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**PAPER**

# Apocarotenoids in the sexual interaction of *Phycomyces blakesleeanus*†

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A simple genetic test allowed us to carry out the first systematic study of the apocarotenoids in the Mucorales. We have identified 13 apocarotenoids in the culture media of the fungus *Phycomyces blakesleeanus* (Mucoromycota, Mucorales). Three of these compounds were novel apocarotenoids: (2*S*,8*R*,*E*)-8,14-epoxycyclofarnesa-4,6,9-triene-2,11-diol (**6**), (2*S*,6*E*,8*E*)-cyclofarnesa-4,6,8-triene-2,10,11-triol (**7**), and its 6*Z* isomer (**8**). Four of the remaining compounds have been reported previously from this fungus and six from other Mucorales. All of them belong to three families, the 18-carbon trisporoids, the 15-carbon cyclofarnesoids, and the 7-carbon methylhexanoids, derived from the three fragments that result when β-carotene is cleaved at its 11′,12′ and 12,13 double bonds. The apocarotenoids were more varied and more abundant in mated cultures of strains of opposite sex than in single cultures. The presence of acetate in the medium blocked the production of many apocarotenoids while having little effect on the concentrations of the remaining ones.

## Introduction

Natural isolates of *Phycomyces blakesleeanus* and other fungi of the order Mucorales belong to either the (+) or the (−) sex,[1] undistinguishable by their morphology, but defined by their interaction. When mycelia of opposite sex grow near each other, their hyphal tips thicken and develop into zygophores, the first structures of the sexual cycle that eventually produces recombinant progeny. Zygophores are induced by an exchange of sex-specific diffusible signals, the first pheromones reported for any organism.[2] The mycelia of *Phycomyces* and other Mucorales contain yellow β-carotene (**1**), whose concentration increases during sexual interaction. β-Carotene is not essential for vegetative life, as shown by the isolation of mutants that cannot synthesize it, such as the white *carB* mutants.[3] These mutants fail to stimulate their mating partner,[4] because the sexual pheromones are apocarotenoids, *i.e.*, they derive from fragments of β-carotene.

Following the isolation[5] of trisporic acid C from mixed cultures of *Blakeslea trispora* of different sex ("mated cultures") and the demonstration that it derives from β-carotene,[6] various apocarotenoids have been reported from *B. trispora*, *Mucor mucedo*, and other Mucorales,[7] some of which increase the carotene content or induce zygophore formation in test strains. These compounds fall into three families, the 18-carbon trisporoids, the 15-carbon cyclofarnesoids, and the 7-carbon methylhexanoids. The three families derived from the three fragments[8] that result from cleavage of the 11′,12′ and 12,13 double bonds of β-carotene by two carotene oxygenases, the products of *Phycomyces* genes *carS* and *acaA*, respectively.[9]

Although *P. blakesleeanus* is the best known of the Mucorales,[10] only four apocarotenoids (**2**–**5** in Fig. 1) have been reported from its culture media: trisporic acid E (**2**),[11] apotrisporine E (**3**),[12] and two isomeric hydroxymethylhexanedienoic acids (**4**, **5**).[8a]

We have analyzed the culture media of cultures of *P. blakesleeanus* wild-type strains under various conditions and have compared them with those of *carB* mutants unable to produce β-carotene, and thus devoid of apocarotenoids.

## Results

### Extraction, isolation and identification of apocarotenoids

The three wild type strains used by us differ by sex and genetic background. NRRL1554 and A56 are (+) strains, while NRRL1555 is (−); the NRRL strains were isolated from nature, while A56 is the product of repeated backcrosses that made its genetic background similar to that of NRRL1555.[13] The culture media of single and mated cultures differed in their ultraviolet

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†Electronic supplementary information (ESI) available: UV and $^{1}$H NMR spectra of all compounds, $^{13}$C NMR spectra of compounds **4**–**8**, **10**, **4a**, **5a**, **9a**, and two-dimensional NMR of **6**, and HPLC chromatograms. See DOI: 10.1039/c2ob07147a

