derivative with methanol or ethanol—acetic acid (250:1). According to UV spectroscopy, this method permits the isolation of 90-100% of the untransformed 1,4-benzodiazepin-2-one and 60% of the desired product formed. The UV spectra of the 7-bromo-5-(o-chlorophenyl)-3-hydroxy-1,2-dihydro-3H-1,4-benzodiazepin-2-one were recorded on an SF-16 spectrophotometer in ethanol, the IR spectra of a Perkin-Elmer 577 instrument in KBr tablets, and the mass spectra on an MKh-1303 instrument with a system for direct introduction at an ionizing energy of 50 eV, an emission current of 1.5 μA, and an inlet temperature 20-40°C below the melting point of the substance.

SUMMARY

1. *Actinomyces roseochromogenes*, VKM A-612, and *Streptomyces viridis* VKM A-607 effect the microbiological hydroxylation of phenazepam.

2. Cells of the actinomycetes immobilized in poly(vinyl alcohol) in the presence of a cosubstrate perform the microbiological synthesis with a 15% yield of 3-hydroxyphenazepam.

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LOW-MOLECULAR-WEIGHT METABOLIES OF WHEAT.

I. COMPONENTS OF AN ETHEREAL EXTRACT OF WHEAT LEAVES

I. V. Egorov, V. N. Gramenitskaya, and N. S. Vul'fson

UDC 63.311+547.992

The chromatographic separation of the components of an ethereal extract of the leaves of "Mironovskaya 808" wheat has been performed. The presence in wheat of phthalic acid and its dimethyl, diethyl, and dibutyl esters has been shown for the first time. Substituted benzoic and cinnamic acids, p-hydroxybenzaldehyde, vanillin, 6-methoxybenzoxazolone, tricin, and aconitic and fumaric acids have also been isolated. The structures of the compounds isolated have been confirmed by IR, UV, mass, and NMR spectra.

Questions of the chemical regulation of the productivity of agricultural crops are connected primarily with an understanding of the inherent endogenous regulation of a plant. It is necessary to know the complex of low-molecular-weight metabolites characteristic for each plant and the changes in its composition taking place in the process of growth and development and to understand the role of each component of this complex. With respect to the most important agricultural crop — wheat — so far the attention of workers has been attracted mainly by questions of changing the composition of this biologically active substances in various periods of ontogenesis (without studying their structure, solely on the basis of histograms; see, for example, [1, 2]). The participation of known compounds widely distributed in plants has also been investigated. Thus, the dynamics of the levels of the most important phytohormones in wheat shoots in vernalization [3], in the developing heads [4], and in the seeds [5] has been studied. It has been shown that the compositions and amounts of free and bound forms of phenolic compounds are linked with lignification processes (see, for example, [6]), with the resistance of the plants to stem rust [7], and with drought resistance [8]. There are reports of considerable fluctuations in the amount

M. M. Shemyakin Institute of Bioorganic Chemistry, Academy of Sciences of the USSR, Moscow. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 783-789, November-December, 1981. Original article submitted April 23, 1981.

 0009-3130/81/1706-0574$07.50

TABLE 1. Compounds Isolated from an Ethereal Extract of the Leaves of Mironovskaya 808 Winter Wheat

Compound	Amount, mg/kg of dry wheat	Form, mp, °C (solvent)	R_f in solvent systems		
			1	4	5
Dibutyl phthalate	0,36	Oil	0,40	0,82	0,94
Diethyl phthalate	Ca.		0,28	0 77	0,87
Dimethyl phthalate	0,34	Oil	0,21	0,69	0 74
Vanillin	0,10	Crystals	0,05	0,46	0,53
p-Hydroxybenzaldehyde	0,08	Crystals	0,05	0,38	0,62
p-Hydroxybenzoic acid	0,40	212–213 (ether)*		0,21	0,58
Syringic acid	0,37	200–202 (ether)*		0,12	0,28
p-Coumaric acid	0,28	204–206 (benzene–acetona)*		0,19	0,52
Ferulic acid	0 35	167–168 (benzene–acetone)*		0,20	0,39
6-Methoxybenzoxazolone	0,37	156 157 (acetone–hexane)†		0,43	0,50
Protocatechuic acid	0,10	Crystals		0,09	0,40
Tricin	5,01	287–289 (methanol)*		0 11	0,15
Phthalic acid	0,10	Crystals		0,14	0,30
Vanillic acid	0,18	Crystals		0,24	0,47
Aconitic acid (mixture of cis and trans isomers)	20,00	145–147 (acetone–benzene)*		0,04	0,11
Fumaric acid	1,00	300–301 sealed capillary (acetone–benzene)*		0,16	0,54

