Design, Synthesis, and Biological Activity of Conformationally Restricted Analogues of Silibinin

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ABSTRACT: Silibinin (Sib), one of the main components of milk thistle extract, has attracted considerable attention because of its various biological activities, which include antioxidant activity and potential effects in diabetes and Alzheimer’s disease (AD). In a previous study, we synthesized catechin analogues by constraining the geometries of (+)-catechin and (−)-epicatechin. The constrained analogues exhibited enhanced bioactivities, with the only major difference between the two being their three-dimensional structures. The constrained geometry in (+)-catechin resulted in a high degree of planarity (PCat), while (−)-epicatechin failed to maintain planarity (PEC). The three-dimensional structure of Sib may be related to its ability to inhibit aggregation of amyloid beta (Aβ). We therefore introduced PCat and PEC into Sib to demonstrate how the constrained molecular geometry and differences in three-dimensional structures may enhance such activities. Introduction of PCat into Sib (SibC) resulted in effective inhibition of Aβ aggregation, α-glucosidase activity, and cell growth, suggesting that not only reduced flexibility but also the high degree of planarity may enhance the biological activity. SibC is expected to be a promising lead compound for the treatment of several diseases.

INTRODUCTION

Oxidative stress is related to the onset of a number of diseases, such as cancer, cerebrovascular disease, and heart disease, among others. Reactive oxygen species (ROS), which are often generated during energy production and nucleotide metabolism, can damage organs and cells and may induce such diseases. Natural antioxidants, such as catechin, resveratrol, and proanthocyanidin, are effective for scavenging ROS to suppress oxidative damage. Although most natural antioxidants exhibit a variety of biological activities, their use as therapeutics remains limited because they do not effectively cure diseases. The flexibility of most antioxidant molecules may weaken their affinity for targeting specific molecules. For example, the structures of flavonoids exhibit a high degree of flexibility between chroman nuclei and phenyl substitution, resulting in a low affinity for specific target sites required for the expression of potent bioactivity.

In a previous study, we synthesized a catechin analogue, namely, “planar catechin (PCat, 3),” in which the geometry of the (+)-catechin molecule was constrained to be planar by a bridge between the 3-hydroxyl group and C6’ on the B-ring.1 PCat exhibited potent radical scavenging activity, which was 5-fold greater than that of (+)-catechin.2 Conformationally constrained epicatechin (PEC, 4) was also synthesized using the same method and showed strong radical scavenging activity that was 4-fold greater than that of both (−)-epicatechin and PCat (unpublished data). Introducing alkyl side chains to constrain the molecular geometry results in an increase in the
electron-donating abilities of catechol rings. Several different biological activities were identified for both PCat and PEC, including the α-glucosidase inhibition activity, as well as antiviral and antitumor activities. The major distinction between PCat and PEC is the three-dimensional structure. PCat exhibits a high degree of planarity, while PEC failed to maintain planarity.

Constraining the geometry of one or both catechin molecules to be planar in procyanidin B3, a dimer of (+)-catechin, was predicted to enhance the biological activity to a degree similar to that of PCat. Indeed, we demonstrated that constraining both catechin molecules in procyanidin B3 enhanced several bioactivities, including the antioxidant activity and the inhibitory effects on $\beta$ aggregation. These findings suggest that constraining the geometry in molecules can enhance their affinity for targeting specific molecules. Generally, when bioactive compounds bind to target molecules, such as receptors or enzymes, they undergo a change in the three-dimensional structure to enhance the affinity for the target molecules via induced-fit binding. Geometrically constrained molecules exhibit a high degree of stability and decreased entropy, which contribute to the increased affinity for target molecules. As a result, such compounds offer the potential for enhanced biological activity compared with compounds that bind to target molecules via induced-fit.

Figure 1. Structures of catechins and their analogues.

Figure 2. Structures of Sib and its analogues.

