JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 219–225

Received September 1, 2004
Accepted October 26, 2004
DOI 10.1211/0022357055371
ISSN 0022-3573

Synthesis of baicalein derivatives as potential anti-aggregatory and anti-inflammatory agents

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Abstract

The direct acylation of trimethoxyphenol (**1**) with substituted cinnamoyl chlorides followed by Fries rearrangement and cyclization afforded a practical route for the synthesis of novel baicalein derivatives **4** functionalized on the B-ring in good overall yields. In the methylthiazoletetrazolium bromide (MTT) assay, none of the synthetic polyhydroxyflavonoids were cytotoxic at concentrations up to 200 μM on lipopolysaccharide (LPS)-activated murine RAW 264.7 macrophages over 24 h, while in the same cells they significantly inhibited NO production. Among the derivatives, **4d** (IC50 = 46.1 ± 0.3 μM) was found to exhibit the most potent activity compared with *N*-nitro-L-arginine methyl ester (L-NAME, IC50 > 300 μM). Compounds **4b**, **4e**, **4f**, **4h** and **4i** remarkably inhibited platelet aggregation induced by arachidonic acid and collagen in rabbit washed platelets compared with aspirin. Analysis of their structure–activity relationships indicated that, in the structural modification on the B-ring of baicalein (**4a**), introduction of appropriate electro-withdrawing substituents such as 2-Cl (**4b**), 4-Cl (**4d**), and 4-phenyl (**4i**) notably increased the potency on the inhibition of LPS-activated NO production and arachidonic acid- and collagen-induced aggregation. Baicalein itself was equally effective in the inhibition of LPS-activated NO production and collagen-induced aggregation but less active against arachidonic acid-induced aggregation. Our in-vitro results suggested that by appropriate structural modification of baicalein it may be possible to develop novel therapeutic agents against platelet-aggregation and inflammation.

Introduction

Nitric oxide (NO), a pro-inflammatory factor, is produced from L-arginine catalysed by nitric oxide synthase (NOS), existing in constitutive and inducible forms. Inducible NOS augments inflammation in the early stages of inflammatory cascades by dual mechanisms of action: dilation and enhancing permeability of blood vessels by the excess of NO generated and over-production of prostaglandins (PGs) via inducing cyclooxygenase-2 (COX-2). Therefore, agents possessing inhibitory activity against NO production have the potential to be anti-inflammatory and cytoprotective (Salvemini et al 1996a, b; Alderton et al 2001).

Baicalein is one of the active polyhydroxyflavonids of *Scutellaria baicalensis* GEORGI and since ancient times has been used for the treatment of allergic and inflammatory diseases in China. A number of flavonoids have shown promise as potential anti-inflammatory agents with very low toxicity by way of diminishing formation of pro-inflammatory mediators such as PGs, leukotrienes, reactive oxygen species (ROS), and NO. Baicalein (**4a**) potently and selectively inhibited the lipoxygenase (LOX) pathway while the whole herb showed preferential activity against COX, which might have been responsible for the in-vivo anti-inflammatory effect of this plant (Kimura et al 1981; Alcarez & Ferrandiz 1987; Chen et al 2001).

In response to the mediators in the initial stages of inflammation, arachidonic acid (AA), a regular esterified constituent of the cell membrane, is released as free AA, a substrate of LOX and COX, forming metabolites with a variety of roles in inflammatory cascades. Aspirin and related non-steroidal anti-inflammatory drugs (NSAIDs) link positively to the inhibition of COX by covalent acetylation of a serine residue in the active centre. As a result, they reduce PG synthesis, leading to pain-relieving and

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Funding: We gratefully acknowledge the research grant supported in part from the National Science Council (NSC 93–2320-B-016-006), the Republic of China and National Defense Medical Center.

anti-rheumatic effects, and inhibit platelet adhesion and aggregation, which play an important role in the pathogenesis of occlusive vascular diseases and atherosclerosis. In clinical uses aspirin has been proved to be extremely effective in the prevention of transient ischaemic attack, stroke, and myocardial infarction (Willard et al 1992). Some flavonoids exert anti-inflammatory effects by an action quite different from that of baicalein. At high concentrations they directly suppress the very tightly regulated enzyme phospholipase A_2 catalysing the hydrolytic release of AA from phospholipids (Welton et al 1988). Consequently, release of AA is diminished, as is the formation of inflammatory mediators of PG and the leukotriene series. Moreover, some flavonoids inhibit platelet adhesion, aggregation, and secretion and exert noteworthy anti-thrombotic activity, probably by the limitation of formation of isoprostanes and the removal of superoxide anions facilitating anti-aggregatory PGI_2 formation (Robak & Gryglewski 1996), which are indicative of these highly prized polyhydroxyflavonoids as promising medical agents.

Previously, we successfully developed a novel synthetic approach for the preparation of the three major naturally-occurring polyhydroxyflavonoids of *Scutellaria baicalensis* GEORGI (baicalein (**4a**), oroxylin A, and wogonin), suitable for use on a large-scale (Huang et al 2003a). The aim of this study was to seek out new compounds related to this class of biologically and pharmacologically active ingredients with a view to systematically studying the structure–activity relationship. Thus, we report the synthesis of novel baicalein derivatives substituted on the B-ring using this methodology, and have evaluated the compounds for their anti-aggregatory and anti-inflammatory effects.

