Pergamon

0031-9422(95)00583-8

Phytochemistry, Vol. 41, No. 1, pp. 55–63, 1996

0031-9422/96 $15.00 + 0.00

SELECTIVE INHIBITION OF CYCLIC AMP-DEPENDENT PROTEIN KINASE BY AMPHIPHILIC TRITERPENOIDS AND RELATED COMPOUNDS

BING HUI WANG*† and GIDEON M. POLYA‡

*Department of Chemistry, ‡Department of Biochemistry, La Trobe University, Bundoora, Victoria, 3083 Australia

(*Received in revised form* 12 *June* 1995)

Key Word Index—Triterpenoids; protein kinase inhibitors; cyclic AMP-dependent protein kinase.

Abstract—A set of plant- and animal-derived amphiphilic triterpenoids have been shown to be potent and selective inhibitors of the catalytic subunit of rat liver cyclic AMP-dependent protein kinase (cAK). Thus plant-derived 18α- and 18β-glycyrrhetinic acid, ursolic acid, oleanolic acid and betulin and animal-derived lithocholic acid, 5-cholenic acid and lithocholic acid methyl ester are inhibitors of cAK with IC_{50} values (concentrations for 50% inhibition) in the range 4–20 μM. These compounds are ineffective or relatively ineffective as inhibitors of various other eukaryote signal-regulated protein kinases namely wheat embryo Ca^{2+}-dependent protein kinase (CDPK), avian calmodulin-dependent myosin light chain kinase (MLCK) and rat brain Ca^{2+}- and phospholipid-dependent protein kinase C (PKC). These naturally occurring triterpenoids have a common structural motif involving polar residues located at opposite ends of an otherwise non-polar triterpenoid nucleus. A variety of triterpenoids not possessing this structural motif are relatively inactive as inhibitors of cAK and of CDPK, PKC and MLCK. The terpenoid amphiphilic compound crocetin is also a potent and relatively selective inhibitor of cAK (IC_{50}value for cAK 3.0 μM). 12-Hydroxystearic acid and 10-hydroxydecanoic acid do not inhibit CDPK, PKC or MLCK but are selective inhibitors of cAK (IC_{50} values 127 and 138 μM, respectively), consistent with a simple model for amphiphile inhibition of cAK involving two polar groups separated by a non-polar region. However, laurylgallate and 15-pentadecanolide are also potent and selective inhibitors of cAK (IC_{50} values 1.5 and 20 μM, respectively) although the structures of both of these compounds involve a large non-polar portion associated with only one polar region. Crocetin and the plant-derived amphiphilic triterpenoids described here are the most potent non-aromatic plant-derived inhibitors of cAK yet found.

INTRODUCTION

Plants elaborate a wide variety of defensive metabolites that defend these sessile organisms against motile microbial pathogens and herbivores [1, 2]. Determination of the molecular basis of plant defense involving such secondary metabolites involves the resolution of the high affinity biochemical sites of action of such compounds. In addition, plants represent a major source of pharmaceuticals and definition of such high affinity sites of action will define possible sites of medicinal action of bioactive compounds as well as providing *in vitro* biochemical screening systems for the detection of bioactive medicinal plant constituents [3]. Well-known examples of plant defensive metabolites for which the site of action are known include oubain (an inhibitor of Na^+, K^+-ATPase), curare (a nicotinic acetylcholine receptor antagonist) and cyanogenic glycosides [1, 4, 5].

A variety of plant defensive compounds are potent inhibitors of protein kinases involved in eukaryote signal transduction pathways. Thus various flavonoids inhibit cyclic AMP-dependent protein kinase (cAK) [6, 7], Ca^{2+}- and phospholipid-dependent protein kinase C (PKC) [8], myosin light chain kinase (MLCK) [6, 9] and plant Ca^{2+}-dependent protein kinase (CDPK) [6]. A range of plant condensed tannins are potent inhibitors of cAK, PKC and CDPK but not of MLCK [10] and several plant-derived xanthones are better inhibitors of cAK and plant CDPK than of MLCK [3]. Various plant-derived anthraquinone derivatives differentially inhibit cAK, PKC and MLCK [11] as do several synthetic analogues including the important clinically employed anti-tumour agent mitoxantrone [11]. A range of naturally occurring gallic acid esters are potent inhibitors of cAK and also inhibit PKC and CDPK [12]. However, the benzophenanthridine alkaloid chelerythrine is a specific inhibitor of PKC [13].

A variety of synthetic and naturally occurring compounds that inhibit PKC also have anti-tumour activity [1, 14–18]. The anti-tumour anthraquinone derivative mitoxantrone inhibits both PKC and MLCK [11] and a variety of anti-tumour acridine derivatives are potent selective inhibitors of MLCK [18]. Particular related aza-acridine derivatives are inhibitors of PKC and other eukaryote protein kinases [18, 19] including plant

†Author to whom correspondence should be addressed.

CDPK [19]. The important anti-malarial halofantrine is a highly selective and potent inhibitor of cAK [20]. While these high affinity interactions of such bioactive compounds with particular protein kinases are not necessarily primarily responsible for the chemotherapeutic (e.g. anti-tumour or anti-protozoal) activities *in vivo* [18, 20], they may nevertheless contribute to the biological effects of such compounds. Such high affinity biochemical interactions may provide the basis for *in vitro* biochemical detection of plant bioactive compounds of potential pharmaceutical use [21].

Triterpenoids represent a very large class of plant secondary metabolites [22–25] of which the acidic triterpenoids represent a particular subset of such compounds for which a variety of biological activities have been described. The reported biological effects of particular acidic triterpenoids include cytotoxic, anti-tumour and anti-inflammatory activities [2, 26–28] and anti-viral activity [29]. In particular, the acidic triterpenoid ursolic acid inhibits lipoxygenase and cyclo-oxygenase *in vivo* [30], histamine release from mast cells [31], mutagenicity [32] and leukaemic cell proliferation [27]. In a plant context, ursolic acid inhibits synthesis of jasmonate-induced proteins in barley leaves [33]. The anti-inflammatory action of ursolic acid and the structurally related triterpenoid oleanolic acid has been related to the activity of these compounds as inhibitors of leukocyte elastase (K_i values 4 and 6 μM, respectively) [26]. However, clearly other biochemical sites of action could also be involved in the biological activity of such acidic triterpenoids. It is clearly important to define biochemical sites of action in order to establish the mechanisms involved in the medicinal and plant defence properties of these bioactive compounds. We have examined the possibility of interactions of such compounds with signal-regulated protein kinases and the present paper describes the potent and highly selective inhibition of cAK by ursolic acid, oleanolic acid and by other structurally related compounds. These bioactive plant compounds are the most potent non-aromatic plant-derived inhibitors of cAK yet found.

