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Discovery, Isolation, and Characterization of Diazeniumdiolate Siderophores

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Abstract

The *C*-diazeniumdiolate (*N*-nitrosohydroxylamine) group in the amino acid graminine (Gra) is a newly discovered Fe(III) ligand in microbial siderophores. Graminine was first identified in the siderophore gramibactin, and since this discovery, other Gra-containing siderophores have been identified, including megapolibactins, plantaribactin, gladiobactin, trinickiabactin (gramibactin B), and tistrellabactins. The *C*-diazeniumdiolate is photoreactive in UV light which provides a convenient characterization tool for this type of of siderophores. This report details the process of genomics-driven identification of bacteria producing Gra-containing siderophores based on selected biosynthetic enzymes, as well as bacterial culturing, isolation and characterization of the *C*-diazeniumdiolate siderophores containing Gra.

Keywords: siderophore natural products, iron acquisition, diazeniumdiolate group, nitrosohydroxylamine, biosynthetic gene cluster, photoreactivity

Contents

1. Introduction

Cells of nearly all forms of life require iron to function. Bacteria and fungi often produce small organic molecules called "siderophores" which have a high affinity for Fe(III). The Fe(III)-siderophore complex is taken up by the cell for metabolic use (Sandy & Butler, 2009). Until recently, the ligands within characterized siderophores fell into the categories of catecholate, hydroxamate, α-hydroxycarboxylate, oxazoline and thiazoline, most commonly. Part of the non-proteogenic amino acid graminine (Gra), the *C*-type diazeniumdiolate functional group (also known as *N*-nitrosohydroxylamine) (Fig. 1) is a newly discovered siderophore ligand, first characterized in gramibactin (Gbt), a siderophore produced by the rhizospheric bacterium, *Paraburkholderia graminis* (Hermenau et al., 2018). Only a few other examples of *C*-type diazeniumdiolates are found in natural products (Dolak et al., 1983; Iimura et al., 1972; Jenul et al., 2018; Murthy et al., 1966; Natori et al., 1997; Nishio et al., 1993; Sieber et al., 2021; Tamura et al., 1967). Since the discovery of gramibactin, several diazeniumdiolate siderophores have been characterized, including gramibactin, trinickiabactin (gramibactin B), megapolibactins, plantaribactin, gladiobactin, and tistrellabactins (Fig. 1). Most of the bacteria producing

these siderophores are found in soil, with the exception of *Tistrella mobilis* KA081020-065, a marine strain which produces the tistrellabactins (Hermenau et al., 2018; Hermenau et al., 2019; Jiao et al., 2020; Makris et al., 2023).

In addition to the ability to coordinate Fe(III), diazeniumdiolate siderophores have also been found to produce nitric oxide (NO) both *in vivo* and *in vitro* (Hermenau et al., 2019; Makris et al., 2022). Nitric oxide is a versatile signaling molecule in bacteria and higher eukaryotes (Tuteja et al., 2004). While synthetic *N*-diazeniumdiolate compounds are well-known NO donors in various chemical systems, little is known about the chemistry of *C*-diazeniumdiolate natural products and their ability to donate NO (Hrabie & Keefer, 2002; Li et al., 2020). It was recently established that upon UV photolysis (e.g., Hg(Ar) pen lamp or sunlight), *C*-diazeniumdiolate siderophores release the equivalent of NO and H, forming an oxime photoproduct, thus providing a pathway for bacteria to produce NO. The photoproduct loses its ability to bind Fe(III), which hints at a potential greater ecological role for these siderophores, and also provides an attractive rationale for the development of related compounds with pharmaceutical applications (Makris et al., 2022; Makris et al., 2023).

[Insert Figure 1 here]

Fig. 1 Characterized *C*-diazeniumdiolate siderophores. The diazeniumdiolate group is shown in red.

Through microbial gene knock-out studies on the biosynthetic gene cluster (BGC) of gramibactin, two enzymes, GrbD and GrbE, were found to be responsible for the biosynthesis of Gra, the non-canonical amino acid that bears the diazeniumdiolate

functional group in diazeniumdiolate siderophores (Hermenau et al., 2019). L-Arg is the precursor of L-Gra based on the results of isotopic labeling studies (Makris et al., 2022). The biosynthesis of *C*-diazeniumdiolate compounds, including that of Gra, is a topic of much current interest. (Jenul et al., 2018; Morgan & Li, 2020; Sieber et al., 2020; Sieber et al., 2021; Wang et al., 2020; Wang & Ryan, 2023).

In this chapter, we detail the process of genomics-driven discovery, bacterial culturing, and the isolation and characterization of the *C*-diazeniumdiolate siderophores containing Gra, to provide researchers with guidance to carry out experiments related to this novel class of siderophores.

2. Bioinformatics and genome mining to predict bacteria producing graminine-containing siderophores

Targeted discovery of new *C*-diazeniumdiolate siderophores is achieved by using multiple bioinformatics tools. Sequence similarity network (SSN) helps visualize the relationships among protein sequences (Atkinson et al., 2009). The SSN of GrbD (or GrbE) is created and parameters are adjusted to select the entries which are likely in the BGCs of diazeniumdiolate siderophores. Accession codes of the certain bacteria strains are gained from NCBI, and the possible secondary metabolites BGCs are predicted using antiSMASH (antibiotics & Secondary Metabolite Analysis Shell) (Blin et al., 2023). By searching for GrbE (or GrbD) homologs and nonribosomal peptide synthetase (NRPS) adenylation (A) domains with the specificity code for Gra, candidate microbe-producing diazeniumdiolate siderophores can be predicted.

