2-Hydroxytorularhodin, a New Xanthophyll from the Red Yeast *Sporobolomyces coprosmae*

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A new hydroxylated carotenoic acid was isolated from the red yeast *Sporobolomyces coprosmae* and unambiguously identified as 2-hydroxytorularhodin (**1**), *i.e.*, (*all-E*)-3′,4′-didehydro-2-hydroxy-β,ψ-caroten-16′-oic acid, by application of extensive 1D and 2D NMR techniques (gCOSY, gHSQC, gHMBC, DQS, gTOCSY, and ROESY). Hydroxylation of carotenoids at C(2) is uncommon in nature, very rare in fungi, and unprecedented for torularhodin.

1. Introduction. – Red yeasts are characterized by the intracellular accumulation of carotenoid pigments. Although taxonomically diverse in belonging to three different groups of Basidiomycota, red yeasts are thought to synthesize only a limited range of carotenoids. The most frequently reported biosynthetic route is that involving the oxidation of γ-carotene *via* torulene to give torularhodin as the most polar final product. Typical representatives of yeasts producing these carotenoids are species of *Sporobolomyces* and *Rhodotorula* which are ubiquitous in nature, especially in epiphytic (phylloplane) habitats [1–3]. In the course of our work on ecophysiological aspects of carotenoid production in phylloplane yeasts, we have recently isolated two strains producing unusual pigments which were more polar than torularhodin. Both strains were identified as *Sporobolomyces coprosmae* HAMAMOTO & NAKASE, a species not previously analyzed for its pigment profile. The present report describes the identification of a hitherto unknown 2-hydroxylated carotenoic acid, 2-hydroxytorularhodin (**1**), as one of these more polar torularhodin derivatives.

2. Results and Discussion. – Yeast strains CAR027 and CAR028 were isolated from living hazel (*Corylus avellana*) leaves collected near Kaiserslautern, Germany, and grew as pinkish-red colonies on a range of agar media. The ITS1–5.8S–ITS2 rDNA sequences of both strains comprised 620 nt including primers ITS5 and ITS4. They differed from each other at position 554 in the substitution of T (CAR027) by C (CAR028). FASTA searches in GenBank and EMBL showed only two close matches (GenBank AB030343 and AF444577), both deposited as *Sporobolomyces coprosmae* and, in fact, both obtained from the same strain (CBS7899). The ITS sequence homologies of CAR027 and CAR028 to these two sequences were 100% and 99.8%, respectively (*i.e.*, 0–1 nt difference). The ITS sequence was considered a crucial criterion for

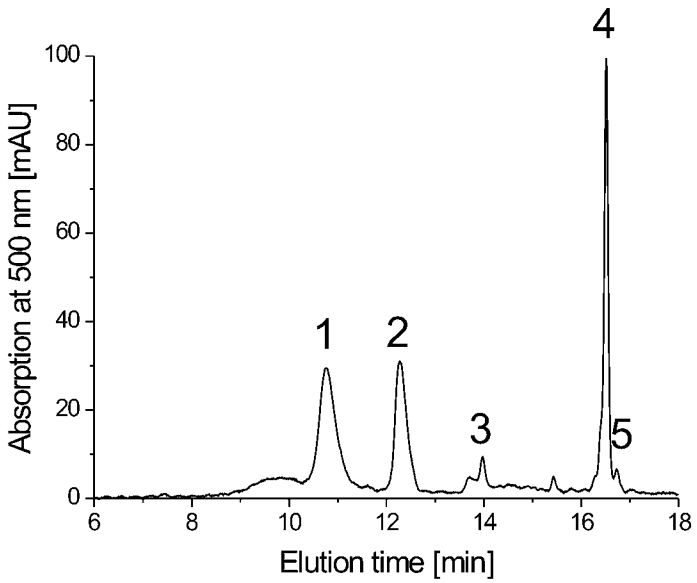

Fig. 1. *Reversed-phase HPLC trace of a carotenoid extract from* Sporobolomyces coprosmae *strain CAR028.* See *Fig. 2* for the structures of **1** and **3**–**5**; the structure of **2** is not yet established.

