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Inhibitory effect of synthetic C–C biflavones on various phospholipase A_2s activity

Tae Chul Moon,[a] Zhejiu Quan,[a] Jeongsoo Kim,[b] Hyun Pyo Kim,[b] Ichiro Kudo,[c] Makodo Murakami,[c] Haeil Park[b,*] and Hyeun Wook Chang[a,*]

[a]*College of Pharmacy, Yeungnam University, Gyeongsan 712-749, Republic of Korea*
[b]*College of Pharmacy, Kangwon National University, Chunchon 200-701, Republic of Korea*
[c]*Department of Health Chemistry, School of Pharmaceutical Sciences, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan*

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Abstract—Several prototypes of C–C biflavones (**a**–**f**) were synthesized and evaluated their inhibitory activity against phospholipase A_2s (PLA_2s) activity. The synthetic C–C biflavones (**a**–**f**) showed rather different inhibitory activity against various PLA_2s. Most synthetic C–C biflavonoids exhibited potent and broad inhibitory activity against various $sPLA_2$s and $cPLA_2$ tested regardless of their structural array. In particular, of natural and synthetic biflavonoids tested, the synthetic C–C biflavonoid (**d**) only showed inhibitory activity against $sPLA_2$ X. None of the natural and synthetic biflavonoids tested showed inhibitory activity against $sPLA_2$ IB. Further chemical modification of these basic structures will be carried out in order to investigate the synthetic C–C biflavones which possess more selective inhibitory activity against isozymes of PLA_2.

1. Introduction

Biflavonoid, flavonoid dimer connected with either a C–C or C–O–C bond, is one of the subclasses of flavonoid. Although many biflavonoids have been discovered from various plants, only a few data on their biological and pharmacological properties have been available so far. Previously, certain biflavonoids were reported to inhibit phosphodiesterase,[1] lens aldose reductase,[2] release of histamine from mast cells[3] and have anticancer activity.[4] In addition, some C–C biflavonoids were synthesized and their antimicrobial activities were reported.[5] During our study to investigate potential anti-inflammatory plant drugs, several biflavones such as amentoflavone, ginkgetin and ochnaflavone (Fig. 1) were demonstrated to be inhibitors of group II secretory phospholipase A_2($sPLA_2$IIA).[6] Moreover, morelloflavone (Fig. 1), a flavone-flavanone dimer, was also reported to be a $sPLA_2$ inhibitor.[7]

PLA_2 is a growing family of distinct enzymes that shows different substrate specificities, cofactor requirements, subcellular localization, and cellular functions.[8] $sPLA_2$ has a low molecular weight (14–18 kDa) with a rigid tertiary structure that is configured by 6–8 disulfide bridges. Thus far, 10 genes encoding structurally related and enzymatically active $sPLA_2$s have been identified in mammals (groups IB, IIA, IIC, IID, IIF, V, X, and XII).[9] $sPLA_2$-IIA involvement in the inflammatory process has also been investigated by administering particular inhibitors or antibodies that are fairly specific to $sPLA_2$-IIA into inflamed sites. Group V $sPLA_2$ ($sPLA_2$-V) is expressed mainly in rats, the human heart, and various mouse tissues.[10,11] This enzyme may compensate for $sPLA_2$-IIA under particular conditions because it is induced in many tissues by pro-inflammatory agents[12,13] in a similar way to $sPLA_2$-IIA. $sPLA_2$-IIA and -V, whose genes are clustered on the same chromosome locus, share several enzymatic and functional features.[14]$sPLA_2$ inhibitors might show favorable anti-inflammatory activity since $sPLA_2$ is a key enzyme that generates arachidonic acid, which is converted to proinflammatory eicosanoids. Indeed, some of these biflavonoids have been found to possess promising anti-inflammatory activity in vivo.[7,15] In a previous

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* Corresponding authors. Tel.: +82 33 250 6920; fax: + 82 33 255 7865 (H.P.); e-mail addresses: haeilp@kangwon.ac.kr; hwchang@yu.ac.kr

