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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and cytotoxic activity of genistein derivatives

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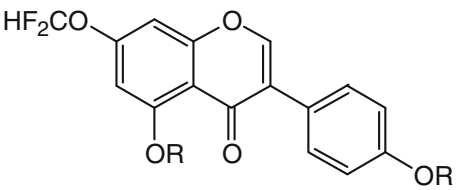
Abstract A series of genistein derivatives was prepared and tested in vitro against leukocythemia (HL-60), colorectal adenocarcinoma (HT-29), and human gastric adenocarcinoma (SGC-7901) cell lines. Among these derivatives, 4′,5-di-*n*-octoxy-7-*gem*-difluoromethylenegenistein, **9f**, had the strongest activity against HL-60, HT-29, and SGC-7901 cells.



9f R = n-C_8H_{17}

Keywords Synthesis · Genistein · Cytotoxicity · MTT assay

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Introduction

Cancer represents one of the most serious health problems in the world. Major advances have been made in early detection, prevention, and treatment. However, anticancer agents generally act on metabolically active or rapidly proliferating cells, and cannot distinguish between cancer and normal cells (Kumar *et al*., 2007; Zhang *et al*., 2005). Thus, toxicity to normal cells limits the amount of drugs that can be given to patients (Kumar *et al*., 2003).Therefore, in recent years, there has been growing interest in the search for anticancer substances with high efficacy, low toxicity, and minimum side effects. Since many clinically successful anticancer drugs either are natural products or have been developed from naturally occurring lead compounds, one approach to finding new anticancer drugs is to search chemicals of plant origin. Although isoflavones enjoy a limited distribution in the plant kingdom, these compounds have received much attention recently due to their interesting biological activities (Gao *et al*., 2003; Peterson and Barnes, 1991). Apart from their important biological property of estrogenic activity, isoflavones possess a broad range of pharmacological properties including antioxidant or possible therapeutic applications in the prevention of coronary heart diseases and cancers (Uckun *et al*., 1995; MerzDemlow *et al*., 2000). It is well known that Asian populations, who have low rates of breast and prostate cancers, consume 20–80 mg of genistein/day, whereas the dietary intake of genistein in the United States is only 1–3 mg/day. A typical isoflavone is the commonly occurring genistein. Its many biological activities have made it the subject of more than 3,600 published studies (listed in Biological Abstracts) in the past 10 years. Most of these studies have focused on the pharmacological activities of genistein as a tyrosine kinase inhibitor, its chemoprotectant activities against cancers and cardiovascular diseases, and its phytoestrogen activities (Dixon and Ferreira, 2002). In the anticancer area, genistein can reduce the risk of breast (Peterson and Barnes, 1996; Kousidou *et al*., 2006), uterus, ovary, stomach, and lung cancers and leukemia and mortality of prostate cancer in humans because it not only inhibits angiogenesis in vitro and in vivo but also inhibits tumor cell growth and tumor cell proliferation (Mitchell *et al*., 2000; Zhou *et al*., 1998). However, its anticancer activities are low. Numerous studies in vitro have shown a close relationship between the chemical structure and the biological activity of genistein derivatives, whereby their basic structure can be modified to increase or decrease their biological activity (Kim *et al*., 2003; Gao *et al*., 2001). As part of our current work (Zheng *et al*., 2003), searching for anticancer substances, we describe herein the synthesis of genistein derivatives and their anticancer activities against leukocythemia (HL-60), colorectal adenocarcinoma (HT-29), and human gastric adenocarcinoma (SGC-7901) cell lines.

Chemistry and biological activity

Melting points were measured on a Kofler apparatus, uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AM 300(300-MHz) spectrometer with Me_4Si as internal standard. ^{19}F NMR spectra were obtained on a Bruker AM-300

spectrometer in $CDCl_3$ with $CFCl_3$ as external standard, downfield shifts being designated as negative. All chemical shifts (δ) are expressed as parts per million, and coupling constants (J) are given as hertz. Mass spectra were recorded on a Finnigan-MAT-8430 instrument using EI ionization at 70 eV. IR spectra were recorded on a Shimadzu IR-440 spectrometer. Elemental analyses (C and H) were performed on an Italian Carlo-Erba 1106 elemental analyzer. All reagents were commercial materials used without further purification.

