78	5.49 (br)
6	5.87	5.97	5.90	—
7	2.16	2.18	2.20	2.20
8	1.92, 1.35	4.15 (d)	1.94, 1.36	1.94, 1.42
9	4.30	4.10 (dd)	4.32	4.31
10	2.26, 1.34	2.36, 1.29	2.28, 1.37	2.28, 1.37
11	0.90	1.46	1.02	1.02
12	2.86	2.88	2.89	2.79
13	6.77	6.79	6.89	6.78
16	3.31	3.31	3.28	3.31
17	3.66	3.66	3.66	3.66
18	1.60	1.58	1.25	1.60
19	1.80, 1.23	1.80, 1.24	1.84, 1.44	1.82, 1.26
20	1.60	1.60	2.10, 2.05	1.60
21	5.04	5.04	5.50	5.04
22	5.30	5.31	5.19	5.31
23	5.66	5.68	4.80	5.68
24	1.99	2.00	1.65, 1.55	2.00
25	0.95	0.96	0.90	0.96
26	1.20	1.19	1.12	1.20

Within the amino sugar family, two compounds (**7**, **18**) were found which were mono-demethylated on the amino function, with trivial changes to the spectra consistent with those found for spinosyn B. However, there was also a new variant in the form of 2″-hydroxyspinosyn α1 (**8**) which was identified from MS and 2D-NMR experiments. In this case, the anomeric 1″ signal was shifted upfield 0.3 ppm with respect to **1**, and was a doublet rather

Table 4
Selected proton chemical shifts for spinosyns demethylated on the C-9 trimethylrhamnose glycoside (600 MHz, $CDCl_3$)

#	α1 (**1**)	2′-ODM-α1[a] (**9**)	3′-ODM-α1 (**10**)
1′	4.86	4.84	4.89
2′	3.50	3.97	3.41
3′	3.46	3.45	3.82
4′	3.11	3.08	2.98
5′	3.55	3.62	3.58
6′	1.28	1.30	1.31
2′-OMe	3.48		3.51
3′-OMe	3.48	3.51	
4′-OMe	3.56	3.58	3.60

[a] ODM = O-demethylated.

Table 5
Selected proton chemical shifts for C-17 glycoside variants (600 MHz, $CDCl_3$)

#	α1 (**1**)	α2 (**12**)	α4 (**11**)	α5 (**16**)	NDM-α6 (**15**)[a]	2″-OH-α1 (**8**)	β3(**14**)
1″	4.42	4.45	4.51	4.80	4.87	4.24	4.46
2″	1.98	3.48	2.09	1.94	1.81	3.51	2.33
	1.46	—	1.51	1.55	1.59	—	1.46
3″	1.86	3.22	1.98	1.88	1.89	2.13	3.25
	1.50	—	1.50	1.70	1.82	1.47	—
4″	2.22	3.60	3.30	2.35	2.52	2.35	2.74
5″	3.50	3.40	3.30	4.30	4.63	3.48	3.21
6″	1.26	3.89	1.30	1.28	1.16	1.27	1.19
		3.82					
NMe	2.23	—		2.30	2.45	2.25	
3″-OMe							3.70
				3.44			
4″-OMe							
				3.56			

[a] NDM = N-demethylated.

than a doublet of doublets. H-2″ resonated at 3.5 ppm, and thus was not directly detected in a ^1H spectrum due to severe signal overlap, however it was determined from examination of gCOSY, HSQC and HMBC experiments. The H-3″ signals were readily identified by these experiments, and were notably more simple multiplets than seen in other spinosyns, with a downfield shift of C-3″ consistent with hydroxylation at the 2″ position. The most upfield proton on C-3″ was identified as the axial proton, and resonated as a quartet-like multiplet, with three large coupling constants. Thus H-2″ was shown to also be axial ($^3J_{2-3}$ = 12 Hz), indicating that the hydroxy group was equatorial. Two epimeric variants of **1** were also identified (these glycosidic units therefore becoming osamines). In spinosyn α5 (**16**), the anomeric proton had shifted downfield by 0.3 ppm, and signals for the dimethylamine protons and H-4″ were broadened somewhat. Additionally, H-5″ had been shifted downfield to 4.3 ppm. These changes were consistent with those found for the 'classical' analog, spinosyn G.[14] Confirmation of the epimerization at C-5″ was made with the acquisition of a selective NOESY experiment on H-1″. This resulted in enhancement of the signals for macrolide H-17, one of the H-2″ signals, and the methyl signals for H-6″ and macrolide H-26, thus confirming that H-6″ was substituted on C-5″ in an axial position. A second epimeric compound was found which had a monomethylamine group on C-4″. *N*-Demethyl spinosyn α6 (**15**), also exhibited a large downfield shift of the anomeric proton. The positions of the other signals on the glycoside were deduced by analysis of gCOSY and TOCSY experiments. The signals for H-3″ were shifted downfield when compared with **1**, but more significant was the signal for H-4″. The width of the H-4″ multiplet in *N*-demethyl spinosyn α1 is 25 Hz (consisting of 2 large axial/axial and one small axial/equatorial coupling constants), whereas in *N*-demethyl spinosyn α6 the width of H-4″ is approx. 8 Hz, indicating the lack of any axial/axial coupling. These findings were consistent with H-4″ being equatorial, thus all the couplings to it being small. To verify this a selective NOESY experiment was performed on H-4″. In the equivalent experiment for regular forosamine containing compounds (e.g., **1**) a strong correlation would be expected between H-4″ to one of the H-2″ signals. However, for **15**, the only enhancement found on irradiating H-4″ was the trivially assigned H-5″.

