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JUST HOW INSOLUBLE ARE MONOTERPENES?

JEFFREY D. WEIDENHAMER,[1,*] FRANCISCO A. MACIAS,[1,3] NIKOLAUS H. FISCHER,[1] and G. BRUCE WILLIAMSON[2]

[1]*Department of Chemistry*
[2]*Department of Botany*
Louisiana State University
Baton Rouge, Louisiana 70803

Abstract—Prior generalizations about the ecological roles of monoterpenes may be misleading if based on the presumed insolubility of monoterpenes in water. We determined the aqueous solubility of 31 biologically active monoterpenes by gas chromatography. While hydrocarbons were of low solubility (<35 ppm), oxygenated monoterpenes exhibited solubilities one or two orders of magnitude higher, with ranges of 155–6990 ppm for ketones and of 183–1360 ppm for alcohols. Many monoterpenes are phytotoxic in concentrations under 100 ppm, well below the saturated aqueous concentrations of oxygenated monoterpenes. Therefore, even dilute, unsaturated solutions of monoterpenes, occurring naturally in plant tissues and soil solutions, may act as potent biological inhibitors.

Key Words—Allelopathy, monoterpenes, *Calamintha ashei*, *Conradina canescens*, ursolic acid, borneol, camphor, juglone, solubility.

INTRODUCTION

Monoterpenes operate as chemical defenses against herbivores (Eisner, 1964) and disease (W.H. Muller, 1965), fragrances attractive to pollinators (Harborne, 1988), and phytotoxins inhibitory to other plants (Muller et al., 1964; Muller and Chou, 1972; Gant and Clebsch, 1975). Chemically, the molecular skeletons of monoterpenes possess 10 carbon atoms derived from two C_5 isoprene units. They exist as hydrocarbons or as oxygenated moieties with aldehyde, alcohol, ketone, ester, and ether functionalities. Furthermore, they may be acyclic,

*To whom correspondence should be addressed. Current address: Department of Chemistry, Ashland University, Ashland, Ohio 44805.
[3]Current address: Departamento de Química Orgánica, Universidad de Cadiz, 11510 Puerto Real, Cadiz, Spain.

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monocyclic, bicyclic or tricyclic in structure (Dev, 1982). Owing to the low molecular weight and nonpolar character of these compounds, the group as a whole has been classified as volatile and assumed to have negligible water solubility compared to other classes of organic compounds. For example, Harborne (1984) claims "terpenoids are generally lipid-soluble" whereas "phenolic substances tend to be water-soluble." Despite two older (Rhode, 1922; Seidell, 1940–41) and one modern (Smyrl and LeMaguer, 1980) citation to the contrary, standard chemical references concur on the aqueous insolubility of monoterpenes: the Merck Index (Budavari, 1989) lists borneol, carvone, and pulegone as "almost insoluble" or "practically insoluble" in water, and the CRC Handbook of Chemistry and Physics (Weast, 1976, 1989) registers camphor, geraniol, and pulegone as insoluble, and borneol, carvone, and menthone as slightly soluble in 1976, but none of these compounds are listed as water soluble in 1989.

In studies of allelopathy in the pine forests of the southeastern coastal plain of the United States, we found evidence for the allelopathic effects of two shrubs, *Calamintha ashei* and *Conradina canescens* (Labiatae), which contain monoterpenes as active constituents (Richardson and Williamson, 1988; Tanrisever et al., 1987, 1988; Macias et al., 1989; Williamson et al., 1989). However, the only likely mechanism of allelochemical release was aqueous leaching of foliage and litter—an apparent incongruity with the reputed insolubility of monoterpenes. Because of the alleged insolubility of these compounds, we have proposed that natural detergents such as the triterpene ursolic acid, which is also present in these two mints, may speed solubilization and increase the solubilities of terpenoid compounds. Our previous experimental studies unambiguously established the formation of micelles in water leachates of these plants (Fischer et al., 1988; Tanrisever et al., 1988), although increases in solubilization rates and solubilities were not determined.