Scheme 1. Reagents and Conditions: (a) Ph$_3$P═CHCOOEt, CHCl$_3$, Reflux, 95%; (b) LiAlH$_4$-AlCl$_3$, THF, 80%; (c) TMSOTf, Acetone, THF, $-10 \, ^\circ C$, 92%; (d) Ag$_2$CO$_3$, Benzene/Acetone, 55 $^\circ C$, 66%; (e) TMSOTf, Acetone, THF, $-20 \, ^\circ C$, 78%; (f) Ag$_2$CO$_3$, Benzene/Acetone, 55–60 $^\circ C$, 41%.
Silibinin (Sib) is a major component of extract from milk thistle seeds and consists of a mixture of two diastereomers, silybin A (7′R, 8′R) and silybin B (7′S, 8′S). Sib exhibits several biological activities, such as anticancer and anti-inflammatory activities, and may improve diabetes, enhance liver functions, and inhibit aggregation of amyloid beta (Aβ) that is thought to coincide with the onset of Alzheimer’s disease (AD). AD is a neurodegenerative disorder with several known pathological characteristics, one of which is the characteristic formation of senile plaques comprising fibrils formed from Aβ aggregates. Aβ peptides are 39–43 amino acid peptides derived from the distributed transmembrane amyloid precursor protein. The length of Aβ peptides varies at the C-terminus depending on the amyloid precursor protein cleavage pattern, with Aβ1–40 being the most toxic. Within plaques, Aβ peptides adopt a β-sheet structure conformation that allows for assembly and polymerization into structurally distinct forms, including fibrils, protofibrils, and polymorphic oligomers. Accumulation of Aβ plaques acts as a pathological trigger for a cascade that includes neurotic injury and formation of neurofibrillary tangles via the tau protein, which leads to neuronal dysfunction and cell death in AD. ROS are generated during the Aβ aggregation process, which induces cytotoxicity to nerve cells. The detailed mechanism of how Sib acts to inhibit Aβ aggregation is not yet clear. Interestingly, Yang et al. reported that planarity of silybin analogues to demonstrate how the constrained geometries in structures (Figure 2). We assessed Sib and the two synthesized analogues to demonstrate how the constrained geometries in catechin molecules allow for assembly and polymerization into structurally distinct forms, including fibrils, protofibrils, and polymorphic oligomers.

RESULTS

Synthesis. The novel analogues were synthesized according to the procedure reported by Yang et al. As shown in Scheme 1, vanillin introduced an ester group on the side chain by the Wittig reaction, which was then reduced by lithium aluminum hydride to form (6). The synthesis of catechin analogues (3) and (4) was accomplished by the Oxa-Pictet–Spengler reaction in which (+)-catechin or (−)-epicatechin and acetone were treated with trimethylsilyl trifluoromethanesulphonate (TMSOTf) in tetrahydrofuran (THF). Finally, SibC (1) was synthesized by an oxidative coupling reaction of 3 with 6 using silver carbonate (Ag2CO3); SibEC (2) was synthesized using a similar method. Based on the reaction mechanism of oxidative coupling, it is predicted that the oxygen molecules of catechol and the vinylc carbons were condensed by a synergetic arrangement. Both the positional relationships in the E-ring and the hydroxyl methyl group were a mixture of (S, R) or (R, S), and we successfully purified the main products.

Antioxidant Activity. In a previous study, we demonstrated that the constrained geometry in catechin molecules enhances their antioxidant activity. We therefore expected SibC and SibEC to also exhibit enhanced antioxidant activity, similar to the catechin analogues. We employed the Fenton reaction to recreate the conditions for hydroxyl radical production in the human body and measured the antioxidative activities of the test compounds by the 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) spin-trapping electron spin resonance spectroscopy (ESR) method. Figure 3 shows the ESR spectra for the Fenton reaction in the presence and absence of Sib and its analogues. The line shape and hyperfine splitting of the signal are typical of the spin adduct formed between DMPO and the hydroxyl radical (•OH). These results suggest that Sib and its analogues exhibit little radical scavenging activity in contrast to SibEC, which exhibits radical scavenging activity.

Furthermore, the inhibitory activity against oxidative damage to DNA caused by •OH, generated by the iron (Fe)-containing system of xanthine oxidase (XO) and hypoxanthine (HX) (FeXO/HX), was measured by electrophoresis. This assay used pBR322, which exhibits a covalently closed circular form (CCC). Upon induction of oxidative damage to pBR322 caused by ROS, a phosphodiester bond in one strand of the double-stranded DNA is cleaved to form an open circular (OC) form. Upon further cleaving of the deoxyribonucleic acid (DNA), the OC form changes to a linear form. Lane 1 in Figure 4 shows untreated pBR322 (CCC), and the OC form in Lane 2 demonstrates hydroxyl radical-induced DNA cleavage by FeXO/HX. Lanes 3 to 9 show the results of oxidative DNA cleavage by the enzyme reaction performed in the presence of test compounds. These results demonstrate that PCat has a protective effect against DNA breakage caused by ROS. Sib and its analogues decrease the inhibition of oxidative damage to DNA compared with PCat.

Calculation of Thermodynamic Parameters. To better understand the chemical structure of Sib and its analogues, we calculated the Gibbs free energies, highest occupied molecular orbitals (HOMOs), and ionization potentials (IPs). HOMO and IP values of SibC and SibEC were calculated utilizing the (S, R) configuration, which are the structures with the lowest Gibbs free energies. The results are shown in Figure 5 and Table 2. In SibC and SibEC, the HOMOs were mainly located on the aromatic ring A of the chroman moiety, whereas the electron densities of silybin A and B were distributed in guaianol E. Furthermore, the most
stable structure in SibC was found to exhibit the highest planarity of all three compounds; SibEC did not maintain planarity relative to silybin A.

