Natural Products Chemistry & Research

Mori et al., Nat Prod Chem Res 2015, 3:3
DOI: 10.4172/2329-6836.1000172

Research Article Open Access

Role of 2,3-*cis* Structure of (–) -Epicatechin-3,5-*O*-digallate in Inhibition of HeLa S3 Cell Proliferation

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Abstract

Flavan-3-ol, which is primarily found in tea, is able to inhibit the proliferation of the human cancer cell line HeLa S3; in this study, we investigate the importance of the 2,3-cis structure in this inhibition. We synthesized six (−)-epicatechin and (+)-catechin analogs modified with a galloyl moiety at either the 3-hydroxyl, 5-hydroxyl, or 3,5-dihydroxyl positions. We then investigated their biological activity, DPPH radical scavenging activity and inhibitory activity on HeLa S3 cell proliferation. Among the six compounds, (−)-epicatechin-3,5-*O*-digallate showed the strongest inhibitory activity on HeLa S3 cell proliferation, whereas (+)-catechin-3,5-*O*-digallate was not active. In addition, there is no relation among the cell proliferation inhibitory activity and DPPH radical scavenging activity. Furthermore non-specific BSA binding ability of synthesized compounds was demonstrated. Improved photoaffinity beads method revealed that there is no difference between (−)-epicatechin-3,5-*O*-digallate and (+)-catechin-3,5-*O*-digallate on the non-specific BSA absorption. These data indicated that the 2,3-cis structure of flavan-3-ol is essential for the inhibition of HeLa S3 cell proliferation.

Keywords: Condensed tannins; Oligomeric flavonoid; Synthesis; Cancer cells proliferation; Inhibitory activity

Abbreviations

DPPH: 2,2-diphenyl-1-picrylhydrazyl; BSA: Bovine Serum Albumin; TBS: *tert*-butyldimethylsilyl; DCC: dicyclohexylcarbodiimide; TBAF: Tetra-*n*-butylammonium Fluoride; EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; THF: Tetrahydrofuran; DMF: *N,N*-dimethylformamide; TFA: Trifluoroacetic Acid; DMAP: *N,N*-dimethyl-4-aminopyridine

Introduction

There is currently great interest in the investigation of compounds from food sources that have strong biological activities; as these compounds are generally considered highly safe if they are already part of the diet. Polyphenols are thought to have various health benefits; and as such; are found in many health foods; as well as vegetables and fruits [1,2]. Furthermore; the investigation of polyphenol compounds is now increasingly important because of their various beneficial biological activities. However; the Structure–Activity Relationship (SAR) of polyphenols is not well understood because they are obtained as a mixture of various analogs in many cases; which makes purification difficult. Therefore; we have developed a simple; versatile; stereoselective and length-controlled synthetic method for various polyphenols containing with (–)-epicatechin (**1**) and (+)-catechin (**2**) analogs (**Figure 1**). We have also demonstrated that the galloyl modification of the hydroxyl groups of flavan-3-ols can enhance their biological activity [3-6].

Binding of polyphenols with proteins is often adopted for determination of the concentration of polyphenols to evaluate astringency of food products [7]; based on non-specific protein binding of polyphenols. This non-specific protein binding is commonly considered to be one of the factors of poor-bioavailability of polyphenol compounds [8,9]. Considerable research; however; indicates that the interactions to proteins are not so non-specific recently. Therefore we are interested in the non-specific binding of our polyphenol compounds to proteins such as serum albumin. Especially; BSA (bovine serum albumin) is abundant as the constituent of the culture medium in the assays using mammalian cells.

Here; we describe the development of a regioselective deprotection of *tert*-butyldimethylsilyl (TBS) protected flavan-3-ols; allowing for modification of the 5-position with various moieties; such as the galloyl group and the SAR studies of 3- or 5-*O*-galloyl-modified (–)-epicatechin (**1**) and (+)-catechin (**2**); as well as the DPPH radical scavenging and inhibitory activities toward HeLa S3 cell proliferation. We also investigated the nonspecific protein binding with synthesized polyphenols using improved photoaffinity beads.

Materials and Methods

General

All commercially available chemicals were used without further purification. All reactions were performed under an argon atmosphere and monitored using thin-layer chromatography (TLC) with 0.25 mm pre coated silica-gel plates (Merck 60F254 Art 5715). An ATAGO AP-300 spectrometer was used to measure optical rotation. ^{1}H-NMR spectra were recorded on an Agilent Inova 500 Spectrometer (500 MHz) and an Agilent DD2 NMR Spectrometer (400 MHz). A JEOL JMS-AX500 mass spectrometer was used to acquire fast atom bombardment (FAB) mass spectra. A Bruker Daltonics micrOTOF focus mass spectrometer was used to acquire electrospray ionization (ESI). The human cervical adenocarcinoma cell line; HeLa S3; were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT; Tsukuba; Japan. Synthesized compounds were dissolved in dimethyl sulfoxide

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Received March 07, 2015; **Accepted** March 26, 2015; **Published** March 28, 2015

Citation: Mori K, Ayano Y, Hamada Y, Hojima T, Tanaka R, et al. (2015) Role of 2,3-cis Structure of (-)-Epicatechin-3,5-O-digallate in Inhibition of HeLa S3 Cell Proliferation. Nat Prod Chem Res 3: 172. doi:10.4172/2329-6836.1000172

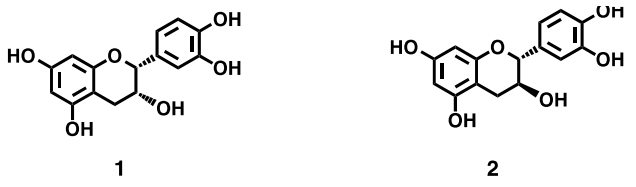


Figure 1: Structure of (−)-epicatechin (1) and (+)-catechin (2).

(DMSO) and stored at −25°C. HPLC purification was conducted on an Ascentis® column (SUPELCO® analytical; Sigma Aldrich Co. USA; 250 × 21.5 mm; 5 µm) using 0.05% HCOOH in CH_3CN as solvent A and 0.05% HCOOH and 10% CH_3CN in H_2O as solvent B. A linear gradient of 20%–100% solvent A in B over 20 min (flow rate; 4.0 ml/min) was used for elution.

