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ANTITUMOR AGENTS, 112.[1] EMARGINATINE B, A NOVEL POTENT CYTOTOXIC SESQUITERPENE PYRIDINE ALKALOID FROM *MAYTENUS EMARGINATA*

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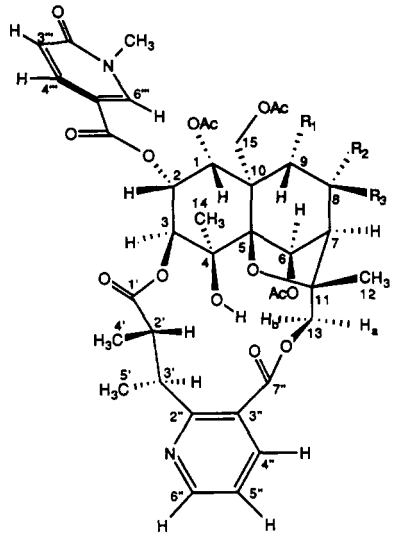
ABSTRACT.—A new sesquiterpene pyridine alkaloid, emarginatine B [**2**] has been isolated, along with maytansine [**3**], from *Maytenus emarginata*. Emarginatine B showed potent cytotoxicity against human KB cells (ED_{50} = 0.4 μg/ml). Spectral correlations established its structure as a 9-benzoate and C-8 epimer of emarginatine A [**1**]. The conformational aspect of the latter compound was elucidated by nmr studies, including the use of 2D-nmr techniques and difference nOe methods.

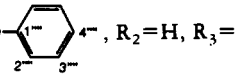
Maytenus emarginata (Willd.) Hou (1) (Celastraceae), previously named *Gymnosporia trilocularis* Hayata (1), is now known as "Lan Yu Lo Shih" to indicate that it is a shrub indigenous to Lan Yu island southeast of Taiwan. Previously we reported potent cytotoxic effects of the EtOH extract of this plant against P-388 lymphocytic leukemic cells (2) and the isolation of a novel cytotoxic sesquiterpene pyridone alkaloid, emarginatine A [**1**] (3), which was elucidated by spectral and X-ray analyses. Further investigation of the same plant has now led to the isolation of a new cytotoxic benzoate analogue of **1**, emarginatine B [**2**], as well as the potent cytotoxic macrolide alkaloid maytansine. With further quantity of compound **1** available, an extensive investigation of 2D correlation spectroscopies, including ^1H-^1H COSY, ^1H-^{13}C heteronuclear COSY, and difference nOe (dnOe) (Figure 1), as well as 2D nOe studies (Figure 2), allowed complete assignments of both ^1H- and ^{13}C-nmr spectra (Tables 1 and 2), based on which the structure of **2** was deduced.

The homonuclear ^1H-^1H COSY study of **1** not only correlated each proton in the spin systems in accordance with spin-spin decoupling studies, but also revealed several interesting correlations via long-range coupling. Thus, the couplings between H-12 and H-13a,b and also between H-14 and the 4-OH allowed unambiguous assignment of the chemical shifts of H-12 and H-14, respectively. W-type couplings were also observed between H-7 and both H-9 and H-13a, which, along with direct coupling of H-6 and H-7, located the chemical shift of H-7 at δ 2.38. Further elaboration of stereochemistry came from dnOe studies. The enhancement of the signals of H-3, H-6, and H-15 protons on irradiation of H-14 suggested that proton H-6 and the C-4

