Nodulisporic Acids D–F: Structure, Biological Activities, and Biogenetic Relationships

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Nodulisporic acids D, E, and F (**7**–**12**) are the newest members of a family of nontremorogenic indole-diterpenoids that are potent, orally bioavailable, antiflea agents derived from a fungus belonging to the genus *Nodulisporium*. The four members of the D series (**7a**–**10a**) are each devoid of an isoprene residue that is present at C-26 in the three nodulisporic acids described originally (the A series, **1a**–**3a**). Nodulisporic acid E (**11a**) has a simpler structure, which lacks not only the isoprene residue at C-26 but also two that form the A/B rings. Nodulisporic acid F (**12**) is the simplest of all nodulisporic acids and is devoid of all three isoprene residues of the indole unit; as such, it represents the earliest biosynthetic intermediate in this series. A biogenetic grid based on mutation studies is proposed that encompasses all the known nodulisporic acids. Structure–activity relationships of the known natural nodulisporic acids have been elucidated. Within a series the most active compound possesses a dienoic acid chain, and overall, the end product of the biogenetic grid, i.e., nodulisporic acid A (**1a**), exhibits the most potent antiflea activity. Additionally, the stereochemistries of C-3′′ and C-4′′ of nodulisporic acid D_2 (**9a**) and therefore of nodulisporic acids A_2 (**3a**), B_2 (**3b**), and C_2 (**6**) have been assigned.

As vectors of viral or bacterial pathogens, ectoparasites such as fleas represent a significant health hazard to companion animals and to the humans who maintain them. Although numerous insecticides are currently marketed for flea control, development of resistance is an issue that makes continued discovery of new chemical entities a necessity. Also, many of these antiflea drugs are applied topically, which can cause wide variability in efficacy as well as environmental problems. Therefore, new systemic drugs, preferably with novel mechanisms of action, are needed.

Nodulisporic acid A (**1a**) is an indole-diterpenoid that was discovered in 1992 and was first reported by us in 1997 as a potent insecticidal agent.[1,2] It was purified from an endophytic fungus, *Nodulisporium* sp. (MF5954), by bioassay-guided isolation using a mosquito-larval assay. Subsequent studies demonstrated that it was an effective systemic ectoparasiticidal agent against fleas on dogs with no apparent mammalian toxicity.[3] Like the avermectins and milbemycins, nodulisporic acid A modulates the glutamate-gated chloride channel in insects, but has no effect on helminths.[4–6] Nodulisporic acid A does not affect other related chloride channels present in both insects and mammals. To date, no report of nodulisporic acid-related vertebrate toxicity has appeared. While nodulisporic acid A exhibited good in vitro and in vivo activity against fleas, its potency and pharmacokinetic properties did not justify its development as a drug, so therefore a medicinal chemistry effort was initiated to optimize the profile of the lead. Chemical modifications were performed on most of the accessible sites, particularly the dienoic acid side chain, leading to a substantial improvement in activity;[7–13] the effort has been briefly reviewed.[14,15]

In parallel with the medicinal chemistry efforts, we sought to find congeners and/or natural analogues of nodulisporic acid A, from the original producer as well as variants derived from it by chemical mutagenesis, in the hope of providing compounds that may not be accessible by the chemical derivatization. This process led to the discovery of nodulisporic acids A_1 (**2a**), A_2 (**3a**),[16] B (**1b**), B_1 (**2b**), B_2 (**3b**),[17] C (**4**), C_1 (**5**), and C_2 (**6**).[18] A continuation of this work with mutant *Nodulisporium* strains has led to the isolation of early biosynthetic intermediates named herein nodulisporic acids D (**7a**), D_1 (**8a**), D_2 (**9a**), D_3 (**10**), and E (**11a**) from ATCC74473, nodulisporic acid F (**12**) from MF6518, and A_4 (**13**), Δ^{23}-A_4 (**14**), and Δ^{23}-C_4 (**15**) from two other mutant cultures. Like nodulisporic acids of the B and C series, some of these compounds were detected in the extracts of the original producer but in quantities too low for purification. Details of the isolation, structure elucidation, and biological activities of these compounds are described in this paper. In addition, we propose a biogenetic grid of all known nodulisporic acids based on the products accumulated in various mutants of the original producer.

Results and Discussion

Isolation of Nodulisporic Acids. Mycelia from a filtered culture of *Nodulisporium* sp. mutant MF6227 (ATCC74473) were extracted with methyl ethyl ketone. The extract was subjected to chromatography on a reversed-phase HPLC, affording nodulisporic acids D (**7a**, 43 mg/L), D_1 (**8a**, 70 mg/L), D_2 (**9a**, 80 mg/L), D_3 (**10a**, 30 mg/L), and E (**11a**, 130 mg/L) as pale powders. At acidic pH (in TFA), particularly at higher concentrations, the $\Delta^{18,19}$-olefin tended to isomerize to $\Delta^{18,22}$-olefin and thus complicated the purification. Methylation with diazomethane followed by purification by reversed-phase HPLC at neutral pH led to the isolation of the corresponding methyl esters **7b**–**11b**. This extract did not contain nodulisporic acids A–C (**1**–**6**). Nodulisporic acid F (**12**) was similarly isolated from fermentations of MF5954 and was the only nodulisporic acid produced by MF6518. Nodulisporic acid A_4 (**13**) was purified from other mutants MF6265 and MF6225. The MEK extraction of the mycelia of a mutant culture of *Nodulisporium* sp. followed by two successive reversed-phase HPLC steps afforded Δ^{23}-nodulisporic acid A_4 (**14**, 18 mg/L). Silica gel chromatography of the same extract

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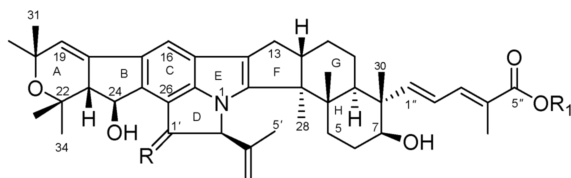
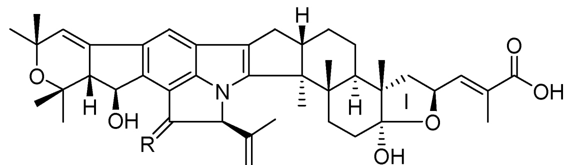
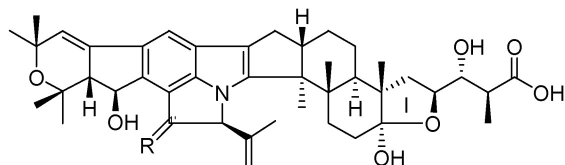


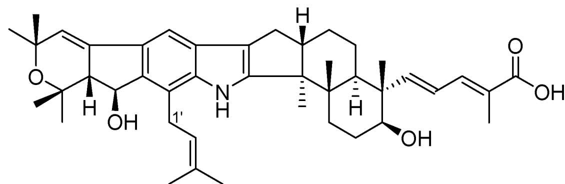
1a: R = O, R_1 = H, Nod acid A
1b: R = H_2, R_1 = H,Nod acid B
1c: R = O, R_1 = Me
1d: R = H_2, R_1 = Me



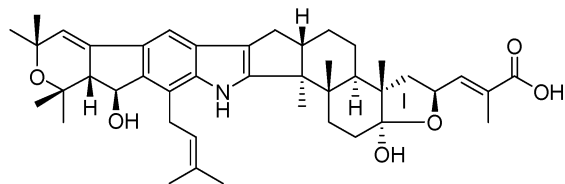
2a: R = O, Nod acid A_1
2b: R = H_2, Nod acid B_1



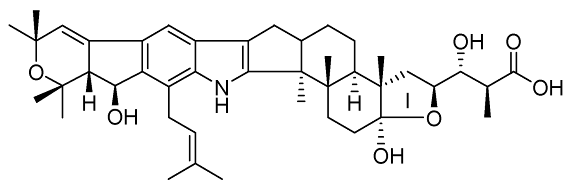
3a: R = O, Nod acid A_2
3b: R = H_2, Nod acid B_2



4: Nod acid C



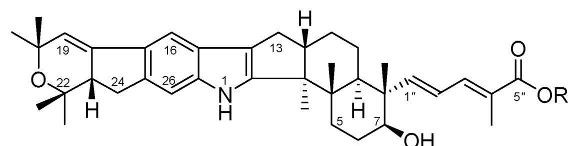
5: Nod acid C_1



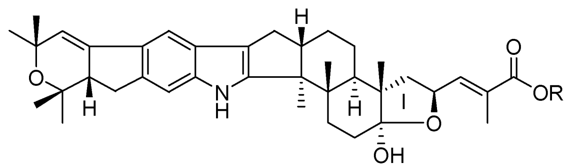
6: Nod acid C_2

furnished Δ^{23}-nodulisporic acid C_4 (**15**, 10 mg/L); both **14** and **15** were obtained as yellow powders. Nodulisporic acids A–A_2 (**1a**–**3a**) were present in significant amounts in the extract of MF6225.

