

Aplysqualenol A Binds to the Light Chain of Dynein Type 1 (DYNLL1)**

Brunilda Vera, Abimael D. Rodríguez,* and James J. La Clair*

Natural products continue to serve as a hotbed for cancer chemotherapy, providing vital leads, probes, and clinical entries.^[1] The advance of spectroscopic methods^[2] and analytical techniques^[3] has led our understanding into the dynamic yet complex structural diversity of marine secondary metabolites. Even given the expanse of this molecular diversity, we have only identified the biomolecular targets of a small subset of the known natural products,^[4] and many of those that have been identified arise owing to the fact that they share common targets (examples include actin or microtubules).^[5] The fact remains that we have only begun to understand the myriad of biological pathways engaged by secondary metabolites.

Despite the fact that a variety of methods exist for evaluating the biological targets of a natural product,^[6] gaps in this knowledge have arisen from a combination of reasons, including a) the fact that many natural products were isolated before the onset of key biological techniques; b) the lack of advanced biological screens during the discovery process; and c) the gradual shift away from natural products as therapeutic leads.^[7] While target identification studies are only one factor in the drug discovery process, the lost of these data has a significant impact on our understanding of the relationship between the structure and function of small molecules, and therefore it plays an essential role.

As isolation efforts are an ongoing process, we focused our program on developing methods that allow the biological targets of a natural product to be screened in conjunction with its isolation and structure elucidation. Recently, we described the use of a bidirectional affinity platform to identify the

binding of the dimeric pyrrole–imidazole alkaloid sceptrin to a prokaryotic homologue to actin, MrB.^[8] This study demonstrated the ability to identify metabolites and their bacterial protein targets in harmony. Herein, we describe a bidirectional approach (Figure 1) to sequentially identify cancer targets and their corresponding natural product ligands.

We recently reported the isolation of the bromotriterpene polyethers aplysqualenol A (**1a**) and B (**1b**) (Scheme 1) from the Caribbean sea slug *Aplysia dactylomela*, collected in Puerto Rico.^[9] Samples of **1a** were submitted to the NCI for evaluation in the NCI-60, one-dose primary anticancer assay. It showed potent in vitro activity against SNB-19 (IC₅₀ value of 0.4 μM), a CNS tumor cell line, and T-47D (IC₅₀ value of 0.4 μM), a breast cancer cell line.^[9] Comparable analyses indicated that **1a** was also active in HCT-116 colon carcinoma

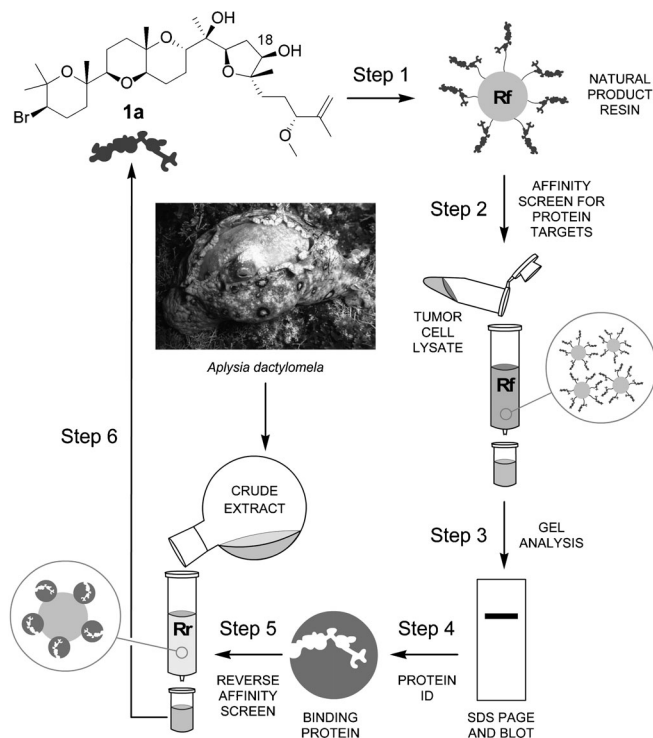
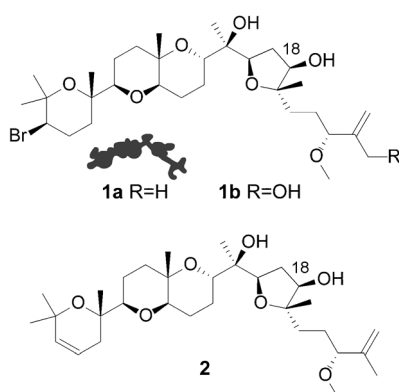


Figure 1. Protocol for bidirectional affinity-guided studies. Step 1: Aplysqualenol A (**1a**) was covalently linked to a resin (Rf). Step 2: Resin Rf was then used to screen for aplysqualenol A (**1a**) binding proteins. Steps 3, 4: The bound proteins were examined by SDS PAGE, identified by proteomic analyses, and validated by Western blot analysis. Steps 5, 6: The isolated binding proteins were then attached to resin and used in a reverse-affinity sense using resin (Rr) to isolate **1a** from its corresponding crude natural-product extract.

