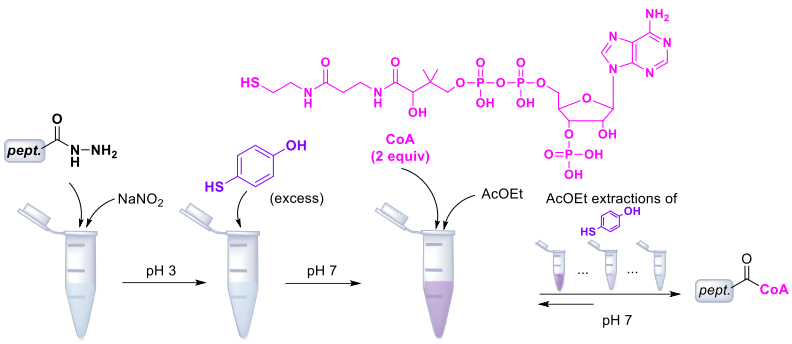
Efficient Peptide Alkyl Thioester Synthesis from Advanced Thiols and Peptide Hydrazides

Julie Di Adamo[1], Nathalie Ollivier[1], Vincent Diemer[1], Oleg Melnyk*[1]

[1]Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 - UMR 9017 - CIIL - Center for Infection and Immunity of Lille, Lille, France

* Corresponding authors



Abstract: Peptide alkyl thioesters are versatile reagents in various synthetic applications, commonly generated from peptide hydrazides and thiols. However, a notable limitation is the need for a substantial excess of the thiol reagent, restricting usage to simple thiols. Here, we introduce an adapted procedure that significantly enhances thioester production with just a minimal thiol excess, facilitating the use of advanced thiol nucleophiles.

Alkyl thioester functionality is characterized by a low hydrolysis rate in neutral aqueous media, minimal propensity for racemization or epimerization, and a strong reactivity towards S-nucleophiles like thiols.[1] These properties make alkyl thioesters particularly attractive in diverse applications such as protein semi or total synthesis,[2-6] the study of protein folding,[7] the design of dynamic combinatorial libraries[8-9] and the production of organic polymers.[10] In particular, peptide alkyl thioesters are popular reagents for the chemical synthesis of proteins using the native chemical ligation (NCL), which consists in reacting a C-terminal peptidyl thioester with an N-terminal cysteinyl (Cys) peptide to produce a larger peptide through the chemoselective formation of a peptide bond to cysteine. Logically, many works were devoted to their synthesis using solid phase, liquid phase or hybrid solid phase-liquid phase approaches.[2] The widespread adoption of the 9-fluorenylmethyloxycarbonyl (Fmoc) solid phase peptide synthesis method by the peptide community has favored the development of the hybrid solid phase-liquid phase approach. This tendency is due to the incompatibility of the thioester functionality with the repeated piperidine treatments used to remove the Fmoc group during the elongation of the peptide sequence on the solid support. Practically, peptide thioesters are frequently prepared in aqueous solution from unprotected precursors produced by conventional Fmoc SPPS.[11] Amide and hydrazide precursors are appreciated for their excellent stability and ease of synthesis.[12-16] In both cases, the thioester group is formed through an activation-displacement mechanism that requires a large excess of an alkylthiol to achieve good yields. Although highly efficient and popular, these methods are restricted to the use of simple and cheap thiols such as sodium 2-ethanesulfonate (MESNa[17]), 3-mercaptopropionic acid (MPA[12-13]) or 3-mercaptopropiosulfonate (MPSNa[18]) owing to the excess of thiol required. Peptide thioesters derived from advanced thiols require the setup of special protocols. For example, peptide thioesters equipped with an oligoarginine tag in the thiol arm are accessible through Boc SPPS.[19]

The production of peptide alkyl thioesters from advanced alkyl thiols is of high interest for modulating peptide segment solubility[19] or thioester group chemical[20] or biochemical reactivity.[21] We show here that one of the most popular method for peptide thioester synthesis, the peptide hydrazide method designed by Liu's group,[15] can be adapted to access various types of alkyl thioesters using nearly stoichiometric quantities of the alkyl thiol nucleophile by exploiting an hallmark of the thiol-thioester exchange process, i.e., its reversibility.[22]