Therefore, as part of our ongoing investigations of allelopathic mechanisms in the Florida scrub, we determined the water solubilities of a variety of monoterpenes, eight hydrocarbons and 23 with oxygenated moieties, and compared them to known phenolic phytotoxins (Davis, 1928; Blum and Dalton, 1985; Tanrisever et al., 1987; Harborne, 1988) that are widely presumed to be water-soluble: juglone, ferulic acid, and hydrocinnamic acid.

METHODS AND MATERIALS

Preparation of Standards. The three *Calamintha ashei* natural products (calaminthone, desacetylcalaminthone, and epievodone) were previously isolated and purified in our laboratories. Other compounds were obtained from

commercial sources (Aldrich Chemical Co., Milwaukee, Wisconsin; Eastman Kodak Co., Rochester, New York; Fluka Chemical Corp., Ronkonkoma, New York; Sigma Chemical Co., St. Louis, Missouri; and SCM Glidden Organics, Jacksonville, Florida). Purities of all monoterpenes were determined by gas chromatography. A 10,000 ppm stock solution of each compound was prepared in ethanol and serially diluted to 1000, 100, and 10 ppm. Standard solutions were stored under refrigeration in crimp-top vials with Teflon seals.

Preparation and Analysis of Saturated Aqueous Solutions of Monoterpenes and Phenolics. Saturated aqueous solutions were prepared by adding an excess of each compound to 1.5 ml of water in crimp-top vials with Teflon seals and sonicating for 30 min in a water bath (25–30°C). Vials were inverted and stored for three days at ambient temperature to permit phase separation of the excess organic compound from the aqueous solution, with the exception of juglone, which was analyzed immediately before oxidation occurred. Solutions prepared from solids were filtered prior to analysis.

The effect of ursolic acid on monoterpene solubility was investigated by determining solubilities using two procedures. In the first, solubility was determined in a saturated aqueous solution of ursolic acid using the methods detailed above. Saturated solutions of ursolic acid were prepared by sonication for 30 min (2 mg/20 ml). The ursolic acid solution was allowed to stand at room temperature for 24 hr and filtered through a 0.2-μm nylon membrane. The second procedure used water containing 0.5 mg solid ursolic acid/1.5 ml. The ursolic acid–monoterpene emulsions were filtered (0.2 μm) and stored inverted in a fresh vial for 72 hr.

Monoterpene concentrations were quantified based on peak area, corrected for compound purity, in a Hewlett Packard 5890 gas chromatograph equipped with a split/splitless injector system, a flame ionization detector, and a Hewlett Packard 3393A integrator. Linear detector response over the range of 10–10,000 ng was verified for each compound. Solubilities of the phenolics were determined by UV absorbance with a Perkin-Elmer Lambda 2 spectrophotometer.

Bioassay Procedure. Five milliliters of five concentrations (25, 10, 5, 1, and 0 ppm) of aqueous solutions of borneol, camphor, and juglone were added to 480 ml glass jars lined with one sheet of Whatman No. 1 filter paper and containing 25 seeds of native *Rudbeckia hirta* or commercial lettuce *Lactuca sativa*. Assays were conducted in the dark at room temperature (23–25°C), replicated three times, and terminated after three to five days. Seed were considered to be germinated if the radicle protruded at least 1 mm. Germination in each treatment is presented as a percent of germination in the control (0 ppm). Responses were compared to the controls ($P = 0.05$) using the least squares means of the general linear models procedure of the Statistical Analysis System (SAS) programs (SAS Institute, Inc., 1985).

RESULTS AND DISCUSSION

Solubility among the monoterpenes was extremely variable, ranging from a low of < 10 ppm (parts per million by weight) to a high of 6990 ppm. The eight hydrocarbon monoterpenes had low solubilities, all under 35 ppm (Figures 1 and 2). However, monoterpenes containing oxygen in the form of a ketone, alcohol, ether, or aldehyde had solubilities 10–100 times greater than hydrocarbons with comparable skeletons. Alcohols were somewhat more soluble than comparable ketones in the monocyclic skeletons (Figure 1). In bridged bicyclic monoterpenes, the ketones were more soluble than comparable alcohols, perhaps due to ring strain favoring formation of the geminal diols—for example, camphor (550 ppm) versus borneol (274 ppm) and verbenone (6990) versus myrtenol (1010 ppm) (Figure 2).