Further structural variation was seen in the 4″-hydroxylated series which are unique to the *S. pogona*-produced spinosyns. Spinosyn α2 (**12**) was particularly noteworthy as it had one less methyl signal at 1.4 ppm than seen for **1**, and there was a pair of double-doublets apparent at 3.8 and 3.9 ppm indicating that the glycoside on C-17 was not 6-deoxy. COSY and HSQC-TOCSY spectra revealed that these signals were part of a fully oxygenated glucose unit with the detection of triplets at 3.6 (4″) and 3.2 (3″) ppm and an anomeric doublet at 4.4 ppm. The signal for H-2″ overlapped with a large number of other signals, including H-5″, and was not directly detected. All coupling constants between these protons were determined to be axial–axial, confirming the β-D-glucose assignment. Also noted was an extra methoxy signal additional to those arising from the rhamnose sugar. This extra methylation was determined to be on the 3″-OH as shown by the upfield shift of H-3″ when compared to a semi-synthetic sample of spinosyn A which had been substituted at C-17 with glucose (H-3″ 3.6 ppm vs 3.22 ppm; C-3″ 76 ppm vs 85 ppm in **12**).

One of the most intriguing compounds in this series, spinosyn β3 (**14**), contained an oleandrose sugar at C-17 in combination with a C-8 hydroxylated (β type) macrocycle. The macrocyclic core of **14** was identified as described above, with differences noted when compared to **3**, including the lack of dimethylamine groups, and addition of two extra methoxy signals. In contrast to spinosyn α2 (**12**), the anomeric signal for the C-17 glycoside in **14** was a doublet of doublets—similar to the signal seen for forosamine, and there was also a methyl doublet at 1.3 ppm implying a 6″-deoxy sugar. COSY and TOCSY experiments revealed oxygen substitution on C-3″ and C-4″ (H-3″ 3.3 ppm, and H-4″ 2.7 ppm), with the vicinal coupling constants suggesting axial–axial coupling between H-3″ and H-4″, as well as H-4″ and H-5″. Again the upfield shifts for H-3″ and H-4″ implied that the oxygen atoms were methylated, thus this sugar was concluded to be 4″-*O*-methyl β-D-oleandrose.

The final compounds in this series were spinosyns α4 (**11**) and β4 (**13**) neither of which showed a methylamine signal, or any extra methoxy groups. COSY and TOCSY experiments revealed that the glycoside signals resembled that of forosamine in **1**, except that H-4″ was shifted downfield to 3.3 ppm with C-4″ (from HMQC) at 72 ppm suggesting hydroxylation. Comparison with a semi-synthetic spinosyn A-related compound having this glycoside confirmed the structures to contain the monohydroxy sugar D-amicetose attached to the macrocycle C-17 hydroxy group.[14]

2.3.9. C-21 variants

While the majority of the factors found in this work were substituted with a but-1-enyl group at C-21, 10 compounds were found that differed in this side chain (Table 6). For example, in

Table 6
Selected proton chemical shifts for various C-21 groups (600 MHz, $CDCl_3$)

#	α1 (**1**)	α1a (**19**)	α1b (**20**)	α1c (**17**)
19	1.80, 1.23	1.85, 1.27	1.81, 1.25	1.84, 1.28
20	1.60	1.62	1.60	1.60
21	5.04	5.11	5.03	5.09
22	5.30	5.57	5.35	5.56
23	5.66	6.17	5.66	5.68
24	1.99	5.56	1.65	4.29
25	0.95	5.21, 5.10		1.20

spinosyns α1a (**19**) and -β1a (**21**) the signals in the olefinic portion of the ^{1}H spectra were substantially different than in **1** or **3**, most notably by the presence of two doublets of doublets at 5.2 and 5.1 ppm which resembled a terminal olefin. COSY and HSQC spectra confirmed that these molecules, as well as the pseudoaglycone spinosyn αa (**28**), consisted of a buta-1,3-diene moiety attached to C-21. In spinosyns α1b (**20**) and -β1b (**22**), the triplet signal usually assigned to H-25 had been replaced with a doublet at 1.55 ppm, which was coupled to an olefinic doublet of quartets at 5.65 ppm, indicating the substitution of a propenyl group at C-21. Spinosyn α1c (**17**) exemplified several compounds showing a new methyl doublet at 1.2 ppm, coupled to a new doublet of quartets at 4.3 ppm which was coupled to an olefinic signal. The carbon associated with this proton signal resonated at 69 ppm, indicating substitution with a hydroxy function, leading to the identification of four compounds (**17**, **18**, **26**, **27**) containing a C-21 3-hydroxy-1-butenyl function. Finally, one example of a saturated alkyl group was found. The H-21 signal for spinosyn αd (**29**) was shifted upfield of the anomeric 1′ signal, reminiscent of spinosyn A. However 2D spectroscopy—in particular HMBC experiments—revealed a 4 carbon subunit attached to C-21. This *n*-butyl group was the only saturated group found in this family. There were some indications by LC–MS that the related compound spinosyn α1d was also produced, but a sufficient sample for full characterization was never isolated.