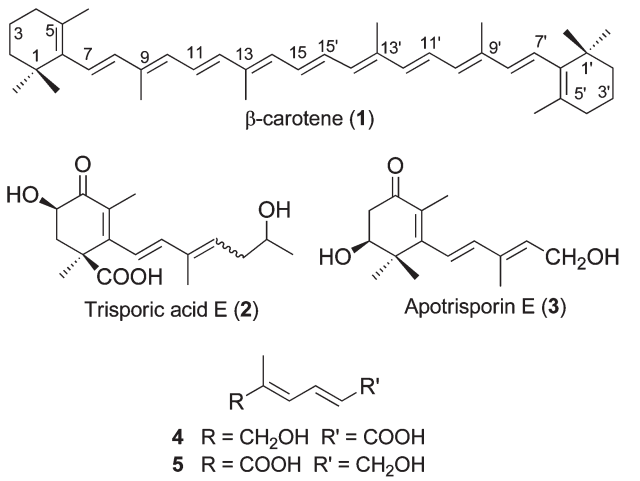



**Fig. 1** β-Carotene and the four apocarotenoids reported from cultures of *Phycomyces blakesleeanus*.

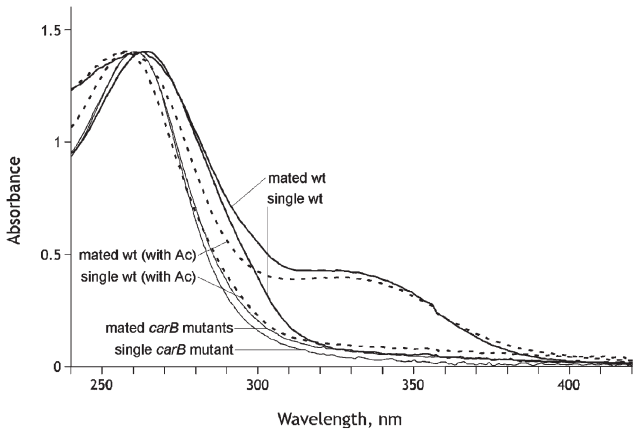



**Fig. 2** Absorption spectra of culture media of mated and single cultures of wild type strains (A56 and NRRL1555) and *carB* mutants unable to produce β-carotene (C5 and S342), all grown on minimal agar for five days. Thick lines, wild types; thin lines, mutants; dotted lines, wild types with sodium acetate added (10 mmol $L^{-1}$).

absorption spectra (Fig. 2). The increased absorption of ultraviolet in mated cultures was due to apocarotenoids, since it did not occur in the *carB* mutants. The relative ultraviolet absorption at 328 and 260 nm provides a simple estimate of the apocarotenoid content. The numerical analysis (Fig. S1 in the ESI†) found no significant differences between minimal and enriched agar cultures and between the two pairs of wild type strains (NRRL1554 × NRRL1555 and A56 × NRRL1555).

The culture media were extracted giving a neutral extract and an acid extract. The compounds in these extracts were separated by HPLC and detected by diodo-array (Table 1, and Fig. S2–S4 in the ESI†). All the compounds detected in the wild types were assumed to be apocarotenoids, as they were completely absent from cultures of the *carB* mutants, and in fact those that were chemically identified turned out to be apocarotenoids.

Mating increased the diversity of apocarotenoids (from **10** to **25** compounds). Eight apocarotenoids were seen in both single and mated cultures: six of them were abundant ones (the four 1,6-cyclofarnesoids and the two methylhexanoids) and two were minor ones (Tn154 (**14**) and an unidentified compound). Their concentrations were always larger in mated cultures than in single ones. Two minor compounds were not found in mated cultures; one of them, Tn153 (**13**), was barely detected in single cultures of strain NRRL1555 only. The four trisporic acids, the methylated trisporic acid Tn144 (**10**), and 12 unidentified compounds were found only in mated cultures; the most abundant one was Tn116 (**2**, trisporic acid E).

A small amount of yeast extract (1 g $L^{-1}$) in the culture media did not affect the chromatograms. A small amount of acetate in the medium (10 mmol $L^{-1}$) diminished the chemical diversity, leading to the disappearance of four compounds from single cultures and ten from mated cultures, with little or no changes in the concentration of the remaining compounds; an apparent exception was Ta102 (**9**, trisporic acid C), down by about two thirds.

Thirteen of the apocarotenoids detected in the chromatograms have been identified (**2–5** in Fig. 1 and **6–14** in Fig. 3) from five-day old mated cultures of strains A56 and NRRL1555. The structure of eleven of them (**2–12**) was determined following their isolation whereas that of two of them **13–14** was established by standards. Neutral and acid extracts obtained after acid–base partition of the culture media were fractionated by semi-preparative, normal-phase HPLC; the acid extracts were methylated with $TMSCHN_2$ before fractionation. The apocarotenoids **2**, **4**, **5**, **9**, **11**, **12**, (as methyl esters), **3**, and **10** were determined by comparing their spectroscopic data with those reported in the literature.[8a,12,14] The standards for compounds **12** and **13** were obtained by chemical synthesis.[15]

The apocarotenoids **6–8** were unknown. These compounds are unstable and decomposed partially during NMR registration and chromatographic fractionation. Their structures were established as follows.

### A heterocyclic apocarotenoid

The structure of apocarotenoid **6** was elucidated after exhaustive analysis of results from MS and 1D NMR and 2D NMR (COSY, HSQC and HMBC) (Fig. 4 and ESI†). The molecular formula of this compound was established as $C_{15}H_{22}O_3$ (five degrees of unsaturation) on the basis of its HRFABMS peak at *m/z* 273.1461 $[M + Na]^+$ (calcd 273.1461). The $^{13}C$ and DEPT NMR spectra revealed 15 carbon signals: three methyl groups, three methylene groups (two oxygenated), five methine groups (three olefinic and two oxygenated) and four quaternary carbons (three olefinic). Thus three unsaturations correspond to three double bonds and then the molecule contains two rings. The $^1H$ NMR spectrum indicated the presence of: (a) three methyl singlets, one of them attached to double bond ($\delta_H$: 1.00 (H-13), 1.05 (H-12) and 1.60 (H-15)); (b) three olefinic protons at $\delta_H$: 5.31 (br s, H-4), 5.50 (s, H-7) and 5.62 (t, $J$ = 6.6 Hz, H-10); (c) two oxygenated methylene groups, one at $\delta_H$: 4.16 (d, $J$ = 6.6 Hz, H-11) and $\delta_H$: 4.19 (d, $J$ = 12.8 Hz, H-14b) and the second at 4.09 (br d, $J$ = 12.8 Hz, H-14a); (d) two oxygenated methine groups ($\delta_H$: 4.55 (s, H-8) and 3.46 (t, $J$ = 4.5 Hz, H-2)). The COSY spectrum showed correlations between H-10 and H11; H-7 and H-8; H2 and H3, H3 and H-4 (Fig. 4). Taking into