Synthesis

3',4',7-Tri-*O*-TBS-(−)-epicatechin (9): To a solution of 5,7,3',4'-tetra-*O*-TBS-(−)-epicatechin (**7**) (440 mg; 0.59 mmol) in CH_2Cl_2 (25 ml); TFA (280 µl; 2.01 mmol) was added dropwise at 0°C to RT. After stirring for 2 h; the pale yellow reaction mixture was quenched with sat. $NaHCO_3$. The aqueous solution was extracted with $CHCl_3$ and the organic phase was washed with water and brine and then dried ($MgSO_4$). Filtration; concentration and silica-gel column purification (hexane/EtOAc; 9:1 to 2:1) afforded 400 mg (0.63 mmol; 84%) of **9** as an amorphous solid. $[\alpha]_D^{24}$ = −58.3 (*c* 0.26; $CHCl_3$); ^{1}H-NMR (400 MHz; $CDCl_3$) 6.97 (1H; s); 6.94 (1H; br s); 6.86 (1H; d; *J* = 8.3 Hz); 6.09 (1H; s); 5.95 (1H; s); 5.22 (1H; br); 4.91 (1H; s); 4.25 (1H; br s); 2.91–2.82 (2H; m); 1.82 (1H; br); 0.990 (9H; s); 0.989 (9H; s); 0.96 (9H; s); 0.20–1.86 (9H; m); ^{13}C-NMR (100 MHz; $CDCl_3$) 155.5; 155.3; 155.0; 147.0; 146.8; 131.0; 121.1; 119.30; 119.28; 100.9; 100.6; 99.7; 78.1; 66.2; 27.5; 25.93; 25.91; 25.6; 18.45; 17.43; 18.1; -4.092; -4.097; -4.11; -4.12; -4.43; -4.45; ESIMS (*m/z*) 633 ([M+H]$^+$; 100); 655 ([M+Na]$^+$; 14);ESIHRMS: m/z (M$^+$+H) Calculated for $C_{33}H_{57}O_6Si_3$; 633.3463 Found; 633.3458.

3',4',7-Tri-*O*-TBS-(+)-catechin (10): To a solution of 5,7,3',4'-tetra-*O*-TBDMS-(+)-catechin (**8**) (107 mg; 0.14 mmol) in CH_2Cl_2 (50 ml) was added dropwise TFA (19 µL; 0.17 mmol) at 0°C. After stirring for 5 h; the pale yellow reaction mixture was quenched with sat. $NaHCO_3$. The aqueous solution was extracted with $CHCl_3$ and the organic phase was washed with water and brine and dried (Na_2SO_4). Filtration; concentration and silica-gel column purification (hexane/EtOAc; 10:1 to 2:1) afforded a 82 mg (0.13 mmol; 90%) of **10** as an amorphous solid. $[\alpha]_D^{24}$ = +87.6 (*c* 1.14; $CHCl_3$); ^{1}H-NMR (500 MHz; $CDCl_3$) 6.93 (1H; br); 6.89 (2H; br); 6.15 (1H; d; *J* = 2.5 Hz); 6.00 (1H; d; *J* = 2.5 Hz); 5.61 (1H; br; OH); 4.73 (1H; d; *J* = 7.0 Hz); 4.08 (1H; ddd; *J* = 5.5; 7.0; 8.5 Hz); 2.96 (1H; dd; *J* = 5.5; 16.0 Hz); 2.66 (1H; dd; *J* = 8.5; 16.0 Hz); 2.09 (1H; br); 1.03 (9H; s); 1.00 (9H; s); 0.99 (9H; s); 0.25 (3H; s); 0.24 (3H; s); 0.22 (6H; s); 0.21 (3H; s); 0.20 (3H; s). ^{13}C-NMR (125 MHz; $CDCl_3$) 155.60; 155.58; 155.1; 147.5; 147.4; 131.1; 121.5; 120.3; 120.0; 101.1; 100.85; 100.79; 81.3; 68.5; 26.9; FABMS (m/z) 634 (20); 633 ([M+H]$^+$; 43); 632 (27); 368 (32); 367 (100); HRFABMS: m/z Calculated for $C_{33}H_{57}O_6Si_3$; 633.3463 Found; 633.3447.

(−)-Epicatechin-3,5-di-*O*-(tri-*O*-benzyl)gallate (12): To a solution of **9** (1.18 g; 1.87 mmol) and (tri-*O*-benzyl)gallic acid (1.99 g; 4.66 mmol) was added DCC (0.96 g; 4.66 mmol) and DMAP (22 mg; 0.18 mmol) in CH_2Cl_2 (75 ml) at 0°C. After stirring for 3 days; the reaction mixture was quenched with water. The aqueous solution was extracted with $CHCl_3$ and the organic phase was washed with water and brine and then dried ($MgSO_4$). Filtration; concentration and silica gel column purification ($CHCl_3$/EtOAc; 200:1 to 2:1 followed by hexane/EtOAc; 10:1 to 2:1) afforded crude **11** as an amorphous solid. A solution of crude **11** in THF (20 ml) was added dropwise to TBAF (3.46 ml; 3.46 mmol; 1M solution in THF) in the presence of AcOH (0.20 ml; 3.46 mmol) at 0°C. Concentration and a short silica-gel column (hexane/EtOAc; 7:1 to 1:5) afforded 413 mg (0.37 mmol; 2 steps 18%) of **12** as an amorphous solid. Data for **11**: [α]24 *D* = −45.9 (*c* 0.13; $CHCl_3$); ^{1}H-NMR (400 MHz; $CDCl_3$) 7.50–7.21 (34H; m); 6.87 (1H; d; *J* = 2.0 Hz); 6.85 (1H; dd; *J* = 2.0; 8.2 Hz); 6.72 (1H; d; *J* = 8.2 Hz); 5.58 (1H; br s); 5.16 (6H; s); 5.12 (1H; s); 5.16 (2H; s); 5.58 (4H; s); 3.02 (1H; dd; *J* = 15.1 Hz); 2.87 (1H; d; *J* = 4.3; 15.1 Hz); 0.98 (9H; s); 0.94 (9H; s); 0.91 (9H; s); 0.23 (6H; s); 0.15 (3H; s); 0.14 (3H; s); 0.09 (3H; s); 0.07 (3H; s); ^{13}C-NMR (100 MHz; $CDCl_3$) 164.9; 163.8; 155.6; 155.2; 152.6; 152.3; 150.2; 146.74; 146.72; 143.1; 142.4; 137.4; 137.3; 136.50; 136.46; 130.5; 128.51; 128.50; 128.4; 128.18; 128.16; 128.01; 127.97; 127.95; 127.90; 127.52; 127.51; 127.50; 124.8; 123.9; 120.9; 119.7; 119.2; 109.7; 109.0; 107.4; 105.9; 105.5; 75.2; 75.1; 71.3; 70.9; 68.0; 60.3; 31.5; 25.9; 25.8; 25.6; 18.4; 18.3; 18.1; −4.1; −4.18; −4.26; −4.28; −4.4; −4.5; ESIMS (*m/z*) 1478 ([M+H]$^+$; 8.7); 1501 ([M+Na]$^+$; 64); ESIHRMS: m/z (M$^+$+H) Calculated for $C_{89}H_{101}O_{14}Si_3$; 1477.6499 Found 1477.6794; Data for **12**: $[\alpha]_D^{24}$ = −18.4 (*c* 0.22; CH_3Cl_3); ^{1}H-NMR (400 MHz; CD_3OD) 7.49–7.19 (34H; m); 6.87 (1H; br s); 6.87-6.67 (2H; m); 6.43 (1H; d; *J* = 2.4 Hz); 6.33 (1H; d; *J* = 2.4 Hz); 5.49 (1H; dd; *J* = 4.3; 4.8 H); 5.13-4.99 (13H; m); 2.96 (1H; dd; *J* = 4.8; 17.0 Hz); 2.76 (1H; dd; *J* = 4.3; 17.0 Hz); ^{13}C-NMR (100 MHz; $CDCl_3$) 164.9; 163.8; 155.6; 155.2; 152.6; 152.3; 150.2; 146.74; 146.72; 143.09; 142.41; 137.4; 137.3; 136.50; 136.46; 130.5; 128.51; 128.50; 128.49; 128.45; 128.18; 128.16; 128.01; 127.97; 127.95; 127.91; 127.52; 127.51; 127.50; 124.8; 123.9; 120.9; 119.7; 119.2; 109.7; 109.0; 107.4; 105.9; 105.5; 75.2; 75.1; 71.3; 70.9; 68.0; 60.3; 31.5; 25.87; 25.85; 25.83; 18.4; 18.3; 18.1; −4.1; −4.2; −4.25; −4.28; −4.45; −4.48; ESIMS (*m/z*) 1135 ([M+H]$^+$; 16.9); 1157 ([M+Na]$^+$; 100); ESIHRMS: m/z (M$^+$+H) Calculated. for $C_{89}H_{101}O_{14}Si_3$; 1157.3724 Found 1157.3722.

