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Biosynthesis of the allene (−)-marasin in *Marasmius ramealis*

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[1-^{14}C]-*E*-dehydromatricaria methyl ester and dimethyl [1-^{14}C]-deca-4,6,8-triyne-1,10-dioate are incorporated into the allene (−)-marasin in *Marasmius ramealis* without scrambling of the ^{14}C label. This and the levels of the incorporations (0.8% and 4.9% respectively) strongly suggests that the above esters, or close relatives, can be converted directly into (−)-marasin in *M. ramealis*, and that the diyne-allene moiety in this latter compound arises by the rearrangement, under enzymic control, of an alkyltriyne moiety.

Introduction

Polyacetylenes, *i.e.* compounds with at least two conjugated acetylenic moieties, occur in many plants, especially Umbelliferae and Compositae, and in many Basidiomycete fungi.[1] The structures of those found in plants are usually significantly different from those found in fungi. Thus, the former are commonly relatively non-polar C_{13}, C_{14} or C_{17} compounds which may contain aromatic rings, whereas the latter are commonly straight-chain C_8, C_9 or C_{10} compounds containing allene and/or alcohol, diol or triol moieties.[1] Some polyacetylenes, however, occur both in plants and fungi, for example, *Z*,*Z*-matricaria ester (**1a**)[2,3] and *E*-lachnophyllum ester (**2**).[3,4]

CH_3-CH^{8}=CH-C≡C-C≡C-CH=^{2}CH-CO_2CH_3

matricaria esters

(1a): *Z,Z*-isomer

(1b): *E,E*-isomer

(1c): *2E,8Z*-isomer

CH_3-CH_2-CH_2-C≡C-C≡C-CH=^{E}CHCO_2CH_3

lachnophyllum ester

(2)

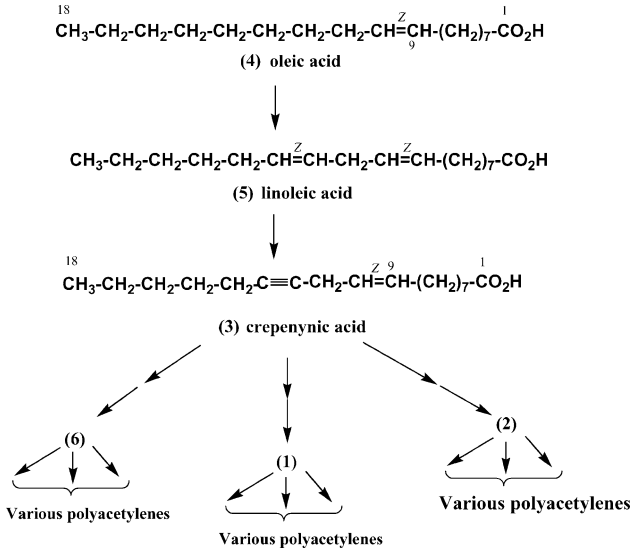
CH_3-C≡C-C≡C-C≡C-CH=^{E}CH-CO_2CH_3

dehydromatricaria ester

(6)

Tracer studies have shown that crepenynic acid (**3**) (or a close biological equivalent such as a thiol ester) is a key intermediate in the biosynthesis of polyacetylenes in both plants and fungi:[1,5–8] see Scheme 1. It is derived from oleic acid (**4**) *via* linoleic acid (**5**). In many fungi crepenynic acid (**3**), after substantial further elaboration, is broken down to a polyunsaturated C_{10} acid or ester such as *E*-dehydromatricaria ester (**6**), *Z*,*Z*-matricaria ester (**1a**) or *E*-lachnophyllum ester (**2**). These esters, derived from C9–C18 of oleic acid, are then elaborated to give many of the other C_9 and C_{10} fungal polyacetylenes.[3,9,10] There is, for example, tracer evidence for (i) the reduction of the C1 ester group to an alcohol;[3,9] (ii) the saturation of the C2=C3 double bond;[9] (iii) the oxidation of the terminal C10 methyl group to give a hydroxymethyl group, then a carboxyl group;[3,9] and (iv) decarboxylation of the acid formed in (iii) to give a C9 compound.[9]

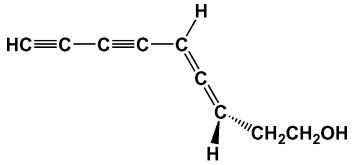
Although many fungal polyacetylenes contain allene moieties, more particularly diyne-allene moieties,[11] there is no tracer evidence to indicate how these moieties are formed. This is the subject of the present paper. Allene moieties are also found in numerous carotenoids,[12] but otherwise naturally occurring allenes are rare.[13]



Scheme 1 Summary of the biosynthetic pathways from oleic acid to many fungal polyacetylenes.

