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Target Discovery |*Hot Paper*|

Chemoproteomic Evaluation of the Polyacetylene Callyspongynic Acid

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Abstract: Polyacetylenes are a class of alkyne-containing natural products. Although potent bioactivities and thus possible applications as chemical probes have already been reported for some polyacetylenes, insights into the biological activities or molecular mode of action are still rather limited in most cases. To overcome this limitation, we describe the application of the polyacetylene callyspongynic acid in the development of an experimental roadmap for characterizing potential protein targets of alkyne-containing natural products. To this end, we undertook the first chemical synthesis of callyspongynic acid. We then used in situ chemical proteomics methods to demonstrate extensive callyspongynic acid-mediated chemical tagging of endoplasmic reticulum-associated lipid-metabolizing and modifying enzymes. We anticipate that an elucidation of protein targets of natural products may serve as an effective guide to the development of subsequent biological assays that aim to identify chemical phenotypes and bioactivities.

Introduction

Bioactive natural products have found widespread applications in chemical biology.[1] Polyacetylenes are a class of natural products that are characterized by one or several alkyne residues and they often display highly lipid-like chemical features.[2] Whereas potent bioactivities and thus potential applications as chemical probes have already been elucidated for some members of this large class of natural products, insights into the biological activity or molecular mode of action of most polyacetylenes are still lacking.[2] Further insights into their bioactivities is, however, hampered by several factors such as their limited availability and the scientific challenge of identifying chemical phenotypes of potentially bioactive compounds; in fact, the identification of small molecule bioactivities beyond 'simple' cytotoxicity determinations is a difficult task that usually requires extensive biological studies.[3]

Given their lipid-like chemical features, a presumable mode of action of many polyacetylenes could be chemical interference with cellular fatty acid/lipid catabolism/metabolism or signaling. These are important biological processes that affect almost all biological activities in living cells, and their dysregulation is often associated with severe diseases such as cancer or inflammatory or metabolic disorders.[4] Despite recent advances, the elucidation of the molecular mechanisms underlying these processes is still in its infancy, owing to, among other factors, a limited repertoire of research methodologies.

Recently, a novel research approach consisting of the use of global profiling methodologies such as chemical proteomics has emerged as a potent approach to study these complex biological processes.[5] These methodologies have, for example, been used to assign functions to diverse lipid and fatty acid metabolizing enzymes or lipid-derived metabolites; they are based on the use of small molecule chemical probes that covalently modify proteins from fatty acid or lipid-associated biological processes in a function-dependent manner with a 'reporter tag'.[6] To generate the required chemical probes, mostly 'rational design' approaches, for example, for obtaining activity-based probes for profiling lipid-related serine hydrolases or for generating alkyne/azide tagged metabolic derivatives of endogenous fatty acids and lipids, have been employed.[7] In addition, some natural products or natural product derivatives such as the lipase inhibitor tetrahydrolipstatin (Orlistat) or natural product-derived chemical probes such as the Orlistat electrophilic warhead, a β-lactone system, has been used in chemical proteomics studies.[8] In all cases, the chemical probe is fused with a reporter tag that enables target protein identification through protein target affinity enrichment prior to mass spectrometry-based identification.[9] Alkyne residues as reporter tags in conjunction with a two-step labeling/enrichment procedure have become one of the major chemical proteomics-based target identification procedures.[10] Given that polyacetylene natural products already contain one or more alkyne residues, the application of chemical proteomics methodologies to study their covalent targets should be a rewarding approach.

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To demonstrate the feasibility of such a chemical proteomics approach for polyacetylenes, we performed a test study with a long-chain fatty acid polyacetylene natural product, callyspongynic acid (**1**, CalA; Figure 1). This natural product was isolated from the marine sponge *Callyspongia truncata* and incorporates as structural peculiarities, and in addition to the alkyne moieties, a propiolic acid and an allyl propargyl alcohol residue.[11] Callyspongynic acid inhibits α-glucosidase moderately in biochemical inhibition assays; however, the cellular bioactivities and molecular mode of action of this compound have yet to be explored.[11] Although a significant number of polyacetylene natural products have been synthesized so far,[2b,12] no chemical synthesis of callyspongynic acid and thus experimental validation of the proposed chemical structure have been reported.

