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Crinatusins, Bioactive Diels–Alder Adducts from *Cyathocalyx crinatus*

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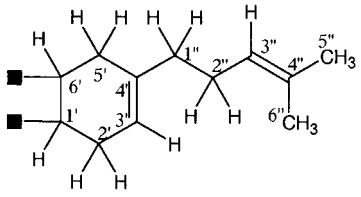
Abstract—From the stems of *Cyathocalyx crinatus*, a rain forest plant in Borneo, six new Diels–Alder adducts, named crinatusins A_1, A_2, B_1, B_2, C_1, and C_2, together with a known chalcone derivative, 4′,6′-dihydroxy-3′,5′-dimethyl-2′-methoxychalcone, have been obtained. Their structures were determined on the basis of spectroscopic data and chemical methods. Crinatusins showed a lethal toxicity against brine shrimp.

Introduction

Borneo is one of the richest areas with species of plants in the world. The studies of the dynamics of the maintenance of species in rain forests is essential to the search for methodology to rehabilitate disturbed rain forests. The objects in those studies are to establish ecological and molecular correlation between lives in vast rain forests and to utilize the physiological and pharmacological knowledge of the peoples living in the forests who use the natural plant resources. In the course of our investigation on bioactive constituents of plants, we found that the methanol extract of *Cyathocalyx crinatus*, a climber belonging to the family Annonaceae, showed lethal toxicity in brine shrimp bioassay the general bioassay tool for bioactive plant constituents.[1] This plant is used sometimes in order to obtain fresh water in jungle and was collected at Miri, Sarawak. Fractionation of the extract by monitoring toxicity to the brine shrimp has now led to the isolation of three new couples of Diels–Alder adducts, designated crinatusins A_1 (**1**) and A_2 (**2**), B_1 (**3**) and B_2 (**4**), and C_1 (**5**) and C_2 (**6**), along with a known chalcone derivative, 4′,6′-dihydroxy-3′,5′-dimethyl-2′-methoxychalcone (**7**).[2] In this paper, we report the structural elucidation and biological activity of these new compounds.

Results and Discussion

Crinatusin A_1 (**1**) was isolated as an optically inactive amorphous solid and showed a molecular ion peak at *m/z* 434.2476 (Δ +1.8 mmu) as molecular formula $C_{28}H_{34}O_4$ in the HREIMS. The IR spectrum for **1** showed hydroxylic absorptions at 3600 and 3480 cm^{-1}. The phenolic nature of the hydroxyl groups was evident from IR absorptions at 1605 and 1495 cm^{-1} and from the positive $FeCl_3$ test. The ^1H NMR spectrum of **1** showed signals due to two vinyl methyls at δ 1.61 and 1.70, two aromatic methyls at δ 1.93 and 2.09, a methoxyl group attached to aromatic ring at δ 3.69, two trisubstituted double bonds at δ 5.12 and 5.51, an unsubstituted phenyl group at δ 7.01, 7.10, and 7.15, and a hydrogen bonded hydroxylic proton at δ 12.65. In addition, the ^{13}C NMR data for **1** exhibited the presence of one ketonic carbon (δ 209.53), together with four methylene carbons, two methine carbons, and six fully substituted sp^2 carbons. The scrutiny of ^1H and ^{13}C NMR data, assisted with ^1H–^1H and ^1H–^{13}C COSY experiments, revealed the presence of a 4,6-dihydroxy-3,5-dimethyl-2-methoxybenzoyl system, the same as that in **7**, and a partial structure **1a** (Fig. 1), in which long-range couplings were observed



1a

Figure 1. Partial structure of **1**.

Keywords: *Cyathocalyx crinatus*; Annonaceae; stems; Diels–Alder adducts; crinatusins A_1, A_2, B_1, B_2, C_1, and C_2; 4′,6′-dihydroxy-3′,5′-dimethyl-2′-methoxychalcone; brine shrimp lethality.