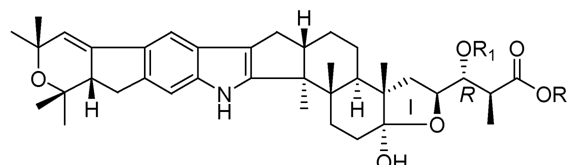
Nodulisporic Acids D, D_1, D_2, and D_3 (7–10). HREIMS of compound **7a** indicated a molecular formula of $C_{38}H_{49}NO_4$, which was supported by the ^{1}H and ^{13}C NMR spectra (Table 1). The UV spectrum of **7a** was different from that of nodulisporic acid A (**1a**) and was similar to that of nodulisporic acids B (**1b**) and C (**4**). It showed



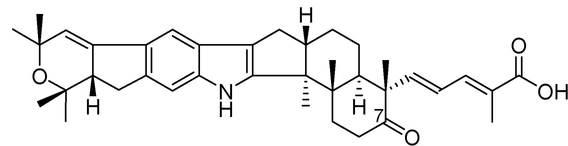
7a: R = H, Nod acid D
7b: R = Me



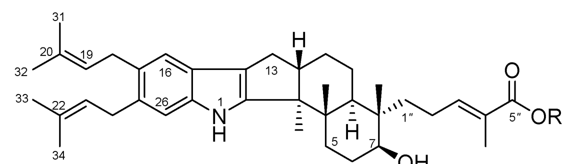
8a: R = H, Nod acid D_1
8b: R = Me



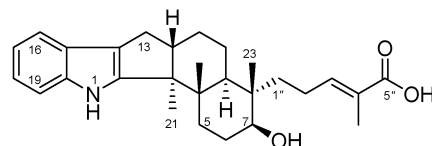
9a: R = R_1 =H, Nod acid D_2
9b: R = Me, R_1 = H
9c: R = Me, R_1 = (*R*)-MTPA
9d: R = Me, R_1 = (*S*)-MTPA



10: Nod acid D_3



11a: R = H, Nod acid E
11b: R = Me



12: Nod acid F

absorption bands at λ_{max} 262, 316, and 336 nm. Comparison of the ^{1}H and ^{13}C NMR spectra of nodulisporic acid D (**7a**) with that of A (**1a**), B (**1b**), and C (**3a**) indicated the absence of the isoprene unit comprising structural fragment C1′–C5′ and the hydroxy group at C-24 accounting for the molecular formula differences. The ^{1}H NMR spectrum of **7a** exhibited an additional aromatic proton singlet at δ_H 7.15 assigned to H-26, which was substantiated by the presence of the corresponding shift for C-26 (δ_C 108.5) in the ^{13}C NMR spectrum and confirmed by an HMQC correlation. The C-24 hydroxy methine proton of nodulisporic acids A–C was substituted by two methylene protons (δ_H 3.07, dd, J = 16.0, 9.2 Hz; δ_H 2.66, ddd, J = 16.4, 7.6, 3 Hz; δ_C 34.2) in the ^{1}H NMR spectrum of **7a**, which showed a COSY correlation to H-23 (δ_H 2.83, dt, J

Table 1. NMR Assignments of Nodulisporic Acids D, D_1, D_2, and D_3 (**7**−**10**)

position	**7a** CD_3OD δ_C	**7a** CD_3OD δ_H	**8a** CD_2Cl_2 δ_C	**8a** CD_2Cl_2 δ_H	**9a** CD_3OD δ_C	**9a** CD_3OD δ_H	**10** CD_2Cl_2 δ_C	**10** CD_2Cl_2 δ_H
1				7.88, brs				7.77, s
2	152.6		151.3		152.5		150.5	
3	54.2		53.1		53.7		53.1	
4	39.9		39.7		40.3		39.2	
5	33.6	~1.81, m	30.3	~2.05, m	31.4	~1.65, m	34.4	2.37, m
				~1.95, m		~1.95, m		1.89, ddd, 12.8, 6.8, 3.6
6	27.8	~1.78, m	30.3	~2.05, m	30.0	~1.90, m	35.5	2.80, m
								2.54, dd, 15.6, 6, 4
7	77.8	3.37, dd, 10.4.8	107.0		106.8		213.3	
8	48.1		49.3		50.4		45.8	
9	45.6	1.61, dd, 12.4, 2.4	40.8	~1.80, m	42.1	1.70, m	42.1	2.29
10	25.6	~1.35, m	25.9[a]	~1.75, m	26.0	~1.58, m	25.0	1.60, m
		~1.15, m		~1.85, m		~1.35, m		1.50, m
11	26.4	~1.45, m	25.2[a]	~1.65, m	26.8	~1.70, m	24.9	1.70, m
		~1.35, m		~1.50, m		~1.58, m		1.50, m
12	50.1	2.42, m	49.5	2.78, m	50.4	2.60, m	49.1	2.80, m
13	28.3	2.56, dd, 12.8, 6.4	27.7	~2.64, m	28.3	~2.58, m	27.6	2.70, dd, 13.6, 6.8
		2.20, dd, 13.2, 10.8		2.30, m		~2.25, m		2.36, m
14	118.1		118.4		118.0		118.7	
15	126.1		125.2		126.1		125.1	
16	110.2	7.38, s	109.7	7.45, s	110.2	7.38, s	109.9	7.46, s
17	138.3		138.4		138.3		138.7	
18	141.3		139.8		141.3		139.7	
19	118.8	5.88, d, 3.2	119.6	5.94, d, 3.2	118.8	5.87, d, 3.2	119.8	5.95, d, 2.8
20	74.3		72.9		74.3		72.8	
22	76.2		74.7		76.2		74.6	
23	50.1	2.83, dt, 2.8, 7.6	49.1	2.90, dt, 2.1, 8.8	50.1	2.84, dt, 2.1, 7.6	49.1	2.90, dd, 8, 3.2
24	34.2	3.07, dd, 16, 9.2	33.6	3.1, dd, 16, 9.2	34.2	3.06, dd, 9.2, 15.6	33.6	3.12, dd, 16.4, 9.6
		2.66, ddd, 16.4, 7.6, 3		2.65, dd, 16, 6		2.65, dd, 16, 8		2.67, dd, 16, 8
25	132.6		132.8		132.6		133.0	
26	108.5	7.15, s	107.8	7.15, s	108.5	7.14, s	107.8	7.17, s
27	143.6		141.8		143.6		141.9	
28	14.6	0.92, s	14.6	0.99, s	14.8	0.92, s	14.6	1.03, s
29	19.3	0.90, s	16.6	1.06, s	17.3[a]	0.99, s	17.2[a]	1.28, s
30	11.9	1.00, s	17.7	1.13, s	17.8[a]	0.99, s	18.8[a]	1.26, s
31	30.2	1.29, s	30.1	1.30, s	30.2	1.30, s	30.1	1.29, s
32	32.2	1.28, s	32.0	1.31, s	32.2	1.30, s	32.0	1.31, s
33	22.6	1.08, s	22.3	1.07, s	22.6	1.08, s	22.2	1.06, s
34	30.5	1.33, s	30.1	1.34, s	30.5	1.35, s	30.2	1.40, s
1″	155.2	5.86, d, 15.6	44.3	~2.30, m	39.0	2.05, m	148.6	6.10, d, 15.6
				~1.72, m		1.78, m		
2″	126.1	6.35, dd, 15.2, 11.2	73.4	4.90, q, 8	78.7	4.21, ddd, 4.4, 7.2, 8.8	125.6	6.36, dd, 15.2, 11.2
3″	140.6	7.26, dd, 11.2, 1.2	146.9	6.95, d, 8.0	75.8	3.83, dd, 4.4, 6.8	140.3	7.32, d, 10.8
4″	126.1		127.2		44.2	2.50, pent, 6.8	125.9	
5″	172.2		172.7		179.2		173.5	
6″	12.9	1.93, d, 1.2	12.6	1.87, brs	14.6	1.18, d, 6.4	12.6	1.95, d, 1.6

= 2.8, 7.6 Hz, δ_C 50.1). The NMR assignment of **7a** was fully corroborated by HMBC experiments and is listed in Table 1. The methylation of **7a** with diazomethane produced ester **7b**.

The structures of nodulisporic acids D_1 (**8a**) and D_2 (**9a**) were elucidated by analogous comparison of ^1H, ^{13}C NMR and mass spectral data with the corresponding data of nodulisporic acids A_1, A_2, B_1, B_2, C_1, C_2, and D. Molecular formulas of $C_{38}H_{49}NO_5$ ($C_{39}H_{51}NO_5$ of methyl ester **8b**) and $C_{38}H_{51}NO_6$ ($C_{39}H_{53}NO_6$ of methyl ester **9b**, IR ν_{max} 1723 cm^{-1}) were determined for **8a** and **9a,** respectively, by HRESI-FTMS. The ^1H and ^{13}C NMR spectra (Table 1) of both of these compounds did not possess isoprene unit C1′−C5′, like nodulisporic acid D. The presence of the hemiketal-containing furan ring side chain (ring I) and the stereochemistry at C-7 and C-2″ of **8a** and **9a** was confirmed by the NMR spectroscopic analysis as previously described for nodulisporic acids A_1, A_2, B_1, B_2, and C_1, C_2. The stereochemistry at C-3″ and C-4″ of **9a** was elucidated as described below, establishing the stereochemistry of these two stereocenters in A_2 (**3a**), B_2 (**3b**), and C_2 (**6**).

Stereochemistry at C-3″ and C-4″. Preparation of Mosher esters[19] (**9c** and **9d**) of the methyl ester of nod-

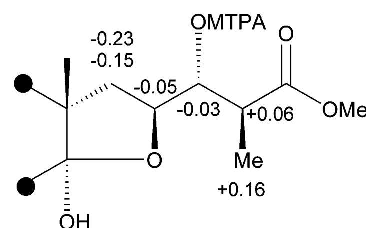
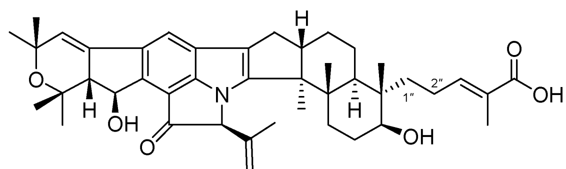
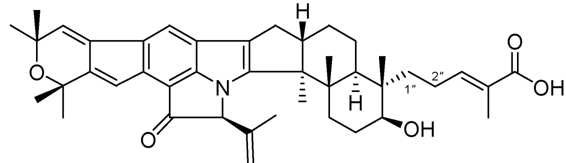


Figure 1. $\Delta\delta_H$ ($\delta S - \delta R$) of MTPA esters of **9b**.

