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ΚΟΝΤΑΞΗ ΤΙΤΙΚΑ

“Σεξουαλική αλληλεπίδραση του Ζυγομύκητα *Blakeslea trispora*. Απομόνωση, δομικός χαρακτηρισμός και βιοσύνθεση τρισποροειδών και παραγώγων”.

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Η παρούσα μεταπτυχιακή διατριβή έχει πραγματοποιηθεί στο Πανεπιστήμιο της Γρανάδας, Σχολή Επιστημών, Τμήμα Οργανικής Χημείας, και έχει κατατεθεί στη Γραμματεία Προγράμματος Μεταπτυχιακών του Τμήματος Βιοχημείας και Βιοτεχνολογίας στην Αγγλική, σύμφωνα με την έγκριση της Γενικής Συνέλευσης Ειδικής Σύνθεσης του Τμήματος Βιοχημείας και Βιοτεχνολογίας.

Η παρούσα μεταπτυχιακή διατριβή πραγματοποιήθηκε στο εργαστήριο του καθηγητή Fernández Barrero, στο Ίδρυμα Βιοτεχνολογίας, Τμήμα Οργανικής Χημείας του Πανεπιστημίο της Γρανάδας. Η ερευνητική ομάδα του Καθηγητή Barrero εξειδικεύεται στη χημική μελέτη δευτερογενών μεταβολιτών οι οποίοι εκκρίνονται από νηματοειδείς μύκητες, σε συνεργασία με την ομάδα του καθηγητή Cerdá-Olmedo του Τμήματος Γενετικής, Πανεπιστήμιο Σεβίλλης. Μεταξύ αυτών των μυκήτων συναντούμε τους Mucorales μύκητες *Phycomyces blakesleeanus* και *Blakeslea trispora* (διαίρεση Zygomycota, κλάση Zygomycetos) στους οποίους το ιδιαίτερο ενδιαφέρον έγκειται στο γεγονός ότι μεταξύ των μεταβολιτών τους συναντούμε τα γνωστά με το όνομα αποκαροτενοειδή (apocarotenoids). Τα τρισπορικά οξέα (trisporic acids-TSAs) αντιπροσωπεύουν μια ιδιαίτερη οικογένεια αποκαροτενοειδών, η οποία παρουσιάζει και το μεγαλύτερο επιστημονικό ενδιαφέρον, καθώς μέχρι σήμερα όλα τα πειραματικά δεδομένα δείχνουν ότι πρόκειται για τις σεξουαλικές φερομόνες, οι οποίες υποκινούν τη σεξουαλική μορφογένεση και σεξουαλική καροτενογένεση αυτών των μυκήτων. Κύριος στόχος της ευρύτερης έρευνας, η οποία πραγματοποιείται πάνω στα Mucorales είναι η εύρεση αυτών των χημικών σημάτων τα οποία προάγουν τη σεξουαλική αλληλεπίδραση των μυκήτων και την υπερπαραγωγή β-καροτενίου, συντελώντας με αυτό τον τρόπο στη βελτίωση της βιομηχανικής παραγωγής β-καροτενίου, λυκοπενίου και άλλων χημικών προϊόντων.

Για τη μελέτη των αποκαροτενοειδών στην παρούσα εργασία, πραγματοποιήθηκε εξαγωγή, διαχωρισμός και ταυτοποίηση των μεταβολιτών, οι οποίοι παράγονται στο μέσο μονής ή μικτής καλλιέργειας στελεχών του μύκητα *Blakeslea trispora*, εμβολιασμένων με σπόρια ενός ή και των δύο φύλων του μύκητα αντίστοιχα.

Για το σκοπό αυτό χρησιμοποιήσαμε τα άγρια στελέχη F986(+) και F921(-) του μύκητα *Blakeslea trispora*. Χρησιμοποιήσαμε δύο τύπους μέσου καλλιέργειας, υγρό με ανάδευση και στερεό. Οι μονές καλλιέργειες πραγματοποιήθηκαν σε υγρό αλλά και σε στερεό μέσο ενώ οι μικτές πραγματοποιήθηκαν μόνο σε στερεό, καθώς σε αυτό επιτυγχάνεται καλύτερα ο συνδυασμός των δυο φύλων. Όλες οι καλλιέργειες επωάστηκαν στους 30^{o} C, σε σκοτάδι και η συγκέντρωση των σπόρων με την οποία

πραγματοποιήθηκε ο κάθε εμβολιασμός, ανα 25 mL μέσου καλλιέργειας, ήταν 50 μL συγκέντρωση 10^6 spores/ml του στελέχους F986(+) και 100 μL συγκέντρωση 10^6 spores/ml του στελέχους F921(-).

Για τις καλλιέργειες σε στερεό μέσο, η περίοδος επώασης ήτανε 3 μέρες, ενώ για τις καλλιέργειες σε υγρό μέσο πραγματοποιήθηκε μελέτη για την εύρεση της βέλτιστης περιόδου επώασης για μέγιστη παραγωγή τρισποροειδών. Σε συμφωνία και με τα πειραματικά δεδομένα από μονές καλλιέργειες του *Phycomyces blakesleeanus* (βέλτιστη περίοδος επώασης 5 μέρες), αρχικά πραγματοποιήθηκαν μονές καλλιέργειες για 5 μέρες, στις οποίες όμως δεν είχαμε παραγωγή τρισποροειδών. Λόγω του αποτελέσματος, προχωρήσαμε σε επόμενο πείραμα όπου η περίοδος επώασης παρατάθηκε σε 10 ημέρες. Μετά την επεξεργασία των καλλιεργειών δεν παρατηρήθηκε επίσης παρουσία τρισποροειδών στα ^{1}H NMR και HPLC φάσματα τα οποία πραγματοποιήθηκαν.

Ελλείψει τρισποροειδών στις υγρές καλλιέργειες, συνεχίσαμε με μονές καλλιέργειες των δυο στελεχών σε στερεό μέσο, προκειμένου να εξεταστεί εάν ο τύπος του μέσου έχει επιπτώσεις στην παραγωγή τρισποροειδών από το μύκητα. Τα αποτελέσματα που προέκυψαν ήταν ίδια με εκείνα των καλλιεργειών σε υγρό μέσο, δηλαδή δεν είχαμε παραγωγή τρισποροειδών.

Στη συνέχεια προχωρήσαμε σε μικτές καλλιέργειες των δύο στελεχών, οι οποίες πραγματοποιήθηκαν κάτω από τις ίδιες συνθήκες με αυτές των μονών καλλιεργειών και στο ίδιο μέσο. Προκειμένου να επιβεβαιωθεί η παρουσία ή όχι τρισποροειδών, μετά το τέλος της περιόδου επώασης πραγματοποιήθηκε HPLC και ^{1}H NMR ανάλυση, η οποία έδειξε την παρουσία τρισποροειδών στο μέσο.

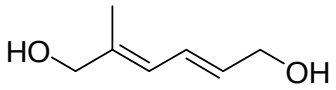
Συνεχίζοντας την έρευνα, πραγματοποιήθηκε GC-MS ανάλυση προκειμένου να προσδιοριστεί η ταυτότητα των κυρίως μεταβολιτών, οι οποίοι παρουσιάστηκαν στο μέσο καλλιέργειας των μονών καλλιεργειών και στις οποίες δεν είχαμε παραγωγή τρισποροειδών. Με την ολοκλήρωση της διαδικασίας κατέστη δυνατός ο προσδιορισμός του 4-υδροξυκινναμικού οξέος (p-κουμαρικού οξέος) 4-hydroxycinnamic acid (p-coummaric acid) και της 2-4 υδροξυφαινυλαιθανόλης (τυροσόλης) 2-(4-hydroxyphenyl)ethanol (Tyrosol).

Οι μεταβολίτες αυτοί, αν και έχουν περιγραφεί στο παρελθόν για άλλους μύκητες, ωστόσο είναι η πρώτη φορά που περιγράφονται για τον *Blakeslea trispora*.

Στη συνέχεια προχωρήσαμε στην ταυτοποίηση των τρισποροειδών τα οποία παρήχθησαν στο μέσο των μικτών καλλιεργειών.

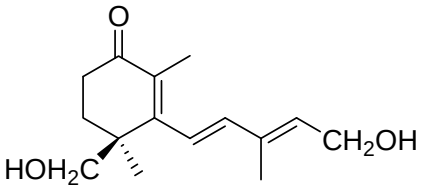
Αρχικά, από την ανάλυση του ουδέτερου κλάσματος μέσω ημιπαρασκευαστικής HPLC κανονικής-φάσης, κατέστη δυνατή η απομόνωση 2 αποκαροτενοειδών.

Για το **αποκαροτενοειδές 1**, βάση των HPLC και ^{13}C NMR φασμάτων τα οποία προέκυψαν, προτάθηκε για αυτό η δομή του (2*E*,4*E*)-2-methyl-2,4-hexadiene-1,6-diol και το όνομα **Blakeslediol**. Η παραπάνω ένωση αποτελεί καινούργιο φυσικό προϊόν το οποίο ανήκει σε μια νέα οικογένεια αποκαροτενοειδών, η οποία ανακαλύφθηκε πρόσφατα από την ερευνητική ομάδα.



(2*E*,4*E*)-2-methyl-2,4-hexadiene-1,6-diol

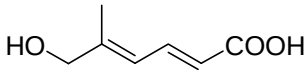
Για το **αποκαροτενοειδές 2,** κατέστη δυνατή η ταυτοποίηση του με τη βοήθεια των ανάστροφης φάσης-HPLC, ^{1}H NMR, UV και MS, τα οποία έδειξαν ότι πρόκειται για το γνωστό από προηγούμενες έρευνες **Apotrisporol**.



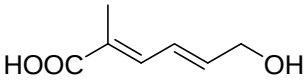
Apotrisporol

Η ταυτοποίηση των αποκαροτενοειδών του Όξινου κλάσματος, το οποίο αποκτήθηκε από τις μικτές καλλιέργειες, πραγματοποιήθηκε με τη βοήθεια ^{1}H NMR και αναλυτικής ανάστροφης-φάσης HPLC. Με τον τρόπο

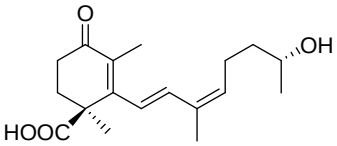
αυτό κατέστη δυνατή η ταυτοποίηση των Blakesleoic acids A and B, του Trisporic acid C (9*Z*) και Trisporic acid C (9*E*).



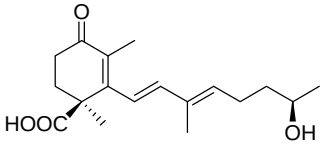
Blakesleoic acid A



Blakesleoic acid B

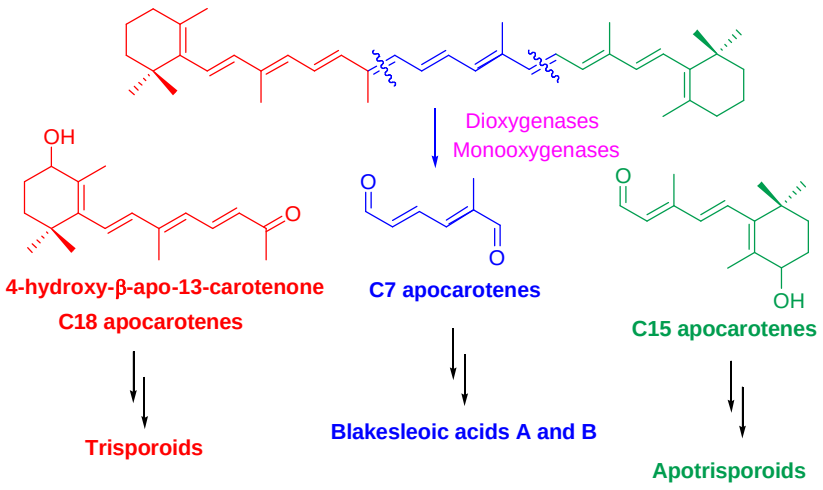


Trisporic acid C (9*Z*)



Trisporic acid C (9*E*)

Βάση των ανωτέρω αποτελεσμάτων και κυρίως μετά την απομόνωση του Blakesleδiol 1 και των Blakesleoic acids A and B από τις μικτές καλλιέργειες, μπορούμε να προτείνουμε για τον *Blakselsea trispora* ένα νέο βιοσυνθετικό μονοπάτι για την παραγωγή τρισποροειδών και αποτρισποροειδών, ανάλογο με αυτό που έχει προταθεί από την ερευνητική ομάδα για τον *Phycomyces blakeselaunus*. Το νέο βιοσυνθετικό μονοπάτι προτείνει την παραγωγή των ενώσεων αυτών μέσω διπλής οξειδωτικής σχάσης του β-καροτενιού, όπως φαίνεται στο σχήμα που ακολουθεί:



UNIVERSITY OF GRANADA

FACULTY OF SCIENCES

Department of Organic Chemistry

"Sexual interaction in the Zygomycete *Blakeslea trispora*. Isolation, structural elucidation and biosynthesis of trisporoids and derivatives"

Master Thesis

TITIKA KONTAXI

Granada 2010

“Sexual interaction in the Zygomycete *Blakeslea trispora*. Isolation, structural elucidation and biosynthesis of trisporoids and derivatives”

Report presented by **Titika Kontaxi** for the completion of Master Studies in Biotechnology - Quality Assessment in Nutrition and the Environment.

Granada, 22 of March 2010

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I. INTRODUCTION

The research group of Professor Fernández Barrero is working inside the Biotechnology field more than fifteen years now, in collaboration with the group of Professor Cerdá-Olmedo of the Department of Genetics, University of Sevilla, on the chemical study of secondary metabolites excreted to the culture broth of filamentous fungi. Among these fungi are the Mucorales *Phycomyces blakesleeanus* and *Blakeslea trispora* (division Zygomycota, class Zygomycetos) greatly interest because they produce the named "Apocarotenoids" metabolites. Trisporic Acids (TSAs) represent a family group of "Apocarotenoids" which seem to be one of the most interest, because until today all the experimental evidences indicate that they are the sexual pheromones of these fungi stimulating their sexual morphogenesis and sexual carotenogenesis.

1. Zygomycota: General characteristics, sexuality and sexual reproduction

Within the Eumycota, the Zygomycota form one of the basal groups (of more than 1000 species). The Zygomycota received their name by analogy to the other fungal groups based on the overt morphology of their sexual apparatus. Zygote formation in this group occurs within a structure arranged between two suspensor hyphae, and the whole apparatus typically resembles an oxen yoke (from the Greek ζυγός [zygos]). Sexuality is based on the interaction of two thalli of different mating types in the heterothallic species and on the interaction of two hyphae or hyphal branches in the same mycelium in homothallic species[1]. Zygomycota is distinguished from all other Eumycota by its ability to reproduce sexually by zygospores following gametangial fusion.

[1] Schipper, M.A. and Stalpers J.A. (1980) Various aspects of the mating system in Mucorales. Persoonia 11: 53-63.

2. *Blakeslea trispora*: the organism of this work

Blakeslea is a saprophyte that performs its vegetative cycle of spores, filamentous mycelia, fruiting bodies, and again spores on chemically defined media in the laboratory. Mycelia of this organism belong to either the (+) or the (-) mating type or sex[2].

Blakeslee[3] postulated that Zygomycetes belong to one of only two mating types, (+) and (-), a view which he was able to verify subsequently by more extensive studies[4]. None of the occasionally occurring features such as preference for a certain location for progametangial fusion or the actual size of the progametangia and gametangia could be unequivocally attributed to one of the mating types in any species[5]. Therefore, as no distinguishing morphological mark exists to define mating type, classification of newly isolated strains still requires mating reactions with defined tester strains.

Blakeslea, like Mucorales generally, undergoes two life circles, asexual (or vegetative) and sexual, that consist of a succession of different phases in morphology, biochemistry, physiology and behavior (Figure 1).

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[2] Mehta J.B.; Obraztsova N.I.; Cerdá-Olmedo E. (2003) Mutants and Intersexual Heterokaryons of *Blakeslea trispora* for Production on β-Carotene and Lycopene. Applied & Environmental Microbiology: 4043-4048.

[3] Blakeslee, A.F. (1904) Sexual reproduction in the *Mucorineae*. Proc. Am. Acad. Arts Sci. 40: 205-319.

[4] (a) Blakeslee, A.F. and Cartledge J.L. (1927) Sexual dimorphism in Mucorales. II. Interspecific reactions. Bot. Gaz. 84: 51-57. (b) Blakeslee, A. F.; Cartledge, J.L.; Welch, D.S. and Bergner, A.D. (1927) Sexual dimorphism in Mucorales. I. Intraspecific reactions. Bot. Gaz. 84: 27-50.

[5] Satina, S. and Blakeslee, A.F. (1930) Imperfect sexual reactions in homothallic and heterothallic Mucors. Bot. Gaz. 90: 299-311.

