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Drug-like Properties: Guiding Principles for the Design of Natural Product Libraries

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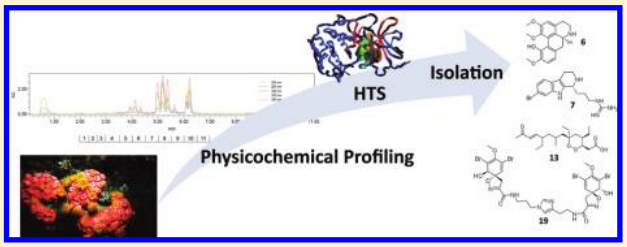
Supporting Information

ABSTRACT: While natural products or their derivatives and mimics have contributed around 50% of current drugs, there has been no approach allowing front-loading of chemical space compliant with lead- and drug-like properties. The importance of physicochemical properties of molecules in the development of orally bioavailable drugs has been recognized. Classical natural product drug discovery has only been able to undertake this analysis retrospectively after compounds are isolated and structures elucidated. The present approach addresses front-loading of both extracts and subsequent fractions with desired physicochemical properties prior to screening for drug discovery. The physicochemical profiles of natural products active against two neglected disease targets, malaria and African trypanosomiasis, are presented based on this strategy. This approach can ensure timely development of natural product leads at a hitherto unachievable rate.



A bottleneck in lead discovery for pharmaceutical research and for chemical biology lies in the identification of new, biologically relevant substances.[1] Lead generation requires the discovery of new molecules that possess the ability to reach and modify a biological target. This, in turn, requires the identification of compounds that are complementary to biological structure space.[1] Natural products (NPs) produced by biosynthetic enzymes possess an imprint of biology space (protein fold topology) as identified by the similarity in recognition of NPs in binding both to their biosynthetic enzymes and to a class of therapeutic targets (e.g., flavonoid biosynthetic pathway/protein kinases) even though the proteins are of disparate folds.[2,3] An investigation of structural motifs absent in screening libraries by Shoichet et al. found that 83% (12 977) of core ring scaffolds present in NPs were absent from commercially available molecules and, by extension, the majority of screening libraries.[4] They concluded that the inclusion of molecules into screening libraries containing scaffolds present in NPs, but absent from commercial collections, would improve hit rates.[4] NPs have continued to play a vital role in providing new therapeutic agents.[5−11] By way of example, in the period from January 1981 to mid October 2008, there were 1024 small-molecule new chemical entities (NCEs) introduced as drugs in Western medicine, and, overall, 50% of these were NPs, modified NPs, or synthetic compounds with a NP pharmacophore.[11]

Even if the 12 977 ring scaffolds derived from NPs unrepresented among commercially available molecules are restricted to a molecular weight (MW) of <350 and two or fewer stereocenters, there still remains 1891 unique scaffolds that can be enumerated into libraries. Given these large numbers, it is clear that scaffold synthesis and library generation will not, in the foreseeable future, cover the breadth of NP scaffolds. In previous work, we have demonstrated that it is possible to prepare a screening library of highly diverse NPs that are drug-like in their physicochemical parameters.[12] However, this delivers a limited subset of NP chemical space and, again, will fail to deliver a comprehensive coverage of NP diversity. The exposure of NP scaffolds (and related libraries) can be achieved only via a comprehensive sampling of the chemical diversity found in nature using high-throughput screening (HTS).

A number of recent reviews highlight the past achievements of NPs in affording new drugs, while at the same time providing a realistic evaluation of commitment within the pharmaceutical industry in the area of discovering new NP drugs.[13,14] The review by Harvey concludes that there is a clear need to develop new approaches to overcome the perceived disadvantages of NPs, such as (i) the difficulty in access and supply, (ii) the complexities of NP chemistry, (iii) the inherent slowness of working with NPs, (iv) concerns about intellectual property rights, and (v) aspirations associated with the use of collections of compounds prepared by combinatorial chemistry methods.[13] Williams reaches a similar conclusion regarding the future of NP drug discovery by commenting that overcoming the paradigm shift away from NP-based drug discovery to alternate strategies, such as combinatorial chemistry or fragment-based drug design, will require the development of new strategies and resources.[14]

A protocol that front-loads an extract with lead- and drug-like molecules could address the need for novel approaches to screening libraries based on NP motifs. The approach would

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ideally be guided by the physicochemical profiling of small-molecule libraries that occurs in the pharmaceutical industry and that has become an intrinsic part of the design and selection process. Importantly, such a scheme should also afford a screening set that captures new (analogue of a known motif) and novel (previously unreported skeleton) structures that comply with the lead- and drug-like concepts first articulated by Oprea[15] and Lipinski,[16] respectively, and further refined by Leeson and Davis.[17] Ideally, the set would also benefit from intrinsic structure−activity relationships (SAR) given that structurally related analogues of the most potent actives are often present and can be rapidly identified and assessed for activity without recourse to synthesis.

The usefulness of any molecule as a lead is generally dependent upon the compound being able to permeate cells in order to modulate cellular signaling pathways or to have wider systems biology effects. Much emphasis is placed on addressing cell permeability by examining the physicochemical properties of biologically active small molecules early in the current drug discovery paradigm.[18] Lipinski's seminal investigation leading to the "rule-of-five" has become one of the most widely used tools to assess the relationship between structures and drug-like properties.[16] More often though, screening libraries are comprised of lead-like molecules to identify compounds that provide the initial lead *en route* to the drug. Molecules that satisfy lead-like or reduced complexity screening set parameters as defined by Hann and Oprea[15] are smaller and have more stringent physicochemical property criteria than drug-like molecules. More recently, Leeson and Davis have proposed that the most important physicochemical properties of drugs are those that remain most consistent over time.[17] Examination of drug approvals prior to and after 1983 showed that the median log *P*, percent polar surface area (% PSA), and number of H-bond donors (HBD) were virtually unaffected, while other physicochemical properties such as MW increased steadily over time in a statistically significant manner.[17] Of the three most constant physicochemical properties, log *P* lends itself most readily to a generic chromatography-based protocol that can be used in tandem with NP drug discovery. This is somewhat fortuitous, as log *P* is also considered the "Lord of the Rules" for drug discovery and development.[19] With this in mind, a conscious decision was made to enrich crude extracts containing components with desirable log *P* rather than apply some sort of chemical (e.g., functional group) filter or MW cutoff, as pharmacokinetic acceptable chemical space exists beyond the rule-of-five guidelines dictated by MW, hydrogen-bond donors (HBD), or hydrogen-bond acceptors (HBA).

Herein, a NP drug discovery strategy is reported that, for the first time, addresses the need to front-load a screening library with molecules having desirable physicochemical properties with the flexibility to incorporate a downstream MW filter in the triage following screening. Two screening campaigns using a library generated by the above approach have been analyzed with respect to the drug-like properties of the isolated active molecules.

