The Bacterial Degradation of Flavonoids

OXIDATIVE FISSION OF THE A-RING OF DIHYDROGOSSYPETIN BY A *PSEUDOMONAS* SP.

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Cell-free extracts prepared from a *Pseudomonas* sp., grown on (+)-catechin, oxidized dihydrogossypetin (3',4',5,7,8-pentahydroxyflavanonol) by cleaving the A-ring to form oxaloacetic acid from C-5, C-6, C-7 and C-8 together with 5-(3,4-dihydroxyphenyl)-4-hydroxy-3-oxovalero- δ -lactone. The structure of this lactone was confirmed by synthesis of related phenylvalerolactones.

The metabolism of a number of related flavonoids has been examined in animals. Feeding with rutin or quercetin (Booth *et al.*, 1956), taxifolin (Booth & DeEds, 1958) or catechin (Watanabe, 1959) resulted in either total loss of the A-ring or fission of the A-ring with loss of carbon atoms from positions C-5 to C-8. Das & Griffiths (1969), using (+)-catechin selectively labelled in the A-ring were able to detect only radioactive CO₂ from these four positions. Use of antibiotics has confirmed Watanabe's (1959) suggestion that the above oxidations are caused by intestinal bacteria (Das & Griffiths, 1968).

In the preceding paper (Jeffrey *et al.*, 1972), dihydrogossypetin was established as a metabolite in the bacterial degradation of (–)-taxifolin, which has itself been shown to be the first product of the degradation of (+)-catechin (I. S. Farr & W. C. Evans, unpublished work). Further oxidation of dihydrogossypetin by cell-free extracts from the same *Pseudomonas* sp. is now reported.

Materials and Methods

Synthesis of [4-¹⁴C](±)-taxifolin

The route employed was the biosynthesis of quercetin from radioactive phenylalanine followed by chemical reduction to taxifolin. A modification of the procedure of Watkin *et al.* (1957) was used in the preparation of [4-¹⁴C]quercetin. [1-¹⁴C]-DL-Phenylalanine (50 μ Ci; specific radioactivity, 48 Ci/mol), together with 0.9 mmol of sodium acetate to dilute any labelling of the acetate pool, was administered to 50 g (9 g dry wt.) of buckwheat (*Fagopyrum tataricum*) seedlings. During the isolation of quercetin (120 mg), magnesol was replaced by binder-free Kieselgel H,

which had first been washed with conc. HCl and then with water. The radiochemical purity of the quercetin was determined by sodium amalgam reduction under the conditions described by Jeffrey *et al.* (1972); phloroglucinol and resorcinol contained less than 10% of the total radioactivity when compared with the major radioactive components, which were catechols.

Dilution of the [4-¹⁴C]quercetin with 500 mg of carrier, followed by reduction with alkaline dithionite (Geissman & Lischner, 1952), gave 290 mg of crude taxifolin. After a threefold recrystallization, the resulting [4-¹⁴C](±)-taxifolin (129 mg; specific radioactivity, 0.89 mCi/mol) appeared to be, by t.l.c. on cellulose in solvent (a), chemically and radiochemically pure.

Lactone synthesis

In taxifolin the protons on C-2 and C-3 are *trans* to each other. The synthesis shown in Scheme 1 therefore requires *erythro*-3-phenylglyceric acid (II), which was prepared from *trans*-cinnamic acid by the method of Böseken (1922): m.p. 120°C (lit. 121–122°C); n.m.r. in (C²H₅)₂CO: H₍₂₎ and H₍₃₎, δ 4.38, δ 4.95, J_{2-3} = 5.1. The acetone (III) was prepared by causing 16.26 g of compound (II) to react with 50 ml of 2,2-dimethoxypropane and 0.1 ml of conc. HCl for 1.5 h. Removal of excess of reagent at reduced pressure left an oil with an n.m.r. spectrum showing two non-equivalent methyl groups, δ 1.50 and 1.75; H₍₂₎, δ 4.79, H₍₃₎, δ 5.43, J_{2-3} = 7.8. The *p*-nitrophenylester (IV) was obtained by directly adding 12.4 g of *p*-nitrophenol in 100 ml of methylene chloride to compound (III) followed by 18.4 g of *NN'*-dicyclohexylcarbodi-imide in methylene chloride and, after 1 h,

