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In vitro chemopreventive potential of fucophlorethols from the brown alga *Fucus vesiculosus* L. by anti-oxidant activity and inhibition of selected cytochrome P450 enzymes

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ABSTRACT

Within a project focusing on the chemopreventive potential of algal phenols, two phloroglucinol derivatives, belonging to the class of fucophlorethols, and the known fucotriphlorethol A were obtained from the ethanolic extract of the brown alga *Fucus vesiculosus* L. The compounds trifucodiphlorethol A and trifucotriphlorethol A are composed of six and seven units of phloroglucinol, respectively.

The compounds were examined for their cancer chemopreventive potential, in comparison with the monomer phloroglucinol. Trifucodiphlorethol A, trifucotriphlorethol A as well as fucotriphlorethol A were identified as strong radical scavengers, with IC_{50} values for scavenging of 1,1-diphenyl-2 picrylhydrazyl radicals (DPPH) in the range of 10.0–14.4 μg/ml. All three compounds potently scavenged peroxyl radicals in the oxygen radical absorbance capacity (ORAC) assay. In addition, the compounds were shown to inhibit cytochrome P450 1A activity with IC_{50} values in the range of 17.9–33 μg/ml, and aromatase (Cyp19) activity with IC_{50} values in the range of 1.2–5.6 μg/ml.

1. Introduction

Cancer chemoprevention describes the use of nutrients and/or pharmaceutics to block, inhibit, or reverse tumor development at various steps during the initiation, promotion, or progression phase of carcinogenesis (Surh, 2003). To identify novel chemopreventive agents, we have established a series of *in vitro* test systems which cover a broad spectrum of mechanisms relevant for the prevention of cancer in humans, including radical scavenging effects and anti-oxidant mechanisms, the modulation of phase 1 and 2 drug metabolism, anti-hormonal properties, anti-inflammatory activities, and anti-proliferative mechanisms (Gerhäuser et al., 2003). Several of the reported biological activities of algal phenols, such as anti-oxidant potential (Shin et al., 2006; Chkhikvishvili and Ramazanov, 2000; Jimenez-Escrig et al., 2001; Kang et al., 2003, 2005; Kim et al., 2004; Cerantola et al., 2006), anti-inflammatory activity (Shin et al., 2006; Shibata et al., 2003), and activation of Nrf2-mediated enzyme induction (Kang et al., 2007) have been associated with cancer chemopreventive potential. To explore the therapeutic potential of phlorotannins in detail pure compounds were isolated during the current study from the extract of *Fucus vesiculosus* L.

The brown alga *F. vesiculosus* is a member of the family Fucaceae, belonging to the order Fucales. It grows in the rocky mid-littoral and intertidal temperate coasts of Europe and North America (Lüning, 1985). Brown algae of the order Fucales contain three types of phlorotannins, all merely consisting of phloroglucinol subunits. Depending on the type of connection between the subunits these phlorotannins are termed fucols, where phloroglucinol units are connected by aryl-aryl bonds, fucophlorethols with ether and aryl–aryl bonds (Fig. 2) and phlorethols with only ether bonds present. From *F. vesiculosus* 15 fucophlorethols and four fucols with three to eight and two to four units of phloroglucinol, respectively, were described (Ragan and Glombitza, 1986). During most of these studies extracts were derivatized and the compounds subsequently isolated in their acetylated form (Glombitza et al., 1975, 1977; Preuss, 1983). The isolation of the free phenols is due to their instability a challenging task and was reported from *F. vesiculosus*

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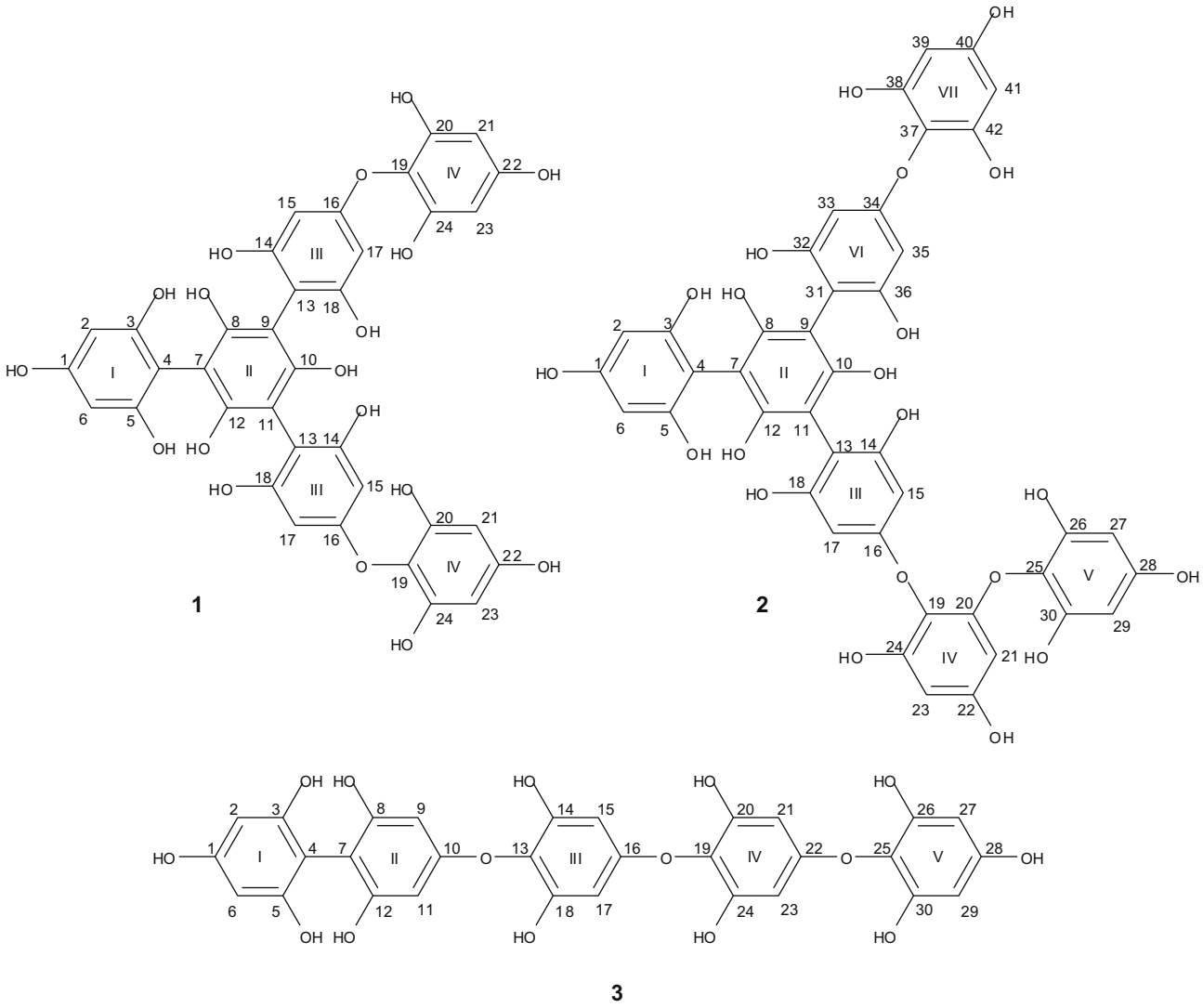

Fig. 1. Structures of compounds **1**–**3**.

