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Synthesis and anti-inflammatory activities of 4*H*-chromene and chromeno[2,3-*b*]pyridine derivatives

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Abstract Several derivatives of 4*H*-chromene and chromeno[2,3-*b*]pyridine were efficiently prepared under microwave irradiation in a one-pot reaction, and their anti-inflammatory activities were evaluated. Six synthetic products (**1b**, **1c**, **1h**, **2d**, **2j**, and **2l**) exhibited more powerfully inhibited the production of tumor necrosis factor-α-induced nitric oxide (NO) than quercetin and exhibited comparable cell viability in both human and porcine chondrocytes. In particular, **2d** at dosages of 10 and 20 mg/kg had a very potent anti-inflammatory effect by suppressing the formation of carrageenan-induced rat paw edema and prostaglandin E_2. The results herein suggest that these compounds may have potential as structural templates in the design and development of new anti-inflammatory drugs.

Keywords 4*H*-chromene · Chromeno[2,3-*b*]pyridine · NO production · Carrageenan-induced rat paw edema · Anti-inflammatory

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Introduction

Inflammation is a general response to infection and injury and has been associated with such diseases arthritis, asthma, allergy, diabetes, atherosclerosis, and cancer, which affect people globally [1–6]. Patients with rheumatoid arthritis and osteoarthritis commonly describe swelling, pain, and stiffness as significant symptoms, which reduce health-related quality of life [7]. Many investigations have demonstrated that certain important inflammatory mediators, such as cytokines, nitric oxide (NO) and prostaglandin E_2 (PGE_2), have a significant pathophysiological role in the development of inflammation [8–10]. Numerous anti-inflammatory drugs have been approved for treating inflammation, including aspirin, corticosteroids, indomethacin, diclofenac, ibuprofen, pranoprofen, celecoxib, and roficoxib [7, 11]. Of these, nonsteroidal anti-inflammatory drugs (NSAIDs) are effective anti-inflammatory agents and analgesics that inhibit the biosynthesis or release of prostaglandins from arachidonic acid by suppressing cyclooxygenases [7]. Unfortunately, since these drugs inhibit the production of prostaglandins, they can cause serious side effects, such as gastrointestinal irritation, ulceration, hepatotoxicity, acute renal failure, hypertension, and even heart failure [12–15]. The need to develop new and safer therapeutic agents motivated the design and synthesis herein of two series of substituted 4*H*-chromene and chromeno[2,3-*b*]pyridine derivatives as potential anti-inflammatory agents.

Recently, derivatives of 4*H*-chromene and chromeno[2,3-*b*]pyridine (Fig. 1) have been found to have various potential biological activities, including anticancerous, anti-proliferative, anti-inflammatory, anti-rheumatic, anti-allergic, apoptosis-inducing, antibacterial, and anti-tubercular activities [16–23]. For example, pranoprofen, a safe and effective NSAID with a chromeno[2,3-*b*]pyridine scaffold, has been approved to treat conjunctivitis topically by inhibiting prostaglandin synthesis [24–26]. Pranoprofen has also determined to be as active as diclofenac sodium in reducing pain and inflammation following strabismus surgery [25, 26]. Interestingly, derivatives of both 4*H*-chromene and chromeno[2,3-*b*]pyridine comprise a benzopyran nucleus, and most have a 2-amino-3-nitrilo functionality. To the best of our knowledge, only a few studies have focused on structural modifications of such compounds that are associated with potential anti-inflammatory activity. The conventional syntheses of those compounds by the condensation of aromatic aldehydes, malononitrile and phenols with or without a catalyst in a solvent or neat are typically time-consuming (normally >24 h) [27, 28]. Recent investigations have presented the use of a multicomponent reaction (MCR) to synthesize certain pharmacologically important heterocyclic compounds in a few steps or in a one-pot procedure [19, 29]. Accordingly, MCR has been identified as time-effective and economically favorable for a large number of manufacturing processes. Microwave methods have enabled in the rapid synthesis of target compounds, the optimization of chemical reactions, and green chemistry. The combination of MCR and microwave-assisted synthesis appears to be a fascinating means of preparing active pharmaceutical ingredients.

Our earlier efforts to design and prepare novel anti-inflammatory agents has led to the discovery of some unique 4*H*-chromene derivatives synthesized by MCRs, with

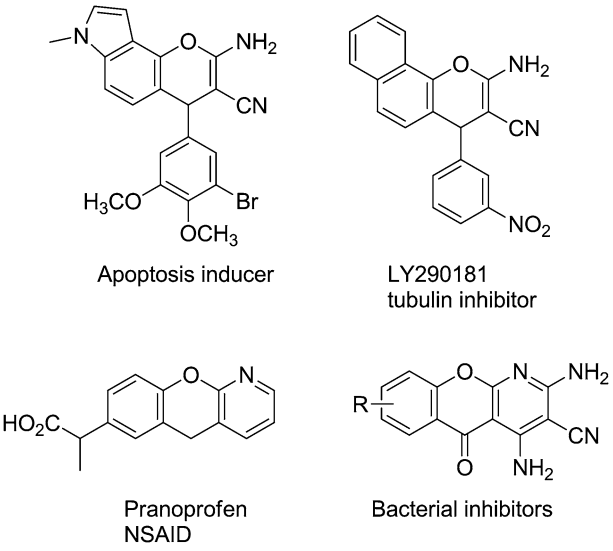
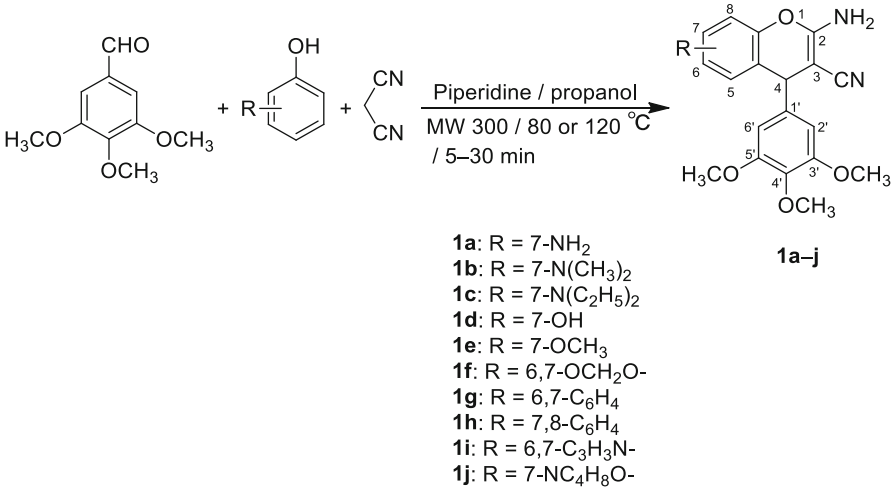
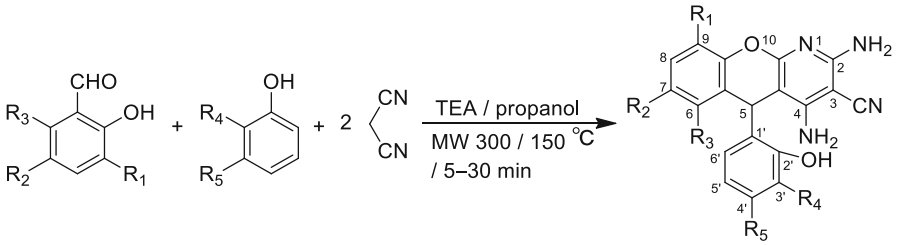


Fig. 1 Derivatives of 4*H*-chromene and chromeno[2,3-*b*]pyridine as privileged medicinal scaffolds



Scheme 1 Synthesis of compounds **1a**–**j**

moderate anti-inflammatory activity [30]. In this work, two novel series of substituted 4*H*-chromenes (**1a**–**j**, Scheme 1) and chromeno[2,3-*b*]pyridines (**2a**–**l**, Scheme 2) were obtained by one-pot, microwave-assisted synthesis in 5–30 min with good yields. A systematic investigation of the structures of the aforementioned 4*H*-chromenes and chromeno[2,3-*b*]pyridines revealed that all of the synthetic products, **1a**–**j** and **2a**–**l**, significantly inhibited the production of NO in both human and porcine chondrocytes in vitro. The anti-inflammatory activity of selected products, evaluated using a carrageenan-induced rat hind paw edema assay [31, 32], indicated that **2d** exhibited very potent anti-inflammatory effect in vivo.



