Biological Evaluation of Proanthocyanidin Dimers and Related Polyphenols

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A series of dimeric procyanidins (**1**−**9**) and some related polyphenols (**10**−**15**) were chosen as model compounds in a comparative investigation for various biological activities in order to obtain structure−activity relationships. Antiviral [herpes simplex virus (HSV) and human immunodeficiency virus (HIV)], antibacterial, superoxide radical-scavenging, and complement-modulating properties were assessed. In general, more pronounced activities were seen with epicatechin-containing dimers for anti-HSV, anti-HIV, and radical-scavenging effects, while the presence of *ortho*-trihydroxyl groups in the B-ring was important in compounds exhibiting anti-HSV and radical-scavenging effects and complement classical pathway inhibition. Double interflavan linkages gave rise to interesting antiviral effects (HSV and HIV) and complement inhibition. The influence of the degree of polymerization or the type of interflavan linkage (4→6 or 4→8) differed in the different biological systems evaluated. Only minor or moderate antibacterial effects were observed for the compounds under investigation.

Condensed tannins or proanthocyanidins are widely distributed in nature and are, in many cases, the active compounds of the medicinal plants from which they can be isolated.[1] Reports of several in vitro assays demonstrate potentially significant interactions with biological systems, such as antiviral, antibacterial, molluscicidal, enzyme-inhibiting, antioxidant, and radical-scavenging properties.[2] Their tendency to interfere with biological systems is, at least in part, due to a characteristic ability to form complexes with macromolecules, combined with a polyphenolic nature.[1,2] To investigate the possible influence of individual structural and configurational parameters on some biological systems, the properties of a series of proanthocyanidin dimers, together with some related polyphenols, namely procyanidin B_1 or epicatechin-(4β→8)-catechin (**1**), procyanidin B_2 or epicatechin-(4β→8)-epicatechin (**2**), procyanidin B_3 or catechin-(4α→8)-catechin (**3**), procyanidin B_4 or catechin-(4α→8)-epicatechin (**4**), procyanidin B_5 or epicatechin-(4β→6)-epicatechin (**5**), procyanidin B_6 or catechin-(4α→6)-catechin (**6**), procyanidin B_8 or catechin-(4α→6)-epicatechin (**7**), proanthocyanidin A_1 or epicatechin-(4β→8,2β→*O*→7)-catechin (**8**), proanthocyanidin A_2 or epicatechin-(4β→8, 2β→*O*→7)-epicatechin (**9**), procyanidin C_1 or epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin (**10**), (−)-epicatechin (**11**), (+)-catechin (**12**), epigallocatechin (**13**), (+)-taxifolin (**14**), and gallocatechin-(4′→*O*→7)-epigallocatechin) (**15**) (Chart 1), were evaluated in a panel of biological screening assays, comprising antibacterial, antiviral [anti-herpes simplex (HSV) and anti-human-immunodeficiency virus (HIV)], complement modulation, and radical-scavenging test systems.

Results and Discussion

Compounds **1**−**9**, **14**, and **15** were obtained from various plant sources or by biomimetic synthesis,[3−5] and compounds **11** and **12** were obtained from a commercial source. A comparative NMR study of the procyanidin dimers will be published elsewhere in due course.[3] Procyanidin C_1 (**10**) was isolated from *Theobroma cacao*, and epigallocatechin (**13**) from "dragon's blood", the latex of a South American *Croton* species.[6]

The screening system used to evaluate complement modulation was based on the hemolytic properties of the complement system and involved the spectrophotometric determination of the amount of hemoglobin that was released. The 50% inhibitory concentrations (IC_{50}) for the classical and alternative pathways of the complement system are presented in Table 1. The results of inhibition on the classical pathway clearly showed that the dimers tested were more active than the monomeric flavonoids. The trimer procyanidin C_1 (**10**) had a still more pronounced activity. The flavanoid epigallocatechin (**13**) was an important exception, having an IC_{50} value of 19.6 μM. Comparing the dimers with single interflavanoid linkage, differences were not very pronounced, although there was a tendency for 4→6-dimers to have a better inhibitory activity than their respective 4→8-counterparts. Procyanidins B_6 (**6**) (IC_{50} 18.5 μM) and B_8 (**7**) (IC_{50} 19.7 μM) proved the most active. The doubly linked proanthocyanidin A_2 (**9**) exhibited potent inhibitory effect on classical complement (IC_{50} 11.6 μM). The *Bridelia ferruginea* biflavanoid gallocatechin-(4′→*O*→7)-epigallocatechin (**15**) also showed a relatively low IC_{50} value (14.6 μM). The *ortho*-trihydroxyl group is probably responsible for the potent complement inhibition, as also demonstrated for other interactions of procyanidins and related compounds in biological systems, such as radical scavenging activity.

