LVII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

LVI. LUTEOLEERSIN AND ALBOLEERSIN; METABOLIC PRODUCTS OF *HELMINTHOSPORIUM LEERSII* ATKINSON. A POSSIBLE OXIDATION- REDUCTION SYSTEM

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IN a series of investigations carried out in collaboration with Prof. R. Robinson of the University of Oxford, a number of crystalline metabolic products, isolated from the mycelium of different species of moulds in the genus *Helminthosporium*, have been isolated. These include four hitherto undescribed hydroxyanthraquinones: (a) helminthosporin (2-methyl-4:5:8-trihydroxyanthraquinone) from *H. gramineum* Rabenhorst and *H. catenarium* Drechsler [Charles *et al.* 1933; Raistrick *et al.* 1934]; (b) catenarin (a β -(hydroxymethyl)-1:5:8-trihydroxyanthraquinone) from *H. catenarium*, *H. gramineum* and *H. velutinum* Link [Raistrick *et al.* 1934]; (c) cynodontin (2-methyl-1:4:5:8-tetrahydroxyanthraquinone) from *H. cynodontis* Marignoni and *H. euchlaenae* Zimmermann [Raistrick *et al.* 1933] and (d) tritisorin (6(or 7)-hydroxymethyl-1:3:5:8-tetrahydroxyanthraquinone) from *H. tritici-vulgaris* Nisikado [Raistrick *et al.* 1934]. More recently a substituted xanthone, ravenelin (3-methyl-1:4:8-trihydroxy-xanthone) was isolated in a crystalline form from cultures of *H. Ravenelii* Curtis and *H. turcicum* Passerini [Raistrick *et al.* 1936].

It has now been found that the mycelium of *H. leersii* Atkinson contains considerable amounts of two different, but chemically closely related, metabolic products which have not been described previously and for them the names *luteoleersin* and *alboleersin* are proposed.

The fungus *H. leersii* was first reported by Atkinson [1897], who isolated it from the leaves of *Leersia virginica* Willd. (= *Homolocenchrus virginicus* (Willd.)) Britton at Auburn, Alabama in September 1891. It is widely distributed in America and was found by Drechsler [1923] as a parasite on the leaf-blades of *L. virginica* in Connecticut in 1920 and in Washington in 1921. It forms dark spots on the leaves. When grown on artificial media it develops a dense grey aerial mycelium.

Luteoleersin, $C_{26}H_{38}O_7$, M.P. 135° , $[\alpha]_{5461}^{18^\circ} + 214^\circ$ ($c=0.456$ in alcohol) was obtained as beautiful yellow rods or blunt-ended needles; alboleersin, $C_{26}H_{40}O_7$, M.P. $215-216^\circ$, $[\alpha]_{5461}^{18^\circ} + 274^\circ$ ($c=0.430$ in alcohol) as long, colourless needles. Luteoleersin contains two active hydrogen atoms and alboleersin three. The empirical formulae for both substances are advanced tentatively at present, since, although they are based on a large number of C and H determinations and on mol. wt. estimations, it has not been found possible to confirm them by the analysis of derivatives as no crystalline derivatives have in fact been isolated.

It is hoped to obtain further evidence for these empirical formulae from further work which is in progress on these substances and will be reported later.

The biochemical significance of luteoleersin and alboleersin lies we believe in their relationship to each other. They are very readily interconvertible *in vitro*, and while both substances have been isolated from all cultures of *Helminthosporium leersii* examined, their relative proportions have varied in different preparations. Luteoleersin may be readily reduced to alboleersin by catalytic reduction with palladium-charcoal-hydrogen, or by heating with phenylhydrazine. Alboleersin may be smoothly and almost quantitatively oxidized to luteoleersin by treatment in the cold with FeCl_3 . Luteoleersin behaves as a quinone or semiquinone and alboleersin as the corresponding phenol. It is believed that these two substances offer an example of an oxidation-reduction system in the life processes of the mould *H. leersii*. Several other examples have recently been encountered in these laboratories and will be reported at a later date, and one similar case was suggested by Birkinshaw & Raistrick [1932] in the two metabolic products given by both *Penicillium puberulum* Bainier and *P. aurantio-virens* Biourge, i.e. puberulic acid $\text{C}_8\text{H}_6\text{O}_6$, and the unnamed acid $\text{C}_8\text{H}_4\text{O}_6$, which were shown by Barger & Dorrer [1934] to be interconvertible.

EXPERIMENTAL

History of the culture used

The culture of *Helminthosporium leersii* Atkinson used throughout this work was purchased from the Centraalbureau voor Schimmelcultures, Baarn, Holland in June 1933. It was received by them from Nisikado who isolated it from diseased leaf blades of *Leersia virginica*. It bears the L.S.H.T.M. catalogue number Ag 132.

