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A Convolutional Neural Network-based approach for the Rapid Annotation of Molecularly Diverse Natural Products

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Abstract

This report describes the first application of the novel NMR-based machine learning tool 'Small Molecule Accurate Recognition Technology' (SMART 2.0) for mixture analysis and subsequent accelerated discovery and characterization of new natural products. The concept was applied to the extract of a filamentous marine cyanobacterium known to be a prolific producer of cytotoxic natural products. This environmental *Symploca* extract was roughly fractionated, and then prioritized and guided by cancer cell cytotoxicity, NMR-based SMART 2.0, and MS^2-based molecular networking. This led to the isolation and rapid identification of a new chimeric swinholide-like macrolide, symplocolide A, as well as the identification of swinholide A, samholides A-I and several new derivatives. The planar structure of symplocolide A was confirmed to be a structural hybrid between swinholide A and luminaolide B by 1D/2D NMR and $LCMS^2$ analysis. A second example applies SMART 2.0 to the characterization of structurally novel cyclic peptides, and compares this approach to the recently appearing 'atomic sort' method. This study exemplifies the revolutionary potential of combined traditional and deep learning-assisted analytical approaches to overcome longstanding challenges in natural products drug discovery.

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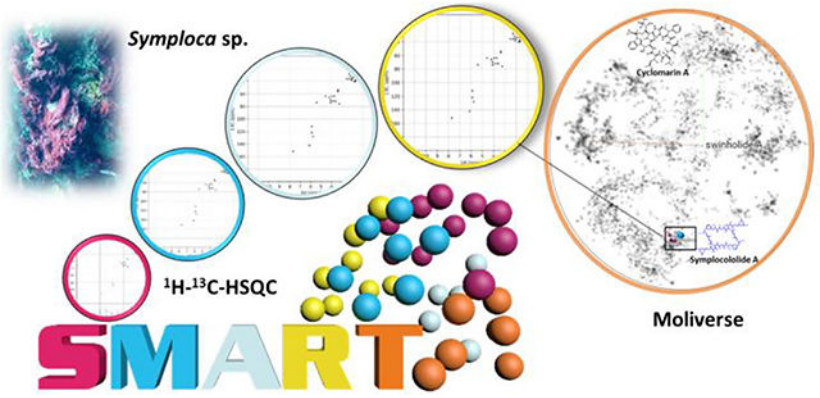
The authors declare no conflict of interest. Chen Zhang, Garrison W. Cottrell, and William H. Gerwick are the cofounders of NMR Finder LLC. Mingxun Wang is the founder of Ometa Labs LLC.

Graphical Abstract



Natural Products (NPs) of terrestrial and marine organisms have been a highly valuable source of leads for biomedical applications. About 50% of FDA approved drugs can trace their origin to NPs, and notably, marine NPs have proportionately produced a much higher success rate than other sources of drug leads (e.g. 19 approved marine derived or inspired agents are on the market today).[1–3] Filamentous marine cyanobacteria are especially rich in structurally-diverse and biologically active NPs,[4,5] such as those showing differential cytotoxicity against various cancer cell lines[6,7]. In this regard, the genus *Symploca* is a prolific producer of cytotoxic NPs such as dolastatin 10[8,9], symplocamide A[10], and others[11–16].

Drug discovery and development is expensive ($2 billion on average), time-consuming (13–15 years from concept to market), and risky (clinical success rate of 12%).[17] Informatic tools are therefore being developed to make the discovery of new leads more efficient, to repurpose known agents, to target new metabolites on the basis of genomic analysis, to rapidly reveal mechanisms of action, and to optimize the pharmaceutical properties of drug leads. For example, the emerging research area of genome mining[18] featuring bioinformatic tools such as AntiSMASH 5.0[19] and BiGSCAPE[20] provide an orthogonal approach to NP discovery,[21] although an approved drug discovered by this approach has not yet been marketed. The rapid identification of molecular structure, either known or new, is a significant challenge in NP discovery, and a variety of partial solutions are emerging using disparate analytical approaches.

