SECONDARY METABOLITES OF Ulocladium chartarum. Ulocladols A AND B – NEW PHYTOTOXINS OF TERPENOID NATURE

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Ulocladols A and B – new exometabolites having a mixed sesquiterpenoid-polyketide nature that are structurally close to the host-specific toxins of Alternaria citri – have been isolated from the deuteromycete Ulocladium chartarum. Both substances inhibit the growth of cotton seeds.

Among the Fungi Imperfecti attacking plants a prominent place is occupied by species of the genus Alternaria and also of the genera Stemphyllium and Ulocladium, which are close to it and which cause diseases in a broad spectrum of agricultural crops. These fungi are known as a rich source of phytotoxins, and a number of reviews on host-specific and nonspecific toxins of fungi of the genus Alternaria have been published [1].

In preliminary experments on the study of the toxic action of culture filtrates of fungi of these genera isolated from cotton-plant leaves and bolls, strain 108F-1 of the fungus Ulocladium chartarum (Pr.) Simmons (Alternaria chartarum) [2] was selected for further investigations. This strain caused blight on cotton-plant leaves both in experiments with isolated leaves and also on plants grown in a hothouse. A culture filtrate possessed toxic properties in relation to Paramecium caudatum, inhibited the growth of cotton seeds, and caused the development of plants with completely or partially yellow cotyledons and first leaves. In this paper we describe the isolation of two metabolites of this fungus and the determination of their structures.

The ethyl acetate extraction of the culture liquid, acidified to pH 6.0, gave a material with which the subsequent work was conducted. The fresh, undried, mycelium was comminuted and was steeped in 95% ethanol. A more detailed description of the course of the operations is given in the Experimental part. Preliminary investigations using the TLC method showed that the culture liquid contained a more diverse set of metabolites, and our investigations were therefore concentrated on it. We give the results of the investigations of two substances isolated from the culture liquid.

These substances, which have been called ulocladols A and B, were isolated with yields of 2.1 and 1.7% on the weight of the extract in the course of chromatographic separation on silica gel and Sephadex LH-20 (see the Experimental part). Both substances were isolated in the form of colorless oils, but it was found possible to crystallize ulocladol B from aqueous methanol in the form of acicular crystals with mp 53-54°C.

The two substances had similar mass spectra and the same composition, $C_{21}H_{30}O_3$. The UV spectra of the ulocladols were also identical. At the same time, the $[\alpha]_D^{20}$ values of +213° and +124° for ulocladols A and B, respectively, and the considerable differences in their IR and NMR spectra, and also their different chromatographic mobilities in TLC indicated that they were different, isomeric, compounds.

The PMR spectra of ulocladols A (I) and B (II) were largely similar, and therefore a number of the subsequent considerations for ulocladol A, in the spectrum of which the multiplets scarcely overlapped, apply in equal measure to ulocladol B. The strong-field region of the spectrum of (I) contained the signals of four methyl groups: doublet at 0.95 ppm (Table 1) from a methyl at a tertiary carbon; singlet at 1.49 ppm due to a methyl at a quaternary carbon linked with an oxygen function; and doublets at 1.55 and 1.66 ppm corre-

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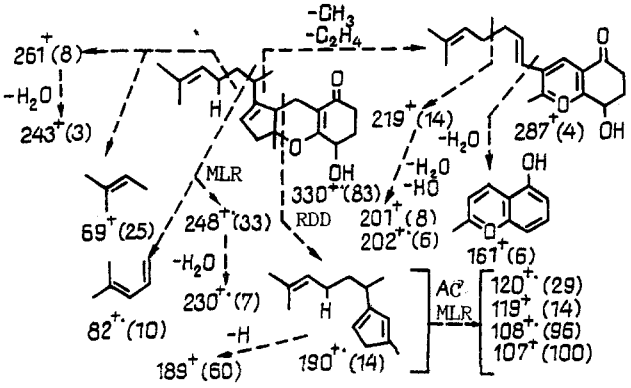

Fig. 1. AC) Allyl bond cleavage; MLR) McLafferty rearrangement; RDD) retrodiene decomposition.

