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TIO_2-CATALYZED SYNTHESIS OF A PRIMARY AMINE NONOATE

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HNO induces vascular and myocardial activities, exhibits anticancer and antioxidant activities and inhibits platelet aggregation. However, the mechanism by which it functions is still poorly understood. Primary amine NONOates are HNO donors that can be used to study and understand the chemical properties and physiological effects of HNO. However, its synthesis involves very high pressure and low temperature conditions and requires specialized glassware and equipment.

The aim. *In this study, a method for the synthesis of an HNO donor, a primary amine NONOate based on cyclohexylamine, that does not involve specialized glassware and high pressure is developed.*

Materials and methods. *The synthesis of primary amine NONOate was performed through the reaction of cyclohexylamine and NO gas, in the presence of sodium methoxide and TiO_2, for 24 h. The resulting product was isolated and characterized spectrophotometrically to determine its identity, decomposition kinetics, and HNO release profile at physiological pH. The synthesis method was further optimized based on reaction time, solvent, catalyst loading, and temperature.*

Results*. The TiO_2-catalyzed method yielded the desired product which was characterized spectrophotometrically. The product shows typical electronic spectrum of a NONOate (λ_{max} = 250 nm), followed first-order decomposition kinetics, and released both HNO and NO at physiological pH, which are characteristics of primary amine NONOate. The method yielded 5.98 mg cyclohexylamine NONOate which is equivalent to a percent yield of 0.0550%. The yield of the method was low but comparable to the yield from conventional method (1–20%) which requires –78°C and 50 psi NO. Furthermore, the yield of this method is sufficient for chemical and biological assays.*

Conclusion*. This study proposed a possible alternative method for synthesizing cyclohexylamine NONOate using TiO_2 catalysis at ambient conditions. Although the yield is lower than that of conventional method, the result is quite sufficient for chemical and biological assays. This approach eliminates the requirement of using specialized high-pressure and low-temperature equipment, making the study of HNO donors more feasible*

Keywords*: HNO donor, TiO_2 catalyst, NONOate*

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1. Introduction

Nitroxyl (HNO) has recently emerged as an important pharmacological agent, particularly in cardiovascular system and in cancer biology [1–4]. Studies of HNO require the use of HNO donors, due to the rapid irreversible dimerization: $2HNO \rightarrow [HONNOH] \rightarrow N_2O + H_2O$. Donors of HNO have been shown to induce pharmacological responses, including enhancement of myocardial contractility [5], preconditioning against ischemia/reperfusion injury [6, 7], induction of apoptosis and suppression of tumor angiogenesis [8], and inhibition of alcohol metabolism [9–11]. The clinical efficacy of HNO donors has been demonstrated with cyanamide (NH_2CN) in the treatment of alcohol abuse [9, 11]. The more recent discovery that HNO donors may also be beneficial in the treatment of heart failure [12] has stimulated the development of a variety of structurally diverse HNO donors and related prodrugs [13–15].

One such class of HNO donor is primary amine diazeniumdiolates or NONOates [16, 17], which are adducts of the NO dimer and primary amine. Primary amine NONOates spontaneously decompose at physiological conditions to release HNO [18]. To date, only two primary amine NONOates have been reported to be stable. These are based on isopropylamine and cyclohexylamine. These NONOates provide a useful basis to develop new donors of HNO.

NONOates are synthesized by the direct reaction of NO with an amine [19]. The method may sound simple but special conditions are needed for the reaction to take place. The conventional preparation of NONOates needs specialized equipment due to the high pressure and low temperature requirements [20]. The reaction is slightly slow due to the low temperature condition [21]. Moreover, low yields are obtained from the conventional preparation. Because of these reasons, a catalytic method is highly desirable. The group of Huang et al. have used titanium dioxide (TiO_2) as catalyst for the synthesis of secondary amine NONOates. This study adapts the study of Huang et al. [22] for the preparation of primary amine NONOates. A primary amine NONOate from cyclohexylamine was synthesized under ambient conditions in the

presence of TiO_2 catalyst. Optimization of the synthetic procedure was also performed. TiO_2 has been widely used as a catalyst for different types of chemical reactions due to its high chemical stability, low toxicity, and high surface area when used in the nanometer size scale [23]. The high surface area of TiO_2 has been shown to activate gas molecules such as NO [24]. Activation of NO increases its reactivity and makes the reaction with amine at room temperature and pressure plausible [22].

2. Planning (methodology) of research

1. Utilize the use of NO gas generated from a modified apparatus setup for the synthesis of primary amine NONOates.

2. Determine the optimal reaction conditions to achieve maximum yield and purity of primary NONOates using TiO_2 as a catalyst.

3. Investigate the properties of primary amine NONOates through spectrophotochemical analysis, focusing on the first-order decomposition kinetics, and the release of both HNO and NO under physiological pH conditions.

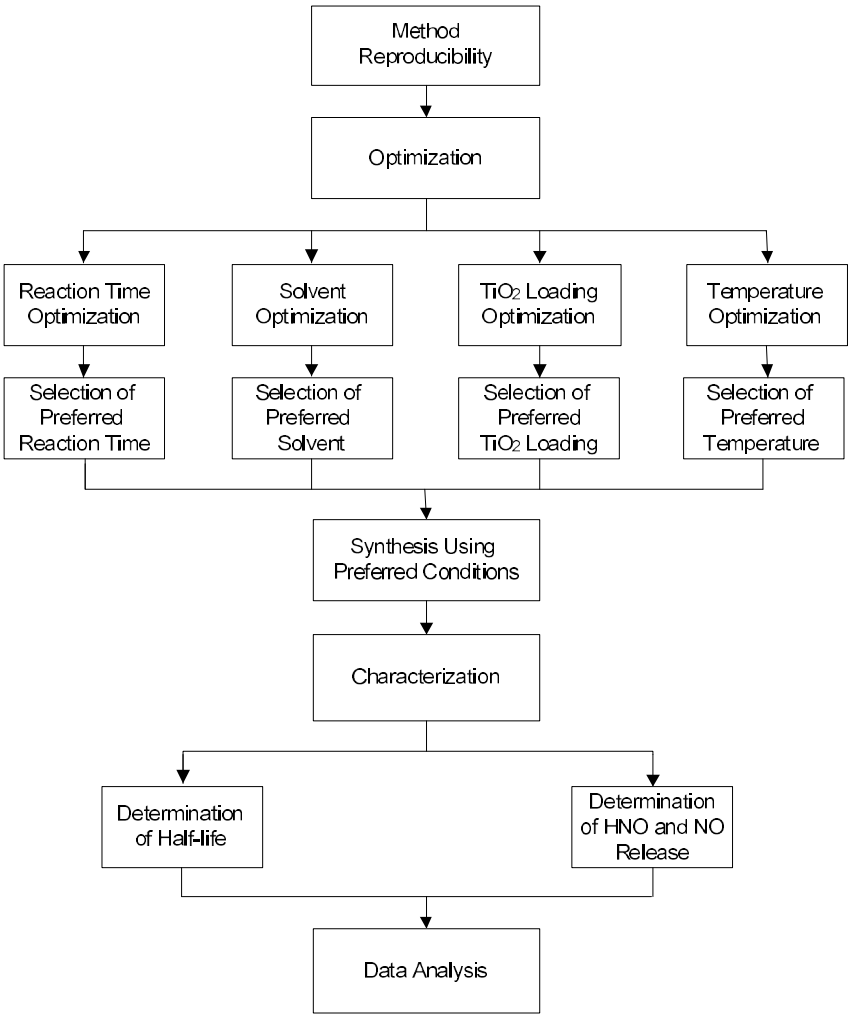


Fig. 1. Overall flowchart of the experiment

3. Materials and methods

Fig. 1 shows the overall experimental design. Method reproducibility assessment was first conducted to obtain a baseline experimental condition. This was followed by optimization where four experimental variables were examined. Characterization of the NONOate was done next.