RESULTS AND DISCUSSION

Lead-like Enhanced Extracts: Enhancing Crude Extracts for Lead- and Drug-like Property Profiles. To achieve a lead-like enhanced (LLE) extract, we first explored readily available solid-phase extraction (SPE) adsorbents to identify one that would retain components with log $P > 5$ and elute components with log $P < 5$. Four different adsorbents were evaluated: chemically modified octadecylsilica gel (i.e., Davisil C_{18}-bonded silica); polystyrene (i.e., Diaion HP-20); cross-linked poly(styrene-divinylbenzene) (i.e., Amberlite XAD-16); and the cross-linked poly(divinylbenzene-*N*-vinylpyrrolidone) (DVB-NVP) copolymer (i.e., Waters Oasis HLB). Reversed-phase high-performance liquid chromatography (RP-HPLC) was used to analyze the eluate because log *P* is known to correlate to RP-HPLC retention times.[20] For calibration of the RP-HPLC retention times, 14 NP drugs and bioactives were used (Figure S1, Supporting Information) and 115 isolated NPs with calculated log *P* and experimentally determined log P_{oct} (Elog P_{oct}) values.[12]

A total of 221 crude extracts derived from plant and marine invertebrates were used to develop a generic SPE protocol that filtered on log *P*. Dried crude extracts obtained from dichloromethane (CH_2Cl_2) and methanol (MeOH) (see Experimental Section) were dissolved in the eluting solvent (two bed volumes) and loaded onto the various adsorbents in a ratio of 20:1 (mass adsorbent:mass dried extract). A further two to three bed volumes (dependent on adsorbent) of the eluent was then passed through the SPE cartridge. The eluate was collected in a test tube, dried, and analyzed by RP-HPLC (Table 1), using a photodiode array (PDA) detector and evaporative light scattering detector (ELSD) connected in series. Retention of the undesirable high log *P* components was increased in the presence of 1% trifluoroacetic acid (TFA) for all four adsorbents (Table 1), with complete retention of the components with log $P > 5$ achieved only with the cross-linked DVB-NVP copolymer. Presumably, molecules such as fatty acids, which can be major components of crude extracts, are maintained in the neutral form in the presence of acid that facilitates retention.[21] Under neutral conditions, ionization can occur and compounds elute more readily.[21]

Table 1. Percentage of Log $P > 5$ Components of Extracts Retained by SPE Adsorbents

eluting solvent	SPE adsorbent			
MeOH/H_2O	C_{18}[a]	HP-20[b]	XAD-16[c]	HLB[d]
70:30	91	73	65	100
80:20	75	52	57	94
70:30:TFA (1%)	94	82	79	100
80:20:TFA (1%)	88	87	82	100

[a]Reversed-phase C_{18}-bonded silica. [b]Polystyrene matrix. [c]Cross-linked poly(styrene-divinylbenzene). [d]Cross-linked poly(divinylbenzene-*N*-vinylpyrrolidone) (DVB-NVP) copolymer.

Elution of constituents with calculated log $P < 5$, while still maintaining retention of constituents with calculated log $P > 5$, was achieved with the DVB-NVP (HLB) copolymer when the crude extract was initially solubilized in three bed volumes of 70:30 MeOH/H_2O containing 1% TFA, then eluted with a further seven bed volumes of the same solvent followed by elution in the same manner with 90:10 MeOH/H_2O containing 1% TFA (see Experimental Section).

The recovery of NP drugs using the above solvent system and DVB-NVP copolymer was next investigated in an effort to gauge the overall applicability of the method. Recoveries of above 50% were observed for all NPs apart from tetracycline and penicillin G (Figure S1, Supporting Information). This recovery would be sufficient to allow detection of the compounds in HTS campaigns. Tetracycline suffered from lack of solubility in the eluent. β-Lactams appear too sensitive

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to any combination of acid, adsorbent, or drying conditions to be isolated via this method.

Finally, various crude extracts were spiked with brucine, theophylline, and clotrimazole to determine if such substances would behave in a similar manner in an extract. Initially, six crude extracts derived from three randomly selected plant and marine organisms were spiked with these three compounds that were chosen because of their low cost and availability. It was gratifying to observe that each compound had the same recovery from an extract as when in pure form. This prompted a further set of experiments in which two previously isolated NPs,[22] eupomatenoid 5 (**1**) and eupomatenoid 3 (**2**) (Figure 1), with Elog P_{oct} values of 6.15 and 6.45, respectively, were added to the same six extracts in conjunction with the three standards. Compounds **1** and **2** were retained on the DVB-NVP copolymer adsorbent using 70:30 MeOH/H_2O containing 1% TFA as the eluent. Subsequent elution using 90:10 MeOH/H_2O resulted in the elution of 14%, on average, of **1**, while compound **2** was completely retained as determined by downstream RP HPLC-UV-ELSD analysis. On the basis of these data, solubilization of extracts in 70:30 MeOH/H_2O containing 1% TFA and further elution with the same solvent was chosen to prepare 18 453 LLE extracts. The adsorbent and solvent conditions were tuned for retention of compounds with calculated log P > 5, while the eluate contained those components with lead- and drug-like log P values.

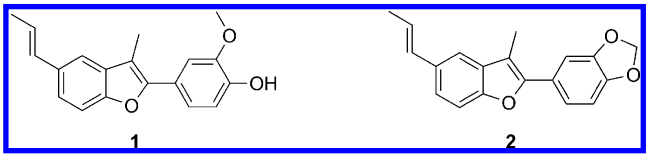

Figure 1. High log P natural products, eupomatenoid 5 (**1**) and eupomatenoid 3 (**2**).

Lead-like Enhanced Fractions: Fractionation of the LLE Extracts to Afford a Screening Set. Having satisfied the log P criteria with the LLE extract generation, we next set out to simplify the complex NP mixture by chromatographic methods. Fractionation of each LLE extract was accomplished using reversed-phase solvent conditions (MeOH/H_2O/0.1% TFA) and a Phenomenex C_{18} Monolithic HPLC column (4.6 mm × 100 mm). Eleven fractions were collected per LLE extract within the region of the chromatogram corresponding to log P < 5 (Figure 2).

This fractionation provides a second log P filter allowing any remaining high log P components to be excluded. It is noted that, in our experience, there was very limited carryover of high log P components in the LLE extract. The fractionation process delivers lead- and drug-like molecules separated into mixtures of a small number of compounds to facilitate the rapid identification of active molecules. Following screening to identify active fractions, reinjection of the LLE extract under the LLE HPLC conditions was used to confirm that the chromatogram is identical to the original and that activity is retained in the same fraction. This also provides an aliquot of the active fraction for subsequent HPLC-mass spectrometry (LC-MS) to resolve the constituents. The MW of an individual constituent can be used to make a decision about progressing the isolation of constituents. Considerations such as lead-like (MW < 350) and drug-like (MW < 500) constituents, the desirable MW for central nervous system (CNS)-active components, or other considerations related to the drug target profile can then be implemented (via mass-directed isolation). This saves time by concentrating resources on constituents of interest to the target profile.

Not only does the combined information have the potential to shed light on the structural classes present in the bioactive fraction(s) but, more importantly, the presence of known compounds can be predicted on the basis of searches of databases of known compounds. Semipreparative HPLC fractionation of the crude extracts is monitored using the HPLC retention time, UV profile, and mass spectrometric information as a guide.

The biota repository used to construct the LLE extracts and fractions contains over 45 000 samples of macro plant and marine organisms collected from three mega-diverse countries, namely, Australia, Papua New Guinea, and mainland China. All specimens were collected in accordance with the U.N. Convention on Biological Diversity. A subset of 18 453 biota was used to prepare the same number of LLE extracts, which were subsequently fractionated by analytical reversed-phase C_{18} HPLC (Onyx Monolithic) to yield 202 983 LLE fractions.

