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Letter

Origin of the Urease Inhibition of *Metschnikowia pulcherrima* Extracts: Comparative Assays with Synthetic Pulcherriminic Acid and Cyclo-dileucine

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ABSTRACT: The objective of this work was to determine whether pulcherriminic acid was responsible for the urease inhibition activity of the extracts of the yeast *Metschnikowia pulcherrima*. Pulcherriminic acid was synthesized through a seven-step pathway from L-leucine, starting with the thermal cyclodimerization of L-leucine to the corresponding 2,5-diketopiperazine, followed by oxidation to the 2,5-dichloropyrazine through three consecutive steps without purification of the intermediates, oxidation to the corresponding di-*N*-oxide, dechlorination by nucleophilic aromatic substitution with benzyloxide, and deprotection with trifluoroacetic acid without isolation of an intermediate. The urease inhibition assay showed 57 ± 2.3% inhibition of the urease activity at 500 ppm of pulcherriminic acid, much lower than the percent inhibition obtain with the extract, in which pulcherriminic acid was not detected. The cyclic dimer of L-leucine was present in the extract, and its inhibitory capacity was also tested, showing a percent inhibition of 56.1 ± 6.11% of the urease activity at 400 ppm, again much lower than the percent inhibition of the extract. This work demonstrates that the inhibitory capacity of the extracts of the yeast *M. pulcherrima* is not due to either only pulcherriminic acid or only its cyclic dipeptide precursor.

KEYWORDS: *urease inhibitor, pulcherriminic acid, multistep synthesis*

1. INTRODUCTION

Urea is one of the most widely used nitrogen-based fertilizers worldwide. In 2022, global urea production reached 183.2 million metric tons.[1] This is due to its main advantages over other nitrogen-based fertilizers: high nitrogen content (46%), low cost, easy management, and water solubility. However, the main disadvantage of urea is its loss of efficacy (>50%), which is mainly caused by ammonia volatilization,[2,3] which occurs by the hydrolysis of urea upon its application to soil. Urea hydrolysis is catalyzed by urease, a nickel-dependent metalloenzyme, released by microorganisms in the soil to form ammonium, which can be converted into volatile ammonia depending on the pH of the medium.[4−6] Therefore, there is not only a loss of nitrogen and, therefore, a loss of efficiency but also a negative environmental impact. The use of urease inhibitors is one of the most widely used strategies for preventing the loss of nitrogen from ammonia volatilization.[4,7,8] Urease inhibitors slow the transformation of urea into ammonia and carbon dioxide through its interaction with the active site of the urease. Therefore, the use of urease inhibitors reduces the amount of ammonia released and, thus, improves the effectiveness of urea-based fertilizers.[9]

A wide variety of compounds, like thiols, hydroxamic acids, or amides and esters of phosphoric acid, can be used as urease inhibitors.[10] The amides and esters of phosphoric acid are classified as stronger urease inhibitors, and *N*-(*n*-butyl) thiophosphoric triamide (NBPT) is among the most commonly used due to its high efficacy as a urease inhibitor.[4,7,11,12] The main problem with phosphoramides as urease inhibitors is their short degradation half-life. Their degradation half-life is dependent on the soil pH. For example, the value for NBPT at 25 °C is reported to be 58 min at pH 3.0, 92 days at pH 7.0, and 16 days at pH 11.0.[13] Furthermore, the degradation of NBPT under slightly acidic−alkaline conditions (pH 5.1−7.6) mainly due to chemical hydrolysis into *n*-butylamine and phosphoradiamine has been reported.[14] Natural products, like extracts from plants and microorganisms, have been investigated for this purpose; however, either they irreversibly inhibit urease, or their production is not economically viable.[7] Thus, it would be of great interest to find a natural extract with urease inhibitory capacity that is economically viable, effective at low concentrations, chemically stable, compatible with urea, and nontoxic. These natural extracts are mainly composed of secondary metabolites, which are described as bioactive compounds, with potential applications as pharmaceuticals, nutraceuticals, and agrochemicals. Some of these bioactive compounds result from protein hydrolysis; for example, a peptide derived from spinach Rubisco and its analogues has been synthesized and evaluated

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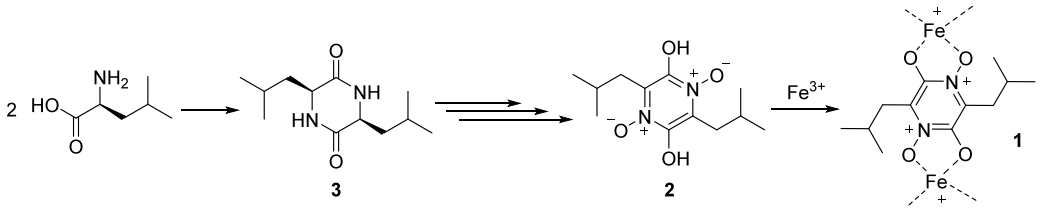
Scheme 1. Biosynthesis of Pulcherrimin (1)



to validate their biological properties as antioxidant, α-tyrosinase inhibitors of G protein stimulation.[15,16]

In this way, Fertinagro Biotech has found an extract from the natural yeast *Metschnikowia pulcherrima* that exhibits urease inhibitory capacity.[17] In the literature, the antifungal and antimicrobial properties of *M. pulcherrima* have been described.[18−20] According to the literature, these properties are caused by the characteristic red pigment found in this yeast, named pulcherrimin. Pulcherrimin (**1**) is a polymeric Fe(III) chelate of pulcherriminic acid (**2**).[21,22] *M. pulcherrima* biosynthesizes pulcherriminic acid from the cyclic dipeptide of L-leucine (**3**). In the presence of Fe(III), a non-enzymatic reaction converts pulcherriminic acid into the red pigment pulcherrimin (Scheme 1).[18,23]

Thus, pulcherriminic acid (**2**), the precursor of the characteristic red pigment present in *M. pulcherrima*, might be responsible for the urease inhibitory capacity of the yeast extract. In addition, pulcherriminic acid has a molecular structure analogous to that of hydroxamic acids, known for their ability to chelate metals[24] and inhibit metalloenzymes, such as urease.[25,26] In this paper, we report the synthesis of pulcherriminic acid from L-leucine, the analysis of the *M. pulcherrima* extracts, and the urease inhibition assays to confirm this hypothesis.

