Ningalins A−D: Novel Aromatic Alkaloids from a Western Australian Ascidian of the Genus *Didemnum*

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Four aromatic alkaloids, ningalins A−D (**1**−**4**), three of which possess new carbon skeletons, were isolated from an undescribed ascidian of the genus *Didemnum* collected in Western Australia near Ningaloo Reef. The structures of these new alkaloids were elucidated by interpretation of overall spectral data and by 2D NMR correlation methods. Ningalins A−D are composed of C_{18}, C_{25}, C_{32}, and C_{40} condensed aromatic systems with the unifying theme that all appear derived via the condensation of the amino acid 3,4-dihydroxyphenylalanine (DOPA). In these highly condensed alkaloids, steric crowding forces several catechol rings into twisted configurations, resulting in unexpected shielding and deshielding of several proton shifts in their NMR spectra. Because the ningalins are related to other metal binding *o*-catechols, it is conceivable that they too participate in the metal chelation phenomena characteristic of this class of marine invertebrates.

Marine ascidians or tunicates (subphylum Hemichordata, class Ascidiacea) are prominent producers of metabolites derived from amino acids.[1] The amino acid DOPA [2-amino, 3-(3′,4′-dihydroxyphenyl)propionic acid], in particular, appears to play an important role in the metabolism of these marine invertebrates, serving as the apparent precursor of several alkaloidal metabolites isolated from this source. DOPA metabolic products range from peptides to polycyclic alkaloids. Examples of peptide products are the tetrapeptides, halocyamines,[2] and the tripeptides known as the tunichromes.[3] Alkaloids apparently derived via DOPA metabolism include the lamellarins,[4] highly condensed isoquinoline DOPA alkaloids isolated from various *Didemnum* spp.,[5] and the lukianols[6] and polycitrins,[7] metabolites isolated from an unidentified tunicate from Palmyra Atoll and a species of marine ascidian genus *Polycitor*, respectively. Other ascidian metabolites such as rigidin, isolated from *Eudistoma rigida*,[8] and the rubrolides, isolated from *Ritterella rubra*,[9] appear to be derived from tyrosine by similar amino acid condensation reactions.

DOPA-derived metabolites have also been isolated from many ascidians which accumulate massive quantities of ionic vanadium and iron from seawater, and suggested to participate in this process.[10] The accumulation of vanadium has been documented among species of the orders Phlebobranchia and Aplousobranchia, whereas ascidians of the order Stolidobranchia concentrate smaller amounts of vanadium, but high levels of iron.[11] The families Polyclinidae and Didemnidae sequester iron at concentrations as much as 18 million times greater than that of seawater.[12] While an understanding of this process appears incomplete, the metal-binding phenomenon has been partially explained by complexation with *o*-catechol functionalities. One mechanism that has been proposed involves the chelation of metals such as Fe^{3+} and V^{5+} by DOPA or 3,4,5-trihydroxyphenylalanine (TOPA) residues. At pHs greater than 4, the vicinal hydroxyl substituents of catechol and pyrogallol rings confer potent chelating properties toward vanadium and iron.[13] Some formation constants of these ligands toward V^{IV}, V^{III}, Fe^{III}, and Fe^{II} are greater than 10^{10}, and several surpass 10^{28}.

As part of our continuing interest in the chemistry and chemical ecology of marine ascidians,[14] we have focused considerable attention on an ascidian-rich habitat near

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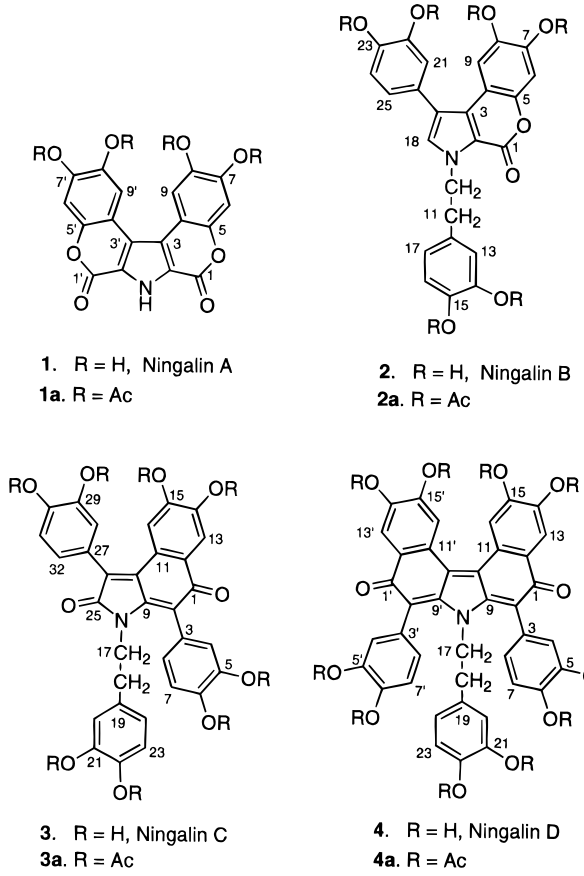
Chart 1



the Ningaloo Reef region at the northwest cape of Western Australia. In this paper we report the results of our studies of an undescribed, dark purple *Didemnum* sp.[15] collected in this environment. This interesting animal was found to contain four polar, highly-colored alkaloids, ningalins A−D (**1**−**4**), three of which possess highly unsaturated, new carbon skeletons. The ningalins appear to be derived via the condensation of DOPA amino acid units (Chart 1). NMR analyses, and subsequently computer modeling studies, illustrated that the phenyl rings in ningalins B, C, and D adopt low-energy conformations at approximately 90° to the planes of the molecules. Although this conformation appears to restrict rotation, creating the possibility of optical activity, the molecules appear to be racemic. The ningalins possess replicate *o*-catechol functionalities similar to the vanadium-binding metabolite tunichrome.[3] Thus, it seems feasible that these molecules could also participate as organic substrates in metal chelation phenomena.

Specimens of the dark purple *Didemnum* sp. were collected using SCUBA (−10 m) in December 1990 and immediately frozen. The samples were subsequently lyophilized and extracted twice with 70% methanol in chloroform and then methanol. The combined extracts were concentrated under vacuum and partitioned between hexane and methanol, and the methanol-soluble material was further partitioned between ethyl acetate and water. Gel filtration of the ethyl acetate-soluble material using Sephadex LH-20 (methanol) resulted in the separation of highly colored bands ranging from yellow, red-orange, and dark red to purple and black. Samples were combined based upon similar TLC and NMR profiles and purified by reversed-phase HPLC to give yellow ningalin A (**1**) at this point as an impure solid, pure samples of dark yellow ningalin B (**2**, 60 mg, 0.017% dry wt), red ningalin C (**3**, 23.4 mg, 0.0068%), and dark red ningalin D (**4**, 36 mg, 0.01%), each as noncrystalline solids. Final purification of ningalin A (**1**, 20 mg, 0.0058%) required an additional gel permeation chromatography on Spectra Gel HW-40 using methanol as the eluent.

(15) The ascidian, voucher numbers WA90-19 and WA90-59, was indentified as an undescribed *Didemnum* species by Dr. Françoise Monniot. The dark purple animal possesses a tough leathery surface and forms encrusting mats to 100 cm^2 in area and to 0.5 cm thick.

