Pergamon

PII: S0031-9422(96)00664-4

Phytochemistry, Vol. 44, No. 5, pp. 787–796, 1997

0031-9422/97 $17.00+0.00

SPECIFIC INHIBITION OF CYCLIC AMP-DEPENDENT PROTEIN KINASE BY WARANGALONE AND ROBUSTIC ACID

BING HUI WANG, BELA TERNAI* and GIDEON POLYA†

School of Biochemistry, La Trobe University, Bundoora, Victoria 3083 Australia, *Department of Chemistry, Chulalongkorn University, Bangkok 10330 and Chiang Mai University, Chiang Mai, 50002, Thailand

(*Received in revised form* 28 *August* 1996)

Key Word Index—*Derris scandens*; Leguminosae; protein kinase inhibitors; warangalone; robustic acid.

Abstract—The prenylated isoflavone warangalone from the insecticidal plant *Derris scandens* is a selective and potent inhibitor of rat liver cyclic AMP-dependent protein kinase catalytic subunit (cAK) (IC_{50} 3.5 μM). The inhibition of rat liver cAK by warangalone is non-competitive with respect to both ATP and the synthetic peptide substrate (LRRASLG) employed in this study. Warangalone is a poor inhibitor of avian calmodulin-dependent myosin light chain kinase (MLCK), rat brain Ca^{2+}- and phospholipid-dependent protein kinase C (PKC) and wheat embryo Ca^{2+}-dependent protein kinase (CDPK). The related plant derived prenylisoflavones are also potent cAK inhibitors. Thus, 8-γ-γ-dimethylallylwighteone, 3′-γ-γ-dimethlallylwighteone and nallanin are inhibitors of cAK with IC_{50} values in the range 20–33 μM. The prenyl-substituted isoflavones tested in this study are ineffective or poor as inhibitors of PKC. Thus nallanin is a poor PKC inhibitor (IC_{50} value of 120 μM). The related isoflavones biochanin A and genistein are poor inhibitors of cAK (IC_{50} values 100 μM and 126 μM, respectively). Genistein inhibits MLCK (IC_{50} value 14 μM) but biochanin A is a poor MLCK inhibitor (IC_{50} value 300 μM). The *D. scandens* prenyl-isoflavones and related isoflavones are ineffective inhibitors of wheat embryo Ca^{2+}-dependent protein kinase (CDPK). The 4-methoxy-3-phenyl-coumarin robustic acid is a potent inhibitor of rat liver cAK (IC_{50} value 10 μM) but is a poor inhibitor of rat brain PKC, avian MLCK and wheat embryo CDPK. The coumarins 5-methoxypsoralen and 4,4′-di-*O*-methyl scandenin are poor cAK inhibitors (IC_{50} values 240 and 248 μM, respectively). All of the non-prenylated coumarins examined are ineffective as inhibitors of the eukaryote signal-regulated protein kinases cAK, MLCK, PKC and CDPK. The selective, high affinity interaction of warangalone and robustic acid with cAK may contribute to their biological effects *in vivo* and to the insecticidal activity of the plant *D. scandens*.

INTRODUCTION

A wide variety of bioactive secondary metabolites are produced by plants. Such compounds are involved in their defence against animal herbivores and microbial pathogens [1–7]. In addition, some plant secondary metabolites have been shown to have allelopathic effects enabling defence against competing plants [1, 3]. The nature of high affinity sites of action of many plant secondary metabolites (or the metabolic products of such compounds) can be inferred in many situations when such compounds have animal hormone or pheromone properties or have evident physiological effects related to their taste or smell [1]. High-affinity biochemical sites of action of a variety of toxic plant secondary metabolites have been demonstrated, well known examples including inhibition of $(Na^+ + K^+)$ATPase by cardiac glycosides, interaction of curare with the nicotinic acetylcholine receptor and inhibition of cytochrome oxidase by CN^- derived from plant cyanogenic glycosides [1, 2]. Nevertheless, there remains a massive task of defining high-affinity biochemical sites of action of a large number of plant defensive secondary metabolites in target microorganisms and animal herbivores.

Many plant defensive secondary metabolites interact with protein kinases involved in signal transduction in eukaryotes. Thus particular plant-derived anthraquinones variously inhibit animal Ca^{2+}- and phospholipid-dependent protein kinase C (PKC), Ca^{2+}- and calmodulin-dependent myosin light chain kinase (MLCK) and cyclic AMP-dependent protein kinase catalytic subunit (cAK) as well as plant Ca^{2+}-dependent protein kinase (CDPK) [8]. Several plant-derived xanthones inhibit MLCK and are potent inhibitors of cAK and CDPK [9]. Particular flavonoids inhibit MLCK [10, 11], PKC [12–17], cAK

† Author to whom correspondence should be addressed.

[18] and tyrosine kinase [19]. A range of flavonol compounds, notably oligomeric procyanidins, are potent inhibitors of cAK, PKC and of plant CDPK [20] as are a variety of gallic acid esters [21, 22] including hydrolysable tannins [21]. The isoflavone genistein is a potent tyrosine kinase inhibitor [23] as is the stilbene derivative piceatannol [24]. Certain non-aromatic plant defensive compounds are selective inhibitors of cAK including various amphiphilic, acidic triterpenoids [22] and the carotenoid crocetin [22].

The benzophenanthridine natural product chelerythrine is a potent and selective inhibitor of PKC [25] and a number of structurally related synthetic phenanthrene derivatives are protein kinase inhibitors [26]. A number of microbial indole derivatives (notably staurosporine) are potent protein kinase inhibitors [27–29] and various synthetic bis-indolyl compounds are potent and selective PKC inhibitors [30, 31]. A series of synthetic isoquinoline derivatives are potent and selective cAK inhibitors [32–34]. The structure/activity relations of naturally occurring protein kinase inhibitors may be useful for the development of synthetic chemotherapeutic agents that are protein kinase inhibitors. Conversely, knowing that particular chemotherapeutic agents interact with particular protein kinases may assist in the detection of naturally occurring bioactive compounds with similar properties. Thus the synthetic phenanthrene-based antimalarial halofantrine is a selective inhibitor of cAK [26] and a variety of anti-tumour compounds are inhibitors of PKC [8, 35–38] or of MLCK [8, 38].

