Review

The Chemistry of Antipredator Defense by Secondary Compounds in Neotropical Lepidoptera: Facts, Perspectives and Caveats

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Defesas químicas em lepidópteros contra predadores têm sido observadas desde o século XIX. O caso clássico de proteção química contra predadores é o da borboleta monarca, *Danaus plexippus*, cuja larva seqüestra cardenolidas de sua planta hospedeira *Asclepias curassavica* e transfere-as para os adultos tornando-os impalatáveis para pássaros. Entretanto diversas outras substâncias podem estar envolvidas na proteção química de lepidópteros neotropicais (glicosídeos iridóides, glicosídeos cianogênicos, glicosinolatos, alcalóides pirrolizidínicos e tropânicos, ácidos aristolóquicos, inibidores de glicosidase, pirazinas). Esses compostos podem ser seqüestrados da planta hospedeira larval, obtidos de fontes vegetais visitadas por adultos ou biossintetizados *de novo*. Os lepidópteros conhecidos como impalatáveis para predadores vertebrados e/ou invertebrados são as borboletas Troidini (Papilionidae), Pierinae (Pieridae), Eurytelinae, Melitaeinae, Danainae, Ithomiinae, Heliconiinae e Acraeinae (Nymphalidae) e mariposas Arctiidae. Entretanto informações sobre as substâncias que são responsáveis pela impalatabilidade e como elas são adquiridas nem sempre são obtidas. Esse artigo de revisão aborda principalmente observações de campo e laboratório sobre a rejeição de borboletas e mariposas neotropicais por predadores, correlações entre impalatabilidade e substâncias químicas encontradas nos insetos e bioensaios que demonstrem a atividade dessas substâncias contra predadores. Perspectivas são sugeridas para esses tópicos.

Chemical defense against predation in butterflies and moths has been studied since nineteenth century. A classical example is that of the larvae of the monarch butterfly *Danaus plexippus*, which feed on leaves of *Asclepias curassavica* (Asclepiadaceae), sequestering cardenolides. The adults are protected against predation by birds. Several other substances may be involved in chemical defense, such as iridoid glycosides, cyanogenic glycosides, glucosinolates, pyrrolizidine and tropane alkaloids, aristolochic acids, glycosidase inhibitors and pyrazines. The acquisition of these substances by lepidopterans can be due to sequestration from larval or adult host plants or *de novo* biosynthesis. Many Lepidoptera are known to be unpalatable, including the butterflies Troidini (Papilionidae), Pierinae (Pieridae), Eurytelinae, Melitaeinae, Danainae, Ithomiinae, Heliconiinae and Acraeinae (Nymphalidae), and Arctiidae moths, but knowledge of the chemical substances responsible for property is often scarce. This review discusses mainly three topics: field and laboratory observations on rejection of butterflies and moths by predators, correlation between unpalatability and chemicals found in these insects, and bioassays that test the activity of these chemicals against predators. Perspectives and future directions are suggested for this subject.

Keywords: pyrrolizidine alkaloids, tropane alkaloids, aristolochic acids, cardenolides, cyanogenic glycosides, glucosinolates

Introduction

Chemical defense against predation in insects, particularly in Lepidoptera, is a well studied subject in chemical ecology with several reviews available[1-10]. As defined by Brower[8], "chemical defense can be suggested when individual prey organisms contain one or more noxious chemical substances which facilitate proximal and/or distal rejection[a] by predators; rejection can occur after a predator partially to completely ingests one or more prey individuals, or after the predator simply smells or tastes the prey".

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[a] Proximal rejection involves contact with the prey in order to taste or smell it, while in distal rejection the predator perceives the prey from a distance due to odor cues, avoiding physical contact. In the later case, visual or acoustic cues are involved in mimicry systems.

The subject of chemical defense involves various areas of biology and chemistry. From a biological perspective, reports of prey rejection by predators have appeared since the nineteenth century. Bates[11] and Müller[12] were the first authors to propose that brightly colored butterflies were unpalatable to visually oriented predators, and that similarly conspicuous coloration in other palatable[b] or unpalatable[c] Lepidoptera evolved in order to enhance their protection through predator learning. Poulton[13] pointed out that the unpalatability of butterflies was derived from their larval host plants. In the last 60-80 years chemical defense has been repeatedly tested against both vertebrate and invertebrate predators[1,8,14-17]. Evolutionary explanations for the reason why insects acquired noxious chemicals from host plants (so-called substances of secondary metabolism) began to take form after the seminal paper of Ehrlich & Raven[18], who proposed a theory of "radiation and escape between plants and butterflies"[d]. In their scenario, three main steps promoted the diversification of both based mainly on evolution of protective chemicals in the plants: 1. plants with random mutations and recombinations could produce several chemical compounds not directly related to their basic metabolic pathways; 2. some of these compounds, by chance, would protect plants against attack by herbivores; the plants would then enter a new adaptive zone, promoting evolutionary radiation; 3. if insects had also random mutations and recombinations that enabled them to explore these new plant groups, selection would carry them into a new adaptive zone, where they would be free from competitors and natural enemies, promoting again an evolutionary radiation.

