Developments in Applied Microbiology and Biotechnology

ENDOPHYTIC FUNGI

The Full Story of the Untapped Treasure

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CHAPTER

4

In silico prediction and characterization of secondary metabolites from endophytic fungi

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4.1 Introduction

Endophytic fungi belong to the phyla of fungi, and they reside in the inner tissues or underneath the plant epidermal cell layer. Plants such as shrubs, marine algae, lichens, palm, and grasses, seagrasses, medicinal plants, and trees have been known to harbor endophytic fungi (Ganesh Kumar and Mongolla, 2018; Rungjindamai et al., 2008; Thi Minh Le et al., 2019), being a rich source of bioactive secondary metabolites, enzymes, peptides, and biocontrol agents with numerous applications (Harper et al., 2003; Yadav et al., 2019). Hence, a lot of research has been focused on endophytic fungi and resources. Apart from these, endophytic fungi are finding applications in biofuels and agricultural crop products (Sharma et al., 2016). Especially secondary metabolites isolated from plant endophytic fungi have been given much attention. Excessive and inappropriate usage of antibiotics has led to the emergence of drug-resistant pathogens. The different treatment strategies applied for these drug-resistant pathogens have proven futile. Hence, there is a continuous attempt at finding new antagonistic compounds that will help in treating the new emerging and multi-drug-resistant infections. In this regard, the endophytic fungi are one of the natural sources of bioactive metabolites novel to science and holding promise (Castillo et al., 2002; Choi et al., 2005; Sadrati et al., 2013; Rani et al., 2017; Wang et al., 2007). Research on the metabolites of endophytic fungi has shown its applicability not only in antimicrobial bioactive compound production but also as producers of antioxidants and anticancer compounds (Harper et al., 2003; Lee et al., 1996; Wani et al., 1971; Strobel et al., 2002). These secondary metabolites isolated from endophytic

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fungi exhibit high biological activities; hence, they are prospective and promising lead compounds in drug discovery.

The process of novel drug molecule selection comprises many vital steps including the removal of compounds with side effects and those with the possibility of interaction with other drugs. To design novel drugs, pharmaceutical companies employ in silico drug designing software. The drug designing software and programs play a major role in the molecular modeling of the genes responsible for drug synthesis, gene expression, gene sequence analysis, and 3-D structure of proteins or drug molecules. In silico methods have been of great importance in prediction of novel drugs, their structure as well as drug target identification. These are breakthrough methodologies applied to microbiomes of plants for natural bioactive compound discovery. The methods comprise bioprospecting of endophytic microorganisms, prediction of novel biomolecules from endophytes, and regulatory control of endophytic biosynthetic machinery. The use of these strategies has resulted in the fruitful exploitation of the enormous databases available (genomic, metabolomic, regulomic, and chemical), thus helping in discovering or help discovering the enormous untouched treasure of natural products. This chapter gives an insight into the endophytic fungal secondary metabolite characterization and its structure prediction using in silico methods for efficient drug discovery.

4.2 Secondary metabolites produced by endophytic fungi

Plant microbiomes, especially the endophytic microbiome, are the most promising microorganisms for their secondary metabolite production. Fungal endophytic species are endosymbiosis fungi that live inside plants without causing physical harm to their hosts (Kumar and Kaushik, 2012). Plants harboring these endophytes produce specific compounds during their growth inside the plants. These compounds are secondary metabolites that are produced at the site of fungal growth. Long-evolved close interactions of the microbiome help protect the plants from pathogens while the plants provide nutrition and shelter. They play a major role in the chemical defense of plants (White and Torres, 2010; Hiruma et al., 2018; Munir et al., 2020). In order to protect itself and its host from harmful invaders or hostile interactions, the endophytic microbiome resorts to chemical defense. Hence, endophytes show higher incidences of defense roles, such as antimicrobial and antiviral, as compared to free-living microbes (Gange et al., 2019; Gundel et al., 2020). The endophytic fungi residing in plants, especially medicinal plants, play a role in the alterations of metabolite production and higher amounts of active compounds in medicinal plants. Fungi are known to produce a wide array of natural compounds that are secondary metabolites (SMs), which are responsible for a number of biological activities. An often-cited literature survey showed that of the 1500 compounds that have been isolated from fungi between 1993 and 2001, more than half displayed antibacterial, antifungal, or antitumor activity. A newer review covering fungal natural products that were discovered between 2009 and 2013 confirms the enormous potential of the fungal secondary metabolome (Pelaez, 2005; Schueffler and Anke, 2014; Keller, 2019). There is a record of 224 novel compounds being identified from endophytic fungi in a span of 3 years from 2014 to 2017 (Li et al., 2018). At least a few hundred of these compounds