Cultural conditions

Batches of 100 flasks of Czapek-Dox solution (glucose, 50 g.; NaNO_3 , 2.0 g.; KH_2PO_4 , 1.0 g.; KCl, 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; distilled water, 1 l.), 350 ml. in each litre conical flask, were sown with a suspension of a culture of *Helminthosporium leersii*, Ag. 132, and incubated in the dark at 24° . The rate of growth was slow and incubation was continued for 90 days at which time the glucose had almost completely disappeared. All the flasks presented an even appearance. The mycelium was dark grey in colour, with a black margin and a black reverse. The filtered metabolism solution was peach-coloured. The mycelium was separated by filtration, washed with cold water, pressed, dried in a vacuum oven at $45\text{--}50^\circ$ and weighed. (Wt. of mycelium, from 100 flasks in 3 representative experiments, 299 g.; 316 g.; 323 g.)

Extraction of alboleersin and luteoleersin

The dried mycelium was ground in a coffee mill and thoroughly extracted for 3–4 working days in a Soxhlet apparatus with light petroleum, B.P. $40\text{--}50^\circ$, giving a yellow solution. At the end of each day the extraction flask was placed in the cold room and replaced the following day by a new flask containing fresh solvent. There separated from the light petroleum extracts on standing a greyish white product consisting essentially of crude alboleersin, the M.P. of which was usually $195\text{--}205^\circ$. The mother liquors from the crude alboleersin were combined, evaporated to a small volume and kept. There thus separated crude luteoleersin as a bright yellow crystalline powder, the M.P. of which, while very

variable, was between 120 and 135°. Considerable quantities of crude fat remained after evaporation to dryness of the final light petroleum mother liquors. The yields of crude alboleersin obtained in 3 representative experiments, using 100 flasks in each case, were 0.58, 1.95 and 1.55 g., while the corresponding yields of crude luteoleersin were 6.80, 4.39 and 5.46 g.

After exhaustive extraction with light petroleum, the mycelium was extracted with ether, giving considerable amounts of a dark brown extract, and then with CHCl_3 which gave smaller amounts of a dark green extract. No crystalline substances have, at present, been isolated either from the ether or CHCl_3 extracts.

Purification of the metabolic products

(a) *Alboleersin*. Some of the fractions of crude alboleersin were very impure. The different fractions, together with small amounts obtained in purifying the crude luteoleersin, were twice crystallized from benzene (charcoal). The purified alboleersin was thus obtained as practically colourless thin, pearly plates, M.P. 208–209° (decomp.). This product was then repeatedly crystallized from 65–70 % methyl alcohol (aqueous, by volume) to give clusters of long colourless, silky needles with a constant M.P. of 215–216°. The average yield of purified alboleersin obtained per 100 flasks was about 1 g.

(b) *Luteoleersin*. The crude luteoleersin was dissolved in a minimum volume of boiling light petroleum (B.P. 80–100°) and was separated from undissolved alboleersin. The luteoleersin which crystallized out on cooling the filtrate was purified to constant M.P. by recrystallization from light petroleum (B.P. 60–80°) and charcoal. The substance was finally obtained as beautiful yellow rods or blunt-ended needles, M.P. 135°. The average yield of purified luteoleersin obtained per 100 flasks was rather more than 2 g.

Analysis and general properties of alboleersin and luteoleersin

A number of different samples of both substances, dried to constant weight at 80–90° in a high vacuum, were analysed in duplicate by two different analysts with the following results.

(a) *Alboleersin*. Four samples analysed by Weiler: C, 67.44 % (average of 8 analyses—limits 67.25–67.72 %); H, 8.54 % (average of 8 analyses—limits 8.25–8.80 %). Two samples analysed by Schoeller: C, 67.00 % (average of 4 analyses—limits 66.86–67.10 %); H, 8.54 % (average of 4 analyses—limits 8.48, 8.65 %). $\text{C}_{26}\text{H}_{40}\text{O}_7$ requires C, 67.20; H, 8.68 %.

(b) *Luteoleersin*. Two samples analysed by Weiler: C, 67.45 % (average of 4 analyses—limits 67.35–67.60 %); H, 8.23 % (average of 4 analyses—limits 8.19–8.28 %). Duplicate portions of the same two samples analysed by Schoeller: C, 67.54 % (average of 4 analyses—limits 67.44–67.66 %); H, 8.18 % (average of 4 analyses—limits 8.00–8.35 %). $\text{C}_{26}\text{H}_{38}\text{O}_7$ requires C, 67.50; H, 8.28 %.

Neither substance contains N, S, P, Cl or methoxyl.

