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Tetrahedron Letters 45 (2004) 1075–1078
Tetrahedron Letters

Laetiporic acid, a new polyene pigment from the wood-rotting basidiomycete *Laetiporus sulphureus* (Polyporales, Fungi)

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Received 16 October 2003; revised 12 November 2003; accepted 17 November 2003

Abstract—A new pigment, laetiporic acid, has been isolated from fruit-bodies of the wood-rotting fungus *Laetiporus sulphureus* (sulfur shelf). Structure elucidation by application of extensive 2D NMR techniques permitted its identification as a polyene of non-isoprenoid origin. Laetiporic acid, which represents the main pigment in *L. sulphureus* basidiocarps, bears an unprecedented decaene skeleton as part of its chromophore and, interestingly, contains double bonds with a stable *cis* configuration.

Laetiporus sulphureus (Bull.: Fr.) Murr. (Polyporales, Fungi) is a wood-rotting basidiomycete growing on several tree species and producing shelf-shaped fruit-bodies of pink-orange colour, except for the fleshy margin which is bright yellow. This remarkable pigmentation accounts for its specific epithet, and for the trivial name under which this bracket fungus is known, sulfur shelf.

Early studies on the nature of the pigments responsible for this colour led to the isolation of an orange substance with acidic properties whose UV–vis spectrum closely resembled that of a carotenoid.[1] The presence of a novel carotenoid pigment was therefore suspected, to which the trivial name laetiporxanthin was given, even though a molecular structure could not be envisaged at that time. Later, the structure of 8′-apo-β-caroten-8′-oic acid was suggested, but no experimental evidence was presented to support the proposed structure,[2] so laetiporxanthin remained an unidentified carotenoid pigment.[3] Despite subsequent chemical investigations on *Laetiporus* species, which were found to contain a number of lanostane triterpenoids[4] and other metabolites,[5,6] apparently no further attempts were made to shed light on the nature of laetiporxanthin. It is now recognised that UV–vis spectra cannot be used as the sole criterion for identification, especially in the field of fungal pigments.[3] Recently, pigment production by *L. sulphureus* in submerged culture has been investigated, but no additional information was reported as to the chemical identity of these pigments, which were still thought to be of carotenoid nature.[7,8]

As part of our ongoing research on fungal carotenoids[9] and pigments,[10] laetiporxanthin was chosen as an attractive candidate for more detailed chromatographic and spectroscopic investigations. In the present paper we describe the isolation and structure elucidation of the main pigment from fruit-bodies of *L. sulphureus*, for which the name laetiporic acid **1** is proposed. Unexpectedly, the pigment was not an acidic carotenoid as proposed by Valadon and Mummery[1] and Valadon,[2] but is a new polyene of non-isoprenoid origin.

Fruit-bodies of *L. sulphureus* growing on old oak stumps that had been uprooted about 10 years previously were collected on 2 September 2002 in Forstgut Wiegersen near Buxtehude (Lower Saxony, Germany),[11] freeze-dried (109.2 g) and thoroughly extracted with MeOH (3×1 L). The solvent was rotary evaporated, the residue partitioned between ethyl acetate and 0.03 M aqueous HCl and repeatedly extracted with AcOEt to afford an orange-red solid (4.86 g). HPLC analysis[12] of the crude extract revealed the presence of a major orange pigment (t_R 4.1 min), along with a few minor ones, all displaying UV–vis spectra with a fine peak structure typical of carotenoids.

Keywords: Fungi; *Laetiporus sulphureus*; Fruit-bodies; Pigments; *Cis* polyenes; Structure elucidation.
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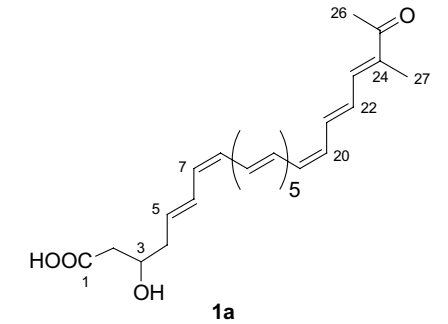
0040-4039/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2003.11.073

The crude extract (1.43 g) was applied onto a silica gel column (Merck 60, 63–200 μm; 240×25 mm) and eluted with cyclohexane/ethyl acetate/acetic acid 50:50:1, yielding 65.4 mg of an enriched fraction. A chromatographically pure pigment was obtained by means of preparative HPLC on a Merck LiChrosorb® RP-18 column (7 μm particle size; 250×25 mm; gradient: water/acetone (1:1) to acetone over 60 min at a flow rate of 5 mL min^{-1}). A total of 3.46 mg of pure main pigment was obtained as a dark red amorphous solid, which was only sparingly soluble in most organic solvents (around 1 mg/mL).

