Relative Toxicity of *Amanita* Amino Acids

Dennis C. Drehmel* and W. Scott Chilton

Abstract

Nine non-protein amino acids found in genus *Amanita* were tested for toxicity to the bacterium *Erwinia amylovora* (a plant pathogen) and the insect *Oncopeltus fasciatus* (milkweed bug). The most toxic to the bacterium was a cyclopropyl amino acid from *A. cokeri* common in the southeastern United States, and the most toxic to the insect was a doubly unsaturated (allenic) amino acid from *A. smithiana* frequently found in western North America. The famous hallucinogen ibotenic acid from *A. muscaria* common in many parts of the United States was also highly effective against the insect by means of paralysis. Presence of a highly toxic amino acid in a particular species implies a serious threat of accidental poisoning to mycophagous foragers.

Introduction

WHILE ALL FUNGI must contain amino acids as part of primary metabolism, the presence of amino acids from secondary metabolism can vary greatly. These non-protein amino acids often give fungi toxic properties and possible protection against predation. However, the production of secondary metabolites can be for a variety of reasons including that they are waste products, reserve food, or breakdown products of macromolecules. The genus *Amanita* Pers. per Hook. is the source of many secondary metabolites (Drehmel et al., 1999) and many non-protein amino acids in particular (Chilton, 1982). The purpose of this study is to compare toxicity and hence the potential efficacy of an amino acid to protect a species of *Amanita* against predation. Moreover, it will be possible in some cases to predict the relative risk to mycophagous human foragers.

In previous studies toxicity of mushroom nonprotein amino acids has been assessed with bacteria (Meek and Rowe, 1955; Harding and DeShazo, 1967; Moriguchi et al., 1987), renal epithelial cells (Pelizzari et al., 1994), fungal spores (Ohta et al., 1986), lettuce seeds (Yoshimura, 1999), fruit fly larva (Besl et al., 1987), mice (Yamaura et al., 1986), and guinea pigs (Chilton et al., 1973). Because the immediate focus was to be on protection against predation, this study selected a plant pathogenic bacterium, *Erwinia amylovora*, and a milkweed specialist insect, *Oncopeltus fasciatus*, for test organisms. *Oncopeltus fasciatus* had been used previously by other mycologists to study toxicity of mushroom extracts (Dowd and Miller, 1990). These species are not known to be natural predators of amanitas and would not have occasion to develop tolerance to amanita secondary metabolites. This is in contrast to mycophagous *Drosophila* wherein species have developed tolerance to amanita toxin (Jaenike, 1985). While it is true that there are potential test organisms which are predators of "non-toxic" mushrooms, it was determined that complete fairness in comparing mushroom amino acids (one of which is considered non-toxic) could be achieved only by avoiding organisms which have had any relationship to mushrooms.

Amino acids selected for this study are shown in Tables 1a and 1b along with the authority that has determined that a particular amanita species does contain the amino acid. It is apparent from Table 1a that 2-amino-4,5-hexadienoic acid (4&5H) is found in many amanita species, of which at least two are frequently collected in the United States. Furthermore, 4&5H has already been identified by Pelizzari et al. (1994) as an important toxin. Hence it was necessary to include 4&5H in the testing. Another well-known toxin is ibotenic acid (IBO), which serves as a good reference amino acid. Along with muscimol (MUS), IBO provides the intoxication of *A. muscaria* and *A. patherina* which are found across North

* Department of Botany, North Carolina State University; e-mail: dennis_drehmel@ncsu.edu.

Table 1a
Occurrence of Straight or Branched Chain Amino Acids Tested

AMINO ACID (short name for text)	SOURCE SPECIES	REFERENCE
4-Dehydronorvaline (**4NV**)	*A. pseudoporphyria*	Hatanaka et al. (1985)
5-Dehydronorleucine (**5NL**)	*A. gymnopus*	Hatanaka (1992)
2-amino-4,5-hexadienoic acid (**4&5H**)	*A. smithiana*	Chilton et al. (1968)
	A. pseudoporphyria	Hatanaka (1975)
	A. neoovoidea	Hatanaka and Kawakami (1980)
	A. abrupta	Yamaura et al. (1986), Ohta et al. (1987)
	A. gymnopus	Hatanaka (1992)
	A. miculifera	Hatanaka et al. (1998)
2-amino-5-chloro-4-pentenoic acid (**5Cl4P**)	*A. cokeri*	Drehmel and Chilton (2002)
	A. virgineoides	Ohta et al. (1995)
	A. castanopsidis	Yoshimura et al. (1999)
2-amino-5-chloro-4-hexenoic acid (**5Cl4H**)	*A. smithiana*	Chilton and Tsou (1972)

America (Lampe, 1979). As an amino acid for which no toxicological data has been reported, stizolobic acid (STZ) was added to the study since it is an abundant amino acid in toxic *A. muscaria* and *A. patherina* (Chilton et al., 1974; Chilton and Ott, 1976). Other amino acids were added to cover typical included chemical groups such as single unsaturation, covalent chlorine, or a cyclopropyl group.

