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The synthesis of morusin as a potent antitumor agent

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ABSTRACT

Morusin, which can be isolated from Chinese herbal medicine, is achieved in which the longest linear sequence is only 13 steps in 12% overall yield from commercially available phloroglucinol. In addition, the metal/EtSH reagents for regioselective demethylation of polymethylated morusin were described. Consequently, this strategy provided a concise route to synthesize the morusin analogues as well. Further biological studies of morusin, it exhibited strong antitumor effects with IC_{50} ~3.0 μM on three cancer cells.

1. Introduction

Our research group has exhibited a long-standing interest in the synthesis and bioassay study of a number of unique natural products containing flavonoids, camphorataimides, and benzofurans.[1] In previous works, we have shown that tumor suppressor protein p53 and p38 MAPK (mitogen activated protein kinase) play a prominent role in the caffeic acid phenethyl ester (CAPE) induced-apoptosis of C6 glioma cells.[2] Accordingly, Barron et al. reported that a library of 42 natural and synthetic flavonoids has been screened for their effect on cell proliferation and apoptosis in a human colonic cell line (HT29).[3] In addition, studies on the constituents of the Chinese crude drug 'Sang-bai-pi' (Morus root bark) have been recorded in books of traditional Chinese herbal medicines, which are used as an antiphlogistic, a diuretic, an expectorant, and *anti*-hepatitis B activity.[4] Recently, Kim et al.[5] reported that the effects of 19 naturally occurring prenylated flavonoids on cyclooxygenase (COX)-1 and COX-2 and on 5-lipoxygenase (5-LOX) and 12-LOX were investigated. Generally, the inhibitory activity of prenylated flavonoids against 5-LOX was much stronger than against 12-LOX.

Arachidonic acid (AA) is released from membrane phospholipids during normal and pathological processes and is metabolized primarily by the COX, LOX and cytochrome P450 (CYP450) enzyme system. The arachidonic acid metabolizing enzymes have been implicated in the development of a variety of human cancers including colon, prostate, and breast and lung cancers and in mesothelioma.[6] Additionally, mammalian 5-LOX is particularly important because of its unique ability to convert AA to leukotrienes that act as potent mediators of inflammation, apoptosis, and tumorigenesis.[7] Since the LOX pathway appears to be particularly implicated in tumor growth,[8] the development of selective inhibitors of arachidonate 5-lipoxygenase has been a subject of medicinal chemical interest. Recently several studies have reported the various biological effects of hop prenylflavonoids in vitro.[8] In addition, for investigating the effects of morusin and himanimides[9] (Fig. 1) on COXs and LOXs, five known compounds, such as NS-398 (COX-2 inhibitor), indomethacin (a COX-1/COX-2 inhibitor), NDGA (an LOX inhibitor), celecoxib (a COX-2 inhibitor), and zileuton (a 5-LOX inhibitor), were used as the inhibitors[10] of enzyme references (Fig. 2). With the prenyl structural relative among other flavonoid natural products, morusin[4] occupies an unique position in the realm of flavonoid chemistry. Nonetheless, despite the structural novelty and pronounced biological activity of the morusin, its total synthesis has not previously been recorded. Herein, we undertook the total synthesis of the morusin and study its biological activities.

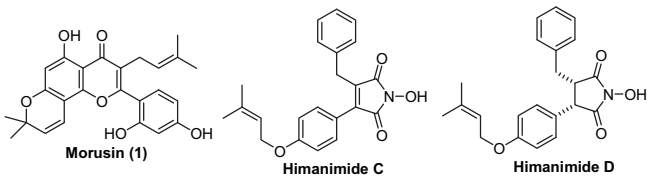

Figure 1. Structures of the naturally occurring products.

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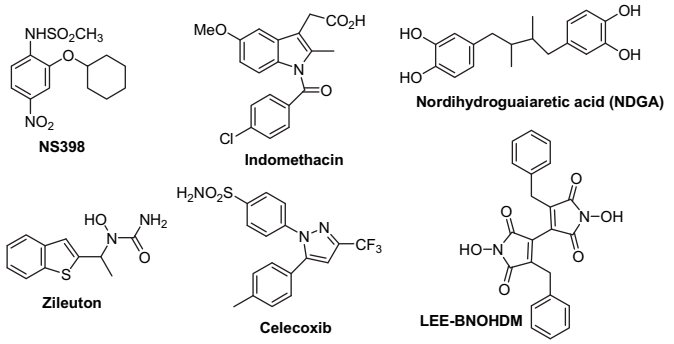
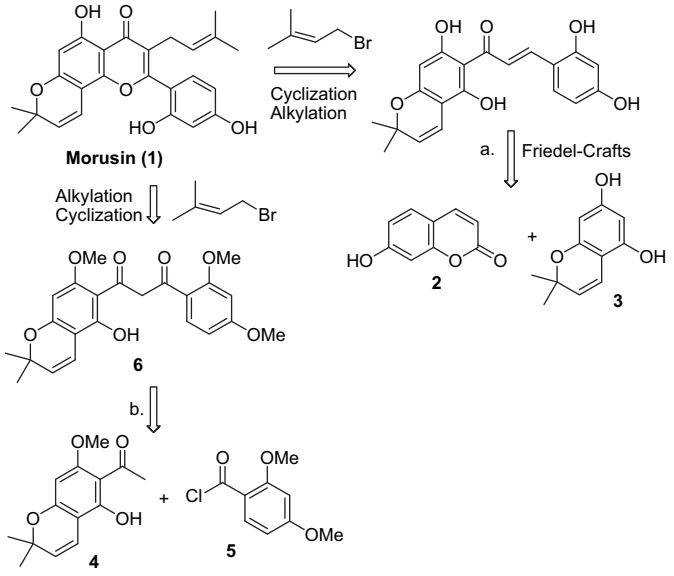


Figure 2. Structural features of LOXs and COXs inhibitors.

