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Study of the Structural Changes on the Antimicrobial Activity of [3.1.1.]-Bicyclics

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Abstract

α-Pinene, a rigid bicyclic monoterpene, was altered by functional group transformations and the antimicrobial activity of the resulting compounds was studied using thin layer chromatography-autobiographic assay to determine the effect of structure on antimicrobial activity. Except for α-pinanone (and α-pinene oxide in two cases), all oxygenated α-pinene derivatives showed greater antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Micrococcus luteus* and *Candida albicans* than α-pinene. Conformationally flexible methyl cyclohexene and some of its oxygenated derivatives were also tested to evaluate the role of carbon skeleton in determining the antimicrobial activity. The results indicate that both functional groups and carbon skeleton affect the antimicrobial activity of the compound and adding nitrogen to the carbon framework increases the antimicrobial activity. The results also confirm that introduction of oxygen function to the carbon framework increases antimicrobial activity.

Key Word Index

[3.1.1.]-bicyclic monoterpenes, pinene, antimicrobial activity, autobiographic assay, structure function.

Introduction

Terpenes and terpenoids, compounds that are found in essential oils, exhibit varying degrees of antimicrobial activity (1-5). The antimicrobial activity of a compound increases with the presence of an oxygen containing functional group (6-10) indicating a relationship between structure and antimicrobial activity. To date, only one detailed study has examined the link between structure and antimicrobial properties of terpenes and terpenoids with only a few [3.1.1.]- bicyclics studied (11). The current study further examines the structure activity relationship of these compounds.

Specifically, this study focuses on the rigid [3.1.1.] bicyclic monoterpene, α-pinene, a component of pine oil that exhibits antimicrobial activity (12). The site of action of terpenes and terpenoids is the cell membrane (13-16). α-Pinene has been found to affect the structural and functional properties of artificial membranes (17) by permeating the membranes and causing them to swell, thus inhibiting respiratory enzymes and causing partial dissipation of the pH gradient and electrical potential (18).

The antimicrobial activity of a set of oxygenated α-pinene derivatives and structurally related compounds that are constituents of several essential oils was evaluated *in vitro* against four microorganisms by thin layer chromatography TLC-agar overlay assay (19). TLC-agar overlay method combines TLC with a bioassay *in situ* and allows localization of compounds in the silica matrix. With this method, previously chromatographed compounds were transferred from the stationary phase to the agar phase by diffusion (20). TLC bioautography is a very simple, convenient and rapid method for testing crude extracts as well as pure compounds and gives results similar to the broth dilution method. In addition, the method is visual and stability of the compound on the plate can be easily verified.

The test microorganisms included the gram positive bacteria *Micrococcus luteus* and *Staphylococcus aureus*, the gram negative bacterium *Escherichia coli* and the unicellular fungus *Candida albicans*. Comparison of the test results of different chemical classes of compounds enabled an examination of the relationship between chemical structure and antimicrobial activity.

In addition to the study of [3.1.1.]-bicyclics, conformationally flexible methyl cyclohexene and some of its oxygenated derivatives were also studied. This was prompted by a report (21) that concluded that oral bioavailability of a molecule increased with reduced molecular flexibility. Comparison of the test results of rigid bicyclic α-pinene series and conformationally flexible methyl cyclohexene series enabled an examination of the role of molecular rigidity in antimicro-

