CHEMICAL INVESTIGATION OF THE BITTER SUBSTANCES OF THE FRUIT OF *Lonicera caerulea*

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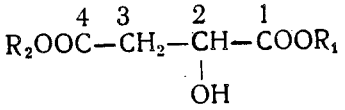
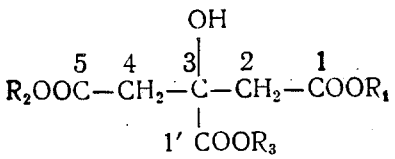
Esters of malic and citric acids have been isolated from an ethyl acetate fraction of an ethanolic extraction of Lonicera caerulea L.: dibutyl malate (I), 4-butyl 1-methyl malate (II), 1-butyl 4-methyl malate (III), tributyl citrate (IV), 1,5-dibutyl citrate (V), 1,1'-dibutyl citrate (VI), 1,5-dibutyl 1'-methyl citrate (VII), 1,1'-dibutyl 5-methyl citrate (VIII), 1-butyl citrate (IX), 1'-butyl citrate (X), 1'-butyl 1,5-dimethyl citrate (XI), and 1-butyl 1',5-dimethyl citrate (XII). Compounds (I-IV, VII, VIII, XI, and XII) had a bitter taste and were the main components of the bitter substances of the fruit of Lonicera caerulea. The structures of compounds (I-XII) were established on the basis of their ^1H and ^{13}C NMR spectra, by FAB mass spectrometry and chromato-mass spectrometry.

The flavor properties of the fruit of sweetberry honeysuckle Lonicera caerula L. are largely due to the presence of a bitter principle in them. A knowledge of the chemical nature of the bearers of this property is of value for the selection and the technology of the processing of the honeysuckle as a food raw material. We have previously [1] detected the iridoid glucoside 7-oxologanin as a bitter component of the fruit of L. caerulea. However, it is only a minor component of the bitter complex. In the present paper we describe the isolation and composition of the main bitter substances of the fruit L. caerulea.

On the chromatographic separation of the ethyl-acetate-soluble material from an ethanolic extract of the fruit we obtained intensely bitter fractions of the substances. Their IR spectra had a band at 1720 cm^{-1} showing the presence of an ester group. By repeated chromatography on silica gel it was possible to obtain several individual compounds and mixtures enriched with one of the components. As was found in the process of study, the main compound of the bitter fractions were esters of malic acid (I-III) and of citric acid (IV-XII):



(I) dibutyl malate; (II) 4-butyl 1-methyl malate; (III) 1-butyl 4-methyl malate;



(IV) tributyl citrate; (V) 1,5-dibutyl citrate; (VI) 1,1'-dibutyl citrate; (VII) 1,5-dibutyl 1'-methyl citrate; (VIII) 1,1'-dibutyl 5-methyl citrate; (IX) 1-butyl citrate; (X) 1'-butyl citrate; (XI) 1'-butyl 1,5-dimethyl citrate; (XII) 1-butyl 1',5-dimethyl citrate.

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Column and thin-layer chromatography did not give a clear separation of the mixtures under investigation. The course of elution of the substances from the columns was monitored with the aid of FAB mass spectrometry. This was due to the fact that citric acid esters do not give the peaks of the molecular ions in EI mass spectra, while the intensity of such ions for malic acid derivatives is low (0.5-1.5%). In the FAB mass spectra, malates are revealed by less intense signals than citric acid derivatives. To determine the compositions of the mixtures and to identify components in the individual case we used chromato-mass spectrometric analysis in combination with NMR spectroscopy.

The main component of the bitter fractions was dibutyl malate (I), which was obtained in the pure form and was identified by spectral methods. The double-resonance method showed an interconnection of the protons of the CH and OH groups (4.48 ppm, m, and 3.57 ppm, d, respectively) in compound (I). The positions of the signals of the protons in the PMR spectrum were confirmed by the change in the nature of the splitting and the magnitudes of the CSs when (I) was acylated with trichloroacetyl isocyanate (TAI): the signal of the proton of the CH group shifted downfield by 1.08 ppm and those of the CH_2 group by 0.2 ppm [2]. 4-Butyl 1-methyl malate (II) and 1-butyl 4-methyl malate (III) were obtained in the form of a mixture. On the chromato-mass-spectrometric analysis of the mixture differences were observed in the fragmentation of (II) and (III). In the spectrum of (III) an ion with m/z 103 had the maximum intensity, and for this the structure ($\overset{+}{HO}{=}CHCH_2CO_2CH_3$) is suggested. In the spectrum of (II) an ion with m/z 89, possibly ($\overset{+}{HO}{=}CHCH_2CO_2H$), had the maximum intensity, and there was no m/z 103 signal. In the PMR spectra of (II) and (III) the signals of the methyl protons resonated at 3.80 and 3.71 ppm, respectively, for the methoxy groups at C-1 and C-4.