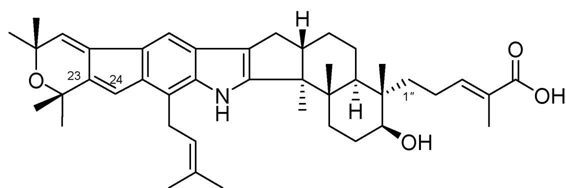
ulisporic acid D_2 (**9b**) followed by differential analysis (Figure 1) revealed *R*-stereochemistry at C-3″ of D_2 (**9a**). Once the stereochemistry at C-3″ was established, only the stereochemistry at C-4″ remained to be established. To accomplish this goal, we used a conformational analysis approach with the introduction of dihedral angle constraints obtained from the coupling constants between H2″−H3″ and H3″−H4″. Conformational analyses were carried out for the two isomers, i.e., 3*R*,4*S* and 3*R*,4*R*, of nodulisporic acid D_2 (**9a**) and corresponding methyl esters (**9b**). Approximately 200 conformations for each compound were generated and energy minimized. All unique confor-



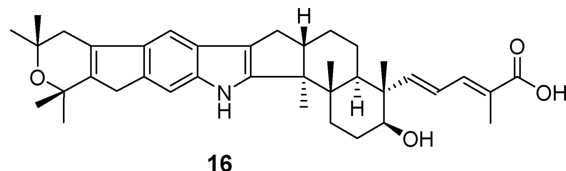
13: Nod acid A_4



14: Δ^{23}-Nod acid A_4



15: Δ^{23}-Nod acid C_4



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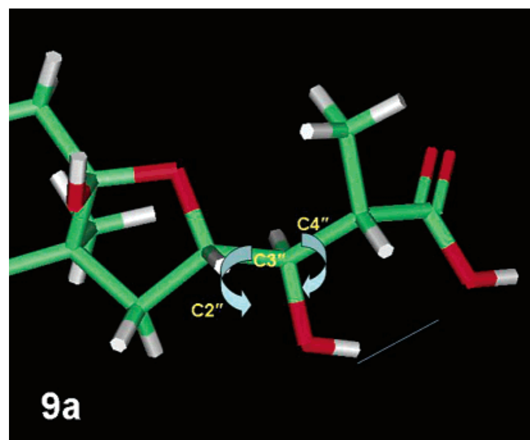
mations were selected, and the dihedral angles of H2″–H3″ and H3″–H4″ were measured for each conformation. The results showed that these two dihedrals (θ) sample wide range of angles. On the basis of the assumption that the hydrogen bond between OH at C-3″ and the oxygen of the ester (or free acid) would limit the freedom of two dihedrals, such conformers were selected, as shown in Table 2 and Figure 2. From the experimental coupling constants, the experimental dihedral angles (θ) were derived using the Karplus equation. For the ester (**9b**), experimental dihedrals of H2″–H3″ are ±55° (J = 3.2 Hz) and H3″–H4″ are ±160° (J = 8.8 Hz), similar to 60° and 160° of the calculated *R*,*S* conformers 1, 3, and 4. Also for the free acid (**9a**), the dihedrals of the calculated conformers 5 and 6 with *R*,*S* configuration (60° and 160°) are close to the experimental values [±42° (J = 4.4 Hz), ±140° (J = 6.8 Hz)]. Similar 60° and 160° dihedrals were obtained for conformers with a six-atom-centered hydrogen bond using carboxyl ketone. Therefore, it is likely that both compounds possess the *S*-configuration at the C-4″ position. Thus, a



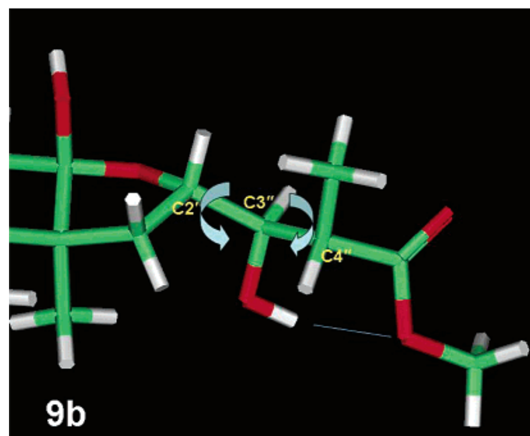


Figure 2. Selected 3*R*,4*S*-conformations of side chain fragments of **9a** and **9b** showing a six-atom-centered H-bond with an acid/ester oxygen.

Table 2. Experimental and Calculated Dihedral Angles (θ) of H2″–H3″ and H3″–H4″ of 3*R*- and 4″-(*R*,*S*)-nodulisporic Acid D_2 (**9a**) and Methyl Ester (**9b**)

compound	conformer #	$\theta_{H2''-H3}$	$\theta_{H3''-H4}$	$J_{H2''-H3''}$ (obsd)	$J_{H3''-H4''}$ (obsd)	energy (kcal/mol)
9a	experimental	±42	+/−140	4.4	6.8	
R,*R*	conformer 1	175	71			178
	conformer 2	176	75			179
	conformer 3	−78	75			181
	conformer 4	64	75			183
	conformer 5	64	77			184
R,*S*	conformer 1	174	59			177
	conformer 2	174	56			179
	conformer 3	−171	−159			180
	conformer 4	−172	−164			181
	conformer 5	−64	−168			182
	conformer 6	66	−163			188
9b	experimental	±55	+/−160	3.2	8.8	
R,*R*	conformer 1	176	75			183
	conformer 2	−78	75			186
	conformer 3	69	−71			190
R,*S*	conformer 1	−64	−161			186
	conformer 2	73	61			187
	conformer 3	66	163			190
	conformer 4	−64	169			193

3″*R*- and 4″*S*-configuration was suggested for **9a** and accordingly to nodulisporic acids A_2, B_2, and C_2.

Mass spectral analysis indicated that nodulisporic acid D_3 (**10a**) had a molecular formula of $C_{38}H_{47}NO_4$, i.e., that it possessed two fewer hydrogens as compared to nodulisporic acid D (**7a**). The ^1H and ^{13}C NMR spectra of **10a** were identical to the corresponding spectra of **7a** except for the substitution of the C-7 methine (δ_H 3.37, δ_C 77.8) with a ketone group in D_3 (δ_C 213.3). The structure was fully corroborated by HMBC experiments, and assignment is listed in Table 1.

Treatment of **7a** with TFA in $CDCl_3$ or CH_3CN led to instantaneous isomerization of the $\Delta^{18,19}$ olefin and to the $\Delta^{18,23}$ olefin, leading to the compound **16**. The ^1H NMR spectrum of **16** lacked the doublet of the olefinic proton for H-19 and the doublet of triplets of methine H-23 and revealed the presence of two singlets for the new methylene protons (H_2-24, δ_H 3.54, and H_2-19 δ_H 2.60). The UV spectra of **16** experienced a 4−6 nm hypsochromic shift compared to **7a**.

Nodulisporic Acid E (11a). The mass spectral analysis of **11a** afforded a molecular formula of $C_{38}H_{53}NO_3$, indicating that it was related to nodulisporic acid D but has lost an oxygen atom and possessed two additional hydrogen atoms. Like other nodulisporic acids, methylation of **11a** with diazomethane gave a monomethyl ester, **11b** ($C_{39}H_{55}NO_3$). The ^1H and ^{13}C NMR spectra of **11a** (Table 3) were essentially identical to the corresponding spectra of D (**7a**) except for the shifts arising from the western hemisphere of the molecule and the absence of one of the dienoic acid olefins. The NMR shifts of oxygenated carbons C-20 (δ_C 74.3) and C-22 (δ_C 76.2) were absent and instead were replaced by two additional olefinic carbons, indicating the loss of oxygen-21. In addition, the C31−C34 methyl singlets of nodulisporic acid D were shifted downfield to the olefinic methyl region (Table 3). The COSY spectrum of **11a** showed the spin systems indicative of the 1″,2″-dihydro-enoic acid residue and also indicated the presence of two dimethyl allyl units connected to the phenyl group which were established at C-17 and C-25 by the HMBC correlations of H-16 (δ_H 7.14) to C-18 (δ_C 32.3) and H-26 (δ_H 7.07) to C-24 (δ_C 32.2) and thus confirming the *seco* A/B structure **11a** for nodulisporic acid E.

Nodulisporic Acid F (12). HREIMS analysis of **12** produced a molecular formula of $C_{28}H_{37}NO_3$, which was corroborated by the ^{13}C NMR spectrum. The UV spectrum of **12** showed absorption maxima at 230 and 282 nm and was similar to the spectrum of **11a** and was different from the other nodulisporic acids **1a**−**10**. It was obvious from analyses of the ^1H and ^{13}C NMR spectra of **12** that the two isoprene units represented by the dimethylallyl groups present at C-17 and C-25 of **11** were absent in **12** and were replaced with the two protons H-17 and H-18 appearing as a doublet of triplets at δ_H 6.91 (J = 1.2, 7.2 Hz) and δ_H 6.95 (J = 2.0, 7.2 Hz), respectively, confirming the presence of an unsubstituted phenyl ring of the indole unit. The structure of the remainder of the molecule of **12** was identical to **11a**. Full NMR assignment of **12** is listed in Table 4, which was fully substantiated by the COSY, HMQC, and HMBC experiments.

