Structure of Ramulosin, a Metabolic Product of the Fungus *Pestalotia ramulosa*

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Brewer (1958) described the isolation of a number of fungi from slime samples taken from pulp and paper mills in Eastern Canada, one of the organisms being identified as *Truncatella ramulosa* (v. Beyma) Steyaert (Syn. *Pestalotia ramulosa* v. Beyma). In 1959, during a study of a subculture of this organism, we observed crystals in old cultures grown on malt-extract-agar. In a preliminary note Benjamin & Stodola (1960) reported the isolation from such cultures of a pure product to which the name ramulosin was given. We now present evidence that this compound is the enol chelate of perhydro-3-methyl-1,8-dioxo-2-benzopyran (I). (A name based on isocoumarin would be 3,4,4a,5,6,7-hexahydro-8-hydroxy-3-methylisocoumarin; one in conformance with the practice of *Chemical Abstracts* is 3,4,4a,5,6,7-hexahydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran.)

(I)
Ramulosin

Ramulosin is produced slowly at 15° in amounts of about 3–4 g./l. of liquid medium; it is easily isolated in the pure state by filtration of the culture liquor, solvent extraction of the dried mycelium and crystals, and recrystallization. Ramulosin has the molecular formula $C_{10}H_{14}O_3$, m.p. 120–121° and $[\alpha]_D^{20} + 18^\circ$. A Kuhn–Roth determination showed one *C*-methyl group. At room temperature ramulosin is moderately soluble (100–800 mg./ml.) in chloroform, dichloromethane, pyridine, benzene, dimethylformamide, acetone, acetonitrile, acetic acid and ethyl acetate, somewhat less so (40–90 mg./ml.) in carbon disulphide, carbon tetrachloride,

methanol, butan-1-ol, 95 % (v/v) ethanol and diethyl ether, and very slightly soluble in light petroleum and water. It dissolves readily in sodium hydroxide solution but not in sodium carbonate solution. A violet colour is produced with ferric chloride in ethanol; this evidence for a phenol or enol structure was in accordance with the strong ultraviolet absorption, a single peak being observed at 264 $m\mu$ (ϵ 10 120). In 0.1 *N*-potassium hydroxide this peak was shifted to 291 $m\mu$ (ϵ 16 600), a change comparable with that reported for ethyl acetoacetate (Kögl & DeBruin, 1950). The preparation of *O*-benzoyl derivatives of ramulosin confirmed the presence of the enolic hydroxyl group. Acid and alkaline hydrolysis of ramulosin yielded 1 mole of carbon dioxide/mole, thereby accounting for all the oxygen of the molecule in the form of either a β -oxo ester or a β -oxo lactone.

Prolonged treatment of ramulosin with 50 % (w/v) potassium hydroxide solution at room temperature resulted in the formation of a $C_9H_{10}O_2$ compound. Since the only carbon atom lost in this reaction was that of carbon dioxide, it followed that ramulosin must be a lactone rather than an ester. The lactone nature of ramulosin was further demonstrated as follows. A solution of ramulosin in 5 equiv. of 1 *N*-potassium hydroxide was saturated after 1 hr. with carbon dioxide to pH 8; because no solid separated, conversion into the salt of the β -oxo acid must have occurred. Acidification with hydrochloric acid, followed by filtration, gave a 90 % yield of ramulosin.

Attempts to prepare carbonyl derivatives of ramulosin with various reagents were unsuccessful; hence it appeared that the compound must be fixed in the enol form, presumably through the formation of a stable chelate ring. Chelation was further indicated by infrared-spectroscopic studies, which showed no distinct hydroxyl band absorption, but did reveal a strong band at 1653 cm^{-1} (6.05μ) characteristic of a 'conjugated chelate', as first demonstrated by Rasmussen, Tunnicliff & Brattain (1949). Recent examples of natural products showing this shift of the carbonyl band are atrovenetin (Neill & Raistrick, 1957), mangostin (Yates & Stout, 1958) and rubrofusarin (Stout, Dreyer &

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Jensen, 1961). The possibility of a chelate ring in ramulosin was indicated also by the preparation of a copper chelate salt soluble in benzene.

The formation of a metal chelate (Eistert & Reiss, 1954) ruled out the possibility that the enolic hydroxyl group and the lactone group are in the same ring, and, since the molecular formula of ramulosin required two rings, the basic structure of ramulosin was tentatively formulated as (II), in view of the preponderance of 6-membered rings in Nature.