Scheme 1. *Synthesis of phenylvalerolactones*

R = 4-Nitrophenyl.

discarding the precipitate. The ester was recrystallized from CHCl_3 -diethyl ether (m.p. 163.5°C , 75% yield). The n.m.r. spectrum had methyls δ 1.55 and 1.80; $\text{H}_{(2)}$ and $\text{H}_{(3)}$, δ 5.07 and 5.60, $J_{2-3} = 7.1$. To 25 ml of dimethoxyethane, dried by distillation from sodium hydride, was added 15 mmol of t-butyl acetoacetate (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.), and 15 mmol of NaH followed by 15 mmol of compound (IV). The reaction mixture was heated to 80°C for 1 h, during which time a pale-yellow precipitate formed. The reaction mixture was cooled and filtered, and to the precipitate was added 25 ml of CHCl_3 , 10 ml of water, and sufficient acetic acid to bring the

pH to 6.0. The aqueous phase was re-extracted with more CHCl_3 and the combined organic fractions were dried and concentrated under reduced pressure to yield 4.79 g (82%) of the crude compound (V), which contained some nitrophenol. The n.m.r. spectrum of compound (V) showed 12 protons, δ 1.5; the down-field methyl group of the acetonide, δ 1.73; acetyl methyl, δ 2.00; $\text{H}_{(4)}$ and $\text{H}_{(5)}$, δ 5.52 and 5.82, $J_{4-5} = 8.0$; $\text{H}_{(2)}$ = 13.6 (broad). The material was added to 25 ml of aq. 8M- NH_3 soln., shaken for 1 h and extracted twice with 20 ml of diethyl ether to give, after drying and evaporation at reduced pressure, 2.7 g of compound (VI) (66%), now free from residual nitro-

phenol. The n.m.r. spectrum showed the *t*-butyl group at δ 1.37; the acetonide methyls δ 1.50 and 1.77; the protons on $C_{(2)}$ as a non-equivalent AB pattern δ 2.70 and 3.08, $J_{AB} = 16.0$; $H_{(4)}$ and $H_{(5)}$, 4.83 and 5.40, $J_{4-5} = 8.0$; aromatic protons at δ 7.28. The acetonide group was removed by treating compound (VI) (2.7g) with 75% trifluoroacetic acid in methanol for 0.5h. The n.m.r. spectrum of the crude compound (VII) in $C^2H_3O^2H$ indicated removal of the acetonide protons and a concomitant upfield shift of the protons on $C_{(4)}$ and $C_{(5)}$ to 4.35 and 4.83, $J_{4-5} = 5.8$. Elimination of the *t*-butyl group and cyclization were achieved by treatment with trifluoroacetic acid at 0°C for 0.5h. Preparative t.l.c. gave 750mg (43%) of the major u.v.-absorbing component (R_F 0.35, solvent *h*), which was first methylated with diazomethane in ether-methanol (9:1, v/v; 5h) and then acetylated with acetyl chloride for 2h. The two major products were separated by preparative t.l.c. with solvent (*i*). The product subsequently assigned the structure (Xd) had R_F 0.56 (yield 41 mg); the one subsequently assigned the structure (Xa) had R_F 0.67 (yield 35 mg).

