



Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 2497–2509

BIOORGANIC &
MEDICINAL
CHEMISTRY

Cysteinyl-flavan-3-ol Conjugates from Grape Procyanidins. Antioxidant and Antiproliferative Properties

J. L. Torres,^{a,*} C. Lozano,^{a,c} L. Julià,^b F. J. Sánchez-Baeza,^b J. M. Anglada,^b
J. J. Centelles^c and M. Cascante^c

^a*Department of Peptide and Protein Chemistry, Institute for Chemical and Environmental Research (IIQAB-CSIC),
Jordi Girona 18-26, 08034 Barcelona, Spain*

^b*Department of Biological Organic Chemistry, Institute for Chemical and Environmental Research (IIQAB-CSIC),
Jordi Girona 18-26, 08034 Barcelona, Spain*

^c*Department of Biochemistry and Molecular Biology, University of Barcelona, Martí i Franquès 1-11, 08028 Barcelona, Spain*

Received 7 December 2001; accepted 2 April 2002

Abstract—New bio-based antioxidant compounds have been obtained by depolymerisation of grape polymeric flavanols in the presence of cysteine. Their preparation and purification, as well as their antiradical/antioxidant and antiproliferative properties are reported. 4 β -(*S*-cysteinyl)epicatechin **5**, 4 β -(*S*-cysteinyl)catechin **6** and 4 β -(*S*-cysteinyl)epicatechin 3-*O*-gallate **7** were efficiently purified from the crude depolymerised mixture by cation-exchange chromatography and preparative reversed-phase chromatography. The new compounds were more efficient than the underivatised (–)-epicatechin **1** as scavengers of the 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) and weak growth inhibitors of human colon carcinoma HT29 cells. The order of antiradical and antiproliferative efficiency was **7** > **5** ~ **6** > **1**, the same for both assays. © 2002 Elsevier Science Ltd. All rights reserved.

Introduction

Much attention is currently being paid to the putative benefits of plant polyphenols as complements to the organism's antioxidant defence systems. While evolving from anaerobic microorganisms, aerobic life forms had to establish a delicate balance between the benefits and risks of using oxygen to obtain energy. The respiratory chain inevitably produces reactive intermediates such as the superoxide anion radical (O₂^{•−}) which lead to other reactive species potentially harmful to biological molecules such as DNA, lipids and proteins.¹ Life organisms have developed enzymatic systems to regulate the levels of all these reactive oxygen species (ROS). Superoxide dismutase, catalase and glutathione peroxidase which scavenge the superoxide anion, H₂O₂ and organic hydroperoxides, respectively, are major detoxifying enzymes.¹ Occasionally, the defence systems are overwhelmed by an excess of ROS produced by illnesses, aging or by external factors such as air pollution, smoking or UV radiation.

In humans, the excess of ROS, which can be either radicals or non-radical compounds, has been related to numerous diseases and dietary plant antioxidants such as carotenoids and flavonoids are considered to help the internal defence mechanisms against unwanted oxidations.^{1,2} Particularly flavanols from tea, grape or pine bark are potent free radical scavengers of interest as preventative agents against cancer and cardiovascular diseases.^{3–7} The capacity to arrest the cell cycle and/or promote apoptosis may contribute to the chemopreventative effect of some members of the flavanoid family.^{8,9}

The major flavanols in green tea are monomeric catechins, that is (–)-epigallocatechin 3-*O*-gallate, (–)-epigallocatechin, (–)-epicatechin (**1**) and (–)-epicatechin 3-*O*-gallate (**3**).¹⁰ Grape and pine bark extracts also contain monomers and are rich in oligomeric (~2–7 residues) and polymeric (>7 residues) flavanols.^{7,11–14} Polymerised flavanols are called proanthocyanidins because they yield anthocyanidins upon depolymerisation under acidic conditions. In the presence of a strong nucleophile, proanthocyanidins yield two kinds of compounds: flavan-3-ols and their derivatives at position 4, coming from the polymer's terminal and extension

*Corresponding author. Tel.: +34-93-400-61-12; fax: +34-93-204-59-04;
e-mail: jltqbp@iiqab.csic.es

units, respectively¹⁵ (Scheme 1). This procedure, with toluene- α -thiol as nucleophile, is currently used to estimate the degree of polymerisation and galloylation of proanthocyanidins.^{11,12,16,17}

The acid cleavage of proanthocyanidins in the presence of other thiols (2-hydroxyethylthiol, 2-aminoethylthiol, Scheme 1) has been recently used to obtain new compounds with antiradical/antioxidant activity and modified physico-chemical properties.^{18,19} The combination of antioxidant polyphenols with other natural products is a promising approach in the search for new safe compounds with diversified applications.

Here, we describe the preparation and purification of a new family of bio-based thio-derivatives of flavan-3-ols (**5–7**) obtained by depolymerisation of procyanidins²⁰ in the presence of the natural amino acid cysteine. The reaction also yielded the underivatised terminal units **1–3**. The cleavage conditions used were essentially those described before for aminoethylthio-derivatives such as **4**.¹⁹ The antiradical/antioxidant activity in the DPPH assay and the antiproliferative activity on a human colon carcinoma cell line of the new derivatives are evaluated.

Results

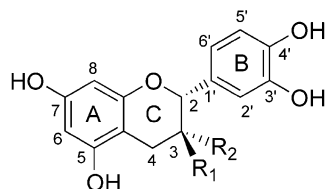
Purification

The cysteinyl flavan-3-ols were separated from the crude depolymerisation mixture on a strong cation-exchange resin (MacroPrepTM High S 50 μ m) by taking advantage

of the amino function on the cysteinyl moiety. The goal was to have the underivatised monomers and the cysteinyl conjugates elute in two separate groups by means of two consecutive isocratic steps, namely elution with initial buffer and with NaCl containing buffer, respectively.

First, the elution conditions were optimised at semi-preparative scale (6 mL bed volume). The buffers pH was adjusted to 2.25 to make sure that the carboxylic acid function was completely protonated, and the conjugates were retained on the resin through their net positive charges. Hydrophobic interactions with the stationary phase were eliminated with 30% (v/v) EtOH added as co-solvent. More ethanol resulted in early release of the charged conjugates. Moreover, the amount of salt necessary to elute the charged conjugates was minimised. The final optimal conditions were: [A], 4.75 bed volumes of 20 mM sodium phosphate pH 2.25 buffer/EtOH (7:3) for the elution of monomers; [B], 4.75 bed volumes of 20 mM sodium phosphate pH 2.25 buffer/EtOH (13:7) 100 mM NaCl for the elution of the conjugates together with the excess of cysteine from the cleavage reaction.

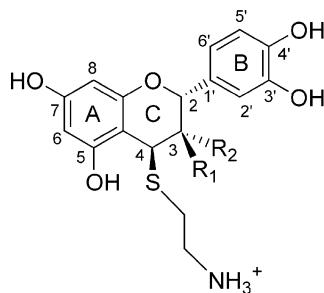
The cation-exchange separation was scaled-up on a glass column (21 $\times$ 2.5 i.d., 105 mL bed volume) packed with the same stationary phase. The maximum load was found to be 500 mL (ca. 500 mg GAE, gallic acid equivalents by the Folin–Ciocalteu's method, initial polyphenols) of crude cleavage mixture which was processed in seven repetitive runs. Subsequently, compounds **5–7** were purified by preparative reversed-phase



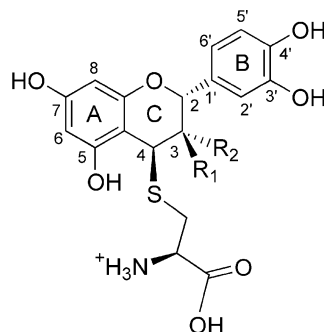
1 R₁=H, R₂=OH, (-)-epicatechin

2 R₁=OH, R₂=H, (+)-catechin

3 R₁=H, R₂=Gal, (-)-epicatechin 3-O-gallate



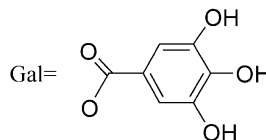
4 R₁=H, R₂=OH, 4β-(2-aminoethylthio)epicatechin