2.1 Materials

2.1.1 Equipment (Software)

- NCBI (https://www.ncbi.nlm.nih.gov/)
- EFI – Enzyme similarity tool (EST) (https://efi.igb.illinois.edu/efi-est/)
- Cytoscape (https://cytoscape.org/)
- yFiles Layout Algorithms for Cytoscape (https://www.yworks.com/products/yfiles-layout-algorithms-for-cytoscape)
- antiSMASH (https://antismash.secondarymetabolites.org/)

2.2 Constructing an SSN

1. Obtain the sequence of GrbD (Accession code WP_006051176.1) or GrbE (Accession code WP_006051175.1) on NCBI website.
2. To create an SSN, first go to EFI – EST website.
3. Copy and paste the protein sequence into “Query Sequence” under “Sequence BLAST”.
4. Edit UniProt BLAST query e-value under “BLAST Retrieval Options. The default value is set to 5. Increasing the e-value can help to retrieve more divergent homologs; decreasing the e-value gives more similar homologs. The value was set to 5 for creating SSN for GrbD (Fig. 2).
5. Edit other parameters (e.g., maximum number of sequences retrieved, sequence database, etc), if necessary.
6. Enter job name and email address to receive job updates by email.

7. There will be one email notification for successfully submitting the job and another email after the initial calculation is complete. Click on the link to finalize the SSN.
8. Go to the “Dataset analysis” tab and look for “percent identity vs. alignment score” graph. The alignment score corresponding to 35% percent identity is a good starting point to enter in “Alignment Score Threshold” under “SSN Finalization.” The higher the alignment score, more smaller clusters will be obtained; when the alignment score is lower, there will be less clusters and the clusters will be bigger. For finalizing SSN of GrbD, 80 was entered.
9. An email is sent when the SSN is completed and available for download. Click on the link in the email and go to “Network Files”.
10. Download the full network or representative node networks. In the representative node networks, the sequences with higher than a certain percent identity are put into the same node. The computer will process the SSN faster with fewer nodes in it.
11. Unzip the file and drag the .xgmml file into Cytoscape, which is an open-source software platform to visualize various networks.
12. Under “Layout”, choose “yFiles Organic Layout”.
13. Analyze the clusters; look for the cluster of which the sequences in BGCs of characterized diazeniumdiolate siderophores are in. Adjust the parameters to create SSN if necessary.
14. Taking the SSN of GrbD as an example (Fig. 2), the GrbD homologs which are in BGCs of characterized diazeniumdiolate siderophores are in the same cluster, and

SznF, the N-N bond forming enzyme of streptozotocin, which does not have a diazeniumdiolate group, is in a separate cluster. Thus, the sequences in the former cluster are candidate GrbD homologs involved in the biosynthesis of diazeniumdiolate siderophores.

Place Figure 2 here

Fig. 2 SSN of GrbD (e-value = 5, alignment score threshold = 80)

2.3 Identifying bacterial strains of interest:

1. Find the NCBI IDs of the sequence of interest in SSN.
2. Search for the NCBI ID on NCBI website.
3. Choose “Identical Protein Groups”; look for the bacteria strain name.
4. Search for the bacterial strain name on NCBI website; choose “Nucleotide.”
5. Acquire the accession code (“Accession:”) under the entry name containing “whole genome shotgun sequencing project.” Sort the results by sequence length at the bottom of this page.
6. In antiSMASH, select “Get from NCBI”; enter the accession code. Change parameters if necessary and submit the job.
7. After the job is completed, download the log file by clicking “Download” at the top right of the page and recheck results in a certain period of time without re-running the job. To recheck results, enter the log file name under “Results for existing job” at the start page.

8. Go through the predicted regions and look for the BGC candidates which may produce new diazeniumdiolate siderophores. The predicted type of the region is usually “NRPS” or “NRP-metallophore”, and sometimes the name of the most similar known cluster contains the name of a characterized diazeniumdiolate siderophore. Click on regions of interest.
9. Look for the GrbD homolog – the homolog of GrbD can usually be found since the strain information is obtained from the SSN created for GrbD. At the bottom half of the page, check through the list of genes. GrbD homolog is usually labeled as “hypothetical protein” or “peptide synthetase”, the function is “biosynthetic”, and the amino acid sequence length is between 450 to 500. Click on the possible gene, the “Gene details” at the top right of the page contains more information on this gene, in which “GrbD” is usually found.
10. Look for GrbE homolog – the homolog of GrbE is usually next to the GrbD homolog or very close to it. It is sometimes a stand-alone gene, predicted to be either “YqcI/YcgG protein” or “hypothetical protein”, and in other cases fuses with a NRPS. The amino acid sequence length is around 290. If it is fused with a NRPS, “GrbE” will be listed in the “Gene details” of that NRPS.
11. To check the prediction of the amino acid building blocks of this possible siderophore, click on “NRPS/PSK” modules at the bottom half of the page. Compare the predictions to the characterized siderophores to check its novelty. A “CAL” (CoA ligase) module suggests that this possible siderophore may have a fatty acid tail besides the peptide backbone. If a module is predicted to be “X” (or “D-X”),

click on “NRPS/PKS substrates” at the middle-right of the page, and click on “+” and check the prediction of this amino acid. If the prediction is Gra (specificity code DVHRTGLVAK), this may be a new diazeniumdiolate siderophore.