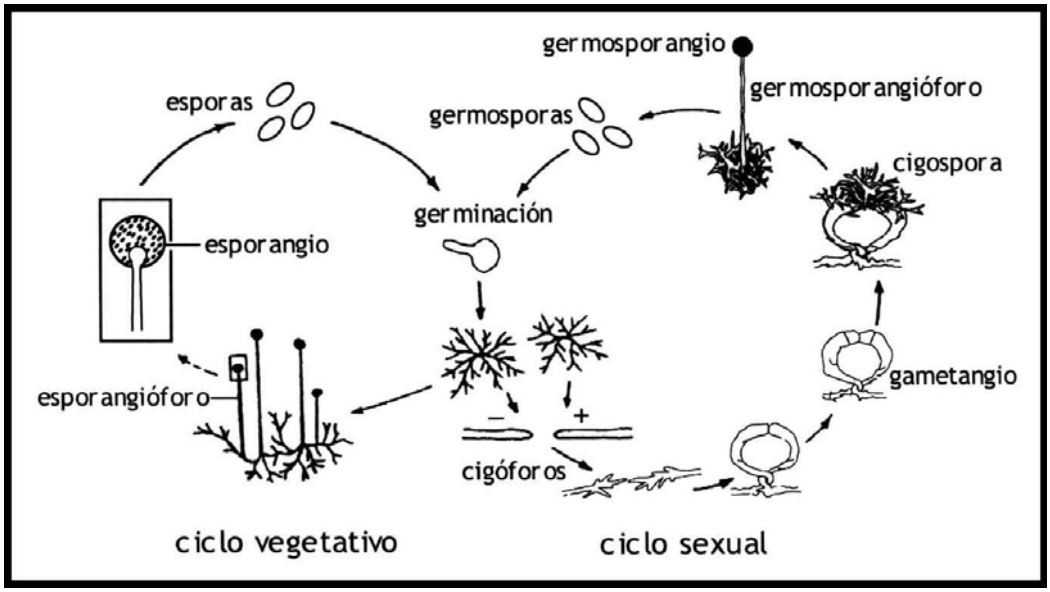

Figure 1: *Blakeslea's* life circle. The spores germinate to produce an hyphal branched mycelium. The sporangiophores excel of the mycelium and maintain at their distant end a sporangium that contains spores. Two sexes exist, when mycelia of each sex found, a series of biochemical and morphological events are triggered, leading to the formation of zygospore. After a latency period of several months, zygospore germinates producing a germosporangiophoro with his germosporangium and its germosporas.

When mycelia of the opposite sexes of many Mucorales meet on solid medium, they become bright yellow and produce a succession of special structures, particularly zygospores. The enhanced coloration is due to increases accumulation of β-carotene, which is termed sexual carotenogenesis to distinguish it from the vegetative carotenogenesis that occurs in single strain cultures. Sexual carotenogenesis occurs in mated cultures that contain mycelia of the two sexes, in single-strain cultures of either sex exposed to natural or synthetic trisporates[6], in single-strain cultures of intersexual heterokaryons, whose mycelia contain nuclei of both sexes[5,7] and in single-strain cultures of intersexual diploids[8].

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[6] Govind, N.S. and Cerdá-Olmedo E. (1986) Sexual activation of carotenogenesis in *Phycomyces blakesleeanus*. J. Gen. Microbiol. 132: 2775-2780.

[7] (a) Murillo, F.J.; Calderón, I.L.; López-Díaz, I. and Cerdá-Olmedo E. (1978) Carotene-superproducing strains of *Phycomyces*. Appl. Environ. Microbiol. 36: 639-642.

[8] Mehta, B.J. and Cerdá-Olmedo E. (2001) Intersexual partial diploids of *Phycomyces*. Genetics 158: 635-641.

3. The chemistry of sexual interaction

The compounds coordinating and synchronizing sexual reproduction have been isolated and identified from members of the order Mucorales, namely *Blakeslea trispora*, *Phycomyces blakesleeanus*, *Zygorhynchus moelleri* and *Mucor mucedo*. In all cases, the bioactive molecules belonged to the large family of trisporic acids (TSAs) representing a special group of "Apocarotenoids".

As "Apocarotenoids" ("apo" means "from") are defined the metabolites originated by carotene degradation, by means of loss of carbon atoms of the linear chain through oxidating reactions. The enormous number of "Apocarotenoids" that we meet in nature is due to the great number of their precursors "carotenoids" (more than 600 different naturally occurring carotenoids have been identified so far), to the different site of oxidation in the chain and the consequent functional modifications[9]. Apocarotenoids are widely distributed in nature and serve important biological functions. Examples of biologically active apocarotenoids include retinoids in animals, trisporic acid in fungi, and abscisic acid in higher plants.

Trisporoids, as mentioned above, are biosynthetically derived from β-carotene. The connection between β-carotene and trisporic acid was established early in trisporoids research, when firstly, trisporic acid was established as a substance produced by mated cultures of (+) and (-) strains of *B. trispora* and *M. mucedo*[10] enhancing β-carotene synthesis[11]. The

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[9] Schwartz, S.H.; Qin, X.; Zeevaart, J.A.D. (2001) Characterization of a novel carotenoid cleavage dioxygenase from plants. J. Biol. Chem. 276: 25208-25211.

[10] (a) van den Ende, H. (1967) Sexual factors of the Mucorales. Nature 215: 211-212. (b) Gooday, G. W. (1968). Hormonal control of sexual reproduction in *Mucor mucedo*. New Phytol. 67: 815-821.

[11] (a) Caglioti, A.L.; Cainelli, G.; Camerino, B.; Mondelli, R.; Prieto, A.; Quilico, A.; Salvatori T.; Selva, A. (1966) The structure of trisporic-c acid. Tetrahedron Suppl. 7: 175-187. (b) Vail, W.J.; Morris, C.; Lilly, V. G. (1967) Hormone-like substances which increase carotenogenesis in + and – sexes of *Choanephora cucurbitarum*. Mycologia 59: 1069-1074. (c) van den Ende, H. (1968) Relationship between sexuality and carotene synthesis in *Blakeslea trispora*. J. Bacteriol. 96: 1298-1303. (d) Govind, N.S.; Cerdá-Olmedo, E. (1986) Sexual activation of carotenogenesis in *Phycomyces blakesleeanus*. J. Gen. Microbiol. 132: 2775-2780.

biosynthetic connection was founded on the observations that reduction of the amount of β-carotene by either mutation[12] or treatment with diphenylamine[13] results in reduced trisporic acid synthesis. Moreover, radioactivity from ^{14}C-labeled β-carotene was found to be incorporated into trisporic acids at a higher ratio than that from ^{14}C-labeled mevalonate, allowing the conclusion that β-carotene as precursor of trisporoids is more likely than de novo synthesis via the isoprenoid synthesis pathway[14]. These findings imply that the first step in trisporoids synthesis is the oxidative cleavage of β-carotene.

Pioneering analytical work of van den Ende[15], Werkmann[16], Austin[17] and Sutter[18] with *B. trispora*, *P. blakesleeanus* and *M. mucedo* revealed a chemical dialogue between the sexual partners that required an exchange of early trisporoids as precursors for the bioactive trisporic acids[19].

The production of the different series of bioactive TSAs requires an enzymatic contribution from both mating partners, since the TSAs and their

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[12] (a) Sutter, R. P. (1975) Mutations affecting sexual development in *Phycomyces blakesleeanus*. Proc. Natl. Acad. Sci. USA 72: 127-130. (b) Sutter, R.P.; Grandin, A.B.; Moore, W.R. (1996) (-) Mating type-specific mutants of *Phycomyces* defective in sex pheromone biosynthesis. Fungal Genet. Biol. 20: 268-279.

[13] (a) Austin, D.J., Bu'Lock, J.D., Gooday, G.W. (1969) Trisporic acids: sexual hormones from *Mucor mucedo* and *Blakeslea trispora*. Nature 223: 1178-1179. (b) Bu'Lock, J.D.; Jones, B.E.; Taylor, D.; Winskill, N.; Quarrie, S.A. (1974b) Sex hormones in *Mucorales.* The incorporation of C_{20} and C_{18} precursors into trisporic acids. J. Gen. Microbiol. 80: 301-306.

[14] Austin, D.J.; Bu'Lock, J.D.; Drake, D. (1970) The biosynthesis of trisporic acids from β-carotene via retinal and trisporol. Experientia 26: 348-349.

[15] van den Ende H.; Werkman B.A.; van den Briel M.L. (1972) Trisporic acid synthesis in mated cultures of the fungus *Blakeslea trispora*. Archiv für Mikrobiologie 86: 175-184.

[16] Werkman T. A. and van den Ende H. (1973) Trisporic acid synthesis in *Blakeslea trispora*. Interaction between plus and minus mating types. Archiv für Mikrobiologie 90: 365-374.

[17] Austin D.J.; Bu'Lock J.D.; Drake D. (1970) The biosynthesis of trisporic acids from β-carotene via retinal and trisporol. Experientia 26: 348-349.

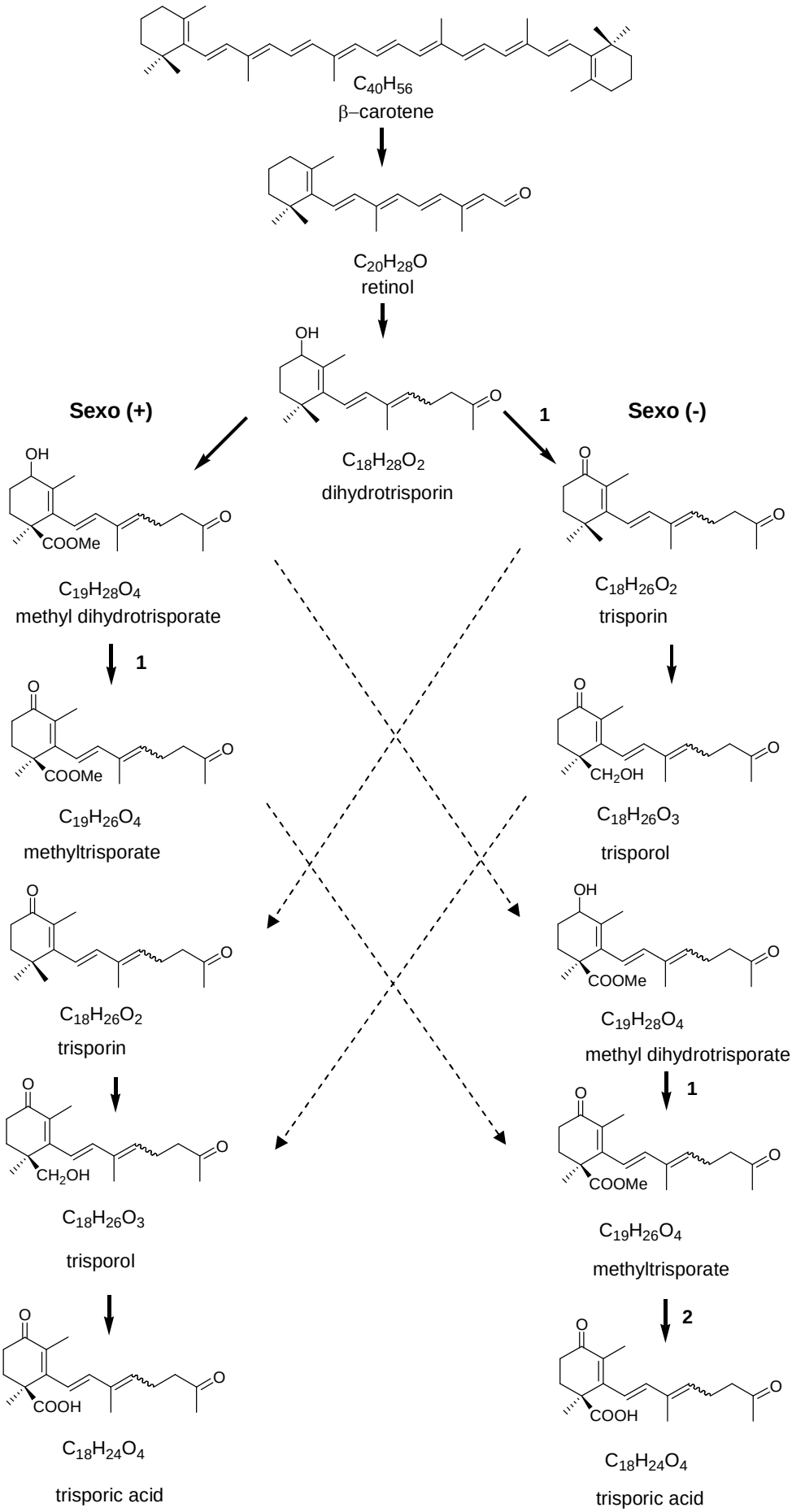
[18] Sutter R.P.; Harrison T.L.; Galasko G. (1974) Trisporic acid biosynthesis in *Blakeslea trispora* via mating type-specific precursors. Journal of Biological Chemistry 249: 2282-2284.

[19] (a) Sutter, R.P. (1986) Apotrisporin-E: A new sesquiterpenoid isolatedfrom *Phycomyces blakesleeanus* and *Blakeslea trispora*. Exp. Mycol. 10: 256–258. (b) Sutter, R.P.; Dadok, J.; Bothner-By, A.A.; Smith, R.R.; Mishra, P.K. (1989) Cultures of separated mating types of *Blakeslea trispora* make D and E forms of trisporic acids. Biochemistry 28: 4060–4066.

early precursor β-carotene are accumulated in the contact area of their hyphae.

As it is outlined in Scheme 1, both mating types are able to convert β-carotene to 4-dihydrotrisporin but lack the ability to generate significant amounts of the downstream metabolites required for the production of hormones, such as TSAs. Instead, the biosynthesis of the late trisporoids was assumed to require a different exchange of “prohormones” between the mating partners and results in a “cooperative biosynthesis” that offers additional regulatory possibilities and molecular diversification at the level of metabolites[20].

[20] Schachtschabel, D.; David, A.; Menzel, K.-D.; Schimek, C.; Wostemeyer, J. and Boland W. (2008) Cooperative Biosynthesis of Trisporoids by the (+) and (-) Mating Types of the Zygomycete *Blakeslea trispora*. ChemBioChem 9: 3004-3012.



Scheme 1: Biosynthetic pathway model for TSAs. It is observed a pheromone interchange between both sexes and some metabolic reactions associated with the sexual development. The arrows represent enzymatic reactions, whereas the interrupted lines the diffusion of the compounds between organisms. The numbers represent enzymes partially characterized: (1) dehydrogenase and (2) esterase[19,21].

4. Biologic effects of trisporoids

Certain trisporoids and specially TSAs and many of its isoforms and precursors are used from Zygomycota, as a communication system. As a part of their involvement is the recognition of mating partners, induce the first steps of sexual differentiation maintain the development of sexual structures and probably mediate the recognition between certain zygomycetes and mycoparasites, such as *Parasitella parasitica*[22].

At the moment, all the experimental evidences indicate that they are the sexual pheromones that stimulate the carotenogenesis and sexual morphogenesis of these fungi.

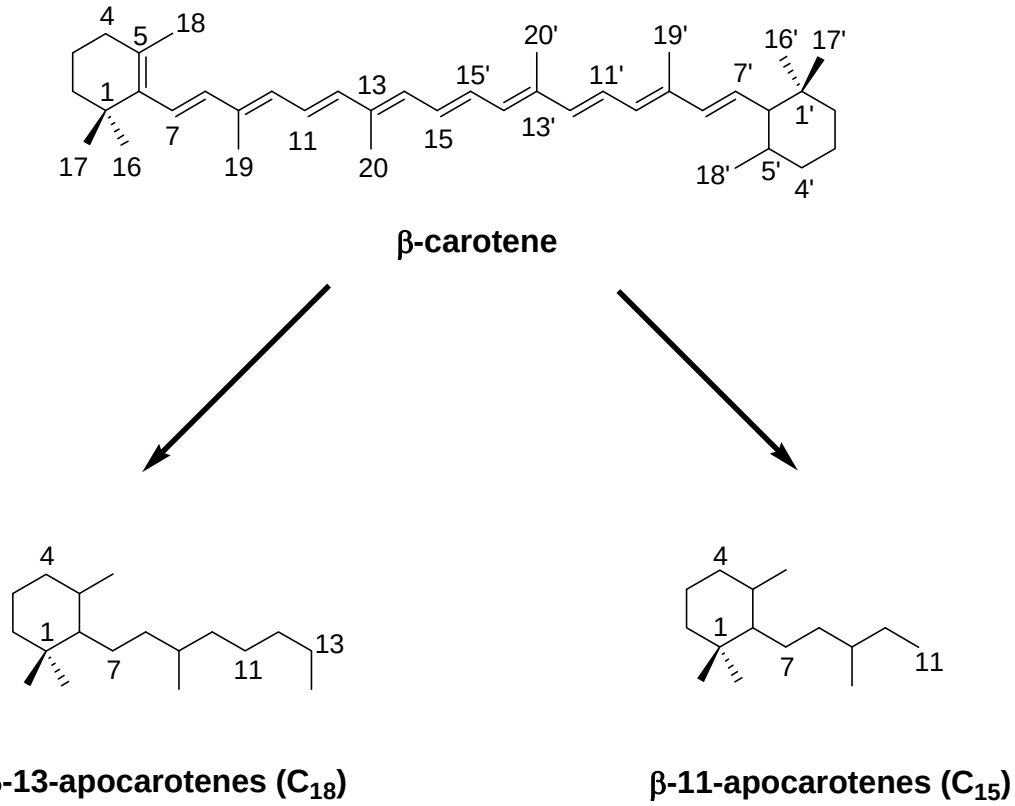
5. Classification of trisporoids

From the point of view of their chemical structure, exists two groups of Trisporoids (Scheme 2)

- Trisporoids that contain 18 (19 if a methyl ester group is present) carbon atoms, and are β-13-apocarotenes.
- Monocyclofarnesanes or Apotrisporoids, containing 15 carbon atoms and denominated β-11-apocarotenes.

