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An Alkaloid from Scorpion Venom: Chemical Structure and Synthesis

Shibdas Banerjee,[†,‡] Elumalai Gnanamani,[†] Stephen R. Lynch,[†] Fernando Zamudio Zuñiga,[§] Juana María Jiménez-Vargas,[§] Lourival Domingos Possani,[§] and Richard N. Zare*[,†]

[†]Department of Chemistry, Stanford University, Stanford, California 94305, United States

[‡]Indian Institute of Science Education and Research Tirupati, Karakambadi Road, Tirupati-517507, India

[§]Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autonoma de Mexico, Avenida Universidad 2001, Apartado Postal 510-3, Cuernavaca, Morelos 62210, Mexico

Supporting Information

ABSTRACT: While most scorpion venom components identified in the past are peptidic or proteinic in nature, we report here a new alkaloid isolated from the venom of the Mexican scorpion *Megacormus gertschi*. Nuclear magnetic resonance and mass spectrometric investigations elucidate the structure of the alkaloid as (*Z*)-*N*-(2-(1*H*-imidazol-4-yl)ethyl)-3-(4-hydroxy-3-methoxyphenyl)-2-methoxyacrylamide (**1**). A chemical method of synthesizing this alkaloid is also described. Although abundant in venom, the above alkaloid was not found to have insecticidal activity. Structural analysis suggests that this venom alkaloid might be of potential interest for evaluating its medicinal effect.

Although scorpion envenomation represents a major health problem including death, components from scorpion venom have been used in traditional medicine since the emergence of ancient cultures, mainly in Asia and Africa.[1,2] Recent studies[3] focusing on the therapeutic potential of several venom components from scorpions have heightened our interest to investigate this important natural resource. With the advent of novel methodologies, separation techniques, and biological assays to characterize venom components, it has become evident that along with toxins[4] several biologically active components are present in the scorpion venom, which could be valuable natural products in drug development for the treatment of many diseases including cancer.[3,5−8]

While scorpion venoms are found to be highly complex mixture of peptides, enzymes, mucoproteins, amino acids, nucleotides, lipids, and inorganic salts, mainly proteins are used by scorpions for defense and capture of prey.[3] At least 800 toxic components of proteinic nature are listed in data banks (Pfam and Interprot) of scorpion venoms, but almost nothing is known on the other components that are not made of amino acids.[9] Venom components of scorpions belonging to several families are also poorly understood. With respect to the biodiversity of this arthropod, less than 1% of the total expected number of biomolecules from scorpion venom is known to date.[3] In this regard, venom from the Mexican scorpion *Megacormus gertschi* was recently studied[10] as a first approach to examine the venom components from a species belonging to the family Euscorpiidae. The data certainly showed that this venom is distinctly different from all other scorpion species described so far in the literature. Surprisingly, the venom of *M. gertschi* was found to be largely composed of a mysterious molecular species of low molecular weight (317 Da), the structure of which remained unknown.[10] As part of an ongoing program aimed at discovering novel bioactive compounds from the venom of *M. gertschi*, we report here the isolation and structural characterization of the aforementioned major venom component. A comprehensive mass spectrometry and nuclear magnetic resonance study revealed the structure as an alkaloid that could be synthesized from vanillin and histamine precursors. A detailed method for chemical synthesis of the above alkaloid is also described here.

RESULTS AND DISCUSSION

High-performance liquid chromatography (HPLC) of the extracted soluble venom from *M. gertschi* enabled us to recover 80 distinct fractions of different molecular species (Figure S1). A fraction that eluted at 21.40 min accounted for nearly 32% of the total light (λ = 230 nm) absorption recorded in the entire chromatogram (Figure S1). Although use of this absorption at 230 nm for relative quantitation of venom components is probably not the best method, the fraction at 21.40 min, being most intense in the chromatogram (Figure S1), is likely to be one of the major components of the soluble venom. Herein, our study is limited to this venom compound. After isolation, this fraction was dried and obtained as a white powder, which was then subjected to an HRMS study.