12. Some bacteria strains can be obtained from sources like DSMZ (https://www.dsmz.de/), ATCC (https://www.atcc.org/), etc.

3. Bacterial growth conditions for production of siderophores

3.1 Materials

3.1.1 Equipment

- Freezer (-80°C)
- Autoclave
- Orbital shaking incubator
- UV-Visible spectrophotometer

3.1.2 Reagents and supplies

- Large Erlenmeyer or Fernbach flask for bacterial culturing
- Hydrochloric acid (4 M)
- Ultrapure (e.g., MilliQ, 18Ω) water
- Growth nutrients and salts
- Magnetic stirrer
- Stir bar
- Aluminum foil
- Ethanol to sterilize surfaces and gloves

- Bunsen burner to maintain sterile conditions
- Large petri dishes
- Agar
- Luria-Bertani (LB) broth base or other nutrient-dense broth base
- Long-term storage cryogenic tubes for freezer stocks
- Sterile culture tubes
- Inoculation loops
- Plastic cuvettes
- Sterile pipette tips
- Micropipette

3.2 Protocols for bacterial culturing

Once bacterial strains have been selected and obtained, prepare glycerol freezer stocks as instructed by the bacterial supplier. Store the glycerol stocks at -80°C.

All bacteria should be grown up in media, temperature, and pH conditions best suited for the specific strain. Bacterial growth conditions are usually listed on the purchasing site.

3.2.1 Preparation of iron-deficient media

Siderophore production is stimulated by iron deficiency. To achieve iron-limited conditions, all glassware used for growth should first be rinsed with 4 M hydrochloric acid to remove adsorbed Fe(III). Bacterial media should provide the nutrients necessary for the specific strain to grow but should not include a source of iron. Small batch (~150-500 mL)

cultures can be grown to test media conditions before selecting a growth medium that is suitable for growth. For terrestrial bacteria that produce diazeniumdiolate siderophores, iron-deficient M9 minimal medium is a good place to start, and supplementation with sodium pyruvate may accelerate growth. To grow marine bacteria, artificial seawater lacking iron can be used, though some adjustments to specific salt concentrations may be necessary.

1. Rinse an Erlenmeyer or Fernbach flask twice the volume of desired culture (i.e., for a 1 L culture, use a 2 L flask) with 4 M HCl. Rinse out excess acid with ultrapure (e.g., MilliQ, 18Ω) water.
2. Add all but 10 mL of the intended final volume of ultrapure water. The remaining water will be used to rinse excess salt off of the side of the flask.
3. Add salts and other nutrients.
4. Rinse inside of the flask to ensure all salts are in solution.
5. Stir the media until salts are mostly dissolved.
6. Cover the flask with aluminum foil.
7. Autoclave for 20 minutes at 121°C to sterilize.
8. Once the media has cooled to room temperature, add remaining salts and nutrients as needed (e.g., steri-filtered glucose solution) under sterile conditions.
9. Allow media to stand at room temperature for at least 24 hours prior to inoculation; an increase in turbidity of the medium may indicate bacterial contamination.

3.2.2 Culture inoculation and monitoring

1. Under sterile conditions, streak bacteria on a nutrient-rich agar plate. Incubate the plate at the temperature suggested for ideal growth conditions (often approximately 30°C) until single colonies are visible. Store plate at 4°C until use.
2. To make a starter culture, dispense desired volume of liquid nutrient-dense broth such as Luria-Bertani (LB) broth or tryptic soy broth (TSB) into a sterile culture tube (for a 1 L culture, prepare a 5-7 mL starter culture).
3. Under sterile conditions, use an inoculation loop to transfer a single colony from the plate into the liquid nutrient broth and briefly stir.
4. Incubate the starter culture at suggested temperature while shaking at 180 rpm.
5. Once the starter culture is cloudy, decant the starter culture under sterile conditions into the prepared growth media solution. Cover, and incubate at suggested temperature while shaking at 180 rpm.

3.2.3 Measurement of optical density

Microbial growth can be monitored by measuring the optical density (OD_{600nm}), or the degree of light scattering caused by the bacteria in the culture, at 600 nm. This wavelength is chosen because it is minimally damaging to the suspended cells. To maximize siderophore production, it is advisable to track the OD_{600nm} of the culture throughout the growing period, noting the time since inoculation, to obtain a growth curve. Bacterial growth curves typically progress through a series of consecutive phases including lag phase, log phase, stationary phase, and decline. For maximal siderophore yield, bacterial cultures should be grown until late log/early stationary phase.

1. Swirl bacterial culture to suspend cells.
2. Under sterile conditions, pipette 1 mL of bacterial culture into a cuvette. Cover culture flask and return to incubation conditions.
3. Measure the optical density at 600 nm with a spectrophotometer.
4. Generally, an OD_{600} absorbance of around 1.0 au indicates that a culture has reached late log/early stationary phase.