**Table 1** Apocarotenoids in single and mated cultures, with and without added acetate[a]

| Compound | | $\lambda_{max}$ | Mass in extract % | | Absorption in chromatogram AUs | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Single | Mated | Single | Mated | Single Ac | Mated Ac |
| Xn85 | | 337 | — | — | — | 0.05 | — | 0.08 |
| Xn92 | | 336 | — | — | — | 0.39 | — | 0.57 |
| Xn100 | | 308 | — | — | — | 0.71 | — | 0.60 |
| Xn109 | | 311 | — | — | — | 0.16 | — | 0.25 |
| Cn113 | **6** | 248 | 26.4 | 17 | 1.29 | 8.81 | 2.75 | 10.42 |
| Cn115 | **3** | 307 | — | 7.2 | 0.06 | 3.15 | 0.03 | 3.46 |
| Xn118 | | 299 | — | — | — | 0.19 | — | 0.20 |
| Cn122 | **7** | 282 | 10.6 | 3.6 | 2914 | 9570 | — | — |
| Cn123 | **8** | 286 | 5.0 | 2.0 | 1797 | 4892 | — | — |
| Xn129 | | 329 | — | — | — | 0.06 | — | — |
| Xn130 | | 329 | — | — | — | 0.15 | — | 0.20 |
| Xn135 | | 329 | — | — | — | 0.20 | — | 0.13 |
| Xn142 | | 329 | — | — | — | 0.17 | — | — |
| Tn144 | **10** | 330 | — | 3.6 | — | 0.41 | — | — |
| Xn145 | | 293 | — | — | 0.01 | — | — | — |
| Xn147 | | 332 | — | — | — | 0.04 | — | — |
| Xn150 | | 345 | — | — | 0.04 | 0.16 | — | — |
| Tn153 | **13** | 312 | <0.1 | — | 0.03 | — | — | — |
| Tn154 | **14** | 328 | — | <0.1 | 0.03 | 0.09 | — | — |
| Ma26 | **4** | 265 | 30.0 | 21.7 | 1.64 | 18.3 | 1.90 | 18.97 |
| Ma26 | **5** | nd | 14.8 | 11.2 | * | * | * | * |
| Xa81 | | 325 | — | — | — | 0.11 | — | 0.15 |
| Xa91 | | 333 | — | — | — | 0.62 | — | 0.60 |
| Ta96 | **12** | 332 | — | 1.4 | — | 0.56 | — | 0.36 |
| Ta102 | **9** | 338 | — | 8.6 | — | 2.79 | — | 0.80 |
| Ta109 | **11** | 331 | — | 2.7 | — | 1.08 | — | 0.87 |
| Ta116 | **2** | 333 | — | 14.9 | — | 4.66 | — | 2.50 |

[a] Apocarotenoids are named according to their carbon skeleton (T, trisporane; C, cyclofarnesane; M, methylhexane; X, unknown), followed by "n" for neutral compounds and "a" for acid compounds, and the peak chromatographic retention time in tens of seconds. The mass percentage (estimated by $^1$H NMR in separate neutral and acid dry extracts) was determined in NRRL1555 single cultures and A56 × NRRL1555 mated cultures. The absorption (the integral of the product of the absorbance units and the retention time in seconds) was the mean for samples of 20 µL obtained from 4 to 34 independent experiments of single cultures (A56, NRRL1554 and NRRL1555) and mated cultures (A56 × NRRL1555 and NRRL1554 × NRRL1555). Cn113 (**6**) was measured at 254 nm, Ma26 (**4**) and Ma27 (**5**) at 280 nm, and the others at 328 nm. The asterisk indicates a result pooled with that of the compound in the preceding line. The result for Tn153 (**13**) refers to single cultures of strain NRRL1555, as it was not found elsewhere.



account in addition the HMBC correlations of C-8 ($\delta_C$: 80.2) with H-7/H-10/H-14, of C-1 ($\delta_C$: 38.7) with H-3/H-12/H-13, and of C-10 ($\delta_C$: 127.5) with H-8/H-11/H-15, compound **6** must contain the frameworks A and B (Fig. 4).

Both frameworks were connected thanks to the HMBC correlations of C-6 ($\delta_C$: 140.0) with H-8/H-12/H-13/H-14, of C-4 ($\delta_C$: 117.0) with H-14, and of C-5 ($\delta_C$: 129.9) with H-4/H-14. Thus, the structure of **6** is 8,14-epoxy-1,6-cyclofarnesa-4,6,9-triene-2,11-diol. The *E* stereochemistry for the double bond on the side chain was deduced from the chemical shifts at C-11 ($\delta_C$: 59.2) and C-15 ($\delta_C$: 12.9) in the $^{13}$C NMR spectrum.[16] The relative *syn* stereochemistry between the OH and the side chain of **6** was established from the pattern of multiplicity and *J* values of H-8 (s) and H-2 (t, $J$ = 4.5 Hz). These values are only possible if H-2 adopted a pseudo-equatorial orientation, and H-8 a pseudo-axial orientation in the minimal energy conformation (Fig. 5). This is the first apocarotenoid of the Mucoromycotina that contains a heterocyclic framework.