4′,5-Dihydroxy-7-methoxygenistein (**2**)

A mixture of 1 mmol (270 mg) **1**, 980 mg K_2CO_3, 6 mmol MeI, and 10 ml acetone was stirred at room temperature for 24 h, and then the mixture was filtered to remove inorganic salts. After distillation of the acetone, 7-methoxygenistein, **2** (4.1%), was obtained by column chromatography on silica gel and elution with 98:8 petroleum ether:ethyl acetate. MS (EI, 70 eV) *m/z*: 284 (M^+, 100.00), 166 (23.73), 167 (20.38), 285 (20.07), 138 (17.21), 95 (12.47), 118 (12.18), 255 (11.72). ^{1}H NMR (300 MHz, DMSO-d_6): 3.830 (3H, s, OC$\underline{H_3}$), 6.366 (1H, d, J = 2.4 Hz, H-6), 6.604 (1H, d, J = 2.4 Hz, H-8), 6.798 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 7.361 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 8.358 (1H, s, H-2), 9.561 (1H, s, O$\underline{H}$-4′), 12.890 (1H, s, O$\underline{H}$-5).

General procedures for preparation of **3a**–**3d**, **4a**, and **4b**

A mixture of 1 mmol (270 mg) **1**, 980 mg K_2CO_3, 6 mmol RX, and 10 ml acetone was stirred at reflux for 6–24 h, and then the mixture was filtered to remove inorganic salts. After distillation of the acetone, dialkylated products **3a**–**3d** and trialkylated **4a** and **4b** were isolated by column chromatography on silica gel and elution with 98:2 or 90:10 petroleum ether:ethyl acetate.

*4′,7-Dimethoxy-5-hydroxygenistein (**3a**)*

Yield, 83.5%. MS (EI, 70 eV) *m/z*: 298 (M^+, 100.00), 271 (34.39), 132 (21.39), 299 (19.86), 166 (19.24), 283 (12.00), 138 (11.48), 89 (10.61). ^{1}H NMR (300 MHz, $CDCl_3$): 3.381 (3H, s, OC$\underline{H_3}$), 3.900 (3H, s, OC$\underline{H_3}$), 6.407 (1H, d, J = 2.4 Hz, H-6), 6.422 (1H, d, J = 2.4 Hz, H-8), 7.003 (2H, d, J = 9.9 Hz, H-3′ and H-5′), 7.484 (2H, d, J = 9.9 Hz, H-2′ and H-6′), 7.893 (1H, s, H-2), 12.890 (1H, s, O$\underline{H}$).

*4′,7-Diallyloxy-5-hydroxygenistein (**3b**)*

Yield, 77.5%; m.p., 117–119.5°C. MS (EI, 70 eV) *m/z*: 349 (100.00), 308 (37.62), 350 (M^+, 23.86), 275 (17.25), 291 (16.94), 41 (14.77), 293 (14.48). IR υmax (cm^{-1}, KBr): 1649 (C=O), 2954 (OH). ^{1}H NMR (300 MHz, $CDCl_3$): 4.556–5.087 (4H, m, 2 × C$\underline{H_2}$O), 5.283–5.485 (4H, m, 2 × C$\underline{H_2}$), 5.928–6.114 (2H, m, 2 × C$\underline{H}$), 6.372 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 6.982 (1H, d, J = 2.1 Hz, H-6), 7.428 (1H, d, J = 2.1 Hz, H-8), 7.833 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 7.862 (1H, s, H-2), 12.981 (1H, s, O$\underline{H}$). ^{13}C NMR($CDCl_3$): 180.824 (C=O, C-4), 164.461 (C-7),

162.722 (C-5), 158.816 (C-4′), 157.877 (C-9), 152.675 (C-2), 133.077 (allyl-4′, CH), 132.047 (allyl-7, CH), 130.056 (2C, C-2′ and C-6′), 123.633 (C-3), 123.076 (C-1′), 118.507 (allyl-4′, CH_2), 117.797 (allyl-7, CH_2), 114.906 (2C, C-3′ and C-5′), 114.654 (C-10), 98.772 (C-6), 93.134 (C-8), 69.120 (allyl-4′, OCH_2), 68.868 (allyl-7, OCH_2). Anal. Calcd. for $C_{21}H_{18}O_5$: C, 71.99, H, 5.18. Found: C, 71.86, H, 5.16.

