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Antioxidant Properties of Catechins and Proanthocyanidins: Effect of Polymerisation, Galloylation and Glycosylation

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A range of catechins and oligomeric procyanidins was purified by high performance liquid chromatography (HPLC) from grape seed, apple skin, lentil and almond flesh. Catechins, galloylated epicatechin, glycosylated catechin, procyanidin dimers, galloylated dimers, trimer, and tetramer species were all identified, purified and quantified by HPLC, LC-MS and NMR. The antioxidant properties of these compounds were assessed using two methods: (a) inhibition of ascorbate/iron-induced peroxidation of phosphatidylcholine liposomes; (b) scavenging of the radical cation of 2,2′-azinobis(3-ethyl-benzothiazoline-6-sulphonate) (ABTS) relative to the water-soluble vitamin E analogue Trolox C (expressed as Trolox C equivalent antioxidant capacity, TEAC). Antioxidant activity in the lipid phase decreased with polymerisation in contrast with antioxidant action in the aqueous phase which increased from monomer to trimer and then decreased from trimer to tetramer. Galloylation of catechin and dimeric procyanidins decreased lipid phase and increased aqueous phase antioxidant activity. Glycosylation of catechin demonstrated decreased activity in both phases.

Keywords: Catechin, procyanidin, flavonoid, antioxidant, TEAC, lipid peroxidation

INTRODUCTION

It has been established that a diet rich in fruit and vegetables is responsible for reducing the risk of chronic diseases such as cardiovascular disease and certain forms of cancer.[1,2] These protective effects have been attributed to compounds such as vitamin C, carotenoids and plant phenolics, such as the flavonoids. One group of flavonoids is the flavan-3-ols (catechins and proanthocyanidins), which are found widely in the plant kingdom. Proanthocyanidins are characterised by the anthocyanidins (e.g. cyanidin) which are formed upon acid hydrolysis. Anthocyanins derived from these and other anthocyanidins

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are the pigments present in flowers and fruit and more rarely in wood and bark from certain trees. In plants, proanthocyanidins occur as a mixture of oligomers and polymers and therefore their structural analysis requires a prior fractionation stage usually involving Sephadex LH20 chromatography and high performance liquid chromatography (HPLC).