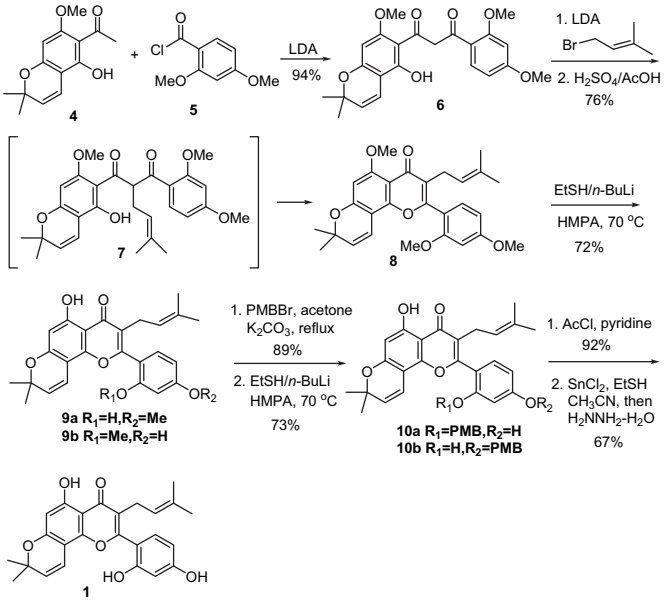
2. Results and discussion

Retrosynthetic analysis suggested two possible routes for the synthesis of this natural product: routes a and b as shown in Scheme 1. Two approaches will be pursued to determine, which strategy is viable. The first approach involves the Friedel–Crafts reaction of coumarin **2** and **3** catalyzed by Lewis acid or base to provide the precursor chalcone, followed by subsequent cyclization and C-3 alkylation of flavone with allyl bromide to afford the desired **1**. The other way employs the enolate acylation[11] of the known[12] 2-hydroxyl acetophenone **4** and benzoyl chloride **5** to obtain the key intermediate diketone **6**, followed by subsequent alkylation and cyclization to achieve the target molecule **1**.



Scheme 1. Retrosynthetic analysis of morusin.

The initial proposal was to use the phenolic **3** and the commercially available 7-hydroxycoumarin **2** via the Friedel–Crafts reaction for Morusin synthesis. Although the chromene **3** seems a simple compound, but it has not been reported in the literature. We started with the known[12] 5,7-dihydroxy-2,2-dimethyl chroman-4-one by reduction and dehydration with $NaBH_4$ and 4 M HCl to generate the desired **3** in 93% yield. In the case of **2** and **3**, however, the Friedel–Crafts reaction catalyzed with $AlCl_3$, $AlBr_3$, $ZnCl_2$, $SnCl_4$, BF_3OEt_2, pyridine, piperidine, pyrrolidine, and KOH to give the precursor chalcone or flavone was unsuccessful. To overcome these technical difficulties, we switched to using the simple acylation of ketone **4** and acyl chloride **5** with LDA (Scheme 2).



Scheme 2. Total synthesis of the morusin.

With known[12] precursors **4** and **5** in hands, followed by the procedure of Banerji and Luthriathe underwent the Baker–Venkataraman rearrangement to give diketo **6** in high yield (94%). The alkylation of **6** with allyl bromide provided the prenylated intermediate **7**, and followed by cyclization with H_2SO_4/AcOH to generate the expected methylated morusin **8** in 76% yield over two steps. At this point, the final step of the synthesis was focused on the demethylation of **8**, however, attempts to treat with Me_3SiI, $AlCl_3$, BBr_3, BBr_3/NaI, $SiCl_4$/NaI, pyridinium hydrohalides, or EtSLi to give morusin (**1**) were unsuccessful. The major product was 5-hydroxyl-2′,4′-dimethoxy morusin, the product from forming more stable with hydrogen bond on 5-demethylation of **8**. Consequently, we sought to manipulate the deprotection of **8** in a separate step, to our delight, **8** was undergone smoothly with EtSLi/HMPA to obtain in 72% yield the desired isomer **9**. Continuously, in the case of **9**, we attributed our failure to achieve **1** due to the free 2-or 4-hydroxy group interfered with EtSLi/HMPA. This problem was overcome by protection of **9** with *p*-methoxybenzyl bromide (PMBBr) and subsequent treatment with EtSLi/HMPA to give **10**. It is striking that cleaving PMB group of **10** to **1** could not be achieved despite our efforts in methods either by oxidizing agents or Lewis acids for cleaving PMB with 1,2-dichloro-4,5-dicyanoquinone (DDQ),[13] $CeCl_3$–$7H_2O$/NaI,[14] cerium(IV) ammonium nitrate (CAN),[15] $ZrCl_4$,[16] BF_3–Et_2O/$NaBH_3CN$.[17] In all case, they were proceeded with decomposed and recovered starting material **10**. Obviously, the target morusin was unstable under acidic and oxidizing conditions, but it can make a detour by the first protection with AcCl and subsequent deprotection with mild $SnCl_2$/EtSH[18] and NH_2NH_2 to provide **1** in moderate yield 62%. It is worth noting that the reported method is the best among the well-established methods by comparing thoroughly with other methods. In addition, we also failed to achieve **1** by the use of other starting materials **5** with easily removal protecting groups, such as isopropyl, MOM, benzyl, acetyl, and silyl groups instead of methyl group of **5**.