Inhibition of α-Glucosidase Activity. It is well known that Sib exhibits hypoglycemic activity, but the detailed mechanisms are not clear. We previously demonstrated that the constrained geometry in catechin molecules enhances their inhibitory activity of α-glucosidase, which is one of the enzymes responsible for breaking down carbohydrates into smaller sugar particles such as glucose. SibC and SibEC are also expected to enhance this activity, similar to the catechin analogues. By reacting α-glucosidase from Saccharomyces cerevisiae and 4-nitrophenyl-α-D-glucopyranoside, the absorbance of 4-nitrophenol released from 4-nitrophenyl-α-D-glucopyranoside at 405 nm was measured. The concentration of the inhibitors required to inhibit 50% of α-glucosidase activity under the assay conditions was defined as the IC50 value. Sib and its analogues were found to exhibit α-glucosidase inhibitory activity (Table 2), suggesting that the hypoglycemic effect of Sib in diabetes is related to its α-glucosidase inhibitory activity. SibC exhibits a nearly 8-fold higher α-glucosidase inhibitory activity than Sib, while SibEC failed to enhance the inhibitory activity relative to Sib and SibC.

Bioactivity Assays Assessing Activity toward Aβ Aggregation. Sib is known to inhibit Aβ aggregation and

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Table 1. Gibbs Energies and IPs in Silybin A, Silybin B, SibC, and SibEC

|            | silybin A | silybin B | SibC (R, S) | SibC (S, R) | SibC (R, S) | SibC (S, R) | SibEC (R, S) | SibEC (S, R) |
|------------|-----------|-----------|-------------|-------------|-------------|-------------|-------------|-------------|
| ΔG (kJ/mol)| −4,510,770.57 | −4,510,942.50 | −4,622,968.72 | −4,622,997.31 | −4,622,986.86 | −4,622,990.67 |
| IP (eV)    | 6.91      | 6.94      | 6.72        | 6.73        | 6.59        | 6.64        |

Table 2. IC50 Values of Sib and Its Analogues against α-Glucosidase

|            | Sib   | SibC  | SibEC | acarbose |
|------------|-------|-------|-------|----------|
| IC50 (μM) | 16.0  | 2.4   | 28.1  | <1.0     |

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Figure 4. Effects of Sib and its analogues on DNA breakage induced by *OH via an enzymatic reaction (HX/XO). Assays were performed in phosphate buffer, pH 7.4, containing pBR322 DNA (0.05 μg/μL), 1.57 μM ferric chloride—ethylenediaminetetraacetic acid (FeCl3−EDTA), 63 μM HX, 2.00 × 10−2 U/mL XO, and test compounds.

Figure 5. Optimized geometries and HOMOs of silybin A, SibC (S, R), and SibEC (S, R).
neurotoxicity induced by Aβ. Yang et al. demonstrated that the most stable structure of Sib exhibits a high degree of planarity and suggested that the planar structure may be related to its inhibitory activities against Aβ aggregation. However, the details of this mechanism are not yet clear. We predicted that SibC and SibEC should show results similar to those reported by Yang et al. and that the three-dimensional structures of these compounds should correlate with inhibitory activity against Aβ aggregation. First, inhibitory activities of Sib and its analogues, as well as of (+)-catechin, on Aβ aggregation were assessed using a thioflavin-T (Th-T) assay. Th-T is a fluorescent dye that demonstrates high specificity for Aβ fibrils. Binding of Th-T to Aβ fibrils can be observed by an increase in fluorescence intensity. We investigated the time-dependent variation in fluorescence intensity upon incubation of 15 μM Aβ1-42 with or without 10 μM of each analogue. As shown in Figure 6, Sib and its analogues demonstrated inhibitory activity against Aβ aggregation, with levels similar to those of (+)-catechin. SibEC demonstrated a slightly stronger inhibitory effect than both Sib and (+)-catechin. The most potent inhibitory activity was observed for SibC, which was found to almost completely inhibit Aβ1-42 aggregation.

To confirm that SibC is the most potent inhibitor of Aβ1-42 aggregation of all the tested analogues, the circular dichroism (CD) spectrum of SibC was measured to determine the effects of SibC on the secondary structure of Aβ1-42. It is well known that a negative CD signal with a negative maximum at 218 nm is indicative of the β-sheet structure of Aβ1-42. Compared with the CD spectrum of Aβ1-42 alone, the CD spectrum of Aβ1-42 with SibC shows a more decreased negative maximum, indicating that the inhibitory activity of SibC on Aβ1-42 aggregation is due to inhibition of the conversion of Aβ1-42 to a highly β-sheet-rich form (Figure 7). The inhibitory effect of SibC on Aβ1-42 fibril formation was also confirmed by transmission electron microscopy (TEM). Aβ1-42 fibrils in the absence of treatment were observed to be very thick and firm. Fibrils treated with SibC, on the other hand, appeared very thin, and there were fewer fibrils observed compared with Aβ1-42 alone (Figure 8).