[*] B. Vera, Prof. A. D. Rodríguez
Department of Chemistry, University of Puerto Rico
P.O. Box 23346, UPR Station
San Juan, 00931–3346 (Puerto Rico)
E-mail: abimael.rodriguez1@upr.edu
Dr. J. J. La Clair
Xenobe Research Institute
P.O. Box 3052, San Diego, CA 92163-1052 (USA)
E-mail: i@xenobe.org
Homepage: <http://www.xenobe.org>

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Scheme 1. Structures of aplysqualenol A (**1a**), aplysqualenol B (**1b**), and elimination product **2**.

cells (IC_{50} value for **1a** in HCT-116 cells of $20.6 \mu\text{M}$ by NCI screening and 12.2 ± 0.3 by an in house MTT screen).^[10]

We began by developing methods for the isolation of proteins that bound to **1a** by preparing resins with covalently linked **1a** (resin **Rf**; Figure 1). While methods existed for the installation of a tag at the hindered secondary hydroxy group at C18,^[9] these methods contain semisynthetic steps to convert the natural product into a functional derivative.^[11] To minimize the use of **1a**, we examined procedures that allowed the natural product to be attached in one step.

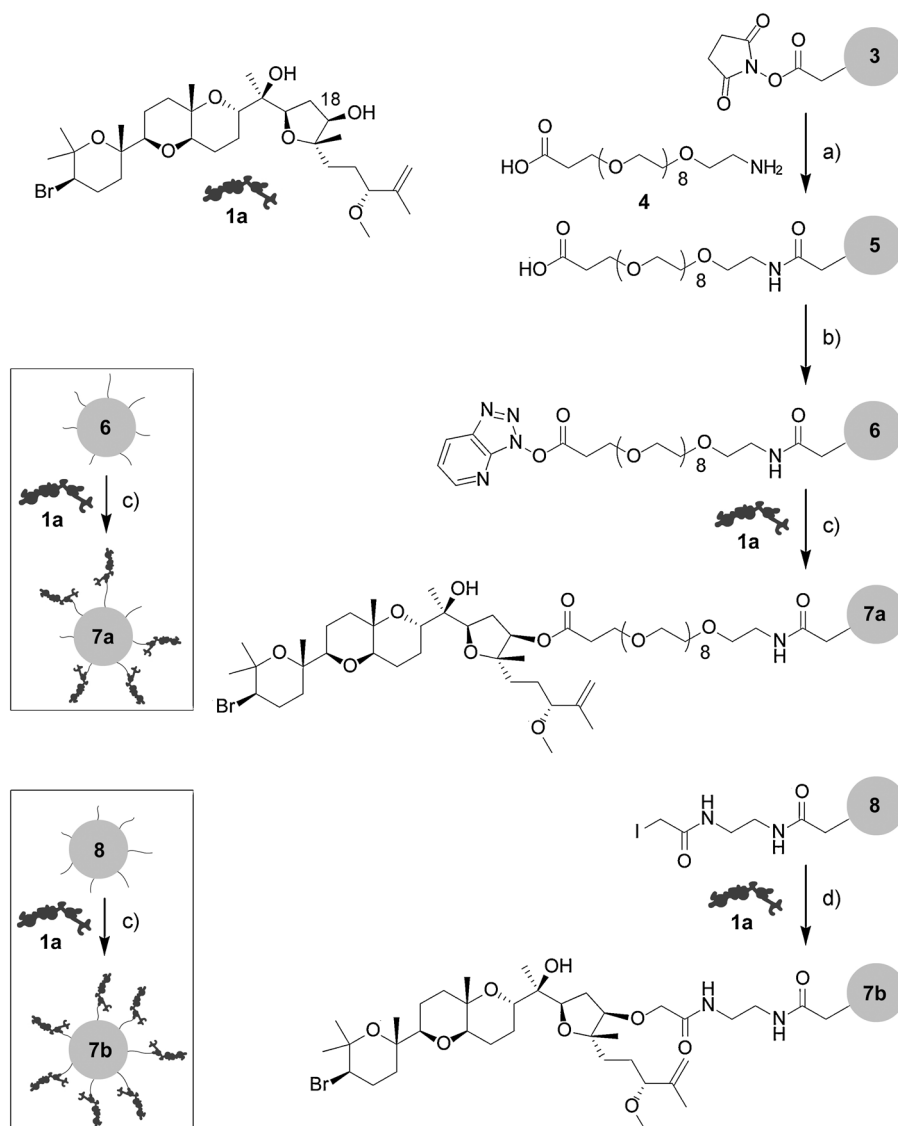
Our studies focused on preparation of two resins **7a** and **7b** (Scheme 2). Preparation of resin **7a** began with the installation of spacer **4**^[12] onto Affigel 10 resin **3** to afford resin **5**. We then activated resin **5** by treatment with HATU and coupled **1a** onto the activated resin **6** under DMAP catalysis.^[13] While the yield was modest ($(0.5 \pm 0.1) \text{ mg mL}^{-1}$),^[14] we were able to confirm the loading of **1a** on resin **7a** hydrolytically.^[15]