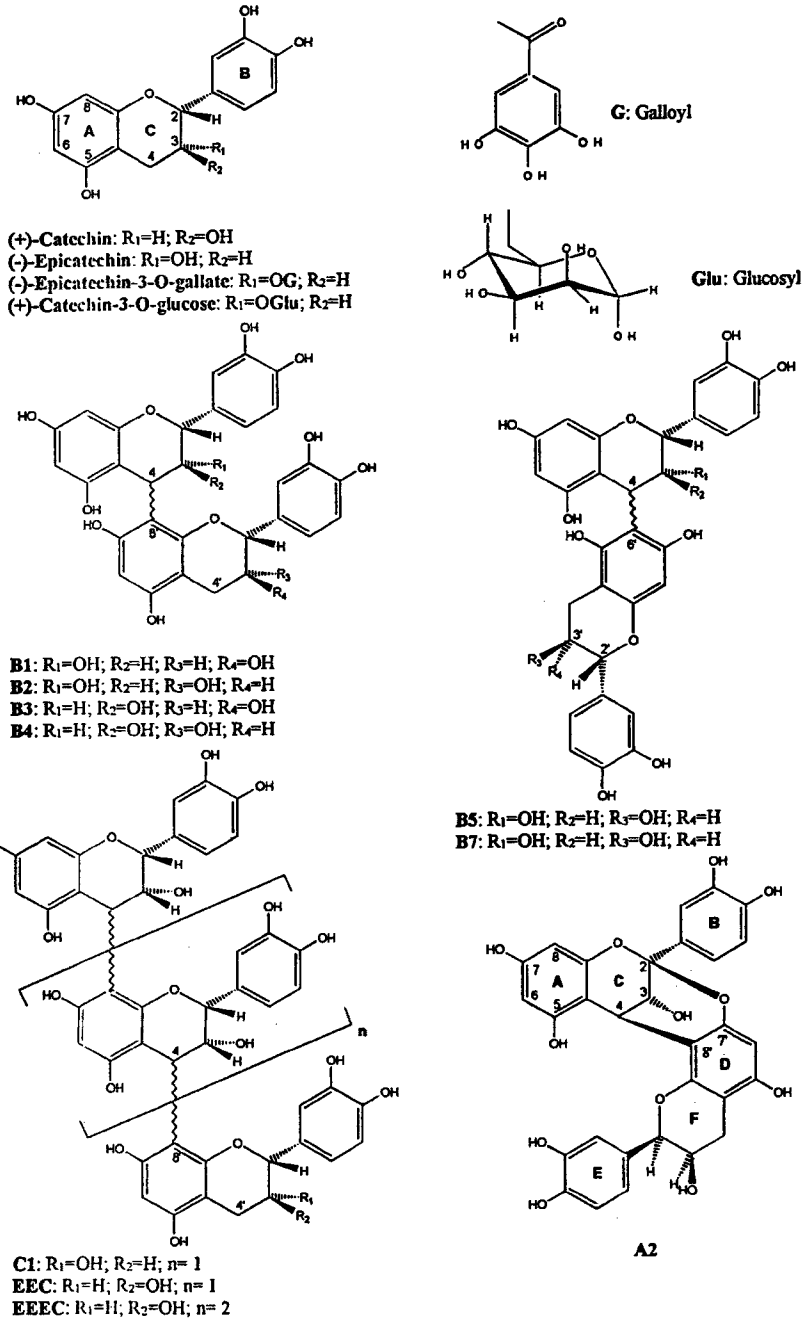

FIGURE 1 Structure of catechins and procyanidins.

Many studies have focused on the biological activities of catechins and galloylated catechins which are commonly found in green teas. They have been shown to be potent direct antioxidants,[3,4] effective inhibitors of phase I enzymes,[5] and demonstrate inhibition of tumour initiation and promotion in skin and other organs.[6,7] Catechins and proanthocyanidins from other sources, such as (+) catechin, (−) epicatechin and dimers B1, B2 and B5 (see Figure 1), have antibacterial and angioprotective properties.[8] In addition, the proanthocyanidins C1 and EEC (see Figure 1) have exhibited the ability to inhibit platelet aggregation.[9]

Previous studies analysing the direct antioxidant action of catechins in lipid phase systems such as erythrocyte membranes,[10] rat liver microsomes[11] and phospholipid bilayers[4] show that catechin and epicatechin are as potent as the well characterized flavonol quercetin. This is in contrast to the action in the aqueous phase, where quercetin is twice as effective as catechin and epicatechin.[12] In this paper we report the isolation and antioxidant properties of a range of catechins and proanthocyanidins in both the lipid and aqueous phase systems.

MATERIALS AND METHODS

Compounds were obtained from several plant sources: (+)-catechin (cat), (−)-epicatechin (Ec) and (−)-epicatechin-3-O-gallate (EcG), procyanidin dimers B2, B3, B4 and B5 and galloylated derivatives were from grape seeds; procyanidins of the B1 series (B1, B7, trimer Ec-Ec-cat and tetramer Ec-Ec-Ec-cat) were from almond fruit flesh; dimer A2 was from horse chestnut shells; and the catechin-3-O-glucose (cat-Glu) from lentil.

Preparation of Catechins and Proanthocyanidins

Frozen samples were ground and extracted with cold methanol containing 0.05% ascorbic acid (−20°C). The mixture was sonicated for 15 min and then centrifuged (15000 rpm) at −10°C. The supernatant was collected and the residue processed a further four times as described above. Water was added and the methanol eliminated in a vacuum. The aqueous extract was washed with n-hexane and the catechins and proanthocyanidins extracted with ethyl acetate and subsequently lyophilised. Further fractionation was performed using a Sephadex LH-20 column with 96% ethanol; the collection of fractions was monitored at 280 nm. Pure compounds were isolated from the fractions by HPLC using a Waters 600 Controller pump and a Nova-pak C18 6 μm (25 × 100 mm) radial compression cartridge. Solvents used were 2% acetic acid (A) and methanol (B); the gradient employed was 5% B isocratic over 5 min, 5–50% B over 25 min (linear) at a flow rate of 18 ml/min, although slight modifications were introduced for the isolation of some co-eluting compounds. Detection was at 280 nm.

