Stereochemical Assignment and Absolute Abundance of Nonproteinogenic Amino Acid Homoarginine in Marine Sponges

Ipsita Mohanty, Samuel G. Moore, Jason S. Biggs, Christopher J. Freeman, David A. Gaul, Neha Garg, and Vinayak Agarwal*

ABSTRACT: Together with arginine, the nonproteinogenic amino acid homoarginine is a substrate for the production of vasodilator nitric oxide in the human body. In marine sponges, homoarginine has been postulated to serve as a precursor for the biosynthesis of pyrrole-imidazole alkaloid and bromotyrosine alkaloid classes of natural products. The absolute abundance of homoarginine, its abundance relative to arginine, and its stereochemical assignment in marine sponges are not known. Here, using stable isotope dilution mass spectrometry, we quantify the absolute abundances of homoarginine and arginine in marine sponges. We find that the abundance of homoarginine is highly variable and can far exceed the concentration of arginine, even in sponges where incorporation of homoarginine in natural products cannot be rationalized. The [homoarginine]/[arginine] ratio in marine sponges is greater than that in human analytes. By derivatization of sponge extracts with Marfey’s reagent and comparison with authentic standards, we determine the L-isomer of homoarginine to be exclusively present in sponges. Our results shed light on the presence of the high abundance of homoarginine in marine sponge metabolomes and provide the foundation to investigate the biosynthetic routes and physiological roles of this nonproteinogenic amino acid in sponge physiology.

INTRODUCTION

Homoarginine (1, Figure 1A) is a nonproteinogenic amino acid present in the human metabolome. Together with L-arginine (2), 1 is a substrate for nitric oxide synthase leading to the production of vasodilator nitric oxide (NO).1 Due to its role in NO production, the abundance of 1 in the human blood plasma is negatively correlated with cardiovascular risk and renal dysfunction.2,3 The abundance of 1 increases during pregnancy with proposed roles in increasing the blood volume and vasodilation.4 The enzyme arginine/glycine amidotransferase catalyzes the amidino group transfer from the chain primary amine of lysine (3) leading to the production of 1 (Figure 1B).5

Although the function of 1 and its relevance as a disease biomarker in mammalian physiology are well validated, the presence, abundance, and role(s) of 1 in other biomes have received lesser attention. We recently reported the detection of 1 in marine sponges.6,7 Sponges are benthic invertebrate metazoans and are prolific producers of bioactive small organic molecules called natural products.8,9 Molecule 1 was rationalized to be a biosynthetic precursor of bromotyrosine alkaloid natural products10 aplysiamisin e11 (4, Figure 1C) and aerophobin12 (5) that are detected in Aplysina and Aiolochroia spp. sponges7 and a precursor of poly brominated pyrrole-imidazole alkaloid natural products13 such as oroidin (6) that are detected in the metabolome of the Stylissa sp. sponge (Figure 1C,D).6 In concert with the above-mentioned biochemical activity of arginine/glycine amidotransferase which converts 3 to 1, radiolabeled 3 was found to be incorporated in 6, conceivably involving the intermediate 1.4,15 The construction of 4–6 from 1 is expected to proceed via hydroxylation, followed by oxidative intramolecular dehydration to furnish the aminoimidazole heterocycle akin to enduracididine biosynthesis.6,7,16 Some marine sponges such as Ianthella sp. that do not possess natural products that can readily be rationalized to be derived from 1 also bear high concentrations of 1.7 The marine sponge eukaryotic host harbors a symbiotic microbiome; the presence of 1 is independent of the microbiome architecture of the sponge holobiont. Although Aplysina and Aiolochroia spp. sponges are high microbial diversity and high microbial abundance sponges, Stylissa and Ianthella spp. are low microbial diversity and low microbial abundance sponges.7

Received: October 11, 2021
Accepted: November 16, 2021
Our prior detection of 1 in marine sponge metabolomes was bereft of the stereochemical assignment as the analytical methods employed did not differentiate between L-homoarginine (1a, Figure 1A) and D-homoarginine (1b). Moreover, the absolute abundance of 1 in sponge metabolomes was not determined. In this study, we query the stereochemistry at the α site and determine the absolute abundance of 1a and compare that to the abundance of 2 in *Aplysina*, *Aiolochroia*, *Stylissa*, and *Ianthella* spp. sponges. We find the proteinogenic amino acid 2 to be uniformly abundant in these phylogenetically and geographically dispersed sponges. However, the abundance of 1a was variable and was found to be several folds higher than 2 even in the *Ianthella* sp. sponge that does not contain natural products derived from 1. Our results now set the stage for investigating the physiological role(s) potentiated by the high concentration of 1 in marine sponges.