A second resin **7b** was prepared by adapting methods developed by Mitcheson, Walzack, and co-workers.^[17] Affigel 10 resin **3** was converted into iodoacetamide resin **8** (Scheme 2) by sequential treatment with ethylenediamine and iodoacetic acid *N*-hydroxysuccinimide ester. Aplysqualenol A (**1a**) was then coupled to resin **8** by incubation in dry dioxanes followed by capping with 2-mercaptoethanol. This procedure

was far more effective, providing resins with $(1.8 \pm 0.3) \text{ mg mL}^{-1}$ of **1a**.^[14]

We then applied resins **7a** and **7b** to screen tumor cell lysates for binding proteins. Using lysates freshly prepared from HCT-116 cells (Figure 2a, lane L1), we identified a band from a $< 10 \text{ kDa}$ protein over multiple repetitions using either resin **7a** or **7b**. After optimization, a single band could be obtained in the bound fractions (Figure 2a, lanes L2 or L3). While both resins were functional, resin **7b** provided nearly twice the return.

Samples of these bands were excised and submitted for protein identification. Trypsin digestion followed by LC-MS/MS analyses indicated that this protein was the light chain of dynein type 1 (DYNLL1) with coverage by three peptides (blue sequences in Figure 2a). We validated this identification



Scheme 2. Preparation of aplysqualenol A resins **7a** and **7b**. Reagents and conditions: a) PEG spacer **4**, H_2O , pH 8.5, 4°C , 8 h; b) HATU, 1,4-dioxane, 12 h, 4°C to RT; c) **1a**, DMAP, 1,4-dioxane, 18 h, 4°C to RT, followed by capping with 0.1 M glycine methyl ester in PBS pH 8.0; d) **1a**, 1,4-dioxane, 18 h, 4°C to RT, followed by capping with 0.05 M 2-mercaptoethanol in PBS pH 8.0. PEG = poly(ethylene glycol), HATU = *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate, DMAP = 4-(dimethylamino)pyridine, PBS = phosphate-buffered saline.