2.3.10. Pseudo-aglycones

Nine compounds were found which had no glycoside substituted at C-17 based on their ^{1}H NMR and mass spectra. All 12-membered ring macrocycle variations were represented in these cases [spinosyns α (**23**), 6-Me-β (**25**), δ (**24**)] with the greatest structural variation seen in the substituent on C-21. In addition to the butadiene and hydroxy-butene groups discussed above [spinosyns αa (**28**) and αc (**26**)], one compound (**29**) was found with no double bonds in the tail as described above.

2.4. Biological activities of selected spinosyns

Table 7 summarizes the biological activities of some of the new compounds isolated during this work. In the tobacco budworm (TBW) neonate assay, spinosyn α1 (**1**) was approximately as active as spinosyn A, whereas the 6-methylated analog, spinosyn δ1 (**2**) was more active than its 21-ethyl analog, spinosyn D. Replacement of the forosamine sugar with D-amicetose gave spinosyn α4 (**11**), which had comparable TBW activity to the parent compound. Adding an 8-hydroxy function to this compound, to give spinosyn β4 (**13**), resulted in a slight reduction in TBW activity. Further, replacement of the forosamine in **1** with 3-*O*-methyl-glucose gave spinosyn α2 (**12**), which was 10–50× less active than the parent compounds. Conversely, spinosyn β3 (**14**), where the forosamine had been replaced with a methyl-oleandrose unit, was the most TBW-active of the new compounds. While the lower level of its activity was not defined, it was at least 3–5× more active than **1** and spinosyn A. All the new compounds showed some activity against cotton aphid at 50 ppm, and of the few tested, in the cases where whitefly activity was seen, it generally paralleled the order seen on TBW.

Table 7
Summary of insecticidal activity[a] of selected spinosyns

Compound	Tobacco budworm neonate		Cotton aphid	Whitefly
	LC_{50}	LC_{90}	LC_{50}	LC_{50}
Spinosyn α1 (**1**)	0.29	0.55	11	2
Spinosyn δ1 (**2**)	0.30	1.16	2	nd[b]
Spinosyn α4 (**11**)	0.21	0.39	6	5
Spinosyn β4 (**13**)	0.07	0.97	nd	nd
Spinosyn α2 (**12**)	>25	25	17	nd
Spinosyn β3 (**14**)	<0.1	<0.1	18	nd
Spinosyn A	0.31	0.57	18–50	5
Spinosyn D	0.80	nd	65	2

[a] ppm.
[b] nd = not determined.

The novel spinosyns reported here had three main areas of significance. First, when viewed in the context of their insecticidal activities (Table 7), they significantly enhanced our understanding of the spinosyn structure–activity relationship. In particular, they demonstrated for the first time that structural modifications to the C21 side chain and C17 sugar moiety, including replacement with neutral sugars, as well as macrolide ring-hydroxylation and macrolactone enlargement need not be deleterious to bio-activity. Secondly, through directly providing new synthetic handles on the base spinosyn molecule and *via* the production of mutants furthering this approach,[18,19] they enabled the synthesis of a range of new analogs.[20] Third, they stimulated further interest in the genetics and enzymology leading to the assembly of this class of molecules. This work showed that the gene sequence for these new spinosyns code for one extra polyketide synthase module, compared with the original spinosyn sequence, and that this module is responsible for the two additional carbon atoms in the C21-side chain.[13,21] The genetic work also suggested that the *S. pogona* forosaminyl-transferase, *Bus*P, may occupy a unique biosynthetic space through its ability to catalyze attachment of such a diverse range of sugars to the spinosyn C17-hydroxyl group. Finally, it was hypothesized that the *S. pogona* genes may represent the *parental* gene structure which may have been naturally engineered to create the *S. spinosa* spinosyn genes.