### Trihydroxy apocarotenoids

Apocarotenoid **7** was assigned the molecular formula $C_{15}H_{24}O_3$ (four degrees of unsaturation) on the basis of its HRFABMS peak at *m/z* 275.1626 $[M + Na]^+$ (calcd 275.1623). Its $^1$H and $^{13}$C NMR spectra (Table 2) were similar to those of **6** with signals corresponding to the same 1,6-cyclofarnesane framework but without those corresponding to the heterocyclic ring. Thus the NMR analysis of **7** (Table 2) revealed the following differences: the presence of a conjugated triene system (C-4/C-5, C-6/C-7 and C-8/C-9); the existence of a methyl group at the double bond C-4/C-5, instead of the oxygenated allylic methylene, and the presence of three hydroxyls at C-2, C-10 and C-11. The structure of **7** is established as cyclofarnesa-4,6,8-triene-2,10,11-triol. The pseudoaxial disposition of the hydroxyl group at C-2 was inferred from the observed values of $J_{H2–H3a} = J_{H2–H3b}$ = 4.5 Hz and the *E* stereochemistry of the 6,7 and 8,9 double bonds were deduced from the observed NOE effects between H-7 and H-12/H-14, and H-8 and H-13 (Fig. 6).

The structure of compound **8** have been elucidated through spectroscopic data from a mixture with **7** (see Experimental and Table 2), revealing that the minor component (**8**) was a geometric stereoisomer of **7**. The observed NOE effects (Fig. 6) between H-7 and H-14/H-15, and H-8 and H-12/H-13 determined a *Z* stereochemistry for the C-6/C-7 double bond and an *E* stereochemistry for the C-8/C-9 double bond.

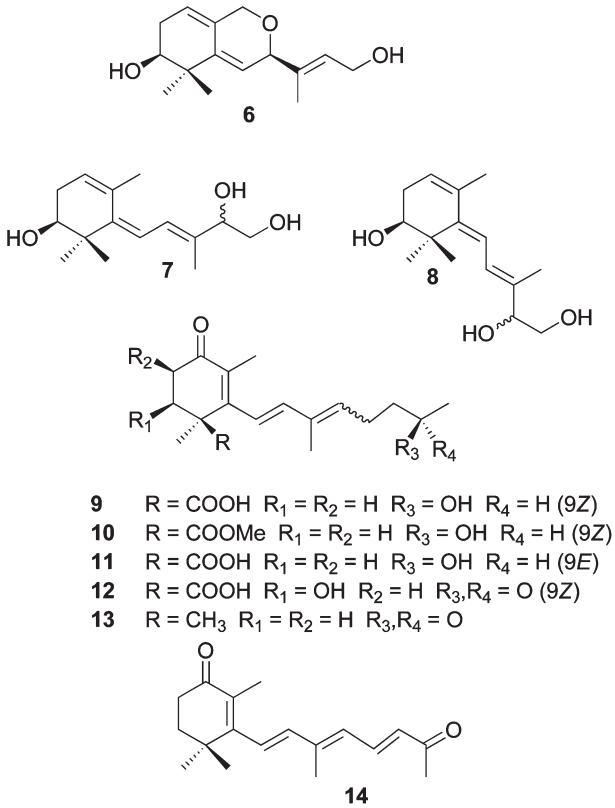



**Fig. 3** New apocarotenoids from *P. blakesleeanus* identified in this work (**6**–**14**).

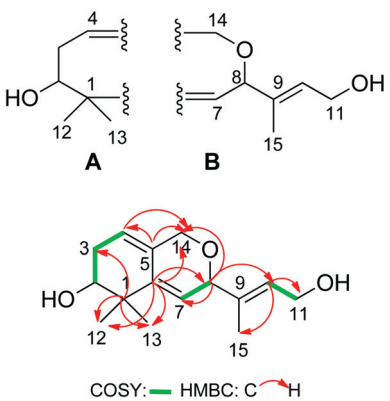



**Fig. 4** Key COSY and HMBC correlations for **6**.

## Discussion

### Diversity of the apocarotenoids

The apocarotenoids of *P. blakesleeanus* are much more diverse than could be anticipated from the previous identification of just four compounds. Mated cultures contain more than two dozen apocarotenoids, 13 of which, the most abundant as suggested by their absorbance in UV-visible detection in HPLC, have been identified (Table 1). There are seven C18 trisporoids (**2**, **9**–**12**), four C15 cyclofarnesoids (**3**, **6**–**8**), and two C7 methylhexanoids **4** and **5**. All of them can be viewed as modifications of the three fragments that result when β-carotene is cleaved at its 11′,12′ and 12,13 double bonds by the *CarS* and *AcaA* gene products.[8a,9]

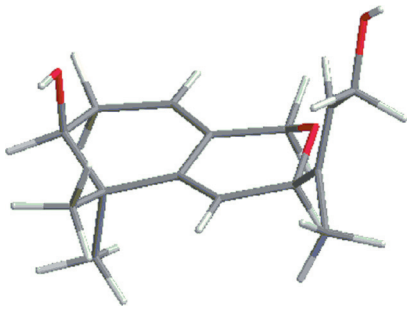


**Fig. 5** Conformation of apocarotenoid **6**.

The different genetic backgrounds of the strains NRRL1554 and A56, both (+), had little influence on apocarotenoid production. There were some quantitative differences between cultures of different sex. Mated cultures NRRL1554 × NRRL1555 and A56 × NRRL1555 had very similar apocarotenoid profiles. Compound Tn153 (**13**) was found as traces only in single cultures of NRRL1555, our (−) strain.