Numerous species of *Derris* and *Lonchocarpus* have been used as insecticides and have been examined for insecticidal compounds and especially for their rotenone content [39]. The climbing shrub *Derris scandens* which occurs throughout S. E. Asia and N. Australia, belongs to the sub-section *Brachyterum* of the genus *Derris*, to which *D. robusta* also belongs. The roots were first examined by Clarke [40] who isolated scandenin, together with lonchocarpic acid, previously obtained by Jones from a species of *Lonchocarpus* [41, 42]. Various 4-hydroxy-3-phenylcoumarins and prenylisoflavones have been isolated and structurally characterized [43–48]. We have investigated the possible interaction between these bioactive plant defense compounds and protein kinases involved in signal transduction in eukaryotes in order to identify possible biochemical sites of action. The present paper describes the specific inhibition of rat liver cAK by warangalone and robustic acid from the insecticidal plant *D. scandens* and the interaction of certain other related compounds with cAK and MLCK.

RESULTS

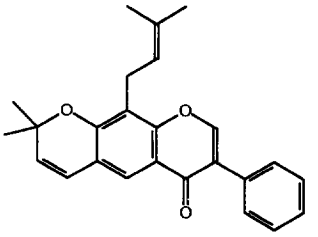
Inhibition of protein kinases by warangalone and related isoflavones

Four signal-related eukaryote protein kinases were screened for inhibition by the prenyl isoflavones and other isoflavones targeted in this investigation. The protein kinases were assayed employing synthetic oligopeptides as substrates rather than high molecular weight protein substrates to avoid possible interactions of the test compounds with such proteins [11, 18]. Rat liver cyclic AMP-dependent protein kinase catalytic subunit (cAK) was assayed using kemptide (LRRASLG) as the polypeptide substrate and rat brain Ca^{2+}- and phospholipid-dependent protein kinase C (PKC) was assayed with the epidermal growth factor receptor-based peptide EGFRP (VRKRTLRRL-NH_2) as substrate. Both wheat embryo Ca^{2-}-dependent protein kinase (CDPK) and avian gizzard calmodulin-dependent myosin light chain kinase (MLCK) were assayed using the myosin light chain-based peptide MLCP (KKRAA-RATSNVFA-NH_2) as substrate. Wheat CDPK was included in this protein kinase screening analysis since various compounds that inhibit non-plant protein kinases such as MLCK, PKC or cAK also inhibit wheat CDPK [8, 9, 11, 18, 20–23]. Further, it was of interest to see if defensive isoflavone secondary metabolites are ineffective as inhibitors of plant signal-regulated CDPK as found for a variety of flavonoids that inhibit cAK [11, 18].

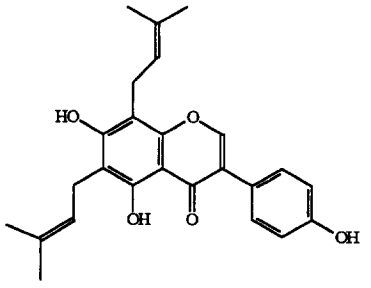
The various prenylated isoflavones (**1**–**4**, **7**) in this study were isolated from *D. scandens*, a plant used as a traditional insecticide [39]. Four related isoflavones (**5**, **6**, **8**, **9**) were also examined as potential inhibitors of various protein kinases. Warangalone (**1**) is a potent inhibitor of rat liver cAK (IC_{50} 3.5 μM) (Table 1, Fig. 1). However, warangalone at high concentration does not inhibit PKC, MLCK or CDPK (Table 1). There is notable specificity in the inhibition of cAK by warangalone and in the lack of inhibition of other protein kinases by this compound. Further, a variety of related compounds are ineffective or much less effective as inhibitors of cAK and of other protein kinases examined (Table 1).

Other isoflavones, namely compounds **2**–**5**, are also relatively specific inhibitors of rat liver cAK, although they are much poorer inhibitors than **1** (Table 1). Thus 8-γ-γ-dimethylallylwighteone (**2**) (IC_{50} value for cAK 20 μM), 3′-γ-γ-dimethylallylwighteone (**3**) (IC_{50} 24 μM), nallanin (**4**) (IC_{50} 33 μM) and biochanin A (**5**) (IC_{50} 100 μM) inhibit cAK but are not inhibitors or are relatively poor inhibitors of the other protein kinases tested (Table 1, Fig. 2). Genistein (**6**) is a relatively poor cAK inhibitor (IC_{50} for cAK 126 μM) (Table 1, Figs 1 and 2) but is a much more effective inhibitor of MLCK (IC_{50} for MLCK 14 μM) (Table 1, Fig. 3). Eturunagarone (**7**) from *D. scandens* is a relatively poor inhibitor of cAK (IC_{50} 248 μM). Genistein (**8**) and 2-carbethoxy-5,7-dihydroxy-4′-methoxyisoflavone (**9**) are ineffective as inhibitors of cAK and of the other protein kinases examined (Table 1).

