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BIOCHEMICAL ACTIVITIES OF BERBERINE, PALMATINE AND SANGUINARINE MEDIATING CHEMICAL DEFENCE AGAINST MICROORGANISMS AND HERBIVORES

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Key Word Index—chemical defence; radio receptor assay; neuroreceptors; cholinergic; serotoninergic; adrenergic receptors; DNA intercalation; DNA synthesis; reverse transcriptase; *in vitro* translation; membrane permeability.

Abstract—The alkaloids berberine, palmatine and sanguinarine are toxic to insects and vertebrates and inhibit the multiplication of bacteria, fungi and viruses. Biochemical properties which may contribute to these allelochemical activities were analysed. Acetylcholine esterase, butyrylcholinesterase, choline acetyl transferase, alpha$_1$- and alpha$_2$-adrenergic, nicotinergic, muscarinergic and serotonin$_2$ receptors were substantially affected. Sanguinarine appears to be the most effective inhibitor of choline acetyl-transferase (IC_{50} 284 nM), while the protoberberines were inactive at this target. Berberine and palmatine were most active at the α_2-receptor (binding with IC_{50} 476 and 956 nM, respectively). Furthermore, berberine and sanguinarine intercalate DNA, inhibit DNA synthesis and reverse transcriptase. In addition, sanguinarine (but not berberine) affects membrane permeability and berberine protein biosynthesis. In consequence, these biochemical activities may mediate chemical defence against microorganisms, viruses and herbivores in the plants producing these alkaloids. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

The benzophenanthridine alkaloid sanguinarine and the protoberberine alkaloids berberine and palmatine occur in several genera of the families Papaveraceae (e.g. *Argemone*, *Bocconia*, *Chelidonium*, *Dicranostigma*, *Eschscholtzia*, *Glaucium*, *Hunnemannia*, *Hylomecon*, *Hypecoum*, *Macleaya*, *Meconopsis*, *Papaver*, *Platystemon*, *Romneya*, *Sanguinaria*, *Stylomecon* and *Stylophorum*), Berberidaceae (*Berberis* and *Mahonia*), Fumariaceae (*Corydalis*, *Dicentra* and *Fumaria*) and less abundantly in Menispermaceae (*Jateorhiza*), Ranunculaceae (*Thalictrum*), Rutaceae (*Zanthoxylum*), Sapindaceae (*Pteridophyllum*) and other taxa. In some species, sanguinarine and derivatives are formed after elicitation with microbial cell-wall fractions, as in *Papaver somniferum* and *Eschscholtzia californica* [1, 2].

These alkaloids interfere with the growth of *Lepidium* and *Lactuca* seedlings [3], are cytotoxic [4], insect deterrent and insecticidal [5, 6], and toxic to vertebrates (LD_{50} in mice is 23 mg kg^{-1} [i.p.] for berberine, and 16 mg kg^{-1} [i.v.] for sanguinarine [7–9]). Furthermore, these alkaloids inhibit the multiplication of trypanosomes [10], phages [11], fungi [12, 13], different *Vibrio* species [14] and other bacteria [15–18]; for further review, see refs [9, 19]. In addition, they exhibit a wide range of pharmacological effects, including anti-inflammation, respiratory stimulation, transient hypotension, convulsion, uterine contraction, antiarrhythmic, inotropic, adrenocorticotropic and analgesic activities [9, 10, 20–22]. Since the plants that produce these alkaloids are usually well protected against herbivores and microorganisms and because of the allelochemical activities of these alkaloids, it appears likely that they serve as chemical defence compounds. Due to their quaternary nitrogen, polycyclic and planar structure, sanguinarine, berberine and palmatine can react with nucleophilic and anionic groups of amino acids in several biomolecules, receptors and enzymes; e.g. these alkaloids bind to microtubules [23], inhibit several enzymes [24–30], including Na^+,K^+-ATPase [31–33], uncouple oxidative phosphorylation [34] and intercalate in GC-rich regions of DNA [35, 36].

In this study we have analysed a wider set of molecular and cellular targets, including neuroreceptors, acetylcholine-related enzymes, DNA, RNA and related enzymes, protein biosynthesis and membrane permeability in order to understand the mechanisms which could mediate chemical defence against a heterogeneous group of organisms, ranging from viruses and microorganisms to insects and vertebrates.

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Table 1. Binding of the studied alkaloids to neuroreceptors (displacement of the specifically bound radioligand) and inhibition of enzymes involved in acetylcholine metabolism (IC_{50} in μM). Adrenergic = $alpha_1$ and $alpha_2$, serotoninergic = $5\text{-}HT_2$, nicotinic = nAChR, muscarinic = mAChR, acetylcholine esterase = AChE, butyrylcholine esterase = BChE, choline acetyl transferase = ChAT

Alkaloid	$Alpha_1$	$Alpha_2$	$5\text{-}HT_2$	mAChR	nAChR	AChE	BChE	ChAT
Berberine	3.2	0.476	1.9	1.0	35.5	167.4	55.8	n.a.†
Palmatine*	5.8	0.956	2.9	4.1	n.a.	124.5	425.6	n.a.
Sanguinarine	33.6	6.4	91.7	2.4	11.8	10.9	17.4	0.284

*The maximum concentration of palmatine that could be tested on ChAT was 100 μM. Alkaloids were dissolved in ethanol in esterase assays.

†n.a. = not active, i.e. $IC_{50} > 500$ μM.

