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Actions of picrodendrin antagonists on dieldrin-sensitive and -resistant *Drosophila* GABA receptors

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1 A series of terpenoid compounds, recently isolated from *Picrodendron baccatum*, share a picrotoxane skeleton with picrotoxinin, an antagonist of ionotropic GABA receptors. Referred to as picrodendrins, they inhibit the binding of [^{35}S]-*tert*-butylbicyclophosphorothionate (TBPS) to rat $GABA_A$ receptors. Hitherto, their effects on GABA receptors have not been investigated electrophysiologically. Under two-electrode voltage-clamp, the actions of picrodendrins and related terpenoids have been assayed on homo-oligomeric GABA receptors formed by the expression of a *Drosophila* GABA receptor subunit (RDL_{ac}) in *Xenopus* oocytes.

2 All the terpenoids tested, dose-dependently antagonized currents induced by 30 μM (EC_{50}) GABA.

3 Tutin and its analogues (dihydrotutin and isohyenanchin) differ in the structure of their axial C4 substituents. Of these compounds, tutin, which bears an isopropenyl group at this carbon atom, was the most potent antagonist of RDL_{ac} homo-oligomers, whereas isohyenanchin, which bears a hydroxyisopropyl group, was the least potent antagonist tested.

4 Picrodendrins differ mainly in the structure of their C9 substituents. The IC_{50}s of picrodendrins ranged from 17 ± 1.3 nM (picrodendrin-Q) to 1006 ± 1.3 nM (picrodendrin-O). As such, the most potent picrodendrins (Q, A and B) were approximately equipotent with picrotoxinin as antagonists of RDL_{ac} homo-oligomers.

5 Certain picrodendrin compounds effected a use-dependent blockade of RDL_{ac} homo-oligomers. Such a biphasic block was not observed with tutin analogues.

6 Picrotoxin-resistant RDL_{ac}^{A3025} homo-oligomers, which have a single amino acid substitution (A302S) in the 2nd transmembrane region, were markedly less sensitive to picrodendrin-O than the wild-type, dieldrin-sensitive, homo-oligomers.

7 The relative potency of tutin analogues demonstrates that the structure-activity relationship of the C4 substituent of picrotoxane-based compounds is conserved in vertebrates and insects. However, the relative order of potency of picrodendrins on RDL_{ac} homo-oligomers is distinctly different from that observed in previous radioligand binding studies performed on vertebrate $GABA_A$ receptors. As picrodendrin compounds differ in the structure of their C9 substituents, these data suggest that the optimal convulsant pharmacophores of vertebrate $GABA_A$ receptors and RDL_{ac} homo-oligomers differ with respect to this substituent.

Keywords: Picrodendrins; tutin; dihydrotutin; GABA receptor; *Drosophila melanogaster*

Introduction

Ionotropic γ-aminobutyric acid (GABA) receptors are widespread in the nervous systems of vertebrates and invertebrates (Sattelle, 1990). The activity of these receptors is antagonized by a range of structurally distinct compounds which include picrotoxinin (Smart & Constanti, 1986; Twyman *et al.*, 1989; Newland & Cull-Candy, 1992) and TBPS (*tert*-butylbicyclophosphorothionate; van Rentergehm *et al.*, 1987). Data from radioligand binding experiments and electrophysiological studies strongly suggest that many insecticidal compounds, which antagonize vertebrate and insect GABA receptors, interact with the picrotoxinin and TBPS binding sites (Kadous *et al.*, 1983; Matsumura & Ghiasuddin, 1983; Cohen & Casida, 1986; Lummis & Sattelle, 1986; Olsen *et al.*, 1989; Wafford *et al.*, 1989a,b; Lummis *et al.*, 1990; Deng *et al.*, 1993; Anthony *et al.*, 1994). Such compounds include cyclodienes (e.g. dieldrin), hexachlorocyclohexanes (e.g. lindane), and bicycloorthobenzoates (e.g. EBOB: ethynylbicycloorthobenzoate), and are collectively termed convulsant antagonists.

Picrodendrins and tutin analogues are a series of terpenoids which have been isolated from the bark and stems of the Euphorbiaceae plant, *Picrodendron baccatum*; (L) Krug & Urban (Ohmoto *et al.*, 1989a,b; Koike *et al.*, 1991a,b, 1994; Suzuki *et al.*, 1992). In the Dominican Republic this plant is used to kill bedbugs and lice and is known as 'mata becerro' (calf killer) (Hayden *et al.*, 1984). These terpenoids share a polycyclic lactone 'picrotoxane skeleton' with picrotoxinin (Figure 1), suggesting that they may underlie the convulsant properties of *P. baccatum*, and like picrotoxinin they displace [^{35}S]-TBPS from rat brain $GABA_A$ receptors (Ozoe *et al.*, 1994). However, the effects of these compounds on GABA receptors have yet to be assayed electrophysiologically.

Homo-oligomeric GABA receptors formed by the heterologous expression of a *Drosophila* GABA receptor subunit, RDL_{ac} (ffrench-Constant *et al.*, 1991), are providing to be a convenient model for investigating the pharmacology of insect GABA receptors. RDL_{ac} subunits readily form GABA-gated chloride-channels in a variety of expression systems (ffrench-Constant *et al.*, 1993a; Lee *et al.*, 1993; Buckingham *et al.*, 1994; Millar *et al.*, 1994; Hosie & Sattelle, 1996) where they faithfully mimic several of aspects of the characteristic pharmacology of native insect GABA receptors (Sattelle, 1990).

