Published in final edited form as:

ACS Chem Biol. 2016 August 19; 11(8): 2328–2336. doi:10.1021/acscchembio.5b00924.

Thiol Probes To Detect Electrophilic Natural Products Based on Their Mechanism of Action

Gabriel Castro-Falcón, Dongyup Hahn, Daniela Reimer, and Chambers C. Hughes*

Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92093, United States

Abstract

New methods are urgently needed to find novel natural products as structural leads for the development of new drugs against emerging diseases such as cancer and multiresistant bacterial infections. Here we introduce a reactivity-guided drug discovery approach for electrophilic natural products, a therapeutically relevant class of natural products that covalently modify their cellular targets, in crude extracts. Using carefully designed halogenated aromatic reagents, the process furnishes derivatives that are UV-active and highly conspicuous via mass spectrometry by virtue of an isotopically unique bromine or chlorine tag. In addition to the identification of high-value metabolites, the process facilitates the difficult task of structure elucidation by providing derivatives that are primed for X-ray crystallographic analysis. We show that a cysteine probe efficiently and chemoselectively labels enone-, β -lactam-, and β -lactone-based electrophilic natural products (parthenolide, andrographolide, wortmannin, penicillin G, salinosporamide), while a thiophenol probe preferentially labels epoxide-based electrophilic natural products (triptolide, epoxomicin, eponemycin, cyclomarin, salinamide). Using the optimized method, we were able to detect and isolate the epoxide-bearing natural product tirandalydigin from *Salinispora* and thereby link an orphan gene cluster to its gene product.

Graphical Abstract

*Corresponding Author: chughes@ucsd.edu.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchembio.5b00924.

Bioinformatic analysis of the tirandalydigin biosynthetic gene cluster, detailed experimental procedures, chromatograms of reaction mixtures, UV/vis and MS spectra, and tabulated spectroscopic data including original ^1H , ^1H - ^1H COSY, HSQC, and HMBC spectra for **1**, **4**, **6**, **8**, **16**, **17**, **21**, **23**, **26**, **28**, **30**, and **33** (PDF)

The authors declare no competing financial interest.

Historically, natural products have had a tremendous influence on the development of new drugs for cancer, antibacterial infections, immunosuppression, and immunostimulation.^{1,2} Bioinformatic analyses of bacterial genomes clearly show the presence of many more biosynthetic pathways encoding natural products than actual isolated compounds.^{3,4} This disparity signals a need for the development of new methods for natural products research. Some of these “orphan” gene clusters are “silent” and require a genetics-based solution for production of a metabolite.^{5,6} However, the primary reason for the large quantity of orphan gene clusters is that a natural product extract, in general, consists of miniscule amounts of the desired metabolite in a hodgepodge of medium components and primary metabolites such as amino acids, sugars, and lipids. Thus, new chemistry-based methods to access the hidden potential of bacterial genomes are needed.

One promising approach that complements traditional bioactivity-guided isolation utilizes the inherent reactivity of a metabolite to inform the detection and isolation process. One version uses soluble reagents to label natural product functional groups or structural features in an extract. Although the original metabolites are irreversibly modified, the resulting adducts often have enhanced visibility using UV-detected liquid chromatography/mass spectrometry (LC/MS) instruments. Marfey’s reaction, in which amino acids are chemoselectively modified to give adducts with strongly absorbing chromophores, is an early application of this method.⁷ Another method uses reactive resins to immobilize and selectively elute natural products on the basis of chemical functionality.^{8–11} In either case, only simple functional groups have been targeted, including dehydroalanine,^{12,13} ketones and aldehydes,^{8,9} carboxylic acids,^{8,9} amines,^{7–9} thiols,^{8,9} alcohols,¹⁰ terminal alkynes,^{11,14} and azides.¹⁵ Until recently,¹⁶ reactivity-guided isolation has not been employed for the identification of natural product pharmacophores that would lead directly to biologically active metabolites with a high potential for therapeutic development.

An important class of natural products owe their potent biological activity to an electrophilic moiety that engages the cellular target of the molecule in a covalent linkage.^{17,18} On the basis of the misconceived notion that they would be too toxic, the drug industry has historically avoided the development of covalent drugs, despite the unparalleled success of aspirin and penicillin. Recently, however, there has been a resurgence of interest in covalent drugs.¹⁹ In addition to the newly approved covalent proteasome inhibitor carfilzomib, whose structure is based on the natural product epoxomicin, several covalent kinase inhibitors with

striking selectivity and potency are in clinical trials or have been approved by the FDA.²⁰ Interestingly, the electrophilic mechanism of action of all 39 FDA-approved covalent drugs was discovered serendipitously only after they were selected and developed for their biological activity.¹⁹

RESULTS AND DISCUSSION

We set out to apply a reactivity-guided approach for the deliberate discovery of electrophilic natural products. Given the reactivity of thiolates with naturally occurring electrophilic moieties,^{21–30} which has previously been exploited to classify certain electrophilic natural products,³¹ we wished to design and synthesize a thiol-based probe that would tag these compounds in a crude extract. These “pharmacophore probes” consist of three parts: (1) a highly chemoselective reagent that reacts covalently with a specified pharmacophore, (2) an easily identifiable UV/vis or mass spectrometric tag, and (3) a linker that joins the reagent and the tag. We tested several different probe designs. Two probes proved superior to the others in terms of reactivity, chemoselectivity, and stability (Figure 1). Cysteine probe **1** was synthesized in three steps from L-cystine, while thiophenol probe **2** was obtained commercially. These probes possess each of the characteristics of an ideal pharmacophore probe. In addition to the thiol functionality, both have a chromophore due to the aromatic ring and a conspicuous MS isotopic pattern (⁸¹Br:⁷⁹Br = 1:1 and ³⁵Cl:³⁷Cl = 3:1). Though UV-active halogenated metabolites are not entirely uncommon, prior analysis of an extract can rapidly identify these endogenous molecules and thereby discount them as labeled electrophilic natural products. The rationale for different halogen atoms on probes **1** and **2** will be discussed later.

