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Novel nonadride, heptadride and maleic acid metabolites from the byssochlamys acid producer *Byssochlamys fulva* IMI 40021 — an insight into the biosynthesis of maleidrides

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The filamentous fungus *Byssochlamys fulva* strain IMI40021 produces (+)-byssochlamys acid 1, its novel dihydroanalogue 2 and four related secondary metabolites. Agnestadrides A, 17 and B, 18 constitute a novel class of seven-membered ring, maleic anhydride-containing (hence termed heptadride) natural products. The putative maleic anhydride precursor 5 for both nonadride and heptadride biosynthesis was isolated as a fermentation product for the first time and its structure confirmed by synthesis. Acid 5 undergoes facile decarboxylation to anhydride 6. The generic term maleidrides is proposed to encompass biosynthetically-related compounds containing maleic anhydride moieties fused to an alicyclic ring, varying in size and substituents.

The collective name “nonadrides” was introduced by Barton and Sutherland for fungal metabolites containing a nine-membered ring fused to one or two maleic anhydride moieties in 1965. The first of these, glaucanic acid 3 and glauconic acid 4 were isolated from *Penicillium glaucum*, by Wijkman and reported in 1931. Soon afterwards the pseudo-symmetrical analogue, byssochlamys acid 1 was isolated from the common food contaminant *Byssochlamys fulva* by Raistrick and coworkers. However the structures of 1 and 4 were only established some 30 years later by X-ray analysis. Barton and coworkers subsequently reisolated 4 from *Penicillium purporogenum* and began considering the biosynthesis of the nonadrides. The cis relative stereochemistry of the ethyl and propyl groups of byssochlamys acid was confirmed by X-ray analysis of the bis-p-bromophenylhydrazide. The absolute configuration of 1 was established by classical degradative methods, and subsequently confirmed by enantiomeric synthesis of (+)-byssochlamys acid.

All three metabolites, 1, 3 and 4, were hypothesised to be biosynthesised via alternative dimerisation modes of a C6/C10 maleic acid precursor 5 and/or 6, and evidence for this was obtained by feeding studies with radiolabelled 6. Many more nonadride metabolites have since been reported, e.g. heveadrides and phomoidrides A and B. Cornexistin 14, is a nonadride unique in having a single anhydride unit. *Viburspiran* 15 and *zopfiellin* 16, feature 8-membered carbo cyclic rings and so have been classed as octadrides. Notably, all of the compounds belonging to this family reported so far have been isolated from fungi.

Pursuing a general interest in the biosynthesis of nonadrides and other related fungal compounds, we re-analysed the secondary metabolite profile of *B. fulva* to assess its nonadride-related metabolites in greater detail. This has led to isolation and characterisation of a new analogue, dihydro-byssochlamys acid 2, two novel metabolites agnestadride A 17 and agnestadride B 18.
Both containing 7-membered rings which we have therefore termed heptadrides; and as a natural product for the first time, the proposed nonadride precursor, maleic anhydride 5 along with its decarboxylated derivative 6.

B. fulva, was grown as described by Raistrick in static culture in Czapek-Dox liquid medium with glucose as the sole carbon source for between 7 and 30 days. The crude ethyl acetate extracts were analysed by LCMS. Byssolchamic acid 1 (C_{19}H_{22}O_6) was detectable after 7 days of static fermentation (Figure 1, 15.4 min.) and large amounts (> 50 mg L^{-1}) could be isolated after 4 weeks. Its identity was confirmed as (+)-byssolchamic acid by comparison of 1D ^1H and ^13C NMR spectra, and optical rotation comparison with literature values. Detailed analysis of 2D NMR spectra (COSY, HSQC and HMBC) allowed all signals in the ^1H and ^13C NMR spectra to be fully assigned for the first time (Table S1).