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The RDL_{ac} subunit is one of four splice variants of the *Rdl* gene (ffrench-Constant & Rocheleau, 1993), the products of which are widely distributed throughout the central nervous system of *Drosophila melanogaster* (Aronstein & ffrench-Constant, 1995; Harrison *et al.*, 1996). The substitution of a single amino acid in the second transmembrane (M2) region of *Drosophila Rdl*-encoded subunits (A302S or A302G; ffrench-Constant *et al.*, 1993b) confers resistance to picrotoxinin, dieldrin, and a range of insecticidally active compounds in native (Bloomquist, 1994; Zhang *et al.*, 1994), and heterologously expressed, *Drosophila* GABA receptors (ffrench-Constant *et al.*, 1993a; Belelli *et al.*, 1995; Hosie *et al.*, 1995a,b). Homologues of *Rdl*-encoded subunits have also been identified in cyclodiene-sensitive and -resistant insects from three orders (Thompson *et al.*, 1993a,b; Kaku & Matsumura, 1994; Miyazaki *et al.*, 1995). In all cases, the substitution by serine or glycine of the alanine residue found at the equivalent of *Drosophila* RDL^{A302} was observed in cyclodiene-resistant strains of these insects. These data suggest that RDL-like subunits may be constituents of many insect GABA receptors.

In the present study, we have investigated the structure-activity relationship of picrodendrins and related terpenoids (corianin and tutin analogues) on wild-type (dieldrin-sensitive) RDL_{ac} homo-oligomers expressed in *Xenopus* oocytes. The effect of the A302S substitution on the potency of a representative picrodendrin was also assayed. The potency of tutin analogues on RDL_{ac} homo-oligomers demonstrates that certain aspects of the structure-activity relationship of picrotoxane-based antagonists are conserved in insect and vertebrate GABA receptors. However, there are marked differences in the relative potency of picrodendrins on RDL_{ac} homo-oligomers and vertebrate $GABA_A$ receptors, which can be ascribed to differences in the structure of specific substituents of the picrotoxane skeleton. Further, we find that the mutation in M2 greatly reduced the potency of the representative picrodendrin.

Methods

cRNA synthesis

The cloning and subcloning of the cDNAs encoding the wild-type and dieldrin-resistant forms of RDL_{ac} has been described elsewhere (ffrench-Constant *et al.*, 1991; 1993b; Hosie *et al.*, 1995a). Plasmid pNB14.1, containing the wild-type cDNA was linearised with the restriction endonuclease *Not*I and $m^7G(5')ppp(5')G$ capped cRNA was synthesized with SP6 RNA-polymerase (Promega) using a standard protocol (Sambrook *et al.*, 1989). Plasmid pHARRT (Hosie *et al.*, 1995a) was used as a template for the synthesis, by T7 RNA polymerase, of capped cRNA encoding the dieldrin-resistant form of RDL_{ac} (RDL_{ac}^{A302S}). As pHARRT has a T7 RNA-polymerase termination sequence 3′ to the cDNA insert, it was not linearised.

Oocyte preparation and cRNA injection

Stage V and VI oocytes were removed from mature *Xenopus laevis* and manually defolliculated after a 40 min incubation with collagenase type IA (2 mg ml^{-1}) in a low-calcium version of standard oocyte saline, (standard saline composition, μM: NaCl 100, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5; pH 7.6). Each oocyte was injected with 40–50 ng of cRNA in 25–50 nl and incubated at 17–18°C in saline supplemented with penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}), gentamycin (50 μg ml^{-1}) and 2.5 mM sodium pyruvate. Electrophysiological examination was performed 18–72 h after injection.

Electrophysiological investigation

Oocytes were secured in a 90 μl Perspex recording chamber and continuously perfused with saline (5 ml min^{-1}). All drugs were applied dissolved in the perfusate. Terpenoids were initially dissolved in dimethyl sulphoxide (DMSO) at concentrations which ensured that the final solvent concentration in the perfusate never exceeded 0.01% v/v. The concentrations of solvent used had no effect on the current required to clamp the membrane at −60 mV. In all experiments the membrane potential was clamped at −60 mV and membrane currents were monitored under two-electrode voltage-clamp using 3 M KCl filled electrodes (1–10 MΩ) and an Oocyte Clamp OC-725C amplifier (Warner Instruments). Signals were displayed on an oscilloscope (Trio) and recorded onto computer using a TL-1 interface and Axotape software (both from Axon instruments).

When determining the dose-effect relationship of terpenoids each oocyte was challenged with 3 mM GABA (EC_{100}) and, following recovery, with at least 3 applications of 30 μM GABA to ascertain that the response to 30 μM GABA was approximately EC_{50} and that the preparation was stable. Each oocyte was pre-incubated for 2 min in the lowest concentration of the picrodendrin under test prior to co-application of the terpenoid and 30 μM GABA. Following wash with saline, the oocyte was pre-incubated in the next highest dose of terpenoid, and the process repeated. Thus GABA was applied at intervals of approximately 3.5 min. As recovery from terpenoid blockade is, in many cases, only partial, only one terpenoid compound was tested per oocyte.

All averaged data are presented as the mean ± the standard error of the mean. GraphPad Prism (GraphPad Software) was used to fit the following four parameter logistic equation (1) which describes a sigmoid curve of variable slope, to the averaged, normalised data:

$$\frac{I}{I_{max}} = \frac{I_{min}}{I_{max}} + \frac{I_{max} - I_{min}}{[1 + 10^{(\log IC_{50}[ant])^* n_H}]I_{max}} \quad (1)$$

where $\%I_{max}$ is the current induced by a given concentration of antagonist ([ant]) expressed as a percentage of I_{max}, the amplitude of the control GABA response. I_{min} is the minimal agonist response, IC_{50} is the concentration of antagonist predicted to block half the control response and n_H is the slope (Hill) coefficient. Estimated IC_{50} values are shown with their 95% confidence interval (95% CI).