Pure procyanidins obtained were characterised by LC-MS using a Finnigan MAT LCQ ion trap detector with an electrospray ionisation probe[13] and employing selective cleavages in the presence of phloroglucinol[13] and phenylmethanethiol[14] with subsequent desulphuration of the thioethers.[15] Characterisation of glycosylated catechin was accomplished by MS and 2D-NMR.[16] Purity of the compounds was checked by HPLC-DAS[17] and LC-MS. All fractions were lyophilised and stored under argon prior to antioxidant studies.

Preparation of Galloylated Dimers

A grape seed tannin extract (prepared from grape seeds, *Vitis vinifera*, var Alicante Bouchet, as described previously[14]) was dissolved in 2% acetic acid (200 mg/20 ml). Caffeine (100 mM) was added to the resulting tannin solution to precipitate contaminating coloured material (oxidation products) and subsequently decaffeinated by three successive extractions with 100 ml

chloroform. The decaffeinated extract was then chromatographed using a HPLC system including two gradient pumps (M306, head pump 200Wti, Gilson), an injection pump (M306, head pump 10SC, Gilson), a hydraulic compression column (500 × 4 mm i.d., ISA Jobin-Yvon), and a UV detector (M875-UV, Jasco) set at 289 nm. The column was filled with Toyopearl TSK HW-40F (500 ml), under 7 bar pressure and eluted with 50% ethanol in water (7 ml/min). Fractions containing the galloylated dimers were collected and concentrated individually to 1 ml and repurified on HPLC (semi-preparative scale) employing a reverse phase Lichospher RP-18 column (5 μm packing, 125 × 22 mm i.d., Merck). Solvent A was CH_3CN–H_2O–HCOOH (80 : 18 : 2, v); solvent B was H_2O–HCOOH (98 : 2, v). Elution commenced with 3% solvent A isocratically for 5 min, continuing with linear gradients from 3% to 20% solvent A in 15 min, from 20% to 30% solvent A in 10 min and from 30% to 50% solvent A in 5 min followed by washing and reconditioning of the column.

The retention times of all compounds collected were compared to those obtained previously.[18] Chromatographically pure compounds were identified by thiolysis followed by HPLC analysis and LC-MS as described previously.[19] Galloylated dimers were lyophilised and stored under argon prior to antioxidant studies.

Lipid Phase Antioxidant Activity

Phospholipid liposomes (final concentration 1 mg/ml) were suspended in 150 mM KCl containing 0.2 mM $FeCl_3$ and test compound at a range of concentrations. Peroxidation was started as described previously[20] with ascorbate (final concentration 0.05 mM), in a final volume of 0.4 ml. Samples were incubated at 37°C for 40 min and the reactions terminated by the addition of 0.8 ml of 20% (w/v) trichloroacetic acid (TCA)/0.4% (w/v) thiobarbituric acid (TBA)/0.25 N HCl and 0.01 ml of butylated hydroxytoluene in ethanol. The production of thiobarbituric acid reactive substances (TBARS) was measured after incubation at 80°C for 20 min.[20] Results are expressed as % inhibition of peroxidation, where 100% inhibition is defined as baseline peroxidation of liposomes without added iron/ascorbate, and 0% inhibition is defined as peroxidation of liposomes with added iron/ascorbate. Calculation of IC_{50} values were performed by fitting a third order polynomial curve to the data.

Aqueous Phase Antioxidant Activity

The Trolox equivalent antioxidant capacity (TEAC) was measured by the method of Salah *et al.*[21] Values are expressed relative to a standard of Trolox C, the water-soluble analogue of vitamin E. The assay is based on the relative ability of antioxidants to scavenge the radical cation of 2,2′-azinobis(3-ethyl-benzothiazoline-6-sulphonate) (ABTS). Since the radical is generated by interaction with activated metmyoglobin and H_2O_2 then the assay is also influenced by how well the test compound inhibits formation of the radical. The extent of quenching of the ABTS radical is measured spectrophotometrically at 734 nm and compared to standard amounts of Trolox C. Quercetin was used as a positive control. The TEAC values obtained for quercetin, (−) epicatechin and (+) catechin agree well with published data.[12]

Measurement of Partition Coefficient

The method for measuring partition coefficients was performed essentially according to Foti *et al.*[22] Each procyanidin was dissolved in n-octanol and kept at 50°C for 1 h. A UV spectrum was recorded, and the value of the absorbance at the maximum was measured (A_0). An equal volume of organic solution and water were then mixed and UV spectra of the organic layer run at regular intervals until the absorbance value reached equilibrium (A_x). The partition coefficient ($\log P$) was calculated according

to the relationship:

$$P = \frac{A_x}{A_0 - A_x}.$$

A solution of n-octanol saturated with water was used as the blank.