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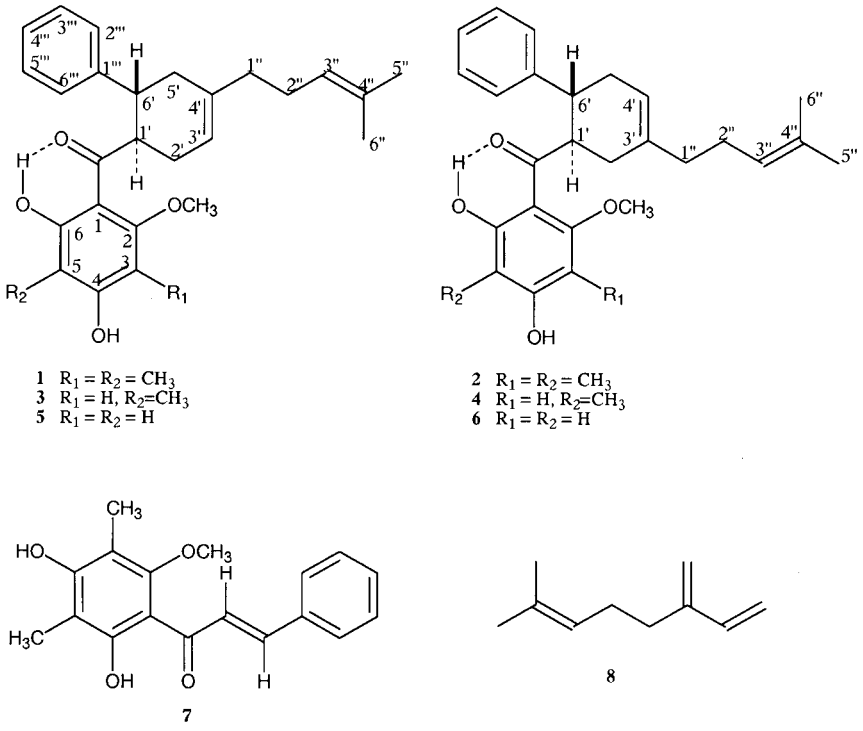

Figure 2. The structures of **1**–**8**.

between 2′-H_2 and 5′-H_2, between 3′-H and 5′-H_2, and between 3′-H and 1″-H_2. Observations of COLOC cross peaks for 6′-H to C-1‴ and for 2‴-H/6‴-H to C-6′ and NOEs between 5′-H_2 and 2‴-H/6‴-H and between 6′-H and 2‴-H/6‴-H established the location of the phenyl group at C-6′, and hence the linkage of the 4,6-dihydroxy-3,5-dimethyl-2-methoxybenzoyl system at C-1′, leading to a gross structure **1** for crinatusin A_1. The relative stereochemistry at C-1′ and C-6′ was deduced from the *J*-value between 1′-H and 6′-H (10.4 Hz) as shown in structure **1** (Fig. 2).

Crinatusin A_2 (**2**), $C_{28}H_{34}O_4$, was also isolated as an optically inactive amorphous solid and displayed the spectral data quite similar to those of **1**. The ^1H and ^{13}C NMR spectra of **2** differed from those of **1** essentially in the resonances due to the cyclohexene moiety. Examination of the proton connectivity by the same techniques described above established the location of the 4-methyl-3-pentenyl side chain at C-3′. Thus, the structure **2** was assigned to crinatusin A_2.

Biosynthetically, crinatusins A_1 and A_2 can be regarded as products resulted from Diels–Alder-like cyclization of 4′,6′-dihydroxy-3′,5′-dimethyl-2′-methoxychalcone (**7**) and the acyclic monoterpene myrcene (**8**). Actually, **7** and **8**, when refluxed in benzene under nitrogen atmosphere overnight, gave a 2:1 mixture of **1** and **2**. In view of the regioselectivity of the Diels–Alder reaction by molecular-orbital considerations,[3] structure **1** is reasonably assigned to the major adduct and structure **2** to the minor one, respectively.

