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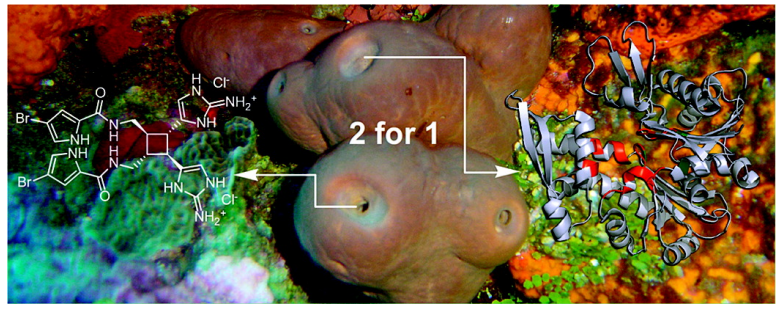

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Identification of the Binding of Sceptrin to MreB via a Bidirectional Affinity Protocol

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While the structures of a myriad of natural products have been elucidated from marine sponges, the biological targets for only a small percentage of these materials are known.[1] Arguably, understanding the target of these compounds can be as essential to clinic entry as the structure.[2] In an effort to expand the scope of affinity data,[3] we developed a system that identifies protein targets in parallel with natural product isolation. Our study focused on evaluating sponge extracts for natural products that bind to *Escherichia coli* proteins as a model.

We began by designing a method that employed affinity resins as a vehicle for tandem natural product isolation and chemical biological evaluation (Figure 1). The concept was to use affinity resins in both a reverse sense, to isolate natural products using resin-bound *E. coli* protein lysate, and a forward sense, to identify proteins in the *E. coli* lysate that were targeted by the isolated natural products. To this end, we engineered two types of resins. The first type (resins **R1**−**R5**) contained surface-conjugated proteins from the *E. coli* proteome. It served as the first step in purification by returning natural products that have affinity to *E. coli* proteins (Steps 2 and 3, Figure 1). We then turned to label the crude mixtures of natural products with an IAF tag to allow for subsequent protein target identification efforts (Step 4, Figure 1).

A second type of resin containing a surface-bound antibody (resin **R6**) was used to expedite probe development by selectively extracting the natural products labeled with **1** (Steps 5 and 6, Figure 1). The materials obtained through resin **R6** were then used to co-immunoprecipitate the natural product binding proteins from protein lysates (steps 9 and 10, Figure 1). As outlined in Figure 1, the sequential implementation of resins **R1**−**R6** allows the identification of natural products and their binding partners. Presently, we demonstrate how this system can be used to identify a natural product and associated protein target without prior knowledge of the natural product's structure, a goal that was viewed as the ultimate test for this protocol.

To validate this bidirectional affinity approach, we began by quantitatively evaluating the reverse affinity procedure (steps 2 and 3, Figure 1) using the serine threonine protein phosphatase $PP2A_c$.[4] (Figure 2) as a model system. Resin **R8** was prepared bearing active $PP2A_c$ and evaluated for its ability to retrieve both a potent and a weak protein phosphatase inhibitor, **4**[5] and **5**[5] (Figure 3), respectively, from a crude sponge extract.

A crude extract **E1** (954 mg) was prepared by soaking a 0.35 kg (dry weight) specimen of the sponge *Agelas conifera*[6] collected near Mona Island, Puerto Rico (Figure 1), in a 1:1 mixture of MeOH and CH_2Cl_2 for 12 h at 4 °C. The solution was filtered, concentrated, and defatted by partitioning between hexanes and acetonitrile. Aliquots of