[21] van de Ende, H. (1987) Sexual morphogenesis in the *Phycomycetes* in the filamentous fungi (ed. Smith J.E. and Berry D.R.), Wiley, New York 3: 256-274.

[22] Schultze, K.; Schimek, C.; Wostemeyer, J.; Burmester, A. (2005) Sexuality and parasitism share common regulatory pathways in the fungus *Parasitella parasitica*. Gene 348: 33-44.



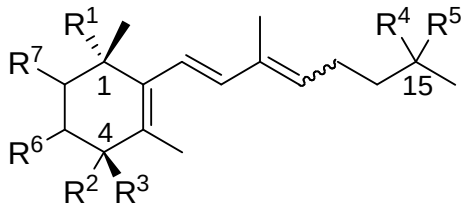
Scheme 2: Classes of Trisporoids

6. Structure of naturally existing Trisporoids

As mentioned above, as Trisporoids are denominated the compounds with 18 (19 if a methyl ester group is present) carbon atoms.

Trisporoids are grouped within A, B, C, D and E families according to their substitution pattern at R^4, R^5, R^6, and R^7 and according to further oxidative functionalization of the ring system (R^1, R^2, R^3) in trisporols, trisporic acids, methyl trisporates, methyl 4-dihydrotrisporates, and trisporins (Scheme 3)[23].

[23] Schachtschabel, D. and Boland, W. (2007) Efficient Generation of a Trisporoid Library by Combination of Synthesis and Biotransformation. J.Org.Chem. 72: 1366-1372.



R^1=CH_2OH,COOH,$COOCH_3$,CH_3

R^2,R^3=H,OH

R^2,R^3=O

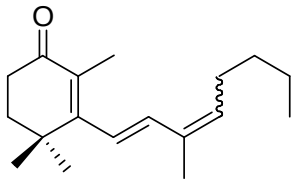
Family	**R^4**	**R^5**	**R^6**	**R^7**
A	H	H	H	H
B	=O		H	H
C	H	OH	H	H
D	=O		H	OH
E	H	OH	OH	H

Scheme 3: Functionalization patterns of Trisporoids

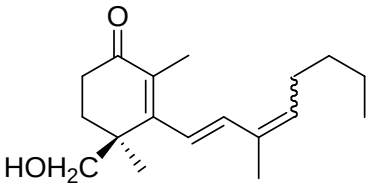
The B and D families have a ketone group at C13, and the C and E families have a hydroxyl group. The A family has no oxygenated function at C2, C3 and C13. The D and E families have also a hydoxyl group at C2 and C3, respectivily.

All trisporoids have *E* stereochemistry in C7, C8 double bond and *E* or *Z* stereochemistry in C9, C10 double bond.

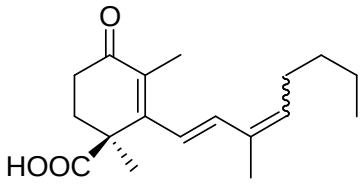
Trisporoids of A-family



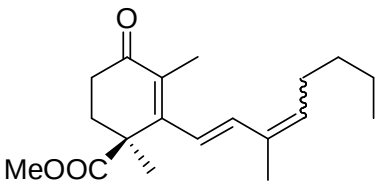
trisporin A



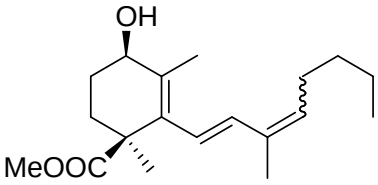
trisporol A



trisporic acid A

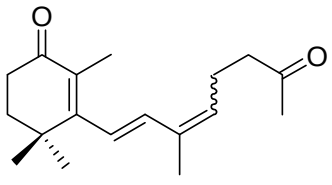


methyl trisporate A

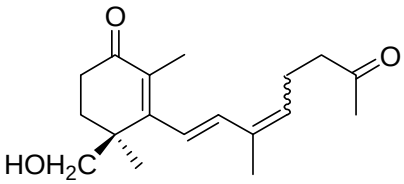


methyl 4-dihydrotrisporate A

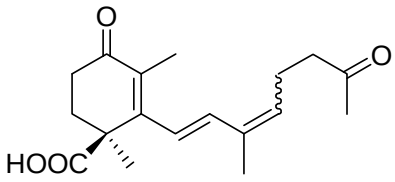
Trisporoids of B-family



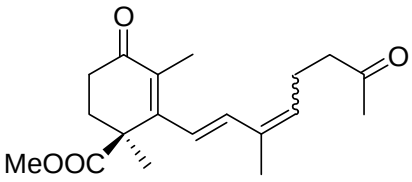
trisporin B



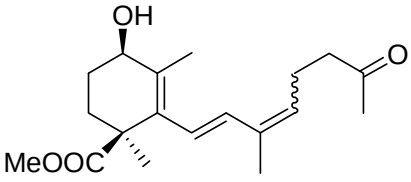
trisporol B



trisporic acid B

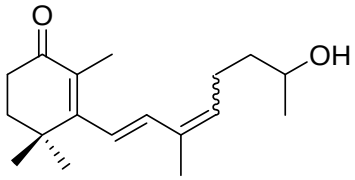


methyl trisporate B

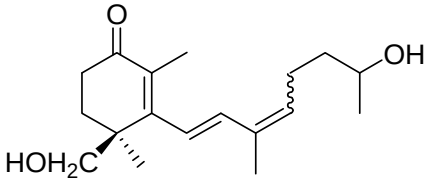


methyl 4-dihydrotrisporate B

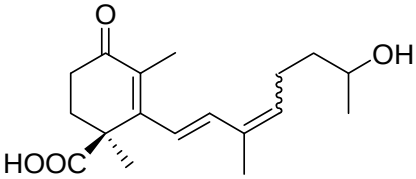
Trisporoids of C-family



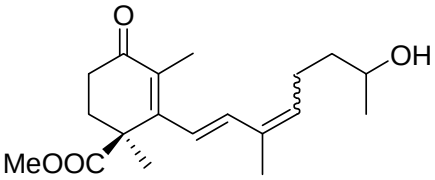
trisporin C



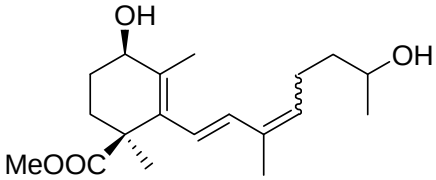
trisporol C



trisporic acid C

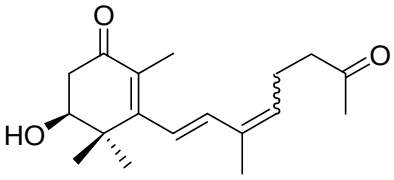


methyl trisporate C

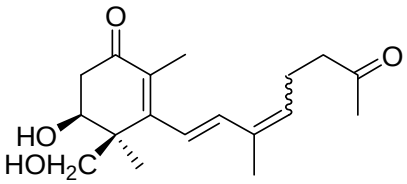


methyl 4-dihydrotrisporate C

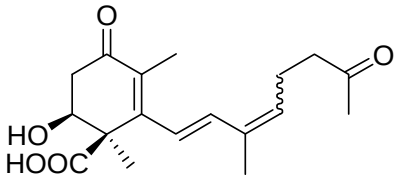
Trisporoids of D-family



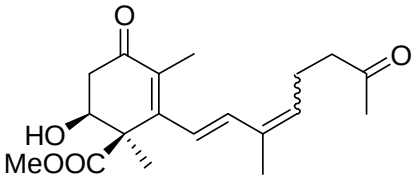
trisporin D



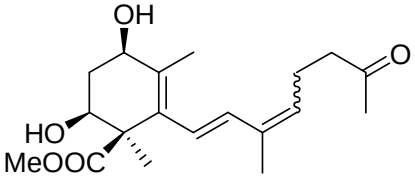
trisporol D



trisporic acid D

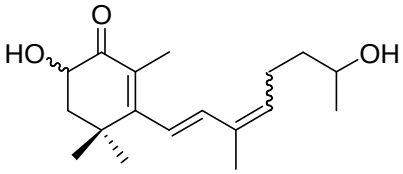


methyl trisporate D

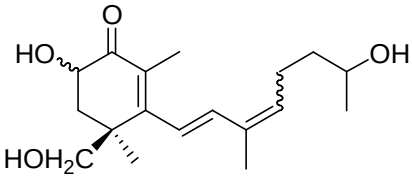


methyl 4-dihydrotrisporate D

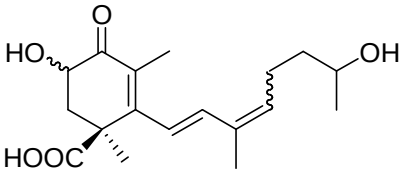
Trisporoids of E-family



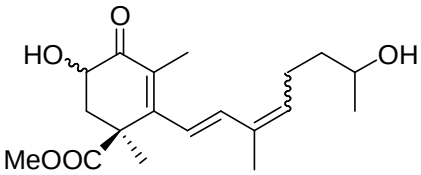
trisporin E



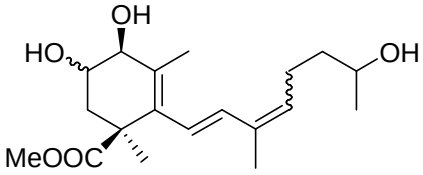
trisporol E



trisporic acid E

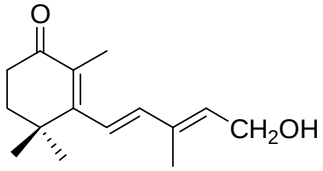


methyl trisporate E

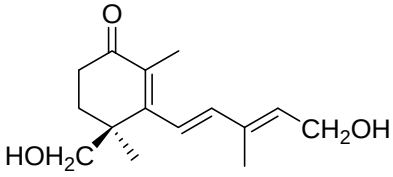


methyl 4-dihydrotrisporate E

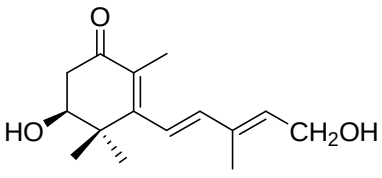
Apotrisporoids or Monocyclofarnesanes



apotrisporin



apotrisporol



apotrisporin E

7. Interest of sexual interaction on Mucorales

As it is mentioned above, in mated cultures of (+) and (-) mating type of the Mucorales has been observed the formation of important amounts of trisporoids, obtained from the culture medium. Certain trisporoids, especially trisporic acids and methyl trisporates, have excellent biological activities. Among these, one of their most interesting biological properties is their capacity to induce the formation of zygophoros, the first of the steps in the sexual process of Mucorales. This result, as well as a considerable increase in the production of β-carotene in these fungi (carotenogenesis), confers to trisporoids a great industrial interest.

To date, carotenoids are produced mainly artificially by chemical synthesis, but due to the increasing preference for more ecological production, the extraction of carotenoids from natural sources has become an important alternative. For this reason, sexual interaction of Zygomycetes has gained a great industrial interest, with special concern in the production of β-carotene from *Blakeslea trispora*. *Blakeslea* increase their carotenogenesis during sexual interaction and after exposure to β-ionone[2]. The accumulation of β-carotene production in *B. trispora* linked to sexual interaction of these fungi, increases 13- to 15- fold during mating of (-) and (+) strains[5].

Carotenoids in general have antioxidant properties and protect organisms from reactive oxygen species[24]. They are also thought to have anti-tumour effects[25] and have been considered useful in the prevention of many chronic diseases[26].

Furthermore, β-carotene is one of the carotenoids more appreciated by the agro-alimentary, pharmaceutical and cosmetic industries, due to its coloring properties. Additional β-carotene is the precursor of Vitamin A and

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[24] Bartley, G.E.; Scolnik, P.A. (1995) Plant carotenoids: pigments for photoprotection, visual attraction and human health. Plant Cell 7: 1027-1038.

[25] Mayne S.T. (1996) Beta-carotene, carotenoids and disease prevention in humans. FASEB J. 10: 690-701.

[26] Smith TAD. (1998) Carotenoids and cancer: prevention and potential therapy. Br.J. Biomed Sci 55: 268-275.

displays great antioxidant properties. Also it contributes to the delay or prevention of cardiovascular and chronic diseases like cancer, arthritis, cataract, etc[27].

As has been mentioned above, apocarotenoids are natural products derived from the oxidative cleavage of carotenoids. These reactions are catalysed by a family of proteins denominated generally “Carotenoid cleavage dioxygenases” (CCDs)[28]. This type of enzymes is a class of non-heme iron enzymes, which seems to have high specificity concerning the point of carotene cleavage.

One of these is β-carotene-15,15´-dioxygenase (BCDO1, EC 1.14.99.36) which acts at specific double bonds of the carotenoid molecule (β-carotene) resulting to the production of two molecules of retinal. The three-dimensional structure of this enzyme it is showed in Figure 2.

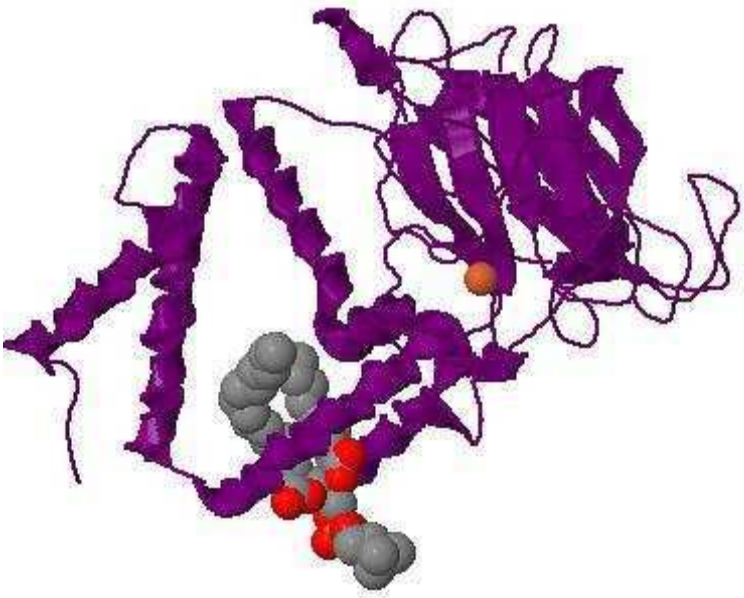


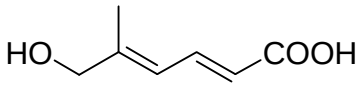
Figure 2: Three-dimensional structure of β-carotene-15,15´-dioxygenase

[27] (a) Ross, A.; Ternus, C.; Maureen, E. (1993) Vitamin A as a hormone: Recent advances in understanding the actions of retinal, retinoic acid and beta-carotene. Journal of the American Medical Association 93: 1285-1290. (b) Bertram, J.S. (1994) The chemoprevention of cancer by dietary carotenoids: studies in mouse and houman cells. Pure & Appl. Chem. 66: 1025-1032.

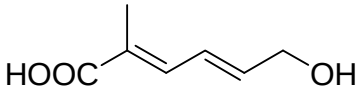
[28] Bouvier, F.; Isner, J.C.; Dogbo, O. and Camara, B. (2005) Oxidative tailoring of carotenoids: a prospect towards novel functions in plants. Trends Plant Sci 10: 187-194.

8. Objective of this Thesis

Continuing our researches on biotechnology of filamentous fungi,[29] the principal objective of this research is to find the chemical signals that trigger the sexual interaction in Mucorales and the overproduction of β-carotene, helping to improve the industrial production of β-carotene, lycopene and other chemical products. In this sense the group of Prof. Cerdá-Olmedo has demonstrated recently that both effects are independent and therefore different mechanisms must exist for its development.[30] As part of this research have been isolated from mated cultures in solid medium of the A56(+) and NRRL1555(-) strains of *Phycomyces blakesleeanus,* the Blakesleoic acids A and B (C7 apocarotenoids), new natural products which form a new family of apocarotenoids, together with different trisporoids (C18 apocarotenoids) and apotrisporoids (C15 apocarotenoids).



