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Thiol probes to detect electrophilic natural products based on their mechanism of action

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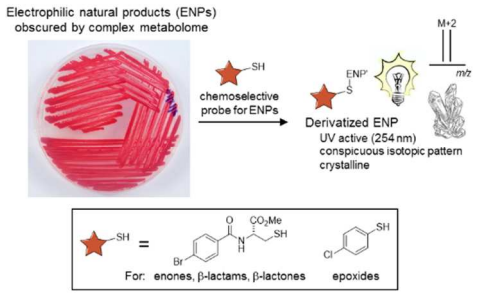

ABSTRACT

New methods are urgently needed to find novel natural products as structural leads for the development of new drugs against emerging diseases like cancer and multi-resistant bacterial infections. Here, we introduce a reactivity-guided drug discovery approach for electrophilic natural products, a therapeutically-relevant class of natural products that covalently modifies 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), whilst 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.

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, consist of miniscule amounts of the desired metabolite in a hodgepodge of primary metabolites such as amino acids, sugars, lipids, natural products and media components. 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. The Marfey’s reaction is an early application of this method, in which amino acids

are chemoselectively modified to give adducts with strongly absorbing chromophores.[7] Another method uses reactive resins to immobilize and selectively elute natural products based on 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] Due to 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 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, whilst 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 owing to the aromatic ring and a conspicuous MS isotopic pattern (^{81}Br:^{79}Br 1:1 and ^{35}Cl:^{37}Cl 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 produce an adduct that is inherently more crystalline. The cysteine probe, furthermore, contains a defined *R*-stereocenter that facilitates determinations of absolute stereochemistry.

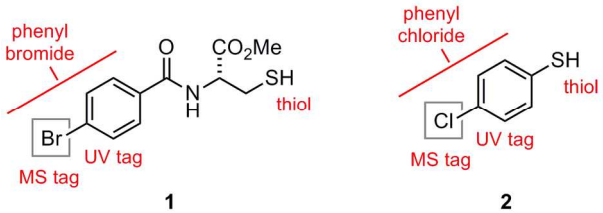

Figure 1. Pharmacophore probes **1** and **2**.

We relied initially on four substrates to assess the coupling efficiency of our thiol probes and optimize reaction conditions. Parthenolide (**3**) from feverfew (*Tanacetum parthenium*) contains a reactive butenolide moiety that is known to interact covalently with Cys171 of Iκβ 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 and so inhibits NF-κB 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 the major 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 a ring-opened structure. 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 the amino acid **10** proved 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 *S. 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 and then retrosynthetically disconnected to 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 α,β,γ,δ-unsaturated enone, a β-lactam or 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 Figure 2, **12**–**15**).

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 allowed for the unambiguous assignment of the absolute configuration of **17** and, hence, the natural product (**18**).[46]