the delimitation of *S. coprosmae* against the most closely related species, *S. oryzicola*, by the depositors of AB030343 [4] and AF444577 [5]. In our sequence searches, both available sequences of *S. oryzicola* were 98.4–99.0% identical to those of CAR027 and CAR028, with the next closest sequences being considerably more diverse ($\leq$93.5% identity) and belonging to other *Sporobolomyces* spp. The identification of our strains as *S. coprosmae* extends the known habitat and distribution of this species which has so far been isolated only once, from a leaf of *Coprosma tenuifolia* in New Zealand [6].

Extracts of *S. coprosmae* CAR027 and CAR028 were analyzed by reversed-phase HPLC as described previously [3]. Both strains revealed an essentially identical and characteristic pigment profile consisting of at least five different carotenoids (**1**–**5**; see *Figs. 1* and *2*). Torulene (=3′,4′-didehydro-β,ψ-carotene; **4**; t_R 16.5 min) represented the main pigment, and torularhodin (=3′,4′-didehydro-β,ψ-caroten-16′-oic acid; **3**; t_R 14.0 min) was also present, along with a trace of γ-carotene (=β,ψ-carotene; **5**; t_R 16.7 min). These three pigments were identifiable on the basis of on-line DAD UV/VIS spectra (DAD = diode array detector), t_R and HPLC/APCI-MS data (APCI = atmospheric pressure chemical ionization) [3]. The bicyclic β-carotene (=β,β-carotene) could not be detected in either strain. However, two additional major peaks, **1** and **2**, eluting at a shorter retention time (t_R 10.8 and 12.3 min, resp.) than **3**, were also detected (*Fig. 1*). Their on-line spectra (λ_{max} 492 nm) suggested the presence of a tridecaene chromophore conjugated with a terminal carboxylic group, as for torularhodin (**3**).

For **1**, which was the more polar of these two pigments, HPLC/APCI-MS analysis indicated an M_r of 580 (*m/z* 580 and 581 in the negative and positive mode of APCI-MS, resp.), which would be compatible with the formula $C_{40}H_{52}O_3$. A difference of 16

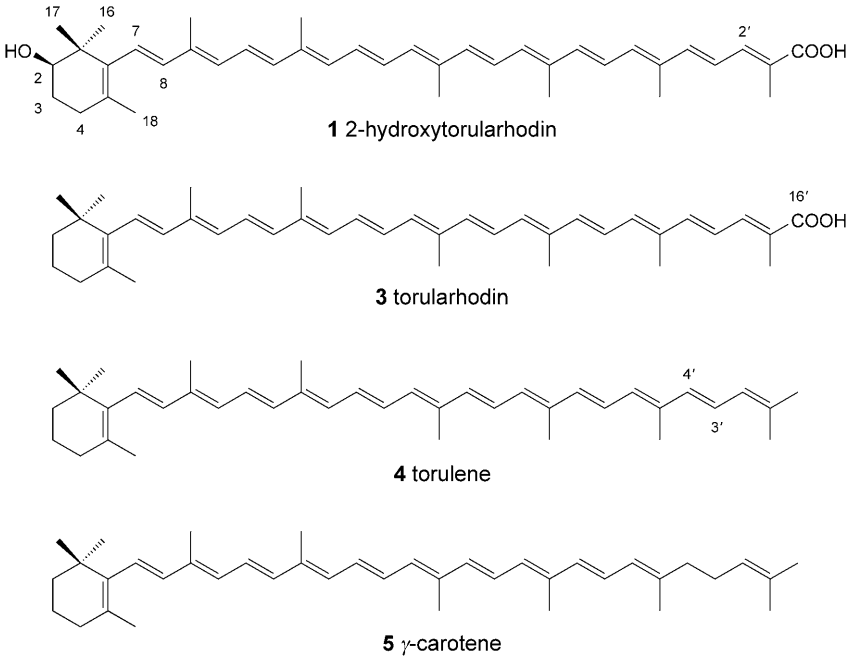

Fig. 2. *Structure of carotenoids* **1** *and* **3**–**5** *from* Sporobolomyces coprosmae *strain CAR028*

mass units with respect to torularhodin would suggest the presence of an OH group in the molecule, which is well supported by the fragment peak at *m/z* 563 in the APCI-MS (pos. mode), accounting for loss of H_2O.

