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Privileged Structures: Efficient Chemical "Navigators" toward Unexplored Biologically Relevant Chemical Spaces

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ABSTRACT: In the search for new therapeutic agents for currently incurable diseases, attention has turned to traditionally "undruggable" targets, and collections of drug-like small molecules with high diversity and quality have become a prerequisite for new breakthroughs. To generate such collections, the diversity-oriented synthesis (DOS) strategy was developed, which aims to populate new chemical space with drug-like compounds containing a high degree of molecular diversity. The resulting DOS-derived libraries have been of great value for the discovery of various bioactive small molecules and therapeutic agents, and thus DOS has emerged as an essential tool in chemical biology and drug discovery. However, the key challenge has become how to design and synthesize drug-like small-molecule libraries with improved biological relevancy as well as maximum molecular diversity. This Perspective presents the development of privileged substructure-based DOS (pDOS), an efficient strategy for the construction of polyheterocyclic compound libraries with high biological relevancy. We envisioned the specific interaction of drug-like small molecules with certain biopolymers via the incorporation of privileged substructures into polyheterocyclic core skeletons. The importance of privileged substructures such as benzopyran, pyrimidine, and oxopiperazine in rigid skeletons was clearly demonstrated through the discovery of bioactive small molecules and the subsequent identification of appropriate target biomolecule using a method called "fluorescence difference in two-dimensional gel electrophoresis". Focusing on examples of pDOS-derived bioactive compounds with exceptional specificity, we discuss the capability of privileged structures to serve as chemical "navigators" toward biologically relevant chemical spaces. We also provide an outlook on chemical biology research and drug discovery using biologically relevant compound libraries constructed by pDOS, biology-oriented synthesis, or natural product-inspired DOS.

1. INTRODUCTION

Novel bioactive small molecules can serve as useful research tools for probing complex biological systems through perturbation of the function of biopolymers, which results in phenotypic changes.[1−3] Compared with traditional genetic approaches using gene modulation or knockout, chemical genetics employing small-molecule modulators to dissect living systems provides the following advantages: (1) a high degree of rapid, usually reversible, and temporal control in a dose-dependent manner[4] and (2) specific perturbation of a single function among multiple roles of proteins of interest.[5] Once the chemical biology approach reveals the effect and mechanism of action of bioactive small molecules at the molecular level, first-in-class drug candidates can be rationally developed for biopolymers with therapeutic potential including traditionally "undruggable" targets.[6,7]

Phenotype-based screening is a preferred approach for the discovery of first-in-class drugs in the fields of chemical biology and medicinal chemistry, because new bioactive chemical entities can be identified through the monitoring of phenotypic changes in living cells or whole organisms.[8] Bioactive small molecules identified by phenotypic screening can open the door to the development of new therapeutic agents targeting currently "undruggable" targets, including protein−protein interactions, protein-nucleic acid interactions.[9,10] A recent analysis revealed that more than half of 50 first-in-class small-molecule drugs approved by the U.S. Food and Drug Administration between 1999 and 2008 were discovered using a phenotype-based approach,[11] which confirms phenotypic screening as the most successful approach for discovering novel therapeutic agents with new modes of action. One of the big hurdles in the phenotype-based approach is the identification of target proteins after the discovery of new bioactive chemical entities, which we have been extensively exploring using a new target identification method, called "fluorescence difference in two-dimensional gel electrophoresis" (FITGE).[12] In addition, molecular diversity is extremely important for the successful discovery of novel small-molecule ligands using phenotypic screening.[13,14] A collection of structurally diverse drug-like small molecules is an essential element in the conventional target-based approach as well as in the phenotype-based approach because of the lack of structural and functional knowledge of target biopolymers or ligands.

For identification of bioactive small molecules against specific druggable targets, a focused library is rationally designed on the basis of structural information about the target protein and its known ligands, which allows efficient maturation of hit compounds toward drug candidates with high affinity for the target protein. However, since this approach grants incremental improvements on existing knowledge regarding target proteins, it is quite difficult to obtain novel skeletons with new modes of action.[15] Therefore, molecular diversity in a screening collection of drug-like small molecules is extremely important for new breakthroughs using the target-based approach.[16] Molecular diversity is even more important in phenotype-based

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drug discovery owing to the lack of knowledge of a target's structure and its ligands. To tackle this issue, the construction of structurally and functionally diverse drug-like small molecules has become a prerequisite for successful discovery of new bioactive compounds with a range of biological and pharmacological activities in the target-based approach as well as the unbiased phenotypic approach.[13,14,16]

Owing to their superb complexity and diversity, natural products are prime candidates for high-throughput screening (HTS) to identify new chemical entities.[17−19] However, the current collection of natural products is limited by the sophisticated isolation steps or lengthy chemical syntheses required to produce these compounds. To bypass these issues with natural products, combinatorial chemistry, a synthetic technique that enables the preparation of a large number of drug-like compounds, was developed and has been widely used in pharmaceutical industry in particular.[20] Combinatorial small-molecule libraries are usually constructed by systematic addition of various building blocks to well-characterized scaffolds with bioactivity and good synthetic accessibility to reduce the time and cost of drug candidate development.

Combinatorial chemistry has made a significant contribution to the drug discovery process, particularly the lead optimization of hit compounds.[21,22] However, most combinatorial chemistry approaches have been focused on the late stage of drug discovery and traditional druggable targets. An analysis of the sources of new drugs indicates that combinatorial chemistry has not significantly improved the generation of newly approved chemical entities, especially molecularly diverse drugs for so-called "undruggable" targets.[15] This analysis implies that a combinatorial library, starting from a limited number of bioactive core skeletons, mainly emphasizes the number of compounds and thus fails miserably in the creation of new chemical skeletons that cover a wide range of chemical space. Consequently, the focus has now shifted toward the quality of compounds in a library rather than their quantity.

To maximize the quality of a chemical library, which is dictated by the molecular diversity of its members along with their physicochemical and biological properties, Schreiber and co-workers initiated a new synthetic strategy, called diversity-oriented synthesis (DOS),[9,23−25] which aims to populate new chemical space with novel drug-like compounds with a high degree of molecular diversity (e.g., skeletal,[26] stereochemical,[27,28] or building-block diversity[29,30]) and biological relevancy. The resulting DOS-derived libraries contain complex and diverse structures with a high fraction of sp^3-hybridized carbon atoms and more stereogenic centers; consequently, they have been highly valuable for the discovery of bioactive small molecules and therapeutic agents for diverse biological targets.[10,31−36] Therefore, DOS has emerged as an essential tool to promote the identification of novel hit compounds using HTS, especially in phenotype-based high-content screening.

