EBC-219: A New Diterpene Skeleton, Crotinsulidane, from the Australian Rainforest Containing a Bridgehead Double Bond**

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Dedicated to Professor Armin de Meijere on the occasion of his 75th birthday

Abstract: *EBC-219 (4), isolated from Croton insularis (Baill), was established by spectroscopic and DFT methods as the first member of a new diterpene skeletal class, uniquely defined by the presence of a bicyclo[10.2.1] bridgehead olefin. The proposed biogenetic pathway to 4 from the co-isolated natural products EBC-131 (1), EBC-180 (2) and EBC-181 (3) is highly likely. EBC-180 (2) and EBC-181 (3) showed moderate to strong cytotoxic activity against various cancer cell lines.*

Croton is a well-known plant genus, which has delivered many classes of bioactive compounds, for example phorbols,[1] *ent*-kauranes,[2] clerodanes,[3] and halimanes[4] to name a few. This rich history prompted our interest in pursuing the minor constituents of croton, particularly *Croton insularis* (Baill),[5,6] from Australia's rain forest.[7] We herein report the isolation of a novel diterpene, EBC-219 (**4**) (Figure 1), which defines a new diterpene skeletal class, in addition to possessing a rare naturally occurring bridgehead olefin (anti-Bredt system). The term anti-Bredt, arising from the work of Julius Bredt in the early 1900s,[8] is bestowed upon unsaturated bridgehead bicyclic caged systems.[8] This rule, although initially derived by Bredt utilizing monoterpenes, has been mostly contested by synthetic and physical organic chemists.[9] Nevertheless, there are some rare, but famous, natural product examples, such as taxol[10] and the phomoidrides,[11] along with other uniquely sized ring systems[12,13] that have appeared in the literature. Lastly, reassignments of natural product structures
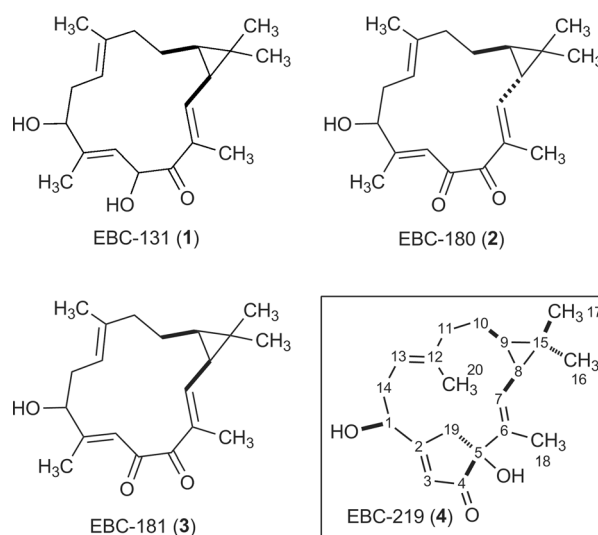

Figure 1. EBC-131 (**1**), EBC-180 (**2**), EBC-181 (**3**), and EBC-219 (**4**). Numbering of the casbane system used for **4**.

based on Bredt's rule have been made by our group[14] and by Fraga et al.[15]

EBC-131 (**1**), EBC-180 (**2**), EBC-181 (**3**), and EBC-219 (**4**) (Figure 1) were extracted from the stems of *Croton insularis* (Baill) and separated by silica gel chromatography followed by HPLC, as guided by a combination of bioassays and ^{1}H NMR spectroscopy.

The high-resolution mass spectrum of EBC-131 (**1**) gave the molecular formula $C_{20}H_{30}O_3$ suggesting six ring and/or double-bond equivalents (RDBE). The ^{1}H NMR spectrum revealed two methyl groups situated on double bonds ($\delta_H = 1.58$, 1.69 ppm), and a third which was adjacent to a carbonyl group ($\delta_H = 1.93$ ppm). This, together with ^{13}C NMR shifts, corresponded to the data of a casbane reported by Filho et al.[16] EBC-180 (**2**), EBC-181 (**3**) and EBC-219 (**4**) all possess the same molecular formula ($C_{20}H_{28}O_3$), which constitutes seven RDBE. For EBC-180 (**2**) the ^{1}H NMR and ^{13}C NMR spectra revealed three methyl groups ($\delta_H = 1.57$, 1.85, and 1.97 ppm) each placed on a double bond, one of which is isolated while the other two are adjacent to two carbonyls, suggesting the remainder of the skeleton to be bicyclic. Considering the similarity to EBC-131 (**1**), comparison to literature data confirmed EBC-180 (**2**) as another known casbane.[17] However, a slight reassignment of the reported structure containing a 6-*Z* double bond was required

