Two Complex Proline Esters from the Sea Hare *Stylocheilus longicauda*

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Investigation of *Stylocheilus longicauda* led to the isolation of chlorinated metabolites, makalika ester (**1**) and makalikone ester (**2**). Also reported is the isolation of lyngbyatoxin A acetate (**3**). The structures of the new compounds are based on spectroscopic data obtained from 1D and 2D NMR experiments.

While unsuccessfully attempting to recollect the sea hare *Aplysia parvula* at Black Point, Oahu, in August 1996, and again in February 1999, we observed large numbers of the sea hare *Stylocheilus longicauda*. Although we had studied the chemistry of this animal,[2−4] all earlier collections were from Kaneohe Bay, and it seemed worthwhile to check its chemistry at this site, as the chemical constituents of the animal are correlated to the source of its algal diet. This proved to be fortunate. Not only did we fail to reisolate known constituents, but we also succeeded in characterizing the makalika esters, unprecedented *N*-methylprolines esterified with a *tert*-butyl and chloro-substituted octatrienol, which are the subjects of this report.

Extraction of the freeze-dried animals with methanol yielded a green gum that was solvent−solvent partitioned with hexane and dichloromethane. Makalika ester (**1**) was obtained as a colorless oil (7.3 mg) after repeated chromatography of the hexane partition on silica, reversed-phase C_{18} and Sephadex LH-20 gels. Makalika ester (**1**) was found to have an elemental composition $C_{19}H_{30}ClNO_2$ on the basis of high-resolution mass spectrometry (340.2043 Da, $[M + H]^+$). Of the five double-bond equivalents implied by this molecular formula, ^{13}C NMR data provided evidence for the presence of six olefinic carbon atoms (113.9−144.4 ppm), thus three double bonds. The presence of a carbonyl group was inferred from the ^{13}C NMR spectrum (173.0 ppm); hence, the remaining degree of unsaturation was due to a ring. The infrared spectrum of **1** exhibited a strong band at 1741 cm^{-1}, characteristic of an ester carbonyl. Weaker signals at 1582 and 1650 cm^{-1} corresponded to the olefinic bonds.

The connectivity pattern of the molecule was determined by analysis of the observed *J* values, $^1H-^1H$ COSY, HMQC, and HMBC experiments (Table 1) in order to provide partial structures (a), (b), (c), and (d) (Figure 1).

The ^1H NMR spectrum of **1** exhibited downfield signals for two pairs of mutually coupled vinylic methines at 6.12 (H-8′) and 6.43 (H-7′) ppm (J = 13.3 Hz) and 5.66 (H-5′) and 6.01 (H-6′) ppm (J = 15.0 and 15.2 Hz, respectively). Further coupling was observed between methines at 6.01 (H-6′) and 6.43 ppm (H-7′) (J = 10.8 Hz), suggesting that a conjugated diene system was present [fragment (a)]. Based on the magnitude of the coupling constants, the bonds were deduced to be *trans*-oriented.[5] COSY coupling of the H-5′ methine to the resonance at 2.83 ppm (H-4′) established fragment (a), while HMBC correlation of the latter signal to the downfield olefinic carbon at 144.4 ppm established a point of attachment between fragments (a) and (b).

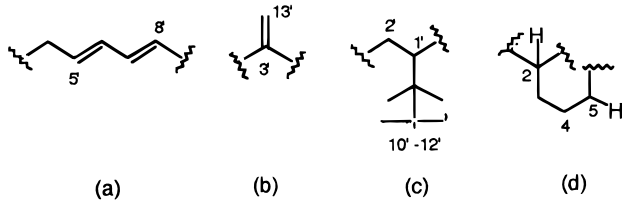

Figure 1. Partial structures (a)−(d) based on HMBC and COSY data of makalika ester (**1**).

The oxymethine proton at 4.93 ppm (H-1′) exhibited extensive COSY and HMBC coupling to adjacent atoms (Table 1). This proton (H-1′) shared a common coupling constant with the proton at 2.15 ppm (J = 10.9 Hz) and displayed cross coupling to the quaternary carbon signal at C-9′ (34.5 ppm). The ^1H NMR spectrum displayed signals for a 9H singlet (0.91 ppm) consisting of three CH_3 groups, which were HMBC-coupled to the quaternary C-9′ carbon, to delineate a *tert*-butyl moiety, thus establishing fragment (c). Further, coupling of the oxymethine to C-3′ afforded the linkage to fragment (b). Cross couplings were also observed between H-1′ and the carbonyl group (C-6) of the ester linkage.