The hydrazide-based peptide thioester synthesis method reported by Liu and colleagues starts with the activation of the peptide hydrazide **H** into an acyl azide **A** in aqueous medium by treatment with an excess of sodium nitrite at pH 3 (Fig. 1A). Subsequently, an excess of aryl[15] or alkyl[17] thiol R-SH is added to the peptide acyl azide **A** under neutral pH conditions, resulting in the formation of a peptide thioester **T**. The excess of thiol is essential to displace the azide anion and neutralize the excess of nitrous acid. We hypothesized that employing nearly stoichiometric amounts of an advanced thiol R_A-SH could be achieved by initially quenching the excess of nitrous acid with a readily available and cost-effective thiol R_Q-SH before introducing R_A-SH (Fig. 1B). While classical Liu's method anticipates the production of a thioester intermediate derived from R_Q-SH, $\mathbf{T_Q}$, the subsequent addition of thiol R_A-SH is expected to lead to thioester $\mathbf{T_A}$ formation via a thiol-thioester exchange process. Nevertheless, our expanded understanding of the thiol-thioester exchange reaction allows us to foresee that, despite selecting thiol R_Q-SH as a superior leaving group compared to R_A-SH, the significant excess of R_Q-SH will inevitably bias the formation towards thioester $\mathbf{T_Q}$ over $\mathbf{T_A}$. We reasoned that this problem might be solved by removing selectively the excess of thiol R_Q-SH after having added R_A-SH, in order to displace the equilibrium toward $\mathbf{T_A}$ formation according to Le Chatelier's principle.

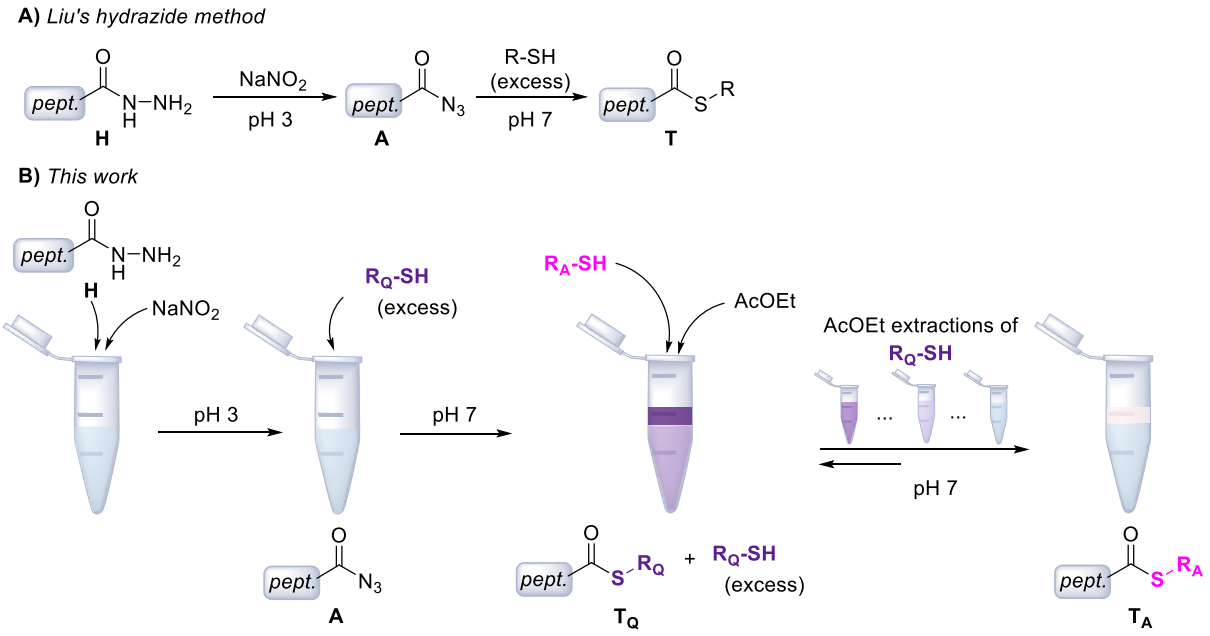


Figure 1. Peptide thioester synthesis using hydrazide method. A) The classical hydrazide activation method proceeds in two steps: i) the conversion of the hydrazide into an acyl azide by nitrosation, ii) the acyl azide thiolysis step in the presence of excess thiol RSH. B) Adaptation of the hydrazide method to the synthesis of alkyl peptide thioesters using nearly stoichiometric quantities of an advanced thiol R_A-SH.

