«Πρόγραμμα Μεταπτυχιακών Σπουδών του Τμήματος Βιοχημείας και Βιοτεχνολογίας»

«ΒΙΟΤΕΧΝΟΛΟΓΙΑ - ΠΟΙΟΤΗΤΑ ΔΙΑΤΡΟΦΗΣ ΚΑΙ ΠΕΡΙΒΑΛΛΟΝΤΟΣ»

ΚΙΤΣΙΔΗ ΠΟΛΥΤΙΜΗ

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

2010

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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 μελέτη αποτελεί μέρος ενός ευρύτερου ερευνητικού πεδίου που ασχολείται με τη βιοτεχνολογία των νηματωειδών μυκήτων και έχει αναπτυχθεί με τη συνεργασία των ερευνητικών ομάδων του καθηγητή A. F. Barrero (Τμήμα Οργανικής Χημείας, Πανεπιστήμιο Γρανάδας) και του καθηγητή Cerdá-Olmedo (Τμήμα Γενετικής, Πανεπιστήμιο Σεβίλλης). Μεταξύ αυτών των μυκήτων συναντούμε τους Mucorales μύκητες *Phycomyces blakesleeanus* και *Blakeslea trispora* (διαίρεση Zygomycota, κλάση Zygomycetos) και ιδιαίτερα την άκρως ενδιαφέρουσα κατηγορία μεταβολιτών τους τα γνωστά 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. Το βασικό αντικείμενο αφορά στον εντοπισμό των χημικών σημάτων που ευθύνονται για τη σεξουαλική αλληλεπίδραση των Mucorales και την υπερπαραγωγή του β-καροτενίου, βοηθώντας στη βελτίωση της βιομηχανικής παραγωγής του ιδίου, του λυκοπενίου και άλλων χημικών προιόντων.

Το αντικείμενο της μεταπτυχιακής διατριβής που παρουσιάζεται αφορά στη χημική μελέτη των αποκαροτενοειδών σε μονές και μεικτές καλλιέργειες άγριων στελεχών A56(+) και NRRL1555(-), του *Phycomyces blakesleeanus* με σκοπό την απομόνωση νέων που θα χρησιμοποιηθούν σε βιολογικά τεστ, ως πιθανά χημικά σήματα που επάγουν τη σεξουαλική αλληλεπίδραση στα Mucorales ή την υπερπαραγωγή του β-καροτενίου.

Έλαβαν χώρα, μονές καλλιέργειες άγριων στελεχών A56(+) και NRRL1555(-), σε υγρό μέσο και συνθήκες, ανάδευσης στις 200 στροφές/λεπτό κατά την επώαση, θερμοκρασίας 22°C και σκοταδιού, μεικτές καλλιέργειες των ιδίων στελεχών σε στερεό μέσο, σε ίδιες συνθήκες καλλιέργειας, εκτός της ανάδευσης και τέλος, μονές και μεικτές καλλιέργειες μεταλλαγμένων στελεχών που δεν παράγουν καροτένιο (*carB10*), C5(-) και S342(+), σε στερεό μέσο στις ίδιες συνθήκες, εκτός ανάδευσης, που θα χρησιμοποιηθούν ως τυφλά. Η διάρκεια της επώασης για τις μεικτές καλλιέργιες καθορίστηκε βάσει βιβλιογραφικών δεδομένων στις 5 μέρες ενώ για τις απλές σε υγρό μέσο ακολουθήθηκε η παρακάτω πορεία.

Βάσει των δομών διαφορετικών οικογενειών των τρισποροειδών καθέστει δυνατή η ανίχνευση της παρουσίας τους σε απλές καλλιέργειες υγρού μέσου του *Phycomyces blakesleeanus* μέσω της σπεκτρομετρίας UV. Η παρουσία διαφορετικών χρωμοφόρων στα τρισποροειδή (καρβονύλια με συζευγμένους διπλούς δεσμούς, διένια κ.α) τα οποία και απορροφούν επιλεκτικά σε αρκετά μήκη κύματος όπως 328, 300, 280, 250, 230 nm, επιτρέπει την ανίχνευση την αρχικής παραγωγής των τρισποροειδών των μυκήτων, την παρακολούθηση της εξελιξής τους και τελικά την εκτίμηση της μέγιστης συγκέντρωσης τους

Λήφθηκαν μετρήσεις 5-6 ημερών και παρατηρήθηκε ότι οι τιμές των απορροφήσεων στα διάφορα μήκη κύματος σταθεροποιήθηκαν την $5^{η}$-$6^{η}$ μέρα. Σε αυτό το σημείο σταμάτησε και η επώαση συνεχίζοντας με την επεξεργασία των καλλιεργειών. Η παραγωγή των τρισποροειδών παρακολουθούνταν επίσης διαμέσου οπτικών παρατηρήσεων ελέγχοντας την αλλαγή του χρώματος της βιομάζας κατά τη ροή του χρόνου. Ο χρωματισμός άλλαζε από λευκό ($2^{η}$ μέρα) σε κίτρινο ($3^{η}$ μέρα) και μέρα με τη μέρα γινόταν όλο και πιο έντονος εως την $5^{η}$ -$6^{η}$ μέρα. Το κίτρινο χρώμα οφείλεται στην παραγωγή β-καροτενίου που βαδίζει παράλληλα με αυτή των τρισποροειδών.

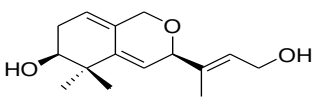
Στη συνέχεια πραγματοποιήθηκε εκχύλιση και κλασματοποίηση ακολουθώντας κάθε φορά κατάλληλη μεθοδολογία, καθώς η ανάπτυξη του μύκητα διαφέρει ανάλογα με τον τύπο της καλλιέργειας. Στις μεν υγρές καλλιέργειες με ανάδευση, ο μύκητας σχηματίζει μηκυλιακή μάζα χωρίς σποραγγειοφόρα, ενώ στις δε στερεές καλλιέργειες το μηκύλιο εμφανίζεται ως μια συλλογή υφών που απλώνεται σε όλη την επιφάνεια του άγαρ από την οποία εξέχουν μεγάλα και κάθετα σποραγγειοφόρα.

Προκειμένου να προχωρήσουμε σε ταυτοποίηση αποκαροτενοειδών, στις υγρές καλλιέργειες, αρχικά, διαχωρίστηκε η βιομάζα από το μέσο καλλιέργειας μέσω φιλτραρίσματος υπο κενό. Το τελευταίο υπέστη οξινο-βασική κλασμάτωση. Αρχικά αλκαλοποιήθηκε με 2N NaOH μέχρι pH=8 και εκχυλίστηκε με οξικό αιθυλεστέρα για να συγκροτήσει το βασικό κλάσμα. Η εναπομείνουσα υδατική φάση υπέστη οξίνιση έως pH=2 με 2N HCl και εκ νέου εκχυλίστηκε με AcOEt συγκροτώντας το όξινο κλάσμα.

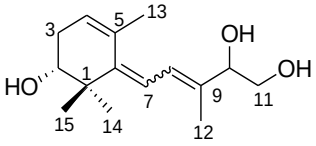
Στις στερεές καλλιέργειες τα τρυβλία καταψύχθηκαν στους –20ºC για ένα βράδυ, αφέθηκαν σε θερμοκρασία δωματίου για 1 ώρα για να αποψυχθούν και στη συνέχεια εφαρμόστηκε πίεση για να διευκολυνθεί η εξαγωγή του εκχυλίσματος. Κατόπιν, υπέστησαν, με την ίδια ακριβώς μεθοδολογία που περιγράφηκε ανωτέρω οξινοβασική κλασμάτωση.

Η ανάλυση των βασικών κλασμάτων που λήφθηκαν από τις μονές καλλιέργειες σε υγρό μέσο και τις μεικτές καλλιέργειες σε στερεό μέσο των στελεχών NRRL1555(-) και A56(+) του *P. blakesleeanus,* επιτεύχθει με ^{1}H NMR και αναλυτική HPLC αντίστροφης φάσης. Οι παραπάνω αναλύσεις έδειξαν ότι όλα τα βασικά κλάσματα διαφορετικών μονών καλλιεργειών έχουν το ίδιο αποκαροτενοειδές περιεχόμενο.

Μείγμα λοιπόν αυτών υπέστει ημιπαρασκευαστική HPLC κανονικής φάσης χρησιμοποιώντας ως εκλούτη *t*-BuOMe. Δυο καινούρια αποκαροτενοειδή (**1** και **2**) ανιχνεύτηκαν μαζί με το τρισποξάνιο (**3**), αποκαροτενοειδές που είχε απομονωθεί και χαρακτηριστεί δομικά στο πλαίσιο άλλων ερευνών του εργαστηρίου. Ανάλυση GC-MS μείγματος **1-2** αποκάλυψε ότι πρόκειται για μείγμα ισομερών με $[M]^{+}$ στα 252 m/z. Αυτό το δεδομένο σε συνδυασμό με τα αντίστοιχα των ^{1}H και ^{13}C NMR οδήγησαν στη μοριακή φόρμουλα $C_{15}H_{24}O_3$. ^{1}H NMR του μείγματος 1,2 αποκάλυψε για το μικρότερης συγκέντρωσης **2,** ότι αποτελεί γεωμετρικό στερεοισομερές του **1**. Τα 2 συστατικά ονομάστηκαν **τρισποτριενόλη Α και Β.**



trispoxane, **3**



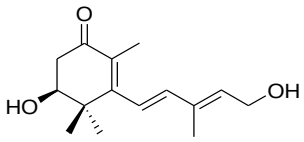
trispotrienol 1, 2

Επιπλέον, ανάλυση των βασικών κλασμάτων των μονών και μεικτών καλλιεργειών επιτελέσθηκε με αναλυτικό HPLC αντίστοφης φάσης με τη βοήθεια προτύπων από διαφορετικές καλλιέργειες των Mucorales ή/και με σύθεση. Τα ακόλουθα αποκαροτενοειδή ταυτοποιήθηκαν και συγκρίθηκαν οι σχετικές αναλογίες τους στις μονές και μεικτές καλλιέργειες.

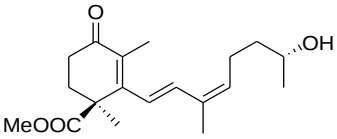
Τρισποτριενόλη Α και Β: εμφανίζουν 2 κορυφές στα 20.0 και 20.3 min στο χρωματογράφημα HPLC με μέγιστη απορρόφηση στο UV στα λ = 300 nm. Εμαφνίζονται και στις μεικτές καλλιέργειες αλλά σε μικρότερη συγκέντρωση. **Επίσης,** το φάσμα ^{1}H NMR έδειξε ότι αποτελεί το μέγιστο συστατικό του βασικού κλάσματος στις μονές και μεικτές καλλιέργειες.

Τρισποξάνιο: ταυτοποιήθηκε σε όλα τα βασικά κλάσματα μονών και μεικτών καλλιεργειών με κορυφή στατ 19.6 min του HPLC χρωματογραφήματος και απορρόφηση στο UV στα λ = 250 nm. Αφθονεί στις μεικτές καλλιέργειες.

Αποτρισπορίνη Ε: ταυτοποιήθηκε στο βασικό κλάσμα των μεικτών καλλιεργειών με κορυφή στα 18.9 min με κορυφή στατ 19.6 min του HPLC χρωματογραφήματος και μέγιστο απορρόφησης στο UV στα λ = 300 nm.



Μεθυλ-τρισπορικός εστέρας C (9Z): ταυτοποιήθηκε στο βασικό κλάσμα των μεικτών καλλιεργειών με κορυφή στα 23.7 min του HPLC χρωματογραφήματος και μέγιστο απορρόφησης στο UV στα λ = 328 nm.

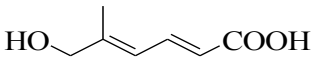


Η ανάλυση των βασικών κλασμάτων των μονών και μεικτών καλλιεργειών των στελεχών C5(-) και S342(+) του *P. blakesleeanus* έδειξε απουσία αποκαροτενοειδών σε αυτά τα κλάσματα. Περαιτέρω η συνθεσή τους δε μελετήθηκε**.**

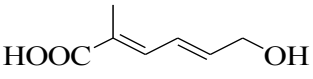
Η μελέτη των όξινων κλασμάτων των μεικτών καλλιεργειών των στελεχών NRRL1555(-) και A56(+) επιτελέστηκε με την ίδια μεθοδολογία που ακολουθήθηκε και για τα βασικά, με τη χρήση προτύπων. Σε αυτά ταυτοποιήθηκαν τα εξής αποκαροτενοειδή:

Μπλακεσλοϊκά οξέα Α και Β: ταυτοποιήθηκαν σε όλα τα όξινα κλάσματα και εμφανίζονται ως μια κορυφή στα 2.4 min του HPLC χρωματογραφήματος και

δίνουν μέγιστο απορρόφησης στο UV στα λ = 250 nm. Πρόκειται για αποκαροτενοειδή C7 που απομονώθηκαν σε προηγούμενη έρευνα του εργαστηρίου και συντέθηκαν χημικά. Εμφανίζονται σε αφθονία σε μονές και μεικτές καλλιέργειες.

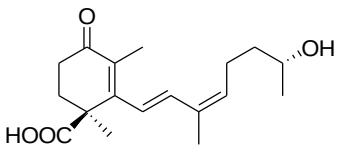


Blakesleoic A acid

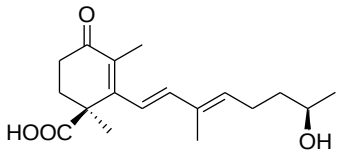


Blakesleoic B acid

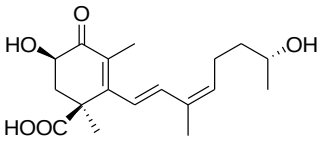
Τρισπορικό οξύ C (9*Z*): ταυτοποιήθηκε στο όξινο κλάσμα των μεικτών καλλιεργειών με κορυφή στα 15.1 min του HPLC χρωματογραφήματος και μέγιστο απορρόφησης στο UV στα λ = 328 nm.



Τρισπορικό οξύ C (9*E*): ταυτοποιήθηκε στο όξινο κλάσμα των μεικτών καλλιεργειών με κορυφή στα 15.3 του HPLC χρωματογραφήματος και μέγιστο απορρόφησης στο UV στα λ = 328 nm.



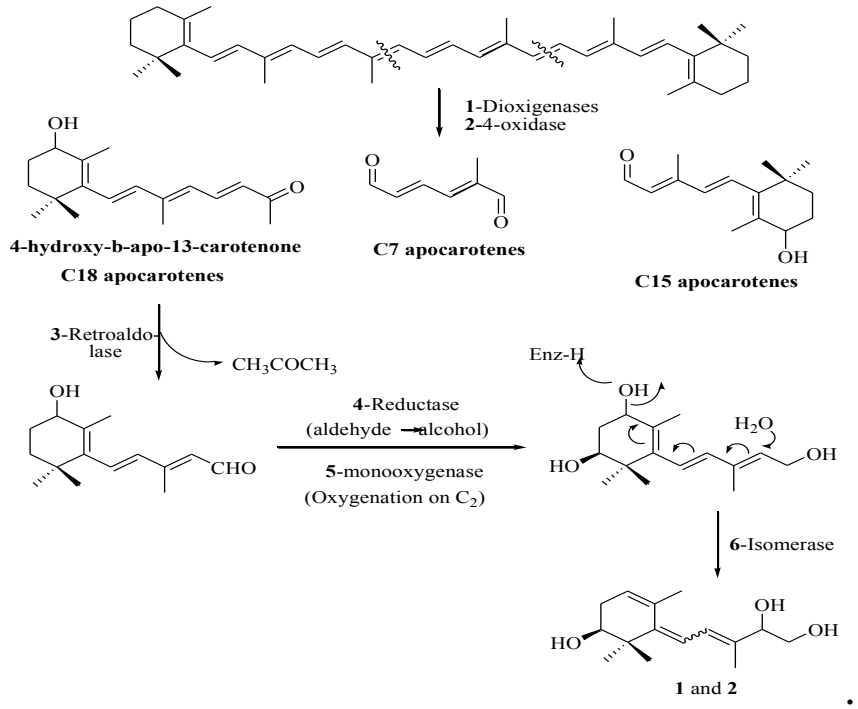
Τρισπορικό οξύ E (9*Z*): ταυτοποιήθηκε στο όξινο κλάσμα των μεικτών καλλιεργειών με κορυφή στα 16.7 min του HPLC χρωματογραφήματος και μέγιστο απορρόφησης στο UV στα λ = 328 nm.



