Hydroxymethylation of Rutin Induced by Radiolysis as Novel α-Glucosidase Inhibitors

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Rutin, a major flavonoid glycoside found in many higher plants, was exposed at 25 kGy of γ-ray and produced three new hydroxymethylated products 2–4 in the C-ring by γ-irradiation. Structures of the new compounds, including absolute configurations, were elucidated based on spectroscopic interpretation (NMR, UV, [α]_D, MS, and circular dichroism (CD)). The new unusual rutin derivatives 2 and 3 exhibited significantly enhanced inhibitory effects against α-glucosidase with IC₅₀ values of 23.1±1.2 and 11.2±0.7 μM, respectively, when compared to the parent rutin.

Key words γ-irradiation; rutin; radiolysis; α-glucosidase; hydroxymethylation

Diabetes mellitus, one of the most common chronic diseases worldwide, is a polygenic complex metabolic disease caused by high blood glucose levels. This undesirable disorder constitutes one of the most challenging health problems in recent years and is closely associated with cardiovascular complications.1,2) Serious complications associated with type 2 diabetes mellitus, include peripheral vascular disease, diabetic neuropathy, amputations, renal failure, stroke, and blindness.3) The most effective treatment for type 2 diabetes mellitus is based on achieving optimal blood glucose levels after meals. Recently, delay of dietary monosaccharide absorption via inhibition of α-glucosidase activity was shown to be a beneficial strategy for treatment of type 2 diabetes mellitus.4) Despite numerous studies on the development of α-glucosidase inhibitors such as acarbose and voglibose, only a few are currently available, and all are sugar mimics with tedious preparation steps as well as serious gastrointestinal side-effects.5) Thus, development of effective non-sugar inhibitors of α-glucosidase from natural products has become a current focus of research.6,7)

γ-Irradiation has been demonstrated as an advanced technology applied in food processing and is also known to have major role in the destruction of micro-organisms, because it contains an abundance of reactive species and free radicals such as methoxy (CH₃O·), hydroxy alkyl (·CH₂OH), hydroxyl ion (OH⁻), peroxyl (OOH ·) radicals, and superoxide anion (O₂⁻) radicals, which showed a pseudomolecular ion peak at M+ [M+H]⁺. Its molecular formula was determined to be C₂₉H₃₄O₁₉ (MeOH). Its molecular formula was determined to be C₂₉H₃₄O₁₉ using negative high resolution (HR)-FAB-MS, which showed a pseudomolecular ion peak at m/z 641.1710 [M−H]⁻. The absorption maxima at 290 nm in the UV spectrum were attributed to a dihydroflavonol nucleus.10) The sugar composition was determined to be α-glucose and l-rhamnose by microscale acid hydrolysis and directed HPLC analysis of the phenylisothiocyanate derivatives using UV detector based on previously reported convenient method.11) The presence of the dihydroflavonol skeleton was further suggested by the ¹H-NMR spectrum of 2 (Table 1) for diagnostic signals at δ_H 5.45 (1H, s, H-3), an ABX system at δ_H 7.13 (1H, d, J=1.8 Hz, H-2'), 6.68 (1H, dd, J=8.4, 1.8 Hz, H-6'), and 6.65 (1H, d, J=8.4 Hz, H-5'). and two meta-coupled AB system at δ_H 6.00 (1H, d, J=1.8 Hz, H-6) and 5.80 (1H, d, J=1.8 Hz, H-8). The ¹³C-NMR spectrum of 2 confirmed the presence of a dihydroflavonol skeleton for this molecule, with characteristic C-ring resonances at δ_C 197.1 (C-4), 89.3 (C-2), and 76.4 (C-3).10) Ad-
ditional characteristic signals of two anomeric protons at $\delta^1_H$ 4.85 (1H, d, $J=7.8$ Hz, H-1") and 4.76 (1H, d, $J=1.8$ Hz, H-1'), as well as 10 oxygen-bearing signals at $\delta^1_H$ 3.97–3.37, along with a doublet methyl proton at $\delta^1_H$ 1.19 supported the presence of a rutinoside moiety in the molecule. The $^1$H-NMR spectroscopic data of 2 also revealed resonances corresponding to a hydroxymethyl substituent at $\delta^1_H$ 3.98 (1H, d, $J=12.6$ Hz, H-11a) and 3.65 (1H, d, $J=12.6$ Hz, H-11b). Consistent with these $^1$H-NMR observations, the $^{13}$C-NMR and heteronuclear single quantum coherence (HSQC) spectra of 2 closely resembled those of the parent rutin, except for the presence of an oxygenated hydroxymethyl (H-11) and oxymethine (H-3) signals. The linkage point of the hydroxymethyl residue at C-2 in 2 was determined unambiguously from the key heteronuclear multiple bond connectivity (HMBC) spectrum, which showed H-11/C-2, 3, 1 correlations (Fig. 2). The relative stereostructures of the pseudo-axial catechol on C-2 and the pseudo-equatorial sugar moiety on C-3 positions in the C-ring were characterized by the nuclear Overhauser effect spectroscopy (NOESY) spectrum (Fig. 2). The spectrum of 2 displayed correlations between H-3/H-11, 1", 2", indicating a trans-relationship between the phenyl group and the rutinosyl

