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GIBBERELLINS AND SUBSTANCES RELATED TO THEM

V. Isolation of Gibberellins and Substances Related to Them from Wastes from the Industrial Biosynthesis of Gibberellin

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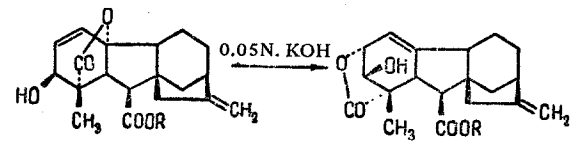
Production of individual gibberellins in preparative amounts and also the isolation and purification of the metabolites accompanying them is an urgent methodical task in the chemistry of the gibberellins. Various modifications of adsorption and partition column chromatography are usually used for its solution.

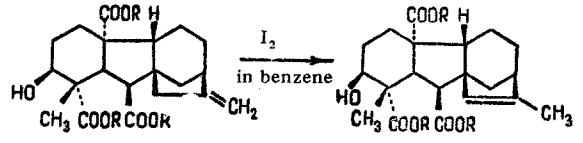
Our method for isolating the individual gibberellins by the chromatography of an industrial preparation on a column of KSK silica gel buffered at pH 6.2 [1] has been applied to the separation of the gibberellins contained in the waste resin from factory production. This resin consists of the evaporated ethyl acetate mother solution from the crystallization of the gibberellins preparation obtained under factory conditions from the PG-7 strain on Fusca's medium [2].

By the chromatography of the acid fraction of this resin we have isolated: fujenal; a substance A with 202-203° C; a hydroxylactone acid (II) isomeric with gibberellin A_7 (I); gibberellin A_4 (V); giberellin A_7 (I); a substance B with mp 145-147° C; gibberellin A_{13} (III); and azelaic acid. In addition we have obtained fractions in which spots of gibberellins A_3 and A_9 and also a number of unknown components have been detected by thin-layer chromatography. The separation of gibberellins A_4 (V), A_7 (I), and the hydroxylactone acid (II) isomeric with A_7 is very difficult because of the closeness of their physicochemical properties and was carried out previously by repeated chromatography on columns of various adsorbents [3,4]. The degree of difficulty of the isolation of a particular gibberellin depends in each case on the qualitative composition of the initial mixture and the quantitative ratio of its components. However, in our case even a single chromatographic separation led to the isolation of the compounds mentioned, which shows the effectiveness of a column with buffered KSK silica gel.

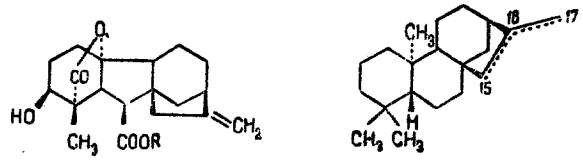
The fujenal was identified by comparison with a specimen obtained previously [5]. The constants of substance A (mp 202-203° C, $[\alpha]_D$ −84°) agree with those of allogibberic acid. However, the IR spectra of these substances are different and a mixture of them gives a depression of the melting point. Gibberellins A_7 (I) and A_4 (V) were identified on the basis of their constants and the IR spectra of the acids and methyl esters, and also by the biotest (active in cucumber). The hydroxylactone acid (II) isomeric with gibberellin A_7 is, like the corresponding isomer of gibberellin A_3, an artefact formed in fermentation [4]. Since the chromatographic behavior and the constants of the substance that we had isolated differed from those described in the literature [4], we decided to identify it chemically. The isomerization of the methyl ester of A_7 (Ia) in an alkaline medium by a known method [4] gave a product identical in respect of its constants and IR spectrum with the methyl ester of the hydroxylactone acid that we had isolated (IIa). Isomerization was accompanied by the appearance in the IR spectrum of the lactone ester (IIa) of a strong absorption band at 960 cm^{-1} which is absent from the spectrum of the methyl ester of A_7 (Ia).



I. R=H; Ia. R=CH$_3$; A_7 II. R=H; IIa. R=CH$_3$



III. R=H; IIIa. R=CH$_3$; A_{13} IV. R=H; IVa. R=CH$_3$



V. R=H; Va. R=CH$_3$; A_4 VI. Δ^{16}; VII. Δ^{17}

Substance B issued from the colum before the fractions containing gibberellins A_3 and A_{13}. Less than 2 mg of this substance was obtained, and therefore it was possible to characterize it only by the UV spectrum. Then gibberellin A_{13} with mp 194–196° C and $[\alpha]_D$ −45° was isolated. The action of diazomethane on gibberellin A_{13} gave two crystalline products: (a) with mp 153–158° C, $[\alpha]_D$ −50.8°, and (b) with mp 120–122° C, $[\alpha]_D$ −39.0°. It was found by the Zeisel-Zabrodina method that the methyl ester (b) contained three methoxy groups. An isomer of the methyl ester of gibberellin A_{13} (IIIa) is known [6] in which the double bond has shifted into ring D (IVa). According to the literature, the methyl ester of IIIa has mp 117–119° C, while its isomer (IVa), obtained by the action of sulfuric acid in acetone on the ester IIIa, melts at 121–122° C.

