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New clerodane diterpenoids from the twigs and leaves of *Croton euryphyllus*

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ABSTRACT

Three new nor-clerodane diterpenoids, crotoeurins A–C (**1**–**3**), together with four known ones were isolated from the twigs and leaves of *Croton euryphyllus*. Among them, crotoeurin A (**1**) is a new nor-clerodane diterpenoid dimer with a unique cyclobutane ring via a [2+2] cycloaddition. Their structures were elucidated by spectroscopic analyses and the stereochemistry of **1** was confirmed by single-crystal X-ray diffraction analysis. Compounds **1**–**3** exhibited neurite outgrowth-promoting activity on NGF-mediated PC12 cells at concentration of 10 μM.

Clerodane diterpenoids, possessing a 6,6-bicyclic skeleton, are a large and diverse group of naturally occurring organic compounds, mainly occurred in the families Compositae and Labiatae.[1] Since the first sample of clerodane series, clerodin, was isolated from the leaves of *Clerodendrum infortunatum* (Verbenaceae) in 1961, more than one thousand clerodane diterpenoids with different oxygenations and cleavage patterns were discovered from plant origin so far. Moreover, clerodane diterpenoids have attracted wide attention due to their wide variety of biological properties such as antifeedant,[2] cytotoxic,[3] antimicrobial,[4] anti-inflammatory,[5] and NGF-potentiating activities.[6] The genus *Croton* (Euphorbiaceae) has been reported to be another rich source of clerodane diterpenoids,[1] which is still of interest to natural product scientists.[7,8] *Croton euryphyllus* W. W. Smith is a shrub widely distributed in southwest China especially in the Karst region of Guangxi Province,[9] and its roots have been used as a traditional medicine for the treatment of rheumatism, traumatic injury, and related diseases.[10] The phytochemical constituents of *Croton euryphyllus* had never been investigated unless our recent report on the volatile constituents of this plant.[11] Our continuous phytochemical investigation of this plant has led to the isolation of three new nor-clerodane diterpenoids, crotoeurins A–C (**1**–**3**) (Fig. 1), and four known ones, teucvin (**4**),[12] teucvidin (**5**),[13] isoteucvin (**6**),[14] and isocrotocaudin (**7**).[15] Crotoeurin A (**1**) is the first nor-clerodane type diterpenoid dimer with a cyclobutane ring formed through [2+2] cycloaddition. Additionally, the neurite outgrowth-promoting effects of compounds **1**–**3** on NGF-mediated PC12 cells were measured. This Letter deals with structural elucidation of these new compounds and their NGF-potentiating activity on PC12 cells.

The air-dried twigs and leaves of *Croton euryphyllus* (15 kg) were extracted with 95% EtOH and then successively partitioned with petroleum ether, EtOAc, and *n*-BuOH. The EtOAc-soluble partition (400 g) was chromatographed over various columns to obtain compounds **1** (36 mg), **2** (50 mg), and **3** (65 mg) (see detailed experimental procedures in the Supporting information).

Compound **1**[16] was isolated as colorless crystals. Its molecular formula $C_{38}H_{36}O_{10}$ was determined by the HR-EI-MS at *m*/*z* 652.2305 $[M]^+$, corresponding to 21 degrees of unsaturation. The IR spectrum showed the presence of a lactone carbonyl (1770 cm^{-1}) functionality. The ^1H NMR spectrum of compound **1** showed the presence of a secondary methyl [δ_H 1.09 (d, J = 7.0 Hz)], two oxymethines [δ_H 4.71 (br dd, J = 9.0, 6.0 Hz) and 5.58 (t, J = 8.5 Hz)], and a typical β-substituted furan ring [δ_H 6.42 (br d, J = 1.0 Hz), 7.46 (t, J = 1.5 Hz), and 7.49 (s)]. The ^{13}C NMR spectrum (Table 1) displayed 19 carbon signals, with the aid of DEPT and HSQC spectra, were assigned to one methyl, four methylene, seven methine (including two oxygenated at δ_C 72.6 and 76.3 and three olefinic at δ_C 108.0, 139.4, and 144.2), five quaternary (including three olefinic at δ_C 124.8, 131.4, and 136.5) and two carbonyl (δ_C 173.3 and 176.1) carbons.

