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ANTIFUNGAL ACTIVITY OF BICYCLIC MONOTERPENOIDS AND TERPENESULFIDES

L. E. Nikitina,[1*] V. A. Startseva,[1] L. Yu. Dorofeeva,[1]
N. P. Artemova,[1] I. V. Kuznetsov,[1]
S. A. Lisovskaya,[2] and N. P. Glushko[2]

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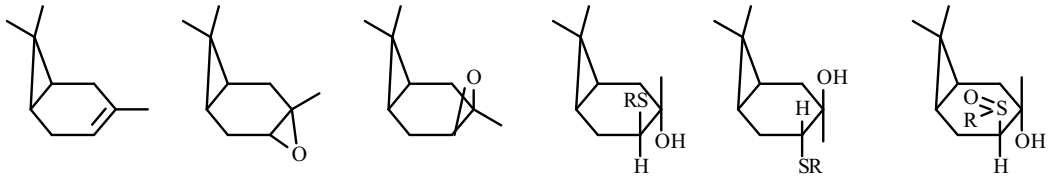
A total of 33 compounds including bicyclic monoterpenes, oxides, allyl alcohols, and S*-containing pinane and carane derivatives were screened for antifungal activity against mycelial and yeast-like fungi. The structure–activity relationship was studied. The most active antimycotics were found.*

Keywords: carane and pinane monoterpenoids, antifungal activity.

We performed for the first time a systematic study of the antifungal activity of series of pinane and carane monoterpenoids with a broad set of *S*-containing functional groups and studied the structure–activity relationship to find the most active antimycotics.

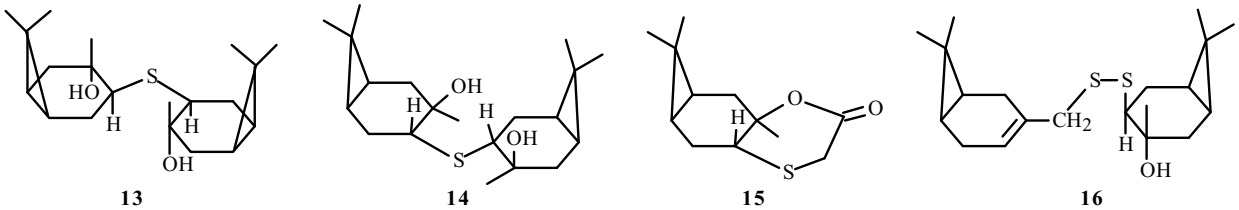
At the start of our work, the literature contained data on the antifungal activity of α- and β-pinenes in addition to the antifungal effect of essential oils containing (+)-3-carene (**1**), verbenols, and myrtenol [1–5]. However, the antifungal activity of *S*-containing terpenoids was not previously investigated.

Carane thioterpenoids **4**–**15** were synthesized by reacting stereoisomeric 3-carene oxides (**2** and **3**) with thiols (**4**, **5**, **10**, **11**, **15**), isothiuronium salts (**6**–**9**, **13**, **14**), and thiourea (**16**) in the presence of sodium ethoxide [6–9]. Sulfoxide **12** was obtained by oxidation of **6** with H_2O_2 in glacial acetic acid. The products were isolated by column chromatography. The structures were established using spectral data.



1 **2** **3** **4, 6, 8** **5, 7, 9 - 11** **12**

R = CH_2 (**4, 5**), $CH_2CH{=}CH_3$ (**6, 7, 12**), CH_2Ph (**8, 9**), *iso*-Bu (**10**), *n*-Bu (**11**)



13 **14** **15** **16**

1) Kazan State Medical University, 420012, Republic of Tatarstan, Kazan, e-mail: nikit@mi.ru; 2) Kazan Institute of Epidemiology and Microbiology, 420015, Republic of Tatarstan, Kazan, e-mail: farrus@mail.ru. Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 27–30, January–February, 2010. Original article submitted January 19, 2009.

