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Intermediates in the oxidative pathway from torulene to torularhodin in the red yeasts *Cystofilobasidium infirmominiatum* and *C. capitatum* (Heterobasidiomycetes, Fungi)

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Abstract

Two red *Cystofilobasidium* spp. isolated from spring sap-flows of *Betula pendula* were analysed for their carotenoid content. In *Cystofilobasidium infirmominiatum*, three unusual pigments were detected and identified by structure elucidation as oxidised torulene derivatives. These included 16′-hydroxytorulene and torularhodinaldehyde, two carotenoids known so far only from chemical synthesis or as postulated biosynthetic intermediates *en route* to torularhodin. Unprecedented formation of β-apo-2′-carotenal was also observed. The production of these pigments in pure culture was dependent on enhanced oxidative stress caused by cultivation in well-aerated (indented) flasks with or without 2% ethanol (16′-hydroxytorulene), or with 100 μM duroquinone (torularhodinaldehyde and β-apo-2′-carotenal). Among these three pigments, only 16′-hydroxytorulene was detected in *C. capitatum*. Torularhodin, a common end product of carotenoid oxidation in red yeasts, was not produced by either species under any incubation conditions. Biosynthetic aspects of incomplete oxidation of torulene by these *Cystofilobasidium* spp. are discussed.

Keywords: Apocarotenoids; Carotenoids; *Cystofilobasidium capitatum*; *Cystofilobasidium infirmominiatum*; 16′-Hydroxytorulene; Oxidative stress; Red yeasts; Sap-flow; Torularhodinaldehyde

1. Introduction

Sap-flows exuding from fresh wounds of deciduous trees in spring quickly become colonised by fungal consortia comprising a planktonic phase of yeast cells and a biofilm of yeasts in a matrix of fungal hyphae. This phenomenon has been particularly well-characterised for sap-flows of birch (*Betula* spp.) which may acquire striking pink or orange colours if the heterobasidiomycetous yeast *Xanthophyllomyces dendrorhous* dominates the consortium (Golubev et al., 1977a,b; Weber, 2006). Considerable research interest has been devoted to this species together with a closely related anamorph, *Phaffia rhodozyma*, since these are the only yeasts so far shown to produce the carotenoid astaxanthin which is of commercial importance as a pigment in salmon farming and as a nutraceutical (Johnson and An, 1991). Our detailed analyses of sap-flow consortia in Germany (Weber et al., 2006) and Italy (Weber and Davoli, 2005) have also yielded red yeasts producing non-astaxanthin carotenoids. Two species were distinguished, *viz.* the abundant *Cystofilobasidium infirmominiatum* and the less common *C. capitatum*. The former has also been reported occasionally from birch sap-flows in Japan (Phaff et al., 1972). These *Cystofilobasidium* spp. can cause red pigmentation of sap-flows even in the absence of *Xanthophyllomyces* (Weber and Davoli, 2005).

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Schroeder and Johnson (1995) have provided evidence by means of mutant studies that astaxanthin can protect *X. dendrorhous* against oxidative stress in its natural substrate, birch juice. Such oxidative stress may be caused by an unidentified photosensitising molecule which generates reactive oxygen species (ROS), especially singlet oxygen ($^1O_2$), upon stimulation by near-UV light around 365 nm. Under artificial cultivation conditions, oxidative stress in *Xanthophyllomyces*/*Phaffia* and certain other red yeasts results in a biosynthetic switch from β-carotene to oxygen-containing carotenoids (xanthophylls). This can be triggered by a high degree of aeration in combination with ROS inducers such as duroquinone, H_2O_2 or ethanol (Schroeder and Johnson, 1993; Sakaki et al., 2000; Davoli et al., 2004; Madhour et al., 2005).

Since initial assays indicated an unusual spectrum of carotenoid pigments for *C. infirmominiatum* and *C. capitatum*, we elucidated their structure by NMR analysis and examined their biosynthesis under oxidative stress. The results, described in the present paper, reveal two partially oxidised torulene derivatives as major pigments, *viz.* 16′-hydroxytorulene (**4**; see Fig. 4) in *C. capitatum* and *C. infirmominiatum* and torularhodinaldehyde (**5**) only in the latter species. These pigments have not previously been identified unambiguously in wild-type yeasts. In addition, in *C. infirmominiatum* grown under enhanced oxidative stress a third pigment was isolated and identified as β-apo-2′-carotenal (**7**). This represents the first apocarotenoid ever isolated and characterized from red yeasts. The biosynthetic pathway of these uncommon carotenoids and some chemotaxonomic implications resulting from the close phylogenetic relationship between their producers and *Xanthophyllomyces* are discussed.

