

Inhibition of Plant Asparagine Synthetase by Monoterpene Cineoles¹

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Asparagine (Asn) synthetase (AS) is the key enzyme in Asn biosynthesis and plays an important role in nitrogen mobilization. Despite its important physiological function, little research has been done documenting inhibitors of plant AS. Plant growth inhibition caused by the natural monoterpene 1,4-cineole and its structurally related herbicide cinmethylin was reversed 65% and 55%, respectively, by providing 100 μM Asn exogenously. Reversion of the phytotoxic effect was dependent on the concentration of Asn. The presence of either 1,4-cineole or cinmethylin stimulated root uptake of [¹⁴C]Asn by lettuce (*Lactuca sativa*) seedlings. Although the physiological responses suggested that both compounds affected Asn biosynthesis, biochemical analysis of AS activity showed that the natural monoterpene was a potent inhibitor (I_{50} = approximately 0.5 μM) of the enzyme, whereas the commercial product was not inhibitory up to levels of 10 mM. Analysis of the putative metabolite, 2-hydroxy-1,4-cineole, showed that the cis-enantiomer was much more active than the trans-enantiomer, suggesting that the hydroxyl group was involved in the specific ligand/active site interaction. This is the first report that AS is a suitable herbicide target site, and that cinmethylin is apparently a proherbicide that requires metabolic bioactivation via cleavage of the benzyl-ether side chain.

Asn synthetase (AS; EC 6.3.5.4) is the primary enzyme involved in the production of Asn in plants. It catalyzes a reaction where Asn is biosynthesized from Asp using an ATP-dependent reaction with either Gln or ammonia as the nitrogen source. There are two principal classes of AS: The first class is found in prokaryotes and employs ammonia as the sole source of nitrogen. The second class includes eukaryotic ASs that use either Gln or ammonia as the nitrogen source (Richards and Schuster, 1998). The gene coding for Gln-dependent AS has been isolated from several organisms, including bacteria (Reitzer, 1983), yeast (Ramos and Wiame, 1979), plants (Tsai and Coruzzi, 1990; Lam et al., 1994), and mammalian sources (Andrulis et al., 1987). Sequence analysis has determined that Gln-dependent AS is a member of the Class II Gln amidotransferase (GAT) superfamily (Richards and Schuster, 1998). Gln-dependent AS is the primary source of Asn in vascular plants.

ASs also play a major role in the remobilization of nitrogen in germinating seeds, particularly in legumes such as soybean (*Glycine max*) and lupine (*Lupinus albus*; Rognes, 1975, 1980), where storage proteins are converted to Asn for transport to growing apices. For example, in lupine (*Lupinus* spp.) up to 87% of stored nitrogen is converted to Asn (Lea et al., 1978). The important physiological roles of AS make this enzyme a putative site for potential inhibitors. Numerous compounds have been found to in-

hibit AS. However, inhibitors of AS are generally substrate analogs and their affinity for AS is usually not very high (e.g. K_i s in the millimolar range [Cooney et al., 1976]).

Monoterpene cineoles are commonly found as components of essential oils from aromatic plants such as *Laurus nobilis* L. (Hogg et al., 1974), *Salvia* spp. (Muller et al., 1964; Muller, 1965), *Eucalyptus* spp. (Birch et al., 1959; Orihara and Furuya, 1994), *Xanthoxylum rhetsa* D.C. (Naves and Ardizio, 1950), *Cunila spicata* (Manns, 1995), and *Artemisia* spp. (Haagen-Smit, 1958; Ahmad and Misra, 1994). Many classes of volatile monoterpenes inhibit plant growth (e.g. Muller and Muller, 1964). Field studies have indicated that growth inhibition of grasses by volatile monoterpenes released by *Salvia leucophylla* (including 1,8-cineole) is most effective during seedling development and establishment (Muller et al., 1964). One of the major constituents of these plant essential oils is 1,8-cineole (eucalyptol; 1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane), which has been identified as one of the most potent allelochemicals released by *Artemisia* spp. (Halligan, 1975) and *Eucalyptus* spp. (Kumar and Motto, 1986). Muller (1965) originally reported phytotoxic activity of 1,8-cineole in the field, and suggested that this natural product may be involved in allelopathy. Laboratory studies (Vaughn and Spencer, 1993; Romagni et al., 2000) also have shown that soil-applied 1,8-cineole suppressed the growth of several weeds. However, several field reports demonstrated that 1,8-cineole alone has poor herbicidal activity (Halligan, 1975; Heisey and Delwiche, 1984).