RESULTS

Inhibition of protein kinases by triterpenoids

Four signal-regulated eukaryote protein kinases were screened for inhibition by the triterpenoids targeted in this investigation. The protein kinases were assayed employing synthetic oligopeptide substrates rather than high M_r protein substrates to avoid possible interactions of the test compounds with such proteins [6, 7]. Both

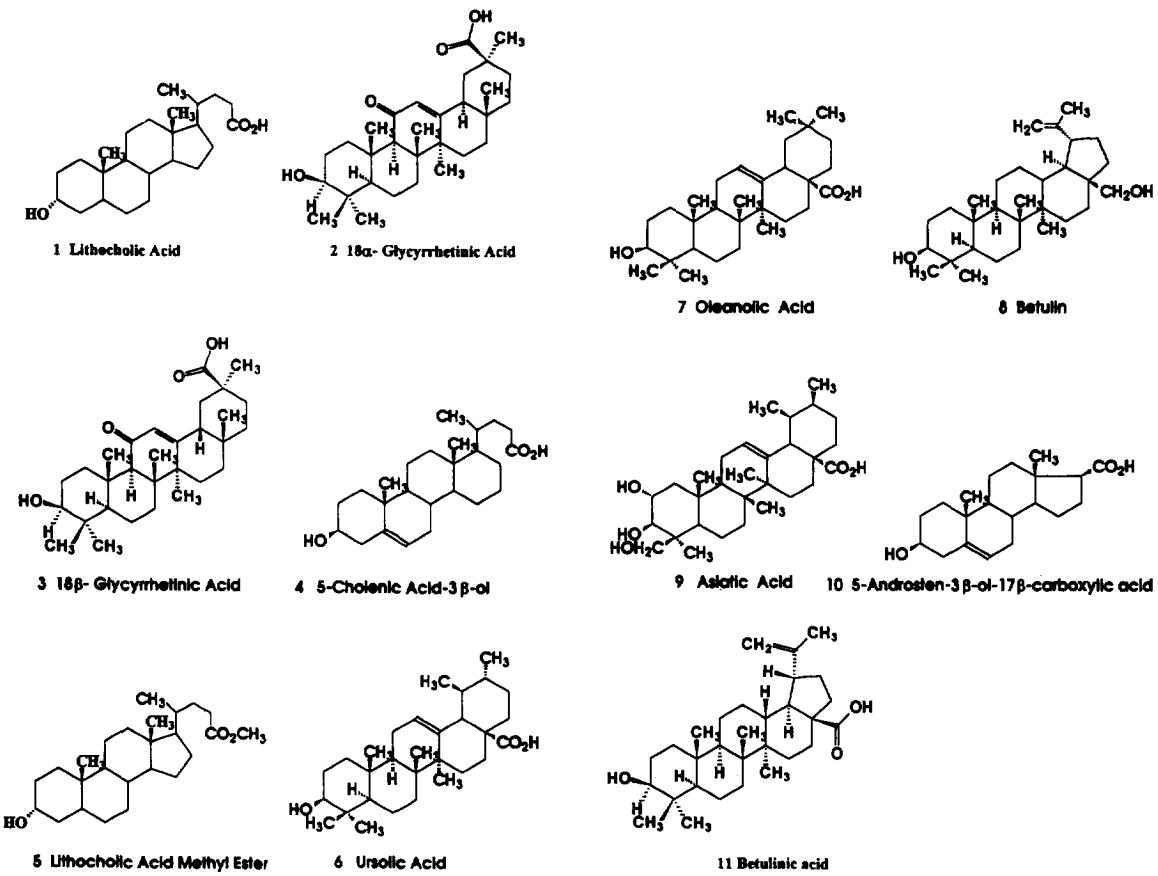

Fig. 1. Structures of triterpenoid protein kinase inhibitors.

wheat embryo CDPK and avian gizzard calmodulin-dependent MLCK were assayed using the myosin light chain-based peptide MLCP (KKRAARATSNVFA-NH_2) as substrate. Rat liver cAK was assayed using kemptide (LRRASLG) as substrate and rat brain Ca^{2+}- and phospholipid-dependent PKC was assayed with the epidermal growth factor receptor-based peptide EGFRP (VRKRTLRRL-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 [3, 6, 7, 10, 11, 19]. Further, it was of interest to see if plant defensive triterpenoid secondary metabolites were ineffective as inhibitors of plant signal-regulated CDPK as found for a variety of flavonoids that inhibit cAK [6, 7]. The structures of the most potent triterpenoid inhibitors found in this study are presented in Fig. 1.

The most potent plant-derived triterpenoid inhibitors of cAK found here are 18α-glycyrrhetinic acid (**2**) (IC_{50} 6.2 μM), 18-β-glycyrrhetinic acid (**3**) (IC_{50} 6.5 μM), ursolic acid (**6**) (IC_{50} 9.0 μM), oleanolic acid (**7**) (IC_{50} 12 μM), betulin (**8**) (IC_{50} 20 μM) and asiatic acid (**9**) (IC_{50} 22 μM). In addition, 5-androsten-3-β-ol-17β-carboxylic acid (**10**) and betulinic acid (**11**) also inhibit cAK (IC_{50} values 42 μM and 45 μM, respectively) (Table 1; Fig. 2). However, lithocholic acid (**1**), an animal bile-derived triter penoid, is the most potent triterpenoid inhibitor of the cAK found in this study (IC_{50} 4.2 μM; Table 1; Fig. 2).

None of compounds **1**–**11** cause significant inhibition of MLCK at 0.3 mM and these compounds are either inactive or are relatively poor inhibitors of PKC and CDPK (Table 1). Thus, asiatic acid (**9**) inhibits CDPK(IC_{50} 40 μM) and the only other moderately effective CDPK inhibitors are α-hederin (**18**), betulinic acid (**11**), ursolic acid (**6**), oleanolic acid (**7**) and betulin (**8**) (IC_{50} values 64, 84, 71, 112 and 75 μM, respectively) (Table 1). A number of the triterpenoids examined inhibit PKC and are listed below (IC_{50} values indicated in parenthesis): lithocholic acid (**1**; 57 μM), betulinic acid (**11**; 145 μM), 18α-glycyrrhetinic acid (**2**; 159 μM), 18β-glycyrrhetinic acid (**3**; 121 μM), ursolic acid (**6**; 106 μM), oleanolic acid (**7**; 250 μM) and asiatic acid (**9**; 400 μM)