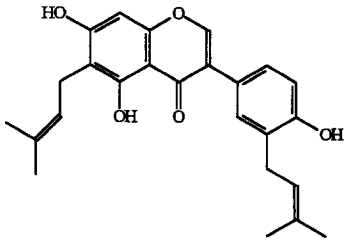
None of compounds **2**–**9** cause significant inhibition of PKC at 0.3 mM but **4** is a weak inhibitor of PKC (IC_{50} value 120 μM) (Table 1). With the exception of genistein (**6**), the isoflavones studied here are either



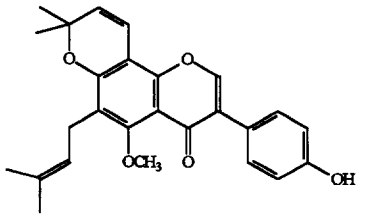
1 Warangalone



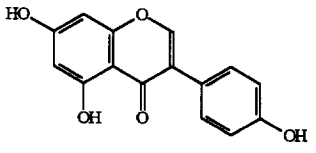
2 8-γ-γ-Dimethyllylwighteone



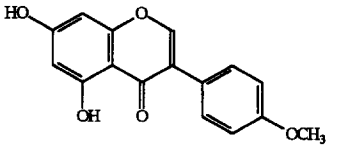
3 3'-γ,γ-Dimethylallylwighteone



4 Nallanin



5 Biochanin A



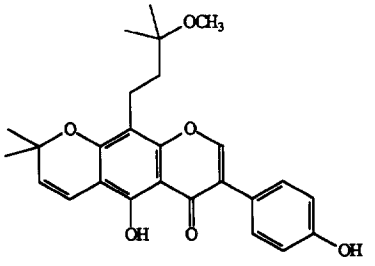
6 Genistein

inactive or are relatively poor inhibitors of CDPK and MLCK (Table 1). The isoflavone genistein (**6**) is an effective inhibitor of MLCK (IC_{50} 14 μM) (Table 1, Fig. 3) as well as being an inhibitor of protein tyrosine kinase (IC_{50} about 22–29 μM) [23]. With the exception of genistein (**6**), isoflavones (**1–7**) are relatively selective inhibitors of cAK (Table 1). The most potent inhibitors of cAK are isoflavones **1–4** having 1- or 2-position prenyl substituents (Fig. 1) However, it is notable that the most potent protein kinase inhibitors *per se* in this series are warangalone (**1**) (IC_{50} for cAK 3.5 μM) and genistein (**6**) (IC_{50} for MLCK 14 μM) (Table 1), these two compounds differing from the other isoflavones in lacking a polar substituent on the *p*-position of the isoflavone phenyl ring.

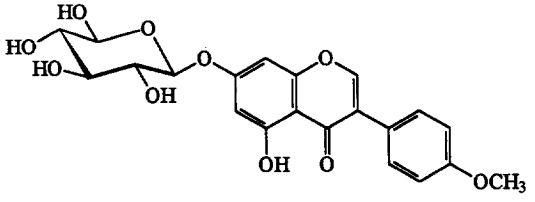
Inhibition of protein kinases by robustic acid and related coumarins

Since the isoflavone warangalone is such a potent and specific inhibitor of cAK (Table 1, Fig. 1), the interaction with eukaryote protein kinases of structurally related coumarins from the same plant source and of some other coumarins was examined. The 4-methoxy-3-phenyl-coumarin robustic acid (**10**) is a potent and selective inhibitor of cAK (IC_{50} 10 μM) (Table 2, Fig. 4). Robustic acid at high concentration does not inhibit PKC, MLCK and CDPK (Table 2, Fig. 4). The other structurally related coumarins studied here (**11–25**) are inactive or relatively ineffective as inhibitors of cAK (Table 2). There is a notable specificity in the inhibition of cAK by robustic acid in both the lack of inhibition of the protein kinases by this compound and the ineffectiveness or relative ineffectiveness of a range of other coumarin derivatives as inhibitors of cAK and other protein kinases examined (Table 2).

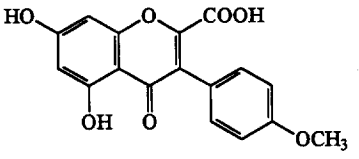
While robustic acid is a potent and specific cAK inhibitor, other coumarins namely, 7-hydroxy-4-methylcoumarin (**15**) 7-hydroxycoumarin (**19**), psoralen (**12**), 3-[2-(diethylamino)ethyl]-7-hydroxy-4-methylcoumarin (**20**) and coumarin (**18**) exhibit relatively weak activity as inhibitors of MLCK with IC_{50} values of 167, 197, 267, 287, and 317 μM, respectively (Table 2). Compounds **10–25** do not inhibit PKC or CDPK (Table 2).



7 Eturunagarone



8 Genistin



9 2-Carbethoxy-5,7-dihydroxy-4'-methoxyisoflavanone

Mechanism of inhibition of cAK by warangalone

Lineweaver–Burk double reciprocal plots of v_0^{-1} [(initial velocity)$^{-1}$] vs [substrate]$^{-1}$ from enzyme kinetic data obtained in the presence or absence of warangalone (**1**) indicate that **1** is a non-competitive inhibitor of cAK with respect to the synthetic peptide substrate kemptide (Fig. 5(A)). Thus the inclusion of **1** decreases the V_{max} but the K_m remains approximately the same (Fig. 5(A)). The K_i for cAK of **1** with respect to kemptide substrate is 4.7 ± 2.5 μM (mean $\pm$ S.D. from three determinations). Compound **1** is also a non-competitive inhibitor with respect to ATP with a K_i of 2.0 ± 0.5 μM (Fig. 5(B)). The K_i estimate for warangalone (**1**) as a non-competitive inhibitor of cAK is similar to the IC_{50} value for warangalone inhibition of rat liver cAK determined in the standard assay conditions (3.5 μM) (Table 1, Fig. 1). We have previously found that cAK is inhibited non-competitively with respect to both ATP and peptide substrate by a potent polycyclic, aromatic, catechin-derived inhibitor [20], various azaacridine inhibitors [38] and by the antimalarial halofantrine [26]. Genistein, while having a structural similarity to warangalone, is an inhibitor of protein tyrosine kinase (IC_{50} values 22–29 μM) [23]. Genistein is not a specific protein tyrosine kinase inhibitor as it also inhibits MLCK (IC_{50} 14 μM) (Table 1, Fig. 3). The prenyl substitution of positions 6 and 8 of the isoflavone ring may contribute to the specific inhibition of cAK by warangalone as compared to the specificity of genistein for protein tyrosine kinase [23] and MLCK (IC_{50} 14 μM) (Table 1, Fig. 3). The prenyl substitution of position 6 of coumarin may well contribute to the specificity of the inhibition of cAK by robustic acid as neither cAK nor any of the other protein kinases tested