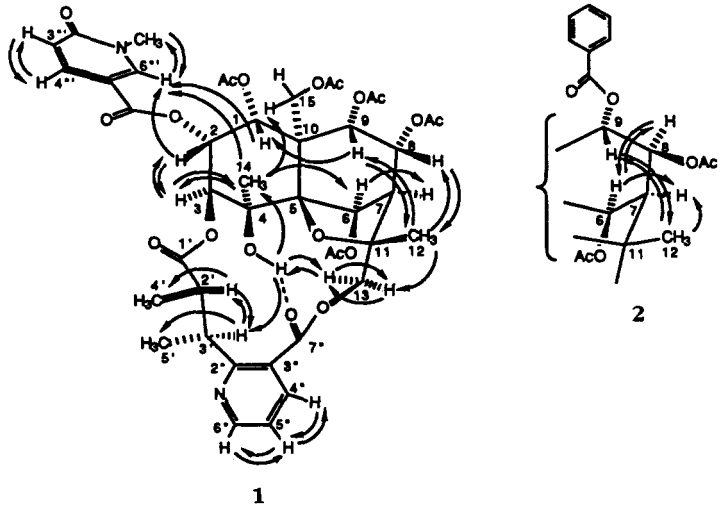
[1]For part 111, see K.H. Lee, S.A. Beers, M. Mori, Z.Q. Wang, Y.H. Kuo, L. Li, S.Y. Liu, and Y.C. Cheng, *J. Med. Chem.*, in press.



1 $R_1=R_2=OAc$, $R_3=H$

2 $R_1=$  , $R_2=H$, $R_3=OAc$

methyl group are both α to the β-agarofuran ring. Irradiation of the C-12 methyl protons caused enhancement of both H-8 and H-9 proton signals, indicating that these protons are β to the same ring. On irradiation of the 4-OH proton, the signals of H-3′ and H-13b were enhanced. This would not only suggest their nearness in space, but would also imply that the 4-OH proton is held by an H-bond with the C-7″ keto group and that the macrocyclic diester bridge assumes a conformation in solution similar to that in solid state established by X-ray study (3). It is worth noting in this regard that the chemical shift of the 4-OH proton always appears near δ 4.5 among analogues of euonymine-type alkaloids (4–7). Finally, the enhancement of the H-6‴ signal by irradiation of protons H-1, H-2, and H-14 serves to confirm the C-2α substitution of the pyridone ring.



1 **2**

FIGURE 1. Difference nOe of emarginatine A [**1**] and emarginatine B [**2**].