For example, liquid chromatography tandem mass spectrometry-based (LCMS/MS) dereplication tools, such as the Global Natural Products Social molecular networking (GNPS; http://gnps.ucsd.edu), MS2LDA, and SIRIUS[22–28] represent paradigm shifts in NP research. These new tools are facilitating the targeted isolation of new NPs as well as rapidly dereplicating known ones. Nevertheless, unambiguous identification of new NPs still requires characterization by NMR spectroscopy to comply with the minimum standards for novel metabolite annotation.[30,31]. Recent efforts have focused on combining various NMR techniques with mass spectrometry and *in silico* data bases.[32,33] Previously. we reported a novel approach that involved training a deep convolutional neural network (CNN) of siamese architecture with 2,048 ^{1}H–^{13}C HSQC spectra mined from the supporting

information sections of the Journal of Natural Products.[34] This trained system was used to analyze new spectra, place them within 10-dimensional SMART cluster space, and accelerate the structure elucidation of a series of cyclic lipopeptides.[34,35]

Encouraged, we subsequently developed automated pipelines to produce constructed HSQC spectra from data tables as well as predicted HSQC spectra from published structures. SMART 2.0 was trained on 25,434 HSQC spectra from NPs of the JEOL database (https://www.j-resonance.com/en/nmrdb/). The spectra were mapped into a 180-dimensional embedding space using a CNN (SqueezeNet).[36] We mapped an additional 27,642 spectra that were computed using the ACD Labs predictor to create the HSQC spectra (see page S6 for details) from randomly chosen, mainly marine NPs (e.g. NP Atlas[37] and NPASS), into the 180-D space. The resulting 53,076 NP HSQC spectra cover approximately 15% of the currently known NPs. Further experiments demonstrated the remarkable robustness of the SMART analysis towards different NMR solvents (Figure S1), and the value of calculated HSQC spectra in the SMART analysis (Figure S2).

Here, we demonstrate this unique cheminformatic tool to automatically characterize a complex NP from a cyanobacterial extract mixture for the first time. Resultantly, the extract was fractionated and the isolation of novel NPs undertaken guided by NMR-based SMART mixture analysis, MS^2 molecular networking, and cytotoxicity against NCI-H460 human lung cancer cells *in vitro*. This led to the discovery of a new swinholide class of NP, named 'symplocolide A' (compound **1**, Scheme 1, Scheme S1), as well as the description of several known compounds in this structural class.

Tufts of a filamentous marine cyanobacterium, morphologically identified as *Symploca* sp., were collected near American Samoa. The preserved collection was repetitively extracted (2/1 CH_2Cl_2/MeOH) and fractionated using vacuum liquid chromatography (VLC) to obtain nine fractions of increasing polarity (A-I). All fractions and the crude extract were screened for cytotoxicity to H-460 human lung cancer cells.[38] Besides the crude extract, only fraction H showed strong cytotoxicity, while fraction I was moderately active (Figures 1a and S3). For initial SMART 2.0 analysis of the most cytotoxic crude fraction H, a NUS-ASAP-HSQC[39–41] was recorded in 13 minutes on about 1 mg material in a 1.7 mm TCI MicroCryoProbe™ (599.10 MHz)s to obtain the correlations of the major components of that fraction. After auto-peak picking of the 45 most intense features, 11 out of the top 12 structures returned by SMART 2.0 were macrolides including the known cyanobacterial metabolites swinholide A and isoswinholide A (Figures 1b–d).

Targeting these macrolides for isolation, fraction H was purified by C-18 solid phase extraction into sub-fractions H1–H7; these were analyzed using SMART 2.0 mixture analysis as well as MS^2-based molecular networking. The latter revealed a highly abundant and distinct cluster of four nodes with the major *m/z* 1395.9 in active fraction H4 (Figure 1e). Isolation and structure elucidation of this compound led to the discovery of a new cytotoxic macrolide (Scheme 1), named symplocolide A (**1**), representing a structural chimera between swinholide A (**4**) and luminaolide B (**3**).[43] Analysis of the MS^2 spectra of metabolites from these fractions using GNPS led to identification of swinholide A (**4**), the glycosylated samholides A-I[44] (**5–13**, Scheme 1 Figures S12–22), as well as several

putatively new analogs swinholides L and M, samholides J-M, and symplocolides B-D (Figures S23–30).

Similarities in the MS^2 fragmentation patterns of **1** and **4** (Figures 1f and g)[29] suggested that they differed in their carbon backbones (see blue part of the feature *m/z* 939.49, Figures 1g, S4). This hypothesis was confirmed following isolation and unambiguous structure determination by 2D NMR analysis and HRMS (Figures S5–S11, Table S1). These analyses disclosed **1** to possess the backbone skeleton of **3** and the side chain structure of **4**, thus constituting a chimera of these two macrolides.