4. Detection and isolation of the siderophores

4.1 Materials

4.1.1 Equipment

- Top loading balance scale to balance centrifuge bottle masses
- High speed centrifuge that can accommodate at least 1 L total volume
- Refrigerator (4°C)
- Freezer (-20°C)
- Orbital shaker
- Rotary evaporator
- Liquid chromatography system for semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC) equipped with a UV-Visible detector
- C18-AQ, 20 x 250 mm HPLC Column
- Ultra-performance liquid chromatography/mass spectrometry (UPLC-MS, Waters Xevo G2-XS QTOF with positive mode electrospray ionization coupled to an AQUITY UPLC-H-Class system) instrument

- Lyophilizer
- Analytical balance scale to determine yield

4.1.2 Reagents and supplies

- Ultrapure water
- Hexadecyltrimethylammonium bromide (HDTMA)
- Iron chloride hexahydrate ($FeCl_3 \cdot 6H_2O$)
- Hydrochloric acid
- Chrome azurol S (CAS)
- 0.5 M piperazine buffer (pH 5.6)
- 96 well plate
- Micropipette
- Sterile pipette tips
- Ethanol to sterilize surfaces
- Bunsen burner to maintain sterile conditions
- Centrifuge bottles
- Bleach
- Large Erlenmeyer or Fernbach flasks
- Amberlite™ XAD-4 resin
- Borosilicate glass funnel
- Stainless-steel filter
- HPLC grade methanol

- Glass chromatography column (diameter ≈ 2.5 cm) equipped with base fitted with cheesecloth filter
- 1L round-bottom flask
- 20-60 mL plastic syringe
- 20 µm syringe filter
- 50 mL centrifuge tubes
- Trifluoroacetic acid
- 1 mL Hamilton syringe
- Acetonitrile (CAN)
- Formic acid
- Polypropylene beaker
- Liquid nitrogen

4.2 CAS assay for general siderophore detection

Siderophores in a growing bacterial culture can be detected *via* the Chrome Azurol S (CAS) assay (Schwyn & Neilands, 1987). This colorimetric method results in a color change when siderophores scavenge Fe(III) from an Fe-CAS-hexadecyltrimethylammonium bromide complex (blue), liberating the CAS dye (red-orange, positive assay response). CAS assays can be performed on agar plates, with the Fe-CAS complex added to the plate medium, or in solution, as detailed here. Unlike agar plate CAS assays, solution CAS assays can provide a quantitative siderophore production measurement. A positive CAS assay response is a simple way to rapidly test for the presence of siderophores. The CAS assay

will only detect the presence of metal-free, or apo, siderophores. Perform a CAS assay approximately once per day during bacterial growth to monitor siderophore production. It is convenient to perform the CAS assay at the same time as measuring the optical density, using the separated aliquot of culture solution (see above section, “Optical density”).

4.2.1 Preparation of Fe-CAS-hexadecyltrimethylammonium bromide solution

1. Combine the following solutions to achieve final assay conditions of 15 µM $FeCl_3$, 150 µM CAS, 600 µM HDTMA, in 0.5 M piperazine buffer (pH 5.6).
    a. 1.202 mM hexadecyltrimethylammonium bromide (HDTMA) solution
    b. 1 mM $FeCl_3 \cdot 6H_2O$ (4.2 mM HCl)
    c. 2 mM Chrome azurol S in ultrapure water.
    d. Piperazine buffer solution (0.5 M, pH 5.6).
2. Solution will be maroon. Store at room temperature overnight. Solution should be blue within 24 hours.

4.2.2 CAS Assay procedure

1. Dispense 200 µL bacterial culture into one well of a 96 well plate.
2. Add 100 µL ferric CAS solution, using the micropipette to gently mix the solutions.
3. Wait up to 24 hours. Diazeniumdiolate siderophores often exhibit a color change from blue to red within 20 minutes, while hydroxamate siderophores scavenge Fe(III) from the Fe-CAS complex more slowly.

4.3 Extraction of siderophores

Once the culture has reached late log/early stationary phase and is CAS positive, the supernatant can be separated from the cells by centrifugation. Siderophores are excreted from the bacterial cells, so they are present in the supernatant solution. Note that some siderophores have fatty acid tails, and they may adhere to the cell pellet, requiring further methanolic extraction. To extract low molecular weight organic compounds, including siderophores, from the supernatant solution, Amberlite XAD-4 polymeric resin can be utilized as detailed below.

4.3.1 Solid phase extraction of siderophores from bacterial culture

2. Pour bacterial culture evenly into centrifuge bottles, ensuring that the masses of each bottle are balanced. Centrifuge the culture at 6000 rpm and 4°C.
3. Decant the supernatant solution into an acid-washed flask. Unless further extraction of the cell pellet is needed, the cell pellets can be bleached and discarded.
4. Prepare fresh XAD-4 resin:
    a. Pour about 300-400 g (300-400 mL volume) dry XAD-4 resin into a funnel fitted with a screen, over a large flask to collect rinse waste.
    b. Rinse resin with 3 L ultrapure water. Dispose of rinse waste, but do not remove resin from the filter funnel.
    c. Repeat with 3 L methanol, then 1 L ultrapure water, and then 1 L methanol.
    d. Store resin in methanol at 4°C until use.
5. Prepare XAD-4 resin for use:
    a. Pour XAD-4 resin into the glass funnel and flask.

b. If reusing XAD-4 resin, rinse with 400 mL methanol to remove any residual contaminants. If using freshly prepared resin, skip ahead to the next step.
c. Rinse resin with 1 L ultrapure water.