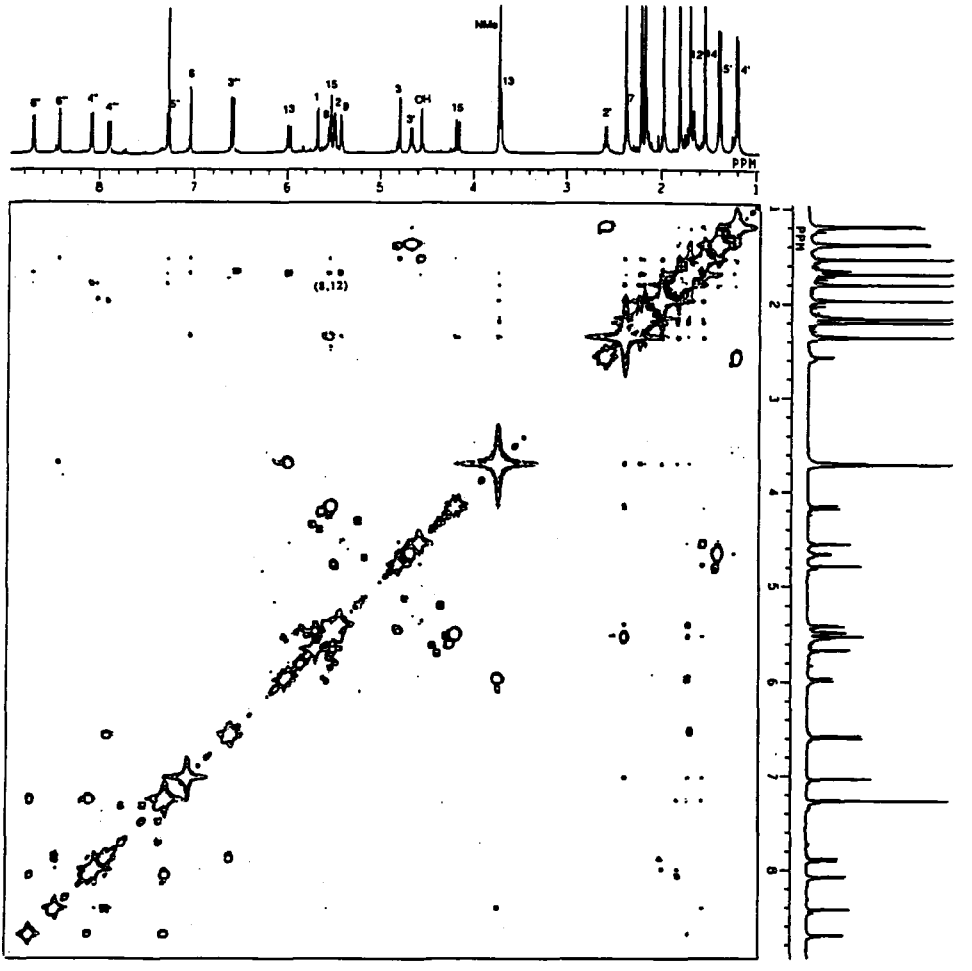


FIGURE 2. 2D nOe spectrum of emarginatine A [1].

With the chemical shifts of each proton firmly established, the assignment of carbon resonances of emarginatine A [1] was achieved by heteronuclear ^{1}H-^{13}C correlation spectroscopy, including both one-bond and long-range correlations. Except for the signals of acetate carbons, chemical shifts of all carbons are unambiguously assigned (Table 2). This allows, for the first time, the assignment of chemical shift values of the skeletal carbinyl carbons at C-1 to C-3, C-6 to C-9, and, as well, those carbons on the evoninate and pyridone fragments. In the evoninate bridge, the shift values established for the carbinyl carbons C-2′ and C-3′ and the methyl carbons C-4′, C-5′, C-12, and C-14 are different from literature values (5) for other similar analogues, which need to be reassigned. Particular attention is drawn to the correlation between C-7″ and H-13a,b, which established one connection point of the evoninate diester bridge at C-13. Also worth noting is that two acetate carbonyl carbons at the C-6 and C-9 positions were ascertained by their correlations with the H-6 and H-9 protons, respectively, and the methyl proton signals of these two substituents were, therefore, indirectly located. The chemical shift values established for methyl protons, H-12 and H-14, are different from literature values (6–8) for similar analogues, which need to be revised.

Emarginatine B [2], $[\alpha]_D$ +26° ($c = 0.033$, $CHCl_3$), was isolated as needles. Its eims showed a molecular ion at *m/z* 960 corresponding to a composition of

TABLE 1. ^{1}H-nmr (400 MHz) Data for Emarginatine A [**1**] and Emarginatine B [**2**].

Proton	Compound	
	1	**2**
H-1	5.67 (d, 4.2)	6.02 (d, 3.6)
H-2	5.48 (dd, 4.2, 2.4)	5.57 (dd, 3.6, 2.6)
H-3	4.78 (d, 2.4)	4.89 (d, 2.6)
H-6	7.04 (s)	6.66 (s)
H-7	2.38 (d, 4.2)	2.51 (d, 3.2)
H-8	5.54 (dd, 4.2, 6.1)	5.53 (dd, 3.2, 9.8)
H-9	5.42 (d, 6.1)	5.89 (d, 9.8)
H-13	3.72, 5.98 (ABq, 11.6)	3.71, 6.02 (ABq, 12.6)
H-15	4.16, 5.54 (ABq, 13.5)	4.53, 5.40 (ABq, 12.9)
H-4″	8.08 (dd, 1.8, 7.8)	8.10 (dd, 1.8, 7.9)
H-5″	7.32 (dd, 4.8, 7.8)	7.28 (dd, 4.5, 7.9)
H-6″	8.70 (dd, 1.8, 4.8)	8.73 (dd, 1.8, 4.5)
H-3‴	6.59 (d, 9.6)	6.59 (d, 9.6)
H-4‴	7.90 (dd, 2.5, 9.6)	7.82 (dd, 2.6, 9.6)
H-6‴	8.42 (d, 2.5)	8.35 (d, 2.6)
H-2′	2.57 (q, 6.6)	2.66 (q, 7.1)
H-3′	4.67 (q, 7.0)	4.71 (q, 7.0)
Me-4′	1.20 (d, 6.8)	1.27 (d, 7.1)
Me-5′	1.39 (d, 7.0)	1.43 (d, 7.0)
Me-12	1.71 (s)	1.63 (s)
Me-14	1.57 (s)	1.58 (s)
Me-N	3.72 (s)	3.72 (s)
OAc	1.81 (C-9)[a]	—
	1.98	1.40 (C-1)[b]
	2.18 (C-6)[a]	1.98
	2.22	2.24
	2.38	2.39
H-2‴, -6‴	—	7.70 (d, 8.9)
H-3‴, -5‴	—	7.38 (t, 8.9)
H-4‴	—	7.52 (t, 8.9)

