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Antimitotic diterpenoids from *Erythropodium caribaeorum*: isolation artifacts and putative biosynthetic intermediates

Robert Britton,[a] Michel Roberge,[b] Hans Berisch[c] and Raymond J. Andersen[a,*]

[a]*Departments of Chemistry and Oceanography (EOS), University of British Columbia, Vancouver, BC, Canada V6T 1Z1*
[b]*Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada V6T 1Z3*
[c]*Ross University Medical School, Commonwealth of Dominica*

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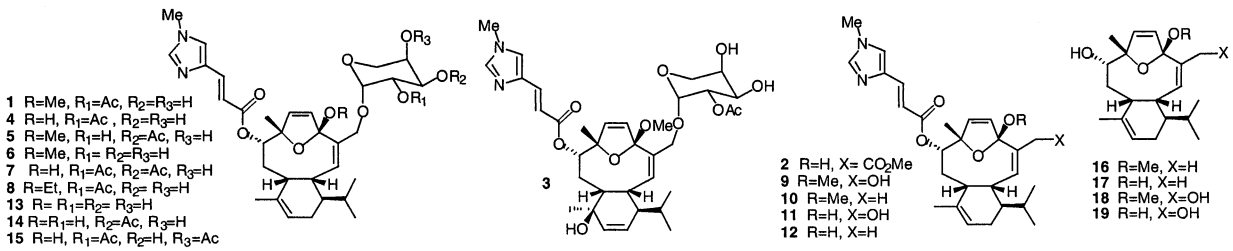
Abstract—Two new natural products, caribaeorane (**12**) and 15-hydroxycaribaeorane (**11**), have been identified in *Erythropodium caribaeorum* extracts by isolation of their C-4 methylketals **10** and **9**. It has been demonstrated that eleutherobin (**1**) is an isolation artifact. A proposal for the late stages of the biosynthetic pathway to the *E. caribaeorum* antimitotic diterpenoids is presented.

Eleutherobin (**1**), isolated by Fenical and coworkers from the Western Australian soft coral *Eleutherobia* sp., belongs to a small family of soft coral diterpenoids that are microtubule-stabilizing antimitotic agents.[1] A large amount of effort has been directed towards the total synthesis of eleutherobin and other members of this family.[2] Although these efforts have culminated in the total synthesis of eleutherobin (**1**), its preclinical evaluation as an anticancer drug has been stalled by the limited supply of material available for testing.[3] Recently, we reported that the relatively abundant Caribbean soft coral *Erythropodium caribaeorum* is a good source of eleutherobin (**1**) and a number of analogs including sarcodictyin A (**2**), caribaeoside (**3**), *Z*-eleutherobin, desmethyleleutherobin (**4**), isoeleutherobin A (**5**), and desacetyleleutherobin (**6**).[4,5] *E. caribaeorum* could provide adequate quantities of material for preclinical evaluation of this promising family of anticancer drug leads and even clinical trials should the compounds progress that far.

Initial collections of *E. caribaeorum* examined by our laboratory were extracted with MeOH, resulting in the isolation of highly variable ratios of eleutherobin (**1**) and desmethyleleutherobin (**4**). Ketzinel et al. have reported isolating the eleuthosides (e.g. **7**), which are all hemiketals at C-4, from *Eleutherobia aurea* by using non-alcohol solvents such EtOAc.[6] These observations raised the possibility that eleutherobin (**1**) was actually an isolation artifact. To resolve this issue, fresh specimens of *E. caribaeorum* were collected in Dominica and one portion of the sample was extracted with EtOH and a second portion was extracted with MeOH. The MeOH extracted sample yielded eleutherobin (**1**), desmethyleleutherobin (**4**), and sarcodictyin A (**2**) as the major components along with minor amounts of **3**, **5**, and **6** as before,[5] while the EtOH extracted sample yielded the C-4 ethylketal **8** along with **2** and **4** as the major components. Eleutherobin (**1**) could not be detected by analytical HPLC or NMR analysis of the chromatography fractions from the EtOH extract.



* Corresponding author.