Because the alkyl thiols R_A-SH examined in this work are highly hydrophilic (MPA, coenzyme A, peptide or protein thiols), extracting the quenching thiol R_Q-SH during the thiol-thioester exchanged step was envisioned as a simple mean for its removal from the reaction mixture. Liu's hydrazide method was initially developed using an excess of the aromatic thiol 4-mercaptophenylacetic acid (MPAA) to effect the thiolysis of the acyl azide intermediate. Although MPAA is known to be an excellent leaving group compared to alkyl thiols,[23] it is not a suitable candidate as a quenching thiol in this work. Indeed, this compound is highly water-soluble at neutral pH, a property that precludes its extraction with an

organic solvent under the working conditions of the thiol-thioester exchange step. Therefore, more hydrophobic arylthiols such as thiophenol or *p*-hydroxythiophenol were considered instead.

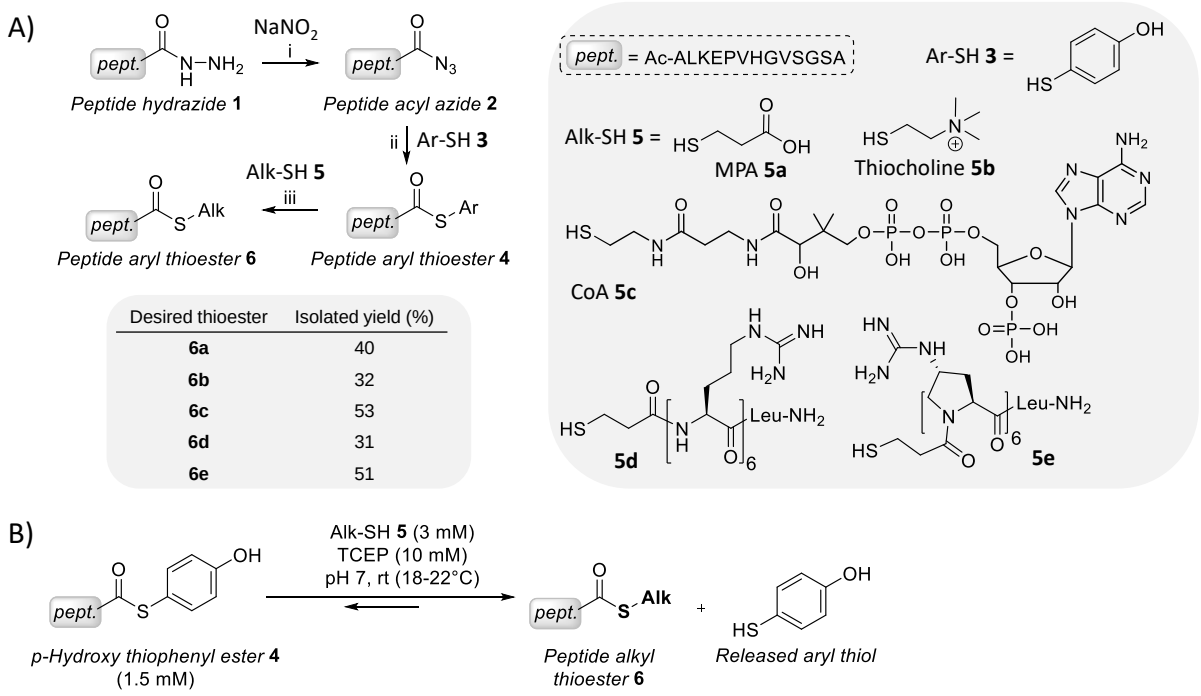
The procedure for converting peptide hydrazides into the corresponding peptide alkyl thioesters, based on this concept, is outlined in detail in Fig. 2A. Initially, peptide acyl azide **2** was generated from peptide hydrazide **1** at pH 3 and – 15°C. Subsequently, the thiolysis step, leading to peptide aryl thioester **4**, was initiated by adding an excess of aryl thiol **3** and adjusting the pH to 7. After 15 minutes at room temperature (rt), the thiol-thioester exchange reaction leading to the formation of peptide alkyl thioester **6** was triggered by adding alkyl thiol **5** (2 equiv) and TCEP (*tris*(2-carboxyethyl)phosphine). The extraction of the aryl thiol **3** was performed using either diethyl ether or ethyl acetate. The progression of the reaction was monitored by HPLC with UV detection at 215 nm. Peak areas for peptide aryl thioester **4** were corrected for the absorbance of the mercaptoaryl moiety to determine the fraction of peptide alkyl thioester **6** formed over time (see Supplementary Information). Moreover, we investigated the time course of the thiol-thioester exchange reaction between pre-synthesized peptide thioester **4** and different alkyl thiols, mimicking the conditions of the final step in the modified hydrazide method, for comparative analysis (Fig. 2B).