Other naturally occurring monoterpenes, such as camphor, pulegone, and α - and β -pinene, have sub-

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sequently been found to be phytotoxic to crop and weed species (Asplund, 1968; Duke and Abbas, 1995). Natural monoterpene analogs of 1,8-cineole, such as 1,4-cineole, are minor components of some of the same plant extracts (Kumar and Motto, 1986), but little is known about their biological activities. We recently determined that 1,4-cineole was phytotoxic and that the difference in the placement of the oxygen bridge, relative to 1,8-cineole, imparts different modes of action for these two structural analogs (Romagni et al., 2000).

As a part of a continuing search for novel herbicide sites of action, we have focused this research on the elucidation of the mode of action of natural phytotoxins. Biologically active natural products tend to have mechanisms of action different from those of synthetic herbicides (Duke and Abbas, 1995; Dayan et al., 1999). In this study, the structural similarities between portions of cinmethylin and the natural monoterpene 1,4-cineole have provided a rather unique situation where synthetic and natural product chemistry overlap.

The commercial herbicide cinmethylin (Fig. 1A) is a 2-benzyl ether substituted analog of the monoterpene 1,4-cineole (Fig. 1B) (1-methyl-4-(1-methylethyl)-7-oxabicyclo[2.2.1]heptane). This compound was discovered and partially developed by Shell Chemicals (Grayson et al., 1987), ostensibly as a derivative of the allelopathic natural monoterpene, 1,8-cineole (Devine et al., 1993). The benzyl ether substitution ap-

pears to decrease the volatility of the cineole ring by several orders of magnitude thereby rendering it more suitable for herbicide use (Vaughn and Spencer, 1996). Cinmethylin is a moderately effective growth inhibitor used for monocot weed control (Russell et al., 1991). Despite the fact that it has been used commercially in both Europe and Japan and has been studied experimentally for several decades, the mechanism of action of this herbicide has remained unknown (DiTomaso and Duke, 1991; Baum et al., 1998).

In this study we report that cinmethylin has a novel mechanism of action involving the inhibition of AS. To our knowledge, the Asn biosynthetic pathway has never been identified as a molecular target site for herbicides. Furthermore, we demonstrate that although the *in vivo* phytotoxic effects of cinmethylin and 1,4-cineole are similar, AS is not inhibited by cinmethylin directly, but is highly sensitive to the natural plant monoterpene 1,4-cineole. Thus, cinmethylin is apparently a proherbicide, which requires metabolic cleavage of the benzyl ether substitution to release the highly AS-inhibiting monoterpene component.

RESULTS

The I_{50} of cinmethylin and 1,4-cineole on lettuce (*Lactuca sativa*) seedlings, defined as the concentration required for 50% inhibition of root growth, were approximately 0.4 and 0.04 μM , respectively (Fig. 1). Under the controlled conditions of this experiment, where volatilization of 1,4-cineole was minimized by sealing the plates, the natural cineole was 1 order of magnitude more active than the synthetic derivative.

These experiments also allowed the calculation of the lowest-complete-inhibition concentration (LCIC), which reflects the lowest concentration required for maximum phytotoxic effect. Determination of this concentration was important because of the possible stoichiometric effects involved in reversion studies that were performed later (i.e. reversion of growth inhibition may not be physiologically possible if extremely high concentrations of inhibitor are used). The LCIC of cinmethylin and 1,4-cineole on lettuce were 1.0 and 0.1 μM , respectively.

Using similar dose-response assays performed in Petri dishes, we determined that the structurally related 1,8-cineole was not toxic to lettuce or barley at concentrations up to 100 μM , or to corn or velvetleaf at concentrations up to 10 mM (data not shown). However, further investigation found that 1,8-cineole could affect germination and seedling development at 10 $\mu\text{g g}^{-1}$ sand (Romagni et al., 2000). 1,4-Cineole was also phytotoxic in this assay, but symptoms of 1,8-cineole were different from either cinmethylin (using the standard assay) or 1,4-cineole, therefore making comparisons of the relative potency of these compounds difficult.

