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DESIGN, SYNTHESIS AND INVESTIGATION OF MECHANISMS OF ACTION OF NOVEL PROTEIN KINASE C INHIBITORS: PERYLENEQUINONOID PIGMENTS

ZHENJUN DIWU,*† JÜRG ZIMMERMANN,‡ TH. MEYER‡ and J. WILLIAM LOWN*§

*Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2; and ‡Ciba-Geigy Ltd., Basel, Switzerland

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Abstract—A series of perylenequinonoid pigments (PQPs) and related compounds were synthesized and screened for the inhibition of protein kinase C (PKC), a key enzyme involved in cellular differentiation and proliferation, and a potential target for anticancer and antiviral chemotherapeutic drugs. This study has established PQPs as efficient PKC inhibitors, and elucidated aspects of the light-enhanced action mode of the PKC inhibitors. Comparative studies between natural and synthetic PQPs led to the recognition of the effect of certain structural features of PQPs on PKC inhibition, including the skeleton of the 3,10-dihydroxy-4,9-perylenequinonoid chromophore and the configuration of the two side chains at positions 1 and 12. Calphostin C was identified as a superior PKC inhibitor of the PQP class, and with the latter as a representative structure, we investigated the mechanism of PKC inhibition by PQPs via electron paramagnetic resonance spectroscopy in conjunction with the spin-trapping technique, absorption and fluorescence spectroscopy, photochemical and photobiological studies, and enzyme methodology. Multiple modes of action are suggested for PKC inhibition, comprising the following steps: (1) the binding of PQPs to the PKC regulatory domain via complexation; (2) the photobonding between mercapto groups of PKC cysteine residues and the PQP quinonoid moiety; and (3) the PQP-sensitized photodamage of PKC via Type I and/or Type II photosensitization.

Key words: protein kinase C, anticancer agents, antiviral agents, perylenequinones, photosensitization, semiquinone radical, singlet oxygen, and reactive oxygen species

Protein kinase C (PKC)‖ has attracted considerable attention since it plays a pivotal role in signal transduction for activation of numerous cellular functions and for control of cellular differentiation and proliferation [1–3]. PKC activity is dependent upon intracellular calcium and diacylglycerol, both of which are regulated by phosphatidylinositol hydrolysis [1]. The enzyme is also stimulated by several natural products that act through the diacylglycerol site, including phorbol esters, telecidin and bryostatin [2]. Molecular cloning analysis has shown that PKC is a family of multiple subspecies having closely related structures [2]. These proteins consist of two parts. The carboxyl terminal region corresponds to the catalytic domain, which contains the ATP binding region and the protein substrate binding region. The other amino terminal fragment corresponds to the regulatory domain bearing the targets for phospholipids, diacylglycerol and phorbol esters [2]. Recently, PKC has been recognized as a significant target for anticancer and antiviral drugs [1, 4]. Therefore, potent specific PKC inhibitors would be very useful both as pharmacological tools to investigate the cellular responses of PKC itself and as potential anticancer and antiviral agents.

Although the catalytic domain-acting PKC inhibitors such as staurosporine and other indolocarbazoles strongly inhibit PKC, these compounds show low specificity for PKC since they also inhibit other protein kinases such as protein kinase A and tyrosine-dependent protein kinase [3]. While the regulatory domain-acting PKC inhibitors, such as Adriamycin®, polymixin B, chlorpromazine and trifluoperazine, are generally considered to be specific for PKC, their PKC-inhibitory activities are low. Significantly, calphostin C (CC), a new member of the perylenequinonoid pigment (PQP) family, has been demonstrated recently to be both a potent and a specific PKC inhibitor [3, 5, 6]. Interestingly, its PKC-inhibitory activity is light dependent [7]. Therefore, it is worthwhile to elucidate the mode of action of PKC inhibition by CC and its analogs, on the basis of which superior PKC inhibitors of the PQP class might be developed.

† Present address: Molecular Probes, Inc., 4849 Pitchford Ave., Eugene, OR 97402.

§ Corresponding author. Tel. (403) 492-3646; FAX (403) 492-8231.

‖ Abbreviations: CC, calphostin C; $CC^{-\cdot}$, semiquinone radical of CC; CP, cercosporin; DABCO, 1,4-diazabicyclo[2,2,2]octane; DMSO, dimethyl sulfoxide; DPA, 9,10-diphenylanthracene; HA, hypocrellin A; HB, hypocrellin B; HC, hypericin; HIV, human immunodeficiency virus; HPD, hematoporphyrin derivatives; $^1O_2$, singlet oxygen; DiC8, 1,2-dioctanoyl-syn-glycerol; OOPS-Na, 1,2-dioleoyl-syn-glycero-3-phospho-L-serin, sodium salt; PDT, photodynamic therapy; PKC, protein kinase C; PQP, perylenequinonoid pigment; TEMP, 2,2,6,6-tetramethyl-4-piperidone; and TEMPO, 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl radical.

CC belongs to a general class of 4,9-dihydroxy-3,10-perylenequinones, which comprise a relatively small but growing group of natural pigments, and they possess some unique chemical and biological properties [8–10]. The natural PQPs of this class identified to date include hypocrellins, cercosporin, phleichrome, elsinochromes, erythroaphins and calphostins. Most of them are produced by a wide variety of molds and act as the photodynamic phytotoxins of their hosts, with the exception of erythroaphins that are isolated from aphids [8]. Weiss *et al.* have reviewed the general chemical properties of PQPs [8], and we have summarized their photosensitizations and biological activities [9, 10].

Besides acting as potent specific PKC inhibitors, PQPs have also shown promising properties for photodynamic therapy (PDT) of human malignancies [9, 10], anticancer [6], and antihuman immunodeficiency virus [11, 12]. The latter property is also light dependent as in the case of PKC inhibition (*vide supra*) [10]. More significantly, as new potential PDT photosensitizers for tumors, PQPs have proven to possess quite different properties from porphyrins and phthalocyanines, the most widely used PDT agents [13]. Preliminary data have shown that some PQPs exert much stronger photodynamic action on EMT6/ED tumor cells *in vitro* compared with photofrin-II.* Hypocrellins, prototypes in the PDT application of PQPs, exhibit several advantages over the presently widely used hematoporphyrin derivatives (HPD), i.e. ready preparation and easy purification relative to HPD, small aggregation tendency (which decreases PDT efficiency in the case of HPD), strong red light absorptivity and significantly reduced normal tissue photosensitivity because of their fast metabolism *in vivo* [9, 10]. As a result, hypocrellins have been employed successfully in the clinical PDT treatment of certain skin diseases, such as white lession of vulva, keloid, vitiligo, psoriasis, tinea capitis and lichen amyloidosis, without observing the prolonged normal-tissue photosensitivity that occurs with porphyrin photosensitizers [9]. In the meantime, increasing interest is being shown in both the action and inhibition of protein kinase C [14, 15].

These significant biological activities have renewed attempts to synthesize functionalized PQPs, promoting the chemical and biological studies of PQPs [9, 10]. Although the parent compound of PQPs, 4,9-dihydroxy-3,10-perylenequinone, was prepared as early as 1954, and afterwards several unsuccessful attempts were made to synthesize natural PQPs [10, 16], until recently only a few functional PQPs have been synthesized [10]. Our reported one-step double coupling reaction of 1,2-naphthoquinone opens a facile entry to PQPs [17].

Among PQPs, hypocrellins and cercosporin (CP) have received the most attention. It was observed that PQPs are toxic to bacteria, viruses, plant cells and mice only when illuminated, which implies that they might be useful as photodynamic agents [9, 10]. Recently we have shown that PQPs are a new class of efficient singlet oxygen ($^1O_2$) generators, and that they demonstrate some advantages over the established $^1O_2$ sensitizers (such as porphyrins, rose bengal and methylene blue), including high molar extinction coefficients, wide UV-VIS absorption, high quantum yield of $^1O_2$ generation, high stability, good solubility, and small solvent and concentration effects [18].

