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Chemo-enzymatic synthesis of NPN cofactor taking advantage of ADP-ribosyl cyclase and LarC cyclometallase promiscuous activities

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ABSTRACT

The nickel-pincer nucleotide cofactor (NPN) is a widespread organometallic cofactor required for lactate racemase (LarA) and for α-hydroxy acid racemases and epimerases of the LarA superfamily. Its biosynthesis, which starts with nicotinic acid adenine dinucleotide (NaAD), requires three enzymes: LarB, LarC, and LarE, and can be performed *in vitro* with purified enzymes. Nevertheless, as LarE and LarC are single turnover enzymes, the *in vitro* NPN biosynthesis requires huge amounts of enzymes (particularly 2 equivalents of LarE), which hampers the study of NPN and of NPN-dependent enzymes. By using adenosine diphosphate (ADP)-ribosyl cyclase (ARC), we exchanged the nicotinamide moiety in NAD^+ with synthetic pyridine-3,5-bisthiocarboxylic acid in order to synthesize the novel intermediate pyridinium-3,5-bisthiocarboxylic acid adenine dinucleotide (P2TAD). The latter could be produced at a multimilligram scale allowing its characterization by Nuclear Magnetic Resonance (NMR) and mass spectrometry. Interestingly, P2TAD could directly be used by LarC in order to generate the NPN cofactor, bypassing both LarB and LarE. Globally, a new chemoenzymatic route towards NPN was developed via the intermediate P2TAD, which should facilitate the biochemical and biotechnological investigations on NPN binding enzymes.

1. Introduction

Discovered in 2015 in lactate racemase (LarA), the nickel-pincer nucleotide (NPN) cofactor combines the properties of a scaffold derived from nicotinic acid, which enables the cofactor to capture a hydride, with a nickel ion coordinated by two sulfur atoms and forming a nickel-carbon bond with the pyridinium ring (Fig. 1) [1]. This cofactor is required for the activity of α-hydroxy acid racemases and epimerases of the LarA superfamily [2] and probably for other yet undiscovered enzymes, given its wide occurrence in bacteria and archaea, even in absence of any LarA homolog [3].

As illustrated in Fig. 1, the biosynthesis of NPN starts with nicotinic acid adenine dinucleotide (NaAD) and requires the three enzymes LarB, LarC, and LarE. LarB starts by catalyzing the carboxylation of the nicotinic ring accompanied by hydrolysis of the phosphoanhydride bond, forming pyridinium biscarboxylic acid mononucleotide (P2CMN) [4]. LarE then converts both carboxylate groups of P2CMN into thiocarboxylate groups by an adenosine triphosphate (ATP)-dependent sacrificial sulfur insertion, forming pyridinium bisthiocarboxylic acid mononucleotide (P2TMN) [4]. As the two sulfur atoms inserted into

Abbreviations: ADP, adenosine diphosphate; AMP, adenosine monophosphate; ARC, ADP ribosyl cyclase; ATP, adenosine triphosphate; CTP, cytidine triphosphate; DIPEA, diisopropylethylamine; HPLC, high performance liquid chromatography; HRMS, high resolution mass spectroscopy; LarA, lactate racemase; NaAD, nicotinic acid adenine dinucleotide; NAD^+, nicotinamide adenine nucleotide; NMR, nuclear magnetic resonance; NPN, nickel pincer nucleotide; P2CMN, pyridinium biscarboxylic acid mononucleotide; P2T, 3,5-pyrididinebisthiocarboxylic acid; P2TAD, pyridinium-3,5-bisthiocarboxylic acid adenine dinucleotide; P2TCD, pyridinium-3,5-bisthiocarboxylic acid cytidine dinucleotide; P2TMN, pyridinium bisthiocarboxylic acid mononucleotide; PyBOP, benzotriazole-1-yl-oxytripyrrolidino-phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; TrtSH, triphenylmethanethiol.

