JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

New Approach for the Synthesis and Isolation of Dimeric Procyanidins

NILS KÖHLER,[†] VICTOR WRAY,[‡] AND PETER WINTERHALTER*,[†]

Institute of Food Chemistry, University of Braunschweig—Institute of Technology, Schleinitzstrasse 20, 38106 Braunschweig, and Helmholtz Centre for Infection Research, Inhoffenstrasse 7, 38124 Braunschweig, Germany

A semisynthetic approach for the strategic formation of various procyanidins has been developed. Procyanidin-rich grape seed extracts were reacted with flavan-3-ols under acid catalysis. The reaction enables the formation of dimeric procyanidins and the elimination of higher oligomeric and polymeric procyanidins through degradation. An easy and fast method for the isolation of large amounts of procyanidins after semisynthetic formation by high-speed countercurrent chromatography is presented. Dimeric procyanidins (B1, B2, B3, B4, B5, and B7) were obtained and isolated. Furthermore, galloylated dimeric procyanidins [(−)-epicatechin-3-*O*-gallate-4β→8-(+)-catechin, (−)-epicatechin-3-*O*-gallate-4β→8-(−)-epicatechin, (−)-epicatechin-3-*O*-gallate-4β→6-(−)-epicatechin, and (−)-epicatechin-4β→8-(−)-epicatechin-3-*O*-gallate], as well as trimeric procyanidins [C1, (−)-epicatechin-4β→6-(−)-epicatechin-4β→8-(−)-epicatechin, and (−)-epicatechin-4β→6-(−)-epicatechin-4β→6-(+)-catechin] were obtained and isolated as side products. This approach also afforded gambiriins A1 and A2, which were all isolated and unambiguously identified, and the novel 3-(2,4,6-trihydroxyphenyl)-1-(3,4-dihydroxyphenyl)-propan-2-ol-1β→8-(−)-epicatechin (gambiriin A4).

KEYWORDS: HSCCC; countercurrent chromatography; procyanidins; synthesis; chalcane flavan-3-ols; gambiriins

INTRODUCTION

Procyanidins, commonly known as condensed tannins, occur widely in nature and are found as prominent compounds in foods (e.g., fruits, cereals, nuts, seeds, and chocolate) and beverages (e.g., wine, beer, fruit juice, and green tea). Because of their chemical interactions with proteins, they have an important impact on taste (e.g., astringency) (*1–4*).

Many publications have reported the procyanidin content of different natural products (*4–9*). However, these compositional data were often determined by nonselective methods (e.g., Porter assay and vanillin assay), which vary significantly and only provide incomplete information about the real procyanidin content.

In recent years, procyanidins have also increasingly attracted attention due to their biological effects, for example, antioxidant, anti-inflammatory, and anticancer activities (*3, 5, 10, 11*). Until today, detailed knowledge on the bioavailability of procyanidins is still sparse as studies have only used complex procyanidin mixtures due to a lack of appropriate reference compounds (*10, 12–14*). Only a few procyanidins are commercially available, such as procyanidin B1 and B2. Consequently, a supply of authentic standard compounds would improve their analyses and enable more thorough biological studies.

The isolation of pure procyanidins on a preparative scale is still a challenging task. Column chromatography (e.g., gel permeation chromatography and solid-phase extraction chromatography) has been used for preparative fractionation of procyanidins from complex natural extracts (*15–19*). Such isolation procedures are often time-consuming and bear the risk of irreversible adsorption of the target compounds on the solid-phase materials. As an alternative method, high-speed countercurrent chromatography (HSCCC) has already been successfully used for the isolation of procyanidins, although coelution of compounds was observed, which hindered the isolation of pure compounds in a single run (*20*).

The goal of this work was the development of a strategy for the preparation and isolation of dimeric procyanidins on a large scale. Various synthetic approaches for the formation of procyanidins have already been reported and indicate that procyanidins can be synthesized stereoselectively from flavan-3-ol thioether with silver tetrafluoroborate ($AgBF_4$) either under acid (*21*) or basic conditions (*22*). Procyanidins can also be produced from taxifolin after reduction with sodium borohydride to flavan-3,4-diols, which are then reacted with flavan-3-ols to form dimeric procyanidins. Both methods exhibit several disadvantages. In the case of taxifolin, the reduction is not stereoselective, with the 3,4-*cis*- and 3,4-*trans*-flavandiol inter-

* To whom correspondence should be addressed. Tel: +49-531-391-7200. Fax: +49-531-391-7230. E-mail: p.winterhalter@tu-bs.de.
[†] University of Braunschweig—Institute of Technology.
[‡] Helmholtz Centre for Infection Research.

10.1021/jf7036505 CCC: $40.75
Published on Web 06/10/2008

mediates causing the formation of various structural isomers (*23–25*).

Alternatively, dimeric procyanidins can be formed directly by the reaction of procyanidin-rich extracts with flavan-3-ols. This approach was first applied to the synthesis of procyanidins with C2 epimers (*26*) using harsh reaction conditions (e.g., 95 °C for 22 h). This method has now been modified, and more gentle reaction conditions have been applied for the stereoselective formation of 3,4-*trans*-configured procyanidins. This approach enables a cheap and rapid generation of primarily dimeric procyanidins. Isomer formation is suppressed, and separation by countercurrent chromatography is improved due to reduced coelution.

MATERIAL AND METHODS

Reagents. Grape seed extract OPC-40 was supplied by Breko (Bremen, Germany). The mean degree of polymerization was 4.25 with a monomeric content of 9.0% flavan-3-ols. Chemicals and solvents were as follows: sodium acetate (anhydrous), p.a. (Merck, Darmstadt, Germany); phloroglucinol, p.a. (Merck); hydrochloric acid, 37% (Riedel-de-Haën, Seelze, Germany); and ascorbic acid, pure (Merck). Solvents for high-performance liquid chromatography (HPLC) analysis were as follows: acetonitrile, HPLC quality (Sigma, Steinheim, Germany); acetic acid (Mallinckrodt Baker B. V., Deventer, Holland); and water (bidistilled). Solvents used for HSCCC and solvent precipitation were as follows: ethyl acetate (Acros Organics, Geel, Belgium); methanol (distilled, industrial quality); hexane (Acros Organics); pentane (distilled, industrial quality); water (bidistilled); 1-butanol, p.a. (Fisher Scientific, Loughborough, United Kingdom); *tert*-butylmethyl ether (distilled, industrial quality) and ethanol (distilled, industrial quality); and 2-propanol, p.a. (Sigma).

Semisynthetic Formation of Procyanidins. *Optimization.* For the ratio of substrates, the grape seed extract OPC-40 (7.05 mg/ mL) was reacted with different ratios of flavan-3-ol (7.05, 14.10, and 21.14 mg/ mL; this corresponds to ratios of 1:1, 2:1, and 3:1 of flavan-3-ol to grape seed extract) in 0.1 N methanolic HCl solution for 20 min at 40 °C, respectively. For the temperature, the grape seed extract OPC-40 (7.05 mg/mL) was reacted with flavan-3-ol (14.10 mg/mL) in 0.1 N methanolic HCl solution for 20 min at 30, 35, 40, and 50 °C, respectively. For the reaction time, the grape seed extract OPC-40 (7.05 mg/ mL) was reacted with flavan-3-ol (14.10 mg/ mL) in 0.1 N methanolic HCl solution at 40 °C for 10, 15, 20, and 30 min, respectively. For all solutions, the reaction was terminated by adding 5 times the volume of aqueous sodium acetate solution (40 mM).

Preparative Formation. One gram of (+)-catechin and 1.0 g of grape seed extract for CCC-1, 1.0 g of (+)-catechin and 0.5 g of grape seed extract for CCC-5, and 0.5 g of (−)-epicatechin and 0.5 g of grape seed extract for CCC-3 and CCC-7 were used. The reactants were dissolved in 0.1 N methanolic HCl (50 mL), and the reaction was carried out at 40 °C for 20 min. The solutions were neutralized with 0.5 N sodium hydrogen carbonate solution (10 mL). The solvents were evaporated with a rotary evaporator, and the residual aqueous solution was freeze-dried.

Solvent Precipitation. According to a published protocol (*20*), the freeze-dried reaction mixture was dissolved in ethanol (50 mL). Pentane (130 mL) was slowly dropped into the solution for successive precipitation of nonreacted higher oligomeric and polymeric procyanidins. The suspension was filtered and dried under vacuum using a rotary evaporator.

HPLC-Diode Array Detector (DAD). A HPLC system from Jasco (Gross-Umstadt, Germany), consisting of a PU-980 pump combined with a DG-980-50 degasser and LG 980-02 ternary gradient unit, and MD-910 DAD were used. HPLC conditions: column, 250 mm × 4.6 mm i.d., 5 μm Aqua (Phenomenex, Aschaffenburg, Germany), 2% aqueous acetic acid (v/v, solvent A), and acetonitrile (solvent B); gradient, initial 97% A and 3% B in 25 min to 90% A and 10% B, in 20 min to 65% A and 35% B, in 5 min to 25% A and 75% B, and in 5 min to 97% A and 3% B; detection at 280 nm; and flow rate, 0.8 mL/ min. For analysis of isolated HSCCC fractions, the yield was calculated as the relative absorbance according to the UV trace.

Preparative HPLC. A HPLC system from Knauer (Berlin, Germany) consisting of a WellChrom K-1001 HPLC pump, K-1500 solvent organizer and degasser, K-2600 UV detector, and a preparative HPLC column [250 mm × 16 mm i.d., 5 μm, Hypersil ODS C-18 (Phenomenex, Aschaffenburg, Germany), equipped with a guard column] were used. Conditions: water (solvent A) and acetonitrile (solvent B); gradient 1 (for compounds with t_R < 30 min by HPLC method): initial 6% B in 40 min to 25% B; gradient 2 (for compounds with t_R > 30 min): initial 10% B in 40 min to 30% B. The flow rate was adjusted to 6.0 mL/min, and the fractions were monitored at 280 nm.

Liquid Chromatography−Electrospray Ionization (ESI)−Tandem Mass Spectrometry (MS/MS). A HP Series 1100 HPLC pump (Hewlett-Packard, Waldbronn, Germany) and a L-4000 UV/vis detector from Merck Hitachi (Tokyo, Japan) were connected in series with an Esquire HPLC-MS/MS from Bruker GmbH (Bremen, Germany). MS parameters: negative mode; capillary, 3000 V; end plate, −500 V; capillary exit, −120 V; skim 1, −40 V; skim 2, −8.0 V; dry gas, 325 °C; gas flow, 9.0 L/min; and nebulizer, 40 psi. HPLC conditions were the same as those used for HPLC-DAD analysis.

Phloroglucinolysis. The analysis was carried out according to published data (*20*). The isolated procyanidins (0.1 mg) were dissolved in 100 μL of reaction solution containing 50 mg/mL phloroglucinol and 10 mg/mL ascorbic acid in 0.1 N methanolic HCl.

Analysis of Selected Compounds. Reaction conditions were as follows: 5 min at 30 °C (method A, mild incomplete cleavage of interflavanoid bond) and 5 min at 50 °C (method B, for complete degradation). Immediately after the exposure, the reaction was terminated by adding 500 μL of a sodium acetate solution (40 mM). The standards for quantification were isolated by a published protocol (*27*).