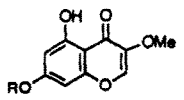
The photodynamic action of hypericin (HC), a plant phenanthroperylenequinonoid pigment structurally related to PQPs, was investigated earlier [19]. However, only recently has HC been demonstrated to photoinactivate viruses [especially human immunodeficiency virus (HIV)] and PKC, both of which are also light dependent as in the case of PQPs (*vide supra*) [11, 20–22]. Recently De Riccardi *et al.* [23] have isolated novel kinds of HC analogs: gymnochromes from the stalked crinoid *Gymnocrinus richeri*, but unfortunately no studies of their biological properties have been reported at the present time.

As discussed above, some significant biological activities of PQPs including PKC inhibition are light dependent; therefore, studies on PQP photosensitizations are urgently needed. Thus far, several lines of evidence indicate the key role of $^1O_2$ in the photosensitization of PQPs [9, 10]. Nevertheless, it appears that some chemical and biological phenomena are not caused only by $^1O_2$. In the presence of some substrates, or under certain biological environments, PQP-derived radicals and additional activated oxygen species, including superoxide anion radical, hydroxy radical and hydrogen peroxide, may play supplemental roles; in some cases, their roles even appear to supervene over that of $^1O_2$ [24, 25]. Therefore, the multiple modes of action of PQP photosensitizations need to be investigated in detail.

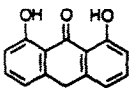
Thus far, although CC has been identified as a potent specific PKC inhibitor, a detailed explanation of the mechanism of PKC inhibition by PQPs is still lacking [10]. Bruns *et al.* [7] attempted to relate the photoinactivation of PKC by CC to the photogeneration of $^1O_2$ by CC, but the critical and expected suppressing effect of sodium azide, an efficient $^1O_2$ scavenger, on the PKC inactivation was not observed. In this report, we designed, synthesized, and screened a series of PQPs for PKC inactivation, and investigated the structural effect of PQPs on the PKC-inhibitory activities; on the basis of such structural effects superior PQPs could be designed and synthesized for PKC inhibition. By means of EPR spectroscopy, absorption and fluorescence spectrometric studies, and enzyme methodology, we have examined and elucidated some aspects of the modes of action of PQPs for PKC inhibition.

* Estey EP, Miller GG, Diwu ZJ, Tulip J, Lown JW and McPhee MS, *In vitro* screening of hypocrellins as photodynamic therapy (PDT) agents. Presented at *The Alberta Cancer Board Research Initiative Program Annual Scientific Meeting*, Kananaskis, Alberta, 1991.

Miller GG, Brown KM, Estey EP, Diwu ZJ, Lown JW, Tulip J and McPhee MS, *In vitro* toxicology of hypocrellin compounds as novel photodynamic therapy agents. In: *The 40th Annual Meeting of the Radiation Research Society*, p. 109, Salt Lake City, UT, 1992.



1. R = H
2. R = -CH_2Ph



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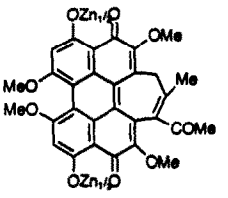

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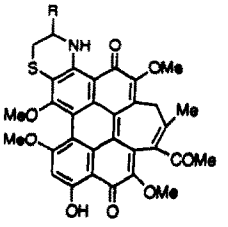
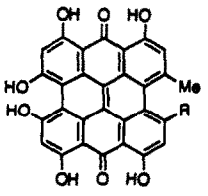
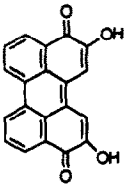


11. R = H
12. R = COOH



4. hypericin (HC), R = Me
5. pseudoHC R = CH_2OH



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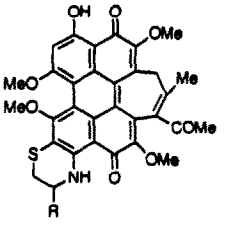
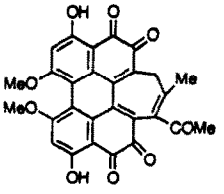


13. R = H
14. R = COOH



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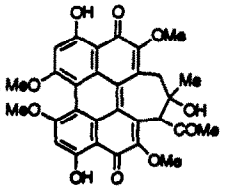
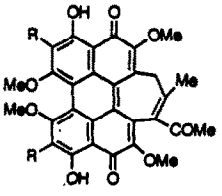
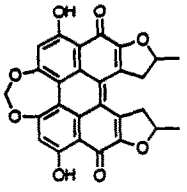


7. hypocrellin A



8. hypocrellin B, R = H
9. R = Br



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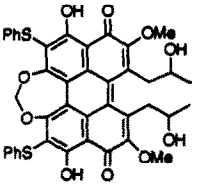

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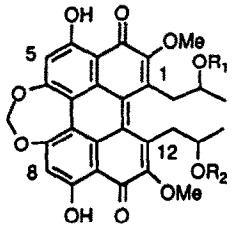

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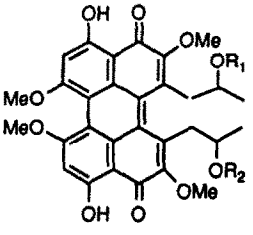


19-24

Code	R_1	R_2
18 (cercosporin)	H	H
*19 (phleichrome)	H	H
20 (calphostin A)	COPh	COPh
21 (calphostin B)	H	COPh
22 (calphostin C)	COPh	COO(*p*-OH-Ph)
*23 (calphostin D)	H	H
24 (calphostin I)	CO(*p*-OH-Ph)	COO

*They are stereoisomers.

Fig. 1. Chemical structures of natural and synthetic perylenequinonoid pigments, and related compounds.

MATERIALS AND METHODS

Chemicals, biochemicals and equipment

Mercaptoethanol, 9,10-diphenylanthracene (DPA), kynurenine, α-reduced nicotinamide adenine dinucleotide, hydroquinone, benzoquinone, pyrogallol, reduced glutathione, ascorbic acid, sodium azide, 1,4-diazabicyclo[2,2,2]octane (DABCO), 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl radical (TEMPO) and all amino acids were purchased from the Aldrich Chemical Co. (Milwaukee, WI), and were used without further purification. Tetramethylethylene, 2,5-dimethylfuran, 2,2,6,6-tetramethyl-4-piperidone (TEMP) and all the amines used, which were also purchased from the Aldrich Chemical Co., were redistilled before use. 5,5-Dimethyl-1-pyrroline-1-oxide (DMPO) of the same source was purified before use by the procedure of Buettner and Oberley [26]. The catalytic domain peptide of PKC was obtained from the Sigma Chemical Co. (St. Louis, MO) without further purification. [γ-^{32}P]ATP (10 Ci/mol) was purchased from Amersham (Little Chalfont, U.K.), and 1,2-dioctanoyl-syn-glycerol (DiC8) was obtained from Novabiochem (Laufelfingen, Switzerland) [27]. The required high purity solvents were prepared by further purification of the commercial products [28], and no impurities were detected by either absorption or fluorescence spectroscopies. *N'*-Formyl-kynurenine, 2 - carboxy - 3a - hydroxy - 1,2,3,3a,8,8a - hexahydropyrrolo[2,3*b*] - indole and 2 - carboxy - 3a - hydroperoxy - 1,2,3,3a,8,8a - hexahydropyrrolo - [2,3*b*]indole were prepared according to the procedure of Diwu and Lown [9]. P81 chromatography paper was obtained from Whatman (Maidstone, U.K.). Merck silica gel 60 was used for column chromatography and commercial Kiesegel 60 F254 plates were used for TLC.