Materials and Methods

Chemistry

Melting points were taken in open capillary tubes on a Buchi-530 melting point apparatus and were uncorrected. UV-vis spectra were recorded on a Shimazu UV-160A UV-Visble recording spectrophotometer. IR spectra were recorded on a Perkin-Elmer FTIR 1610 series infrared spectrophotometer in KBr discs. ^{1}H and ^{13}C NMR spectra were determined on a Varian Gemini-300 NMR instrument. Chemical shifts (δ) were reported in parts per million (ppm) relative to tetramethylsilane (TMS) as an internal standard, and coupling constants (J) were given in hertz (Hz). Fast atom bombardment (FAB) mass spectra were recorded using a Finnigan MAT 95S (GC/MS) mass spectrometer. All reactions were routinely monitored by TLC on Merck F254 silica gel plates. Merck silica gel (70–230 mesh) was used for chromatography. Elemental analyses for carbon, hydrogen and nitrogen were performed in the Instrument Center of the National Science Counsel at the National Taiwan University using Perkin-Elmer CHN-2400. All the solvents and reagents were obtained from commercial sources and purified before use if necessary.

General procedures for preparation of substituted cinnamoyl chlorides ***2***

To a solution of substituted cinnamic acid (20 mmol) in dichloromethane (50 mL) at 0°C was added oxalyl chloride (2 mL, 22 mmol) and *N*,*N*-dimethylformamide (4 drops). The reaction mixture was stirred at 0°C for 5 min and then allowed to stir at room temperature for 1 h, and finally concentrated under reduced pressure to dryness. The mixture was used for further synthesis without purification.

The products of trimethoxyflavones **3** and baicalein derivatives **4** were synthesized according to our published procedure with slight modifications (Huang et al 2003a, b).

General procedures for the synthesis of etherated baicalein and related derivatives ***3***

The direct Fries acylation of trimethoxyphenol (**1**, 20 mmol) with substituted cinnamoyl chlorides (**2**, 20 mmol) was reacted in boron trifluoride-etherate (BF_3-Et_2O) complex (20 mL) and heated to reflux for 15 min, monitored by TLC (hexane:EtOAc = 3:1), and then quenched with an excess of water. Filtration and recrystallization from hexane and EtOAc (3:1) afforded chalcone intermediates 1-(6-hydroxy-2,3,4-trimethoxyphenyl)-3-(substituted phenyl)propen-1-ones which, without purification, were then submitted to oxidative cyclization by treatment with I_2 (cat.)/DMSO at reflux for 2 h. After cooling, the mixture was poured onto crushed ice (200 g). The precipitate was filtered and thoroughly washed with 20% Na_2SO_3. Purification from flash column chromatography (SiO_2, hexane:EtOAc = 3:1) procured trimethoxyflavones **3**.

Pale yellow etherated baicalein (**3a**) was prepared by the procedure outlined above in 78% overall yield (Huang et al 2003a).

*2-(2′-Chlorophenyl)-5,6,7-trimethoxychromen-4-one (**3b**)*. Pale yellow powder, 2.31 g (33% yield): mp 143–144°C. R_f: 0.40 (hexane:EtOAc = 3:1). ^{1}H NMR (DMSO-d_6) δ: 3.89 (3H, s), 3.94 (3H, s), 3.97 (3H, s), 6.91 (1H, s), 7.32 (1H, s), 7.59 (1H, t, J = 8.1 Hz), 7.69 (1H, t, J = 8.1 Hz), 7.87 (1H, d, J = 8.4 Hz), 8.03 (1H, d, J = 8.4 Hz). IR (KBr) cm^{-1}: 3190, 3090, 1645, 1598. FAB-MS m/z (%): 347 (MH^+, 47), 331 (100). Anal. Calcd. for $C_{18}H_{15}ClO_5$: C, 62.35; H, 4.36. Found: C, 61.93; H, 4.31.

*2-(3′-Chlorophenyl)-5,6,7-trimethoxychromen-4-one (**3c**)*. Pale yellow powder, 2.63 g (38% yield): mp 127–128°C. R_f: 0.45 (hexane:EtOAc = 3:1). ^{1}H NMR (DMSO-d_6) δ: 3.83 (3H, s), 3.87 (3H, s), 3.95 (3H, s), 6.91 (1H, s), 7.30 (1H, s), 7.58 (1H, d, J = 7.5 Hz), 7.64 (1H, t, J = 7.5 Hz), 8.04 (1H, d, J = 7.5 Hz), 8.15 (1H, s). IR (KBr) cm^{-1}: 3192, 3093, 1647, 1589. FAB-MS m/z (%): 347 (MH^+, 43), 331 (100). Anal. Calcd for $C_{18}H_{15}ClO_5$: C, 62.35; H, 4.36. Found: C, 61.99; H, 4.33.

*2-(4′-Chlorophenyl)-5,6,7-trimethoxychromen-4-one (**3d**)*. Pale yellow crystals, 1.25 g (18% yield): mp 165–166°C. R_f: 0.46 (hexane:EtOAc = 3:1). ^{1}H NMR (DMSO-d_6) δ: 3.91 (3H, s), 3.95 (3H, s), 3.99 (3H, s), 6.97 (1H, s),

7.65 (2H, d, $J = 8.4$ Hz), 8.11 (2H, d, $J = 8.4$ Hz). IR (KBr) cm^{-1}: 3188, 3121, 2998, 1645, 1597. FAB-MS *m/z* (%): 347 (MH^+, 77), 331 (100). Anal. Calcd for $C_{18}H_{15}ClO_5$: C, 62.35; H, 4.36. Found: C, 62.41; H, 4.38.