Figure 1 shows the positive ion mode electrospray ionization (ESI) mass spectrum. We find ion signals of the singly protonated monomer (m/z 318.1450) and the noncovalent dimer (m/z 635.2828) of the venom compound in the gas phase. A high mass accuracy and isotopic distribution pattern (Figure 1) suggests the chemical formula $C_{16}H_{19}N_3O_4$ for the

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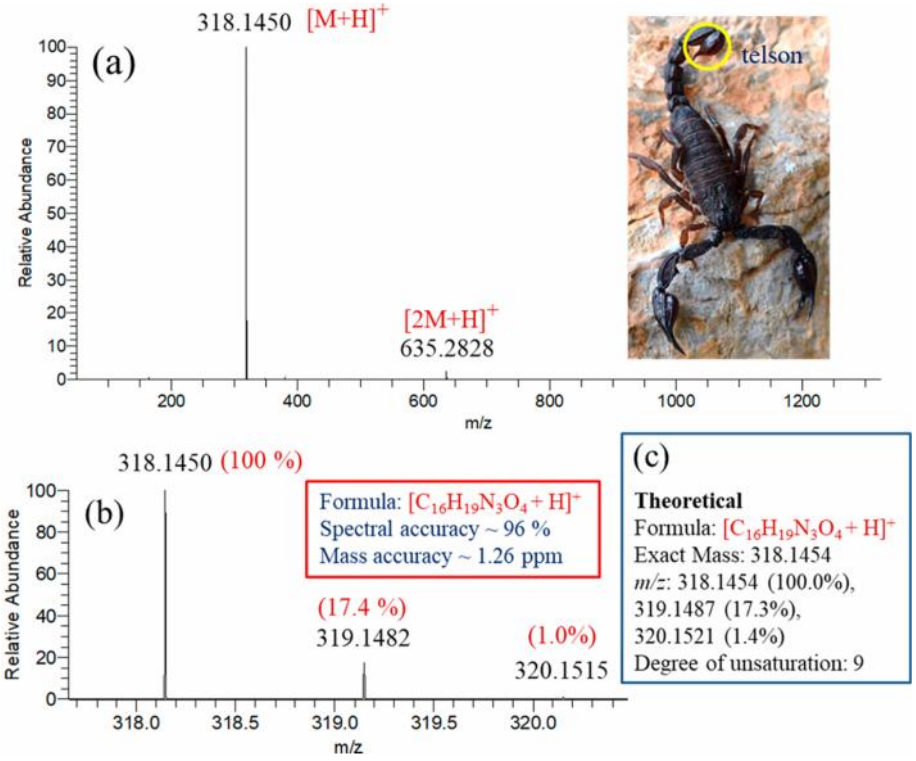

Figure 1. High-resolution positive ion mode ESI mass spectrum of the methanolic solution of the venom compound from *M. gertschi* (shown in the inset of (a)), eluted at the retention time 21.40 min from HPLC (Figure S1), showing its (a) protonated ion signals and (b) isotopic distribution of the protonated ion signals of $[M + H]^+$. Inset of (b) suggests the empirical formula $C_{16}H_{19}N_3O_4$, with high accuracy for the venom component, which corresponds well with the theoretical *m/z* and isotopic distribution of the protonated species (c).

compound, which was further accurately (mass accuracy 0.094 ppm) verified by calibrating the mass spectrum with an internal standard (Figure S2). The molecular formula indicates nine degrees of unsaturation in the chemical structure. The high ion current ($\sim 10^8$) of the protonated species in the mass spectrum (Figure 1) suggests that the compound is basic; however, the negative result in the ninhydrin test indicated that there is no primary amine in the structure (data not shown). A hydrogen−deuterium exchange (HDX) experiment with the compound detected three intrinsic exchangeable protons (e.g., −OH, −NH) in the molecule (Figure S3). The ion signal of the deprotonated species in the negative ion mode ESI mass spectrum (Figure S4) was found to be very weak, informing us that there are no strong acid functional groups in the molecule.

After acquiring preliminary information about the molecular structure of the venom compound from mass spectrometry, we made a detailed investigation of the compound using various nuclear magnetic resonance (NMR) spectroscopic techniques. Use of ^{1}H NMR, COSY, HSQC, HMBC, and NOE collectively suggested a structure of the venom compound as shown in Figure 2, which is similar but not identical to the natural product feruloylhistamine found in ephedra roots.[11] ^{1}H NMR spectra recorded in methanol-d_4 and DMSO-d_6 indicated the presence of one phenolic −OH, two −OMe, one $-CH_2-CH_2-$ moiety, three exchangeable protons, and the possibility of an imidazole ring in the molecule (Figures S5 and S6). Study with gCOSY (Figure S7) showed the ^{1}H−^{1}H coupling correlation in the molecule. The ^{13}C NMR could not be recorded with the small amount of wild-type compound (~1 mg) available to us. However, we recorded the individual carbon chemical shifts from HMBC and HSQC experiments

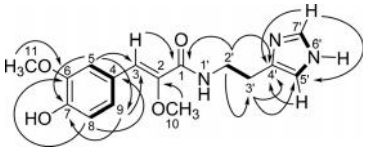

Figure 2. Structure of the extracted venom component **1** determined by NMR experiments showing observed COSY in bold lines and key HMBC correlations in arrows.