To probe accessibility of the active sites of P38 MAPK, c-Met tyrosin kinase and neurokinin (NK) receptors of hepatocyte growth factor/scatter factor (HGF/SF), and COXs/LOXs, the binding affinity of protein–ligand complexes were first examined by automated docking method (Table 1). The crystal structures of proteins were from the Brookhaven Protein Data Bank (PDB)

(http://www.rcsb.org/pdb/), for instance, 5-lipoxygenase entry code (2Q7M). From the presented docking, CAPE and himanimides showed significant binding on P38 kinase, c-Met and NK1 receptors, and COX-1, however, morusin gave preference to the tight bound of COX-2 and 5-lipoxygenase. Moreover, it is worth noted that morusin can show more specific inhibition of 5-LOX than that of COX-2 because all 10 docking poses are clustered within 1.1 Å (rmsd), indicating a strong consensus for a single binding mode. The lowest-energy pose are shown in Figure 3A. The active site for docking was defined with the amino acids Leu135, Phe131, Ala128, Val127, and Leu124 of both B and F chains in the 5-LOX falling within 7 Å radius of the cocrystallized ligand. The 2′- and 4′-hydroxy groups of morusin formed hydrogen bonds to Val127 of both B and F (2.95 and 2.09 Å), respectively. Additional analytical information, the isopropyl group of the residue Val127 (B chain) was positioned to make hydrophobic contact with 3-prenyl group of morusin, and the tricyclic core of morusin was aligned into the groove of the residues Ala128, Val127, Leu124, Leu135, and Phe131 for active site recognition (Fig. 3B).

higher than that of the original inhibitor (NDGA=115 μM). Thus, these results imply that the presence of prenylated flavonoids and *N*-hydroxyl imides assist in binding with 5-LOX. Consistently, their inhibitory effect of CAPE, himanimide C, and morusin on 5-LOX in vitro was also examined with an IC_{50} of 50.5, 26.8, and 8.7 μM, respectively. Finally, the combination of hydrogen bond and hydrophobic pocket was validated with the designed three C2 symmetric dimmers, and LEE-BNOHDM provided K_i value (263.98 nM) for the strongest inhibitor of 5-LOX (Fig. 3C). The present work demonstrates the ability of prenylated flavonoid chemistry to quickly generate a novel series of arachidonate 5-LOX inhibitors, showing features of high potency and selectivity, as well as ability to inhibit inducible NO synthase (iNOS) expression in RAW 264.7 cells.[20] Additionally, Lin et al. reported[21] that morusin inhibited platelet aggregation induced by platelet-activating factor (PAF), which did not cause thromboxane formation in rabbit platelet. The combination of docking and Lin's results enabled us to propose that morusin may become a lead candidacy as therapeutic implications for atherosclerosis and vascular diseases.

Table 1
Compound K_i values and the lowest binding energies from AutoDock Lamarckian Genetic Algorithm

Ligand	Protein(PDBentry) Binding Energy (Kcal/mol)/K_i(μM)					
	COX-1 (2OYE)	COX-2 (1DDX)	5-LOX (2Q7M)	P38 MAPK (1P38)	NK1/HGF (1NK1)	c-Met/HGF (2RFN)
CAPE	−7.36/4.06	−4.57/445.4	−5.15/166.52	−7.56/2.86	−7.06/6.7	−5.86/50.61
Himanimide C	−7.64/2.51	−5.24/144.41	−7.37/3.95	−7.91/1.6	−7.91/1.59	−6.19/28.94
Morusin	38.44/NA	−5.88/48.95	−6.76/11.08	0.57/NA	−3.69/1970	−5.74/61.67
Himanimide D	−4.35/652.0	−4.83/286.68	−6.44/19.01	−4.22/813.4	−7.33/4.26	−5.44/102.65

NA:Unavailable.