Results and discussion

In 1959 Bendz described the isolation and characterization of the antibiotic (−)-marasin (**7**) from the fungus *Marasmius ramealis*.[14] The (+)-enantiomer, accompanied by at least ten other polyacetylenes, was subsequently isolated from *Aleurodiscus roseus* by Jones *et al.*,[15] and the same research group isolated the (−)-enantiomer, together with two other polyacetylenes, from *Cortinellus berkeleyanus*.[11] Since (−)-marasin (**7**) is the only major polyacetylene isolated from *M. ramealis*, this organism was selected for the present studies.



(7): (-)-marasin

RO_2C−C≡C-C≡C-C≡C−$CH_2CH_2CO_2R$

(8a): R = H
(8b): R = CH_3

Comparing polyacetylene structures suggests (−)-marasin (**7**) is derived from *E*-dehydromatricaria ester (**6**). To test this [1-^{14}C]-*E*-dehydromatricaria ester (**6**), available from the earlier

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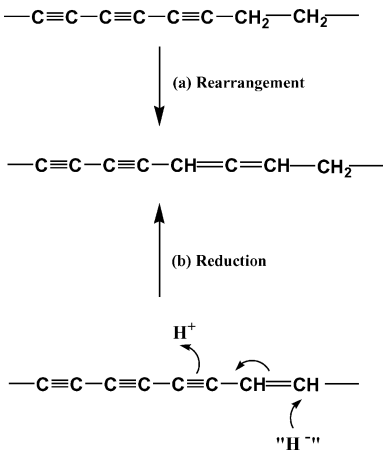
studies,[9] was fed to cultures of *M. ramealis* when the rate of polyacetylene production was considered to be high. The uptake was monitored by UV spectroscopy. After three days <5% of the labelled ester **6** remained. (−)-Marasin (**7**) was then isolated and purified, first by counter current distribution then by column chromatography. The purity of the marasin, an unstable oil,[14–16] was checked by thin layer chromatography (TLC) (two different systems) and by radioautographs of the TLCs. The radioactivity of the marasin (**7**) corresponded to an incorporation of 0.8%. This is somewhat lower than the incorporations of ester **6** into other C_9 or C_{10} compounds (3.4%–46%).[9] To locate the site of the label, the marasin was diluted with nonanol, the mixture hydrogenated and the product oxidized to nonanoic acid.[9] A portion of this was converted into the crystalline *p*-bromophenacyl ester derivative and this was recrystallized to constant activity [assigned relative molar activity (RMA) = 1.00]. Another portion of the nonanoic acid was subjected to a Schmidt decarboxylation and the carbon dioxide collected as barium carbonate.[17] It had a RMA = 0.97. Thus, the [1-^{14}C]-*E*-dehydromatricaria ester (**6**) is incorporated into (−)-marasin (**7**) without scrambling of the label. This result provides strong evidence that (−)-marasin (**7**) is produced directly in *M. ramealis* from *E*-dehydromatricaria ester (**6**) *via* reactions of the types (i), (iii) and (iv) mentioned in the Introduction, plus the step(s) to generate the allene moiety.

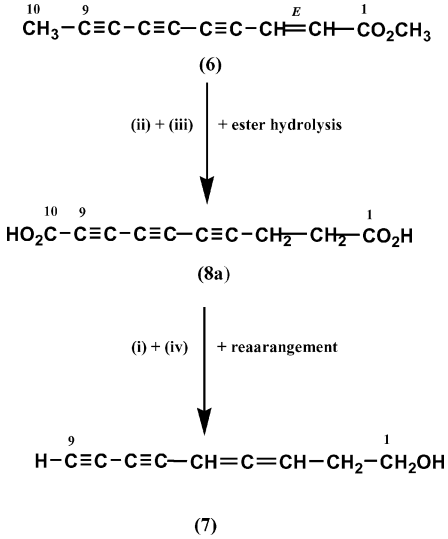
It is conceivable that the allene group is formed (a) by a simple rearrangement of an alkylacetylene moiety following saturation of the C2=C3 double bond; (b) by a combined reduction–rearrangement of a conjugated eneyne moiety, see Scheme 2a and b; (c) by an elimination reaction;[18] or (d) by some other method. It is known that reduction of certain allylic alcohols containing the eneyne unit with lithium aluminiumhydride affords allenes.[19]



Scheme 2 Two potential routes to polyacetylenes containing the diyne-allene moiety.

