Novel Inhibitors of Prolyl 4-Hydroxylase; Solid-phase Synthesis of 2,2-Dimethyl-3,4-Dialkoxy-Substituted 6-Aminobenzopyran Derivatives

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2,2-Dimethyl-3,4-dialkoxy-substituted 6-aminobenzopyran analogues (*eg*., **7** and **8**) were identified as prolyl 4-hydroxylase inhibitors via a screening process using HSC-T6 and LI 90 cells that express an immortalized rat hepatic stellate cell line and as part of a test of the type I collagen contents employing the ELISA method. A subsequent lead optimization effort based on solid-phase parallel synthesis led to the identification of 2,2-dimethyl-3,4-dialkoxy-substituted 6-aminobenzopyrans as potent inhibitors of prolyl 4-hydroxylase.

Key Words : Prolyl 4-hydroxylase, Collagen contents, Solid-phase synthesis, 2,2-Dimethyl-3,4-dialkoxy-substituted 6-aminobenzopyrans

Introduction

Hepatic fibrosis is a wound-healing process that takes place in response to a variety of chronic stimuli. This cellular response results in the activation of hepatic stellate cells (HSC) which are responsible for the enhancement of synthesis and deposition of the extracellular matrix. In the liver, type I collagen is the extracellular matrix.[1] In this organ, the fibrosis caused by an imbalance between type I collagen production and degradation leads to accumulation of the collagen and progressive impairment of liver function (*i.e*. liver cirrhosis).[2] Collagen synthesis requires several posttranslational processes, including hydroxylation of lysine and proline residues, triple helix formation, and glucosylation. The crucial step of the collagen processing is the formation of hydroxyproline residues by the enzyme, prolyl 4-hydroxylase (P4H), resulting in the production of the thermally stable triple helical procollagen molecules. Inhibition of prolyl 4-hydroxylase underhydroxylated newly synthesized procollagen peptides, which is not stable at body temperature.[3] Thus, P4H has long been recognized as an ideal target for the phamarcological control of excessive collagen biosynthesis.[4]

Prolyl 4-hydroxylase is a member of the 2-oxoglutarate and non-heme-Fe(II)-dependent dioxygenase family and it requires Fe^{2+}, 2-oxoglutarate, O_2 and ascorbate for the catalytic process. In the catalytic cycle, 2-oxoglutarate is stoichiometrically decarboxylated and one oxygen atom of O_2 is incorporated into succinate and the other into the hydroxy group of the formed hydroxyproline residue. Ascorbate is not consumed stoichiometrically in the process, and prolyl 4-hydroxylase can catalyze a number of reaction cycles in the absence of this substance. However, even in at extremely high peptide substrate concentrations, prolyl 4-hydroxylase also promotes uncoupled decarboxylation of 2-oxoglutarate, *i.e*. decarboxylation without subsequent hydroxylation of a proline residue. Ascorbate is consumed stoichiometrically and acts as an alternative oxygen acceptor in the uncoupled decarboxylation cycle.[5]

Although the exact chemical mechanism for catalytic process of the prolyl 4-hydroxylase is not known, iron(II) is assumed to participate by forming a 5-membered chelate complex with substrates and/or cofactors. Recently, it was reported that some 3,4-disubstituted benzopyran derivatives formed chelate complex with iron[6] through the participation of 3-hydroxyl and 4-keto of quercetin (Fig. 1). This observation suggests that the 3,4-disustituted benzopyran structure could serve as a platform for the discovery of new types of iron chelating ligands that might have prolyl 4-hydroxylase inhibitory properties. As part of a research program guided by this proposal, we required a synthetic approach for the preparation of benzopyran derivatives that would be applicable to a combinatorial protocol.[7] Below, we report the results of a lead optimization effort that uses solid-phase parallel synthesis to identify and optimize novel benzopyran anti-fibrotic agents.

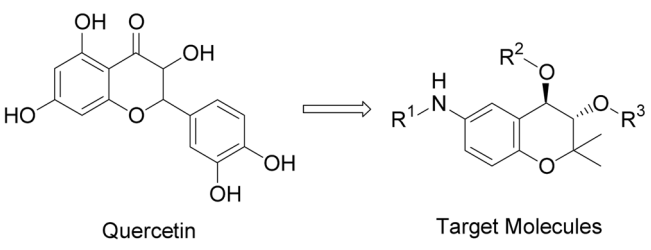


Figure 1. Structure of quercetin and target molecules.

Results and Discussion

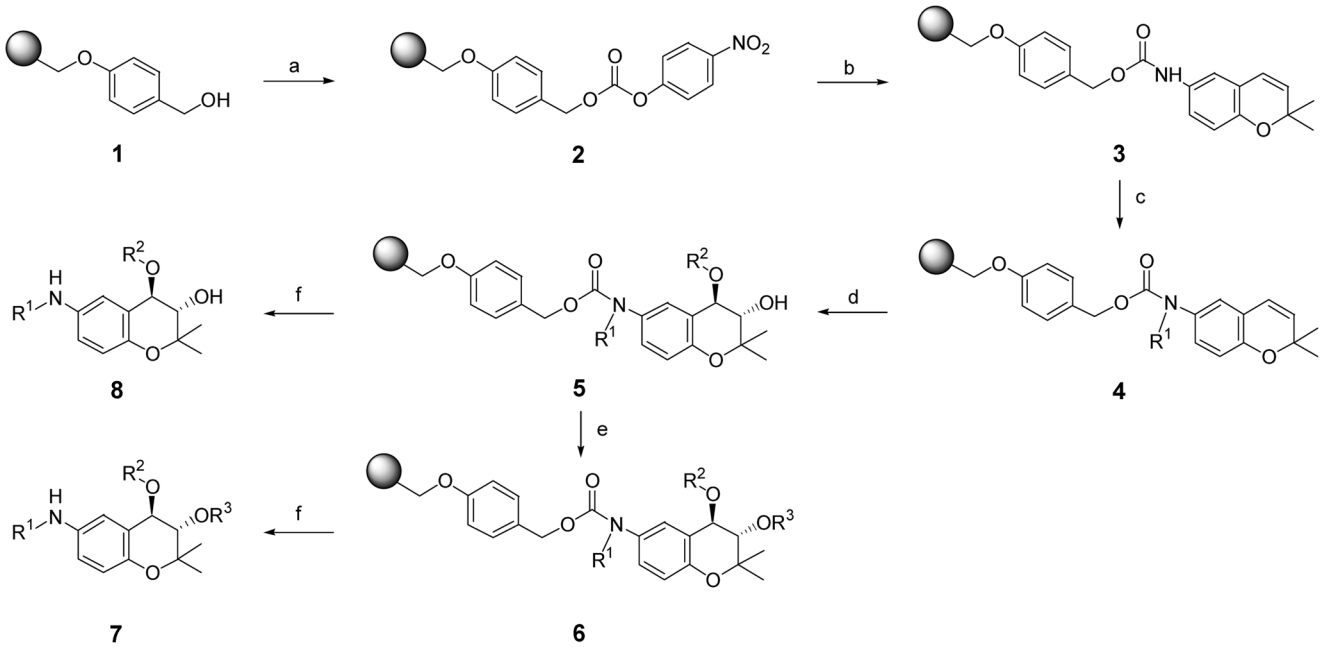
Chemistry. In an earlier publication, we described a solid-phase synthetic approach to the construction of 6-amino-2,2-dimethyl-3,4,6-trisubstituted-2*H*-1-benzopyran libraries that employs a carbamate linker strategy.[8] For the solid-phase parallel syntheses described below, the Wang resin **1** was used as a polymer support since hydroxy groups present in this resin enable ready introduction of the 6-amino-2,2-dimethylchromene **9** through the carbamate linker. The carbamate group in the conjugate also serves as an efficient amine protecting group needed in the subsequent oxidation and alkylation reactions. The key carbamate resins **5** are prepared by using the four-step procedure shown in Scheme 1 starting from the Wang resin **1**. The desired benzopyran products, **7** and **8**, are liberated from the respective resins **5** and **6** by treatment with trifluoroacetic acid (TFA). The progress of the reactions was monitored by using attenuated total reflection (ATR)-FTIR[9] on single beads and high-resolution magic-angle spinning (HR-MAS) NMR.[10]