All known swinholide-type compounds, irrespective of origin (sponge, cyanobacteria, algae, nudibranch), possess a highly analogous monomeric carbon skeleton and identical configurations at comparable chiral centers (Scheme 1); this has been confirmed in two cases by X-ray crystallography[45,46] as well as total synthesis.[47] These swinholide-type metabolites are produced by highly similar *trans* AT polyketide synthase biosynthetic gene clusters.[29,43] Thus, the stereochemistry of 1 is assigned by analogy given the close similarities between the ^{1}H and ^{13}C NMR chemical shifts and coupling constants of compound **1** with those of previously reported swinholide-like macrolides (Tables S2, S3, Scheme S1).[42–44,48]

Recently, Duggan et al. developed an ‘atomic sort’ method wherein HSQC spectra from two different NP extracts were compared with a database of 1,207, mostly human primary metabolites using Euclidian distance scores, and the method was able to detect novel structural features of marine NPs.[49] This approach yielded a new analog of a known antiprotozoal polyketide (gracilioether L, see SMART results in Figure S32).[50] They also demonstrated the method with re-isolation of known antibacterial cyclic heptapeptide, cyclomarin A.[51–53] However, three of four “novel features” detected by this method in cyclomarin A were false positives in terms of structural novelty (e.g. regular methyl group, methylene next to primary alcohol and alpha-carbon methine of *N*-methylleucine).

To demonstrate SMART 2.0 for mixture analysis and early prioritization for rare structural motifs, we used the NMR table from the SI in Duggan et al.[49] to evaluate the HSQC data of the crude extract as well as pure cyclomarin A (not in the SMART database). Intriguingly, the top two hits for cyclomarin A and top twenty hits for the crude extract, consisting of 289 C-H correlations in the HSQC spectrum, revealed the closely related ilamycins C1 and C2 as candidate structures.[54] Ilamycins harbor a very similar and rare *N*-(1,1-dimethyl-2,3-epoxypropyl)-tryptophan moiety as cyclomarin A [substructure search of NP Atlas database, (https://www.npatlas.org/) returned no additional NPs with this amino acid moiety].[37] Furthermore, ilamycins contain a similar 4-hexenoic acid residue as cyclomarin A (Figures 2, S33). This example underscores that while features such as heteroatoms and quaternary carbons are not directly measured in the ^{1}H-^{13}C HSQC experiment, CNNs can detect these features indirectly via analyzing chemical shift patterns as well as the relative positions of correlations. Thus, CNNs can extract information from non-peak areas whereas spectroscopists mainly rely on visible peaks for interpretation. This aspect likely explains the extraordinary robustness of SMART 2.0, as it very accurately predicts structures with chemical moieties that are not directly visible in the HSQC spectrum. Finally, each analysis

performed by SMART, from NMR data acquisition to table construction and structure prediction, took less than 30 minutes (the SMART structure prediction takes approximately 8 seconds). This compares favorably with the time-scale of LCMS measurements and is a great improvement over the weeks to months typically required for the structural analysis of highly modified cyclic peptides such as cyclomarin A.

In this report we introduce a highly improved user friendly version of the SMART tool and demonstrate its remarkable ability to rapidly recognize natural product structure types. SMART 2.0 is now available to the academic community in Python (https://smart.ucsd.edu/classic).