Screening against Malaria and Trypanosome Parasites. The LLE fraction library was first used in HTS campaigns aimed at identifying new leads or drugs for the tropical parasitic diseases malaria and human African trypanosomiasis (HAT). From the HTS campaign of the 202 983 LLE fractions, 60 compounds were isolated following the malaria screen and 58 for HAT. Activity ranged from good (<10 μM; 48 malaria, 30 HAT) to moderate (10−50 μM; 4 malaria, 10 HAT) to poor (>50 μM; 8 malaria, 18 HAT). An analysis of these compounds in terms of physicochemical profiles according to Lipinski's rule-of-five[16] compared to NPs in the *Dictionary of Natural Products*[23] (DNP) was undertaken (Figure 3). An initial examination of the rule-of-five violations in each set of compounds indicated an improvement of predicted oral bioavailability relative to the DNP in both

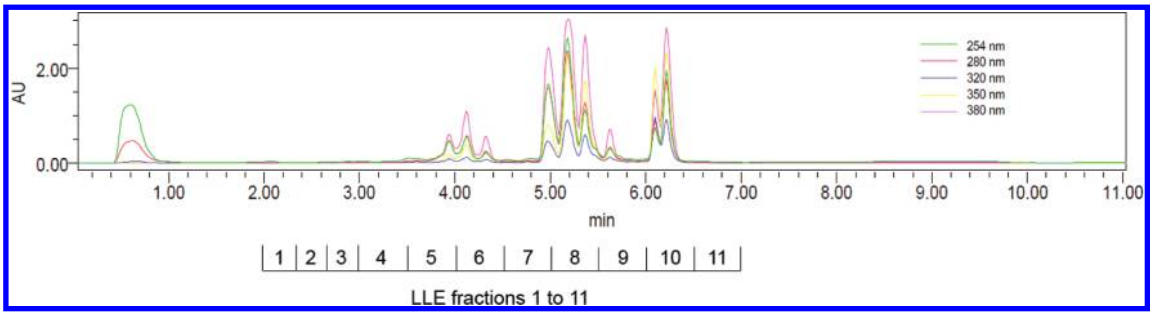

Figure 2. Example of a lead-like enhanced extract HPLC chromatogram.

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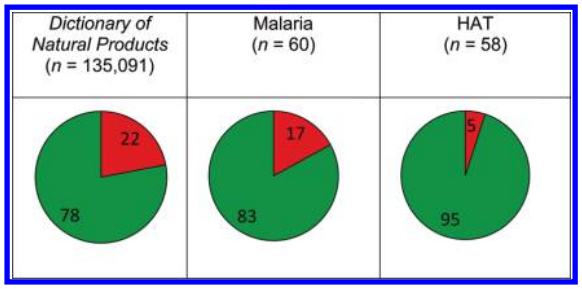

Figure 3. Lipinski's rule-of-five profiles. Comparison of Lipinski's rule-of-five profiles for the two screening projects using the LLE fractions and the *Dictionary of Natural Products* (unique structures). The red shaded area shows the percentage of compounds in each data set with undesirable property profiles (>1 violation). All physicochemical properties were calculated using InstantJChem 3.0.4. [Instant JChem 3.0.4, 2009, ChemAxon Ltd. (http://www.chemaxon.com)].

screens where the LLE process had been implemented. This demonstrates how the initial enrichment of the screening set, based on physicochemical profiling, can translate into isolation of bioactive lead- and drug-like molecules and not hydrophobic molecules that may be present in higher concentrations or exhibit an increase in binding by virtue of their highly lipophilic character.[24]

The reasons for violations of Lipinski's rule-of-five were examined in the set of isolated compounds in the malaria and HAT projects. As shown in Figure 4, both screening campaigns afforded small numbers of compounds with high log *P* (>5) values, which was somewhat unexpected, as the LLE process was essentially designed as a log *P* filter. More careful consideration suggests that the inclusion of TFA in the LLE process would lead to ionization of NPs containing basic moieties, which can lead to misleading results, as log *P* calculations specifically predict the *n*-octanol/H_2O partitioning of neutral (i.e., un-ionized) species. It is obvious that the distribution coefficient (log *D*) is a more appropriate measure, as it considers the distribution of both ionized and un-ionized species at a given pH. Indeed, it has been proposed that log *D* at pH 5.5 (log $D_{5.5}$), which is the pH of the small intestine where oral drug absorption occurs, provides a better description of the lipophilic nature of drug-like molecules under physiological conditions.[25] The log $D_{5.5}$ criteria were applied to all isolated compounds, and it was observed that no compounds exceeded the cutoff value (>5) in the malaria screening campaign (Figure 4A), while only one compound exceeded the cutoff value (>5) in the HAT screening campaign (Figure 4B). Since the single compound violating log $D_{5.5}$ was located in the last LLE fraction, it is possible that the violation resulted from slight inaccuracies in the prediction of log $D_{5.5}$.

Since the process used allows MW to be considered at the isolation stage following MS analysis of active LLE fractions, the data were reanalyzed to exclude those compounds with MW > 500 (Figure 5). Excluding MW > 500 compounds removed simultaneously most compounds with a high HBD or HBA count.

In summary, the isolated compounds from both campaigns were enhanced in lead- and drug-like profiles compared to the DNP. This indicates that initial tuning of the LLE extract within a desirable log *P* range, and subsequent fractionation used in tandem with a MW decision point, facilitates selection and isolation of compounds that occupy physicochemical space considered essential for oral bioavailability/cell permeability. The LLE fraction library yielded new and novel molecules in addition to previously known compounds. Several new compounds following screening campaigns against malaria and HAT have now been reported, i.e., the azafluorenone alkaloid **3**,[26] (+)-7-bromotrypargine (**7**),[27] psammaplysin G (**8**),[28] 12-deoxyascididemin (**10**),[29] plakortide polyketide peroxides **13** and **14**,[30] and convolutamines I (**17**) and J (**18**).[31] The bromotyrosine **19** is an example of a novel molecule with a previously unreported carbon skeleton that was isolated as part of the HAT campaign.[32] The known compounds **4**,[26] **6**,[33] **9**,[28] **11** and **12**,[29] and **16**[34] have newly

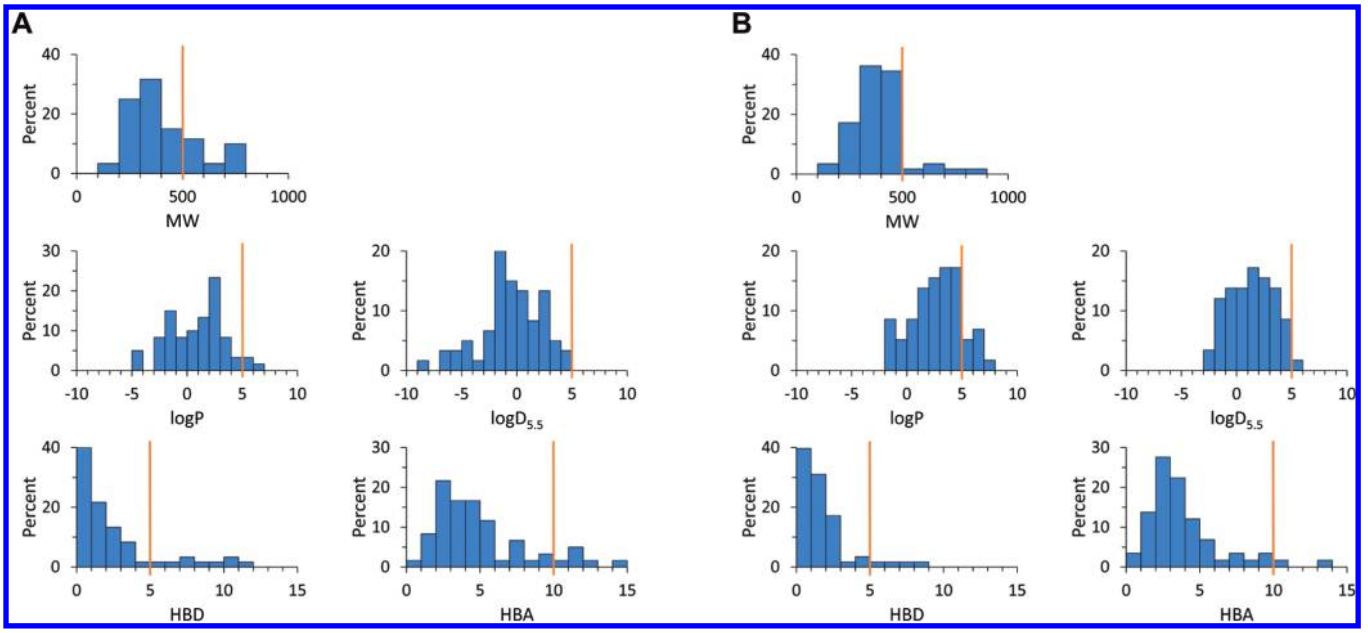

Figure 4. Physicochemical property histograms. MW, calculated log *P*, calculated distribution coefficient at pH 5.5 (log $D_{5.5}$), hydrogen bond donors (HBD), and hydrogen bond acceptors (HBA) for active compounds resulting from (A) malaria and (B) human African trypanosomiasis screening projects. In each case the orange line indicates the maximum desirable value for oral bioavailability.

