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Synthesis and Biological Activity of Monoterpenoids Belonging to Menthane Series

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Abstract

Antifungal activity has been studied for monoterpenoids from menthane series and their thio derivatives obtained by the reactions of corresponding monoterpenoids with thiols in the presence of Lewis acids. Basing on (+)-limonene-1,2-oxide and methyl ether of mercaptoacetic acid in the presence of sodium methylate we have synthesized an active antimicotic agent with a wide spectrum of action. Data are presented concerning the toxicity of some monoterpenoids.

Key words: monoterpenoids of menthane series, the synthesis of terpenesulphides, antifungal activity, toxicity

INTRODUCTION

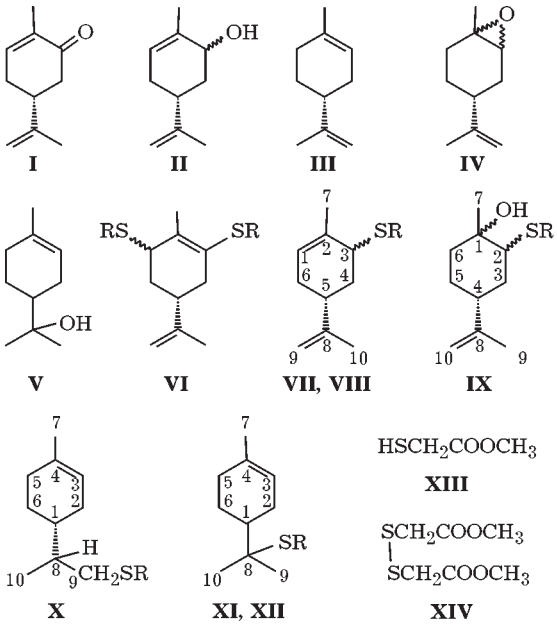
Compounds of terpene series represent an important source for obtaining biologically active compounds with a wide spectrum of action [1–8]. Aiming at a possible increase in the efficiency of the biological action of naturally occurring monoterpenes we have performed their chemical modification *via* the introduction of biogenic sulphide groups low-toxic for warm-blooded animals, into these molecules.

There are scarce data in the literature concerning the biological activity of sulphur-containing monoterpenoids. So, a moderate antimalarial action was revealed for epimeric terpenesulphides obtained basing on (+)-carvone and R-(+)-limonene; sulphonamide with anesthetizing properties has been synthesized from (+)-carvone [9, 10].

To continue the studies concerning the search of biologically active compounds with antifungal activity [11, 12] we have been obtained sulphur-containing derivatives basing on monoterpenoids of menthane series. Target products were isolated by means of column chromatography on silica gel; their structure has been established employing a combination of spectral methods. According to NMR spectroscopy and chromatography/mass spectrometry data, thioterpenoids **VI–XII** represent an inseparable mixture of isomers.

The reaction between R-(−)-carvone **I** and ethanethiol under the conditions of catalysis zinc chloride proceeds with the participation of diastereotropic fragments of the molecule (conjugated endocyclic double bond and oxo group of carvone) resulting in the formation of compound **VI** as a mixture of three adducts with a considerable predominance of bis-sulphide with the axial arrangement of RS group [13] (Scheme 1).

Carveol **II** obtained *via* the reduction of R-(−)-carvone **I** by sodium tetraborate employing known technique [14] represents a mixture of *cis*- and a *trans*-isomers at a ratio of ~12 : 1. The reactions of carveol **II** with ethanethiol and isopropylmercaptane in the presence of BF_3



Scheme 1.

etherate proceed with substitution of OH group by sulphide function resulting in the formation of compounds **VII**, **VIII** as a mixture stereoisomers at a ratio of ~6 : 1. The presence of the signal of an equatorially located methine proton (H-3) as a broadened singlet in the ^{1}H NMR spectrum within the range of ~3.2 ppm indicates an axial arrangement of sulphide group in the prevailing isomer. According to the data of chromatography/mass spectrometry, the minor isomer of compounds **VII**, **VIII** exhibits an identical character of fragmentation with respect to the basic isomer, differing from it only in the intensity of "debris". Basing on this one might assume that the isomer contains equatorially arranged RS group, which is in an agreement with literature data [15, 16].

The reaction between (+)-limonene **III** and methyl ester of mercaptoacetic acid **XIII** under the conditions of catalysis by BF_3 etherate occurs at the exocyclic double bond with a high level of chemo- and regioselectivity with finishing by the formation of sulphide **X** in the form of an equimplar mixture of two diastereomers. The course of the reaction in a similar route has been fixed earlier in our experiments for the reaction between limonene and thiols [17].