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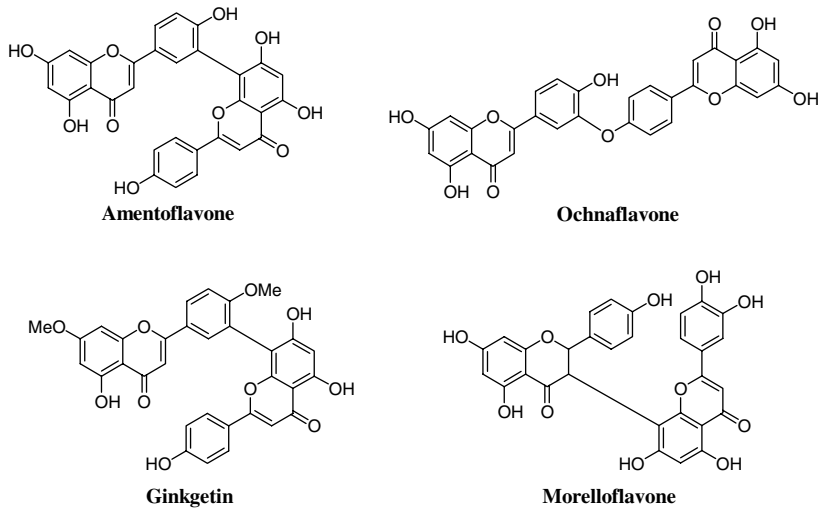


Figure 1. Structures of naturally occurring biflavonoids.

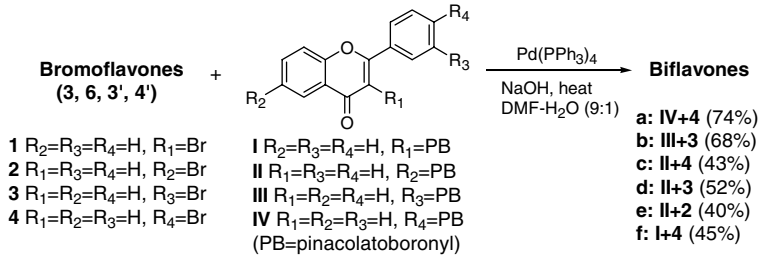
report, various biflavonoids were synthesized and their PLA inhibitory activity was examined.[16] In this respect, this study evaluated several synthetic biflavonoids for their $sPLA_2$-IIA enzyme inhibitory activity.

2. Chemistry

Six C–C biflavones (**a**–**f**) were prepared following the synthetic pathway (Scheme 1) and evaluated for the inhibitory activity against various secretoryvPLA_2s.[16] Treatment of bromoflavones (**1**–**4**) with commercially available bis(pinacolato)diboron in the presence of catalytic $PdCl_2$(dppf) and K_2CO_3 in DMF at 90 °C provided the corresponding pinacolato boronates (**I**–**IV**) as described in the previous literature.[16] Suzuki coupling reactions of the pinacolato boronates **I**–**IV** (120 mol%) with bromoflavones (**1**,**2**,**3**,**4**; 100 mol%) in standard conditions [$Pd(PPh_3)_4$ (5 mol%), NaOH (400 mol%), and DMF-water (9:1), 100 °C] gave C–C biflavones (**a**, **b**, **c**, **d**, **e**, **f**) in 74%, 68%, 43%, 52%, 40%, and 45% yields, respectively. Thus six C–C biflavones (**a**–**f**, Fig. 2) were prepared and evaluated for their inhibitory activity against PLA_2s.

3. Results and discussion

Extracellular $sPLA_2$-IIA activity has been reported in the inflamed sites of several experimental animal models,[17–19] as well as in human diseases such as pancreatitis and rheumatoid arthritis.[20–22] The correlation between the $sPLA_2$-IIA levels and the disease status has led to the development of various pharmacological agents that can inhibit the $sPLA_2$-IIA activity, which would be beneficial for mitigating various diseases. As a result of the ongoing efforts aimed at developing new $sPLA_2$ inhibitors from natural products through an activity-guided isolation procedure, new $sPLA_2$-IIA biflavonoids were isolated from natural products.[6,7] Figure 1 shows the chemical structures of naturally occurring biflavonoids. In an attempt to develop more potent sPLA2 inhibitors than natural biflavonoids, various C–C type biflavonoids were designed and synthesized (Scheme 1 and Fig. 2). The synthetic biflavones showed somewhat different inhibitory activity against $sPLA_2$-IIA.[16] Among them, a biflavone with a C–C 4′-4‴ (**4′,4‴-BF, a**) linkage showed 7 times stronger inhibition than the natural biflavonoid.[16] However, the effect of the synthetic biflavonoids on other $sPLA_2$ isozymes was not described. Therefore, a comparative test was carried out to determine out how the synthetic biflavonoids affect various mammalian $sPLA_2$s isozymes. As shown in Table 1, the synthetic C–C biflavonoids (**a**–**f**) were generally more potent and showed broad inhibitory activity profiles against the PLA_2s tested compared with those of amentoflavone, a natural C–C biflavonoid. Natural biflavonoids, amentoflavone and ochnaflavone, only showed moderate inhibitory activity against $sPLA_2$ V, whereas synthetic C–C biflavonoids **a**–**f** showed inhibitory activity against $sPLA_2$ V and $cPLA_2$. In particular, among natural and synthetic biflavonoids tested, only