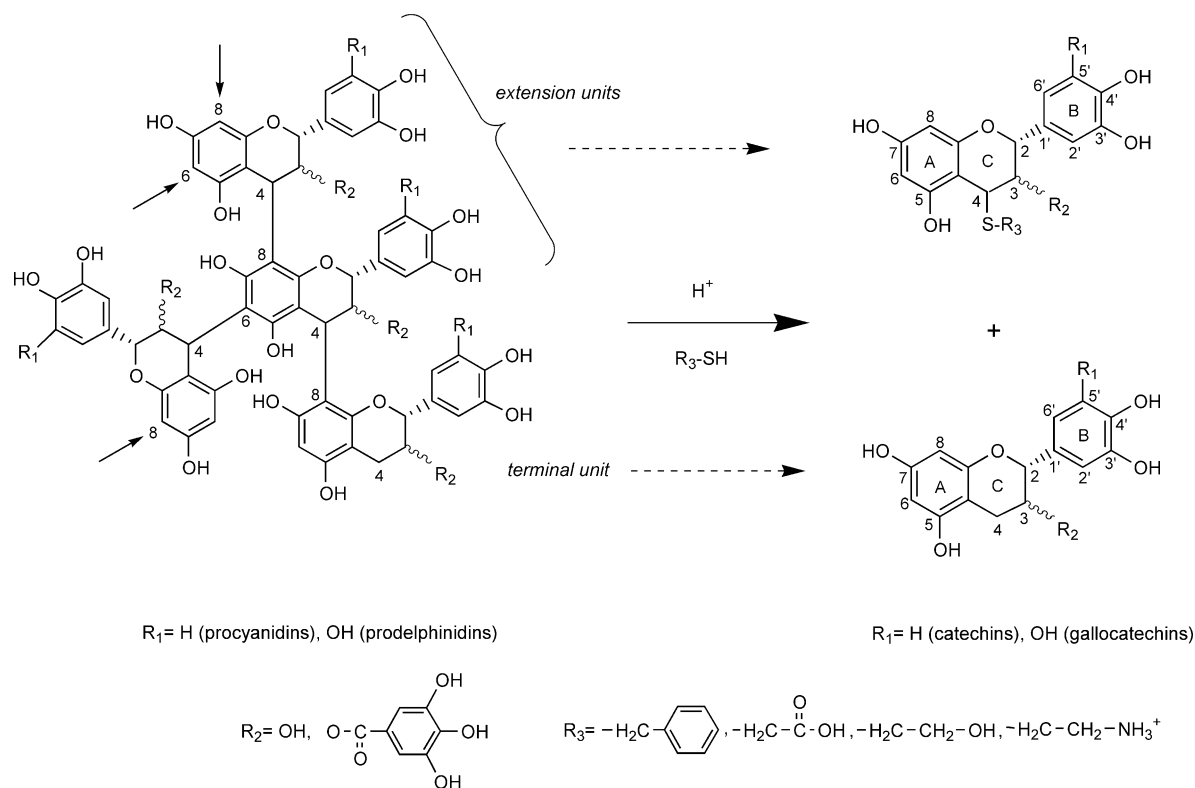


5 R₁=H, R₂=OH, 4β-(S-cysteinyl)epicatechin

6 R₁=OH, R₂=H, 4β-(S-cysteinyl)catechin

7 R₁=H, R₂=Gal, 4β-(S-cysteinyl)epicatechin 3-O-gallate





Scheme 1. Depolymerisation of proanthocyanidins (polymeric flavan-3-ols). The arrows indicate putative polymerisation positions.

high-performance liquid chromatography (RP-HPLC) as stated in the experimental section. Briefly, the mixture obtained by cation exchange was desalted and fractionated by a CH_3CN gradient in 0.1% (v/v) trifluoroacetic acid (TFA). Then, each fraction, rich in one of the cysteinyl derivatives, was re-chromatographed with CH_3CN gradients in triethylamine phosphate (TEAP) buffers and 0.1% (v/v) TFA to yield 99.5% pure (by RP-HPLC) compounds. The stereochemistry at C-2, C-3 and C-4 of compounds **5–7** was assigned from the hydrogen coupling constants measured by ^1H NMR and following Haslam and co-workers.¹⁵ In

agreement with the literature, the 4β derivatives were the major isomers obtained irrespective of 2,3-stereochemistry.^{15,21}

Free radical scavenging activity

The new cysteinyl flavan-3-ol derivatives are potent free radical scavenging agents (Fig. 1) in the 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) assay.^{22,23} The results are expressed as ED_{50} as defined in the experimental section. The inverse of ED_{50} is a measure of the antiradical power (ARP). By multiplying the ED_{50} by two, the stoichiometric value (theoretical concentration of antioxidant to reduce 100% of the DPPH) is obtained. The inverse of this value represents the moles of DPPH reduced by one mole of antioxidant and gives an estimate of the number of hydrogen atoms involved in the process. Table 1 summarises the parameters obtained from the DPPH assay. The new conjugates were more efficient than (–)-epicatechin **1** and Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), being the order of antiradical power

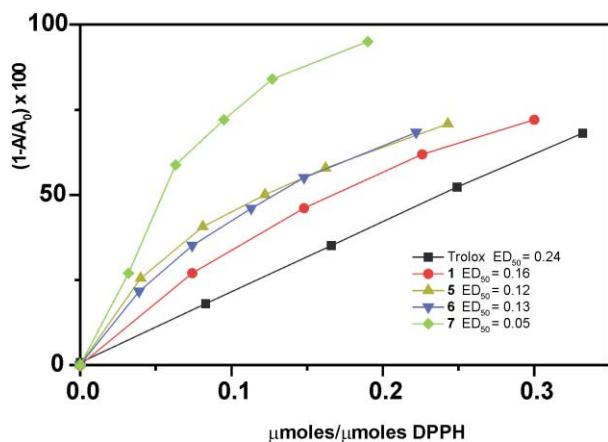


Figure 1. Free radical scavenging activity of the cysteinyl derivatives of flavan-3-ols in the DPPH assay. Absorbance (A) at 517 nm is a measure of the amount of free radical remaining in solution. $(1-A/A_0) \times 100$ represents the percentage of DPPH already reacted with the antioxidant. Each point is the mean of three determinations.

Table 1. Antiradical power and stoichiometry from the DPPH assay

Compound	ARP ($1/\text{ED}_{50}$)	Stoichiometric value	H atoms per molecule ^a
Trolox	4.2	0.48	2.1
1	6.2	0.32	3.1
5	8.3	0.24	4.2
6	7.7	0.26	3.8
7	20	0.10	10

^aMoles reduced DPPH per mole antioxidant.

$7 > 5 \sim 6 > 1 > \text{Trolox}$.²⁴ We obtained a stoichiometric value of 0.32 for (–)-epicatechin, corresponding to the reduction of ca. three DPPH molecules per molecule of **1**. The cysteinyl derivatives of epicatechin (**5**) and catechin (**6**) were able to reduce roughly one more molecule of DPPH than the underivatised (–)-epicatechin **1**. Gallate containing compound **7** is clearly the most efficient of the new molecules.

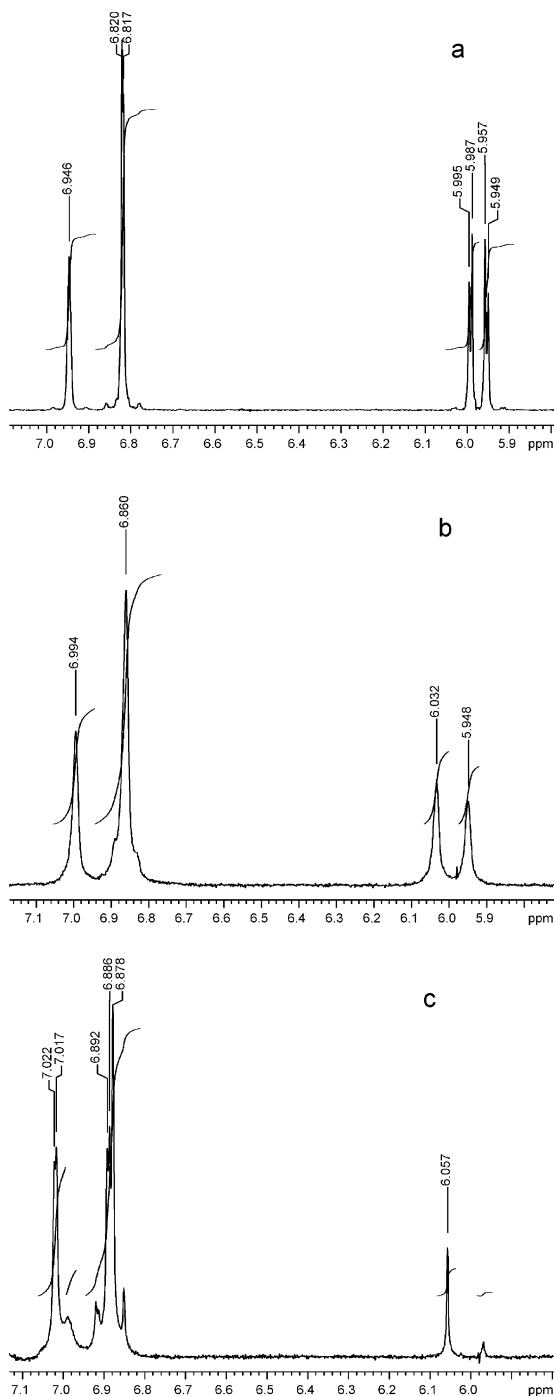


Figure 2. ¹H NMR spectra expansion of the aromatic hydrogen region. (a) Compound **1**; (b) compound **4** and (c) compound **5**. All three spectra were recorded under identical conditions after 6 h in D₂O/acetone-*d*₆ (3:1 v/v) at pD=6 (ammonium chloride buffer). Signal at ca. 6.05 ppm corresponds to H-6 and signal at ca. 5.95 ppm corresponds to H-8.^{25,26} The signals at lower field and insensitive to the deuterium exchange correspond to H-2', H-5' and H-6'.