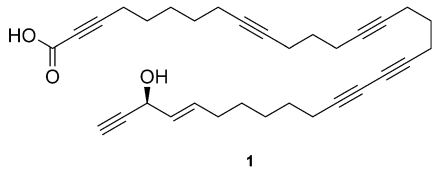

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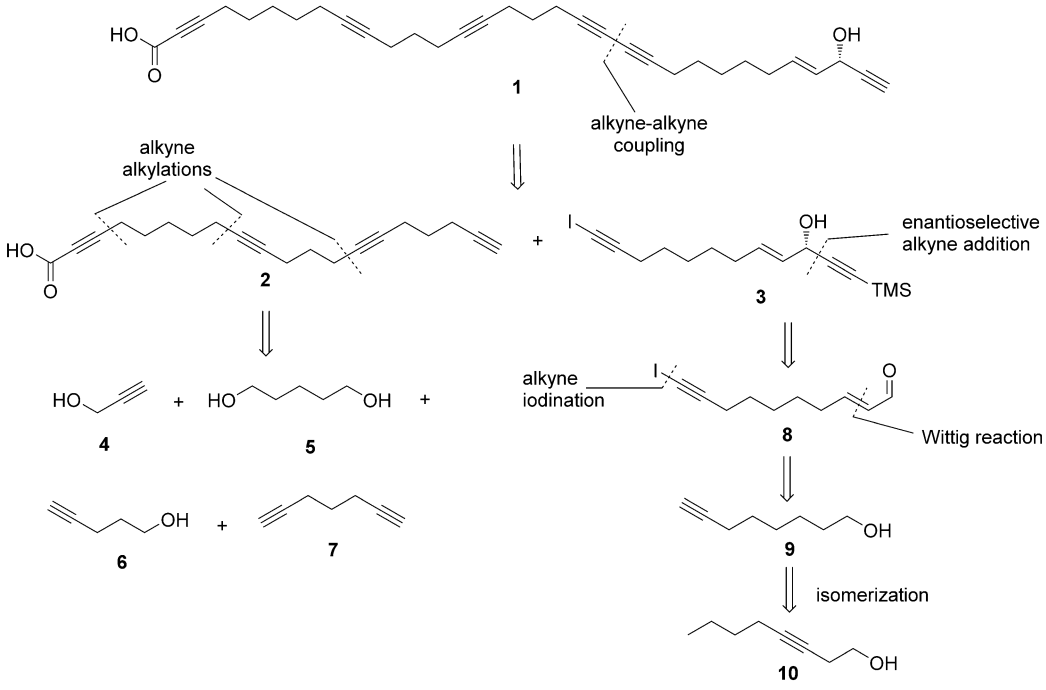
Figure 1. Chemical structure of callyspongynic acid (**1**, CalA).

Results and Discussion

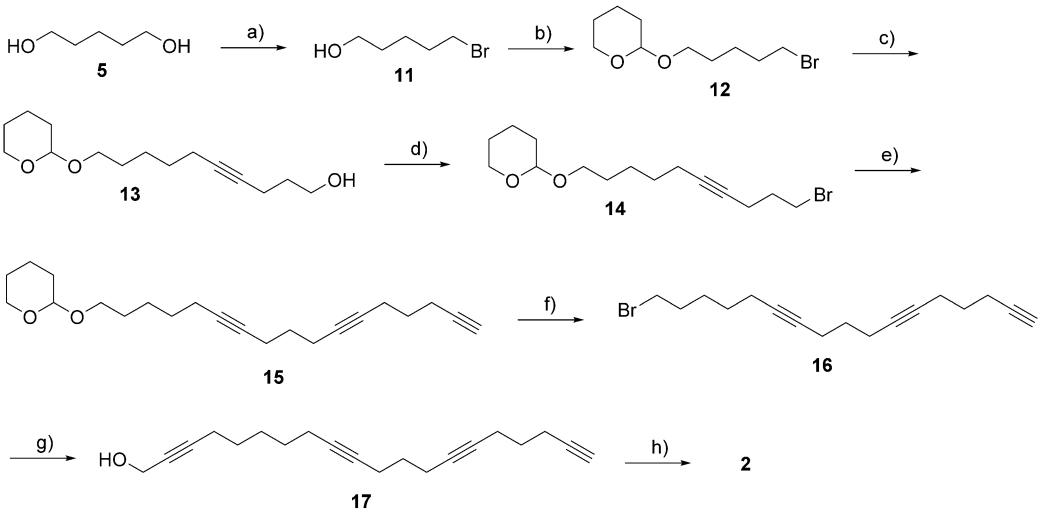
Synthesis of callyspongynic acid

To obtain a sample of **1** for further investigations, we first devised a total synthesis of callyspongynic acid. The corresponding retrosynthetic analysis (Scheme 1) relied on late-stage formation of the central bis-alkyne moiety through a Cadiot–Chodkiewicz reaction, thereby generating two fragments, **2** and **3**, of comparable structural complexity. The alkyne-rich, left-hand portion should then be available by iterative alkyne alkylations, disconnecting this building block to the prospective starting materials propargyl alcohol (**4**), 1,5-pentanediol (**5**), 4-pentyn-1-ol (**6**), and 1,6-heptadiyne (**7**). To establish the secondary allyl propargyl alcohol on building block **3**, an enantioselective alkyne addition to an α,β-unsaturated aldehyde was envisaged, leading to intermediate **8**. This compound could be further dissected by an alkyne iodination and Wittig reaction, resulting in **9**, which can be easily obtained by isomerization of the commercially available starting material 3-octyn-1-ol (**10**).