In the inseparable mixture of three isomers – the products of the reactions of α-terpineol **V** with 2-mercaptoethanol (~4 : 2 : 1, **XI**) and ethanedithiol (~7 : 3 : 1, **XII**), there are two prevailing regioisomeric terpenesulphides with a ratio ~4 : 2 (**XI**) and ~7 : 3 (**XII**). Their formation could be caused by α-terpineol dehydration occuring in the presence of $BF_3 \cdot Et_2O$ and by the subsequent electrophilic reaction of catalytic thiol addition to the exocyclic double bond of terpinolene. The spectral data available do not allow one to draw a conclusion concerning the structure of the minor isomer. However, one could assume that it is formed as the result of α-terpineol dehydration in an opposite manner with respect to the Zaitsev rule, and the subsequent addition of thiol to the exocyclic bond of limonene in an opposite manner with respect to the Markovnikov rule, which is in a good agreement with the data from the literature [17, 18].

Compound **IX** in the form of the mixture of two stereoisomers at a ratio ~7 : 1 (the prevailing isomer being with the axial arrangement of the sulphide group) have been obtained basing on (+)-1,2-limonene oxide **IV** (the mixture of *cis*- and *trans*-isomers at a ratio ~1 : 1) and methyl ester of mercaptoacetic acid **XIII**, in the presence of sodium methylate. In the ^{1}H NMR spectrum of the prevailing stereoisomer, the signal of the methyne proton (H-2) represents a doublet of doublets within the range of 2.92 ppm, SFCC = 3.6 Hz ($H_{2e}H_{3e}$) and 5.4 Hz ($H_{2e}H_{3a}$), which indicates an axial orientation of the RS group. Disulphide **XIV** is formed in the course of a side reaction due to the secondary transformations of thiol **XIII** within the basic medium.

R-(−)-carvone **I**, carveol **II**, (+)-limonene **III**, (+)-1,2-limonene oxide IV, α-terpineol **V**, as well as sulphur-containing compounds **VI**–**XIV** have been investigated for antifungal activity with respect to filamentous and yeastlike fungi.

EXPERIMENTAL

Chemical section

NMR spectra were registered on a Varian Unity spectrometer (USA) with the operation frequency of 300 and 75.43 MHz for ^{1}H and ^{13}C nuclei, respectively. TMS was used as the internal standard, $CHC1_3$ being used as a solvent. Chromatography/mass spectral profiles

were obtained using Turbo Mass Gold mass spectrometer (Perkin Elmer, USA), with a capillary column 30 m long, 320 μm in diameter, U_{He} = 1.2 mL/min. IR spectra were registered employing Tensor-27 Fourier-transform spectrometer (Bruker) within the range of wave number values of 4000–400 cm^{-1} (a sample being pressed between KBr plates).

Spectral data for compounds **VII–XII** are presented for the prevailing isomer. For the isolation and purification of reaction products we employed an adsorption chromatography technique, with the use of silica gel L (100/160 μm). As an eluent, we used hexane, as well as hexane–diethyl ether mixture. The monitoring of the reactions and the quality of separating reaction mixtures was carried out by means of TCL technique on the plates Silufol (developers: I_2 and ethanol–sulphuric acid–anisic aldehyde mixture at a ratio of 90 : 5 : 5).

The following compounds were used in the work: (–)-carvone **I** (Fluka), $[\alpha]_D^{20} = (-61.5\pm2)^o$; (+)-limonene **III**, $[\alpha]_D^{20} = +115^o$ (c = 10, EtOH); (+)-1.2-limonene oxide **IV** (97 % mixture of *cis*- and *trans*-isomers), n_D^{20} 1.4700; α-terpineol **V**, n_D^{20} = 1.4813 (Acros Organics); sulphur-containing reagents (Aldrich). Carveol **II** (n_D^{20} = 1.4969) was obtained by means of technique described in [14]; the synthesis technique and spectral data for compound **VI** are presented in [13]. The purification and drying of solvents was carried out according to commonly known techniques [19].

General technique for obtaining thio derivatives VII, VIII, X–XII. To 0.02 mol of monoterpenoid **II**, **III**, **V** at a room temperature and under stirring was added 0.04 mol of corresponding thiol in 30 mL CH_2Cl_2, and then was added freshly calcined $ZnCl_2$ on a scalpel tip or $BF_3 \cdot OEt_2$ in the catalytic amounts. After completing the reaction (duration being from 1 h to 3 days), we added 200 mL of water to the reaction mixture, then extracted it with CH_2Cl_2 and dried over $MgSO_4$. The products were purified with the help of column chromatography on silica gel (as an eluent, hexane, diethyl ether being used). The yield of compounds **VI–VIII**, **X–XII** amounted to 55–60 %.