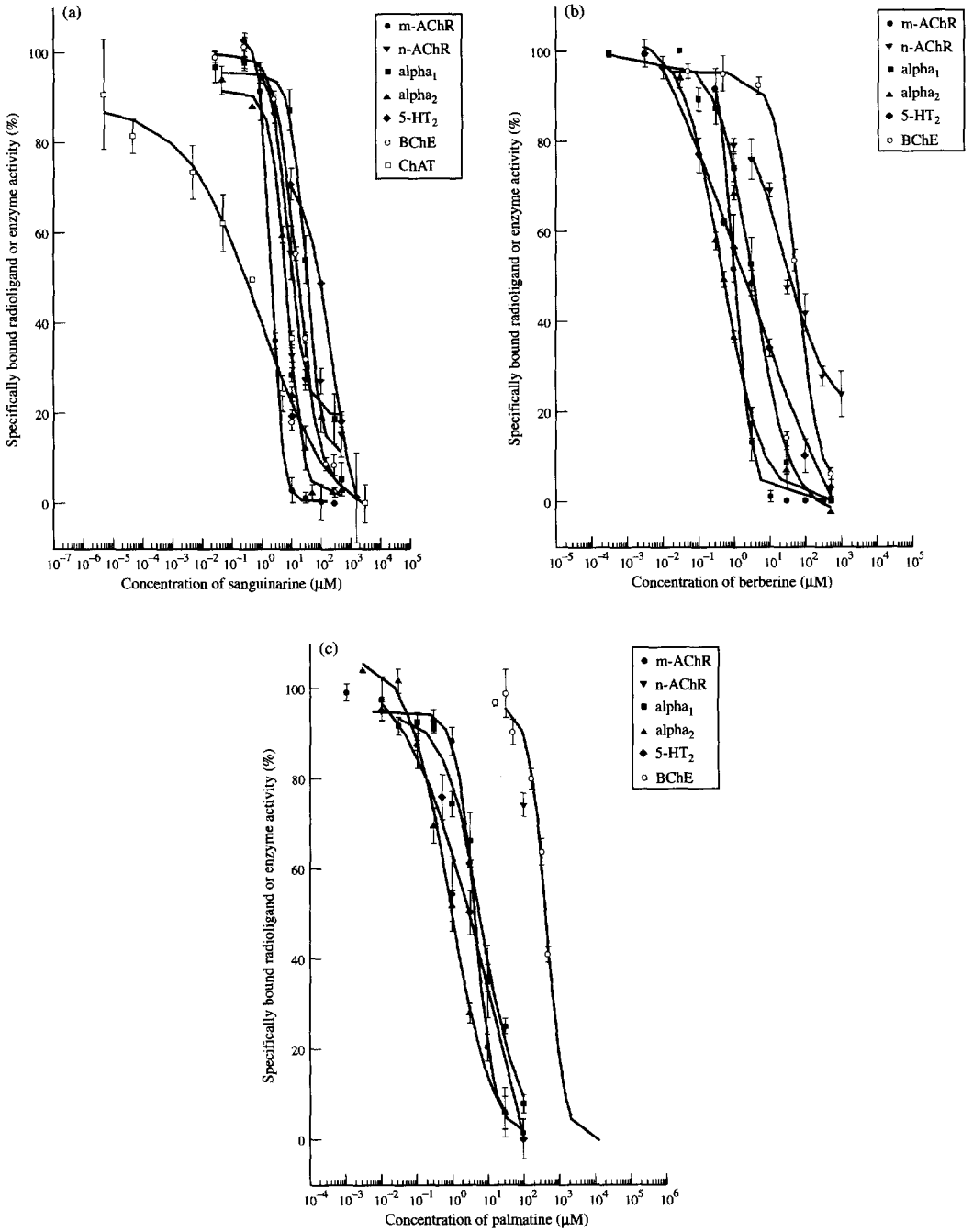


Fig. 1. Binding of (A) sanguinarine, (B) berberine and (C) palmatine to neuroreceptors and inhibition of acetylcholine-related enzymes. $Alpha_1$ and $alpha_2$ = adrenergic, $5\text{-}HT_2$ = serotoninergic, nAChR = nicotinic, mAChR = muscarinic, AChE = acetylcholine esterase, BChE = butyrylcholine esterase, ChAT = choline acetyl transferase; values are means±s.d. ($n = 3$).

RESULTS AND DISCUSSION

Animal-related targets

Signalling in nerve cells and at synapses is an important molecular target restricted to animals. Any agonistic or antagonistic activity of a natural product at this target will usually affect the physiology of the corresponding animal substantially.

Binding to neuroreceptors

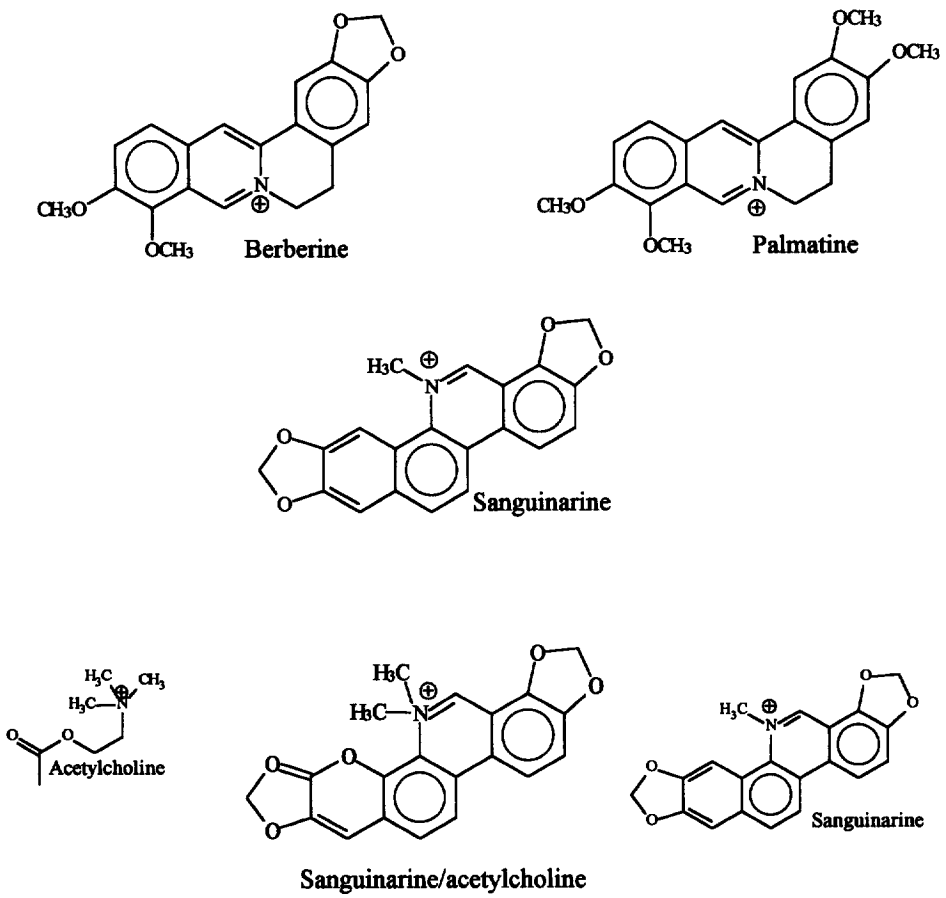
Radio receptor assays were established for alpha$_1$- and alpha$_2$-adrenergic, nicotinergic (nAChR), muscarinergic (mAChR) and serotonin$_2$ (5-HT$_2$) receptors [37, 38], which are important for neuronal signal transduction in insects and vertebrates. Sanguinarine, berberine and palmatine clearly bind to all the receptors, with IC_{50} values ranging from 0.47 to 91.7 μM (Table 1; Fig. 1). The affinity of berberine and palmatine was *ca* 5–45 times higher than that of sanguinarine at adrenergic and serotonin receptors (Table 1). In the case of the cholinergic receptors the affinity was nearly equal for all three alkaloids.

