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Synthesis of *gem*-difluoromethylenated daidzein analogues and their inhibitory effect on U2OS cell cycle

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Received April 27, 2005, accepted July 7, 2005

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Pharmazie 61: 648–649 (2006)

gem-Difluoromethylenated daidzein analogues **2** and **4** were obtained by the insertion reaction of difluorocarbene. A series of alkylated fluorine-containing daidzein analogues **3a–f** and **5a–f** were synthesized. All the compounds were tested for the inhibitory effect on U2OS cell cycle *in vitro*. The results showed that 7-octyloxy-3-(4-difluoromethoxyphenyl)-chromen-4-one **5d** was the most active inhibitor.

The synthesis and anti-tumor activities of chrysin derivatives and B-ring trifluoromethylated flavonoids have earlier been reported (Zheng et al. 2003a, 2003b). Continuing of our ongoing efforts to synthesize fluorine-containing flavonoids as anti-tumor agents, *gem*-difluoromethylenated daidzein analogues **2**, **4** and their alkylated compounds **3a–f** and **5a–f** were synthesized. All the compounds were screened as inhibitors of U2OS cell line.

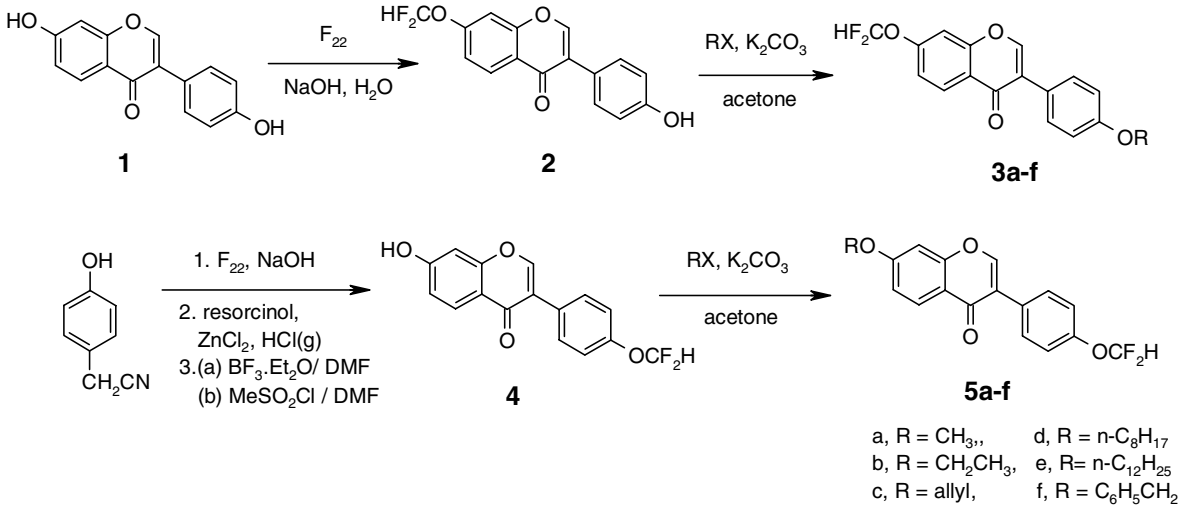
7-*gem*-Difluoromethylenated daidzein (**2**) was prepared by selective difluorocarbene insertion reaction of daidzein (**1**) in the presence of chlorodifluoromethane (Freon 22, F_{22}) and sodium hydroxide, due to the different acidity of 7- and 4′-OH groups of daidzein ($pK_a = 7.6$ and 11.2) (Gao 2001). On the other hand, 4′-*gem*-difluoromethylenated daidzein (**4**) was prepared starting from 4-hydroxyphenylacetonitrile by treatment with F_{22} and sodium hydroxide, condensation with resorcinol in the presence of zinc chloride and gaseous hydrochloride followed by a literature ring closure (Bass et al. 1976; Chang et al. 1994). *gem*-Difluoromethylenated daidzein (**2**) and (**4**) were then alkylated with a series of alkyl halides including methyl iodide, ethyl bromide, allyl bromide, *n*-octyl bromide, *n*-dodecyl bromide and benzyl bromide in the presence of potassium carbonate in refluxing acetone to give compounds **3a**–f and **5a–f** respectively (Scheme). The new compounds were characterized by ^1H and ^{19}F NMR, MS, IR and elemental analysis data.