Source of reagents

Picrodendrin and tutin analogues were prepared as described elsewhere (Ohmoto *et al.*, 1989a,b; Koike *et al.*, 1991a,b; Suzuki *et al.*, 1992). The structures of the terpenoids tested in the present study are shown in Figure 1. All reagents for RNA synthesis were purchased from Promega (U.K.) except for $m^7G(5')ppp(5')G$ cap analogue, which was obtained from NEB (U.K.). Collagenase type IA, and GABA were obtained from Sigma (U.K.). cDNAs encoding the two forms of RDL_{ac} were gifts of R.T. Roush (Cornell University, U.S.A.).

Results

All the terpenoids suppressed, dose-dependently, the response to 30 μM GABA of oocytes injected with cRNA encoding wild-type RDL_{ac} subunits. Two phases of blockade were observed with certain terpenoid antagonists. During the prolonged (e.g. 20 s) application of GABA, picrodendrins A, B, G, O and Q (and to a lesser extent, picrodendrin-F) resulted in a reduction in the initial peak amplitude of GABA-responses, and accelerated current decay to a steady state (Figure 2a,b). This decay was in addition to the desensitization observed with GABA alone, as the fraction of the control GABA response observed at peak was consistently greater than that observed 15 s after co-application of GABA and the antagonist. With 0.2 μM picrodendrin-G, the mean peak current elicited by 30 μM GABA was 52 ± 1% ($n=4$) of the peak control response, whereas that observed after 15 s application of GABA

response was 33±1% ($n=4$) of that seen 15 s into the control response. This increase in the rate of current decay, beyond that seen with GABA alone, leads to a slight increase in the apparent potency of such picrodendrins during the course of a GABA response (Figure 2c). For example, the concentrations for half-maximal inhibition (IC_{50}s) of the response to 30 μM by picrodendrin-G were 193 nM (95% CI: 146–258 nM) at the peak and 140 nM (95% CI: 115–171 nM) 15 s into the GABA response ($n=3$). By contrast, in the presence of the corianin and the tutin analogues, the amplitude of the initial GABA response was suppressed but little or no further decay of the GABA-induced currents was observed over 20 s (Figure 2d).

Structure-potency relationship of tutin analogues

When examining relative potency, the amplitudes of current recorded in the absence and presence of terpenoids 15 s after the onset of the GABA response were compared. This was found to give more consistent results than those obtained using the initial peak of the GABA response.

Tutin, dihydrotutin and isohyenanchin are distinguished by their carbon-4 (C4) alkyl substituents. Reductions in antagonist potency were observed when the electronegativity, hydrophobicity or planarity of this substituent were altered (Figure 3). Thus tutin, which bears an isopropenyl group at C4, was 5 fold more potent as an antagonist of responses to 30 μM GABA than dihydrotutin, the C4 isopropyl group of which is more hydrophobic, but less electronegative and non-planar, by virtue of its saturation. The substitution of a hydroxyisopropyl group at C4 yielded isohyenanchin which was the least potent of these analogues, being 36 fold less potent than tutin. The estimated IC_{50}s of these compounds are given in Table 1.

Structure-potency relationship of picrodendrin terpenoids

The picrodendrin compounds tested here, differ mainly in the structures of their electronegative substituents at C9 (structures shown in Figure 1). Picrodendrin dose-inhibition curves are shown in Figure 4, and their estimated IC_{50}s in Table 1. Picrodendrin-Q (IC_{50} 17 nM, 95% CI: 7–39 nM) was the most potent of these compounds, as it is in radioligand binding studies on $GABA_A$ receptors (Ozoe *et al.*, 1994), whereas picrodendrin-O was the least potent (IC_{50} 1006 nM, 95% CI: 339–2981 nM). Although the picrodendrins tested here all bear an isopropyl group at C4 (like dihydrotutin), they were all potent antagonists of RDL_{ac} homo-oligomers. Picrodendrins differ from dihydrotutin in the structure of their C9 and C2