HSCCC. A high-speed countercurrent chromatograph model CCC-1000 manufactured by Pharma-Tech Research Corp. (Baltimore, MD) was equipped with three preparative coils connected in series (total volume, 850 mL). The separation was carried out at a revolution speed varying from 900 to 1000 rpm with flow rates between 2.5 and 3.0 mL/min. The lower aqueous phase was employed as the mobile phase in the head to tail elution mode. A Biotronic HPLC pump BT 3020 was used. All samples were dissolved in a 1:1 mixture of upper and lower phase and injected into the system by an injection loop (25 mL). Elution was monitored with a Knauer UV/vis detector (280 nm) and recorded using a Knauer L 250 E plotter. Chromatograms were digitalized with a scanner. Fractions were collected with a fraction collector (Pharmacia LKB Super Frac). Solvent systems and sample loads used are given in the text.

Nuclear Magnetic Resonance (NMR) Spectroscopy. One-dimensional ^{1}H and ^{13}C NMR and two-dimensional ^{1}H−^{1}H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond correlation (HMBC) experiments were performed on Avance DMX 600 or DMX 300 spectrometers from Bruker (Rheinstetten, Germany). The chemical shifts were calibrated with respect to the solvent signals and are in ppm. Coupling constants are in Hz.

Circular Dichroism. The experiments were carried out with a J-715 spectropolarimeter from Jasco (Gross-Umstadt, Germany) at a temperature of 20 °C. The solvent was methanol.

Isolated Compounds. The structures of isolated compounds are summarized in **Figure 1**.

Gambiriin A1. (1*R*,2*S*)-3-(2,4,6-Trihydroxyphenyl)-1-(3,4-dihydroxyphenyl)propan-2-ol-1α→8-(+)-catechin (**5**). Electrospray LC-MS [M − H]$^-$ *m/z* 579. CD (MeOH): $[\theta]_{280}$ −3.4 × 10^3, $[\theta]_{230}$ −3.9 × 10^4, $[\theta]_{215}$ −3.6 × 10^4. ^{1}H NMR (300 MHz, acetone-d_6, 300 K): δ 6.85 (d, 1H, *J* = 1.6 Hz, H2′t), 6.81 (d, 1H, *J* = 1.8 Hz, H2′u), 6.67 (m, 4H, H5′t, H6′t, H5′u, H6′u), 6.09 (s, 1H, H6t), 5.93 (s, 2H, H6u, H8u), 4.75 (d, 1H, *J* = 4.3 Hz, H1u), 4.68 (m, 1H, H2u), 4.61 (d, 1H, *J* = 5.6 Hz, H2t), 3.95 (m, 1H, H3t), 2.95 (m, 2H, H3αu, H4αt), 2.59 (dd, 1H, *J* = 8.3 Hz, *J* = 16.2 Hz, H4βt), 2.56 (dd, 1H, *J* = 10.1 Hz, *J* = 13.9 Hz, H3βu) ppm. ^{13}C NMR (75 MHz, acetone-d_6, 300 K): δ 158.6 (C5u, C9u), 158.4 (C7u), 156.8 (C7t), 156.1 (C5t), 155.5 (C8at),

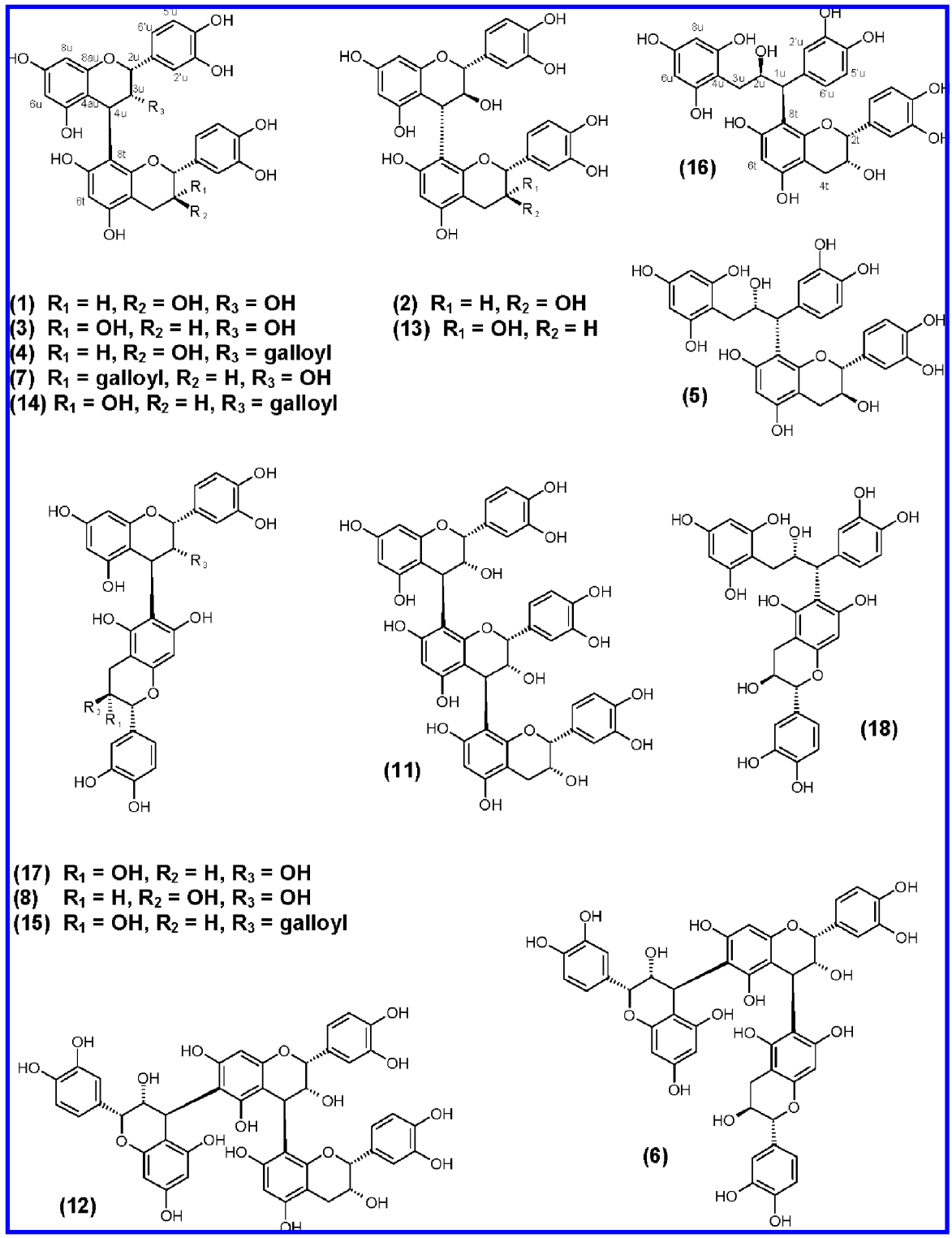

Figure 1. Structure of procyanidins isolated by HSCCC: B1 (**1**), B2 (**3**), B3 (**2**), B4 (**13**), B5 (**17**), and B7 (**8**); galloylated dimeric procyanidins: ECG-4β→8-C (**4**), ECG-4β→8-EC (**14**), ECG-4β→6-EC (**15**), and EC-4β→8-ECG (**7**); trimeric procyanidins: C1 (**11**), EC-4β→6-EC-4β→8-EC (**12**), and EC-4β→6-EC-4β→6-C (**6**); and chalcane flavan-3-ols: gambiriin A1 [3-(2,4,6-trihydroxyphenyl)-1-(3,4-dihydroxyphenyl)propan-2-ol-1α→8-(+)-catechin] (**5**), gambiriin A3 [3-(2,4,6-trihydroxyphenyl)-1-(3,4-dihydroxyphenyl)propan-2-ol-1α→6-(+)-catechin] (**18**), and gambiriin A4 [3-(2,4,6-trihydroxyphenyl)-1-(3,4-dihydroxyphenyl)propan-2-ol-1β→8-(−)-epicatechin] (**16**, tentative).

146.2−144.4 (C3′t, C4′t, C3′u, C4′u), 136.2 (C1′u), 132.9 (C1′t), 121.4−120.6 (C6′t, C6′u), 117.4 (C2′u), 116.3−116.1 (C5′t, C5′u), 115.8 (C2′t), 107.9 (C8t), 106.2 (C4u), 101.1 (C4at), 98.2 (C6t), 96.7 (C6u, C8u), 83.3 (C2t), 77.6 (C2u), 69.0 (C3t), 46.8 (C1u), 31.0 (C3u), 30.3 (C4t) ppm.

Gambiriin A3. (1*R*,2*S*)-3-(2,4,6-Trihydroxyphenyl)-1-(3,4-dihydroxyphenyl)propan-2-ol-1α→6-(+)-catechin (**18**). Electrospray LC-MS [M − H]$^-$ *m/z* 579. CD (MeOH): $[\theta]_{289}$ 0.8 × 10^3, $[\theta]_{275}$ −2.0 × 10^3, $[\theta]_{235}$ −1.1 × 10^4, $[\theta]_{212}$ −6.9 × 10^4. ^{1}H NMR (600 MHz, acetone-d_6 + 5% D_2O, 300 K): 6.92 (d, 1H, J = 1.7 Hz, H2′t), 6.82 (s, 1H, H2′u), 6.79 (d, 1H, J = 8.1 Hz, H5′t), 6.76 (dd, 1H, J = 1.8 Hz, J = 8.1 Hz, H6′t), 6.66 (s, 2H, H5′u, H6′u), 6.01 (s, 1H, H8t), 5.97 (s, 2H, H6u, H8u), 4.86 (d, 1H, J = 3.2 Hz, H1u), 4.63 (m, 1H, H2u), 4.48 (d, 1H, J = 8.5 Hz, H2t), 3.97 (ddd, 1H, J = 5.3 Hz, J = 8.5 Hz, J = 9.2 Hz, H3t), 2.97 (dd, 1H, J = 1.4 Hz, J = 14.5 Hz, H3αu), 2.96 (dd, 1H, J = 5.3 Hz, J = 16.1 Hz, H4αt), 2.59 (dd, 1H, J = 10.1 Hz, J = 14.2