(*vide infra*). Detailed analysis of carbon−hydrogen correlations in the HSQC and HMBC spectra revealed that compound **1** is alkaloid in nature, containing histamine and vanillic moieties connected through a 2-methoxyacrylamide bridge (Figure 2, Table 1). An NOE study also revealed the proximity of two aromatic protons (δ_H 7.36 for H-5 and 7.14 for H-9) to the −OMe functional group (δ_H 3.6), confirming the *Z*-configuration of the molecule across the olefinic double bond.

The proposed structure (Figure 2) was further verified by tandem mass spectrometry (MS/MS) using collision-induced dissociation.[10,12] For having an amide bond in the structure, the compound was fragmented in the gas phase to produce ladder ions (*a*, *b*, *c* and *x*, *y*, *z*; Figure 3) analogous to peptide bond fragmentation.[12] Accurate measurement of *m/z* values of those different fragments and corresponding neutral loss of CH_3OH, H_2O, CO, and $H_3C^{\bullet}$ further supported the proposed structure (Figure 3).

The proximity (*ortho*-position) of the −OMe functional group to the phenolic −OH is also evident by CID of the deuterated species $[M + D]^+$ in the gas phase (Figure S11). Finally, the structure of this venom alkaloid was unambigu-

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Table 1. Chemical Shifts of Different Protons and Carbons As Labeled in the Structure of 1[a]

position	δ_C,[b] type	δ_H, multiplicity (J in Hz)
1	166, CO	
2	147, C	
3	122, C	6.83, s
4	126, C	
5	125, CH	7.14, dd (8.3, 1.8)
6	115, C	6.80, d (8.3)
7	148, C	
8	148, CH	
9	113, CH	7.36, d (2.0)
10	58, CH_3	3.61, s
11	55, CH_3	3.87, s
1′ (NH)[c]		8.26,[d] t
2′	38, CH_2	3.63, t (6.6)
3′	25, CH_2	3.00, t (6.6)
4′	133, C	
5′	117, CH	7.37, d (1.4)
6′ (NH)[c]		14.06[d], broad singlet
7′	134, CH	8.81, d (1.4)
7 (OH)[c]		9.36,[d] s

[a]The chemical shifts are for the TFA salt of compound **1**. [b]Ascertained from HSQC and HMBC experiments (Figures S8 and S9). [c]Hydrogen−deuterium exchange. [d]Obtained from DMSO-d_6 solvent (Figure S6).

ously confirmed by comparing the NMR and MS data with its synthetic standard (*vide infra*, Figures S12−S16).

Based on retrosynthetic analysis, we began the synthesis of the venom alkaloid **1** from vanillin (Scheme 1). The first step of the synthesis is a Claisen−Schmidt condensation reaction between vanillin (**2**) and methyl-2-methoxy acetate. While several initial approaches using different types of catalytic bases (KOH, NaOMe, Na in dry methanol, *etc.*) failed to make this condensation happen, finally we succeeded using the strong base lithium diisopropylamide (LDA), which was generated *in situ* by reacting *n*-butyllithium and diisopropylamine. LDA catalyzed the above condensation reaction, which was then treated with benzenesulfonyl chloride to form the addition product **3**. This intermediate **3** was subjected to an *in situ* elimination reaction catalyzed by triethylamine base to yield an unsaturated *Z*-carboxylic ester (**4**), which was hydrolyzed in the next step to form the unsaturated carboxylic acid **5**. Finally, the alkaloid **1** was synthesized in 73% yield by amidating the carboxylic acid **5** using histamine. The detailed method of the chemical synthesis of alkaloid **1** is given in the Experimental Section. The purified synthetic alkaloid **1** was characterized using NMR and mass spectrometry, which were compared with that of wild-type venom alkaloid (Figure 2) and found to be identical.

