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Modification of the butenyl-spinosyns utilizing cross-metathesis

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ABSTRACT

The discovery of a strain of *Saccharopolyspora* sp. that produced a number of spinosyn analogs that had not before been seen gave an ideal opportunity for extending our knowledge of that SAR of these highly efficacious insecticides. In particular, these compounds contained a butenyl group connected to C-21 which in the regular spinosyns was substituted with a simple ethyl group. The double bond therefore gave us a handle to further modify this position allowing us to substitute different groups there. In this paper we show one of our approaches to this modification using olefin cross-metathesis. Even though the spinosyns were not highly efficient substrates for metathesis reactions, we were nevertheless successful in extending their chemistry accordingly.

1. Introduction

The spinosyns are an important family of natural products that are highly efficacious against a number of different insect pests.[1] The compounds are extracted from a fermentation of the actinomycete *Saccharopolyspora spinosa*—originally isolated from a soil sample collected in a disused rum distillery on a Caribbean Island—from which over 20 factors were isolated. They are marketed under the trade mark Tracer™, which is primarily a mixture of the two major factors, spinosyns A and D.[2] During the development stage, much chemistry was undertaken on the spinosyns in an effort to discover potentially more active compounds, in particular by manipulating both glycoside units and, where possible, undertaking substitutions at other parts of the molecule.[3] This effort was rewarded with the discovery of Spinetoram, a semi-synthetic product derived from fermentation of a strain of *S. spinosa* that produces spinosyn J and L which are both missing one methyl group on the rhamnose moiety. Ethylation of this remaining hydroxyl group followed by selective hydrogenation of the isolated double bond of spinosyn J yields a spinosyn derivative that has a better activity profile than the parent.[4]

Amongst the many synthetic manipulations of the spinosyn skeleton undertaken, it was not possible to make significant changes to the ethyl group substituted *alpha* to the lactone oxygen (the 'tail' group on C-21). Early structure activity relationship (SAR) studies were, therefore limited to the few variations found in the naturally occurring molecules. Some limited success was found utilizing enzyme approaches both in our labs and others by which alcohol groups could be incorporated onto the tail,[5] but no progress was made using classical chemical techniques. Manipulation of the genome did give access to simple variants including branched alkyl and cycloalkyl groups through splicing of the starter unit from the avermectins into that of spinosyn allowing some access to new derivatives. However none of these could be further manipulated by synthesis.[6]

The tail SAR was significantly advanced with the discovery of a closely related organism *Saccharopolyspora pogona* which produced spinosyns extended with an extra polyketide synthase module at the start of the biosynthetic pathway.[7] The group at C-21 was predominately but-1-enyl [for example, the major factor spinosyn α1 (**1**)], with minor variants including butadienyl, and 3-hydroxybut-1-enyl which contained an allylic alcohol group. The presence of these new functional groups gave potential for further manipulation of the side chain through reduction, substitution, oxidation, etc. However of particular interest was the presence of the olefin on the C21-tail which opened up the opportunity for further modifications using cross-metathesis (CM).[8] Closer examination of the overall structure raised concerns about the chances of success for this chemistry though; basic nitrogen groups have been shown to affect metathesis chemistry,[9] and the two ring-olefins present in the spinosyn molecule could undergo ring-opening metathesis. One example of natural product manipulation by CM which drew our attention was the derivitization of erythromycin, particularly considering the presence of a 3-amino glycoside in the starting material.[10] This gave hope that the forosamine sugar in spinosyn derivatives would not hinder the cross-metathesis reaction.

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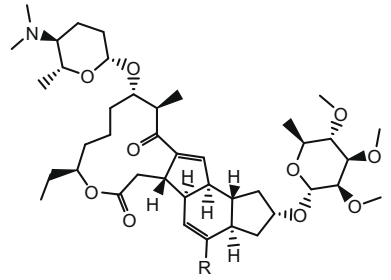
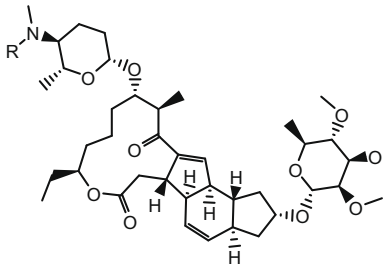
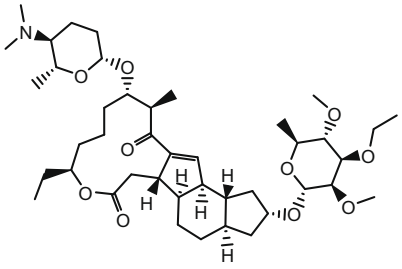


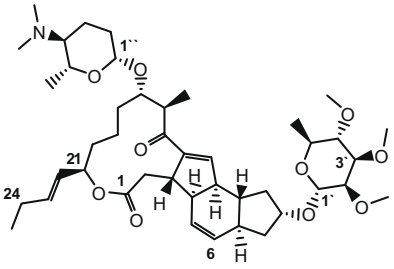
R = H Spinosyn A
R = Me Spinosyn D



R = Me Spinosyn J
R = H Spinosyn M



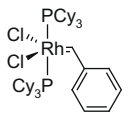
Spinetoram - main factor



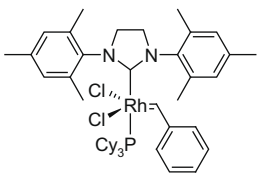
1: Spinosyn α1

2. Results and discussion

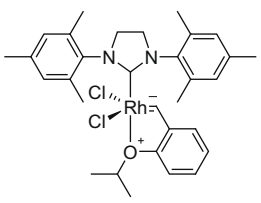
There are many catalysts available for metathesis chemistry covered by a massive body of publications since development has taken place over recent decades for both CM and ring-closing metathesis (RCM) chemistry.[8] During this work a number of these catalysts were utilized for example the so called 'first generation' Grubbs catalyst (**A**), the more recent 'second generation' Grubbs (**B**) and the phosphine-free Grubbs/Hoyveda catalyst (**C**). The first two catalysts require displacement of a phosphine ligand to allow coordination of an olefin to the metal center whereas the third one removes that requirement, while retaining the selectivity and functional group compatibility of the earlier catalysts. Ability to coordinate to the metal complexes is key to the success of metathesis reactions, and is dependent on electronic and steric properties of the reactants and products. These were unknown factors for the spinosyns prior to this study.



