Cytotoxic Polyketides

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Franklinolides A–C from an Australian Marine Sponge Complex: Phosphodiesters Strongly Enhance Polyketide Cytotoxicity**

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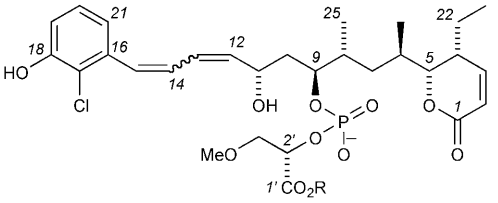
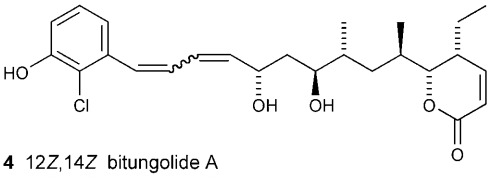
During investigations into cytotoxic metabolites as potential anticancer agents from Australian marine invertebrates and algae, our attention was drawn to a sponge sample (CMB-01989) collected during deepwater (−105 m) scientific trawling operations in the Great Australian Bight. The aqueous EtOH extract of CMB-01989, a massive *Geodia* sp. thinly encrusted with a *Halichondria* sp., returned a *n*BuOH partition that displayed noteworthy cytotoxicity against human colon (HT-29), prostate (DU145), ovary (JAM and C180-13S), and lung (A549) cancer cell lines ($GI_{50} < 5$–$10\ \mu g\,mL^{-1}$), with slightly lower potency against breast (MDA-MB-231) and skin (SK-MEL-128) cancer cell lines ($GI_{50} \approx 30\ \mu g\,mL^{-1}$). Fractionation of the aqueous EtOH extract yielded franklinolides A–C (**1**–**3**) as unprecedented polyketide phosphodiesters that are prone to esterification, isomerization, and hydrolysis on exposure to protic solvents and/or elevated temperatures in acidic media. Complete absolute configurations were assigned to the franklinolides based on detailed spectroscopic analyses, chemical derivatization and degradation, and comparison to natural and synthetic model compounds. What follows is an account of the isolation, characterization, and structure elucidation of **1**–**3**, together with an assessment of chemical stability and cytotoxic properties.

A portion of the aqueous EtOH extract from CMB-01989 was subjected to solvent partitioning and sequential trituration, followed by gel chromatography and normal (silica) and reverse phase (C_8 and C_{18}) SPE and HPLC, to yield franklinolides A–C (**1**–**3**). Examination of the NMR ([D_4]MeOH) data for **1**–**3** revealed a high degree of similarity with data reported for the sponge metabolites bitungolides A–D (**4**–**7**). First described in 2002 from an Indonesian sponge *Theonella* cf. *Swinhoei*, the bitungolides **4**–**7** are chlorophenol polyketides that weakly inhibit dual-specificity phosphatase VHR, and exhibit modest cytotoxicity (ca. 20 μM) against rat normal fibroblast 3Y1 cells.[1] The structure and absolute stereochemistry of bitungolide A (**4**; and by inference the co-metabolites **5**–**7**) was secured by single-crystal X-ray analysis.[1]



1 12*Z*,14*Z* and R = H franklinolide A
1a 12*Z*,14*Z* and R = CD_3 franklinolide A deuteromethyl ester
1b 12*Z*,14*Z* and R = CH_3 franklinolide A methyl ester
2 12*E*,14*E* and R = H franklinolide B
3 12*E*,14*Z* and R = H franklinolide C



4 12*Z*,14*Z* bitungolide A
5 12*E*,14*E* bitungolide B
6 12*Z*,14*E* bitungolide C
7 12*E*,14*Z* bitungolide D

