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Hydrolysis of Grape Monoterpenyl β-D-Glucosides by Various β-Glucosidases

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The efficiency of hydrolysis of monoterpenyl β-D-glucosides by various plant (grape and sweet almond) and fungal (*Aspergillus niger*) β-glucosidases was found to be dependent on the structure of the aglycon and the origin of the enzyme. Plant β-glucosidases hydrolyzed only β-D-glucosides of primary alcohols, such as geraniol, nerol, and citronellol. β-D-Glucosides of tertiary alcohols such as linalool and α-terpineol could be hydrolyzed efficiently only by one of the two fungal β-glucosidases studied. Diastereoisomeric monoterpenyl β-D-glucosides (linalool, α-terpineol, citronellol) and isomeric monoterpenyl β-D-glucosides (geraniol, nerol) were hydrolyzed at different rates.

The role of monoterpenes in Muscat grapes and wine flavors is now well established (Bayonove and Cordonnier, 1971; Ribereau-Gayon et al., 1975; Williams et al., 1980). A major fraction of these compounds is present in grapes under the form of diglycosides consisting of 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides and 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides. The corresponding aglycons are mainly geraniol, nerol, linalool, linalool oxides, 3,7-dimethylocta-1,5-diene-3,7-diol, and to a lesser extent α-terpineol and citronellol (Williams et al., 1982a; Günata et al., 1985). These precursors are unaffected during wine making and represent an important potential source of aroma (Günata et al., 1986).

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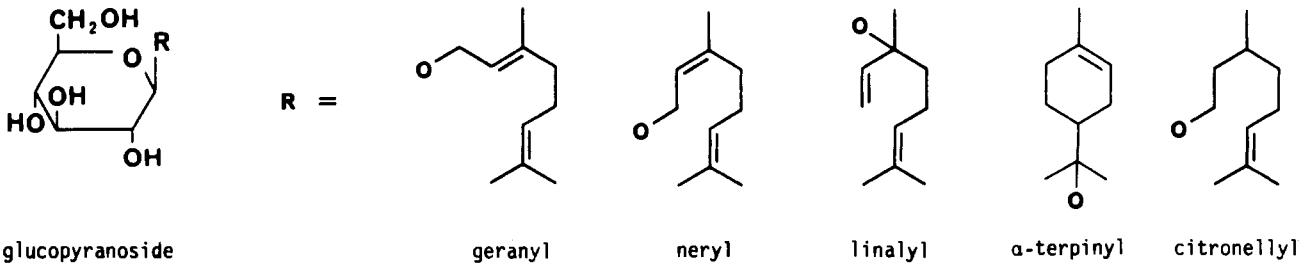

Figure 1. Structure of grape monoterpenyl β-D-glucopyranosides.

These odorless monoterpenyl glycosides can be hydrolyzed by acids or enzymes to release volatile aromatic compounds. Enzymic hydrolysis was considered a more natural method that would not modify the natural monoterpene distribution (Cordonnier and Bayonove, 1974; Williams et al., 1982b; Di Stefano, 1982; Günata et al., 1985). Recent studies demonstrated that enzymic hydrolysis of grape monoterpenyl diglycosides proceeds in two steps: First, the inter-sugar linkage is cleaved by either α-L-arabinofuranosidase or α-L-rhamnosidase regardless of the structure of aglycon moiety and the corresponding monoterpenyl β-D-glucosides are released. The liberation of the aglycon moiety can only take place during the second step, which consists in the action of a β-D-glucosidase on the previous monoterpenyl β-D-glucosides (Günata et al., 1988, 1990a). The hydrolysis of grape glycoside extracts by various plant and commercial fungal enzymes showed that some enzymes could not liberate the tertiary alcohols, such linalool and α-terpineol (Güanta et al., 1985; Aryan et al., 1987; Cordonnier et al., 1989). As the action of β-D-glucosidase enables the release of monoterpenols from grape monoterpenyl β-D-glucosides (Figure 1), the hydrolytic activity of various plant and fungal β-glucosidases toward these compounds was studied. This work was carried out in a way to determine the possible application of enzymes for the enhancement of the flavor of grape juice and wine.