An increasing number of processes has been associated with enhanced free-radical production, including inflammation, radiation damage, anti-cancer reaction, immunity, arteriosclerosis, myocardial ischemia, and aging. The superoxide scavenging activity of the test compounds was expressed as the IC_{50} value for the production of superoxide anions in an enzymatic system. The IC_{50} values are listed in Table 1. When comparing the results of (−)-epicatechin (**11**), (+)-catechin (**12**), epigallocatechin (**13**), and (+)-taxifolin (**14**), similar conclusions, as in a previous study on flavonoids, could be reached.[7] Epigallocatechin showed a pronounced activity. Three B-ring hydroxyl groups; a hydroxyl at C-3; a saturated C-2, C-3 bond, and the absence

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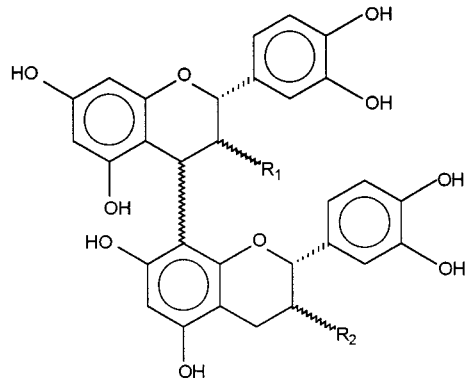
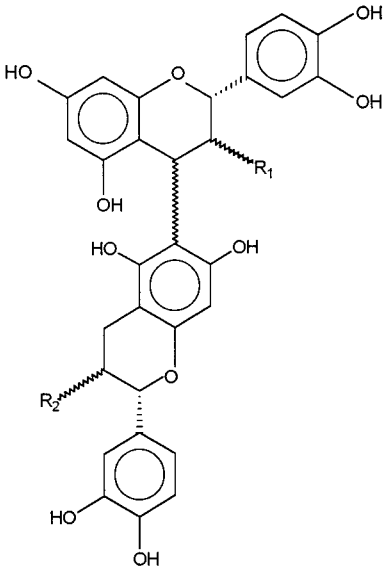
Chart 1





R_1	R_2	interflavan bond :		
OH	OH		Procyanidin B_1 or epicatechin-(4 β→8)-catechin	**1**
OH	OH		Procyanidin B_2 or epicatechin-(4 β→8)-epicatechin	**2**
OH	OH		Procyanidin B_3 or catechin-(4 α→8)-catechin	**3**
OH	OH		Procyanidin B_4 or catechin-(4 α→8)-epicatechin	**4**

R_1	R_2	interflavan bond :		
OH	OH		Procyanidin B_5 or epicatechin-(4 β→6)-epicatechin	**5**
OH	OH		Procyanidin B_6 or catechin-(4 α→6)-catechin	**6**
OH	OH		Procyanidin B_8 or catechin-(4 α→6)-epicatechin	**7**

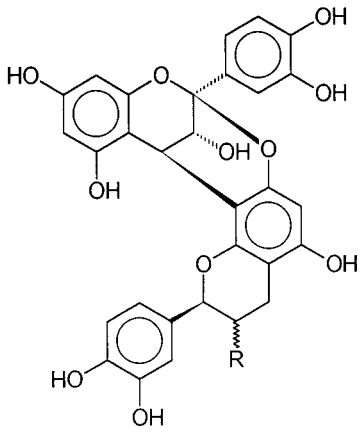
Table 1. Biological Activities of Compounds **1**−**15**