(II)

Two compounds of this type have already been isolated from fungi: one is mellein (III), obtained by Nishikawa (1933a, b) from *Aspergillus melleus* and by Yabuta & Sumiki (1933) from *A. ochraceus*; the other is 8-hydroxy-3-methylisocoumarin (IV), isolated by Bendz (1959) from the mushroom *Marasmius ramealis*. These structures suggested a probable location for the methyl group in ramulosin and permitted a tentative assignment of structure (I); proof of the correctness of this formulation was provided by the following degradation studies.

Attention was first directed to the liquid $C_9H_{16}O_2$ compound mentioned above as the product of the action of strong alkali on ramulosin. On the basis of formula (I) it would be expected to have structure

(V). The presence of a hydroxyl group in the $C_9H_{16}O_2$ compound was demonstrated by the formation of a *p*-nitrobenzoate (m.p. 103–104°). The presence of a carbonyl group was shown in two ways: (1) the *p*-nitrobenzoate was converted into an oxime melting at 113–114°; (2) the $C_9H_{16}O_2$ compound was reduced by sodium borohydride to a diol of composition $C_9H_{18}O_2$ (m.p. 113–114°), which formed a bis-*p*-nitrobenzoate (m.p. 125–126°). The $C_9H_{16}O_2$ ketol therefore exhibited the behaviour to be expected of the decarboxylation product of an acid formed by the opening of a β -oxo lactone ring.

Next, the $C_9H_{16}O_2$ compound was freed of its carbonyl group by means of a Wolff-Kishner reaction. The liquid $C_9H_{18}O$ alcohol thus obtained, which should have structure (VI), was characterized as a *p*-nitrobenzoate (m.p. 47–48°) with $[\alpha]_D^{25} - 53^\circ$. The racemic form of 1-cyclohexylpropan-2-ol was then synthesized by the following series of reactions in which R = cyclohexyl:

The synthetic alcohol had the same infrared-absorption spectrum as the $C_9H_{16}O$ product; moreover, the *p*-nitrobenzoate (m.p. 69–70°) of the synthetic alcohol showed the same infrared-absorption spectrum as the corresponding derivative of the natural $C_9H_{16}O$ alcohol.

Further structural confirmation was obtained as follows. The natural $C_9H_{16}O$ alcohol was oxidized with *tert*.-butyl hypochlorite to the corresponding ketone, which gave a 2,4-dinitrophenylhydrazone (m.p. 117–118°) indistinguishable from the corresponding derivative made from synthetic cyclohexylacetone.

(III)
Mellein (IV)
8-Hydroxy-3-methylisocoumarin

(V)

(VI)

(VII)

(VIII)

(IX)

Finally, the natural $C_9H_{18}O$ alcohol was subjected to the following series of reactions:

The final product proved to be the expected *N*-(cyclohexylmethyl)benzamide.

These studies establish with reasonable certainty the formula (I) assigned to ramulosin. Also, the nuclear-magnetic-resonance spectrum is in accordance with this formula. Compounds with related structures have been isolated by Sondheimer (1957) (VII) and by Yamamoto, Nitta & Yamamoto (1961, 1962) (VIII and IX).

Hendershot, Hesseltine, Pridham, Benedict & Jackson (1962) have shown that ramulosin inhibits the germination of various seeds and the spores of certain micro-organisms. Ramulosin can be regarded as an $\alpha\beta$ -unsaturated lactone, examples of which have long been known to affect seeds similarly, the best known being coumarin.

EXPERIMENTAL

Cultures

The ramulosin-producing culture was received as *Truncatella ramulosa* (v. Beyma) Steyaert from Dr D. Brewer, Atlantic Regional Laboratory, National Research Council, Halifax, Nova Scotia, Canada, who had isolated it from the kraft tailing trough of a paper mill in New Brunswick. This culture, for which we prefer the designation *Pestalotia ramulosa* v. Beyma, has not shown sporulation on a number of media tried in this Laboratory. The organism, which has the designation NRRL 2826 in the ARS Culture Collection here, is kept on agar slants and transferred monthly. Two cultures of *P. ramulosa* v. Beyma obtained from The Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, showed sporulation typical of *Pestalotia* but did not produce ramulosin (Hesseltine, Benjamin, Bradle & Hendershot, 1963).