A, 1st generation
Grubb's catalyst



B, 2nd generation

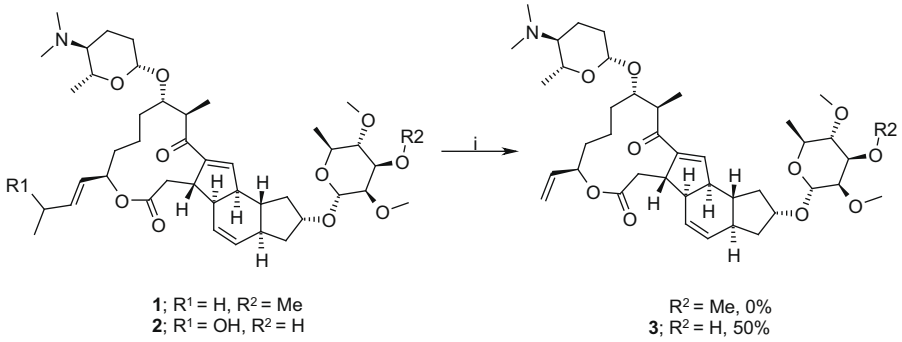


C, Grubbs/Hoyveda Catalyst

2.1. Choice of starting material[†]

There was substantial interest in spinosyns that contained incomplete alkylation of the rhamnose glycoside. Since some effort had been made to produce more demethylated 'J' type spinosoids with alkenyl groups substituted on C-21, selection of precursors for this study was largely determined by the greater availability of these butenyl 'J' factors. For purposes of biological comparisons, C21-modified analogs prepared in this study were subsequently converted into their 3′-O-ethylated derivatives and evaluated relative to both spinosyn A and the corresponding 3′-*O*-alkyl spinosyn analog. There were some fully methylated derivatives available though, and initial experiments were undertaken with them. Unfortunately, reaction of **1** with either generation of Grubbs catalyst (**A** or **B**) either ethylene gas or *cis*-1,4-diacetoxy-2-butene in a sealed vial at reflux temperature in methylene chloride (CH_2Cl_2) returned only starting material. On the other hand, when the 'J' derivative 3′-*O*-demethyl-24-hydroxy spinosyn α1c (**2**) was reacted with catalyst **B** in ethylene saturated CH_2Cl_2, substantial conversion to the 22,23-dehydro spinosyn J (**3**) was observed (Scheme 1). In several instances throughout this investigation, it was noted that the 24-hydroxy compounds appeared to be more reactive metathesis partners than the unadorned butenyl groups, suggesting either a positive influence from the free hydroxyl group or that allylic alcohols are more reactive than simple internal olefins—an effect that has been noted in RCM reactions.[11] In any event, the incomplete conversion of starting material suggested that, as with steric or electronic factors, the basic amine group can serve to make a poor metathesis partner even worse, while not precluding metathesis occurring altogether. Therefore it was decided to explore protection procedures for the nitrogen function that would allow higher-yielding metathesis chemistry to occur

[†] Naming of the spinosyns follows the conventions described in the literature. Compounds with 2 or fewer carbon atoms attached to C-21 are described as derivatives of the 'classical' spinosyns (A, D, J, etc.)[1] whereas those with three or more are named according to the nomenclature described in the preceeding paper on the butenyl compounds.[7]



Scheme 1. General scheme for CM of unprotected spinosyns. Reagents and conditions: (i) catalyst **A** or **B** (10–20 mol %), ethylene gas, DCM or dichloroethane.

either by protection of the secondary amine, or by formation of amine salt derivatives.

