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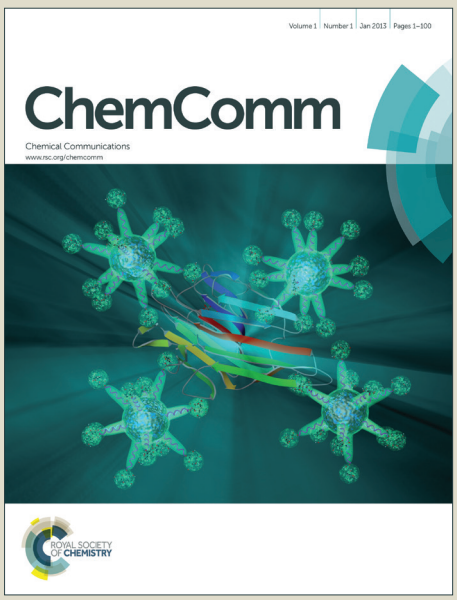

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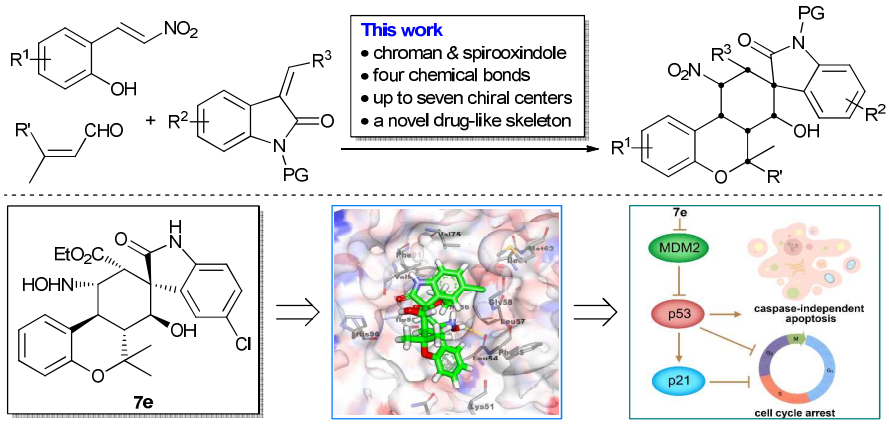

Enantioselective preparation of pharmacologically interesting chroman-fused spirooxindole derivatives is described. The most potent compound **7e** induced caspase-independent apoptosis and cell cycle arrest in MCF-7 breast cancer cells by interfering with the p53-MDM2 interaction and downstream pathways.

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Organocatalytic cascade reaction for asymmetric synthesis of novel chroman-fused spirooxindoles that potently inhibit cancer cell proliferation†

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Enantioselective preparation of pharmacologically interesting chroman-fused spirooxindole derivatives is described based on an organocatalytic multicomponent cascade reaction. Compounds synthesized using this method potently inhibited proliferation of various cancer cell lines. The most potent compound (7e) induced caspase-independent apoptosis and cell cycle arrest in MCF-7 breast cancer cells by interfering with the p53-MDM2 interaction and downstream pathways.

Asymmetric organocatalytic domino/cascade reactions can assemble simple starting materials into complex chiral frameworks in high yield and often with excellent stereoselectivity under environmentally friendly conditions.[1] Such cascade reactions have become increasingly important for total synthesis,[2] natural product synthesis[3] and construction of chiral molecular libraries.[4] Nevertheless, many possibilities remain unexplored for harnessing organocatalytic cascade reactions to generate optically active molecules for medicinal chemistry.[5]

Recent years have seen intense efforts to develop organocatalytic multicomponent cascade reactions to form highly optically active derivatives of chroman[6] or spiro-oxindole.[7] Chiral chroman and spirooxindole scaffolds are considered "privileged structures" because they occur widely in biologically active natural products and pharmaceuticals (Fig. 1). For example, the chroman ring is present in tocopherols and tocotrienols, two subgroups of the vitamin E family. These compounds show promise for adjuvant drug treatment of cancer.[8] Recently, the spirooxindole derivative MI-77301 was found to inhibit murine double minute 2 (MDM2), and the compound entered its second Phase I clinical trial in 2013.[9] We

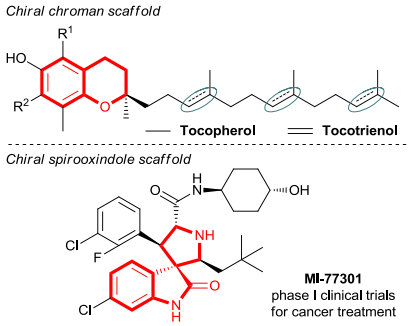

Fig. 1 Examples of chiral chroman and spirooxindole scaffolds in natural product and therapeutics.