It is not possible to decide from these experiments whether the binding of alkaloids to these neuroreceptors produces an agonistic or antagonistic effect *in vivo*. However, pharmacological studies [7, 20, 39, 40] imply that berberine, palmatine and sanguinarine are antagonistic at the α-receptors and agonistic at the cholinergic receptors. The pharmacology at the serotonin receptor still needs to be established.

Inhibition of acetylcholine esterase and choline acetyltransferase

Acetylcholine esterase (AChE) is important for the breakdown of acetylcholine when it is released from its receptor, whereas choline acetyl transferase (ChAT) is responsible for the biosynthesis of acetylcholine. Besides nAChR and mAChR, these enzymes are important targets in animals. Sanguinarine was the most effective of the alkaloids studied, inhibiting all three enzymes; especially the ChAT with an IC_{50} value of 284 nM. Both protoberberine alkaloids were significantly less active or even inactive (Table 1, Fig. 1(B) and (C)).

The structural similarity of sanguinarine and acetylcholine (Scheme 1) appears to explain the esterase inhibition (Fig. 1). This explanation does not agree with



Scheme 1. Structures of sanguinarine, berberine and palmatine as compared to acetylcholine. In the bottom line structures of sanguinarine (right) and acetylcholine (left) are compared; the central structure is simply an overlay of the sanguinarine and acetylcholine structures to visualize structural similarities.

the kinetic studies [26, 27, 41], indicating that the alkaloid binding site differed from the active site of AChE. The different effects seen at the protein level (enzymes or receptors) might be due to interactions of these alkaloids with sulphhydryl groups [42, 43] or aromatic amino acids [44] in the ligand or substrate binding sites.

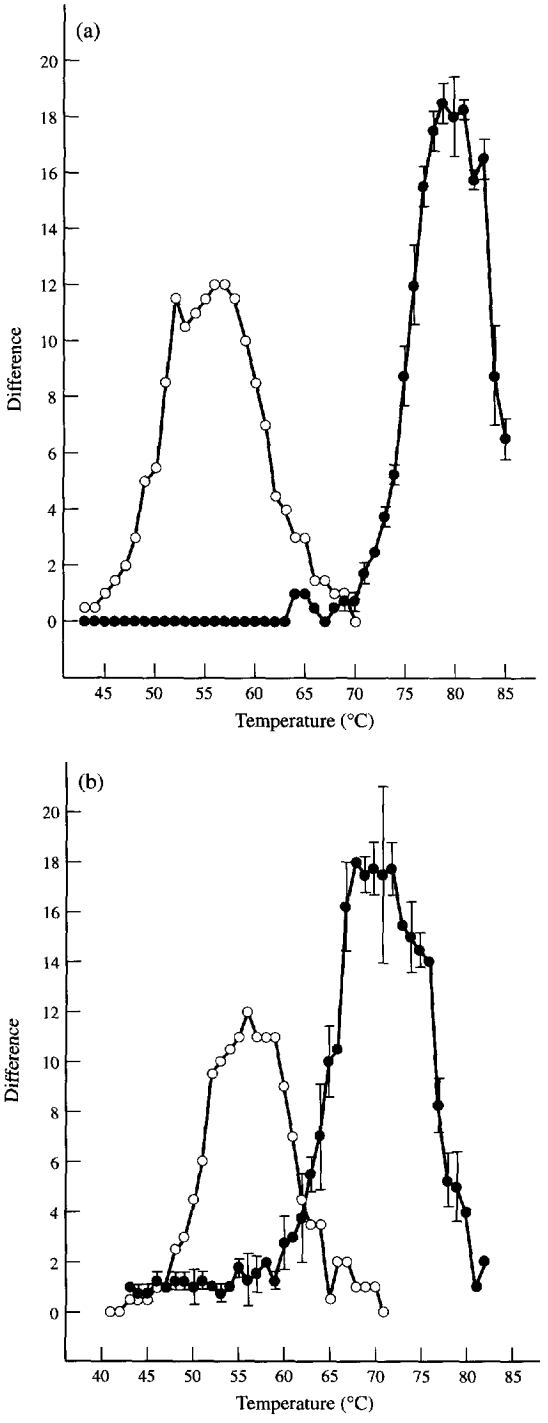


Fig. 2. Influence of (a) sanguinarine and (b) berberine on DNA melting temperature. Open circles refer to pure DNA and filled circles to DNA plus alkaloid. Alkaloids which intercalate lead to stabilization of DNA and thus to an increase of DNA melting temperature.

Targets common to animals, microorganisms and viruses

To determine any interaction at basic molecular targets common to all cells (and viruses) we employed assays for DNA intercalation, inhibition of DNA synthesis, reverse transcriptase, protein biosynthesis and membrane stability.

Interaction with DNA, RNA and related enzymes

We have established a number of assays to measure the potential interaction of natural products with DNA. Determination of the melting temperature (*T*m) of DNA gave most reproducible results [3]. Sanguinarine and berberine increased the melting temperature of *Sinapis* DNA by 25 or 15°, respectively (Fig. 2). A strong association between DNA and these alkaloids was also evident from assays with methyl green (Fig. 3; note that sanguinarine again provoked a stronger effect than berberine) or when DNA–alkaloid complexes were separated from unbound alkaloids by gel filtration or agarose gel electrophoresis (Latz-Brüning and Wink, unpublished). These results are in agreement with those of earlier studies [35, 36] which showed that sanguinarine intercalates GC-rich DNA regions. The structurally related alkaloids canadine and chelidonine also