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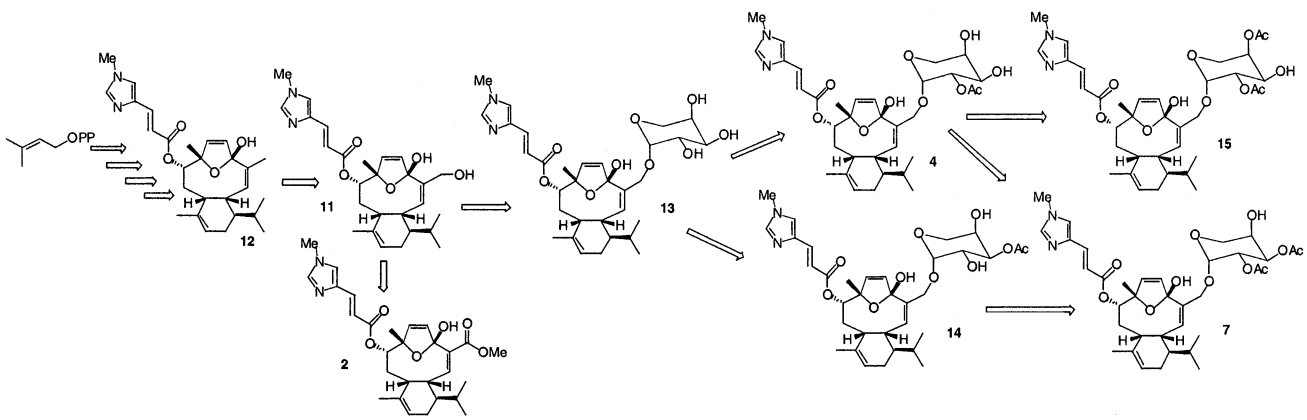
These results show that the C-4 methyl ketal in eleutherobin (**1**) isolated from *E. caribaeorum* is indeed an artifact.

During the course of fractionating a large scale *E. caribaeorum* MeOH extract in order to obtain sufficient eleutherobin (**1**) for biological testing, the eleutherobin aglycon **9**[7] and methylcaribaeorane (**10**) were isolated as very minor components along with sarcodictyin A (**2**) from the least polar fractions eluting from silica gel with EtOAc:MeOH (85:15). The eleutherobin aglycon **9** has previously been reported as a synthetic intermediate.[2a] Comparison of the spectroscopic data collected on the material isolated from *E. caribaeorum*[7] with the literature data for the synthetic compound confirmed that they were identical. The eleutherobin aglycon **9** is presumed to be an artifact formed from the corresponding hemiketal natural product 15-hydroxycaribaeorane (**11**) during the MeOH extraction.

Methylcaribaeorane (**10**),[8] isolated as a white amorphous solid, gave an $[M+H]^+$ ion at m/z 467.2912 in the HRFABMS appropriate for a molecular formula of $C_{28}H_{38}N_2O_4$ that differed from the molecular formula of the eleutherobin aglycon **9** simply by the loss of one oxygen atom. Examination of the ^1H and ^{13}C NMR data obtained for methylcaribaeorane (**10**) revealed the presence of two olefinic methyl groups (^1H NMR, C_6D_6: δ 1.65, s, 3H; 1.80, s, 3H) and the absence of resonances that could be assigned to an allylic hydroxymethyl fragment. These observations indicated that methylcaribaeorane (**10**) was simply missing the C-15 allylic alcohol found in the eleutherobin aglycon **9**. HMBC correlations observed between a methyl resonance at δ 3.18 and a carbon resonance at δ 117.4 assigned the carbon resonance to the C-4 ketal. The olefinic methyl resonance at δ 1.80 showed HMBC correlations to the C-4 ketal carbon resonance at δ 117.4 and to the C-2 and C-3 olefinic carbon resonances at δ 131.3 and 134.0, respectively, confirming that there was an allylic methyl at C-15. The remaining NMR data for methylcaribaeorane was completely consistent with the structure **10**.[8] Methylcaribaeorane (**10**) is also presumed to be an isolation artifact formed from the corresponding hemiketal natural product caribaeorane (**12**). The eleutherobin aglycon **9** and methylcaribaeorane (**10**) were active in a cell-based assay for antimitotic activity at 1 and 10 μM, respectively.[5]