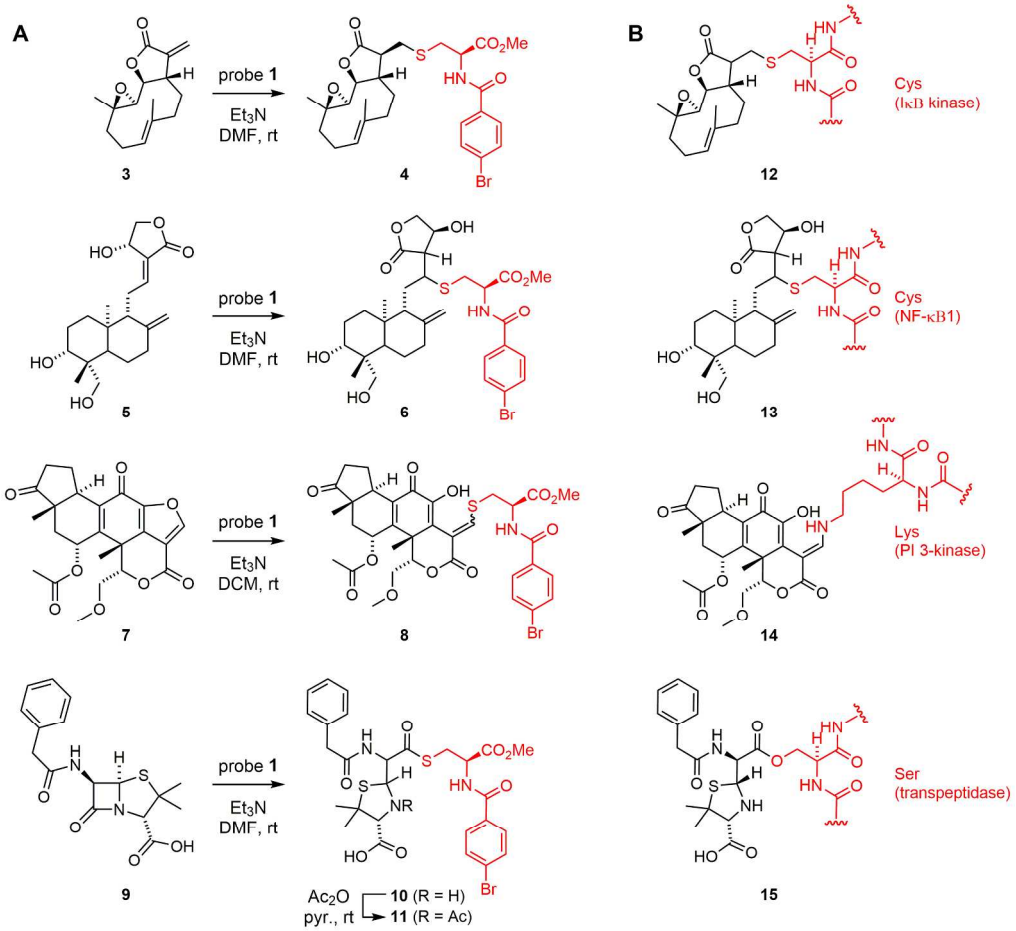

Figure 2. (A) Reaction of probe **1** with enone- and β-lactam-based electrophilic natural products parthenolide (**3**), andrographolide (**5**), wortmannin (**7**), penicillin G (**9**). Et_3N = triethylamine, DMF = *N*,*N*-dimethylformamide, DCM = dichloromethane, Ac_2O = acetic anhydride, pyr. = pyridine, rt = room temperature. (B) Natural products bound to the key nucleophilic amino acid residue of their respective cellular targets.