The first step in these sequences involves preparation of the *p*-nitrophenyl carbonate resin **2** by reaction of the Wang resin **1** and *p*-nitrophenyl chloroformate in CH_2Cl_2. The formation of the carbonate resin **2** was confirmed by the prominent carbonate band at 1765 cm^{-1} by ATR-FTIR. Treatment of carbonate resin **2** with 6-amino-2,2-dimethylchromene and *N*,*N*-diisopropylethylamine (DIPEA) in *N*,*N*-dimethylacetamide (DMA) affords the resin **3,** which was also confirmed by the appearance of the carbamate band at 1725 cm^{-1}. Initial alkyl group variation on the amino group in the chromene system was introduced by using nucleophilic substitution reactions of **3** with alkyl halides in the presence of lithium *t*-butoxide as a base in dimethylsulfoxide (DMSO).

We hypothesized that chromene epoxidation reactions carried out in the presence of nucleophiles, would lead to nucleophilic ring opening of the *in situ* formed epoxides. To our delight, nucleophilic alkoxides, derived from benzyl alcohol, substituted benzyl alcohols, and primary alcohols, add to the initially formed epoxides to produce the hydroxyalkoxy resins **5**. To confirm the success of these processes, the methoxyl-hydroxyl resin **5b** is treated with 25% TFA in CH_2Cl_2 for 3 h to obtain the desired benzopyran derivative **8a** in high yield. By using this approach, various alkoxyl-hydroxyl substituted chromenes **8** are generated in relatively high five-step yields.

In order to introduce diversity *via* the hydroxyl group of the resin **5**, we examined the ether forming reactions of this resin with alkyl halides. For this purpose, resins **5** are reacted with various alkyl and benzyl halides in the presence of lithium *t*-butoxide in DMF. The reactions proceed smoothly to provide the ether containing resins **6**, which are subsequently treated with 25% TFA in CH_2Cl_2 for 3 h to produce the desired 6-alkylamino-2,2-dimethyl-3,4-dialkoxy-substituted-2*H*-1-benzopyrans **7** in high overall yields. By using these techniques, a 200-member library of the 6-amino-2,2-dimethyl-3,4-dialkoxy-substituted 6-amino-benzopyran analogues was constructed. The identity of each substance (racemates) in this library was evaluated by using 200, 300, and 500 MHz NMR spectroscopy. In addition, all of the substances were purified by parallel chromatography prior to their biological evaluation.

Biological activity test. Initial screening for prolyl-4-hydroxylase inhibitory activity was performed by determin-

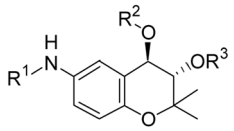


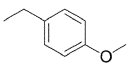
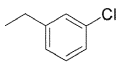
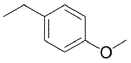
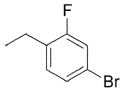
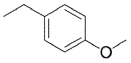
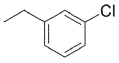
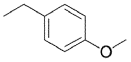
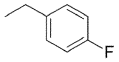
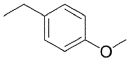
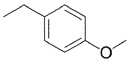
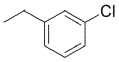
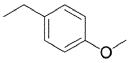
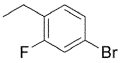
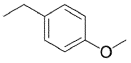
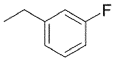
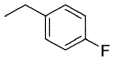
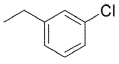
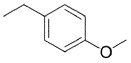
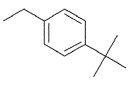
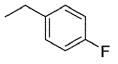
Scheme 1. *Reagents and conditions*: (a) *p*-nitrophenyl chloroformate, pyridine, CH_2Cl_2; (b) 6-amino-2,2-dimethyl chromene **9**, DIPEA, DMA; (c) alkyl or benzyl halide, ^{t}BuOLi, DMSO; (d) *m*-CPBA, alcohol, CH_2Cl_2; (e) alkyl or benzyl halide, ^{t}BuOLi, DMF; (f) TFA/CH_2Cl_2 (1 : 3); **5**, **6**, **7**, and **8** are racemates.

ing the hydroxyproline content in HSC-T6 which is an immortalized rat hepatic stellate cells. The screen resulted in the identification of 3,4-dialkoxy-subutituted 6-aminobenzopyrans, such as **7**, as potent inhibitors of prolyl-4-hydroxylase.

To explore the SAR of **7** and optimize inhibitory activities, parallel libraries (Scheme l) were designed to allow systemic variation of three regions of **7**, including (1) alkyl and benzyl group variation of R^1 in the left portion, (2) methyl and benzyl group variation of R^2 in the center, and (3) alkyl and various benzyl group variation of R^3 in the right portion. Several interesting conclusions can be drawn from inspecting the SAR (Table 1) obtained from study of the library **7**. Firstly, the corresponding methyl, benzyl, 4-fluorobenzyl, and 4-methylbenzyl group containing analogs, **7a-m**, are about half as potent inhibitors. On the other side, introduction of 4-methoxybenzyl substituents (as in **7n-z**) in the R^1 group generally enhanced inhibitory activity except **7t** and **7u** as compared with the corresponding methyl, benzyl, 4-fluorobenzyl, and 4-methylbenzyl group containing ana-

