Discovery of the Development Candidate *N-tert*-Butyl Nodulisporamide: A Safe and Efficacious Once Monthly Oral Agent for the Control of Fleas and Ticks on Companion Animals†

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Received October 22, 2008

Nodulisporic acid A (**1**) is a structurally complex fungal metabolite that exhibits systemic efficacy against fleas via modulation of an invertebrate specific glutamate-gated ion channel. In order to identify a nodulisporamide suitable for monthly oral dosing in dogs, a library of 335 nodulisporamides was examined in an artificial flea feeding system for intrinsic systemic potency as well as in a mouse/bedbug assay for systemic efficacy and safety. A cohort of 66 nodulisporamides were selected for evaluation in a dog/flea model; pharmacokinetic analysis correlated plasma levels with flea efficacy. These efforts resulted in the identification of the development candidate *N-tert*-butyl nodulisporamide (**3**) as a potent and efficacious once monthly oral agent for the control of fleas and ticks on dogs and cats which was directly compared to the topical agents fipronil and imidacloprid, with favorable results obtained. Multidose studies over 3 months confirmed the in vivo ectoparasiticidal efficacy and established that **3** lacked overt mammalian toxicity. Tissue distribution studies in mice using [^{14}C]-labeled **3** indicate that adipose beds serve as ligand depots, contributing to the long terminal half-lives of these compounds.

Introduction

Infestations of blood sucking ectoparasites, such as fleas and ticks, have long plagued both humans and animals. To alleviate the morbidity and discomfort associated with these infestations, considerable resources have been directed at identifying safe and effective cidal agents.[1] In part, this focus reflects a need associated with prodigious levels of pet ownership; the American Veterinary Medical Association currently estimates that 2007 U.S. pet ownership exceeds 71 million dogs and 81 million cats, a substantial increase from 61 and 70 million dogs and cats, respectively, in 2001.[2] Over the years, a remarkable pharmacopeia of topical agents (mostly flea treatments) or to a much lesser extent oral agents (to date, with flea control only) were developed to meet these needs; these substances are extensively detailed elsewhere.[3−6] As blood feeding ectoparasites are efficient vectors of disease transmission (e.g., Lyme disease, ehrlichiosis, Rocky Mountain spotted fever, etc.) and sources of human allergens, effectively reducing flea and tick populations will confer ancillary benefits for humans.

In the context of the search for products with durable antiparasitic efficacy and strong potential for mammalian or environmental safety and to mitigate against the development of resistance, identification of novel antiparasitic agents continues unabated. While multiple topical ectoparasiticidal agents are available, including selamectin,[7,8] market share is currently governed by two main groups of ectoparasiticides: fipronil and imidacloprid.[9] Imidacloprid is a fast acting, topical spot-on agent which controls fleas for 1 month but lacks acaracidal activity. Fipronil, also topically applied, is more effective, controlling not only fleas for up to 3 months but also ticks for 4 weeks.

With respect to comparably efficacious and nontoxic oral and *systemically* active ectoparasiticides, the record is considerably more modest, as long-acting oral cidal agents with suitable efficacy and commensurate safety profiles remain elusive despite a preference among some consumers for orally administered agents.[6] In principal, the dosing afforded by orally ingested agents should ensure uniform coverage over a pet's body and its efficacy should not be mitigated by environmental exposure to rain or bathing while also eliminating the potential for contact exposure-based liabilities to humans. Ideally, an oral agent also should confer immediate results, expeditiously killing both fleas and ticks, thereby protecting against new infestations by preventing oviposition or shedding of larvae, with reduced propensity for flea or tick allergic dermatitis as sequelae. These requirements, coupled with concerns associated with the potential for resistance development to existing agents and the desire to identify novel agents with benign mammalian and environmental profiles, further spur drug discovery efforts. In late 2007, however, a preliminary report appeared describing a promising new systemically active pulicide.[10] Spinosad, a mixture of spinosyns A and D, is a systemically active cidal agent with rapid onset of activity and the ability to confer 1 month's protection against fleas only (no tick claims) following a single oral dose.

The systemic ectoparasiticide activity of a natural product was discovered in a *Cimex lectularius* mouse assay.[31] Subsequently, a remarkable and structurally complex natural product was identified as nodulisporic acid A[11] (**1**, NsA A, Figure 1, R = OH), produced by the endophytic fungus *Nodulisporium* sp. (MF5954). It has the potential to usher in a new era in the treatment of ectoparasitic infestations for companion animals.[12] This fungal metabolite, despite close structural relationships to

† This manuscript is dedicated to the memory of Dr. Michael H. Fisher (1926−2005).

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[a] Abbreviations: BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; HOBT, *N*-1-hydroxybenzotriazole; HPLC, high pressure liquid chromatography; PK, pharmacokinetics; po, per os.

10.1021/jm801334v CCC: $40.75
Published on Web 05/15/2009

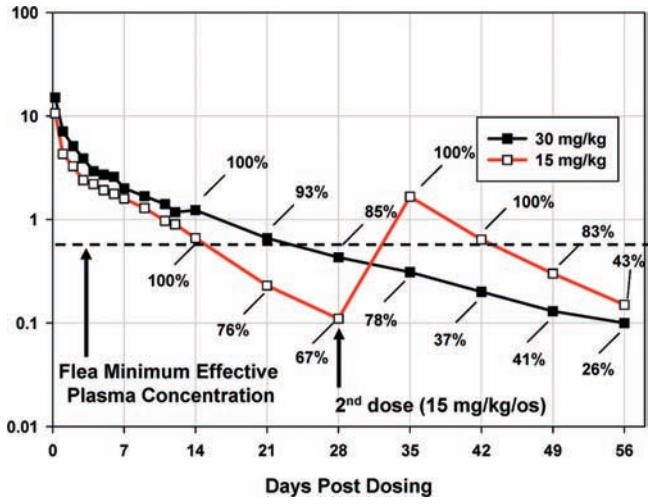


Figure 1. Plasma disposition profile and flea efficacy (percent control) of nodulisporamide **2** in dogs.

a large family of tremorgenic indole diterpenes,[13] exhibits unique properties: potent systemic efficacy against fleas on dogs with rapid onset of action while devoid of acute mammalian toxicity.[14] NsA A is the preeminent member of a larger cohort with multiple NsA fermentation congeners identified;[15−18] these natural products have broad worldwide distribution.[19] To date, none has shown biologically superior characteristics to those possessed by NsA A itself.

Mechanism of action investigations established that this indole diterpenoid modulates the function of a subset of the channels targeted by agents such as ivermectin: more specifically, invertebrate-specific glutamate-gated chloride ion channels.[20] These channels, for which no mammalian homologue exists, are vital to neurotransmission in insects, and their disruption results in paralysis, thereby providing a biological basis for NsA A's potent invertebrate toxicity without concomitant vertebrate liabilities. No activity of NsA A on ligand gated channels (GABA, kainite, glycine) or Na and K voltage gated channels was observed. These discoveries were of critical importance for any systemically long-acting agent, as plasma drug exposures will likely be considerable.

Because of their considerable structural complexity, this indole diterpene class of antiparasitic agents largely has resisted efforts at total synthesis. To date, only a diminutive member of the nodulisporanes, (+)-NsA F, has succumbed to the elegant and pioneering efforts of the Smith group,[21] extending their prior notable achievements on closely related indole alkaloids, including paspalline[22] and penetrum D.[23] Prodigious efforts directed toward total syntheses of more complex NsA family members continue to emerge unabated from the Smith laboratories.[24,25]

Preliminary medicinal chemistry efforts delineated that the core of NsA poorly tolerated structural modification, whereas dienoic acid side chain manipulations yielded derivatives (e.g., **2**) with improved in vitro[26] and in vivo efficacy.[27] Further research established that incorporating heterocyclic surrogates for the 3″,4″-olefin produced analogues that exhibited prolonged systemic activity against both fleas and ticks on dogs.[28] Despite these attractive qualities, however, simple economic imperatives due to synthetic complexity associated with heterocycle incorporation precluded the ready advancement of these derivatives into clinical development.

These present studies were directed toward the identification of a safe, economically viable nodulisporamide derivative with potent, systemic flea and tick cidal efficacy suitable for once monthly oral dosing. To satisfy key criteria for a once monthly agent using a clinically meaningful dosage, new NsA derivatives initially were evaluated for intrinsic systemic activity against the flea in vitro, then screened in a murine model using a blood sucking ectoparasite (bedbug) prior to testing suitable derivatives in dogs (against fleas and ticks) and in cats (against fleas only).

Results and Discussion

The animals were cared for in accordance with federal animal welfare regulations, and the studies were approved by the respective institutional animal care and use committees. All dogs and cats used in the studies described herein were in excellent health at the beginning of each study, and at no instance did any dog or cat show any sign of adverse reaction during the studies described herein.

A previously published flea efficacy study in dogs established that while *N*-(4-methoxy)benzyl nodulisporamide (**2**, Table 2) fully protected against flea infestations for approximately 2 weeks, it maintained residual efficacy for an additional 3 weeks.[14] Therefore, a 9 week flea efficacy study in dogs using **2** was initiated, and the goal was to compare the effect of a single higher dosage (30 mg/kg po[a]) on flea control versus two consecutive lower dosages (15 mg/kg po) punctuated by 28 days. The results of this pilot study are shown in Table 1 and Figure 1; efficacy was determined by challenging the dogs with 100 live fleas at various time points followed by removal and quantitation of any live fleas 48 h postchallenge. Consistent flea challenges were noted for the two vehicle-treated dogs held throughout the 56 day observation period. For the 15 mg/kg po dosing arm, the flea control response replicated with good fidelity the earlier report by Shoop et al.[14] More specifically, **2** conferred 100% flea control for the first 2 weeks after initial (or second) dosing, followed by progressively diminishing efficacy at weeks 3 and 4 after dosing; this efficacy profile was reproduced following the second oral dose as well. Minimal evidence of drug accumulation based on either flea efficacy or plasma pharmacokinetic analysis was detected following the second monthly dose. For the dog treated at 30 mg/kg po, statistically significant reductions relative to control occurred through week 5, although 100% control was only noted for the first 14 days with breakthrough commencing at day 21. At weeks 6 and 7 (days 42 and 49), some residual control of fleas was seen followed by complete loss of ectoparasite control at week 8. Simple modeling of pharmacokinetic disposition curves suggested that a projected monthly dose of **2** would be approximately 200 mg/kg po, a likely economically untenable dosage. Clearly, identification of an analogue with improved intrinsic potency and longer duration of action was integral to program success.

