

Marine Natural Product Libraries for High-Throughput Screening and Rapid Drug Discovery

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There is a need for diverse molecular libraries for phenotype-selective and high-throughput screening. To make marine natural products (MNPs) more amenable to newer screening paradigms and shorten discovery time lines, we have created an MNP library characterized online using MS. To test the potential of the library, we screened a subset of the library in a phenotype-selective screen to identify compounds that inhibited the growth of BRCA2-deficient cells.

Historically, natural products and their derivatives have been a major source of pharmaceutical leads and therapeutic agents.¹ In particular, marine natural products (MNPs) have demonstrated exceptional potency and potential as anticancer therapeutics.^{2,3} Natural products have evolved over millions of years to elicit precisely tuned biological effects. As a result, many natural products occupy chemical space that is different from and complementary to that in synthetic libraries, making natural product libraries attractive for drug discovery.^{4,5}

Large screening campaigns have demonstrated the need for high-quality molecular libraries in order to promote drug discovery.⁶ However, crude natural product extracts are not well suited for high-throughput screening especially in single protein assays.^{5,7} In the pharmaceutical industry, difficulties encountered with natural products in high-throughput screening (HTS) campaigns have resulted in a decreased emphasis in natural products drug discovery.⁵ In an attempt to fully exploit the chemical diversity present in sponges and ascidians, we have developed methodology to produce high-purity natural product libraries for high-throughput screening and phenotype-selective screens.

A major goal for developing libraries was to address the issues that have made natural products less amenable to the high-throughput paradigm. These include the complexity of crude extracts, dereplication time, and a lag in prioritization of lead compounds compared to synthetic libraries. However, advances in approaches to natural products drug discovery incorporating new technologies have helped to overcome these difficulties.^{8,9} The pioneering work by researchers at Sequoia Sciences set the stage for combining MS analyses with the generation of large natural product libraries.^{10,11} More recently, the potential for marine natural product libraries to accelerate drug discovery was demonstrated using a yeast halo assay.¹²

We have developed a two-dimensional chromatographic strategy that includes an automated HPLC-MS fractionation protocol to generate natural product libraries that are sufficiently pure for HTS and characterized by accurate mass during production to expedite dereplication of known compounds and identification of novel chemotypes. The final fractionation is directed into 96-well format with each well being mapped to an MS chromatogram. Through the use of monolithic HPLC column technology, we can obtain sufficient material in each well from a single HPLC injection not only for performing multiple screens but also for NMR studies. Using this approach to generate screening libraries, we have performed selective screens, confirmed the structures of active leads, and identified a likely mechanism of action directly from the library.

Effectively, this methodology has the potential to eliminate bioassay-guided fractionation.

We utilized HP20SS for initial separation followed by C18 RP-HPLC. This two-dimensional approach using orthogonal techniques afforded essentially unit resolution. Since the marine invertebrate extracts contained high amounts of inorganic salts, we needed to effectively desalt the extract prior to any LCMS analysis to avoid suppressing ionization. This was effectively performed by drying extracts onto HP20SS and performing solid-phase extraction (SPE). Washing the extract-loaded resin with 100% H₂O efficiently removed salts, while the organics were separated using a four-step elution (see Experimental Section). The eluates were subsequently plated in 96-well plates for use in an autosampler, dried in a centrifugal evaporator, and resolubilized in DMSO prior to HPLC. The effluent from the HPLC was split after a photodiode array detector with the majority going to a fraction collector and a small amount to a Q-tof Micro equipped with lockspray to enable accurate mass measurements. Fractions were obtained using one-minute time slices and were mapped on both the mass and PDA chromatograms using FractionLynx to correlate the contents of each well in the collection plate.

One difficulty was to effectively separate a wide variety of natural product mixtures in a slug of DMSO while processing enough material for screening and follow-up NMR studies. Since our fraction collection was limited to <2 mL volumes due to the capacity of a deep-well collection plate, we had to achieve separation without resorting to traditional semipreparative chromatography. This problem was solved by utilizing a Phenomenex 3 mm × 100 mm Onyx C18 monolithic HPLC column. Although this column is much smaller than a traditional semipreparative column, we were able to obtain efficient separation of most HP20SS fractions on 2 mg dissolved in 200 μL of DMSO. Monolithic columns have demonstrated increased efficiency and higher capacity with decreased back pressure when compared to traditional packed HPLC columns.^{13,14} After HPLC, the contents of the collection plate were split to make two identical daughter plates, one for screening and one for a material archive. We have now processed 768 HP20SS fractions to generate a 15 360-membered marine natural products library for HTS and phenotype-selective screens.