(−)-Epicatechin-3,5-di-*O*-gallate (3): A solution of **12** (413 mg; 0.37 mmol) in THF/MeOH/H_2O (20:1:1; 11 ml) was hydrogenated over 20% $Pd(OH)_2$/C (2 mg) for 12 h at RT. Filtration and concentration afforded a pale brown solid; which was purified using HPLC purification to give 128 mg of pure **3** (0.21 mmol; 59%) as a pale brown powder [10-12]. $[\alpha]_D^{24}$ = −27.2 (*c* 0.09; MeOH) {lit.[10] $[\alpha]_D^{23}$ = −5.4 (*c* 2.17; MeOH)}; ^{1}H-NMR (400 MHz; CD_3OD) 7.17 (2H; s); 6.93 (2H; s); 6.93 (1H; d; *J* = 1.9 Hz); 6.81 (1H; dd; *J* = 1.9; 8.2 Hz); 6.69 (1H; d; *J* = 8.2 Hz); 6.36 (1H; d; *J* = 2.4 Hz); 6.26 (1H; d; *J* = 2.4 Hz); 5.50 (1H; br s); 5.10 (1H; s); 3.02 (1H; dd; *J* = 4.4; 15.4 Hz); 2.77 (1H; d; *J* = 15.4 Hz); ^{13}C-NMR (100 MHz; CD_3OD) 174.4; 173.3; 165.1; 164.2; 158.9; 153.6; 153.2; 153.0; 152.9; 147.5; 146.8; 137.9; 128.1; 127.1; 126.3; 122.9; 122.0; 117.5; 117.1; 111.8; 110.7; 108.9; 85.7; 76.2; 34.1; ESIMS (*m/z*) 595 ([M+H]$^+$; 3); 617 ([M+Na]$^+$; 17); ESIHRMS Calculated for $C_{29}H_{22}O_{14}Na$; 617.0902; Found; 617.0914.

(+)-Catechin-3;5-di-*O*-(tri-*O*-benzyl)gallate (14): To a solution of **10** (681 g; 1.07 mmol) and (tri-*O*-benzyl)gallic acid (1.99 g; 4.64 mmol) was added DCC (962 mg; 4.66 mmol) and DMAP (23 mg; 0.19 mmol) in CH_2Cl_2 (75 ml) at 0°C. After stirring for 3 days; the reaction mixture was quenched with water. The aqueous solution was extracted with $CHCl_3$ and the organic phase was washed with water and brine and then dried ($MgSO_4$). Filtration; concentration and silica-gel column purification ($CHCl_3$/EtOAc; 200:1 and then hexane/EtOAc; 10:1 to 2:1) afforded crude **13** as an amorphous solid. A solution of crude **13** in

THF (20 ml) was added dropwise to TBAF (3.64 ml; 3.64 mmol; 1M solution in THF) in the presence of AcOH (0.20 ml; 3.64 mmol) at 0°C. Concentration and a short silica gel column (hexane/EtOAc; 7:1 to 1:5) afforded 762 mg (0.65 mmol; 2 step 60%) of **14** as an amorphous solid. Data for **13**: $[\alpha]_D^{24}$ −24.1 (*c* 0.13; $CHCl_3$); ^{1}H-NMR (400 MHz; $CDCl_3$) 7.84–7.50 (34H; m); 7.26 (1H; d; *J* = 1.7 Hz); 7.19–7.14 (2H; m); 6.85 (1H; d; *J* = 2.3 Hz); 6.78 (1H; d; *J* = 2.3 Hz); 5.81 (1H; ddd; *J* = 4.9; 6.0; 6.4 Hz); 5.57 (1H; d; *J* = 6.0 Hz); 5.49–5.37 (12H; m); 3.24 (1H; dd; *J* = 4.9; 16.6 Hz); 3.05 (1H; dd; *J* = 6.4; 16.6 Hz); 1.34 (9H;s); 1.32 (9H; s); 1.29 (9H; s); 0.59 (6H; s); 0.53 (3H; s); 0.52 (3H; s); 0.49 (3H; s); 0.47 (3H; s); ^{13}C-NMR (100 MHz; $CDCl_3$) 165.0; 163.7; 155.3; 155.0; 152.6; 152.3; 149.9; 147.1; 146.9; 143.0; 142.4; 137.3; 137.2; 136.5; 136.4; 130.8; 128.44; 128.73; 128.41; 128.40; 128.12; 128.09; 127.94; 127.91; 127.89; 127.85; 127.5; 127.4; 124.7; 123.9; 121.1; 119.6; 118.8; 109.5; 108.9; 107.3; 105.9; 105.6; 75.1; 75.0; 71.1; 70.9; 69.3; 34.8; 25.83; 25.81; 25.5; 18.4; 18.3; 18.1; −4.32; −4.49; −4.50; ESIMS (*m/z*) 1477.7 ([M+H]$^+$; 12); 1499.6 ([M+Na]$^+$; 81); ESIHRMS Calculated for $C_{89}H_{100}O_{14}Si_3Na$; 1499.6313; Found; 1499.6318; Data for **14**: $[\alpha]_D^{24}$ = +40.8 (*c* 0.42; $CHCl_3$) ^{1}H-NMR (400 MHz; CD_3OD) 7.50–7.17 (34H; m); 6.96 (1H; d; *J* = 1.6 Hz); 6.81 (1H; d; *J* = 8.2 Hz); 6.72 (1H; dd; *J* = 1.6; 8.2 Hz); 6.49 (1H; d; *J* = 2.3 Hz); 6.23 (1H; d; *J* = 2.3 Hz); 5.37–5.35 (1H; m); 5.30 (1H; d; *J* = 3.7 Hz); 5.13–5.03 (12H; m); 2.69 (1H; br d; *J* = 14.8 Hz); 2.63 (1H; br d; *J* = 14.8 Hz); ^{13}C-NMR (100 MHz; $CDCl_3$) 166.2; 165.4; 155.6; 155.1; 152.7; 152.4; 149.4; 144.5; 143.8; 142.6; 137.2; 137.0; 136.5; 136.2; 129.8; 128.53 (Cx3); 128.5; 128.22; 128.20; 128.11; 128.08; 128.01; 127.55 (Cx2); 127.54 (Cx2); 124.6; 122.7; 118.8; 115.2; 112.1; 110.0; 109.2; 105.1; 103.0; 101.8; 77.8; 75.2; 75.1; 71.4; 71.1; 69.0; 21.5; ESIMS (*m/z*) 1135 ([M+H]$^+$; 7.8); 1157 ([M+Na]$^+$; 100); ESIHRMS Calculated for $C_{71}H_{59}O_{14}$; 1135.3905; Found; 1135.3899.