**MATERIALS AND METHODS**

*Marine Sponges Used in the Study.* Phylogeny and natural product chemical classes present in marine sponges used in this study are delineated in Table 1. References 7, 6, and 17.

**Synthesis of 1b.** The procedure for synthesis of 1b was adopted from the literature. Dipropylamine (1.41 mL, 10.04 mmol) was added to a stirred solution of D- N-α-Boc-lysine (485 mg, 1.97 mmol) in 10 mL of MeOH at room temperature, followed by the addition of the guanidinylating reagent N,N′-bis-Boc-1-guanyl pyrazole (1.63 g, 5.28 mmol). The reaction mixture was stirred at room temperature for 3 h. The reaction mixture was concentrated under vacuum. Deprotection of the Boc functional group was achieved by dissolving the guanidinylated product (100 mg) from the previous step in 3 mL of DCM, followed by the dropwise addition of 2 mL of trifluoroacetic acid. The reaction mixture was stirred at room temperature for 16 h and concentrated under vacuum. Cation-exchange chromatography was performed using the DOWEX resin, and the pure molecule 1b was eluted using 1 M aqueous ammonium hydroxide as the mobile phase. The reaction mixture was concentrated under vacuum. A blue precipitate of cupric chloride (61 mg, 0.32 mmol) in 1.2 mL of 1 M NaOH, a solution of CuSO4 (48 mg, 0.19 mmol) in 3 mL of water was added. The reaction mixture was stirred at room temperature for 24 h. A blue precipitate of cupric–lysine complex was obtained after filtration and was dissolved in saturated ethylenediaminetetraacetic acid and stirred overnight at room temperature. The white precipitate thus obtained was collected by filtration and was dissolved in MeOD-D2O (2 mL) and stirred for 5 h. The guanidinylating reagent N,N′-bis-Boc-1-guanyl pyrazole (139 mg, 0.45 mmol) and NaHCO3 (53 mg, 0.63 mmol) were added. The reaction mixture was stirred at room temperature for 3 h. The reaction mixture was concentrated under vacuum. The reaction mixture was stirred at room temperature for 24 h. A blue precipitate of cupric–lysine complex was obtained after filtration and was dissolved in saturated ethylenediaminetetraacetic acid and stirred overnight at room temperature. The white precipitate thus obtained was carried forward for deprotection of the Boc groups by acid treatment as abovementioned and characterized using 1H NMR.

**Synthesis of the Isotopic Standard of 1a.** The isotopic standard of 1a was synthesized based on the literature procedure. To a stirred solution of 13C, 15N-labeled L-lysine chloride (61 mg, 0.32 mmol) in 1.2 mL of 1 M NaOH, a solution of CuSO4 (48 mg, 0.19 mmol) in 3 mL of water was added. The reaction mixture was stirred at room temperature for 5 h. The guanidinylating reagent N,N′-bis-Boc-1-guanyl pyrazole (139 mg, 0.45 mmol) and NaHCO3 (53 mg, 0.63 mmol) were added. The reaction mixture was stirred at room temperature for 24 h. A blue precipitate of cupric–lysine complex was obtained after filtration and was dissolved in saturated ethylenediaminetetraacetic acid and stirred overnight at room temperature. The white precipitate thus obtained was carried forward for deprotection of the Boc groups by acid treatment as abovementioned and characterized using 1H NMR.