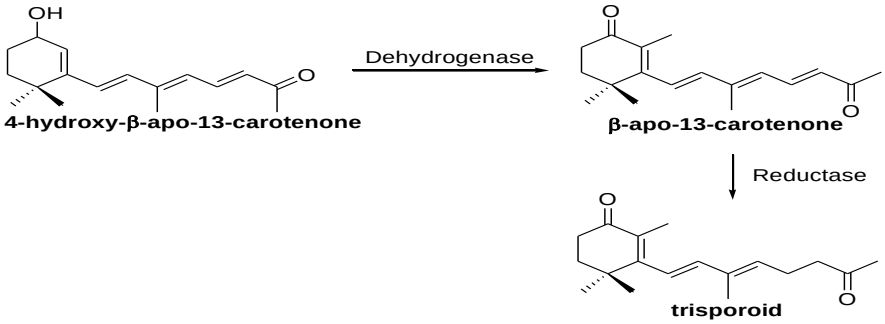
Η ανάλυση των όξινων κλασμάτων των μονών και μεικτών καλλιεργειών των στελεχών C5(-) και S342(+) του *P. blakesleeanus* έδειξε απουσία αποκαροτενοειδών σε αυτά τα κλάσματα. Περαιτέρω η συνθεσή τους δε μελετήθηκε**.**

Τα αποτελέσματα αυτής της έρευνας καταδεικνύουν ότι στις μονές καλλιέργειες των στελεχών NRRL1555(-) και A56(+) , σχηματίζονται αποκαροτένια

C15 (**1**-**3**), αποκαροτένια C7 (μπλακεσλοϊκά A και B), αλλά όχι αποκαροτένια C18. Η παρουσία των αποκαροτενίων C7 σε αυτές τις καλλιέργειες και η απουσία τους στις μονές καλλιέργειες των στελεχών C5(-) και S342(+) που δεν παράγουν καροτένιο, με μετάλλαξη στο *carB10*, δείχνει ότι η παραγωγή αποκαροτενοειδών στις μονές καλλιέργειες ξεκινά από το β-καροτένιο και επιτυγχάνεται μέσω διπλής οξειδωτικής σχάσης, (2 διοξυγενάσες, ενζυματικό σύστημα **1**). Ακολουθεί 4-υδροξυλίωση (ένζυμο **2**). Έτσι δημιουργούνται 3 οικογένειες αποκαροτενοειδών C18, C15 με ένα υδροξύλιο στην θέση C4 και C7. Τα αποκαροτένια C18 υπόκεινται σε ρετροαλδολική μετατροπή (ένζυμο **3**) χάνοντας ένα μόριο ακετόνης και σχηματίζουν το αποκαροτενοειδές C15 με μία αλδευδομάδα. Τελικά, αναγωγή της καρβονυλομάδας και ισομερίωση (ένζυμα **4** και **5**) καταλήγουν στο σχηματισμό των αποτριενολών **1** και **2** όπως φαίνεται στο παρακάτω σχήμα.



Στις μεικτές καλλιέργειες των άγριων στελεχών όταν η βιοσύνθεση φθάσει στη στην 4-υδρόξυ-απο-καροτενόνη, μια δεϋδρογενάση μετατρέπει το υδροξύλιο του C4 σε κετόνη και στη συνέχεια μια ρεδουκτάση υδρογονώνει τις α, β θέσεις της μεθυλ-κετόνης δίνοντας μία σταθεροποιητική δράση ώστε τα C18 να αποφύγουν την περαιτέρω διάσπαση σε C15.



UNIVERSITY OF GRANADA

FACULTY OF SCIENCES

Department Of Organic Chemistry

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

Master Thesis

Polytimi Kitsidi

Granada, 2010

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

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

Granada, 22nd of March, 2010

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INDEXES

I. INTRODUCTION & OBJECTIVES

The present study forms part of a research line on Biotechnology of filamentous fungi, which is developing more than fifteen years ago by the research group of Prof. A. F. Barrero (Dept. of Organic Chemistry, University of Granada), in collaboration with the group of Prof. Cerdα-Olmedo (Dept. of Genetics, University of Sevilla). The research field concerns the Chemistry and Biology of the Mucorales fungi and between these, *Phycomyces blakesleeanus* (division Zygomycota, class *Zygomycetes*) and specifically its apocarotenoid production.

"Apocarotenoids" (apo- meaning "from") are defined as the metabolites originated by degradation of carotenes by the loss of carbon atoms of the linear chain through oxidative reactions. The large number of apocarotenoids found in nature is due to the large amount of carotenoid precursors (more than 600 have been identified), variations on the site of oxidation of the chain, and the consequent functional changes.[1]. Some of the most interesting apocarotenoids are those named "Trisporoids"[2] (related to the fungus *Blakeslea trispora*). These compounds are metabolites originated by degradation of β-carotene during the sexual interaction.

Carotenoids are naturally occurring ubiquitous pigments, belong to the large and heterogenic family of terpenoids, synthesized as hydrocarbons (carotene; e.g., lycopene, α-carotene, and β-carotene) or their oxygenated derivatives (xanthophylls; e.g., lutein, α-cryptoxanthin and β-cryptoxanthin, zeaxanthin, canthaxanthin and astaxanthin) by plants, fungi, algae and bacteria.

Industrially, carotenoids are used as pharmaceuticals, antioxidants, and animal feed additives, as well as colorants in cosmetics and foods. Scientific interest in dietary carotenoids has increased in recent years because of their beneficial effects on

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[1]Schwartz S.; Qin X.; Zeevart, J. *J. Biol. Chem.* **2001**, *276*, 25208-25211.
[2](a) Avalos, J.; Cerdα-Olmedo, E.; Arora, D. K.; Dekker, M. *Handbook of fungal biotechnology* **2004**, (b) Ciegler, A. *Adv. Appl. Microbiol.* **1965**, *7*, 1-34

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human health, such as lowering the risk of cancer and enhancement of immune system function, which are attributed to their antioxidant potential. The increasing interest in fungal sources of carotenoids is related to consumer preferences for natural additives and the potential cost effectiveness of creating carotenoids via fungal biotechnology is gaining more and more ground as an environmental friendly method compared to respective chemicals[3].

β-carotene, the most well-known precursor of vitamin A, is accumulated as intracellular storage pigment in the fungal class *Zygomycetes*, and it displays one of the highest carotenogenic powers. *Zygomycetes* synthesizes predominantly *trans*-β-carotene, the most potent biological form[4]. The biological production of β-carotene originates from the sexual interaction of the mentioned above fungal class, provokes the apparition of yellow (*Phycomyces blakessleeanus*) or orange (*Blakeslea*) coloration, present in the young aged mycelia, sporangiophores and zones of sexual interaction.

3 (a) Armstrong, G.A, *J.Bacteriol* **1994,** *176*, 4795–4802. (b)Armstrong, G.A. *Annu. Rev. Microbiol.* **1997,** *51*, 629-659. (c) Sandmann, G. *Arch. Biochem. Biophys.* **2001,** *385*, 4-12

4 Ciegler, A. *Adv. in Apl. Microbiol.* **1965**, *7*, 1-34

1. ZYGOMYCETES

Fungi are heterotrophic organisms that are omnipresent in our environment. With few exceptions, fungi have filamentous bodies enclosed by cell walls, are nonmotile and reproduce both sexually and asexually by spores.

The second Eumycotan phylum, zygomycota—a group of more than 1000 species— are largely unseptate and their cell walls partially consists of chitosan, the deacetylation product of chitin. Many members of the group are saprotrophs living as primary colonizers on carbon-rich substrates (class *Zygomycetes*), whereas others are obligate entomopathogenic endoparasites (class *Trichomycetes*)[5].

The Zygomycota received their name by analogy to the other fungal groups based on the overt morphology of their sexual apparatus which makes it distinguish from all other eumycota by its ability to reproduce sexually by zygospores following gametangial fusion. Zygote formation in this group occurs within a structure arranged between two suspensor hyphae which make the whole apparatus resemble to an oxen yoke (from the greek word zygos)[6].

1.1 Aspects of mating reactions in Zygomycete Fungi

The morphological features of sexual development are basically identical in all zygomycetes. The process culminates in the formation of a characteristic fusion zone between the two parent hyphae where the mature, name-giving zygospore develops. It is contained within a thick-walled, often pigmented and ornamented structure, the zygosporangium, (**Figure 1**).

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5 Deacon, J. *Fungal Biology*, Blackwell, Oxford, **2006**, p. 24

6 Wφstemeyer, J.; Schimek, C. *Trisporic acid and mating in zygomycetes*. In: *Sex in Fungi: Molecular Determination and Evolutionary Implications*. Heitman, J.; Kronstad, J. W.; Taylor, J.W.; Casselton, L. (Eds.). **2007** ASM Press, Washington, pp. 431-443

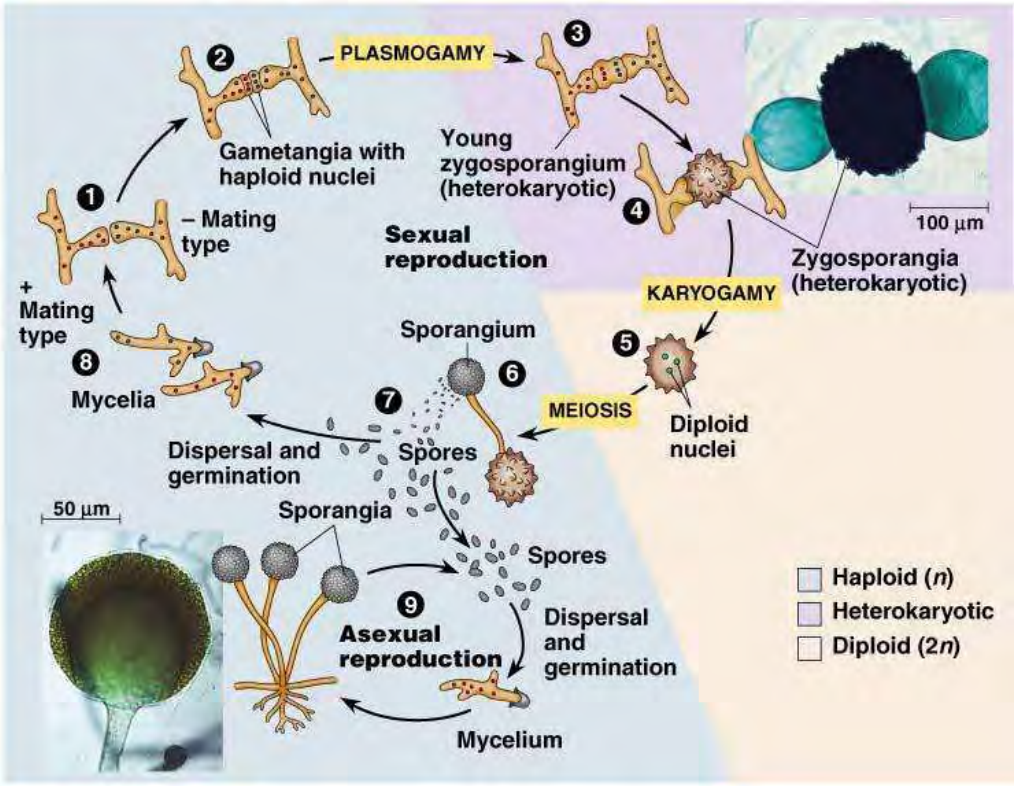

Figure 1. Sexual and Asexual cycle of *Phycomyces blakesleeanus*. When mycelia of opposite sex are close, develop specialized hyphae which called zygophores. These hyphae undergo identical parallel morphological changes and fuse to form the zygospores that containing cytoplasm and nuclei from the two species. After a dormancy period of several months, develops a germsporangiophore with germsporangium containing about ten thousand germospores prepared to initiate asexual life cycles. The germspores are active phenomena resulted of genetic recombination.

As zygomycetes are coenocytical fungi without regular septation, a large number of nuclei are finally incorporated into the zygospore. During zygospore maturation, the number of nuclei declines, and subsequently nuclear fusion and meiosis will take place.

Heterothallic species comprise two mating types designated (+) and (-). Blakeslee (1904)[7] managed to divide the Mucorales strains into two sexual types called (+) and (-). The two types are morphologically indistinguishable and only can be identified because of their reaction in the presence of the opposite sex.

When hyphae of the different mating types meet, a series of successive morphological changes takes place, bringing first the hyphal tips into close contact with each other and leading subsequently to the formation of swollen hyphal tips, gametangia, and finally the fusion zone. In homothallic species, these events will take place between branches of the same primary hypha.

Best studied in its physiological aspects is the heterothallic development, namely in the three species *Blakeslea trispora (Choanephoraceae), P. blakesleeanus (Phycomycetaceae)* and*Mucor mucedo (Mucoraceae*). In all three species, distinct sexually committed hyphae, the zygophores, are formed, but only in *M. mucedo* they rise above the substrate mycelium and are therefore a good marker for sexual induction (**Figure 1**).

Chemical recognition stands at the beginning of all sexual reactions and takes place both between the outermost hyphae in mycelial colonies approaching each other, and between the individual hyphae, may they be zygophores or hyphae branching out from the aerial mycelium in other species.

A sexual reproduction leads to the formation of sporangia at the tip of another type of aerial hyphae, the sporangiophores. Sexual development and asexual

[7] Blakeslee, A. F. *Proc. Amer. Acad. Arts Sci*. **1904**, *40*, 203.

development are often separated spatially within the mycelium and in these areas are mutually exclusive in some species[5] (**Figure 1**).

1.2 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 a part of their involvement in the recognition of mating partners, they 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*.[8]

Nevertheless the most interesting biological property of trisporic acids is its capacity to induce zygophores formation, the first crucial step in the sexual process of Mucorales. This originates, in turn, great increased in the production of β-carotene (carotenogenesis) in these fungi, giving consequently large industrial interest to these molecules. The production of β-carotene is observed by an intensification of the orange-red coloration in the sexual area when two mycelia of different sexes are located nearby. Currently, all experimental evidence suggests that they are pheromones responsible for these sexual processes[9].

The regulation of sexual development and their trisporoid´s precursors is not limited to members of Mucorales, also occurs within the order Mortierellales[10]. Furthemore, overproduction of carotenoids is observed for other apocarotenoids

8 Schultze, K.; Schimek, C.; Wφstemeyer, J.; Burmester, A. *Gene* **2005**, *348*, 33-44.

9 Austin, D. J.; Bu`Lock, D. J.; Drake, D. *Experiencia* **1970**, *26*, 348-349.

10 Schimek, C.; Kleppe, K.; Saleem A. *Mycol. Res.* **2003**, *107*, 736-7

structurally related to trisporoids. According to the stimulating effect, the following order was established: Trisporoids > ABA (abscisic acid) > β-ionone > α ionone > vitamin A[11].

2. CLEAVAGE OF β-CAROTENE: THE INITIAL STEP IN SEXUAL COMMUNICATION

The connection between β-carotene and trisporic acid was established early in trisporoids research, when, first, trisporic acid was established as a substance produced by mated cultures of (+) and (-) strains of *B. trispora* and *M. mucedo*[12] enhancing β-carotene synthesis[13]. The biosynthetic connection was founded on the observations that reduction of the amount of β-carotene by either mutation[14] or treatment with diphenylamine[15], results in reduced trisporic acid synthesis.

Moreover, radioactivity from ^{14}C-labeled β-carotene – administering to growing cultures. *B. trispora*, *M. mucedo* and *P. blakesleeanus* in particular, with respect to the diverse active substances they contain, during the sexual reproduction cycle[16] -, 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

11 (a) Dankear, S.; Modi, V.V.; Jani, U.K. *Phytochemistry* **1980**, *19*, 795-798. (b) Corrochano, L.M.; Cerdα-Olmedo, E. *Trends Genet*. **1992**, *8*, 268-274

12 (a) Van den Ende, H. *Nature* **1967,** *215*, 211-212. (b) Gooday, G.W. *New Phytol*. **1968**, *67*, 815-821.

13 (a) Caglioti, A.L.; Cainelli, G.; Camerino, B.; Mondelli, R.; Prieto, A.; Quilico, A.; Salvatori,T.; Selva, A. *Tetrahedron Suppl.* **1966,** *7*, 175-187. (b) Vail, W.J.; Morris, C.; Lilly, V.G. *Mycologia* **1967**,*59*, 1069-1074 (c) van den Ende, H. *J. Bacteriol*. **1968**, *96*, 1298-1303. (d) Govind, N.S.; Cerda-Olmedo, E. *J. Gen. Microbiol.* **1986**, *132*, 2775-2780.

