GIBBERELLINS AND SUBSTANCES RELATED TO THEM

III. On the Presence of a Physiologically Active Isomer of Gibberellic Acid in the Culture Liquid of Fusarium Moniliforme

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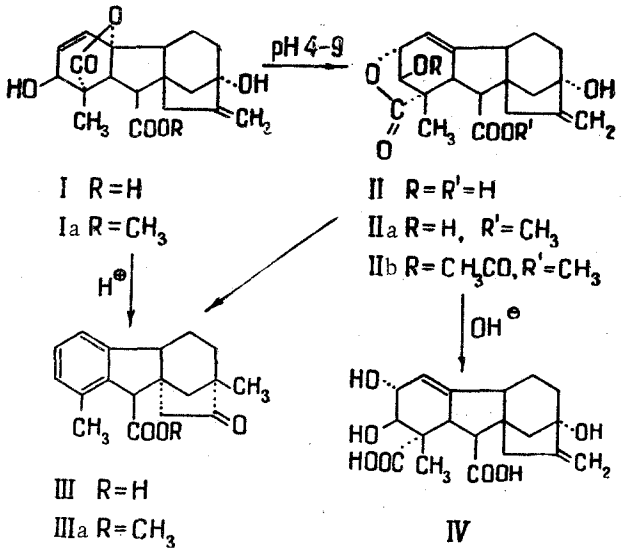
Khimiya Prirodnykh Soedinenii, Vol. 2, No. 2, pp. 114–120, 1966

In the literature it is stated that gibberellic acid (I) is readily isomerized to the 1,3-lactone of 2β, 3α, 7α-trihydroxy-1β-methyl-8-methylene-Δ^4-gibbene-1α, 10β-dicarboxylic acid (II). This isomerization (see scheme) takes place predominantly in a weakly alkaline medium [1–3], but is also possible in weakly acidic aqueous solutions [3, 4]. A study of the hydrolytic conversions of gibberellic acid (I) in buffer solutions at 50° C and 100° C has shown that the 1,3-lactone isomeric with it (II) is the first transformation product of gibberellic acid both in alkaline and in acid media [3].

However, up to the present time the 1,3-lactone (II) has not been isolated from the culture liquid of the producing fungus, although the metabolite composition of Fusarium moniliforme has been investigated repeatedly and in great detail.

In a study of the composition of the gibberellins synthesized by F. moniliforme in a deep culture in a medium containing sunflower oil and ammonium nitrate, we found a gibberellin-like substance differing from gibberellic acid on paper chromatography in the butan-1-ol–1.5 N NH_3 (3:1) system and having R_f 0.25. This substance, which we initially called "component no. 5" [5], is produced in the largest amount by the F-6 strain. Consequently, we used this strain for subsequent work. Component No. 5 is produced in the culture liquid after 6 days' fermentation and is clearly detectable on the 9th day. Its concentration reaches a maximum on about the 12–15th day of fermentation.

Attempts to carry out a preparative separation of gibberellic acid and component No. 5 by means of column or thin-layer partition chromatography on silica gel, on a mixture of silica gel and Celite 545, and on cellulose in various systems of solvents proved fruitless. The best separation of component No. 5 from gibberellic acid was achieved by chromatography on paper in the butan-1-ol–methyl ethyl ketone–7 N NH_3 (1:38:6) system for 36 hr in a beaker at room temperature.



Component No. 5 was isolated from the culture liquid of the F-6 strain by paper chromatography with subsequent zonal elution and adsorption on carbon. Two liters of culture liquid gave 22 mg of an amorphous sample of component No. 5. We identified the latter as the 1,3-lactone (II) on the basis of the following results:

1) The physical constants of component No. 5 and the authentic 1,3-lactone (II), and also the constants of their derivatives are extremely close to one another or coincide, and their IR spectra are practically identical.

2) The nature of the physiological activity of component No. 5 and that of an authentic sample of the 1,3-lactone (II) are similar when they are tested on three biotest plants (table).