Rapid structure prediction of major constituents from crude extracts and fractions greatly assists with prioritization of structurally novel or otherwise interesting NPs for further study. Because knowledge of the structural class of an unknown NP can greatly accelerate its structure elucidation, this tool can overcome several of the bottlenecks currently present in NP research. We applied SMART 2.0 on crude fractions from a cyanobacterial extract that helped target the isolation of a new chimeric macrolide, symplocolide A, as well as dereplicate swinholide A and related compounds. The hybridic nature of symplocolide A suggests a novel biosynthetic pathway, an aspect that is under continuing study as the genetic basis for molecular diversification in a compound class is of great interest.[43,55] Further, we demonstrated here that SMART 2.0 can detect rare structural features from crude extracts and fractions. We envision that the continued development of SMART 2.0 can revolutionize aspects of NP drug discovery, especially when combined with orthogonal information derived from genome mining of the biosynthetic gene clusters, accurate mass and fragmentation data, and fingerprint analyses of molecular substructures via the GNPS platform, MS2LDA and Sirius.[22,27,28]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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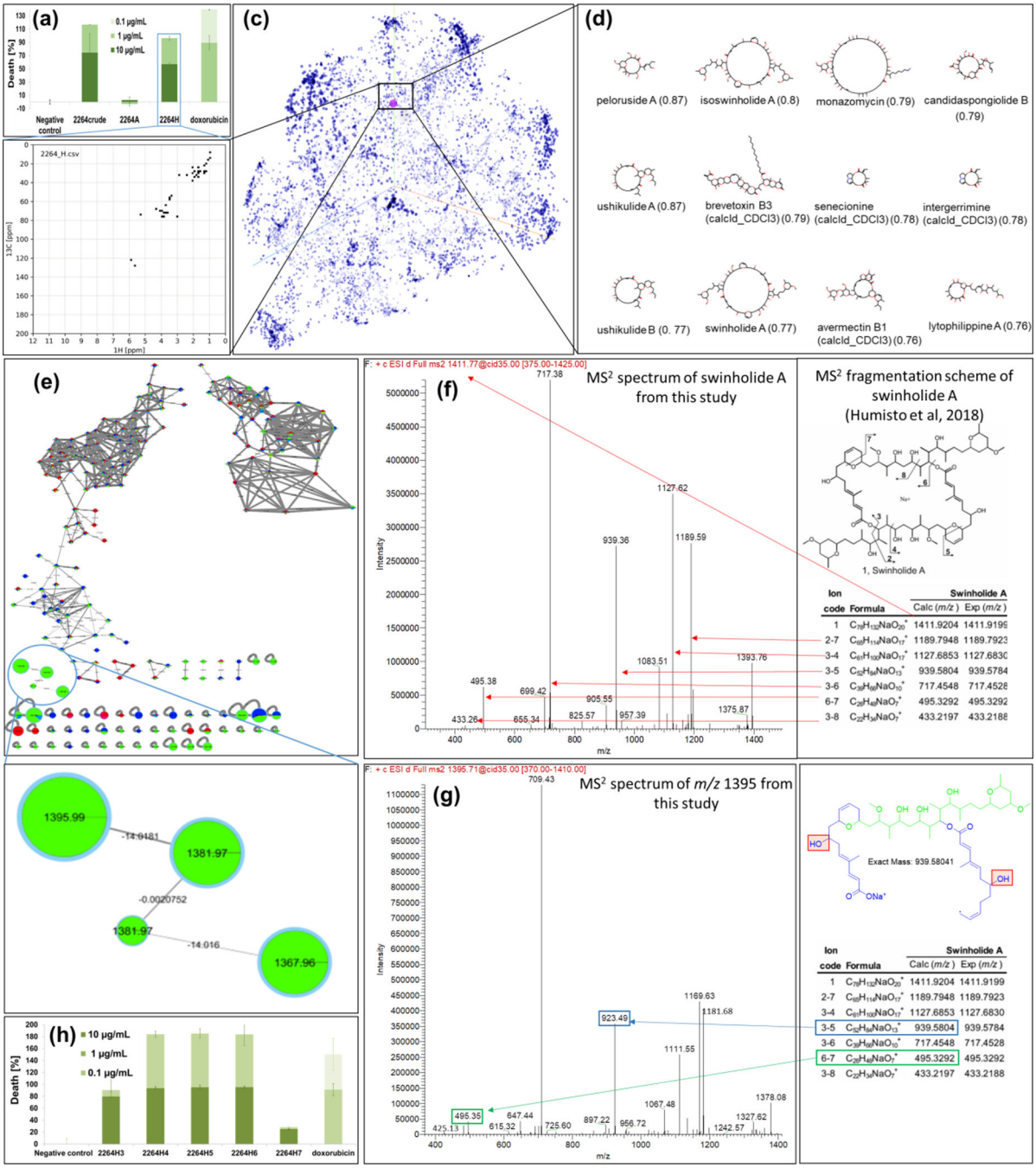


Figure 1.

