322. *The Biosynthesis of Polyacetylenes. Part III.* Polyacetylenes and Triterpenes in* Polyporus anthracophilus.

By J. D. Bu'Lock, D. C. Allport, and W. B. Turner.

The matricaria ester (I) which occurs in *P. anthracophilus* is derived from acetate by head-to-tail linkage. The polyacetylenes in this species are in a dynamic state, with synthesis *de novo* accompanying their disappearance. The incorporation of acetate into triterpenes proceeds simultaneously and may be very efficient; in different strains of the fungus competition between triterpene and polyacetylene synthesis is apparent.

Over a hundred polyacetylenes have been isolated from two principal sources—plants (Compositae) and higher fungi (Agaricales).[1] Each type of source generally affords a distinctive variety of polyacetylene, but members of the important C_{10} group have been found in both plants and fungi. It seems probable, on structural grounds, that all the

* Part II, Bu'Lock and Gregory, *Biochem. J.*, 1959, **72**, 322.

[1] Reviewed by Jones, *Proc. Chem. Soc.*, 1960, 199.

known polyacetylenes have similar biogenetic origins, and in a search for useful parallels to the experimentally demonstrated derivation of nemotinic acid from acetate [2] the biogenesis of the matricaria ester (I) seemed an appropriate subject, since stereoisomers of the ester (I) occur frequently in Compositae [3] and in certain Basidiomycetes. Moreover, the fungus *Polyporus anthracophilus* Cke, from which *trans-trans*-ester (I) was first isolated [4] has a complex metabolism which itself seemed to merit further study.

In extracts from surface cultures of *P. anthracophilus* made at various times, at least 17 polyacetylenes, have been detected, of which 13 have been characterised, all but one being C_{10} substances with oxygen groups at one or both ends of the molecule. Besides

$CH_3 \cdot CH{=}^{t}CH \cdot C{\equiv}C \cdot C{\equiv}C \cdot CH{=}^{t}CH \cdot CO_2Me$ (I)

$CH_3 \cdot CH{=}^{t}CH \cdot C{\equiv}C \cdot C{\equiv}C \cdot CH{=}^{t}CH \cdot CH_2 \cdot OH$ (II)

$HO \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot C{\equiv}C \cdot C{\equiv}C \cdot CH{=}CH \cdot CO_2Me$ (III)

$HO_2C \cdot CH{=}^{t}CH \cdot C{\equiv}C \cdot C{\equiv}C \cdot CH{\equiv}^{t}CH \cdot CO_2H$ (IV)

matricaria ester (I), the major metabolites are the alcohol (II), the hydroxy-ester (III), and the diacid (IV) (and its diester). The proportions of these components vary as the cultures develop.[4]

FIG. 1. *Production of the major metabolites (I—III) from quantitative analyses of successive flasks. The full curves show the general trends found in* 1956 *and* 1959, *the broken curves the detailed effects found only in* 1956.

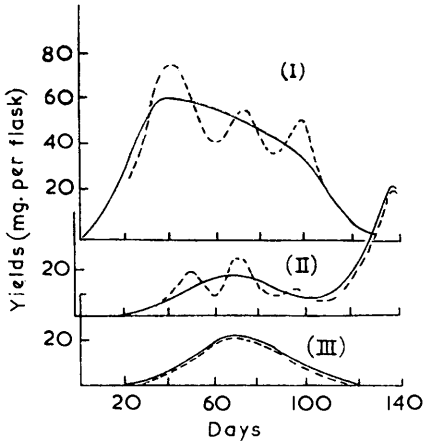
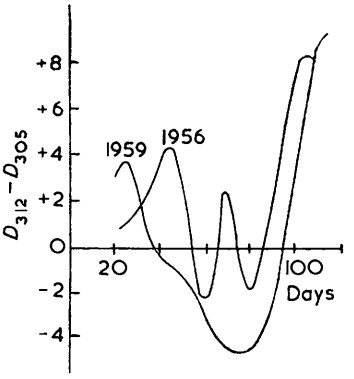


FIG. 2. *Relative amounts of products (II) and (III) in the aqueous medium of single flasks, from the optical-density difference, showing typical results from the* 1956 *and* 1959 *series.*



These variations have now been examined in more detail by two methods, with complementary results. In the first, samples of the medium from a given flask were examined spectroscopically, giving a qualitative picture not affected by uncontrolled differences between flasks. In the second, the entire contents of successive culture-flasks were analysed quantitatively by multi-component spectroscopic assay; the accuracy of this assay is limited, and moreover to follow the sequence of changes the data from different flasks must be used; the agreement between the two sets of data is, however, significant.