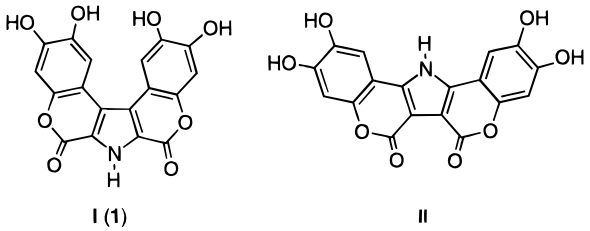
Table 1. NMR Assignments for Ningalin A (1)[a]

C no.	^{13}C[b]	^{1}H[c]	HMBC (8 Hz)[c,d]
1, 1′	154.8 (C)		
2, 2′	110.3 (C)		
3, 3′	122.0 (C)		
4, 4′	108.6 (C)		
5, 5′	144.4 (C)[d]		
6, 6′	104.2 (CH)	6.89 (s, 2H)	C4, C4′, C5, C5′, C7, C7′, C8, C8′
7, 7′	146.3 (C)[e]		
8, 8′	142.7 (C)[e]		
9, 9′	110.3 (CH)	7.79 (s, 2H)	C3, C3′, C5, C5′, C7, C7′, C8, C8′
OH		9.41 (bs, 2H)	
OH		9.88 (bs, 2H)	
NH		14.0 (bs, 1H)	

[a] Spectra were recorded in DMSO-d_6. Assignments were aided by DEPT sequence experiment and HMQC experiments. Chemical shifts are reported in δ units (downfield of TMS). [b] ^{13}C NMR spectrum was recorded at 50 MHz. [c] Spectra were recorded at 500 MHz. [d] The HMBC experiment was optimize for 8 Hz couplings to observe $^{3}J_{CH}$ correlations. [e] Assignments may be interchanged.

Ningalin A (**1**) analyzed by HRFABMS and combined spectral methods for $C_{18}H_9NO_8$, a molecular formula possessing 15 units of unsaturation and indicating a highly unsaturated aromatic structure. Since only five proton resonances were observed in the ^{1}H NMR spectrum of **1**, a symmetry plane incorporating the nitrogen and N-H proton was required to be present in the molecule. Identical $C_9H_4O_4$ fragments were thus required to be placed on each side of the symmetry plane. The aromaticity and extended conjugation generating the color of **1** was demonstrated by its UV spectrum which showed absorptions at 262, 303, 325, and 370 nm. Strong IR absorptions at 3377, 3186, and 1702 cm^{-1}, coupled with carbon signals at δ 142.7, 144.4, 146.3, and 154.8, suggested that there were numerous aromatic hydroxyl groups, an N-H, and a conjugated ester present in the molecule. A pronounced bathochromic shift (from 370 to 393 nm) upon addition of base, and no change in the UV spectrum on addition of acid, confirmed the phenolic nature of ningalin A and suggested the nitrogen atom was not a basic amine.

Of the five signals observed in the ^{1}H NMR spectrum of ningalin A (**1,** Table 1), three were exchangeable with D_2O. Four of the signals, except a proton resonance at δ 14.0 (bs, 1H), integrated for two protons each. Since coupling was not observed between the two proton signals at δ 6.89 (s, 2H) and 7.79 (s, 2H), and these protons were later confirmed, by HMQC and HMBC experiments, as substituents on the same benzene ring, these protons were placed in *para* positions. HMBC experiments established the presence of a 4,5-disubstituted catechol moiety. Two hydroxyl groups were assigned at the two most deshielded carbons, δ 146.3 and 142.7 (C-7 and C-8), while the adjacent carbons were assigned at δ 104.2 (C-6) and 110.3 (C-9), consistent with their predicted higher



I (1) II

Figure 1. Two possible structures for ningalin A (**1**) possessing a symmetry element.

field shifts. Carbon resonances at δ 122.0 and 108.6 were assigned to C-3 and C-4 since $^4J_{CH}$ couplings are rarely observed in HMBC experiments recorded at 8 Hz. Considering the oxygenation defined, and the degree of unsaturation in **1**, the four oxygen atoms composing each side of the symmetry plane were then assigned to a lactone functionality and to an *o*-catechol.

Although NMR data fully defined half of the molecule, there were two plausible ways to combine these part structures (**I** and **II**, Figure 1) with retention of a symmetry plane bissecting the NH bond. Plausible structure **II** for ningalin A was ultimately excluded on the basis of the ^{13}C NMR features of this metabolite. The C-3 (C-3′) resonance was confidently assigned by HMBC spectral methods at δ 122.0. If this carbon possessed a nitrogen substituent, as in **II**, it would be expected to resonate at much lower field, most likely lower than δ 150. In addition, the ^{1}H NMR spectrum of ningalin A showed unusual proton deshieldings consistent with structure **I**. Proton H-9 (H-9′) at δ 7.79 (s, 2H) was much more deshielded than proton H-6 (H-6′) at δ 6.89 (s, 2H). These observations could be rationalized by inspection of the molecular model for **I**, but not for **II**. Because of the helical nature of **1**, the phenyl rings are forced into a stacked conformation in which the catechol rings overlap above and below the plane of the pyrrole ring. In this conformation, H-9 and H-9′ are significantly deshielded by the adjacent aromatic rings. The structure of ningalin A (**1**) was also indicated to be structure **I** based on NMR experiments performed with ningalin B (**2**, see below). The close correspondence in the carbon resonances of the tricyclic fused lactone subunit of **1** with the shifts confirmed for **2**, and the observed NOESY correlations from H-9 to H-21 and H-25 in **2** confirmed the structure as in **I.** Thus, on the basis of NMR features, the structure for ningalin A (**1**) was established as **I**. Confirmation of the four phenolic hydroxyl groups, and support for the overall structure, was obtained by acetylation to yield the expected tetraacetate **1a** which analyzed for the expected molecular formula $C_{26}H_{18}NO_{12}$ [HRFABMS, obsd $(M + H)^+$ m/z 536.0837, $C_{26}H_{18}NO_{12}$ dev 1.5 ppm]. Consistent with the element of symmetry, the ^{1}H NMR spectrum of tetraacetate **1a** showed two acetate methyl signals in the ratio of 1:1.

Ningalin B (**2**), a dark yellow solid, analyzed for $C_{25}H_{19}NO_8$ by high-resolution FAB mass spectrometry in conjunction with ^{1}H and ^{13}C NMR data. Unlike ningalin A, all protons and carbons were clearly observed as magnetically nonequivalent bands in the ^{1}H and ^{13}C NMR spectra, indicating a lack of symmetry in this alkaloid (Table 2). The 17 units of unsaturation indicated by the molecular formula were further illustrated by the UV characteristics of ningalin B (λ_{max} = 332, 289, 236, and 204 nm). A bathochromic shift in the UV spectrum upon addition of base, coupled with analysis of ^{13}C NMR data (8 resonances at lower field than δ 140), suggested that ningalin B also contained multiple phenolic functional groups. A strong IR absorption at 1698 cm^{-1}, in conjunction with a carbon resonance at δ 156.0 (C), indicated a conjugated ester was present. The ^{1}H NMR spectrum of ningalin B (**2**) revealed the presence of two mutually coupled methylenes [δ 2.94 (t, 2H, J = 7.3 Hz) and 4.55 (t, 2H, J = 7.3)], two 1,2,4-trisubstituted benzene rings [δ 6.47 (dd, 1H, J = 8, 1.5 Hz), 6.60 (d, 1H, J = 1.5), 6.67 (d, 1H, J = 8), 6.70 (dd, 1H, J = 8, 1.5), 6.84 (d, 1H, J = 1.5), and 6.86 (d, 1H, J = 8)], and three noncoupled aromatic protons [δ 6.80 (s, 1H), 7.06 (s, 1H), and 7.13