Table 1. Inhibition of protein kinases by prenylated isoflavones and related isoflavones

Compound	IC_{50} [μM] or [% control]*			
	cAK	PKC	MLCK	CDPK
1 warangalone	3.5	(78%)	(91%)	(93%)
2 8-γ,γ-dimethylallylwighteone	20	(89%)	(100%)	(90%)
3 3'-γ,γ-dimethylallylwighteone	24	(88%)	(89%)	(99%)
4 nallanin	33	120	(75%)	(73%)
5 biochanin A	100	(92%)	303	(75%)
6 genistein	126	(111%)	14	(73%)
7 eturunagarone	248	(84%)	(88%)	(93%)
8 genistin	(60%)	(99%)	(54%)	(73%)
9 2-carbethoxy-5,7-dihydroxy-4'-methoxyisoflavone	(86%)	(113%)	(83%)	(104%)

* Rat liver cAK, wheat germ CDPK, avian MLCK and rat brain PKC were assayed as described in the Experimental in the presence or absence of increasing concentrations of the test compounds. Test compounds were added dissolved in 10% (v/v) DMSO to give 2% (v/v) final DMSO concentration (cAK and CDPK assays) or 1.7% final DMSO concentration (PKC and MLCK assays). Concentrations for 50% inhibition (IC_{50} values) were determined by interpolation from plots of protein kinase activity vs inhibitor concentration. Where relatively little inhibition was observed with inclusion of 400 μM inhibitor (cAK or CDPK assays) or 333 μM inhibitor (PKC or MLCK assays), the protein kinase activity observed is presented as % of control (no added inhibitor) in parentheses.

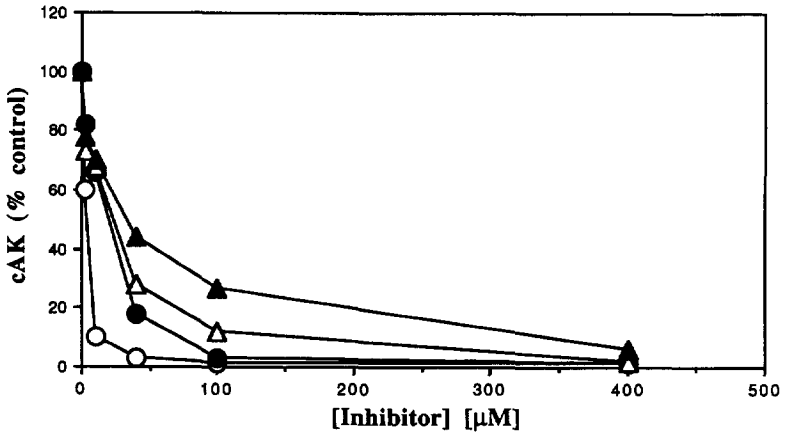


Fig. 1. Inhibition of cAK by isoflavones. ○ – ○, **1**; ● – ●, **2**; △ – △, **3**; ▲ – ▲, **4**.

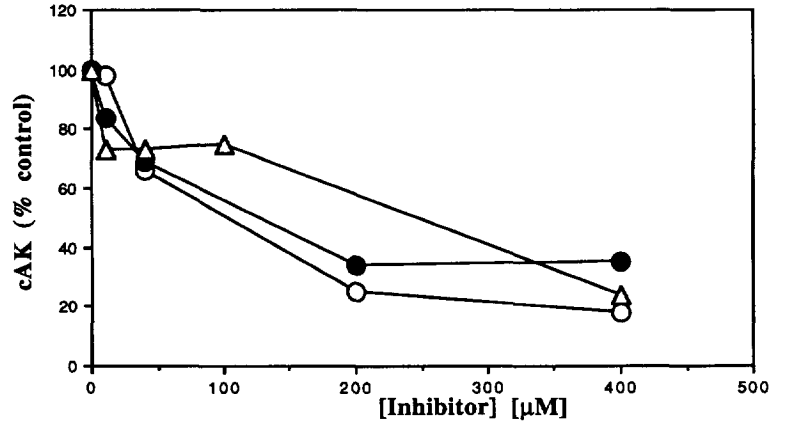


Fig. 2. Inhibition of cAK by isoflavones. Rat liver cAK was assayed with 20 μM kemptide as substrate in the standard assay containing 2% (v/v) DMSO and increasing concentrations of test inhibitor. The cAK activity is expressed as % control (no added inhibitor). ○ – ○, **5**; ● – ●, **6**; △ – △, **7**.

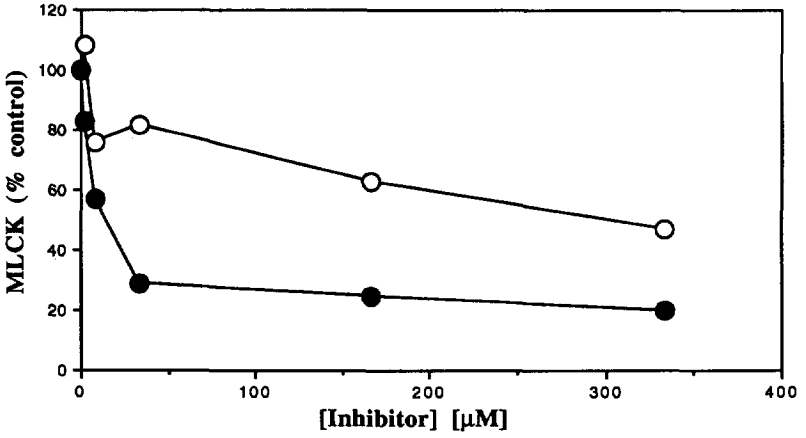
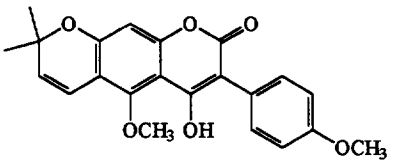


Fig. 3. Inhibition of MLCK by genistein and biochanin A. MLCK was assayed as described in the Experimental and MLCK activity is presented as % control activity (no added inhibitor). ○ – ○, Biochanin A; ● – ●, genistein.