6. With ultrapure water, rinse about 100 g (or 100 mL volume) per liter of culture XAD-4 resin into the flask containing the supernatant solution.
7. Place the flask on an orbital shaker at 150 rpm at room temperature and shake for 2-4 hours.
8. Remove the flask from the shaker. Swirl contents to create a slurry. Pour supernatant and XAD-4 resin into the borosilicate glass funnel and flask.
9. Rinse with 1 L ultrapure water. Bleach and then dispose of the filtrate.
10. Preparation of column:
    a. Rinse a glass chromatography column (diameter ≈ 2.5 cm) and collection flask (a 1 L round bottom flask works well) with 4 M HCl.
    b. Rinse column and flask with ultrapure water and then with methanol.
    c. Attach column base fitted with a cheesecloth filter.
    d. Replace collection flask with a waste container.
11. Running the column extraction:
    a. Pack the column: use ultrapure water to transfer the rinsed XAD resin into the column. Allow excess water to drip into the waste container.
    b. Once drips have slowed, replace the waste container with the collection flask.
    c. Pour 250 mL 100% methanol into the column to extract adsorbed organic compounds. (Note: The concentration of methanol can be adjusted to the

specific elution conditions of the siderophore of interest. Use HPLC elution time, as described below, to estimate an appropriate eluent concentration).

d. Collect the eluate. Remove the collection flask and replace it with the waste container.
e. To rinse XAD resin, add 50-100 mL 100% methanol to the column. Once all of the solvent has passed through the column, use methanol to transfer the XAD back into its storage container for future use. New XAD resin should be prepared for different bacterial strains, however the existing XAD resin may be reused many times for the same bacterial strain.

12. Add 30 mL of water to the eluate.
13. Concentrate the extracted eluate solution to about 15-30 mL by rotary evaporation, avoiding water bath temperatures above 40°C. Use a plastic syringe with a 20 µm syringe filter to filter and transfer the concentrated extract to a 50 mL centrifuge tube. Store extract at -20°C until HPLC separation. Thaw extract before use.

4.4 Purification of siderophores

Once an extract has been prepared, the siderophore can be isolated by semipreparative reverse phase high performance liquid chromatography (HPLC) with a 20 x 250 mm C18-AQ column and UV-Visible detector.

For diazeniumdiolate siderophores, it is advisable to monitor the absorbance of HPLC injections at the peptide bond absorption wavelength, 215 nm, as well as the diazeniumdiolate bond absorption wavelength, 250 nm. The eluted compounds can be

analyzed by UPLC-MS to determine the peak which corresponds to the siderophore of interest.

4.4.1 Collecting fractions and identifying the siderophore HPLC peak

1. Prepare 3-4 L each of:
    a. Ultrapure water (0.05% trifluoroacetic acid)
    b. HPLC grade methanol (0.05% trifluoroacetic acid)
2. To develop a new HPLC method, start by running a linear gradient of 10% methanol to 80% methanol over 40 minutes.
3. Inject a small volume (<100 µL) of the extract as a test run.
4. Adjust the method gradient to maximize the peak separation.
5. Inject a larger volume of the extract to achieve high absorbance intensities, without surpassing the detector maximum. Isolate appropriate fractions based on their absorption at 215 and 250 nm.
6. Concentrate fractions to about 10% of their original volume.
7. To test for the presence of general siderophores, CAS assays can be employed following the same procedure as above.
8. Run the concentrated fractions on UPLC-MS. Depending on the system, a linear gradient of 0-100% ACN over ten minutes should be effective for siderophore analysis.
9. See the mass spectrometry section below for siderophore characterization information.

10. Once a potential siderophore mass has been identified, continue to use HPLC separation to collect the peak of interest as it elutes.
11. Concentrate the collected fractions to <10 mL by rotary evaporation.
12. If necessary, ultra-purify the concentrated fraction by repeating steps 10 and 11.

4.4.2 Lyophilization

Upon HPLC ultra-purification of the siderophore, the collected fractions can be combined, concentrated, and dried to yield a solid.

1. Combine ultra-purified fractions in a round bottom flask at least twice the total volume of the combined fractions.
2. Concentrate to a final volume of 5-10 mL by rotary evaporation, avoiding water bath temperatures above 40°C.
3. Transfer concentrated solution into a pre-weighed 50 mL centrifuge tube. Do not seal the tube.
4. In a polypropylene beaker, flash freeze the contents of the tube in liquid nitrogen.
5. Lyophilize for 24-48 hours or until the sample is a fluffy solid (usually white; yellow solids can indicate the presence of ferric siderophores). A 1 L culture will generally yield 3-15 mg of siderophore, though this yield varies based on the strain and growth conditions.

5. Identification and characterization of Graminine-containing siderophores

C-Diazeniumdiolate siderophores are readily identifiable by their pH-dependent absorbance band (in gramibactin: λ_{max} = 248 nm, pH 8; λ_{max} = 220 nm, pH 2 and by their

characteristic MS ionization pattern (i.e., loss of 30 mu corresponding to the ionization-induced loss of ^{14}NO) (Makris et al., 2022). Upon identifying a diazeniumdiolate siderophore, UPLC-ESI-MS, Marfey's amino acid analysis, and NMR spectroscopy can be employed to characterize the compound's structure.