2. MATERIALS AND METHODS

2.1. Synthesis of Pulcherriminic Acid. *2.1.1. Materials.* L-Leucine (99%), phosphorus(V) oxychloride (99%), pyridine anhydrous (>99.5%), sodium hydroxide (98%), trifluoroacetic acid (99%), hydrochloric acid (37%), ethylene glycol (99%), dichloromethane (≥99.8%), diethyl ether (99%), ethyl acetate (≥99.8%), and methanol (HPLC grade) were purchased from Fisher Chemicals. A sodium bisulfite solution (40 wt % in water), a hydrogen peroxide solution (50 wt % in water), *m*-chloroperbenzoic acid (≤77%), potassium *tert*-butoxide (≥98.0%), anisole (99%), silica gel technical grade (60 Å pore size, 40−63 μm particule size), absolute ethanol (≥99.8%), dimethyl sulfoxide anhydrous (≥99%), and tetrahydrofuran (≥99%) were purchased from Merck. Anhydrous sodium bicarbonate (99.8%), anhydrous sodium carbonate (99.8%), and hexane were purchased from Scharlab. Anhydrous magnesium sulfate was purchased from LabKem, and benzyl alcohol (99%) was purchased from Cymit.

The products were characterized by nuclear magnetic resonance (NMR) using a Bruker AV-400 spectrometer (400 MHz). $CDCl_3$ (δ = 7.26 ppm for ^{1}H, and δ = 77.16 ppm for ^{13}C) and DMSO-d_6 (δ = 2.50 ppm for ^{1}H, and δ = 39.58 ppm for ^{13}C) were used as deuterated solvents and a reference. The chemical shifts of the different signals are listed in parts per million, and the coupling constants (J) in hertz. Multiplicities are given as s (singlet), d (doublet), m (multiplet), and br (broad signal).

High-resolution mass spectra (HRMS) of the products were recorded by using a Bruker MicroTof-Q mass spectrometer with electrospray ionization (ESI) at atmospheric pressure and a high-resolution hybrid Q-TOF analyzer. The products were dissolved in methanol for the analysis.

*2.1.2. Synthetic Methods. 2.1.2.1. 3,6-Diisobutylpiperazine-2,5-dione (**3**).* First, 8.0 g of L-leucine (60.98 mmol) and 50 mL of dry ethylene glycol were added to a 100 mL round-bottom flask. The mixture was stirred for 20 h at 180 °C under an argon atmosphere. Then, the mixture was cooled to room temperature and 20 mL of cooled ethanol was added. The precipitate was filtered off and recrystallized from a hot ethanol/water mixture (5:1). The solid was filtered and dried under vacuum at 80 °C overnight to give **3** as a white needle-like solid (3166.2 mg, 46%): ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.18 (br, 1H), 8.01 (br, 1H), 3.78−3.68 (m, 2H), 1.88−1.74 (m, 2H), 1.65−1.40 (m, 4H), 0.89 (d, 6H, J = 6.4 Hz), 0.86 (d, 6H, J = 6.4 Hz); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 168.81, 168.48, 52.72, 52.46, 43.69, 41.33, 23.68, 23.63, 23.08. 22.90, 21.98. 21.72.

*2.1.2.2. 3-Chloro-2,5-diisobutylpyrazine (**7**).* First, 1.50 g of **3** (6.63 mmol), 2.04 g of $POCl_3$ (13.28 mmol), and 560.0 mg of pyridine (6.63 mmol) were added to a 20 mL PTFE vial. The mixture was stirred for 2 h at 160 °C under an argon atmosphere. Then, the mixture was cooled to room temperature and transferred to a 100 mL round-bottom flask and 50 mL of an ice/water mixture was added. The mixture was stirred for 30 min. The aqueous solution was extracted with CH_2Cl_2 (3 × 50 mL), and the combined organic phases were washed with 1 M NaOH (2 × 100 mL) and brine (100 mL), dried with anhydrous $MgSO_4$, and filtered. The solvent was evaporated under reduced vacuum to give an orange oil, which was purified by column chromatography eluting with an ethyl acetate/hexane mixture (1:9) to give **7** as a pale yellow oil (653.4 mg, 43%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.23 (s, 1H), 2.80 (d, 2H, J = 7.2 Hz), 2.60 (d, 2H, J = 7.2 Hz, 1H), 2.28−2.15 (m, 1H), 2.15−2.02 (m, 1H), 0.96 (d, 6H, J = 6.7 Hz), 0.93 (d, 6H, J = 6.6 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 154.31, 152.60, 148.33, 141.96, 43.67, 43.33, 29.02, 28.13, 22.57, 22.42.

*2.1.2.3. 2,5-Dichloro-3,6-diisobutylpyrazine (**4**).* First, 3.0 g of **3** (13.26 mmol), 4.07 g of $POCl_3$ (26.52 mmol), and 1.05 g of pyridine (13.26 mmol) were added to a 100 mL PTFE vial. The mixture was stirred for 2 h at 160 °C under an argon atmosphere. Then, the mixture was cooled to room temperature and transferred to a 250 mL round-bottom flask and 100 mL of an ice/water mixture was added. The mixture was stirred for 30 min. The aqueous solution was extracted with CH_2Cl_2 (3 × 100 mL), and the combined organic phases were washed with 1 M NaOH (2 × 200 mL) and brine (200 mL), dried with anhydrous $MgSO_4$, and filtered. The solvent was evaporated under reduced vacuum to give an orange oil (2.85 g). For the second step, the crude mixture and 5.58 g of 70% mCPBA (22.62 mmol) were added to a 250 mL round-bottom flask. The mixture was dissolved in 100 mL of dry CH_2Cl_2, and the solution was stirred for 3 h at 40 °C. Then, the solution was washed with a 10% $NaHSO_3$ solution (3 × 100 mL), a $NaHCO_3$-saturated solution (3 × 100 mL), and brine (100 mL). The organic layer was dried with anhydrous $MgSO_4$ and filtered. The solvent was evaporated under vacuum to give a pale orange solid/liquid (2.78 g). For the final step, the crude mixture was dissolved in 15 mL of $POCl_3$ in a 100 mL PFTE vial. The mixture was stirred at 100 °C overnight. Then, the mixture was cooled to room temperature and transferred to a 250 mL round-bottom flask and 100 mL of an ice/water mixture was added. The mixture was stirred for 30 min. The aqueous solution was extracted with CH_2Cl_2 (3 × 100 mL), and the combined organic phases were washed with 1 M NaOH (2 × 200 mL) and brine (200 mL), dried with anhydrous $MgSO_4$, and filtered. The solvent was evaporated under reduced vacuum to give an orange oil, which was purified by column chromatography eluting with an ethyl acetate/hexane mixture (1:9) to give **4** as a pale yellow oil (1601.8 mg, 46%): ^{1}H NMR ($CDCl_3$, 400