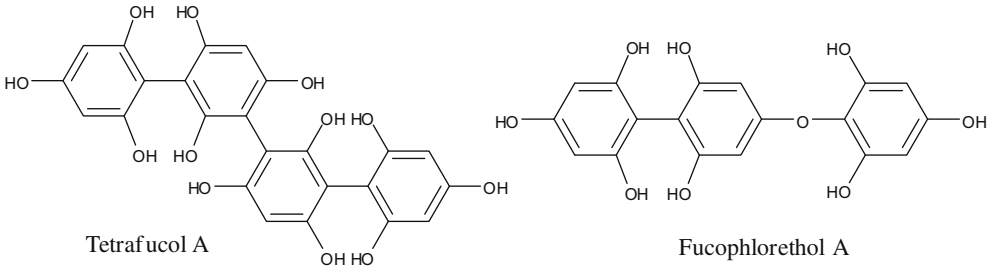

Fig. 2. Phlorotannins of *F. vesiculosus*.

only once (Craigie et al., 1977). Furthermore, cleavage products of high molecular weight phenols in *F. vesiculosus* were isolated and analyzed (Glombitza and Lentz, 1981).

Phlorotannins have been shown to be of ecological importance. They protect the producing organism against UV irradiation (Pavia et al., 1997; Swanson and Druehl, 2002), deter herbivores (Ragan and Glombitza, 1986; Pavia et al., 1997; Boettcher and Targett, 1993; Schoenwaelder, 2002) and act against pathogens (Ragan and Glombitza, 1986). Several pharmacological activities have been reported for this class of compounds including enzyme inhibition, anti-oxidative activities and antibacterial effects (Shin et al., 2006; Chkhikvishvili and Ramazanov, 2000; Jimenez-Escrig et al., 2001; Kang et al., 2003, 2005; Kim et al., 2004; Linares et al., 2004; Shibata et al., 2003; Nagayama et al., 2002). Sandsdalen et al. (2003) determined the antibacterial effects of compounds isolated from an extract of *F. vesiculosus*. One of these antibacterial compounds, i.e. fucodiphlorethol A is composed of four units of phloroglucinol and belongs to the class of fucophlorethols.

However, in general the ecological as well as pharmacological activities of *F. vesiculosus* were estimated employing crude extracts or fractions (Geiselman and McConnell, 1981; Cerantola et al., 2006; Collen and Davison, 1999).

RP-HPLC separations of the bioactive ethanolic extract of *F. vesiculosus* yielded three phlorotannins (Fig. 1), whose structures were elucidated mainly based on NMR spectra, and identified as trifucodiphlorethol A (**1**), trifucotriphlorethol A (**2**) as well as the known fucotriphlorethol A (**3**) (Glombitza et al., 1977; Rauwald, 1976). The latter compound was to date only described in its acetylated form. The purified compounds were tested, in comparison to the monomer phloroglucinol, in the above mentioned test system to investigate their chemopreventive potential.

2. Results and discussion

After the extraction of frozen algal material of *F. vesiculosus* with ethanol, the lipophilic compounds were removed from the aqueous phase by liquid–liquid partitioning. Preparative HPLC of the hydrophilic extract yielded 10 fractions, of which fraction 4 was chosen for further detailed chemical investigation due to its pronounced radical scavenging activity in the DPPH assay, with a halfmaximal scavenging concentration of 11.0 ± 1.1 μg/ml (data not shown). From the bioactive fraction 4, two new (**1**, **2**) and one known compounds (**3**) were isolated (Fig. 1S-A Supplementary data) and evaluated for their chemopreventive effects.

HPLC-DAD separation of fraction 4 resulted in six sub-fractions (Fig. 1S-B, Supplementary data), of which three were pure compounds (**1**–**3**). Compounds **1**, **2**, and **3** all had UV adsorption spectra characteristic for phenolic compounds. Further characterization of compounds **1**–**3** was done using ^{1}H and ^{13}C NMR, DEPT 135, COSY, HSQC and HMBC spectra. The chemical shifts observed in ^{1}H and ^{13}C NMR experiments confirmed the polyphenolic structures as phlorotannins (Tables 1, 2 and 3), since resonance signals were in the range of δ_H 5.8–6.3 and δ_C 95–163, respectively. The quaternary carbons have characteristic resonances depending on the type of bonding, i.e. they are either involved in direct carbon–carbon bonding between phloroglucinol units, in ether-linked phloroglucinol units, or are attached to hydroxyl groups, as shown exemplarily for compound **3** (Fig. 3). Hence, compounds **1**–**3** were determined to be fucophlorethols. Calculated spectra for fucophlorethols using ACD software (ACD/Chem Sketch; ACD/Labs release: 9.00; Product Version: 9.08) confirmed this estimation. LC–ESI-MS analyses of compounds **1**–**3** indicated fucophlorethols with five to seven units of phloroglucinol.

Table 1
NMR spectral data for compound **1**.

Ring	Carbon	^{13}C[a,b,c] (δ in ppm)	^{1}H[a] (δ in ppm, int., mult., J in Hz)	HMBC[a,d]
I	1	160.1 (C)		
	2/6	95.8 (CH)	6.03, 2H, *s*	1, 3/5, 4
	3/5	158.4 (C)		
	4	99.7 (C)		
II	7	99.4 (C)		
	8/12	158.3 (C)		
	9/11	99.4 (C)		
	10	158.1 (C)		
III	13	101.2 (C)		
	14/18	158.7 (C)		
	15/17	95.4 (CH)	6.11, 4H, *s*	9/11, 13, 14/18, 16
	16	162.1 (C)		
IV	19	124.8 (C)		
	20/24	152.3 (C)		
	21/23	96.3 (CH)	6.01, 4H, *s*	19, 20/24, 22
	22	156.3 (C)		

[a] MeOD, 300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR.
[b] Assignments are based on extensive 1D and 2D NMR measurements (HMBC, HSQC).
[c] Implied multiplicities by DEPT.
[d] Numbers refer to carbon resonances.