3. Experimental

3.1. General experimental procedures

All NMR spectra were recorded on a Bruker DRX600 spectrometer operating at 600.13 MHz (^{1}H), and 150.62 MHz (^{13}C). Full proton NMR data can be found for all compounds with two glycoside groups in the Supplementary data. Additionally Tables 2–6 summarize various aspects of the NMR data for these compounds. LC/MS was performed on a Micromass Platform single-quadrupole mass spectrometer in both positive electrospray (+ESI) and negative electrospray (−ESI) modes. Table 1 summarizes the +ESI-MS data for the isolated factors. Flash column chromatography was performed on a variety of stationary phases obtained from Biotage, Inc. (Charlottesville, VA, USA). Analytical HPLC was performed on an HP1050 system with quaternary pump and HP1040 Series-II diode array detector. When needed, a portion of the post-column eluent was split off and collected as fractions for bioassay in a 96-well microtiter plate using a Gilson FC204 fraction collector. Most preparative HPLC was accomplished using a Waters system comprising 600E quaternary pump and Waters 991 diode array detector run in Powerline mode. Samples were introduced to the solvent stream by manual injection using a Rheodyne 7125 injector with 2-mL loop.

3.2. Bacterial fermentation conditions

S. pogona strain NRRL 30141 was grown by the methods previously described.[10]

3.3. Chromatographic methods

Silica gel flash chromatography was performed in one of two ways. For separations up to several grams in scale, using 40-series Biotage silica gel cartridges, eluent was delivered to the column

using an LC pump with multi-step gradient elution. Fractions were collected using an Isco Retriever-IV fraction collector, typically 60–100 fractions per run. For separations in the tens of grams range, using 75-series Biotage cartridges, solvent was delivered under gas pressure in batch mode, with several changes of eluent during the run. The separation conditions used and referred to in the Metabolite Isolation section and in the Supplementary Figures were as follows (flow rate = 20 mL/min unless stated otherwise).

B1: Multi-step linear gradient of A (0 min) to B (10 min) to B:C (50:50; 15 min then held to 20 min) to D (20.01 min) to E (30 min) to F (40 min) to G (50 min and held to 70 min); collected 140 × 0.5-min fractions; solvents: A = hexane; B = toluene; C = ethyl acetate; D = hexane–DEA (99.5:0.5); E = toluene–DEA (99.5:0.5); F = EtOAc–DEA (99.5:0.5); G = EtOAc–MeOH–H_2O–DEA (70:20:9.5:0.5).

B2: Multi-step linear gradient of A (0–5 min) to A:B (60:40; 15 min) to B (25 min and hold to 40 min); collected 80 × 0.5-min fractions; solvents: A = hexane–DEA (99.5:0.5); B = hexane–isopropanol–DEA (75:25:2).

B3: Run under gas pressure with sequential batches of solvent as follows: hexane (2 L; fraction 1); toluene (1 L; fraction 2); toluene–EtOAc (1:1; 1 L; fraction 3); hexane–DEA (99:1; 2 L; fraction 4); toluene–DEA (99:1; 2 L; fraction 5); EtOAc–DEA (99:1; 4 × 500 mL; fractions 6–10); EtOAc–MeOH–H_2O–DEA (70:20:10:2; 10 × 400 mL; fractions 11–20); EtOAc–MeOH–H_2O–DEA (45:45:10:2; 5 × 400 mL; fractions 21–25).

B4: Linear gradient from toluene (0 min) to toluene–isopropanol (90:10; 30 min and hold to 40 min); collected 80 × 0.5-min fractions.

B5: Linear gradient from 25 mM NH_4OAc–MeOH–MeCN (50:25:25; 0 min) to MeOH–MeCN (50:50; 20 min and hold to 50 min); collected 100 × 0.5-min fractions.

Preparative HPLC separations were most commonly achieved using Hypersil C_{18}-BDS or C_8-BDS columns using either isocratic or gradient solvent conditions. Isocratic elution conditions are summarized in Table 8. Additionally, the following two gradient elution systems were used. Gradient G-1 consisted of a 3 mL/min flow rate of solvent delivered to a Hyperprep-C_8-BDS (250 × 10 mm; 8 μm particle size) column. The gradient profile was 10 mM NH_4OAc–MeOH–MeCN (60:20:20; 0–5 min), followed by a linear ramp to MeOH–MeCN (1:1) at 25 min, held under these conditions for 5 min, then ramped back to initial conditions over 2 min and held there for a further 8 min. Gradient G-2 consisted of a 13 mL/min flow rate of solvent delivered to a Rainin-RP-C18 (200 × 25 mm; 10 μm particle size) column. The gradient profile was H_2O–MeOH (30:70 at 0 min, then linearly ramped to 12:88 at 40 min).

For very large-scale chromatography, separation of extracts was achieved using a ProChrom Prep LC-110 System with a column (31.5 cm long × 11 cm i.d.) of Kromasil-C_{18} stationary phase (2.0 kg; spherical, 10 μm, 100 A pore size; Eka Nobel). The flow rate was 590 mL/min and the load/run was approximately 4 g. The mobile phase used was CH_3CN–CH_3OH–0.5% NH_4OAc (42.5:42.5:15). Eluent was monitored by UV absorbance at 260 nm and peaks were manually collected using this profile as a guide.