Being one of the major components of venom,[10] we thought that this alkaloid might be used by *M. gertschi* to paralyze their prey. However, when we performed pilot experiments on insecticidal assays by injecting this alkaloid with different dosages (up to 50 μg/animal) in a few crickets, to our surprise, we found that none of the crickets became paralyzed by this alkaloid. This result suggests that this alkaloid, although abundant in venom, is probably not used by the scorpion for paralyzing their prey. At present, the exact biological role of this alkaloid in the venom of *M. gertschi* is far from understood and requires further exploration. However, variations of this

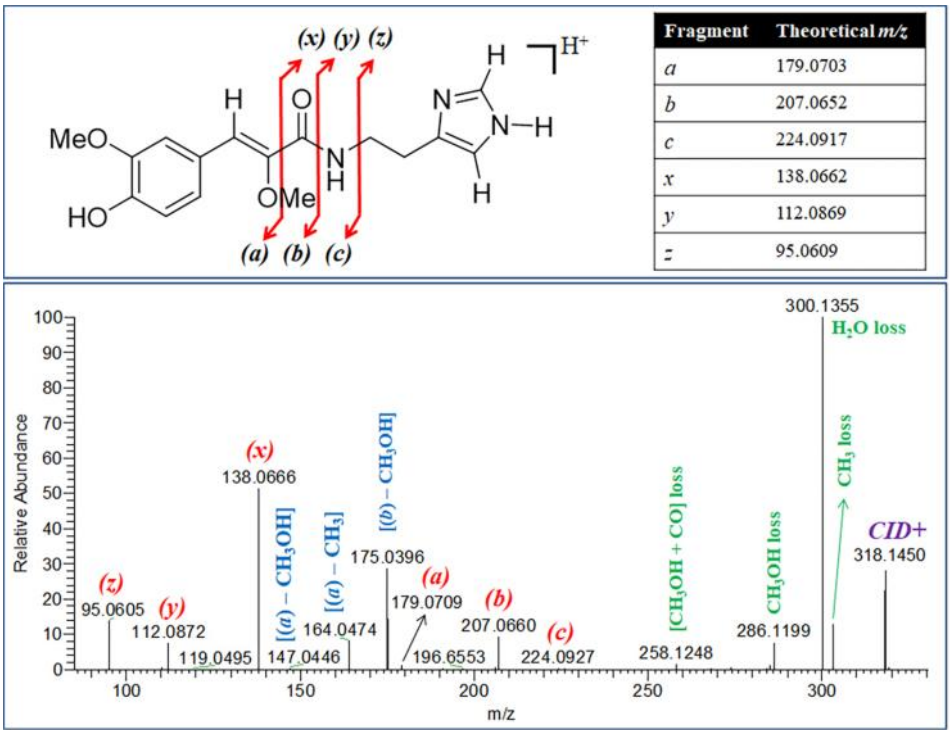

Figure 3. Analogous to the peptide bond fragmentation in CID, the upper panel depicts the anticipated olefinic fragments (a, b, c) and complementary imidazolic fragments (x, y, z) with their theoretical m/z values for the venom component **1**. The lower panel presents the positive ion mode CID spectrum, confirming the structure of the venom alkaloid detected at m/z 318.1450 (Figure 1).

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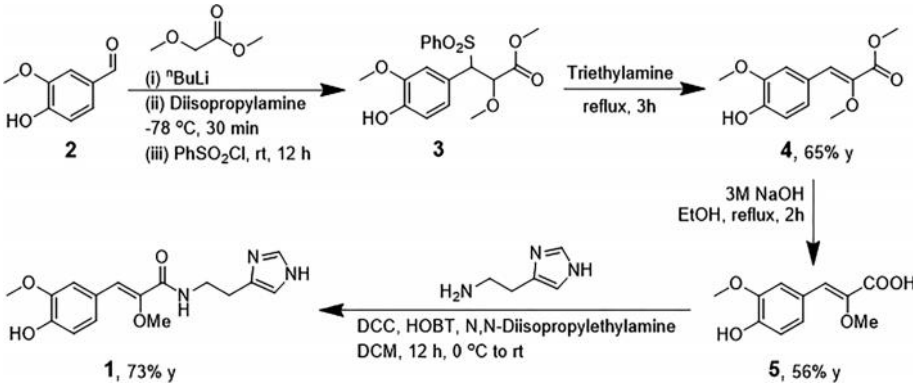
Scheme 1. Chemical Synthesis of the Venom Alkaloid 1



compound have been determined by us to demonstrate antimicrobial activity (unpublished results in progress).