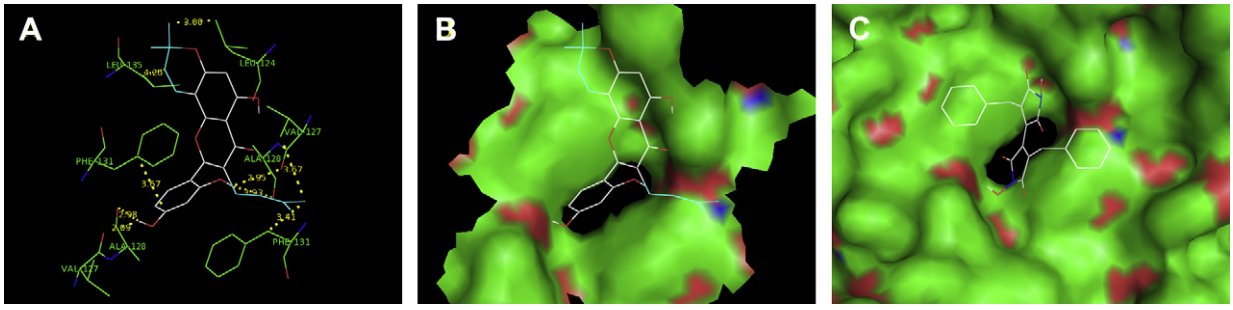


Figure 3. Binding orientation of protein-ligand predicted by the AutoDock Lamarckian Genetic Algorithm on PyMOL viewer (A) LOX-5 (PDB entry 2Q7M)/morusin is shown as a line model, (B) LOX-5/morusin is shown as a surface model, and (C) LOX-5/LEE-BNOHDM is shown as a surface model.

Once the docking protocol was confirmed, the other protein–ligand complexes under analysis were docked using the same strategy. In addition, it is well known that the 5-LOX isoenzyme play crucial roles in the development of human cancers.[19] Therefore, the influence of prenylated flavonoids on the binding affinity of 5-LOX for thirty-six compounds and five reference inhibitors (COXs/LOXs) was assessed to further test the docking models. The K_i values for compounds, **1**, himanimide C and D that were estimated by automated docking on 5-LOX were 11.0, 3.95, and 19.0 μM, respectively. In addition, the K_i values of compound **1**, himanimide C and D that were evaluated were found to range from 30–fold lower to >6–fold

To further test cell line-dependent cytotoxicity, the effect of morusin on tumor cell proliferation/viability was examined in MDA-MB-231 and MCF 7 breast tumor cells and in A549 lung cancer cell (Table 2). Following 24 h incubation, morusin inhibited MDA-MB-231 and MCF 7 cell proliferation/viability with an IC_{50} of 3.2 and 3.4 μM, respectively, and inhibited A549 cells with an IC_{50} of 3.1 μM. In addition, in comparison with results of CAPE and camphorataimides has clearly shown the cytotoxicity of morusin on above three cancer cell lines. Therefore, it is worthy to further investigate the chemopreventative or antitumorigenesis mechanisms of morusin in those cancer cell lines.

Table 2
Cytotoxicity was estimated by MTT assay (24 h). The comparative cytotoxicity is expressed the 50% half maximal concentration (IC_{50} μM) for Morusin and camphorataimides[9]

	Camphorataanhydride A	Camphorataimide B	Camphorataimide C	Himanimide A	Morusin
MDA-MB-231[a]	>200	11.4	51.6	19.6	3.2
MCF-7[a]	>200	10.6	33.9	21.2	3.4
A549[b]	>200	15.4	60.3	30.3	3.1

[a] MDA-MB-231 and MCF-7 are breast cancer cell lines.
[b] A549 is lung cancer cell.

3. Conclusion

In summary, the first total synthesis route to morusin has been achieved in which the longest linear sequence is only 13 steps in 12% overall yield from commercially available phloroglucinol. In addition, it demonstrated the usefulness of the metal/EtSH reagents for regioselective demethylation of polymethylated morusin. Furthermore, it demonstrated the usefulness of the automated docking method for a drug lead discovery. Consequently, the rationally optimized and designed LEE-BNOHDM was identified as a potentially important inhibitor of the arachidonate 5-lipoxygenase. Moreover, morusin exhibited strong antitumor effects in three cancer cells. Finally, the synthetic morusin is currently underway to evaluate the efficacy as a 5-LOX inhibitor or an antitumor agent, and its biological activity will further be investigated in vitro/vivo as well.

4. Experimental

4.1. General experimental procedures

Melting points were determined on a Mel Temp II melting point apparatus and are uncorrected. IR spectra recorded with an Equinox 55 (FTIR) spectrometer. ^{1}H NMR and ^{13}C NMR spectra were obtained using a Varian-300 spectrometer. Chemical shifts are reported in parts per million (δ, ppm) using $CHCl_3$ (δ_H 7.26) as an internal standard. Low-resolution mass spectra (MS) and high-resolution mass spectra (HRMS) were determined on a JEOL JMS-HX 110 mass spectrometer from National Chung-Tsing University, Taichung. Solvents were freshly distilled prior to use from phosphorus pentoxide or CaH_2. THF was from sodium diphenyl ketyl. All reactions were carried out under nitrogen atmosphere unless otherwise stated. Silica gel (Silica gel 60, 230–400 mesh, Merck) was used for chromatography. Organic extracts were dried over anhydrous $MgSO_4$.