2a–l

2a: R_1 = H, R_2 = H, R_3 = H, R_4 = H, R_5 = NH_2
2b: R_1 = H, R_2 = H, R_3 = H, R_4 = H, R_5 = $N(CH_3)_2$
2c: R_1 = H, R_2 = H, R_3 = H, R_4 = H, R_5 = $N(C_2H_5)_2$
2d: R_1 = H, R_2 = H, R_3 = H, R_4, R_5 = C_6H_4
2e: R_1 = OCH_3, R_2 = H, R_3 = H, R_4 = H, R_5 = NH_2
2f: R_1 = OCH_3, R_2 = H, R_3 = H, R_4 = H, R_5 = $N(CH_3)_2$
2g: R_1 = OCH_3, R_2 = H, R_3 = H, R_4 = H, R_5 = $N(C_2H_5)_2$
2h: R_1 = OCH_3, R_2 = H, R_3 = H, R_4, R_5 = C_6H_4
2i: R_1 = H, R_2, R_3 = C_6H_4, R_4 = H, R_5 = NH_2
2j: R_1 = H, R_2, R_3 = C_6H_4, R_4 = H, R_5 = $N(CH_3)_2$
2k: R_1 = H, R_2, R_3 = C_6H_4, R_4 = H, R_5 = $N(C_2H_5)_2$
2l: R_1 = H, R_2, R_3 = C_6H_4, R_4, R_5 = C_6H_4

Scheme 2 Synthesis of compounds **2a–l**

Experimental

Chemistry

All the chemicals were purchased from Aldrich-Sigma Chemical Company (St. Louis, MO, USA) and Alfa-Aesar Chemical Company (Heysham, LA32XY, England) and purified before use if necessary. All reactions were carried out in a CEM Discover Benchmate TM microwave apparatus (CEM Corp., Italy). All reactions were routinely monitored by TLC on Merck F254 silica gel plates. Melting points were measured on a Büchi-530 melting point apparatus. UV–Vis spectra were recorded on a Shimazu UV-160 A UV–visible recording spectrophotometer. IR spectra were registered on a Perkin-Elmer FTIR 1610 series infrared spectrophotometer in KBr discs. The ^{1}H- and ^{13}C-NMR spectra were determined on a Varian Gemini-400 NMR instrument in DMSO-d_6 unless otherwise noted. Chemical shifts (δ) were reported as parts per million (ppm) downfield from tetramethylsilane (TMS) as the internal standard and coupling constants (J) were given in hertz (Hz). High-resolution mass spectra (HR-MS) were performed in the Instrument Center of the National Science Counsel at the National Tsing-Hua University, Taiwan, using a Finnigan MAT-95XL.

General procedure for synthesis of 4*H*-chromene derivatives 1a–j

To a solution of 3,4,5-trimethoxybenzaldehyde (1.0 mmol), malononitrile (1.0 mmol) and substituted phenols (1.0 mmol) in propanol (3.0 mL) was added piperidine

(0.5 mmol). The mixture was subject to microwave irradiation (300 W) at 80 or 120 °C for 5–30 min. The residue was filtered and washed with ethanol and hexane. Re-crystallization from ethanol afforded pure **1a–j**.

*2,7-Diamino-4-(3,4,5-trimethoxyphenyl)-4H-chromene-3-carbonitrile (**1a**)*

Conditions: 80 °C, 5 min. yield: 85 %. mp: 208–210 °C (lit. 205–208 °C) [19]. UV (MeOH): 269 (5.64), 214 (5.21). IR (KBr): 3416, 3356, 3329, 3175 (NH_2), 2195 (CN), 1647. ^{1}H-NMR (400 MHz, DMSO-d_6): 3.61 (*s*, 3H), 3.70 (*s*, 6H), 4.48 (*s*, 1H), 5.21 (*s*, 2H), 6.18 (*s*, 1H), 6.27 (*dd*, $J = 8.4$, 2.0 Hz, 1H), 6.44 (*s*, 2H), 6.69 (*d*, 1H), 6.75 (*s*, 2H). ^{13}C-NMR (100 MHz, DMSO-d_6): 55.8, 56.1, 59.9, 99.9, 104.5, 109.8, 111.0, 120.9, 129.3, 136.1, 142.4, 148.7, 148.8, 152.8, 160.5. HR-EI-MS: 353.1377 (M^+, $C_{19}H_{19}N_3O_4^+$; calc. 353.1376).

*2-Amino-7-(dimethylamino)-4-(3,4,5-trimethoxyphenyl)-4H-chromene-3-carbonitrile (**1b**)*

Conditions: 80 °C, 5 min. yield: 89 %. mp: 180–182 °C (lit. 179–181 °C) [27]. UV (MeOH): 268 (5.68). IR (KBr): 3460, 3327, 3198 (NH_2), 2193 (CN), 1663. ^{1}H-NMR (400 MHz, DMSO-d_6): 2.85 (*s*, 6H), 3.61 (*s*, 3H), 3.70 (*s*, 6H), 4.55 (*s*, 1H), 6.21 (*sd*, $J = 2.4$ Hz, 1H), 6.44 (*sd*, $J = 2.4$ Hz, 1H), 6.47 (*s*, 2H), 6.79 (*s*, 2H), 6.86 (*d*, $J = 8.4$ Hz, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 55.8, 56.0, 59.9, 98.5, 104.6, 109.3, 110.3, 120.9, 129.4, 136.2, 142.2, 148.8, 150.2, 152.9, 160.5. HR-EI-MS: 381.1681 (M^+, $C_{21}H_{23}N_3O_4^+$; calc. 381.1689).

*Amino-7-(diethylamino)-4-(3,4,5-trimethoxyphenyl)-4H-chromene-3-carbonitrile (**1c**)*

Conditions: 80 °C, 5 min. yield: 92 %. mp: 169–171 °C. UV (MeOH): 267 (5.73). IR (KBr): 3456, 3360, 3198 (NH_2), 2189 (CN), 1653. ^{1}H-NMR (400 MHz, DMSO-d_6): 1.04 (*t*, $J = 7.0$ Hz, 6H), 3.27 (*q*, $J = 7.2$ Hz, 4H), 3.61 (*s*, 3H), 3.71 (*s*, 6H), 4.53 (*s*, 1H, CH), 6.14 (*s*, 2H), 6.39 (*dd*, $J = 8.4$, 2.0 Hz, 1H), 6.48 (*s*, 2H), 6.77 (*s*, 2H), 6.85 (*d*, $J = 8.4$ Hz, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 12.3, 43.7, 55.8, 56.0, 60.0, 97.5, 104.6, 108.5, 109.2, 120.9, 129.6, 136.2, 142.2, 147.3, 149.1, 152.9, 160.6. HR-EI-MS: 409.2000 (M^+, $C_{23}H_{27}N_3O_4^+$; calc. 409.2002).

*Amino-7-hydroxy-4-(3,4,5-trimethoxyphenyl)-4H-chromene-3-carbonitrile (**1d**)*

Conditions: 80 °C, 5 min. yield: 94 %. mp: 243–245 °C. UV (MeOH): 270 (5.65), 210 (5.13). IR (KBr): 3410, 3333, 3219 (NH_2), 2193 (CN), 1653. ^{1}H-NMR (400 MHz, DMSO-d_6): 3.61 (*s*, 3H), 3.71 (*s*, 6H), 4.57 (*s*, 1H), 6.38 (*sd*, $J = 2.4$, Hz, 1H), 6.46 (*s*, 2H), 6.49 (*dd*, $J = 8.4$, 2.0 Hz, 1H), 6.84 (*s*, 2H), 6.88 (*d*, $J = 8.4$ Hz, 1H), 9.67 (*s*, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 55.8, 56.0, 60.0, 102.1, 104.6, 112.3, 113.5, 120.7, 129.8, 136.2, 141.9, 148.7, 152.9, 157.0, 160.3. HR-EI-MS: 354.1217 (M^+, $C_{19}H_{18}N_2O_5^+$; calc. 354.1216).