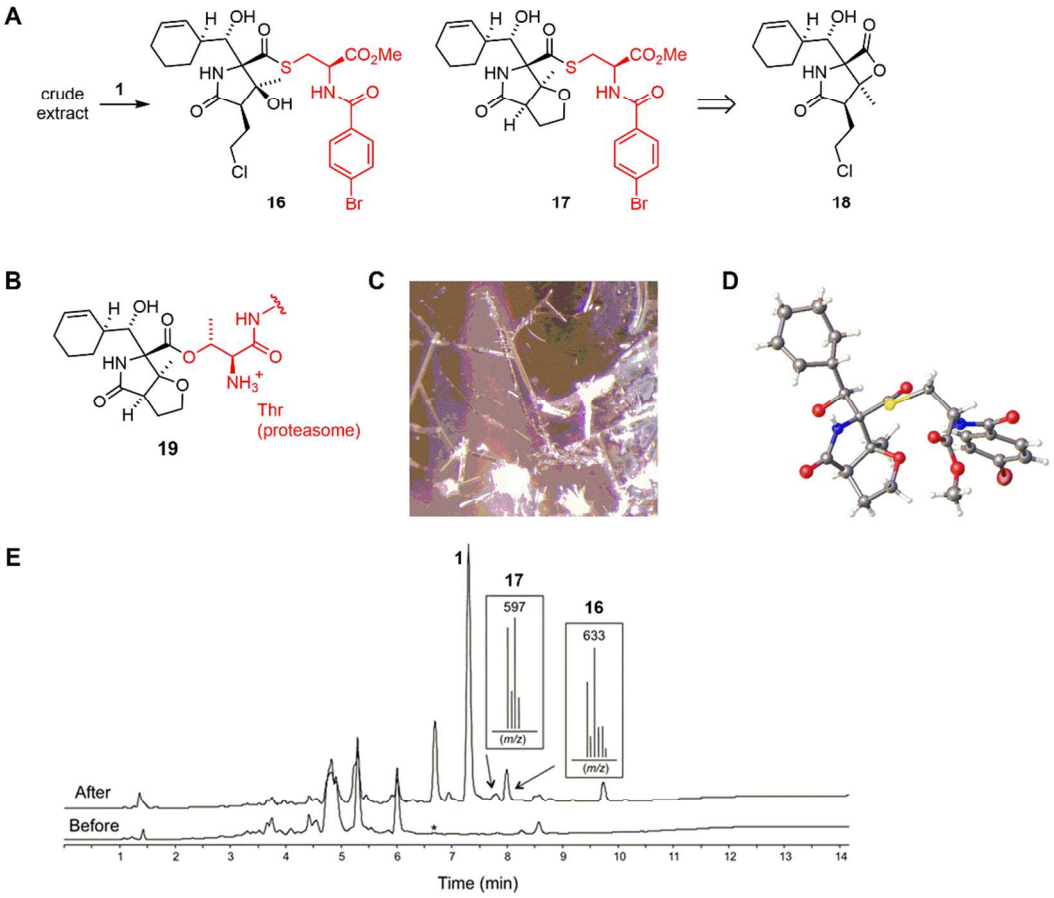

Figure 3. Reactivity-guided isolation of salinosporamide A (**18**). A) Reaction of probe **1** with the crude extract of strain CNB-392 produces brominated products **16** and **17**, which can be retrosynthetically traced back to salinosporamide A (**18**). (B) Salinosporamide A bound to the proteasome. (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.

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 triptolide (**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, epoxomicin (**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 co-crystal structure of **22** and the proteasome, which like other covalent proteasome inhibitors involves reaction with the *N*-terminal threonine residue (**25**).[51]

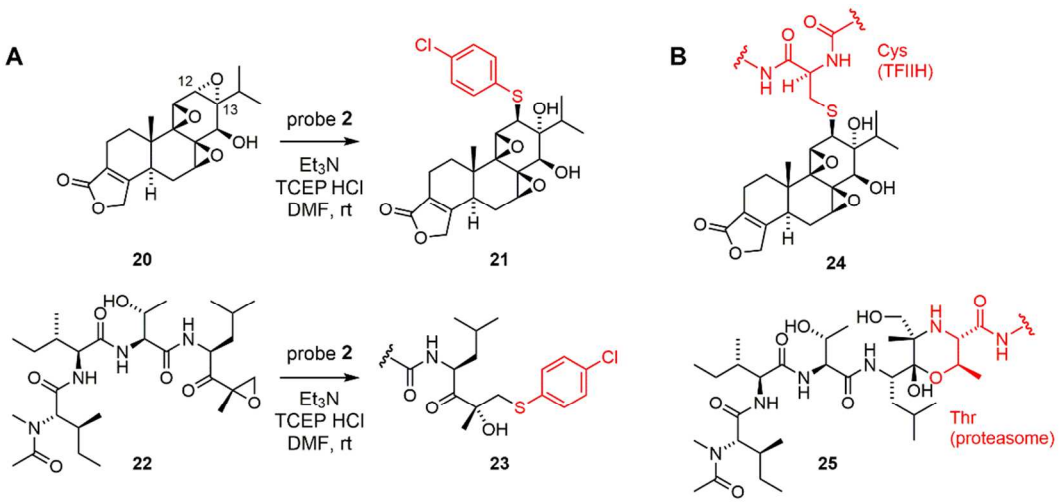

Figure 4. (A) Reaction of probe **2** with epoxide-based electrophilic natural products triptolide (**20**) and epoxomicin (**22**). Et_3N = triethylamine, TCEP HCl = tris(2-carboxyethyl)phosphine hydrochloride, DMF = *N*,*N*-dimethylformamide, rt = room temperature. (B) Natural products bound to the key nucleophilic amino acid residue of their respective cellular targets.

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 the proteasome inhibitor eponemycin (**27**), in addition to several minor chlorinated compounds from eponemycin analogues (Figure 5).[52,53] Like epoxomicin (**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** leads to the discovery of epoxide-based electrophilic natural products, like eponemycin, as well as natural products that contain epoxides that do not appear involved in the mechanism of action, like 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 labelled 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**) whilst chlorinated probe **2** alone reacted with the epoxide in salinamide A (**31**) (See Supporting Information). Interestingly, some chlorinated products formed from reaction of salinosporamide A (**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 a ~1:1 mixture of brominated and chlorinated parthenolide adducts was produced.