were found to display significant bioactive activities as well as therapeutic properties, such as antibiotic, antiparasitic, anticancer, antidiabetic, antiviral, neuroprotective, and immunosuppressive effects (Keller et al., 2005).

The large-scale production of these compounds is possible by isolating and growing the endophyte in the laboratory, and this method is much easier than chemical synthesis of these compounds. Furthermore, many of these useful compounds cannot be produced chemically due to the complexity of their molecular structures (Keller, 2019). Studies have shown the presence of multiple endophytic fungi can inhabit a host plant at a given time. For example, 56 endophytic fungi were isolated from roots and leaves of *Salvia abrotanoides* (Teimoori-Boghsani et al., 2020). They reported secondary metabolite profiles of endophytic fungi isolated from *S. abrotanoides* plants obtained different geographically distinct sites and showed site-specificity and root-dominated colonization. Since fungal species may be present in numerous host plants such as *Verticillium dahliae* a plant pathogen has been found to infect 200 host plants worldwide. This endophyte has yet to be studied for its secondary metabolite composition (Shi-Kunne et al., 2019).

A total of 134 journal articles (from 2017 to 2019) were reviewed by Zheng et al. (2021), and the authors have summarized the chemical structures of 449 new metabolites, including polyketides, terpenoids and steroids, as well as various biological activities and structure-activity relationship of some compounds were also described.

The SMs of fungal origin are classified into four main groups based on core enzymes and precursors involved in their biosynthesis. The four groups are polyketides (e.g., aflatoxin), nonribosomal peptides (e.g., penicillin), terpenes (e.g., carotene), and indole alkaloids (Keller et al., 2005). The core enzyme for polyketide production is polyketide synthases (PKSs), which are responsible for their chemical scaffold. These are further divided into sub-types, and types I and II are found in fungi (Cox, 2007; Gallo et al., 2013; Hashimoto et al., 2014). Similarly, the enzymes nonribosomal peptide synthetases (NRPSs) are responsible for nonribosomal peptide synthesis, while terpene cyclases and dimethylallyl tryptophan synthases bring about the synthesis of terpenes and indole alkaloids, respectively. Both the enzymes, PKS and NRPS, are studied extensively in fungi (Cox, 2007; Erken et al., 2021). Boettger and Hertweck (2013) reported hybrid enzymes such as PKS-NRPSs that are also involved in the secondary metabolite synthesis by the fungal strains.

Genomic studies have shown the genes that are involved in secondary metabolite biosynthesis to be located in close proximity to each other, forming secondary metabolite clusters (Keller and Hohn, 1997; Brakhage and Schroeckh, 2011; Wiemann and Keller, 2014). These genes are expressed by plant-pathogenic fungi during infection, secreting the SMs to enable establishment in the plant tissues, for instance, as cytotoxic compounds. A single biosynthetic core gene is present in the cluster along with other genes whose products are required for modifying the SMs. These genes include transporter proteins, transcription factors, and genes encoding tailoring enzymes that flank the biosynthetic core genes (Keller and Hohn, 1997; Keller et al., 2005). It can be said that the study of fungal SMs is aided and accelerated by the application and advent of new genomic tools (Wiemann and Keller, 2014; Medema and Fischbach, 2015). Application of genomic knowledge, that is, the phylogenetic analysis and comparison of the genomes, has indeed helped to identify gene clusters that are involved in the production of SMs that have been categorized or studied in other fungal species

(Shi-Kunne et al., 2019). This know-how is thus a tool for the prediction of identical or related compounds that a particular fungal species is capable of producing (Medema et al., 2013; Cairns and Meyer, 2017).