Cultural conditions

A number of 300 ml. Erlenmeyer flasks, plugged with cotton wool, were prepared, each containing 100 ml. of malt-extract medium (malt extract, 20 g.; peptone, 1 g.; glucose, 20 g.; distilled water, 1000 ml.). After sterilization for 15 min. at 15 lb./in.² pressure each flask was inoculated

with a block of mycelium on agar and incubated at 15°. In about 26 days, crystals began to appear in the culture liquor.

Isolation of ramulosin

After 85 days the mycelial mat and crystals from five flasks were removed by filtration and dried in air (wt. 3.52 g.). Repeated extraction with boiling acetone and finally with ether gave an extract, which was concentrated *in vacuo* to about 25 ml. Dilution with 200 ml. of water yielded 1.58 g. of crude crystalline ramulosin. A small amount of colour was removed with decolorizing charcoal in acetone, the filtrate was concentrated to a small volume, and the colourless platelets of m.p. 120–121° were separated. Recrystallization from hexane gave no change in melting point.

General properties of ramulosin

Ramulosin crystallizes readily from aqueous acetone in the form of plates, m.p. 120–121° (capillary and corrected); infrared-absorption peak (KBr disk) at 1653 cm^{-1} (6.05 μ) (hydrogen-bonded and conjugated CO group); nuclear-magnetic-resonance signal at -3.1τ (enolic proton), a doublet at $+8.6\tau$ corresponding to a methyl group influenced by a proton, and a multiplet at 5.7τ indicative of one proton influenced by one methyl group and one methylene group.

Ramulosin contains no N, S or halogen (Found: C, 66.1; H, 7.8. $C_{10}H_{14}O_3$ requires C, 65.9; H, 7.8%). A Rast molecular-weight determination gave a value of 179 (calc. for $C_{10}H_{14}O_3$, 182); by the Signer method (acetone and benzoic acid) a value of 193 was obtained. Small and variable amounts of alkyl iodide were observed in the alkoxy determination (1.7 and 3.0% calc. as OMe; calc. for 1 OMe, 17.0%). In the Kuhn–Roth determination 0.85 mole of *C*-Me/mole was indicated. Ramulosin showed $[\alpha]_D^{25} + 18 \pm 2^\circ$ (c 2.9 in ethanol). With $FeCl_3$ in aqueous ethanol ramulosin gives a deep-purple colour. Ramulosin is readily soluble in a NaOH solution but not in Na_2CO_3 . No reaction was observed with phenylhydrazine, semicarbazide or hydroxylamine.

At the boiling point of solvent, 1 g. of ramulosin dissolved in 1.45 ml. of acetone, 2.69 ml. of light petroleum (b.p. 94–97°), or 284 ml. of water.

In absolute ethanol, ramulosin shows a single peak at 264 $m\mu$ (ϵ 10 120), which is shifted in 0.1 *N*-KOH solution in absolute ethanol to 291 $m\mu$ (ϵ 16 600). In aq. 0.1 *N*-KOH the peak at 264 $m\mu$ disappears to leave only end absorption; acidification brings about a slow return of the peak, indicating re-formation of an $\alpha\beta$ -unsaturated lactone.

The lactone behaviour of ramulosin was further demonstrated as follows. Ramulosin was dissolved in 5 equiv. of 1 *N*-KOH and allowed to stand for 1 hr. at room temperature.

The solution was then saturated with CO_2 to pH 8; no ramulosin was precipitated, showing conversion into the potassium salt of the β -oxo acid. Acidification with HCl and filtration gave a 90% recovery of ramulosin. The remaining 10% of ramulosin was recovered by ether extraction.

Derivatives of ramulosin

Benzoate. Ramulosin (200 mg.) was dissolved in dry pyridine (1 ml.), benzoyl chloride (0.40 ml.) was added and the solution was allowed to stand at room temperature overnight. The addition of excess of K_2CO_3 solution gave a precipitate of crude crystalline product, which was separated and crystallized from ethanol-hexane after treatment with decolorizing charcoal. Recrystallization from aqueous ethanol yielded 169 mg. of colourless needles of *O*-benzoate, m.p. 82–83° (Found: C, 71.4; H, 6.5. $\text{C}_{17}\text{H}_{18}\text{O}_4$ requires C, 71.3; H, 6.3%).