Scheme 1. Synthesis of Biflavones **a**–**f** by Suzuki C–C cross-coupling reaction.

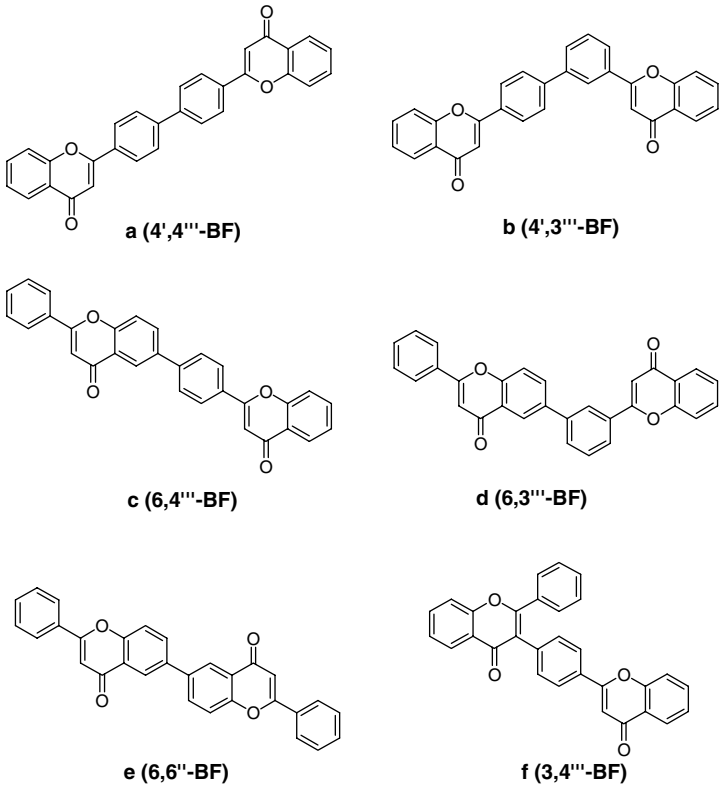


Figure 2. Structures of synthesized C–C biflavones (**a**–**f**).

Table 1. IC_{50} values of synthetic biflavones on various PLA_2s activity

Compounds	$sPLA_2$ IB	$sPLA_2$ IIA[a]	$sPLA_2$ V	$sPLA_2$ X	$cPLA_2$
a (4′,4′-BF)	>100	3.0 ± 0.90	8.7 ± 3.77	>100	6.0 ± 1.92
b (4′,3′-BF)	>100	15.7 ± 3.7	23.9 ± 5.21	>100	38.7 ± 6.3
c (6,4′-BF)	>100	63.9 ± 4.2	10.8 ± 2.56	>100	11.9 ± 3.07
d (6,3′-BF)	>100	19.9 ± 4.6	12.8 ± 3.08	27.6 ± 4.3	16.8 ± 3.74
e (6,6-BF)	>100	69.3 ± 5.7	50.0 ± 10.1	>100	48.1 ± 10.6
f (3,4′-BF)	>100	23.2 ± 3.1	6.50 ± 3.41	>100	8.42 ± 5.08
Amentoflavone	>100	23.8 ± 3.4	41.8 ± 5.2	>100	>100
Ochanflavone	>100	3.5 ± 0.6	17.6 ± 2.41	>100	>100

[a] These results were already published.[16] All data are arthmetic means ± SD ($n = 3$).

the C–C biflavonoid **d** showed inhibitory activity against $sPLA_2$ X. None of the natural and synthetic C–C biflavonoids tested showed any inhibitory activity against $sPLA_2$ IB. The synthetic C–C biflavonoid **a** showed equipotent inhibitory activity against $sPLA_2$ IIA[16] and more potent inhibitory activity against $sPLA_2$ V compared with those of ochnaflavone, a natural C–O–C biflavonoid that is known to be the most potent natural biflavonoid against $sPLA_2$ IIA and $sPLA_2$ V. At this moment, we do not have any reasonable ideas to explain the selectivity differences between synthetic and natural biflavones. We assume that phenol groups in natural biflavone structures may play roles on selective interactions with PLA_2 isoforms.