Nuclear magnetic resonance (NMR) deuterium exchange

When recording the standard ¹H NMR spectra of derivatives **4–7** using acetone-*d*₆/D₂O as solvent, we observed a systematic discrepancy in the area corresponding to the signals of hydrogen atoms at positions 6 and 8 which we attributed to deuterium exchange with the solvent. In order to confirm this, we recorded the ¹H and ¹³C NMR spectra of two freshly prepared samples, one using regular compound **4** and a second sample using a preparation of the same compound generated by three successive operations of dilution in D₂O and lyophilisation. The spectrum corresponding to the first sample showed the normal signals with only a little decrease in the integral for H-6 and H-8. In the second sample no signal for H-6, H-8, C-6 and C-8 was detected while the rest of the spectrum did not show any other change. A closer look at the ¹³C NMR spectrum revealed two low intensity signals with the characteristic pattern for monodeuterated carbons near the positions of C-6 and C-8.

The observed hydrogen exchange was unexpectedly fast and we decided to follow the kinetics of the deuteration process by ¹H NMR under controlled conditions (solvent, concentration, temperature, pD) using (–)-epicatechin **1** as a control. The spectra were obtained using a mixture of D₂O/acetone-*d*₆ buffered at pD=6 (see details in Experimental). For compounds **4** and **5** the deuterium exchange at positions H-6 and particularly H-8 was very fast compared to that of **1** (Figs 2 and 3). The deuteration first order pseudokinetic data were fitted using a simple exponential decay model and a good correlation (*R*² higher than 0.96) for the six sets of data was obtained. The observed half life for H-8 and H-6 signals was respectively of 3851 and 5369 min for compound **1**; 511 and 911 min for compound **4**; 57 and 150 min for compound **5**. In all cases the exchange process proceeded until total hydrogen exchange.

Bond dissociation enthalpies (BDE)

We have performed theoretical calculations at B3LYP level of theory^{27,28} to estimate the BDEs of several X–H

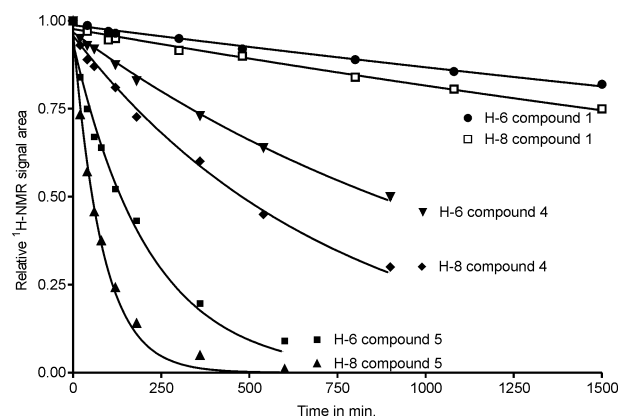


Figure 3. Time evolution for the H-6 and H-8 ¹H NMR signal areas for compounds **1**, **4**, **5**. Relative signal is the signal area referred to the average one-hydrogen area for the further hydrogen signals. Data were adjusted using a single exponential decay. *R*² was higher than 0.96 in all cases.

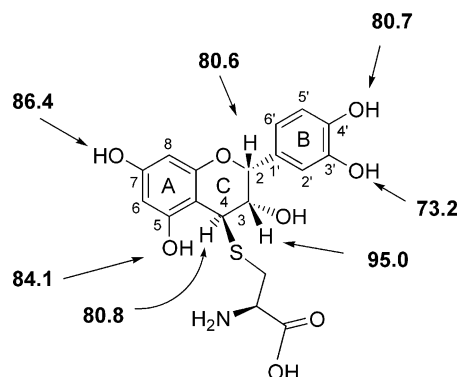


Figure 4. Calculated bond dissociation enthalpies for compound **5** obtained at B3LYP level of theory.

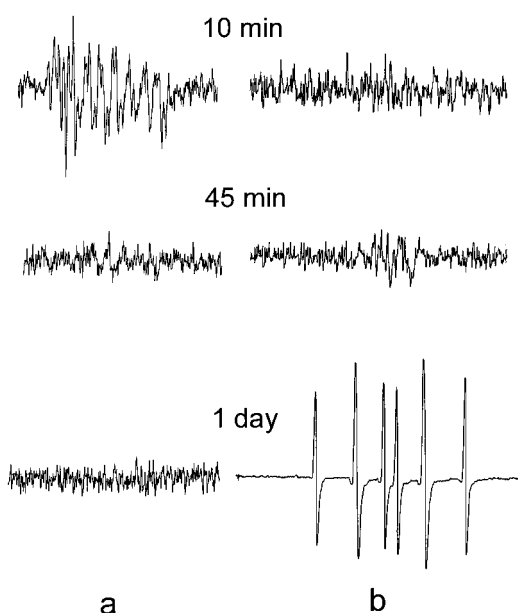


Figure 5. EPR spectra of NaOH (1 M) aqueous solutions of (–)-epicatechin **1** (10 mM) (a) and 4β-(S-cysteinyl)epicatechin **5** (10 mM) (b) at different times.

bonds of compound **5**. These BDEs are expected to be directly related to the ability of flavanols to scavenge reactive oxygen species and radicals such as DPPH by donating hydrogen atoms. Our computed values for compound **5** are displayed in Figure 4, which shows that the BDEs at the C-2 and C-4 positions (80.6 and 80.8 kcal/mol, respectively) are of the same order than the BDE of the OH at C-4' (80.7 kcal/mol).

Formation of free radicals

Flavanols are air oxidised under aqueous basic conditions and the intermediate radicals formed may be detected by electron paramagnetic resonance (EPR).^{29–31} The EPR spectra of diluted (10 mM) solutions of (–)-epicatechin **1** and its cysteinyl derivative **5** in 1 M aqueous NaOH, obtained at different times, revealed significant differences (Fig. 5). While **1** generated

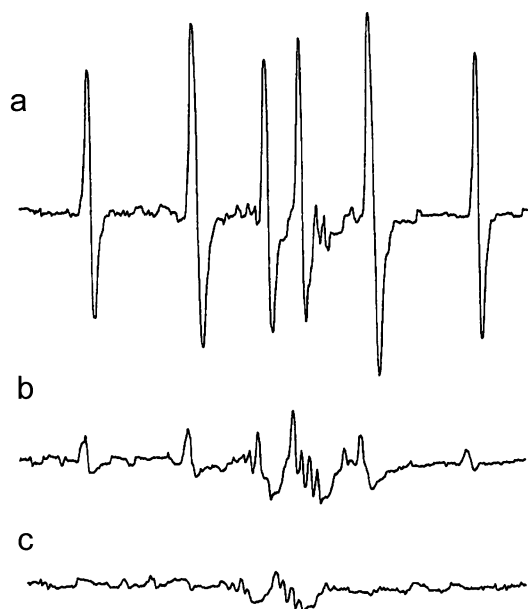


Figure 6. EPR spectra of the stable radical generated from 4β-(S-cysteinyl)epicatechin **5** (10 mM) by a 1-day treatment with 1 M aqueous NaOH (a) and after ultraviolet irradiation with a high-pressure mercury lamp for 2 min (b) and 10 min (c).

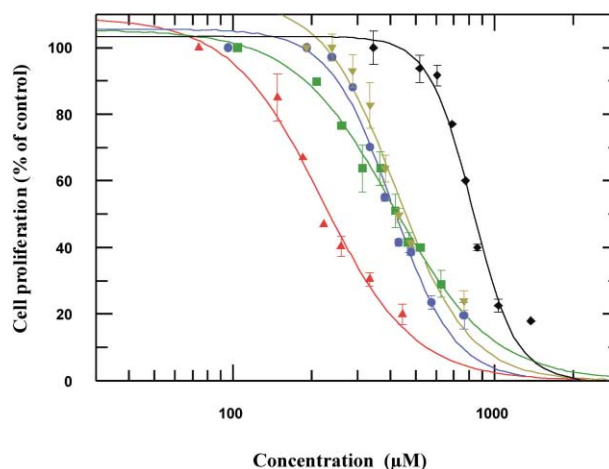


Figure 7. HT29 colorectal adenocarcinoma cell proliferation in response to treatment with increasing doses (*X* axis) of 2-aminoethylthio and cysteinyl derivatives of flavan-3-ols. ■ (4), ● (5), ▼ (6), ▲ (7) and ♦ (1). The viability and proliferation are expressed as per cent of untreated control cells (mean ± SD).

radicals detected within minutes which rapidly disappeared and were coincident with those observed by other authors²⁹ (Fig. 5a), **5** generated within hours a simple and much more stable radical which presents a six-line pattern with relative intensities that correspond to hfs constants from three protons with values of 2.67, 1.55 and 1.62 G (Fig. 5b). These signals slowly decreased over a period of several days and were reduced significantly upon irradiation with UV light (Fig. 6). The same radical was detected for the 2-aminoethylthio derivative **4**. In no case, this paramagnetic species was detected from (–)-epicatechin **1**.

Table 2. Antiproliferative potency against human colorectal adenocarcinoma HT29 cells

Compound	<i>n</i> ^a	Mean IC ₅₀ (μ M)	SD
1	4	825.7	12.2
4	6	408.0	11.6
5	6	406.6	21.0
6	6	421.2	26.5
7	6	218.9	6.7

^a*n*, number of experiments performed; SD, standard deviation.