Table 1. Inhibition of protein kinases by amphiphilic triterpenoids*

Compound	IC_{50} (μM or % control)			
	cAK	CDPK	PKC	MLCK
1. Lithocholic acid	4.2	(81%)	57	(131%)
2. 18α-Glycyrrhetinic acid	6.2	(142%)	159	(83%)
3. 18β-Glycyrrhetinic acid	6.5	(57%)	121	(91%)
4. 5-Cholenic acid-3β-ol	7.8	(81%)	(90%)	(120%)
5. Lithocholic acid methyl ester	9.0	(85%)	(106%)	(105%)
6. Ursolic acid	9.0	71	106	(89%)
7. Oleanolic acid	12	112	250	(106%)
8. Betulin	20	75	(70%)	(104%)
9. Asiatic acid	22	40	400	(90%)
10. 5-Androsten-3-β-ol-17β carboxylic acid	42	(105%)	(96%)	(83%)
11. Betulinic acid	45	84	145	(90%)
12. Uvaol	165	(67%)	(89%)	(101%)
13. Asiaticoside	190	(58%)	(85%)	(96%)
14. Chenodeoxycholic acid	221	(96%)	(91%)	(87%)
15. Glycyrrhyzic acid	360	(74%)	(93%)	(83%)
16. 3-β-Hydroxychol-5-en-24-oic acid methyl ester	369	(90%)	(108%)	(92%)
17. Taurodeoxycholic acid	(49%)	(79%)	(104%)	(115%)
18. α-Hederin	(50%)	64	(61%)	(78%)
19. Taurocholic acid	(53%)	(89%)	(104%)	(103%)
20. Taurochenodeoxycholic acid	(54%)	(81%)	(125%)	(101%)
21. Ouabagenin	(55%)	(93%)	(107%)	(103%)
22. Ouabain	(57%)	(87%)	(92%)	(117%)
23. Deoxycholic acid	(57%)	(75%)	(86%)	(95%)
24. Glycochenodeoxycholic acid	(62%)	(97%)	(83%)	(64%)
25. Glycocholic acid	(71%)	(97%)	(114%)	(113%)
26. Dihydrocholesterol	(91%)	(82%)	(117%)	(99%)
27. Dehydrocholic acid	(94%)	(102%)	(106%)	(106%)
28. Cholic acid methyl ester	(101%)	(86%)	(120%)	(84%)
29. Cholic acid	(107%)	(100%)	(122%)	(95%)

*Rat liver cAK, wheat germ CDPK, avian MLCK and rat brain PKC were assayed as described in 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). % Control activity (no added inhibitor) with 0.3 mM inhibitor (PKC and MLCK assays) and 0.4 mM inhibitor (cAK and CDPK assays) is given in parentheses.

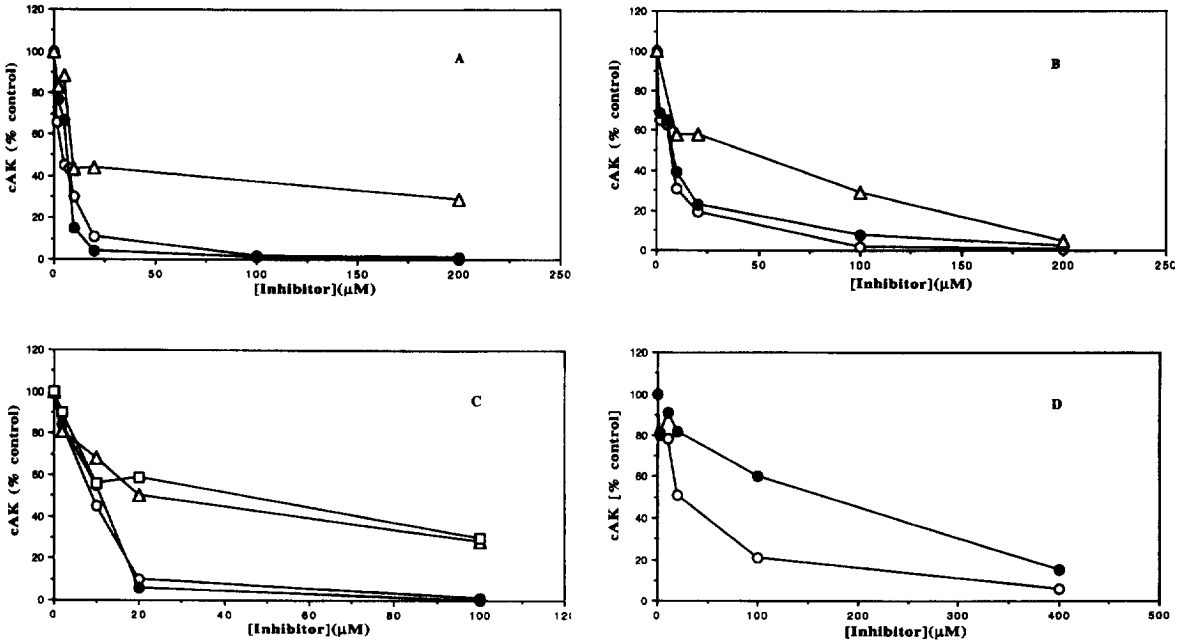

Fig. 2. Inhibition of cAK by amphiphilic triterpenoids. Rat liver cAK was assayed with 20 μM kemptide as substrate in the standard assay conditions containing 2% (v/v) DMSO and increasing concentrations of triterpenoid inhibitors. The cAK activity is expressed as % control (no added inhibitor). (A) ○, Lithocholic acid (**1**); ●, 18α-glycyrrhetinic acid (**2**); △, lithocholic acid methyl ester (**5**). (B) ○, 18β-Glycyrrhetinic acid (**3**); ●, 5-cholenic acid-3-β-ol (**4**); △, 5-androsten-3-β-ol-17β-carboxylic acid (**10**) ○, Ursolic acid (**6**); ●, Oleanolic acid (**7**); △, Betulin (**8**); □, Betulinic acid (**11**). (D) ○, Asiatic acid (**9**); ●, uvaol (**12**).

(Table 1). However, the IC_{50} values for PKC in all cases are 10 to 20 times higher than the IC_{50} values with respect to cAK (Table 1). Thus the amphiphilic triterpenoids **1**–**11** are relatively specific inhibitors of cAK.