3) Paper chromatography in the butan-1-ol–1.5 N ammonium hydroxide (3:1) and the butan-1-ol–methyl ethyl ketone–7 N ammonium hydroxide (1:38:6) systems gave identical R_f values for component No. 5 and an authentic sample of the 1,3-lactone (II).*

4) On paper chromatography in the benzene–methanol–water (2:1:1) system and in a thin layer of neutral alumina in several systems, the R_f values of the methyl ester of component No. 5 and the methyl ester of the 1,3-lactone (IIa) were identical. The R_f values of the acetylation products of the two methyl esters also coincided.

5) As in the case of the methyl ester of the 1,3-lactone (IIa), heating the methyl ester of component No. 5 with 3 N hydrochloric acid gave methyl gibberate (IIIa). The same product was obtained from gibberic acid formed by the acid degradation of gibberellic acid (I).

Physiological Activity of Gibberellic Acid (I), the 1,3-Lactone (II), and Component No. 5.

Test plant	Dose, γ/plant	Substance (I)		Substance (II)	Component no. 5	
		Length of the plant, %				
		of control	of (I)		of control	of (I)
Dwarf pea "pioner"	0.001	125.9	103.0	—	101.0	—
	0.01	172.5	125.6	72.8	127.3	73.7
	0.1	210.2	155.3	73.8	155.3	73.8
Lettuce "berlinskii"	0.001	129.7	102.8	—	102.8	—
	0.01	169.0	124.8	73.8	129.6	76.6
	0.1	195.3	152.0	77.8	151.5	77.5
Cucumbers "podmoskovnye"	0.1	103.4	103.9	—	102.3	—
	1.0	121.1	121.5	100.3	117,2	96.6

A study of the dynamics of the fermentation of F. moniliforme has shown that the accumulation of the gibberellins is accompanied by a fall in the pH of the medium from 5.5 to 5. Since the conversion of gibberellic acid (I) into the 1,3-lactone (II) is possible not only in a weakly alkaline but also in a weakly acid medium [3, 4], the question arises as to whether component No. 5 is a natural product of the vital activity of the fungus or whether it consists of an artefact, i.e., arises as the result of the nonenzymatic isomerization of the gibberellic acid. We carried out the following experiment to answer this question.

A solution of pure gibberellic acid in 0.1 M phthalate buffer (pH 4.8) was shaken in a rotary shaking device for six days at 26° C. The fermentation of the F-6 strain was carried out simultaneously on the same rocking device. The final pH was 4.9 (6-day culture). After the lapse of the time mentioned, butanolic extracts of the buffer solution of gibberellic acid and filtrates of the culture liquid were chromatographed on paper. The 1,3-lactone (component No. 5) was found in the chromatograms of both butanolic extracts. The content of the 1,3-lactone (II) was considerably higher in the buffer solution of gibberellic acid than in the filtrate of the culture liquid. We explain this by the fact that in the buffer solution, containing 400 mg/l of gibberellic acid, isomerization took place during the whole of the experiment, while in the culture liquid there was no gibberellic acid at the beginning of the experiment and isomerization could begin only after it had been formed by the producing agent. The results of the experiment showed that under the conditions of fermentation the isomerization of gibberellic acid (I) into the 1,3-lactone (II) takes place by a purely chemical mechanism without the participation of the producing agent.

To determine the ratio of gibberellic acid (I) and component No. 5 (II) in the culture liquid of the F-6 strain, the corresponding fraction obtained in the group separation of the gibberellins on a column of buffered silica gel was used [6]. This fraction was converted into the mixture of methyl esters which could be separated without difficulty on a column of neutral alumina. It was found that the methyl ester of gibberellic acid (Ia) and the methyl ester of component No. 5 (IIa) were present in a ratio of $\approx 8:1$. The results of a specially-performed experiment show that the pure methyl ester of gibberellic acid (Ia) is not converted into the methyl ester of component No. 5 (IIa) when it is passed through a column of neutral alumina. Consequently, the ratio of the corresponding acids in the culture liquid must be approximately 8:1.

*A sample of the 1,3-lactone (II) was obtained by the isomerization of gibberellic acid in a weakly alkaline medium by a somewhat modified method [1].

In a study of the physiological activity of the 1,3-lactone (II) on three test-plants by the method described by Murontsev [7], it was found that this substance stimulates the growth not only of the pea [2] but also of lettuce and cucumbers (see table).

