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Structural diversity and defensive properties of diterpenoid alkaloids

Matías Reina · Azucena González-Coloma

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Abstract Diterpenoid alkaloids are compounds of pharmacological interest. Forty four C_{19} norditerpenoid (NDAs) and 23 C_{20} diterpenoid (DAs) alkaloids isolated from *Aconitum*, *Delphinium* and *Consolida* species were tested for their insecticidal effects (antifeedant and toxic) on *Spodoptera littoralis* and *Leptinotarsa decemlineata*, their cytotoxicity on tumoral cell lines with several multidrug resistance mechanisms, and their antiparasitic effects against *Trypanososma cruzi* and *Leishmania infantum*. Overall, C_{19} norditerpene alkaloids (NDAs) resulted better insect antifeedants and post-ingestive toxicants than the related C_{20} diterpene alkaloids (DAs). Their antifeedant or insecticidal potencies did not parallel their reported nAChR binding activity, but did correlate with the agonist/antagonist insecticidal/ antifeedant model proposed for nicotininc insecticides. Among the most potent antifeedants ($EC_{50} < 0.2$ μg/cm^2) are the NDAs 1,14 diacetylcardiopetaline (**10**), 18-hydroxy-14-*O*-methylgadesine (**34**) and 14-*O*-acetyldelectinine (**28**) (to CPB) and the DA 19-oxodihydroatisine (**55**) (to *S. littoralis*). DAs had strong antiparasitic effects with molecular selectivity while NDAs were inactive. Delphigraciline (**53**), 15,22-*O*-Diacetyl-19-oxo-dihydroatisine (**56**), azitine (**64**) and isoazitine (**65**) were active against *L. infantum* promastigotes and had a moderate effect on *T. cruzi* epimastigotes, while atisinium chloride (**59**) and 13-oxocardiopetamine (**48**) had a potent effect on *T. cruzi* epimastigotes. These compounds were not toxic to the host cell, significantly reduced parasite infection capacity and severely affected the multiplication of their extracellular forms. Several NDAs exhibited selective cytotoxicity to cancerous cells and some of these had irreversible effects on SW480, HeLa and SkMel25 cell lines (neoline **5**, pubescenine **16**, 14-deacetylajadine **26**, lycoctonine **27**, dehydrotakaosamine **35**, and ajadelphinine **38**). These cytotoxic effects could be related to the inhibition of ATP production.

Keywords *Aconitum* · *Consolida* · *Delphinium* · Diterpenoid alkaloids · Insecticidal · Cytotoxic · Antiparasitic activity · Structure–activity relationships

Abbreviations

CPB	Colorado potato beetle
DA	Diterpenoid alkaloid

M. Reina
Instituto de Productos Naturales y Agrobiología, IPNA, CSIC, La Laguna-Tenerife, Spain

A. González-Coloma (✉)
Instituto de Ciencias Agrarias, ICA-CCMA, CSIC, C/ Serrano 115 dpdo., 28006 Madrid, Spain
e-mail: azu@ccma.csic.es

NDA Norditerpenoid alkaloid
PBO Piperonyl butoxide

Introduction

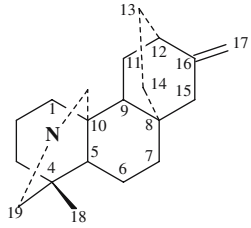
Plant species of the genera *Aconitum*, *Delphinium* and *Consolida* (Ranunculaceae) are almost the exclusive known sources of C_{19}-norditerpenoid and C_{20} diterpenoid alkaloids (NDAs and DAs, respectively) and are widely distributed over the temperate regions of the northern hemisphere (Atta-ur-Rahman and Choudary 1999).

These compounds have attracted considerable interest because of their complex structure, pharmacological effects (Dzhakhangirov et al. 1997; Ulubelen et al. 2001; Ameri 1998) and economic importance due to cattle poisoning (Panter et al. 2002).

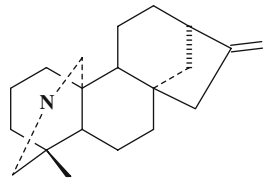
The atisine or veatchine system is considered the biogenetic origin of C_{20} DAs, while aconitine or lycoctonine systems give rise to the C_{19} NDAs, depending on the oxygen position (C-8 or C-7 and C-8, respectively). NDAs are highly oxygenated, more abundant (420 compounds isolated until the year 2000) and therefore more studied than the related DAs. However, a large number of DAs have been recently reported (281 compounds isolated until the year 2000) and a structural classification of the atisine and veatchine-derived structures has been proposed by Wang and Liang (2002).

NDAs act as potent nicotinic cholinergic receptor (nAcChR) agonists and antagonists in invertebrates, including insects, and vertebrates (see Panter et al. 2002; Ameri 1998; Seitz and Ameri 1998). The insecticidal and antifeedant activity of NDAs (Jennings et al. 1986; Ulubelen et al. 2001; González-Coloma et al. 2004a) suggest a plant defensive role played by these compounds and are well known pharmacologically for their anti-inflammatory, analgesic, antiarrythmia and antifungal actions (Ameri 1998; Atta-ur-Rahman and Choudhary 1999). However, the biological actions of DAs are less known. There are a few reports on their plant defensive and pharmacological properties (Bessonova and Shaidkhozaeva 2000; González-Coloma et al. 1998, Li et al. 2002a, b; Ulubelen et al. 2001), however, their neurotoxic effects are unknown.

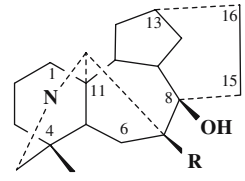
In this article, we present a comparative overview of the insecticidal effects (antifeedant and toxic) on *Spodoptera littoralis* and *Leptinotarsa decemlineata*, the cytotoxicity on several tumoral cell lines with varying multidrug resistance mechanisms (CT26, SW480, HeLa, SkMel25 and SkMel28), and the antiparasitic effects against *Trypanososma cruzi* and *Leishmania infantum* of 67 diterpenoid alkaloids (44 NDAs, and 23 DAs) from several chemical classes (González-Coloma et al. 1998, 2004a, b; González et al. 2005, 2006; De Inés et al. 2006), isolated from *Aconitum*, *Delphinium* and *Consolida* species (citations in González-Coloma et al. 1998, 2004a; De la Fuente and Reina 1990).