Fragment (d) was defined based on $^1H-^1H$ COSY and HMBC correlations. The relatively downfield nature of the signals at C-2 (3.09, 67.2 ppm) and C-5 (3.19, 55.7 ppm) indicated that these carbons were linked to a heteroatom, nitrogen, based on the magnitude of the chemical shifts and the odd molecular mass of the molecule. Both C-2 and C-5 showed cross-peak connectivity to a three-proton methyl singlet (2.36 ppm), deduced to be attached to the nitrogen atom, thus affording an *N*-methyl pyrrolidine ring, which was attached to the remainder of the molecule via the C-2 methine.

The position of the chlorine atom remained to be determined. The point of attachment could be either on the ester carbonyl to form a chloroformate (expected chemical shift of about 150 ppm)[6] or to the alkene at C-8′. Based on the magnitude of the carbonyl resonance (173.0 ppm), the chloro residue was concluded to be attached to the terminal methine carbon at C-8′. The *N*-methylpyrrolidine ring was therefore deduced to be attached to the carbonyl of C-6, hence defining an *N*-methylproline residue.

The stereochemistry of the *N*-proline residue was determined by hydrolysis of the ester.[7] The amino acid was purified, and the optical rotation was found to be comparable with that of L-*N*-methylproline.

Makalikone ester (**2**) was obtained as a colorless oil. The high-resolution DCIMS data gave an $[M + H]^+$ peak at 354.1836 for a molecular formula of $C_{19}H_{28}ClNO_3$, indicating that it possessed six degrees of unsaturation, one more than makalika ester (**1**). The ^1H and ^{13}C NMR spectra of **2**

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Table 1. ^{1}H NMR, ^{13}C NMR, and HMBC Correlation Data for Makalika Ester (**1**)[a–c]

position	^{13}C (ppm)	^{1}H (ppm)	HMBC	COSY
2	67.2	3.09 (1H, br s)	C-3	H-3a, 3b
3	29.8	1.95 (1H, br s)		H-2
		2.20 (1H, br s)		H-2, H-4b
4	22.8	1.85 (1H, m)		H-5a, 5b
		1.95 (1H, m)		H-3b
5	55.7	3.19 (1H, m)	C-3	H-4a, H-5b
		2.45 (1H, m)	C-4	H-4a, H-5a
6	173.0			
1′	78.2	4.93 (1H, dd, J = 10.9, 2.1 Hz)	C-3′, C-9′, C-10′-12′, C-6	H-2′a
2′	36.8	2.15 (1H, dd, J = 13.9, 10.9 Hz)	C-3′	H-1′, H-2′b
		2.26 (1H, d, J = 13.8 Hz)	C-3′	H-2′a
3′	144.4			
4′	38.6	2.80 (1H, dd, J = 16.1, 7.6 Hz)	C-3′, C-5′, C-6′	H-5′
		2.88 (1H, dd, J = 16.0, 6.6 Hz)		H-5′
5′	132.7	5.66 (1H, dt, J = 15.0, 7.3 Hz)	C-4′, C-7′	H-4′, H-6′
6′	128.1	6.01 (1H, dd, J = 15.2, 10.8 Hz)	C-4′, C-7′	H-5′, H-7′
7′	133.4	6.43 (1H, dd, J = 13.3, 10.8 Hz)	C-5′	H-6′, H-8′
8′	119.3	6.12 (1H, d, J = 13.3 Hz)	C-6′, C-7′	H-7′
9′	34.5			
10′−12′	25.9	0.91 (9H, s)	C-1′, C-9′	
13′	113.9	4.75 (1H, d, J = 1.3 Hz)		
		4.79 (1H, s)		
N-CH$_3$	40.0	2.36 (3H, s)	C-2, C-5	

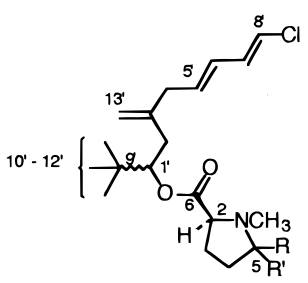
[a] NMR spectra were recorded in $CDCl_3$. [b] The multiplicites were determined by DEPT experiment. [c] HMBC and COSY data obtained from spectra recorded in CD_3CN; ^{1}H−^{13}C connectivities assigned by HMQC.