After DOS was introduced to the synthetic chemistry community, the key challenge became how to design and synthesize drug-like small-molecule libraries with improved biological relevancy along with maximum molecular diversity. Because the chemical space encompassing all possible small molecules with a molecular mass of less than 500 Da is enormous (about 10^{63} distinct molecules!),[37] it is impossible to fully populate this chemical space. Instead, researchers have focused on occupying a small fraction of the whole chemical space through the construction of biologically relevant chemical space. Therefore, in addition to exhibiting a high degree of molecular diversity, the chemical space occupied by DOS libraries needs to be maximally overlapped with biologically relevant chemical space. However, until now, the boundary between bioactive and nonbioactive chemical spaces has not been specified, and thus there are great challenges to navigating the unexplored bioactive chemical space.

As stated earlier, natural products have inherent bioactivities and high bioavailability, probably because of their specific interactions with target macromolecules in living organisms. Thus, the chemical space defined by natural products may nicely overlap with biological space. Therefore, structural motif and core skeletons from bioactive natural products can serve as chemical "navigators" for the synthesis of novel core skeletons with high biological relevancy and lead us to fruitful bioactivities identified through HTS in an unbiased manner.[17,38] In particular, a DOS strategy inspired by the discriminated core structures of natural products can play a critical role in identifying bioactive compounds that specifically interact with target biopolymers. Therefore, core skeletons of natural products or their mimetics have been utilized for the construction of natural product-like DOS libraries to rapidly access the unexplored bioactive chemical space (see Figure 1).[17,39] To ensure relevancy to nature, Waldmann and co-workers proposed biology-oriented synthesis (BIOS) and used the core structures derived from bioactive natural products as synthetic scaffolds for the construction of a drug-like compound library.[40,41] It is worth mentioning that BIOS offers a guiding strategy for library design with pre-validated fractions of biologically relevant chemical space. Since DOS and BIOS have been extensively reviewed elsewhere,[41,42] detailed descriptions are not provided in this Perspective.

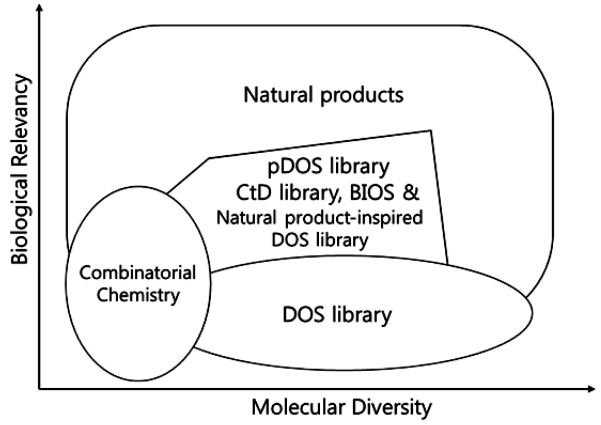

Figure 1. Comparison of natural products, combinatorial chemistry, DOS and pDOS/CtD/BIOS libraries in terms of molecular diversity and biological relevancy.

Meanwhile, Hergenrother and co-workers developed a new approach, the complexity-to-diversity (CtD) strategy, for the efficient construction of natural product-like small-molecule collections starting from commercially available natural products such as abietic acid, adrenosterone, gibberellic acid, and quinine.[43,44] As shown in Figure 2, this strategy would provide a valuable collection of natural product-like small molecules with structural uniqueness and complexity, which might cover unexplored biologically relevant chemical space. Owing to the structural complexity and defined stereochemistry of naturally occurring chemical skeletons, diverse and discrete core skeletons could be generated through relatively simple

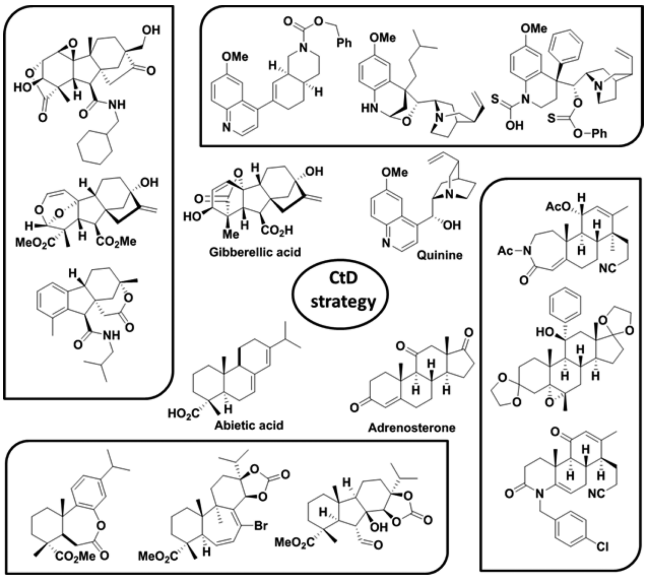

Figure 2. Representative diverse scaffolds constructed from natural products such as gibberellic acid, adrenosterone, quinine, and abietic acid through the CtD strategy. Reproduced with permission from refs 43 and 44. Copyright 2013 Nature Publishing Group, and 2014 Wiley-VCH, respectively.

chemical transformations, such as ring expansion, contraction, breaking, and aromatization. Even though the CtD strategy efficiently diversifies the core skeletons of abundant natural products through chemo-, regio-, and stereoselective chemical transformations, the biological relevancy of the resulting core skeletons must be demonstrated by specific interactions with various biopolymers in a selective manner.

To complement these synthetic endeavors to enhance biological relevancy, we proposed a privileged-substructure-based DOS (pDOS) strategy for the efficient construction of novel polyheterocyclic core skeletons through the creative reconstruction of privileged substructures frequently observed in natural products or bioactive small molecules. In this Perspective, we will introduce some examples of the pDOS strategy and the subsequent identification of new bioactive small-molecule modulators via HTS of pDOS libraries. Then, we will provide an outlook on synthetic strategies to allow convenient access to unexplored tracts of bioactive chemical space.

2. CONCEPT OF PRIVILEGED-SUBSTRUCTURE-BASED DIVERSITY-ORIENTED SYNTHESIS

The pDOS strategy aims to explore the unexploited bioactive regions in chemical space by using privileged substructures from natural products as chemical navigators. "Privileged structure" was first proposed by Evans and co-workers in 1988 as "a single molecular framework able to provide high-affinity ligands for more than one type of receptor."[45] Privileged substructural motifs are frequently observed in a wide variety of bioactive natural products and therapeutic agents and have been recognized as significant elements for ensuring good drug-like properties.[46,47] Therefore, a small-molecule library embedded with privileged substructures might have high potential for the discovery of bioactive compounds or drug candidates. However, the simple modification of privileged structures only increases the number of analogues without increasing skeletal diversity, which contradicts the fundamental purpose of the DOS approach.