The ratio of crinatusin A_1 to A_2 (2:1) in the extract from *C. crinatus* is in fair agreement with that of the products (2:1) obtained by Diels–Alder reaction, and the optical polarities in both compounds are 0. These results suggested that crinatusins A_1 and A_2 might be artifacts. The Diels–Alder reaction of chalcone **7** and myrcene **8**, however, could be achieved only under reflux condition in benzene solution, whereas the isolation process of natural products was carried out at ambient temperature. Similar compounds, e.g., shefflerin,[4] isoshefflenin,[4] and panduratins,[5,6] Diels–Alder adducts of chalcones and b-ocimene, were reported as natural products.

Crinatusins B_1 (**3**) and B_2 (**4**) were obtained as an inseparable 5:2 mixture and had a common molecular formula $C_{27}H_{32}O_4$ deduced from the HREIMS (*m/z* 420.2307, M^+, Δ +0.6 mmu). The ^1H and ^{13}C NMR spectra for the mixture were very similar to those of the mixture of **1** and **2** except for the lack of one of the aromatic methyls present in **1** and **2**; a proton attached to the meta position of the benzoyl moiety was observed at δ 5.86 (1H, s). Observation of an NOE between the proton and the methoxyl and of COLOC cross peaks for the proton to the C-2 and C-4 secured the location of the proton at C-3. From the evidence outlined above, we proposed the structure **3** for crinatusin B_1, the major one, and the structure **4** for crinatusin B_2, the minor one.

Crinatusin C_1 (**5**) and C_2 (**6**) were also obtained as an inseparable 5:2 mixture with the molecular formula of $C_{26}H_{30}O_4$ judging from EIMS (*m/z* 406, M^+) and the presence of 26 carbons observed in ^{13}C NMR spectrum, and showed the spectral data very similar to those of both mixtures of **1** and **2**, and of **3** and **4**. The ^1H and ^{13}C NMR spectra of the mixture of **5** and **6** did not contain any aromatic methyl signal and, instead, showed the presence of two aromatic protons attached to the benzoyl moiety at δ 5.83 and 5.86 (1H each, d, *J*=2.3 Hz). Observation of an

Table 1. ^{1}H NMR spectral data for compounds **1**–**6** (^{1}H NMR spectra were obtained at 400 MHz and recorded in $CDCl_3$ at room temperature. Coupling constants (*J* in Hz) in parentheses)

H	1	2	3 and 4	5 and 6
3	–	–	5.86 s	5.86 d (2.3)
5	–	–	–	5.83 d (2.3)
1′	4.26 dt (10.4, 7.5)	4.32 dt (10.4, 5.2)	4.28, 4.33 dt (10.7, 5.3)	4.24, 4.30 dt (10.7, 5.1)
2′	2.41 m	2.28, 2.38 m	2.25, 2.45 m	2.26, 2.44 m
3′	5.51 m	–	5.50 m	5.51 m
4′	–	5.52 m	5.50 m	5.51 m
5′	2.24 m	2.31 m	2.23 m	2.23 m
6′	3.18 dt (10.4, 6.9)	3.12 dt (10.4, 6.0)	3.28, 3.23 dt (10.7, 5.5)	3.27, 3.22 dt (10.7, 5.5)
1″	2.02 brt (6.7)	2.0 brt (6.8)	2.03 brt (6.8)	2.02 brt (6.7)
2″	2.11 brq (6.7)	2.12 brq (6.8)	2.11 brq (6.8)	2.12 brq (6.7)
3″	5.12 tsept (6.7, 1.3)	5.13 tsept (6.8, 1.4)	5.12 tsept (6.8, 1.4)	5.12 tsept (6.8, 1.4)
5″	1.70 brs	1.70 brs	1.70 brs	1.70 brs
6″	1.61 brs	1.62 brs	1.61 brs	1.62 brs
2‴ and 6‴	7.15 dt (7.0, 1.8)	7.14 dt (7.0, 1.8)	7.15 m	7.15 m
3‴ and 5‴	7.10 td (7.0, 1.8)	7.09 td (7.0, 1.8)	7.15 m	7.15 m
4‴	7.01 tt (7.0, 1.8)	7.00 tt (7.0, 1.8)	7.06 tt (7.0, 1.8)	7.06 tt (7.0, 1.8)
2-OMe	3.69 s	3.69 s	3.86 s	3.88 s
3-Me	2.09 s	2.09 s	–	–
5-Me	1.93 s	1.94 s	1.92 s	–
6-OH	12.65 s	12.64 s	13.86 s	–

NOE between the signal at δ 5.86 and the signal of the methoxyl and of COLOC peaks between the signal at δ 5.83 and carbon signals of C-1, C-3, and C-6 defined the position of these protons at C-3 and C-5. Accordingly, crinatusin C_1, the major one, can be represented by the structure **5**, and crinatusin C_2, the minor one, by the structure **6**.