Most of the compounds isolated from the *E. caribaeorum* MeOH extract had C-4 methylketals, which are artifacts. Interestingly, sarcodictyin A (**2**) was only isolated as the C-4 hemiketal. Since methylketal transformations clearly take place during the extraction process, it seemed possible that the eleutherobin aglycon **9** might also be an artifact formed from eleutherobin (**1**) by hydrolysis/methanolysis of the glycosidic linkage during extraction. An investigation of the ketal transformations was undertaken in order to confirm that glycoside hydrolysis was not occurring during extraction and to gain insight into the lack of ketal formation in sarcodictyin A (**2**). First, it was found that desmethyleleutherobin (**4**) can be converted quantitatively to eleutherobin (**1**) by treatment with a catalytic amount of pyridinium *p*-tolunesulfonate (PPTS) in methanol at room temperature.[2] The reverse transformation, **1** to **4**, can be quantitatively effected using PPTS and water/CH_2Cl_2. Neither of these transformations result in glycoside hydrolysis. Saracodictyin A (**2**) could also be converted quantitatively to its methylketal using PPTS and MeOH at rt. The combination of the extraction and the laboratory observations indicate that the MeOH extraction conditions are able to readily convert the C-4 hemiketal to the methylketal when there is a methyl, hydroxymethyl, or glycosidic functionality at C-15, but that they cannot effect methylketal formation when there is a ester at C-15. The PPTS in MeOH or PPTS in water/CH_2Cl_2 quantitatively interconverts all C-4 hemiketals and methylketals, but still does not bring about glycoside hydrolysis. Therefore, it is apparent that the milder MeOH extraction conditions will not cleave the glycoside linkage, confirming that 15-hydroxycaribaeorane (**11**) is indeed a natural product.

The discovery of methylcaribaeorane (**10**) and the eleutherobin aglycon **9** as minor constituents in the MeOH extracts of *E. caribaeorum* along with the previously reported compounds **1** to **6**, and the demonstration that the C-4 methyl ketal functionalities in these



Scheme 1.

compounds are isolation artifacts, led to the biosynthetic proposal presented in Scheme 1. This proposal suggests that geranylgeranylpyrophosphate undergoes cyclization and oxidative functionalization to give the diterpenoid core which is esterified at the C-8 hydroxyl with the urocanic acid residue to give caribaeorane (**12**), the first known intermediate in the pathway. Caribaeorane (**12**) would then be oxidized to 15-hydroxycaribaeorane (**11**) and the arabinose residue would be added to give desacetyldesmethyleleutherobin (**13**), which if monoacetylated at C-2′ would give desmethyleleutherobin (**4**) or at C-3′ would give desmethylisoeleutherobin A (**14**). Further acetylation of either **4** or **14** would give eleuthoside A (**7**), while acetylation of desmethyleleutherobin (**4**) at C-4′ would give eleuthoside B (**15**). In this proposal, sarcodictyin A (**2**) represents a shunt metabolite formed by further oxidation of 15-hydroxycaribaeorane (**11**) to the 15-carboxylic acid followed by SAM methylation to give the methyl ester. It should be noted that there is no evidence for the occurrence of either **7** or **15** in the *E. caribaeorum* extract, however, their biosynthesis in *Eleutherobia aurea* would presumably follow the same pathway.

One significant aspect of the hypothesis presented in Scheme 1 is the proposal that the urocanic ester residue is added to the diterpenoid core before the C-15 alcohol required for glycoside formation is introduced. In an attempt to provide further evidence for this suggestion, the urocanic acid residue was removed from methylcaribaeorane (**10**) by hydrolysis with a few drops of 5N NaOH in MeOH overnight at rt to give **16**,[9] the methyl ketal analog of one of the potential biosynthetic precursors **17** of caribaeorane (**12**). Careful examination of the *E. caribaeorum* MeOH extract chromatography fractions by TLC and GC analysis using **16** as a reference failed to show any evidence for its presence. The corresponding hydrolysis product **18**[10] of the eleutherobin aglycon **9** was also prepared and once again TLC and GC analysis failed to show any evidence for its presence in the *E. caribaeorum* MeOH extract. This negative evidence does not eliminate **17** as the immediate biosynthetic precursor to caribaeorane (**12**) or rule out the possibility that **19** is the direct precursor to **11**, but it does raise the interesting possibility that the urocanic ester is formed before all the diterpenoid functionality, such as the C-7 tertiary alcohol or the C-4 ketone, are in place.