Purification of extracts from strain CAR028 by means of column chromatography followed by reversed-phase prep. HPLC led to the isolation of pigment **1** as remarkably stable dark red crystals in sufficient quantity for extensive homonuclear and heteronuclear 1D and 2D NMR characterization (see *Table*). In particular, gCOSY, gHSQC, gHMBC, DQS, gTOCSY, and ROESY experiments were performed and allowed us to assign unambiguously to pigment **1** the structure of 3′,4′-didehydro-2-hydroxy-β,ψ-caroten-16′-oic acid, to which the semisystematic name 2-hydroxytorularhodin is given.

In the ^{1}H-NMR spectrum of **1**, most downfield, a characteristic *d* at δ 7.39 ($^3J = 11.5$ Hz) featured as a diagnostic signal in the olefinic region, whereas 17 protons resonated in the crowded δ 6.1–6.7 range instead. Application of the gTOCSY technique, which is known to yield correlations of all scalarly coupled protons within a given spin system (see, *e.g.*, *Bross-Walch et al.* [7]), was most useful and indicated the presence of six different spin systems (*Fig. 3*,*a*). By means of gCOSY, gHSQC, and gHMBC experiments, these olefinic protons could be identified as belonging to an unsymmetrical polyene chain of isoprenoid origin and were assigned accordingly, along with the corresponding C-atoms. In particular, the signal at δ(H) 7.39 (δ(C) 140.8) showed gHMBC correlation with a C-atom at δ(C) 170.9, and well corresponded to an olefinic proton deshielded by an adjacent carboxylic group, *i.e.*, to H–C(2′) in torularhodin. Moreover, this proton coupled with an H-atom resonating at δ(H) 6.51 (H–C(3′), *dd*) which, in turn, displayed coupling with a signal at δ(H) 6.67 (*d*, $^3J = 15.1$ Hz), thus confirming in pigment **1** the presence of the 3′,4′-didehydro-16′-oic ψ-end group which is also found in torularhodin (**3**). Likewise, the ^{1}H-NMR data for the remaining portion of the polyene chain closely matched those reported for **3** [8]. At the same time, the whole set of ^{13}C-NMR resonances for the polyolefinic system assigned by gHSQC

Table. *^{1}H- and ^{13}C-NMR Data ($CDCl_3$) of 2-Hydroxytorularhodin* (**1**) *at 400.13 and 100.61 MHz, respectively.* δ in ppm, J in Hz.