*2-Amino-7-methoxy-4-(3,4,5-trimethoxyphenyl)-4H-chromene-3-carbonitrile (**1e**)*

Conditions: 120 °C, 30 min. yield: 80 %. mp: 226–227 °C (lit. 228–230 °C) [19]. UV (MeOH): 269 (5.67), 211 (5.17). IR (KBr): 3422, 3337, 3215 (NH_2), 2183 (CN), 1651. ^{1}H-NMR (400 MHz, DMSO-d_6): 3.61 (*s*, 3H), 3.71 (*s*, 6H), 3.72 (*s*, 3H), 4.63 (*s*, 1H), 6.48 (*s*, 2H), 6.54 (*sd*, $J = 2.8$, Hz, 1H), 6.66 (*dd*, $J = 8.8$, 2.8 Hz, 1H), 6.89 (*s*, 2H), 7.00 (*d*, $J = 8.8$ Hz, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 55.4, 55.8, 55.9, 59.9, 100.9, 104.6, 111.2, 115.2, 120.6, 129.9, 136.3, 141.8, 148.7, 152.9, 158.9, 160.3. HR-EI-MS: 368.1378 (M^+, $C_{20}H_{20}N_2O_5^+$; calc. 368.1372).

*6-Amino-8-(3,4,5-trimethoxyphenyl)-8H-[1,3]dioxolo[4,5-g]chromene-7-carbonitrile (**1f**)*

Conditions: 80 °C, 5 min. yield: 97 %. mp: 247–249 °C. UV (MeOH): 271 (5.67). IR (KBr): 3441, 3327, 3209 (NH_2), 2186 (CN), 1661. ^{1}H-NMR (400 MHz, DMSO-d_6): 3.62 (*s*, 3H), 3.72 (*s*, 6H), 4.56 (*s*, 1H), 5.94, 5.99 (both *d*, on $J = 0.8$ Hz, 1H), 6.49 (*s*, 2H), 6.63 (*s*, 1H), 6.66 (*s*, 1H), 6.86 (*s*, 2H). ^{13}C-NMR (100 MHz, DMSO-d_6): 40.9, 55.4, 55.8, 59.9, 97.7, 101.6, 104.5, 107.3, 115.4, 120.6, 136.3, 141.6, 142.5, 144.0, 146.7, 153.0, 160.5. HR-EI-MS: 382.1159 (M^+, $C_{20}H_{18}N_2O_6^+$; calc. 382.1165).

*2-Amino-4-(3,4,5-trimethoxyphenyl)-4H-benzo[g]chromene-3-carbonitrile (**1g**)*

Conditions: 120 °C, 30 min. yield: 85 %. mp: 181–183 °C. UV (MeOH): 228 (5.20). IR (KBr): 3477, 3356, 3196 (NH_2), 2187 (CN), 1655. ^{1}H-NMR (400 MHz, DMSO-d_6): 3.57 (*s*, 3H), 3.63 (*s*, 6H), 5.25 (*s*, 1H), 6.46 (*s*, 2H), 6.95 (*s*, 2H), 7.33 (*d*, $J = 8.8$ Hz, 1H), 7.41–7.49 (*m*, 2H), 7.90–7.94 (*m*, 3H). ^{13}C-NMR (100 MHz, DMSO-d_6): 55.8, 57.8, 59.9, 104.3, 115.5, 116.8, 120.5, 123.7, 125.0, 127.1, 128.4, 129.5, 130.3, 130.8, 136.2, 141.4, 146.8, 152.9, 159.8. HR-EI-MS: 388.1420 (M^+, $C_{23}H_{20}N_2O_4^+$; calc. 388.1423).

*2-Amino-4-(3,4,5-trimethoxyphenyl)-4H-benzo[h]chromene-3-carbonitrile (**1h**)*

Conditions: 80 °C, 5 min. yield: 95 %. mp: 184–185 °C (lit. 185–186 °C) [33]. UV (MeOH): 224 (5.30). IR (KBr): 3396, 3321, 3198 (NH_2), 2185 (CN), 1663. ^{1}H-NMR (400 MHz, DMSO-d_6): 3.61 (*s*, 3H), 3.70 (*s*, 6H), 4.86 (*s*, 1H), 6.57 (*s*, 2H), 7.13 (*s*, 2H), 7.19 (*d*, $J = 8.4$ Hz, 1H), 7.54–7.64 (*m*, 3H), 7.89 (*d*, $J = 8.0$ Hz, 1H), 8.22 (*d*, $J = 8.4$ Hz, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 41.1, 55.9, 56.0, 59.9, 105.0, 117.7, 120.5, 120.8, 122.7, 123.8, 126.2, 126.6, 126.8, 127.7, 132.7, 136.4, 141.3, 142.5, 153.0, 160.3. HR-EI-MS: 388.1430 (M^+, $C_{23}H_{20}N_2O_4^+$; calc. 388.1423).

*2-Amino-4-(3,4,5-trimethoxyphenyl)-4H-pyrano[3,2-h]quinoline-3-carbonitrile (**1i**)*

Conditions: 120 °C, 25 min. yield: 80 %. mp: 226–228 °C. UV (MeOH): 234 (5.19). IR (KBr): 3447, 3377, 3198 (NH_2), 2195 (CN), 1664. ^{1}H-NMR (400 MHz,

DMSO-d_6): 3.62 (*s*, 3H), 3.70 (*s*, 6H), 4.92 (*s*, 1H), 6.59 (*s*, 2H), 7.14 (*s*, 2H), 7.30 (*d*, $J = 8.4$ Hz, 1H), 7.58–7.61 (*m*, 1H), 7.65 (*d*, $J = 8.4$ Hz, 1H), 8.33 (*d*, $J = 7.6$ Hz, 1H), 8.92 (*sd*, $J = 3.2$ Hz, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 41.3, 55.7, 59.9, 60.0, 105.0, 120.5, 121.8, 122.2, 123.5, 126.9, 127.7, 136.0, 136.5, 137.5, 141.2, 142.8, 150.2, 153.0, 160.4. HR-EI-MS: 389.1382 (M^+, $C_{22}H_{19}N_3O_4^+$; calc. 389.1376).

*2-Amino-7-morpholino-4-(3,4,5-trimethoxyphenyl)-4H-chromene-3-carbonitrile (**1j**)*

Conditions: 120 °C, 15 min. yield: 90 %. mp: 227–229 °C. UV (MeOH): 216 (5.25). IR (KBr): 3431, 3331, 3209 (NH_2), 2199 (CN), 1666. ^{1}H-NMR (400 MHz, DMSO-d_6): 3.06 (*s*, 4H), 3.61 (*s*, 3H), 3.69 (*s*, 4H), 3.71 (*s*, 6H), 4.59 (*s*, 1H), 6.45 (*s*, 1H), 6.48 (*s*, 2H), 6.69 (*d*, $J = 8.8$ Hz, 1H), 6.85 (*s*, 2H), 6.93 (*d*, $J = 8.8$ Hz, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 48.0, 55.8, 59.9, 66.0, 101.4, 104.6, 111.9, 113.4, 120.8, 129.4, 136.2, 142.0, 148.7, 150.9, 152.9, 160.5. HR-EI-MS: 423.1788 (M^+, $C_{23}H_{25}N_3O_5^+$; calc. 423.1794).

General procedure for synthesis of chromeno[2,3-*b*]pyridine derivatives 2a–l

Two drops of TEA were added to a mixture of substituted salicylaldehydes (1.0 mmol), substituted phenols (1.0 mmol), and malononitrile (2.0 mmol) in propanol (5.0 mL). The mixture was subjected to microwave irradiation (300 W) at 150 °C for 5–30 min. The residue was filtered and washed with ethanol and hexane. Re-crystallization from ethanol afforded pure **2a–l**.