Following this retrosynthesis, we started by generating the left-hand building block **2** (Scheme 2). 1,5-Pentanediol (**5**) was converted in 85% yield into 1-bromo-pentan-5-ol (**11**) by using a two-phase system consisting of aqueous 48% HBr solution and toluene to assure monobromination.[13] The remaining hydroxy group was subsequently protected as a tetrahydropyranyl (THP) ether (**12**). Intermediate **12** was employed in the first alkyne alkylation, using 4-pentyn-1-ol (**6**) as the nucleophile, resulting in the formation of **13** in 94% yield. Conversion of the alcohol into bromide **14** through bromination with *N*-bromosuccinimide (NBS)/triphenylphosphane set the stage for the second alkyne alkylation, which was performed with 1,6-heptadiyne (**7**). The corresponding intermediate **15** was obtained in 71% yield. To prepare this intermediate for the next alkylation, **15** was converted into bromine **16**, which could be obtained in a single reaction with bromine and triphenylphosphane without prior THP deprotection, in 92% yield. Direct introduction of propargylic acid failed in the next step, so a two-step procedure was used instead: **16** was first converted into propargyl derivative **17** by alkylation with propargyl alcohol (**4**), fol-



Scheme 1. Retrosynthetic analysis: A convergent approach using two fragments with comparable structural complexity is followed.

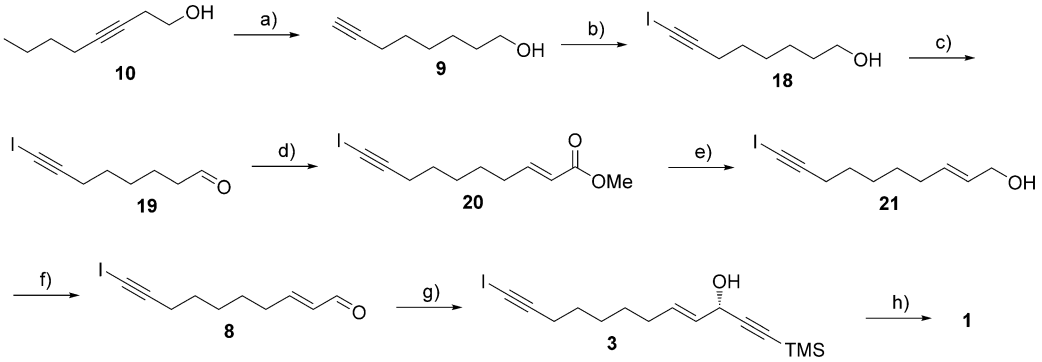


Scheme 2. Synthesis of left-hand building block **2**. Reagents and conditions: a) 48% aq. HBr, toluene, reflux, 12 h, 85%; b) dihydropyran (1.25 equiv), HCl (0.2 equiv), CH_2Cl_2, RT, 4 h, 93%; c) **6** (2 equiv), *n*BuLi (4.5 equiv), HMPA (3 equiv), THF, 0 °C to RT, 12 h, 94%; d) NBS (1.3 equiv), PPh_3 (1.3 equiv), DMF, 0 °C, 2 h, 91%; e) **7** (1 equiv), *n*BuLi (2.6 equiv), HMPA (2 equiv), THF, 0 °C to RT, 12 h, 71%; f) PPh_3 (1.1 equiv), Br_2, CH_2Cl_2 (1:20), RT, 30 min, 92%; g) **4** (2 equiv), *n*BuLi (4.5 equiv), HMPA (3 equiv), THF, 0 °C to RT, 12 h, 48%; h) CrO_3 (2.2 equiv), aq. H_2SO_4 (23 wt% in H_2O), acetone, 0 °C, 1 h, 92%.

lowed by Jones oxidation to establish the propargylic acid residue of building block **2**.

The synthesis of the right-hand building block **3** started from commercially available 3-octyn-1-ol (**10**), which was isomerized by base catalysis to 7-ocytn-1-ol (**9**) in 96% yield (Scheme 3). Subsequent alkyne iodination to give **18** was performed by following the Denmark protocol.[14] In this procedure, iodination is achieved with inexpensive and convenient reagents, I_2 and KOH as base in methanol, thereby rendering the use of silver salts or strong bases unnecessary. A subsequent oxidation with Dess–Martin periodinate delivered aldehyde **19** in 87% yield, which was immediately used in a Wittig reaction with methyl 2-(triphenylphosphoranylidene)acetate to give **20** in 94% yield. Diisobutylaluminum hydride (DIBAL-H) mediated reduction then delivered allyl alcohol **21**, which was reoxidized to the α,β-unsaturated aldehyde **8** with activated MnO_2 in 87% yield. Subsequent asymmetric alkyne addition was performed by following the Trost approach, using dimethyl zinc and trimethylsilylacetylene as the nucleophile and (*R*,*R*)-ProPhenol as the enantioselective catalyst, to afford **3** in a yield of 76%.[15] The enantiomeric excess (*ee*) of **3** was 96%, as determined by Mosher ester analysis.