Method reproducibility assessment and baseline conditions

The reproducibility of the method was examined by synthesizing cyclohexylamine NONOate in three trials using the modified method from Huang et al. [22], outlined in Fig. 2. The standard deviation was calculated to assess method reproducibility.

The experimental conditions used for the assessment of method reproducibility were selected based on literature [20, 21] and the ease of execution in the laboratory. These conditions (Table 1) are designated as baseline conditions.

NO gas is not commercially available in the Philippines, so NO was generated in situ using a home-made laboratory equipment (Fig. 3). The generation of NO was adapted from Aga and Hughes [25], which is based on the disproportionation of nitrous acid (Equation 1) under acidic condition

$$3HNO_2 \rightarrow 2NO + HNO_3 + H_2O. \quad (1)$$

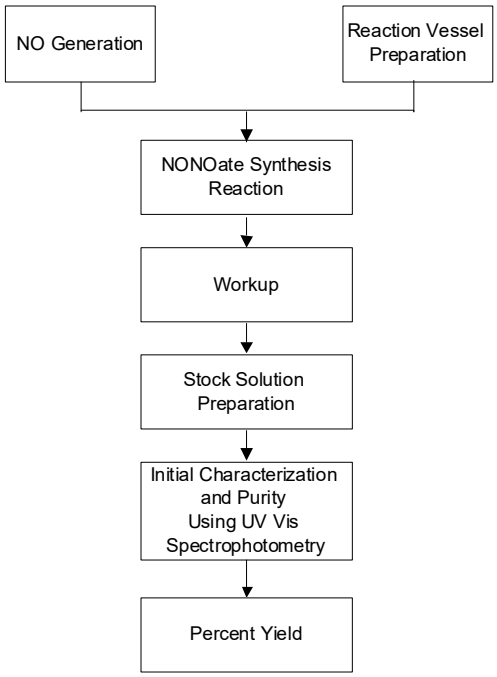


Fig. 2. Flowchart for general method of NONOate synthesis

A concentrated solution of sodium nitrite (120 g in 200 mL water) was added dropwise from a burette into a

l-L filter flask containing sulfuric acid (200 mL, 6 *M*) to produce NO. The filter flask was covered with a bored rubber stopper. The tip of the burette was fitted into the hole of the rubber stopper and secured with Teflon tape to ensure airtight system. Clear plastic tubing was attached to the side arm of the filter flask which led the produced NO gas into a sequence of six 500-mL filter flasks, each containing a corresponding solution, which served as a trap to purify the NO gas. In each case, the transfer of NO into the succeeding flask was through a clear plastic tubing connected to a Pasteur pipette submerged into the solution to generate small bubbles of NO gas. Similar with the 1-L reaction vessel, a bored rubber stopper was used to seal the filter flasks and the Pasteur pipette was fitted into the hole which was secured with Teflon tape to ensure airtight system. The rubber stopper of the filter flasks may rise upon increased pressure in the system, so the rubber stopper was fixed to the filter flask using cable ties.

Table 1

Baseline conditions for method reproducibility assessment

Parameter	Baseline Condition
Reaction Time	24 h
Solvent	methanol
TiO_2 Loading	50 mg
Temperature	room temperature

Note: NO Generation [25].

Fig. 3. NO-generating setup

The first filter flask was empty, intended to collect any suck back from the second filter flask. The second and third filter flasks contained 1 *M* NaOH solution to trap other nitrogen oxide species and to neutralize acid carried over from the reaction flask. The third and fourth filter flasks contained distilled water to trap droplets of NaOH. The final flask contained desiccant to dry the outgoing gas. Before generation of NO commenced, argon was passed through the entire experimental system for at least 3 h to deoxygenate the whole apparatus and the solutions. This was achieved by introducing argon through the burette from which it passes through the rest of the apparatus. The NO gas was verified by examining its IR spectrum. The NO-generating setup was then used to bubble NO gas directly into the reaction vessel for the synthesis of cyclohexylamine NONOate.

NONOate synthesis [20]

A 100-mL three-necked flask was used as the reaction vessel for the synthesis of cyclohexylamine NONOate. Cyclohexylamine (6.86 mL, 0.06 mol), 50 mg titanium dioxide, 1 g sodium methoxide and 50 mL methanol were mixed in the reaction vessel and homogenized using sonicator. To provide an anaerobic atmosphere, the flask was sealed by covering the two outer necks with rubber septum. The two outer necks also served as the inlet and outlet of NO and Ar through a stainless-steel needle wire punctured through the rubber septum. The middle neck was covered with a balloon, secured by a cable tie, which equilibrates the system with atmospheric pressure. The sealed flask was purged with argon for 30 min to deoxygenate the reaction vessel.

The reaction was started by bubbling NO from the NO-generating setup into the reaction mixture, with constant stirring. After 24 h, the reaction was terminated by disconnecting the reaction vessel from the NO-generating setup. The reaction vessel was then purged with argon for 30 min to vent off the excess NO before opening the vessel.

To collect the crude product, the reaction mixture was transferred to a 500-mL filter flask. Cold diethyl ether (200 mL) was then added to the reaction mixture to precipitate NONOate. The resulting precipitate was filtered to remove solvent and excess reactant, washed with diethyl ether, and dried to give a white powder. The white powder was dissolved in 20 mL methanol, magnetically stirred for 3 min, and filtered to remove TiO_2. Finally, cold diethyl ether (200 mL) was added to the methanol solution to precipitate the product which was then filtered to collect the crude product. While in the filter paper, the crude product was dried in an evacuated container placed in the freezer for 24 h.

Stock solution and storage.

The crude product was weighed by difference by transferring the dried product into a pre-weighed stoppered vial. From this, stock solution was prepared by dissolving crude product (0.1 g) in NaOH (1 mL, 0.1 *M*) in a stoppered vial. Both vials were stored in freezer.

Initial characterization and determination of purity.