5.1 Materials

5.1.1 Equipment

- Analytical balance scale
- UPLC-MS (Waters Xevo G2-XS QTOF with positive mode electrospray ionization coupled to an AQUITY UPLC-H-Class system)
- Waters BEH C18 UPLC column
- Liquid chromatography system for analytical reversed-phase high-performance liquid chromatography (RP-HPLC)
- Oven (110 °C)
- Freezer (-20 °C)
- High field (e.g., 500 MHz) nuclear magnetic resonance (NMR) instrument
- UV-Visible spectrophotometer

5.1.2 Reagents and supplies

- $^{15}NH_4Cl$ or other source of ^{15}N
- 99.8% NMR-grade D_2O
- Ultrapure water
- Glass ampoules

- Micropipette
- Pipette tips
- Hydrochloric acid
- Propane tank
- Tweezers
- 1.5 mL centrifuge tubes
- Heated water bath (40°C)
- Amino acid standards of all relevant stereochemical configurations
- Marfey's reagent
- Acetone
- Sodium bicarbonate ($NaHCO_3$)
- Triethylamine
- Phosphoric acid
- Acetonitrile
- 150 μL Hamilton syringe
- Formic acid
- 5 mM aqueous buffer (MOPS or phosphate, pH 8.0)
- 3 mL quartz cuvette
- 75 mm stir bar
- Mercury (argon) spectral calibration pen lamp
- Magnetic stirrer
- NMR tube

- Quartz NMR tube
- Deuterated dimethyl sulfoxide (DMSO-d_6)
- 5 mM phosphate buffer in D_2O, pD 8.0

5.2 Mass spectrometry and stable isotope labeling

The mass of the siderophore, in conjunction with the NRPS prediction of the structure, are a starting point for the structural characterization of the compound. In the UPLC-MS chromatogram, search for a peak whose mass is in the range of the mass of the NRPS-predicted structure. Siderophores often will display an *m/z* $[M - 2H + Fe]^+$ ionized mass, corresponding to the Fe(III)-siderophore complex. For each diazeniumdiolate ligand present, the siderophore mass spectrum will exhibit a characteristic *m/z* $[M + H - 30]^+$ ion corresponding to a mass loss of 30 Da consistent with ionization of the N–N bond in the Gra residue. Note that the formula mass may include a sodium adduct ion (*m/z* $[M + Na]^+$).

5.2.1 ^{15}N labeling

The number of nitrogen atoms present in the molecule can be determined by stable isotope labeling. To achieve a fully ^{15}N-labeled siderophore structure, a new culture should be grown, replacing the nitrogen source with ^{15}N (e.g., in M9 media, using $^{15}NH_4Cl$ instead of $^{14}NH_4Cl$ as the sole nitrogen source), so that all nitrogen atoms incorporated into the bacterial metabolites are isotopically labeled. Repeat the culturing and isolation methods, using new XAD-4 resin for the solid phase extraction of organic compounds. Note that ^{15}N-labeled cultures often grow slightly slower. Once the labeled siderophore has been isolated, it should be run on MS under the same conditions as the unlabeled ^{14}N

siderophore. The difference in mass between the labeled and unlabeled siderophore ($M_{labeled}$–$M_{unlabeled}$) is equivalent to the number of nitrogen atoms present in the structure, which provides valuable information about the number and structure of the constituent residues.

5.2.2 Deuterium(^{2}H) labeling

Similarly to the ^{15}N-labeling detailed above, deuterium exchange can be employed to determine the number of exchangeable protons in the siderophore structure. Exchangeable protons are those which are bound to heteroatoms.

1. Dissolve a small (spatula-tip's worth) aliquot of solid siderophore in 100 µL 99.8% NMR-grade D_2O.
2. Lyophilize to dryness.
3. Add a second aliquot of 100 µL 99.8% NMR-grade D_2O. Lyophilize.
4. Repeat step 3 four more times. Exchangeable protons should now have been replaced by deuterium atoms.
5. Analyze the deuterated siderophore by direct injection mass spectrometry.

5.2.3 Tandem MS/MS

Tandem MS/MS can serve an important role in the structural characterization of linear siderophores (Pluháček et al., 2016). In many cases, however, diazeniumdiolate-containing siderophores are relatively large and often cyclic, convoluting the MS/MS fragmentation pattern. It is therefore recommended to use MS/MS as a useful check for the determined structure, rather than the primary method of characterization. NMR

spectroscopy is much more elucidating in these cases. See below for NMR spectroscopic structural characterization details.

5.3 Marfey's amino acid analysis

To unambiguously determine the stereocenter configuration of the amino acid building blocks of the siderophore, Marfey's analysis is performed (Marfey, 1984). HCl is used to hydrolyze the siderophore at elevated temperature, into component L- and D-amino acids. The amino acids in the hydrolysate are derivatized with Marfey's reagent (i.e., 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide), forming diastereomers which can be separated. Analytical HPLC and/or UPLC-MS can then be used to analyze the derivatized hydrolysate in comparison to amino acid standards. The presence of an amino acid with a certain stereo-configuration is indicated by superposition of the peak of the derivatized hydrolysate and the derivatized standard amino acid when co-injected.

5.3.1 Hydrolysis of the siderophore

1. Dissolve 1 mg siderophore in 200 µL ultrapure water.
2. Divide the solution into two glass ampoules.
3. Add 100 µL concentrated HCl to each ampoule to achieve a final concentration of 6 M HCl.
4. Seal ampoules using a propane tank and tweezers.
5. Place the sealed ampoules in an oven at 110°C for 20-24 hours. The solution will be light yellow.

6. Crack open the ampules and combine the contents in a small (1.5 mL) plastic centrifuge tube.
7. Use a hot water bath at 40°C and gentle air flow to dry the hydrolysate. When nearly dry, add 700 µL ultrapure water. Repeat the evaporation and water addition three times.
8. After final water addition, allow the hydrolysate to evaporate to dryness. Store the dry hydrolysate at -20°C until use.

