Review

Approaches to Neural Stem Cells and Cancer Cells Based on Natural Products

Midori A. Arai

Graduate School of Pharmaceutical Sciences, Chiba University; 1–33 Yayoi-cho, Inage-ku, Chiba 263–8522, Japan.
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We have developed "protein- and cell-based screen methods" for the isolation of new natural products. By using VDR (vitamin D receptor) immobilized magnetic beads, two new natural products were isolated rapidly. To find inhibitors of Hes1 protein, which is one of the important transcriptional factors in neural stem cells, a Hes1 dimer plate assay was developed, and then first Hes1 dimer inhibitors were found from our natural products library. As a "cell-based screen method," a reporter gene assay for screening Hh (hedgehog) signaling inhibitors was constructed. New Hh signaling inhibitors were isolated from our natural extracts library. We evaluated their Hh inhibitory activity in Hh related protein synthesis and cytotoxicity against cancers, in which Hh signaling is aberrantly activated. In addition to the naturally made compounds library (extracts library), "small molecules based on natural products" were synthesized. The total synthesis of Melleumin A, B, which were isolated by our group, was achieved. From their synthetic derivatives, inhibitors of the Wnt signal, which has been reported to cause colon cancers, were discovered. Moreover, flavanone and chromone were selected as natural product scaffolds. Many flavonoids and chromones with diverse heterocyclic units were constructed using our efficient synthetic method.

Key words natural product; cancer cell; neural stem cell; protein magnetic bead; hedgehog signaling; Wnt signaling

Introduction

Isolation and identification of novel naturally occurring bioactive products has played an important role in drug discovery. Because most new drugs are related to natural products or related compounds,[1] discovery of the active compounds based on natural products would be a powerful way to approach to human diseases.

We have developed a "protein-based screen method" for the quick isolation of new natural products. Targeting of drugs to specific "proteins," for the treatment of specific diseases, is also of great clinical importance. On the other hand, isolation by the guided biological activity of a "cell-based screen method" is also an attractive method for obtaining desired phenotypes by small molecules. A reporter gene assay using cells has been constructed allowing the isolation of new hedgehog (Hh) signaling inhibitors, which is an aberrant signal in several type of cancers.

Synthesis of small molecules based on their scaffolds would be of chemical relevance to living cells and organs, protein binding, absorption, distribution, metabolism, and excretion.[2,3] We are interested in making "synthetic small molecules based on natural products." Small molecules which have a scaffold of natural products would have a big chance to be candidates as new drugs. To create such bioactive molecules, we focused on the new natural products, Melleumin A, B, which were isolated by our group. Flavonoids which were known to have broad bioactivity were also used as a template.

This review describes our recent efforts in approaches to cancer cells and neural stem cells, by the above three core: "protein based screen," "cell based screen," and "naturally made or synthetic compounds library." (Fig. 1)

1. Protein-Based Approaches

1.1. A Method for the Rapid Discovery of Naturally Occurring Products Using Proteins Immobilized on Magnetic Beads and Reverse Affinity Chromatography[4]

There is a strong demand for the "quick" discovery of novel natural products which are potential candidates for new medicines. To this end, we developed a method for the rapid discovery of natural products using protein magnetic beads. By using a combination of protein immobilized beads, followed by HPLC analysis, the time required to isolate of target compounds can be greatly shortened. Magnetic nanoparticles have been widely utilized in a range of fields including biology, chemistry, and molecular imaging.[5] Immobilization of proteins on magnetic beads has facilitated protein purification,[6] the detection of protein–protein interactions,[7] cancer-cell detection (using immobilized antibody),[8] and isolation of the target protein of small molecules.[9] Handa *et al.* have described a method for the separation of FK506 from extracts by using FKBP (FK506 binding protein) immobilized magnetic beads.[10]

Magnetic beads are particularly useful for the immobilization of proteins, as bead-bound compounds can be easily separated from other soluble components by using a magnet, thereby lending themselves to high-throughput system (HTS) applications. Compared with the many centrifuge steps that would be required for separation if sepharose beads were used, the simple collection of magnetic beads on the wall of

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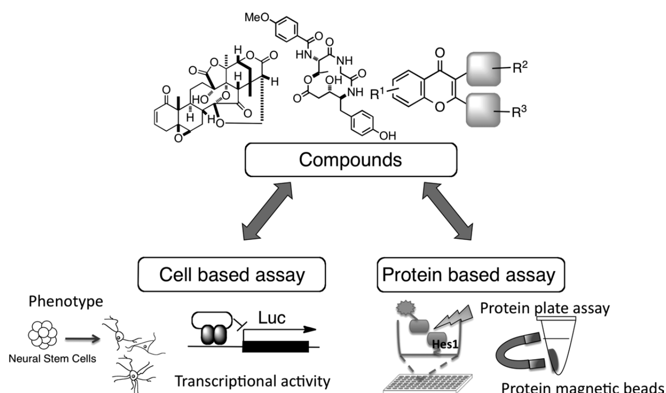


Fig. 1. Approaches to Stem Cells and Cancer Cells Based on Natural Products

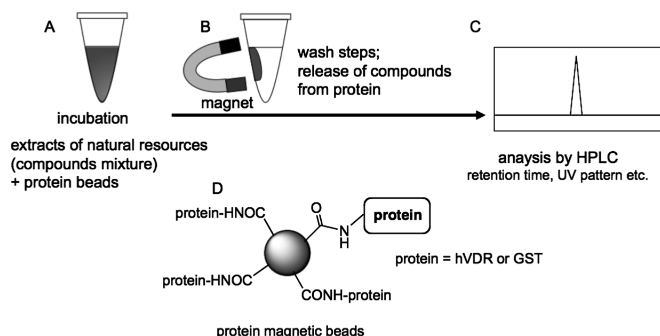


Fig. 2. Protocol for Screening of Natural Compounds by Protein Beads

(A) Incubation of the extracts of natural resources (a mixture of compounds) with protein beads; (B) washing of the beads using a magnet and release of the compounds bound to the bead-bound-protein; (C) analysis of the released compounds by HPLC; (D) outline of the protein magnetic beads.

the tube by use of a magnet, followed by removal of the supernatant, is a much easier and far less time-consuming method (Fig. 2). To establish a method for the isolation of naturally occurring small molecules from the complex mixtures composed of extracts of natural resources (such as plants, marine organisms, actinomycetes, mold and myxomycetes), we first selected human vitamin D receptor (hVDR), a member of the superfamily of nuclear receptors, as the target protein. This receptor and its ligands have been well studied. It is known that 1α,25-dihydroxyvitamin D_3 [$1\alpha,25(OH)_2VD_3$] (compound **1**) is an endogenous cellular ligand of hVDR, and that vitamin D_3 (VD_3) (compound **2**), which is the precursor of compound **1**, also binds but with much lower affinity than compound **1**.