Atisine type



Veatchine type



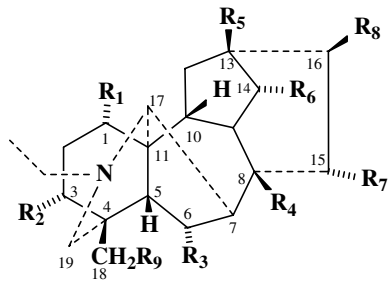
Aconitine type R= H

Lycoctonine type R = OH

Test compounds

Alkaloids are shown in Figs. 1–7. The isolation and identification on these compounds has

Fig. 1 Aconitine-type structures



Aconitine (**1**); $R_1 = R_3 = R_8 = R_9 = OMe$; $R_2 = R_5 = R_7 = OH$; $R_4 = OAc$; $R_6 = OBz$

3-Acetylaconitine (**2**); $R_1 = R_3 = R_8 = R_9 = OMe$; $R_2 = R_4 = OAc$; $R_5 = R_7 = OH$; $R_6 = OBz$

8-O-Ethyl-14-Benzoylaconine (**3**); $R_1 = R_3 = R_8 = R_9 = OMe$; $R_2 = R_5 = R_7 = OH$; $R_4 = OCH_2CH_3$; $R_6 = OBz$

8-O-Ethylaconine (**4**); $R_1 = R_3 = R_8 = R_9 = OMe$; $R_2 = R_5 = R_6 = R_7 = OH$; $R_4 = OCH_2CH_3$

Neoline (**5**); $R_1 = R_4 = R_6 = OH$; $R_2 = R_5 = R_7 = H$; $R_3 = R_8 = R_9 = OMe$

8-O-Methylcolumbianine (**6**); $R_1 = R_6 = R_9 = OH$; $R_2 = R_3 = R_5 = R_7 = H$; $R_4 = R_8 = OMe$

Karakoline (**7**); $R_1 = R_4 = R_6 = OH$; $R_2 = R_3 = R_5 = R_7 = R_9 = H$; $R_8 = OMe$

8-O-Methylkarakoline (**8**); $R_1 = R_6 = OH$; $R_2 = R_3 = R_5 = R_7 = R_9 = H$; $R_4 = R_8 = OMe$

Cardiopetaline (**9**); $R_1 = R_4 = R_6 = OH$; $R_2 = R_3 = R_5 = R_7 = R_8 = R_9 = H$

1,14-Diacetylcardiopetaline (**10**); $R_1 = R_6 = OAc$; $R_4 = OH$; $R_2 = R_3 = R_5 = R_7 = R_8 = R_9 = H$

been reported in Ruiz Mesia et al. 2002, Reina et al. 1997; González-Coloma et al. 2004a; Reina et al. in press, and references cited therein.

Antifeedant and insecticidal effects

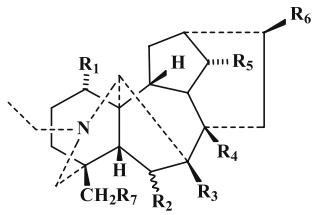
The antifeedant effects of NDAs **1–44** were structure- and species-dependent (Table 1). Overall, *L. decemlineata* (CPB) responded to a larger number of compounds than *S. littoralis* (67% and 46%, respectively), according to their different feeding adaptations. The most active CPB antifeedants were compounds **10** and **34** ($EC_{50} < 0.2$) followed by **13**, **28**, **7**, **9** ($Ec_{50} < 0.5$), **19**, **14**, **22**, **25** and **6** ($EC_{50} < 1$). *S. littoralis* showed the strongest response to **25**, followed by **19** > **20** > **30** ($EC_{50} < 3$) (Table 1).

Among the insect toxins, 50% and 19% of the tested compounds significantly increased CPB mortality or negatively affected *S. littoralis* larval performance, respectively (Table 1), indicating species-dependent tolerance to these alkaloids. The most toxic compound to CPB was aconitine (**1**, 100% mortality), followed by **11** (%mortality > 60), **15**, **18**, **20**, **26**, **31** (%mortality > 45), **13**, **14**, **30**, **34**, **37**, **39**, and **42** (%mortality > 30). All the moderate toxicants were behavioral antifeedants in choice tests, indicating that these compounds act at both the peripheral and central nervous system and suggesting a negative correlation between antifeedant and toxic effects on CPB (González-Coloma et al. 2004a).

Orally injected *S. littoralis*larvae were negatively affected by **1**, **9**, **11**, **12**, **13**, **15**, **42**, **44**, with varying degrees. A covariance analysis of food consumption (ΔI) and biomass gains (ΔB) indicated that alkaloids **12**, **13**, and **15** were post-ingestive toxins without delayed antifeedant effects. Compounds **1**, **11**, **42**, **44** had post-ingestive antifeedant effects while **9** also had further toxic action (González-Coloma et al. 2004a). Similar effects of neuroactive β-carboline alkaloids on *Trichoplusia ni* growth and consumption have been attributed to their interference with neurochemical mechanisms regulating food intake (Heinz et al. 1996).

A few compounds (18%) randomly distributed among the chemical classes had selective

Fig. 2 Lycoctonine-type structures



Cardiopetalidine (**11**); $R_1 = R_3 = R_4 = R_5 = OH$; $R_2 = R_6 = R_7 = H$

1,14-O-Acetylcardiopetalidina (**12**); $R_1 = R_5 = OAc$; $R_3 = R_4 = OH$; $R_2 = R_6 = R_7 = H$

8-O-Methylconsolarine (**13**); $R_1 = R_3 = R_5 = OH$; $R_2 = \alpha OH$; $R_4 = R_6 = OMe$; $R_7 = H$

18-O-Demethylpubescenine (**14**); $R_1 = R_3 = R_7 = OH$; $R_2 = \alpha OH$; $R_4 = R_6 = OMe$; $R_5 = OAc$

14-Deacetylpubescenine (**15**); $R_1 = R_3 = R_5 = OH$; $R_2 = \alpha OH$; $R_4 = R_6 = R_7 = OMe$

Pubescenine (**16**); $R_1 = R_3 = OH$; $R_2 = \alpha OH$; $R_4 = R_6 = R_7 = OMe$; $R_5 = OAc$

Consolidine (**17**); $R_1 = R_3 = OH$; $R_2 = \alpha OH$; $R_4 = R_5 = R_6 = R_7 = OMe$

18-O-Benzoyl-18-O-Demethyl-14-O-Deacetylpubescenine (**18**); $R_1 = R_3 = R_5 = OH$; $R_2 = \alpha OH$; $R_4 = R_6 = OMe$; $R_7 = OBz$

14-O-Acetyldeltatsine (**19**); $R_1 = R_3 = OH$; $R_2 = \beta OMe$; $R_5 = OAc$; $R_4 = R_6 = R_7 = OMe$

14-O-Acetyldelcosine (**20**); $R_1 = R_3 = R_4 = OH$; $R_2 = \beta OMe$; $R_5 = OAc$; $R_6 = R_7 = OMe$