4.2. 6-Acetyl-5-hydroxy-7-methoxy-2,2-dimethylchrom-3-ene (4)

The 5-hydroxy-7-methoxy-2,2-dimethylchroman-4-one was prepared as previous procedure.[12] The ketone (5.8 g, 26 mmol) was dissolved in MeOH (200.0 mL), and added Zn (5.1 g, 78 mmol) in one portion at room temperature for 15 min. The suspension was followed by addition of conc. HCl (5 mL) dropwise and stirred at room temperature for 1 h. The resulting mixture was filtered and the filtrate was extracted with H_2O (150.0 mL) and ethyl acetate (3×400 mL). The organic layer was concentrated in vacuo, and then subjected to column chromatography (Silica gel ether:hexane 1:1) to obtain the desired chromane (5.0 g, 93%) as a yellow solid: mp 74–75 °C (lit.[22] 78–79 °C). To a solution of chromane (5.0 g, 24 mmol) in CH_2Cl_2 (50 mL) was added pyridine (2.0 mL, 25 mmol) at room temperature, and then treated with AcCl (2.0 mL, 28 mmol) dropwise. The mixture was stirred at room temperature for 0.5 h. The resulting suspension was filtered and washed with CH_2Cl_2, and the filtrate was subjected to column chromatography (SiO_2, CH_2Cl_2:hexane 1:2) to give the expected acetoxy chromane (5.7 g, 96%) as an oil. ^{1}H NMR δ 1.30 (s, 6H), 1.72 and 2.47 (t, J=6.8 Hz, 4H), 2.24 (s, 3H), 3.69 (s, 3H), 6.20 and 6.26 (d, J=2.5 Hz, 2H); ^{13}C NMR δ 16.7, 20.4, 26.3, 31.8, 55.0, 74.2, 99.6, 100.3, 106.3, 149.6, 155.2, 158.7, 168.6.

A mixture of the acetoxy chromane (5.7 g, 23 mmol) and $AlCl_3$ (9.1 g, 68 mmol) in anhydrous dioxane (50.0 mL) was heated to 120 °C for 5 h. The solvent was removed and neutralized with $NaHCO_3$ (80.0 mL), then followed by extraction with CH_2Cl_2 (3×100 mL), and the organic layer was concentrated in vacuo. The residue was subjected to column chromatography (Silica gel ether:hexane 1:2) to provide the Fries product (4.8 g, 85%) as a white solid: 95–96 °C (lit.[23] 92–93 °C). To a mixture of the Fries product (4.8 g, 19 mmol) and DDQ (4.9 g, 22 mmol) in acetic acid (50.0 mL) was heated to 120 °C for 4 h, and then the suspension was filtered. The filtrate was directly subjected to column chromatography (SiO_2, ether:hexane 1:4) to obtain the desired **4** (3.9 g, 81%) as a yellow solid: mp 125–126 °C (lit.[24] 128–129 °C), IR (KBr) ν_{max}: 3450, 2973, 1634, 1600, 1265, 1124, 850 cm^{-1}.

4.3. 1-(2,4-Dimethoxyphenyl)-3-(5-hydroxy-7-methoxy-2,2-dimethylchromen-6-yl)-propane-1,3-dione (6)

This compound was prepared as a yellow solid from **4** and 2,4-dimethoxybenzoyl chloride (**5**) according to the procedure of Banerji and Luthria[11] mp 107–108 °C (lit.[11] mp 107–110 °C), IR (KBr) ν_{max}: 2978, 2938, 1634, 1600, 1265, 1124, 800 cm^{-1}.

4.4. 2-(2,4-Dimethoxyphenyl)-5-methoxy-8,8-dimethyl-3-(3-methyl-but-2-enyl)-pyrano[2,3-f]chromen-4-one (8)[25]

To a solution of **6** (1.0 g, 2.4 mmol) in anhydrous THF (50.0 mL) was cooled down to -78 °C, and then added LDA (2.0 M 1.3 mL, 2.6 mmol) dropwise at that temperature for stirring 0.5 h. The mixture was warmed up to −25 °C for 1 h and cooled down again to −78 °C, and followed by addition of allyl bromide (290 μL, 2.5 mmol) dropwise for 1 h. The resulting solution was quenched by addition of 1 N HCl (1.0 mL), and extracted with ether (3×200 mL). The organic layer was concentrated in vacuo, and the crude product **7** was directly used no further purification. A solution of **7** (0.98 g, 0.2 mmol) in acetic acid (15.0 mL) was added conc. H_2SO_4 (0.02 mL) and stirred at room temperature for 15 min. The resulting mixture was added dist. H_2O (15.0 mL) and extracted with CH_2Cl_2 (3×200 mL). The solvent was removed and the residue was subjected to column chromatography (SiO_2, ether:hexane 1:1) to afford the desired **8** (0.85 g, 76%) as a white solid: mp 153–154 °C (lit.[26] mp 152–154 °C), IR (KBr) ν_{max}: 3050, 2973, 2928, 2851, 1639, 1280, 1164, 850 cm^{-1}, HRMS (FAB) calcd for $C_{28}H_{31}O_6$ {$[M+H]^+$} 463.2121, found 463.2126.

