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DERIVATIVES OF UNSATURATED AROMATIC ALCOHOLS IN PROPOLIS AND STYRAX BENZOIN

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UDC 547.915

The benzoate of trans-coniferyl alcohol and the benzoate of trans-p-coumaryl alcohol have been obtained from propolis and the styrax benzoin, this being the first time that the latter has been described.

The basis of propolis consists of the resinous secretions of certain species of woody plants, including the axillary buds of the birch, poplar, and pine [1, 2]. One of the main components of propolis collected from pine buds is, as we have shown previously [2], a substance with a molecular weight of 284 and the empirical formula $C_{17}H_{16}O_4$. It is readily detected by thin layer chromatography on silica gel in the form of a cherry-red spot when the plates are sprayed with concentrated H_2SO_4 and is convenient for the purposes of standardization.

The isolation of this compound in the pure form is associated with considerable difficulties in view of its instability in the presence of atmospheric oxygen. The best results in the purification of the substance were achieved with preliminary separation of the extract on a column of alumina (Brockman activity grade V) followed by separation of the enriched fraction on a column of silica gel and its final purification by preparative thin-layer chromatography (PTLC). To prevent subsequent oxidation, a large part of the fraction isolated was acetylated by the action of Ac_2O in pyridine. The substance isolated in this way (I) and its acetate were investigated by the methods of physicochemical analysis.

M. M. Shemyakin Institute of Bioorganic Chemistry, Academy of Sciences of the USSR, Moscow. Translated from Khimiya Prirodnykh Soedinenii, No. 2, pp. 152-160, March-April, 1984. Original article submitted February 22, 1983.

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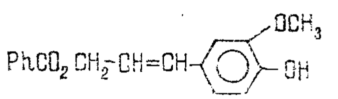
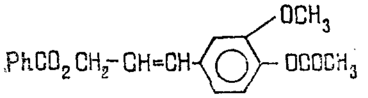
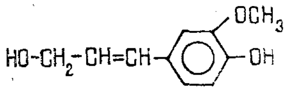
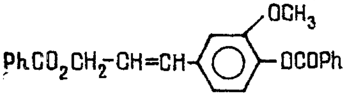
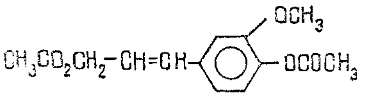
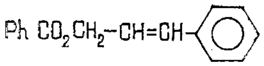
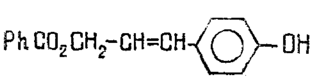
TABLE 1. Features of the NMR Spectra of Derivatives of Unsaturated Aromatic Alcohols Isolated from Plant Sources and of a Number of Model Compounds

Compound	Formula	Solvent	Protons, ppm			
			OCH_3	$OCOCH_3$	OCH_2	CH=CH–Ar
I		CCl_4	3,82	—	4 98 J=6	6,58 J=16
I–Ac		CCl_4	3,80	2,22	4,90 J=6	6,62 J=16
II		$(CD_3)_2CO$	3,88	—	4,21 J=6	6,52 J=16
III		$(CD_3)_2CO$	3,87	—	5,03 J=6	6,88 J=16
IV		CCl_4	3,80	2,22	4,64 J=6	6,56 J=16
V*		$CDCl_3$	—	—	4,96 J=6	6.74 J=16
VI		$(CD_3)_2CO$	—	—	4,97 J=6	6,78 J=16

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*The specimen was obtained by the action of benzoyl chloride on cinnamyl alcohol in the presence of pyridine.

The NMR spectrum of the acetate of (I) (CCl_4) (Table 1 and Fig. 2) showed the presence of, in addition to the signals of the three protons of the OAc group at 2.22 ppm and the three protons of a OMe group at 3.80 ppm, the signals of another twelve protons in the weak-field region. A doublet two-proton signal with J = 6 Hz at 4.90 ppm corresponds to a methylene grouping in an α,β-unsaturated alcohol. In this case, a one-proton doublet signal at 6.62 ppm with J = 6 Hz can be assigned to an α-methine proton present in the trans position to a neighboring H atom.