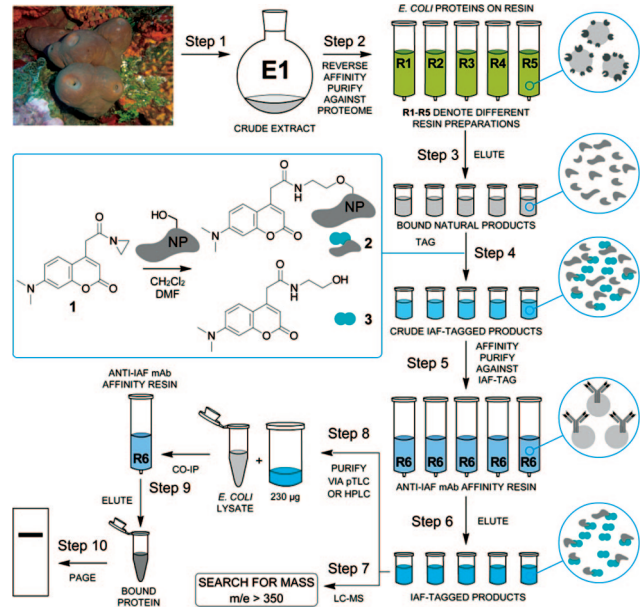

Figure 1. Protocol for bidirectional affinity guided isolation. (Step 1) Preparation of extract **E1** from a sample of *Agelas conifera*. (Step 2) Purification of protein-binding natural products using resin-bound *E. coli* protein lysate. Five reverse affinity resins **R1**−**R5** were evaluated. Samples of the crude extract **E1** were passed through resins **R1**−**R5**. (Step 3) Resins **R1**−**R5** were washed with media, and the bound natural products were extracted with ethanol and dried. (Step 4) The extracted natural product fractions were then tagged with the immunoaffinity fluorescent (IAF) tag **1** in THF to produce a mixture of natural product analogues **2** and the hydrolysis product **3**. (Step 5) The crude IAF-tagged products were purified by an immunoaffinity column with Affi-Gel Hz resin **R6** bearing a monoclonal antibody (mAb) against the IAF tag. (Step 6) The purified IAF-tagged products were extracted from the **R6** resin. (Step 7) LC/MS analysis was used to screen for extracts containing materials with a mass greater than tag **1**. (Step 8) Materials with mass greater than *m*/*z* 270 were purified by pTLC and then (Steps 9 and 10) used to co-immunoprecipitate their target protein. Resins are denoted as (**R1**) Affi-Gel 10 with 15 mg/mL of protein lysate, (**R2**) Affi-Gel 10 with 10 mg/mL of protein lysate, (**R3**) Affi-Gel 10 with 5 mg/mL of protein lysate, (**R4**) Affi-Gel Hz with 10 mg/mL of protein lysate, (**R5**) Affi-Gel Hz with 5 mg/mL of protein lysate, (**R6**) Affi-Gel Hz bearing 1 mg/mL of XRI-TF35 mAb and displayed potent activity to the IAF tag as illustrated by its affinity ($K_d = 0.95 \pm 0.35$ nM) to **3**.

the **E1** extract (25 mg) were doped with 125 μg of **4** or **5** and dissolved in 50 mL of PBS containing 1% DMSO. This solution was continuously pumped through columns loaded with control resin **R7** capped with ethyl glycine ester (15 mL) and active resin **R8** bearing $PP2A_c$ (15 mL) in series for 12 h at 4 °C (Figure 2A). Resins **R7** and **R8** were then washed successively with PBS (3 × 50 mL) and deionized water (50 mL). The bound materials were then eluted from the column by washing with 95% EtOH warmed to 50 °C. LC/MS analysis

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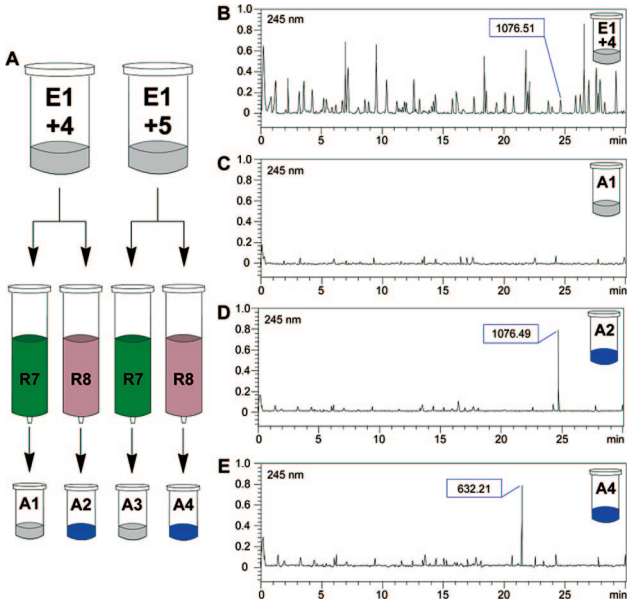