As can be seen from the table, the physiological activity of component No. 5 and a synthetic sample of the 1,3-lactone (II) are practically identical. On cucumbers, the 1,3-lactone has the same effect as gibberellic acid, but on peas and lettuce its effect is considerably weaker. The minimum dose of the 1,3-lactone (II) stimulating the growth of these plants is approximately ten times higher than the corresponding dose of gibberellic acid. This nature of the physiological activity of the 1-3 lactone (II) is of interest in virtue of the fact that generally any kind of change in ring A of gibberellic acid leads to a marked decrease or the complete loss of its activity [8].

Experimental

The chromatography was carried out on Schleicher and Schüll 2043a paper and also on type "B" paper of the Leningrad mill No. 2, on silica gel of type KSK freed from traces of iron (150–250 mesh) and on neutral alumina [Brockmann activity grade III/IV (150–250 mesh)]. The thin-layer chromatography was carried out, as a rule, without supporting the layer. The paper chromatograms and the plates of silica gel were revealed with 70% sulfuric acid [5], and the alumina plates in a chamber saturated with the vapors of iodine and water. All the melting points were determined on a "Boetius" stage, and the angles of rotation in ethyl acetate on a "Hilger" polarimeter.

The experiments were carried out with chromatographically pure gibberellic acid with mp 235°–236° C and $[\alpha]_D$ +86° C.

Isolation of component No. 5 from the culture liquid. The F-6 strain was cultured in a medium of the following composition, %: sunflower oil, 10: NH_4NO_3, 0.3; KH_2PO_4, 0.02; $MgSO_4 \cdot 7H_2O$, 0.2. Mixture of trace elements, 1 mg; distilled water, 1 *l*.

The fermentation was carried out for 15 days at 26° C with shaking on a rotary shaking device (210 rpm) in 0.75-*l* flasks containing 150 ml of the nutrient medium.

The filtrate of the culture liquid (2 *l*) was acidified with hydrochloric acid to pH 2.5 and was twice extracted with butan-1-ol (400 and 200 ml). The extract was concentrated under vacuum (40° C, 25 mm) and was deposited on sheets of Schleicher and Schull 2043a paper. Chromatography was carried out in the butan-1-ol–methyl ethyl ketone–7 N NH_3 system (1:38:5, upper phase) at 18°–20°C for 36 hr in a beaker. The distance from the start to the center of the zone of component No. 5 was 26 cm and the distance between the zones of component No. 5 and that of gibberellic acid, in front of it, was 3 cm. The zones of component No. 5 (more accurately, its ammonium salt) were cut out and eluted with water. The total weight of paper taken was 800 g and the volume of the aqueous eluate was 1 *l*. Elution was carried out in a homogenizer until the paper ceased to fluoresce in UV light after its treatment with 70% sulfuric acid. The aqueous eluate was acidified to pH 2.8 and was treated successively with four portions of OU-B activated carbon (1.2, 1.2, 2.4, and 0.6 g; moisture content of the carbon, 24%). After the third treatment, there was a considerable weakening of the fluorescence of the eluate in UV light (sample on paper).

The carbon was filtered off and desorption was carried out with 58% aqueous acetone. The first two portions of carbon were treated with 40 ml of acetone, the third with 40 ml, and the fourth with 20 ml. After extraction (4 hr at 26° C), the carbon was filtered off, combined, and extracted for 12 hr with 40 ml of 78% acetone. The aqueous acetonic eluate was evaporated under vacuum (40° C, 25 mm). The aqueous residue (15 ml) was acidified to pH 2.5 and extracted with ethyl acetate (17 × 4 ml, until the fluorescence had weakened considerably). The extract was dried over Na_2SO_4 and was concentrated under vacuum to a volume of 3 ml. When 15 ml of petroleum ether (bp 40°–60° C) was added, an amorphous precipitate deposited, and this was reprecipitated with benzene from acetone solution. This gave 22 mg of an amorphous white powder with mp 148°–156° C, $[\alpha]_D^{20}$ +126.8° C (c 0.35); IR spectrum (ν_{KBr}): 3386, 1762, 1714, 1249, 1114, 1092, 1052, 960, 819, and 804 cm^{-1}. This substance gave only one spot on chromatography on paper in the butan-1-ol–1.5 N NH_3 (3:1) and the butan-1-ol–methyl ethyl ketone–7 N NH_3 (1:38:6) systems.