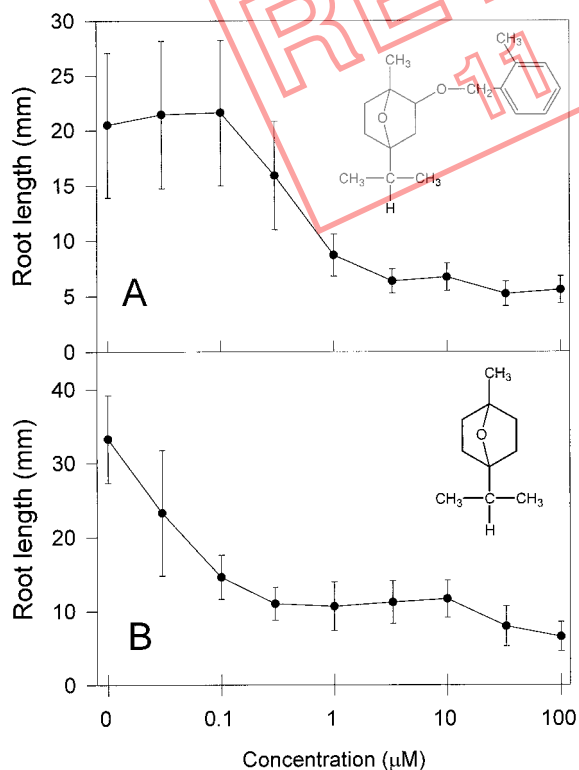


Figure 1. Dose-response curves and structures for cinmethylin (A) and 1,4-cineole (B). Error bars represent ± 1 SD.

Reversal of the effect of cinnemethylin using the 20 common amino acids was attempted. Growth inhibition caused by cinnemethylin was reversed with 100 μM Asn ($P < 0.0001$) (Fig. 2). Gln also alleviated some of the inhibition ($P < 0.01$) but not to the degree of Asn. The remaining 18 amino acids did not significantly reverse the effect of cinnemethylin.

An Asn dose-response study established that reversion of the effect of cinnemethylin was concentration dependent and that reversal could be attained at levels as low as 1 μM (Fig. 3). However, optimum Asn concentration required for reversal was 50 μM (55% reversal). Higher levels ($\geq 100 \mu\text{M}$) were found to inhibit growth. A concurrent Asn dose response was performed with the LCIC concentration of 1,4-cineole (0.1 μM). Asn also reversed the phytotoxic effect of 1,4-cineole (approximately 65% reversal), further demonstrating that both inhibitors have the same target site. A separate series of dose-response assays were done using Gln, Gln plus Asp, and Asp to determine which AS binding site might be affected. Gln and Gln plus Asp did not differ significantly, reversing inhibition 17% and 20%, respectively (data not shown). Asp alone did not reverse inhibition.

Root uptake of radiolabeled Asn was greater in treated plants than in the controls (Fig. 4). Increased

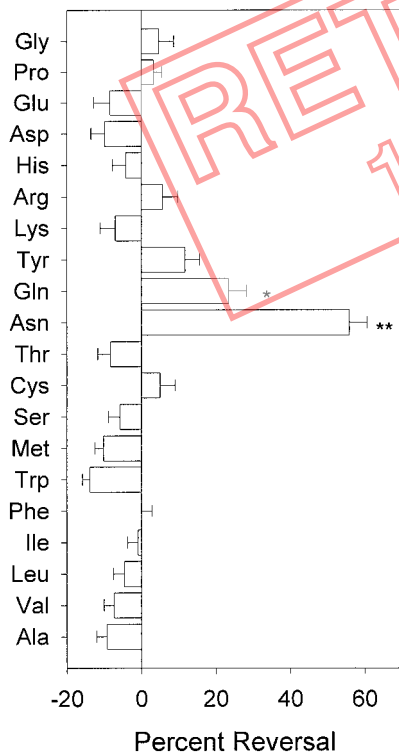


Figure 2. Percent reversal of cinmethylin-induced root growth inhibition by exogenous supply of amino acids (see "Materials and Methods"). All 20 essential amino acids were tested individually at 100- μM final concentrations (except for Tyr, Trp, and Met at 50 μM). Asterisks denote significance (**, $P \leq 0.0001$; *, $P \leq 0.01$); error bars = 1 SD.