*4′,7-Dioctyloxy-5-hydroxygenistein (**3c**)*

Yield, 75.6%; m.p., 85.5–87°C. MS (EI, 70 eV) *m/z*: 494 (M^+, 100.00), 270 (73.92), 495 (34.42), 382 (28.34), 242 (18.08), 271 (18.07), 43 (16.34), 269 (15.52). IR υmax (cm^{-1}, KBr): 1666 (C=O), 3128 (OH). ^{1}H NMR (300 MHz, $CDCl_3$): 0.867–2.171 (30H, m, 2 × n-C_7H_{15}), 3.957-4.028 (4H, m, 2 × CH_2O), 6.358 (1H, d, J = 2.1 Hz, H-6), 6.380 (1H, d, J = 2.1 Hz, H-8), 6.960 (2H, d, J = 8.4 Hz, H-3′ and H-5′), 7.437 (2H, d, J = 8.4 Hz, H-2′ and H-6′), 7.845 (1H, s, H-2), 12.857 (1H, s, OH). ^{13}C NMR($CDCl_3$): 180.839 (C=O, C-4), 165.125 (C-7), 162.283 (C-5), 159.373 (C-4′), 157.946 (C-9), 152.576 (C-2), 130.041 (2C, C-2′ and C-6′), 123.671 (C-3), 122.733 (C-1′), 114.670 (2C, C-3′ and C-5′), 106.126 (C-10), 98.566 (C-6), 92.844 (C-8), 68.693 (C-1‴), 68.113 (C-1″), 31.809 (C-6‴), 31.786 (C-6″), 29.345-28.933 (6C, C-2″ and C-2‴, C-4″ and C-4‴, C-5″ and C-5‴), 26.026 (C-3‴), 25.927 (C-3″), 22.647 (2C, C-7″ and C-7‴), 14.088 (2C, C-8″ and C-8‴). Anal. Calcd. for $C_{31}H_{42}O_5$: C, 75.27, H, 8.56. Found: C, 75.12, H, 8.47.

*4′,7-Didecyloxy-5-hydroxygenistein (**3d**)*

Yield, 73.3%; m.p., 89–90°C. MS (EI, 70 eV) *m/z*: 550 (M^+, 100.00), 270 (60.92), 551 (38.64), 410 (26.75), 271 (17.81), 43 (16.01), 242 (15.18), 269 (14.23). IR υmax (cm^{-1}, KBr): 1665 (C=O), 3123 (OH). ^{1}H NMR (300 MHz, $CDCl_3$): 0.863–1.827 (38H, m, 2 × n-C_9H_{19}), 3.982–4.031 (4H, m, 2 × CH_2O), 6.364 (1H, d, J = 2.4 Hz, H-6), 6.381 (1H, d, J = 2.4 Hz, H-8), 6.963 (2H, d, J = 8.4 Hz, H-3′ and H-5′), 7.439 (2H, d, J = 8.4 Hz, H-2′ and H-6′), 7.847 (1H, s, H-2), 12.859 (1H, s, OH). ^{13}C NMR($CDCl_3$): 180.832 (C=O, C-4), 165.125 (C-7), 162.283 (C-5), 159.373 (C-4′), 157.938 (C-9), 152.560 (C-2), 130.041 (2C, C-2′ and C-6′), 123.664 (C-3), 122.733 (C-1′), 114.662 (2C, C-3′ and C-5′), 106.126 (C-10), 98.566 (C-6), 92.837 (C-8), 68.693 (C-1‴), 68.113 (C-1″), 31.885 (2C, C-8″ and C-8‴), 29.558–28.933 (10C, C-2″ and C-2‴, C-4″ and C-4‴,C-5″ and C-5‴, C-6″ and C-6‴, C-7″ and C-7‴), 26.026 (C-3‴), 25.920 (C-3″), 22.670 (2C, C-9″ and C-9‴), 14.111 (2C, C-10″ and C-10‴). Anal. Calcd. for $C_{35}H_{50}O_5$: C, 76.33, H, 9.15. Found: C, 76.12, H, 9.15.

*4′,5,7-Trimethoxygenistein (**4a**)*

Yield, 10.1%. MS (EI, 70 eV) *m/z*: 312 (100.00), 313 (75.82), 266 (29.62), 267 (25.53), 132 (22.94), 283 (20.88), 281 (19.92), 282 (17.92). ^{1}H NMR (300 MHz, DMSO-d_6): 3.759 (3H, s, OCH_3), 3.806 (3H, s, OCH_3), 3.857 (3H, s, OCH_3), 6.484 (1H, d, J = 2.4 Hz, H-6), 6.635 (1H, d, J = 2.4 Hz, H-8), 6.940 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 7.416 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 8.167 (1H, s, H-2).

