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TRADITIONAL MEDICINAL PLANTS OF THAILAND, IX. 10-HYDROXY-11-METHOXYDRACAENONE AND 7,10-DIHYDROXY-11-METHOXYDRACAENONE FROM *DRACAENA LOUREIRI*[1]

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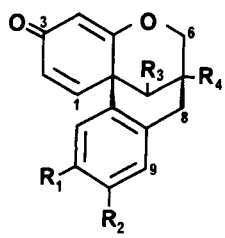
ABSTRACT.—Examination of *Dracaena loureiri*, a Thai medicinal plant possessing antibacterial activity, has led to the isolation of two new representatives, **1** and **2**, of a rare skeleton of homoisoflavans. Proton assignments of the two isolates were aided by extensive 2D-homonuclear chemical shift correlation and nOe difference spectroscopy. Carbon assignments were completed through the utilization of simple and sensitive one-dimensional techniques such as selective population transfer via one-bond (CSCM 1D) and selective polarization transfer via long range coupling (selective INEPT) experiments. Conformational assignments were proposed through nOe difference spectroscopy and have been established by X-ray crystallography. The absolute configuration is proposed based on the octant rule and a biogenetic pathway for this type of homoisoflavan is briefly discussed.

Phytochemical study of the genus *Dracaena* (Agavaceae) has previously led to the isolation of a variety of flavonoids (2), and biologically, extracts of *Dracaena mannii* have shown antibacterial activity against Gram-negative and Gram-positive organisms (3). Investigation of the $CHCl_3$ extract of the Thai medicinal plant *Dracaena loureiri* Gagnep, which has traditionally been employed for many infectious diseases, as well as an antipyretic (4) and for gastrointestinal disturbances, showed in vitro antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis*. Using a procedure for the isolation of antibacterial agents from higher plants, we have carried out an activity-directed fractionation (5) of the stem wood of *D. loureiri*. This led to the isolation of several flavonoids, two of which, **1** and **2**, were found to contain a rare type of homoisoflavan skeleton. The recent disclosure of the structure of caesalpin J [**3**] from *Caesalpinia sappan* L. (6,7) prompts us to report our work at this time.

The long wave absorption maxima of **1**, which appeared at 285 nm, were consistent with a homoisoflavonoid nucleus, and the band at λ max 239 nm, shifting to 268 nm

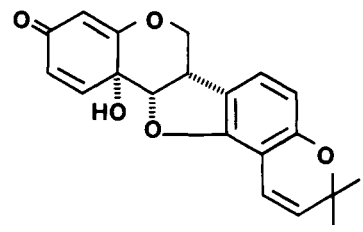


1 R_1=OMe, R_2=OH, R_3=R_4=H
2 R_1=OMe, R_2=R_4=OH, R_3=H
3 R_1=R_2=R_4=OH, R_3=OMe
7 R_1=R_2=R_3=R_4=H

[1]For the previous paper in this series, see Hamburger *et al.* (1).

with NaOMe, suggested the presence of a phenolic hydroxyl group. Furthermore, the ir spectrum also showed the presence of a typical saturated homoisoflavonoid (8) (1650, 1617, and 1593 cm^{-1}). The eims displayed, in addition to a molecular ion peak at *m/z* 284, a tropylium ion at *m/z* 137, characteristic of a 3-benzylchromane derivative.

The ^{1}H-nmr spectra, however, differed in certain respects from the typical spectra of 3-benzylchroman-4-ones. Analyses of the ^{1}H-nmr and COSY spectra indicated that a phenolic proton appeared at 9.10 ppm, and a doublet at 6.86 ppm assigned to H-1 was coupled to a resonance at 6.36 ppm which was in turn coupled to a high field sp^2 proton resonance at 5.45 ppm. These were assigned to H-2 and H-4, respectively. The unusual high field of H-4 was thought to be due to the anisotropic effects of the conjugated carbonyl group, as well as additional *ortho* shielding by the oxygen of the pyran ring as noted previously in 1a-hydroxyphaseollone [**4**] (9). Two apparently aromatic singlets at 6.63 and 6.34 ppm were then assigned to either H-9 or H-12. The simplicity of these signals confirmed their *para* orientation on a ring with methoxyl (3.59 ppm) and phenolic (9.10 ppm) substituents. Three pairs of nonequivalent methylene protons were geminal-coupled to each other, i.e., 4.34 and 4.13 ppm, 3.13 and 2.89 ppm, and 2.17 and 2.04 ppm. They were assigned to H-6, H-8, and H-13, respectively, and three methylene carbons were noted in the APT spectrum of **1**. The homonuclear COSY spectrum of **1** revealed the presence of strong *W* couplings between H-6β and 13β, between H-6α and 8α, and between H-8β and 13α. Only one of each methylene