The genetic test considers apocarotenoids all compounds present in the wild types and missing in the *carB* mutants that are wholly devoid of β-carotene. The possibility that the absence of β-carotene might block the appearance of a compound not derived from it cannot be excluded, but this has not been observed. The test would detect compounds that might be derived from lycopene and other precursors of β-carotene, but not those that might be derived from phytoene, because this is plentiful in the *carB* mutants.

The variety of apocarotenoids is not exclusive to *Phycomyces*. Eight of the 13 apocarotenoids identified in *Phycomyces* have been reported from other Mucorales, but we have not identified ten apocarotenoids known from other Mucorales.[7] Even strains of the same species, *Blakeslea trispora*, differ widely in their panoply of apocarotenoids.[8b]

Many new apocarotenoids are likely to be found in the Mucoromycotina when this ancient and diverse taxon receives the attention that it deserves. The systematic chemical names are complex, to give each new compound a trivial name would be impractical, and some of the trivial names already given are misleading. In the face of the same problem, the gibberellins are named by the letters GA followed by an ordinal number. In Table 1 we have used a somewhat more informative designation for the apocarotenoids of the Mucoromycotina, using a letter to indicate their carbon skeleton, another for their prevalence in neutral or acid extracts, and a number for the chromatographic retention time. The alphanumeric order within each group corresponds roughly to their polarity.

It is worth pointing out that apocarotenoids are much more abundant than β-carotene in our five-days-old cultures. Thus, the medium of a mated culture (see Experimental) yielded 711 mg of dry apocarotenoids, neutral and acidic; the mycelium in that medium contained about 5 mg of β-carotene.

### New apocarotenoids and their biosynthesis

Five apocarotenoids have been found so far in *Phycomyces* only; two of them (Cn115 (**3**) and Ta116 (**2**)) were known already and

**Table 2** $^{1}H$ and $^{13}C$ NMR data of **6–8**

| | **6**[a] | | **7**[a] | | **7**[b] | | **8**[b] | |
|---|---|---|---|---|---|---|---|---|
| C/H | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 1 | | 38.7 | 42.1 | | | 42.6 | | 41.0 |
| 2 | 3.46 t (4.5) | 74.3 | 3.42 t (4.5) | 74.8 | 3.44 t (4.5) | 74.0 | 3.42–3.45 m | 75.6 |
| 3a | 2.19 dd (4.5, 17.9) | 31.0 | 2.09 dd (4.5, 17.8) | 31.1 | Overlapped by acetone signal | 33.9 | 2.15 br d (17.8) | 32.2 |
| 3b | 2.42 dd (3.9, 17.9) | | 2.43 dd (4.0, 17.8) | | 2.43 br d (17.9) | | 2.34 br d (17.8) | |
| 4 | 5.31 br s | 117.0 | 5.44 br s | 125.7 | 5.48 br s | 126.7 | 5.55 t (4.5) | 124.3 |
| 5 | | 129.9 | | 132.1 | | —[c] | | —[c] |
| 6 | | 140.0 | | 136.9 | | 133.0 | | 144.7 |
| 7 | 5.50 s | 123.2 | 6.18 d (11.4) | 120.9 | 6.26 d (11.4) | 120.7 | 6.31 d (11.7) | 122.0 |
| 8 | 4.55 s | 80.2 | 6.62 d (11.4) | 124.1 | 6.67 d (11.4) | 124.2 | 6.83 d (11.7) | 123.7 |
| 9 | | 138.1 | | 138.3 | | 138.6 | | 135.0 |
| 10 | 5.62 t (6.6) | 127.5 | 4.16 t (6.3) | 80.3 | 4.08–4.12 m | 78.3 | 4.12–4.17 m | 78.3 |
| 11a | 4.16 d (6.6) | 59.2 | 3.52 br d (10.0) | 65.5 | 3.49 dt (2.0, 5.2) | 66.2 | 3.42–3.45 m | 66.3 |
| 11b | | | 3.63 dd (4.9, 10.0) | | 3.55–3.59 m | | 3.52–3.55 m | |
| 12 | 1.05 s | 22.6 | 1.04 s | 22.7 | 1.13 | 21.9 | 1.35 s | 21.8 |
| 13 | 1.00 s | 25.2 | 1.02 s | 25.5 | 1.03 | 25.3 | 1.21 s | 26.8 |
| 14a | 4.09 d (12.8) | 67.2 | 2.01 s | 25.3 | 2.03 | 25.3 | 1.85 s | 21.8 |
| 14b | 4.19 d (12.8) | | | | | | | |
| 15 | 1.60 s | 12.8 | 1.76 s | 12.9 | 1.76 | 12.9 | 1.76 s | 13.0 |
| OH | 1.56 br s | | 1.50 br s | | 3.85 t (3.4) | | 3.94 t (3.4) | |

[a] Spectra carried out in $Cl_3CD$. [b] Spectra carried out in $(CD_3)_2CO$. [c] C-5 signal not observed. $\delta$ in ppm. $J$ in Hz in parentheses.