*4′,5,7-Triallyloxygenistein (**4b**)*

Yield, 11.6%; m.p., 101–104°C. MS (EI, 70 eV) *m/z*: 390 (M^+, 100.00), 361 (95.87), 293 (88.82), 41 (45.92), 321 (44.10), 349 (37.56), 252 (37.22), 362 (34.97). IR υmax (cm^{-1}, KBr): 1644 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): 4.546-4.637(6H, m, 3 × $C\underline{H}_2O$), 5.262–5.688(6H, m, 3 × $C\underline{H}_2$), 6.002–6.110 (3H, m, 3 × $C\underline{H}$), 6.383(2H, d, J = 2.1 Hz, H-6), 6.425(1H, d, J = 2.1 Hz, H-8), 6.939(2H, d, J = 8.7 Hz, H-3′ and H-5′), 7.440(2H, d, J = 8.7 Hz, H-2′ and H-6′), 7.719 (1H, s, H-2). ^{13}C NMR($CDCl_3$): 175.179 (C=O, C-4), 162.546 (C-7), 160.303 (C-5), 159.762 (C-4′), 158.419 (C-9), 149.970 (C-2), 133.237 (allyl-5, $\underline{C}H$), 132.108 (2C, allyl-7, allyl-4′, $\underline{C}H$), 130.361 (2C, C-2′ and C-6′), 126.028 (C-3), 124.548 (C-1′), 118.545 (allyl-5, $\underline{C}H_2$), 118.003 (allyl-4′, $\underline{C}H_2$), 117.614 (allyl-7, $\underline{C}H_2$), 114.555 (2C, C-3′ and C-5′), 110.268 (C-10), 97.803 (C-6), 93.592 (C-8), 69.837 (allyl-5, $O\underline{C}H_2$), 69.158 (allyl-4′, $O\underline{C}H_2$), 68.799 (allyl-7, $O\underline{C}H_2$). Anal. Calcd. for $C_{24}H_{22}O_5$: C, 73.83, H, 5.68. Found: C, 73.87, H, 5.72.

4′,7-Diacetoxy-5-hydroxygenistein (**5**) and 7-acetoxy-4′,5-dihydroxy-genistein (**6**)

A mixture of 1 mmol (270 mg) **1**, 10 ml acetic anhydride and a drop of pyridine was heated at reflux temperature for 7 h and poured into ice-cold water. The yellow solid precipitate was filtered, and **5** and **6** were isolated by column chromatography on silica gel and elution with CH_2Cl_2 or 98:2 CH_2Cl_2:MeOH.

*4′,7-Diacetoxy-5-hydroxygenistein (**5**)*

Yield, 87.2%; m.p., 200–202°C. MS (EI, 70 eV) *m/z*: 270 (100.00), 312 (38.98), 43 (18.93), 269 (17.18), 354 (16.99), 271 (16.51), 153 (9.27), 246 (7.99). IR υmax (cm^{-1}, KBr): 1656 (C=O), 3076 (OH). ^{1}H NMR (300 MHz, $CDCl_3$): 2.338 (3H, s, $C\underline{H}_3COO$), 2.346 (3H, s, $C\underline{H}_3COO$), 6.610 (1H, d, J = 1.8 Hz, H-6), 6.783 (1H, d, J = 1.8 Hz, H-8), 7.199 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 7.563 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 7.982 (1H, s, H-2), 12.748 (1H, s, O$\underline{H}$).

*7-Acetoxy-4′,5-dihydroxygenistein (**6**)*

Yield, 5.3%; m.p., 265°C. MS (EI, 70 eV) *m/z*: 284 (100.00), 166 (22.33), 167 (16.82), 138 (14.94), 255 (11.75), 312 (M^+, 10.48), 118 (9.26), 95 (9.23). IR υmax (cm^{-1}, KBr): 1670 (C=O), 3013 (OH), 3384 (OH). ^{1}H NMR (300 MHz, DMSO-d_6): 2.863 (3H, s, $C\underline{H}_3COO$), 6.404 (1H, d, J = 2.1 Hz, H-6), 6.649 (1H, d, J = 2.1 Hz, H-8), 6.827 (2H, d, J = 8.4 Hz, H-3′ and H-5′), 6.391 (2H, d, J = 8.4 Hz, H-2′ and H-6′), 7.400 (1H, s, H-2), 8.597 (1H, s, O$\underline{H}$-4′), 11.951 (1H, s, O$\underline{H}$-5). Anal. Calcd. for $C_{17}H_{12}O_6$: C, 65.39, H, 3.87. Found: C, 65.46, H, 3.93.

7-*gem*-Difluoromethylenated genistein (**7**) and 4′,7-di-*gem*-difluoromethylenated genistein (**8**)

A mixture of 51 mmol genistein, 20 g NaOH, 12.5 ml H_2O, and 15 ml dioxane was prepared in a four-necked flask equipped with thermometer, stirrer, gas-inlet tube extending below the liquid surface, and reflux condenser. The temperature was adjusted to 70–75°C and HCF_2Cl was introduced for 8 h. The mixture was allowed to cool to room temperature, then diluted with 50 ml H_2O, 10 ml ether was added, and the mixture was filtered to remove precipitated inorganic salts. The water layer was extracted with three 10-ml portions of ether and dried over anhydrous Na_2SO_4. After distillation of the ether, purification of the residue by column chromatography on silica gel and elution with 95:5 petroleum ether:ethyl acetate gave compound **7** in a 42.0% yield and **8** in a 15.8% yield.