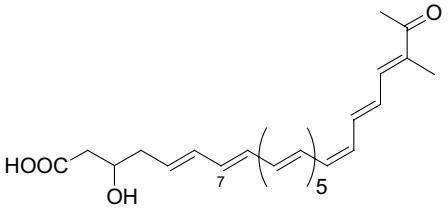
The main pigment displayed UV–vis absorption maxima at λ_{max} (MeOH) 419 (sh), 442 (log ε 4.426) and 461 (sh) nm. APCI-PI and -NI mass spectra showed peaks at m/z 421 and 419, respectively, thus indicating a molecular weight of 420. A fragment peak at m/z 403 was also detected in the APCI-PI mass spectrum, accounting for loss of water. Since these data were not in agreement with the structure of 8′-apo-β-caroten-8′-oic acid proposed by Valadon,[2] a complete structure elucidation by means of NMR spectroscopy was carried out.

At first inspection, the ^{1}H NMR spectrum (in $CDCl_3$) revealed the presence of only two methyl signals, along with a few other aliphatic signals, whereas the majority of protons resonated in the 5.7–7.2 ppm region. Such a NMR pattern is not compatible with a carotenoid molecule, for which a greater number of aliphatic protons would be expected, but would rather suggest a polyene structure of non-isoprenoid origin. Extensive homonuclear and heteronuclear characterisation through DQF-COSY, *J*-resolved, ^{1}H,^{1}H-DQS, ROESY, HMQC and HMBC experiments allowed us to establish unambiguously the structure of 3-hydroxy-24-methyl-25-oxo-hexacosa-5,7,9,11,13,15,17,19,21,23-decaenoic acid for the main *L. sulphureus* pigment, to which the name laetiporic acid was given. In addition, NMR analysis showed that the purified sample was actually a mixture of two main geometric isomers **1a** and **1b** in an approximate 60:40 ratio, which could not be separated under any of the HPLC conditions employed. The two stereoisomers differ in the geometry around the double bond C-7–C-8, with the major isomer **1a** bearing the *cis* configuration.

The terminal carbonyl carbon (C-25) was detected through 27-CH_3 and 26-CH_3, whereas the carboxylic group was detected through 2-CH_2 in the HMBC experiments. The presence of an oxygen-bearing aliphatic carbon (C-3) was evident from the HMQC correlation between the proton signal at 4.13 ppm and the carbon signal at 67 ppm [$^1J(H,C) = 145$ Hz)]. Only one set of signals was found for the methylcarbonyl terminal group and for the protons up to H-17, for which coupling correlations were easily followed. Interestingly, ROESY, *J*-resolved and COSY experiments clearly showed that the double bond at C-19 must be in the *cis* configuration (ROE between H-21 and H-18, and two ^3J values lower than 12 Hz for both H-19 and H-20), whereas those at C-21 and C-23 are in the *trans* configuration (ROE between H-20 and H-22, H-21 and H-23, H-22 and 27-CH_3). Similarly, the carbonyl group is in the *s-trans* conformation, as confirmed by the ROE between 26-CH_3 and H-23. Two sets of protons were evident and corresponded to the aliphatic end group, indicating a *cis*/*trans* isomerism which can be attributed to the second double bond of the polyene chain. Unfortunately, it was not possible to follow completely the two major correlation patterns in the NMR spectrum, owing both to strong coupling and partial overlapping of proton signals. The assigned data for the major isomer **1a**, along with selected ROE and HBMC correlations, are reported in Table 1. As far as the minor isomer **1b** is concerned, most of the NMR signals were overlapped with those of the major isomer, except for the aliphatic terminus for which a second set of proton resonances could be extracted and assigned accordingly (Table 2).