To demonstrate the potential of the library for drug discovery, the library was screened in a paired cell line phenotype assay to identify leads that demonstrated selectivity toward BRCA2-deficient tumor cells. Optimal cancer chemotherapy would allow the selective elimination of tumor cells over normal cells by exploiting the molecular differences between tumor cells and normal cells. The genetic susceptibility to breast cancer caused by mutations in BRCA1 and BRCA2 provides an opportunity to identify small molecules that selectively inhibit the growth of BRCA2-deficient cells. Both BRCA1 and BRCA2 proteins are important for the repair of double-strand DNA breaks. As a result, BRCA2-deficient cells

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Table 1. Differential Cytotoxicity

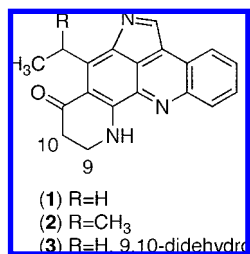
	IC ₅₀ , μ M		SI ^a	compound(s)
	VC-8	BAC29		
MPI-0454126	0.30	0.99	3.29	plakinidines A (1), B (2), and C (3) ^b
MPI-0454127	0.04	0.16	5.47	plakinidines A (1) and C (3) (~2:1)
MPI-0454128	0.02	0.14	7.90	plakinidines A (1) and C (3) (~2:1)
MPI-0454141	0.17	1.10	5.91	plakinidine B (2)
MPI-0453967	0.23	1.37	5.91	plakinidines A (1), B (2), and C (3) (~1:1:8)
mitomycin C	0.005	0.4	292	

^a The fold change in the IC₅₀ values of proficient, BAC29, and deficient V-C8 cell lines was reported as the selectivity index, SI. ^b Ratio could not be determined due to a poor baseline in the ¹H spectrum.

have an elevated need for utilizing error-prone DNA repair pathways and are hypersensitive to ionizing radiation and DNA cross-linking agents.^{15,16}

In order to identify novel small-molecule inhibitors with specificity for cells compromised in BRCA2 function, we designed a phenotypic screen. This approach utilized a matched set of hamster cell lines, including one that was mutated at the BRCA2 locus, V-C8, and therefore deficient for BRCA2. The other line, BAC29, was wild-type for BRCA2; the BRCA2 protein was restored by complementation with a mouse BRCA2 gene.¹⁵

Initially, a portion of the library (5000 wells) was screened for toxicity in V-C8 cells. All samples showing >75% reduction in cell viability were subsequently screened in V-C8 and isogenic BAC29 cells. In order to validate the assay performance and the biological response of the BRCA2-deficient and -proficient cell lines, they were treated with the DNA alkylating agent mitomycin C. The viability of cells was measured using the WST1 reagent three days after compound exposure. Mitomycin C was cytotoxic in both cell lines, exhibiting more than 200-fold selectivity for BRCA2-deficient cells (see Table 1). Of the 252 cytotoxic fractions from the MNP library, only five showed 3- to 8-fold selectivity for V-C8 cells over BAC29 cells (see Table 1). Four of the five were from the same sponge, *Plakortis quasiamphiaster* Díaz and van Soest, 1994 (family Plakinidae), and the remaining sample was from a *Crella spinulata* (Hentschel, 1911) (family Crellidae).



Once the activity of the five prioritized wells was confirmed, we extracted the MS data from the chromatogram that corresponded to each well. On the basis of accurate mass measurements and taxonomy of the *P. quasiamphiaster*, we were able to quickly propose the presence of plakinidines A, B, and C (1–3).^{17–19} A literature search on *C. spinulata* suggested compounds such as crellastatin A,²⁰ which was reported to be a colorless compound and a potent inhibitor of cell growth. However, this class was not supported by the MS data, and the active fraction was reddish-purple. In order to confirm the structures of the compounds, the contents of the prioritized wells from the archive plate were transferred directly to an NMR tube for analyses. On the basis of equal distribution, we anticipated that each well in the archive plate would contain approximately 50 μ g and NMR analyses might be difficult. However, we were able to obtain data using a 600 MHz cryoprobe (see Figure 1, Supporting Information). We were able to obtain gCOSY and gHSQC spectra on each sample and confirm the contents of each well.

Interestingly, the NMR data for the active sample from *C. spinulata* indicated it was a mixture of plakinidines, which have

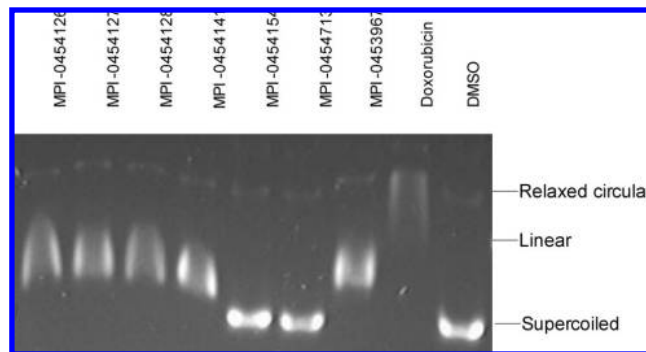


Figure 1. Five samples (MPI-0454126, -0454127, -0454128, -0454141, and -0453967) reduce the electrophoretic mobility of negatively supercoiled DNA, indicative of DNA intercalation. MPI compounds (including two unrelated samples, MPI-0454154 and -00454713, as negative controls) were incubated with DNA at a concentration of 33 μ M prior to electrophoresis. Doxorubicin, a known intercalator, was included at 50 μ M as a positive control. Relaxed circular and linear DNA markers were included on the gel for reference.