7-gem-Difluoromethylenated genistein (7)

M.p., 194–195°C. MS (EI, 70 eV) *m/z*: 320 (M^+, 100.00), 319 (27.42), 203 (22.57), 118 (18.14), 321 (17.72), 202 (7.10), 89 (5.25), 51 (4.90). IR υmax (cm^{-1}, KBr): 1662 (C=O), 3319 (OH), 3528 (OH). ^{1}H NMR (300 MHz, $CDCl_3$): 6.599 (1H, d, J = 2.4 Hz, H-6), 6.820 (2H, d, J = 12.0 Hz, H-3′ and H-5′), 6.867 (1H, d, J = 2.4 Hz, H-8), 7.371 (2H, d, J = 12.0 Hz, H-2′ and H-6′), 7.427 (1H, t, J = 72.9 Hz, $\underline{H}F_2CO$), 8.244 (1H, s, H-2), 9.550 (1H, s, O$\underline{H}$-4′), 12.994 (1H, s, O$\underline{H}$-5). ^{19}F NMR (300 MHz): −84.372 (d, J = 72.9 Hz). Anal. Calcd. for $C_{16}H_{10}F_2O_5$: C, 60.08, H, 3.15. Found: C, 60.07, H, 2.94.

4′,7-Di-gem-difluoromethylenated genistein (8)

m.p., 103–106°C. MS (EI, 70 eV) *m/z*: 370 (M^+, 100.00), 253 (50.40), 369 (26.68), 202 (22.67), 371 (18.52), 118 (11.40), 155 (10.85), 174 (10.81). IR υmax (cm^{-1}, KBr): 1653 (C=O), 3074 (OH)· ^{1}H NMR (300 MHz, $CDCl_3$): 6.561 (1H, t, J = 73.8 Hz, $\underline{H}F_2CO$), 6.590 (1H, d, J = 2.4 Hz, H-6), 6.638 (1H, t, J = 72.3 Hz, $\underline{H}F_2CO$), 6.684 (1H, d, J = 2.4 Hz, H-8), 7.229 (2H, d, J = 9.0 Hz, H-3′ and H-5′), 7.551 (2H, d, J = 9.0 Hz, H-2′ and H-6′), 7.975 (1H, s, H-2), 12.774 (1H, s, O$\underline{H}$). ^{19}F NMR (300 MHz): −80.953 (d, J = 73.8 Hz), −2.414 (d, J = 72.3 Hz). Anal. Calcd. for $C_{17}H_{10}F_2O_5$: C, 55.31, H, 2.74. Found: C, 55.40, H, 2.53.

General procedures for preparation of **9a–9g**

A mixture of 1 mmol (320 mg) **7**, 980 mg K_2CO_3, 6 mmol RX, and 10 ml acetone was stirred at reflux for 6–2 4 h, then the mixture was filtered to remove inorganic salts. After distillation of the acetone, purification of the residue by column chromatography on silica gel and elution with 95:5 or 90:10 petroleum ether:ethyl acetate gave compounds **9a–9g** in a 81–95% yield, respectively.

*4′,5-Dimethoxy-7-gem-difluoromethylenated genistein (**9a**)*

Yield, 92.1%; m.p., 136–138°C. MS (EI, 70 eV) *m/z*: 348 (M^+, 100.00), 347 (41.07), 302 (25.34), 349 (19.45), 132 (18.11), 319 (15.70), 317 (13.69), 331 (11.19). IR υmax (cm^{-1}, KBr): 1637 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): 3.834 (3H, s, $OC\underline{H}_3$), 3.970 (3H, s, $OC\underline{H}_3$), 6.546 (1H, d, J = 2.1 Hz, H-6), 6.642 (1H, t, J = 72.3 Hz, $\underline{H}F_2CO$), 6.724 (1H, d, J = 2.1 Hz, H-8), 6.940 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 7.472 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 7.811 (1H, s, H-2). ^{19}F NMR (300 MHz): −82.150 (d, J = 72.3 Hz). Anal. Calcd. for $C_{18}H_{14}F_2O_5$: C, 62.07, H, 4.05. Found: C, 62.26, H, 4.15.