To obtain more information on the processes occurring in *M. ramealis*, dimethyl [1-^{14}C]-deca-4,6,8-triyne-1,10-dioate (**8b**), biosynthetically produced in the earlier work,[9] was also fed to cultures of *M. ramealis.* The (−)-marasin (**7**) was isolated, purified and counted as before. The activity corresponded to an incorporation of 2.0% but the amount of marasin and its specific activity were judged to be insufficient for satisfactory degradation to locate the site(s) of the activity. In a second experiment, carried out on a larger scale, the activity corresponded to an incorporation of 4.9%. When it was degraded as before (*i.e.* in the experiment using active ester **6**), but without any dilution, 98% of the radioactivity was found to be present at C1. This strongly suggests that diester **8b** is directly converted into marasin and hence that the allene moiety is formed simply by rearrangement of an alkylacetylene moiety: see Scheme 2a. As the marasin isolated from *M. ramealis* is chiral the rearrangement evidently occurs under enzymic control.

It is tempting to suggest that the combined tracer results imply that the sequence of events in the conversion of *E*-dehydromatricaria ester (**6**) into (−)-marasin (**7**) in *M. ramealis* is reactions of the type (ii) and (iii) (as mentioned in the Introduction) (sequence of reactions involved not known) plus ester hydrolysis, followed by reactions (i) and (iv) plus the rearrangement of the tryiyne to a diyne-allene (sequence not known): see Scheme 3. However, very little is known about the specificity of the enzymes involved in these types of conversions. The fact that often many very closely related polyacetylenes are found in a given fungus (for example, at least 11 C_9–C_{11} polyacetylenic compounds in *A. roseus*)[15] strongly suggests that many of the enzymes involved have only limited specificity. This is consistent with the results obtained with the two cell-free systems studied to date: one a system that brings about the decarboxylation of acetylenic acids [*i.e.* reaction (iv)],[20] and one that catalyses the oxidation of polyacetylenic alcohols to the corresponding carboxylic acids [*i.e.* the reverse of conversion (i)].[21] Further evidence for limited specificity comes from the observation that if labelled ester **6** is fed to cultures of *Serpula lacrymans* the ester is converted, without scrambling of the label, into diacid **8a**,[9] although other tracer experiments[22] suggest that in this organism the ester **6** is not actually on the normal pathway from crepenynic acid (**3**) to the diacid **8a**. Thus, we believe that the conversions that elaborate the various C_{10} esters are to some extent achieved *via* "metabolic grids" that provide various routes between certain metabolites.[7,23] Clearly the relative importance of the routes depends on the specificities of the enzymes.[7,23] If this is so compound **6**, for example, may be converted into compound **7** by more than one reaction sequence, just as compound **8b** may be converted into compound **7** by more than one reaction sequence. Indeed compounds **8a** and/or **8b** may not even be on the *major pathway* by which compound **6** is converted into compound **7**.



Scheme 3 Apparent pathway from ester **6** to marasin (**7**) *via* diester **8b**. Reactions over arrows refer to those discussed in the Introduction.

The above results do, however, strongly support the view that *M. ramealis* contains an enzyme system that can catalyse the rearrangement of appropriate alkyltriynes to diyne-allenes (Scheme 2a). It may be that the enzyme involved is not highly specific and it would be interesting to investigate if *M. ramealis*, or a cell-free extract, can be used to prepare other chiral allenes. Other researchers have shown that 3-ynoyl thioesters are converted by rearrangement into the 2,3-dienoylester, although in these cases one of the allenic double bonds becomes conjugated with the ester group.[24] Different routes to allenes operate in other organisms. For example, there is evidence that the allenic moiety

in certain carotenoids arises by a ring-opening elimination of an epoxy-olefin.[18]

Experimental

Experimental details and counting methods were as described previously.[3,9] Activities determined by scintillation counting are expressed in bequerel (disintegrations per second) per mM (Bq mM^{-1}) and those determined by counting "infinitely thick" films as μC mM^{-1}. RMA = relative molar activity.