1a



1b

Laetiporic acid **1** represents a new fungal polyene of non-isoprenoid origin that closely resembles piptoporic acid, a pigment isolated from the bright orange fruit-bodies of *Piptoporus australiensis* (Wakefield) Cunningham,[13] which is probably related to *L. sulphureus*. Both pigments share the same terminal motif, that is the 1-methyl-2-oxo-1-propylidene group conjugated with a polyene chain, which in piptoporic acid is represented by seven double bonds, whilst laetiporic acid features an unprecedented decaene system as part of its chromophore. In contrast, however, piptoporic acid bears an α,β-unsaturated acid moiety as the opposite end group, thus lacking the hydroxy function in position 3,[14] even though its 3*R*-acetoxy derivative was characterised as a minor component of *P. australiensis*.[13] Similary, from a biosynthetic viewpoint, β-elimination of water from laetiporic acid would afford the corresponding 2-dehydro-3-deoxy analogue, which has been detected as a minor pigment in our *L. sulphureus* extracts by HPLC (t_R 5.2 min)[12] and identified on the basis of LC–APCI-MS data.[15]

Table 1. ^{1}H and ^{13}C NMR spectral data of the major isomer **1a** of laetiporic acid (400.13 and 100.61 MHz, $CDCl_3$, δ ppm, TMS as reference; J_{HH} in Hz)

Position	δ_H (J_{HH})	ROE (H → H#)	HMBC (H → C#)	δ_C
1	—			174.0
2	2.61 dd (16.3, 2.2) 2.52 dd (16.3, 8.4)	3	1, 3	40.0
3	4.13 m	2, 4, 5		67.0
4	2.41 t (7.0)	3, 6	3	40.0
5	5.75 dt (15.0, 7.0)	7		130.0
6	6.64 dd (11.7, 15.0)	4, 9	6/7	129.0
7	6.02 m[b]	5		128.7
8	6.05 m[b]			129.5
9	6.74 m	6, 11		128.7
10	6.11 m	[a]		130.7
11	6.83 dd (11.7, 15.0)	9[a]		130.2
12–15	6.35–6.40			134
16	6.38 m			133.6
17	6.46 dd (12.0, 15.0)	19		135.7
18	6.80 dd (12.0, 15.0)	21		128.4
19	6.26 t (11.5)	17		134.0
20	6.17 t (11.7)	22		129.0
21	7.08 dd (11.9, 14.9)	18, 23	23	134.8
22	6.60 dd (11.7, 15.0)	20, 27		129.0
23	7.14 d (11.7)	21, 26	21, 25, 27	139.0
24	—			136.5
25	—			199.5
26	2.36 s	23	25	25.5
27	1.92 d (1.5)	22	23, 24, 25	11.5

Owing to the small amount of sample available in solution, the ^{13}C resonances were not directly acquired, but measured in the inverse-detection mode with a spectral resolution of ±0.5 ppm.
[a] ROE with a proton at 6.38.
[b] *J*-resolved indicates second order.

Table 2. Selected ^{1}H NMR spectral data for the aliphatic end group of the minor isomer **1b** of laetiporic acid

Position	δ_H (J_{HH})	ROE (H → H#)
2	2.60 dd (16.3, 3.2) 2.51 dd (16.3, 8.4)	3
3	4.11 m	2, 4, 5
4	2.38 t (7.0)	3, 6
5	5.75 dt (15.0, 7.0)	7
6	6.25 m[a]	4
7	6.26 m[a]	5
8	6.66 m	6

[a] *J*-resolved indicates second order.

Fungal pigments such as falconensones, produced by the mycelium of the ascomycete *Emericella falconensis* Horie et al.,[16] and melanocrocin, isolated from basidiocarps and mycelial cultures of *Melanogaster broomeianus* Berk.,[17] also contain the same ketonic terminus in conjugation with an all-*trans* polyene chain of different length (five and seven conjugated double bonds, respectively), but completely differ from laetiporic acid **1** in the remaining portion of the molecule. In comparison, however, the ^{1}H and ^{13}C chemical shifts of the C-22–C-27 portion in **1** are in perfect agreement with those reported for falconensones[16] and melanocrocin,[17] and strongly support our structure elucidation. In addition, as further confirmation of our *cis* assignments, the H-21 proton in **1** resonates at about 0.5 ppm downfield from the value which would be expected if the double bond between C-19 and C-20 were in the *trans* configuration as for melanocrocin[17] and falconensones.[16] This deshielding effect matches nicely with that described in the literature for *cis* carotenoids.[18] Similarly, the H-6 proton in **1a** is deshielded in comparison to **1b** due to the adjacent C-7–C-8 double bond of *cis* configuration.