Antiproliferative activity

The effect of compounds **4–7** and (–)-epicatechin **1** on the proliferation of a human carcinoma cell line (HT29) using an MTT assay was examined. The results showed a reduction in cell proliferation in a dose-dependent fashion after treatment with the flavan-3-ols at the reported concentrations for 72 h (Fig. 7). Table 2 shows the mean IC₅₀ values and corresponding SD obtained. It should be noted that IC₅₀ was calculated with respect to the total number of cells in the control after 72 h of proliferation (20,000 cells/well). At the IC₅₀, the final number of treated cells was approximately 10,000 cells/well, which was 4 times higher than the number of cells at the beginning of the experiment. Thus, the IC₅₀ calculated in this paper is indicative of the concentration of product that inhibits the proliferation by 50%. The lower the IC₅₀, the more potent the compound is. The order of antiproliferative potency followed the same pattern obtained from the antiradical activity assay: **7** > **4** ~ **5** ~ **6** > **1**.

Discussion

Purification

The efficient cation-exchange separation of the cysteinyl conjugates from the complex crude cleavage mixture depends on several factors, particularly on how successfully the hydrophobic interactions with the stationary phase are handled. If no interaction other than electrostatic were to take place, the underivatized monomers together with any other uncharged material would be washed away with the starting aqueous buffer while positively charged amine containing derivatives would be retained and subsequently eluted with salt. In many instances though, some amount of an organic co-solvent is needed to either lessen or eliminate hydrophobic interactions. In our hands, when no solvent was added to the eluents all molecules were hydrophobically retained on the resin. Particularly, more than 10 bed volumes of washing buffer were needed to release the uncharged (–)-epicatechin 3-*O*-gallate **3**. Moreover, other materials, including coloured species from the grape extract, were also retained and gradually released over the entire chromatographic process. Addressing this point is crucial for preparative purposes when volumes must be kept to a minimum and cleaner mixtures mean easier purifications. We have previously

reported the use of an agarose-based gel (SP SepharoseTM high performance) and acetonitrile (CH₃CN) as co-solvent for the separation of flavanol conjugates with cysteamine.¹⁹ The monomers were washed away with 10% (v/v) CH₃CN in the starting eluent and the amine containing conjugates were eluted with a gradient of salt to eliminate the electrostatic interaction with the resin and a simultaneous gradient of CH₃CN. The co-solvent gradient allowed the separation of the epicatechin and catechin derivatives from the more hydrophobic gallate containing conjugate. This facilitated the subsequent purification of the products. The main disadvantage of this procedure is the high eluting volumes needed to obtain a baseline separation. In the present case we decided to minimise the eluting volume by raising the amount of co-solvent at the cost of having all conjugates eluting together. Further modifications introduced in this step were the use of a bulk stationary phase (MacroPrepTM High S 50 μ m) based on a methacrylate co-polymer and EtOH instead of CH₃CN. The appropriate amount of co-solvent in the starting eluent was found to be 30% (v/v) EtOH. Less co-solvent resulted in unacceptable retention of (–)-epicatechin 3-*O*-gallate **3** and more EtOH resulted in early elution of the charged conjugates **5** and **6**. This effect has also been observed in the cation-exchange purification of hydrophobic cationic surfactants³² and might be due to the influence of the co-solvent on the stabilisation of ions pairs with the buffer. After elution of the monomers we took advantage of this effect and increased the amount of EtOH to help recover the conjugates in the presence of salt (100 mM NaCl). At the end we came out with a set of conditions that yielded two groups of compounds, namely underivatized monomers and cysteinyl conjugates, with minimum buffer, co-solvent and salt consumption.

Antiradical/antioxidant activity

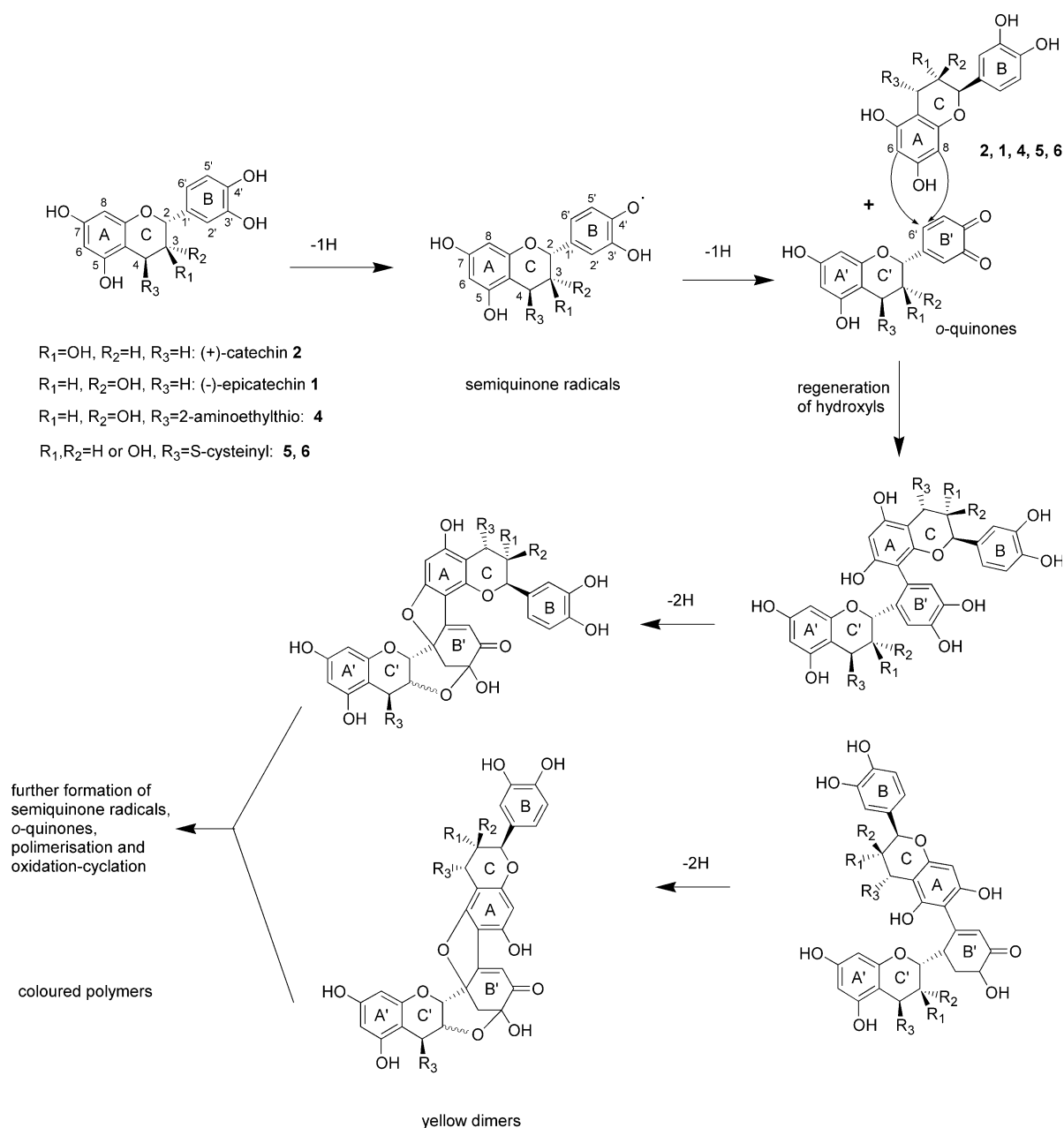
In the present paper, we have focused on the free radical scavenging activity of the cysteinyl derivatives in solution using a well known stable free radical (DPPH). While the overall efficiency of polyphenolic antioxidants against lipid and protein oxidation may depend on many factors including the physico-chemical properties of the environment, the DPPH test provides a simple measure of intrinsic antioxidant efficiencies.^{23,33,34}

It has been described that the oxidation of (+)-catechin **2** by DPPH takes place in two kinetic steps, one fast step when the most labile hydrogen atoms are abstracted and simple dimers formed, and a second slow step when dimers are further oxidised and polymerised (Scheme 2, catechin).³³ The electron-rich positions 6 and 8 are putatively involved in the polymerisation process by nucleophilic additions on flavanol derived *o*-quinones.³³ Altogether the process results in the reduction of more than two molecules of DPPH per molecule of (+)-catechin. Regeneration of phenolic hydroxyls (Scheme 2, third step) by polymerisation appears to be the reason for the high number of H atoms or electrons involved in the scavenging activity of polyphenols under a variety of other experimental setups such as enzyme catalysed oxidation followed by

either product purification³⁵ or EPR measurements,³⁰ and electrochemical oxidation.³⁶