Chemical defense in insects involve several research areas and the investigations generally assume interdisciplinary feature. Exemplifying this multiplicity we can find studies on physiological mechanisms of biosynthesis and sequestration of defensive compounds by Lepidoptera[21,22], evolution of warning coloration associated with unpalatability[23-26] and techniques for isolation and identification of the defensive chemicals[27].

The purpose of this review is to examine the progress in studies of secondary compounds thought to be involved in the chemical defense of Neotropical Lepidoptera. I organized it by classes of chemical compounds, focusing on three aspects: 1. field and laboratory observations on rejection of butterflies and moths by predators, 2. correlation between unpalatability and chemicals found in these insects, and 3. bioassays that test the activity of these chemicals against predators. Perspectives and directions for further research on the subject are suggested.

Chemical Compounds Acting as Defense in *Neotropical Lepidoptera*

Most organisms have alternate metabolic pathways in addition to those of primary metabolism that involve polysaccharides, lipids, proteins and nucleic acids. The natural products coming from such pathways are called "substances of secondary metabolism"[28]. In plants, from which butterflies and moths often sequester many of these substances, there are three principal building blocks for these compounds: 1. acetate, which *via* the mevalonate pathway leads to mono-, sesqui-, and diterpenes, iridoid glycosides and cardenolides; 2. amino acids, leading to cyanogenic glycosides, glucosinolates, pyrrolizidine alkaloids, tropane alkaloids and glycosidase inhibitors; and 3. shikimic acid, the precursor of many aromatic compounds such as furanocoumarins, aristolochic acids and β-carboline alkaloids (*via* aromatic amino acids). These substances take part in the chemical defenses in Lepidoptera and their roles will be discussed in detail in the next sections.

Iridoid glycosides

Iridoid glycosides[29] (Figure 1, **1**) are cyclopentenoid-monoterpene derived compounds in which the glycoside often occurs as an O-linked glycoside at C-1. They occur in about 57 plant families, and more than 600 iridoids structures have been described[29].
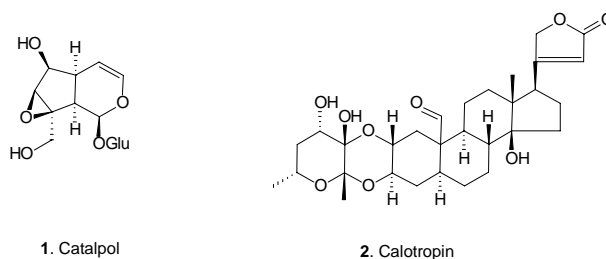

1. Catalpol   **2.** Calotropin
Figure 1. Glycoside iridoids (**1**) and cardenolides (**2**).

These compounds have only been investigated in North American butterflies and moths. They are sequestered from their host plants by larvae of the nymphalid *Euphydryas*

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[b] Batesian mimicry: mimicking of brightly colored, or distinctively patterned, unpalatable species by palatable ones, protecting the latter against visual orientated predators by resemblance.

[c] Müllerian mimicry: similarity in appearance of one species of animal to that of another, where both are unpalatable to predators. Both gain from having the same warning coloration, since the predator learns to avoid both species after tasting either one or the other.

[d] For a criticism on this theory see Futuyma and Keese[19] and Schoonhoven and coworkers[20], and references therein.

phaeton (host plants: *Chelone glabra, Aureolaria flava* – Scrophulariaceae and *Plantago lanceolata* – Plantaginaceae), *E. chalcedona* (*Scrophularia californica* – Scrophulariaceae), *E. anicia* (*Besseya plantaginea, Castilleja integra* – Scrophulariaceae), *Poladryas arachne* (*Penstemon virgatus* – Scrophulariaceae) and *Junonia coenia* (*Plantago lanceolata*), the pterophorid moth *Ptatyptila pica* (*Castilleja sulphurea*), the geometrid moth *Meris alticola* (*Besseya plantaginea*) and the sphingid moth *Ceratomia catalpae* (*Catalpa bignonioides*)[10,29,30]. *Euphydryas* and *Poladryas* retain the iridoids through the adult stages, while in the remaining species these compounds seem to be lost in the pupal stage[10,29,30]. Both adult and larva are warningly colored in *Euphydryas*, while in *Junonia* and *Ceratomia* larvae are conspicuous but the adults cryptic, suggesting that in the former both stages would be protected against predators, and in the latter only larvae would. Bowers and collaborators[31-33] postulated that due to sequestration of iridoid glycosides from host plants the adults of the genus *Euphydryas* are generally unpalatable to birds. Bioassays with ants and spiders also demonstrated the role of iridoid glycosides in the chemical protection of larvae[34-37].