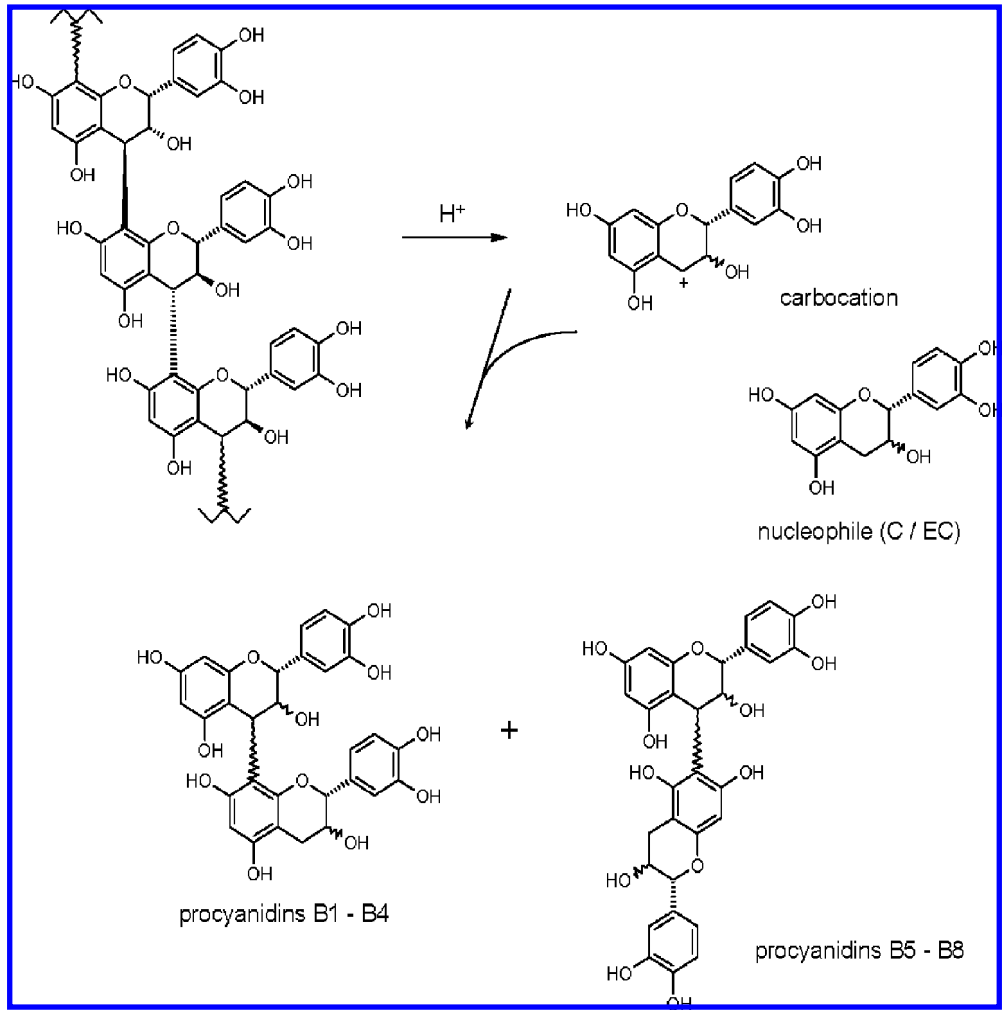

Figure 2. Semisynthetic pathway for the formation of dimeric procyanidins starting from oligomeric precursors.

Hz, H3βu), 2.56 (dd, 1H, J = 9.2 Hz, J = 16.1 Hz, H4βt) ppm. The upper B ring showed no characteristic coupling of the protons due to overlap of H5′u and H6′u. After addition of 5% benzene-d_6, an anisotropic effect was obtained that enabled determination of the coupling of the B ring protons. ^{1}H NMR (600 MHz, acetone-d_6 + 5% D_2O + 5% C_6D_6, 300 K): 6.88 (d, 1H, J = 1.5 Hz, H2′u), 6.71 (dd, 1H, J = 1.5 Hz, J = 8.1 Hz, H6′u), 6.70 (d, 1H, J = 8.1 Hz, H5′u) ppm. ^{13}C NMR (150 MHz, acetone-d_6 + 5% D_2O, 300 K): 158.2 (C5u, C7u, C9u), 156.6 (C7t), 156.4 (C5t), 155.5 (C8at), 146.3 (C3′, C4′), 146.2 (C3′, C4′), 145.9, (C4′u*) 144.2 (C3′u*), 135.6 (C1′u), 132.5 (C1′t), 120.8 (C6′t, C6′u), 117.0 (C2′u), 116.2 (C5′t), 116.1 (C2′t, C5′u), 108.3 (C6t), 105.9 (C4u), 102.3 (C4at), 96.3 (C6u, C8u), 96.1 (C8t), 83.3 (C2t), 78.2 (C2u), 69.1 (C3t), 46.6 (C1u), 31.0 (C4t), 30.4 (C3u) ppm (* exchangeable).

Gambiriin A4. Tentative, (1*S*,2*R*)-3-(2,4,6-Trihydroxyphenyl)-1-(3,4-dihydroxyphenyl)propan-2-ol-1β→8-(−)-epicatechin (**16**). Electrospray LC-MS [M − H]$^-$ m/z 579. CD (MeOH): $[\theta]_{289}$ −1.6 × 10^3, $[\theta]_{270}$ 0.6 × 10^3, $[\theta]_{235}$ 1.0 × 10^4, $[\theta]_{212}$ −1.3 × 10^5. ^{1}H NMR (300 MHz, acetone-d_6, 300 K): δ 6.99 (d, 1H, J = 1.0 Hz, H2′t), 6.88 (dd, 1H, J = 1.0 Hz, J = 7.9 Hz, H6′t), 6.77 (s, 1H, H2′u), 6.76 (d, 1H, J = 8.1 Hz, H5′t), 6.66 (d, 1H, J = 7.8 Hz, H6′u), 6.59 (d, 1H, J = 8.1 Hz, H5′u), 6.09 (s, 1H, H6t), 5.98 (s, 2H, H8u, H6u), 4.90 (d, 1H, J = 2.8 Hz, H1u), 4.83 (s, 1H, H2t), 4.76 (ddd, 1H, J = 3.9 Hz, J = 3.9 Hz, J = 7.9 Hz, H2u), 4.21 (m, 1H, H3t), 3.69 (d, 1H, J = 3.4 Hz, OH3t), 3.14 (dd, 1H, J = 3.7 Hz, J = 14.3 Hz, H3βu), 2.96 (dd, 1H, J = 4.7 Hz, J = 17.0 Hz, H4αt), 2.82 (dd, 1H, J = 2.3 Hz, J = 17.1 Hz, H4βt), 2.67 (dd, 1H, J = 8.6 Hz, J = 13.8 Hz, H3αu) ppm. ^{13}C NMR (75 MHz, acetone-d_6, 300 K): δ 158.5 (C5u, C9u), 158.2 (C7u), 156.7 (C5t, C7t), 155.7 (C8at), 145.9−144.3 (C3′t, C4′t, C3′u, C4′u), 136.2 (C1′u), 133.0 (C1′t), 121.2 (C6′u), 120.1 (C6′t), 117.3 (C2′u), 116.3 (C5′t), 116.1 (C5′u), 115.4 (C2′t), 107.9 (C8t), 106.5 (C4), 99.9 (C4at), 98.2 (C6t), 97.1 (C6u, C8u), 80.2 (C2t), 77.8 (C2u), 67.6 (C3t), 46.3 (C1u), 31.0 (C3u), 30.3 (C4u) ppm.

RESULTS AND DISCUSSION

The semisynthetic approach involves the degradation of procyanidins under acid conditions. Conditions applied are similar to those used for the characterization of procyanidin-rich extracts by thiolysis (*28–30*) and phloroglucinolysis (*28, 31*). The major difference is that the intermediate cleavage products, that is, the positively charged carbocations, further react with an excess of a flavan-3-ol to form dimeric procyanidins (**Figure 2**). This allows an elimination of higher oligomeric procyanidins by acid-catalyzed degradation, an enrichment of certain dimeric procyanidins that contain the added flavan-3-ol in the terminal position, and the application of HSCCC for the preparative isolation of synthesized dimeric procyanidins.

Optimization of Reaction Conditions. Beside the desired procyanidin formation, we observed the formation of various byproducts. LC-MS analysis of the newly formed compounds revealed a pseudo molecular ion [M − H]$^-$ at m/z 579. These compounds were isolated and unambiguously identified as chalcane flavan-3-ols. During the reaction process, the acidic conditions cause protonation at the oxygen of the C ring of the flavan-3-ols followed by ring opening and the formation of the positively charged chalcanes. These carbocations are competing with the cleaved flavan-3-ol carbocations for the nucleophiles. As a result, formation of chalcan-flavan-3-ol derivatives, so-

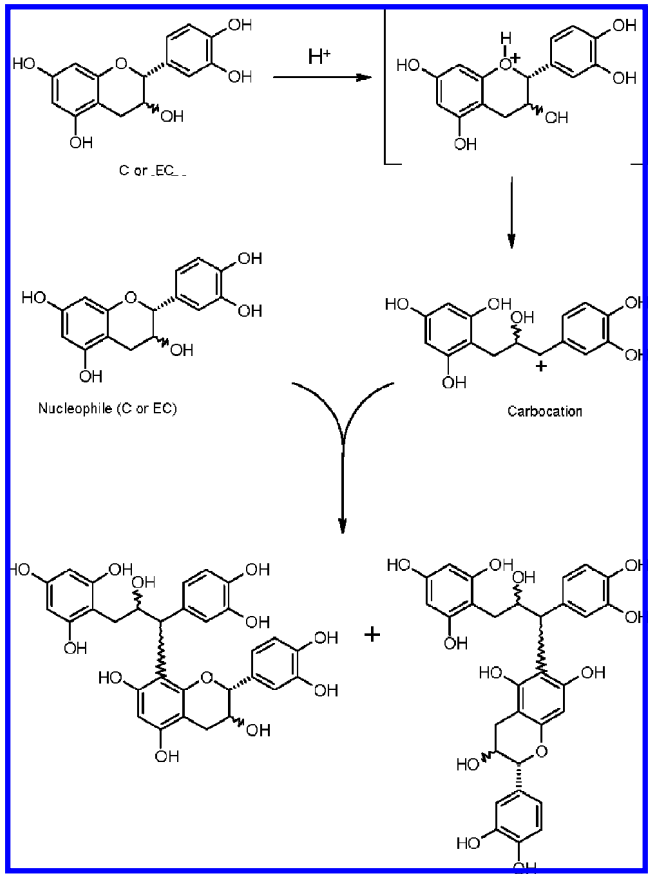

Figure 3. Pathway of chalcane flavan-3-ol adduct formation.

called gambiriins (**Figure 3**), takes place. The gambiriins are known compounds from leaves of *Uncaria gambir* (*32, 33*).

To find optimal reaction conditions, the influence of reaction time, temperature, and ratio of reactants on product composition was examined (**Figure 4**). Optimization of the ratio of reactants shows an increase in the formation of procyanidin dimers upon increasing the ratio of flavan-3-ol. Simultaneously, the concentration of the chalcane flavan-3-ols also increases. The total amount of procyanidins exceeds the total content of chalcane flavan-3-ols under all selected conditions. Because of likely coelutions (e.g., B7 and gambiriin A3), reaction conditions with minimum gambiriin formation should be selected. The temperature dependence shows a maximum for procyanidin formation between 35 and 40 °C, although formation of chalcane flavan-3-ols also increases with increasing temperature.

The reaction time has different effects on the product formation. In the case of (+)-catechin as a nucleophile, the yield of procyanidins increases slightly and the yield of chalcane flavan-3-ols increases significantly. With (−)-epicatechin, there is nearly no effect on procyanidin formation, although the yield of chalcane flavan-3-ols is again increased.

Considering the outcome of these experiments, a compromise between maximum procyanidin formation and a minimum formation of chalcane flavan-3-ols (gambiriins) have been chosen for the semisynthetic preparation of dimeric procyanidins. Furthermore, the cost of reactants must be taken into account. Hence, conditions chosen for synthetic studies were 20 min at 40 °C with substrate ratios of 1:1 and 2:1.