14 (a) Sutter, R.P. *Proc. Natl. Acad. Sci. USA* **1975**, *72*, 127-130. (b) Sutter, R.P.; Grandin, A.B. Moore, W.R. *Fungal Genet. Biol.* **1996,** *20*, 268-279

15 (a) Austin, D.J.; Bu'Lock, J.D.; Gooday, G.W. *Nature* **1969**, *223*, 1178-1179. (b) Bu'Lock, J.D.; Jones, B.E.; Taylor, D.; Winskill, N.; Quarrie, S.A. *J. Gen. Microbiol*. **1974**, *80*, 301-306.

16 Sutter, R. P.; Harrison, T. L.; Galasko G. *J. Biol. Chem*. **1974**, 249, 2282-2284

pathway[17]. All the above findings imply that the first step in trisporoid synthesis is the oxidative cleavage of β-carotene.

The cleavage of β-carotene is generally carried out by a β-carotene mono- or dioxygenase acting at specific double bonds in the carotene molecule and arise either two or three cleavage products. Two putative carotene oxygenase genes named TSP3 and TSP4, have been identified in the first available zygomycete genome obtained from a clinical isolate of *Rhizopus oryzae*[18]. The functionality of the higher conserved gene, TSP3 was shown by heterologous expression in a β-carotene producing strain of *Escherichia coli*. Their connection to mating reactions or in trisporoids synthesis, shown with Northern hybridization studies that revealed mating-related expression only for the TSP3. Its mRNA is only detectable in sexually induced mycelia sufficiently developed to undergo sexual development. Similar observations were made for the correspondent TSP3 of *B. trispora*. The transcription levels increased only in mated cultures and the displayed amounts of the transcriptional activity varied not only between the different sexes of the strains but also among the same sex of the same strain.

In the meantime, were identified the corresponding genes, by BLAST search, in the newly available genomes of *P. blakesleeanus* and *Mucor circinelloides* and comparison to the *B. trispora* carotene oxygenase, showed identity values of the derived amino acid sequences of about 80% in *M. circinelloides*, *R. oryzae* and *P. blakesleeanus*.

Moreover a second putative β-carotene oxygenase gene identified in *P. blakesleeanus* that has 31% identical amino acids with TSP3 and thus exhibits the same identity ratio as found between TSP3 and TSP4 in *R. oryzae*. These fundamental differences in primary structure, and the observed differences in transcription regulation, assume different functions of the respective gene products of TSP3[19].

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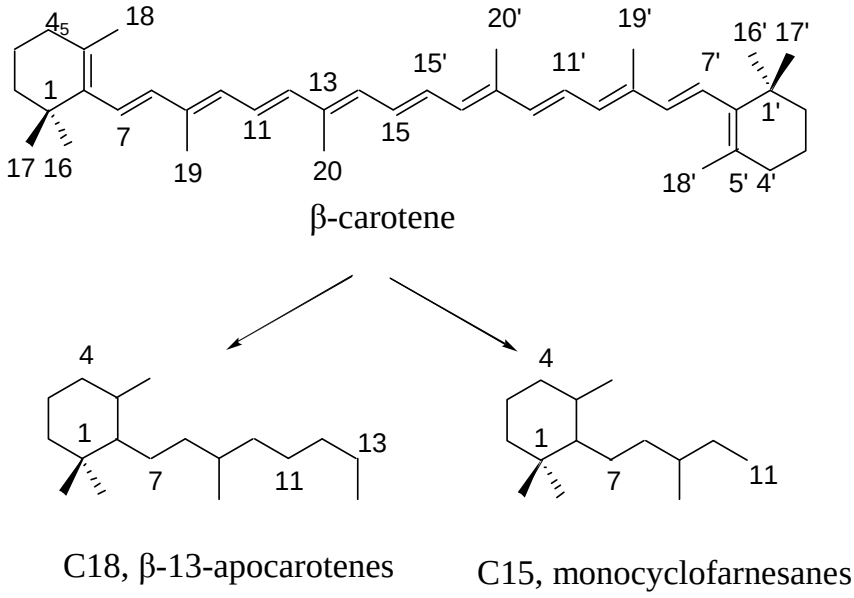
17 Austin, D.J.; Bu'Lock, J.D.; Drake, D. *Experientia* **1970**, *26*, 348-349.
18 Kloer, D.P.; Ruch, S.; Al-Babili, S.; Beyer, P.; Schulz, G.E. *Science* **2005**, *308*, 267-269.
19 Burmester, A.; Richter, M.; Schultze, K.; Voelz, K.; Schachtschabel, D.; Boland, W.; Wφstemeyer, J.; Schimek, C. Fungal Genet. Biol. **2007**, *44*, 1096-1108.

3. CLASSIFICATION OF TRISPOROIDS

Based on their chemical structure, trisporoids (family of isoprenoid compounds), are divided into two groups, as shown in **Schema 1**:

- Trisporoids containing 18 carbon atoms, named, β-13-apocarotenes
- Monocyclofarnesanes or apotrisporoids, containing 15 carbon atoms, named, β-11-apocarotenes

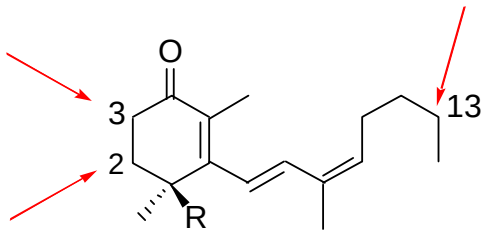


Schema 1. Classes of Trisporoids

Five trisporoid families are known designed by the letters A-E characterized by specific substitute pattern at C2, C3 and C13, (**Figure 2**)[20]. Within each family are described five groups (trisporins, trisporols, trisporic acids, methyl trisporates, methyl 4-dihydrotrisporates) according to R. In all groups there is a carbonyl group in

[20] Cerdα-Olmedo, E.; Lipson, E.D. *Phycomyces*, Cerdα-Olmedo, E.; Lipson, E.D. (eds.). Cold Spring Harbor Laboratory, Cold Spinrg Harbor, New York, **1987**.

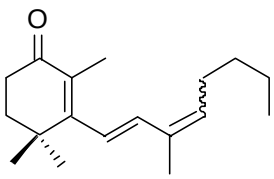
position 4, except in the group of methyl 4-dihydrotrisporates that have is a hydroxyl group in this position.



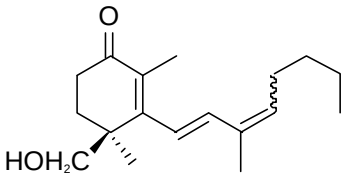
Families	C2	C3	C13
A	H,H	H,H	H,H
B	H,H	H,H	=O
C	H,H	H,H	H,OH
D	H,OH	H,H	=O
E	H,H	H,OH	H,OH

Figure 2: General structure of trisporoids.

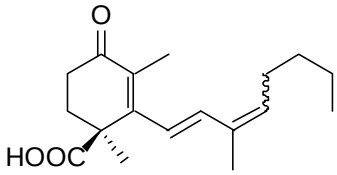
TRISPOROIDS OF A FAMILY



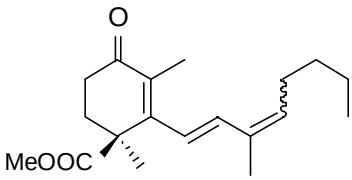
Trisporin A



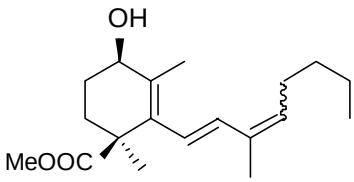
Trisporol A



Trisporic A Acid

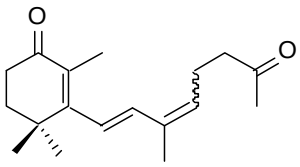


Methyl-trisporate A

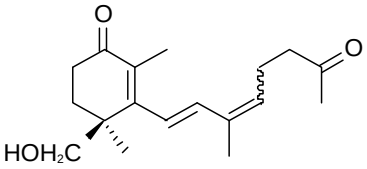


Methyl 4-Dihydrotrisporate A

TRISPOROIDS OF B FAMILY



Trisporin B



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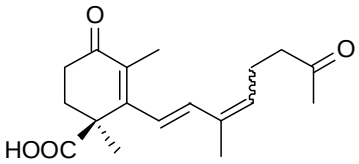
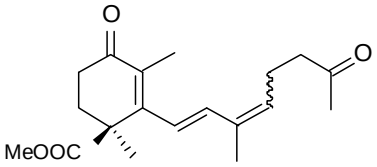
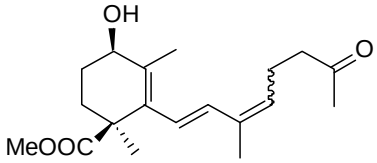


Trisporic B Acid

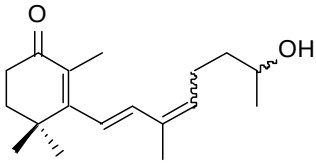


Methyl-trisporate B

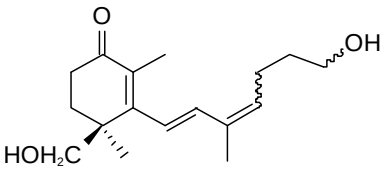


Methyl 4-Dihydrotrisporate B

TRISPOROIDS OF C FAMILY



Trisporin C



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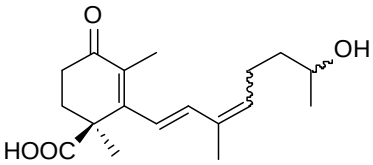

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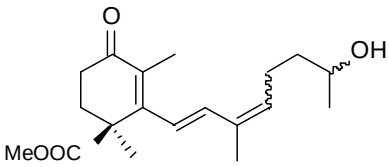

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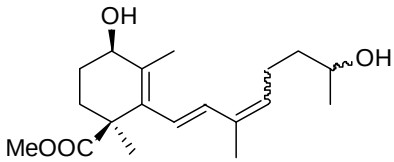
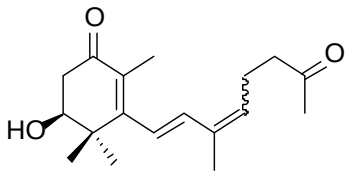


Methyl 4-Dihydrotrisporate C

TRISPOROIDS OF D FAMILY



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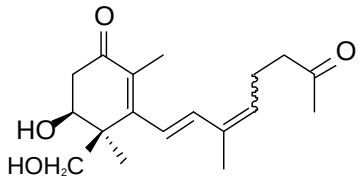
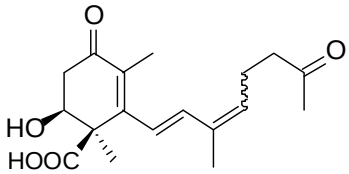
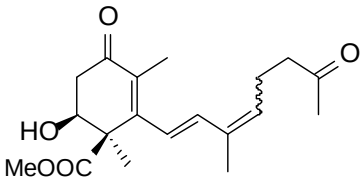


Trisporol D



Trisporic D Acid



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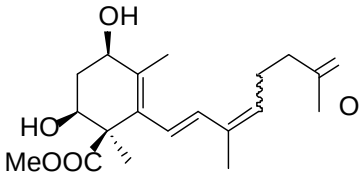
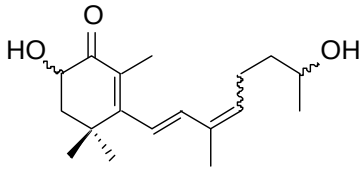


Methyl 4-Dihydrotrisporate D

TRISPOROIDS OF E FAMILY



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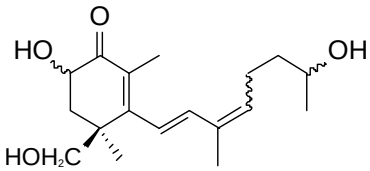
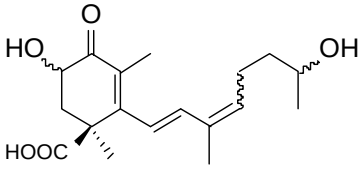
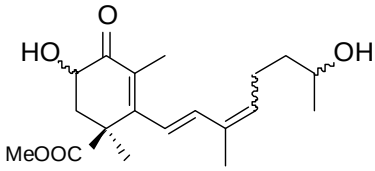


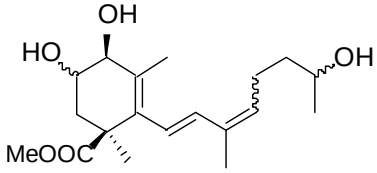
Trisporol E



Trisporic E Acid



Methyltrisporate E



Methyl 4-Didydrotriporate E

The stereochemistry of the disubstituted double bond in all trisporoids identified is *E*, while the double bond trisubstituted can have *E* or *Z* stereochemistry.

4. OBJECTIVES

Continuing our researches on biotechnology of filamentous fungi,[21] 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, licopene and other chemical products. In this sense the group of Prof. Cerdα-Olmedo has demonstrated recently that both effects are independent and therefore different mechanisms must exist for its development.[22]

In this report, is presented the chemical study of apocarotenes in the single and mated cultures of the wild strains, A56(+) and NRRL1555(-), of *Phycomyces blakesleeanus*. The methodologies optimizated by Barrero *et al.*[23] will be used for this study and the single and mated cultures of the mutants carB10, C5(-) and S342(+), devoid of β-carotene will also be used as blanks. This research will allow, if necessary, the isolation of new apocarotenes for their biological test as potential chemical signals that induce the sexual interaction in Mucorales or the overproduction of β-carotene.

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21 a) Avalos, J.; Cerda-Olmedo, E.; Reyes, F.; Barrero, A. F. *Current Organic Chemistry* **2007**, *11*, 721-737. b) Barrero, A.F.; Oltra, J.E.; Cabrera, E.; Reyes, F.; Alvarez, M. *Phytochemistry* **1999**, *50*, 1133-1140. c) Weinkove, D.; Poyatos, J.A.; Greiner, H.; Oltra, J.E.; Avalos, J.; Fukshansky, L.; Barrero, A.F.; Cerda-Olmedo, E. *Fungal Genetics and Biology* **1998**, *25*, 196-203. d) Barrero, A.F.; Oltra, J.E.; Poyatos, J.A.; Jimenez, D.; Oliver, E. *Journal of Natural Products* **1998**, *61*, 1491-1496. e) Barrero, A.F.; Oltra, J.E.; Poyatos, Juan A. *Phytochemistry* **1996**, *42*, 1427-1433. f) Fernandez-Martin, R.; Reyes, F.; Domenech, C.E.; Cabrera, E.; Bramley, P.M.; Barrero, A.F.; Avalos, J.; Cerda-Olmedo, E. *Journal of Biological Chemistry* **1995**, *270*, 14970-4.

22 Kuzina, V.; Cerdα-Olmedo, *E. Appl. Environ. Microbiol.* **2006**, *72*, 4917-4922.

23 Fernαndez-Lopez, J.A.; Tesis de Master **2008,** Universidad de Granada

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II. LITERATURE OVERVIEW

The name trisporoids is derived from the corresponding acids-molecules oxidatively modified at both the ring moiety and the isoprenoid side chain-, whose presence came into light in 1966, with the work of Cagliotti *et al.*[1].They managed to isolate and identify trisporic acids B and C from acid fraction of *Blakeslea trispora* cultures, as compounds that stimulate carotenogenesis in separate cultures of every sex and also as substances increasing β-carotene production in mated cultures of this fungus.

Only one year later, Cainelli *et al.*[2] isolated and identified apotrisporol and apotrisporin from neutral phase of *Blakeslea trispora* cultures.

Heisenberg and Cerdα-Olmedo[3] demonstrated that mutant *car* of *Phycomyces blakesleeanus*, defective in the gene encode for carotene, didn't show any sexual activity and Austin *et al.*[4] observed that mated cultures of *B. trispora* fed with radiolabeled β-carotene, produced also radioladeled trisporic acids. So, the correlation between β-carotene, trisporoids and morphogenetic factors like the induction of zygospores, came along really soon.