The signals of the other protons present in the weak-field region were assigned to two aromatic rings the structures of which were elucidated in the following way.

In the mass spectrum of the initial ester strong peaks were observed with m/z values of the ions formed of 122 and 123, which are characteristic for benzoic acid derivatives [3]. Measurement of the high-resolution mass spectra confirmed the compositions of these ions as $C_7H_6O_2$ and $C_7H_7O_2$. On the other hand, the mass spectrum also contained a strong ion at 179 m/z, which is characteristic for a residue of a coniferyl alcohol residue, as was confirmed by its composition, $C_{10}H_{11}O_3$, measured by high-resolution mass spectrometry (Tables 2 and 3).

The combination of these facts permits the assumption that the compound isolated was the benzoate of trans-coniferyl alcohol (I).

TABLE 2. Empirical Formulas and R_f Values of Compounds (I) and (VI) Isolated from Propolis and Styrax Benzoin

Compound	Empirical formula*	Molecular weight		TLC results†	
		calc.	meas.	R_f	coloration of the spot
I	$C_{17}H_{16}O_4$	284,1049	284,1018	0.6	Cherry-red
VI	$C_{16}H_{14}O_3$	254,0943	254,0944	0.5	Deep blue

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*According to the results of high-resolution mass spectrometry.
†On Silufol in the benzene—acetone (9:1) system, detected with concentrated sulfuric acid.

TABLE 3. Compositions of the Characteristic Ions of the Esters (I) and (VI) from the Results of High-Resolution Mass Spectrometry*

Compound	a		b		c	
	calc.	meas.	calc.	meas.	calc.	meas.
I	105,0340	105,0332	122,0368	122,0364	179,0708	179,0712
VI	105,0340	105,0332	122,0368	122,0358	149,0602	149,0606

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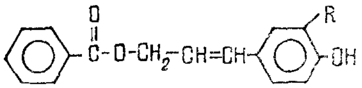
*For the designations of the ions and the fragmentation pathway, see Figs. 1 and 3.

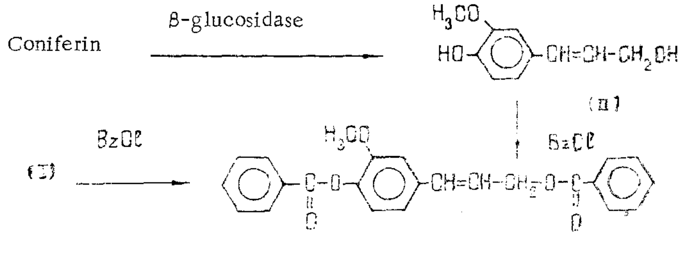


(I) R=OCH$_3$; (VI) R=H

This hypothesis was in good agreement with the characteristics of the NMR spectra of compounds (II-V), which are related to substance (I), including the benzoate of cinnamyl alcohol (V) and a diacetate of trans-coniferyl alcohol (IV) (Table 1). Thus, in the NMR spectrum of the acetate of trans-coniferyl alcohol taken in the same solvent (CCl_4) as acetate (I), the protons of the aromatic ring formed a three-proton singlet peak at 6.88 ppm, and the signal of the α-methine proton of the $-CH{=}CH-CH_2-O$ group was represented by a doublet at 6.5 ppm with J = 16 Hz. The signals of the methyl and acetyl groups in compounds (II-IV) had the same chemical sequence as in the acetate of (I) (Table 1). So far as concerns the groups of signals in the weak field represented by a two-proton quadruplet doublet with J = 2 and 9 Hz at 8.0 ppm and a three-proton unresolved group of peaks at 7.38 ppm, they were present in the range of signals of protons of the aromatic ring of benzoic acid in a synthetic sample of the dibenzoate of trans-coniferyl alcohol (III) that we obtained (at 8.10 ppm with J = 2 and 9 Hz, respectively, and a group of peaks at 7.38 ppm).

In order to achieve a definitive confirmation of the structure of the compound isolated (I), it was converted by the action of benzoyl chloride into the corresponding dibenzoate (III), which in its turn, was obtained from an authentic sample of natural coniferin by its subsequent enzymatic hydrolysis with β-glucosidase to trans-coniferyl alcohol and the benzylation of the latter in the presence of pyridine (scheme).