Table 2. NMR Assignments for Ningalin B (2)[a]

C no.	^{13}C	^{1}H	HMBC (8 Hz)	NOESY
1	156.0 (C)			
2	114.8 (C)			
3	127.6 (C)			
4	110.6 (C)			
5	146.0 (C)			
6	104.0 (CH)	6.80 (s, 1H)	C4, C5, C7, C8	
7	142.9 (C)			
8	146.8 (C)			
9	109.3 (CH)	7.13 (s,1H)	C3, C5, C7, C8	H21, H25
10	50.9 (CH2)	4.55 (t, 2H, J = 7.3)	C2, C11, C12, C18	H11, H13, H17, H18
11	37.9 (CH2)	2.94 (t, 2H, J = 7.3)	C10, C12, C13, C17	H10, H13, H17, H18
12	130.2 (C)			
13	116.9 (CH)	6.60 (d, 1H, J = 1.5)	C14, C15, C17	H10, H11
14	145.9 (C)			
15	144.4 (C)			
16	116.1 (CH)	6.67 (d, 1H, J = 8)	C12, C14, C15	H17
17	120.6 (CH)	6.47 (dd, 1H, J = 8, 1.5)	C13, C15	H10, H11, H16
18	133.5 (CH)	7.06 (s, 1H)	C2, C3, C19, C20	H10, H11, H25
19	120.0 (C)			
20	126.4 (C)			
21	117.7 (CH)	6.84 (d, 1H, J = 1.5)	C19, C23, C25	H9
22	145.8 (C)			
23	145.5 (C)			
24	116.4 (CH)	6.86 (d, 1H, J = 8)	C20, C22	H25
25	112.7 (CH)	6.70 (dd, 1H, J = 8, 1.5)	C19, C21, C23, C24	H9, H18

[a] Spectra were acquired in a DMSO-d_6/CD_3OD mixture (5:1) with internal TMS. J values are reported in hertz, and chemical shifts are given in δ units. Assignments were aided by DEPT, COSY, and XHCORR experiments. All experiments, except for XHCORR (200 MHz), were performed at 500 MHz.

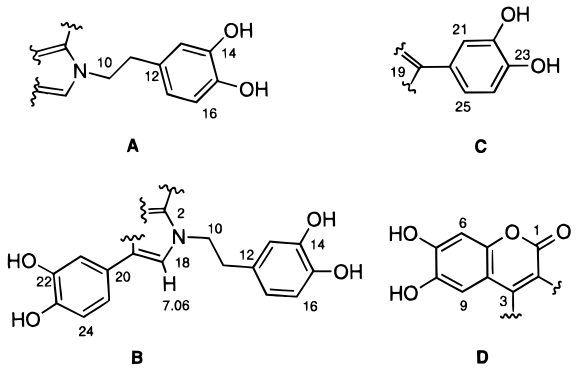


Figure 2. Partial structures of ningalin B (**2**) based on 2D NMR correlation methods.

(s, 1H)] in the molecule (Figure 2). A 1,2,4-trisubstituted benzene ring (substructure **A,** Figure 2) was constructed from analysis of ^{1}H NMR coupling constants [δ 6.47 (dd, 1H, J = 8, 1.5 Hz), 6.60 (d, 1H, J = 1.5), and 6.67 (d, 1H, J = 8)] and on the basis of COSY and HMBC experiments. The proton signals at δ 6.60 (d, 1H, J = 1.5 Hz) and 6.67 (d, 1H, J = 8) showed HMBC correlations to two quaternary carbons signals at δ 144.4 and 145.9. However, the proton signal at δ 6.47 (dd, 1H, J = 8, 1.5 Hz) showed correlations to only the carbon signal at δ 144.4 (C). Again, since $^4J_{CH}$ couplings were rarely observed in HMBC experiments performed with J values at 8 Hz, the carbon signal at δ 144.4 was assigned to C-15. On the basis of chemical shifts, two hydroxyl groups were placed at carbons C-14 and C-15, which was supported by the high-field shifts of the two adjacent carbons, C-13 (δ 116.9) and C-16 (δ 116.1). The mutually coupled proton signals at δ 2.94 (t, 2H, J = 7.3 Hz) and 4.55 (t, 2H, J = 7.3) directly correlated to the carbon signals at δ 37.9 (CH_2) and 50.9 (CH_2), respectively. The chemical shift of C-10 (δ 50.9) suggested that the nitrogen atom was located at this position. In the HMBC spectrum of ningalin B, the H-10 proton at δ 4.55 (t, 2H, J = 7.3 Hz) correlated to two carbons at δ 133.5 and 114.8, assigned as C-18 and C-2, respectively. The adjacent C-11 protons [δ 2.94 (t, 2H, J = 7.3 Hz)] showed correlations to carbon signals at δ 130.2 (C-12), 116.9 (C-13), and 120.6 (C-17). Since these latter carbon atoms were established by HMBC data as part of a 3,4-dihydroxyphenyl group, these data defined a dopamine residue forming the pyrrole nucleus (as in substructure **B**).

The presence of another 1,2,4-trisubstituted benzene ring (as in substructure **C**) was also established by comprehensive HMBC and 2D NMR data. By subtraction of the structural features defined above, ningalin B

Table 3. NMR Assignments for Ningalin C (3)[a]

C no.	^{13}C	^{1}H	HMBC (8 Hz)	NOESY
1	182.8 (C)			
2	118.7 (C)			
3	123.4 (C)			
4	118.4 (CH)	6.72 (d, 1H, J = 1.5)	C2, C5, C6, C8	H17, H18
5	144.6 (C)			
6	145.4 (C)			
7	115.0 (CH)	6.79 (d, 1H, J = 7.8)	C3, C5, C6	H8
8	122.1 (CH)	6.58 (dd, 1H, J = 7.8, 1.5)	C4, C6	H7, H17, H18
9	146.4 (C)			
10	122.9 (C)			
11	122.3 (C)			
12	133.1 (C)			
13	113.4 (CH)	7.33 (s, 1H)	C1, C11, C14, C15	
14	147.8 (C)			
15	149.6 (C)			
16	112.3 (CH)	7.12 (s, 1H)	C10, C12, C14, C15	H16, H32
17	42.6 (CH2)	3.30 (dt, 1H, J = 14.7, 7.8) 3.23 (dt, 1H, J = 14.7, 7.8)	C9, C18, C19, C25	H4, H8, H18, H20, H24
18	33.9 (CH2)	2.24 (t, 2H, J = 7.8)	C17, C19, C20, C24	H4, H8, H17, H20, H24
19	128.7 (C)			
20	116.0 (CH)	6.31 (d, 1H, J = 1.5)	C18, C21, C22, C24	H17, H18
21	144.9 (C)			
22	143.6 (C)			
23	115.3 (CH)	6.48 (d, 1H, J = 7.8)	C19, C21, C22	H24
24	119.1 (CH)	5.95 (dd, 1H, J = 7.8, 1.5)	C18, C20, C22	H17, H18, H23
25	170.4 (C)			
26	129.5 (C)			
27	121.5 (C)			
28	116.8 (CH)	6.84 (d,1H, J = 1.5)	C26, C29, C30, C32	H16
29	145.6 (C)			
30	146.6 (C)			
31	115.9 (CH)	6.86 (d, 1H, J = 7.8)	C27, C29, C30	H32
32	121.2 (CH)	6.74 (dd, 1H, J = 7.8, 1.5)	C26, C28, C30	H16, H31
OH		8.68 (s, 1H)[b]		
OH		9.14 (s, 1H)[b]		
OH		9.82 (s, 1H)[b]		
OH		9.99 (s, 1H)[b]		
OH		9.09 (s, 1H)[b]		
OH		8.64 (s, 1H)[b]		
OH		9.24 (s, 1H)[b]		
OH		9.42 (s, 1H)[b]		

[a] Spectra were obtained in DMSO-d_6. Assignments were aided by DEPT, COSY, and HMQC experiments. J values are reported in hertz, and chemical shifts are given in δ units. All experiments were performed at 500 MHz. [b] All values are reported in DMSO-d_6 with trace amounts of TFA with reference to internal TMS.