Nodulisporic Acid A_4 (13). The mass spectral analysis of **13** revealed a molecular formula of $C_{43}H_{55}NO_6$, indicating the presence of two additional hydrogens compared to **1a**. The ^1H and ^{13}C NMR spectra of the two compounds were identical except for the absence of the 1″,2″-olefin of the dienoic acid of **1a**. The 1″,2″-olefin signals were replaced by a pair of methylene (δ_H 1.3, 1.70; δ_C 37.6; δ_H 2.15, 2.0, δ_C 23.3) groups (Table 5). Thus the 1″,2″-dihydronodulisporic acid A (**13**) structure was assigned for nodulisporic acid A_4.

Table 3. NMR Assignments of Nodulisporic Acid E (**11a**) and Methyl Ester (**11b**) Recorded in CD_2Cl_2 at 400 MHz

position	**11a** δ_C	**11a** δ_H	**11b** δ_C	**11b** δ_H
1		7.66, brs		7.72, brs
2	150.7		150.7	
3	53.5		53.4	
4	39.7		39.6	
5	33.7	~1.90, m	33.7	~1.90, m
		~1.50, m		~1.50, m
6	25.5	~1.85, m	25.5	~1.85, m
		~1.75, m		~1.75, m
7	73.5	3.54, dd, 9.6, 5.6	73.5	3.57, dd, 9.2, 5.6
8	41.6		41.6	
9	40.4		40.4	
10	23.1	~1.60, m	23.1	~1.61, m
		~1.45, m		~1.46, m
11	28.1	~1.75, m	27.9	~1.77, m
		~1.65, m		~1.66, m
12	49.1	2.70, m	49.1	2.72, m
13	27.8	2.62, dd, 13.2, 6.4	27.8	2.63, dd, 13.6, 6.4
		2.27, dd, 10.8, 13.2		2.29, dd, 10.8, 12.8
14	117.9		117.9	
15	123.9		124.0	
16	118.5	7.14, s	118.5	7.16, s
17	132.1		132.0	
18	32.3[a]	3.37, d, 6.8	32.3[b]	3.39, d, 6.4
19	124.7[b]	5.28, m	124.7[a]	5.31, m
20	131.6		131.6[c]	
22	131.6		131.5[c]	
23	124.3[b]	5.28, m	124.3[a]	5.31, m
24	32.2[a]	3.37, d, 6.8	32.2[b]	3.39, d, 6.8
25	132.8		132.8	
26	111.6	7.07, s	111.7	7.01, s
27	139.5		139.6	
28	14.6	0.99, s	14.6	1.01, s
29	19.2	1.08, s	19.3	1.10, s
30	16.5	0.83, s	16.6	0.85, s
31	25.8	1.75, brs	25.9	1.77, brs
32	17.9	1.72, brs	18.0	1.74, brs
33	17.9	1.73, brs	18.0	1.75, brs
34	25.9	1.76, brs	25.9	1.75, brs
1″	36.3	~1.65, 1.35	36.1	~1.60, 1.40
2″	22.7	2.10, m	22.9	2.10, m
		2.20, m		2.20, m
3″	143.0	6.77, t, 6.4	145.6	6.93, t, 7.2
4″	127.7		127.3	
5″	168.8		173.2	
6″	12.5	1.86, d, 1.2	12.2	1.88, brs
OCH_3	51.9	3.71, s		

Δ^{23}-Nodulisporic Acid A_4 (14). The molecular formula of $C_{43}H_{53}NO_5$ was determined for **14** by HRMS, which indicated the loss of a molecule of H_2O as compared to **13**. The ^1H and ^{13}C NMR spectra of **13** and **14** were identical except for replacement of the C-23 and C-24 methines with an olefin appearing as a singlet at δ_H 6.87 (Table 5). As a result of the loss of H_2O, the UV spectrum of **14** exhibited a 33 nm bathochromic shift of the UV maxima (λ_{max} 396 to 429 nm) due to extended conjugation and caused the change of color to deep yellow. Thus, structure **14** was assigned to Δ^{23}-nodulisporic acid A_4.

Δ^{23}-Nodulisporic Acid C_4 (15). HRMS of **15** afforded a molecular formula of $C_{43}H_{57}NO_4$, which was corroborated by the ^{13}C NMR spectrum (Table 5). The ^1H and ^{13}C NMR analysis of **15** indicated that it was related to nodulisporic acid C (**4**) and it lacked a 1″,2″-olefin and C-23, C-24 methines (Table 5). Similar to the structural assignments of **13** and **14**, a structure of Δ^{23}-1″,2″-dihydronodulisporic acid C was assigned to **15**, named herein Δ^{23}-nodulisporic acid C_4.

Biogenetic Relationships. It has been known for two decades that the isoprenyl units of fungal indole-diterpenes

Table 4. ^{1}H and ^{13}C NMR Assignment of Nodulisporic Acid F (**12**) Recorded in CD_3OD

position	δ_C	δ_H
2	152.2	
3	54.5	
4	40.5	
5	33.0	~1.80, m
		~1.80, m
6	28.5	~1.90, m
		~1.75, m
7	74.0	3.55, dd, 9.6, 4.4
8	42.7	
9	41.5	
10	24.2	~1.60, m
		~1.40, m
11	26.5	~1.70, m
		~1.60, m
12	50.5	2.73, m
13	28.4	2.61, dd, 13.2, 6.4
		2.28, dd, 10.8, 13.2
14	118.1	
15	126.4	
16	118.8	7.28, brd, 7.2
17	120.8	6.91, dt, 1.2, 7.2
18	119.8	6.95, dt, 2.0, 7.2
19	112.7	7.28, brd, 7.2
20	142.2	
21	15.0	1.0, s
22	19.6	1.1, s
23	17.4	0.85, s
1″	37.2	1.70, m, 1.35, m
2″	23.6	2.10, m
		2.20, m
3″	144.1	6.80, t, 7.2
4″	129.0	
5″	172.2	
6″	12.6	1.85, brs

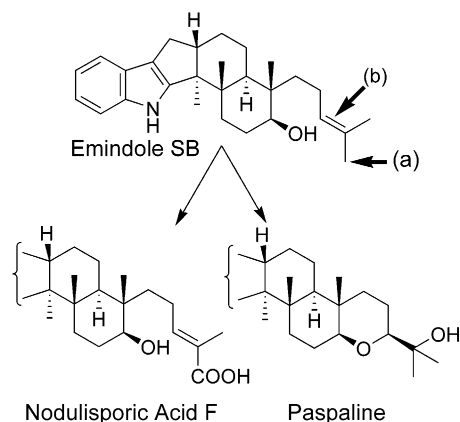


Figure 3. Biogenetic relationships of emindole SB, nodulisporic acid F (oxidation, arrow a), and paspaline (oxidation–cyclization, arrow b).

arise through the classical mevalonate pathway,[20] and it has been presumed that the first pathway intermediate is made by condensation of the C20 isoprenyl chain of geranylgeranyl diphosphate with an indole donor such as tryptophan.[21,22] Recently Byrne et al.[23] have presented evidence that indole-3-glycerol phosphate rather than tryptophan serves as the indole donor in the formation of nodulisporic acid and, by inference, for other indole-diterpenes such as paxilline and aflatrem. Formally emindole SB (Figure 3) may be considered as the product of the initial condensation[21,24] leading to a simple indole-diterpenoid, although it may not occur as an unbound intermediate in all indole-diterpene-producing fungi. Oxidation of the distal isoprenyl unit of emindole SB may be the key event distinguishing the biosynthetic pathway for nodulisporic acids from those of other indole-diterpenes: conversion of a terminal methyl to a carboxyl moiety provides nodulisporic acid F, whereas hydration of the subterminal double bond is needed for production of paspaline, the precursor proposed for a wide variety of indole-diterpenes including paxilline and aflatrem.[22,24] Biosyntheses of nodulisporic acids appear to start with nodulisporic acid F (**12**), and the known compounds can be arranged on the basis of increasing complexity into a hypothetical biosynthetic pathway that is fully consistent with the pattern of production observed in the mutant cultures (Figure 4, for extended Figure 4, see Supporting Information). Mutants that no longer make compounds of the A series (A, A_1, A_2) accumulate the B (B, B_1, B_2) or C (C, C_1, C_2) series of compounds (**1**–**6**) or, as shown in the present work, the D (**7**–**10**) series together with nodulisporic acids E (**11**) and F (**12**), nodulisporic acid A_4 (**13** and **14**), and Δ^{23}-nodulisporic acid C_4 (**15**). The scheme shows an initially linear pathway that becomes a metabolic grid once nodulisporic acid D is formed. Nodulisporic acid F, the basic indole-diterpenoid intermediate, is bis-prenylated on the aromatic ring, producing nodulisporic acid E (**11**), and oxidation of the dimethylallyl group at C-17 and a subsequent cyclization step(s) produces a putative precursor for the eventual synthesis of the advanced nodulisporic acids (D → A). The alternative oxidation of the dimethylallyl group at C-25 followed by analogous cyclizations would be expected to produce the janthetrem type of cyclopentyl-pyrenosyl rings. The putative missing precursor could take one of the two pathways. In one, the first step would be an allylic oxidation at C-2″, producing a C-2″ hydroxy derivative, which could also take two routes: (i) dehydration to produce the dienoic acid series (D → A) or (ii) cyclization to produce tetrahydrofuran ring I of the $D_1 \rightarrow A_1$ series of nodulisporic acids [the latter implies that the C-7 hydroxy group is also simultaneously oxidized to a ketone (e.g., nodulisporic acid D_3)]. In the second hypothetical pathway involving the missing precursor, oxidation, dehydration, or cyclization steps could take place after the last prenylation step at C-26 and advance through $C_4 \rightarrow A_4$ and sideways to $C_4 \rightarrow$ C and $A_4 \rightarrow$ A and so on (Figure 4). It appears that the hydroxylation at C-24 occurs after the last prenyl group has been added (e.g., absence of the C-24 hydroxy group in D series nodulisporic acids). In addition, the oxidation of C-1′ to a keto group appears to be one of the last steps of the biosynthesis. We have also found mutants deficient in the A_2–D_2 series (MF6224) and both the A_1–D_1 and A_2–D_2 series (MF6224) series, supporting the lineage of A → A_1 → A_2. Although no other indole-diterpene-producing fungus has been subjected to a mutational analysis to this degree, the recent[25] cloning of a cluster of genes mediating paxilline synthesis in *Penicillium paxillii* presents the opportunity for conducting systematic studies of indole-diterpene pathways through targeted gene inactivation.[26] Homologies between proteins of known function and those predicted from the DNA sequences of paxilline genes have already suggested the types of enzymes involved. As for the presumed origin of the C20 isoprenyl moiety, it is noteworthy that a gene for a geranylgeranyl diphosphate synthase was found in the paxilline cluster and that the activity of this gene is required for paxilline production but not the vegetative growth of *P. paxillii*. Further insights into indole-diterpene biosynthesis can be expected from functional analyses of the products of other pathway genes. Nodulisporic acids resemble other insecticidal indole-diterpenes such as the janthitrems,[27] shearinines,[28] and others (see ref 15 for a comprehensive review). The resemblance is all the more striking with the D series of compounds.