The presence of NONOate in the crude product was initially verified by scanning its UV-Vis spectrum. The appearance of peak at λ_{max} = 250 nm suggests the presence of NONOate [26]. Furthermore, the extinction coefficient (ε_{250} = 8,000 $M^{-1}cm^{-1}$) of NONOates at this wavelength was used to calculate the purity of the crude product. Stock solution (20 µL) was mixed with NaOH (0.98 mL, 0.01 *M*) in a 1-mL cuvette. The percent yield (NONOate) of the synthesis was calculated based on the ratio of the amount of NONOate obtained and the theoretical stoichiometric amount (equation (2)

$$\%\ \text{yield}\left(\text{NONOate}\right) = \\ = \frac{\text{actual mass of NONOate}}{\text{theoretical mass of NONOate}} \times 100\%. \quad (2)$$

The amount of NONOate in the crude product was determined using the absorbance at 250 nm (ε_{250} = 8,000 $M^{-1}cm^{-1}$) according to equation (3)

$$\text{actual mass of NONOate} = \\ = \left(\frac{A_{250}}{\varepsilon_{250}}\right)\left(V_{mL}\right)\left(f\right)\left(M\right), \quad (3)$$

where A_{250} is the absorbance at 250 nm, V_{mL} is the volume of the solution in the cuvette, f is the dilution factor (typically 100) and M is the molar mass of the sodium salt of cyclohexylamine (181.16 g/mol).

Optimization of NONOate synthesis.

The synthesis of cyclohexylamine NONOate was optimized in terms of the following parameters: reaction time, solvent, TiO_2 loading and reaction temperature. Each parameter consists of a set of optimization conditions listed in Table 2. For each parameter, the baseline condition (Table 1) was replaced with the optimization conditions, one at a time, while maintaining the baseline conditions for other parameters constant. The percent yield was determined for each variation made. The optimization condition with the highest yield was selected for each parameter and designated as the preferred condition.

Table 2

Optimization conditions and baseline conditions

Parameter	Conditions
Reaction time	6, 12, 24, 48 h
Solvent	methanol, diethyl ether, 1:1 methanol-ether mixture
TiO_2 loading	10, 50, 100 mg
Temperature	0°C, room temperature

Reaction time optimization.

The duration of the synthesis was optimized by examining the effect of varying the reaction time on the yield while maintaining other parameters at baseline conditions. The reaction time used for the optimization were 6, 12, 24 and 48 h. The reaction time with the highest yield was designated as the preferred reaction time.

TiO_2 loading optimization.

The amount of TiO_2 used for the synthesis was optimized by examining the effect of varying the TiO_2 loading on the yield while maintaining other parameters at baseline conditions. The amount of TiO_2 used for the optimization were 10, 50 and 100 mg. The TiO_2 loading with the highest yield was designated as the preferred TiO_2 loading.

Solvent optimization.

The solvent used for the synthesis was optimized by examining the effect of varying the solvent on the yield while maintaining other parameters at baseline conditions. The solvent used for the optimization were methanol, diethylether and 1 : 1 methanol-diethylether mixture. The solvent that gave the highest yield was designated as the preferred solvent.

Temperature optimization.

The temperature used for the synthesis was optimized by examining the effect of varying temperature on the yield while maintaining other parameters at baseline conditions. The temperatures used for the optimization were room temperature and around 0°C. The temperature with the highest yield was designated as the preferred temperature. The temperature around 0°C was achieved using an ice bath which contains saturated NaCl solution at –5°C. The solution was replaced when the temperature rose to 5°C.

Synthesis using preferred conditions.

The preferred conditions from the optimization were employed in the synthesis of cyclohexylamine NONOate to determine the yield of the method. The product from this synthesis was used for partial characterization.

NONOate partial characterization.

The widely used method for the characterization of NONOates is UV-Vis spectrophotometry. The crude product was initially characterized by the presence of peak at λ_{max} = 250 nm. Further characterization includes kinetics study of NONOate decomposition and detection of HNO and NO release by reductive nitrosylation of metMb.

The product obtained from the synthesis of cyclohexylamine NONOate using the preferred conditions was used in the preparation of stock solution. Crude product (0.2 g) was dissolved in NaOH (1 mL, 0.01 M) in a stoppered vial. Stock solutions were similarly prepared for myoglobin (0.5 mM), glutathione (25 mM) and DTPA (10 mM). The stock solutions prepared were purged with argon for 15 min while in an ice bath.

Kinetics study of NONOate decomposition.

The product was characterized by comparing the half-life (pH 7.4, room temperature) of decomposition with literature. The decomposition profile of the product was obtained by monitoring the decrease in absorbance at 250 nm, which is characteristic of NONOate functionality. Phosphate buffered saline (PBS, 2.4723 mL, 7.4 final pH) and DTPA (12.5 µL, 50 µM final) were mixed in a cuvette and was used to blank the UV-Vis spectrophotometer. The decomposition was initiated by introducing NONOate (15.2 µL stock solution, 100 µM final) into the assay solution which was quickly mixed using pipettor. The decomposition profile was obtained by reading the UV-Vis spectrum (200–700 nm) of the solution every two minutes until the absorbance at λ_{max} (250 nm) is steady. The half-life of decomposition was calculated by fitting the profile to a first-order decomposition kinetics.

Reductive nitrosylation of MetMb.

The HNO and NO releasing ability of the product was studied through reductive nitrosylation of metMb. The formation of MbNO (λ_{max} = 502 nm, 630 nm) from metMb (λ_{max} = 543 nm, 575 nm) produces spectral shift in the UV-Vis spectrum which qualitatively determines the release of HNO, according to equation (4)

$$\text{metMb} + \text{HNO} \rightarrow \text{MbNO} + \text{H}^+. \quad (4)$$

Reductive nitrosylation was done in a deoxygenated environment using a cuvette with graded seal tube. PBS (2.3375 mL, 7.4 final pH) and DTPA (12.5 µL, 50 µM final) were mixed in the cuvette and sealed with rubber septum. The solution in the cuvette was purged by gently bubbling argon into the solution through a stainless-steel canula. MetMb (0.1 mL, 50 µM final) was added to the solution using airtight Hamilton syringe and mixed by agitating the cuvette. The bubbles formed were removed by carefully swirling the cuvette to pop the bubbles. The reaction was started by introducing NONOate (0.05 mL, 330 µM final), then carefully agitating the cuvette to mix the solution without forming bub-

bles. The spectral shift was observed by obtaining the UV-Vis spectrum (400–700 nm) of the solution every two minutes until the absorbance at 543 nm and 575 nm are constant (Table 3).

Table 3

λ_{max} and extinction coefficient (E) of MetMb and MbNO [27].

Parameter	λ_{max}, nm	Extinction Coefficient (ε), $M^{-1}cm^{-1}$
MetMb	502	10200
	630	3900
MbNO	543	11600
	575	10500

Reductive nitrosylation assay was also performed in the presence of glutathione (GSH) using the above procedure. GSH (25 µL, 250 µM final) was added to the solution together with DTPA (12.5 µL, 50 M final). Succeeding process follows the same procedure as above (metMb = 0.1 mL, 50 *M* final; NONOate = 0.05 mL, 330 µM final).

Instrumentation.

UV-Vis spectrophotometry was performed using Lasany UV-Vis Spectrophotometer Double Beam LI-2800. The Nicolet iS50 FT-IR equipped with gas cell accessory was used for IR spectroscopy. Fisher Accumet pH meter was used for reading the pH of solutions.

3. Results

Method feasibility and reproducibility.

The method adapted from Huang et al. was doable in the laboratory. However, modifications were made due to the unavailability of some equipment. For instance, NO was generated in the laboratory because NO cylinders are not commercially available in the Philippines. As such, strict measures were observed to ensure the purity of the NO produced. There were more difficulties encountered in the execution of the method. Based on the characterization of the crude product, the method yielded cyclohexylamine NONOate.