Later, Bu'Lock *et al.*[5] also from *Blakeslea trispora* identified trisporic acids A, C and its 9-*cis* isomers. Its aim was to obtain trisporoids by biotransformation, incorporating into the culture medium substrate methylether of retinol, among others. As result they proposed the following biosynthetic pathway, which depicts the degradation of β-carotene to trisporoids and apotrisporoids and moreover it is accepted until present (**Scheme 1**).

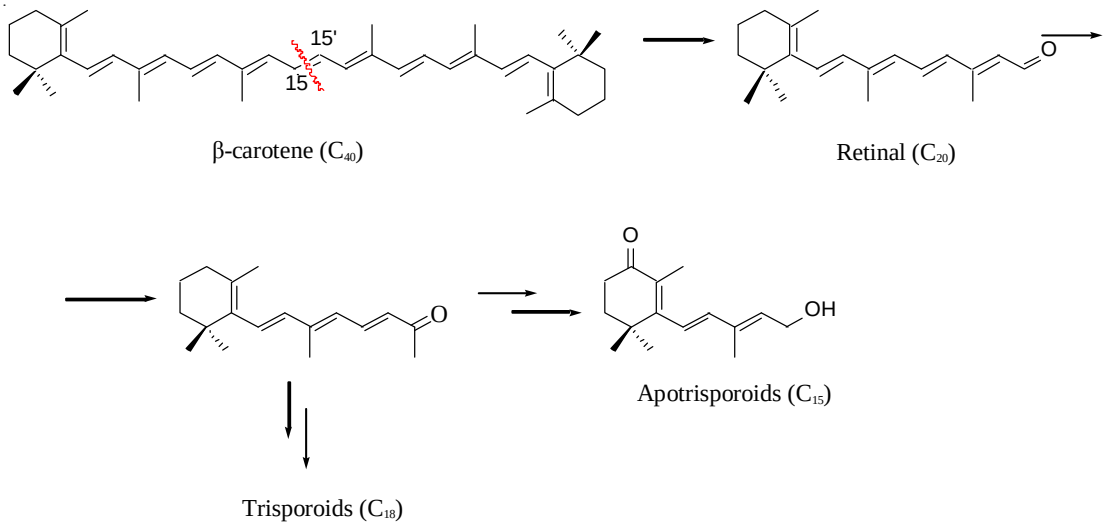
1 Caglioti, L.; Cainelli, G. *Tetrahedron Supplement* **1966**, *7*, 175-187.

2 Cainelli, G.; Grasselli, P.; Selva, A. *Chimica e l'Industria* **1967**, *49*, 748-751

3 Heisenberg ,:M., Cerdα –Olmedo, E. *J. Gen. Microbiol* **1968**, *132*, 2775-2780

4 Austin, D. J.; Bulock, J. D.; Drake, D. *Experientia* **1970**, 26, 348-349

5 Bu'lock, J. D.; Drake, D.; Winstanley, D. J. *Phytochemistry* **1972**, *11*, 2011-2018



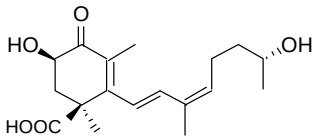
Scheme 1. Trisporoids Biosynthesis. It is presented a key biosynthetic step of enzymatic degradation of β-carotene to retinal (C20), that is later on metabolized to β-apo-13-carotenone (C18) from which, through new biotransformations originate trisporoids (C18) and apotrisporoids (C15).

From pioneering analytical work of van den Ende *et.al.*,[6] Werkmann *et.al*[7] Austin *et al.*[4] and Sutter *et al.*[8] with the fungi *Mucor mucedo*, *P. blakesleeanus* and the trisporoid-overproducing species *B. trispora* was envisaged that the production of the bioactive trisporoids demands a putative chemical dialogue between sexual partners (cooperative synthesis), which involves an exchange of early trisporoids as precursors[9]. Both the precursors and the end product trisporic acid, display physiological activity in the mating process.

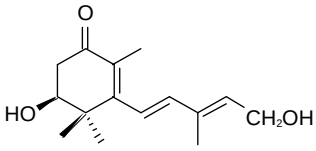
It was then, when Sutter *et al.*[10], identified trisporic E acid in *P. blakesleeanus* and found that the methylester of trisporic E acid produces excellent biological activities by inducing the development of zygophores and other sexual responses in

6 Van den Ende, H.; Werkman, B.A.; Van den Briel, M. L. *Arch. Mikrobiol* **1972**, *86*, 175-184.
7 Werkman, T. A.; Van den Ende, H. *Arch. Mikrobiol.* **1973**, *90*, 365-374
8 Sutter, R. P.; Harrison, T. L.; Galasko, G. *J. Biol. Chem.* **1974**, *249*, 2282-2284.
9 (a) Sutter, R. P. *Exp. Mycol*. **1986**, 10, 256-258. (b) Sutter, R. P.; Dadok, J.; Bothnerby, A. A.; Smith, R. R.; Mishra, P. K. *Biochemistry* **1989**, *28*, 4060-4066.
10 Matthew, L. M.; Sutter, R. P. *J. Biol. Chem.* **1984**, *159*, 6420-6422.

Mucorales. Two years after the identification of apotrisporine E advocated the same result[11].

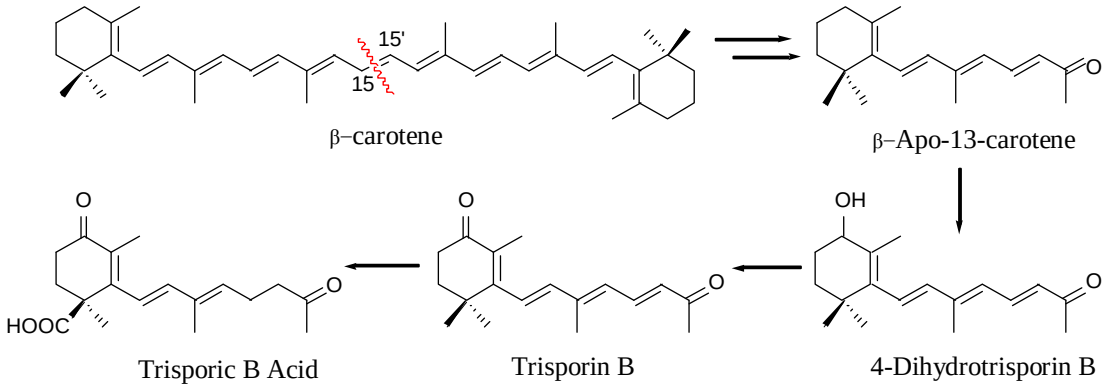


Trisporic E Acid



Apotrisporine E

The group Belozerskaya *et al.*[12] years later, found the enzymatic degradation of β-carotene up to obtainment of trisporic acids (**Scheme 2**).



Scheme 2. Degradation of β-carotene

Later on found evidence that the regulation of sexual development by trisporic acid and its precursors is not limited to the members of the order *Mucorales* but also occurs within the order *Mortierellale*s.[13]

Physiological activities of trisporoid compounds and derivatives were measured at various levels. For zygophore induction, the most important features are the dimension of the longer side chain, the number of conjugated double bonds in this

11 Sutter, R. P. *Experimental Mycology* **1986**, 10, 256-8.

12 Gessler, N. N.; Sokolov, A.V.; Belozerskaya, T. A. *Appl. Biochem. Microbiol* **2002**, *38*, 536-543

13 Schimek, C.; Kleppe, K.; Saleem, A.; Voigt, K.; Burmester, A.; Wφstemeyer, J. *The British Mycological Society* **2003**, *107*, 736-747

side chain and the polarity of the functional groups at C(4) and C(13)[14] were found. In other approaches, the rate of β-carotene synthesis or the effect of the different compounds on the expression or activity of trisporoid synthesis enzymes is measured.

One year after, Kuzina et al.[15] demonstrated that sexual interaction which leads to zygospore development and sexual carotenogenesis are two uncoupling processes which function by different mechanisms. The addition of small carboxylic acid to mated cultures of *B. trispora* and *P. blakesleeanus* stimulates the morphogenetic response but inhibits the metabolic response.

Recently, Boland *et al*.[16] have carried out the chemical synthesis of natural trisporoids and analogous products managing to obtain trisporic acids and trisporols through biotransformations of trisporins. The advantage of their concept was the small number of steps required for the synthesis, the flexibility to produce early and late trisporoids along the same protocol using common intermediates and finally the rapid access to deuterium-labeled compounds as valuable tools for the trisporic acid biosynthesis.

The same scientific group[17]- complementing the previous model proposed firstly from Van den Ende[18] and modified later from Sutter[9]-, advocated a cooperative biosynthetic pathway of trisporoids between the (+), (-) mating types of *B trispora* originated from β-carotene (**Scheme 3**) and unified experimental results from different zygomycetes (extension of this route to trisporic acids A, C, D and E).

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14 Schachtschabel, D.; Schimek, C.; Wφstemeyer, J.; Boland, W. *Phytochemistry* **2005,** *66*, 1358-1365.

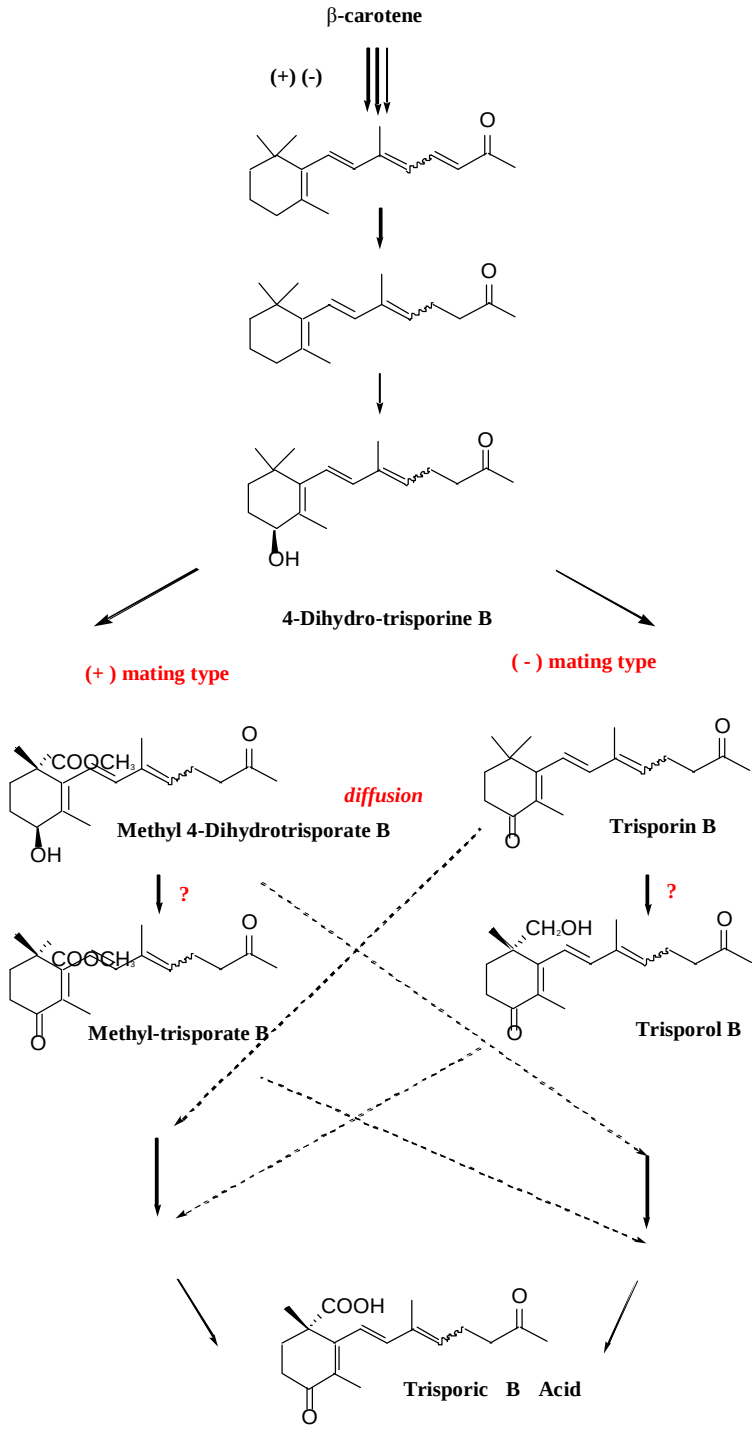
15 Kuzina, V.; Cerdα-Olmedo, E. *Enviromental Microbiology* **2006**, *72*, 4917-4922

16 Schachtschabel, D.; Boland, W. *J. Org. Chem*. **2007**, *72*, 1366-1372.

17 Schachtschabel, D.; David, A.; Menzel, K.D.; Schimek, C.; Wφstemeyer, J.; Boland, W. *ChemBioChem* **2008**, *9*, 3004-3012.

18 Van den Ende, H. *Sexual morphogenesis in the Phycomycetes*. in *The filamentous fungi*. Smith, J.E.; Berry, D.R. (Eds), *Wiley New York* **1979**, Vol. *3*, pp. 256-274

In it, the first intermediates are common to both sexes, with 4-dihydrotrisporin B the last common intermediate. For the occurrence of further transformations, mating partners were supposed to exchange early metabolites, such as trisporin B and methyl 4-dihydrotrisporate B, with their complementary mating partners. According to this concept, trisporic B acid (TSA B) is generated in the (-) type by the saponification of methyltrisporate B or, in the (+) mating type, by the oxidation of trisporol. They also find evidence that both mating types are able to produce all early intermediates en route to trisporol B albeit with different degrees of efficiency.



Scheme 3. Postulated cooperative biosynthetic pathway for TSA B. Its production required the exchange of the intermediates between mycelia of the different mating types. The details of the biosynthesis scheme were composed from experiments with radiolabeled precursors with compounds isolated from the mixed growing cultures and the logic of enzymatic transformations connecting the metabolites in a functional biosynthesis pathway.

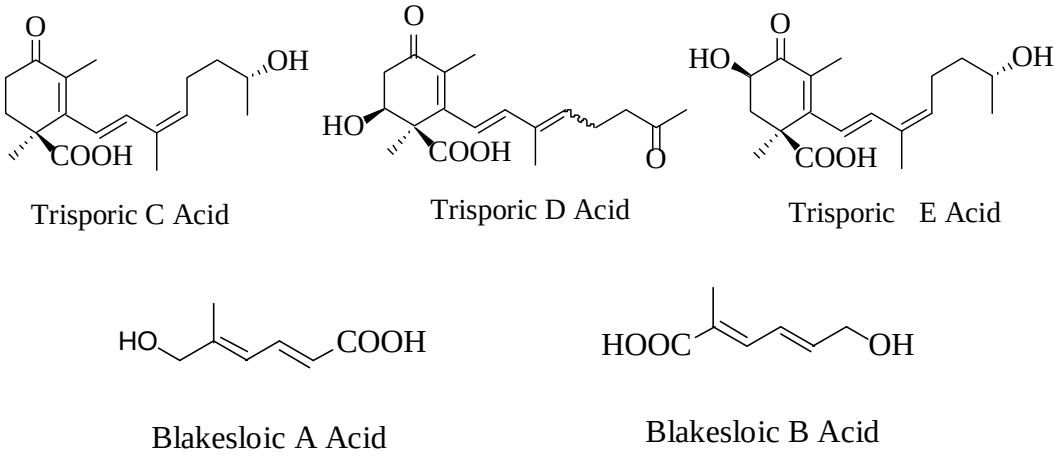
The same group revealed that the physiologically highly active methyl 4-dihydrotrisporate probably originates from another synthesis route and is apparently not a major intermediate for the production of trisporic acid (mated cultures of *B. trispora*). Instead, it gives rise to 4-dihydrotrisporic acid in the (-) mating type, a compound never isolated before from zygomycete cultures. The role of 4-dihydrotrisporic acid in sexual recognition and regulation is not yet known, but may well play a key role as signal molecule in mating reactions

Idnurm *et al.*[19] and his group, made the research into the basis of sexual differences finally possible, when they found a sex-specific region in the *Phycomyces blakesleeanus* genome. The sex loci unique for each mating type, sexM and sexP, both encode high mobility group (HMG) domain transcription factors with yet unknown function. Studies on their expression and the range of their regulatory activities will provide new insights into the mechanisms of the mating process. Still, apart from the basic determination of mating type by these sex loci, the strict complementarity of the sexual communication system relying on the participation of both mating types in the production of the regulatory pheromones would be alone sufficient to govern the sexual differentiation programme.