RESULTS

Figure 2 shows the efficacy of catechin and dimeric/trimeric/tetrameric procyanidins (all with the same interflavan linkages) to inhibit lipid peroxidation of phosphatidyl choline vesicles. From this data, IC_{50} values were calculated (Table I). Catechin and the Ec-cat dimer are both highly effective antioxidants in this system, and compare favourably with the authenticated antioxidants quercetin and butylated hydroxytoluene. "Adding" further epicatechins to the molecule to form the trimer and tetramer species decreases the potency of inhibition comensurately. To determine whether these differences were due to the antioxidant properties of the procyanidins and not simply a function of their ability to partition into the lipid phase, their partition coefficients were measured (see Table II). The tetramer possessed the highest partition coefficient, indicating it is the most likely to partition into the lipid phase. The monomer, dimer and trimer molecules exhibited much lower values suggesting the results from lipid peroxidation were not solely a function of the ability of the procyanidin molecules to enter the lipid bilayer.

The trend of decreasing antioxidant activity with polymerisation was not observed in the

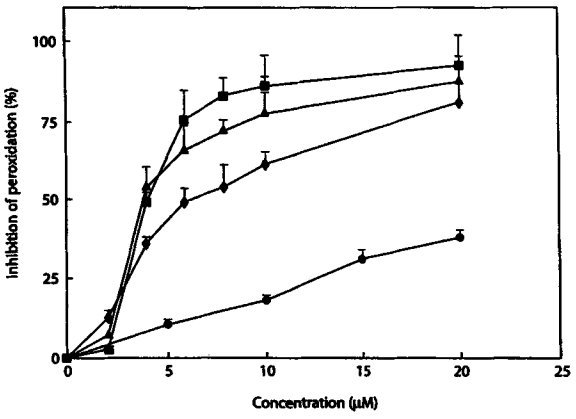

FIGURE 2 Influence of polymerisation of procyanidins on inhibition of iron/ascorbate induced lipid peroxidation. Peroxidation was performed in the presence of catechin (▲), Ec-cat dimer (■), Ec-Ec-cat trimer (◆) and Ec-Ec-Ec-cat tetramer (●). Each point represents the mean and standard deviation of three determinations.

TABLE I IC_{50} values from lipid peroxidation assay and TEAC values for catechins, procyanidins and control compounds

Procyanidin	IC_{50} value (μM)	TEAC
Catechin (cat)	3.4 ± 0.5	2.47 ± 0.02*
Epicatechin (Ec)	5.30 ± 0.3	2.23 ± 0.02*
Epicatechin gallate (EcG)	25.2 ± 3.6	5.02 ± 0.05*
Glycosylated catechin (cat-Glu)	28.7 ± 1.1	2.17 ± 0.11
Dimer B1 (Ec 4-8 cat)	4.1 ± 1.0	4.73 ± 0.03
Dimer B2 (Ec 4-8 Ec)	3.2 ± 1.2	4.39 ± 0.04
Dimer B3 (cat 4-8 cat)	4.3 ± 1.1	4.61 ± 0.02
Dimer B4 (cat 4-8 Ec)	4.7 ± 2.1	4.50 ± 0.02
Dimer B5 (Ec 4-6 Ec)	4.6 ± 1.7	4.47 ± 0.02
Dimer B7 (Ec 4-6 cat)	4.8 ± 1.1	4.52 ± 0.04
Dimer A2 (Ec 4-8, 2-0-7 Ec)	4.1 ± 0.4	3.79 ± 0.04
Galloylated dimer (cat-EcG)	12.9 ± 1.2	5.16 ± 0.04
Galloylated dimer (EcG-cat)	15.6 ± 0.1	5.34 ± 0.08
Trimer (Ec-Ec-cat)	6.5 ± 1.0	4.87 ± 0.04
Tetramer (Ec-Ec-Ec-cat)	40.3 ± 1.8	3.02 ± 0.03
Gallic acid	68.9 ± 5.7	2.91 ± 0.04
BHT	5.0 ± 0.2	N.D.
Trolox C	12.6 ± 1.4	1.00 ± 0.02
Quercetin	7.7 ± 0.3	4.70 ± 0.10*

*Consistent with published data.[12] N.D. – Not determined.