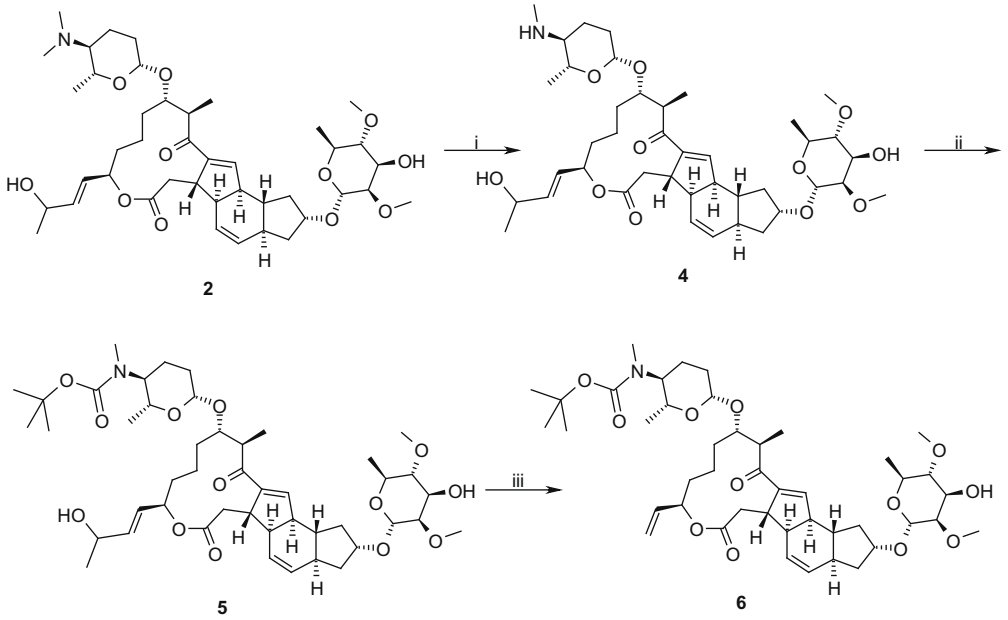
2.2. Protection of amine

In the first example, the dimethyl amino group of **2** was monodemethylated (**4**)[12] and protected as the *tert*-butyl carbamate (Boc) (**5**, Scheme 2). Exposure of **5** to conditions similar to those described in Scheme 1, led to complete conversion of the starting material providing Boc protected 21-vinyl derivative (**6**) in 65% yield.

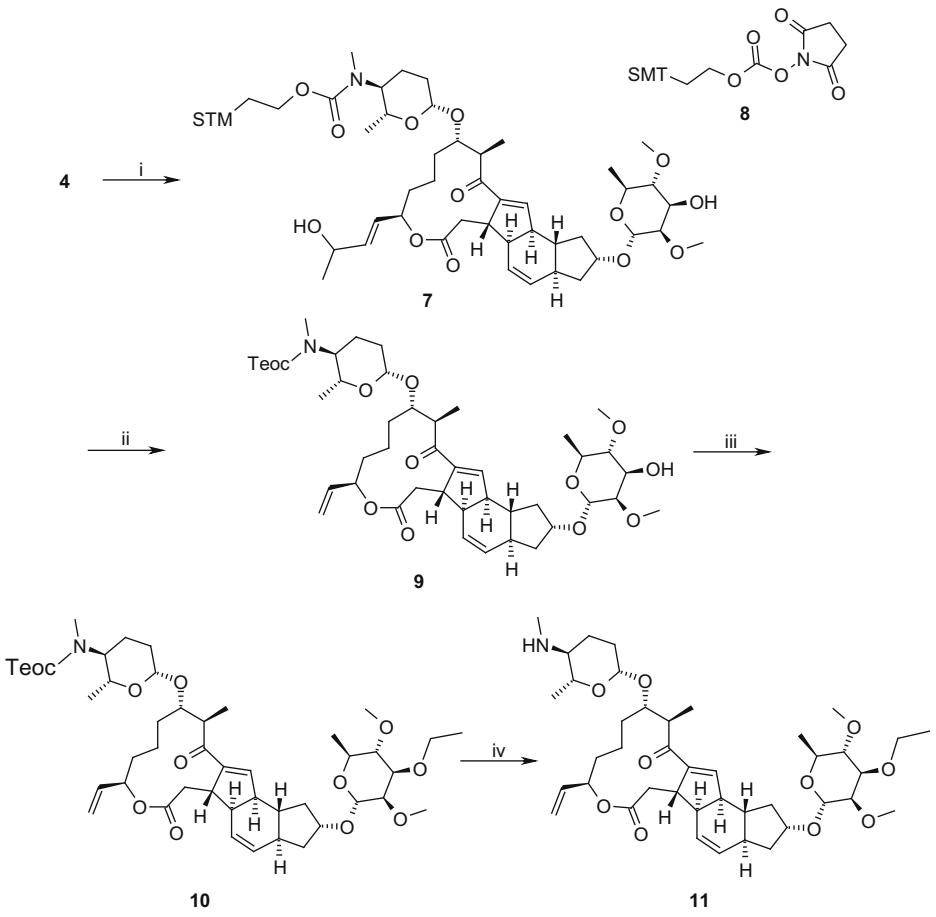
Unfortunately, all attempts to remove the Boc protecting group from **6** resulted in loss of the forosamine sugar, thus negating the usefulness of this strategy. A second protecting group strategy involved use of 2-(trimethylsilyl)ethyl carbamate group (Teoc) in place of Boc (**7**, Scheme 3). As with the Boc protected substrate, metathesis with ethylene went smoothly giving rise to the Teoc protected 21-vinyl compound (**9**) in 88% yield. After capping the 3′-hydroxyl group as an ethyl ether (**10**), the substrate was exposed to tetrabutylammonium fluoride (TBAF) causing slow conversion to 21-vinyl-3′-*O*-Et-spinosyn M (**11**) in moderate yield.

While protecting the amine as a carbamate allowed for clean conversion in this simple metathesis reaction, the process required three additional steps. A far simpler approach to removing the effects of the basic amine was to protect it as a salt prior to the metathesis reaction.[9a] Several salts and the N-oxide were prepared and their reactivity examined in the metathesis reaction with ethylene. The results of this study are shown in Table 1.

There are a number of observations to be made regarding the data in Table 1. Interestingly, the metal complex seemed to serve as an oxidation catalyst in the presence of the N-oxide (**2a**), deoxygenating the N-oxide and oxidizing the allylic alcohol at C-24. Protecting the amine as the tetrafluoroborate (**2b**) or acetate salt (**2c**) offered no benefit over **2**. In all three cases, some conversion to the vinyl product was observed but starting material remained even after prolonged reaction time indicating degradation of the catalyst. Use of hydrochloric acid (HCl) or trifluoroacetic acid (TFA) to protonate the amine proved optimal. With either salt, **2** was completely consumed giving rise to reasonable yields of **3** which was readily ethylated to give the required Spinetoram derivative (**12**). As mentioned previously, there appeared to be some benefit from using the 24-hydroxy butenyl spinosyn **2** rather than the non-hydroxylated **1** in metathesis reactions. Thus even as the HCl or TFA salt, incomplete conversion was observed with **1** as the substrate (**1a** and **1b**). One caveat to using the HCl or TFA salts was their sensitivity to moisture and subsequent degradation to the 17-pseudo aglycone. Addition of ethereal HCl to a solution of a spinosyn in ether produced a fine white precipitate which was isolated by filtration or concentration. If the salt was not used immediately or carefully protected from moisture, noticeable loss of forosamine occurred, severely affecting yields. Even with fresh salt samples however, some loss of forosamine was observed with



Scheme 2. Protection of amine with Boc group. Reagents and conditions: (i) NaOAc, I_2, MeOH, NaOH (aq); (ii) Boc_2O, $NaHCO_3$, EtOH, 70%; (iii) ethylene, **B**, DCM, reflux, 65%.