[a]Assigned by ^{1}H-^{13}C long-range correlations.
[b]Assignment of this signal explained in text.

$C_{48}H_{52}N_2O_{19}$. The uv spectrum indicated a band at 266 nm (pyridone), and ir spectra displayed absorptions at 3400 (OH), 1740 (ester), and 1660 (pyridone) cm^{-1}, which resembled closely those of emarginatine A. Cross comparisons of its ^{1}H- and ^{13}C-nmr spectra (Table 1 and 2) with those of emarginatine A [**1**] suggested that **2** is also a similar sesquiterpene pyridone alkaloid. All carbon and proton signals on the β-agarofuran skeleton, evoninate diester bridge, and pyridone substituents showed close correspondence with those of emarginatine A [**1**], except that an acetate signal was missing and replaced by a set of signals corresponding to a benzoate group. These include the aromatic carbon signals of C-1‴″ (δ 129.37), C-2‴″, -6‴″ (δ 128.44), C-3‴″, -5 ‴″ (δ 129.28), C-4‴″ (δ 133.34), and benzoyl carbonyl (δ 164.55), and the proton signals at δ 7.70 (d), 7.52 (t), and 7.38 (t), constituting an A_2M_2X spin system. This is also supported by the eims fragment ion at *m/z* 105, representing a benzoate group, and, in addition, a base peak ion at *m/z* 136 from the *N*-methylpyridone group, and the ion at *m/z* 206, reflecting the evoninate diester bridge. Comparing the chemical shifts of carbinyl protons of **2** vs. **1** revealed pronounced downfield shifts for H-9 and H-1 (Δδ 0.47 and 0.35 ppm, respectively), which would place the benzoate group at C-1 or C-9 positions. On the other hand, a similar comparison of the carbinyl carbon signals for both

TABLE 2. ^{13}C-nmr (100 MHz) Data[a] for Emarginatine A [1] and Emarginatine B [2].

Carbon	1	^{1}H-^{13}C Connectivities[b]	2
C-1	73.12 (d)	H-3, H-9	72.24 (d)
C-2	69.38 (d)	H-1, H-3	69.48 (d)
C-3	75.65 (d)	H-2, H-14	74.86 (d)
C-4	70.45 (s)	H-2, H-14	70.37 (s)
C-5	94.09 (s)	H-3, H-7, H-14, H-15a	94.29 (s)
C-6	73.75 (d)	H-7, H-8	73.63 (d)[c]
C-7	50.74 (d)	H-12	49.33 (d)
C-8	68.95 (d)	H-7	73.62 (d)[c]
C-9	70.63 (d)	H-7	74.49 (d)
C-10	52.15 (s)	H-1, H-2, H-15a	51.17 (s)
C-11	84.42 (s)	H-6, H-12, H-13b	85.99 (s)
C-12	18.73 (q)	—	19.45 (q)
C-13	70.02 (t)	H-12	69.92 (t)
C-14	23.42 (q)	4-OH	24.16 (q)
C-15	60.52 (t)	H-1, H-9	61.34 (t)
C-2′	45.13 (d)	H-4′, H-5′	44.86 (d)
C-3′	36.54 (d)	H-5′	36.46 (d)
C-4′	9.87 (q)	—	9.91 (q)
C-5′	11.99 (q)	—	12.02 (q)
C-2″	165.72 (s)	H-6″	165.46 (s)
C-3″	125.13 (s)	—	124.92 (s)
C-4″	137.95 (d)	H-6″	137.76 (d)
C-5″	121.31 (d)	H-6″	121.11 (d)
C-6″	151.73 (d)	H-4″	151.60 (d)
C-3‴	120.00 (d)	H-4‴	119.99 (d)
C-4‴	139.13 (d)	H-6‴	138.68 (d)
C-5‴	108.35 (s)	H-3‴	108.06 (s)
C-6‴	144.22 (d)	H-4‴, NC*H*$_3$	143.89 (d)
NCH$_3$	38.34 (q)	H-6‴	38.32 (q)
Ac*Me*	20.59, 20.68		20.23, 20.81
	21.24, 21.49		21.17, 21.53
	21.81		
C-7‴	162.65	H-4‴, H-6‴	162.25
C-2‴	163.17	H-6‴, NC*H*$_3$	162.89
C-7″	168.64	H-4″, H-13a,b	168.49
C-1′	174.02	H-4′	173.89
MeCOO	169.01		169.46, 169.78
MeCOO-C-6	170.17, 170.32	H-6	169.83
	171.18		170.98
MeCOO-C-9	162.67	H-9	—
BzCO	—		164.55
C-2‴, -6‴	—		128.44
C-3‴, -5‴	—		129.28
C-4‴	—		133.34
C-1‴	—		129.37