We then focused on the Cadiot–Chodkiewicz coupling between **2** and **3**, which turned out to be much more difficult than originally anticipated. In test studies with various precursors, the allyl propargyl alcohol moiety proved to be rather unstable, irrespective of the alcohol protecting group in **3**. We therefore chose to employ a Pd-catalyzed version of the Cadiot–Chodkiewicz reaction, using **2** and **3** as coupling partners together with $PdCl_2(PPh_3)_2$ and CuI as catalysts, and DIEA as a base.[16] Although this approach significantly reduced the problems with the stability of the alcohol moiety, partial TMS removal occurred under these conditions. Moreover, the resulting product turned out to be very difficult to purify from the



Scheme 3. Synthesis of right-hand building block **3** and final assembly to callyspongynic acid (**1**). Reagents and conditions: a) NaH (4 equiv), ethylendiamine, 65 °C, 1 h, 96%; b) I_2 (1.1 equiv), KOH (2.5 equiv), RT, 3 h, 84%; c) Dess–Martin periodinane (1 equiv), RT, 1 h, 87%; d) methyl 2-(triphenylphosphoranylidene) acetate (1.2 equiv), CH_2Cl_2, RT, overnight, 94%; e) DIBAL-H (2.5 equiv), hexane, −78 °C, 1 h, 93%; f) MnO_2 (20 equiv), CH_2Cl_2, RT, overnight, 87%; g) trimethylsilylacetylene (2.8 equiv), Me_2Zn (2.95 equiv), (*R*,*R*)-ProPhenol (10 mol%), 0 to 4 °C, 4 d, 76%, 96% *ee*; h) i. **2** (1.2 equiv), CuI (0.03 equiv), $PdCl_2(PPh_3)_2$ (0.03 equiv), DIEA (1.8 equiv), RT, 12 h; ii. 1 M NaOH (1.05 equiv), MeOH, RT, overnight, 24% (two steps).

catalyst residues and other side products. We therefore decided to partially purify this coupling product and to subject the crude mixture to the subsequent TMS deprotection step, leading to the final product callyspongynic acid (**1**). To this end, 1 M aqueous NaOH solution was used, thereby obtaining the desired natural product in a moderate yield of 24% over two steps. The synthesized natural product displayed an $[\alpha]_D^{22}$ value of $+5.8^\circ$ ($c=0.5$, EtOH), thus closely matching the reported value of $+5.4^\circ$ ($c=0.5$, EtOH) from the isolated compound and thereby proving the original stereochemical assignment.[11]

First in vitro assays with callyspongynic acid in human cell cultures

With synthetic **1** in hand, we initiated the first chemical proteomics studies on this natural product. We started with the determination of optimal labeling conditions by measuring labeling efficiencies at different concentrations of **1** in two cell lines routinely cultured in our laboratories, HeLa (cervical cancer) and HEK293 (human embryonic kidney) cells. To this end, the cells were incubated for 6 h with **1** at different concentrations, washed, and lysed, and tagged proteins in the lysate were ligated to the secondary capture reagent (AzTB)[17] through copper-catalyzed alkyne–azide cycloaddition (CuAAC).[10a] AzTB contains a fluorophore that allows convenient analysis of tagged protein band patterns by in-gel fluorescence. Thus, the labeled proteins could be directly visualized in gel following separation by SDS PAGE (see the Supporting Information, Figure 1). Overall, the pattern appeared to be similar for both HeLa and HEK293, with very few proteins tagged at lower concentrations of the probe and an optimal tagging efficiency at 5 μM in both cell lines. At higher concentrations, tagging intensities decreased again, possibly due to the low solubility of **1**, which may cause aggregation at these concentrations. We therefore continued our experiments at 5 μM concentration of **1**.

Target Identification by using a SILAC Cell Line and Quantitative Mass Spectrometry

To achieve maximum coverage and confidence for the covalently bound protein targets of **1**, we performed target identification studies in a quantitative, gel-free manner. To this end, we repeated the labeling experiment in heavy isotope labeled (Stable Isotope Labeling of Amino acids in Cell culture, SILAC) HeLa cell lines.[18] Accordingly, a triplicate set of HeLa cells grown in the presence of either light or heavy amino acids were incubated with 5 μM **1** or with dimethyl sulfoxide (DMSO) (negative control) for 6 h, washed and lysed. We then combined one heavy extract with one light extract (Figure 2A), and the three heavy/light mixes (H1/L1, H2/L2, and H3/L3) were subjected to CuAAC with AzTB, which contains a biotinyl moiety in its structure to allow for selective enrichment of tagged proteins by NeutrAvidin affinity purification.[17] After the purification of putative targets on NeutrAvidin–agarose beads and on-bead reduction and alkylation, captured proteins were digested on-bead with trypsin. The peptide mixtures