5.3.2 Derivatization of the hydrolysate and amino acid standards

1. Dissolve the siderophore hydrolysate and amino acid standards in ultrapure water to yield 1 mg/100 µL solutions of each.
2. To each solution, add 150 µL of 5 mM Marfey's reagent in acetone and 20 µL of 1 M $NaHCO_3$.
3. Heat the solutions in a hot water bath at 40°C for one hour.
4. Quench the reaction by adding 15 µL of 4 M HCl to each sample. The solutions should be bright yellow.
5. Dilute each solution with 100 µL ultrapure water.
6. Store solutions at -20°C until use.

5.3.3 Analytical HPLC

1. Prepare the solvents

a. Solvent A (50 mM triethylamine phosphate, pH 3.0): To 494.84 mL ultrapure water, add 3.47 mL TEA (triethylamine) and 1.69 mL phosphoric acid. Adjust to pH 3.0 with phosphoric acid.
b. Solvent B (Acetonitrile)

2. Prepare the solution to dilute samples before injection (dilution solution – v:v 10 acetone: 9 H_2O).
3. Analytical HPLC method on a C18 column (250 × 4.6 mm YMC C18-AQ column) – 10% to 40% solvent B over 45 minutes.
4. With a Hamilton syringe, inject derivatized amino acid standards (30 µL standard + 45 µL dilution solution), derivatized siderophore hydrolysate (30 µL hydrolysate + 45 µL dilution solution) respectively, and separately co-inject the two solutions (30 µL standard + 30 µL hydrolysate + 15 µL dilution solution). The absorbance is monitored at 215 nm and 340 nm.
5. In the resulting HPLC traces, look for the unreacted Marfey's reagent peak, the standard amino acid peaks, and peak increase in the co-injection to determine the chirality of amino acids in the siderophore.

5.3.4 UPLC-MS

1. The amino acid standards, siderophore hydrolysate, and a mixture of these two, can also be run on a UPLC-MS (Waters Xevo G2-XS QTOF with positive mode electrospray ionization coupled to an AQUITY UPLC-H-Class system with a Waters BEH C18 column) to confirm the identity of the peaks.
2. Solvent A – ultrapure water + 0.1% formic acid

3. Solvent B – Acetonitrile + 0.1% formic acid
4. LC method – 15% to 50% Solvent B over 15 minutes

5.4 Photoreactivity of the diazeniumdiolate group

Diazeniumdiolate-containing siderophores are photoactive, absorbing at approximately 250 nm at pH 8. The diazeniumdiolate absorbance band is pH dependent, shifting to shorter wavelengths at lower pH ($\lambda_{max,\ pH\ 8} \approx 250$ nm shifts to $\lambda_{max,\ pH\ 2} \approx 220$ nm) as the ligand is protonated, as is characteristic of the diazeniumdiolate (*N*-nitrosohydroxylamine) group (Gama et al., 2021). The diazeniumdiolate absorbance band of a siderophore can be determined by dissolving the purified siderophore into an aqueous buffer solution (pH 8) and measuring its absorbance by UV-Visible spectrophotometry. Note: Choosing an appropriate aqueous buffer will affect the results of any spectrophotometric analyses because many buffers absorb in the 250 nm range.

UV irradiation destroys the diazeniumdiolate group, causing each Gra residue in the siderophore to lose the equivalent of NO + H. The photolysis reaction of the siderophore can be monitored spectrophotometrically, and its reaction products can be analyzed by UPLC-mass spectrometry and/or NMR spectroscopy. (Note: Direct UV radiation exposure is harmful, so photolysis studies are best performed in a photolysis box or under a closed fume hood with the glass covered.)

5.4.1 Photolysis of diazeniumdiolate siderophores

1. Dissolve siderophore sample in aqueous buffer (e.g., 5 mM MOPS or phosphate buffer, pH 8.0).
2. Transfer solution to a 3 mL quartz cuvette, with a 75 mm stir bar.
3. Measure the absorbance of the "dark" solution before photolysis.
4. In timed intervals, expose the solution to UV light using an Hg(Ar) pen lamp. Keep the cuvette on a stir plate, stirring gently during the photolysis reaction. After each timed interval, measure the absorbance of the solution. The 250 nm band should decrease in absorbance intensity as the photolysis reaction progresses.
5. Analyze the photoproduct by UPLC-MS.
6. *For NMR analysis of the photoproduct, use 99.9% purity D_2O instead of ultrapure H_2O, buffered at pD 8.0 and carry out the photolysis in a quartz NMR tube.

[Insert Figure 3 here]

Fig. 3 UV photolysis of apo-Gbt. (a) 254 nm irradiation of apo-Gbt (44 μM) over 6 h in 5 mM MOPS pH 8.0). Inset shows the isosbestic point over the first hour of photolysis; (b) MS of apo-Gbt; (c) MS of the photoproduct of Gbt (Makris et al., 2022).

5.4.2 MS detection of NO mass loss

The photolysis of diazeniumdiolate siderophores forms *E/Z* oxime photoproducts, which differ from the unphotolyzed siderophore by 31 mass units per Gra residue (Hermenau et

al., 2018). For a siderophore that contains two Gra residues, like gramibactin, the photoproduct will therefore be 62 mass units lower than the unphotolyzed gramibactin mass (Gbt *m/z* 835 $[M+H]^+$).