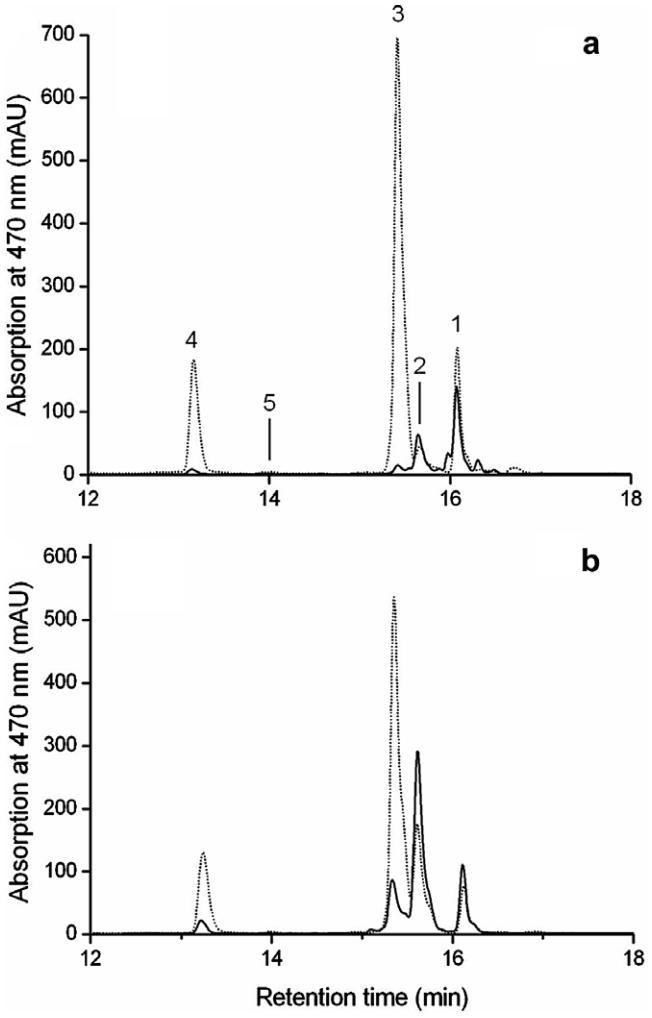

Fig. 1. HPLC carotenoid profiles of *Cystofilobasidium infirmominiatum* Car230 (a) and *C. capitatum* Car243 (b) in cells grown in aeration-limited cultures in straight flasks (straight line) and in indented flasks with improved aeration (dotted lines). Chromatograms were recorded at 470 nm. The following pigments were identified: β-carotene (**1**), γ-carotene (**2**), torulene (**3**), and 16′-hydroxytorulene (**4**). The position of torularhodinaldehyde (**5**) is also indicated. For chemical structures, see Fig. 4. For HPLC conditions, see Section 4.

2. Results

2.1. Identification of yeasts

In addition to *Xanthophyllomyces dendrorhous*, two further red yeast species were isolated from spring sap-flows of cut birch trees. A FASTA search of their ITS1–5.8S rDNA–ITS2 sequences with GenBank data revealed 100% sequence identity between isolate Car230 and *Cystofilobasidium infirmominiatum* (GenBank AF444400, AY264716), and between isolate Car243 and *C. capitatum* (AY052489, AY052492, AY052493) as well as *C. lari-marini* (AY052491, AY052494), the latter now being regarded as a carotenoidless mutant strain of *C. capitatum* (Sampaio et al., 2001). Confirmation of identity of both species was obtained by their C and N utilisation spectra (not shown) which were similar to those described by Barnett et al. (2000).

2.2. Identification of carotenoids

At first glance, the carotenoid profiles of both yeast species (Fig. 1) showed high similarities to those of other red yeasts, notably *Sporobolomyces roseus* and *Rhodotorula glutinis* (see Davoli et al., 2004). In straight flasks providing a low degree of aeration, β-carotene (**1**) predominated whereas more highly oxidised carotenoids were formed in flasks with indentations. The most polar pigment, **4**, was produced by both species in indented flasks with or without added ethanol (2%) and eluted at the same time ($t_R \sim 13$ min) as torularhodin (**6**) but showed an online UV/visible spectrum identical to torulene (**3**), with a shoulder at 462 nm, main peak at 489 nm and sub-peak at 520 nm. This pigment differed from torularhodin also in its molecular weight as shown by HPLC-MS, which gave an APCI-negative $[M]^-$ peak at m/z 550 and an APCI-positive $[M+H]^+$ peak at m/z 551, compatible with the formula $C_{40}H_{54}O$. A difference of 16 mass units with respect to torulene would suggest the presence of a hydroxy group

in the molecule, as confirmed by the base peak at *m/z* 533 in the APCI-PI-MS, accounting for loss of water.

A less polar pigment, **5** ($t_R \sim 14$ min), showing a UV/vis spectrum shifted to higher wavelengths ($\lambda_{max} = 503$ nm) with a poorly resolved peak fine-structure, was produced only by *C. infirmomininatum* growing in well-aerated flasks with added duroquinone (100 μM); its APCI peaks were $[M]^-$ at *m/z* 548 in the negative mode and $[M+H]^+$ at *m/z* 549 in the positive mode. The bathochromic shift in the UV/vis spectrum and the difference of two mass units with respect to **4** hinted at the presence of a more highly oxidised, sp^2-type carbon atom, such as an aldehyde group, in conjugation with the polyene chain.

In addition, a third pigment, **7** ($t_R \sim 14$ min), was detected in extracts of *C. infirmominiatum* after prolonged cultivation in 100 μM duroquinone. Pigment **7** displayed a UV/vis absorption spectrum that was deceptively similar to **5**, though at slightly lower wavelengths ($\lambda_{max} = 498$ nm). Initially thought to be identical to **5**, HPLC-MS analysis gave an unexpected molecular weight of only 508 mass units (peaks at *m/z* 508 and 509 in the APCI-negative and -positive ionisation mode, respectively), raising the question whether pigment **7** contained a shortened polyolefinic chain relative to **5**.

Since we could not safely establish the identity of these three pigments solely on the basis of UV/visible and HPLC-MS data, we purified them for a complete structure elucidation which was carried out by means of homonuclear and heteronuclear mono- and bidimensional NMR techniques such as gCOSY, gTOCSY, ROESY, gHSQC and gHMBC.

