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Synthesis of biflavones having a 6-O-7″ linkage and effects on cyclooxygenase-2 and inducible nitric oxide synthase

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Dedicated to Prof. Moon Woo Chun on the occasion of his retirement.

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ABSTRACT

In order to establish anti-inflammatory potential of biflavonoids, 17 biflavone derivatives having a 6-O-7″ linkage were synthesized and their effects on cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) were evaluated. The basic molecule (6-O-7″ biflavone) potently inhibited COX-2-mediated PGE_2 production (IC_{50}: < 2 μM), being less active on iNOS-mediated NO production (IC_{50}: > 50 μM) from lipopolysaccharide-treated RAW 264.7 cells, a mouse macrophage cell line. Generally, the hydroxyl/methoxyl substitution(s) on the basic biflavone (6-O-7″) reduced the inhibitory activity of PGE_2 production, while the effects on NO production were varied. It is suggested that the basic biflavone (6-O-7″) may have a potential for new anti-inflammatory agent.

Inducible isoforms of cyclooxygenase (COX-2) and nitric oxide synthase (iNOS) produce large amount of prostaglandins (PG) and nitric oxide (NO), respectively, which have an important role to provoke and maintain inflammatory response.[1] The compounds having inhibitory capacity of these enzymes may show anti-inflammatory effect.

Biflavonoids belong to flavonoid family and they are dimers of monomeric flavonoid. Many different chemical structures of biflavonoids have been isolated from plants and some of them were found to possess anti-inflammatory activity in vivo and in vitro. Their anti-inflammatory action mechanisms include inhibition of mast cell histamine release,[2] lymphocyte proliferation[3] and phospholipase A_2 (PLA_2),[4] and down-regulation of proinflammatory molecule expression such as COX-2 and iNOS.[5,6] Recently, several C–C biflavones were chemically synthesized and some of them showed promising anti-inflammatory activity.[7,8] To extend these findings and to find structurally optimized derivatives, 17 C–O–C biflavone derivatives having 6-O-7″ linkage (Fig. 1) were prepared and their effects on cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) were evaluated.

The 6-O-7″ biflavones were prepared via intermolecular Ullmann ether syntheses[9,10] of 6-bromoflavone analogs and 7-hydroxyflavone analogs as key intermediates. 6-Halogenoflavone and 7-hydroxyflavone analogs were prepared from 5-bromo-2-hydroxyacetophenone following the general procedures as described in the earlier publications in our group.[11] The mixture of a 6-bromoflavone analog (100 mol%), a 7-hydroxyflavone analog (100 mol%), CuI (20 mol%), *N,N*-dimethylglycine (8 mol%), cesium carbonate (8 mol%) in dry dioxane was reacted at 100 °C for 24 h. Purification of the crude product by column chromatography ($CHCl_3$:MeOH = 100 ~ 20:1) gave biflavones (671-1, 672-1, 673-1, 674-1, 675-1, 676-1, 677-1 and 678-1) as white solid in 10–16% yields, respectively. Demethylation of the C–O–C biflavones with BBr_3 in $CHCl_3$ (2.0–8.0 equiv) at 0 °C provided crude products. The crude products were purified by recrystallization in MeOH gave the desired biflavones (671-2, 672-2, 673-2, 674-2, 675-2, 676-2, 677-2 and 678-2) as pale yellow solid in 65–75% yields, respectively (Scheme 1).