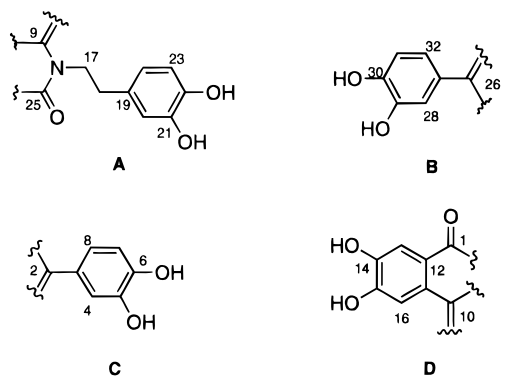


Figure 3. Partial structures of ningalin C (**3**) based on 2D NMR correlation methods.

was recognized to contain a C_9 residue (as in substructure **D**, C-1 to C-9) found by NMR analysis to possess the identical substructure as that in ningalin A. Evaluation of 2D NMR data allowed this unit to be linked to the other structural fragments, leading to the final structure assignment of ningalin B as **2**. As expected, acetylation yielded the hexaacetate **2a** [HRFABMS, obsd $(M + H)^+$ m/z 714.1811, $C_{37}H_{32}NO_{14}$ dev −1.7 ppm] which demonstrated the presence of six nonequivalent hydroxyl groups.

Ningalin C (**3**) was obtained as a dark-red amorphous powder. The molecular formula, $C_{32}H_{23}NO_{10}$, indicating 22 units of unsaturation, was determined by HRFABMS and combined NMR methods (Table 3). Even longer wavelength absorptions (λ_{max} = 450, 355, 301, 289 and 204 nm) were observed in the UV−vis absorption spectrum of ningalin C, suggesting a more complex aromatic chromophore to be present than found in **2**. A bathochromic shift was observed upon addition of base, while addition of acid did not effect the absorptions of this molecule. These data indicated **3** possessed similar hydroxyl and nonbasic nitrogen structural elements as in **1** and **2**. As in **2**, all protons and carbons were magnetically distinct; thus ningalin C (**3**) lacked a symmetry plane. In contrast to **1** and **2**, however, ningalin C possessed strong IR absorptions at 1699 and 1623 cm^{-1} and carbonyl carbon resonances at δ 182.8 (C-1) and 170.4 (C-4), indicative of the presence of a conjugated ketone and an amide functionality. Interpretation of ^1H and ^{13}C NMR data for ningalin C (**3**), including HMQC and HMBC heterocorrelation spectra, led to the assignment of all protons and carbons and confirmed several subunits in the molecule (Figure 3). The ^1H and COSY NMR spectra of ningalin C revealed the presence of four different spin systems, a 1,2,4,5-tetrasubstituted benzene and three 1,2,4-trisubstituted benzene rings, one of which was included in a dopamine unit as in **2**. Heterocorrelation NMR data showed that the dopamine nitrogen formed part of an amide group [C-25 at δ 170.4 (s)] (substructure **A**). These data further allowed a quaternary carbon (C-26) at δ 129.5 to be connected to another 4-monosubstituted catechol moiety as in substructure **B** (C-27−C-32). The same connectivity was established between C-2 (δ 118.7) and C-3 (δ 123.4) using $^1H-^{13}C$ correlation methods to lead to substructure **C**. The last substructure, **D**, composed of a 3,4-disubstituted catechol moiety (C-11−C-16), was constructed by analogous interpretation of NMR data. Thus, four partial structures were firmly established.

The long-wavelength UV absorption of **3** (450 nm), coupled with the presence of an unsaturated ketone carbonyl, suggested the presence of a quinoidal chromophore. Subsequently, a quinone methide moiety was assigned involving six carbons, C-1 (δ 182.8), C-2 (δ 118.7), C-9 (δ 146.4), C-10 (δ 122.9), C-11 (δ 122.3), and C-12 (δ 133.1), requiring connectivities between C-10 and C-26, and between C-26 and C-25. The structure assigned for ningalin C (**3**) was also supported by data derived from NOESY NMR experiments. NOESY correlations from H-17 to both H-4 and H-8 indicated that one of the catechol rings (C-3−C-8) was flanking the dopamine moiety. The observed NOE correlations from H-16 to protons H-28 and H-32 suggested that the other catechol ring (C-27−C-32) was also in proximity of the 4,5-disubstituted catechol moiety (**D**). The ^1H NMR spectrum of **3**, recorded in DMSO-d_6 with a trace amount of trifluoroacetic acid, showed eight sharp signals, each of which represented an OH proton. Acetylation of ningalin C (**3**) yielded the expected octaacetate **3a** [HRFABMS obsd $(M + H)^+$ m/z 918.2186, $C_{48}H_{40}NO_{18}$ dev −6.4 ppm].

Once again, analysis of molecular models for ningalin C suggested that restricted rotation of the catechol rings was to be expected on the basis of steric crowding. Indeed, a significant upfield shift of the C-17 and C-18 protons in the dopamine moiety, compared to those values observed for the same protons in ningalin B (**2**), provided further proof that one of the catechol rings (C-3−C-8) was in close spatial proximity. These results were fully consistent with the proposed structure for ningalin C (**3**).

Ningalin D (**4**), the largest of these alkaloids, was obtained as a dark-red amorphous solid. The HRFAB mass spectrum of ningalin D, in combination with NMR spectral data, established the molecular formula as $C_{40}H_{27}NO_{12}$ (28 units of unsaturation). The ^1H NMR spectrum of **4** (Table 4) showed eight aromatic signals, five of which represented two protons by integration. Three signals at δ 5.95 (dd, 1H, J = 7.5, 1.5 Hz), 6.12 (d, 1H, J = 1.5), and 6.30 (d, 1H, J = 7.5) integrated for one proton each. In addition, two aliphatic signals, integrating for two protons each, were observed at δ 2.13 (t, 2H, J = 6.5 Hz) and 2.95 (t, 2H, J = 6.5). Consistent with these ^1H NMR data, only 24 signals could be observed in the ^{13}C NMR spectrum of ningalin D. This information showed that there was a symmetry plane in the molecule, analogous to the situation in ningalin A. As in ningalin C, the presence of a dopamine unit was readily assigned and shown by overall NMR features to fall within the symmetry plane. Subtracting the dopamine component left identical $C_{16}H_9O_5$ structures on each side of the plane.