hVDR, which was prepared from *Escherichia coli*, was immobilized on COOH-coated magnetic beads (Dynabeads

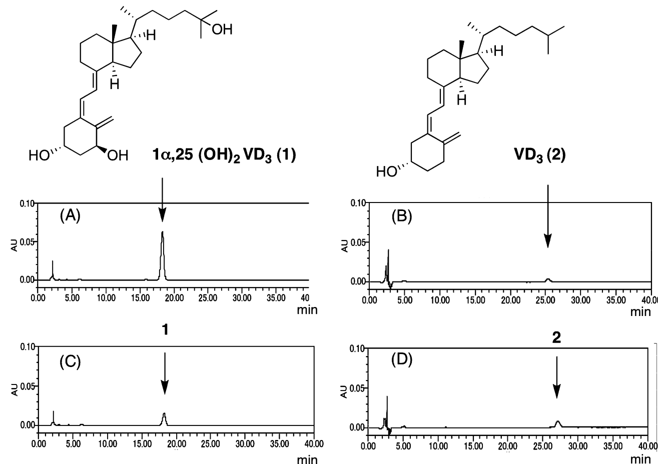


Fig. 3. HPLC Profiles of Compounds Eluted from the Protein Magnetic Beads

(A) $1\alpha,25(OH)_2$ VD_3 (**1**) eluted from hVDR beads. 3.0 nmol of **1** was detected; (B) VD_3 (**2**) eluted from hVDR beads; (C) compound **1** eluted from GST beads; (D) compound **2** eluted from GST beads; all samples are the final EtOH-eluted solution obtained following incubation of compounds **1** or **2** with protein beads, washing of the beads with buffer and release of the bound compound from the bead-bound-protein by EtOH. Assay condition: protein beads (1 mg) in NET-N buffer (225 μl) and compounds (25 nnoml) in EtOH 25 μl were incubated. HPLC condition: column; CAPCELL PAK C18 MGII (5 μm, ϕ4.6×250 mm), CH_3CN+0.1% TFA/H_2O=60/40 (for **1**) or 100/0 (for **2**) with isocratic method, 1 ml/min, UV 254 nm detection.

M-270 carboxylic acid, Invitrogen, Norway) that had been treated with *N*-hydroxy succinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (Fig. 2D). A suspension of hVDR- or glutathione *S*-transferase (GST)-bound magnetic beads and ligand **1** was gently mixed at 4 °C for 2 h (Fig. 2A), after which excess ligand solution was easily removed after the beads were gathered on the wall of the tube using a magnet (Fig. 2B). The HPLC profile of the eluted EtOH solution is shown in Fig. 3A, and compound **1** bound to hVDR can be clearly detected. Compound **1** binding to negative control GST-bound magnetic beads was similarly examined, and showed a very small peak for **1** (Fig. 3C). When binding of the weak compound **2** was assayed, the peak of **2** bound to hVDR was very small, as shown in Fig. 3B, and was similar to the peak obtained following incubation with control GST magnetic beads (Fig. 3D).

Based on this result, we then screened several plant extracts, and determined that an extract of *Limocharis flava* (the aerial part) yielded HPLC peaks following binding to and elution from hVDR beads. We therefore proceeded to purify and identify "peak" as follows. The methanol extract of *L. flava* (2.2 g) was partitioned between H_2O, and *n*-hexane and ethyl acetate. Further purification by consecutive steps of silica gel column chromatography and reversed-

Midori A. Arai, born in 1972 in Miyazaki, Japan. The University of Tokyo (BS, MS, Ph.D. 2000; Prof. M. Shibasaki). JSPS fellow in Prof. Schreiber's group at Harvard University (2001—2002). Special postdoctoral researcher at RIKEN (2003—2004), Assistant Prof. Teikyo University (2004—2006), then Associate Prof. Chiba University (2006—present). She received the Sankyo Award in Synthetic Organic Chemistry, Japan, 2001, the poster award at the 22nd Naito Conference, Japan, 2008, and the Pharmaceutical Society of Japan Award for Young Scientists in 2010. Her current research interests include chemical biology, natural products chemistry, and combinatorial chemistry.

Midori A. Arai

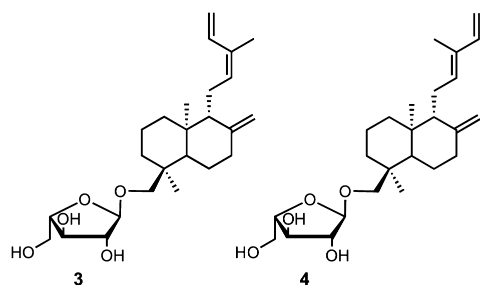


Fig. 4. Chemical Structures of Compounds **3** and **4**

phase HPLC yielded two new labdane type diterpenes (compounds **3** and **4**) (Fig. 4). Assay of the binding of the isolated pure compounds to hVDR beads revealed stronger binding of compound **4** than compound **3**.

A method of screening for naturally occurring products that bind to a specific target protein that is immobilized on magnetic beads was established. This method was proven to be applicable to the identification of new natural products, which interact with a specific protein immobilized on the beads, from an extract of a natural resource. This method has the further advantages that it can identify the HPLC peak corresponding to the target compound for isolation, as well as provide important UV, circular dichroism (CD) or MS profile information.

1.2. Inhibitors of Hes1 Dimer Formation from Natural Products[11] The second example of a "protein-based screen" is the Hes1 dimer plate screen. Recent studies have revealed that the adult mammalian brain has some capacity for neuronal regeneration after insult.[12—14] Therefore, neural stem cells (NSCs) are of great clinical interest regarding the treatment of neural diseases. Basic-helix-loop-helix (bHLH) repressor and activator genes play an essential role in embryogenesis, neurogenesis and the development of many organs.[15,16] bHLH activators, such as Mash1 and E47, control the differentiation of NSCs into neurons by the activation of neural-specific gene expression. Hes factors (Hes1, Hes3 and Hes5) regulate cell proliferation and differentiation in the nervous system, acting as bHLH repressors by either active or passive suppression of bHLH activator activity. Active suppression comprises Hes binding to N box (CACNAG) as homodimers to suppress bHLH activator transcription (Fig. 5). We hypothesized that the inhibition of Hes1 dimer formation would result in the acceleration of bHLH activator transcription, which may generate the differentiation of NSCs into neurons. Small molecules that can modulate the activity of Hes1 would be candidates for a medicine to treat neural disease, especially as 'regenerative medicine' for new neurons. As the precise mechanisms of NSC proliferation and differentiation contain many unknown factors, small molecules that control the roles of bHLH factors are potentially important chemical tools.