Figure 5A shows the HPLC analysis of the grape seed extract OPC-40. Certain low molecular compounds could be assigned, and a significant amount of higher oligomeric and polymeric procyanidins was found as a broad unresolved peak in the chromatogram.

After reaction with (+)-catechin, the "polymer peak" nearly disappeared and the concentration of dimeric compounds increased (**Figure 5B**). A similar result was observed for the formation of procyanidins with (−)-epicatechin (**Figure 5C**). The highest yields were observed for 4→8-connected procyanidin dimers. Procyanidins B1 and B2, with (−)-epicatechin as the upper unit, were obtained in highest yields. This is due to the flavan-3-ol composition of the oligomeric and polymeric procyanidins of the grape seed extract used for the semisynthesis. In the phloroglucinolysis of the applied grape seed extract, (−)-epicatechin (70%) dominated followed by (+)-catechin (15%) and (−)-epicatechin-3-*O*-gallate (15%). Consequently, this composition determines the composition of the corresponding newly formed dimeric procyanidins. The 4→6-connected procyanidins are formed in a significant smaller amount.

Fractionation of the Reaction Mixture of the Semisynthesis Carried out with (+)-Catechin. The target compounds of the reaction with (+)-catechin are B1 (**1**), B3 (**2**), B6, and B7 (**8**). **Figure 6A** (CCC-1) shows the results of the separation of the reaction mixture by HSCCC using the solvent system I (ethyl acetate/1-butanol/water, 14:1:15, v/v/v), which has been demonstrated to be suitable for the separation of procyanidins B1 (**1**) and B3 (**2**) (*20*). The formation of dimer B6 was not observed. According to the synthesized procyanidins, 151.5 mg of B1 (**1**) (purity 75.0%) was obtained in fraction I. Fraction II contained mainly trimeric procyanidins. B3 (**2**, 46.0%) was found in fraction III (67.6 mg) accompanied by B2 (**3**, 15.0%). The latter was formed from the significant amounts of monomeric (−)-epicatechin, which were present in the grape seed extract.

Compounds that remained on the CCC system with *K* values higher than 2 were fractionated with the more lipophilic solvent system II, composed of hexane/ethyl acetate/methanol/water (0.75: 10: 0.75: 10, v/v/v/v), in which they were expected to have adequate *K* values (between 0.5 and 1.5) and which was presumed to be suitable for the isolation of B7 (**8**). The CCC separation is shown in **Figure 6B** (CCC-2).

Fraction I contained 25.5 mg of galloylated dimeric procyanidin (−)-epicatechin-3-*O*-gallate-4β→8-(+)-catechin (**4**) (purity 67.1%). Fraction II consisted of 17.3 mg of gambiriin A1 (**5**), (−)-epicatechin-4β→6-(−)-epicatechin-4β→6-(+)-catechin (**6**), and (−)-epicatechin-4β→8-(−)-epicatechin-3-*O*-gallate (**7**), in concentrations of 19.0, 18.4, and 3.0%, respectively. In fraction III (54.0 mg), a coelution of B7 (**8**, 48.3%) and (−)-epicatechin (**9**, 40.0%) took place due to very similar partition coefficients. The nonreacted (+)-catechin (**10**) was found in fraction IV (375.0 mg, purity >98%).

Fractionation of the Reaction Mixture of the Semisynthesis Carried out with (−)-Epicatechin. Under these conditions, a formation of dimeric procyanidins bearing (−)-epicatechin units in the terminal position was expected. Target compounds of the reaction are B2 (**3**), B4 (**13**), B5 (**17**), and B8 (not detected). Solvent system III (ethyl acetate/2-propanol/water, 20: 1: 20, v/v/v) was selected because it was successfully used for isolation of procyanidins B2 (**3**) and B4 (**13**) (*20*).

The results of the separation are shown in **Figure 6C** (CCC-3). A complex mixture (43.6 mg) of the trimeric procyanidin C1 (**11**, 18.0%), (−)-epicatechin-4β→6-(−)-epicatechin-4β→8-(−)-epicatechin (**12**, 15.2%), and B1 (**1**, 5.6%) was found in fraction I. Although synthesis of **1** was not intended, it was formed from small amounts of catechin present in the grape seed extract. Fraction II (71.8 mg) consisted of 68.6% pure B2 (**3**). Fraction III (32.8 mg) contained B4 (**13**, 31.8%) ac-

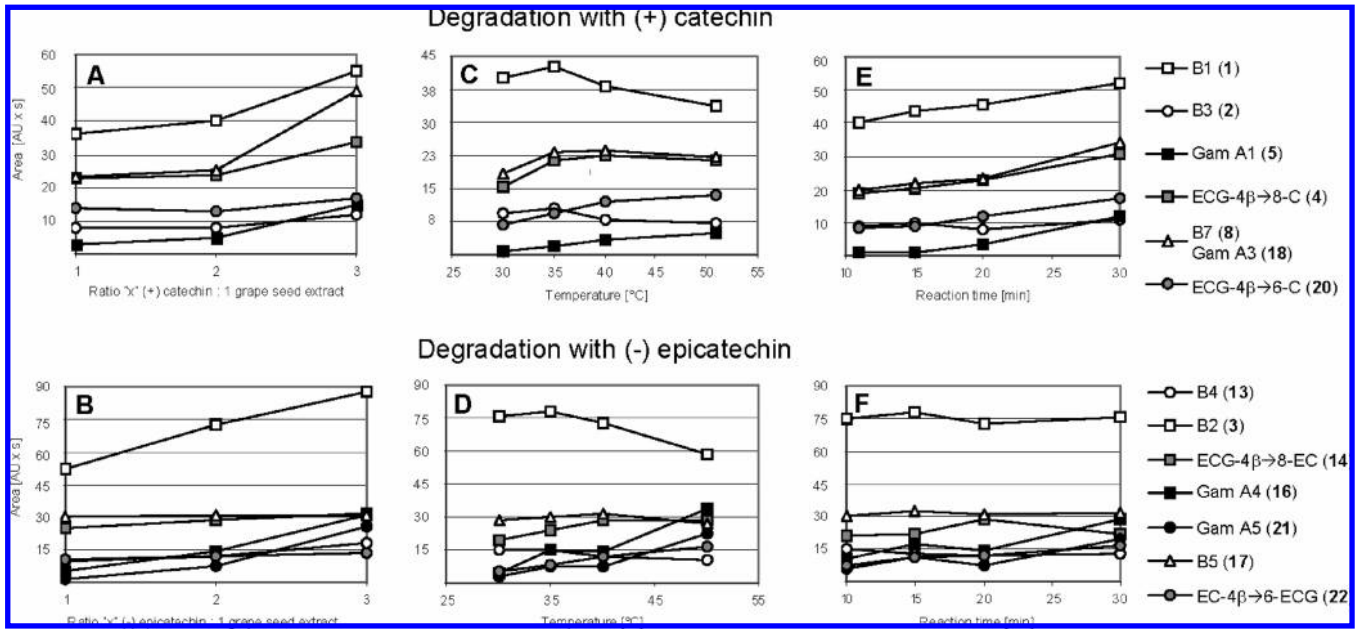

Figure 4. Optimization of semisynthetic preparation of dimeric procyanidins: (**A**) dependence of the substrate ratios (+)-catechin to grape seed extract for 20 min at 40 °C; (**B**) dependence of the substrate ratios (−)-epicatechin to grape seed extract for 20 min at 40 °C; (**C**) dependence of the temperature for the ratio 2:1 [(+)-catechin to grape seed extract] for 20 min; (**D**) dependence of the temperature for the ratio 2:1 [(−)-epicatechin to grape seed extract] for 20 min; (**E**) dependence of the reaction time at 40 °C for the ratio 2:1 [(+)-catechin to grape seed extract]; and (**F**) dependence of the reaction time at 40 °C for the ratio 2:1 [(−)-epicatechin to grape seed extract]; gam = gambiriin.

companied by B2 (**3**, 6.2%). The galloylated dimeric procyanidin (−)-epicatechin-3-*O*-gallate-4β→8-(−)-epicatechin (**14**, 30.4 mg) was found in fraction IV in a purity of 66.7%.

Compounds that remained on the coil due to *K* values >2.4 were fractionated with the more lipophilic solvent system II, composed of hexane/ethyl acetate/methanol/water (0.75: 10: 0.75: 10, v/v/v/v), in which they have sufficiently different *K* values for the isolation of B5 (**17**). The results are shown in **Figure 6D** (CCC-4). Fraction I (10.8 mg) contained (−)-epicatechin-3-*O*-gallate-4β→8-(+)-catechin (**4**), (−)-epicatechin-4β→8-(−)-epicatechin-3-*O*-gallate (**7**), and (−)-epicatechin-3-*O*-gallate-4β→6-(−)-epicatechin (**15**). Gambiriin A4 (**16**) was found in fraction II (15.7 mg, 29.6% purity). Fraction III (30.1 mg) contained 60.4% pure B5 (**17**), and the nonreacted (−)-epicatechin (**9**) was recovered in >98% purity in fraction IV (326.4 mg).

Optimization of the Solvent System for the Isolation of B7. Procyanidin B7 (**8**) and (−)-epicatechin (**9**) have very similar partition coefficients (*K* values) in solvent system II (hexane/ethyl acetate/methanol/water, 0.75:10:0.75:10, v/v/v/v), thus hindering the isolation of pure B7 in a single CCC run (**Figure 6B**).

For the separation of **8** and **9**, three different solvent systems were therefore compared (**Table 1**), as small changes of the solvent composition can afford significant changes in the *K* values. Solvent system II causes coelution as demonstrated in CCC-2. Solvent system IV enables a partial separation of **8** and **9** due to a difference of 0.1 in the *K* values. The highest difference in *K* value for **8** and **9** is observed with solvent system V, which was later successfully applied to the separation of B7 and (−)-epicatechin.

Isolation of Procyanidins after Synthesis and Cleanup by Solvent Precipitation. The semisynthetic approach can be directly applied for the separation of dimeric procyanidins by HSCCC. However, the low retention of stationary phase (R_s) of only 35−40% indicates that the separations still have a potential for improvement to increase the purity of isolated compounds. R_s is an important factor for high separation efficiency (*34*), which can be negatively affected by compounds with a high interfacial activity (e.g., polymeric procyanidins). High sample loads and the presence of nonreacted higher oligomeric procyanidins cause a loss of stationary phase due to a reduced countercurrent motion of the biphasic solvent system (emulsification) and, hence, a loss in peak resolution. A simple cleanup step by solvent precipitation allowed the elimination of these nonreacted polymeric compounds, which has been shown to be useful for the isolation of procyanidins from grape seed extract (*20*).

The semisynthesis was therefore repeated. In the case of the reaction with catechin, the ratio of reactants was changed to 2:1 to increase the degradation of oligomeric and polymeric procyanidins. After solvent precipitation, the same solvent system as selected for CCC-1 was used for the fractionation (**Figure 7A**, CCC-5).

The retention of stationary phase increased from 39.8 to 71.5% as indicated by the breakthrough of mobile phase after 1.3 h. The intensity of the first polymeric peak decreased significantly and improved the separation efficiency considerably. The purity of procyanidin B1 (**1**) increased to 95.7% in fraction I (55.5 mg) as well as of B3 (**2**) to 66.4% in fraction III (17.9 mg).