Furthermore, we assessed the potential neuroprotective effects of Sib and its analogues against Aβ1-42-induced toxicity in SH-SYSY cells, a human neuroblastoma cell line. We first assessed the cytotoxicity of the compounds in SH-SYSY cells. Sib and its analogues showed higher cytotoxicity than expected. However, upon treatment with 4 μM of the test compounds, minimal cytotoxicity was observed (Figure 9A). Next, the effects of the test compounds on Aβ1-42-induced neurotoxicity were investigated at a concentration of 6.3 μM,
which is nontoxic to SH-SY5Y cells. The results are shown in Figure 9B. Treatment of SH-SY5Y cells with 20 μM Aβ1-42 showed 50% cell viability. All test compounds at concentrations of 6.3 μM in the presence of 20 μM Aβ1-42 failed to protect against Aβ1-42-induced cytotoxicity.

Inhibition of Cell Proliferation. As the test analogues are cytotoxic to SH-SY5Y cells, even at low concentrations, it was speculated that it might inhibit the growth of cancer cells. It was previously reported that Sib induces the apoptosis of MCF-7 and MDA-MB-231 cells.25 We assessed the cytotoxicity of the test compounds in MCF-7 (human breast cancer) and HCT-116 (human colorectal cancer) cells. Upon treatment of HCT-116 cells, all test compounds inhibited cell growth; Sib analogues were more cytotoxic than Sib, with SibC being the most cytotoxic. The results were similar in MCF-7 cells following treatment with the test compounds (Figure 10). To elucidate details of the inhibitory mechanism, we evaluated the effects of SibC on cell cycles. Fawn et al. previously reported that Sib arrests the G1 phase of the cell cycle in HCT-116 cells.26 Our results also show that Sib arrests the G1 phase of the cell cycle more strongly (Figure 10).
**DISCUSSION**

In this study, we synthesized SibC and SibEC by constraining the geometry of Sib to demonstrate how the three-dimensional structure affects the respective bioactivities of the compounds. We calculated the most stable structure in Sib and demonstrated its high degree of planarity, consistent with the report of Yang et al. SibC exhibits a higher degree of planarity than silybin A and B, while SibEC fails to maintain its planarity. Most bioactivities evaluated were related to the three-dimensional structure. In particular, SibC resulted in enhanced bioactivities, while SibEC did not. The IC_{50} value of SibC for \( \beta \)-glucosidase was one-eighth of that observed for Sib, while the activity of SibEC was lower than that of Sib. Alasmary et al. reported details of the interaction between the flavonoid quercetin and an active site of \( \beta \)-glucosidase, including a stacking interaction in the aromatic rings (A and B rings) of quercetin and the amino acid residue Trp432 in \( \alpha \)-glucosidase, hydrogen bonds between the hydroxyl group in quercetin and residues Asp568, His626, and Asp357 in \( \alpha \)-glucosidase, and hydrophobic interactions between the hydroxyl group in quercetin and residues Phe601 and Trp329 in \( \alpha \)-glucosidase.

As quercetin exhibits a planar structure from the conjugated system with an \( \alpha,\beta \)-unsaturated ketone, it can bind to the active site of \( \alpha \)-glucosidase. As SibC exhibits a highly planar structure and a molecular size similar to that of quercetin, we predicted that SibC could fit easily into the active site of \( \alpha \)-glucosidase. In this study, SibC did not demonstrate enhanced activity compared with Sib and SibEC because SibEC failed to maintain its planarity, which is essential for binding to the active site of \( \alpha \)-glucosidase.

Molecular planarity was also found to be related to the inhibitory activities against \( A\beta_{1-42} \) aggregation. The results of the Th-T assay demonstrated that Sib exhibits inhibitory activity against \( A\beta_{1-42} \) aggregation and that SibC is even more potent than Sib. Irie et al. demonstrated that some natural compounds, such as curcumin and datiscine, have inhibitory activity against \( A\beta_{1-42} \). These compounds, which contain noncatechol rings and highly planar structures, intercalate into the intermolecular \( \beta \)-sheet structures of \( A\beta_{1-42} \) peptides to inhibit fibril formation. It is suggested that SibC, with its high degree of planarity and a noncatechol structure, can also intercalate into the \( \beta \)-sheet structures, leading to the inhibition of \( A\beta_{1-42} \) aggregation. This is consistent with the reduction in \( A\beta_{1-42} \) fibrils observed by TEM and inhibition of \( \beta \)-sheet formation of \( A\beta_{1-42} \) based on CD spectra. Although SibC exhibits potent inhibitory activity against \( A\beta_{1-42} \) aggregation, it was unable to protect against \( A\beta_{1-42} \)-induced neurotoxicity in SH-SY5Y cells because 6.3 \( \mu \)M SibC, which shows no cytotoxicity, did not sufficiently inhibit \( A\beta_{1-42} \) aggregation. SibC exhibits potent cytotoxicity, not only toward SH-SY5Y cells but also against MCF-7 and HCT-116 cells, suggesting that cytotoxicity may also be related to the molecular planarity. Sib was previously reported to induce apoptosis in MCF-7 and MDA-MB-231 cells, and Sib is known to arrest the G1 phase of the cell cycle in HCT-116 cells. In this study, Sib was also found to arrest the G1 phase in HCT-116 cells, while SibC was found to be even more effective than Sib. The enhanced cytotoxicity of SibC and SibEC may be attributed to the higher lipophilicity due to structural constraints, resulting in increased permeability through the cell membrane. The cLogP value shown in Table 3 indicates that SibC and SibEC are more lipophilic than Sib. However, SibC showed a degree of higher cytotoxicity than SibEC in the present study. It is suggested that SibC may intercalate into DNA to induce cytotoxicity because of the high degree of molecular planarity.