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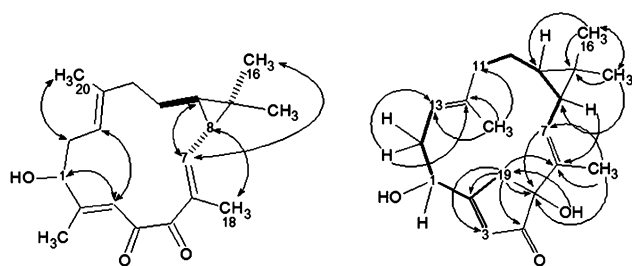EcoBiotics Limited, Yungaburra (Australia)

Figure 2. Left: Selected NOESY correlations for EBC-180 (**2**). Right: Selected COSY (bold bonds) and HMBC (curved arrows) correlations for EBC-219 (**4**).

connection, that between C4 ($\delta_C = 209.74$ ppm) and C5, completed the skeleton. Additional multiple HMBC cross-peaks from 19-H (δ_H 2.61 ppm) to C2–C6 confirmed the cyclopentenone location and structure (Figure 2).

The relative stereochemistry of EBC-219 (**4**) was determined from NOESY correlations in conjunction with DFT calculations (Figure 3). These data unmasked the presence of
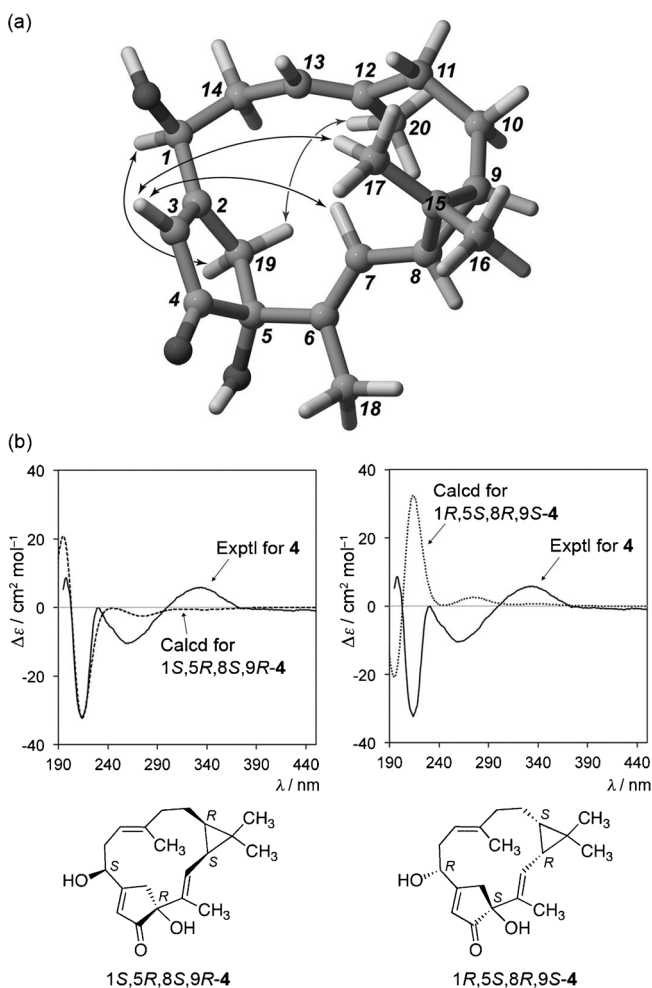
as the NOESY spectra confirmed the cyclopropane ring and all double bonds to be *trans*-configured (Figure 2). The key feature to this realization was a NOESY cross-peak between Me-18 ($\delta_H = 1.85$ ppm) and 8-H ($\delta_H = 1.09$ ppm) and the absence in our data of a cross-peak Me-18/7-H ($\delta_H = 5.85$ ppm) reported in the literature data.[17] Furthermore, *Z*-configured double bonds are rather rare within the casbane skeleton; in the few instances that have been confirmed unequivocally by X-ray crystallography, the double bonds have distinct δ_C values.[18]

In ^{13}C NMR and DEPT experiments EBC-181 (**3**) had entirely the same multiplicity characteristics to suggest the same structure as EBC-180 (**2**). However, a difference in the δ_H and δ_C values was apparent for the cyclopropyl moiety [for example, 9-H (**3**) $\delta_H = 1.23$ cf. 9-H (**2**) $\delta_H = 0.71$ ppm; C8 (**3**) $\delta_C = 28.5$ cf. C8 (**2**) $\delta_C = 33.1$ ppm].[19] NOE spectra correlations 7-H/10-H and 8-H/10-H then confirmed that the cyclopropane was *cis*-configured.