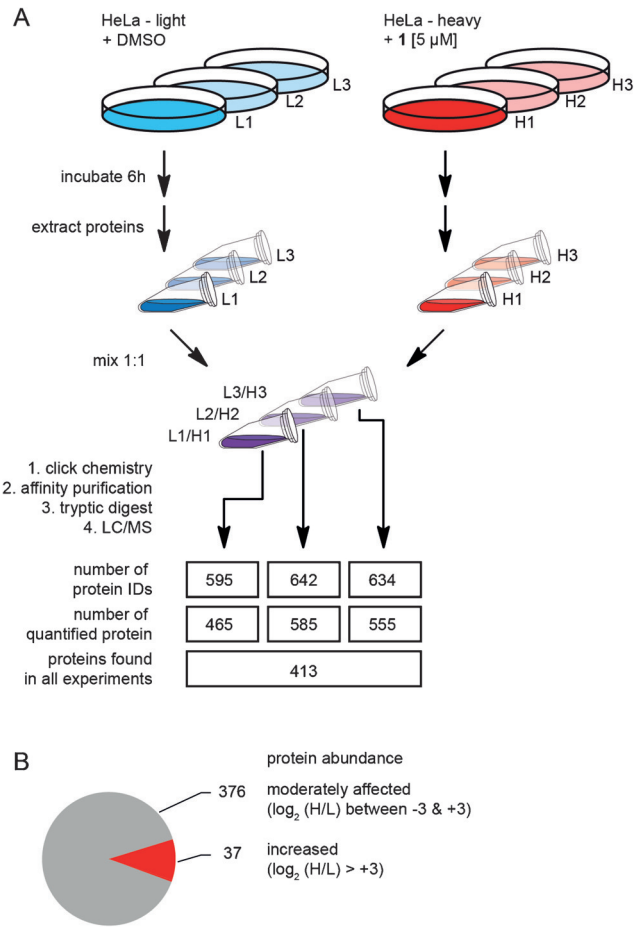


Figure 2. Workflow and results of SILAC experiments with live HeLa cells. A) Three independent SILAC-based enrichment measurements led to the identification of 413 protein groups. B) Most of the identified targets were either only moderately enriched or depleted (with $\log_2$ (H/L) ratios between $-3/+3$), whereas 37 proteins displayed higher enrichment ratios ($\log_2$ (H/L) >3).

were then separated by nanoscale liquid chromatography coupled to mass spectrometry (MS)/MS analysis on a high-resolution accurate-mass Q Exactive mass spectrometer, and the recorded RAW files were searched against the Uniprot human protein database using the Andromeda search engine as implemented in the MaxQuant quantitative proteomics software package.[19] MaxQuant analysis yielded quantitation (H/L SILAC ratio) for an impressive range of protein groups (between 465 and 585) in each heavy/light sample (Figure 2A and Supporting Information, Table 1). For further analysis, a set of 413 protein groups quantified in all the samples was selected. Most of these proteins were, however, only moderately affected by treatment with callyspongynic acid (376 protein groups, fold increase smaller than $\log_2$ H/L <3; see Figure 2B and the Supporting Information, Figure 2). Such an outcome is typical for affinity enrichment methods coupled to high-sensitivity mass spectrometry, and is the result of nonspecific binding of highly abundant proteins to the beads. By applying a stringent cutoff ($\log_2$H/L >3), 37 protein groups were identified as confident targets of callyspongynic acid in HeLa cells (see the Supporting Information, Table 2 and Figure 3).

Analysis of Callyspongynic Acid Targets

We then performed an analysis of gene ontology (GO) term enrichment within our data set; GO terms are biologically relevant expressions that are attributed to proteins based on experimental data and bioinformatic analysis.[20] The 37 gene names for significantly enriched protein groups were searched against the GO annotation database for the human proteome, and the GO term enrichment for biological process, molecular function and cellular component were extracted. The observed number of terms was compared (Fisher exact test) with the expected number of hits for a naïve (i.e., unenriched) dataset, which is a function of the number of terms in the original annotation database of the human proteome. A close inspection of the data showed a significant enrichment of terms connected to the degradation or metabolism of lipids and fatty acids (see Figure 3 and the Supporting Information, Table 3). Almost half of the putative protein targets are enzymes that may mediate metabolism and degradation of callyspongynic acid, and we hypothesize that this would result in their labeling with the natural product. One example is the enzyme ALDH3A2, which is a fatty aldehyde dehydrogenase that is involved in the detoxification of polyunsaturated fatty acids.[21] Another example is NCEH1 (KIAA1363, neutral cholesterol ester hydrolase 1), which is a serine hydrolase associated with tumor invasiveness.[22] It was shown that this enzyme catalyzed the conversion of 2-acetyl monoalkylglycerol ethers into monoalkylglycerol ethers.[23] Although callyspongynic acid does not contain a 2-acetyl group, a possible mechanism could be that the free allylic OH group is modified in cells to a substrate for NCEH1,