Preparation of the 1,3-lactone (II) from gibberellic acid. A solution of 1.0 g of pure gibberellic acid in 200 ml of 0.11 N NH_3 was allowed to stand at room temperature for 6 days, after which the solution was concentrated under vacuum (40° C, 25 mm) to a volume of 20 ml, and was acidified to pH 2.4 and extracted with ethyl acetate (5 × 50 ml). The extract was dried over Na_2SO_4 and evaporated to dryness. A spongy residue was produced. Yield 690 mg; $[\alpha]_D$ +97°. It was dissolved in 75 ml of boiling ether, and the solution was filtered from insoluble impurities and diluted with 40 ml of hexane. A white precipitate was deposited (310 mg), $[\alpha]_D$ +93°. The filtrate was diluted with 110 ml of hexane, and a second fraction precipitated (342 mg) with mp 151°–156° C and $[\alpha]_D$ +103°. Published data for the pure 1,3-lactone (II), gives mp 150°–160° C, $[\alpha]_D$ +104° [1]. However, on paper chromatography in the butan-1-ol–1.5 N NH_3 (3:1) system, the sample obtained gave three spots: a main spot with R_f 0.25, a considerable

spot with R_f 0.08, and a weak spot with R_f 0.31. These spots corresponded to the 1,3-lactone (II), to the dicarboxylic acid (IV) formed from substance (II) in an alkaline medium ([1], see scheme), and to the initial gibberellic acid (I).

The second fraction was freed from gibberellic acid (I). For this purpose it was dissolved in 50 ml of 0.25 N NH_3 and the solution was left at room temperature for 6 days. Repetition of the operations mentioned led to a simple (yield 214 mg) with mp 150°–157° C which, from the results of paper chromatography, contained only components (II) and (IV). The product isolated was transferred to a preparative plate of silica gel (24 × 22 × 0.2 cm) and was chromatographed in the chloroform–methanol (92:8) system. The zone with R_f 0.35–0.45 was collected on a porous filter and eluted with acetone (5 × 15 ml). The eluate was evaporated under vacuum, the residue was dissolved in 25 ml of ether, and the solution was treated with 50 ml of hexane. The resulting white amorphous precipitate was filtered off and dried under vacuum at 75° C. This formed a pure sample of the 1,3-lactone (II). Yield 57 mg, mp 143°–148° C, $[\alpha]_D$ +114.2° C (c 1.13); The IR spectrum and R_f values of this sample coincided with those for component No. 5.

Methyl ester of the 1,3-lactone (IIa). A. The fraction obtained in the group separation of the gibberellins on a column of buffered silica gel [6] and containing a mixture of gibberellic acid and component No. 5 (1.720 g) was treated with a distilled ethereal solution of diazomethane, and the volatile products were distilled off under vacuum. The residue was dissolved in acetone and transferred to 5 g of alumina (with evaporation under vacuum). The resulting powder was transferred to a column of alumina (100 g, 3 × 17 cm). Elution was carried out and 60-ml fractions were collected. Fractions 1–10 (benzene) and 11–15 [benzene–ethyl acetate (9:1)] proved to be blank; 16–20 [benzene-ethyl acetate (5:1)] gave 19 mg of crystals with mp 203°–219° C and $[\alpha]_D^{20}$ +71° C; 21–25 [benzene–ethyl acetate (4:1)] gave 1.429 g of crystals with mp 206°–210° C and $[\alpha]_D^{20}$ + 81° C (c 1.10); 26–30 [benzene–ethyl acetate (4:1)] gave 14 mg of a semicrystalline residue; 31–35 [benzene–ethyl acetate (7:3)] gave 179 mg of a white foam with $[\alpha]_D^{20}$ + 109.3°; 36–40 [benzene–ethyl acetate (7:3)] gave 10 mg of a yellowish foam; fractions 41–45 [benzene–ethyl acetate (6:4)] and 46–50 [benzene–ethyl acetate (1:1)] gave, respectively, 40 and 11 mg of a colorless oil.