Blakesleoic acid A



Blakesleoic acid B

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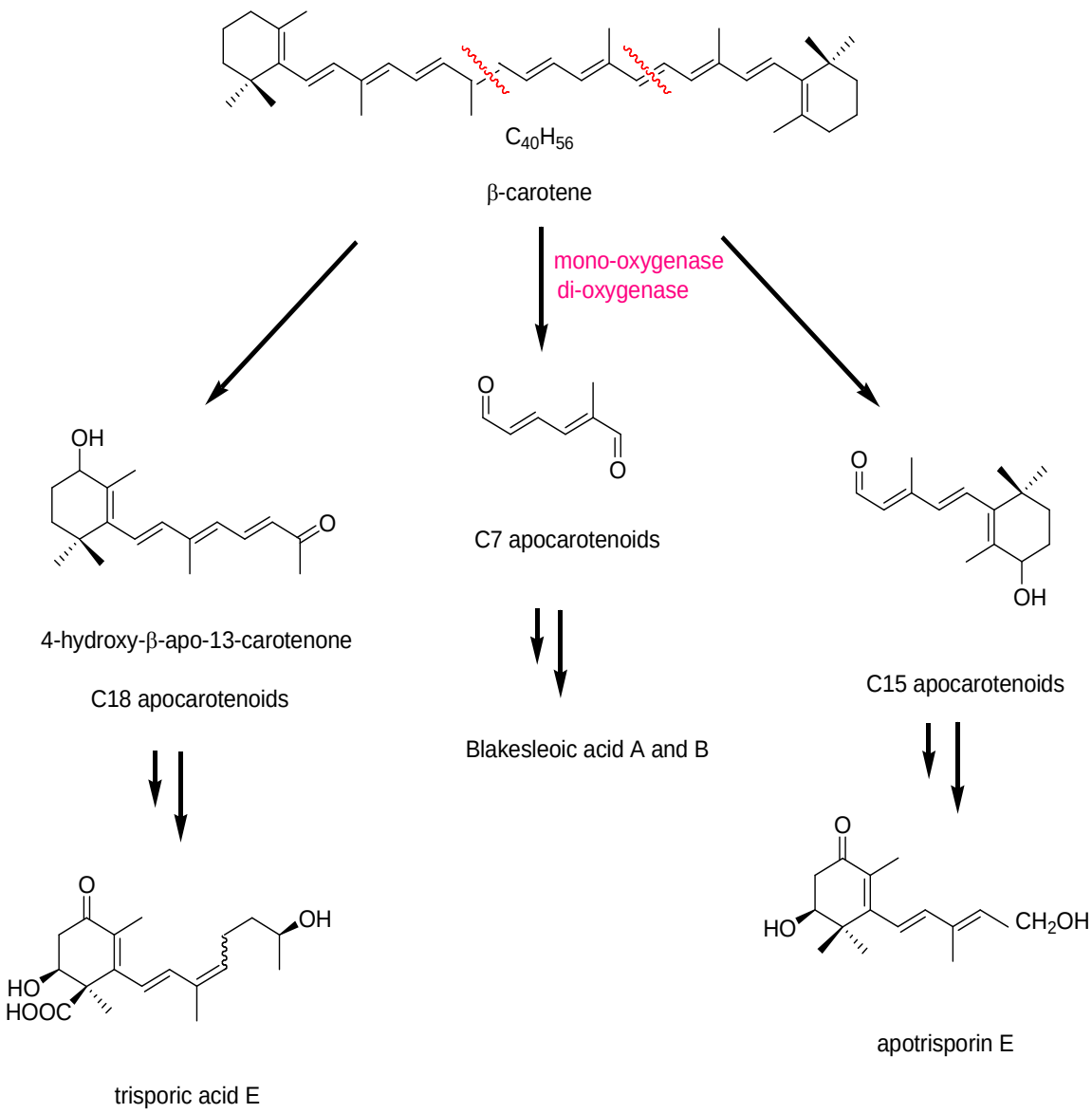
[29] a) Avalos, J.; Cerdá-Olmedo, E.; Reyes, F.; Barrero, A. F. (2007**)** Gibberellins and Other Metabolites of *Fusarium fujikuroi* and Related Fungi. Current Organic Chemistry 11: 721-737. b) Barrero, A.F.; Oltra, J.E.; Cabrera, E.; Reyes, F.; Alvarez, M. (1999) Metabolism of gibberellins and ent-kaurenoids in mutants of *Gibberella fujikuroi.* Phytochemistry 50: 1133-1140. c) Weinkove, D.; Poyatos, J.A.; Greiner, H.; Oltra, J.E.; Avalos, J.; Fukshansky, L.; Barrero, A.F.; Cerdá-Olmedo, E. (1998) Mutants of *Phycomyces* with Decreased Gallic Acid Content. Fungal Genetics and Biology 25: 196-203. d) Barrero, A.F.; Oltra, J.E.; Poyatos, J.A.; Jimenez, D.; Oliver, E. (1998) Phycomysterols and other sterols from the fungus *Phycomyces blakesleeanus*. Journal of Natural Products 61: 1491-1496. e) Barrero, A.F.; Oltra, J.E.; Poyatos, Juan A. (1996) Acidic metabolites from *phycomyces blakesleeanus*. Phytochemistry 42: 1427-1433. f) Fernandez-Martin, R.; Reyes, F.; Domenech, C.E.; Cabrera, E.; Bramley, P.M.; Barrero, A.F.; Avalos, J.; Cerdá-Olmedo, E. (1995) Gibberellin biosynthesis in *gib* mutants of *Gibberella fujikuroi*. Journal of Biological Chemistry 270: 14970-4.

[30] Kuzina, V. and Cerdá-Olmedo, E. (2006) Modification of Sexual Development and Carotene Production by Acetate and Other Small Carboxylic Acids in *Blakeslea trispora* and *Phycomyces blakesleeanus*. Appl. Environ. Microbiol. 72: 4917-4922.

As consequence of these results Barrero *et al*. have proposed a new biosynthetic pathway for this type of compounds in this fungus by oxidative double cleavage of β-carotene (Scheme 4).[31]



Scheme 4: New biosynthetic proposal of apocarotenoids in *Phycomyces blakesleeanus*.

[31] Barrero, A. F.; Herrador, M. M.; Arteaga, P. (2009) 2º Congreso del GEQPN-RSEQ. La Palma.

The new proposed biosynthetic pathway consists of a double cleavage of β-carotene, carried out by a β-carotene dioxygenase acting at specific double bonds, C13, C14 and C11', C12', giving rise to the formation of the three previous fragments. The three compound types are formed in equimolecular quantities.

The chemical study of apocarotenoids in the single and mated cultures of the wild strains, F986(+) and F921(-), of *Blakeslea trispora* is presented in this manuscript, approached principally on the identification of C7 apocarotenoids together with C18 and C15 apocarotenoids. The identification of these metabolites will indicate that our scheme for splitting β-carotene into C18, C15 and C7 apocarotenoids in *P. blakesleeanus* is valid for *Blakeslea trispora* as well.

This research is part of the Projects CTQ2006-15575-C02-01 (Ministerio de Educación y Ciencia) and of Excelencia 2005/CVI-910 (Junta de Andalucía).

II. ADVANCES IN THE STUDIES ON TRISPOROIDS

1. Advances in the studies on trisporoids

Studies on trisporoids began in 1966 with the work of Caglioti *et al*[32]., who managed to isolate and identify trisporic acids B and C from Acid fractions of *Blakeslea trispora's* mated cultures. Only one year later, Cainelli *et al*[33]*.* isolated and identified apotrisporol and apotrisporin from neutral fraction of *Blakeslea trispora* cultivations.

Few years later, in 1972, Bu´Lock *et al*[34]., identified trisporic acid A, the separate 9-*cis* and 9-*trans* isomers of trisporic acids B and C and trisporol C, all obtained from mated cultures of *Blakeslea trispora*. Only two years later, in 1974, the group of Bu´Lock *et al*[35]*,* proposed a biosynthetic route from β-carotene to trisporic acid, in *Blakeslea trispora*, but leaving some uncertainties as to the sequence of later steps in the process (Scheme 5). This biosynthetic pathway is accepted until to date.

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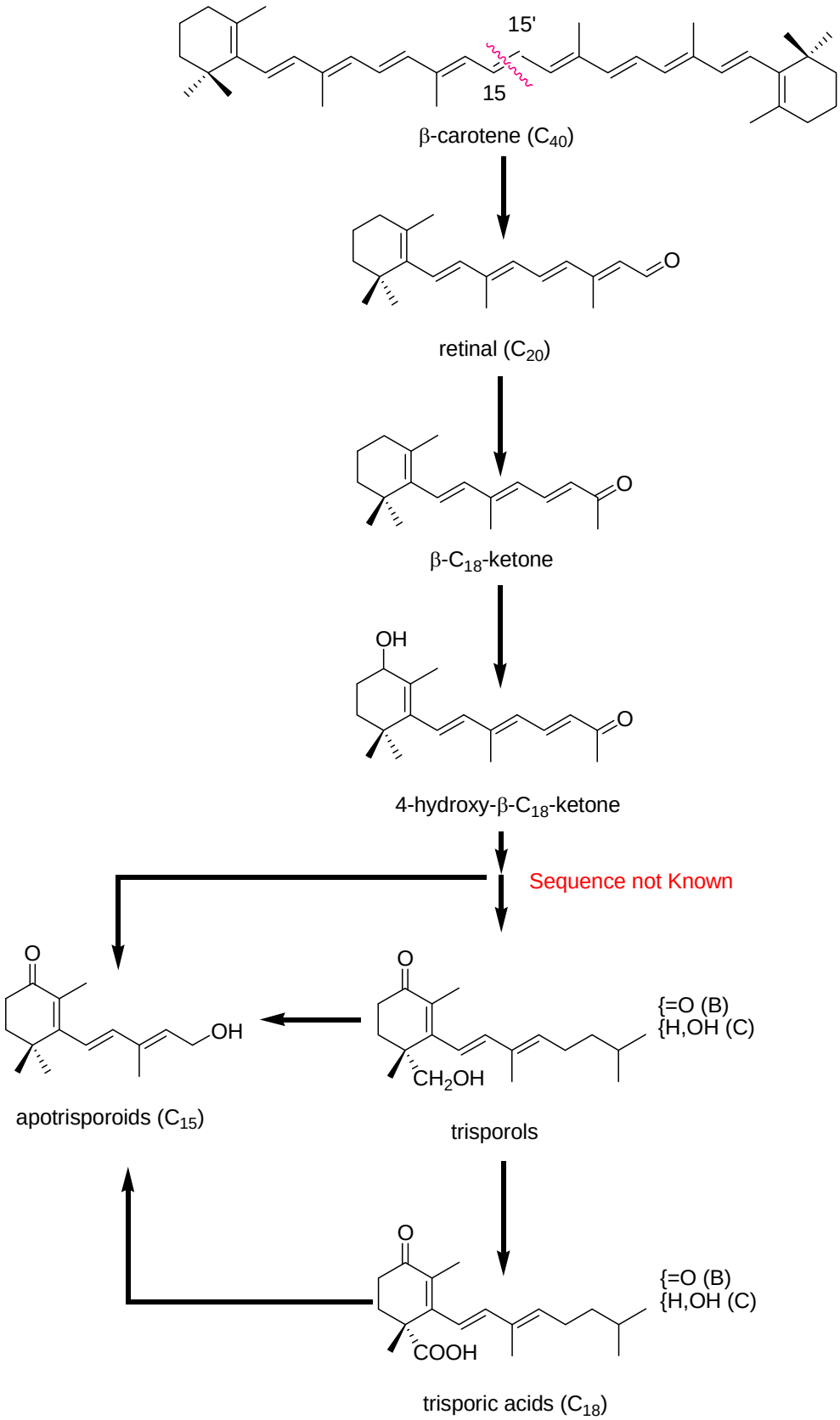
[32] Caglioti, A.L.; Cainelli, G.; Camerino, B.; Mondelli, R.; Prieto, A.; Quilico, A.; Salvatori, T.; Selva, A. (1966) The structure of trisporic-c acid. Tetrahedron Suppl. 7: 175-187.

[33] Cainelli, G.; Camerino, B.; Grasselli, P.; Mondelli, R.; Morrocchi, S.; Prieto, A.; Quilico, A., Selva, A. (1967a) Struttura del trisporone e dell'anidrotrisporone. Chim. Ind. 49: 748-751.

[34] Bu´Lock, J.D.; Drake, D.; Winstanley, D. J. (1972) Specificity and transformations of the trisporic acid series of fungal sex hormones. Phytochemistry 11: 2011-2018.

[35] Bu´Lock, J.D.; Jones, B. E.; Taylor, D.; Winskill, N. and Quarrie, S.A. (1974) Sex Hormones in Mucorales. The Incorporation of C_{20} and C_{18} precursors into Trisporic Acids. Journal of General Microbiology: 80: 301-306.



Scheme 5: Biosynthesis of trisporic acids in mated cultures of *Blakeslea trispora* through a 15,15'-cleavage of β-carotene.

The suggested biosynthetic pathway for trisporic acid formation in mated *Blakeslea trispora* cultures, presents as key step the enzymatic degradation of β-carotene into two retinal (C_{20}) molecules, which subsequently undergo transformation into β-C_{18}-ketone (C_{18}) and it is transformed finally to trisporic acids (C_{18}) and apotrisporoids (C_{15}) through several steps not well known.

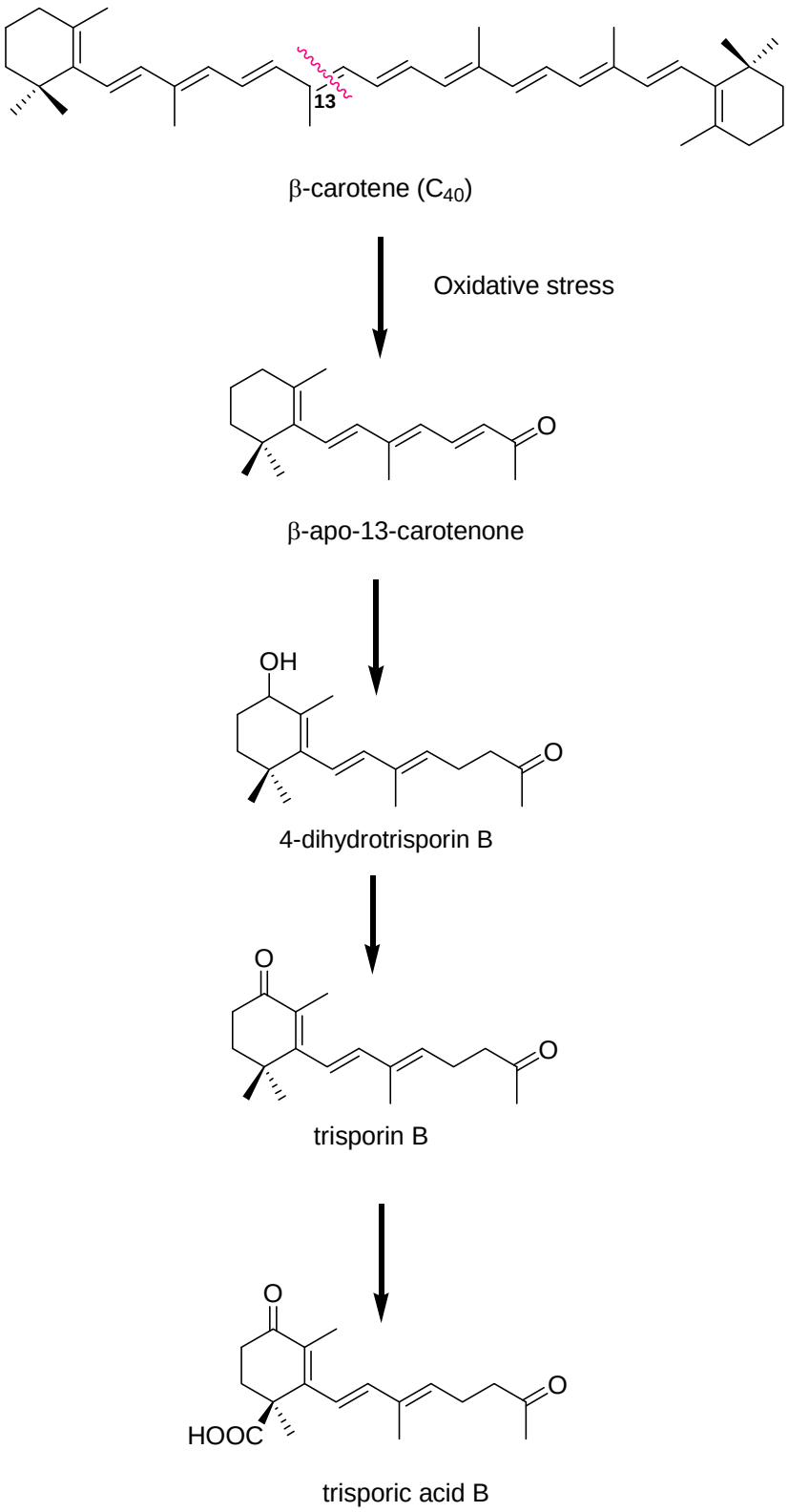
More ahead, Sutter *et al.*[36] in 1986 identified apotrisporin E in mated cultures of *Blakeslea trispora* and three years later, in 1989, the same group of Sutter et *al*.[37], managed to isolate and identify trisporic acids D and E in mated cultures of *Blakeslea trispora*.

