Title: Untargeted identification of alkyne containing natural products using ruthenium catalyzed azide alkyne cycloaddition reactions coupled to LC-MS/MS.

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Introduction:

Acetylenic natural products have been identified in extracts of plants, insects, algae, fungi, and bacteria. One of the first acetylenic natural products isolated was dehydromatricaria ester from *Artemisia* sp., in 1826.¹ The majority of previously described acetylenic natural products have been identified in plants, fungi, algae, and insects including lentinamycin (**1**), an acetylenic natural product displaying antibiotic properties produced from the fungus *Lentinula edodes* (shiitake mushroom), faltarinol (**2**) an alkyne natural product displaying anticancer activity from the plant *Daucus carota* (wild carrot), and (Z)-13-hexadecen-11-ynoic acid (**3**), a sex pheromone from the moth *Thaumotopoea pityocampa*.²⁻⁴

Comparatively, acetylenic natural products characterized from bacteria are seemingly less prevalent.⁵ Many of the currently described bacterial acetylenic natural products, such as the cyanobacterial natural products jamaicamide B (**4**)⁶, anaephene B (**5**),⁷ and georgamide (**6**)⁸, feature a single terminal alkyne moiety. Much less common are bacterial conjugated polyacetylenic natural products (polyynes), as to date only a handful have been isolated and structurally characterized.⁹ The low frequency of discovered polyynes can be attributed to the fact that some polyynes have been described to undergo decomposition during purification and characterization of these compounds is difficult.⁹ This lead us to the hypothesis that that they may be underrepresented in databases of characterized natural products, which is supported by the prevalence of proteins putatively involved in the biosynthesis of alkynes in bacterial genomes.¹⁰⁻¹¹ Recently, copper catalyzed azide-alkyne cycloaddition reactions (CuAAC), also referred to as copper click chemistry, have been employed to stabilize polyynes containing a terminal alkyne moiety by generating a 1,4-disubstituted triazole from the coupling of the terminal alkyne moiety with an azide. The cycloadducts are typically more stable than the unreacted polyynes, which allows isolation and structural characterization of the resulting 1,2,3-triazole.⁵ This approach was recently utilized for isolation and structure determination of caryoynencin A (**7**)⁵, collimonin A (**8**)⁹, and related compounds. CuAAC has also been utilized to aid in the initial discovery of acetylenic natural products from crude extracts by coupling CuAAC with fluorescence or LC-MS based analyses.¹²⁻¹³

While CuAAC has undoubtedly aided in the study and identification of several acetylenic natural products, it neglects a subset of acetylenic natural products, those containing internal alkyne moieties as these compounds do not undergo a copper catalyzed cycloaddition. Examples of natural products containing internal alkynes include fischerellin A (**9**),¹⁴⁻¹⁵ microcarbonin A (**10**)¹⁶, and ergoyne A (**11**).¹⁷

Ruthenium mediated azide-alkyne cycloaddition reactions (RuAAC) were first described in 2005¹⁸ and can catalyze the cycloaddition of azides with both terminal and internal alkynes to generate the 1,5-disubstituted and 1,4,5-trisubstituted triazoles respectively (Fig. 1) in contrast to the 1,4-disubstituted-1,2,3-triazole

product formed during CuAAC reactions. While RuAAC has a broader scope of substrate reactivity, it has not been previously utilized in natural products discovery, most likely due to byproducts produced under reaction conditions.¹⁹

In this paper, we report a method utilizing RuAAC for the untargeted discovery of acetylenic natural products from unfractionated bacterial extracts, which relies solely on the unique fragmentation pattern of a *p*-bromobenzyl triazole. As a proof of concept, both fischerellin A and B were located in the unfractionated extract of the cyanobacterium *Fischerella muscicola* PCC 9339 using this method. Coupling the RuAAC with the genome mining of acetylenases allowed us to develop a workflow to identify acetylenic natural products that we describe in the manuscript. Through genome mining approaches followed by the described RuAAC method, two acetylenic natural products produced by the plant protective bacterium *Pseudomonas protegens* Pf-5 were identified, which have recently been

reported by the Kai group as protegenin A and C²⁰. This discovery demonstrates the advantage of utilizing RuAAC as a powerful tool to screen unfractionated extracts for acetylenic natural products discovery.