To know whether this alkaloid exclusively exists in the telson (venom bulb) or in any other region of the scorpion, we conducted a mass spectrometric study on the hemolymph extracted from the junction of the femur and patella (Figure S17). No ion signal corresponding to the venom alkaloid (m/z 318.1450; Figure 1) was detected from the hemolymph (Figure S17), suggesting that this alkaloid is probably very selectively produced in the telson of *M. gertschi*. We also found the HPLC profile of the venom from *M. gertschi* maintained alive in the laboratory for 30 days feeding on crickets is identical to that of venom extracted just after collection from the field (data not shown). These two results suggest that this alkaloid originates from the scorpion's metabolic process and not from directly what the scorpion ingests.

In conclusion, we have identified a new alkaloid, (*Z*)-*N*-(2-(1*H*-imidazol-4-yl)ethyl)-3-(4-hydroxy-3-methoxyphenyl)-2-methoxyacrylamide (**1**), from the venom of the scorpion *M. gertschi*. Interestingly, the alkaloid was found to be composed of vanillin and histamine moieties, which are often used in many pharmaceuticals. Recent trends of using scorpion venom components as potential candidates for drug development inspire us to target this alkaloid for screening its biological effects as a pharmaceutical agent. As the structure of the alkaloid **1** is very close to the hypotensive agent feruloylhistamine,[11] compound **1** can be investigated in the future to determine whether or not it would show a similar hypotensive property. Notably, as the venom from *M. gertschi* was found to be nontoxic to mice and humans,[10] we are optimistic about the nontoxicity of this venom component too if used as a pharmaceutical drug in the future. With this being a potential candidate for evaluating its medicinal effect, we have developed a cost-effective method for chemically synthesizing this alkaloid from vanillin and histamine precursors. This compound is the first alkaloid ever identified from a scorpion.

EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra were acquired at 600 MHz (^{1}H NMR), 500 MHz (^{1}H NMR), 300 MHz (^{1}H NMR), 125 MHz (^{13}C NMR), and 75 MHz (^{13}C NMR) in methanol-d_4, DMSO-d_6, or acetone-d_6 solution, using Varian Inova-600, Varian Inova-500, and Varian Inova 300 spectrometers. NMR data for compound **1** were recorded using a 300 MHz spectrometer. The 2D NMR experiments (gCOSY, HSQC, HMBC, NOESY) were also performed using a Shigemi NMR tube in a Varian Inova-600 spectrometer with standard pulse sequences.

The electrospray ionization mass spectrometric (ESIMS) studies were performed on a high-resolution mass spectrometer (Thermo Scientific LTQ Orbitrap XL Hybrid Ion Trap-Orbitrap mass spectrometer) using a home-built ESI source.[13,14] Nitrogen (120 psi) was used as a sheath gas. Electrospray of the analyte solution was performed in either positive (+5 kV) or negative (−5 kV) ion mode. The heated transfer capillary (MS inlet) temperature and voltage were maintained at 275 °C and 45 V, respectively. Helium was used as the collision gas (buffer gas) in the collision-induced dissociation cell (CID cell; an ion trap). CID spectra (MS/MS) were acquired using an isolation width of 0.9 m/z units with activation Q and activation time set to 0.25 and 30 ms, respectively. All experiments were carried out under identical conditions, unless otherwise stated. The ion optics were tuned to obtain the maximum ion current. Data acquisition was performed using XCalibur software (Thermo Fisher Scientific). Spectral accuracy was measured using MassWorks software.[15]

For organic synthesis, all reagents were obtained from commercial sources unless otherwise stated. Reactions were performed using clean and oven-dried glassware. Air- and moisture-sensitive liquids and solutions were transferred by using syringe or stainless-steel cannula. Organic solutions or reaction mixtures were concentrated under reduced pressure by a rotary evaporator. Chromatography was performed on Silicycle Silia-P silica gel (40−63 μm). Thin-layer chromatography was performed on either Whatman Partisil K6F silica gel 60 Å plates (250 μm) or EMD Chemicals silica gel (250 μm).