All of the more potent triterpenoid inhibitors of cAK (compounds **1**–**11**) have the common structural features of having an amphiphilic character defined by a 3-hydroxy and a polar residue at the distal end of the molecule with no polar groups in between. In all cases, except for lithocholic acid methyl ester (**5**) and betulin (**8**), the polar residue well removed from the 3-hydroxy group is a carboxyl group. However, lithocholic acid methyl ester (**5**; IC_{50} 9.0 μM) is effective as an inhibitor albeit less so than lithocholic acid (**1**). Betulin (**8**; IC_{50} 20 μM) which has a 17-hydroxymethyl substituent is much less effective than betulinic acid (**11**) (the 17-carboxy analogue of betulin) (Table 1). However, uvaol (**12**) (IC_{50} 165 μM for cAK and having a 17-hydroxymethyl substituent) is much less effective as an inhibitor of cAK than ursolic acid (**6**), the 17-carboxy-substituted analogue of betulin (IC_{50} 9.0 μM). 3-β-Hydroxychol-5-en-24-oic acid methyl ester (**16**) (IC_{50} 369 μM) is much less effective than the non-methylated analogue 5-cholenic acid-3-β-ol (**4**) (IC_{50} 7.8 μM) (Table 1). Dihydrocholesterol (**26**), which has a 3-hydroxy but does not have an additional distal polar group, is inactive as a cAK inhibitor (Table 1).

Substitution of the 3-hydroxy group also greatly decreases cAK inhibitory effectiveness. Thus glycyrrhyzic acid (**15**) (IC_{50} value 360 μM) is a very poor inhibitor in comparison with the corresponding analogues having an unsubstituted 3-hydroxy group, namely 18α- and 18β-glycyrrhetinic acids (**2**, **3**; IC_{50} values 6.2 and 6.5 μM, respectively). Similarly the 3-*O*-glycosylated triterpenoid α-hederin (**18**) (IC_{50} about 400 μM) is much less active than otherwise structurally similar triterpenoids with a non-substituted 3-hydroxy such as ursolic acid (**6**), oleanolic acid (**7**) and asiatic acid (**9**) (IC_{50} values 9.0, 12.0 and 22 μM, respectively) (Table 1). Dehydrocholic acid (**27**) which has a 23-carboxy and a 3-oxo substituent is a very poor inhibitor of cAK (Table 1).

A variety of triterpenoids having a 3-hydroxy and a distal polar group but also having hydroxy group(s) located in between on the triterpenoid nucleus are ineffective as cAK inhibitors, namely chenodeoxycholic acid (**14**), taurodeoxycholic acid (**17**), taurocholic acid (**19**), taurochenodeoxycholic acid (**20**), ouabagenin (**21**), deoxycholic acid (**23**), glycochenodeoxycholic acid (**24**), glycocholic acid (**25**), cholic acid methyl ester (**28**) and cholic acid (**29**). Ouabain (**22**), a 3-*O*-glycosyl derivative of ouabagenin (**21**), is also relatively ineffective as an inhibitor of cAK (Table 1). It is notable that while compounds having hydroxy groups located between the 3-hydroxy and the requisite distal group are inactive, an 11-oxo substituent does not preclude inhibitory effectiveness. Thus, 18α-glycyrrhetinic acid (**2**) and 18β-glycyrrhetinic acid (**3**), which both have an 11-oxo group, are among the most potent triterpenoid inhibitors of cAK found here (Table 1).

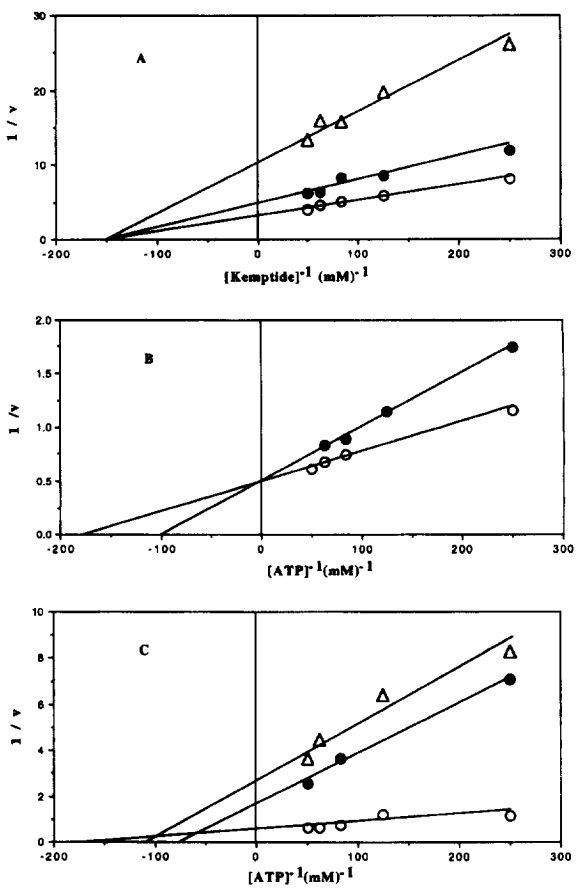

Fig. 3. Non-competitive inhibition of rat liver cAK by ursolic acid. (A) Rat liver cAK was assayed as described in Experimental with 20 μM ATP and at various concentrations of kemptide in the absence or presence of ursolic acid (**6**). A double-reciprocal plot of the data is presented (v^{-1} is in arbitrary units): ○, no added **6**; ●, 2 μM **6**; △, 16 μM **6**. (B) Rat liver cAK was assayed in the standard conditions with 20 μM kemptide and at various concentrations of ATP in the presence or absence of ursolic acid (**6**); ○, no added **6**; ●, 2 μM **6**. (C) Rat liver cAK was assayed in the standard conditions with 20 μM kemptide and at various concentrations of ATP in the presence or absence of ursolic acid (**6**). ○, No added **6**; ●, 10 μM **6**; △, 16 μM**6**.

Lineweaver–Burk double-reciprocal plots of v_0^{-1} versus [substrate]$^{-1}$ from kinetic experiments revealed that the K_m for kemptide is the same in the absence of inhibitor or in the presence of various concentrations of ursolic acid (**6**) (Fig. 3). Thus, ursolic acid inhibits cAK in a fashion that is apparently non-competitive with respect to the peptide substrate. The K_i for ursolic acid determined from these experiments is 3.9 ± 2.5 μM (mean $\pm$ standard deviation from four estimations). However, while ursolic acid when present at 2 μM is apparently competitive with respect to ATP (K_i 2.6 μM), it is apparently non-competitive with respect to ATP at higher concentration (5, 10 and 16 μM) (Fig. 3) (K_i 6.0 ± 1.1 μM; mean $\pm$ standard deviation from three estimations). The K_i estimates for ursolic acid as a non-competitive inhibitor of cAK are similar to the IC_{50} value for ursolic acid (8 μM) (Table 1).

