Splitting of β-carotene in the sexual interaction of *Phycomyces*†

Silvia Polaino,[a] M. Mar Herrador,[b] Enrique Cerdá-Olmedo[a] and Alejandro F. Barrero*[b]

Received 25th June 2010, Accepted 23rd July 2010
DOI: 10.1039/c0ob00321b

Two new 7-carbon compounds, 1 and 2, have been found in the culture medium of *Phycomyces blakesleeanus*. A genetic test showed that they derive from β-carotene. These new molecules represent the missing link that proves that β-carotene is split into fragments of 18, 15 and 7 carbon fragments, each head of a separate family of apocarotenoids.

Phycomyces blakesleeanus is a filamentous, saprophytic fungus (former phylum Zygomycota, order Mucorales; now the subphylum Mucoromycotina) used in many studies of physiology, environmental sensing, genetics and metabolism.[1] The yellow color in the hyphae is due to all-*trans* β-carotene (**3** in Fig. 1) with other isomers and biosynthetic precursors present in small amounts. *Phycomyces* strains belong to either the (+) or the (−) sex, which are not distinguished by their morphology, but by their interaction: when hyphae of opposite sex come in the vicinity of each other, their color is enhanced by increased carotene synthesis, and their tips thicken as they develop into zygophores, the first structures of the sexual cycle.

The sexual interaction of *Phycomyces* and other Mucorales was the first effect attributed to diffusible hormones in any organism, early in the previous century.[2] The signals are derivatives of β-carotene, because mutants devoid of β-carotene do not stimulate their wild type partners, but are stimulated by them.[3] This action is attributed to a family of 18-carbon apocarotenoids, the trisporoids, which include trisporic acids, found in mixed cultures of strains of opposite sex ("mated cultures") of various Mucorales.[4] A related family of 15-carbon compounds, found in the same circumstances, are usually called apotrisporoids, because they were thought to derive from trisporoids by removing a 3-carbon fragment.[5] Although *P. blakesleeanus* is the species of Mucorales most often used in research, only trisporic acid E, **4**, and apotrisporin E, **5**, have been reported from its cultures.[4e,6] Several synthetic trisporoids enhance carotenogenesis when added to single cultures of *Phycomyces*.[7]

Here we report two new 7-carbon natural compounds, **1** and **2**, and show that β-carotene is split into three fragments of 18, 15, and 7 carbon atoms, each head of a separate family of apocarotenoids.

Two novel 7-carbon compounds **1** and **2** (Fig. 1) were found in single and mated cultures of three *Phycomyces* strains, the standard (−) strain, NRRL1555, the (+) strain, NRRL1554, and A56, a (+) strain largely isogenic with the first. For the purification and structural characterization of the new compounds, the culture medium was extracted and the acid extract was methylated with $TMSCHN_2$ and fractionated by semipreparative normal-phase HPLC. Thus, we obtained a mixture of **1m** and **2m**, the methyl esters of **1** and **2**, present in a ratio of about 2 : 1.

The structures of **1**, **2**, **1m** and **2m** were established by GC-MS, HRMS, ^{1}H and ^{13}C NMR analyses. The mixture of **1m** and **2m** was resolved by GC-MS. Both present a molecular formula $C_8H_{12}O_3$ deduced from its HRFABMS. Their λ_{max} at 252 nm corresponds to an α,β,γ,δ,-diunsaturated chromophore ester.[8] The ^{13}C NMR clearly shows two groups of eight signals, one group for each isomer, easily assigned according to their size (Table 1). For the major component **1m** there are signals for a methyl ester, a primary alcohol and a methyl group, and four signals for disubstituted and trisubstituted double bonds. The 2-methyl-2,4-hexadiene skeleton is established by the direct coupling of the three olefinic protons in the ^{1}H NMR spectrum. The *E* configuration of the disubstituted double bond is evident by its coupling constant (15.2 Hz) and that of the trisubstituted one by the chemical shift at C-6 and C-7 in the ^{13}C NMR spectrum.[9] The chemical shift at 7.59 ppm indicates that the proton occupies a β position with respect to the carbonyl of the ester group. Because of this, and the lack of direct coupling between the primary carbinol proton and the other protons, the structure of the major component **1m** is methyl (2*E*,4*E*)-6-hydroxy-5-methyl-2,4-dienoate.