Although silibinin is a known natural antioxidant, the antioxidant activity of Sib analogues was not significantly increased in this study, despite their constrained three-dimensional structures. The results of antioxidant assays reported herein suggest that the \( \bullet \)OH scavenging activity of Sib is very weak. As such, the constrained geometry in the Sib analogues was unable to enhance the direct \( \bullet \)OH scavenging activity. Unlike catechin, Sib does not have a catechol structure, so it is considered that the antioxidant activity is not enhanced even if the geometry is constrained. Sib and SibEC did not show significant differences in antioxidant activity, while SibEC demonstrated minimal antioxidant activity. PEC (4), which is the flavonoid skeleton of SibEC, was found to have the most potent antioxidant activity among the catechins and its analogues (3, 4); it is suggested that SibEC, which contains PEC in its structure, results in similar enhanced antioxidative activity. The relationship between the antioxidant activity and the IP value (which is an index of

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**Figure 11.** Quantitative results of cell cycles for HCT-116 cells treated with 25 \( \mu \)M of Sib or SibC for 48 h. Paclitaxel (PTX) is a typical anticancer agent that arrests the G2 phase of the cell cycle and was used as a positive control.

**Table 3.** cLogP Values of Sib, SibC, and SibEC

|          | silybin A | silybin B | SibC (S, R) | SibEC (S, R) |
|----------|-----------|-----------|-------------|-------------|
| cLogP    | 1.53      | 1.53      | 3.04        | 3.04        |
antioxidant activity) was also evaluated in this study and showed that the IP of SibEC was the lowest of the three compounds. While a slight increase in antioxidant activity was observed with the Sib analogues, the compounds did not exhibit sufficient antioxidant activity to protect against oxidative damage to DNA.

Finally, constraining the geometry of flavonoid structures (e.g., Sib) to be planar was found to enhance the bioactivity. SibC may be a potential candidate for treatments targeting a number of different diseases. Other flavonoid compounds with similarly constrained structures are currently under investigation in our laboratory.

**METHODS**

**General.** Unless otherwise specified, all commercially available compounds were used as provided without further purification. Solvents for chromatography were of technical grade. Reactions were monitored by thin-layer chromatography (TLC) carried out on silica gel plates (TLC silica gel 60 F254, Merck). Column chromatography was performed using silica Gel 60 (spherical, 63–210 μm, KANTO CHEMICAL). 

1H and 13C nuclear magnetic resonance (NMR) spectra were recorded with a JEOL JNMAL-400 (400 MHz) NMR and a Varian 600 MHz NMR spectrometer, respectively, using acetone-6 or dimethyl sulfoxide (DMSO)-δ as the solvent and internal reference. Mass spectra were measured with a JMS-700V (JEOL) mass spectrometer. Sib, the mixture of silybin A and silybin B, was purchased from Sigma Aldrich, Inc. Th-T and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) were purchased from Sigma-Aldrich, Inc. AcOH (hydrochloride salt) was purchased from Wako Pure Chemical Industries, Ltd. AcOH-δ (trifluoroacetic acid salt) was purchased from Peptide Institute, Inc. Prior to experiments, AcOH-δ was completely dissolved in HFIP using sonication. After dispensing the solution into appropriate volumes, the solvent was evaporated and the resultant AcOH-δ was stored at ~80 °C before use. Reagents for cell culture were used as provided without further purification.