Both probes incorporate a halogenated aromatic ring. The introduction of this moiety, especially bromobenzoyl substituents, is a long-established method for obtaining crystalline derivatives of natural products that are amenable to X-ray crystallographic analysis.^{32–36} Thus, the probes not only react with the desired pharmacophore but also produce an adduct that is inherently more crystalline. Furthermore, the cysteine probe contains a defined *R* stereocenter that facilitates determinations of absolute stereochemistry.

We relied initially on four substrates to assess the coupling efficiency of our thiol probes and optimize the reaction conditions. Parthenolide (**3**) from feverfew (*Tanacetum parthenium*) contains a reactive butenolide moiety that is known to interact covalently with Cys171 of I κ B kinase and inhibit this enzyme (**12**) (Figure 2).³⁷ Treatment of **3** with probe **1** in triethylamine and *N,N*-dimethylformamide (DMF) afforded **4** as a single isomer in 36% yield after HPLC purification. The addition of triethylamine was essential for the reaction to proceed at an appreciable rate, presumably via formation of the corresponding thiolate. Andrographolide (**5**) from the plant *Andrographis paniculata* contains a substituted butenolide that reacts with Cys62 of p50, thereby inhibiting NF- κ B activation (**13**).³⁸ The probe efficiently reacted with **5** as well to give **6** as a mixture of diastereomers. The formation of diastereomeric products is not desired since it complicates the analysis, but we were able to purify a single diastereomer using reversed-phase HPLC. Via reaction with Lys802, wortmannin (**7**) from the fungus *Talaromyces wortmanii* is a covalent inhibitor of phosphoinositide 3-kinase (**14**).^{39–42} Metabolite **7** reacted with thiol **1** in a manner

completely analogous to the natural product's mechanism of action, producing ring-opened structure **8**. Importantly, this particular reaction was conducted in dichloromethane (DCM) since undesired elimination reactions occurred in DMF. The most famous electrophilic natural product, penicillin G (**9**), contains a β -lactam ring that is essential for its antibacterial activity. The mechanism of action of **9** involves acylation of a key serine residue of the bacterial transpeptidase enzyme (**15**).⁴³ Thiol **1** engaged in an efficient coupling reaction with **9** to cleanly produce a pair of diastereomers. Purification and NMR analysis of amino acid **10** proved to be difficult, so the labeling reaction was immediately followed by acetylation to give **11**.

We then screened a number of strains from the *tropical* marine bacterial genus *Salinispora* in order to demonstrate the method in crude extracts. Upon reaction of **1** with the extract of *Salinispora tropica* strain CNB-392, we observed the formation of two new compounds with UV absorption at 254 nm and brominated isotope patterns (Figure 3). Surprisingly, of the scores of other metabolites in the crude extract, none appeared to react with the probe. The first adduct, thioester **16**, was purified and analyzed by NMR spectroscopy and then retrosynthetically disconnected to obtain salinosporamide A (**18**), a well-known covalent proteasome inhibitor from *Salinispora*.⁴⁴ The second adduct, thioester **17**, is highly reminiscent of the threonine adduct in the crystal structure of the enzyme–inhibitor complex (**19**).⁴⁵ Thus, whether the electrophilic moiety is an α,β -unsaturated enone, an $\alpha,\beta,\gamma,\delta$ -unsaturated enone, a β -lactam, or a β -lactone, the thiol probe simultaneously tags the compound and elucidates its mode of action by mimicking the key nucleophilic residue on the cellular target responsible for covalent binding. This occurs whether the key amino acid residue on the target is cysteine, lysine, serine, or threonine (see **12–15** in Figure 2).

Notably, a saturated solution of thioester **17** in benzene produced crystals amenable to X-ray analysis within 12 h (see Figure 3). A common characteristic of molecules containing a “heavy” bromine atom and a key feature of the method, a near-zero Flack parameter of 0.068 was readily obtained and enabled the unambiguous assignment of the absolute configuration of **17** and hence the natural product **18**.⁴⁶

Although probe **1** is capable of engaging enone-, β -lactam-, and β -lactone-based electrophilic metabolites, its reactivity with epoxide-based electrophilic natural products was poor. Using triptolide (**20**), originally discovered from thunder god vine (*Tripterygium wilfordii*), and the bacterial metabolite epoxomicin (**22**) as model substrates, we determined that thiophenol probe **2** displayed remarkable reactivity toward epoxides (Figure 4). It has been shown that **20** exerts its biological effects via inhibition and covalent modification of Cys342 of the transcription factor TFIIA (**24**).^{47–49} In a manner that predicted the particular epoxide that is modified by the enzyme, the probe reacted with the 12,13-epoxide of **20** to yield **21**.⁴⁹ Likewise, **22**, a covalent proteasome inhibitor with an epoxy ketone functionality, gave **23** under the same conditions.⁵⁰ Unlike adducts formed from **1** and enone-, β -lactam-, and β -lactone-based electrophilic natural products, the structure of **23** does not mimic the morpholino adduct observed in the cocrystal structure of **22** and the proteasome, which like other covalent proteasome inhibitors involves reaction with the N-terminal threonine residue (**25**).⁵¹

In order to demonstrate the effectiveness of probe **2** in crude extracts, we first treated extract from *Streptomyces hygroscopicus* strain ATCC 53709 with probe **2**. This gave a major chlorinated adduct, **26**, which was retrosynthetically disconnected to obtain the proteasome inhibitor eponemycin (**27**) in addition to several minor chlorinated compounds from eponemycin analogues (Figure 5).^{52,53} Like that in **22**, the epoxide moiety in **27** is essential for the molecule's potent irreversible inhibition of the proteasome. When probe **2** was treated with extract from *Streptomyces* sp. strain CNB-382, a suite of chlorinated compounds was produced. The major adduct, **28**, can be traced back to cyclomarin A (**29**).⁵⁴ The other chlorinated compounds were derived from new cyclomarin analogues. In terms of its purported activity against *Mycobacterium tuberculosis*, which involves inhibition of the ClpC1 subunit of the caseinolytic protease,^{55,56} and its activity against malaria,⁵⁷ the epoxide is dispensable. Treatment of extract from *Streptomyces* sp. strain CNB-091 with probe **2** yielded a single chlorinated product, **30**, which is derived from salinamide A (**31**), in addition to the chloride-containing analogue salinamide B.^{58,59} Again, the epoxide ring is not required for inhibition of bacterial RNA polymerase.^{60,61} Thus, reactivity-guided isolation of epoxide-containing natural products with probe **2** led to the discovery of epoxide-based electrophilic natural products (e.g., eponemycin) as well as natural products containing epoxides that do not appear to be involved in the mechanism of action (e.g., cyclomarin and salinamide).