EXPERIMENTAL SECTION

Substrates. Diastereoisomeric mixtures of linalyl, α-terpinyl, and citronellyl β-D-glucopyranosides on one hand, (*S*)-(+)-linalyl, (*R*)-(+)-α-terpinyl, (*S*)-(+)-citronellyl β-D-glucopyranosides and neryl and geranyl β-D-glucopyranosides on the other hand (Figure 1) were synthesized according to the modified Köenigs and Knorr method (Flowers, 1972), and the structure were confirmed by NMR.

Enzymes. Several enzyme preparations were used for their β-D-glucosidase activities. This activity, together with the α-L-arabinofuranosidase and α-L-rhamnosidase activities were determined with appropriate *p*-nitrophenyl glycosides (Günata et al., 1988).

β-D-Glucosidase from Sweet Almond (Koch-Light, England). It was devoid of α-L-arabinofuranosidase and α-rhamnosidase activities and used without further purification.

β-D-Glucosidase from Grape. Sound mature grapes (cv. Muscat of Alexandria) were harvested from the vine collections of the Agronomy Research Centre in Montpellier, France. They were immediately stored in a freezer at −20 °C. The deseeded freezed berries (500 g) were then immersed in liquid nitrogen and ground in the presence of liquid nitrogen. The subsequent powder was used to prepare a crude acetone powder that was, in turn, used for the preparation of a crude enzymic extract (Biron et al., 1988). The β-glucosidase activity was isolated by ion-exchange chromatography on a column (1.6 × 25 cm) of DEAE-Sepharose-CL-6B (Pharmacia, Sweden) by means of a linear gradient of NaCl from 0 to 0.5 M in a 0.025 M imidazole-HCl buffer (pH 7.5) at 43 mL·h^{-1}. The β-glucosidase activity was eluted at 0.06 M NaCl. The combined fractions, which exhibited β-glucosidase activity, were free of α-arabinofuranosidase and α-rhamnosidase activities.

β-D-Glucosidases from Fungi. Two different commercial crude powder enzyme preparations from *Aspergillus niger*, marketed Hemicellulase REG2 (Gist Brocades, France) and Pectinol VR (Röhm, West Germany), were studied for isolation of their β-D-glucosidase activity. A 130-mg portion of each preparation was submitted to gel filtration on a column (1.6 × 90 cm) of Ultrogel AcA 44 (IBF, France). The β-glucosidase activity of each preparation was eluted at 1.3 void volume with 0.1 M citrate–phosphate buffer (pH 7.2) at 8 mL·h^{-1}. The combined fractions exhibited less than 5% α-arabinofuranosidase and α-rhamnosidase activity compared to the β-glucosidase activity.

Enzyme Assays. A substrate solution was prepared consisting of geranyl (5 mM) and neryl (5 mM) β-D-glucosides and diastereoisomeric mixtures of (±)-linalyl (10 mM), (±)-α-terpinyl (10 mM), and (±)-citronellyl (10 mM) β-D-glucosides in 0.1 M acetate buffer (pH 4.4). The substrate concentrations used here were more than 100-fold those found in grape juice and wine (Günata et al., 1985; Wilson et al., 1986).

The enzyme assay was carried out by incubating 40 μL of each enzyme solution (showing 9 nkat of β-glucosidase activity) in 400 μL of the substrate solution at 40 °C for various times up to 36 h. Controls runs were performed by addition of heat-inactivated enzymes (95 °C, 30 min).

Analysis. At different times of incubation, 90 μL of 1 mM *p*-nitrophenyl β-D-glucopyranoside (Sigma) solution in ethyl acetate used as internal standard was added to 18 μL of enzymic reaction mixture. The solution was then evaporated to dryness in a small vial at 40 °C under nitrogen. Trimethylsilyl derivatives were prepared by adding a Tri-sil reagent (50 μL, Pierce) and allowing the mixture to stand for 20 min at 40 °C. Analytical gas chromatography was performed with a Varian 3300 gas chromatograph under the following conditions: fused silica capillary column, 50 m × 0.32 mm (i.d.), bonded with OV-1 (DB-1, J&W Scientific, 0.20-μm film thickness), operated from 125 to 305 °C at 5 °C·min^{-1}. Hydrogen was used as carrier gas (inlet pressure 60 kPa), and the fid temperature was 300 °C. The on-column injection was performed, and the injector was programmed from 90 to 150 °C at 60 °C·min^{-1} and then to 260 °C at 10 °C·min^{-1}.