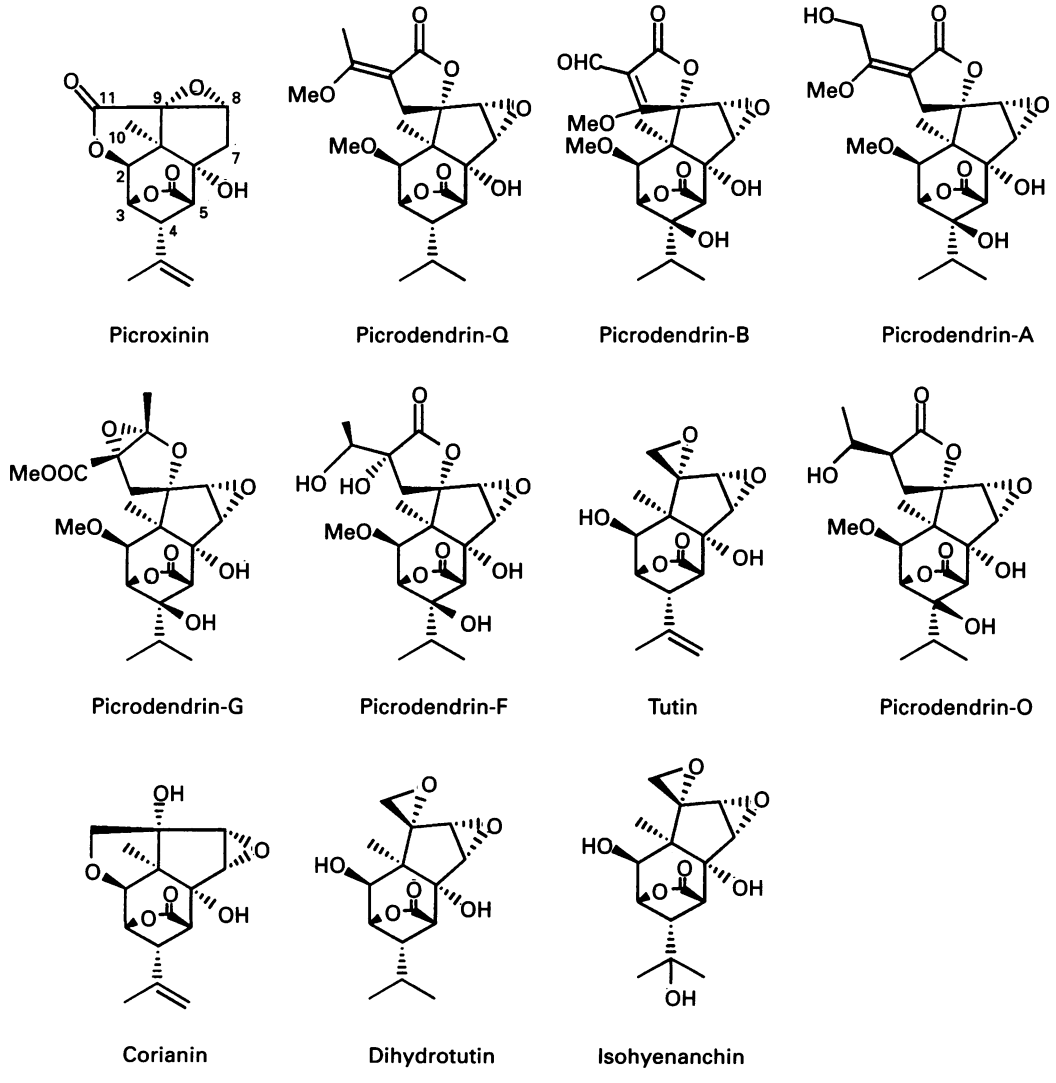

Figure 1 Structures of picrodendrins and tutin analogues. The structures of the compounds used in the present study are shown together with that of picrotoxinin. The numbering system used to identify the carbon atoms of the picrotoxane skeleton is illustrated on the structure of picrotoxinin (cf. Jarboe *et al.*, 1968). The picrodendrins and tutin analogues are ordered by their potency as antagonists of RDL_{ac} homo-oligomers; thus, picrodendrin-Q, the most potent antagonist is shown top left, while isohyenanchin, which was the least potent of the compounds tested is shown bottom right.