The introduction of sulphur atom containing moieties into position 4 of the flavan system have resulted in derivatives (**4–7**) able to reduce at least one more molecule of DPPH than (–)-epicatechin **1**. The differences in antiradical efficiencies may be related to an enhanced nucleophilic character of positions 6 and 8 in ring A. Our evidence comes from NMR studies. While the ¹H NMR spectrum of (–)-epicatechin **1** remained almost unaltered in acetone-*d*₆/D₂O, the signals corresponding to protons 6 and 8 from compounds **4**, **5** disappeared progressively over a period of 1–72 h, suggesting that these protons were exchanged by deuterium. To rule out detection problems arising from long relaxation times of those atoms or due to a dynamic chemical exchange

process, we introduced changes in the appropriate acquisition parameters with the same outcome: H-6 and H-8 signals had completely vanished from the old samples. The exchange was corroborated by ¹³C NMR with a sample of **4** previously lyophilised in the presence of D₂O as described in Experimental and by a kinetic study at controlled pD for compounds **1**, **4** and **5** (Figs 2 and 3). It is known that phloroglucinol derivatives are sensitive to the presence of Lewis and protic (low pH) acids reactive towards electrophiles.³⁷ When spectra of those compounds were recorded in solvents with interchangeable deuterium atoms (e.g., D₂O) the aromatic ring hydrogen atoms could be exchanged by deuterium. In flavan-3-ols hydrogen atoms at positions 6 and 8 have similar properties but deuterium exchange is more difficult.³⁷ This exchange may be related to the well-known aromatic electrophilic substitution or keto-enol

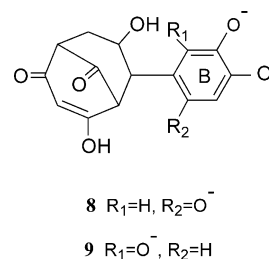


Scheme 2.

tautomerism applied to the case of aromatic phenol rings with highly increased π -electron density. Our data show clearly that the substitution of the 4 β hydrogen of flavanols by cysteamine or cysteine through a thioether bond exerts a dramatic influence over the properties of the contiguous aromatic ring A. In the pseudo first order kinetic conditions used (great excess of the electrophile reagent), the difference in exchange half-lives may be directly related to variations in the activation energy barriers. This effect may be mediated by the formation of intramolecular hydrogen bonds between the phenol groups and the functional groups on the sulphur containing moiety. Other mechanisms directly related with the sulphur atom may also be involved. In any case our results show that positions 6 and particularly 8 of compounds **4–7** possess an increased ability to react with electrophilic species (e.g., water). This may result in a higher capacity to regenerate polyphenolic hydroxyls through polymerisation (Scheme 2) that would explain why derivatives **4–6** are able to provide more H atoms than (–)-epicatechin **1**. Compound **7** is the most effective of the conjugates as expected by the presence of the pyrogallol group containing gallate ester moiety, which provides additional H atoms and polymerisation positions.^{29,38}

An alternative explanation for the enhanced capacity of the new derivatives to transfer hydrogen atoms would involve the substituted position 4 on ring C. Mechanistic studies on radical oxidation of catechins by 2,2'-azobis(2-aminopropane)hydrochloride (AAPH) and semiempirical calculations show that hydrogen atoms other than phenolic (i.e., H-2 on ring C) may be abstracted from flavanols.^{38,39} Using more accurate theoretical calculations, which have been shown to compare quite well with the experimental values,²⁸ we obtained a BDE for the C–H bond at position 4 of compound **5** similar to the values for the C–H bond at position 2 and the O–H bond at position 4' (Fig. 4). This result is suggesting that the hydrogen atom on C-4 may be abstracted by oxidising agents such as DPPH and might contribute to the enhanced antiradical power of the thiol containing derivatives.

EPR experiments of spontaneous air oxidation of **4**, **5** and (–)-epicatechin **1** in 1 M aqueous NaOH also evidenced differences between derivatised and underivatized flavanols. The multiplet (Fig. 5b), with a Landé factor $g = 2.0041$, detected when **4** and **5** (10 mM) were analysed corresponds to the coupling of the unpaired electron with three protons with constant values of 2.67, 1.55 and 1.62 G. In a very extensive work by Jensen and Pedersen³¹ about oxidation of (–)-epicatechin **1**, (+)-catechin **2** and catechinic acid in basic solutions they concluded that oxidation of (–)-epicatechin led to the stable radicals **8** and **9** in alkaline aqueous DMSO solution, and the generation of only **8** in alkaline aqueous EtOH solution. These radicals appeared to be species resulting from hydroxylation on ring B and rearrangement reactions on rings A and C of (–)-epicatechin. We have confirmed that oxidation of (–)-epicatechin **1** in concentrated (50 mM) 1 M aqueous NaOH solution generates radical **8**, and in no case gives the six-line



pattern multiplet coming from **4** and **5**. This pattern differs from the spectra of radicals generated from 1-substituted 2,3,4-trihydroxybenzenes, 1-substituted 3,4,6-trihydroxybenzenes and 1-substituted 3,4,5-trihydroxybenzenes.⁴⁰ The stable radical detected for the sulphur containing derivatives might alternatively be formed on ring A or C. In view of the bond dissociation energies of the different carbon-hydrogen bonds obtained by theoretical calculations (Fig. 4), a radical on C-4 is likely to be formed under our experimental conditions. This radical would be coupled to three protons at positions 3, 6 and 8 which might explain the EPR pattern recorded (Figs 5b and 6a). This point is currently being looked into in our Laboratories. Apart from those described so far, other reactions may occur in an aqueous basic medium, namely oxidation to sulfoxides and nucleophilic attack of the amino group onto quinones, which may influence the formation and/or stabilisation of radicals. In any case the thio derivatives tend to generate relatively stable radicals that last for days and remain sensitive to further oxidation triggered by UV light (Fig. 6) and, presumably, by reactive oxygen species.

Antiproliferative activity

It has been reported that flavan-3-ols are active against proliferation of cancer cells including colon carcinoma cell lines, being (–)-epicatechin **1** and (+)-catechin **2** the least efficient of the monomers.^{4,41,42} The novel 2-aminoethylthio and cysteinyl derivatives **4–7** proved to be more efficient than **1** in HT29 human carcinoma cells. It has been proposed that the antiproliferative activity of catechins (flavanols) and other flavonoids comes from the inhibition of the kinase activity of proteins involved in cell cycle regulation^{43–46} rather than any interaction with any antioxidant defence mechanism. The conjugates might have higher affinity than intact flavan-3-ols for the appropriate protein binding site. Alternatively, conjugation might influence the transport of the flavanols into the cell to reach putative intracellular binding sites. This point is also being looked into in our Laboratories. Curiously, the order of efficiency of the molecules on both the antiradical and antiproliferative assays was identical. Whether this is just a coincidence or not is something that will have to be clarified. Thorough studies on cell cycle disruption and apoptosis are also under way.

Conclusions

Potent cysteine conjugates with natural antioxidants of the flavanol type have been obtained by acid cleavage of polymeric procyanidins followed by cation-exchange

and RP-HPLC purification. The derivatisation appears to improve the free radical scavenging capacity of flavanols by fostering the regeneration of active hydroxyl groups upon polymerisation and/or by facilitating the abstraction of the hydrogen atom geminal to the sulphur atom. The new conjugates are also weak inhibitors of colon carcinoma cells proliferation. The cysteinyl flavanols, together with the previously described cysteamine conjugates incorporate chemical functions (amine, carboxylic acid) and include activated positions (8 and 6 on ring A) which may facilitate the preparation of other products with improved properties.

Experimental

Materials

The primary source of procyanidins was the byproduct from pressing destemmed Parellada grapes (*Vitis vinifera*) (Bodegas Miguel Torres, S.A., Vilafranca del Penedès, Spain) which were extracted and fractionated as described.^{19,20} Water and solvents used were: analytical grade MeOH (Panreac, Montcada i Reixac, Spain) for the acid cleavage reaction and DPPH assay; deionised water and bulk EtOH (Momplet y Esteban, Barcelona, Spain) for semipreparative and preparative cation-exchange chromatography; milli-Q[®] water and HPLC grade CH₃CN (E. Merck, Darmstadt, Germany) for analytical RP-HPLC; deionised water and preparative grade CH₃CN (Scharlau, Barcelona, Spain) for preparative and semipreparative RP-HPLC; Milli-Q[®] water for electron paramagnetic resonance (EPR) studies. Deuterated solvents for nuclear magnetic resonance (NMR) were from SDS (Peypin, France). Cysteine hydrochloride (Aldrich, Steinheim, Germany) was of synthesis grade. Acetic acid, 37% HCl (E. Merck) and NaCl (Carlo Erba, Milano, Italy) were of analytical grade. Triethylamine (E. Merck) was of buffer grade. Trifluoroacetic acid (TFA, Fluorochem, Derbyshire, UK) biotech grade was distilled in-house. 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH) (95%) and gallic acid (97%) were from Aldrich (Gillingham-Dorset, UK), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) (97%) from Aldrich (Milwaukee, USA) and (–)-epicatechin from Sigma Chemical CO, (Saint Louis, MO, USA).

Fetal bovine serum (FBS) was purchased from Gibco (Invitrogen, Carlsbad, CA, USA). Dulbecco's phosphate buffer saline (PBS) and trypsin-EDTA solution C (0.05% trypsin & EDTA 1:5000 in PBS) were from Biological Industries (Kibbutz Beit Haemek, Israel). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and DMSO (dimethyl sulfoxide) were from Sigma Chemical CO (Saint Louis, MO, USA).