In summary, the synthetic C–C biflavonoids (**a**–**f**) examined in this study have potent and broad inhibitory activity against various PLA_2s tested regardless of their structural array. We also found that only the C–C biflavonoid **d** showed inhibitory activity against $sPLA_2$ X. Further chemical modification of these basic structures will be carried out in order to investigate the synthetic C–C biflavones which possess more selective inhibitory activity against isozymes of $sPLA_2$.

4. Experimental

4.1. Materials and methods

All chemicals were obtained from commercial suppliers and used without further purification. All solvents used for reaction were freshly distilled from proper dehydrating agent under nitrogen gas. All solvents used for chromatography were purchased and directly applied without further purification. ^1H NMR spectra were recorded on a Varian Gemini 2000 instrument (200 MHz) and a Bruker DPX 400 (400 MHz) spec-

trometers. Chemical shifts are reported in parts per million (ppm) downfield relative to tetramethylsilane as an internal standard. Peak splitting patterns are abbreviated as m (multiplet), s (singlet), bs (broad singlet), d (doublet), bd (broad doublet), t (triplet), dd (doublet of doublets), and ddd (doublet of double doublet). ^{13}C NMR spectra were recorded on a Bruker DPX 400 (100 MHz) spectrometer, fully decoupled, and chemical shifts are reported in parts per million (ppm) downfield relative to tetramethylsilane as an internal standard. Melting points were recorded on a Fisher-Johns microscopic scale melting point apparatus. EI mass spectra were recorded on an Autospec M363. Analytical thin-layer chromatography (TLC) was performed using commercial glass plate with silica gel $60F_{254}$ purchased from Merck. Chromatographic purification was carried out by flash chromatography using Kieselgel 60 (230–400 mesh, Merck).

4.2. General procedure for synthesis of flavone pinacolato boronates (I-IV)

The reaction mixture of bis(pinacolato)diboron (100 mol%), $PdCl_2$(dppf) (5 mol%), and anhydrous potassium carbonate (400 mol%) in freshly distilled DMF was degassed by vacuum pump for 10 min and saturated with nitrogen gas. The reaction was conducted with stirring at 90 °C for 10 h. The mixture was cooled to room temperature, filtered through a filtering reagent, and the filtrate was extracted with ethyl acetate. The organic layer was washed with brine and water, dried over anhydrous $MgSO_4$, and filtered with a glass filter. The filtrate was concentrated in a reduced pressure, and the crude residue was purified by column chromatography. The product was further purified by recrystallization in hot methyl alcohol to afford the target compound (**I**–**IV**).

4.2.1. 3-Pinacolatoboronflavone (I). 78%; ^1H NMR (200 MHz, $CDCl_3$) δ 8.30 (d, 1H, J = 1.6 Hz, H5), 7.86 (d, 2H, J = 2.2 Hz, 4.4 Hz, H2′, H6′), 7.75 (t, 1H, J = 1.8 Hz, 8.8 Hz, H7), 7.53–7.57 (m, 3H, H3′, H4′, H5′), 7.49 (t, 1H, J = 3.6 Hz, 4.4 Hz, 8.0 Hz, H6), 7.42 (d, 1H, J = 1.4 Hz, H8), 1.37 (s, 12H, 4*CH_3).

4.2.2. 6-Pinacolatoboronflavone (II). 65%; ^1H NMR (200 MHz, $CDCl_3$) δ 8.35 (s, 1H, H5), 7.92 (d, 2H, J = 3.2 Hz, 4.4 Hz, H2′, H6′), 7.79 (d, 1H, J = 2.6 Hz, H7), 7.45–7.56 (m, 4H, H8, H3′, H4′, H5′), 6.88 (s, 1H, H3), 1.38 (s, 12H, 4*CH_3).