*According to the literature, see [31].
†According to the literature, see [29].

of acids of the Krebs cycle [9] and of the influence of these acids on metabolic processes [10]. Information is also known about the influence of the temperature on the composition of the sterols of wehat [11], and its changes in spring in the period of ripening [12].

There are considerably fewer publications on the isolation and deciphering of the structures of individual compounds of wheat. They include reports of the isolation of tricin [13], of derivatives of apigenin, of luteolin, and of cyanidin [13, 14], of diferulic acid [15], and also of a number of protective substances – benzoxazinone derivatives [16] and the 2-hydroxyputrescinamides of ferulic and p-coumaric acids [17].

We have begun a systematic study of the complex of low-molecular-weight metabolites of wheat roots and leaves with the aim of a more detailed investigation of the processes of chemical regulation of this crop. The present communication gives the results of the chromatographic separation and identification of the components of an ethereal extract of the leaves of Mironovskaya 808 winter wheat collected in the period when the processes determining the sexual differentiation of the head are taking place. Chromatography on a column of silica gel followed by purification with the aid of TLC permitted the isolation of several substituted benzoic and cinnamic acids, p-hydroxybenzaldehyde, vanillin, 6-methoxybenzoxazolone, tricin, and aconitic and fumaric acids (Table 1), which were identified by IR, NMR, mass, and UV spectroscopy and, in the majority of cases, by comparison with authentic samples. In addition to the compounds mentioned, the presence of which in other varieties of wheat was known previously, we have also isolated phthalic acid and its dimethyl, diethyl, and dibutyl esters which have not hitherto been detected in cereal crops. There is only sparse information in the literature on the isolation of phthalic esters from plants (see, for example, [18-23]). In view of the wide use of phthalates, in particular, as plasticisers the question usually remains open as to whether they have passed into an extract from the plant material or were present in the solvents used for extracting the plants. Japanese authors have isolated from pea pods an oil the saponification of which gave phthalic acid and a set of low-molecular-weight alcohols. However, the results were not reproduced when redistilled methanol was used for extraction [24]. To check the results that we have obtained, using redistilled solvents (in which the possible presence of phthalates was checked) we have carried out a chromatographic separation of the weakly polar fraction of an extract of another sample of leaves collected in the same period of development of wheat as the first. In this case, likewise, we isolated dimethyl, diethyl, and dibutyl phthalates, which

were identified by their characteristic mass spectra and the agreement of their R_f values with those of authentic samples on chromatography in three different solvent systems (see Table 1).

Since in the experiments described above, methanol was used for extraction and it could be assumed that the dimethyl phthalate was an artifact, we have also used the procedure of Yamanishi et al. [20] for the isolation of phthalates. These authors evaporated the water with which pea leaves had been covered, and from an ethereal extract of the aqueous distillate obtained they isolated dibutyl phthalate. In the analogous treatment of wheat leaves, stems, and roots we detected (chromatographically) the presence of dimethyl, diethyl, and dibutyl phthalates only in the leaves and stems. It must also be mentioned that these compounds were not found in an ethereal extract of a control experiment in which all the operations were repeated without the plant material.

Thus, the results given above permit us to speak with some confidence of the presence of the dimethyl, diethyl, and dibutyl esters of phthalic acid in wheat leaves and stems. It may also be assumed that the phthalic acid that we have isolated is not an artifact but is present in wheat leaves in the free state, since the freshly collected plant material was fixed at a low temperature and the extracts obtained from it were not subjected to alkaline treatment.