Biological Material. Scorpion specimens (*Megacormus gertschi*) were collected in El Salto Jacala, Hidalgo, Mexico, in October 2013 and May 2016. The permits for collection were issued by SEMARNAT (SGPA/DGVS/02483 of March 18, 2005, and Scientific Permit FAUT-0175 granted to Oscar Francke, see Acknowledgments). The scorpions were maintained in plastic boxes with a permanent water supply and were routinely fed with crickets. The specimens were classified based on the available literature.[16] Species names follow current classification.[17,18]

Venom Extraction and Molecular Fractionation. Venom from *M. gertschi* was obtained in the laboratory by electrical stimulation (15−25 V for 3 s) applied at the articulation of the telson of the specimen. The venom was collected in Eppendorf Lo-bind tubes and dissolved in double-distilled H_2O followed by centrifugation at 14000*g* for 15 min at 4 °C. The soluble supernatant was either lyophilized or stored at −20 °C and later separated by HPLC (Figure S1) as described earlier.[10] Briefly, whole soluble venom was injected onto a C4 reversed-phase semipreparative column (Vydac) and separated by using a linear gradient from solvent A (0.12% trifluoroacetic acid, TFA, in H_2O) to 60% solvent B (0.10% TFA in CH_3CN) for 90 min. The fraction of interest (retention time 21.40 min) obtained by HPLC was dried on a vacuum concentrator, and the corresponding purified white powder (~1 mg) was stored at −20 °C before subjecting it to analytical studies (characterization).

(Z)-N-(2-(1H-Imidazol-4-yl)ethyl)-3-(4-hydroxy-3-methoxyphenyl)-2-methoxyacrylamide (1): white powder; ^{1}H and ^{13}C NMR data,

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Table 1; HRESIMS m/z 318.1450 $[M + H]^+$ (calcd for $C_{16}H_{20}N_3O_4$, 318.1454).

Chemical Synthesis of the Venom Component (Scheme 1). *Synthesis of (Z)-3-(4-Hydroxy-3-methoxyphenyl)-2-methoxyacrylic Acid (5).* The unsaturated carboxylic acid **5** was synthesized by slight modification of the literature report.[19] In a flame-dried flask, diisopropylamine (2.03 mL, 14.5 mmol) was dissolved in tetrahydrofuran (THF, 20 mL) and cooled to −78 °C. ^{n}BuLi (2.5 M in hexane, 5.6 mL, 14 mmol) was added slowly, and the reaction mixture was stirred for 30 min at −78 °C. After being stirred for 30 min, a solution of methyl-2-methoxy acetate (1.19 g, 11.4 mmol) in 10 mL of THF was added dropwise. The mixture was further stirred for another 20 min at −78 °C and then allowed to warm to −30 °C followed by dropwise addition of a 10 mL solution of vanillin (1.52 g, 10 mmol) in THF. Then, benzenesulfonyl chloride (1.66 mL, 13.0 mmol) was added, and the resulting mixture was allowed to warm to room temperature (rt) and was stirred overnight. The reaction was then quenched with brine solution and extracted with EtOAc.

The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under vacuum. The crude product (**3**) was utilized for next step synthesis without purification. The crude residue was mixed with 5 mL of triethylamine (excess) and heated under reflux in an oil bath for 3 h. After cooling to rt, the mixture was concentrated, and 3 M HCl was added. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were then dried over anhydrous Na_2SO_4 and concentrated under vacuum. The crude product was purified by flash column chromatography to yield 65% of the unsaturated carboxylic ester (**4**) as a colorless liquid.

To a stirred solution of **4** (952 mg, 4 mmol) in 10 mL of EtOH was added 10 mL of NaOH (1.2 g in water). The mixture was stirred for 2 h under reflux. The reaction mixture was cooled and concentrated followed by addition of ether (100 mL) and 3 M HCl (50 mL) at 0 °C. The organic phase was separated, and the aqueous phase was extracted twice with ether. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under vacuum. The crude product was purified by flash column chromatography to yield 56% of the unsaturated carboxylic acid **5** (Z-isomer): ^{1}H NMR (300 MHz, acetone-d_6) δ 11.20 (br s, 1H), 8.09 (br s, 1H), 7.58−7.51 (m, 1H), 7.29 (ddd, J = 8.3, 2.0, 0.5 Hz, 1H), 6.97 (s, 1H), 6.86 (d, J = 8.3 Hz, 1H), 3.88 (s, 3H), 3.78 (s, 3H); ^{13}C NMR (75 MHz, acetone-d_6) δ 165.6, 148.7, 148.2, 144.6, 126.5, 125.2, 124.8, 115.9, 113.9, 59.1, 56.1.