TABLE 1. PMR Spectra of (I) and (II) (400 MHz, $CDCl_3$)

Position	I		II	
	δ_H, ppm	SSCC, Hz	δ_H, ppm	SSCC, Hz
1	2,23 br.ddd	17,0; 6,7; 3,0	1,95 ddd	17,3; 6,9; 4,1
	2,67 dddd	17,0; 3,2; 1,6; 1,6	2,56–2,64 m	—
2	2,74 br.dd	6,7; 3,2	2,78 br.dd	7,0; 3,0
4	2,42 dddd	16,0; 1,7; 1,7; 1,7	2,45 dddd	16,3; 1,8; 1,8; 1,8
	2,60 br.dddd	16,0; 2,5; 2,0	2,56–2,64 m	—
5	5,32 br.s	—	5,32 br.s	—
7	2,03 br.sext	7,0	1,85 m	—
8	1,30 ddt	13,5; 9,0; 6,7	1,29 ddt	13,5; 9,0; 6,7
	1,48 ddt	13,5; 9,0; 6,7	1,46 ddt	13,5; 9,0; 6,7
9	1,88 m	—	1,88 m	—
10	5,03 t.q.q	7,0; 1,4; 1,4	5,02 t.q.q	7,1; 1,4; 1,4
12	1,66 d	1,4	1,66 d	1,4
13	1,55 d	1,4	1,55 d	1,4
14	0,95 d	6,9	0,94 d	6,8
15	1,43 s	—	1,49 s	—
4′	2,38 br.dd	18,0; 5,5	4,33 br.dd	8,0; 4,8
	2,51 ddddd	18,0; 12,5; 5,0; 3,0; 1,6	—	—
5′	1,73 dddd	13,0; 12,5; 12,5; 5,5	1,95 ddd	18,0; 8,0; 5,2
	2,32 dddd	12,5; 5,5; 5,0; 2,2	2,31–2,16 m	—
6′	4,03 ddd	18,0; 5,5; 1,4	2,31–2,16 m	—
	—	—	2,56–2,64 m	—
4′-OH	—	—	3,02 br.s	—
6′-OH	3,93 d	1,4	—	—

sponding to methyls at a double bond. Double resonance showed that they had an allyl SSCC with an olefinic proton giving a signal at 5.03 ppm, which, in its turn, was coupled with a methylene group giving a signal at 1.88 ppm. Protons of a methylene group corresponding to signals at 1.30 and 1.48 ppm also had spin–spin coupling with the above-mentioned methylene group and with a methine giving a signal at 2.03 ppm. The fact that the 0.95 ppm methyl had a SSCC with the same methine permitted the conclusion that ulocladol A (I) contained a $(CH_3)_2C{=}CHCH_2CH_2CH(CH_3)-$ grouping. Ions with m/z 261, 248, 247, and 219 in the mass spectra of (I) and (II), corresponding to the cleavage of this chain (a scheme of fragmentation is given in Fig. 1) also confirmed its presence.

In addition to this grouping, (I) and (II) also contain two three-spin systems each with shifts of 2.74, 2.76, and 2.23 ppm, and 5.32, 2.24, and 2.60 ppm, respectively (the shifts are given for ulocladol A) due to $-CH_2\underset{|}{C}H-$ and $=CHCH_2-$ groups. These systems are coupled with one another and with other protons of the molecule by allyl and homoallyl SSCCs; however, not all these SSCCs could be measured and a large number of them were observed as broadenings of the lines of multiplets.

A consideration of the ^{13}C NMR spectra in the 50-100 ppm region permitted the detection of signals with shifts at 88.0 and 70.62 ppm and at 88.44 and 66.29 ppm for (I) and (II), respectively (Table 2). The weaker-field signal in each case corresponded to a quaternary-carbon, which, in combination with the absence of other quaternary-carbon signals indicated