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MHz) δ 2.77 (d, 4H, J = 7.2 Hz), 2.29−2.13 (m, 2H), 0.97 (d, 12H, J = 6.7 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 152.71, 146.01, 42.82, 28.25, 22.51.

*2.1.2.4. 2,5-Dichloro-3,6-diisobutylpyrazine-1,4-dioxide (**5**).* First, 1601.8 mg of **5** (6.13 mmol) was dissolved in 12 mL of TFA in a 25 mL round-bottom flask. Then, 3.34 mL of 50% H_2O_2 (58.0 mmol) was added dropwise, and the mixture was stirred for 3 h at 50 °C. The mixture was cooled to room temperature and slowly poured onto an ice-cold 20% $NaHSO_3$ solution (100 mL). Then, the mixture was neutralized with 1 M NaOH (60 mL) and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic phases were washed with brine (100 mL), dried with anhydrous $MgSO_4$, and filtered. The solvent was evaporated under reduced vacuum to give a pale yellow solid, which was purified by column chromatography eluting with dichloromethane to give **5** as a pale yellow solid (1258.0 mg, 70%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.02 (d, 4H, J = 7.3 Hz), 2.41−2.25 (m, 2H), 1.01 (d, 12H, J = 6.7 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 146.11, 139.70, 37.86, 26.24, 22.60.

2,5-Dichloro-3,6-diisobutylpyrazine-1-oxide (**9**) was also isolated (339.8 mg, 21%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.95 (d, 2H, J = 7.3 Hz), 2.77 (d, 2H, J = 7.3 Hz), 2.36−2.24 (m, 1H), 2.24−2.13 (m, 1H), 0.98 (d, 6H, J = 5.8 Hz,), 0.96 (d, 6H, J = 5.7 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 154.13, 147.35, 144.95, 138.74, 43.72, 36.88, 28.06, 26.11, 22.80, 22.43.

*2.1.2.5. 2,5-Dihydroxy-3,6-diisobutylpyrazine-1,4-dioxide [pulcherriminic acid (**2**)].* First, 1756.9 mg of **5** (6.0 mmol) and 2356.4 mg of KO^{t}Bu (21.0 mmol) were dissolved in 50 mL of dry THF. Then, 2.19 mL of benzylic alcohol (21.0 mmol) was added dropwise under an argon atmosphere, and the mixture was stirred overnight at room temperature. The reaction was quenched by the addition of H_2O (100 mL), and the mixture extracted with CH_2Cl_2 (3 × 100 mL). The combined organic phases were washed with brine (100 mL), dried with $MgSO_4$, and filtered. The solvent was evaporated under reduced vacuum to give an orange/brown oil (2638.6 mg). For the next step. the crude mixture was dissolved in 20 mL of a TFA/anisole mixture (1:1) and the mixture was stirred for 3 h at room temperature under an argon atmosphere. The solvent was evaporated under reduced vacuum, and diethyl ether was added to the mixture. The precipitate was filtered off, washed with cooled dichloromethane, and dried under reduced vacuum. Pulcherriminic acid (**2**) was obtained as a yellow solid (292.0 mg, 19%): ^{1}H NMR (DMSO-d_6, 400 MHz) δ 2.67 (d, 4H, J = 7.2 Hz), 2.16−2.06 (m, 2H), 0.89 (d, 12H, J = 6.6 Hz); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 145.53, 129.63, 33.41, 26.22, 22.39.

*2.1.2.6. 2-Benzyloxy-5-chloro-3,6-diisobutylpyrazine (**8**).* First, 1000.0 mg of **4** (3.8 mmol) was dissolved in 15 mL of dry THF. Then, 1730.0 mg of sodium benzyloxide (13.3 mmol) was added under an argon atmosphere, and the mixture was stirred overnight at room temperature. The reaction was quenched by the addition of H_2O (20 mL), and the mixture extracted with CH_2Cl_2 (3 × 20 mL). The combined organic phases were washed with brine (60 mL), dried with $MgSO_4$, and filtered. The solvent was evaporated under reduced vacuum to give an orange/yellow oil, which was purified by column chromatography eluting with an ethyl acetate/hexane mixture (0.5:9.5) to give **8** as a yellow oil (495.8 mg, 39.2%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.45−7.43 (m, 2H), 7.39−7.36 (m, 2H), 7.34−7.29 (m, 1H), 5.39 (s, 2H), 2.70 (d, 2H, J = 5.4 Hz), 2.68 (d, 2H, J = 5.6 Hz), 2.74−2.14 (m, 2H), 0.96 (d, 6H, J = 5.6 Hz), 0.95 (d, 6H, J = 6.0 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.39, 148.19, 144.11, 138.32, 137.01, 128.52, 127.98, 127.86, 68.23, 42.73, 40.46, 27.92, 27.88, 22.63, 22.59; found (ESI+) for $C_{19}H_{25}ClN_2NaO$ ($M + Na^+$) 355.1548 (100%), 356.1579 (21.6%), 357.1524 (34.4%), 358.1551 (7.1%); calcd (ESI+) 355.1547 (100%), 356.1579 (21.6%), 357.1524 (34.4%), 358.1552 (7.1%).