2.2.1. Structure elucidation of pigment *4*

The presence of an unsymmetrical polyene chain of isoprenoid origin bearing similarities to that of torularhodin derivatives but lacking the typical downfield signal at 7.4 ppm for deshielded H-2′ (Weber et al., 2005) was easily recognised in the ^{1}H NMR spectrum of pigment **4**. Application of gCOSY, gTOCSY and ROESY techniques enabled a safe assignment of the olefinic protons. The corresponding carbon atoms were assigned accordingly through gHSQC and gHMBC experiments. In particular, gTOCSY indicated that a peculiar spin system featuring three protons at 6.17 (*d*; δ_C 125.7 from gHSQC), 6.48 (*dd*) and 6.33 ppm (*d*) also included three methyl protons at 1.85 ppm (δ_C 14.2). In the gCOSY experiment, this methyl group correlated through long-range coupling with methylene protons resonating at 4.11 ppm (*d*, $^3J = 5.4$ Hz) which, in turn, coupled with a triplet at 1.44 ppm (–OH) and showed also gHSQC correlations with a carbon atom at 68.5 ppm, thus confirming the presence of a $–CH_2OH$ group in the molecule. These data were in accordance with δ_H and δ_C values reported in the literature for structurally comparable end groups bearing a hydroxymethyl functionality (Englert, 1995), and were strongly suggestive of the localisation of the hydroxy group at C-16′. Diagnostic ROEs between the signal at 4.11 ppm and those at 1.85 and 6.17 ppm (H-17′ and H-2′, respectively; see Fig. 2a) safely confirmed in pigment **4** the presence of the 3′,4′-didehydro-16′-ol ψ end group, which was further corroborated by key gHMBC correlations between the methyl protons at 1.85 ppm (H-17′) and carbon atoms at 125.7 and 68.5 ppm (C-2′ and C-16′, respectively). For the remaining portion of the polyene chain, the whole set of ^{1}H and ^{13}C resonances was in agreement with data for torularhodin derivatives reported in the literature (Englert, 1995; Weber et al., 2005), and secured the identity of the chromophore in **4**. The configurations of the double bonds of the polyolefinic system were determined as all-*trans* on the basis of $^3J_{H,H}$ values (see Table 1) and were further corroborated by gCOSY data.

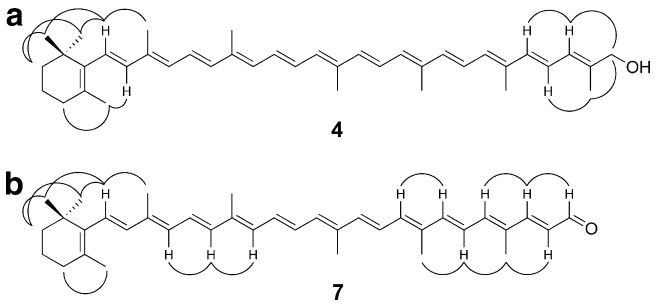

Fig. 2. Selected ROESY correlations in pigments **4** (a) and **7** (b).

In the aliphatic region of the ^{1}H NMR spectrum, the magnetic equivalence of two geminal methyl groups at δ_H 1.03 ppm (δ_C 28.8) was strongly suggestive of the presence of a β-ionone moiety at the opposite side of the molecule. This was indeed confirmed by gCOSY, gTOCSY, ROESY, gHSQC and gHMBC experiments, which provided a set of δ_H and δ_C values in perfect agreement with those reported in the literature for atoms C-1–C-6 and C-16–C-18 belonging to a standard β end group (Englert, 1995; Madhour et al., 2005). Therefore, pigment **4** can be identified unambiguously as all-*trans*-3′,4′-didehydro-β,ψ-caroten-16′-ol, to which the semisystematic name 16′-hydroxytorulene is given. ^{1}H and ^{13}C NMR spectral data of carotenol **4** are summarized in Table 1.

2.2.2. Structure elucidation of pigment *5*

In the ^{1}H NMR spectrum of pigment **5**, a singlet at 9.43 ppm was diagnostic of the presence of the aldehyde function that had been suspected from APCI-MS data. A downfield signal at 6.93 ppm (*d*) coupled with a *dd* resonating at 6.65 ppm which, in turn, displayed *trans* coupling with another olefinic proton at 6.74 ppm (*d*). These data perfectly matched those reported in the literature for the acyclic 3′,4′-didehydro-16′-al ψ end group (Englert, 1995). Likewise, δ_H values for the remaining olefinic signals and for the aliphatic protons which were assigned with the aid of gCOSY, gTOCSY and ROESY experiments were in excellent agreement with literature data available for synthetic torularhodinaldehyde (Englert, 1995) and allowed us to assign unequivocally to pigment **5** the structure of

Table 1
^{1}H and ^{13}C NMR spectral data (400.13 and 100.61 MHz, respectively) of 16′-hydroxytorulene (**4**), and ^{1}H NMR data of torularhodinaldehyde (**5**) and β-apo-2′-carotenal (**7**) from *Cystofilobasidium infirmominiatum* Car230 in $CDCl_3$ solution (δ in ppm downfield from TMS as reference; $J_{H,H}$ in Hz)