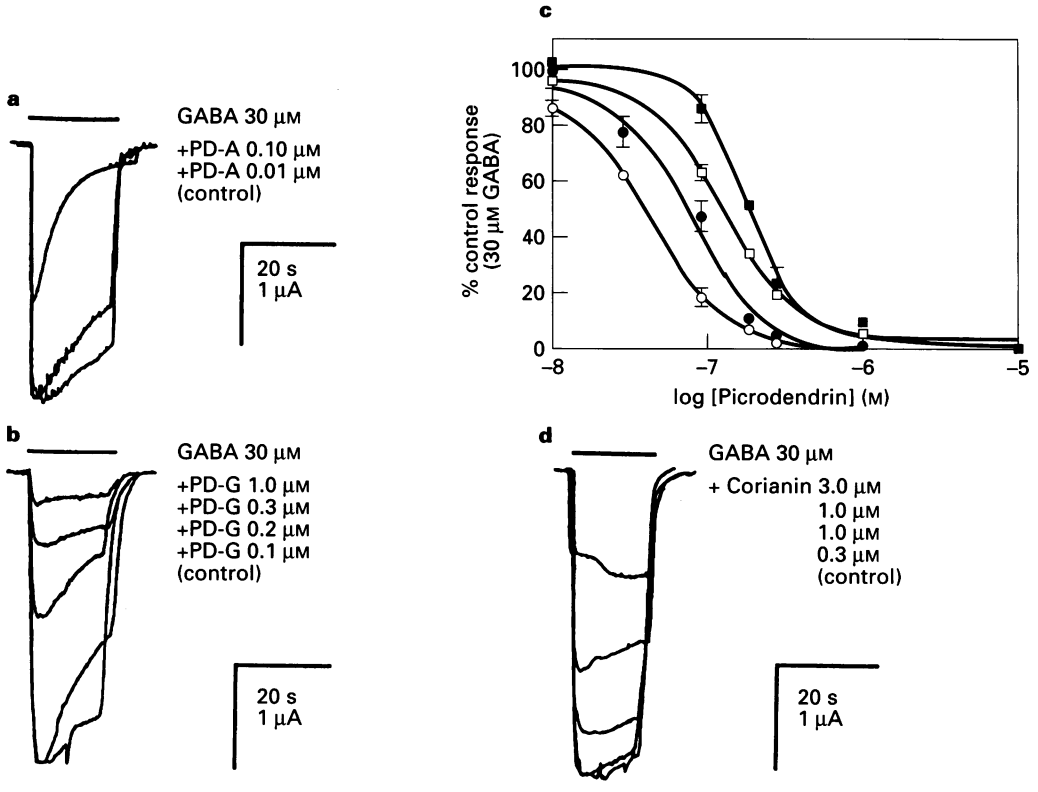

Figure 2 Terpenoid blockade of the GABA response of RDL_{ac} compounds effected a biphasic blockade of the response of RDL_{ac} homo-oligomers to 30 μM GABA, reducing the amplitude of the peak response and accelerating the decay of GABA-induced currents. Examples of biphasic block effected by a range of concentrations of (a) picrodendrin-A and (b) picrodendrin-G are shown. (c) Dose-inhibition curves for picrodendrins-A and -G were determined at the peak (●PD-A, ■PD-G), and 15 s (○PD-A, □PD-G) after the onset of the response to 30 μM GABA. The use-dependency of these compounds resulted in an increase in their apparent potency during an application of GABA. Thus, the estimated IC_{50}s of picrodendrin-A were respectively 83 nM and 46 nM at the peak and 15 s after the onset of the GABA response. Similarly, the IC_{50} for picrodendrin-G decreased from 193 nM at the peak of the response to 140 nM after a 15 s application of GABA. Each point plotted is the mean of 3–5 observations and is shown ± s.e.mean. (d) By contrast, corianin and the tutin analogues did not increase the rate of decay of the GABA responses of RDL_{ac} homo-oligomers.

substituents. The least potent picrodendrin (picrodendrin-O) was approximately equipotent with tutin and corianin.

The descending order of terpenoid potency on RDL_{ac} homo-oligomers is shown in Table 2.

Effects of A302S substitution

The substitution of serine for alanine at residue 302 has previously been shown to reduce the potency of a number of convulsant and insecticidal compounds, including picrotoxinin, dieldrin and fipronil (ffrench-Constant *et al.*, 1993a; Hosie *et al.*, 1995a,b). Homo-oligomers composed of the dieldrin-resistant form of RDL_{ac} (RDL_{ac}^{A302S}) were markedly less sensitive to picrodendrin-O than those containing the wild-type (RDL_{ac}) (Figure 5).

Discussion

The tutin and picrodendrin terpenoids tested in this study share a polycyclic lactone structure similar to that of picrotoxinin: i.e. a picrotoxane skeleton. Analogues of picrotoxinin, which differ in the nature of their substituents at various carbon atoms on the common picrotoxane skeleton, have been the subject of a number of electrophysiological and radioligand binding studies which yield insights into the structural requirements for potent convulsant antagonism (Jarboe *et al.*, 1968; Miller *et al.*, 1979; Klunk *et al.*, 1983; Kudo *et al.*, 1984; Anthony *et al.*, 1993; 1994). Such data led Ozoe & Matsumura

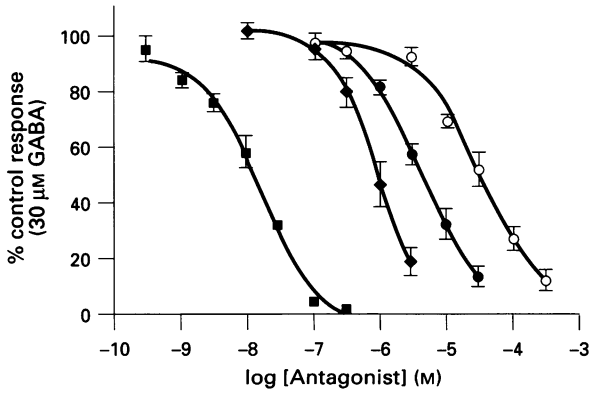

Figure 3 Dose-inhibition curves of tutin analogues on wild-type RDL_{ac} homo-oligomers. Tutin(◆) was the most potent antagonist in this series. Dihydrotutin(●), which differs from tutin only in the presence of a saturated C4 isopropyl substituent (as opposed to an isopropenyl group) was less potent whereas the presence of a hydroxyisopropyl group at C4 further reduced the potency of tutin analogues, as demonstrated by isohyenanchin(○). The dose-inhibition curve of picrodendrin-Q is shown for comparison(■). Oocytes were incubated for 2 min in the antagonist prior to co-application of GABA and the antagonist. Data were normalized to the response elicited from each oocyte 15 s into a 20 s application of 30 μM GABA alone. Each point is the mean of 3–5 observations and is shown ± s.e.mean.

(1986) to suggest that a picrotoxane-based molecule's convulsant activity is dependent on the presence of at least two of the following structures: a bulky lipophilic group and two or more electronegative centres. In picrotoxinin the lipophilic moiety is provided by the isopropenyl group located at C4, while the electronegative centres are formed by the lactone group that bridges C3 - C5, the epoxide group between C8 and C9, and carbonyl group at C11, which is part of a γ-butyrolactone moiety. The bridgehead hydroxyl group at C6 may also act as an electronegative centre (Jarboe *et al.*, 1968; Kudo *et al.*, 1984; Anthony *et al.*, 1993; 1994). Higher activity has been observed in picrotoxinin analogues where the lipophilic C4 moiety is *trans* to the lactone (Miller *et al.*, 1979; Ozoe & Matsumura, 1986; Ozoe *et al.*, 1993), as it is in picrotoxinin and the terpenoids. A comparison of the structures illustrated in Figure 1, demonstrates the presence of all these features in the picrodendrin and tutin terpenoids studied here, although the positions of the electronegative centres of the terpenoids tested here differ slightly from those of picrotoxinin.