The UV spectrum of ningalin D showed long wavelength absorption at λ_{max} = 508 nm (ϵ 9900) with additional absorptions at 404 (sh), 294 (17 000), 276 (18 000), and 206 nm (41 000), indicating an even more complex chromophore to be present. A bathochromic shift was observed upon addition of base, suggesting once again the phenolic nature of ningalin D (**4**). The addition of acid produced no modifications in the UV absorptions of ningalin D, indicating as in the other ningalins that the nitrogen is not in a basic form. The IR spectrum of ningalin D showed a strong hydroxyl band at 3700−3000 cm^{-1} and a carbonyl absorption at 1665 cm^{-1}. These data, in conjunction with a carbon signal at δ 185.4 (s), indicated that ningalin D possessed an α,β-unsaturated ketone. The NMR data for **4** [specifically two mutually coupled methylenes at δ 2.13 (t, 2H, J = 6.5 Hz) and 2.95 (t, 2H, J = 6.5), and three aromatic signals at δ 5.95 (dd,

Table 4. NMR Assignments for Ningalin D (4)[a]

C no.	^{13}C[b]	^{1}H[b,c]	HMBC (8 Hz)[b,c]	NOESY[d]
1, 1′	185.4 (C)			
2, 2′	118.9 (C)			
3, 3′	126.1 (C)			
4, 4′	119.4 (CH)	6.82 (d, 2H, J = 1.5 Hz)[e]	C2, C2′, C5, C5′ C6, C6′, C8, C8′	H17, H18
5, 5′	146.1 (C)			
6, 6′	146.5 (C)			
7, 7′	116.2 (CH)	6.83 (d, 2H, J = 8)[e]	C3, C3′ C5, C5′, C6, C6′	H8, H8′
8, 8′	124.2 (CH)	6.68 (dd, 2H, J = 8, 1.5)[e]	C2, C2′, C4, C4′, C6, C6′	H7, H7′, H17, H18
9, 9′	156.4 (C)			
10, 10′	126.4 (C)			
11, 11′	125.0 (C)			
12, 12′	132.2 (C)			
13, 13′	115.0 (CH)	7.45 (s, 2H)	C1, C1′, C11, C11′, C15, C15′	
14, 14′	148.9 (C)			
15, 15′	150.2 (C)			
16, 16′	114.2 (CH)	7.85 (s, 2H)	C10, C10′, C12, C12′, C14, C14′, C15, C15′	
17	47.5 (CH2)	2.95 (t, 2H, J = 6.5)	C9, C9′, C18, C19	H4, H4′, H8, H8′, H18, H20, H24
18	34.5 (CH2)	2.13 (t, 2H, J = 6.5)	C17, C19, C20, C24	H4, H4′, H8, H8′, H17, H20, H24
19	130.5 (C)			
20	117.1 (CH)	6.12 (d, 1H, J = 1.5)	C18, C21, C22, C24	H17, H18
21	145.5 (C)			
22	144.6 (C)			
23	115.7 (CH)	6.30 (d, 1H, J = 7.5)	C19, C21, C22	H24
24	121.4 (CH)	5.95 (dd, 1H, J = 7.5, 1.5)	C18, C20, C22	H17, H18, H23

[a] ^{13}C NMR data were recorded at 125 MHz. Assignments were aided by DEPT, COSY, and HMQC experiments. [b] Spectra were obtained in MeOH-d_4. [c] Experiments were carried out at 500 MHz. [d] The NOESY experiment was performed at 500 MHz in DMSO-d_6. [In this experiment, the ^{1}H NMR spectrum was also recorded at 500 MHz in DMSO-d_6 (protons H4, H7, and H8 appeared at δ 6.76 (s, 2H), 6.72 (d, 2H, J = 8 Hz), and 6.59 (d, 2H, J = 8), respectively).]

1H, J =7.5, 1.5 Hz), 6.12 (d, 1H, J = 1.5), and 6.30 (d, 1H, J = 7.5)] showed the presence of the familiar dopamine moiety. As in ningalins B and C, HMQC and HMBC experiments established the connectivity between C-18 (δ 34.5) and C-19 (δ 130.5). The proton signal at δ 2.95, assigned as H-17, showed HMBC correlations to a quaternary carbon at δ 156.4, assigned as C-9, via three-bond coupling through the nitrogen atom. Another quaternary carbon at δ 118.9, assigned as C-2, showed correlations to H-4 (δ 6.82) and H-8 (δ 6.68) in the HMBC spectrum, thus showing a connection to a 4-monosubstituted catechol ring (C-3−C-8). A 4,5-disubstituted catechol moiety (C-11−C-16) analogous to that in **1**−**3,** but identical to that in **3**, was also identified by correlations of H-13 (δ 7.45) to C-1 (δ 185.4), C-11 (δ 125.0) and C-15 (δ 150.2), and by correlations of H-16 (δ 7.85) to C-10 (δ 126.4), C-12 (δ 132.2), C-14 (δ 148.9) and C-15 (δ 150.2). The large difference in chemical shift between C-2 (δ 118.9) and C-9 (δ 156.4) could be rationalized only if C-2 was α and C-9 β to the conjugated carbonyl (C-1). Although numbered differently, these data defined half of ningalin D as identical to part of ningalin C. Establishing the remaining half resulted, by definition, in a quinone methide structure which was recognized to justify the color and long-wavelength absorptions of this molecule. The structure assigned to ningalin D, **4**, was also fully supported by NOESY NMR experiments. NOESY correlations from H-17 (δ 2.95) and H-18 (δ 2.13) to H-4 and H-4′ (δ 6.82), and H-8 and H-8′ (δ 6.68) confirmed that two catechol rings (C-3−C-8 and C-3′−C-8′) were flanking the dopamine moiety.

Ningalin D possesses a unique functionality, a biphenylene quinone methide. This functional group is clearly responsible for the color of this molecule providing a chromophore with highly extended conjugation. A similar molecule, purpurone, recently isolated from a sponge, is closely related in that it possesses one less hydroxyl group on the phenethyl aromatic ring. Indeed, the overall spectral data of **1** were in close accord with those reported for purpurone.[16]

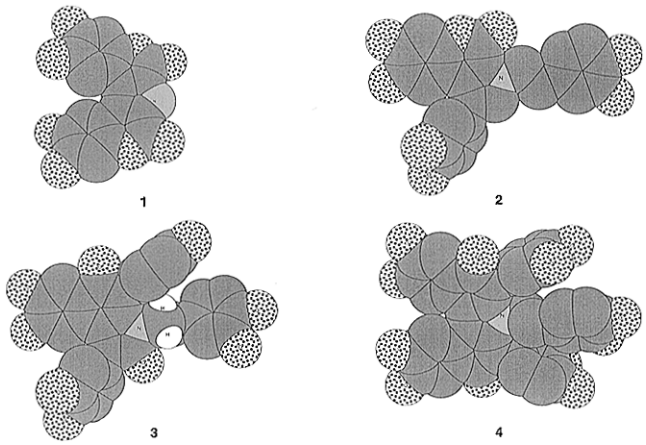


Figure 4. Computer-generated perspective drawings of one of the lowest energy forms for the ningalins A−D (**1**−**4**). Hydrogen atoms, except for those at C-17 in ningalin C (**3**) (shown to illustrate their diastereotopic character), are omitted for clarity.

By virtue of their condensed aromatic structures, ningalins A−D incorporate severe steric crowding resulting in interesting conformational restrictions. These conformations result in proton NMR shift anomalies and are predictable based upon molecular models. Computer analysis[17] of the four alkaloids was performed, and one of the lowest energy forms, as illustrated using van der Waals radii models, is shown in Figure 4. In order to avoid severe steric interactions, the pentacyclic carbon skeletons of ningalin A (**1**) and ningalin D (**4**) are

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(17) Computer analysis of the low-energy isomers of ningalins A−D was performed utilizing pcmodel, version 4.51, released from Serena Software and the display was mediated by Chem-3D from Cambridge Scientific Computing.