According to R_f values and NMR and mass spectrometry, the two benzoates were completely identical.



The structure of the benzoate of trans-coniferyl alcohol (I) was previously ascribed by Reinitzer to the main components of the Styrax benzoin, which possesses a number of valuable

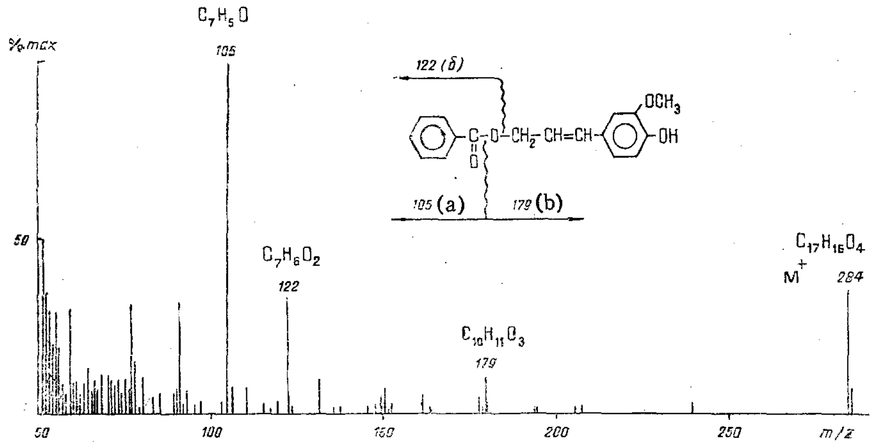


Fig. 1. Mass spectrum of compound (I).

properties and is exported to Europe from the countries of Southeast Asia for the needs of the perfumery and pharmaceutical chemical industry in considerable amounts. This resin is collected from the trunks of trees, from which it runs in response to an artificial injury [8].

According to Reinitzer [5], compound (I) makes up 75-80% of the weight of Styrax benzoin. The resin also contains a considerable amount of free benzoic acid (10-12%), cinnamyl benzoate (2-3%), and a number of other components [5, 6].

In fact, from a sample of this resin by PTLC we isolated compound (I) as the main component, this proving on the basis of R_f values and mass and NMR spectra to be completely identical with the substance that we had isolated from propolis (Figs. 1 and 2).

By chromatographing an extract of the natural resin, we detected in it another compound, related to substance (I), which was then isolated by TLC on silica gel in the benzene–acetone (9:1) system. Its structure was determined on the basis of the results of NMR spectroscopy and of high- and low-resolution spectrometry (Tables 1, 2, and 3, and Figs. 3 and 4). The compound has the empirical formula $C_{16}H_{14}O_3$, and its fragmentation under the action of electron impact was accompanied by the ejection of ions with m/z 149, 122, and 185, which, according to the results of high-resolution mass spectrometry, corresponded to residues of p-coumaryl alcohol and of benzoic acid.

The NMR spectrum of this substance taken in trideuteroacetone solution indicated the presence in the molecule of a methylene grouping of an α,β-unsaturated alcohol, the protons of the CH_2 group of which had a chemical shift of 4.97 ppm and a spin–spin coupling constant of 6 Hz. Analysis of the signals of the other weak-field protons showed that the molecule contained at least two aromatic rings, one of which was monosubstituted and the other p-disubstituted (two doublets of two-proton signals at 6.84 and 7.36 ppm with J = 9 Hz). The chemical shift of the signal of the protons of the methylene group in the compound under investigation proved to be the same as in the model compound cinnamyl benzoate (4.97 and 4.96 ppm, respectively) and differed sharply from that in trans-coniferyl alcohol (II), where the primary alcohol group is unsubstituted (4.21 ppm) (Table 1). This shows that the benzoic acid residue in the molecule of the natural ester is attached to the aliphatic OH group and the substance isolated therefore has the structure (VI).