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TABLE 1. Antifungal Activity of Carane Compounds

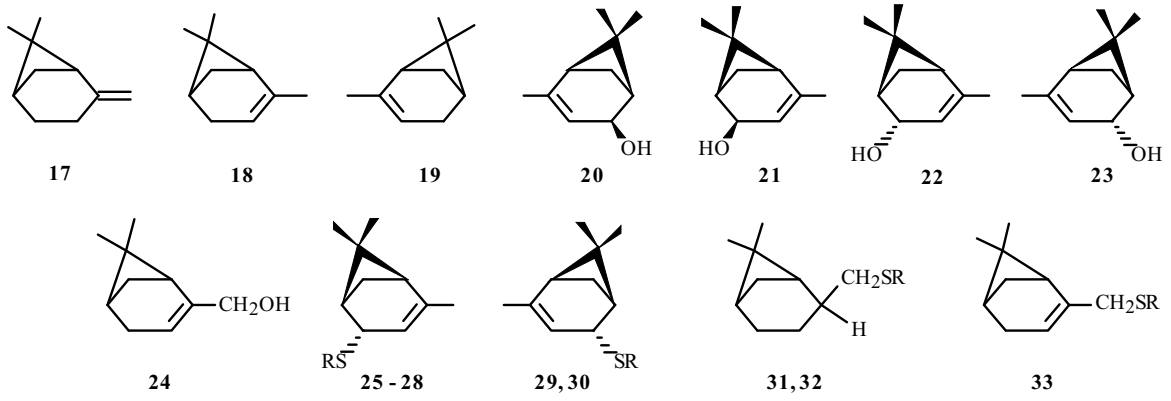
Compound	1 nonpath.	1 path.	2	3	4	5	6	7	8	9
1	3+	2+	2+	2+	2+	2+	2+	2+	+	2+
2	–	–	–	–	–	–	+/–	–	–	+/–
3	–	–	2+	+	–	–	2+	+	+/–	–
4	2+	2+	+/–	+/–	2+	+	2+	3+	+	2+
5	+/–	+/–	+/–	+/–	2+	+/–	+	+	+	2+
6	–	–	–	–	–	–	–	–	–	–
7	+	–	+/–	–	+	–	–	–	–	–
8	–	–	–	–	+/–	–	–	–	+/–	–
9	+/–	+/–	+	+	–	+/–	+/–	–	–	+
10	–	–	+/–	+/–	2+	+/–	+/–	–	–	–
11	–	+/–	+/–	–	–	–	+/–	–	–	–
12	+/–	–	+/–	–	–	+	–	–	n.d.	n.d.
13	–	–	+/–	–	+/–	+	+	–	–	2+
14	–	n.d.	n.d.	–	2+	n.d.	–	–	n.d.	n.d.
15	–	–	+/–	–	–	–	+/–	–	–	–
16	–	–	–	–	–	–	–	–	–	+/–

1 – *Candida albicans,* 2 – *Candida parapsilosis*, 3 – *Rhodotorula rubra*, 4 – *Aspergillus niger*, 5 – *Penicillium tardum*, 6 – *Candida kruzei*, 7 – *Epidermophyton floccosum*, 8 – *Aspergillus fumigatus*, 9 – *Penicillium chrysogenum*.
n.d. – no data.

(+)-3-Carene (**1**), 3-carene α-oxide (**2**), 3-carene β-oxide (**3**), and *S*-containing carane derivaties **4**–**16** were tested for antifungal activity against mycelial and yeast-like fungi. Table 1 presents the test results. The "+" sign denotes a growth inhibition zone of 1–3 mm (weak activity); "2+", of 3–5 mm (moderate activity); "3+", >5 mm (high activity). A lack of activity is denoted by a "–" sign. The "+/–" sign denotes a growth inhibition zone of up to 1 mm (very weak activity). The negative controls were plates without compounds that were treated in the same manner with solvent. The positive controls were disks with Polisept "4+" fungicide.

Screening for antifungal activity of 16 carane compounds showed that (+)-3-carene (**1**) and 4β-methylthiocaran-3α-ol (**4**) had the highest activity (Table 1). A comparison of the antifungal activity of **6**, **7**–**9**, **12**–**16** with that of starting oxides **2** and **3** showed that the antifungal effect of the terpenesulfides was lower whereas adding an S-CH_3 group to 3-carene oxide increased the antifungal activity of the thioterpenoids.

Thus, **4**, which was synthesized from 3-carene α-oxide, exhibited moderate activity (2+) against *Candida albicans* (nonpath.), *C. albicans* (path.), *Aspergillus niger*, *C. krusei*, and *Penicillium chrysogenum* and high activity (3+) against *Epidermophyton flocossum*. Also, stereoisomer **5**, which was prepared from 3-carene β-oxide, exhibited moderate activity (2+) against only *A. niger* and *P. chrysogenum*. Thus, the steric structure of the molecule and the structure of the added group affected the manifestation of antifungal activity by the compounds.