wondered whether using organocatalytic multicomponent cascade reactions to combine chroman and spirooxindole motifs might generate novel anticancer drug candidates. To our knowledge, the asymmetric synthesis of chroman-fused spirooxoindoles has never been reported.[10]

As part of our on-going interest in developing efficient methodologies for stereoselective assembly of multiple substrates into synthetically important cyclic molecules,[11] we hypothesized that the drug-like spirooxindole chroman scaffold could be generated via a four-step organocatalytic relay cascade (Scheme 1). The protocol would begin with a secondary amine-catalyzed tandem oxa-Michael-Michael reaction of **1** and **2** to yield **3**. It is worth noting that the β,β-disubstituted enal **2** was chosen especially. This is because the quaternary carbon center at the 2-position occurs widely in a variety of natural chromans exhibiting various biological activities.[12] Meanwhile, the branched **2** bearing steric hindrance could decrease the side reaction of intermediate **3** and excess enal.[6a,h,i] The resulting chiral intermediate **3** would then participate directly in the second catalytic cycle by serving as the donor to induce asymmetric Michael reaction with the electron-deficient olefinic oxindole **4**. Subsequent cyclization via an intramolecular Aldol condensation would form the desired product **5**.

Here we report our development of this cascade approach which allowed the enantioselective synthesis of chroman-

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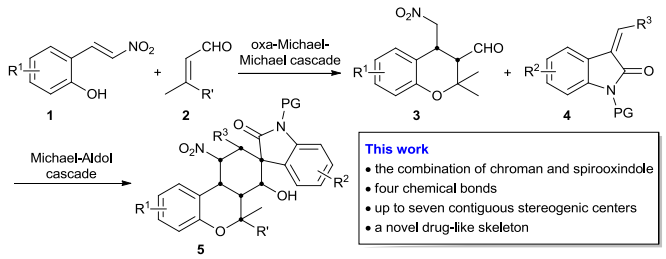
‡ These authors contributed equally to this work.



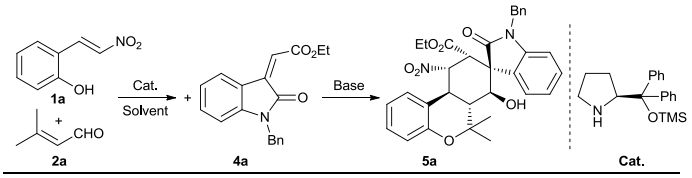
Scheme 1 Asymmetric synthesis of chiral chroman-fused spirooxindoles via organocatalytic multicomponent cascade reaction

fused spirooxindoles with six contiguous stereogenic centers and diverse substitutions. We also evaluated the ability of these compounds to inhibit the proliferation of various cancer cell lines. Our hope is that this molecular library will serve as the basis for developing antitumor drugs.

We selected 2-nitrovinyl phenol (**1a**), 3-methyl-2-butenal (**2a**) and olefinic oxindole (**4a**) as the model substrates to examine the feasibility of our approach. The first oxa-Michael-Michael reaction proceeded in the presence of Hayashi-Jørgensen proline-type catalyst (10 mol%) in toluene at room temperature for 3 h. Adding a toluene solution of oxindole derivative **4a** and triethylamine in a one-pot operation led to successive Michael and aldol reactions. To our satisfaction, the tandem reaction proceeded smoothly to afford the desired product **5a** with good diastereoselectivity and excellent enantioselectivity, albeit in moderate yield (Table 1, entry 1).

Screening of reaction conditions showed that the reaction medium was crucial for improving efficiency (entries 2-4), with acetonitrile emerging as the best choice. Different bases led to different diastereoselectivities in the second Michael-Aldol step (entries 5-7), with K_2CO_3 giving the best results. We further improved yield and enantioselectivity by lowering the reaction temperature, which led to 64% yield of **5a** with 99% ee (entry 8). The absolute configuration of **5a** was assigned as 9S,10R,11S,12S,13R,14S based on X-ray crystallographic analysis.[13]

Table 1 Optimization of reaction conditions[a]