compound	superoxide scavenging activity [IC_{50} (μM) ± SD]	complement classical pathway [IC_{50} (μM)]	complement alternative pathway [IC_{50} (μM)]	anti-HIV IC_{50} (μg/mL)	anti-HIV CC_{50} (μg/mL)	anti-HIV SI[a]
1	n.t.[b]	31.3	72.0	n.t.	n.t.	
2	10.5 ± 0.1	58.0	n.a.[c]	17.2	135.1	8
3	41.3 ± 5.4	37.7	n.a.	27.0	117.8	5
4	30.0 ± 2.0	45.5	n.a.	>0.3	0.3	<1
5	11.4 ± 0.9	51.7	n.a.	8.3	53.5	9
6	27.3 ± 1.8	18.5	n.a.	29.9	141.2	5
7	32.9 ± 2.9	19.7	83.7	19.6	126.6	8
8	14.7 ± 1.0	57.1	105.0	14.0	144.3	10
9	17.8 ± 0.7	11.6	112.8	5.8	137.6	24
10	10.7 ± 0.5	6.0	85.5	4.3	16.5	5
11	18.3 ± 0.7	655.5	n.a.	34.1	136.2	4
12	13.1 ± 0.1	647.2	n.a.	>86.8	86.8	<1
13	3.4 ± 0.3	19.6	179.4	>16.1	16.1	<1
14	24.2 ± 1.5	850.3	n.a.	>25.4	25.4	<1
15	2.0 ± 0.1	14.6	86.0	n.t.	n.t.	
positive control substance	$(4.9 \pm 0.2)10^{-4}$ [d]	21.9[e]	408.3[e]	0.0002[f]	2.5813[f]	12906

[a] SI = selectivity index (CC_{50}/IC_{50}). [b] n.t. = not tested. [c] n.a. = not active. [d] Manganese superoxide dismutase (*E. coli*). [e] Rosmarinic acid. [f] Zidovudine.

of any carbonyl at C-4 contributed to a good superoxide scavenging activity. The best results for dimeric proanthocyanidins were obtained for procyanidins B_2 (**2**) and B_5 (**5**). The procyanidins B_3 (**3**), B_4 (**4**), B_6 (**6**), and B_8 (**7**) were about three times less active. For the dimeric procyanidins with single linkage, those with two epicatechin units (B_2 and B_5) gave markedly better results than products containing a catechin unit. The type of linkage had no consistent significant influence on superoxide scavenging activity. Procyanidin C_1 (**10**) produced no higher scavenger properties than its dimeric analogue procyanidin B_2. The biflavanoid gallocatechin-(4′→*O*→7)-epigallocatechin (**15**) displayed a prominent activity. The doubly linked proanthocyanidins A_1 (**8**) and A_2 (**9**) also showed a good activity, although they were not as potent as procyanidins B_2 or B_5. The superoxide radical-scavenging properties observed were in line with the observations of Ricardo da Silva et al. who stated for the first time that it was not the degree of polymerization as such, but the number of hydroxyl groups (preferably *ortho*-trihydroxyl) (also increasing with the degree of polymerization) that was important for the activity.[8] Our results did not show a pronounced difference in superoxide radical-scavenging activity between 4→6- and 4→8-linked dimers.

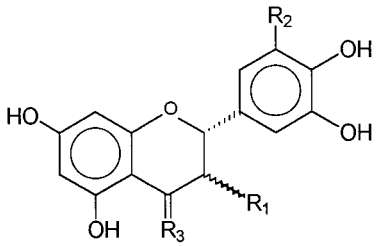
Chart 2



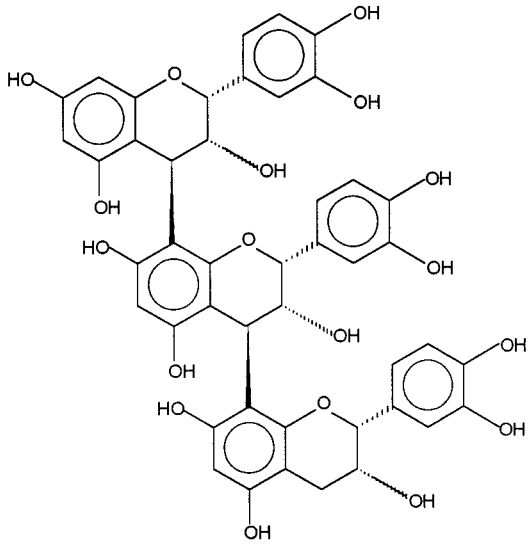
R

OH — Proanthocyanidin A_1 or epicatechin-(4 β→8, 2β→*O*→7)-catechin **8**

OH — Proanthocyanidin A_2 or epicatechin-(4 β→8, 2β→*O*→7)-epicatechin **9**

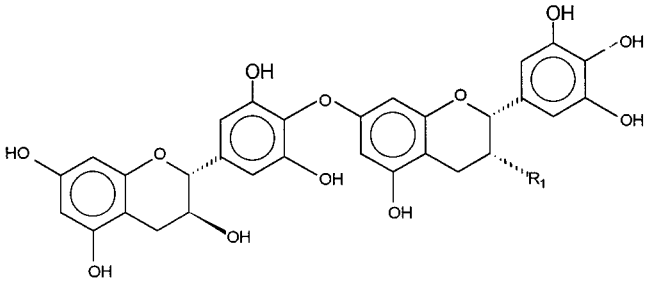


R_1	R_2	R_3		
OH	H	H_2	(-)-Epicatechin	**11**
OH	H	H_2	(+)-Catechin	**12**
OH	OH	H_2	Epigallocatechin	**13**
OH	H	O	(+)-Taxifolin	**14**