17 **18** **19** **20** **21** **22** **23**

24 **25 - 28** **29, 30** **31, 32** **33**

R = *i*-Pr (**25**), $CH_2CH_2SCH_2CH_2SH$ (**26**), CH_2COOH (**27**, **29**), CH_2COOCH_3 (**28**, **30**, **32**, **33**), CH_2CH_2SH (**31**)

TABLE 2. Antifungal Activity of Pinane Compounds

Compound	1 nonpath.	1 path.	2	3	4	5	6	7	8	9
17	+/–	+/–	+	–	–	–	–	+/–	–	+/–
18	+	+/–	2+	2+	–	+/–	2+	+/–	+/–	2+
19	–	–	+	–	–	+/–	–	–	–	2+
20	+	+	+	–	+	+	3+	–	3+	+
21	n.d.	+/–	+/–	–	2+	n.d.	n.d.	n.d.	2+	n.d.
22	3+	2+	3+	2+	–	2+	+	+	+/–	2+
23	+/–	–	+/–	+	–	+	+	–	–	+
24	+	+	+/–	–	+	+	–	n.d.	+/–	+
25	–	–	–	–	+/–	–	–	+/–	–	+/–
26	n.d.	+/–	–	–	–	–	n.d.	n.d.	–	n.d.
27	n.d.	–	+/–	–	2+	–	–	n.d.	+/–	n.d.
28	+	2+	+	+	3+	2+	3+	2+	–	3+
29	+/–	+/–	–	2+	+/–	+	+/–	–	+/–	+
30	+/–	+/–	+	2+	2+	2+	+/–	2+	2+	2+
31	–	+/–	+/–	–	n.d.	n.d.	n.d.	–	n.d.	n.d.
32	+/–	+/–	+/–	+/–	–	+	+/–	+/–	+/–	3+
33	+/–	–	+	–	–	+	–	n.d.	+/–	+

1 – *Candida albicans,* 2 – *Candida parapsilosis*, 3 – *Rhodotorula rubra*, 4 – *Aspergillus niger*, 5 – *Penicillium tardum*, 6 – *Candida kruzei*, 7 – *Epidermophyton floccosum*, 8 – *Aspergillus fumigatus*, 9 – *Penicillium chrysogenum*.
n.d. – no data.

A comparison of the antifungal activity of structural isomers, **10** and **11**, which contain butyl and *iso*-butyl groups, found that **10** was better than **11** and had moderate activity against *A. niger* whereas **11** had weak antifungal activity. Thus, the degree of branching in the hydrocarbon chain of the substituent affects the antifungal activity.

Pinane terpenesulfides **25**–**33** were synthesized by methods developed by us [10–12]. Allyl alcohols **20**–**22** and **24** were reacted with mono- and bifunctional thiols in the presence of $ZnCl_2$ with replacement of the OH group by sulfide. This produced **25**–**30** and **33**. The main product in every instance from reactions of (–)-β-pinene (**17**) with thiols was the pinane *cis*-isomer (**31** and **32**).

We also screened 17 pinane compounds for antifungal activity. These were bicyclic monoterpenes (–)-β-pinene (**17**), (+)- and (–)-α-pinenes (**18** and **19**), allyl alcohols (+)- and (–)-*cis*-verbenols (**20** and **21**), (+)- and (–)-*trans*-verbenols (**22** and **23**), and (+)-myrtenol (**24**) and *S*-containing derivatives **25**–**33** prepared from them. They were tested against mycelial and yeast-like fungi. Table 2 presents the test results.

If pinane compounds with a hydroxyl group such as *cis*- and *trans*-verbenols **20**–**23** are compared with α- and β-pinenes **17**–**19**, then an increase of antifungal activity is noted for the pinane alcohols. However, introducing a hydroxyl in and of itself is not the only factor that helps to increase the antifungal activity of the molecules. The most active of all starting monoterpenoids was (+)-*trans*-verbenol (**22**).

Introducing sulfide groups such as S–*i*Pr, –S–$(CH_2)_2$SH, S–$(CH_2)_2S(CH_2)_2$SH, and –S–CH_2–COOH into the pinane structure produces compounds **25**–**27** and **29** with practically no antifungal activity.