*4′,5-Diethoxy-7-gem-difluoromethylenated genistein (**9b**)*

Yield, 89.7%; m.p., 96–97°C. MS (EI, 70 eV) *m/z*: 376 (M^+, 100.00), 361 (97.06), 303 (42.10), 358 (32.81), 304 (32.33), 118 (27.28), 375 (26.81), 377 (21.98). IR υmax (cm^{-1}, KBr): 1613 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): 1.386–1.572 (6H, m, 2 × $C\underline{H}_3$), 4.023–4.181 (4H, m, 2 × $OC\underline{H}_2$), 6.525 (1H, d, J = 2.4 Hz, H-6), 6.628 (1H, t, J = 72.6 Hz, $\underline{H}F_2CO$), 6.699 (1H, d, J = 2.4 Hz, H-8), 6.931 (2H, d, J = 8.4 Hz, H-3′ and H-5′), 7.436 (2H, d, J = 8.4 Hz, H-2′ and H-6′), 7.780 (1H, s, H-2). ^{19}F NMR (300 MHz): −82.085 (d, J = 72.6 Hz). Anal. Calcd. for $C_{20}H_{18}F_2O_5$: C, 63.82, H, 4.82. Found: C, 63.69, H, 4.77.

*4′,5-Di-n-propoxy-7-gem-difluoromethylenated genistein (**9c**)*

Yield, 86.3%. MS (EI, 70 eV) *m/z*: 375 (100.00), 43 (39.12), 404 (M^+, 26.74), 41 (26.73), 118 (25.13), 304 (23.05), 376 (22.01), 303 (19.05). IR υmax (cm^{-1}, KBr): 1651 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): 0.972–1.120 (4H, m, 2 × $C\underline{H}_2$), 1.157 (3H, t, J = 7.5 Hz, $C\underline{H}_3$), 1.223 (3H, t, J = 7.5 Hz, $C\underline{H}_3$), 3.987–4.164 (4H, m, 2 × $C\underline{H}_2O$), 6.643 (1H, d, J = 2.4 Hz, H-6), 6.761 (1H, t, J = 72.6 Hz, $\underline{H}F_2CO$), 6.807 (1H, d, J = 2.4 Hz, H-8), 7.053 (2H, d, J = 9.0 Hz, H-3′ and H-5′), 7.547 (2H, d, J = 9.0 Hz, H-2′ and H-6′), 7.886 (1H, s, H-2). ^{19}F NMR (300 MHz): –82.142 (d, J = 72.6 Hz). Anal. Calcd. for $C_{22}H_{22}F_2O_5$: C, 65.34, H, 5.48. Found: C, 65.56, H, 5.66.

*4′,5-Dibenzyloxy-7-gem-difluoromethylenated genistein (**9d**)*

Yield, 94.7%. MS (EI, 70 eV) *m/z*: 91 (100.00), 500 (M^+, 9.06), 92 (8.20), 65 (8.09), 57 (6.32), 43 (4.67), 89 (4.12), 71 (3.91). IR υmax (cm^{-1}, KBr): 1647 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): 4.636 (4H, s, 2 × $C\underline{H}_2O$), 7.314–7.342 (18H, m, 2 × $C_6\underline{H}_5$, $\underline{H}F_2CO$, H-6, H-8, H-3′, H-5′, H-2′ H-6′, H-2). ^{19}F NMR (300 MHz): –82.161 (d, J = 72.2 Hz). Anal. Calcd. for $C_{30}H_{22}F_2O_5$: C, 71.99, H, 4.34. Found: C, 72.03, H, 4.52.

*4′,5-Di-n-heptoxy-7-gem-difluoromethylenated genistein (**9e**)*

Yield, 80.9%. MS (EI, 70 eV) *m/z*: 431 (100.00), 516 (M^+, 60.10), 517 (56.18), 41 (33.58), 57 (32.98), 43 (29.15), 432 (29.02), 320 (23.46). IR υmax (cm^{-1}, KBr):

1652 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): 0.906–2.016 (26H, m, 2 × n-$C_6\underline{H_{13}}$), 3.950 (2H, t, J = 6.6 Hz, $C\underline{H_2}O$), 4.047 (2H, t, J = 6.6 Hz, $C\underline{H_2}O$), 6.512 (1H, d, J = 2.1 Hz, H-6), 6.648 (1H, d, J = 2.1 Hz, H-8), 6.764 (1H, t, J = 72.9 Hz, $\underline{H}F_2CO$), 6.893 (2H, d, J = 9.0 Hz, H-3′ and H-5′), 7.412 (2H, d, J = 9.0 Hz, H-2′ and H-6′), 7.759 (1H, s, H-2). ^{19}F NMR (300 MHz): −82.897 (d, J = 72.9 Hz). Anal. Calcd. for $C_{30}H_{38}F_2O_5$: C, 69.75, H, 7.41. Found: C, 69.68, H, 7.36.

