Nodulisporic Acids C, C_1, and C_2: A Series of D-Ring-Opened Nodulisporic Acids from the Fungus *Nodulisporium* sp.

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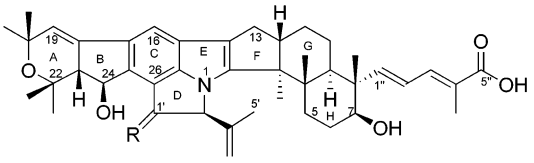
Nodulisporic acids C (**4a**), C_1 (**5a**), and C_2 (**6a**), a series of D-ring-opened nodulisporic acids, were isolated from fermentations of a mutant culture of *Nodulisporium* sp. MF5954. Nodulisporic acid C, the most potent of the three, showed good activity against fleas with an LD_{90} of 10 μg/mL. These compounds tend to be unstable in the free acid form and were isolated as stable sodium salts.

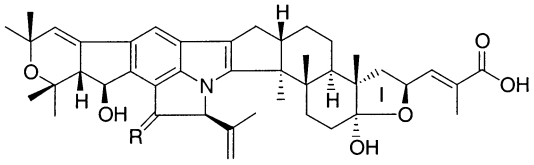
Ectoparasites such as fleas cause significant health hazards to companion animals, and because of the close association between companion animals and humans, they can also cause significant health hazards to humans by either infesting them directly or transmitting viral or bacterial pathogens to them. Although there are numerous insecticides that are currently in the market to control fleas, development of resistance is an issue that makes continuous 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 significant environmental problems. Therefore, new systemic drugs, preferably with new mechanisms of action, are needed to counter both of these problems.

In the past few years we reported on the discovery of nodulisporic acid A (**1a**)[1] and its congeners A_1 (**2a**) and A_2 (**3a**)[2] from an endophytic fungus, *Nodulisporium* sp. MF5954, as potent insecticidal agents.[3,4] Nodulisporic acid A is reported to be an effective systemic ectoparasiticidal agent against fleas on dogs with no apparent mammalian toxicity.[3] Nodulisporic acid A modulates the glutamate-gated chloride channel in insects similar to avermectins and milbemycins, but has no effect on helminths.[5] These channels are present only in insects, and thus compounds active against these channels should show no mammalian toxicity.

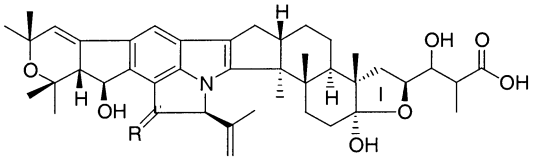
During large-scale isolation of nodulisporic acid A, prepared for purposes of a medicinal chemistry effort aimed at improving potency and duration of action, a number of related compounds were isolated by the targeted chemical and analytical screening of various extracts. From these studies we recently reported[6] the discovery of a series of 1′-deoxy nodulisporic acid derivatives, namely, nodulisporic acids B (**1b**), B_1 (**2b**), and B_2 (**3b**). Continued screening led to the isolation of the D-ring-opened nodulisporic acid congeners systematically named herein nodulisporic acids C (**4a**), C_1 (**5a**), and C_2 (**6a**). The isolation, structure elucidation, and biological activities of these compounds are described herein.



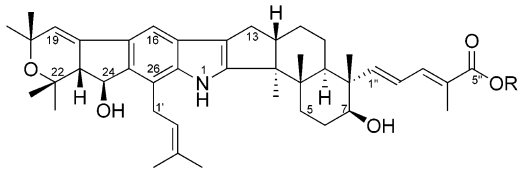
1a: R = O, Nod acid A
1b: R = H_2, Nod acid B