The majority of citric acid esters were isolated in the form of mixtures. To identify the citrates in the mixtures containing malates we used the TAI method, and also the spectral characteristics of trimethyl, monobutyl, and dibutyl citrates and their methylated derivatives that had been isolated previously [3]. In the case of the citrate isomers we made use of considerations of molecular symmetry and, correspondingly, the magnetic equivalence or nonequivalence of diagnostic signals in the NMR spectra, and also the presence of intense signals of $[M - COOR_3]^+$ in the chromato-mass spectra of (IV)-(XII).

The chemical shifts of the protons in the PMR spectra of the butyl citrates (IV-VI), (IX), and (X) were the same, and they differed only by the integral intensities of the signals of the butyl groups. We have reported the determination of the structures of (V) and (IX) [3]. The methylation with diazomethane of (V), (VI), (IX), and (X) gave the corresponding bitter derivatives (VII) and (VIII), (XI), and (XII), which were also detected in the native bitter fractions. The different positions of the signals of the protons of the methoxy groups at C-1 and C-5 (3.67-3.69 ppm) and at C-1' (3.81-3.82 ppm) in the PMR spectra of (VII), (VIII), (XI), and (XII) confirmed their structures and the structures of the corresponding precursors. In the EI mass spectra, the fragmentation of (IV), (VII), (VIII), (XI), and (XII) also agreed with their structures.

It is possible to draw certain conclusions concerning the connection of the structures of the malates and citrates with bitterness. The malic acid esters (I)-(III) possessed an intensely bitter taste. Of the citric acid esters, the completely esterified compounds containing at least one butyl radical were bitter. The presence of free carboxy groups in the substances isolated imparted an acidic taste to them. The butyl and dibutyl citrates did not possess a bitter taste.

We have found no information on the presence of the esters (I)-(XII) in plants; compounds (I) and (IV) have been obtained by synthesis [4, 5].

EXPERIMENTAL

PMR and ^{13}C NMR spectra were obtained on a Jeol FX-90Q spectrometer (^1H, 89.55 MHz; ^{13}C, 22.49 MHz) in $CDCl_3$ (δ, 0 – TMS), and IR spectra on a UR-20 instrument using thin layers of the substances. Mass spectra were obtained on a LKB-2091 instrument with an Iontech FAB-ion source by ionization with Xe atoms having an energy of 6 kEV at a discharge current of 1 mA without a matrix. Chromato-mass spectrometric analysis was performed in the same instrument. Capillary column containing SE-30, column temperature 135-240°C (8 deg/min), separator at 290°C, ion source at 300°C, ionizing voltage 70 eV. Angles of optical rotation were determined on a Polarmat instrument.

Extraction and Primary Separation of the Extract. The dry honeysuckle fruit (moisture content 12.7%) collected in Sverdlovsk province (1.5 kg) was extracted five times with 70% ethanol. The extracts obtained were concentrated in vacuum to a syrupy consistency. This gave 730 g of extract (~56% on the absolutely dry weight), which was diluted with water and treated successively with hexane (yield 1.2% on the absolutely dry weight), chloroform (0.4%), ether (0.8%), and ethyl acetate (5.6%). The water-soluble residue was chromatographed on polyamide, and from aqueous eluates a bitter fraction containing oxologanin [1] was obtained. The bitter ethyl acetate fraction was chromatographed on silica gel in the chloroform–methanol system. Chloroform eluted intensely bitter liquid fractions 1-4 with yields of 3.2, 1.2, 2.5, and 2.7 g, respectively. The total yield was ~0.9% on the absolutely dry weight of the fruit.