EXPERIMENTAL

IR spectra were recorded on a UR-20 spectrophotometer (GDR), and UV spectra on a Cary-15 Becmar spectrophotometer (USA). Mass spectra were obtained on an MKh-1309 instrument with direct introduction into the ion source, and the high-resolution mass spectrum on a Varian MAT CH-5 instrument (GFR). The ^1H and ^{13}C NMR spectra were recorded on a Varian XL-100 instrument (USA) — with partial proton decoupling, in the case of ^{13}C resonance. Chemical shifts are given in parts per million (ppm) in the δ scale from tetramethylsilane (internal standard), and spin–spin coupling constants (J) in Hertz (Hz); abbreviations: s — singlet; d — doublet; t — triplet; m — multiplet. Below, in comparing the spectra obtained with those published previously only references to the literature source are given.

Melting points were determined on a Boëtius micro heated stage.

Analytical TLC was carried out on Silufol plates (Czechoslovakia), the substances being revealed in iodine vapor and by spraying with concentrated H_2SO_4, and also in UV light at 254 and 360 nm.

For preparative TLC, glass plates (20 × 20 cm) were coated with a nonfixed layer of silica gel (0.2 mm) of type LL_{254} 5/40 μ. For column chromatography we used silica gel of type L 100/160 (Czechoslovakia). The following solvent systems were used for chromatography on plates: 1) heptane–benzene–chloroform–ethyl acetate (10:2:2:1); 2) heptane–dioxane–acetic acid (40:8:1); 3) diisopropyl ether–acetic acid (20:1); 4) heptane–hexane–dioxane–methyl ethyl ketone–acetic acid (4:30:16:10:1); 5) diisopropyl ether–acetic acid (10:1); and 6) benzene–dioxane–acetic acid (15:8:1).

Leaves of Mironovskaya 808 winter wheat (110 kg) collected in May, 1978 on an experimental plot of the Botanical Gardens of Moscow State University were fixed with solid carbon dioxide, ground, and repeatedly extracted with methanol until the material had been decolorized. The methanol was evaporated off in a rotary evaporator (30°C, 25 mm) until a precipitate of chlorophyll deposited, and then the precipitate was filtered off, the methanol was evaporated off and the residual aqueous solution was extracted with ether (the completeness of extraction was checked by evaporating the successive extracts). Evaporation of the ether yielded 7.0 g of dry residue which was then separated on a column (4 × 100 cm) filled with silica gel in a benzene–acetone gradient. The fraction volume of the solutions taken from the column was 20 ml. Then, on the basis of analysis with the aid of TLC in system 4, selected solutions were added to one another to form 15 combined fractions. By chromatography on silica gel plates in systems 1 and 2, the first five fractions yielded esters of phthalic acid.

Dibutyl phthalate; IR (film of the substance, ν, cm^{-1}): 3440, 1730, 1480, 1390, 1350, 1250; ^1H NMR (CCl_4): 0.90 (6 H, m, J 7.0, CH_3); 1.52 (8 H, m, CH_2); 4.20 (4 H, m, J 7.0, OCH_2); 7.54 (4 H, m, $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ 5.7; $J_{2,4}$ and $J_{3,5}$ 3.5; $J_{2,5}$ 1.0; ArH). For the mass spectrum see [25].

Diethyl phthalate, for the mass spectrum, see [25].

Dimethyl phthalate; IR (film of the substance, ν, cm^{-1}): 1730, 1600, 1500, 1300, 1130, 1080; ^1H NMR (CCl_4): 3.98 (6 H, s, CH_3); 7.56 (4 H, m, $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ 5.8; $J_{2,4}$ and $J_{3,5}$ 3.6; $J_{2,5}$ 1.2; ArH). For the mass spectrum, see [25].

By chromatography on plates in system 3, fractions 6-9 yielded vanillin; IR (KBr, ν, cm^{-1}: 3200, 1670, 1570, 1270, 1170, 1160; ^1H NMR [$(CD_3)_2CO$]: 3.94 (3 H, s, OCH_3); 7.08 (1 H, d, J 8.0, 5-H); 7.49 (1 H, d, J 1.0, 2-H); 7.52 (1 H, q, $J_{5,6}$ 8.0, $J_{2,6}$ 1.0, 6-H); 8.64 (1 H, s, OH); 9.88 (1 H, s, CHO). For the mass spectrum, see [25, 26].