*2,4-Diamino-5-(4-amino-2-hydroxyphenyl)-5H-chromeno[2,3-b]pyridine-3-carbonitrile (**2a**)*

Conditions: 10 min. yield: 87 %. mp: 297–299 °C. UV (MeOH): 215 (4.63). IR (KBr): 3444, 3358, 3348, 3241, 3208 (NH_2), 2203 (CN), 1617. ^{1}H-NMR (400 MHz, DMSO-d_6): 4.91 (*s*, 2H), 5.17 (*s*, 1H), 5.90 (*d*, $J = 8.4$ Hz, 1H), 6.06 (*s*, 1H), 6.16 (*s*, 2H), 6.35–6.38 (*m*, 3H), 6.97–7.18 (*m*, 4H), 9.72 (*s*, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 70.6, 91.4, 100.2 106.9, 116.1, 116.5, 119.6, 123.9, 125.7, 127.4, 129.3, 129.6, 148.3, 150.0, 153.1, 156.6, 158.6, 159.2. HR-EI-MS: 345.1230 (M^+, $C_{19}H_{15}N_5O_2^+$; calc. 345.1226).

*2,4-Diamino-5-(4-(dimethylamino)-2-hydroxyphenyl)-5H-chromeno[2,3-b]pyridine-3-carbonitrile (**2b**)*

Conditions: 30 min. yield: 92 %. mp: 286–288 °C. UV (MeOH): 229 (4.72). IR (KBr): 3497, 3433, 3390, 3348, 3241 (NH_2), 2192 (CN), 1653, 1636, 1624. ^{1}H-NMR (400 MHz, DMSO-d_6): 2.76 (*s*, 6H), 5.22 (*s*, 1H), 6.08 (*dd*, $J = 8.4$, 2.4 Hz, 1H), 6.15–6.16 (*m*, 3H), 6.36 (*s*, 2H), 6.55 (*d*, $J = 8.8$ Hz, 1H), 6.97–7.19 (*m*, 4H), 9.86 (*s*, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 40.0, 70.6, 91.2, 98.9, 105.4, 116.1,

116.5, 120.1, 123.9, 125.4, 127.5, 129.3, 129.6, 150.0, 150.1, 153.1, 156.6, 158.7, 159.2. HR-EI-MS: 373.1532 (M^+, $C_{21}H_{19}N_5O_2^+$; calc. 373.1539).

*2,4-Diamino-5-(4-(diethylamino)-2-hydroxyphenyl)-5H-chromeno[2,3-b]pyridine-3-carbonitrile (**2c**)*

Conditions: 10 min. yield: 85 %. mp: 278–279 °C. UV (MeOH): 231 (4.71). IR (KBr): 3497, 3444, 3390, 3348, 3241 (NH_2), 2192 (CN), 1662, 1617. ^{1}H-NMR (400 MHz, DMSO-d_6): 1.01 (*t*, $J = 6.8$ Hz, 6H), 3.18 (*q*, $J = 6.8$ Hz, 4H), 5.19 (*s*, 1H), 6.00 (*dd*, $J = 8.8$, 2.4 Hz, 1H), 6.11 (*s*, 1H), 6.12 (*s*, 2H), 6.35 (*s*, 2H), 6.50 (*d*, $J = 8.8$ Hz, 1H), 6.97–7.19 (*m*, 4H), 9.75 (*s*, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 12.6, 43.6, 70.6, 91.3, 97.7, 104.3, 116.2, 116.5, 118.9, 123.9, 125.6, 127.5, 129.3, 129.8, 147.1, 150.0, 153.4, 156.6, 158.7, 159.2. HR-EI-MS: 401.1855 (M^+, $C_{23}H_{23}N_5O_2^+$; calc. 401.1852).

*2,4-Diamino-5-(1-hydroxynaphthalen-2-yl)-5H-chromeno[2,3-b]pyridine-3-carbonitrile (**2d**)*

Conditions: 5 min. yield: 93 %. mp: 285–287 °C (lit. 283–284 °C) [28]. UV (MeOH): 229 (5.19). IR (KBr): 3410, 3358, 3250 (NH_2), 2203 (CN), 1614, 1601. ^{1}H-NMR (400 MHz, DMSO-d_6): 5.73 (*s*, 1H), 6.20 (*s*, 2H), 6.45 (*s*, 2H), 6.92–8.30 (*m*, 10H), 10.08 (*s*, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 70.7, 90.2, 116.3, 116.4, 121.3, 122.3, 124.1, 124.5, 125.5, 126.0, 126.3, 127.3, 127.6, 128.0, 129.3, 133.0, 146.9, 150.0, 156.7, 158.9, 159.5. HR-EI-MS: 380.1269 (M^+, $C_{23}H_{16}N_4O_2^+$; calc. 380.1273).

*2,4-Diamino-5-(4-amino-2-hydroxyphenyl)-9-methoxy-5H-chromeno[2,3-b]pyridine-3-carbonitrile (**2e**)*

Conditions: 10 min. yield: 83 %. mp: 310–312 °C. UV (MeOH): 235 (4.74). IR (KBr): 3485, 3443, 3368, 3229 (NH_2), 2203 (CN), 1614. ^{1}H-NMR (400 MHz, DMSO-d_6): 3.81 (*s*, 3H), 4.90 (*s*, 2H), 5.14 (*s*, 1H), 5.89 (*dd*, $J = 8.4$, 2.0 Hz, 1H), 6.05 (*sd*, $J = 2.0$ Hz, 1H), 6.15 (*s*, 2H), 6.33 (*s*, 2H), 6.35–6.93 (*m*, 4H), 9.71 (*s*, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 55.8, 70.6, 91.5, 100.3, 107.0, 110.0, 116.6, 119.6, 120.5, 123.6, 126.3, 129.6, 139.6, 147.4, 148.3, 153.1, 156.6, 158.6, 159.2. HR-EI-MS: 375.1323 (M^+, $C_{20}H_{17}N_5O_3^+$; calc. 375.1331).

*2,4-Diamino-5-(4-(dimethylamino)-2-hydroxyphenyl)-9-methoxy-5H-chromeno[2,3-b]pyridine-3-carbonitrile (**2f**)*

Conditions: 30 min. yield: 90 %. mp: 306–308 °C. UV (MeOH): 232 (4.85). IR (KBr): 3476, 3337, 3229, 3165 (NH_2), 2203 (CN), 1624. ^{1}H-NMR (400 MHz, DMSO-d_6): 2.76 (*s*, 6H), 3.81 (*s*, 3H), 5.19 (*s*, 1H), 6.07–6.15 (*m*, 4H), 6.35 (*s*, 2H), 6.53–6.93 (*m*, 4H), 9.85 (*s*, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 40.0, 55.7, 70.6, 91.1, 98.9, 105.5, 110.0, 116.5, 120.0, 120.4, 123.5, 126.0, 129.6, 139.5, 147.3,

150.1, 153.0, 156.6, 158.6, 159.2. HR-EI-MS: 403.1651 (M^+, $C_{22}H_{21}N_5O_3^+$; calc. 403.1644).

*2,4-Diamino-5-(4-(diethylamino)-2-hydroxyphenyl)-9-methoxy-5H-chromeno[2,3-b]pyridine-3-carbonitrile (**2g**)*

Conditions: 20 min. yield: 80 %. mp: 296–298 °C. UV (MeOH): 232 (5.02), 216 (3.96). IR (KBr): 3425, 3325, 3229 (NH_2), 2214 (CN), 1618. ^{1}H-NMR (400 MHz, DMSO-d_6): 1.01 (*t*, $J = 6.8$ Hz, 6H), 3.18 (*q*, $J = 6.8$ Hz, 4H), 3.81 (*s*, 3H), 5.16 (*s*, 1H), 6.00 (*d*, $J = 8.0$ Hz, 1H), 6.10 (*s*, 1H), 6.15 (*s*, 2H), 6.34 (*s*, 2H), 6.48–6.93 (*m*, 4H), 9.74 (*s*, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 12.6, 43.7, 55.8, 70.6, 91.4, 97.7, 104.4, 110.0, 116.6, 118.8, 120.5, 123.6, 126.2, 129.8, 139.6, 147.2, 147.4, 153.3, 156.6, 158.7, 159.2. HR-EI-MS: 431.1962 (M^+, $C_{24}H_{25}N_5O_3^+$; calc. 431.1957).