***p*-Nitrobenzoate.** Ramulosin (200 mg.) and *p*-nitrobenzoyl chloride (610 mg.) were heated at 100° in pyridine (1 ml.) for 5 min. The solution was cooled and treated with excess of K_2CO_3 solution. The solid was separated and crystallized from ethanol-hexane and from aqueous acetone to yield 202 mg. of very-pale yellow needles of *O*-*p*-nitrobenzoate, m.p. 135–136° (Found: C, 61.6; H, 5.3; N, 4.4. $\text{C}_{17}\text{H}_{17}\text{NO}_6$ requires C, 61.6; H, 5.2; N, 4.2%).

***p*-Phenylazobenzoate.** Ramulosin (182 mg.) and *p*-phenylazobenzoyl chloride (978 mg.) were heated at 60° for 5 min. in pyridine (7 ml.). The reaction mixture was allowed to stand at room temperature until a clear solution resulted, which was heated on a steam bath for 3 min. with water (200 mg.). Water and ether were added; the ether solution was separated and washed successively with dilute NaOH, dilute H_2SO_4 and water. The removal of ether left 301 mg. of crude product, which was crystallized from ethanol and from acetone-hexane to give *O*-*p*-phenylazobenzoate as orange needles (168 mg.), m.p. 137–138° (Found: C, 70.5; H, 5.9; N, 7.1. $\text{C}_{23}\text{H}_{22}\text{N}_2\text{O}_4$ requires C, 70.8; H, 5.7; N, 7.2%).

4-Dimethylamino-3,5-dinitrobenzoate. Ramulosin (99 mg.) and 4-dimethylamino-3,5-dinitrobenzoyl chloride (298 mg.) were dissolved in warm pyridine (1.4 ml.). The solution, after standing overnight at room temperature, was treated with excess of K_2CO_3 solution and ether. The ether solution was separated and freed of pyridine with dilute H_2SO_4 . The removal of ether gave 218 mg. of crude product, which was crystallized from 65% ethanol and from 95% ethanol-acetone to yield *O*-4-dimethylamino-3,5-dinitrobenzoate as orange-yellow needles, m.p. 180–181° (Found: C, 54.6; H, 5.3; N, 10.5. $\text{C}_{19}\text{H}_{21}\text{N}_3\text{O}_8$ requires C, 54.4; H, 5.1; N, 10.0%).

Copper chelate. Ramulosin (91 mg.; 0.5 m-mole) and copper chelate of acetoacetic ester (79.9 mg.; 0.25 m-mole) were refluxed for 3 hr. in dry benzene. The resulting solution was allowed to evaporate in a beaker to a crystalline mass, which gave the *copper chelate* as pale-blue needles on crystallization from benzene-hexane, m.p. 219–220° (Found: C, 56.2; H, 6.2; Cu, 14.7. $\text{C}_{20}\text{H}_{26}\text{CuO}_6$ requires C, 56.4; H, 6.2; Cu, 14.9%).

Degradative studies on ramulosin

Quantitative hydrolysis of ramulosin. A solution of ramulosin (182 mg.) in ethanol (3 ml.) and 3*N*- H_2SO_4 (3 ml.) was refluxed, and the liberated CO_2 swept through

an Ascarite tube with N_2 . In 2.5 hr. a 96% yield (42.2 mg.) of CO_2 was obtained. An alkaline hydrolysis similarly conducted (5 hr. refluxing with 1*N*-NaOH, followed by acidification) gave a 95% yield of CO_2 .

Preparation of the C_9 ketol. Ramulosin (209 mg.) was added to water (1 ml.) containing KOH (380 mg.). The solid dissolved on standing overnight at room temperature, and in 2 days oily droplets began to appear. After a total reaction time of 2 weeks, the mixture was diluted with water and extracted continuously with ether for 2 hr. The removal of ether and drying to constant weight gave 169 mg. of the *ketol* as an almost odourless, colourless oil (calc. 166 mg., after correction for 26 mg. of recovered ramulosin) (Found: C, 68.8; H, 10.5. $\text{C}_9\text{H}_{16}\text{O}_2$ requires C, 69.2; H, 10.3%). The compound showed no characteristic ultraviolet absorption, and a Kuhn-Roth determination indicated 0.87 mole of *C*-Me/mole. Strong infrared-absorption bands for hydroxyl and carbonyl groups were obtained.