Position	δ(H)	δ(C)	Position	δ(H)	δ(C)
C(1)	–	39.3	C(1′)	–	124.3
H–C(2)	3.57 (*dd*, *J* = 3.3, 9.0)	75.7	H–C(2′)	7.39 (*d*, *J* = 11.5)	140.8
CH_2(3)	1.74 (*m*, H_{ax}), 1.84 (*m*, H_{eq})	26.3	H–C(3′)	6.51 (*dd*, *J* = 11.5, 15.1)	122.7
CH_2(4)	2.15, 2.18 (2*m*)	29.8	H–C(4′)	6.67 (*d*, *J* = 15.1)	145.0
C(5)	–	128.3	C(5′)	–	134.9
C(6)	–	136.3	H–C(6′)	6.37_4 (*d*, *J* = 11.5)	136.6
H–C(7)	6.13[a])	126.0	H–C(7′)	6.63 (*dd*, *J* = 11.5, 15.0)	124.7
H–C(8)	6.13[a])	138.4	H–C(8′)	6.48 (*d*, *J* = 15.0)	140.4
C(9)	–	135.7	C(9′)	–	134.9[b])
H–C(10)	6.16 (*d*, *J* = 11.5)	131.1	H–C(10′)	6.31 (*d*, *J* = 11.5)	133.9
H–C(11)	6.66 (*dd*, *J* = 11.5, 15.0)	124.9	H–C(11′)	6.64 (*dd*, *J* = 11.5, 15.0)	124.7
H–C(12)	6.37_0 (*d*, *J* = 15.0)	137.2	H–C(12′)	6.43 (*d*, *J* = 14.9)	138.9
C(13)	–	136.7[b])	C(13′)	–	135.7[b])
H–C(14)	6.27 (*d*, *J* = 11.2)	132.4	H–C(14′)	6.30 (*d*, *J* = 11.5)	133.7
H–C(15)	6.65 (*m*)	130.1	H–C(15′)	6.64 (*m*)	130.1
Me(16)	1.08 (*s*)	26.7	C(16′)	–	170.9
Me(17)	1.04 (*s*)	22.1	Me(17′)	2.01 (*s*)	12.6
Me(18)	1.72 (*s*)	21.2	Me(18′)	2.00 (*s*)	12.6
Me(19)	1.98 (*s*)	12.6	Me(19′)	2.00 (*s*)	12.6
Me(20)	1.98–1.99 (*s*)	12.6	Me(20′)	1.98–1.99 (*s*)	12.6

[a]) Strongly coupled. [b]) Tentatively assigned from long-range gHMBC correlations with Me groups, by analogy with the corresponding δ(C) of torularhodin ethyl ester reported by *Englert* [8].

and gHMBC was in excellent agreement with literature data for torularhodin ethyl ester in *Englert*'s compilation [8], and established unequivocally the identity of the chromophore in **1**. The configurations of the C=C bonds of the polyene chain in **1** were determined as (*all-E*) on the basis of the ^3J(H,H) coupling constants and were further corroborated by gCOSY and ROESY data. In particular, the ROESY experiment (*Fig. 3*,*b*) revealed all through-space interactions between protons on the same side of the olefinic chain that were to be expected if the geometry of all C=C bonds was *trans* (*E*). This technique, therefore, demonstrated its utility in the carotenoid field where the conventional NOESY approach fails [8][9].

In the aliphatic region of the ^{1}H-NMR spectrum of **1**, a *dd* at δ(H) 3.57 (^3J = 3.3 and 9.0 Hz) showing gHMBC correlation with a C-atom at δ(C) 75.7 was diagnostic for the presence of a CH proton geminal to an OH group and coupled vicinally to two nonequivalent CH_2 protons of a cyclohexene ring (δ(H) 1.84 and 1.74) which, in turn, correlated with a signal at δ(C) 26.3 in the gHMBC experiment. The magnitude of these ^3J values would be compatible with an axial,equatorial and an axial,axial coupling, respectively, and suggested that the OH group was equatorial. This was indeed confirmed by ROEs between δ(H) 3.57 and the CH_2 *m* at δ(H) 1.84 and 1.74 (*Fig. 3*,*b*), a stronger ROE being observed with δ(H) 1.84, as expected for adjacent protons in a *cis* relationship within a cyclohexene ring. In contrast, only weaker ROEs with the remaining methylene protons at δ(H) 2.15 and 2.18 were detected.

The magnetic nonequivalence of the two geminal Me–C(1) (δ(H) 1.04 and 1.08; δ(C) 22.1 and 26.7) suggested that the asymmetric centre should be located at an adjacent position, *e.g.*, at C(2), which was in perfect accordance with literature data for caroten-2-ols [8] [10]. Diagnostic ROEs between H–C(2) (δ(H) 3.57) and the two Me–C(1) (δ(H) 1.04 and 1.08; *Fig. 3*,*b*), featuring a stronger intensity for the latter signal, confirmed the localization of the OH function at position C(2) with equatorial orientation.

