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Epicoccamide, a novel secondary metabolite from a jellyfish-derived culture of *Epicoccum purpurascens*

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From the inner tissue of the jellyfish *Aurelia aurita* a marine strain of the fungus *Epicoccum purpurascens* was obtained. After mass cultivation the fungus was investigated for its secondary metabolite content and found to contain the new, and most unusual tetramic acid derivative, epicoccamide (**1**). Epicoccamide is quite unusual since it is composed of three biosynthetically distinct subunits; glycosidic, fatty acid and tetramic acid (amino acid). The structure of the new compound was elucidated using spectroscopic methods, mainly 1D and 2D NMR, ESI-MS, and chemical degradations.

Introduction

Marine microorganisms are a new source of biologically active metabolites with novel chemical structures.[1,2] Fungi investigated from the marine environment are mostly derived from sediments,[3–5] wood,[6] algae,[7–9] or, as in the case of *Epicoccum purpurascens*, from marine animals.[10–17] In the current study the isolation of the fungus *E. purpurascens* from the North Sea jellyfish *Aurelia aurita* is described. This fungal strain produces a most unusual glycosylated tetramic acid containing secondary metabolite, epicoccamide. Tetramic acid derived natural products are interesting due to their pronounced biological activities. Thus ascosalipyrrolidinone, a desoxy tetramic acid derivative produced by the obligate and algae associated marine fungus *Ascochyta salicorniae*, was recently patented due to its antimicrobial activity.[7] Though no obligate marine species of the fungus *Epicoccum* have been described representatives of this genus are found on beached algae and other beach wrack material.[18] Terrestrial species of the genus *Epicoccum* have been studied for their natural products content with epicorazines A and B,[19] epirodin,[20] and triornicin,[21] all being produced by *E. nigrum* (*E. purpurascens*),[22] indicating some members of the genus *Epicoccum* to have a highly developed and diverse secondary metabolism.

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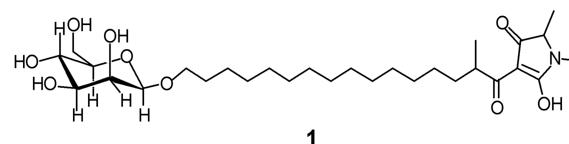
Results and discussion

After isolation, the fungus *E. purpurascens* was cultivated on a solid malt soya medium containing added artificial sea salt. Successive fractionation of the EtOAc extract of the fungus and medium by vacuum–liquid chromatography (VLC), and normal (Si-60) and reversed (RP-C_{18}) phase HPLC yielded the most unusual new natural product epicoccamide (**1**).



The molecular formula of epicoccamide (**1**) (Fig. 1) was found to be $C_{29}H_{51}NO_9$ by accurate mass measurement (HRFAB +ve). Of the five elements of unsaturation implied by the molecular formula of **1**, three were present as carbonyl (enol) groups [δ 175.3 (C-1, s), 197.6 (C-3, s), 201.3, (C-7, s)] as deduced from the ^{13}C NMR and IR spectra of the molecule. This deduction enabled three of the nine oxygen atoms within the molecule to be accounted for, and also indicated the molecule to be bicyclic since no other multiple bonds were present. From the ^{1}H and ^{13}C NMR data it was evident that **1** was composed of three distinct fragments; a hexose sugar, an aliphatic chain, and a tetramic acid moiety. After all protons