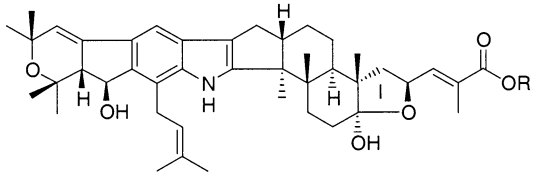
2a: R = O, Nod acid A_1
2b: R = H_2, Nod acid B_1



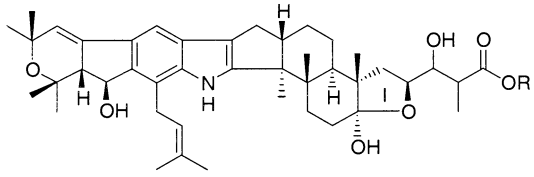
3a: R = O, Nod acid A_2
3b: R = H_2, Nod acid B_2



4a: R = H, Nod acid C
4b: R = Na



5a: R = H, Nod acid C_1
5b: R = Na



6a: R = H, Nod acid C_2
6b: R = Na

Methyl ethyl ketone extract of *Nodulisporium* sp. MF6222 (ATCC74383) was processed as described for the isolation of nodulisporic acid A[1]. Dehydration of the C-24 hydroxy

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Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Assignments of Nodulisporic Acids C (**4b**), C_1 (**5b**), and C_2 (**6b**) in Acetone-d_6

	4b		**5b**		**6b**	
carbon	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
2	153.0		153.0		153.1	
3	54.3		53.7		53.7	
4	39.7		40.1		40.0	
5	33.4	1.85 (m)	30.5	1.90 (m, 2H)	30.5	1.90 (m, 2H)
6	27.6	1.78 (m), 1.81 (m)	27.9	1.75 (m, 2H)	27.9	1.75 (m, 2H)
7	76.9	3.45 (dd, J = 4.5, 10.5)	106.6		105.7	
8	47.9		49.7		49.6	
9	45.0	1.75 (m)	41.1	1.90 (m)	41.5	1.80 (m)
10	25.4	1.46 m, 2H)	25.6	1.58 (m, 2H)	25.5	1.75 (m), 1.9 (m)
11	26.0	1.60 (m, 2H)	26.4	1.65 (m, 2H)	26.4	1.50 (m), 1.75 (m)
12	49.7	2.75 (m)	50.0	2.80 (m)	50.0	2.80 (m)
13	27.9	2.25 (dd, J = 10.5, 13.0)	28.0	2.28 (brt, J = 13.0)	28.0	2.30 (m)
		2.60 (dd, J = 6.5, 13.0)		2.62 (dd, J = 6.4, 13.0)		2.60 (m)
14	118.8		118.8		118.7	
15	127.4		127.4		127.3	
16	107.6	7.31 (s)	107.5	7.31 (s)	107.5	7.30 (s)
17	132.2		132.2		132.2	
18	137.2		137.2		137.2	
19	119.8	5.96 (d, J = 3.0)	119.7	5.95 (d, J = 6.0)	119.7	5.94 (brs)
20	72.7		72.7		72.7	
22	74.2		74.2		74.2	
23	60.7	2.70 (dd, J = 3.0, 6.0)	60.7	2.68 (dd, J = 3.0, 6.0)	60.6	2.68 (brs)
24	76.5	5.00 (d, J = 6.0)	76.5	5.02 (d, J = 6.0)	76.3	5.05 (brs)
25	137.1		137.2		137.5	
26	123.3		123.2		123.2	
27	142.1		142.1		142.0	
28	14.7	1.05 (s)	14.9	1.02 (s)	14.9	1.00 (s)
29	19.4	1.13 (s)	17.7	1.15 (s)	17.7	1.12 (s)
30	11.7	1.07 (s)	17.0	1.06 (s)	17.4	1.05 (s)
31	30.3	1.26 (s)	30.4	1.27 (s)	30.4	1.26 (s)
32	32.3	1.27 (s)	32.4	1.25 (s)	32.4	1.25 (s)
33	23.1	1.00 (s)	23.1	1.00 (s)	23.1	1.00 (s)
34	30.3	1.40 (s)	30.4	1.40 (s)	30.4	1.40 (s)
1′	26.6	3.70 (dd, J = 6.0, 15.0)	26.4	3.70 (dd, J = 6.0, 15.0)	26.4	3.70 (brm)
		4.00 (dd, J = 8.0, 15.0)		4.00 (dd, J = 8.0, 15.0)		4.00 (brm)
2′	124.4	5.30 (t, J = 6.0, 8.0)	124.4	5.30 (m)	124.4	5.29 (m)
3′	131.7		132.2		131.1	
4′	25.4	1.80 (s)	26.4	1.82 (s)	25.8	1.82 (s)
5′	18.1	1.67 (s)	18.1	1.67 (s)	18.1	1.66 (s)
1″	155.2	5.96 (d, J = 15.0)	45.0	1.73 (m), 2.34 (m)	31.3	1.60 (m), 2.0 (m)
2″	125.6	6.38 (dd, J = 11.5, 15.0)	74.2	4.94 (m)	78.4	4.32 (m)
3″	140.1	7.22 (d, J = 11.5)	146.0	6.90 (brd)	74.6	3.85 (m)
4″	125.4		123.2		44.5	2.35 (m)
5″	170.0		170.5		174.8	
6″	12.7	1.90 (d, J = 1.2)	12.7	1.82 (d, J = 1.3)	12.5	1.16 (brs)
NH		9.00 (brs)		9.00 (brs)		9.00 (brs)