Inhibition of PKC by triterpenoids

The only triterpenoids found here to inhibit PKC have a 3-hydroxy group and also have a carboxyl substituent well removed from the 3-hydroxy group (Fig. 4; Table 1). Thus while lithocholic acid (**1**) inhibits PKC (IC_{50} value 57 μM), lithocholic acid methyl ester (**6**) is ineffective at 0.3 mM (Table 1). Betulinic acid (**11**) inhibits PKC but betulin (**8**) (which has a 28-hydroxymethyl substituent as compared to the 28-carboxy of betulinic acid (**11**)) is ineffective. Similarly, while ursolic acid (**6**) inhibits PKC (IC_{50} 106 μM), uvaol (**12**) (which has a 28-hydroxymethyl group instead of the 28-carboxy group of ursolic acid) is ineffective. Dihydrocholesterol (**26**) which has a 3-hydroxy group but no distal carboxyl substituent, does not inhibit PKC (Table 1). While 18α- and 18β-glycyrrhetinic acids (compounds **2** and **3**) inhibit PKC (IC_{50} values 159 μM and 121 μM, respectively), the glycyrrhetinic acid 3-*O*-glycosylated derivative glycyrrhyzic acid (**15**) is ineffective. Dehydrocholic acid (**27**) (3,7,12-trioxo-5β-cholan-24-oic acid) lacks a 3-hydroxy group and does not inhibit PKC. We conclude that inhibition of PKC by triterpenoids requires a 3-hydroxy group and a carboxyl substituent at the distal part of the molecule.

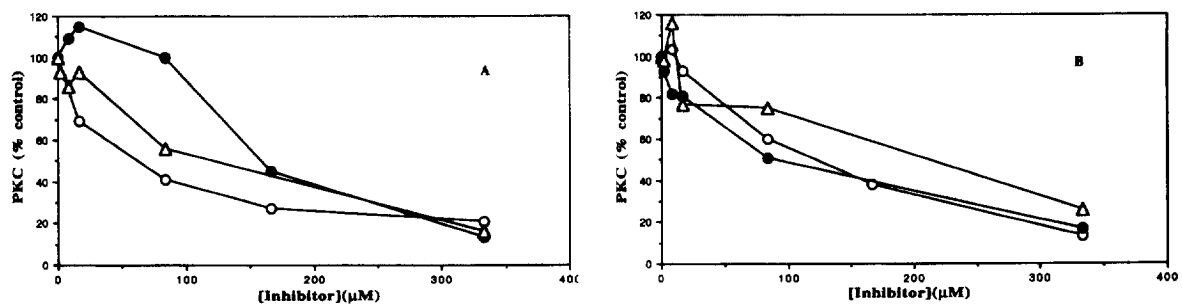

Fig. 4. Inhibition of PKC by amphiphilic triterpenoids. Rat brain PKC was assayed with 3.5 μM EGFRP as substrate in the standard assay conditions containing 1.7% DMSO and increasing concentrations of triterpenoid inhibitors. The PKC activity is expressed as % of control (no added inhibitor). (A) ○, lithocholic acid (**1**); ●, 18α-glycyrrhetinic acid (**2**); △, betulinic acid (**11**). (B) ○, 18β-glycyrrhetinic acid (**3**); ●, ursolic acid (**6**); △, oleanolic acid (**7**).

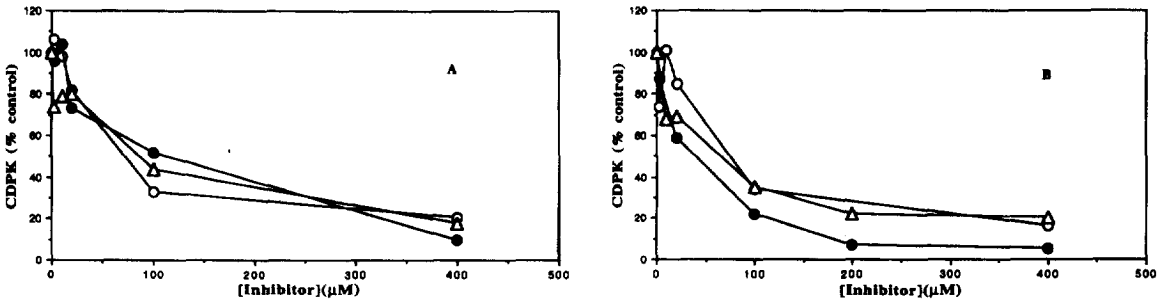

Fig. 5. Inhibition of wheat embryo CDPK by amphiphilic triterpenoids. Wheat embryo CDPK was assayed with 20 μM MLCP as substrate in the standard assay conditions containing 2.0% DMSO and increasing concentrations of triterpenoid inhibitors. CDPK activity is expressed as % of control (no added inhibitor). (A) ○, Ursolic acid (**6**); ●, oleanolic acid (**7**); △, betulinic acid (**11**). (B) ○, Betulin (**8**); ●, asiatic acid (**9**); △, α-hederin (**18**).

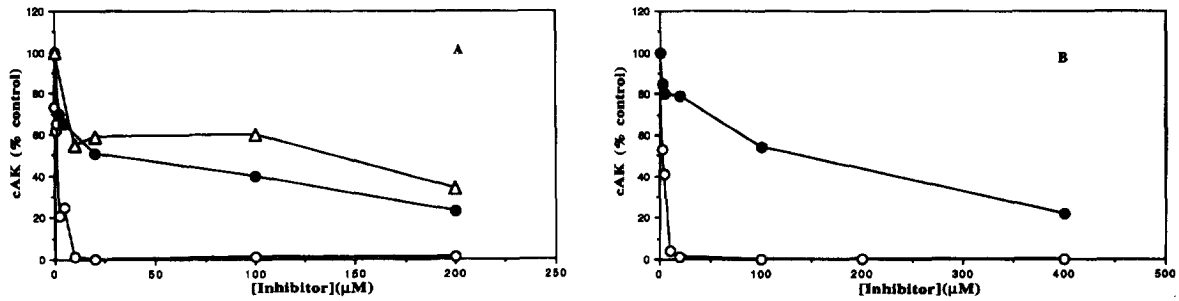

Fig. 6. Inhibition of cAK by non-triterpenoid amphiphilic compounds. Rat liver cAK was assayed with 20 μM kemptide as substrate in the standard assay conditions containing 2% (v/v) DMSO and increasing concentrations of inhibitors. The cAK activity is expressed as % of control (no added inhibitor). (A) ○, Laurylgallate (**30**); ●, 15-pentadecanolide (**32**); △, 10-hydroxydecanoic acid (**34**). (B) ○, crocetin (**31**); ●, 12-hydroxystearic acid (**33**).

Triterpenoids having a 3-hydroxy group and a distal carboxyl but with a polar substituent in between (compounds, **14**, **17**, **19**–**25**, **28** and **29**) are poor inhibitors of PKC (Table 1).