**Table 1. Marine Sponges Used in This Study**

| sponge genus | collection site | dereplicated natural product chemical class | refs |
|-------------|----------------|-------------------------------------------|------|
| *Aplysina*  | Florida Keys   | bromotyrosine alkaloids                   | Reference 7 |
| *Aiolochroia* | Florida Keys  | bromotyrosine alkaloids                   | Reference 7 |
| *Stylissa*  | Guam           | pyrrole–imidazole alkaloids               | Reference 6 |
| *Ianthella* | Guam           | bromotyrosine alkaloids                   | References 7 and 17 |

**Synthesis of 1b.** The procedure for synthesis of 1b was adopted from the literature. Diisopropylamine (1.41 mL, 10.04 mmol) was added to a stirred solution of D- N-α-Boc-lysine (485 mg, 1.97 mmol) in 10 mL of MeOH at room temperature, followed by the addition of the guanidinylating reagent N,N′-bis-Boc-1-guanyl pyrazole (1.63 g, 5.28 mmol). The reaction mixture was stirred at room temperature for 3 h. The reaction mixture was concentrated under vacuum. Deprotection of the Boc functional group was achieved by dissolving the guanidinylated product (100 mg) from the previous step in 3 mL of DCM, followed by the dropwise addition of 2 mL of trifluoroacetic acid. The reaction mixture was stirred at room temperature for 16 h and concentrated under vacuum. Cation-exchange chromatography was performed using the DOWEX resin, and the pure molecule 1b was eluted using 1 M aqueous ammonium hydroxide as the mobile phase. The reaction mixture was concentrated under vacuum. A blue precipitate of cupric chloride (61 mg, 0.32 mmol) in 1.2 mL of 1 M NaOH, a solution of CuSO4 (48 mg, 0.19 mmol) in 3 mL of water was added. The reaction mixture was stirred at room temperature for 24 h. A blue precipitate of cupric–lysine complex was obtained after filtration and was dissolved in saturated ethylenediaminetetraacetic acid and stirred overnight at room temperature. The white precipitate thus obtained was carried forward for deprotection of the Boc groups by acid treatment as abovementioned and characterized using 1H NMR.

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**Derivatization of Standards and Sponge Extracts.** The derivatization for derivatizing 1a and 1b standards was adapted from the literature. To a 50 μL aqueous solution of 50 mM
standards, 20 μL of 1 M NaHCO₃ was added, followed by the addition of 100 μL of 1% (w/v) 1-fluro-2,4-dinitrophenyl-5-L-alanine amide (Marfey’s reagent) in acetone. The solution was vortexed and then incubated at 37 °C for 1 h. The reactions were quenched by the addition of 20 μL of 1 N HCl. The samples were diluted by the addition of 810 μL of MeCN and chromatographed on a Thermo Scientific Accucore C₁₈ reversed phase LC column (250 × 2.1 mm, 2.6 μm particle size) coupled to a Thermo Fisher Scientific Orbitrap ID-X Tridrib mass spectrometer operating in the negative ionization mode with an electrospray ionization source. The chromatographic method for sample analysis involved elution with water with 10 mM ammonium acetate and 0.1% acetic acid (mobile phase solvent A) and 90:10 isopropanol/water with 10 mM ammonium acetate (mobile phase solvent B) using the following gradient program: 0 min 8% B; 5 min 25% B; 7 min 90% B; 7.4 min 100% B; 10.5 min 100% B; 10.7 min 5% B; and 12 min 8% B. The flow rate was 0.4 mL/min. The column temperature was set to 40 °C, and the injection volume was 0.5 μL. The MeOH extracts of pulvzerized sponge tissues were derivatized using the same protocol as described above, with the only deviation being the use of 200 μL of sponge extract instead of 50 μL of aqueous solution of standards.