4.5. 2-(2-Hydroxy-4-methoxyphenyl)-5-hydroxy-8,8-dimethyl-3-(3-methyl-but-2-enyl)-pyrano[2,3-f]chromen-4-one (9a)[27]

A solution of EtSH (0.8 mL, 10.8 mmol) in HMPA (6.0 mL) was cooled down to 0 °C for 10 min, and then added n-BuLi (2 M, 10.8 mmol) under N_2 at that temperature for stirring 30 min. Subsequently, **8** (1.20 g, 2.4 mmol) was introduced a fresh solution of EtSLi in HMPA, and the resulting solution was warmed at 70 °C under N_2. After stirring at 70 °C for 8 h, the reaction mixture was cooled and quenched with a saturated solution of NH_4Cl (4.0 mL) and extracted with ethyl acetate (3×20.0 mL). The combined organic extracts were washed with saturated aqueous LiCl, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by flash column chromatography (SiO_2, hexane:ethyl acetate 3:1) to provide **9a** (0.68 g, 60%) and **9b** (135 mg,12%); **9a** as yellow powder: mp 162–164 °C (lit.[27] mp 162–164 °C), IR (KBr) ν_{max}: 3400, 2986, 2925, 1654, 1620, 1580, 1265, 1184, 850 cm^{-1}, ^{1}H NMR [$(CD_3)_2CO$] δ 1.41 (s, 3H), 1.42 (s, 6H), 1.55 (s, 3H), 3.10 (d, J=3.3 Hz, 2H), 3.82 (s, 3H), 5.09 (t, J=3.3 Hz, 1H), 5.63 (d, J=5.4 Hz, 1H), 6.14 (s, 1H), 6.57 (d, J=5.1 Hz, 1H), 6.60 (d, J=5.1 Hz, 1H), 6.61 (s, 1H), 7.33 (d, J=5.4 Hz, 1H), 9.00 (br s, 1H), 12.79 (br s, 1H); ^{13}C NMR δ 17.7, 24.3, 25.6, 28.2, 28.2, 55.5, 78.0, 99.9, 100.9, 102.1, 104.8, 107.2, 112.2, 114.7, 120.8, 121.3, 127.1, 131.3, 133.3, 152.0, 155.1, 159.3, 159.4, 161.4, 162.8, 182.0, HRMS (EI) calcd for $C_{26}H_{26}O_6$ (M^+) 434.1729, found 434.1728.

4.6. 2-(4-Hydroxy-2-methoxyphenyl)-5-hydroxy-8,8-dimethyl-3-(3-methyl-but-2-enyl)-pyrano[2,3-f]chromen-4-one (9b)[27]

Compound **9b** as yellow powder: mp 195–197 °C (lit.[27] mp 198–199 °C), IR (KBr) ν_{max}: 3400, 2975, 2928, 1650, 1620, 1580, 1265, 1184, 850 cm^{-1}, ^1H NMR [$(CD_3)_2CO$] δ 1.38 (s, 3H), 1.42 (s, 6H), 1.55 (s, 3H), 3.02 (d, J=3.3 Hz, 2H), 3.80 (s, 3H), 5.06 (t, J=3.3 Hz, 1H), 5.62 (d, J=5.1 Hz, 1H), 6.14 (s, 1H), 6.54 (d, J=5.1 Hz, 1H), 6.58 (dd, J=5.1, 1.2 Hz, 1H), 6.64 (d, J=1.2 Hz, 1H), 7.27 (d, J=5.1 Hz, 1H), 9.09 (br s, 1H), 13.2 (br s, 1H); ^{13}C NMR [$(CD_3)_2CO$] δ 16.7, 23.7, 24.9, 27.3, 27.3, 55.1, 77.8, 98.9, 99.2, 100.7, 104.6, 107.3, 113.1, 114.4, 120.8, 121.5, 127.2, 131.2, 131.4, 152.3, 158.7, 159.1, 161.0, 161.4, 161.8, 182.2, HRMS (EI) calcd for $C_{26}H_{26}O_6$ (M^+) 434.1729, found 434.1723.

4.7. 2-[2-(4-Methoxybenzyloxy)-4-hydroxyphenyl]-5-hydroxy-8,8-dimethyl-3-(3-methyl-but-2-enyl)-pyrano [2,3-f]chromen-4-one (10a)