Influence of the C4 substituents on picrotoxane antagonist potency

Tutin and its analogues (dihydrotutin and isohyenanchin) differ only in the nature of their C4 substituent. Of these compounds, tutin was the most potent antagonist of RDL_{ac} homo-oligomers. Substitution of the isopropenyl group of tutin with an isopropyl group yielded dihydrotutin and a 5 fold reduction in potency on RDL_{ac} homo-oligomers. This substitution, represents a slight increase in the hydrophobicity of the C4 substituent, but reduces its electronegativity and abolishes the planarity of the C4 group found in the unsaturated isopropenyl group of tutin. Isohyenanchin, which was the least potent tutin analogue, bears a hydroxyisopropyl group at C4, which is markedly less hydrophobic than an isopropenyl group and also non-planar. The structure-activity relationship of tutin analogues confirms the results of a previous study in which we demonstrated that decreases in the planarity and hydrophobicity of the C4 substituents of picrotoxinin analogues had similar effects on their potency as antagonists of RDL_{ac} homo-oligomers (Shirai *et al.*, 1995). Like tutin, picrotoxinin possesses an isopropenyl group at C4. Reductions in C4-substituent hydrophobicity and planarity were represented by picrotin which like isohyenanchin contains a hydroxyisopropyl group, and α-picrotoxinone (C4 acetyl group). Both these compounds are markedly less potent antagonists of RDL_{ac} homo-oligomers than picrotoxinin.

The structure-activity relationship of the C4 substituent of picrotoxane-based antagonists observed for RDL_{ac} homo-oligomers appears to be preserved on native GABA receptors of insects and vertebrates. Thus, the relatively greater potency of tutin over dihydrotutin observed in the present study (i.e. 5 fold), was very similar to that observed in binding studies on rat $GABA_A$ receptors (4 fold; Ozoe *et al.*, 1994). The decrease in C4-substituent planarity effected by replacing an isopropenyl group with an isopropyl group had similar effects on the potency of dihydropicrotoxinin (isopropyl group) relative to picrotoxinin on a variety of vertebrate and insect preparations (Jarboe *et al.*, 1968; Miller *et al.*, 1979; Olsen *et al.*, 1989; Deng *et al.*, 1991; Anthony *et al.*, 1993; 1994). Decreases in C4-substituent hydrophobicity, which in the present study was represented by isohyenanchin, had similar effects on the potency of picrotoxinin analogues. Picrotin (which bears a C4 hydroxyisopropyl group) and α-picrotoxinone (C4 acetyl group) are weaker antagonists of RDL_{ac} homo-oligomers (Shirai *et al.*, 1995) and of native vertebrate and insect GABA receptors than is picrotoxinin (Jarboe *et al.*, 1968; Miller *et al.*, 1979; Kudo *et al.*, 1984; Olsen *et al.*, 1989; Deng *et al.*, 1991;

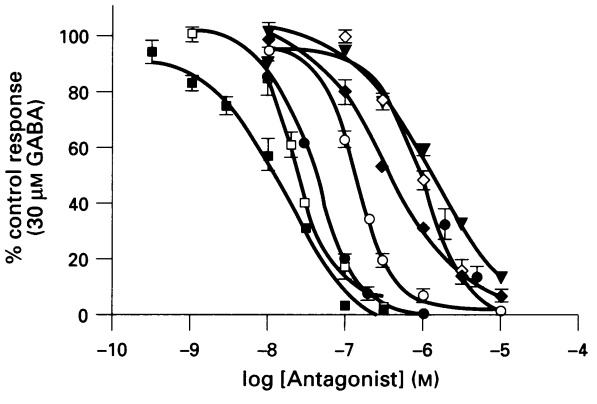

Figure 4 Dose-inhibition curves of picrodendrin antagonists for wild-type RDL_{ac} homo-oligomers. Oocytes were incubated for 2 min in the antagonists prior to co-application of GABA and the antagonist. The responses 15 s after the co-application of GABA and the picrodendrins were normalised to the response elicited from each oocyte 15 s after the application of 30 μM GABA alone. Each point is the mean of observations from 3–5 oocytes and is shown ± s.e.mean. Picrodendrin-Q(■) was the most potent antagonist of RDL_{ac} homo-oligomers, whereas picrodendrin-O(◇) and corianin(▼) were the least potent of these compounds. Data for other picrodendrins are denoted as follows: picrodendrin-A (●), -B (□), -F (◆), -G (○).

Table 1 Terpenoid IC_{50}s estimated from data obtained 15s after the onset of the response to 30 μM GABA

Compound	*IC_{50} (nM)*	n	*Compound*	*IC_{50} (nM)*	n
PD-A	44 (39–55)	3	PD-Q	17 (8–39)	5
PD-B	23 (15–35)	4–5	Corianin	1445 (295–7082)	4–6
PD-F	317 (177–573)	3–5	Tutin	880 (716–1080)	4–6
PD-G	140 (115–170)	4	Dihydrotut.	4468 (4372–4567)	4–6
PD-O	1006 (338–2986)	5	Isohyen.	31890 (12940–78570)	4–5

Data are presented as the mean, with 95% CI, of *n* observations. PD, DiH-Tutin and IH refer to picrodendrin, dihydrotutin and isohyenanchin respectively.