Position	**4**		**5**	**7**
	δ_H ($J_{H,H}$)	δ_C	δ_H ($J_{H,H}$)	δ_H ($J_{H,H}$)
1	–	34.2	–	–
2	1.46 *m*	39.4	1.47 *m*	1.47 *m*
3	1.62 *m*	19.1	1.62 *m*	1.62 *m*
4	2.02 *m*	32.9	2.02 *m*	2.02 *m*
5	–	129.3	–	–
6	–	137.8	–	–
7	6.17 *d* (15.9)	126.5	6.17 *d* (15.8)	6.19 *d* (16.0)
8	6.13 *d* (15.9)	137.6	6.13 *d* (15.8)	6.13 *d* (16.0)
9	–	n.d.	–	–
10	6.15 *d* (11.0)	130.7	6.14 *d* (11.0)	6.15 *d* (11.0)
11	6.65 *dd* (11.0, 14.8)	124.8	6.66 *dd* (11.0, 15.0)	6.68 *dd* (11.0, 15.0)
12	6.35 *d* (14.8)	137.0	6.35 *d* (15.0)	6.35 *d* (15.0)
13	–	n.d.	–	–
14	6.25[a] *m*	133.0	6.25 *m*	6.26 *d* (11.0)
15	6.63–6.64 *m*	130.0	6.66 *m*	6.66 *m*
16	1.03 *s*	28.8	1.03 *s*	1.03 *s*
17	1.03 *s*	28.8	1.03 *s*	1.03 *s*
18	1.71 *s*	21.6	1.71 *s*	1.72 *s*
19	1.97 *s*	12.6	1.97_5 *s*	1.98 *s*
20	1.97 *s*	12.6	1.98 *s*	1.98 *s*
1′	–	137.1	–	–
2′	6.17 *d* (11.1)	125.7	6.93 *d* (11.0)	9.58 *d* (7.8)
3′	6.48 *dd* (11.1, 15.0)	123.3	6.65 *dd* (11.0, 15.0)	6.18 *dd* (7.8, 15.5)
4′	6.33 *d* (15.0)	137.4	6.74 *d* (15.0)	7.17 *d* (15.5)
5′	–	n.d.	–	–
6′	6.22[b] *d* (11.0)	132.4[e]	6.42 *d* (11.0)	6.60[g]
7′	6.62[c] *dd* (11.0, 15.0)	124.6[f]	6.62 *dd* (11.0, 15.0)	6.60[g]
8′	6.38 *d* (15.0)	138.0	6.50 *d* (15.0)	6.60[g]
9′	–	n.d.	–	–
10′	6.24[b] *d* (11.0)	132.3[e]	6.31 *d* (11.0)	6.37 *d* (11.0)
11′	6.64[c] *dd* (11.0, 15.0)	124.7[f]	6.62 *dd* (11.0, 15.0)	6.64 *dd* (11.0, 15.0)
12′	6.38 *d* (15.0)	138.0	6.43 *d* (15.0)	6.46 *d* (15.0)
13′	–	n.d.	–	–
14′	6.27[a] *m*	133.0	6.28 *m*	6.32 *d* (11.0)
15′	6.63–6.64 *m*	130.0	6.63 *m*	6.64 *m*
16′	4.11 *d* (5.4)	68.5	9.43 *s*	–
17′	1.85 *s*	14.2	1.90 *s*	–
18′	1.97[d] *s*	12.6	2.03 *s*	1.98 *s*
19′	1.98_5[d] *s*	12.6	1.98 *s*	2.01 *s*
20′	1.97 *s*	12.6	1.98 *s*	1.98 *s*
OH	1.44 *t* (5.4)	–	–	–

[a–f] Tentative assignments: resonances may be interchangeable.
[g] Strongly coupled. n.d. = not detected.

all-*trans*-3′,4′-didehydro-β,ψ-caroten-16′-al. ^{1}H NMR resonances of natural **5** are listed in Table 1.

2.2.3. Structure elucidation of pigment 7

Despite the strong similarity of their UV/vis spectra, the presence of a polyene system which differed from that of carotenal **5** was immediately recognised in the ^{1}H NMR spectrum of pigment **7**. Most downfield, an isolated *d* at 9.58 ppm ($^{3}J = 7.8$ Hz) featured as a characteristic signal and coupled with an olefinic proton at 6.18 ppm (*dd*, $^{3}J = 7.8$, 15.5 Hz) which, in turn, displayed *trans* coupling with a deshielded *d* at 7.17 ppm. Such an unusual pattern would well correspond to an aldehyde group adjacent to a *trans*-disubstituted double bond (Englert, 1995; Schmidt et al., 2006) and hinted at the presence of a polyolefinic chain that had been somehow shortened, as indicated by APCI-MS data. In particular, we reasoned that if the C-1′,2′ double bond in torulene was cleaved and the C-2′ atom oxidised to aldehyde, a 2′-apocarotenal with the formula $C_{37}H_{48}O$ would be obtained which would match the observed molecular weight of 508 mass units. Examination of the remaining olefinic signals in the ^{1}H NMR spectrum of **7** with the aid of gCOSY and gTOCSY techniques revealed the presence of four additional spin systems whose δ_H values closely resembled those already seen in pigments **4** and **5** for protons H-7 to H-15 and H-10′ to H-15′ belonging to an unsymmetrical all-*trans* conjugated polyene chain. A marked difference, however, was represented by a conspicuous broad *s* at 6.60 ppm integrating for three protons, which suggested that the missing H-6′ to H-8′ atoms were strongly coupled and resonated at the same frequency. Evidence of a similar behaviour has been noted in the ^{1}H NMR spectrum of the C_{37} apocarotenoid gelliodesxanthin (3-hydroxy-4-oxo-3′,4′-didehydro-2′-apo-β,ψ-caroten-2′-al) by Tanaka and Inoue (1987), even though a full assignment of δ_H values was not performed by these authors. In the case of pigment **7**, gratifyingly, such an assignment was indeed confirmed by key ROE correlations between the intense signal at 6.60 ppm and protons at 6.37 (H-10′) and 7.17 ppm (H-4′), as well as with methyl groups at 1.98 and 2.01 ppm (H-18′ and H-19′, respectively) (see Fig. 2b). By analogy to **4** and **5**, the all-*trans* nature of double bonds in the polyene chain in **7** was established on the basis of $^{3}J_{H,H}$ values (see Table 1) and confirmed by gCOSY and ROESY (Fig. 2b) data.

At the opposite side of the molecule, all proton resonances assigned through gCOSY, gTOCSY and ROESY (Fig. 2b) experiments were essentially superimposable on those observed for pigments **4** and **5**, and were in perfect agreement with δ_H values reported in the literature for a standard β end group (Englert, 1995; Madhour et al., 2005), thus allowing us to secure unambiguously for pigment **7** the structure of all-*trans*-3′,4′-didehydro-2′-apo-β,ψ-caroten-2′-al, for which the semisytematic name of β-apo-2′-carotenal is used. ^{1}H spectral data of apocarotenal **7** are given in Table 1.