The structure was confirmed by a 1D TOCSY experiment in the ^{1}H NMR spectrum. Thus, when the double doublet at 7.59 ppm (H-3) was irradiated selectively, there was a strong response of the directly coupled protons H-2 and H-4, and a weaker one of the homoallylic coupled protons H-6 and H-7. The structure and the stereochemistry of the minor compound **2m** was established alike, taking into account the remaining signals in the NMR spectra.

A mixture of the hydroxyacids **1** and **2** was obtained by saponification of the mixture of their methyl esters with 1 N NaOH in EtOH. The spectral data assigned to the new natural products **1** and **2** were concordant with their proposed structures (Table 1).

The structure of **1** was unequivocally established by chemical synthesis (Scheme 1). The starting material was **6** (3-methylbut-2-en-1-ol), a commercial prenol. Protection of its primary hydroxyl and allylic oxidation gave rise to a mixture of **7** and **8**; the yield of **7** was increased by soft reduction of the carbonyl group of **8**. Acetylation of **7** and removal of the silyl protection led to **9**. Oxidation to aldehyde and Wadsworth–Emmons olefination produced stereoselectively the *E* stereoisomer **10**. A final saponification led to our target, **1**, whose NMR data coincided with those assigned to the natural product.

The 2-methylhexa-2,4-diene framework of the new compounds **1** and **2** coincides exactly with the 7-carbon fragment expected if β-carotene is cut at its 13,14 and 11′,12′ double bonds (Fig. 1). This observation suggested that compounds **1** and **2** actually derive from β-carotene. For confirmation of this hypothesis the use of isotopic labelled precursors is not appropriate because it has been

[a]*Department of Genetics, Faculty of Biology, University of Sevilla, Reina Mercedes, s/n, 41071 Sevilla, Spain. E-mail: eco@us.es; Tel: +34 954624107*
[b]*Department of Organic Chemistry, Institute of Biotechnology, University of Granada, Avda. Fuente Nueva, s/n, 18071 Granada, Spain. E-mail: afbarre@ugr.es; Fax: +34 958243318; Tel: +34 958243318*

† Electronic supplementary information (ESI) available: Experimental procedures and NMR spectra of new compounds. See DOI: 10.1039/c0ob00321b

Table 1 ^{1}H and ^{13}C NMR data of the compounds **1**, **2**, **1m** and **2m**. δ in ppm

	1		**2**		**1m**		**2m**	
C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	—	167.1	—	168.1	—	167.9	—	169.0
2	5.86	119.9	—	126.0	5.87	120.4	—	127.3
3	7.59	140.3	7.23	141.7	7.59	140.3	7.18	139.7
4	6.34	120.5	6.69	137.8	6.25	121.6	6.58	137.6
5	—	148.7	6.25	125.2	—	147.5	6.17	125.6
6	4.09	66.1	4.24	61.6	4.14	67.5	4.29	63.1
7	1.86	13.6	1.92	11.7	1.87	14.6	1.95	12.7
OMe	—	—	—	—	3.74	51.6	3.75	51.9