Insect screening: High throughput lepidopteran and tobacco budworm neonate diet assays were run as previously described.[22,23] Sweet potato whitefly (*Bemisia tabaci*) and cotton aphid (*Aphis gossypii*) foliar spray assays were run as follows. Cotton plants (21 days old) were infested with *B. tabaci* eggs and hand-sprayed with test compound dissolved in water–acetone (90:10) containing 0.025% Tween 20. Four replicate plants were sprayed per dose. The treated plants were held for 14 days in a growth chamber at 27 °C, with 14 h light/10 h dark photoperiod, at which time percent whitefly mortality was recorded. In similar fashion, squash plants (7 days old) were infested with a mixed population (nymphs and adults) of *A. gossypii* and after 24 h were hand-sprayed with test compound dissolved in water–acetone (90:10) containing 0.025% Tween 20. Four replicate plants were sprayed per dose. The treated plants were held for 3 days in a growth chamber at 27 °C, with 14 h light/10 h dark photoperiod, at which time percent aphid mortality was recorded.

3.4. Metabolite isolation (flow charts SF1–SF3 in Supplementary data)

3.4.1. Four litre batch

The total culture, that is, cells plus broth, from fermentation of 5 L of inoculated medium had a total volume of 4 L after fermentation was complete. This sample was extracted with an equal volume of denatured EtOH by shaking vigorously then allowing to stand at room temperature for 2 h. The cell debris was removed by centrifugation and the total soluble extract was partitioned using DCM (2 × 7 L). The DCM extract was concentrated to give a pale yellow oil (3.3 g). The oil was dissolved in MeOH and divided into two equal aliquots, each of which was chromatographed on a silica gel column (4 × 15 cm; 32–63 μm). Each column was eluted using solvent gradient B-1 then flushed with a final aliquot of solvent G (100 mL). The collected fractions were analyzed by HPLC and, on the basis of the results, were pooled as follows: SI-1 (fractions 92–97), SI-2 (104–112), SI-3 (114–120), SI-4 (121–140) and SI-5 (flush fraction). These pooled fractions were dried in vacuo and redissolved in MeOH before further fractionation as described below.

Table 8
Summary of isocratic elution systems used for preparative separation

System #	Stationary phase	Particle size (μm)	Dimensions (mm)	Flow rate (mL/min)	Solvent A[a] (%)	Solvent B (%)
I-1	Hyperprep-C_8-BDS	8	250 × 10	3	NH_4OAc (30%)	AcCN–MeOH (1:1; 70%)
I-2	Hyperprep-C_8-BDS	8	250 × 10	3	NH_4OAc (20%)	AcCN–MeOH (1:1; 80%)
I-3	Hyperprep-C_8-BDS	8	250 × 10	3	NH_4OAc (85%)	AcCN–MeOH (1:1; 15%)
I-4	HS-Hyperprep-C_{18}-BDS	8	250 × 22	25	NH_4OAc (25%)	MeOH (75%)
I-5	HS-Hyperprep-C_8-BDS	8	250 × 22	25	NH_4OAc (20%)	AcCN–MeOH (1:1; 80%)
I-6	HS-Hyperprep-C_{18}-BDS	8	250 × 22	25	NH_4OAc (15%)	AcCN–MeOH (1:1; 85%)
I-7	HS-Hyperprep-C_{18}-BDS	8	250 × 22	25	NH_4OAc (20%)	MeOH (80%)
I-8	HS-Hyperprep-C_{18}-BDS	8	250 × 22	25	NH_4OAc (30%)	MeOH–THF (3:1; 70%)
I-9	HS-Hyperprep-C_8-BDS	8	250 × 22	25	NH_4OAc (25%)	AcCN–MeOH (1:1; 75%)
I-10	HS-Hyperprep-C_{18}-BDS	8	250 × 22	25	NH_4OAc (15%)	MeOH (85%)
I-11	HS-Hyperprep-C_{18}-BDS	8	250 × 22	25	NH_4OAc (10%)	AcCN–MeOH (1:1; 90%)
I-12	Hyperprep-C_{18}-BDS	5	250 × 10	5	NH_4OAc (40%)	MeOH–THF (3:1; 60%)
I-13	Hyperprep-C_{18}-BDS	5	250 × 10	5	NH_4OAc (30%)	MeOH–THF (3:1; 70%)
I-14	HS-Hyperprep-C_8-BDS	8	250 × 22	25	NH_4OAc (30%)	AcCN–MeOH (1:1; 70%)

[a] 10 mM NH_4OAc.

Aliquots of fraction SI-1 (73 mg) were chromatographed by semi-preparative isocratic HPLC (system I-1; Table 8). Fractions eluting between 14–15.5, 17.5–18.5 and 21.5–22.5 min were pooled and dried in vacuo to give **23** (14.9 mg), **24** (1.4 mg) and **11** (4.2 mg), respectively.