4.3 Characterization of endophytic fungal secondary metabolites

The SMs produced by fungal endophytes have an extensive range of biological activities and can be structurally categorized into several groups including alkaloids, steroids, flavonoids, glycosides, xanthones, quinones, phenylpropanoids, aliphatic metabolites, terpenoids, and lactones (Zheng et al., 2006). Most of the studies involving the characterization and identification of SMs are carried out using analytical methods and instruments (Ebrahimi et al., 2021).

To unravel the SM production potential of *Verticillium dahlia,* in silico predictions and in-depth analyses of its secondary metabolite clusters were carried out. Using distinctive traits of gene clusters and the conserved signatures of core genes, 25 potential SM gene clusters were identified (Shi-Kunne et al., 2019). Fungal endophytes associated with antimalarial medicinal plant *Artemisia annua* were studied to produce SMs having antimalarial activity. The study was carried out by using Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS)-based metabolomics and multivariate analyses and resulted in the identification of eight potentially active metabolites (Alhadrami et al., 2021).

Different analytical methods can be applied to separate and detect SMs. Some of these methods involve the use of thin layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC), ultra-high-performance liquid chromatography, micellar capillary electrophoresis, flow injection-electrospray mass spectrometry, ultraviolet diode array detection and nuclear magnetic resonance detection (Wadood et al., 2013).

For drug discovery, microbial cultures are grown, and the extracts of the microbial cells are intracellular while culture supernatants are used for extracellular secondary metabolite (Cragg et al., 1997; Shu, 1998). In addition to the routine practice of screening microbial fermentation extracts for antibiotic activity, extracts can now be routinely screened with a variety of new assays such as functional, receptor binding, enzyme inhibition, and protein-protein interaction assays (Wadood et al., 2013). The process starts with identifying the collections of microorganisms that have the ability to produce the SMs, standardizing culture conditions that support maximal secondary metabolite production, and analyzing methods for identifying the metabolite and checking its efficacy. Fermentation conditions highly influence the production of SMs by microorganisms by eliciting production, significantly changing product concentrations as well as production of rare metabolites have been reported (Yarbrough et al., 1993; Monaghan et al., 1995). Identification and activity checking require the preparation of fermentation extracts for analysis by or application to automated high-throughput screening assay systems, which is one strategy to align natural product drug discovery.

Schmid et al. reported the analyses of fermentation broth by a multistage automated solid-phase extraction system (Schmid et al., 1999). The high-throughput screening assays involve making the fermentation extracts compatible and thus become cost intensive (Higgs et al., 2001) and not advisable as in most fermentation extracts, the amount of product may be

too insufficient for detection. Thus, direct chemical detection assays or measurements such as methods for detecting the SMs used for classification and characterizing microbes can be used (Erhard et al., 1997; Feistner, 1994; Filtenborg et al., 1983; Frisvad et al., 1989; Smedsgaard and Frisvad, 1996). Frisvad et al. (1989) reported the characterization of fungal isolates by detecting SMs produced using high-performance liquid chromatography (HPLC) diode array detection (DAD) and flow injection analysis together with electrospray ionization mass spectrometry (ES-MS). Similar reports of analytical work on fungal metabolites have been reported by other workers (Filtenborg et al., 1983; Frisvad et al., 1989; Julian et al., 1998; Smedsgaard and Frisvad, 1996).

With chromatographic systems that combine two or more analytical techniques such as optimum performance laminar chromatography (OPLC), HPLC-DAD, gas chromatography-mass spectroscopy (GC-MS), liquid chromatography-mass spectrometry (LC-MS), or direct infusion-electrospray ionization-mass spectrometry (DI-ESI-MS), allow developing rapid and effective analytical methods in order to define the quality and safety of plant-derived products. Accelerated solvent extraction or microwave-assisted extraction in combination with hyphenated techniques such as GC-MS and LC-MS represents a modern approach to performing fast and reproducible analytical methods for the quality control of secondary metabolite production. Several types of SMs may remain unnoticed because of inefficient extraction procedures, low analytical sensitivity, or low reproducibility under different growth and metabolic conditions.