Figure 1. Catalytic mechanisms of CuAAC (left) and RuAAC (right) reactions.

Results and discussion:

Evaluating alkyne reactivity and MS/MS fragmentation. As some acetylenic natural products contain conjugated alkyne moieties (either in conjugation with an alkene or alkyne) and the reactivity of conjugated alkynes has not been extensively evaluated with RuAAC, we synthesized a small library of conjugated alkynes for testing (Fig.2 and Supplementary Figure 1). The synthetic compounds with internal diyne moieties were then subject to a RuAAC reaction containing *p*-bromobenzyl azide and Cp*RuCl(PPh₃)₂ in 1,4-dioxane. The catalyst Cp*RuCl(PPh₃)₂ was chosen as it has been shown to be one of the more stable ruthenium catalysts in aerobic environments.²¹ 1,4-Dioxane was used as the solvent as previous research has shown success with employing 1,4-dioxane as the solvent with the Cp*RuCl(PPh₃)₂

catalyst.²¹ We chose *p*-bromobenzyl azide as the azide for three reasons. First, the bromine atom is easily traceable via mass spectrometry due to the characteristic isotope pattern. Second, we anticipated that the *p*-bromobenzyl moiety would result in a common fragment produced from the resulting cycloadducts (vide infra). Third, we envisioned that the *p*-bromobenzyl group would aid in crystallization of the cycloadducts in later steps. With all of our synthetic alkynes we observed the formation of a triazole and the resulting cycloadducts were purified, structurally characterized, and subjected to MS/MS analysis. The synthetic triazoles all showed a consistent fragmentation pattern to yield the brominated tropylium ion ($[M]^+$ of 168.96/170.96) as well as the non-brominated tropylium ion with an $[M]^+$ of 90.04 (Fig. 2 and Supplementary Figure 1). Since the 168.96/170.96 signals were present in all MS² spectra with the highest abundance, we suspected that this fragment ion would serve as an excellent reporter ion for the detection of derivatized alkynyl natural products in extracts.

Detecting standard compounds in an extract. We then tested to see if the Cp*RuCl(PPh₃)₂ catalyst was compatible for RuAAC reactions in 60% aq. methanol extracts of cyanobacterial cells. We chose to use *Anabaena* sp. PCC 7120 as our organism to generate our test extract for the following reasons. *Anabaena* sp. PCC 7120 is a fairly fast-growing cyanobacterium, and it is not known to produce acetylenic natural products. It also contains chlorophyll a, carotenoids, and other typical cellular compounds that could poison the catalyst or complicate downstream LC-MS/MS analysis. We extracted a lyophilized cell mass of *Anabaena* sp. PCC 7120 with 60% aq. methanol. The particulate was removed via filtration and the extract was concentrated to dryness. The resulting dried material was resolubilized in 1,4-dioxane and diphenyl acetylene was spiked into the extract at a concentration of 10 µg/ml. Compound **12** was clearly observed in the RuAAC reaction mixture using an extracted ion chromatogram (Fig. 2D) but was not observed in any of the control reactions lacking catalyst, azide, or alkyne (Fig. 2E and Supplementary Figure 2). This suggested that RuAAC could be adapted for the discovery of acetylenic natural products in extracts without fractionation or purification.