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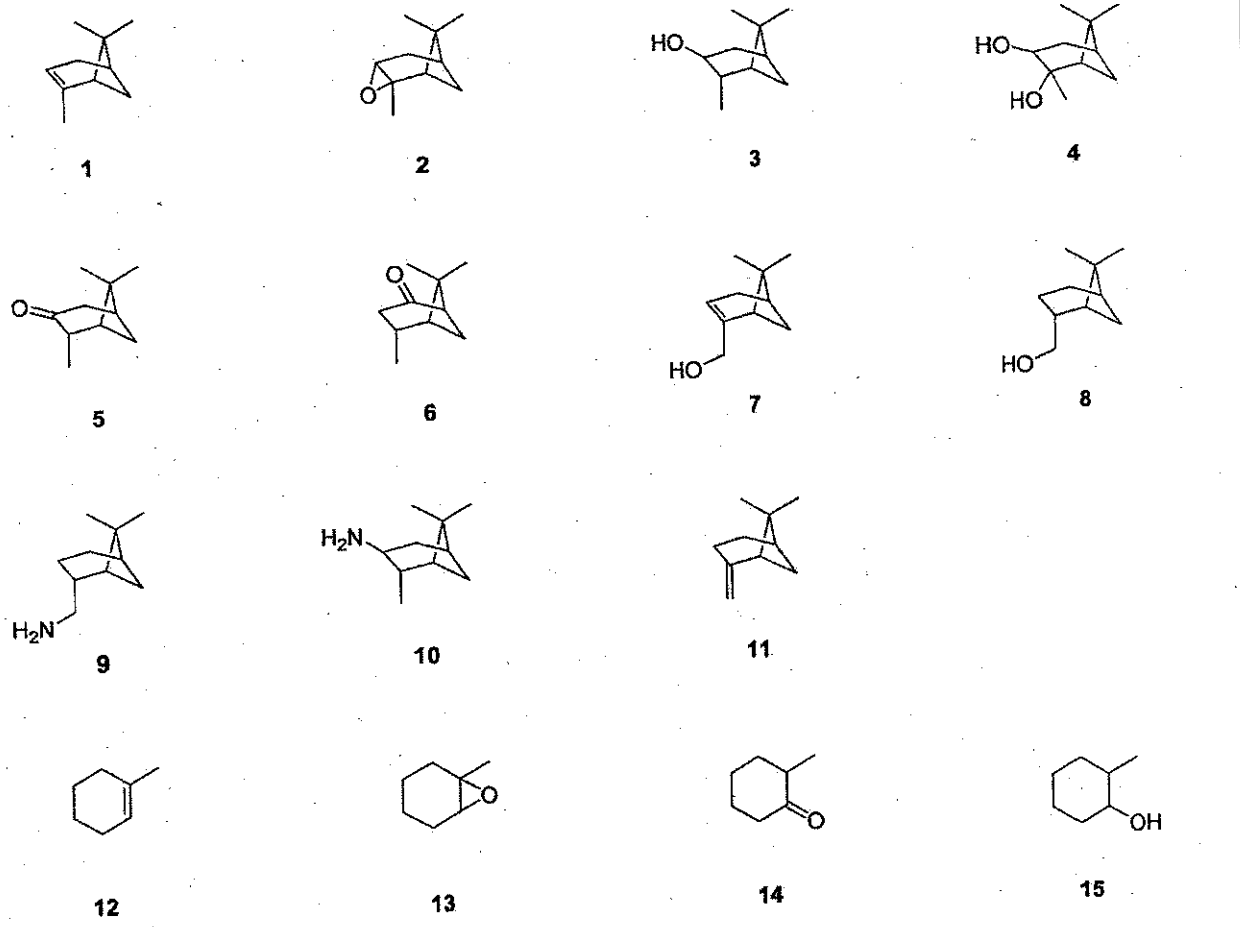


Figure 1. Compounds used in this study. 1 = (1R)-(+)-α-pinene; 2 = α-pinene oxide; 3 = (1R,2R,3R,3S)-(-) pinanol; 4 = (1R,2R,3R,5S)-(+) pinanediol; 5 = α-pinanone; 6 = (1S)-(-) verbenone; 7 = (1R)-(-) myrtenol; 8 = (1S,2S,5S)-(-) myrtanol; 9 = (-) *cis*-myrtanyl amine; 10 = (1R,2R,3R,5S)-(-) isopinocampheyl amine; 11 = (1S)-(-)-β-pinene; 12 = methyl cyclohexene; 13 = methylcyclohexene epoxide; 14 = methylcyclohexanone; 15 = methylcyclohexanol

bial activity. A listing of the compounds studied is given in Figure 1.