*2,4-Diamino-5-(1-hydroxynaphthalen-2-yl)-9-methoxy-5H-chromeno[2,3-b]pyridine-3-carbonitrile (**2h**)*

Conditions: 5 min. yield: 92 %. mp: 300–302 °C (lit. 319–321 °C) [28]. UV (MeOH): 234 (5.18), 213 (4.19). IR (KBr): 3443, 3358, 3240 (NH_2), 2214 (CN), 1614. ^{1}H-NMR (400 MHz, DMSO-d_6): 3.83 (*s*, 3H), 5.70 (*s*, 1H), 6.19 (*s*, 2H), 6.43 (*s*, 2H), 6.71–8.29 (*m*, 9H), 10.06 (*s*, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 55.8, 70.7, 90.2, 110.5, 116.4, 120.5, 121.3, 122.4, 123.8, 125.2, 125.5, 125.6, 126.0, 126.4, 127.3, 127.7, 133.1, 139.6, 146.9, 147.5, 156.7, 158.9, 159.5. HR-EI-MS: 410.1374 (M^+, $C_{24}H_{18}N_4O_3^+$; calc. 410.1379).

*9,11-Diamino-12-(4-amino-2-hydroxyphenyl)-12H-benzo-[5,6]chromeno[2,3-b]pyridine-10-carbonitrile (**2i**)*

Conditions: 10 min. yield: 84 %. mp: 303–305 °C. UV (MeOH): 237 (5.01). IR (KBr): 3443, 3368, 3240 (NH_2), 2214 (CN), 1614. ^{1}H-NMR (400 MHz, DMSO-d_6): 4.89 (*s*, 2H), 5.61 (*s*, 1H), 5.82 (*dd*, $J = 8.4$, 2.0 Hz, 1H), 6.09 (*s*, 1H), 6.35–6.38 (*m*, 3H), 6.56 (*s*, 2H), 7.33–8.03 (*m*, 6H), 10.33 (*s*, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 70.7, 92.3, 99.7, 107.3, 116.6, 117.5, 117.9, 118.9, 123.0, 124.5, 126.9, 128.5, 128.6, 129.6, 130.5, 130.9, 148.0, 148.3, 151.9, 156.8, 158.1, 159.1. HR-EI-MS: 395.1392 (M^+, $C_{23}H_{17}N_5O_2^+$; calc. 395.1382).

*9,11-Diamino-12-(4-(dimethylamino)-2-hydroxyphenyl)-12H-benzo[5,6]chromeno[2,3-b]pyridine-10-carbonitrile (**2j**)*

Conditions: 30 min. yield: 91 %. mp: 337–339 °C. UV (MeOH): 233 (5.12). IR (KBr): 3443, 3346, 3240 (NH_2), 2203 (CN), 1612. ^{1}H-NMR (400 MHz, DMSO-d_6): 2.72 (*s*, 6H), 5.67 (*s*, 1H), 6.01 (*dd*, $J = 8.8$, 2.4 Hz, 1H), 6.18 (*sd*, $J = 8.8$ Hz, 1H), 6.39 (*s*, 2H), 6.52–6.54 (*m*, 3H), 7.35–8.04 (*m*, 6H), 10.49 (*s*, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 39.9, 70.7, 92.1, 98.2, 105.85, 116.5, 117.5, 117.7, 119.2,

122.9, 124.5, 126.9, 128.5, 128.7, 129.7, 130.5, 130.9, 148.0, 150.0, 151.9, 156.8, 158.1, 159.1. HR-EI-MS: 423.1687 (M^+, $C_{25}H_{21}N_5O_2^+$; calc. 423.1695).

*9,11-Diamino-12-(4-(diethylamino)-2-hydroxyphenyl)-12H-benzo[5,6]chromeno[2,3-b]pyridine-10-carbonitrile (**2k**)*

Conditions: 20 min. yield: 83 %. mp: 327–329 °C. UV (MeOH): 235 (5.06). IR (KBr): 3476, 3358, 3231 (NH_2), 2203 (CN), 1620. ^{1}H-NMR (400 MHz, DMSO-d_6): 0.97 (*t*, $J = 6.8$ Hz, 6H), 3.13 (*q*, $J = 6.8$ Hz, 4H), 5.65 (*s*, 1H), 5.93 (*dd*, $J = 8.8$, 2.0 Hz, 1H), 6.14 (*sd*, $J = 2.0$ Hz, 1H), 6.39 (*s*, 2H), 6.49 (*d*, $J = 8.4$ Hz, 1H), 6.55 (*s*, 2H), 7.35–8.05 (*m*, 6H), 10.36 (*s*, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 12.5, 43.5, 70.7, 92.2, 96.9, 104.7, 116.5, 117.5, 117.8, 118.0, 123.0, 124.5, 126.9, 128.5, 128.6, 129.9, 130.5, 130.9, 147.1, 148.0, 152.2, 156.7, 158.1, 159.1. HR-EI-MS: 451.2006 (M^+, $C_{27}H_{25}N_5O_2^+$; calc. 451.2008).

*9,11-Diamino-12-(3-hydroxynaphthalen-2-yl)-12H-benzo[5,6]chromeno[2,3-b]pyridine-10-carbonitrile (**2l**)*

Conditions: 5 min. yield: 97 %. mp: 315–317 °C (lit. 317–319 °C) [28]. UV (MeOH): 237 (5.20). IR (KBr): 3497, 3352, 3379, 3250 (NH_2), 2214 (CN), 1618. ^{1}H-NMR (400 MHz, DMSO-d_6): 6.16 (*s*, 1H), 6.47 (*s*, 2H), 6.57 (*s*, 2H), 6.95–8.36 (*m*, 12H), 10.79 (*s*, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): 70.8, 91.1, 116.3, 116.9, 117.6, 121.4, 122.1, 122.8, 124.4, 124.6, 125.6, 125.9, 126.0, 126.5, 127.1, 127.7, 128.6, 129.2, 130.5, 130.9, 133.0, 145.9, 148.3, 156.9, 158.4, 159.4. HR-EI-MS: 430.1432 (M^+, $C_{27}H_{18}N_4O_2^+$; calc. 430.1430).

Biological activity

Isolation and culture of human and porcine chondrocytes

Human cartilage from osteoarthritis patients receiving total knee joint replacement was obtained aseptically with prior approval of the Institutional Review Board (IRB), Tri-Service General Hospital, Taipei, Taiwan. The porcine cartilage was obtained from the hind leg joints of pigs. The preparation of chondrocytes from cartilage was performed according to our previous report [34]. After enzymatic digestion of articular cartilage with 2 mg/mL protease in serum-free Dulbecco's modified Eagle's medium (DMEM) containing antibiotics and 10 % fetal bovine serum (FBS), the specimens were then digested overnight with 2 mg/mL collagenase I and 0.9 mg/mL hyaluronidase in DMEM/antibiotics. The cells were collected, passed through a cell strainer (Beckton Dickinson, Mountain View, CA, USA) and cultured in DMEM containing 10 % FBS and antibiotics for 3–4 days before use.

In vitro cytotoxicity analysis

Potential cytotoxic effects of **1a**–**2l** were evaluated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assays, as described elsewhere [34]. Briefly, chondrocytes were incubated in the presence or absence of **1a**–**2l** for 48 h. Then, 25 μL of MTT (1 mg/mL in H_2O) was added and cells were incubated at 37 °C for 5 h, prior to adding 100 μL of lysis buffer containing 20 % sodium dodecyl sulfate and 50 % dimethylformamide. Following incubation at 37 °C for another 2 h, 100 μL of dimethyl sulfoxide (DMSO) was added and the absorbance was measured at a wavelength of 590 nm using an ELISA reader (Dynatech, Chantilly, VA, USA).