*2.1.2.7. 2-Chloro-5-hydroxy-3,6-diisobutylpyrazine-1,4-dioxide (**11**).* First, 100.0 mg of **5** (0.34 mmol) and 5 mL of aqueous 0.4 M NaOH (2.04 mmol) were added to a 15 mL PTFE vial. The mixture was stirred for 4 h at 120 °C. Then, the mixture was cooled to room temperature, and 37% HCl was added dropwise until the pH was ≤3. The solid was filtered and dried under vacuum at 60 °C overnight to give **11** as a yellow solid (32.4 mg, 35%): ^{1}H NMR (DMSO-d_6, 400 MHz) δ 2.71 (d, 2H, J = 7.2 Hz), 2.21−2.05 (m, 2H), 0.95 (d, 6H, J = 6.4 Hz), 0.88 (d, 6H, J = 6.8 Hz); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 152.99, 139.81, 138.34, 121.31, 36.65, 34.44, 27.03, 25.13, 22.62, 22.07; found (ESI+) for $C_{12}H_{19}ClN_2NaO_3$ ($M + Na^+$) 297.0976 (100%), 298.1007 (14.0%), 299.0950 (33.5%), 300.0977 (4.6%); calcd (ESI+) 297.0976 (100%), 298.1007 (14.1%), 299.0950 (33.5%), 300.0979 (4.6%).

2.2. Assays of Urease Inhibition. *2.2.1. Materials.* Jack bean urease was purchased from Toyobo Biosystems. A 0.1 M carbonate-bicarbonate buffer solution (pH 9.06), Tween 20, urea, KCl (≥99%), HCl (32%), NaOH (≥97%), sodium salicylate (≥99.5%), sodium nitroprusside, sodium dichloroisocyanurate, and ammonium sulfate (≥99%) were purchased from Merck.

Absorbance was measured with a microplate reader (BioTek Synergy HTX, Agilent) at 690 nm.

2.2.2. M. pulcherrima Extract. The yeast cell culture was centrifuged at 5000 rpm for 10 min at 4 °C, and a cell pellet was obtained. The cell pellet was washed twice with ultrapure water and weighed. A methanol/water mixture (1:1) was added in a solid:liquid ratio of 1:4. The mixture was incubated at room temperature for 6 days. Then, it was centrifuged under the same conditions used previously. The supernatant was collected, and methanol was evaporated in a water bath until the volume was decreased by half. The dry matter content was determined by freeze-drying.

2.2.3. Method. The urease assay was performed in 0.1 M carbonate-bicarbonate buffer (pH 9.06), and 0.5 μg/mL jack bean urease (Toyobo Biochemichal URH-201) was used. The initiation of the enzymatic reaction was preceded by a 30 min preincubation of the enzyme with the products to be tested to promote interaction between enzyme E and inhibitor I and formation of enzyme inhibitor complex EI* if it is attained. The reaction was initiated by addition of a urea solution to the reaction mixture at a final concentration of 50 mM. The assay was performed for 30 min at 25 °C, and reaction was stopped with a 1 M KCl-HCl solution. Reactions were performed in duplicate, with three independent repeats. Acetohydroxamic acid (CH_3CO-NHOH) was used as a standard inhibitor. Enzyme activity was determined by measuring the concentration of ammonia released by the colorimetric Berthelot reaction[27] and a modification of the procedure of Cordero et al.[28] Briefly, the experiment was performed with two different reagents: an oxidation solution (1 mg/mL dichloroisocyanuric acid sodium salt dehydrate) and a color reagent [a 2:1 (v:v) mixture of solutions A and B mixed just before use]. Solution A consisted of 0.15 M NaOH. Solution B consisted of 170 mg/mL sodium salicylate and 1.278 mg/mL sodium nitroprusside dehydrate. For ammonia measurement, each well received 75 μL of the color reagent followed by 30 μL of the oxidation solution. The plate was read after a 30 min incubation at room temperature in the dark by the absorbance at 690 nm in a microplate reader (Synergy HTX). Colorimetric reaction was achieved in duplicate for each sample point. The inhibitory capacity was determined by the percentage of inhibition and was calculated as follows:

$$\%\ \text{inhibition}: 100 - \frac{\text{test tube } NH_4^+ \times 100}{\text{control tube } NH_4^+}$$

2.3. UPLC-MS Analysis. *2.3.1. Materials.* Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Fisher Chemicals. Formic acid (98%) was purchased from Merck.

2.3.2. Method. The *M. pulcherrima* extract and the synthesized pulcherriminic acid **2** and cyclic dipeptide of leucine **3** were analyzed by ultraperformance liquid chromatography with mass spectrometry (UPLC-MS). The UPLC Waters Acquity instrument coupled to a Waters Acquity QDA instrument as the mass spectrometry detector was employed. The method is based on that described by Gore-Lloyd et al. with minor modifications.[23] The following solvent gradient was used (A, acetonitrile and 0.1% formic acid; B, H_2O and 0.1% formic acid) at a flow rate of 0.5 mL/min on an ACE Excel 3 Super C18 column (3 μm, 150 mm × 4.6 mm):

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time (min)	A (%)	B (%)
0	5	95
2	20	80
20	98	2
25	98	2
27	5	95
30	5	95

The MS instrument was operated in positive ionization mode with a scan range of m/z 130−800. The capillary and cone voltages were set to 3.5 kV and 10 V, respectively. The desolvation and column temperatures were set to 600 and 40 °C, respectively.

2.3.3. Quantification of the Cyclic Dipeptide of Leucine. The standards were prepared from a synthetic cyclic dipeptide of leucine (**3**). In a 100 mL volumetric flask, 10.0 mg of **3** was added. Then, **3** was dissolved in 50 mL of acetonitrile. Acetonitrile was added up to the volume line to obtain a 100 ppm standard solution of **2**. From a 100 ppm solution, 0.1, 0.3, 0.5, 1.0, and 1.2 mL were transferred to a 10 mL volumetric flask, and acetonitrile was added up to the volume line for each. Finally, ∼2.0 mL of each solution was filtered through a syringe filter into an HPLC vial and 5 μL was injected into the UPLC-MS instrument following the conditions described previously. Once the chromatograms were obtained, an ion at m/z 227 was filtered and the peak area was measured for a peak with a retention time of 10.80 min. The calibration curve was obtained by representing area versus concentration (Figure 1).