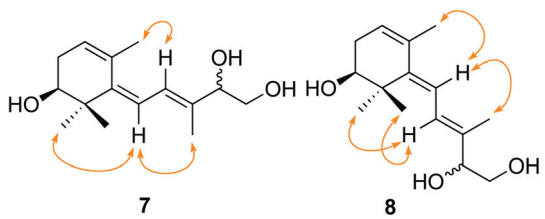



**Fig. 6** NOE effects of **7** and **8**.



the other three are new natural compounds. Compound Cn113 (**6**) is the first heterocyclic apocarotenoid of the Mucoromycotina. This compound and the isomers Cn122 (**7**) and Cn123 (**8**) have no oxygen atom bound to the C4 atom, a trait that has not been found previously in the Mucoromycotina. The three new compounds are likely to be biosynthesized from a hypothetical intermediate trihydroxy cyclofarnesoid that has not been identified (**A** in Scheme 1). The existence of this intermediate **A** is supported by the results of Sutter and Whitaker,[17] which describe the isolation of anhydro derivatives of methyl 4-dihydrotrisporate C, and methyl 4-dihydrotrisporate B from single cultures of *Phycomyces blakesleeanus*. These derivatives are formed by the corresponding 4-dihydro derivatives in slightly acidic solutions. This hypothetical intermediate would be formed from the original C15 fragment of β-carotene, following hydroxylation at C4 and C2 and the reduction of the aldehyde group to alcohol. Compound Cn113 (**6**) would result from hydroxylation of methyl 14 and after heterocyclization with concomitant hydroxyl elimination at ring level following a $S_N2'$ mechanism. An enzyme-mediated attack of water at C10 of **A** would give rise to the triol Cn122 (**7**) and its isomer Cn123 (**8**) with hydroxyl elimination at ring level following also a $S_N2'$ mechanism (Scheme 1).

### Environmental dependence of the apocarotenoids

We have reported on the apocarotenoid content under two conditions that modify sexual behavior: mated cultures and presence of acetate in the culture medium (Table 1). We investigated five-day-old cultures because they are close to the onset age for sexual morphogenesis. The apocarotenoids observed at that time probably differ in quality and in amount from those that were synthesized because of differences in the metabolism and the stability of the different compounds.

The main qualitative change induced by mating was the formation of trisporic acids. The quantitative changes are due to increased biosynthesis of β-carotene ("sexual carotenogenesis") and to increased transcription levels of genes for its further metabolism, as shown for the β-carotene-cleaving oxygenases.[9]

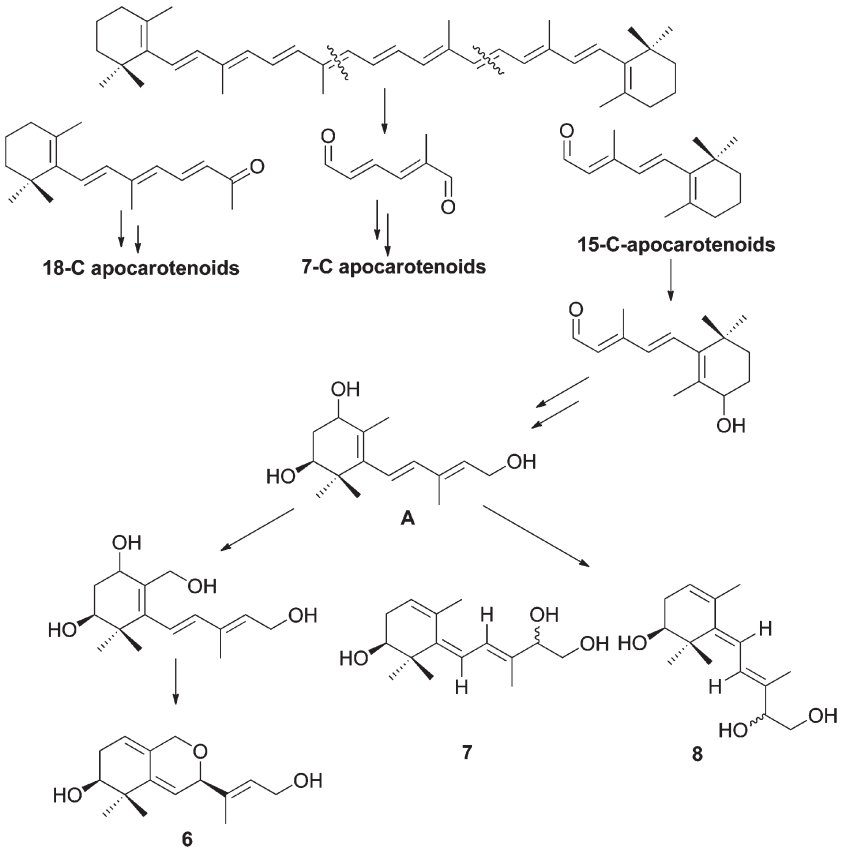
The presence of acetate in the medium diminished the chemical diversity, leading to the disappearance of several compounds. The most notable effect was the disappearance of two of the most abundant compounds, the triols Cn122 (**7**) and Cn123 (**8**), from both single and mated cultures. This suggests that acetate blocks the attack of water on C10 of precursor **A** (Scheme 1).

### Functions of the apocarotenoids

Three different functions are mediated by apocarotenoids in *Phycomyces*: feed-back inhibition of carotenogenesis, active in single cultures; induction of sexual morphogenesis by sex-specific pheromones; and induction of sexual carotenogenesis in mated cultures.

The feedback inhibitor is an early apocarotenoid, probably one of the products of the first cleavage of β-carotene.[9] It must be present in the cells of single cultures, and it is unlikely to be secreted to the medium, because the deep yellow color of the *carS* mutant mycelia that lack the inhibition does not decrease in the vicinity of wild-type cultures.