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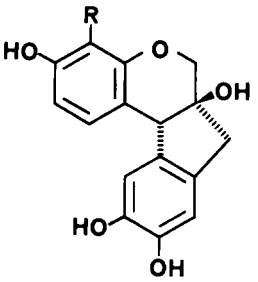
proton-pair of H-6, H-8, and H-13, i.e., H-6α, H-8α, and H-13α, was coupled to H-7, suggesting that the other members of the pairs were disposed at 90° to H-7.

The unambiguous assignments of H-9 and H-12, and the location of the methoxyl and hydroxyl groups were determined by a nOe difference experiment. Both nOe and control were acquired by interleaving simultaneously under identical conditions apart from the frequency of irradiation; the power level and time of irradiation were the same in both spectra to avoid phase shift of drift problem (10). Subsaturating power levels were selected to achieve adequate frequency selectivity, and the delay time following a 90° pulse was set ten times the T_1 to ensure complete recovery of the equilibrium magnetization for all nuclei of interest (11). Irradiation of H-8β afforded a large geminal nOe enhancement of H-8α at 3.13 ppm (27.2%), of H-7 at 2.39 ppm (12.6%), H-6β at 4.34 ppm (6.4%), and of an aromatic proton at 6.63 ppm (16.4%) which must be H-9. A long-range coupling between H-8 and H-9 was evident from the homonuclear COSY spectrum in a similar manner as the brazilin [**5**] or haematoxylin [**6**] types (12). Irradiation of the methoxyl resonance at 3.59 ppm afforded an nOe effect on the aromatic singlet at 6.34 ppm (12.6%) which was consequently assigned to H-12.

The COSY and nOe difference experiments established that **1** possessed a different skeleton from the other homoisoflavans that in C-6′ is joined to C-4a. This ring system, 7,8-dihydro-6*H*-7,12b-methano-3*H*-dibenz[*b,d*]oxocin-3-one [**7**], was given the trivial name dracaenone, and **1** was defined as 10-hydroxy-11-methoxydracaenone. The complete ^{1}H-nmr assignments of the isolate **1** are presented in Table 1.



5 R=H
6 R=OH

A standard APT pulse sequence was employed to delineate the ^{13}C signals due to one methoxyl carbon, three methylene carbons, six methine carbons, and seven quaternary carbons of **1**. With the previously deduced proton assignments, a selective population transfer (SPT) via one-bond coupling experiment (CSCM 1D) then permitted a detailed analysis of the hydrogen-bearing carbons (13). Carbon-13 magnetization transferred from the downfield ^{13}C satellite of H-1, H-9, and H-4 gave positive resonances at 150.11 ppm (C-1), 116.02 ppm (C-9), and 107.58 ppm (C-4), respectively. Magnetization transferred from a high-field satellite of H-12 gave rise to two negative absorption lines at 109.76 ppm (C-12) and 128.85 ppm (C-2) of lower intensity. Magnetization transferred from a low-field satellite of H-6β confirmed the oxymethylene carbon C-6 to be at 77.32 ppm, and magnetization transferred from an upfield satellite of H-8β, which also fell into a downfield satellite of H-7, gave a negative methylene resonance at 33.75 ppm (C-8) and a positive methine signal at 28.04 ppm (C-7). Irradiation of a high-field satellite of H-13α yielded a negative methylene resonance at 32.25 ppm (C-13).