Synthesis of (Z)-N-(2-(1H-Imidazol-4-yl)ethyl)-3-(4-hydroxy-3-methoxyphenyl)-2-methoxyacrylamide (1). In a flame-dried flask, unsaturated carboxylic acid **5** (200 mg, 0.89 mmol) was dissolved in CH_2Cl_2 (2 mL), and the reaction mixture was cooled to 0 °C followed by addition of *N,N′*-dicyclohexylcarbodiimide (DCC, 220 mg, 1.07 mmol), 1-hydroxybenzotriazole (144 mg, 1.07 mmol), and diisopropylethylamine (0.201 mL, 1.28 mmol) in CH_2Cl_2 (1 mL) slowly. The reaction mixture was stirred for 10 min at 0 °C, and after that a solution of histamine (1.19 g, 11.4 mmol) in 2 mL of CH_2Cl_2 was added dropwise. The mixture stirred for another 30 min at 0 °C, followed by warming the resulting mixture to rt, and then it was stirred overnight. After the starting material completion, evidenced through TLC, the solid materials were filtered off and the mother liquor concentrated under vacuum. The crude product was purified by flash column chromatography to yield 73% (207 mg) final product **1** as a white solid,[19] which was then treated with TFA before the product was confirmed through ^{1}H NMR, ^{13}C NMR, and HRMS. ^{1}H NMR (600 MHz, methanol-d_4) δ 8.81 (d, J = 1.4 Hz, 1H), 7.37 (d, J = 1.4 Hz, 1H), 7.36 (d, J = 2.0 Hz, 1H), 7.14 (dd, J = 8.3 and 1.8 Hz, 1H), 6.83 (s, 1H), 6.80 (d, J = 8.3 Hz, 1H), 3.87 (s, 3H), 3.64 (t, J = 6.6 Hz, 2H), 3.61 (s, 3H), 3.00 (t, J = 6.0 Hz, 2H); ^{13}C NMR (125 MHz, methanol-d_4) δ 167.0, 148.97, 148.93, 147.8, 134.9, 132.9, 126.3, 125.2, 122.0, 117.6, 116.3, 113.9, 59.7, 56.3, 39.2, 25.9; HRESIMS m/z 318.1452 $[M + H]^+$ (calcd for $C_{16}H_{20}N_3O_4$, 318.1454); 340.1271 $[M + Na]^+$ (calcd for $C_{16}H_{19}NaN_3O_4$, 340.1268).

The NMR characterization data for the product **1** without treating with TFA are also given in Figure S19. ^{1}H NMR (500 MHz, methanol-d_4) δ 7.62 (d, J = 1.2 Hz, 1H), 7.38 (d, J = 2.0 Hz, 1H), 7.14 (ddd, J = 8.4, 2.0, 0.6 Hz, 1H), 6.94−6.87 (m, 1H), 6.83 (s, 1H), 6.80 (d, J = 8.2 Hz, 1H), 3.87 (s, 3H), 3.58 (s, 3H), 3.58−3.53 (m, 2H), 2.94−2.83 (m, 2H); ^{13}C NMR (126 MHz, methanol-d_4) δ 166.7, 148.9, 148.8, 148.1, 136.1, 126.5, 125.2, 121.6, 118.1, 118.0, 116.3, 113.9, 59.6, 56.3, 40.4, 27.7; HRESIMS m/z 318.1452 for $[M + H]^+$ (calcd 318.1454).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b00527.

HPLC data, ESIMS, HDX, and tandem mass spectra (MS/MS), ^{1}H NMR, ^{13}C NMR, gCOSY, HSQC, HMBC, and NOESY data, and experimental section with synthesis procedure (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: zare@stanford.edu.

ORCID

Shibdas Banerjee: 0000-0002-3424-8157
Elumalai Gnanamani: 0000-0003-3699-3350
Fernando Zamudio Zuñiga: 0000-0001-7920-410X
Juana María Jiménez-Vargas: 0000-0001-9508-9332
Lourival Domingos Possani: 0000-0001-6357-2661
Richard N. Zare: 0000-0001-5266-4253

Notes

The authors declare no competing financial interest.

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