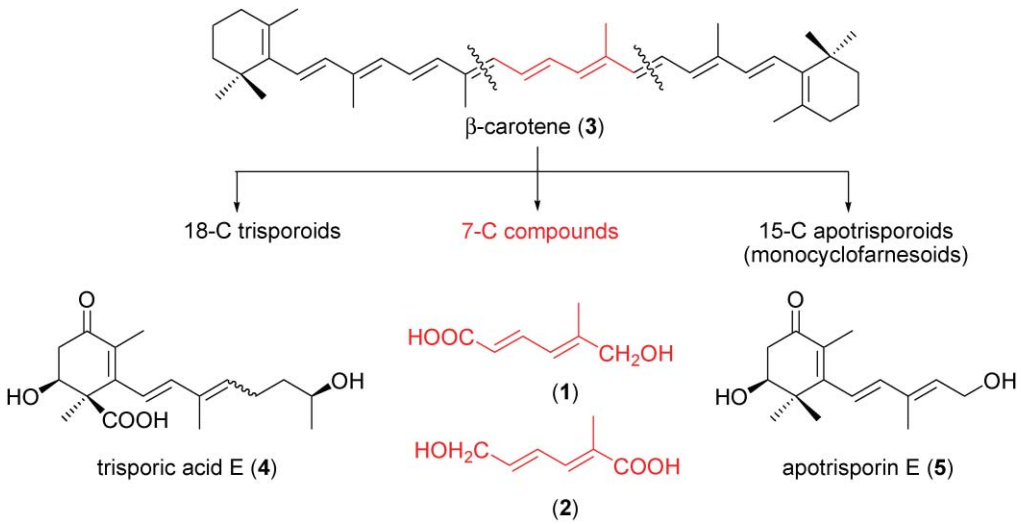
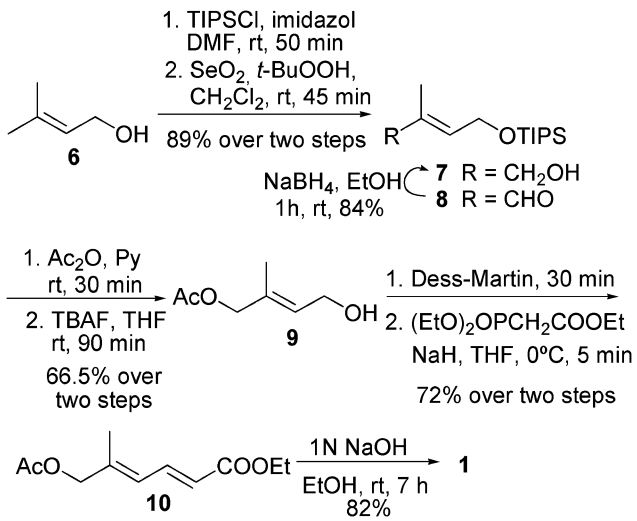


Fig. 1 The three families of apocarotenoids in *Phycomyces*. According to our results, β-carotene, **3**, must be split at the points indicated by the wavy lines to produce compounds with 18 carbons (left), 7 carbons (center), and 15 carbons (right). The examples given for each group are two compounds previously reported from *Phycomyces* under the names of trisporic acid E, **4**, and apotrisporin E, **5**, and the new compounds reported here, (2*E*,4*E*)-6-hydroxy-5-methylhexa-2,4-dienoic acid, **1**, and its 2-methyl isomer, **2**.



Scheme 1 Synthesis of the new natural compound **1**.

reported that the dilution of the label from mevalonate leads to uncertain results.[10] Thus, we have proven this by a genetic approach that allows recognition of the apocarotenoids among all other organic compounds present in the culture medium of *Phycomyces*.

We compared the presence or absence of the C7 compounds in wild type strains, which produce β-carotene, and white mutant strains totally devoid of lycopene, β-carotene and other colored carotenes. The *carB* mutants are genetically defective in phytoene dehydrogenase, which carries out the four dehydrogenations that convert phytoene to lycopene, but conserve all the other enzymes.[11] When a *carB* mutant strain meets a wild type of opposite sex, only the mutant responds sexually (Fig. 2), as expected from a strain that, lacking β-carotene, cannot produce sexual hormones to stimulate its partner. As mutants of opposite sex we chose strains C5 and S342 because both carry the same mutation, *carB10*, thus precluding even the possibility of functional metabolic complementation in mated cultures. Compounds **1** and **2** were completely absent from the single and the mated cultures of the *carB* mutants according the ^{1}H NMR spectra of acid extracts of the culture medium.