To maximize the unbiased coverage of chemical space with high biological relevancy, we envisioned the creative construction of polyheterocycles embedded with privileged substructures by means of complexity-generating reactions. Through the incorporation of privileged substructures into rigid core skeletons, we expect to enhance the interaction of small molecules with specific biopolymers. We have been quite interested in the discovery of small-molecule modulators of complex biological targets including protein−protein interactions, which would require a large enough surface area to interrupt the large interface between protein partners. Therefore, we have designed and synthesized three-dimensionally well-defined rigid polyheterocyclic skeletons, rather than flexible linear compounds, as substrate analogues. The rigid skeletons restrict conformational flexibility in pDOS compounds, and this prepaid entropic penalty leads to superior specificity toward target biopolymers, as observed in numerous bioactive natural products. In this regard, a pDOS strategy that constructs diverse drug-like polyheterocyclic compounds with high molecular diversity and complexity as well as high biological relevancy has been the focus of several investigations. The construction of diverse polyheterocycles using a pDOS strategy has been pursued through two distinct approaches: one is to design diverse core skeletons containing common privileged substructures, and the other is to use common key intermediates amenable to divergent construction of various privileged polyheterocycles.

2.1. Benzopyran- and Pyrimidine-Based pDOS Pathways. The benzopyran motif is a molecular framework frequently observed in natural products and synthetic drugs, and there are a handful of reports regarding the construction of combinatorial libraries with benzopyrans.[48−50] However, previous studies have focused on the simple modification of benzopyran core skeletons with various substituents.[51] To explore the untapped chemical space with benzopyranyl polyheterocycles, we developed a new pDOS pathway starting from a simple 2,2-disubstituted chroman-4-one moiety as a key intermediate (see Figure 3). Owing to the unique acidic nature of the α-proton adjacent to carbonyl moiety, we designed distinct polyheterocycles with maximum skeletal diversity. The creative reconstruction of core skeletons embedded with a privileged benzopyranyl motif might ensure the high biological relevancy of the resulting pDOS library.

As shown in Figure 3, we emphasized the skeletal diversity of benzopyranyl polyheterocycles in three-dimensional (3-D) space; this is described in more detail in our previous report.[52−54] We also focused on diversification of polar surface charge by introducing heteroatoms at various positions of three-dimensionally similar molecular frameworks, since non-covalent interactions such as dipole−dipole interactions, electrostatic interactions, and hydrogen bonding play a pivotal role in specific interactions of small molecules with biopolymers.[55] Thus, polyheterocyclic compounds with diverse distributions of polar surface charge might have discrete interactions with various proteins. To access the molecular diversity in polar surface charge, we developed an efficient synthetic method for the generation of benzopyran-fused polyheterocyclic core skeletons with distinct orientations of heteroatoms using an *s-cis* enone as a key intermediate.[56,57] With this pDOS pathway, we successfully synthesized benzopyranyl core skeletons fused with diverse heterocycles

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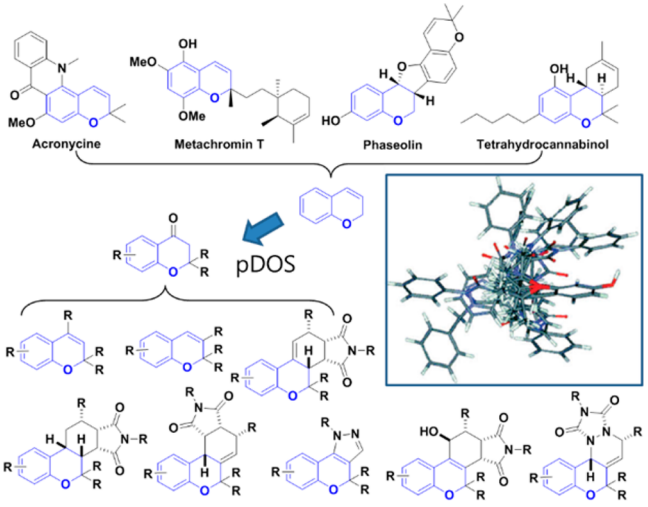

Figure 3. pDOS pathways for the construction of diverse polyheterocycles embedded with a privileged benzopyran motif and the overlay of energy-minimized conformers with alignment of the arene region of the benzopyran motif. Reproduced with permission from refs 52 and 56. Copyright 2006 Royal Society of Chemistry, and 2008 American Chemical Society, respectively.

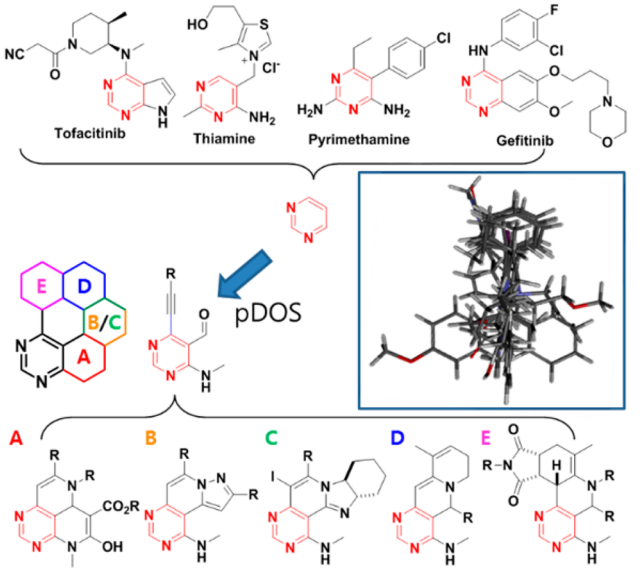

Figure 4. pDOS pathways for the construction of five unique pyrimidine-embedded core skeletons and the overlay of energy-minimized conformers with alignment of the pyrimidine substructure. Reproduced with permission from ref 61. Copyright 2013 American Chemical Society.

such as pyridine, pyrimidine, pyrazole, and pyrazolopyrimidine with different distributions of polar surface areas.