Probe **1** reacts much more readily with β -lactam- and β -lactone-based electrophilic natural products, and probe **2** reacts much faster with epoxide-containing natural products. This orthogonal reactivity allows them to be employed in extracts simultaneously. Since probe **1** is brominated and probe **2** is chlorinated, the isotopic patterns of labeled natural products point toward their structural origin. Competition experiments using probes **1** and **2** indicated that brominated probe **1** reacted exclusively with the β -lactam in penicillin G (**9**) and the β -lactone in salinosporamide A (**18**) while chlorinated probe **2** alone reacted with the epoxide in salinamide A (**31**) (see the Supporting Information). Interestingly, some chlorinated products were formed from the reaction of **18** and probe **2** via a substitution reaction with the alkyl chloride functionality in **18**. This result was validated using salinosporamide B, which lacks an alkyl chloride and shows no reactivity with probe **2**. Probe reactivity was not exclusive with parthenolide (**3**), which bears a highly reactive exocyclic enone, as an approximately 1:1 mixture of brominated and chlorinated parthenolide adducts was produced.

Although genome mining techniques that can predict natural product structures have progressed considerably, detecting and isolating gene products produced in minute quantities still remains a challenge. As reactivity-guided isolation directly probes molecular structure, it is an ideal method for enabling genome mining efforts. For instance, the genome of *Salinispora pacifica* strain CNT-003 contained fragments of an orphan gene cluster that showed similarity to the tirandamycin gene cluster (Figure 6).⁶² While screening *Salinispora* extracts with the probes, we noticed the production of a distinct chlorinated adduct, **32**, upon reaction of **2** with the extract of strain CNT-003. We suspected that we had labeled the gene product of the tirandamycin-like cluster, as many of these metabolites contain epoxides. In this case, the probe–natural product adduct was not isolated. Instead, the molecular formula

of the original metabolite was calculated to be $C_{22}H_{27}NO_6$, and the compound was then directly isolated from the crude extract using its calculated mass and predicted UV properties. The 1H NMR spectrum clearly matched tirandalydigin (**33**), a compound closely related biosynthetically and structurally to the tirandamycins.^{63,64} Like cyclomarin A (**28**) and salinamide A (**31**), the epoxide in **33** does not appear to be essential to its activity as a bacterial RNA polymerase inhibitor.

Given the central role that the electrophilic centers play in the natural products' biological activity, we examined the relative cytotoxicities of the natural products and their corresponding thiol adducts (Table 1). Against the HCT-116 colon cancer cell line, enones **3**, **5**, and **7** as well as their corresponding thiol adducts **4**, **6**, and **8** showed only moderate cytotoxicity ($IC_{50} = 11\text{--}32\text{ }\mu M$). Salinosporamide A (**18**) ($IC_{50} = 41\text{ nM}$) was converted into thioester **16**, which maintained remarkable activity ($IC_{50} = 14\text{ nM}$), and thioester **17**, which showed little cytotoxicity ($IC_{50} = 14\text{ }\mu M$). Like lactacystin and other salinosporamide-based thioesters,^{65,66} **16** is capable of reforming the β -lactone ring that is crucial to proteasome inhibition, while **17** cannot undergo this transformation.^{67,68} The cytotoxicities of the epoxide-based electrophilic natural products **20**, **22**, and **27** are greatly diminished (100–1000-fold) upon ring-opening thioether formation, highlighting the importance of this reactive pharmacophore. Metabolites **9**, **29**, and **31**, which have demonstrated antibacterial activity and prokaryotic targets, and their derivatives were not tested in the HCT-116 bioassay.

Conclusions.

The tremendous potential for the discovery of natural products that inspire new drugs has not been realized because there is a shortage of new methods. Reactivity-guided isolation, a hybrid of traditional natural products chemistry and synthetic organic chemistry, was invented to help access the high-value chemical space of natural product extracts. This approach, which can be applied to bacterial, fungal, and plant extracts alike, could transform the field of natural products.

METHODS

Preparation of Cysteine Thiol Probe 1.

To cooled methanol (150 mL) at 0 °C was slowly added thionyl chloride (7.60 mL, 105 mmol) followed by L-cystine (10.0 g, 41.6 mmol), and the reaction mixture was heated at reflux for 12 h. The solution was concentrated until the volume was reduced to 15 mL. Diethyl ether (30 mL) was added, and 12.4 g of cystine dimethyl ester precipitated out of solution. To a white heterogeneous solution of the dimethyl ester (3.64 g, 13.6 mmol) in acetonitrile (150 mL), 4-bromobenzoyl chloride (6.22 g, 28.7 mmol) and triethylamine (7.50 mL, 54.3 mmol) were added. The reaction mixture was left to stir for 2 h. A saturated ammonium chloride solution (300 mL) was added, and the resulting solution was extracted with ethyl acetate (2 × 200 mL). The combined organic extracts were washed with a saturated sodium bicarbonate solution (400 mL) and brine (200 mL), dried over Na_2SO_4 , and concentrated. A saturated solution of the product in dichloromethane (50 mL) was prepared. Then hexanes (30 mL) was added to the solution, and dibromobenzoyl disulfide