According to the optimization for the isolation of procyanidin B7 (**8**), the coil fraction of this fractionation was separated in CCC-6 (**Figure 7B**) using solvent system V composed of hexane/ethyl acetate/methanol/water (1:10:1:10, v/v/v/v). The mobile phase elutes after 1.5 h. The retention of stationary phase increased from 46.2 to 68.5% due to reduced emulsification. Compounds **8** and **9** were nearly baseline separated, and the purities increased to 82.9% (fraction III, 17.2 mg) for **8** and >98% (fraction IV, 45.6 mg) for **9**. The purity of **5** was increased from 19.0 to 67.0% in fraction II (19.5 mg). A 684.7 mg amount of the remaining (+)-catechin was isolated in fraction V (purity >98%). Furthermore, fraction VI contained

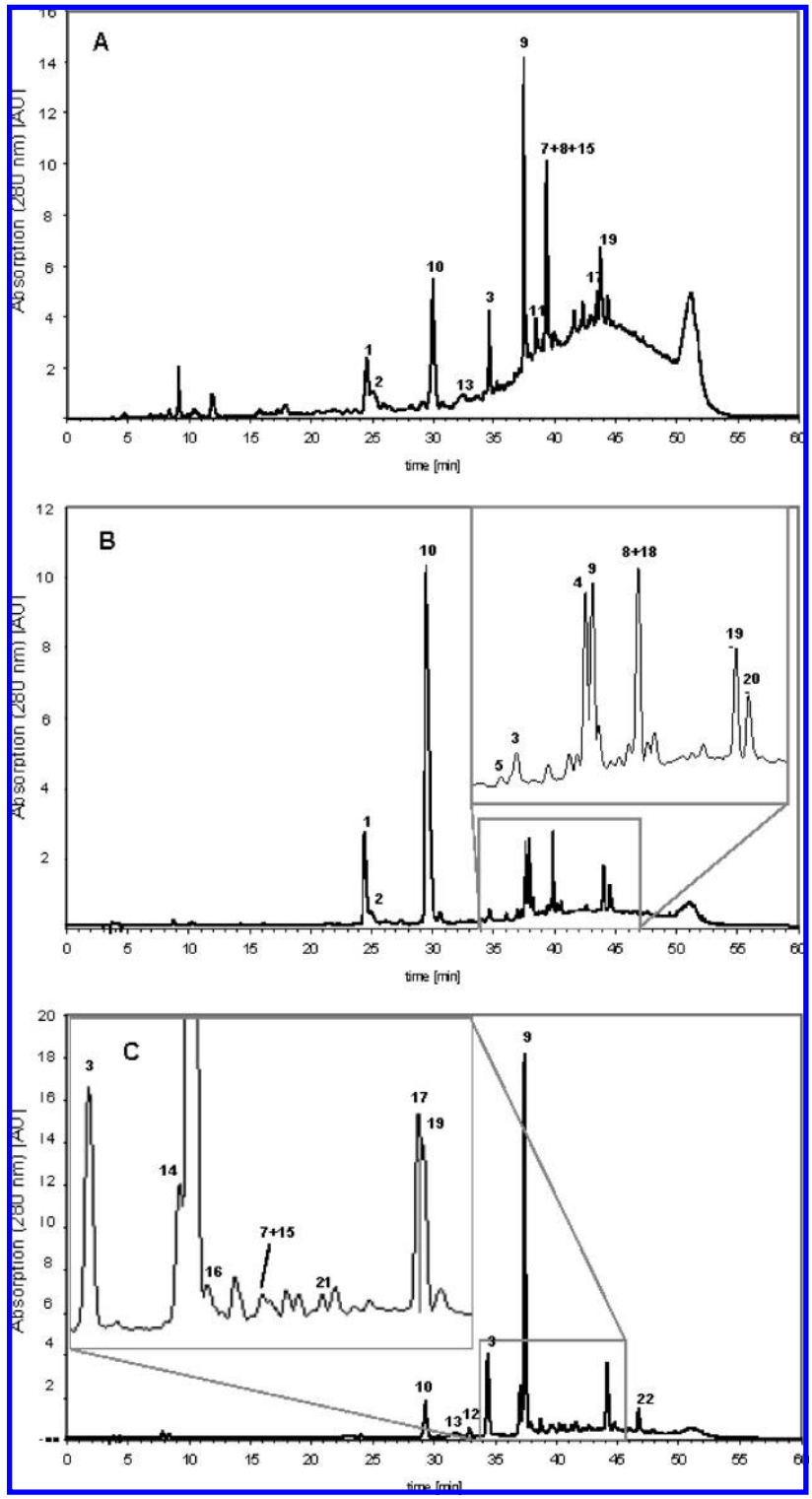

Figure 5. HPLC-UV (280 nm) chromatograms of (**A**) the grape seed extract (OPC-40), (**B**) chromatogram of semisynthesis with (+)-catechin, and (**C**) of semisynthesis with (−)-epicatechin (for compounds cf. **Figure 1**).

12.7 mg of 78.4% pure gambiriin A3 (**18**). A 13.8 mg amount of 62.0% pure **4** was found in fraction I.

The formation of procyanidins with (−)-epicatechin was carried out under the same conditions as for CCC-3, prior to solvent precipitation as cleanup step. The separation (CCC-7) is shown in **Figure 7C**. The retention of stationary phase rose significantly from 35.3 to 55.4%. Whereas this increase is not as high as that for the separation of the reaction mixture with (+)-catechin (cf. CCC-5), the intensity of the first peak that contains residues of oligomeric and polymeric procyanidins as well as the baseline level caused by tailing of the first peak are reduced significantly.

In fraction I (28.8 mg), the enrichment of **1** increased to 8.1%, of **11** to 23.3%, and of **12** to 18.8%. Compound **3** increased to 77.7% in fraction II (67.7 mg) as well as **13** to 44.3% in fraction III (25.3 mg). The purity of **14** in fraction IV (25.2 mg) remained nearly unchanged at 65.3%. The coil residue fractionation is shown in CCC-8 (**Figure 7D**). The separation was improved, which was indicated by a higher retention and a better peak resolution for fractions III and IV. Therefore, the purity of **17**

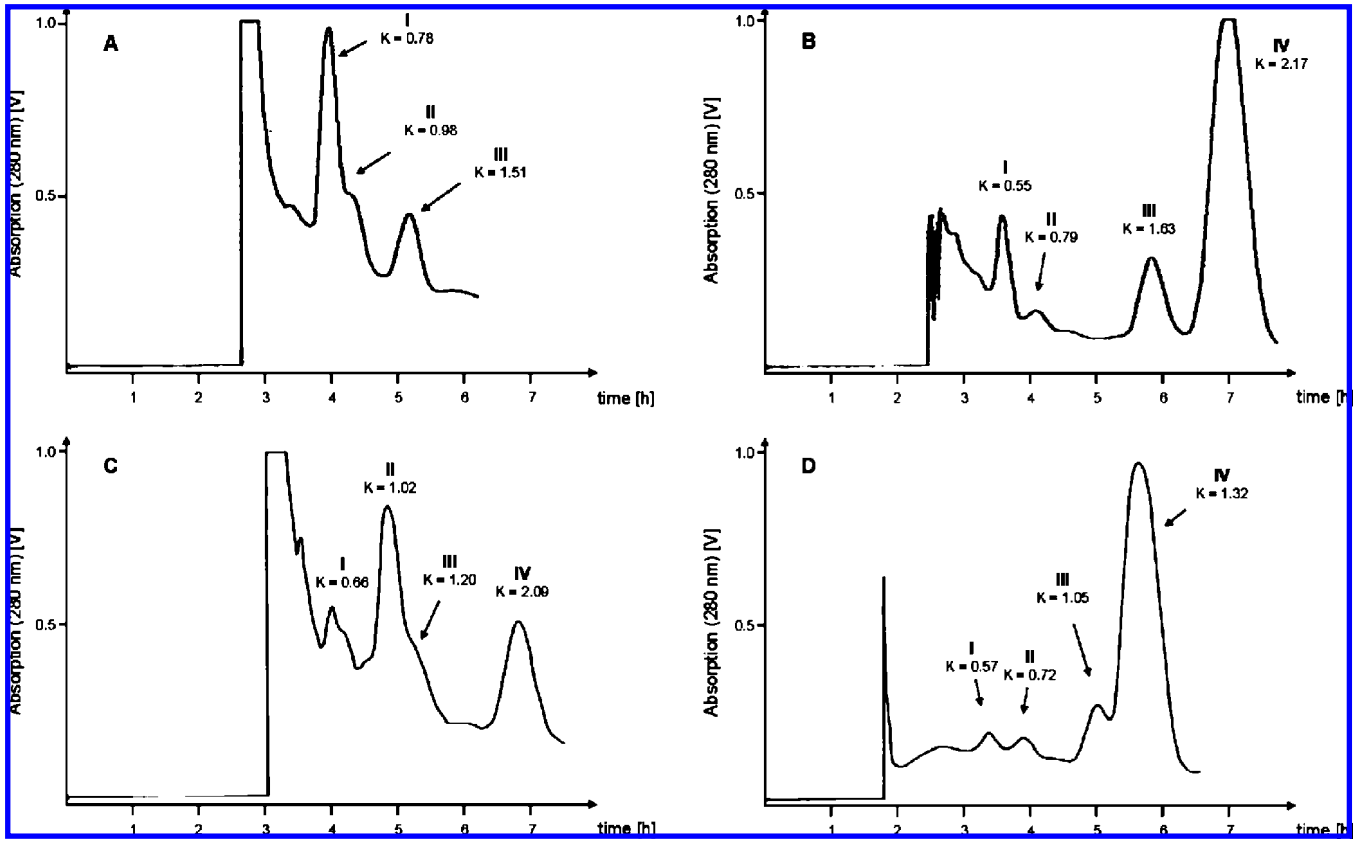

Figure 6. HSCCC separations of semisynthetic reaction mixtures. (**A**) CCC-1: separation of reaction products of OPC-40 and (+)-catechin; solvent system, ethyl acetate/1-butanol/ water (14:1:15, v/v/v); flow rate, 2.5 mL/min; 2.3 g sample load; 900 rpm; and R_s = 39.8%. (**B**) CCC-2: separation of the coil fraction of CCC-1; solvent system, hexane/ethyl acetate/methanol/water (0.75:10:0.75:10, v/v/v/v); flow rate, 3.0 mL/min; 700 mg sample load; 800 rpm; R_s = 46.2%. (**C**) CCC-3: separation of reaction products of OPC-40 and (−)-epicatechin; solvent system, ethyl acetate/2-propanol/water (20:1:20, v/v/v); flow rate, 2.9 mL/min; 1.3 g sample load; 1000 rpm; R_s = 35.3. (**D**) CCC-4: separation of the coil fraction of CCC-3; solvent system, hexane/ethyl acetate/methanol/water (0.75:10:0.75:10, v/v/v/v); flow rate, 3.0 mL/min; 600 mg sample load; 1000 rpm; R_s = 57.8%.