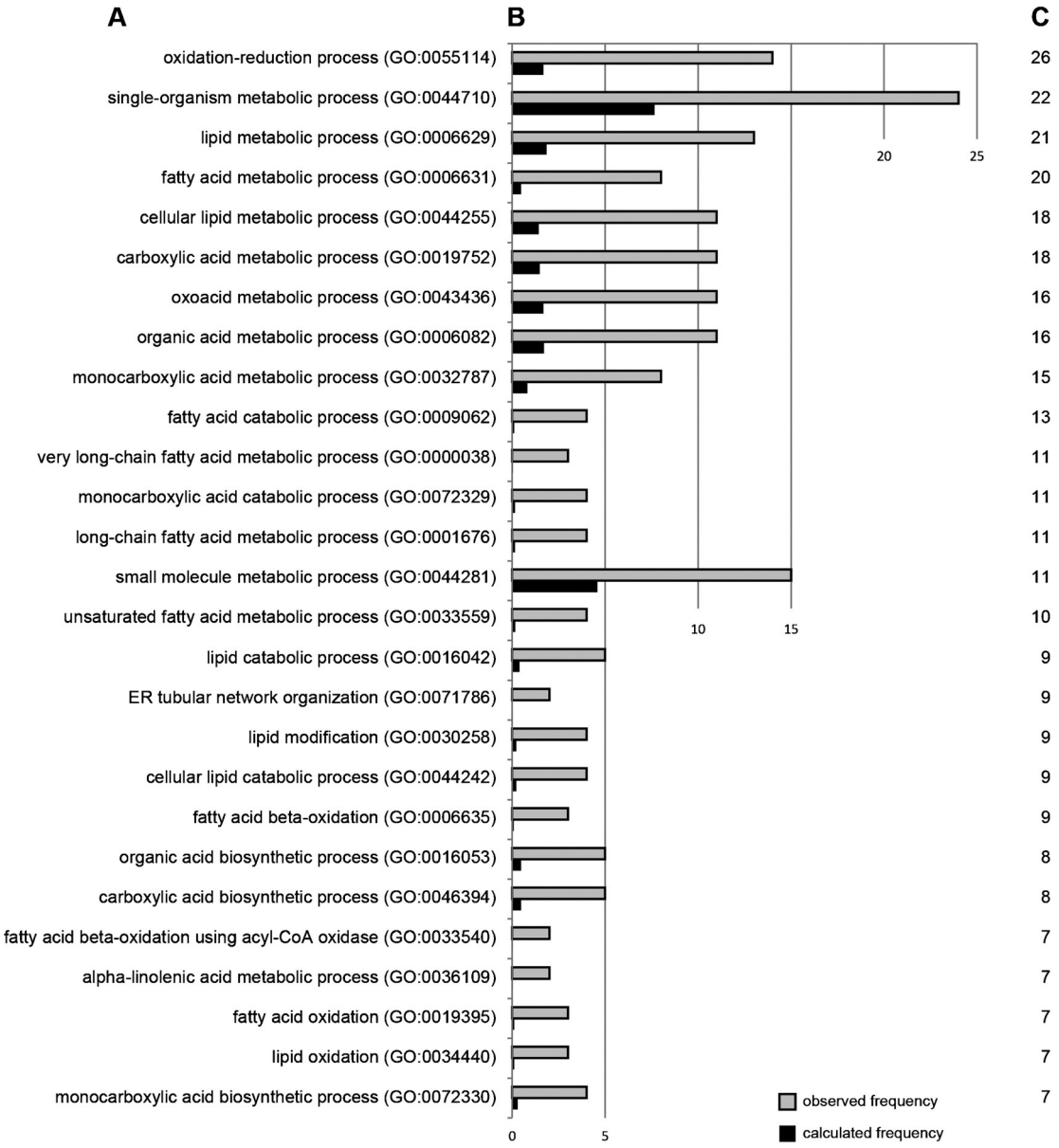


Figure 3. Analysis of gene ontology (GO) term enrichment. A) GO terms tested; B) calculated versus observed numbers in the dataset; C) the $-\log_2$(p-value) of the enrichment (note: higher values correlate with higher confidence).

for example, by an acetyltransferase such as SOAT1, which was also identified as a target of callyspongynic acid.[24]

The other half of the putative targets is predicted to lack enzymatic activity. Some targets such as VDAC2 (voltage-dependent anion-selective channel protein 2)[25] or SLC6A8 (sodium- and chloride-dependent creatine transporter 1)[26] are supposed to be involved in cellular trafficking, and at first sight might not be obvious targets for callyspongynic acid. However, the GO annotation enrichment by cellular compartment reveals that almost all putative targets are mainly membrane-associated.

Altogether, the observed enrichment of the enzymes and membrane-associated proteins seems to result from direct labeling with callyspongynic acid, for example, by a 'metabolic labeling' in which callyspongynic acid is used as a long-chain fatty acid derivative or by chemical reaction with target proteins, for example, after chemical modification (e.g., acylation) of the hydroxy moiety, which converts this residue into a leaving group for S_N2'-type reactions. Alternatively, the enrichment of the membrane-associated proteins could be explained by an 'unspecific' detergent-like perturbation of membrane structure from callyspongynic acid application, resulting in an increased 'release' of membrane-associated proteins.