Further, after the isolation and identification of the metabolites, it is important to check the effectiveness of the metabolites as bioactive agents. This is checked by activity assays involving in vivo assays like antimicrobial activities, anticancer activities, etc. The antibacterial, cytotoxic, and antidiabetic activities of endophytic *Diaporthe eres* (SPEF004) were carried out after profiling the bioactive molecules produced by the strain. The antimicrobial assay using the ethyl acetate extract of the fungal biomass showed activity in the range of 9.06–27.5 mm against Gram-positive and Gram-negative bacteria. The fungal extract also showed the presence of antioxidant activity, amylase inhibitory activity, α-glucosidase activity, as well as anti-proliferation activity toward human breast cancer cell line (MDA MB231). GC-MS analysis and docking experiments with breast cancer-related heat shock protein (HSP90) were used to study structure and activity prediction (Saravanakumar et al., 2021). This example shows that the use of in silico prediction methods will accelerate the process of drug discovery and design, thereby making treatments available for diseases like cancer, infections caused by drug-resistant pathogens, and re-emerging infections.

4.4 In silico methods for secondary metabolite selection and activity prediction

Plant fungal microbiomes are the major source of SMs, waiting to be discovered and utilized for the treatment of diseases not curable by available bioactive. Yet, most endophytic microbes within plants appear to be uncultivable. The cultivable microbiome of the plants produces the SMs upon activation of the biosynthetic genes, which are under the control of regulatory silencing. Recently, numerous interdisciplinary approaches, including multi-omics methods, have been explored and applied to study the host-microbiome interactions

along with computational approaches, which helped in finding the functional distribution of the microbiome. These approaches have been successful in natural product discovery from plant-associated microbes. Possibly, the key to characterizing and exploiting the biometabolite bulk depends on a novel, systematic approach to characterize the signals that trigger the biosynthesis of SMs. Recently, in the designing of the T cell epitopes-based peptide vaccine from envelope protein of 2019-nCoV as a target, potential peptide selection was carried out by using several techniques employing a combination of immune-informatics approach and comparative genomic approach (Abdelmageed et al., 2020).

Two techniques are utilized for predicting the interaction site and dynamics of the interaction, namely, molecular docking and molecular dynamic simulation, respectively. These techniques help to select the biological activity of varied potentially active compounds in a cost- and time-effective in silico manner (Tazikeh-Lemeski et al., 2020; Mandal et al., 2009). Molecular modeling methods are nowadays applied in various fields of science, especially in drug design and discovery (Ansari et al., 2018; Moradi et al., 2019a,b). For example, using docking and molecular dynamic simulation, the interactions and potent inhibitory effects of some important fungal SMs were investigated on SARS-CoV-2 nsp12 (Wang et al., 2020). In this study, after two steps of molecular docking, the more potent compounds were evaluated in terms of their effects on the structure and dynamic of the protein. Finally, after the exploration of their pharmacological aspects, the best metabolites are introduced for further experimental studies (Wang et al., 2007; Zhou et al., 2020; Shah et al., 2020; Wang and Guan, 2021; Wang, 2020). Hence, these strategies have increased the pace of finding effective treatment in the form of SMs.

4.4.1 Molecular docking

Molecular docking, one of the sub-techniques of molecular modeling, is a key tool in structural molecular biology and computer-aided drug design. This study involves finding the fit ratio between two or more molecular structures (e.g., drug and enzyme or protein) (Kirkpatrick, 2004; Dar and Mir, 2017). It is a molecular modeling technique that is used to predict how a protein (enzyme) interacts with small molecules called ligands. Depending upon the interaction of the protein (enzyme) with ligands forming a supramolecular complex, the dynamics of the protein and, thus, its functional role will be either enhanced or inhibited. The interaction of the ligand with the target protein and its positioning and fit within binding pockets is determined by molecular docking. The correct pose of the ligand in the binding pocket of a protein predicts the affinity between the ligand and the protein. Based on the types of ligand, docking can be classified as protein-small molecule (ligand) docking, protein-nucleic acid docking, and protein–protein docking. Protein-small molecule (ligand) docking symbolizes a simpler end of the complex spectrum, and the number of available programs executes particularly well in predicting SMs that may potentially inhibit proteins.

4.4.2 Docking procedure

Docking is performed by insertion of the rigid molecules or fragments, that is, ligands, into the protein's active site using different approaches like clique-searching, geometric hashing,

or pose clustering. The docking performance depends upon the search algorithms that are used such as Monte Carlo (MC) methods, genetic algorithms (GAs), Tabu searches, fragment-based methods, and distance geometry methods. Scoring functions employed can also affect docking performance, such as force-field (FF) methods and empirical free energy scoring functions. Fig. 4.1 is a flowsheet depicting the molecular docking procedure.