In Neotropical environments Chai[38] verified that adults of *Thessalia ezra*, a melitaeini butterfly that feed on Acanthaceae, was sight- and taste-rejected by birds, but no iridoid glycoside analyses were done. The investigation of all developmental stages of *Thessalia* and other butterflies that also feed on Acathaceae (e.g. *Siproeta, Ortilia, Eresia* and *Anameca*) and Plantaginaceae (e.g. *Junonia*) will be necessary to elucidate the role of iridoid glycoside in Neotropical species.

Cardenolides

Cardenolides or cardiac glycosides (Figure 1, **2**) are, together with pyrrolizidine alkaloids, one of the best studied chemical defense system in insects, particularly in Lepidoptera[39]. The biosynthetic pathway of these compounds is not completely understood; cholesterol and β-sistosterol are metabolized in plants to pregnenolone, progesterone, and thence to cardenolides[28]. These compounds are found in 202 plant species in 55 genera and 12 Angiosperm families[39].

The sequestration of cardenolides by North American *Danaus* and the rejection of these butterflies by birds have been studied for more than 40 years since the Browers[40,41] showed that birds rejected the monarch butterfly *D. plexippus*. The presence of cardenolides in butterflies was shown to be highly effective against predation by Blue Jays (*Cyanocitta cristata bromia*, Corvidae). When fed with adults of *D.*

plexippus reared as larvae upon a cardenolide plant, *Asclepias curassavica* (Asclepiadaceae), the birds exhibited typical effects of cardenolide poisoning, including repeated vomiting[42]. Monarchs reared on plants bearing cardenolides were much more emetic (= causing vomiting) than those reared on an asclepiad species lacking cardenolides[41].

Some questions remain open about this system. For example, studies on the role of cardenolides in chemical protection of larvae against predators have received little attention. The presence of two kinds of chemical defense, cardenolides and pyrrolizidine alkaloids, in *Danaus* species[44,45] is poorly explored from either a mechanistic or an evolutionary point of view. The dynamics of cardenolides in Neotropical species of *Danaus* need to be studied in relation to those found in the North American species.

Cyanogenic glycosides

Cyanogenic glycosides[46] (Figure 2, **3-7**) are O-β-glycosides of α-hydroxynitriles (cyanohydrins) biosynthetically derived from amino acids; these compounds have intermediate polarity and are water-soluble. They are accumulated in vacuoles in the plant and maybe to be so in animal cells. They generally co-occur with β-glycosidases and hydroxynitrile lyases, which are compartmentalized in other cells. The enzymatic cleavage of cyanogenic glycosides releases HCN plus sugar and ketones or aldehydes. The distribution of these compounds includes at least 2,650 plants (more than 550 genera and 130 families), with Passifloraceae as one of the main families. These compounds are also found in butterflies belonging to the Neotropical genera *Heliconius* (Nymphalidae, Heliconiinae), and *Actinote, Altinote* and *Abananote* (Nymphalidae, Acraeinae)[47,48].