group occurred during the standard isolation procedure of the C-series nodulisporic acids resulting in Δ^{23} derivatives, as had been observed during the purification of the nodulisporic acid B series.[6] Therefore, the modified isolation procedure that was used for the isolation of nodulisporic acid B was also adopted for the isolation of these compounds, as described in the Experimental Section.

HRESI-FTMS of compound **4b** indicated a molecular formula of $C_{43}H_{57}NO_5$, which was supported by the ^{1}H and ^{13}C NMR spectra. The UV spectrum of **4b** was different from that of nodulisporic acid A (**1a**)[1] and similar to that of nodulisporic acid B (**1b**).[6] It showed absorption bands at λ_{max} 262, 267, 316, and 336 nm. The comparison of ^{1}H and ^{13}C NMR spectra of nodulisporic acid A (**1a**), the sodium salt of B (**1b**), and **4b** indicated the absence of the C-1′ carbonyl carbon and the 4′-vinyl protons and the presence of two new vinyl methyl singlets at δ 1.67 and 1.80 and a olefinic methine triplet at δ 5.30 (δ_C = 124.40) in the spectrum of **4b**. The two new methyl signals correlated to δ 18.10 and 25.40, respectively, in the HMQC spectrum of **4b** (Table 1). The olefinic methine proton correlated to the methylene protons at δ 3.7 and 4.0 in the COSY spectrum. Compound **4b** also showed a free NH signal at δ 8.95 in the ^{1}H NMR spectrum recorded either in CD_2Cl_2 or in acetone-d_6. This indicated the absence of the C3′−N1 bond and the dihydropyrrole ring. Thus, nodulisporic acid C (**4a**) was elucidated as the dihydropyrrole ring-opened analogue of nodulisporic acid B with an isopentenyl group at C-26. Comparison of the ^{1}H and ^{13}C NMR spectra of **4b** to **1a** and **1b** suggested that it had identical stereochemistry.