Table 2
NMR spectral data for compound **2**.

Ring	Carbon	^{13}C[a,c,d] (δ in ppm)	^{1}H[b] (δ in ppm, int., mult., J in Hz)	^{1}H–^{1}H-COSY[a,d]	HMBC[a,e]
I	1	160.0 (C)			
	2/6	95.8 (CH)	6.02, 2H, *s*		1, 3/5, 4
	3/5	158.7 (C)			
	4	99.9 (C)			
II	7	99.4 (C)			
	8/12	158.4 (C)			
	9/11	99.4 (C)			
	10	158.0 (C)			
III	13	101.1 (C)			
	14/18	158.3 (C)			
	15/17	95.8 (CH)	6.25, 2H, *s*		
	16	162.2 (C)			
IV	19	125.4 (C)			
	20	154.0 (C)[f]			
	21	94.9 (CH)	5.80, 1H, *d*, 2.5	H-23	20
	22	156.2 (C)[f]			
	23	97.9 (CH)	6.11, 1H, *d*, 2.5	H-21	19, 22, 24
	24	152.5 (C)[f]			
V	25	124.7 (C)			
	26/30	152.2 (C)			
	27/29	96.3 (CH)	5.95, 2H, *s*		25, 26/30, 28
	28	156.4 (C)			
VI	31	101.4 (C)			
	32/36	158.8 (C)			
	33/35	95.4 (CH)	6.11, 2H, *s*		31, 32/36, 34
	34	162.1 (C)			
VII	37	124.8 (C)			
	38/42	152.3 (C)			
	39/41	96.2 (CH)	5.99, 2H, *s*		37, 38/42, 40
	40	156.3 (C)			

[a] MeOD, 75 MHz for ^{13}C NMR.
[b] MeOD, 500 MHz for ^{1}H NMR.
[c] Assignments are based on extensive 1D and 2D NMR measurements (HMBC, HSQC, and COSY).
[d] Implied multiplicities by DEPT.
[e] Numbers refer to carbon resonances.
[f] Resonances interchangeable.

Positive and negative ESI-MS measurements showed the pseudomolecular ion of **1** at *m/z* 747 $[M+H]^+$ and at *m/z* 745 $[M-H]^-$, respectively (Fig. 2S, Supplementary data). This indicated a polyphenolic compound composed of six units of phloroglucinol. After acetylation the FAB (fast atom bombardment) MS spectrum gave pseudomolecular ions at *m/z* 1441 $[M+Na]^+$ and *m/z* 1419 $[M+H]^+$ (Fig. 3S, Supplementary data), indicating 16 acetyl groups. Further, it showed an elimination series of fragments with a mass of *m/z* 42 characteristic for the loss of ketene (C_2H_2O) which is typical for compounds with acetylated tertiary hydroxyl groups (Ragan and Glombitza, 1986). A series of 11 consecutive eliminations was determined originating from the pseudomolecular ion at *m/z* 1419 (→ 957). Furthermore, three fragments resulted from loss of water (*m/z* 1167 → 1149, *m/z* 1041 → 1023, *m/z* 957 → 939). Such fragments are generated in molecules containing biphenyl moieties with *ortho, ortho′*-hydroxyl groups (rings I, II, and III) (Ragan and Glombitza, 1986; Glombitza et al., 1975). Therefore, the elimination of three times water indicated three carbon–carbon linkages between phenyl rings.

The ^{1}H NMR spectrum (Table 1) recorded in deuterated methanol (MeOD) contained two singlet resonances at δ 6.01 and δ 6.11, each showing twice the intensity when compared to the third singlet signal at δ 6.03. Couplings between the aromatic protons were lacking indicating a symmetrical substitution and two magnetically equivalent protons on ring I, III, and IV. The ^{13}C NMR spec-

Table 3
NMR spectral data for fucotriphlorethol A (**3**).

Ring	Carbon	^{13}C[a,b,c] (δ in ppm)	^{1}H[a] (δ in ppm, int., mult., J in Hz)	HMBC[a,d]
I	1	161.3 (C)		
	2/6	96.0 (CH)	6.11, 2H, *s*	1, 3/5, 4
	3/5	158.6 (C)		
	4	102.1 (C)		
II	7	100.5 (C)		
	8/12	158.2 (C)		
	9/11	96.2 (CH)	6.01, 2H, *s*	4[f], 7, 8/12, 10
	10	159.5 (C)		
III[e]	13	126.1 (C)		
	14/18	152.2 (C)		
	15/17	95.5 (CH)	6.11, 2H, *s*	13, 14/18,16
	16	157.8 (C)		
IV[e]	19	126.1 (C)		
	20/24	152.3 (C)		
	21/23	96.0 (CH)	6.09, 2H, *s*	19, 20/24, 22
	22	158.0 (C)		
V	25	124.9 (C)		
	26/30	152.5 (C)		
	27/29	96.2 (CH)	5.99, 2H, *s*	25, 26/30, 28
	28	156.3 (C)		

[a] MeOD, 300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR.
[b] Assignments are based on extensive 1D and 2D NMR measurements (HMBC, HSQC).
[c] Implied multiplicities by DEPT.
[d] Numbers refer to carbon resonances.
[e] Position of rings III and IV in the molecule could be interchanged.
[f] Weak correlation.

trum displayed 16 carbon signals between δ 95.4 and δ 162.1. A DEPT experiment evidenced the signals at δ 95.4, δ 95.8, and δ 96.3 to be methine groups, each resonance accounting for two carbon signals. Furthermore, 13 signals for quaternary carbon atoms were present (Table 1), several of which had to account for more than one carbon. Altogether, results of FAB-MS analysis and NMR data, pointed to a phlorotannin consisting of six units of phloroglucinol and having a symmetrical structure with one fully substituted aromatic moiety (ring II). ^{13}C NMR and HMBC spectra displayed carbon resonances for two ether bonds (C-19, δ 124.8) and for three aryl–aryl bonds (C-9/C-11, δ 99.4; C-7, δ 99.4; C-4, δ 99.7; C-13, δ 101.2). HMBC correlations between H-15/H-17 and C-13 and C-9/11 led to the aryl-aryl linkage between rings II and III. A further aryl-aryl bond was deduced to be between C-4 (δ 99.7) and C-7 (δ 99.4) based on the symmetry within the molecule. The connection of rings III and IV was also established for symmetry reasons and based on the chemical shifts of C-19 (δ 124.8) and C-16 (δ 162.1), which are characteristic for an ether bond (Table 1). After acetylation ^{1}H and ^{13}C NMR spectra of compound **1** were measured in $CDCl_3$ and acetone-d_6 (Table 3S). ^{13}C NMR shifts were compared with shifts calculated with increments developed by Forster (1979) and Wegner-Hambloch (1983) and found to be close to identical. For compound **1** the name trifucodiphlorethol A is proposed.