4.2.3. 3′-Pinacolatoboronflavone (III). 86%; ^1H NMR (200 MHz, $CDCl_3$) δ 8.36 (d, 1H, J = 8.0 Hz, H5), 8.24 (d, 1H, J = 6.6 Hz, H4′), 8.00 (t, 1H, J = 8.0 Hz, H7), 7.50–7.78 (m, 4H, H8, H3′, H2′, H5′), 7.43 (t, 1H, J = 6.0 Hz, H6), 6.88 (s, 1H, H3), 1.38 (s, 12H, 4*CH_3).

4.2.4. 4′-Pinacolatoboronflavone (IV). 84%; ^1H NMR (200 MHz, $CDCl_3$) δ 8.25 (d, 1H, J = 6.0 Hz, H5), 7.94–7.95 (m, 4H, H2′, H6′, H7, H8), 7.66 (d, 2H, J = 5.0 Hz, 6.4 Hz, H3′, H5′), 7.44 (t, 1H, J = 6.4 Hz, 5.4 Hz, H6), 6.89 (s, 1H, H3), 1.38 (s, 12H, 4*CH_3).

4.3. General synthetic procedure for Suzuki C–C cross-coupling reaction

The reaction mixture of tetrakis(triphenylphosphine)-palladium (5 mol%), a flavone pinacolato boronate (**I**–**IV**, 120 mol%), an aryl bromide (**1**–**4**, 100 mol%), and NaOH (400 mol%) in a mixed solution of DMF and water (9:1) was degassed by vacuum pump for 10 min and saturated with nitrogen gas. The reaction was conducted with stirring at 100 °C for 14 h. The mixture was cooled to room temperature, filtered through a filtering reagent, and the filtrate was extracted with ethyl acetate. The organic layer was washed with brine and water, dried over anhydrous $MgSO_4$, and filtered with a glass filter. The filtrate was concentrated in a reduced pressure, and the crude residue was purified by column chromatography. The product was further purified by recrystallization in hot methyl alcohol to afford the target compound (**a**–**f**).

4.3.1. [4′,4‴]-Biflavone (4′,4‴-BF; a). The product was obtained as a white solid in 74% yield; mp >300 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 8.25–8.28 (dd, 2H, J = 1.5 Hz, 7.9 Hz, H5, H5″), 8.06–8.08 (d, 4H, J = 8.4 Hz, H3′, H3‴, H5′, H5‴), 7.82–7.84 (d, 4H, J = 8.4 Hz, H2′, H2‴, H6′, H6‴), 7.72–7.76 (m, 2H, H7, H7″), 7.61–7.63 (d, 2H, J = 8.1 Hz, H8, H8″), 7.44–7.47 (t, 2H, J = 7.4 Hz, H6, H6″), 6.92 (s, 2H, H3, H3″); ^{13}CNMR (100 MHz, $CDCl_3$) δ 178.84 (C-4, C-4″), 163.46 (C-2, C-2″), 156.72 (C-9, C-9″), 143.31 (C-4′, C-4‴), 134.46 (C-7, C-7″), 131.79 (C-1′, C-1‴), 128.17 (C-3′, C-3‴, C-5′, C-5‴), 127.45 (C-2′, C-2‴, C-6′, C-6‴), 126.20 (C-6, C-6″), 125.88 (C-5, C-5″), 124.25 (C-10, C-10″), 118.54 (C-8, C-8″), 107.99 (C-3, C-3″); m/z 442.12 (M^+, 100).

4.3.2. [4′,3‴]Biflavone (4′,3‴-BF; b). The product was obtained as a white solid in 68% yield; mp 267–269 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 8.25–8.27 (dd, 2H, J = 1.5 Hz, 7.9 Hz, H5, H5″), 8.19–8.20 (d, 1H, J = 1.4 Hz, H2′), 8.07–8.09 (d, 2H, J = 8.4 Hz, H3‴, H5‴), 7.96–7.98 (d, 1H, J = 7.9 Hz, H6′), 7.81–7.83 (d, 3H, J = 8.4 Hz, H2‴, H6‴, H4′), 7.72–7.76 (ddd, 2H, J = 1.7 Hz, 7.8 Hz, 1.3 Hz, H7, H7″), 7.61–7.68 (m, 3H, H5′, H8, H8″), 7.44–7.47 (t, 2H, J = 7.5 Hz, H6, H6″), 6.92–6.93 (s, 2H, H3, H3″); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.82 (C-4, C-4″), 163.45 (C-2), 163.28 (C-2″), 156.71 (C-9, C-9″), 143.66 (C-4‴), 141.24 (C-3′), 134.35 (C-7″), 134.31 (C-7), 133.11 (C-1′), 131.70 (C-1‴), 130.70 (C-2′), 130.23 (C-4′), 128.22 (C-3‴, C-5‴), 127.41 (C-2‴, C-6‴), 126.40 (C-6′), 126.20 (C-6, C-6″), 125.83 (C-5″), 125.77 (C-5′), 125.44 (C-5), 124.41 (C-10, C-10″), 118.55 (C-8), 118.54 (C-8″), 108.45 (C-3), 108.13 (C-3″); m/z 442 (M^+, 100), 441 (23), 322 (14), 221 (14), 202 (21), 92 (14), 57 (22).