The new compounds **1** and **2**, as well as the trisporoids and apotrisporoids, were much more abundant in mated cultures than in single cultures. From their ^{1}H NMR data we estimated the 7C **1** and **2** total respective concentrations of about 40 mg L^{-1} and about 10 mg L^{-1}. These values agree with those obtained by purification and the difference is due to the stimulation of the enzymatic splitting of β-carotene as a consequence of the sexual interaction between (+) and (−) strains.

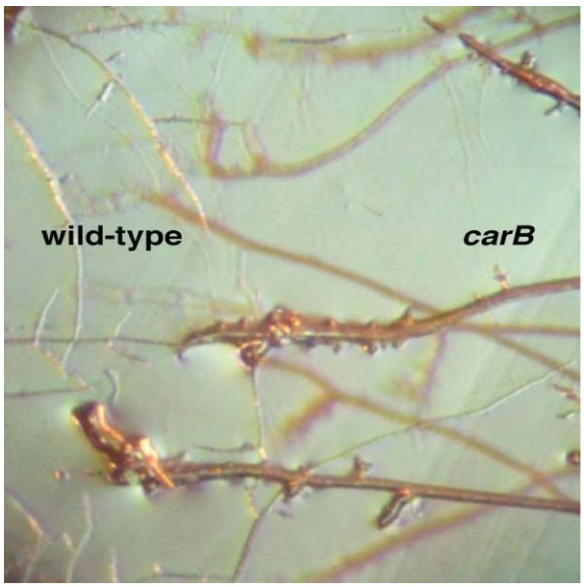


Fig. 2 Sexual interactions in *Phycomyces* require β-carotene. Mycelia of the wild type A56 and the *carB* mutant strain C5, unable to produce β-carotene, grow towards each other on glutamate agar. Thick hyphae, called zygophores, develop in the mutant, but not in the wild type (photograph by D. Pérez de Camino).

No physiological effects of the new natural compounds on the sexual interaction have been detected. The synthetic compound **1** and the mixture of **1** and **2**, purified from acid extracts of mated cultures, did not increase the carotene concentration nor modify the hyphal morphology of single and mated wild type cultures. Concentrations of up to 15 mg L^{-1} and spot amounts of 25 μg were tested.[6,11b] The same amounts of dimethyl phthalate, used as a control, strongly accentuated the yellow color of the mycelia.

Additionally, the culture media where **1** and **2** were found contained many other compounds of interest, including the previously known trisporic acid E, **4**, and apotrisporin E, **5**, identified by comparison with authentic samples.

The presence of the new 7-carbon compounds **1** and **2** together with the known **4** and **5** provides a new insight into the degradation of β-carotene and forces a reconsideration of apocarotenoid biosynthesis in *Phycomyces*. The two cuts in Fig. 1 leave three fragments, easily recognized as the heads of three families of apocarotenoids. This class of double β-carotene degradation is unknown, and only in another Mucoral, *Blakeslea trispora*, has been postulated the existence of a C13–C14 degradation.[12] We assume that initially the 18-carbon fragment has a ketone end and the other fragments have aldehyde ends, because this is the way of action expected from carotene oxygenases from various organisms[13] on β-carotene. Variations of the 18-carbon fragment that includes one of the β rings are trisporic acid E, **4**, from *Phycomyces* and other 18-carbon compounds from other Mucorales, collectively called trisporoids. Variations of the 15-carbon fragment that includes the other β ring are apotrisporin E, **5**, from *Phycomyces* and other 15-carbon compounds that can no longer be called apotrisporoids, because they are not derived from trisporoids. Their carbon skeleton is that of the monocyclofarnesoids. The central fragment is modified to produce compounds **1** and **2** with the 2-methylhexa-2,4-diene skeleton.