The results are given as percent of variation in the peak area of each component compared with that of the internal standard.

RESULTS AND DISCUSSION

The GC analysis of trimethylsilyl derivatives of linalyl, α-terpinyl, and citronellyl β-D-glucosides led to two peaks for each compound corresponding to both diastereoisomers, which were identified by coinjection of one of them (see the Experimental Section).

The results of hydrolysis of monoterpenyl β-D-glucosides by plant (sweet almond and grape) and fungal (*A. niger*) β-glucosidases are given in Figures 2 and 3. The rate and the efficiency of hydrolysis depended on the source of the enzyme and on the structure of the aglycon.

β-D-Glucosides of primary alcohols such as geraniol, nerol, and citronellol were easily hydrolyzed by all the β-glucosidases studied here, contrary to those of tertiary alcohols (linalool, α-terpineol). The rate of hydrolysis of the latter did not change significantly even when the hydrolysis was performed on an individual solution. This dif-

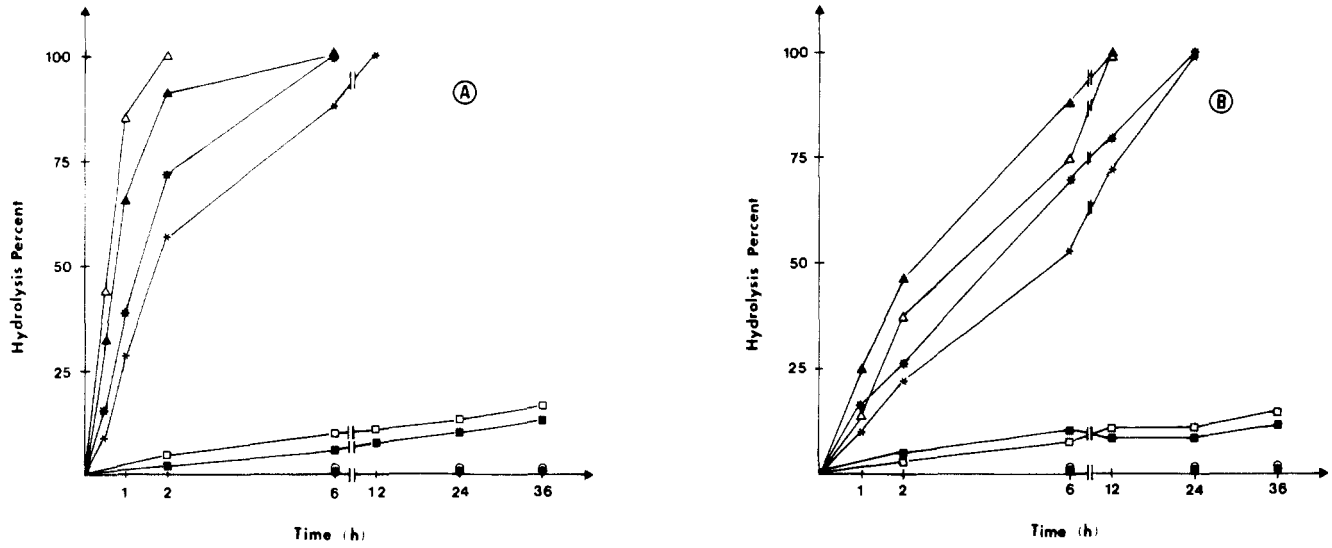

Figure 2. Hydrolyses of (*R*)-(−)-citronellyl (△), (*S*)-(+)-citronellyl (▲), (*R*)-(−)-linalyl (□), (*S*)-(+)-linalyl (■), (*S*)-(−)-α-terpinyl (○), (*R*)-(+)-α-terpinyl (●), geranyl (★), and neryl (*) β-D-glucosides by plant β-glucosidases. Key: A, sweet almond; B, grape.