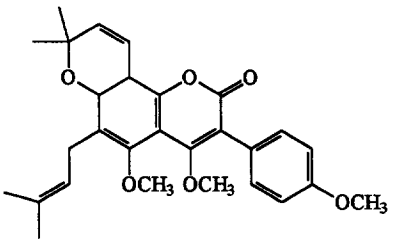
exhibit a comparable affinity for a range of other related coumarins. We conclude that the prenyl substituent of warangalone and robustic acid may contribute to the high affinity and selectivity of the interactions of these compounds with cAK.

DISCUSSION

The present paper demonstrates that the prenylated isoflavone warangalone and the 4-methoxy-3-phenyl coumarin robustic acid are potent inhibitors of the



10 Robustic acid



11 4, 4'-Di-*O*-methyl scandenin

34], H-88 and H-89 [33, 34] which are potent and relatively selective inhibitors of cAK. While the K_i of warangalone for rat liver cAK (3.5 μM) is comparable to the K_i values of H-7 and H-8 for cAK (3.0 and 1.2 μM, respectively) [32], it is much higher than the K_i values of H-88 and H-89 for cAK (0.4 μM and 0.05 μM, respectively) [33]. However, while warangalone does not inhibit PKC even at 0.3 μM (Table 1), the K_i values, of H-7, H-8, H-88 and H-89 for PKC are 6, 14, 76 and 32 μM, respectively [32, 33]. While warangalone inhibits CDPK and MLCK by less than 10% at 0.4 mM and 0.33 mM, respectively (Table 2), the K_i values of H-89 (the most potent and selective isoquinoline sulphonamide inhibitor of cAK) for MLCK, PKC, casein kinase I and Ca^{2+}-calmodulin-dependent protein kinase II are all about 30 μM [33].

While warangalone is a specific inhibitor of cAK, the related isoflavone genistein inhibits cAK while also inhibiting MLCK (Table 1) and protein tyrosine kinase [23]. Genistein inhibits avian MLCK with an IC_{50} of 14 μM, noting that this is about half of the IC_{50} value for the inhibition of protein tyrosine kinase (22–29 μM) [23]. Thus in comparison with genistein, warangalone has both a high specificity as well as a high affinity as an inhibitor of cAK. The high affinity

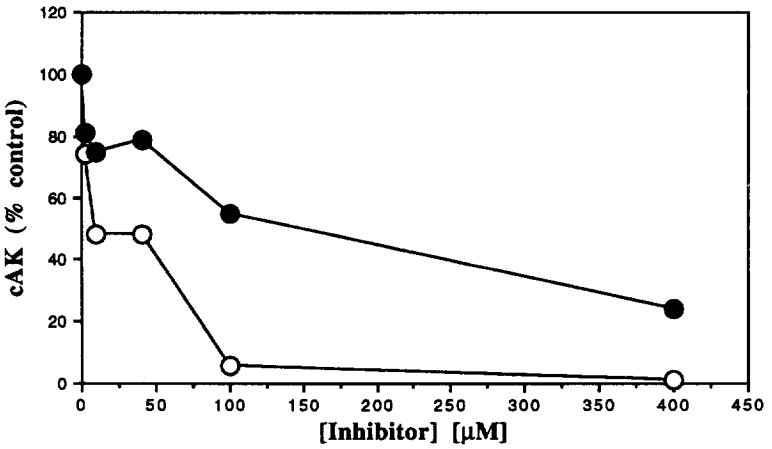


Fig. 4. Inhibition of cAK by robustic acid and 4, 4'-di-*O*-methyl scandenin. Other details as for the legend to Fig. 2. ○ – ○, Robustic acid; ● – ●, 4, 4'-di-*O*-methyl scandenin.

catalytic subunit of cyclic AMP-dependent protein kinase (cAK). This inhibition is very specific in that of the protein kinases tested only cAK is inhibited by low concentrations of warangalone and robustic acid (the IC_{50} values for warangalone and robustic acid being in the range from 10^{-5} to 10^{-6} M). Neither cAK nor any of the other protein kinases tested exhibit a comparable affinity and specificity for any of a range of related compounds examined.

Warangalone can be usefully compared with the synthetic isoquinoline sulphonamides H-7, H-8 [32, and specificity of warangalone as a cAK inhibitor indicates a potential for this compound as a pharmacological tool for examining cAK-mediated biological processes.

While the 4-methoxy-3-phenylcoumarin robustic acid is also a potent inhibitor of cAK, none of the other related coumarins tested in this study are effective as inhibitors of rat liver cAK (Table 2). Robustic acid has little effect, if any, on the other protein kinases examined here (Table 2). Since robustic acid is the only prenylated coumarin in this series, we conclude

