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-butylmethyisilyl; DCC: dicyclohexylcarbodiimide; TBAF: Tetra-Butyldimethylsilyl; 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 (−)-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 astragining of food products [7]; based on non-specific protein binding of polyphenols. This non-specific protein binding 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 (−)-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].

Here; we describe the development of a regioselective deprotection of tert-butylmethyisilyl (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 non-specific 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 precoated silica-gel plates (Merck 60F254 Art 5715). An ATAGO AP-300 spectrometer was used to measure optical rotation. 1H-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) mass spectra. 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. © 2015 Mori K, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
(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 CH₃CN as solvent A and 0.05% HCOOH and 10% CH₃CN in H₂O 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'-tetra-O-TBS-(−)-epicatechin (7) (440 mg; 0.59 mmol) in CH₂Cl₂ (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. NaHCO₃. The aqueous solution was extracted with CHCl₃ and the organic phase was washed with water and brine and then dried (MgSO₄). Filtration; concentration and silica-gel column purification (CHCl₃/MeOH; 20:1) afforded 413 mg (0.37 mmol; 2 steps 18%) of 12 as an amorphous solid. Data for 11: [α]_D24 = −45.9 (c 0.13; CHCl₃); [M+Na]⁻ (400 MHz; CDCl₃) 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); 3.03 (1H; dd; J = 1.5 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 (1H; s); 0.14 (3H; s); 0.09 (3H; s). ¹³C-NMR (100 MHz; CDCl₃) 164.9; 163.8; 156.9; 155.5; 155.3; 150.0; 147.6; 146.7; 130.1; 131.2; 131.1; 119.30; 112.28; 109.9; 100.6; 99.7; 78.1; 66.2; 27.5; 25.93; 25.91; 25.6; 18.45; 17.34; 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; ESIHRRMS: m/z (M'+H) Calculated for C₃₀H₂₅O₁₉Si; 633.4364 Found; 633.4358.

3',4',7-Tri-O-TBS-(−)-catechin (10): To a solution of 5,7,3'-tetra-O-TBDS-(−)-catechin (8) (107 mg; 0.14 mmol) in CH₂Cl₂ (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. NaHCO₃. The aqueous solution was extracted with CHCl₃ and the organic phase was washed with water and brine and then dried (Na₂SO₄). Filtration; concentration and silica-gel column purification (hexane/MeOH; 9:1 to 2:1) afforded 404 mg (0.63 mmol; 84%) of 9 as an amorphous solid. [α]_D24 = −58.3 (c 0.26; CHCl₃), ¹H-NMR (400 MHz; CDCl₃) 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); 0.99 (9H; s); 0.96 (9H; s); 0.20−1.86 (9H; m); ¹³C-NMR (100 MHz; CDCl₃) 155.5; 155.3; 150.0; 147.6; 146.7; 130.1; 131.2; 119.30; 112.28; 109.9; 100.6; 99.7; 78.1; 66.2; 27.5; 25.93; 25.91; 25.6; 18.45; 17.34; 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; ESIHRRMS: m/z (M'+H) Calculated for C₃₀H₂₅O₁₉Si; 633.4364 Found; 633.4358.

(−)-Epicatechin-3,5-di-O-gallate (3): A solution of 12 (413 mg; 0.37 mmol) in THF/MeOH/H₂O (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]. [α]_D24 = −27.2 (c 0.09; MeOH) lit.[⁵] [α]_D24 = −5.4 (c 2.17; MeOH); [M+Na]⁻ (400 MHz; CDCl₃) 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); ¹³C-NMR (100 MHz; CDCl₃) 174.4; 173.3; 165.1; 162.4; 158.9; 153.6; 153.2; 153.0, 149.7; 145.6; 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; ESIHRRMS Calculated for C₃₉H₂₆O₁₉Na; 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 CH₂Cl₂ (75 ml) at 0°C. After stirring for 3 days; the reaction mixture was quenched with water. The aqueous solution was extracted with CHCl₃ and the organic phase was washed with water and brine and then dried (MgSO₄). Filtration; concentration and silica-gel column purification (CHCl₃/MeOH; 20:1) and then hexane/MeOH; 1:1 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) at 0°C. Concentration and a short silica gel column (hexane/EtOAc; 7:1 to 1.5) afforded 762 mg (THF) in the presence of AcOH (0.20 ml; 3.64 mmol) at 0°C. A THF/MeOH/H₂O (20:1:1) mixture was evaporated to dryness at 0°C. The residue was dissolved in a small amount of THF and concentrated under vacuum. The residue was then treated with 10% aqueous CH₃OH solution (1 ml) and the mixture was stirred for 1 h at 0°C. The solution was concentrated under vacuum and the residue was treated with CHCl₃ (40 ml). The mixture was concentrated under vacuum and the residue was treated with CHCl₃ (40 ml). The mixture was concentrated under vacuum and the residue was treated with CHCl₃ (40 ml). The mixture was concentrated under vacuum and the residue was treated with CHCl₃ (40 ml). The mixture was concentrated under vacuum and the residue was treated with CHCl₃ (40 ml). 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The residue was purified by silica-gel column chromatography (CHCl₃/CH₂O = 1:1) to a solution of photoaffinity amino linker [16] (10 mg; 0.02 mmol) in 108.5; 90.0; 75.1; 35.6; ESIMS (+)[M+Na]⁺; 465 ([M+Na]+; 94); ESIHRMS Calculated for C₃₄H₂₇O₁₉Na; 465.0792; Found; 465.0779.

**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 100 µL of DMSO and was incubated for 12 h at 4°C. After removal of the supernatant; 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 H₂O (1 µL; 1 mg/mL) 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 using 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 flavan-3-ols; however; Mambu series compounds are present in the same plant; making SAR studies more difficult. Therefore; we synthesized galloyl analogs derived from (−)-epigallocatechin-3-0-gallate (6) 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 hydroxy groups of (−)-epicatechin (1) and (+)-catechin (2) were protected with TBS using a previously reported
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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% yield, 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% yield, respectively. Subsequent hydrogenation afforded (-)-epicatechin-3-O-gallate (5) and (+)-catechin-5-O-gallate (6) in 52% and 67% yield, 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 inhibition.
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 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-3,5-0-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.
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.

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