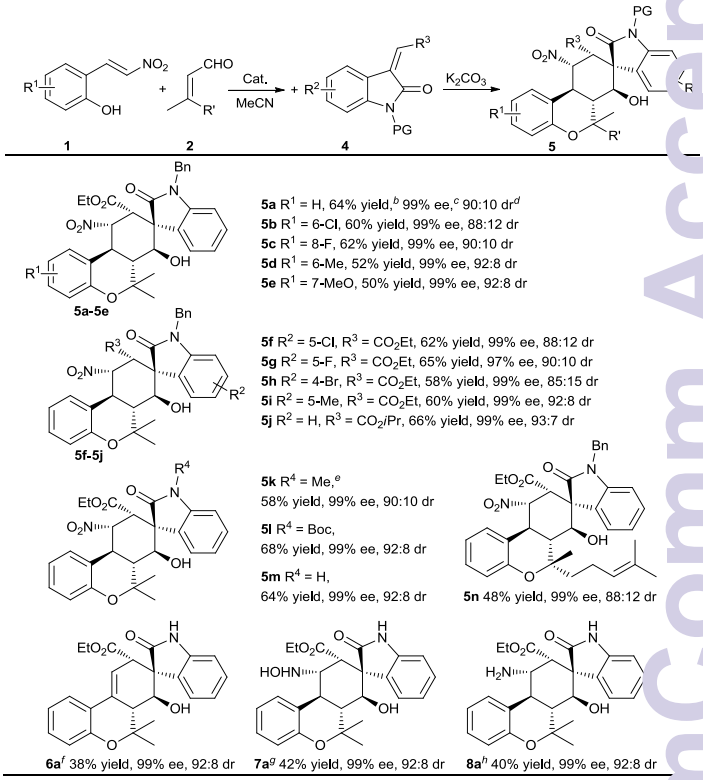


Entry	Solvent	Base	Yield[b] (%)	dr[c]	ee[d] (%)
1	toluene	TEA	45	80:20	96
2	CH_2Cl_2	TEA	38	75:25	96
3	THF	TEA	40	78:22	97
4	MeCN	TEA	55	82:18	97
5	MeCN	DBU	55	70:30	95
6	MeCN	NaOH[e]	48	80:20	96
7	MeCN	K_2CO_3[e]	58	88:12	97
8[f]	MeCN	K_2CO_3[e]	64	90:10	99

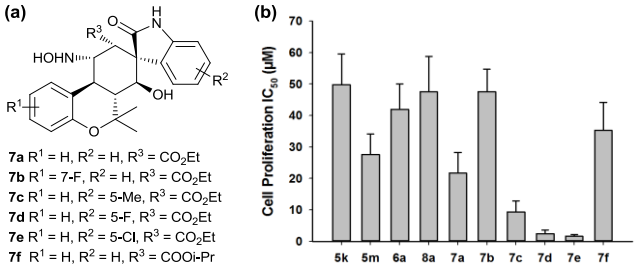
[a] Unless noted otherwise, reactions were performed with 0.5 mmol of **1a**, 0.5 mmol of **2a**, 10 mol% of catalyst and AcOH in 2 mL solvent at rt for 3 h, after which **4a** (0.4 mmol) and base (0.2 mmol) were added. [b] Isolated yield of pure diastereomer **5a**. [c] Based on ^{1}H NMR analysis of the crude reaction mixture. [d] Based on chiral HPLC analysis. [e] 0.2 mmol of base in 0.4 mL H_2O. [f] Reaction performed at 0 °C.

With these optimized conditions in hand, we examined the substrate scope for asymmetrically synthesizing poly-substituted chroman-fused spirooxindoles (Table 2). All reactions progressed smoothly to give the corresponding products **5** in moderate to good yields (up to 68%) with high diastereoselectivities (dr > 85:15) and excellent enantioselectivities (up to 99% ee). The nature of the functional group at R^1 in substrate **1** slightly affected the reaction. Products were generated in higher yield from 2-nitrovinyl phenols bearing neutral or electron-withdrawing groups (**5a**-**5c**) than from those bearing electron-donating groups (**5d**-**5e**). On substrate **2**, the functional group at R^2 on the oxindole core and the ester group at R^3 at the double bond slightly affected the reaction (**5f**-**5j**). Various protecting groups were well tolerated, such as benzyl, methyl and Boc groups (**5k** and **5l**), and the Boc group of **5l** was easily removed to afford **5n**. Using the asymmetric β,β-disubstituted enal as substrate allowed us to construct a new chiral center at the 2-position of the chroman core (**5n**).[14] To optimize the drug potential of these compounds according to Lipinski's rules, we transformed the nitro group and removed the protecting group, thereby ensuring a reasonable number of hydrogen bond receptors / donors and a favorable lipo-hydro partition coefficient. The elimination product (**6a**) and selective reduction products (**7a** and **8a**) were successfully produced.

