ISOLATION OF FUSICOCCIN FROM A CULTURE FILTRATE OF *Fusicoccum amygdali*

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Fusicoccin A has been isolated from a culture filtrate of the filtrate of the fungus *Fusicoccum amygdali* Del., and it has been characterized as an α-glycoside of a tricyclic diterpenoid. The trimethylsilyl derivative of fusicoccin A has been investigated by combined GLC and mass spectrometry.

Fusicoccin is a compound of terpenoid nature of microbial origin which possesses a high physiological activity of flowering plants. The producing agent of the substance is the phytopathogenic fungus *Fusicoccum amygdali* Del., which causes canker of stone-fruit plants [1]. Fusicoccum A — the main metabolite produced by the fungus — consists of an α-glycoside of a tricyclic diterpenoid (Fig. 1). The carbotricyclic skeleton of the aglycone of fusicoccin is also possessed by a number of other metabolites of fungal origin — ophiobolins, cotylenins, and ceroplastans, which also possess physiological activity.

A method for isolating fusicoccin from a liquid culture of the fungus has been suggested by Ballio et al. [2]. A culture filtrate was extracted with butyl acetate, the extract was evaporated, and the residue was extracted with chloroform. The chloroform extract was evaporated, and the residue was dissolved in methanol, diethyl ether, and hexane, the insoluble fractions being discarded. The subsequent isolation of fusicoccin was carried out on columns of Florisil with elution by chloroform containing 10 vol.% of acetone, and the substance isolated was crystallized from ethyl acetate.

Later, this method was simplified [3]. The culture filtrate was treated with activated carbon and the fusicoccin was desorbed with water and acetone. The acetone eluates were separated, and the substance was extracted from the residue with chloroform. After the evaporation of the chloroform extract, a concentrate of fusicoccin was obtained which was dissolved in ethyl acetate, and from this solution pure fusicoccin was isolated. The method was used to isolate fusicoccin from large volumes of culture liquid but it is associated with considerable losses of the substance.

In our laboratory of the physiology and biochemistry of microorganisms under the direction of Academician of FASKhNIL [Lenin All-Union Academy of Agricultural Sciences] G. S. Muromtsev a culture liquid has been obtained by the deep cultivation of the producing fungus *Fusicoccum amygdali* Del. in flasks on a shaking maching on a semisynthetic medium. A filtrate of the culture liquid contained an average of 200 mg/liter of fusicoccin. The amount of fusicoccin in the CL was determined by a spectrophotocolorimetric method [4].

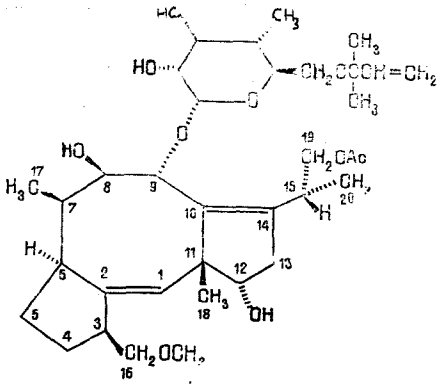

Fig. 1. Fusicoccin A.

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The culture filtrate was extracted twice with half-volumes of chloroform. The chloroform extracts were combined and dried over calcined sodium sulfate. The extract was evaporated and the residue, in the form of a viscous yellow-brown oil, was chromatographed on a column of silica gel or Florisil in a ratio of substance to sorbent of 1:30.

The column was eluted with solvents or mixtures of them, beginning with hexane, and then benzene, mixtures of benzene with chloroform in various ratios (from 2:8 to 3:7), chloroform, and mixtures of chloroform and isopropanol in ratios of 19:1 and 9:1. The last mixtures were the eluates with which the fusicoccin issued.

The method that we have proposed for isolating fusicoccin under laboratory conditions permits numerous extractions of the culture liquid with various solvents to be avoided, and the selected system of eluents for column chromatography provides the possibility of a sharp isolation of the fraction containing the bulk of the fusicoccin.