A few years later, in 2002, the group of Gessler *et al*[38]., suggested that under oxidative stress, the oxidative degradation of β-carotene into β-apo-13-carotenone results in the formation of trisporic acids. The proposed Scheme (Scheme 6), demonstrated the connection between activation of trisporic acids synthesis with the development of oxidative stress.

[36] Sutter, R.P. (1986) Apotrisporin-E: A New Sesquiterpenoid Isolated from *Phycomyces blakesleeanus* and *Blakeslea trispora*. Experimental Mycology 10:256-258.

[37] Sutter, R.P.; Dadok, J.; Bothner-By, A.A.; Smith, R.R.; Mishra, P.K. (1989) Cultures of separated mating types of *Blakeslea trispora* make D and E forms of trisporic acids. Biochemistry 28: 4060–4066.

[38] Gessler, N.N.; Sokolov, V.A.; Belozerskaya, A.T. (2002) Initial stages of Trisporic Acid Synthesis in *Blakeslea trispora*. Appl. Biochem. Microbiol. 38: 536-543.



Scheme 6: Initial stages of TSA B synthesis from β-carotene in *Blakeslea trispora.*

In 2006, Kuzina V. and Cerdá-Olmedo E.[39], demonstrated that the morphogenetic and metabolic changes induced by sexual interaction in cultivations of *Blakeslea trispora* and *Phycomyces blakesleeanus*, are not tightly coupled as a single response. The increase in carotene biosynthesis and the induction of sexual morphogenesis that lead to zygospore production, demonstrated that are two processes that do not follow a common mechanism. The result is significant for understanding sexuality in Zygomycetes and for practical application of this sexuality in the carotene industry.

Recently, in 2007, Schachtschabel *et al.*[40] carried out the chemical synthesis of natural and similar trisporoids and has experimentally verified the obtaining of trisporic acids and trisporols by means of biotransformation of trisporina.

Besides, the same year, 2007, the group of Burmester *et al*.[41], identified the genes that encode the carotene oxygenase that is responsible for the cleavage of β-carotene as the first step in trisporoids synthesis, verifying with this the enzymatic cleavage of β-carotene as the first step is sexual hormone synthesis in Zygomycetes.

Finally, and up to day, the group of Schachtschabel *et al.* [42] proposed in 2008 a cooperative biosynthesis of trisporoids (Scheme 7) by both mating partners, which was reinvestigated in this contribution by the administration of specifically labeled precursors to individual cultures of the (-) and (+) strains

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[39] Kuzina, V. and Cerdá-Olmedo, E. (2006) Modification of Sexual Development and Carotene Production by Acetate and Other Small Carboxylic Acids in *Blakeslea trispora* and *Phycomyces blakesleeanus*. Applies and Environmental Microbiology 72: 4917-4922.

[40] Schachtschabel, D. and Boland, W. (2006) Efficient Generation of a Trisporoid Library by Combination of Synthesis and Biotransformation. J.Org.Chem. 72: 1366-1372.

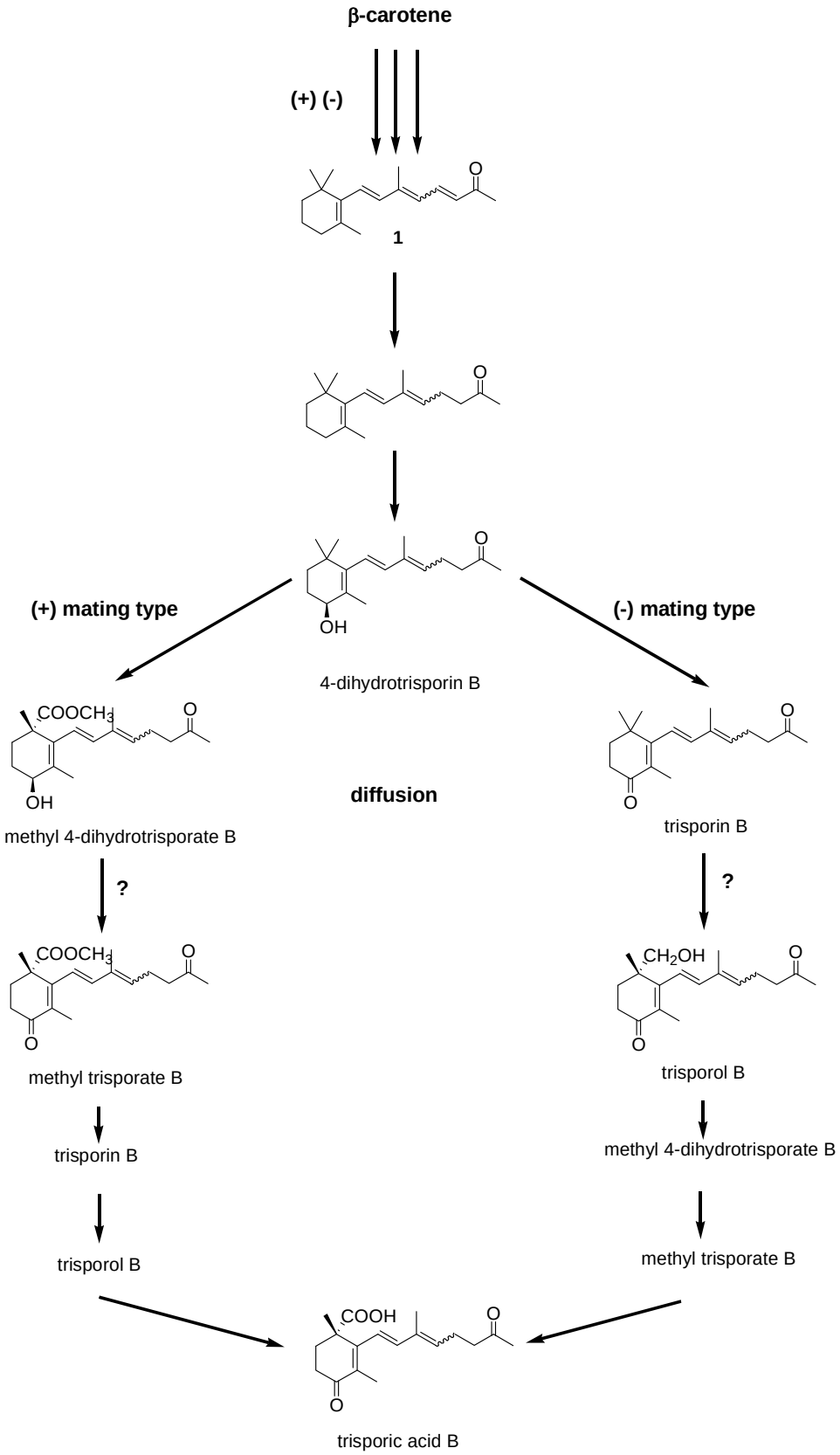
[41] Burmester, A.; Richter, M.; Schultze, K.; Voelz, K.; Schachtschabel, D.; Boland, W.; Wostemeyer, J.; Schimek, C. (2007) Cleavage of β-carotene as the first step in sexual hormone synthesis in zygomycetes is mediated by a trisporic acid regulated β-carotene oxygenase. Fungal Genetics and Biology 44: 1096-1108.

[42] Schachtschabel, D.; David, A.; Menzel, K-D.; Schimek, C.; Wostemeyer, J.; Boland, W. (2008) Cooperative Biosynthesis of Trisporoids by the (+) and (-) Mating Type of Zygomycete *Blakeslea trispora*. ChemBioChem. 9: 3004-3012.

as well as to mixed cultures of *Blakeslea trispora*. This new biosynthetic pathway validates the previously postulated pathway for trisporic acids biosynthesis in *Blakeslea trispora* through β-carotene enzymatic cleavage. As it is shown in Scheme 7, both mating types are able to convert β-carotene to 4-dihydrotrisporin B, which is the last common intermediate of both sexes, but lack the ability to generate significant amounts of the downstream metabolites required for the production of hormones such as trisporic acid B. Therefore for further transformation to occur, mating partners are supposed to exchange early metabolites, such as trisporin B and methyl 4-dihydrotrisporate B, with their complementary mating partners. According to this concept, trisporic acid B is generated in the (-) type by the saponification of methyl trisporate B or in the (+) mating type by the oxidation of trisporol.

Although the sequence of Scheme 7 apparently matches currently known aspects of sexual recognition and differentiation in Zygomycetes, many details of the elegant and convincing Scheme remain speculative, such as the β-carotene cleavage product 1, which have never been isolated or characterized.

The same biosynthetic route can be extended for the production of trisporic acids A, C, D and E.



Scheme 7: Postulated cooperative biosynthetic pathway for trisporic acid B in cultivations of *Blakeslea trispora*. The production of TSA B requires the exchange of intermediates between the mycelia of the different mating types.

2. Trisporoids identified in *Blakeslea trispora*

In Figure 3 are presented trisporoids that have been so far isolated and identified in cultivations of *Blakeslea trispora.*

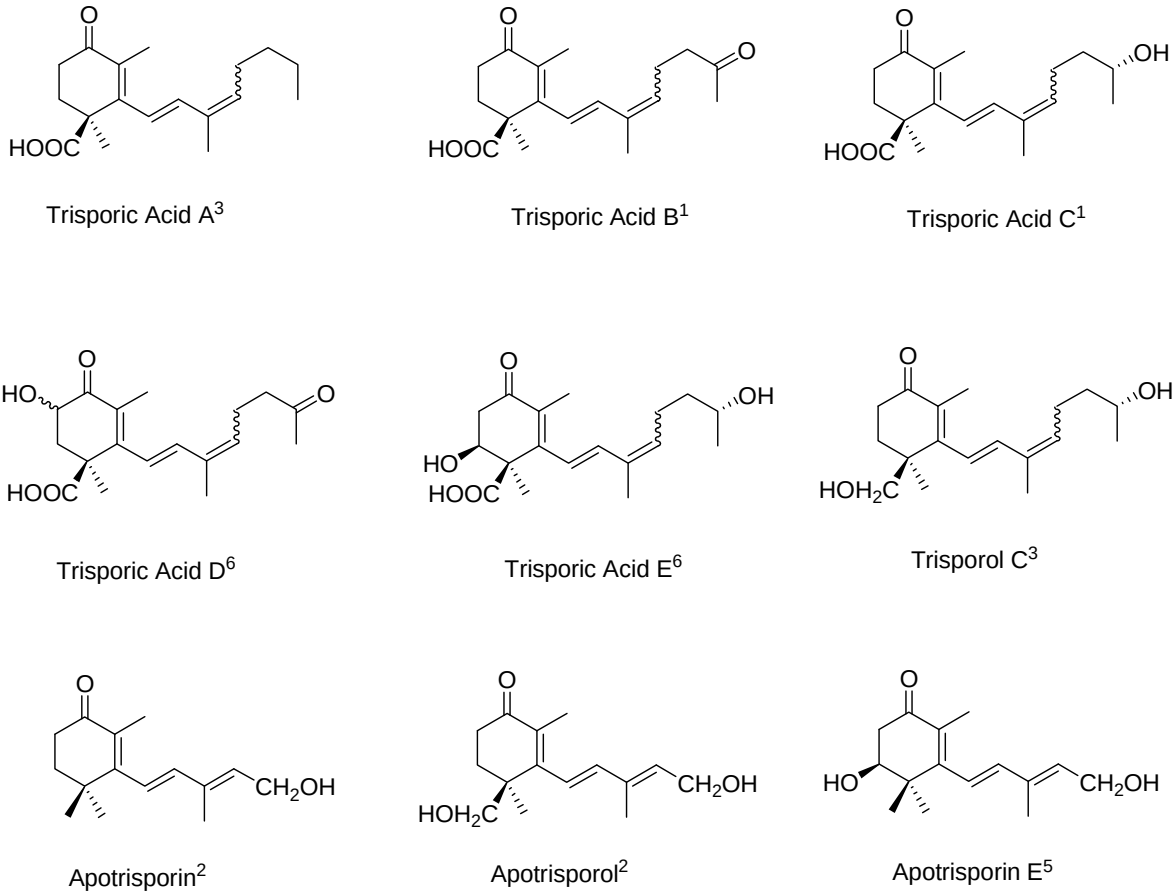


Figure 3: Trisporoids identified in cultivations of *Blakeslea trispora*

III. RESULTS AND DISCUSSION

The study of apocarotenoids involves the extraction, separation and identification of the metabolites that appear at the medium of single or mated cultures of strains of *Blakeslea trispora,* inoculated with spores from one or both sexes of the fungus respectively.

For this purpose we used the F986(+) and F921(-) wild strains of *B. trispora*. We used two types of culture media, liquid with shaking and solid. The single cultures were carried out in liquid medium as in solid medium, while the mated cultures were only carried out in solid medium where the crossing of both sexes is greater than in liquid medium.[43] All cultures were incubated at 30ºC in darkness and the spore concentration inoculated per each 25 mL of culture medium was 50 μL from 10^6 spores/ml of the F986(+) strain and 100 μL from 10^6 spores/ml of the F921(-) strain.

In the cultures in solid medium, the incubation period was for 3 days, whereas in the cultures in liquid medium, a study of the optimal incubation period for a maximum production of trisporoids was carried out. This study was realized by an analysis of the absorption in the UV at different λ (230, 250, 280, 300 & 328 nm) where the different trisporoids present in the culture medium must absorb in accordance with existing chromophores in their structure (carbonyl with conjugated double bonds, dienes, etc). In accordance with the results obtained from single cultures of *Phycomyces blakesleeanus* (optimum incubation period: 5 days), it was carried out a first culture of the F986(+) strain at 200 rpm for 5 days. The work up of the culture led to an extract where no trisporoids were identified. Due to this result the incubation period was prolonged to 10 days taking aliquots every 24 hours, starting from the fourth day and determining the absorption in the UV. This same study was carried out for the F921(-) strain. The results are shown in Table 1.

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[43] López Fernández, J.A. (2008) Tesis de Master: Trisporoids en *Phycomyces blakesleeanus* y *Blakeslea trispora,* Instituto de Biotecnología, Universidad de Granada.

Table 1: Absorbance results of single strain F986(+) & F921(-) cultures of *B. trispora* in liquid medium

Strain	Incubation Period (days)	λ	Absorbion (A)	^{1}HNMR
F986(+)	4th	328	0.051	No
		300	0.138	
		280	1.357	
		250	2.322	
		230	1.562	
	5th	328	0.077	
		300	0.186	
		280	1.703	
		250	2.812	
		230	1.967	
	6th	328	0.100	
		300	0.218	
		280	1.473	
		250	2.705	
		230	2.222	
	7th	328	0.139	
		300	0.336	
		280	1.724	
		250	2.994	
		230	2.833	
	10th	328	0.230	
		300	0.584	
		280	1.062	
		250	1.666	
		230	3.034	
F921(-)	4th	328	0.000	No
		300	0.038	
		280	0.387	
		250	0.770	
		230	1.081	
	5th	328	0.042	
		300	0.130	
		280	1.100	
		250	1.975	
		230	1.907	
	6th	328	0.052	
		300	0.166	
		280	1.733	
		250	2.951	
		230	2.169	
	10th	328	0.130	
		300	0.461	
		280	2.238	
		250	3.058	
		230	2.864	

As it is shown in Table 1, the results in both strains are similar and the absorption in the UV was stabilized or decreased at the tenth day of incubation. When the cultures were manipulated after ten days of incubation, the presence of trisporoids was not observed in the ^{1}H NMR spectrum realized to obtained extracts.

In the absence of trisporoids in the liquid cultures, single cultures of both strains in solid medium were performed to verify if the type of medium affected the trisporoids production by the fungus as was shown previously for mated cultures.[1] The results obtained were identical to those in cultures in liquid medium.

The mated cultures of both strains were carried out under the same conditions that the single cultures and in the same medium.

The aspect that shows the cultures in liquid (five days) and solid medium (three days) are shown in Figures 4 and 5, respectively.

Figure 4: Liquid cultures: On the left it is shown a culture of F921(-) strain, to the right a culture of the F984(+) strain and in the middle a mated culture of both strains.