Characterization of NO gas.

The NO gas used for the synthesis of cyclohexylamine NONOate must be free of contaminants to avoid formation of side products. Specifically, the system must be oxygen-free to avoid the oxidation of NO to NO_2, which may interfere with the synthesis. The IR spectrum of NO from the NO-generating setup was examined for characterization and purity.

The IR spectrum of NO from the NO-generating setup agrees with literature (Fig. 4). The bands at ν_{NO} = 1815–1840 cm^{-1} and 1385–1340 cm^{-1} correlates with the NIST based IR spectrum of NO gas. Furthermore, there is no apparent presence of contaminants. Specifically, comparing the IR spectrum of the produced NO gas with the NIST based IR spectrum of NO_2 suggests the absence of NO_2.

Nanometer-size TiO_2 as catalyst.

The group of Huang et al. used nanometer-size TiO_2 (AEROXIDE® TiO_2 P25) from Evonik Degussa company as catalyst in synthesizing NONOates.[33] Nanometer-size TiO_2 was also used as catalyst in this study, purchased from Sigma-Aldrich (TiO_2 nanopowder, 21 nm primary particle size). An attempt to synthesize NONOate using normal TiO_2 was conducted but was unsuccessful. White powder was obtained as the crude product. However, the UV-Vis spectrum of the crude product did not exhibit a peak near 250 nm.

Properties and purity of the crude product.

The TiO_2-catalyzed synthesis of cyclohexylamine NONOate produced a white powder as the crude product. No further purification was done on the crude product due to the unstable nature of NONOate, which is not admissible to chromatographic methods of separation [26]. The crude product is soluble in water and methanol and precipitates in diethyl ether. UV-Vis spectrum exhibited λ_{max} = 253 nm which correlates with literature. The product easily absorbs moisture from air and must be stored in the freezer.

The purity of the crude product was determined using UV-Vis spectrophotometry and results showed that actual absorbance is very small compared with theoretical absorbance. The average ratio of actual to theoretical absorbance means that only 1.09% of the crude product is NONOate and the rest is impurity.

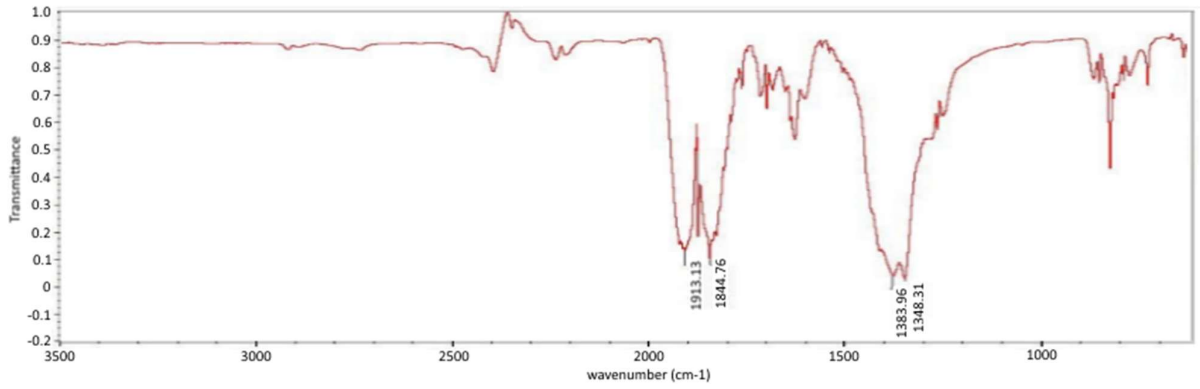
The synthesis method was subjected to blank analysis, i. e. following the same procedure but in the absence of NO, and yielded white powder as the crude product. UV-Vis spectrum of the crude product showed no peak at 250 nm. Based on the reagents present in the reaction mixture, the crude product is mostly composed of sodium methoxide.

The yield of the synthesis was very low, but reasonable. The yield using conventional method usually ranges from 1–20%, with maximum yield of 50% [26]. Conventional method of synthesis is usually done in –78°C, 40–50 psi NO and lasts for 5 days [26]. Considering these factors, the percent yield of the method in this study is justified. Furthermore, the yield from this method is sufficient for performing chemical and biological assays.

Using purity in the assessment of method reproducibility.

Due to the low purity of the crude product, the use of percent yield based on the weight of the crude product can lead to incorrect interpretation of data and conclusion, especially in the optimization. A different means of expressing the yield must be used to interpret the data correctly (Table 4).

Using the percent yield based on the weight of the crude product (i.e. not accounting for purity) lead to incorrect data interpretation in the reaction time optimization (Table 5). The yield was expected to increase with reaction time, but this is not reflected in the result. This data can lead to an incorrect conclusion that increasing the reaction time does not enhance the yield of the reaction. This means that the percent yield (crude product) cannot be used in the analysis of data. However, the amount of NONOate in the crude product (calculated using equation (3)) increases with reaction time. The increase in the amount of NONOate was outweighed by the amount of impurity in the crude product which led to the incorrect conclusion.



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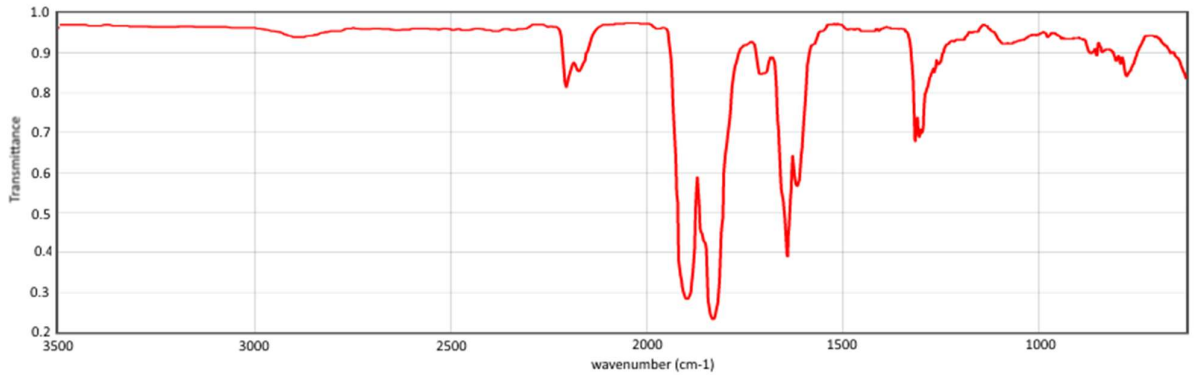