![Fig. 1. Structures of New Degraded Products 2–4 of Rutin](image-url)

### Table 1. $^1$H- and $^{13}$C-NMR Shifts of Compounds 2–4<sup>a</sup>

| Position | $\delta^1_H$ (J in Hz) | $\delta^1_C$, multit. | $\delta^1_H$ (J in Hz) | $\delta^1_C$, multit. | $\delta^1_H$ (J in Hz) | $\delta^1_C$, multit. |
|----------|------------------------|-----------------------|------------------------|-----------------------|------------------------|-----------------------|
| 2 | — | 89.3 | — | 89.3 | — | 82.3 |
| 3 | 5.45 (s) | 76.4 | 5.45 (s) | 78.0 | 5.86 (d, 2.4) | 98.6 |
| 4 | — | 197.1 | — | 195.9 | — | 154.5 |
| 5 | — | 162.2 | — | 161.5 | — | 159.3 |
| 6 | 6.00 (d, 1.8) | 97.3 | 6.01 (d, 1.8) | 96.6 | 5.81 (d, 2.4) | 97.1 |
| 7 | — | 168.2 | — | 167.4 | — | 155.9 |
| 8 | 5.80 (d, 1.8) | 96.8 | 5.81 (d, 1.8) | 96.0 | 5.81 (d, 2.4) | 94.6 |
| 9 | — | 163.8 | — | 162.9 | — | 104.6 |
| 10 | — | 102.4 | — | 101.3 | — | 159.3 |
| 11a | 3.98 (d, 12.6) | 76.4 | 3.92 (d, 12.6) | 66.5 | 4.17 (d, 12.6) | 65.8 |
| 11b | 3.65 (d, 12.6) | 3.72 (d, 12.6) | 4.10 (d, 12.6) | 4.10 (d, 12.6) | 5.07 (d, 12.0) | 56.9 |
| 12a | — | — | — | — | — | — |
| 12b | — | — | — | — | — | — |
| 1' | — | — | — | — | — | — |
| 2' | 7.13 (d, 1.8) | 115.5 | 7.11 (d, 1.8) | 114.5 | 7.00 (d, 2.4) | 115.3 |
| 3' | — | 145.1 | — | 144.7 | — | 145.9 |
| 4' | — | 145.9 | — | 145.1 | — | 146.1 |
| 5' | 6.65 (d, 8.4) | 115.8 | 6.66 (d, 8.4) | 115.0 | 6.71 (d, 8.0) | 115.2 |
| 6' | 6.68 (dd, 8.4, 1.8) | 119.7 | 6.86 (dd, 8.4, 1.8) | 118.8 | 6.80 (dd, 8.0, 2.4) | 120.1 |
| 1" | 4.85 (d, 7.8) | 103.8 | 4.77 (d, 7.8) | 105.2 | 4.59 (d, 7.8) | 100.8 |
| 2" | 3.50 (m) | 77.0 | 3.53 (m) | 76.2 | 3.50 (m) | 76.0 |
| 3" | 3.37 (t, 9.6) | 73.5 | 3.46 (t, 9.6) | 74.2 | 3.44 (t, 9.6) | 73.6 |
| 4" | 3.52 (m) | 73.4 | 3.51 (m) | 75.5 | 3.47 (m) | 74.2 |
| 5" | 3.57 (m) | 76.7 | 3.54 (m) | 76.2 | 3.34 (m) | 77.4 |
| 6"a | 3.97 (dd, 12.0, 2.4) | 67.1 | 4.09 (dd, 12.0, 2.4) | 67.1 | 3.85 (m) | 71.7 |
| 6"b | 3.73 (dd, 12.0, 4.8) | 67.1 | 3.50 (dd, 12.0, 4.8) | 3.70 (m) | 101.1 |
| 1"" | 4.76 (d, 1.8) | 101.7 | 4.73 (d, 1.2) | 100.8 | 4.78 (d, 1.2) | 101.1 |
| 2"" | 3.85 (dd, 3.6, 1.8) | 71.4 | 3.94 (dd, 3.6, 1.8) | 70.3 | 3.88 (m) | 71.2 |
| 3"" | 3.69 (m) | 69.2 | 3.66 (m) | 68.5 | 3.55 (m) | 67.6 |
| 4"" | 3.40 (m) | 71.9 | 3.38 (t, 9.6) | 72.4 | 3.33 (m) | 71.2 |
| 5"" | 3.45 (m) | 70.6 | 3.56 (m) | 71.0 | 3.67 (dd, 9.6, 6.0) | 69.3 |
| 6"" | 1.19 (d, 6.6) | 18.1 | 1.19 (d, 6.0) | 17.2 | 1.25 (d, 6.0) | 18.1 |