In the first step of docking, all possible compositions of conformations and orientations of the protein paired with the ligand are generated. The second step involves the scoring function that indicates the favorable interaction of the ligand with the protein, which is referred to as the dock score (Ewing and Kuntz, 1997). Selection of the required X-ray cocrystallized structure from the protein data bank (PDB) is performed, followed by identification of the active site of the protein by extracting the bound ligand. A particular pose of the ligand within the protein binding site can be evaluated by counting the number of favorable intermolecular interactions such as hydrogen bonds and hydrophobic contacts. Various poses are evaluated based on their compatibility with the target in terms of shape and properties such as electrostatics. The most favorable pose is recognized based on the energy level, and the corresponding dock score is generated. A good dock score for a given ligand signifies that it is potentially a good binder. Before starting the docking studies, identification of the bound ligand in the co-crystal structure is important as the knowledge about its interaction with the corresponding protein's amino acid residues.

4.4.3 Essential requirements of docking

1. Receptor crystal structures: Crystal structure of the receptor is required. This can be determined by X-ray crystallography or nuclear magnetic resonance and can be easily downloaded from the PDB (http://www.rcsb.org/pdb/home/home.do).
2. Receptor homology modeling and threading techniques (Peitsch et al., 2002; Zimmer, 2002): these are methods that can be used in case crystal structure is not available.
3. A set of ligands of interest: useful in docking techniques to apply to the crystal structure.

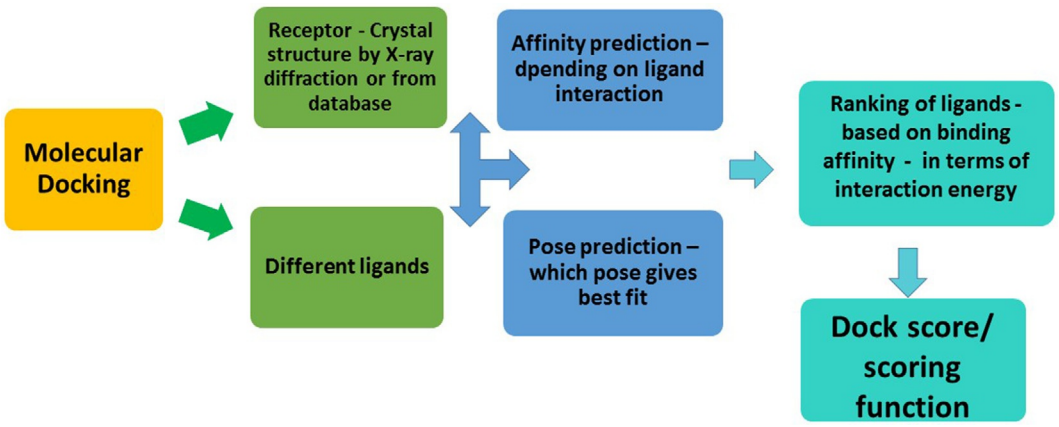

FIG. 4.1 Flowchart for molecular docking studies.

4.4.4 Difficulties faced in docking studies (Kroemer, 2007; Teague, 2003)

Considerable work has been done in molecular docking; however, some difficulties or drawbacks need to be resolved.

(a) Water molecules in protein: interaction energy of the protein-ligand binding is dependent on the water molecules. Holding water molecules affects the binding and affinity of the ligand. Thus, it is important to locate or assign a position to the water molecules from which it will interact with the protein and ligand.

(b) Tautomers and protomers: the molecules can adopt different tautomeric and protomeric states. Prior to docking, the molecules need to be ionized, and tautomer generation followed by decision on tautomer to be used is important.

(c) Docking into flexible receptors: docking with flexible receptors is another challenge in molecular docking, as it can change its conformation.

4.4.5 Applications of docking in the drug discovery process

Molecular docking plays a major role in the lead drug identification process, as well as in potential target identification against different infections (Kroemer, 2007).