Heliconius uses *Passiflora* species (Passifloraceae) as larval food plants[47], and both larvae and adults biosynthesize *de novo*, from the amino acids valine and isoleucine, simple cyanogenic glycosides (linamarin and lotaustralin, **3** and **4**, respectively – Figure 2)[49]. *Passiflora* species have a vast array of different cyanogenic glycosides, varying from simple aliphatic and aromatic compounds to sulphates and cyclopentenoid derivatives[46,47] (Figure 2, **6** and **7** respectively). It has recently been demonstrated that a monoglycoside cyclopentenyl cyanogen was sequestered by *Heliconius sara* fed on *Passiflora auriculata*[50]. Moreover, it was found that *H. sara* has saurauriculatin (**8**), a thiol derivate from the cyclopentenoid cyanogenic glycoside epivolkenin (**7**), suggesting that the replacement of the nitrile group by a thiol would prevent cyanide release from the host plant[50].
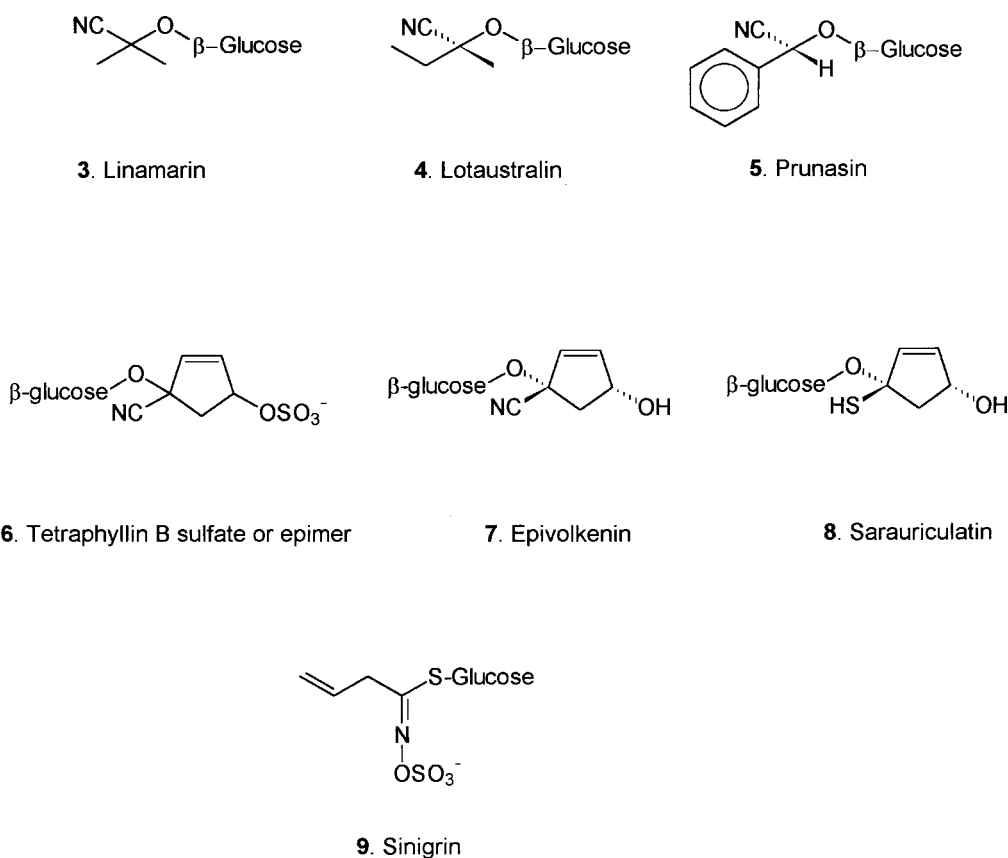
3. Linamarin   **4**. Lotaustralin   **5**. Prunasin

6. Tetraphyllin B sulfate or epimer   **7**. Epivolkenin   **8**. Sarauriculatin

9. Sinigrin

Figure 2. Cyanogenic glycosides (**3-8**) and glucosinolates (**9**).

Into the neotropical acraeines, Brown and Francini[48] showed that 16 species of *Actinote*, 12 of *Altinote* and one of *Abananote* may biosynthesize *de novo* these compounds in all developmental stages, since their larval host plants (mostly *Eupatorium* and *Mikania*, Asteraceae) do not have cyanogenic glycosides.

Heliconius species, together with *Danaus* (Nymphalidae: Danainae), are among the most studied species in relation to unpalatability. Several tests have demonstrated that they are unpalatable to vertebrate predators[38,41,51-53]. Chai[38] verified that *Actinote anteas* and *A. lapihta* were sight-rejected by birds. However, there is much speculation in relation to the role of cyanogenic glycosides in chemical defense. The activity of these compounds against predators is poorly understood.

Glucosinolates

Glucosinolates (Figure 2, **9**) are sulfur- and nitrogen-containing compounds biosynthesized through amino acid metabolism and are found mainly in the order Capparales (e.g. Cruciferae and Capparidaceae)[54]. Glucosinolates are known for their deterrent activity in plants against generalist herbivores and other natural enemies[54]. Their volatile derivatives are used as cues by specialist herbivores in the search of host plants and by parasitoids that attack insects feeding on glucosinolate-containing plants[54]. There are sparse data in the literature showing sequestration of glucosinolates by butterflies or moths from host plants and their role against predators.