The structures of nodulisporic acids C_1 (**5a**) and C_2 (**6a**) were elucidated by analogous comparison of ^{1}H NMR, ^{13}C NMR, and mass spectral data of the corresponding sodium salts **5b** and **6b** with the corresponding data of nodulisporic acids A_1, A_2 and B_1, B_2. Molecular formulas of $C_{43}H_{57}NO_6$ and $C_{43}H_{59}NO_7$ were determined for **5b** and **6b**, respectively, by HRESI-FTMS. Like nodulisporic acid C, the ^{1}H and ^{13}C NMR spectra (Table 1) of both of these two compounds showed the presence of an isopentenyl group. The NMR spectral data confirmed the presence of the hemiketal-containing furan ring side chain (ring I) as well as stereochemistry similar to acids A_1 (**2a**) A_2 (**3a**) and B_1 (**2b**) B_2 (**3b**), therefore establishing the structures for **5a** and **6a** as nodulisporic acids C_1 and C_2, respectively.

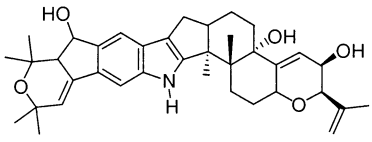
Table 2. Biological Activities of the Nodulisporic Acids and Other Insecticides

compound	LD_{90} *A. aegypti*, ng/mL	LD_{90} *L. sericata*, ng/mL	LD_{90} flea, μg/mL
nodulisporic acid A (**1a**)	500	300	1
nodulisporic acid A_1 (**2a**)	200	300	5
nodulisporic acid A_2 (**3a**)	800	600	10
nodulisporic acid C (**4b**)	10 000	10 500	10
nodulisporic acid C_1 (**5b**)	NT[a]	NT	>100
nodulisporic acid C_2 (**6b**)	NT	NT	>100
paraherquamide	50 000	50 000	>100
ivermectin	5	40	10

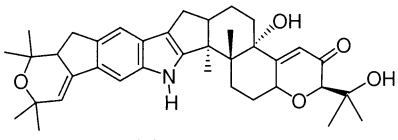
[a] NT (not tested).

The stable sodium salts of nodulisporic acids C (**4b**), C_1 (**5b**), and C_2 (**6b**) were tested in an ex-vivo flea (*Ctenocephalides felis*) artificial membrane feeding assay.[3,7] The data are compared with the A-series of nodulisporic acids and ivermectin and are shown in Table 2. In addition, these compounds were also evaluated for their ability to kill various other insects such as mosquito larvae (*Aedes aegypti*)[8] and blowfly larvae (*Lucilia sericata*).[9] Paraherquamide,[10] an anthelmintic agent, and ivermectin,[11] a commercially used anthelmintic and insecticidal agent, were also evaluated in these assays for comparison. Nodulisporic acid C (**5a**) was 10-fold less active than nodulisporic acid A, exhibiting an LD_{90} of 10 μg/mL against fleas, and was as active as nodulisporic acid A_2 and ivermectin. The sodium salts of nodulisporic acids C_1 (**5b**) and C_2 (**6b**) were not active in the flea assay up to 100 μg/mL. Nodulisporic acid A_1 was the most potent (LD_{90} = 200 ng/mL) in the mosquito larvae assay, while nodulisporic acid C was significantly less active (LD_{90} = 10 000 ng/mL) in this assay. Both nodulisporic acids A and A_1 were equipotent in the blowfly larvae assay, exhibiting LD_{90} values of 300 ng/mL, and were less active than ivermectin in this assay. Similar to the mosquito larvae assay, nodulisporic acid C was significantly less active in the blowfly larvae assay. Paraherquamide was found to be uniformly less active than nodulisporic acids in all three assays. Ivermectin is very potent against blowfly and mosquito larvae; however it is 10-fold less effective in killing fleas when compared with nodulisporic acid A.

Nodulisporic acids resemble other insecticidal indole diterpenes such as the janthitrems,[12] shearinines,[13] and lolitrems.[14] The resemblance is all the more striking with the C-series of compounds.