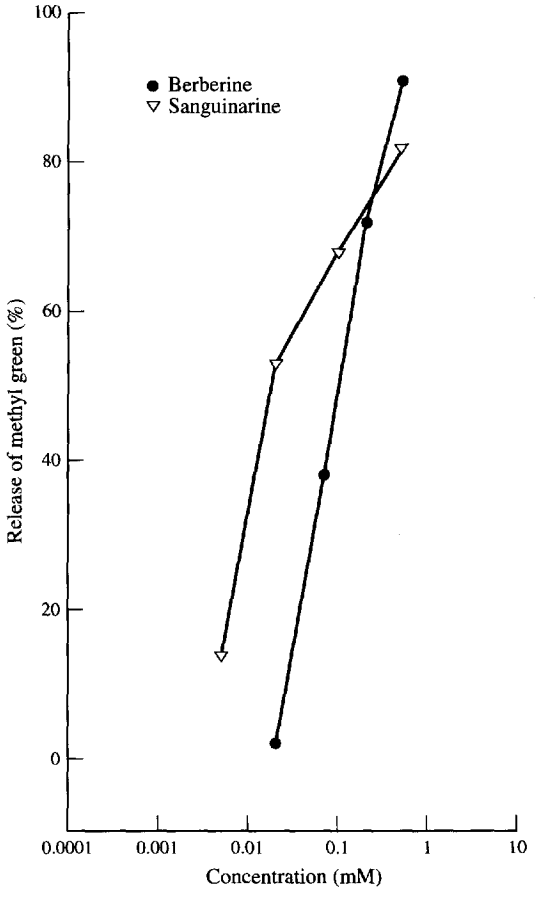
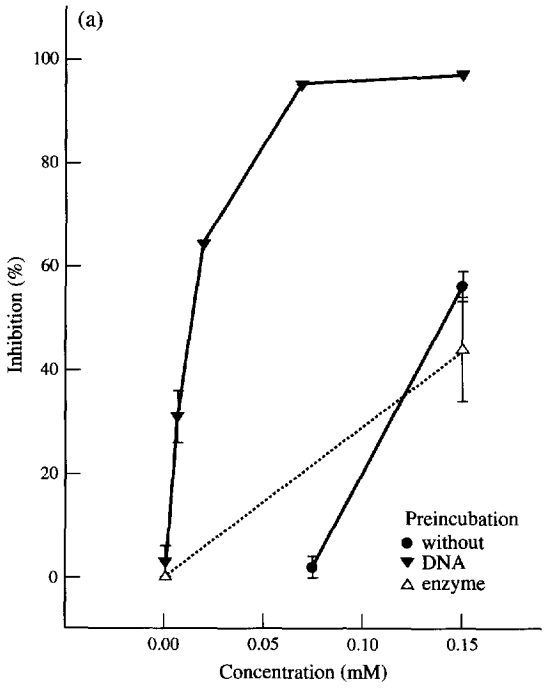


Fig. 3. Release of methylgreen from DNA by intercalating alkaloids.



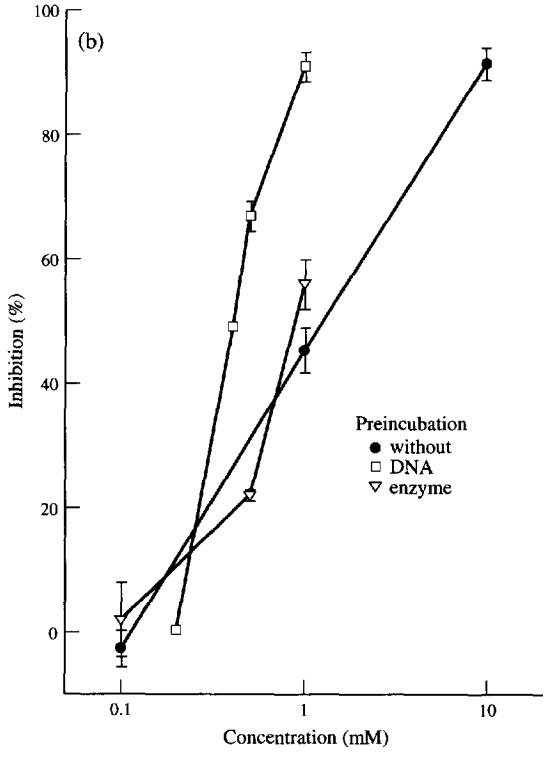
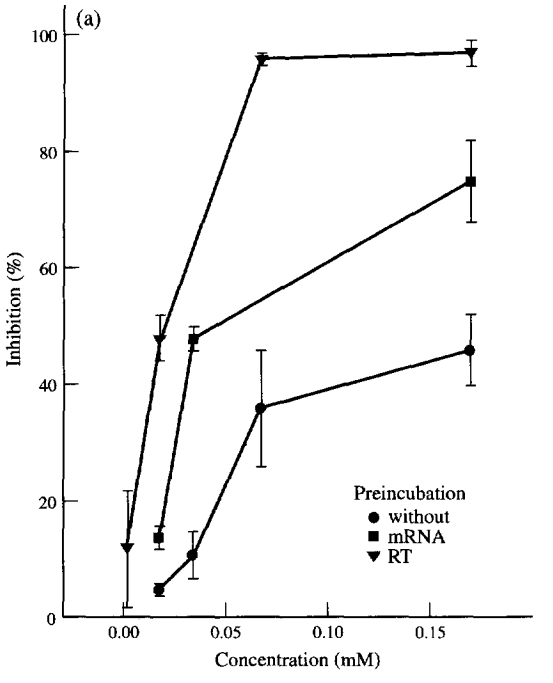


Fig. 4. Inhibition of the DNA synthesis ('nick translation' with DNA polymerase I) by (a) sanguinarine and (b) berberine. Assays were performed with three modifications: (1) alkaloids were preincubated with DNA before adding the enzyme (▼, □); (2) alkaloids were preincubated with the polymerase before adding the DNA (△, ▽); (3) alkaloids were added to the assay mixture containing DNA and polymerase so that the reaction started immediately (●).