Melting points were determined on a Thermovar MHT Kofler-Block, and are uncorrected. UV-VIS absorption spectra were conducted on a Hewlett–Packard 8542A diode array spectrometer. IR absorption spectra were recorded on a Nicolet 7199 FT spectrometer by chloroform cast. ^{1}H-NMR spectra were run on either Bruker WH-300 or WH-400 spectrometers in deuterated chloroform (unless otherwise stated) with tetramethylsilane as internal standard. Mass spectra were performed either on an Associated Electrical Industries (AEI) MS-50 for electron impact (EI) ionization or an AEI MS-9 for fast atom bombardment (FAB).

Synthesis of PQPs and related compounds

Compounds **1–6** were obtained from commercial sources and were purified prior to use. The preparation and purification of hypocrellins A (**7**) and B (**8**) (HA and HB), compound **9** and compounds **11–16** have been described [9, 25, 29, 30]. All these compounds have ^{1}H-NMR, IR and MS spectral data consistent with the assigned structures in Fig. 1. The pleichrome and calphostins **19–24** were obtained from the Sigma Chemical Co.

Preparation of compound **17**. CP (100 mg, 0.187 mmol) was dissolved in dichloromethane (2.5 mL), to which thiophenol (0.041 mL, 0.4 mmol) and triethylamine (0.056 mL, 0.4 mmol) were added, and the reaction mixture was stirred at room temperature for 16 hr. After this, another portion of thiophenol (0.2 mL) was added, and stirring continued further for 5 min. Evaporation and TLC separation (chloroform:methanol, 100:5) of the reaction mixture afforded pure compound **17** (62 mg, 44.3%). Melting point: 127.5 to 129°; λ_{max} (ε) (ethanol): 230 (48960), 252 (46720), 267 (46680), 508 (22680), 590 (sh); ^{1}H-NMR (DMSO-d_6): 15.90 (s, 2H), 7.13–7.32 (m, 10H), 5.89 (s, 2H), 4.63 (d, 2H), 4.06 (s, 6H), 3.51 (m, 2H), 3.23 (m, 2H), 2.73 (m, 2H) and 0.38 ppm (d, 6H); *m/z*: 750 (M).

Photochemical reaction of hypocrellin A with mercaptoethanol

A solution (7 mL) of mercaptoethanol (20 mg) in 0.1 M NaH_2PO_4–Na_2HPO_4 buffer (pH = 9.0) was added to 3 mL of a methanol solution of HA (10 mg). The mixture was introduced into a photochemical reactor and irradiated with a sunlamp (500 W) while nitrogen gas of high purity was bubbled through the solution, during which time the reaction was followed by TLC. The reaction mixture was extracted with chloroform, and the extract washed with water and evaporated *in vacuo* to dryness. The red solid obtained was chromatographed on Sephadex LH-20 using acetone as an eluent to afford the crude products, which were further purified on TLC using petroleum ether:ethyl acetate:ethanol (8:1:1) as developing agent.

Compounds **25** and **26**: λ_{max} ($CHCl_3$): 494, 551 and 592 nm; γ_{max}: ~3400, 1698 and 1594 cm^{-1}; ^{1}H-NMR: 16.01 (s, 1H, exchangeable with D_2O), 15.99 (s, 1H, exchangeable with D_2O), 6.60 (s, 1H), 4.10 (s, 9H), 3.76 (m, 5H), 3.52 (s, 1H), 3.51, 2.62 (dd, J_{AB} = 12 Hz, 2H), 3.21 (m, 2H), 2.50 (broad, 2H, exchangeable with D_2O), 1.92 (s, 3H) and 1.71 ppm (s, 3H); *m/z*: 622 (M).

Compound **27**: λ_{max} ($CHCl_3$): 518 and 592 nm; γ_{max}: ~3452, 1700 and 1600 cm^{-1}; ^{1}H-NMR: 16.08 (s, 1H, exchangeable with D_2O), 16.00 (s, 1H, exchangeable with D_2O), 4.13 (s, 3H), 4.10 (s, 3H), 3.86 (s, 6H), 3.74 (m, 4H), 3.54 (s, 2H, exchangeable with D_2O), 3.52, 2.63 (dd, J_{AB} = 12 Hz, 2H), 3.45 (s, 1H), 3.26 (m, 4H), 1.93 (s, 3H) and 1.70 ppm (s, 3H); *m/z*: 698 (M).

Calphostin C-sensitized photooxidations of amino acids

A solution of CC (0.1 mg) and the amino acid (5 mg) in 1:1 methanol:water (4 mL) was irradiated with the sunlamp while oxygen was bubbled through the solution. The progress of the reaction was followed by absorption spectroscopy, and the products were isolated by TLC using butanol:acetic acid:water (4:1:1) as developing agent. The structures of the products were elucidated by comparison with standard samples purchased or synthesized (*vide supra*) via spectrometric and chromatographic methods. The identified products were shown in Equations 2–5 (*vide infra*).

Determination of composition and association constants of CC–Ca^{2+} chelate

The methods of slope ratio and continuous molar fraction variation were used to determine the

Table 1. Quantum yields of $^1O_2$ generation and IC_{50} values for PKC inhibition of PQPs and related compounds

Entry	Quantum yields of $^1O_2$ generation	IC_{50}* (μM)
1		>100
2		>100
3		>100
4	0.72	3.4
5		29
6	ND†	>100
7	0.84	3.6
8	0.76	9.0
9	0.62	6.5
10	ND	10
11 (or 13)	0.31	34
15	0.42	>100
16	0.63	>100
17	0.48	11.3
18	0.81	0.85
19	0.75	10
20	0.76	0.25
21		1.04
22		0.05
23		6.36
24		0.14

* The IC_{50} data of compounds **4** and **5**, and compounds **19–24** come from Refs. 11, 3 and 6, respectively.
† ND = not detected.

Selectivity data

	PKC	PKA	PPK
		IC_{50} (μM)	
Calphostin C	0.46	>100	>100
Cercosporin	1.32	>500	180

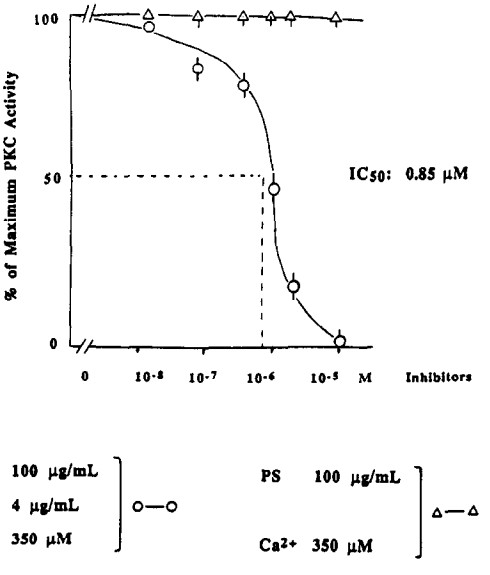


Fig. 2. Effect of CGP 49090 (cercosporin) on PKC activity.