Mol. wt. (Dr A. E. Oxford); cryoscopic in dioxane. (a) *Alboleersin*. Found 389. $\text{C}_{26}\text{H}_{40}\text{O}_7$ requires 464. This estimation has probably given too low a result since the solution, which was colourless at the start, had become bright yellow at the end of the experiment. This is probably due to oxidation, since alboleersin when in solution is rather quickly oxidized to luteoleersin.

(b) *Luteoleersin*. Found 449. $\text{C}_{26}\text{H}_{38}\text{O}_7$ requires 462.

Active hydrogen estimations (Roth). (a) *Alboleersin* gave in pyridine 2.87 active H atoms per molecule at 21° and 3.00 at 95° calculated on the formula $\text{C}_{26}\text{H}_{40}\text{O}_7$.

(b) Luteoleersin gave in pyridine 2.0 active H atoms per molecule at 21° and 2.0 at 95° calculated on the formula $C_{26}H_{38}O_7$.

Optical rotations. (a) Alboleersin. $[\alpha]_{5461}^{18^\circ} + 274^\circ$, $[\alpha]_{5790}^{18^\circ} + 242^\circ$ ($c = 0.4304$ in alcohol).

(b) Luteoleersin. $[\alpha]_{5461}^{18^\circ} + 214^\circ$, $[\alpha]_{5790}^{18^\circ} + 232^\circ$ ($c = 0.4560$ in alcohol).

The abnormal fact that the specific rotation of luteoleersin is greater for the mercury yellow line (5790) than for the mercury green line (5461) was checked by independent observers.

Alboleersin and luteoleersin are fairly soluble in most organic solvents but are almost insoluble in water. Luteoleersin is in general more soluble than alboleersin. Alboleersin may be readily crystallized from benzene, 50% aqueous alcohol, or, better, from 65–70% aqueous methyl alcohol, and luteoleersin from light petroleum, B.P. 60–80°.

On heating in air (e.g. in a m.p. determination) alboleersin begins to darken and decompose at about 150–160° and finally melts to a dark red liquid at 215–216° (decomp.). Alboleersin behaves in a characteristic way on heating in a high vacuum at 90°. The colourless crystals turn a beautiful mauve colour which remains stable overnight *in vacuo* but the crystals again become colourless on short exposure to air. No detectable change in weight occurred during this colour change. Alboleersin slowly turns yellow on continued exposure to air, probably due to oxidation to luteoleersin. Luteoleersin melts smoothly at 135° and is quite stable in air.

Reactions of alboleersin and luteoleersin

Alboleersin dissolves in cold conc. H_2SO_4 to give a pale yellow colour which changes to dark brown on standing overnight. With the same reagent luteoleersin gives a pale blue solution changing quickly to green and then to a dirty yellowish green.

An alcoholic solution of alboleersin gives with $FeCl_3$ a bright yellow colour. Luteoleersin gives no reaction.

With aqueous NaOH alboleersin slowly dissolves to give a soapy yellow solution, changing quickly in colour to pinkish mauve and on standing becoming nearly colourless. Luteoleersin is insoluble in aqueous NaOH. With alcoholic NaOH a characteristic purple colour is developed after a few seconds.

Luteoleersin is readily reduced in alcoholic solution with Zn and HCl to give a colourless solution.

Both substances instantly reduce $KMnO_4$ in acetone, and neither of them gives a sterol reaction.

Luteoleersin gives the reaction, characteristic of quinones, with substances containing an active methylene group. Thus, on adding to a very dilute solution of luteoleersin in 50% pyridine one drop of ethyl acetoacetate together with a few drops of 2N NaOH, a beautiful pale blue coloration is obtained. If the ethyl acetoacetate is omitted from this test, only the characteristic purple coloration is produced.

Conversion of luteoleersin into alboleersin

(a) *By heating with phenylhydrazine.* A mixture of luteoleersin (0.3 g.), anhydrous sodium acetate (0.3 g.), alcohol (12 ml.), water (3 ml.) and phenylhydrazine (0.2 g.) was heated under a reflux condenser for 1 hr. There was evolution of gas, presumably nitrogen. The mixture was cooled, diluted with water and carefully acidified with HCl. The buff-coloured solid which separated was recrystallized first from benzene and then from 70% aqueous methyl

alcohol. Yield, 70 % of theoretical. Long colourless silky needles, m.p. 215–216° decomp., alone or mixed with authentic alboleersin. $[\alpha]_{5461}^{18^\circ} + 274^\circ$, $[\alpha]_{5790}^{18^\circ} + 243^\circ$ ($c=0.5184$ in alcohol). (Found (Weiler): C, 67.35, 67.32; H, 8.34, 8.49 %. Found (Schoeller): C, 67.36, 67.17; H, 8.57, 8.47 %. $C_{26}H_{40}O_7$ requires C, 67.20; H, 8.68 %.)