EBC-219 (**4**), possessed the same molecular formula as EBC-180 (**2**) and EBC-181 (**3**), however, the NMR data (Table 2 in the Supporting Information) showed three double bonds and only one carbonyl indicating a tricyclic structure. Analysis of ^{13}C and DEPT NMR data implied four methyl groups, a carbonyl, four methylenes, three double bond methines, three aliphatic methines, and six quaternary carbons. According to the ^{1}H NMR spectrum, however, two methyl groups ($\delta_H = 1.62$ and 1.85 ppm) were connected to double bonds, both having allylic coupling constants, $^4J = 1.4$ and 1.5 Hz, respectively. Analysis of the COSY spectrum provided the following sequential connectivities, from C7 to C11, from C13 to C3 and to C19 (Figure 2). HMBC correlations of Me-20 ($\delta_H = 1.62$ ppm) with C11 ($\delta_C = 39.95$ ppm), C12 ($\delta_C = 140.19$ ppm), and C13 ($\delta_C = 117.42$ ppm), and Me-18 ($\delta_H = 1.85$ ppm) with C5 ($\delta_C = 82.89$ ppm), C6 ($\delta_C = 136.99$ ppm), and C7 ($\delta_C = 124.21$ ppm) provided two connections to the COSY-determined fragments (Figure 2). The HMBC correlations of Me-16 ($\delta_H = 1.04$ ppm) and Me-17 ($\delta_H = 0.85$ ppm) displayed the characteristic *gem*-dimethyl cross-peak patterns arranging the cyclopropane moiety. The mode of the five-carbon ring closure was resolved on the basis of the ^{13}C NMR chemical shifts of C2 ($\delta_C = 180.65$ ppm) and C3 ($\delta_C = 125.99$ ppm) presenting the C2–C4 enone system. The HMBC correlation of OH-5 with C19 ($\delta_C = 43.76$ ppm) allowed the connection of C5 ($\delta_C = 82.89$ ppm) to C19 as a single bond. The last bond

Figure 3. a) Geometry of the lowest-energy conformer of EBC-219 (**4a**) and key NOE correlations. b) Comparison of calculated and experimental CD spectra of **4** in acetonitrile. Calculated spectra were simulated at the TD-RI-B2PLYP/TZVP//B3LYP/6-31G(d) level of theory in acetonitrile (COSMO).

distinct northern and southern hemispheres, the former occupied by the cyclopentenone C3–C4 fragment and the *gem*-dimethyl group, and the latter containing the groups CH$_2$-19, Me-18, and Me-20. Assignment of the northern grouping was made on the basis of a key NOE correlation between cyclopentenone 3-H and cyclopropyl Me-17, while the southern grouping was inferred from correlations between 19-H and both Me-18 and Me-20. DFT calculations (B3LYP-D3/6-31G(d)) revealed that the macrocyclic ring can adopt four low-energy conformations, which feature either in-plane or perpendicular alignments of the alkene groups

(Figure A in the Supporting Information). The perpendicular conformers [for example, **4a** (Figure 3), **4d**, **4k** (Figure A)] support the strongest 19-H/Me-18 and 19-H/Me-20 NOEs, while all conformers support the 3-H/Me-17 correlation. Taken together, these results indicate a 5*R**,8*S**,9*R** relative stereochemistry for EBC-219 (**4**); the configuration at the remaining stereocenter (C1) is assigned as *S** based on the presence of a NOESY cross-peak between 1-H and 19-H and the absence of a cross-peak between 1-H and 3-H.
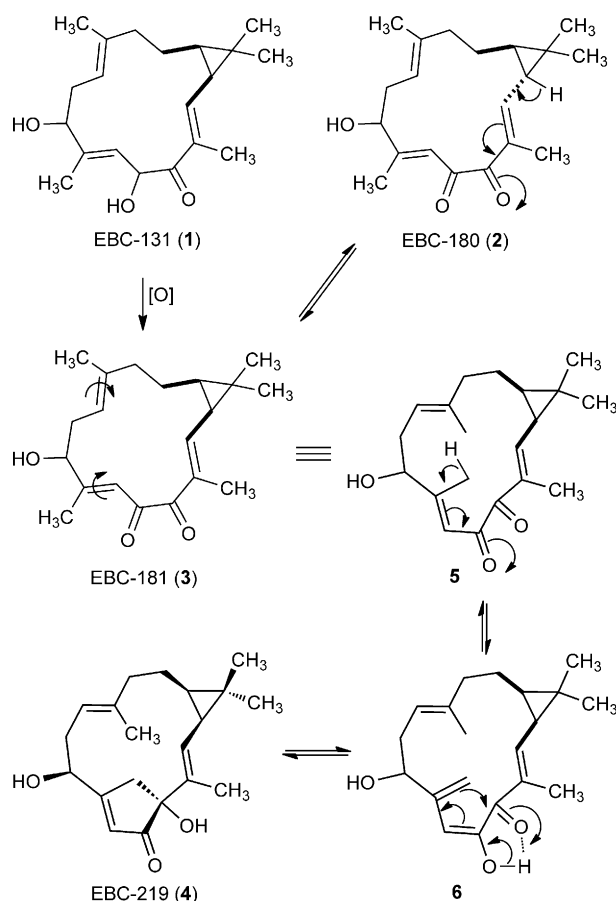
The absolute configuration of EBC-219 (**4**) was deduced from comparison of experimental and calculated CD spectra. Following a conformational search of EBC-219 (**4**) at the B3LYP/6-31G(d) level, the conformers' free energies in acetonitrile were computed by adding to the gas-phase free energies a B3LYP-D3 dispersion energy correction, and a PCM solvation energy. Time-dependent DFT calculations on the important conformers ($\Delta G \leq 3$ kcal mol^{-1}) at the RI-B2PLYP/TZVP level in acetonitrile (COSMO) afforded Boltzmann-weighted spectra for the enantiomers of **4**, which are compared to the experimental CD spectrum[20,21] (Figure 3). Good qualitative agreement between the calculated spectrum of the 1*S*,5*R*,8*S*,9*R* enantiomer and experiment permit the assignment of the absolute configuration of EBC-219 as 1*S*,5*R*,8*S*,9*R*-**4**.