TABLE 2. ^{13}NMR Spectra of Compounds (I-IV), δ_C, ppm

Position	I	II	III	IV
1	15,42 t	15,54 t	15,9	14,9
2	46,23 d	46,72 d	47,4	46,9
3	88,00 s	88,44 s	89,7	89,2
4	44,73 t	44,87 t	45,8	45,0
5	119,69 d	119,71 d	121,2	119,6
6	150,22 s	160,45 s	151,0	150,3
7	32,20 d	32,34 d	33,2	30,9
8	34,86 t	34,94 t	35,9	34,5*
9	25,71 t	25,47 t	26,3*	24,8*
10	124,26 d	124,40 d	126,4	124,9
11	130 97 s	131,14 s	135,9	135,2
12	25,37 q	25,47 q	68,8	68,5
13	17,38 q	17,52 q	13,7	13,6
14	19,94 q	20,08 q	20,6	19,7
15	23,23 q	23,65 q	23,1	23,5
1′	197,32 s	196,52 s	199,4	196,9
2′	105,13 s	108,01 s	106,6	107,7
3′	171,78 s	169 81 s	173,9	170,7
4′	27,57 t	66,29 d	28,7	66,5
5′	29,31 t	29,02 t	30,8*	28,7*
6′	70,62 d	33,29 t	72,1	33,4*

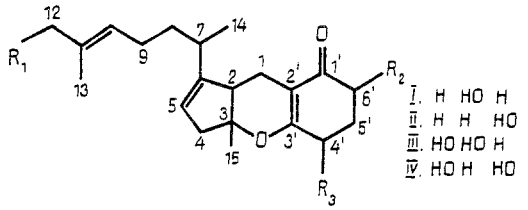

Fig. 2. On the right of the formula in the vertical columns are given the values of R_1, R_2, and R_3, respectively.

the presence of a $-C(CH_3)(O-)-$ grouping. The stronger-field signal in each case corresponded to a tertiary carbon. The proton attached to this carbon had a shift of 4.03 ppm for (I) and 4.33 ppm for (II). Exchange with heavy water and double resonance revealed the signals of hydroxy groups at 3.93 and 3.02 ppm, respectively, and their coupling with the multiplets at 4.03 and 4.33 ppm. The mass spectra of ulocladols A and B lacked $(M - H_2O)$ ions; however, a number of secondary ions were accompanied by fragments corresponding to the ejection of H_2O, and this permitted them to be regarded as secondary alcohols. A difference in the nature of the hydroxy groups in (I) and (II) must be mentioned: In the case of (I) the hydroxyl signal at 3.93 ppm had the form of a narrow doublet with a SSCC of 1.4 Hz, while for (II) the corresponding signal, at 3.02 ppm, was a broad singlet. This difference showed that in (I) the hydroxyl was more accessible for exchange. As was revealed after the establishment of the structure of (I), it is bound by a weak hydrogen bond to a keto group.

Analysis of the weak-field part of the ^{13}C spectrum showed the presence of, in addition to signals corresponding to trisubstituted isolated double bonds, signals at 197.32, 105.13, and 171.78 ppm for (I) and at 196.52, 108.01, and 169.81 ppm for (II). These signals permitted the assumption of the presence in (I) and (II), in each case, of a grouping formed by an ether of the enolic form of a diketone system $-COC{=}C-O-$, which was also confirmed by the presence of a single λ_{max} at 264 nm for each ulocladol. Calculation by the Woodward–Fieser rule for this system gave a value of 267 nm [3].

The combination of results obtained showed the terpenoid nature of the compounds isolated. A study of the literature on the metabolites of fungi showed that ulocladols A and B were new compounds and, at the same time, were very close to the ACTG toxins – host-specific toxins of the fungus Alternaria citri, parasitic on the mandarin [4]. A comparison of the spectral characteristics of the ulocladols with those of the ACTG toxins (III) and (IV) showed that the ACTG toxins are hydroxy derivatives of ulocladols A and B, respectively. The good correlation of the NMR spectra of (I), (II), (III), and (IV) enables us to assume the identity of the stereochemistries of these compounds, which, on the whole, remains unknown. The $[\alpha]_D^{20}$ values for (I) and (III) are +213° and +165°, respectively, and for (II) and (IV) +124° and +83°, which likewise does not contradict this hypothesis.