TABLE II Partition coefficient values for quercetin, catechins and procyanidins

Compound	Partition coefficient (log P)
Quercetin	1.23 ± 0.04
Epicatechin	0.63 ± 0.02
Epicatechin gallate	0.58 ± 0.01
Dimer (Ec-cat)	0.60 ± 0.02
Galloylated dimer (EcG-cat)	0.65 ± 0.02
Trimer (Ec-Ec-cat)	0.72 ± 0.03
Tetramer (Ec-Ec-Ec-cat)	1.40 ± 0.05

aqueous phase antioxidant assay (Table I). The TEAC value of catechin was approximately doubled when an epicatechin unit was "added" forming the Ec-cat dimer. Therefore the TEAC value per catechin molecule remained the same (Figure 3). The "addition" of a further epicatechin unit to form the trimer Ec-Ec-cat increased the TEAC value by a small amount but a further "addition", as in the tetramer, showed a significant decrease in the TEAC value ($P < 0.05$) and a large decrease in the TEAC value per catechin molecule.

Procyanidin dimers differ in their catechin moieties and the kind of interflavan linkages between them. We have analysed seven different dimers: six of the B series, linked 4–8 or 4–6, and one of the A series linked at two positions (4–8 and 2–0–7) (see Figure 1). There are no major differences between the two series of dimers in their action in the lipid peroxidation assay. However, in the TEAC assay, the A2 dimer has a TEAC value significantly lower than the B series dimers ($P < 0.01$).

The effect of "adding" a gallic acid unit to catechin and dimeric procyanidins on inhibition of lipid peroxidation is shown in Figure 4 (IC_{50} values shown in Table I). In both cases galloylation results in a reduced ability to prevent peroxidation of phosphatidyl choline vesicles. These changes cannot be explained by differences in partitioning into the lipid bilayer, since these two molecules possess very similar partition coefficients (see Table II). In contrast, in the aqueous phase TEAC assay, galloylation of these two procyanidins resulted in a significant increase in TEAC values. The TEAC value obtained for epicatechin was approximately doubled after galloylation to epicatechin gallate. Similarly the TEAC value of the Ec-cat dimer was increased from 4.73 ± 0.03 to 5.34 ± 0.08 after galloylation. The same trend in both assays was observed for the cat-Ec dimer and its galloylated ester (results not shown).

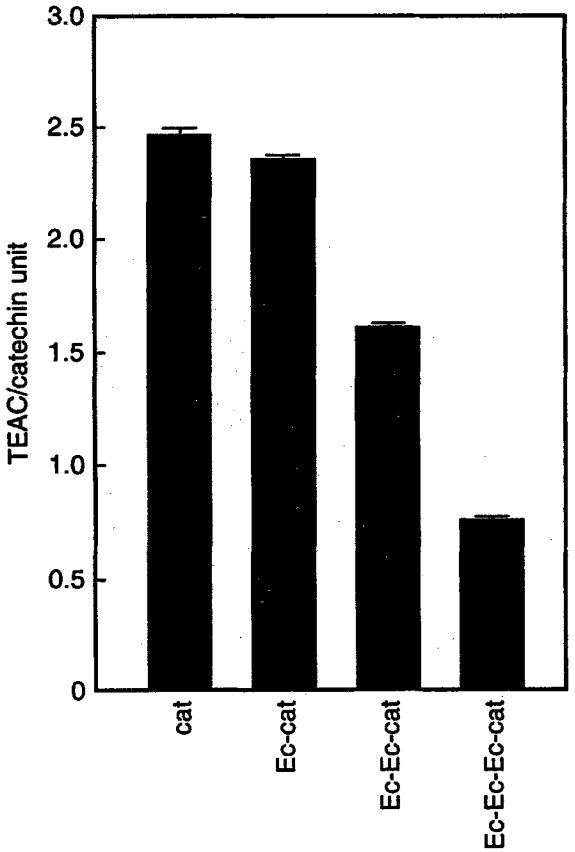

FIGURE 3 Influence of polymerisation of procyanidins on TEAC values. Values are expressed as TEAC value per catechin residue for each compound. Each value represents the mean and standard deviation of four determinations.