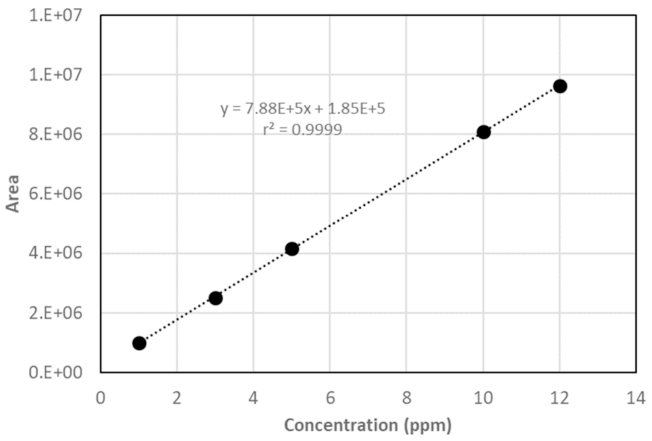


Figure 1. Calibration curve obtained for quantification of the cyclic dipeptide of leucine (**3**).

The samples were prepared following the next procedure. In a 125 mL separatory funnel, 1.0 mL of *M. pulcherrima* extract and 20 mL of deionized water were added. Then, the cyclic dipeptide of leucine was extracted with ethyl acetate (3 × 20 mL). The solvent of the combined organic phases was evaporated under reduced pressure, and the remaining solid residue was dissolved in 5 mL of acetonitrile. The solution was transferred to a 10 mL volumetric flask, and acetonitrile was added up to the volume line. Finally, ∼2.0 mL of each solution was filtered through a syringe filter into an HPLC vial and 30 μL was injected into the UPLC-MS instrument following the conditions described previously. The concentration of the cyclic dipeptide of leucine (**3**) in *M. pulcherrima* extract was calculated by using the following equation:

$$C_{CDPL} = \frac{C_i V_i F}{V}$$

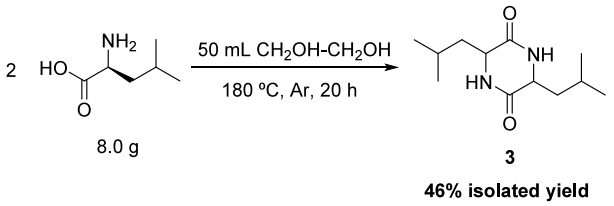
where C_i is the concentration of the cyclic dipeptide of leucine calculated by the calibration curve (parts per million), V_i is the dilution volume (10 mL), F is the dilution factor (5/30), and V is the volume of *M. pulcherrima* extract (2 mL).

3. RESULTS AND DISCUSSION

3.1. Synthesis of Pulcherriminic Acid. The synthesis of pulcherriminic acid (**2**) was based on the synthetic pathway (Scheme 2) previously described.[29,30]

First, cyclo-(L-leucyl-L-leucyl) **3** was synthesized from L-leucine. Most of the methods described in the literature require the protection of one amino acid as a methyl ester and the other one protected in the nitrogen with a formyl[31] or *tert*-butoxycarbonyl[32] group. However, the procedures that do not require the protection of L-leucine are more interesting from a practical point of view. For this reason, we synthesized **3** via a thermal method from L-leucine (Scheme 3).[33] In our hands,

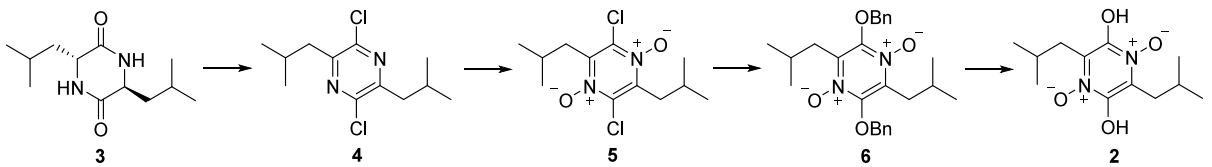
Scheme 3. Thermal Cyclodimerization of L-Leucine



the pure enantiomer cyclo-(L-leucyl-L-leucyl) was not obtained. The ^{13}C NMR spectrum showed double signals probably because of the racemization of the L-leucine during the reaction. This was not a problem given that chirality at the α carbon of leucine is lost in the final pulcherriminic acid (**2**). We managed to scale up the reaction to 8.0 g and optimize the amount of solvent (ethylene glycol), and a mixture of diastereomers of **3** was obtained in 46% isolated yield (Scheme 3).

The next step is the aromatization of the 2,5-diketopiperazine ring to 2,5-dichloropyrazine. The methods described in the literature use an excess of phosphorus oxychloride ($POCl_3$).[34−36] One of the problems of these methods is their low selectivity due to partial chlorination, leading to a mixture of chloropyrazine (**7**) (as the main product) and dichloropyrazine (**4**) (Scheme 4). Total isolated yields (**4** + **7**) ranged from 30% to 60%, with an only 17% yield of **4** at most. Using only 2 eq of $POCl_3$ and 1 eq of pyridine at 160 °C for 2 h,[36] the total isolated yield was improved to 67% and the yield of **4** to 24% when the reaction was carried out under an argon atmosphere.

Scheme 2. Synthetic Pathway Described for the Synthesis of 2 from 3



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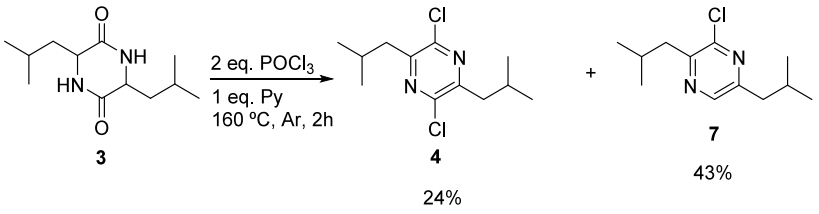
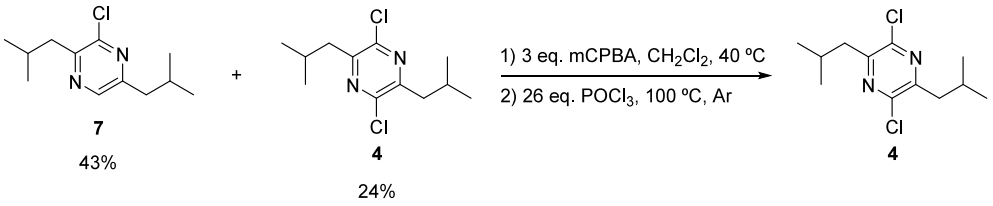
Scheme 4. Aromatization of 7 with $POCl_3$