Acknowledgements

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7. Eleutherobin aglycon **9**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.52, d, J=15.4 Hz (H-3′), 7.45, s (H-7′), 7.08, s (H-5′), 6.54, d, J=15.4 Hz (H-2′), 6.21, d, J=6.0 Hz (H-6), 6.02, d, J=6.0 Hz (H-5), 5.56, d, J=9.4 Hz (H-2), 5.25, m (H-12), 4.80, d, J=7.4 Hz (H-8), 4.16, d, J=12.0 Hz (H-15), 3.85–3.95, m (H-15, H-1), 3.69, s (H-9′), 3.23, s (H-21), 2.67, m (OH), 2.59, m (H-10), 2.31, m (H-13), 1.98, m (H-13), 1.50–1.65, m (H-9, H-18), 1.50, s (H-17), 1.45, s (H-16), 1.25–1.40, m (H-9, H-14), 0.97, d, J=6.5 Hz (H-19), 0.91, d, J=6.5 Hz (H-20); HRFABMS [M+H]$^+$ calcd for $C_{28}H_{39}N_2O_5$: 483.2859, found: 483.2860; $[\alpha]_D$ −24.2°.
8. Methylcaribaeorane (**10**): ^{1}H NMR (C_6D_6, 500 MHz) δ 7.95, d, J=15.4 Hz (H-3′), 7.26, d, J=15.4 Hz (H-2′), 6.72, s (H-7′), 6.00, s (H-5′), 5.95, d, J=5.8 Hz (H-6), 5.78, d, J=5.8 Hz (H-5), 5.49, d, J=9.7 Hz (H-2), 5.33, m (H-12), 5.25, d, J=7.3 Hz (H-8), 4.30, m (H-1), 3.18, s (H-21), 2.95, m (H-10), 2.37, m (H-13), 2.25, s (H-9′), 2.00, m (H-9), 1.98, m (H-13), 1.81, m (H-9), 1.80, s (H-15), 1.65, s (H-17), 1.57, m (H-18), 1.46, s (H-16), 1.28, m (H-14), 1.06, d, J=6.5 Hz (H-19), 0.89, d, J=6.5 Hz (H-20); ^{13}C NMR (C_6D_6, 100 MHz) δ 167.1 (C-1′), 139.2 (C-4′), 139.2 (C-7′), 137.3 (C-3′), 134.9 (C-11), 134.8 (C-6), 134.0 (C-3), 131.3 (C-2), 130.1 (C-5), 122.6 (C-5′), 121.6 (C-12), 117.4 (C-4), 116.5 (C-2′), 90.5 (C-7), 82.2 (C-8), 49.5 (C-21), 43.3 (C-14), 39.6 (C-10), 34.6 (C-1), 32.3 (C-9), 31.9 (C-9′), 29.4 (C-18), 24.9 (C-16), 24.8 (C-13), 22.4 (C-15), 22.3 (C-17), 22.2 (C-20), 20.8 (C-19); HRFABMS [M+H]$^+$ calcd for $C_{28}H_{39}N_2O_4$: 467.2910, found: 467.2912.
9. Compound **16**: ^{1}H NMR (C_6D_6, 400 MHz) δ 5.99, d, J=5.9 Hz (H-6), 5.74, d, J=5.9 Hz (H-5), 5.41, dd, 1H, J=9.3, 1.3 Hz (H-2), 5.36, m (H-12), 4.15, m (H-1), 3.47, m (H-8), 3.19, s (H-21), 2.35, m (H-10), 2.28, m (H-13), 1.97, m (H-13), 1.80, s (H-15), 1.62, s (H-17), 1.50, s (H-16), 1.50–1.72, m (H-9, H-18, H-14, OH), 1.27, m (H-9), 0.98, d, J=6.5 Hz (H-19), 0.93, d, J=6.5 Hz (H-20); HRDCI$^+$MS calcd for $C_{21}H_{32}O_3$: 332.2351, found: 332.2351.

10. Compound **18**: ^{1}H NMR (C_6D_6, 400 MHz) δ 5.94, d, $J=5.9$ Hz (H-6), 5.68, d, $J=5.9$ Hz (H-5), 5.67, d, $J=9.5$ Hz (H-2), 5.31, m (H-12), 4.00–4.20, m (H-1, H-15, H-15), 3.44, bd, $J=6.1$ Hz (H-8), 3.00, s (H-21), 2.23–2.38, m (OH, H-10, H-13), 1.93, m (H-13), 1.59, s (H-17), 1.43, s (H-16), 1.42–1.62, m (H-9, H-18, OH), 1.30, m (H-14), 1.25, m (H-9), 0.93, d, $J=6.9$ Hz (H-19), 0.90, d, $J=6.7$ Hz (H-20); HRDCI$^+$MS calcd for $C_{21}H_{32}O_4$: 348.2301, found: 348.2303.