2.3. Production of carotenoids under oxidative stress

When *C. infirmominiatum* growing in indented flasks was subjected to oxidative stress by addition of inducers of ROS, changes in pigment composition were observed relative to control flasks without additions (Fig. 3). Whereas 2% ethanol stimulated the production of γ-carotene (**2**), a putative precursor of **4**, **5** and **6**, the biosynthesis of **5** was selectively enhanced in cultures to which 100 μM duroquinone had been added at the time-point of inocula-

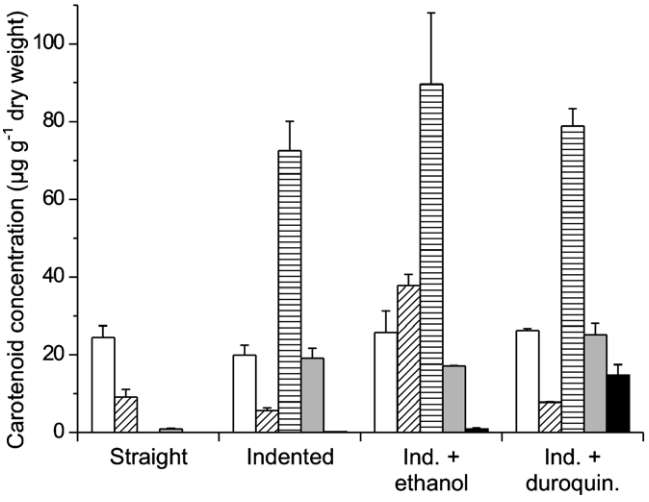

Fig. 3. Production of β-carotene (**1**; empty bars), γ-carotene (**2**; sloped lines), torulene (**3**; horizontal lines), 16′-hydroxytorulene (**4**; grey bars) and torularhodinaldehyde (**5**; black bars) by *Cystofilobasidium infirmominiatum* Car230 in straight and indented flasks with or without ROS inducers (2% ethanol or 100 µM duroquinone). Results are shown as average ± SD of three independent replicates. For chemical structures, see Fig. 4.

tion. The yet more highly oxidised pigment torularhodin (**6**) was not observed under any incubation conditions. Addition of the lycopene cyclase inhibitor nicotine (10 or 20 mM) resulted in the production of lycopene as the dominant pigment.

3. Discussion

Red yeasts comprise members of three subclasses of Basidiomycota, *viz.* Heterobasidiomycetes, Urediniomycetes and Ustilaginomycetes (Fell et al., 2001; Webster and Weber, 2007). The carotenoids β-carotene (**1**), γ-carotene (**2**) and torulene (**3**) are among the most common in red yeasts, being found in all three subclasses. Numerous red yeasts respond to oxidative stress caused by high aeration and/or the addition of ROS-generating reagents to the culture medium by switching their carotenoid biosynthesis from β-carotene to xanthophylls such as plectaniaxanthin (*Dioszegia takashimae*; Madhour et al., 2005), canthaxanthin and astaxanthin (*Xanthophyllomyces* and *Phaffia*; Schroeder and Johnson, 1993), or torularhodin (**6**). The latter is frequently encountered in well-examined species such as *Rhodotorula glutinis* or *Sporobolomyces roseus* (see Fig. 4; Sakaki et al., 2001; Davoli et al., 2004). However, if torularhodin is absent, torulene may be the most highly oxidised pigment, as found e.g. in *Sakaguchia dacryoidea* Car118 (A. Madhour and R.W.S. Weber, unpublished).

The oxidation of torulene to torularhodin has been postulated to proceed *via* 16′-hydroxytorulene (**4**) and torularhodinaldehyde (**5**) (Bonaly and Malenge, 1968). Although both pigments have been synthesised chemically (Rüegg et al., 1959; Schwieter et al., 1966), neither has been unequivocally demonstrated from natural sources prior to