The HRESI(−) mass spectrum for franklinolide A (**1**) displayed a highest mass ion cluster (*m/z* 629/631) measuring for an anion of composition $C_{29}H_{39}ClO_{11}P$ (Δmmu + 1.2), and consistent with a $C_4H_6O_6P$ adduct to one of the isomeric bitungolides **4**–**7**. Supportive of a close structure relationship between franklinolides and bitungolides, the NMR ([D_4]MeOH) data for **1** (see Supporting Information Table S1 and S2 and Figure S1 and S2, acid form) were very similar to those for **4** (Table S4 and Figure S4),[1] with diagnostic values for $J_{12,13}$ and $J_{14,15}$ (11.1 and 11.1 Hz) confirming a common 12*Z*,14*Z* double-bond geometry. Differences in the NMR data between **1** and **4** were limited to 1) a deshielded chemical shift and broadened multiplicity for H-9 (**1** $\delta_H = 4.55$, br; **4** $\delta_H = 3.73$, ddd (10.0, 4.7, 2.3)) and 2) the appearance of new resonances in **1** consistent with a deshielded oxymethine ($\delta_H = 4.89$, br, H-2′) coupled to a methoxymethylene ($\delta_H = 3.83$, br, H_2-3′; $\delta_H = 3.40$, s, 3′-OCH_3). Further interpretation of the NMR data for **1** were complicated by broadening of key resonances.

Concerned that the initially purified **1** was either a mixture of co-eluting or equilibrating isomers, and/or was undergoing structure modification on storage, we undertook to carefully reanalyze **1**. An NMR sample of **1** (stored in [D_4]MeOH at RT for several days) was examined by HPLC-

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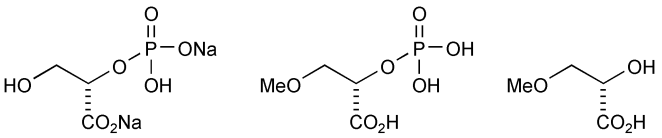
ESI(−)MS to reveal a chromatogram analyzing for ca. 45% **1** (*m*/*z* 629/631), but featuring two additional later-eluting components, **1a** (*m*/*z* 646/648, ca. 40%) and **4** (*m*/*z* 447/449, ca. 15%)—neither of which were apparent in the originally isolated sample. Significantly, over time the abundance of these later-eluting components continued to increase, suggesting that under these mild storage conditions **1** was chemically unstable. Semipreparative HPLC fractionation returned pure samples of **1**, **1a**, and **4**. Spectroscopic analysis of **4** proved that it was identical in all respects (UV/Vis, MS, NMR, $[\alpha]_D$) to bitungolide A,[1] and its formation was attributed to hydrolysis of **1**. In contrast, while NMR analysis for **1a** (Table S1a and Figure S1a) showed almost identical data with those for **1** (with reduced broadening), we were perplexed by the MS data for **1a** (17 amu heavier than **1**). The solution to this problem only became evident when attempts to re-isolate **1** from the crude extract using a H_2O:MeOH:TFA gradient (TFA = trifluoroacetic acid), rather than the H_2O:MeCN:TFA gradient HPLC method employed initially (the practical consequence of a worldwide shortage of MeCN), yielded **1** and **1b** (ESI(−)MS *m*/*z* 643/645) instead of **1a**. Taken together these observations suggested that **1a** and **1b** were the deuteromethyl and methyl derivatives of **1**, respectively. Moreover, giving that **1** was stable to storage in the crude extract, in aqueous EtOH at −30°C over a period of 15+ years, the appearance of hydrolysis (**4**) and methylation (**1a** and **1b**) products during fractionation were attributed to the use of TFA, MeOH, and elevated temperatures during handling.

Alert to chemical instability at low pH values, but also acknowledging that TFA was an essential modifier to achieve HPLC fractionation, we established optimal conditions for franklinolide purification. These conditions consisted of gradient C_8 MeCN:H_2O (0.01% TFA) HPLC followed by immediate postcolumn neutralization with aqueous $NaHCO_3$ prior to in vacuo concentration and subsequent storage in DMSO (dimethyl sulfoxide) at −30°C. An unanticipated bonus from these handling protocols, in particular post HPLC neutralization, was a very significant decrease in the broadening of the NMR signals. Applying this optimized protocol facilitated purification and characterization of **1a** (Table S1a and Figure S1a) and **1b** (Table S1b and Figure S1b), with the latter exhibiting a HMBC correlation linking the ester methyl protons ($\delta_H = 3.76$ ppm) to the C-1′ carbonyl carbon atom ($\delta_C = 172.4$ ppm), necessitating that the $C_4H_6O_6P$ adduct "subunit" in **1** incorporated a carboxylic acid moiety.