Conclusion

We have presented a combined experimental approach to characterize potential targets of a polyacetylene natural product. To this end, we described the first enantioselective chemical synthesis of the polyacetylene natural product callyspongynic acid, thereby proving the correct structural assignment from the original isolation. By using an inherent feature of the compound (that is, the presence of multiple alkyne groups amenable to the click chemistry approach), we subsequently investigated the putative protein targets of this compound in human cell cultures. To this end, we affinity-enriched putative targets after in-cell tagging and used quantitative mass spectrometry to identify 37 proteins that bind callyspongynic acid. Notably, 90% of these putative targets turned out to be localized within the membrane. Based on GO annotations, about 60% of the targets were classified as enzymes, and most of these are involved in lipid and fatty acid metabolism.

Our findings thus indicate that callyspongynic acid might represent a valuable chemical tool to perturb and to profile activity of multiple different membrane-associated proteins, although further biological studies are required to determine the overall biological impact of these perturbations. In addition, our studies indicate that callyspongynic acid may target organelles featuring large-membrane structures such as the endoplasmic reticulum (ER), which comprises the largest membrane-bound structure in the cell and harbors many lipid-modifying enzymes. These findings indicate that the so far unknown chemical phenotype and thus bioactivity of callyspongynic acid could, for example, be perturbation of ER biology.

On a broader view, our study provides a general roadmap to characterize putative targets of polyacetylene natural products (as well as other alkyne-containing natural products). The coupling of a synthesis (or isolation) of an alkyne-containing natural product with a 'native' chemical proteomics approach, in combination with a GO term analysis, is a straightforward methodology to gain insights into the potential molecular mechanism of a compound and thus may enable the direct search for 'chemical phenotypes' of natural products. We therefore anticipate that such an approach may find widespread application in the future.

Experimental Section

Supplementary figures and synthetic procedures for the synthesis of callyspongynic acid (**1**) can be found in the Supporting Information.

Live cell tagging and preparation of samples for in-gel fluorescence detection

HeLa cells in DMEM (6 cm round culture dishes, 80% confluent, 10% CO_2, 37 °C, 3 mL media from Gibco supplemented with 10% FBS from Sigma) were incubated for 6 h with **1** at 0, 1, 2, 5, 10, and 20 μM. Cells were washed with PBS (3×2 mL), and then harvested on ice in 100 μL lysis buffer (1×PBS, 0.1% SDS, 1% Triton X-100, 1×EDTA-free complete protease inhibitor cocktail from Roche Diagnostics). Lysates were kept on ice for 20 min and centrifuged at 17000×g for 20 min to remove insoluble material. Protein concentration was determined by using the Bio-Rad DC Protein Assay. Proteins (100 μg) were diluted with lysis buffer to a final concentration of 1.0 mg mL^{-1} (94 μL final volume). The click reaction was then started by adding TBTA (final concentration 0.1 mM), capture reagent AzTB (final concentration 0.1 mM), $CuSO_4$ (final concentration 1 mM), and TCEP (final concentration 1 mM). The sample was vortex-mixed for 1 h before the click reaction was stopped by adding 2 μL 0.5 M aqueous EDTA (final concentration 10 mM). To precipitate all proteins and remove unreacted capture reagent, methanol–chloroform precipitation was performed. Briefly, 200 μL MeOH, 50 μL $CHCl_3$, and 100 μL H_2O were added to the click reaction. The samples were then centrifuged at 17000×g for 5 min and the pellets were washed with 1 mL MeOH and dried on air. SDS (30 μL, 2% in PBS) was added to dissolve the proteins by vigorous vortex-mixing and the samples were diluted with 10 μL 4 times sample loading buffer to 2.5 mg mL^{-1}. Proteins (25 μg) were loaded onto 12% polyacrylamide bis-tris gels and run at 150 V for 90 min. Proteins were fixed (40% MeOH, 10% acetic acid, 50% water) for 5 min and the gels were washed with water (3×). In gel fluorescence was detected with an Ettan DIGE Imager (GE Healthcare) and the protein loading was checked by Coomassie staining.

Live cell tagging and preparation of samples for proteomics

HeLa cells in R10K8 DMEM (three 10 cm round culture dishes, cells 80% confluent pre-cultured in R10K8 for 7 cell doublings, 10% CO_2, 37 °C, 10 mL media from Dundee Cell Products supplemented with 10% dialysed FBS from Sigma) were incubated for 6 h with **1** at 5 μM, whereas control HeLa cells in R0K0 DMEM supplemented with 10% dialysed (M_w = 10000 Da) FBS were incubated for 6 h with DMSO (10 μL, same amount the probe was dissolved in). Cells were then washed with PBS (3×5 mL), and then harvested on ice in the lysis buffer (1×PBS, 0.1% SDS, 1% Triton X-100, 1×EDTA-free complete protease inhibitor cocktail from Roche Diagnostics). Lysates were kept on ice for 20 min and centrifuged at 17000×g for 20 min to remove insoluble matter. Protein concentration was