Many Neotropical pierine butterflies (*Appias*, *Ascia*, *Leptophobia*, *Itaballia*, *Pieriballia*, *Perrhybris*)[55,56] use Cruciferae and Capparidaceae as host plants, many of which may contain glucosinolates. Chai[38,53] observed that the Neotropical Pierinae *Melete*, *Appias*, *Perrhybris* and *Ascia* were sight- and/or taste-rejected by birds. In experiments carried out in our laboratory it was verified that larvae of *Ascia monuste*, which feed on the crucifer *Brassica oleracea*, were taste-rejected by chicks. In both cases no chemical analyses were carried out to verify if glucosinolates were responsible for this activity.

Pyrrolizidine alkaloids

Pyrrolizidine alkaloids are probably the best studied defensive compounds in insects, especially in Lepidoptera. Many reviews on the activity of pyrrolizidine alkaloids in chemical defense and the role of these alkaloids in pheromone biosynthesis in Lepidoptera are available[21,22,57-62].

Pyrrolizidine alkaloids are a diverse class of natural compounds based on a [3.3.0] azabicyclo ring, generally occurring as esters of a "necine base" with "necic acids" as mono- or diesters (Figure 3, **10-13**)[63]. These alkaloids are known mainly from Asteraceae (tribes Eupatorieae and Senecioneae), Boraginaceae, Fabaceae (mainly in *Crotalaria*), Apocynaceae (subfamily Echitoideae, tribe Parsonsieae) and Orchidaceae (a few genera)[21,60,63,65]. They are postulated to occur in plants and Lepidoptera as N-oxides[21,66], but recent work has discovered more polar metabolites in Ithomiinae butterflies[67], similar to glycosylated pyrrolizidine alkaloids that have been characterized in Chrysomelidae bettles[68].

Eisner[14] was the first to point out the role of pyrrolizidine alkaloids as responsible for chemical defense of the arctiid moth *Utetheisa ornatrix* against the orb-weaving spider *Nephila clavipes*. Vasconcellos-Neto and Lewinsohn[69] observed that the spider released, unharmed, Ithomiinae and Danainae butterflies from their webs. Brown[15-17] found that pyrrolizidine alkaloids acquired from plants visited by adults[e] were responsible for this activity, since most Ithomiinae and Danainae do not fed as larvae on plants containing pyrrolizidine alkaloid. Other authors have shown the activity of pyrrolizidine alkaloids in other butterflies and moths against spiders[74-78], lizards[79] and birds[79,80]. Pure pyrrolizidine alkaloids were bioassayed against spiders[81] and birds[79,80]; N-oxides were shown to be more active than free bases[81-83]. Corroborating the activity of pyrrolizidine alkaloids against predators, it is known that predators avoid or taste-reject danaine and ithomiine butterflies[38,41,53]. However, the role of glycosilated alkaloids against predators remains unknown.

Aristolochic acids

Aristolochic acids (Figure 4, **14**) have been found only in plants belonging to the family Aristolochiaceae; biosynthetically, they are nitrophenanthrenes derived from aporphine alkaloids[84]. The unpalatability of these compounds has been postulated by several authors, but only one bioassay has been done with pure aristolochic acid, where the Japanese tree sparrow *Passer montanus* rejected rice grains treated with these compounds[85,86]. However, the authors pointed out that aristolochic acids
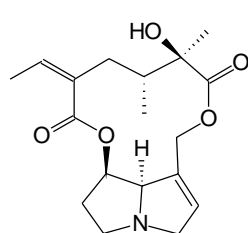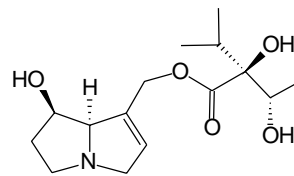

10. Senecionine
(Arctiidae, Danainae)
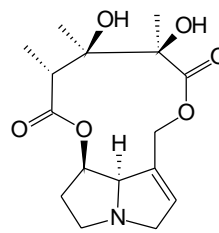
11. Monocrotaline
(*Utetheisa*)
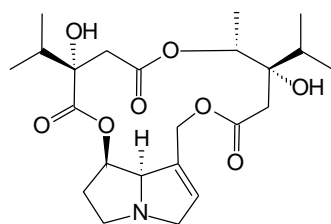
12. Parsonsine
(*Tithorea*)

13. Lycopsamine
(Arctiidae, Danainae, Ithomiinae)

Figure 3. Pyrrolizidine alkaloids and organisms where they occur.

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[e] Plants were visited by adults in order to obtain food (pyrrolizidine-containing or not)[70] or withered plants (only pyrrolizidine plants)[71,72]. Visits to the latter were defined as pharmacophagy[73] (a syndrome where insects actively search for and take up secondary plant substances independent of their nutritional requirements and use them to increase their fitness).

alone have lower activity than that the total osmeterium secretion from the Asiatic Troidini *Atrophaneura alcinous*, which also contains sesquiterpenes and a complex mixture of more polar components, possibly sequestered from the host plant (*Aristolochia debilis*).