Isotope Standard Spiking in Sponge Tissues. Three biological replicates for each sponge species were used in this study. The isotopic standard for 2 was obtained commercially. In a 2 mL Eppendorf safe-lock tube, lyophilized sponge tissues were homogenized with two tungsten carbide beads in a QIAGEN TissueLyser II at 20 Hz for 20 min, in two cycles of 10 min each. The pulverized sponge tissue was weighed in Eppendorf tubes and known concentrations of stable isotope standards, 20 μL of 1 M NaHCO₃, were added, followed by the addition of 100 μL of 1% (w/v) 1-fluro-2,4-dinitrophenyl-5-L-alanine amide (Marfey’s reagent) in acetone. The solution was vortexed and then incubated at 37 °C for 1 h. The reactions were quenched by the addition of 20 μL of 1 N HCl. The samples were diluted by the addition of 810 μL of MeCN and chromatographed on a Thermo Scientific Accucore C₁₈ reversed phase LC column (250 × 2.1 mm, 2.6 μm particle size) coupled to a Thermo Fisher Scientific Orbitrap ID-X Tridrib mass spectrometer operating in the negative ionization mode with an electrospray ionization source. The chromatographic method for sample analysis involved elution with water with 10 mM ammonium acetate and 0.1% acetic acid (mobile phase solvent A) and 90:10 isopropanol/water with 10 mM ammonium acetate (mobile phase solvent B) using the following gradient program: 0 min 8% B; 5 min 25% B; 7 min 90% B; 7.4 min 100% B; 10.5 min 100% B; 10.7 min 5% B; and 12 min 8% B. The flow rate was 0.4 mL/min. The column temperature was set to 40 °C, and the injection volume was 0.5 μL. The MeOH extracts of pulvzerized sponge tissues were derivatized using the same protocol as described above, with the only deviation being the use of 200 μL of sponge extract instead of 50 μL of aqueous solution of standards.

Development of LC–MS/MS Method. LC/MS data were acquired using a Waters Corporation ACQUITY UPLC BEH Amide column (2.1 × 150 mm, 1.7 μm particle size) coupled to a high-resolution accurate mass Orbitrap ID-X Tridrib mass spectrometer. The chromatographic method for sample analysis involved elution with 20:80 water/MeCN with 10 mM ammonium formate and 0.1% formic acid (mobile phase A) and MeCN and 0.1% formic acid (mobile phase B) using the following gradient program: 0 min 5% A; 0.5 min 5% A; 8 min 60% A; 9.4 min 60% A; 9.5 min 5% A; and 12 min 5% A. The flow rate was set at 0.4 mL/min. The column temperature was set to 40 °C, and the injection volume was 1 μL. The mass spectra were acquired on the Orbitrap ID-X tridrib spectrometer with full scan and targeted MS. Full scan data were collected in the positive mode from 100 to 600 m/z with a resolution of 30 000 and the targeted MS² data were collected with an isolation window of 0.8 m/z and HCD precursor activation of 40%. The product ions were collected in the Orbitrap at a resolution of 30 000. Inclusion lists including 1a, 2, and their respective isotope standards were employed for acquiring the MS² data. The raw data files were processed using Xcalibur 4.3.73.11 (Thermo Fisher Scientific) and manually curated to extract peak areas for the metabolites of interest.

Limit of Detection. The limit of detection (LOD) is defined here as the lowest concentration of a metabolite in a sample detected using the mass spectrometer. Samples of different concentrations for the synthetic 1a and 2, ranging from 50 nM to 10 μM, were prepared by serial dilution. Separate calibration curves were generated for 1a and 2 by plotting the response factor (peak areas) against corresponding metabolite concentrations. The LOD was calculated from the external calibration curves based on the standard deviation of the response (σ) and the slope (s) using the equation; LOD = 3.3σ/s.

Calculations for the Abundance of 1a and 2. The ratio of peak areas of endogenous 1a and 2 to the peak areas of spiked isotopic standards (along y-axes) versus the amount of isotopic standard added per milligram of sponge tissue (along x-axes) were plotted. Data points on these plots were fitted to linear functions. Equating the value of “y” as 1 in the linear equation of the calibration curves for 1a and 2 delivered their corresponding absolute concentrations in the sponge tissue on the x-axes.

RESULTS AND DISCUSSION

Stereochemical Assignment of 1. We have previously reported the detection of 1 in Aplysina, Aiolochroia, Styllisa, and Ianthella spp. sponges (Figure 1D, Table 1). However, the stereochemistry at the 1-Cα remained indeterminate. A standard for the l-isomer, 1a, was obtained commercially. The d-isomer, 1b, was synthesized by guanidinylation of the side chain ε-amine of d-lysine. Both standards were derivatized by Marfey’s reagent yielding a pair of diastereomers (Figure 2A).
The stereochemical assignment based on the data presented in this study is consistent with adducts of the MS<sup>1</sup> parent ions and the MS<sup>2</sup> product ions observed for (A) 1a and its respective isotopic standards and (B) 2 and the respective isotopic standard. The MS<sup>2</sup> EIC (top) and SRM chromatogram (bottom) observed for (C) 1a, (D) 2, (E) isotopic standard of 1a, and (F) isotopic standard of 2. Retention times and peak profiles of the MS<sup>2</sup> EICs are identical to that of the respective SRM chromatograms. 13C isotopes are represented as red dots, 15N isotopes are denoted by green boldface letter “N”. 