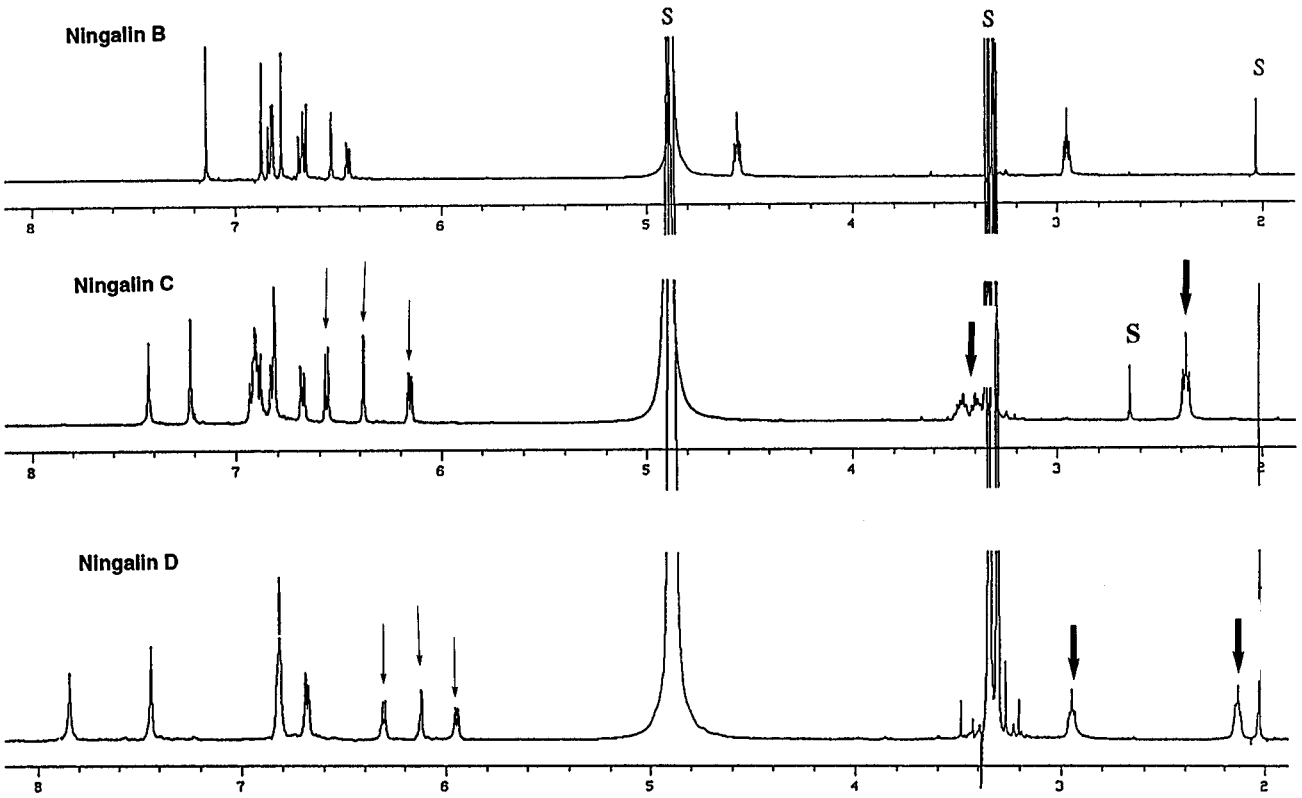

Figure 5. Proton NMR spectra (500 MHz) of ningalins B−D (**2**−**4**) illustrating the effects of adjacent catechol functionalities on the chemical shifts of the dopamine methylene and aromatic protons. The bold arrows indicate the additive shielding effects observed for the dopamine methylene protons while the narrow arrows illustrate the same phenomenon for the adjacent three catechol ring protons of the dopamine moieties. S refers to solvent (MeOH-d_4, HDO, or DMSO) peaks.

predicted to adopt twisted conformations. In ningalins B (**2**), C (**3**), and D (**4**), the phenyl rings of the catechol moieties were predicted to be pseudoorthogonal to the planes of the molecules, by analogy to the structures of the lamellarins which also possess these functionalities.[4,5] On the basis of computer analysis and inspection of simple molecular models, it was clear that the rotations of the catechol rings would be restricted in ningalins B−D. The methylene proton signals at C-17 in **3** were predicted to be magnetically nonequivalent due to their asymmetric environment. In **4**, however, the C-17 protons are magnetically equivalent based upon the presence of a symmetry plane. Only one of the isomeric forms of **4** is presented in Figure 4.

On the basis of the restricted rotation predicted above, and because of the apparent helicity in ningalins A and D, the ningalins could be chiral. Several of the related lamellarins are chiral by virtue of their restricted rotations.[5c,d] Similar pentacyclic aromatic systems appear sufficiently helical to generate optical isomerism.[18] From a theoretical perspective, the rotational complexity of ningalin D (**4**) generates at least four possible diastereomeric rotamers with different orientations of the catechol rings. In addition, the issue of helical chirality is superimposed on these isomeric structures. Consideration of the behaviors of synthetic helicenes gave insight into the possibity of helical chiralty in the ningalins. While pentahelicene can be resolved,[18a] the thermal racemization of helicenes[18a,19] through Cs-twisted transition states[20] has been readily observed. The half-life ($t_{1/2}$) for thermal racemization of pentahelicene is only 63.7 min at 57 °C, a temperature which approaches that required for solvent removal.[21] Furthermore, racemization was observed to greatly accelerate as five-membered rings were substituted for phenyl rings.[22] In such a case, the $t_{1/2}$ of a hexahelicene possessing three thiophene rings was only 13 min at 25 °C.

Ningalins A−D showed zero rotation at the sodium D line, indicating that they are racemic. On the basis of the behavior of the lamellarins,[5c] and the helicenes, it would appear that the energy barriers to rotation and helical racemization are low and that racemization is a facile process.

The more stable, pseudoorthogonal conformations of the catechol rings adjacent to the dopamine moiety generated significant proton NMR shielding effects of the dopamine ethylene protons (Figure 5). The shielding and deshielding effect of the phenyl ring is well-known,[23] and this is utilized in the Mosher ester method to assess absolute stereochemistry.[24] The shielding of protons near a benzene ring is a function of distance along the orthogonal axis from the center of the benzene ring, but the deshielding of the protons is a function of distance along the hexagonal axis.[23] In **2**, the C-10 and C-11