2-(2′,4′-Dichlorophenyl)-5,6,7-trimethoxychromen-4-one (***3e***). Yellow crystals, 2.65 g (35% yield): mp 131–132°C. R_f: 0.43 (hexane:EtOAc = 3:1). ^1H NMR (DMSO-d_6) δ: 3.76 (3H, s), 3.79 (3H, s), 3.95 (3H, s), 6.83 (1H, s), 6.89 (1H, s), 7.69 (1H, dd, $J = 8.4$, 2.1 Hz), 7.83 (1H, d, $J = 8.4$ Hz), 7.93 (1H, d, $J = 2.1$ Hz). IR (KBr) cm^{-1}: 3187, 3091, 2993, 1647, 1593. FAB-MS *m/z* (%): 381 (MH^+, 95), 365 (100). Anal. Calcd for $C_{18}H_{14}Cl_2O_5$: C, 56.71 ; H, 3.70. Found: C, 56.83; H, 3.72.

2-(3′,4′-Dichlorophenyl)-5,6,7-trimethoxychromen-4-one (***3f***). Pale yellow powder, 2.36 g (31% yield): mp 200–201°C. R_f: 0.47 (hexane:EtOAc = 3:1). ^1H NMR (DMSO-d_6) δ: 3.76 (3H, s), 3.79 (3H, s), 3.95 (3H, s), 6.96 (1H, s), 7.32 (1H, s), 7.83 (1H, d, $J = 8.4$ Hz), 8.07 (1H, dd, $J = 8.4$, 1.8 Hz), 8.36 (1H, d, $J = 1.8$ Hz). IR (KBr) cm^{-1}: 3168, 3087, 2993, 1647, 1586. FAB-MS *m/z* (%): 381 (MH^+, 60), 365 (100). Anal. Calcd for $C_{18}H_{14}Cl_2O_5$: C, 56.71 ; H, 3.70. Found: C, 56.91; H, 3.59.

2-(3′-Bromo-4′-fluorophenyl)-5,6,7-trimethoxychromen-4-one (***3g***). White powder, 2.03 g (25% yield): mp 232–235°C. R_f: 0.45 (hexane:EtOAc = 3:1). ^1H NMR (DMSO-d_6) δ: 3.73 (3H, s), 3.79 (3H, s), 3.91 (3H, s), 6.95 (1H, s), 7.32 (1H, s), 7.59 (1H, t, $J = 8.5$ Hz), 8.17 (1H, d, $J = 8.5$ Hz), 8.46 (1H, s). IR (KBr) cm^{-1}: 3185, 3098, 2997, 1645, 1589. FAB-MS *m/z* (%): 409 (MH^+, 45), 393 (100). Anal. Calcd for $C_{18}H_{14}BrFO_5$: C, 52.83; H, 3.45. Found: C, 52.76; H, 3.41.

2-(4′-Carboxyphenyl)-5,6,7-trimethoxychromen-4-one (***3h***). Pale yellow crystals, 0.98 g (14% yield): mp 236–238°C. R_f: 0.39 (hexane:EtOAc = 3:1). ^1H NMR (DMSO-d_6) δ: 3.83 (3H, s), 3.91 (3H, s), 4.07 (3H, s), 6.94 (1H, s), 7.33 (1H, s), 8.13 (2H, d, $J = 7.8$ Hz), 8.31 (2H, d, $J = 7.8$ Hz), 13.24 (1H, s, br). IR (KBr) cm^{-1}: 3454, 1709, 1586. FAB-MS *m/z* (%): 357 (MH^+, 47), 297 (100). Anal. Calcd for $C_{19}H_{16}O_7$: C, 64.04; H, 4.53. Found: C, 63.93; H: 4.51.

2-(1,1′-Biphenyl-4-yl)-5,6,7-trimethoxychromone-4-one (***3i***). Yellow powder, 1.94 g (25% overall yield): mp 131–132°C. R_f: 0.49 (hexane:EtOAc = 3:1). ^1H NMR (DMSO-d_6) δ: 3.83 (3H, s), 3.89 (3H, s), 4.11 (3H, s), 6.95 (1H, s), 7.31 (1H, s), 7.51 (1H, t, $J = 7.2$ Hz), 7.67 (2H, t, $J = 7.2$ Hz), 7.83 (2H, d, $J = 7.2$ Hz), 7.92 (2H, d, $J = 8.3$ Hz), 8.17 (2H, d, $J = 8.3$ Hz). IR (KBr) cm^{-1}: 3165, 3091, 2989, 1635, 1587. FAB-MS *m/z* (%): 389 (MH^+, 100), 373 (67). Anal. Calcd for $C_{24}H_{20}O_5$: C, 74.21; H, 5.19. Found: C, 73.97; H, 5.08.