Finally, the non-protonated carbons were assigned by the use of the selective INEPT experiment (14), and this technique also verified some other ambiguous assignments. Selective INEPT transfer from H-1 (J=9 Hz) showed one carbonyl carbon at

TABLE 1. ^1H-nmr Assignments of 10-Hydroxy-11-methoxydracaenone [**1**] and 7,10-Dihydroxy-11-methoxydracaenone [**2**][a]

H	**1** in DMSO-d_6			**2** in 25% $CDCl_3$+CD_3OD		
	chemical shift	mult	coupled proton(s) (J, Hz)	chemical shift	mult	coupled proton(s) (J, Hz)
1	6.86	d	2(9.8)	6.88	d	2(9.9)
2	6.36	dd	1(9.8), 4(1.6)	6.46	dd	1(9.9), 4(1.6)
4	5.45	d	2(1.6)	5.58	d	2(1.6)
6α	4.13	dd	6β(10.9), 7(2.5), 8α(<1)	3.85	dd	6β(10.7), 8(1.7)
6β	4.34	d	6α(10.9), 13β(<1)	4.24	dd	6α(10.7), 13β(1.9)
7	2.39	m	—			
8α	3.13	ddd	8β(16.1), 7(6.3), 6α(<1), 9(<1)	3.16	AB	
8β	2.89	d	8α(16.1), 13α(<1), 9(<1)	3.10		
9	6.63	s	8α,8β(<1)	6.65	s	8(<1)
12	6.34	s		6.43	s	
13α	2.04	dd	13β(12.6), 7(2.9), 8β(<1)	1.99	dd	13β(11.8), 8(1.4)
13β	2.17	d	13α(12.6), 6β(<1)	2.33	dd	13α(11.8), 6β(1.9)
OH	9.10	s		7.81	s	
OCH_3	3.59	s		3.69	s	

[a]Obtained at 360 MHz, δ TMS=0 ppm.

187.62 ppm (C-3), one oxygen-bearing sp^2 carbon at 177.57 ppm (C-4a), and a ring-junction carbon at 42.27 ppm (C-12b). The disappearance of three-bond scalar coupling from H-1 through C-12a and C-13 can be explained on the basis of the change in a dihedral angle which was no longer in the same planar system. This feature confirmed the presence of the 7,12b-methano-3*H*-dibenz [*b,d*] oxocin-3-one ring system as well as the presence of a carbonyl group at C-3, instead of C-2, due to the appearance of an oxygenated sp^2 carbon (C-4a). Selective INEPT transfer from H-9 (J=8 Hz) yielded two oxygen-bearing aromatic carbons at 146.38 ppm (C-10) and 145.75 ppm (C-11), one ring-junction aromatic carbon at 125.06 ppm (C-12a), and one methylene benzylic carbon at 33.75 ppm (C-8). On transfer from H-9, residual SPTs from neighboring protons, H-1, H-2, and H-12 were also observed (15). Three-bond scalar coupling from the methoxyl protons (J=4 Hz) across the oxygen atom to the aromatic carbon at 145.75 ppm was also obtained from this selective INEPT experiment (16) which firmly established the assignment of C-11. Complete ^{13}C-nmr assignments for **1** are shown in Table 2.

TABLE 2. ^{13}C-nmr Assignments of 10-Hydroxy-11-methoxydracaenone [**1**] and 7,10-Dihydroxy-11-methoxydracaenone [**2**][a]

Carbon	δ_C	
	1 in DMSO-d_6	**2** in 25% $CDCl_3$+CD_3OD
1	150.11	150.61
2	128.85	129.42
3	187.62	190.69
4	107.58	108.33
4a	177.57	178.58
6	77.32	80.57
7	28.04	66.28
8	33.75	43.14
8a	129.09	129.61
9	116.02	116.54
10	146.38	147.44
11	145.75	147.23
12	109.76	109.55
12a	125.06	124.88
12b	42.27	47.44
13	32.25	40.86
OCH_3	55.71	56.28

[a]Obtained at 90.8 MHz, δ TMS=0 ppm.

