GIBBERELLINS AND SUBSTANCES RELATED TO THEM

II. The Isolation of Some Metabolites of Fusarium moniliforme

E. P. Serebryakov, V. F. Kucherov, and G. S. Muromtsev

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We have previously studied the qualitative composition of the gibberellins and gibberellin-like substances produced by some strains of Fusarium moniliforme in various media and have also carried out a group separation of the metabolites into acidic and neutral fractions (see Communication I). We have used the culture liquid of strain F-6 grown on a medium containing sunflower oil and ammonium nitrate for a more detailed analysis of the metabolite composition.

The mixture of neutral substances was chromatographed on a column of neutral alumina which led to the isolation first of the known 12-epi-(−)-manoyl oxide [1], and then β-sitosterol contaminated with sitostanol. For identification, the sample obtained was oxidized by the Oppenauer method which enabled the proportion of sterols in the mixture to be evaluated from the proportion of ketones formed [2]. It was shown by thin-layer chromatography that β-sitosterol is included in the neutral fraction of the initial nutrient medium.

The acidic products extracted with ethyl acetate from the bicarbonate solution acidified to pH 2.5 were chromatographed on a column of silica gel by a method described previously [3]. This yielded the known fujenal [1], and then amorphous or semicrystalline fractions containing gibberellin A_9, a mixture of gibberellins A_4 and A_7, and a mixture of gibberellins A_3 and A_1 with the 1, 3-lactone of 2β-3α, 6α-trihydroxy-1β-methyl-8-methylene-Δ^4 gibbene-1d, 10β-dicarboxylic acid (see Communication III). Gibberellenic acid was found in the later fractions. On referring the weight of the chromatographically purest fractions to the volume of the initial culture filtrate it was shown that it contained about 5 mg/*l* of a mixture of gibberellins A_4 and A_7, and nearly 650-680 mg/*l* of gibberellins A_3 and A_1 and the 1, 3-lactone isomeric with gibberellin A_3 combined. Judging from the value of $[\alpha]_D$ of the last fraction, its content of gibberellin A_1 was low.

R_f Values of the Gibberellins and Accompanying Substances in a Thin Layer of Adsorbent in Standard Solvent Systems

Substance	Solvent system and R_f				
	on KSK silica gel			on Al_2O_3	
	1	2	3	3	4
1, 3-Lactone	0.01	0.40	0.00	0.00	0.00
Gibberellins A_1 and A_3	0.01	0.40	0.00	0.00	0.00
Gibberellins A_4 and A_7	0.11	0.60	0.03	0.00	0.00
Gibberelins A_9	0.60	1.0	0.32	0.00	0.00
n-β-Sitosterol	0.55	0.95	0.48	0.18	0.02
13-Epi-(−)-manoyl oxide	1.0	1.0	0.70	0.95	0.53
Fujenal	0.68	1.0	0.85	0.05	0.00

Note. Systems: 1) Benzene-CH_3COOH-water (8:3:5), upper phase; 2) chloroform-CH_3OH (92:8); 3) benzene-ethyl acetate (9:1); 4) heptane-toluene (4:1). The spots of gibberellins A_4 and A_7 are partially separated when the run is repeated four times.

For the present work, the qualitative analysis of mixtures of gibberellins and gibberellin-like substances was carried out not only by paper chromatography but also by chromatography on a thin free layer of silica gel (cf. [4-6]). In a comparison of the mobilities of the biologically active components on paper and on silica gel it was found that the sequence of their migration was the same; it was possible to separate the neutral substances from the acidic substances by means of alumina (table). Thin-layer chromatography did not enable gibberellins A_3 and A_1 and the 1, 3-lactone of 2β, 3α, 7α-trihydroxy-1β-methyl-8-methylene-Δ^4-gibberene-1α, 10β-dicarboxylic acid isomeric with gibberellin A_3 to be separated, but it was very convenient for the group separation of the gibberellins and also for the analysis of the components of low polarity having high R_f values on paper (see table).

Experimental

Silica gel of grade KSK free from traces of iron and washed to complete neutrality (150-250 mesh for plates and 60-100 mesh for columns) and neutral alumina [Brockmann activity grade III/IV (150-250 mesh)] were used for the

chromatography. The silica gel plates were developed with 70% sulfuric acid and the alumina plates with iodine vapor. The thin layer chromatography on plates and column chromatography on buffered silica gel were carried out by the procedure developed previously [3]. All melting points were determined on a Boetius stage and the angles of rotation in ethyl acetate in a Hilger polarimeter.

Separation and isolation of metabolites of the F-6 strain. A filtrate of the culture liquid (6 *l*) was extracted with pH 2.5 with two 600-ml portions of butan-1-ol. The extract was concentrated under vacuum to a volume of 180 ml (40°C, 25 mm) and was treated with a 5% solution of $KHCO_3$ (6 × 150 ml). The bicarbonate extract was shaken with benzene (2 × 200 ml) and ethyl acetate (2 × 200 ml). The extracts were combined with the butanol layer, and washed with small portions of water; the water washings were combined with the bicarbonate extract.