Material and Methods

Amino Acid Isolation

Total amino acids were isolated from common United States (US) amanita species by 70% ethanol extraction of fresh, dried or frozen mushrooms, absorption onto a cation exchange resin (Dowex 50H+, 1x10 cm), and elution of the total amino acid fraction with dilute ammonia. Individual amino acids were purified by descending paper chromatography in 1-butanol: acetic acid:water 12:3:5 (BAW). Identity and purity of amino acids was verified by NMR and by thin layer chromatography (solvent: BAW) using color variation from the detection agents ninhydrin and isatin (Smith, 1960). Two of the amino acids, 4-dehydronorvaline (4NV) and 5-dehydronorleucine (5NL) were prepared synthetically (Chilton et al., 1973); the rest of the amino acids were extracted from collections in the US.

Bacterial Testing

The plant pathogenic bacterium *Erwinia amylovora* (Burrill) Winslow, Broadhurst, Buchanan, Krumwiede, Rogers & Smith was grown in potato dextrose broth preculture. Culture plates of potato dextrose agar (PDA) were inoculated with the preculture and soft PDA. After the PDA had hardened, sterile 3MM paper disks (7mm dia.) were placed on the culture plate. The disks were prepared by adding a solution of the amino acid and drying the disk in a sterile environment. The dose was 0.5 mg except for STZ where solubility limited the dose to 0.1mg. After 48 hours, the plate was checked for any zone of inhibition. Each plate had both a negative and a positive control disk. The negative control was a sterilized disk that had no further treatment. The positive control disk was treated with 0.05 mg of tetracycline.

Insect Testing

The insect used was *Oncopeltus fasciatus* Dallas also known as milkweed bug. Both juvenile (2nd & 3rd instars) and fully adult insects were tested

Table 1b
Occurrence of Hetereocycle or Cyclopropyl Amino Acids Tested

AMINO ACID (short name for text)	*SOURCE SPECIES*	*REFERENCE*
Ibotenic acid(**IBO**)	*A. muscaria*	Eugster et al. (1965)
	A. pantherina	Benedict et al. (1966), Chilton and Ott (1976)
Cyclopropylalanine(**CPA**)	*A. polypyramis*	Chilton and Drehmel (2001)
	A. virgineoides	Ohta et al. (1986)
2-amino-3-cyclopropyl butanoic acid(**3CPB**)	*A. cokeri*	Chilton and Drehmel (2001)
	A. castanopsidis	Yoshimura et al. (1999)
Stizolobic acid(**STZ**)	*A. pantherina*	Chilton et al. (1974)

by adding an amino acid to the water supply. A 2 ml microcentrifuge tube fitted with a paper wick provided water. Also provided were raw, shelled sunflower seeds. For each group tested with an amino acid, there was always a negative control group with untreated water being tested at the same time. In all groups, water was replenished every third day because of evaporation and consumption. Because dosing of the treated insects was only once at the beginning of the test, the water used for replenishment was only water for all groups. Treated and untreated groups were observed for 14 days, at which time the experiment was ended. In the case of juvenile insects, it was necessary to carefully examine debris from the test because dried dead insects were similar in appearance to molted exoskeletons. Adult insects were easier to observe but had to be tested in smaller groups than the juveniles because of the greater water consumption by adult insects. A typical group of juveniles was 20 individuals; a typical group of adults was only 8 individuals.

Results

For a majority of the amino acids tested, no zone of inhibition (ZOI) and hence no antibiotic activity was detected. In contrast, both of the amino acids containing cyclopropyl groups did inhibit *Erwinia amylovora*. The ZOI for 3CPB was 29 mm and that for CPA was 12 mm. The one remaining amino acid with antibiotic activity was 5Cl4P which has both a covalently bonded chlorine and a single unsaturation; the ZOI was 14 mm. The homologous unsaturated chloroamino acid did not show any activity. The ZOI for the positive control (tetracycline) was 37 mm.