The spectral data of **2** were very similar to those of **1**, except that the ir spectrum indicated an additional hydroxyl group (ν max 3504 cm^{-1}), and hrms indicated a formula of $C_{17}H_{16}O_5$ with a molecular ion at m/z 300, having the same fragmentation pattern as **1** including a tropylium ion at m/z 137. The ^{1}H-nmr and homonuclear COSY spectra of **2** were almost identical to **1**, especially in the low-field region where five sp^2 hydrogens and a phenolic proton were located. The signal of H-7 was not observed, and H-8 at 3.13 ppm was now an AB system. Confirmation of the location of the methoxyl protons was made by irradiation of H-8 which resulted in nOe enhancements of H-9 at 6.65 ppm, of H-6β at 4.24 ppm, and of H-13β at 2.33 ppm. Irradiation of the methoxyl protons at 3.69 ppm gave an enhancement of H-12 at 6.43 ppm. On this basis **2** was defined as 7,10-dihydroxy-11-methoxydracaenone. This was confirmed by the APT spectrum where C-7 was moved downfield to 66.28 ppm, and C-6, C-8, and

C-13 were shifted to 80.57, 43.14, and 40.86 ppm, respectively, due to deshielding by the hydroxyl group at C-7.

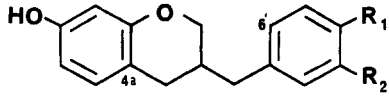
CSCM 1D spectroscopy was used to confirm the assignments of protonated carbons of **2**, and selective INEPT spectroscopy was used for the unambiguous assignment of the non-protonated carbons. The complete ^{1}H and ^{13}C assignments of **2** are shown in Tables 1 and 2, respectively.

It is requisite that rings B and C of dracaenone are *cis*-fused. There are, however, two possible conformations such that ring B can have either a chair or a boat conformation. Evidently, the ring system is fairly rigid with rings A and D flattened. The dihedral angles between H-6β and H-7, and between H-8β and H-7, are about 90°, and show strong *W*-coupled patterns between H-6β and H-13β and between H-6α and H-8α. These torsion angles indicated that ring B was in a slightly distorted chair form whereby the steric interaction between the oxygen in pyran ring and H-13 protons is relieved. Preirradiation of H-1 gave moderate nOe enhancement of H-2 (4.3%), together with two weak nOe enhancements of H-12 (0.9%) and H-13β (0.4%). The stronger nOe observed for H-12 than for H-13β eliminated the possibility of a boat-conformation for ring B. The ring fusion of rings B and C is, therefore, established as *cis*-fused with a chair conformation for ring B and a sofa conformation for ring C.

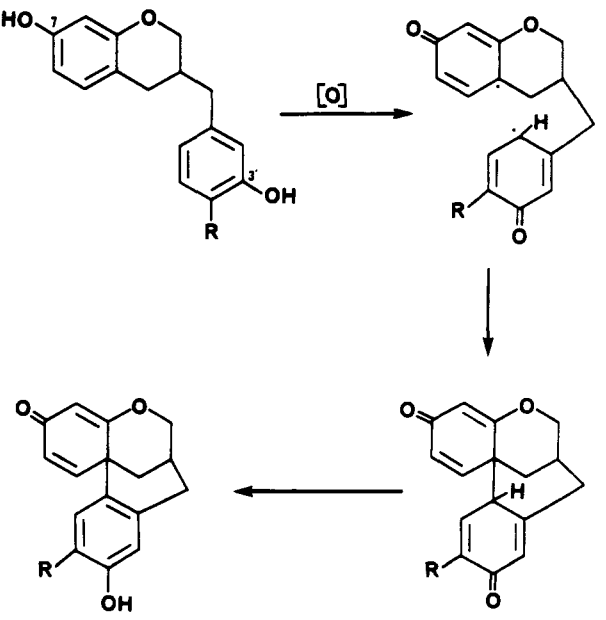
It was not possible to employ the cd exciton chirality method (17) to establish the absolute stereochemistry at C-7 and C-12b of a nondegenerate system of **1** and **2**. The probable absolute configuration was deduced by the application of the octant rule using the chiroptical technique (18,19). The dracaenone derivative **1** showed $[\alpha]_D -411.3°$, and its cd bands at 314 nm and the second cd band at 274 nm corresponding to the $n-\pi^*$ and the $\pi-\pi^*$ transitions of the dienone, respectively. Dreiding models suggested two possible conformations and yielded their octant projections. In both conformations the relative geometry of the oxygen *p*-orbital of the 11-OCH_3 and the π-system of the dienone group was such that an overlap of the involved orbitals should be expected leading to a bathochromic shift for $\pi-\pi^*$ transition (18), as was observed. The distribution of the electron density, as well as the π-donation of a long-pair orbital of the OCH_3 substituent connected to the carbonyl group through an effective coupling path of six C-C bonds (19) appeared in the right upper rear octant in one conformation which should lead to a negative cd band. Experimentally, the values were $\Delta\epsilon -707.9$ and -451.1 at 314 and 274 nm, respectively. On this basis, the absolute configurations of **1** and **2** are proposed as (7*S*, 12b*R*) and (7*R*, 12b*R*), respectively, as shown. Caesalpin J would then have the opposite absolute configuration since it showed $[\alpha]_D +445°$ (6). The structure and conformation of **2** were confirmed through single crystal X-ray analysis, details of which were reported elsewhere (19).