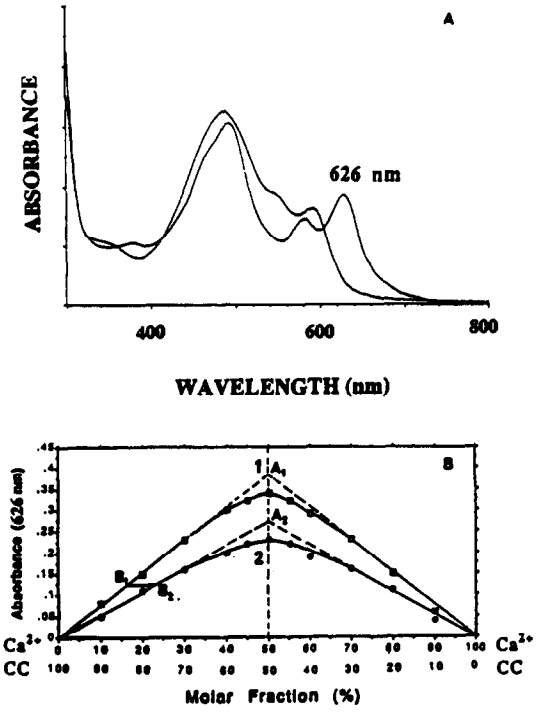


Fig. 3. Absorption spectra of Ca^{2+}-CC chelate (A) and absorption spectrometric titration of CC with Ca^{2+} (B) in ethanol:water (1:1).

composition and association constants of the complex [29]. In the chelation of CC with Ca^{2+}, if the total concentration of metal ion and PQP were maintained constant, the molar fraction ratio of CC to Ca^{2+} corresponding to the maximum concentration of the complex must be the coordination number of the complex as the molar fractions of CC and Ca^{2+} were varied continuously. The formation of the complex can be followed spectrometrically by the absorption peak longer than 600 nm (see Fig. 3) where neither CC nor Ca^{2+} has any absorptivity. The maximum concentrations of the complexes are points A_1 and A_2 in Fig. 3 in which line 1 was obtained by plotting the longer wavelength absorbance of the complex (626 nm) against a series of different molar fractions of CC and Ca^{2+} with the same total concentration of CC and Ca^{2+}. The above-mentioned solutions were equally diluted to afford line 2 with the lower total concentration of CC and Ca^{2+} than that of line 1. On the basis of Beer's Law, the isoabsorptivity points B_1 and B_2 on lines 1 and 2 must have the same concentration of the complex. Therefore, an equation

$$K = x/(a_1 - x)^m(b_1 - x)^n = x/(a_2 - x)^m(b_2 - x)^n$$

[where a_1, b_1, a_2 and b_2, respectively, represent the molar concentrations of CC corresponding to point B_1 and B_2 in Fig. 3 and those of Ca^{2+}, and can be read out directly from Fig. 3], can be derived from the proposed reaction scheme: m/n PQP + M = $1/n$ M_n(PQP)$_m$ [where M and m/n, respectively, represent Ca^{2+} and the coordination number of the complex, and the latter can be spectrometrically

Table 2. Association constants of PQPs with Ca^{2+}*

Ligand (PQP)	K ($\times 10^4$) (M^{-1})
Hypocrellin A	2.7
Hypocrellin B	1.0
Cercosporin	2.3
Calphostin C	2.8

* The association constants of hypocrellins A and B and cercosporin with Ca^{2+} come from Ref. 32.

determined (*vide supra*)]. From the equation, the association constants of the complex can be derived, and are listed in Table 2.

Purification and assay of PKC

PKC from porcine brain was partially purified (purity 50–70%) as described by Borner *et al.* [31]. PKC displayed a specific activity of 5000 U/mg. One unit of PKC activity was defined as the amount of enzyme transferring 1 nmol ^{32}P from [γ-^{32}P]ATP (Amersham; 10 Ci/mol) to histone H1 (Sigma, type V-S) in 1 min/mg protein. PKC activity was assayed essentially as described by Fabbro *et al.* [32]; the assay was carried out in a reaction mixture containing a final volume of 100 μL, 20 mM Tris–HCl, pH 7.4, 200 μg/mL histone H1 (type III-S), 350 μM $CaCl_2$, 10 mM $Mg(NO_3)_2$, 100 μg/mL OOPS-Na [27], 4 μg/mL DiC8, 10 μM ATP (0.25 μCi [γ-^{32}P]ATP), 1.2 to 1.4 U of partially purified enzyme and various concentration of inhibitors. Aliquots of 50 μL were analyzed for substrate phosphorylation [33] using P81 chromatography paper (Whatman). Measurements of enzyme inhibition in all kinase assays were performed in at least two independent experiments. Each single-point determination represents the mean of at least triplicates with a standard deviation $<10\%$.

The activities measured upon stimulation with the single agents phospholipid (PS) (100 μg/mL) or DiC8 (4 μg/mL) were 873 ± 77 and 15 ± 6 pmol/min/mg, respectively. A combination of PS (100 μg/mL)/DiC8 (4 μg/mL) revealed an activity of 1315 ± 37 pmol/min/mg. Consequently, the difference of the activities (PS/DiC8-PS) was calculated to be 442 pmol/min/mg and was attributed to the stimulatory effect exerted by DiC8. The increase in PKC activity related to DiC8 was defined as 100%, and IC_{50} values (half-maximum inhibition) of reported compounds refer to this value. Attention was paid to the relatively narrow delta (PS/DiC8-PS), but efforts made to increase this value were rendered rather difficult due to experimental particularities: cercosporin and cercosporin-like compounds exerted maximum inhibitory effects at calcium concentrations >300 μM. At calcium concentrations >300 μM, PS (100 μg/mL) stimulated PKC to 60–70% of the activity observed with PS (100 μg/mL)/DiC8 (4 μg/mL). Nevertheless, IC_{50} values of the reported compounds seem to be valid since cercosporin(s) up to 100 μM did not inhibit or only marginally inhibited PS-stimulated PKC activity (see Fig. 2) but exerted concentration-dependent inhibitory effects on DiC8-related PKC activation. In the inhibitory assay, PS was substituted by the synthetic phospholipid OOPS-Na [27]. cAMP-dependent protein kinase and phosphorylase kinase PKA and PPK were assayed exactly as described by Meyer *et al.* [34].

Determination of generation of $^1O_2$ by PQPs

The DPA bleaching method was used to determine the $^1O_2$-generating quantum yields, the details of which were described in an earlier report [18]. The photooxidations of DPA sensitized by PQPs were carried out on a "merry-go-round" where the samples were illuminated by 436 nm light, obtained from the combination of a high pressure vapor lamp of the ACE-Hanovia Co. with a narrow band interference filter model 54300 (maximum transition at 440 nm = 40%) of the Oriel Corp. The irradiation intensity that the samples received was measured by a YSI radiometer model 65A to be 23 J cm^{-2} sec^{-1}. The spin-trapping of $^1O_2$ by TEMP was used as an alternative method to determine the formation of $^1O_2$ by PQPs (*vide infra*). These two methods gave consistent results. cAMP-dependent protein kinase and phosphorylase kinase PKA and PPK were assayed exactly as described by Meyer *et al.* [34].

EPR studies

EPR spectra were recorded at room temperature using a Bruker ER-400 EPR spectrometer operating at 9.5 Hz, x-band, with 100 KHz field modulation fitted with a Varian V3601 12-inch magnet. The line widths of the EPR spectra were measured by a digital NMR gauss meter of the Alpha Co. (model 3093), and the microwave frequency was measured by a frequency meter of the Hewlett–Packard Co. (model X532B). The value of the *g*-factor was determined using a 98% sulfuric acid solution of perylene as a reference ($g = 2.002578$) [35]. The simulations of the experimental EPR spectra were performed on a Bruker Analytische Messtechnik GMBH computer (model Aspect 2000).

Photoinduced EPR spectra were obtained from the samples introduced into an aqueous flat quartz cell and illuminated directly inside the microwave cavity using 460 nm light, which matches the maximum absorption peak of CC in the visible region (see Fig. 3), from a 1000 W high pressure xenon arc lamp of the Optical Radiation Corp. (model LPS 255HR), fixed in a lamp housing of the Spectral Energy Corp. (model LH 151), which was coupled to a grating monochromator of the Spectral Energy Corp. (model GM 252). The incident fluence of the cavity window was measured by the radiometer to be 18 J m^{-2} sec^{-1}.

The spin-trapping of $^1O_2$ by TEMP was performed according to a modification of the method of Lion *et al.* [36]. Typically the reaction solution consisted of 3.0×10^{-5} M CC and 10 mM TEMP.