Since the constants of our esters did not agree with those given in the literature, we performed the isomerization of the ester (a) in benzene solution by the method [7] proposed for the conversion of kaurene (VI) into isokaurene (VII). In an analysis of the ester (a) by gas-liquid chromatography, in addition to the main peak, a peak with a shorter retention time was found. The product of the isomerization of the ester (a) corresponded to the substance with the shorter retention time, and the main peak of the initial ester had decreased. Thus, the ester (a) contains an exo-methylene group and, like kaurene (VI), issues from the column later, and the ester (b) possesses an endocyclic double bond and, like isokaurene (VII) is characterized by a shorter retention time. Consequently, the ester (a) may be ascribed structure (IIIa) and the ester (b) structure (IVa). The two esters have the same molecular weight (120, mass spectrometry) and peaks corresponding to the detachment of three methoxycarbonyl groups.

The fractions eluted from the column with ethyl acetate (on standing) yielded 2.5 mg of azelaic acid.

The method of partition column chromatography which we used for the separation and isolation of the gibberellins has proved to be more effective than the method using adsorption columns [3,4]. We have shown previously that different ratios between the neutral diterpene metabolites elaborated by F. moniliforme (strain F-6, laboratory fermentation) arise according to the conditions of its cultivation, the relative stimulation of the biosynthesis of the gibberellins on a medium containing fats correlating with the relative suppression of the biosynthesis of highly oxidized neutral diterpenoids (7β-hydroxykaurenolide, 7β,18-dihydroxykaurenolide, and fujenal). Having available the resins obtained by the industrial fermentation of the PG-7 strain on two media—Fusca's carbohydrate medium [8] and a medium containing whale oil (source of C) and ammonium nitrate (source of N)—we decided to make a comparison of the metabolite composition of the neutral fractions of these resins and to determine whether the correlation between the relative stimulation of the biosynthesis of the gibberellins and the relative suppression of the biosynthesis of highly oxidized diterpenoids found previously is possible.

The scheme for the isolation and purification of the gibberellin samples was such that the neutral metabolites passed from the culture liquid into the resin without appreciable losses. We analyzed the resins obtained under similar technological conditions of the purification and the isolation of gibberellic acid, and therefore the ratio of the neutral metabolites in the source used by us probably does not differ very markedly from their true ratio in the culture liquid.

In the resin obtained in the cultivation of the industrial PG-7 strain on Fusca's medium (7–8 days) we found kaurene (VI), epimanoyl oxide, 7β-hydroxykaurenolide, 7β,18-dihydroxykaurenolide, and fujenal; in the resin isolated from the fermentation of the same strain on a medium containing whale oil and ammonium nitrate (12–14 days) we found kaurene, epimanoyl oxide, kauran-16α-ol, fujenal, and traces of 7β,18-dihydroxykaurenolide. No 7β-hydroxykaurenolide was found in this medium. The content of kaurene was appreciably higher in the medium containing whale oil; it is an indicative fact that only in this medium was kauran-16α-ol found. The contents of fujenal in both types of media was fairly high. There was no point in giving a quantitative evaluation of the contents of diterpene metabolites in the resin, since the latter may contain various foreign substances (for example, unassimilated components of the whale oil) as inert ballast. In a qualitative comparison of the metabolite composition of the two resins the same pattern is found as in a comparison of media 1 and 2 (see part IV) [9].

Thus, in the case of the PG-7 strain there is the same correlation between the yield of gibberellins and the concentration of kaurene (VI) and of highly oxidized diterpenes (kaurenolides and fujenal) as in the case of the F-6 strain. A medium with a substrate which is assimilated gradually (whale oil) promotes the formation of a large amount of gibberellins and is unfavorable for the biosynthesis of highly oxidized diterpenes. Conversely, Fusca's carbohydrate medium stimulates the formation of the latter which, to some extent, weakens the metabolic flow in the direction of the biosynthesis of the gibberellins.