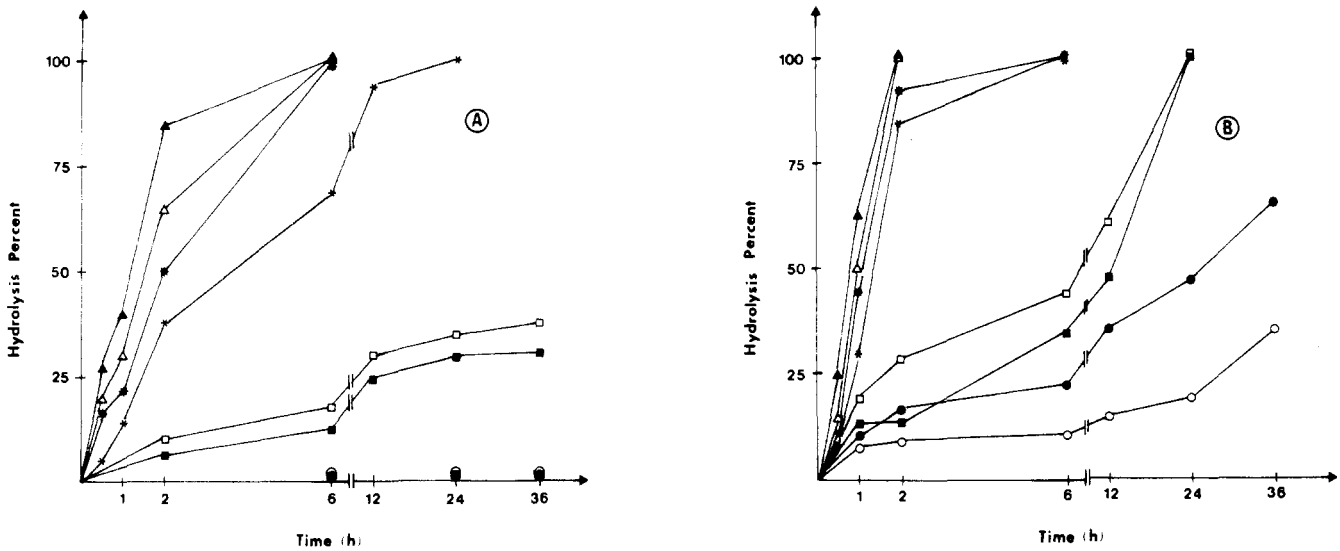

Figure 3. Hydrolysis of (*R*)-(−)-citronellyl (△), (*S*)-(+)-citronellyl (▲), (*R*)-(−)-linalyl (□), (*S*)-(+)-linalyl (■), (*S*)-(−)-α-terpinyl (○), (*R*)-(+)-α-terpinyl (●), geranyl (★), and neryl (*) β-D-glucosides by fungal (*A. niger*) β-glucosidases. Key: A, pectinol preparation; B, hemicellulase preparation.

ference is likely to be ascribed to steric effects due to the structure of the aglycon moiety. Moreover, the ^{1}H and ^{13}C NMR spectra showed that both glucoside series differ in the orientation of the β-D-glucopyranosidic linkage (Voirin et al., 1990). The rates of hydrolysis of β-D-glucosides of primary monoterpenols were found to be higher than those of β-D-glucosides of tertiary monoterpenols. These results are consistent with those obtained for the hydrolysis of primary and tertiary alkyl β-D-glucosides by almond β-glucosidase (Pigman, 1946). It should be noticed that opposite results were obtained when the acid-catalyzed hydrolysis of these substrates was performed (Pigman, 1946; Capon, 1969).

Considering β-D-glucosides of primary alcohols, (*S*)-(+)- and (*R*)-(−)-citronellyl β-D-glucosides were hydrolyzed more rapidly than geranyl and neryl β-D-glucosides. The rate of hydrolysis of (*S*)-(+)-citronellyl β-D-glucoside by β-glucosidases was higher than that of the other isomer except when almond β-glucosidase was used. On the other hand, the replacement of geraniol by its cis isomer (i.e., nerol) on the aglycon moiety caused an important decrease in hydrolysis rate.

The highest rates of hydrolyses of β-D-glucosides of primary alcohols were obtained by action of β-glucosidase from the Hemicellulase preparation and from sweet almond, whereas the lowest rates were obtained with grape β-glucosidase. For example, neryl β-D-glucoside, which was hydrolyzed more slowly than the other substrates, disappeared after 6 h of incubation with β-glucosidase from Hemicellulase preparation, but disappeared only after 24 h of incubation with grape β-glucosidase.