These biflavonoids were evaluated for their anti-inflammatory activity as followings. RAW 264.7 cells obtained from American Type Culture Collection (ATCC) were cultured with DMEM supplemented with 10% FBS and 1% antibiotics (penicillin, 100 U/ml and streptomycin, 100 μg/ml) under 5% CO_2 at 37 °C and activated with LPS based on the previously described procedures.[12] Briefly, cells were plated in 96-well plates (2×10^5 cells/well). After pre-incubation for 2 h, test compounds and LPS (1 μg/ml) were added and incubated for 24 h, unless otherwise specified. Test com-

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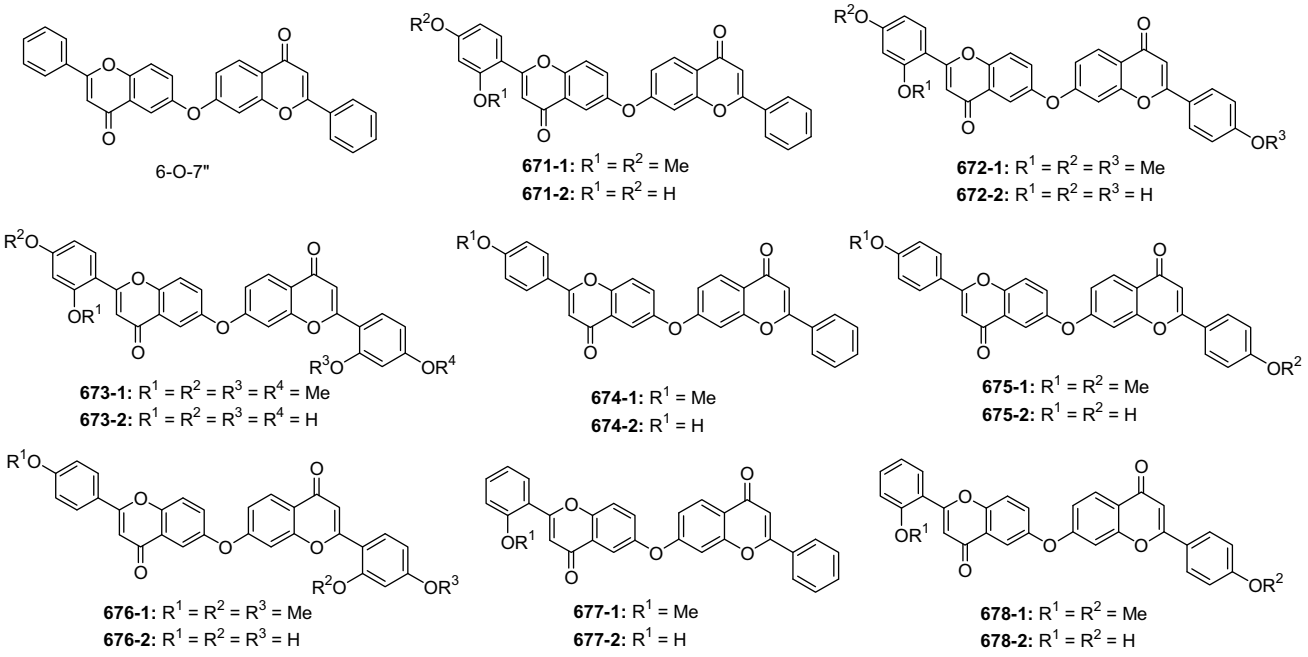
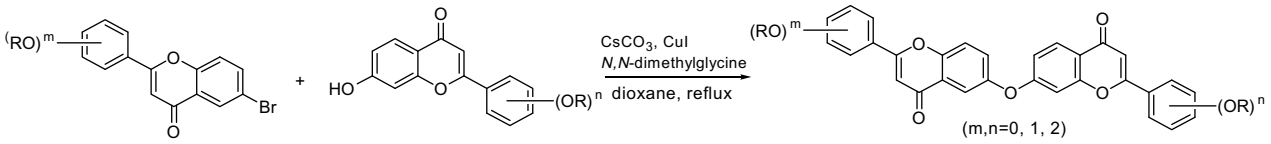


Figure 1. Synthetic biflavones with 6-O-7″ linkage.