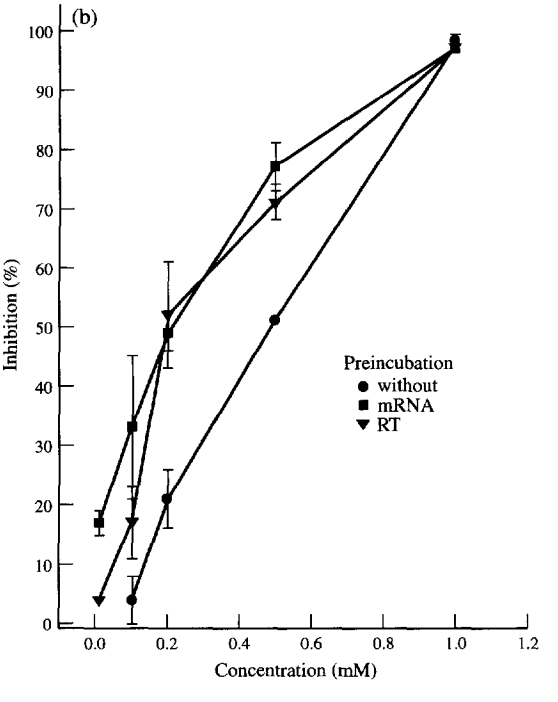


Fig. 5. Inhibition of reverse transcription by (a) sanguinarine and (b) berberine. Assays were performed with three modifications: (1) alkaloids were preincubated with mRNA before adding the enzyme (■); (2) alkaloids were preincubated with reverse transcriptase (RT) before adding the mRNA (▼); (3) alkaloids were added to the assay mixture containing mRNA and RT so that the reaction started immediately (●).

interacted with DNA: the increase of T_m was quite low, i.e. 1.8 or 1.6°, respectively, and methylgreen release was 21% at 0.7 or 1.3 mM (Latz-Brüning and Wink, unpublished).

DNA intercalation might be responsible for the mutagenic and even carcinogenic effects of sanguinarine [43]. This alkaloid is used as an antiplaque agent in mouthwash and toothpaste preparations, but the FDA has classified the drug Sanguinaria, which is rich in sanguinarine, as unsafe for use in foods, beverages and drugs [21].

Compounds which strongly intercalate DNA are potential inhibitors of DNA synthesis (repair and replication). As an experimental system for DNA synthesis we employed a modified 'nick translation' assay using DNA polymerase I (Fig. 4). Inhibition was most pronounced when sanguinarine or berberine were preincubated with DNA before adding DNA polymerase, indicating that the DNA–alkaloid complex was responsible for inhibition and not an alkaloid–protein interaction. The chemically related alkaloids canadine and chelidonine inhibited this reaction only weakly, i.e. 12% at 0.5 mM (Latz-Brüning and Wink, unpublished).

We used a reverse transcription assay with poly A^+ mRNA to determine the effects of these alkaloids at the RNA level (Fig. 5). Preincubation experiments indicate that sanguinarine and berberine appear to interact strongly with both the mRNA and the enzyme; thus confirming earlier results from other laboratories [29, 45]. Canadine was inactive at 0.5 mM, whereas chelidonine inhibited RV by 24% at 0.67 mM (Latz-Brüning and Wink, unpublished).

Inhibition of protein biosynthesis

As an experimental system we employed a reticulocyte lysate to which mRNA was added (Fig. 6). Berberine inhibited *in vitro* translation completely at 1 mM concentration, whereas sanguinarine was inactive at 0.014 mM (because of solubility problems, higher concentrations were not tested). However, sanguinarine had been found to inhibit protein biosynthesis and Phe–tRNA binding at 1.3 mM in a previous study [46]. Chelidonine inhibited protein biosynthesis by 20% at 0.14 mM (Latz-Brüning and Wink, unpublished).

Induction of membrane leakage

We have chosen erythrocytes as an assay system to measure haemolysis and the efflux of 3-*O*-methyl-D-[1 − ^{3}H]-glucose (Fig. 7). Only sanguinarine (berberine was inactive) promoted the efflux of 3-*O*-methyl-D-[1 − ^{3}H]-glucose; since haemolysis did not occur concomitantly, sanguinarine either affected the glucose transporter or only produced very small pores, which allowed the diffusion of 3-*O*-methyl-D-[1 − ^{3}H]-glucose, but not that of haemoglobin.

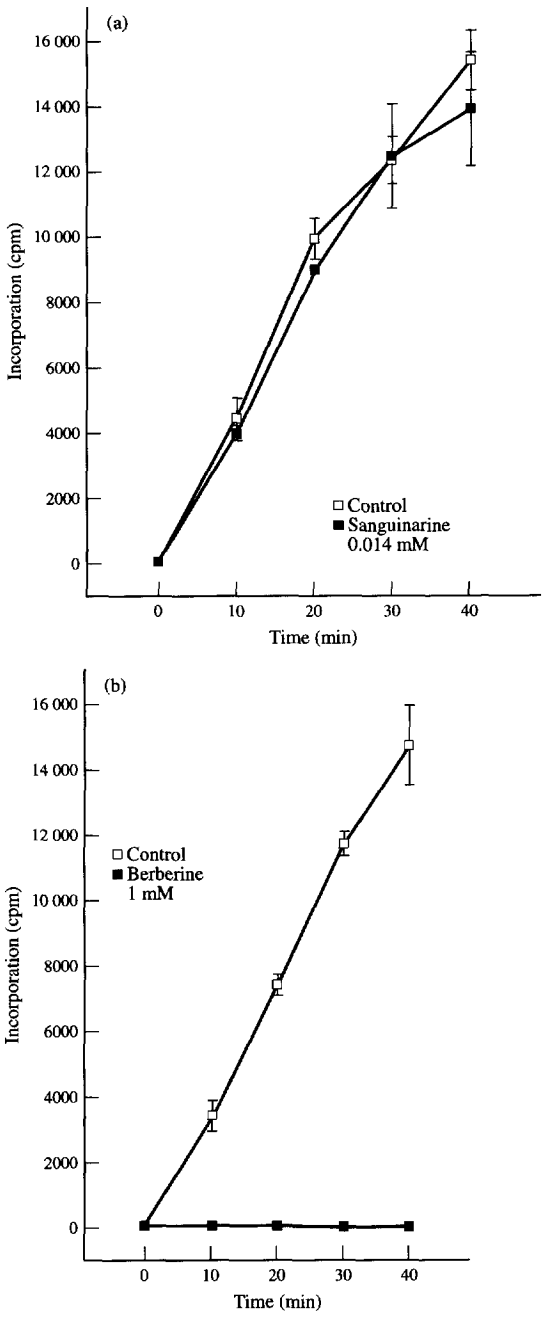


Fig. 6. Inhibition of protein biosynthesis ('*in vitro* translation') by (a) sanguinarine and (b) berberine. Control = without alkaloid.