Delsoline (**21**); $R_1 = R_3 = R_4 = OH$; $R_2 = \beta OMe$; $R_5 = R_6 = R_7 = OMe$

Takaosamine (**22**); $R_1 = R_3 = R_4 = R_5 = R_7 = OH$; $R_2 = \beta OMe$; $R_6 = OMe$

Gigactonine (**23**); $R_1 = R_3 = R_4 = R_7 = OH$; $R_2 = \beta OMe$; $R_5 = R_6 = OMe$

Delcosine (**24**); $R_1 = R_3 = R_4 = R_5 = OH$; $R_2 = \beta OMe$; $R_6 = R_7 = OMe$

Ajadine (**25**); $R_1 = R_6 = OMe$; $R_2 = \beta OMe$; $R_3 = R_4 = OH$; $R_5 = OAc$; $R_7 = OCOPhNHAc$

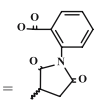
14-Deacetylajadine (**26**); $R_1 = R_6 = OMe$; $R_2 = \beta OMe$; $R_3 = R_4 = R_5 = OH$; $R_7 = OCOPhNHAc$

Lycoctonine (**27**); $R_1 = R_5 = R_6 = OMe$; $R_2 = \beta OMe$; $R_3 = R_4 = R_7 = OH$

14-O-Acetyldelectinine (**28**); $R_1 = R_6 = OMe$; $R_2 = \beta OMe$; $R_3 = R_4 = R_7 = OH$; $R_5 = OAc$

Browniine (**29**); $R_1 = R_6 = R_7 = OMe$; $R_2 = \beta OMe$; $R_3 = R_4 = R_5 = OH$

Delphatine (**30**); $R_1 = R_5 = R_6 = R_7 = OMe$; $R_2 = \beta OMe$; $R_3 = R_4 = OH$

Methyllicaconitine (**31**); $R_1 = R_5 = R_6 = OMe$; $R_2 = \beta OMe$; $R_3 = R_4 = OH$; $R_7 =$ 

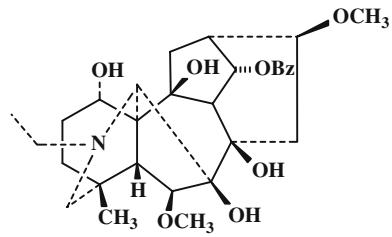
cytotoxic effects to insect-derived Sf9 cells (none of these compounds was cytotoxic to mammalian CHO cells). This cytotoxicity indicates a mode of action other than neurotoxic. Compound **15** was the most active. Some of these cytotoxic compounds were also toxic to CPB (**14**, **15**, **20**, **37**, **39**, **41**) and/or *S. littoralis* (**15**, **42**); therefore their insecticidal effects could be the result of neurotoxicity and/or cytotoxicity.

The antifeedant effects of DAs **45–67** (Figs.5–7) were also structure- and species-dependent (Table 2). The most-active CPB antifeedant was compound **66**, while *S. littoralis* showed the strongest response to **55** (González-Coloma et al. 2004b). Overall, CPB responded to a larger number of compounds than *S. littoralis* (75% and 45%, resp.), according to their different feeding adaptations as previously shown for NDAs (González-Coloma et al. 2004a). However, *S. littoralis* had a stronger response to the active compounds than CPB (Table 2). Additionally, the DAs tested here had lower antifeedant effects on CPB than these previously reported for NDAs, suggesting species- and structure-related differences in taste receptor binding to these two classes of diterpenoid alkaloid (González-Coloma et al. 2004b).

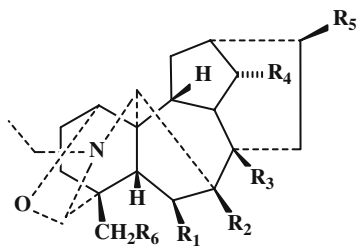
Their overall toxic effects were also lower than these of NDAs. Alkaloid **52** had moderate post-ingestive antifeedant effect. Compound **48** was cytotoxic to Sf9 and mammalian CHO cells. Compounds **55** and **59** were cytotoxic to Sf9 cells (González-Coloma et al. 2004b).

Two new weak base highly oxygenated hetisine-type DAs, delphigraciline (**53**), 14-hydroxyhetisinone N-oxide (**54**) and the NDA 8-methoxykarakoline (**8**) have been recently isolated from a neutral extract

Fig. 3 Gadesine-type structures



Gadenine (**32**)



14-O-Benzoylgadesine (**33**); $R_1 = R_5 = OMe$; $R_2 = R_3 = OH$; $R_4 = OBz$; $R_6 = H$

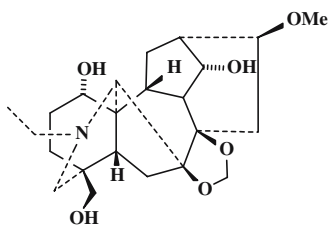
18-Hydroxy-14-O-Methylgadesine (**34**); $R_1 = R_4 = R_5 = OMe$; $R_2 = R_3 = R_6 = OH$

Dehydrotakaosamine (**35**); $R_1 = R_5 = OMe$; $R_2 = R_3 = R_4 = R_6 = OH$

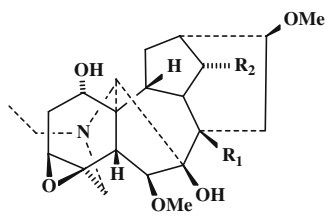
18-O-Methoxygadesine (**36**); $R_1 = R_5 = R_6 = OMe$; $R_2 = R_3 = R_4 = OH$

Dehydrodelsoline (**37**); $R_1 = R_4 = R_5 = R_6 = OMe$; $R_2 = R_3 = OH$

Fig. 4 Miscellaneous structures



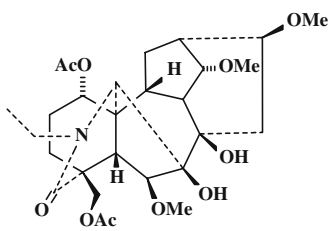
Ajadelphinine (**38**)



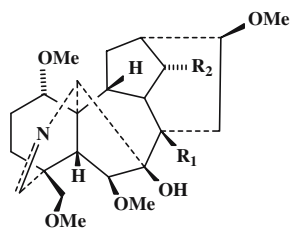
Tuguaconitine (**39**); $R_1 = OH$, $R_2 = OMe$