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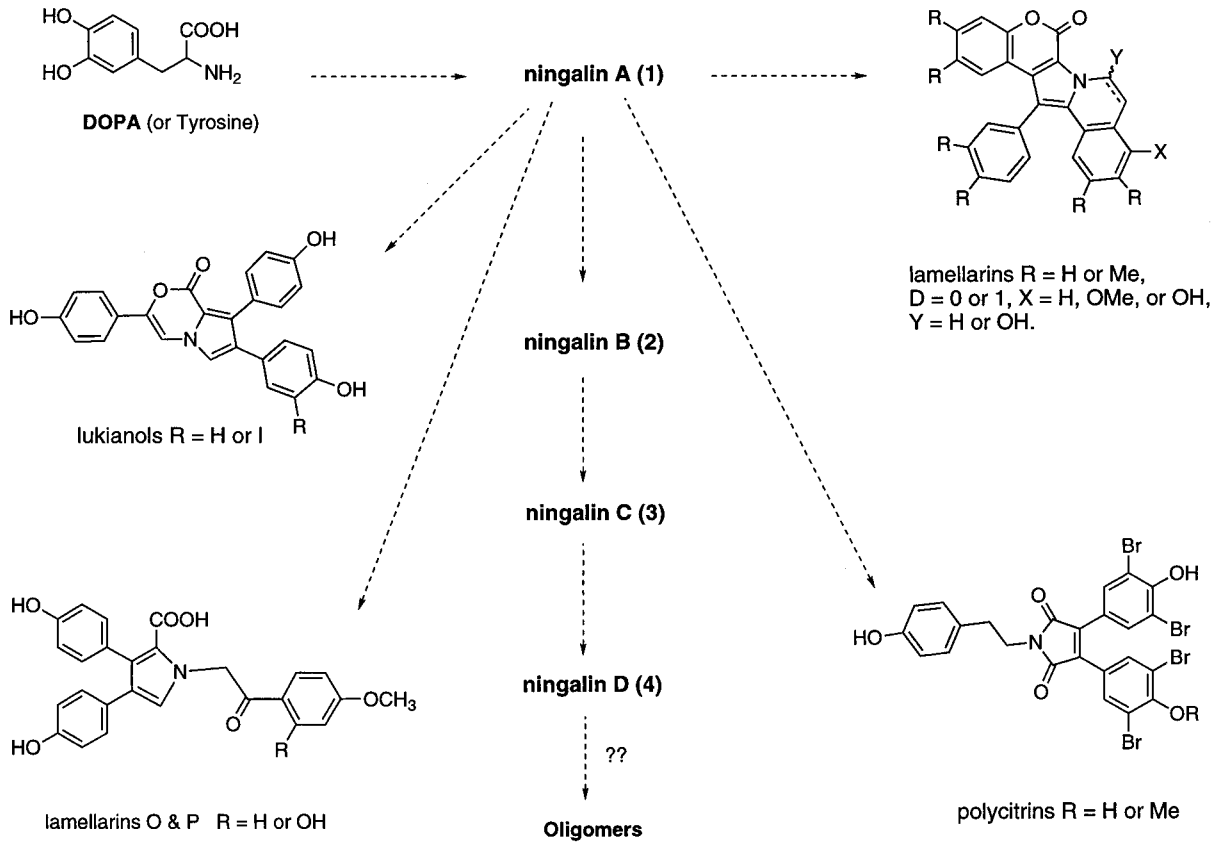

Figure 6. Possible biosynthetic pathways for several apparently DOPA-derived alkaloids from marine ascidians. Lamellarins O and P, isolated from the sponge, *Dendrilla cactos*, were included in the biosynthetic scheme due to their close analogy to the ascidian-derived lukianols.

methylene protons in the dopamine moiety resonated at δ 4.55 and 2.94, respectively. The same protons in **3,** however, appeared at δ 2.24, and 3.23 and 3.30 which were significantly shielded compared to those in **2** (Figure 5). All shielding effects observed in **3** could be rationalized by the diamagnetic anistropy of the adjacent catechol ring at C-2. The effect of the catechol rings to the ethylene moiety is even more conspicuous in **4**. Two flanking catechol rings at (C-2 and C-2′) significantly shielded the dopamine protons by as much as 1.60 ppm compared to those of **2**.

Although the biosynthesis of the ningalins remains to be demonstrated, these alkaloids appear to be condensation products of the amino acid DOPA. The ningalins appear derived from the condensation of two, three, four, and five DOPA precursors, respectively (Figure 6). Interestingly, the apparent mechanism by which these alkaloids are produced is consistent in the ningalins, even though each has a completely different carbon skeleton. Other ascidian compounds which are apparently produced by similar condensations are the lamellarins,[4,5,25] polycitrins,[7] and lukianols.[6] The close analogy between purpurone,[16] isolated from the sponge *Iotrochota* sp., and ningalin D indicate that DOPA condensation chemistry is biologically more widespread.

During the purification process, we also observed a highly insoluble black substance which possessed the same aromatic NMR characteristics as **1**−**4**. In the initial solvent partitions, a black powder was obtained at the interface of the 1-butanol−water mixture. The powder was filtered and found to be only very sparingly soluble in warm DMSO. Adding a trace of trifluoroacetic acid improved the solubility such that a poorly resolved proton NMR spectrum could be recorded. Although more complex than those of the ningalins A−D, the spectrum showed the same aromatic proton resonances and overall features of these DOPA-derived metabolites. This compound, although unworkable because of its poor solubility, may be a major component in this animal. On the basis of its proton NMR features, the compound appears to be a higher homologue of DOPA condensation.

Although a potential role for these alkaloids in metal sequestration has not been demonstrated, very similar ascidian-derived catechols appear to be involved in iron accumulation.[22] In didemnid ascidians, vanadium accumulation is negligible, but iron accumulation is a prominent feature generating *in vivo* iron concentrations up to 10^7 times higher than that found in seawater.[12] Since iron is known to chelate with catechols,[13] these compounds could conceivably function in this interesting process.

Experimental Section

Collection, Extraction, and Isolation Procedures. The purple encrusting ascidian collections, both identified as the same *Didemnum* sp. (our sample numbers WA90-19 and WA90-58) were collected using SCUBA (−10 m) near Exmouth, Western Australia, in December 1990. The specimens were immediately frozen after collection. The lyophilized animals were extracted twice with 70% MeOH/$CHCl_3$ and MeOH. The combined extract was concentrated and partitioned between hexane and methanol, and the methanol-soluble material was further partitioned between ethyl acetate and water. Gel

(25) Urbon, S.; Butler, M. S.; Capon, R. J. *Aust. J. Chem.* **1994**, *47*, 1919.

filtration of the ethyl acetate-soluble material through Sephadex LH-20 with methanol as the eluent gave a semipure ningalin A (**1**) and several fractions which contained ningalins B−D (**2**−**4**). Further purification of the ningalins was accomplished by reversed-phased HPLC (ODS-silica). Pure samples of ningalins B and C (**2**, **3**) were obtained when a 35% CH_3CN−5% methanolic water solution was used as the eluting solvent. The most polar compound ningalin D (**4**) was eluted using 30% CH_3CN−5% methanolic water as the solvent. Final purification of ningalin A (**1**) required an additional gel permeation chromatography on Spectra Gel HW-40 using MeOH as the eluent. Yields of ningalins A−D (**1**−**4**) were 20 mg (0.0058% dry wt), 60 mg (0.017% dry wt), 23.4 mg (0.0068% dry wt), and 36 mg (0.010% dry wt), respectively.

Ningalin A (1): amorphous yellow solid; $[\alpha]_D = 0°$ (*c* 0.5, 10% DMSO/MeOH); HRFABMS, obsd $(M + H)^+$ *m*/*z* 368.0420, $C_{18}H_{10}NO_8$, dev 3.7 ppm; IR (NaCl) 3377, 3186, 1702, 1617, 1494, 1375, 1256, 1153 cm^{-1}; UV (10% DMSO/MeOH) λ_{max} 370 nm (ϵ 2160), 325 (sh), 303 (8450), 262 (8650); UV (10% DMSO/MeOH + NaOH) λ_{max} 393 nm (ϵ 1970), 350 (sh), 314 (9150), 274 (8450).

Ningalin B (2): amorphous dark yellow solid; $[\alpha]_D = 0°$ (*c* 0.5, MeOH); HRFABMS, obsd $(M + H)^+$ *m*/*z* 462.1189, $C_{25}H_{20}NO_8$ dev −0.109 ppm; IR (NaCl): 3700−3000, 1698, 1602, 1561, 1527, 1414, 1281, 1158, 1023, 984 cm^{-1}; UV−vis (MeOH) λ_{max} 332 nm (ϵ 8700), 289 (10 000), 236 (sh), 204 (46 000); UV−vis (MeOH + NaOH) 363 nm (ϵ 8100), 292 (10 000), 245 (sh), 204 (62 000).