Feeding of [1-^{14}C]-*E*-dehydromatricaria methyl ester (6) to *Marasmius ramealis*

M. ramealis was grown as surface cultures as reported previously.[14] [1-^{14}C]-*E*-Dehydromatricaria methyl ester (**6**) (5.0 mg, 248 μC mM^{-1})[9] in ethanol (12 ml) was distributed between 12 penicillin flasks of *M. ramealis* supported on glass wool 15 days after innoculation. The uptake was monitored by ultraviolet spectroscopy. Three days later, when the mycelium had taken up >95% of the added ester, the culture fluids were decanted and continuously extracted with ether (500 ml) for 30 h. The marasin (25 mg, estimated spectroscopically) present in the extracts was purified using ether and water in a 50 tube Craig counter-current apparatus (volumes of upper and lower phases 40 ml). The marasin appeared in tubes 10–23. The contents of these tubes were combined and the aqueous layer continuously extracted with ether (750 ml) for 24 h. The upper layer and extracts were combined. The solvent was evaporated off and the residue adsorbed onto alumina (20 ml) from 5% methylene dichloride in hexane. The fractions containing marasin (eluted with 50% methylene dichloride in hexane) were evaporated and the residue dissolved in ether (250 ml). The amount of marasin present (17 mg) was estimated spectroscopically. It had λ_{max} (ether) 278, 263,249, 237 μm (relative absorbance 2.1, 2.8, 1.7, 1.0).[16] An aliquot of the solution (containing 0.50 mg of marasin) was evaporated and the residue dissolved in scintillator solution (7 ml). It had a count rate (neglecting quenching) corresponding to the marasin having an activity of 1.00×10^4 Bq mM^{-1}. To confirm the purity, the ethereal solution of marasin was evaporated to dryness and the residue adsorbed from 5% ether in hexane onto alumina (20 ml). Elution with 50% ether in hexane gave marasin. The marasin (9 mg) was found to have, within experimental error (within 2%), the same activity as before. This corresponds to an incorporation of 0.81%.

Thin layer chromatography of the marasin on Kieselgel "HF" plates showed one main spot when developed with ether (R_f 0.49; authentic marasin had R_f 0.46) or with methylene dichloride (R_f 0.18, authentic marasin had R_f 0.20). The spots were detected by ultraviolet light and by spraying with iodine. Radioautographs of the chromatogram showed that in each case more than 95% of the activity was associated with the marasin spots and that <1% of the activity could be due to labelled ester **6**.

Marasin (3.0 mg) and nonanol (49.5 mg) in ether (40 ml) were hydrogenated over 10% palladium on charcoal. The nonanol (51.5 mg) in acetone (6 ml) was cooled to 0 °C and 8 N chromic acid was added dropwise with shaking until the solution remained yellow for 4 min.[9] Water (28 ml) was added and the mixture extracted with ether (4 × 20 ml). The extracts were washed with water (2 × 10 ml), dried, and evaporated to give an oil (55 mg). The oil was dissolved in methanolic potassium hydroxide (20 ml, 5%) and water (10 ml) and the mixture heated under reflux for 45 min.[9] The nonanoic acid (45 mg) was isolated as a colourless oil. The infrared spectrum was identical to that of an authentic sample. A portion (20 mg) of the nonanoic acid was converted into the *p*-bromophenacyl ester.[25] This was recrystallized to a constant activity of 1.54×10^{-2} μC mM^{-1} (defined as RMA 1.00). Another portion of the nonanoic acid (20 mg) was subjected to a Schmidt decarboxylation using Phares procedure[17] except that the carbon dioxide was collected as barium carbonate and washed and dried in a centrifuge tube; it (16 mg) has an activity of 1.50×10^{-2} μc mM^{-1} (RMA 0.97).

Feeding of dimethyl [1-^{14}C]deca-4,6,8-triyne-1,10-dioate (8b) to *M. ramealis*: isolation of marasin (7)

Two experiments were carried out.

Experiment A. The experiment was carried out as described above except that biosynthetic dimethyl [1-^{14}C]deca-4,6,8-triyne-1,10-dioate (**8b**) (3.8 mg, 4.45 μC mM^{-1})[26] was added instead of [1-^{14}C]dehydromatricaria methyl ester (**6**). The 12 flasks of *M. ramealis* were from the same batch as those above. The marasin (18 mg) was isolated and purified as before. It had an activity of 434 Bq mM^{-1} corresponding to an incorporation of *ca.* 2.0%.

Experiment B. The experiment was carried out as described above but dimethyl [1-^{14}C]deca-4,6,8-triyne-1,10-dioate (**8b**) (10.0 mg, 4.45 μC mM^{-1})[26] was added to fresh growths of *M. ramealis* in 20 penicillin flasks. The marasin (41 mg) was isolated and purified as described above. After the first column chromatography it had an activity of 1315 Bq mM^{-1}, and after the second an activity of 1325 Bq mM^{-1}. This corresponds to an incorporation of 4.9%. The marasin was purified and, without dilution, degraded as above. The *p*-bromophenacyl derivative of the nonanoic acid had an activity of 3.57×10^{-2} μC mM^{-1} (defined as RMA 1.00). This gave barium carbonate with a RMA of 0.98.

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