14-Demethyltuguaconitine (**40**); $R_1 = R_2 = OH$

14-Demethyldelboxine (**41**); $R_1 = OMe$; $R_2 = OH$



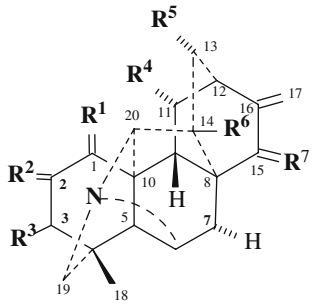
1,18-O-Diacetyl-19-oxo-gigactonine (**42**)



Olividine (**43**); $R_1 = OMe$; $R_2 = OAc$

Olivimine (**44**); $R_1 = OH$; $R_2 = OMe$

of *Delphinium gracile* (Reina et al. in press). Alkaloid **54** was a post-ingestive toxin to *S. littoralis* larvae in the presence of the insecticide synergist piperonyl butoxide (PBO) (ΔB decreased from 92% to 67 % in the presence of PBO). The lack of insect toxicity of **54** in the absence of PBO suggest an oxidative-mediated



Hetisinone (**45**); $R^2 = O$; $R^1 = R^3 = R^6 = R^7 = H$; $R^4 = R^5 = OH$

Cardiopetamine (**46**); $R^2 = O$; $R^1 = R^3 = R^6 = H$; $R^5 =$ OH; $R^7 =$ ▬ OH; $R^4 = OBz$

15-Acetylcardiopetamine (**47**); $R^2 = O$; $R^1 = R^3 = R^6 = H$; $R^4 = OBz$; $R^5 = OH$; $R^7 =$ ▬ OAc

13-oxo-cardiopetamine (**48**); $R^2 = R^5 = O$; $R^1 = R^3 = R^6 = H$; $R^7 =$ ▬ OH; $R^4 = OBz$

13-acetyl-15-oxo-cardiopetamine (**49**); $R^2 = R^7 = O$; $R^1 = R^3 = R^6 = H$; $R^5 = OAc$; $R^4 = OBz$

15β-Hydroxy-hetisinone (**50**); $R^2 = O$; $R^1 = R^3 = R^6 = H$; $R^4 = R^5 = OH$; $R^7 =$ ▬ OH

Cardiodine (**51**); $R^2 =$ ''''' $OCOCH(CH_3)CH_2CH_3$; $R^1 =$ ▬ OAc; $R^3 = R^4 = OAc$; $R^5 = OBz$; $R^7 = H$; $R^6 = OH$

Glandulosine (**52**); $R^2 =$ ''''' $OCOCH(CH_3)CH_2CH_3$; $R^3 = R^4 = R^5 = OAc$; $R^1 = R^7 = H$; $R^6 = OH$

Delphigraciline (**53**); $R^1 = R^3 = R^4 =$ ▬ OAc; $R^2 = R^5 = OBz$; $R^6 = OH$; $R^7 = H$

14-Hydroxyhetisinone N-Oxide (**54**); $R^1 = R^3 = R^7 = H$; $R^4 = R^5 = R^6 = OH$; $R^2 = O$; N→O

Fig. 5 Hetisine-type structures

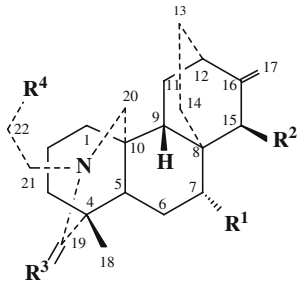
detoxification of this alkaloid. Hepatic P450 enzymes in mammals apparently do not metabolize some NDAs (Panter et al. 2002), suggesting differences in insect P450 enzymatic system ability to detoxify DAs.

The action of NDAs on the voltage-dependent sodium channels can be separated into activators (alkaloids with a benzoyl substituent at C-14) with extremely high toxicity in mammals, and blockers (Friese et al. 1997). Among the Na^+ channel agonists are aconitine (**1**) and 3-acetylaconitine (**2**) (Seitz and Ameri 1998), while several lycoctonine-type alkaloids (including lycoctonine, **27**; and methyllycaconitine, **31**) are competitive antagonists at the muscular and/or insect nAcChR junction (Jennings et al. 1986; Dobelis et al. 1999). The intensity of the nAChRs inhibition by NDAs is structure-dependent. The active core is the lycoctonine skeleton. The methylsuccinylanthranoyl ester at C-18 and the quaternary amine are important factors of the neuromuscular blocking effect (see Panter et al. 2002). In addition, the C-14 functionalities, the pattern of oxygenation and the electronic nature of the oxygen bearing functionalities appear to enhance the potency (Kukel and Jennings 1994; Hardick et al. 1996; Dobelis et al. 1999).

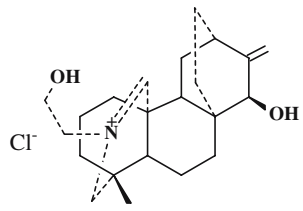
Neither the antifeedant nor the toxic activity of the compounds studied followed the expected structure–activity relationship from their reported receptor binding activity. The C-14 benzoyl group of agonists **1** and **2** and related compounds **3** and **33** resulted in null or low CPB and *S. littoralis* taste regulation respectively, while aconitine (**1**) was a strong toxin to both insects. The C-18 methylsuccinylanthranoyl substituent in methyllycaconitine (**31**) did not result in a potent antifeedant action, in contrast to the C-18 benzoyl (**25**). In addition, their antifeedant effects did not correlate with toxicity. This activity pattern could be related to the mode of action of these compounds on nAChRs (agonists vs. antagonists).

Previous studies have shown that agonists of insect nAChRs were in general insecticidal (toxic) whereas antagonists, such as imidacloprid, were antifeedants (Nauen et al. 1999). However, there is no evidence of the direct link between the antifeedant effects and the antagonistic action of compounds on insect nAChRs.

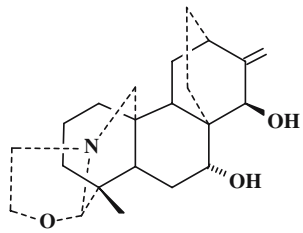
A GABA-mediated taste regulation has been proposed for chrysomelids and aphids (Mullin et al. 1997; González-Coloma et al. 2002; Reina et al. 2002). However, given the structural diversity of



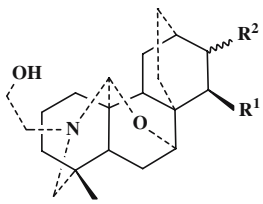
(**55**) 19-Oxodihydroatisine: $R^1 = H$, $R^2 = R^4 = OH$, $R^3 = O$
(**56**)15,22-O-Diacetyl-19-oxo-dihydroatisine: $R^1 = H$, $R^2 = R^4 = OAc$, $R^3 = O$
(**57**) Dihydroajaconine: $R^1 = R^2 = R^4 = OH$, $R^3 = H$
(**58**)15-Epi-dihydroatisine: $R^1 = R^3 = H$, $R^2 = OH$, $R^4 = OH$