The initial experiments employing thiophenol as the quenching thiol and MPA **5a** as a representative alkyl thiol were unsuccessful, primarily due to significant thioester hydrolysis observed over time (see Supplementary Information). In contrast, employing *p*-hydroxythiophenol as the quenching thiol resulted in a satisfactory yield of the target MPA-derived peptide thioester **6a** (HPLC yield 78%, 40% isolated). This success was achieved by introducing the alkyl thiol to the peptide thioester before extracting *p*-hydroxythiophenol. Notably, delaying the addition of the alkyl thiol until after the extraction process led to significant thioester hydrolysis. By adhering to this precautionary step, the reaction was complete in about 35 minutes after the addition of MPA (Fig. 3A, red dots).

In the control experiment where no extraction was performed during the final step, peptide alkyl thioester **6a** was obtained in a low yield (Fig. 3A, green dots), highlighting the significance of eliminating the excess *p*-hydroxythiophenol to attain yields suitable for practical applications. Figure 3A further illustrates that the thiol-thioester exchange process between pre-synthesized peptide aryl thioester **4** and MPA **5a** progressed at a slower rate compared to the adapted hydrazide method, reaching similar equilibrium position after more than 500 minutes of reaction (Fig. 3A, black dots). Intriguingly, performing the thiol-thioester exchange reaction between **4** and **5a** while extracting the released *p*-hydroxythiophenol had a minor yet positive impact on the rate (see Supplementary Information). All these experiments demonstrate that while it is essential to extract the excess *p*-hydroxythiophenol during the final step of the adapted hydrazide method to achieve satisfactory yields in peptide alkyl thioester, the extraction procedure alone does not explain the rapid rate of its formation.

Because the hydrazide method leads to the release of one equivalent of azide anion in situ during the acyl azide thiolysis step, the effect of adding one equivalent of sodium azide to the thiol-thioester exchange reaction on the rate of peptide alkyl thioester **6a** formation was examined (see Supplementary Information). Sodium azide was found to catalyze the formation of peptide alkyl thioester **6a**, but the rate achieved was again below that recorded for adapted hydrazide method, whether the reaction mixture was extracted or not. The reaction was also accompanied by byproduct formation which was not clearly identified. Our data clearly suggest that hydrazide method generates in situ a type of compound able to catalyze the thiol-thioester exchange process and those exact nature remains to be established.

We next examined the capacity of the adapted hydrazide method described in Fig. 2A to access peptide alkyl thioesters derived from various thiols such as thiocholine **5b**, coenzyme A (CoA) **5c**, MPA-derived oligoarginine peptide **5d**, as well as its analog **5e**, where the arginine residues were substituted with *trans*-4-guanidinoproline residues. Thiocholine **5b** (with a pK_a SH of 7.8) was specifically included in this investigation to assess the adapted hydrazide method's ability to produce peptide alkyl thioesters derived from thiols that are more acidic than MPA (with a pK_a SH of 10.5).[24] CoA **5c** is a negatively charged thiol that plays an essential role in cell metabolism through the formation of thioesters derived from carboxylic acids.[25] Peptide thioesters derived from CoA, i.e. peptidyl-CoA, have been prepared in the past from protected peptide precursors using complex procedures.[21] Peptide thiols **5d** and **5e** carry a positive charge and are recognized for their ability to enhance the solubility of peptide thioesters in aqueous environments, owing to their pronounced hydrophilicity.[19] Moreover, recent findings have highlighted their improved reactivity toward negatively charged S-nucleophiles, rendering them valuable substrates for accelerating the NCL reaction.[20] All the tested thiols furnished the corresponding thioesters in good yield (Fig. 2A), showing the broad applicability of the method. Particularly noteworthy and as already observed for peptide alkyl thioester **6a**, the formation of the peptide alkyl thioesters **6c,d** using the adapted hydrazide method occurred at a faster rate than anticipated, surpassing the rate observed in the isolated study of the final thiol-thioester step (Fig. 3B,C).