Table 1. Representative Inhibitors of Prolyl 4-hydroxylase



No	R^1	R^2	R^3	No	R^1	R^2	R^3
7a	CH_3	CH_3	CH_3	**7o**			
7b		CH_3	CH_3	**7p**			
7c		CH_3		**7q**			
7d		CH_3		**7r**			$(CH_2)_4CH_3$
7e		CH_3		**7s**			$CH_2C{\equiv}CH$
7f				**7t**		CH_3	$CH_2(CH)_2CH_3$
7g				**7u**		CH_3	$CH_2C{\equiv}CH$
7h				**7v**		CH_3	
7i				**7w**		CH_3	
7j				**7x**		CH_3	
7k				**7y**		CH_3	
7l				**7z**		CH_3	CH_2CHCH_2
7m				**8a**		CH_3	H

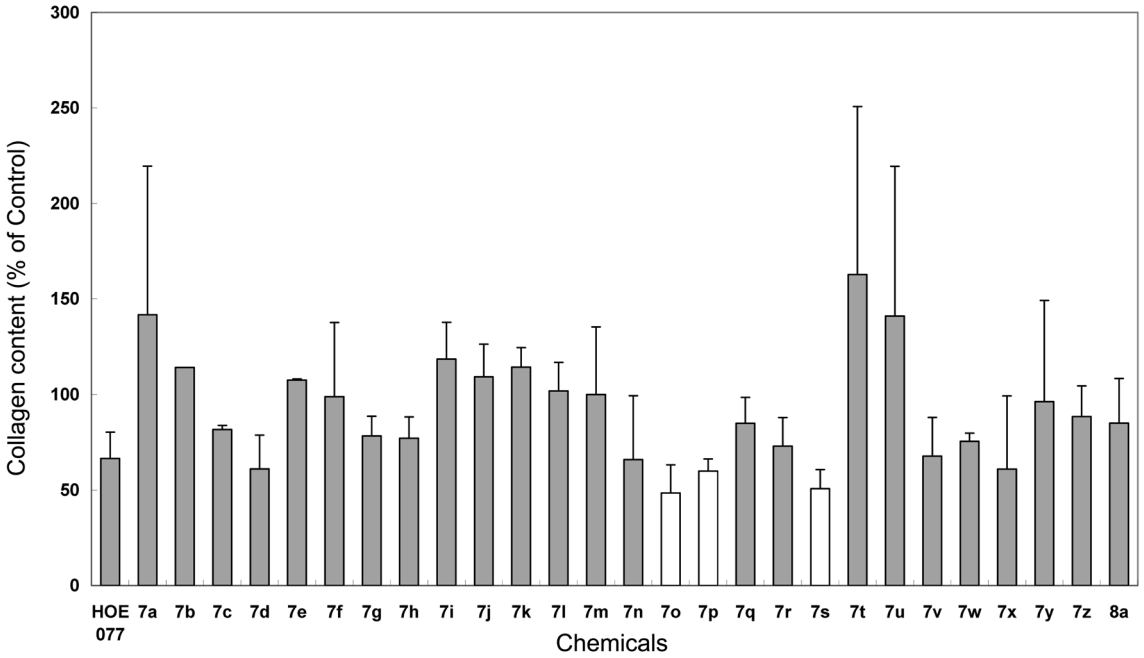
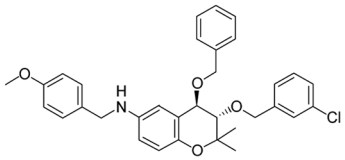
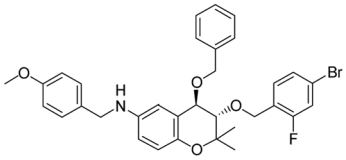
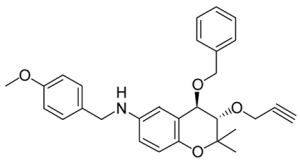


Figure 2. Effects of synthetic P4H inhibitors on the collagen synthesis in the HSC-T6 cells.

logs, **7a**-**m**. Secondly, though benzyl group at R^2 are slightly less potent than methyl group when R^1 is benzyl group and R^3 are equal (**7c** *vs* **7f** and **7d** *vs* **7g**), it enhance inhibitory activity when the R^1 is replaced as 4-methoxybenzyl group. Especially, the methyl group at R^2 is about half as potent inhibitors when the R^1 is 4-methoxybenzyl group and R^3 is acetylene as shown in Figure 2 (**7s** *vs* **7u**). Finally, substances with R^3 being some halogens (F, Cl and Br as in **7d**, **7n**, **7o**, **7p**, **7v** and **7x**) and acetylene group (as in **7s**) seem to be more potent than HOE077 or similarly potent. On the other hand, compounds with a benzyl and methyl group at R^3 are low potent. For example, the 3-chlorbenzyl group (as in **7o**) is two times more potent compared with benzyl group (as in **7f**).

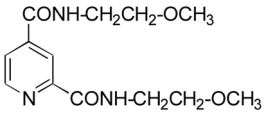
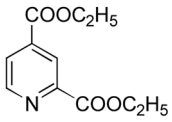
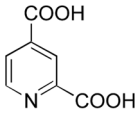
The ultimate aim of this study was to discover novel anti-fibrotic agents. Collagen composed with unusual amino acid 4-hydroxyproline about 8%. Thus, the hydroxyproline content in HSC-T6 cells incubated in the presence of 10 μg/mL of each substance for 24 h was measured in order to select the first hit compounds. The compounds which were

Table 2. Effects of the Hit Compounds on the Collagen Content in HSC-T6 Cells

No	Structure	Collagen content (% of Control)	Cytotoxicity (% of Control)
7o		48.4 ± 14.8	92.1 ± 3.81
7p		60.0 ± 6.37	99.2 ± 6.65
7s		50.8 ± 10.0	71.0 ± 2.38

HSC-T6 cells were treated with 10 mM chemicals for 24 h.

Table 3. Effects of HOE077 and its Metabolites on the Collagen Content in HSC-T6 Cells

Compound	Structure	Collagen content (% of Control)	Cytotoxicity (% of control)
HOE077		71.0 ± 2.10	95.358 ± 2.39
Mys-yl-3		67.9 ± 3.30	93.32 ± 4.32
Pyridine 2,4-dicarboxylic acid		74.7 ± 2.86	90.92 ± 3.86

HSC-T6 cells were treated with 100 mM HOE077 or its metabolites for 24 h.