The data obtained show that citronellyl, geranyl, and neryl β-D-glucosides are good substrates for the grape, the almond, and the fungal β-glucosidases studied here. However, the hydrolysis of grape monoterpenyl glycoside extracts by these plant β-glucosidases liberated much smaller amounts of geraniol and nerol when compared to fungal glycosidases (Günata et al., 1985; Aryan et al., 1987). This can be ascribed to the lack or to the low levels of α-arabinofuranosidase and α-rhamnosidase activities in almond and grape β-glycosidases (Aryan et al., 1987; Cordonnier et al., 1989) and also to the presence of high levels of these activities in fungal enzymic preparations (Günata et al., 1988, 1990a; Cordonnier et al., 1989) since they are involved, along with β-glucosidase activity, in the hydrolysis of grape monoterpenyl glyco-

sides (Günata et al., 1988). This also explains why some authors could not observe an increase in the amounts of monoterpenols released from grape glycoside extracts when almond β-glucosidase was supplemented with a α-glucosidase (Aryan et al., 1987).

Grape and almond β-glucosidases possess much lower activities toward β-D-glucosides of tertiary alcohols than *A. niger* β-glucosidases. These results confirm our previous data (Günata et al., 1985) and those recently reported (Aryan et al., 1987). Additionally, we found that grape and almond β-glucosidases exhibit a glucosyl transferase activity for geraniol and nerol but not for linalool (unpublished data), which correlates with their hydrolytic action toward β-D-glucosides of these monoterpenols.

It is interesting to notice that *A. niger* β-glucosidases from two different commercial preparations do not show the same hydrolysis rates toward linalyl β-D-glucoside. After 24 h of incubation, β-glucosidase from the Hemicellulase preparation hydrolyzed this substrate completely, whereas β-glucosidase from the Pectinol preparation hydrolyzed only one-third of it. The lower rate of liberation of linalool from the grape monoterpenyl glycoside extracts by Pectinol than by Hemicellulase (Cordonnier et al., 1989) could be a result of this difference of activity toward linalyl β-D-glucoside, since both preparations possess rich enough α-L-arabinofuranosidase and α-L-rhamnopyranosidase activities (Cordonnier et al., 1989). Furthermore, plant and fungal β-glucosidases were slightly more efficient on (*R*)-(−)-linalyl β-D-glucoside than on (*S*)-(+)-linalyl β-D-glucoside.

α-Terpinyl β-D-glucoside was a substrate neither for plant β-glucosidases nor for *A. niger* β-glucosidase from Pectinol preparation. This substrate was not hydrolyzed even when incubated alone with each of these enzymes in order to eliminate possible inhibitory effects due to the high substrate concentrations in our enzyme assays. The cyclic form of α-terpineol probably causes a steric hindrance for these β-glucosidases. However, this substrate was hydrolyzed by *A. niger* β-glucosidase from the Hemicellulase preparation, contrary to β-glucosidase from the Pectinol preparation. Such variations in both commercial enzymic preparations are probably due to the use of differents strains of *A. niger*. (*R*)-(+)-α-Terpinyl β-D-glucoside is cleaved more rapidly than (*S*)-(−)-α-terpinyl β-D-glucoside. In contrast to linalyl β-D-glucoside, α-terpinyl β-D-glucoside was not hydrolyzed completely even after 36 h of incubation by β-glucosidase from Hemicellulase. Besides, yeast β-glucosidases from *Candida* strains were found to be active on α-terpinyl β-D-glucoside (Günata et al., 1990b).

Almond β-glucosidase (Pigman, 1946; Heyworth and Walker, 1962; Lalégerie, 1974) and fungal β-glucosidases (Pigman, 1946; Sadana et al., 1983) are known to be nonspecific for the aglycon moiety of some alkyl and aryl β-D-glucosides. However, the data given here show that the activity of plant and fungal β-glucosidases, particularly the former, seems to be influenced by the aglycon moiety of monoterpenyl β-D-glucosides. Some plant β-glucosidases were reported to possess a narrow aglycon specificity toward some cyanogenic glucosides (Hösel and Conn, 1982).

Furthermore, the great difference in the hydrolysis of α-terpinyl β-D-glucoside by two *A. niger* β-glucosidases could be used to characterize β-glucosidases from different fungi strains. The ortho and para substitutions in the aglycon moiety of aryl β-D-glucosides have been used to characterize enzymes from different sources (Pigman, 1946).