Aliquots of fraction SI-2 (88 mg) were chromatographed by semi-preparative gradient HPLC (system G-1). Fractions eluting between 16.5–17.5, 19.5–20, 22.5–23, 23–23.5 and 26–26.75 min were pooled and dried in vacuo to give **26** (11.5 mg), **25** (3.9 mg), **13** (2.0 mg), **12** (4.9 mg) and **14** (2.3 mg), respectively. Fractions eluting between 8 and 9 min were pooled and dried in vacuo to give of a mixture of the two 24-epimeric isomers of compound **27** (15 mg). This mixture was dissolved in MeOH and chromatographed by semi-preparative isocratic HPLC (system I-3; Table 8). Fractions eluting between 65–68 and 69–72 min were pooled and dried in vacuo to give **27-1** (9.8 mg) and **27-2** (4.7 mg), respectively.

Pooled fraction SI-3 (310 mg) was dissolved in MeOH and chromatographed on a silica gel column (4 × 7.5 cm; 32–63 μm) eluted using method B-2. Based on HPLC analysis, fractions 46–50 were pooled and dried in vacuo. The dried sample (48 mg) was dissolved in MeOH and chromatographed by semi-preparative isocratic HPLC (system I-2; Table 8). Fractions eluting between 20–22.5 and 25.5–27 min were pooled and dried in vacuo to give **1** (24.3 mg) and **2** (1.5 mg), respectively.

Pooled fraction SI-4 (405 mg) was dissolved in MeOH and chromatographed by semi-preparative gradient HPLC (system G-2). Fractions eluting between 21.25 and 21.75 min were pooled and dried in vacuo to give crude **1** (5.8 mg), as were fractions eluting between 22.75 and 23.75 min, which contained crude **3** (7.3 mg). The pooled, dried fractions from above were each dissolved in MeOH and re-chromatographed by semi-preparative isocratic HPLC (system I-2; Table 8). Fractions eluting between 15.5 and 16.5 min were pooled and dried in vacuo to give **3** (3.1 mg). Fractions eluting between 35.5 and 38.5 min were pooled and dried in vacuo to give a further 3.2 mg pure **1**.

The dried column flush fraction SI-5 (53 mg) was dissolved in MeOH and chromatographed by semi-preparative isocratic HPLC (system I-2; Table 8). Fractions eluting between 16 and 18 min were pooled and dried in vacuo to give **7** (3.5 mg).

3.4.2. 140 Litre batch

One hundred and forty litres of fermentation broth was extracted with 2,6-dimethylheptan-4-one (DMH; 2 × 35 L) and the extract was dried in vacuo as an aqueous azeotrope. The residue (60 g) was dissolved in acetone–hexane (1:1) and filtered through a small plug of silica gel, before being chromatographed on a silica gel column (7.5 × 15 cm; 32–63 μm). The column was eluted using method B-3. Based on HPLC analysis, fractions 10 and 11 were pooled and dried in vacuo to give SI-6 (5 g) as were fractions 14–17 to give SI-7 (21 g).

Fraction SI-6 was chromatographed on a silica gel column (4 × 15 cm; 32–63 μm), eluted using method B-4. Collected fractions were pooled as follows based on HPLC analysis: fractions 52–70 (SI6-1; 2.1 g) and 71–80 (SI6-2; 830 mg). Fraction SI6-1 was dissolved in MeOH and chromatographed using preparative gradient HPLC under conditions G-2. Four regions were collected in this step, yielding **11** (100 mg), **23** (300 mg), **28** (5 mg) and a mixture (61 mg). The latter mixture was re-chromatographed by preparative isocratic HPLC using conditions I-4 (Table 8). Fractions eluting between 27–29, 36–39 and 40–42.5 min were pooled and dried to give **23** (2.3 mg), **24** (17.3 mg) and **29** (12.4 mg), respectively. Fraction SI6-2 from above was dissolved in MeOH and dried on a small aliquot of Diaion HP20SS (75–150 μm). The sample was then chromatographed using a Diaion HP20SS column (4 × 15 cm; 75–150 μm), using method B-5. Following HPLC analysis, fractions 57–63 were pooled and dried (103 mg). This sample was dissolved in MeOH and chromatographed by preparative isocratic HPLC using conditions I-5 (Table 8). Fractions eluting between 6–6.5, 8–9 and 10.5–11.5 min were pooled and dried to give a mixture of **30** and **31** (4.0 mg, not further separated), **23** (10.7 mg) and **14** (7.0 mg), respectively.