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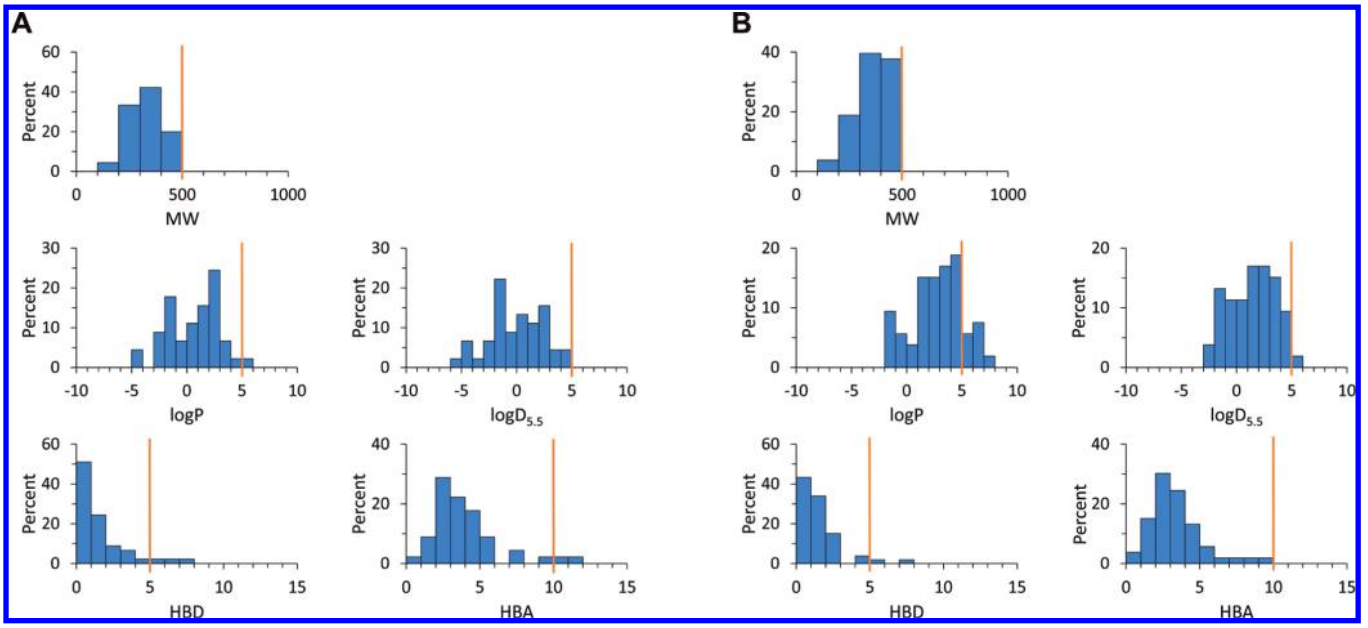

Figure 5. Physicochemical property histograms. MW, calculated log *P*, calculated distribution coefficient at pH 5.5 (log $D_{5.5}$), hydrogen bond donors (HBD), and hydrogen bond acceptors (HBA) for active compounds resulting from (A) malaria and (B) human African trypanosomiasis screening projects where MW < 500. In each case the orange line indicates the maximum desirable value for oral bioavailability.

described biological function (Figure 6 and Table 2). Though limited to two compounds in each series, the demonstration of SAR across four series is noteworthy. Comparing **4** (IC_{50} 9.9 μM 3D7, 11.4 μM Dd2) and **3** (IC_{50} 87% inhibition at 120 μM 3D7, 80% at 120 μM Dd2); **9** (IC_{50} 870 nM 3D7) and **8** (80% inhibition at 40 μM); **13** (IC_{50} 48 nM *T. b. brucei*) and **14** (IC_{50} 943 nM *T. b. brucei*); **17** (IC_{50} 1.1 μM *T. b. brucei*) and **18** (IC_{50} 13.7 μM *T. b. brucei*), there is around 10-fold reduction of potency in all series. The introduction of a hydroxy group into the aromatic ring of **4** to produce **3** increases MW and also decreases potency. The secondary amine of **9** is more potent than the *N*-methyl urea derivative **8**, perhaps indicating the need for a H-bond donor at the nitrogen. The HAT-active compounds **13** and **14** provide significant starting points since the most potent compound has an IC_{50} of 48 nM. The peroxide moiety in these molecules is stable. The open-chain substructure of convolutamine I (**17**) confers greater activity than the ring-closed analogue convolutamine J (**18**).

Examples of two HPLC chromatograms for the fractionation of the LLE extract of the rainforest tree *Mitrephora diversifolia* and the marine sponge *Ancorina* sp. showing the active fractions from HTS and reinjection of isolated compounds are included in Figure S2, Supporting Information. Other structures and biological activity will be published in due course.

The results are indicative of how the method filters initially on log *P* and then uses MW, in conjunction with biological data and some preliminary structural information as a second filter, to assess the relative merits of an active fraction before committing time and resources to isolation.

Returning to our strategy motivation; it has previously been reported that 60% of the DNP's unique compounds have no violations of Lipinski's rule-of-five.[12] Indeed, two independent studies using the DNP and BioscreenNP databases indicated 80% of NPs have two or less violations.[12,35] The strategy to purify as many components of a NP extract as possible and determine the structures of the compounds is therefore attractive to various groups pursuing NP drug discovery.[12,36,37] This approach has several obvious advantages, as the isolated compounds can be treated the same as any other substance in a library and immediate assessment of the potential of a compound can be made during hit evaluation, thus eliminating the time delay between hit extract identification and isolation of the active NP. With a structure in-hand, it is possible to exclude compounds from the screening set that do not conform to the physicochemical parameters of choice or have chemical alerts associated with the structures. However, it is not feasible to isolate every minor component in an extract library due to the underlying logistics and cost. Limited throughput associated with purification restricts the ability to build a library representative of all NP chemical space. Ignoring minor components would lead to an inevitable loss of chemical diversity, and, as such, a pure NP library approach does not lead to a comprehensive nor generic approach to front-loading NP discovery with only those NPs that are drug-like in their physicochemical properties. In an analogous fashion, the 12 977 unique core ring scaffolds present in NPs cannot be sampled comprehensively by synthetic libraries generated around these scaffolds.

However, the classical bioassay-guided isolation for NP drug discovery is not suited to current lead generation time lines, especially when competing with HTS of pure compound libraries. Isolation of active constituents from bioactive biota following the screening of an extract library is time-consuming, and, importantly, bioassay-guided isolation allows the determination of the physicochemical parameters of a molecule only retrospectively after the elucidation of the structure. Compound libraries, on the other hand, can be assembled from lead-like synthetic compounds, ensuring tractable starting points for lead generation.