Table 5. NMR Assignments of Nodulisporic Acids **13**−**15**

position	**13** CD_3OD δ_C	**13** CD_3OD δ_H	**14** $CDCl_3$ δ_H	**15** $CD_3OD + CDCl_3$ δ_C	**15** $CD_3OD + CDCl_3$ δ_H
1					
2	156.1			151.1	
3	57.1			54.3	
4	40.4			40.6	
5	33.6	~1.7, m; ~1.90, m	~1.7, m; ~1.90, m	34.6	~1.37, m; ~1.65, m
6	28.0	~1.70, m; 1.72, m	~1.76, m	28.5	~1.71, m; 1.76, m
7	73.8	3.50, m	3.56, dd, 9.0, 5.0	74.1	3.37, m
8	41.8			42.5	
9	42.8	1.6, m	1.76, m	41.3	1.56, m
10	23.8	~1.65, m; ~1.50, m	~1.62, m; ~1.5, m	24.1	~1.60, m; ~1.50, m
11	26.5	~1.6, m; ~1.8, m	~1.60, m; ~1.84, m	26.4	~1.4, m; ~1.6, m
12	48.5	2.81, m	2.85, m	49.5	2.56, m
13	28.5	2.74, dd, 13.6, 7.0	2.76, dd, 14, 7.0	28.7	2.50, dd, 13, 6.5
		2.30, dd, 13.6, 10.8	2.34, dd, 14, 10		2.17, dd, 12.4, 10.0
14	117.6			120.5	
15	123.1			123.9	
16	117.1	7.75, s	7.80, s	110.0	7.31, s
17	137.1			129.6	
18	135.3			135.4	
19	122.0	6.12, d, 2.8	6.78, brs	129.6	6.36, s
20	74.2			74.1	
22	75.5			74.5	
23	60.3	2.75, dd, 2.8, 6.0		142.5	
24	75.8	5.14, d, 6.0	6.87, brs	120.5	6.35, s
25	138.6			136.6	
26	114.5			112.7	
27	163.3			140.7	
28	15.5	0.97, s	0.95, s	15.7	0.85, s
29	20.1	1.11, s	1.13, s	20.3	0.95, s
30	17.5	0.81, s	0.87, s	17.6	0.69, s
31	30.3	1.35, s	1.59, s	32.0	1.38, s
32	32.2	1.32, s	1.60, s	32.0	1.38, s
33	23.7	1.12, s	1.51, s	32.6	1.47, s
34	30.3	1.45, s	1.52, s	32.6	1.47, s
1′	197.9			28.8	3.47, t, 6.0
2′	77.1	5.15, brs	4.96, brs	124.5	5.22, t, 6.4
3′	141.6			133.0	
4′	117.4	5.16, brs; 4.9, brs, m	5.16, brs; 4.96, brs	19.2	1.73, brs
5′	18.5	1.49, brs	1.48, brs	26.3	1.60, brs
1″	37.6	1.3, m; 1.70, m	1.47, m; 1.70, m	37.2	1.20, m; 1.60, m
2″	23.3	2.15, m; 2.0, m	2.20, m; 2.0, m	23.7	2.05, m; 1.95, m
3″	137.8	6.45, t, 6.8	6.93, t, 7.0	144.8	6.67, t, 7.2
4″	134.2			128.4	
5″	172.0			171.9	
6″	13.9	1.83, brs	1.90, brs	134	1.70, brs

Biological Activities. The new nodulisporic acids were all tested in an ex-vivo flea (*Ctenocephalides felis*) artificial membrane feeding assay.[3] The activities of new nodulisporic acids D−F (**7**−**12**) as well as the nodulisporic acids A−C (**1**−**6**) were also evaluated for their abilities to replace the binding of an [^{35}S]-labeled nodulisporic amide derivative in a competition binding assay using *Drosophila melanogaster* head membrane.[6] This assay provided more sensitive and direct comparison of the activities of these compounds. The data are presented in Table 6. Nodulisporic acid A (**1a**) showed the best activity in both flea ex-vivo and binding assays. It exhibited an LD_{90} of 1.5 μM in the flea assay and an IC_{50} of 0.00027 μM in the binding assay. The nodulisporic acids that contain the dienoic acid chain showed better activities when compared within its own series. For example, **1a** was more active than **2a** and **3a**; **1b** was more active than **2b** and **3b**; and **4** was more active than **5** and **6**. The only exception appears to be in the D series where the binding activity of **9a** was significantly better than **7a** and **8a**; this observation was repeated twice. The structural simplicity of nodulisporic acids caused significant dimunition of all biological activities. For example, D, E, and F (**7a**, **11a**, and **12**) were 62-, 12-, and 30-fold less active than **1a**, respectively, in the flea assay. The structural simplicity had a more significant adverse effect on the binding activity. For example, E and F exhibited >8000- and >37 000-fold loss in the activity, respectively, compared to nodulisporic acid A. The nodulisporic acid A methyl ester (**1c**) was 10-fold less active in the flea assay and only 2-fold less active in the binding assay. The result was somewhat different for nodulisporic acid B methyl ester (**1d**), where the flea activity was unchanged compared to **1b** but the binding activity was 8-fold better. It should be recalled that the binding assay was run with *D. melanogaster* membranes, versus the flea activity, and thus the two assays may have slightly different structure−activity relationships. In general, Δ^{23} 1″,2″-dihydro nodulisporic acids were less active than corresponding nodulisporic acids of the same class.

In conclusion, we have reported three new classes of nodulisporic acids: namely, D, E, and F (**7**−**12**) and three new examples of the A (**13**, **14**) and C series of nodulisporic acids (**15**). In total, nine new examples of nodulisporic acids have been described that were isolated from the cultures generated by chemical mutagenesis of *Nodulisporium* sp. Nodulisporic acid D series are devoid of one of the isoprene residues at C-26, and nodulisporic acid E is the penultimate biosynthetic precursor of all known nodulisporic acids and