(+)-Catechin-3,5-di-*O*-gallate (4): A solution of **14** (0.74 g; 0.66 mmol) in THF/MeOH/H_2O (20:1:1; 11 ml) was hydrogenated over 20% $Pd(OH)_2$/C (2 mg) for 12 h at RT. Filtration and concentration afforded a pale brown solid; which was purified using HPLC purification to give 193 mg of pure **4** (0.32 mmol; 50%) as a pale brown powder [13]. $[\alpha]_D^{24}$ = +16.8 (c 0.18; $CHCl_3$); {lit.[13] [α]20 *D* = +2.84 (*c* 0.5; MeOH)}; ^{1}H-NMR (400 MHz; CD_3OD) 7.16 (2H; s); 7.00 (2H; s); 6.83 (1H; d; *J* = 1.5 Hz); 6.74 (1H; d; *J* = 8.2 Hz); 6.72 (1H; dd; *J* = 1.5; 8.2 Hz); 6.36 (1H; d; *J* = 2.4 Hz); 6.27 (1H; d; *J* = 2.4 Hz); 5.38 (1H; ddd; *J* = 4.8; 5.3; 10.4 Hz); 5.19 (1H; d; *J* = 5.3 Hz); 2.75 (1H; dd; *J* = 4.8; 16.6 Hz); 2.66 (1H; dd; *J* = 5.44; 16.6 Hz); ^{13}C-NMR (100 MHz; CD_3OD) 174.4; 173.3; 165.3; 163.4; 158.7; 153.6; 153.35; 153.31; 153.27; 147.6; 146.9; 138.0; 128.1; 127.1; 125.9; 123.3; 121.1; 117.5; 117.1; 112.1; 110.6; 108.6; 86.3; 77.3; 30.9; ESIMS (*m/z*) 595 ([M+H]$^+$; 2); 617 ([M+Na]$^+$; 15); ESIHRMS Calculated for $C_{29}H_{23}O_{14}$; 595.1082; Found; 595.1059.

3',4',7-Tri-O-TBS-(−)-epicatechin-5-*O*-(tri-*O*-benzyl)gallate (15): To a solution of **9** (577 mg; 0.91 mmol) and (tri-*O*-benzyl)gallic acid (1.02 g; 2.40 mmol) was added EDC (372 mg; 2.40 mmol) and DMAP (30 mg; 0.25 mmol) in CH_2Cl_2 (75 ml) at 0°C. After stirring for 17 h; the reaction mixture was quenched with water. The aqueous solution was extracted with $CHCl_3$ and the organic phase was washed with water and brine then dried ($MgSO_4$). Filtration; concentration and silica gel column purification (hexane/EtOAc; 10:1 to 2:1) afforded 900 mg (0.90 mmol; 93%) of **15** as an amorphous solid. $[\alpha]_D^{24}$ = +10.1 (*c* 0.40; $CHCl_3$); ^{1}H-NMR (400 MHz; $CDCl_3$) 7.52–7.26 (17H; m); 6.98 (1H; d; *J* = 1.8 Hz); 6.95 (1H; dd; *J* = 1.8; 8.2 Hz); 6.88 (1H; d; *J* = 8.2 Hz); 6.43 (1H; d; *J* = 2.3 Hz); 6.37 (1H; d; *J* = 2.3 Hz); 5.16 (6H; s); 4.95 (1H; s); 4.18 (1H; br s); 2.85 (1H; dd; *J* = 4.0; 14.6 Hz); 2.77 (1H; d; *J* = 14.6 Hz); 1.77 (1H; br s); 1.00 (18H; s); 0.99 (9H; s); 0.23 (6H; s); 0.217 (6H; s); 0.216 (6H; s); ^{13}C-NMR (100 MHz; $CDCl_3$) 163.8; 155.2; 155.0; 152.5; 150.3; 146.9; 146.7; 142.9; 137.2; 136.4; 130.6; 128.43; 128.39; 128.08; 127.90; 127.86; 127.39; 124.0; 121.0; 119.14; 119.10; 109.5; 107.5; 105.9; 105.5; 78.1; 75.0; 71.1; 65.8; 27.9; 25.83; 25.79; 25.5; 18.33; 18.29; 18.0; −4.20; −4.21; −4.24; −4.57; −4.59; ESIMS (*m/z*) 1055 ([M+H]$^+$; 100); 1077 ([M+Na]$^+$; 45); ESIHRMS Calculated for $C_{61}H_{79}O_{10}Si_3$; 1055.4976; Found; 1055.5010.

3',4',7-Tri-O-TBS-(+)-catechin-5-*O*-(tri-*O*-benzyl)gallate (16): To a solution of **10** (836 mg; 1.32 mmol) and (tri-*O*-benzyl)gallic acid (909 mg; 2.06 mmol) was added EDC (333 mg; 2.14 mmol) and DMAP (22 mg; 0.18 mmol) in CH_2Cl_2 (75 ml) at 0°C. After stirring for 17 h; the reaction mixture was quenched with water. The aqueous solution was extracted with $CHCl_3$ and the organic phase was washed with water and brine and then dried ($MgSO_4$). Filtration; concentration and silica-gel column purification (hexane/EtOAc; 10:1 to 2:1) afforded 1.13 g (1.07 mmol; 62%) of **16** as an amorphous solid. $[\alpha]_D^{24}$ = +3.4 (*c* 0.30 $CHCl_3$); ^{1}H-NMR (400 MHz; $CDCl_3$) 7.53-7.26 (17H; m); 6.93 (1H; s); 6.88 (2H; s); 6.43 (1H; s); 6.39 (1H; s); 5.17 (2H; s); 5.15 (4H; s); 4.71 (1H; d; *J* = 6.7 Hz); 4.01 (1H; ddd; *J* = 5.4; 6.7; 8.5 Hz); 2.88 (1H; dd; *J* = 5.4; 16.1 Hz); 2.60 (1H; dd; *J* = 8.5; 16.1 Hz); 1.87 (1H; br); 1.02 (9H; s); 1.00 (9H; s); 0.99 (9H; s); 0.23 (12H; s); 0.21 (6H; s); ^{13}C-NMR (100 MHz; $CDCl_3$) 163.9; 155.3; 155.1; 152.6; 150.5; 147.0; 146.9; 143.0; 137.3; 136.5; 130.8; 128.54; 128.50; 128.2; 128.02; 127.98; 127.5; 124.1; 121.1; 119.25; 119.22; 109.6; 107.6; 106.0; 105.7; 78.2; 75.2; 71.2; 66.0; 28.0; 25.94; 25.91; 25.6; 18.5; 18.4; 18.1; −4.09; −4.10; −4.12; −4.45; −4.46; ESIMS (*m/z*) 1055 ([M+H]$^+$; 55); 1077 ([M+Na]$^+$; 51); ESIHRMS Calculated for $C_{61}H_{78}O_{10}Si_3Na$; 1077.4795; Found; 1077.4761.