RESULTS AND DISCUSSION

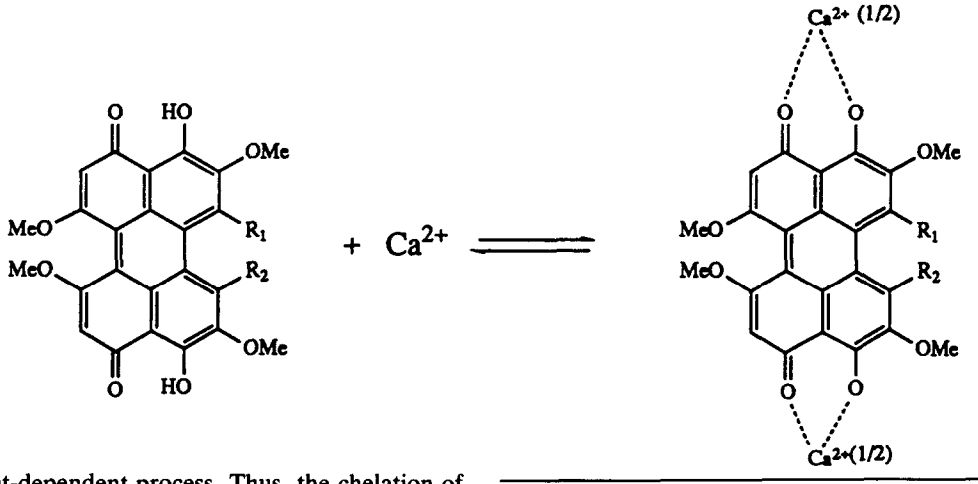
PQPs as a new class of PKC inhibitors

It can be seen from Fig. 2 that strong activation of PKC was stimulated with the single agents PS (100 μg/mL) or DiC8 (4 μg/L) in the presence of 350 μM Ca^{2+}. It can also be seen from Table 1 that there exists a strong structural effect of PQPs on the

PKC-inhibitory activity. Simple flavones (compounds **1** and **2**), anthrone (compound **3**) and 3,9-perylenequinone (compound **6**) did not exhibit any inhibition against PKC. All the tested natural PQPs, which contain a 4,9-dihydroxy-3,10-perylenequinonoid chromophore, were demonstrated to inhibit PKC strongly; these included calphostins (compounds **20–24**), CP (compound **18**), HA (compound **7**), HB (compound **8**), HC (compound **4**), pseudo-HC (compound **5**) and phleichrome (compound **19**). No clear relationship between PQP structures and their PKC-inhibitory activities was observed at this stage. However, it appears that the destruction of the 4,9-dihydroxy-3,10-perylenequinonoid chromophore and modification of the configuration of two side chains at positions 1 and 12 of PQPs markedly influenced the PKC-inhibitory activities. For example, calphostin D (compound **23**) and phleichrome (compound **19**) displayed a big difference in PKC inhibition, although their only difference is the opposite configurations of the side chains. The effects of the PQP side chains on PKC inhibition are also demonstrated by a series of calphostins and CP derivatives (compounds **18–24**).

Light-enhanced chelation of PQPs with Ca^{2+} *in photoinactivation of PKC by PQPs*

As shown in Fig. 3, the spectrometric titration of CC by Ca^{2+} indicated that a complex is formed between CC and Ca^{2+} in a 1:1 stoichiometric ratio as shown in Equation 1. Interestingly, the chelation is a light-dependent process. Thus, the chelation of CC with Ca^{2+} will decrease the local Ca^{2+} concentration surrounding PKC and result in PKC inhibition since Ca^{2+} is an essential element for activating PKC [1, 2]. However, this process might not play a critical role in PKC inhibition because PQPs are not particularly strong Ca^{2+} chelators, as judged from the association constants of PQPs with Ca^{2+} listed in Table 2.



(1)

Complex formation and light-enhanced bonding between mercapto groups of cysteine-rich residues in the PKC regulatory domain and PQP quinonoid moiety as a specific photoaffinity mode of PQPs for PKC

As shown in Fig. 4A, an isobestic point appeared when CC was spectrometrically titrated by PKC, and which was accompanied by a slight hypsochromic shift of the CC absorption spectrum. Moreover, a new absorption band at a longer wavelength (~710 nm) was observed when a higher concentration of CC was used (see Fig. 4B). These phenomena indicated that a complex is formed between CC and PKC, which may be interpreted by molecular orbital considerations [37]. The interaction between PKC and CC increases the energy difference between HOMO and LUMO of PKC ($\Delta E'_p > \Delta E_p$) and that of CC ($\Delta E'_c > \Delta E_c$), which leads to the observed hypsochromic shift of the CC absorption spectrum (the change of the absorption spectrum of PKC was not visible because PKC absorption falls within UV range, which is overlapped with the absorption spectrum of CC). The new longer absorption band originates from the charge transfer complex of PKC and CC, the energy difference between HOMO and LUMO of which ($\Delta E'_{pc}$) is smaller than those of PKC and CC ($\Delta E'_{pc} < \Delta E_c < \Delta E_p$). The complex formation between PKC and CC is also evidenced by the non-Sterm–Volmer fluorescence quenching mode (non-linear relationship of F_0/F vs CC concentration) (data not shown) [38, 39]. Similar phenomena were also observed for HA, an analog of CC (see Fig. 1). When the PKC catalytic domain peptide was used to replace the entire PKC protein, no complex was observed by absorption spectroscopy, suggesting that CC might selectively bind to the PKC regulatory domain, which is unique for PKC and different from other protein kinases [1, 2]. The fact that PQPs only inhibit the synergistic part caused by DiC8 suggests that they do bind at the regulatory site. There was only marginal inhibition of PKC in the absence of DiC8 (see Fig. 5). The statement is further supported by binding studies, which demonstrated that calphostin C inhibits protein kinase C by competing at the binding site for diacylglycerol and phorbol esters [7]. This suggestion was further supported both by the high specificity of CC for the PKC inhibition, and by the fact that CC inhibits the entire PKC instead of the PKC catalytic domain [3].

It has been proven that HA photosensitization causes the dramatic destruction of cysteine of the erythrocyte membrane, indicating that cysteine is a

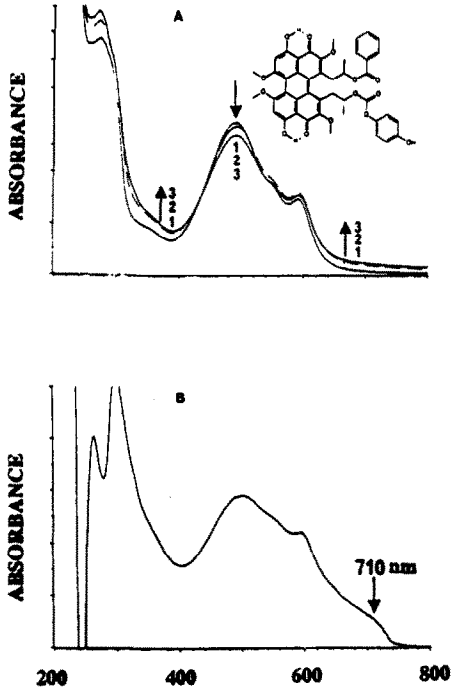


Fig. 4. Absorption spectrometric titration of CC with PKC in (98:2) 0.1 M Na_2HPO_4-NaH_2PO_4 (pH = 7.0):DMSO. [CC] = 10 mM. (A) 1. [PKC] = 0; 2. [PKC] = 1.5 mg/L; 3. [PKC] = 3.0 mg/L. (B) [PKC] = 18.0 mg/L.