The solubility of monoterpenes was not enhanced in saturated ursolic acid solutions or in emulsions of ursolic acid with the monoterpenes (data not shown). Because no increase in solubility was observed with a group of 11 monoterpenes (borneol, camphene, (+)-camphor, cineole, *p*-cymene, *d*-limonene, myrcene, α-pinene, β-pinene, sabinene, and α-terpinene), no other determinations were made. When solid ursolic acid was present, the solubility of monoterpenes was, in fact, reduced. The ursolic acid apparently adsorbed the bulk of the added monoterpene, similar to the action of a solid-phase adsorbent. The two ursolic acid procedures were originally chosen because of uncertainty about how ursolic acid might act to increase monoterpene solubility. Lacking a sensitive analytical method for ursolic acid in aqueous solution, there was doubt about whether the small but unquantifiable amount of ursolic acid present in a saturated solution would be sufficient to have an effect. In the natural situation, the leaves of *Calamintha* and *Conradina* contain large quantities of ursolic acid, hence the treatment with 0.5 mg ursolic acid per vial was included for comparison.

Overall, the prior generalization that monoterpenes are insoluble in water is shown by these results to be invalid. As a class, monoterpenes exhibit a range of solubilities comparable to the common phenolics tested here—juglone (52 ppm), ferulic acid (174 ppm), and hydrocinnamic acid (3490 ppm). Since the true difference between monoterpenes and phenolics is the biochemical pathway of origin, the mevalonic acid and shikimic acid pathways, respectively, the reported generalizations about solubility differences may be false. Given that variation in solubility within the monoterpenes and within the phenolics is greater than differences between the classes, the generalizations about their biochemical activities and ecological functions based on putative differences in aqueous solubilities need to be reexamined. For example, one conclusion of Tukey's (1969) classic foliar leaching studies is that "carbon dioxide, ethylene and terpenes" are released as volatiles, while rain and dew leach "mineral nutrients, carbohydrates, amino and organic acids, and growth regulators." Characterizing the