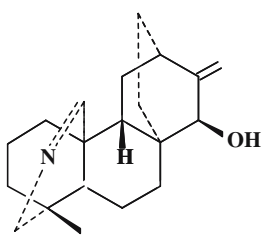
(**59**) Atisinium chloride



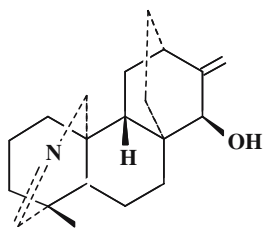
(**60**) 7- Hydroxy-isoatisine



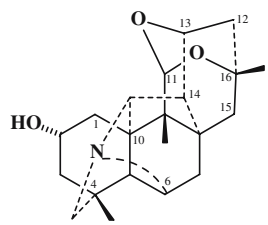
(**61**) Ajaconine: $R^1 = OH$, $R^2 = CH_2$
(**62**) Alkaloid A: $R^1 = =O$, $R^2 = CH_3$
(**63**) Alkaloid B: $R^1 = =O$, $R^2 = CH_3$



(**64**) Azitine

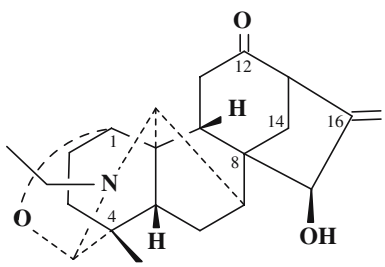


(**65**) Isoazitine



66) Hetisine rearranged

Fig. 6 Atisine-type structures



(**67**) Songoramine

Fig. 7 Veatchine-type structures

plant natural products and the increasing evidence of peripheral neuroreception involved in insect taste regulation (Bloomquist 2001; Cohen et al. 2002; Sanes et al. 1977), we propose a species-dependent multireceptor/channel mechanism for insect taste mediation tuned according to their feeding adaptations and involving nAChRs among others.

Antiparasite effects

From a total of 44 NDAs and 23 DAs tested, only three atisine-type DAs showed in vitro

Table 1 Effective antifeedant doses (EC_{50} and 95% confidence limits), and mortality (%M, 72 h, data corrected according to Abbot, 1925) of the NDAs on adult *L. decemlineata.* Consumption (ΔI) and biomass gain (ΔB) of orally injected *S. littoralis* L6 larvae, expressed as percent of the control. Cytotoxic effects on *S. frugiperda* Sf9 cells

Compound	Type	*L. decemlineata*		*S. littoralis*			Sf9
		EC_{50} (μg/cm^2)	%M	EC_{50} (μg/cm^2)	ΔB	ΔI	LD_{50} (μg/ml)
1	Aconitine	>100[a]	100	32.3 (19.6,45.0)[a]	34[b]	67[b]	>100
2		>50	0	≈50	94	84[b]	>100
3		>50	8	8.29 (8.17,8.42)	99	99	>100
4		2.57 (0.44,14.88)	0	5.37 (3.14,45.47)	91	90	>100
5		≈50	15*	≈50	90	90	>100
6		0.99 (0.97,1.02)	nt	>50	111	99	>100
7		0.44 (0.20,0.97)	32*	>50	89	96	>100
8		>50	nt	>50	nt	nt	nt
9		0.42 (0.40,0.43)	4	≈50	26[b]	70[b]	>100
10		0.11 (0.01,1.72)	0	21.84 (4.32,51.27)	90	112	30.39 (25.23,36.61)
11	Lycoctonine	>50	61*	>50	45[b]	71[b]	>100
12		6.00 (1.96,18.42)	0	>50	69[b]	112	>100
13		0.23 (0.04,1.29)	34*	>10	79[b]	94	>100
14		0.60 (0.18,2.01)	37*	>50	111	104	29.17 (21.40,40.67)
15		≈50	47*	17.99 (17.70,18.30)	78[b]	95	0.38 (0.22,0.66)
16		12.53 (2.71,57.85)[ns]	1	>50	94	90	>100
17		≈50	21*	9.86 (4.83,20.16)	105	115	>100
18		nt	47*	nt	98	101	>100
19		0.54 (0.53,0.56)	11	0.84 (0.82,0.86)	107	104	>100
20		>50	41*	1.51 (1.48,1.51)	106	99	14.88 (5.02,44.08)
21		2.22 (0.96,5.08)	0	>50	89	93	>100
22		0.66 (0.64,0.68)	7	5.29 (5.18,5.42)	91	96	>100
23		13.02 (12.77,13.28)	0	9.31 (9.09,9.91)	100	128	>100
24		1.11 (0.34,3.58)	1	3.53 (3.46,3.60)	92	97	32.37 (17.20,58.09)
25		0.84 (0.82,0.85)	24*	0.42 (0.41,0.44)	96	95	>100
26		nt	47*	nt	80	87	>100
27		>50	0	>50	115	105	>100
28		0.29 (0.04,1.82)	14*	5.63 (5.54,5.72)	100	111	>100
29		nt	27*	nt	107	103	>100
30		2.97 (2.94,3.02)	32*	2.72 (2.68,2.76)	82	91	>100
31		2.78 (2.72,2.85)	47*	17.77 (5.88,53.66)	90	93	>100
32		11.93 (3.14,45.47)	0	>50	97	103	>100
33	Gadesine	≈60	1	13.61 (13.42,13.81)	86	87	>100
34		0.13 (0.01,1.42)	34*	>50	110	109	>100
35		1.49 (0.31,7.24)	11	14.29 (8.50,24.08)	82	93	>100
36		6.36 (2.16,18.76)	0	>50	88	88	>100
37		12.2 (20,73.82) [ns]	31*	nt	99	96	18.89 (9.36, 38.17)
38	Miscellaneous	4.43 (1.54,12.73)	12	>50	101	102	>100
39		3.31 (1.10,9.94)	37*	11.79 (11.70,11.89)	97	98	1.83 (1.18,2.83)
40		2.36 (0.47,11.80)	25*	5.38 (1.43,20.37)	91	88	>100
41		1.92 (0.66,5.54)	31*	≈50	106	96	6.27 (3.26,12.05)
42		>50	nt	>50	61[b]	61[b]	29.45 (17.46,49.67)
43		3.62 (3.54,3.69)	nt	3.33 (1.07,10.39)	118	112	>100
44		10.92 (10.75,11.10)	nt	>50	76[b]	69[b]	>100

nt, not tested (insufficient compound available). ns, not significant dose–response relationship, $P > 0.05$. *Significantly different from the control, $P < 0.05$, contingency table analysis