*4′,5-Di-n-octoxy-7-gem-difluoromethylenated genistein (**9f**)*

Yield, 82.0%. MS (EI, 70 eV) *m/z*: 445 (100.00), 446 (28.67), 320 (19.66), 544 (M^+, 15.24), 459 (12.97), 321 (12.45), 43 (11.29), 333 (10.55). IR υmax (cm^{-1}, KBr): 1651 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): 0.860–2.181 (30H, m, 2 × n-$C_7\underline{H_{15}}$), 3.985–4.064 (4H, m, 2 × $C\underline{H_2}O$), 6.378 (1H, d, J = 2.1 Hz, H-6), 6.423 (1H, d, J = 2.1 Hz, H-8), 6.531 (1H, t, J = 74.1 Hz, $\underline{H}F_2CO$), 7.155 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 7.543 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 7.748 (1H, s, H-2). ^{19}F NMR (300 MHz): −80.555 (d, J = 74.1 Hz). Anal. Calcd. for $C_{32}H_{42}F_2O_5$: C, 70.56, H, 7.77. Found: C, 70.67, H, 7.85.

*4′,5-Di-n-decoxy-7-gem-difluoromethylenated genistein (**9g**)*

Yield, 81.2%; m.p., 60–62°C. MS (EI, 70 eV) *m/z*: 473 (100.00), 43 (73.36), 57 (44.90), 320 (37.26), 55 (34.87), 41 (33.91), 474 (31.13), 600 (M^+, 24.62). IR υmax (cm^{-1}, KBr): 1658 (C=O). ^{1}H NMR (300 MHz, $CDCl_3$): 0.848–1.783 (38H, m, 2 × n-$C_9\underline{H_{19}}$), 3.966 (2H, t, J = 6.6 Hz, $C\underline{H_2}O$), 4.040 (2H, t, J = 6.6 Hz, $C\underline{H_2}O$), 6.513 (1H, d, J = 2.1 Hz, H-6), 6.627 (1H, t, J = 72.3 Hz, $\underline{H}F_2CO$), 6.674 (1H, d, J = 2.1 Hz, H-8), 6.925 (2H, d, J = 8.4 Hz, H-3′ and H-5′), 7.423 (2H, d, J = 8.4 Hz, H-2′ and H-6′), 7.753 (1H, s, H-2). ^{19}F NMR (300 MHz): −82.052 (d, J = 72.3 Hz). Anal. Calcd. for $C_{36}H_{50}F_2O_5$: C, 71.97, H, 8.39. Found: C, 72.13, H, 8.57.

Biological activity

HL-60, HT-29, and SGC-7901 cell lines were cultured and passaged in RPMI-1640 medium, supplemented with 10% (v/v) inactive calf bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin in a humidified incubator at 37°C under an atmosphere of 5% CO_2. Stock solutions of tested compounds were prepared in DMSO and were used for serial dilutions in culture medium. The cytotoxicity assay was performed by a modification of the MTT method (Mosmann, 1983).

Results and discussion

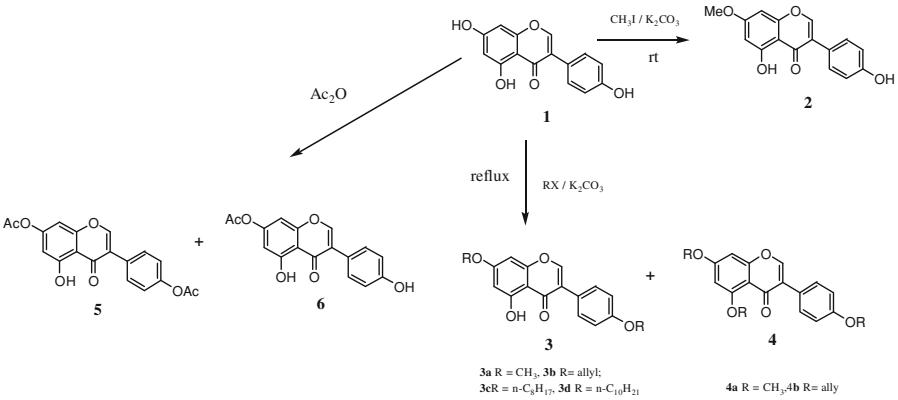
The readily available genistein (**1**) was used as the starting material for preparation of derivatives of genistein. Upon treatment of genistein (**1**) with CH_3I in the presence of K_2CO_3 in acetone at room temperature, 7-methoxygenistein (**2**) was obtained as the single product in a very low yield, together with the recovery of genistein. The reason might be that the acidity of the 7- and 4′-hydroxy groups of

genistein are different enough to allow selective ionization of the 7-OH group at relatively low temperatures. However, when the reaction mixture was stirred at reflux, dialkylated products (**3a**–**3d**) were isolated, as well as trialkylated products (**4a** and **4b**) in the case of CH_3I and allyl bromide. The ratios of **3a/4a** and **3b/4b** were about 8/1 and 9/1, respectively. Tri-*n*-octyloxygenistein and tri-*n*-decyloxygenistein were not obtained, probably due to the steric hindrance of octyl and decyl groups. A mixture of **1**, acetic anhydride (used as both reactant and solvent), and a drop of pyridine was heated at reflux temperature for **7** h to provide **5** and **6** (Scheme 1).