(6.96 g, 81% yield) precipitated out of the solution. To a solution of the disulfide (6.0 g, 9.4 mmol) in a 2:1 dichloromethane/methanol mixture were added tris(2-chloroethyl)phosphine hydrochloride salt (4.0 g, 14 mmol) and triethylamine (5.0 mL, 38 mmol). The reaction mixture was left for 3 h. Flash chromatography of the crude material on silica gel (hexanes/ethyl acetate, 4:6) yielded cysteine thiol probe **1** (5.4 g, 90% yield) as a white solid. UV/vis: $\lambda_{\text{max}} = 241$ nm. $[\alpha]_{\text{D}} = +3.3$ (c 0.020, CHCl_3). IR (KBr): $\tilde{\nu} = 3323, 1742, 1635, 1524, 1219$ cm^{-1} . ^1H NMR: δ 7.71 (d, $J = 8.6$ Hz, 2H), 7.61 (d, $J = 8.6$ Hz, 2H), 7.01 (br d, 1H), 5.07 (ddd, $J = 7.1, 4.0, 4.0$ Hz, 1H), 3.84 (s, 3H), 3.14 (dd, $J = 9.1, 4.0$ Hz, 2H), 1.38 (t, $J = 9.1$ Hz, 1H). ^{13}C NMR: δ 170.3, 165.8, 132.1, 131.7, 128.5, 126.6, 53.7, 52.8, 26.7. HR-ESI-Q-TOF-MS: m/z ($\text{M} + \text{H}$) $^+$ 317.9800 calcd for $\text{C}_{11}\text{H}_{13}^{79}\text{BrNO}_3\text{S}$, found 317.9784.

Generation of Crude Extracts.

Streptomyces hygroscopicus ATCC53709 was grown in ISP1 medium (5 g L^{-1} tryptone and 3 g L^{-1} yeast extract in deionized water), while *Streptomyces* spp. CNB-091 and CNB-382 and *Salinispora* spp. CNB-392 and CNT-003 were grown in A1 seawater medium (10 g L^{-1} starch, 4 g L^{-1} yeast extract, and 2 g L^{-1} peptone in 75% seawater and 25% deionized water). From cryovials, these strains were first grown in starter cultures (25 mL), and after 7 days the whole volume was used to inoculate precultures (1 L). After 3–4 days, 25 mL aliquots of the precultures were used to inoculate producing cultures (1 L). The cultures were incubated at 27 °C with rotatory shaking at 150 rpm for 3–7 days, after which Amberlite XAD-16N resin (~ 20 g L^{-1}) was added and the cultures were left to shake for 2 h. The resin was filtered through cheesecloth, washed with deionized water, and extracted with acetone. The acetone was removed under reduced pressure, and the resulting aqueous layer was extracted with ethyl acetate. The combined organic extracts were concentrated.

Labeling Reaction.

To pure natural product or crude extract, probe **1** or **2** (1.2–1.5 equiv), and triethylamine (2.0–4.2 equiv) was added dry *N,N*-dimethylformamide that had been previously sparged with gaseous nitrogen for 10 min. The reaction vessel was kept under an inert atmosphere using a nitrogen gas source and bubbler. The reaction was conducted with or without added tris(2-chloroethyl)phosphine hydrochloride salt (1.2–1.4 equiv) to prevent unwanted dimerization of the probes. The progress of the reaction was monitored on an analytical Agilent 1100 Series HP system (1.0 mL min^{-1}) with UV (210 and 254 nm) and ELS detection and also on an analytical Agilent 1260 Infinity Series LC system coupled to a 6530 Series Q-TOF mass spectrometer, both using a C18(2) Phenomenex Luna column (5 μm , 100 mm $\times$ 4.6 mm) with a 10 min solvent gradient from 10% to 100% acetonitrile + 0.1% formic acid in water. When HPLC analysis indicated complete conversion (1.5–24 h), the mixture was concentrated. The adducts were then purified by isocratic reversed-phase HPLC (acetonitrile and water + 0.1% trifluoroacetic acid) using a C8(2) Phenomenex Luna column (5 μm , 250 mm $\times$ 10 mm) with UV detection (254 nm). Preparative reversed-phase HPLC purifications were performed with a C18(2) Phenomenex Luna column (10 μm , 250 mm $\times$ 21.2 mm) with UV detection (254 nm).

Cytotoxicity Assays.

The human colorectal cancer cell line HCT-116 (ATCC CCL-247) was cultured in McCoy's 5A medium (Life Technologies) supplemented with 10% fetal bovine serum (Invitrogen), 30 mM HEPES (Fisher Scientific), and 100 $\mu\text{g mL}^{-1}$ penicillin/streptomycin (Invitrogen) and incubated in tissue culture flasks with a surface area of 75 cm^2 at 37 °C in a 5% CO_2 atmosphere. Routine passaging of cells was conducted every 3–4 days. The cell layer was briefly rinsed in 10 mL of Dulbecco's phosphate-buffered saline (DPBS) with Ca^{2+} and Mg^{2+} (Life Technologies) and detached from the culture flask using 2 mL of 0.25% (w/v) trypsin-EDTA (Life Technologies) at 37 °C. For the subcultivation, 8 mL of complete McCoy's 5A medium was added to the detached cells, and the cells were aspirated by gentle pipetting and then transferred into a new culture flask (1 mL into 20 mL of fresh medium). Cytotoxicities as measured by half-maximal inhibitory concentrations (IC_{50}) of the natural products (**3**, **5**, **7**, **18**, **20**, **22**, and **27**) and their adducts (**4**, **6**, **8**, **16**, **17**, **21**, **23**, and **26**) were tested using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) bioassay. HCT-116 cells (2.5×10^4 cells mL^{-1}) were incubated for 24 h at 37 °C in a 5% CO_2 atmosphere in flat-bottom 96-well plates. Compounds in dimethyl sulfoxide (DMSO) were added at a concentration of 10, 1, or 0.1 mg mL^{-1} on the basis of their activity, serially diluted, and incubated for an additional 72 h. As a reference, the standard anticancer drug etoposide VP-16 (Sigma-Aldrich) with an IC_{50} of 0.49–4.9 μM at a concentration of 4 mg mL^{-1} in DMSO and DMSO (ATCC) were tested as positive and negative controls, respectively. IC_{50} values were determined after a 3 h incubation of the colorimetric indicator MTS (1.9 mg mL^{-1} in DPBS, Promega) in the presence of phenazine methosulfate (0.044 mg mL^{-1} in DPBS, Sigma-Aldrich). MTS is reduced by living cells into a formazan product with an absorbance maximum at 490 nm in phosphate-buffered saline. Quantification of the formazan product at 490 nm representing the proportional cell survival rates and the calculation of the IC_{50} values were determined by an E^{Max} microplate reader (Molecular Devices) using the analysis software SoftMax Pro.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was primarily supported by startup funds from the Scripps Institution of Oceanography. C.C.H. acknowledges the UCSD Academic Senate for a Research Award (RN143S-HUGHES) and the Hellman Foundation for a generous fellowship. G.C.-F. thanks SIO for a San Diego Fellowship (2T32GM067550–10) as part of The Training Program in Marine Biotechnology (NIH GM067550). D.R. thanks the Deutsche Forschungsgemeinschaft (RE 3704/1–1) for postdoctoral funding. Genome sequencing was conducted by the U.S. Department of Energy Joint Genome Institute and supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02–05H11231. An HRLC-MS instrument was provided by the National Institutes of Health (S10 OD0106400).