When the column was eluted with chloroform–methanol (19:1), bitter liquid fractions 5 and 6 were obtained with yields of 8.1 and 6.7 g, respectively (total yield ~1.1% on the absolutely dry weight) and acid-bitter fractions 7-10 with yields of 6.2, 5.6, 4.2, and 4.0 g, respectively (total yield ~1.9% on the absolutely dry weight). Then, as the proportion of methanol in the eluting mixture was increased fractions of substances containing no bitter principle were isolated.

Isolation of Esters of Malic and Citric Acids. The further purification of the bitter fractions was carried out by chromatography on silica gel using as eluents mixtures of hexane and chloroform (system 1), hexane and acetone (system 2), and benzene and acetone (system 3) with increasing proportions of the polar component from 0 to 100%. Analysis of the fractions separated on chromatography was carried out by FAB mass spectrometry and chromato-mass spectrometry.

When fraction 1 (3.2 g) was chromatographed in system 1, 0.02 g of (IV), 0.12 g of a mixture of (I) and (IV), 0.26 g of (I), 0.40 g of a mixture of (I) and (VIII) (3:1), and fractions containing more complex mixtures of esters differing in their qualitative and quantitative compositions were obtained.

The chromatography of fraction 2 (1.2 g) in chloroform yielded 0.67 g of a mixture of (I)-(III) (totaling ~80%) and (IV), (VII), (VIII), (XI), and (XII) (totaling ~20%), and 0.27 g of a mixture of (II), (III), (XI), and (XII). The rechromatography of these fractions in system 3 yielded 0.04 g of a mixture of (I), (VII), and (VIII), 0.30 g of a mixture of (II), (III), (XI), and (XII), and 0.11 g of a mixture of (II) and (III).

By the chromatography of fraction 5 (0.2 g) in system 2 we isolated 0.06 g of (V) [3] and 0.08 g of a mixture of (V) and (VI). When these fractions were methylated with diazomethane, (V) yielded (VII) and the mixture of (V) and (VI) yielded (VII) and (VIII).

The chromatography of fraction 8 (0.22 g) in system 3 yielded 0.07 g of (IX) [3] and 0.09 g of a mixture of (IX) and (X). When these fractions were methylated with diazomethane, (IX) yielded (XI), and the mixture of (IX) and (X) yielded (XI) and (XII).

Dibutyl malate (I). $C_{12}H_{22}O_5$, $(M + H)^+$ m/z 247 (FAB – MS), n_D^{20} 1.4397 $[\alpha]_{546}^{20}$ –8.6° ($CHCl_3$, c 3.97). ^1H NMR spectrum (δ, ppm): 0.93 (6H, t), 1.45 (8H, m), 4.18 (4H, m) (Bu at C-1 and C-4), 3.27 (1H, d, J = 6 Hz, OH), 4.48 (1H, m, CH–), 2.77 and 2.83 (2H, CH_2–C–). ^{13}C NMR (δ, ppm): 13.29; 18.81; 30.40; 65.34 (BuOOC-1), 13.29; 18.81; 30.40; 64.48 (BuOOC-4), 38.75 (C-3), 67.29 (C-2), 170.17 (C-4); 173.15 (C-1). EI mass spectrum (m/z, %): $(M + H)^+$ 247 (1.5), 173 (7), 145 (29), 117 (2), 89 (100), 71 (23), 57 (50), 41 (27), 29 (26).

4-Butyl 1-methyl malate (II) [in a mixture with (III)], $C_9H_{16}O_5$, $(M + H)^+$ m/z 205 (FAB – MS). ^1H NMR spectrum (δ, ppm): 0.93 (3H, t), 1.44 (4H, m), 4.16 (2H, m) (BuOOC-4), 2.79 and 2.85 (CH_2–CO), 3.80 (3H, s, OCH_3), 4.51 (1H, m, CH–), 3.36 (1H, d, 5.4 Hz). ^{13}C NMR (δ, ppm): 13.65; 19.07; 30.55; 65.88 (Bu); 38.68 (C-3), 67.34 (C-2), 52.77 (OCH_3), 170.70 or 170.97 (C-4), 173.50 or 173.85 (C-1). EI mass spectrum (m/z, %): 205 (0.5), 173 (2), 145 (16), 117 (92), 89 (100), 71 (45), 57 (39).