The co-occurence of **1** and **2** with 7-hydroxy-3-(4-hydroxybenzyl)chromane [**8**] in *D. loureiri* suggested that its close relative, 7-hydroxy-3-(3-hydroxy-4-methoxybenzyl) chromane [**9**], might cyclize by *para-para* phenolic oxidative coupling to a tetracyclic intermediate which can undergo dienone-phenol rearrangement to the dracaenone skeleton as shown in Scheme 1. Synthetic studies, including the synthesis of **1** and **2** in racemic form, will be reported elsewhere (20).

The antimicrobial activity of the isolates was determined by the microdilution method against *S. aureus*, *B. subtilis*, and *E. coli*. No useful activity was observed (MIC>250 μg/ml).



8 R_1=OH, R_2=H
9 R_1=OMe, R_2=OH



SCHEME 1. Biogenesis of dracaenone nucleus

EXPERIMENTAL

All solvents were redistilled. Adsorption column chromatography was performed with Si gel 60 (70-230 mesh) (E. Merck, Darmstadt, Germany). Tlc was performed with Si gel GHLF uniplates (layer thickness 0.25 mm) from Analtech, Inc. Visualization of the tlc chromatograms was conducted with 1% vanillin in MeOH and 50% aqueous H_2SO_4 spray reagents or by exposure to uv light.

Melting points were determined on a Kofler-type hot-stage apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer Model 241 polarimeter. Cd spectra were recorded on a Jasco J-40A automatic recording spectropolarimeter using a quartz cell of 20 mm length and 3.5 ml volume. Uv spectra were recorded by employing a Beckman model DU-7 spectrophotometer, and ir spectra were obtained with a Nicolet MX-1 interferometer. Mass spectra were determined with a Varian MAT 112S double focusing mass spectrometer operating at 80 eV. The ^{1}H-nmr spectra (DMSO-d_6, $CDCl_3$, and CD_3OD solutions with TMS as internal standard) were obtained with Nicolet NMC 360 (360 MHz) and Varian XL-300 (300 MHz) spectrometers. All ^{13}C-nmr spectra were measured at 90.8 MHz using a Nicolet NMC 360 spectrometer and are reported in ppm downfield from TMS.

For the nmr studies, approximately 16 mg of **1** was dissolved in 0.5 ml of DMSO-d_6, and for **2** a solution of 9 mg in 0.5 ml of CD_3OD-$CDCl_3$ (3:1) was transferred to 5-mm nmr tubes.

Homonuclear COSY spectra of **1** and **2** were recorded at 1K with a Varian XL 300 spectrometer. Standard Varian pulse programs were used. NOe difference spectra for **1** and **2** were measured with the previously described concentration on a Nicolet NMR 360 spectrometer. The samples were degassed by using a repeated freeze-pump-thaw cycle and then closed under N_2 (subaseal). Data sets of 16 K covering a spectral width of 2000 Hz were acquired. A 2.0 Hz line broadening was applied to the data prior to Fourier transformation.