Rejection by birds of aposematic adult Troidini whose larvae feed on *Aristolochia* was described 35 years ago[38,41,53] and aristolochic acids were found in several members of this tribe[87-89]. Chicks and ants also taste-rejected the aposematic larvae of the swallowtail butterfly *Battus polydamas*, but other invertebrate predators such as the reduviid bugs *Arilus* sp. and *Montina confusa* did not[90]. It is interesting to note that *Aristolochia* plants have other nitrophenanthrene derivatives, such as aristolactams (**15**) and benzoisoquinoline alkaloids (**16**)[84], that have not yet been tested.

Glycosidase inhibitors

Glycosidase inhibitors are widespread in plants and can be sequestered by Lepidoptera, for whom they probably serve for defense by making the insects indigestible to a range of potential predators[91,92]. A very interesting case is reported for the uraniid *Urania fulgens*, a colorful, day-flying moth native to the tropical regions of Central America[93]. The larvae feed on *Omphalea* (Euphorbiaceae), particularly the liana *O. diandra*. Leaves of *O. diandra* contain polyhydroxypyrrolidine and a piperidine alkaloid analog (Figure 5, **17** and **18**), sequestered by larvae and

transferred to adults through the pupal stage; eggs also contained these alkaloids[92]. These azafuranose and azopyranose alkaloids, analog of hexose and heptose sugars, are potent inhibitors of glycosidases[94]. Adults of the ithomiine *Mechanitis polymnia* also show glycosidase inhibitors (polyhydroxylated nortropane alkaloids – calystegines A_3 and B_2, **19** and **20**)[92,95], but their host plants (*Solanum* spp – Solanaceae) were not analyzed. Although defensive functions have been proposed for these compounds, no bioassays have been carried out to show the activity of these substances against predators.

Pyrazines

Pyrazines are substances widespread in the plant and animal kingdoms and include some of the most powerful odors detected. The pyrazine nucleus comprises a six-membered aromatic ring containing two *para*-orientated tertiary nitrogen atoms[96,97]. Alkyl-substituted pyrazines are known to serve as trail-laying pheromones[98] or alarm pheromes[99] in some ants. In Lepidoptera, 2-methoxy-3-alkylpyrazines (Figure 6, **21**) were found in several taxa of aposematic butterflies and moths, and sometimes in their larval host plants[100,101]. These substances potentiate the rejection response of rats and chickens when they drink an unpalatable quinine-water solution[96,102,103]. As suggested by these authors[96,102,103], pyrazines might promote predation-learning of aposematic insects, since they have an extremely potent odor and a very low olfactory threshold.
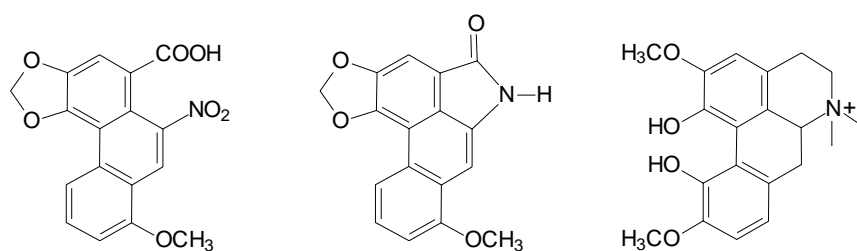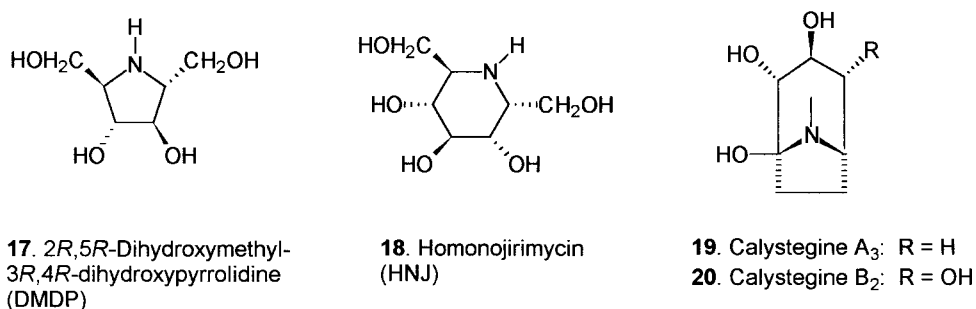

14. Aristolochic acid I   **15**. Aristolactam I   **16**. Magnoflorine

Figure 4. Aristolochic acids (**14**), aristolactams (**15**) and benzoisoquinoline alkaloids (**16**).