CONCLUSIONS

Berberine, palmatine and sanguinarine affect several molecular targets at the same time. Some of these allelochemical activities, such as DNA intercalation (Fig. 2), inhibition of DNA synthesis (Fig. 4), protein biosynthesis (Fig. 6), membrane permeability (Fig. 7) and uncoupling of oxidative phosphorylation [34], will modulate all type of cells. It is likely that these

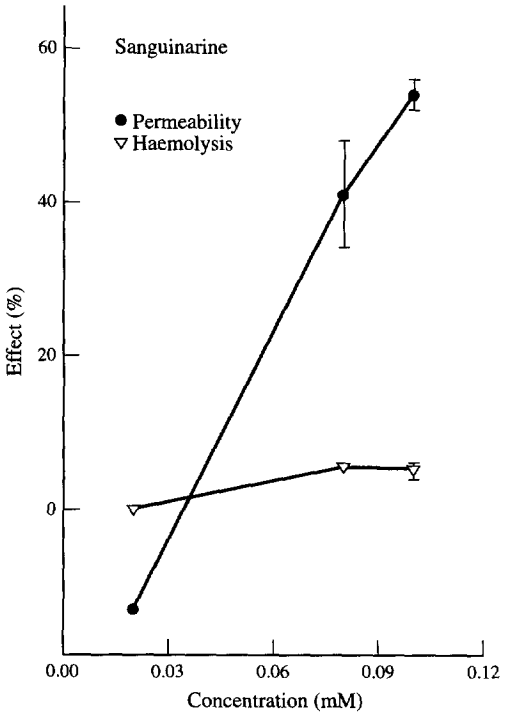


Fig. 7. Induction of membrane instability (permeability and haemolysis) by sanguinarine. Erythrocytes were preloaded with 3-*O*-methyl-D-[1-^{3}H]-glucose, and its efflux was measured after adding the alkaloid; the release of haemoglobin was determined concomitantly.

interactions contribute to the allelochemical and toxic effects observed against bacteria, fungi, other plants, insects and vertebrates. DNA intercalation and inhibition of reverse transcription could be responsible for the inhibition of phages and other viruses [11]. The interactions with neuroreceptors (Fig. 1), the inhibition of acetylcholine related enzymes (Fig. 1), and Na^+, K^+-ATPase [31–33], and the binding to microtubules [23] should mainly affect insects and vertebrates. Although the list of targets is already quite long, it is likely that additional targets are modulated by these alkaloids. These alkaloids are usually present in relatively high (i.e. millimolar) concentrations, often stored in laticifers (latex immediately spills out upon wounding), and the wide variety of affected molecular targets (which are inhibited in nano- and micromolar concentrations) leads to our conclusion that berberine, palmatine and sanguinarine may mediate the chemical defence of plants against viruses, bacteria, fungi, other plants, insects and vertebrates.

EXPERIMENTAL

Membrane preparation for receptor binding studies. Porcine brains, which were obtained within 30 min after death of the animals from a local slaughterhouse, were used to prepare receptor-rich membranes. The brains were immediately frozen in liquid N_2; 50 g brain tissue per 200 ml ice-cold buffer (0.32 M sucrose, 10 mM K-Pi buffer, pH 7.0, 1 mM EDTA) were homogenized ×2 for 15 sec in a blender and then for 1 min with an ultraturrax. The homogenate was centrifuged ×3 for 15 min at 1400 *g* and 4° to separate cellular debris. The supernatant was spun down at 100 000 *g* for 60 min. The resulting pellet was resuspended in buffer (as above, but without sucrose). Aliquots were stored frozen at −80°. Protein content was determined by the Lowry method, using BSA as standard [37, 38].

Radio receptor binding assays. Binding assays (×3) were performed using a rapid filtration technique, essentially as described in ref. [47].

Muscarinic receptor (mAChR). Membrane prepns adjusted to 500 μg protein in a final vol. of 500 μl buffer were incubated with [^{3}H]-quinuclidinyl benzilate (52.3 Ci mmol^{-1}; Dupont NEN) for 1 hr at 20° in the absence and presence of alkaloids, employing 20 μM atropine as a positive control. The incubation was stopped with 3 ml ice-cold 0.9% NaCl solution and filtered (by suction) through Whatman GF/C glass fibre filters. The filters were washed ×3 with 3 ml 0.9 % NaCl, placed in vials and dried for 30 min at 60°. Their radioactivity was measured in a liquid scintillation counter (RackBeta, Pharmacia) using 'Ultima-Gold' (Packard) as scintillation cocktail.

Nicotinic receptor. [^{3}H]-Nicotine (85 Ci mmol^{-1}; Amersham) was used to assay specific binding of alkaloids to the nACh receptor. The membrane prepn was incubated for 40 min with different concentrations of alkaloids or 1 mM nicotine as positive control. The GF/C filters were presoaked with PEG 8000 (5% in H_2O) for 3 hr to reduce non-specific binding of [^{3}H]-nicotine. Further procedures were the same as described above for mAChR.

Alpha$_1$ receptor. [^{3}H]-Prazosine (78 Ci mmol^{-1}; DuPont NEN) was used to assay specific binding of alkaloids to the alpha$_1$ receptor. The membrane prepn was adjusted to 400 μg in a final vol. of 500 μl and incubated for 45 min at 20° with different concns of alkaloids or 400 μM phentolamine as positive control. Further procedures were the same as described above for mAChR.

Alpha$_2$ receptor. [^{3}H]-Yohimbine (81 Ci mmol^{-1}; DuPont NEN) was used instead of [^{3}H]-prazosine; other conditions as in the alpha$_1$ receptor assay.

Serotonin$_2$ receptor. [^{3}H]-Ketanserine (85.1 Ci mmol^{-1}; DuPont NEN) was used to assay specific binding of alkaloids to the (5-HT$_2$) receptor. The membrane prepn was adjusted to 400 μg in a final vol. of 500 μl and incubated for 40 min at 20° with different concns of alkaloids or 100 μM mianserine as positive control. Further procedures were the same as described above for mAChR.

Esterase and transferase assays. Assays (×3) of the esterases were based on a photometric method. ChAt activity was assayed essentially as described in refs [48, 49].

Acetylcholine esterase. The activity of AChE was measured according to the Ellman method. We used AChE type VI-S from the electric eel (400 U mg^{-1}, Sigma). Different concns of alkaloids were incubated at room temp. with butyrylthiocholine iodide (18 mM), 5,5′-dithiobis(2-nitrobenzoic acid) (0.75 mM) and 50 μl enzyme soln in a final vol. of 300 μl on microtitre plates. The enzyme reaction was stopped with 30 μl physostigmine (20 mM) after *ca* 10 min. The coloured product formed was measured at 405 nm using an autoreader (Virion). All solns were prepd in K-Pi buffer (pH 7.2, 0.5 mM).

Butyrylcholine esterase (BChE). Procedures were comparable to those described for AChE. We used BChE from horse serum (11.6 U mg^{-1}, Sigma) for assays.