Table: Effect of cell cycle of U2OS *in vitro*

Compds	Content of cell cycle (%)		
	G_1	S	G_2/M
Control[a]	48.21	41.90	9.89
1	42.07	56.11	1.83
2[b]	–	–	–
4	51.74	48.13	0.13
3a	50.38	38.34	11.28
3b	48.85	43.54	7.62
3c	56.35	34.98	8.67
3d	48.21	41.90	11.57
3e	46.27	41.78	11.95
3f	50.72	39.58	9.70
5a	44.59	55.27	0.14
5b	50.01	42.48	7.51
5c	56.47	37.82	5.71
5d	46.01	53.99	0.00
5e	54.34	35.90	9.75
5f	50.62	39.41	9.97

[a] negative control added 1% DMSO
[b] in the concentration, all the cells died

Scheme



All the compounds were screened as inhibitors of U2OS cells. The results are summarized in Table. Daidzein (**1**) showed some activity to inhibit the U2OS cells at G_2/M phase. Compound **2** and **4** had a significant effect on the cell cycle. Compound **2** had cytotoxicity so strong that all the cells were killed at the test concentration. Compound **4** showed much stronger activity than the parent compound **1** did. Alkylation of compound **2** decreased its cytotoxicity, however the alkylated compounds **3a–f** gave weaker activities than compound **1**. On the other hand, compounds **5a** and **5d** had stronger activities than compound **1**. Compound **5d** was identified as the most active inhibitor in the series.

In conclusion, two *gem*-difluoromethylenated daidzein analogues **2** and **4**, and their alkylated products were synthesized. The preliminary biological tests indicated that **5d** was the most active inhibitor of U2OS cells.

Experimental

1. Chemistry

^{1}H NMR spectra were recorded on a Bruker AM 400 (400 MHz) and Bruker AM 300 (300 MHz) spectrometers with Me_4Si as the internal standard. ^{19}F NMR spectra were obtained on Bruker AM 400 (376 MHz) and Bruker AM 300 (282 MHz) spectrometers using trifluoroacetic acid as the external standard, downfield shifts being designated as negative. All chemical shifts (δ) were expressed in ppm, coupling constants (J) were given in Hz. Mass spectra were recorded on a Finnigan-MAT-8430 instrument using EI ionization at 70 eV. IR spectra were recorded on a NEXUS-670 spectrometer. All the chemicals were used as purchased, daidzein (**1**) was prepared according to the literature (Bass et al. 1976). The results of elemental analyses were in an acceptable range.

1.1 7-gem-Difluoromethoxy-4′-hydroxyisoflavone (**2**)

Daidzein (**1**, 1.27 g, 5 mmol) was dissolved in dioxane (10 mL), a 10% (w/w) aqueous sodium hydroxide solution (10 mL) was added and the Freon 22 was bubbled in. The reaction mixture was maintained at 60–70 °C for 4 h, then extracted with ether. The organic phase was dried over anh. sodium sulfate and concentrated. The residue was purified by chromatography on silica gel and recrystallized from methanol to give white crystals as compound **2** (0.31 g, 20%). Mp 192 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.59 (s, 1 H), 8.47 (s, 1 H), 8.19 (d, 1 H, J = 9.0 Hz), 7.52 (d, 1 H, J = 3.0 Hz), 7.52 (t, 1 H, J = 72.0 Hz, OCF_2H), 7.43 (d, 2 H, J = 9.0 Hz), 7.32 (dd, 1 H, J = 9.0, 3.0 Hz), 6.84 (d, 2 H, J = 9.0 Hz). ^{19}F NMR (282 MHz, DMSO-d_6) δ −96.44 (d, 2 F, 72.0 Hz). IR (KBr): 3262 (OH), 1626 (C=O) cm^{-1}. MS (EI, 70 eV) m/z: 304 [M$^+$]. Anal. calcd for $C_{16}H_{10}O_4F_2$: C, 63.16; H, 3.29. Found: C, 63.08; H, 3.53.