[a]Multiplicities were obtained from DEPT spectra.

[b]^{1}H-^{13}C long-range correlation corresponding to 2-bond or 3-bond connectivities.

[c]These assignments may be interchanged.

compounds revealed downfield shifts of C-9, C-8 in **2** ($\Delta\delta$ 3.86, 4.67 ppm, respectively), suggesting C-9 or C-8 benzoate substitution. A close inspection of the acetate signals of both compounds revealed that the C-9 acetate carbonyl carbon (δ 162.67) and its methyl proton singlet (δ 1.81) are missing from **2**; this clearly established **2** as a 9-benzoate ester analogue of **1**. Previous workers (8–10) have observed an unusual

diamagnetic shift of one of the acetate methyl signals to δ 1.5–1.7 ppm from normal range of δ 1.9–2.3 ppm when an equatorially oriented acetate on C-1 is shielded by an aromatic ester on C-9. The appearance of a high field acetate methyl singlet at δ 1.4 in **2** clearly suggested that it is an equatorial C-1 acetate flanked by a C-9 benzoate group. This point is further substantiated by dnOe of **2**, which showed an enhancement on C-1 acetate methyl singlet upon irradiation of the doublet at δ 7.70 (H-2'''', -6'''') of benzoate ring. Further inspections revealed configurational difference as well. Thus, the coupling constant between H-8 and H-9 ($J_{8,9}$ = 9.8 Hz) in **2** is considerably larger than the same coupling in **1** ($J_{8,9}$ = 6.1 Hz), which would suggest a diaxial orientation for both H-8 and H-9 in **2** and an α configuration for H-8. This is further corroborated by dnOe studies (Figure 1) for compound **2**, which showed mutual enhancement between H-8 and H-6 in accord with an α-oriented H-8. On the contrary, both the dnOe and 2D nOe (Figure 2) of compound **1** showed a mutual enhancement between H-8 and H-12, where the H-8 proton is β-oriented. As a result of this inversion of configuration at C-8 in **2**, an upfield shift for H-6 (Δδ 0.38 ppm) and a downfield shift for H-7 (Δδ 0.13 ppm) were observed in comparison with **1**. An obvious downfield shift of the C-8 carbon signal is also noted (Δδ 4.67 ppm), which reflects the combined effect of C-9 benzoylation as well as the configurational change at C-8. A quite large downfield shift for H-15b in **2** (Δδ 0.37 ppm from **1**) is consistent with the deshielding effect of the C-9 benzoate group. Thus, summarizing all the evidence, emarginatine B is the 9-benzoate and 8-epimer of emarginatine A, as represented by structure **2**. Compounds **1** and **2** represent the first two examples of a euonymine-type polyester sesquiterpene alkaloid bearing a 5-carboxy-*N*-methylpyridonyl substituent.