Table 2 Order of potency of terpenoids on RDL_{ac} homo-oligomers and rat $GABA_A$ receptors

Receptor	*Order of potency of terpenoids*
(RDL_{ac})	Q> B> A> G> F> Tutin> O> Corianin> DiH-tutin> IH
($GABA_A$)	Q> A> Tutin> DiH-tutin> O> B> Corianin> F=G=IH

The terpenoids are ordered by their IC_{50}s for antagonism of the response of RDL_{ac} homo-oligomers to 30 μM GABA (upper row), and inhibition of [^{35}S]-TBPS binding to rat $GABA_A$ receptors as determined by Ozoe *et al.* (1994) (lower row). Letters A, B, F, G, O, Q refer to the various picrodendrins. DiH-tutin and IH refer to dihydrotutin and isohyenanchin respectively.

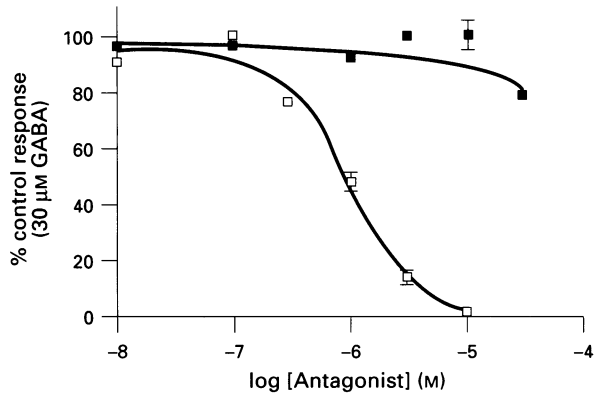

Figure 5 Effect of the A302S substitution on the potency of picrodendrin-O. Dose-inhibition curves are shown for antagonism by picrodendrin-O of wild-type(□) and dieldrin-resistant(■) RDL_{ac} homo-oligomers. Oocytes were pre-incubated for 2 min in the presence of the antagonist prior to co-application of GABA and the antagonist. The responses 15 s after the co-application of GABA and the picrodendrins were normalized to the response elicited from each oocyte 15 s after the application of 30 μM GABA alone. Each point is the mean of observations from 3 oocytes and is shown ± s.e.mean. The A302S substitution greatly reduced the potency of picrodendrin-O.

Anthony *et al.*, 1993; 1994; Ozoe *et al.*, 1994). Thus, the potency of picrotoxane-based antagonists of RDL_{ac} homo-oligomers and native vertebrate and insect GABA receptors is increased by a planar, hydrophobic alkyl group on C4.

Influence of electronegative substituents on terpenoid potency

Previous studies have demonstrated that the IC_{50} of picrotoxinin block of the response to EC_{50} GABA in homo-oligomers composed of RDL_{ac}, or its splice variant DRC 17-1-2, is approximately 50 nM (ffrench-Constant *et al.*, 1993; Chen *et al.*, 1994). Thus, only picrodendrins Q, B and A (IC_{50}s 17 nM, 23 nM and 44 nM respectively) were more potent or equipotent with picrotoxinin. All the picrodendrins tested in this study have an isopropyl group at C4 instead of the isopropenyl group found in picrotoxinin which would be expected to reduce their potency slightly, as discussed above. However, there are also a number of marked differences in the location and structure of the electronegative centres of picrotoxinin and the picrodendrin and tutin terpenoids which presumably contribute to the differential potency of these compounds (cf. Figure 1). In picrotoxinin, an epoxy group bridges C8–C9, whereas the epoxy groups of the compounds tested here bridge C7–C8. A carbonyl group found at C11 of picrotoxinin, is absent in all the terpenoids tested here. The low potency of corianin (IC_{50}: 1.44 μM), which is approximately 30 fold less potent than picrotoxinin, demonstrates that the positions of these electronegative substituents are important determinants of the potency of picrotoxane-based compounds as antagonists of RDL_{ac} homo-oligomers. Corianin differs from picrotoxinin only in the absence of the carbonyl group at C11, the position of its epoxy group (C7-8 in corianin), and the presence of a hydroxyl group at C9.

Picrodendrin compounds differ from each other mainly in the structures of their C9 substituents. With the exception of picrodendrin-G, where the C9 substituent is a spiro tetrahydrofuran ring, all the picrodendrins tested here possess a spiro-γ-butyrolactone ring attached to C9. The substituents on this ring vary, but are all electronegative. These substituents strongly affect the potency of picrodendrins on $GABA_A$ receptors (Ozoe *et al.*, 1994) and RDL_{ac} homo-oligomers. However, the considerable variation in the structure of these groups prevents a detailed understanding of the structural requirements of high-potency antagonists of RDL_{ac} homo-oligomers. Yet some features common to the most potent terpenoid antagonists of RDL_{ac} homo-oligomers (i.e., picrodendrins Q, B, A and G) can be identified. By virtue of olefinic bonding in these rings, the side chains in picrodendrins A, B and Q will be nearer the plane of the spiro-γ-butyrolactone rings than in the less potent picrodendrins. The only differences between picrodendrins F and O, which must therefore account for the three fold difference in their IC_{50}s, is hydroxyl substitutions at C4 and the α position of the C9 lactone of the former.