A modular program to prepare a library of nodulisporamides from which to identify an analogue suitable for once monthly dosing therefore was initiated. Briefly, newly prepared derivatives initially were evaluated for intrinsic systemic efficacy in the cat flea, *Ctenocephalides felis* (EC_{50} for lethality) in an artificial membrane in vitro feeding system.[29] Ultimately, approximately 335 newly prepared nodulisoramides were titrated in this assay against fleas. This artificial membrane feeding assay identified multiple analogues with useful intrinsic systemic efficacy comparable or superior to that observed for the parent natural product, NsA A. As evaluation of all potent compounds in dogs for systemic flea efficacy was impractical, a murine model for in vivo systemic activity was instituted as an intermediate screening model, reducing compound requirements

Table 1. Flea Efficacy of *N*-(4-Methoxy)benzyl Nodulisporamide (**2**) in Dogs at 15 or 30 mg/kg po

			no. of live fleas recovered[a] at indicated week postdose							
compd	dog no.	dose (mpk)	1	2	3	4	5	6	7	8
vehicle	1	0	48	51	56	73	56	31	37	44
vehicle	2	0	31	71	53	71	51	46	62	65
2	3	30[b]	0	0	4	11	12	24	29	40
2	4	15[c]	0	0	13	24	0	0	8	31

[a] Counts were done blindly. [b] Dosed at 30 mg/kg po on day 1. [c] Dosed at 15 mg/kg po on day 1 and day 28.

Table 2. Nodulisporamide Structures and Biological Activity Profiles

entry	R group	flea (LC_{50}, ppm)	bedbug (ED_{50}, mg/kg)
1	$-OH$	0.7	1.0
2	$-NHCH_2Ph(4\text{-}OCH_3)$	0.1	0.5
3	$-NHC(CH_3)_3$	0.1	0.125
4	$-NHC(CH_3)_2CC{\equiv}H$	0.1	0.5
5	$-NHCH(CH_3)_2$	1.0	0.5
6	$-NHCH_2C(CH_3){=}CH_2$	1.0	1.0
7	$-NHCH_2CF_3$	0.1	0.5
8	$-NHCH_2CH(CH_3)_2$	0.1	1.0
9	$-NHC(CH_3)_2CO_2CH_3$	1.0	0.25
10	$-NHC(CH_3)_2C(O)CH_3$	1.0	0.5
11	$-NHCH_2$-cyclopropyl	1.0	1.0
12	$-NHCH_2CH_2CF_3$	1.0	0.5
13	$-NHC(CH_3)_2C(OH)(CH_3)_2$	1.0	
14	$-NHCH(CH_2F)_2$	0.1	

and simultaneously minimizing assay cycle times. Analogues with suitably useful activity in the artificial membrane flea assay subsequently were screened using a rodent model against a blood sucking ectoparasite: the common bedbug, *Cimex lectularis*.[31] The *C. lectularis* assay served to identify not only intrinsic systemic efficacy (ED_{50} for efficacy) but also functioned as a potential preliminary filter for overt mammalian toxicity (none was observed).

Of the 335 analogues thus tested, approximately 66 semisynthetic analogues met the selection criteria of appropriate activity in both models (flea $LC_{50} \leq 1.0$ ppm, bedbug $ED_{50} \leq 1.0$ mg/kg) to merit resynthesis for flea efficacy studies and pharmacokinetic analysis in dogs. Flea systemic efficacy assay results, *C. lectularis* mouse assay data, and flea efficacy and half-life in dogs are provided for these 66 nodulisporamides (Supporting Information, Tables I−IV). Critically, testing numerous new nodulisporamides in dogs at potential monthly use levels was impractical because of multiple factors, including long in vivo assay cycle times as established for **2** (vide supra) and, equally significantly, because of severe limitations in parent NsA A availability. As a consequence, novel nodulisporamides initially were screened in dogs at 5 mg/kg po, and as superior analogues were identified, subsequent analogues were benchmarked at 2.5 mg/kg po dosages, both to spare material and further mitigate the long, requisite in vivo assay cycle time. These studies confirmed prior observations that the plasma levels of compound required for flea activity correlated well with intrinsic flea systemic activity and that plasma half-lives of the new compounds differed substantially from one another, indicating the potential for identifying a once monthly agent was viable. These preliminary results established that the optimal compounds bore small, aliphatic substituents on the 5″-amide. A combination of in vitro potency, in vivo efficacy, and terminal half-lives in dogs served to winnow the list of 66 nodulisporamides to more tractable cohorts that were resynthesized for head-to-head evaluation in dogs against fleas (*C. felis*) and ticks (*Dermacentor variabilis*) and in cats against fleas (*C. felis*) at a potentially economically viable once monthly use dose (10 mg/kg po); these promising amide analogues (**3**−**14**) are shown in Table 2.

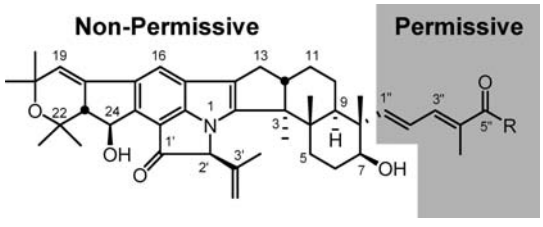


Figure 2. Permissive and nonpermissive regions of nodulisporic acid.

A dog flea and tick efficacy study of 8 weeks duration using 12 nodulisporamides (**3**−**14**) was initiated; all compounds were dosed once at a single oral dose of 10 mg/kg po (Table 3). Weekly flea challenges were continued until efficacy dropped below 60%. Again in this study, consistent flea challenges in the control dogs were observed. Repeated flea challenges (100 live unfed fleas/challenge) were made on the fifth day of each week postdosing with flea combing occurring 48 h later on the seventh day of each week for the 8 weeks of the study; an additional flea challenge was made on the 29th day following dosing with flea combing occurring on the 31st day postdosing. Excluding nodulisporamides **7** and **11**, for the first 2 weeks of this study, 100% efficacy against fleas was noted. By week 3, amides **10**, **11**, and **13** were exhibiting decreased effectiveness in their ability to control flea infestations with **7**, **9**, and **14** showing breakthrough in one dog each. At week 4, for compounds **7**, **9**, **10**, **11**, **13**, and **14**, at least one dog from each pairing was showing substantial lack of flea efficacy. No monthly flea challenges were made for dogs for which significant loss of flea efficacy was observed at week 4 (e.g., **9**, **10**, **11**, **13**, and **14**); one last confirmatory challenge was made at week 5 for each of these compounds, then further testing on these animals was eliminated. In general, an initial break in coverage for a given analogue was inevitably followed by a more precipitous loss of ectoparasite control in subsequent weeks. Remarkably for three derivatives (**3**, **4**, and **6**), at both 1 month and 5 weeks postdose, at least one dog was noted with 100% control of fleas. However, ectoparasite control for **12** failed at week 5 and at week 7 for **4**. Further examination of the data from weeks 6 and 7 established the clear superiority of **3** in controlling fleas with minimal breakthrough for 7 weeks postdose. Remarkably, only at week 8 was a significant loss of coverage observed for compound **3**.

Simultaneously, in the above dog 8-week flea efficacy study using nodulisporamides **3**−**14**, the ability to also control *Dermacentor variabilis* ticks was probed using weekly challenges (50 ticks per challenge) for the first 31 days. Immediately following dosing of the dogs with **3**−**14**, the first tick challenge was performed, with comb-counts performed 72 h later. Cognizant of typical feeding behavior for *D. variabilis* ticks, where blood meal ingestion routinely initiates by day 3 and terminates after engorgement on days 7−10 (Table 4), the comb-counts were made 72 h postchallenge. Because of concerns associated with potential for dog flea and/or tick allergy

Table 3. Comparison of 12 Nodulisporamides at 10 mg/kg po in Dogs Using Fleas and Ticks

				fleas on dogs[b,c] at indicated week									ticks on dogs[b,d] at indicated week			
compd ($n = 2$)	$T_{1/2}$ [a](day)	bedbug (mg/kg)	flea (ppm)	1	2	3	4	M*	5	6	7	8	0	1	2	M*
vehicle				43	47	42	47	71	67	46	54	49	2	11	10	27
				40	56	42	46	59	56	41	29	44	1	16	9	13
3	8.7	0.5	0.1	0	0	0	0	0	0	1	4	25	1	7	0	15
				0	0	0	0	0	0	0	0	13	0	9	1	15
4	8.4	0.5	0.1	0	0	0	0	2	7	30	42		0	2	0	25
				0	0	0	0	0	0	1	14		0	0	0	6
5	5.8	0.5	1.0	0	0	0	4	20	19	38			2	2	1	12
				0	0	0	1	10	18	42			0	2	1	21
6	6.0	1.0	1.0	0	0	0	7	23	22	39	35		0	3	0	13
				0	0	0	0	0	0	4	14		0	1	0	4
7	7.2	0.5	0.1	0	0	0	0	5	4	13			2	2	1	11
				0	1	5	25	36	28	52			4	15	3	18
8	5.9	0.1	0.1	0	0	0	3	5	5	15			0	3	0	27
				0	0	0	0	3	3	9			1	1	0	22
9	3.9	0.5	1.0	0	0		21		21				2	6	4	
				0	0	1	12		45				1	4	0	
10	2.6	0.5	1.0	0	0	23	29		61				4	14	16	
				0	0	31	44		73				2	15	12	
11	5.0	1.0	1.0	0	0	0	6		26				0	5	0	
				0	3	23	34		58				0	13	0	
12	4.0	0.5	1.0	0	0	0	0	4	15	15	38		2	4	0	18
				0	0	0	0	3	10	9	27		2	2	0	19
13	4.6		1.0	0	0	29	40		45				5	14	12	
				0	0	3	36		45				9	14	13	
14	4.6		1.0	0	0	5	22		28				0	2	0	
				0	0	0	20		20				1	0	0	

[a] Half-lives determined using days 4−21 data only; full pharmacokinetic data is provided (Supporting Information, Table V). [b] All counts were done blindly. M* = 1 month. [c] 100 (fleas/dog)/challenge. [d] 50 (ticks/dog)/challenge.