Compounds **1**–**6** showed lethal toxicity in the brine shrimp (*Artemia salina*) bioassay[7] (LD_{50} **1**: 30 ppm, **2**: 9 ppm, the mixture of **3** and **4**: 15 ppm, and the mixture of **5** and **6**: 24 ppm), while compounds 7 and 8 were inactive at 50 ppm.

Table 2. ^{13}C NMR spectral data for compounds **1**–**6** (^{13}C NMR spectra were obtained at 100 MHz and recorded in $CDCl_3$ at room temperature)

C	1	2	3 and 4	5 and 6
1	109.90	109.87	106.56	106.80
2	158.53	158.54	160.66	163.04
3	108.18	108.19	90.29	90.93
4	158.43	158.44	159.81	162.33
5	106.09	106.09	103.16	96.63
6	160.43	160.49	164.84	167.01, 167.05
C=O	209.53	209.42	208.91	208.83, 208.64
1′	50.60	50.88	50.63, 50.96	50.57, 50.91
2′	30.69	33.61	30.65, 33.54	30.52, 33.41
3′	119.31	136.58	119.60, 136.81	119.46, 136.75
4′	137.45	120.33	137.41, 120.47	137.41, 120.42
5′	37.54	34.35	38.11, 35.01	38.05, 34.92
6′	44.16	43.88	43.14, 42.83	43.15, 42.85
1″	37.36	37.52	37.35, 37.52	37.32, 37.51
2″	26.52	26.57	26.52, 26.62	26.49, 26.59
3″	124.28	124.28	124.30	124.28
4″	131.54	131.62	131.51	131.53
5″	25.68	25.70	25.70	25.68
6″	17.73	17.76	17.74	17.73
1‴	144.77	144.64	145.56	145.41
2‴	127.62	127.65	127.38	127.35
3‴	128.03	127.98	128.23, 128.17	128.26, 128.20
4‴	125.97	125.94	125.92	126.01
5‴	128.03	127.98	128.23, 128.17	128.26, 128.20
6‴	127.62	127.65	127.38	127.35
2-OMe	62.70	62.60	55.68	55.82
3-Me	8.58	8.61	–	–
5-Me	7.43	7.43	6.92	–

Experimental

General method

NMR: 400 MHz (^{1}H) and 100 MHz (^{13}C), $CDCl_3$, TMS as int. standard. CC: silica gel (Kieselgel 60, Merck) and Lobar B LiChroprep Si 60 and Lobar B LiChroprep RP18 (Merck). TLC: precoated silica gel 60 F_{254} and RP-8 F_{254} plates (Merck). Spots were visualized by UV (254 nm) and 2% $CeSO_4$ in H_2SO_4 after heating. HPLC: Waters 6000 A for pump and TSK-GEL LS-410 KG (ODS, TOSOH) for column.

Plant material

The stems of *Cyathocalyx crinatus* were collected in Lambir National Park, Sarawak, Malaysia, in August 1992. A voucher specimen was deposited at the Herbarium of Forest Research Branch, Forest Department, Sarawak State, Malaysia.