Proanthocyanidin C_1 or epicatechin-(4 β→8)-epicatechin- (4β→8)-epicatechin **10**

Chart 3



Gallocatechin-(4' →*O*→7)- epigallocatechin **15**

Condensed tannins exhibit antiviral activities as shown by different research groups.[9−11] Galloylation and polymerization potentiated the antiviral activities markedly. However, no structure−activity differences within an isomeric group of proanthocyanidins have been investigated before. In the virucidal test system, the test compounds are preincubated with the virus suspension, after which the residual infectious virus is titrated. Results of the antiviral and virucidal assays with HSV are presented in Table 2. The antiviral assay demonstrated antiherpetic activity for the majority of the compounds tested. Antiviral activity was most prominent for the doubly linked proanthocyanidins A_1 (**8**) and A_2 (**9**), for the trimeric procyanidin C_1 (**10**), and for the 4→6-coupled dimeric procyanidins B_5 (**5**) and B_6 (**6**). Comparing the procyanidin pairs B_2 (**2**)/B_5 (**5**) and B_3 (**3**)/B_6 (**6**), each consisting of a 4→8-linked dimer and its 4→6-linked isomer, a gain of 10^2 in reduction factor (at 100 μg/mL) was noted for the 4→6 coupled dimers, with procyanidin B_5 (epicatechin-(4β→6)-epicatechin) as the most active one. Only a tenfold difference could be observed between procyanidin B_4 (**4**) and B_8 (**7**). Further elongation of procyanidin B_2 (**2**) to procyanidin C_1 (**10**) increased the reduction factor with 10^2 at 100 μg/mL. Epigallocatechin (**13**) had a weak antiherpetic activity, although still better than some dimers [B_3 (**3**), B_4 (**4**)], confirming that *ortho*-trihydroxyl groups potentiated activity. Other potentiating factors were polymerization and mode of linkage, with a double linkage or 4→6 linkage being preferred. The antiherpetic activity−dependence of condensed tannins on the degree of condensation was already described by Takechi et al. in 1985.[12] In the extracellular (virucidal) assay, no activities could be observed at concentrations up to 200 μg/mL against HSV.

Table 2. Antiviral Activity on HSV of Compounds **1**−**15**

	antiviral activity	
compound	conc. (μg/mL)	RF[a]
2	100	10
	50	1
3	100−1	1
4	100−1	1
5	100	10^3
	50	1
6	100	10^2
	50	1
7	100	10
	50	1
8	100	10^4
	50	10
	25	1
9	100	10^4
	50	1
10	100	10^3
	50	1
13	100	10
	50	1
14	100−1	1

[a] RF = reduction factor.

For comparison of anti-HIV potencies, the selectivity indices (SI = CC_{50}/IC_{50}) were considered; they are listed in Table 1. The most interesting activity was seen for the doubly linked proanthocyanidin A_2 (**9**) with a SI of 24. For its analogue with a (+)-catechin terminal unit, proanthocyanidin A_1 (**8**), the SI was more than halved, but was still larger than for the procyanidins with single linkage. The monomeric compounds tested showed no particularly significant activity except for (−)-epicatechin (**11**), with a selectivity index of 4. Looking at the procyanidin dimers with a single linkage, a general tendency of higher anti-HIV activity for (−)-epicatechin-containing compounds is apparent. No significant difference was observed for the different types of interflavanoid linkage. Procyanidin B_4 (**4**), however, was an exception in both regards. The observations of Kakiuchi et al. on reverse transcriptase inhibition also indicated the importance of the presence of (−)-epicatechin units.[13] Further elongation to form procyanidin C_1 (**10**) did not increase the SI. Although the IC_{50} value was rather small, the 50% cytotoxic concentration (CC_{50}) was very low. Because with a higher degree of polymerization both cytotoxicity and HIV inhibition appeared to increase, there might be an optimum with maximum selectivity index.