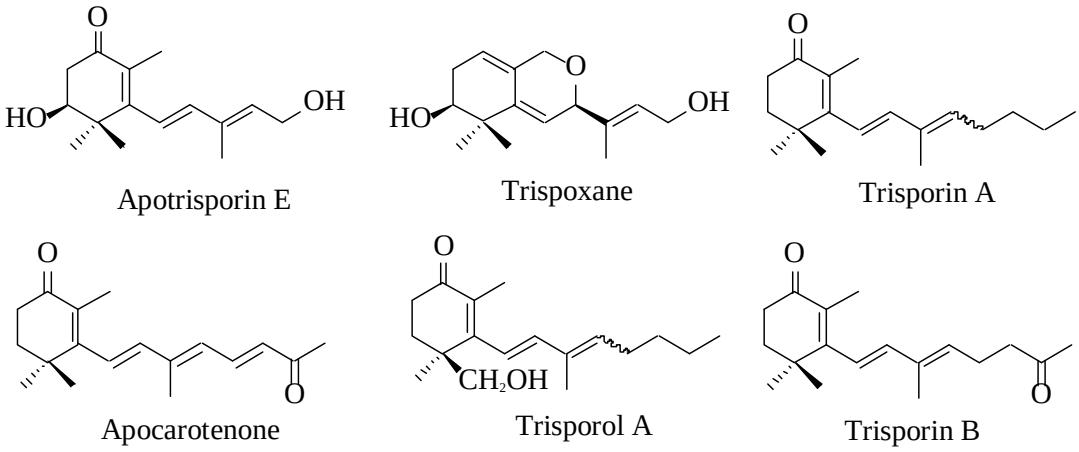
Barrero *et al.*[20] have identified in mated cultures of NRRL15555(-) and A56(+) strains of *P. blakesleeanus* the following trisporoids and apotrisporoids:

19 Idnurm, A.; Walton, F.J.; Floyd, A.; Heitman, J. *Nature* **2008**, *451*, 193-196.

20 Barrero, A. F.; Herrador, M. M.; Arteaga, P. *2ΙCongreso del GEQPN-RSEQ*. La Palma **2009**.



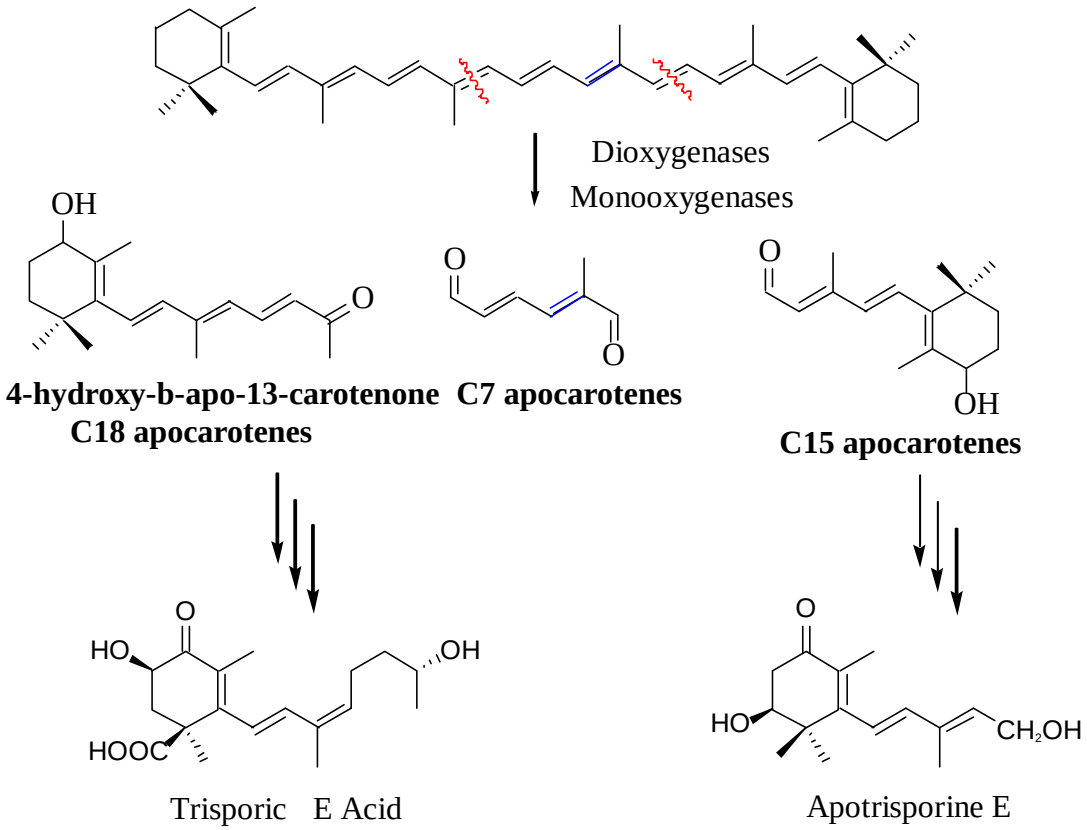
(a)



(b)

Figure 1. (a) Acid metabolites of mated solid cultures of *Phycomyces blakesleeanus*, (b) neutral metabolites of mated solid cultures of *Phycomyces blakesleeanus.*

Based on the results of their researches, the isolation of the C7 apocarotenes blakesleoic A and B acids, led this group to propose a new biosynthetic pathway for trisporoids and apotrisporoids by double oxidative cleavage of β-carotene in mated cultures of *Phycomyces blakesleeanus*. This proposal involves a different pattern of oxidative cleavage of β-carotene to the accepted (**Scheme 4)**.



Schema 4. New proposed biosynthetic pathway for trisporoids (C18 apocarotenes) and apotrisporoids (C15 apocarotenes).

III. RESULTS & DISCUSSION

1. OPTIMIZATION OF THE INCUBATION CONDITIONS FOR THE TRISPOROIDS BIOSYNTESIS IN SINGLE CULTURES IN LIQUID MEDIUM.

Considering the structures of different families of trisporoids that have just been previously reviewed, we firstly thought to detect their presence in the single cultures in liquid medium of *Phycomyces blakesleeanus* via UV spectrometry. The presence of different chromophores in trisporoids (carbonyl with conjugated double bonds, dienes, etc) that absorb at wavelengths quite selective at 328, 300, 280, 250, 230 nm, it should allow the detection of initial production of trisporoids by fungus, the monitoring of their evolution and finally, the estimation of the maximum concentration by UV spectrophotometry.

The measurements of absorbance were carried out in single liquid cultures of NRRL1555(-) and A56(+) *Phycomyces blakesleeanus* strains, of identical concentration, using distilled water as blank. Aliquots of medium (4-6 ml) were taken from the 3rd-4th day every 24 hours and the absorbance were determined at 328, 300, 280, 250 and 230 nm. The measurements in the two first days were not carried out, because of the previous results of our group.[1] The experimental results are shown below (**Table 1**).

[1] Fernαndez-Lopez, J.A.; Tesis de Master **2008,** Universidad de Granada

Table1. Measurements of absorbance of the single liquid cultures of *Phycomyces blakesleeanus* strains at different days of incubation.

STRAINS					
NRRL1555 (-)			A 56 (+)		
Incubation Days	λ (nm)	Absorbance values	Incubation Days	λ (nm)	Absorbance values
3rd	328	0.167	4th	328	0.217
	300	0.503		300	0.659
	280	1.434		280	1.662
	250	2.162		250	2.620
	230	2.635		230	3.304
4th	328	0.262	5th	328	0.389
	300	0.769		300	1.206
	280	1.923		280	2.653
	250	2.728		250	3.326
	230	3.010		230	3.544
5th	328	0.350	6th	328	0.512
	300	0.907		300	1.338
	280	2.103		280	2.744
	250	2.931		250	3.418
	230	2.981		230	3.541

As it is shown in the table 1, the absorbance was stabilized at 5th-6th of incubation at all wavelengths. This was the point that we've stopped the incubation, carrying out the work up of the cultures.

We could also observe the trisporoids production at first sight, looking at the color change of the biomass in process of time. The coloration changed of white (2nd day) to yellow (3rd day) and day by day, was getting slightly more intensive up to the 5-6th day. The yellow color is due to β-carotene production, which runs parallel to that of trisporoids.

2. CULTURES, EXTRACTION & FRACTIONATION.

It has been carried out in single cultures of the NRRL1555(-) and A56(+) *Phycomyces blakesleeanus* strains in liquid medium with orbital shaking, in single and mated cultures of the mutants C5(-) and S342(+) in solid medium, and in mated culture of NRRL1555(-) and A56(+) strains in solid medium. The incubation conditions are shown below (**Table 2**).

Table 2. Culture conditions.

Strain	Medium	Temperature (IC)	Shaking (rpm)	Incubation time (days)
NRRL1555(-)	liquid	22	200	5
A56(+)	liquid	22	200	5
C5(-)	solid	22	0	5
S342(+)	solid	22	0	5
C5(-) X S342(+)	solid	22	0	5
NRRL1555(-) X A56(+)	solid	22	0	5

As the grow of the fungus is different according to the culture type (in liquid cultures with shaking, the fungus forms a mycelia mass without sporangiophores, while in solid cultures the mycelium appears as a collection of hyphae, widespread by the agar surface, from which come out large sporangiophores vertically), the work up for the metabolite extraction has been carried out in every case by the adequate methodology.

2.1 Cultures in liquid medium.

The methodology followed for the analysis of cultures in liquid medium that is represented in **Figure 1**, was based on the experience of our research group in the metabolite isolation from culture media of different fungi[2] and to the structures of the trisporoid isolate from Mucorales[3].

[2] (a) Fernandez, M. R.; Reyes, F.; Domenech, C. E.; Cabrera, E.; Bramley, P. M.; Barrero, A. F.; Avalos, J.; Cerda-Olmedo, E. *Journal of Biological Chemistry* **1995**, *270*,14970-4. (b) Barrero, A. F.; Oltra, J. E.; Poyatos, J.A. *Phytochemistry* **1996**, *42*,1427-1433. (c) Weinkove, D.; Poyatos, J.A.; Greiner, H.; Oltra, E.; Avalos, J.; Fukshansky, L.; Barrero, A. F.; Cerda-Olmedo, E. *Fungal, genetics and biology* **1998**, *25*, 196-203. (d) Barrero, A. F.; Oltra, J. E.; Poyatos, J. A.; Jimenez, D. O. *Journal of natural products* **1998**, *61*,1491-6. (e) Barrero, A. F.; Oltra, J. E.; Cabrera, E.; Reyes, F.; Alvarez, M. *Phytochemistry* **1999**, *50*,1133-1140. (f) Avalos, J.; Cerda-Olmedo, E.; Reyes, F.; Barrero, A. F. *Current Organic Chemistry* **2007**, *11*,721-737

[3] Schachtschabel, D.; Schimek, C.; Woestemeyer, J.; Boland, W. *Phytochemistry* **2005**, *66*,1358-1365.

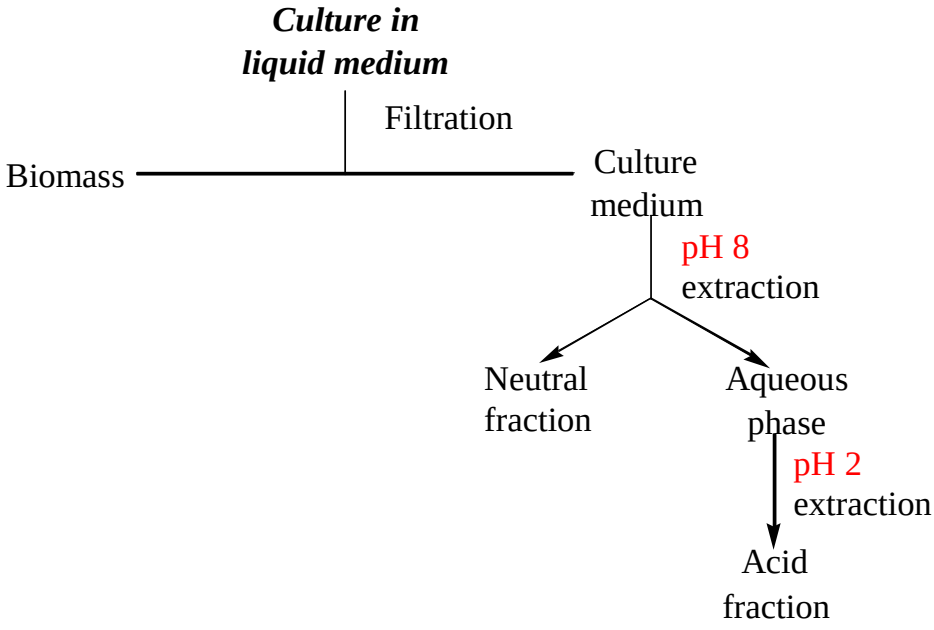


Figure 1. Methodology of extraction and fractionation in liquid cultures.

The first step for the apocarotenoids identification from the cultures in liquid medium was a filtration to eliminate the biomass of the culture medium. The latter was subjected to an acid-base fractionation. For it, the culture media was alkalized with 2N NaOH up to pH=8 and extracted with acetyl acetate (AcOEt) to obtain the ***Neutral Fraction***. Afterwards, the remaining aqueous phase was acidified to pH=2 with 2N HCl and newly extracted with AcOEt to obtain the ***Acidic Fraction*****.**

Table 3 shows the amounts of biomass (g), neutral and acid fractions (mg) from a culture medium volume (mL) obtained from single cultures in liquid medium of the applied strains NRRL1555(-), A56(+) of *P.blakesleeanus*.

Table 3.

Strain Culture	medium (mL)	Biomass (g)	Neutral fraction (mg)	Acid fraction (mg)
NRRL1555(-)	3600 40		40	60
A56(+)	3600 30		47	78

2.2 Cultures in solid medium.

The cultures in solid medium were extracted and fractionated following the methodology shown in **Figure 2.**

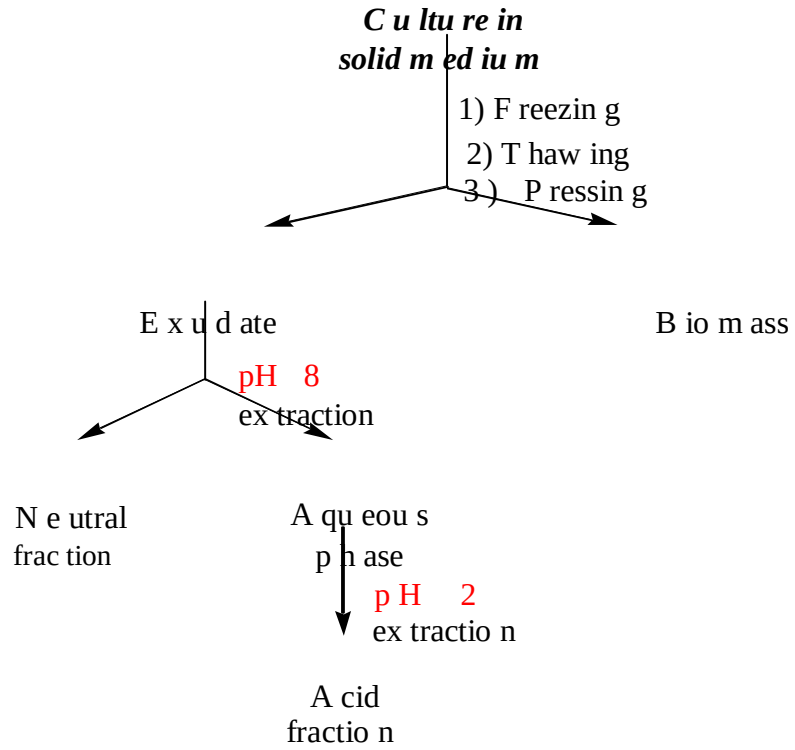


Figure 2. Methodology of extraction and fractionation in solid cultures.

The first step for the apocarotenoids identification from the cultures in solid medium was the preparation of the exudate. For it, the plates were frozen at –20ΊC for one night, and then left thawing at room temperature for one hour, in order to allow the obtainment of the exudate, whose yielding was increased by pressing the agar.

The exudate was subjected to an acid-base fractionation, equal of which indicated for the culture in liquid medium as to obtain the neutral and acid fractions.

Table 4 shows the amounts of neutral and acid fractions (mg) from an exudate volume (ml), obtained from single and mated cultures, in solid cultures of the applied strains C5(-), S342(+),C5(-) X S342(+), NRRL1555(-) X A56(+) of *P.blakesleeanus*.