Detection of known acetylenic natural products in extracts. With our test case successful, we then turned our attention to the detection of known natural products. Extraction of the cyanobacterium *Fischerella muscicola* PCC 9339, which is a known producer of the internal diyne-ene containing secondary metabolites fischerellin A¹⁴⁻¹⁵ and B,²² and hapalindole-like compounds,²³ was performed with 60% aq. methanol as this has been reported to extract both fischerellins and the hapalindole-like compounds. After concentration to dryness, the extract was resuspended/redissolved in 1,4-dioxane and the RuAAC reaction was performed as described above. Following the RuAAC derivatization of the extract, LC-MS/MS data was collected using the AutoMS/MS setting (data independent MS/MS) for both the RuAAC reaction and the underivatized extract. We were able to observe the formation of the expected triazole product (**14**, Fig. 3) in the RuAAC reaction, but not in the control reactions (Supplementary Figure 3), although not all the fischerellin A in the extract was consumed. However, in the initial LC-MS/MS run, **14** was not selected by the computer for fragmentation. We hypothesized that the intensity of **14** was never one of the top five (5) ions in the MS (one of our program parameters) and was therefore not chosen for fragmentation. Therefore, the generation of an exclusion list would aid in the detection of lower abundance cycloadduct products.

We examined the chromatogram produced by identifying the precursor ions that generated the brominated tropylium fragment. We noted the presence of multiple brominated compounds throughout the chromatogram (Supplementary Figure 7b, top panel). Based on the exact masses we propose that these compounds were produced by off-pathway reactions of *p*-bromobenzyl azide with the ruthenium catalyst. To reduce the background of unwanted byproducts of the RuAAC reaction, we tested various conditions including various solvents (monitored by TLC, data not shown), diluting the catalyst, and reducing the azide concentration (Supplementary Figures 5 and 6). However, the reaction was shown to work best in 1,4-dioxane and dilution of *p*-bromobenzyl azide did not produce a lower ratio of unwanted byproduct compared to triazole formation. Efforts were then put into identifying the major byproducts of the reaction and generating an exclusion list so they would be excluded from fragmentation during LC-MS/MS analysis. To do this, the major byproducts were identified and manually added to an exclusion list. The derivatized extract was re-analyzed by LC-MS/MS using the manually curated exclusion list of the major byproducts. However, the mass corresponding to the fischerellin A cycloadduct was still not

fragmented during the analysis. This posed a major problem as there were too many byproducts yielding the signature ion fragment during LC-MS/MS fragmentation to manually identify.

We then utilized iterative MS/MS to analyze our samples. Iterative MS/MS automatically adds ions selected for fragmentation to an exclusion list for subsequent analyses. In this way an operator can delve deeper into the data and fragment molecular ions of lower abundance in subsequent runs with minimal operator involvement thereby increasing automation, while simultaneously performing fragmentation over the entire elution period. Using iterative MS/MS, **14** was successfully fragmented in the second iteration of the derivatized extract producing an MS/MS spectrum for **14**. However, the peak for **14** was not easily identifiable in the EIC of precursor ions that generated the brominated tropylium fragment (Fig. 3D and Supplementary Fig. 4). While obtaining an MS/MS spectrum for the fischerellin A cycloadduct was considered a success and indicated that iterative MS/MS was a potential solution, we needed to further optimize our analytical method.

To optimize our workflow, we utilized iterative MS/MS of a reaction containing $Cp^*RuCl(PPh_3)_2$ and *p*-bromobenzyl azide to generate an exclusion list. Using three successive iterative MS/MS runs of the reaction containing $Cp^*RuCl(PPh_3)_2$ and *p*-bromobenzyl azide (no *F. muscicola* extract) generated a large list of byproducts from the reaction that could then be programmed into an exclusion list for subsequent runs. The RuAAC-derivatized extract of *F. muscicola* PCC 9339 was analyzed once again using this method in conjunction with celite filtering, and the fischerellin A cycloadduct is a prominent peak in the chromatogram (Fig. 4). We also observed a peak which corresponded to the predicted $[M+H]$ of the fischerellin B cycloadduct, which had not been observed previously. The appearance of the fischerellin B cycloadduct in our EIC indicates that the multiple iterations allowed for more compounds to be identified (Supplementary Figure 7). The successful identification of both fischerellin A and B demonstrated that this method was ready to screen bacteria with putative alkyne producing biosynthetic gene clusters.