Table 2 Studies of cascade reaction scope[a]



[a] See entry 8 and footnote *a* in Table 1. [b] Isolated yield of pure diastereomer **5**. [c] Based on chiral HPLC analysis. [d] Based on ^{1}H NMR analysis of the crude reaction mixture. [e] The absolute configuration of **5k** was determined by X-ray analysis, and other products were assigned by analogy. [f] **5l** was reacted with DBU in DMF at 60 °C for 4 h, after which the Boc group was removed to yield **6a**. [g] **5m** was reduced with Fe and NH_4Cl at 40 °C for 1 h to yield **7a**. [h] **5m** was reduced with Fe and NH_4Cl at 60 °C for 5 h to yield **8a**.



7a R^1 = H, R^2 = H, R^3 = CO_2Et
7b R^1 = 7-F, R^2 = H, R^3 = CO_2Et
7c R^1 = H, R^2 = 5-Me, R^3 = CO_2Et
7d R^1 = H, R^2 = 5-F, R^3 = CO_2Et
7e R^1 = H, R^2 = 5-Cl, R^3 = CO_2Et
7f R^1 = H, R^2 = H, R^3 = COOi-Pr

Fig. 2 (a) Structures of compounds **7a-7f**. (b) Comparison of antiproliferative activity of selected compounds on MCF-7 cells.

Having synthesized a variety of chiral chroman-fused spirooxindole derivatives, we performed preliminary studies of their anticancer activity. Cancer cell lines A549, HepG2, MCF-7, HCT116 and U87 were treated for 48 h with the compounds in Table 2 at various concentrations up to 50 μM, and cell viability was determined by MTT assay (see Supplementary Tables S1-2). MCF-7 breast cancer cells showed the greatest sensitivity to most of the compounds: **5k**, **5m**, **6a**, **7a** and **8a** inhibited MCF-7 proliferation in a time- and dose-dependent manner, showing nearly 50% inhibition at respective concentrations (μM) of 49.8, 27.6, 41.9, 21.8 and 47.5. These results suggest that removing the protecting group and converting the nitro group to hydroxylamine (**7a**) significantly enhance the library's antitumor activity. Hydroxylamine derivatives **7b**-**7f** were prepared for further screening (Fig. 2). These compounds showed moderate to good antiproliferative activity in MCF-7 cultures; **7e** showed the lowest IC_{50} value at 1.7 μM.

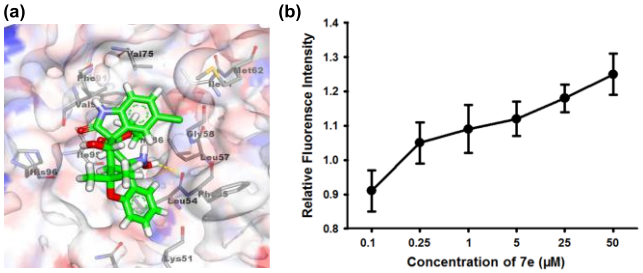


Fig. 3 (a) Potential modes of **7e** binding to MDM2; side chains of key binding residues in the docked complex are shown. (b) Competition assay in which increasing **7e** concentrations were monitored for their ability to block binding of a fluorescent probe to the p53-MDM2 complex.

Since many spirooxindole derivatives have been shown to exert their antitumor activity by inhibiting MDM2,[15] we wondered whether the same would be true of chroman-fused spirooxindole **7e**. First, we performed molecular docking studies to examine the possibility that the compound might bind in the enzyme active site. The docking studies suggest that it is feasible that the chroman fragment might deeply bind to the active site through hydrophobic interactions and π-π stacking with side chains on an alpha-helix (Leu54-Tyr67) and in part of a beta-sheet (Phe91-His96) (Fig. 3a). The hydroxyl-amine fragment might also form hydrogen bonds with the main chain atoms of Leu54 (Fig. 3a). Consistent with the possibility of **7e** binding to MDM2, **7e** competed with the binding of a fluorescent probe[16] to the p53-MDM2 complex in the cytoplasm of MCF-7 cells (Fig. 3b and S1). This suggests that **7e** may efficiently interfere with the p53-MDM2 interaction, like the known MDM2 inhibitor Nutlin-3.