The chemical comparison of wild types with mutants impaired in carotene synthesis can be generalized to identify apocarotenoids in *Phycomyces* and other organisms. The comparative analysis of extracts from wild types and white mutants is leading us to a systematic study of over twenty apocarotenoids present in the cultures of *Phycomyces* and other Mucorales. Preliminary observations indicate that apocarotenoid production depends on genetic background, down to the strain level. Thus, generalizations for species, genera and families of the Mucorales are dangerous. Nevertheless, detection of **1** and **2** in cultures of *Blakeslea trispora* indicates that our scheme for splitting β-carotene into apocarotenoids is not unique to *Phycomyces*.

This research was partially supported by Junta de Andalucía (Grants CVI 910 and P08-CVI-03901), and the Spanish Government (Grants CTQ-2006-15575-C01 and C02).

Notes and references

1 (*a*) *Phycomyces*, ed. E. Cerdá-Olmedo and E. D. Lipson, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA, 1987; (*b*) E. Cerdá-Olmedo, *FEMS Microbiol. Rev.*, 2001, **25**, 503.
2 H. Burgeff, *Bot. Abh.*, 1924, **4**, 1.
3 R. P. Sutter, *Proc. Natl. Acad. Sci. U. S. A.*, 1975, **72**, 127.
4 (*a*) D. J. Austin, J. D. Bu'Lock and D. Drake, *Experientia*, 1970, **26**, 348; (*b*) L. Caglioti, G. Cainelli, B. Camerino, R. Mondelli, A. Prieto, A. Quilico, T. Salvatori and A. Selva, *Tetrahedron*, 1966, **22**, 175; (*c*) H. van den Ende, *Nature*, 1967, **215**, 211; (*d*) D. J. Austin, J. D. Bu'Lock and G. W. Gooday, *Nature*, 1969, **223**, 1178; (*e*) M. L. Miller and R. P. Sutter, *J. Biol. Chem.*, 1984, **259**, 6420; (*f*) D. Schachtschabel and W. Boland, *J. Org. Chem.*, 2007, **72**, 1366; (*g*) C. Schimek and J. Wöstemeyer, *Phytochemistry*, 2009, **70**, 1867.
5 (*a*) R. P. Sutter and J. P. Whitaker, *Naturwissenschaften*, 1981, **68**, 147; (*b*) R. P. Sutter and P. D. Zawodny, *Experimental Mycology*, 1984, **8**, 89.
6 R. P. Sutter, *Experimental Mycology*, 1986, **10**, 256.
7 N. S. Govind and E. Cerdá-Olmedo, *J. Gen. Microbiol.*, 1986, **132**, 2775.
8 E. Pretsch, T. Clerc, J. Seibl and W. Simon, in *Tabellen zur Strukturaufklärung organischer Verbindungen mit spektroskopischen Methoden*. Springer-Verlag, Berlin, Germany, 1990 (Spanish enlarged edition 1998).
9 F. W. Wehrli and T. Nishida, in *Progress in the Chemistry of Organic Natural Products*. Springer-Verlag, Wien, Austria, 1979.
10 E. R. Bejarano and E. Cerdá-Olmedo, *FEBS Lett.*, 1992, **306**, 209.
11 (*a*) A. P. Eslava and E. Cerdá-Olmedo, *Plant Sci. Lett.*, 1974, **2**, 9; (*b*) C. M. G. Aragón, F. J. Murillo, M. D. de la Guardia and E. Cerdá-Olmedo, *Eur. J. Biochem.*, 1976, **63**, 71.
12 A. Burmester, M. Richter, K. Schulte, K. Voelz, D. Schachtschabel, W. Boland, J. Wöstemeyer and C. Schimek, *Phytochemistry*, 2007, **44**, 1096.
13 D. P. Kloer and G. E. Schulz, *Cell. Mol. Life Sci.*, 2006, **63**, 2291.