Pyrimidine has been extensively used for the development of numerous bioactive small molecules, especially kinase inhibitors and adenosine receptor modulators, because of its unique hydrogen-bonding ability.[58−60] However, since the design strategy for pyrimidine analogues has mainly focused on their role as nucleoside mimetics, the pyrimidine-embedded core skeletons are usually limited to monocyclic or bicyclic structures as kinase substrate analogs. Therefore, the structural diversification of molecular frameworks around pyrimidine is an unexplored sector in chemical space, and the molecular diversity obtained with pyrimidine-embedded polyheterocycles with unique 3-D structures would provide useful chemical resources to explore the untapped biological space. In order to expand the molecular diversity beyond monocyclic and bicyclic pyrimidine skeletons, we developed a new pDOS pathway starting from 4-alkynylpyrimidine-5-carbaldehyde as a key intermediate.[61] As shown in Figure 4, we could deliver five distinct pyrimidine-embedded polyheterocycles with unique 3-D structures and diverse electrostatic distributions through silver- or iodine-mediated tandem cyclization. The diverse projections of substituents and different ring sizes were visualized by the structural alignment of energy-minimized 3-D conformers of the resulting scaffolds.

Both pDOS approaches allowed the creative reconstruction of polyheterocycles embedded with benzopyran or pyrimidine as the privileged substructure and afforded a collection of core skeletons with unique 3-D structures and diverse orientations of polar residues to ensure high biological relevancy through non-covalent interactions with biopolymers.

2.2. Divergent Synthesis of Natural Product-like Polyheterocycles Embedded with Privileged Structures Using Cyclic Iminium Ions as Key Intermediates. In this section, we introduce a pDOS pathway for the divergent synthesis of various natural product-like non-aromatic polyheterocycles embedded with different privileged substructures using a single reactive intermediate, which is different from pDOS pathways for skeletal diversity based on a single privileged substructure, such as benzopyran or pyrimidine.

Natural products have been rich sources of challenge to the synthetic chemistry community owing to their tremendous complexity and diversity; their unique architectures are an essential feature for diverse bioactivities with excellent specificity because of their high fractions of sp^3-hybridized carbon atoms and stereogenic centers.[17,38] However, their lack of synthetic amenability might result in the limited application of natural products in drug discovery and chemical biology research. In addition, according to a recent analysis by Shoichet, 83% of the core ring skeletons present in natural products are absent among available synthetic molecules.[62] Thus, there is a great demand for the efficient synthesis of diverse drug-like compounds containing natural product-like core ring skeletons to access unexploited chemical space with high biological relevancy.

To address this demand, we developed pDOS strategies for the divergent synthesis of natural product-like non-aromatic polyheterocycles (types I–IV) and mimetics of protein turn structures (types V and VI) using cyclic iminium ions as common key intermediates (see Figure 5).[63] Even though six novel core skeletons were inspired by architectures of natural products or β/γ-turn mimetic structures, we significantly improved the synthetic accessibility and incorporated natural product-like structural features into the pDOS library. Diaza-bridged bicyclic structures were extracted and simplified from naturally occurring bioactive polycyclic products such as Yondelis (ET-743),[64] Saframycin A,[65] and Cribrostatin IV[66] (see Figure 5a). We also incorporated additional privileged substructures such as indole (type I) or L-DOPA (L-3,4-dihydroxyphenylalanine, type II) into the diaza-bridged bicyclic motif to afford novel polyheterocycles with a high degree of complexity and conformational rigidity.[67] Tetrahydro-β-carboline[68] and benzodiazepine[69] are other privileged core skeletons often found in complex natural products with a wide range of biological activities. To harness these prominent privileged core

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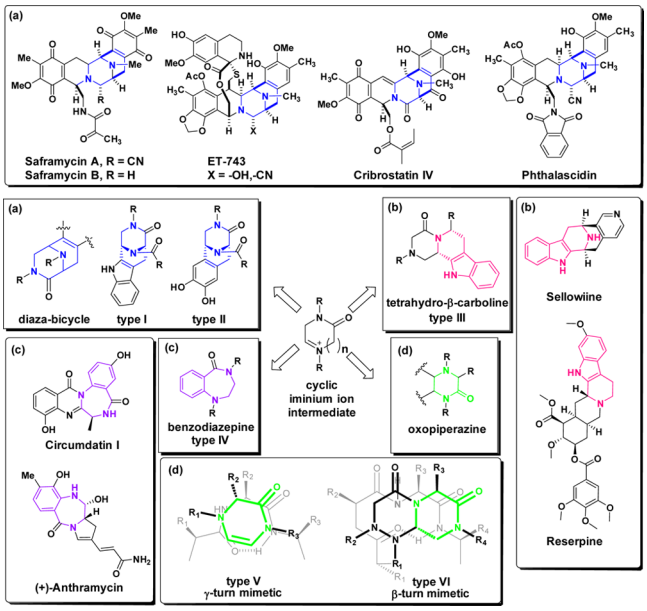


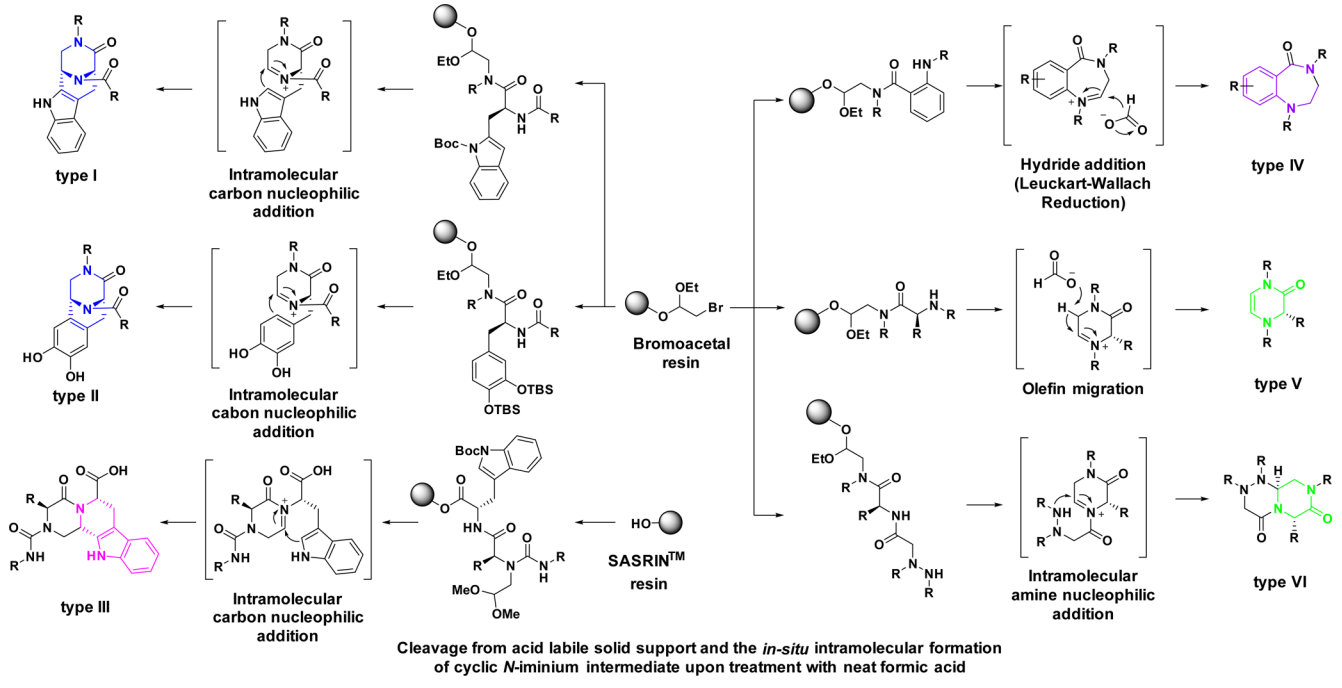
Figure 5. Six novel core scaffolds extracted or designed from natural products and peptide mimetics: (a) diaza-bridged heterocycles (types I and II), (b) tetrahydro-β-carboline alkaloid (type III), (c) tetrahydro-1,4-bezodiazepine (type IV), and (d) Δ^5-2-oxopiperazines (types V and VI). Reproduced with permission from refs 63 and 73. Copyright 2012 Wiley-VCH, and 2014 American Chemical Society, respectively.