Since both the esters isolated proved to be benzoic acid derivatives, we analyzed the acid fraction of propolis to determine the presence of this acid. The fraction containing the aromatic acids was extracted from an ethyl acetate extract by an aqueous solution of sodium bicarbonate. After the usual working up, the acids were subjected to the action of N,O-bis(trimethylsilyl)acetamide, and the resulting TMS esters were separated by TLC on a column containing 3% of OV-17. The esters were identified in comparison with authentic samples by a procedure that we have described previously [7].

It was found that the propolis extract contained, in addition to benzoic acid, smaller amounts of other aromatic acids, including p-coumaric, ferulic, and caffeic (Fig. 5).

Characteristic for benzoic acid is a considerable antifungal activity which is exhibited at acid pH values [8]. We have shown previously [9] that a fraction obtained by the chromato-

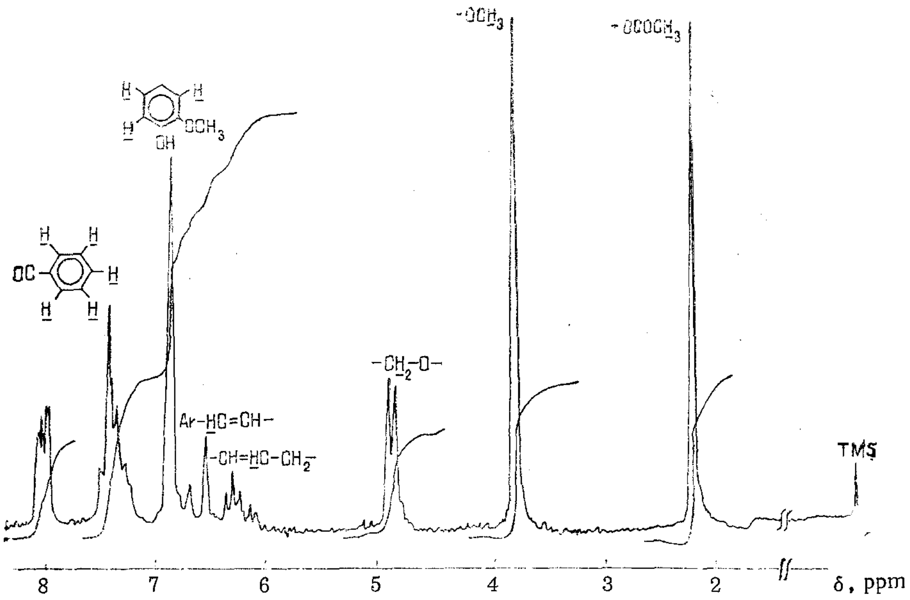


Fig. 2. NMR spectrum of the acetate of (I).

graphic separation of an ethanolic extract of propolis and possessing an increased antimicrobial activity also contained this acid. It is interesting to note that benzoic acid is the main protective substance in the nectar of sweet clover or honeysuckle (*Hedysarum*). In the honey collected from this source, as one of us has shown previously, it is present in a concentration of 1000 γ/ml, which proves to be sufficient for preventing the development of various fungi and microorganisms in it [10]. According to Brown and Swinburne [11], in unripe apples this substance exhibits the properties of a phytoalexin, being formed only in response to an infection of the fruit with the pathogenic fungus *Nectria galligena* and prevents its further development.

Biological trials of compounds (I) performed in the All-Union Scientific-Research Institute of Chemical Agents for Plant Protection have shown that the compound exhibits a considerable growth-inhibiting activity, suppressing the development of culture cells of *Chlorella vulgaris* in a concentration of 10^{-4} M.

Schneidewind et al., investigating one of the samples of propolis collected from the apiaries of the GDR, has also detected the presence in it of various esters of unsaturated aromatic acids, including derivatives of p-coumaric and caffeic acids [13]. The authors were unable to establish the structures of these compounds accurately, but Schneidewind gave information indicating their considerable antifungal activity. We have previously reported the identification in propolis of a number of related substances [12].