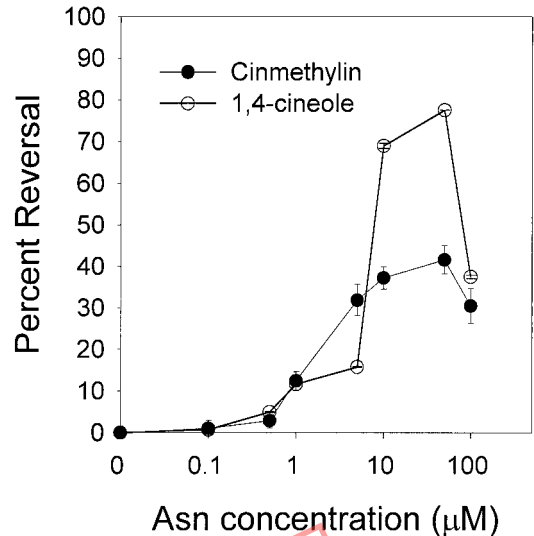


Figure 3. Dose-response curve illustrating the reversal effect of exogenous Asn on the phytotoxic effect of 1 μM cinmethylin and 1,4-cineole. Error bars = 1 SD.

uptake of Asn was not due to increased root permeability since the uptake of [^{14}C]Asp was not affected by either the presence or absence of cinmethylin. Levels of [^{14}C]Asp remained at 5,000 dpm for all treatments (data not shown).

Using an HPLC protocol that measured the enzymatic conversion of [^{14}C]Asp into [^{14}C]Asn, we determined that in vitro AS activity was not inhibited by cinmethylin at concentrations up to 10 mM. However, and most importantly, we found that 1,4-cineole inhibited AS at concentrations as low as 0.5 μM (Fig. 5). In comparison, Gln analogs (e.g. albizzine and 6-diazo-5-oxo-nor-Leu) known to inhibit mammalian AS had LCIC values as much as 3 orders

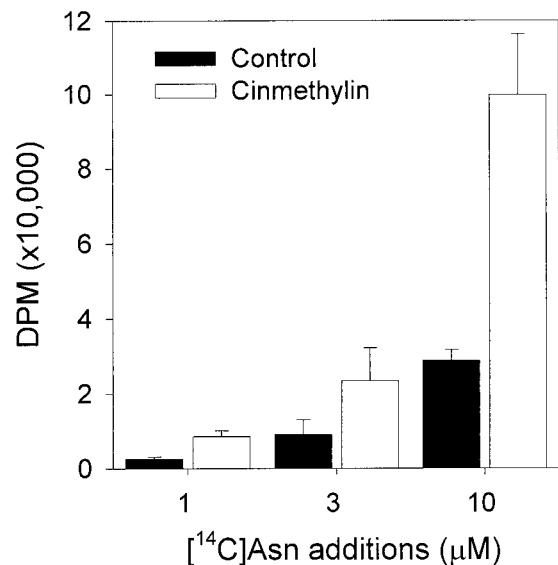


Figure 4. Root uptake of [^{14}C]Asn (DPM $\times 10,000$) by untreated seedlings and 1 μM cinmethylin. Error bars = 1 SD.

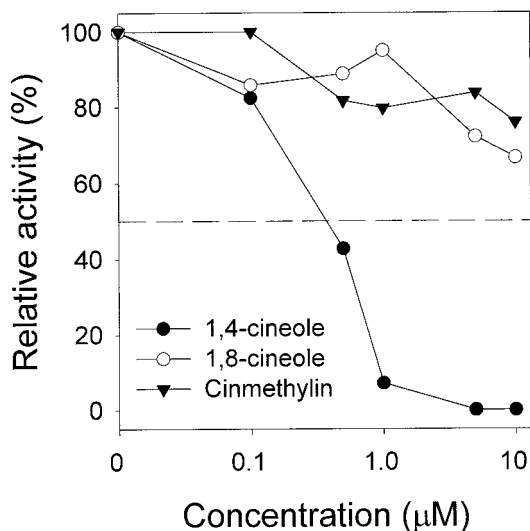


Figure 5. Relative activity (percent of control) of AS at different concentrations of 1,4-cineole, 1,8-cineole, and cinmethylin. See "Materials and Methods" for assay conditions. AS activity varied between 1,400 and 1,600 nmol mg⁻¹ h⁻¹ in the control treatments.

of magnitude higher than 1,4-cineole (2 mM albizzine and 100 μM 6-diazo-5-oxo-norleucine [Cooney et al., 1976]).