***p*-Nitrobenzoate of the C_9 ketol.** The *ketol* (64 mg.) and *p*-nitrobenzoyl chloride (550 mg.) in pyridine (2 ml.) were heated at 50° for 5 min. After the addition of water and ether, *p*-nitrobenzoic acid was removed from the ether with NaHCO_3 solution, and the pyridine with dilute H_2SO_4 . The removal of ether gave a crude product, which on crystallization from dilute acetic acid and from acetone-hexane gave the *p*-nitrobenzoate as pale-yellow blades, m.p. 103–104° (Found: C, 62.9; H, 6.2; N, 4.4. $\text{C}_{16}\text{H}_{16}\text{NO}_5$ requires C, 62.9; H, 6.3; N, 4.6%).

***Oxime of the ketol p*-nitrobenzoate.** The *p*-nitrobenzoate (63 mg.) was refluxed for 10 min. with an aqueous-ethanolic solution of hydroxylamine acetate. The crystals that appeared on cooling were separated and recrystallized from methylene dichloride-hexane to give the *oxime* (blades), m.p. 113–114° (Found: C, 60.3; H, 6.1. $\text{C}_{16}\text{H}_{20}\text{N}_2\text{O}_5$ requires C, 60.0; H, 6.3%).

Reduction of the C_9 ketol with sodium borohydride. The *ketol* (102 mg.) and NaBH_4 (31 mg.) were dissolved in propan-2-ol (2 ml.) and refluxed for 2 hr. Methanol (1 ml.) was added and the refluxing continued for 10 min. The solvent was removed, the residue treated with 2*N*-NaOH (3 ml.), and the solution extracted for 6 hr. with ether. The removal of ether left crude crystalline material from which the higher melting *diol* was obtained as colourless bars by three crystallizations from benzene, m.p. 113–114° (Found: C, 68.1; H, 11.4. $\text{C}_9\text{H}_{18}\text{O}_2$ requires C, 68.3; H, 11.5%).

***Bis*-*p*-nitrobenzoate of the diol.** The *diol* (16 mg.) and *p*-nitrobenzoyl chloride (77 mg.) were heated at 100° in pyridine (0.5 ml.) for 5 min. After the addition of water and ether, *p*-nitrobenzoic acid was removed with Na_2CO_3 solution, and pyridine with dilute H_2SO_4 . The removal of ether gave a crude product from which pure *bis*-*p*-nitrobenzoate was obtained by two crystallizations (needles) from ethanol, m.p. 125–126° (Found: C, 60.5; H, 5.2; N, 5.9. $\text{C}_{23}\text{H}_{22}\text{N}_2\text{O}_8$ requires C, 60.5; H, 5.3; N, 6.1%).

Conversion of the C_9 ketol into the C_9 alcohol. The *ketol* (215 mg.) and dry hydrazine were heated for 2 hr. at 100° in diethylene glycol (1 ml.). After the addition of powdered KOH (200 mg.), the reaction mixture was heated for 1 hr. at 155–160°, at which temperature there was vigorous evolution of gas. After heating for 1 hr. at 195° the gas evolution ceased. The product was treated with conc. HCl and extracted with benzene. The removal of benzene and drying in a vacuum desiccator for 0.5 hr. left the fragrant liquid *C}_9* *alcohol* (180 mg.). Its infrared-absorption spectrum

was identical with that of 1-cyclohexylpropan-2-ol, the synthesis of which is described below.

p-Nitrobenzoate of the C_9 alcohol. The C_9 alcohol (180 mg.) and *p*-nitrobenzoyl chloride (500 mg.) were heated in pyridine (1.5 ml.) at 100° for 5 min. After the addition of water and ether, *p*-nitrobenzoic acid was removed with Na_2CO_3 solution, and pyridine with dilute H_2SO_4 . The removal of ether gave a crude crystalline product, which on recrystallization from dilute acetic acid yielded *p*-nitrobenzoate, m.p. 47–48° and $[\alpha]_{25}^D -53^\circ$ (*c* 3.2 in ethanol) (Found: C, 66.5; H, 7.3; N, 5.0. $C_{16}H_{21}NO_4$ requires C, 66.0; H, 7.3; N, 4.8%). This derivative had an infrared-absorption spectrum identical with that of the corresponding derivative, m.p. 69–70°, prepared from synthetic 1-cyclohexylpropan-2-ol.