The microbial battery included representatives of most human pathogenic bacteria as well as those causing food infections and intoxications. The test was limited to microorganisms only requiring a standard growth medium. None of the compounds tested was active against *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella paratyphi*, *Enterobacter cloaca*, *Mycobacterium fortuitum*, *Staphylococcus aureus*, or *Candida albicans* (MIC and MBC > 100 μg/mL). The results revealed a moderate antibacterial activity for certain compounds against *Streptococcus pyogenes*, *Bacillus cereus*, *Klebsiella pneumoniae*, and *Proteus vulgaris* at concentrations ≤ 100 μg/mL. These encompassed representatives of Gram-positive cocci, Gram-positive spore-forming rods, and Gram-negative, facultatively anaerobic rods. *S. pyogenes* proved most susceptible to several of the test compounds: procyanidin B_4 (**4**) (MIC 25 μg/mL, MBC 50 μg/mL); procyanidin A_2 (**9**) (MIC and MBC 50 μg/mL); procyanidins B_2 (**2**), B_5 (**5**), B_6 (**6**), and C_1 (**10**) (MIC and MBC 100 μg/mL). Epigallocatechin (**13**) inhibited the Gram-negative bacteria *P. vulgaris* (MIC 50 μg/mL, MBC 100 μg/mL) and *K. pneumoniae* (MIC and MBC 100 μg/mL). In addition, procyanidin A_2 (**9**) was active against *B. cereus* (MIC 100 μg/mL) and procyanidin A_1 (**8**) against *P. vulgaris* (MIC 50 μg/mL).

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a Varian Unity 400 instrument (^{1}H, 399.9 MHz; ^{13}C, 100.6 MHz), using 5-mm tubes, and locked to the deuterium resonance of the solvent, Me_2CO-d_6 (99.8%) or MeOH-d_4 (99.5%). Chemical shifts are reported in parts per million on the δ scale; spectra are referenced to the solvent. The temperature was maintained at 303 K. MS were recorded on a VG 70-SEQ hybrid mass spectrometer. Unless mentioned otherwise, negative FAB with NOBA/HMPT (*m*-nitrobenzyl alcohol−hexamethylphosphorthionamide, 1:1 v/v) matrix or positive FAB with magic bullet (eutectical mixture of dithiothreitol−dithioerythritol, 5:1 w/w) matrix were applied.

A standard TLC system used precoated Merck Kieselgel $60F_{254}$ plates eluted with the organic layer of the following developing solvent: EtOAc 30 mL, H_2O 8 mL, HCOOH 1.2 mL, CH_3COOH 0.8 mL. Spots were detected under UV light (254 nm) and subsequently sprayed with 5% H_2SO_4 or HCl and 1% vanillin in MeOH. Proanthocyanidins emerged immediately after spraying as red to pink-red spots; occasionally heating at 110 °C was required for visualization. Unless mentioned otherwise, column chromatography was carried out on Sephadex LH-20 (Pharmacia) using a gradient from 75% 1-propanol−25% MeOH to 100% MeOH. High-performance liquid chromatography (HPLC) was carried out using Gilson equipment: pump model 303, manometric module 802C, dynamic mixer 811, and data master 621. The chromatograms were detected with a Hewlett−Packard 1030B variable wavelength UV detector. For proanthocyanidin analysis, a Merck Lichrosorb RP-18 7-μm (250 × 4 mm) column was used with a mobile phase of 5% HCOOH (A) and MeOH (B) with the following elution profile: 0−1 min: 2% B, 1−30 min: linear from 2% to 40% B, 30−37 min: linear from 40% to 90% B, 37−40 min: 90% B. Flow was 1.5 mL/min. Signals were detected at 280 nm.

Plant Material. A *Theobroma cacao* L. (Sterculiaceae) extract ("EC-powder") obtained as a byproduct in cacao production (Cacao De Zaan, Koog aan de Zaan, The Netherlands) was obtained as described elsewhere.[3] "Sangre de drago" ("dragon's blood") (*Croton* sp.) (Euphorbiaceae), produced in 1989 (batch no. L8908101), was purchased from Quimica Universal, Lima (Peru).

Extraction and Isolation. The isolation from plant material as well as the biomimetic synthesis of compounds **1**−**9**, **14**, and **15** have been reported before.[3−5] (+)-Catechin (**11**) and (−)-epicatechin (**12**) were obtained from Sigma.