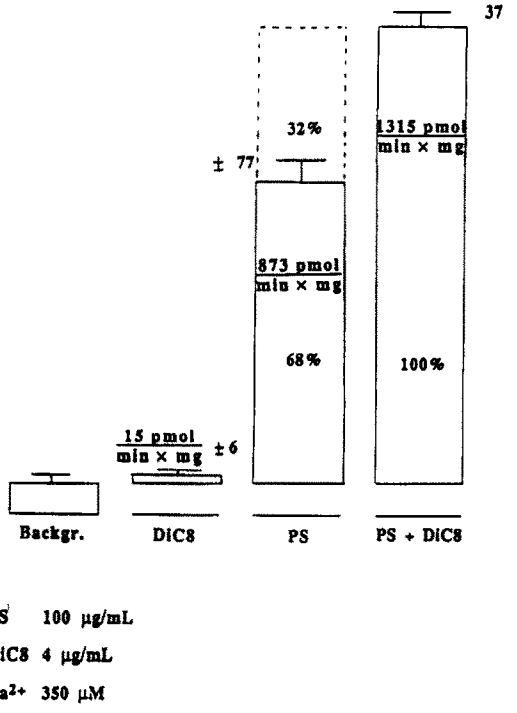
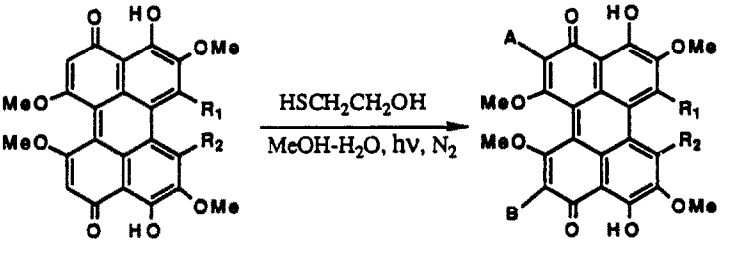


Fig. 5. Activation of PKC by single agents PS, DiC8 and PS plus DiC8 in the presence of 350 μM Ca^{2+}.

significant target for PQP photosensitization in biological systems [9]. On the other hand, the PKC regulatory domain is rich in cysteine units, which are required for PKC activity [40]. Therefore, it is necessary to investigate the effect of PQP photosensitization on the cysteine residues of PKC.

It was observed that free cysteine can react with hypocrellins to afford compounds **11–14**. The reaction was promoted by light irradiation (Diwu Z and Lown JW, unpublished results). Because amino groups of cysteine residues in PKC are blocked by the peptide chain, and are not available for the further cyclization observed in the reaction of free cysteine with hypocrellins, mercaptoethanol was selected as a model compound to mimic the reaction of the blocked cysteine residue of the protein with PQPs. The reaction of mercaptoethanol with HA gave compounds **25–27** shown in equation 2, and that of reduced glutathione, a closer mimic compound for PKC cysteine residue, was demonstrated by absorption spectroscopy to give similar products. However, the products of the latter reaction are much more difficult to purify, and further studies are still in progress. As in the case of free cysteine, the reactions of mercaptoethanol and reduced glutathione with hypocrellins are also greatly promoted by light irradiation. These studies suggest that bonding between mercapto groups of PKC cysteine residues and the PQP quinonoid moiety occurs as a possible specific photoaffinity mode of PQPs for PKC, which is displayed in Fig. 6. This suggestion is supported by the decrease in anti-PKC activity of CP by blocking the reactive positions 5 and 8 (compound **17**) and the increase in anti-PKC activity of HB by activating these positions



Compound **25** A = H, B = $-SCH_2CH_2OH$
Compound **26** A = $-SCH_2CH_2OH$, B = H
Compound **27** A = B = $-SCH_2CH_2OH$

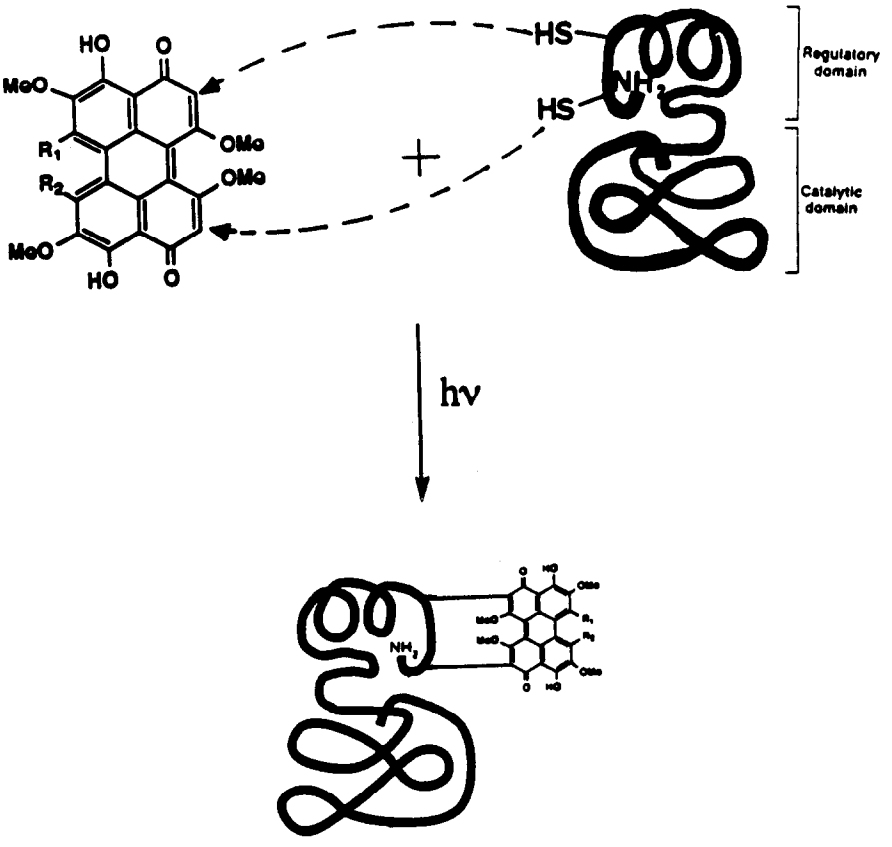


Fig. 6. Proposed bonding of PKC cysteine residues with PQP quinonoid moiety.

(compound **9**) (compare compound **17** with compound **18**, and compound **8** with compound **9**). The bonding process destroys the zinc-finger structure of the PKC regulatory domain supported by cysteine residues, and results in PKC inactivation since the zinc-finger structure is essential for PKC activity [40, 41]. However, this is evidently not the only pathway of inactivation of PKC since compound **17**, which cannot react with thiol compounds, also exhibited some PKC-inhibitory activity, implying that alternative ways responsible for the PKC photoinactivation must exist.

On the other hand, the bonding between mercapto groups of PKC cysteine residues and the PQP quinonoid moiety might also enhance PQP photosensitization efficiency of PKC amino acid residues, since PQPs (photosensitizers) are intramolecularly positioned and oriented towards the amino acids (substrates) much better than in the free state of solution (intermolecularly). The PQP-sensitized photooxidation of the amino acids is independently responsible for PKC photoinactivation (*vide infra*).

PQP semiquinone radical as a possible active species for the photoinactivation of PKC by PQPs

It has been observed by EPR spectroscopy that HA, HB and CP readily generate the corresponding semiquinone radicals upon illumination. As shown in Fig. 7, a strong and well-defined EPR signal appeared upon illuminating the solution of CC in deaerated dimethyl sulfoxide (DMSO). This spectrum displayed 11 lines, and the two outermost components on the wings were very weak; this became more apparent when higher modulation amplitude and gain were used. The control experiments indicated that irradiation was indispensable for the generation of the CC radical (compare lines A, B and C in Fig. 7). The EPR signal intensity of the CC radical depended on CC concentration, oxygen, irradiation time and intensity, and was greatly intensified by the addition of electron donors, including amines (ethylamine, diethylamine and triethylamine), polyphenols (hydroquinone and pyrogallol) and some typical biological substrates (NADH, histidine, methionine, cysteine, tryptophan, reduced glutathione, ascorbic acid and 5-hydroxytryptophan). These reduced substrates, more electron-rich than CC, may directly donate an electron to excited CC and thus efficiently enhance the concentration of the semiquinone radical ($CC^{-\cdot}$). These phenomena are similar to those exhibited by HA, HB and CP semiquinone radicals. Therefore, the observed EPR spectrum was considered to originate from $CC^{-\cdot}$.