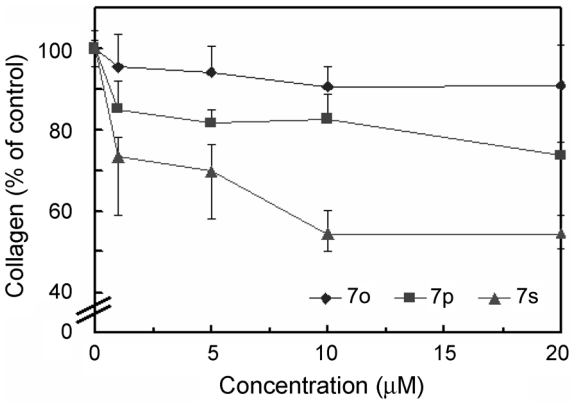


Figure 3. Effect of three first hits on the collagen synthesis in LI 90 cells.

inhibited the collagen synthesis more potently than HOE077 (100 μg/mL) were selected as first hits. As the data given in Table 2, **7o**, **7p**, and **7s** have the highest potencies of all the compounds tested without the significant cytotoxicity. They were inhibited more strongly comparing with HOE077 and its metabolites (Table 3). These hits were confirmed in human hepatic stellate cell line, LI 90 cells, using ELISA assay for a secretion of collagen which relate to the perturbation of collagen synthesis by inhibition of P4H. Although all of these three hits were dose-dependently inhibited the collagen synthesis at 1-20 μM, only **7s** were significant (Figure 3).

Conclusion

In summary, we have succeeded in preparing novel 6-amino-3,4,6-trisubstituted benzopyrans hit compounds, which have been identified as prolyl 4-hydroxylase inhibitors by employing a screening protocol, using a HSC-T6 and LI 90 cells, which express an immortalized rat and human hepatic stellate cell line, to determine type I collagen contents. A subsequent solid-phase parallel synthesis driven lead optimization effort resulted in the discovery of the 6-amino-3,4,6-trisubstituted benzopyran analogues, **7o**, **7p** and **7s**, as novel, highly potent anti-fibrotic agents.

Experimental Section

Chemistry

Materials and Methods. The polystyrene Wang resin (1.0 mmol/g, 1% cross-linking, 100-200 mesh) was obtained from NovaBiochem. Quaternary ammonium styrene divinylbenzene scavenger resin was obtained from Alltech (particle size: 45-150 m, exchange capacity 1.2 meq/mL). Solvents were purchased from Merck and were anhydrous and HPLC grade. Reactions, filtration and washings were carried out on a Quest210 synthesizer (Agronaut Technology) and a Mini-Block (Bohdan). Solvent evaporation was performed on a GeneVac Atlas HT-4 centrifugal evaporator. Crude products were purified by parallel chromatography using QuadFlash silica-cartridge (Biotage catalog No. QK0-1107-1504L). All of the solid bound intermediate resins were monitored by ATR-FTIR (SensIR Technology) or HR-MAS-NMR (Bruker Advance 500FT-NMR) spectroscopy. The structures of the final products were confirmed by ^{1}H NMR (Bruker DPX-300FT-NMR, Varian GEMINI-200FT-NMR, and Bruker AMX-500 FT NMR) and HRMS (Micromass Auto Spec MS), MS (Hewlett-Packard 5971A, Shimadzu QP5050) spectroscopy. LC/MS data were recorded on a Waters ZQ electrospray mass spectrometer (EI) equipped with PDA (200-600 nm) detection using XTerraMS column (C_{18}, 5 μM, 4.6 × 100 mm) from Waters (U.K.). Typical gradient were 5-95% MeCN/H_2O containing 0.1% trifluoroacetic acid.

Representative Procedure for the Synthesis of *p*-Nitrophenyl Carbonate Wang Resin (2). Wang resin **1** (10.00 g, 10.00 mmol) was swollen in dry CH_2Cl_2 under Ar gas for 30 min. After filtration of the solvent, a solution of *p*-nitrophenyl chloroformate (10.08 g, 50.00 mmol) was added in dry CH_2Cl_2 (40 mL), followed by slow addition of a

solution of dry pyridine (7.91 g, 100.00 mmol) in dry CH_2Cl_2 (40 mL). The suspension was shaken for 48 h at room temperature under Ar. Carbonate resin **2** was filtered and washed with DMF (2 × 100 mL), MeOH (2 × 100 mL), MeOH/CH_2Cl_2 (1 : 1; 2 × 100 mL), CH_2Cl_2 (2 × 100 mL), MeOH/CH_2Cl_2 (1 : 1; 2 × 100 mL), and MeOH (2 × 100 mL) and dried under high vacuum. FTIR (cm^{-1}): 1765, 1595, 1349, 1214.

Representative Procedure for the Synthesis of 6-Amino-2,2-dimethyl-2*H*-1-benzopyran Carbamate Wang Resin (3). Carbonate resin **2** (8.00 g, 8.00 mmol) was suspended in dry DMA (50 mL), and 6-amino-2,2-dimethyl-2*H*-1-benzopyran (2.80 g, 16.00 mmol) and DIPEA (6.97 mL, 40.0 mmol) were successively added. The mixture was shaken for 10 h at room temperature. Carbamate resin **3** was filtered and washed with DMF (2 × 80 mL), MeOH (2 × 80 mL), MeOH/CH_2Cl_2 (1 : 1; 2 × 80 mL), CH_2Cl_2 (2 × 80 mL), MeOH/CH_2Cl_2 (1 : 1; 2 × 80 mL), and MeOH (2 × 80 mL) and dried under high vacuum. FTIR (cm^{-1}): 1725, 1374, 1055.

Representative Procedure for the First Generation Step by *N*-Alkylation Reaction; 6-Benzylamino-2,2-dimethyl-2*H*-1-benzopyran Resin (4b). The 6-amino-2,2-dimethyl-2*H*-1-benzopyran resin **3** (6.00 g, 3.00 mmol) was suspended in dry DMSO (40 mL) and benzyl bromide (1.54 g, 9.00 mmol) and ^{t}BuOLi (1.20 g, 15.00 mmol) were successively added. The mixture was shaken for 12 h at room temperature. The desired resin **4b** was filtered and washed with DMF (2 × 50 mL), MeOH (2 × 50 mL), MeOH/CH_2Cl_2 (1 : 1; 2 × 50 mL), CH_2Cl_2 (2 × 50 mL), MeOH/CH_2Cl_2 (1 : 1; 2 × 50 mL), and MeOH (2 × 50 mL) and dried under high vacuum. FTIR (cm^{-1}): 1699, 1251, 1134.

Representative Procedure for the Second Generation Step by Hydroxy-alkoxylation Reaction; 6-Benzylamino-2,2-dimethyl-3-hydroxy-4-methoxy-2*H*-1-benzopyran Resin (5b). The 6-benzylamino-2,2-dimethyl-2*H*-1-benzopyran Resin **4b** (5.00 g, 2.50 mmol) was suspended in dry CH_2Cl_2 (30 mL), MeOH (30 mL) and *m*-CPBA (2.16 g, 12.50 mmol) were successively added. After the mixture was shaken for 24 h at room temperature, the solvent was filtered off and washed with DMF (2 × 30 mL), MeOH (2 × 30 mL), MeOH/CH_2Cl_2 (1 : 1; 2 × 30 mL), CH_2Cl_2 (2 × 30 mL), MeOH/CH_2Cl_2 (1 : 1; 2 × 30 mL), and MeOH (2 × 30 mL) and dried under high vacuum.