The main feature of the tricyclo[11.2.1^{1,13}.0^{4,6}]hexadecane structure of EBC-219 (**4**) is the one-carbon C2–C5 bridge creating the double-bond terminus at the bridgehead position (anti-Bredt system). Although, Bredt himself realized towards the end of his career that stable molecules could exist that violate his rule,[22] a philosophical perspective, based on the guidelines laid down by Fawcett,[9a] Prelog,[23] Wiseman,[9g] and Schleyer,[9h] could imply that natural products struggle to adhere to the rule.[24] Nevertheless, the bridging ring combination seen in EBC-219 (**4**) would be considered a stable system (e.g. Fawcett *S* value ≥ 9; EBC-219 $S = 13$). Therefore, from a biosynthetic perspective it is plausible to consider that an intramolecular extended enol/enolate (see **5** and **6**) is formed from EBC-181 (**3**), which is perfectly positioned, not to mention entropically favored, to attack the ketone at C5 giving rise to EBC-219 (**4**) (Scheme 1). The starting point, for this likely biosynthetic cascade, would arise from biological oxidation of EBC-131 (**1**) with probable γ enolization of the product [that is, EBC181 (**3**)] leading to EBC-180 (**2**) as a competing process.

Scheme 1. Proposed biosynthetic pathway starting from EBC-181 (**3**) and leading to EBC-219 (**4**) and the interrelationship with EBC131 (**1**) and EBC-180 (**2**).

The cytotoxicity of the isolated diterpenes against human fibroblasts and five human cancer lines was evaluated and is summarized in Table 1. Among the tested compounds, EBC-180 (**2**) and EBC-181 (**3**) showed moderate to strong cytotoxic activity against some of the cancer cell lines, compared to that against the NFF strain.

In conclusion, the bicyclo[10.2.1] structural motif seen in EBC-219 (**4**) is a first to class in the rare natural product *anti*-Bredt arena, and represents a new diterpene structure class, which we herein name crotinsulidane (**7**) (Figure 4).
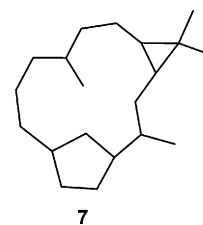

Figure 4. Crotinsulidane skeleton.

Table 1: Inhibition of cell growth by EBC-131, EBC-180, EBC-181, and EBC-219 (**1–4**). [a]

Compound	IC$_{50}$ [μM]					
	HeLa[b]	HT29[c]	MCF7[d]	MM96L[e]	NFF[f]	K562[g]
EBC-131 (**1**)	100	100	60	80	140	>100
EBC-180 (**2**)	8	8	18	7	20	50
EBC-181 (**3**)	2.5	8	30	4	60	9.5
EBC-219 (**4**)	50	80	80	60	80	100

[a] Mean concentrations for 50% growth inhibition (replicate values); compounds showing values >30 μM are considered inactive. [b] HeLa (cervical carcinoma). [c] HT29 (colon cancer). [d] MCF7 (breast cancer). [e] MM96L (melanoma). [f] NFF (normal fibroblasts). [g] K562 (leukemia).

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