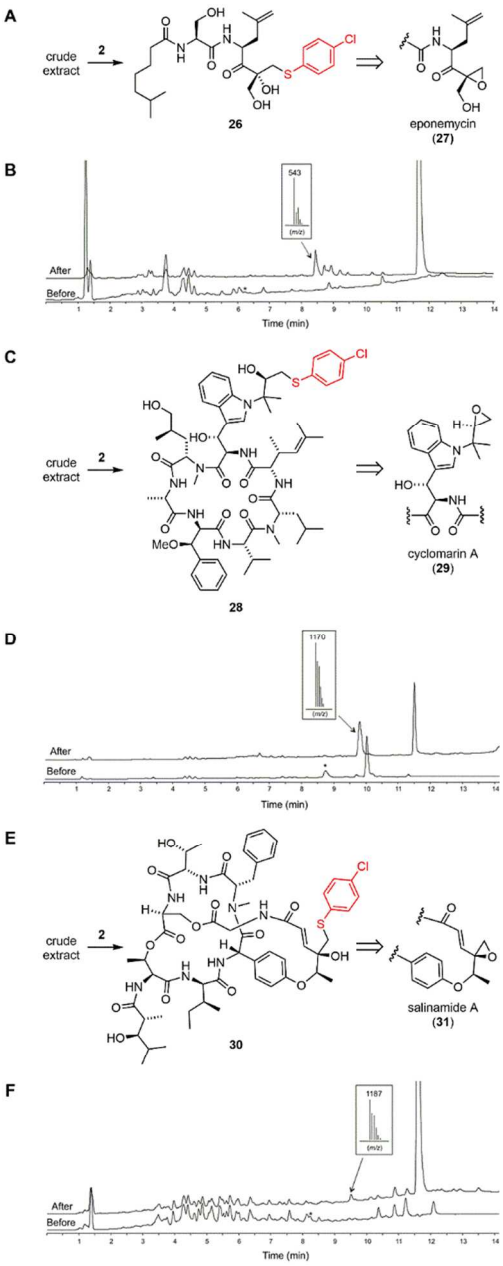

Figure 5. Reactivity-guided isolation of eponemycin (**27**), cyclomarin (**29**), and salinamide (**31**). Reaction of probe **2** with the crude extract of (A) strain ATCC 53709 produces chlorinated product **26**, (C) strain CNB-382 gives chlorinated product **28**, and (E) strain CNB-091 gives chlorinated 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 an asterisk.

Though 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 *Salinospora pacifica* strain CNT-003 contained an orphan gene cluster (Table S1) that was predicted to encode a member of the tirandamycin family of natural products (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 labelled the gene product of the tirandamycin 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 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 cytotoxicity 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 (11–32 μM). Salinosporamide (**18**) (IC_{50} = 41 nM) was converted into thioester **16**, which maintained remarkable activity (IC_{50} = 14 nM), and thioester **17**, which showed little cytotoxicity (IC_{50} = 14 μM). 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 cytotoxicity of the epoxide-based electrophilic natural products **20**, **22**, and **27** is 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.