skeletons to attain improved bioavailability and bioactivity, we envisioned the systematic construction of drug-like compound libraries based on non-aromatic polyheterocyclic scaffolds embedded with tetrahydro-β-carboline (type III, Figure 5b)[70] and benzodiazepine (type IV, Figure 5c).[71]

Finally, oxopiperazine moieties are constrained dipeptide mimetics commonly used as privileged structures for the construction of drug-like compound libraries. Specifically, they mimic the turn structures of proteins,[72,73] which are crucial recognition elements in protein−protein interactions, and thus can serve as an interesting molecular framework for the discovery of small-molecule modulators of protein−protein interactions.[74,75] Therefore, we developed new pDOS pathways for the synthesis of a trisubstituted Δ^5-2-oxopiperazine that mimics the seven-membered hydrogen-bonding ring motif and the three side-chain residues in γ-turn structures (type V, Figure 5d).[76] Distinct from γ-turns, β-turn structures are composed of a 12-membered hydrogen-bonding ring motif and four side-chain residues. To mimic the β-turn structure and key residues, we designed and synthesized a bicyclic β-turn mimetic with two fused six-membered rings and four substituents (type VI, Figure 5d).[77] It is worth mentioning that the orientations of individual substituents of the synthesized γ-turn or β-turn mimetics are well aligned with those of the side chains in the peptide turn structures.

As shown in Scheme 1, the versatile pDOS strategies were developed by employing solid-phase technology for parallel synthesis of six novel core skeletons. Each synthetic route was initiated by the incorporation of a primary amine into an acid-labile bromoacetal resin through a simple substitution reaction (except in the case of the type III product, where SASRIN resin was used), and the resulting resin-bound secondary amines were modified through a series of chemical reactions. In the final step, multiple transformations were carried out simultaneously upon a single treatment with neat formic acid to generate cyclic *N*-iminium intermediates through cleavage of products from the solid support. The aldehyde intermediates generated *in situ* can react with nitrogen atom of amide or aniline to form cyclic *N*-iminium ions, which enabled further chemical transformations, such as intramolecular nucleophilic addition (types I, II, III, and VI), hydride addition (the Leuckart−Wallach reduction, type IV), and olefin migration

Scheme 1. Divergent Synthesis of Six Discrete Polyheterocyclic Scaffolds from Cyclic *N*-Iminium Intermediates[a]



[a]Reproduced with permission from ref 63. Copyright 2012 Wiley-VCH.

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(type V), with high yields and excellent stereo- and regioselectivity. In all cases, the cyclic *N*-iminium ion was the common key intermediate and was transformed into the six discrete types of natural product-like non-aromatic polyheterocycles or mimetics of β/γ-turn structure. The robustness and practicality of our novel solid-phase synthetic methods were validated by the successful construction of a drug-like small-molecule library of more than 1000 members containing six discrete natural product-like core skeletons or privileged substructures in excellent overall yields and purities without any purification steps. This pDOS strategy enabled the construction of natural product-like compound collections with a high degree of skeletal and appendage diversity as well as high biological relevancy.

3. IDENTIFICATION OF BIOLOGICALLY ACTIVE SMALL MOLECULES DERIVED FROM PRIVILEGED-SUBSTRUCTURE-BASED DIVERSITY-ORIENTED SYNTHESIS PATHWAYS

The polyheterocyclic compound library constructed by pDOS is expected to have high biological relevancy with excellent specificity, since the pathways were designed to incorporate privileged substructures in conformationally rigid natural product-like polyheterocycles. To demonstrate the substantial utility of the pDOS strategy, the following section will focus on the identification of bioactive small molecules derived from pDOS pathways. Our in-house pDOS library, consisting of more than 4000 members, was subjected to phenotypic screening in an unbiased manner. As shown in Figure 6, we have identified bioactive polyheterocycles embedded with a privileged benzopyranyl motif in four different biological evaluations:[78] (1) a nonsteroidal androgen receptor antagonist (P01F01) for the treatment of antiandrogen-resistant prostate cancer;[79] (2) a small-molecule modulator (P10E05) with insulin-independent antidiabetic and antiobesity effects mediated through activation of AMP-activated protein kinase;[80−82] (3) a specific inhibitor of receptor activator of nuclear factor κB ligand (RANKL)-induced osteoclastogenesis (P24A05);[83] and (4) a small-molecule anabolic activator of osteogenic activity (P07A05).[84] These examples demonstrate that a pDOS library with a high degree of skeletal diversity derived from the creative reconstruction of privileged substructure is a fruitful resource for identifying diverse small-molecule modulators with a wide range of biological targets.

In the following discussion, we provide three more examples of the discovery of novel bioactive compounds from the pDOS library, which highlights the great potential of the pDOS strategy for the construction of novel drug-like compound libraries to address therapeutic challenges. In addition, a diverse collection of natural product-like polyheterocycles derived by the pDOS strategy can be a powerful research tool to specifically dissect biological processes and illuminate new modes of action, which is an essential element in the study of chemical biology and in stem cell research.