Table 2. ^{1}H NMR, ^{13}C NMR, COSY, and HMBC Correlation Data for Makalikone Ester (**2**)[a,b]

position	^{13}C (ppm)	^{1}H (ppm)	HMBC	COSY
2	62.1	4.06 (1H, dd, J = 3.03, 8.87 Hz)	C-3, 5	H-3a, 3b
3	23.0	2.06 (1H, m),	C-4, 5	H-2, H-4a, 4b
		2.33 (1H, m)	C-6	H-2, 4
4	29.3	2.33 (1H, m),	C-5	H-3a, 3b
		2.45 (1H, m)		
5	175.3			H-3a
6	171.5			
1′	79.1	4.94 (1H, dd, J = 2.0, 11.3 Hz)	C-6, 3′, 9, 10′−12′	H-2′a
2′	36.8	2.16 (1H, dd, J = 11.3, 14.1 Hz)	C-1′, 3′, 4′, 13′	H-1′
		2.31 (1H, m)	C-3′	H-13′
3′	144.4			
4′	38.4	2.78 (1H, m),	C-3′, 6′, 7′, 13′	H-5′
		2.89 (1H, dd, J = 6.5, 15.8 Hz)		H-5′, 4′a
5′	132.4	5.64 (1H, ddt, J = 0.9, 6.9, 15.2 Hz)	C-4′, 7′	H-4′a, 4′b, 6′
6′	128.3	6.01 (1H, dd, J = 15.4, 10.8 Hz)		H-7′, 5′
7′	133.3	6.44 (1H, ddd, J = 0.4, 13.2, 10.8 Hz)	C-5′, 8′	H-6′, 8′
8′	119.6	6.13 (1H, d, J = 13.21 Hz)	C-6′, 7′	H-7′
9′	34.5			
10′−12′	26.0	0.92 (12H, s)	C-1′, 9′, 10′−12′	
13′	113.9	4.78 (2H, d, J = 4.1 Hz)	C-2′, 4′	H-2′b
N-CH$_3$	29.0	2.81 (3H, s)	C-2, 5	H-4a, 4b

[a] NMR spectra were recorded in $CDCl_3$. [b] The multiplicites were determined by HMQC experiment.

(Table 2) bore close resemblance to makalika ester (**1**), suggesting that both compounds possessed the same gross structure. The most prominent difference in the ^{1}H NMR spectrum was a downfield shift of the proton signal at 4.06 ppm in **2** (H-2) from a corresponding resonance at 3.09 ppm for **1**. Also, the doublet resonance of the exomethylene (4.75 and 4.79 ppm, H-13′) of makalika ester (**1**) was replaced by a single resonance centered at 4.78 ppm. The appearance of a second carbonyl resonance at 175.3 ppm confirmed that **2** represented an oxidized analogue of **1**.



(1) R, R' = H, makalika ester
(2) R, R' = O, makalikone ester

Fragment (a), connected from COSY correlations (Figure 1), was reminiscent of makalika ester (**1**), with characteristic *trans*-couplings observed between the protons at H-8′ and H-7′ (J = 13.2 Hz), H-7′ and H-6′ (J = 10.8 Hz), and H-6′ and H-5′ (J = 15.4 and 15.2 Hz, respectively). As with **1**, the chlorine atom was attached to the vinylic methine of H-8′.

Fragment (c), also comparable to **1** (Figure 1), was identified by the COSY correlation between the oxygen-linked H-1′ at δ 4.94 and H-2′, and an HMBC correlation between the *tert*-butyl moiety, and the oxymethine at H-1′ confirmed its position with respect to the *tert*-butyl group. The cross-peak between H-1′ and C-6 in the HMBC spectrum established the ester linkage.