REFERENCES

- (1). Newman DJ, and Cragg GM (2012) Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J. Nat. Prod* 75, 311–335. [PubMed: 22316239]
- (2). Cragg GM, and Newman DJ (2013) Natural products: A continuing source of novel drug leads. *Biochim. Biophys. Acta, Gen. Subj* 1830, 3670–3695.

- (3). Bentley SD, Chater KF, Cerdeño-Tárraga A-M, Challis GL, Thomson NR, James KD, Harris DE, Quail MA, Kieser H, Harper D, Bateman A, Brown S, Chandra G, Chen CW, Collins M, Cronin A, Fraser A, Goble A, Hidalgo J, Hornsby T, Howarth S, Huang C-H, Kieser T, Larke L, Murphy L, Oliver K, O'Neil S, Rabbinowitsch E, Rajandream M-A, Rutherford K, Rutter S, Seeger K, Saunders D, Sharp S, Squares R, Squares S, Taylor K, Warren T, Wietzorrek A, Woodward J, Barrell BG, Parkhill J, and Hopwood DA (2002) Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* 417, 141–147. [PubMed: 12000953]
- (4). Udvarý DW, Zeigler L, Asolkar RN, Singan V, Lapidus A, Fenical W, Jensen PR, and Moore BS (2007) Genome sequencing reveals complex secondary metabolome in the marine actinomycete *Salinispora tropica*. *Proc. Natl. Acad. Sci. U. S. A* 104, 10376–10381. [PubMed: 17563368]
- (5). Zhang H, Boghigian BA, Armando J, and Pfeifer BA (2011) Methods and options for the heterologous production of complex natural products. *Nat. Prod. Rep* 28, 125–151. [PubMed: 21060956]
- (6). Ongley SE, Bian X, Neilan BA, and Müller R (2013) Recent advances in the heterologous expression of microbial natural product biosynthetic pathways. *Nat. Prod. Rep* 30, 1121–38. [PubMed: 23832108]
- (7). Marfey P (1984) Determination of D-amino acids. II. Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. *Carlsberg Res. Commun* 49, 591–596.
- (8). Carlson EE, and Cravatt BF (2007) Enrichment tags for enhanced-resolution profiling of the polar metabolome. *J. Am. Chem. Soc* 129, 15780–15782. [PubMed: 18052286]
- (9). Carlson EE, and Cravatt BF (2007) Chemoselective probes for metabolite enrichment and profiling. *Nat. Methods* 4, 429–435. [PubMed: 17417646]
- (10). Odendaal AY, Trader DJ, and Carlson EE (2011) Chemoselective enrichment for natural products discovery. *Chem. Sci* 2, 760–764. [PubMed: 24926410]
- (11). Jeon H, Lim C, Lee JM, and Kim S (2015) Chemical assay-guided natural product isolation via solid-supported chemodosimetric fluorescent probe. *Chem. Sci* 6, 2806–2811. [PubMed: 28706669]
- (12). Miles CO, Sandvik M, Nonga HE, Rundberget T, Wilkins AL, Rise F, and Ballot A (2012) Thiol derivatization for LC-MS identification of microcystins in complex matrices. *Environ. Sci. Technol* 46, 8937–8944. [PubMed: 22834560]
- (13). Cox CL, Tietz JJ, Sokolowski K, Melby JO, Doroghazi JR, and Mitchell DA (2014) Nucleophilic 1,4-additions for natural product discovery. *ACS Chem. Biol* 9, 2014–2022. [PubMed: 24937678]
- (14). Ross C, Scherlach K, Kloss F, and Hertweck C (2014) The molecular basis of conjugated polyyne biosynthesis in phytopathogenic bacteria. *Angew. Chem., Int. Ed* 53, 7794–7798.
- (15). Pérez AJ, Wesche F, Adihou H, and Bode HB (2016) Solid-phase enrichment and analysis of azide-labeled natural products: Fishing downstream of biochemical pathways. *Chem. - Eur. J* 22, 639–645. [PubMed: 26626278]
- (16). Rudolf GC, Koch MF, Mandl FAM, and Sieber SA (2015) Subclass-specific labeling of protein-reactive natural products with customized nucleophilic probes. *Chem. - Eur. J* 21, 3701–3707. [PubMed: 25605563]
- (17). Drahl C, Cravatt BF, and Sorensen EJ (2005) Protein-reactive natural products. *Angew. Chem., Int. Ed* 44, 5788–5809.
- (18). Gersch M, Kreuzer J, and Sieber SA (2012) Electrophilic natural products and their biological targets. *Nat. Prod. Rep* 29, 659–682. [PubMed: 22504336]
- (19). Singh J, Petter RC, Baillie TA, and Whitty A (2011) The resurgence of covalent drugs. *Nat. Rev. Drug Discovery* 10, 307–317. [PubMed: 21455239]
- (20). Sanderson K (2013) Irreversible kinase inhibitors gain traction. *Nat. Rev. Drug Discovery* 12, 649–51. [PubMed: 23989776]
- (21). Corey EJ, Clark DA, Goto G, Marfat A, Mioskowski C, Samuelsson B, and Hammarstrom S (1980) Stereospecific total synthesis of a "slow reacting substance" of anaphylaxis, leukotriene C-1. *J. Am. Chem. Soc* 102, 1436–1439.