3.1. A Potent Antitumor Agent That Functions via Inhibition of Tubulin Polymerization. As stated earlier, phenotype-based HTS is a method of choice for the efficient discovery of novel first-in-class therapeutic agents. However, to facilitate phenotype-based drug discovery, it is essential to identify the target proteins of hit compounds discovered through by unbiased phenotypic screening. Thus, there is a great demand for the development of new and robust methods

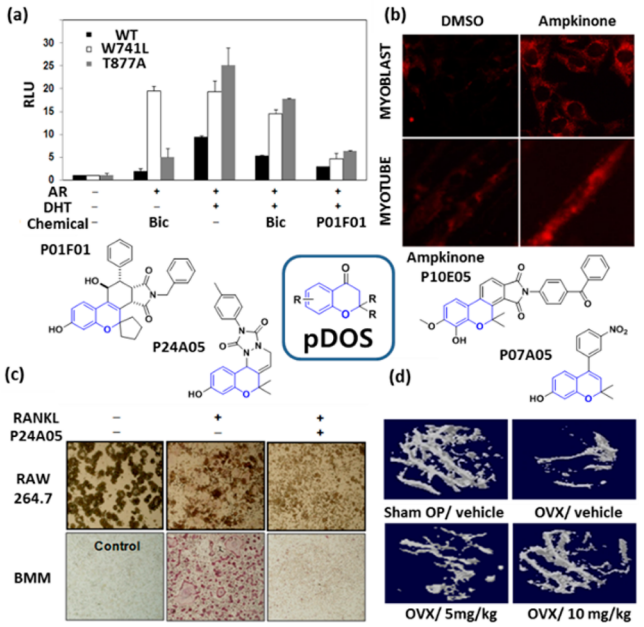

Figure 6. Identification of biologically active compounds containing benzopyranyl substructure. (a) Antagonistic activities of bicalutamide (Bic) and P01F01 (10 μM) in a cell-based reporter gene assay in 293T cells cotransfected with androgen receptor-binding element−luciferase and androgen receptor plasmids (DHT = dihydrotestosterone). Reproduced with permission from ref 79. Copyright 2010 Wiley-VCH. (b) Fluorescence microscopic images of C2C12 cells 24 h after treatment with ampkinone (P10E05) using a fluorescent glucose bioprobe. Reproduced with permission from refs 81 and 82. Copyright 2010 American Chemical Society, and 2014 Wiley-VCH, respectively. (c) Inhibitory efficacy of P24A05 in RANKL-induced osteoclastogenesis confirmed by tartrate-resistant acid phosphatase staining. Reproduced with permission from ref 83. Copyright 2010 American Chemical Society. (d) Three-dimensional microtomography reconstruction of the proximal femur of ovariectomized (OVX) C57BL/6 mice after 4 weeks of oral treatment with P07A05. Reproduced with permission from ref 84. Copyright 2011 Royal Society of Chemistry.

for target identification to shine a light on new molecular mechanisms of action in various diseases.

To address this unmet need, we developed a new target identification method called "fluorescence difference in two-dimensional gel electrophoresis" (FITGE), which involves covalent coupling of target proteins to a photoactivatable group.[12] With this approach, specific target proteins are effectively differentiated from abundant nonspecific proteins by covalent labeling of proteomes with either a positive probe or a negative probe upon UV irradiation. Then, two different fluorophores, Cy5 and Cy3, are incorporated into proteomes labeled with a positive or a negative probe, respectively, through bioorthogonal Click chemistry for fluorescent visualization of proteins on two-dimensional polyacrylamide gels. As shown in Figure 7a, the merged fluorescence images of the Cy5 and Cy3 channels provide three distinct colors: red for proteins labeled with a positive probe, green for proteins labeled with a negative probe, and yellow for proteins labeled with both probes. Therefore, FITGE can efficiently differentiate target proteins (in red) from nonspecifically labeled proteins (in green and yellow). It is worth mentioning that FITGE can harness target proteins that might be missed by conventional target identification methods, mainly affinity chromatography, owing to the covalent labeling of the target protein with a

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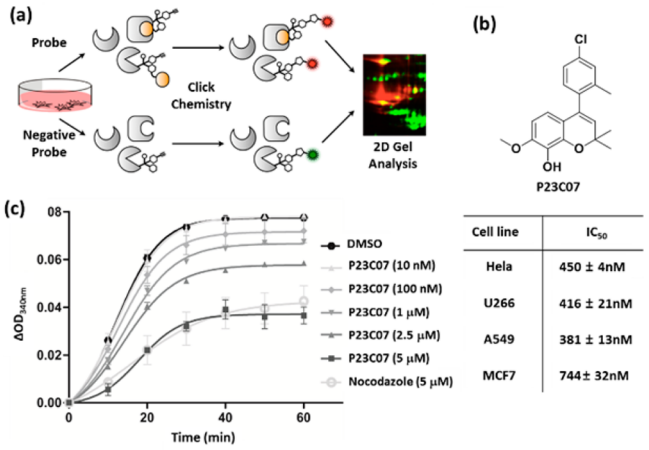

Figure 7. (a) FITGE strategy for identification of target proteins of bioactive small molecules in live cells. (b) An antiproliferative agent, P23C07, discovered from a pDOS chemical library and its biological activity. (c) *In vitro* tubulin polymerization assay showing the dose-dependent inhibitory effect of P23C07. Reproduced with permission from ref 12. Copyright 2012 Wiley-VCH.