Figure 3. SRM transitions for 1a and 2, and their respective isotopic standards. MS<sup>1</sup> parent ions and MS<sup>2</sup> product ions observed for (A) 1a and its respective isotopic standard and (B) 2 and the respective isotopic standard. The MS<sup>2</sup> EIC (top) and SRM chromatogram (bottom) observed for (C) 1a, (D) 2, (E) isotopic standard of 1a, and (F) isotopic standard of 2. Retention times and peak profiles of the MS<sup>2</sup> EICs are identical to that of the respective SRM chromatograms. 13C isotopes are represented as red dots, 15N isotopes are denoted by green boldface letter “N”. 

To query the absolute abundance of 1a and 2 in marine sponges, we observed oxidative decarboxylation followed by imine hydrolysis to yield a MS<sup>2</sup> Ca-aldehyde product ion. Thus, for 1a, the SRM is based on the MS<sup>1</sup> m/z 189 → MS<sup>2</sup> m/z 144 transition (Figure 3A). For 2, the corresponding SRM is based on the MS<sup>1</sup> m/z 175 → MS<sup>2</sup> m/z 130 transition (Figure 3B). For 1a and 2 standards, EICs for MS<sup>1</sup> m/z 189 and MS<sup>1</sup> m/z 175 (corresponding to MS<sup>1</sup> ions detected for 1a and 2, respectively) and for SRM transitions m/z 189 → m/z 144 (for 1a) and m/z 175 → m/z 130 (for 2) demonstrated identical retention times and chromatographic profiles (Figure 3C,D), respectively. Next, an isotopic standard for 1a was synthesized by guanidinylation of commercially available isotopically labeled 3. An isotopic standard for 2 was commercially obtained. For isotopic standards of 1a and 2, EICs for MS<sup>1</sup> m/z 197 and MS<sup>1</sup> m/z 185 (corresponding to MS<sup>1</sup> ions detected for isotopic standards for 1a and 2, respectively) and for SRM transitions m/z 197 → m/z 150 and m/z 185 → m/z 138 demonstrated identical retention times and chromatographic profiles (Figure 3E,F), respectively. For sponge extracts, areas under the SRM chromatograms were used for quantification of the abundance of 1a and 2.

To minimize matrix effects, different amounts of isotopic standards were directly added to lyophilized and pulverized sponge tissues, followed by extraction and quantification. Assuming identical ionization of 1a and 2 as compared to their respective isotopic standards, the relative peak area ratios (the SRM chromatogram peak area for 1a divided by peak area of its isotopic standard; similarly for 2) were plotted against the concentration of isotopic standard added to the sponge tissue (Figures S3–S10). When the SRM peak area ratio was unity, the amount of isotopic standard added to the sponge tissue would translate to the abundance of 1a and 2 in sponge tissues. Using this methodology, the absolute abundance of 1a and 2 determined in different sponge tissues is illustrated in Figure 4. Calculated LODs for 1a and 2 were lower than the concentrations of 1a and 2 detected in sponge tissues used in this study (Figures S11 and S12).

The abundance of the proteinogenic amino acid 2 ranged from the 68.7 ng/mg sponge tissue to 9.1 ng/mg sponge tissue (7.5-fold variation) with the maximum concentration recorded in Aplysina sp. and the minimum in Stylissa sp. sponge (Figure 4). The variation in the abundance of 1a was much greater. The highest concentration of 1a was recorded in Stylissa sp. (453.5 ng/mg sponge tissue) and the lowest in Aplysina sp.
are both substrates for the production of NO, a metabolite with important consequences on marine invertebrate physiology and development. This study provides the framework for further investigating the biosynthesis and role of L-homoarginine in marine sponge holobionts.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c05685.