1-Butyl 4-methyl malate (III) [in a mixture with (II)] $C_9H_{16}O_5$, $(M + H)^+$ m/z 205 (FAB – MS). ^1H NMR spectrum (δ, ppm): 3.71 (OCH_3); other proton signals as in (II). ^{13}C NMR spectrum (δ, ppm): 52.01 (OCH_3), 170.97 or 170.70 (C-4), 173.85 or 173.50 (C-1). EI mass spectrum (m/z, %): 205 (1), 173 (2), 145 (19), 131 (21), 103 (100), 89 (60), 71 (80), 61 (33), 57 (49).

Tributyl citrate (IV), $C_{18}H_{32}O_7$, $(M + H)^+$ m/z 361 (FAB – MS). ^1H NMR spectrum (δ, ppm) 0.93 (9H, t), 1.48 (12H, m), 4.18 (6H, m) (BuOOC-1, C-5, C-1'), 2.84 (4H, br.s, 2 × CH_2–C).

^{13}C NMR spectrum (δ, ppm): 13.65; 19.07; 30.50; 64.90 (BuOOC-1 and BuOOC-5); 13.65; 19.07; 30.50; 65.88 (BuOOC-1'); 43.34 (C-2, C-4); 170.54 (C-1, C-5); 173.47 (C-1'). EI mass spectrum (m/z, %): 259 (43), 185 (100), 129 (71), 57 (38).

The dibutyl citrates (V) and (VI), $C_{14}H_{24}O_7$, $(M + H)^+$ 305 (FAB – MS). ^1H NMR spectrum as for (IV) with different intensities of the protons of the Bu groups.

1,5-Dibutyl 1'-methyl citrate (VII), $C_{15}H_{26}O_7$, $(M + H)^+$ m/z 319 (FAB – MS). ^1H NMR spectrum (δ, ppm): 3.81 (3H, OCH_3 at C-1'), 2.82 and 2.85 (4H, s, CH_2–C). Signals of the protons of the Bu groups as in (IV). ^{13}C PMR spectrum (δ, ppm): 13.50; 18.92; 30.40; 64.75 (Bu); 43.24 (C-2, C-4); 52.88 (OCH_3 at C-1'); 73.20 (C-3), 169.74 (C-1, C-5); 173.80 (C-1'). EI mass spectrum (m/z, %): 259 (23), 231 (27), 185 (100), 157 (52), 129 (79).

1,1'-Dibutyl 5-methyl citrate (VIII) [in a mixture with (I) or (VII)], $C_{15}H_{26}O_7$, $(M + H)^+$ m/z 319 (FAB – MS). ^1H NMR spectrum (δ, ppm): 3.67 (OCH_3 at C-5). ^{13}C NMR spectrum (δ, ppm): 51.75 (OCH_3 at C-5). EI mass spectrum (m/z, %): 259 (8), 217 (54), 143 (100), 129 (51).

The butyl citrates (IX) and (X), $C_{10}H_{16}O_7$, $(M + H)^+$ m/z 249 (FAB – MS). ^1H PMR spectra as for (IV), differing only by the integral intensities of the signals of the protons of the Bu groups.

1'-Butyl 1,5-dimethyl citrate (IX) [in a mixture with (XII)], $C_{12}H_{20}O_7$, $(M + H)^+$ m/z 277 (FAB – MS). ^1H NMR spectrum (δ, ppm): 3.69 (6H, OCH_3). ^{13}C NMR spectrum (δ, ppm): 51.85 (OCH_3 at C-1 and C-5). EI mass spectrum (m/z, %): 217 (20), 185 (38), 175 (10), 143 (100), 129 (29).

1-Butyl 1',5-dimethyl citrate (XII) [in a mixture with (XI)], $C_{12}H_{20}O_7$, $(M + H)^+$ m/z 277 (FAB – MS). ^1H NMR spectrum (δ, ppm): 3.81 and 3.69 (3H each, OCH_3 at C-1' and C-5); ^{13}C NMR spectrum (δ, ppm): 53.10 (OCH_3 at C-1'); 51.85 (OCH_3 at C-5). EI mass spectrum (m/z, %): 217 (3.5), 185 (4), 175 (24), 143 (100), 129 (2), 101 (4).

SUMMARY

Esters of malic and citric acids have been isolated from an ethyl acetate fraction of an extract of the fruit of L. caerulea. It has been established that the completely esterified compounds containing not less than one butyl residue are the main components forming the bitter taste of the fruit.

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