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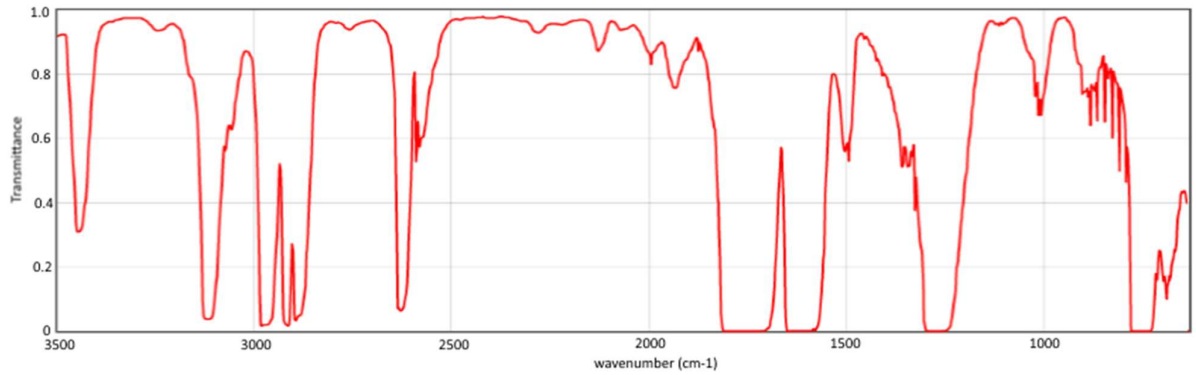

c

Fig. 4. IR Spectra of NO: *a* − experimental; *b*− literature [28] and NO_2 *c* − literature [29]

Table 3

Percent yield using the crude product weight

Trial	Crude product weight, g	Percent yield, % (crude product)
1	0.4285	3.94
2	0.4366	4.02
3	0.4263	3.92

Table 4

Percent yield (crude product) and the amount of NONOate in the reaction time optimization

Reaction time, h	Percent yield, % (crude product)	Amount of NONOate, mg
6	4.22	3.96
12	4.15	4.10
24	3.96	5.15
48	4.33	6.59

Percent yield based on the actual amount of NONOate in the crude product (Table 6) was another option to express the yield. The trend of the percent yield (NONOate) in the optimization mostly coincided with literature. However, close examination of data shows that the percent yield is greatly affected by the amount of crude product recovered from the workup. Higher crude product resulted in higher percent yield (NONOate), which gave a trend for solvent optimization that does not agree with literature (diethyl ether < methanol-ether mixture < methanol) [30]. The percent yield (NONOate) can lead to incorrect interpretation of data because it does not consider the variabilities in the amount of crude product recovered. Therefore, percent yield (NONOate) cannot be used in the analysis of data.

Table 5

Crude product weight, amount of NONOate and percent yield (NONOate) from the optimization

Parameter	Condition	Crude product weight, g	Amount of NONOate, mg	Percent yield (NONOate), %
Reaction Time, h	6	0.4595	3.96	0.0365
	12	0.4511	4.10	0.0378
	24	0.4305	5.15	0.0475
	48	0.4710	6.59	0.0606
Solvent	methanol	0.4305	5.15	0.0475
	1:1 methanol – ether	0.4933	5.39	0.0496
	ether	0.3243	1.73	0.0159
TiO_2 Loading, mg	10	0.4372	4.93	0.0453
	50	0.4305	5.15	0.0475
	100	0.4085	3.99	0.0367
Temperature	0°C	0.4571	6.29	0.0578
	Room temperature	0.4305	5.15	0.0475

The purity of the crude product is a better choice for data analysis. Unlike percent yield, the calculation of purity considers the amount of NONOate in the crude product and the total product recovered.

$$\text{purity} = \frac{\text{NONOate}}{\text{crude product}} = \frac{\text{NONOate}}{\text{NONOate} + \text{impurity}} = \frac{\text{NONOate}}{\text{impurity}}.$$

Analysis of the synthesis method in terms of purity of the product is logical because we observed that the amount of NONOate formed increases with increasing amount of crude product. Furthermore, since both NONOate and the impurity went through the same process in the workup procedure, the ratio of NONOate to impurity would be unaffected by the variation in the amount of crude product recovered. The purity of the crude product was expressed in parts per thousand (ppt). Purity will be referred to as "yield based on purity", "yield (purity)" or "yield" throughout the discussion.

Method reproducibility. Cyclohexylamine NONOate was synthesized in triplicate using the baseline conditions shown in Table 1. The synthesis method is shown to be reproducible (Table 7) with a standard deviation of 0.4662, which corresponds to a relative standard deviation (RSD) equal to 3.88%.

The standard deviation is proportional to the amount of error in the synthesis method. The standard deviation of the method was used in the optimization to determine whether the change in yield was cause by the variation of conditions or by the errors in the experiment [31, 32]. Changes in the yield greater than 1SD, 2SD and 3SD means a significant change caused by varying the condition. On the other hand, changes in the yield less than 1SD means an insignificant change caused by the error in the synthesis method. Only one trial was performed in the optimization due to limited amount of cyclohexylamine.

Table 7

Yield (Purity) and the amount of NONOate from the method reproducibility assessment

Trial	Yield (purity), ppt	Amount of NONOate, mg
1	12.10	5.19
2	11.50	5.02
3	12.42	5.30

Optimization.

From the optimization process, summarized in Fig. 5, the preferred conditions are 48 h reaction time, methanol as solvent, 50 mg TiO_2 at 0°C. To determine whether the yields of the optimization are significantly different from the yield of baseline conditions, comparison was made.

Only two conditions generated yields greater than the baseline conditions (Fig. 6), namely 48 h (reaction time optimization) and 0°C (temperature optimization), which are 3SD greater than the baseline conditions. The rest of the optimization conditions generated lower yields than the baseline conditions, with yields ranging from –1SD (10 mg TiO_2), –2SD (1 : 1 methanol-ether mixture) and –3SD (6 h, 12 h, ether, 100 mg TiO_2) from the baseline conditions. No condition resulted to a yield lower than 1SD. This means that the changes in yield in the optimization are due to the effect of varying the conditions rather than experimental errors.

Reaction time optimization.

The baseline condition used for the reaction time parameter was 24 h. In the optimization, longer (48 h) and shorter (6 and 12 h) reaction time were used.

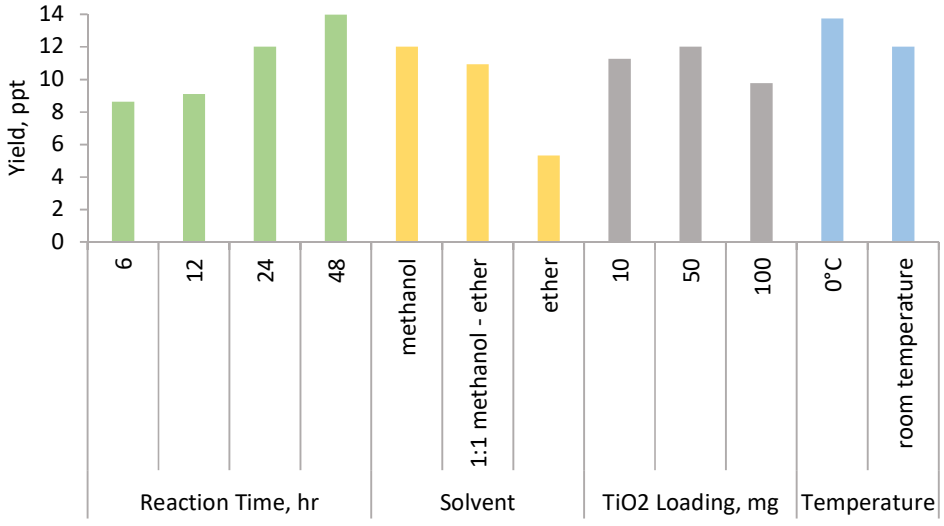

Fig. 5. Summary of yield from optimization

Fig. 6. Difference of optimization yield from baseline conditions

The preferred reaction time is 48 h with a yield of 13.99 ppt (6.59 mg), which is greater by 3SD than the baseline condition. This is expected because formation of product increases with time. Lowering the reaction time decreased the yield to 8.62 ppt (3.96 mg, –3SD) and 9.10 ppt (4.10 mg, –3SD) for 6 h and 12 h, respectively.