The aliphatic region in the ^{1}H-NMR spectrum also included signals belonging to Me protons (δ(H) 1.98–2.01) at olefinic C-atoms and displaying gHMBC correlations with C-atoms at δ(C) 12.6, which would account for Me groups attached to a polyene chain of isoprenoid nature. More importantly, for a *s* at δ(H) 1.72 (δ(C) 21.2), gTOCSY (*Fig. 3*,*a*) showed long-range correlations with methylene protons at δ(H) 1.74 and 1.84 and δ(H) 2.15 and 2.18 (CH_2(3) and CH_2(4), resp.), with H–C(2) at δ(H) 3.57, and with the two strongly coupled olefinic H–C(7) and H–C(8) at δ(H) 6.13; these correlations corresponded well to the Me(18) group of a 2-sub-

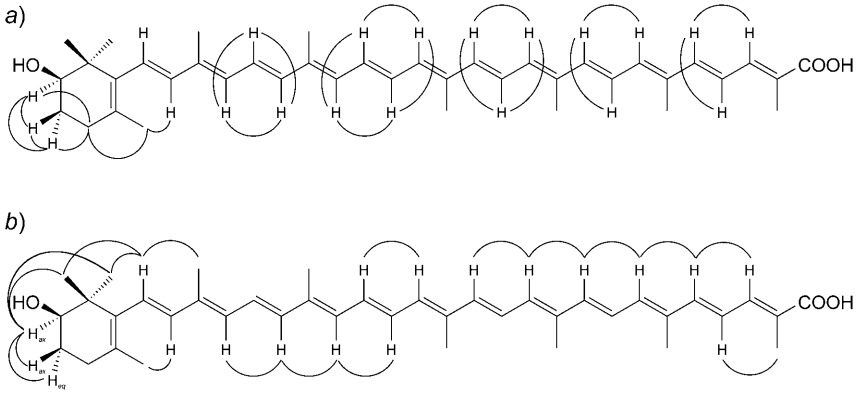

Fig. 3. a) *Key gTOCSY correlations and* b) *selected ROESY correlations in 2-hydroxytorularhodin* (**1**). ROE cross-peaks between in-chain Me groups (δ 1.98–2.00) and olefinic H-atoms resonating in the δ 6.51–6.66 range (*i.e.*, H–C(11), H–C(15), H–C(15′), H–C(11′), H–C(7′), and H–C(3′)) were also observed. However, since these ROEs could not been assigned individually, they are omitted in *Fig. 3,b*, also for the sake of clarity.

stituted β-end group. Comparison of the δ(C) values for the C(2) to C(4) atoms assigned by gHMBC with those compiled by *Englert* [8] safely established the 2-hydroxy-β-ionone cyclic nature of the second end group and secured unequivocally for pigment **1** the structure of 2-hydroxytorularhodin (*Fig. 2*).

2-Hydroxytorularhodin (**1**) bears a stereogenic centre at C(2). The CD spectrum of **1** displayed positive maxima at 246, 317, and 382 nm, and negative maxima at 224, 276, and 355 nm, thus indicating that the pigment occurs in nature in optically active form. In the absence of configurationally known reference compounds sharing the same chromophore or bearing close structure similarities to **1**, any determination of the absolute configuration at C(2) by comparison to CD data available in the literature must remain questionable. Nonetheless, on the assumption that general carotenoid CD rules [11] should hold also for **1**, which displayed a conservative CD spectrum, it is tempting to compare it with hydroxylated monocyclic carotenoids having a somewhat related chromophore, *e.g.*, (2*R*)-deinoxanthin [12] or (3*S*)-flexixanthin [12][13]. Such an approach would suggest that **1** has the (2*R*) absolute configuration. Similarly, whether **1** occurs in the optically pure form or partially racemized, as is the case with some caroten-2-ols (see below), remains a topic for future investigations. A rigorous CD, chemical correlations, or even a total synthesis may have to be carried out before a safe and unambiguous configurational assignment can be issued.