5-Isopropenyl-3-isopropylthio-2-methylcyclohexene-1 (VII). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 1.25 d, 1.29 d (6H, H-12,13, 7.2 Hz), 1.68 s, 1.73 s (6H, H-7,10), 2.95 m (1H, H-11, 7.2 Hz), 3.18 s (1H, H-3), 4.70 s (2H, H-9), 5.50 s (1H, H-1). Chromatography/mass spectral analysis: 240 (M^+, 1), 209 (1), 197 (10), 167 (40), 133 (50), 119 (48), 99 (45), 91 (38), 77 (75), 55 (80), 41 (100).

Methyl[(5-isopropenyl-2-methylcyclo-hex-2-en-3-yl)thio]ethanoate (VIII). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 1.67 s, 1.76 s (6H, H-7,10), 3.17 s (1H, H-3), 3.39 s (2H, H-11), 3.70 s (3H, H-13), 4.70 s (2H, H-9), 5.50 m (1H, H-1). ^{13}C NMR spectrum, δ, ppm: 195.0 (C-12), 144.5 (C-8), 127.4, 128.3 (C-1,2), 109.8 (C-9), 53.0 (C-13), 42.5 (C-11), 36.0–20.0 (C-3,4,5,6,7,10).

Methyl{2-[4-methylcyclohex-3-en-1-yl)propyl]thio}ethanoate (X). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 0.92 d (3H, H-10, 6.7 Hz), 1.6 s (3H, H-7), 2.4–2.8 m (2H, H-9), 3.2 s (2H, H-11), 3.7 s (3H, H-13), 5.3 s (1H, H-3). Chromatography/mass spectral analysis: 258 (M^+, 4), 241 (13), 226 (7), 185 (16), 152 (70), 135 (90), 107 (65), 93 (78), 67 (41), 55 (63), 43 (100).

2-{[1-Methyl 4-methylcyclohex-3-en-1-yl)ethyl]thio}ethanol (XI). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 1.1 s, 1.3 s (6H, H-9,10), 1.8 s (3H, H-7), 2.7 m (2H, H-11), 3.2 s (1H, OH), 3.65 m (2H, H-12), 5.35 s (1H, H-3). ^{13}C NMR spectrum, δ, ppm: 120.0, 122.1 (C-1,2), 60.2 (C-12), 40.8 (C-11), 44.2 (C-4), 26.2 (C-8), 17.4–33.5 (C-3,5-7,9,10). Chromatography/mass spectral analysis: 214 (M^+, 1), 169 (1), 136 (95), 121 (97), 93 (80), 81 (100), 69 (50), 41 (84), 27(30).

2-{[1-Methyl-1-(4-(methylcyclohex-3-en-1-yl)ethyl]thio}ethanethiol (XII). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 0.86 s, 0.89 s (6H, H-9,10), 1.62 s (3H, H-7), 2.65 m, 2.85 m (4H, H-11,12), 3.25 s (1H, SH), 5.2 s (1H, H-3). Chromatography/mass spectral analysis: 230 (M^+, 2), 169 (1), 137 (60), 95 (30), 81 (100), 41 (50), 27 (25).

Reaction between (+)-1,2- limonene oxide and methyl ester of mercaptoacetic acid. To a solution of sodium methylate in an absolute methanol (0.3 g, 0.013 mol Na, 50 mL CH_3OH) were added 1.38 g (0.013 mol) of mercaptoacetic acid methyl ester and 2 g (0.013 mol) of (+)-1.2- limonene oxide **IV**. The reaction mixture was heated (80 °C) under stirring for 4.5 h, diluted with water, acidified by 3 % HCl solution up to pH 3, extracted with methylene chloride, dried over $MgSO_4$. After removing the solvent

TABLE 1

Antifungal activity level for monoterpenoids **I–V** and thio derivatives **VI–XIV**