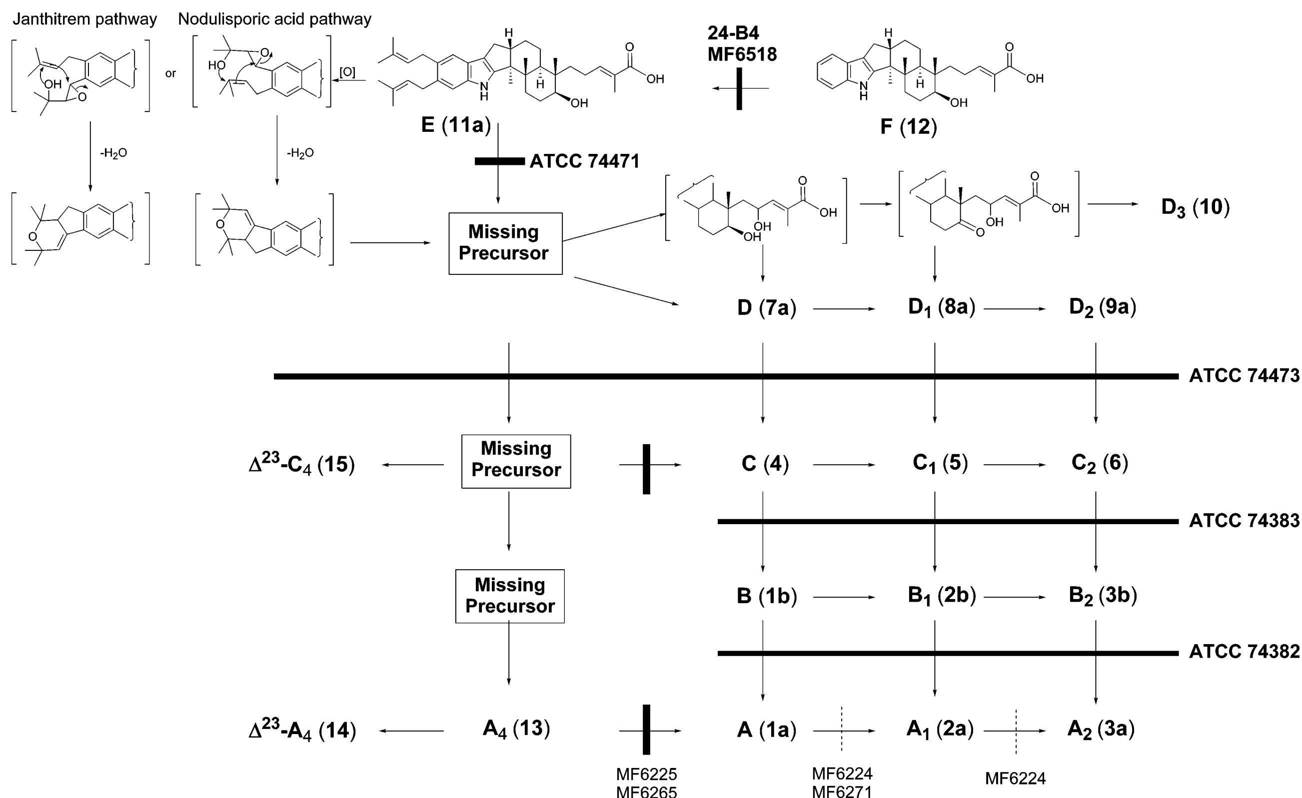


Figure 4. Proposed biogenetic pathway for nodulisporic acids starting with nodulisporic acid F. The point where each of the mutants defective in nodulisporic acid production is blocked or partially blocked is shown by a bar or dotted line, respectively, crossing an arrow that represents an enzymatic step or steps in the pathway.

Table 6. Biological Activities of Nodulisporic Acids

compound	flea LD_{90} μM	nod acid binding IC_{50} μM
1a	1.5	0.00027
1b	150	0.0174
1c	14.4	0.00043
1d	147.1	0.0026
2a	7.3	0.0105
2b	>147.7	0.121
3a	14.0	0.0700
3b	>142.9	0.101
4	15.0	0.0052
5	>146.2	0.0523
6	>142.5	0.0715
7a	92.6	>1.0
7b	NT	>0.10
8a	NT	>10.0
8b	NT	>0.10
9a	NT	0.0221
9b	NT	>10.0
10	NT	>1.0
11a	17.5	2.143
11b	NT	>0.10
12	45.9	>10.0
13	14.7	0.0024
14	>15.1	0.023
15	15.3	>0.10

is simplified by the opening of the A/B rings of dihydronodulisporic acid D. Nodulisporic acid F is the simplest member of the nodulisporic acids and lacks all three of the isoprene residues of the indole unit. As such, it represents the simplest known biosynthetic intermediate of this class. Based on the systematic discovery of nodulisporic acids with varying degrees of complexities, a biogenetic grid has been proposed that suggests the biogenetic relationships of all nodulisporic acids. An antiflea structure–activity relationship of all natural nodulisporic acids has been presented. It indicates that in general the compounds that contain a dienoic acid chain are most active within the given series and that the end of the biosynthetic pathway, i.e., nodulisporic acid A, is the most active compound of all. Additionally, the stereochemistries of the C-3″ and C-4″ of nodulisporic acid D_2 and therefore of nodulisporic acids A_2, B_2, and C_2 have been assigned.

Experimental Section

General Experimental Procedures. Optical rotation was determined on a Perkin-Elmer 241 polarimeter. UV/vis spectra were recorded on a Beckman DU 70 spectrometer. The IR spectrum was recorded on a Perkin-Elmer Spectrum One spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity 500 or 400 MHz NMR spectrometer operating at 500 and 400 MHz for ^{1}H and 125 and 100 MHz for ^{13}C nuclei, respectively. Solvent peaks were used as a reference standard. All NMR assignments were verified by COSY, HMQC, and HMBC experiments. Mass spectra were measured on a LCQ, Thermo Quest HRFTMS, and/or Bruker APEX II FTMS. A Hewlett-Packard HP1100 was used for analytical HPLC. Trace elements used in fermentation media consisted of $FeSO_4 \cdot 7H_2O$, 0.5 g/L, $ZnSO_4 \cdot 7H_2O$, 0.5 g/L, $MnSO_4 \cdot H_2O$, 0.1 g/L, $CuSO_4 \cdot 5H_2O$, 0.05 g/L, and $CoCl_2 \cdot 6H_2O$, 0.04 g/L.

Fungal Isolation and Fermentation of Producing Organism for the Production of Nodulisporic Acids D–E (7–11). *Nodulisporium* sp. MF6227 (ATCC 74473) was derived by mutation of the original culture MF5954 (ATCC 74245), which was isolated as an endophytic fungus from the woody plant tissue of *Bontia daphnoides* collected from Hawaii. The mutants were generated by treating mycelia with *N*-methyl-*N*′-nitro-1-nitrosoguanidine (NTG) and screening progeny for altered nodulisporic acid production by HPLC. The new mutant of *Nodulisporium* sp. was cultured in shake flasks from vegetative mycelial growths on a rotary shaker for 22 days in darkness in a medium consisting of glycerol (75 g/L), glucose (10 g/L), ardamine pH (5 g/L), $(NH_4)_2SO_4$ (2 g/L), soybean meal (5 g/L), tomato paste (5 g/L), sodium citrate (2 g/L), and distilled H_2O at pH 7.0 at 29 °C.

Isolation of Nodulisporic Acids D (7a), D_1 (8a), D_2 (9a), D_3 (10), and E (11a). A 2 L fermentation broth of ATCC 74473 was filtered, and mycelia were extracted with 2000 mL of methyl ethyl ketone by shaking for 30 min and were concentrated to afford 2.2 g of dark-colored solid. A 25 mg aliquot of the gum was chromatographed on a Waters reversed-phase symmetry column (9.4 × 250 mm) and was eluted with 60% aqueous CH_3CN + 0.1% TFA for 10 min followed by a 30 min gradient to 70% CH_3CN + 0.1% TFA at a flow rate of 4 mL/min. This chromatographic procedure was repeated five times, and identical fractions were pooled, diluted with four volumes of H_2O, and extracted with EtOAc. The organic layer was washed with H_2O, dried (Na_2SO_4), and concentrated to furnish nodulisporic acids D_2 (**9a**, 9.1 mg, 80 mg/L), D (**7a**, 4.9 mg, 43 mg/L), D_3 (**10**, 3.4 mg, 30 mg/L), D_1 (**8a**, 7.9 mg, 70 mg/L), and E (**11a**,14.7 mg, 130 mg/L), all as pale solids.

Nodulisporic acid D (7a): $[\alpha]^{23}_{578}$ −5.2° (*c* 1.5, MeOH); UV (MeOH) λ_{max} 206 (ϵ 18 900), 262 (58 380), 316 (sh), 336 (sh) nm; IR (ZnSe) ν_{max} 3356, 2970, 2928, 1683, 1457, 1376, 1282, 1249, 1205, 1144, 1072, 1029, 982, 831 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; ESIMS *m/z* 584 $[M + H]^+$; HRESI-FTMS *m/z* 584.3729 (calcd for $C_{38}H_{49}NO_4$+H, 584.3735).

Nodulisporic acid D_1 (8a): $[\alpha]^{23}_{578}$ −3° (*c* 2.0, MeOH); UV (MeOH) λ_{max} 214 (ϵ 27 280), 260 (46 150), 312 (6918), 324 (6000) nm; IR (ZnSe) ν_{max} 3356, 2970, 2928, 1692, 1456, 1376, 1350, 1249, 1139, 1077, 1033, 980, 920, 831 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; ESIMS *m/z* 600 $[M + H]^+$, 582 $[M - H_2O]^+$; HRESI-FTMS *m/z* 600.3682 (calcd for $C_{38}H_{49}NO_5$+H, 600.3684); HREIMS *m/z* 599.3538 (calcd for $C_{38}H_{49}NO_5$, 599.3611).

Nodulisporic acid D_2 (9a): $[\alpha]^{23}_{578}$ −15.7° (*c* 2.9, MeOH); UV (MeOH) λ_{max} 210 (ϵ 18 050), 260 (45 250), 310 (7400), 336 (sh) nm; IR (ZnSe) ν_{max} 3333, 2969, 2925, 1679, 1457, 1376, 1351, 1249, 1203, 1105, 1078, 1022, 981, 915, 833, 801, 724 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; ESIMS *m/z* 618 $[M + H]^+$, 616 $[M - H]^-$; HRESI-FTMS *m/z* 618.3787 (calcd for $C_{38}H_{51}NO_6$+H, 618.3889).

Nodulisporic acid D_3 (10): $[\alpha]^{23}_{578}$ −53.8° (*c* 0.13, MeOH); UV (MeOH) λ_{max} 208 (ϵ 14 550), 262 (51 060), 315 (sh), 330 (sh) nm; IR (ZnSe) ν_{max} 3475, 2971, 2929, 2859, 1693, 1456, 1378, 1353, 1248, 1211, 1145, 1113, 1034, 973, 832, 725 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; ESIMS *m/z* 582 $[M + H]^+$; HRESI-FTMS *m/z* 582.3571 (calcd for $C_{38}H_{47}NO_4$+H, 582.3578); EIMS *m/z* 581.3513 (calcd for $C_{38}H_{47}NO_4$, 581.3505).