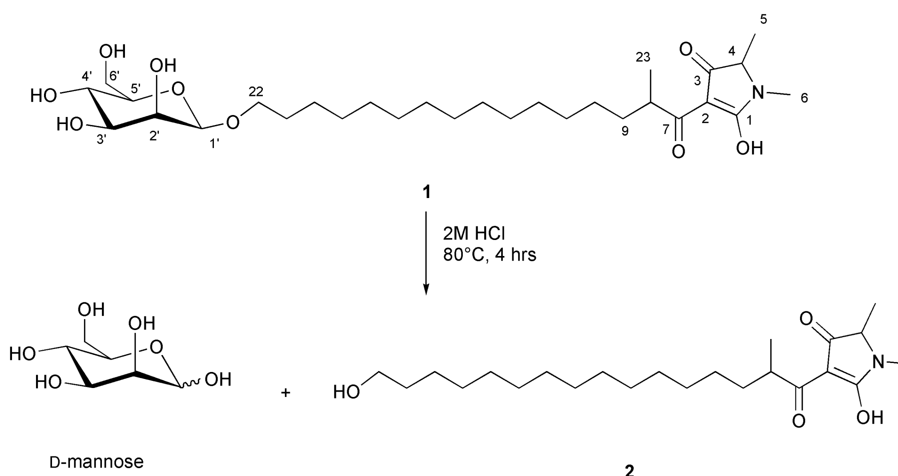


Fig. 1 Structures of epicoccamide (**1**) and its hydrolysis products, **2** and D-mannose.

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had been associated with their directly bonded carbon atoms *via* a ^{1}H–^{13}C 2D NMR shift-correlated measurement (HMQC) it was possible to develop the sugar and aliphatic moieties from the ^{1}H–^{1}H COSY data. Thus, crosspeaks seen between the resonances for H-1′, the anomeric proton, and H-2′, between the resonances for H-2′ and H-3′, those for H-3′ and H-4′, H-4′ and H-5′, and between the resonances for H-5′ to those for H_2-6′, revealed the complete ^{1}H–^{1}H spin system within the sugar moiety. The coupling constant between H-5′ and H-4′ (J = 9.5 Hz) showed them to have a *trans*-diaxial disposition, as do H-3′ and H-4′ (J = 9.5 Hz). The coupling constant (J = 3.2 Hz) between H-2′ and H-3′ showed H-2′ to be equatorial, the hexose moiety was thus concluded to be mannose. With the mannose moiety established it was then necessary to determine the stereochemistry of the glycosidic linkage, and if D- or L-mannose were present in epicoccamide. From the magnitude of the C–H coupling constant between C-1′ and H-1′ (J = 156.4 Hz) it was evident that **1** contained β-mannose since β-pyranoses show J_{CH} values of around 160 Hz, and α-pyranoses values of around 170 Hz.[23] Acid hydrolysis of **1** (see Experimental section and Fig. 1) enabled the sugar and **2** to be isolated. The optical rotation of the sugar moiety was $+18.4 \times 10^{-1}$ deg cm^2 g^{-1}, which showed epicoccamide to contain D-mannose. The mannose moiety accounted for all of the remaining oxygen within the molecule as well as for one of the two rings within **1**. At this stage of the structural analysis 3 × CH_3, 14 × CH_2, 3 × CH groups, and one nitrogen, as deduced from the MS, ^{1}H and ^{13}C NMR data of **1**, remained to be accounted for. Evident in the ^{13}C NMR spectrum of epicoccamide were four broad resonances at δ 201.3 (C-7, s), 197.6 (C-3, s), 175.3 (C-1, s), and 101.6 (C-2, s), which were best assigned to a tetramic acid moiety.[24,25] From the ^{1}H–^{1}H COSY crosspeaks observed between the resonances for H_3-5 and H-4, and between the resonances for H_3-23 and H-8, and between the resonances for H-8 and H_2-9, it was evident that CH_3-5 is bonded to C-4, and that CH_3-23 is bonded to C-8, which further bonded to C-9. As the resonance for C-8 was broad in the ^{13}C NMR spectrum it was likely that this group was also affected by the keto–enol tautomerism of the tetramic acid moiety (Fig. 2). In the ^{1}H NMR spectrum of **1** the signal for H-8 (δ 3.80, m) was notably deshielded, an effect which is characteristic of tetramic acid systems.[24] CH_3-6 was assigned as an N–CH_3 on the basis of its ^{1}H and ^{13}C NMR chemical shifts. Finally, long-range ^{1}H–^{13}C 2D NMR correlations (HMBCs) observed between the resonances for H_3-6 and C-1 and C-4, between those associated with H_3-5 and C-3 and C-4, and between those of H_3-23 and C-2, C-7, C-8 and C-9 clearly showed C-1 and C-4 to bond to the N–CH_3, C-3 to bond to C-4, C-7 to bond to C-8 and C-2, and by deduction C-2 to bond to both C-1 and C-3, thus completing the tetramic acid moiety and the second ring within the molecule. The remaining 14 × CH_2 groups were assigned to a single aliphatic chain connected to C-8 at one end and to the mannose *via* C-22 at the other. From the long-range correlation observed between the resonances for H_2-22 and that of C-1′ it was obvious that the aliphatic chain was linked *via* oxygen to the anomeric carbon C-1′. The electrospray (ESI) mass spectrum (negative mode) of epicoccamide (**1**) contained the highest mass signal at *m/z* 556, consistent with $[M - H]^-$. Collision-induced dissociation-tandem mass spectrometry (CID-MS/MS, negative mode) showed the 556 peak and a base peak at *m/z* 394 corresponding to the molecular ion less the mass of mannose. Additionally, a *m/z* 126 peak was present in the ESI CID-MS/MS (negative mode) for the molecular fragment resulting from the α-cleavage of the tetramic acid moiety. In the ESI (+) mass spectrum *m/z* 580 ($[M + Na]^+$) was the base peak. A CID-MS/MS (positive mode) showed the formation of a *m/z* 418 base peak which represents the sodium adduct of **1** less the mannose. Increasing the collision energy from −39 eV to −59 eV resulted in a loss of mannose and water from the molecule and gave a base peak at *m/z* 400. Attempts to resolve the stereochemistry of **1** at C-4 and C-8 by comparison of its CD spectra with those of similar compounds yielded no unambiguous results.[26,27] Epicoccamide (**1**) is quite an unusual low molecular weight natural product since it is composed of three biosynthetically distinct subunits; glycosidic (mannose), fatty acid (presumably a hexa- or heptadecanoic acid derivative), and amino acid (tetramic acid).