Chromatographic equipment and columns

Semi-preparative cation-exchange chromatography was performed on a FPLC[®] system (Amersham-Pharmacia Biotech, Uppsala, Sweden) fitted with a Omnifit (Cambridge, UK) column (8 × 1 cm i.d., ca. 6 mL bed

volume) packed in-house with MacroPrep[™] High S 50 µm (BioRad Laboratories, Hercules, CA, USA). For preparative purposes, the same stationary phase was packed into a flash chromatography type glass column (21 × 2.5 cm i.d., ca. 105 mL bed volume). Preparative RP-HPLC chromatography was performed on a Waters (Milford, USA) Prep LC 4000 pumping system with a Waters PrepPack[®] 1000 module fitted with a PrepPack[®] Waters cartridge (30 × 4.7 cm i.d.) filled with VYDAC[™] (The Separations Group, Hesperia, USA) C₁₈, 300 Å pore size, 15–20 µm particle size stationary phase. A flow splitter was placed after the column and detection was done by an analytical Merck-Hitachi (Darmstadt, Germany) L-4000 UV detector. Semipreparative runs were performed on the same pumping system fitted with a Perkin-Elmer C₁₈ column (25 × 2 cm i.d.). Analytical RP-HPLC was performed on either a Kontron Analytical system (Kontron Instruments, Basel, Switzerland) fitted with a VYDAC[™] C₁₈, 300 Å pore size, 5 µm particle size, 250 × 4.6 mm i.d. column, or a Smart[®] System (Amersham-Pharmacia Biotech) equipped with a µ Peak Monitor (Amersham-Pharmacia Biotech) and fitted with a µRPC C2/C18 SC 2.1/10 (100 × 2.1 mm i.d.) column (Amersham-Pharmacia Biotech).

Mass spectrometry, NMR and EPR measurements

Electrospray-mass spectrometry (ES-MS) analyses were recorded on a VG-Quattro[®] system from Fisons Instruments (Altricham, UK). The carrier solution was water/CH₃CN (1:1) containing 1% (v/v) formic acid. ¹H and ¹³C NMR spectra were observed with a Unity-300 spectrometer from Varian (Palo Alto, CA, USA) for acetone-*d*₆/D₂O solutions using standard acquisition conditions at a regulated temperature of 25 °C. Controlled deuterium exchange experiments were performed in D₂O/acetone-*d*₆ (100% deuteration degree, 3:1 v/v) buffered at pD=6 (measured with standard pH electrode) with deuterated ammonium chloride (150 mM final concentration in the solvent mixture). All the glass material used in the experiment was thoroughly washed with the deuterated solvent mixture to remove all traces of labile hydrogen. Compounds (ca. 5 mg) were dissolved in 0.75 mL of the buffered solvent mixture (concentration between 15 and 20 mM) just before starting the NMR acquisition. Spectra were acquired at 30 °C using short pulse widths (15°) and 32 scans (for each kinetic data point) with 4 s interpulse time in order to assure a similar integral to concentration factor for all spectral signals. In the case of deuterium substituted samples, the carbon spectra were acquired using an interpulse delay of 5 s and a pulse of 25° until a minimum signal:noise ratio of 40:1 for the quaternary aromatic ring carbons signals was achieved.

EPR measurements were performed on a Varian (Palo Alto, CA, USA) E-109 spectrometer. The freshly prepared basic solutions (1 M NaOH) of the samples were degassed and transferred to a quartz capillary tube, fitted into the cavity of the spectrometer working in the X band at room temperature, and the changes in the spectra were recorded with time. *g* Values of the radicals were

determined with DPPH ($g=2.0037$) as standard. Measurement conditions: microwave power, 5 mW; modulation amplitude, 0.1 G; modulation 100 kHz; scan width, 20 G. High values of gain were necessary to detect very low concentrations of magnetic species.

Theoretical calculations

The program Gaussian 98⁴⁷ was used to perform all calculations described in this paper. The bond dissociation enthalpies were computed following the theoretical approach pointed out by Wright et al.²⁸ In a first step, we carried out geometry optimisations and vibration frequencies calculations for X–H and X radicals by using the semiempirical PM3 method. In a second step, the electronic energies were obtained by performing single point calculations at the optimised geometries by means of density functional theory (DFT) using the B3LYP functional.²⁷ The bond dissociation enthalpies at 298 K were then computed as the difference between the B3LYP energy of the X+H radicals minus the B3LYP energy of the XH molecule, plus the corresponding thermochemical corrections obtained at the PM3 level of theory. The B3LYP calculations were performed using the 6–31G(d,p) basis set,⁴⁸ with the p-exponent on hydrogen changed to 1.0 as optimised by Wright et al.²⁸ Moreover, in computing the thermochemical corrections, we have also scaled the zero point energy (ZPE) by 0.947 as suggested by Wright et al.²⁸ It is worth noting that our results show that the PM3 derived BDEs are systematically underestimated in the 8–30 kcal/mol range, with respect to the more accurate B3LYP values. We obtained computed BDEs of 69.2, 65.9, 60.3, 65.0, 67.5, 71.4 and 71.7 kcal/mol for positions 2, 3, 4, 3', 4', 5 and 7, respectively, at PM3 level of theory, which are lower than those displayed in Fig. 4, calculated at B3LYP level of theory. The reason for this discrepancy is that the high level of delocalisation of the radicals is not accurately described at the semiempirical level of theory.

Treatment of procyanidins with acid in the presence of cysteine

The solvent (water saturated with EtOAc) was eliminated from an aliquot (400 mL, 4 g GAE, 6 g estimated polyphenols by weight, coming from 3.2 kg of grape byproduct) of the source of procyanidins.²⁰ The pellet was then dissolved in MeOH (400 mL) and dried. This operation was repeated three times in order to eliminate moisture. The resulting syrupy residue was dissolved in MeOH (400 mL) and a solution of cysteine hydrochloride (20 g) and 37% HCl (10 mL) in MeOH (400 mL) was added. The mixture was kept at 65 °C for 20 min under agitation. The reaction was then quenched with cold water (3.2 L).

Cation exchange separation of the cysteinyl derivatives from the depolymerised mixture and fractionation by preparative RP-HPLC

Semipreparative runs on a 6 mL bed volume column loaded with MacroPrepTM High S resin were used to

set-up the separation conditions at milligram scale. The preparative separations were performed on a 105 mL bed volume column loaded with the same stationary phase. The eluents were [A]: 20 mM sodium phosphate, pH 2.25 buffer/EtOH (7:3) and [B]: 20 mM sodium phosphate, pH 2.25 buffer/EtOH (13:7), 100 mM NaCl. The column was equilibrated with eluent [A], loaded with the quenched depolymerised mixture (500 mL) and washed with [A] (500 mL, 4.75 bed volumes). The retained flavan-3-ol-derivatives were released with 500 mL (4.75 bed volumes) of eluent [B]. The column was then washed with 7.14 bed volumes (750 mL) of 20 mM sodium phosphate buffer, pH 2.25/EtOH (3:2), 1 M NaCl. The operation was repeated (7 times total) until the whole mixture was consumed. The separation process was monitored by analytical RP-HPLC on a VYDACTM C₁₈ column eluted with a binary system, [C] 0.10% (v/v) aqueous TFA, [D] 0.09% (v/v) TFA in water/CH₃CN (2:3) under isocratic conditions 14% [D] (**5** and **6**), 22% [D] (compound **7**), at a flow rate of 1.5 mL/min and detection at 215 nm, 0.016 absorbance units full scale (aufs). The eluates containing compounds **5–7** were pooled (3.5 L), the solvent volume was reduced under vacuum down to 1.8 L, and water was added up to a final volume of 2.6 L. The RP-HPLC profile of the pooled eluates as well as the initial and final washing steps were recorded on the same analytical system under gradient conditions 8–23% [D] over 45 min at a flow rate of 1.5 mL/min with detection at 215 nm.

The mixture containing compounds **5–7** was fractionated on a preparative RP-HPLC cartridge filled with VYDACTM C₁₈ stationary phase by a CH₃CN gradient in 0.1% (v/v) aqueous TFA (2–14% CH₃CN over 45 min). The solution (2.6 L) was processed in three portions of ca. 900 mL each. Fractions enriched in each of the three compounds were obtained: fraction **I**, 2–5% CH₃CN, compound **6**; fraction **II**, 5–8% CH₃CN, compound **5**; fraction **III**, 10–13% CH₃CN, compound **7**.

Purification of the S-cysteinyl derivatives

The 4β-(S-cysteinyl)flavan-3-ols were purified from fractions **I–III** by preparative RP-HPLC and identified by mass spectrometry and nuclear magnetic resonance.