17. 2*R*,5*R*-Dihydroxymethyl-3*R*,4*R*-dihydroxypyrrolidine (DMDP)

18. Homonojirimycin (HNJ)

19. Calystegine A_3: R = H
20. Calystegine B_2: R = OH

Figure 5. Glycosidase inhibitors (**17-20**).

It is necessary to investigate the presence of pyrazines in other aposematic Lepidoptera (including all developmental stages) and compare them with cryptic ones. In addition, antipredator bioassays on pyrazines alone and together with other protective substances could give more information to draw a picture of the role of pyrazines in this context.
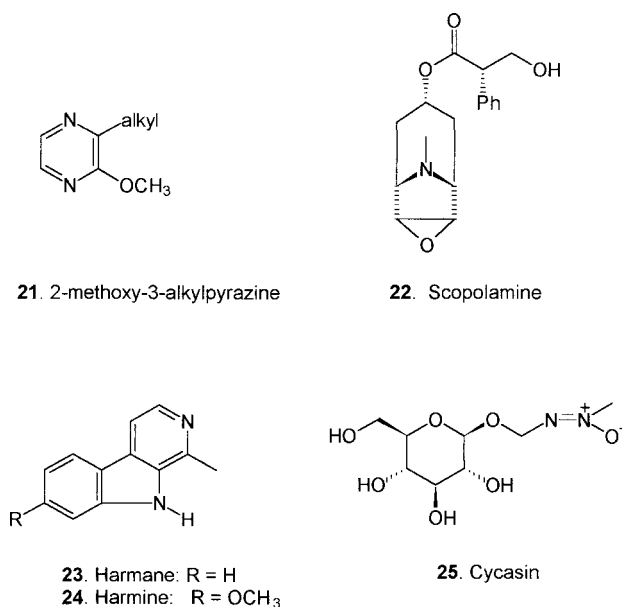

21. 2-methoxy-3-alkylpyrazine   **22**. Scopolamine

23. Harmane: R = H
24. Harmine: R = OCH$_3$

25. Cycasin

Figure 6. Pyrazines (**21**), tropane alkaloids (**22**), β-carboline alkaloids (**23** and **24**) and β-glycoside of methylazoxymethanol (**25**).

Other substances

Aposematic lycaenid larvae *Eumaeus* (Eumaeinae) are found in the Amazonian region feeding on *Zamia* sp. (Cycadaceae)[104]. It is known in North America that *E. atala*, whose larvae feed on the cycad *Zamia floridana*, is protected against ants (larvae) and birds (adults) by cycasin, a β-glycoside of methylazoxymethanol[105] (Figure 6, **25**).

The β-carboline alkaloids (Figure 6, **23** and **24**) are present in tissues of larvae and adults of *Heliconius ismenius* (Heliconiinae), and are sequestered from their larval host plant *Passiflora costaricensis* (Passifloraceae)[106]. The role of these alkaloids in chemical defense of *Heliconius* species is unknown.

Tropane alkaloids (Figure 4, **22**) were found in aposematic larvae and adults of *Placidula euryanassa* and sequestered from the larval host plant *Brugmansia suaveolens*. The cryptic larva of *Miraleria cymothoe*, which feed on the same host plant excretes these alkaloids[77]. A biossay were carried out using *Nephila* spider as predator, but tropane were not active[77]. Further studies are needed to elucidate their role in the chemical defense.

Perspectives, future directions and caveats

Question on the antipredator role of secondary substances can be discussed in two main ways: "how" and "why" questions, similar to those discussed in animal behaviour[107], and other biological areas. "How questions" can be summarized concerning the activity of the substances against predators and their action mechanisms. "Why questions" lead to evolutionary and ecological questions.

Focusing on "how questions" some problems remain to be solved in relation to chemical defense. The responses of predators to aposematic Neotropical butterflies and moths, behaviors such as liberation, rejection or non-attack are not always related to chemical compounds. Nevertheless, in some butterfly groups there is a close relationship between chemicals of larval or adult host plants and unpalatability. It is important to stress that correlation does do not mean a cause-effect relationship. It is necessary in most cases to isolate the chemicals from the insects and test them against natural predators. As examples, iridoid glycosides were tested only against spiders and ants, cardenolides against birds, pyrrolizidine alkaloids against ants, beetles, spiders, birds and lizards, but no bioassays with the other compounds were done. Other intriguing point is: are the substances *per se* or their metabolic and/or catabolic products responsible for antipredator activity? For example, cyanogenic glycosides are substances postulated to be unpalatable. These compounds are subject to metabolism by enzymes giving HCN, sugars and ketones or aldehydes[44]. The following questions rise from it: what compounds are really active against predators? Is there any synergistic interaction among them? For example, Petersen and collaborators[108] showed that benzaldehyde is more active than HCN against ants but no bioassay with prunasin (Figure 2, **5**), the parent compound, was done.