Table 4. *D. variabilis* Adult Female Tick Weights Days Postchallenge

day	weight (mg)
0	10
3	13
5	42
7−10	680

dermatitis, weeks 3 and 4 tick challenges were not performed. As the data in Table 3 illustrate, excluding the day 31 (M*) comb-count, recovery of live ticks on control dogs was modest relative to the numbers used in each challenge for reasons that remain unclear. Tick challenges for **9**−**11**, **13**, and **14** were terminated because of loss of flea control; the remaining test compounds displayed full tick efficacy at 2 weeks but failed to control the ticks at the end of the study.

A parallel flea efficacy study in cats of 4 weeks duration also was performed using nodulisporamides **3**−**14** (10 mg/kg po, 100 fleas per challenge, Table 5). These assays are more challenging, as cats typically exhibit superior grooming characteristics relative to dogs. All cats were in good health at the beginning of the study, and no adverse effects in any animal were noted at any juncture throughout the course of the study. Consistent flea counts were noted in the control cats; the lower numbers of recovered fleas reflect the superior grooming talents of cats relative to dogs. As in the previous dog studies, flea challenges were discontinued for analogues when loss of efficacy was noted. Generally speaking, nodulisporamides **3**−**14** manifested lesser parasite control in cats, yielding results rather distinct from prior dog studies. While all compounds exhibited robust flea control at week 1, several derivatives (**6**, **7**, **8**, **14**) lost appreciable efficacy at week 2, and excluding nodulisporamide **3**, essentially all failed to control fleas at week 3. Interestingly, nodulisporamide **3**, while in one animal efficacy was modest at week 2 and completely abated by week 3, the second cat showed 100% control of fleas to week 3. Little efficacy remained at week 4 for this derivative in this cat.

Table 5. Comparison of Flea Efficacy in Fed Cats for 12 Nodulisporamides (10 mg/kg po)

		fleas on cats at indicated week[b]			
compd ($n = 2$)	$T_{1/2}$ [a] (days)	1	2	3	4
vehicle		53	56	30	40
		56	61	52	36
3	6.6	0	2	0	19
		1	25	37	79
4	3.2	0	12	44	60
		0	9	12	43
5	4.4	1	6	30	
		0	9	54	
6	2.6	6	60	47	
		1	65	45	
7	6.2	2	17	34	
		0	16	39	
8	2.2	0	45	48	
		0	45	61	
9	4.2	0	0	32	
		0	0	43	
10	3.2	0	0	31	
		0	0	60	
11	4.0	0	3	18	
		0	1	58	
12	4.3	0	0	17	87
		0	0	20	41
13	3.8	0	0	8	25
		0	31	16	72
14	7.6	3	18	20	
		0	37	47	

[a] Half-lives determined using days 4−21 data only; full pharmacokinetic data is provided (Supporting Information, Table VI). [b] All counts were done blindly. 100 (fleas/cat)/challenge.

On the basis of the flea efficacy results from the head-to-head study of compounds **3**−**14** in dogs, clearly nodulisporamides **3**, **4**, **8**, and **12** were among the most efficacious agents. Incorporating the tick efficacy results from the same study failed to winnow the list further, as all but **10** and **13** had attractive tick activity at week 2. In addition, with the absence of potentially discriminating weeks 3 and 4 tick challenges, a broad

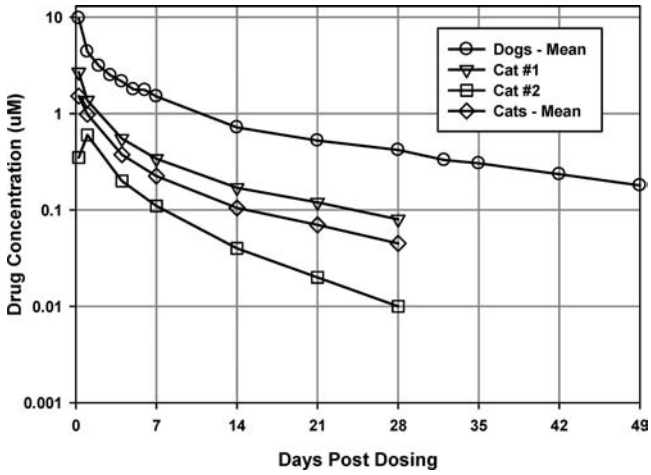


Figure 3. Comparison of plasma disposition profiles of **3** in dogs and cats.

loss of efficacy by day 31 (M*) made compound selection a challenge that was further exacerbated by the modest recovery of live *D. variabilis* ticks from control animals. Fortunately, however, when the additional results from the corresponding study in cats were assimilated, the aggregate data were sufficiently unambiguous that of the 12 compounds profiled only *N-tert*-butyl nodulisporamide (**3**) displayed the desired optimal balance of ectoparasitic activity in both dogs and cats.

A comparison of the PK profiles of **3** in both cats and dogs clarifies the origin of the reduced feline efficacy. In cats, the shorter $t_{1/2}$ (6.6 vs 8.7 days) and lower C_{max} (1.52 $\pm$ 1.65 vs 9.8 $\pm$ 0.28 μM) values contribute to the observed differential (Figure 3). It should be noted, however, that the cat for which the greatest flea efficacy (100% at week 3) was measured was the animal exposed to the highest drug levels of **3**. Overall, the plasma disposition curves for **3** are similar in dogs and cats. In addition, preliminary data suggest that the oral bioavailability in cats is similar to that of dogs (75−85%) and independent of dose. Plasma concentrations of the compound in both species again correlate well with observed flea efficacy with levels of 0.15−0.20 μg/mL being required for 100% flea control. Nodulisporamide **3** has multicompartment pharmacokinetics, best described with a triexponential equation, where the third exponential rate constant represents the "apparent" elimination phase. The first two exponential terms presumably represent a rapid and a slow distribution sequence, suggesting that the majority of the compound is partitioned into both highly and poorly perfused tissues. The "apparent" elimination half-life of **3** is ~19 days. At the proposed doses, plasma levels are low enough at 30 days so that no significant drug accumulation is anticipated following repeated monthly treatment. These results also indicate that minimal metabolism occurs.

As a consequence of the above conclusion, sufficient **3** was resynthesized to support multiple efficacy studies to more completely profile the qualities of this remarkable material. First, a larger confirmatory study to evaluate the efficacy of **3** against fleas on dogs was initiated (Table 6). In this 9 week study, two single dosages of **3** were utilized: 5 and 10 mg/kg po ($n = 4$ per arm) to determine if a lower dosage would confer 1 month's protection against fleas and ticks. As before, no adverse effect of **3** in the dogs was detected in this study. Again, consistent live flea counts were seen throughout this study on the control animals. Remarkably, following a single oral dose of **3** at 10 mg/kg po, 100% flea control was observed for 6 weeks. Partial loss of efficacy was observed in weeks 7 and 8 and not until week 9 did loss of efficacy approach 50%. For the lower dosage of **3**, 100% and 98% flea control was noted at weeks 3 and 4, respectively. By weeks 5 and 6, this dosing arm produced diminished efficacy. The pharmacokinetic analysis of **3** from this study was consistent with the observation that excellent ectoparasite control is maintained until systemic plasma drug levels decrease to approximately $\leq$0.20 μg/mL. Large numbers of dead fleas were combed from the dogs in the first month of this study for both dosing arms of **3** and up to weeks 7 and 8 for the 10 mg/kg po arm, whereas all fleas from control dogs were viable. In addition, the hair coats of these treated dogs were largely free of flea feces until weeks 7 and 8 for this higher dosage. Only in the ninth week did fleas on these treated dogs begin to approach the size of those on control dogs, did females begin to survive, and did significant levels of flea feces begin to manifest in the dog hair coats.

Currently, fipronil and imidacloprid are the most popular topical agents marketed for companion animals. In order to rigorously assess the systemic ectoparasitic efficacy of **3** versus that of these two useful topical molecules, a direct comparison was made (Table 6). Fipronil and imidacloprid were applied as per their labeling to the backs of dogs ($n =$ 3 per arm), using their optimized commercial formulations at their recommended use levels. In this head-to-head study, the efficacy profile of the orally active nodulisporamide **3** compared favorably to the efficacy profiles of both topical agents. In this four arm study, only the 5 mg/kg po dosing arm for **3** exhibited less than 100% flea efficacy at 1 month postdosing. Breakthrough in parasite control occurred earliest for imidacloprid at week 5 and progressed further in week 6. Imidacloprid exhibited 100% efficacy at 1 month (M*), with breakthrough occurring in week 5 and further erosion of efficacy at week 6. The topical agent fipronil, on the other hand, controlled 100% of fleas at week 5, with >90% efficacy persisting to the end of the study at week 9. Nodulisporamide **3**, at 10 mg/kg po, conferred 100% control of fleas at week 6 and >90% flea control in weeks 7 and 8 before significant erosion of activity manifested itself at week 9.