Finally, the inhibitory activity of the putative metabolite of cinmethylin, *cis*-2-hydroxy-1,4-cineole, and its enantiomer, *trans*-2-hydroxy-1,4-cineole, was tested. HPLC analyses of AS activity showed that *cis*-2-hydroxy-1,4-cineole has an I₅₀ of 0.03 μM, over 1 order of magnitude lower than that of 1,4-cineole (Fig. 6). Conversely, the I₅₀ of the *trans*-2-hydroxy-enantiomer was 2 orders of magnitude higher (approximately 10 μM) than the *cis*-enantiomer, suggesting that the orientation of the hydroxyl group plays a significant role in interaction of the cineole molecule with the enzyme.

DISCUSSION

The symptomatology of the lettuce dose-response assays was similar for both 1,4-cineole and cinmethylin, with inhibition of root development being most evident. We have shown previously that 1,4-cineole was also active against several weeds when applied to soil (Romagni et al., 2000). However, commercial use of this natural product is probably not feasible because of rapid volatilization occurring under natural conditions. This problem was addressed with the herbicide cinmethylin by reducing the vapor pressure of the cineole moiety with the presence of the benzyl ether side chain at position 2 of the monoterpene ring.

We developed a hypothesis that both inhibitors have a similar mechanism of action that results in a depletion of the cytoplasmic pool of Asn and that exogenously applied Asn reverses the effect by providing an external source of this amino acid. Furthermore, the binding site of the inhibitors on AS may be

at or near the binding site of Gln. Increasing the pool of Gln may favor the stoichiometry of the reaction toward completion, therefore providing partial reversion of the effect of the inhibitors. On the other hand, the lack of reversion observed with Asp suggests that the binding of the inhibitor is not affected by increasing levels of Asp. All of these physiological clues pointed to the enzyme AS as the molecular target site of both cinmethylin and 1,4-cineole.

Asn is important because it is a major nitrogen transport and storage compound found in all plants (Sieciechowicz et al., 1988). In developing seeds and leaves, Asn is actively metabolized, and in mature leaves where excess nitrogen is not needed, it is re-exported to regions of active growth (Joy et al., 1983; Sieciechowicz et al., 1988). The primary enzyme involved in the production of Asn is AS. This enzyme catalyzes an ATP-dependent transfer of the amide group of Gln to Asp, to yield Asn. Two forms of the enzyme are known to exist in plants, AS-A, which may use either NH₃ or Gln as the amine substrate, and AS-B, a Gln-dependent form.

Since Gln provided some degree of reversal of growth inhibition (Fig. 2), we hypothesized that the mechanism of action of these inhibitors affected, either directly or indirectly, the binding of Gln on AS. To test this, we attempted to reverse cinmethylin inhibition with Asn, Asp, and Gln alone (100 μM each), and a combination of Asp plus Gln (100 μM each). If the binding site of Gln was affected, then the combined effect of Asp plus Gln would not be significantly different from Gln alone. If another site was affected, this combination might then have an additive effect. We found that the results supported the first hypothesis. The greatest reversal was obtained with Asn (54%), and the effect of Asp plus Gln

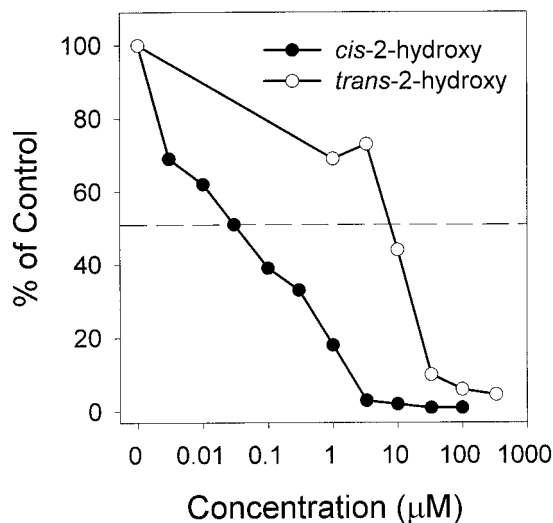


Figure 6. Relative activity (percent of control) of AS at different concentrations of *cis*- and *trans*-2-hydroxy-1,4-cineole. See "Materials and Methods" for assay conditions. AS activity varied between 1,400 and 1,600 nmol mg⁻¹ h⁻¹ in the control treatments.

was similar to Gln alone, suggesting that the binding of Gln only on AS is affected by the inhibitor.