We developed a rapid *in vitro* HTS for identifying Hes1 inhibitors using fluorophore-labeled Hes1 and Hes1 immobilized on microplates (Fig. 6). In the presence of an inhibitor compound, the level of Hes1 dimerization was reduced, as detected by a corresponding reduction in fluorescence. Using this system, we identified the first Hes1 dimer inhibitors from our natural products library. Hes1 protein was immobilized on a microplate by chemical linkage. To form the homodimer

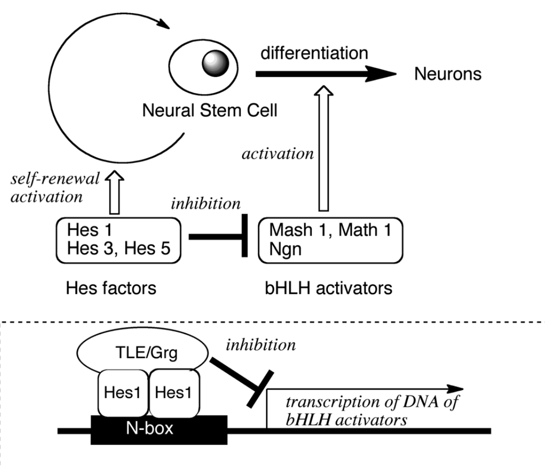


Fig. 5. The Roles of Hes Factors and bHLH Activators in the Differentiation of Neural Stem Cells

Hes factors, which act as bHLH repressors, suppress bHLH activator activity. A Hes1 dimer bound to an N box suppresses the transcription of the bHLH activator.

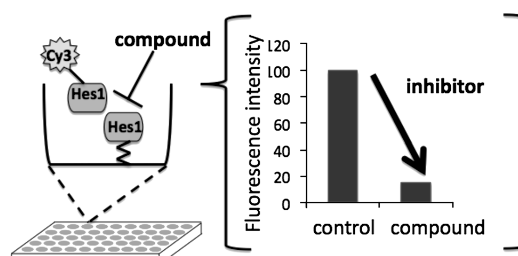


Fig. 6. High-Throughput Screening System for Hes1 Dimer Inhibitors

In the presence of the Hes1 dimer inhibitors, there was decreased fluorescence from dimer complexes between Hes1 bound on the microplate and Cy3-labelled-Hes1.

of Hes1, Cy3-dye-labeled Hes1 was added to the Hes1-immobilized well. After 1 h incubation at room temperature, the well was washed and the fluorescence was measured by a microplate reader. The dimer complex between Hes1 and Cy3-Hes1 was detected successfully. We reported previously on the development of a HTS of vitamin D receptor-co-activator interaction using vitamin D derivatives.[17] In the study, we found the HCHO-fixation method was effective in detecting protein–protein interaction. Although the fluorescence intensity of the Hes1 dimer complex was greatly improved with HCHO-fixation, the risk of structural modification of some natural compounds, especially quinone and hydroquinone type compounds, was observed. Therefore we did not use the HCHO-fixation method in the present study. The compounds of our natural product library were evaluated using this constructed HTS system. This library consists of natural compounds isolated by our research group, including terpenoids, flavonoids, phenylpropanoids, their glycosides and bisindole alkaloids. We identified six natural compounds as inhibitors of Hes1 dimer formation (Fig. 7) with the following IC_{50} values: lindbladione (**5**),[18,19] IC_{50}, 4.1 μM isolated from the myxomycetes *Lindbladia tubulina*; two bisindole alkaloids, lycogarubin B (**6**),[20] 16.5 μM and lycogaric acid A (**7**),[21] 6.0 μM isolated from the myxomycetes *Lycogala epidendrum*; demethyl-2″-epifraxamoside (**8**),[22] 7.8 μM isolated from *Jasminum grandiflorum*; and, two lignan glycosids, lyoniside (**9**),[23] 48.1 μM and nudiposide (**10**),[24] 9.6 μM isolated from

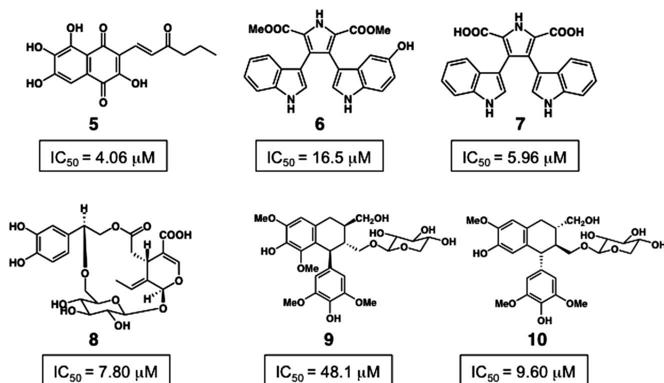


Fig. 7. Chemical Structures of Inhibitors of Hes1 Dimer Formation

Saraca asoca. Compounds **5** to **7** are natural products isolated by the Ishibashi group from myxomycetes (true slime molds), which are an unusual group of primitive organisms that can be assigned to one of the lowest classes of eukaryotes. We subsequently examined the intracellular inhibition activity of these compounds. Hes1 dimer binds to N box sequences in the target promoter of bHLH activators, thus suppressing their transcription. A cell-based reporter assay for measuring the effect on Hes1-induced repression[25] was performed. Of the inhibitors identified by protein-based screening, **5** and **7** showed dose-dependent inhibition of the Hes1-mediated suppression of gene expression. With compounds **5** and **7**, transcription recovered to around 60% at 50 μM.

2. Cell-Based Approaches

To discover the inhibitors of signaling in cancer cells or neural stem cells, we have been trying to create cell based assays, which use a stable cell line with a target gene. For neural stem cells, the promoters of bHLH factor were used to find the modulators of bHLH factors expression. For cancer cells, the aberrant signaling was targeted. In this review, we would like to describe the development of a cell-based assay for Hh signaling inhibitors.

2.1. Discovery of Hh Signaling Inhibitors from Natural Products The hedgehog (Hh)/GLI signaling pathway has recently been implicated in several tumors,[26,27] such as those in the skin, brain, prostate, digestive tract, pancreas, and lung. The vertebrate Hh family includes three members: sonic Hh (Shh), desert Hh, and indiana Hh, which all bind to the same receptor, Patched (PTCH). In the absence of a Hh ligand, PTCH interacts with Smoothend (Smo) to inhibit its function, preventing the activation of the downstream signaling cascade. Upon Hh binding, Smo inhibition is released, resulting in the activation of the downstream signaling cascade through the release of the transcriptional factor GLI from a macromolecular complex on microtubules (Fig. 8). In some types of human tumors, Hh/GLI signaling is constitutively activated because of mutations in PTCH or SMO, leading to tumor formation and progression. Thus, targeted Hh/GLI signaling is anticipated as an effective cancer therapeutic strategy.[28] Cyclopamine has been identified as an inhibitor of Hh signaling by binding to SMO,[29,30] and other types of SMO antagonists have been reported, such as Cur-61414[31] and SANTs.[32] In addition, several small-molecule inhibitors of Hh signaling, including GLI-mediated transcription inhibitors (GANTs),[33] inhibitor of class IV alcohol dehydrogenase (JK184)[34] and a small molecule that binds Shh protein (robotnikinin),[35] have been reported, but there is still an urgent need to identify different types of GLI-mediated transcriptional inhibitors.