Choline acetyltransferase. We used ChAT from bovine brain (12 U mg^{-1}, Sigma) to assay inhibitory effects of alkaloids. Different alkaloid concns were incubated at 37° in a water bath with [^{3}H]-acetyl-Co A (1 Ci $mmol^{-1}$, DuPont NEN), 25 μl choline (100 mM) and 50 μl enzyme soln in a final vol. of 500 μl. The incubation was stopped with 300 μl TFA. The resulting [^{3}H]-acetylcholine was pptd with 1 ml hydroxylamine hydrochloride (1 mM), 1 ml acetylcholine (10 mg ml^{-1}) and 1 ml sodium tetraphenyl borate (50 mM) overnight at 4°. The ppt. was filtered through Whatman GF/C glass fiber filters with suction, washed ×3 with 2 ml EtOH, dried for 30 min at 60° and dissolved in 4 ml BzOH–MeCN (1:1). Radioactivity was measured in a liquid scintillation counter (RackBeta, Pharmacia) using 'Ultima-Gold' as scintillation cocktail.

Inhibition of DNA-polymerase I. To measure the activity of DNA-polymerase I, we modified a 'nick translation assay' [50]. The assay buffer contained 50 mM Tris–HCl (pH 7.5), 10 mM $MgSO_4$, 0.1 mM dithiothreitol (DTT), 500 ng linearized plasmid (pUC19), 625 μM dNTPs, 0.01 μCi α[^{32}P]-dCTP, 1 U DNA-polymerase I, 25 pg DNAse I and up to 10 mM alkaloids. The reaction was started by adding DNAse I; after 15 min at 37°, the reaction was terminated by adding 100 mM EDTA (pH 8.0). Two variations were carried out: (1) a preincubation of DNA polymerase I with alkaloids for 15 min, prior to adding plasmid DNA, and (2) a preincubation of DNA and alkaloids for 15 min before adding enzymes in order to differentiate between alkaloidal effects on DNA polymerase I and on DNA. Incorporated radioactivity was removed from the unincorporated α[^{32}P]-dCTP by gel filtration on Sephadex G 50 (Pharmacia) and measured in a liquid scintillation counter.

Inhibition of reverse transcriptase (RT). To measure activity of RT, a protocol for synthesis of cDNA was modified [50]. mRNA was isolated from rat liver according to standard protocols [50]. mRNA (500 ng) and 500 ng random primer (Boehringer Mannheim) were denatured at 70° for 5 min and immediately cooled afterwards in ice–water, then 0.3 mM dNTPs, 0.01 μCi α[^{32}P]-dCTP, 6 U AMV RT (Promega) and RT buffer (50 mM Tris–HCl, pH. 7.8, 10 mM $MgCl_2$, 80 mM KCl, 10 mM DTT) were added and incubated 30 min at 42°. The reaction was terminated by adding 100 mM EDTA; the incorporated α[^{32}P]-dCTP was measured as described before in the DNA-polymerase assay. Again, the two preincubation strategies as described before were used.

Inhibition of protein biosynthesis. An *in vitro* reticulocyte translation assay (Boehringer Mannheim) was modified to determine inhibition of translation by alkaloids. An assay (total vol. 25 μl) contained 2 μl 12.5 x translation mix (Boehringer), 10 μl reticulocyte lysate, 200 mM KOAc, 1.5 mM $Mg(OAc)_2$, 0.25 μCi L-[4,5-^{3}H(N)]-leucine, 0.5 μg TMV-RNA (Boehringer) and up to 5 mM alkaloids (buffered to pH 7). The mixt. was incubated at 30°; reactions were terminated after 0, 10, 20, 30 and 40 min. The radiolabelled protein was pptd by adding 200 μl ice-cold TCA (50%, w/v) and after 30 min filtered through GF 34 filters (Schleicher–Schüll), which binds proteins. After washing the filters ×3 with 50% TCA, they were dried at 85°. Radioactivity of the filters was determined in a liquid scintillation counter.

Influence of alkaloids on membrane permeability. Sheep erythrocytes were purified and incubated in 50 μl PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1 l H_2O). Then 8 μCi 3-*O*-methyl-D-[1 – ^{3}H]-glucose was added, which enters the cells with the aid of a glucose transporter. After 30 min at 37°, the erythrocytes were centrifuged for 3 min at 2000 *g* and suspended in 7 ml PBS (with or without 1 μM cytochalasine B, an inhibitor of outwardly directed glucose transport). Alternatively, purified sheep erythrocytes in PBS were incubated in 1 μCi α[^{35}S]-dATP and treated by electroshock ('electroporation'). Loaded erythrocytes were incubated for 15 min at 10° together with up to 5 mM alkaloids. Erythrocytes were then pptd by centrifugation (4 min at 2000 *g*) and radioactivity of the supernatant was determined by liquid scintillation counting. Haemoglobin released from erythrocytes was determined photometrically at 543 nm.

Interaction of alkaloids with DNA. Melting point determination. If compounds intercalate with DNA, then the mp is shifted to higher temps [51, 52]. *Sinapis* DNA (70 μM) was incubated in TE buffer (10 mM Tris–HCl [pH 7.4], 1 mM EDTA) with 70 μM alkaloids for 30 min at 22°. The temperature was then increased by 1° min^{-1} to 90° and absorbance was continuously determined at 256 nm.

Methyl green (MG) assay. MG binds to DNA and bound MG displays an absorption maximum at 642 nm, whereas free MG shows no absorption at this wavelength. [35, 53]. When an alkaloid binds or intercalates with DNA, then MG is released, which can be measured as a decrease of OD at 642 nm. DNA–MG (70 μM Sigma) was incubated in the dark in 20 mM Tris–HCl (pH 7.4), together with up to 5 mM alkaloids. After 24 hr the OD_{642} of untreated controls and treated samples was determined.