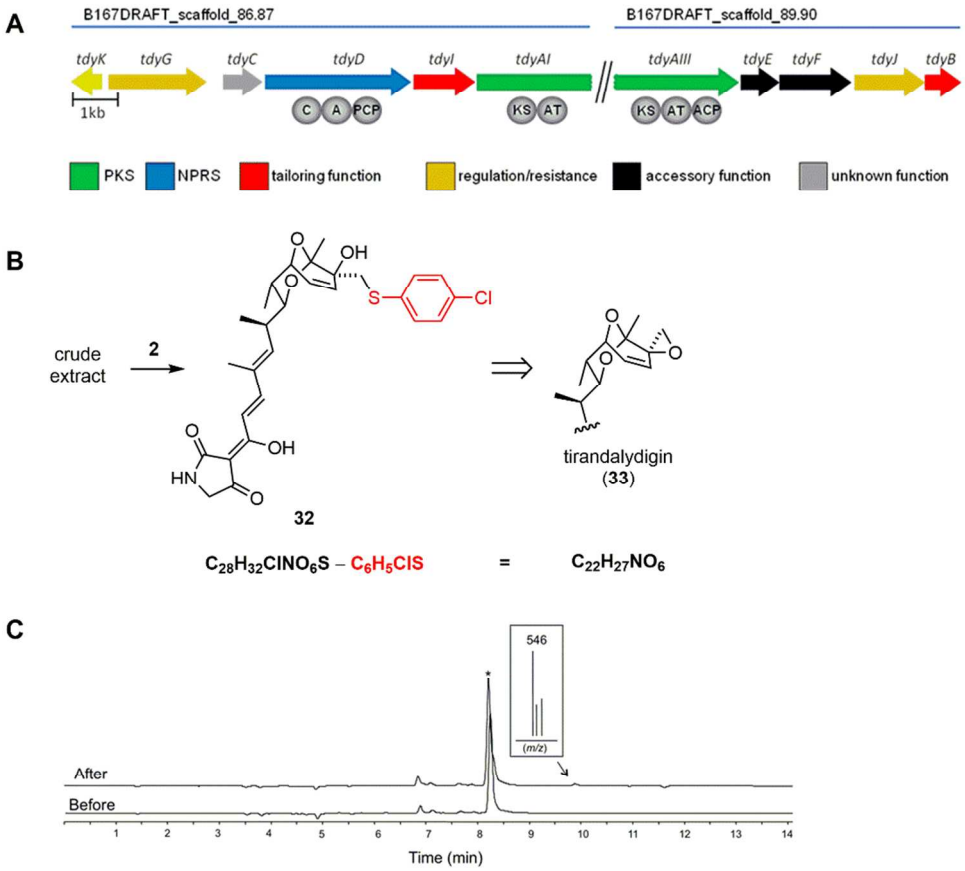

Figure 6. Using reactivity-guided isolation to validate genome mining efforts: A) A partial NRPS-PKS hybrid gene cluster found in *Salinispora pacifica* strain CNT-003 and analyzed

bioinformatically to code for a natural product in the tirandamycin family (Tdy). The gene cluster is located on two contigs and is missing 6 PKS modules; B) Reaction of probe **2** with crude CNT-003 extract gave a single labelled compound (**32**). The molecular formula culated for the original natural product was $C_{22}H_{27}NO_6$, which matched tirandalydigin (**33**). Epoxide **33** was then detected and isolated from the extract using its calculated mass. 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. Cytotoxicity of selected compounds against the human colon cancer line HCT-116

natural product	cytotoxicity (IC_{50}, μM)		thiol adduct	cytotoxicity (IC_{50}, μM)
18	0.041		**16**	0.014
			17	14
20	0.0080		**21**	62
22	0.0054		**23**	85
27	0.17		**26**	24

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 left stirring for 2 h. A saturated ammonium chloride solution (300 mL) was added, and the resulting solution was extracted with ethyl acetate (2 x 200 mL). The combined organic extracts were washed with a saturated sodium bicarbonate solution (400 mL), 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 6.96 g (81%) of dibromobenzoyl disulfide precipitated out of solution. To a solution of the disulfide (6.0 g, 9.4 mmol) in a 2:1 dichloromethane-methanol mixture, tris(2-chloroethyl)phosphine hydrochloride salt (4.0 g, 14 mmol) and triethylamine (5.0 mL, 38 mmol) were added. The reaction was left for 3 h. Flash chromatography of the crude material on silica gel (hexanes-ethyl acetate, 4:6) yielded 5.4 g (90%) of cysteine thiol probe (**1**) as a white solid. UV/Vis: λ_{max} = 241 nm; $[\alpha]_D$ = +3.3 (*c* 0.020, $CHCl_3$); IR (KBr) $\tilde{\nu}$ =3323, 1742, 1635, 1524, 1219 cm^{-1}; ^1H NMR 7.71 (d, 8.6 Hz, 2H), 7.61 (d, 8.6 Hz, 2H), 7.01 (br d, 1H), 5.07 (ddd, 7.1, 4.0, 4.0 Hz, 1H), 3.84 (s, 3H), 3.14 (dd, 9.1, 4.0 Hz, 2H), 1.38 (t, 9.1 Hz, 1H); ^{13}C NMR 170.3, 165.8, 132.1, 131.7, 128.5, 126.6, 53.7, 52.8, 26.7; HRESI-Q-TOF-MS: *m/z* $(M+H)^+$ 317.9800 calcd for $C_{11}H_{13}{}^{79}BrNO_3S$, found 317.9784.