Scheme 3. Teoc protection. Reagents and conditions: (i) **8** Et_3N, DCM, EtOH, 54%; (ii) **B**, ethylene, DCM, reflux, 88%; (iii) EtBr, KOH/Bu_4NI (10:1) 88%; (iv) Bu_4NF, THF, 66%.

nearly all these reactions and contributed to the relatively modest yields.

2.3. Extension of the alkenes

2.3.1. Terminal olefins

We first investigated the use of terminal olefins to extend the C-21 side chain, with **2d** or **2e** as starting materials (Table 2).

Under the conditions tried, most reactions gave mixtures of the desired metathesis product and the 21-vinyl analog. Presumably this is due, in part, to the reluctance of the spinosyn-based olefin to undergo metathesis as well as the facility with which these simple terminal dienes undergo homocoupling to the symmetrical dimer. This was indicated by the substantial amount of stilbene recovered when using styrene as the coupling partner. It was further expected that the internal olefin of the homodimer would not be as reactive as its terminal counterpart. Also, as the reactions became dark in color and began to precipitate solids, it appeared that the catalyst was decomposing under the reaction conditions no matter how much care was taken to exclude air and moisture, further indicating that spinosyn is a reluctant metathesis partner at best.[13] Lastly, attempts to use vinyl cyclohexane in the reaction gave only trace amounts of the 21-vinyl analog while allyl cyclohexane coupled readily pointing to the influence of steric interactions on the outcome of the reaction.

2.3.2. Symmetrical olefins

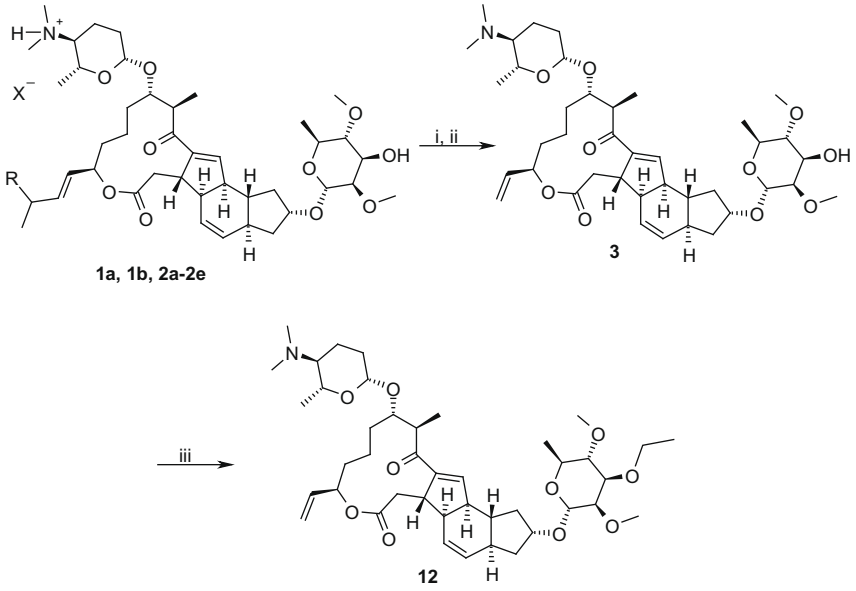
Derivatives of *cis*-2-butene-1,4-diol and certain other symmetric internal olefins are reported to be excellent cross-metathesis partners—often providing higher yields and greater *trans* selectivity than the cross coupling utilizing the corresponding terminal olefin.[14] Therefore the metathesis of butenyl spinosyn analogs and various butene analogs was examined (Table 3).

The reaction with allyl acetate (entry 4) was perplexing. It was assumed that this material would homo-dimerize to the 1,4-butene diol derivative faster than productive cross-metathesis would occur. However, it is obvious that the dimer itself participated in the reaction (entries 2 and 3), thus the lack of conversion to the desired product was surprising. This could simply point to a relatively short half life for the catalyst under these conditions. Perhaps not unexpectedly, the Boc protected 21-vinyl compound (**5**) was an excellent substrate for cross-metathesis, at least when compared to other spinosyns. This result supported the idea that while protecting basic amines as salts can be effective, it is not an ideal solution and the salts themselves may have a detrimental effect on the longevity of the catalyst or catalyst precursor. The low conversion observed with the 21-vinyl-3′-OEt spinosyn J (**12**) was also intriguing. This result may lend further support to the aforementioned idea that a free hydroxyl group somehow facilitated the metathesis reaction and that substrates lacking a free hydroxyl were not as well suited for the reaction.[11]

2.3.3. Incorporation of polar functionalities onto the C-21 spinosyn side chain

Methyl acrylate and acrylic acid were two additional cross-metathesis substrates of interest as we envisioned a dramatic change in physical properties that would accompany introduction of an acidic functional group on the C21 side chain. A variety of conditions and catalysts were screened using methyl acrylate as the metathesis partner. As with the butene-1,4-diol derivative dis-

Table 1
Comparison of amine salts of butenyl spinosyns in cross-metathesis with ethylene



Reagents and conditions: i. Cross metathesis (see Table for conditions); ii $NaHCO_3$; iii EtBr, KOH/Bu_4NI (10:1).