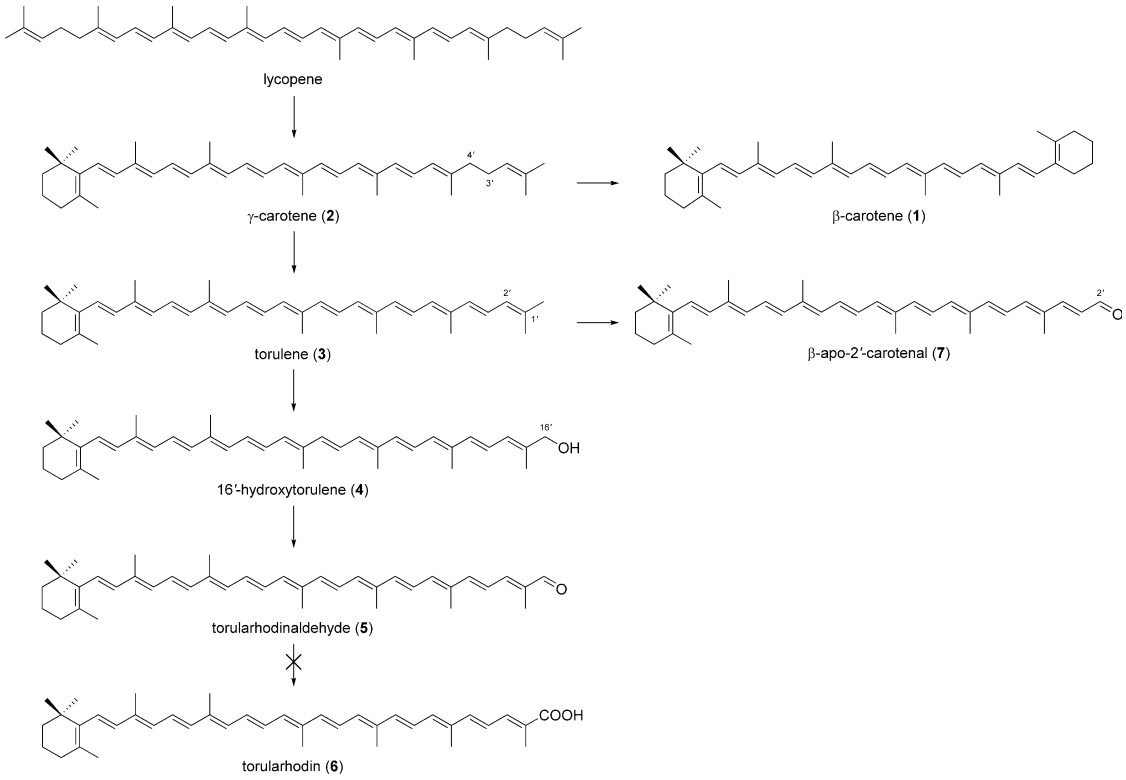

Fig. 4. Postulated carotenoid biosynthetic pathway in *Cystofilobasidium infirmominiatum* Car230. Early biosynthesis results in lycopene and γ-carotene (**2**). At low oxidative stress, β-carotene (**1**) may accumulate, whereas high oxidative stress leads to an accumulation of torulene (**3**), 16′-hydroxytorulene (**4**), torularhodinaldehyde (**5**) and β-apo-2′-carotenal (**7**). Torularhodin (**6**), a common end product of carotenoid oxidation in red yeasts, was not produced by *C. infirmominiatum*.

the present report. Evidence coming from a mutant of *Rhodotorula mucilaginosa* was based merely on UV/vis absorption spectra and chromatographic properties (Bonaly and Villoutreix, 1965; Bonaly and Malenge, 1968), and this was considered insufficient by Britton et al. (2004). Likewise, more recently, 16′-hydroxytorulene has been identified only tentatively as a minor carotenoid in extracts of *R. glutinis* and *R. rubra* by HPLC analysis on the basis of online DAD UV/vis absorption spectra (Squina and Mercadante, 2003, 2005). The lack of these intermediates in nature has been explained by the hypothesis that the same enzyme is responsible for all three steps of torulene oxidation (Goodwin, 1980; Britton, 1998). Based on evidence showing incorporation of one atom of atmospheric molecular oxygen into the carboxyl group of torularhodin (Simpson et al., 1964), the action of a mixed-function oxidase was postulated to be responsible for the oxidation of the 16′ methyl group in torulene to afford eventually torularhodin *via* transient intermediates 16′-hydroxytorulene and torularhodinaldehyde (Goodwin, 1980; Britton, 1998). In a recent study on the influence of selected inhibitors on the carotenoid composition of *Rhodotorula glutinis* and *R. rubra*, Squina and Mercadante (2005) failed to detect any torularhodinaldehyde formation and concluded that oxidation of 16′-hydroxytorulene to torularhodin was likely to occur in a single step without aldehyde accumulation. We were therefore surprised to detect both postulated intermediates **4** and **5** as the most highly oxidised carotenoids in *Cystofilobasidium infirmominiatum*, suggesting a deficiency in the torulene-oxidising enzyme system in this species. Torularhodin, in fact, was never produced by either *C. infirmominiatum* or *C. capitatum* under any incubation conditions. More intriguingly still, the differential accumulation of 16′-hydroxytorulene and torularhodinaldehyde in *C. infirmominiatum* subjected to different causes of oxidative stress indicated that more than one enzyme may be involved in torulene oxidation.

The isolation of β-apo-2′-carotenal (**7**) from *C. infirmominiatum* in response to enhanced oxidative stress is even more surprising and puzzling. Although apocarotenal **7** has long been known from chemical synthesis (Rüegg et al., 1959; Schwieter et al., 1966), its natural occurrence in red yeasts is unprecedented. It has been reported from saponified extracts of the sea molluscs *Aplysia depilans* and *A. rosea* (Czeczuga, 1984), but this claim was based on UV/vis data and chromatographic properties only, and natural and synthetic compounds were not compared. Metabolism of synthetic **7** in chickens has been thoroughly investigated (Schiedt et al., 1991) and methods for the isolation and the separation of the numerous products of its metabolic breakdown have been reported (Schiedt et al., 1995). Gelliodesxanthin, a closely related C_{37} apocarotenal sharing the same acyclic end group but bearing a 3-hydroxy-4-keto β-ionone moiety at the opposite side of the molecule, has been described from the sea sponge *Gelliodes callista* (Tanaka and Inoue, 1987). Carotenal **7** therefore represents the first apocarotenoid ever isolated and characterized unequivocally from red yeasts. The parent β-apo-2′-carotenol (3′,4′-didehydro-2′-apo-β,ψ-caroten-2′-ol) was reported to occur as a minor carotenoid from a mutant of *Rhodotorula mucilaginosa* (Bonaly and Malenge, 1968), but sufficient evidence for identification was not provided (Britton et al., 2004). Biosynthetically, β-apo-2′-carotenal (**7**) is likely to arise from torulene by oxidative cleavage of the C-1′,2′ double bond through the action of a carotenoid cleavage oxygenase. Under enhanced oxidative stress, the failure of *Cystofilobasidium* to produce torularhodin as the most highly oxidised end product may trigger the action of a specific carotenoid cleavage oxygenase, thus channelling biosynthesis towards β-apo-2′-carotenal. Carotenoid cleavage oxygenases are widespread in nature and are responsible for the biosynthesis of most apocarotenoids from C_{40} carotenoid precursors (for overviews, see Camara and Bouvier, 2004; Bouvier et al., 2005; Kloer and Schulz, 2006). In addition, production of apocarotenoids as a response to biotic or abiotic stress has been observed in various organisms (see Bouvier et al., 2005; Walter et al., 2007). Recently, the involvement of a carotenoid cleavage oxygenase in the biosynthesis of the torulene-derived apocarotenoic acid neurosporaxanthin (4′-apo-β,ψ-caroten-4′-oic acid) in *Fusarium fujikuroi* has been postulated (Thewes et al., 2005). The question whether a carotenoid cleavage monooxygenase or dioxygenase is involved in the biosynthesis of β-apo-2′-carotenal in *C. infirmoniniatum* must remain unanswered at present and should be a topic for future investigations. Of course, the possibility that β-apo-2′-carotenal is formed by the action of some other unspecific carotenoid-degrading enzyme(s) (see Kloer and Schulz, 2006) or by non-enzymatic oxidation of torulene cannot be ruled out, though the formation of a single major cleavage product in *C. infirmominiatum* would argue against such a scenario. In fact, although formation of apocarotenals and epoxycarotenoids by degradation of β-carotene or lycopene under oxidative stress has been documented in model systems (Handelman et al., 1991; Henry et al., 2000; Rodriguez and Rodriguez-Amaya, 2007) as well as processed foods (Rodriguez and Rodriguez-Amaya, 2007), complex mixtures of autoxidation products were always detected. Furthermore, since we did not observe any conspicuous formation of carotenoid degradation products in other carotenoid-producing red yeasts such as *Dioszegia*, *Rhodotorula* or *Sporobolomyces* spp. grown under similar conditions of enhanced oxidative stress (Davoli et al., 2004; Madhour et al., 2005; Weber et al., 2005, 2007), β-apo-2′-carotenal is likely to represent a genuine product of *C. infirmominiatum* metabolism rather than an artifact arising from autoxidation of torulene. Clearly, the nature and catalytic properties of the oxidising enzyme(s) in *C. infirmominiatum* warrant further biochemical investigations.