(a,h) Cytotoxicity assay against H460 lung cancer cells reveals VLC fraction H (a) and subfractions H4–H6 (h), respectively, as the most potent at 1 and 10 µg/mL (see also Figure S3). (b) Digitized HSQC spectrum of most cytotoxic fraction H. (c) t-SNE embedding of the HSQC spectrum into the 180 dimensional cluster space of 53,076 nodes (reduced here to 10,000 for clarity) representing natural products trained on the basis of their ^{1}H-^{13}C-HSQC spectra. Swinholide A and its closest neighbors are highlighted (purple nodes, black rectangle). (d) SMART 2.0 results (top12 structures based on cosine similarity score) of

fraction H suggests that it contains macrolides from the swinholide class. (e) Molecular networking analysis of cyanobacterial subfractions H3–H7. Highlighted cluster with putatively new *m/z* feature 1395.9 (H4, green nodes). (f) MS^2 analysis of swinholide A[29] and comparison to (g) the feature detected as new *m/z* 1395.9 (symplocolide A) show fragmentation patterns that reveal structural insights (same fragment highlighted in green, distinctive fragment highlighted in blue) for both compounds.

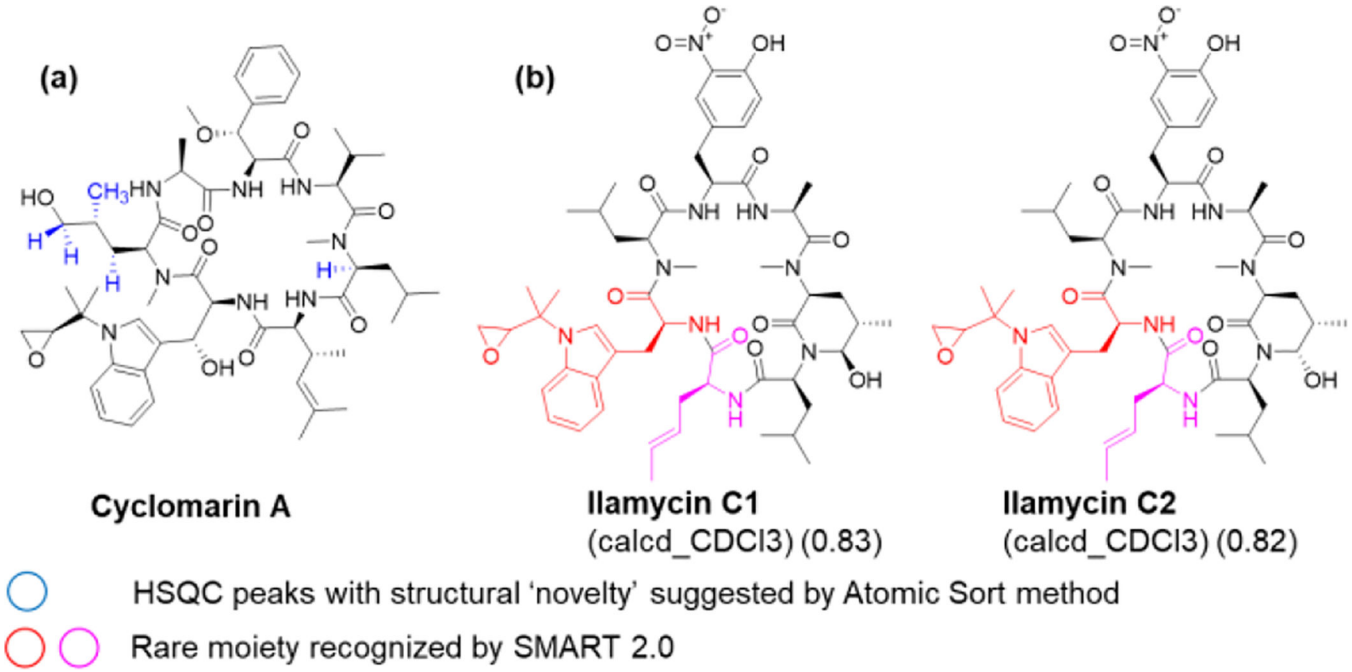
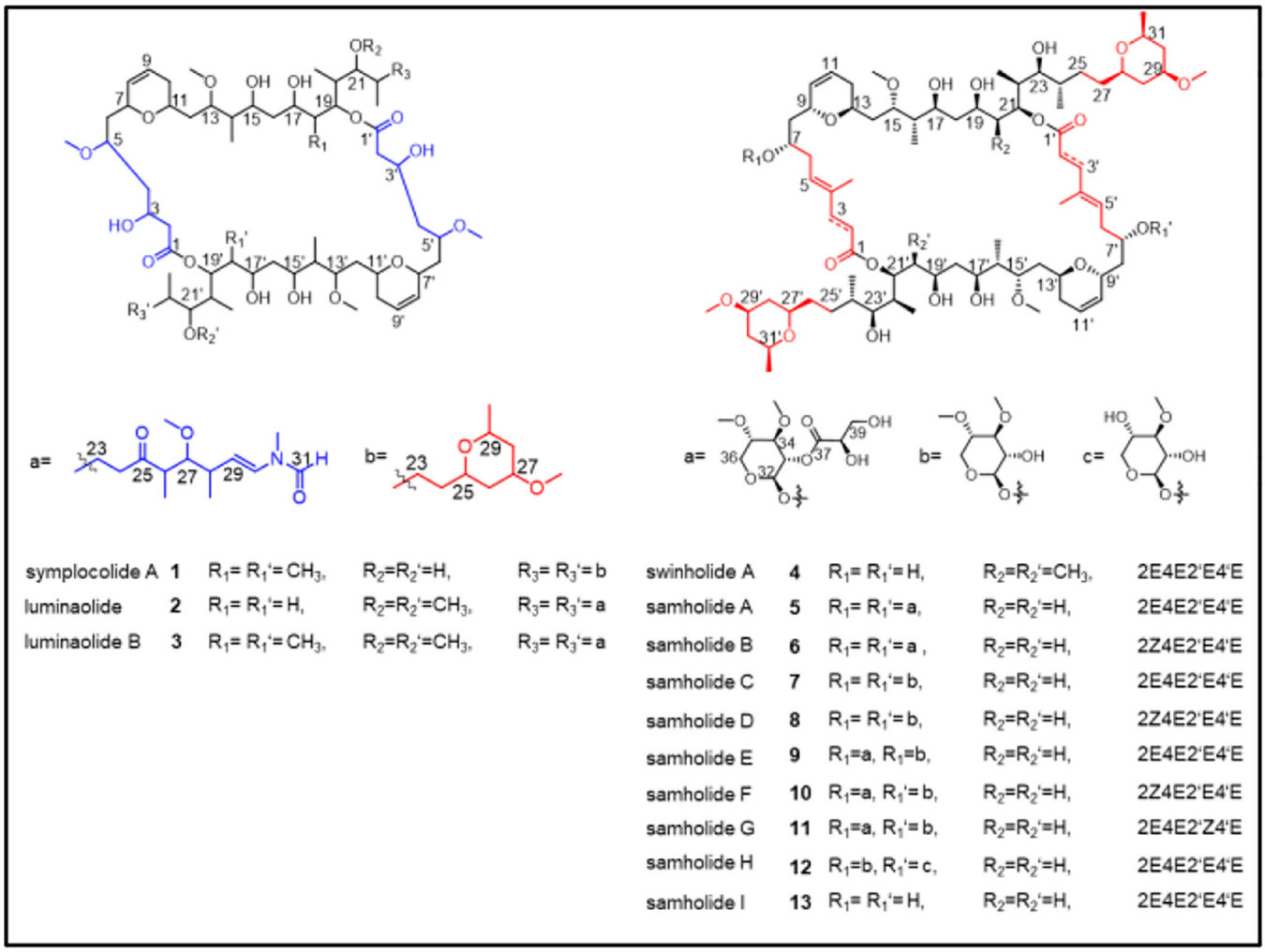


Figure 2.

Comparison of the 'atomic sort' method and SMART 2.0 to detect novel/rare structural elements. (a) HSQC correlations suggesting structural novelty by 'atomic sort' method (highlighted in blue). (b) Top two results of SMART 2.0 analysis querying cyclomarin A (experimental HSQC data from reference[49]) to detect related compounds that include rare structural moieties (highlighted in red, purple).



Scheme 1.

Structures of symplocolide A (**1**), luminaolide (**2**)[42], luminaolide B (**3**)[43], swinholide A (**4**), and samholides A-I (**5–13**).[44] All compounds except **2** and **3** were detected in this study.