Table 4.

Strain	Exudate (mL)	Neutral fraction (mg)	Acid fraction (mg)
C5(-)	700	17	37
S342(+)	650	51	31
C5(-) X S342(+)	650	18	63
NRRL1555(-) X A56(+)	2800	387	324

3. IDENTIFICATION OF APOCAROTENOIDS.

3.1 Identification of the Neutral apocarotenoids.

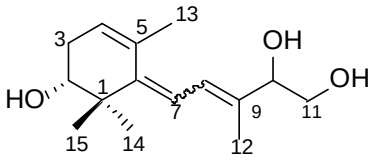
The analysis of the neutral fractions obtained from the single cultures in liquid medium of the NRRL1555(-) and A56(+) strains of *P. blakesleeanus* and that obtained from mated culture in solid medium, was carried out initially by ^{1}H NMR and analytical reverse-phase HPLC. These analyses showed that all neutral fractions obtained from different single cultures had the same apocarotenoid composition.

Apocarotenoid mixture obtained from single cultures was subjected to semipreparative normal-phase HPLC using *t*-buthylmethylether (*t*-BuOMe) as eluent. Two new apocarotenoids (**1** and **2**) were detected together with trispoxane (**3**). This

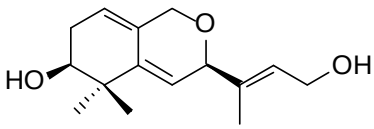
last apocarotenoid had been isolated and structurally elucidated previously for other researchers at our laboratory.[4]

Apocarotenoids **1** and **2** were detected as a 4:3 mixture in small quantity. Apocarotenoid **1** also was separated together with trispoxane (**3**) as a 1:1 mixture, moreover a greater quantity of **1** and **2** was separated in a mixture with **3**. GC-MS analysis of **1-2** mixture revealed that it was a mixture of isomers with $[M]^+$ at m/z 252. This datum together with ^{1}H and ^{13}C NMR data led to the molecular formula $C_{15}H_{24}O_3$.

The ^{13}C NMR and DEPT spectra of the **1-3** mixture showed for **1**, signals of four quaternary carbons (three olefinic), five methine groups (two oxygenated and three olefinic), two methylene groups (one oxygenated) and four methyl groups corresponding to an apotrisporoid structure very related to trispoxane **3**.



structure **1, 2**



trispoxane, **3**

Its ^{1}H NMR spectra (**Table 5**) revealed for **1**, in addition to signals due to a conjugated triene system at δ 5.44 (1H, br s), 6.18 (1H, d, J = 10.0 Hz), 6.62 (1H, d, J = 10.0 Hz), one oxygenated methine group at δ 4.12-4.20 (m) coupled with one oxygenate methylene group at δ 3.52 (br d, J = 10.0 Hz) and 3.63 (dd, J_1 = 4.9 Hz, J_2 = 10.0 Hz). Also signals corresponding to other oxygenated methine group located in position 2 of the ring on the basis of the chemical shift (δ 3.42) and multiplicity (t, J = 4.5 Hz), and to four methyl groups: two of them located on double bond (δ 1.76 and 2.01) and the other two were angular methyls (1.02 and 1.04). On the basis of these data it has been confirmed for **1** the structure of 5-hydroxy-2,6,6-trimethylcyclohex-2-enylidene)-3-methylpent-3-ene-1,2-diol, that we have named **trispotrientriol A**.

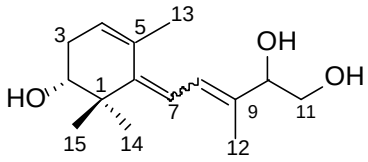
[4] Barrero, A. F.; Herrador, M. M.; Arteaga, P. *2ª Congreso del GEQPN-RSEQ*. La Palma **2009**, p.85

Table 5. ^{1}H and ^{13}C NMR data of **1** and **2**.

C	1		2
	δ_H	δ_C	δ_H
1		42.1	
2	3.42 t (4.5)	74.8	3.36 t (4.5)
3	3α: 2.09 br d (17.9)	31.1	3α: 2.09 br d (17.9)
	3β: 2.43 dd (2.9, 17.9)		3β: 2.43 dd (2.9, 17.9)
4	5.44 br s	125.7	5.51 br s
5		132.1	
6		136.9	
7	6.18 d (10.0)	124.1	6.32 d (10.0)
8	6.62 d (10.0)	120.9	6.72 d (10.0)
9		138.3	
10	4.12-4.20 m	59.4	4.12-4.20 m
11	11a: 3.52 br d (10.0)	65.5	11a: 3.52 br d (10.0)
	11b: 3.63 dd, (4.9, 10.0)		11b: 3.63 dd, (4.9, 10.0)
12	1.76 s	12.9	1.76 s
13	2.01 (3H, s)	25.3	1.86 s
14	1.04 s	25.4	1.32 s
15	1.02 s	27.1	1.20 s
OH	1.50 br s		

(Values in parentheses are coupling constants in Hz.)

The axial orientation of the hydroxyl group at C-2 was established in the basis of the observed values of $J_{2,3}\alpha = J_{2,3}\beta = 4.5$ Hz. The stereochemistry at C-10 and that of the double bonds C6/C7 and C8/C9 has not been possible to determine because of the great instability of compound (Compounds 1 and 2 partially decomposed during NMR spectra recorder).



The[1]H NMR spectrum (**Table 5**) of the **1-2** mixture revealed that the minor component (**2**) of the mixture was a geometric stereoisomer of **1**. The relative stereochemistry of **2** was not determined because of its instability. This compound has been named **Trispotrientriol B**. **Trispotrientriol A** and **B** are new apocarotenoids and constitute a new trisporoid family.

More complete analysis of the neutral fraction of the single and mated cultures was carried out by analytical reverse-phase HPLC with the assistance of standards obtained previously from different cultures of Mucorales[4] or/and by synthesis.[5] The following apocarotenoids have been identified and their relative proportions in single and mated cultures are comparated:

 Trispotrientriol A and **B**. These compounds showed two peaks at 20.0 and 20.3 min in the HPLC chromatogram (Figure 3) with absorbance maximum in the UV at $\lambda = 300$ nm. These compounds appeared in the mated culture also, but in lesser concentrations.

[5] Sαnchez-Lσpez, C.; Cervantes-Hernαndez, C.; Rosales, A.; Corral-Alvarez, M.; Dorado-Muρoz, M.; Garcva-Rodrvguez, I. *Tetrahedron* **2009**, *65*, 9542-9549.

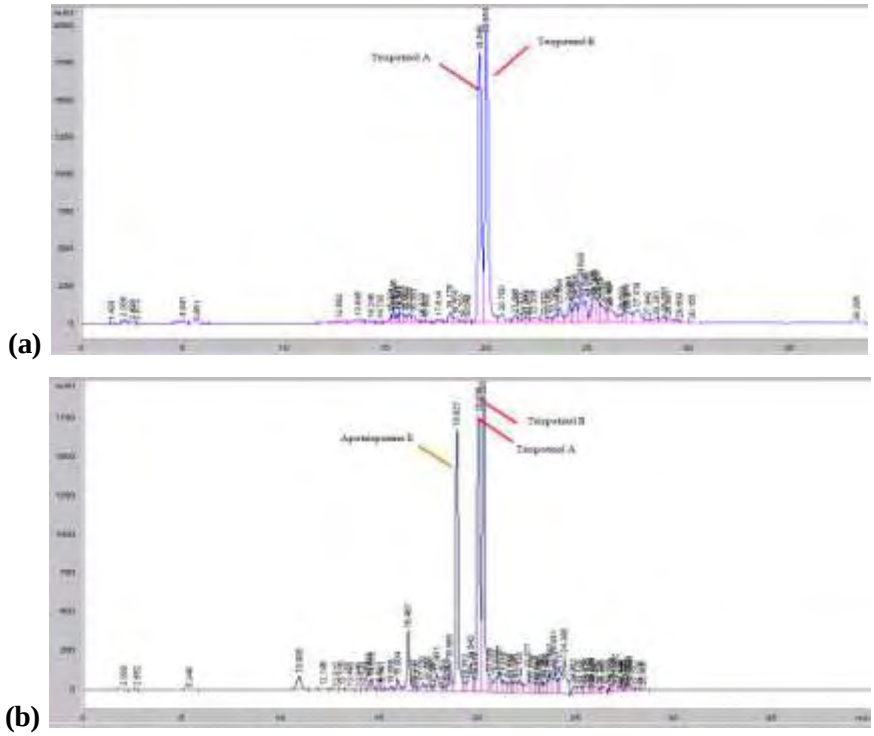
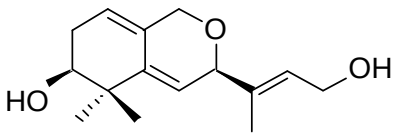


Figure 3. (a) Chromatogram of analytical reverse-phase HPLC of neutral fraction, at 300nm, acquired by **single** cultures of NRRL1555 (-) *Phycomyces blakesleeanus* strain. (b) Chromatogram of analytical reverse-phase HPLC of neutral fraction, at 300nm, acquired by **mated** cultures of NRRL1555 (-) X A56(+) *Phycomyces blakesleeanus* strains.

 Trispoxane: was identified in all the neutral fractions of the single and mated cultures. This compound showed one peak at 19.6 min in the HPLC chromatogram (**Figure 4**) with absorbance maximum in the UV atλ = 250 nm. Its abundance is greater in mated cultures.



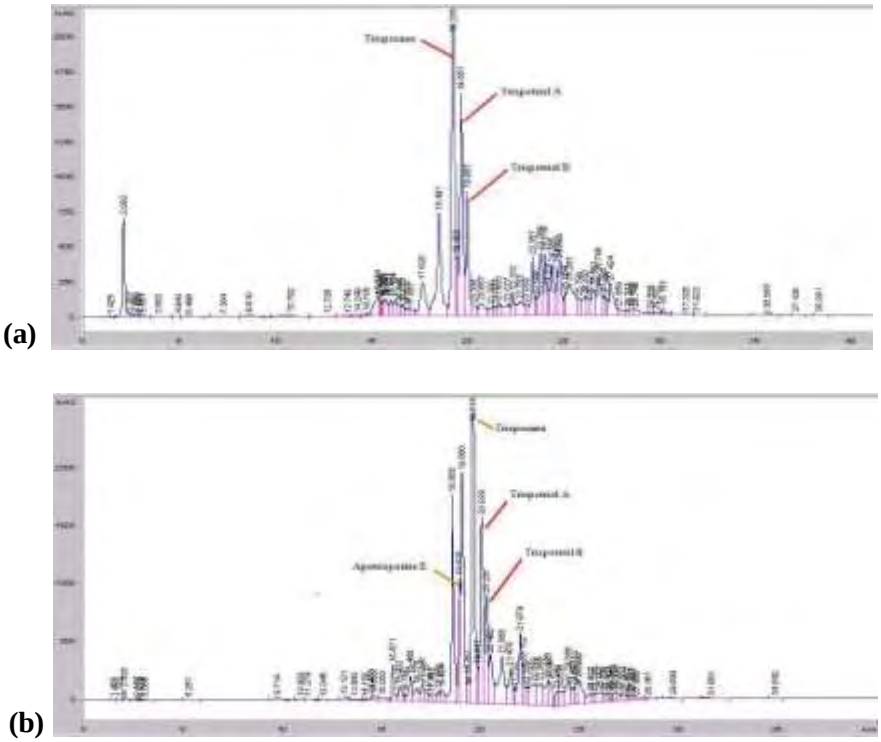
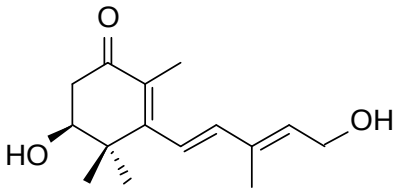
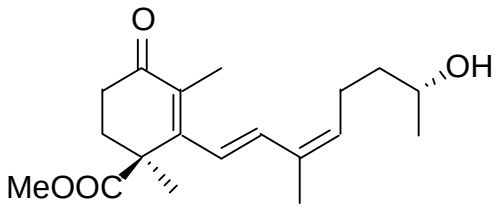


Figure 4. (a) Chromatogram of analytical reverse-phase HPLC of neutral fraction of single culture, at 250 nm, acquired by NRRL1555 (-) *Phycomyces blakesleeanus* strain**.** (b) Chromatogram of analytical reverse-phase HPLC of neutral fraction of mated culture, at 250 nm, acquired by NRRL1555(-) X A56(+) *Phycomyces blakesleeanus* strains**.**

 Apotrisporine E: Only was identified in the neutral fraction obtained from mated culture. This compound showed one peak at 18.9 min in the HPLC chromatogram (Figure 5) with absorbance maximum in the UV at λ = 300 nm.



 Methyl trisporate C (9Z): was identified in the neutral fraction obtained from mated culture. This compound showed one peak at 23.7 min in the HPLC chromatogram (**Figure 5**) with absorbance maximum in the UV at λ = 328 nm.



The presence of some apocarotenoids in neutral fractions was confirmed by ^{1}H NMR spectroscopy. The ^{1}H NMR spectra 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 1.22 (d, *J* = 6.0 Hz) due to **methyl trisporate C (9Z)**,[6] at δ 5.62 (t, *J* = 6.6 Hz), 5.50 (s), 5.31 (br s), 4.55 (s), 4.07-4.21 (m), 3.46 (t, *J* = 4.5 Hz), 1.60 (s), 1.05 (s) and 1.00 (s) due to **trispoxane**[4] and at δ 6.17 (d, *J* = 16.5 Hz), 6.12 (d, *J* = 16.5 Hz), 5.70 (1H, t, *J* = 7.0 Hz), 4.35 (d, *J* = 7.0 Hz), 3.89 (dd, J_1 = 4.0 Hz, J_2 = 9.5 Hz), 2.78 (1H, dd, J_1 = 4.0 Hz, J_2= 17.0 Hz) and 2.61 (1H, dd, J_1 = 9.5 Hz, J_2= 17.0 Hz) due to **apotrisporine E**.[7]. Also the ^{1}H NMR spectra established that **Trispoxane** was the major component of the neutral fraction in both single and mated cultures.

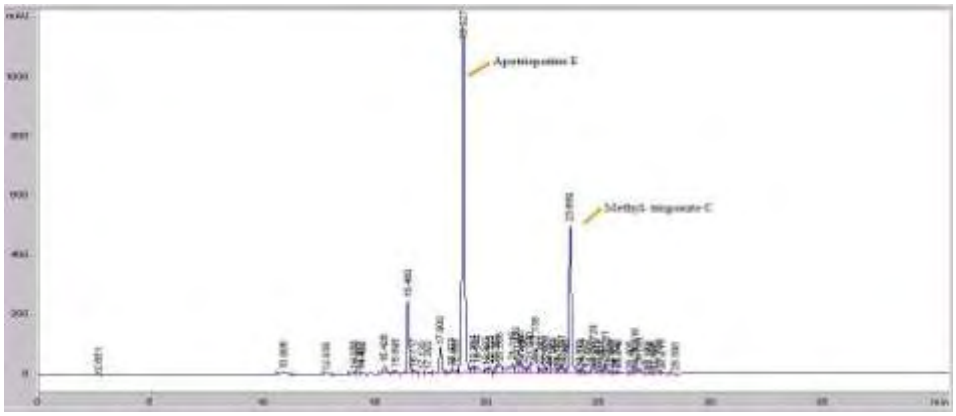


Figure 5. Chromatogram of analytical reverse-phase HPLC of neutral fraction of **mated** culture, at 328 nm, acquired by NRRL1555(-)XA56(+) *Phycomyces blakesleeanus* strains.

6 Schachtschabel, D.; Boland, W. *J. Org. Chem.* **2007**, *72*, 1366-1372.

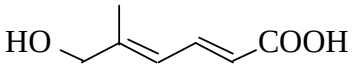
7 Sutter, R. P. *Experimental Mycology* **1986**, *10*, 256-258.

The analysis of the neutral fractions obtained from the **single and mated cultures of C5(-) and S342(+) strains** of *P. blakesleeanus* showed the absence of apocarotenoids in these fractions. Thus their composition was not studied.

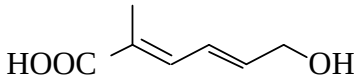
3.2 Identification of the Acid apocarotenoids.