The structure elucidation of compound **2** was mainly based on mass spectrometric measurements. Positive and negative ESI-MS spectra of **2** (Fig. 4S, Supplementary data) revealed pseudomolecular ions at *m/z* 871 $[M+H]^+$ and 869 $[M-H]^-$, respectively, pointing to a polyphenolic compound consisting of seven units of phloroglucinol. After acetylation a pseudomolecular ion at *m/z* 1649 $[M+Na]^+$ was found in the FAB-MS spectrum (Fig. 5S, Supplementary data), which evidenced clearly the presence of 18 hydroxyl groups in **2**. The difference in mass between compounds **1** and **2** after acetylation was *m/z* 208 indicating that compound **2** is a homolog of **1**, differing only by one phloroglucinol unit linked by an ether bond. A ketene elimination series was observed between *m/z* 1543 and *m/z* 1165, equivalent to the loss of 9 ketenes. An MS fragment at *m/z* 917 was found in the FAB-MS spectrum for a tetraphenyl fragment (rings I–IV), a substructure identical to the equivalent rings (I, II, and 2 × III) in compound **1** (Fig. 6S, Supplementary data). In addition, three MS-fragments were obtained after loss of water indicating three aryl–aryl bonds within the molecule.

An ^{1}H NMR spectrum measured in MeOD showed seven signals including five singlet and two doublet signals. The integration of these resonances demonstrated that the singlet signals (δ 6.25, δ 6.11, δ 6.02, δ 5.99, and 5.95) had twice the intensity of the doublet resonances at δ 5.80 and δ 6.11. The latter coupled with each other

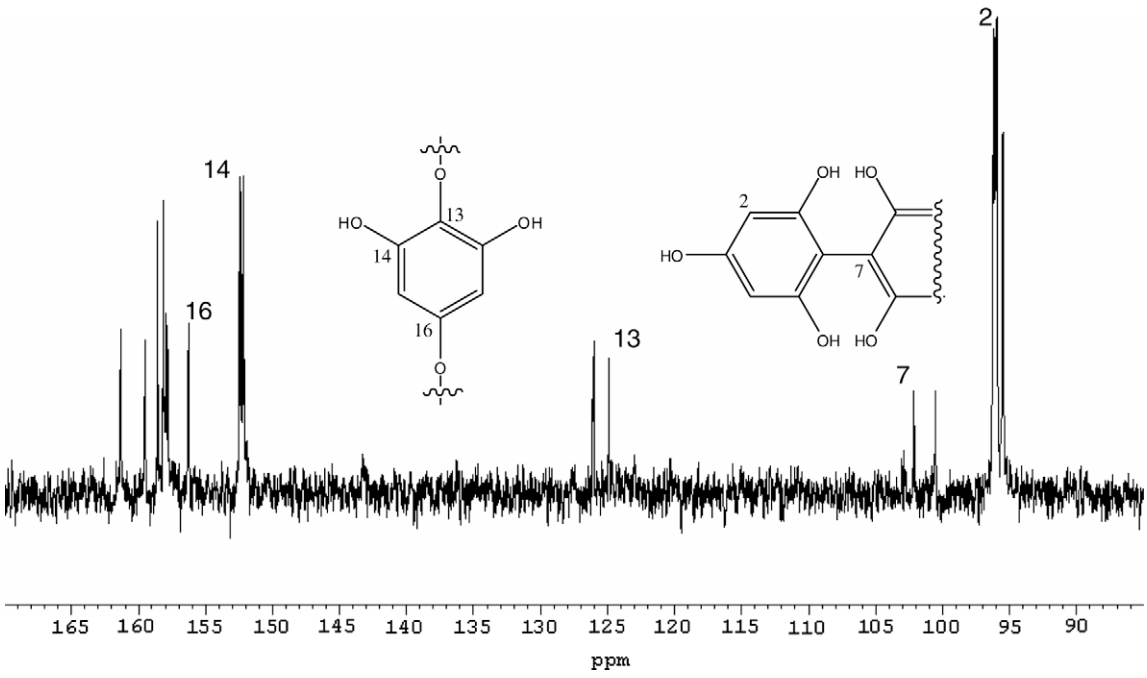

Fig. 3. ^{13}C NMR spectrum of **3** recorded in MeOH-d_4.

(2.5 Hz) suggesting that they are located in meta position to each other. ^{13}C NMR and DEPT spectra of **2** included seven resonance signals for methine groups, five of which had an account for two carbon atoms. Of the 22 resonance signals for quaternary carbon atoms some had to be equivalent to more than one carbon atom as well (Table 2). Comparison of ^{13}C NMR data of compound **2** with those of **1** showed that ^{13}C NMR shifts of rings I, II, VI (in **1** ring III), VII (in **1** ring IV) were close to identical. Together with mass spectral data this secured a partial structure of **2** comprising rings I, II, III, VI, and VII. For ring II in compound **2** it was noticed that ^{13}C NMR shifts of C-9 (δ 99.36) and C-11 (δ 99.40) differed slightly, which is due to the non-symmetrical nature of **2**. At this point of the structure elucidation two phloroglucinol units still had to be attached to the partial structure of **2**. They had to be bonded by ether bonds, since only five phenolic groups were left of the overall 18 hydroxyl groups. Additionally, ^{13}C NMR shifts of C-19 (δ 125.4) and C-25 (δ 124.7) pointed towards ether-linked phenyl moieties. One of these two phloroglucinol units was ring IV including the methylene groups CH-21 and CH-23. Due to the differing ^{1}H and ^{13}C NMR chemical shifts of these methylene groups ring IV had to be substituted asymmetrically, i.e. ring IV had to be attached to the oxygen on C-20 (or C-24). Rings III and V had to be substituted symmetrically, thus clearly positioning the C-16/C-19 and C-20/C-25 phenoxy-bridges. As the carbon atoms of the rings V and VII displayed similar ^{13}C NMR chemical shifts both of these aromatic moieties were confirmed to be the identical terminal structural elements of **2**. Compound **2** was named trifucotriphlorethol A.