Desired thioester	Isolated yield (%)
6a	40
6b	32
6c	53
6d	31
6e	51

Figure 2. Peptide alkyl thioester synthesis. A) Adapted hydrazide method. i) $NaNO_2$ (6.7 equiv), 0.2 M sodium phosphate, 6 M Gn.HCl, pH 3, - 14-16 °C, 15 min. Intermediate peptide concentration: 4.5 mM. ii) Aryl thiol (67 equiv), pH 3, - 14-16 °C -> rt then adjusted to pH 7 using NaOH 6 M, 15 min. Intermediate peptide concentration: 3.0 mM. iii) Alkyl thiol (2 equiv), TCEP (6.7 equiv), 0.2 M sodium phosphate, 6 M Gn.HCl, pH 7, rt (18-22 °C).Extraction using AcOEt. Final peptide concentration: 1.5 mM. B) Thiol-thioester exchange method.

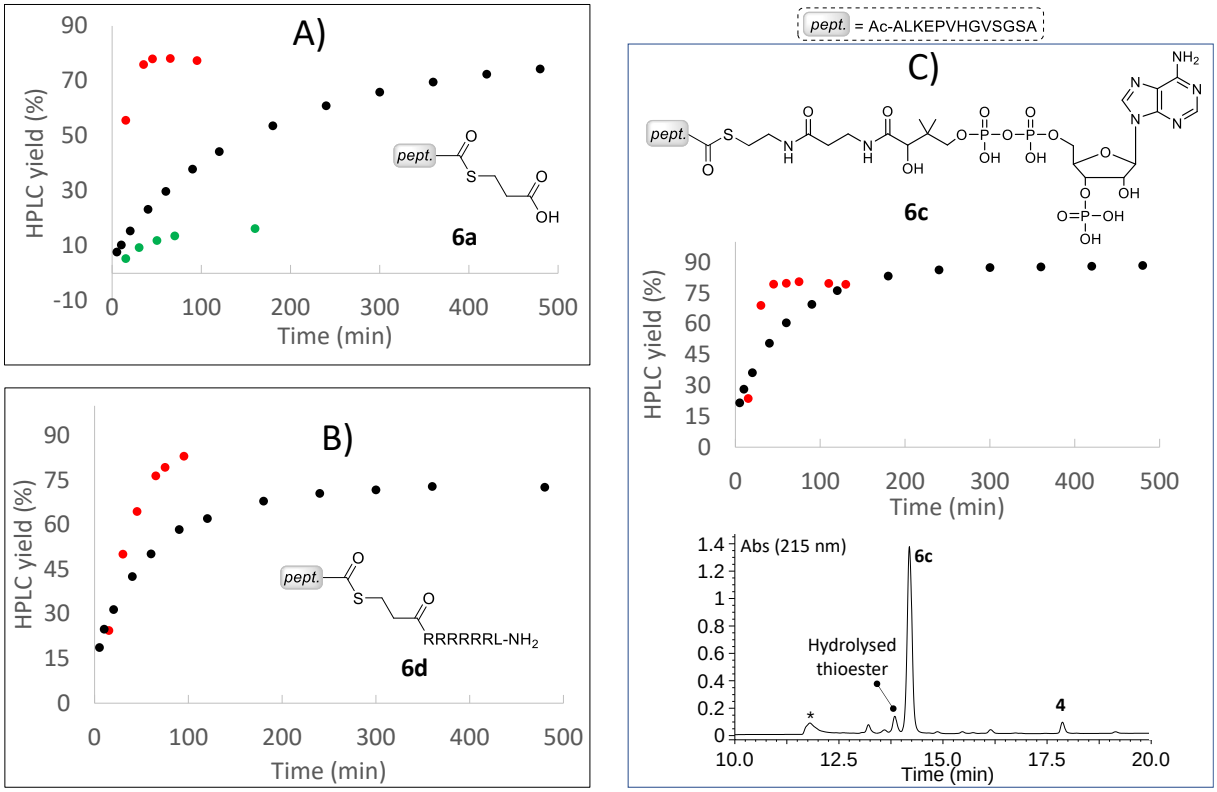


Figure 3. Time course of peptide alkyl thioester **6** formation using the adapted hydrazide method (red dots) or the thiol-thioester exchange method using peptide aryl thioester **4** (black dots). A) **6a**. Green dots are for the adapted hydrazide method where no extraction was performed during the final step. B) **6d**, C) **6c**. Kinetic data and HPLC chromatogram of the crude reaction mixture for the adapted hydrazide method at t = 2 h 10 min. Elution conditions : C18 BEH XBridge 300 Å, 3.5 µm, 2.1 × 100 mm column, eluent A 0.1 % TFA in water, eluent B 0.1 % TFA in CH_3CN, gradient 0-5 % B in 2 min, then 5-20 % B over 15 min and finally 20-30 % B over 3 min, 0.4 mL/min, T = 50 °C. * Non peptidic.