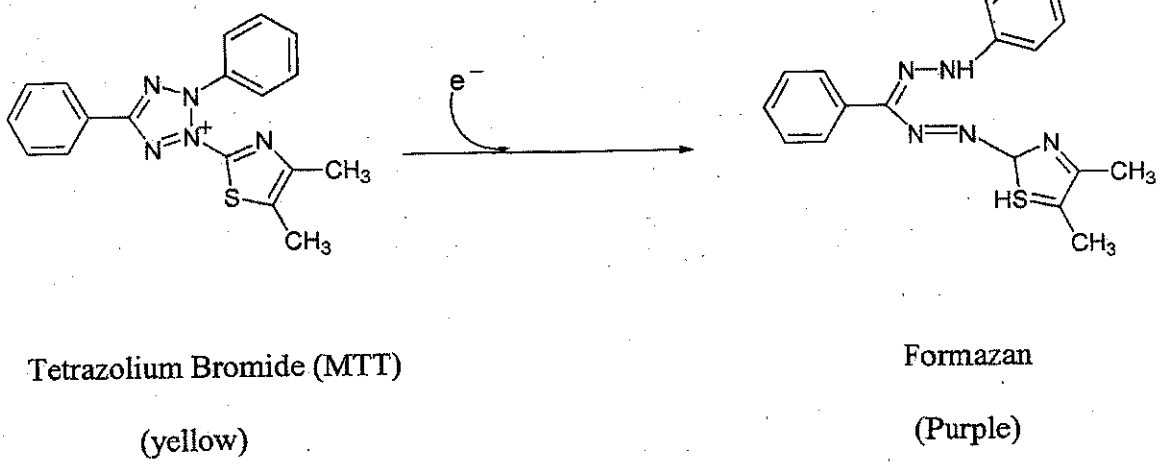
Experimental

General: Silica gel 60A 230-400 mesh ASTM from Aldrich was used for gravity column chromatography. IR spectra were recorded on a Perkin Elmer 1600 FTIR spectrophotometer and ^{1}H- and ^{13}C-NMR spectra were recorded at 300 and 75 MHz, respectively on a Jeol NMR instrument. All ACS grade solvents and dry solvents were purchased from Aldrich and used as such. Most compounds were purchased from Aldrich/Fluka and two were synthesized using standard chemical procedures (22). Synthesized compounds were found to have the same IR and NMR as reported in the literature. Purity of all the compounds was checked using ^{1}H-NMR.

Microorganisms and culture media: The microorganisms, *M.luteus*, *E. coli* and *S. aureus*, were clinical isolates and were received as a gift from Katherine Griner, chair of the medical technology program at Marist College; *C. albicans* (ATCC 90294) was purchased from Sigma. The bacterial strains and the yeast were grown in Luria Bertani (LB) broth and LB agar plates at 37°C and the yeast was maintained on Sabaraud's agar (SAB). All media were autoclaved at 120°C for 20 min.

Inoculum preparations: Bacterial and yeast cultures were subcultured in LB medium at 37°C for 12-14 h (25°C and 48-72 h for *M. luteus*) at 100 RPM and then used for the test. The optical density at 600 nm (OD_{600}) of the culture was measured with an UV/VIS Beckman 650 spectophotometer (an OD_{600} = 1 corresponds to approximately 10^{9} cells/mL) (23).

Bioautographic analysis: All solvents and silica TLC plates containing the fluorescent indicator were purchased from Aldrich. The same amount of each compound (2.5 mg) was spotted on the silica plates. In order to accurately spot the same amount of each compound on the plate, a stock solution of the compound (1 mg/10 µL of methylene chloride) was prepared and 25 µL of this solution was spotted on the TLC plate, taking care that the spots were of the same size. The developing system used was either ethylacetate/hexane (80:20, v/v) or ethylacetate/hexane (90:10, v/v). Antimycotic and an-



Tetrazolium Bromide (MTT)

(yellow)

Formazan

(Purple)

Figure 2. Conversion of MTT to formazan

tibacterial solution purchased from Sigma (A 9909) was used as the positive control and methylene chloride was used as the negative control. After the plates were developed, they were subjected to agar overlay.

Agar overlay TLC autography: The log phase microbes were sprayed (~10 mL) on developed TLC plates until the plates were evenly wet but not running. Immediately following the microbial spray, half strength hot (~55°C) LB agar was sprayed over each plate (~10 mL). The plate was then placed on cork supports above damp tissues in a sealed plastic box to allow 100% humidity. The tray was left in the incubator at 37°C (25°C for *M. luteus*) overnight to allow the bacteria to grow (19). An aqueous solution of MTT (5 mg/mL) was sprayed onto the bacteria covered plate (1-2 mL) and the plate was then placed back in the incubator at 37°C for several hours in a humid chamber until purple color appeared.