It must also be mentioned that the absence of one hydroxy group in (I) and (II), as compared with (III) and (IV), leads to a considerable decrease in the intensity of the molecular ions in their mass spectra. The presumed fragmentation pathways of the molecular ion are shown for ulocladol A in Fig. 1. The main pathway of the breakdown of M^+ is a retrodiene decomposition with the formation of a m/z 190 ion, which, in its turn, loses a hydrogen atom with the formation of a cyclopentadienyl ion having m/z 189. A number of ions are formed through the McLafferty rearrangement and allyl cleavages of the side chain. The strongest ions, with m/z 107 and 108, are formed in the McLafferty rearrangement of ions with m/z 189 and 190. Allyl cleavage, leading to the splitting out of methyl followed by the elimination of ethylene, leads to a pyrylium ion with m/z 287 and a number of other ions.

The undoubted closeness of ulocladols A and B, on the one hand, and the ACTG toxins, on the other hand, indicates the closeness of A. citri and U. chartarum. According to Joly [5], the most frequent and most serious disease of mandarin oranges is caused by A. chartarum, a species which today is assigned to Ulocladium. He considers that although a species is frequently described as A. citri (Ellis et Pierce) Bliss et Fawcett, this is a collective name for a number of Alternaria species parasitic on citrus fruits and, most frequently, for A. chartarum. The results that we have obtained, which have shown the closeness of the secondary metabolism of U. chartarum, isolated from the cotton plant, and of A. citri – the producing agent of the ACTG toxins – show the validity of this point of view.

Neither of the compounds obtained possessed antibiotic activity against Escherichia coli, Staphylococcus aureus, or Candida tropicalis. In concentrations of $3.3 \cdot 10^{-2}$ and $6.6 \cdot 10^{-3}$ mg/ml ulocladol A caused the death of P. caudatum 3 and 20 minutes after treatment. A similar activity was observed for ulocladol B. When cotton seeds were steeped in solutions of compounds (I) and (II) for a day, 100% inhibition of growth was observed at concentrations of 0.12-0.14 mg/ml for both compounds.

EXPERIMENTAL

Mass spectra were recorded on an LKB-9000 instrument (Sweden) with the use of device for direct introduction. IR spectra were taken on a Shimadzu IR-435 spectrometer in a film, and UV spectra on a Shimadzu-260 spectrophotometer. Specific optical rotations were determined on a Perkin-Elmer spectropolarimeter (United Kingdom). PMR spectra were obtained on a Bruker AC-80 and WM-400 spectrometers (FRG), and ^{13}C NMR spectra on the Bruker AC-80.

The following systems were used for chromatography: 1) hexane–chloroform–methanol (6:4:1); 2) the same (5:4:2); 3) ethyl acetate–benzene (1:1); 4) the same (3:7); and 5) chloroform–methanol (19:1).

On the performance of TLC the substances were detected visually with ordinary illumination and under UV light (254 and 366 nm; Cromato-Vue UV lamp) and also after treatment with a 1% solution of vanillin in H_2SO_4 followed by heating to 110°C.

Cultivation. U. chartarum was isolated from bolls of a cotton plant of the variety 108F. The species affiliation of the fungus was determined by L. M. Lekina of the Moscow State University Department of Mycology and Algology. The fungus was grown on Czapek liquid medium under stationary conditions at room temperature for five weeks.

Working Up of the Biological Material. After the mycelium had been filtered off, the culture liquid was acidified with 20% phosporic acid to pH 6 and was extracted twice with ethyl acetate (2:1 by volume). The combined extracts were evaporated at 40°C in vacuum, which gave a brown oil with a sharp acetic acid odor. Elimination of the volatile acids by fivefold evaporation of a suspension of the oil in water (1:10-1:20) at 40°C led to 7.0 g of a dark yellow oil, odorless and with a distinct separation into two phases.

The crude mycelium was homogenized in 20 liters of 95% ethanol and steeping was continued for one week. Then the extract was evaporated to a volume of 1.2-1.6 liters (at this stage it consisted of a dark green solution with drops of a bright red oil), and the concentrate so obtained was extracted with hexane (3 × 0.5 liter). Evaporation of the hexane yielded 20 g of a bright red mobile oil. The aqueous alcoholic residue was evaporated to a volume of 0.5 liter, the pH was brought to 6.0, and it was treated with 2.0 liters of ethyl acetate, the evaporation of which, after drying over Na_2SO_4, gave 3.14 g of a dark purple oil with a black solid. The aqueous phase was filtered through a layer of Celite and was evaporated to the state of a syrup. The cooling of a hot solution of the syrup in 70% ethanol gave 9 g of a colorless crystalline substance. This substance gave a positive reaction for sugars and was chromatographically identical with trehalose.