Monocyclic torulene-derived carotenoids bearing oxygen-containing groups at the acyclic end (and, sporadically, also at the cyclic end; see Liu et al., 1973; Weber et al., 2005) have been described from numerous species scattered

throughout basidiomycete yeasts. Among Urediniomycetes, they have been found e.g. in *Rhodosporidium babjevae* (Sperstad et al., 2006), *Rhodotorula aurantiaca* (Liu et al., 1973), *R. glutinis* (Sakaki et al., 2000, 2001), *Sporobolomyces coprosmae* (Weber et al., 2005) and *S. roseus* (Davoli et al., 2004), whereas in Heterobasidiomycetes they are produced by *Cryptococcus flavescens* (Bae et al., 1971) and *Dioszegia takashimae* (Madhour et al., 2005). Their characterisation in *Cystofilobasidium infirmominiatum* and *C. capitatum* is remarkable because recent phylogenetic schemes (Fell et al., 2000; Scorzetti et al., 2002) have grouped these species together with *Xanthophyllomyces* and *Phaffia* which synthesise bicyclic oxidised carotenoids, chiefly astaxanthin. Clearly, therefore, carotenogenesis is a highly variable character among basidiomycete yeasts, and new or interesting carotenoids may be discovered in any red yeast, whether closely related to a previously characterised producer or not.

4. Experimental

4.1. Identification of strains and cultivation conditions

Cystofilobasidium infirmominiatum was isolated from a spring slime-flux on *Betula pendula* in Kaiserslautern (Germany) in April 2005, and *C. capitatum* from the same tree species in Modena (Italy) in March 2006. These strains have been incorporated into our Culture Collection (IBWF, Kaiserslautern, Germany) under the accession numbers Car230 (Weber et al., 2006) and Car243 (Weber and Davoli, 2005), respectively. Species identification was performed by means of a FASTA search in GenBank of the complete ITS1–5.8S rDNA–ITS2 sequences which were obtained as described previously (Schwarz et al., 2004). Carbon and nitrogen utilisation patterns were determined according to Yarrow (1998), growing the yeasts in 1.5 ml volumes in shaken 24-well plates at 22 °C. A second isolate of *C. infirmominiatum*, strain Car239 from Modena, was identical to Car230 in its ITS sequence and carotenoid profile and is not further described here.

In order to characterise pigment production under various conditions of oxidative stress, Car230 and Car243 were grown in 100 ml aliquots of yeast extract-sucrose (YES) medium (20 g sucrose, 4 g yeast extract, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7\ H_2O\ l^{-1}$) in 500 ml conical flasks with one indentation, and fitted with a loose cotton bung in order to improve aeration. Control cultures were grown in non-indented flasks fitted with firm bungs. Flasks were inoculated with a loopful of cells taken from an actively growing YES agar culture. Duroquinone (100 μM), ethanol (2% v/v) or (−)-nicotine (10 or 20 mM), all of analytical grade, were added at the time-point of inoculation as required. Flasks were incubated on an orbital shaker (120 rpm) for 5 d at 22 °C in dim room light; pigment production relative to biomass was reduced both at lower (18 °C) and higher (27 °C) incubation temperatures (data not shown). All experiments were carried out as triplicate flasks.

For pigment purification, strain Car230 was grown in 20 conical flasks (1 l) with four indentations, each fitted with a loose cotton bung and containing 200 ml YES medium augmented with 2% ethanol for production of **4**, or 100 μm duroquinone for production of **5** and **7**. Incubation conditions were as described above.

4.2. Extraction and purification of pigments

For analysis and quantification of pigments in crude extracts, cells were harvested from 40 ml aliquots of liquid cultures by centrifugation (10 min at 15,000 rpm) followed by freezing, DMSO–acetone extraction, phase separation in light petroleum, rotary evaporation of the organic phase and redissolution in acetone to 1% of the original volume (see Weber and Davoli, 2003; Weber et al., 2007). In order to improve chromatographic separation, the crude extracts thus obtained were frozen at −20 °C for precipitation of colourless lipid material. The remaining clear red solution was separated from these deposits. Dry-weight biomass of cells was determined in pre-weighed Eppendorf tubes to which 1.5 ml aliquots of culture were added, followed by centrifugation (5 min at 13,000 rpm) and drying at 60 °C for 3 d.