It is expected from the CC structure (see Fig. 1) that the hyperfine EPR spectrum of $CC^{-\cdot}$ results from the coupling interaction of an unpaired electron with up to five kinds of protons, including aromatic protons, phenolic hydroxy protons, methylene protons and the protons of the methoxy groups and

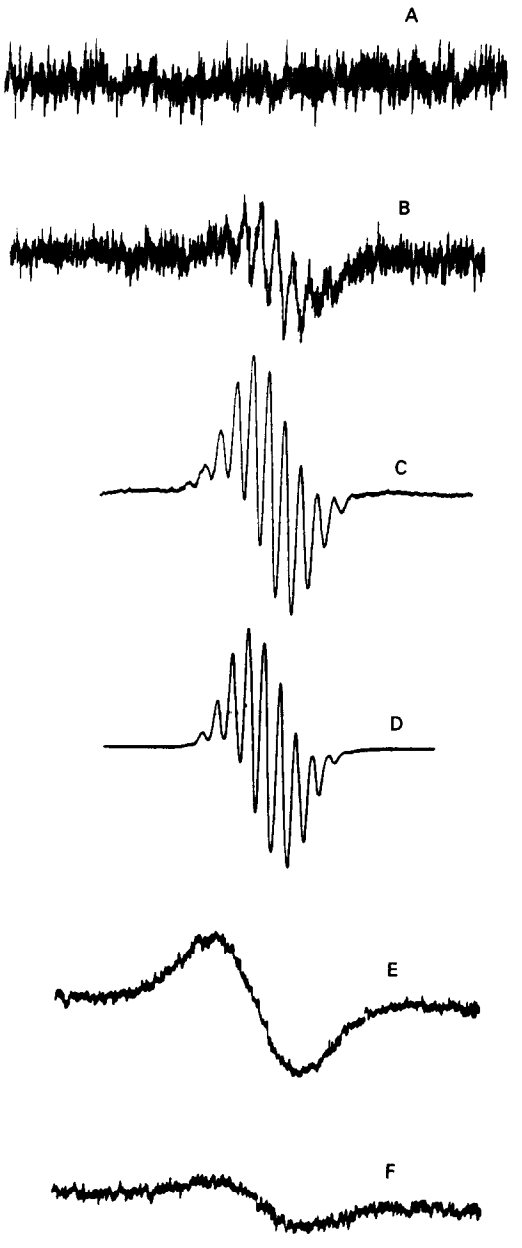


Fig. 7. EPR spectra of semiquinone radical of CC (0.1 mM) in deaerated DMSO. A–D modulation amplitude: 0.08 G and in deaerated (98:2) 0.1 M Na_2HPO_4-NaH_2PO_4 (pH = 7.0):DMSO. E and F modulation amplitude: 0.32 G. The spectrometer settings: microwave power, 15 dB; time constant, 0.2 sec; scan rate, 500 sec; scan width, 15 G. (A) In the dark, receiver gain: 8.0×10^5. (B) On illumination, receiver gain: 8.0×10^5. (C) After 5 min of irradiation, receiver gain: 6.3×10^4. (D) The simulated EPR spectrum using the following parameters: $a_1^H = 0.57$ G (2H); $a_2^H = 0.43$ G (8H); line width = 0.19 G. (E) After 5 min of irradiation in the absence of PKC with receiver gain: 5.0×10^5. (F) After 5 min of irradiation in the presence of PKC (18 mg/L) with receiver gain: 5.0×10^5.

the side chains. It was observed that the replacement of the aromatic protons by bromine drastically altered the EPR spectral shape. In contrast, demethylation of the methoxy groups and deuteration of phenolic hydroxy groups influenced the EPR spectral hyperfine structure to a lesser extent than the substitution of aromatic protons. These effects indicated that aromatic, methoxy and phenolic hydroxy protons of $CC^{-\cdot}$ might couple with the unpaired electron, and the coupling constant of the aromatic protons is larger than those of methoxy and phenolic hydroxy protons. Modifications of the side chains had relatively little effect on the EPR hyperfine structure, indicating that the protons of the side chains are not involved in the splittings of the EPR spectrum to a significant extent.

On the basis of the above-mentioned analysis, three different kinds of spins are involved in the EPR spectral splitting of $CC^{-\cdot}$, and the theoretical number of hyperfine components of the EPR spectrum should be $3 \times 3 \times 7 = 63$ lines, but, owing to the extensive overlap, the observed number of lines is reduced significantly. Spectral simulation was conducted so as to produce the best fit to the experimental spectrum according to the parameters listed in Fig. 7, using a Lorentzian line shape. The two equivalent protons with the largest splitting constant, i.e. 0.57 G, were assigned to aromatic protons. Two of eight protons with a splitting constant of 0.43 G were attributed to phenolic hydroxy protons, and consequently six protons of two identical methoxy groups must be involved in the coupling with the unpaired electron.

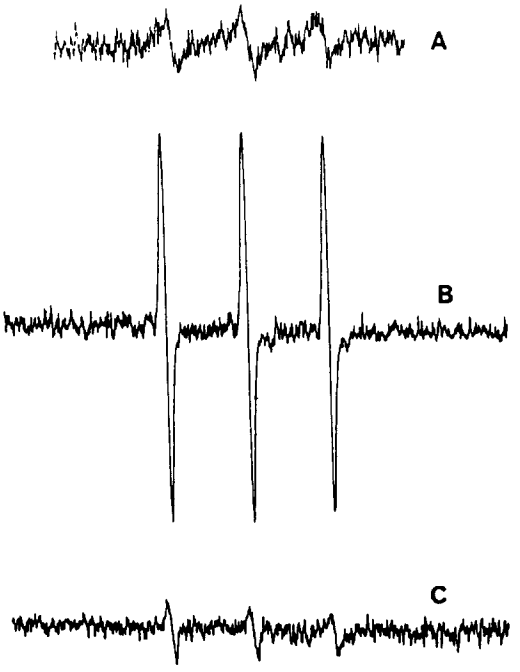


Fig. 8. Formation of TEMPO during irradiation of CC solution in the presence of TEMP at room temperature in aerated (98:2) 0.1 M Na_2HPO_4-NaH_2PO_4 (pH = 7.0):DMSO. The spectrometer settings: microwave power, 15 dB; modulation amplitude, 0.1 G; time constant, 0.2 sec; scan rate, 500 sec; scan width, 150 G; receiver gain, 1.6×10^5. (A) In the dark. (B) After 5 min of irradiation in the absence of PKC. (C) After 5 min of irradiation in the presence of PKC (18 mg/L).

As shown in Fig. 7E, $CC^{-\cdot}$ was also detected in aqueous solution, but its EPR signal resolution was lost because of the water dielectrical effect [42]. It is evident that the EPR signal intensity of $CC^{-\cdot}$ was greatly suppressed by the addition of PKC (compare line E with line F in Fig. 7), indicating that $CC^{-\cdot}$ might react with PKC. The radical induced photooxidation of PKC via the Type I pathway [24, 43]. It has also been demonstrated by EPR studies that, in addition to $CC^{-\cdot}$, CC-sensitized photooxidation of thiol compounds also produces the substrate radical (RS·), which would plausibly act as an alternative reactive intermediate to induce Type I photosensitization of PKC.

Singlet oxygen as another possible active species for the photoinactivation of PKC by PQPs

Several lines of evidence derived from chemical reactions and biological results have revealed that PQPs can produce $^1O_2$ during irradiation [9, 18]. We used the TEMP spin-trapping method to detect the photoproduction of $^1O_2$ by CC. As shown in Fig. 8, the EPR spectrum of three equal intensity lines, characteristic of a nitroxide radical, was recorded when the aerated solution of CC and TEMP was irradiated at room temperature. The *g* factor and hyperfine splitting constant of the radical photogenerated by CC were found to be identical with those of the authentic TEMPO. Under similar detection conditions, irradiation of CC in the presence of TEMP gave the identical EPR spectrum to that of the authentic TEMPO. Therefore, the EPR signal during irradiation of the solution containing CC and TEMP was attributed to TEMPO.