To explore in greater detail the potential mechanism(s) by which **7e** may exert antitumor activity, its effects on cell cycle progression, morphology, and expression of p53, MDM2 and apoptotic markers were examined in MCF-7 cells using flow cytometry, fluorescence microscopy and immunoblotting. Treating MCF-7 cells with a low concentration of either **7e** or Nutlin-3 caused slight arrest in G2 phase, while treating them with a high concentration of either compound caused arrest in both G2 and S phases (Fig. 4a and S2). Propidium iodide (PI) staining of MCF-7 cells after exposure to **7e** or Nutlin-3 revealed morphological evidence of mitotic inhibition and apoptotic body formation (Fig. S3).[17] Flow cytometry analysis using Annexin V/PI showed that **7e** substantially stimulated MCF-7 apoptosis, much more than Nutlin-3 did (Fig. 4b). Immunoblotting for several proteins of the p53 pathway, such as MDM2, p53, p21 and Caspase-3, in MCF-7 cells after exposure to **7e** showed marked down-regulation of MDM2 expression and up-regulation of p53 and p21 (Fig. 4c). In fact, **7e** appeared more effective than Nutlin-3 at up-regulating p53 and p21. Simultaneous up-regulation of p53 and down-regulation of MDM2 is consistent with the idea that **7e** may interfere with the p53-MDM2 interaction, and up-regulation of p21 may induce cell cycle arrest (Fig. 4d).

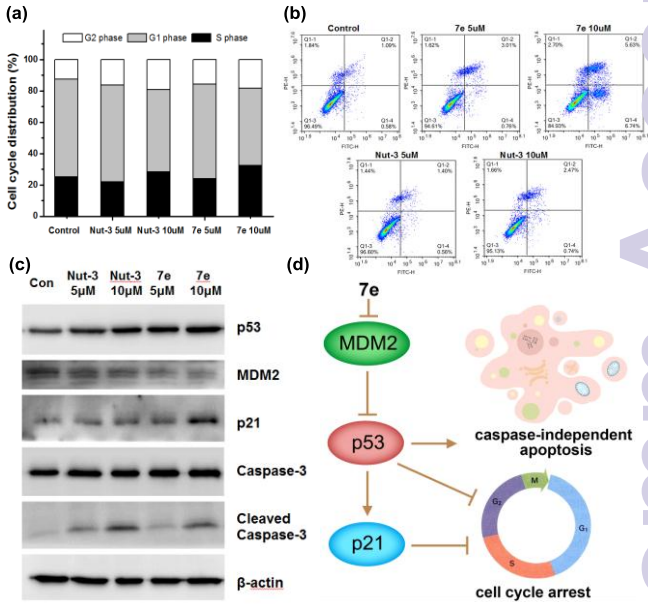


Fig. 4 (a) Cell cycle analysis of MCF-7 breast cancer cells treated with **7e** or Nutlin-3. (b) Compound **7e** induced a greater degree of apoptosis than Nutlin-3 in MCF-7 cells. (c) Comparison of the ability of **7e** and Nutlin-3a to down-regulate MDM2, up-regulate p53 and p21, and trigger caspase-3 cleavage in MCF-7 cells. (d) A model of how **7e** may induce apoptosis and cell cycle arrest in MCF-7 cells.

Immunoblotting experiments showed that Nutlin-3 triggered caspase-3 cleavage more strongly than **7e** did. To examine more directly whether **7e**-induced cell death involved caspase, MCF-7 cells were pretreated with the pan-caspase inhibitor Z-VAD-FMK (5 μM), then incubated with **7e** (Fig. S4).

Z-VAD-FMK pretreatment did not reduce **7e**-induced apoptotic cell death. These results suggest that **7e** induces apoptosis of MCF-7 cells in a caspase-independent manner.

In conclusion, we have developed a flexible and simple organocatalytic cascade reaction involving an oxa-Michael-Michael-Michael-Aldol relay, and we have used it to assemble a functionalized chiral chroman-fused spirooxindole scaffold. Starting from 2-nitrovinyl phenol, β,β-disubstituted enal and olefinic oxindole, we obtained a library of bioactive products in moderate to good yield with high stereoselectivity. Member **7e** of this library showed the most potent antitumor activity, and docking studies with MDM2 as well as culture experiments suggest that **7e** may interfere with the interaction between p53 and MDM2, leading to p53 up-regulation and ultimately to caspase-independent apoptosis. Flow cytometry and immunoblotting experiments also suggest that **7e** induces G2/S cell cycle arrest by up-regulating p53 and p21. These findings indicate that chiral chroman-fused spirooxindoles may serve as a novel scaffold for building small-molecule inhibitors of the p53-MDM2 interaction with potential for cancer chemotherapy.

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