Scheme 1. Typical intermolecular Ullmann ether syntheses for 6-O-7″ biflavones.

pounds dissolved in DMSO were diluted with serum-free DMEM into appropriate concentrations. Final concentration of DMSO was adjusted to 0.1% (v/v). Cell viability was assessed with MTT assay as described previously.[13] For determination of NO concentration, the stable conversion product of NO, nitrite (NO_2^-), was measured using Griess reagent and optical density was checked at 550 nm. PGE_2 concentration in the medium was measured using ELISA kit for PGE_2 (Cayman Chem. Co.) according to the manufacturer's recommendation. *N*-[2-Cyclohexyloxy-4-nitrophenyl]-methane sulfonamide (NS-398) was obtained from Biomol (Plymouth Meeting, PA). 2-Amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine hydrochloride (AMT) was purchased from Tocris Cookson, Ltd (UK). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lipopolysaccharide (LPS, *Escherichia coli* 0127:B8) were purchased from Sigma Chem. (St. Louis, MO). LipofecAMINE PLUS, DMEM and other cell culture reagents including FBS were products of Gibco BRL (Grand Island, NY). Protein assay kit was purchased from Bio-Rad Lab. (Hercules, CA).

It is well established that RAW 264.7 cells, a mouse macrophage cell line, induce COX-2 and iNOS which produce large amounts of PGE_2 and NO, respectively, when activated with LPS. As in Table 1, LPS treatment (1 μg/ml) to RAW cells for 24 h increased PGE_2 and NO production. Under this condition, synthetic 6-O-7″ biflavones showed more or less inhibitory activity on these two parameters. For COX-2-mediated PGE_2 production, the basic molecule (6-O-7″ biflavone without any substitution) showed most potent inhibitory activity. The hydroxyl/methoxyl substitutions on the basic biflavone abolished/diminished inhibitory activity. Only 671-1 and 674-1 possessed slightly less inhibitory activity compared to the basic molecule. For iNOS-mediated NO production, all compounds tested did not show potent inhibition. Some of them possessed weak inhibition of NO production. Among the derivatives, 678-2 possessed slightly higher inhibitory activity than the basic molecule. It is sig-

Table 1
Inhibition of PGE_2 and NO production from LPS-treated RAW264.7 cells.

Compound[14]	IC_{50} (μM)	
	PGE_2 production	NO production
6-O-7″	<2	>50
671-1*	2–10	—
671-2	>50	—
672-1	>50	>50
672-2	>50	>50
673-1	50	>50
673-2*	10–50	10–50
674-1	2–10	—
674-2*	—	—
675-1	—	—
675-2	>50	—
676-1	>50	>50
676-2*	>50	50
677-1	>50	—
677-2	>50	—
678-1	—	—
678-2	>50	10–50

IC_{50} values were calculated by linear regression analysis using the inhibition data of three concentrations of test compounds (2, 10 and 50 μM). PGE_2 and NO concentrations of the control group without LPS were 1.0 ± 0.3 nM and 1.2 ± 0.1 μM, respectively, while the LPS-treated control group produced 105.4 ± 3.2 nM of PGE_2 and 32.5 ± 1.8 μM of NO (arithmetic mean ± SD, $n = 3$). As reference compounds, NS-398 (selective COX-2 inhibitor) showed 99.3% inhibition of PGE_2 production at 0.1 μM and AMT (iNOS inhibitor) showed 92.5% inhibition of NO production at 1.0 μM. *MTT bioassay indicated that 671-1, 673-2, 674-2 and 676-2 reduced cell viability of 2%, 50%, 3% and 9% at 50 μM, respectively.

nificant to note that the potent PGE_2 inhibitory biflavonoids are methoxylated derivatives (671-1, 673-1 and 674-1) among the substituted derivatives while meaningful NO inhibitory biflavonoid is hydroxylated derivative (678-2).

In the present investigation, anti-inflammatory activity of the biflavonoids having a 6-O-7″ linkage with/without substituents at C-2′, 2‴, 4′ and 4‴ was examined in vitro. Although various substitutions were tried, their anti-inflammatory activities were not satisfactory. Only the basic molecule has the potential for new ant-inflammatory agent. The substitutions at different position should be tried further.

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