Experimental

Acid metabolites of the PG-7 strain (Fusca's medium). The resin was separated into an acidic and a neutral fraction by the method described previously [5]. The acidic products were evaporated to constant weight and 10.5 g of the acid fraction of the resin was transferred to a column of 500 g of silica gel previously treated with phosphate buffer having

pH 6.4. Elution was carried out first with mixtures of petroleum ether and chloroform and then with mixtures of chloroform and ethyl acetate, the content of the more polar component being increased gradually from 0 to 100%. Fractions of 250 ml were collected. Elution with petroleum ether (fractions 1–7) gave 110 mg of a yellow-green oil.

On further elution with petroleum ether, fractions 8–10 yielded 130 mg of a crystalline product with mp 154–156° C which, after purification, was identified as fujenal (mp 167–169° C).

Fractions 11–20 (10% of chloroform, 65 mg) gave resins in which the main component was fujenal.

Fractions 21–33 (20% of chloroform, 13 mg) consisted of a chromatographically inhomogeneous resin.

Fractions 34–50 (20–70% of chloroform, 65 mg) consisted of a resin containing a small amount of fujenal and a number of unidentified components.

Fractions 51–54 (80–90% of chloroform, 120 mg) formed a resin. By thin-layer chromatography (TLC) a component with a bright "gibberellin" fluorescence in UV light after the plate had been kept in sulfuric acid vapor was found (R_f, 0.68 in the benzene–ethyl acetate (9 : 1) system; A_9 has R_f 0.40 on this system).

Fractions 55–72 (90% of chloroform in petroleum ether–20% of ethyl acetate in chloroform, 530 mg) formed a resin which was rechromatographed on a column with buffered KSK silica gel. Elution with mixtures of petroleum ether and chloroform yielded 276 mg of an oil which, according to TLC, contained gibberellin A_9. Further elution with mixtures of chloroform with 10 and 15% of ethyl acetate gave 88 mg of an oil. On standing it partially crystallized. The product was washed and crystallized from acetone and petroleum ether. This gave 27 mg of crystals (substance A) with mp 202–203° C, $[\alpha]_D^{23}$ −83.8° (c 1.0; methanol). IR spectrum, ν_{KBr}: 3380 cm^{-1}, 2950, 1710, 1690, 1130, 985 and 880 cm^{-1}; no absorption maxima were found in the UV spectrum. On a plate, the substance gave no coloration with H_2SO_4, fluoresced very feebly in UV, and was shown up poorly by iodine.

Fractions 73 and 74 (20% of ethyl acetate, 170 mg) gave a noncrystallizing oil. The main component, judging from chromatographic data, was the isomer A_7.

Fraction 75 (25% of ethyl acetate, 180 mg) was an oil which partially crystallized on standing. The crystals were washed and recrystallized from acetone and petroleum ether, mp 212–215° C, $[\alpha]_D^{23}$ +38° (c 1.0; methanol).

Found, %: C 69.05; H 6.75. Calculated for $C_{19}H_{22}O_5$, %; C 69.01; H 6.7; mol. wt. 330.4.

The methyl ester had mp 214–215° C, $[\alpha]_D^{23}$ +70° (c 1.0; methanol). Literature data for the 1,3-lactone (II)—mp 186–190° C, $[\alpha]_D^{20}$ +59°; for the methyl ester (IIa): mp 226–228° C; $[\alpha]_D^{20}$ +78°. The methyl ester of A_7 (Ia) (mp 165–167° C; $[\alpha]_D^{20}$ +32°) was subjected to alkaline isomerism by a known method [4]: 16 mg of Ia was dissolved in 3 ml of methanol and the solution was shaken with 3.3 ml of a 0.05 N solution of KOH for 3 hr. Water was added to the flask until an opalescence appeared. The methanol was distilled off and the small crystals that had deposited were filtered off and dried. This gave 7.3 mg of crystals with mp 213–214° C, $[\alpha]_D^{22}$ +70°. The IR spectrum (KBr) of the methyl ester of the product that we had isolated and of the isomer obtained from the methyl ester of A_7 (Ia) completely coincided (ν_{KBr}, 3490 cm^{-1}, 1775, 1710, 1670, 960, 868 and 835 cm^{-1}). A mixture of the isolated ester and the isomerized ester gave no depression of the melting point.

Fraction 76 (25% of ethyl acetate, 150 mg) consisted of a resin two crystallizations of which from acetone and petroleum ether yielded 22 mg of crystals with mp 185–187° C, $[\alpha]$ +18.4° (c 1.0; methanol). When run on a plate five times [upper phase of the benzene–acetic acid–water (8 : 3 : 5) system], the substance gave a spot at the level of gibberellin A_4 and a spot at the level of gibberellin A_7 (isomer A_7).