- Geometrical studies of ligand-receptor complexes.
- Modify lead molecules for potency optimization.
- Designing libraries and generation of data banks.
- Screening for the side effects as a result of ligand-protein interactions.
- Specificity checking of potential drugs against homologous proteins.
- Protein-protein interaction predictions.
- Create knowledge of the molecular association involved in, for example, biometabolite biosynthetic pathways.
- Explore potential pharmacological targets.
- Database search for leads in novel drug discovery.

Number of workers have reported the application of in silico methods for detection and activity prediction of SMs from endophytic fungal strains (Table 4.1).

4.5 Conclusion and future prospects

Fungal endophytes are a major source of antagonistic biomolecules and other medically important compounds. Detection and characterization of these compounds pose a problem due to their highly complex chemical makeup. Thus, utilizing cheminformatics tools such as metabolomics and computer-aided modeling is of great help in dealing with such complexity and selecting the most probable bioactive candidates (Irwin and Shoichet, 2016; Alhadrami et al., 2021).

In silico methods have been of great importance in target identification and in prediction of novel drugs. Drug discovery and development is a very complicated, time-consuming process, and there are many factors responsible for the failure of different drugs such as lack of

TABLE 4.1 Activity prediction of endophytic fungal secondary metabolites by in silico methods.

Bioactive compound	Target	Activity	System	Reference
(Pyrrolidine-5-one, 2-[3-hydroxypropyl]-)	Chaperone protein HSP90	regulate the breast cancer-related proteins such as ERα, PR, EGFR, mTOR, tumor suppressor p53 protein, Akt, Raf-1 MAP kinase, receptor tyrosine kinases, and angiogenesis transcription factor HIF-1alpha in erbB family	Autodock VINA	Zagouri et al. (2013); Powers and Workman (2006); Hoter et al. (2018); Verma et al. (2016)
4,8-Dihydroxy-3,4-dihydronaphthalen-1(*2H*)-one	C-terminal agrA DNA binding domain	Antibacterial activity	AutoDock Vina 4.05 was used to simulate docking	Trott and Olson (2010)
Dankasterone B and pyrrocidine A	New coronavirus RNA-dependent RNA polymerase	Anti-coronavirus	MGLtools package Lamarckian genetic algorithm	Ebrahimi et al. (2021)
Heptadecanoic acid, 16 methyl-, methyl ester; 9,12-octadecadienoic acid; cis-9-octadecenoic acid	Skin cancer protein (Hsp90)	–	–	Kandasamy et al. (2012)
Emodin and physcion		Antimalarial	Neural-network-based prediction software PASS	Irwin and Shoichet (2016)
1,6,8-Trihydroxy-4-benzoyloxy-3-methylanthraquinone (23)	Human cyclin-dependent cytotoxic potential toward kinase 2 (CDK-2), human DNA topoisomerase II (TOP-2), and matrix metalloprotinase 13 (MMP-13)	–	–	Youssef and Singab (2021)

effectiveness, side effects, poor pharmacokinetics, and marketable reasons. The expenditure of this process has amplified ominously during the past 34 years. Presently, the cost involved in the drug discovery process ranges between $800 million to $1.8 billion (Irwin et al., 2002).

The in silico drug design is a vast field in which the different sides of basic research and practice are combined and inspire each other (Bernard et al., 2005). Modern techniques such

as QSAR/QSPR, structure-based design, combinatorial library design, cheminformatics, bioinformatics, and the increasing number of biological and chemical databases are used in the field. Furthermore, large numbers of the available tools provide a much-developed basis for the design of ligands and inhibitors with preferred specificity (Chothia and Lesk, 1986). The establishment of the Computer-Aided Drug Design Centre has further aided in facilitating the in silico drug designing process (Taft et al., 2008). These developments have made it possible to dock libraries of 10 million molecules against targets over several days or weeks. As a result it is possible to get to the market a large number of screened bioactive molecules and test them for potential drug leads. Although docking does have its disadvantages, it can distinguish likely from unlikely ligands, often with hit rates above 10% (Irwin and Shoichet, 2016). Docking-based virtual HTS is less expensive than normal HTS and faster than conventional screening (Kunal et al., 2015). As in the case of endophytic fungi, both cultivable as well as uncultivatable, computational learning-based chemical structure prediction will be especially helpful for overcoming the need for isolation and synthesis, but also such approaches can narrow the search for targets for downstream experimental unsilencing.

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