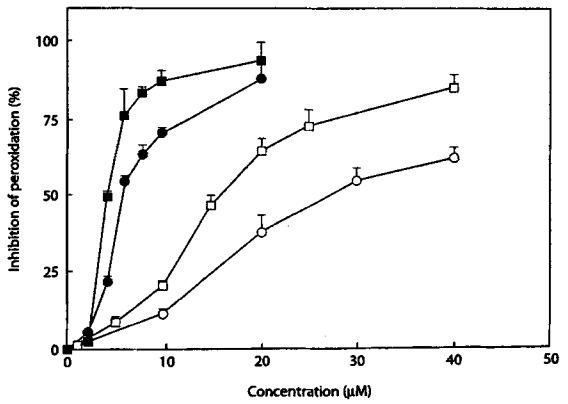

FIGURE 4 Influence of galloylation on inhibition of iron/ascorbate induced lipid peroxidation. Peroxidation was performed in the presence of epicatechin (●), epicatechin gallate (○), Ec-cat dimer (■) and EcG-cat dimer (□). Each point represents the mean and standard deviation of three determinations.

The effect of glycosylation is shown in Table I. "Adding" a sugar unit to a catechin molecule causes a reduction in TEAC value and a pronounced increase in the IC_{50} value of catechin in the lipid phase assay.

DISCUSSION

The efficacy of catechin in the lipid phase is equivalent to that of quercetin, which is consistent with previous studies.[4,10,11] These two molecules differ in that quercetin has a free –OH group at the 3-position attached to a 2,3 double bond and adjacent to a 4-carbonyl function in the C ring. These features increase the potency of antioxidant action in the aqueous phase TEAC assay[12] facilitating the delocalisation of electrons from the aryloxyl radical on the B ring to the A ring; quercetin therefore is a superior antioxidant to catechin. However these structural variables result in no differences in their action in the lipid peroxidation assay.

The potency of catechin in the lipid phase was substantially decreased by the "addition" of a galloyl ester at the 3-OH group in the C ring. This is unsurprising since gallic acid is an extremely poor inhibitor of lipid peroxidation. In the TEAC assay, there appears to be a trend to increasing TEAC value with the number of free hydroxyls. Therefore "adding" a gallic acid unit to a catechin molecule increases the hydrogen-donating ability by both increasing the number of free hydroxyls and by the introduction of carbonyl function adjacent to a double bond by the ester linkage (analogous to quercetin).

The effect of adding a sugar moiety to catechin on the antioxidant action described herein is consistent with previous studies on the glycosylation of quercetin.[23,24] As mentioned previously, for maximum effectiveness of antioxidant activity in the aqueous phase, there is a requirement for the 3-hydroxyl group in the C ring to be unblocked. A glucose molecule attached to the catechin molecule at this position reduces the ability of the B ring hydroxyls to donate hydrogen. This structural difference also affects the action in the lipid phase assay in the same manner.

Polymerisation of catechin molecules from monomer to tetramer substantially diminished the ability to prevent free radical damage in the lipid system (despite the fact that the larger oligomers were more likely to partition into the lipid phase) whilst increasing aqueous phase antioxidant activity from monomer to trimer (subsequently decreasing from trimer to tetramer). Clearly these compounds are exerting their antioxidant effect by different methods in the two assays; antioxidant effectiveness is therefore not simply a function of the ability to donate hydrogen via phenolic hydroxyls.

This paper allows identification of the more active components of this class of compounds present in fruits and beverages that may protect against free radical damage, LDL oxidation (implicated in the pathogenesis of coronary heart disease) and DNA damage and cancer. Future quantitative analysis of foods can now be used to detect those foods which are rich in these components, for human intervention studies and as an aid in the development of natural food additives with desired antioxidant properties.

Acknowledgements

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