Fraction 77 (25% of ethyl acetate, 150 mg) partially crystallized on evaporation. After two recrystallizations from a mixture of acetone and petroleum ether, 42 mg of gibberellin A_4(V) with mp 213–214° C, $[\alpha]_D^{22}$ −19.8° (c 1.01; methanol) was obtained; the methyl ester had mp 176–178° C, $[\alpha]_D^{22}$ −6.7° (c 0.75; methanol).

Fractions 78–80 (25% of ethyl acetate, 250 mg) consisted of an oil which partially crystallized on standing. After two recrystallizations of the washed crystals from acetone–petroleum ether, 51 mg of crystals was obtained with mp 205–208° C and $[\alpha]$ −18° (c 1.0; methanol). On distribution in a thin layer of KSK [benzene–acetic acid–water 8 : 3 : 5) system], spots were obtained at the level of gibberellins A_4 and A_7.

Fractions 81–83 (25% of ethyl acetate, 250 mg) gave a resin containing gibberellin A_7 as the main component.

Fractions 84–87 (30% of ethyl acetate, 350 mg) formed a semicrystalline product. After two recrystallizations from acetone and petroleum ether, 66 mg of crystals was obtained with mp 174–176° C, $[\alpha]_D^{23}$ +28° (c 1.0; methanol); the methyl ester had mp 165–167° C, $[\alpha]_D^{23}$ +32° (c 0.85); TLC, biotests, and the mass spectrum of the methyl ester showed that this substance is gibberellin A_7 (I).

Fractions 88–90 (40–50% of ethyl acetate, 290 mg), and fractions 91–95 (50 and 60% of ethyl acetate, 690 mg) consisted of inhomogeneous noncrystallizing oils.

Fractions 96–98 (70% of ethyl acetate, 440 mg) gave 1.7 mg of crystals (substance B) with mp 145–147° C (viscous melt liquifying at 198–204° C). IR spectrum, ν_{KBr}: 3400–3100 cm^{-1}, 2940, 2870, 1730, 1705, 1425, 1375, 1360, 1300, 1240, 1220, 1130, 1070, 985, 890 and 820 cm^{-1}.

Fractions 99–102 (80 and 90% of ethyl acetate, 2.0 g) partially crystallized on standing. Recrystallization from acetone–petroleum ether gave about 500 mg of crystals with mp 194–196° C $[\alpha]_D^{23}$ −45° (c 1.3; methanol).

Found*, %: C 62.6; 62.6; H 6.7; 6.8. Calculated for $C_{20}H_{26}O_7$, %: C 63.5; H 6.9; mol. wt. 378.

Treatment with diazomethane and fractional crystallization yielded crystals (a) with mp 153–158° C, $[\alpha]_D^{20}$ −50.8°; IR spectrum, ν_{KBr}: 3460 cm^{-1}, 1730, 1701, 1665, 885 and 825 cm^{-1} (b) with mp 120–122° C, $[\alpha]_D^{20}$ −39°; IR spectrum, ν_{KBr}: 3520 cm^{-1}, 1745, 1720, 1665 and 825 cm^{-1}.

Found, %: C 65.53; H 7.91; OCH_3 22.03. Calculated for $C_{23}H_{32}O_7$; %: C 65.7; H 7.7; 3 OCH_3 22.1.

It was shown by gas-liquid chromatography that when a benzene solution of the ester (a) was heated in the presence of iodine [8], the ester (b) was formed. This gives grounds for considering that the ester (a) has the structure IIIa and the ester (b) the structure IVa. The mass spectra of the two esters differed only in the region of ions with low mass numbers. The main ions were: M 420, m/e 402 (M−H_2O), 388 (M−CH_3OH), 360 (M−$HCOOCH_3$), 328 (M−CH_3OH−$HCOOCH_3$), 300 (M−2$HCOOCH_3$), 268 (M−CH_3OH−2$HCOOCH_3$), 222 (M−3$HCOOCH_3$−H_2O).

Fractions 103–107 (90–100% of ethyl acetate, 113 mg) gave a resin from which, on standing, 2.5 mg of crystals with mp 104–107° C deposited. The substance was identified as azelaic acid (IR spectrum and mixed melting point).

Analysis of the neutral metabolites of the PG-7 strain. Medium 1 (Fusca's medium). The neutral fraction obtained from 15 g of industrial resin deposited 1 g of fujenal on standing in the refrigerator, and after recrystallization from a mixture of ethyl acetate and hexane this had mp 167–169° C and $[\alpha]_D^{20}$ −72°. The remainder of the neutral fraction (2.40 g) was chromatographed on 120 g of alumina (activity grade III/IV), 100-ml fractions being collected.