Control experiments indicated that CC, oxygen and light were all essential for the production of TEMPO (Fig. 8), indicating that the formation of the nitroxide radical is a photodynamic process. Thus, $^1O_2$ is plausibly involved in the formation of TEMPO. The EPR signal was suppressed by $^1O_2$ scavengers, such as DABCO and 2,5-dimethylfuran, and intensified by deuteration of the solvent. These two powerful tools, diagnostic for $^1O_2$, suggested that TEMPO is derived from the reaction of TEMP with $^1O_2$ generated by the irradiation of CC [44]. Comparative studies between CC and well-known $^1O_2$ sensitizers (such as rose bengal, meso-porphyrin IX dimethyl ester and fluorescein) indicated that CC is a potent $^1O_2$ generator (see Table 1).

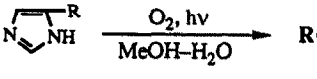
It has been proven that both natural PQPs and the PQPs bonded with thiol compounds are potent $^1O_2$ generators [18]. As seen from Fig. 8, $^1O_2$, generated from the illumination of CC, was suppressed dramatically by the addition of PKC, indicating that $^1O_2$ might effectively induce photooxidation of PKC via a Type II pathway [43], and act as a significant factor for PKC photoinactivation. Indeed, as shown in Table 1, all the potent PKC inhibitors of the PQP class were good $^1O_2$ generators, and decreasing PKC-inhibitory activities were accompanied by reduced $^1O_2$-generating capacities [compare compounds **11** (or **13**) and **15** with their parent compound **8** in Table 1]. However, $^1O_2$ is not the only factor responsible for the PKC photoinactivation because compound **10**, which cannot produce detectable amounts of $^1O_2$, was almost as potent as its parent compound **8**, which is a strong $^1O_2$ generator (see Table 1).

PQP-sensitized photooxidations of sensitive amino acid residues in PKC as an alternative mode for the photoinactivation of PKC by PQPs

As discussed above, both Type 1 (mediated by PQP semiquinone radical and/or substrate radical) and Type II (mediated by $^1O_2$) photosensitization can cause photodamage of the PKC protein. The screening studies for CC-sensitized photooxidation of the amino acids that exist in PKC (including alanine, valine, leucine, isoleucine, serine, threonine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, hydroxylysine, histidine, argine, phenylanine, tyrosine, tryptophan, thyroxine, cysteine, methionine, proline and hydroxyproline) have led to the conclusion that cysteine, methionine, histidine and tryptophan are sensitive targets for the CC-sensitized photoinactivation of PKC. This result is consistent with previous studies with the erythrocyte membrane, in which the four above-mentioned amino acids were also demonstrated as major photodamaging targets [9]. The CC-sensitized photooxidation of cysteine yields cystine and cysteic acid as major products, as shown in Equation 3. A result of this reaction in PKC will lead to the formation of an S–S bond from the cysteine residues, which will result in the structural destruction of the protein. The CC-sensitized photooxidation of methionine produces its corresponding sulfoxide as shown in Equation 4, and that of histidine generates aspartic acid as a major product, as shown in Equation 5. The CC-sensitized photooxidation of tryptophan is more complicated, and affords kynurenine derivatives and other unidentified products shown in Equation 6, which is similar to the HA-sensitized photooxidation of

$$\mathrm{RSH} \xrightarrow[\mathrm{MeOH\text{–}H_2O}]{\mathrm{O_2,\ h\nu}} \mathrm{RSSR} + \mathrm{RSO_3H} \qquad (3)$$

$$\mathrm{RCH_2SMe} \xrightarrow[\mathrm{MeOH\text{–}H_2O}]{\mathrm{O_2,\ h\nu}} \mathrm{RCH_2SOMe} \qquad (4)$$



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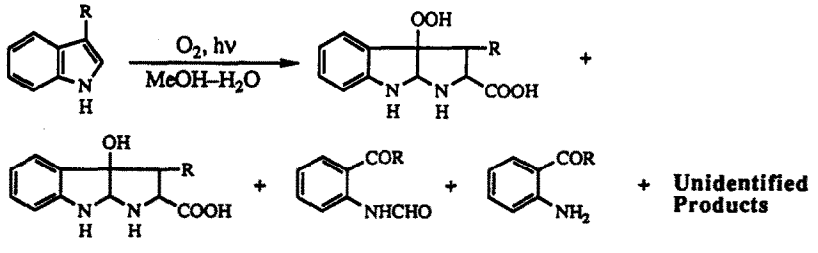

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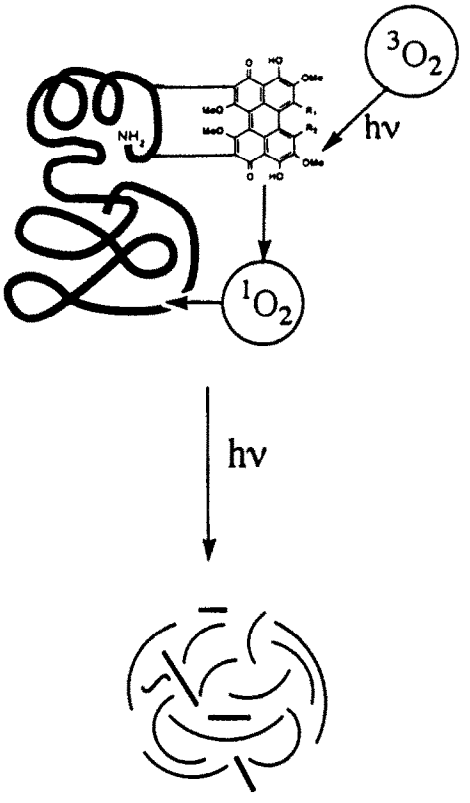

Fig. 9. Photodamage of PKC by PQP-induced photosensitization. A PQP binds to the PKC regulatory domain via complexation, and then is covalently coupled with the PKC fragment via the photosubstitution of the PQP with cysteine residues rich in the fragment. The bonded PQP photogenerates singlet oxygen and other reactive species (such as the semiquinone radical), which readily attack the sensitive amino acid residues in PKC (including cysteine, methionine, histidine and tryptophan). The photosensitization results in the destruction of the protein.

tryptophan and its derivatives [43–46]. The CC-sensitized photooxidations of the other three amino acids are almost similar in mechanism and product distribution to those induced by HA [9, 43]. The photooxidations are mediated by both Type I and Type II pathways, and generally the latter is the major process [43].

In summary, both the photoreaction of PQPs with mercapto groups of PKC cysteine residues and photoproduction of PQP semiquinone radicals and $^1O_2$ may damage the protein via the PQP-induced reactions of certain amino acid residues. The possible processes involved are shown in Fig. 9.

CONCLUSIONS

This study has established certain PQPs as efficient PKC inhibitors, and elucidated some aspects of the light-enhanced action mode of the PKC inhibitors. There exist certain effects of PQP structures on PKC inhibition, among which the skeleton of 3,10-dihydroxy-4,9-perylenequinonoid chromophore and the configuration of the two side chains at positions 1 and 12 act as major structural factors for PKC inhibition. The inhibition of PKC by PQPs is a multiple action mode, including (1) binding of PQPs to the PKC regulatory domain via complexation; (2) photobonding between mercapto groups of the PKC cysteine residues [47, 48] and the PQP quinonoid moiety; and (3) the PQP-sensitized photodamage of PKC via Type I and/or Type II photosensitization. However, the high selectivity of certain PQPs (such as CC) for PKC (Table 1) still cannot be satisfactorily explained when based only on the above-mentioned mechanisms, since other cysteine-rich proteins may also react with PQPs, and activated oxygen species and the semiquinone radicals may also photodamage other biological molecules such as lipids and nucleic acids. The study on the selectivity of PQPs for PKC inhibition is still in progress and will be reported in due course.

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