not been previously reported from this sponge. For the *C. spinulata*, a bright red sponge, only one colored metabolite has been reported, benzothiocrellidone, a bright yellow solid.²¹ We believe that the selective activity of the plakinidines in *C. spinulata* would have been masked in a crude extract by the cytotoxic crellastatins. This example clearly illustrates the power of screening purified MNP libraries in phenotype-selective assays.

On the basis of the pyridoacridine structure and selective cytotoxicity in BRCA2-deficient cells, we hypothesized that the plakinidines were DNA intercalators. The preferential toxicity of these compounds could be explained by interaction of these compounds with DNA and the inability of the BRCA2-deficient cells to efficiently repair the resulting DNA damage that ensues during transcription or replication. To test this hypothesis, samples were taken directly from the screening plate and incubated at a concentration of 33 μ M with 250 ng of pRYG negatively supercoiled plasmid DNA (Topogen, Port Orange, FL) for 21 h. Samples were then subjected to electrophoresis on 1% agarose gels in the absence of ethidium bromide. Following electrophoresis, the gels were stained with 0.5 μ g mL⁻¹ ethidium bromide. Intercalation between bases in DNA reduces negative supercoiling, which was readily monitored by alteration in electrophoretic mobility of plasmid DNA in agarose gels. The reduced electrophoretic mobility in the presence of samples from all five wells indicated a reduction of the negative superhelicity of the plasmid DNA (see Figure 1).

We have demonstrated that high-quality MNP libraries of sufficient purity for HTS and phenotype-selective screening can be generated. In most cases, we observed three compounds or less per well. For the example presented here, our method has eliminated bioassay-guided fractionation and allowed rapid identification of lead structures while providing enough sample for multiple screens. Additionally, we have been able to identify a mechanism of action for the cytotoxicity of the plakinidines without the need to re-extract

the source organism. Our methods also allowed identification of the plakinidines from a sponge that was previously reported to produce cytotoxic compounds, but not the plakinidines. The prevalence of the cytotoxic crellastatins²⁰ could easily mask the presence of the minor plakinidines using traditional bioassay-guided isolation. These studies indicate that our methodology can uncover components that have been previously masked by major bioactive compounds.

Overall, we were able to create a large MNP library consisting of pure and semipure samples for drug discovery. Online mass measurements expedited the identification of known compounds, and we anticipate that the MS data will facilitate the identification of novel chemotypes in conjunction with taxonomy of the producing organism and database queries.

Experimental Section

General Experimental Procedures. All NMR data were obtained at 600 MHz on a Varian INOVA equipped with a cryogenically cooled ¹H channel. For gCOSY, gHMBC, and gHSQC experiments, standard vendor-supplied pulse sequences were used. Samples were dissolved in 250 μ L of DMSO-*d*₆ (Cambridge Isotope Laboratories) and placed in a 5 mm, DMSO-matched Shigemitsu tube. Spectra were referenced to DMSO resonances (δ_{H} 2.49 ppm; δ_{C} 39.5 ppm). All solvents used for HPLC were Optima LCMS grade (Fisher Scientific). All NMR resonances matched those previously published for plakinidines A (1), B (2), and C (3) in DMSO-*d*₆.¹⁹

Biological Material. Sponge specimens were collected in Fiji near Naukathuvu Island using scuba. Voucher specimens for the *Plakortis quasiampfiaster* (88-5-110) and the *Crella spinulata* (88-5-92) are housed at the University of Utah.

Extraction and Chromatography. All invertebrates were extracted with MeOH. The MeOH extract (24 mL) was loaded onto HP20SS (200 mg) and subsequently dried using a centrifugal evaporator. After the sample was dried, the "loaded" HP20SS was transferred to a 2.5 \times 9 cm polypropylene column fitted with a frit. Fifteen milliliters of each solvent (100% H₂O, 25% IPA/H₂O, 50% IPA/H₂O, 75% IPA/H₂O, and 100% MeOH) were pushed through the sample using a 60 mL syringe fitted to the top of the column to generate five eluates (FW, F1, F2, F3, F4, respectively). The solvents were removed from all samples using a centrifugal evaporator. The 100% H₂O fraction was not subjected to LCMS fractionation since we were attempting to create a library consisting of "drug-like" molecules, and it contained predominately sea salt. Each of the remaining four HP20SS eluates were dissolved in an appropriate volume of 3:1 MeOH/H₂O (F1), MeOH (F2 and F3), or ethanol (F4). Two milligrams of each HP20SS fraction was transferred into polypropylene 96-well plates (Greiner, 330 μ L) for direct use in the LCMS autosampler and subsequently dried in a centrifugal evaporator, while the remaining material was aliquoted into a deep 96-well plate (Greiner, 2 mL) for storage. Two hundred microliters of DMSO was added to the LCMS autosampler plate and shaken for 1 h to dissolve the sample.