(−)-Epicatechin-5-*O*-(tri-*O*-benzyl)gallate (17): A solution of **15** (900 mg; 0.85 mmol) in THF (20 ml) was added dropwise to TBAF (2.98 ml; 2.98 mmol; 1M solution in THF) in the presence of AcOH (0.17 ml; 2.98 mmol) at 0°C. Concentration and a short silica gel column purification (hexane/EtOAc; 7:1 to 1:5) afforded 350 mg (0.49 mmol; 57%) of **17** as an amorphous solid. $[\alpha]_D^{24}$ = +13.8 (*c* 0.22; MeOH); ^{1}H-NMR (400 MHz; CD_3OD) 7.53–7.24 (17H; m); 6.97 (1H; br s); 6.78–6.75 (2H; m); 6.32 (1H; br s); 6.26 (1H; br s); 5.18-5.09 (6H; m); 4.12 (1H; br s); 2.78 (1H; d; *J* = 16.6 Hz); 2.58 (1H; dd; *J* = 2.6; 16.6 Hz); ^{13}C-NMR (100 MHz; CD_3OD) 172.5; 164.9; 164.4; 160.9; 158.9; 152.9; 152.8; 150.9; 145.6; 145.0; 138.7; 136.6; 136.49; 136.48; 136.1; 136.02; 136.00; 135.7; 126.3; 122.8; 122.2; 117.3; 112.5; 110.4; 109.0; 87.0; 83.1; 79.1; 73.7; 36.5; ESIMS (*m/z*) 713 ([M+H]$^+$; 5); 735 ([M+Na]$^+$; 46); ESIHRMS Calculated for $C_{43}H_{37}O_{10}$; 713.2381; Found; 713.2398.

(−)-Epicatechin-5-*O*-gallate (5): A THF/MeOH/H_2O (20:1:1; 11 ml) solution of **17** (350 mg; 0.49 mmol) was hydrogenated over 20% $Pd(OH)_2$/C (2 mg) for 12 h at RT [12]. Filtration and concentration afforded a pale brown solid; which was purified using HPLC purification to give 145 mg of pure **5** (0.25 mmol; 52%) as a pale brown powder. [α]24 *D* = −126.3 (*c* 0.12; MeOH); {lit.[12] $[\alpha]_D^{23}$ = +16.4 (*c* 0.45; MeOH)}; ^{1}H-NMR (400 MHz; CD_3OD); 7.20 (2H; s); 6.97 (1H; d; *J*= 1.6 Hz); 6.79 (1H; dd; *J* = 1.6; 8.2 Hz); 6.75 (1H; d; *J* = 8.2 Hz); 6.31 (1H; d; *J*= 2.4 H); 6.23 (1H; d; *J* = 2.4 Hz); 5.01-4.80 (1H; m); 4.16 (1H; br s); 2.85 (1H; dd; *J* = 4.4; 16.6 Hz); 2.64 (1H; dd; *J* = 2.5; 16.6 Hz); ^{13}C-NMR (100 MHz; CD_3OD) 173.4; 164.8; 164.4; 159.1; 153.6; 152.9; 152.8; 147.5; 138.7; 127.3; 126.3; 122.8; 122.2; 117.5; 112.7; 110.5; 108.8; 87.0; 73.8; 36.5; ESIMS (*m/z*) 443 ([M+H]$^+$; 16); 465 ([M+Na]$^+$; 100); ESIHRMS Calculated for $C_{22}H_{18}O_{10}Na$; 465.0792; Found; 465.0770.

(+)-Catechin-5-*O*-(tri-*O*-benzyl)gallate (18): A solution of **16** (1.00 g; 0.95 mmol) in THF (20 ml) was added dropwise to TBAF (2.93 ml; 2.93 mmol; 1M solution in THF) in the presence of AcOH (0.17 ml; 2.93 mmol) at 0°C. Concentration and a short silica gel column (hexane/EtOAc; 7:1 to 1:5) afforded 275 mg (0.62 mmol; 65%) of **18** as an amorphous solid. $[\alpha]_D^{24}$ = +26.9 (*c* 0.15; MeOH); ^{1}H-NMR (400 MHz; CD_3OD) 7.50–7.19 (15H; m); 6.83 (1H; d; *J* = 2.0 Hz); 6.76 (1H;

d; *J* = 8.1 Hz); 6.69 (1H; dd; *J* = 2.0; 8.1 Hz); 6.28 (2H; s); 5.10 (4H; s); 5.05 (2H; s); 4.64 (1H; d; *J* = 7.2 Hz); 3.97 (1H; ddd; *J* = 5.3; 7.2; 8.0 Hz); 2.67 (1H; dd; *J* = 5.3; 16.0 Hz); 2.23 (1H; dd; *J* = 8.0; 16.0 Hz); ^{13}C-NMR (100 MHz; CD_3OD) 172.5; 165.0; 163.9; 160.9; 158.5; 153.3; 150.9; 145.6; 145.0; 138.7; 135.7; 136.00; 136.02; 136.1; 136.47; 136.48; 136.6; 132.4; 126.8; 123.1; 122.0; 117.3; 113.2; 110.4; 108.7; 89.9; 83.1; 79.1; 75.0; 35.3; ESIMS (*m/z*) 735 ([M+Na]$^+$; 57); ESIHRMS Calculated for $C_{43}H_{37}O_{10}$; 713.2387; Found; 713.2381.

(+)-Catechin-5-*O*-gallate (6): A solution of **20** (50 mg; 0.07 mmol) in THF/MeOH/H_2O (20:1:1; 11 ml) was hydrogenated over 20% $Pd(OH)_2$/C (2 mg) for 12 h at RT. Filtration and concentration afforded a pale brown solid; which was purified using HPLC purification to give 21 mg of pure **6** (0.04 mmol; 67%) as a pale brown powder [14,15]. $[\alpha]_D^{24}$ = +26.9 (*c* 0.15; MeOH) {lit.[14] $[\alpha]_D^{20}$ = +2.7 (*c* 3.0; acetone)}; ^{1}H-NMR (400 MHz; CD_3OD) 7.19 (2H; s); 6.83 (1H; d; *J* = 1.9 Hz); 6.76 (1H; d; *J* = 8.1 Hz); 6.71 (1H; dd; *J* = 1.9; 8.1 Hz); 6.25 (1H; d; *J* = 2.4 Hz); 6.22 (1H; d; *J* = 2.4 Hz); 4.64 (1H; d; *J* = 7.4 Hz); 3.99 (1H; ddd; *J* = 5.3; 7.4; 8.2 Hz); 2.75 (1H; dd; *J* = 5.3; 16.0 Hz); 2.49 (1H; dd; *J* = 8.2; 16.0 Hz); ^{13}C-NMR (100 MHz; CD_3OD) 173.4; 165.0; 163.9; 158.6; 153.6; 153.3; 153.2; 147.5; 138.7; 127.2; 126.9; 123.0; 122.1; 117.5; 113.4; 110.4; 108.5; 90.0; 75.1; 35.6; ESIMS (*m/z*) 443 ([M+H]$^+$; 13); 465 ([M+Na]$^+$; 94); ESIHRMS Calculated for $C_{22}H_{18}O_{10}Na$; 465.0792; Found; 465.0779.