Another item concerning to "how questions" is the structure *versus* activity of chemical substances against predation. It is possible that chemical manipulation to extract and isolate these substances for bioassays produces non-natural by-products. Very recent examples of these are the characterization of N-oxides and glycosides of pyrrolizidine alkaloids, which have been found in plants and insects, respectively. Both were in past reports transformed to and isolated as free bases, using the usual chemical methodology: acid-base treatment, followed by reduction of N-oxides with Zn, may also hydrolyzed the glycosides. As stated above, N-oxides seems to be more active as free bases, but what is the role of the presumed glycosides of pyrrolizidine alkaloids? Studies on incorporation of pyrrolizidines into the integument of Neotropical Lepidoptera together with chemical defense activity of different pyrrolizidine chemical states (free bases, N-oxides, glycosides) must be done in order to better understand pyrrolizidine alkaloid activity against predation.

In relation to "why questions" we can formulate intriguing questions, some times very difficult to answer at light of the present knowledge. Are natural enemies the selective force responsible for the acquisition or biosynthesis of compounds by the prey? Or, are the substances sequestered or biosynthesized *de novo* due to other kinds of selective pressure, such as physiological restrictions? The recognition of substances by predators is not evidence of contact between them and prey containing these substances along of evolutionary time. As pointed out by Williams and collaborators[109] if receptors are as conservative, as the genetic code or molecules such as histamine, the recognition of any molecules by them could be due to past interactions with ancient organisms such as microorganisms. Ecological relevance is easier to point out than evolutionary ones. For example, sympatric occurrence between prey and predators could be a signalization to ecological relevance. The best information would be the observation of predators releasing prey in the field and the utilization of those in a bioassay.

In addition to the relevant "how and why questions" the lack of knowledge of the natural history of a vast array of Neotropical butterflies and moths leaves us with a virtually unexplored field to study chemical defense. In groups such as Pierinae (e.g. *Ascia* and *Melete*)[38,53] and Nymphalidae (e.g. *Hamadryas, Diaethria, Callicore* and *Biblis*)[38,53,110-112] aversive response by predators was observed. Reports of rejection of skippers and other butterflies by the captive lion marmoset monkeys *Leontopithecus rosalia* (Callitrichidae) include *Urbanus proteus* and *Astraptes creteus* (Hesperiidae), and the nymphalids *Caligo beltrao* (Brassolinae), *Morpho* spp. (Morphinae) and *Nica flavilla* (Biblidinae)[113]. Data of Collins and Watson[114] on field observations of bird predation on Neotropical moths suggest that the Geometridae are more unpalatable than Arctiidae, being the late a classical case of aposematic moths·. The causes and chemicals involved in the unpalatability of these groups have not been studied.

Finally, studies of chemical defense in Lepidoptera were done using mainly adults, but there is evidence that chemical defensive strategies may differ between the two actively feeding developmental stages of Lepidoptera (larvae and adults). As larvae suffer the constraints of single host plant and relative immobility they might have a wider array of defensive strategies than the free feeding and mobile adults. Unpalatable larvae have several mechanisms such as (1) stinging or irritating hairs or spines, (2) osmeteria and other eversible glands, (3) regurgitation, (4) presence of toxic leaf material in the gut, and (5) sequestration of chemicals from the host plant or *de novo* biosynthesis[10]. For example, fatty

acids and sesquiterpenes, sometimes liberated in the hairs of larvae of *Dione juno* and *Abananote hylonome* are active against ants[115]; compounds whose biogenesis is unknown. Sequestered compounds can also be lost in the change from larvae to pupa, due to the metabolic cost to handle them[9-10]. Therefore, larvae could use a different set of chemicals, or different defensive strategies from those of adult.

The items pointed above presented some problems involving the role of secondary substances in the chemical protection of Neotropical butterflies and moths. The investigation of these topics, here directed at Neotropical Lepidoptera (these comments can also be addressed to aposematic insects in general), will rise with the increase of studies in this area, can help us to understand "how and why chemical substances are used by insects".

Acknowledgments

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