Oxidation of the C_9 alcohol to cyclohexylacetone. The C_9 alcohol (56 mg.), *N*-bromosuccinimide (63 mg.), pyridine (28 mg.) and carbon tetrachloride (700 mg.) were heated for 4 hr. at 78° in a sealed tube. After cooling, the tube was opened and the solution shaken with 3 drops of 2*N*-HCl and 1 ml. of water. The carbon tetrachloride solution was filtered and concentrated to a fragrant oil, which was converted into 46 mg. of crude 2,4-dinitrophenylhydrazone. Recrystallization from 90% ethanol gave 31 mg. of dense-orange crystals, m.p. 117–118°. On admixture with the 2,4-dinitrophenylhydrazone of synthetic cyclohexylacetone (m.p. 119–120°) the m.p. was 117–118°, and the infrared-absorption spectra of the two compounds were indistinguishable.

*Conversion of the C_9 alcohol into *N*-(cyclohexylmethyl)-benzamide.* The C_9 alcohol (71 mg.) was dissolved in *tert*-butyl hypochlorite (0.85 ml.) and allowed to stand for 3 hr. in the dark. The excess of the reagent was removed *in vacuo*, and the resulting ketone was converted into the oxime, which was heated in polyphosphoric acid at 120° for 10 min. The resulting *N*-(cyclohexylmethyl)acetamide was refluxed in 6*N*-HCl (2 ml.) for 5.5 hr. to give the hydrochloride of cyclohexylmethylamine. Benzoylation yielded 40 mg. of product, m.p. 106–107° after two crystallizations (18 mg.). It was shown by mixed m.p. and infrared-absorption spectra to be identical with an authentic sample of *N*-(cyclohexylmethyl)benzamide (Hückel, Neunhoeffer, Gereke & Frank, 1930).

Syntheses

Cyclohexylacetic acid. Cyclohexanone was converted into ethyl cyclohexylcyanoacetate in 73% yield by the method of Alexander & Cope (1944). The free acid was obtained by saponification with aqueous KOH as follows. The ester (74.5 g.) was added slowly to a stirred solution of 2*N*-KOH (500 ml.). A clear solution resulted about 3 min. after the addition was completed. After 5 more min., conc. HCl (150 ml.) was added. Ether extraction gave 61.5 g. (96% yield) of crude cyclohexylcyanoacetic acid, which was purified by crystallization from benzene–hexane, m.p. 85–86°. Ives & Sames (1943) report m.p. 81–82°.

The cyano acid (24 g.) was refluxed for 8 hr. with a solution of water (120 ml.) and H_2SO_4 (120 ml.). After neutralization of most of the H_2SO_4 , the reaction mixture was extracted with ether to give an oil, which was freed of water by azeotropic distillation with hexane. The residue (20.6 g.) of cyclohexylacetic acid crystallized, m.p. 30–31°. Venus-Danilowa (1928) reported m.p. 31°.

Cyclohexylacetone. Cyclohexylacetic acid was converted by using thionyl chloride into the acid chloride, which was

treated with ethoxymagnesiummalonic ester prepared by the method of Bowman (1950). The acid chloride (20.6 g.) was dissolved in benzene (25 ml.) and the solution added dropwise to the ethoxymagnesiummalonic ester with cooling and stirring. After 1 hr. of refluxing the solution was filtered and decomposed with dilute acid, after which the esters were extracted with benzene. Benzene and excess of malonic ester were removed *in vacuo*, and the residue of crude cyclohexylmalonic ester (19.5 ml.) was refluxed for 3 hr. with propionic acid (84.6 ml.) containing conc. H_2SO_4 (0.70 ml.). Then 4*N*- H_2SO_4 (11 ml.) was added and the refluxing continued for another 2 hr. After the addition of water and excess of NaOH, cyclohexylacetone was extracted with ether. Fractionation gave 15.3 g. (76% yield) of pure product, b.p. 196–199° at 755 mm. Hg. Adams, Abramovitch & Hauser (1943) reported b.p. 196–200°.