The study of the acid fractions obtained from mated cultures of NRRL1555(-) and A56(+) strains was carried out following a similar methodology as for the neutral fractions with the aid of standards. In these fractions were identified the following apocarotenoids:

 Blakesleoic A and blakesleoic B acids: were identified in all acid fractions. These compounds showed one peak at 2.4 min in the HPLC chromatogram (**Figure 6**) with absorbance maximum in the UV at λ = 250 nm. These are C7 apocarotenoids identified by our group in precedent work[8]

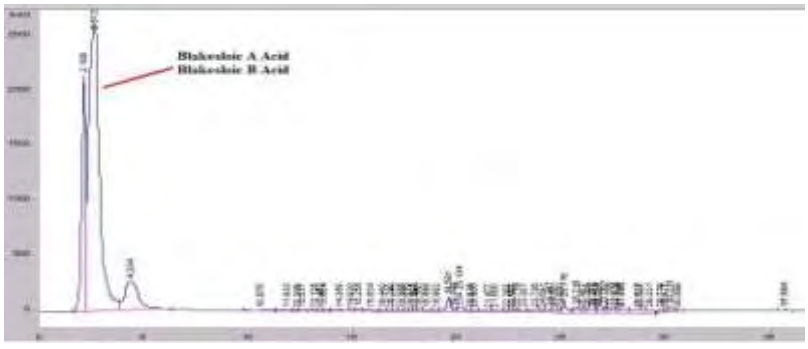


Blakesleoic A acid



Blakesleoic B acid

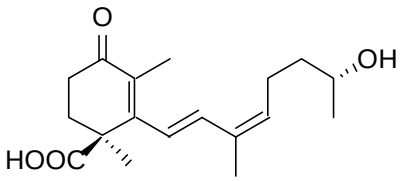
(a)



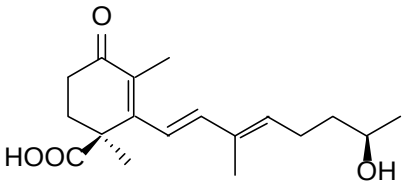
[8]Μυρoz, H.J.; Tesis de Master **2009,** Universidad de Granada

Figure 6.(a) Chromatogram of analytical reverse-phase HPLC of acid fraction of **single** culture, at 250 nm, acquired by NRRL1555 (-) *Phycomyces blakesleeanus* strain. (b) Chromatogram of analytical reverse-phase HPLC of acid fraction of **mated** culture, at 250 nm, acquired by NRRL1555 (-) X A56(+) *Phycomyces blakesleeanus* strains.

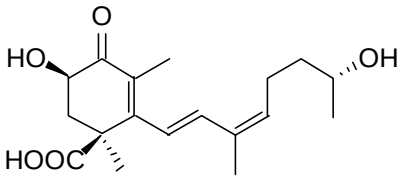
 Trisporic C acid (9*Z*): was identified in the acid fraction obtained from mated culture. This compound showed one peak at 15.1 min in the HPLC chromatogram (**Figure 7**) with absorbance maximum in the UV at λ = 328 nm.



 Trisporic C acid (9*E*): was identified in the acid fraction obtained from mated culture. This compound showed one peak at 15.3 min in the HPLC chromatogram (**Figure 7**) with absorbance maximum in the UV at λ = 328 nm.



 Trisporic E acid (9*Z*): was identified in the acid fraction obtained from mated culture. This compound showed one peak at 16.7 min in the HPLC chromatogram (**Figure 7**) with absorbance maximum in the UV at λ = 328 nm.



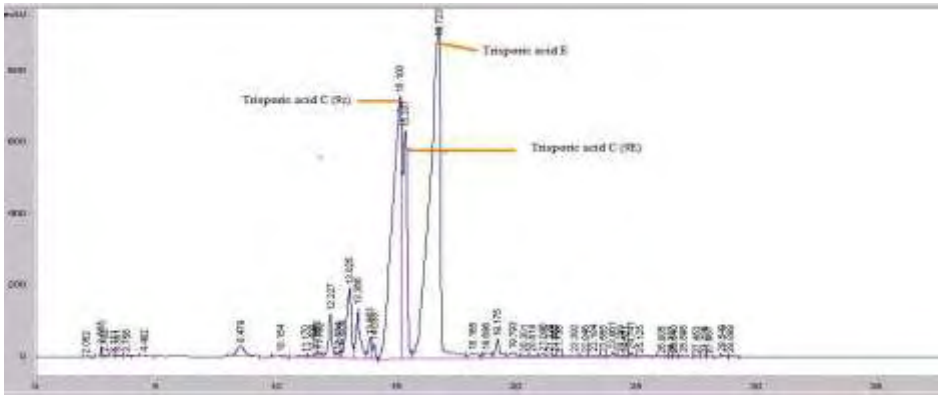


Figure 7. Chromatogram of analytical reverse-phase HPLC of acid fraction of **mated** culture, at 328 nm, acquired by NRRL1555 (-) X A56(+) *Phycomyces blakesleeanus* strains.

The presence of these apocarotenoids in acid fractions 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 **(9*Z*)-trisporic C acid**,[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 **(9*E*)-trisporic C acid**,[6] at δ .6.92 (d, *J* = 16.4 Hz), 6.38 (d, *J* = 16.4 Hz), 5.68 (t, *J* = 8.0 Hz), 4.32 (dd, J_1 = 6.3 Hz, J_2 = 13.0 Hz), 3.77-3.81 (m) and 1.23 (d, *J* = 6.0 Hz) due to **(9*Z*)-trisporic E acid**,[9] 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 A acid** 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

9 Sutter, R. P.; Dadok, J.; Bothnerby, A. A.; Smith, R. R.; Mishra P. K. *Biochemistry* **1989**, *28*, 4060-4066.

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 B acid**.

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-8 signal should appear downfield relative to that of the H-8 of the (9*E*)-isomer[10].

In single cultures only were detected **Blakesleoic A** and **B acid** being the first the major component of the acid fraction in both single and mated cultures.

The analysis of the acid fractions obtained from the single and mated cultures of C5(-) and S342(+) strains of *P. blakesleeanus* showed the absence of apocarotenoids in these fractions. Their composition was not studied.

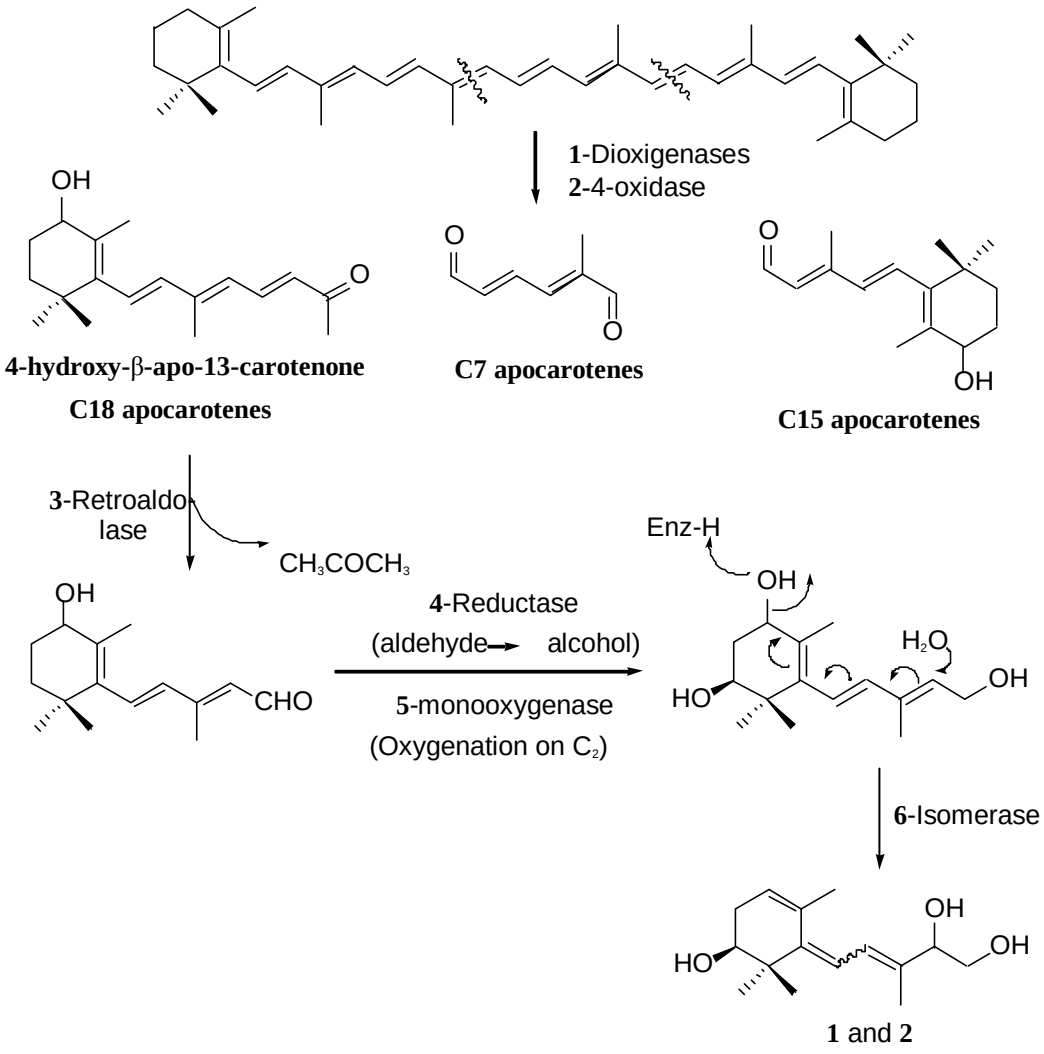
4. BIOSYNTHETIC PROPOSAL FOR TRISPOTRIOL A and B.

The results obtained in this research indicate that in the single culture of the NRRL1555(-) and A56(+) strains, were formed the new C15 apocarotenes (**1**-**3**), C7 apocarotenes (Blakesleoic acids A and B), but no C18 apocarotenes.

The presence of C7 apocarotenes in these cultures and their absence in the single culture media of the C5(-) and S342(+) strains, with a mutation *carB10,* devoid of β-carotene, indicate that the apocarotenoid production in the single strains starts from β-carotene and it is accomplished by a double oxidative cleavage.

On the basis to these results we propose the following biosynthetic pathway for **1** and **2** (**Scheme 1**).

[10] (a) White, D. J. *J. Org. Chem*. **1985**, *50*, 5233-5244. (b) Bu'Lock, J. D.; Drake, D.; Winstanley, D. J. *Phytochemistry* **1972**, *11*, 2011-2018.

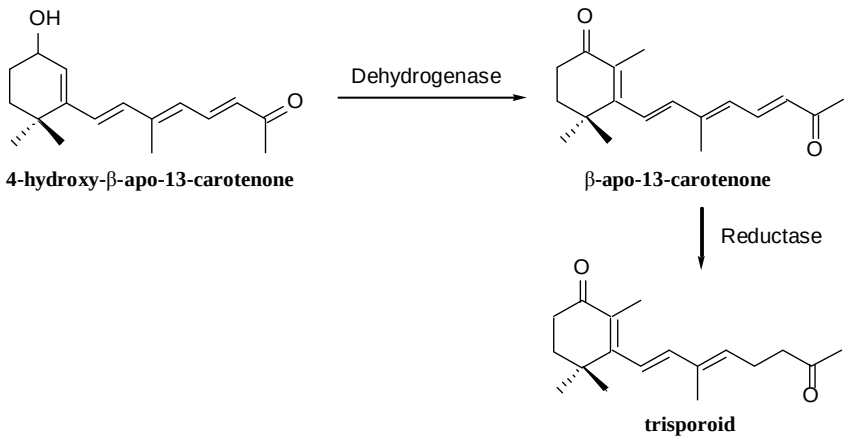


Scheme 1. Biosynthetic pathway for **1** and **2** in single cultures of *P.blakesleeanus*. The first step in the biosynthesis of these compounds is a double oxidative cleavage of **β-carotene from which are formed precursors of C18, C15 and C7 apocarotenes.**

The biosynthesis begins with double degradation of carotene (two dioxygenases, enzymatic system **1**) followed of a 4-hydroxylation (enzyme **2**). Thus are resulted three apocarotenoid families C18 and C15 with a hydroxyl at C4 position and the C7. Apo-carotenone C18 suffers a retroaldolic transformation (enzyme **3**) giving an aldehyde C15 apocarotenoid and losing acetone. Finally, reduction of carbonyl group and isomerization (enzymes **4** and **5**) drives to apotrientriols **1** and **2.**

In the mated cultures of the wild strains when the biosynthesis arrives to 4-hydroxy-apo-carotenone, a dehydrogenase which dehydrogenates the hydroxyl group

at C4 to ketone group and a reductase which hydrogenates the α, β positions of the methylketone in the third and fourth steps, respectively are activated (**Scheme 2**). Consequently these enzymes may have a stabilizing effect which prevents the C18 to C15 degradation.



Scheme 2. Third and fourth steps in the biosynthetic pathway of the trisporoid formation in mated cultures of *Phycomyces blakesleeanus*.

IV. EXPERIMENTAL PART

1. STRAINS & CULTURES

1.1 Strains

In this experimental work the strains of *Phycomyces blakesleeanus* used, (**Table 1**) were provided by the professor Dr. D. E. Cerdα-Olmedo of Genetic Department of the University of Sevilla.

Table 1. Strains used. The symbols (+), (-) designate the sexes. The genotypes like *car*, *geo*, indicate the mutations that affect the carotenogenesis and the geotropism respectively. Furthermore, the nicA101 is an auxotrophic mutation induced by the nicotinic acid and NG indicate the exposition in N-methyl-N'-nitro-N-nitrosoguanidine **(**MNNG) before the isolation of the mutant.

Strain	**Genotype**	**Origin**
NRRL1555	(-)	Unknown
A56	(+)	Isogenic NRRL1555[1]
C5	*car*B10geo-10(-)	NRRL1555 (NG)[2]
S342	*car*B10nicA101(+)	NG and crossings

The nomenclature of the strains has been given according to the origin of the corresponding strain and always with one or more capital letters[3]. For example NRRL1555(-) strains were taken from the Northern Regional Research Laboratory (that is called nowadays National Center for Agricultural Utilization Research, Peoria, Illinois, US). The strain A56 derives from the collection of the Professor A. Purez Eslava, University of Salamanca. The strain C5 comes from the collection of the Prof. Max. Delbróck, California, Institute of Technology (Pasadena, California, U.S.), and finally the strain S342 proceeded from the collection of the Genetic Department, University of Sevilla.

All the stains were conserved in a 50% glycerol solution and stored at -20° C in a concentration of about 10^6-10^7spores/ml.

1 Alvarez, M. I.; Eslava, A. P. *Genetics* **1983**, *105*, 873-879
2 Cerdα-Olmedo, E.; Reau, P. *Mutat. Res* **1970**, *9*, 369-384
3 Demerec, M.;Adelberg, E. A.; Clark, A. J.; Hartman, P. E. *Genetics* **1966**, *54*, 61-76

1.2 Preparation of the culture media

They were used minimal media, which were prepared as it is below indicated.

1.2.1 Preparation Of The Cultures In Liquid Minimal Medium

One liter of **MINIMAL** medium contains:

SIVA: 2 g of L- Asparagine

5 g of KH_2PO_4

0.5 g of $MgSO_4.7H_2O$

10 ml of the calcium Sutter 100x

10 ml of the solution Sutter 100x

480 ml of H_20

SIVB: 20 g of Glucose

500 ml of H_20

The solutions calcium Sutter 100x and Sutter 100x are formed by following constituents:

- **Calcium Sutter 100x solution (250 ml)**

  0.7 g of $CaCl_2.2H_20$

  H_20 up to final volume of 250ml.

- **Sutter 100x solution (250 ml)**

  0.025 g of thiamine

  0.05 g $C_6H_8O_7.H_2O$ (monohydrate citric acid)

  0.0375 g of $Fe(NO_3)_3.9H_2O$

  0.025 g of $ZnSO_4.H_2O$

  0.0075 g of $MnSO_4.H_2O$

  0.00125 g of $CuSO_4.5\ H_2O$

  0.00125 g of $Na_2MoO_4.2\ H_2O$

  H_2O up to final volume of 250ml

The compounds were added one by one and in that order in the Sutter 100x solution.

All solutions were sterilized in an autoclave at 120°C for 20 min and kept at room temperature until their use. The Calcium Sutter 100x solution must protect against the light.

Both SIV (A and B) solutions were mixed in a sterilized atmosphere. This medium (150 ml) was added in every of the 24 Erlenmeyer flaks (500 ml) which were then covered with cotton grass.