A pinene terpenesulfide that had a methyl ester of mercaptoacetic acid (**28**) exhibited high antifungal activity (3+) against *A. niger*, *C. krusei*, and *P. chrysogenum*.

Furthermore, the most active compounds **1**, **4**, **22**, and **28** exhibited moderate activity against the especially dangerous pathogenic fungus *C. albicans*, which causes serious systemic infections in man [13].

Carane and pinane monoterpenoids that were tested for antifungal activity were slightly toxic compounds (LD_{50} = 950–5000 mg/kg) (mouse, i.p.). Studies of monoterpenoids for mutagenicity and genotoxicity indicated that they were not direct mutagens. Therefore, there is little probability that they will exhibit carcinogenic properties.

EXPERIMENTAL

NMR spectra were measured in $CDCl_3$ on a Varian Unity (300 and 75.43 MHz) spectrometer with TMS internal standard; GC–MS, in a Turbo Mass Gold mass spectrometer (Perkin–Elmer) with a capillary column (30 m length, 320 μm diameter, ν_{He} = 1.2 mL/min). Reaction products were isolated and purified using adsorption chromatography on silica gel "L" (100/160 μm). We used S-containing reagents from Aldrich. Terpene alcohols **20**–**24** were prepared at the Institute of Chemistry, Komi Scientific Center, Ural Branch, RAS (Syktyvkar) [14]. Synthetic methods and spectral data of **4**–**15** [6–9], of **25**–**28** [10, 11], and of **31** and **32** [12] have been published. Solvents were purified and dried according to the usual methods [15].

Oxidation of Sulfide 6 to Sulfoxide 12. General Method. Sulfide **6** (0.07 g, 0.031 mol) and glacial acetic acid (0.02 g, 0.031 mol) were stirred, cooled on ice, treated with H_2O_2 (27.5%, 0.035 g, 0.031 mol), and left overnight at room temperature. The absence of sulfide in the mixure signaled completion of the reaction. The mixture was diluted with H_2O and extracted with $CHCl_3$ (3 × 10 mL). The extract was washed with H_2O, aqueous K_2CO_3 solution, and water again and dried over $MgSO_4$. The product was purified by column chromatography (hexane:Et_2O, 5:1). Yield of **12**, 75%.

3α-Hydroxy-4β-allylsulfinylcarane (12). PMR spectrum (δ, ppm, J/Hz): 0.75 (1H, m, H-1,6), 1.02 (s), 1.05 (6H, s, H-8,9), 1.42 (3H, s, H-10), 2.10 (2H, m, H-2), 2.55 (2H, m, H-5), 3.50 (1H, d, ^3J = 7.55, H-4), 3.68 (2H, m, H-11), 5.40 (1H, m, H-12), 5.80 (2H, m, H-13). IR spectrum (ν, cm^{-1}): 3640–3040 (OH), 1016 (S=O). GC–MS (*m*/*z*, I_{rel}, %): 243 (1) $[M + 1]^+$, 152 (8), 135 (30), 119 (12), 109 (41), 93 (36), 81 (19), 71 (22), 67 (21), 55 (15).

(2,6,6-Trimethylbicyclo[3.1.1]hept-2-en-*trans*-4-ylthio)ethanoic Acid (29). PMR spectrum (δ, ppm, J/Hz): 0.8 (s), 1.3 (6H, s, H-8,9), 1.35 (1H, d, J = 9.1, H_β-7), 1.62 (3H, dd, $J_{H\text{-}10\ H\text{-}1}$ = 1.7, $J_{H\text{-}10\ H\text{-}3}$ = 1.8, H-10), 2.0–2.4 (3H, m, H-1,5, H_α-7), 3.1 (2H, m, SCH_2), 3.45–3.51 (1H, m, H-4), 5.25–5.30 (1H, m, H-3), 11.1 (1H, s, COOH). ^{13}C NMR spectrum (δ, ppm): 177.15 (C=O), 147.18 (C-2), 117.18 (C-3), 49.41 (SCH_2), 48.50 (C-5), 47.20 (C-1), 46.15 (C-4), 33.30 (C-7), 29.30 (C-6), 27.51 (C-10), 23.80, 21.70 (C-8,9).