Inhibition of plant CDPK by triterpenoids

A small set of triterpenoids have been found here to inhibit wheat embryo CDPK, namely betulinic acid (**11**; IC_{50} 84 μM), ursolic acid (**6**; IC_{50} 71 μM), oleanolic acid (**7**, IC_{50} 112 μM), betulin (**8**; IC_{50} 75 μM), asiatic acid (**9**; IC_{50} 40 μM) and α-hederin (**18**; IC_{50} 64 μM) (Fig. 5; Table 1). All other triterpenoids tested are inactive or very poor inhibitors of plant CDPK (Table 1). Of the effective CDPK inhibitors found all have a 3-hydroxy group and a distal carboxyl substituent with the exception of betulin (**9**) which has a 3-hydroxy substituent and a 17-hydroxymethyl substituent. Betulinic acid (**11**) and the 17-hydroxymethyl analogue betulin (**8**) have comparable activity as inhibitors of CDPK (IC_{50} values for CDPK 84 μM and 75 μM, respectively) and of cAK (IC_{50} values for cAK 45 μM and 20 μM, respectively (Table 1).

Inhibition of cAK by some non-triterpenoid amphiphilic compounds

Various amphiphilic compounds resembling the best triterpenoid cAK inhibitors in having two polar groups separated by a non-polar spacer were examined as potential inhibitors of cAK (Fig. 6; Table 2). Of such compounds tested crocetin (**31**) is the most potent cAK inhibitor found (IC_{50} for cAK, 3.0 μM). Crocetin also inhibits PKC (IC_{50} 80 μM) but is a poor inhibitor of CDPK and MLCK. In crocetin (**31**), two polar groups are separated by a non-polar 14-carbon atom spacer involving 7 conjugated double bonds. 12-Hydroxystearic acid (12-hydroxyoctadecanoic acid) (**33**) and10-hydroxydecanoic acid (**34**) also inhibit cAK albeit less effectively than crocetin (IC_{50} values 127 μM and 138 μM, respectively). Various dicarboxylic acids in which the carboxyls are separated by 5, 6 or 7 methylenes namely pimelic acid (heptanedioic acid) (**35**), suberic acid (octanedioic acid) (**36**) and azelaic acid (nonanedioic acid) (**37**), respectively, are not active as protein kinase inhibitors (Table 2). Evidently some non-triterpenoid amphiphilic compounds that resemble the amphiphilic triterpenoid protein kinase inhibitors in having two polar

Table 2. Inhibition of protein kinases by amphiphilic compounds*

Compound	IC_{50} (μM or % control)			
	cAK	CDPK	PKC	MLCK
30. Laurylgallate	1.5	(85%)	(50%)	(104%)
31. Crocetin	3.0	(65%)	80	(63%)
32. 15-Pentadecanolide	20	(85%)	(140%)	(64%)
33. 12-Hydroxystearic acid	127	(92%)	(82%)	(94%)
34. 10-Hydroxydecanoic acid	138	(82%)	(97%)	(92%)
35. Pimelic acid	(74%)	(75%)	(127%)	(100%)
36. Suberic acid	(81%)	(90%)	(121%)	(73%)
37. Azelaic acid	(83%)	(95%)	(93%)	(117%)

*For details see Table 1.

regions separated by a non-polar spacer of appropriate length, can be protein kinase inhibitors. However, laurylgallate (**30**), the gallic acid ester of lauryl alcohol (*n*-dodecanol; $CH_3(CH_2)_{10}CH_2OH$), is a potent inhibitor of cAK (IC_{50} 1.5 μM) while not inhibiting CDPK or MLCK and being a very poor inhibitor of PKC (IC_{50} about 0.3 mM) (Table 2). 15-Pentadecanolide (**32**), the lactone of 15-hydroxypentadecanoic acid ($HO(CH_2)_{14}COOH$), is also a potent inhibitor of cAK (IC_{50} 20 μM) and is ineffective as an inhibitor of PKC, MLCK and CDPK (Table 2). Laurylgallate (**30**) and 15-pentadecanolide (**32**) represent examples of amphiphilic compounds that are relatively selective inhibitors of cAK but which have only one polar region associated with a large non-polar structural domain.

DISCUSSION

The present paper shows that a variety of amphiphilic triterpenoids are selective and potent inhibitors of cAK, these compounds having a common structural motif involving polar residues located at opposite ends of an otherwise non-polar triterpenoid structure. Most of the effective compounds have a 3-hydroxy group and a distal carboxyl group and also inhibit PKC but with IC_{50} values for PKC 10–20 times greater than for cAK. Some non-triterpenoid compounds having two polar groups separated by a non-polar spacer of appropriate size — notably crocetin (**31**) — are also selective inhibitors of cAK. Nevertheless, laurylgallate (**30**), having only one polar region associated with a large non-polar domain, is also a selective and potent inhibitor of cAK. In this connection, we have previously shown that a variety of hydrolysable tannins that are gallic acid esters are potent inhibitors of cAK while being, like the amphiphilic triterpenoids, less effective as inhibitors of MLCK, PKC and of plant CDPK [12]. Increasing potency of these inhibitors correlates with increasing number of galloyl residues but gallic acid (3,4,5-trihydroxybenzoic acid) is ineffective as an inhibitor of cAK or of these other protein kinases [12].

The plant-derived amphiphilic triterpenoids shown here to be potent inhibitors of rat liver cAK are biologically active in various animal systems. 18-α-Glycyrrhetinic acid (**2**), 18-β-glycyrrhetinic acid (**3**), ursolic acid (**8**), oleanolic acid (**7**), betulinic acid (**11**) and the α-hederin (**18**) aglycone hederagenin have anti-inflammatory activity [2, 26]. Leukocyte elastase is inhibited by ursolic acid (**6**) and oleanolic acid (**7**) (K_i values 5 μM and 6 μM, respectively) and by related amphiphilic triterpenoids such as erythrodiol, uvaol (**12**), hederagenin and 18-β-glycyrrhetinic acid (**3**) [26]. It has been suggested that inhibition of leucocyte elastase by such compounds may contribute to their anti-inflammatory effects but clearly other mechanisms may also be involved [26]. Thus, inhibition of histamine release [31] and of prostaglandin oxidation [34] have been suggested as possible anti-inflammatory mechanisms for such compounds. The anti-inflammatory effect of glycyrrhizin (**15**) and glycyrrhetinic acid (**2**, **3**) [35–37] can be explained in terms of intestinal conversion of glycyrrhizin to glycyrrhetinic acid and the potent inhibition of 11β-hydroxysteroid dehydrogenase by glycyrrhetinic acid (K_i 8 nM) [38]. The consequent inhibition of the conversion of cortisol to cortisone results in enhanced cortisol binding to glucocorticoid receptor and consequent anti-inflammatory effects [28].