2-(4′-Nitrophenyl)-5,6,7-trimethoxychromone-4-one (***3j***). Brown powder, 2.18 g (31% yield): mp 243–245°C. R_f: 0.46 (hexane:EtOAc = 3:1). ^1H NMR ($CDCl_3$) δ: 3.94 (3H, s), 4.00 (3H, s), 4.011 (3H, s), 6.77 (1H, s), 6.84 (1H, s), 8.8.07 (2H, d, $J = 8.7$ Hz), 8.38 (2H, d, $J = 8.7$ Hz). IR (KBr) cm^{-1}: 3177, 3016, 1627, 1593. FAB-MS *m/z* (%): 358 (MH^+, 59), 343 (100). Anal. Calcd for $C_{18}H_{15}NO_7$: C, 60.50; H, 4.23; N, 3.92. Found: C, 60.61; H, 4..27; N, 3.91.

General procedures for the synthesis of baicalein derivatives ***4***

Demethylation of **3** was achieved by heating with 47% HBr/AcOH over 18–24 h, monitored by TLC (EtOAc:hexane = 1:3 → CH_2Cl_2:EtOAc = 5:1). The solution was carefully poured onto crushed ice (200 g). The resulting yellow precipitate was filtered and collected. Recrystalization from hexane–EtOAc afforded the corresponding polyhydroxyflavones **4** (baicalein derivatives).

Bright yellow baicalein (**4a**) was prepared by the procedure outlined above in 70% yield (Huang et al 2003a).

2-(2′-Chlorophenyl)-5,6,7-trihydroxychromen-4-one (***4b***). Dark brown crystals, 1.28 g (21% overall yield): mp 204–205°C. R_f: 0.20 (hexane:EtOAc = 3:1). ^1H NMR (DMSO-d_6) δ: 6.62 (1H, s), 6.93 (1H, s), 7.54 (1H, t, $J =$ 7.9 Hz), 7.62 (1H, t, $J = 7.9$ Hz), 7.76 (1H, d, $J = 8.1$ Hz), 7.89 (1H, d, $J = 8.1$ Hz), 8.93 (1H, s, br), 10.63 (1H, s, br), 12.57 (1H, s); ^{13}C NMR (DMSO-d_6) δ: 88.6, 93.7, 107.5, 123.3, 124.5, 125.7, 128.8, 130.4, 132.3, 135.2, 146.4, 155.7, 160.0, 169.9, 181.6. IR (KBr) cm^{-1}: 3435, 3186, 1652, 1562. UV λ_{max} (EtOH) nm (log ε): 272 (3.74), 215 (4.25). FAB-MS *m/z* (%): 305 (MH^+, 43), 154(100). Anal. Calcd for $C_{15}H_8ClO_5$: C, 59.13; H, 2.98. Found: C, 58.74; H, 2.95.

2-(3′-Chlorophenyl)-5,6,7-trihydroxychromen-4-one (***4c***). Yellow powder, 1.46 g (24% overall yield): mp 175–178°C. R_f: 0.27 (hexane:EtOAc = 3:1). ^1H NMR (DMSO-d_6) δ: 6.53 (1H, s), 6.91 (1H, s), 7.46 (3H, m), 7.59 (1H, s), 8.91 (1H, s, br), 10.57 (1H, s, br), 12.53 (1H, s); ^{13}C NMR (DMSO-d_6) δ: 91.7, 97.2, 104.3, 127.4, 129.3, 130.3, 131.2, 134.1, 135.6, 136.3, 146.3, 153.8, 160.6, 162.3, 183.4. IR (KBr) cm^{-1}: 3406, 3196, 1652, 1576. UV λ_{max} (EtOH) nm (log ε): 278 (3.85), 216 (4.32). FAB-MS *m/z* (%): 305 (MH^+, 20), 154 (100). Anal. Calcd. for $C_{15}H_8ClO_5$: C, 59.13; H, 2.98. Found: C, 58.91; H, 3.01.

2-(4′-Chlorophenyl)-5,6,7-trihydroxychromen-4-one (***4d***). Dark brown crystals, 0.85 g (14% overall yield): mp 212–214°C. R_f: 0.24 (hexane:EtOAc = 3:1). ^1H NMR (DMSO-d_6) δ: 6.62 (1H, s), 6.97 (1H, s), 7.62 (2H, d, $J = 8.6$ Hz), 8.08 (2H, d, $J = 8.6$ Hz), 8.96 (1H, s, br), 10.67 (1H, s, br), 12.65 (1H, s); ^{13}C NMR (DMSO-d_6) δ: 95.1, 105.1, 107.5, 129.2, 129.9, 130.8, 131.0, 131.6, 138.1, 139.7, 155.1, 162.9, 183.4. IR (KBr) cm^{-1}: 3368, 1652, 1590. UV λ_{max} (EtOH) nm (log ε): 336 (3.86), 283 (4.13), 222 (4.21). FAB-MS *m/z* (%): 305 (MH^+, 95), 154 (100). Anal. Calcd. for $C_{15}H_8ClO_5$: C, 59.13; H, 2.98. Found: C, 58.92; H, 2.92.

2-(2′,4′-Dichlorophenyl)-5,6,7-trihydroxychromen-4-one (***4e***). Brown powder, 2.31 g (34% overall yield): mp 180–182°C. R_f: 0.20 (hexane:EtOAc = 3:1). ^1H NMR

(DMSO-d_6) δ: 6.51 (1H, s), 6.55 (1H, s), 7.64 (1H, dd, $J=8.4$, 2.1 Hz), 7.79 (1H, d, $J=8.4$ Hz), 7.87 (1H, d, $J=2.1$ Hz), 8.83 (1H, s, br), 10.64 (1H, s, br), 12.44 (1H, s). ^{13}C NMR (DMSO-d_6) δ: 95.4, 105.6, 111.4, 129.3, 129.4, 130.5, 130.9, 133.9, 134.2, 137.9, 148.4, 151.6, 155.3, 162.7, 182.9. IR (KBr) cm^{-1}: 3378, 1662, 1590. UV λ_{max} (EtOH) nm (log ε): 276 (4.26), 215 (4.53). FAB-MS m/z (%): 339 (MH^+, 100), 154 (68). Anal. Calcd for $C_{15}H_8Cl_2O_5$: C, 53.12; H, 2.38. Found: C, 52.90; H, 2.44.