Numerous fractionation strategies have been reported in an effort to improve the quality of screening data and shorten time lines associated with downstream bioassay-guided isolation.[37−43] Approaches are broadly scoped with reports ranging from the preparation of four fractions to 200 fractions per

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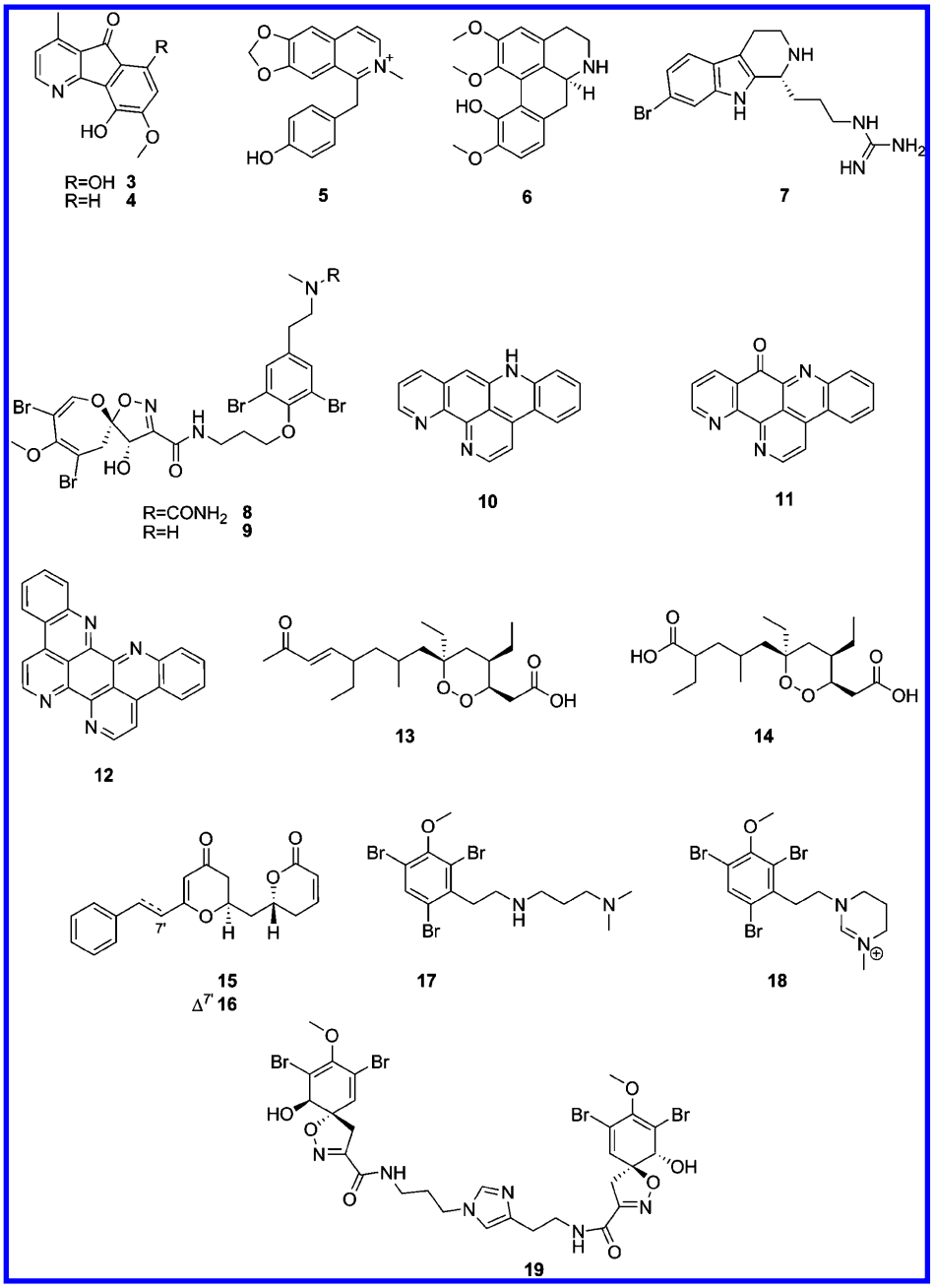

Figure 6. Chemical structures of published antimalarial (**3**–**9**) and antitrypanosomal (**10**–**19**) compounds isolated from screening of the LLE fractions.

sample. MerLion has taken the approach of applying RP C_{18} HPLC to extracts derived from microbial cultures. Four fractions were collected, with collection commencing after the solvent front peak.[39] Sequoia performed an organic extraction on plant samples exclusively, followed by silica flash chromatography, and an aqueous extract followed by C_{18} flash chromatography. Subsequent HPLC collected 40 fractions per each of four flash fractions from the organic extract. The aqueous extract was passed through C_{18}, polyamide, and a 3000 Da cutoff filter, and the single aqueous flash fraction subjected to HPLC, again collecting 40 fractions. The HPLC fractions that contained quantifiable compounds, approximately 60% of the total, constituted the library. This process led to a library of 36 000 fractions containing 1−5 compounds/well.[38] Wyeth has used prefractionation to generate 10 fractions plus crude from the combined extract from at least two different culture conditions.[42] The Ireland group has explored marine organisms and used SPE (HP-20SS) to initially desalt the crude extract. The desalted extract was then subjected to HPLC to generate 80 fractions per sample.[43] A recent report focused on NP drug discovery from plants in which the focus was on removal of interfering polyphenolic tannins using a polyamide adsorbent to achieve efficiency of fractionation (24 fractions per extract).[44]

The presence of tannins in NP extracts can lead to a significant number of false positives, which can be time-consuming to dereplicate. These compounds are notorious for interfering with many assay formats and screening technologies.[39] Their removal consequently affords extracts that are more compatible with NP drug discovery in the HTS paradigm.

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Table 2. Physicochemical Profiling and Biological Activity of Isolated Natural Products 3−19

	physicochemical parameters[a]						IC_{50} (μM) or % inhibition		
compound	MW	log *P*	HBA	HBD	% PSA	TPSA	3D7[b]	Dd2[c]	*T. b. brucei*[d]
Antimalarial Compounds									
3	257	2.67	5	2	23.5	79.6	87%[e]	80%[e]	
4	241	2.33	4	1	18.1	59.4	9.9	11.4	
5	294	−1.38	3	1	10.2	42.6	3.0	4.4	
6	327	1.80	5	2	12.4	59.9	17.0	19.0	
7	350	1.87	4	5	20.6	89.7	3.5	5.4	
8	790	3.14	8	3	19.5	144.9	80%[f]	[g]	
9	747	3.74	8	3	15.8	110.6	0.87	1.38	
Antitrypanosomal Compounds									
10	269	3.40	3	1	10.9	37.8			0.077
11	283	2.98	4	0	16.1	55.7			0.032
12	356	4.70	4	0	11.8	51.6			1.33
13	368	5.29	5	1	11.2	72.8			0.048
14	344	4.33	6	2	15.6	93.1			0.943
15	312	3.33	3	0	11.9	52.6			2.8
16	310	3.21	3	0	12.7	52.6			5.3
17	473	4.05	3	1	4.88	24.5			1.1
18	470	0.29	2	0	3.37	15.5			13.7
19	898	0.62	11	4	21.2	178.1			80%[h]

[a]In silico calculations performed using Instant JChem 3.0.4 software, MW = molecular weight, HBA = H-bond acceptors, HBD = H-bond donors. [b]3D7 = *Plasmodium falciparum* (chloroquinine-sensitive) strain. [c]Dd2 = *Plasmodium falciparum* (chloroquinine-resistant) strain. [d]*Trypanosoma brucei brucei*. [e]At 120 μM. [f]At 40 μM. [g]Not tested. [h]At 80 μM.

Marine invertebrates do not contain tannins but may have a high salt content that can dilute compounds of interest, thereby reducing the likelihood of detection. Salts can also suppress ionization in downstream LC-MS analysis, making identification of the active component more challenging.[43] Fatty acids and lipids can be found in virtually all crude NP extracts and are also known to form aggregates that interfere with certain types of assays.[45,46] MerLion does not collect the later eluting fractions from HPLC to prevent these compounds from being exposed to HTS.