Figure 5: Solid cultures: On the left is shown a culture of the F986(+) strain, in the middle a culture of F921(-) strain and to the right a mated culture of both strains.

As it is observed in both figures, the mated cultures present a much more intense orange coloration than the single ones, due to β-carotene overproduction which is related to trisporoids production. Thus in the case of mated cultures, the trisporoids production can be seen with naked eye.

1. Extraction and Fractionation of apocarotenoids

After the incubation period, proceeded to the extraction and fractionation of the apocarotenoids formed, following an appropriate methodology to the culture type.

1.1 Extraction and Fractionation of cultures in Liquid Medium

The methodology that was followed for the extraction and fractionation of the cultures in medium liquid is presented in Figure 6. First, was removed the mycelia biomass by filtration. Then the liquid culture was adjusted to pH 8.0 by the addition of NaOH 2N and extracted with ethyl acetate -EtOAc- (Fraction Neutral)**.** Then, the aqueous phase of the extraction was adjusted to

pH 2.0 by addition of HCl 2N and extracted with the same solvent –ethyl acetate- (Fraction Acid). The remnants of the aqueous phase were bound to anhydrous sodium sulphate and removed by filtration.

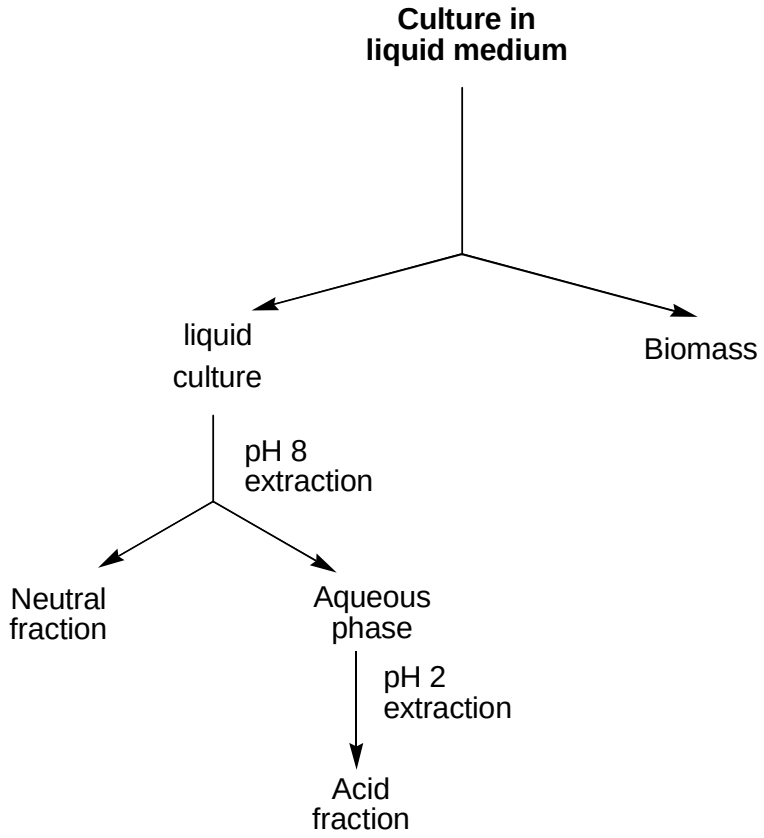


Figure 6: Methodology of extraction and fractionation of liquid cultures**.**

In Table 2 are presented the amounts of biomass, of Neutral and Acid fractions from a culture medium volume.

Table 2

Strain	Culture medium (mL)	Biomass (g)	Neutral fraction (mg)	Acid fraction (mg)
F921(-)	1500	5	49	62
F986(+)	1500	5	50	57

Once the procedure was completed, the two Fractions, Neutral and Acid, were analyzed immediately with reverse-phase HPLC, ^{1}H NMR and GC-MS.

1.2 Extraction and Fractionation of cultures in Solid Medium

After the completion of the incubation period of the cultures in solid medium, we proceeded to extraction and fractionation of the cultures (Figure 7), beginning by freezing the petri dishes at -40^{o}C for one night, their thawing at room temperature for one hour allow to obtain an exudate, whose yielding was increased by pressing of the agar, and centrifuged at 400 rpm for 15 minutes. After filtering, using a nitrocellulose filter of 0.45 μm pores diameter, the exudate was subjected to an acid-base fractionation following the same methodology as described above for the cultures in liquid medium. The resulting fractions were analyzed by HPLC, NMR and GC-MS.

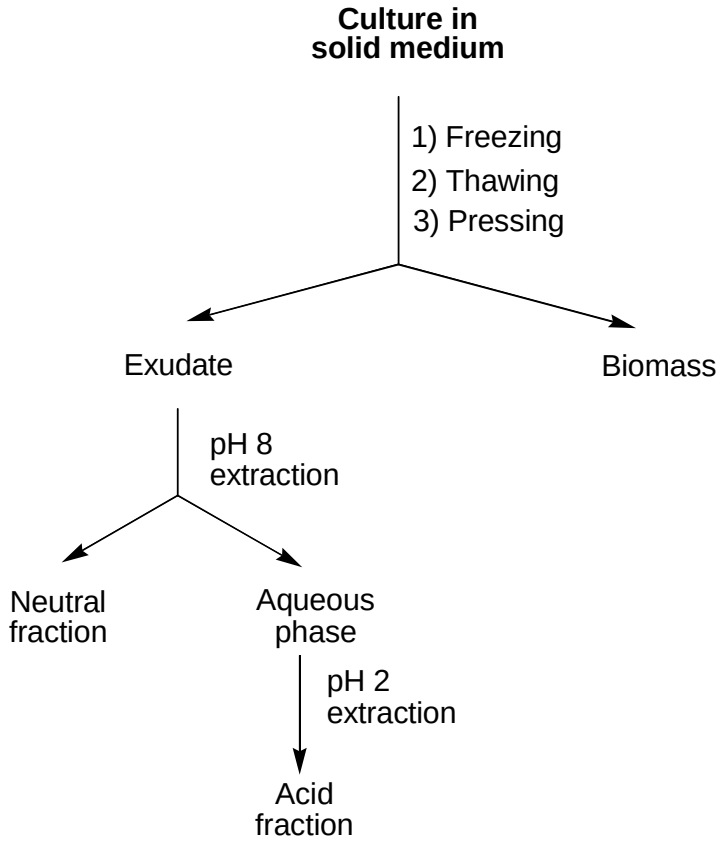


Figure 7: Methodology of extraction and fractionation of solid cultures.

In Table 3 are presented the amounts of biomass, Neutral and Acid fractions from an exudate volume.

Table 3

Strain	Exudate (mL)	Neutral fraction (mg)	Acid fraction (mg)
F921(-)	600	15	27
F986(+)	600	18	41
F921(-) x F986(+)	500	70	75

2. Detection and Identification of Trisporoids using HPLC and ^{1}H NMR

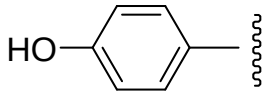
As mentioned above, from the UV measurements we can't be sure that the increase of the UV radiation absorbance is because of the presence of trisporoids or of other metabolites with similar chromophores in our medium. In order to confirm the presence or not of trisporoids, we proceed to HPLC and ^{1}H NMR analysis. These analyses were carried out by the use of standards and compilations of HPLC chromatograms with profiles of trisporoids, and HPLC and ^{1}H NMR spectra of pure apocarotenoids existing in our laboratory of University of Granada.

For the HPLC analysis of the samples we used analytical reverse-phase Column with UV detector adjusted at wavelengths (230, 250, 280, 300 & 328 nm), employing as eluent mixture of MeOH:H_2O in gradient and flow 1ml/min.

The analysis of the acidic fractions obtained from single cultures in liquid and solid medium showed that they had the same composition not showing the presence of trisporoids. A similar result was obtained from the analysis of the neutral fractions. Signals assignable to aromatic compounds were observed in the ^{1}H NMR spectra performed to these fractions.

The corresponding analysis of the acid and neutral fractions obtained from mated cultures showed the trisporoids presence in both fractions.

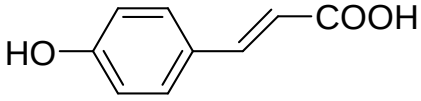
The compounds identification in the acid and neutral fractions obtained from single cultures was realized by GC-MS. For it, aliquots of both fractions were taken. The aliquot of the acid fraction was derivatized with SigmaSil A (Sigma-Aldrich Quimica, S.A.) and the mixture of trimethylsilyl (TMS) derivatives was subjected to GC-MS. In this mixture was identified **4-hydroxycinnamic acid (p-coummaric acid)** as monotrimethylsilylderivative on the basis to the following data obtained from its mass spectrum: the molecular ion was showed at m/z 236 together the intense fragment ions at m/z 221 $[M - CH_3]^+$, 177 $[221 – CO_2]^+$ and 119 $[M - Si(CH_3)_3 – CO_2]^+$ pointing to a carboxylic acid. The loss of CO from the fragment ion at m/z 177 indicated the presence of on aromatic ring.[44] According to these fragments, we know the extremes of the molecule:



m/z = 93

m/z = 117

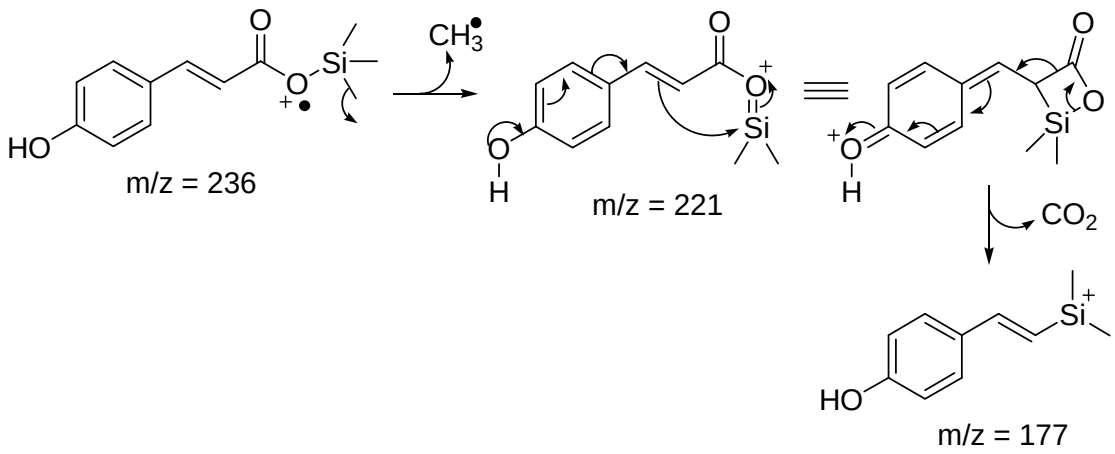
The remaining 26 units of mass must correspond to a disubstituted ethylene group (–CH=CH–).



p-coummaric acid

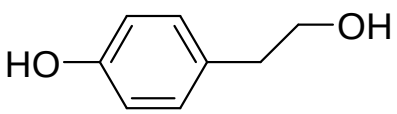
[44] Seibl, J. (1973) Espectrometría de masas. Ed. Alhambra, Madrid.

The formation of the fragment ion at m/z 177 ($[M - CH_3 - CO_2]^+$) can be explained due to conjugation of double bond with the aromatic ring which allows the participation of the substituent on position 4 in the elimination of CO_2 (Scheme 8).



Scheme 8: Formation of the fragment ion at m/z 177.

Another compound found in the acid fraction, as well as monotrimethylsilylderivative was **2-(4-hydroxyphenyl)ethanol (Tyrosol)** on the basis to the following data obtained from its mass spectrum: the molecular ion was shown at m/z 210 and the base peak at m/z 179 $[M - CH_2OH]^+$. Another intense fragment ion appeared at m/z 107 by successive loss of 72 (SiC_3H_8) and 31 (CH_2OH) mass units. The easy loss of 31 mass units resulting in very stable fragments [m/z 179 (100) and m/z 107 (92)] indicated us that this compound must be a derivative of 2-phenylethanol. The presence of aromatic ring was confirmed by the fragment ion at m/z 77.



Tyrosol

Tyrosol was also identified in the neutral fraction on the basis to the following data obtained from its mass spectrum: The molecular ion was shown at m/z 138 together with the base peak at m/z 107 $[M - CH_2OH]^+$. The presence of aromatic ring was evidenced by the fragment ion at m/z 77.

These metabolites, although described in other fungi,[45] they are described by first time in *Blakeslea trispora*.

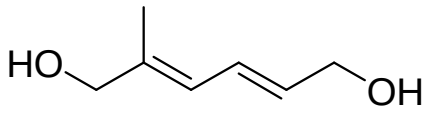
Apocarotenoid mixture obtained from neutral fraction of the mated cultures was subjected to semipreparative normal-phase HPLC using *t*-buthylmethylether (*t*-BuOMe) as eluent. Two **apocarotenoids** (**1** and **2**) were isolated.

Apocarotenoid 1 presented in analytical reverse-phase HPLC a peak at 10.4 min (λ = 250 nm) and a molecular ion at *m/z* 128 in its EIMS spectrum. The ^{13}C NMR spectrum showed seven signals due to two primary alcohol signals at 63.6 and 68.3 ppm, a methyl signal at 14.1 ppm, and four signals for disubstituted and trisubstituted double bonds at 137.9 ppm (C), 131.8 ppm (CH), 127.1 ppm (CH) and 123.8 ppm (CH). The 2-methyl-2,4-hexadiene skeleton was established by the direct coupling of the three olefinic protons in the ^1H NMR spectrum at 6.43 ppm (dd, J_1 = 11.0 Hz, J_2 = 15.1 Hz), 6.02 ppm (d, J = 11.0 Hz), 5.78 ppm (dt, J_1 = 5.5 Hz, J_2 = 15.1 Hz). The stereochemistry of the double bonds was determined as E on the basis to the coupling constant (15.1 Hz) for the disubstituted double bond and to the chemical shifs at C6 and C7 (68.3 ppm and 14.1 ppm, respectively) in the ^{13}C NMR spectrum[46]. Because of these data we have proposed for apocarotenoid 1 the structure of **(2*E*,4*E*)-2-methyl-2,4-hexadiene-1,6-diol** and the name

[45] (a) Barrero, A.F.; Oltra, J.E.; Poyatos, J.A. (1996) Acidic metabolites from *Phycomyces blakesleeanus*. Phytochemistry 42: 1427-1433. (b) Reyes Benítez, J.F. (1995) Tesis doctoral: Estudios de la Producción y Biosíntesis de Giberelinas en *Gibberella fujikuroi*, Granada. (c) Cross, B.E.; Galt, R.H.B.; Hanson, J.R.; Curtis, P.J.; Grove, J.F.; Morrison, A. (1963) New metabolites of *Gibberella fujikuroi*. Part II. The isolation of fourteen new metabolites. J. Chem. Soc.: 2937-43. (d) Trisuwan, K.; Rukachaisirikul, V.; Sukpondma, Y.; Preedanon, S.; Phongpaichit, S.; Sakayaroj, J. (2009) Pyrone derivatives from the marine-derived fungus *Nigrospora* sp. PSU-F18. Phytochemistry 70: 554-557.

[46] Wehrli, F.W.; Nishida, T. (1979) Progress in the Chemistry of Organic Natural Products. Herz, W.; Grisebach, H.; Kirby, G.W. Eds., Springer-Verlag: Wien 36: 1-229.

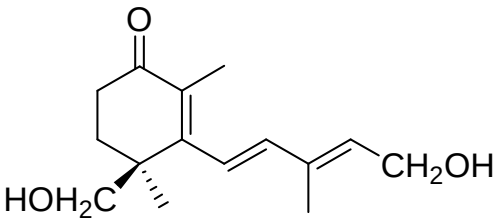
Blakeslediol. This compound is a new natural product belonging to a new apocarotenoid family recently discovered by our group.



(2*E*,4*E*)-2-methyl-2,4-hexadiene-1,6-diol

Apocarotenoid **1** presented in analytical reverse-phase HPLC a peak at 10.4 min (λ = 250 nm).

Apocarotenoid **2** was identified by analytical reverse-phase HPLC, ^{1}H NMR, UV and MS. This compound presented a peak at 19.35 min in HPLC chromatogram with two absorbance maximum in the UV at 230 nm and 300 nm. The molecular ion was shown at m/z = 250 in the mass spectrum. In the ^{1}H NMR spectrum, we observed two signals of olefinic protons which were coupled to each other at 6.26 ppm (1H, d, *J* = 15.8 Hz) and 6.24 ppm (1H, d, *J* = 15.8 Hz), one triplet at 5.77 ppm (1H, *J* = 7.0 Hz) due to a proton on trisustituted double bond which was coupled with a doublet at 4.32 ppm (2H, *J* = 7.0 Hz) due to two protons on oxygenated carbon, two doublets which were coupled to each other at 3.73 ppm (1H, *J* = 11.0 Hz) and 3.43 ppm (1H, *J* = 11.0 Hz) due to two geminal protons on oxygenated carbon and three singlets at 1.87, 1.85 y 1.14 ppm due to two methyl groups on double bond (1.87 ppm and 1.85 ppm) and another on quaternary sp^3 carbon (1.14 ppm). These data allowed us to identify apocarotenoid **2,** as the known apotrisporol[47].