There is evidence from ligand binding studies of differences in the picrotoxinin binding sites of insect and vertebrate ionotropic GABA receptors (Sattelle *et al.*, 1991; Cole & Casida, 1992). Of all the terpenoids, picrodendrin-Q had the highest potency on both RDL_{ac} homo-oligomers and rat $GABA_A$ receptors (Ozoe *et al.*, 1994), with a similar IC_{50} on each preparation (17 nM on RDL_{ac} homo-oligomers; 7.5 nM on rat $GABA_A$ receptors). Yet, with the exception of picrodendrin-Q, all the terpenoids were less potent inhibitors of [^{35}S]-TBPS binding to rat membranes than they were antagonists of the GABA responses of RDL_{ac} homo-oligomers. For example, picrodendrin-A, which was one of the most potent ligands on either preparation, was 2 fold less potent than picrodendrin-Q on RDL_{ac} homo-oligomers, and 24 fold less potent than Q on rat $GABA_A$ receptors. Similarly, picrodendrin-O was 59 fold less potent than picrodendrin-Q on RDL_{ac} homo-oligomers, but 720 fold less potent on rat membranes. Furthermore, there was a re-arrangement in the order of terpenoid potency on RDL_{ac} compared to that seen on $GABA_A$ receptors (Table 2). Thus, picrodendrins F and G which were weak antagonists of radioligand binding to $GABA_A$ receptors, and almost equipotent with isohyenanchin (Ozoe *et al.*, 1994), had much higher activities on RDL_{ac}, being more potent than picrodendrin-O and just 8–18 fold less potent than picrodendrin-Q. By contrast, tutin and dihydrotutin, which were amongst the most potent displacers of [^{35}S]-TBPS from $GABA_A$ receptors and more potent than picrodendrin-O, were, with isohyenanchin, the weakest antagonists of RDL_{ac} homo-oligomers. As picrodendrins differ mainly in their C9 substituents, it appears that this electronegative centre differentiates the optimal picrotoxane antagonist pharmacophore of RDL_{ac} homo-oligomers and rat $GABA_A$ receptors.

Biphasic block of RDL_{ac} homo-oligomers

A use-dependent, biphasic blockade of RDL_{ac} homo-oligomers was effected by the picrodendrins, but not by either corianin or the tutin analogues. This involved a decrease in the amplitude of the initial GABA response, followed by a further decrease to a plateau. It is interesting that there is a structural correlate to use-dependent block of RDL_{ac} homo-oligomers, as it raises the possibility that further investigation of the structure-function relationships of terpenoids may reveal the structural basis of use-dependent blockade. Similar biphasic blockade of vertebrate $GABA_A$ receptors has been observed with picrotoxin (Newland & Cull-Candy, 1992; Yoon *et al.*, 1993; Dillon *et al.*, 1995a), TBPS (van Renterghem *et al.*, 1987; Dillon *et al.*, 1995a), lindane (Nagata & Narahashi 1995) and U93631 (Dillon *et al.*, 1995b). A striking feature of the biphasic picrotoxin block of certain $GABA_A$ receptors is that the second phase of blockade, the relaxation to plateau, can be selectively prevented by αIMGBL (α-isopropyl-α-methyl-γ-butyrolactone; Yoon *et al.*, 1993), while initial depression of the GABA response effected by picrotoxin is unaffected by αIMGBL. When applied alone, αIMGBL does not have any effect on GABA-induced currents and thus appears to antagonize the effects of picrotoxinin (Holland *et al.*, 1990). These data suggest that such antagonists utilise two mechanisms to block $GABA_A$ receptor-mediated currents, one of which is antagonized by αIMGBL. It will be of interest to see if the biphasic block of RDL_{ac} homo-oligomers effected by picrodendrins is also susceptible to pharmacological manipulation.

Effects of the A302S substitution

In the present study, the A302S substitution, which engenders resistance to dieldrin and picrotoxinin (ffrench-Constant *et al.*, 1993a) decreased the potency of picrodendrin-O as an antagonist of RDL_{ac} homo-oligomers. This result was not entirely surprising given the similarities between the structures of picrodendrin-O and picrotoxinin. The substitution of the residue A302 in homo-oligomers composed to RDL_{ac} and the splice variant of the *Rdl* gene, DRC 17-1-2, has been observed to reduce the potency of all convulsant antagonists tested to date. Such compounds include TBPS, heptachlor-epoxide and lindane (Belelli *et al.*, 1995) and the novel convulsants, fipronil (Hosle *et al.*, 1995a) and BIDN (Hosie *et al.*, 1995b). Similarly, the potency of GABA receptor antagonists on neurones cultured from *Drosophila* homozygous for the Rdl^{A302S} allele is reduced relative to wild-type (Zhang *et al.*, 1994). These authors have suggested that residue 302 engenders resistance by a novel, dual mechanism, altering the structure of the convulsant binding site(s) (cf. Cole *et al.*, 1995; Lee *et al.*, 1995), and by reducing the rate of receptor desensitization, thus decreasing the probability of convulsants stabilizing the receptor in an agonist-bound closed state.

The present study demonstrates that picrodendrins antagonized a model insect GABA receptor, and strongly suggests that these compounds underlie the insecticidal activity of *P. baccatum*. The structure-activity relationship of tutin analogues illustrates that alterations in the C4 substituents of a picrotoxane skeleton have the same effects on RDL_{ac} homo-oligomers as they do on native insect and vertebrate GABA receptors. However, the different orders of picrodendrin potency observed on RDL_{ac} homo-oligomers and rat $GABA_A$ receptors suggest the convulsant antagonist pharmacophore of RDL_{ac} homo-oligomers differs from that of vertebrates insofar as the optimal structure of C9 substituents of the picrotoxane skeleton is concerned. There is evidence from radioligand binding studies that the convulsant binding sites of insect and vertebrate ionotropic GABA receptors may be structurally distinct (Sattelle *et al.*, 1991; Cole & Casida, 1992; Ozoe, 1995). Thus the RDL_{ac} homo-oligomer may prove to be a useful model for investigating the structural basis of these differences, aided in part by the differential potency of picrodendrins and tutin analogues observed on vertebrate and invertebrate preparations.

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