Representative Procedure for the Cleavage Step from the Second Generated Resin (5b); 6-Benzylamino-2,2-dimethyl-3-hydroxy-4-methoxy-2*H*-1-benzopyran (8a). The hydroxy-alkoxylated resin **5b** (0.20 g, 0.10 mmol) was treated with 4 mL of cleavage cocktail (TFA/CH_2Cl_2; 1 : 3). After the mixtures were shaken at room temperature for 3 h, the resin was filtered off and washed with CH_2Cl_2 (3 × 1 mL) followed by MeOH (1 mL). The combined filtrates were evaporated and purified by SAX resin and silica gel column chromatography (25% ethyl acetate in hexane; using Quad3$^+$) to yield (19.4 mg, 62%) 6-benzylamino-2,2-dimethyl-3-hydroxy-4-methoxyxy-2*H*-1-benzopyran **8a** (97% purity, determined by HPLC). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.35-7.28 (m, 5H), 6.69-6.61 (m, 3H), 4.29 (d, 1H, J = 7.5 Hz), 4.27 (s, 2H), 3.81 (d, 1H, J = 7.5 Hz), 3.40 (s, 3H), 1.42 (s, 3H), 1.26 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 145.95, 141.11, 138.59, 128.59, 127.92, 127.39, 121.35, 116.25, 112.82, 78.01, 77.53, 71.91, 55.67, 49.39, 25.73, 19.96; LC/MS (ESI) *m/z* 314 $[M+H]^+$; HRMS (EI^+) *m/z* 313.1663 found, 313.1678 calculated for $C_{19}H_{23}N_1O_3$.

Representative Procedure for the Third Generation Step by 3-Etherfication Reaction; 6-Benzylamino-3-methoxy-2,2-dimethyl-4-methoxy-2*H*-1-benzopyran Resin (6b). The 6-benzylamino-2,2-dimethyl-3-hydroxy-4-methoxy-2*H*-1-benzopyran resin **5b** (0.20 g, 0.10 mmol) was suspended in dry DMF (5 mL), and ^{t}BuOLi (0.08 mg, 1.00 mmol) was added, and the mixture was shaken for 30 min. Iodomethane (0.14 g, 1.00 mmol) was added, the mixture shaken for 12 h at room temperature, and the solvent was filtered off and washed with DMF (2 × 10 mL), MeOH (2 × 10 mL), MeOH/CH_2Cl_2 (1 : 1; 2 × 10 mL), CH_2Cl_2 (2 × 10 mL), MeOH/CH_2Cl_2 (1 : 1; 2 × 10 mL), and MeOH (2 × 10 mL) and dried under high vacuum.

Representative Procedure for the Cleavage Step from the Third Generated Resin (6b); 6-Benzylamino-3,4-dimethoxy-2,2-dimethyl-2*H*-1-benzopyran (7b). The resin **6b** (0.20 g, 0.10 mmol) was treated with 4 mL of cleavage cocktail (TFA/CH_2Cl; 1 : 3). After the mixtures were shaken at room temperature for 3 h, the resin was filtered off and washed with CH_2Cl_2 (3 × 1 mL), followed by MeOH (1 mL). The combined filtrates were evaporated to and purified by SAX resin and silica gel column chromatography (15% ethyl acetate in hexane using Quad3$^+$) to yield (16.3 mg, 50%) 6-(Benzylamino)-3,4-dimethoxy-2,2-dimethyl-2*H*-1-benzopyran **7b** (92% purity, determined by HPLC). ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.26-7.37 (m, 5H), 6.63-6.65 (m, 3H), 4.28 (d, 1H, J = 7.3 Hz), 4.27 (s, 2H), 3.61 (s, 3H), 3.50 (s, 3H), 3.33 (d, 1H, J = 7.3 Hz), 1.41 (s, 3H), 1.20 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 145.57, 141.36, 138.98, 128.55, 127.29, 122.44, 117.64, 115.80, 112.52, 82.60, 77.79, 77.69, 60.32, 57.06, 49.76, 26.07, 20.24; LC/MS (ESI) *m/z* 328 $[M+H]^+$; HRMS (EI^+) *m/z* 327.184319 found, 327.183444 calcd for $C_{20}H_{25}N_1O_3$.

3,4-Dimethoxy-2,2-dimethyl-6-(methylamino)-2*H*-1-benzopyran (7a). ^{1}H NMR (200 MHz, $CDCl_3$): δ 6.68-6.48 (m, 3H), 4.33 (d, 1H, J = 7.2 Hz), 3.62 (s, 3H), 3.55 (s, 3H), 3.35 d, 1H, J = 7.2 Hz), 2.80 (s, 3H), 1.42 (s, 3H), 1.20 (s, 3H);); LC/MS (ESI) *m/z* 252 $[M+H]^+$.

6-Benzylamino-3-benzyloxy-2,2-dimethyl-4-methoxy-2*H*-1-benzopyran (7c). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40-7.30 (m, 10H), 6.65 (d, 2H, J = 8.7 Hz), 6.55 (dd, 1H, J = 8.7 Hz, J = 2.2 Hz), 4.90 (d, 1H, J = 11.5 Hz), 4.72 (d, 1H, J = 11.5 Hz), 4.41 (d, 1H, J = 7.3 Hz), 4.28 (s, 2H), 3.60 (d, 1H, J = 7.3 Hz), 3.44 (s, 3H), 1.40 (s, 3H), 1.23 (s, 3H); LC/MS (ESI) *m/z* 404 $[M+H]^+$.

6-Benzylamino-3-(3-chlorobenzyloxy)-2,2-dimethyl-4-methoxy-2*H*-1-benzopyran (7d). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.38-7.28 (m, 9H), 6.70-6.66 (m, 2H), 6.56 (dd, 1H, J = 8.5 Hz, J = 2.2 Hz), 4.85 (d, 1H, J = 11.2 Hz), 4.69

(d, 1H, J = 11.2 Hz), 4.45 (d, 1H, J = 7.3 Hz), 4.29 (s, 2H), 3.61 (d, 1H, J = 7.3 Hz), 3.45 (s, 3H), 1.44 (s, 3H), 1.26 (s, 3H); LC/MS (ESI) *m/z* 438 $[M+H]^+$.

6-Benzylamino-4-methoxy-2,2-dimethyl-3-(4-fluorobenzyloxy)-2*H*-1-benzopyran (7e). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38-7.29 (m, 6H), 7.09-7.00 (m, 3H), 6.69-6.63 (m, 2H), 6.59 (dd, 1H, J = 8.5 Hz, J = 2.2 Hz), 4.88 (d, 1H, J = 11.0 Hz), 4.69 (d, 1H, J = 11.0 Hz), 4.40 (d, 1H, J = 7.3 Hz), 4.27 (s, 2H), 3.60 (d, 1H, J = 7.3 Hz), 3.43 (s, 3H), 1.39 (s, 3H), 1.22 (s, 3H); LC/MS (ESI) *m/z* 422 $[M+H]^+$.