Results for testing with milkweed bugs are shown in Table 2. For many of the amino acids the entries in the table are in pairs because both types of insects were used; namely, juvenile insects only and adults insects only. In all cases, the type of insect used is indicated by a "J" or an "A" following the name of the amino acid. The table shows effectiveness for a given amino acid over a range of concentration. The effectiveness was characterized by one of three categories: these are 1) little to no effect, 2) moderate effect, and 3) strong effect. In order to determine the category, it was first necessary to note the percentage of insects killed by a particular amino acid and concentration and then compare that value to arbitrary limits established for each category. Moderate effect began at 30% mortality, and strong effect began at 70% mortality.

At low concentrations of less than 0.2%, only two amino acids showed significant effectiveness. In the case of 2-amino-4,5-hexadienoic acid (4&5H), there was no difference in effectiveness when used against juveniles and adults because it was strongly effective in both. However, ibotenic acid, the other amino acid effective at low concentration, was markedly more effective against juveniles than adults. Part of the difference can be attributed to late recovery of the adults from ibotenic acid intoxication. After adult milkweed bugs drank treated water, they would lie on their backs with little motion. However, these bugs

Table 2
Results of Milk Weed Bug Tests

Key to results: 0 = little to no effect, i.e. less than 30% insect death
+ = moderate effect, between 30 and 70% insect death
++ = strong effect, greater than 70% insect death
( ) = includes paralyzed insects in the count

	Concentration range, %			
Amino acid tested/Juvenile versus Adult bugs tested	***0.05-0.2***	***0.2-0.4***	***0.4-0.6***	***>0.6+***
4&5H				
2-amino-4,5-Hexadienoic acid/J	++			
2-amino-4,5-Hexadienoic acid/A	++			
IBO				
Ibotenic Acid/J	++			
Ibotenic Acid/A	0(+)		+(++)	
3CPB				
2-amino-3-Cyclopropylbutanoic/J	0	++		
2-amino-3-Cyclopropylbutanoic/A	0	++		
CPA				
Cyclopropylalanine/J			++	
Cyclopropylalanine/A	0		+	
5Cl4P				
2-amino-5-Chloro-4-pentenoic acid/J	0	+		
2-amino-5-Chloro-4-pentenoic acid/A	0	+		
5Cl4H				
2-amino-5-Chloro-4-hexenoic acid/A		0		++
4NV				
4-Dehydronorvaline/J	0			
5NL				
5-Dehydronorleucine/A				0
STZ				
Stizolobic Acid/J				0

would respond to prodding by moving one or more legs but could not change position. After four or five days, some of the intoxicated insects would fully recover with complete range of motion and ability to climb. Naturally, those which did not recover died. The category of effectiveness was set as before by the percentage killed. In recognition of the power to paralyze but not kill using ibotenic acid, a second calculation was made. Adding the percentage paralyzed to the percentage killed raised the category of effectiveness as shown in the table with results given parenthetically after the normal calculation.

At the next concentration range of 0.2 to 0.4%, only 2-amino-3-cyclopropylbutanoic acid (3CPB) can be added to the list of effective amino acids. As with 4&5H, 3CPB does not demonstrate any difference in the effectiveness between use on juvenile and on adult insects. The other cyclopropyl amino acid does not become effective

until the 0.4 to 0.6% concentration range where one of the chlorinated amino acids first demonstrates activity. The other chlorinated amino acid requires even higher concentrations. Stizolobic acid, 4-dehydronorvaline, and 5-dehydronorleucine never do demonstrate significant activity against milkweed bugs even at concentrations up to 1%.

Discussion

The relative toxicity of amanita amino acids to milkweed bug is the inverse of the concentration needed to elicit an adverse response. Using Table 3 as a guide the ranking of relative toxicity from most to least is 4&5H, IBO, 3CPB, CPA, 5Cl4P, 5Cl4H, (4NV, 5NL, and STZ). The last three are tied for last with no response each. Comparison to the ranking based on the bacteria test data is incomplete because only three amino acids gave a zone of inhibition. Thus the ranking from most to least would be 3CPB, 5Cl4P, CPA, (the rest). High on both lists is 2-amino-3-cyclopropylbutanoic acid (3CPB) found in *A. cokeri* which is common in the southeastern US. Low on both lists are the mono-unsaturated amino acids and stizolobic acid (STZ) found in a number of species common in many parts of the US. In both tests, 5Cl4P (found in *A. cokeri*) was more effective than its homolog 5Cl4H (found in *A. smithiana*) although these chemicals differ by only a single carbon atom with hydrogens. If the antibiotic activity of these chlorinated amino acids is as an anti-metabolite as found by Moriguchi et al (1987) with 2-amino-4-chloro-4-pentenoic acid, small differences in shape could greatly affect the toxicity.