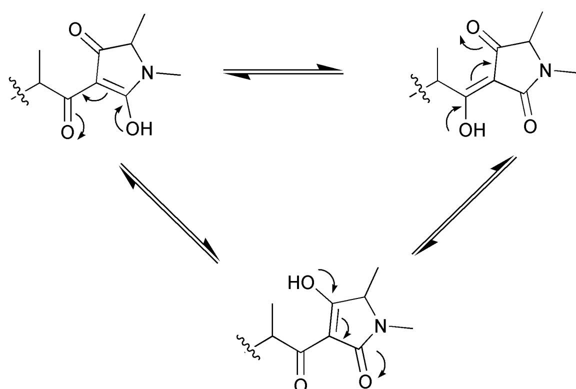

Fig. 2 Tautomeric forms of the tetramic acid part of **1** and **2**.

A number of natural products with a tetramic acid moiety are known, *e.g.*, ancorinoside A,[26] and its magnesium salt,[27] from the marine sponge *Ancorina* sp., both of which inhibit star fish embryo blastulation. Aflastatin A produced by *Streptomyces* sp. was found to inhibit aflatoxin biosynthesis.[25] The tetramic acid glycosides aurantosides A and B from the marine sponge *Theonella* sp., show cytotoxicity towards P-388 and L-1210 leukemia cells.[23] From the sponge *Halichondria cylindrata* cylindramide, a tetramic acid lactam, was isolated and found to have cytotoxic effects towards B 16 melanoma cells.[24] Fischerellin A, isolated from a culture of the cyanobacterium *Fischerella muscicola*, had antifungal, and herbicidal activities, as well as being a potent photosystem-II-inhibitor.[28] As many of the reported tetramic acid derivatives discussed above exhibited pharmacological activities, in particular cytotoxicity, it was surprising to find that the new and unusual natural product epicoccamide (**1**) had no detectable activity in any of the applied assay systems (see Experimental section).