Methyl (2,6,6-trimetylbicyclo[3.1.1]hept-2-en-*trans*-4-ylthio)ethanoate (30). PMR spectrum (δ, ppm, J/Hz): 0.79 (s), 1.21 (6H, s, H-8,9), 1.25 (1H, d, J = 9.6, H_β-7), 1.60 (3H, dd, $J_{H\text{-}10\ H\text{-}1}$ = 1.7, $J_{H\text{-}10\ H\text{-}3}$ = 1.8, H-10), 1.92 (1H, td, $J_{H\text{-}1\ H\text{-}3}$ = 1.2, $J_{H\text{-}1\ H\text{-}7\alpha}$ = 5.5, $J_{H\text{-}1\ H\text{-}7\beta}$ = 10.4, H-1), 2.10–2.15 (1H, m, H-5), 2.18–2.21 (1H, m, J = 5.5, H_α-7), 3.14 (2H, m, SCH_2), 3.45-3.51 (1H, m, H-4), 3.60 (3H, s, OCH_3), 5.22-5.26 (1H, m, H-3). ^{13}C NMR spectrum (δ, ppm): 170.05 (C=O), 150.10 (C-2), 118.15 (C-3), 53.25 (SCH_2), 48.55 (C-5), 47.50 (C-1), 46.45 (C-4), 33.77 (C-7), 29.65 (C-6), 27.35 (C-10), 23.69, 21.65 (C-8,9). Mass spectrum (*m*/*z*, I_{rel}, %): 240 (1) $[M]^+$, 167 (20), 147 (22), 134 (50), 119 (70), 105 (25), 93 (100), 77 (55), 69 (68), 43 (98), 27 (31).

Methyl (6,6-dimethylbicyclo[3.1.1]hept-2-en-10-methylthio)ethanoate (33). PMR spectrum (δ, ppm, J/Hz): 0.79 (s), 1.22 (6H, s, H-8,9), 1.13 (1H, m, H-7), 2.08 (1H, m, H-7), 2.16–2.25 (3H, m, H-4, H-5), 2.36, 2.41 (2H, J_{AB} = 12, H-10, AB centers), 2.17 (1H, td, $J_{H\text{-}1\ H\text{-}5}$ = 1.3, $J_{H\text{-}1\ H\text{-}7\alpha}$ = 5.8, $J_{H\text{-}1\ H\text{-}7\beta}$ = 11.2, H-1), 3.15 (2H, m, SCH_2), 3.48 (2H, dd, $J_{H\text{-}4\ H\text{-}5}$ = 2.7, $J_{H\text{-}4\ H\text{-}3}$ = 5.7, H-4), 3.70 (3H, s, OCH_3), 5.40–5.43 (1H, m, H-3). ^{13}C NMR spectrum (δ, ppm): 170.15 (C=O), 150.30 (C-2), 118.15 (C-3), 53.05 (SCH_2), 48.73 (C-5), 50.14 (C-1), 49.41 (C-4), 33.77 (C-7), 37.85 (C-6), 27.35 (C-10), 32.06, 21.32 (C-8,9). Mass spectrum (*m*/*z*, I_{rel}, %): 240 (1) $[M]^+$, 197 (1), 167 (30), 147 (40), 135 (75), 119 (100), 105 (50), 93 (80), 69 (90), 55 (35), 43 (95).

Starting monoterpenoids **1**–**3** and **17**–**24** and S-containing carane and pinane derivatives **4**–**16** and **25**–**33** were screened for antifungal activty against mycelial and yeast-like fungi by a disk-diffusion application method on modified Sabureau agar. Innoculations of test cultures (spore suspension) were calculated for 1 million CFU/dish. Cultures were incubated for 8 d at 28°C.

Test compounds were dissolved in volatile solvents (ethanol, acetone) and placed on paper disks calculated for 1 mg of compound per disk. Disks were dried under sterile conditions for complete removal of solvent. The effectiveness of the antifungal activity was estimated from the lysis zone (using the distance from the edge of the disk with the test compound to the fungus growth zone) according to the usual method [16].

Strains preserved in the Kazan Institute of Epidemiology and Microbiology [*A. niger* BKM F-412, *A. fumigatus* BKM F-219, *P. tardum* BKM F263, *P. chrysogenum* BKM F-347, *C. albicans* Y-4] and strains of yeast-like fungi and dermatomycetes isolated from patient skin and mucous mycoses [*C. albicans* 228, *C. parapsilosis*, *C. krusei*, *Rhodotorula rubra*, *E. floccosum*] [17, 18] were used for the biotests.

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