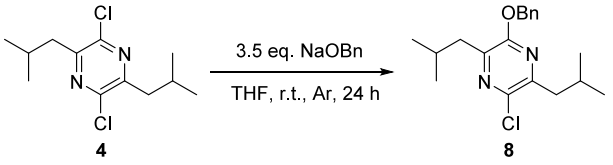
Scheme 5. Synthesis of Dichloropyrazine 4 from 3



Monochloropyrazine **7** can be converted into dichloropyrazine **4** in a two-step process: oxidation of the nitrogen at position 1 followed by a second chlorination.[35] In this work, dichloropyrazine **4** was obtained without purification of the mixture obtained from the chlorination of **3** (Scheme 5). Using NMR spectroscopy, we observed that **4** does not react under these oxidation conditions. The overall yield of **4** from **3** in three steps was 46%, significantly better than the value of 36% previously described.[29]

In the literature, the preparation of pulcherriminic acid **2** from 2,5-dichloropyrazine **4** is described through the oxidation of the nitrogen groups followed by nucleophilic aromatic substitution (S_NAr) of chlorine atoms.[29,30] In this study, we tested S_NAr on **4** before the oxidation step. The substitution was carried out with sodium benzyloxide (generated prior to the reaction with sodium hydride and benzyl alcohol)[37] under the conditions described by Göturk et al.[34] However, 2-benzyloxy-5-chloropyrazine **8** was detected as the only product of the reaction (Scheme 6). Product **8** was purified by column

Scheme 6. Nucleophilic Aromatic Substitution of the Chloro Groups of 4 with Sodium Benzyloxide



chromatography, and its structure was confirmed by NMR and mass spectrometry analysis. No reaction was observed between **8** and sodium benzyloxide under the same conditions. Therefore, the oxidation of nitrogen groups seems to be necessary to increase the reactivity of the 2,5-dichloropyrazine ring and complete the displacement of the chloro groups.

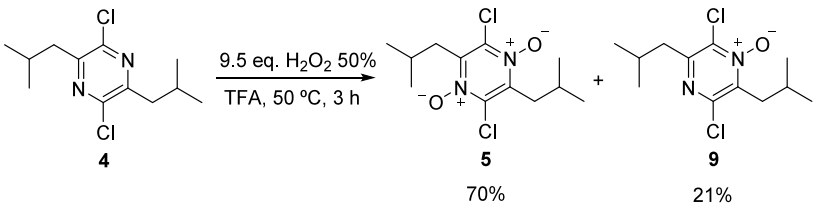
The oxidation of the nitrogen groups of **4** to obtain the *N*-oxide groups was carried out under the conditions described by Usui et al. (Scheme 7).[35] The selectivity for the di-*N*-oxide was similar to that previously described,[29] leading to a 70% yield of dichloro-di-*N*-oxide-pyrazine **5**. For this reason, a second oxidation to recycle **9** was unnecessary.

Finally, pulcherriminic acid **2** could be obtained from **5** in a two-step process, displacement of the chloro groups with potassium benzyloxide (generated *in situ* using potassium *tert*-butoxide) via nucleophilic aromatic substitution followed by deprotection of the benzyloxy groups in acidic medium (Scheme 8),[34] without isolation or purification of **6** and **10**. A 19% isolated yield of **2** was obtained, and its structure was confirmed by NMR. This reproducible yield was lower than that previously described (34%),[29] so we tried several options to improve it.

On one hand, we attempted to displace the chloro groups of 2,5-dichloropyrazine-1,4-dioxide **5** with previously prepared sodium benzyloxide[37] instead of generation *in situ*. The next step, deprotection of the benzyloxy groups of **6** and **10** without their isolation and purification, was carried out under the same conditions cited above.[34] However, a similar isolated yield of pulcherriminic acid **2** was obtained (19%), indicating that the generation of benzyloxide does not influence the yield of pulcherriminic acid **2**.

On the other hand, the direct nucleophilic aromatic substitution of 2,5-dichloro-3,6-dimethylpyrazine-1,4-dioxide with aqueous 0.5 M NaOH at room temperature has been reported.[38] 2-Chloro-5-hydroxy-3,6-dimethylpyrazine-1,4-dioxide was obtained as the only product under these reaction conditions. It was described that heating either this product or

Scheme 7. Oxidation of 4 with H_2O_2



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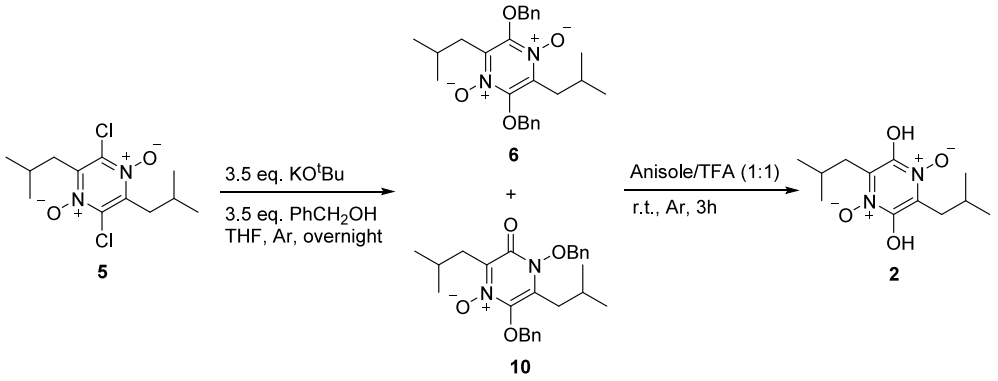
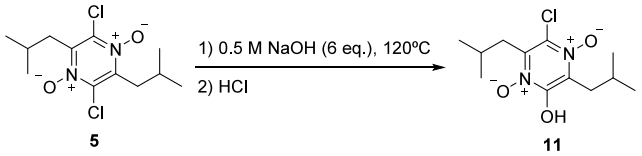
Scheme 8. Nucleophilic Aromatic Substitution Followed by Cleavage of *O*-Benzyl Groups to Obtain 2 from 5



the starting material at 100 °C with aqueous 2.0 M NaOH resulted in the formation of a product mixture, which gave a negative $FeCl_3$ test. A similar result was obtained for 2,5-dichloropyrazine-1,4-dioxide **5** with aqueous 0.4 M NaOH at 120 °C. After a 4 h reaction, a yellow solid precipitated upon acidification. The product was identified by NMR and MS as 2-chloro-5-hydroxy-3,6-diisobutylpyrazine-1,4-dioxide **11** (Scheme 9). Any precipitate was obtained by using aqueous