The compositions of the fractions were checked by TLC. When the plates were sprayed with sulfuric acid and heated for a few minutes at 120°C, the fractions containing fusicoccin acquired a pink-crimson color. The qualitative composition of all the fractions was checked additionally by mass spectrometry making use of the schemes of fragmentation of fusicoccin A and its analogs that are now known [5-7]. By the TLC method, the fusicoccin obtained appeared in the form of a single spot the R_f value of which corresponded to that of an authentic sample used as marker (0.44).

To analyze the fusicoccin by the method of combined GLC and mass spectroscopy we obtained its silyl derivative containing four trimethylsilyl groupings. The fusicoccin obtained was characterized by its melting point and its IR, PMR, and mass spectra, which agreed completely with those given in the literature [8, 9]. The mass spectrum of fusicoccin has, in addition to the molecular ion with m/e 680, the peak of an ion with m/e 722 which was previously ascribed to the product of the trans-acetylation reaction of the fusicoccin molecule in the ion source [8]. Recently, among the minor products of the culture filtrate the 12-O-acetyl derivative of fusicoccin, the molecular ion of which has a mass number of m/e 722, has been isolated from the culture filtrate [5].

Thus, the fusicoccin that we obtained included a small amount of the 12-O-acetyl derivative as impurity. The fragmentation of the molecular ions of these compounds takes place in precisely the same way, but the m/e values of the corresponding fragments differ by 42 mass units. In view of the fact that the substances under consideration possess practically the same volatility under the conditions of mass-spectrometric determination, it may be assumed on the basis of the ratios of the intensities of the peaks of the ions with m/e 680 and 722 that fusicoccin and its 12-O-acetyl derivative were present in the mixture in a ratio of 6:1, respectively. The presence of a small amount of the 12-O-acetyl derivative as impurity was also shown by the PMR spectrum of the sample of fusicoccin under investigation through the appearance of a small signal at δ 2.04 ppm (s, 3 H) of a third acetyl group in the molecule.

The biological activity of the fusicoccin obtained in tests on the elongation of apical segments of maize root and on the growth of radish and lettuce seeds did not differ from that described in the literature (results obtained by laboratory worker V. M. Koreneva) [10].

The fractions eluted from the column were studied by mass-spectrometric methods. The fraction eluted by hexane, with R_f 0.94 — the largest in amount — had a weak pink coloration but its mass spectrum resembled that of a hydrocarbon. Peaks of ions with mass numbers differing by 14 mass units up to m/e 686, which may correspond to an unsaturated hydrocarbon $C_{49}H_{98}$, were present. From the benzene fraction with R_f 0.71 was isolated elementary sulfur in the mass spectrum of which the ions S_1^+, $S_2^+ \ldots S_8^+$ with m/e from 32 to 256 were distinguished.

The broad benzene-chloroform fraction with R_f 0.69, 0.56, and 0.48 contained substances of nonfusicoccin and of fusicoccin natures. The substances of nonfusicoccin nature were most probably the $C_{40}H_{82}$ and $C_{39}H_{78}$ hydrocarbons, since their spectra contained a number of peaks belonging to ions differing from one another by 14 mass units up to m/e 562 and 546, respectively. The peaks of ions with m/e 514 and 650 correspond to substances of fusicoccin nature. The peak of the ion with m/e 514 characterizes an unknown oxygen-containing compound for which the subsequent ejection of several molecules of water is characteristic. The ion peak with m/e belongs to a compound on the fragmentation of which peaks appeared that are characteristic for the ions formed in the fragmentation of the fusicoccin aglycone and the peaks of ions characterizing a glycosyl residue were absent.

The chloroform fractions contained a substance with R_f 0.48 in the mass spectrum of which the ion with the highest mass number was that with m/e 390, corresponding to a molecule similar in structure to the aglycone moiety of the fusicoccin molecule.