6-Benzylamino-3,4-dibenzyloxy-2,2-dimethyl-2*H*-1-benzopyran (7f). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.34-7.25 (m, 15H), 6.66 (d, 1H, J = 8.8 Hz), 6.58 (s, 1H), 6.54 (d, 1H, J = 8.8 Hz), 4.89 (d, 1H, J = 11.4 Hz), 4.78-4.73 (m, 3H), 4.66 (d, 1H, J = 11.4 Hz), 4.20 (s, 2H), 3.70 (d, 1H, J = 7.1 Hz), 1.43 (s ,3H), 1.27 (s, 3H); LC/MS (ESI) *m/z* 480 $[M+H]^+$.

6-Benzylamino-4-benzyloxy-3-(3-chlorobenzyloxy)-2,2-dimethyl-2*H*-1-benzopyran (7g). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36-7.25 (m, 14H), 6.66 (d, 1H, J = 8.7 Hz), 6.59-6.59 (m, 2H), 4.85 (d, 1H, J = 11.8 Hz), 4.70-4.62 (m, 4H), 4.20 (s, 2H), 3.68 (d, 1H, J = 7.3 Hz), 1.44 (s, 3H), 1.27 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 145.09, 140.38, 139.53, 138.37, 134.25, 129.71, 129.64, 128.59, 128.49, 127.84, 127.78, 127.72, 127.64, 127.21, 125.61, 122.54, 117.81, 117.70, 115.51, 111.64, 81.56, 77.74, 76.64, 73.53, 72.04, 49.28; LC/MS (ESI) *m/z* 514 $[M+H]^+$.

6-Benzylamino-4-benzyloxy-3-(4-bromo-2-fluorobenzyloxy)-2,2-dimethyl-2*H*-1-benzopyran (7h). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36-7.22 (m, 13H), 6.65 (d, 1H, J = 8.6 Hz), 6.57-6.55 (m, 2H), 4.85 (d, 1H, J = 12.0 Hz), 4.75-4.67 (m, 3H), 4.59 (d, 1H, J = 7.0 Hz), 4.20 (s, 2H), 3.69 (d, 1H, J = 7.2 Hz), 1.42 (s, 3H), 1.23 (s, 3H); LC/MS (ESI) *m/z* 576 $[M+H]^+$, 578 $[M+2+H]^+$.

4-Benzyloxy-3-(3-chlorobenzyloxy)-2,2-dimethyl-6-(4-fluorobenzylamino)-2*H*-1-benzopyran (7i). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.34-7.17 (m, 11H), 7.04-6.96 (m, 2H), 6.66 (d, 1H, J = 8.4 Hz), 6.55-6.51 (m, 2H), 4.86 (d, 1H, J = 12.0 Hz), 4.71-4.61 (m, 4H), 4.18 (s, 2H), 3.68 (d, 1H, J = 7.3 Hz), 1.45 (s, 3H), 1.28 (s, 3H); LC/MS (ESI) *m/z* 532 $[M+H]^+$.

4-Benzyloxy-3-(4-bromo-2-fluorobenzyloxy)-2,2-dimethyl-6-(4-fluorobenzylamino)-2*H*-1-benzopyran (7j). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.33-7.21 (m, 10H), 7.00 (m, 2H), 6.66 (d, 1H, J = 9.1 Hz), 6.54-6.50 (m, 2H), 4.87 (d, 1H, J = 12.0 Hz), 4.81 (d, 1H, J = 12.0 Hz), 4.74-4.68 (m, 2H), 4.59 (d, 1H, J = 7.2 Hz), 4.17 (s, 2H), 3.69 (d, 1H, J = 7.2 Hz), 1.42 (s, 3H), 1.24 (s, 3H); LC/MS (ESI) *m/z* 594 $[M+H]^+$, 596 $[M+2+H]^+$.

4-Benzyloxy-3-(3-chlorobenzyloxy)-2,2-dimethyl-6-(4-methylbenzylamino)-2*H*-1-benzopyran (7k). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.34-7.12 (m, 13H), 6.65 (d, 1H, J = 8.7 Hz), 6.59-6.55 (m, 2H), 4.85 (d, 1H, J = 11.8 Hz), 4.70-4.61 (m, 4H), 4.16 (s, 2H), 3.68 (d, 1H, J = 7.3 Hz), 2.33 (s, 3H), 1.44 (s, 3H), 1.27 (s, 3H); LC/MS (ESI) *m/z* 528 $[M+H]^+$.

4-Benzyloxy-3-(4-bromo-2-fluorobenzyloxy)-2,2-dimethyl-6-(4-methylbenzylamino)-2*H*-1-benzopyran (7l). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.34-7.12 (m, 12H), 6.65 (d, 1H, J = 8.7 Hz), 6.57-6.55 (m, 2H), 4.86 (d, 1H, J = 12.0 Hz), 4.75-4.67 (m, 3H), 4.60 (d, 1H, J = 7.2 Hz), 4.15 (s, 2H), 3.68 (d, 1H, J = 7.2 Hz), 2.33 (s, 3H), 1.42 (s, 3H), 1.24 (s, 3H); LC/MS (ESI) *m/z* 590 $[M+H]^+$, 592 $[M+2+H]^+$.

4-Benzyloxy-2,2-dimethyl-6-(4-methylbenzylamino)-3-(4-trifluoro-methylbenzyloxy)-2*H*-1-benzopyran (7m). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.58 (d, 2H, J = 8.1 Hz), 7.43 (d, 2H, J = 8.1 Hz), 7.34-7.31 (m, 5H), 7.23 (d, 2H, J = 8.1 Hz), 7.11 (d, 2H, J = 8.1 Hz), 6.66-6.56 (m, 3H), 4.93 (d, 1H, J = 11.8 Hz), 4.74(d, 1H, J = 11.8 Hz), 4.67 (s, 2H), 4.63 (d, 1H, J = 7.4 Hz), 4.15 (s, 2H), 3.69 (d, 1H, J = 7.4 Hz), 2.31 (s, 3H), 1.44 (s, 3H), 1.27 (s, 3H); LC/MS (ESI) *m/z* 562 $[M+H]^+$.