Figure 2. Validation of the reverse affinity protocol. (A) The parent extract **E1** was doped with 5 μg of **4** or **5** per mg. (B) LC/MS trace of **E1** doped with **4**. Samples of these doped fractions were passed through columns containing 10 mL of resin **R7** and **R8** in series. After washing each resin individually, aliquots **A1**−**A4** were obtained by extracting the resin with warm EtOH. LC/MS analysis provided traces of (C) A1, (D) A2, and (E) A4. Resins are denoted as (**R7**) Affi-Gel 10 capped with ethyl glycine ester and (**R8**) Affi-Gel 10 with 2.1 mg of $PP2A_c$ per mL.

confirmed the presence of **4** in **E1** (Figure 2B), and selective extraction of both **4** (Figure 2E vs Figure 2C) and **5** (Figure 2D vs Figure 2C) by the resin containing the $PP2A_c$ (**R8**). Quantification of the process was accomplished using the fluorescence from the IAF tag with excitation at 350 nm with emission at 450 nm.[5] When scaled up to 100 mg of **E1** extract with 5 μg of **4** or **5** per mg of **E1**, we were able to isolate 246 μg (49% yield) of **4** and 101 μg (20% yield) of **5** from resin containing 31.5 mg of $PP2A_c$, indicating that 27 and 19% of the $PP2A_c$ was involved in the extraction of **4** and **5**, respectively.

We then returned to evaluate the **E1** extract using the bidirectional affinity approach (Figure 1). Immobilized *E. coli* lysates **R1**−**R5** were prepared using protocols for preparing reverse affinity matrices (i.e., immobilized *E. coli* lysate, Pierce 44938).[7] A stock of protein lysate was prepared by freeze shocking and pressing *E. coli* K12 in PBS containing 10 mM $MgCl_2$ and a protease inhibitor cocktail (P8465, Sigma-Aldrich) through a French press three times at 12 000 lb/in.[2] followed by concentration of the supernate on a 9 kDa MWCO iCON to deliver stocks with 5 mg/mL in net protein. Five resins were prepared from this lysate as given by 5−15 mg/mL via *N*-hydroxysuccinimide coupling to Affi-Gel 10 (resins **R1**−**R3**) and hydrazide coupling to Affi-Gel Hz (resins **R4** and **R5** after periodate oxidation of the lysate).

A 250 mg sample of the **E1** extract in 100 mL of PBS containing 1% DMSO was pumped through five columns containing 25 g of resins **R1**−**R5** (Step 2, Figure 1). Columns containing 25 mL of resin were linked in series from **R5** to **R1** such that a single sample of extract could be passed through each column. After loading, the columns were separated and washed with PBS (3 × 50 mL) and deionized water (50 mL), and the bound materials were eluted by washing each column with 95% EtOH at 50 °C (Step 3, Figure 1). The five resulting extracts were dried, dissolved at 1 mg/mL in an anhydrous mixture of CH_2Cl_2 and DMF (1:1 v/v), and treated with 0.5 mg/mL of IAF tag **1** (Step 4, Figure 1). The solutions were then dried, dissolved in DMSO (1 mL), and diluted in 25 mL of RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS).