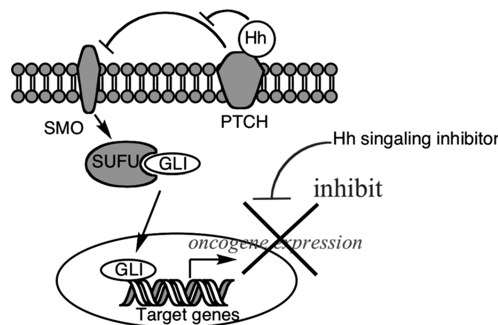


Fig. 8. Hedgehog/GLI-Mediated Signaling Pathway

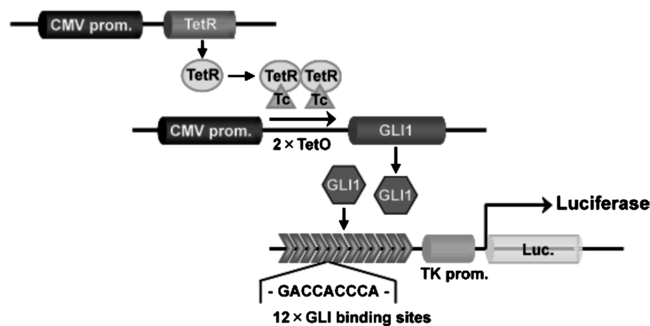


Fig. 9. The Assay System with T-REx (Tetracycline-Regulated Expression System)

pcDAN3.1-GLI1 expresses exogenous GLI1 protein by a tetracycline-regulated CMV promoter, and GLI1 binds to the GLI binding site on pGL4-GLI BS. Tetracycline removes TetR to start GLI1 expression.

To discover inhibitors of the Hh/GLI signaling pathway from natural resources, we have recently reported the construction of a cell-based screening assay system for the Hh/GLI signaling pathway (Fig. 9).[36] This is an assay using a GLI-dependent luciferase reporter in human keratinocyte cells (HaCaT) expressing GLI1 under tetracycline control (T-REx system). The 12 consecutive GLI-binding sites (12× GACCACCCA) and the TK promoter were inserted into pGL4.20 plasmid (Promega, U.S.A.). The constructed plasmid, pGL4-GLI BS, was stably transfected into HaCaT cells expressing exogenous GLI1 protein under tetracycline control. During the screening of our isolated natural products library and natural resource libraries, including plant extracts and actinomycete extracts with the assay system, we identified some natural products and natural plant extracts as GLI1-mediated transcriptional inhibitor samples.

From our natural products library including terpenoids, flavonoids, phenylpropanoids, their glycosides and bisindole alkaloids, sesquiterpene zerumbone (**11**; IC_{50} 7.1 μM) and several bisindole alkaloids including staurosporinone (**12**; IC_{50} 1.8 μM) were identified as Hh signaling inhibitors (Fig. 10). From the natural plant extracts which exhibited GLI1-mediated transcriptional inhibitory activities, natural products were isolated as Hh signaling inhibitors. The methanol extract of *Physalis minima* (2.7 g) was partitioned between

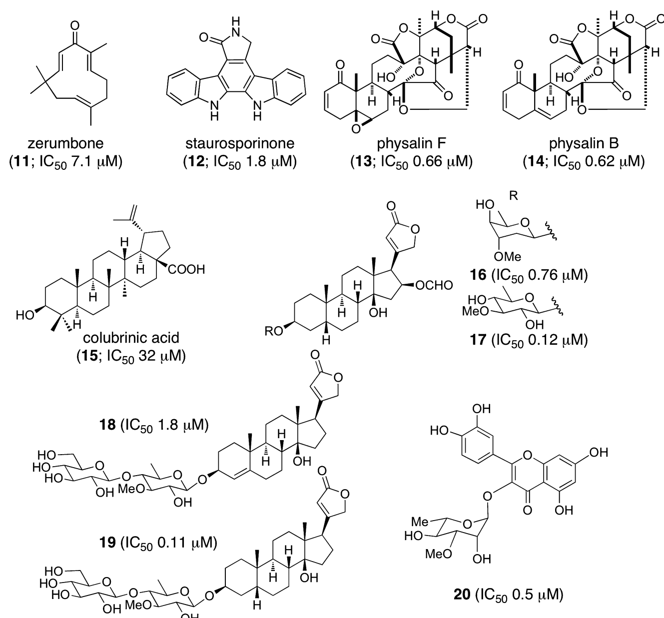


Fig. 10. Isolated Hh Inhibitors

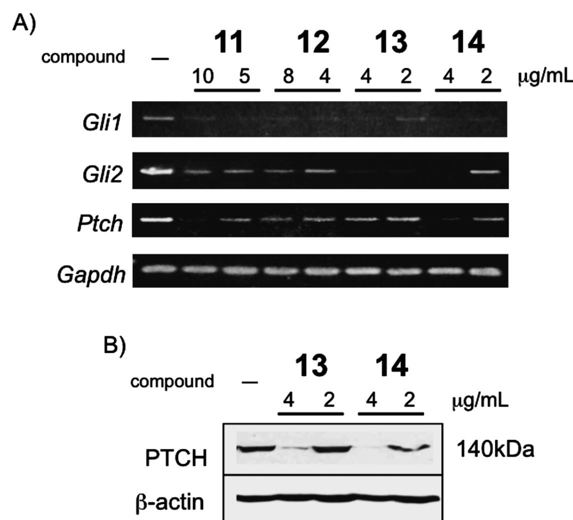


Fig. 11. Effect of Inhibitors on Hh/GLI Signaling Component Expressions in PACN1 Cells

(A) Semiquantitative RT-PCR. PANC1 cells were incubated with or without each inhibitor (**11**—**14**) for 24 h. mRNA expression of *Gli*1, *Gli*2, and *Ptch* in untreated and inhibitor-treated cells were shown with that of *Gapdh*. (B) Western blotting analysis of PTCH in PANC1 cells. PANC1 cells were treated with inhibitors (**13**, **14**) for 24 h, and PTCH protein expression was analyzed by Western blotting. The β-actin was used as a control.