Ningalin C (3): amorphous red solid; $[\alpha]_D = 0°$ (*c* 0.2, MeOH); HRFABMS, obsd $(M + H)^+$ *m*/*z* 582.1385, $C_{32}H_{24}NO_{10}$ dev −2.6 ppm; IR (NaCl) 3700−3000, 1699, 1623, 1593, 1515, 1431, 1393, 1360, 1292, 1200, 1117, 1022, 983 cm^{-1}; UV−vis (MeOH) λ_{max} 450 nm (sh), 355 (ϵ 5800), 301 (sh), 289 (9400), 204 (34 000); UV−vis (MeOH + NaOH) 530 nm (sh), 351 (sh), 309 (ϵ 12 000), 204 (43 000).

Ningalin D (4): amorphous dark-red solid; $[\alpha]_D = 0°$ (*c* 0.025, MeOH); HRFABMS (thioglycerol), obsd $(M + 2H)^+$ *m*/*z* 715.1718, $C_{40}H_{29}NO_{12}$ dev −3.9 ppm); LRFABMS (NBA), obsd $(M + H)^+$ *m*/*z* 714 and $(M + Na)^+$ *m*/*z* 736; IR (NaCl) 3700−3000, 1665, 1562, 1296 cm^{-1}; UV−vis (MeOH) λ_{max} 508 (ϵ 9900), 404 (sh), 294 (17 000), 276 (18 000), 206 (41 000); UV−vis (MeOH + NaOH) λ_{max} 518 (ϵ 4800), 314 (17 000), 274 (19 000), 206 (98 000).

Preparation of Acetates 1a−4a. In a typical procedure, acetic anhydride (0.5 mL) was added to a solution of **3** (5 mg) in pyridine, and the solution was stirred for 2 h at room temperature. The reaction was monitored by TLC. Reagents were evaporated under high vacuum and the residue was partitioned sequentially between ethyl acetate and ice water, between ethyl acetate and 0.1 $NaHCO_3$ solution, and between ethyl acetate and brine. The product was dried with $MgSO_4$, the solids were filtered, and the solvent was removed under vacuum. The residue was purified by reversed-phased HPLC (ODS-silica) using 70% CH_3CN−6% methanolic water to give the octaacetate **3a** (3 mg) as a yellow-green amorphous solid. In all cases, the yields of the acetates **1a**−**4a** were nearly quantitative.

Tetraacetate 1a: yellow, amorphous solid; LRFABMS, obsd $(M + H)^+$ *m*/*z* 536, $(M + Na)^+$ *m*/*z* 558; HRFABMS, obsd $(M + H)^+$ *m*/*z* 536.0837, $C_{26}H_{18}NO_{12}$ dev 1.5 ppm; ^1H NMR (500 MHz, CD_2Cl_2/MeOH-d_4 = 3/2) δ 2.37 (s, 6H), 2.39 (s, 6H), 7.38 (s, 2H), 8.32 (s, 2H).

Hexaacetate 2a: dark yellow oil; HRFABMS, obsd $(M + H)^+$ *m*/*z* 714.1811, $C_{37}H_{32}NO_{14}$ dev −1.7 ppm; ^1H NMR (500 MHz, $CDCl_3$) δ 2.26 (s, 6H), 2.28 (s, 3H), 2.29 (s, 3H), 2.31 (s, 3H), 2.32 (s, 3H), 3.16 (t, 2H, *J* = 8 Hz), 4.67 (t, 2H, *J* = 8), 6.83 (s, 1H), 6.97 (d, 1H, *J* = 8), 6.98 (s, 1H), 7.09 (d, 1H, *J* = 8), 7.21 (d, 1H, *J* = 8), 7.25 (d, 1H, *J* = 8), 7.27 (s, 2H), 7.62 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 20.6 (CH_3, 6C), 37.4 (CH_2), 50.8 (CH_2), 112.4 (CH), 115.8 (C), 116.4 (C), 117.9 (CH), 118.9 (C), 123.6 (CH), 123.9 (CH, 2C), 124.7 (CH), 125.5 (C), 127.1 (CH), 127.7 (CH), 132.0 (C), 132.3 (CH), 136.4 (C), 138.3 (C), 141.0 (C), 141.4 (C), 141.7 (C), 142.0 (C), 142.1 (C), 148.9 (C), 154.5 (C), 168.0 (C), 168.2 (C, 2C), 168.3 (C, 2C), 168.6 (C).

Octaacetate 3a: yellow-green, amorphous solid; HRFABMS, obsd $(M + H)^+$ *m*/*z* 918.2186, $C_{48}H_{40}NO_{18}$ dev −6.4 ppm; ^1H NMR (500 MHz, $CDCl_3$) δ 2.15 (s, 3H), 2.17 (s, 3H), 2.18 (s, 3H), 2.22 (s, 3H), 2.23 (s, 3H), 2.24 (s, H), 2.26 (s, 3H), 2.27 (s, 3H), 2.46 (t, 2H, *J* = 8 Hz), 3.60 (t, 2H, *J* = 8), 6.16 (dd, 1H, *J* = 8, 2), 6.66 (d, 1H, *J* = 2), 6.95 (d, 1H, *J* = 8), 7.18 (dd, 1H, *J* = 8, 2), 7.20 (d, 1H, *J* = 2), 7.25 (d, 1H, *J* = 8), 7.31 (d, 1H, *J* = 8), 7.38 (d, 1H, *J* = 2), 7.42 (dd, 1H, *J* = 8, 2), 7.66 (s, 1H), 7.89 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 20.5 (4C), 20.62, 20.65, 20.69, 20.73, 34.4, 42.8, 121.5, 123.3, 123.5, 123.7, 124.2, 125.3, 126.5, 127.3, 127.6, 127.9, 128.2, 128.6, 128.8, 129.0, 129.1, 130.1, 130.6, 133.7, 136.1, 140.7, 141.7, 141.8, 142.2, 142.6, 143.6, 144.1, 145.5, 147.4, 167.6, 167.72 (2C), 167.76, 167.82, 168.1 (2C), 168.3, 169.6, 181.6.

Decaacetate 4a: dark-green noncrystalline solid; HRFABMS, obsd $(M + 2H)^+$ *m*/*z* 1135.2708, $C_{60}H_{49}NO_{22}$ dev −3.4 ppm; ^1H NMR (500 MHz, $CDCl_3$) δ 2.13 (s, 3H), 2.14 (s, 3H), ~2.24 (2H), 2.28 (s, 6H), 2.30 (s, 6H), 2.33 (s, 6H), 2.35 (s, 6H), 3.23 (m, 2H), 6.43 (d, 1H, *J* = 1.5 Hz), 6.49 (dd, 1H, *J* = 8, 1.5), 6.75 (d, 1H, *J* = 8), 7.26 (d, 2H, *J* = 1.5), 7.28 (d, 2H, *J* = 8), 7.29 (dd, 2H, *J* = 8, 1.5), 7.98 (s, 2H), 8.18 (s, 2H).

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Supporting Information Available: ^1H and ^{13}C NMR and related NOESY, HMBC, XHCORR, and selected HMQC NMR spectra, and IR and LR-FAB-MS spectra for ningalins A−D (**1**−**4**) and their corresponding acetate derivatives (**1a**−**4a**) (41 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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