Nodulisporic acid E (11a): $[\alpha]^{23}_{578}$ +11.2° (*c* 1.87, MeOH); UV (MeOH) λ_{max} 234 (ϵ 41 540), 280 (9300) nm; IR (ZnSe) ν_{max} 3374, 2927, 2857, 1686, 1643, 1562, 1454, 1375, 1281, 1248, 1160, 1097, 1073, 1025, 852 cm^{-1}; ^1H and ^{13}C NMR data, Table 3; ESIMS *m/z* 572 $[M + H]^+$; HREIMS *m/z* 571.4028 (calcd for $C_{38}H_{53}NO_3$, 571.4025).

Methyl Esters of Nodulisporic Acids D−E (7−11). To a cooled (0 °C) solution of 700 mg of crude solid of nodulisporic acid mixture in 20 mL of CH_2Cl_2 was added an ethereal solution of diazomethane, and the mixture was stirred for 10 min. A 100 mg portion of this dried material was chromatographed on a Zorbax RX C-8 (21 × 250 mm) column and eluted with a 30 min 70−90% gradient of aqueous CH_3CN (without TFA) at a flow rate of 8 mL/min to afford methyl esters of nodulisporic acids D_2 (**9b**, 25 min, 6.7 mg), D_1 (**8b**, 32 min, 5.9 mg), D (**7b**, 34 min, 4.0 mg), and E (**11b**, 45−46 min, 14.5 mg) as pale solids.

Nodulisporic acid D methyl ester (7b): $[\alpha]^{23}_{578}$ −16.7° (*c* 0.3, MeOH); UV (MeOH) λ_{max} 210 (ϵ 18 500), 264 (65 000), 312 (sh), 330 (sh) nm; IR (ZnSe) ν_{max} 3379, 2971, 2932, 2861, 1700, 1633, 1456, 1376, 1353, 1288, 1247, 1226, 1147, 1109, 1072, 1029, 978, 831, 751 cm^{-1}; ^1H NMR (CD_2Cl_2), only distinct signals are listed, δ 7.94 (1H, brs, NH), 7.47 (1H, s), 7.25 (1H, d, *J* = 11.2 Hz), 7.18 (1H, s), 6.40 (1H, dd, *J* = 15.6, 11.2 Hz), 5.96 (1H, d, *J* = 2.8 Hz, H-19), 5.89 (1H, d, *J* = 15.2 Hz), 3.76 (3H, s, OCH_3), 3.43 (1H, m, H-7), 3.12 (1H, dd, *J* = 16.0, 9.2 Hz, H-24), 2.92 (1H, ddd, *J* = 9.2, 7.6, 2.8 Hz), 2.74 (1H, m), 2.68 (2H, m), 2.30 (1H, dd, *J* = 13.2, 10.4 Hz), 1.97 (3H, d, *J* = 1.2 Hz), 1.35 (3H, s), 1.32 (3H, s), 1.31 (3H, s), 1.15 (3H, s), 1.09 (3H, s), 1.08 (3H, s), 1.03 (3H, s); HRESI-FTMS *m/z* 598.3884 (calcd for $C_{39}H_{51}NO_4$+H, 598.3891).

Nodulisporic acid D_1 methyl ester (8b): $[\alpha]^{23}_{578}$ −24.1° (*c* 1.33, MeOH); UV (MeOH) λ_{max} 218 (ϵ 23 810), 262 (41 380), 314 (6580), 324 (6210) nm; IR (ZnSe) ν_{max} 3388, 2971, 2937, 2844, 1705, 1456, 1438, 1375, 1349, 1250, 1191, 1160, 1135, 1109, 1076, 1055, 1020, 1011, 830 cm^{-1}; ^1H NMR (CD_2Cl_2), only distinct signals are listed, δ 7.75 (1H, brs, NH), 7.49 (1H, s), 7.20 (1H, s), 6.83 (1H, dd, *J* = 8.5, 1.5 Hz, H-3″), 5.98 (1H, d, *J* = 3.0 Hz, H-19), 4.92 (1H, q, *J* = 8.0 Hz, H-2″), 3.76 (3H, s, OCH_3), 3.15 (1H, dd, *J* = 16.5, 8.5 Hz), 2.93 (1H, ddd, *J* = 9.5, 8.0, 3.0 Hz, H-23), 2.85 (1H, m, H-12), 2.71 (1H, dd, *J* = 13.5, 6.5 Hz), 2.69 (1H, m), 2.37 (2H, m), 1.89 (3H, d, *J* = 1.0 Hz), 1.38 (3H, s), 1.334 (3H, s), 1.332 (3H, s), 1.19 (3H, s), 1.11 (3H, s), 1.10 (3H, s), 1.04 (3H, s); 1; HRESI-FTMS *m/z* 614.3834 (calcd for $C_{39}H_{51}NO_5$, 614.3840); HREIMS *m/z* 613.3693 (calcd for $C_{39}H_{51}NO_5$, 614.3767), 595.3643 (calcd for $C_{39}H_{49}NO_4$, 595.3662).

Nodulisporic acid D_2 methyl ester (9b): $[\alpha]^{23}_{578}$ −21.7° (*c* 1.8, MeOH); UV (MeOH) λ_{max} 210 (ϵ 15 650), 262 (39 820), 314 (6070), 324 (5860) nm; IR (ZnSe) ν_{max} 3376, 2971, 2931, 1723, 1567, 1457, 1374, 1350, 1249, 1200, 1133, 1110, 1077, 1033, 918, 831, 760 cm^{-1}; ^1H NMR (CD_2Cl_2), only distinct signals are listed, δ 7.96 (1H, brs, NH), 7.47 (1H, s), 7.18 (1H, s), 5.95 (1H, d, *J* = 2.8 Hz, H-19), 4.28 (1H, ddd, *J* = 9.6, 7.6, 2.8 Hz, H-2″), 3.98 (1H, dd, *J* = 8.8, 3.2 Hz, H-3″), 3.72 (3H, s, OCH_3), 3.12 (1H, dd, *J* = 16.4, 9.2 Hz, H-24), 2.91 (1H, ddd, *J* = 10, 7.6, 3.2 Hz, H-23), 2.80 (1H, m, H-12), 2.67 (2H, m, H-24, H-13), 2.49 (1H, pentet, *J* = 8 Hz, H-4″), 2.33 (1H, dd, *J* = 13.2, 10.8 Hz, H-13), 1.82 (1H, dd, *J* = 12.8, 9.2 Hz, H-1″), 1.71 (1H, dd, *J* = 12.8, 10 Hz, H-1″), 1.35 (3H, s), 1.32 (3H, s), 1.31 (3H, s), 1.20 (3H, d, *J* = 7.2 Hz, H-6″), 1.14 (3H, s), 1.08 (3H, s), 1.06 (3H, s), 0.99 (3H, s); HRESI-FTMS *m/z* 632.3939 (calcd for $C_{39}H_{53}NO_6$+H, 632.3946).

Nodulisporic acid E methyl ester (11a): $[\alpha]^{23}_{578}$ +18.6° (*c* 0.7, MeOH); UV (MeOH) λ_{max} 234 (ϵ 45 520), 284 (9120) nm; IR (ZnSe) ν_{max} 3399, 2929, 2858, 1698, 1646, 1564, 1454, 1375, 1282, 1222, 1192, 1155, 1100, 1075, 1027, 850, 725 cm^{-1}; ^1H and ^{13}C NMR data, Table 3; ESIMS *m/z* 586 $[M + H]^+$; HRESI-FTMS *m/z* 586.4250 (calcd for $C_{39}H_{55}NO_3$+H, 586.4255); HREIMS *m/z* 585.4186 (calcd for $C_{39}H_{55}NO_3$, 585.4182).

Isolation of Nodulisporic Acid F (12). Large-scale fermentation of MF 5954 that produced nodulisporic acid A as a major product was dewatered, extracted with isopropyl alcohol, and passed through an SP-207 column. The material was eluted with MeOH and partitioned with an equal volume of hexane. The MeOH solution was concentrated, charged to a 70 L silica gel column, and eluted with one column volume each of isopropyl acetate followed by 10%, 20%, 30%, 50%, and 100% MeOH in isopropyl acetate. A portion of the 50% MeOH fraction was concentrated to dryness under reduced pressure, was fractionated on a 2 L Sephadex LH20 funnel, and eluted with MeOH. The late eluting fraction containing **12** was concentrated to dryness, rechromatographed onto a 1 L Sephadex LH20 column, and eluted with MeOH. The fractions eluting at about one column volume were combined, chromatographed on a Zorbax RX C-8 (21 × 250 mm) column, and eluted with a 32 min gradient of 20−90% aqueous CH_3CN + 0.1% TFA at a flow rate of 12 mL/min. Lyophilization of fractions eluting at 30 min gave 2.6 mg of colorless powder, **12**. A similar isolation from the shunted mutant MF6518 by using Sephadex LH20 and reversed-phase HPLC gave almost exclusively **12**: $[\alpha]^{23}_{578}$ +7.2° (*c* 1.7, MeOH); UV (CH_3CN-H_2O + 0.1% TFA) λ_{max} 230, 282 nm; IR (ZnSe) ν_{max} 3334, 2935, 2861, 1678, 1445, 1385, 1300, 1185, 1144, 1071, 1022, 919, 741 cm^{-1}; ^1H and ^{13}C NMR data, Table 4; ESIMS *m/z* 436 $[M + H]^+$, 434 $[M - H]^-$; HREIMS *m/z* 435.2751 (M^+, calcd for $C_{28}H_{37}NO_3$, 435.2773), 420.2539 (calcd for $C_{27}H_{34}O_3N$, 420.2539), 250.1571 (calcd for $C_{15}H_{22}O_2$, 250.1569), 182.0970 (calcd for $C_{13}H_{12}N$, 182.0970).