Identification of acetylenic natural products produced by Pseudomonas protegens Pf-5 using RuAAC. We next identified putative producers of alkynyl natural products through genome mining. As described in previous studies, some acetylenic natural products, such as polyacetylenes, lack a polyketide synthase, non-ribosomal peptide synthetase, and other enzymes characteristic of natural products biosynthesis.⁵ Coupled with the fact that only a handful of polyene natural product biosynthetic gene clusters (BGCs) have been identified to date makes it difficult to identify putative polyene producing organisms using antiSMASH.²⁴ Previous studies have been conducted on the biosynthesis of bacterial acetylenic natural

products.^{5, 25-26} These studies have determined that a desaturase is most likely responsible for the formation of the alkyne bond. Along with a desaturase, an acyl carrier protein, and an AMP-dependent ligase are thought to play important roles in the biosynthesis of the alkyne moiety.²⁵ The sequence of ColB, a desaturase involved in alkyne biosynthesis of the collimonin group of polyynes from the bacterium *Collimonas fungivorans* Ter6, was used as a BLAST query in the EFI-EST web tool to generate a network of similar enzymes based on sequence homology (Fig. 5). In this network, putative desaturase protein sequences from many of the characterized bacterial polyacetylenic producers clustered

together (Cluster 2, Fig. 5A). Within this cluster were also sequences of putative desaturases from various other bacteria in which polyacetylenes have not been characterized, including *Pseudomonas protegens* Pf-5. The putative polyne BGC in *P. protegens* Pf-5 (PFL0261-0268) was shown to be very similar to that of other polyacetylenic gene clusters such as the caryoynencins and collimonins (Fig. 5B).²⁷ As we have previously researched the secondary metabolites produced by *P. protegens* Pf-5, we chose this organism as our initial candidate for screening with the described RuAAC-LC-MS/MS method.

Utilizing the described RuAAC method yielded two prominent peaks with [M+H]⁺ values of 480.1277 and 478.1122 producing the signature brominated tropylium ion as seen in the MS/MS chromatogram (Fig. 6). These masses of the underivatized compounds were calculated by subtracting the mass of the *p*-bromobenzyl azide tag from the observed masses in the derivatized extract resulting in the calculated [M+H]⁺ values of alkynes as 269.1532 and 267.1377, which corresponded to the molecular formulae of C₁₈H₂₀O₂ and C₁₈H₁₈O₂ respectively. These masses were shown to be present in the underivatized extract (Fig. 6 and Supplementary Figure 9). To demonstrate that these compounds were the product of the PFL0261-0268 biosynthetic gene cluster, a deletion mutant was constructed. Protegenin A and C were not identified in the extract of the deletion mutant, thereby demonstrating that these compounds were biosynthesized by the BGC (Supplementary Figure 9). While this manuscript was being finalized, a publication was released reporting the isolation and structure characterization of protegenin A-D (**16-19**) from *P. protegens* Cab57 (aka MAFF 212077).²⁰ These compounds were isolated and their structure determined via bioassay guided fractionation. The compounds identified in this study are protegenin A (*m/z* 267.1) and protegenin C (*m/z* 269.1). We did not identify protegenins B and D. The lack of protegenin B can be explained by the fact that it is most likely not a biosynthetic product as the compound was isolated as a racemic mixture. It is possible that the lack of signal corresponding to protegenin D was due to the extremely low titers of production of this compound in *P. protegens* Pf-5, or the different cultivation methods (liquid vs agar plates) utilized in this study. A second publication was released characterizing protegenin A.²⁸ However, in this study, failure to detect protegenins C and D is expected based on the use of CuAAC click chemistry which only reacts with terminal alkyne groups. This demonstrates the effectiveness of utilizing RuAAC for the discovery of acetylenic natural products in an untargeted manner.