In the confirmatory 9 week investigation described above, brown dog ticks (*Riphicephalus sanguineus*) also were included, as they are the most prevalent dog tick in the U.S. For each challenge, 50 ticks were placed on each dog, and 72 h later the dogs were combed to remove and count any remaining live ticks. To avoid compromising the flea study, tick challenges were interspersed between flea challenges. In this study, the 10 mg/kg po dose of **3** was 80%, 78%, and 41% efficacious at 12, 19, and 26 days, respectively. In contrast, fipronil was 91% and 77% effective at days 19 and 26, respectively. As imidacloprid lacks activity against ticks, it did not merit inclusion in this aspect of the investigation.

A corresponding dose confirmation study in cats using nodulisporamide **3** also was performed (Table 7). In recognition that less robust efficacy had been noted in the prior cat study, the utility of higher dosages (specifically, 15 and 20 mg/kg po) was probed in this larger study. No adverse event of **3** in any cat was noted during the course of this 6-week study. Also, consistent numbers of live fleas were recovered from the control animals throughout the course of this 6-week study. In this investigation, a single oral dose of 15 mg/kg po conferred 100% efficacy for 2 weeks, with breakthrough occurring at week 3 and progressive loss of efficacy evident in weeks 4, 5, and 6. At the higher dosage of 20 mg/kg po, however, 100% efficacy against fleas was observed at week 3, with 97% and 94% control at weeks 4 and 5, respectively. Only in the final, sixth week

Table 6. Dose Confirmatory Study in Dogs Using Nodulisporamide **3** against Fleas and *R. sanguineus* Ticks

fleas on dogs[a,b] at indicated week postdosing										*R. sanguineus* ticks[b,c] at indicated day			
1	2	3	4	M*	5	6	7	8	9	3	12	19	26
Vehicle ($n = 4$)													
33	60	51	54	55	71	41	61	62	56	27	12	19	23
64	47	54	61	71	61	54	62	62	59	21	14	17	15
70	44	55	34	74	51	53	63	83	58	18	2	11	25
48	40	40	57	61	40	45	44	35	35	13	6	11	11
3 (10 mg/kg po) ($n = 4$)													
0	0	0	0	0	0	0	2	1		0	2	4	11
0	0	0	0	0	0	0	0	0	26	0	3	0	10
0	0	0	0	0	0	0	4	7	15	0	2	5	17
0	0	0	0	0	0	0	0	1	11	0	0	4	6
3 (5 mg/kg po) ($n = 4$)													
0	0	1	0	1	5	15				0	8	29	
0	0	0	0	0	10	12				0	5	7	
0	0	0	1	0	1	0				0	8	6	
0	0	0	3	5	5	7				0	9	14	
Imidacloprid, Spot-On (2.5 mL, $n = 3$)													
1	0	0	1	0	6	21							
1	0	0	0	0	4	6							
0	0	0	0	0	2	3							
Fipronil Spray (3.3 Pumps/kg, $n = 3$)													
0	0	0	0	0	0	3	2					3	13
0	0	0	0	0	0	0	0	1	0			0	0
0	0	0	0	0	0	0	0	1	0			1	0

[a] Challenged with 100 fleas/dog 48 h prior to counting. M* = 1 month. [b] All counts were performed blindly. [c] Challenged with 50 ticks/dog 72 h prior to counting.

Table 7. Dose Confirmation Study in Fed Cats Using Nodulisporamide **3**

fleas on cats[a] at indicated week postdosing					
1	2	3	4	5	6
Vehicle ($n = 4$)					
26	20	27	34	31	20
31	38	37	36	39	29
36	44	39	37	53	29
52	43	56	51	38	60
3 (15 mg/kg po) ($n = 4$)					
0	0	1	14	15	14
0	0	1	1	6	15
0	0	0	2	10	41
0	0	5	19	18	6
3 (20 mg/kg po) ($n = 4$)					
0	0	0	1	0	10
0	0	0	2	0	3
0	0	0	1	4	6
0	0	0	0	5	14
Imidacloprid Spot-On ($n = 3$)					
0	1	0	1	2	0
0	0	0	0	1	1
0	0	0	1	0	1
Fipronil Spray ($n = 3$)					
0	0	0	1	0	1
0	0	0	0	5	6
0	0	0	0	8	16

[a] Challenged with 100 fleas at 48 h prior to counting. All counts were performed blindly.

did the efficacy for **3** decrease substantially, to 76%. As with the prior dog study (Table 6), until systemic plasma drug exposures diminished to 0.2−0.3 μg/mL (Supporting Information, Table X), full control of the blood-sucking fleas was observed. Both topical agents (fipronil and imidacloprid) showed excellent duration of action in cats, providing essentially full efficacy against fleas at week 4, with relatively modest loss of efficacy detected at weeks 5 and 6 for imidacloprid and more substantial breakthrough in flea control observed for fipronil.

The prior tick efficacy study (Table 6) using dogs dosed at 5 and 10 mg/kg po established that higher dosages of **3** were integral to achieve 1 month's coverage of ticks. As a consequence, a larger study was initiated using 30 mg/kg po dosages of **3**, and to further extend our understanding of the ability of **3** to control ticks, a second species, *Amblyoma americanum* (Lone Star ticks), also was introduced (Table 8). In this study, consistent numbers of both species of ticks were recovered from the control animals. At day 14, **3** conferred 100% protection from both species of ticks. Despite the higher dosage, breakthrough was seen against both tick species at days 21 (85% and 87% control) and 28 (84% and 92% control) for *R. sanguineus* and *A. americanum*, respectively, suggesting that robust tick efficacy is manifest only while plasma drug concentrations exceed 1 μg/mL levels (Supporting Information, Figure II). Flea efficacy also was determined for **3** at two time points in this study: days 61 and 90 postdosing (Table 8). The observed flea control was 100% and 23% at these respective time points. Systemic plasma exposures of **3** were ~0.17 μM on day 61 and ~0.05 μM on day 90, establishing that a single oral dose of **3** can confer >60 days, but not 90 days, of full protection against fleas (Supporting Information, Figure II).

Blood Sampling and Plasma Analysis. A significant concern with compounds with long half-lives is that the potential for accumulation subsequent to multiple dosing exists. Shown below in Figure 4 is the pharmacokinetic profile of nodulisporamide **3** dosed at 10 mg/kg po for 4 consecutive months. No flea challenges were performed in this study to minimize flea allergic dermatitis risk to the control animals. From this semilog plot, it is apparent that consistent mean exposures and C_{max} and C_{trough} levels were attained between animals for each dosage across the 4 month dosing window, suggesting minimal risk of accumulation of drug in a chronic dosing setting. Again, no signs of behavioral changes or toxicity were noted in the animals throughout the duration of this study.

As part of the routine characterization of potential development candidates, nodulisporamide **3** was tested in a battery of

Table 8. Efficacy of Nodulisporamide **3** in Dogs Orally at 30 mg/kg po against Fleas and Ticks

R. sanguineus[a,b] on indicated day						*A. americanum*[a,b] on indicated day						fleas[b,c] on indicated day	
3	7	14	21	28	32	3	7	14	21	28	32	61	90
Vehicle ($n = 4$)													
27	10	10	18	20	20	24	25	22	26	39	22	69	79
21	27	14	16	25	11	36	29	27	24	44	26	80	72
20	14	15	20	23	27	29	25	28	16	46	20	77	90
27	25	11	26	35	23	26	20	11	30	25	33	81	90
3 ($n = 4$)													
0	0	0	3	2	7	0	0	0	4	2	7	0	77
0	0	0	0	5	4	0	0	0	2	8	12	0	63
0	0	0	3	6	8	0	0	0	3	3	20	0	96
0	0	0	6	3	9	0	0	0	3	0	33	0	34

[a] Challenged with 50 ticks of each species per dog at 72 h prior to counting. [b] All counts were performed blindly. [c] Challenged with 100 fleas per dog 48 h prior to counting.

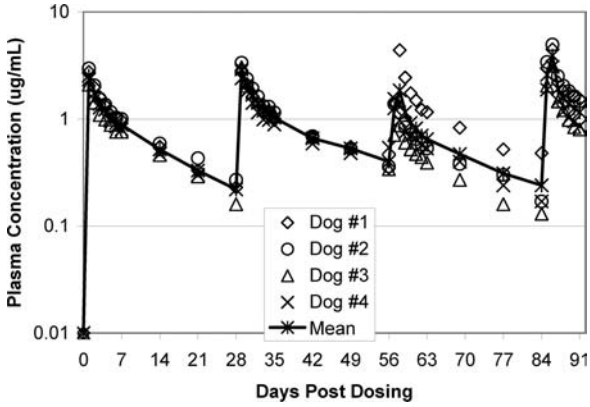


Figure 4. Pharmacokinetic profile of **3**: multiple monthly dosing at 10 mg/kg po (91 day study).