*2-(3′,4′-Dichlorophenyl)-5,6,7-trihydroxychromen-4-one (**4f**)*. Yellow powder, 1.81 g (27% overall yield): mp 220–222°C. R_f: 0.23 (hexane:EtOAc = 3:1). ^{1}H NMR (DMSO-d_6) δ: 6.66 (1H, s), 7.06 (1H, s), 7.80 (1H, d, $J=8.4$ Hz), 8.03 (1H, d, $J=8.4$ Hz), 8.34 (1H, s, br), 10.63 (1H, s, br), 12.50 (1H, s); ^{13}C NMR (DMSO-d_6) δ: 95.6, 105.8, 106.9, 127.7, 129.4, 130.9, 132.6, 132.9, 133.6, 135.8, 148.3, 151.2, 155.2, 161.7, 183.3. IR (KBr) cm^{-1}: 3368, 1661, 1580. UV λ_{max} (EtOH) nm (log ε): 328 (3.87), 281 (4.26), 216 (4.47). FAB-MS m/z (%): 339 (MH^+, 60), 154 (100). Anal. Calcd for $C_{15}H_8Cl_2O_5$: C, 53.12; H, 2.38. Found: C, 52.94; H, 2.44.

*2-(3′-Bromo-4′-fluorophenyl)-5,6,7-trihydroxychromen-4-one (**4g**)*. Pale yellow powder, 1.61 g (22% overall yield): mp 252–255°C. R_f: 0.20 (hexane:EtOAc = 3:1). ^{1}H NMR (DMSO-d_6) δ: 6.65 (1H, s), 7.02 (1H, s), 7.56 (1H, t, $J=8.5$ Hz), 8.14 (1H, d, $J=8.5$ Hz), 8.44 (1H, s), 8.80 (1H, s, br), 10.60 (1H, s, br), 12.36 (1H, s). ^{13}C NMR (DMSO-d_6) δ: 92.9, 95.6,106.7, 110.0, 118.9, 129.5, 129.6, 131.6, 132.9, 147.5, 155.1, 156.1, 162.0, 163.3, 183.4. IR (KBr) cm^{-1}: 3445, 1662, 1586. UV λ_{max} (EtOH) nm (log ε): 322 (3.82), 280 (4.27), 216 (4.51). FAB-MS m/z (%): 367 (MH^+, 26), 154 (100). Anal. Calcd for $C_{15}H_8BrFO_5$: C, 49.07; H, 2.20. Found: C, 48.86; H, 2.22.

*2-(4′-Carboxyphenyl)-5,6,7-trihydroxychromen-4-one (**4h**)*. Yellow crystals, 0.5 g (8% overall yield): mp 236–238°C. R_f: 0.19 (hexane:EtOAc = 3:1). ^{1}H NMR (DMSO-d_6) δ: 6.64 (1H, s), 7.03 (1H, s), 8.06 (2H, d, $J=7.6$ Hz), 8.18 (2H, d, $J=7.6$ Hz), 8.85 (1H, s, br), 10.64 (1H, s, br), 12.57 (1H, s). ^{13}C NMR (DMSO-d_6) δ: 95.5, 105.9, 107.2, 127.9, 128.1, 130.9, 131.1, 131.2, 134.7, 136.2, 148.4, 151.3, 155.2, 163.1, 167.9, 183.4. IR (KBr) cm^{-1}: 3454, 1709, 1586. UV λ_{max} (EtOH) nm (log ε): 332 (4.12), 287 (4.45), 216 (4.27). FAB-MS m/z (%): 315 (MH^+, 33), 154 (100). Anal. Calcd for $C_{16}H_{10}O_7$: C, 61.15; H, 3.21. Found: C, 60.93; H: 3.23.

*2-(1,1′-Biphenyl-4-yl)-5,6,7-trihydroxychromone-4-one (**4i**)*. Yellow powder, 1.46 g (21% overall yield): mp 210–212°C. R_f: 0.26 (hexane:EtOAc = 3:1). ^{1}H NMR (DMSO-d_6) δ: 6.65 (1H, s), 6.99 (1H, s), 7.43 (1H, t, $J=7.0$ Hz), 7.49 (2H, t, $J=7.0$ Hz), 7.77 (2H, d, $J=7.0$ Hz), 7.87 (2H, d, $J=8.3$ Hz), 8.15 (2H, d, $J=8.3$ Hz), 8.85 (1H, s),10.61 (1H, s), 12.68 (1H, s). ^{13}C NMR (DMSO-d_6) δ: 95.4, 105.8, 128.2, 128.3, 128.6, 129.7, 130.5, 131.3, 140.2, 144.6, 148.5, 151.3, 155.1, 164.0, 183.4. IR (KBr) cm^{-1}: 3330 (O-H), 1657 (C=O), 1580 (C=C), 1028 (C-O). UV λ_{max} (EtOH) nm (log ε): 335 (4.47), 295 (4.51), 221 (4.65). FAB-MS m/z (%): 347 (MH^+, 100), 154 (23). Anal. Calcd for $C_{21}H_{14}O_5$: C, 72.83; H, 4.07. Found: C, 72.66; H, 4.09.