CuAAC derivatization of *P. protegens* extract. The results from the RuAAC derivatization of the *P. protegens* Pf-5 extract show the presence of two naturally occurring acetylenic natural products, subsequently named protegenin A and C. To acquire additional structural information, we subjected the *P. protegens* Pf-5 extract to CuAAC. Processing the LC-MS/MS data by searching for fragmented ions that produce the brominated tropylium fragment ion identified the cycloadduct of **16** ([M+H]⁺/([M+2+H]⁺ = 478.11/480.11) as a prominent peak in the computer-generated chromatogram but not **18** ([M+H]⁺/([M+2+H]⁺ = 480.12/482.13). We were unable to detect cycloadduct of **18** by an extracted ion chromatogram but were able to clearly observe the [M+H]⁺ = 269.13 (**18**) in the reaction mixture. This shows that while both compounds were present in the original extract and react during the RuAAC reaction, only **16** reacted during the CuAAC reaction. This confirms that **18** contains a terminal alkene group compared to the terminal alkyne group found in **16**, which is supported by the increase in mass of +2 Da and the co-occurrence of terminal alkyne/terminal alkene natural products in other organisms (e.g. viequeamide B and C²⁹).

Comparison of RuAAC and CuAAC. In the case of *P. protegens* both RuAAC and CuAAC reactions were able to derivatize **16** but only RuAAC was able to form the desired cycloadduct with **18**. This suggests that RuAAC could be utilized to identify a greater number of natural products than CuAAC, for example fischerellin A and B described earlier in the manuscript. The largest downside to utilizing RuAAC is the generation of azide derived byproducts. This necessitates the creation of an exclusion list from the reaction containing Ru catalyst and azide prior to analyzing the RuAAC reaction mixture. In contrast, the identification of **16** in the CuAAC reaction was accomplished without the need for an exclusion list, thereby shortening the total analysis time. While we were able to circumvent this issue, this suggests optimization of the RuAAC reaction or Ru catalyst would be a worthwhile investment to allow greater convenience for end users. However, the discovery of protegenin C by RuAAC and not CuAAC demonstrates that RuAAC is a powerful tool for identifying acetylenic natural products in an extract and that CuAAC can be used in conjunction for preliminary structural assignments on a small-scale culture before isolation.

Conclusion:

Here we describe a workflow for the untargeted identification of acetylenic natural products from bacterial extracts. This was demonstrated with the cyanobacterium *Fischerella muscicola* PCC 9339 locating fischerellin A and B as a proof of concept. This method was then employed with the bacterium *P. protegens* Pf-5 resulting in the discovery of two new acetylenic natural products which have been identified recently as protegenin A and C. As this method was shown to be successful in locating uncharacterized alkyne natural products from a crude extract, it has potential to be used to screen various other organisms including fungi and plants in the future to discover natural products with both terminal and internal alkyne moieties as well as further modifying the method for a high throughput approach to screen organisms with both sequenced and un-sequenced genomes from various culture collections.

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Figure 6. Results of RuAAC reaction with *Pseudomonas protegens* Pf-5 extract. A. Chromatogram of the ions that produced the brominated tropylium [M+H]⁺ fragment shows two prominent peaks, determined to be cycloadducts of protegenin A and C in the extract of *P. protegens* Pf-5 wild type subjected to RuAAC reaction (trace I), Chromatogram of the ions that produced the brominated tropylium [M+H]⁺ fragment in the underivatized extract of *P. protegens* Pf-5 wild type (trace II), Chromatogram of the ions that produced the brominated tropylium [M+H]⁺ fragment in the extract of *P. protegens* Pf-5 LK164 subjected to RuAAC reaction (trace III). B. MS spectrum of the protegenin C cycloadduct peak. This spectrum shows a brominated peak with a mass of 480.1277. Calculated mass of the underivatized natural product [M+H]⁺ ($480.1277 - 210.9745 = 269.1532$). C. MS spectrum of the protegenin A cycloadduct peak. This spectrum shows a brominated peak with a mass of 478.1122. Calculated mass of the underivatized natural product [M+H]⁺ ($478.1122 - 210.9745 = 267.1377$).

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