In all polyacetylene-producing strains of *P. anthracophilus* the same general picture was found, shown in the full curves of Fig. 1. The matricaria ester (I) predominates during growth of the cultures and up to 15 weeks after inoculation, the alcohol (II) being present in smaller amounts. Both begin to disappear from the cultures after 5—9 weeks, but whereas the loss of the ester is continued, the amount of alcohol (II) suddenly increases

[2] Bu'Lock and Gregory, *Biochem. J.*, 1959, **72**, 322.
[3] Sörensen, *Chem. and Ind.*, 1953, 240.
[4] Bu'Lock, Jones, and Turner, *J.*, 1957, 1607.

sharply, reaches a rather high level, and then finally disappears. This pattern was shown by parent strains in 1956 and by descendant strains three years later. However, with the 1956 strains more complex variations were superimposed on this general pattern.

In the aqueous culture medium, the concentration of esters (I) soon reaches the solubility limit, and thereafter the main changes are in the proportions of compounds (II) and (III); since these have their longest-wavelength absorption maxima at 312 and 305 mμ respectively, the difference between optical densities at these wavelengths is a qualitative index of the relative amounts present. In the 1956 experiments, successive measurements on contents of the same flask consistently showed marked fluctuations, with the alcohol (II) and the hydroxy-ester (III) predominating alternately, in addition to the general phenomena already described (cf. Fig. 2). After allowance for uncertainties the full analyses for three series of flasks containing the same strains confirmed this unusual picture, and, as shown by the broken lines in Fig. 1, the fluctuations involved both the matricaria ester (I) and the alcohol (II), whereas the concentration of the hydroxy-ester (III) varied more simply; the latter observation proves incidentally that the apparent variations as regards components (I) and (II) are not due to the analytical method. With descendant strains, in 1959, these quasi-periodic short-term variations were not observed, as shown by the qualitative data in Fig. 2; the alcohol (II) predominated over the hydroxy-ester (III) in the early and the late phase, but was a minor component in the intermediate phase. The intimate mechanism of these fluctuations, and the related question of our failing to reproduce them at will, are beyond the scope of present information, but all the observations show clearly that in *P. anthracophilis* the polyacetylenes are not merely inert products but are also broken down and synthesised anew.

The incorporation of [1-^{14}C]acetate into matricaria ester (I) by a strain not showing the short-term effects was next studied. When the acetate was added 27 days after inoculation and the ester isolated 7 days later, good incorporation was observed (see Table). As would be expected for a C_{10} chain formed from 5 similar C_2 units, $C_{(1)}$ of ester (I) was found to contain almost exactly one-fifth of the total activity and in view of the earlier comprehensive study of nemotinic acid [2] no more detailed degradation was deemed necessary. When the incubation with [1-^{14}C]acetate was 42—63 days after inoculation the amount of the ester (I) isolated was much smaller; nevertheless some incorporation had occurred, though proportionally much less (Table). The incorporation represented strictly synthesis *de novo*, since $C_{(1)}$ still acquired just one-fifth of the total labelling; this shows not only that synthesis *de novo* of the ester (I) continues during its net destruction, but also that any interconversions which occur amongst the polyacetylenes, such as might explain the data of Figs. 1 and 2, do not involve equilibration with the symmetrical substances such as (IV) which are simultaneously present.

By incubation at a still later period incorporation into the alcohol (II) was to be studied, but with these cultures the phase when this alcohol predominates was rather short and, on working up, no polyacetylenes could be isolated (Table).

Incorporation of [1-^{14}C]*acetate into matricaria ester.*

Flasks	^{14}C added (days from inoculation)	Worked up (days from inoculation)	Triterpenes (mg.)	^{14}C in triterpenes (%)	Ester (I) (mg.)	^{14}C in (I) (%)	Proportion of ^{14}C in $C_{(1)}$ of (I)
1, 2	27	35	500	41	56	6·0	1·01/5
3, 4	42	63	570	7·0	22	0·2	1·01/5
5, 6	96	108	370	7·2	1	0·1	—

The stock cultures available to us differed, not only in detailed aspects of polyacetylene synthesis, but also in their overall capacity to produce such compounds, some indeed having quite lost this capacity of the parent strain. It was, moreover, observed that these " defective " strains produced conspicuously larger amounts of eburicoic acid, the major triterpene of this species.[5] Thus, for example, the parent (1956) strain afforded after

[5] Gascoigne, Holker, Ralph, and Robertson, *J.*, 1951, 2346.