Although the data provided circumstantial evidence that the growth inhibition of this class of chemistry was associated with impaired synthesis of Asn, definite proof that cinmethylin and 1,4-cineole inhibit AS required further investigation. Unfortunately, direct assay of AS activity proved to be a difficult task since none of the substrates or products have chromophores, and the enzyme is relatively unstable. We initially designed a simple experiment to test root uptake of [^{14}C]Asn and [^{14}C]Asp in treated (1 μM cinmethylin) and untreated lettuce. Results confirmed our hypothesis that the presence of cinmethylin should lead to an increased absorption of exogenously applied [^{14}C]Asn to compensate for the decreased production of free Asn resulting from inhibition of AS, whereas [^{14}C]Asp uptake should not be affected.

Finally, we developed a convenient and reliable HPLC protocol (as mentioned in "Materials and Methods") for measuring AS activity in crude plant extracts. 1,4-Cineole had a level of activity on AS that is, to our knowledge, unsurpassed by any other known inhibitors of this enzyme. Furthermore, our results suggest that cinmethylin must be bioactivated by plants by cleaving the benzyl-ether side chain, probably releasing a 1,4-cineole-like molecule such as 2-hydroxy-1,4-cineole intermediate reported as the initial metabolite in animals (Lee et al., 1986) and insects (Richards and Schuster, 1998).

The most likely metabolite, *cis*-2-hydroxy-1,4-cineole, was over 1 order of magnitude more effective against AS than 1,4-cineole alone. The addition of the alcohol renders the molecule less volatile, therefore allowing more of the compound to react with the site of inhibition. Furthermore, the higher level of *in vitro* activity observed with this enantiomer of 1,4-cineole is in agreement with the higher herbicidal activity of the *cis*-2-benzyl ether cinmethylin, relative to the *trans*-analog observed in greenhouse studies (Dr. W. Taylor, DuPont Agricultural Products, Wilmington, DE, personal communication).

Little is known about the specific mechanisms of the reaction catalyzed by AS-B (Richards and Schuster, 1998). The reaction involves a ternary complex where ATP and Asp bind to AS to form an aspartyl-AMP intermediate. The enzyme catalyzes the reaction where this intermediate accepts an amide group transferred from Gln, to yield Asn, Glu, pyrophosphate, and AMP. The order in which the aspartyl-AMP complex and Gln bind has not yet been definitively determined (Richards and Schuster, 1998). AS was commonly thought to behave much like the related and well-characterized enzyme Gln synthetase. If this were true, the reaction catalyzed by AS is achieved via a ternary random mechanism, and the binding of one of the substrates does not influence the binding of the others. However, recent kinetic

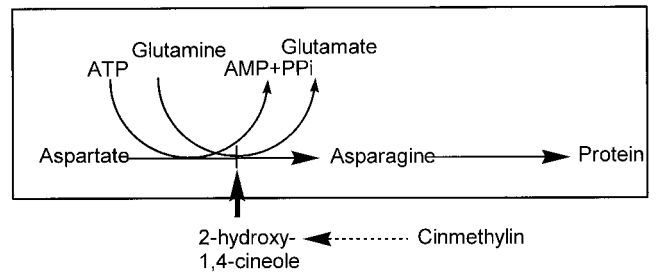


Figure 7. Model illustrating the proposed mechanism of action of cinmethylin and monoterpene cineoles.

data suggests that a ping-pong mechanism may be involved (Ireland and Lea, 1999).

Reversal of the effects of inhibitors of amino acid synthesis by supplying exogenous amino acids is rarely complete and may even be unobservable (e.g. Amagasa et al., 1994; Singh and Shaner, 1995; Vaughn and Spencer, 1996). This is due to several possible factors, including differential accumulation and compartmentalization of the inhibitors and the exogenous amino acids, massive accumulation of substrate, resulting in loss of carbon and nitrogen from other metabolic processes, disrupted metabolic regulation, and accumulation of toxic precursors.

In conclusion, the mechanism of action of cinmethylin is novel and can be summarized as shown in Figure 7. After being absorbed by plants, the benzyl ether moiety of cinmethylin must be cleaved to release a 1,4-cineole-like intermediate (most likely *cis*-2-hydroxy-1,4-cineole) to inhibit AS. Whereas the presence of the hydroxyl group probably enhances the level of interaction with the enzyme by decreasing the volatility and increasing the hydrophilicity of the inhibitor, the stereospecificity observed between the *cis*- and *trans*- analogs suggests that the hydroxyl group is also involved in the specific interaction between the ligand and the active site. Further research is currently being conducted to investigate the activity of the 2-hydroxy-1,4-cineole and related analogs. Although the benzyl ether side chain was apparently added to improve the physical properties of the herbicide by reducing its propensity to volatilize and possibly to improve translocation, this bulky group apparently impaired activity of the monoterpene backbone.

