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METHYLENEDIOXYPHENYL-CONTAINING ALKALOIDS AND AUTOSYNERGISM

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Key Word Index—Autosynergism; monooxygenase inhibitors; alkaloids; methylenedioxyphenyl; berberine; chelidonine; cryptopine; narceine.

Abstract—Of four methylenedioxyphenyl-containing alkaloids tested, only chelidonine, was an inhibitor of microsomal monooxygenases *in vitro* while berberine, cryptopine and narceine were not. Berberine toxicity to the corn earworm, *Heliothis zea* could be synergized by the monooxygenase inhibitor myristicin, further evidence that berberine is not a monooxygenase inhibitor or an autosynergist. Autosynergism is not sufficient to explain the common occurrence of methylenedioxyphenyl substituents on toxic alkaloids.

INTRODUCTION

Many compounds containing a methylenedioxyphenyl (MDP) substituent are inhibitors of microsomal monooxygenases [1], important detoxication enzymes in insects and other organisms [2]. MDP-containing phytochemicals, which are distributed among many classes of plant secondary compounds, can function naturally as synergists of co-occurring toxins (phytosynergists) [3–5]. Some synthetic carbamate insecticides containing MDP substituents are autosynergists, compounds which in addition to their toxicity, inhibit the detoxication enzymes that would otherwise metabolize them [6]. Many toxic, plant produced alkaloids have MDP substituents, leading to speculation that such compounds are autosynergists [3, 7, 8]. Indeed, it has been suggested that the autosynergistic properties of MDPs have led to the large number of plant toxins containing the MDP substituent [7, 8]. However, autosynergism has not yet been demonstrated for plant allelochemicals.

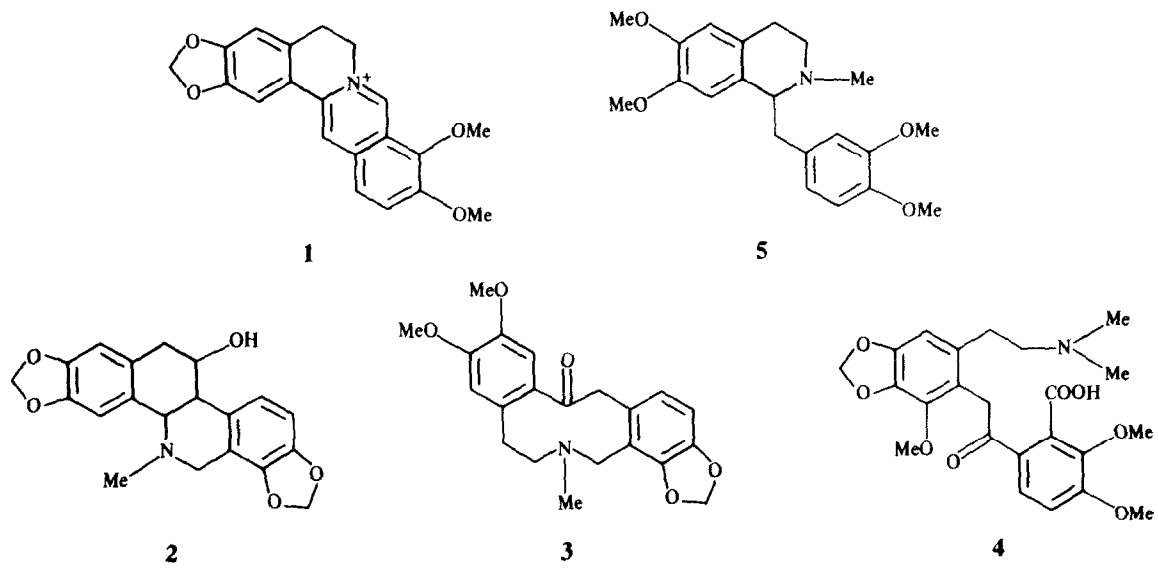
Autosynergists are characterized by inhibition of detoxication enzymes *in vitro* and by the inability of enzyme inhibitors to further synergize their *in vivo* toxicity [6]. Four toxic MDP-containing plant alkaloids were tested for activity as autosynergists. They were berberine (**1**), a protoberberine alkaloid that is found in over 17 plant families, chelidonine (**2**), a benzophenanthridine alkaloid found in plants of the Papaveraceae, cryptopine (**3**), a protopine alkaloid found in plants of the families Fumariaceae, Papaveraceae and Ranunculaceae and narceine (**4**), a phthalideisoquinoline alkaloid found in *Papaver* species.