For purification of pigments **4**, **5** and **7**, cells from 4 l aliquots of cultures were extracted as described above. The crude extracts were redissolved in 20 vol. acetone. Lipids were removed by precipitation at −20 °C and −80 °C. The acetone was evaporated and extracts were redissolved in cyclohexane. In total 317.6 mg crude extract enriched in **4** was obtained from 19.8 g cells (dry wt), 268.9 mg **5**-enriched extract from 22.8 g cells and 685.2 mg **7**-enriched extract from 24.8 g cells.

The second purification step consisted of silica gel chromatography (Merck silica gel 60; 63–200 μm particle size; 150–270 × 25 mm bed size). Following removal of apolar carotenoids in pure cyclohexane and cyclohexane–ethyl acetate (97:3 to 95:5, v/v), pigment **4** eluted at 80:20 (v/v), and pigments **5** and **7** at 90:10 (v/v). For pigment **5**, 3.4 mg violet solid were obtained after silica gel chromatography, and were used as such for structure elucidation. Final purification of pigments **4** and **7** was achieved using a Gilson Model 302 preparative HPLC fitted with a Merck LiChrosorb RP-18 column (7 μm particle size; 250 × 25 mm) and a variable wavelength detector set to 480 nm. In a gradient from 70% to 100% acetone in 60 min followed by washing at 100% acetone at a flow rate of 5 ml min^{-1}, both pigments eluted at approx. 67 min. Altogether 4.4 mg pure **4** and 1.0 mg pure **7** were obtained.

4.2.1. all-E-3′,4′-Didehydro-β,ψ-caroten-16′-ol (16′-hydroxytorulene, 4)

Orange-red amorphous solid. UV/visible λ_{max} (*n*-hexane) nm: 461, 485, 516; % III/II = 31. UV/vis λ_{max} (MeOH) nm: 460, 484, 515; % III/II = 25. IR (KBr) ν_{max} cm^{-1}: 3437 s br, 2926 s, 2855 s, 1741 s, 1663 m, 1456 m, 1379 m, 1171 m, 968 s, 722 w. For ^{1}H and ^{13}C NMR spectra,

see Table 1. APCI-MS, PI: *m/z* 551 ($[M+1]^+$, 23), 533 ($[(M-18)+1]^+$, 100); NI: *m/z* 550 ($[M]^-$, 100).

4.2.2. all-E-3′,4′-Didehydro-β,ψ-caroten-16′-al (torularhodinaldehyde, 5)

Violet solid. UV/vis λ_{max} (*n*-hexane) nm: 506; UV/vis (MeOH) nm: 505. For ^{1}H NMR spectrum, see Table 1. APCI-MS, PI: *m/z* 549 ($[M+1]^+$, 100); NI: *m/z* 548 ($[M]^-$, 100), 473 (12).

4.2.3. all-E-3′,4′-Didehydro-2′-apo-β,ψ-caroten-2′-al (β-apo-2′-carotenal, 7)

Violet solid. UV/vis λ_{max} (*n*-hexane) nm: 489; UV/vis (MeOH) nm: 487. IR (KBr) ν_{max} cm^{-1}: 3407 s br, 2952 m, ~2800 m/w, 1648 m, ~1400 w, 1385 w, ~1200 w, 1021 s, 675 m br. For ^{1}H NMR spectrum, see Table 1. APCI-MS, PI: *m/z* 509 ($[M+1]^+$, 100), 403 ($[(M-106)+1]^+$, 12); NI: *m/z* 508 ($[M]^-$, 100), 402 ($[M-106]^-$, 19).

4.3. HPLC chromatography and spectroscopic methods

For HPLC analysis of crude extracts, a Hewlett Packard HP1090 Series I instrument fitted with a LiChrospher 100 RP-18 column (Merck; 5 μm particle size; 250 × 4 mm) was run with a gradient from 70% to 100% acetone in 15 min at a flow rate of 1 ml min^{-1} (Weber et al., 2007). Carotenoids were quantified at 450 nm by reference to a pure β-carotene standard. Calibration curves for the other pigments were calculated at 450 nm relative to that of β-carotene based on the published correction factors for γ-carotene and torulene (Britton, 1995; Davoli and Weber, 2002), and assuming that the intensity of UV/visible light absorption of pigments **4** and **5** relative to torulene was little affected by oxidation at C-16′. All quantification data were obtained from triplicate cultures.

Mass spectra of carotenoids in crude extracts and as pure compounds were obtained with a Hewlett-Packard Series 1100LC-MSD liquid chromatograph–mass spectrometer fitted with a LiChroCART Superspher 100 RP-18 column (Merck; 4 μm particle size; 125 × 2 mm). The gradient was from 50% to 100% acetone in 15 min at a flow rate of 0.5 ml min^{-1}. The fragmentor voltage was set to 140 V in the APCI-positive and -negative ionisation modes. IR spectra were recorded in KBr using a Perkin Elmer One spectrometer, and UV/vis spectra in methanol and *n*-hexane using a Perkin-Elmer Lambda 20 spectrophotometer.

^{1}H and ^{13}C NMR spectra of pigments were recorded in $CDCl_3$ with a Bruker Avance 400 spectrometer at 400.13 and 100.61 MHz, respectively, equipped with a ^{1}H, ^{13}C high resolution magic angle spinning (HR-MAS) probe. Samples were dissolved in about 50 μl $CDCl_3$ and filled into a 4-mm ZrO_2 rotor which was spun at 4 kHz at 295 K. The following homonuclear and heteronuclear two-dimensional NMR techniques were utilized to aid in the assignment of ^{1}H and ^{13}C signals: gCOSY, gTOCSY, ROESY, gHSQC and gHMBC. Due to the paucity of material, ^{13}C resonances of pigments **5** and **7** could not be detected.

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