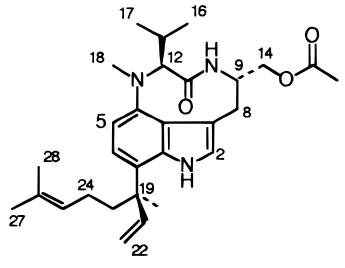
Observed HMBC links between the exomethylene protons at H-13′ and methylenes at H-2′ and H-4′ served to

link fragments (a) and (c) through fragment (b). The fourth fragment (d) contained the *N*-methyl group. HMBC correlations linked the C-2 resonance with the three-proton singlet of the *N*-methyl group. Further, COSY correlations with the H-3 protons established the position of these methylene protons with respect to the H-2 methine. HMBC cross-peaks were observed between H-3a and the amide carbonyl, C-5, confirming the location of the carbonyl group. Attachment of this *N*-methylpyrrolidone residue to the ester carbonyl C-6 was substantiated by HMBC coupling with the H-3b proton, thus establishing the structure of makalikone ester as **2**. Makalikone ester (**2**) displayed moderate activity (IC_{50} 2.5−5 μg/mL) against the cancer cell lines tested.

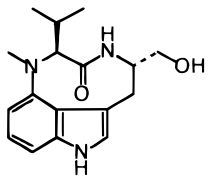
The presence of a *tert*-butyl moiety is atypical in natural product chemistry. This *tert*-butyl functionality, however, has been identified in isolates of *Lyngbya majuscula* and *Lyngbya bouillonii*.[8−11] Although these are new compounds, identical structural elements occur in the macrolide laingolide, which was isolated from *L. bouillonii*.[11] Recurring features include the *tert*-butyl residue connected through the oxymethine carbon to the ester linkage and the *N*-methyl functionality.

As *S. longicauda* is known to feed on *L. majuscula*,[2] isolates akin to those of this blue-green alga have also been identified in the extracts of the sea hare. These include malyngamide A[12,13] and majusculamides A and B.[14] Another group of compounds, the lyngbyatoxins,[14,15] have been isolated from this marine cyanophyte. The first lyngbyatoxin isolated, lyngbyatoxin A,[15] is a potent inflammatory agent and tumor-promoter.

As part of this study of *S. longicauda*, lyngbyatoxin A acetate (**3**), was isolated as a colorless oil. The HREIMS of **3** gave a molecular peak at *m*/*z* 479.3148, consistent with the molecular formula of $C_{29}H_{41}N_3O_3$ implying 11 degrees of unsaturation. Examination of the ^{13}C NMR data revealed the presence of 12 olefinic signals and two carbonyl groups, accounting for eight degrees of unsaturation. The remaining three double-bond equivalents could therefore be attributed to rings. The aromatic resonances suggested that an indole ring[6] was present, the third double-bond equivalent being attributable to the lactam ring. The structure was elucidated with the aid of conventional 2D NMR techniques and comparison of resonances with those of lyngbyatoxin A and indolactam V (**4**).[17]



(**3**)



(**4**)

The *ortho* relationship between the aromatic protons at H-5 and H-6 (J = 8.25 and 7.98 Hz, respectively, for conformer A) was corroborated by observed COSY correlations. The 1,5-dimethyl-1-vinyl-4-hexenyl (linalyl) side chain was located at C-7, while the *tert*- *N*-amide (2.73 and 2.92 ppm) occupied the C-4 position on the indole ring. HMBC and COSY correlations for H-15, H-16, and H-17 delineated the isopropyl residue. HMBC spectra of the conformers served to link the acetoxy group (2.02, 2.09 ppm) with the corresponding carbonyl (171.09, 170.88 ppm) resonances, hence confirming the attachment to the C-14 oxygen-linked methylene.

Two conformers occurring in an approximate 5:1 ratio had been observed in the initial isolation of lyngbyatoxin A.[14] A similar mixture of conformers exists in the acetate, in an approximate 1:1 ratio. Conformer A, by comparison with the data obtained with indolactam V, represents the *cis*-amide (twist conformation), while conformer B is the *trans*-amide in the sofa conformation.[16] The greatest disparity between the conformers was observed at C-12, in which the proton of conformer A resonated at 4.31 ppm, while that of conformer B resonated much farther upfield at 2.97 ppm. The ^1H and ^{13}C NMR data for both conformers of **3** are listed in Table 3.

A variable-temperature experiment was attempted at −35 °C, but no significant change in the population of the conformers was observed at that temperature. This corroborates the findings by Endo and co-workers,[16] who observed little change in conformer populations at −40 °C when samples had been prepared at room temperature.

Although lyngbyatoxin A acetate has been synthesized from lyngbyatoxin A,[14] this is the first isolation, to the best of our knowledge, of this compound from a natural source. This derivative of lyngbyatoxin A exhibits potent activity (IC_{50} 0.05 μg/mL) against the cancer cell lines tested.