The optimization of reaction time resulted to the greatest increase in yield among the conditions used. Therefore, it is recommended to use longer reaction time for the experiment. However, optimization must be done to determine the maximum reaction time possible, since longer reaction time might lead to NONOate decomposition.

Solvent optimization.

The baseline condition used for the solvent parameter was methanol. This was adapted from the method of [22]. Conventional synthesis usually use diethyl ether as solvent [19], which was also used in the optimization. A 1:1 methanol-ether mixture was also used.

The solvent which gave the highest yield is methanol (12.01 ppt, 5.15 mg), which is also the baseline condition. NO is more soluble in methanol than in ether [30], which could explain this trend. Greater amount of dissolved NO in the reaction mixture results to higher collision frequency and yield. Both 1 : 1 methanol-ether mixture and ether generated lower yields than methanol, equal to 10.93 ppt (5.39 mg, –2 SD) and 5.32 ppt (1.73 mg, –3 SD), respectively.

TiO_2 loading optimization.

The baseline condition used for the TiO_2 loading parameter was 50 mg TiO_2. This was adapted from the method of [22]. In the optimization, one low (10 mg) and one high (100 mg) TiO_2 loading were used.

The preferred TiO_2 loading is 50 mg TiO_2 (12.01 ppt, 5.15 mg), which is also the baseline condition. This coincides with the optimization done in the study of Huang et al. [22]. Higher and lower amounts of TiO_2 resulted to lower yields of 11.27 ppt (4.93 mg, –1SD) and 9.77 ppt (3.99 mg, –3SD), respectively. The yield increased with the amount of catalyst used. However, extremely high TiO_2 loading eventually decreased the yield.

Temperature optimization.

The baseline condition used for temperature parameter was room temperature. This is the most convenient temperature to be used as the baseline condition. Only lower temperature (around 0°C) was used in the temperature optimization because higher temperature results to NONOate degradation [19]. The actual temperature of the setup was 0 ± 5°C, which was achieved by immersing the reaction vessel in an ice bath saturated with table salt.

The preferred temperature is 0°C with a yield of 13.76 ppt (6.29 mg) which is +3SD from the baseline condition. The formation of NONOate is an exothermic reaction [19]. Therefore, lower temperature increases the yield of the reaction, in accordance with Le Chatelier's Principle. Moreover, lower temperature slows down the decomposition of NONOate [19]. It is recommended to further optimize the method using lower temperature. Specifically, different solutions could be utilized for the ice bath, aside from NaCl solution.

Sodium methoxide optimization.

The amount of sodium methoxide was not optimized due to limited cyclohexylamine. Sodium methoxide was added to the reaction mixture for direct formation of NONOate sodium salt, instead of ammonium salt [22]. Sodium methoxide also shifts equilibrium towards the product [22]. Huang et al. used an equimolar amount of sodium methoxide with the theoretical NONOate yield. However, the synthesis method in this study yielded very low amount of NONOate, resulting to excessive amount of sodium methoxide in the crude product. Therefore, the amount of sodium methoxide should be optimized to achieve higher purity.

Synthesis using preferred conditions.

Cyclohexylamine NONOate was synthesized using the preferred conditions from the optimization to determine the effect of combining the preferred conditions and to assess the efficiency of the method. The yield for this synthesis is 15.26 ppt (Fig. 7), corresponding to 5.98 mg NONOate and a percent yield (NONOate) of 0.0550%.

Characterization of product.

The widely used method for the characterization of NONOates is UV-Vis spectrophotometry. The crude product was initially verified to contain NONOate by the presence of peak at λ_{max} = 253 nm (Fig. 8). Further characterization includes kinetics study of NONOate decomposition and detection of HNO and NO release by reductive nitrosylation of metMb.

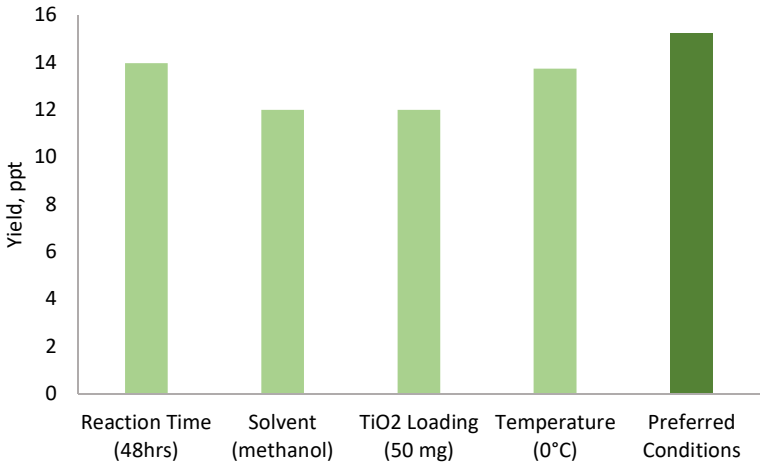

Fig. 7. Yield of individual and combination of preferred conditions

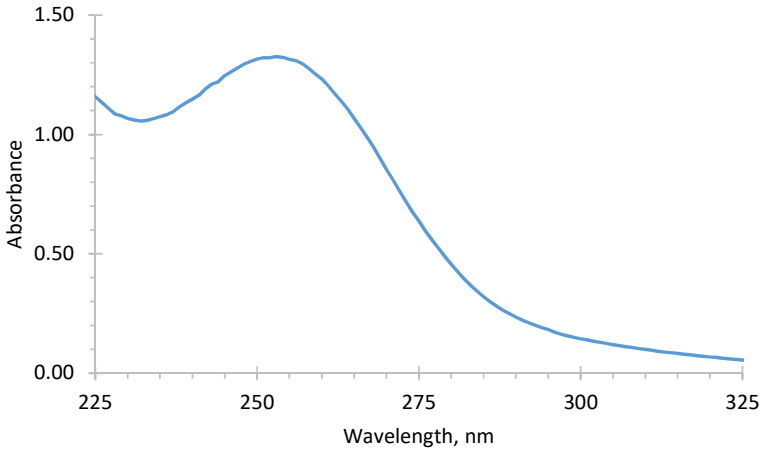

Fig. 8. The UV-Vis spectrum of the crude NONOate product

Kinetics of cyclohexylamine NONOate decomposition.

The decomposition kinetics of the crude product was studied by monitoring the decrease in absorbance at λ_{max} = 253 nm (Fig. 9). This was done by reading the UV-Vis spectrum (200–700 nm) of the solution every 2 min until the absorbance at λ_{max} was steady. The absorbance – time data was fitted to a first-order exponential decay which resulted to a half-life of 16.6 min.