	R	X	Catalyst	Solvent	Time (h)	Yield of **3**	Comments
2	OH	Free base	20 mol % B	CH_2Cl_2	14	ND	Incomplete conversion
2a	OH	(N-Oxide)	10 mol % B	CH_2Cl_2	16	ND	50% Conversion to the 24-keto derivative
2b	OH	BF_4	20 mol % B	CH_2Cl_2	3	ND	Incomplete conversion
2c	OH	Acetate	20 mol % B	$ClCH_2CH_2Cl$	2	ND	Incomplete conversion
2d	OH	Cl	10 mol % B or C	CH_2Cl_2	4	65%	Complete conversion
1a	H	Cl	20 mol % B	CH_2Cl_2	14	ND	Incomplete conversion
2e	OH	TFA	20 mol % B	$ClCH_2CH_2Cl$	2	83%	Complete conversion
1b	H	TFA	20 mol % B	CH_2Cl_2	4	ND	Incomplete conversion

ND = Not determined.

cussed above, all reactions employing methyl acrylate led to a mixture of *E/Z* isomers of the desired compound (**26**) and the 21-vinyl spinosyn J (**3**). The best result was obtained using the Grubbs/Hoyveda catalyst (**C**) (Scheme 4).

Several attempts were made to saponify the methyl ester of **26** and the 3′-O-alkylated derivative **27** but these were unsuccessful, leading to degradation of the substrate. Unfortunately, metathesis reactions using acrylic acid itself met with no success.

A second approach to modify the physical properties of the spinosyn template through metathesis chemistry involved incorporation of a glycine residue into the reaction. There are reports of suitably protected allyl glycine derivatives participating in olefin cross-metathesis reactions.[15]

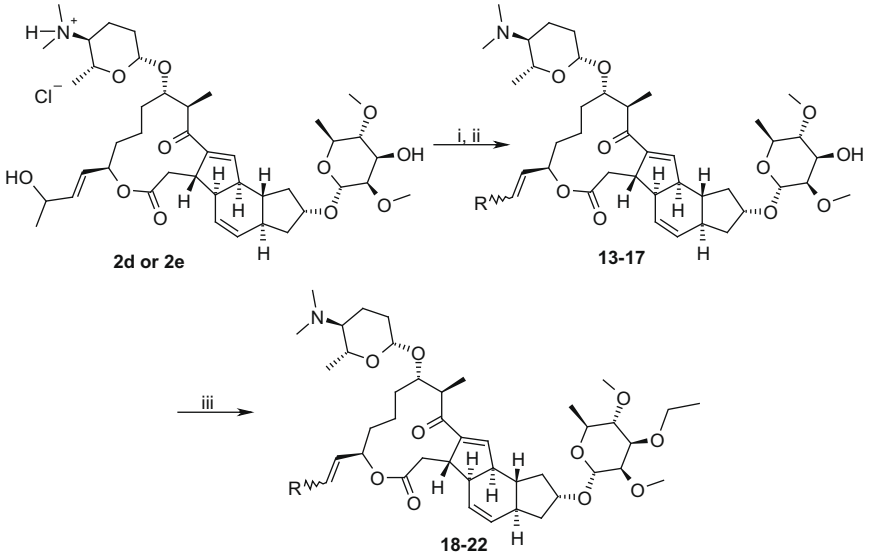
Cross-metathesis was attempted on Fmoc protected allyl glycine (**28**)[16] with several different C21-butenyl and vinyl spinosyns. Success was achieved with the HCl salt of **12** using catalyst **C** (Scheme 5) to give **29**. As with many other metathesis reactions discussed here, the reaction did not proceed to completion and approximately 50% of the Fmoc protected allyl glycine ethyl ester was isolated as the homodimer. Exposure of this homodimer to a separate cross-metathesis using styrene indicated that it is a viable metathesis partner thus again suggesting that catalyst decomposition may contribute to incomplete conversion in metathesis reactions using spinosyns. Exposure of the product mixture of the Fmoc derivative (**29**) and unreacted 21-vinyl starting material to morpholine in tetrahydrofuran (THF) led to clean removal of the protecting group and allowed for facile separation of the deprotected glycine analog (**30**), from the 21-vinyl species.

One compound that could not be made under any conditions we tried was from reaction with acrylonitrile. This starting material is known to be recalcitrant, and subsequently much work has gone into devising procedures for successful CM with it.[17] Application of a variety of these procedures to the cross-metathesis of acrylonitrile and various spinosyns failed to yield any of the required product. A possible explanation is that while butenyl and vinyl spinosyns can enter into productive cross-metathesis reactions, they are not necessarily good alkylidene donors or very nucleophilic and when matched with other olefins that are not particularly well suited for cross-metathesis, no reaction is observed.[13] Also, the structural complexity of the spinosyns, including the presence of two additional double bonds that can enter into the metathesis cycle, may lead to non-productive metathesis events that contribute to catalyst degradation. Other factors including the presence of the amine salts and free hydroxyl groups may also play a role in the success or lack thereof in these reactions.

2.4. Purification of spinosyn γ1 (32)

Olefin cross-metathesis found an additional use within the spinosyn project, enabling the chemical transformation of a mixture of factors to allow for their chromatographic separation. During the course of the purification of a large scale butenyl spinosyn fermentation, several grams of a mixture comprised mainly of spinosyn γ1 (**32**) and spinosyn δ1 (**31**) along with several minor factors was set aside as no chromatographic method, normal or reverse phase, was found that allowed for their separation (Scheme 6).[7]

Table 2
Olefin cross-metathesis with butenyl spinosyns and terminal olefins



Reagents and conditions: i Olefin (see Table), **B**, DCM; ii $NaHCO_3$; iii, EtBr, KOH/Bu_4NI (10:1).