Experimental Section

General Experimental Procedures. ^1H and ^{13}C spectra were recorded on either a General Electric GN Omega 500 spectrometer or a Varian Unity INOVA 400 MHz instrument. Ultraviolet spectra were recorded on a Hewlett-Packard 8452A diode array spectrometer. Mass spectral data were measured on either a VG 70ZAB2SE or a VG 7070 mass spectrometer. Optical rotations were determined on a JASCO DIP-370 polarimeter. Infrared spectra were obtained on a Perkin-Elmer 1600 FTIR instrument.

Animal Material. The sea hare, *S. longicauda*, was collected by snorkeling at Black Point, Oahu in August 1996, and February 1999, where the animals were seen mating in huge numbers. The animals were freeze-dried prior to extraction. The sea hare was identified by Professor E. Alison Kay, Department of Zoology, University of Hawaii.

Biological Assays. Assays were performed to determine IC_{50} values (μg/mL) for the isolated compounds on selected cancer cell lines. The compounds were tested against mouse lymphoma (P-388, ATCC: CCL 46), human lung carcinoma (A-549, ATCC: CCL 8), and human colon carcinoma (ATCC: HTB 38).

Extraction and Isolation. The freeze-dried animals (327.5 g lyophilized wt) were exhaustively extracted with MeOH to yield a green gum (127.4 g) on evaporation of the solvent in vacuo. A portion (30.8 g) of the dried residue was dissolved in MeOH/H_2O (9:1) followed by partitioning with hexane (150 mL × 4). The solution was then diluted to MeOH/H_2O (6:4) and further partitioned with CH_2Cl_2 (150 mL × 3). The hexane extract was fractionated using vacuum liquid chromatography on Si gel employing a stepwise gradient of hexane/EtOAc with final elution with acetone and MeOH. Eluted material was monitored by TLC and similar fractions combined to yield 10 major fractions. The fraction eluting with EtOAc/hexane (6:4) was subjected to further chromatographic separation utilizing a gravity column eluting with $CHCl_3$/hexane (6:4). Further purification was effected with reversed-phase HPLC from which four main peaks (A−D) were culled. SepPak and reversed-phase HPLC (Ultracarb, 80% MeCN/H_2O) of peak A delivered makalikone ester (**2**, 1.0 mg). The components of peak C were separated by size exclusion chromatography on Sephadex LH-20 in MeOH to afford four main fractions. Makalika ester (**1**, 7.3 mg) was obtained from the third fraction. Fraction 2 was subjected to purification on reversed-phase HPLC [Luna, MeCN/H_2O/MeOH (8:1:1)] from which

Table 3. ^{1}H and ^{13}C NMR Data for Conformers A and B of Lyngbyatoxin A Acetate (**3**)[a,b]

	conformer A		conformer B	
position	^{13}C	^{1}H	^{13}C	^{1}H
2	121.05	6.82, 1H, br s	124.58	6.89, 1H, br s
3	118.76		ND	
3a	127.73		128.36	
4	146.24		144.01	
5	107.50	6.50, 1H, d, J = 8.25 Hz	122.55	7.00, 1H, d, J = 7.97 Hz
6	120.14	6.98, 1H, d, J = 7.98 Hz	120.50	7.09, 1H, d, J = 7.72 Hz
7	113.23		ND	
7a	137.60		137.33	
8	34.35	3.06, 1H, m	ND	
		3.22, 1H, d, J = 17.55 Hz		
9	53.12	4.52, 1H, m	52.12	4.58, 1H, m
11	173.20		ND	
12	71.32	4.31, 1H, d, J = 10.11 Hz	77	2.97, 1H, d, J = 10.90 Hz
14	65.99	3.97, 1H, dd, J = 8.51, 11.43 Hz	63.93	3.85, 2H, d, J = 6.91 Hz
		4.22, 1H, dd, J = 3.72, 11.44 Hz		
15	28.62	2.60, 1H, m	24.22	2.40, 1H, m
16	19.61	0.66, 3H, d, J = 6.84 Hz	19.61	1.25, 3H, d, J = 6.65 Hz
17	21.59	0.93, 3H, d, J = 6.12 Hz	19.61	0.94, 3H, d, J = 5.85 Hz
18	33.09	2.92, 3H, s	35.74	2.73, 3H, s
19	43.31		ND	
20	17.46	1.44, 3H, s	ND	
21	148.38[c]	6.21, 1H, dd, J = 6.92, 17.56 Hz	148.07[c]	6.11, 1H, dd, J = 6.91, 17.82 Hz
22	112.52[d]	5.31, 1H, d, J = 12.76 Hz	112.78[d]	5.31, 1H, d, J = 12.76 Hz
		5.36, 1H, d, J = 14.89 Hz		5.36, 1H, d, J = 14.89 Hz
23	38.48		ND	
24	23.08	1.80−2.00, 2H, m	ND	
25	124.31	5.06, 1H, m	124.62	5.06, 1H, m
26	131.49[e]		131.67[e]	
27	25.65	1.65, 3H, s	ND	
28	17.54[f]	1.44, 3H, s	17.46[f]	1.44, 3H, s
29CO*CH*$_3$	171.09		170.88	
30CO*CH*$_3$	20.82	2.09, 3H, s	20.86	2.02, 3H, s
1-*N*H		8.52, 1H, br s		8.75, 1H, br s
10-*N*H		6.09, 1H, br s		