H_2O, and *n*-hexane and ethyl acetate. Cells treated with 10 μg/ml of the *n*-hexane and ethyl acetate fractions showed 51% and 20% reporter activity, respectively, and >95% cell viability. Because TLC analysis of the *n*-hexane and ethyl acetate-soluble fractions revealed the presence of nearly the same compounds, we combined and further purified them by consecutive steps of silica gel column chromatography, sephadex LH-20 column chromatography, and reversed-phase HPLC. This gave two compounds, which were identified as physalin F (**13**),[37] and physalin B (**14**),[38] by comparison of their spectral data with reported values. Physalins F (**13**) and B (**14**) dose-dependently inhibited GLI1-mediated transcriptional activity with little or no effect on cell viability. The IC_{50} values of these two compounds in this assay were 0.66 μM and 0.62 μM, respectively. In addition, it should be emphasized that the compounds did not reduce the viability of normal HaCaT human keratinocytes near their IC_{50} concentrations. PANC1, a human pancreatic cancer cell line, expresses numerous Hh/GLI signaling pathway components, including Shh, PTCH, suppressor of fused, GLI1, and GLI2, indicating that the Hh/GLI signaling pathway is activated in PANC1 cells. We confirmed the effect of compounds (**11**—**14**) on the expression of Hh/GLI signaling components in PANC1 cells by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis (Fig. 11A). These compounds decreased the mRNA expression of Gli1, Gli2, and Ptch genes. These results indicate that these compounds inhibit the expression of these components at the transcriptional level. We further examined the effect of **13** and **14** on the protein expression of PTCH in PANC1 cells (Fig. 11B). These inhibitors apparently decrease the PTCH expression in PANC1 cells at 4 μg/ml. We investigated the effects of isolated Hh inhibitors on the viability of PANC1 cells using a fluorimetric microculture cytotoxicity assay (FMCA). Compounds **11**, **12**, **13** and **14** were cytotoxic to PANC1 cells with IC_{50} values of 40, 68, 2.6, and 5.3 μM, respectively.

Our continuous study also led to the isolation of several potent Hh inhibitors from natural resources. Bioassay-guided fractionation of *Zizyphus cambodiana* extract led to the isolation of three active pentacyclic triterpenes, including colubrinic acid (**15**).[39] Potent inhibitors were isolated from *Acacia pennata*,[40] *Adenium obesum*[41] and *Excoecaria agallocha*.[42] From *Adenium obesum*, 17 cardiac glycosides, including 3 new compounds, 16-formylgitoxigenin β-D-sarmentoside (**16**; IC_{50} 0.76 μM), 16-formylgitoxigenin β-D-thevetoside (**17**; IC_{50} 0.12 μM), canariengenin β-D-glucosyl-(1→4)-β-D-thevetoside (**18**; IC_{50} 1.8 μM), were isolated. These 17 compounds showed strong inhibitory activities, especially IC_{50} of digitoxigenin β-D-glucosyl-(1→4)-β-D-thevetoside (**19**) is 0.11 μM.

We recently found a new type of Hh inhibitor from *Excoecaria agallocha*.[42] A new flavonoid glycoside **20** inhibits Hh signaling by arresting the translocation of GLI into the nucleus of PANC1. Moreover, it was revealed that this inhibition was not affected by SMO. Even in the SMO knocked down C3H10T1/2 cells, **20** inhibited PTCH mRNA expression, which is regulated by the Hh signaling pathway. Most known Hh inhibitors, including cyclopamine, repress the pathway by antagonizing SMO activation; however, one of the oncogenic SMO mutants is apparently resistant to cyclopamine, and most SMO inhibitors were not effective against medullablastoma and cancer associated with downstream lesions. Therefore, a new mechanism of Hh inhibition, other than SMO inhibitors, is very important for clinical use.

3. Synthesis and Evaluation of Small Molecules Based on Natural Products

3.1. Total Synthesis and Evaluation of Wnt Signal Inhibition of Melleumin A, B and Their Derivatives[43] A novel peptide lactone, melleumin A (**21**), and its *seco* acid methy ester, melleumin B (**22**) were discovered by the Ishibashi group in 2005 from cultured plasmodium of the myxomycete *Physarum melleum* (Fig. 12).[44] The stereo-

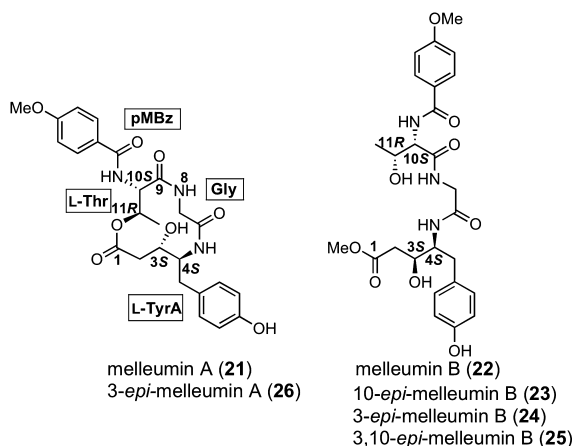


Fig. 12. Structure of Melleumin A, B and Their Derivatives

chemistry of **21** and **22** at the chiral centers was established to be 3*S*, 10*S*, and 11*R*-configurations. Because the quantity of isolated **21** and **22** was very small, we decided to synthesize those compounds to evaluate their biological activity. The absolute stereochemistry of the C-4 position was determined to be *S* by total synthesis of melleumin B (**22**).[45] The 10*R*-epimer (**23**), 3*R*-epimer (**24**) and (3*R*,10*R*)-epimer (**25**) of **22** showed moderate Wnt signal inhibitory activity.[45,46]

Wnt signaling exhibits aberrant activation in many cancer cells, especially in colon cancers.[47] The aberrant activation of this signal is caused by the mutation or loss of function in β-catenin, T-cell factor/lymphoid enhancer factor (TCF/LEF), and glycogen synthase kinase 3β (GSK3β) and other things. Small molecules that act on certain molecular components in the Wnt signal pathway would be potential candidates for treating cancer. Several Wnt signal inhibitors have been previously reported. However, there are not many effective Wnt signal inhibitors. We have also reported natural products or their derivatives that act as Wnt signal inhibitors, such as bisindole alkaloid *cis*-dihydroarcyriarubin C,[48] and the new natural products eleutherinoside B—E.[49]

Luo *et al.* reported the first total synthesis of **21**, 4*R*-epimer and other derivatives by intramolecular lactamization at the N5-C6 amide bond,[50] and found moderate Wnt inhibition in 4-*epi*-melleumin B and 4-*epi*-deoxymelleumin A. We also succeeded in the total synthesis of melleumin A (**21**) and its 3*R*-epimer (**26**). In order to analyze Wnt inhibitory activities on cyclic compounds related to **21**, melleumin A-like compounds that do not contain a lactone subunit that is easily cleaved in cells were designed and synthesized. These compounds showed moderate Wnt inhibitory activity.