4-Benzyloxy-3-(2-chlorobenzyloxy)-2,2-dimethyl-6-(4-methoxybenzylamino)-2*H*-1-benzopyran (7n). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.50-7.09 (m, 12H), 6.91 (dd, 1H, J = 8.7 Hz, J = 2.4 Hz), 6.79-6.69 (m, 3H), 4.95 (d, 1H, J = 12.6 Hz), 4.75 (d, 1H, J = 12.6 Hz), 4.72 (s, 2H), 4.59 (d, 1H, J = 6.7 Hz), 4.12 (s, 2H), 3.67 (s, 3H), 3.66 (d, 1H, J = 6.7 Hz), 1.43 (s, 3H), 1.31 (s, 3H); LC/MS (ESI) *m/z* 544 $[M+H]^+$.

3-Benzyloxy-4-(3-chlorobenzyloxy)-2,2-dimethyl-6-(4-methoxybenzylamino)-2*H*-1-benzopyran (7o). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.34-7.20 (m, 11H), 6.87-6.85 (m, 2H), 6.65 (d, 1H, J = 8.7 Hz), 6.60-6.54 (m, 2H), 4.85 (d, 1H, J = 11.8 Hz), 4.71-4.62 (m, 4H), 4.13 (s, 2H), 3.78 (s, 3H), 3.68 (d, 1H, J = 7.3 Hz), 1.44 (s, 3H), 1.26 (s, 3H); LC/MS (ESI) *m/z* 544 $[M+H]^+$.

4-Benzyloxy-3-(4-bromo-2-fluorobenzyloxy)-2,2-dimethyl-6-(4-methoxybenzylamino)-2*H*-1-benzopyran (7p). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.36-7.21 (m, 10H), 6.85 (d, 2H, J = 8.8 Hz), 6.69-6.59 (m, 3H), 4.85 (d, 1H, J = 12.0 Hz), 4.73-4.66 (m, 3H), 4.59 (d, 1H, J = 7.1 Hz), 4.13 (s, 2H), 3.78 (s, 3H), 3.69 (d, 1H, J = 7.1 Hz), 1.42 (s, 3H), 1.24 (s, 3H); LC/MS (ESI) *m/z* 606 $[M+H]^+$, 608 $[M+2+H]^+$.

4-Benzyloxy-2,2-dimethyl-6-(4-methoxybenzylamino)-3-(naphthalene-2-ylmethoxy)-2*H*-1-benzopyran (7q). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.86-7.77 (m, 4H), 7.51-7.44 (m, 3H), 7.36-7.19 (m, 7H), 6.86 (d, 2H, J = 8.5 Hz), 6.71 (d, 1H, J = 2.3 Hz), 6.67-6.59 (m, 2H), 5.04 (d, 1H, J = 11.5 Hz), 4.88 (d, 1H, J = 11.5 Hz), 4.73 (d, 2H, J = 5.0 Hz), 4.66 (d, 1H, J = 6.7 Hz), 4.14 (s, 2H), 3.78 (s, 3H), 3.76 (d, 1H, J = 6.7 Hz), 1.46 (s, 3H), 1.31 (s, 3H); LC/MS (ESI) *m/z* 560 $[M+H]^+$.

4-Benzyloxy-2,2-dimethyl-6-(4-methoxybenzylamino)-3-pentyloxy)-2*H*-1-benzopyran (7r). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.41-7.28 (m, 5H), 7.22 (d, 2H, J = 8.6 Hz), 6.81 (d, 2H, J = 8.6 Hz), 6.78-6.65 (m, 3H), 4.79 (d, 1H, J = 11.0 Hz), 4.73 (d, 1H, J = 11.0 Hz), 4.49 (d, 1H, J = 7.2 Hz), 4.12 (s, 2H), 3.83-3.76 (m, 1H), 3.75 (s, 3H), 3.60-3.56 (m, 1H), 3.47 (d, 1H, J = 7.2 Hz), 1.60-1.57 (m, 2H), 1.43 (s, 3H), 1.40-1.26 (m, 4H), 1.24 (s, 3H), 0.93-0.87 (m, 3H); LC/MS (ESI) *m/z* 490 $[M+H]^+$.

4-Benzyloxy-6-(4-methoxybenzylamino)-2,2-dimethyl-3-(prop-2-ynyloxy)-2*H*-1-benzopyran (7s). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.43-7.30 (m, 5H), 7.08 (d, 2H, J = 8.6 Hz), 7.06 (d, 1H, J = 2.3 Hz), 6.73 (d, 1H, J = 8.8 Hz), 6.69 (d,

2H, J = 8.6 Hz), 6.39 (m, 1H), 4.74 (d, 1H, J = 11.2 Hz), 4.73 (d, 1H, J = 11.2 Hz), 4.48 (d, 1H, J = 6.1 Hz), 4.32 (s, 2H), 4.11 (s, 2H), 3.72 (d, 1H, J = 6.1 Hz), 3.61 (s, 3H), 2.46 (m, 1H), 1.44 (s, 3H), 1.29 (s, 3H); LC/MS (ESI) *m/z* 458 $[M+H]^+$.

3-(But-2-en-1-oxy)-4-methoxy-6-(4-methoxybenzylamino)-2,2-dimethyl-2*H*-1-benzopyran (7t). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.29 (d, 2H, J = 8.5 Hz), 6.87 (d, 2H, J = 8.5 Hz), 6.66-6.62 (m, 2H), 6.52 (dd, 1H, J = 8.1 Hz, J = 2.4 Hz), 5.73-5.63 (m, 2H), 4.35-4.19 (m, 5H), 3.80 (s, 3H), 3.50 (s, 3H), 3.49 (d, 1H, J = 7.3 Hz), 1.72 (d, 3H, J = 5.7 Hz), 1.40 (s, 3H), 1.21 (s, 3H); LC/MS (ESI) *m/z* 398 $[M+H]^+$.

2,2-Dimethyl-4-methoxy-6-(4-methoxybenzylamino)-3-(prop-2-ynyloxy)-2*H*-1-benzopyran (7u). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.28 (d, 2H, J = 8.6 Hz), 6.86 (d, 2H, J = 8.6 Hz), 6.67-6.55 (m, 3H), 4.45-4.40 (m, 3H), 4.19 (s, 2H), 3.79 (s, 3H), 3.73 (d, 1H, J = 7.3 Hz), 3.47 (s, 3H), 2.45 (m, 1H), 1.43 (s, 3H), 1.22 (s, 3H); LC/MS (ESI) *m/z* 382 $[M+H]^+$.

2,2-Dimethyl-3-(3-fluorobenzyloxy)-4-methoxy-6-(4-methoxybenzylamino)-2*H*-1-benzopyran (7v). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.31-7.26 (m, 6H), 6.86 (d, 2H, J = 8.6 Hz), 6.67 (d, 1H, J = 2.2 Hz), 6.65 (d, 1H, J = 8.5 Hz), 6.53 (dd, 1H, J = 8.5 Hz, J = 2.2 Hz), 4.88 (d, 1H, J = 11.0 Hz), 4.69 (d, 1H, J = 11.0 Hz), 4.41 (d, 1H, J = 7.3 Hz), 4.20 (s, 2H), 3.79 (s, 3H), 3.60 (d, 1H, J = 7.3 Hz), 3.45 (s, 3H), 1.42 (s, 3H), 1.24 (s, 3H); LC/MS (ESI) *m/z* 452 $[M+H]^+$.