RESULTS AND DISCUSSION

All four alkaloids were tested for their ability to inhibit a microsomal monooxygenase catalysed reaction, *O*-demethylation of *p*-nitroanisole (pNA), *in vitro*. The inhibitor constant (K_i) and the alkaloid concentrations that inhibited *O*-demethylation of 500 μM pNA by 50% (I_{50}) were measured. Only chelidonine with an I_{50} of 33 μM and a K_i of 11 μM was a good inhibitor of *O*-demethylase *in vitro* (Fig. 1). Berberine is a weak inhibitor of *O*-demethylase activity, with an I_{50} value of 4400 μM, *ca* 10-fold higher than the substrate binding constant (K_m) for pNA (400 μM). No inhibition was noted with concentrations of narceine or cryptopine up to 2500 and 119 μM, respectively. At greater concentrations, narceine and cryptopine were not completely soluble. In this assay, only chelidonine shows the properties expected of an autosynergist.

Monooxygenases are a suite of enzymes [2], and some inhibitors are enzyme specific, that is they inhibit some substrate reactions but not others [9]. Although three of the alkaloids did not inhibit one enzyme of this suite (*O*-demethylase), it is possible that these alkaloids could inhibit other monooxygenases that were not tested. Therefore, the most toxic of the non-inhibitory alkaloids, berberine, was tested for inhibition of its metabolism *in vivo* using larvae of the polyphagous insect *Heliothis zea*, the corn earworm. The MDP-containing monooxygenase inhibitor, myristicin, was compared as a synergist of the toxicity of berberine and laudanosine (**5**), an alkaloid that does not inhibit monooxygenases and is similar in structure to berberine but lacks an MDP substituent. Thus, if berberine is an autosynergist, then myristicin should have a synergistic effect on laudanosine, but not berberine.

No mortality was observed for larvae fed on artificial diet or diet containing 1000 ppm myristicin. Myristicin at 1000 ppm in an artificial diet had a synergistic effect on both berberine and laudanosine, significantly reducing the LC_{50} of berberine from 6300 to 600 ppm and the LC_{50} of laudanosine from 29000 to 8400 ppm (Fig. 2). These differences are statistically significant by virtue of the non-overlapping 95% confidence intervals in the probit analysis. The synergistic ratios (LC_{50} of the toxin alone divided by the LC_{50} of toxin with synergist) for the addition of myristicin are 10.5 and 3.4. for berberine and laudanosine, respectively. The synergistic effect of myristicin, a monooxygenase inhibitor, on both berberine and laudanosine is evidence that both compounds are



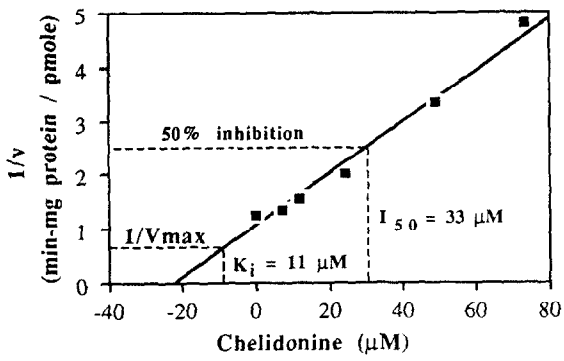


Fig. 1. *In vitro* inhibition of *p*-nitroanisole *O*-demethylase by chelidonine.