The "EC-powder" obtained from *T. cacao* was treated as described elsewhere for the isolation of procyanidin B_2 (**2**).[3] The EtOAc phase was evaporated to dryness in vacuo, which afforded a brown solid. An aliquot (2 g) of this solid was chromatographed on Sephadex LH-20, with 15-mL fractions being collected and monitored by TLC. After repeated chromatography on Sephadex LH-20, fraction 8 afforded 48 mg of a brown amorphous compound. HPLC analysis showed a major signal at t_R 29.5 min. This compound was identified as the trimeric procyanidin C_1 or epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin (**10**).[14]

Epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin (procyanidin C_1) (10): ^{13}C NMR (100.6 MHz, MeOH-d_4) δ 29.7 [C-4 bottom unit (b)], 37.4 [C-4 middle(m) and top(t) unit], 66.7 (C-3 b), 72.9 (C-3 m), 73.4 (C-3 t), 77.1 (C-2 m and t), 79.7 (C-2 b), 96.3 (C-8 t), 96.6 (C-6 m and t), 97.5 (C-6 b), 100.7 (C-4a b, m and t), 107.6 (C-8 b and m), 115.3 (C-2′ b, m and t), 116.0 (C-5′ b, m and t), 119.2 (C-6′ b, m and t), 132.1 (C-1′), 132.7 (C-1′), 143.1 (C-3′ b, m, and t), 145.8 (C-4′ b, m and t), 154.5, 156.4, 156.8, 157.2, 157.8, 158.3 (quaternary carbons rings A, D, G); positive FABMS *m*/*z* 867 $[M+H]^+$.

A portion (1.25 L) of "sangre de drago" was acidified with citric acid to a final concentration of 2%, filtered, and extracted with *n*-hexane. The *n*-hexane extract was evaporated to dryness under reduced pressure. The aqueous phase was then extracted with Et_2O, EtOAc and *n*-BuOH. The EtOAc extract was concentrated under reduced pressure to a final volume of 150 mL, dried over anhydrous sodium sulfate, filtered, and evaporated to dryness under reduced pressure.[6] TLC and subsequent spraying with an $FeCl_3$ solution of the EtOAc fraction afforded dark blue spots, indicative of tannins containing *ortho*-trihydroxyl groups. The EtOAc fraction (8 g) was chromatographed on Sephadex LH-20 and eluted with 1-propanol−MeOH (3:1). Fractions were monitored by TLC, and 12 pooled fractions were collected. Fraction 4 (2.6 g) yielded after two consecutive separations on Sephadex LH-20 one fraction that afforded green spots (R_f 0.79) upon spraying with $FeCl_3$ reagent. The next fraction (R_f 0.80) stained blue with $FeCl_3$ and was submitted to 2D TLC on precoated cellulose plates with mobile phase *n*-BuOH−H_2O−CH_3COOH (14:5:1) in the first dimension and 6% CH_3COOH in the second dimension. This revealed two spots: R_{f1} 0.47, R_{f2} 0.43 and R_{f1} 0.35, R_{f2} 0.33. A final separation on Sephadex LH-20 with MeOH−H_2O (2:1) yielded three fractions, of which the first was identified by NMR and mass spectrometry as epigallocatechin (**13**).[15]

Complement-Modulating Activity. The screening system used to evaluate modulating effects of compounds **1**−**15** on the complement system was based on its hemolytic properties, involving a spectrophotometric measurement of the hemo-

globin released at λ 414 nm, in absence and presence of the compounds tested. The experimental conditions have been described earlier.[4,16−18]

Superoxide-Scavenging Activity. The superoxide-scavenging activity of compounds **1**−**15** was measured by the nitrite method after generation of the superoxide anions by a hypoxanthine−xanthine oxidase system.[19]

Antiviral Activity. The in vitro antiviral screening method estimated the inhibition of the cytopathic effects (CPE) of compounds **1**−**15** on a host cell monolayer (VERO cells) infected with HSV, after incubation in the presence of the test compound.[20] Antiviral activity is expressed as a reduction factor (RF), being the ratio of the viral titers in the virus control and in the presence of the maximal nontoxic dose (MNTD) of test substance. A microtray assay evaluated the protection of compounds **1**−**15** against the CPE of HIV.[21,22] The colorimetric assay described monitors the exclusive ability of metabolically active cells to take up and reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to a blue formazan product, which is measured spectrophotometrically at 540 nm. Determination of cell viability has the advantage that both antiviral and cytotoxic activities can be determined in parallel. Comparison of the effects on HIV-infected and mock-infected MT-4 cells allows calculation of the SI.

Antimicrobial Activity. For the evaluation of the antimicrobial activity of compounds **1**−**15**, the minimum inhibitory concentration and the minimum bactericidal concentration were determined.[20]

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