*2-(4′-Nitrophenyl)-5,6,7-trihydroxychromone-4-one (**4j**)*. Dark brown crystals, 1.9 g (30% overall yield): mp 296–297°C. R_f: 0.24 (hexane:EtOAc = 3:1). ^{1}H NMR (DMSO-d_6) δ: 6.68 (1H, s), 7.15 (1H, s), 8.37 (2H, d, $J=2.4$ Hz), 8.40 (2H, d, $J=2.4$ Hz), 8.46 (1H, s), 10.30 (1H, s), 12.46 (1H, s). ^{13}C NMR (DMSO-d_6) δ: 92.5, 95.6, 106.2, 125.3, 128.8, 129.0, 129.4, 130.6, 146.4, 147.3, 149.9, 153.2, 154.5, 172.3, 184.0. IR (KBr) cm^{-1}: 3340, 1648, 1581, 1343, 1043. UV λ_{max} (EtOH) nm (log ε): 355 (3.78), 296 (3.87), 217 (4.12). FAB-MS m/z (%): 316 (MH^+, 5), 154 (100). Anal. Calcd for $C_{15}H_9NO_7$: C, 57.15; H, 2.88; N, 4.44. Found: C, 56.89; H, 2.77; N, 4.37.

In-vitro evaluations

Cell culture

The mouse BALB/c macrophage cell line RAW 264.7 (identical ATCC number: TIB-71) was obtained from Bioresource Collection and Research Center, Taiwan. Cells were routinely grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum and 10% penicillin–streptomycin according to the conventional procedures as detailed previously (Ralph & Nakoinz 1977; Raschke et al 1978). The cells were cultured in 50 cm^2 plastic flasks (Nunc A/S, Roskilde, Denmark) and passed medium renewal every three days.

Determination of cell viability by the methylthiazoletetrazolium bromide (MTT) assay

To evaluate cell viability, the MTT assay was conducted by the standard method as described previously (Green et al 1984; Denizot & Lang 1986). Incubation was performed and pretreated with a combination of the polyhydroxyflavonoids, with concentrations ranging from 25, 50, 100, and up to 200 μM in dimethyl sulfoxide (DMSO), and lipopolysaccharide (LPS) (100 ng mL^{-1}) in normal saline for 24 h. Cells without treatment of flavonoids were used as control.

Nitrite quantification

The cell culture was performed and treated with or without the polyhydroxyflavonoids ranging from 25, 50, 100, and up to 200 μM as described above. The production of NO was determined by measuring the accumulated levels of nitrite in culture supernatants with the Griess reagent in LPS-stimulated macrophage cells. A quantity of 100-μL samples were mixed with 100 μL Griess reagent (0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% phosphoric acid) in a 96-well plate and incubated at 25°C for 10 min. The absorbance was measured on a Dynatech ELISA reader at 550 nm. $NaNO_2$ was used as the standard to calculate the nitrite concentration.

Evaluation of antiplatelet aggregation activity

The inhibitory potency of platelet aggregation was evaluated according to Born (1962) and Born & Cross (1963) – details of the procedure are given in Hsu et al (1995).

Statistics

Dose–response relationships were constructed by linear regression of the percent inhibition of parameters derived in the preceding sections against log drug concentrations. Each value was expressed as the mean ± s.e.m. IC50 values were calculated from the regression lines. The statistical significance of difference was assessed with an analysis of variance followed by Tukey's test (Jones 2002). Differences with P values of less than 0.05 were considered statistically significant.

Results and Discussion

Chemistry

The baicalein derivatives **4** as the targets were prepared in multi-step synthesis based on our published procedure with slight modifications (Huang et al 2003a, b; Figure 1). At the outset, during the preparation of substituted cinnamoyl chlorides **2** (Figure 2) by converting the corresponding acids with thionyl chloride and a catalytic amount of pyridine, we encountered difficulties due to the intramolecular cyclization of some but not all of the intermediates at reflux to form 3-chloro-2-chlorocarbonylbenzo[*b*]thiophenes. Similar results had been reported and its mechanism proposed (Higa & Krubsack 1975). Thionyl chloride proved to be the main culprit. The difficulties were overcome by using an equimolar amount of oxalyl chloride and a catalytic amount of *N,N*-dimethylformamide (4 drops) in dichloromethane at 0°C and all the reactions were completed in 1 h to furnish the desired substituted cinnamoyl chlorides **2** in satisfactory yields (Carling et al 1993).