4β-(S-Cysteinyl)epicatechin 5. Fraction **II** (6.7 L) from reversed-phase fractionation was concentrated (5 L final volume) under vacuum to eliminate most of the CH₃CN, loaded onto the preparative cartridge and eluted using a CH₃CN gradient (0–12% over 60 min) in triethylamine phosphate pH 2.25 buffer, at a flow rate of 100 mL/min, with detection at 230 nm. Compound **5** eluted at 4–7% CH₃CN. Analysis of the fractions was accomplished under isocratic conditions in 0.10% (v/v) aqueous TFA/CH₃CN using the VYDACTM C₁₈ column, solvent system, flow rate and detection described above with isocratic elution at 14% [D]. The pure fractions were pooled, diluted with water (1:1) and re-chromatographed on the same cartridge by a CH₃CN gradient (2 to 11% over 30 min) in 0.10% (v/v) aqueous TFA. After combining the eluates and lyophilization, the

resulting solid (0.9 g) was dissolved in triethylamine phosphate buffer pH 5.54 and loaded again onto the preparative cartridge previously equilibrated in the same buffer. Compound **5** was eluted by a CH₃CN gradient (0–12% over 90 min). The pure fractions were pooled and desalted by a fast CH₃CN gradient in 0.1% (v/v) aqueous TFA on the same cartridge. 4β-(*S*-cysteinyl)epicatechin **5** (550 mg) was obtained as the trifluoroacetate by lyophilisation. ES–MS positive ions, m/z 410.0 ($M+1$)⁺, calculated for C₁₈H₂₀N₁O₈S₁ ($M+H$)⁺ 410.1. ¹H NMR (acetone-*d*₆ + 3 drops D₂O, 300 MHz): δ 3.98 (1H, d $J=2.0$ Hz, H-4 3, 4-*trans* configuration); 4.08 (1H, dd $J=2.0, 0.9$ Hz, H-3); 4.45 (1H, m, S–CH₂–CH<); 5.09 (1H, s, H-2 2, 3-*cis* configuration); 5.89 (1H, d $J=2.1$ Hz, H-8); 6.10 (1H, d, $J=2.1$ Hz, H-6); 6.77–6.86 (2H, m, H-5', H-6'); 7.07 (1H, d $J=2.1$ Hz, H-2'). Purity (>99.5%) was ascertained by RP-HPLC on a μRPC C2/C18, 3 μm column; elution, [C] 0.10% (v/v) aqueous TFA, [D] 0.09% (v/v) TFA in water/CH₃CN (2:3), gradient 8 to 23% [D] over 45 min at a flow rate of 200 μL/min with simultaneous detection at 214, 280 and 320 nm.

4β-(*S*-Cysteinyl)catechin **6**

Fraction **I** from reversed-phase fractionation was concentrated as stated for **5**, loaded onto the preparative cartridge and eluted using a CH₃CN gradient (0–12% over 90 min) in triethylamine phosphate pH 2.25 buffer, at a flow rate of 100 mL/min, with detection at 230 nm. Analysis of the fractions was accomplished under isocratic conditions in 0.10% (v/v) aqueous TFA/CH₃CN using the VYDACTM C₁₈ column, solvent system, flow rate and detection described above with isocratic elution at 10% [D]. The best fractions were pooled, diluted, re-loaded onto the cartridge and eluted with a CH₃CN gradient (0–12% over 90 min) in triethylamine phosphate pH 5.30 buffer. The purest fractions were pooled, desalted with a steep CH₃CN gradient in 0.1% (v/v) aqueous TFA and lyophilised. Then the preparation was re-chromatographed on a semipreparative Perkin–Elmer C18 cartridge eluted with 6 and 9% CH₃CN in 0.10% (v/v) aqueous TFA under isocratic conditions. After pooling the best fractions and lyophilization, 4β-(*S*-cysteinyl)catechin **6** (74 mg) was obtained as the trifluoroacetate. ES–MS, positive ions, m/z 409.9 ($M+1$)⁺, calculated for C₁₈H₂₀N₁O₈S₁ ($M+H$)⁺ 410.1. ¹H NMR (acetone-*d*₆ + 3 drops D₂O, 300 MHz): δ 4.15 (1H, 2d, $J=9.7, 4.5$, H-3); 4.28 (1H, d $J=4.5$, H-4 3, 4-*cis* configuration); 4.4 (1H, m, S–CH₂–CH<); 4.80 (1H, d $J=9.7$ Hz, H-2 2, 3-*trans* configuration); 5.76 (1H, d $J=2.1$ Hz, H-8); 6.12 (1H, d, $J=2.1$ Hz, H-6); 6.78 (2H, m, H-5', H-6'); 6.93 (1H, d $J=1.2$ Hz, H-2'). Purity (99.9%) was ascertained by RP-HPLC on the system described for compound **5**.

4β-(*S*-Cysteinyl)epicatechin 3-*O*-gallate **7.** Fraction **III** from reversed-phase fractionation was concentrated as stated for **5**, loaded onto the preparative cartridge and eluted using a CH₃CN gradient (2–14% over 60 min) in triethylamine phosphate pH 2.25 buffer, at a flow rate of 100 mL/min, with detection at 230 nm. Fractions were analysed under isocratic conditions in 0.10% (v/v)

aqueous TFA/CH₃CN using the column, solvent system, flow rate and detection described above with elution at 19% [D]. The best fractions were pooled, diluted, re-loaded onto the cartridge and eluted with a CH₃CN gradient (2–14% over 60 min) in triethylamine phosphate pH 5.46 buffer. The purest fractions were pooled and re-chromatographed with a CH₃CN gradient (9–21% over 30 min) in 0.1% (v/v) aqueous TFA. After lyophilisation, 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate **7** (75 mg) was obtained as the trifluoroacetate. ES–MS, positive ions, m/z 561.9 ($M+1$)⁺ calculated for C₂₅H₂₄N₁O₁₂S₁ ($M+H$)⁺ 562.1. ¹H NMR (acetone-*d*₆ + 3 drops D₂O, 300 MHz): δ 4.15 (1H, d $J=2.1$ Hz, H-4 3, 4-*trans* configuration); 4.37 (1H, m, S–CH₂–CH<); 5.28 (1H, 2m, H-3); 5.39 (1H, bs, H-2 2, 3-*cis* configuration); 6.01 (1H, d $J=2.1$ Hz, H-8); 6.13 (1H, d, $J=2.1$ Hz, H-6); 6.78 (1H, d $J=8.1$ Hz, H-5'); 6.90 (1H, dd $J=2.1, 8.1$ Hz, H-6'); 6.97 (2H, s, galloyl H); 7.11 (1H, d $J=2.1$ Hz, H-2'). Purity (>99.5%) was ascertained by RP-HPLC on the system described for compound **5**.

Antiradical activity

The free radical scavenging activity was evaluated by the DPPH method.^{22,23} The samples (0.1 mL) were added to aliquots (3.9 mL) of a solution made up with 4.8 mg DPPH in 200 mL of MeOH and the mixture incubated for 1 h at room temperature. The initial concentration of DPPH, approximately 60 μM, was calculated for every experiment from a calibration curve made by measuring the absorbance at 517 nm of standard samples of DPPH at different concentrations. The equation of the curve was $Abs_{517nm} = 11,345 \times C_{DPPH}$ as determined by linear regression. The results were plotted as the degree of absorbance disappearance at 517 nm $[(1-A/A_0) \times 100]$ against μmoles of sample divided by the initial μmoles of DPPH. Each point was acquired in triplicate. A dose-response curve was obtained for every product. The results were expressed as the efficient dose ED₅₀ given as μmoles of product able to consume half the amount of free radical divided by μmoles of initial DPPH.

Cell culture

HT29 cells (colorectal adenocarcinoma) were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine and antibiotics: 100 U/mL penicillin and 100 mg/mL streptomycin (Biological Industries). Cells were grown in an isolated 37°C–5% CO₂ tissue incubator compartment and the medium was changed every 3 days. HT29 cell cultures used in this study were free of mycoplasma infection as shown by the Gen-probe rapid mycoplasma detection system prior to the treatments with the samples.

Proliferation assay

Cell growth was determined using the MTT assay.⁴⁹ Cells were seeded into 96-well plates at 2.5×10^3 cells/well density and incubated for 24 h prior to addition of

the samples, which were prepared by serial dilutions from a concentrated solution made up in Dulbecco's phosphate buffered saline (PBS). Control cultures were treated with equal volume of PBS as the treated cultures. After 3 days of culture, the supernatant was aspirated and 100 μ L of filtered MTT (0.2 mg/mL in cell culture medium) was added to each well. The plates were incubated at 37 °C–5% CO₂ during 4 h. The supernatant was removed, and the blue MTT formazan precipitated was dissolved in DMSO (100 μ L) and optical density (OD) measured at 550 nm on a multi-well reader (Merck ELISA System MIOS®).

For each compound, a minimum of four experiments measuring the growth inhibition was conducted and the mean $\pm$ SD of OD data from the replicated wells was calculated for each concentration tested. The inhibitory effect of the products at each concentration was expressed as a percentage [(mean OD treated cells after 72 h of incubation with the product/mean OD of control cells after 72 h of incubation with extra-medium instead of product) $\times$ 100]. The IC₅₀ or sample concentration causing a 50% reduction in the mean OD value relative to the control at 72 h of incubation, was estimated using GraFit 3.00 (Data Analysis and Graphics Program, Erithacus Software Ltd. Microsoft Corp., Surrey, UK) curve option: IC₅₀ curve — start at 0.