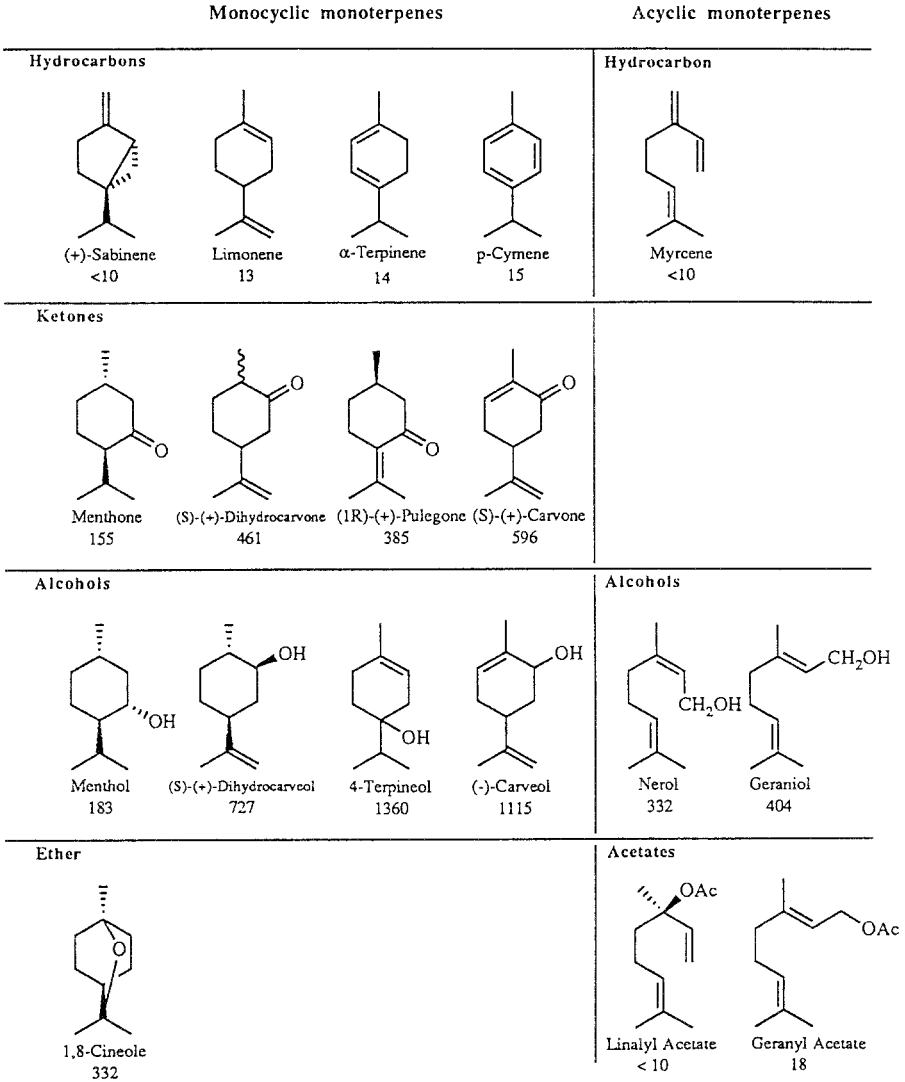

FIG. 1. Monocyclic and acyclic monoterpenes with their aqueous solubilities (ppm).

role of secondary metabolites in litter decomposition, Horner et al. (1988) claimed that "Leaching losses of fairly water soluble components (e.g., most simple phenolics, phenylpropanoids, flavonoids, and tannins) should exceed those of components that are only slightly or negligibly soluble in water (e.g., terpenes and lignin, respectively)." In regard to allelopathy, numerous authors,

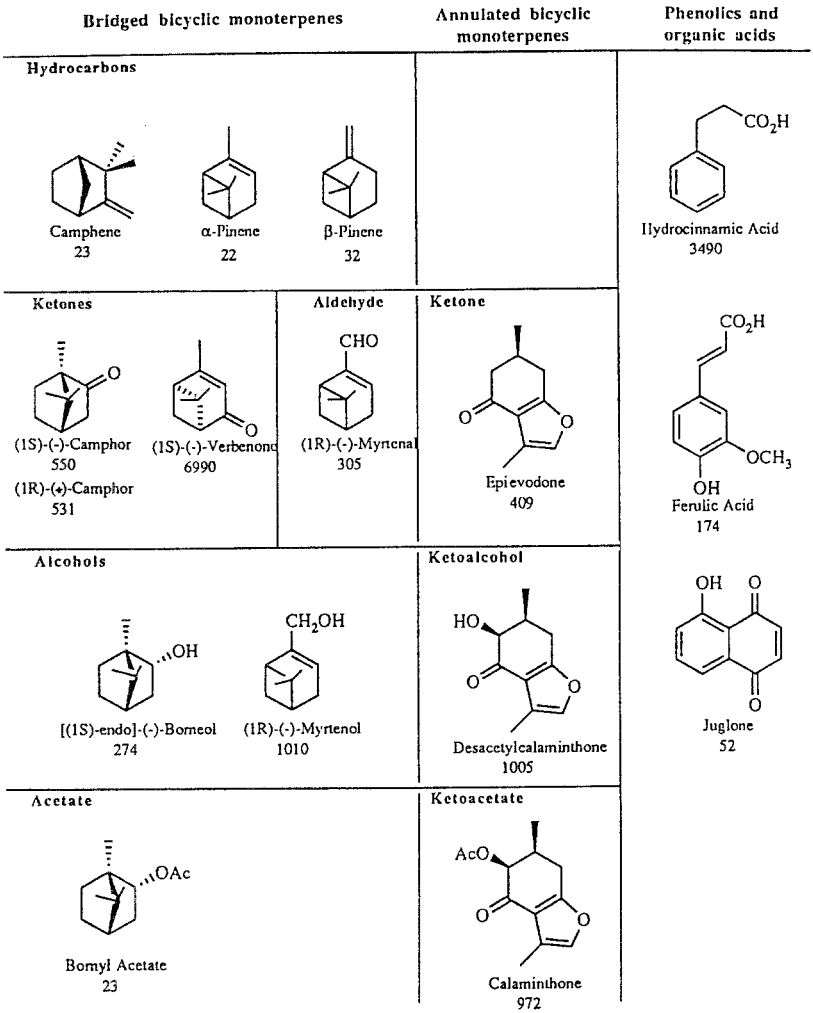

FIG. 2. Bicyclic monoterpenes and phenolic and organic acids with their aqueous solubilities (ppm).