Synthesis of the photoaffinity linker (21): NHS-LC-Biotin (Thermo Fisher Scientific Inc. IL; USA) (14 mg; 0.03 mmol) was added to a solution of photoaffinity amino linker [16] (10 mg; 0.02 mmol) in CH_2Cl_2 (1 ml) at RT. After stirring for 2 h; it was concentrated *in vacuo*. The residue was purified by silica-gel column chromatography ($CHCl_3$/MeOH = 10:1) to give photoaffinity linker **21** (7.0 mg; 0.01 mmol; 42%) as a pale yellow oil. ^{1}H-NMR (400 MHz; $CDCl_3$): 7.90 (1H; br); 7.88 (2H; d; *J* = 8.5 Hz); 7.41 (1H; br); 7.25 (2H; d; *J* = 8.5 Hz); 6.55 (1H; br); 5.98 (1H; br); 5.46 (1H; br); 4.93 (1H; br); 4.50 (1H; br); 4.32 (1H; br); 3.64–3.53 (15H; m); 3.53–3.51 (2H; m); 3.42–3.40 (2H; m); 3.24–3.23 (2H; m); 3.16–3.15 (1H; m); 3.94–2.90 (1H; m); 2.74–2.72 (1H; m); 2.19–2.16 (4H; m); 1.68–1.64 (11H; m); 1.50–1.48 (4H; m); 1.35–1.34 (2H;m); ^{13}C-NMR (100 MHz; $CDCl_3$) 173.2; 173.0; 166.3; 163.5; 135.6; 131.9; 127.7; 126.33; 126.32; 77.1; 70.5; 70.38; 70.37; 70.36 (Cx2); 70.34; 70.32; 70.0; 69.9; 69.8; 69.7; 61.7; 60.0; 55.4; 40.5; 39.9; 39.0; 36.0; 35.7; 28.9; 27.9; 27.8; 26.3; 25.4; 25.0; ESIMS (*m/z*) 832 ([M+H]$^+$; 52); 854 ([M+Na]$^+$; 100); ESIHRMS Calculated for $C_{37}H_{57}F_3N_7O_9S$; 832.3885; Found; 832.3907.

DPPH radical scavenging activity

DPPH radical scavenging activity was measured with following a general procedure [17]. A solution of DPPH radical in EtOH (30 μM; 1.0 ml) was added to 1 μL of each of the synthesized compounds in DMSO; and incubated at 30°C for 30 min. The scavenging activity was estimated with a microplate reader (Filter Max F5 multi-mode microplate reader; Molecular Devices; Downingtown; PA; USA) to measure the OD at 515 nm. Negative controls were prepared at the same time by adding 1 μL of DMSO to 1.0 ml of EtOH.

Inhibitory activity of cell proliferation

10^4 cells per well with 100 μL of medium in a 37°C incubator equilibrated under an atmosphere of 5% CO_2 and 95% humidified air. D-MEM (Dulbecco's Modified Eagle's Medium; Gibco® (Life technologies; Grand Island; NY; USA) supplemented with 5% fetal bovine serum and 1% Pen-Strep; Invitrogen™ (Life technologies; Grand Island; NY; USA) was used. After 24 h of incubation; 1 μL of the six synthesized compounds in DMSO were added and incubated for 48 h. A well containing only a medium added with DMSO were used as negative controls and were prepared at the same time. After the medium was removed and the cells were washed with PBS; 90 μL of the new medium and 10 μL of the MTT solution (3-(4;5-dimethylthiazol-2-yl)-2;5-diphenyltetrazolium bromide; 5 mg/ml) were added to each well and incubated at 37°C for 2.5 h. After incubation; the reaction medium was removed and 100 μL of DMSO was added to each well and mixed thoroughly with a pipette. Viable cells were then assessed using a microplate reader (Filter Max F5 multi-mode microplate reader; Molecular Devices; Downingtown; PA; USA) to measure the OD at 570 nm.

Detection of non-specific binding between polyphenols and BSA

A mixture of test sample (**3**; **4**; **5**; **6**; **19**; or **20**) (100 μL; 10 mM in MeOH) and photoaffinity linker **21** (5 μL; 10 mM in MeOH) was dried *in vacuo* and irradiated with 365-nm-UV light; using a CL-1000 L ultraviolet cross-linker (UVP Inc.; CA; USA). The irradiation energy amounted to 2.8 J cm^{-2}. The mixture was dissolved in DMSO (100 μL) and PBS (400 μL). A mixture of the DMSO solution (10 μL) and Streptavidin Agarose (40 μL; Novex; USA) was added to PBS (500 μL; 0.1% Triton X-100) and incubated for 12 h at 4°C. The beads were washed with PBS (800 μL; 0.5% Triton X-100; 5 times) and PBS (800 μL; 0.1% Triton X-100; once) and resuspended in PBS (500 μL; 0.1% Triton X-100). To the beads solution; a solution of BSA in H_2O (1 μL; 1 mM) was added and incubated for 12 h at 4°C. After removal of the supernatant; the beads were washed with PBS (800 μL; 0.1% Triton X-100; 4 times) and remained BSA on beads was separated by SDS-PAGE and followed silver staining of gel to give each BSA band. Determination of the band intensity was estimated by LI-COR; Image Studio Digits Ver 4.0.

Results and Discussion

Synthesis

Galloyl-modified flavan-3-ols can be isolated from various plants. Among them; (−)-epigallocatechin-3-*O*-gallate (EGCG) is the most well-known compound as it is a multi-functional small molecule extracted from green tea. In green tea; however; small amounts of various other galloyl-modified compounds are present as minor components; the health functions of which are unclear. Large amounts of each pure compound are required to elucidate their functions; however; the isolation and purification of these compounds for biological assays is difficult. Therefore; we have developed a simple; regioselective; and efficient synthesis of these compounds.

Figure 2 shows four galloyl-modified flavan-3-ols (**3**–**6**) synthesized using our regioselective deprotection approach. There have been no reports on the selective galloyl modification of the 5-position of flavan-3-ols; however; Mambu *et al.* reported the only semi-synthesis of (+)-catechin-5-*O*-gallate (**6**) and its derivative. They reported the antiplasmodial and cytotoxic activities of this compound against human diploid embryonic lung cell line MRC-5 [15]. The isolation of 3-; 5-; and 3,5-*O*-digallate derivatives of (−)-epicatechin (**1**) and (+)-catechin (**2**) and their biological activities have been described in several reports [10-14]. However; it is a rare case that (−)-epicatechin and (+)-catechin series compounds are present in the same plant; making SAR studies more difficult. Therefore; we synthesized galloyl analogs derived from (−)-epicatechin (**1**) and (+)-catechin (**2**); for elucidating their SAR.