Melleumin A (**21**) and B (**22**) consist of four residues: *p*-methoxybenzoic acid (pMBz), L-threonine (L-Thr), glycine (Gly), and an unusual amino acid, a tyrosine-attached acetic acid (TyrA). Because compound **22** is a *seco* acid methyl ester of **21**, we attempted the intramolecular lactone formation of the *seco* acid of **22** that was obtained by hydrolysis of synthesized **22**. However, several conditions prevented cyclization. Therefore, we changed strategies to synthesize **21** by N8-C9 amide bond formation as a cyclization step (Chart 1). Compound **27** could be prepared from dipeptide **28** (TyrA-Gly unit) and *N*-*p*-methoxybenzoyl-L-threonine **32** (pMBz-L-Thr unit). Dipeptide **28** could be obtained from

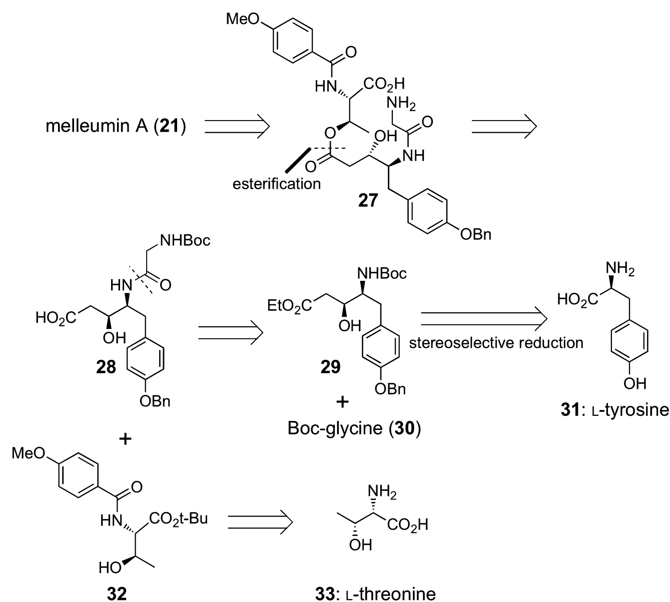
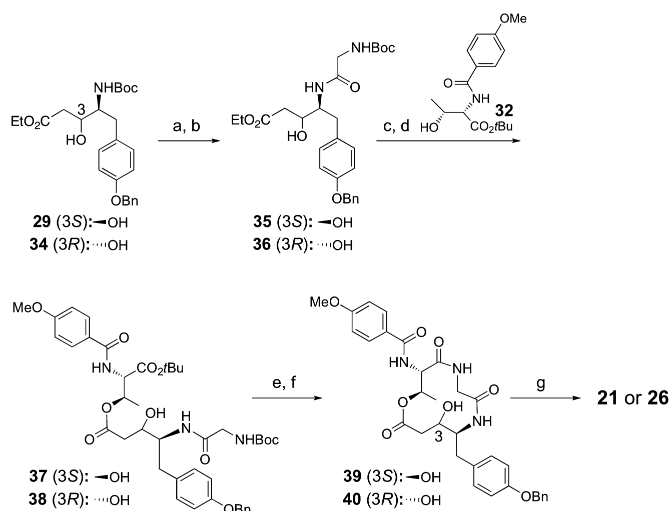


Chart 1. Retrosynthetic Approach to Melleumin A (**21**)



Reagents and conditions: (a) TFA or HCl; (b) Boc-Gly, EDC, HOBt, DMAP, 80% (**35** (3*S*), 2 steps from **29**), 61% (**36** (3*R*), 2 steps from **34**); (c) LiOH, 0 °C; (d) **32**, DCC, DMAP, 50% (**37** (3*S*), 2 steps from **35**), 36% (**38** (3*R*), 2 steps from **36**); (e) TFA; (f) FDPP, 28% (**39** (3*S*), 2 steps from **37**), 22% (**40** (3*R*), 2 steps from **38**); (g) H_2, 10% Pd/C, 97% (**21**), 77% (**26**).

Chart 2. Synthesis of Melleumin A (**21**) and 3-*epi*-Melleumin A (**26**)

(3*S*,4*S*)-β-hydroxy ester **29** and Boc-glycine (**30**).

To obtain (3*S*,4*S*)-TyrA unit **29** stereoselectively, the reduction of β-ketoester was carried out. Desired (3*S*,4*S*)-β-hydroxy ester **29** was obtained in 69% yield with a ratio of **29**/**34** (3*R*,4*S*)=93/7, with K-selectride. When $NaBH_4$ was used, (3*R*,4*S*)-TyrA unit **34** was predominately obtained as expected, which was used for the synthesis of 3-*epi*-melleumin A (**26**).

After deprotection of the Boc group, coupling with *N*-Boc-glycine gave **35** in good yield (Chart 2). Compound **35** was hydrolyzed by lithium hydroxide to give a desired carboxylic acid that was used in the next step without further purification. Compound **32** (*p*MBz-L-Thr unit) and *p*-methoxybenzoyl chloride, and the aforementioned carboxylic acid, were coupled using dicyclohexylcarbodiimide (DCC) to give com-

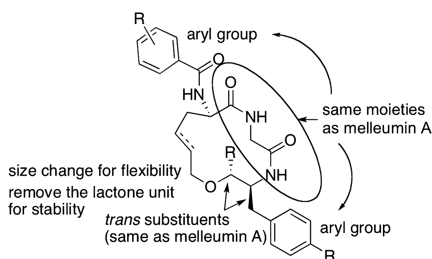


Fig. 13. Design of Melleumin A-Like Compounds

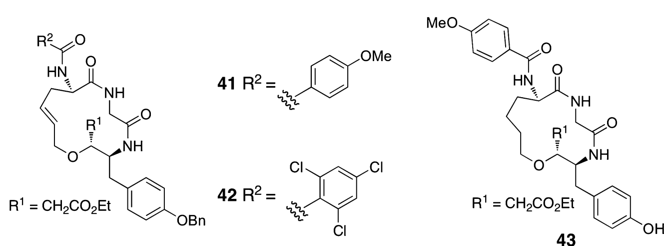


Fig. 14. Melleumin A-Like Compounds

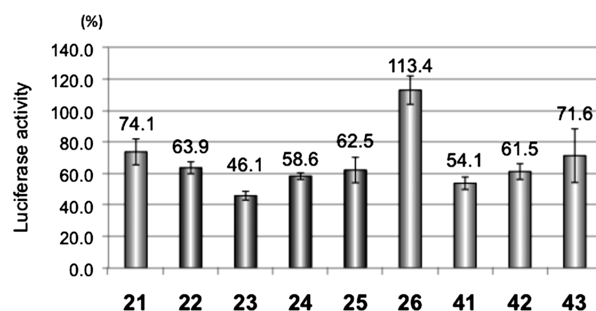


Fig. 15. Comparison of Wnt Signal Inhibitory Activities of Melleumin A, B, Their Derivatives and Melleumin A-Like Compounds at 50 μM

Fold activation of Super TOP-Flash (solid columns). STF/293 cells (3×10^4) were split into 96-well plates and 24 h later cells were treated with 15 mM LiCl and testing samples (DMSO solution). $n=3$, Bars=S.D. The activity of the untreated cells was defined as 100%.