Scheme 9. Nucleophilic Aromatic Substitution of 5 with NaOH



2.0 M NaOH. It has been described that pulcherriminic acid **2** decomposes quickly in aqueous 1.0 M NaOH,[29] so this result is consistent with the reported stability, which may be also responsible for the low yield obtained through any method.

Therefore, attempts to improve the yield of the last step in the synthesis of **2** from **5** (substitution of chlorine for hydroxyl groups) were not successful. As a consequence, a 3% overall yield of pulcherriminic acid (**2**) from L-leucine was obtained.

3.2. Jack Bean Urease Inhibition Assays. The capacity of synthesized pulcherriminic acid **2** to inhibit jack bean urease activity *in vitro*was tested. An enzyme inhibition assay was carried out, and ammonia released from urea hydrolysis was quantified through Berthelot's reaction. The difference between the ammonium released during reaction in the presence and absence of pulcherriminic acid **2** determines its urease inhibitory capacity, and percentages of inhibition were calculated (see Materials and Methods).

The main problem in testing pulcherriminic acid **2** as a urease inhibitor was its low solubility. Pulcherriminic acid **2** has been described as being insoluble in water and a range of organic solvents, such as methanol, chloroform, dichloromethane, acetonitrile, and tetrahydrofuran. It is sparingly soluble in DMSO and totally soluble in formic and trifluoroacetic acids.[21,29] In our hands, we obtained solutions of pulcherriminic acid **2** in methanol (500 ppm) and DMSO (6000 ppm). However, methanol can inhibit urease, and a more concentrated solution is needed to mask the effect of the solvent. DMSO is a very potent urease inhibitor that cannot be used in this kind of an assay.

Pulcherriminic acid **2** can be dissolved in basic aqueous solutions. In 1.0 M NaOH, pulcherriminic acid decomposes quickly,[29] so a buffer solution containing 0.1 M $NaHCO_3$ and 0.01 M Na_2CO_3 (pH 9.06) was used to dissolve it and to perform the assay of urease inhibition. A buffered solution of pulcherriminic acid **2** at 2500 ppm was tested for urease inhibition. The results are listed in Table 1. Both the control

Table 1. Jack Bean Urease Inhibition by Pulcherriminic Acid (2), *M. pulcherrima* Extract, and the Cyclic Dipeptide of Leucine (3)

inhibitor	concentration[a] (ppm)	% inhibition[b] (mean ± SD)
pulcherriminic acid (**2**)	500	57.0 ± 2.3
	250	40.4 ± 1.8
	125	25.4 ± 2.4
	25	12.2 ± 2.2
M. pulcherrima extract[c]	174.0	89.4 ± 1.33
	130.5	88.3 ± 1.80
	87.0	87.3 ± 1.91
	43.5	84.0 ± 1.98
	34.8	82.8 ± 3.14
	17.4	77.5 ± 1.98
	8.7	61.7 ± 0.90
	4.35	39.4 ± 4.54
	1.74	15.1 ± 4.19
cyclodipeptide (**3**)	530.0	63.6 ± 2.92
	400.0	56.1 ± 6.11
	260.0	44.5 ± 4.60
acetohydroxamic acid	3.5	59.5 ± 1.7

[a]Concentration in the enzymatic reaction. [b]Percentages calculated from the control enzyme reaction in the absence of an inhibitor. [c]Dry matter content.

and the reaction were performed in duplicate, with three independent repeats. An inhibition of 57% of urease activity was obtained at 500 ppm pulcherriminic acid **2**. The inhibitory capacity decreased to 40.4% when the concentration was halved (250 ppm). Weak inhibition was observed below 125 ppm.

In addition, a *M. pulcherrima* extract was tested for urease inhibitory capacity to correlate with pulcherriminic acid **2** inhibitions. The *M. pulcherrima* extract was obtained by incubating the cell pellet with a methanol/water mixture (1:1) for 6 days. The supernatant was recovered, and methanol evaporated; the obtained water solution was named *M. pulcherrima* extract. Different concentrations of *M. pulcherrima*

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extract were evaluated for urease inhibitory capacity, and the results are listed in Table 1 and Figure 2. An IC_{50} of 5.8 ppm

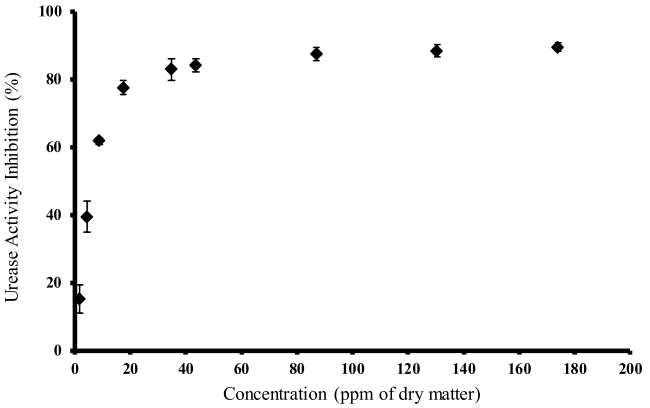

Figure 2. Urease inhibitory capacity of *M. pulcherrima* extract (% inhibition vs concentration).

was calculated by plotting the dry matter logarithm versus the percentage of inhibition, and this was validated by the experimental assay affording 53.3 ± 2.95% inhibition with a dry matter content of 5.4 ppm. This extract exhibited significant urease activity inhibition compared with the standard inhibitor acetohydroxamic acid. Acetohydroxamic acid shows 59.5 ± 1.7% urease inhibition at 3.5 ppm (46.62 μM), barely different from the extract IC_{50}, considering that the extract is composed of many substances and the standard is a single pure compound.