Measurement of NO production

The release of NO was measured determining the concentration of its stable end product, nitrite, in the supernatants [34]. The *Griess* reaction was performed and the obtained concentrations of nitrite were measured by a spectrophotometer. In brief, an aliquot (100 μL) of culture supernatant was incubated with 50 μL of 0.1 % sulfanilamide in 5 % phosphoric acid and 50 μL of 0.1 % *N*-1-naphthylethylene-diamine dihydrochloride. After 10 min of incubation at room temperature, the absorbance was measured at a wavelength of 550 nm using a plate reader (Tecan, Grodig, Australia).

Animals

Adult male *Wister* rats (290 ± 20 g) were provided by BioLasco Taiwan Co., Ltd. (Taipei, Taiwan) and housed in the Laboratory Animal Center of National Defense Medical Center, Taipei, Taiwan. Rats were maintained on a 12 h light/dark cycle, at a temperature of 23 ± 2 °C and a humidity of 60 ± 5 %; they were given free access to water and food. The rats were cared for according to the Guide for the Care and Use of Laboratory Animals, and the relevant ethics regulations of our Medical Center.

In vivo anti-inflammatory activity

The in vivo anti-inflammatory activity of the synthetic products was evaluated using the carrageenan-induced rat hind paw edema assay [32, 33]. Rats were separated at random into five groups ($n = 6$). A suspension of the tested compounds **1h** and **2d** and reference drugs (quercetin and ibuprofen) in 1 % DMSO was administered intraperitoneally (10 and 20 mg/kg) 1 h before 0.05 mL of carrageenan (1 % solution in normal saline) was injected into the plantar tissue of the right hind paw. Control animals were similarly treated with 1 mL of 1 % DMSO. The volume of paw edema was measured hourly for 5 h using a plethysmometer (Ugo Basile Srl, Italy). The edema rate (E %) was calculated as $E\ \% = (V_t - V_0)/V_0 \times 100$, where V_0 denotes the mean volume of the hind paw before the administering of 1 % carrageenan and V_t is the mean volume of the hind paw t h after the administering of

1 % carrageenan. The percentage inhibition (I %) was given by determined as $E\ \% = (E_c - E_t)/E_c \times 100$, where E_c is the edema rate of the control group and E_t is the edema rate of the test groups at t h.

Assay of PGE_2 in inflammatory rat paw

The paws of rats were cut 0.5 cm away from the ankle joints immediately after the rats had been euthanized by an intraperitoneal injection of chloral hydrate (400 mg/kg). The paws were pulverized in liquid nitrogen using a mortar and pestle. The tissue homogenates [35] were centrifuged at 12,000 rpm at 4 °C for 30 min, and the resulting supernatant was collected and maintained at −80 °C until further analysis. Protein levels were quantified by the Bradford protein assay (Bio-Rad, 500-0006). PGE_2 concentrations were measured using an ELISA kit (MyBioSource, San Diego, CA, USA).

Statistical analysis

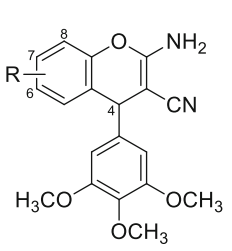
Results are expressed as mean ± SD. Unpaired Student's t test was used to determine statistical differences. $p < 0.05$ was considered to indicate statistical significance, unless noted otherwise.

Results and discussion

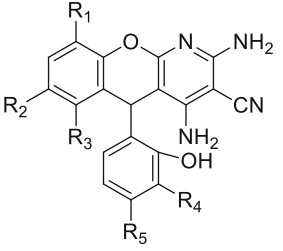
Chemistry

Schemes 1 and 2 present the synthesis and molecular structures of the titled compounds. Of the products, **1a**, **1b**, and **1e** are reportedly obtainable with mediocre yields by conventional synthesis by heating at reflux or stirring at room temperature [19, 27]. In the preparation herein of those compounds, one-pot microwave-assisted synthesis was used, and all yields exceeded 80 %. The one-pot preparation of **1h** from 3,4,5-trimethoxybenzaldehyde, malononitrile, and 1-naphthol in the presence of 4-dimethylaminopyridine as a catalyst under microwave irradiation (MWI) gave an 89 % yield [33], which, in the modified method, was improved to 95 % substituting 4-dimethylaminopyridine with piperidine. The conventional synthesis of compounds **2d**, **2h**, and **2l** involves heating at 110 °C under solvent- and catalyst-free conditions [28]. However, bumping frequently occurs during the reactions, even in the presence of boiling chips, and the resulting charring of mixtures rendered their purification extremely time-consuming. On the contrary, our rather mild reaction conditions of MWI afforded **2d**, **2h**, and **2l** in a relatively short time (5–30 min). Table 1 compares the modified reaction conditions and yields of the known products obtained herein with those of the aforementioned conventional methods. In addition, none of the aforementioned compounds have ever had reported anti-inflammatory activity. This study demonstrates that **1h** and **2d** have highly potent anti-inflammatory effects, both in vitro and in vivo.

Table 1 Comparison of experimental conditions and yields with conventional methods



1a: R = 7-NH_2
1b: R = 7-$N(CH_3)_2$
1e: R = 7-OCH_3
1h: R = 7,8-C_4H_4



2d: R_1 = H, R_2 = H, R_3 = H, R_4, R_5 = C_4H_4
2h: R_1 = OCH_3, R_2 = H, R_3 = H, R_4, R_5 = C_4H_4
2l: R_1 = H, R_2, R_3 = C_4H_4, R_4, R_5 = C_4H_4

Cpd	Catalyst		Solvent		Temperature (°C)		Time (min)		Yield (%)	
	Modified	Lit.	Modified	Lit.	Modified	Lit.	Modified	Lit.	Modified	Lit.
1a	Piperidine	TEA[a]	Propanol	EtOH[a]	80[e]	Reflux[a]	5	–[a,h]	85	53[a]
1b	Piperidine	Piperidine[b]	Propanol	EtOH[b]	80[e]	rt.[b,f]	5	120[b]	89	78[b]
1e	Piperidine	TEA[a]	Propanol	EtOH[a]	120[e]	Reflux[a]	30	–[a,h]	80	12[a]
1h	Piperidine	DMAP[c]	Propanol	No solvent[c]	80[e]	–[c,g]	5	90[c]	95	89[c]
2d	TEA	No catalyst[d]	Propanol	No solvent[d]	150[e]	110[d]	5	20[d]	93	90[d]
2h	TEA	No catalyst[d]	Propanol	No solvent[d]	150[e]	110[d]	5	20[d]	92	88[d]

Table 1 continued

Cpd	Catalyst		Solvent		Temperature (°C)		Time (min)		Yield (%)	
	Modified	Lit.	Modified	Lit.	Modified	Lit.	Modified	Lit.	Modified	Lit.
2l	TEA	No catalyst[d]	Propanol	No solvent[d]	150[e]	110[d]	5	20[d]	97	88[d]

[a] Lit. [19]

[b] Lit. [27]

[c] Lit. [33]

[d] Lit. [28]

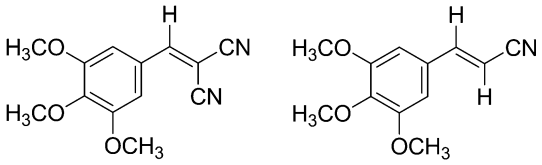
[e] Under microwave irradiation (MWI) at 300 W

[f] rt.: room temperature

[g] –: under MWI; temperature not described

[h] –: time not specified; only the range of reaction time (5–30 min) was given

Fig. 2 Structures of **1k** and **1l**



1k **1l**

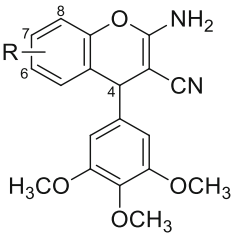
As outlined in Scheme 1, the derivatives of 4*H*-chromene were prepared using equimolar 3,4,5-trimethoxybenzaldehyde, malononitrile, and various substituted phenols in a one-pot reaction in propanol in the presence of piperidine under MWI (80 or 120 °C, 300 W, 5–30 min) to yield target compounds **1a–j** in favorable overall yields (80–97 %). The desired final targets were formed in the *Knoevenagel* condensation of malononitrile with 3,4,5-trimethoxybenzaldehyde, followed by the *Michael* addition of phenols, followed by intramolecular cyclization. A singlet peak at 4.48–5.25 ppm in ^{1}H-NMR was associated with pyran-CH in **1a–j**.