Table 2. Inhibition of protein kinases by coumarins

Compound	IC_{50} [μM] or [% control]*			
	cAK	CDPK	PKC	MLCK
10 robustic acid	10	(93%)	(87%)	(99%)
11 4,4′-di-*O*-methylscandenin	150	(90%)	(88%)	(104%)
12 psoralen	(74%)	(111%)	(97%)	267
13 5-methoxypsoralen	240	(118%)	(99%)	(66%)
14 8-methoxypsoralen	(83%)	(77%)	(106%)	(74%)
15 7-hydroxy-4-methylcoumarin	(87%)	(75%)	(120%)	167
16 DL-3(α-acetonyl-4-chlorobenzyl)-4-hydroxy-coumarin	(93%)	(65%)	(95%)	(52%)
17 coumarin 152	(95%)	(96%)	(119%)	(75%)
18 coumarin	(101%)	(90%)	(98%)	317
19 7-hydroxycoumarin	(104%)	(89%)	(88%)	197
20 3-[2-(diethylamino)ethyl-]-7-hydroxy-4-methyl coumarin hydrochloride	(112%)	(99%)	(102%)	287
21 coumarin-3-carboxylic acid	(114%)	(92%)	(79%)	(67%)
22 troflox	(116%)	(88%)	(90%)	(72%)
23 7-hydroxycoumarin-4-acetic acid	(119%)	(83%)	(106%)	(65%)
24 (±)-warfarin	(134%)	(93%)	(92%)	(86%)
25 4-hydroxycoumarin	(135%)	(85%)	(91%)	(66%)

* Rat liver cAK, wheat germ CDPK, avian MLCK and rat brain PKC were assayed as described in the Experimental in the presence or absence of increasing concentrations of test compounds. All other details are as for Table 1.

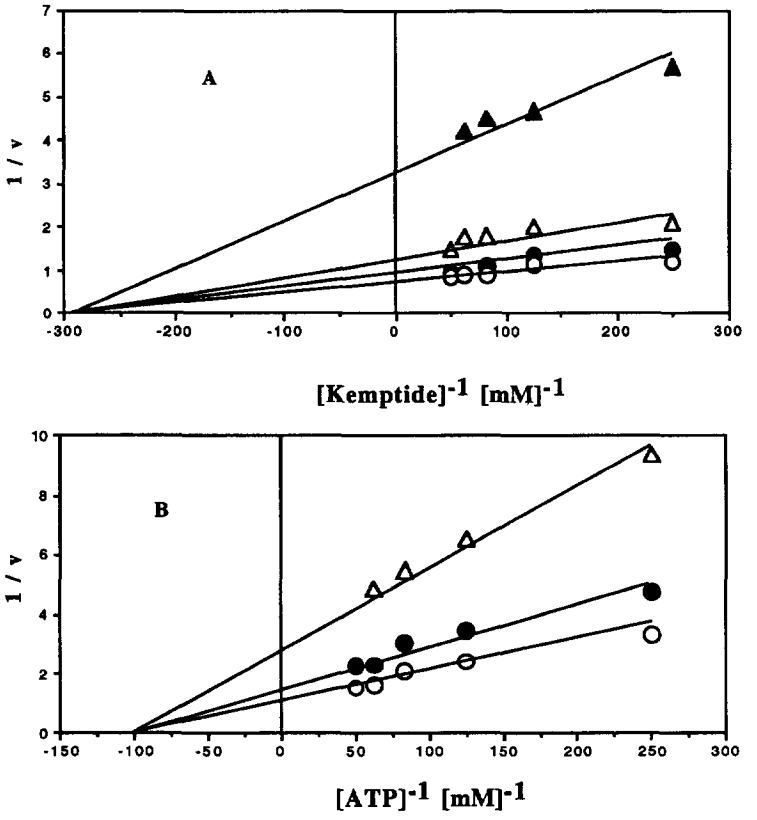


Fig. 5. Non-competitive inhibition of cAK by warangalone. Rat liver cAK was assayed at various concentrations of kemptide (A) or of ATP (B) in the presence or absence of various concentrations of warangalone. Double reciprocal plots of the data are presented (v^{-1} is in arbitrary units). (A) ○ – ○, no added inhibitor; ● – ●, 2 μM warangalone, △ – △, 4 μM warangalone; ▲ – ▲, 8 μM warangalone; (B) ○ – ○, no added inhibitor; ● – ●, 2 μM warangalone; △ – △, 4 μM warangalone.

that the dimethylallyl (prenyl) group contributes to this potency and specificity.

It is significant that all of effective cAK inhibitors found here, namely warangalone (**1**), 8-γ,γ-dimethylallylwighteone (**2**), 3′-γ,γ-dimethylallylwighteone (**3**), nallanin (**4**) and robustic acid (**10**) (IC_{50} values 3.5, 20, 24, 33 and 10 μM, respectively) are prenylated. It is notable that certain prenylated xanthones, namely mangostin and γ-mangostin, are also potent inhibitors of cAK [9]. These prenylated xanthones have a lower affinity for CDPK and a much lower affinity for MLCK [9].

Prenylation will contribute to the hydrophobicity of the effective inhibitors and hence would promote interaction of such ligands with a hydrophobic region of cAK. While warangalone (**1**) is a non-competitive inhibitor of cAK with respect to both ATP and peptide substrate (Fig. 5), this does not exclude the possibility that this compound binds close to the active site of cAK. Thus a key hydrophobic site on cAK is a hydrophobic pocket determined by β strand elements β1 and β2 and which is involved in binding the purine moiety of ATP [49].

The synthetic antimalarial halofantrine [26] and particular synthetic isoquinolines [30–32] are highly selective inhibitors of cAK. We have previously found that certain acidic triterpenoids and the naturally-occurring carotenoid crocetin are potent and selective inhibitors of cAK [22]. The present paper demonstrates that particular prenylated isoflavones and a prenylated coumarin can also be selective inhibitors of cAK. However, noting that cAK is one member of a large protein kinase family [49], it is quite possible that there are other high-affinity sites of action of these compounds. The interaction with cAK described here can conceivably contribute to the defence properties of these plants bioactive compounds.

The levels of warangalone and robustic acid in *D. scandens* roots can be estimated to be 3 μmol g^{-1} [46] and about 0.1 μmol g^{-1} [41], respectively. These levels correspond to concentrations of 3000 μM and 100 μM, respectively, as compared to the cAK IC_{50} values of 3.5 μM (Table 1) and 10 μM (Table 2), respectively. Accordingly one can envisage these compounds having a substantial effect on cAK in an insect feeding on this plant.