Spectral data for these two compounds are shown in Tables 1 and 2. Infrared spectra did not show sufficient differences in carbonyl frequencies to differentiate between the four possible isomers (Xa)–(Xd). However, since the n.m.r. spectrum of the component with R_F 0.56 shows long-range coupling, it must have either structure (Xa) or (Xd), and the mass and i.r. spectra (Rosenbrook & Carney, 1970) support the γ -lactone structure (Xd). This assignment is in agreement with the results from reduction in acetic acid (H_2 , 10% Pd on carbon), which shows, from the chemi-ionization mass spectrum of the product, the addition of 1 mol of H_2 and hydrogenolysis of the acetate group, which occurs more readily under these conditions when it is in a benzylic position (Augustine, 1965). The component of R_F 0.67, whose i.r. and mass spectra (Achenbach & Wittmann, 1970) support structure (Xa), absorbs 2 mol of H_2 , but does not lose the acetate group, a result only consistent with structure (Xa).

Characterization of oxaloacetate and pyruvate

Dnp-hydrazones of keto acids were prepared by adding an excess of Dnp-hydrazine as a saturated solution in 2M-HCl to solutions of the keto acids. After 1 h the Dnp-hydrazone derivatives were isolated by extraction with an equal volume of ethyl acetate, extraction from the ethyl acetate into 2% (w/v) $NaHCO_3$, acidification and re-extraction into ethyl acetate and separated by t.l.c. on cellulose (solvent *j*).

Identity of oxaloacetate and pyruvate Dnp-hydrazones, each of which separated into two components by t.l.c., was established by electrolytic reduction to the corresponding amino acid by the

method of Smith & Smith (1960). The amino acids produced were indistinguishable by two-dimensional t.l.c. (cellulose, solvents *k* and *l* and *m* and *n*, detection by ninhydrin) from both authentic amino acids and those obtained by this procedure starting with authentic pyruvate and oxaloacetate.

General methods

Enzyme preparations. The organism was grown and enzyme preparations were made as described by Jeffrey *et al.* (1972).

Lactic dehydrogenase (rabbit muscle, type I) was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. and alcohol dehydrogenase (yeast) from Boehringer Corp. (London) Ltd., London, W.5, U.K.

Thin-layer chromatography. The methods and solvents used were those described by Jeffrey *et al.* (1972); the support used was silica gel unless otherwise stated. The following additional solvents (by vol. unless otherwise indicated) were used: (*i*) benzene-dioxan-2-methylbutane-acetic acid (125:25:25:4); (*j*) butan-1-ol-ethanol-0.75M-(NH_4) $_2$ CO $_3$ -aq. 1.5M-NH $_3$ soln. (8:2:1:1); (*k*) ethanol-aq. NH $_3$ soln. (sp.gr. 0.88)-water (18:1:1); (*l*) phenol-aq. NH $_3$ soln. (sp.gr. 0.88)-water (160:1:40, w/v/v), used in the dark; (*m*) butan-1-ol-acetic acid-water (12:3:5); (*n*) butan-1-ol-pyridine-water (1:1:1); (*o*) light petroleum (b.p. 40–60°C)-diethyl ether-formic acid (80:20:1); (*p*) benzene-ethyl acetate (20:1).

Detection and measurement of radioactivity. Radioactive areas on two-dimensional t.l.c. plates were detected with Ilflex non-screen X-ray film (Ilford Ltd., Ilford, U.K.). Radioactive compounds were counted in a liquid-scintillation spectrometer, type NE 8304 (Nuclear Enterprises, Edinburgh, U.K.), with NE 220 scintillator fluid.

Nuclear-magnetic-resonance spectra. These were obtained on Varian 60, 100 or 200 MHz spectrometers in deuteriochloroform, except where otherwise stated. Line positions are given in δ relative to internal tetramethylsilane, and coupling constants are in Hz.

Mass spectra. Mass spectra were obtained on either an AEI MS902 or an LKB9000 mass spectrometer at 70 eV, by electron-impact or chemi-ionization with isobutane, as indicated.

Infrared spectra. These were obtained in chloroform on a Perkin-Elmer 421 spectrophotometer.