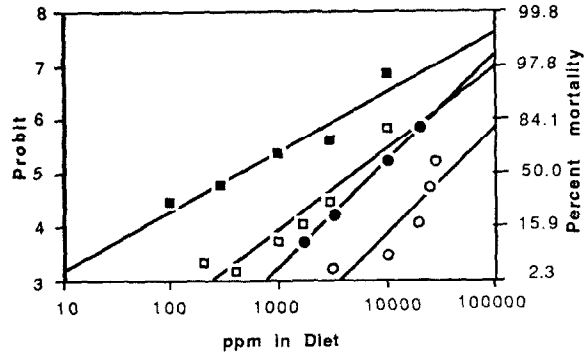


Fig. 2. Synergism of berberine and laudanosine toxicity to first instar *Heliothis zea* by addition of 1000 ppm myristicin. Berberine with (■) and without (□) myristicin; laudanosine with (●) and without (○) myristicin. No mortality was observed with myristicin alone ($N = 30$).

detoxified by monooxygenases in *H. zea* and that neither compound inhibits the monooxygenases responsible for its own detoxication.

An MDP substituent alone is not sufficient for monooxygenase inhibition. MDP-containing compounds possessing a carboxyl or a phenolic group are inactive as monooxygenase inhibitors possibly because they do not penetrate the lipophilic environment of the membrane bound monooxygenase due to their polarity [10]. Both berberine, with a charge on the nitrogen, and narceine, with a carboxylic acid substituent, may not be able to reach the membrane bound, lipophilic environment of the monooxygenase enzymes [10]. Steric properties also influence inhibitor activity [9] and may be responsible for the lack of inhibition by cryptopine, which carries no charge.

It must be concluded that autosynergism is not the only factor that has led to a large number of toxic alkaloids that contain MDP substituents. Reactivity with enzymes other than monooxygenases, electron donating properties, and effect on size and shape are all factors which MDP substituents contribute to a molecule that may be essential for toxicity. Perhaps it is the combination of properties uniquely contributed by a MDP group which has led to its common occurrence as a substituent of toxic phytochemicals.

EXPERIMENTAL

In vitro inhibition. All compounds were obtained from commercial sources. Microsomes were prepd as described in refs [11, 12] from *Heliothis zea* larvae fed pentamethylbenzene [12]. *O-Demethylation of pNA and inhibition by alkaloids was measured* by the method of refs [13, 14]. *Ca* 0.8 mg microsomal protein was incubated for 30 min at 31° in 10 ml Erlenmeyer flasks containing 0.5 mM $NADP^+$, 2.5 mM G6P, 7.5 mM $MgCl_2$, 0.4 units GDH, 50 mM Tris and pNA in a final vol. of 1.6 ml. The reaction was stopped by adding 0.4 ml 1 N HCl and pNP extd into 2 ml CH_2Cl_2. After centrifuging to separate the layers, pNP was

extracted from a 1.2 ml portion of the CH_2Cl_2 with 1.2 ml of 0.5 N NaOH and quantified by measuring A at 400 nm. The K_m (0.4 mM) and V_{max} (2.1 pmol/min/mg protein) for pNA *O*-demethylase were obtained from a Lineweaver–Burk plot of $1/v$ against $1/S$.

For measuring inhibition, alkaloids were dissolved in methyl cellusolve prior to adding (0.02 ml) to the incubation mixt. All reactions were run in duplicate and replicated at least once. I_{50} values were determined with 0.5 mM pNA by linear regression of the average % inhibition on the log of the inhibitor concn [14]. The inhibitor constant, K_i, was determined from plots of reciprocal velocity versus inhibitor concn [15].

In vivo *synergism. H. zea* neonates from a lab culture [16] were used to determine toxicity of berberine and laudanosine incorporated into a meridic diet [4]. Myristicin (1 mg) per g of diet was used for tests of synergism. Larvae were reared in the absence of UV light [5]. The LC_{50} values for first instars were determined by log dose/probit analysis.

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