Acknowledgements

Financial support (research grants PPQ2000–0688–C05–03 and–04, and doctoral fellowship to C.L.) from the Spanish Ministry of Science and Technology is acknowledged. We are thankful to Dr. Irene Fernández from the *Servei d'Espectrometria de Masses* of the University of Barcelona for the mass spectrometry analyses, Ms. Avencia Díez from the *Servei d'Espectrometria de Ressonància Paramagnètica Electrònica* at IIQAB-CSIC for the EPR analyses and Ms. Montserrat Sindreu from the *Servei d'Espectrometria de Ressonància Magnètica Nuclear* at IIQAB-CSIC for the NMR analyses. The calculations described in this work were performed at the *Centre de Supercomputació de Catalunya* (CESCA).

References and Notes

- Diplock, A. T.; Charleux, J. L.; Crozier-Willi, G.; Kok, F. J.; Rice-Evans, C.; Roberfroid, M.; Stahl, W.; Viña-Ribes, J. *Br. J. Nutr.* **1998**, *80*, S77 (Suppl 1).
- Bravo, L. *Nutr. Rev.* **1998**, *56*, 317.
- Yang, C. S.; Lee, M.-J.; Chen, L.; Yang, G. *Environ. Health Perspect.* **1997**, *105* (Suppl. 4), 971.
- Yang, G. Y.; Liao, J.; Kim, K.; Yurkow, E. J.; Yang, C. S. *Carcinogenesis* **1998**, *19*, 611.
- Soleas, G. J.; Diamandis, E. P.; Goldberg, D. M. *J. Clin. Lab. Anal.* **1997**, *11*, 287.
- Ruf, J. C. *Drug Exp. Clin. Res.* **1999**, *25*, 125.
- Packer, L.; Rimbach, G.; Virgili, F. *Free Rad. Biol. Med.* **1999**, *27*, 704.
- Ahmad, N.; Feyes, D. K.; Nieminen, A. L.; Agarwal, R.; Mukhtar, H. *J. Nat. Cancer Inst.* **1997**, *89*, 1881.
- Tan, X. H.; Hu, D. R.; Li, S. R.; Han, Y.; Zhang, Y. L.; Zhou, D. Y. *Cancer Lett.* **2000**, *158*, 1.
- Harbowy, M. E.; Balentine, D. A. *Crit. Rev. Plant Sci.* **1997**, *16*, 415.
- Prieur, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. *Phytochemistry* **1994**, *36*, 781.
- Souquet, J.-M.; Cheynier, V.; Brossaud, F.; Moutounet, M. *Phytochemistry* **1996**, *43*, 509.
- Souquet, J. M.; Labarbe, B.; LeGuerneve, C.; Cheynier, V.; Moutounet, M. *J. Agric. Food Chem.* **2000**, *48*, 1076.
- Cheynier, V.; Souquet, J.-M.; Le Roux, E.; Guyot, S.; Rigaud, J. *Method. Enzymol.* **1999**, *299*, 178.
- Thompson, R. S.; Jacques, D.; Haslam, E.; Tanner, R. J. N. *J. Chem. Soc., Perkin Trans. 1* **1972**, 1387.
- Rigaud, J.; Pérez-Ilzarbe, J.; Ricardo da Silva, J. M.; Cheynier, V. *J. Chromatogr.* **1991**, *540*, 401.
- Sanoner, P.; Guyot, S.; Marnet, N.; Molle, D.; Drilleau, J. F. *J. Agric. Food Chem.* **1999**, *47*, 4847.
- Tanaka, T.; Kusano, R.; Kouno, I. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1801.
- Torres, J. L.; Bobet, R. *J. Agric. Food Chem.* **2001**, *49*, 4627.
- Polymeric procyanidins came from the residue generated after extraction of antioxidant polyphenols from grape pomace. Briefly, the total extract in 70% EtOH was dried, suspended in water and partitioned with ethyl acetate. The residual aqueous layer used in this work contained procyanidins with a mean degree of polymerisation of 8.5 and a content of gallate esters (galloylation) of 11%, calculated by thiolysis with cysteamine and HPLC analysis (Torres, J. L.; Lozano, C. *Chromatographia* **2001**, *54*, 523).
- Kolodziej, H. *Phytochemistry* **1990**, *29*, 1671.
- Blois, M. S. *Nature* **1958**, *181*, 1199.
- Brand-Williams, W.; Cuvelier, M. E.; Berset, C. *Lebensm.-Wis. u.-Technol.* **1995**, *28*, 25.
- Free radical scavenging efficiency of **4** was reported in ref 19. ED₅₀ was 0.11 (ARP 9.1). The corresponding stoichiometry value is 0.22 and the moles DPPH reduced by mole antioxidant is 4.5.
- Shen, C. C.; Chang, Y.-S.; Ho, L.-K. M. *Phytochemistry* **1993**, *34*, 843.
- Hemingway, R. W.; Tobiasson, F. L.; McGraw, G. W.; Steynberg, J. P. *Mag. Res. Chem.* **1996**, *34*, 424.
- Becke, A. D. *J. Chem. Phys.* **1993**, *98*, 5648.
- Wright, J. S.; Carpenter, D. J.; McKay, D. J.; Ingold, K. U. *J. Am. Chem. Soc.* **1997**, *119*, 4245.
- Guo, Q. N.; Zhao, B. L.; Li, M. F.; Shen, S. R.; Xin, W. J. *Biochim. Biophys. Acta-Lipid Lipid Met.* **1996**, *1304*, 210.
- Bors, W.; Michel, C.; Stettmaier, K. *Archiv. Biochem. Biophys.* **2000**, *374*, 347.
- Jensen, O. N.; Pedersen, J. A. *Tetrahedron* **1983**, *39*, 1609.
- Torres, J. L.; Piera, E.; Infante, M. R.; Clapes, P. *Prep. Biochem. Biotechnol.* **2001**, *31*, 259.
- Dangles, O.; Fargeix, G.; Dufour, C. *J. Chem. Soc., Perkin Trans. 2* **1999**, 1387.
- Dangles, O.; Fargeix, G.; Dufour, C. *J. Chem. Soc., Perkin Trans. 2* **2000**, 1653.
- Guyot, S.; Vercauteren, J.; Cheynier, V. *Phytochemistry* **1996**, *42*, 1279.
- Hotta, H.; Sakamoto, H.; Nagano, S.; Osakai, T.; Tsujino, Y. *Biochim. Biophys. Acta-Gen. Subjects* **2001**, *1526*, 159.
- Kolar, G. F. *J. Label. Compd.* **1971**, *7*, 409.
- Kondo, K.; Kurihara, M.; Miyata, N.; Suzuki, T.; Toyoda, M. *Free Rad. Biol. Med.* **1999**, *27*, 855.
- Kondo, K.; Kurihara, M.; Miyata, N.; Suzuki, T.; Toyoda, M. *Archiv. Biochem. Biophys.* **1999**, *362*, 79.
- Stone, T. J.; Waters, W. A. *J. Chem. Soc.* **1965**, 1488.
- Valcic, S.; Timmermann, B. N.; Alberts, D. S.; Wächter, G. A.; Krutzsch, M.; Wymer, J.; Guillén, J. M. *Anti-Cancer Drugs* **1996**, *7*, 461.

42. Chung, L. Y.; Cheung, T. C.; Kong, S. K.; Fung, K. P.; Choy, Y. M.; Chan, Z. Y.; Kwok, T. T. *Life Sci.* **2001**, *68*, 1207.
43. Liang, Y. C.; Lin-shiau, S. Y.; Chen, C. F.; Lin, J. K. *J. Cell. Biochem.* **1997**, *67*, 55.
44. Ahmad, N.; Gali, H.; Javed, S.; Agarwal, R. *Biochem. Biophys. Res. Commun.* **1998**, *247*, 294.
45. Liang, Y. C.; Chen, Y. C.; Lin, Y. L.; Lin-Shiau, S. Y.; Ho, C. T.; Lin, J. K. *Carcinogenesis* **1999**, *20*, 733.
46. Liberto, M.; Cobrinik, D. *Cancer Lett.* **2000**, *154*, 151.
47. Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Zakrzewski, V. G.; Montgomery, J. A., Jr; Stratmann, R. E.; Burant, J. C.; Dapprich, S.; Millam, J. M.; Daniels, A. D.; Kudin, K. N.; Strain, M. C.; Farkas, O.; Tomasi, J.; Barone, V.; Cossi, M.; Cammi, R.; Mennucci, B.; Pomelli, C.; Adamo, S.; Clifford, S.; Ochterski, J.; Petersson, G. A.; Ayala, P. Y.; Cui, Q.; Morokuma, K.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Cioslowski, J.; Ortiz, J. V.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; González, C.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Andrés, J. L.; González, C.; Head-Gordon, M.; Replogle, E. S.; Pople, J. A. *Gaussian 98*, Revision A.6; Gaussian Inc: Pittsburg, PA, 1998.
48. Hariharan, P. C.; Pople, J. A. *Theoret. Chim. Acta* **1973**, *28*, 213.
49. Mosmann, T. *J. Immunol. Meth.* **1983**, *55*.