[Insert Figure 4 here]

Fig. 4 Photolysis scheme of gramibactin (Gbt). ^{14}N-Gra-enriched-Gbt ehibits a mass loss of 62 in the photoproduct. When isotopically labeled, ^{15}N-Gra-enriched-Gbt observes a mass loss of 64 in the photoproduct.

5.5 NMR Spectroscopy characterization of the *C*-diazeniumdiolate and the photoproducts

Complete elucidation of *C*-diazeniumdiolate siderophores is achieved through high field NMR spectroscopy. DMSO-d_6 is a suitable solvent for solubilization and data collection. Characterization of *C*-diazeniumdiolate siderophore photoproducts requires buffered D_2O (50 mM Na_2HPO_4, pD 8.0) as the solvent. Spectra are indirectly referenced by the residual solvent peak or ^{2}H lock.

The *C*-diazeniumdiolate in Gra is conclusively identified through ^{15}N-enrichment of the functional group, as described previously. Gra has a distinct ^{1}H-^{15}N HMBC fingerprint, with correlations of Cδ methylene protons to each ^{15}N in the *C*-diazeniumdiolate and Cγ methylene protons to the proximal hydroxylated ^{15}N only (Fig. 5, adapted from (Makris et al., 2023). Chemical shifts of the proximal and distal ^{15}N residues fall in the range of 316-

317 ppm and 367-368 ppm, respectively. Without ^{15}N isotopic enrichment, Gra shares a similar NMR fingerprint to ornithine. At the Cδ position in Gra, ^{1}H and ^{13}C resonances are observed at approximately 4.1 ppm and 61 ppm in DMSO-d_6.

[Insert Figure 5 here]

Fig. 5 ^{1}H–^{15}N HMBC NMR spectrum of tistrellabactins A and B showing the presence of C-diazeniumdiolate amino acid Gra. Spectrum collected in DMSO-d_6. Figure adapted from Makris et al., 2023.

The *C*-diazeniumdiolate group undergoes a photoreaction in the presence of UV light, yielding *E*/*Z* oxime isomers. The conversion to *E*/*Z* oxime isomers can be followed by NMR, in which the disappearance of Cδ resonances and subsequent appearance of two deshielded protons at 6.9 ppm and 7.5 ppm and ^{13}C resonances in the range of 152-153 ppm are observed (Fig. 6, adapted from (Makris et al., 2022). The transformation is most easily observed with an ^{1}H-^{13}C HSQC experiment, which illustrates the disappearance of ^{1}H-^{13}C Cδ Gra residues and the correlation of new ^{1}H and ^{13}C resonances in the photoproduct. TOCSY NMR also may be used to demonstrate the new ^{1}H resonances in the photoproduct are within the same proton spin system as the other Gra ^{1}H resonances.

[Insert Figure 6 here]

Fig. 6 $^{1}H–^{13}C$ HSQC NMR spectra show the formation of *E* and *Z* oxime isomers upon photolysis of $^{13}C^{15}N$-Gra-enriched-Gbt (pD 8, 6.2 mM, P_i in 99.9% D_2O). (a) $^{1}H–^{13}C\delta$ correlations in Gra residues of apo-Gbt at 4.20 ppm, 61.66 ppm and 4.22 ppm, 61.93 ppm; (b) region of HSQC showing disappearance of the $^{1}H–^{13}C\delta$ correlations in the photoproduct; (c) new downfield $^{1}H–^{13}C$ HSQC correlations in photolyzed $^{13}C^{15}N$-Gra-enriched Gbt, with chemical shifts consistent with *E*/*Z* oxime isomers; (d) scheme showing release of the equivalent of NO + H from Gra yields *E* and *Z* oxime isomers but not a nitroso photoproduct. Figure adapted from Makris et al., 2022.

6. Conclusions and future outlook

The *C*-diazeniumdiolate group is a newly discovered ligand in siderophores, produced mainly by root-associated bacteria, with one known example discovered in a marine bacterial species. Besides binding Fe(III) with high affinity, the diazeniumdiolate group in Gra is also a potential donor of NO, an important biological signaling molecule. Discovery of novel diazeniumdiolate siderophores is of great interest to study bacterial pathogeny, symbiosis between bacteria and other organisms, and the potential of drug design based on the diazeniumdiolate moiety. In this chapter, we describe methods to discover new diazeniumdiolate siderophores *in silico* using bioinformatics tools together with the known information on characterized siderophore BGCs, isolate the siderophores of interest in the lab, structurally characterize this class of siderophores, and study its photoactivity. We anticipate further exciting developments in the future, including elucidation of the biosynthesis of the diazeniumdiolate group in Gra, as well as the

potential incorporation of other diazeniumdiolate-containing amino acids (e.g., alanosine or other new amino acids) into siderophore structures.

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Figure Captions

Fig. 1 Characterized *C*-diazeniumdiolate siderophores. The diazeniumdiolate group is shown in red.

Fig. 2 SSN of GrbD (e-value = 5, alignment score threshold = 80)

Fig. 3 UV photolysis of apo-Gbt. (a) 254 nm irradiation of apo-Gbt (44 μM) over 6 h in 5 mM MOPS pH 8.0). Inset shows the isosbestic point over the first hour of photolysis; (b) MS of apo-Gbt; (c) MS of the photoproduct of Gbt (Makris et al., 2022).

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