including ourselves, have differentiated the "volatile terpenes" from the "water-soluble" phenolics and aromatic acids (Whittaker, 1971; National Research Council, 1971; Harborne, 1988; Fischer et al., 1989; Williamson et al., 1992)—all apparently based on the pioneering research of C.H. Muller (Muller et al., 1964; C.H. Muller, 1965; McPherson et al., 1971; Muller and Chou, 1972) who found several monoterpenes emitted as volatiles from *Salvia leucophylla*

(Labiatae) and *Artemisia californica* (Asteraceae) and several phenols and organic acids washed from the leaves of *Adenostoma fasciculatum* (Roasaceae) and *Arctostaphylos glandulosa* (Ericaceae).

For monoterpenes, biological activities are as variable as their solubilities, but in many cases compounds are active at apparently low concentrations, i.e., well below their aqueous solubilities (Fischer, 1991). Here, we present the results of bioassays for allelopathy with two monoterpenes, borneol and (+)-camphor, and the phenolic, juglone, the active constituent of *Juglans nigra* (Davis, 1928; Harborne, 1988). Significant ($P < 0.05$) inhibition of germination in both test plants was common at 10 and 25 ppm, less so at 5 ppm, and only rarely at 1 ppm for juglone, borneol, and camphor (Figure 3). At 5 ppm, both borneol and camphor significantly reduced germination of *Rudbeckia*, while juglone had no effect. Thus, in numerous cases, the biological activity of the monoterpenes matched or exceeded that of juglone, the well-known allelopathic agent of black walnut, while the solubility of the two monoterpenes exceeded that of juglone. Like many other monoterpenes (Fischer, 1991), borneol and camphor are active biological inhibitors in concentrations well below their

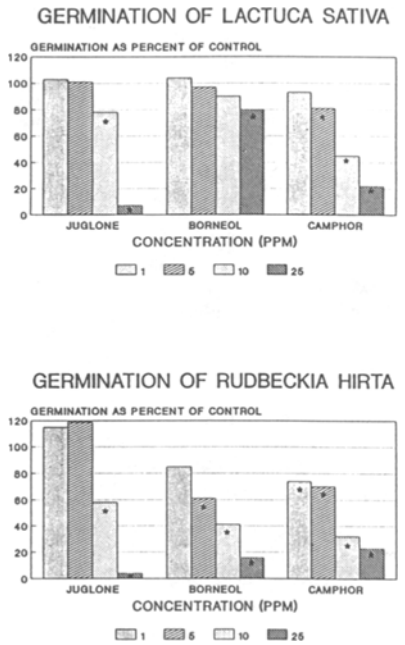

FIG. 3. Germination of *Lactuca sativa* and *Rudbeckia hirta* in 1, 5, 10, and 25 ppm aqueous solutions of juglone, borneol, and camphor, as percentages of the water controls (0 ppm).

aqueous solubilities, 274 and 550 ppm, respectively. Rather than measuring solubility in absolute terms, it is perhaps most appropriate, for biologically active molecules, to consider solubility relative to concentrations needed for biological activity.

Our prior hypothesis of the importance of micelles formed by biological detergents and monoterpenes needs revision on several counts: first, ursolic acid did not increase the water solubilities of monoterpenes; and second, monoterpene solubilities alone are sufficient for biological activity. Effects of ursolic acid on solubilization rate were not investigated in this study. Our preliminary studies (unpublished) using sonication to solubilize the monoterpenes show no effect on solubilization rate. The effect of ursolic acid on the biological activity of monoterpenes was also not investigated in this study. In previous bioassays (Fischer et al., 1988), epievodone (250 ppm) in a saturated ursolic acid solution strongly inhibited *Schizachyrium* germination, while epievodone in aqueous solution at the same concentration was strongly stimulatory. The results of this earlier study suggest that ursolic acid may play a role in facilitating transport of monoterpenes to target seeds or seedlings. Clearly, the ecological significance of the formation of micelles in water leachates of allelopathic shrubs of the Florida scrub community (Fischer et al., 1988; Tanrisever et al., 1988) needs further investigation.

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