The mixture of **9a** (2.10 g, 4.8 mmol) and K_2CO_3 (3.00 g, 21.7 mmol) in acetone (50.0 mL) was added PMBBr (0.5 mL, 5.3 mmol) at room temperature, and then refluxed at 70 °C for 1 h by TLC monitoring. After cooling, the solution was evaporated in vacuo, and the brown residue was subjected to flash chromatography (SiO_2, hexane:ethyl acetate 4:1) to give the protected product (2.39 g, 89%). Subsequently, the protected product was treated by adding a solution of EtSLi in HMPA (6.0 mL) at room temperature, and the resulting mixture was heated at 70 °C under N_2. After stirring at 70 °C for 2 h, the reaction mixture was cooled and quenched with a saturated solution of NH_4Cl (4.0 mL) and extracted with ethyl acetate (3×20 mL). The combined organic extracts were washed with saturated aqueous LiCl, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by flash column chromatography (SiO_2, hexane:ethyl acetate 3:1) to afford **10a** (1.70 g, 73%) as an oil: IR (KBr) ν_{max}: 3400, 2972, 2932, 1654, 1620, 1580, 1265, 1184, 850, 780 cm^{-1}, ^1H NMR δ 1.41 (s, 3H), 1.46 (s, 6H), 1.59 (s, 3H), 3.09 (d, J=5.4 Hz, 2H), 3.75 (s, 3H), 4.99 (s, 2H), 5.11 (t, J=5.4 Hz, 1H), 5.48 (d, J=8.1 Hz, 1H), 6.26 (s, 1H), 6.53 (dd, J=9.9, 2.1 Hz, 1H), 6.60 (d, J=9.9 Hz, 1H), 6.63 (d, J=2.1 Hz, 1H), 6.80 (d, J=8.4 Hz, 2H), 7.20 (d, J=8.4 Hz, 2H), 7.21 (d, J=8.1 Hz, 1H), 13.19 (br s, 1H); ^{13}C NMR ($CDCl_3$) δ 17.6, 24.3, 25.6, 28.1, 28.1, 55.2, 70.2, 77.9, 99.6, 100.9, 100.9, 105.1, 107.6, 113.9, 114.6, 115.0, 121.1, 121.4, 126.7, 128.2, 128.6, 131.6, 132.3, 152.4, 157.7, 159.2, 159.3, 161.2, 161.2, 161.4, 182.6, ^{13}C NMR [$(CD3)_2CO$] δ 16.8, 23.8, 24.9, 27.4, 27.4, 54.5, 69.8, 77.8, 98.9, 100.6, 100.8, 104.6, 107.5, 113.5, 113.7, 114.5, 120.7, 121.6, 127.1, 128.6, 128.9, 131.4, 131.5, 152.2, 157.9, 159.1, 159.5, 160.9, 161.6, 161.8, 182.2, HRMS (EI) calcd for $C_{33}H_{32}O_7$ (M^+) 540.2148, found 540.2158.

4.8. 2-[2-Hydroxy-4-(4-methoxybenzyloxy)phenyl]-5-hydroxy-8,8-dimethyl-3-(3-methyl-but-2-enyl)-pyrano-[2,3-f]chromen-4-one (10b)

This compound was prepared as light yellow oil according to the previous procedures. IR (KBr) ν_{max}: 3400, 2975, 2924, 1650, 1620, 1580, 1265, 1184, 850, 780 cm^{-1}, ^1H NMR δ 1.36 (s, 3H), 1.41 (s, 6H), 1.60 (s, 3H), 3.11 (d, J=6.6 Hz, 2H), 3.81 (s, 3H), 5.01 (s, 2H), 5.08 (t, J=6.6 Hz, 1H), 5.48 (d, J=8.4 Hz, 1H), 6.15 (s, 1H), 6.58 (d, J=9.9 Hz, 1H), 6.63 (dd, J=9.9, 2.1 Hz, 1H), 6.64 (d, J=2.1 Hz, 1H), 6.80 (br s, 1H), 6.92 (d, J=8.4 Hz, 2H), 7.22 (d, J=8.4 Hz, 1H), 7.36 (d, J=8.4 Hz, 2H), 13.19 (br s, 1H); ^{13}C NMR ($CDCl_3$) δ 17.7, 24.2, 25.6, 28.2, 28.2, 55.3, 69.9, 78.1, 99.7, 101.1, 103.0, 104.8, 107.9, 112.5, 114.0, 114.8, 120.9, 121.3, 127.0, 128.4, 129.3, 131.3, 133.0, 152.1, 155.3, 159.3, 159.5, 159.9, 161.1, 161.9, 182.2, ^{13}C NMR [$(CD_3)_2CO$]δ 16.8, 23.7, 24.9, 27.3, 27.3, 54.6, 69.5, 77.9, 98.8, 100.7, 102.6, 104.7, 106.4, 112.9, 113.8, 114.4, 120.9, 121.5, 127.1, 128.8, 129.4, 131.4, 131.5, 152.3, 156.4, 159.1, 159.6, 161.2, 161.8, 161.9, 182.3, HRMS (EI) calcd for $C_{33}H_{32}O_7$ (M^+) 540.2148, found 540.2150.

4.9. 2-(2,4-Dihydroxyphenyl)-5-hydroxy-8,8-dimethyl-3-(3-methyl-but-2-enyl)-pyrano[2,3-f]chromen-4-one (1)[28]