Microbial growth inhibition measurements: Microbial growth inhibition was determined by measuring the diameter of the inhibition zones in four different directions (0°, 45°, 90° and 135°) as described in the literature (24). The inhibition diameter was taken as an average of four measurements per TLC plate.

Statistical analysis: The results of the TLC agar overlay assay were evaluated by measuring the inhibition zone diameters after incubation. All the experiments were replicated three to five times and the mean (M) and standard deviation (Std. Dev.) were calculated for the inhibition zone diameters. The results were analyzed by Kruskal-Wallis and Mann-Whitney *U* tests, and significance was tested at $p < 0.05$.

Results and Discussion

Assessment of the *in-vitro* antimicrobial activity of compounds from α-pinene as well as the methyl cyclohexene series was investigated using TLC-bioautographic assay. The zones of inhibition were visualized using 3-[4,5-dimethylthiazol-2yl] 2,5-diphenyl tetrazolium bromide (MTT). Actively growing bacteria break down MTT (yellow) to formazan (purple) and therefore pale yellow inhibition zones appear against a purple background (Figure 2).

For all four microorganisms, the oxygenated derivatives of α-pinene (except α-pinanone and in two cases α-pinene oxide) were found to be more antimicrobial than α-pinene itself, though the sensitivities towards the various microorganisms were different. Zones of inhibition were used to quantitatively evaluate the antimicrobial efficacy of a compound toward a particular microorganism as the same amount of each compound was spotted on the plate. The descriptive results of the study are summarized in Table I. Comparisons have been made based on statistical analyses.

In the α-pinene series, comparison of the zones of inhibition for compounds α-pinan-3-ol [3] and α-pinan-2,3-diol [4] showed that they have similar antimicrobial activity for all microorganisms except *E. coli*. Compound [4] differed from [3] in just one extra hydroxyl group, indicating that this hydroxyl group is important for the antimicrobial activity against *E.coli*. Further studies need to be done to confirm this observation. Overall, in the α-pinene series, the order in which the compound tested for antimicrobial activity (most to least) is α-pinan-2,3 diol [4], α-pinan-3-ol [3], α-pinene [1], α-pinene epoxide [2], and α-pinanone [5]. Compound [5] is the least antimicrobial and showed antimicrobial activity only against *E.coli*, which also seemed to be the most susceptible of all the microorganisms tested. Among the test microorganisms, α-pinene [1] gave significantly larger inhibition zones with *C. albicans*. *Candida albicans* had a simple layer of chitin (NAM polymer) that appeared to be readily penetrated by this compound. The larger inhibition zone for more water soluble compound [4] against gram negative *E. coli*, may be due to the ability of this compound to more readily cross over the thin layer of peptidoglycan found in this bacterium (25). The gram positive bacteria have a thicker layer of peptidoglycan (E-1,4-acetylglucosamine NAG acetyl muramic acid NAM) and were more resistant to this compound.

Table I. Zone of inhibition (diam., cm), means (M) and standard deviations (Std Dev) of test compounds against four microorganisms

	Microorganism							
	S. aureus ave.		*E. coli* ave.		*M. luteus* ave.		*C. albicans* ave.	
Compound	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
α-pinene [1]	0.43	0.05	0.86	0.05	0.36	0.15	1.56	0.03
α-pinene oxide [2]	0.89	0.01	1.53	0.07	0.00	0.00	0.00	0.00
α-pinanol [3]	2.10	0.15	2.33	0.25	2.75	0.18	2.66	0.24
α-pinane diol [4]	2.19	0.18	3.51	0.17	2.62	0.17	2.76	0.12
α-pinanone [5]	0.00	0.00	0.90	0.16	0.00	0.00	0.00	0.00
verbenone [6]	0.19	0.16	2.29	0.40	1.89	0.07	2.13	0.01
myrtenol [7]	2.06	0.63	2.16	0.78	2.43	0.25	2.57	0.18
myrtanol [8]	1.74	0.09	2.24	0.42	2.31	0.28	2.34	0.14
myrtanylamine [9]	2.53	0.06	2.91	0.07	2.50	0.07	2.34	0.15
isopino-campheylamine [10]	3.47	0.44	3.62	0.44	2.82	0.1	3.34	0.07
β-pinene [11]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
methyl cyclohexene [12]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
methyl cyclohexene epoxide [13]	1.19	0.02	0.97	0.24	1.24	0.01	2.13	0.18
methyl cyclohexanone [14]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
methyl cyclohexanol [15]	0.73	0.04	1.80	0.02	0.89	0.10	0.00	0.00