The present approach differs in that SPE is used to specifically filter on log *P* to afford the LLE extract based on the front-loading of drug-like physicochemical profiling. This step allows the selective enrichment for compounds within the extracts that have a much higher probability of being drug-like. Front-loading for desired physicochemical properties obviates the necessity to accomplish this at a later stage, where it is more time-consuming. In addition, the high log *P* of interfering lipid and fatty acid type molecules facilitates their removal in the present approach. Large polyphenolic tannins are effectively removed from plant extracts in the protocols reported here via initial use of polyamide SPE prior to the SPE step that filters for log *P* (see Experimental Section). Salts from marine organisms are eliminated using a prewash on the HPLC column prior to fractionation.

The SPE process removes, on average, 10% of the mass of a crude extract from marine organisms and 16% of that of plants. Maximum reductions in crude mass were 30% for marine invertebrates and 50% for plants, and this was observed in approximately 3% of the total biota samples. Given the good to excellent recovery of known NPs and other drugs during validation of the method, the majority of mass that has been removed is due to highly lipophilic material. The subsequent HPLC separation is significantly improved by the removal of these constituents. Not only is the resolution in the region of interest improved, but the highly lipophilic material does not elute through successive injections, thus minimizing false positives in HTS. Removal of high log *P* constituents also prevents overloading of columns and gives significantly longer column life.

HTS data have been used to evaluate previously reported fractionation strategies. Wyeth has reported that, following nine HTS campaigns on a microbial NP library, 79.9% of the activity was found only in the fractions, 12.5% of the activity was detected only in the crude extract, and 7.6% was found in both fractions and crude extract from 1882 active cultures.[42] A similar result was achieved using the simpler four fractions per extract approach of MerLion. Of the 1700 active fractions from 11 HTS screens, 80% of the primary screening hits from fractionated samples did not show activity in the associated crude extract.[39]

During previous bioassay-guided isolation, we observed a correlation between lipophilicity as measured by the calculated log *P* and retention time on a C_{18} reversed-phase HPLC column.[47] We have now been able to apply lessons learned from previous endeavors in isolating drug-like NPs toward a generic approach that could be applied to both major and minor constituents within crude or semipurified extracts. The innovative approach involves the initial optimization of the extracts to lead- and drug-like space with a particular emphasis on log *P*, the most important of the physicochemical parameters, followed by fractionation to afford a HTS-friendly NP screening library.

This methodology permits MW, structural data, and retest of active pure compounds to be acquired in a highly efficient manner directly following primary screening, the point at which little to no chemical information is generally known about bioactive constituents in the bioassay-guided isolation paradigm. In our work stream, confirmation is obtained by reinjection of the LLE extract under precisely the same HPLC conditions, followed by LC-MS to further resolve the individual constituents and obtain retention time, UV spectra,

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and MW information on each of the constituents in the active LLE fractions. Scale-up of separation is then based on the chromatographic retention time, UV spectra, and MW of the constituents of the active LLE fraction. Acquisition of LC-MS data is also used for dereplication and as a decision point to pursue a full-scale isolation project. Projects can move forward despite actives having higher MW if the supporting information warrants further effort, e.g., potent activity relative to screening concentration and ligand efficiency.[48] This decision gate allows NP drugs that satisfy the log *P* criteria, but may fail other lead- and drug-like parameters, such as Taxol and cyclosporin, to still be isolated on the basis of potency or new/novel structural motifs (which carry the possibility of novel modes of action).

In summary, it has been shown that it is possible to front-load NP extracts with lead- and drug-like molecules to facilitate drug discovery programs. The innovative optimization process affords a LLE extract that is then fractionated to yield LLE fractions of low chemical complexity per fraction while retaining chemical diversity space. Since log *P* is employed as the primary filter, a second filter based on MW is used to determine whether the active component will be pursued. A concern about the inherent slowness of working with NPs has been addressed,[13] which arises due to the classical bioassay-guided isolation methodology, using mass-directed isolation to rapidly isolate active components. The results from the isolation projects demonstrate convincingly that, when compared to classical NP drug discovery, which can undertake physicochemical analysis only retrospectively after compounds are isolated and structures elucidated, the present approach allows front-loading of the desired physicochemical properties at the extract and fraction stages.

EXPERIMENTAL SECTION

General Experimental Procedures. All solvents used for SPE, HPLC, and MS were Lab-Scan HPLC grade, and the H_2O was Millipore Milli-Q PF filtered. Dimethyl sulfoxide (DMSO, 99.9%) and trifluoroacetic acid (TFA, 99%) were obtained from Fluka. H_2O was purified with a Milli-Q system from Millipore and used for all aqueous solutions. Unpacked 3 mL SPE cartridges fitted with a frit (20 μm pore size) and additional polyethylene frits (20 μm pore size) were from Phenomenex. All adsorbents used in developing the SPE protocols were purchased in bulk (1 kg) quantities. Davisil Octadecylsilica (C_{18}-bonded silica, 30−40 μm, 60 Å) and polyamide CC-6 were from Alltech. Amberlite XAD-16 and Diaion HP-20 were from Supelco. Oasis HLB was obtained from Waters. Commercially available Oasis HLB cartridges (400 mg) were employed to generate the 18 453 LLE extracts.

A Waters 600 pump (with 225 μL pump-heads) equipped with a Waters 966 PDA detector under the control of Waters Millenium[32] software (versions 4.0) was used for the analytical HPLC LLE extract fractionation work. LLE extract injections and subsequent LLE fraction collection were performed using a Gilson 215 liquid handler (5 mL syringe, 200 μL Rheodyne sample loop), which was controlled by Gilson 735 software (version 6.00). Polypropylene lockable 96-tube racks filled with 2D barcoded polypropylene tubes (1.2 mL) (Abgene cat. # AB-1148) were used to store LLE extracts and as injection racks for the Gilson 215 liquid handler during the LLE fractionation process. LLE fractions were collected into clear Flexi-tier polystyrene racks, which each contained 96 conical glass vials (1.5 mL) without liners (Analytical Sales and Services Inc. cat. # 96BS15-C). A Christ Beta-RVC centrifugal evaporator equipped with aluminum swing-out rotor (Christ cat. # 124322) and one set of aluminum carriers (Christ cat. # 124448) was used for drying the LLE fractions.

Reference Compounds. Fifteen reference compounds were purchased from Sigma-Aldrich without further purification as representative bioactive molecules with calculated log *P* values (in parentheses) spanning the range from −4.41 to 5.84. These consisted of 13 NP drugs, artemisinin (3.11) (Sigma cat. # 361593), capsaicin (3.75) (Sigma cat. # M2028), cyclosporin (3.64) (Sigma cat. # C3662), forskolin (1.93) (Sigma cat. # F6886), galantamine (1.16) (Sigma cat. # G1660), mycophenolic acid (3.53) (Sigma cat. # M5225), penicillin G (1.08) (Sigma cat. # P7794), quinine (2.51) (Fluka cat. #22640), reserpine (3.53) (Sigma cat. # R0875), paclitaxel (3.54) (Sigma cat. # T7402), tetracycline (−2.04) (Sigma cat. # T3258), theophylline (−0.77) (Sigma cat. # T1633), and vancomycin (−4.41) (Aldrich cat. # 861987); one bioactive NP, brucine (0.61) (Aldrich cat. # 399027); and one synthetic drug, clotrimazole (5.84) (Sigma cat. # C6019). This set contained a variety of structural classes ranging in complexity from the relatively simple (theophylline) to the densely functionalized (paclitaxel and vancomycin).