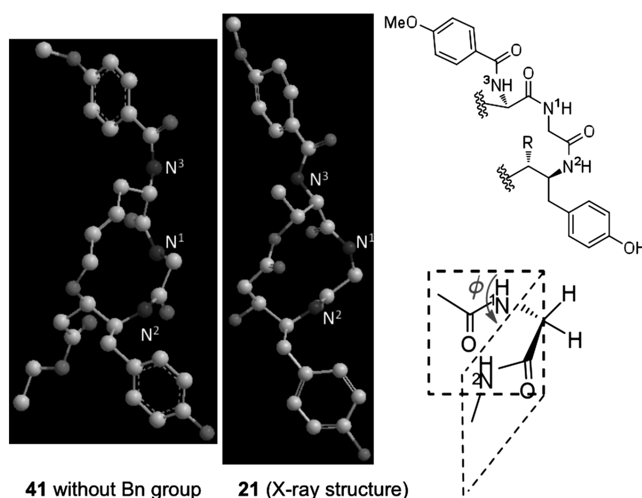


Fig. 16. Structural Comparison of **41** (without Bn) and **21**

Hydrogen atoms have been omitted for clarity.

pound **37** in 50% yield in 2 steps. The Boc group and *tert*-butyl ester of **37** were deprotected to give compound **27**. Macrolactamization of **27** was investigated for various coupling reagents. Pentafluorophenyl diphenylphosphinate (FDPP) and *O*-(7-azabenzotriazol-l-yl)-*N*,*N*,*N′*,*N′*-tetramethyluronium hexafluorophosphate (HATU) provided **39** in 28% and 24% yield (2 steps), respectively. The benzyl group was finally removed by hydrogenolysis to give melleumin A (**21**). The ^{1}H- and ^{13}C-NMR spectral data of the synthetic material were identical to those of the natural product.[1] The optical rotation of synthetic melleumin A (**21**) showed the same sense as the natural product $\{[\alpha]_D^{18}$ +16 (c=0.97, MeOH); $[\alpha]_D^{18}$ +27 (c=0.15, MeOH) lit.[44]$\}$. X-Ray crystallographic analysis of synthetic melleumin A (**21**) succeded. This result strongly confirmed the absolute structure of melleumin A (**21**). Starting from (3*R*,4*S*)-β-hydroxy ester **34**, 3-*epi*-melleumin A (**26**) was also synthesized as shown in Chart 2.

Next, we examined the biological activity of melleumin A (**21**) and 3-*epi*-melleumin A (**26**). Unfortunately, melleumin A (**21**) had weak Wnt signal inhibitory activity and 3-*epi*-melleumin A (**26**) had no inhibitory activity. However, we had previously explored and found that melleumin B derivatives became Wnt signal inhibitors. Therefore, we thought it possible to add Wnt inhibition to melleumin A-like compounds. We designed melleumin A-like compounds as shown in Fig. 13. Although the target molecule of active melleumins is unknown at this stage, most melleumins show moderate or weak Wnt inhibition. Therefore, we hypothesized that two amides in the ring and aryl groups could be important as a common structure, same moieties as melleumin A. Because melleumin B derivatives, which are open chain analogues of melleumin A, have moderate Wnt inhibitory activity, greater flexibility would be important to fit the target molecule (larger ring). The lactone moiety was removed for compound stability in the cells. At this time, a 13-membered ring was chosen instead of the 12-membered ring of melleumin A. By using a ring-closing metathesis (RCM) reaction as a key step, we synthesized three melleumin-like compounds (**41**—**43**) (Fig. 14).

Next we examined the Wnt signal inhibitory activity of the synthesized compounds using a luciferase reporter gene assay. Wnt signaling activates gene transcription by forming a complex between β-catenin and TCF/LEF, a DNA-binding protein. Super TOP-Flash, a reporter plasmid with multiple TCF-binding sites (CCTTTGATC), was stably transfected into 293 cells. Super FOP-Flash has eight mutated TCF-binding sites (CCTTTGGCC); therefore, a selective inhibitor would not affect transcription in Super FOP-Flash-transfected cells. Figure 15 shows the comparison of the Wnt signal inhibitory activities of melleumin A, B, their derivatives and melleumin A-like compounds (50 μM). Natural products, melleumin A (**21**) and melleumin B (**22**), reduced Wnt transcriptional activity to 74% and 64%, respectively. Of them, 10-*epi*-melleumin B (**23**) seemed to be the most active (46%). Regarding cyclic-peptide type (melleumin A type) compounds, 3-*epi*-melleumin A (**26**) lost activity; however, melleumin A-like compound (**41**) showed 20% more inhibition (54%) compared with **21** (74%).

To evaluate the structural differences between melleumin A (**21**) and melleumin A-like compounds, density functional theory (DFT) calculation of **41**, the most active of the three melleumin A-like compounds, was carried out (Fig. 16). The DFT calculation at the level of B3LYP/6-31G* suggested a

slight difference in the three-dimensional orientation of two amide units in the ring. The two amide torsion angle (ϕ) was changed to 86.7° (**41**) from 112.4° (**21**). The distances of N1—N2 and N1—N3 were changed to 3.35 Å (**41**) from 2.93 Å (**21**), and 3.64 Å (**41**) from 3.71 Å (**21**), respectively. Since two amides in the ring and aryl groups are common structures in bioactive melleumins, the Wnt inhibition of **41** might be due to this conformational change in the structure.

The total synthesis of melleumin A (**21**), melleumin B (**22**), and their derivatives were achieved. Moreover, we designed and synthesized melleumin A-like compounds to seek a possible Wnt signaling inhibitor. We succeeded to add Wnt inhibitory activity to a melleumin A-like compound, though natural product **21** showed weak activity. Since few small molecules are known to be Wnt signal inhibitors, and their clinical use has received great attention, we believe that peptide-based inhibitors will be worth synthesizing to evaluate their potential.