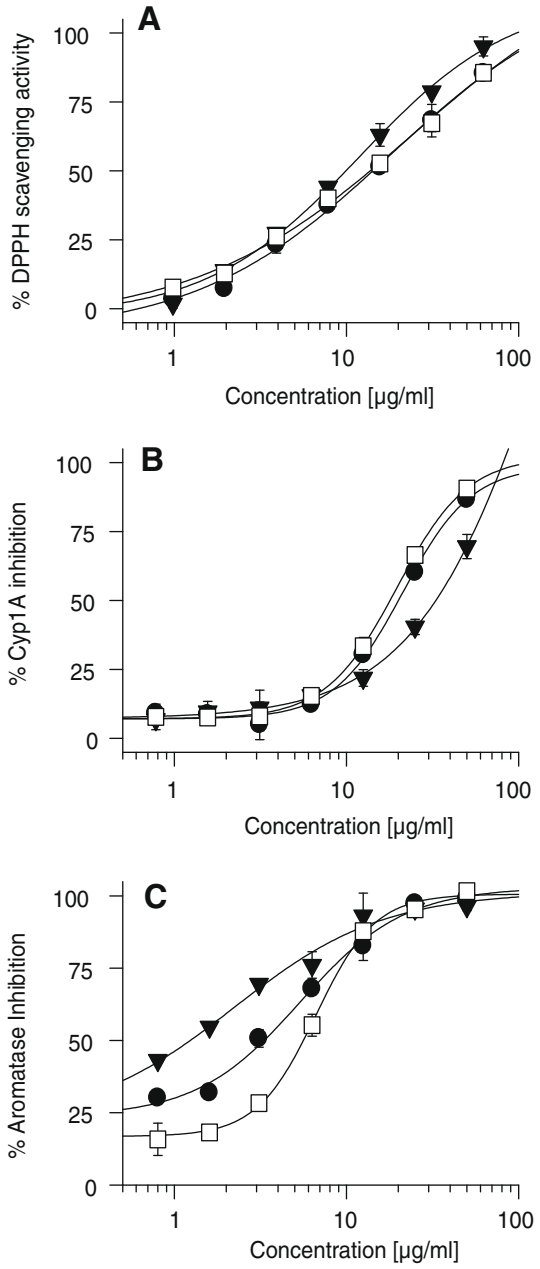

Fig. 4. Dose-dependent chemopreventive activities of trifucodiphlorethol A (compound 1, ●), trifucotriphlorethol A (compound 2, □), and fucotriphlorethol A (compound 3, ▼). (A) Scavenging of DPPH radicals. Vitamin C was used as a positive control with an IC_{50} value of 3.6 ± 0.31 µM (n = 4). (B) Inhibition of Cyp1A enzymatic activity. Cyp1A activity was measured with β-naphthoflavone-induced H4IIE rat hepatoma cell homogenates by dealkylation of 3-cyano-7-ethoxy-coumarin to fluorescent 3-cyano-7-hydroxy-coumarin. Activities of β-naphthoflavone-induced controls were in the range of 63–13 nmol/min/mg of protein. α-Naphthoflavone used as a positive control inhibited Cyp1A activity with an IC_{50} value of 4.2 ± 0.6 nM (n = 4). (C) Inhibition of aromatase activity. Aromatase activity was measured using human recombinant aromatase (human Cyp 19 + P450 reductase Supersomes) and *O*-benzylfluorescein benzyl ester as a substrate. Ketoconazole was used as a positive control with an IC_{50} value of 1.0 ± 0.2 µM (n = 4).

Positive ESI-MS of **3** showed the pseudomolecular ion $[M+H]^+$ at *m/z* 623 (Fig. 5S-A, Supplementary data). Together with the pseudomolecular ion in the negative ESI-MS spectrum at *m/z* 621 $[M-H]^-$ (Fig. 5S-B, Supplementary data) this pointed to a polyphenol having five units of phloroglucinol. After acetylation EI-MS measurement gave the molecular ion at *m/z* 1126, confirming the above results. Furthermore, it indicated the presence of 12 acetyl groups. MS-fragments with a mass of 42 pointed towards the elimination of ketene. A series of 6 consecutive ketene eliminations can be seen between the molecular ion *m/z* 1126 and the fragment ion *m/z* 874. Elimination of ketene up to the molecular ion of the free phenol (*m/z* 622) was not observed, since this series was overlapped by a second one starting at *m/z* 918 and ending at the fragment ion at *m/z* 540, equivalent to the loss of 9 ketenes. These EI-MS data were compared with those of reported values for fucotriphlorethol-A-dodecaacetate (Glombitza et al., 1977; Rauwald, 1976) and matched well.

The ^{1}H NMR spectrum of **3** in MeOD contained several singlet resonances between δ 5.99 and δ 6.11. Integration of these signals revealed that the resonance at 6.11 ppm showed twice the intensity compared to the other signals. Furthermore, coupling between the aromatic protons was lacking due to the symmetrical substitution of two magnetically equivalent protons on each ring i.e. H-2/H-6, H-9/H-11, H15/H-17, H-21/H-23, and H-27/H-29. An AB-system was not determined and compound **3** was estimated to be a linear phlorotannin. The ^{13}C NMR spectrum displayed 20 resonances, some of which must account for two carbon atoms (Table 3, Fig. 3). The aromatic rings I–V were established on the basis of HMBC correlations. For each aromatic moiety correlations between the aromatic protons and the quaternary carbon atoms were observed, e.g. for ring I correlations between H-2/H-6 and C-1, C-3/C-5, and C-4. The position of the aryl–aryl bond of rings I and II was identified between C-4 (δ 102.1) and C-7 (δ 100.5) because of a weak HMBC correlation between H-9/H-11 (δ 6.01) and C-4 (δ 102.1). Comparison with published data supports this deduction (Craigie et al., 1977). The position of moieties I and II in the structure of **3** was evident from the characteristic shifts of H-2/H-6 (δ 6.11) and H-9/H-11 (δ 6.01). Rings II-V had to be connected by ether bonds because of characteristic ^{13}C NMR shifts of C-13 (δ 126.1), C-19 (δ 126.1) and C-25 (δ 124.9). The resonance for H-27/29 (δ 5.99) proved the terminal position of ring V in the molecule. This way the precise position of the aromatic rings III and IV could not be established. After acetylation of compound **3** ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ and acetone-d_6

(Table 5S). The resulting data were compared with previously published data for fucotriphlorethol-A-dodecaacetate (Glombitza et al., 1977) and proved compound **3** to be fucotriphlorethol A. This is the first report of this compound in its non-acetylated form.