4.3.3. [6,4‴]Biflavone (6,4‴-BF; c). The product was obtained as a white solid in 43% yield; mp 291–293 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 8.53 (s, 1H, H5), 8.24–8.26 (d, 1H, J = 7.8 Hz, H5″), 8.04–8.06 (d, 2H, J = 7.8 Hz, H3‴, H5‴), 8.00–8.02 (d, 1H, J = 8.8 Hz, H7), 7.96–7.97 (d, 2H, J = 5.3 Hz, H2′, H6′), 7.84–7.86 (d, 2H, J = 7.9 Hz, H2‴, H6‴), 7.70–7.75 (m, 2H, H8, H7″),

7.61–7.63 (d, 1H, J = 8.4 Hz, H8″), 7.55–7.57 (d, 3H, J = 5.1 Hz, H3′, H4′, H5′), 7.43–7.46 (t, 2H, J = 7.5 Hz, H6″), 6.89–6.90 (d, 2H, J = 4.5 Hz, H3, H3″); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.74 (C-4, C-4″), 163.01 (C-2), 163.23 (C-2″), 156.70 (C-9″), 156.55 (C-9), 142.79 (C-4‴), 137.26 (C-6), 134.26 (C-7″), 132.86 (C-7), 132.21 (C-1′), 132.02 (C-5), 131.55 (C-1‴), 129.54 (C-4′), 128.12 (C-3‴, C-5‴), 127.36 (C-2‴, C_β-6‴), 126.76 (C-3′, C-5′), 126.16 (C-6″), 125.72 (C-5″), 124.71 (C-10), 124.31 (C-10, C-2′, C-6′), 119.41 (C-8), 118.56 (C-8″), 108.12 (C-3 , C-3″); m/z 442 (M^+, 100), 441 (8), 340 (6), 322 (6), 220 (5).

4.3.4. [6,3‴]Biflavone (6,3‴-BF; d). The product was obtained as a yellowish solid in 52% yield; mp 250–252 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.52 (s, 1H, H5), 8.25–8.26 (d, 1H, J = 7.7 Hz, H5″), 8.22 (s, 1H, H2‴), 7.93–8.01 (m, 4H, H7, H2′, H6′, H6‴) 7.86–7.86 (d, 1H, J = 7.5 Hz, H4‴), 7.71–7.75 (t, 2H, J = 6.8 Hz, 7.7 Hz, H7″, H8), 7.62–7.66 (t, 2H, J = 8.0 Hz, 7.5 Hz, H5‴, H8″), 7.55–7.57 (d, 3H, J = 5.8 Hz, H3′, H4′, H5′), 7.43–7.47 (t, 2H, J = 7.4 Hz, H6″), 6.90–6.92 (s, 2H, H3, H3″); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.83 (C-4″), 178.74 (C-4), 163.04 (C-2), 163.51 (C-2″), 156.73 (C-9″), 156.43 (C-9), 140.82 (C-3‴), 137.73 (C-6), 134.32 (C-7″), 133.09 (C-1‴), 133.05 (C-7), 132.22 (C-1′), 132.04 (C-5), 130.82 (C-2‴), 130.21 (C-4‴), 129.55 (C-4′), 126.79 (C-3′, C-5′), 126.17 (C-6″,C-6‴), 125.79 (C-5‴), 125.45 (C-5″), 124.66 (C-10), 124.42 (C-10″), 124.35 (C-2′, C-6′), 119.43 (C-8), 118.69 (C-8″), 108.49 (C-3″), 108.10 (C-3); m/z 442 (M^+, 100), 340 (17), 220 (11), 192 (9), 163 (12).