To a solution of **10a** (0.30 g, 0.6 mmol) in pyridine (4.0 mL) was added acetyl chloride (39 μL) with dropwise at room temperature, and followed by stirring for 1 h. The resulting reaction mixture was quenched with H_2O (0.2 mL) and extracted with CH_2Cl_2 (3×5.0 mL). The combined organic extracts were dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by flash column chromatography (SiO_2, hexane:ethyl acetate 3:1) to obtain the desired product (0.30 g, 92%) as an oil. Subsequently, a mixture of acetoxy product and $SnCl_2$ (0.11 g, 0.6 mmol) in acetonitrile (5.0 mL) was added EtSH (0.2 mL) at room temperature under N_2, and monitored by TLC. The solvent was removed and the residue was subjected to column chromatography (SiO_2, ether:hexane 1:1) to give the demethoxybenzyl acetoxy product isomers, which was dissolved in THF (5.0 mL), and followed by deacetylation with NH_2NH_2–H_2O (29 μL, 0.6 mmol) at room temperature. The mixture was stirred for 20 min, and the resulting solution was diluted with CH_2Cl_2 and filtered by silica gel. The organic solvent was concentrated in vacuo, and the residue was purified by flash column chromatography (SiO_2, ether:CH_2Cl_2 1:20) to achieve **1** (156 mg, 67%) as light yellow solid: mp 149–150 °C (ether/hexane) (lit.[29] 147–149 °C), IR (KBr) ν_{max}: 3400, 2978, 2928, 1648, 1620, 1580, 1265, 1184, 790 cm^{-1}, ^1H NMR [$(CD_3)_2CO$] δ 1.42 (s, 9H), 1.55 (s, 3H), 3.10 (d, J=6.9 Hz, 2H), 4.55 (br s, 1H), 4.58 (br s, 1H), 5.11 (t, J=6.9 Hz, 1H), 5.63 (d, J=8.1 Hz, 1H), 6.13 (s, 1H), 6.50 (dd, J=9.6, 2.4 Hz, 1H), 6.55 (d, J=2.4 Hz, 1H), 6.58 (d, J=9.6 Hz, 1H), 7.23 (d, J=8.1 Hz, 1H), 13.2 (br s, 1H); ^{13}C NMR δ 17.6, 24.2, 25.6, 28.1, 28.1, 78.1, 99.8, 101.0, 103.8, 104.9, 108.3, 112.3, 114.7, 120.8, 121.3, 127.1, 131.6, 133.2, 152.1, 155.2, 159.2, 159.3, 159.9, 161.2, 182.3, HRMS (EI) calcd for $C_{25}H_{24}O_6$ (M^+) 420.1573, found 420.1566.

4.10. Computational methods. Preparation of ligand and protein complexes input structures

First, protein structures were extracted from the Protein Databank (pdb) file (containing only non-hydrogen atoms). The protein's pdb files were edited with AutoDockTools-1.5.1 (on Linux system) to protonate polar hydrogen atoms, add Kollman charges, and save as a pdbqt file. Furthermore, ligand structures were optimized by Chem 3D Ultra version 8.0 (Run MOPAC minimize energy to minimum rms Gradient 0.001) to get the structure (pdb file). Alternatively, the X-ray structure of morusin was extracted from Cambridge Chemical Database (pdb file). The ligand file was input into AutoDockTools-1.5.1 program, detected the root of molecule, and output as a pdbqt file as well.

4.10.1. Grid and docking studies. To predict the energetically favorable positions of ligand molecules in the interior structure of the protein target, a rectangular grid box of 21.75×21.75×21.75 $Å^3$ with grid points separated by 0.333 Å, centered on the midpoint of the ligand binding pocket. It was essential to modify the AutoGrid program to enable docking in the presence of metals and heteroatoms. Subsequently, Automated docking studies were performed with Lamarckian Genetic Algorithm to provide the binding energies and conformations of protein–ligand complexes.

4.11. 5-Lipoxygenase assay

Possible inhibition of 5-lipoxygenase activity was determined by the method of Sircar et al.[30] and modified by Evans.[31] All concentrations refer to final concentrations in 3 mL cuvettes maintained at 25 °C in a thermostated bath. The assay mixture contained 10 μL of various chemicals (including CAPE, himanimide C, and morusin) dissolved in DMSO, 0.1 M potassium phosphate buffer

(pH 6.3,2.95 mL) prepared with analytical grade reagents and 100 μM linoleic acid (≥99%) from Fluka. The reaction was initiated with the addition of 35 U isolated 5-lipoxygenase (Cayman) diluted with equal volume potassium phosphate buffer maintained at 4 °C. The increase in absorbance at 234 nm was recorded for 10 min with a single beam spectrophotometer (Hitachi U-2000). The percentage inhibition of enzyme activity was calculated by comparison with the negative control (DMSO). The concentration of each chemical that caused 50% enzyme inhibition (IC_{50}) was determined.

4.12. Cytotoxicity assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay was developed to monitor mammalian cell survival and proliferation. MTT assay was measured by dehydrogenase activity as described by Mosmann,[32] with minor modification (Alley et al.).[33] The MTT solution was prepared in 5 mg/mL of phosphate-buffered saline just before use and filtered through a 0.22 μM filter. Cells were seeded 5×10^4 cells per well into 24-well culture plates and incubated for 24 h. After a 24 h incubation in various concentrations of morusin or synthetic compounds, 10 μL of MTT solution was added to each well. Plates were incubated in a CO_2 incubator for 4 h, and then the blue crystals, which are the metabolized product of MTT were extracted by isopropanol. Absorbance at 563 nm was determined and used for the measurement of the proportion of surviving cells. The percentage of the dehydrogenase activity at each concentration, compared with that of the control, was calculated from the absorbance values.

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tet.2009.12.002.

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