Cyclohexylacetone 2,4-dinitrophenylhydrazone. Cyclohexylacetone (125 mg.) was added to a solution of 2,4-dinitrophenylhydrazine (187 mg.) in diethylene glycol dimethyl ether (5 ml.). After 10 min. at room temperature, 3 drops of conc. HCl were added to the reaction mixture. The addition of 2*N*-HCl to give cloudiness followed by cooling gave 253 mg. of crude product, which was recrystallized from ethanol to give the 2,4-dinitrophenylhydrazone in the form of orange flakes, m.p. 119–120° (Found: C, 56.2; H, 6.2; N, 17.8. $C_{16}H_{20}N_4O_4$ requires C, 56.2; H, 6.3; N, 17.5%).

Reduction of cyclohexylacetone with potassium borohydride. Cyclohexylacetone (2.70 g.) and KBH_4 (1.04 g.) were dissolved in a mixture of methanol (45 ml.) and 0.5*N*-NaOH (40 ml.). After 26 hr. at room temperature the reaction mixture was treated with 4*N*- H_2SO_4 (10 ml.) to decompose the excess of KBH_4 . The solution was made alkaline with 0.5*N*-NaOH and concentrated *in vacuo* to remove methanol. Continuous ether extraction gave racemic 1-cyclohexylpropan-2-ol, which was dried by azeotropic distillation with hexane; the yield was 2.42 g. The *p*-nitrobenzoate had m.p. 69–70°.

SUMMARY

1. Ramulosin, a $C_{10}H_{14}O_3$ metabolic product of the fungus *Pestalotia ramulosa*, gives ultraviolet-absorption and infrared-absorption spectra that indicate the presence of a β -oxo lactone grouping.

2. This grouping was confirmed by the liberation of 1 mole of carbon dioxide on hydrolysis.

3. The formation of a copper chelate salt showed that the two functional groups are not in the same ring.

4. These findings and a consideration of the structures of analogous fermentation products suggested that ramulosin is the enol chelate of perhydro-3-methyl-1,8-dioxo-2-benzopyran.

5. This structure was confirmed by the conversion of ramulosin into a $C_9H_{16}O_2$ ketol, which yielded the expected 1-cyclohexylpropan-2-ol on removal of the carbonyl group.

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Effects of Insulin and Starvation on the Metabolism of Acetate and Pyruvate by the Perfused Rat Heart

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Studies of myocardial metabolism *in vivo* by means of catheterization of the coronary sinus have revealed the prominent role of fatty acids and ketone bodies as metabolic fuels for energy production in the intact human and dog heart (Bing, 1954–55). Extraction of carbohydrates has been shown to be predominant only in the post-absorptive stage after a high-carbohydrate meal, and to be greatly decreased in the starved and diabetic states (Olson & Piatnek, 1959; Goodale, Olson & Hackel, 1959). Investigations of the metabolism of the isolated perfused rat heart have likewise shown that ketone bodies (Williamson & Krebs, 1961) or fatty acids (Shipp, Opie & Challoner, 1961) are readily utilized as respiratory fuels when supplied to the heart as the sole substrate. Lactate and glucose, on the other hand, are relatively poorly utilized by hearts from normal fed rats, even in the presence of insulin (Williamson, 1962a). When mixtures of substrates are added to the perfusion fluid, glucose competes unfavourably with fatty acids and ketone bodies for oxidation, and tends to be metabolized by alternative pathways to lactate and glycogen.

Hearts from starved rats demonstrate a decreased utilization of glucose and lactate, but aceto-

acetate utilization is unchanged (Williamson & Krebs, 1961; Williamson, 1962a). An impaired utilization of glucose and pyruvate by muscle tissues from diabetic rats has been observed by many groups of investigators (Krahl & Cori, 1947; Pearson, Hsieh, Dutoit & Hastings, 1949; Vilee & Hastings, 1949; Kipnis, Helmreich & Cori, 1959; Morgan, Cadenas, Regen & Park, 1961), and a decreased rate of pyruvate decarboxylation has been suggested as a factor contributing to the abnormal response of diabetic subjects to glucose and pyruvate tolerance loads (Fry & Butterfield, 1962). Insulin has generally not been observed to restore fully the rate of glucose phosphorylation by muscle from diabetic animals, but has been reported to increase to normal the oxidation of pyruvate by diaphragms from alloxan-diabetic rats (Pearson *et al.* 1949; Vilee & Hastings, 1949). These studies suggest that insulin may have a permissive role in the rate of pyruvate entry into the citric acid cycle in muscle from diabetic animals.

The present investigation is concerned with a study of the effect of insulin on pyruvate and acetate utilization by perfused hearts from fed and starved rats and the metabolic competitions