Compounds	*Candida albicans*		*Candida parapsilosis*	*Rhodotorula rubra*	*Aspergillus niger*	*Penicillium tardum*	*Candida kruzei*
	non-pathogenic	pathogenic					
I	+/−	+	+/−	+	−	+/−	2+
II	2+	2+	+/−	+/−	+/−	2+	+
III	2+	+	2+	2+	2+	3+	+
IV	+	+/−	+/−	+	−	+/−	−
V	2+	+	2+	3+	+/−	+	+
VI	−	no data	no data	−	−	no data	−
VII	−	−	+/−	+/−	+	−	+/−
VIII	+/−	−	2+	+	−	+/−	+
IX	no data	3+	no data	3+	3+	no data	no data
X	+/−	no data	no data	+/−	no data	no data	−
XI	−	−	−	+/−	+/−	+/−	−
XII	+/−	−	+	−	−	+/−	−
XIII	2+	2+	2+	2+	2+	2+	2+
XIV	2+	2+	2+	2+	3+	2+	+/−

Note. 1. Sign "+" denotes the zone of 1–3 mm growth inhibition (low activity level); sign "2+" denotes the zone of sign "3+" denotes the zone of growth inhibition ≥5 mm, which corresponds to a high activity level; "−" denotes of growth inhibition less than 1 mm (very low activity level).

the product was isolated employing a column chromatography technique on silica gel (hexane–diethyl ether, 10 : 1). The yield amounted to 60 % for compound **IX** and 30 % for compound **XIV**.

Methyl[2-(1-hydroxy-4-isopropenyl-1-methylcyclohexyl-2-thio)]ethanoate (IX). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 1.29 s (3H, H-7), 1.66 s (3H, H-10), 2.4 s (1H, OH), 2.92 dd (1H, H-2, J_{H2eH3e} = 3.6 Hz, J_{H2eH3a} = 5.4 Hz), 3.12 s (2H, H-11), 3.66 s (3H, H-13), 4.70 s (2H, H-9). Chromatography/mass spectral analysis: 258 (M^+, 3), 226 (7), 185 (10), 169 (15), 152 (30), 135 (45), 107 (90), 93 (50), 81 (40), 69 (62), 55(60), 43 (100). IR spectrum (ν, cm^{-1}): 3200–3500 (OH).

Dimethyl-2,2′-dithiodiyldiacetate (XIV). ^{1}H NMR spectrum ($CDCl_3$), δ, ppm: 2.52 s (4H, 2C$\underline{H}_2$), 3.68 s (6H, 2C$\underline{H}_3$). Chromatography/mass spectral analysis: 210 (19), 178 (31), 151 (23), 119 (14), 106 (10), 95(8), 74 (12), 59 (28), 45 (100).

Biological section

For the experiments concerning the studies on toxicity we used eugamic mice 18–20 g in mass, each group consisted of 10 animals. The compounds under investigation were introduced intragastrically at a dozen not exceeding 0.5 mL. The results of the experiment were estimated in 72 h. The parameter of acute toxicity (LD_{50}) was calculated according to the Litchfield and Wilkinson method.

In order to carry out biological tests for antifungal activity we used strains maintained in the collections of the Kazan Institute of Epidemiology and Microbiology: *Aspergillus niger* VKM F-412, *Aspergillus fumigatus* VKM F-219, *Penicillium tardum* VKM F-263, *Penicillium chrysogenum* VKM F-347, *Candida albicans* Y-4, as well as the strains of yeastlike fungi and dermatomycetes isolated from patients suffering from skin and mucous tunic mycosis: *Candida albicans* 228, *Candida parapsilosis, Candida kruzei, Rhodotorula rubra, Epidermophyton floccosum* [20, 21].

The screening studies on the antifungal activity of compounds **I–XIV** were carried out using an application disc-diffusion technique on the modified Saburo agar. The inoculation of test-cultures (spore suspension) was performed at a rate of 1 million/dish. The inoculation ma-

Epidermophyton floccosum	*Aspergillus fumigatus*	*Penicillium chrysogenum*
2+	+	–
no data	–	+/–
+	2+	2+
–	–	+/–
+/–	2+	3+
+/–	no data	no data
–	+/–	+/–
+	–	+
3+	3+	3+
+/–	no data	no data
–	–	2+
–	–	+/–
no data	2+	2+
no data	2+	3+

3–5 mm growth inhibition (moderate activity level); the absence of activity; sign "+/–" denotes the zone

terial was incubated during 8 days at 28 °C [22]. The substances under investigation were dissolved in volatile solvents (ethanol, acetone) and then were applied onto paper disks at a rate of 1 mg of a substance for a disk. The disks were dried under sterile conditions up to complete removal of the solvent.

RESULTS AND DISCUSSION

The results of testing the antifungal activity of compounds **I–XIV** are presented in Table 1. As a negative reference, we used disks without preparations, treated by the solvent in a similar manner. As a positive reference we used disks with a fungicidal preparation "Polysept" (4+).