LCMS Fractionation. The 96-well plate containing the dissolved HP20SS fractions was placed in the autosampler rack of a Waters 2795 HT HPLC. A sample experiment report can be found in the Supporting Information, which describes all parameters for HPLC, MS, and FractionLynx. MassLynx 4.1 was used to control the 2795 HPLC and the Micromass Q-tof Micro. The only parameter changed for each HP20SS fraction was the curve applied to the gradient. For F1, a #7 curved gradient was used to achieve better separation of polar metabolites, while a #5 curved gradient was used to achieve better separation of nonpolar metabolites. For F2 and F3, a linear gradient (#6) was used. HPLC separations were performed on a 3 mm \times 100 mm Phenomenex Onyx C18 column equipped with a guard column at a flow rate of 1.5 mL min⁻¹. Typically, a back pressure of ~1500 psi was observed at 10% CH₃CN/H₂O.

The HPLC effluent was split after the PDA using an adjustable splitter (Upchurch) that directed 30 μ L min⁻¹ to the Q-tof Micro. Collection was directed to each polypropylene deep-well plate (Greiner,

2 mL) in the fraction collector in an identical pattern to that required for screening, so that each well was appropriately mapped on the chromatogram by FractionLynx. In our case, columns 1 and 12 were empty.

The Waters fraction collector III was controlled by FractionLynx, and the details of collection are in the Supporting Information. Collection plates were dried in a centrifugal concentrator. The contents of each well were dissolved in 500 μ L of MeOH. Two daughter plates were generated by transferring 250 μ L to polypropylene shallow-well 96-well plates (Greiner, 330 μ L). One plate was stored dry at -80 $^{\circ}$ C for NMR analysis, while the other plate was resolubilized with 100 μ L of DMSO to make a 1 mM screening plate. The concentration was based on an average molecular mass of 500 Da and an estimate that each well contains 50 μ g.

For accurate mass measurements, leucine enkephalin was used as a reference mass [M + H]⁺ *m/z* 556.2771. This mass does not account for the loss of an electron, but is consistent with the molecular formula generator in MassLynx 4.1. All data were centroided, and the lock mass correction was applied during acquisition in order to reduce the file size. Accurate mass measurements were obtained directly from the MS data acquired during automated fractionation. Each was averaged over an area where the signal intensity was less than 0.1 ion per pusher pulse to ensure that the detector was not saturated.

Plakinidine A (1): HRESIMS [M + H]⁺ *m/z* 303.1243, calcd 303.1246 for C₁₈H₁₅N₄O (Δ -1.0 ppm).

Plakinidine B (2): HRESIMS [M + H]⁺ *m/z* 317.1395, calcd 317.1410 for C₁₉H₁₇N₄O (Δ -2.2 ppm).

Plakinidine C (3): HRESIMS [M + H]⁺ *m/z* 301.1075, calcd 301.1089 for C₁₈H₁₃N₄O (Δ -4.6 ppm).

Cell-Based Assay for Differential Cytotoxicity. The V-C8 mutant cell line derived from the Chinese hamster V79 cells and the BRCA2-complemented BAC29 cell line have been described previously.¹⁵ Cells were routinely cultured in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), 1 mM HEPES, 1 mM pyruvate, 1 mM glutamate, and nonessential amino acids. The BAC29 cells were also maintained in medium containing G418. Cells were maintained at 37 $^{\circ}$ C in a humidified 5% CO₂ atmosphere. In a preliminary experiment the optimal seeding density for each cell line and compound concentration range was determined. For the selectivity screen, V-C8 cells were seeded at 4000 cells/well and BAC29 cells at 3500 cells/well in 96-well clear-bottom plates (Greiner). Both cell lines were treated with compound, in triplicate, and allowed to grow for 3 days. After 3 days of compound exposure, the WST1 cell viability assay was performed and the absorbance read at A₄₉₀. Dose-response curves were generated with the help of Prism software by plotting percentage of A490 reduction in compound-treated relative to vehicle- or DMSO-treated cells.

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Supporting Information Available: Sample NMR spectra and a complete list of instrument settings are available via the Internet at <http://pubs.acs.org>.

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