Results

Because of the sensitivity of dihydrogossypetin towards autoxidation, determinations of O_2 consumption and acid production during its enzymic degradation were made by preparing dihydrogossypetin *in situ* from taxifolin and taxifolin hydroxylase

and then adding dihydrogossypetin oxygenase. The enzyme fractions used were those eluted from DEAE-cellulose columns (Jeffrey *et al.*, 1972); dihydrogossypetin oxygenase was in 10% excess. With limiting taxifolin and an NADH-generating system, 2.2mol of acid was released/mol; with limiting NADH the release was 1.1mol/mol. Since taxifolin is monoanionic at the pH used, and 1mol of oxaloacetate was shown to be released, the final product must also be monoanionic. Preparation of dihydrogossypetin *in situ* with taxifolin hydroxylase, was accompanied by an O₂ uptake of 0.9mol/mol (Jeffrey *et al.*, 1972); the addition of dihydrogossypetin oxygenase, doubled the O₂ uptake to 1.8mol of O₂/mol of taxifolin. Although both enzymes required O₂ only the taxifolin hydroxylase needed a reduced nicotinamide nucleotide for activity.

The production of oxaloacetic acid from the A-ring of taxifolin was demonstrated by treating [4-¹⁴C]-(±)-taxifolin with crude cell-free extract. The system used was the taxifolin hydroxylase assay system scaled up 25-fold, with an alcohol dehydrogenase NADH-regenerating system and with the addition of 2mM-EDTA to stabilize the 273nm-absorbing products (see below). Progress of the reaction was followed spectrophotometrically and, when completed, the Dnp-hydrazones were prepared. Separation by t.l.c. gave compounds corresponding in *R_F* and colour, when sprayed with alkali, to oxaloacetate and pyruvate Dnp-hydrazones. The former was the major component. After rechromatography the specific radioactivities of the oxaloacetate and pyruvate Dnp-hydrazones were only 4.0% and 6.5% respectively of that which would have been observed had they included the C atom from position 4 of the taxifolin. The identity of the Dnp-hydrazones was confirmed by reducing them to amino acids.

To determine the stoichiometry of oxaloacetate and pyruvate production, spectrophotometric assays were used, based on the quantitative oxidation of NADH by these acids, catalysed by lactic dehydrogenase. The ratios of the two acids could be determined by the difference in rates of reaction; reduction of oxaloacetate was at only 4% of the rate with pyruvate. The reaction mixtures contained, in 2.4ml: potassium phosphate buffer, pH 7.7, 200μmol; NADH, 0.5μmol; EDTA, 5 or 50μmol; crude cell-free extract, 200μg of protein. After measurement of the absorption at 340nm, taxifolin (0.1ml, 0.15μmol) was added. This temporarily increased the extinction, but when all the taxifolin was metabolized the change in NADH concentration could be calculated since the products of degradation of the flavanonol did not absorb at 340nm. Lactate dehydrogenase (5μl, 8 units) was then added and the rate and total change in NADH concentration were measured. There was no initial rapid change in the absorbance, indicative of the absence of pyruvate, and the rate was identical

Table 1. Mass spectra of synthetic and enzymic products

All mass spectra were obtained by electron-impact at 70eV, except where stated. Brackets indicate transitions supported by metastable ions.

Compound	At 10eV		By chemi-ionization		(X _d)		A		B		C		E		F	
	(% of base peak)	(% of base peak)	(% of base peak)	(% of base peak)	(% of base peak)	(% of base peak)	m/e	% of base peak	m/e	% of base peak	m/e	% of base peak	m/e	% of base peak	m/e	% of base peak
m/e																
263	0.0	0.3	80	0.2	100		280*	23	322	30	322	59	324	25	326	11
262	0.0	0.2		0.1			262	1.3	262	100	262	9	264	7	266	45
203	4	13	100	7	68		167	75	234	75	209	15	223	4	264	19
202	25	86		15			166	15	219	23	167	80	223	5	268	35
174	27	42		3.0			165	10	167	17	156	21	210	9	207	80
156	17	67		82			139	23	149	17	149	31	209	13	175	25
149	1.0	3.0		48			124	3.7	139	11	139	24	167	100	167	19
114	100	100		98			114	100	114	64	144	100	166	40	152	40
107	4.0	2.0		20			86	43	85	26	85	42	151	50	151	100
86	22	1.0					156	43	83	38	83	58	139	22	97	28

* C₁₄H₁₆O₆ by peak matching.