The second-most abundant component of the extract eluted at 11.3 min. (Figure 1). The new compound had a UV absorption characteristic for nonadrides (λ_max 211, 260 nm), ionized well in the negative ESIMS mode and had the same fragmentation pattern as byssolchamic acid 1 – the only difference being that all of the peaks appeared at m/z values two units higher. HRESIMS analysis confirmed the molecular formula to be C_{19}H_{22}O_6, corresponding to a reduced form of 1. Dihydro-byssochlamic acid 2 was purified by mass-directed preparative HPLC. The NMR spectra (Table S1) were compared with those of byssolchamic acid 1. A new peak at 95.9 ppm in the ^13C NMR spectra correlated (HSQC) to H-10 at 5.71 ppm confirmed the presence of a hemi-acetal in 2. Consistent with the loss of the carbonyl, the resonances of C-11 and C-9 appeared at higher chemical shifts (172.2 and 160.7 ppm respectively), and the C-8 resonance moved upfield to 130.6 ppm. Key HMBC signals were seen from the butenyl H-4 (6.08 ppm) to C-5, C-6 and C-8, and from H-16 (3.54 ppm) to C-7, and 9-CH_3 (3.01/2.66 ppm) to C-5, C-7 and C-8 to firmly locate the hydroxylactone ring. Similarly, correlations from 9-CH_3 to C-10 (20.6 ppm) and C-11, and 10-CH_3 (3.05/2.52 ppm) to C-11, C-12 and C-13 locate the maleic anhydride moiety.

A less polar new compound was detected (18.4 min. in Figure 1) and was isolated (1mg L^{-1}) using mass-directed preparative HPLC. It had a mass of 314 (peak of m/z 313 [M-H]^{-} in the negative ESIMS spectrum), corresponding to a dehydrated derivative of 17. The formula was confirmed by HRESIMS analysis to be C_{19}H_{22}O_6. In contrast with other colourless metabolites isolated from B. fulva compound 18 was bright yellow. The ^1H NMR spectrum revealed that 18 also contained a pendant 1-butenyl side chain. A total of 18 protons were observed in the ^1H NMR spectrum (Table S2). There were three ^13C NMR resonances (166.2, 164.6 and 163.1 ppm) characteristic for anhydride/unsaturated lactone carbonyls, and 6 quaternary olefinic carbons. The C-7 acetal and C-15 methine hydrogens attached to carbon, suggesting that the other belonged to a transannular C-C bond, and in phomoidride B a full acetal due to further linkage to a side chain hydroxyl. LCMS chromatograms of extracts from older cultures of B. fulva (ca. 30 days) showed a new peak at 13.3 min. with the same molecular weight as byssolchamic acid 1 but a slightly different UV spectrum.

It was isolated by HPLC (~1 mg L^{-1} culture) and HRESIMS confirmed it to have the same molecular formula [C_{19}H_{22}O_6] as byssolchamic acid. However, ^1H NMR analysis (Table S2) showed the presence of a 1-butenyl side chain. The HSQC spectrum revealed only nineteen hydrogens attached to carbon, suggesting that the other belonged to a hydroxyl. The presence of a further propyl substituent linked to an otherwise uncoupled CH, and two mutually coupled methylenes were obvious from the COSY spectrum (Figure 2a). The characteristic signals for one maleic anhydride were apparent at 143.3 (C-11), 165.3 (C-12), 164.8 (C-13) and 144.9 (C-14) ppm. The remaining 4 carbons resonances at 168.5 (C-6), 127.0 (C-5), 153.4 (C-8) and 104.0 (C-7) ppm were indicative of an α,β-unsaturated ester/lactone and an acetal, consistent with the hydroxylactone moiety indicated in bold in Figure 2a. Further detailed analysis of the HMBC data (Figure 2b) was consistent with the seven-membered carbocycle, which we name agnestadride A 17. The key correlations were seen from the butenyl H-4 (6.08 ppm) to C-5, C-6 and C-8, and from H-16 (3.54 ppm) to C-7, and 9-CH_3 (3.01/2.66 ppm) to C-5, C-7 and C-8 to firmly locate the hydroxylactone ring. Similarly, correlations from 9-CH_3 to C-10 (20.6 ppm) and C-11, and 10-CH_3 (3.05/2.52 ppm) to C-11, C-12 and C-13 locate the maleic anhydride moiety.

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Figure 2. (a) COSY correlations of three isolated spin systems of 17. (b) HMBC correlations.
Present in the $^1$H NMR spectrum of the impure naturally occurring 5 matched those of the synthetic analogue (Table S3). Interestingly anhydride 6 did not show any signal by ELS (Evaporative Light Scattering) detection and attempts to purify it by HPLC resulted in yields much lower than anticipated. This was attributed to volatility of 5, and was confirmed by HPLC of a sample that had been allowed to evaporate at room temperature. On re-addition of solvent HPLC analysis showed that ca. 96% of the compound had evaporated.