In former studies with extracts of *F. vesiculosus* fucols as well as fucophlorethols were isolated (Glombitza et al., 1975, 1977). In contrast to these reports, in the present study ^{1}H NMR, MALDI-TOF-MS analysis and the isolation of compounds **1**–**3** only point to fucophlorethols as the predominant structural type. As fucols show ^{1}H NMR resonance signals distinguishable but very similar to those of fucophlorethols, small amounts of fucols in our sample cannot be excluded, however higher concentrations of fucols were clearly not detectable by MALDI-TOF-MS analysis (data not shown).

Fucophlorethols **1**–**3** were tested in a series of bioassays indicative of cancer chemopreventive activities in comparison to the monomer phloroglucinol (Table 4). Reactive oxygen species play a role in the initiation and promotion phase of carcinogenesis. To detect radical-scavenging potential, we used the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). Radical-scavengers react with DPPH to produce 1,1-diphenyl-2-(2,4,6-trinitrophenyl)hydrazine, which is indicated by the degree of discoloration at 515 nm. With all three fucophlorethols we observed dose-dependent scavenging of DPPH radicals in a concentration range of 1–50 μg/ml (Fig. 4A). From these data, IC_{50} values in the range of 10–14 μg/ml were computed (Table 4). The monomer phloroglucinol was used for comparison and as a positive control and demonstrated equal potential to scavenge DPPH radicals as the phloroglucinol oligomers, with an IC_{50} value of 13.2 μg/ml. This was in agreement with an earlier study investigating the anti-oxidant potential of *Ecklonia stolonifera*, which reported an IC_{50} value of phloroglucinol of 55.7 μg/4 ml (Lee et al., 1996). We also investigated anti-oxidant potential by scavenging of superoxide anion radicals generated by oxidation of hypoxanthine to uric acid by xanthine oxidase. Compounds **1** and **3** as well as phloroglucinol did not show any activity at concentrations up to 250 μg/ml. Compound **2** was weakly active with an IC_{50} value of 152.9 μg/ml (Table 4). We also determined oxygen radical absorbance capacity (ORAC), using 2,2-azobis-(2-amidinopropane) dihydrochloride (AAPH) as a peroxyl-radical generator. At a concentration of 1 μg/ml, all three compounds were >3-fold potent in scavenging peroxyl radicals than the water soluble vitamin E analogue trolox used as a reference compound. Phloroglucinol was even more active in peroxyl radical scavenging activity, with 5.4 ORAC units at a 1 μg/ml concentration.

Subsequently the potential of compounds **1**–**3** to modulate the activity of drug-metabolizing enzymes, i.e. phase 1 cytochromes P450 (Cyp) enzymes and phase 2 detoxifying enzymes was investigated. During phase 1 of xenobiotic metabolism, xenobiotics are activated by addition of functional groups which render these compounds more hydrophilic. Although this step in drug metabolism is often essential for complete detoxification, phase 1 enzyme activity is in many cases induced by xenobiotics and may contribute to the activation of carcinogens. During phase 2 of xenobiotic metabolism, enzymes generally conjugate activated xenobiotics to endogenous ligands like glutathione (GSH), glucuronic-, acetic-, or sulfuric-acid, thus facilitating their excretion in form of these conjugates (Köhle and Bock, 2007). Cyp1A, involved in the metabolic activation of polycyclic aromatic hydrocarbons, aflatoxin B1, and mutagens derived from cooked food including IQ, Glu-P1 and PhIP, was selected as a phase 1 marker enzyme. As indicated in Fig. 4B, all three compounds dose-dependently inhibited Cyp1A activity, using homogenates of β-naphthoflavone-induced rat hepatoma cells as an enzyme source. Compounds **1** and **2** were about twofold more active than compound **3**. IC_{50} values were in the range of 18–34 μg/ml (Table 4). In contrast to the oligomeric fucophlorethols **1**–**3**, phloroglucinol did not inhibit Cyp1A activity at concentrations up to 50 μg/ml. This may indicate that the activities observed with compounds **1**–**3** result from an unspecific "tanning"-like interaction with proteins rather than from mechanism-based inhibition. Unfortunately, addition of an excess of bovine serum albumin to protect the enzyme from unspecific protein binding disturbed fluorimetric detection in the assay system, and therefore, specificity could not been determined (data not shown).

NAD(P)H:quinone oxidoreducatese (QR) activity, which is induced through transcription factor Nrf2-mediated activation of the anti-oxidant-response element in the promoter region of QR and other phase 2 enzymes like glutathione *S*-transferases (Köhle and Bock, 2007; Surh et al., 2008) was investigated as a representative for phase 2 enzymes in murine hepatoma cell culture. In contrast to phloroglucinol, which resulted in a dose-dependent induction of QR activity with a CD value (concentration required to induce the specific activity of QR) of 18.1 ± 0.9 μg/ml, all three tested fucophlorethols were inactive with this respect at concentrations up to 50 μg/ml (Table 4). This may be due to limited cellular uptake of these high molecular weight compounds. Interestingly, triphlorethol A, an open-chain trimer of phloroglucinol isolated from *Ecklonia cava* was identified as an inducer of the phase 2 enzyme heme oxygenase-1 through a Nrf2-mediated mechanisms at 5–30 μM concentrations (corresponding to 1.9–11.2 μg/ml) (Kang et al., 2007).

In a simplified scheme, tumor promotion can be regarded as the second step of carcinogenesis (Surh, 2003). Although cellular changes acquired during tumor promotion are generally reversible, repeated contact of initiated cells with tumor promotors finally leads to irreversible tumor progression. Processes such as chronic inflammation contribute to tumor promotion through the action of prostaglandins as inflammatory mediators and have been estimated to be associated to 15% of malignancies (Khor et al., 2008). In addition, life-long exposure to estrogens is regarded as one of the major risk factors of breast cancer by tissue specific mechanisms mediated through the estrogen receptors (Oseni et al., 2008). Consequently, cyclooxygenases, the key enzymes in prostaglandin biosynthesis, and aromatase (Cyp19), catalyzing the conversion of androgens into estrogens, are targets of

Table 4
Summary of potential cancer chemopreventive activities of compounds **1**–**3**.