The one-dimensional heteronuclear ^{1}H-^{13}C shift correlation (CSCM 1D) and selective INEPT experiments were performed on a Nicolet NMC 360 spectrometer. Data sets of 16K covering a spectral width of 10,000 Hz were acquired. Proton pulse widths were calibrated using a sample of HOAc in 10% C_6D_6 (^1J=6.7 Hz) in a 5-mm nmr tube (21). The radiofrequency field strength of the soft proton pulse was on the order of 25 Hz for these experiments.

PLANT MATERIAL.—Dried stem material of *D. loureiri* was collected at Prachuabkirikhan Province, Thailand, in January 1982. The plant material was identified by the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand. A herbarium specimen is deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

EXTRACTION AND ISOLATION.—The chipped stems were dried at 50° for 48 h and ground (630 g).

They were extracted with petroleum ether, $CHCl_3$, and EtOH, successively, in a Soxhlet apparatus for 48 h. Evaporation of the solvents gave residues of 11.8, 152.7, and 104.4 g, respectively. The $CHCl_3$ fraction displayed antibacterial activity by conventional paper disc method against *S. aureus* and *B. subtilis* (MIC 10 mg/ml, paper disc method). The $CHCl_3$-soluble extract (152 g) was subjected to Si gel-60 column chromatography (8×100 cm; 1-liter fractions were collected), eluting with mixtures of petroleum ether and EtOAc, EtOAc, and MeOH of increasing polarity.

PURIFICATION OF 10-HYDROXY-11-METHOXYDRACAENONE [**1**].—Combined fractions 59-66 (9.7 g) were chromatographed on Si gel-60 (5.5×45 cm; 250-ml fractions) eluting with solvents of gradually increasing polarity from $CHCl_3$ to MeOH. 10-Hydroxy-11-methoxydracaenone [**1**] was eluted from the column with $CHCl_3$ and crystallized from a $CHCl_3$/petroleum either mixture to give rectangular prisms (18.6 mg) mp 263-265°; $[\alpha]^{23}D-411.3°$ (c=0.0248, MeOH); cd uv (MeOH) λ max 212 (log ε 3.69), 239 (log ε 3.48), 283 nm (log ε 3.02); ir (KBr) ν max 3175, 2984, 2842, 1650, 1617, 1593, 1511, 1270, 1165, 1108, 873 cm^{-1}; eims (80 eV) *m/z* (rel. int.) 284 (M^+, 100), 269 (2), 256 (2), 241 (4), 147 (5), 137 (25). For ^1H- and ^{13}C-nmr data see Tables 1 and 2, respectively.

PURIFICATION OF 7,10-DIHYDROXY-11-METHOXYDRACAENONE [**2**].—Combined fractions 69-81 (8.8 g) were chromatographed on Si gel 60 (5×78 cm; 500 ml fractions) using 3% MeOH in $CHCl_3$ as the mobile phase. Fractions 32-35 gave 7,10-dihydroxy-11-methoxydracaenone (10.2 mg, **2**) as rectangular prisms: mp 262° C; $[\alpha]^{23}D-465.9°$ (c=0.0088, MeOH); UV (MeOH) λ max 214 (log ε 4.31), 239 (log ε 4.13), 283 (log ε 3.64) nm; ir (KBr) ν max 3504, 3367, 3111, 3026, 2965, 2876, 1655, 1612, 1593, 1284, 1171, 1067, 870 cm^{-1}; eims (80 eV), *m/z* (rel. int.) 300 (M^+, 100), 285 (4), 269 (1), 257 (4), 243 (7), 163 (3), 137 (10); mass measurement found 300.0997; calcd for $C_{17}H_{16}O_5$, 300.0998. ^1H- and ^{13}C-nmr data are presented in Tables 1 and 2, respectively.