Fractions 1–5 (heptane) gave 60 mg of an oil from which, after rechromatography on 5 g of alumina (activity grade II) was obtained 11 mg of a mixture of kaurene (VI) and isokaurene (VII) in a proportion of 45 : 55 (by gas-liquid chromatography), mp 50–51.5° C (from methanol), $[\alpha]_D^{22}$ −62° (c 0.70; chloroform). IR spectrum: strong band at 820 cm^{-1}.

Fractions 6–15 [heptane and a mixture of heptane with benzene (9 : 1)] consisted of 29 mg of a colorless resin; the resin was filtered through 1 g of alumina (activity grade II), giving 6 mg of epimanoyl oxide with mp 92–97° C (from methanol), identical with the sample described previously [5].

Fractions 16–41 (from 30% of benzene in heptane up to 10% of ethyl acetate in benzene) were chromatographically very inhomogeneous.

Fractions 42–47 (20 and 30% of ethyl acetate in benzene) consisted of 24 mg of a semicrystalline mass; by chromatography on a preparative silica gel plate we isolated from it 7β-hydroxykaurenolide with mp 183–187° C (from a mixture of hexane and ethyl acetate), identical with the sample described previously [8].

Fractions 51–57 (50% of ethyl acetate in benzene gave 197 mg of a semicrystalline residue; by chromatography on a preparative silica gel plate we isolated from it 47 mg of pure 7β,18-dihydroxykaurenolide with mp 219–221° C (from a mixture of hexane and ethyl acetate), $[\alpha]_D^{20}$ −40° (c 0.60; methanol) identical with the sample described by Fusca et al. [8]. Further elution with more polar mixtures led to a resin not susceptible to working up.

Medium 2 (whale oil and ammonium nitrate). On standing in the refrigerator, the neutral fraction obtained from 28.5 g of industrial resin deposited 605 mg of crystals with mp 156–162° C; recrystallization from methanol gave pure fujenal with mp 167–169° C, identical with that described previously [5]. The neutral resin (4.50 g) remaining after the isolation of the fujenal was chromatographed on 220 g of silica gel, 200-ml fractions being collected.

Fractions 1–3 (hexane) gave 105 mg of a semicrystalline residue from which, after rechromatography on alumina and crystallization, 35 mg of pure kaurene (VI) with mp 48–50° C (from methanol), $[\alpha]_D^{20}$ −77°, was isolated.

Fraction 5 (hexane) yielded 22 mg of an oil from which, after purification on alumina and crystallization, 7 mg of epimanoyl oxide with mp 93–97° was obtained.

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* As mentioned previously [6], no good analyses for gibberellin A_{13} have been obtained. Literature data: mp 194–196° C, $[\alpha]_D^{17}$ −48°. Found, %: C 61.4; 61.8; H 7.1; 7.0.

Fractions 12–17 (15 and 20% of ethyl acetate in hexane) consisted of 274 mg of a resin from which, by chromatography on preparative alumina plates and crystallization, 9 mg of pure kauran-16α-ol with mp 213–217° C (from ethyl acetate) was obtained, it was identical with sample described previously [8].

Fractions 30–32 (35% of ethyl acetate in hexane) formed 25 mg of a mass which partially crystallized. This was chromatographed on a preparative silica gel plate, giving 3 mg of pure 7β,18-dihydroxykaurenolide with mp 216–219° C (from a mixture of hexane and ethyl acetate), identical with the sample described above. Further elution of the column with more polar mixtures yielded resins not susceptible to working up.

Conclusions

1. The resin is a convenient source of the isolation of the individual gibberellins and other acidic and neutral metabolites.

2. By means of partition chromatography in a column of KSK silica gel treated with phosphate buffer having pH 6.4, it has been possible to separate gibberellins A_4 and A_7 and an isomer of gibberellin A_7—the hydroxylactone acid (II).

3. Gibberellin A_{13} has been isolated from the resin remaining after the evaporation of the mother solutions from the crystallization of industrial gibberellin, and the structure of the isomeric trimethyl esters obtained from it has been elucidated.

4. For the PG-7 strain, the connection observed previously for the F-6 strain between the relative stimulation of the biosynthesis of the gibberellins and kaurene on the one hand, and the relative suppression of the biosynthesis of highly oxidized diterpenes (kaurenolides and fujenal) on the other hand, has been confirmed qualitatively.

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