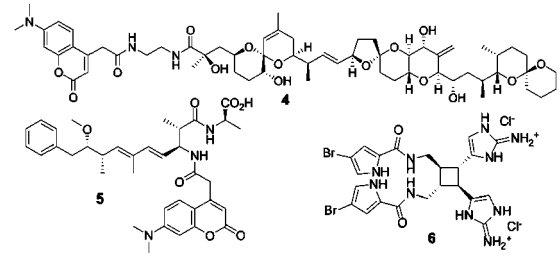

Figure 3. Structures of IAF-tagged okadaic amide **4** and microcystin analogue **5** and the isolated natural product sceptrin (**6**).

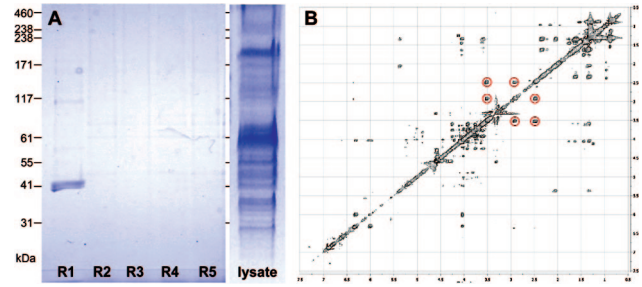

Figure 4. Identification of the binding between sceptrin (**6**) and MreB. (A) Gel-Code Blue-stained SDS page gels depicting co-IP proteins using IAF-tagged materials from resins **R1**−**R5** as compared to *E. coli* lysate. (B) TOCSY spectrum of the bound natural product extract after Step 3 (Figure 1). Peaks attributed to sceptrin (**6**) are circled.

The five crude IAF-tagged products were then passed through affinity columns containing 1 mL of resin **R6** (Step 5, Figure 1). After pumping for 12 h at 4 °C, the resins were washed with RIPA buffer (3 × 50 mL) and deionized water (50 mL). The bound materials were eluted by washing each column with 95% EtOH at 50 °C (Step 6, Figure 1). LC/MS analysis (Step 7, Figure 1) indicated that the extract from resins **R2**−**R5** contained predominantly **3**, while the **R1** extract contained a second peak distribution of mass ions about *m/z* 891[890 (50%), 891 (21%), 892 (100%), 893 (99%), 894 (21%), and 895 (4%)]. Purification by preparative TLC (Step 8, Figure 1) on a 10 × 10 cm, 250 μm C8 reversed-phase column returned 260 μg of IAF-tagged natural product **7** from the 250 mg of initial **E1** extract.

We then turned to use the IAF-tagged material **7** to identify an associated protein-binding partner by applying an affinity matrix in the forward direction (i.e., by using the small molecule to recruit a protein). Co-immunoprecipitation (co-IP) was conducted with resin **R6** with the goal of sequestering binding proteins by formation of an antibody sandwich complex with the IAF-tagged natural product (Step 9, Figure 1). The co-IP precipitate of the parent *E. coli* lysate with **7** from resin **R1** was compared to the crude products from resins **R2**−**R5**. A single band at ~40 kDa was returned from the precipitation of 5 mg of lysate with 50 μg of **7** (Figure 4A). Comparable bands were not observed when using the IAF-tagged fractions from the experiments using resins **R2**−**R5** (Figure 4A) or from precipitation with **3**, suggesting that isolation of the 40 kDa band arose from the natural product moiety within **7**. The band in lane R1 (Figure 4A) was excised and submitted for trypsin digest LC/MS/MS protein ID analysis, suggesting the actin equivalent, MreB, with 52% sequence coverage.

With a binding protein identified, we turned to characterize the material by NMR analysis. We used a combination of 1D and 2D NMR analysis (Figure 4B) to identify **6** as sceptrin[9] (HRMS calcd for $C_{22}H_{24}Br_2N_{10}O_2$ [M + H^+] *m/z* 619.0523, found 619.0541) as the source of **7** (HRMS calcd for $C_{37}H_{40}Br_2N_{12}O_5$ [M + H^+] *m/z* 891.1161, found 891.1157). This was verified after purification of 1.2 mg of an authentic sample of **6** from 250 mg of the parent **E1** extract.