Judging from the literature, derivatives of this type of compounds are widely represented in other resins possessing biological activity. In particular, cinnamyl alcohol derivatives are present in balsam of Tolu and balsam of Peru, which enables us to judge their relatively wide distribution in natural sources:

	Derivatives of the alcohol			
Source	Cinnamyl	p-Coumaryl	Coniferyl	Literature
Propolis	—	+	+*	[12]
Styrax benzoin	+	+*	+	
Balsum of Peru	+	—	—	[4, 5, 6]
Balsum of Tolu	+	—	—	[14]
Secretion of axillary stem buds	—	+	+	[12]

*Present communication.

The identification of the esters described in propolis expands the list of compounds responsible for its many-sided biological activity and permits the use of some of them — in particular, substance (I) — as a convenient marker for the purposes of standardizing propolis preparations.

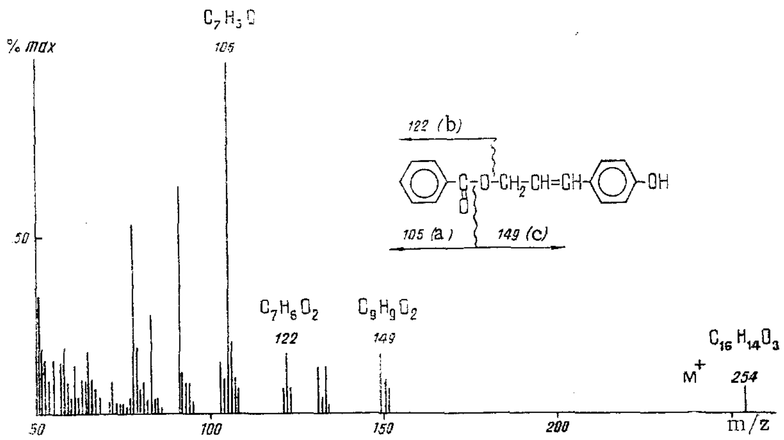


Fig. 3. Mass spectrum of compound (VI).

EXPERIMENTAL

Propolis ("aspen" type [1, 2]) was collected in the apiary of the experimental apiarist A. P. Soldatkin, located close to the village of Vostryakovo, Domodevevo region, Moscow province. Gum benzoin (Styrax benzoin) was provided by the "Novaya Zarya" Moscow perfumery factory, a sample of trans-coniferyl alcohol β-D-glycoside (coniferin) was obtained from the V. I. Vernadskii Institute of Geochemistry of the Academy of Sciences of the USSR, the caffeic acid was of "chda" ["pure for analysis"] grade (USSR), and the p-coumaric and ferulic acids were from Sigma (USA).

The low-resolution mass spectra were taken on a MKh-1309 instrument at 80-100°C with an ionization potential of 70 V. High-resolution mass spectra were taken on an Varian-MAT-44 instrument, NMR spectra on a Varian-XL 100 instrument with TMS as internal standard, and IR spectra on a UR-20 instrument. GLC was performed on a Tsvet-101 instrument with a flame-ionization detector using 3000 × 3 mm columns filled with 3% of OV-17 on Chromosorb W in the temperature interval from 100 to 300°C at the rate of 5°C/min.

All the extracts and eluates of the fractions were evaporated in vacuum at a temperature not exceeding 40°C, and the sorbents used for column chromatography were silica gel L 100-160 μ (Chemapol, Czechoslovakia) and alumina "for chromatography," TU-6-09-3916-75 (USSR). Glass plates with dimensions of 20 × 20 cm with silica gel LL_{254} 5-40 μ (Chemapol, Czechoslovakia) deposited on them by hand were used for preparative thin-layer chromatography, and analytical thin-layer chromatography was carried out on Silufol UV_{254} plates (Czechoslovakia) in the benzene—acetone (9:1) system. The spots of the compounds were revealed by spraying with concentrated H_2SO_4.