[a] From González-Coloma et al. (1998)

[b] Significantly different from the control, $P < 0.05$, LSD test

Table 2 Effective antifeedant doses (EC_{50} and 95% confidence limits) of the DAs on adult *L. decemlineata*. Consumption (ΔI) and biomass gain (ΔB) of orally injected *S. littoralis* L6 larvae, expressed as percent of the control. Cytotoxic effects on *S. frugiperda* Sf9 cells

Compound	Type	*L. decemlineata*	*S. littoralis*			Sf9	CHO
		EC_{50} (μg/cm^2)	EC_{50} (μg/cm^2)	ΔB	ΔI	ED_{50} (μg/ml)	
45	Hetisine	13.1 (5.7,30.2)	>50	93	111	>100	>100
46		22.5 (19.7,25.3)[a]	5.5 (3.0,7.9)[a]	92	103[a]	nt	nt
47		12.9 (0.2,25.6)[a]	>100[a]	100[a]	99[a]	nt	nt
48		nt[b]	>100[a]	106[a]	97[a]	5.3 (8.1, 3.5)	12.5 (17.4,8.6)
49		27.2 (22.9,31.5)[a]	>100[a]	88[a]	114[a]	>100	>100
50		≈100[a]	23.7 (19.4,27.9)[a]	104[a]	106[a]	>100	>100
51		2.2 (2.2, 2.3)	4.4 (1.9,10.0)	98	119	>100	>100
52		4.0 (1.3,13.0)	>50	83	73[b]	>100	>100
53		>50	>50	nt	nt	nt	nt
54		>50	>50	92	102	nt	nt
55	Atisine	>50	0.1 (0.1, 1.0)	90	92	11.4 (7.0,18.5)	>100
56		>50	6.1 (2.4, 15.6)	91	85	>100	>100
57		5.0 (4.9, 5.1)	>50	80	97	>100	>100
58		2.9 (2.8, 2.9)	>50	98	96	>100	>100
59		3.4 (1.4, 8.1)	2.4 (0.5, 10.2)	123	103	38.2 (24.9,58.4)	>100
60		3.4 (3.4, 3.5)	>50	93	119	>100	>100
61		5.1 (5.0, 5.2)	8.2 (2.8,23.5)	80	104	>100	>100
62		5.4 (5.3, 5.5)	≈50	112	115	>100	>100
63		3.6 (3.6, 3.7)	>50	101	120	>100	>100
64		>50	1.1 (0.2, 6.3)	109	99	>100	>100
65		6.9 (4.1, 11.6)	4.1 (1.6,10.0)	115	100	>100	>100
66		1.73 (1.7,1.8)	≈50	89	121	>100	>100
67	Veatchine	≈50	nt	nt	nt	>100	>100

nt, not tested (insufficient compound available). ns, not significant dose–response relationship, $P > 0.05$

[a] From González-Coloma et al. (1998)

[b] Significantly different from the control, $P < 0.05$, LSD test

leishmanicidal activity against promastigote *L. infantum* (Table 3) (González et al. 2005). Compound **65** exhibited the highest toxicity to the extracellular *L. infantum* parasites. This leishmanicidal activity was associated with a lack of toxicity to murine macrophages by compounds **65** and **64** and only weak toxicity by **56** (Table 3). Delphigraciline (**53**) was also leishmanicidal with stronger potency than compound **65** (IC_{50} of 7.3 μg/ml at 48 h) (Reina et al. in press).

The percentage of parasitism and the number of amastigotes in macrophages infected with drug-treated promastigotes were strongly inhibited by compound **65**. Similar results were found for compound **64**. Alkaloid **56** showed the lowest action (Table 4). When the macrophages were infected before the addition of the alkaloids, the percentage of parasitism was not significantly affected. Nevertheless, the number of amastigotes was significantly reduced by the three products tested, indicating that there was a direct action on the intracellular forms and their multiplication. Furthermore, morphological studies showed that compound **56** was the most harmful to *L. infantum* promastigotes. This compound acts fundamentally at the level of the cytoplasmic membrane of the parasites, although alterations were detected also particularly in the mitochondria and kinetoplast (González et al. 2005).

Compounds **56**, **64** and **65** are very active in vitro both against the extracellular as well as against the intracellular forms of *L. infantum*. The in vitro growth rate of *L. infantum* was lowered, its capacity to infect cells was negatively affected, and the multiplication of the amastigotes was strongly reduced.

Five C_{20} alkaloids were active on *T. cruzi* (compounds **49**, **56**, **59**, **64**, **65**, Figs. 5, 6), while none of the C_{19} structures affected this parasite. The in vitro activity of these compounds against

Table 3 In vitro activity of *Leishmania infantum* promastigotes to compounds

Compound	IC_{50} (μg/ml)			Toxicity IC_{50} (μg/ml)[a]
	24 (h)	48 (h)	72 (h)	
Pentostam	–	–	11.32	
56	24.58	15.74	12.80	74.28
64	26.30	15.35	10.12	>200
65	13.38	9.70	7.39	>300

[a] On J774.2 macrophages at 72 h of culture

IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the K_c values at the concentrations tested (1, 10, 25, 50 and 100 μg/ml)

Note: Average of four separate determinations; nd, not determined

Table 4 Effects of the drugs (at 5 μg/ml) on the infection rate of J774A.1 macrophages and on the average number of *Leishmania infantum* amastigotes per infected macrophage during 8 days of culture, under different conditions

Treatment	(%) Mϕs[a]				IP/C[b]			
	48 h	96 h	144 h	192 h	48 h	96 h	144 h	192 h
None (Control)	78.4	79.2	77.6	79.2	16.8 ± 4.7	15.9 ± 2.1	15.1 ± 2.6	14.8 ± 1.1
Mϕ + Li + **56**[c]	64.0	68.0	67.2	63.2	6.0 ± 0.7	6.8 ± 1.1	7.3 ± 1.3	7.3 ± 0.7
[Mϕ – Li] + **56**[c]	74.4	76.0	76.8	72.0	8.6 ± 1.4	7.9 ± 1.5	8.6 ± 1.7	8.7 ± 1.2
Mϕ + Li + **64**[c]	68.0	32.0	29.6	25.4	3.5 ± 0.5	2.8 ± 0.4	2.9 ± 1.0	2.8 ± 0.9
[Mϕ – Li] + **64**[c]	76.0	78.4	76.0	75.2	8.3 ± 1.4	8.1 ± 0.9	8.1 ± 1.3	7.4 ± 1.2
Mϕ + Li + **65**[c]	36.8	14.4	16.4	11.3	2.4 ± 0.6	2.8 ± 1.6	4.8 ± 1.4	4.1 ± 1.1
[Mϕ – Li] + **65**[c]	75.2	73.2	76.4	69.6	4.8 ± 1.3	4.2 ± 0.6	4.9 ± 1.0	6.2 ± 0.6