Carotenoids with the 2-hydroxy-substituted β-end group (caroten-2-ols) are rare in nature when compared to common 3-hydroxy-substituted xanthophylls such as zeaxanthin and the like [14][15]. β,β-Caroten-2-ol was first isolated from the green alga *Trentepohlia iolithus*, along with β,ε-caroten-2-ol and β,β-caroten-2,2′-diol [10] and related epoxides [16]. Their absolute configuration was established as (2*R*) (and (2′*R*)) [17] which were later confirmed by total synthesis [18]. The occurrence of 2-hydroxy-substituted β-type carotenoids has also been reported in insects [19] and crustaceans [20], where they are partially racemized [19a,b] [20b]. More recently, deinoxanthin (=3′,4′-didehydro-1′,2′-dihydro-2,1′-dihydroxy-β,ψ-caroten-4-one), a new 2-hydroxy-

lated 4-keto monocyclic carotenoid, has been characterized from the radioresistant bacterium *Deinococcus radiodurans* [12][21], and its absolute configuration has been assigned as (2*R*) on the basis of comparative CD data [12]. Among pigmented yeasts, hydroxylation at C(2) has been reported only for 2-hydroxyplectaniaxanthin (=3′,4′-didehydro-1′,2′-dihydro-β,ψ-carotene-2,1′,2′-triol) produced by *Rhodotorula aurantiaca* [22], but its absolute configuration at C(2) has remained undetermined.

Only one derivative of torularhodin has been reported in the literature, *viz.* torularhodin methyl ester, which most probably was an extraction artifact [15]. Therefore, 2-hydroxytorularhodin (**1**) bears a substitution pattern which is unprecedented for torularhodin and generally rare among yeasts. It also represents the first substituted torularhodin isolated from a natural source.

From a biosynthetic viewpoint, the exact mechanism for the introduction of the OH function at C(2) in carotenoids bearing a β-end group has not yet been clarified. Although a direct cyclization promoted by HO^+ cannot be ruled out *a priori* [14], hydroxylation at C(2) after cyclization is considered more likely on general grounds, even though support from critical biochemical or genetic studies is lacking [23]. In this respect, 2-hydroxytorularhodin (**1**) and its putative derivative **2** (*Fig. 1*) from our *S. coprosmae* strains may be useful objects for future biochemical studies to shed light on the formation of 2-hydroxy-substituted β-end-group carotenoids. The purification and identification of the additional pigment **2** from *S. coprosmae* strains CAR027 and CAR028 are currently being pursued in our laboratories.

We are grateful to Mrs. *Anja Meffert*, Lehrbereich Biotechnologie, Technische Universität Kaiserslautern, for HPLC/APCI-MS analysis of *S. coprosmae* extracts and for recording the mass spectra of 2-hydroxytorularhodin (**1**). Thanks are due to Prof. *Arrigo Forni*, Dipartimento di Chimica, Università di Modena, for kindly measuring the CD spectrum of **1**. We also thank *Fondazione Cassa di Risparmio di Modena* for funding the purchase of the 400-MHz-NMR spectrometer.

Experimental Part

General. HPLC carotenoid analyses: *Hewlett-Packard 1090-II* HPLC system, equipped with a photodiode-array detector (DAD) and fitted with a *LiChrospher 100 RP-18* column (5 μm particle size; 250×4 mm; *Merck*, Darmstadt, Germany); 70 → 100% (*v*/*v*) acetone/H_2O in 15 min at a flow rate of 1 ml/min, as described in [3]. UV/VIS Spectra: *Perkin-Elmer Lambda-16* spectrophotometer; λ_{max} in nm (log ε). CD Spectra: EPA (Et_2O/isopentane/EtOH 5:5:2) soln. at r.t.; *Jasco J-710* spectropolarimeter (rectangular cuvette, optical pathlength 0.1 cm); λ in nm ($\Delta\varepsilon$ in M^{-1} cm^{-1}). IR Spectra: in KBr; *Bruker IFS-48* FT-IR spectrometer; $\tilde{\nu}$ in cm^{-1}. ^1H- and ^{13}C-NMR Spectra: $CDCl_3$ solns., *Bruker Avance-400* spectrometer at 400.13 and 100.61 MHz, resp.; chemical shifts δ in ppm downfield from $SiMe_4$ as reference, coupling constants *J* in Hz; owing to the small amount of sample available, the δ(C) were acquired by the inverse-detection mode with a spectral resolution of ±0.5 ppm, in particular, by a gradient-enhanced HSQC experiment (gHSQC) in case of CH resonances. HPLC/APCI-MS: *Hewlett-Packard 1100LC-MSD* instrument fitted with a *LiChroCART Superspher-100 RP-18* column (4 μm particle size; 125×2 mm; *Merck*), similar acetone/H_2O gradient as for HPLC; atmospheric-pressure chemical ionization (APCI) MS in the positive and negative mode (see [1]); in *m/z* (rel. intensity in %).