Table 1. *K* Values of Procyanidin B7 (**8**) and Epicatechin (**9**) in Three Different Solvent Systems

	solvent system	B7 (**8**)	EC (**9**)
II	hexane/ethyl acetate/methanol/water (0.75:10:0.75:10, v/v/v/v)	1.63	1.63
IV	*tert*-butylmethyl ether/1-butanol/water (4.75:0.25:5, v/v/v)	0.92	1.02
V	hexane/ethyl acetate/methanol/water (1:10:1:10, v/v/v/v)	1.02	1.23

rose to 71.7%. Consequently, the purity of isolated compounds increased considerably but was not as high as those of CCC-5. Hence, it can be generally recommended that a substrate ratio of 2:1 is used for the semisynthesis of dimeric procyanidins.

Semisynthetic techniques combined with countercurrent chromatographic separations offer a novel and efficient approach for the preparation of dimeric procyanidins. Under appropriate conditions, higher oligomeric procyanidins from natural sources can be converted by the addition of flavan-3-ols into desired dimeric products. Solvent precipitation then affords a fast and easy sample cleanup, which eliminates any remaining higher oligomeric and polymeric procyanidins. Subsequent preparative separation by countercurrent chromatography enables isolation of individual procyanidins in good purities.

Increased flavan-3-ol concentration enables a more efficient degradation of the polymeric procyanidins and allows a product directed synthesis. Although gambiriin formation increases more quickly than procyanidin formation as a consequence of the increase of the flavan-3-ol ratio (see **Figure 5**), these compounds do not interfere with the isolation of procyanidins as they exhibit different *K* values in the solvent systems selected. Therefore, it appears reasonable to use an excess of flavan-3-ols for the formation of procyanidins to improve the separation efficiency. Nevertheless, it should be kept in mind that an increase of flavan-3-ol will increase operational expenses. Furthermore, all chromatographic methods are limited by their sample load capacity, although this is very high for countercurrent chromatography. The absolute concentration of procyanidins increases with an increase of the flavan-3-ol ratio, but a large excess of flavan-3-ol decreases the relative procyanidin concentration relative to the same amount of injected reaction mixture. Thus, a ratio 2:1 of flavan-3-ol to procyanidin-rich natural extract should not be exceeded for the formation of semisynthetic procyanidins. The semisynthetic approach is a compromise between maximal degradation of polymeric procyanidins, maximal formation of dimeric procyanidins, and minimal flavan-3-ol consumption.

It is recommended that monomeric flavan-3-ols are removed from the grape seed extract by solvent precipitation before the semisynthesis. Otherwise, undesired procyanidins will be formed. In the case of the semisynthesis with (−)-epicatechin, remaining (+)-catechin will generate procyanidins B1, B3, and B7 and enlarge the number of isomers found. Generally, different extracts contain several flavan-3-ols. Grape seed extracts contain (−)-catechin, (−)-epicatechin, and (−)-epicatechin-3-*O*-gallate. Furthermore, the yields of dimeric procyanidins are mainly based on the flavan-3-ol composition of the oligomeric and polymeric

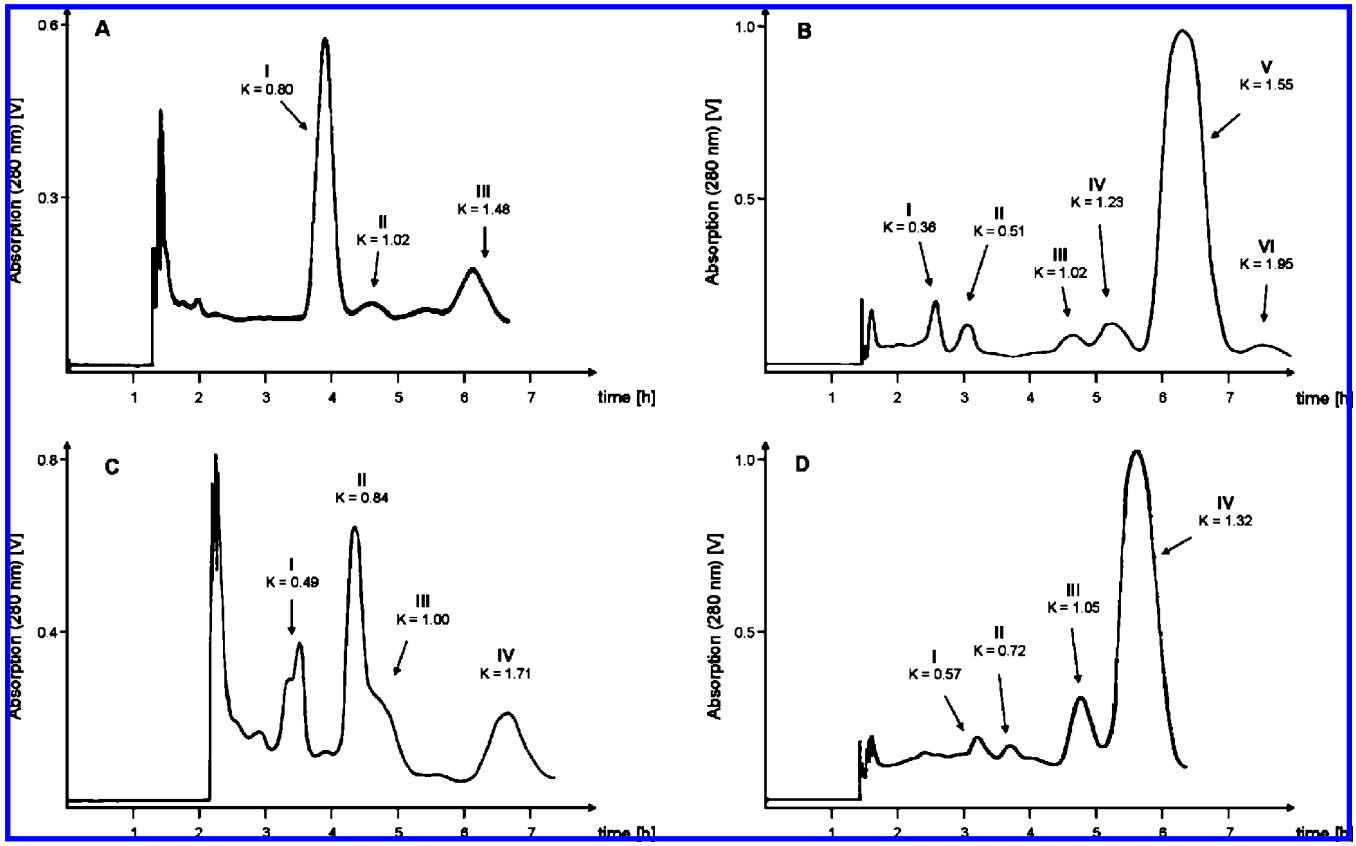

Figure 7. HSCCC separations of semisynthetic reaction mixtures after cleanup by solvent precipitation. (**A**) CCC-5: separation of reaction products of OPC-40 and (+)-catechin cleaned up by solvent precipitation; solvent system, ethyl acetate/1-butanol/water (14:1:15, v/v/v); flow rate, 3.0 mL/min; 1.15 g sample load; 1000 rpm; R_s = 71.5%. (**B**) CCC-6: separation of the coil fractions of CCC-5; solvent system, hexane/ethyl acetate/methanol/water (1:10:1:10, v/v/v/v); flow rate, 3.0 mL/min; 980 mg sample load; 1000 rpm; R_s = 68.5%. (**C**) CCC-7: separation of reaction products of OPC-40 and (−)-epicatechin cleaned up by solvent precipitation; solvent system, ethyl acetate/2-propanol/water (20:1:20, v/v/v); flow rate, 2.9 mL/min; 1.0 g sample load; 1000 rpm; R_s = 55.4%. (**D**) CCC-8: separation of the coil fractions of CCC-7; solvent system, hexane/ethyl acetate/methanol/water (0.75:10:0.75:10, v/v/v/v); flow rate, 3.0 mL/min; 600 mg sample load; 1000 rpm; R_s = 68.9%; composition of fractions cf. text.

procyanidins selected for the synthesis. Consequently, the extract has a strong influence on the final procyanidin composition, and its previous analysis is essential.

Structural Elucidation of Isolated Compounds. Isolated compounds were purified by preparative HPLC before structural elucidation. Dimeric B type procyanidins **1**, **2**, **3**, **8**, **13**, and **17** as well as the trimeric procyanidin **11** were unequivocally identified by comparison of NMR, MS, and phloroglucinolysis with literature data (*20*). Galloylated dimeric procyanidins and two further trimeric procyanidins were identified by LC-MS, phloroglucinolysis, and CD analysis. Chalcane flavan-3-ols were characterized by LC-MS, one- and two-dimensional NMR, and CD spectroscopy.

Galloylated Dimeric Procyanidins. LC-MS analysis shows pseudo molecular ions $[M - H]^-$ at *m/z* 729 and characteristic fragmentations at *m/z* 603, 577, 451, 425, 407, 289, 287, and 245. The phloroglucinolysis has been unequivocally shown to be useful for the determination of monomeric flavan-3-ol composition and allocation of interflavanoid linkages under gentle conditions (*20*). 4→8 bonds were nearly completely cleaved (85−90%) in dimeric procyanidins, whereas 4→6 bonds are cleaved at half the rate (45−50%). This method can also be applied for the determination of interflavanoid bonds in galloylated dimeric procyanidins. Four different galloylated procyanidin dimers were isolated by preparative HPLC. Compound **4** was obtained from fraction I of CCC-2, **14** from fraction IV of CCC-3, **7** from fraction II of CCC-2, and **15** (accompanied by **7**) from fraction I of CCC-4. The latter is not separable by preparative HPLC due to coelutions. The flavan-3-ol composition was determined by complete phloroglucinolysis (method B).