[a] Percent macrophage parasitism. Values are means ± standard deviations of four separate determinations

[b] Number of amastigotes per macrophages infected. Values are means ± standard deviations of four separate determinations

[c] Details are in González et al. (2005)

Table 5 In vitro activity of alkaloids **48**, **56**, **59**, **64** and **65** on *Trypanosoma cruzi* epimastigotes

Compound	IC_{50} (μg/ml)			Toxicity IC_{50} (μg/ml)[a]
	24 (h)	48 (h)	72 (h)	
Benznidazole	nd	nd	4.12	
64	nd	nd	67.74	
65	nd	nd	>100	
56	nd	nd	98.36	
59	13.91	9.37	5.46	>300
48	35.05	20.53	12.17	>200

[a] Vero cells at 72 h. IC_{50} = Concentration required to give 50% inhibition, calculated by linear regression analysis from the K_c values at the concentrations used (1, 10, 25 and 100 μg/ml). *Note*: Average of four separate determinations

T. cruzi epimastigotes is shown in Table 5. Compound **59** exhibited the highest toxicity against *T. cruzi* epimastigotes with IC_{50} values within the range of the reference drug. Compound **48** was also active, with lower potency than **59**. This antitrypanocidal activity was not associated to host cell toxicity. Compounds **64** and **65** were moderately active (González et al. 2006).

A previous screening showed that *T. cruzi* epimastigote mortality increased with 13-oxo-cardiopetamine (**48**) and 15,22-*O*-diacetyl-19-oxo-dihydroatisine (**56**) while azitine (**64**) and isoazitine (**65**) were inactive. These authors did not detect any activity for atisinium chloride (**59**) probably due to the different method used to detect parasite viability (MTT method),

(González-Coloma et al. 2004a). Furthermore, compounds **59** and **48** inhibited host cell infection rate, amastigote replication, and trypomastigote propagation with varying potencies. When the parasites were preincubated with **59**, the number of amastigotes/cell was reduced suggesting a direct action of this compound on the parasite (González et al. 2006).

Leishmania infantum was more sensitive to DAs **64**, **65** and **56** than *T. cruzi*, suggesting species-related selectivity for the antiparasitic action of these compounds (González et al. 2005). However, none of the 43 NDAs tested on *T. cruzi* or *L. infantum* affected parasite viability (González-Coloma et al. 2004a; González et al. 2006), indicating a strong molecular selectivity for the trypanocidal and leishmanicidal effect of DAs (C_{20} vs. C_{19} alkaloids).

Cytotoxicity

The cytotoxic effects of 44 NDAs have been reported against the tumor cell lines CT26 (murine colon adenocarcinoma), SW480 (human colon adenocarcinoma), HeLa (human cervical adenocarcinoma), SkMel25 (human melanoma) and SkMel28 (human malignant melanoma). These cell lines express different resistance mechanisms including the multidrug resistance phenotype (MDR), due to the overexpression of any of the energy-dependent drug efflux transmembrane proteins such as the *P*-glycoprotein (Pgp), or the multidrug resistance protein (MRP1) (see De Inés et al. 2006), and the intracellular glutathione/glutathione *S*-transferase detoxification system (GSH/GST) which protects and detoxifies cells from highly reactive free radicals and organic peroxides and metabolizes xenobiotics (see De Inés et al. 2006).

Overall CT26 and SW480 were sensitive to the largest number of compounds (33%) followed by SkMel25 (31%), HeLa (24%) and SkMel 28 (12%). HeLa showed the lowest MIC value. The different cellular range of action of these compounds could be related to factors such as intracellular transportation, metabolism, inactivation and receptor geometry (Table 6) (De Inés et al. 2006).

The cytotoxicity of the test alkaloids followed different patterns for each chemical class. The most active alkaloids were found among the gadesine-type. The selective cytotoxic effects of some structures indicate that these compounds

Table 6 Minimal inhibitory concentration (MIC) of the active test compounds, classified by chemical type, on several mammalian cell lines

Compound[a]	Type	MIC (μg/ml)					
		CHO	CT26	SW480	HeLa	SkMel25	SkMel28
5	Aconitine	> 100	25	12.5	6.25	25	> 100
6		> 100	50	50	> 100	50	> 100
10		> 100	100	100	> 100	> 100	> 100
12	Lycoctonine	100	100	100	> 100	100	100
14		> 100	> 100	> 100	25	50	50
15		> 100	> 100	> 100	> 100	50	> 100
16		> 100	100	25	50	50	> 100
25		50	50	50	> 100	> 100	50
26		> 100	> 100	100	50	100	> 100
27		> 100	50	50	> 100	> 100	> 100
30		> 100	> 100	> 100	100	> 100	> 100
31		12.50	12.50	50	50	100	100
35	Gadesine	> 100	6.25	6.25	0.40	6.25	25
36		25	50	25	25	25	> 100
37		6.25	12.50	12.50	12.50	25	6.25
38	Miscellaneous	> 100	50	25	12.50	25	> 100
42		25	50	100	> 100	100	> 100

[a] Compounds **1–4**, **7**, **9**, **11**, **13**, **17–24**, **28**, **29**, **32–34**, **39–41**, **43** and **44** had MIC values > 100 for all the cell lines tested

can act on biological targets other than neuroreceptors with strong molecular selectivity as previously demonstrated for several alkaloids belonging to different chemical classes (Wink et al. 1998). The cytotoxic activity of the compounds studied here did not follow the expected structure–activity relationship from their reported receptor binding activity (Kukel and Jennings 1994; Hardick et al. 1996; Dobelis et al. 1999; Panter et al. 2002). The C-14 benzoyl group of nAcChR agonists **1** and **2** and related compound **3**, **32** and **33** resulted in null cytotoxicity. The C-18 methylsuccinylanthranoyl substituent in the antagonist methyllycaconitine (**31**) resulted in a more potent cytotoxic action than that of the C-18 benzoyl (**25** or **26**).