Janthitrem B



Shearinine B

In conclusion, we have reported here three new natural members of the nodulisporic acid family without the dihydropyrrole ring. Nodulisporic acid C is only 10-fold less active than nodulisporic acid A in the flea assay, whereas C_1 and C_2 are more than 100-fold less active, implying the importance of the side chain for the biological activity. Like the B-series of nodulisporic acids, the C-series were also unstable and showed dehydration tendencies to Δ^{23} derivatives. However, the rate of the dehydration was somewhat slower. The C-series could be potential biosynthetic precursors of the nodulisporic acid A series via a potential intermediacy of the B-series of nodulisporic acids.

Experimental Section

General Experimental Procedures. Optical rotation was determined on a Perkin-Elmer 241 polarimeter. The IR spectrum was recorded on a Perkin-Elmer Spectrum One spectrometer. A Hewlett-Packard HP1100 was used for analytical HPLC. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a Varian Unity 500 NMR spectrometer using solvent peaks as a reference standard. MS spectra were measured on a Thermo Quest HRFTMS. UV/vis spectra were recorded on a Beckman DU 70 spectrometer.

Fungal Isolation of Culture. *Nodulisporium* sp. MF6222 (ATCC 74383) was derived from mutations of MF5954, 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 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 a nutrient 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 water at pH 7.0 at 29 °C.

Isolation of Nodulisporic Acids C (4b), C_1 (5b), and C_2 (6b). A 1 L fermentation broth of ATCC 74383 was diluted with an equal volume of MeOH and stirred for 0.5 h and filtered through filter aid. This solution was brought up to pH 9.0 with the addition of an aqueous solution of $NaHCO_3$, charged to a 200 cm^3 SP207 column, and eluted with a step gradient of 2 column volumes (cv) each from 40 to 100% MeOH. The fractions eluting with 100% MeOH (2 cv) contained the nodulisporic acids. These fractions were concentrated under reduced pressure and lyophilized to a pale yellow solid. A portion of this solid was dissolved in MeOH and chromatographed by preparative HPLC (Zorbax RX C-8; 22 × 250 mm) with a gradient from 60 to 90% aqueous CH_3CN and a flow rate of 8 mL/min at neutral pH to yield sodium salts of nodulisporic acids C (14 mg, 42 mg/L), C_1 (15 mg, 45 mg/L), and C_2 (8 mg, 24 mg/L) as off-white powders.

Nodulisporic acid C (4b): $[\alpha]^{23}_D$ −42.5° (*c*, 0.4, MeOH); UV (MeOH) λ_{max} 262 (ϵ 72,400), 267 (78,800), 316 (7240), 336 (6460) nm; IR (ZnSe) ν_{max} 3442, 2973, 2903, 1681, 1454, 1376, 1282, 1248, 1154, 1075, 1027, 1002, 834 cm^{-1}; ^{1}H and ^{13}C NMR data are shown in Table 1; HRESI-FTMS *m*/*z* 668.4321 (calcd for $C_{43}H_{57}NO_5$ + H, 668.4315).

Nodulisporic acid C_1 (5b): $[\alpha]^{23}_D$ −30° (*c*, 0.4, MeOH); UV (MeOH) λ_{max} 260 (ϵ 51,980), 266 (57,300), 316 (6830), 336 (6,400) nm; IR (ZnSe) ν_{max} 3378, 2975, 2889, 1677, 1452, 1376, 1251, 1153, 1135, 1077, 1020, 979 cm^{-1}; ^{1}H and ^{13}C NMR data are shown in Table 1; HRESI-FTMS *m*/*z* 684.4260 (calcd for $C_{43}H_{57}NO_6$ + H, 684.4264).

Nodulisporic acid C_2 (6b): $[\alpha]^{23}_D$ −42.5° (*c*, 0.4, MeOH); UV (MeOH) λ_{max} 262 (ϵ 56,560), 266 (61,100), 316 (7240), 336 (6340) nm; IR (ZnSe) ν_{max} 3446, 3000, 2882, 2838, 1697, 1572, 1437, 1376, 1288, 1247, 1134, 1027, 981, 736 cm^{-1}; ^{1}H and ^{13}C NMR data are shown in Table 1; HRESI-FTMS *m*/*z* 702.4370 (calcd for $C_{43}H_{59}NO_7$ + H, 702.4356).