<sup>a</sup> $^1$H-NMR measured at 600 MHz, $^{13}$C-NMR measured at 150 MHz; obtained in acetone-$d_6$+D$_2$O with TMS as internal standard. Assignments based on HSQC and HMBC NMR spectra. <sup>b</sup> $J$ values (Hz) are given in parentheses.
moiety. While, other possible pseudo-equatorial sugar moiety on C-3 positions can be discarded by the observed NOESY correlation of H-3/H-2. Furthermore, an energy-minimized model generated by Chem3D Ultra 10.0 based on a presumed configuration matched well with the obtained NOESY correlations (Fig. 2). The circular dichroism (CD) spectrum of 2 showed positive and negative Cotton effects at 291 and 331 mm, respectively, indicating that the absolute configuration of 2 was the 2R,3S-configuration. Therefore, the absolute structure of 2 was deduced as radiorutinol, which is a new radiolysis product as shown in Fig. 1.

The HR-FAB-MS analysis of compound 3 showed a molecular ion peak at m/z 641.1721 [M−H]−, indicating a molecular formula of C28H34O17, which is the same as that of 2. The NMR and UV spectral data of 3 were also nearly identical to those of 2 suggesting that these two compounds are diastereoisomers. The absolute configurations of the component monosaccharides were determined as D-glucose and L-rhamnose by HPLC analysis of their arylthiocyanate derivatives using C18 reversed-phase column.15) The location of the aliphatic residue was unambiguously elucidated by key HMBC correlations of H-11/C-2, 3, 1′ correlations (Fig. 2). As shown in Fig. 2, the 2,3-trans stereochemistry of the pseudo-equatorial catechol on C-2 and the pseudo-axial sugar moiety on C-3 positions in the aglycone moiety were also tentatively proposed by NOESY correlations of H-3/H-2′, 1′. The absolute stereochemistry of 2S,3R were determined on the basis of negative and positive Cotton effects at 301 and 332 mm, respectively, in the CD spectra based on a comparison with the authentic analogs.18) Accordingly, the structure of 3 was assigned as isoradiorutinol, which is unknown in the literature.

Compound 4 was obtained as a colorless oil, [α]D20 +21.4 (MeOH). The HR-FAB-MS of 4 had a molecular ion peak at m/z 655.1879 [M−H]−, which is consistent with the molecular formula C29H36O17. Similarities between the 1D NMR spectra of 4, including complete analysis of the HSQC, HMBC, and NOESY experiments, and those of 2 and 3 indicated that 4 is also a rutin analogue.17) Also, absolute configuration of the component monosaccharides were determined after acid hydrolysis as d-glucose and l-rhamnose by convenient sugar analytical method.15) Comparison of the 1H- and 13C-NMR data of 4 with those of 2 and 3 (Table 1) indicated the presence of an extra hydroxymethyl functionalities at δH 5.07 (1H, d, J=12.0 Hz, H-12a) and 4.00 (1H, d, J=12.0 Hz, H-12b). Further, C-3 and C-4 were found to be unsaturated in 4, which was implied by the replacement of 13C-NMR signals at δC 76.4 (C-3) and 197.1 (C-4) in 2 by signals at δC 143.9 (C-3) and 123.7 (C-4) in 4. These spectral features suggest that 4 is an unusual flavonoid with two hydroxymethyl groups in its dihydropyran ring. The hydroxymethyl groups were located at C-2 and C-4, based on the key HMBC correlations (Fig. 2). Based on the above evidence, the planar structure of new 4 possessing two hydroxymethyl groups modified by γ-irradiation was deduced as radiorutindiol, which comprises the unusual 2H-pyran C-ring system.