Scheme 1 shows the synthesis of 3,5-*O*-digalloyl compounds **3** and **4**. The four phenolic hydroxyl groups of (−)-epicatechin (**1**) and (+)-catechin (**2**) were protected with TBS using a previously reported

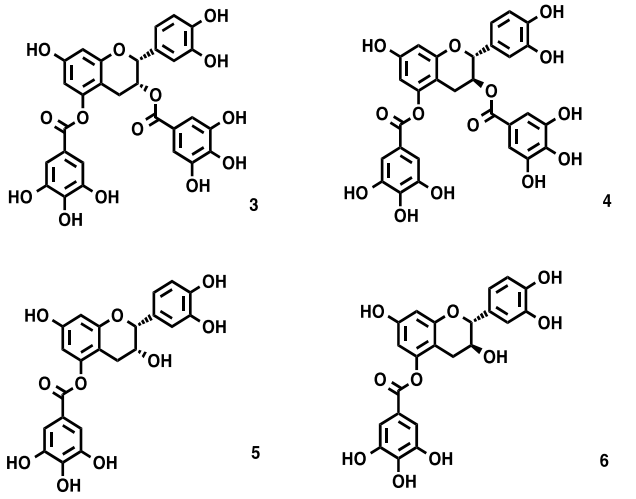
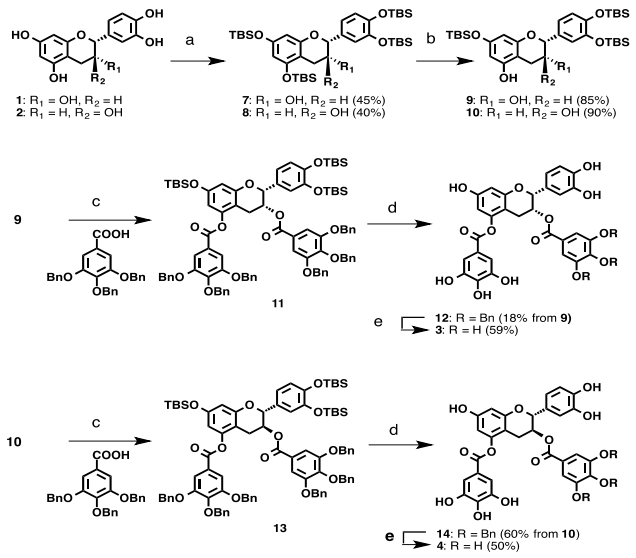


Figure 2: Structure of 5-O-galloyl flavan-3-ols.



Scheme 1: Synthesis of 3,5-*O*-digalloyl compounds 3 and 4.
Reagents and conditions: (a) imidazole, TBSCl, THF/DMF (1:1), (b) TFA, CH_2Cl_2, (c) DCC, DMAP, CH_2Cl_2, (d) TBAF, AcOH, THF, (e) $Pd(OH)_2/C$, H_2, THF/MeOH/H_2O (20:1:1).

procedure [18]. Various examinations of the reactive properties of protected flavan-3-ols led us to discover that the 5-*O*-TBS groups of **7** and **8** could be regioselectively removed with TFA to give **9** and **10** in 85% and 90% yield; respectively. The structure of the 5-OH products **9** and **10** were confirmed by the HMBC experiments. Esterification of dihydroxyl compounds **9** and **10** using benzyl-protected gallic acid and DCC proceeded smoothly to provide digalloyl compounds **11** and **13**. The TBS groups of **11** and **13** were then removed with TBAF in the presence of AcOH to afford **12** and **14** in 18% and 60% yields (over 2 steps); respectively. Hydrogenation of the benzyl groups; which protect the phenolic hydroxyl groups on the galloyl moiety; gave **3** and **4** in 59% and 50% yields; respectively.

5-*O*-Galloyl derivatives **5** and **6** were synthesized as shown in **Scheme 2**. 5-Hydroxyl compounds **9** and **10** were esterified with benzyl protected gallic acid using EDC as a condensation reagent to give **15** and **16** in 88% and 66% yields; respectively.

Deprotection of the TBS groups gave **17** and **18** in 57% and 65% yields; respectively. Subsequent hydrogenation afforded (−)-epicatechin-5-*O*-gallate (**5**) and (+)-catechin-5-*O*-gallate (**6**) in 52% and 67% yields; respectively. As the control compounds (−)-epicatechin-3-*O*-gallate (**19**) and (+)-catechin-3-*O*-gallate (**20**) were also synthesized (**Figure 3**) [3,4].

DPPH radical scavenging activity

Polyphenols are known as strong antioxidants and radical scavengers [17]. In previous papers [3-6]; we investigated the DPPH radical scavenging activity of synthesized procyanidin oligomers; and 3-*O*-galloyl dimers. In this study; we determined the SC_{50} values (the concentration at 50% scavenging activity) of compounds **3**; **4**; **5**; **6**; **19** and **20** to be 1.8; 2.6; 4.2; 2.3; 2.7; and 5.2 μM; respectively. From these SC_{50} values and **Figure 4**; it appears that the synthesized galloyl-modified flavan-3-ols have significant radical scavenging activity; however; this is not affected by the number of galloyl moieties present on the molecule; contrary to our expectations. In addition; the 2;3-structure of flavan-3-ols appears to have minimal impact on the scavenging activity.

Cervical epithelioid carcinoma cell line; HeLa S3; proliferation inhibitory activity

The inhibitory activity of the synthetic galloyl-modified flavan-3-ols against HeLa S3 cell proliferation is shown in **Figure 5**. While no inhibitory effect were observed for EGCG; **4**–**6**; **19**; or **20**; (−)-epicatechin-3;5-*O*-digallate (**3**) inhibited proliferation of HeLa S3 cells quite strongly; based on our assay protocol (IC_{50} value: 12.0 μM; IC_{50}: the concentration at 50% inhibitory activity). From the data for (+)-catechin-3;5-*O*-digallate (**4**); we can deduce that the stereochemistry at the 3-position is critically important for this

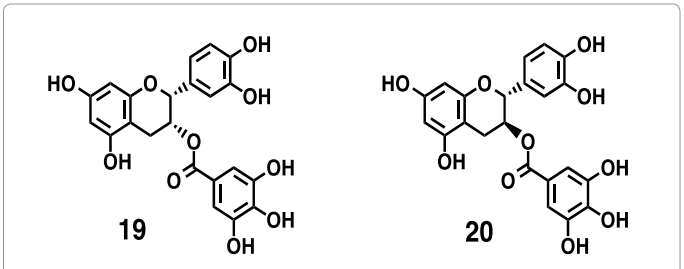
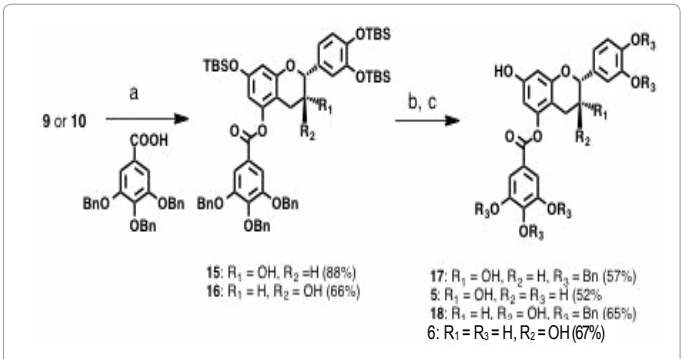


Figure 3: Structure of 3-*O*-galloyl flavan-3-ols.



Scheme 2: Synthesis of 5-*O*-galloyl compounds 5 and 6.