determined by using the Bio-Rad DC Protein Assay. Proteins (400 μg of light and 400 μg of heavy) were pooled together and diluted with lysis buffer to a final concentration of 1.5 mg mL^{-1} (540 μL final volume). The click reaction was then started by adding TBTA (final concentration 0.1 mM), capture reagent AzTB (final concentration 0.1 mM), $CuSO_4$ (final concentration 1 mM), and TCEP (final concentration 1 mM). The sample was vortex-mixed for 1 h before the click reaction was stopped by adding 12 μL 0.5 M aqueous EDTA (final concentration 10 mM). To precipitate all proteins and remove unreacted capture reagent we performed a methanol–chloroform precipitation.[27] Briefly, 800 μL MeOH, 200 μL $CHCl_3$, and 200 μL H_2O were added to the click reaction. The samples were then centrifuged at 17000×g for 5 min and the pellets were washed with 1 mL MeOH and dried on air. SDS (80 μL, 2% in PBS) was added to dissolve the proteins by vigorous vortex-mixing, and then 720 μL PBS was added and samples were centrifuged at 17000×g for 5 min to precipitate any insoluble matter. 760 μL was then carefully removed from the top and added to NeutrAvidin–agarose beads from Fisher (40 μL of slurry, 3×pre-washed with 0.2% SDS in PBS). After 2 h incubation, the supernatant was removed and the beads were extensively washed (3 times with 1% SDS in PBS, 2 times with 4 M urea in 50 mM ABC (ammonium bicarbonate) and 5 times with 50 mM ABC). Each wash was performed with 0.5 mL of washing solution; the beads were gently vortexed for 3 min, centrifuged at 3,000 ×g for 3 min and the supernatant aspirated. Beads were spun at 3000×g for 3 min and the excess liquid discarded. For a beads suspension of 50 μL: samples were reduced by adding 2.5 μL of 100 mM dithiothreitol in 50 mM ABC and heating to 55 °C for 30 min. After the beads cooled to RT, the beads were washed with 0.5 mL 50 mM ABC. Cysteines were then alkylated by adding 2.5 μL of 100 mM iodoacetamide in 50 mM ABC at RT for 30 min in the dark. The beads were then washed twice with 0.5 mL 50 mM ABC. Trypsin digestion was then started by adding 2 μg sequencing grade modified trypsin from Promega to each set of beads. The digestions were performed at 37 °C for 16 h. The samples were then centrifuged and the supernatant was transferred into clean tubes. The beads were washed with ABC (50 μL) and the wash was combined with the supernatant. The tryptic peptide mixtures were cleaned by stage-tipping,[28] reconstituted in 0.5% TFA/2% MeCN/H_2O, and analyzed by LC-MS with an Easy-nLC1000-Q-Exactive system.

LC-MS/MS analysis

The analysis was performed by using a reverse-phase Acclaim PepMap RSLC column 50 cm×75 μm inner diameter (Thermo Fisher Scientific) using a 100 min acetonitrile gradient (2–27%) in 0.1% formic acid at a flow rate of 250 nL min^{-1}. An Easy nLC-1000 instrument was coupled to a Q Exactive mass spectrometer via an easy-spray source (all Thermo Fisher Scientific). The Q Exactive was operated in data-dependent mode with survey scans acquired at a resolution of 75000 at *m/z* 200 (transient time 256 ms). Up to the top 10 most abundant isotope patterns with charge +2 or higher from the survey scan were selected with an isolation window of 3.0 *m/z* and fragmented by HCD with normalized collision energies of 25. The maximum ion injection times for the survey scan and the MS/MS scans (acquired with a resolution of 17500 at *m/z* 200) were 200 and 80 ms, respectively. The ion target value for MS was set to 10^6 and for MS/MS to 10^5, and the under fill ratio was 0.1%.

Proteomics data analysis

Data were processed with MaxQuant version 1.3.0.5, and the peptides were identified from the MS/MS spectra searched against SwissProt human (+ isoforms) database using the Andromeda search engine.[19,29] Cysteine carbamidomethylation was used as a fixed modification and methionine oxidation and protein N-terminal acetylation as variable modifications. For the identification, the false discovery rate was set to 0.01 for peptides, proteins, and sites, the minimum peptide length allowed was 7 amino acids, and the minimum number of unique/razor peptides allowed was set to one. Other parameters were used as pre-set in the software. "Unique and razor peptides" mode was selected; this calculates ratios from unique and razor peptides (razor peptides are nonunique peptides assigned to the protein group with the highest number of other peptides). Data were further analyzed in Perseus ver. 1.4.0.20.

GO-term enrichment analysis

Gene names for the 37 significantly enriched proteins were submitted to the Enrichment Analysis service of the Gene Ontology Consortium (http://geneontology.org/page/go-enrichment-analysis) and searched against the subdatabases "biological process", "molecular function", and "cellular compartment" of the *H. sapiens* GO term database. The results were copied and saved in an Excel spreadsheet (see the Supporting Information, Table 3).

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