PURIFICATION OF 7-HYDROXY-3-(4-HYDROXYBENZYL)CHROMANE [**8**].—Combined fractions 28-32 (14.6 g) were chromatographed on Si gel-60 PF 254 (5.5×47 cm; 50 ml fractions) eluting with mixtures of $CHCl_3$ and MeOH of increasing polarity. Fractions 74-83 afforded 7-hydroxy-3-(4-hydroxybenzyl)chromane [**8**] (582.2 mg) as pink blunt needles mp 123-124°; $[\alpha]^{24}D+62.9°$ (c=0.12, MeOH); uv (MeOH) λ max 215 (log ε 4.13), 225 (4.15), 281 (3.72) nm; ir (KBr) ν max 3592, 3357, 3025, 2975, 2914, 2854, 1617, 1601, 1511, 1474, 1458, 1266, 1241, 1152, 1124, 1033 cm^{-1}; ^1H nmr (360 MHz, CD_3OD) δ 6.96 (2H, d, J=8.6 Hz, H-2′ and H-6′), 6.75 (1H, d, J=8.1 Hz, H-5), 6.72 (2H, d, J=8.6 Hz, H-3′ and H-5′), 6.29 (1H, dd, J=8.1, 2.4 Hz, H-6), 6.20 (1H, d, J=2.4 Hz, H-8), 4.04 (1H, ddd, J=10.6, 2.9, 1.5 Hz, H-2 eq), 3.66 (1H, dd, J=10.6, 8.5 Hz, H-2 ax), 2.60 (1H, dd, J=15.9, 4.9, H-4 eq), 2.51 (1H, dd, J=12.3, 7.6 Hz, H-9), 2.45 (1H, dd, J=12.3, 7.6 Hz, H-9′), 2.32 (1H, dd, J=15.9, 8.7 Hz, H-4 ax), 2.09 (1H, m, H-3 ax); ^{13}C nmr (90.8 MHz, CD_3OD) δ 156.93 (C-7), 156.18 (C-8a), 156.13 (C-4′), 131.63 (C-1′), 131.35 (C-5), 130.86 (C-2′ and C-6′), 115.98 (C-3′ and C-5′), 113.96 (C-4a), 108.94 (C-6), 103.59 (C-8), 70.72 (C-2), 37.82 (C-9), 35.48 (C-3), 31.11 (C-4); eims (80 ev) *m/z* (rel. int.) 256 (M^+, 100), 162 (12), 161 (12), 150 (12), 149 (46), 148 (64), 147 (38), 134 (10), 133 (19), 123 (25), 121 (9).

BIOASSAY.—The bioautography technique for the localization of antibacterial activity of each fraction of $CHCl_3$ extract of *D. loureiri* and assay against *S. aureus* ATCC 25923 and *B. subtilis* ATCC 6633 were carried out as previously described (4) with a modification. A 4×5 cm tlc plate was used and dipped into a 20 ml bacterial suspension (ca. 10^9 colonies per ml) in a petri dish. The plates were dried in a stream of cold air, just sufficiently to remove any film of H_2O and give a translucent appearance, before being incubated at 37° overnight in a petri dish lined with a moist filter paper continuously supplied with H_2O from a small capillary tube to maintain humidity. An aqueous solution of INT (*p*-iodonitrotetrazolium violet, Sigma, 2 mg/ml) was soaked on the tlc plate and again dried and incubated at 37° in the petri dish. A clear zone was detected against a red background if the activity was present.

Antibacterial susceptibility testing for the pure isolates was performed by the microdilution method.

To prepare the inoculum, bacteria were subcultured on nutrient agar slants and incubated at 35° overnight until good growth was obtained. Five similar colonies were then used to inoculate 5 ml of tripticase soy broth. The inoculated broth was incubated at 35° until the turbidity was equal to or greater than 10^9 colony forming units per ml (0.5 MacFarland standard is approximately equal to 1.5×10^8 organisms). A sample (0.05 ml) of this standardized bacterial suspension was diluted with sterile distilled H_2O (15 ml) supplemented with 0.02% Tween 80 and shaken on a vortex mixer before being tested.

The pure isolates were dissolved in DMSO and serial twofold dilutions with Muller-Hinton broth were prepared and transferred to broth microdilution plates to achieve final concentrations ranging from 500 to 3.9 μg/ml. The plates were then inoculated with the prepared bacterial suspension, and the final inoculum size was between 10^4 and 10^5 (cfu/ml). Inoculated plates were incubated at 35° in a non-CO_2 incubator for 15-18 h. Sterility and growth control as well as a positive control with tetracycline were performed.

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