Isolation of the Benzoate of trans-Coniferyl Alcohol (I) and the Preparation of Its Acetate. A. From Propolis. Propolis shavings (28 g) were steeped in 500 ml of ethanol at 20°C for 24 h, and then the mixture was filtered and evaporated in vacuum. The residue obtained (21.5 g) was dissolved in 100 ml of chloroform, 10-15 g of silica gel was added, and evaporation to dryness in vacuum was repeated. The new residue obtained was ground in a mortar and deposited on a column (70 cm × 100 mm) filled in benzene with 300 g of alumina (Brockman activity V). The column was eluted with 1 liter of benzene—acetone (7:3) which yielded fractions with R_f 0.6-0.7 (which were characterized by a cherry-red color after spraying with concentrated H_2SO_4). After evaporation in vacuum, the residue (5.78 h) was rechromatographed on a column (70 × 100 mm) containing 140 g of silica gel filled in n-heptane. Elution was carried out with a n-heptane—ethyl acetate gradient system. Fractions were collected with R_f 0.6 and were evaporated in vacuum to give 250 mg of dry residue. Part of it (50 mg) was additionally purified for the purposes of physicochemical analysis and biological trials by the PTLC method on silica gel in the benzene—acetone (9:1) system, and the remainder (200 mg) was dissolved in 10 ml of pyridine and acetylated by the addition of 5 ml of Ac_2O. The reaction mixture was kept at 20°C for 12 h, after which it was poured into water, the acetate formed was extracted with ethyl acetate, and the extract was washed with dilute HCl, with 5% sodium bicarbonate solution, and with water and was dried with magnesium sulfate. After filtration and evaporation of the solution in vacuum, the residue was additionally purified by PTLC on silica gel, and 110 mg of the acetate of (I) was obtained in the form of an oil.

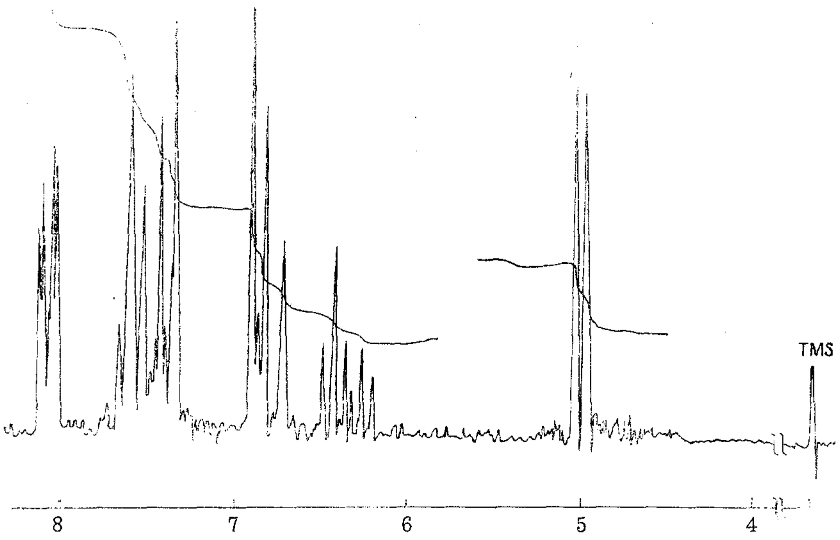


Fig. 4. NMR spectrum of compound (VI).

The mass spectrometric and NMR spectroscopic characteristics of (I) and its acetate are given in Table 1 and in Figs. 1 and 2.

B. From Styrax Benzoin. Compound (I) was isolated from 300 mg of the plant resin Styrax benzoin as described by Freudenberg [15]. This gave 200 mg of a crystalline substance with mp 71°C (ether–petroleum ether); according to [15], mp 72°C.

Isolation of Aromatic Acids from Propolis of the Aspen Type. An extract of the propolis obtained as described previously (0.5 g) was dissolved in 30 ml of ethyl acetate and the solution was extracted with 5% aqueous $NaHCO_3$ (3 × 50 ml). The bicarbonate layer was separated off, acidified with dilute HCl to pH 2, and extracted with ethyl acetate (2 × 50 ml); the extract was dried over $MgSO_4$ and evaporated in vacuum. From the 200 mg of residue of the acid fraction so obtained, 10 mg was treated with 0.4 ml of N,O-bis(trimethyl)acetamide. The resulting esters were analyzed by GLC, being identified by comparison with authentic TMS esters.