Separation of the Extract of the Culture Liquid. A solution of 7 g of the extract in benzene–MeCN was deposited on 15 g of silica gel 40/100 μm (Chemapol). The material obtained was deposited on the top of a column containing the same silica gel (80 g; 4.5 × 9.0 cm), and, with the collection of 100-ml fractions, elution was performed with petroleum ether (6 fractions), petroleum ether–benzene (1:1) (6 fractions), benzene (6 fractions), benzene–ethyl acetate (95:5) (8 fractions), (90:10) (6 fractions), (80:20) (9 fractions), and (50:50) (7 fractions), ethyl acetate (6 fractions), ethyl acetate–95% ethanol (6 fractions), and 95% ethanol (2 fractions). The course of separation was monitored by TLC in systems 3, 4, and 5 on Kieselgel-60 plates. Fractions having similar compositions according to TLC were combined, giving the final fractions U1-U13. Fractions U1 (8-9, 0.09 g), U2 (10-11, 0.02 g), and U3 (12-17, 1.7 g) contained lipophilic substances and were not further investigated. Fractions U12 (55-56, 1.3 g) and U13 (57-62, 0.3 g), containing mainly resinous substances, were not investigated, either.

Isolation of Ulocladols A and B. Fraction U9 (41-44, 0.90 g) was chromatographed on Sephadex LH-20 in 95% ethanol: After the combination of fractions of similar composition, this gave fractions U9/1-U9/11. The analogous treatment of U8 (35-40, 0.41 g) gave fractions U8/1-U8/8. Chromatography of the combined fractions U8/1 and U9/1 on silica gel 5/40 μm in $CHCl_3$, followed by chromatography on LH-20 in system 1, and, finally, filtration through 0.5 g of activated carbon (Norit III, Sigma, USA) in methanol gave 120 mg of ulocladol B (II) in the form of a colorless viscous oil.

The chromatography of U5 (23-26, 0.40 g) on LH-20 in system 1 and then on silica gel 5/40 μm in the ethyl acetate–benzene (3:97) system and final purification by filtration through activated carbon in methanol led to 150 mg of ulocaldol A (I) in the form of a colorless viscous oil.

Ulocladol A (I). Colorless viscous oil with $[\alpha]_{max}^{20}$ (nm): +213° (589; D line of Na), +223° (578), +257° (546), +487° (435), +567° (406), +949° (365) (c 0.97; MeOH). UV spectrum, λ_{max}: 264 nm (log ε: 4.21). IR spectrum, ν_{max}, cm^{-1} (film): 3400, 2890, 1635 sh, 1610 s, 1440, 1370 s, 1300 w, 1260, 1200 w, 1165 w, 1145, 1075 s, 980, 910, 875, 825, 745 w.

The PMR and ^{13}C NMR spectra are given in Tables 1 and 2.

Mass spectrum, m/z (%): 330 (M^+, 55), 287(5), 261(10), 256(3), 249(15), 248(64), 247(5), 243(3), 230(7), 221(8), 219(13), 215(4), 203(5), 202(7), 201(9), 199(4), 190(14), 189(74), 187(9), 175(6), 173(8), 171(9), 161(9), 159(7), 147(29), 145(11), 141(10), 134(8), 133(29), 131(8), 129(9), 123(14), 121(41), 120(26), 119(21), 109(25), 108(79), 107(100). 105(38), 95(21), 93(27), 91(45), 85(11), 83(10), 82(10), 81(19), 79(20), 77(19), 69(45), 67(18), 65(10), 57(15), 55(45), 53(14).

R_f 0.67 (system 4).