The half-lives of NONOates are distinct, depending on the type of amine used [21]. Changing the amine affects the rate of NONOate decomposition due to the changes in the steric and electronic properties of the NONOate [33]. This makes the half-life a viable tool to characterize NONOates. The half-life of cyclohexylamine NONOate based on literature is 3.6 min [17] and 6.1 min [34]. The half-life of NONOate in this study is 3–4 times larger than in literature.

The difference of the experimental half-life from the literature value can be attributed to several factors. The main factor is temperature. The temperature used in the literature (37°C) [26, 34] is higher than the experimental temperature (25°C), which resulted to a lower decomposition rate in the experiment. Most studies perform kinetics assay at 37°C which is the biological temperature. A thermostat accessory for the UV-Vis spectrophotometer is needed to achieve this temperature. However, the Lasany UV-Vis Spectrophotometer Double Beam LI-2800, which was used in the experiment, does not have a thermostat accessory so the assay was performed at room temperature. The presence of impurity, particularly sodium methoxide, could be another factor for the difference in the half-life. Sodium methoxide may affect stability of the product which slowed down its decomposition [22]. The formation of a side-product could also be a possibility which could account for the difference in half-life.

The difference in the assay conditions and the low purity of the product caused the discrepancy in the experimental half-life. Therefore, similar assay conditions and purer product could be used for conclusive results.

Detection of HNO and NO release.

The detection of HNO and NO release from donor compounds is usually done indirectly and qualitatively [35, 36]. The widely used method in qualitatively determining the release of HNO and NO is by reductive nitrosylation of MetMb.

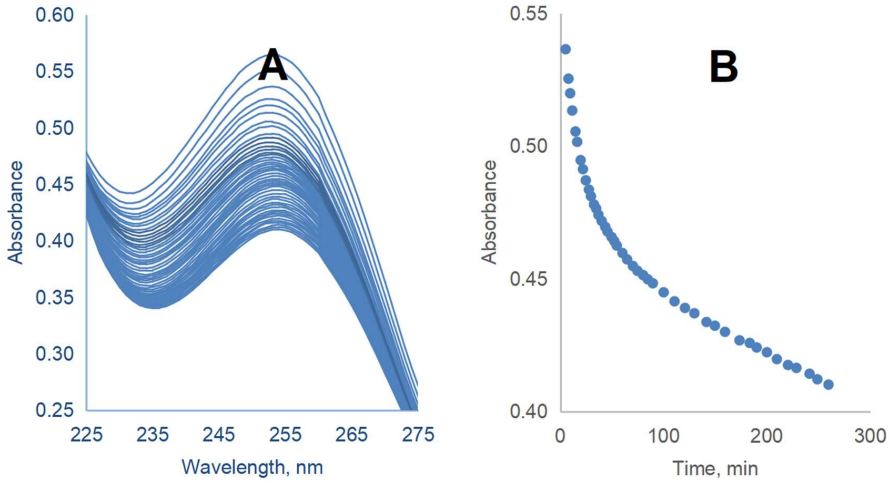

Fig. 9. The Decomposition profile of the crude product: A – scanning; B – single wavelength (253 nm)

Formation of MbNO (λ_{max} = 543 nm, 575 nm) [27] from metMb (λ_{max} = 502 nm, 630 nm) [27] results to a spectral shift, which qualitatively indicates the release of HNO (equation (5)). Furthermore, the high extinction coefficient of MbNO and metMb (Table 3) reduces interference from other absorbing species and allows for

convenient spectrophotometric monitoring to low micromolar concentrations [26]:

$$metMb + HNO \rightarrow MbNO + H^+, \quad (k = 8 \times 10^5\ M^{-1}s^{-1})\ [37]; \qquad (5)$$

$$metMb + NO \leftrightarrow metMbNO, \quad (k = 4.8 \times 10^4\ M^{-1}s^{-1},\ k_{-1} = 43\ s^{-1})\ [38]; \qquad (6)$$

$$GSH + HNO \rightarrow GSNHOH, \quad (k = 2 \times 10^6\ M^{-1}s^{-1})\ [37]; \qquad (7)$$

$$metMbNO + GSH \rightarrow MbNO + products, \quad (k = 47\ M^{-1}s^{-1})\ [39]. \qquad (8)$$

Reductive nitrosylation was done by obtaining the spectrum (400–700 nm) of the assay solution every 2 min until the absorbance at 543 nm and 575 nm are stable. Results (Fig. 10) showed that the product releases HNO, depicted by the decrease of absorbance at 502 nm and 630 nm, shifted to 543 nm and 575 nm (equation (3)). The release of NO is not detected in this assay because it reacts with metMb (equation (4)) to form metMbNO, which has the same λ_{max} as metMb (no spectral shift) [38].

Reductive nitrosylation was also performed in the presence of GSH. HNO reacts with GSH (equation (7)) at a faster rate compared with MetMb to form a UV-Vis-silent product, GSNHOH (Fig. 11) [37]. As a result, the spectral shift due to the release of HNO is quenched [37]. Any spectral shift exhibited in this assay is due to NO. Under deaerated condition, GSH enhances MbNO formation from metMbNO (equations (6) and (8)) [38]. The spectral shift exhibited in the assay shows that the product is also capable of NO release.

Comparing both spectra, the spectral shift was more pronounced from NO. However, this does not necessarily mean that the product releases more NO than HNO. The dimerization of HNO competes with Equation 3 which could account for lesser MbNO formed from HNO. Reductive nitrosylation does not quantitatively determine the amount of HNO and NO release.