	DPPH	X/XO	ORAC	Cyp1A	QR	AR	Cox-1
1	14.4 ± 2.0	>250	3.5 ± 0.2	20.0 ± 0.4	>50	3.3 ± 0.1	39
2	13.8 ± 1.3	152.9	3.2 ± 0.2	17.9 ± 1.0	>50	5.6 ± 0.3	39
3	10.0 ± 0.6	>250	3.3 ± 0.3	33.7 ± 3.0	>50	1.2 ± 0.1	44
Phloroglucinol	13.2 ± 0.8	>250	5.4 ± 0.2	>50	18.1 ± 0.9[a]	>25	90[b]

Test systems: DPPH: DPPH scavenging (SC_{50} in μg/ml); X/XO: $O_2^{-\bullet}$-scavenging (SC_{50} in μg/ml); ORAC: $ROO^{\bullet}$-scavenging (ORAC units at 1 μg/ml); Cyp1A: Cyp1A inhibition (IC_{50} in μg/ml); QR: QR induction (CD values, concentration required to double the specific activity of QR, in μg/ml); AR: Aromatase (Cyp19) inhibition (IC_{50} in μg/ml); Cox-1: Cox-1 inhibition (% inhibition at 100 μg/ml).

[a] Some signs of toxicity were visible, and an IC_{50} value for toxicity of 28.9 μg/ml was determined.

[b] An IC_{50} value of 3.8 μM (corresponding to 0.48 μg/ml) was determined, as described earlier (Bohr et al., 2005)

chemoprevention and were selected as marker systems for antitumor-promoting potential.

We used human recombinant aromatase (Cyp19) as an enzyme source in a fluorimetric test system to sensitively detect aromatase inhibitors. As indicated in Fig. 4C, all three compounds dose-dependently inhibited the enzymatic activity of aromatase at concentrations below 10 μg/ml. Compound **3** was identified as the most potent inhibitor, with an IC_{50} value of 1.2 μg/ml, followed by compounds **1** and **2** (Table 4). Similar to the effects on Cyp1A activity, phloroglucinol did not inhibit aromatase activity. From these results we can not exclude that aromatase inhibition observed with fucophlorethols **1**–**3** might be due to unspecific interaction with the enzyme.

In contrast, phloroglucinol was identified as a potent inhibitor of Cox-1 activity. At a concentration of 100 μg/ml, we observed a 90% inhibition of Cox-1 enzymatic activity using microsomes of ram seminal vesicles as an enzyme source, and we determined an IC_{50} value of 3.8 μM (corresponding to 0.48 μg/ml) as described previously (Bohr et al., 2005). All three fucophlorethol derivatives moderately inhibited Cox-1 activity, with about 40% inhibition at a 100 μg/ml concentration. Cox-1 contains two catalytic sites, a cyclooxygenase site, which is located within a long hydrophobic channel in the core of the protein, and a peroxidase site containing a heme moiety located on the surface of the enzyme (Chandrasekharan and Simmons, 2004). Previous studies with resveratrol (3,4′,5-trihydroxystilbene) indicated that the compound inhibits both the cyclooxygenase and the peroxidase activity of Cox-1 and identified the m-hydroquinone moiety, which is also found in phloroglucinol, as the structural element essential for the irreversible inhibition of Cox-1 (Szewczuk et al., 2004). Based on these findings, we assume that the low molecular weight phloroglucinol may also inhibit both catalytic sites of Cox-1, whereas the high molecular weight fucophlorethols might either react with the heme moiety to inhibit the peroxidase-catalysed step in the formation of prostaglandins or unspecifically interact with the protein.

3. Conclusions

We have tested potential chemopreventive activities of three fucophlorethols from *F. vesiculosus*. All three compounds were identified as potent radical scavengers, and may contribute to the prevention of carcinogenesis by inhibition of cytochrome P450 enzymes like CYP1A, involved in carcinogen activation, and aromatase, essential for estrogen biosynthesis. In addition, all three compounds moderately inhibited Cox-1 activity as an indication of anti-inflammatory potential. Since we can not exclude that these enzyme inhibitory effects are due to unspecific protein binding, chemopreventive potential need to be further investigated in subsequent animal studies.

4. Experimental

4.1. General experimental procedures

UV and IR spectra were obtained employing Perkin-Elmer Lambda 40 and Perkin-Elmer Spectrum BX instruments, respectively. All NMR spectra of fractions and pure compounds were recorded on a Bruker Avance 300-DPX spectrometer operating at 300 MHz (^{1}H) or 75 MHz (^{13}C) or using a Bruker Avance 500-DRX spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C), respectively. Spectra were referenced to residual solvent signals with resonances at $\delta_{H/C}$ 3.35/49.0 (CD_3OD), 2.04/29.8 (acetone-d_6), 7.26/77.0 ($CDCl_3$) and 4.68 (^{1}H: HDO in D_2O). NMR data were processed using Bruker XWIN-NMR Version 3.5 or Topspin 1.3. HPLC was undertaken using a Waters system controlled by Waters millennium software consisting of a Waters controller 600 with in-line degasser, an autosampler 717 plus, a 996 photodiode array detector and a fraction collector III. HPLC–ESI-MS measurements were performed using an Agilent 1100 Series HPLC including DAD (250 nm) coupled with an API 2000, Triple Quadrupole, LC/MS/MS (Applied Biosystems/MDS Sciex) and ESI source. Stationary phase was a reversed phase C_{18} column (Nucleodur 100, 125 × 2 mm, 5 μm, Macherey-Nagel, Düren). For the chromatography a linear gradient was used (from MeOH/H_2O 10/90 to MeOH/H_2O 100/0 in 20 min, each with 2 mM NH_4Ac). MALDI-TOF-MS spectra were recorded on a VOYAGER DE-PRO-time of flight mass spectrometer (Applied Biosystems; Foster City, USA). As matrix 2,5-dihydroxybenzoic acid in acetonitrile/0.05% trifluoroacetic acid was used. EI- and FAB-MS measurements were performed using a Finnigan MAT 95 XL spectrometer or a Kratos Concept 1H spectrometer, respectively. HR MALDI-TOF-MS measurements were performed using an ABI Voyager system 4312 MALDI-TOF-MS. As matrix cyano-4-hydroxycinnamic acid was used.

4.2. Algal material

The brown alga *F. vesiculosus* was harvested in February 2003 by G.M. König and K.-W. Glombitza. The collecting site was Corniche Armorique, St. Efflam, France. The samples used in this study were identified by Glombitza. The fresh algal material was deep frozen and stored at −20 °C. Voucher specimens of *F. vesiculosus* have been deposited at the herbarium of Institute for Pharmaceutical Biology, University of Bonn, Germany.