**Scheme 1** A proposal on the biosynthesis of the new apocarotenoids (**6–8**).

The sexual pheromones are sex specific and must be present in the medium of single mycelia.[2,8a,10a] Sexual carotenogenesis occurs in mated cultures only. Acetate, at the concentration used, blocks sexual carotenogenesis and increases sexual interaction enormously with a copious production of zygospores.[18] Acetate and other small carboxylic acids with the same effects could act on the receptors, but their drastic effects on apocarotenoid composition suggest an action on the signals.

The active signals are not necessarily among the compounds that we have detected because they may too unstable, or be present and active at concentrations under our detection thresholds, or not present at the time of our analyses. The multiplicity of apocarotenoids makes it possible for each function to be carried out by several compounds with different affinity for the presumed receptors. Finally, different Mucoromycotina should not be assumed to use the same signals; the contrary may have been favored by speciation.

## Experimental section

### Materials

Two strains of *Phycomyces blakesleeanus* Bgff. used in this work were originally isolated in nature: NRRL1555, the standard (−) wild type and NRRL1554, a (+) wild type, both from the Northern Regional Research Laboratory (now National Center for Agricultural Utilization Research, Peoria, IL). Strain A56 is a (+) wild type isogenic to NRRL1555.[13] Strain C5, genotype *carB10* (−), is a mutant derived from a spore of NRRL1555 exposed to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.[19] Strain S342, genotype *carB10 nicA101* (+), was derived from C5 after several crosses.[20]

*Phycomyces* was grown in minimal medium with glucose, L-asparagine, thiamine and mineral salts,[4] enriched with yeast extract (1 g $L^{-1}$) when indicated. Solid media contained agar (15 g $L^{-1}$). For the *nic* mutants nicotinic acid was added (1 mg $L^{-1}$). Spores were harvested from 5-day-old cultures and kept in water at 4 °C for up to about one week. Spores were suspended in water or molten water agar (8 g $L^{-1}$) at 48 °C for 15 min before plating ($10^4$ spores in 2.5 mL water agar per plate with 25 mL agar medium or $10^4$ spores in 2.5 mL water per Erlenmeyer flask with 25 mL of liquid medium) and incubated for 5 days at 22 °C in the dark. Mated cultures were inoculated with a 1 : 1 mixture of spores of two strains of opposite sex.

### Instrumental analysis

NMR spectra ($^1$H and $^{13}$C) were recorded with a 500 ($^1$H 500 MHz/$^{13}$C 125 MHz) spectrometers. For high-resolution MS we used an Autospec-Q VG-Analytical mass spectrometer. For semi-preparative normal-phase HPLC, aliquots (0.5 mL) of the neutral and methylated acid extracts dissolved in *t*-BuOMe (20 g dry extract per L) were injected into a column (10 by 250 mm, 5 μm silica particles); with a 15 mm refillable guard pre-column filled with the same material in a Series 1100 liquid

chromatograph (Agilent). The column was eluted at room temperature at a flow rate of 2 mL $min^{-1}$ for 25 min with *t*-BuOMe and monitored with a refractometer. For the methylated acid extracts the eluent was n-hexane–*t*-BuOMe (1 : 4, v/v). For reverse-phase HPLC aliquots (20 μL) of the neutral and acid extracts dissolved in methanol (1 mL for the extract from each 25 mL culture) were loaded with a G1313A autosampler (Hewlett-Packard) into a C18 column (4.6 by 250 mm; 5 μm octyldecylsilane particles; Spherisorb-ODS2, Waters) with a 12.5 mm refillable guard pre-column filled with the same material (Agilent) in a liquid chromatograph (Series 1100, Hewlett-Packard). The column was eluted at room temperature at a flow rate of 1 mL $min^{-1}$ with methanol–water (1 : 4) during the first 6 min and successively with a lineal gradient up to pure methanol at 25 min, with pure methanol until 40 min and with a lineal gradient down to the initial mixture at 45 min. The outflow was monitored with a diode array detector at 328, 280, 254, 230 and 210 nm.

### Extraction and fractionation of apocarotenoids

The initial extracts for apocarotenoid isolation and analysis were obtained by freezing (−20 °C for at least 2 h) and thawing (22 °C for 1 h) the agar media and centrifuging the liquid (4000 × *g*, 15 min) or by filtration of the liquid cultures. Neutral extracts were obtained by adjusting the initial extracts to pH 8.0 with 2 N NaOH and extracting thrice with EtOAc. Acid extracts were obtained by adjusting the remaining aqueous phase to pH 2.0 with 2 N HCl and extracting with EtOAc. Water was removed by mixing with anhydrous $Na_2SO_4$ and filtering; the organic solvent was removed by evaporation under low pressure. For the sake of chemical stability, all procedures were carried out under dim light.

An initial extract (1 L) of mated cultures A56 × NRRL1555 (80 minimal agar plates) yielded 387 mg dry neutral and 324 mg dry acid extract. The neutral extract was fractionated by semi-preparative HPLC. Compound **10** was the major component of the fraction $15.4 < t_R < 19.6$ min (11.8 mg); compound **3** that of the fraction $21.9 < t_R < 23.5$ min (8.2 mg); compound **6** that of the fraction $23.5 < t_R < 29.0$ min (21.6 mg); a 3 : 1 mixture of **6** and **7** was present in the fraction $29.0 < t_R < 31.8$ min (3.4 mg); a 1 : 1 mixture of **7** and **8** was present in the fraction $31.8 < t_R < 32.5$ min (1.9 mg).