A variety of amphiphilic triterpenoid cAK inhibitors are cytotoxic or have anti-tumour properties, namely lithocholic acid (**1**) [39], betulinic acid (**11**), 18-β-glycyrrhetinic acid (**3**), ursolic acid (**6**), oleanolic acid (**7**) and betulin [2]. Inhibition of lipoxygenase by ursolic acid (**6**) has been implicated in the inhibition of leukaemic cell growth by this triterpenoid [27]. A variety of amphiphilic triterpenoid cAK inhibitors have hepatoprotective effects, namely 18-α-glycyrrhetinic acid (**2**), ursolic acid (**6**), oleanolic acid (**7**) and uvaol (**12**) [40]. Protection from Cd^{2+}-induced liver damage afforded by α-hederin (**18**), ursolic acid (**7**), oleanolic acid (**8**) and uvaol (**12**) may derive from an increase of hepatic metallothionein levels induced by these compounds [40]. Similarly, the skin healing properties of asiatic acid (**10**) may be due in part to the promotion of collagen synthesis [41]. The inhibition by lithocholic acid (**1**) of the expression of HLA class I genes in colon cells may contribute to loss of immune surveillance and to tumourigenesis [42]. The inhibition

of cAK by the plant- and animal-derived amphiphilic triterpenoids described here could conceivably contribute to such biological effects of these compounds. Thus, cAK is involved in the regulation of a variety of cellular processes including metabolism, cell division, specific gene expression and development [43–45].

The potent triterpenoid cAK inhibitors described here (Table 1) and structurally related compounds variously interact with certain other proteins. Thus, leukocyte elastase is inhibited by ursolic acid (**6**), oleanolic acid (**7**) uvaol (**12**) and 18β-glycyrrhetinic acid (**3**) (IC_{50} values 4, 6, 16 and 185 μM, respectively) [26]. Glycyrrhetinic acid (**2, 3**) is a potent inhibitor of 11β-hydroxysteroid dehydrogenase (K_i 8 nM) [38]. Lithocholic acid (**1**) binds tightly to a variety of liver cytoplasmic proteins including glutathione-*S*-transferases (K_i values 0.5–5 μM), fatty acid binding proteins (K_d values 5–10 μM) and various 30–35 kDa proteins [46] including a protein homologous to 3α-hydroxysteroid dehydrogenase (K_d 1 μM [47]). The IC_{50} values of the better triterpenoid cAK inhibitors for cAK (Table 1) are similar to the K_d values for these triterpenoid–protein interactions (with the exception of that for the glycyrrhetinic acid—11β-hydroxysteroid dehydrogenase interaction).

A variety of triterpenoid analogues of betulinic acid (**11**) inhibit HIV replication and several of these triterpenoids inhibit PKC (IC_{50} values about 50 μM) [48]. The present paper represents the first report of non-aromatic plant defensive compounds (namely triterpenoids and crocetin) as potent inhibitors of cAK. We have previously shown that cAK is inhibited by particular flavonoids [6], xanthones [3], anthraquinones [11], gossypol [7], condensed tannins [10] and gallic acid esters [12]. Gossypol and the effective flavonoid, xanthone and anthraquinone inhibitors of cAK have a common general feature in that all of these compounds have planar, hydroxylated, polycyclic structures. The non-aromatic triterpenoid cAK inhibitors may have a similar structural feature. Thus the amphiphilic terpenoid hederagenin (3β,23-dihydroxyolean-12-en-28-oic acid) is structurally very similar to ursolic acid (7; 3α-hydroxyurs-12-en-28-oic acid). The structure of hederagenin has been determined by X-ray crystallography [49]. The four alicyclic rings of hederagenin between the 3-hydroxy group and the 18-carboxyl group adopt an extended, approximately planar arrangement with only the fifth ring of this pentacyclic triterpenoid being substantially out of this plane [49]. We have previously argued that the apparent absence of cAK from higher plants could make this enzyme a suitable target for non-lethal plant defensive compounds [6, 12]. The present paper shows that a particular sub-set of acidic terpenoids are, like a wide variety of polyphenolic plant defensive compounds, potent inhibitors of cAK. However, it is clear that other eukaryote proteins may also have high affinities for such triterpenoids [26, 38] and polyphenolics [12]. Crocetin and the amphiphilic triterpenoids described here are the only non-aromatic plant-derived compounds yet shown to be potent inhibitors of cAK. The high affinity interactions of these compounds with cAK may contribute to the biological effects *in vivo* of these bioactive plant secondary metabolites.

EXPERIMENTAL

Materials. [γ-^{32}P]ATP(4000 Ci mmol^{-1}) was obtained from Bresatec, Adelaide, Australia. Kemptide (LRRASLG), epidermal growth factor receptor-derived synthetic peptide (EGFRP; VRKRTLRRL-NH_2) and MLCP (KKRAARATSNVFA-NH_2) were obtained from Auspep (Melbourne, Australia). Laurylgallate, 12-hydroxystearic acid, 10-hydroxydecanoic acid, betulinic acid, ursolic acid, oleanolic acid, betulin, dehydrocholic acid and uvaol were obtained from Aldrich. All other compounds used were obtained from the Sigma Chemical Co.

Protein kinase isolation and assay. Rat brain PKC (specific activity 0.6 μmol min^{-1} mg protein mol^{-1} with 3.5 μM EGFRP as substrate), chicken gizzard MLCK (specific activity 0.05 μmol min^{-1} mg protein^{-1} with 20 μM MLCP as substrate), wheat embryo CDPK (specific activity 0.01 μmol 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 0.3μmol min^{-1} mg protein^{-1} with 20 μM kemptide as substrate) were extensively purified and assayed in standard assay conditions as described previously [12].

Inhibitor IC_{50} values (concentrations for 50% inhibition of particular protein kinases in the standard assay conditions) were determined from interpolation of plots of protein kinase activity versus inhibitor concentration. 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 the mean values. Inhibitor compounds were routinely dissolved in 10% (w/v) DMSO and added to protein kinase assays to give the following DMSO concentrations: 1.7% (w/v) (MLCK and PKC assays) and 2% (w/v) (cAK and CDPK assays). Control protein kinase assays (without added inhibitor) were conducted with inclusion of the appropriate concentration of DMSO.

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

REFERENCES

1. Harborne, J. B. (1988) *Introduction to Ecological Biochemistry* (3rd edn). Academic Press, London.
2. Harborne, J. B. and Baxter, H. (1993) *Phytochemical Dictionary. A Handbook of Bioactive Compounds from Plants*. Taylor and Francis, London.