Extraction and isolation

Air-dried and powdered stems (1.1 kg) of *Cyathocalyx crinatus* were immersed in MeOH (8 l) at room temperature for two weeks. The MeOH extract was evaporated in vacuo to yield a residue which was partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2 soluble portion (6.27 g) was subjected to silica gel CC using a step-gradient of hexane–EtOAc (90:10⟶70:30) to give 10 fractions (I-X) in Whichi fractions IV and VII showed 100% lethal toxicity against brine shrimp lavae at 100 ppm. Rechromatography of fraction IV (1.66 g) on Lobar B LiChroprep RP18 column using MeOH–H_2O (85:15) as the eluent, followed by reverse phase HPLC (MeOH–H_2O 80:20), yielded crinatusins A_1 (**1**, 124 mg) and A_2 (**2**, 70 mg). Fraction V (862 mg) was purified by CC on silica gel 60 (Merck) using CH_2Cl_2 as solvent to give 4′,6′-dihydroxy-3′,5′-dimethyl-2′-methoxychalcone (**7**, 675 mg). Fraction VII (264 mg) was chromatographed on silica gel using $CHCl_3$–acetone (95:5) reverse phase HPLC (MeOH–H_2O 85:15) to afford a 5:2 mixture of

crinatusins B_1 (**3**) and B_2 (**4**) (57 mg) and a 5:2 mixture of crinatusins C_1 (**5**) and C_2 (**6**) (45 mg). Both mixtures could not be separated in our any effort.

Crinatusin A_1 (1). Optically inactive amorphous solid, $FeCl_3$ test (+); IR ν_{max} (CCl_4) cm^{-1}: 3600, 3480, 1610, 1495; UV (EtOH) λ_{max} nm (log ϵ): 296.4 (4.16), 205.4 (4.49); ^1H NMR: Table 1; ^{13}C NMR: Table 2; HRMS *m/z* 434.2476, $C_{28}H_{34}O_4$ requires 434.2458.

Crinatusin A_2 (2). Optically inactive amorphous solid, $FeCl_3$ test (+); IR ν_{max} (CCl_4) $cm^{-1:}$ 3610, 3580, 1605, 1510, 1490; UV (EtOH) λ_{max} nm (log ϵ): 296.0 (4.11), 206.0 (4.35); ^1H NMR: Table 1; ^{13}C NMR: Table 2; HRMS *m/z* 434.2471, $C_{28}H_{34}O_4$ requires 434.2458.

Mixture of crinatusins B_1 (3) and B_2 (4). Optically inactive amorphous solid, $FeCl_3$ test (+); IR ν_{max} (CCl_4) $cm^{-1:}$ 3600, 3250, 1615, 1500; UV (EtOH) λ_{max} nm (log ϵ): 298.4 (4.13), 205.2 (4.38); ^1H NMR: Table 1; ^{13}C NMR: Table 2; HRMS *m/z* 420.2307, $C_{27}H_{32}O_4$ requires 420.2301.

Mixture of crinatusins C_1 (5) and C_2 (6). Optically inactive amorphous solid, $FeCl_3$ test (+); IR ν_{max} (CCl_4) cm^{-1}: 3600, 3250, 1620, 1595, 1500; UV (EtOH) λ_{max} nm (log ϵ): 294.8 (4.22), 205.4 (4.43); ^1H NMR: Table 1; ^{13}C NMR: Table 2; EIMS *m/z* (rel. int.): 406 [M^+] (46), 337 (10), 319 (13), 238 (26), 195 (24), 168 (100), 152 (27), 141 (12), 124 (13), 91 (27), 69 (21).

4′,6′-Dihydroxy-3′,5′,-dimethyl-2′-methoxychalcone (7). The crude substance (675 mg) was recrystallized from ether to give orange needles (358 mg), mp 129.5–130.5, which showed spectral data identical with those in Ref. 1.

Preparation of crinatusins A_1 (1) and A_2 (2). A solution of 4′,6′-dihydroxy-3′,5′-dimethyl-2′-methoxychalcone (**7**) (33 mg) and myrcene (**8**) (0.5 ml) in benzene (3 ml) was refluxed overnight under nitrogen atmosphere. The reaction mixture was evaporated in vacuo and the resulting product was subjected to silica gel CC using petroleum ether–EtOAc (85:15) as solvent and subsequently purified by reverse phase HPLC (MeOH–H_2O 80:20) to yield crinatusins A_1 (**1**) (15 mg) and A_2 (**2**) (8 mg).

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