To determine if the cytotoxic effects of the selective compounds (cytotoxic to tumoral cells vs. CHO cells) were reversible, the recovery of sensitive tumoral cells was studied (Table 7). Compound **16** had irreversible effects on all treated cell lines followed by **35** which affected 3 of 5 cell lines, with SW480 being the most sensitive of all. Alkaloids **26**, **27** and **5** had a selective strong effect on the recovery of SW480 with **26** being the most potent. Alkaloid **38** selectively

Table 7 Reversibility of the cytotoxic effect of selective compounds on cell viability

Compound	Days	Reversibility (%)[a]					
		CHO	CT26	SW480	HeLa	SkMel25	SkMel28
5	0	61 ± 4	12 ± 1	2 ± 0	16 ± 1	17 ± 1	–
	3	91 ± 9	106 ± 15	1 ± 0	55 ± 12	17 ± 1	–
	6	104 ± 0	–	33 ± 1	113 ± 38	73 ± 9	–
6	0	55 ± 12	13 ± 1	19 ± 1	–	19 ± 7	–
	3	72 ± 4	115 ± 5	83 ± 0	–	30 ± 3	–
	6	111 ± 0	–	–	–	101 ± 2	–
10	0	100 ± 10	6 ± 0	2 ± 0	–	–	–
	3	–	38 ± 5	61 ± 6	–	–	–
	6	–	100 ± 1	93 ± 0	–	–	–
14	0	94 ± 0	–	–	2 ± 0	18 ± 3	20 ± 2
	3	–	–	–	108 ± 2	56 ± 3	98 ± 3
	6	–	–	–	–	85 ± 0	–
15	0	41 ± 6	–	–	–	21 ± 3	–
	3	98 ± 0	–	–	–	91 ± 8	–
	6	–	–	–	–	–	–
16	0	45 ± 6	–	15 ± 0	6 ± 2	28 ± 0	–
	3	60 ± 3	–	13 ± 1	4 ± 0	17 ± 3	–
	6	89 ± 0	–	3 ± 0	6 ± 1	14 ± 4	–
26	0	54 ± 8	–	8 ± 1	0	18 ± 1	–
	3	80 ± 9	–	4 ± 0	38 ± 3	32 ± 1	–
	6	–	–	8 ± 0	104 ± 5	94 ± 0	–
27	0	60 ± 3	20 ± 0	9 ± 1	–	–	–
	3	78 ± 5	114 ± 15	3 ± 1	–	–	–
	6	–	–	24 ± 1	–	–	–
29	0	106 ± 0	22 ± 4	6 ± 1	–	–	–
	3	–	100 ± 8	40 ± 5	–	–	–
30	0	59 ± 4	–	–	16 ± 6	–	–
	3	106 ± 1	–	–	59 ± 8	–	–
	6	–	–	–	89 ± 10	–	–
35	0	74 ± 8	17 ± 2	8 ± 1	5 ± 0	19 ± 0	20 ± 1
	3	97 ± 0	109 ± 13	1 ± 0	6 ± 0	18 ± 0	14 ± 0
	6	–	–	3 ± 0	23 ± 1	35 ± 2	68 ± 0
38	0	67 ± 1	21 ± 24	15 ± 0	3 ± 0	20 ± 1	–
	3	93 ± 1	79 ± 5	11 ± 0	13 ± 1	22 ± 0	–
	6	–	–	74 ± 0	40 ± 10	89 ± 13	–

Cells were incubated with their respective MIC value for each compound (Table 6)

[a] Percentage cell viability (percent absorbance of the respective untreated control cells). Represented are mean values ± SE

acted on HeLa cells with moderate potency (De Inés et al. 2006).

In order to gain insights about the mechanism of action of the irreversibly cytotoxic compounds, the viability of the sensitive cells was determined by the MTT and the AP methods (Table 8). The viability of SkMel25 cells incubated with Taxol® which blocks normal microtubule dynamics and cell division (Schiff and Horwitz 1980), was similar when measured by both methods, as expected for a compound that has no effect on cellular respiration and ATP generation. However, incubation of SkMel25 with rotenone which interrupts mitochondrial electron transfer at the NADH dehydrogenase–ubiquinone junction of the respiratory chain (Palmer et al. 1968), resulted in significantly different results for cell viability when measured by both methods. The incubation of the sensitive lines with **5**, **16**, **26**, **27**, **35** and **38** gave higher cell viability values when measured with the AP method (Table 8). Therefore, the mode of action of these compounds could be related to the inhibition of ATP production. This will explain why SW480 (Pgp+) cells, with higher energy demand related to their resistance mechanism, were the most sensitive to most of these compounds (**5**, **16**, **26**, **27** and **35**). HeLa, and SkMel25 were the following more sensitive lines, suggesting that these cells have a high ATP demand maybe related to their resistance mechanism and/or metabolism (De Inés et al. 2006).

Conclusions

A wide array of NDAs and DAs act as insect antifeedants and toxicants, supporting their plant protection role and suggesting nAChR mediation in insect taste regulation. Among the most potent antifeedants are the NDAs 1,14 diacetylcardiopetaline (**10**), 18-hydroxy-14-*O*-methylgadesine (**34**) and 14-*O*-acetyldelectinine (**28**) (to CPB) and the DA 19-oxodihydroatisine (**55**) (to *S. littoralis*). Their potencies did correlate with the agonist/antagonist insecticidal/antifeedant model proposed for nicotininc insecticides, therefore supporting nAChR mediation in insect taste regulation, and opening a new field for insect control strategies.

DAs delphigraciline (**53**), 15,22-*O*-diacetyl-19-oxo-dihydroatisine (**56**) azitine (**64**) and isoazitine (**65**) exhibit promising antileishmanial and/or trypanocidal properties. However, none of the NDAs tested resulted active against these parasites indicating a strong molecular selectivity for these effects (C_{20} vs. C_{19} alkaloids).

Neoline (**5**), pubescenine (**16**), 14-deacetylajadine (**26**), lycoctonine (**27**), dehydrotakaosamine (**35**), and ajadelphinine (**38**) had irreversible cytotoxic effects to several cancerous cell lines. The mode of action of these cytotoxic compounds could be related to low ATP levels.

None of these compounds had ester bounds at C-14 or C-18, primarily responsible for high mammalian toxicity (Ameri 1998).

Table 8 Comparative cytotoxicity of the irreversible compounds on the sensitive cell lines, determined with the AP and MTT methods

Compound	MIC (μg/ml)	Cell line	Viability (%)[a]	
			MTT	AP
Taxol®	0.01	SkMel25	3 ± 0	5 ± 1
Rotenone	0.01	SkMel25	10 ± 1	42 ± 1
5	12.50	SW480	5 ± 0	16 ± 2
16	25	SW480	10 ± 1	19 ± 2
26	100	SW480	na	na
27	50	SW480	7 ± 2	20 ± 1
35	6.25	SW480	5 ± 0	20 ± 4
38	12.50	HeLa	4 ± 0	22 ± 1

[a] Percentage cell viability (percent absorbance of the respective untreated control cells)

Represented are mean values ± SE

na, not enough compound available

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