- (22). Corey EJ, Albright JO, Barton AE, and Hashimoto S (1980) Chemical and enzymic syntheses of 5-HPETE, a key biological precursor of slow-reacting substance of anaphylaxis (SRS), and 5-HETE. *J. Am. Chem. Soc* 102, 1435–1436.
- (23). Llinfás A, Donoso J, Vilanova B, Frau J, Muñoz F, and Page MI (2000) Thiol-catalysed hydrolysis of benzylpenicillin. *J. Chem. Soc., Perkin Trans 2*, 1521–1525.
- (24). Martinelli MJ, Vaidyanathan R, Khau VV, and Staszak MA (2002) Reaction of cryptophycin 52 with thiols. *Tetrahedron Lett.* 43, 3365–3367.
- (25). Oda K, and Yoshida A (1997) Side chain substitution reaction of 2-arylsulfinyl and 2-arylsulfonyl intermediates for 1 β -methylcarbapenems. *Tetrahedron Lett.* 38, 5687–5690.
- (26). Naidu BN, Sorenson ME, Connolly TP, and Ueda Y (2003) Michael addition of amines and thiols to dehydroalanine amides: A remarkable rate acceleration in water. *J. Org. Chem* 68, 10098–10102. [PubMed: 14682706]
- (27). Matsuura F, Peters R, Anada M, Harried SS, Hao J, and Kishi Y (2006) Unified total synthesis of pteriatoxins and their diastereomers. *J. Am. Chem. Soc* 128, 7463–7465. [PubMed: 16756299]
- (28). Taubinger AA, Fenske D, and Podlech J (2008) Synthesis of β,β -diamino acids from α -amino acid derived β -lactams. *Synlett* 2008, 539–542.
- (29). Schoof S, Pradel G, Aminake MN, Ellinger B, Baumann S, Potowski M, Najajreh Y, Kirschner M, and Arndt HD (2010) Antiplasmodial thioestrepton derivatives: Proteasome inhibitors with a dual mode of action. *Angew. Chem., Int. Ed* 49, 3317–3321.
- (30). Csuk R, Heinold A, Siewert B, Schwarz S, Barthel A, Kluge R, and Ströhl D (2012) Synthesis and biological evaluation of antitumor-active arglabin derivatives. *Arch. Pharm. (Weinheim, Ger.)* 345, 215–222.
- (31). Avonto C, Taglialatela-scafati O, Pollastro F, Minassi A, Di Marzo V, De Petrocellis L, and Appendino G (2011) An NMR spectroscopic method to identify and classify thiol- trapping agents: Revival of Michael acceptors for drug discovery? *Angew. Chem., Int. Ed* 50, 467–471.
- (32). Gerwick WH, Fenical W, Van Engen D, and Clardy J (1980) Isolation and structure of spatol, a potent inhibitor of cell replication from the brown seaweed *Spatoglossum schmittii*. *J. Am. Chem. Soc* 102, 7991–7993.
- (33). Pettit GP, Herald DL, Gao F, Sengupta D, and Herald CL (1991) Antineoplastic agents. 200. Absolute configuration of the bryostatins. *J. Org. Chem* 56, 1337–1340.
- (34). Ondeyka JG, Helms GL, Hensens OD, Goetz MA, Zink DL, Tsiouras A, Shoop WL, Slayton L, Dombrowski AW, Polishook JD, Ostlind DA, Tsou NN, Ball RG, and Singh SB (1997) Nodulisporic acid A, a novel and potent insecticide from a *Nodulisporium* sp. Isolation, structure determination, and chemical transformations. *J. Am. Chem. Soc* 119, 8809–8816.
- (35). Shigemori H, Komaki H, Yazawa K, Mikami Y, Nemoto A, Tanaka Y, Sasaki T, In Y, Ishida T, and Kobayashi J (1998) Brasilicardin A. A novel tricyclic metabolite with potent immunosuppressive activity from actinomycete *Nocardia brasiliensis*. *J. Org. Chem* 63, 6900–6904. [PubMed: 11672311]
- (36). Brady SF, Bondi SM, and Clardy J (2001) The guanacastepenes: A highly diverse family of secondary metabolites produced by an endophytic fungus. *J. Am. Chem. Soc* 123, 9900–9901. [PubMed: 11583556]
- (37). Kwok BHB, Koh B, Ndubuisi MI, Elofsson M, and Crews CM (2001) The anti-inflammatory natural product parthenolide from the medicinal herb Feverfew directly binds to and inhibits I-kappa B kinase. *Chem. Biol* 8, 759–766. [PubMed: 11514225]
- (38). Xia Y-F, Ye B-Q, Li Y-D, Wang J-G, He X-J, Lin X, Yao X, Ma D, Slungaard A, Hebbel RP, Key NS, and Geng J-G (2004) Andrographolide attenuates inflammation by inhibition of NF-kappa B activation through covalent modification of reduced cysteine 62 of p50. *J. Immunol* 173, 4207–4217. [PubMed: 15356172]
- (39). Arcaro A, and Wymann MP (1993) Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem. J* 296, 297–301. [PubMed: 8257416]
- (40). Norman BH, Shih C, Toth JE, Ray JE, Dodge JA, Johnson DW, Rutherford PG, Schultz RM, Worzalla JF, and Vlahos CJ (1996) Studies on the mechanism of phosphatidylinositol 3-kinase

inhibition by wortmannin and related analogs. *J. Med. Chem* 39, 1106–1111. [PubMed: 8676346]