Much work remains to be done to fully understand the physiological ramifications of inhibiting AS in plants and to understand the precise mechanism of inhibition of 1,4-cineole. We are currently investigating the metabolism of cinmethylin to elucidate the true nature of the toxophore. Furthermore, we have initiated a study on the interaction between 1,4-cineole and AS at the molecular level.

MATERIALS AND METHODS

Materials

Soybean (*Glycine max* L. Merr. var DP3588) and lupine (*Lupinus albus* var Victoria) were grown in moist commer-

cial potting soil (Miracle Grow) for 6 d at 27°C in the dark. 1,4-Cineole (>99%) was obtained from Fluka (Milwaukee, WI). 1,8-Cineole (>99%) and all other chemicals were obtained from Sigma-Aldrich (St. Louis), except for the radiolabeled [U-¹⁴C]Asp (200 mCi/mmol) and [U-¹⁴C]Asn (195 mCi/mmol) (Moravek Biochemicals, Brea, CA) and cinmethylin (American Cyanamid, Princeton). The cis- and trans-2-hydroxy-1,4-cineole compounds were gifts from DuPont Agricultural Products.

Dose-Response Studies

Twenty-five lettuce (*Lactuca sativa* cv Iceberg) seeds were placed on sterile 5.5-cm-diameter filter paper fitted to 60- × 15-mm Petri dishes. Filter paper was premoistened with 3 mL of test solutions. A 90× dilution series of cinmethylin dissolved in acetone was made to obtain final concentrations of 100, 33, 10, 3, 1, 0.3, 0.1, and 0.03 μM in water. Controls received equivalent amounts of acetone (1% [v/v]). Seeds were germinated for 6 d. Root growth of the lettuce seedlings was measured in millimeters. Each treatment consisted of three replicates with 25 seeds per replicate and the experiment was duplicated. A similar protocol was followed for testing the natural monoterpenes 1,8-cineole and 1,4-cineole.

Amino Acid Growth Reversal Studies

Methods were modified from Singh and Shaner (1995). All 20 essential amino acids were tested at 100-μM final concentrations (except for Tyr, Trp, and Met at 50 μM) in an attempt to reverse the growth inhibition of 1 μM cinmethylin. Reversal was calculated based on untreated controls for each amino acid. A multiple comparison ANOVA was performed using SAS (SAS Institute, Cary, NC). The same procedure was followed in an attempt to reverse the growth inhibition of 0.1 μM 1,4-cineole. Each treatment consisted of three replicates with 25 seeds per replicate and the experiment was duplicated.

Asn Dose-Response Studies

Reversal of the phytotoxic effects of cinmethylin obtained in the previous experiment with Asn was further characterized by performing a dose-response curve with 0.1, 0.5, 1, 5, 10, 50, and 100 μM Asn in the presence of 1 μM cinmethylin. Appropriate controls without cinmethylin were included. Each treatment consisted of three replicates with 25 seeds per replicate and the experiment was duplicated. A similar experiment was performed in the presence of 0.1 μM 1,4-cineole.

The nature of the reversion obtained with Asn was investigated in more detail by testing Asn, Gln, and Asp individually as well as a Gln/Asp mixture. Each amino acid was tested at 100 μM in the presence or absence of 1 μM cinmethylin.

[¹⁴C]Asp/Asn Incorporation by Roots

A 24-well cell culture plate was divided into two equal sections of 12 wells (control and cinmethylin). Filter paper