A further two compounds, eupomatenoid 5 (**1**) and eupomatenoid 3 (**2**), were isolated in-house and added to the reference set as NPs with Elog $P_{oct} > 5$ to facilitate optimization of the lead-like enhanced extraction and fractionation protocols. The calculated log *P* for **1** was 4.88, and that for **2** was 4.96, while the Elog P_{oct} values were 6.15 and 6.45, respectively, using the method reported by Lombardo et al.[20] The MS and NMR spectra for **1** and **2** were identical to those previously reported.[22] Dipyridamole was also obtained from Sigma-Aldrich and used without further purification as a reference compound for internal quality control during scale-up production of optimized extracts.

Crude Extracts. Dried plant and marine invertebrate biota (300 mg) were extracted with a range of organic solvents to afford the crude extract. Marine invertebrates were extracted with *n*-hexane (7 mL). The *n*-hexane extract was discarded, and each sample then extracted with 80:20 CH_2Cl_2/MeOH (7 mL) and dried. A second extract using MeOH (13 mL) was collected in the same glass test tube and dried to afford the crude extract. Plant biota were extracted in a similar manner using *n*-hexane (7 mL), CH_2Cl_2 (7 mL), and MeOH (13 mL). In a slight variation for marine invertebrates, the combined and dried CH_2Cl_2 and MeOH plant extracts were reconstituted in MeOH (4 mL) before being loaded onto a cartridge of polyamide gel (900 mg). The cartridge was washed with MeOH (2 × 4 mL), and the combined MeOH washes were dried to afford the crude plant extract. A standard biota sample was extracted with each batch of 96 biota and checked by reversed-phase HPLC to ensure consistency of the extraction process.

Lead-like Enhanced (LLE) Extracts. A borosilicate glass tube containing the dried crude extract was presolubilized in 70:30 MeOH/H_2O containing 1% TFA (4 mL) and loaded onto a SPE cartridge (Waters Oasis HLB, 400 mg) and eluted with a further 8.5 mL of the same solvent. The eluant was dried and reconstituted in DMSO (1.2 mL) to yield each LLE extract. For QC purposes, a DMSO mixture consisting of brucine (0.1 mg/mL), capsaicin (0.5 mg/mL), and dipyridamole (0.1 mg/mL) was processed with each batch of LLE extracts and checked for recovery by reversed-phase HPLC.

Lead-like Enhanced (LLE) Fractions. A 96-tube rack containing the LLE extracts and the Flexi-tier fraction plates were placed on the Gilson 215 liquid handler tray (Figure S3, Supporting Information). The LLE extracts (18 453) were processed in batches of 96 with 100 μL injected for each sample. HPLC separations were performed on a Phenomenex C_{18} Monolithic HPLC column (4.6 mm × 100 mm) using conditions that consisted of a linear gradient (curve #6) from 90% H_2O (0.1% TFA)/10% MeOH (0.1% TFA) to 50% H_2O (0.1% TFA)/50% MeOH (0.1% TFA) in 3 min at a flow rate of 4 mL/min; a convex gradient (curve #5) to MeOH (0.1% TFA) in 3.50 min at a flow rate of 3 mL/min, held at MeOH (0.1% TFA) for 0.50 min at a flow rate of 3 mL/min, held at MeOH (0.1% TFA) for a further 1.0 min at a flow rate of 4 mL/min; then a linear gradient (curve #6) back to 90% H_2O (0.1% TFA)/10% MeOH (0.1% TFA) in 1 min at a flow rate of 4 mL/min, then held at 90% H_2O (0.1% TFA)/10% MeOH (0.1% TFA) for 2 min at a flow rate of 4 mL/min. The total run time for each LLE extract injection was 11 min, and 11 fractions were collected between 2.0 and 7.0 min, i.e., fraction 1 (time = 2.00−2.33 min), fraction 2 (time = 2.34−2.66 min), fraction 3 (time = 2.67−3.00 min), fraction 4 (time = 3.01−3.50 min), fraction 5 (time = 3.51−4.00 min), fraction 6 (time = 4.01−4.50 min), fraction 7 (time = 4.51−5.00

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min), fraction 8 (time = 5.01−5.50 min), fraction 9 (time = 5.51−6.00 min), fraction 10 (time = 6.01−6.50 min), and fraction 11 (time = 6.51−7.00 min). After evaporation of solvent, each glass vial containing an LLE fraction was resuspended in 100 μL of DMSO and transferred to a 384-well microtiter plate using a Biomek FX (Beckman Coulter) liquid handler.

After injection of every eight LLE extracts, a standard mixture consisting of methyl 4-hydroxybenzoate 99% (Aldrich cat. # W271004), ethyl 4-hydroxybenzoate 99% (Aldrich cat. # 111988), benzophenone 99% (Aldrich cat. # B9300), and uracil 99% (Sigma cat. # U0750) (all 0.125 mg/mL in DMSO) was injected as a positive control for the LLE fractionation process.