The apparent absence of cAK from plants [50] could make this enzyme, which is present in other eukaryotes including insects [50–52], an appropriate target for non-lethal plant defensive compounds [11, 21]. Elevated cyclic AMP concentration is a basic "hunger signal" in prokaryotes and in eukaryotes other than higher plants [53]. Activation of cAK is a major consequence of cyclic AMP elevation in eukaryotes [50–53]. Accordingly, inhibition of cAK in fungal pathogens and in insects and other herbivores by plant defensive compounds would effectively block cAK-mediated cellular responses to a catabolite deficit [53] in these plant-consuming organisms. In addition it should be noted that the absence of functional cAK in insects can lead to developmental as well as metabolic impairment [52].

EXPERIMENTAL

Materials. The compounds from *D. scandens* (**1–4**, **7**, **10** and **11**) were kindly supplied by Professor G. Srimannarayana, Department of Chemistry, Osmania University, Hyderabad 500 007, India. The isolation and identification of these compounds was as previously reported [48].

Genistein, genistin and 2-carbethoxy-5,7-dihydroxy-4′-methoxyisoflavone were obtained from Sigma. All other isoflavones and coumarins tested here were obtained from Aldrich [γ-^{32}P] ATP (specific activity 4000 Ci $mmol^{-1}$) was obtained from Bresatec, Adelaide, Australia. Kemptide (LRRASLG), epidermal growth factor receptor-derived synthetic peptide (EGFRP; VRKRTLRRL-NH_2) and myosin light chain-based synthetic peptide (MCLP; KKRAA-RATSNVFA-NH_2) were obtained from Auspep, Melbourne, Australia.

Protein kinase isolation and assay. Rat brain PKC (specific activity 0.6 mmol min^{-1} mg $protein^{-1}$) with 3.5 μM EGFRP as substrate), chicken gizzard myosin light chain kinase (MLCK) (specific activity 0.05 mmol min^{-1} mg $protein^{-1}$ with 20 μM MLCP as substrate), wheat embryo CDPK (specific activity 0.01 mmol min^{-1} mg $protein^{-1}$ with 1.0 mg ml^{-1} histone type III-S as substrate) and rat liver cyclic AMP-dependent protein kinase catalytic subunit (cAK) (specific activity 0.3 mmol min^{-1} mg $protein^{-1}$ with 20 μM kemptide as substrate) were extensively purified and assayed in standard assay conditions as described previously [8, 11, 18]. PKC, MLCK, CDPK and cAK were assayed using 3.5 μM EGFRP, 17 μM MLCP, 20 μM MLCP and 20 μM kemptide as substrates, respectively.

Inhibitor IC_{50} values (concns for 50% inhibition of particular protein kinases in the standard assay conditions) were determined from interpolation of plots of protein kinase activity versus inhibitor concn. Control protein kinase activity (no added inhibitor) was routinely determined in sextuplet and assays with inhibitor included were determined in duplicate. All assay results were corrected by subtraction of blank values from assays conducted in the absence of added protein kinase. The standard deviations associated with control protein kinase assays were about 10% of mean values. Inhibitor compounds were routinely dissolved in 10% (v/v) DMSO and added to protein kinase assays to give the following DMSO concentrations: 1.7% (v/v) (MLCK and PKC assays) and 2% (v/v) (cAK and CDPK assays). Control protein kinase assays (without added inhibitor) were conducted with inclusion of the appropriate concentration of DMSO. The absolute values of the control activities (nmol phosphoryl transferred min^{-1} ml^{-1}) were typically about 0.2 (cAK and PKC assays), 0.4 (CDPK assays) and 0.05 (MLCK assays), noting

that 20 μl of enzyme preparation was included in all protein kinase assays and that the final assay volumes were 100 μl (cAK and CDPK assays) and 120 μl (PKC and MLCK assays).

Acknowledgements—This research was supported by a grant to G.M.P. from the Australian Research Council and B.H.W. was supported by a La Trobe University Postgraduate Scholarship.