63 days *ca.* 120 mg. of triterpenes and *ca.* 110 mg. of matricaria ester (I) per flask, whilst the strain used in 1959 for the tracer experiments produced, again after 63 days, *ca.* 280 mg. of triterpenes and only 29 mg. of the ester (I). As shown in the Table, the triterpenes appear most rapidly during the earlier incubation, with extremely high acetate incorporation (41%), but appreciable synthesis persists for much longer. The triterpenes are known to be formed from acetate by a pathway diverging at an early stage from that which leads to fatty acids and (probably) polyacetylenes; somewhat similar cases of reciprocity between triterpene and fatty acid synthesis are well known for moulds.[6]

Experimental

Culture Conditions.—Inocula were taken from subcultures of the parent strain, Forest Products Laboratory No. 327, made at 6-monthly intervals from 1956 to 1959 and stored under mineral oil at 4°, after plating-out on malt agar. The fungus was grown as surface cultures of 230 cm.^{2} on 700 ml. of glucose–salts–cornsteep medium supported on washed glass wool.

Analysis.—(a) *By repeated sampling.* Samples (10 ml.) were taken every 3—7 days from the aqueous medium of individual flasks in a batch of ten or twelve, some flasks being used only at the beginning or end of the series to minimise the effect of depletion by sampling; the samples were equilibrated with equal volumes of ethyl acetate or methylene dichloride, and the extracts examined spectroscopically between 250 and 360 mμ.

(b) *By total extraction.* Pairs of flasks were taken at weekly intervals from a large batch; the mycelium and medium were separated and each was extracted exhaustively with ether. The combined extracts were washed with aqueous sodium hydrogen carbonate to remove small amounts of acids, and then diluted with ethanol for spectroscopic examination. If D_{332}, D_{312}, D_{305} are optical densities at 332, 312, and 305 mμ respectively, then for a mixture of products (I), (II), and (III) in amounts W_1, W_2, W_3 mg. in V ml. of extract, calculations from measured absorption spectra of the pure components and mixture of known composition give that

$$W_1 = \tfrac{1}{2}V(11{\cdot}09D_{332} - 0{\cdot}664D_{312} + 0{\cdot}09D_{305})$$
$$W_2 = \tfrac{1}{2}V(8{\cdot}77D_{312} - 2{\cdot}93D_{305} - 7{\cdot}38D_{332})$$
$$W_3 = \tfrac{1}{2}V(11{\cdot}55D_{305} - 8{\cdot}15D_{332} - 3{\cdot}32D_{312})$$

With known mixtures these equations give W_1 and W_3 within ±5%, W_2 within ±10%, the uncertainty arising mainly from the steepness of the absorption curves.

(c) *General.* The results in the Figures are typical of those obtained in several experiments, *e.g.*, three for each curve in Fig. 1, eight for the curves of Fig. 2.

Incorporation of [1-^{14}C]*Acetate.*—To each of two culture-flasks in a batch of six, prepared as above, [1-^{14}C]acetate was added as the sodium salt (70—100 μc) at the time shown in the Table; 7—20 days later (cf. Table) the contents of the two flasks were removed and worked up as previously described,[4] by ether-extraction and chromatography on alumina. The triterpene fraction (eburicoic and dehydroeburicoic acid [5]) was washed free from fats by benzene and ether before counting. The matricaria ester (I) was weighed (22—56 mg.) and purified by recrystallisation with pure inactive material (220 mg.) and chromatography on alumina. Part of the product (*ca.* 50 mg.) was hydrogenated in light petroleum over 10% palladium–strontium carbonate, and the hydrogenation product saponified with ethanolic potassium hydroxide to give an oil (37 mg.) which was diluted with inactive hexanoic acid (270 mg.) before reversed-phase chromatography,[7] giving the purified hexanoic acid (249 mg.). Part of the purified acid (39 mg.) was converted by way of the acid chloride into the *p*-bromoanilide, m. p. 105—106°, purified by recrystallisation to constant activity. Another portion (27 mg.) was degraded by the Schmidt reaction,[8] giving barium carbonate from $C_{(1)}$. Radiochemical methods were as described previously,[9] and the results are summarised in the Table.

The University, Manchester, 13. [*Received, November 3rd*, 1960.]

[6] See, *e.g.*, Prill, Wenck, and Peterson, *Biochem. J.*, 1935, **29**, 21.
[7] Crombie, Comber, and Boatman, *Biochem. J.*, 1955, **59**, 309.
[8] Blomstrand, *Acta Chem. Scand.*, 1954, **8**, 1487.
[9] Allport and Bu'Lock, *J.*, 1960, 654.