The characteristic features of ^1H and ^{13}C NMR data together with the molecular formula indicated **1** was a dimeric nor-clerodane diterpenoid with a highly symmetrical skeleton. The NMR

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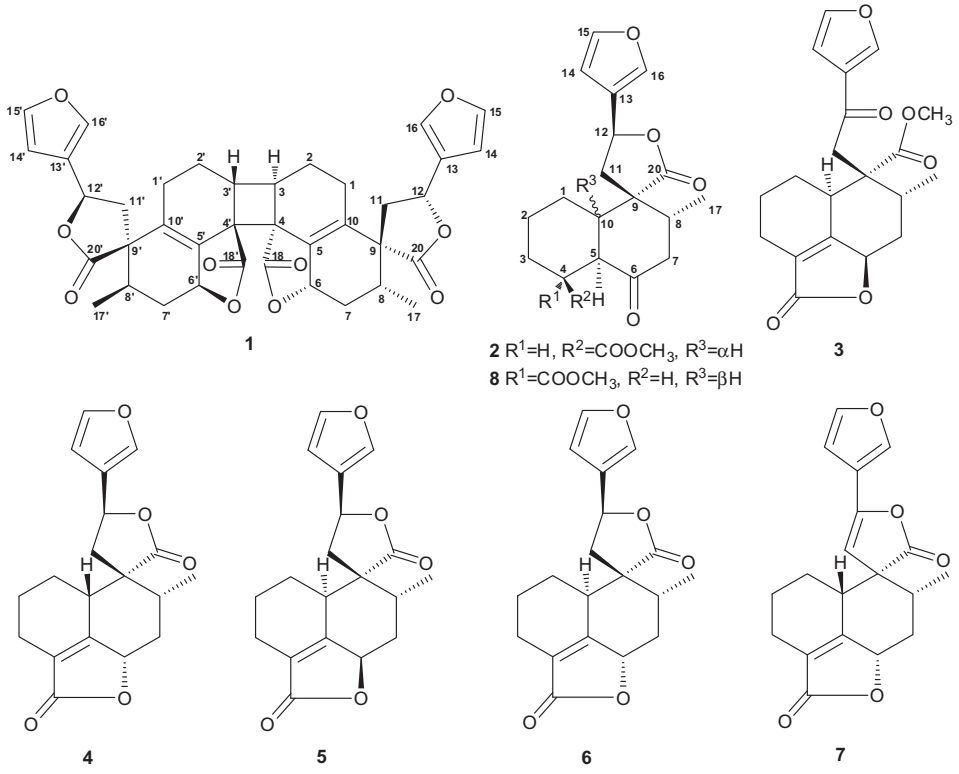

Figure 1. Structures of compounds **1**–**7** and reference compound **8**.

Table 1
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for compounds **1**, **2** and **3** in $CDCl_3$

Position	1		2		3	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1(1′)	21.4 t	2.65 ddd (4.5, 4.0, 3.5)	23.1 t	1.76 m	25.2 t	1.16 m
		2.01 m[a]		2.01 m		2.02 m
2(2′)	22.3 t	1.80 m[a]	25.6 t	1.97 m	21.6 t	1.48 m
		1.27 br dd (14.0, 13.0)		1.26 m		1.69 m
3(3′)	30.9 d	2.69 br d (3.0)	25.0 t	1.03 m	20.2 t	2.23 m[a]
				1.76 m		2.04 m
4(4′)	49.9 s		42.3 d	2.16 m[a]	128.1 s	
5(5′)	131.4 s		47.5 d	3.89 t (3.6)	163.9 s	
6(6′)	76.3 d	4.71 br dd (9.0, 6.0)	208.8 s		76.5 d	4.97 m
7(7′)	32.6 t	2.02 m[a]	45.5 t	3.10 dd (15, 2.4)	34.4 t	1.70 m
		2.02 m[a]		2.23 dd (15, 5.4)		2.23 m[a]
8(8′)	34.9 d	1.82 m[a]	32.9 d	2.16 m[a]	38.9 d	2.24 m[a]
9(9′)	53.4 s		51.7 s		51.1 s	
10(10′)	136.5 s		42.6 d	2.20 ddd (12.6, 3.6, 3.0)	35.3 d	3.45 m
11(11′)	39.9 t	2.92 dd (14.0, 8.5)	36.5 t	2.57 dd (13.8, 10.8)	45.5 t	3.02 s
		2.29 dd (14.0, 8.5)		2.34 dd (13.8, 6.6)		3.02 s
12(12′)	72.6 d	5.58 t (8.5)	70.9 d	5.44 dd (10.8, 6.6)	191.1 s	
13(13′)	124.8 s		124.0 s		126.9 s	
14(14′)	108.0 d	6.42 br d (1.0)	108.2 d	6.41 br s	108.6 d	6.69 d (1.8)
15(15′)	144.2 d	7.46 t (1.5)	144.3 d	7.44 br s	144.5 d	7.44 t (1.8)
16(16′)	139.4 d	7.49 s	140.1 d	7.49 br s	147.1 d	8.01 s
17(17′)	16.0 q	1.09 d (7.0)	17.0 q	1.04 d (6.0)	15.6 q	1.04 d (6.6)
18(18′)	173.3 s		173.8 s		173.2 s	
20(20′)	176.1 s		177.0 s		174.5 s	
OCH_3			51.6 q	3.62 s	52.1 q	3.71 s