- (41). Wymann MP, Bulgarelli-Leva G, Zvelebil MJ, Pirola L, Vanhaesebroeck B, Waterfield MD, and Panayotou G (1996) Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol. Cell. Biol* 16, 1722–1733. [PubMed: 8657148]
- (42). Walker EH, Pacold ME, Perisic O, Stephens L, Hawkins PT, Wymann MP, and Williams RL (2000) Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine. *Mol. Cell* 6, 909–919. [PubMed: 11090628]
- (43). Yocum RR, Waxman DJ, Rasmussen JR, and Strominger JL (1979) Mechanism of penicillin action: penicillin and substrate bind covalently to the same active site serine in two bacterial D-alanine carboxypeptidases. *Proc. Natl. Acad. Sci. U. S. A* 76, 2730–2734. [PubMed: 111240]
- (44). Feling RH, Buchanan GO, Mincer TJ, Kauffman CA, Jensen PR, and Fenical W (2003) Salinosporamide A: A highly cytotoxic proteasome inhibitor from a novel microbial source, a marine bacterium of the new genus *Salinospora*. *Angew. Chem., Int. Ed* 42, 355–357.
- (45). Groll M, Huber R, and Potts BCM (2006) Crystal structures of salinosporamide A (NPI-0052) and B (NPI-0047) in complex with the 20S proteasome reveal important consequences of β -lactone ring opening and a mechanism for irreversible binding. *J. Am. Chem. Soc* 128, 5136–5141. [PubMed: 16608349]
- (46). Flack HD (1983) On enantiomorph-polarity estimation. *Acta Crystallogr., Sect. A: Found. Crystallogr* 39, 876–881.
- (47). McCallum C, Kwon S, Leavitt P, Shen DM, Liu W, and Gurnett A (2007) Triptolide binds covalently to a 90 kDa nuclear protein. Role of epoxides in binding and activity. *Immunobiology* 212, 549–556. [PubMed: 17678712]
- (48). Titov DV, Gilman B, He Q-L, Bhat S, Low W-K, Dang Y, Smeaton M, Demain AL, Miller PS, Kugel JF, Goodrich JA, and Liu JO (2011) XPB, a subunit of TFIIH, is a target of the natural product triptolide. *Nat. Chem. Biol* 7, 182–188. [PubMed: 21278739]
- (49). He Q-L, Titov DV, Li J, Tan M, Ye Z, Zhao Y, Romo D, and Liu JO (2015) Covalent modification of a cysteine residue in the XPB subunit of the general transcription factor TFIIH through single epoxide cleavage of the transcription inhibitor triptolide. *Angew. Chem., Int. Ed* 54, 1859–1863.
- (50). Kim KB, Myung J, Sin N, and Crews CM (1999) Proteasome inhibition by the natural products epoxomicin and dihydroeponemycin: Insights into specificity and potency. *Bioorg. Med. Chem. Lett* 9, 3335–3340. [PubMed: 10612595]
- (51). Groll M, Kim KB, Kairies N, Huber R, and Crews CM (2000) Crystal structure of epoxomicin: 20S proteasome reveals a molecular basis for selectivity of α,β -epoxyketone proteasome inhibitors. *J. Am. Chem. Soc* 122, 1237–1238.
- (52). Meng L, Kwok BHB, Sin N, and Crews CM (1999) Eponemycin exerts its antitumor effect through the inhibition of proteasome function. *Cancer Res.* 59, 2798–2801. [PubMed: 10383134]
- (53). Schorn M, Zettler J, Noel JP, Dorrestein PC, Moore BS, and Kaysser L (2014) Genetic basis for the biosynthesis of the pharmaceutically important class of epoxyketone proteasome inhibitors. *ACS Chem. Biol* 9, 301–309. [PubMed: 24168704]
- (54). Renner M, Shen Y, Cheng X, Jensen PR, Frankmoelle W, Kauffman CA, Fenical W, Lobkovsky E, and Clardy J (1999) Cyclomarins A-C, new anti inflammatory cyclic peptides produced by a marine bacterium (*Streptomyces* sp.). *J. Am. Chem. Soc* 121, 11273–11276.
- (55). Schmitt EK, Riwanto M, Sambandamurthy V, Roggo S, Miault C, Zwingelstein C, Krastel P, Noble C, Beer D, Rao SPS, Au M, Niyomrattanakit P, Lim V, Zheng J, Jeffery D, Pethe K, and Camacho LR (2011) The natural product cyclomarin kills mycobacterium tuberculosis by targeting the ClpC1 subunit of the caseinolytic protease. *Angew. Chem., Int. Ed* 50, 5889–5891.
- (56). Vasudevan D, Rao SPS, and Noble CG (2013) Structural basis of mycobacterial inhibition by cyclomarin A. *J. Biol. Chem* 288, 30883–30891. [PubMed: 24022489]
- (57). Bürstner N, Roggo S, Ostermann N, Blank J, Delmas C, Freuler F, Gerhartz B, Hinniger A, Hoepfner D, Liechty B, Mihalic M, Murphy J, Pistorius D, Rottmann M, Thomas JR, Schirle M, and Schmitt EK (2015) Gift from nature: Cyclomarin A kills mycobacteria and malaria parasites by distinct modes of action. *ChemBioChem* 16, 2433–2436. [PubMed: 26472355]