3. Jinsart, W., Ternai, B., Buddhasukh, D. and Polya G. M. (1992) *Phytochemistry* **31**, 3711.
4. Rang, H. P. and Dale, M. M. (1991) *Pharmacology* (2nd edn). Churchill Livingstone, London.
5. Nogrady, T. (1985) *Medicinal Chemistry. A Biochemical Approach.* Oxford University Press, Oxford.
6. Jinsart, W., Ternai, B. and Polya, G. M. (1991) *Biol. Chem. Hoppe-Seyler* **372**, 819.
7. Jinsart, W., Ternai, B. and Polya, G. M. (1992) *Biol. Chem. Hoppe-Seyler* **373**, 205.
8. Ferriola, P. C., Cody, V. and Middleton, E. (1989) *Biochem. Pharmacol.* **38**, 1617.
9. Rogers, J. C. and Williams, D. L. (1989) *Biochem. Biophys. Res. Commun.* **164**, 419.
10. Polya, G. M. and Foo, L. Y. (1994) *Phytochemistry* **35**, 1399.
11. Jinsart, W., Ternai, B. and Polya, G. M. (1992) *Biol. Chem. Hoppe-Seyler* **373**, 903.
12. Polya, G. M., Wang, B. H. and Foo, L. Y. (1995) *Phytochemistry* **38**, 307.
13. Herbert, J. M., Augereau, J. M., Gleye, J. and Maffrand, J. P. (1990) *Biochem. Biophys. Res. Commun.* **172**, 993.
14. Gescher, A. and Dale, I. L. (1989) *Anticancer Drug Des.* **4**, 93.
15. Powis, G. (1991) *Trends Pharmacol. Sci.* **12**, 188.
16. Powis, G. and Kozikowski, A. (1991) *Clin. Biochem.* **24**, 385.
17. Takeuchi, N., Nakamura, T., Takeuchi, F., Hashimoto, and Yamamura, H. (1992) *J. Biochem.* **112**, 762.
18. Chen, Q., Deady, L. W. and Polya, G. M. (1994) *Biol. Chem. Hoppe-Seyler* **375**, 223.
19. Chen, Q., Deady, L. W. and Polya, G. M. (1994) *Phytochemistry* **36**, 1153.
20. Wang, B. H., Ternai, B. and Polya, G. M. (1994) *Biol. Chem. Hoppe-Seyler* **375**, 527.
21. Larkin, M., Brazier, J., Ternai, B. and Polya, G. M. (1993) *Planta Medica* **59**, 485.
22. Basu, N. and Rastogi, R. P. (1967) *Phytochemistry* **6**, 1249.
23. Agarwal, S. K. and Rastogi, R. P. (1974) *Phytochemistry* **13**, 2623.
24. Das, M. C. and Mahato, S. B. (1983) *Phytochemistry* **22**, 1071.
25. Mahato, S. B., Nandy, A. K. and Roy, G. (1992) *Phytochemistry* **31**, 2199.
26. Ying, Q.-L., Rinehart, A. R., Simon, S. R. and Cheronis, J. C. (1991) *Biochem J.* **277**, 521.
27. Simon, A., Najiid, A., Chulia, A. J., Delage, C. and Rigaud, M. (1992) *Biochim. Biophys. Acta* **1125**, 68.
28. Stormer, F. C., Reistad, R. and Alexander, J. (1993) *Food Chem. Toxicol.* **31**, 303.
29. Hudson, J. B. (1990) *Antiviral Compounds from Plants.* CRC Press, Boston.
30. Najiid, A., Simon, A., Cook, J., Chable-Rabinovitch, H., Delage, C., Chulia, A. J. and Rigaud, M. (1992) *FEBS Lett.* **299**, 213.
31. Tsuruga, T., Chun, Y.-T., Ebizuka, Y. and Sankawa, V. (1991) *Chem. Pharm. and Bull.* **39**, 3276.
32. Niikawa, M., Hayashi, H., Sata, T., Nagase, and Kito, H. (1993) *Mutat. Res.* **319**, 1.
33. Wasternack, C., Atzora, R., Blume, B., Leopold, J. and Parthier, B. (1994) *Phytochemistry* **35**, 49.
34. Lewis, D. A. (1992) *Chemistry in Britain* **February**, 141.
35. Finney, R. S. H. and Somers, G. F. (1958) *Pharmacology* **10**, 613.
36. Tangri, K. K., Seth, P. K., Parmar, S. S. and Bhargava, K. P. (1965) *Biochem. Pharmacol.* **14**, 1277.
37. Teelucksingh, S., Mackie, A. D. R., Burt, D., McIntyre, M. A., Brett, L. and Edwards, C. R. W. (1990) *Lancet* **335**, 1060.
38. Monder, C., Stewart, P. M., Lakshmi, V., Valentino, R., Burt, D. and Edwards, C. R. W. (1989) *Endocrinology* **125**, 1046.
39. Latter, R. K., Fiander, H. Ross, N. W., Simpson, C. and Schreider, H. (1993) *Cancer Lett.* **70**, 167.
40. Liu, J., Liu, Y., Mao, Q. and Klaassen, C. D. (1994) *Fundament. Appl. Toxicol.* **22**, 34.
41. Bonte, F., Domas, M., Chaudagne, C. and Meybeck, A. (1994) *Plan Med.* **60**, 133.
42. Arvind, P., Papavassiliou, E. D., Tsioulias, G. J., Duceman, B. W., Lovelace, C. I. P., Geng, W., Staino-Coico, L. and Rigas, B. (1994) *Molec. Immunol.* **31**, 607.
43. Edelman, A. M., Blumenthal, D. K. and Krebs E. G. (1987) *Ann. Rev. Biochem.* **56**, 567.
44. Cohen, P. (1989) *Ann. Rev. Biochem.* **58**, 453.
45. Karin, M. and Sneal, T. (1992) *TIBS* **17**, 418.
46. Stolz, A., Takikawa, H., Ookhtens, M. and Kaplowitz, N. (1989) *Ann. Rev. Physiol.* **51**, 161.
47. Stolz, A., Hammond, L., Lou, H., Takikawa, H., Ronk, M. and Shively, J. E. (1993) *J. Biol. Chem.* **268**, 10448.
48. Fujioka, T., Kashiwada, Y., Kilkuskie, R. E., Cosentino, L. M., Ballas, L. M., Jiang, J. B., Janzen, W. P., Chen, I.-S. and Lee, K.-H. (1994) *J. Nat. Prod.* **57**, 243.
49. Roques, P. R., Druet, D. and Comeau, L. C. (1978) *Acta Crystallogr. Sect. B*, **34**, 1634.