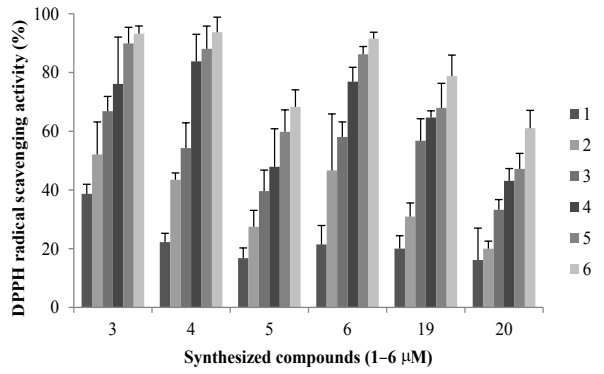


Figure 4: DPPH radical scavenging activity of the synthesized compounds. Results were given as % radical scavenging activity. Values represent mean ± SD (n = 8).

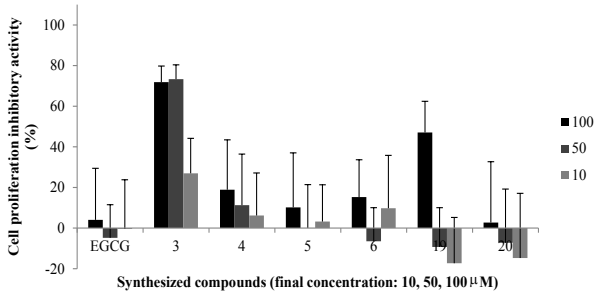


Figure 5: Inhibitory activity of synthetic galloyl-modified flavan-3-ols against HeLa S3 cell proliferation.

activity because the only difference between compounds **3** and **4** is the 2;3-structure. In addition; these results suggest that the biological activity of flavan-3-ols depends not only on the number of phenolic hydroxyl groups or galloyl moieties but also on other factors such as their structure.

HeLa S3 cells were incubated with a solution of each compound in DMSO for 48 h. All error bars represent standard deviations of the mean (n>8).

Non-specific binding assay of synthetic compounds with BSA

Polyphenols show both specific and non-specific protein interactions [19]. Discoveries of EGCG specific receptors made a powerful impact on the field of polyphenol studies [20]. However various EGCG biological activities thought not to be due to specific interaction with its receptors have been reported. We have thought that the multifunctional properties of polyphenols are one of the most important features for functional food ingredients. But it often makes analysis of biological assay and elucidation of functionalities of polyphenols complicated. It is expected that non-specific bindings of polyphenol to protein especially affect biological assay such as cell proliferation inhibitory activity because of containing appreciable quantities of proteins. (−)-Epicatechin-3,5-*O*-digallate (**3**) inhibited proliferation of HeLa S3 cells quite strongly; but (+)-catechin-3,5-*O*-digallate (**4**) and other compounds were not. If the non-specific protein binding abilities of synthesized compounds are different; our assays may not evaluate correctly the activity of the compounds.

To elucidate the non-specific binding ability of polyphenols; we immobilized the synthesized polyphenols on beads in a "functional group-independent" manner using a modified photoaffinity linker [15,21,22]. We synthesized photoaffinity-biotin linker **21** (**Figure 6A**); which was then mixed with each of the compounds; dried; and irradiated with a 365 nm UV light. In this protocol; polyphenols are immobilized on the linker through a highly reactive carbene species generated from aryl diazirine upon UV irradiation. A biotin-streptavidin interaction could then occur between the polyphenol-bound linkers and streptavidin sepharose beads. The interaction analysis between BSA; a protein present in large amounts in cell culture medium; and the polyphenol immobilized beads is shown in **Figures 6B and 6C**.

The binding assay of the beads showed that the non-specific binding property of compound **3**; the strongest cell proliferation inhibitor; was low. The non-active compounds **4**; the stereoisomer of **3** also did not interact with BSA. This data suggests that non-specific binding to BSA didn't affect the activity difference between **3** and **4**. In addition; (−)-epicatechin galloyl derivatives **5** and **19**; the compounds that one of two galloyl moieties of **3** is removed; bound to BSA stronger than other compounds. These data also reveals that adsorption behavior of polyphenols to BSA is different depending on each structure. Further investigations to clarify the mechanisms of the inhibitory activity against HeLa S3 cell proliferation of **3** are now underway.

Conclusion

We synthesized galloyl-modified flavan-3-ols to elucidate their SAR with DPPH radical scavenging activity; HeLa S3 cells proliferation

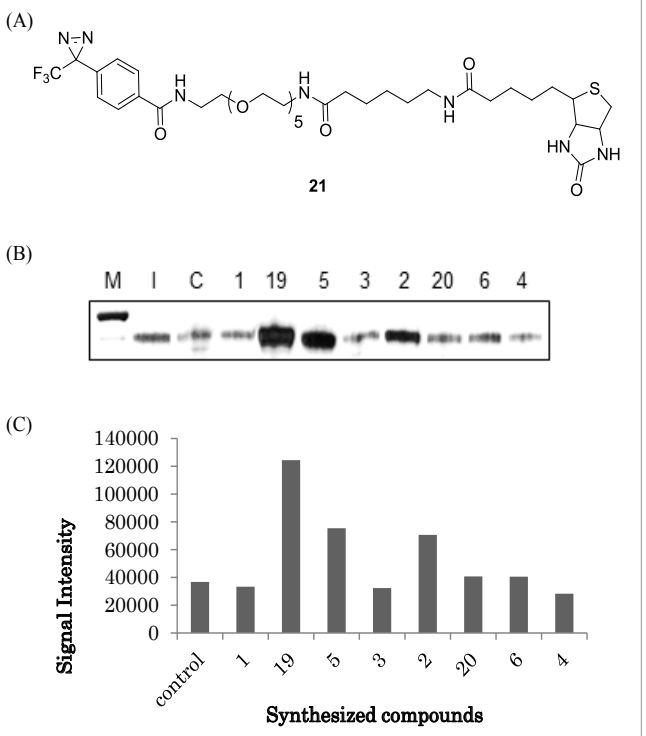


Figure 6: Nonspecific binding between polyphenols and BSA

(A) Structure of photoaffinity biotin linker (21)
(B) SDS-PAGE/silver-stain analysis of BSA binding to the beads on which each compound is immobilized. M: marker, I: input, C: control beads.
(C) Determination of band intensity by LI-COR, Image Studio Digits Ver 4.0.

inhibitory activity; and non-specific binding ability with BSA. Among the synthesized six compounds; (−)-epicatechin-3;5-*O*-digallate (**3**) showed the strongest inhibitory activity against HeLa S3 cell proliferation; but this activity did not have any relation to the DPPH radical scavenging activity or the non-specific protein binding property. Consequently; we determined that the 2,3-*cis*-structure of flavan-3-ols is critical for the inhibitory activity against HeLa S3 cell proliferation.

Acknowledgment

This work was supported by BRAIN: Program for Promotion of Basic and Applied Research for Innovations in Bio-oriented Industry (to A. Saito); technology research promotion program for agriculture; forestry; fisheries; food industry (to A. Saito); JSPS KAKENHI Grant Number 23780123 (Grant-in-Aid for Young Scientist (B) to A. Saito) and Grant Number 24590014 (Grants-in-Aid for Scientific Research (C) to N. Nakajima).

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