Interestingly, when the reactions were carried out at or below 80 °C, the isolated products were consistently proved to be the intermediate 2-(3,4,5-trimethoxybenzylidene)malononitrile (**1k**, Fig. 2). Only when conducted in the range of approximately 80–120 °C did the reaction run to completion. The structure of **1k** was elucidated using ^{1}H-NMR (DMSO-d_6: δ 3.80, *s*, 3H; 3.81, *s*, 6H; 7.36, *s*, 2H; 8.38, *s*, 1H) and HR-EI-MS: 244.0842 (M^+, $C_{13}H_{12}N_2O_3^+$; calc. 244.0848).

When 4-aminophenol was used as a *Michael* addition reagent, after 1 h of reaction under 300 W of MWI, regardless of temperature and even under neat conditions, the synthesis failed and an unexpected product was obtained, the structure of which was (*E*)-3-(3,4,5-trimethoxyphenyl)acrylonitrile (**1l**, Fig. 2), determined by spectral analysis by ^{1}H-NMR (DMSO-d_6: δ 3.68, *s*, 3H; 3.81, *s*, 6H; 6.81, *d*, $J = 16.0$ Hz, 1H; 7.05, *s*, 2H; 7.55, *d*, $J = 16.0$ Hz, 1H) and HR-EI-MS: 219.0892 (M^+, $C_{12}H_{13}NO_3^+$; calc. 219.0895). While the mechanism of formation of **1l** is yet to be determined, the intermediate that firstly formed might have quickly degraded to (*E*)-3-(3,4,5-trimethoxyphenyl)acrylonitrile (**1l**), preventing it from reacting further. The microwave irradiation power appeared to be critical in this one-pot reaction, as a similar reaction in the literature that used a higher power (540–720 W) gave yields of 70–80 % [29].

To identify the most favorable conditions for synthesizing chromeno[2,3-*b*]pyridine derivatives, the microwave-assisted reaction of substituted salicylaldehydes, substituted phenols, and malononitrile in a ratio of 1:1:2 was performed neat or in one of various solvents, such as H_2O, toluene, EtOH, or propanol, using one of two catalysts (piperidine or TEA) under MWI with a maximum power of 300 W. The reaction in propanol in the presence of TEA at 150 °C for 5–30 min turned out to be most satisfactory, so these conditions were used for all reactions hereafter. To the best of our knowledge, the proposed method is the first efficient and convenient method for preparing the derivatives of chromeno[2,3-*b*]pyridine. This one-pot reaction may have proceeded via an initial *Knoevenagel* condensation of salicylaldehyde and malononitrile, followed by subsequent intramolecular cyclization (the *Pinner* reaction), the product of which was then attacked by phenol to form

Table 2 In vitro IC_{50} of **1a–j** in human and porcine chondrocytes by MTT assay and NO production



Compound	R	MTT		NO	
		IC_{50} (μM)		IC_{50} (μM)	
		Human	Porcine	Human	Porcine
1a	7-NH_2	>100	>100	13.4 ± 2.9	19.9 ± 1.7
1b	7-$N(CH_3)_2$	>100	>100	9.6 ± 0.1	9.5 ± 0.3
1c	7-$N(C_2H_5)_2$	>100	>100	9.1 ± 1.8	8.7 ± 0.5
1d	7-OH	>100	>100	12.5 ± 2.0	20.8 ± 2.0
1e	7-OCH_3	>100	>100	10.4 ± 0.2	17.0 ± 1.5
1f	6,7-OCH_2O–	>100	>100	10.4 ± 1.7	18.6 ± 1.1
1g	6,7-C_6H_4–	>100	>100	15.3 ± 0.8	15.8 ± 0.6
1h	7,8-C_6H_4–	>100	>100	8.1 ± 0.6	8.0 ± 0.2
1i	7,8-C_3H_3N–	>100	>100	13.1 ± 0.2	12.4 ± 1.9
1j	7-NC_4H_8O–	>100	>100	14.0 ± 1.4	24.1 ± 1.7
Quercetin		>100	>100	65.7 ± 3.5	63.4 ± 5.5

Cell lines were treated with different concentrations of the compounds for 48 h as described in the experimental section

IC_{50} (μM) values are indicated as the mean ± standard deviation ($n = 6$)

a 4*H*-chromene intermediate, which subsequently reacted with a second molecule of malononitrile to generate the final products. A singlet peak at 5.14–6.16 ppm in ^1H NMR was characteristic of the pyran-CH in **2a–l**.

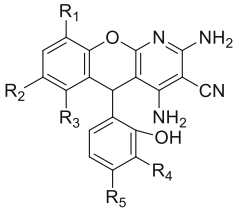
All of the crude compounds were purified by re-crystallization from ethanol. The structures of the end products were established spectroscopically.

Cytotoxicity and inhibition of NO production in human and porcine chondrocytes

The cytotoxic effects of the two new series compounds were tested by performing the MTT assay in human and porcine chondrocytes [34]. Tables 2 and 3 present the resulting IC_{50} values of cytotoxicity. None of these compounds exhibited significant cytotoxicity in either human or porcine chondrocytes.

After stimulation of human and porcine chondrocytes with TNF-α for 12 h, the inhibitory effects of **1a–j** and **2a–l** on the production of NO were measured by

Table 3 In vitro IC_{50} of **2a–l** in human and porcine chondrocytes by MTT assay and NO production



Compound	R_1	R_2	R_3	R_4	R_5	MTT		NO	
						IC_{50} (μM)		IC_{50} (μM)	
						Human	Porcine	Human	Porcine
2a	H	H	H	H	NH_2	>100	>100	25.7 ± 3.0	12.2 ± 2.1
2b	H	H	H	H	$N(CH_3)_2$	>100	>100	23.0 ± 2.8	9.1 ± 1.0
2c	H	H	H	H	$N(C_2H_5)_2$	>100	>100	26.5 ± 1.2	14.2 ± 1.9
2d	H	H	H	$-C_6H_4-$		>100	>100	6.9 ± 0.3	6.1 ± 1.0
2e	OCH_3	H	H	H	NH_2	>100	>100	35.2 ± 4.1	26.6 ± 2.3
2f	OCH_3	H	H	H	$N(CH_3)_2$	>100	>100	12.0 ± 1.8	11.2 ± 3.0
2g	OCH_3	H	H	H	$N(C_2H_5)_2$	>100	>100	13.9 ± 1.6	9.9 ± 3.1
2h	OCH_3	H	H	$-C_6H_4-$		>100	>100	34.3 ± 3.5	18.7 ± 2.3
2i	H	$-C_6H_4-$	H	NH_2		>100	>100	36.2 ± 0.4	17.5 ± 0.7
2j	H	$-C_6H_4-$	H	$N(CH_3)_2$		>100	>100	7.6 ± 1.3	8.8 ± 1.1
2k	H	$-C_6H_4-$	H	$N(C_2H_5)_2$		>100	>100	34.0 ± 4.1	17.9 ± 2.0
2l	H	$-C_6H_4-$	$-C_6H_4-$			>100	>100	7.6 ± 0.1	8.0 ± 0.1
Quercetin						>100	>100	65.7 ± 3.5	63.4 ± 5.5

Cell lines were treated with different concentrations of the compounds for 48 h as described in the experimental section

IC_{50} (μM) values are indicated as the mean ± standard deviation ($n = 6$)

Table 4 Anti-inflammatory activity of **1h** and **2d** using carrageenan-induced paw edema in rats