- (58). Trischman JA, Tapiolas DM, Jensen PR, Dwight R, Fenical W, Mckee TC, Ireland CM, Stout TJ, and Clardy J (1994) Salinamides A and B: anti-inflammatory depsipeptides from a marine Streptomyces. *J. Am. Chem. Soc* 116, 757–758.
- (59). Moore BS, Trischman JA, Seng D, Kho D, Jensen PR, and Fenical W (1999) Salinamides, antiinflammatory depsipeptides from a marine streptomycete. *J. Org. Chem* 64, 1145–1150.
- (60). Degen D, Feng Y, Zhang Y, Ebright KY, Ebright YW, Gigliotti M, Vahedian-Movahed H, Mandal S, Talaue M, Connell N, Arnold E, Fenical W, and Ebright RH (2014) Transcription inhibition by the depsipeptide antibiotic salinamide A. *eLife* 3, e02451. [PubMed: 24843001]
- (61). Hassan HM, Degen D, Jang KH, Ebright RH, and Fenical W (2015) Salinamide F, new depsipeptide antibiotic and inhibitor of bacterial RNA polymerase from a marine-derived Streptomyces sp. *J. Antibiot* 68, 206–209. [PubMed: 25227504]
- (62). Ziemert N, Lechner A, Wietz M, Millán-Aguinaga N, Chavarria KL, and Jensen PR (2014) Diversity and evolution of secondary metabolism in the marine actinomycete genus Salinispora. *Proc. Natl. Acad. Sci. U. S. A* 111, E1130–E1139. [PubMed: 24616526]
- (63). Brill GM, McAlpine JB, and Whittern D (1988) Tirandalydigin, a novel tetramic acid of the tirandamycin-streptolydigin type. II. Isolation and structural characterization. *J. Antibiot* 41, 36–44. [PubMed: 3346191]
- (64). Karwowski JP, Jackson M, Theriault RJ, Barlow GJ, Coen L, Hensey DM, and Humphrey PE (1992) Tirandalydigin, a novel tetramic acid of the tirandamycin-streptolydigin type. I. Taxonomy of the producing organism, fermentation and biological activity. *J. Antibiot* 45, 1125–1132. [PubMed: 1517158]
- (65). Omura S, Fujimoto T, Otoguro K, Matsuzaki K, Moriguchi R, Tanaka H, and Sasaki Y (1991) Lactacystin, a novel microbial metabolite, induces neuritogenesis of neuroblastoma cells. *J. Antibiot* 44, 113–116. [PubMed: 1848215]
- (66). Reed KA, Manam RR, Mitchell SS, Xu J, Teisan S, Chao TH, Deyanat-Yazdi G, Neuteboom STC, Lam KS, and Potts BCM (2007) Salinosporamides D-J from the marine actinomycete Salinispora tropica, bromosalinosporamide, and thioester derivatives are potent inhibitors of the 20S proteasome. *J. Nat. Prod* 70, 269–276. [PubMed: 17243724]
- (67). Dick LR, Cruikshank AA, Grenier L, Melandri FD, Nunes SL, and Stein RL (1996) Mechanistic studies on the inactivation of the proteasome by lactacystin. *J. Biol. Chem* 271, 7273–7276. [PubMed: 8631740]
- (68). Dick LR, Cruikshank AA, Destree AA, Grenier L, McCormack TA, Melandri FD, Nunes SL, Palombella VJ, Parent LA, Plamondon L, and Stein RL (1997) Mechanistic studies on the inactivation of the proteasome by lactacystin in cultured cells. *J. Biol. Chem* 272, 182–188. [PubMed: 8995245]

Figure 1.
Pharmacophore probes **1** and **2**.

Figure 2.

(A) Reactions of probe 1 with the enone- and β -lactam-based electrophilic natural products parthenolide (3), andrographolide (5), wortmannin (7), and penicillin G (9). Abbreviations: Et₃N, triethylamine; DMF, *N,N*-dimethylformamide; DCM, dichloromethane; Ac₂O, acetic anhydride; pyr., pyridine; rt, room temperature. (B) Natural products bound to the key nucleophilic amino acid residues of their respective cellular targets.

Figure 3.

Reactivity-guided isolation of salinosporamide A (**18**). (A) Reaction of probe **1** with the crude extract of strain CNB-392 produces brominated products **16** and **17**, which can be retrosynthetically traced back to **18**. (B) Salinosporamide A bound to the proteasome. (C) Crystals of **17** formed via slow evaporation from benzene. (D) X-ray crystal structure of **17**. (E) LC/MS chromatogram (254 nm) of the crude extract before and after treatment with **1**, including the conspicuous MS isotope clusters of **16** and **17**. The retention time of **18** is indicated with an asterisk.

Figure 4.

(A) Reaction of probe 2 with the epoxide-based electrophilic natural products triptolide (**20**) and epoxomicin (**22**). Abbreviations: Et₃N, triethylamine; TCEP HCl, tris(2-carboxyethyl)phosphine hydrochloride; DMF, *N,N*-dimethylformamide; rt, room temperature. (B) Natural products bound to the key nucleophilic amino acid residues of their respective cellular targets.

Figure 5.

Reactivity-guided isolation of eponemycin (**27**), cyclomarin (**29**), and salinamide (**31**). (A, C, E) Reactions of probe **2** with crude extracts: (A) reaction with strain ATCC 53709 produces chlorinated product **26**; (C) reaction with strain CNB-382 gives chlorinated product **28**; (E) reaction with strain CNB-091 gives chlorinated product **30**. (B, D, F) LC/MS chromatograms (254 nm) of the crude extracts before and after treatment with **2**, including the conspicuous MS isotope clusters of **26**, **28**, and **30**. The retention times of **27**, **29**, and **31** are indicated with asterisks.

Figure 6.

Use of reactivity-guided isolation to validate genome mining efforts. (A) Partial NRPS–PKS hybrid gene cluster found in *Salinispora pacifica* strain CNT-003 and analyzed bioinformatically to code for a natural product in the tirandamycin family (Tdy). The gene cluster is located on two contigs and is missing six PKS modules. (B) Reaction of probe **2** with crude CNT-003 extract gave a single labeled compound, **32**. The molecular formula calculated for the original natural product was $C_{22}H_{27}NO_6$, which matched that of tirandalydigin (**33**). Epoxide **33** was then detected and isolated from the extract using its calculated mass. Abbreviations: NRPS, nonribosomal peptide synthetase; PKS, polyketide synthase; KS, ketosynthase domain; AT, acyltransferase domain; ACP, acyl carrier protein; C, condensation domain; A, adenylation domain; PCP, peptidyl carrier protein.

Table 1.

Cytotoxicities of Selected Compounds against the Human Colon Cancer Line HCT-116

natural product	cytotoxicity (IC ₅₀ , μ M)	thiol adduct	cytotoxicity (IC ₅₀ , μ M)
18	0.041	16	0.014
		17	14
20	0.0080	21	62
22	0.0054	23	85
27	0.17	26	24