Judging from the $[\alpha]_D$ value, fractions 16–20 consisted of a mixture of the methyl ester of gibberellin A_1 with methyl gibberellate (Ia) in which the latter predominated. After crystallization from a mixture of methyl acetate and hexane, fractions 21–25 gave methyl gibberellate (I) with mp 206°–208° C and $[\alpha]_D^{20}$ + 80.2° C (c 1.30), identical with respect to its constants and chromatographic characteristics with an authentic sample. On paper chromatography in the benzene–methanol–water (2:1:1) system, and in a thin layer of alumina in the chloroform–methanol (95:5) system, it gave the same R_f values as an authentic sample of methyl gibberellate (0.85 and 0.46, respectively).

After two reprecipitations with hexane from ethyl acetate, fractions 31–35 gave a substance with mp 106°–112° C and $[\alpha]_D^{20}$ + 111.4° C. An analytical sample isolated by recrystallization from a mixture of ether and hexane, after drying under vacuum at 75° C, had mp 106°–109° C/169°–170° C and $[\alpha]_D^{20}$ +112.3° C (c 0.86). IR spectrum (ν_{CHCl_3}): 3400, 1785, 1738, 1637, 1043, 960, 799 cm^{-1}.

Found, %: C 66.24; H 6.87. Calculated for $C_{20}H_{24}O_6$, %: C 66.65; H 6.71.

The methyl ester obtained from the authentic 1,3-lactone (II) had mp 104°–107° C/165°–170° C and $[\alpha]_D$ +114°. On paper chromatography in the benzene-methanol-water (2:1:1) system and in a thin layer of alumina in the chloroform-methanol (95:5) system, the two samples of the 1,3-lactone (IIa) gave the same R_f values (0.74 and 0.35–0.38, respectively). Literature data for the pure ester (IIa) gives mp 102°–106° C (hydrate) and 172° C, $[\alpha]_D^{20}$ +125° [1].

B. A stirred suspension of 30 mg of pure methyl gibberellate (Ia) in 60 ml of 1% NH_3 was left at room temperature for 6 days. The resulting solution was concentrated under vacuum (40° C, 25 mm) to a volume of 10 ml and was extracted with ethyl acetate (3 × 20 ml). The extract was dried over Na_2SO_4 and concentrated under vacuum. The concentrate so obtained was applied to a preparative plate of alumina (24 × 5 × 0.2 cm) and was chromatographed in the chloroform-methanol (95:5) system. The zone with R_f 0.30–035, corresponding to the bulk of the substances, was collected on a porous filter and eluted with acetone (5 × 10 ml). The residue from the evaporation of the eluate was dissolved in ether and precipitated with hexane. The crystals formed (11 mg) with mp 103°–108° C/163°–167° C and $[\alpha]_D^{20}$ +109.7° C (c 0.38) were identical with the ester (IIa) described above.

When substance (IIa) was acetylated with acetic anhydride in pyridine and the product was purified by preparative thin-layer chromatography [chloroform-methanol (95:5) system], the monoacetate (IIb) was obtained with mp 161°–168° C and the IR spectrum (ν_{max}) 3540, 1770, 1730, and 1714 cm^{-1}, which correspond with literature data [1].

Summary

1. By means of preparative paper chromatography, an isomer of gibberelin A_3, the 1,3-lactone of 2β,3α,7α-trihydroxy-1β-methyl-8-methylene-Δ^4-gibbene-1α,10β-dicarboxylic acid (II) has been isolated from the culture liquid of Fusarium moniliforme grown in a medium containing sunflower oil and ammonium nitrate.

2. The formation of this product under fermentation conditions takes place hydrolytically by a nonenzymatic mechanism.

3. A study of the physiological activity of the 1,3-lactone (II) has shown that the change in the position of the lactone cycle in ring A leads to a considerable decrease of the growth activity of this substance with respect to dwarf pea and lettuce, while it is retained with respect to cucumbers (as compared with the activity of gibberellin A_3).

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14 June 1965

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