In the [3.1.1.]-bicyclics, the level of saturation was not found to be significant. Antimicrobial activity of myrtenol [7] was compared to myrtanol [8] and it was found that the less saturated and more water soluble [7] (11), had similar antimicrobial activity as [8] for all the test microorganisms.

Comparing the antimicrobial activities of α-pinene [1] (having an endocyclic double bond) and β-pinene [11] (having an exocyclic double bond), showed that the position of the double bond in [3.1.1.]-bicyclics did affect the antimicrobial activity (Table 1). α-Pinene [1] was found to be significantly more antimicrobial than β-pinene [11]. β-Pinene [11] was inactive against all microorganisms tested. To see the affect of ring substitution versus side chain substitution of a given functional group on the antimicrobial activity, α-pinanol [3] and myrtanol [8] were compared. Myrtanol [8] has a hydroxyl group as a terminal side chain substituent and was not as effective as α-pinanol [3], which has a hydroxyl group as a ring substituent. Both compounds [3] and [8] had similar activity towards *E. coli*, which seemed to be susceptible to the number of hydroxyl groups present in a compound and not their positions as observed earlier.

Similarly, myrtanylamine [9] and isopinocampheylamine [10] were compared. Compound [9] has a side chain amino substituent and compound [10] has an amino group as a ring substituent. In both cases, [3] versus [8] and [9] versus [10], it was found that ring substitution compounds were more antimicrobial than their corresponding side chain substitution compounds. Terpenoid amines appeared to be more antimicrobial than their corresponding oxygenated terpenoids as also observed earlier (11).

To see how the position of the functional group on the ring affects the antimicrobial activity of a [3.1.1.]-bicyclic, α-pinanone [5] was compared with verbenone [6]. Both compounds have a ring keto group and differ from each other only in the position of this keto group. Compound [6] was found to be significantly more antimicrobial than compound [5]. Both of these compounds showed no or little activity against the gram positive *S. aureus*.

Methyl cyclohexene epoxide [13] and methyl cyclohexanol [15] were the only two compounds that were antimicrobial in the methylcyclohexene series. Compound [13] was the most active of all the compounds tested in this series. The pattern in the antimicrobial activity of these compounds did not correlate with that found in the corresponding oxygenated α-pinene series and this observation suggests that a combination of the two factors (functional groups as well as the carbon skeleton) determine the overall antimicrobial activity of a compound.

Preliminary results show that for similarly substituted systems, a rigid system (α-pinene series) usually is more antimicrobial than a flexible one (methyl cyclohexene series). In the methyl cyclohexene series, the epoxide was the most antimicrobial and has a more rigid structure than the other methyl cyclohexene derivatives tested.

In conclusion, this study shows that in the [3.1.1.]-bicyclics, the presence of a functional group as a ring substituent renders the compound more antimicrobial than when the same functional group is a side chain substituent. [3.1.1.]-Bicyclics with an amino functional group exhibited the maximum antimicrobial activity followed by compounds with an alcoholic functional group. With a few exceptions, addition of the oxygen and nitrogen containing functional groups to the α-pinene framework increased the antimicrobial activity, especially if the functional groups could function as both H-bond donors and acceptors. This may be due to an increase in the water solubility of these compounds (10). Nitrogen containing terpenoids were found to be more antimicrobial than their oxygen containing counterparts. The antimicrobial activity trend in the methylcyclohexene series did not correspond with that found in the α-pinene series, indicating that a combination of both (carbon skeleton and functional groups) factors determine the antimicrobial activity of a compound.

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