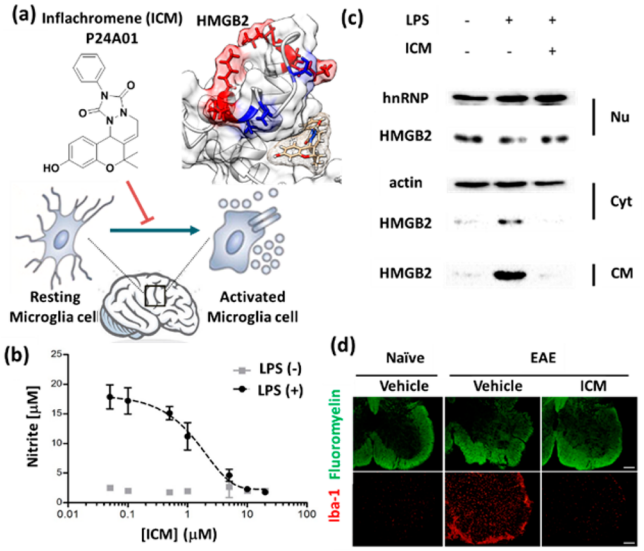

Figure 8. (a) Discovery of inflachromene (ICM, P24A01), a novel microglial inhibitor with anti-inflammatory effects mediated through direct binding to high mobility group box 2 (HMGB2). (b) Dose-dependent inhibitory effect of ICM on BV-2 microglial cells in the absence or presence of lipopolysaccharide (LPS) stimulation. The level of nitrite in the extracellular milieu was measured by Griess assay, 24 h after treatment. (c) Inhibitory effect of ICM on the cellular translocation of HMGB2 from the nucleus (Nu) to the cytoplasm (Cyt) and extracellular milieu in conditioned medium (CM). BV-2 cells were treated with ICM (10 μM) prior to treatment with LPS (200 ng/mL). (d) Histological analysis of ICM effects in an EAE model. Lumbar spinal cords from each group were removed at the disease peak time (day 15 of EAE induction). Frozen sections of lumbar spinal cords were stained with fluoromyelin for myelin (*upper*) and anti-Iba-1 antibody for microglial activation (*lower*); comparison is with pre-immunized mice (Naïve). Reproduced with permission from ref 89. Copyright 2014 Nature Publishing Group.

photoactivatable group. Therefore, FITGE allows identification of targets of hit compounds with a wide spectrum of efficacy.

As shown in Figure 7b, we identified P23C07, a pDOS-derived heterocyclic compound embedded with a benzopyran motif, as an antiproliferative agent with a submicromolar IC_{50} through a phenotype-based cell viability assay against various cancer cell lines such as HeLa, U266, A549, and MCF7. The subsequent FITGE analysis revealed that tubulin was the target protein of P23C07. Moreover, tubulin is one of the validated drug targets for anticancer agents. Therefore, we performed an *in vitro* assay which confirmed that treatment of cells with P23C07 disrupted tubulin polymerization and led to cell cycle arrest in a dose-dependent manner (see Figure 7c). Based on the similar mode of action of nocodazole,[85] a known inhibitor of tubulin polymerization, we confirmed that the benzopyran-embedded polyheterocyclic P23C07 is a potent antitumor agent that exerts its effect through specific inhibition of tubulin polymerization.

3.2. A Novel Microglial Inhibitor with Anti-inflammatory Effects Mediated through Direct Binding to High Mobility Group Box 2 (HMGB2). Neuroinflammation mediated by activation of microglia leads to neuronal damage and death, which might result in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis.[86−88] Therefore, there is a great demand for novel therapeutic agents to inhibit microglial activation for the treatment of neuroinflammatory diseases. When stimulated by immunogens or the bacterial endotoxin lipopolysaccharide (LPS), microglia in the brain release various neurotoxic factors such as IL-1β, TNF-α, prostaglandin E_2, nitric oxide, and superoxide anion (O_2^-).[88] To identify anti-neuroinflammatory agents that specifically inhibit microglial activation, we subjected our pDOS library to phenotype-based screening against nitrite production by LPS-induced BV-2 mouse microglial cells. Using HTS, we identified P24A01, which contains a tetracyclic core skeleton embedded with a benzopyran motif, as a novel microglial inhibitor with anti-inflammatory effects and named it inflachromene (ICM; see Figure 8a).[89]

As shown in Figure 8b, ICM efficiently blocked LPS-induced nitrite release in a dose-dependent manner without any toxicity in BV-2 microglial cells. After confirming the inhibitory effect of ICM on microglia, we applied FITGE technology and identified a nuclear protein, high mobility group box 2 (HMGB2), as a target of ICM. Recently, HMGBs have attracted attention for their roles as inflammatory cytokines; however, the cellular functions of HMGB isoforms other than HMGB1 remain unclear.[90,91] When cytoplasmic HMGB1 is released into the extracellular space, it can function as a proinflammatory cytokine and induce the activation of inflammatory signaling pathways.[92,93] Based on their structural similarity to HMGB1, other HMGBs are expected to be late mediators of inflammation.[94] Therefore, the cellular translocation of HMGB2 was measured upon treatment of BV-2 cells with ICM. As shown in Figure 8c, ICM effectively suppressed the LPS-stimulated accumulation of HMGB2 in the cytoplasm as well as the subsequent release of HMGB2 from the cytoplasm to the extracellular milieu. Subsequent biochemical studies revealed that ICM inhibited the extracellular release of HMGB2 by interfering with its post-translational modification. In addition, ICM effectively suppressed chronic neuroinflammation in the spinal cord of an experimental autoimmune encephalitis (EAE) animal model as well as LPS-stimulated microglial activation in mouse brain (see Figure 8d), which confirms the neuroprotective effect of ICM and its therapeutic

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potential against microglia-mediated neurotoxicity via specific inhibition of post-translational modification of HMGBs.

We previously reported that the benzopyranyl tetracycle P24A05, an analogue of ICM, inhibits the nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways in mouse macrophages and bone marrow monocytes, resulting in suppression of RANKL-induced osteoclastogenesis (see Figure 6c). Interestingly, NF-κB and MAPK signaling pathways are also related to the regulation of HMGB-induced inflammation. Since HMGB1 plays crucial roles in arthritis as well as the activation of macrophages, the inhibitory effect of ICM on NF-κB and MAPK signaling pathways in microglia is closely associated with an anti-inflammatory effect of macrophages. Therefore, the previously observed therapeutic effect of P24A05 in osteoporosis could be explained by identification of the target of ICM and the subsequent mechanism of action studies.

3.3. A Potent Small-Molecule Inducer of Chondrogenic Differentiation of Mesenchymal Stem Cells.

Osteoarthritis (OA) is one of the most prevalent chronic diseases caused by cartilage loss among middle-aged and elderly people worldwide.[95] For treatment of OA, the restoration of cartilage defects is essential; therefore, mesenchymal stem cells (MSCs),[96] which can differentiate into several cell types such as cartilage, bone, and adipose tissue, have attracted considerable attention.[97] In the presence of members of the transforming growth factor-β (TGF-β) superfamily, which act as chondrogenic inducers in defined medium, MSCs can differentiate into cartilage balls with limited specificity.[98] Unfortunately, treatment with TGF-β may induce undesirable side effects such as synovial fibrosis and osteophyte formation.[99−101] The identification of potent small-molecule modulators that induce specific chondrogenic differentiation of MSCs can shed light on the mechanism of developmental chondrogenesis of MSCs and lead to the discovery of novel therapies for patients with OA.[102,103] To this end, our pDOS-derived small-molecule collection was subjected to phenotype-based medium-throughput screening with primary human bone marrow-derived mesenchymal stem cells (hBM-MSCs) to measure changes in cell morphology and the contents of glycosaminoglycan (GAG), a biomarker of chondrogenic extracellular matrix. After an extensive search, we discovered chondrogenamine (P20B09), a novel small-molecule modulator that contains the Δ^5-2-oxopiperazine molecular framework (a γ-turn mimetic).[104]