3-(4-*tert*-Butylbenzyloxy)-2,2-dimethyl-4-methoxy-6-(4-methoxybenzylamino)-2*H*-1-benzopyran (7w). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.39 (d, 2H, J = 8.3 Hz), 7.30 (d, 2H, J = 8.3 Hz), 7.20 (d, 2H, J = 8.8 Hz), 7.13 (d, 1H, J = 2.2 Hz), 6.90 (dd, 1H, J = 8.5 Hz, J = 2.2 Hz), 6.78 (d, 1H, J = 8.8 Hz), 6.69 (d, 1H, J = 8.5 Hz), 4.85 (d, 1H, J = 11.2 Hz), 4.66 (d, 1H, J = 11.2 Hz), 4.31 (d, 1H, J = 6.9 Hz), 4.17 (s, 2H), 3.72 (s, 3H), 3.56 (d, 1H, J = 6.9 Hz), 3.50 (s, 3H), 1.40 (s, 3H), 1.32 (s, 9H), 1.24 (s, 3H); LC/MS (ESI) *m/z* 490 $[M+H]^+$.

3-(4-Bromo-2-fluorobenzyloxy)-4-methoxy-6-(4-methoxybenzylamino)-2,2-dimethyl-2*H*-1-benzopyran (7x). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.34-7.22 (m, 5H), 6.86 (d, 2H, J = 8.7 Hz), 6.71-6.60 (m, 3H), 4.89 (d, 1H, J = 11.9 Hz), 4.72 (d, 1H, J = 11.9 Hz), 4.39 (d, 1H, J = 7.2 Hz), 4.20 (s, 2H), 3.80 (s, 3H), 3.59 (d, 1H, J = 7.2 Hz), 3.47 (s, 3H), 1.40 (s, 3H), 1.21 (s, 3H); LC/MS (ESI) *m/z* 530 $[M+H]^+$.

3-(3-Chlorobenzyloxy)-2,2-dimethyl-4-methoxy-6-(4-methoxybenzylamino)-2*H*-1-benzopyran (7y). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.41-7.27 (m, 6H), 6.87 (d, 2H, J = 9.0 Hz), 6.66 (d, 1H, J = 2.8 Hz), 6.65 (d, 1H, J = 8.8 Hz), 6.53 (dd, 1H, J = 8.8 Hz, J = 2.8 Hz), 4.88 (d, 1H, J = 11.0 Hz), 4.69 (d, 1H, J = 11.0 Hz), 4.40 (d, 1H, J = 7.3 Hz), 4.20 (s, 2H), 3.80 (s, 3H), 3.60 (d, 1H, J = 7.3 Hz), 3.46 (s, 3H), 1.41 (s, 3H), 1.23 (s, 3H); LC/MS (ESI) *m/z* 468 $[M+H]^+$.

3-Allyloxy-2,2-dimethyl-4-methoxy-6-(4-methoxybenzylamino)-2*H*-1-benzopyran (7z). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.36 (d, 1H, J = 2.6 Hz), 7.14 (d, 2H, J = 8.7 Hz), 7.08 (dd, 1H, J = 8.6 Hz, J = 2.6 Hz), 6.73 (d, 2H, J = 8.7 Hz), 6.72 (d, 1H, J = 8.6 Hz), 6.03-5.87 (m, 1H), 5.30 (dd, 1H, J = 17.3 Hz, J = 1.6 Hz), 5.20 (dd, 1H, J = 10.4 Hz, J = 1.6 Hz), 4.32-4.11 (m, 5H), 3.68 (s, 3H), 3.54 (s, 3H), 3.43 (d, 1H, J = 7.1 Hz), 1.40 (s, 3H), 1.24 (s, 3H); LC/MS (ESI) *m/z* 384 $[M+H]^+$.

Biological Assay

Cell Culture. HSC-T6 cells, an immortalized rat hepatic stellate cell line, which have stable phenotype and biochemical characteristics, were kindly provided by Dr. Friedman SL (Mount Sinai School of Medicine, NY). LI 90 cells, human hepatic mesenchymal tumor cells, were purchased from Japan Health Sciences Research Resources Bank (Osaka, Japan). Both cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco-BRL, Gaithersburg, MD, USA), 100 IU/mL penicillin and 50 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 + 95% air.

Cytotoxicity. The cells were applied to a 96 well microplate at an initial loading of 2×10^5 cells per well for HSC-T6 cells and 1×10^4 cells per well for LI 90 cells. After 24 h cells were added to the serum free DMEM containing various concentrations of the organic substances, penicillin (100 units/mL) and streptomycin (100 μg/mL), incubated for 24 h and then diluted with MTS solution (Cell Titer 96 non-radioactive cell proliferation assay kit, Promega). The plate was incubated under 5% CO_2 at 37 °C for 90 min and the absorbance was measured by using a Molecular Dynamics plate reader at 490 nm.

Hydroxyproline Content. The organic substances were added to HSC-T6 cells in the 100 mm plate and the resulting mixtures were allowed to stand for 24 h. The hydroxyproline content in HSC-T6 cells was then determined to assess the collagen changes in the manner previously described.[7]

Assay of type I Collagen in the Media Using ELISA Method. LI 90 cells were incubated with serum free DMEM containing various concentrations of the organic substances for 48 h and the supernatants were collected and assayed for type I collagen contents using the ELISA method. Medium samples (100 μL) were coated in 96-well plates in carbonate-bicarbonate buffer at 4 °C and allowed to stand overnight. After aspirating, each well was incubated with 200 μL skim milk buffer at room temperature for 4 h. After washing 3 times with PBS containing 0.05% Tween 20, each well was incubated with 100 μL of 1:4000 dilution of rabbit polyclonal antibody to type I collagen (AB Chem Co., UK) in PBS at 37 °C for 2 h. After washing 3 times with PBST, each well was incubated with 100 μL of 1:8000 dilution of goat anti-rabbit IgG-HRP at 37 °C for 1 h. Then 40 μL TMB, 4 μL of 3% H_2O_2 and 4 mL of 50 mM sodium acetate (pH 5.1) were added for 15 min at room temperature. The reaction mixtures were quenched by adding 1 M H_2SO_4, and the plate was read by using a microplate reader (model 3550-UV, BioRad, USA) at 450 nm.

Supplementary Information. Analytical data ^{1}H NMR of the entire compounds; ^{13}C NMR spectra of representative

compounds **7b**, **7g**, and **8a**, and HRMS of **7b** and **8a** and analytical data (LC/MS) **7g**, **7h**, **7j**, **7o**, and **7p**.

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