Table 2. Proton-magnetic-resonance and infrared spectra

Proton-magnetic-resonance spectra of compounds (Xa) and (Xd) were run at 60 MHz, compounds B and C at 100 MHz, and compound A at 220 MHz in deuteriochloroform solution. Chemical shifts are expressed as δ (p.p.m.) relative to tetramethylsilane and coupling constants (J) in Hz with $m = \text{multiplet}$, $d = \text{doublet}$ and $s = \text{singlet}$. Infrared spectra were obtained in chloroform and values of the vinyl and carbonyl region are given as cm^{-1} . For mixture A the minor component (about 33%) absorbs at 4.17(s), 5.29(s) and 5.83(broad), together with corresponding signals in the aromatic and methoxyl regions (as judged from the integration).

Mixture A		(Xa)		Compound B		(Xd)		Compound C	
Group	δ	Group	δ	Group	δ	Group	δ	Group	δ
Proton-magnetic-resonance spectrum									
CH ₃ O-	3.87(s)	CH ₃ O-	3.76(s)	CH ₃ O-	3.74(s)	CH ₃ O-	3.83(s)	CH ₃ O-	3.80(s)
	3.83(s)				3.86(s)				3.86(s)
					3.87(s)				3.87(s)
Aromatic									
	6.79-								
	7.03(m)								
H ₍₂₎	5.22(s)	H ₍₂₎	5.34(s)	H ₍₂₎	5.34(s)	H ₍₂₎	4.88(d)	H ₍₂₎	5.42(s)
							1.1 Hz		
H ₍₄₎ and H ₍₅₎	4.53(d)	H ₍₄₎ and H ₍₅₎	5.62(d)	H ₍₄₎ and H ₍₅₎	5.64(d)	H ₍₄₎	5.20(dd)	H ₍₄₎ and H ₍₅₎	5.52(d)
	5.13(d)		5.68(d)		5.71(d)	H ₍₅₎	6.12(d)		5.68(d)
J ₄₋₅	9.0 Hz	J ₄₋₅	4.8 Hz	J ₄₋₅	5.2 Hz	J ₄₋₅	3.2 Hz	J ₄₋₅	2.5 Hz
Infrared spectrum									
	cm^{-1}		cm^{-1}		cm^{-1}		cm^{-1}		cm^{-1}
	1630, 1700		1625, 1640		1625, 1642		1637		1640
	1708(shoulder)		1705-15, 1740		1710-20, 1745		1700(broad)		1710-20, 1750

with that obtained for oxaloacetate under similar conditions. The result was 1.02 ± 0.035 s.d. (four determinations) mol of oxaloacetate/mol of taxifolin. It seems very likely that oxaloacetate is the product at this stage and that the pyruvate, found as its Dnp-hydrazone, arose by decarboxylation of oxaloacetate during the extraction procedure.