As shown in Figure 9a, chondrogenamine can robustly induce the chondrogenic differentiation of hBM-MSCs into chondrocyte balls. The cellular content of GAG is also significantly increased upon treatment with chondrogenamine (see Figure 9b). In particular, chondrogenamine improved the cellular GAG/DNA ratio, which is associated with the degree of chondrogenic differentiation, compared with the defined medium only. In addition, chondrogenamine upregulated the expression of SOX9 and aggrecan (chondrogenesis specific markers) relative to a hypertrophic chondrocyte-specific marker (collagen type X) and osteogenic markers (collagen type I, RUNX2 and MMP13) (see Figure 9c). In contrast, TGF-β3 drastically upregulates collagen type X and MMP13, which confirms that TGF-β3 might induce the non-specific differentiation of hBM-MSCs by facilitating osteogenesis of MSCs along with chondrogenesis. Therefore, unlike TGF-β3, chondrogenamine can promote selective chondrogenic differentiation of hBM-MSCs without osteogenic differentiation.

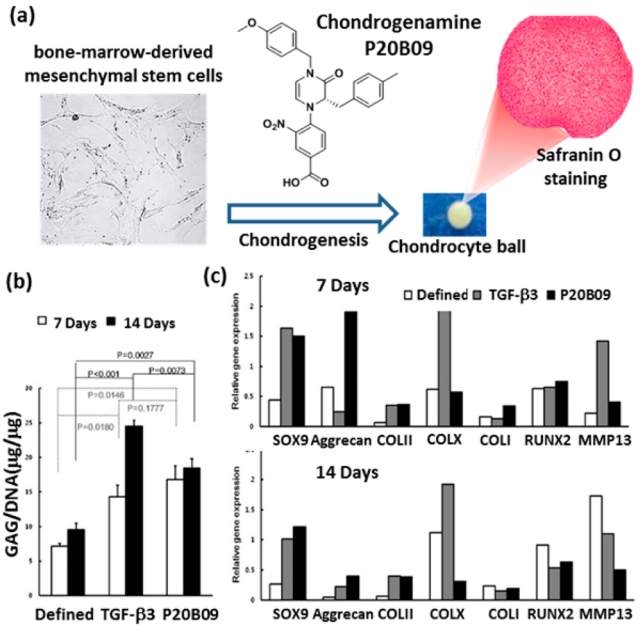

Figure 9. (a) Chondrogenic differentiation of hBM-MSCs into chondrocyte balls upon treatment with chondrogenamine (P20B09, 10 μM) and a representative image for Safranin-O staining. (b) Measurement of glycosaminoglycan (GAG) content at day 7 and day 14. hBM-MSCs were treated with TGF-β3 (10 ng/mL) or chondrogenamine (10 μM) in defined medium (n = 3). (c) Expression levels of mRNA for various biomarkers, including chondrogenic markers (SOX9, aggrecan, and collagen type II), a hypertrophic chondrocyte-specific marker (collagen type X), and osteogenic markers (collagen type I, RUNX2, and MMP13). Reproduced with permission from ref 104. Copyright 2012 Royal Society of Chemistry.

4. OUTLOOK AND CONCLUDING REMARKS

Efficient methods to obtain diverse collections of drug-like small molecules are essential for the discovery of new bioactive small molecules. To address this issue, Schreiber et al. developed DOS, a synthetic strategy to populate new chemical space with novel drug-like compounds with a high degree of molecular diversity.[9,23−25] For the successful application of DOS in the fields of chemical biology and drug discovery, two issues must be addressed. The first issue is how to increase the molecular diversity of compound libraries so as to maximize coverage of the chemical space. Since the introduction of DOS, many different strategies have been developed to generate diverse compound libraries with a high degree of molecular diversity in terms of appendage and functional group diversity, skeletal diversity, and stereochemical diversity. These DOS strategies can be categorized as either reagent-based or substrate-based approach.

The second issue is how to design and synthesize molecular skeletons with improved biological relevancy. This issue has received relatively little attention in the synthetic chemistry community because it is more difficult to enhance the biological relevancy of a chemical library than to increase its molecular diversity. Unlike the modification of known bioactive natural products or bioactive drug-like compounds, the construction of novel molecular frameworks with high biological relevancy is quite challenging because of the undefined boundary between biologically relevant and non-bioactive chemical spaces. To guide navigation of the unexplored biologically relevant chemical space, strategies for library design have been

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developed based on pre-validated regions of bioactive space. Through a combination of diversity-generating reactions and biologically relevant structural units such as natural products or their core skeletons, BIOS and natural product-inspired DOS generate novel collections of natural product-like small molecules for the discovery of new bioactive small molecules that can perturb specific biological pathways.

In this Perspective, we introduced pDOS, a subclass of DOS, which generates polyheterocycles containing privileged substructures that serve as chemical "navigators" to efficiently access unexplored tracts of bioactive chemical space. The capability of privileged substructures to serve as chemical "navigators" was demonstrated using specific examples of novel bioactive small-molecule modulators that were discovered using pDOS strategy. It is worth mentioning that the excellent specificity of bioactive pDOS-derived compounds discussed in this Perspective is probably due to the unique three-dimensional structures of polyheterocycles, which emphasizes the importance of privileged substructures as chemical "navigators" of biologically relevant chemical space. We have been particularly interested in the construction of non-traditional polyheterocyclic drug-like molecules because we believe that their large surface area and limited conformational flexibility might allow specific disruption of biomacromolecular interactions such as protein−protein and protein−DNA/RNA interactions. Along with BIOS and natural product-inspired DOS, pDOS can generate collections of novel small molecules with high molecular diversity and biological relevancy that are highly useful for the discovery of novel therapeutic agents and the study of untapped biological processes. In these endeavors, privileged structures can serve as chemical "navigators" for the efficient construction of biologically relevant small-molecule libraries with diverse structural features. Therefore, the pDOS strategy will be an essential tool in chemical biology research and drug discovery aimed at modulating diverse biological pathways including traditionally "undruggable" targets.

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Notes

The authors declare no competing financial interest.

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