(b) *By catalytic reduction.* Luteoleersin (0.2 g.) dissolved in absolute alcohol (12 ml.) was reduced in an atmosphere of hydrogen with a Pd charcoal catalyst. The reduction was complete in 1–2 min. The alcoholic solution, now colourless, was filtered from the catalyst and alboleersin was precipitated by the addition of water. It was recrystallized from 70 % aqueous methyl alcohol. Yield about 80 %. Long colourless silky needles, m.p. 215° decomp. alone or mixed with authentic alboleersin.

Conversion of alboleersin into luteoleersin

Alboleersin (0.2 g.) dissolved in alcohol (4 ml.) was treated in the cold with 3 ml. of a solution containing anhydrous $FeCl_3$ (50 g.) in water (110 ml.). The solution, initially colourless, became momentarily dark green and finally deep golden in colour. After 5 min. it was diluted with water when luteoleersin separated as a yellow jelly which was filtered, washed, dried and recrystallized from light petroleum, b.p. 60–80°. Yield nearly quantitative. Yellow rods, m.p. 134–136°, alone or mixed with authentic luteoleersin. $[\alpha]_{5461}^{18^\circ} + 215^\circ$, $[\alpha]_{5790}^{18^\circ} + 231^\circ$ ($c=0.4928$ g. in alcohol). (Found (Schoeller): C, 67.51, 67.54; H, 8.17, 8.25 %. $C_{26}H_{38}O_7$ requires C, 67.50; H, 8.28 %.)

Luteoleersin, in strongly acid (HCl) alcoholic solution, liberates iodine from potassium iodide. When carried out on a quantitative basis, this method, which gave theoretical results with benzoquinone, yielded with luteoleersin a figure which was about 10 % too high. The reaction is complex, since the product which was isolated was not alboleersin, as would be expected, and further the same product was obtained by merely dissolving alboleersin in a mixture of alcohol and concentrated HCl. Luteoleersin (0.1021 g.) was dissolved in 95 % alcohol (10 ml.). To the solution was added a mixture of 95 % alcohol (20 ml.), 15 % KI (10 ml.) and conc. HCl (10 ml.). The iodine liberated required 3.95 ml. $N/10$ thiosulphate, corresponding to a mol. wt. for luteoleersin of 512. ($C_{26}H_{38}O_7$ requires 462.) The solution from this and a number of similar titrations was diluted with water and the resultant precipitate was dried and crystallized from 65 % aqueous methyl alcohol. Colourless masses of plates, m.p. 171–173°. The same product was obtained from alboleersin by dissolving it in 95 % alcohol and adding concentrated HCl (using the same concentrations as for the titration but omitting KI). The resultant product, after recrystallization, melted at 174°, and had a mixed m.p. with the product from luteoleersin of 173–174°. (Found (Weiler), on the material from alboleersin: C, 67.22, 67.35; H, 8.11, 8.28 %. $C_{26}H_{40}O_7$ requires C, 67.20; H, 8.68 %.) The substance may be an isomeride of alboleersin. Its solutions readily oxidize in air, rapidly turning pink in colour. It is soluble in NaOH giving a deep yellow solution which rapidly turns red and a reddish orange flocculent substance separates. It is readily oxidized in alcoholic solution by $FeCl_3$ with the formation of a fine reddish colour. The product of the reaction, which is probably a quinone, is difficult to crystallize. It separates from aqueous methyl alcohol in long hair-like needles, reddish in colour, m.p. 129–131°. It gives a pale blue colour with concentrated H_2SO_4 .

Attempts to make derivatives of alboleersin and luteoleersin

Gums or glasses, which were almost invariably extremely soluble in all organic solvents, and could not be crystallized, were given by alboleersin with the following reagents: (a) diazomethane; (b) dimethyl sulphate and alkali in acetone solution; (c) acetic anhydride, sodium acetate and glacial acetic acid; (d) 3:5-dinitrobenzoyl chloride and pyridine; (e) *p*-bromobenzoyl chloride and pyridine; and by luteoleersin with the following reagents: (a) diazomethane; (b) 2:4-dinitrophenylhydrazine; (c) semicarbazide hydrochloride.

SUMMARY

An account is given of the isolation in crystalline form, from the mycelium of *Helminthosporium leersii* Atkinson, grown on a Czapek-Dox glucose medium, of two hitherto undescribed mould metabolic products. These are luteoleersin, $C_{26}H_{38}O_7$, which was obtained in yellow blunt-ended needles, and alboleersin, $C_{26}H_{40}O_7$, which was obtained as colourless silky needles. Luteoleersin is believed to be a substituted quinone or semiquinone, and alboleersin the phenol corresponding to luteoleersin. The two substances are very readily interconvertible, luteoleersin giving alboleersin by simple reduction, and alboleersin giving luteoleersin by simple oxidation. The view is expressed that the two substances may form an oxidation-reduction system in the life processes of *H. leersii*.

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