To investigate the fragments of the heterocyclic and B-rings it was convenient to treat taxifolin with crude cell-free extract in the presence of 2 mM-EDTA. Preliminary experiments showed that this stabilized a product with a pH-dependent absorption maximum at 273 nm. Acidification to pH 3.0 caused the spectrum to change to one with a small peak at 282 nm. Reaction mixtures contained, in 200 ml of 0.1 M-potassium phosphate buffer (pH 7.7), 400 μ mol of EDTA, 50 μ l (300 units) of alcohol dehydrogenase, 10 μ mol of NAD⁺ and 2 ml of crude cell-free extract containing 40 mg of protein. To this was added 5 mM-taxifolin in aq. 5% (v/v) ethanol, the concentration being maintained between 10 and 50 μ M and high local concentrations being avoided until either 100 μ mol had been added or the dihydrogossypetin oxygenase had become inactivated. The activity of these enzymes could be maintained for about 1 h provided that the substrate concentration was maintained at a suitable value: taxifolin hydroxylase assay systems were inhibited at taxifolin concentrations above 0.1 mM, whereas in the absence of taxifolin dihydrogossypetin oxygenase lost activity and dihydrogossypetin accumulated if more taxifolin was then added. Supply of O₂ became rate-limiting as more substrate was added; this was overcome by having a surface area of at least 40 cm²/100 ml, by stirring with a magnetic bar at the maximum rate possible without foaming, and by blowing compressed air over the surface of the solution. The progress of reactions was followed spectrophotometrically by repeat scans over the 300–350 nm region in a sample of the main reaction mixture. The solution was then acidified to pH 2.5 with 4 M-HCl and saturated with NaCl before extraction four times with 150 ml of ethyl acetate. The extract was dried over Na₂SO₄ and evaporated under reduced pressure at 30°C. From two experiments 45 mg of a catechol mixture was obtained, which could be separated analytically by two-dimensional t.l.c. on cellulose with solvents (a) and (g). Methylation of the catechols with diazomethane in 10% methanol in diethyl ether for 1 h gave one major u.v.-absorbing band (A) by t.l.c. (solvent *h*, *R_F* 0.35), which was eluted with 5% methanol in diethyl ether and which crystallized on storage at –25°C. Treatment of 1 mg of material A in 0.5 ml of ethanol with 0.5 ml of 20% (w/v) KOH for 1 h at 120°C gave veratraldehyde (*R_F* 0.29, solvent *o*), and as its Dnp-hydrazone *R_F* 0.62, solvent *p*), and veratric acid (*R_F* 0.25, solvent *o*, and *R_F* 0.46, solvent *k*). The latter when methylated was identical with methyl veratrate

in its retention time on g.l.c. and subsequent mass spectrum. The u.v. spectrum of material A had $\lambda_{\max}$ at 231, 279 and 285 (shoulder) nm, and its i.r. spectrum in CHCl₃ showed peaks at 1630, 1700 (shoulder) and 1708 cm^{–1} [cf. Achenbach & Wittmann (1970); Kimura *et al.* (1971)]. The mass spectrum (Table 1) showed the molecular ion at *m/e* 280, which was peak-matched as C₁₄H₁₆O₆. The major fragment ion (*m/e* 114, 100%) resulted from a retro-Diels–Alder fragmentation, similar to the dihydropyrone isolated by Achenbach & Wittmann (1970). The n.m.r. data (Table 2) suggest two compounds, although these could not be resolved by t.l.c. However, after acetylation with acetyl chloride it was resolved into two components by t.l.c. in solvent (*i*); compound B (crystalline), with *R_F* 0.62 (yield 18 mg), and compound C (oil), with *R_F* 0.57 (yield 12 mg). The two compounds could also be separated by g.l.c. on 4% SE30 at 220°C; compound B had a retention time of 9.0 min and compound C 8.5 min. From the mass-spectral data presented in Table 1 and the n.m.r. and i.r. data in Table 2, compound B appears to be the 3',4'-dimethoxy analogue of compound (Xa), and the absence of long-range coupling of the vinyl proton in compound C indicates that it is the analogue of either compound (Xb) or (Xc). The i.r. data (cf. Casnati *et al.*, 1966) and the peak at *m/e* 209 in the mass spectrum suggest the five-membered ring system of (Xc). Both compounds B and C, in ethanolic solution, were reduced with H₂ and palladium on charcoal for 1 h, yielding compounds D and E respectively; these were purified by t.l.c. From the mass spectra of the products, both absorbed 1 mol of H₂. Compound E still fragmented to give an ion at *m/e* 209, suggestive of a five-membered ring. Compound D was further reduced with H₂ and palladium on charcoal in acetic acid, where it absorbed an additional 1 mol of H₂ but did not lose the acetic group by hydrogenolysis, yielding compound F, the 3',4'-dimethoxy analogue of compound (XIa). This confirmed the identity of compound B as the 3',4'-dimethoxy analogue of compound (Xa).