**Synthesis.** Compounds 5 and 6 were synthesized according to the procedure described by Yang et al.22,29

(6 a S, 1 2 a R)-5, 5-Di m e t h y l-5, 6 a, 7, 1 2 a-tetrahydroisochromeno[4,3-b]chromene-2,3,8,10-tetraol (3). To a solution of 2.0 g of (+)-catechin (6.89 mmol) and 2.03 mL of acetone (27.56 mmol) in 63 mL of dry THF at -10 °C, 1.87 mL of TMSOTf (10.33 mmol) was slowly added. After stirring for 1 h at -10 °C, 100 mL of saturated sodium bicarbonate (NaHCO3) was added to the reaction mixture and the product was extracted with ethyl acetate (AcOEt), which was then washed with brine and dried over Na2SO4, and concentrated to yield 694.5 mg (66% yield) of SibC (1) as a white solid. 1H NMR (600 MHz, DMSO-δ6): δ 9.36 (s, 1H), 9.17 (s, 1H), 9.11 (s, 1H), 7.03 (s, 1H), 7.00 (s, 1H), 6.86 (dd, 1H, J = 1.8, 8.4 Hz), 6.84 (d, 1H, J = 1.8 Hz), 6.77 (d, 1H, J = 8.4 Hz), 5.94 (s, 1H), 5.83 (s, 1H), 4.94 (s, 1H), 4.89 (m, 1H), 4.47 (d, 1H, J = 8.4 Hz), 4.15 (m, 1H), 3.78 (dddd, 1H, J = 5.4, 8.4, 11.4 Hz), 3.76 (s, 3H), 3.51 (m, 1H), 3.33 (m, 1H), 2.83 (dd, 1H, J = 5.6, 15.2 Hz), 2.33 (dd, 1H, J = 10.8, 15.2 Hz), 1.48 (s, 1H), 1.45 (s, 3H); MS (ESI)+ m/z: 509 (M + H)+.

(6 a R, 1 2 a R)-5, 5-Di m e t h y l-5, 6 a, 7, 1 2 a-tetrahydroisochromeno[4,3-b]chromene-2,3,8,10-tetraol (4). To a solution of 415 mg of (−)-epicatechin (1.43 mmol) and 0.42 mL of acetone (5.72 mmol) in 60 mL of dry THF at -20 °C, 0.31 mL of TMSOTf (1.72 mmol) was slowly added. After stirring for 14 h at -20 °C, 50 mL of saturated NaHCO3 was added to the reaction mixture and the product was extracted with AcOEt, which was then washed with brine and dried over Na2SO4. The resulting residue was evaporated to remove the solvent and subsequently subjected to column chromatography over silica gel (200–300 mesh) with toluene/acetone/methanol (7:3:1) as the eluent to yield 368 mg (78% yield) of PEC (4) as a white solid. 1H NMR (400 MHz, acetone-δ6): δ 6.82 (s, 1H), 6.70 (s, 1H), 5.98 (d, 1H, J = 2.4 Hz), 5.77 (d, 1H, J = 2.4 Hz), 4.94 (br s, 1H), 3.65 (m, 1H), 2.88 (dd, 1H, J = 5.2 Hz, J = 17.6 Hz), 2.78 (dd, 1H, J = 15.2 Hz, J = 18.4 Hz), 1.46 (s, 3H), 1.37 (s, 3H); 13C NMR (acetone-δ6): δ 156.7, 156.6, 156.0, 146.2, 143.6, 135.4, 124.0, 116.6, 111.6, 98.1, 95.2, 94.9, 75.1, 70.5, 63.6, 31.3, 27.3, 25.6; mp 184–190 °C; MS (ESI)+ m/z: 331 (M + H)+.

(7aR,13aR)-2-(4-Hydroxy-3-methoxyphenyl)-3-(hydroxy-methyl)-6,6-dimethyl-2,3,6,7a,8,13a-hexahydro-[1,4]-dioxino[2′,3′:6,7]isochromeno[4,3-b]chromene-9,11-diol (SibEC, 2). To a mixture of 0.41 g of Ag2CO3 (1.5 mmol), PEC (4) (0.33 g, 1.0 mmol), and coniferol (6) (0.36 g, 2.0 mmol) in a dry flask, 45 mL of dry acetone and 12 mL of dry benzene were added under an argon atmosphere. The reaction was stirred at 55 °C for 22 h. The reaction mixture was filtered and subsequently evaporated to remove the solvent. The resulting residue was then subjected to column chromatography over silica gel (200–300 mesh) with chloroform/ethyl acetate/acetic acid (CHCl3/ACOEt/CH3CO2H) (50:20:0.25) as the eluent. The combined fractions were then washed with brine, dried over Na2SO4, and concentrated to yield 215.7 mg (41% yield) of SibEC (2) as a white solid. 1H NMR (600 MHz, DMSO-δ6): δ 9.56 (s, 1H), 9.40 (s, 1H), 8.99 (s, 1H), 7.08 (s, 1H), 7.02 (s, 1H), 6.88 (dd, 1H, J = 1.8, 8.4 Hz), 6.83 (d, 1H, J = 1.8 Hz), 6.75 (d, 1H, J = 8.4 Hz), 5.78 (s, 1H), 5.58 (s, 1H), 4.89 (s, 1H), 4.86 (m, 1H), 4.51 (br s, 1H), 4.13 (m, 1H), 3.74 (s, 3H), 3.65 (m, 1H), 3.50 (m, 1H), 3.30 (m, 1H), 2.80 (dd, 1H, J = 5.2, 17.6 Hz), 2.65 (dd, 1H, J = 1.6, 18.4 Hz), 1.44 (s, 1H), 1.35 (s, 3H); MS (ESI)+ m/z: 509 (M + H)+.
Calculation Methods. IPs, the orbital composition of HOMOs, and cLogP were calculated using density-functional theory (DFT) methods with the B3LYP exchange–correlation functional and the 6-31G(d) basis set using the procedure embedded in Wavefunction (Irvine, CA, USA). Spartan 14. IPs were obtained as the difference in energies between the parent molecule and its cation radical formed by a one-electron-transfer reaction.