Fractions 10-14 were chromatographed successively in a column (1.5 × 100 cm) in system 4 and the zones for the same R_f value (according to TLC in system 4) obtained as a result were combined. The combined zones were chromatographed on plates in system 5, which led to the isolation of a series of compounds the results of analyses of which are given below.

p-Hydroxybenzaldehyde; for mass spectrum, see [26].

p-Hydroxybenzoic acid; for the ^1H NMR spectrum, see [27]; for the mass spectrum, see [25].

Syringic acid; IR (mull with paraffin oil, ν, cm^{-1}): 3600-3480, 2570, 1680, 1600, 1500, 900; ^1H NMR [$(CD_3)_2CO$]: 3.88 (6 H, s, OCH_3); 5.56 (2 H, m, OH, COOH); 7.35 (2 H, s, 2-H and 6-H). For the mass spectrum, see [26].

p-Coumaric acid; ^1H NMR [$(CD_3)_2CO$]: 6.48 (1 H, d, J 16.0, α-H); 6.92 (2 H, d, J 8.0, 2-H and 6-H); 7.56 (2 H, d, J 8.0, 3-H and 5-H); 7.66 (1 H, d, J 16.0, β-H); for the mass spectrum, see [28].

Ferulic acid; ^1H NMR [$(CD_3)_2CO$]: 3.94 (3 H, s, OCH_3); 6.42 (1 H, d, J 16.0, α-H); 6.92 (1 H, d, J 8.0, 5-H); 7.20 (1 H, q, $J_{5,6}$ 8.0, $J_{2,6}$ 2.0, 6-H); 7.36 (1 H, d, J 2.0, 2-H); 7.76 (1 H, d, J 16.0, β-H); 8.90 (2 H, m, OH and COOH). For the mass spectrum, see [26].

p-Methoxybenzoxazolone; for the ^1H NMR spectrum, see [29]; mass spectrum, m/z: M^+ measured 165.0423; calculated for $C_8H_7NO_3$, 165.0426; $M^+ - CH_3$ measured 150.0189; calculated for $C_7H_4NO_3$, 150.0191; $M^+ - CH_3 - CO_2$, measured 169.0292; calculated for C_6H_4NO, 106.0293.

Protocatechic acid; for the ^1H NMR spectrum, see [27]; mass spectrum (130°C), m/z (relative intensities, %): M^+ 154 (100), 137 (100), 110 (45), 91 (17), 81 (19), 69 (14), 57 (25), 55 (25).

Tricin; the UV spectra taken in methanol and with the addition of $AlCl_3$ or sodium methanolate, were identical with those given in the literature (see [30]); ^1H NMR [$(CD_3)_2CO$]: 3.89 (6 H, s, OCH_3); 3.34 (2 H, m, 7-OH and 4'-OH); 6.24 (1 H, d, J 2.0, 6-H); 6.59 (1 H, d, J 2.0, 8-H); 6.98 (1 H, s, 3-H); 7.34 (2 H, s, 2'-H and 6'-H); 12.96 (1 H, s, 5-OH); ^1H NMR (CD_3OH): 3.94 (6 H, s); 6.10 (1 H, d J 2.0); 6.32 (1 H, d, J 2.0); 6.54 (1 H, s); 7.20 (2 H, s).

Phthalic acid; IR (mull with paraffin oil, ν, cm^{-1}): 2650, 2530, 1700, 1680, 1580, 900, 740; ^1H NMR [$(CD_3)_2CO$]: 6.72 (2 H, m, COOH); 7.67 (2 H, m, 4-H and 5-H); 7.82 (2 H, m, 3-H and 6-H); for the mass spectrum, see [25]; the mass spectrum of a sample treated with diazomethane agreed completely with the spectrum of dimethyl phthalate taken under the same conditions.

Vanillic acid; ^1H NMR [$(CD_3)_2CO$]: 3.93 (3 H, s, OCH_3); 6.96 (1 H, d, J 9.0, 5-HO; 7.61 (1 H, d, J 2.0, 2-H); 7.66 (1 H, q, $J_{2,6}$ 2.0, $J_{5,6}$ 9.0, 6-H); 8.99 (2 H, m, OH and COOH); for the mass spectrum, see [26].

From a solution in acetone of fraction 15 the addition of benzene yielded aconitic acid, and chromatography of the residue in system 6 yielded fumaric acid.