The comparative analysis of the antifungal activity of initial monoterpenoids **I–V** belonging to menthane series has demonstrated that the least level of the antifungal activity is observed for (+)-1,2-limonene oxide **IV**; a moderate activity level with respect to some fungi species is inherent in carvone **I** and carveol **II**, and the best antifungal properties are observed for α-terpineol **V** and (+)-limonene **III** (see Table 1).

The authors of [23–25] have been studied the antifungal activity of R-(–)-carvone **I** with respect to fungi species such as *Candida albicans, Fusarium solani, Fusarium sulphureum* and *Trychoderma pseudokoningii*. We have established that compound **I** demonstrates moderate activity with respect to fungi *Candida kruzei* and *Epidermophyton floccosum*.

In the literature, data are presented concerning fungistatic activity of α-terpineol **V** with respect to pathogenic fungi such as *Candida albicans, Enterococcus faecalis, Staphylococcus aureus*, *etc.* [26, 27]. Our studies have demonstrated that α-terpineol **V** demonstrates a high (3+) activity with respect to fungi such as *Rhodotorula rubra* and *Penicillium chrysogenum* and moderate activity (2+) with respect to fungi *Aspergillus fumigatus,* non-pathogenic fungi *Candida albicans* and *Candida parapsilosis*.

The minimal inhibiting concentration of limonene with respect to mold fungi *Penicillium chrysogenum, Rhodotorula rubra* MC 12, *Candida albicans* ATCC 1023 and some other species has been established earlier [28, 29]. From Table 1 one can see that antifungal properties of (+)-limonene **III** with respect to fungi *Candida kruzei, Epidermophyton floccosum* and pathogenic fungi *Candida albicans* are poorly pronounced (+), whereas this compound exhibits a moderate activity level (2+) with respect to six fungi species as well as a high activity (3+) level concerning fungi *Penicillium tardum*.

It is commonly known that a wide range of biological activity is inherent in sulphur-containing compounds; some sulphur-containing organic compounds compose antifungal remedies [30]. In this connection one could assume that joining together a terpene fragment and sulphide groups in one molecule could result in obtaining biologically active compounds. However, the functionalization of menthane series monoterpenoids by sulphur-containing reagents (ethanethiol, isoprop0ylmercaptane, 2-mercaptoethanol, ethanedthiiol) results as a whole in decreasing the antifungal activity level for thioterpenoids **VI–VIII**, **X–XII**.

Biological testing the antifungal activity for bicyclic terpenesulphides obtained in our labo-

ratory earlier have demonstrated that the highest activity is exhibited by sulphides with the fragment of mercaptoacetic acid methyl ester [11]. In the course of the studies, of interest was to investigate antifungal properties of menthane series sulphides with an ester fragment.

The introduction of the ester functions into the molecules of carveol **II** and (+)-limonene **III** (sulphides **VII**, **X**) instead of increasing the antifungal activity of the compounds results in a decrease of the value. However, the product of reaction between (+)-1,2-limonene oxide **IV** with methyl ester of mercaptoacetic acid (sulphide **IX**) has demonstrated an extremely high antifungal activity (3+) with respect to seven fungal species under investigation.

It should be noted especially that sulphur-containing derivative **IX** was revealed to exhibit a high activity level concerning pathogenic fungi *Candida albicans* those cause severe system infections in a human organism [31].

At the following stage of the work we carried out a comparative analysis of the antifungal properties of thioterpenol **IX**, initial thiol **XIII** and disulphide **XIV**. However, in spite of a pronounced antifungal action of compounds **XIII**, **XIV**, their practical application is limited due to their high toxicity, unpleasant smell and skin irritant action [32].

At the same time, our further studies have demonstrated that compound **IX** is not only the most efficient antifungal agent, but also exhibits the lowest toxicity among monoterpenoids and their sulphur-containing derivatives. So, when thioterpenoids of the pinane and carane series (including 3-carene) belong to III hazard class compounds (moderately hazardous compounds, intragastral LD_{50} = 950–4000 mg/kg for mice), menthane series thioterpenol **IX** belongs to **IV** hazard class compounds (low-hazardous compounds intragastral LD_{50} = 10000 mg/kg for mice) [33].

CONCLUSION

The studies on antifungal activity of menthane series monoterpenoids and thioderivatives with respect to filamentous and yeast-like fungi has allowed us to reveal an active antimicotic agent with wide-range action. High antifungal activity level, low toxicity and the availability of the product of the reaction between (+)-1,2-limonene oxide and methyl ether of mercaptoacetic acid offer considerable prospects for its further use.

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