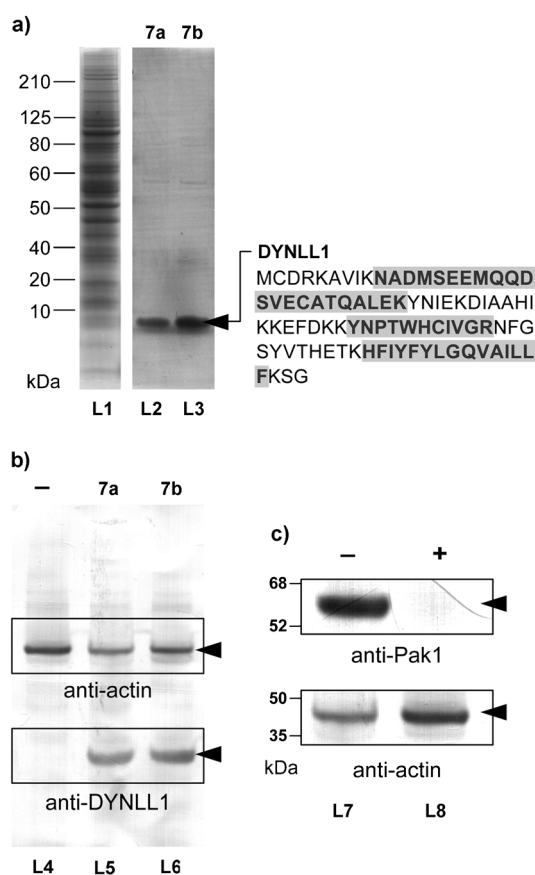


Figure 2. Forward-affinity studies to identify dynein light chain type 1 (DYNLL1) as a target of aplysqualenol A (**1a**). a) SDS-PAGE gels of HCT-116 cell lysate (lane L1) and the fractions that were pulled down with resins **7a** (lane L2) and **7b** (lane L3). A band corresponding to a <10 kDa protein was observed in the bound fractions from both resins **7a** and **7b**. b) Western blot analysis using an anti-DYNLL1 mAb confirms the presence of DYNLL1 protein in the bound fractions from **7a** (lane L5) and **7b** (lane L6). Resins that lack **1a** (lane L4) as given by a sample of resin 5 that was capped with glycine methyl ester did not contain DYNLL1. Actin was added to each fraction as loading control. c) Affigel resin bearing (2.4 ± 0.2) mg mL⁻¹ of DYNLL1 was capable of isolating Pak1 from HCT-116 cell lysate. The Western blots depict the isolation of Pak1 in DYNLL1-affinity fraction of pure lysates (-, lane L7). In contrast, the same lysate treated with 20 μM **1a** lost the ability to sequester Pak1 (+, lane L8), indicating that **1a** blocked the interaction between DYNLL1 and Pak1.^[16]

using three methods. First, western blot analysis using antibodies against human DYNLL1 confirmed the presence of DYNLL1 in the bound fractions (Figure 2b, lanes L5 and L6). Second, isothermal calorimetry (ITC) indicated that **1a** bound to recombinant human DYNLL1^[18] with a K_d value of (0.84 ± 0.31) μM. Finally, we confirmed this binding constant using surface plasmon resonance (SPR) measurements, which returned a comparable K_d of (0.52 ± 0.09) μM (see Supporting Information).

We further validated the binding event by reversing the affinity method. Samples of recombinant DYNLL1 protein^[18] were loaded on Affigel 10 resin at (2.4 ± 0.2) mg mL⁻¹^[19] and screened for their ability to isolate aplysqualenol A (**1a**) from extracts of *A. dactylomela* (Figure 3a). By using 100 μL of this

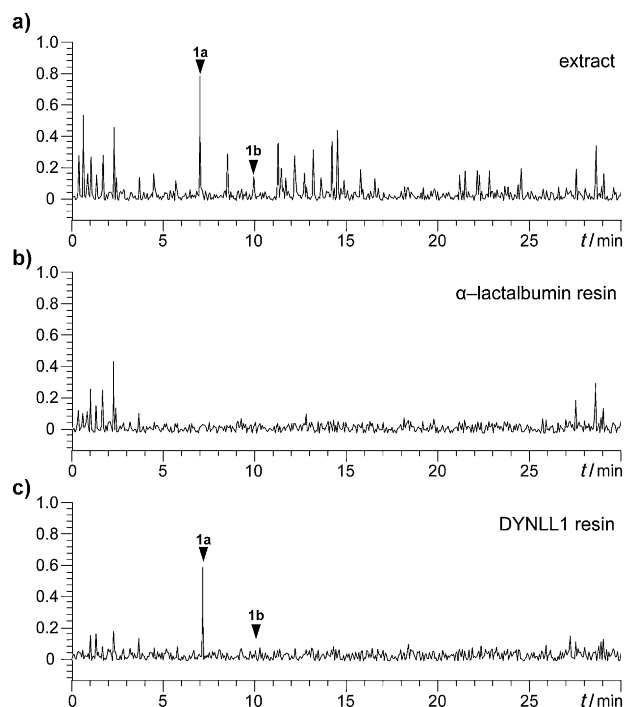


Figure 3. Reverse-affinity studies confirm the interaction between **1a** and DYNLL1. a) Metabolic profile obtained from the crude *A. dactylomela* extract using LC/MS analysis. The y coordinate denotes the net signal detected by the mass spectrometer. Samples of this extract were then incubated with resins containing either b) (3.2 ± 0.1) mg mL⁻¹^[19] of α-lactalbumin^[20] on Affigel 10 resin as a negative control or c) (2.4 ± 0.2) mg mL⁻¹^[19] of DYNLL1 on Affigel 10 resin. Aplysqualenol A (**1a**) and aplysqualenol B (**1b**) are noted by arrows and were confirmed by the presence of [M-HBr]⁺ of 536 and 552 *m/z* for **1a** and **1b**, respectively.

resin, we were able to observe the sequestration of **1a** from 10 mg of crude extract (Figure 3c). Comparable extraction using α lactalbumin,^[20] a negative control, failed to return **1a** (Figure 3b).