Compound **4**revealed (−)-epicatechin-3-*O*-gallate-4β→2-phloroglucinol and (+)-catechin as cleavage products indicating (−)-epicatechin-3-*O*-gallate in the upper and (+)-catechin in the lower unit. Compound **14** and **15** carry (−)-epicatechin-3-*O*-gallate in the upper and (−)-epicatechin in the lower position, due to the cleavage products (−)-epicatechin-3-*O*-gallate-4β→2-phloroglucinol and (−)-epicatechin. Compound **7** released (−)-epicatechin-4β→2-phloroglucinol and (−)-epicatechin-3-*O*-gallate and proved that (−)-epicatechin is located in the upper unit and (−)-epicatechin-3-*O*-gallate in the lower unit. The interflavanoid bond was determined from a gentle phloroglucinolysis (method A). To interpret the degradation rate of **15**, the degradation products of **7** needed to be taken into account. We obtained cleavage rates of 60% for **7**, 25% for **4** and **14**, and 5% for **15**. Cleavage was apparently retarded by galloylation. Thus, the cleavage rate is influenced by the position of galloylation as well as the interflavanoid bond. Compounds **14** and **15** exhibit the same flavan-3-ol composition and differ only in the interflavanoid bond. According to the restricted cleavage of 4→6 bonds, compound **15** must contain a 4→6 interflavanoid linkage. Compound **14** shows a five times faster cleavage rate; therefore, a 4→8 interflavanoid linkage can be assumed. Consequently, the two compounds are (−)-epicatechin-3-*O*-gallate-4β→8-(−)-epicatechin (**14**) and (−)-epicatechin-3-*O*-gallate-4β→6-(−)-epicatechin (**15**). Compound **4** showed the

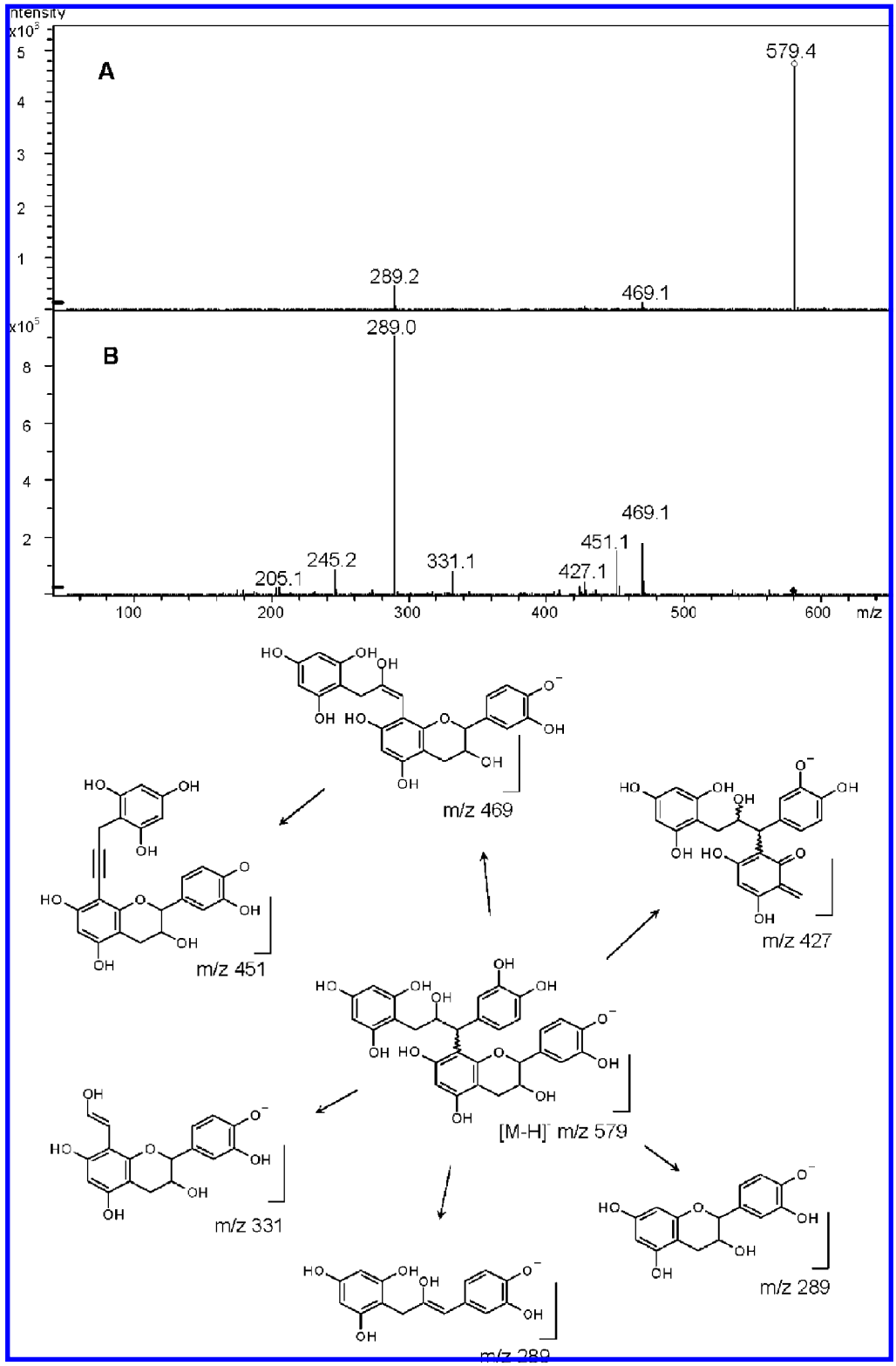

Figure 8. Mass spectra of chalcane flavan-3-ol adducts, MS^1 (**A**) and MS^2 (**B**), and postulated fragmentation of chalcane flavan-3-ol adducts by ESI-MS^n.

same cleavage rate as **14**. The only difference is the absolute configuration at the C3 position of the lower unit. According to the results for **14** and **15**, **4** has a 4→8 linkage and is therefore (−)-epicatechin-3-*O*-gallate-4β→8-(+)-catechin. The reaction rate of **7** is exactly between the rate of 4→8- and 4→6-connected dimeric procyanidins (without any galloylation). Taking this and the galloylation into consideration, compound **7** most likely has a 4→8 linkage and is therefore assigned as (−)-epicatechin-4β→8-(−)-epicatechin-3-*O*-gallate. Generally, galloylations in the upper unit influence the cleavage rate more strongly than in the lower unit. The reason for this can be seen in the hindered attack of the catalytic acid at the interflavanoid bond, which is sterically protected in the case of galloylation in the upper unit.

The 3,4-*trans* configuration was assigned for all compounds by CD experiments, which show positive Cotton effects at 216 nm. Furthermore, sterically controlled formation for the semi-synthetic pathway was demonstrated (*31, 35*).

Two more galloylated dimeric procyanidins were assigned in the LC-MS analyses of the reaction mixtures. For the semisynthetic formation carried out with (+)-catechin, two major galloylated dimeric procyanidins are produced, that is, (−)-epicatechin-3-*O*-gallate-4β→8-(+)-catechin (**4**), which elutes in the HPLC analysis at 37.7 min, and another dimer, which elutes at 44.7 min (**Figure 5B**). The latter remained on the coil in CCC-2 and CCC-6. It is assumed that the compound is (−)-epicatechin-3-*O*-gallate-4β→6-(+)-catechin (**20**), the structural isomer of (−)-epicatechin-3-*O*-gallate-4β→8-(+)-catechin (**4**). For the semisynthesis carried out with (−)-epicatechin, three galloylated dimeric procyanidins, that is, (−)-epicatechin-3-*O*-gallate-4β→8-(−)-epicatechin (**14**), (−)-epicatechin-4β→8-(−)-epicatechin-3-*O*-gallate (**7**), and (−)-epicatechin-3-*O*-gallate-4β→6-(−)-epicatechin (**15**) were isolated and identified (**Figure 5C**), while the fourth remains on the coil in CCC-6 and CCC-8. It is assumed that this compound, which elutes in the HPLC run at 46.8 min, is (−)-epicatechin-4β→6-(−)-epicatechin-3-*O*-gallate (**22**), the missing isomer that completes the homologous series of galloylated dimers, consisting only of flavan-3-ols with 2,3-*cis* configuration [(−)-epicatechin and (−)-epicatechin-3-*O*-gallate].

Trimeric Procyanidins. LC-MS analysis showed characteristic pseudo molecular ions $[M - H]^-$ at *m/z* 865 for trimeric procyanidins with fragment ions at *m/z* 847, 739, 713, 577, 575, 451, 425, 407, 289, and 245. Phloroglucinolysis (method B) indicated two equivalents of (−)-epicatechin-4β→2-phloroglucinol and one equivalent of (+)-catechin for **6**, with a backbone of (−)-epicatechin-(−)-epicatechin-(+)-catechin. Mild phloroglucinolysis (method A) revealed only 30% monomeric cleavage products. Furthermore, 15% **8** and 8% of a compound with a pseudo molecular ion $[M - H]^-$ at *m/z* 701 were found, which was proposed to be B5-4β→2-phloroglucinol. This indicates a molecule containing two 4→6 bonds. Consequently, **6** was identified as (−)-epicatechin-4β→6-(−)-epicatechin-4β→6-(+)-catechin.

Compound **12** contained 2 equiv of (−)-epicatechin-4β→2-phloroglucinol and 1 equiv of (−)-epicatechin from phloroglucinolysis (method B). Mild phloroglucinolysis (method A) revealed 14% nonreacted trimeric procyanidin, 11% of **15** and 8% B5-phloroglucinol as cleavage products. This indicated a 4→6 bond between the upper and the middle flavan-3-ol units and a 4→8 bond between the middle and the lower units. Consequently, compound **12** is (−)-epicatechin-4β→6-(−)-epicatechin-4β→8-(−)-epicatechin.

Byproducts, Chalcane Flavan-3-ols (Gambiriins). LC-MS experiments showed pseudo molecular ions at *m/z* 579, two mass units higher than dimeric procyanidins. According to the published fragmentation of procyanidins (*36–39*) and our experiments, we have postulated the ionization and fragmentation pattern, which enables the identification of chalcane flavan-3-ols by electrospray LC-MS (**Figure 8**).

Structural elucidation was carried out by ^{1}H, ^{13}C, two-dimensional homonuclear (^{1}H−^{1}H COSY) and heteronuclear (HMQC, HMBC) NMR experiments. The assignments of the chemical shifts are summarized in the section below.

The chalcane flavan-3-ols were formed due to protonation and ring opening of the flavan-3-ols followed by further reaction of the flavan-3-ols with the positively charged intermediate carbocations. No isomerization at position 3 of the original flavan-3-ol (later position 2 of chalcane) occurs. Theoretically, four stereoisomers for each reactant can be expected as the chalcane carbocation can attack both of the two electrophilic positions of the flavan-3-ol (C6t and C8t). The orientation of the attached flavan-3-ol may occur in either a *cis* or a *trans* position (α and β orientation). The reaction of the single flavan-3-ol under acid conditions exhibited formation of only two compounds. One of them is 1→8-linked (major product), whereas the other exhibits a 1→6 linkage (minor product). Therefore, we expect a stereoselective formation that is influenced by the hydroxyl group in position 2 of the upper chalcane unit and by steric properties of the aromatic substituents.

Initial data for the chalcane flavan-3-ols (*33*) assumed a 1,2-*trans* orientation through retention of the configuration at C4u after thiolysis (*40*). This is only possible in a static molecule (bond between C2u and C3u). However, the same bond in the open chalcane (here C2u and C1u) can rotate; consequently, the impact of substituents has to be considered. Protons H1u and H2u show coupling constants between 2.8 and 4.3 Hz, indicating that the angle in Newman projection has to be around 60 or 180°. An angle of 180° is sterically hindered due to interactions of the phenolic substituents. Therefore, we assume an angle of 60° with absolute configurations of C1u and C2u being 1*R*,2*S* [for the reaction with (+)-catechin] and 1*S*,2*R* [for the reaction with (−)-epicatechin]. The hydroxyl at C2u and the flavan-3-ol at C1u are *cis* oriented. Most recently, on the basis of hetero- and homonuclear NMR data, the published structures for gambiriins (*33*) have been revised and are in line with our assumption about the absolute configuration (*32*).

A second gambiriin is formed as a byproduct in the semisynthesis with (−)-epicatechin. This compound remained on the coil of CCC-4 and CCC-8. It is assumed that it has a similar stereochemistry to that of gambiriin A4, but the C2 atom of the chalcane is connected to the C6 position of the flavan-3-ol. Therefore, this compound is assumed to be a novel gambiriin that is named gambiriin A5 (**21**).

ACKNOWLEDGMENT

We thank C. Kakoschke and B. Jaschok-Kentner for NMR measurements and W. Lörsch (Breko GmbH, Bremen, Germany) for providing the grape seed extract.