[a] Overlapped or partially overlapped signal.

data of **1** were similar to those of isoteuflin,[17] and the main difference was the presence of a quaternary carbon [δ_C 49.9 (C-4)] in compound **1** instead of the absence of a methylene carbon in isoteuflin, which was proven by the ^{1}H–^{1}H COSY experiment which showed the presence of the fragment CH_2(1)–CH_2(2)–CH(3) and the HMBC correlations of H-3 with C-1, C-4, C-5 and C-18 as well as H-2 with C-4 and C-10 (Fig. 2). The presence of a double bond between C-5 and C-10 was confirmed by HMBC correlations of H-1 with C-5 and C-10 as well as H-6 with C-5 and C-10. Finally, the other correlations in the ^{1}H–^{1}H COSY and HMBC spectra further established the two symmetric units (**1a** and **1b**) for **1**, as shown in Figure 2. The above evidence revealed that the units of **1** were connected through a cyclobutane ring (C-3, C-3′, C-4, and C-4′).

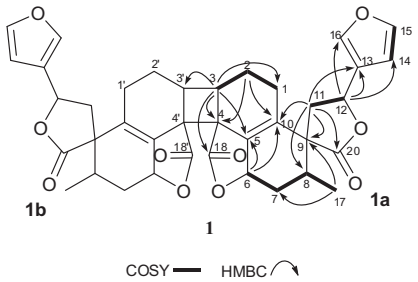

Figure 2. Key ^{1}H–^{1}H COSY and HMBC correlations of **1**.

In the NOESY spectrum, the observed correlations of H-6/H-6′ with H-8/H-8′ indicated H-6 and H-8 as well as H-6′ and H-8′ were in the same orientation (Fig. 3). However, because of the highly symmetrical skeleton, the type of dimerization and the complete stereochemistry of **1** could not be directly determined by NMR spectra including NOESY experiment. Fortunately, a single crystal of **1** was obtained from petroleum ether/acetone, and an X-ray diffraction experiment (CCDC 1000654) using Mo Kα radiation was successfully carried out, which unambiguously confirmed the presence of the cyclobutane ring formed in head-to-head mode but also determined the relative stereochemistry (Fig. 4). Furthermore, on the basis of its small absolute structure Flack parameter [0.01(6)] owe to the anomalous scattering of X-ray by chlorine atom introduced from the solvent $CHCl_3$,[18,19] the absolute configuration of compound **1** was also established to be 3(3′)R, 4(4′)R, 6(6′)S, 8(8′)R, 9(9′)R, 12(12′)S. On the basis of above analysis, compound **1** was identified and named crotoeurin A.

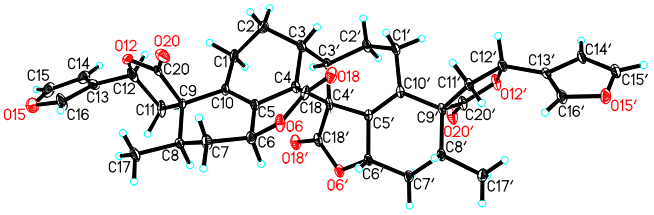

Figure 4. X-ray crystal structure of **1**.

The dimers of clerodane-type diterpenoid via carbon–carbon bond formation are rare in natural products. Up to now, only two dimers of this type compounds were reported, both of which were derived from two different monomers through a six-membered cyclohexane ring via Diels–Alder cycloaddition reactions.[20] However, Compound **1** represents the first nor-clerodane diterpenoid dimer with a cyclobutane ring formed through [2+2] cycloaddition and is, thus, of particular significance for the biosynthesis of clerodane-type diterpenoids.