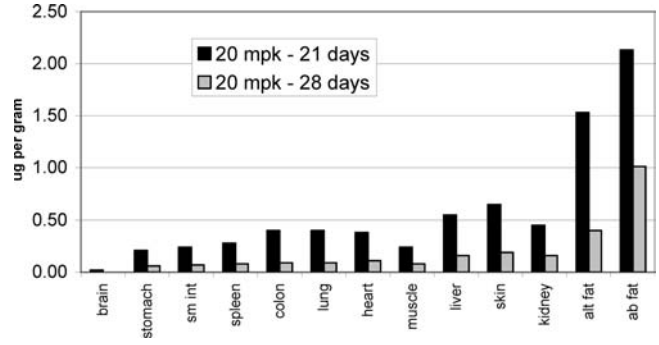


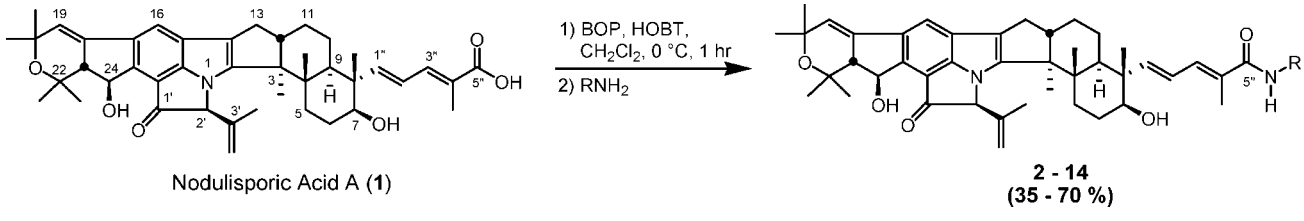
Figure 5. Tissue distribution of [^{14}C]-*N-tert*-butyl nodulisporamide (**3**) in mice.

assays to better understand the qualities and limitations of the molecule. Compound **3** was devoid of meaningful activity at 10 μM in a Panlabs panel of 102 in vitro assays with the exception of 90% inhibition of monoamine uptake at 10 μM and 52% inhibition at 1 μM. No inhibition of dog P450 enzymes was noted at 10 μM. Nodulisporamide **3** was radiolabeled by reacting $H_2N^{14}C(CH_3)_3$ with the previously isolated and purified HOBT ester of nodulisporic acid A (Scheme 1). Covalent binding studies using [^{14}C]**3** in rat and dog liver microsomes were unremarkable, yielding <50 pM equiv/mg protein labeling in the presence or absence of NADPH, GSH (5 mM), or NaCN (1 mM). Nodulisporamide **3** is highly plasma protein bound (99.3%), which also may contribute to both its excellent mammalian safety profile and serve as the drug delivery vehicle for blood sucking ectoparasites.

CD-1 mice were dosed orally with [^{14}C]**3** at 5 and 20 mg/kg. Tissue distribution of [^{14}C]**3** was determined at 8 h, 21 days, and 28 days postdose. Initially, at 8 h postdose, the radiolabeled material is predominantly present in the stomach and gastrointestinal tract (Supporting Information, Figure I for 8 h and 5 mpk data). In contrast, at days 21 and 28 postdose, as illustrated in Figure 5 for the 20 mg/kg dosing arm, the distribution of [^{14}C]**3** is distinct from that seen at prior time points. The histogram indicates that a significant depot effect of [^{14}C]**3** occurs over time with maximal drug absorbing into abdominal and altilateral adipose tissue. Presumably, [^{14}C]**3** gradually leaches out of these adipose depot beds, contributing to the prolonged terminal half-life observed for **3** and its extensive duration of efficacy against ectoparasites.

Chemistry. Nodulisporamides **2**−**14** were derived in a single chemical step from nodulisporic acid A (**1**) in coupling yields ranging from 35% to 70% (Scheme 1).[26] Unsurprisingly, the observed chemical yield was significantly affected by the purity of the starting carboxylic acid, which typically ranged from 80% to 90%. If the NsA A was relatively pure, the corresponding nodulisporamides were readily obtained following flash chromatography on silica gel. Often, however, **1** was heavily contaminated with multiple fermentation congeners (including 2′-epi-NsA A, 3″,4″-Z-NsA A, nodulisporic acids B, C, D, E, and F), raising purification challenges. In those instances, both preparative normal phase and reversed phase HPLC purification were necessary to obtain pure nodulisporamides. Alternatively, if desired, the intermediate NsA HOBT ester could be isolated by column or thin layer chromatography and later be subjected

Scheme 1. General Synthesis of Nodulisporamide Derivatives



to amide bond forming reactions; this ester was stable for weeks at −20 °C under nitrogen.

Conclusion

A modular synthesis effort was utilized to prepare a library of nodulisporamide analogues from which the development candidate *N-tert*-butyl nodulisporamide (**3**) was identified. Through a combination of potency and extended half-life, a single oral dose of **3** can protect dogs (5−10 mg/kg) and cats (15−20 mg/kg) against fleas for an entire month. In addition, at higher dosages, **3** is 100% effective against ticks upon dosing and retains tick activity (84−92%) throughout the month. A higher single oral dose of 30 mg/kg in dogs may protect against flea infestations for greater than 2 months. No commercial product has the unique oral properties of nodulisporamide, and its systemic efficacy profile compares favorably to currently marketed topical agents. Mechanism of action studies provide a biological basis for the safety profile of this systemically active agent and potent cidal activity against blood sucking ectoparasites.

Experimental Section

Artificial Membrane Flea Feeder. Artificial membrane flea feeding devices have been described previously.[29,30] In brief, it is a plexiglass chamber divided into a heated upper and a nonheated lower and has a series of 104 ports (a 25 port variant is sold commercially by Jay Georgi, FleaData, Inc., Freeville, NY). Bovine blood spiked with various drugs and concentrations was introduced into aluminum sleeves with PARAFILM stretched across the sleeve bottom to form a membrane. Each blood-filled sleeve was placed into the heated upper half with the PARAFILM membrane interfacing with 1 of the 104 ports. A small cage with 25 adult fleas fed on the warm bovine blood through the PARAFILM membrane. The blood in the heated chamber was maintained at 40 °C, and the fleas in the bottom half were maintained at 28 °C and 85% relative humidity (RH).

NsA A and the nodulisporamide derivatives were dissolved in polyethylene glycol 400 and dimethyl sulfoxide (2:1) and were added to 10 mL of bovine blood in each aluminum sleeve to achieve concentrations of 10.0, 1.0, and 0.1 μg/mL. The bovine blood had been treated with citrated dextrose as an anticoagulant. After 24 h of feeding, the sleeves were replaced with fresh blood and drug. The fleas were allowed to feed on the fresh drug-treated blood for another 24 h after a total of 48 h of drug exposure. The cages were removed, and the living and dead fleas were counted. Lethal concentrations that killed 50% of fleas (LC_{50}) were calculated using linear regression.

***Cimex lectularis* Efficacy Test.** The *Cimex lectularis* assay has been described previously.[31] Briefly, female CD-1 mice ($n = 3$) were given compounds orally via gavage (four dose levels: 1.0, 0.5, 0.25, and 0.125 mg/kg) 24 h prior to feeding the fourth stage instars. The single orally dosed compounds were dissolved in a mixture of polyethylene glycol 400 and DMSO (1:2, v/v) and administered at a rate of 0.5 mL/100 g, with concentrations adjusted to provide the specified levels in mg/kg. Following treatment (24 h for single oral doses) the mice were restrained in plastic containers from which their tails were put through a small hole. The tail was washed, wiped dry, and inserted into a shell vial containing five unfed fourth instar *C. lectularius*. The vials containing the engorged insects were removed, plugged with a foam stopper, and examined daily until 80% or more of the control instars had molted to the fifth instar. Death, paralysis, or molt delay relative to the placebo-treated controls served as the criteria for activity.

Dog Flea Efficacy Tests. Multiple independent dog flea efficacy studies were performed; each trial used different numbers of animals and had different length but followed the general protocols developed by Shoop et al.[14] With the exclusion of the trial detailed in Table 3, only male beagle dogs were used, and all beagle dogs were of similar weight (10.0−13.5 kg). In all cases, animals were randomly allocated to control or treatment groups, and the groups were allocated randomly to treatment. All dogs were in excellent health at the beginning of each study described below, and no dog showed any sign of adverse reaction during these studies.

Briefly, for the trial described in Table 3, 26 beagle dogs of similar weight (10.0−13.5 kg) were allocated randomly to 1 of 12 groups ($n = 2$, one male and one female per treatment arm), and the groups were allocated randomly to treatment. The treatment arms were nodulisporamides **3**−**14** (Table 1) or vehicle treated control. Each compound was administered per os via a calibrated syringe at 10 mg/kg at 0.2 mL/kg on day 0. The vehicle was dimethyl sulfoxide/propylene glycol/glycerol formal (5:3:2). Flea challenges were made by placing 100 live, unfed fleas, *Ctenocephalides felis*, from our flea colony along the dorsal midline of each dog on the evening before the trial. The fleas were comb-counted 48 h after treatment according to the methods of Zakson et al.[32] Five days after the 48 h comb-count, a fresh batch of 100 unfed fleas was placed onto each dog. This weekly comb-count iteration was continued for the duration of each assay. In two assays, an additional flea challenge was performed on day 31 (M*), where fleas were placed on the dogs. All counts were done in a "blind" fashion.

Cat Flea Efficacy Tests. Two independent cat flea efficacy studies were performed; each trial used different numbers of animals and had different durations but followed the general protocols developed by Shoop et al.[14] as described above using fed cats (males only) instead of dogs. In all cases, animals were randomly allocated to control or treatment groups, and the groups were allocated randomly to treatment. All cats were in excellent health at the beginning of each study, and no cat showed any sign of adverse reaction during these studies.

Dog Tick Efficacy Studies. Three independent tick efficacy studies were performed using three different species of ticks from our colonies, as detailed in Table 3 [*Dermacentor variabilis* (American Dog tick)], Table 4 [*Rhipicephalus sanguineus* (Brown Dog tick)], and Table 7 [*R. sanguineus* and *Amblyoma americanum* (Lone Star tick)]. All dogs were in good health at the beginning of each study described below, and no dog showed any sign of adverse reaction during these studies.

For the trial described in Table 3, concurrent with the flea efficacy study, the beagle dogs dosed with nodulisporamides **3**−**14** and the vehicle treated beagles also were exposed to repeated tick challenges, interleaved at the indicated time points. Tick challenges were made by placing 50 live, unfed *D. variabilis* ticks from our tick colony along the dorsal midline of each dog on day −2 of the trial. The ticks were comb-counted 72 h after treatment according to the methods of Zakson et al. previously developed for counting fleas.[32] Careful inspection of the combed parasites permitted identification and quantification of recovered ticks. Four days subsequent to the initial 72 h comb-count, a fresh batch of 50 unfed *D. variabilis* ticks was placed onto each dog. This weekly comb-count iteration was continued on days 7, 14, and 31 (M*). All counts were done in a "blind" fashion.