Salt	Olefin	R (*E*/*Z*)	Time (h)	Product[a]	Yield[b] (%)	Comments
TFA (**2e**)	1-Dodecene	$CH_3(CH_2)_9$ (10:1)	7	**13** (**18**)	68	Complete conversion
HCl (**2d**)	1-Octene	$CH_3(CH_2)_5$ (3:1)	14	**14 (19)**	76	3:1 Mixture 21-octenyl:21-vinyl
TFA (**2e**)	1-Octene	$CH_3(CH_2)_5$ (6:1)	2	**14 (19)**	68	Complete conversion
HCl (**2d**)	1-Hexene	$CH_3(CH_2)_3$ (8:1)	2	**15 (20)**	44	9:1 Mixture of 21-hexenyl:21-vinyl. (22% 21-hexenyl-17-pseudoaglycone also isolated)
TFA (**2e**)	Styrene	Phenyl (1:0)	3	**16 (21)**	61	1:1.5 Mixture of 21-styryl:21-vinyl. (60% stilbene recovered)
TFA (**2e**)	4-*O*-Et-Styrene	4-*O*-Et-Phenyl (1:0)	6		ND	1:1 Mixture of 21-styryl:21-vinyl
TFA (**2e**)	Allyl benzene	$PhCH_2$	7.5		ND	3:1 Mixture 21-allylbenzyl:21-vinyl
TFA (**2e**)	Allyl cyclohexane	Cyclohexyl-CH_2- (5:1)	5.5	**17 (22)**	61	Complete conversion

ND = not determined.
[a] Figure in brackets corresponds to the final (ethylated) product.
[b] Yield corresponds to that of the metathesis product.

Exposure of this mixture of spinosyns to our standard metathesis protocol using ethylene gas and catalyst **C**, led to conversion of the **31** into the 21-vinyl-6-methyl spinosyn J (**33**) while spinosyn γ1 (**32**) remained unaffected. This subtle change allowed for facile separation of the two major factors by reverse phase HPLC.

2.5. Incorporation of ^{13}C label

Further utility for the cross-metathesis reaction was found in the preparation of a stable isotope derivative of the spinosyns (Scheme 7). Exposure of 24-hydroxy-butenyl spinosyn J (**2**) to ^{13}C labeled ethylene in the presence of catalyst **C** led to modest conversion to **3** containing a ^{13}C label at the terminal olefin carbon atom. The reaction did not go to completion and the yield was further lowered by some loss of the forosamine to give the 17-pseudoaglycone. Nevertheless, this labeled material could be further manipulated to furnish a stable isotope standard for studies of spinosyn (via methylation of the rhamnose alcohol and selective reduction of the terminal olefin) or Spinetoram (via ethylation of the alcohol and reduction of the terminal and the 5,6 olefins).

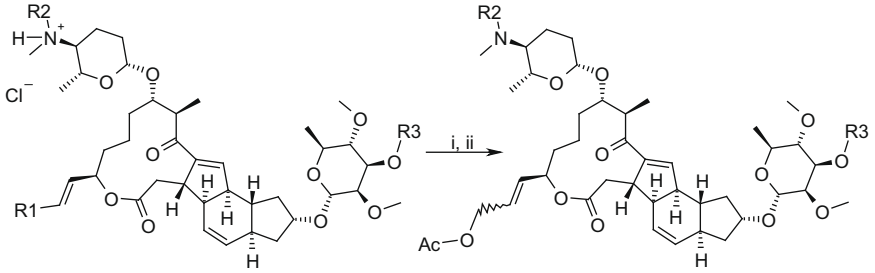
3. Biological considerations

The activity of a selection of these new spinosyn derivatives was compared with spinosyn A and 3′-*O*-ethyl spinosyn J. In the neonate tobacco budworm (TBW) bioassay all compounds tested had comparable activity to the standards (Table 4) except **10**, which was much less active. Testing of some of the other analogs in several other bioassays all pointed to the styrene derivative (**21**) as being the most active—about as active as the 3′-*O*-ethyl analog of spinosyn J.

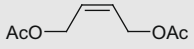
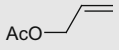
4. Concluding remarks

Increasing the size of the alkyl moiety at the 3′-position of the rhamnose significantly increases the activity of the spinosyns compared to spinosyn A.[18] In contrast, extending the size of the alkyl moiety at the C21-position of the macrocycle produces little improvement in activity relative to spinosyn A against larvae of the tobacco budworm. In the present study, the effect of further modifications to the butenyl moiety at C21 position was investigated via cross-metathesis. The spinosyn derivatives resulting from the cross-metathesis allowed further exploration of the space around the C21 position of the spinosyn macrocycle. With the exception of the large modification to the forosamine nitrogen [Teoc] in compound **10**, all of the resultant analogs exhibited high levels of biological activity. Several analogs possessed activity similar to that of spinosyn J, while a few were similar in activity to the 3′-*O*-ethyl analog of spinosyn A. Because almost all of the cross-metathesis derived analogs had an *O*-ethyl moiety in the 3′-position of the rhamnose, comparison to the 3′-*O*-ethyl analog of spinosyn J or its C21-2-butenyl homolog was most appropriate. As such