Compound **2**[21] was obtained as needle crystals, and had the molecular formula $C_{20}H_{24}O_6$ with nine degrees of unsaturation as deduced from HR-EI-MS m/z 360.1571 $[M]^+$. The IR spectrum revealed the presence of two carbonyl (1742 and 1702 cm^{-1}) groups. The ^{1}H NMR spectrum showed signals of one secondary methyl group at δ_H 1.04 (d, J = 6.0 Hz, H-17), and a furan ring at δ_H 6.41 (br s, H-14), 7.44 (br s, H-15), and 7.49 (br s, H-16). The ^{13}C NMR spectrum (Table 1) displayed 20 carbon signals due to two methyl (including one oxygenated at δ_C 51.6), five methylene, eight methine (including one oxygenated at δ_C 70.9 and three olefinic at δ_C 108.2, 140.1, and 144.3), two quaternary (including

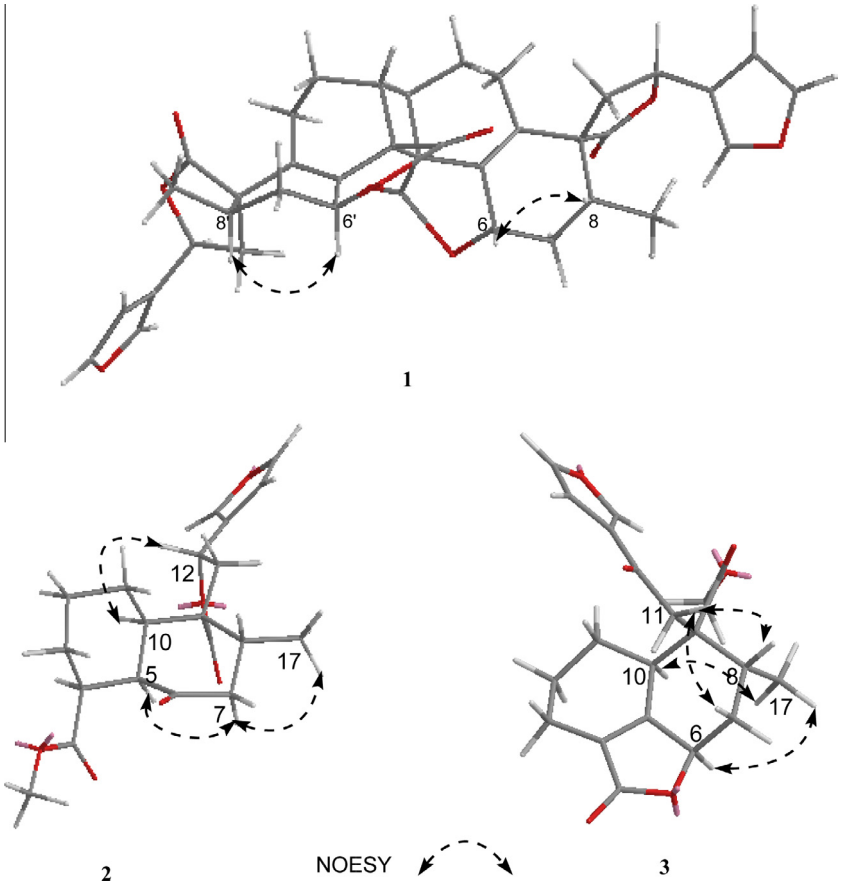

Figure 3. Key NOESY correlations of **1**–**3**.

one olefinic at δ_C 124.0) and three carbonyl (δ_C 173.8, 177.0, and 208.8) carbons. With the aid of routine analyses of ^1H–^1H COSY, HSQC and HMBC spectra, the planar structure of **2** were elucidated as the structure reported previously for the semisynthetic derivative (**8**) of teucvin (**4**).[12] However, the NMR data of **2** were not entirely consistent with those of **8**, especially H-4, H-5 and H-10, suggesting that **2** is a stereoisomer of **8** with regard to the configuration of these positions. The smaller coupling constant ($J_{4,5}$ = 3.6 Hz and $J_{5,10}$ = 3.6 Hz) indicated a *cis*-configuration for H-5/H-4 and H-5/H-10 in **2** in comparison with those ($J_{4,5}$ = 11.6 Hz and $J_{5,10}$ = 11.6 Hz) in **8**, in which the two groups of protons are in a *trans* relationship. The observed NOESY correlations of H-7α with H-5 and H-17 as well as H-10 with H-12 suggested that these protons were also α-orientation (Fig. 3). Therefore, the structure of compound **2** was determined and named crotoeurin B.