For the study detailed in Table 7, nodulisporamide **3** was administered per os via a calibrated syringe at 30 mg/kg at 0.2 mL/kg on day 0 ($n = 4$ male beagle dogs per treatment or vehicle-treated control arm). The vehicle was dimethyl sulfoxide/propylene glycol/glycerol formal (5:3:2). Only male beagle dogs were used, and all beagle dogs were of similar weight (10.0−13.5 kg). In all cases, animals were randomly allocated to control or treatment groups, and the groups were allocated randomly to treatment. Tick challenges were made by placing 50 live, unfed *R. sanguineus* ticks and 50 live, unfed *A. americanum* ticks from our tick colonies along the dorsal midline of each dog on day 0 of the trial. This study was otherwise conducted exactly as described in the preceding paragraph.

Blood Sampling and Plasma Analysis. Blood samples were taken from treated dogs in heparinized VACUTAINER tubes at various times during each study. Concentrations of the administered compounds were determined in plasma using HPLC after analyte extraction by protein precipitation. For the nodulisporamides, 0.5

mL of plasma was diluted with an equal volume of water, and 3.5 mL of acetonitrile was added with gentle vortexing to precipitate the plasma proteins. After removal of solids by centrifugation, the supernatant liquid was reduced to dryness, and the residue was reconstituted with 0.2 mL of 50% aqueous acetonitrile. The resulting solution was analyzed by HPLC with ultraviolet photometric detection using a YMC ODS-AM column (150 mm × 3 mm, 3 μm particle diameter) at 40 °C with an eluant of acetonitrile and water (4:1). The flow rate was 0.4 mL/min, the injection volume was 100 μL, and the detection wavelength was 380 nm.

General Procedure for the Preparation of Nodulisporic Acid A (NsA A, 1). NsA A was fermented, isolated, and purified according to the methods outlined by Ondeyka et al.[11] Briefly, NsA A was purified from methyl ethyl ketone extracts of *Nodulisporium* sp., grown either on solid substrate or in liquid medium. The extracts were dried and portioned between hexane and methanol (1:1), and the methanol soluble fraction was chromatographed over silica gel, followed by gel filtration. Final purification was achieved by preparative reversed-phase high-performance liquid chromatography (HPLC), which provided pure NsA A as a yellow powder.

General Procedure for the Synthesis of Nodulisporamides 2−14. All 66 semisynthetic nodulisporamides, including **2**−**14**, were prepared by Medicinal Chemistry at Merck Research Laboratories (Merck & Co., Inc., Rahway, New Jersey) as described previously[26] as illustrated for *N-tert*-butyl nodulisporamide (**3**) below. Purity for all new nodulisporamides described herein was determined by RP-HPLC and normal phase HPLC analysis (≥97% purity required for samples submitted for in vivo evaluation). To NsA A (**1**, 2.01 g, 2.96 mmol, ∼90% pure) in 50 mL of methylene chloride at 0 °C was added HOBT (0.20 g, 1.48 mmol), triethylamine (1.30 mL, 9.27 mmol), and *tert*-butylamine (3.1 mL, 29.6 mL). To this solution was added benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP, 2.2 g, 4.98 mmol). After aging for 1 h at 0 °C, the reaction mixture was partitioned between saturated sodium bicarbonate and methylene chloride and extracted with methylene chloride (3 × 20 mL). The combined organic layers were washed once with saturated sodium chloride and then dried (sodium sulfate). The solution was filtered, concentrated under reduced pressure, and purified by flash chromatography on silica gel (8 cm × 15 cm column) using gradiant elution. The silica gel was equilibrated with 25:75 EtOAc/hexanes and the reaction crude loaded using 40 mL of methylene chloride. The column was flushed with 25:75 EtOAc/hexanes (1 L, **3** remained at the baseline), 33:67 EtOAc/hexanes (2 L, **3** began to elute), 40:60 EtOAc/hexanes (until **3** began to elute off the column), and then 50:50 EtOAc/hexanes during product collection. Concentration under reduced pressure, yielded impure **3** (1.35 g, ∼80% pure) as a bright-yellow amorphous solid. A 6 cm × 30 cm axial compression HPLC column was slurry-packed with 20 μm silica gel. The column was equilibrated with 25:75 EtOAc/hexanes (flow rate, 220 mL/min; λ = 270 nm). *N*-1-*tert*-Butyl nodulisporamide (1.35 g, 68% crude yield, ∼80% pure) was loaded onto the column with approximately 150 mL of methylene chloride. The column was flushed using 1 L of 25:75 EtOAc/hexanes, and then the product was eluted using 50:50 EtOAc/hexanes. Pure **3** began to elute after 20 min and was collected in 110 mL cuts. After 35 min, all **3** had been eluted from the column. Concentration under reduced pressure yielded pure **3** (0.85 g, 44%). Mp: 196−203 °C (decomposed). TLC: 1:1 EtOAc/hexanes, R_f = 0.38. HPLC (reversed-phase, ZORBAX Rx-C8, 4.6 mm × 25 cm, 8:2 MeCN/H_2O isocratic, 1.5 mL/min, λ = 254 nm) t_R = 6.67 min. HPLC (normal phase, ZORBAX Rx-Sil, 4.6 mm × 25 cm, 1:1 EtOAc/hexanes isocratic, 1.5 mL/min, λ = 254 nm) t_R = 8.52 min. $[\alpha]_D^{20}$ + 17.4° (*c* 0.82, CH_2Cl_2). log *P* = 7.6 (65:35 MeOH/0.04 M phosphate buffer, 37 °C). ^{1}H NMR (500 MHz, $CDCl_3$, ppm) δ 7.64 (s, 1H), 6.84 (d, *J* = 10.7 Hz, 1H), 6.30 (dd, *J* = 11.0, 15.3 Hz, 1H), 6.03 (d, *J* = 2.9 Hz, 1H), 5.72 (d, *J* = 15.5 Hz, 1H), 5.55 (s, 1H), 5.22 (dd, *J* = 2.1, 6.2 Hz, 1H), 5.18 (s, 1H), 5.06 (s, 1H), 4.97 (br s, 1H), 3.68 (s, 1H), 3.36 (br s, 1H), 3.16 (s, 1H), 2.86 (dd, *J* = 2.7, 6.2 Hz, 1H), 2.82 (m, 1H), 2.70 (dd, *J* = 6.5, 14.1 Hz, 1H), 2.28 (dd, *J* = 10.8, 13.7 Hz, 1H), 1.95−1.40 (m, 9H), 1.93 (s, 3H), 1.55 (s, 3H), 1.47 (s, 3H), 1.38 (s, 9H), 1.34 (s, 3H), 1.32 (s, 3H), 1.12 (s, 3H), 1.11 (s, 3H), 1.03 (s, 3H), 0.93 (s, 3H). ^{13}C NMR spectrum (125 MHz, $CDCl_3$, ppm) δ 197.56, 168.52, 154.03, 150.21, 139.29, 137.94, 135.34, 133.62, 132.50, 130.09, 128.16, 125.46, 122.09, 121.49, 121.81, 117.42, 116.45, 112.69, 76.49, 75.95, 74.81, 73.61, 72.26, 57.54, 55.50, 51.06, 47.45, 46.94, 44.82, 38.55, 31.80, 37.74, 29.79, 29.61, 28.67, 27.34, 25.22, 24.08, 23.12, 19.24,17.74,14.95, 13.12, 11.03. IR (film, NaCl) 3348.6, 2972.8, 2936.1, 1707.6, 1655.7, 1509.7, 1509.5, 1452.9, 1378.8, 1229.8, 1075.8, 1022.0, 976.7 cm^{-1}. UV−vis λ_{max} (MeCN) 245, 260, 390 nm. HRMS (ESI, pos mode) calculated for $C_{47}H_{62}N_2O_5$, 734.4658; found *m*/*z* = 735.473 12 $[M + H]^+$.

***N*-(4-Methoxy)benzyl Nodulisporamide (2).** Yield: 320 mg, 54%. TLC, silica gel: R_f = 0.21 (1:2 acetone/hexanes). RP-HPLC (C18 ODS, 1 mL/min, 4:1 MeCN/H_2O, isocratic): t_R = 11.0 min. ^{1}H NMR (500 MHz, $CDCl_3$, ppm) δ 7.71 (s, 1H), 7.25 (d, *J* = 8.4 Hz, 2H), 6.99 (d, *J* = 10.7 Hz, 1H), 6.89 (d, *J* = 8.4 Hz, 2H), 6.35 (dd, *J* = 11.2, 15.4 Hz, 1H), 6.06 (d, *J* = 2.7 Hz, 1H), 5.95 (t, *J* = 5.5 Hz, 1H), 5.79 (d, *J* = 15.6 Hz, 1H), 5.26 (d, *J* = 6.2 Hz, 1H), 5.21 (s, 1H), 5.09 (s, 1H), 5.00 (br s, 1H), 4.48 (d, *J* = 5.5 Hz, 2H), 3.82 (s, 3H), 3.40 (br s, 1H), 2.89 (dd, *J* = 2.8, 6.2 Hz, 1H), 2.84 (br s, 1H), 2.73 (dd, *J* = 6.4, 14.0 Hz, 1H), 2.32 (dd, *J* = 10.9, 13.5 Hz, 1H), 2.00 (s, 3H), 1.95−1.40 (m, 11H), 1.60 (br s, 3H), 1.51 (s, 3H), 1.37 (s, 3H), 1.36 (s, 3H), 1.16 (s, 3H), 1.15 (s, 3H), 1.06 (s, 3H), 0.96 (s, 3H). HRMS (ESI, pos mode) calculated for $C_{51}H_{62}N_2O_6$ 798.4608; found *m*/*z* = 799.463 75 $[M + H]^+$.