The combined organic extracts containing the neutral substances were evaporated under vacuum (40°C, 25 mm) until a dark viscous residue was obtained. This was treated with 100 ml of ether. The amorphous flocs which deposited on standing were filtered off and the filtrate was concentrated under vacuum. This gave a brown resin (1.493 g) which was chromatographed on a column containing 80 g of alumina, 150-ml fractions being collected. Elution with heptane gave 30 mg of crystals with mp 96-98°C (from methanol), $[\alpha]_D^{20}$ −38° (c 0.45). IR spectrum ν_{CHCl_3}: 3060, 1640, 1625, 1461, 1370, 1095, 1058, 966, 926, 909, 842 and 789 cm^{-1}.

Found, %: C 82.45; H 11.80. Calculated for $C_{20}H_{34}O$, %: C82.69; H 11.80.

Literature data for 13-epi-(−)-manoyl oxide: mp 96-98.5°C, $[\alpha]_D$ −37° [1].

Elution with a mixture of heptane and benzene (4:1) gave 162 mg of a crystalline mixture of two substances difficult to separate; elution with benzene gave 45 mg of a semicrystalline mass containing four substances; and elution with a mixture of benzene and ethyl acetate (95:5) gave 223 mg of a crystalline substance. After purification on a preparative alumina plate (24 × 22 × 0.2 cm) in the benzene-ethyl acetate (88:12) system, crystals were obtained with mp 138-140°C (from methanol), $[\alpha]_D^{20}$ −27° (c 2.78). IR spectrum, ν_{KBr}: 3386, 2924, 1471, 1370, 1185, 1125, 1056, 1019, 950, 878, 836 and 798 cm^{-1}.

Found, %: C 83.77; H 11.93; mol. wt. (by mass spectrometry) 426. Calculated for $C_{30}H_{52}O$, %: C 84.04; H 12.23, mol. wt. 428.7. Calculated for $C_{30}H_{50}O$, %: C 84.44; H 11.81; mol. wt. 426.7.

Acetate, mp 124-126°C (from ethyl acetate), $[\alpha]_D^{20}$ −24.8° (c. 085; in chloroform). Literature data for β-sitosterol: mp 136-137°C, $[\alpha]_D$ −35°; for β-sitosterol acetate: mp 125°C, $[\alpha]_D$ −35°[7].

The sample of β-sitosterol that we had obtained (25 mg) was oxidized by the Oppenauer method and the resulting mixture of ketones was chromatographed on a column of alumina. This yielded 3 mg of sitostanone and 6.5 mg of Δ^4-sitosten-3-one, the constants of which (mp, $[\alpha]_D$, λ_{max}, and ε) agreed with those given in the literature [2].

The bicarbonate extract containing the acidic products was acidified to pH 5.5 and concentrated under vacuum (40°C, 25 mm) to a volume of 120 ml. The concentrate was acidified to pH 2.5 and extracted with ethyl acetate (7 × 100 ml). The extract was filtered from resinous impurities, washed with water, and dried under vacuum (40°C, 25 mm). This gave 6.456 g of solid residue.

Half this amount (3.230 g) was chromatographed on a column containing 160 g of silica gel treated with phosphate buffer at pH 6.4 by the method described previously [3]. Elution was carried out with chloroform and mixtures of chloroform and ethyl acetate with a gradually increasing content of the latter. The chloroform elution gave 178 mg of crystals with mp 167-169°C (methanol and ethyl acetate-hexane) $[\alpha]_D^{20}$ −74° (c 1.05). With concentrated sulfuric acid, the substance gave a purple coloration. IR spectrum, ν_{CHCl_3}: 2730, 1868, 1785, 1724, 1663, 1461, 1386, 1004, 885, 864 cm^{-1}. Literature data for fujenal: mp 168-170°C, $[\alpha]_D$ −74° [1].

On further elution with chloroform and with a mixture of chloroform and ethyl acetate (95:5), 47 mg of a solid product was obtained. After purification on a preparative plate of silica gel (24 × 12 × 0.2 cm) in the chloroform-methanol (97:3) system, 15 mg of a semicrystalline mass with $[\alpha]_D^{22}$ −19° (c 0.42) was obtained. From its R_f values in all the systems, this substance was identical with an authentic sample of gibberellin A_9, the literature data for which are mp 208-210°C, $[\alpha]_D$ −22° [1]. Elution with a mixture of chloroform and ethyl acetate (85:15) gave 264 mg of a noncrystallizing solid with $[\alpha]_D^{21}$ +13° (c 0.92). From its R_f values in all systems, this was identical with authentic samples of A_4 and A_7.

The magnitude of $[\alpha]_D$ indicates the predominance of gibberellin A_7 in this product. On elution with a mixture of chloroform and ethyl acetate (2:3) 1.973 g of a solid residue with $[\alpha]_D^{20}$ +77° (c 1.17) was obtained. From its R_f values in all systems, this product was identical with an authentic sample of gibberellin A_3; on paper chromatography in the butan-1-ol-1.5 N ammonia system, the 1, 3-lactone of 2β, 3α, 7α-trihydroxy-1β-methyl-8-methylene-Δ^4-gibbene-1α-10β-dicarboxylic acid was detected in the product.

Summary

1. Column and thin-layer chromatography have been used to analyze the metabolite composition of the culture liquid of Fusarium moniliforme.

2. On fermentation in a medium containing sunflower oil and ammonium nitrate, the content of gibberellins of the A_3-A_1 group was 650-680 mg/*l*, of the gibberellins of the A_4-A_7 group 85-90 mg/*l*, and of gibberellin A_9 about 5 mg/*l*.

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Zelinskii Institute of Organic Chemistry, AS USSR

All-Union Scientific Research Institute for Phytopathology