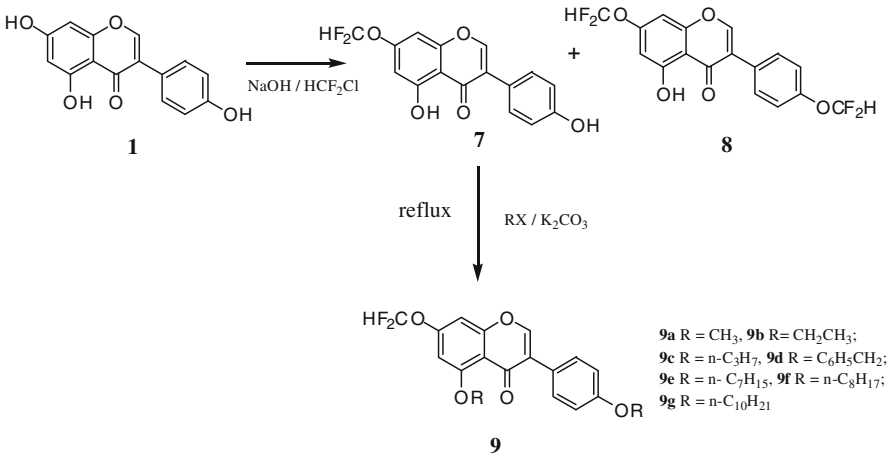
The insertion reaction of difluoromethylene carbene was carried out for the introduction of a *gem*-difluoromethylene group to genistein. Treatment of **1** with HCF_2Cl gas in the presence of NaOH in dioxane/H_2O (Miller and Thanassi, 1960) gave **7** and **8**. The reaction of **7** with RX in the presence of K_2CO_3 at reflux temperature gave **9a**–**9g** (Scheme 2).

Assays were performed in 96-well plates essentially as described by Mosmann (1983). Pharmacological results are summarized in Table 1. It appears that these closely related molecules display remarkable differences in cytotoxicity. As reported in Table 1, we discovered that compounds **2**, **3b**, **3c**, **3d**, **7**, and **9a**–**9g** show stronger cytotoxicity toward HL-60 cells, compounds **9c**, **9d**, and **9f** have stronger cytotoxicity against HT-29 cells, and compounds **2, 3c**, **3d**, **7**, **8**, and **9b**–**9g** possess stronger cytotoxicity toward SGC-7901 cells than genistein **1**. 5,4′-Di-*n*-octoxyl-7-*gem*-difluoromethylene-genistein (**9f**) has the strongest activity against HL-60, HT-29, and SGC-7901 tumor cells.

Although the general structure–activity relationship of these genistein derivatives was not elucidated from these data, the following points are noteworthy. (1) Compounds **2**, **6**, and **7** have stronger activities than **3a**, **5**, and **8** against HL-60 and SGC-7901 tumor cells, respectively. These results show that a 4′-hydroxy moiety in isoflavonoids is necessary. (2) Comparing **9a**–**9g** with **1**, the results show that, apart from **9a**, the 5,4′-dialkylated compounds with a 7-*gem*-difluoromethyleneoxy moiety are more active against HL-60 and SGC-7901 tumor cells than **1**. Moreover,



Scheme 1 General synthesis of **2**, **3a**–**3d**, **4a**, **4b**, and **5**



Scheme 2 General synthesis of **7**, **8**, and **9a–9g**

Table 1 Human cancer cell line growth inhibition values (μM) for the synthesized compounds

Compound	Cancer cell line[a]		
	HL-60	HT-29	SGC-7901
1[b]	4.82	4.11	5.78
2	4.35	5.11	3.94
3a	5.14	4.18	7.78
3b	3.44	14.20	6.51
3c	1.62	10.80	4.82
3d	3.09	11.60	4.27
5	5.86	19.40	11.24
6	4.95	4.21	8.11
7	3.53	4.56	4.59
8	5.50	5.35	5.54
9a	4.61	9.83	6.41
9b	1.75	11.20	5.51
9c	0.54	2.50	2.15
9d	1.29	2.82	4.24
9e	1.41	5.25	3.39
9f	0.47	1.86	1.64
9g	1.63	14.26	3.30

[a] Cancer type: SGC-7901(gastric carcinoma), HT-29 (colon), and HL-60 (promyelocytic leukemia)

[b] Genistein (**1**; 99 + %) from Arcos Organics (USA) as positive control

apart from **9c** and **9g**, the activity against SGC-7901 was enhanced with a longer alkyl chain. In addition, the different alkylated substituents had little effect against HL-60 cells.

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