Discussion

The present investigation has established the structure of the products formed during bacterial oxidation of dihydrogossypetin. The identification of oxaloacetate from positions C-5 to C-8 of dihydrogossypetin suggests why neither Watanabe (1959) nor, in the further investigations of the problem, Das & Griffiths (1969) and Das & Sothy (1971) were able to detect anything other than CO₂ from C-5 to C-8: oxaloacetate formed in the gut of an animal would be rapidly further metabolized.

Identification of the catechol components produced after loss of oxaloacetate was a considerably

Scheme 2. *Proposed scheme for enzymic oxidation of dihydrogossypetin*

R = 3,4-Dihydroxyphenyl; R' = 3,4-dimethoxyphenyl.

more formidable problem. The initial mixture of compounds extracted from the incubation could not be resolved on a preparative scale. However, after methylation a purified fraction (A) was obtained by t.l.c. The mass spectrum of fraction A indicated a molecular formula of $C_{14}H_{16}O_6$, which by n.m.r. appeared to consist of a mixture of two isomers. The most likely structures for these compounds seemed to be trimethylated derivatives of the proposed phenylvalerolactone (Scheme 2). Since Herbst *et al.* (1959) showed that 2,4-pyroneones can be methylated at the enol oxygen of either carbonyl and since Evans *et al.* (1969) demonstrated that ring contraction of lactone esters occurs, four possible structures (3',4'-dimethoxy analogues of compounds Xa–Xd) must be considered. After acetylation of the mixture A, two isomers, B and C, were separated, and their structures assigned by unambiguous synthesis of compounds (Xa) and (Xd). The known *trans*-disposition of the phenyl and hydroxyl groups is in agreement with the J_{4-5} of 9.0 Hz observed in the major component of mixture A and contrasts with 2.3 Hz for a similar ring system reported by Achenbach & Wittmann (1970), where the stereochemistry was assigned as *cis*. The n.m.r. spectrum of compound B, however, shows that acetylation caused a large decrease in the coupling constant to 5.2 Hz, which is probably a reflection of both steric and polar effects. Both i.r. and mass spectra of compound B agree with the proposed structure, the retro-Diels–Alder reaction being the major process (cf. Achenbach & Wittmann, 1970). The structure of compound C is more difficult to establish. The absence of long-range

coupling and the i.r. spectrum tend to eliminate the γ -lactone as a possibility, whereas the presence of an ion at m/e 209 suggests a five-membered ring. The two possibilities (3',4'-dimethoxy analogues of Xb or Xc) cannot be rigorously differentiated with the information available, though the occurrence of an i.r. peak at 1710cm^{-1} suggests 3,4-dimethoxy-(Xc) rather than 3,4-dimethoxy-(Xb) (Casnati *et al.*, 1966). However, the course of the enzymic ring-cleavage is completely defined.

Presumably the metabolism of dihydrogossypetin proceeds via an initial oxidative ring-fission, although no evidence for the product has been obtained, followed by a hydrolase-type reaction to yield oxaloacetic acid (Scheme 2) and 5-(3,4-dihydroxyphenyl)-4-hydroxy-3-oxovalero- δ -lactone. $^{18}\text{O}_2$ experiments would be valuable to clarify the details of the reaction.

The importance of dihydropyroneones as microbial metabolites has only recently been established (e.g. Achenbach *et al.*, 1971; Raistrick & Rice, 1971; Kimura *et al.*, 1971; Rosenbrook & Carney, 1970). Certain of these compounds are weak antibiotics or gibberellin synergists. The biological properties of the lactones described in this paper have not been investigated.

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