Isolation of the Benzoate of trans-p-Coumaryl Alcohol (VI). A sample of Styrax benzoin (250 mg) was dissolved in 10 ml of chloroform, and the resulting mixture was separated by PTLC on silica gel. The resin with R_f 0.5 was eluted with chloroform. After evaporation of the eluate in vacuum, 15 mg of a chromatographically pure substance was obtained in the form of an oil. Its mass and NMR spectra are given in Figs. 3 and 4. IR spectrum (paraffin oil), ν, cm^{-1}: 3380, 1700, 1610, 1590, 1520.

Enzymatic Hydrolysis of the β-D-Glycoside of trans-Coniferyl Alcohol (Coniferin). A solution of 250 mg of coniferin in 6 ml of sodium phosphate-citrate buffer (pH 5) was treated, with stirring, with 2 mg of 92% β-glucosidase (product of the Olaine chemical reagents factory). Stirring was continued until the material had dissolved completely and the solution was kept in a thermostat at 37°C for a day. The resulting reaction mixture was extracted with ether (2 × 30 ml) and the extract was dried with $MgSO_4$ and evaporated in vacuum. This gave 80.3 mg of crystals of trans-coniferyl alcohol (II) with mp 72°C from ether. According to the literature [16], mp 70-71°C. R_f 0.9 (chloroform–methanol (7:3)), 0.2 (benzene–acetone (9:1)), and 0.6 (chloroform–methanol (4:1)) (red coloration on spraying with concentrated H_2SO_4).

The diacetate of trans-coniferyl alcohol (IV) was obtained by the action of acetic anhydride and pyridine on (II) as described above. R_f 0.5 (deep blue coloration on spraying with concentrated H_2SO_4). Mass spectrum, m/z (l): 264 (8) M^+, 222 (75), 180 (30), 179 (48), 131 (100), 103 (62), 91 (87).

Dibenzoate of trans-Coniferyl Alcohol (III). A solution in 3 ml of pyridine of 16.6 mg of a fraction containing substance (I) obtained as described in paragraph A was treated with 2 ml of freshly distilled benzoyl chloride, and the mixture was left overnight. Then it was worked up in the usual way (poured into water, extracted with ethyl acetate, extract washed with dilute HCl, $NaHCO_3$ solution, water, and dried with magnesium sulfate) and was subjected

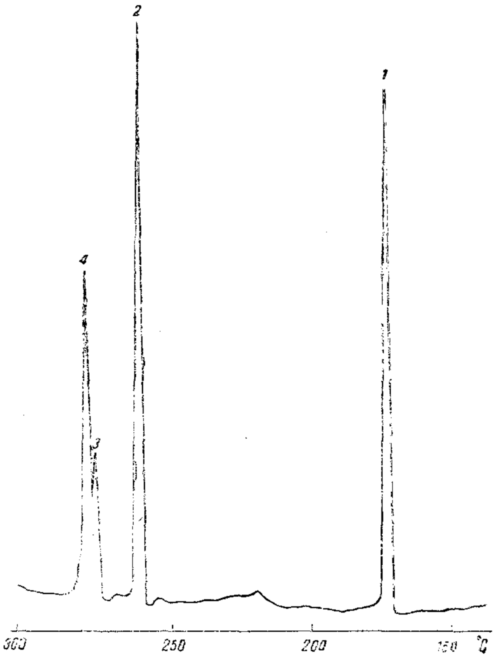


Fig. 5. GLC of the acid fraction of propolis of the aspen type. 1) Benzoic acid; 2) p-coumaric acid; 3) caffeic acid; 4) ferulic acid.

to preparative TLC on silica gel. The dibenzoate formed was eluted from the zone with R_f 0.8 by chloroform. After evaporation in vacuum, 5.5 mg of an oily residue was obtained. Mass spectrum, m/z (l): 388 (92) M^+, 283 (17), 181 (20), 179 (6), 149 (78), 105 (100), 91 (79), 77 (88).

SUMMARY

The benzoates of trans-coniferyl alcohol and of trans-p-coumaryl alcohol have been isolated from propolis and from Styrax benzoin, this being the first time that the latter substance has been described.

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