Ulocladol B (II). Colorless needles with mp 53-54°C (aqueous MeOH), $[\alpha]_{max}^{20}$ (nm): +124° (589; D line of Na), +130° (578), +150° (546), +293° (435), +380° (406), +627° (365) (c 0.98; MeOH). UV spectrum, λ_{max}: 264 nm (log ε: 4.20). IR spectrum, ν_{max}, cm^{-1} (film): 3360, 2890 s, 1605 s, 1435, 1380, 1310, 1255, 1215, 1190, 1160, 1145 sh, 1080 s, 1055 sh, 1005, 950, 925 sh, 915, 870 w, 820, 745.

The PMR and ^{13}C NMR spectra are given in Tables 1 and 2.

Mass spectrum, m/z (%): 330 (M+, 83), 287(4), 261(8), 249(11), 248(33), 247(6), 243(3), 230(5), 221(6), 219(14), 202(6), 201(8), 191(12), 190(14), 189(60), 187(6), 175(5), 173(5), 161(6), 159(5), 147(26), 145(7), 141(10), 135(11), 134(8), 133(23), 123(10), 121(39), 120(29), 119(14), 109(26), 1088(96), 107(100), 105(38), 95(18), 93(29), 91(8), 83(6), 82(10), 81(16), 79(5), 69(25), 67(5), 55(9).

R_f 0.33 (system 4).

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CHEMICAL MODIFICATION OF ESTAFIATIN

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The halogenation, epoxidation, acetylation, and oxidation of the sesquiterpene-γ-lactone estafiatin (I) has given eight derivatives. It has been shown that during the epoxidation of estafiatone (IV) the formation of a δ-lactone takes place as the result of the Bayer-Villiger oxidation of the cyclopentanone fragment. A stereoselective conversion of estafiatin into the known natural guaianolide isozaluzanin C is described. The structures of the derivatives synthesized were established on the basis of their IR, UV, PMR, and mass spectra.

In this paper we present the results of the chemical transformation of a sesquiterpene lactone of the guaiane type – estafiatin (I). Estafiatin is one of the guaianolides that are widely distributed in the natural flora [1-9]. The presence in the molecule of this compound of such structural fragments as an exomethylene group at C-10 and an α-epoxide ring at C-3, 4 has permitted the performance of such relations as halogenation, acetylation, and oxidation.

The reaction of estafiatin (I) with aluminum isopropanolate gave derivative (II). Its IR spectrum showed the presence of a hydroxy group (3500 cm^{-1}) in its molecule, while its PMR spectrum included the signal of a gem-hydroxylic proton – a triplet at 4.68 ppm (1H, J = 8 Hz) – and two broadened singlets with their centers at 5.35 and 5.48 ppm, which are characteristic for the protons of an exomethylene group at C4. These facts permit us to suggest for (II) the structure of 3α-hydroxyguaia-4(15),10(14),11(13)-trien-6,12-olide, identical with isozaluzanin C [10].

On the oxidation of (II) with chromium trioxide in pyridine, derivative (III) was obtained with 95% yield. The molecule of (III) included a keto group conjugated with a double bond, as was unambiguously characterized by its IR, UV, and PMR spectra, and this permitted us to put forward for (III) the structure of 3-oxoguaia-4(15),10(14),11(13)-trien-6,12-olide.

The interaction of estafiatin (I) with boron trifluoride etherate led to the formation of derivative (IV) with a yield of 90%; from its physicochemical constants and its IR and PMR spectra, (IV) proved to be identical with estafiatone [11].

When (IV) was epoxidized with m-chloroperbenzoic acid in chloroform, followed by the separation of the mixture of products by flash column chromatography, two derivatives, (V) and (VI), were obtained with yields of 68 and 16.3%, respectively. The IR spectrum of (V) showed an absorption band at 1170 cm^{-1} characteristic for an epoxy group and one at 1740 cm^{-1} characteristic for a keto group, while the PMR spectrum contained the signals of gem-epoxidic methylene protons: two doublets at 2.42 and 2.62 ppm (1H each, J = 3.5 Hz). On the basis of the spectral features found, for (V) we suggest the structure of 3-oxo-10(14)-epoxyguaia-11(13)-en-6,12-olide. The IR spectrum of the second derivative (VI) contained absorption bands characteristic for an epoxide group, at 1170 cm^{-1}, and for a carbonyl group,

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