1.2. 4′-gem-Difluoromethoxy-7-hydroxyisoflavone (**4**)

4-Hydroxyphenylacetonitrile (2.0 g, 15 mmol) was treated with F_{22} as described above to give an intermediate, which was dissolved in THF (10 mL) with resorcinol (10 mmol) and $ZnCl_2$ (1 mmol). The mixture was cooled in an ice bath and saturated with a stream of anh. HCl gas for 3 h. The reaction mixture was refrigerated for 24 h. After evaporation, the residue was purified by column chromatography on silica gel to give a compound, which was dissolved in DMF (4 mL) and trifluoroboron etherate (1.25 mL, 10 mmol) was added dropwise. The reaction mixture was heated to 50 °C and a solution of methylsulfonyl chloride (0.6 mL) in DMF (1 mL) was added slowly. The reaction mixture was kept at 60–70 °C for 12 h, then cooled and poured into water with quick stirring. A precipitate was formed, collected and recrystallized from ethanol to give the title compound **4** as a white powder (0.35 g). Mp 218 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 9.64 (s, 1 H), 8.26 (s, 1 H), 8.07 (d, 1 H, J = 8.8 Hz), 7.68 (d, 2 H, J = 8.8 Hz), 7.23 (d, 2 H, J = 8.8 Hz), 7.04 (t, 1 H, J = 74.2 Hz, OCF_2H), 7.01 (dd, 1 H, J = 8.8, 1.6 Hz), 6.92 (d, 1 H, J = 1.6 Hz). ^{19}F NMR (376 MHz, acetone-d_6) δ −82.68 (d, 2 F, J = 74.2 Hz). IR (KBr): 3426 (OH), 1633 (C=O) cm^{-1}. MS (EI, 70 eV) m/z: 304 [M$^+$]. Anal calcd for $C_{16}H_{10}O_4F_2$: C, 63.16; H, 3.29. Found: C, 62.73; H, 3.36.

1.3. General procedure for the alkylation of compounds **2** *and* **4**

A mixture of **2** or **4** (0.2 mmol), anh. potassium carbonate (0.33 mmol), alkyl halide (0.8 mmol) and acetone (2 ml) was stirred at reflux for 8 h, then extracted with ether. The combined organic phases were dried over anh. sodium sulfate and concentrated, The residue was purified by recrystallization from ethanol to give the alkylated compounds **3a–f** or **5a–f** (70–98%).

3a (98%): m.p. 130 °C; ^{1}H NMR (400 MHz, CDCl3) δ 8.32 (d, 1 H, J = 8.8 Hz), 7.98 (s,1 H), 7.50 (d, 2 H, J = 8.4 Hz), 7.21–7.17 (m, 2 H), 6.98 (d, 2 H, J = 8.4 Hz), 6.65 (t, 1 H, J = 72.2 Hz), 3.85(s, 3 H). ^{19}F NMR (376 MHz, $CDCl_3$) δ −82.18 (d, 2 F, J = 72.2 Hz). IR (KBr): 2921, 1642 cm^{-1}. MS (EI, 70 eV) m/z: 318 [M$^+$]. Anal calcd for $C_{17}H_{12}O_4F_2$: C, 64.15; H, 3.11. Found: C, 63.82; H, 3.90.

5a (95%): m.p. 153 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21 (d, 1 H, J = 9.2 Hz), 7.95 (s, 1 H), 7.57 (d, 2 H, J = 8.8 Hz), 7.19 (d, 2 H, J = 8.8 Hz), 7.01 (dd, 1 H, J = 9.2, 2.4 Hz), 6.87 (d, 1 H, J = 2.4 Hz), 6.54 (t, 1 H, J = 74.0 Hz), 3.93 (s, 3 H). ^{19}F NMR (376 MHz, $CDCl_3$) δ −80.67 (d, 2 F, J =74.0 Hz). IR (KBr): 2926, 1638 cm^{-1}. MS (EI, 70 eV) m/z: 318 [M$^+$]. Anal calcd for $C_{17}H_{12}O_4F_2$: C, 64.15; H, 3.11. Found: C, 63.65; H, 3.84.

2. Biological activity

All the above compounds were tested for their inhibitory activity against U2OS *in vitro* by FCM Assay. U2OS cells were seeded 3×10^5 cells/well in a 96-well plate. Until the density of cells cultured at 37 °C was 40–50%, the compounds were added. The concentration of compounds was 500 nM. The suspensions were incubated for 24 h. The cell cycle distribution was evaluated with a BD Biosciences FACScan flow cytometer and CellQuest software. (U2OS cell was obtained from American Type Culture Collection (Manassas, VA). Cells were maintained at 37 °C and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum(FBS)).

Acknowledgments: We thank the National Natural Science Foundation of China (Grant 20325210) and the Ministry of Education of China for financial support.

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