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REFERENCES

1. Schumacher, H. M., Gundlach, H., Fiedler, F. and Zenk, M. H., *Plant Cell Reports*, 1987, **6,** 410.
2. Eilert, U., Kurz, W. G. W. and Constabel, F., *Journal of Plant Physiology*, 1985, **119,** 65.
3. Wink, M. and Latz-Brüning, B., in *Allelopathy: Organisms, Processes and Applications*, Vol. 582, eds Inderjit, K. M. M. Dakshini and F. A. Einhellig. American Chemical Society, Washington, DC, 1995, pp. 117–126.
4. Stermitz, F. R., Larson, K. A. and Kim, D. K., *Journal of Medicinal Chemistry*, 1973, **16,** 939.
5. Detzel, A. and Wink, M., *Chemoecology*, 1993, **4,** 8.
6. Wink, M. and Schneider, D., *Journal of Comparative Physiology*, 1990, **B, 160,** 389.
7. Schwartz, H., Becci, P. J., Perry-Barnes, H. H. and Southard, G. L., *Toxicologist*, 1985, **5** (Abstr. 702), 176.
8. Becci, P. J., Schwartz, H., Barnes, H. H. and Southard, G. L., *Journal of Toxicology and Environmental Health*, 1987, **20,** 199.
9. Wink, M., in *The Alkaloids*, Vol. 43 (ed. G. A. Cordell). Academic Press, San Diego, CA, 1993, pp. 1–118.
10. Lenfeld, J., Kroutil, M., Marsalek, E., Slavik, J., Preininger, V. and Simanek, V., *Planta Medica* 1981, **43,** 161.
11. Bodalski, T. and Makower, H., *Dissertationes Pharm.*, 1957, **9,** 273.
12. Greathouse, G. A. and Rigler, N. E., *Phytopathology*, 1940, **30,** 475.
13. Vichkanova, S. A. and Adgina, V. V., *Antibiotiki (Moscow)*, 1971, **16,** 609–612.
14. Nandi, R., Maiti, M., Chaudhuri, K., Mahato, S. B. and Bairagi, A. K., *Experientia*, 1983, **39,** 524.
15. Johnson, C. C., Johnson, G. and Poe, C. F., *Acta Pharmacol. Toxicol.*, 1952, **8,** 71.
16. Vichkanova, S. A., Rubinchik, M. A., Adgina, V. V. and Fedorchenko, T. S., *Farmakol. Toksikol. (Moscow)*, 1969, **32,** 325.
17. Odebiyi, O. O. and Sofowora, E. A., *Planta Medica*, 1979, **36,** 204.
18. Dzink, J. L. and Socransky, S. S., *Antimicrobial Agents in Chemotherapy*, 1985, **27,** 663.
19. Simeon, S., Rios, J. L. and Villar, A., *Plantes Medicinales et Phytotherapie*, 1989, **XXIII,** 202.
20. Meyer, H., *Archives of Experimental Pathology and Pharmacology*, 1892, **29,** 397.
21. *Martindale—The Extra Pharmacopoeia.* Pharmaceutical Press, London, 1993.
22. Budavari, S. (ed.), *The Merck Index*, Merck & Co., Rahway, NJ, 1989.
23. Wolff, J. and Knipling, L., *Biochemistry*, 1993, **32,** 13334.
24. Orgell, W. H., *Lloydia*, 1963, **26,** 36.
25. Nikolskaya, B. S., *Farmakol. Toksikol.*, 1966, **26,** 76.
26. Ulrichova, J., Walterova, D. and Simanek, V., *Acta Univ. Palacki olomue Fac. Med.*, 1984, **106,** 31.
27. Ulrichová, J., Walterová, D., Preininger, V., Slavik, J., Lenfeld, J., Cushman, M. and Simanek, V., *Planta Medica*, 1983, **48,** 111.
28. Kakiuchi, N., Hattori, M., Ishii, H. and Namba, T., *Planta Medica*, 1987, **53,** 22.
29. Tan, G. T., Pezzuto, J. M. and Kinghorn, A. D., *Journal of Natural Products*, 1991, **54,** 143.
30. Tanaka, T., Metori, K., Hirotani, M., Furuya, T. and Kobayashi, S., *Planta Medica*, 1993, **59,** 200.
31. Straub, K. D. and Carver, P., *Biochemistry and Biophysics Research Communications*, 1975, **62,** 913.
32. Cohen, H. G., Seifen, E., Straub, K. D., Tiefenback, C. and Stermitz, F. R., *Biochemical Pharmacology*, 1978, **27,** 2555.
33. Seifen, E., Adams, R. J. and Riemer, R. K., *European Journal of Pharmacology*, 1979, **60,** 373.
34. Vallejos, R. H., *Biochimica Biophysica Acta*, 1972, **292,** 193.
35. Krey, A. K. and Hahn, F. E., *Science*, 1969, **166,** 755.
36. Nandi, R. and Maiti, M., *Biochemical Pharmacology*, 1985, **34,** 321.
37. Schmeller, T., Sauerwein, M., Sporer, F. and Wink, M., *Journal of Natural Products*, 1994, **57,** 1316.
38. Schmeller, T., Sporer, F., Sauerwein, M. and Wink, M., *Pharmazie*, 1995, **50,** 493.
39. Kelentey, B., *Arzneimittelforschung*, 1960, **10,** 135.
40. Ölmez, E. and Ilhan, M., *Arzneimittelforschung*, 1992, **42,** 1095.
41. Ulrichová, J., Walterová, D., Preininger, V. and Simanek, V., *Planta Medica*, 1983, **48,** 174.
42. Sarkar, S. N., *Nature*, 1948, **162,** 265.
43. Hakim, S. A. E., *Indian Journal of Cancer*, 1968, **5,** 183.
44. Dougherty, D. A. and Stauffer, D. A., *Science*, 1990, **250,** 1558.
45. Sethi, M. L., *Canadian Journal of Pharmaceutical Science*, 1981, **16,** 29.
46. Wink, M. and Twardowski, T., in *Allelopathy. Basic and Applied Aspects*, eds S. J. H. Rizvi and V. Rizvi. Chapman & Hall, London, 1992, pp. 129–150.
47. Yamamura, H. I. and Snyder, S. H., *Proceedings of the National Academy of Science of the U.S.A.*, 1974, **71,** 1725.
48. Fonnum, F., *Biochemical Journal*, 1966, **100,** 479.
49. Fonnum, F., *Journal of Neurochemistry*, 1975, **24,** 407.
50. Sambrook, J., Fritsch, E. F. and Maniatis, T.,

Molecular Cloning. A Laboratory Manual, 2nd edn, Cold Spring Harbour Laboratories, Cold Spring Harbour, NY, 1989.
51. Maiti, M. and Chaudhuri, K., *Indian Journal of Biochemistry and Biophysics*, 1981, **18,** 245.
52. Maiti, M., Nandi, R. and Chaudhuri, K., *FEBS Letters*, 1982, **142,** 280.
53. Burres, N. S., Frigo, A., Rasmussen, R. R. and McAlpine, J. B., *Journal of Natural Products*, 1992, **55,** 1582.