4.3.5. [6,6″]Biflavone (6,6″-BF; e). The product was obtained as a white solid in 40% yield; mp > 300 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.53–8.53 (d, 2H, J = 2.3 Hz, H5, H5″), 8.07–8.10 (dd, 2H, J = 2.3 Hz, 8.8 Hz, H7, H7″), 7.96–7.98 (m, 4H, H2′, H2‴, H6′, H6‴), 7.70–7.72 (d, 2H, J = 8.8 Hz, H8, H8″), 7.54–7.57 (m, 6H, H3′, H3‴, H4′, H4‴, H5′, H5‴), 6.89 (s, 2H, H3, H3″); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.71 (C-4, C-4), 164.10 (C-2, C-2″), 156.40 (C-9, C-9″), 136.95 (C-6, C-6″), 133.13 (C-7, C-7″), 132.21 (C-1′, C-1‴), 132.05 (C-5, C-5‴), 129.54 (C-4′, C-4‴), 126.79 (C-3′, C-3‴, C-5′, C-5‴), 124.57 (C-10, C-10″), 124.14 (C-2′, C-2‴, C-6′, C-6‴), 119.44 (C-8, C-8″) , 108.02 (C-3, C-3″); m/z 442 (M^+, 100), 340 (39), 210 (20), 126 (19), 102 (20).

4.3.6. [3,4‴]Biflavone (3,4‴-BF; f). The product was obtained as a white solid in 45% yield; mp 231–233 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.292–8.316 (dd, 1H, J = 1.5 Hz, 8.0 Hz, H5″), 8.11–8.23 (dd, 1H, J = 1.6 Hz, 8.0 Hz, H5), 7.86–7.89 (t, 2H, J = 1.9 Hz, 8.4 Hz, H2′, H6′), 7.66–7.75 (m, 2H, H7, H7″), 7.53–7.57 (dd, 2H, J = 8.2 Hz, H8, H8″), 7.36–7.47 (m, 7H, H3′, H3‴, H5′, H5‴, H2‴, H6‴, H4′), 7.29–7.33 (m 2H, H6, H6″), 6.82 (s, 1H, H3″); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.85 (C-4″), 178.36 (C-4), 163.52 (C-2″), 162.48 (C-2), 156.65 (C-9″), 156.45 (C-9), 137.19 (C-4‴), 134.41 (C-7″), 134.19 (C-7), 133.30 (C-1′), 132.47 (C-3‴, C-5‴), 131.18 (C-1‴), 130.92 (C-2‴, C-6‴), 130.02 (C-4′), 128.78 (C-3′, C-5′), 126.79 (C-6), 126.53 (C-2′, C-6′), 126.10 (C-6″), 125.79 (C-5), 125.63 (C-5″), 124.41 (C-10″), 123.79 (C-10), 118.50 (C-8″), 118.47 (C-8), 107.97 (C-3, C-3″); m/z 442 (M^+, 70), 441 (100), 322 (10), 321 (18), 202 (16), 121 (9).

4.4. Assay of phospholipase A_2 inhibitory activity by synthetic biflavonoids

The cDNA for rat sPLA$_2$-IB, human sPLA$_2$-IIA, sPLA$_2$-V, sPLA$_2$-X, and cPLA$_2$ was cloned into an expression vector and transfected into human embryonic kidney 293 cells (HEK293 cells) using Lipofectamine PLUS (Gibco BRL, Gaithersburg, MD, USA) as described previously.[13,14]

The standard reaction mixture (200 μl) contained 100 mM Tris–HCl (pH 9.0), 6 mM $CaCl_2$, 1% bovine serum albumin, 2.5 μM of radiolabeled 1-acyl-2-[1-^{14}C] arachidonoyl-*sn*-glycerol phosphoethanolamine (48 mCi/mmol, NEN, Boston, MA, USA), and synthetic biflavonoids. The reaction was started by the addition of an aliquot of the culture medium as an enzyme source and carried out at 37 °C for 20 min, and [^{14}C]arachidonic acid released was extracted by the method described previously.[17]

Under these conditions, the reaction mixture without synthetic biflavonoids released 10% free fatty acid. Inhibition was expressed as a percentage. Synthetic biflavonoids were dissolved in dimethylsulfoxide (DMSO) and added to the enzyme assay tubes at 2% of the final volume. Control experiments showed that DMSO at concentrations up to 2% had no effect on enzymatic activity. All determinations were duplicated and the 50% inhibitory concentration was obtained by linear regression analysis at 1–100 μM of biflavones tested.

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