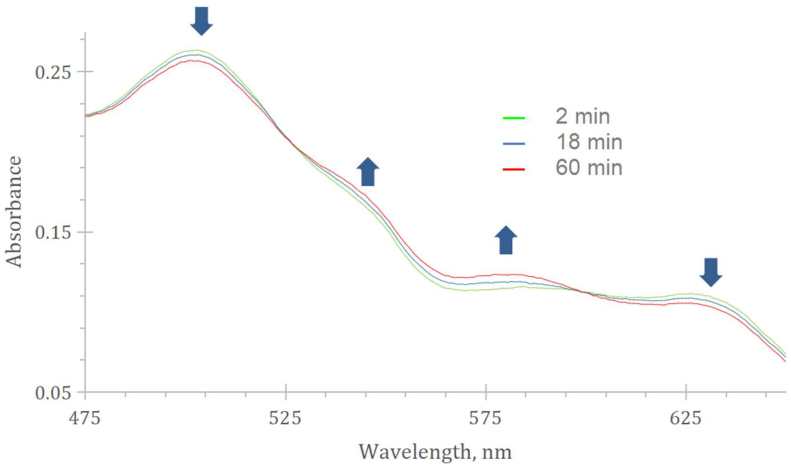

Fig. 10. Reductive nitrosylation of MetMb by the crude product

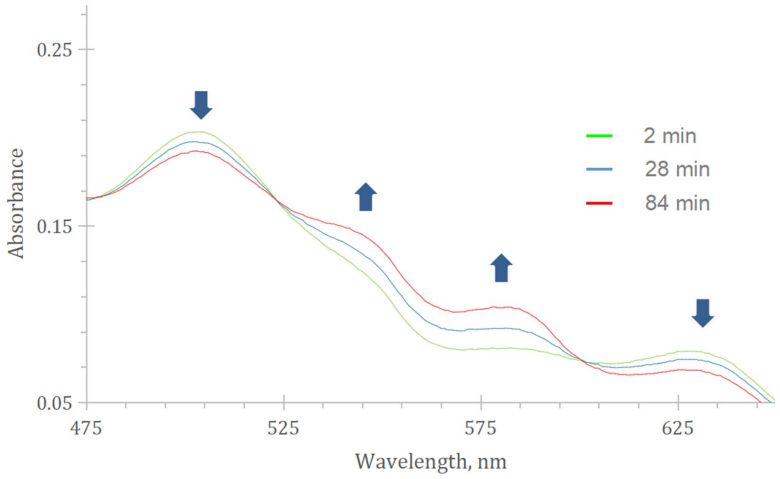

Fig. 11. Effect of GSH on the reductive nitrosylation of MetMb

5. Discussion

The synthesis of cyclohexylamine NONOate was produced using a modified NO-generation setup at a low-pressure method due to the inaccessibility of NO gas in the Philippines. This is based on the work reported by Keefer and co-workers, who demonstrated that *in situ* NO production systems can yield diazeniumdiolates of acceptable purity for synthetic and biological applications using their alternative NO-generation set-up that is comparable to this study [40, 41]. The obtained spectral data between the present study and those reported in earlier works proves to contain a reliable NONOate compound even with improvised NO-generating setup, provided oxygen contamination is minimized.

The morphology of the catalyst was essential for its catalytic activity, as only nanometer-sized TiO_2 has managed to produce a product supporting the formation of NONOates through UV–Vis absorption assays [32, 36]. Fujishima and Honda previously reported their work on sized TiO_2 photocatalysis and showed that nanostructured TiO_2 demonstrates a highly catalytic and photoactive properties than the bulk materials due to the increased of the reactivity of the surface [37, 38]. These findings support earlier reports that nanoscale TiO_2 provides a higher surface area and enhanced catalytic activity in NONOate synthesis.

Although the crude product exhibited very low purity, the UV-Vis data result revealed that only a small fraction of the recovered solid corresponded to cyclohexylamine NONOate, with sodium methoxide identified as the dominant impurity. Comparable challenges have been reported in previous studies in achieving high purity of the compounds, where competing side reactions and solvent-derived impurities generally affect the recovery of the product [42]. While reliance on the weight of the crude product alone would result in misleading conclusions, expressing the yield in terms of purity provides a more reliable basis for

variations in crude product recovery and impurity content. The precision level, given the multistep nature of the synthesis and the sensitivity of NONOates to oxygen, moisture, and temperature, is reasonable.

The optimization process shows that the reaction time and temperature had the greatest impact on the formation of NONOate. Prolonged reaction time and a decrease in temperature significantly increased the yield, which was consistent with the time-dependent nature of integrated NO and the enhanced stability of NONOates at low temperatures. Methanol was the most efficient solvent, which is due to higher NO solubility, whereas TiO_2 loading demonstrated an optimum value wherein the further increase of the amount of catalyst led to diminished yields, which is probably due to accumulation or reduced effective surface area.

Product characterization confirmed the presence of cyclohexylamine NONOate, with a λ_{max} at 253 nm by UV-Vis spectrophotometry, which exhibits the defining features of NONOates. Although the experimentally determined half-life was longer than reported literature values, this discrepancy can be attributed to lower reaction temperature and the presence of stabilizing impurities. The qualitative detection of the release of both HNO and NO through reductive nitrosylation of metmyoglobin promotes validation of the functional behavior of the synthesized compound.

This study expands upon the work of Huang et al. by investigating the synthesis of a primary amine NONOates, which are inherently less stable and more challenging to prepare than the secondary amine NONOates reported previously. Although the isolated yields obtained in this work were modest, the successful synthesis of a primary amine NONOate under ambient conditions is nevertheless significant.

Practical relevance. This work demonstrates the synthesis of cyclohexylamine NONOate using laboratory-accessible conditions, even in the lack of commercial NO gas. The modified NO-generation setup and optimized reaction parameters formed a sufficient amount of primary amine NONOate for chemical characterization and qualitative HNO and NO release assays.

Research limitations. The study was focused on the use of TiO_2 as a catalyst for the synthesis of primary amine NONOate based on cyclohexylamine. Additionally, optimization trials were conducted for each reaction time, solvent, TiO_2 catalyst loading and reaction temperature. The NO pressure was not fully optimized and was applied directly to the reaction vessel containing the starting reagents and catalyst. Furthermore, qualitative detection of HNO and NO release assays were investigated.

Prospects for further research. The application of TiO_2 as a catalyst resulted to the synthesis of cyclohexylamine NONOate. Future work of this method aims on enhancing product yield and purity by further optimizing sodium methoxide loading, NO gas pressure, and temperature regulation. Conducting replicated optimization experiments and exploring alternative solvents could possibly improve yield. Additional quantitative techniques for measuring HNO/NO release and expanding the method to include other amines are recommended to broaden the applicability of this synthesis. The choice of catalyst and its mechanism of action towards NONOate synthesis could also be studied.

6. Conclusions

Cyclohexylamine NONOate was successfully synthesized in ambient temperature and pressure using nanometer-size TiO_2 as catalyst. The use of nanometer-size TiO_2 was essential in this method since the use of normal TiO_2 did not yield the desired product. The yield of the method was enhanced by increasing the reaction time and decreasing the temperature.

The product obtained exhibited a peak at 253 nm, released both HNO and NO at physiological pH and followed a first-order decomposition, which are the characteristics of cyclohexylamine NONOate. The method yielded 5.98 mg cyclohexylamine NONOate which is equivalent to a percent yield of 0.0550%. The yield was very low but reasonable considering the yield of the conventional method (1–20%) which requires –78°C and 50 psi NO. Furthermore, the yield of this method is sufficient for chemical and biological assays.

The yield of this method can be enhanced by further optimizing the reaction time and temperature. Specifically, longer reaction time can be used in the optimization and lower temperature can be achieved using different solvents for the ice bath, aside from table salt. The amount of sodium methoxide could also be optimized to increase the purity of the product.

This method can be directly used to synthesize cyclohexylamine NONOate to be used as HNO donor for chemical and biological assays. This method can also be used to synthesize discover an array of stable primary amine NONOates which can ultimately be used as HNO donors.

Conflict of interest

The authors declare that they have no conflict of interest in relation to this research, whether financial, personal, authorship or otherwise, that could affect the research and its results presented in this article.

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Data availability

Data will be made available on reasonable request.

Use of artificial intelligence

The authors confirm that they did not use artificial intelligence technologies in creating the submitted work.

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Authors' contributions

Nonil Mart S. Aque: Methodology, Investigation, Data curation, Formal analysis, Writing – review & editing; **Maria Distressa G. Billacura**: Validation, Data curation, Writing – review & editing; **Merell P. Billacura**: Validation, Data curation, Writing – review & editing; **Joel H. Jorolan**: Visualization, Data curation, Formal analysis, Writing – review & editing, Funding acquisition.

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