Fraction SI-7 (21 g) was dissolved in Et_2O (200 mL) and extracted using 1 N-HCl (200 mL). The aqueous phase was extracted with an aliquot of Et_2O (200 mL), then adjusted to pH 8.5 using 30% aq NH_4OH and again extracted with Et_2O (2 × 200 mL). The dried (Na_2SO_4) basic organic phase was concentrated to dryness under vacuum (850 mg). This sample (SI-7-ET2) was dissolved in MeOH and chromatographed by preparative isocratic HPLC using conditions I-6 (Table 8). Fractions eluting between 11–11.5, 15–16.5 and 22.5–27.5 min were pooled and dried to give **3** (26.6 mg), crude **8** (7.2 mg) and **1** (250 mg; mp = 110 °C; $[\alpha]_D^{25} = -95$ [*c* 0.01 in EtOH]), respectively. Crude **8** was further purified by preparative isocratic HPLC using conditions I-7 (Table 8), where it eluted between 34 and 37 min, to give pure **8** (1.7 mg). SI-7-ET2 Fractions eluting between 8.5 and 9.5 min were dried (40.4 mg) and chromatographed by preparative isocratic HPLC using conditions I-7 (Table 8), where fractions eluting between 7–8, 14–16.5, 17–18.5 and 22.5–26 min gave, on pooling and drying **18** (1.1 mg), **17** (23 mg), **6** (3.0 mg) and **7** (4.3 mg), respectively. SI-7-ET2 Fractions eluting between 16.5–18 min were dried (10.2 mg) and chromatographed by preparative isocratic HPLC using conditions I-14 (Table 8), where fractions eluting between 44–46 and 46–50 min were pooled and dried to give **9** (2.9 mg) and **10** (3.5 mg), respectively. SI-7-ET2 Fractions eluting between 19–21 min were pooled, dried (19.6 mg) and re-chromatographed by preparative isocratic HPLC using conditions I-8 (Table 8). Fractions eluting between 26–27.5, 27.5–30 and 32–34.5 min were pooled and dried to give spinosyn A (2.8 mg), **20** (4.1 mg) and **19** (2.5 mg).

3.4.3. 400 Litre batch

Four hundred litres of fermentation broth was extracted with DMH (2 × 40 L). The organic phase was extracted with 1 N-H_2SO_4 (40 L), and the aqueous phase adjusted to pH 9 using 1 N-NaOH. The basified aqueous phase was extracted with Et_2O (3 × 20 L), which was dried under vacuum (12 g). The residue was dissolved in MeOH and chromatographed by ProChrom preparative HPLC as described in Section 3.1. This resulted in the collection of seven significant chromatographic regions, with the following dry weights: PC-3 (196 mg), PC-4 (551 mg), PC-5 (46 mg), PC-6 (65 mg), PC-7+8 (155 mg), PC-9+10 (**1**; 1.11 g) and PC-11 (162 mg). Except for PC-9+10, these fractions were further purified by chromatography as described below.

PC-3 was dissolved in MeOH and chromatographed by isocratic preparative HPLC using conditions I-10 (Table 8), where fractions eluting between 14.5 and 18 min were pooled and dried to give **15** (4.9 mg). Fractions eluting between 8.5 and 9.5 min gave a mixture (86.4 mg) which was further separated by re-chromatography with semi-preparative isocratic HPLC using conditions I-12 (Table 8). Fractions eluting between 13–15, 17–19.5 and 20–23 min were pooled and dried to give **17** (53.2 mg), **22** (10.3 mg) and **21** (10.6 mg), respectively. PC-4 was dissolved in MeOH and chromatographed by preparative isocratic HPLC using conditions I-9 (Table 8), where fractions eluting between 14.5–16 and 17–19 min were pooled and dried to give **3** (228 mg) and **7** (126 mg), respectively. PC-5 was chromatographed by preparative gradient HPLC using conditions G-2 (Table 8), which gave **4** (9.5 mg) as the major component. PC-6, chromatographed under the same conditions, gave **16** (24 mg) as the major component.

PC-7+8 was chromatographed by preparative isocratic HPLC using conditions I-8 (Table 8), where fractions eluting between 26–28, 28–31 and 33–36 min were pooled and dried to give spino-

syn A (23.2 mg), **20** (26.1 mg) and **19** (11.3 mg). PC-11 was chromatographed by preparative isocratic HPLC using conditions I-11 (Table 8), where fractions eluting between 16–17 and 17.5–20.5 min were pooled and dried to give additional **1** (7 mg) and a mixture (91.3 mg), respectively. The mixture was further separated by semi-preparative isocratic HPLC using conditions I-13 (Table 8), where fractions eluting between 25–27.5 and 28–30.5 min were pooled and dried to give **5** (42.9 mg) and **2** (29.7 mg), respectively.

Acknowledgements

We gratefully acknowledge the skill and dedication of the Dow AgroSciences fermentation and work-up staff at the Harbor Beach and Midland, MI, research facilities, in particular Ron Genis.

Supplementary data

^{1}H NMR spectra of all fully glycosylated molecules, a full table of ^{1}H NMR assignments for all fully glycosylated compounds, and schemes of isolation protocols are all available. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.02.035.