The culture flasks were inoculated with a concentration of 10^4spores/ml of medium for single cultures [NRRL15555(-) and A56(+)]. Activation of the spores in a water bath at 48°C for 15 min was necessary before every inoculation.

The incubations were carried out in an orbital shaker (Radiber Microtermic I) at 200 rpm in darkness during 5-6 days at 22°C.

1.2.2 Preparation Of The Cultures In Solid Minimal Medium

Was utilized AGAR **SIV$_{MINIMAL}$** of similar composition like the **SIV$_{MINIMAL}$**, with the only emerged difference the addition of 15g of agar in SIVB.

The solid medium was coated in Petri plates (25 ml of medium per plate). Afterwards, the plates were inoculated with $5x10^3$spores/ml of every sex in the case of mated cultures [NRRL15555(-) X A56(+) and C5(-) X 342(+)] and 10^4 spores/ml in the case of single cultures [C5(-) and S342(+)] in a suspension of 3ml of "agar de cobertera" (2g of agar diluted in H_2O up to final volume 250 ml).

The incubations were carried out in darkness during 5 days at 22°C.

2. EXTRACTION & FRACTIONATION

2.1 Cultures in liquid medium

After incubation, the culture media were filtered under vacuum. The neutral fractions were obtained by adjusting the initial filtrates to pH 8 with 2N NaOH and extracting thrice with EtOAc. The acid fractions were obtained by adjusting the remaining aqueous phase to pH 2 with 2N HCl and extracting thrice with EtOAc. The organic phases were dried over anhydrous Na_2SO_4, filtered and evaporated under low pressure. For the sake of chemical stability, all procedures were carried out under dim light and the fractions were kept under Argon atmosphere before their storage in a freezer at -20ΌC until their apocarotenoid analysis.

The biomass was left to dry and weighted.

Table 2 shows the amounts of, biomass (g), neutral and acid fraction (mg) derived from a volume of culture broth (ml) of the applied liquid cultures of NRRL1555(-) and A56(+).

Table 2.

Strains	Culture broth (ml)	Neutral Fraction (mg)	Acid Fraction (mg)	Biomass dry (g)
NRRL1555(-)	3600	40	60	40
A56 (+)	3600	47	132	30

2.2 Cultures in solid medium

After incubation, the exudates were obtained by, freezing (-20°C for 12 hours), thawing (room temperature for 1 hour) the media and centrifuging the liquid (4000 x g, 15 min). Neutral and acid fractions were obtained from initial extracts using the same methodology described for the liquid cultures.

Table 3 shows the amounts of neutral and acid fraction (mg) derived from a volume of culture broth (ml) of the applied solid cultures of NRRL1555(-) X A56(+), C5(-), S342(+) and C5(-) X S342(+) correspondingly.

Table 3.

Strains	Culture Broth (mL)	Neutral Fraction (mg)	Acidic Fraction (mg)
NRRL1555(-) X A56(+)	2800	387	324
C5(-)	700	17	37
S342(+)	650	51	31
C5(-) X S342(+)	650	18	63

3. TECHNIQUES & INSTRUMENTATION

3.1 Chromatography

The solvents employed in all chromatography processes[4] were Hexane (H), t-Butyl-Methyl-Ether (TBME), Acetyl Acetate (AcOEt), Methanol (MeOH) and Dichloromethane (DCM) and their mixtures.

- **Thin Layer Chromatography (TLC)**

Analytical TLC was performed on silica gel Merck DC-Alufolien (Kieselgel 60 F_{254}) layers of 0.25 mm tickness, using ultraviolet light of wavelength 254 nm (Vilber Lourmat lamp) or/and a 7% phosphomolibdic acid solution in ethanol for compound visualization.

- **Analytical reverse-phase HPLC**

The neutral and acid fractions were dissolved in methanol (1mL for 10 mg of fraction). Aliquots (20 μL) were loaded into a C18 column (4.6 by 250 mm; 5 μm octyldecylsilane particles; Spherisorb-ODS2, Waters Corporation, Milford, MA) with a 12.5 mm refillable guard pre-column filled with the same material

4 Martvnez Grau, M. A.; Csαky, A. G. En *"Tιcnicas experimentales en quvmica orgαnica";* Svntesis: Madrid, **1998**.

(Agilent Technologies, Santa Clara, CA) in a liquid chromatograph (Series 1100, Agilent). The column was eluted at room temperature at a flow rate of 1 mL/min with methanol/water (1:4) during the first six min and successively in a lineal gradient until arriving to pure methanol at 25 min, pure methanol until 40 min and in a lineal gradient down to the initial mixture at 50 min. The outflow was monitored with a diode array detector at 328, 300, 280, 250 and 230 nm.

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

The neutral and methylated acid extracts were dissolved in *t*-BuOMe (at 20 g dry extract/L). Aliquots (0.5 mL) were injected into a 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). The column was eluted at room temperature at a flow rate of 2 mL/min for 25 min with *t*-BuOMe and monitored with a refractometer. For the methylated acid extracts the eluent was *n*-hexane/*t*-BuOMe (1:4, v/v).

- **Gas Chromatography-Mass Spectrometry**

GC/MS analyses were carried out in a Hewlett Packard 6890 chromatograph connected to a Hewlett-Packard 5988A mass spectrometer using an ionization voltage of 70 eV. The GC conditions were: HP-1 methyl silicone capillary column (30 m x 0.25 mm X 0.25 microns); the carrier gas was helium at a flow rate of 1.9 ml/min.; the injection and detector heater temperature were 250 ΄IC and 280 ΄IC, respectively; the temperature was increased from 60΄I to 300΄IC at 10 ΄IC/min.

3.2 Spectroscopy

- **UV-Visible Spectroscopy**

The absorbance measurements were performed in a UV-VIS Unicam spectrophotometer (Helios Alfa).

- **Nuclear Magnetic Resonance Spectroscopy**

NMR spectra (^{1}H,^{13}C and 1D TOCSY) were recorded with Varian Direct-Drive 400 (^{1}H 400 MHz/^{13}C 100 MHz) and 500 (^{1}H 500 MHz/^{13}C 125 MHz) spectrometers.

NMR spectra were performed at the Scientific Instrumentation Center of University of Granada.

We used tetramethylsilane (TMS) as internal reference and as solvents: $CDCl_3$or $(CD_3)_2CO$. The chemical shifts (δ) are expressed in parts per million (ppm) and coupling constants (J) in hertz (Hz). The multiplicity of signals is expressed by the following abbreviations: s (singlet), d (doublet), t (triplet), q (quadruplet), quint (quintuplet), sixth (sextuplete), m (multiplet), dd (double doublet), dt (double triplet), tt (triple triplet) dsext (doubles sextuplete, etc.). In the case of broad signals are used the letter "a" beside the abbreviations outlined above. The degree of substitution of the carbons was established using the sequence DEPT pulse.

4. METHODOLOGY OF ANALYSIS

The analysis of all neutral and acid fractions obtained from the different cultures by ^{1}H NMR and analytical reverse-phase HPLC, shown the presence of apocarotenoids, except of those obtained from cultures of the C5 and S342 strains.

5. INDENTIFICATION OF APOCAROTENOIDS

The identification of the apocarotenoids in the neutral and acid fractions obtained from mated cultures and in the acid fraction obtained from single cultures, has been carried out by analytical reverse-phase HPLC and GC/MS, using standards obtained for research group of Prof. Barrero in previous researches.[5]

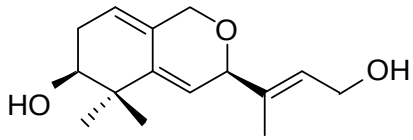
The neutral fractions obtained from single cultures were subjected to semi-preparative normal-phase HPLC and four fractions were formed. Fraction 1 (t_R = 7.6-13.0 min) was constituted by complex mixture of compounds no identified. No apocarotenoids was observed in this fraction. Fraction 2 (t_R = 21.5-24.3 min) was constituted by **trispoxane.** Fraction 3 (t_R = 24.3-27.2 min) was constituted by a mixture of **trispoxane** and **trispotrientriol A** in a ratio 1:1. Fraction 4 (t_R = 27.2-29.1 min) was constituted by a mixture of **trispotrientriol A** and **trispotrientriol B** in a ratio 4:3.

The neutral and acid fractions corresponding to the cultures of the C5 y S342 strains have not been studied.

[5] Barrero, A. F.; Herrador, M. M.; Arteaga, P. **2010**, results without publishing.

Identification of neutral apocarotenoids.

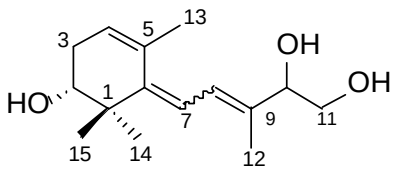
 Trispoxane: was identified in all neutral fractions by analytical reverse-phase HPLC using a standard (t_R = 19.6 min, λ = 250 nm)



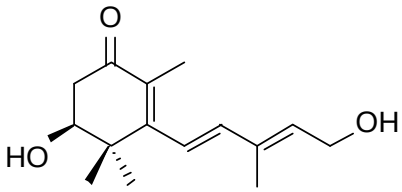
 Trispotrientriol A and trispotrientriol B: were identified in all neutral fractions as 4:3 mixture. Analytical reverse-phase HPLC and UV: t_R = 20.0 and 20.3 min, λ = 300 nm. GC/MS: t_R = 20.2 and 20.3 min, $[M]^+$ 252.

Trispotrientriol A: ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.02 (3H, s, H-15), 1.04 (3H, s, H-14), 1.50 (3H, br s, 3OH), 1.76 (3H, s, H-12), 2.01 (3H, s, H-13), 2.09 (1H, br d, *J* = 17.9 Hz, H-3β), 2.43 (1H, dd, J_1 = 2.9 Hz, J_2 = 17.9 Hz, H-3α), 3.42 (1H, t, *J* = 4.5 Hz, H-2), 3.52 (1H, br d, *J* = 10.0 Hz, H-11a), 3.63 (1H, dd, J_1 = 4.9 Hz, J_2 = 10.0 Hz, H-11b), 4.12-4.20 (1H, m, H-10), 5.44 (1H, br s, H-4), 6.18 (1H, d, *J* = 10.0 Hz, H-7), 6.62 (1H, d, *J* = 10.0 Hz, H-8).^{13}C NMR (100 MHz, $CDCl_3$): δ 12.9 (CH_3, C-12), 25.3 (CH_3, C-13), 25.4 (CH_3, C-15), 27.1 (CH_3, C-14), 31.1 (CH_2, C-3), 42.1 (C, C-1), 59.4 (CH, C-10), 65.5 (CH_2, C-11), 74.8 (CH, C-2), 120.9 (CH, C-8), 124.1 (CH, C-7), 125.7 (CH, C-4), 132.1 (C, C-5), 136.9 (C, C-6), 138.3 (C, C-9).

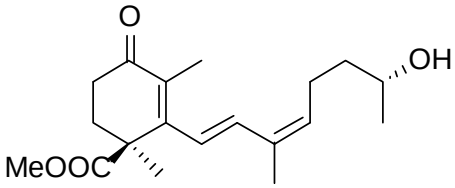
Trispotrientriol B: ^{1}H NMR (500 MHz, ($CDCl_3$): only distintive signals δ 1.20 (3H, s, H-15), 1.32 (3H, s, H-14), 1.86 (3H, s, H-13), 3.36 (1H, t, *J* = 4.5 Hz, H-2), 5.51 (1H, br s, H-4), 6.32 (1H, d, *J* = 10.0 Hz, H-7), 6.72 (1H, d, *J* = 10.0 Hz, H-8).



 Apotrisporin E: was identified in the neutral fraction obtained from the mated culture by analytical reverse-phase HPLC using a standard (t_R = 18.9 min, λ = 300 nm).

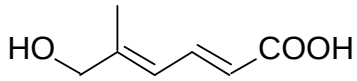


 Methyl trisporate C (9Z): was identified in the neutral fraction obtained from the mated culture by analytical reverse-phase HPLC using a standard (t_R = 23.7 min, λ = 328 nm).

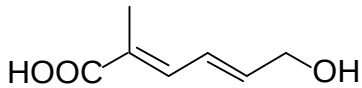


Identification of acid apocarotenoids.

 Blakesleoic A and blakesleoic B acids: were identified in all acid fractions by analytical reverse-phase HPLC using a standard (t_R = 2.4 min, λ = 250 nm) as a 2:1 mixture.

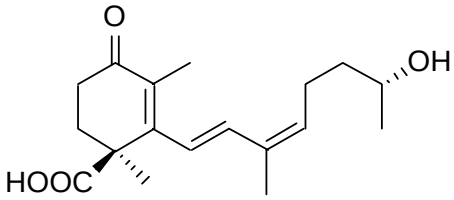


Blakesleoic A acid

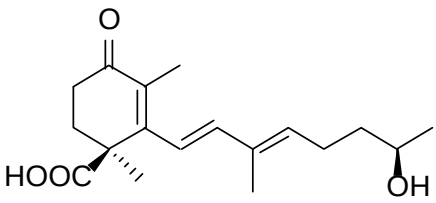


Blakesleoic B acid

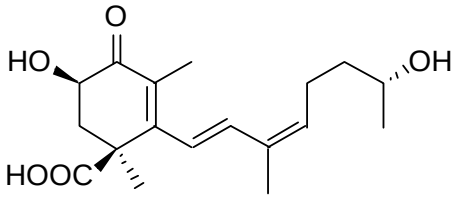
 Trisporic acid C (9Z): was identified in the acid fraction obtained from the mated culture by analytical reverse-phase HPLC using a standard (t_R = 15.1 min, λ = 328 nm).



 Trisporic acid C (9E): was identified in the acid fraction obtained from the mated culture by analytical reverse-phase HPLC using a standard (t_R = 15.3 min, λ = 328 nm).



 Trisporic acid E (9Z): was identified in the acid fraction obtained from the mated culture by analytical reverse-phase HPLC using a standard (t_R = 16.7 min, λ = 328 nm).



V. CONCLUSIONS

1. The composition of both neutral and acid fractions in single liquid cultures of the wild strains NRRL1555(-) and A56(+) of *Phycomyces blakesleeanus* was similar as concerns their apocarotenoid context.

2. Two new apocarotenoids **1** and **2** were isolated and identified as a mixture of isomers in single liquid cultures of *P. blakesleeanus* named correspondingly Trispotrientriol A and B, with the first to be occurred in higher concentration than the second. They present a new apocarotenoid skeleton and also they were observed in mated cultures of the same strains in solid medium, but in amounts much less that in the single ones.

3. Other apocarotenoids identified in *Phycomyces* were Trispoxane, Apotrisporine E, Methyltrisporate C (9*Z*), Blakesleoic A and B acids, Trisporic C Acid (9*Z*), Trisporic C Acid (9*E*) and Trisporic E Acid (9*Z*). Trispoxane and Blakesleoic A and B acids were identified in single cultures as in mated cultures, while Apotrisporine E, Methyltrisporate C (9*Z*), Trisporic C Acid (9*Z*), Trisporic C Acid (9*E*) and Trisporic E Acid (9*Z*) were only identified in mated cultures.

4. Trispoxane and Blakesleoic A and B acids were identified for the first time in the single cultures of *P. blakesleeanus* strains.

5. In the single cultures of the mutants, C5(-) and S342(+) strains, with a mutation *carB10*, devoid of β-carotene, was not found any evidence of the existence of the apocarotenes C7, fact which confirm that their presence in single cultures of wild strain derive from the degradation of β-carotene.

6. Identification of Blakesleoic A and B acids (C7 apocarotenes) in the single cultures of the wild strains allow us to propose that the biosynthesis of C15 and C7 apocarotenes is also carried out by oxidative double cleavage of β-carotene.

7. In the single cultures C18 apocarotenes have not been isolated. Taking into consideration that equimolecular amounts of C18, C15 and C7 apocarotenes resulting from the biosynthetic proposal based on oxidative double cleavage of

β-carotene, we propose that C18 apocarotenes are transformed into C15 apocarotenes by a mechanism of retroaldol condensation.

8. The presence of trispotrientriol A and B and trispoxane and the absence of apocarotenes (C18 and C15) with ketone group on position C4 of the ring, in the **single cultures**, indicate that the dehydrogenase which transforms the hydroxyl group at C4 to ketone group and the reductase which hydrogenates the α,β positions of the methylketone, are not activated in these cultures.