Experimental

General

FAB (positive mode) mass spectra were recorded using a JEOL 102SX A, double focussing sector field instrument. All other general experimental procedures were carried out as previously described.[29,30]

Isolation and taxonomy of the fungus

The jellyfish *Aurelia aurita* was collected from the North Sea, Tönning, Germany. Its inner tissue was cut into small pieces and placed on agar plates containing isolation medium (15 g L^{-1} agar and 1 L sea water from the sample collecting site and the antibiotics benzylpenicillin and streptomycin sulfate, each 250 µg L^{-1}). Fungal colonies growing out of the jellyfish tissue were transferred to the medium for sporulation (1.0 g glucose, 0.1 g yeast extract, 0.5 g peptone from meat, enzymatic digest, 15 g agar, and 1 L sea water, pH 8) in order to enable taxonomy of the isolates. The fungal strain *E. purpurascens*, voucher number N7–9, used in this study was identified by Dr S. Draeger, Institute for Microbiology, Technical University of Braunschweig.

Table 1 ^1H (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data for epicoccamide (**1**) and ^1H (CD_3OD, 300 MHz) and ^{13}C NMR (CD_3OD, 75.5 MHz) data for **2**[a]

Position	δ_C (ppm) (**1**)	δ_H (ppm) (**1**)	J_{CH}/Hz (**1**)	δ_C (ppm) (**2**)	δ_H (ppm) (**2**)
1	175.3 C			171.4 C	
2	101.6 C			Not observed	
3	197.6 C			196.5 C	
4	61.8 CH	3.54 (br)		62.0 CH	3.68 (qm, J = 7.0 Hz)
5	16.0 CH_3	1.31 (d, J = 6.9 Hz)		15.2 CH_3	1.37 (d, J = 7.0 Hz)
6	26.7 CH_3	2.91 (br s)		27.0 CH_3	3.00 (s)
7	201.3 C			191.5 C	
8	40.8 CH	3.80 (m)		40.7 CH	3.87 (q, J = 7.0 Hz)
9	35.0 CH_2	1.27 (m), 1.72 (m)		34.8 CH_2	1.27 (m), 1.74 (m)
10–19	30.6–30.9 CH_2	1.25–1.38 (m)		30.9–31.7 CH_2	1.25–1.38 (m)
20	27.3 CH_2	1.42 (m)		28.4 CH_2	1.42 (m)
21	30.8 CH_2	1.65 (m)		30.6 CH_2	1.55 (m)
22	70.6 CH_2	3.57 (ddd, J = 2.9, 6.8, 9.8 Hz) 3.98 (td, J = 6.8, 6.8, 9.8 Hz)		63.0 CH_2	3.58 (t, J = 6.6 Hz)
23	18.0 CH_3	1.03 (d, J = 6.2 Hz)		17.5 CH_3	1.21 (d, J = 7.0 Hz)
1′	101.7 CH	4.54 (br s)	156.4 Hz		
2′	72.5 CH	3.90 (br d, J = 3.2 Hz)	145.9 Hz		
3′	75.3 CH	3.50 (dd, J = 3.2, 9.5 Hz)	142.0 Hz		
4′	68.5 CH	3.62 (t, J = 9.5, 9.5 Hz)	146.2 Hz		
5′	78.1 CH	3.26 (ddd, J =3.0, 5.6, 9.5 Hz)	140.5 Hz		
6′	62.8 CH_2	3.77 (dd, J = 5.6, 11.8 Hz) 3.95 (dd, J = 3.0, 11.8 Hz)	143.5 Hz		

[a] All assignments are based on extensive 1D and 2D NMR experiments (COSY, HMQC, HMBC).