We confirmed that MreB was the target of sceptrin (**6**) by comparison to recent studies by Shapiro, which examined the binding

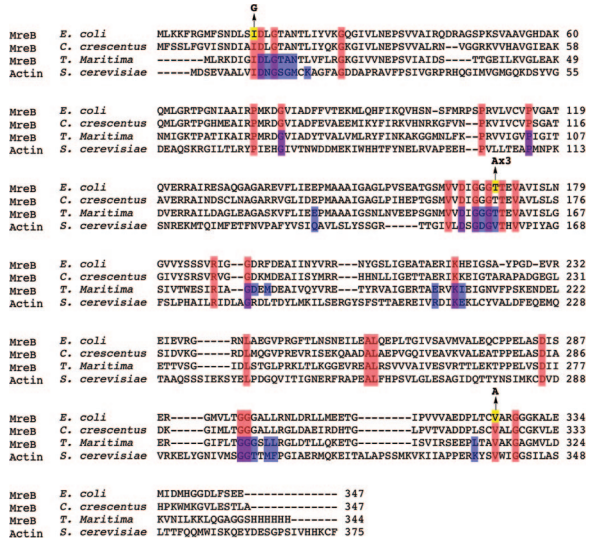

Figure 5. Sceptrin-resistant MreB mutations map to the nucleotide binding pocket of MreB. Clustal W alignment of MreB from *E. coli* K12, *Caulobacter crescentus*, and *Thermotoga maritima*, with actin from *Saccharomyces cerevisiae*. Residues are denoted by homology in red, positioning in the ATP binding site in blue, and mutated for sceptrin resistance in yellow. Point mutations are noted by arrows and residue.

of the small molecule A22 to MreB.[10] Sceptrin-resistant mutants were identified by screening wild-type *E. coli* K12 on large plates containing 50 μg/mL of **6**. We isolated five colonies that repeatedly formed on plates containing 50 μg/mL of **6** and grew in liquid culture containing 50 μg/mL of **6**. Missense point mutations were observed after PCR amplification and sequence analysis of the *mreB* gene of these strains (Figure 5). In agreement with Shapiro,[10] we found single-point mutations at residues associated with the nucleotide binding site of MreB from *T. maritima*.[11]

In summary, we have identified the binding of sceptrin (**6**) to MreB by means of a bidirectional affinity approach. This binding event was in accordance with evidence that indicated that **6** disrupted the bacterial cell wall synthesis, leading to the formation of spheroplasts.[12] The regulation of MreB by the binding of **6** is consistent with cell wall decomposition, as MreB is known to regulate the bacterial cell shape by positioning cell wall synthetic complexes.[13] In addition, recent evidence supports the conclusion that small molecule inhibitors of MreB induce comparable disruption of the bacterial cell wall.[14] MreB due to its role in regulating cell wall assembly has recently been validated as an antibiotic target.[10]

Most importantly, the binding between **6** and MreB was identified without prior knowledge of its structure and needing to characterize the IAF-tagged material (**7**). This evidence now suggests that protein target studies can be conducted in parallel with structure elucidation efforts. Under our current design, the method was limited to natural products that bind to abundant proteins such as MreB. Quantitative studies using a protein phosphatase as a model suggest that 3.2 μg of natural product can be sequestered per milligram of a 30 kDa protein. Subsequent titration experiments with lysate doped with MreB indicate the limit of co-IP purification (Figure 4A) was 250 $\pm$ 50 ng/mL of MreB. These limits suggest that preparing resins containing a single recombinant protein could provide a more effective tool to expand future screening efforts. Given the ready access to plasmids for *E. coli* protein expression,[15] one can consider developing libraries of resins.[16] In a theoretical context, the synergistic construction of a library of affinity resins (one for each protein in a proteome) would provide a facile high-throughput tool to exhaustively screen the connectivity between natural products and proteins.

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Supporting Information Available: Protocols for forward and reverse affinity purification, resin preparation, mutant screening, as well as copies of the spectral data used to characterize sceptrin (**6**). This material is available free of charge via the Internet at http://pubs.acs.org.

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