Among the naturally occurring polyphenols are widely distributed in various food stuffs such as fruits, vegetables, wines, and juices, and divided into several sub classes including flavonoids, phenolic acids, stilbenes, and lignans.19) Flavonoids are one of the largest plant secondary metabolites and this huge class of natural compounds has been shown to possess a wide spectrum of significant biological benefits, such as antioxidant, anti-inflammatory, antiviral, anticancer, and chemopreventive properties.20) Several naturally occurring flavonoids have been transformed into structurally modified products using microbial and enzymatic methods. A previous study demonstrated that the biotransformed metabolites of rutin derived from Aspergillus niger exhibited significantly
improved antiproliferative effects.\textsuperscript{21)}

Managing of glucose level in postprandial plasma is essential in the early treatment of diabetes. Enzyme inhibition like $\alpha$-glucosidase and $\alpha$-amylase, which are played in the carbohydrate digestion, is a valuable strategy for decreasing postprandial hyperglycemia.\textsuperscript{22)} According to several \textit{in vivo} studies, $\alpha$-glucosidase and $\alpha$-amylase are further verified to be one of the most valuable approaches for diabetes.\textsuperscript{23,24)} In our systemic investigation, structural change of rutin using irradiation processing showed potentially improved $\alpha$-glucosidase inhibitory activity. As summarized in Table 2, the degraded rutin analogue isoradiorutinol (3), having $\Delta S,\Delta R$-stereostucture at the chiral centers, was found to exhibit significantly higher $\alpha$-glucosidase inhibitory activity with an IC$_{50}$ value of 11.2$\pm$0.7 $\mu$m than the parent rutin (1). Interestingly, another structurally similar radiorutinol (2), with $2R,3S$-stereostucture, was found to exhibit relatively weaker inhibitory activity in these bioassays than 3. Furthermore, the isolated unusual degraded product, radiorutindiol (4), showed slightly decreased $\alpha$-glucosidase inhibitory activity with an IC$_{50}$ value of 56.2$\pm$3.5 $\mu$m. These findings indicate that the replacement and stereochemy of the hydroxymethyl group at the C-2 position in dihydroflavonol skeleton may have a positive influence on inhibition of $\alpha$-glucosidase.

Methanolic radiolysis in the presence of oxygen could produce various free radicals such as methoxy $(CH_2O)$, hydroxyalkyl (‘CH$_3$OH), hydrogen (H$^\cdot$), superoxide anion $(O_2$)$^-$, and peroxy $(HO_2$)$^-$ radicals, leading to formation of oxygen-bearing by-products.\textsuperscript{25)} In this investigation, free radicals induced by the alchohol radiolysis might have affected donation of hydroxymethyl groups in the molecule. Further systematic investigations are required to verify a hypothesis concerning the reaction mechanism of radiolysis with $\gamma$-irradiation of the related compounds.

The current study verifies that rutin is degraded into three new modified products 2, 3, and 4. The structures of the new hydroxymethyl derived from rutin were established based on the spectroscopic data. The new unusual products 2 and 3 containing a hydroxymethyl group at C-2 position exhibited more potent inhibitory activity against $\alpha$-glucosidase than the parent rutin. Further systematic investigation into the effects of $\gamma$-irradiation on biological properties of other natural flavonoids is now in progress.

### Experimental

**General Experimental Procedures** Rutin and acarbose were purchased from Sigma-Aldrich Corp. (St. Louis, MO, U.S.A.). All other chemicals used in this study were of analytical grade. UV spectra were obtained using a Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) and CD spectra were run on a JASCO J-720W spectrometer (JASCO, Tokyo, Japan). $^1$H- and $^{13}$C-NMR spectra were measured on a Varian VNS600 instrument (Varian, Palo Alto, CA, U.S.A.) operated at 600 and 150 MHz, respectively. Chemical shifts are given in $\delta$ (ppm) values relative to those of the solvent acetone-$d_6$ ($\delta_H$ 2.04, $\delta_C$ 29.8) on a tetramethylsilane (TMS) scale. The standard pulse sequences programmed into the instruments were used for each 2D measurement. The $J_{CH}$ value was set at 8 Hz in the HMBC spectra. FAB-MS were obtained on a Micro Mass Auto Spec OA-TOF spectrometer (Micromass, Manchester, U.K.). Column chromatography was performed using Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan) and YMC GEL ODS AQ 120-50S (YMC Co., Kyoto, Japan). TLC was performed on Kieselgel 60 F$_{25}_4$ plates (0.25 mm layer thickness, Merek, Darmstadt, Germany), and spots were detected by UV irradiation (254, 365 nm) as well as spraying with 10% H$_2$SO$_4$ reagent.