The extract was analyzed by UPLC-MS, and pulcherriminic acid **2** could not be detected (LOD = 0.03 ppm) (Figure 3A). Therefore, the urease inhibitory capacity of pulcherriminic acid **2** is not sufficient to explain the urease inhibition of *M. pulcherrima* extract.

To understand the urease inhibition presented by *M. pulcherrima* extract, another compound of the biosynthetic pathway of pulcherriminic acid, the precursor cyclic dipeptide of leucine (**3**), was tested for urease inhibition under the same conditions. Different concentrations of the purified product were evaluated, and the results are listed in Table 1. A 63% urease activity inhibition was obtained at 530 ppm **3**. The inhibitory capacity decreased to 44.5% when the concentration was decreased at 260 ppm.

The concentration of the cyclic dipeptide of leucine (**3**) in *M. pulcherrima* extract was quantified by UPLC-MS (Figure 3B). A concentration of 4.23 ± 0.48 ppm of the cyclic dipeptide of leucine was calculated. Therefore, the capacity of the cyclic dipeptide of leucine as a urease inhibitor does not seem to explain the urease inhibitory capacity of *M. pulcherrima* extract, and deeper studies are required to identify the molecules responsible for the inhibitory capacity of the extracts.

In conclusion, we have studied the urease inhibitory capacity of pulcherriminic acid **2**, synthesized in a seven-step pathway from L-leucine: (i) thermal cyclodimerization of L-leucine to the corresponding 2,5-diketopiperazine, (ii) aromatization to form 2,5-dichloropyrazine in three consecutive steps (chlorination, oxidation, and chlorination) without purification of the intermediate mixtures, (iii) oxidation to afford the di-*N*-oxide, and (iv) nucleophilic aromatic substitution of the chloro groups with benzyloxide (generated *in situ*) followed by deprotection of the benzyloxy groups without isolation or purification of the dibenzyloxy intermediate. Several of these steps were optimized from the scarce reports described in the literature.[29,30]

The *M. pulcherrima* extract inhibits 61.7% of the urease activity at 8.70 ppm. To understand its capacity as a urease inhibitor, synthetic pulcherriminic acid **2** and the cyclic dipeptide of leucine were tested in the urease inhibition assay. Percent inhibitions of ∼57% of the urease activity at 500 ppm **2** and 63% at 260 ppm **3** were obtained. In addition, the *M. pulcherrima* extract was analyzed by UPLC-MS. Pulcherriminic acid **2** could not be detected (LOD = 0.03 ppm), and a concentration of cyclic dipeptide of leucine **3** of 4.23 ± 0.48 ppm was calculated. Therefore, the urease inhibitory capacity of pulcherriminic acid **2** does not explain the urease inhibitory capacity of *M. pulcherrima* extract or that of the precursor cyclic dipeptide of leucine **3**. Therefore, unequivocal identification of molecules present in the extract and their

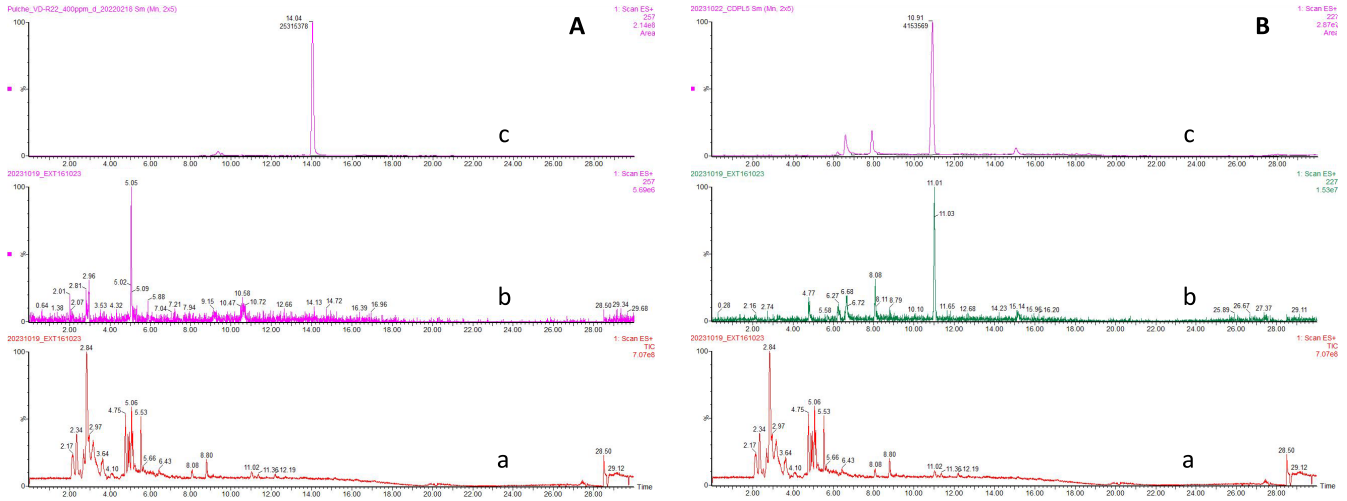

Figure 3. Detection in the *M. pulcherrima* extract of (A) pulcherriminic acid (**2**) and (B) the cyclic dipeptide of leucine (**3**) by UPLC-MS: (a) total ion chromatogram (TIC) of the extract, (b) extracted [M + 1] ions (m/z 257 for **2** and m/z 227 for **3**) from the TIC of the extract, and (c) extracted [M + 1] ions of the pure compounds.

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synthesis would be necessary to determine which are responsible for the main inhibitory activity of the yeast extract.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsagscitech.3c00587.

^{1}H and ^{13}C NMR spectra of all of the synthesized compounds, HMBC spectrum of compound **11**, and mass spectra (ESI+) of compounds **8** and **11** (PDF)

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Notes

The authors declare no competing financial interest.

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