Compound	Dose (mg/kg)	Anti-inflammatory activity (% inhibition)				
		1 h	2 h	3 h	4 h	5 h
Control	0	0	0	0	0	0
1h	10	22.8 ± 0.9*	36.3 ± 1.0*	54.4 ± 1.6**	56.1 ± 0.6**	64.3 ± 0.5**
	20	46.2 ± 2.9**	55.7 ± 1.3*	62.9 ± 2.7**	64.3 ± 2.0**	69.0 ± 0.5**
2d	10	36.3 ± 0.1**	45.9 ± 1.5*	67.3 ± 6.2**	69.0 ± 4.6**	75.4 ± 6.2**
	20	36.2 ± 0.7**	59.4 ± 0.7*	64.3 ± 0.3**	76.6 ± 1.8**	85.4 ± 0.7**
Quercetin	10	6.7 ± 0.9	19.6 ± 1.4	36.7 ± 6.1*	31.5 ± 1.8	30.3 ± 1.2
	20	38.7 ± 1.5	40.1 ± 0.1*	53.3 ± 1.4**	53.8 ± 0.4**	52.2 ± 0.8*
Ibuprofen	10	9.8 ± 0.8	39.0 ± 0.9*	46.2 ± 0.8**	48.5 ± 1.3*	46.6 ± 1.5*
	20	19.5 ± 1.8*	44.7 ± 7.1*	55.9 ± 0.9**	54.8 ± 1.2**	52.0 ± 0.2**

Each value represents the mean ± standard deviation ($n = 6$)

Each statistically significant difference from control was expressed as * $p < 0.05$ and ** $p < 0.01$

analyzing the nitrite concentrations using the *Griesis* reaction [34]. The results thus obtained revealed that all derivatives of 4*H*-chromene and chromeno[2,3-*b*]pyridine behaved similarly in both human and porcine chondrocytes and that their potencies in inhibiting NO production exceeded that of the positive control quercetin. Generally, the derivatives of 4*H*-chromene exhibited still higher potency than those of chromeno[2,3-*b*]pyridine; the most powerful were **1b**, **1c**, **1h**, **2d**, **2j**, and **2l**, with IC_{50} values of around 6.9–9.6 μM in human and 6.1–9.5 μM in porcine chondrocytes. Of the series of 4*H*-chromene derivatives, compounds with amino groups substituted at C(7) (**1a**, **1b**, **1c**) were more potent than the other compounds. Introducing an *N*,*N*-dimethyl (**1b**) or an *N*,*N*-diethyl group (**1c**) resulted in the particularly favorable inhibition of NO, with an activity that was approximately eight times that of quercetin. The most potent compound in this series was **1h**, which had a benzene ring that was fused to C(7)–C(8) and was more potent than the **1i**, in which a pyridine ring was fused at the corresponding C(7)–C(8). However, **1g**, the constitutional isomer of **1h**, did not exhibit a potency comparable with that of **1h** in either human or porcine chondrocytes. Of the derivatives of chromeno[2,3-*b*]pyridine, compounds in which a benzene ring was fused to C(3′)–C(4′) (**2d**) or C(6)–C(7) (**2j**) or both (**2l**) displayed the most potent activity, with IC_{50} values of approximately 6.9–7.6 μM in human and 6.1–8.8 μM in porcine chondrocytes.

Of the two series of products, **1h** and **2d** were selected for further in vivo studies.

Anti-inflammatory activity in carrageenan-induced rat paw edema

Compounds **1h** and **2d** were tested in vivo using a carrageenan-induced edema method to evaluate their anti-inflammatory activities; the method was developed by *Winter* and modified as described in our previous report [32, 33]. Test groups (**1h** and **2d**) and reference groups (quercetin and ibuprofen) of *Wistar* rats received an intraperitoneal injection at a dose of 10 and 20 mg/kg 1 h before the injection of

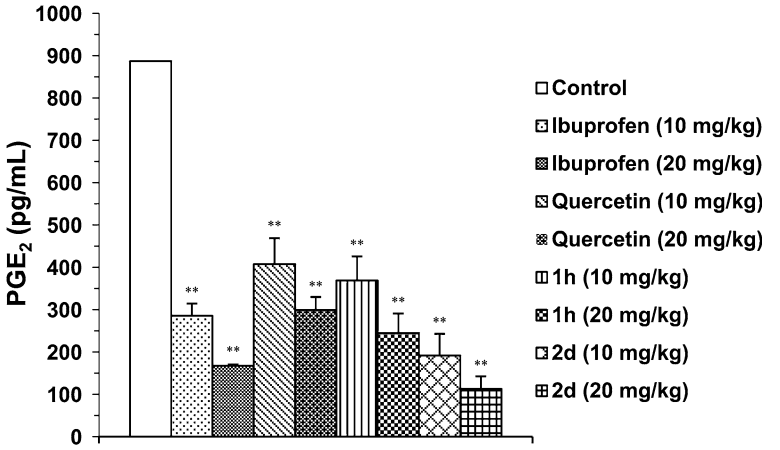


Fig. 3 PGE_2 levels (pg/mL) of test groups (**1h**, **2d**) and reference groups (quercetin and ibuprofen). Each value represents the mean ± standard deviation ($n = 6$). Each statistically significant difference from control was expressed as $^{**}p < 0.01$

0.05 mL of a 1 % solution of carrageenan in normal saline into the right hind paw. The paw volumes were measured at intervals of 1 h for up to 5 h. Table 4 reveals that both **1h** and **2d** exhibited significant and dose-dependent anti-inflammatory activities that were sustained and intensified, respectively, throughout the time course, whereas the anti-inflammatory activities of references quercetin and ibuprofen reached their plateaus at about the third hour, subsequently declining gradually. Additionally, **2d** presented extremely potent anti-inflammatory activity with an inhibitory effect against carrageen-induced edema of 67.3 % (3 h), 69.0 % (4 h), and 75.4 % (5 h) at 10 mg/kg and 64.3 % (3 h), 76.6 % (4 h), and 85.4 % (5 h) at 20 mg/kg.

Inhibition of PGE_2 production in carrageenan-induced rat paw edema

Levels of PGE_2 were evaluated in vivo using the same carrageenan-induced paw edema in rats [35]. In Fig. 3, both **1h** and **2d** produced significantly less PGE_2 than did the reference quercetin, once again, in a dose-dependent manner. Compound **2d** displayed greater PGE_2-suppressing activity (191.7 pg/mL at 10 mg/kg; 112.5 pg/mL at 20 mg/kg) than either **1h** (369.2 pg/mL at 10 mg/kg; 245.0 pg/mL at 20 mg/kg) or ibuprofen (285.4 pg/mL at 10 mg/kg; 167.5 pg/mL at 20 mg/kg).

Conclusion

Novel derivatives of 4*H*-chromenes and chromeno[2,3-*b*]pyridines were successfully prepared by a one-pot, microwave-assisted, three- or four-component condensation; their anti-inflammatory activities both in vitro and in vivo were evaluated. Among them, six compounds (**1b**, **1c**, **1h**, **2d**, **2j**, and **2l**) exhibited

significant anti-inflammatory activities in vitro in both human and porcine chondrocytes. Moreover, **1h** and **2d** exhibited a persistent ability to protect against carrageenan-induced rat paw edema. Compound **2d** even demonstrated superiority over the clinically used ibuprofen in both potency and sustainability. The advantages of the ease of synthesis, simple purification, potent activity and favorable safety profile of **2d** renders it promising for further use in developing novel anti-inflammatory agents. Detailed pharmacological and pharmacokinetic studies are in progress and will be published in the near future.

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Conflict of interest The authors declare no conflicts of interest.

Ethical standard This study was performed in accordance with the criteria of the National Academy of Sciences, and approved by the Institutional Animal Care and Use Committee of National Defense Medical Center, Taipei, Taiwan (Approval Number: IACUC-13-178). All patients enrolled with informed consent in this study were purposively sampled under Tri-Service General Hospital Institution Review Board approval (Approval Number: 1-102-05-091).

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