3.2. Efficient Synthesis of Chromone and Flavonoid Derivatives with Diverse Heterocyclic Units[51] To find new bioactive small molecules based on natural products, an efficient synthesis method in which it is easy to add molecules with structural and/or substitution diversity is important. We focused on chromones and flavonoids, which are important bioactive compounds isolated from a wide range of plants. Many studies have suggested that flavonoids have biological activity, such as antiviral action, anti-inflammatory

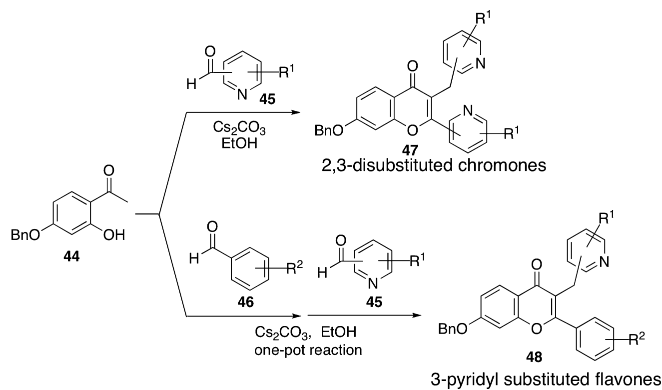


Chart 3. One-Pot Synthesis by Michael–Aldol Reaction of Chromone and Flavonoid Derivatives Bearing Pyridine Units

Table 1. Construction of Chromones Bearing Heterocycles

49 → (50a-l; Cs_2CO_3, EtOH, 40 °C) → 51a-l + 52a-l

Entry[a]	R	Aldehyde	Time (h)	Yield (%)[b]			
				51 (*endo*)		**52** (*exo*)	
1		**50a**	8	**51a**	90	**52a**	0
2		**50b**	47.5	**51b**	65	**52b**	0
3		**50c**	21.5	**51c**	66	**52c**	0
4		**50d**	15.5	**51d**	82	**52d**	0
5		**50e**	26	**51e**	0	**52e**	93
6		**50f**	12	**51f**	90	**52f**	0
7		**50g**	5	**51g**	84	**52g**	0
8		**50h**	42	**51h**	60	**52h**	0
9		**50i**	73	**51i**	91	**52i**	0
10		**50j**	74	**51j**	8	**52j**	71
11		**50k**	144	**51k**	90	**52k**	0
12		**50l**	63	**51l**	84	**52l**	0

a) All reactions were carried out with **50** (10 eq) and Cs_2CO_3 (5 eq) in EtOH (0.16 M). *b*) Isolated yield based on **49**.

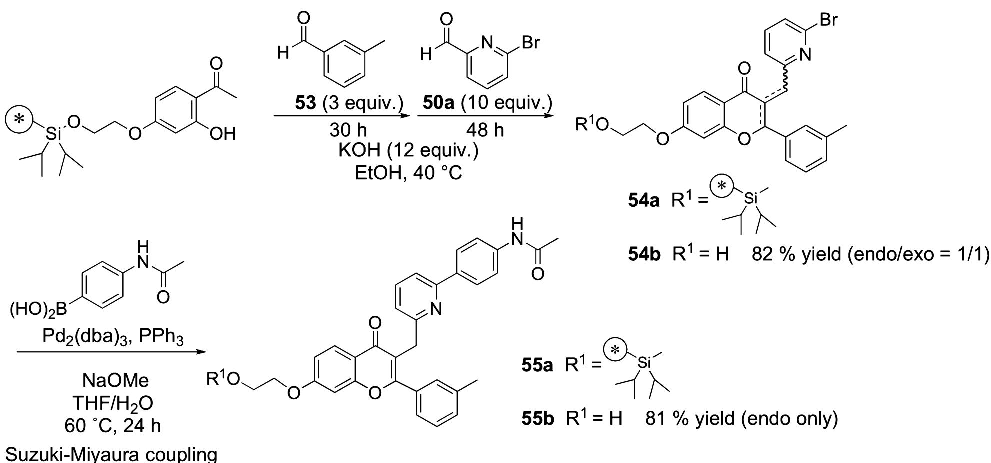


Chart 4. Synthesis of 3-Heterocyclic-Substituted Flavonoids on Solid Phase

effects, and a beneficial influence on multiple cancer-related biological pathways. We envisioned that heterocyclic-substituted chromones or flavonoids would act as new bioactive compounds. Efficient one-pot syntheses methods of flavones with disubstituents at the 2 and 3 positions have yet to be developed. Chawla *et al.* reported the first one-pot synthesis of 3-arylideneflavonoids from 2′-hydroxyacetophenones with aromatic aldehydes in aq. NaOH.[52] Dhara *et al.* reported an improved one-pot synthesis of 3-arylideneflavonoids and 2,3-disubstituted chromanones by using furfural or thiophen-2-aldehyde.[53] The Baker–Venkataraman rearrangement is another important one-pot method for synthesizing 3-acyl flavonoids from 2′-hydroxyacetophenones, and a 3-acyl flavonoid with pyridine units was synthesized in this manner.[54] To construct small molecules with complexity and diversity to increase the number of protein-binding elements, we developed efficient one-pot syntheses methods of 2,3-disubstituted chromones and 3-substituted flavonoids (Chart 3). After the construction of corresponding chalcone from hydroxyacetophenone derivatives **44** and heterocyclic aldehyde **45** or aromatic aldehyde **46**, a tandem type Michael–Aldol reaction gave the 2,3-heterocyclic disubstituted chromones **47** or 3-heterocyclic substituted flavonoids **48**.

One-pot synthesis by Michael–Aldol reaction of chromone derivatives bearing pyridine units was optimized by using 4′-benzyloxy-2′-hydroxyacetophenone **49**. The reaction proceeded smoothly with pyridinecarboxaldehyde **50a**—**h** (Table 1, entries 1—8). Endo olefinic products **51** were obtained in good yield in all cases apart from **50e**. In some cases, *exo* products were obtained when the reactions were quenched in a shorter reaction time. The position of CHO or Br on the pyridine did not affect the yield. Furfurals **50i** and **50j** (entrys 9—10), thiophen-2-aldehydes **50k** and **50l** (entrys 11—12) also gave the desired product. Because the cyclization of chalcones which was made from aromatic aldehyds **46** (Aldehyde A) were very slow, 3-heterocyclic-substituted flavonoids **48** were also obtained in one-pot reaction (Chart 3). Variety of 3-heterocyclic-substituted flavonoids were synthesized.[51]

Next, application of this efficient reaction to solid-phase reactions to construct small molecule libraries was examined. After attaching the acetophenone derivative to (4-methoxyphenyl) diisopropyl-silylpropyl polystyrene beads (novabiochem), a desired reaction smoothly proceeded to give 2,3-heterocyclic-substituted chromones (see ref. 51). In the case of 3-heterocyclic-substituted flavonoids, after optimization of the condition, the desired product **54b** was obtained in 82% (Chart 4). Furthermore, to add diverse substituents on the molecules, we examined Suzuki–Miyaura coupling on a pyridine unit on solid phase. Under several reported conditions, the described combination gave coupling products in good yield. *p*-Acetamidopheny- (**55b**) attached compound was obtained in 81% (*endo* only).

Recently, we succeded in constructing a library of chromones and flavonoids with heterocycles. By the screening of this library, several compounds were found to be Hes1 promoter inhibitors. The application of these inhibitors to neural stem cells is in progress.

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