Cultivation

The micro-organism cultures were grown at 20 °C for 50 days in 4.5 L of solid malt extract soy meal agar (30 g malt extract, Merck, 3 g peptone from soy meal, papain-digest, Merck, 7.6 g agar, 800 mL artificial sea water and 200 mL demineralised water, adjusted to pH 5.5). The composition of the artificial sea water was as described by Höller *et al.*[11]

Biological activity

The activity of compounds was tested in agar diffusion assays against the bacteria *Bacillus megaterium*, *Escherichia coli*, the fungi *Microbotryum violaceum*, *Eurotium repens*, *Fusarium oxysporum*, *Mycotypha microsporum* and the alga *Chlorella fusca*.[30] Assays with *Artemia salina* and *Caenorrhabditis elegans* aimed at assessing cytotoxic and nematicidal activity were also performed.[31] In ELISA based enzyme assays inhibition of tyrosine kinase p56lck and reverse transcriptase HIV-1 was examined.[32,33] Antiplasmodial activity was determined as described by Desjardins *et al.*[34] The activity against *Mycobacterium tuberculosis* was assessed as described by Collins and Franzblau,[35] against hemoflagellates, which cause human sleeping sickness (*Trypanosoma brucei* subsp. *rhodesiense*), and Chagas disease (*Trypanosoma cruzi*), and also the cytotoxic activities were assessed as described by Kaminsky and Brun.[36]

Extraction and isolation

The solid medium and fungal mycelium were diluted with H_2O, blended using the Ultra Turrax T 25 at 8000 min^{-1}, and then extracted with 13.5 L EtOAc. The resultant EtOAc extract (3.9 g) was purified employing a combination of chromatographic techniques. First, it was passed over normal phase silica (vacuum–liquid chromatography, VLC) using a gradient of CH_2Cl_2–EtOAc–MeOH as eluent to yield 13 fractions each of 250 mL. VLC fraction 11 (2.2 g, eluted with MeOH–EtOAc, 60 : 40) was subjected to RP-18 HPLC (Eurospher 100, 5 μm, 8 mm × 25 cm) using MeOH–H_2O, 85 : 15, as eluent to yield compound **1**.

Epicoccamide (1). A white amorphous powder (2.6 mg L^{-1}); $[\alpha]_D^{20}/10^{-1}$ deg cm^2 g^{-1} −10.3 (c 0.10, EtOH); UV (EtOH) λ_{max}/nm (log ε/dm^3 mol^{-1} cm^{-1}) 282 (7940); CD (MeOH, 0.0079 mol L^{-1}) λ/nm 218 ($\Delta\varepsilon$ +0.20), 232 ($\Delta\varepsilon$ −0.34), 269 ($\Delta\varepsilon$ +0.91), 295 ($\Delta\varepsilon$ −0.83); IR (film) ν_{max}/cm^{-1} 3360, 2925, 2850, 1655, 1595, 1480; ^1H and ^{13}C NMR data, see Table 1; HRFAB (+) m/z 580.3379 (−8.3 mmu) $[M + Na]^+$, 596.3126 (−7.5 mmu) $[M + K]^+$; ESI-MS (−) m/z 556 $[M - H]^-$; ESI-MS (+) 580 $[M + Na]^+$; CID-ESI-MS/MS (−) 556 (31), 394 (100), 126 (40); CID-ESI-MS/MS (+) 418 (28), 400 (100).

Hydrolysis of epicoccamide (1). To 8 mg of **1** 2 mL 2 M HCl (methanolic) was added and the resulting solution maintained at 80 °C for 4 h. At the end of this period the residual solvent was removed under reduced pressure and the remaining material partitioned between H_2O and CH_2Cl_2. The aqueous phase contained D-mannose (2.4 mg, $[\alpha]_D^{20}/10^{-1}$ deg cm^2 g^{-1} +18.4 (c 0.24, H_2O), lit.[37] 14.2), and the CH_2Cl_2 contained **2**.

Tetramic acid-aliphatic moiety (2). A light yellow viscous oil (2.6 mg L^{-1}); $[\alpha]_D^{20}/10^{-1}$ deg cm^2 g^{-1} −15.2 (c 0.17, CH_2Cl_2); UV (EtOH) λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 246 (9590), 285 (11 250); IR (film) ν_{max}/cm^{-1} 2924, 2853, 1714, 1652, 1610, 1457; ^1H and ^{13}C NMR data, see Table 1.

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