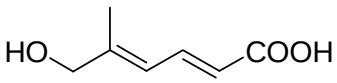
apotrisporol

[47] Sutter, R.P. and Whitaker, J.P. (1981a) Sex pheromone metabolism in *Blakeslea trispora.* Naturwissenschaften 68: 147-148.

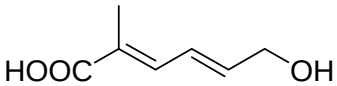
The 1H-NMR spectrum of the neutral fraction indicates that the major component was (2*E*,4*E*)-2-methyl-2,4-hexadiene-1,6-diol.

The identification of apocarotenoids in the acid fraction, obtained from mated cultures, was carried out by ^{1}H NMR and analytical reverse-phase HPLC. The apocarotenoids that follow were identified in this fraction:

Blakesleoic acids A and B : These compounds showed one peak at 2.4 min in the HPLC chromatogram (Figure 8) with maximum absorbance in UV at λ = 260 nm.



Blakesleoic acid A



Blakesleoic acid B

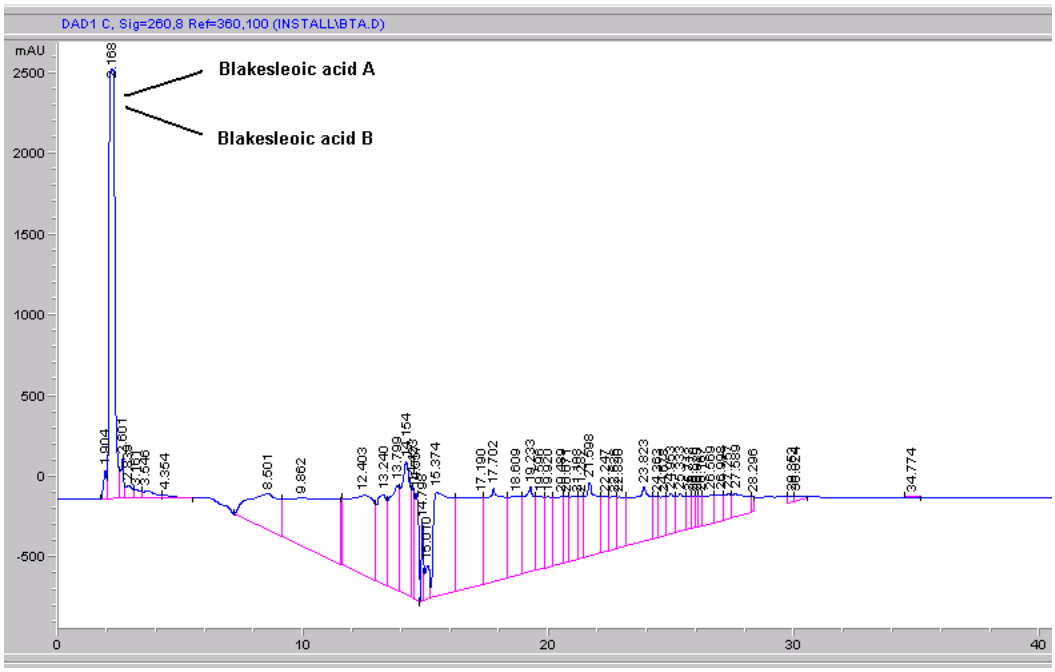
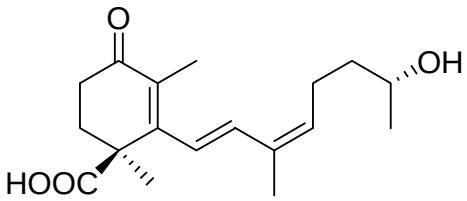


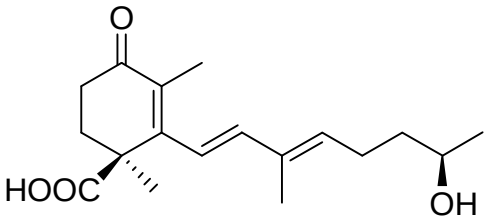
Figure 8: Chromatogram of analytical reverse-phase HPLC of acid fraction obtained from mated culture at 260 nm: Blakesleoic acids A and B (2.4 min).

Trisporic acid C (9*Z*): This compound showed one peak at 15.1 min in the HPLC chromatogram (Figure 9) with absorbance maximum in the UV at λ = 328 nm.



Trisporic acid C (9*Z*)

Trisporic acid C (9*E*): This compound, showed one peak at 15.3 min in the HPLC chromatogram (Figure 9) with absorbance maximum in the UV at λ = 328 nm.



Trisporic acid C (9*E*)

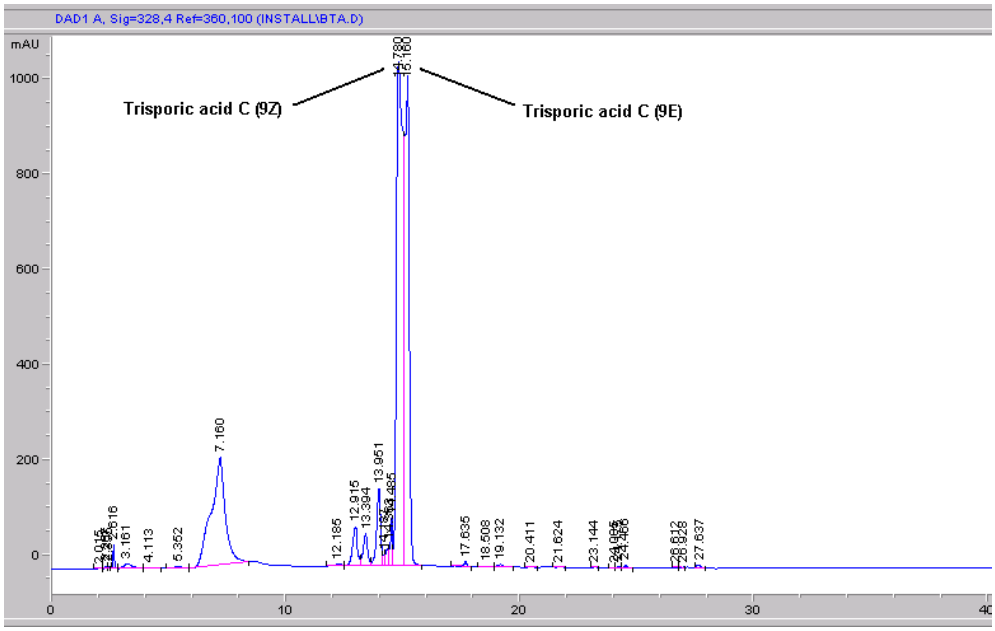


Figure 9: Chromatogram of analytical reverse-phase HPLC of acid fraction obtained from mated culture at 328 nm: trisporic acid C (9*Z*) (15.1 min) and trisporic acid C (9*E*) (15.3 min).

The presence of all these apocarotenoids in acid fraction also was confirmed by ^{1}H NMR spectroscopy. The ^{1}H NMR spectra of the acid fractions showed signals at δ 6.83 (d, *J* = 16.3 Hz), 6.39 (d, *J* = 16.3 Hz), 5.61 (t, *J* = 7.9 Hz), 3.77-3.81 (m), 3.68 (s) and 1.21 (d, *J* = 6.0 Hz) due to **trisporic acid C (9*Z*)**,[6] at δ 6.34 (d, *J* = 16.4 Hz), 6.24 (d, *J* = 16.4 Hz), 5.63 (t, *J* = 7.5 Hz), 3.80-3.84 (m), 3.20 (s), 1.21 (d, *J* = 6.0 Hz) due to **trisporic acid C (9*E*)**,[6] at δ 7.59 (dd, J_1 = 11.8 Hz, J_2 = 15.2 Hz), 6.34 (br d, J = 11.8 Hz), 5.86 (d, J = 15.2 Hz) and 4.09 (s) due to **Blakesleoic acid A**[48] and at δ 7.23 (1H, d, *J* = 11.5 Hz, H-3), 6.69 (1H, ddt, J_1 = 1.8 Hz, J_2 = 11.5 Hz, J_3 = 15.1 Hz, H-4), 6.25 (1H, dt, J_1 = 4.2 Hz, J_2 = 15.1 Hz, H-5) and 4.24 (2H, d, *J* = 4.2 Hz, H-6) due to **Blakesleoic acid B**.[6]

The E or Z stereochemistry of the C9, C10 double bond in trisporic acids was assigned on the basis to value of the chemical shift of H-8. The H-8 of the (9*Z*)-isomer is deshielded by the 11-methylene group and hence the H-

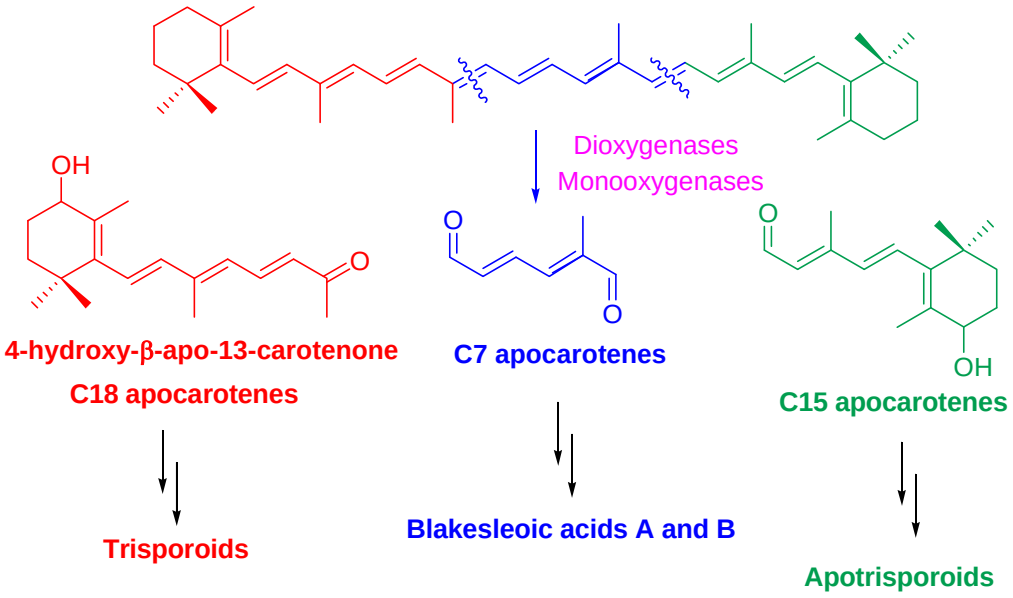
[48] Barrero, A.F.; Herrador, M.M.; Arteaga, P. Unpublished results.

8 signal should appear downfield relative to that of the H-8 of the (9*E*)-isomer[49].

Trisporic acid C (9*Z*) was the major component of the acid fraction in the mated culture.

3. Biosynthetic considerations

The isolation of the diol **1** and of Blakesleoic acids A and B in the mated cultures, allows us to propose for *Blakeslea trispora* the same start step in the biosynthetic pathway for trisporoids and apotrisporoids as for *Phycomyces blakesleeanus*.[50] These compounds were obtained by double oxidative cleavage of β-carotene (Scheme 9).



Scheme 9: New biosynthetic pathway of trisporoids and apotrisporoids.

[49] (a) White J.D.; Takabe K.; Prisbylla M.P. (1985) Stereoselective synthesis of trisporic acids A and B, their methyl esters, and trisporols A and B, hormones and prohormones of mucoraceous fungi. Journal of Organic Chemistry 50: 5233-5244. (b) Bu'Lock, J.D.; Drake, D.; Winstanley, D.J. (1972) Specificity and transformations of the trisporic acid series of fungal sex hormones. Phytochemistry 11: 2011–2018.

[50] Barrero, A.F.; Herrador, M.M.; Arteaga, P. (2009) 2º Congreso del GEQPN-RSEQ. La Palma, p. 85.

In a first step, the action of β-carotene dioxygenases on the double bonds 13,14 and 11',12' lead to three fragments of 18, 7 and 15 carbons, respectively, heads of three families of apocarotenoids. We assume that the initial 18-C fragment has a ketone ends and the other initial fragments have aldehyde ends, because this is the way of action expected from various carotene oxygenases[51] on β-carotene. The 18-C fragment that includes one of the β rings is the head of the family of the trisporoids. The 15-C fragment that includes the other β ring is the head of the family of the apotrisporoids. The central fragment is enzymatically modified to produce **Blakeslediol 1** and **Blakesleoic acids A** and **B** with the methylhexane skeleton. On the other hand, this framework only can be resulted of this double fragmentation pattern.

These results constitutes the second example where acts this biosynthetic pathway and allow us to extend that to other species of Mucorales.

[51] Kloer, D.P. and Schulz, G.E. (2006) Structural and biological aspects of carotenoid cleavage. Cell Mol Life Sci 63: 2291-2303.

IV. EXPERIMENTAL PART

1. Techniques of analysis

1.1 Chromatographic methods

Solvents

The solvents used as eluents during all chromatographic[52] processes were: Hexano (He), *t*-Butyl-Methyl-Ether (TBME), Dichloromethane (DCM), Acetyl Acetate (AcOEt), water (H_2O) and Methanol (MeOH) and their mixtures.

⁕**Thin Layer Chromatography (TLC):** Was performed using plates of silica gel Merck DC-Alufolien (Kieselgel 60 F_{254}) of 0.25mm thick, with the components revealed by exposure to Ultraviolet light of 254 nm (lamp Vilbaste Lourmat) or by immersion in an ethanolic solution of 7% phosphomolybdic acid and immediate warming.

⁕**High Performance Liquid Chromatography (HPLC):** we performed HPLC analysis with an Agilent 1100 Series equipment and using detector a DAD (Diode Array) or RI (Refractive Index).

- **Analytical reverse-phase HPLC**

The analysis of metabolites with structure possible of trisporoids was obtained with HPLC analytical, using a C_{18} column (4.6 x 250 mm, 5 µm octyldecylsilane particles, Spherisorb-ODS2, Waters Corporation, Milford, MA), at a flow rate of 1 ml/min, with gradient elution of a mixture of methanol/water (MeOH:H_2O):

- MeOH:H_2O (20:80) 0-6 min
- MeOH:H_2O (100:0) 6-25 min
- MeOH:H_2O (100:0) 25-40 min
- MeOH:H_2O (20:80) 40-50 min

[52] Martínez Grau, M.A. and Csáky, A.G. (1998) Técnicas Experimentales en Química Orgánica. Síntesis, D.L., Madrid.

Aliquots introduction was made directly by an injection valve, by means of a microsyringe and under a final volume of 20 μL sample, dissolved in methanol (1mL for 10 mg of fraction). The outflow was monitored with a diode array detector at 328, 300, 280, 250 & 230 nm.

- **Semi-preparative normal-phase HPLC**

The separation of trisporoids and other metabolites of our interest made by means of HPLC semi-preparative column (10 by 250 mm, 5 μm silica particles, Agilent) with a 15 mm refillable guard pre-column filled with the same material in a Series 1100 liquid chromatograph (Agilent), at flow rate of 2 ml/min, with isocratic elution. For neutral fraction, *t*-BuOMe was used as eluent.

The final volume of sample's injection was 500 μl dissolved in *t*-BuOMe (at 20 g dry extract/L). The detection was carried out with RI Detector.

* **Gas Chromatography-Mass Spectrometry**

The analysis of the samples with Gas Chromatography were carried out with a Hewlett-Packard 6890 Gas Chromatograph, model G1530A, equipped with an HP-1 methyl silicone capillary column (30 m x 0.25 mm x 0.25 μ thickness film), connected to a Hewlett-Packard 5988A mass spectrometer using an ionization voltage of 70 eV. As carrier gas was used helium with a flow rate of 1.9 ml/min.

The temperature conditions, under which the GC-MS analysis was performed, are presented in Table 4:

Injection Temperature: 260^{o}C
Detector Heater Temperature: 280^{o}C.

Table 4: Temperature program

Initial Temperature (^oC)	Ramp I ($^oC/min^{-1}$)	Next Temperature (^oC)	Ramp II ($^oC/min^{-1}$)	Final Temperature (^oC)	Hold Time (min)
50	10	-	-	300	10
80	5	160	2	300	10
120	5	220	3	280	10

Aliquots introduction was made directly by means of a microsyringe and under a final volume of 3 μL sample, dissolved in Dichloromethane (1mL for 20 mg of fraction).