It is no surprise that ibotenic acid demonstrates very high toxicity to milkweed bug since much has been made of the common and ubiquitous *A. muscaria* being the fly agaric. As a folk method to kill flies, bits of *A. muscaria* were sprinkled with sugar (Rolfe and Rolfe, 1974) or added to milk (Lincoff, 1988). One of two main intoxicants of *A. muscaria* is ibotenic acid. It is also known that *A. muscaria* and ibotenic acid are intoxicants to humans; many authors have reported the effect (Lampe, 1979; Chilton, 1983; Benjamin, 1995). On the other hand, it may be a mild surprise that 4&5H appears to be a consistently better anti-insect amino acid than ibotenic acid. While the poisonous nature of *A. muscaria* and *A. pantherina* has received an appropriately high level of attention, the poisonous nature of the many amanita species containing 4&5H needs to be more widely understood and appreciated. Unfortunately, many members of section *Lepidella* including *A. smithiana* have been shown to contain 4&5H (see Table 1a). In the western United States, *A. smithiana* is frequently found and has been confused with matsutake (*Tricholoma magnivelare* = *Armillaria ponderosa*) with subsequent poisonings (Tulloss and Lindgren, 1992).

In addition to concern for human exposure to 4&5H, there remain questions about the use, if any, for secondary metabolites in fungi. In the case of 4&5H, it appears that this toxic amino acid would be useful for abatement of insect predation. Moreover, *A. smithiana* has been tested with guinea pigs and found to be toxic (Chilton and Tsou, 1972). This implies the possibility that 4&5H could also be useful for abatement of predation by mammals. Lastly, it has been proposed that 4&5H is an important intermediate for manufacture by the fungus of other toxic chemicals. For example, cyclopropylalanine (CPA) could be formed by reductive cyclization of 4&5H (Hatanaka, 1992). The chlorinated amino acid 5Cl4H (also found in *A. smithiana*) could be formed by chlorination of 4&5H (Chilton and Tsou, 1972). Both CPA and 5Cl4H were tested in this study and both are much less toxic to milkweed bugs. However, CPA (found in *A. polypyramis* which is common in the southeastern US) did show some activity against *Erwinia amylovora* while 4&5H showed none. It could be argued that further processing of some of the alleged good insect toxin 4&5H was necessary to provide some protection against bacteria with the amino acid CPA. On the other hand, 5Cl4H provides no apparent improvement in toxicity against milkweed bugs or *Erwinia amylovora*. It may be present in the fruiting body as a natural and unavoidable waste product of 4&5H thereby reducing the level of 4&5H which is useful to the fungus to abate predation.

The other major non-protein amino acid of note is the other cyclopropyl amino acid tested, 3CPB. Of the amino acids compared in this study, this was the most effective against *Erwinia amylovora*. From a previous study, it is known that it is also effective against the bacteria *Agro-*

bacterium tumefaciens and *Xanthomonas campestris* (Drehmel and Chilton, 2002). The previous study also demonstrated that 3CPB was an anti-metabolite for isoleucine. When isoleucine was available in excess, 3CPB had only a slight adverse effect because odds favored isoleucine being incorporated into proteins rather than 3CPB. This has implications for accidental consumption of 3CPB. An individual with good diet may be at small risk of complications because of a high background level of isoleucine. To date, the only known sources of 3CPB are *A. castanopsidis* and *A. cokeri*. Unfortunately, the latter is a common mushroom in the southeastern United States, so accidental poisoning is always a possibility.

The remaining amino acids tested have not shown sufficient toxic effect in the limited range of organisms tested to be of value for defense of the fungus. This is not to say that these amino acids are safe. It is still possible that human consumption of these chemicals even at low concentrations found in a fruiting body could lead to an adverse reaction such as an allergic reaction or other complication. In our determining amino acid profiles for a large number of amanita collections, it is usual to find evidence of stizolobic acid in "American Caesar's mushroom" (*A. jacksonii*) which is generally considered safe for human consumption (Lincoff, 1988; Philips, 1991). However, stizolobic acid has been shown to be biologically active (Ishida and Shinozaki, 1988; Maruyama and Takeda, 1993; Shinozaki and Ishida, 1988). In particular, stizolobic acid shows interactions with different types of excitatory amino acid receptors dependent on the species of animal. Testing with a mammalian model is indicated to verify the safety as food for both *A. jacksonii* and stizolobic acid in the concentrations found in mushrooms.

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