***N*-(1,1-Dimethyl)proparg-2-yl Nodulisporamide (4).** Yield: 74 mg, 67%. TLC, silica gel: R_f = 0.44, 1:1 acetone/hexanes. HPLC (reversed-phase, ZORBAX Rx-C8, 4.6 mm × 25 cm, 6:4 to 8:2 MeCN/H_2O, 20 min gradient, 2.0 mL/min, λ = 254 nm) t_R = 22.4 min. ^{1}H NMR (500 MHz, $CDCl_3$, ppm) δ partial spectrum 7.67 (s, 1H), 6.90 (d, *J* = 10.8 Hz, 1H), 6.30 (dd, *J* = 10.9, 15.4 Hz, 1H), 6.02 (d, *J* = 2.7 Hz, 1H), 5.80 (s, 1H), 5.74 (d, *J* = 15.3 Hz, 1H), 5.21 (dd, *J* = 2.3, 6.2 Hz, 1H), 5.17 (s, 1H), 5.05 (s, 1H), 4.96 (br s, 1H), 3.74 (s, 1H), 3.36 (br s, 1H), 3.18 (d, *J* = 2.1 Hz, 1H), 2.85 (dd, *J* = 2.8, 6.2 Hz, 1H), 2.80 (m, 1H), 2.71 (dd, *J* = 6.5, 13.7 Hz, 1H), 2.60 (s, 1H), 2.28 (dd, *J* = 10.8, 13.8 Hz, 1H), 2.15 (s, 1H), 1.94 (s, 3H), 1.95−1.40 (m, 11H), 1.67 (s, 3H), 1.47 (s, 3H), 1.33 (s, 3H), 1.32 (s, 3H), 1.23 (s, 3H), 1.12 (s, 3H), 1.11 (s, 3H), 1.03 (s, 3H), 0.92 (s, 3H). HRMS (ESI, pos mode) calculated for $C_{48}H_{60}N_2O_5$, 744.450 11; found *m*/*z* = 745.457 39 $[M + H]^+$.

***N*-Isopropyl Nodulisporamide (5).** Yield: 476 mg, 60%. TLC, silica gel: R_f = 0.75 (1:9 MeOH/CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$, ppm) δ 7.67 (s, 1H), 6.91 (d, *J* = 11.0 Hz, 1H), 6.31 (dd, *J* = 11.0, 15.1 Hz, 1H), 6.02 (d, *J* = 2.8 Hz, 1H), 5.75 (d, *J* = 15.3 Hz, 1H), 5.51 (d, *J* = 7.8 Hz, 1H), 5.22 (dd, *J* = 1.7, 6.1 Hz, 1H), 5.18 (s, 1H), 5.06 (s, 1H), 4.97 (br s, 1H), 4.15 (m, 1H), 3.38 (br s, 1H), 3.16 (s, 1H), 2.85 (dd, *J* = 2.8, 6.3 Hz, 1H), 2.81 (m, 1H), 2.71 (dd, *J* = 6.6, 14.0 Hz, 1H), 2.27 (dd, *J* = 11.1, 13.9 Hz, 1H), 1.95 (s, 3H), 1.95−1.40 (m, 10H), 1.55 (s, 3H), 1.47 (s, 3H), 1.34 (s, 3H), 1.32 (s, 3H), 1.18 (s, 3H), 1.17 (s, 3H), 1.13 (s, 3H), 1.11 (s, 3H), 1.03 (s, 3H), 0.93 (s, 3H). HRMS (ESI, pos mode) calculated for $C_{46}H_{60}N_2O_5$, 720.450 57; found *m*/*z* = 721.457 84 $[M + H]^+$.

***N*-(2-Methyl)propen-2-yl Nodulisporamide (6).** Yield: 470 mg, 54%. TLC, silica gel: R_f = 0.72 (1:9 MeOH/CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$, ppm) δ 7.69 (s, 1H), 6.97 (d, *J* = 11.3 Hz, 1H), 6.33 (dd, *J* = 10.9, 15.0 Hz, 1H), 6.03 (d, *J* = 2.9 Hz, 1H), 5.80 (m, 2H), 5.24 (d, *J* = 6.3 Hz, 1H), 5.20 (s, 1H), 5.07 (s, 1H), 4.98 (br s, 1H), 4.84 (d, *J* = 5.8 Hz, 1H), 3.89 (d, *J* = 7.4 Hz, 2H), 3.39 (m, 1H), 3.18 (s, 1H), 2.86 (dd, *J* = 2.7, 6.1 Hz, 1H), 2.80 (m, 1H), 2.70 (dd, *J* = 6.3, 13.9 Hz, 1H), 2.28 (dd, *J* = 10.7, 13.7 Hz, 1H), 1.99 (s, 3H), 1.95−1.40 (m, 11H), 1.74 (s, 3H), 1.54 (s, 3H), 1.45 (s, 3H), 1.36 (s, 3H), 1.34 (s, 3H), 1.13 (s, 3H), 1.11 (s, 3H), 1.03 (s, 3H), 0.93 (s, 3H). HRMS (ESI, pos mode) calculated for $C_{47}H_{60}N_2O_5$, 732.4501; found *m*/*z* = 733.457 40 $[M + H]^+$.

***N*-2,2,2-Trifluoroethyl Nodulisporamide (7).** Yield: 431 mg, 77%. TLC, silica gel: R_f = 0.65 (5:95 MeOH/$CHCl_3$). HPLC (reversed-phase, ZORBAX Rx-C8, 4.6 mm × 25 cm, 6:4 to 1:0 MeCN/H_2O, 20 min gradient, 2.0 mL/min, λ = 254 nm) t_R = 11.4

min. ^{1}H NMR (500 MHz, $CDCl_3$, ppm) δ 7.67 (s, 1H), 6.98 (d, J = 11.2 Hz, 1H), 6.33 (dd, J = 11.0, 15.3 Hz, 1H), 6.03 (d, J = 3.0 Hz, 1H), 5.95 (t, J = 6.3 Hz, 1H), 5.82 (d, J = 15.4 Hz, 1H), 5.22 (d, J = 6.0 Hz, 1H), 5.18 (s, 1H), 5.06 (s, 1H), 4.97 (br s, 1H), 4.00 (m, 2H), 3.38 (br s, 1H), 3.16 (s, 1H), 2.86 (dd, J = 2.8, 6.2 Hz, 1H), 2.81 (m, 1H), 2.71 (dd, J = 6.5, 13.8 Hz, 1H), 2.28 (dd, J = 11.0, 13.8 Hz, 1H), 1.99 (s, 3H), 1.95−1.40 (m, 10H), 1.56 (s, 3H), 1.47 (s, 3H), 1.34 (s, 3H), 1.32 (s, 3H), 1.12 (s, 3H), 1.11 (s, 3H), 1.04 (s, 3H), 0.93 (s, 3H). HRMS (ESI, pos mode) calculated for $C_{45}H_{55}F_3N_2O_5$, 760.4069; found m/z = 761.41428 $[M + H]^+$.

***N*-Isobutyl Nodulisporamide (8).** Yield: 542 mg, 67%. TLC, silica gel: R_f = 0.24 (3:1 hexanes/acetone). HPLC (reversed-phase, ZORBAX Rx-C8, 4.6 mm × 25 cm, 6:4 to 1:0 MeCN/H_2O, 20 min gradient, 2.0 mL/min, λ = 254 nm) t_R = 22.6 min. ^{1}H NMR (500 MHz, $CDCl_3$, ppm) δ 7.68 (s, 1H), 6.94 (d, J = 11.7 Hz, 1H), 6.32 (dd, J = 11.0, 15.1 Hz, 1H), 6.02 (d, J = 2.8 Hz, 1H), 5.74 (m, 2H), 5.22 (dd, J = 2.3, 6.3 Hz, 1H), 5.18 (s, 1H), 5.06 (s, 1H), 4.97 (br s, 1H), 3.68 (s, 1H), 3.39 (m, 1H), 3.16 (t, J = 6.4 Hz, 2H), 2.86 (dd, J = 2.7, 6.1 Hz, 1H), 2.81 (m, 1H), 2.70 (dd, J = 6.5, 14.1 Hz, 1H), 2.28 (dd, J = 10.7, 13.7 Hz, 1H), 1.97 (s, 3H), 1.95−1.40 (m, 11H), 1.54 (s, 3H), 1.47 (s, 3H), 1.34 (s, 3H), 1.32 (s, 3H), 1.13 (s, 3H), 1.11 (s, 3H), 1.03 (s, 3H), 0.93 (s, 3H), 0.92 (d, J = 6.4 Hz, 6H). HRMS (ESI, pos mode) calculated for $C_{47}H_{62}N_2O_5$, 734.4658; found m/z = 735.473 10 $[M + H]^+$.

***N*-(1,1-Dimethyl)carbomethoxymethyl Nodulisporamide (9).** Yield: 17 mg, 42%. TLC, silica gel: R_f = 0.65 (1:1 acetone/hexanes). ^{1}H NMR (500 MHz, $CDCl_3$, ppm) δ 7.72 (s, 1H), 6.99 (d, J = 10.8 Hz, 1H), 6.36 (dd, J = 11.2, 15.1 Hz, 1H), 6.07 (s, 1H), 5.82 (d, J = 15.3 Hz, 1H), 5.27 (d, J = 5.9 Hz, 1H), 5.23 (s, 1H), 5.10 (s, 1H), 5.02 (br s, 1H), 3.93 (s, 3H), 3.73 (s, 1H), 3.41 (br s, 1H), 2.91 (d, J = 3.7 Hz, 1H), 2.89 (m, 1H), 2.76 (dd, J = 6.1, 13.8 Hz, 1H), 2.34 (dd, J = 10.7, 14.0 Hz, 1H), 2.01 (s, 3H), 1.95−1.40 (m, 11H), 1.60 (s, 6H), 1.57 (s, 3H), 1.51 (s, 3H), 1.38 (s, 3H), 1.36 (s, 3H), 1.16 (s, 3H), 1.15 (s, 3H), 1.07 (s, 3H), 0.97 (s, 3H). HRMS (ESI, pos mode) calculated for $C_{48}H_{62}N_2O_7$, 778.455 62; found m/z = 779.462 90 $[M + H]^+$.