Aconitic acid; IR (mull with paraffin oil, ν, cm^{-1}): 3400 – 3300, 2800 – 2600, 1730, 1710, 1700, 1430, 1300, 1230, 910, ^1H NMR [$(CD_3)_2CO$]: 3.94 (2 H, s, CH_2); 6.97 (1 H, s, CH); 10.77 (3 H, m, COOH); ^{13}C NMR (CD_3OD): 35.8 (CH_2); 130.8 (CH); 142.3 (>C=); 168.6 ($-CH_2COOH$); 174.2 (=CHCOOH); mass spectrum (50°), m/z (relative intensity, %): M^+ 156 (10), 139 (12), 138 (45), 130 (7), 128 (9), 112 (45), 105 (30), 84 (68), 77 (8), 69 (13), 68 (27), 67 (19), 66 (10), 55 (14), 53 (10), 45 (60), 44 (100), 43 (26). Found, %: C 41.30; H 3.49; O 55.21. $C_6H_6O_6$. Calculated, %: C 41.39; H 3.47; O 55.14.

Fumaric acid; IR (mull with paraffin oil, ν, cm^{-1}): 3090, 2700, 2500, 1670, 900; ^1H NMR (D_2O): 6.80, (s); mass spectrum (30°C), m/z (relative intensity, %): M^+ 116 (40), 99 (40), 98 (100), 88 (30), 81 (12), 72 (20), 71 (25), 70 (12), 55 (16), 54 (17), 53 (30), 45 (80), 43 (20), 42 (12), 41 (10); the mass spectrum coincides with that of an authentic sample taken under the same conditions.

Experiments Confirming the Presence of Phthalic Acid Esters in the Plant Material. All the solvents used in the experiments described below were redistilled through a column of 25 theoretical plates and the presence of phthalic acid esters in the residue after distillation was checked with the aid of TLC.

a) Leaves of Mironovskaya 808 winter wheat (10 kg) collected in May, 1979, on experimental plots of the Institute of Applied Molecular Biology and Genetics were kept for a day in the refrigerator at −60°C and were extracted with methanol; the methanol extract was evaporated and the chlorophyll was filtered off as described above. The aqueous layer obtained (about four liters) was extracted with carbon tetrachloride (5 × 2 liters), and the extract was filtered through a column (15 × 2 cm) filled with silica gel (previously washed with methanol and dried) to eliminate the last traces of chlorophyll. After evaporation of the solvent, 50.1 mg of a mixture was obtained which was separated on plates in system 1. This gave fractions with R_f values in systems 1, 4, and 5 coinciding with those of dimethyl phthalate (0.2 mg), diethyl phthalate (0.6 mg), and dibutyl phthalate (7.0 mg). These fractions were additionally purified by chromatography on plates in system 1 and the narrow zones of phthalic acid esters isolated were identified with the aid of mass spectrometry.

b) Samples (100 g each) of leaves, stems, and roots of Mironovskaya 808 winter wheat collected in October, 1979, on experimental plots of Moscow State University were kept at −70°C for 4 h and were then ground to the state of a powder and each was covered with a liter of distilled water. The water was distilled off under reduced pressure at a bath temperature of 50°C (see also [20]), after which 300 ml of ether was added to the flask containing the plant material, and this was then distilled off in the same apparatus at atmospheric pressure into the receiver containing the aqueous distillate. After the removal of the ethereal layer the aqueous distillates obtained were each extracted with ether (3 × 300 ml). Similar operations were performed in a control experiment where water was distilled off from a flask containing no plant material. The volume of the total ethereal extract obtained in each experiment was brought to 1 ml by distilling off the ether. Then this extract was analyzed by TLC in comparison with authentic samples of dimethyl, diethyl, and dibutyl phthalates in solvent systems 1, 4, and 5. Analysis showed the presence of the esters mentioned in the extracts with wheat leaves and stems.

SUMMARY

The chromatographic separation of the components of an ethereal extract of the leaves of Mironovskaya 808 winter wheat has been carried out. The presence of phthalic acid and its esters in wheat has been shown for the first time. The substituted benzoic and cinnamic acids, p-hydroxybenzaldehyde, vanillin, 6-methoxybenzoxazolone, and tricin, and also aconitic and fumaric acids have been isolated.

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