Identification of Compounds 1−19. Compounds **1**−**19** were all isolated from plant or marine invertebrates archived at the Eskitis Institute, Griffith University. A brief description of the source biota and the extraction and isolation protocols employed for each compound is detailed below. Eupomatenoid 5 (**1**) and eupomatenoid 3 (**2**) were isolated following chemical investigation of the CH_2Cl_2 extract from the bark of *Eupomatia* sp.[22] Compounds **3** and **4** were isolated from the CH_2Cl_2/MeOH extract of the roots of *Mitrphora diversifolia* following semipreparative C_{18} HPLC (MeOH/H_2O/0.1% TFA).[26] Compounds **5** and **6** were isolated from the CH_2Cl_2/MeOH extract of the leaves of *Doryphora sassafras* following polyamide gel purification and semipreparative C_{18} HPLC (MeOH/H_2O/0.1% TFA).[33] (+)-7-Bromotrypargine (**7**) was purified from the CH_2Cl_2/MeOH extract from the marine sponge *Ancorina* sp. following two rounds of semipreparative C_{18} HPLC (MeOH/H_2O/0.1% TFA).[27] Psammaplysins G (**8**) and F (**9**) were isolated from the CH_2Cl_2/MeOH extract from the marine sponge *Hyattela* sp. after semi-preparative C_{18} HPLC (MeOH/H_2O/0.1% TFA) and preparative silica TLC ($CHCl_3$/MeOH).[28] 12-Deoxyascididemin (**10**), ascidide-min (**11**), and eilatin (**12**) were purified from the combined CH_2Cl_2/MeOH extract from the marine ascidian *Polysyncraton echinatum* after several rounds of semipreparative C_{18} HPLC (MeOH/H_2O/0.1% TFA).[29] The plakortide derivatives **13** and **14** were identified following C_{18} HPLC (MeOH/H_2O/0.1% TFA) of the CH_2Cl_2/MeOH extract from the marine sponge *Plakortis* sp.[30] Mass-directed isolation [C_{18} HPLC (MeOH/H_2O/0.1% TFA)] of the CH_2Cl_2/MeOH extract from the leaves of *Cryptocarya obovata* resulted in the purification of 7′,8′-dihydroobolactone (**15**) and obolactone (**16**).[34] Mass-directed isolation [C_{18} HPLC (MeOH/H_2O/0.1% TFA)] of the CH_2Cl_2/MeOH extract from the marine bryozoan *Amathia tortusa* resulted in the identification of convolutamines I (**17**) and J (**18**).[31] Mass-directed isolation [C_{18} HPLC (MeOH/H_2O/0.1% TFA)] of the CH_2Cl_2/MeOH extract from the sponge *Pseudoceratina* sp. led to the isolation of pseudoceratinazole A (**19**).[32]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Graph showing recovery of marketed natural product drugs using the LLE extraction protocols. LLE and pure compound chromatograms associated with the isolation and purification of structures **3**, **4**, and **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Wess, G.; Urmann, M.; Sickenberger, B. *Angew. Chem., Int. Ed.* **2001**, *40*, 3341−3350.
(2) McArdle, B. M.; Campitelli, M. R.; Quinn, R. J. *J. Nat. Prod.* **2006**, *69*, 14−17.
(3) Kellenberger, E.; Hofmann, A.; Quinn, R. J. *Nat. Prod. Rep.* **2011**, *28*, 1483−1492.
(4) Hert, J.; Irwin, J. J.; Laggner, C.; Keiser, M. J.; Shoichet, B. K. *Nat. Chem. Biol.* **2009**, *5*, 479−483.
(5) Cragg, G. M.; Newman, D. J.; Snader, K. M. *J. Nat. Prod.* **1997**, *60*, 52−60.
(6) Shu, Y.-Z. *J. Nat. Prod.* **1998**, *61*, 1053−1071.
(7) Newman, D. J.; Cragg, G. M.; Snader, K. M. *J. Nat. Prod.* **2003**, *66*, 1022−1037.
(8) Newman, D. J.; Cragg, G. M. *J. Nat. Prod.* **2004**, *67*, 1216−1238.
(9) Lam, K. S. *Trends Microbiol.* **2007**, *15*, 279−289.
(10) Newman, D. J.; Cragg, G. M. *J. Nat. Prod.* **2007**, *70*, 461−477.
(11) Cragg, G. M.; Grothaus, P. G.; Newman, D. J. *Chem. Rev.* **2009**, *109*, 3012−3043.
(12) Quinn, R. J.; Carroll, A. R.; Pham, N. B.; Baron, P.; Palframan, M. E.; Suraweera, L.; Pierens, G. K.; Muresan, S. *J. Nat. Prod.* **2008**, *71*, 464−468.
(13) Harvey, A. L. *Drug Discovery Today* **2008**, *13*, 894−901.
(14) Williams, P. G. *Trends Biotechnol.* **2008**, *27*, 45−52.
(15) Hann, M. M.; Oprea, T. I. *Curr. Opin. Chem. Biol.* **2004**, *8*, 255−263.
(16) Lipinski, C. A.; Lombardo, F.; Dominy, B.; Feeney, P. J. *Adv. Drug Delivery Rev.* **1997**, *23*, 3−25.
(17) Leeson, P. D.; Davis, A. M. *J. Med. Chem.* **2004**, *47*, 6338−6348.
(18) Leeson, P. D.; Davis, A. M.; Steele, J. *Drug Discovery Today: Technol.* **2004**, *1*, 189−195.
(19) Ganesan, A. *Curr. Opin. Chem. Biol.* **2008**, *12*, 306−317.
(20) Lombardo, F.; Shalaeva, M. Y.; Tupper, K. A.; Gao, F.; Abraham, M. H. *J. Med. Chem.* **2000**, *43*, 2922−2928.
(21) Wissiack, R.; Rosenberg, E.; Grasserbauer, M. *J. Chromatogr. A* **2000**, *896*, 159−170.
(22) Bowden, B. F.; Ritchie, E.; Taylor, W. C. *Aust. J. Chem.* **1972**, *25*, 2659−2669.
(23) *Dictionary of Natural Products*; Chapman and Hall/CRC Press: London, UK, 2005 (http://www.crcpress.com).
(24) Leeson, P. D.; Springthorpe, B. *Nat. Rev. Drug Discovery* **2007**, *6*, 881−890.
(25) Bhal, S. K.; Kassam, K.; Peirson, I. G.; Pearl, G. M. *Mol. Pharmaceutics* **2007**, *4*, 556−560.
(26) Mueller, D.; Davis, R. A.; Duffy, S.; Avery, V. M.; Camp, D.; Quinn, R. J. *J. Nat. Prod.* **2009**, *72*, 1538−1540.
(27) Davis, R. A.; Duffy, S.; Avery, V. M.; Camp, D.; Hooper, J. N. A.; Quinn, R. J. *Tetrahedron Lett.* **2010**, *51*, 583−585.
(28) Yang, X.; Davis, R. A.; Buchanan, M. S.; Duffy, S.; Avery, V. M.; Camp, D.; Quinn, R. J. *J. Nat. Prod.* **2010**, *73*, 985−987.
(29) Feng, Y.; Davis, R. A.; Sykes, M. L.; Avery, V. M.; Carroll, A. R.; Camp, D.; Quinn, R. J. *Tetrahedron Lett.* **2010**, *51*, 2477−2479.
(30) Feng, Y.; Davis, R. A.; Sykes, M. L.; Avery, V. M.; Camp, D.; Quinn, R. J. *J. Nat. Prod.* **2010**, *73*, 716−719.
(31) Davis, R. A.; Sykes, M.; Avery, V. M.; Camp, D.; Quinn, R. J. *Bioorg. Med. Chem.* **2011**, *19*, 6615−6619.
(32) Feng, Y.; Davis, R. A.; Sykes, M. L.; Avery, V. M.; Camp, D.; Quinn, R. J. *Tetrahedron Lett.* **2010**, *51*, 4847−4850.
(33) Buchanan, M. S.; Davis, R. A.; Duffy, S.; Avery, V. M.; Quinn, R. J. *J. Nat. Prod.* **2009**, *72*, 1541−1543.
(34) Davis, R. A.; Demirkiran, O.; Sykes, M. L.; Avery, V. M.; Suraweera, L.; Fechner, G. A.; Quinn, R. J. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 4057−4059.
(35) Lee, M. L.; Schneider, G. *J. Comb. Chem.* **2001**, *3*, 284−289.

(36) Bindseil, K. U.; Jakupovic, J.; Wolf, D.; Lavayre, J.; Leboul, J.; van der Pyl, D. *Drug Discovery Today* **2001**, *6*, 840−847.
(37) Abel, U.; Koch, C.; Speitling, M.; Hansske, F. G. *Curr. Opin. Chem. Biol.* **2002**, *6*, 453−458.
(38) Eldridge, G. R.; Veroort, H. C.; Lee, C. M.; Cremin, P. A.; Williams, C. T.; Hart, S. M.; Goering, M. G.; O'Neil-Johnson, M.; Zeng, L. *Anal. Chem.* **2002**, *74*, 3963−3971.
(39) Appleton, D. R.; Buss, A. D.; Butler, M. S. *Chimia* **2007**, *61*, 327−331.
(40) Schmid, I.; Sattler, I.; Grabley, S.; Thiericke, R. *J. Biomol. Screening* **1999**, *4*, 15−25.
(41) Jia, Q. *Stud. Nat. Prod. Chem.* **2003**, *29*, 643−718.
(42) Wagenaar, M. M. *Molecules* **2008**, *13*, 1406−1426.
(43) Bugni, T. S.; Richards, B.; Bhoite, L.; Cimbora, D.; Harper, M. K.; Ireland, C. M. *J. Nat. Prod.* **2008**, *71*, 1095−1098.
(44) Tu, Y.; Jeffries, C.; Ruan, H.; Nelson, C.; Smithson, D.; Shelat, A. A.; Brown, K. M.; Li, X.-C.; Hester, J. P.; Smillie, T.; Khan, I. A.; Walker, L.; Guy, R. K.; Yan, B. *J. Nat. Prod.* **2010**, *73*, 751−754.
(45) Shoichet, B. K. *Drug Discovery Today* **2006**, *11*, 607−615.
(46) Thorne, N.; Auld, D. S.; Inglese, J. *Curr. Opin. Chem. Biol.* **2010**, *14*, 315−324.
(47) Avery, V. M.; Camp, D.; Carroll, A. R.; Jenkins, I. D.; Quinn, R. J. In *Comprehensive Natural Products II. Chemistry and Biology*; Mander, L. N.; Lui, H.-W., Eds.; Elsevier: Oxford, UK, 2010; Vol. 3, pp 177−203.
(48) Hopkins, A. L.; Groom, C. R.; Alex, A. *Drug Discovery Today* **2004**, *9*, 430−431.