REFERENCES

1. Harborne, J. F., *Introduction to Ecological Biochemistry*, 3rd edn. Academic Press, London, 1988.
2. Nogrady, T., *Medicinal Chemistry. A Biochemical Approach*. Oxford University Press, Oxford, 1985.
3. Swain, T., *Annual Reviews of Plant Physiology*, 1977, **28**, 479.
4. Bell, E. A., in *Encyclopedia of Plant Physiology*, Vol. 8, eds E. A. Bell and B. V. Charlwood. Springer, Berlin, p. 11.
5. Bell, E. A., in *The Biochemistry of Plants*, Vol. 7, eds P. K. Stumpf and E. E. Conn. Academic Press, New York, 1981, p. 1.
6. Mann, J., *Secondary Metabolism*, 2nd edn. Clarendon Press, Oxford, 1987.
7. Kúc, J. A., in *Physiological Plant Pathology, Encyclopedia of Plant Physiology New Series*, Vol. 4, eds R. Heitefull and P. H. Williams. Springer, Berlin, 1976, p. 632.
8. Jinsart, W., Ternai, B. and Polya, G. M., *Biological Chemistry Hoppe-Seyler*, 1992, **373**, 903.
9. Jinsart, W., Ternai, B., Buddhasukh, D. and Polya, G. M., *Phytochemistry*, 1992, **31**, 3711.
10. Rogers, J. C. and Williams, D. L., *Biochemical and Biophysical Research Communications*, 1989, **164**, 419.
11. Jinsart, E., Ternai, B. and Polya, G. M., *Biological Chemistry Hoppe-Seyler*, 1991, **372**, 819
12. Gschwendt, M., Horn, F., Kittstein, W. and Marks, F., *Biochemical and Biophysical Research Communications*, 1983, **117**, 444.
13. Gschwendt, M., Horn, F., Kittstein, W., Furstenberger, G., Besemfelder, E. and Marks, F., *Biochemical and Biophysical Research Communications*, 1984, **124**, 63.
14. End, D. W., Look, R. A., Shaffer, N. L., Balles, E. A. and Perisco, F. J., *Research Communications in Chemical Pathology and Pharmacology*, 1987, **56**, 75.
15. Picq, M., Dubois, M., Munari-Silem, Y., Prigent, A. F. and Pacheco, H., *Life Science*, 1989, **44**, 1563.
16. Caulfield, J. J. and Bolander, F. F., *Journal of Endocrinology*, 1986, **109**, 29.
17. Ferriola, P. C., Cody, V. and Middleton, E., *Biochemical Pharmacology*, 1989, **38**, 1617.
18. Jinsart, W., Ternai, B. and Polya, G. M., *Biological Chemistry Hoppe-Seyler*, 1992, **373**, 205.
19. Graziani, Y., Erikson, E. and Erikson, R. L., *European Journal of Biochemistry*, 1983, **135**, 583.
20. Polya, G. M., and Foo, L. Y., *Phytochemistry*, 1994, **35**, 1399.
21. Polya, G. M., Wang, B. H. and Foo, L. Y., *Phytochemistry*, 1995, **38**, 307.
22. Wang, B. H. and Polya, G. M., *Phytochemistry*, 1996, **41**, 55.
23. Akiyama, T., Ishida, J., Nakagawaa, S., Ogawara, H., Watanabe, S. Itoh, N., Shibuya, M. and Fukami, Y., *Journal of Biological Chemistry*, 1987, **262**, 5592.
24. Johnson, M. S., Wolbers, W. B., Noble, J., Fennell, M. and Mitchell, R., *Molecular and Cellular Endocrinology*, 1995, **109**, 69.
25. Herbert, J. M., Augereau, J. M., Gleye, J. and Maffrarnd, J. P., *Biochemical and Biophysical Research Communications*, 1990, **172**, 993.
26. Wang, B. H., Ternai, B. and Polya, G. M., *Biological Chemistry Hoppe-Seyler*, 1994, **375**, 527.
27. Davis, P. D., Hill, C. H., Keech, E., Lawton, G., Nixon, J. S., Sedgwick, A. D., Wadsworth, J., Westmacott, D. and Wilkinson, S. E., *FEBS Letters*, 1989, **259**, 61.
28. Herbert, J. M., Seban, E. and Maffrano, J. P., *Biochemical and Biophysical Research Communications*, 1990, **171**, 189.
29. Kase, H., Iwahashi, K., Nakanishi, S., Matsuda, Y., Yamada, K., Takahashi, M., Murakata, G., Sato, A. and Kareko, M., *Biochemical Biophysical Research Communications*, 1987, **142**, 436.
30. Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriollet, F., Duhamel, L., Charon, D. and Kirilovsky, J., *Journal of Biological Chemistry*, 1991, **266**, 15771.
31. Davis, P. D., Elliott, L. H., Harris, W., Hill, C. H., Hurst, S. A., Keech, E., Kumar, M. K. H., Lawton, G., Nixon, J. S. and Wilkinson, S. E., *Journal of Medical Chemistry*, 1992, **35**, 994.
32. Hidaka, H., Inagaki, M., Kawamoto, S. and Sasaki, Y., *Biochemistry*, 1984, **23**, 5036.
33. Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T. and Hidaka, H., *Journal of Biological Chemistry*, 1990, **265**, 5267.
34. Hidaka, H. and Kobayashi, R., *Annual Review of Pharmacology and Toxicology*, 1992, **32**, 377.
35. Gescher, A. and Dale, I. L., *Anticancer Drug Design*, 1989, **4**, 93.
36. Powis, G., *Trends in Pharmaceutical Science*, 1991, **12**, 188.
37. Powis, G. and Kozikowski, A., *Clinical Biochemistry*, 1991, **24**, 385.
38. Chen, Q., Deady, L. W. and Polya, G. M., *Biological Chemistry Hoppe-Seyler*, 1994, **375**, 223.
39. Roark, J., *Journal of Economic Entomology*, 1933, **26**, 587.
40. Clarke, E. P., *Journal of Organic Chemistry*, 1943, **8**, 489.

41. Jones, H. A., *Journal of the American Chemical Society*, 1934, **56**, 1247.
42. Jones, H. A. and Haller, H. L., *Journal of Organic Chemistry*, 1943, **8**, 493.
43. Johnson, A. P., Pelter, A. and Stainton, P., *Journal of the Chemical Society*, (*C*) *Organic Chemistry*, 1966, 192.
44. Pelter, A. and Stainton, P., *Journal of the Chemical Society*, (*C*) *Organic Chemistry*, 1969, 701.
45. East, A. J., Ollis, W. D. and Wheeler, R. E., *Journal of the Chemical Society*, (*C*) *Organic Chemistry*, 1969, 365.
46. Falshaw, C. P., Harmer, R. A., Ollis, W. D. and Wheeler, R. E., *Journal of the Chemical Society*, (*C*) *Organic Chemistry*, 1969, 374.
47. Srimannarayana, G. and Subba Rao, N. V., *Journal of the Indian Chemical Society*, 1974, **LI**, 83.
48. Narayana Rao, M., Krupadanam, D. G. L. and Srimannarayana, G., *Phytochemistry*, 1994, **37**, 267.
49. Knighton, D. R., Zheng, J., Ten Eyck, L. F., Ashford, V. A., Xuong, N., Taylor, S. S. and Sowadski, J. M., *Science*, 1991, **253**, 407.
50. Polya, G. M., Chung, R. and Menting, J., *Plant Science*, 1991, **79**, 37.
51. Taylor, S. S., Buechler, J. A. and Yonemoto, W., *Annual Review of Biochemistry*, 1990, **59**, 971.
52. Lane, M. E. and Kalderon, D., *Genes Development*, 1993, **7**(7A), 1229.
53. Stryer, L., *Biochemistry*, 4th edn. Freeman, New York, 1995.