Nodulisporic Acid A_4 (13) and Δ^{23}-Nodulisporic acid A_4 (14). A frozen vial of MF6265 was inoculated into 50 mL of SL3 seed medium (consisting of monosodium glutamate (MSG), 10 g/L, amicase, 2 g/L, NH_4Cl, 3 g/L, K_2HPO_4, 1 g/L, lactic acid, 2 g/L, MES buffer, 20 g/L, $MgSO_4\cdot 7H2O$, 0.5 g/L, $CaCO_3$, 1 g/L, trace elements, 20 mL, with 200 mL of glucose, 250 g/L) in a 250 mL Erlenmeyer flask. The flask was incubated in the dark at 29 °C for 2 days while shaking. Two mL aliquots of

this culture were transferred to 20 × 50 mL portions of FFL production medium (consisting of glycerol, 100 g/L, MSG, 10 g/L, amicase, 8 g/L, NH_4Cl, 3 g/L, l-tryptophan, 0.7 g/L, K_2PO_4, 1 g/L, MES buffer, 20 g/L, $MgSO_4·7H2O$, 0.5 g/L, lactic acid, 5 mL, trace elements, 20 mL, $CaCO_3$, 1 g/L, with 200 mL of glucose, 350 g/L) in 250 mL Erlenmeyer flasks. These cultures were fermented for 19 days at 29 °C in the dark. At the harvest, 6 mL of 1 N HCl was added to each flask, pooled, extracted with 800 mL of MEK, and filtered through a Celite filter aid. The layers were separated, and the MEK layer was concentrated to dryness under reduced pressure to afford 2.3 g of semisolid containing 500 mg of compound **13** (titer = 500 mg/L). One half portion of the crude material was dissolved in 7 mL of MeOH and chromatographed on a 200 mL Sephadex LH 20 column. Elution with MeOH eluted **13** in 0.6–0.7 column volumes. A small portion of the LH20 fraction was chromatographed on a Waters reversed-phase C-18 symmetry (21 × 250 mm) column and eluted with 60–90% aqueous MeOH over 60 min and holding for 10 at a flow rate of 8 mL/min. Concentration of fractions eluting at 40–45 and 63–65 min in vacuo followed by lyophilization afforded 42 and 20 mg of **13** and **14**, respectively, as yellowish powders.

Nodulisporic acid A_4 (13): $[\alpha]^{23}_{578}$ +5.9° (*c* 0.5, MeOH); UV (CH_3CN–H_2O+0.1% TFA) λ_{max} 236, 274, 396 nm; IR (ZnSe) ν_{max} 3442, 2974, 2938, 2870, 1704, 1645, 1588, 1456, 1381, 1290, 1228, 1077, 1022, 985, 750 cm^{-1}; ^1H and ^{13}C NMR data, Table 5; ESIMS *m/z* 682 $[M + H]^+$, 680 $[M - H]^-$; HREIMS *m/z* 666.3754 (M – CH_3, calcd for $C_{42}H_{52}NO_6$, 666.3795), 636.4037 (M – CO_2H, calcd for $C_{42}H_{54}NO_4$, 636.4053).

Δ^{23}-Nodulisporic acid A_4 (14): UV (MeOH) λ_{max} 212 (ϵ 27 250), 267 (25 415), 316 (sh), 429 (sh) nm; IR (ZnSe) ν_{max} 3376, 2930, 1686, 1445, 1376, 1257, 1134, 1027 cm^{-1}; ^1H NMR data, Table 5; ESIMS *m/z* 664 $[M + H]^+$.

Δ^{23}-Nodulisporic Acid A_4 (14) and Δ^{23}-Nodulisporic acid C_4 (15). The fermentation broth (2 L) of another mutant culture grown using conditions described above was filtered, and the cells were extracted with MEK (500 mL) and concentrated under reduced pressure to give 1.75 g of residue. A portion (0.9 g) of the residue was purified on a silica gel flash column (50 g) using a step gradient of 5, 10, and 15% of CH_3OH in CH_2Cl_2. Fractions eluting with 10% CH_3OH produced yellow crystals of Δ^{23}-nodulisporic acid C_4, **15** (10 mg).

Another 100 mg portion of the crude residue was purified on a reversed-phase HPLC (Zorbax SB-CN, 22.4 × 250 mm) eluting with 50% aqueous CH_3CN + 0.1% TFA at a flow rate of 8 mL/min. The fractions eluting at 38–42 min were combined, concentrated to dryness, and rechromatographed on a Zorbax SB-CN (9.4 × 250 mm) column eluting with 50% aqueous CH_3CN + 0.1% TFA at a flow rate of 3 mL/min, affording 2 mg of Δ^{23}-nodulisporic acid A_4 (**14**) as a yellow powder.

Δ^{23}-Nodulisporic acid C_4 (15): $[\alpha]^{23}_{578}$ +26.2° (*c* 2.33, MeOH); UV (CH_3CN + H_2O + 0.1% TFA) λ_{max} 212, 284, 306 (sh), 420 (sh) nm; IR (ZnSe) ν_{max} 3356, 2971, 2928, 1686, 1643, 1452, 1375, 1255, 1222, 1134, 1072, 1025, 986, 740 cm^{-1}; ^1H and ^{13}C NMR data, Table 5; ESIMS *m/z* 652 $[M + H]^+$, 650 $[M - H]^+$.

Compound 16. To a solution of **7a** (1 mg) in $CDCl_3$ (150 uL) was added 10 μL of TFA. Product **16** was exclusively formed and was characterized without purification. ^1H NMR ($CDCl_3$+CF_3CO_2D, 15:1), only distinct signals are listed, δ 7.74 (1H, brs, H-26), 7.44 (1H, s, H-16), 7.42 (1H, d, *J* = 11 Hz, H-3″), 6.50 (1H, dd, *J* = 15, 11 Hz, H-2″), 5.99 (1H, d, *J* = 15 Hz, H-1″), 3.79 (1H, m, H-7), 3.54 (2H, s, H_2-24), 3.03 (1H, m, H-12), 2.76 (1H, m, H-13), 2.61 (1H, m, H-13), 2.60 (2H, brs, H_2-19), 2.02 (3H, s, H-5″), 1.613 (3H, s), 1.608 (3H, s), 1.50 (3H, s), 1.48 (3H, s), 1.31 (3H, s), 1.18 (3H, s); HREIMS *m/z* 583.3677 (calcd for $C_{38}H_{49}NO_4$, 583.3662).

(*R*)-MTPA Ester of Nodulisporic Acid D_2-Methyl Ester (9c). To a 1.4 mg solution of **9b** in 0.2 mL of CH_2Cl_2 was added 1 mg of DMAP and 0.050 mL of DIPEA followed by 0.050 mL of a 200 mg/mL solution of (*S*)-MTPA chloride. The solution was stirred at room temperature for 2 h, and the reaction product was directly purified by a reversed-phase HPLC (Zorbax RX C-8, 9.4 × 250 mm) using a 38 min linear gradient of 40–90% aqueous CH_3CN at a flow rate of 4 mL/min that eluted the product between 43 and 44 min, which was lyophilized to give 1.5 mg of (*R*)-MTPA ester as an amorphous powder: ^1H NMR δ (only key resonances are listed) 5.58 (1H, dd, *J* = 4, 9 Hz, H-3″), 4.19 (1H, q, *J* = 9 Hz, H-2″), 2.97 (1H, m, 4″), 2.10 (1H, dd, *J* = 12, 7.5 Hz, H-1″), 1.72 (1H, dd, *J* = 13, 9 Hz, H-1″), 1.10 (3H, d, *J* = 6.8 Hz, H-6″); ESIMS *m/z* 870 $[M + Na]^+$.

(*S*)-MTPA Ester of Nodulisporic Acid D_2-Methyl Ester (9d). The *S*-ester **9d** was prepared and purified similar to the *R*-ester by reaction with (*R*)-MTPA chloride. ^1H NMR δ (only key resonances are listed) 5.55 (1H, dd, *J* = 4, 9 Hz, H-3″), 4.14 (1H, q, *J* = 9 Hz, H-2″), 3.03 (1H, m, 4″), 1.87 (1H, dd, *J* = 12.5, 7 Hz, H-1″), 1.57 (1H, dd, *J* = 12, 9 Hz, H-1″), 1.26 (3H, d, *J* = 7 Hz, H-6″); ESIMS *m/z* 870 $[M + Na]^+$.

***Ex-Vivo* Flea Assay.** For this ex-vivo assay, 20–25 cat fleas (*Ctenocephalides felis*) were used at each titration level. Compounds were dissolved/suspended in bovine blood. The LD_{90} was defined as the concentration that killed >90% fleas. Design of the artificial feeding apparatus has been reported by Pullen et al.[29]

Nodulisporic Acid Binding Assay. Compounds were dissolved in DMSO at 20 mM and were tested using the [^{35}S]-nodulisporic amide binding assay as previously described.[6] Briefly, compounds were tested from 10 μM to 10 pM (0.1 pM for potent compounds) in 0.5 log steps in triplicate. Ligand concentration was 10 pM, and cited values were IC_{50} values. Every compound was assayed two or more times, and geometric means for the IC_{50} values are reported. For compounds that increased the recovery of [^{35}S]-nodulisporic amide, presumably by coprecipitating with the label, the highest concentration at which no "precipitation" was observed is given.

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Supporting Information Available: ^1H NMR spectra of compounds **7a**, **7b**, **8a**, **8b**, **9a**–**9d**, **10**, **11a**, **11b**, and **12**–**15** and ^{13}C spectra of **7a**, **8a**, **9b**, **10**, **11a**, **11b**, **12**, and **13**; and extended Figure 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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