**Sample Preparation and Isolation Procedure** A cobalt-60-$\gamma$-source with an activity of approximately 215 kCi with a dose rate of 25 kGy/h was used for irradiation. A sample solution of rutin (1.0 g) in MeOH (1.0 L) in capped vials was irradiated with $\gamma$-ray 25 kGy (absorbed dose). The irradiated rutin were directly dried and successively monitored by reversed-phase HPLC analysis and exhibited most improved inhibitory activity with an IC$_{50}$ value of 37.1$\pm$2.6 $\mu$g/mL against $\alpha$-glucosidase inhibition assay using a previously reported method.\textsuperscript{26)} A part of the irradiated sample (700 mg) was subjected to column chromatography over a Toyopearl HW-40 column (coarse grade; 2.8 cm i.d.$\times$32 cm) and a YMC GEL ODS AQ 120-50S column (1.5 cm i.d.$\times$36 cm) with H$_2$O containing increasing amounts of MeOH in a stepwise gradient to yield pure new compounds 2 (9.5 mg, t$_R$ 10.3 min), 3 (9.8 mg, t$_R$ 11.6 min), and 4 (1.7 mg, t$_R$ 7.8 min) (Fig. 1). HPLC analysis was carried out on a YMC-Pack ODS A-302 column (4.6 mm i.d.$\times$150 mm; YMC Co., Ltd.) and the solvent system consisted of a linear gradient that was initiated with MeCN in 0.1% HCOOH–H$_2$O (detection: UV 280 nm; flow rate: 1.0 mL/min; at 40°C), increased to 20% MeCN over 15 min, and then increased to 100% MeCN over 20 min.

| Compound | IC$_{50}$ value ($\mu$m)${}^a$ |
|----------|------------------|
| 1        | $>500$           |
| 2        | 23.1$\pm$1.2     |
| 3        | 11.2$\pm$0.7     |
| 4        | 56.2$\pm$3.5     |
| Acarbose | 310.2$\pm$3.6    |

$a$) All compounds were examined in triplicate experiments and means within column with different letters differ significantly ($p<0.05$). b) Used as positive control.
warmed at 60°C for 1h. A 0.1mL solution of o-tolylisothiocyanate (0.5mg) in pyridine was added to the reaction mixture, which was warmed at 60°C for 1h. The mixture was directly analyzed by reversed-phase HPLC. HPLC analysis was carried out on a Cosmosil 5C18-MS-II column (4.6mm i.d.×250mm; Nacalai Tesque Inc., Japan) with isocratic mode of 25% MeCN in 50mM H3PO4 (detection: UV 250nm; flow rate: 0.8mL/min; at 40°C). Under above analytical conditions, authentic D/L-glucose, D/L-rhamnose gave HPLC peaks at tR 17.8/16.4 and 15.7/30.2 min, respectively. The retention times of the reacted sugars obtained by acid hydrolysis gave similar results as those of authentic sugar derivatives.

Inhibitory Effects of α-Glucosidase A previously reported method26) with minor modifications was used to evaluate α-glycosidase inhibitory activities of the compounds. Briefly, the reaction mixture consisted of enzyme solution (0.02 unit of α-glucosidase, 50µL), substrate (1mM p-nitrophenyl-α-D-glucopyranoside, 50µL) in 50mM phosphate buffer (pH 7.0), and test sample in 5% dimethyl sulfoxide (DMSO) (10µL). After incubation at 37.5°C for 20min, 2M NaOH was added to stop the reaction. The amount of released p-nitrophenol was measured at 410nm using a UV microplate reader (Infinite M200; Tecan Austria GmBH, Grödig, Austria). The IC50 value was calculated by linear regression analysis of inhibitory activities under the assay conditions. Acarbose was used as a positive control, and all assays were carried out in triplicate.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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