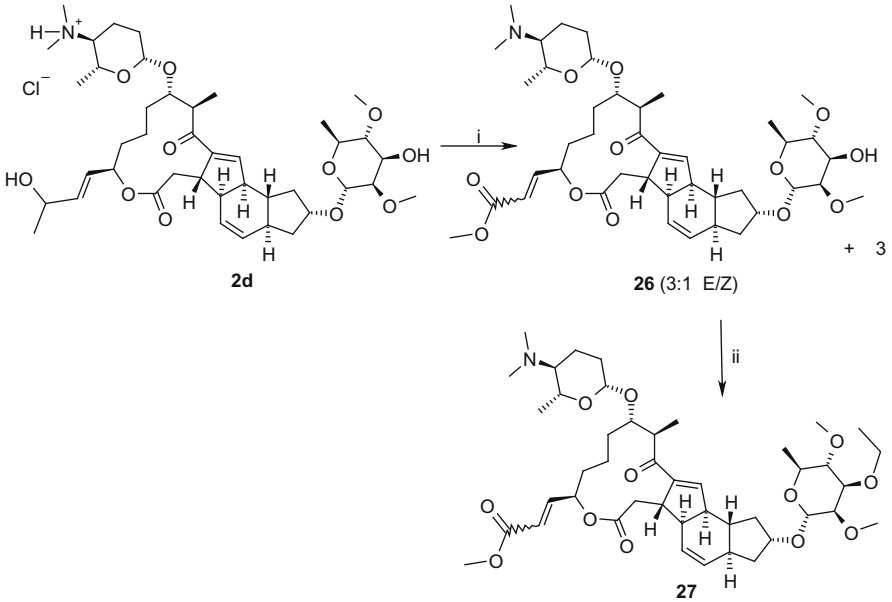
Table 3
Conversion of 21-butenyl and 21-vinyl spinosyns to 24-acetoxy-propenyl spinosyns



Reagents and conditions: i Olefin and catalyst (see Table), DCM; ii $NaHCO_3$

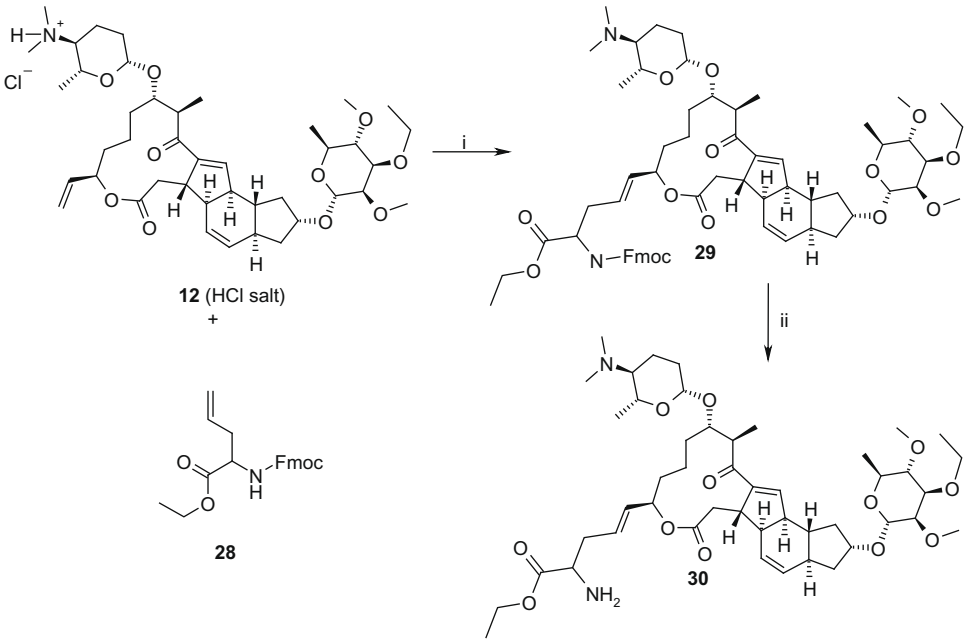
Starting Material	R1	R2	R3	X	Olefin	Catalyst	Product	Yield (*E*/*Z*)	Comments
1	Et	Me	H	Free base		A		ND	No reaction (85% recovered 1)
2d	$CH(OH)CH_3$	Me	H	Cl		B	23	20% (2:1)	Trace 21-vinyl (50% recovered 2d)
2e	$CH(OH)CH_3$	Me	H	TFA		B	23	60% (4:1)	25% Recovered 2e
2e	$CH(OH)CH_3$	Me	H	TFA		B		ND	45% Conversion to 21-vinyl spinosyn J (51% recovered 2e)
5	H	Boc	H	NA		B	24	92% (all *Z*)	Complete conversion
12	H	Me	Et	TFA		B	25	40% (1:1)	50% Recovered 12

ND = not determined.