[a] All data were recorded in $CDCl_3$; ^{1}H−^{13}C connectivities assigned by HMQC experiment. [b] Multiplicities determined by HMQC experiment. [c,d,e,f] Signals may be interchanged; ND not unambiguously determined.

lyngbyatoxin A acetate (**3**, 2.0 mg) was obtained. Malyngamide A was also isolated from the sample.

Makalika ester (**1**): colorless oil; $[\alpha]_D$ −39° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (ϵ) 214 (45 000), 220 (45 000), 223 (11 995), 229 (45 000), 245 (12 807); IR (KBr smear) ν_{max} 2963, 1741, 1650, 1582, 1367 cm^{-1}; ^{1}H and ^{13}C NMR data ($CDCl_3$), see Table 1; DCI/NH_3 *m*/*z* (rel int) 340 (14) $[M + H]^+$, 211 (10), 144 (26), 130 (100), 114 (14), 100 (13), 98 (14), 82 (16), 77 (11), 60 (11), 58 (40); HRDCIMS *m*/*z* 340.2043 (calcd for $[M + H]^+$ $C_{19}H_{30}$ Cl NO_2, 340.2044).

Hydrolysis of Makalika Ester. Makalika ester (1.0 mg) was dissolved in 0.4 mL of 2.4 N HCl. The solution was heated to 90 °C in a sealed ampule with stirring for 12 h. The hydrolysate was dried and partitioned with $CHCl_3$ and H_2O. *N*-Methylproline was obtained from the water layer. The optical rotation of the amino acid, $[\alpha]_D$ −74° (*c* 0.02, H_2O), was found to be comparable to that of the standard *N*-methyl-L-proline obtained from Aldrich ($[\alpha]_D$ −86° [*c* 0.1, H_2O]). The expected alkylated chloro-octadienol was not recovered from the reaction.

Makalikone ester (**2**): colorless oil; $[\alpha]_D$ −23° (*c* 0.14, MeOH); UV (MeOH) λ_{max} (ϵ) 203 (15 909), 207 (13 131), 234 (7575), 357 (4646); IR (film) ν_{max} 3422, 1654, 1165 cm^{-1}; ^{1}H and ^{13}C NMR data ($CDCl_3$), see Table 2; DCI/NH_3 *m*/*z* (rel int) 354 (2), $[M + H]^+$, 268 (11), 161 (14) 144 (100), 100 (8), 98 (60); HRDCIMS *m*/*z* 354.1836 (calcd for $[M + H]^+$ $C_{19}H_{29}ClNO_3$, 354.1837).

Lygnbyatoxin A acetate (**3**): obtained as a colorless oil, $[\alpha]_D$ −4° (*c* 0.4, MeOH,); UV (MeOH) λ_{max} (ϵ) 229 (4261), 297 (1270); IR (film) ν_{max} 3422, 1654 cm^{-1}; ^{1}H and ^{13}C NMR data ($CDCl_3$), see Table 3; FABMS *m*/*z* (rel int) 479 (100), 452 (17), 307 (8), 223 (8), 154 (10), 136 (9), 107 (5); HRFABMS *m*/*z* 479.3148 (calcd for $C_{29}H_{41}N_3O_3$, 479.3148).

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References and Notes

(1) MBRS undergraduate participant 1995−97; MARC Scholar 1997−98; "Makalika" is Hawaiian for "daisy".
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