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Supporting Information Available: ^{1}H, ^{13}C, and COSY NMR spectra for compounds **4b**, **5b**, and **6b**. HMBC spectrum for compound

4b. UV spectra for compounds **4b**, **5b**, and **6b**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Ondeyka, J. G.; Helms, G. L.; Hensens, O. D.; Goetz, M. A.; Zink, D. L.; Tsipouras, A.; Shoop, W. L.; Slayton, L.; Dmbrowski, A. W.; Polishook, J. D.; Ostlind, D. A.; Tsou, N. N.; Ball, R. G.; Singh, S. B. *J. Am. Chem. Soc.* **1997**, *119*, 8809−8816.
(2) Hensens, O. D.; Ondeyka, J. G.; Dombrowski, A. W.; Ostlind, D. A.; Zink, D. L. *Tetrahedron Lett.* **1999**, *40*, 5455−5458.
(3) Shoop, W. L.; Gregory, L. M.; Zakson-Aiken, M.; Michael, B. F.; Haines, H. W.; Ondeyka, J. G.; Meinke, P. T.; Schmatz, D. M. *J. Parasitol.* **2001**, *87*, 419−423.
(4) Ostlind, D. A.; Felcetto, T.; Misura, A.; Ondeyka, J. G.; Smith, S.; Goetz, M.; Shoop, W.; Mickle, W. *Med. Vet. Entomol.* **1997**, *11*, 407−408.
(5) Smith, M. M.; Warren, V. A.; Thomas, B. S.; Brochu, R. M.; Ertel, E. A.; Rohrer, S.; Schaeffer, J.; Schmatz, D.; Petuch, B. R.; Tang, Y. S.; Meinke, P. T.; Kaczorowski, G. J.; Cohen, C. J. *Biochemistry* **2000**, *39*, 5543−5554.
(6) Ondeyka, J. G.; Dahl-Roshak, A. M.; Tkacz, J. S.; Zink, D. L.; Zakson-Aiken, M.; Shoop, W. L.; Goetz, M. A.; Singh, S. B. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2941−2944.
(7) 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. For design of the artificial feeding apparatus see, for example: Pullen, S. R.; Meola, R. W. *Lab Animal* **1996**, *25*, 39−40.
(8) Henrick, C. A.; Staal, G. B.; Siddall, J. B. *J. Agric. Food Chem.* **1973**, *213*, 354−359.
(9) Boisvenue, R. J.; O'Doherty, G. O. P. *J. Econ. Entomol.* **1989**, *82*, 1843−1845.
(10) Ondeyka, J. G.; Goegelman, R. T.; Schaeffer, J. M.; Keleman, L.; Zitano, L. *J. Antibiot.* **1990**, *43*, 1375−1380.
(11) Chabala, J. C.; Mrozik, H.; Tolman, R. L.; Eskola, P.; Lusi, A.; Peterson, L. H.; Woods, M. F.; Fisher, M. H.; Campbell, W. C.; Egerton, J. R.; Ostlind, D. A. *J. Med. Chem.* **1980**, *23*, 1134−1136.
(12) de Jesus, A. E.; Steyn, P. S.; van Heerden, F. R.; Vleggaar, R. *J. Chem. Soc., Perkin Trans. 1* **1984**, 697−701.
(13) Belofsky, G. N.; Gloer, J. B.; Wicklow, D. T.; Dowd, P. F. *Tetrahedron* **1995**, *51*, 3959−3968.
(14) Munday-Finch, S. C.; Wilkens, A. C.; Miles, C. O.; Tomoda, H.; Omura, S. *J. Agric. Food Chem.* **1997**, *45*, 199−204.

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