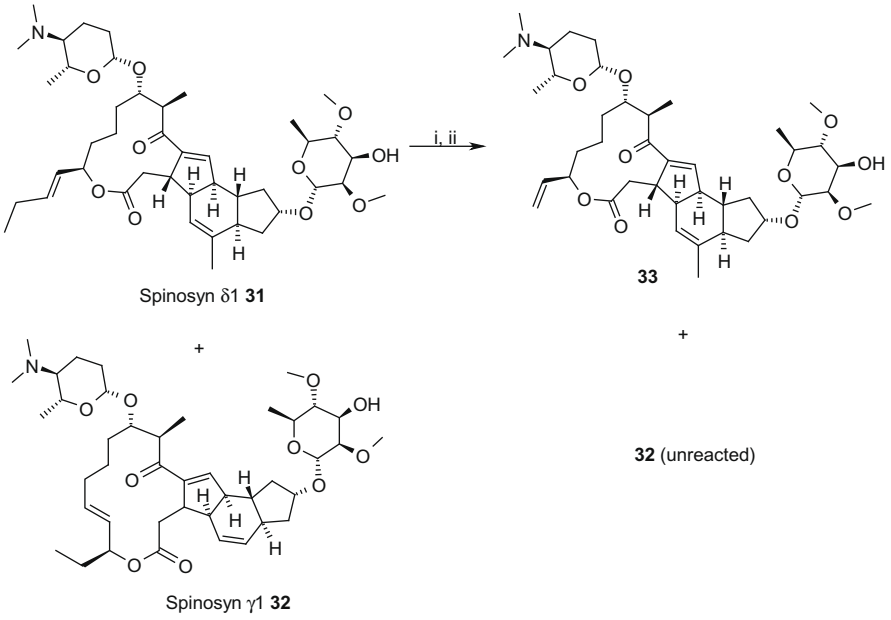


Scheme 4. CM with methyl acylate. Reagents and conditions: (i) methyl acrylate, 20 mol % **C**, DCM, 48 h; (ii) EtBr, KOH/Bu_4NI (10:1).

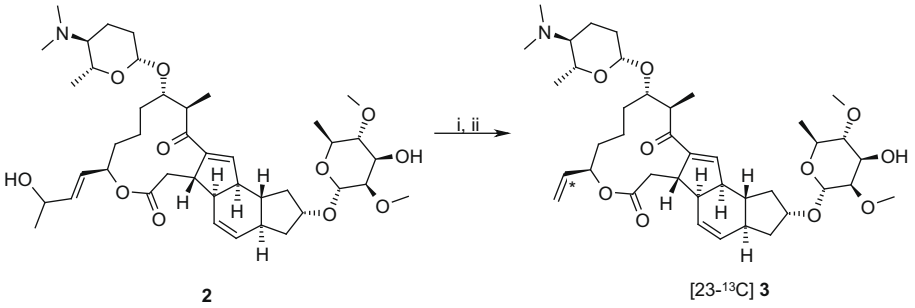
it is interesting that further substitution at the terminus of the C21 most often only resulted in a modest reduction in activity with several of the analogs having activity near that of the reference compounds (i.e., **19**, **20** and **21**). Of particular interest is the styryl derivative (**21**) the activity for which was as good as the 3′-*O*-ethyl analog of spinosyn J, and which is a significant new finding for SAR



Scheme 5. Reagents and conditions: (i) 20 mol % **C**, DCM, 48 h; (ii) morpholine, THF, 70%.



Scheme 6. CM of **31** in presence of **32**. Reagents and conditions: (i) HCl, Et_2O; (ii) 20 mol % **C**, C_2H_4, DCM.



Scheme 7. Preparation of ^{13}C labeled spinosyn J. Reagents and conditions: (i) HCl, Et_2O; (ii) 20 mol % **C**, $^{13}C_2H_4$, DCM.

Table 4
Biological activity of selected novel spinosyns

Compound	Neonate[a] TBW LC_{50} (ppm)	BAW[b] oral LC_{50} (μg/cm^2)	TBW[c] leaf LC_{50} (ppm)	CL[d] leaf, LC_{50} (ppm)
11	0.2	—	—	—
12	0.5	0.018	0.22	0.13
19	—	0.022	1.46	0.12
20	—	<0.012	0.37	0.13
21	—	0.013	0.17	0.05
25	2.0	—	—	—
27	0.2	—	—	—
10	47.2	—	—	—
3′-OEt-J	0.28	0.012	0.15	0.048
Spin A	0.42	0.079	0.43	0.10

[a] Neonate; TBW—tobacco budworm larvae—neonate drench bioassay.
[b] BAW oral—beet armyworm—diet feeding assay.
[c] TBW leaf—TBW leaf disk bioassay.
[d] CL leaf—Cabbage Looper leaf disk bioassay.

development. These observations suggest that there is some reasonable latitude in the size and substitution extending out from the C21 position that does not readily interfere with the biological activity.

5. Experimental

5.1. Spectroscopic methods

NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer operating at 299.7 MHz or a Bruker DRX600 spectrometer operating at 600.13 MHz (1H), and 150.6 MHz (13C). HPLC separations were achieved using a Hewlett–Packard 1100 LC system. LC/MS was performed on a Micromass Platform single-quadrupole mass spectrometer in both positive electrospray (+ESI) and negative electrospray (−ESI) modes.

5.2. Bioassays

Neonate tobacco budworm (TBW, *Heliothis virescens*) larvae were assayed as described previously.[19] Larvae of the beet armyworm (BAW, *Spodoptera exigua*) were assayed using a treated diet assay. Second instar larvae were individually placed (one larva per well) in 128-well diet trays (Bio-Serv, Frenchtown, NJ) that contained artificial diet (1 mL per well) that had been treated with the test compounds (dissolved in 50 μL of 90:10 acetone–water). Each dose had eight replicates, and a range of doses were used to estimate the LC_{50}s. Controls were treated with solvent only. Treated trays were covered with a clear self-adhesive cover and held at 25 °C under a 14:10 light:dark regimen in a light chamber. The average percent mortality for the eight wells for each dose was determined six days after treatment. LC_{50}s were calculated using the method of Finney.[20] Second instar tobacco budworm or cabbage looper (CL, *Trichoplusia ni*) larvae were placed on leaf disks (six per dose, squash) that had been treated (compound in 250 μL of 90:10 acetone–water) and allowed to dry. Controls were treated with solvent only. Infested leaf disks were held in 6-well microtiter plates covered with a plastic lid at 25 °C under a 14:10 light:dark regimen as above. Percent mortality of the six replicates was determined five days after treatment. LC_{50}s were again calculated using the method of Finney.[20]

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Supplementary data

Supplementary data (Details of synthetic methods) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.02.036.

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