Nuclear magnetic resonance spectra and curves demonstrating determination of the abundance of arginine and homoarginine in sponge tissues (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors acknowledge support from the National Science Foundation (NSF, CHE-2004030), the National Institutes of Health (NIH, GM142882), and the Research Corporation for Science Advancement to V.A. and support from the Georgia Institute of Technology’s Systems Mass Spectrometry Core Facility.

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**SUPPLEMENTARY INFORMATION FOR:**

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**SUPPLEMENTARY FIGURES**

![Figure S1. 1H-NMR spectra for 1b in CD$_3$OD.](image-url)
Figure S2. $^1$H-NMR for isotopic standard of 1a in D$_2$O.
Figure S3. Calibration curve generated for the quantification of 1a in the sponge of genus *Aplysina*. 

Equation: 
\[ y = 1.51938 - 14.67331x \]

| Statistic               | Value              |
|-------------------------|--------------------|
| Equation                | \( y = a + bx \)   |
| Plot                    | B                  |
| Weight                  | Instrumental (+1e16) |
| Intercept               | 1.51938 ± 0.09424  |
| Slope                   | -14.67331 ± 1.67656 |
| Residual Sum of Squares | 12.5311            |
| Pearson's r             | -0.9972            |
| R-Square (COD)          | 0.97455            |
| Adj. R-Square           | 0.96163            |
Figure S4. Calibration curve generated for the quantification of 1a in the sponge of genus Aiolochroia.

\[ y = 1.7627 - 13.52361x \]

| Equation | \[ y = a + bx \] |
|----------|------------------|
| Plot     | B                |
| Weight   | Instrumental (=1/s2) |
| Intercept| 1.7627 ± 0.11025 |
| Slope    | -13.52361 ± 1.20006 |
| Residual Sum of Squares | 18.60243 |
| Pearson's r | -0.9921 |
| R-Square (COD) | 0.98426 |
| Adj. R-Square | 0.97639 |
Figure S5. Calibration curve generated for the quantification of 1a in the sponge of genus *Ianthella*. 

\[ y = 2.64202 - 0.7441x \] 

| Equation   | \( y = a + bx \) |
|------------|-------------------|
| Plot       | a                 |
| Weight     | Instrumental (=1/s²) |
| Intercept  | 2.64202 ± 0.06555 |
| Slope      | -0.7441 ± 0.04162 |
| Residual Sum of Squares | 84.13716 |
| Pearson's r | -0.99089 |
| R-Square (COD)      | 0.99378 |
| Adj. R-Square       | 0.99087 |

\( y = 1; x = 2.2067 \)
Figure S6. Calibration curve generated for the quantification of 1a in the sponge of genus *Stylissa.*
Figure S7. Calibration curve generated for the quantification of 2 in the sponge of genus *Aplysina*.
Figure S8. Calibration curve generated for the quantification of 2 in the sponge of genus *Aiolochroia.*
**Figure S9.** Calibration curve generated for the quantification of 2 in the sponge of genus *Stylissa.*
Figure S10. Calibration curve generated for the quantification of 2 in the sponge of genus *Ianthella.*

\[
y = 1.96342 - 4.02071x
\]

| Equation | \( y = a + bx \) |
|----------|------------------|
| Plot     | 8                |
| Weight   | Instrumental (10±2) |
| Intercept| 1.96342 ± 0.11800 |
| Slope    | -4.02071 ± 0.33583 |
| Residual Sum of Squares | 265.11514 |
| Pearson's r | -0.98633 |
| R-Square (COD) | 0.97285 |
| Adj. R-Square | 0.99607 |

\( y = 1; x = 0.239 \)
Figure S11. Calibration curve generated for the linearity test of the analytical standard 1a. LOD calculation is shown in the inset of the figure.
Figure S12. Calibration curve generated for the linearity test of the analytical standard 2. LOD calculation is shown in the inset of the figure.

\[
\text{LOD} = 3.3 \times (\text{Std. error of X-intercept/slope})
\]
\[
= 3.3 \times \left(\frac{4229.1809}{33.32324} \cdot 0.92152\right)
\]
\[
= 418.8200 \text{ nM}
\]
\[
= 0.0418 \text{ nmoles}
\]