The ^{31}P NMR ([D$_4$]MeOH) spectrum of **1** displayed a resonance ($\delta_P = 0.3$, dd ($J_{P\text{-}H} = 8.9$, 8.3 Hz)) comparable with that measured for an authentic sample of commercially available (*S*)-2-phosphoglyceric acid disodium salt (**8**; $\delta_P = 0.8$, d ($J_{P\text{-}H} = 9.5$ Hz)). Pursuing this reasoning, a $J_{P\text{-}H}$ coupling to H-2′ (8.3 Hz) and a broadening of the H-9 resonance in **1** relative to **4** was taken as evidence of a C-9 to C-2′ phosphodiester linkage, consistent with **1** being the 3-*O*-methyl-2-phosphoglyceric acid phosphodiester adduct of **4** as shown. To confirm this assignment a sample of **1** was subjected to acid-catalyzed hydrolysis at 40°C to yield 3-*O*-methyl-2-phosphoglyceric acid (**9**). Further acidic hydrolysis of **9** at 100°C yielded (*S*)-3-*O*-methylglyceric acid (**10**).[2] As the absolute stereochemistry for bitungolide A (**4**) had previously been solved by X-ray spectroscopy,[1] the structure and absolute configuration for franklinolide A (**1**) were assigned as shown.



To further explore chemical stability, samples of **1** (100 μg) were exposed for 5 days to aliquots of different solvents and modifiers (100 μL; Figure 1) to reveal that esterification, isomerization, and hydrolysis of **1** were promoted to varying degrees by exposure to protic solvents and elevated temperatures in acidic media.

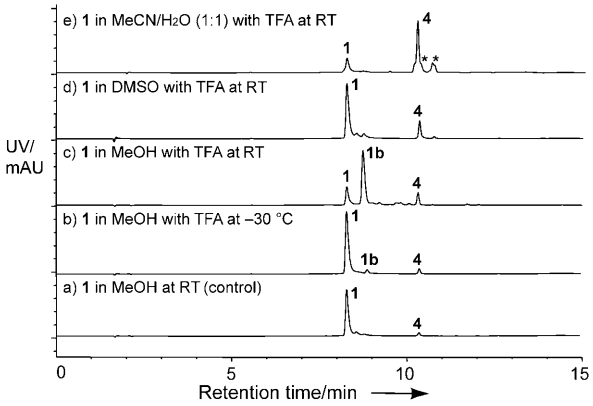


Figure 1. Chemical stability studies of franklinolide A (**1**). The asterisk (*) denotes double bond isomers of **4** (bitungolides B–D). HPLC conditions: Zorbax Eclipse C_8 analytical column, 1 mL min^{-1}, 10–100% MeCN/H_2O (0.01% TFA) over 15 min, with diode array detection (displayed at 270 nm).

HRESI(−)MS measurements on the minor metabolites franklinolides B (**2**) and C (**3**) confirmed them to be isomeric to **1** ($C_{29}H_{39}ClO_{11}P$, Δmmu −0.5 and −2.6, respectively), while comparison of their respective NMR data ([D$_4$]MeOH, Tables S2 and S3) revealed very good correlations with bitungolides B (**5**) and D (**7**), respectively. Furthermore, and consistent with the major co-metabolite **1**, both **2** and **3** displayed spectroscopic signatures for the (*S*)-3-*O*-methyl-2-phosphoglyceric acid phosphodiester moiety, and were assigned the structures as shown.

The franklinolides are polyketides that very likely incorporate a 3-hydroxybenzoyl-CoA starter unit, and are further elaborated through a phosphodiester linkage to the primary metabolite 2-phosphoglyceric acid (Figure 2). Given the stability studies described above it is plausible that franklinolide A (**1**) is the sole direct polyketide biosynthesis product, with the franklinolides B (**2**) and C (**3**) and the bitungoli-

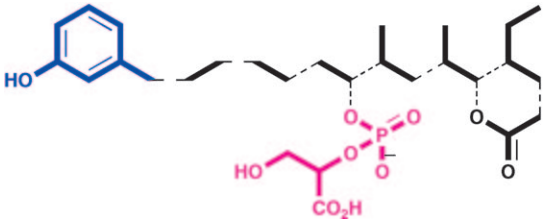


Figure 2. Plausible franklinolide biosynthesis.

des A–D (**4**–**7**) capable of being induced through non-enzymatic isomerization and hydrolysis.