4.3. Extraction

Extracts obtained from *F. vesiculosus* were prepared according to the extraction procedure described before (Parys et al., 2007). For this purpose deep frozen algal fragments (500 g) were pulverized and extracted on ice and under N_2-gassing with 96% EtOH (800 ml) employing an Ultra Turrax (Ika T 25) for 2 h. The solid residue was removed by centrifugation. After evaporation of ethanol under reduced pressure chlorophyll and lipophilic substances were removed by liquid–liquid partitioning thrice to eight times between petroleum ether as well as CH_2Cl_2 (each 300 ml) and the residual aqueous phase until the organic layer was merely slightly yellow. Subsequently, the aqueous phase was freeze dried to yield 18.9 g crude extract. The crude extract was fractionated via semi-preparative HPLC. As stationary phase a reversed phase C_{18} column (Phenomenex aqua, 200 Å, 5 μm, 250 × 10 mm) was employed. The column was eluted using (A) 1% acetic acid in demineralized water and (B) 1% acetic acid in acetonitrile as linear gradient (1% (B) up to 100% (B) in 30 min, 2.5 ml/min) to yield 10 fractions. Fraction 4 eluting between 13 and 15.5 min was further separated on the same column using an isocratic mobile phase (A:B, 90:10, 2.5 ml/min) to get six sub-fractions. One known and two new compounds were isolated and characterized: trifucodiphlorethol A (**1**, $R_t \sim 14.5$ min), trifucotriphlorethol A (**2**, $R_t \sim 18$ min) and fucotriphlorethol A (**3**, $R_t \sim 17$ min).

4.3.1. Trifucodiphlorethol A (1)

brown solid; yield 7.1 mg; UV (MeOH) λ 350–200 nm (br); λ_{max} 209 nm (ε 10417); IR (ATR) ν_{max} (cm^{-1}) 3219, 1703, 1598, 1523, 1434, 1272, 1150, 1047, 1001, 820, 644, 597; ^{1}H and ^{13}C NMR data (see Table 1); LC–ESI-MS: *m/z* 747 $[M+H]^+$, *m/z* 745 $[M-H]^-$.

4.3.2. Trifucodiphlorethol-A-hexadecaacetate

^{1}H and ^{13}C NMR data (see Table 3S); FAB MS: elimination series of ketene (C_2H_2O, *m/z* 42): *m/z* 1441 $[M+Na]^+ \rightarrow$ 1357, *m/z* 1419 $[M+H]^+ \rightarrow$ 957; elimination of water: *m/z* 1167 → 1149, *m/z*

1041 → 1023, *m/z* 957 → 939; HR MALDI-TOF-MS: *m/z* 1441.2734 (calc. for $C_{68}H_{58}O_{34}Na$, 1441.2707).

4.3.3. Trifucotriphlorethol A (2)

brown solid; yield 5.3 mg; UV (MeOH) λ 350–200 nm (br); λ_{max} 210 nm (ε 14033); IR (ATR) ν_{max} (cm^{-1}) 3219, 1599, 1519, 1434, 1271, 1148, 1120, 1044, 999, 818, 650, 588; ^1H and ^{13}C NMR data (see Table 2); LC–ESI-MS: *m/z* 871 $[M+H]^+$, *m/z* 869 $[M-H]^-$.

4.3.4. Trifucotriphlorethol-A-octadecaacetate

^1H NMR data (see Table 4S); FAB MS: *m/z* 1665 $[M+K]^+$, elimination series of ketene (*m/z* 42): *m/z* 1649 $[M+Na]^+$ → 1565, *m/z* 1543 → 1165; *m/z* 1001 → 917, elimination of water and ketene (*m/z* 60): *m/z* 1065 → 1005, *m/z* 1001 → 941, elimination of water: *m/z* 1083 → 1065, tetraphenyl fragment: *m/z* 917; HR MALDI-TOF-MS: *m/z* 1649.2990 (calc. for $C_{78}H_{66}O_{39}Na$, 1649.3079).

4.3.5. Fucotriphlorethol A (3)

brown solid; yield 7.2 mg; UV (MeOH) λ 350–200 nm (br); λ_{max} 216 nm (ε 49626); IR (ATR) ν_{max} (cm^{-1}) 3202, 1607, 1518, 1447, 1372, 1273, 1153, 1112, 1052, 999, 821, 644, 598; ^1H and ^{13}C NMR data (see Table 3); LC–ESI-MS: *m/z* 623 $[M+H]^+$, *m/z* 621 $[M-H]^-$.

4.3.6. Fucotriphlorethol-A-dodecaacetate

^1H and ^{13}C NMR data (see Table 5S); EI MS: elimination series of ketene (*m/z* 42): *m/z* 1126 → 874, *m/z* 918 → 540, *m/z* 710 → 374; HR MALDI-TOF-MS: *m/z* 1149.2206 (calc. for $C_{54}H_{46}O_{27}Na$, 1149.2124).

4.4. Acetylation

Compounds **1**–**3** were acetylated to compare NMR spectra with data of previous studies. The reagent was prepared by mixing equal volumes of pyridine and acetic anhydride. The reagent (300 µl) was added to a small sample (between 1.7 and 3.4 mg) of the freshly freeze dried compound. After a reaction time of 14–16 h the reagent was evaporated.

4.5. Determination of potential cancer chemopreventive activities

Experimental details of most test systems utilized in this study are summarized in (Gerhäuser et al. (2002, 2003)). In brief, radical-scavenging potential was determined photometrically by reaction with 1,1-diphenyl-2 picrylhydrazyl (DPPH) free radicals in a microplate format. Superoxide anion radicals were generated by oxidation of hypoxanthine to uric acid by xanthine oxidase and quantitated by the concomitant reduction of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) adjusted to a 96-well microplate format (X/XO; xanthin/xanthinoxidase). Oxygen radical absorbance capacity (ORAC) using fluorescein and AAPH as a peroxyl-radical generator was quantified as described by Huang et al. (2002). Inhibition of Cyp1A (EC 1.14.14.1) enzymatic activity and induction of NAD(P)H:quinone reductase QR (EC 1.6.99.2) in cultured Hepa1c1c7 cells were assayed as described by Crespi et al. (1997) and Gerhäuser et al. (1997), monitoring the dealkylation of 3-cyano-7-ethoxycoumarin to 3-cyano-7-hydroxycoumarin and the NADPH-dependent menadiol-mediated reduction of MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] to a blue formazan, respectively. Inhibition of aromatase activity was estimated using human recombinant Cyp19 (EC 1.14.14.1) and *O*-benzylfluorescein benzyl ester as a substrate (Stresser et al., 2000). Inhibition of Cox-1 (prostaglandin G/H synthase, EC 1.14.99.1) activity was determined with the system described by Jang et al. (1997), measuring oxygen consumption during the conversion of arachidonic acid to prostaglandins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2009.10.020.

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