Compound **3**,[22] needle crystals, exhibited in the HR-EI-MS analysis a peak at *m*/*z* 358.1410 $[M]^+$, consistent with the molecular formula of $C_{20}H_{22}O_6$. Signals of one methyl group [δ_H 1.04 (d, *J* = 6.6 Hz)], an OCH_3 group [δ_H 3.71 (s)], and a furan ring [δ_H 6.69 (d, *J* = 1.8 Hz), 7.44(t, *J* = 1.8 Hz), 8.01 (s)] were observed in the ^1H NMR spectrum. The ^{13}C NMR spectrum (Table 1) showed 20 carbon signals including one methyl (δ_C 15.6), one OCH_3 (δ_C 52.1), and three carbonyl (δ_C 173.2, 174.5, and 191.1) carbons. The NMR data of **3** (Table 1) were almost similar to those of **5**,[13] except for absence of the signal for an oxygenated methine and presence of the signals for an carbonyl carbon (δ_C 191.1) and a methoxy group (δ_C 52.1; δ_H 3.71, s) in the NMR data of **3**. The carbonyl group was established to be at C-12 on the basis of the HMBC correlation of H_2-11 with C-12. The HMBC correlation of the OCH_3 signal to C-20 suggested the OCH_3 moiety was attached to C-20. In the NOESY spectrum, the strong correlations of Me-17 with H-6 and H-10 suggested that H-6, H-10 and Me-17 were α–oriented. Then the observed NOESY correlations of H_2-11 to H-8 and H-7β confirmed the β-orientation of H-8 and H_2-11 (Fig. 3). Thus, the structure of compound **3** was established and named crotoeurin C.

Considering some clerodane diterpenoids possessed NGF-potentiating effect on PC12 cells,[6,23] compounds **1**–**3** were examined the effect to promote NGF-mediated neurite outgrowth on PC12 cells as previously reported.[24] All the assays were carried out in triplicate. The results showed crotoeurins A–C (**1**–**3**) obviously increased the NGF (5 ng/ml)-induced proportion of neurite-bearing cells after 72 h at the concentration of 10 μM. The percentages of neurite-bearing cells were 9.72%, 14.90%, and 7.14%, respectively, compared with 3.47% of the negative control (5 ng/ml NGF) and 34.72% of the positive control (50 ng/ml NGF). In addition, compounds **1**–**3** showed no cytotoxicity toward the A-549, HL-60, MCF-7, SMMC-7721, and SW-480 human cell lines.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.01.033.

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16. Crotoeurin A (**1**): colorless crystals (Me_2CO); mp 225–228 °C; $[\alpha]_D^{25.1}$ +56.29 (*c* 0.16, MeOH); UV (MeOH) λ_{max} (log ε) 208 (4.00), 251 (3.97); IR (KBr) ν_{max} 3441, 2927, 1770, 1336, 1173, 1022, 975, 874 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; positive ESI-MS *m*/*z* 675 $[M+Na]^+$; positive HR-EI-MS *m*/*z* 652.2305 $[M]^+$ (calcd for $C_{38}H_{36}O_{10}$, 652.2308).
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21. Crotoeurin B (**2**): needle crystals (Me_2CO); mp 132–135 °C; $[\alpha]_D^{24.9}$ +78.60 (*c* 0.22, MeOH); UV (MeOH) λ_{max} (log ε) 206 (3.74); IR (KBr) ν_{max} 3450, 2941, 1742, 1702, 1633, 1384, 1201, 1189, 1018, 874 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; positive EI-MS *m*/*z* 360 $[M]^+$; positive HR-EI-MS *m*/*z* 360.1571 $[M]^+$ (calcd for $C_{20}H_{24}O_6$, 360.1573).
22. Crotoeurin C (**3**): colorless crystals (Me_2CO); mp 145–147 °C; $[\alpha]_D^{24.9}$ −193.16 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.25), 217 (4.31), 256 (3.54); IR (KBr) ν_{max} 3434, 2947, 1749, 1683, 1629, 1392, 1209, 1154, 1041, 873 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; positive EI-MS *m*/*z* 358 $[M]^+$; positive HR-EI-MS *m*/*z* 358.1410 $[M]^+$ (calcd for $C_{20}H_{22}O_6$, 358.1416).
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