In addition to **1** and **2**, the known potent cytotoxic macrolide maytansine (11,12), was also isolated. The presence of this compound accounts for most of the activity of the crude extract. Previously, emarginatine A [**1**] was found to be active against KB cells, with ED_{50} of 4.0 μg/ml. In comparison, emarginatine B [**2**] was found to be much more active against the same cell line, with ED_{50} of 0.4 μg/ml. Evidently, the placement of a benzoate group at C-9 position potentiates the cytotoxicity. This is the first observation of this level of significant cytotoxicity among this type of alkaloids. The detailed structure-cytotoxicity relationship remains to be explored.

EXPERIMENTAL

PLANT MATERIAL.—The stems of *M. emarginata* (1) used in this investigation were from a collection made in January 1986 on Lan Yu Island, Taiwan. A voucher specimen was kept at the Herbarium of the School of Pharmacy, National Defense Medical Center and the School of Agriculture, Chinese Culture University, Taipei, Taiwan.

GENERAL EXPERIMENTAL PROCEDURES.—All melting points were taken on a Fischer-Johns melting point apparatus and are uncorrected. ^{1}H- and ^{13}C-nmr spectra were recorded at 400 MHz and 100.06 MHz, respectively, using a JEOL FX-400 instrument. The usual pulse sequences of JEOL were used in ^{1}H-^{1}H COSY, ^{1}H 2D nOe, and ^{1}H-^{13}C COSY experiments; for the heteronuclear correlations, coupling constants of 140 Hz (one-bond), 5 Hz, and 10 Hz (long-range) were employed in the measurements. Eims were determined on a V.G. Micromass 70-70 instrument at 70 eV with a direct inlet system. Si gel (Merck 70–230 mesh) was used for cc, and precoated Si gel (Merck 60 F-254) plates were employed for tlc. Detection of alkaloid components was performed by spraying with Dragendorff's reagent or by 1% ceric sulfate solution.

ISOLATION OF EMARGINATINE B [**2**].—The dried stems of *M. emarginata* (20 kg) were extracted exhaustively with MeOH. Removal of the MeOH gave a syrup, which was extracted successively with hexane-H_2O (4:1) and $CHCl_3$-H_2O (3:2). The $CHCl_3$ extract [138 g, ED_{50} (KB) = 0.4 μg/ml] was chromatographed on a Si gel (1.5 kg) column and eluted with $CHCl_3$, $CHCl_3$/MeOH, and MeOH. Bioassays with cytotoxicity detected the activity in the $CHCl_3$/MeOH fraction [ED_{50} (KB) = 0.08 μg/ml], which was further chromatographed on a Si gel column and eluted with EtOAc, EtOAc/Me_2CO, and Me_2CO. Similar fractions were combined on the basis of tlc analysis, and the most active portion (210 mg)

[ED_{50} (KB) = 0.04 μg/ml] and next active portion (78 mg) [ED_{50} (KB) = 0.42 μg/ml] were obtained from elution with EtOAc-Me_2CO (3:1). Both active portions were further separated by hplc [Nucleosil 7 C_{18}, MeOH-H_2O (7:3), 3 ml/min] to yield maytansine (16 mg) and emarginatine B [**2**] (10 mg), respectively.

EMARGINATINE B [**2**].—White crystalline needles: mp 191–194°; $[\alpha]_D$ +26 (c = 0.033, $CHCl_3$); uv λ max nm (ε) 266 (12200); ir ν max (KBr) cm^{-1} 3500, 1740, 1660, 1580, 1560, 1440, 1290, 710; eims *m/z* (rel. int.) 961.6 (4.4), $[M]^+$ 960.5 (8.8), 926 (5.4), 867 (1.6), 866 (1.1), 572 (16.9), 220 (12.6), 206 (46.0), 178 (18.0), 136 (100), 107 (56.8), 105 (83.8); ^1H nmr see Table 1; ^{13}C nmr see Table 2.

MAYTANSINE.—Light yellow needles: mp 171–172°; $[\alpha]_D$ −138° (c = 0.055, $CHCl_3$). This compound was identified by spectral (uv, ir, ^1H nmr, and ms) comparisons with literature data (13), and also with an authentic sample in our laboratory.

ACKNOWLEDGEMENTS

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