ESR Measurements. A sample containing 24 μL of Milli-Q, 12 μL of 0.2 M phosphate buffer (pH 7.4), 30 μL of 5 mM Sib and its analogues, 30 μL of 8 mM FeCl3, 2 μL of DMPO, and 24 μL of 500 mM H2O2 was introduced into a flat quartz cell, which was then placed in the ESR sample cavity. ESR spectra were recorded on a JEOL JES-FR30EX ESR spectrometer under the following conditions: modulation frequency, 100 kHz; modulation amplitude, 10 G; scan time, 1 min; microwave power, 4 mW; center field, 3360.50 G; and microwave frequency, 9.394 GHz.

Analysis of DNA Strand Breakage. The effects of novel analogues on DNA strand breakage were measured via conversion of supercoiled pBR322 plasmid DNA to the open circular and linear forms. Reactions were carried out in 25 μL (total volume) of 0.2 M phosphate buffer, pH 7.4, containing pBR322 DNA (0.3 μg), 1.57 μM FeCl3-EDTA, 63 μM hypoxanthine (HX), 2.00 × 10−2 U/mL XO (Roche), and each of the test compounds. Reactions were initiated by adding XO. Following incubation at 37 °C for 15 min, the reaction mixture was treated with 10 μL of loading buffer [100 mM Tris–borate–EDTA (TBE) buffer, pH 8.3, containing 30% glycerol and 0.1% bromophenol blue] and applied to 1.0% agarose gel. Horizontal gel electrophoresis was carried out in 50 mM TBE buffer containing 0.1% ethidium bromide. Gels were destained in water for 30 min and then photographed by UV transillumination.

α-Glucosidase Activity. α-Glucosidase inhibitory activity was determined by a partial modification of the procedure reported by Matsui et al. The reaction mixture for α-glucosidase from S. cerevisiae (TOYOBO CO., LTD.) consisted of 180 μL of 1.11 mM 4-nitrophenyl-α-D-glucopyranoside in 0.1 M phosphate buffer (pH 7.0), 10 μL of flavonoid in DMSO or Milli-Q, and 10 μL of enzyme solution (6.60 × 10−2 U/mL XO). After incubation for 20 min at 37 °C, the absorbance of 4-nitrophenol released from 4-nitrophenyl-α-D-glucopyranoside at 405 nm was measured. The concentration of the inhibitor required to inhibit 50% of α-glucosidase activity (IC50 value) under the assay conditions was determined. The IC50 value was measured graphically by a plot of percent inhibition versus log of the concentration of the test compound. The data presented in this assay were obtained from three independent experiments for each experimental condition.

Th-T Fluorescence Assay. The Th-T fluorescence assay was employed to assess the abilities of the synthesized compounds to inhibit Aβ1−42 aggregation. Aβ1−42 (trifluoroacetic acid salt) was dissolved in 0.3% ammonium hydroxide (NH4OH) at 1 mM under sonication. Test compounds were prepared in 1 mM DMSO. A volume of 25 μL of 0.2 M phosphate buffer (pH 7.4), 64 μL of Milli-Q, 5 μL of 2.0 M sodium chloride (NaCl) solution, 2 μL of test compound, and 100 μL of 20 μM Th-T in glycine–sodium hydroxide buffer (pH 9.0) was added to microfuge tubes in the abovementioned sequence. Then, 4 μL of Aβ1−42 solution and 196 μL of the above mixture were added to a 96-well plate (nonbinding μClear, 96-well black microplate, Greiner Bio-One) and subsequently incubated at 37 °C. After 0, 0.5, 1, 2, 3, 4, 6, 8, 10, and 12 h, the fluorescence intensity was measured at 450 nm excitation and 485 nm emission using a Varioskan LUX microplate reader (Thermo Fisher Scientific). Prior to each measurement, the 96-well plate was shaken for 15 s. The data presented in this assay were obtained from three independent experiments for each experimental condition.