LITERATURE CITED

(1) Brossaud, F.; Cheynier, V.; Noble, A. C. Bitterness and astringency of grape and wine polyphenols. *Aust. J. Grape Wine Res.* **2001**, *7*, 33–39.

(2) Vidal, S.; Francis, L.; Noble, A.; Kwiatkowski, M.; Cheynier, V.; Waters, E. Taste and mouth-feel properties of different types of tannin-like polyphenolic compounds and anthocyanins in wine. *Anal. Chim. Acta* **2004**, *513*, 57–65.

(3) Xie, D.-Y.; Dixon, R. A. Proanthocyanidin biosynthesis—Still more questions than answers. *Phytochemistry* **2005**, *66*, 2127–2144.

(4) Rasmussen, S. E.; Frederiksen, H.; Krogholm, K. S.; Poulsen, L. Dietary proanthocyanidins: occurrence, dietary intake, bioavailability, and protection against cardiovascular disease. *Mol. Nutr. Food Res.* **2005**, *49*, 159–174.

(5) Santos-Buelga, C.; Scalbert, A. Proanthocyanidins and tannin-like compounds—Nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric.* **2000**, *80*, 1094–1117.

(6) de Pascual-Teresa, S.; Santos-Buelga, C.; Rivas-Gonzalo, J. C. Quantitative analysis of flavan-3-ols in spanish foodstuffs and beverages. *J. Agric. Food Chem.* **2000**, *48*, 5331–5337.

(7) Gu, L. W.; Kelm, M. A.; Hammerstone, J. F.; Beecher, G.; Holden, J.; Haytowitz, D.; Prior, R. L. Screening of foods containing proanthocyanidins and their structural characterization using LC-MS/MS and thiolytic degradation. *J. Agric. Food Chem.* **2003**, *51*, 7513–7521.

(8) Gu, L. W.; Kelm, M. A.; Hammerstone, J. F.; Beecher, G.; Holden, J.; Haytowitz, D.; Gebhardt, S.; Prior, R. L. Concentrations of proanthocyanidins in common foods and estimations of normal consumption. *J. Nutr.* **2004**, *134*, 613–617.

(9) Auger, C.; Al Awwadi, N.; Bornet, A.; Rouanet, J. M.; Gasc, F.; Cros, G.; Teissedre, P.-L. Catechins and procyanidins in mediterranean diets. *Food Res. Int.* **2004**, *37*, 233–245.

(10) Dixon, R. A.; Xie, D. Y.; Sharma, S. B. Proanthocyanidins—A final frontier in flavonoid research? *New Phytol.* **2005**, *165*, 9–28.

(11) Cos, P.; De Bruyne, T.; Hermans, N.; Apers, S.; Berghe, D. V.; Vlietinck, A. J. Proanthocyanidins in health care: Current and new trends. *Curr. Med. Chem.* **2003**, *11*, 1345–1359.

(12) Tomas-Barberan, F. A.; Cienfuegos-Jovellanos, E.; Marin, A.; Muguerza, B.; Gil-Izquierdo, A.; Cerda, B.; Zafrilla, P.; Morillas, J.; Mulero, J.; Ibarra, A.; Pasamar, M. A.; Ramon, D.; Espin, J. C. A new process to develop a cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans. *J. Agric. Food Chem.* **2007**, *55*, 3926–3935.

(13) Hirano, R.; Osakabe, N.; Iwamoto, T.; Matsumoto, A.; Natsume, M.; Takizawa, T.; Igarashi, O.; Itakura, H.; Kondo, K. Antioxidant effects of polyphenols in chocolate on low-density lipoprotein both in vitro and ex vivo. *J. Nutr. Sci. Vitaminol.* **2000**, *46*, 199–204.

(14) Baba, S.; Osakabe, N.; Natsume, M.; Yasuda, A.; Takizawa, T.; Nakamura, T.; Terao, J. Cocoa powder enhances the level of antioxidative activity in rat plasma. *Br. J. Nutr.* **2000**, *84*, 673–680.

(15) McMurrough, I.; Madigan, D.; Smyth, M. R. Semipreparative chromatographic procedure for the isolation of dimeric and trimeric proanthocyanidins from barley. *J. Agric. Food Chem.* **1996**, *44*, 1731–1735.

(16) Karchesy, J. J.; Hemingway, R. W.; Foo, Y. L.; Barofsky, E.; Barofsky, D. F. Sequencing procyanidin oligomers by fast atom bombardment mass spectrometry. *Anal. Chem.* **1986**, *58*, 2563–2567.

(17) Gabetta, B.; Fuzzati, N.; Griffini, A.; Lolla, E.; Pace, R.; Ruffilli, T.; Peterlongo, F. Characterization of proanthocyanidins from grape seeds. *Fitoterapia* **2000**, *71*, 162–175.

(18) Sun, B. S.; Belchior, G. P.; Ricardo da Silva, J. M.; Spranger, M. I. Isolation and purification of dimeric and trimeric procyanidins from grape seeds. *J. Chromatogr. A* **1999**, *841*, 115–121.

(19) Sun, B.; Leandro, C.; Ricardo da Silva, J. M.; Spranger, I. Separation of grape and wine proanthocyanidins according to their degree of polymerization. *J. Agric. Food Chem.* **1998**, *46*, 1390–1396.

(20) Köhler, N.; Wray, V.; Winterhalter, P. Preparative isolation of procyanidins from grape seed extracts by high-speed counter-current chromatography. *J. Chromatogr. A* **2008**, *1177*, 114–125.

(21) Steynberg, P. J.; Nel, R. J. J.; Van Rensburg, H.; Bezuidenhoudt, B. C. B.; Ferreira, D. Oligomeric flavanoids. Part 27. Interflavanyl bond formation in procyanidins under neutral conditions. *Tetrahedron* **1998**, *54*, 8153–8158.

(22) Foo, L. Y.; Hemingway, R. W. Condensed tannins—Synthesis of the 1st branched procyanidin trimer. *J. Chem. Soc. Chem. Commun.* **1984**, 85–86.

(23) Botha, J. J.; Ferreira, D.; Roux, D. G. Synthesis of condensed tannins. Part 4. A direct biomimetic approach to [4.6]- and [4.8]-biflavanoids. *J. Chem. Soc. Perkin Trans. 1* **1981**, 1235–1245.

(24) Delcour, J. A.; Serneels, E. J.; Ferreira, D.; Roux, D. G. Synthesis of condensed tannins. 13. The 1st 2,3-*trans*-3,4-*cis*-procyanidins—Sequence of units in a trimer of mixed stereochemistry. *J. Chem. Soc. Perkin Trans. 1* **1985**, 669–676.

(25) Stafford, H. A.; Lester, H. H.; Porter, L. J. Chemical and enzymatic-synthesis of monomeric procyanidins (leucocyanidins or 3‴,4‴,5,7-tetrahydroxyflavan-3,4-diols) from (2*R*,3*R*) -dihydroquercetin. *Phytochemistry* **1985**, *24*, 333–338.

(26) Foo, L. Y.; Porter, L. J. Synthesis and conformation of procyanidin diastereoisomers. *J. Chem. Soc. Perkin Trans. 1* **1983**, 1535–1543.

(27) Köhler, N.; Winterhalter, P. Large-scale isolation of flavan-3-ol phloroglucinol adducts by high-speed counter-current chromatography. *J. Chromatogr. A* **2005**, *1072*, 217–222.

(28) Matthews, S.; Mila, I.; Scalbert, A.; Pollet, B.; Lapierre, C.; Hervé du Penhoat, C. L. M.; Rolando, C.; Donnelly, D. M. X. Method for estimation of proanthocyanidins based on their acid depolymerization in the presence of nucleophiles. *J. Agric. Food Chem.* **1997**, *45*, 1195–1201.

(29) Guyot, S.; Marnet, N.; Laraba, D.; Sanoner, P.; Drilleau, J. F. Reversed-phase HPLC following thiolysis for quantitative estimation and characterization of the four main classes of phenolic compounds in different tissue zones of a french cider apple variety (*Malus Domestica* Var. Kermerrien). *J. Agric. Food Chem.* **1998**, *46*, 1698–1705.

(30) Guyot, S.; Marnet, N.; Drilleau, J. F. Thiolysis-HPLC characterization of apple procyanidins covering a large range of polymerization states. *J. Agric. Food Chem.* **2001**, *49*, 14–20.

(31) Kennedy, J. A.; Jones, G. P. Analysis of proanthocyanidin cleavage products following acid-catalysis in the presence of excess phloroglucinol. *J. Agric. Food Chem.* **2001**, *49*, 1740–1746.

(32) Taniguchi, S.; Kuroda, K.; Doi, K. I.; Tanabe, M.; Shibata, T.; Yoshida, T.; Hatano, T. Revised structures of gambiriins A1, A2, B1, and B2, chalcane-flavan dimers from gambir (*uncaria gambir* extract). *Chem. Pharm. Bull.* **2007**, *55*, 268–272.

(33) Nonaka, G.-I.; Nishioka, I. Novel biflavonoids, chalcan-flavan dimers from Gambir. *Chem. Pharm. Bull.* **1980**, *28*, 3145–3149.

(34) Conway, W. D. *Countercurrent Chromatography: Apparatus, Theory and Applications*; VCH Publishers, Inc.: New York, NY, 1990.

(35) Fletcher, A. C.; Porter, L. J.; Haslam, E.; Gupta, R. K. Plant proanthocyanidins. Part 3. Conformational and configurational studies of natural procyanidins. *J. Chem. Soc. Perkin Trans. 1* **1977**, 1628–1637.

(36) Friedrich, W.; Eberhardt, A.; Galensa, R. Investigation of proanthocyanidins by HPLC with electrospray ionization mass spectrometry. *Eur. Food Res. Technol.* **2000**, *211*, 56–64.

(37) Peng, Z. K.; Hayasaka, Y.; Iland, P. G.; Sefton, M.; Hoj, P.; Waters, E. J. Quantitative analysis of polymeric procyanidins (tannins) from grape (*Vitis vinifera*) seeds by reverse phase high performance liquid chromatography. *J. Agric. Food Chem.* **2001**, *49*, 26–31.

(38) Zhu, Q. Y.; Hammerstone, J. F.; Lazarus, S. A.; Schmitz, H. H.; Keen, C. L. Stabilizing effect of ascorbic acid on flavan-3-ols and dimeric procyanidins from cocoa. *J. Agric. Food Chem.* **2003**, *51*, 828–833.

(39) Hayasaka, Y.; Waters, E. J.; Cheynier, V.; Herderich, M. J.; Vidal, S. Characterization of proanthocyanidins in grape seeds using electrospray mass spectrometry. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 9–16.

(40) Thompson, R. S.; Jacques, D.; Haslam, E.; Tanner, R. J. N. Plant proanthocyanidins. 1. Introduction—Isolation, structure, and distribution in nature of plant procyanidins. *J. Chem. Soc. Perkin Trans. 1* **1972**, 1387–1399.

Received for review December 15, 2007. Revised manuscript received April 1, 2008. Accepted April 3, 2008. We thank the Bundesministerium für Bildung and Forschung (BMBF) for funding part of this work (Förderkennzeichen: 0313828C).

JF7036505