All the synthetic etherated **3** and baicalein derivatives **4** were fully characterized by NMR (^{1}H, ^{13}C), MS and elemental analysis. Baicalein derivatives **4** undergo dark green colorations after treatment with dilute H_2SO_4 on TLC and display specific spectroscopic properties of flavonoid series in maximal UV absorbance at 270–290 nm (band II) and 320–360 nm (band I), respectively (Robak et al 1991). They show a distinct bathochromic shift by the addition of $AlCl_3$/HCl, indicating the presence of a chelated hydroxyl at C-5. The ^{1}H NMR spectrum of compound **4b** in DMSO-d_6, for example, showed three phenolic protons, each as singlet, resonanced at δ 8.93, 10.63, and 12.57, respectively. The very downfield of δ 12.57 indicated there must exist an intramolecular hydrogen bonding, facilitated by a carbonyl group situated in the *ortho* position. In the aromatic region of the spectrum, there were six proton signals appearing as a singlet (1H, δ 6.62) due to the C-8 proton on the A-ring, a singlet (1H, δ 6.93) of C-3 proton on the C-ring, and two triplets (2H, δ 7.54 and 7.62, $J = 7.9$ Hz) and two doublets of the B-ring protons (2H, δ 7.76 and 7.89, $J = 8.1$ Hz), requiring that the chlorine be in the C-2′position. The ^{13}C NMR spectrum of compound **4b** showed that there were 15 signals, all of which were sp^2 carbons due to the flavone skeleton. Among them, the signal at δ 181.6 was assigned as the C-4 carbonyl.

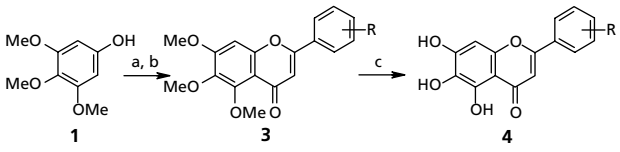

Figure 1 Synthesis of baicalein derivatives **4**. Reagents: a, cinnamoyl chlorides 2, BF_3-Et_2O; b, I_2, DMSO; c, 47% HBr/HOAc.

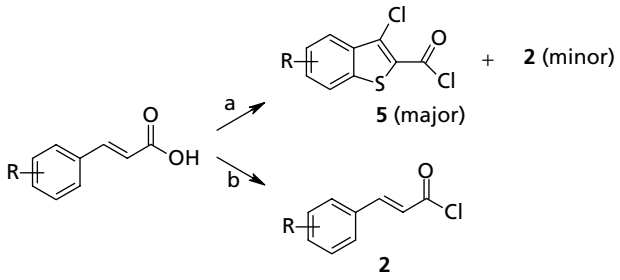

Figure 2 Preparation of substituted cinnamoyl chlorides **4**. Reagents: a, thionyl chloride, cat. pyridine; b, oxalyl chloride, cat. DMF.

Pharmacology

Compounds were evaluated for cell viability and the inhibitory activity on NO production in LPS-activated murine RAW 264.7 macrophages and platelet aggregation induced by AA and collagen, respectively. In the investigations for cytotoxicity, each of the synthetic baicalein derivatives **4**, ranging from 25, 50, 100, and up to 200 μM, were incubated with LPS-activated RAW 264.7 macrophages for 24 h. The results indicated that there was no appreciable cytotoxic effect (cell viability, 85–98%) up to the concentration of 200 μM as measured by the MTT assay (data not shown). When LPS (1 μg mL^{-1}) was added to RAW 264.7 cells, the NO production, measured by nitrite formation, was increased dramatically up to 63.3 ± 3.7 μM (38.7 ± 1.3 nmol/well/10^5 cells in a 96-well plate) for 24 h from the basal level of 2.1 ± 0.4 μM without LPS or from the background level of 1.7 ± 0.7 μM in co-incubation of the respective compound and the Griess reagent.

Table 1 shows that nearly all of the compounds significantly attenuated LPS-stimulated NO production in macrophages at very low concentrations and thus might explain further the related anti-inflammatory action of these polyhydroxyflavonoids. Among them, **4d** (4-Cl; IC50 = 46.1 ± 0.29 μM) exhibited the most potent activity, whereas **4h** (4-COOH), **4i** (4-phenyl) and L-NAME were inactive. Compounds with 2-Cl (**4b**) or 4-Cl (**4d**)

Table 1 Inhibitory effects of polyhydroxyflavonoids **4** on NO production and platelet aggregation

Compounds	Substituents (R)	IC50 (μM)[a]		
		AA[b] (100 μM)	Collagen[b] (10 μg mL^{-1})	Nitrite[c]
4b	2-Cl	24.8 ± 0.5*	10.3 ± 0.5*	53.5 ± 0.3*
4c	3-Cl	>300	>400	68.0 ± 0.3*
4d	4-Cl	109 ± 0.4	80.5 ± 0.2	46.1 ± 0.3*
4e	2,4-$(Cl)_2$	110 ± 0.3	47.5 ± 0.4	88.2 ± 0.2*
4f	3,4-$(Cl)_2$	102 ± 0.6	34.0 ± 0.4	85.4 ± 0.3*
4g	3-Br, 4-F	78.5 ± 0.7	62.4 ± 1.0	87.0 ± 0.3*
4h	4-COOH	>300	40.0 ± 0.8	>100
4i	4-Phenyl	58.0 ± 0.2	7.70 ± 0.9*	>100
4j	4-NO_2	160 ± 0.42	>300	97.0 ± 0.4*
3a–j		>300	>300	>100
Baicalein (**4a**)	H	181 ± 0.4	36.5 ± 0.1	66.4 ± 1.0*
Aspirin[d]		34.7 ± 0.4	50.3 ± 1.3	ND[e]
L-NAME[d]		ND[e]	ND[e]	>300

[a]Each value represents the mean ± s.e.m., n = 6. *$P < 0.05$ compared with the positive controls, respectively. [b]Platelet aggregation was activated by arachidonic acid (AA) and collagen, respectively. [c]The inhibition of NO production was measured by nitrite formation. [d]Aspirin and L-NAME were positive controls for platelet aggregation and NO production, respectively. [e]Not determined.