Byssochlamic acid 1 exists in equilibrium with its mono-hydrated diacid forms which appear together at 9.8 min in the LCMS chromatogram (1a/1b in Figure 1), as indicated by the characteristic [M + H - CO$_2$] ion (m/z 349.2) in the negative ESI mass spectrum. This peak appeared in chromatograms of purified byssochlamic acid 1 and of a purified mixture of 1a/1b. When re-injected, this reforms an equilibrium mixture with byssochlamic acid 1. $^1$H NMR spectra showed that the ring-closed form 1 is preferred in organic solvent, while the ring-open forms 1a/1b are also present in aqueous solvents. Similar behaviour is observed for other maleic anhydride containing metabolites including the compound 5 (see peaks at 11.3 and 4.9 min in Figure 1), and 6 (Figure S3) and dihydrobyssochlamic acid 2, and for related maleic anhydrides isolated from P. variotii. 25

Previous work 26 had provided evidence for biosynthesis of byssochlamic acid via dimersisation of maleic anhydride 6 the decarboxylated form of 5. However, it would appear likely that 5 is the actual precursor, decarboxylation providing the exo-methylene intermediate 28 necessary for cyclisation with a second molecule of 5 as indicated in Scheme 2 (pathway a). Tautomeration of the first formed macrocycle leads to byssochlamic acid 1. The heptadrides, agnastadrides A and B can be accounted for by a different mode of dimerisation of these intermediates as indicated in Scheme 2 (pathway b). Formal Michael addition of the anion derived by decarboxylation of 5 now occurs on the other end of the diene system of 28. In the in vitro studies 26 leading to 19 strong base was required to trigger dimerisation. Although presented in Scheme 2 as synchronous processes, stepwise mechanisms are equally valid at this stage. Interestingly diene 28 has been isolated as waquafranone B, along with waquafranone A 29 and several epihveeadrides from F. aquatica. 18 Also, the exo-methylene compound 30 has been isolated, as tubigenoic acid, from Aspergillus tubigenus, 7 along with the hexyl analogue of 5, itself a metabolite of several Aspergillus species. 26, 28, 25

**Scheme 2.** Proposed biosynthesis of nonadride and heptadrides via common intermediates.

Our work with B. fulva has led to the isolation of 5 new maleic anhydride-containing compounds in addition to byssochlamic acid. These include dihydrobyssochlamic acid, but more significantly, two novel 7-membered ring analogues which we have termed present in the LCMS characteristics with the data collected for the synthetic product. The synthetic compound was observed to undergo decomposition to 6 spontaneously and decomposition was complete in under 48 h. The less polar peak at 20.6 min (Figure S2) had a similar UV spectrum to 5, however it did not ionize well (the -ESI spectrum shows a m/z 165 with a m/z of 209 but only in very concentrated samples), which was consistent with the loss of the ionisable carboxylate group. In addition the chemical shifts of peaks
heptadrides by analogy to nine-membered nonadrides and eight-membered octadrides. Heptadrides have never been reported previously as natural products. In addition, the putative monomeric maleic anhydride intermediate previously proposed to undergo macrocyclisation via dimerisation to give byssochlamic acid and other nonadrides, has also been isolated for the first time. A scheme for the biosynthesis of both the nonadrides and heptadrides is proposed. Work is in progress to establish details of the biosynthesis, as well as the identification of the biosynthetic gene cluster.

To account for diversity among nonadride-like natural products, we propose the generic name maleidrides to denote biosynthetically related compounds, with one (monomaleidrides) or two (bismaleidrides) maleic anhydride units anchored on a diversely substituted ring. Production of the anhydride (bismaleidrides) maleic anhydride units anchored on a diversely substituted ring. Production of the anhydride maleidrides very likely arises not only from diversely substituted rings found in the nonadride and related metabolites.

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Notes and References

‡ Tropolone metabolites, e.g. stipitatonic and puberlionic acids, contain maleic anhydride moieties fused to 7-membered aromatic polyketide-derived rings, in contrast to the alicyclic rings found in the nonadride and related metabolites.