The chloroform–isopropanol fraction contained both fusicoccin itself with R_f 0.44 (M^+ with m/e 680) and also more polar substances of fusicoccin nature with R_f 0.3 and 0.17. This fraction, again according to its mass spectrum, also contained a small amount of high-molecular-weight hydrocarbons.

EXPERIMENTAL

The IR spectrum was recorded on a UR-10 spectrograph in paraffin oil, and the PMR spectrum on a Varian XL-100A instrument at a frequency of 100 Hz in $CDCl_3$. Fusicoccin and its TMS derivative were analyzed on an LKB-9000 instrument at an energy of ionizing electrons of 70 eV and a temperature of the ionizing chamber of 250-270°C. For column chromatography we used silica gel 50-100 μ on Florisil containing 0.05% of Fe, 60-100 mesh. The columns were filled in the solvent with which chromatography was begun. The substance was deposited in the form of a "cushion" – i.e., it was first dissolved in acetone, the sorbent was added, and after the evaporation of the solvent to dryness the material was transferred to the column. Evaporation was carried out under reduced pressure in a rotary evaporator at a water-bath temperature of 40°C. For TLC we used Silufol prepared plates with a layer thickness of 100 μ. The system for TLC was chloroform–isopropanol (9:1). The revealing agent was concentrated H_2SO_4 followed by heating.

Fusicoccin A. The mycelium was separated from the culture liquid by filtration through coarse calico. The culture liquid in an amount of 6 liters was extracted with chloroform (2 × 3 liters) with shaking in a shaking machine for 1 h. The evaporated chloroform extracts yielded 4.4 g of a yellow-brown oil. This was chromatographed on 132 g (270 ml) of Florisil. The column was filled in hexane. The volume of one fraction was 270 ml. On elution with hexane (V = 540 ml), 122 mg of oil was isolated, while benzene (V = 1620 ml) eluted 157 mg of a yellow oil. The broad fraction eluted by various mixtures of benzene and chloroform with a total volume of 2700 ml contained 800 mg of oil. Pure chloroform (V = 1350 ml) eluted 150 mg of a light yellow resinous substance. The fraction eluted from the column by chloroform–isopropanol (19:1) (V = 1350 ml) contained 1115 mg of an oil with crystals.

Recrystallization from a mixture of hexane and ethyl acetate yielded 400 mg of fusicoccin A with mp 155-156°C. IR spectrum, ν_{max} (cm^{-1}): 3400-3700 (OH), 1730, 1750, and 1250 (C=O group in an acetate), 1640 (olefinic double bond), 920 (vinyl group). PMR spectrum, δ, ppm: 0.84 (d, 3 H, J = 7 Hz) (20-CH_3); 1.11 (d, 3 H, J = 7 Hz) (7-CH_3); 1.21 (s, 3 H) (11-CH_3); 1.26 (s, 6 H) ($C\begin{smallmatrix}CH_3\\CH_2\end{smallmatrix}$) 2.04 (s, 3 H) (12-OAc); 2.09 (s, 3 H) (19-OAc); 2.15 (s, 3 H) (3'-OAc); 3.35 (s, 3 H) (16-OCH_3); 3.43 (d, 2H, J = 7 Hz) (17-CH_2); 3.5 (d, 2 H) (6'-CH_2); 5.84 (q, 1 H, J = 10 Hz, J = 18 Hz) ($CH{=}CH_2$, ABX system). Mass spectrum: the spectrum contained ions ranging from the molecular ion to m/e 105:

m/e	I, % of max.	m/e	I, % of max.	m/e	I, % of max.	m/e	I, % of max.
722	0,06	561	0,26	357	25,9	205	70,6
704	0,39	553	0,28	348	9,4	203	9,4
696	0,69	552	0,43	347	14,1	199	20,0
690	0,27	543	0,14	339	7,1	197	29,4
689	0,53	534	0,65	331	11,8	187	72,9
680 (M^+)	0,35	533	0,65	330	31,8	183	28,2
678	0,12	519	0,14	329	47,1	181	24,7
663	0,49	518	0,07	317	44,7	169	31,8
662	0,85	501	0,52	315	7,6	167	31,8
654	0,47	500	0,14	313	11,8	155	52,9
647	0,44	491	0,12	297	30,6	163	9,4
638	0,27	476	0,45	289	17,6	161	30,6
636	0,62	474	0,4	287	11,8	157	23,5
635	0,39	458	0,73	279	17,6	149	52,9
621	0,37	450	0,98	271	23,5	147	23,5
612	0,60	449	0,67	269	22,4	145	58,8
611	0,26	434	1,06	257	23,5	143	23,5
604	0,16	433	1,80	251	17,6	139	17,6
603	0,22	432	2,30	248	29,4	137	29,4
595	0,49	431	1,20	247	100	135	23,5
594	0,94	419	0,31	239	34,1	133	35,3
593	0,48	408	23,5	229	21,2	131	28,2
579	0,53	390	45,9	227	25,9	127	55,3
576	0,39	389	40,0	225	23,5	121	41,2
575	0,59	373	10,6	211	30,6	119	37,6
570	0,24	372	20,0	209	29,4	109	41,2
569	0,16	371	11,8	207	11,8	107	35,3
						105	35,3

2',4',8,12-Tetrakis(trimethylsilyl)fusicoccin. This was obtained by dissolving fusicoccin in an excess of N,N-bis(trimethylsyly1)trifluoroacetamide and allowing the mixture to stand at room temperature for 12 h. The mass spectrum included ions from the molecular ion to m/e 201:

m/e	I, % of max.	m/e	I, % of max.	m/e	I, % of max.	m/e	I, % of max.
968	0,107(M^+)	746	2,68	493	23,3	343	23,7
967	0,216	745	4,50	492	45,7	342	23,7
939	0,32	709	0,42	491	18,7	329	36,8
926	0,97	707	0,42	478	24,8	327	42,1
925	1,39	685	1,50	460	10,5	319	100
907	0,32	677	1,92	459	15,0	311	49,9
896	0,27	674	1,92	449	16,5	301	26,3
894	0,43	673	3,87	433	16,1	289	57,9
867	0,97	656	0,47	432	27,1	283	27,3
866	1,71	617	1,50	431	16,5	269	42,1
836	1 07	613	0,94	420	16,5	267	50,0
802	0 76	605	0,76	419	18,0	251	28,9
801	1,26	582	3,34	417	14,3	247	78 9
777	0,43	581	3 87	407	4 5	239	28,9
776	0,64	577	1,8	403	17,3	229	36,8
768	0,43	565	3,21	402	21,0	222	36,8
763	0,32	564	2,16	401	21,8	213	26,3
762	0,54	549	1,39	399	13,6	201	47,3
676	0,76	537	1,39	387	21,0		
748	0 63	533	1,18	380	39,5		
747	1 24	521	2,90	379	84,2		
		510	27,1	367	18,4		
		509	14,3	359	26,3		

The silyl derivative was introduced into the mass spectrometer through a glass gas-chromatographic column 2 m long and 3 mm in internal diameter containing 3% of SE-30 on Gas-Chrom Q (100-120 mesh) at an evaporator temperature of 240°C under conditions of temperature programing from 240 to 290°C at the rate of 10 deg/min and with a rate of flow of the carrier gas (helium) of 30 ml/min. The substance issued from the column as a single peak at a temperature of 280°C 4 min after the sample had been injected.

SUMMARY

1. Fusicoccin A has been isolated from the culture liquid of the fungus *Fusicoccum amygdali* Del., and the method of its isolation has been modified.

2. The TMS derivative of fusicoccin A has been obtained and the conditions have been found for its gas-chromatographic analysis.

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