1.2 Spectroscopic methods

* UV-Visible Spectrometry

The measurements of UV absorbance of all the samples were carried out with a UV-VIS Unicam spectrofotometer model Helios Alfa.

* Nuclear Magnetic Resonance Spectroscopy (NMR)

- NMR spectra (^1H, ^{13}C) were recorded with Varian Direct-Drive 400 (^1H 400 MHz/^{13}C 100 MHz) and 500 (^1H 500 MHz/^{13}C 125 MHz) spectrometers, and performed at the Scientific Instrumentation Center of the University of Granada.

As internal reference it was used tetramethylsilane (TMS) and as solvents: chloroform ($CDCl_3$) for Neutral Fractions & $(CD_3)_2CO$ for Acid Fractions. The chemical shifts (δ) are expressed in parts per million (ppm) and coupling constants (J) in Hertz (Hz). The multiplicity of the signals is expressed by the following abbreviations: s (singlet), d (doublet), t (triplet), q (quadruplet), sixth (sextuplet), m (multiplet), dd (double doublet), dt (double triplet), tt (triple triplet), dsext (double sextuplete), etc. In the case of broad signals is used the letters “br” beside the abbreviations outlined above.

The substitution grade of the carbons was established using the sequence DEPT pulse.

1.3 Derivation Techniques

Preparation of trimethylsilylderivatives: Silylation of Acid Fractions with SigmaSil A.

SigmaSil A, trimethylchlorosylan:hexamethyldysylizan:pyridine, 1:3:9v/v (0,1mg) was added under stirring to the acid fraction (1 mg). The solution was left for 10 min at room temperature and the solvent was evaporated at low pressure.

2. Biological Materials and Cultivations

2.1 Biological Materials

The wild strains of *Blakeslea trispora* used in this work were: strain F921(-) and strain F986(+), which were obtained from the Professor Dr. D. E. Cerdá-Olmedo, Department of Genetics, University of Sevilla.

These wild strains of *B. trispora* (class Zygomycetos, order Mucorales, family Choanophoracac) were obtained originally from VKM (Vsiesoyuznaya Kollektsiya Mikroorganizmov: All-Russian Collection of Microorganisms, Moscow, Russia[53].

[53] Mehta, J.B.; Obraztsova, N.I. and Cerdá-Olmedo, E. (2003) Mutants and Intersexual Heterokaryons of *Blakeslea trispora* for Production of β-carotene and Lycopene. Applied and Environmental Microbiology 69: 4043-4048.

The chosen strains are not among those with the highest number of nuclei per spore (the spores of *B. trispora* are multinucleate). Strains F986(+) and F921(-) contain average 4.7 & 5.6 nuclei per spore, respectively.

Conservation of the Strains

The strains were conserved in the refrigerator at 20^{o}C, in a 50% glycerol solution, in a concentration of about 10^6 to 10^7 spores/ml.

2.2 Mediums And Cultivations

2.2.1. Cultivations in Liquid Medium

Preparation of Liquid Medium

For the cultures in liquid medium we used the **minimal medium**[54] which consists of the following ingredients per Liter:

SIVB: 20 g D-(+)-glucose and 500 ml of distilled water

SIVA: 2 g L-asparagine, 5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 10 ml calcium Sutter 100 x (0.7 g of $CaCl_2 \cdot 2H_2O$ in 250 ml of distilled water and a few drops of chloroform), 10 ml Solution Sutter 100x (0.025g thiamine, 0.05 g acido citric monohidrato-$C_6H_8O_7 \cdot H_2O$, 0.0375 g $Fe(NO_3)_3 \cdot 9H_2O$, 0.025 g $ZnSO_4 \cdot 7H_2O$, 0.0075 g $MnSO_4 \cdot H_2O$, 0.00125 g $CuSO_4 \cdot 5H_2O$, 0.00125 g $Na_2MoO_4 \cdot 2H_2O$ in 250 ml of distilled water and a few drops of chloroform) and 480 ml of distilled water.

[54] Cerdá-Olmedo, E. (1987) Standard growth conditions and variations, In E. Cerdá-Olmedo and E.D. (Lipson), Phycomyces. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.: 337-339.

After sterilization for 20 min at 120^{o}C, we added SIVA into SIVB in sterilized atmosphere and added 150 ml of Minimal Medium Liquid in 500-ml Erlenmeyer flasks.

Cultivation process in Liquid Medium

The 500-ml Erlenmeyer flasks, filled with 150 ml of Minimal Medium Liquid each, were inoculated with 100 μL per 25 ml of medium from a concentration of 10^6 spores/ml for F921(-) strain cultures and 50 μL per 25 ml of medium from a concentration of 10^6 spores/ml for F986(+) strain cultures.

The culture flasks were covered with cotton and kept in an orbital shaker (Radiber Microtermic I) at 200 rpm, in darkness, for 3-5 days, at 30^{o}C.

2.2.2. Cultivations in Solid Medium

Preparation of Solid Medium

For the cultures in solid medium we used the **minimal agar medium** which consists of the following ingredients per Liter:

SIVB: 20 g D-(+)-glucose, **15 g agar** and 500 ml of distilled water

SIVA: 2 g L-asparagine, 5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 10 ml calcium Sutter 100 x (0.7 g of $CaCl_2 \cdot 2H_2O$ in 250 ml of distilled water and a few drops of chloroform), 10 ml Solution Sutter 100x (0.025g thiamine, 0.05 g acid citric monohidrato-$C_6H_8O_7 \cdot H_2O$, 0.0375 g $Fe(NO_3)_3 \cdot 9H_2O$, 0.025 g $ZnSO_4 \cdot 7H_2O$, 0.0075 g $MnSO_4 \cdot H_2O$, 0.00125 g $CuSO_4 \cdot 5H_2O$, 0.00125 g $Na_2MoO_4 \cdot 2H_2O$ in 250 ml of distilled water and a few drops of chloroform) and 480 ml of distilled water.

After sterilization for 20 min at 120^{o}C of SIVA and SIVB solutions, to add SIVA into SIVB, stir for a few seconds and prepare the petri dishes.

Preparation of petri dishes.

To add 25 ml of **minimal agar medium** in every petri dish carefully and leave the dish open until the medium gets solid and cold. One it is ready, we store the petri dishes in the refrigerator ready for use.

Cultivation process in solid medium

Inoculate each petri dish with addition of:

- 100 μL from a concentration of 10^6 spores/ml for F921(-) strain cultivations
- 50 μL from a concentration of 10^6 spores/ml for F986(+) strain cultivations
- 100 μL [F921(-)] x 50 μL [F986(+)] from a concentration of 10^6 spores/ml for mated cultivations.

The petri dishes were kept in darkness, for 3 days at 30^{o}C. All the process was carried out in a Biological Safety Cabinet Class I, under sterilized conditions.

3. Extraction And Fractionation of Apocarotenoids

3.1. Extraction of cultures in liquid medium

The extraction procedure of the cultivations in liquid medium was performed in two steps, at pH 2 and at pH 8, to allow the best separation of acid and neutral substances. First the mycelia were removed by filtration. The culture filtrate was then adjusted to pH 8.0 with addition of NaOH 2N and extracted with ethyl acetate -EtOAc- **(Neutral extracts)**. The residual culture filtrate was adjusted to pH 2.0 with addition of HCL 2N and extracted with the

same eluent –ethyl acetate- **(Acid extracts)**. The organic phases were dried with anhydrous sodium sulphate and removed by filtration.

The solvent was removed in a rotary vacuum concentrator and the residues were stored at -20^{o}C and under Argon atmosphere, until their analysis.

The biomass of the filtrated mycelia was left to dry in room temperature and weighted.

As trisporoids are susceptible to photodegradation, all procedures were carried out under dim light.

In Table 5 are shown the amounts of biomass, Neutral & Acid extracts that were obtained from the cultures.

Table 5: Summary table of biomass, Neutral & Acid extract results, in liquid medium cultivations.

Cultivation Strain	mL cultivation	Neutral Extract (mg)	Acid Extract (mg)	Biomass (g)
F921 (-)	3600	55	61	15
F921(-)	1500	49	62	5
F986(+)	1500	50	57	5

3.2. Extraction of cultures in solid medium

When the inoculation period of cultures was finished, the extraction procedure started with freezing of the dishes at -80^{o}C for one night. After thawing the dishes at 22^{o}C for 1 hour, the agar was pressed and centrifuged at 400 rpm for 15 minutes, in order to collect all the exudate from the mycelia. Then, the exudate was filtrated with a nitrocelulosa filter of 0.45μm diameter of pores. With the exudate that was collected, we continued to the extraction procedure the same way as described before for the cultures in liquid medium, in two steps, at pH 2 and at pH 8.

In Table 6 are presented the amounts of Neutral & Acid extracts that were obtained from the cultures in solid medium.

Table 6: Summary table of Neutral & Acid extract results, in solid medium cultivations.

Cultivation Strain	mL cultivation	Neutral Extract (mg)	Acid Extract (mg)
F921 (-)	600	15	27
F986(+)	600	18	41
F921(-) x F986(+)	500	70	75

4. Methodology of Analysis

The analysis of all neutral and acid fractions obtained from the different cultures were carried out by ^{1}H NMR, analytical reverse-phase HPLC and/or GC-MS. Analyzes showed the presence of apocarotenoids in mated cultures, but no in single cultures. In addition, the composition of single cultures in solid as in liquid medium was the same.

5. Identification of Trisporoids And other Molecules

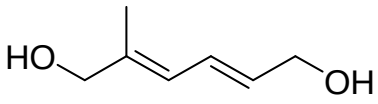
The identification of the apocarotenoids in acid fraction obtained from mated culture has been carried out by analytical reverse-phase HPLC and GC-MS, using standards obtained from our research group in previous researches.[55]

The neutral fraction obtained from mated cultures was subjected to semi-preparative normal-phase HPLC from which obtained three fractions. Fraction 1 (t_R = 10.2-13.0 min) was constituted by complex mixture of compounds no identified. No apocarotenoids were observed in this fraction. Fraction 2 (t_R = 15.4-18.1 min) was constituted by **(2*E*,4*E*)-2-methyl-2,4-hexadiene-1,6-diol.** Fraction 3 (t_R = 18.1-21.5 min) was constituted by a

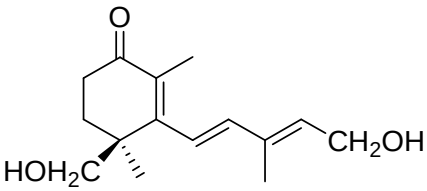
[55] Barrero, A. F.; Herrador, M. M.; Arteaga, P. (2010) results without publishing.

mixture of **(2*E*,4*E*)-2-methyl-2,4-hexadiene-1,6-diol** and **apotrisporol** in a ratio 1:3.

(2*E*,4*E*)-2-methyl-2,4-hexadiene-1,6-diol (Blakesleadiol): Analytical reverse-phase HPLC: λ = 230 nm, t_R = 10.4 min; EIMS (70 eV), m/z: 128 $[M]^+$; IR (film) ν_{max} 3447, 2871, 1440, 1071, 1012, 825, 996 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 6.43 (1H, dd, J_1 = 11.0 Hz, J_2 = 15.1 Hz, H-4), 6.02 (1H, d, J = 11.0 Hz, H-3), 5.78 (1H, dt, J_1 = 5.5 Hz, J_2 = 15.1 Hz, H-5), 4.15 (2H, d, J = 5.5 Hz, H-6), 4.02 (2H, s, H-1), 1.73 (3H, s, H-7); ^{13}C NMR (125 MHz, $CDCl_3$): δ 137.9 (C, C-2), 131.8 (CH, C-4), 127.1 (CH, C-5), 123.8 (CH, C-3), 68.3 (CH_2, C-1), 63.6 (CH_2, C-6), 14.1 (CH_3, C-7).

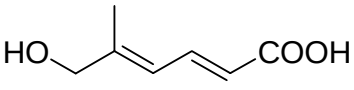


Apotrisporol: Analytical reverse-phase HPLC: t_R = 19.35 min, λ = 300 nm; GC-MS: t_R = 19.84 min, (70 eV), m/z: 250 $[M]^+$; ^1H NMR (400 MHz, $CDCl_3$): δ 1.14 (3H, s), 1.69-1.73 (1H, m), 1.85 (3H, s), 1.87 (3H, s), 2.02 (2H, br s), 2.24-2.28 (1H, m), 2.53-2.58 (2H, m), 3.43 (1H, d, J = 11 Hz), 3.73 (1H, d, J = 11 Hz), 4.32 (2H, d, J = 7 Hz), 5.77 (1H, t, J = 7 Hz), 6.24 (1H, d, J = 15.8 Hz), 6.26 (1H, d, J = 15.8 Hz).

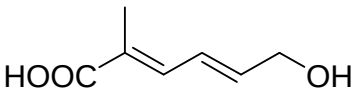


The apocarotenoids that follow were identified in the acid fraction:

Blakesleoic acids A and B: were identified by analytical reverse-phase HPLC using a standard (t_R = 2.4 min, λ = 260 nm) as a 2:1 mixture.

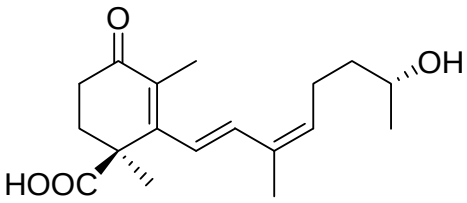


Blakesleoic acid A

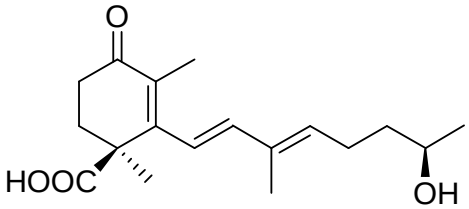


Blakesleoic acid B

Trisporic acid C (9*Z*): was identified by analytical reverse-phase HPLC using a standard (t_R = 15.1 min, λ = 328 nm).

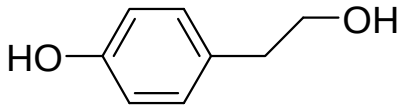


Trisporic acid C (9*E*): was identified by analytical reverse-phase HPLC using a standard (t_R = 15.3 min, λ = 328 nm).

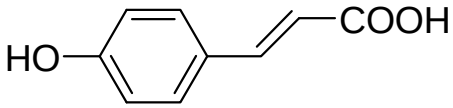


The neutral and acid factions corresponding to single cultures were studied by GC-MS. The acid fraction was previously derivatized, in order to obtain trimethylsilylderivatives. The following compounds were identified:

2-(4-hydroxyphenyl)ethanol (Tyrosol): identified in the neutral fraction. GC-MS: t_R = 8.78 min, m/z (rel. int.): 138 (20) $[M]^+$, 107 (100) $[M - CH_2OH]^+$, 77 (18). This compound was identified also in the acid faction as monotrimethylsilyl derivative. GC-MS: t_R = 17.4 min, m/z (rel. int.): 210 (30) $[M]^+$, 195 (9) $[M - CH_3]^+$, 179 (100) $[M - CH_2OH]^+$, 138 (20) $[M – SiC_3H_8]^+$, 107 (92) $[M - CH_2OH]^+$, 77 (25), 73 (72).



4-hydroxycinnamic acid (p-Coummaric acid): identified in the acid fraction as monotrimethylsilyl derivative. GC-MS: t_R = 13.78 min, m/z (rel. int.): 236 (10) $[M]^+$, 221 (100) $[M - CH_3]^+$, 177 (30) $[221 – CO_2]^+$, 149 (41) $[177 – CO]^+$, 119 (32) $[M - Si(CH_3)_3 – CO_2]^+$, 107 (60) $[135 – CO]^+$, 91 (42) $[119 – CO]^+$, 73 (23).



V. CONCLUSIONS

1. Apocarotenoids has not been identified in the single cultures of wild strains, F986(+) and F921(-), of *Blakeslea trispora* as in liquid medium as in solid medium. In these cultures, have been only identified **p-coummaric acid** and **tyrosol**. These compounds are described for first time in *Blakeslea trispora*.

2. **(2*E*,4*E*)-2-methyl-2,4-hexadiene-1,6-diol**, a new apocarotenoid, named **Blakeslediol** together with **Blakesleoic acids A** and **B**, were identified in mated cultures of wild strain of *Blakeslea trispora*. These compounds form a new family of apocarotenoids.

3. Other apocarotenoids identified in mated cultures of wild strain of *Blakeslea* were **apotrisporol** and **(9*Z*)-trisporic acid C** and **(9*E*)-trisporic acid C**.

4. Identification of the Blakeslediol and of Blakesleoic acids A and B (C7 apocarotenes) in the mated culture allow us to propose for *Blakeslea trispora* the same biosynthetic origin for trisporoids (C18 apocarotenes) and apotrisporoids (C15 apocarotenes) with *Phycomyces blakesleeanus*, that is to say, these compounds are obtained by double oxidative cleavage of β-carotene (double bonds 13,14 and 11',12').