References and notes

1. Kirst, H. A.; Michel, K. H.; Martin, J. W.; Creemer, L. C.; Chio, E. H.; Yao, R. C.; Nakatsukasa, W. M.; Boeck, L. D.; Occolowitz, J. L.; Paschal, J. W.; Deeter, J. B.; Jones, N. D.; Thompson, G. D. *Tetrahedron Lett.* **1991**, *32*, 4839.
2. Salgado, V. L.; Sparks, T. C.. In *Compr. Mol. Insect Sci*; Gilbert, L. F., Iatron, K., Gill, S. S., Eds.; Elsevier Ltd.: Oxford, UK, 2005; 6, pp 137–173.
3. Crouse, G. D.; Dripps, J. E.; Orr, N.; Sparks, T. C.; Waldron, C. In *Modern Crop Protection Compounds*; Kramer, W., Schirmer, U., Eds.; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, 2007; pp 1013–1031.
4. Thompson, G. D.; Michel, K. H.; Yao, R. C.; Mynderse, J. S.; Mosburg, C. T. *Down Earth* **1997**, *52*, 1.
5. Sparks, T. C.; Crouse, G. D.; Dripps, J. E.; Anzeveno, P. B.; Martynow, J.; Gifford, J. *Abstracts of Papers*, 234th ACS National Meeting, Boston, MA, United States, August 19–23, 2007.
6. Lewer, P.; Graupner, P. R.; Hahn, D. R.; Duebelbeis, D. O.; Gilbert, J. R.; Crouse, G. D.; Karr, L. L.; Gifford, J.; Dripps, J. E. *Abstracts of Papers*, Plant Canada Meeting, Saskatoon, Canada, June 10–14, 2007.
7. Gaisser, S.; Martin, C. J.; Wilkinson, B.; Sheridan, R. M.; Lill, R. E.; Weston, A. J.; Ready, S. J.; Waldron, C.; Crouse, G. D.; Leadlay, P. F.; Staunton, J. *Chem. Commun.* **2002**, *6*, 618.
8. Sheehan, L. S.; Lill, R. E.; Wilkinson, B.; Sheridan, R. M.; Vousden, W. A.; Kaja, A. L.; Crouse, G. D.; Gifford, J.; Graupner, P. R.; Karr, L.; Lewer, P.; Sparks, T. C.; Leadlay, P. F.; Waldron, C.; Martin, C. J. *J. Nat. Prod.* **2006**, *69*, 1702.
9. Eberz, G.; Mohrle, V.; Frode, R.; Velten, R.; Jeschke, P.; Ger. Offen., DE 10135550 A1 20030130, 2003.
10. Lewer, P.; Hahn, D. R.; Karr, L. L.; Graupner, P. R.; Gilbert, J. R.; Worden, T. V.; Yao, R.C.; Norton, D.W. U.S. Patent 6,455,504, 2002.
11. Lewer, P.; Hahn, D. R.; Graupner, P. R.; Gilbert, J. R.; Karr, L. L.; Worden, T. V.; Yao, R. C.; Norton, D. W. Pro. 44th Natl Mtg Am. Soc. Pharmacognosy; Book of Abstracts; 0:37, 2003.
12. Lewer, P.; Hahn, D. R.; Huang, X.; Karr, L. L.; Gifford, J.; Duebelbeis, D. O.; Graupner, P. R.; Gilbert, J. R. Pro. 53rd Natl. Mtg. Entomological Soc. America, 2005.
13. Hahn, D. R.; Gustafson, G.; Waldron, C.; Bullard, B.; Jackson, J. D.; Mitchell, J. *J. Ind. Microbiol. Biotechnol.* **2006**, *33*, 94.
14. Graupner, P. R.; Martynow, J.; Anzeveno, P. B. *J. Org. Chem.* **2005**, *70*, 2154.
15. Graupner, P. R.; DeAmicis, C.; Erickson, J.; Paschal, J. W.; Kirst, H. A.; Creemer, L. C.; Fanwick, P. E. *J. Org. Chem.* **2001**, *66*, 8431.
16. Frank, S. A.; Roush, W. R. *J. Org. Chem.* **2002**, *67*, 4316.
17. The implications of the extended macrocycle over regular spinosyns is the subject of current study.
18. Hahn, D. R.; Balcer, J. L.; Lewer, P.; Karr, L. L.; Graupner, P. R.; Gilbert, J. R. Abstract 36th Central Meeting of the Am. Chem. Soc.; Indianapolis, IN, 2004.
19. Hahn, D. R.; Balcer, J. L.; Lewer, P.; Gilbert, J. R.; Graupner, P. R. WO 02/077005 A1.
20. Crouse, G. D.; Hahn, D. R.; Graupner, P. R.; Gilbert, J. R.; Lewer, P.; Balcer, J. L.; Anzeveno, P. B.; Daeuble, J. F.; Oliver, M. P.; Sparks, T. C. WO 2002077004.
21. Hahn, D. R.; Jackson, J. D.; Bullard, B. S.; Gustafson, G. D.; Waldron, C.; Mitchell, J. C. WO 2002079477.
22. Lewer, P.; Graupner, P. R.; Hahn, D. R.; Karr, L. L.; Duebelbeis, D. O.; Lira, J. M.; Anzeveno, P. B.; Fields, S. C.; Gilbert, J. R.; Pearce, C. *J. Nat. Prod.* **2006**, *69*, 1506.
23. Sparks, T. C.; Thompson, G. D.; Kirst, H. A.; Hertlein, M. B.; Larson, L. L.; Worden, T. V.; Thibault, S. T. *J. Econ. Entomol.* **1998**, *91*, 1277.