In summary, the modified peptide hydrazide method outlined in this study simplifies the synthesis of peptide alkyl thioesters sourced from sophisticated thiols. This advancement simplifies the variation of peptide thioester solubility and reactivity, two types of properties that often need to be refined during chemical synthesis of proteins through thioester-based peptide ligation techniques. Furthermore, the kinetic data gathered in this investigation offer valuable insights into the mechanistic intricacies of the hydrazide method.

EXPERIMENTAL SECTION

A typical experimental procedure for the adapted hydrazide method is illustrated with the synthesis of peptide alkyl thioester **6e**.

Peptide hydrazide **1** (4.59 mg, 2.78 µmol, 1.00 equiv) was dissolved in 0.2 M phosphate buffer containing 6 M Gn·HCl pH 3.0 (560 µL). After adjusting the pH to 3.0, the mixture was cooled to -14 °C using a NaCl/ice bath. Then, a solution of 0.5 M $NaNO_2$ (37.1 µL, 18.6 µmol, 6.67 equiv) was added to the reaction mixture which was agitated for 15 min.

Then, *p*-hydroxythiophenol (23.4 mg, 186 µmol, 66.7 equiv) was suspended in 0.2 M phosphate buffer containing 6 M Gn·HCl pH 3.0 (600 µL) and the pH was adjusted to 6.7 using NaOH 6 M (24 µL). At this concentration and pH, *p*-hydroxythiophenol is only partly solubilized. This suspension was then added to the above reaction mixture. The NaCl/ice bath was removed and the pH of the reaction mixture was adjusted to 6.9 using NaOH 6 M (20 µL). The solution is cloudy at this stage.

After 15 min, alkyl thiol **5e** (10.2 mg, 5.57 µmol, 2.00 equiv) and TCEP (5.3 mg, 19 µmol, 6.7 equiv) were solubilized in 0.2 M phosphate buffer containing 6 M Gn·HCl pH 7.0 (580 µL) and the pH of this solution was adjusted to pH 7.1 using NaOH 6 M (12 µL). This solution was added to the reaction mixture and the final pH was adjusted to pH 7.0. At this stage, the final concentrations are: peptide 1.5 mM, *p*-hydroxythiophenol **3** 100 mM, alkyl thiol **5e** 3.0 mM, TCEP 10 mM.

p-Hydroxythiophenol was extracted with AcOEt (0.5 mL, 18 times, typically each min for the first 10 min of reaction, then each 5 min and one before RP-HPLC purification). The extraction of *p*-hydroxythiophenol was monitored by spotting the organic phases on a silica gel plate for thin layer chromatography and reading the plate under 254 nm UV light.

After 1 h 30 min at rt, the reaction was acidified by adding a 10% v/v TFA solution (371 µL) to reach a final TFA concentration of 2% v/v. AcOEt remaining on top of the aqueous solution was carefully removed. Purification of the crude peptide alkyl thioester **6e** by RP-HPLC (BEH C18 column, eluent A 0.25% TFA in water, eluent B 0.25% TFA in CH_3CN, gradient 0-10% B in 6 min and then 10-25% B in 54 min, 6 mL/min, T = 60 °C, λ = 215 nm) furnished the target peptide alkyl thioester **6e** (4.36 mg, 51%) as a white powder after lyophilization.

SUPPORTING INFORMATION.

The Supporting Information is available free of charge at:

https://pubs.acs.org/doi/10.1021/

Experimental procedures and characterization data for all compounds (pdf file). All the data collected during this study can be found in the data source file associated with this article (excel file).

AUTHOR INFORMATION

Corresponding Authors

Oleg Melnyk – CNRS

Center of Infection and Immunity of Lille, Lille, France.

orcid.org/0000-0002-3863-561;

Email: Oleg.melnyk@ibl.cnrs.fr

Authors

Julie Di Adamo – University of Lille

Center of Infection and Immunity of Lille, Lille, France.

Nathalie Ollivier – Institut Pasteur de Lille

Center of Infection and Immunity of Lille, Lille, France.

Vincent Diemer – CNRS

Center of Infection and Immunity of Lille, Lille, France.

orcid.org/0000-0002-5735-4630;

Vincent.diemer@ibl.cnrs.fr

NOTES

The authors declare no competing financial interest.

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