CONCLUSION

The present work shows that the structure of the aglycon moiety of monoterpenyl β-D-glucosides can notably influence the hydrolytic efficiency of plant and fungal β-glucosidases. The tertiary monoterpenol aglycon appears to be a determinant factor in the hydrolytic activity of β-glucosidases.

These results obviously show that the distribution and the amounts of monoterpenols liberated from their glucoside precursors can vary to a large extent according to the origin of the enzyme investigated. Considering the possible use of a β-glucosidase in juice processing and wine making, the enzyme under concern should have, besides a large aglycon specificity, a good stability and tolerance to low pH (3.0–3.5), to glucose (0.5 M in juice), to gluconolactone (Günata et al., 1989), and to ethanol (10–15% in wine).

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Registry No. (*R*)-Citronellyl-β-D-glucopyranoside, 117895-55-3; (*S*)-citronellyl-β-D-glucopyranoside, 99096-57-8; (*R*)-linalyl-β-D-glucopyranoside, 99096-59-0; (*S*)-linalyl-β-D-glucopyranoside, 104154-37-2; (*S*)-α-terpinyl-β-D-glucopyranoside, 89616-07-9; (*R*)-α-terpinyl-β-D-glucopyranoside, 114673-99-3; geranyl-β-D-glucopyranoside, 22850-13-1; neryl-β-D-glucopyranoside, 22850-14-2; β-glucosidase, 9001-22-3.

A Bromine-Based Color Reaction for the Detection of Geosmin

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Geosmin is responsible for natural flavors in beans and beets and for off-flavors in water and fish. This tertiary decalol reacts with bromine in the presence of formic acid to produce a blue complex with maximum absorbance at 650 nm. A method for carrying out this reaction is described, and a possible mechanism is discussed.

Geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol) (Figure 1) has been identified as a musty/earthy odor and off-flavor causative compound in water (Rosen et al., 1969; Person, 1981), beets (Murray et al., 1975), beans (Buttery et al., 1975), and fish (Person, 1981; Lovell and Sackey, 1973; Johnsen and Kuan, 1987). The compound is organoleptically detectable in the parts per billion range (Huang et al., 1984). However, instrumental methods for detection of the compound have been limited to gas chromatographic methods. Most of these are modifications of purge and trap methods utilizing various phase separations based on the volatility and/or hydrophobicity of the tertiary decalol (Buttery et al., 1976; Huang et al., 1984; Johnsen and Kuan, 1987). Such methods, though sensitive and accurate, are slower than spectrophotometric or HPLC assays. Such spectrophotometric and HPLC methods for geosmin have not been practical due to the absence of natural or induceable ultraviolet or visible absorptivity of the compound.

The Tortelli–Jaffe (TJ) reaction is a bromine-based color reaction stated to be specific for compounds that contain either naturally occurring or chemically induced ditertiary double bonds (Tortelli and Jaffe, 1915; Fieser and Fieser, 1959; Hensarling and Jacks, 1983). Since geosmin dehydrates to argosmin (Gerber, 1979), which contains such a ditertiary double bond (Figure 1), it was

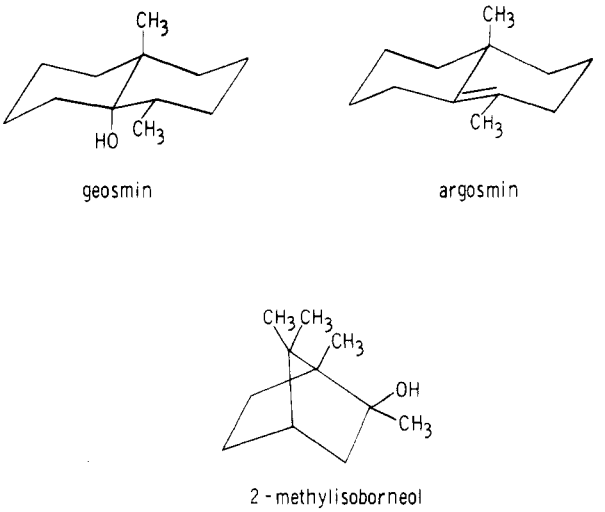

Figure 1. Structure of geosmin, argosmin, and 2-methylisoborneol.

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