CD Spectroscopy. The secondary structure of Aβ1−42 was estimated by CD spectroscopy in a 0.1 mM quartz cell (JASCO). Aβ1−42 (trifluoroacetic acid salt) was dissolved in 0.3% NH4OH at 200 mM concentration under sonication. SibC was prepared in 200 μM acetonitrile and Milli-Q. One hundred microliters of 0.2 M phosphate buffer (pH 7.4), 200 μL of Milli-Q, 20 μL of 2.0 M NaCl solution, 100 μL of SibC solution, and 40 μL of Aβ1−42 solution were added to microfuge tubes in the above order, and the mixture was then incubated at 37 °C. After 4 h, an aliquot of the mixture was loaded into the quartz cell, and CD spectra were recorded in the range of 190–250 nm. The spectrum of the vehicle alone was subtracted from the spectra of Aβ1−42. The spectrum of SibC was subtracted from the spectra of Aβ1−42 in the presence of SibC.

Transmission Electron Microscopy. Aggregates of Aβ1−42 after 48 h of incubation were examined by TEM. Aβ1−42 (trifluoroacetic acid salt) solution with or without SibC was prepared as described above for the Th-T assay and subsequently incubated at 37 °C for 48 h. After centrifugation at 4 °C for 10 min, the supernatant was removed from the pellets and the solution was gently mixed. The suspensions were applied to a film and dried in air before being negatively stained for a few minutes with 2% uranyl acetate. The aggregates were examined with a transmission electron microscope (Hitachi, H7600).

Statistical Analysis. Data were expressed as means ± standard deviation. Differences between samples and paired controls were analyzed by two-tailed Student’s t-test. P-values < 0.01 were considered statistically significant. For multiple comparisons, data were analyzed by one-way ANOVA with posthoc Dunnett or Bonferroni tests to identify data sets that differed from control data.

Cell Culture. Cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) or the Riken BioResource Research Center (Tsukuba, Japan) and maintained according to the supplied instructions (Table 4). Cells were grown in a 5% CO2 atmosphere at 37 °C.

| Table 4. List of Cancer Cell Lines |
|----------------------------------|
| cell line | cell bank | culture method |
|----------|----------|----------------|
| MCF-7    | ATCC HTB-22 | DMEM (4.5 g/L glucose), 10% FBS |
| HCT116   | RCB2979  | DMEM (1.0 g/L glucose), 10% FBS |
| SH-SYSY  | ATCC CRL-2266 | DMEM/Ham’s F12, 10% FBS |

Cell Viability. Cell viability was determined using WST-8 (Dojindo) or calcein-AM. Briefly, cells were plated on 96-well plates and incubated overnight in a CO2 incubator to allow for attachment. After treatment with test compounds in the presence and absence of Aβ1−42 (hydrochloride salt), cells were incubated for 48 h in the CO2 incubator. Cell viability was determined using WST-8 (Dojindo) or calcein-AM according to the manufacturer’s instructions. The data presented in the assay of cytotoxicity were obtained from three or four
independent experiments for each experimental condition. The data presented in the assay of cytotoxicity induced Aβ were obtained from two independent experiments for each experimental condition.

**Cell Cycle Analysis.** After treatment with the test compounds for 48 h, cells were washed with phosphate-buffered saline (PBS) and fixed with 70% ethanol in PBS for 10 min. Cells were washed with PBS and incubated with PI (40 μg/mL) and RNase (0.1 mg/mL) for 30 min at 37 °C, protected from light. After washing, images were acquired for DNA content analysis with a CQ1 quantitative confocal image cytometer (Yokogawa Electric Corporation). DNA histograms were obtained and analyzed in terms of cell cycle distribution with CellPathFinder analysis software (Yokogawa Electric Corporation). The data presented in the assay were obtained from three independent experiments for each experimental condition.

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**Notes**

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**ABBREVIATIONS USED**

*OH, hydroxyl radical
AcOEt, ethyl acetate
AD, Alzheimer’s disease
Asp, aspartic acid
Aβ, amloid beta
CCC, covalently closed circular
CD, circular dichroism
DFT, density-functional theory
DMPO, 5,5-dimethyl-1-pyrroline N-oxide
DMSO, dimethyl sulfoxide
EDTA, ethylenediaminetetraacetic acid
ESR, electron spin resonance
EtOH, ethanol
FBS, fetal bovine serum
Gly, glycine
HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol
His, histidine
HOMO, highest occupied molecular orbital
HX, hypoxantheme
IP, ionization potential
NMR, nuclear magnetic resonance
OC, open circular
PBS, phosphate-buffered saline
Phe, phenylalanine
PI, propidium iodide
PTX, paclitaxel
ROS, reactive oxygen species
STS, staurosorine
TBE, Tris–borate–EDTA
TEM, transmission electron microscopy
Th-T, thioflavin T
THF, tetrahydrofuran
TLC, thin-layer chromatography
TMSOTf, trimethylsilyl trifluoromethanesulfonate
Trp, tryptophan
UV, ultraviolet
XO, xanthine oxidase
WST, water-soluble tetrazolium salts

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