Application of this reverse-affinity process to 25 mg of *A. dactylomela* extract returned sufficient **1a** to be evaluated by capillary NMR analysis (Figure 4).^[21] Standardization with pure **1a** indicated that we obtained (8.4 ± 0.6) μg of **1a**, corresponding to a yield of 85 μg mL⁻¹ resin, and that at least 0.35 mg g⁻¹ of **1a** was in the *A. dactylomela* extract.^[22]

With this evidence in hand, we then established whether the binding of **1a** had any effects on proteins known to interact with DYNLL1. In particular, DYNLL1 was recently described to participate in the nuclear transport of Pak1, a serine/threonine kinase implicated in regulation of cell motility, cell survival, and in the malignant transformation of mammary epithelia cells.^[16,23] Using western blot analysis (Figure 2c), we determined that treatment of HCT-116 cell lysates with **1a** blocked the binding between DYNLL1 and Pak1, as indicated by the loss in the sequestration of Pak1 in lysates treated with **1a** (Figure 2c, lane L7 versus L8).^[24]

In summary, a bidirectional affinity approach was used to identify the binding of marine polyether aplysqualenol A (**1a**) to protein DYNLL1. DYNLL1 is a small cytosolic protein that belongs to a large enzymatic complex, dynein, with molecular

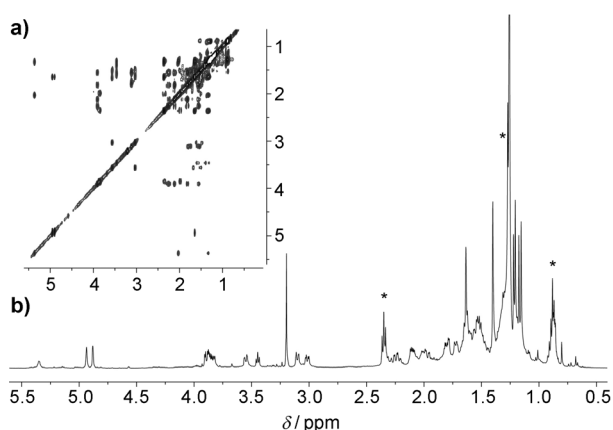


Figure 4. NMR analysis to confirm the isolation of aplysqualenol A (**1a**) from the DYNLL1 reverse-affinity column. a) gCOSY and b) ^1H NMR obtained with a 1.7 mm Bruker BioSpin 600 MHz NMR equipped with a cryoprobe using material eluted from the reverse affinity column. These NMR spectroscopic data were comparable to those published for aplysqualenol A (**1a**) in CDCl_3 .^[9] Lipid impurities are indicated by a *.

mass of about 1.5 MDa.^[25] In the cytoplasm, dynein plays a major role in motion regulating not only cell movement along microtubules but also guiding the positioning of organelles within the cell.^[26] While the function of the heavy and intermediate chains is somewhat established,^[27] that of the light chains such as DYNLL1 is less understood.^[28] The identification of ligands to DYNLL1, such as **1a**, provides a starting point for development of molecular probes.^[29]

While samples of **1a** or **1b** can be obtained naturally they are scarce.^[30] Development of a synthetic entry to **1a** could provide a secondary source of material as well as expand the scope of probe development. Such probes offer immediate potential to examine the complex interactions of DYNLL1 but also could be used to establish the features of DYNLL1 that differentiate its isoforms.^[31] Last, discovery of **1a** as a ligand to DYNLL1 suggests new potential for the development of small-molecule regulators of the dynein complex,^[32] an endeavor that could have applications to cancer treatment,^[33] viral infections,^[34] and neurodegenerative diseases.^[35]

In conclusion, we demonstrated the ability to sequester proteins from small molecules and vice versa. The integration of both approaches into a single tactical (forward and reverse affinity) method provided further support for this target identification and supported its specificity. By combining these studies with mass spectrometry and NMR methods, we were able to rapidly identify the binding event, and suggest the further integration of these methods as primary tools for MOA research. While this approach is intrinsically linked to evaluate individual natural products serially, the adaption of multiplexed approaches such as bead libraries may allow each metabolite to be rapidly integrated within a metabolome with a corresponding binding protein, or set of proteins, in a given proteome.

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