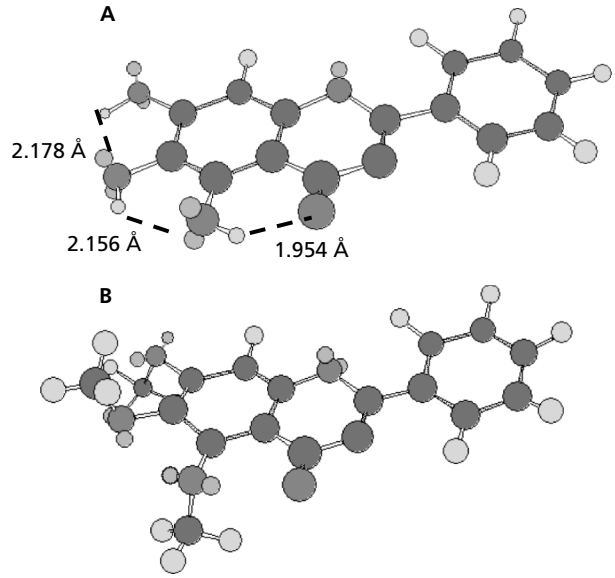

Figure 3 Structural features of **4a** and **3a**. Conformational analysis was performed using MM2 implemented on a BioMedCAChe 6.0 (a molecular modeling software, Fujitsu Ltd, Beaverton, OR). Compound **4a** has stabilization of coplanar feature reinforced by three H-bonds (bond length 1.954, 2.156 and 2.178 Å, respectively), whereas **3a** does not.

substituents flanking at ring-B apparently displayed greater activities. We reasoned that the inhibitory activity of the polyhydroxyflavonoids on LPS-activated NO production might be directly connected with the inhibition of iNOS activity or reduction of iNOS expression. If this was the case, it would make them potential anti-inflammatory agents (Robak & Gryglewski 1996; Chen et al 2001).

To investigate the effect of the compounds on the metabolism of AA, we incubated platelets with AA, which strikingly enhances platelet aggregation as a consequence of conversion to thromboxane A_2 (TXA_2). On the other hand, platelet aggregation induced by collagen is dependent on the formation of TXA_2 from AA by the action of COX and thromboxane synthase. In the experiments, induction of platelet aggregation was achieved by using AA (100 μM) and collagen (10 μg mL^{-1}), respectively. The inhibitory effects are shown in Table 1. Among all the baicalein derivatives, **4b** (2-Cl; IC50 = 24.8 ± 0.54 μM and 10.3 ± 0.52 μM against AA- and collagen-induced aggregation, respectively) and **4i** (4-phenyl; IC50 = 58.0 ± 0.19 μM against AA, 7.70 ± 0.88 μM against collagen, respectively) exhibited the strongest inhibitory effects compared with aspirin as positive control (IC50 = 34.7 ± 0.4 μM and 50.3 ± 1.3 μM against AA- and collagen-induced aggregation, respectively). Compound **4g** (3-Br, 4-F) showed only moderate effects and **4c** (3-Cl) the least. In general, most of the tested compounds, except **4j** (4-NO_2), manifested a preferential effect against collagen-induced to AA-induced aggregation. As Gryglewski et al (1987) pointed out in the antiplatelet activity of flavonoids, the ability to bind to platelet membranes is highly dependent on their chemical structures. Our preliminary results showed that in the structure modification of baicalein (**4a**), by introduction of such appropriate inductive substituents as 2-Cl (**4b**), 4-Cl (**4d**), or 4-phenyl (**4i**) in the B-ring of the flavonoids, we were able to enhance the potency to a significant extent on the inhibition of LPS-activated NO production and platelet aggregation. The coplanar feature of the flavone framework, which might be the critical structural facet for pharmacological activity, was exhibited by a carbonyl group at C-4 and a double bond between C-2 and C-3 and the 5-OH in the essential skeleton (Figure 3). There exist intramolecular H-bonding interactions in the A-ring of the baicalein involving the three hydroxyl groups (C-5,6,7), where each of the hydroxyl H atoms acts as a donor in a polycyclic H-bonding system, further reinforcing the co-planar trait in our flavonoids. The substitution patterns in the B-ring of the flavonoids apparently contributed an additional effect on the molecules by conjugative stabilization via an exocyclic oxygen atom at C-4. Surprisingly, substituted 5,6,7-*O*-trimethylbaicaleins **3**, the fully etherated form of the corresponding baicalein derivatives **4**, were completely void of the inhibitory activity on NO production and platelet aggregation. This was possibly due to the loss of entire intramolecular H-bondings, the prerequisites for inhibitory activity, and thus led to a damage effect on the interaction between **3** and the target macromolecules, even though they were much more hydrophobic. It was noted that even a minor structural modification in the flavonoid skeleton resulted in enormous changes in the potency of the biological activity. The knowledge gleaned from this investigation should provide valuable information for the future development of novel therapeutic agents.

Conclusion

Our methodological development made it feasible to prepare polyhydroxyflavonoids with discrepantly versatile pharmacological functions. The synthetic baicalein derivatives **4** remarkably inhibited platelet aggregation and NO production. The in-vitro results suggested that they were potential anti-aggregatory and anti- inflammatory agents, meriting further study.

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