(1.5 cm diameter) was fitted to the wells and moistened with 0, 1.0, 3.0, and 10.0 μM of either [¹⁴C]Asp or [¹⁴C]Asn in the presence or absence of 1 μM cinmethylin. Unlabeled Asn (10 μM) was added to the [¹⁴C]Asp treatment to ensure that roots would develop in the presence of cinmethylin. Four lettuce seeds were placed into each well. The plate was sealed with laboratory film (Parafilm, American National Can, Greenwich, CT) and incubated in a growth chamber at 25°C 12:12 d/night at a photon flux density of 205 μmol s⁻¹ m⁻². After 6 d of growth, the lettuce roots were excised, washed three times with distilled water, and placed into separate glass test tubes containing 1 mL of ethanol:ddH₂O (50:50, v/v). Eight roots were placed into each tube, homogenized on maximum speed for 15 s using a polytron homogenizer (model PT 3100, Kinematica, Lucerne, Switzerland). The root homogenates were transferred into 1.5-mL microcentrifuge tubes and centrifuged at 20,000g for 10 min to pellet radiolabeled amino acids incorporated into protein. A 900-μL aliquot of the supernatant was added to 18 mL of Ultima Gold scintillation fluid (Packard Instrument, Downers Grove, IL) and the amount of unincorporated [¹⁴C]Asp or [¹⁴C]Asn was determined by liquid scintillation spectrophotometry. Aliquots (100 μL) of the supernatant of each extract were also injected into the HPLC to monitor any enzymatic conversion of [¹⁴C]Asp into [¹⁴C]Asn.

HPLC Methods

The HPLC system (Waters, Milford, MA) consisted of a model 717 autosampler, model 600 controller, and model 996 photodiode spectrophotometric detector. An in-line β-RAM radioisotope detector (IN/US Systems, Tampa, FL) equipped with a 400-μL lithium glass packed dry cell was connected in tandem with the photodiode spectrophotometric detector. The column was a 250- × 4.6-mm Spherisorb S5 SCX cation exchange column (Waters). The isocratic solvent system (10 mM KH₂PO₄, pH 3.0) was developed to obtain chromatographic separation of Asp and Asn. The injection volume was 100 μL and each run lasted 8 min. The amount of radiolabeled Asn synthesized was calculated with a standard curve established using technical grade ¹⁴C[U]Asn.

Enzyme Extraction

Extraction methods are modified from Dembinski et al. (1996) according to Romagni and Dayan (2000). Etiolated cotyledons of 6-d-old lupine were harvested in the dark and homogenized in a Waring blender (three 30-s pulses on high) at 4°C in 2.5:1 ratio (v/w) extraction buffer (100 mM Tris HCl, 0.1 mM EDTA, 10 mM MgCl₂, 0.1 mM ATP, 2 mM Asp, 20% [v/v] glycerol, 0.5 mM DTT, and 67 mM β-mercaptoethanol; pH was adjusted to 7.8 at 4°C). The homogenate was filtered through two layers each of cheesecloth and Miracloth (Calbiochem-Novabiochem, La Jolla, CA), and the resultant filtrate was centrifuged at 16,000g for 15 min at 4°C. Proteins in the supernatant were precipitated from the cell-free crude extract with 65% (NH₄)₂SO₄ for 30

min on ice. The pH of the extraction buffer was carefully monitored and maintained at pH 7.8 with 10 M NaOH during the salting-out process. The precipitate was collected via centrifugation for 15 min at 20,000g at 4°C. The pellet was resuspended in extraction buffer (volume 30% fresh weight) and desalted on a PD-10 Sephadex G-25 M column (Supelco, Bellefonte, PA) that had been pre-equilibrated with extraction buffer. The protein fractions were combined and concentrated using Centricon 30 concentrators (Amicon, Beverly, MA). Protein concentration in the extracts was determined according to Bradford (1976) and adjusted to 15 to 20 mg mL⁻¹. The concentrated extracts were frozen at -25°C overnight and assayed for AS activity the following day, unless otherwise indicated.

Enzyme Assay

AS activity was assayed according to Romagni and Dayan (2000) by mixing 250 µL of concentrated protein extract (normalized to 15 mg protein mL⁻¹) with 200 µL of assay buffer (100 mM Tris-HCl, pH 7.5, 1 mM Gln, 1.6 mM Asp, 10 mM ATP, 10 mM MgCl₂, 1 mM DTT, and 0.25 µM [¹⁴C]Asp [final activity of 0.5 µCi]) in 10-mL glass test tubes. Samples were incubated at 25°C and the conversion of Asp to Asn was monitored over time. The reaction was terminated with the addition of 500 µL of 100% ethanol to the reaction mixture and boiling for 5 min. Samples were transferred to screw cap 1.5-mL microcentrifuge tubes, and the denatured proteins were pelleted from the sample by centrifugation (13,000g) for 15 min. The supernatant was kept for quantitative analysis of Asn by HPLC as described above.

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