In all cases where polyenes of non-isoprenoid origin have been described as fungal pigments,[3,13,16,17,19] the stereochemistry of the conjugated double bonds was reported as all-*trans*, mostly on the basis of ^{1}H NMR data and 2D experiments.[16,17,19] In the case of piptoporic acid, by far the closest analogue of **1**, the absence of a diagnostic '*cis*-peak' in the UV–vis spectrum allowed the assignment of the all-*trans* stereochemistry to the conjugated polyene chain, whose protons were reported to resonate in a broad envelope between 5.6 and 7.2 ppm.[13] Laetiporic acid, in contrast, lacked any conspicuous '*cis*-peak' in the near UV region when dissolved in methanol, but when the solvent was replaced by *n*-hexane, the UV–vis spectral shape changed dramatically and a well-resolved fine structure with several absorption maxima at shorter wavelengths was displayed,[20] which suggests the presence of *cis* double bonds in the polyolefinic chain.

Laetiporic acid **1** bears an unusual polyene chain of 10 conjugated double bonds which has never been reported so far among fungal pigments of non-isoprenoid origin. Moreover, its polyolefinic skeleton contains double bonds bearing stable *cis* configuration(s), which could be unambiguously characterised by means of 2D

NMR techniques such as ROESY, *J*-resolved and COSY experiments, and by comparison with literature data for similar pigments. In this respect, laetiporic acid represents one of the few well-authenticated *cis*-polyenes other than carotenoids.[18] Although we cannot exclude that isomerisation of a putative all-*trans* analogue may have occurred during handling or storage, the ratio between **1a** and **1b** did not change during our prolonged NMR measurements either in chloroform or acetone.

Laetiporic acid is the main pigment responsible for the bright orange colouration of *L. sulphureus* fruit-bodies, where it is found at a concentration of 250 $\mu g\,g^{-1}$ dry weight.[21] A few minor pigments were also detected in the crude extracts by HPLC analysis,[12] one of which could be tentatively identified as 2-dehydro-3-deoxylaetiporic acid on the basis of its APCI-MS fragmentation.[15] Interestingly, laetiporic acid is also produced when *L. sulphureus* is grown in submerged culture, though not as the main pigment. In this respect, our preliminary chromatographic investigations have revealed a different pigment profile displaying a number of apparently related structures, as yet unidentified.[22] Separation and structure elucidation of these pigments, along with studies on their chemotaxonomic significance, are being actively pursued in our laboratories and will be reported soon.

Acknowledgements

We are grateful to Prof. Timm Anke and Mrs. Anja Meffert (Department of Biotechnology, University of Kaiserslautern) for the use of experimental facilities and for recording APCI-MS spectra, respectively. We also thank Dr. Leanid Mishyn (Institute of Microbiology, Belarus National Academy of Sciences, Minsk) for kindly providing bibliographical references. Thanks are due to Prof. Arrigo Forni (Department of Chemistry, University of Modena and Reggio Emilia) for CD measurements.

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11. A voucher specimen and a culture of *L. sulphureus* have been deposited in the Culture Collection, Department of Biotechnology, University of Kaiserslautern, under the accession number 02089.
12. HPLC analysis was carried out with an HP 1090 Series II liquid chromatograph equipped with a diode array detector and a Merck LiChrospher® 100 RP-18 column (5 μm particle size; 250×4 mm). The injection volume was 10 μL, and the flow rate 1 mL min^{-1}. A water/acetone (A/B) gradient was used, running from 70% B to 100% B in 15 min followed by 2 min at 100% B.
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14. The absolute configuration at C-3 in laetiporic acid still remains undetermined. Owing to the strong absorption in the UV–vis region, the optical activity of **1** was not amenable to measurement using a polarimeter. When subjected to circular dichroism spectroscopy, however, our sample displayed a weak CD pattern, showing $\Delta\varepsilon$ (λ_{max}, nm; MeOH) −1.05 (250), +0.59 (286), +1.50 (476).
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20. UV/vis λ_{max} (*n*-hexane) 282, 296, 313, 328, 343, 365, 390, 415, 437 nm.
21. As determined by HPLC analysis[12] of methanolic crude extracts; the pigment was quantified against peak area calibrations obtained from a standard curve at 450 nm, using a chromatographically pure sample of laetiporic acid as the standard.
22. Weber, R. W. S.; Davoli, P., unpublished data.