Preliminary structure–activity relationship (SAR) studies, using in vitro cytotoxicity and cell proliferation assays against stomach (AGS) and colon (HT-29) cancer cell lines, and a noncancerous control cell line (HFF-1; Table 1), confirmed **1**

Table 1: In vitro cytotoxicity[a] data (μM) for compounds **1**–**4**.

No.		HFF-1	AGS	HT-29	SH-SY5Y
1	GI_{50}	0.2	0.3 ± 0.2	0.1 ± 0.1	
	TGI	1.0	1.5 ± 0.8	0.8 ± 0.3	
	IC_{50}	2.5 ± 0.7	1.1 ± 0.1	1.8 ± 1.0	1.1 ± 0.1
1b	GI_{50}	0.3	0.5 ± 0.4	0.2 ± 0.1	
	TGI	0.7	3.0 ± 1.4	1.0 ± 0.6	
	IC_{50}	2.0 ± 0.0	1.6 ± 0.4	1.4 ± 0.7	4.4 ± 0.8
2	GI_{50}	1.5	0.7 ± 0.2	0.5 ± 0.2	
	TGI	3.9	6.0 ± 5.3	2.7 ± 1.5	
	IC_{50}	16 ± 6	8.0 ± 3.5	5.7 ± 1.5	8.4 ± 3.3
3	GI_{50}	10	9.3 ± 4.9	4.7 ± 4.0	
	TGI	23	> 30	≈ 20	
	IC_{50}	> 30	> 30	> 30	> 30
4	GI_{50}	> 30	9.7 ± 1.5	> 30	
	TGI	> 30	25 ± 3	> 30	
	IC_{50}	> 30	≈ 16	> 30	> 30

[a] HFF-1 = human foreskin fibroblast; AGS = gastric adenocarcinoma (stomach); HT29 = colorectal adenocarcinoma (colon); SH-SY5Y neuroblastoma (brain). GI_{50} and TGI values were acquired from cell proliferation assay; IC_{50} values were acquired from MTT cytotoxicity assay. All compounds were tested at least twice in duplicate except in the cell proliferation assay for HFF-1 cell line (once in duplicate).

as the dominant cytotoxic agent (GI_{50} range from 0.1 to 0.3 μM). SAR analysis defined the relative importance of key structural features: 1) a very significant 30- to > 300-fold decrease in cytotoxicity following hydrolysis of **1** to **4**, 2) a 2- to 7-fold decrease on isomerization of **1** to the 12*E*,14*E* isomer **2**, 3) a 30- to 50-fold decrease on isomerization of **1** to the 12*E*,14*Z* isomer **3**, and 4) a limited 1.5- to 2-fold decrease on esterification of **1** to **1b**. A similar pattern of cytotoxicity (IC_{50}) was also observed against a human brain (SH-SY5Y) cancer cell line (Table 1).

In conclusion, this work describes the discovery of three novel compounds with a rare polyketide skeleton incorporating an unusual 3-*O*-methylglyceric acid phosphodiester moiety. To the best of our knowledge the closest natural phosphodiester analogues to the franklinolides are *Streptomyces* derived antibiotic phosphoglycolipids (i.e. moenomycin A,[3] pholipomycin,[4] and noskomycins[5]). The franklinolides are the first examples of polyketide phosphodiesters and, most importantly, our work highlights their relatively fragile nature and very high cytotoxicity compared to the bitungolides. These observations encourage speculation that glyceric acid phosphodiesters may have broader application in enhancing the potency and therefore therapeutic value of other cytotoxic agents.

Experimental Section

Initial cytotoxicity screening on crude extract, general experimental procedures, animal materials collection and taxonomy, extraction and isolation of franklinolides A–C (**1**–**3**), chemical stability studies of **1**, and acid-catalyzed methanolysis and hydrolysis of **1**, as well as tabulated NMR data for compounds **1**–**4**, **1a** and **1b**, full NMR spectra for compound **1**, and ^{1}H NMR spectra for compounds **2**–**4**, **1a**, **1b**, **9**, and **10a** are provided in the Supporting Information.

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