**(2*S*,8*R*,*E*)-8,14-Epoxy-1,6-cyclofarnesa-4,6,9-triene-2,11-diol (6).** Colourless syrup. $[\alpha]_D$ +22.5 ($CHCl_3$, c1); UV (MeOH) $\lambda_{max}$: see Table 1; $\delta_H$ (500 MHz; $CDCl_3$; $Me_4Si$): see Table 2; $\delta_C$ (125 MHz; $CDCl_3$; $Me_4Si$): see Table 2; HRMS [M + $Na^+$] calculated for $C_{15}H_{22}O_3Na$ requires 273.1461, found 273.1461.

**(2*S*,7*E*,9*E*)-2,11-Dihydroxy-1,6-cyclofarnesa-5,7,9-trien-4-one (previously.[12] apotrisporin E) (3).** Colourless syrup. It has been identified by spectroscopic data (see ESI†). These data were consistent with those previously reported.[12]

The acid extract (324 mg) of the mated cultures was dissolved in $C_6H_6$–MeOH (4 : 1 v/v) (10.1 mL) at 0 °C and methylated by adding $TMSCHN_2$ 2 M in $Et_2O$ (1.1 mL) under stirring. The solution was left for 5 min at room temperature and the solvent was evaporated at low pressure to obtain a mixture of methyl ester (350 mg) that was fractionated by semi-preparative HPLC. A 2 : 1 mixture of **4** and **5** (as methyl esters) was present in the fraction $13.4 < t_R < 15.9$ min (63.3 mg); a 2 : 1 mixture of **11** and **9** (as methyl esters) was present in the fraction. $17.5 < t_R < 18.5$ min (12 mg); compound **9** (as methyl ester) was in the fraction $18.5 < t_R < 20.6$ min (25.5 mg); a 3 : 2 mixture of **9** and **2** (as methyl esters) was present in the fraction $20.6 < t_R < 22.2$ min (14.2 mg); a 1 : 9 mixture of **12** and **2** was present in the fraction $22.2 < t_R < 25.1$ min (52.2 mg).

**Hexa-2,4-dienoic acids 4 and 5, and trisporic acids E (2), C (9, *Z*), C (11, *E*), D (12)** (as methyl esters) have been identified by spectroscopic data (see ESI†). These data were consistent with those previously reported.[8a,14]

The filtered minimal medium (2.4 L) of a NRRL1555 single culture yielded 40 mg dry neutral extract and 60 mg dry acid extract. The neutral extract was fractionated by semi-preparative HPLC. Compound **6** was present in the fraction $21.5 < t_R < 24.3$ min (8 mg); a 1 : 1 mixture of **6** : **7** was present in the fraction $24.3 < t_R < 25.7$ min (4 mg). Compound **7** was present in the fraction $25.7 < t_R < 27.2$ min (2 mg); a 1 : 1 mixture of **7** and **8** was present in the fraction $27.2 < t_R < 29.1$ min (2.5 mg).

**(2*S*,6*E*,8*E*)-1,6-Cyclofarnesa-4,6,8-triene-2,10,11-triol (7).** Colourless syrup. UV (MeOH) $\lambda_{max}$: see Table 1; $\delta_H$ (500 MHz, $CO(CD_3)_2$): see Table 2; $\delta_C$ (125 MHz, $CO(CD_3)_2$): see Table 2; $\delta_H$ (500 MHz, $CDCl_3$): see Table 2; $\delta_C$ (125 MHz, $CDCl_3$): see Table 2; HRMS [M + $Na^+$] calculated for $C_{15}H_{24}O_3Na$ 275.1623, found 275.1626.

**(2*S*,6*E*,8*E*)-1,6-Cyclofarnesa-4,6,8-triene-2,10,11-triol (7) + (2*S*,6*Z*,8*E*)-cyclofarnesa-4,6,8-triene-2,10,11-triol (8).** Colourless syrup. HRMS [M + $Na^+$] calculated for $C_{15}H_{24}O_3Na$ requires 275.1623, found 275.1624.

**Compound 8.** UV (MeOH) $\lambda_{max}$: Table 1; $\delta_H$ (500 MHz, $CO(CD_3)_2$): see Table 2; $\delta_C$ (125 MHz, $CO(CD_3)_2$): Table 2; $\delta_H$ (500 MHz, $CDCl_3$): (distintive signals only) 6.72 (1H, d, $J$ = 11.7 Hz, H-8), 6.32 (1H, d, $J$ = 11.7 Hz, H-7), 5.51 (1H, br s, H-4), 3.36 (1H, t, $J$ = 4.5 Hz, H-2), 1.86 (3H, s, H-14), 1.32 (3H, s, H-13), 1.20 (3H, s, H-12).

The acid extract of the single cultures was methylated with $TMSCHN_2$ and fractionated by semi-preparative HPLC. A 2 : 1 mixture of **4** and **5** (as methyl esters) was present in the fraction $13.4 < t_R < 15.9$ min (5 mg).

A similar culture of strain A56 yielded 62 mg of neutral extract and 132 mg of acid extract. These extracts were processed in the same way as those of strain NRRL1555.

## Conclusions

This article presents the first systematic analysis of the apocarotenoids in a species of the Mucoromycotina. In this analysis we have used an effective operational definition of apocarotenoids as compounds that are produced by the wild-type strain and not by the mutants devoid of carotene. The apocarotenoids in mated cultures were much more diverse and abundant than in single cultures. The presence of small amounts of acetate, relative to the total carbon supply in the medium, led to the disappearance of some apocarotenoids with little or no change in the amounts of the others.

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