***N*-(1,1-Dimethyl)-2-oxopropyl Nodulisporamide (10).** Yield: 412 mg, 41%. TLC, silica gel: R_f = 0.52 (0.4:1 acetone/hexanes). RP-HPLC (C18 ODS, 1 mL/min, 4:1 MeCN/H_2O, isocratic): t_R = 6.1 min. ^{1}H NMR (500 MHz, $CDCl_3$, ppm) δ 7.71 (s, 1H), 6.98 (d, J = 10.8 Hz, 1H), 6.70 (s, 1H), 6.35 (dd, J = 11.2, 15.1 Hz, 1H), 6.06 (s, 1H), 5.81 (d, J = 15.3 Hz, 1H), 5.26 (d, J = 5.9 Hz, 1H), 5.22 (s, 1H), 5.09 (s, 1H), 5.01 (br s, 1H), 3.72 (s, 1H), 3.40 (br s, 1H), 2.90 (d, J = 3.7 Hz, 1H), 2.88 (m, 1H), 2.75 (dd, J = 6.1, 13.8 Hz, 1H), 2.33 (dd, J = 10.7, 14.0 Hz, 1H), 2.23 (s, 3H), 2.01 (s, 3H), 1.95−1.40 (m, 10H), 1.60 (s, 6H), 1.57 (s, 3H), 1.51 (s, 3H), 1.38 (s, 3H), 1.36 (s, 3H), 1.16 (s, 3H), 1.15 (s, 3H), 1.07 (s, 3H), 0.97 (s, 3H). HRMS (ESI, pos mode) calculated for $C_{48}H_{62}N_2O_6$, 762.460 79; found m/z = 763.468 00 $[M + H]^+$.

***N*-Cyclopropylmethyl Nodulisporamide (11).** Yield: 87 mg, 47%. TLC, silica gel: R_f = 0.49 (5:95 10% NH_4OH in MeOH/$CHCl_3$). HPLC (reversed-phase, ZORBAX Rx-C8, 4.6 mm × 25 cm, 6:4 to 8:2 MeCN/H_2O, 20 min gradient, 2.0 mL/min, λ = 254 nm) t_R = 11.8 min. ^{1}H NMR (500 MHz, $CDCl_3$, ppm) δ 7.68 (s, 1H), 6.95 (d, J = 11.2 Hz, 1H), 6.32 (dd, J = 11.1, 15.5 Hz, 1H), 6.02 (d, J = 3.0 Hz, 1H), 5.82 (t, J = 5.5 Hz, 1H), 5.76 (d, J = 15.3 Hz, 1H), 5.23 (dd, J = 2.1, 6.2 Hz, 1H), 5.18 (s, 1H), 5.06 (s, 1H), 4.97 (br s, 1H), 3.37 (br s, 1H), 3.17 (m, 2H), 2.86 (dd, J = 2.7, 6.2 Hz, 1H), 2.81 (m, 1H), 2.70 (dd, J = 6.3, 14.1 Hz, 1H), 2.28 (dd, J = 10.9, 13.7 Hz, 1H), 1.98 (s, 3H), 1.95−1.40 (11H, m), 1.59 (s, 3H), 1.47 (s, 3H), 1.34 (s, 3H), 1.32 (s, 3H), 1.12 (s, 3H), 1.11 (s, 3H), 1.04 (s, 3H), 0.93 (s, 3H), 0.51 (q, J = 8.0 Hz, 2H), 0.22 (q, J= 4.8 Hz, 2H). HRMS (ESI, pos mode) calculated for $C_{47}H_{60}N_2O_5$, 732.450 22. found m/z = 733.457 53 $[M + H]^+$.

***N*-(3,3,3-Trifluoro)-*n*-propyl Nodulisporamide (12).** Yield: 401 mg, 33%. TLC, silica gel: R_f = 0.58 (6:4 hexanes/acetone). HPLC (reversed-phase, ODS, 4.6 mm × 25 cm, 7:3 MeCN/H_2O, isocratic, 1.5 mL/min, λ = 254 nm) t_R = 8.02 min. ^{1}H NMR (500 MHz, $CDCl_3$, ppm) δ 7.71 (s, 1H), 6.95 (d, J = 10.9 Hz, 1H), 6.37 (dd, J = 11.1, 15.4 Hz, 1H), 6.02 (d, J = 2.6 Hz, 1H), 6.01 (br s, 1H), 5.80 (d, J = 15.2 Hz, 1H), 5.22 (d, J = 6.1 Hz, 1H), 5.20 (s, 1H), 5.06 (s, 1H), 5.00 (br s, 1H), 3.61 (m, 2H), 3.40 (m, 1H), 3.21 (s, 1H), 2.86 (dd, J = 2.7, 6.1 Hz, 1H), 2.81 (m, 1H), 2.73 (dd, J = 6.3, 13.5 Hz, 1H), 2.40 (m, 1H), 2.28 (dd, J = 10.8, 13.6 Hz, 1H), 1.99 (s, 3H), 1.95−1.40 (m, 11H), 1.57 (s, 3H), 1.47 (s, 3H), 1.34 (s, 3H), 1.32 (s, 3H), 1.13 (s, 3H), 1.12 (s, 3H), 1.05 (s, 3H), 0.93 (s, 3H). HRMS (ESI, pos mode) calculated for $C_{46}H_{57}F_3N_2O_5$, 774.421 96; found m/z = 775.429 73 $[M + H]^+$.

***N*-(1,1,2,2-Tetramethyl)hydroxyethyl Nodulisporamide (13).** Yield: 408 mg, 36%. TLC, silica gel: R_f = 0.51 (1:1 acetone/hexanes). ^{1}H NMR (500 MHz, $CDCl_3$, ppm) δ 7.72 (s, 1H), 6.99 (d, J = 11.1 Hz, 1H), 6.36 (dd, J = 11.0, 14.9 Hz, 1H), 6.05 (s, 1H), 5.82 (s, 1H), 5.80 (d, J = 15.1 Hz, 1H), 5.65 (s, 1H), 5.27 (d, J = 6.1 Hz, 1H), 5.21 (s, 1H), 5.08 (s, 1H), 5.03 (br s, 1H), 3.41 (br s, 1H), 3.18 (m, 1H), 2.91 (d, J = 3.5 Hz, 1H), 2.86 (m, 1H), 2.75 (dd, J = 6.0, 13.6 Hz, 1H), 2.35 (dd, J = 10.3, 14.1 Hz, 1H), 2.02 (s, 3H), 1.95−1.40 (m, 10H), 1.51 (s, 3H), 1.56 (s, 3H), 1.40 (s, 6H), 1.38 (s, 3H), 1.36 (s, 3H), 1.22 (s, 6H), 1.17 (s, 3H), 1.16 (s, 3H), 1.07 (s, 3H), 0.98 (s, 3H). HRMS (ESI, pos mode) calculated for $C_{49}H_{66}N_2O_6$, 778.492 09; found m/z = 779.499 18 $[M + H]^+$.

***N*-(1-Fluoromethyl)-2-fluoroethyl Nodulisporamide (14).** Yield: 449 mg, 40%. TLC silica gel: R_f = 0.29 (1:2 acetone/hexanes). ^{1}H NMR (500 MHz, $CDCl_3$, ppm) δ 7.71 (s, 1H), 7.00 (d, J = 11.0 Hz, 1H), 6.36 (dd, J = 11.0, 15.4 Hz, 1H), 6.06 (s, 1H), 6.05 (d, J = 10.1 Hz, 1H), 5.85 (d, J = 15.3 Hz, 1H), 5.26 (d, J = 6.2 Hz, 1H), 5.21 (s, 1H), 5.09 (s, 1H), 5.01 (br s, 1H), 4.48−4.71 (m, 5H), 3.41 (br s, 1H), 2.90 (dd, J = 2.8, 6.0 Hz, 1H), 2.85 (m, 1H), 2.75 (dd, J = 6.6, 13.9 Hz, 1H), 2.32 (dd, J = 11.0, 13.6 Hz, 1H), 2.01 (s, 3H), 1.95−1.40 (m, 11H), 1.62 (s, 3H), 1.51 (s, 3H), 1.37 (s, 3H), 1.36 (s, 3H), 1.16 (s, 3H), 1.15 (s, 3H), 1.08 (s, 3H), 0.97 (s, 3H). HRMS (ESI, pos mode) calculated for $C_{46}H_{58}F_2N_2O_5$, 756.431 38; found m/z = 737.431 55 $[M - H_2O + H]^+$.

Acknowledgment. We thank colleagues in Merck Research Laboratories' Natural Products group for their considerable efforts dedicated toward the fermentation and isolation of sufficient NsA A to perform the research described herein. We gratefully acknowledge Dr. J. A. Hair, a leading expert in tick biology (NuEra Laboratories, OK), for performing the flea and tick study, whose results are described in Table 8. In addition, we thank Deborah Zink for providing the high resolution mass spectral analyses.

Supporting Information Available: Spectra of **3** (^{1}H, ^{13}C, DEPT NMR spectra, UV−vis and IR spectra) (Figures III−VI); profiles (flea and bedbug activity, $T_{1/2}$ data, and flea efficacy in dogs) for 66 nodulisporamides (Tables I−IV); plasma disposition profiles for nodulisporamides **3**−**14** in dogs (42 day study) and cats (28 day study) (Tables V and VI); plasma disposition profile of **3** in dogs (91 day multiple dosing study) (Table 7); tissue distribution of [^{14}C]**3** in mice at 5 and 20 mg/kg (Figure I); plasma disposition profile of **2** in dogs dosed at 30 mg/kg once or 15 mg/kg twice (Table 8); plasma disposition profile of **3** dosed in dogs at 30 mg/kg (90 day study) (Table IX and Figure II); plasma disposition profile of **3** dosed in cats at 15 or 20 mg/kg (Table X); plasma disposition profile of **3** dosed in dogs at 5 or 10 mg/kg (Table XI). This material is available free of charge via the Internet at http://pubs.acs.org.

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JM801334V