Material and Taxonomy. Strains CAR027 and CAR028 were isolated from fresh leaves of *Corylus avellana* collected on the University of Kaiserslautern Campus on 16 Sept. 2002. Leaves were suspended in sterile H_2O, and the leaf washings were incubated on 2% malt agar from which colored colonies were isolated after 7 d at r.t. Both strains are maintained in the Culture Collection, Department of Biotechnology, University of Kaiserslautern. Yeasts were identified by means of their complete ITS1–5.8S rDNA–ITS2 nuclear ribosomal DNA sequences which were obtained as described previously [24].

Culture Conditions. Fermentations were carried out in a 20-l fermentor (*C6*; *Biolafitte*) containing 15 l of YES medium (sucrose (20 g), yeast extract (4 g), KH_2PO_4 (1 g), and $MgSO_4 \cdot 7H_2O$ (0.5 g)/l tap water) at high aeration (6 l air/min, 250 r.p.m.) at 24°. Cells were harvested by centrifugation after 6 d cultivation.

Extraction and Purification. A crude extract was prepared from cells of *S. coprosmae* strain CAR028 by the standard DMSO/acetone extraction method for yeast carotenoids [3]. Colorless lipids were precipitated by dissolving the crude extract in 20 vol. of acetone and incubating for 3 h at −80°, followed by removal of the colored clear supernatant with a pipette. This step was repeated twice, and the extract thus obtained was separated by column chromatography (silica gel, eluting with pure cyclohexane, then cyclohexane/AcOEt 9:1 → 7:3 → 1:1, and finally pure AcOEt): carotenoids, *i.e.*, γ-carotene (**5**) and torulene (**4**), and pigment **1** (eluted with pure AcOEt). Reversed-phase prep. HPLC (*RP-18*, 50 → 100% H_2O/acetone in 30 min) afforded an enriched fraction which was finally purified by means of prep. TLC (*SIL G/UV*$_{254}$, 0.2 mm thickness (*Merck*), toluene/acetone/AcOH 70:30:1, then partition between H_2O/light petroleum ether): pure **1**.

2-Hydroxytorularhodin (=(*all*-E)-*3′,4′-Didehydro-2-hydroxy-β,ψ-caroten-16′-oic Acid*; **1**). Dark red crystals. UV/VIS (MeOH): 463 (sh), 489 (5.066), 520 (4.991). UV/VIS (hexane): 466, 492, 524. CD (c = 0.3 mg/ml EPA): 224 (−1.4), 246 (+0.33), 276 (−0.17), 317 (+0.67), 355 (−0.05), 382 (+0.16). IR (KBr): 3427*m* br., 2925*s*, 2854*m*, 1735*m*, 1653*w*, 1457*m*, 1377*w*, 1120*m* – *w*, 967*m* – *w*. ^{1}H- and ^{13}C-NMR ($CDCl_3$): *Table*. HPLC/APCI-MS (pos.): 581 (100, $[M+1]^+$), 563 (70, $[M-18]^+$), 501 (7, $[M-80]^+$), 489 (7, $[M-92]^+$), 475 (9, $[M-106]^+$), 457 (8). HPLC/APCI-MS (neg.): 579 (100, $[M-1]^-$), 473 (7, $[M-106]^-$).

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