Generation of crude extracts. *Streptomyces hygroscopicus* ATCC-53709 was grown in ISP1 medium (5 g L^{-1} of tryptone and 3 g L^{-1} of 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} of starch, 4 g L^{-1} of yeast extract and 2 g L^{-1} of 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 pre-cultures (1

L). After 3–4 days, 25 mL aliquots of the pre-cultures 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 left shaking 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 which had been previously sparged with gaseous nitrogen for 10 min. The reaction vessel was kept under 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 x 4.6 mm) with a 10 minute 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 x 10 mm) with UV detection (254 nm). Preparative reversed-phase HPLC purifications were performed with a C18(2) Phenomenex Luna column (10 µm, 250 x 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 (FBS, Invitrogen), 30 mM HEPES (Fisher Scientific) and 100 µg mL^{-1} penicillin/streptomycin (Invitrogen) and incubated in tissue culture flasks with a surface of 75 cm^2 at 37 °C in a 5% CO_2 atmosphere. Routine passaging of cells was conducted every 3 to 4 days. The cell layer was briefly rinsed in 10 mL 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, cells were aspirated by gently pipetting and then transferred into a new culture flask (1 mL into 20 mL fresh medium). Cytotoxicity in half maximal inhibitory concentrations (IC_{50}) of the natural products (**3**, **5**, **7**, **18**, **20**, **22**, **27**) and their adducts (**4**, **6**, **8**, **16**, **17**, **21**, **23**, **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 x 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 DMSO were added in a concentration of 10 mg mL^{-1}, 1 mg mL^{-1} or 0.1 mg mL^{-1} based on their activity, serially diluted, and incubated for additional 72 h. As reference, the standard anticancer drug etoposide VP-16 (Sigma Aldrich) with an IC_{50} of 0.49–4.9 µM in 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 using MTS (1.9 mg mL^{-1} in DPBS, Promega) in the presence of phenazine methosulfate (PMS, 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.

CONCLUSIONS

The tremendous potential for the discovery of natural products that inspire new drugs has not been realized because there is a shortage in the number 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.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: XXXXXXX. 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 ^{1}H, ^{1}H-^{1}H COSY, HSQC, and HMBC spectra for **1**, **4**, **6**, **8**, **16**, **17**, **21**, **23**, **26**, **28**, **30**, **33** (PDF).

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Notes

The authors declare no competing financial interests.

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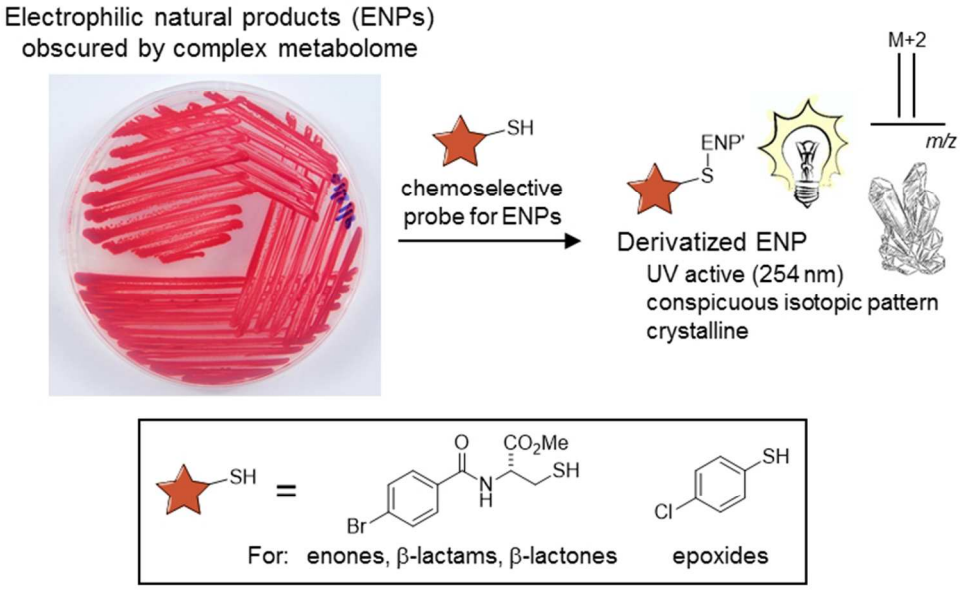

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