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P2CMN are derived from one cysteine of LarE, two LarE proteins are required for the synthesis of one P2TMN [5]. Finally, nickel-containing LarC catalyzes the cytidine triphosphate (CTP)-dependent nickel cyclometallation between one sulfur atom and the C4 of P2TMN, generating NPN. This reaction requires the hydrolysis of CTP into CMP and forms a transient pyridinium-3,5-bisthiocarboxylic acid cytidine dinucleotide (P2TCD) intermediate [6,7]. Intriguingly, LarC behaves as a single turnover enzyme *in vitro*, similarly to LarE [6]. Eventually, in the active site of some LarA enzymes, NPN reacts spontaneously with a lysine, forming a thioamide bond (Fig. 1). The overall biosynthetic pathway can be performed *in vitro* in order to synthesize NPN from purified enzymes and common nucleotides (NaAD, ATP, and CTP). In order to perform mechanistic studies, simple models of this cofactor were synthetized and showed participation of the pincer ligand in the chemical dehydrogenation reaction [8,9]. Nevertheless, for the study of NPN-dependent enzymes, true NPN cofactor is required. The only currently available synthesis pathway for NPN synthesis is the incubation of a stoichiometric amount of LarC and two molar equivalents of LarE, plus LarB and cofactors, and letting LarE and LarC catalyze their single-turnover reactions [10]. This article describes a novel chemoenzymatic sequence designed to bypass LarB and LarE through the generation of pyridinium-3,5-bisthiocarboxylic acid intermediate (P2TAD), a so far unknown adenine dinucleotide. Interestingly, P2TAD can directly be used by LarC for the synthesis of NPN, demonstrating the promiscuity of this cyclometallase (Fig. 2).

2. Materials and methods

Here are described the materials used to realize all the chemical reactions and biological assays. In the supporting information are available the NMR spectrum of the intermediate that yield to **2**, P2T and P2TAD, the HRMS spectra of P2TAD and the enzymatic reactions with P2TAD. The molecular schemes of the chemical reactions are available in the supporting information.

2.1. Materials

The solvents used for chromatography (CH_2Cl_2, cyclohexane, EtOAc and MeOH) were purchased in industrial grade and further distilled before their use. Reagents and chemicals were purchased from Sigma-Aldrich, Acros, Carbosynth, BLD-pharm or ABCR at ACS grade and were used without additional purification. Some anhydrous solvents (THF, DCM and toluene) were collected after a purification on the solvent purifier (MBRAUN, MB-SPS-compact). DMF and 1,2-dichloroethane were bought anhydrous and stocked over MS 4 Å. Thin layer chromatography (TLC) analyses were performed using standard procedures on Kieselgel 60F254 plates (Merck, Kenilworth, NJ, USA). Compounds were visualized using UV light (254 nm) and a solution of phosphomolybdic acid. Melting points were determined with Melting Point M-560 Buchi®. Infra-Red spectrum were recorded with Perkin Elmer Spectrum two 119040 apparatus. Optical rotations were measured on an Anton-Paar MCP 300 polarimeter. High resolution mass spectrometry spectra were recorded on a Bruker MicroTOF-Q II XL apparatus and MALDI-TOF-LD+ on a Waters QTOF1 apparatus by the "Fédération de Recherche" ICOA/CBM (FR2708) platform of Orléans in France in negative mode (voltage 3500 V, sheath gas 350 C at 11 l/min, nebulizer pressure 35 psig and drying gas 200C at 14 l/min). An *m/z* range from 70 to 1200 was acquired with a frequency of 1 per second. Solvents and reagents for LC-MS analysis were from Biosolve, with exception of hexylamine and ammonia which were obtained from Merck.

Column chromatography was performed on silica gel SI 60 (40–63 μm) (Merck). Flash chromatography was performed by Reveleris® apparatus with silica cartridge of 40 μm. ^{1}H, ^{13}C, ^{19}F and ^{31}P NMR spectra were recorded on a JEOL spectrometer JNM EX-400 (400 MHz, 100 MHz, 376 MHz and 162 MHz respectively) or JNM EX-500 (500 MHz, 125 MHz, 471 MHz and 202 MHz respectively).

2.2. Methods

2.2.1. NPN biosynthesis assay

LarC [6] (0.125, 0.25 or 0.5 μM final concentration) was incubated at room temperature in 50 mM Tris-HCl buffer pH 8 with 1 mM $MnCl_2$ (200 eq.), 10 μM $NiCl_2$ (2.0 eq.), 1 mM CTP (200 eq.) (if indicated), and 0.5 μM P2TMN or 5 μM P2TAD. Of this reaction mixture, 5 μL was added after the indicated time to the 95 μL malate racemization assay, a modified L-malic assay (Megazyme Ltd.) composed of the components of the L-malic assay supplemented with 20 mM D-malate (4000 eq.) and 1 μM malate racemase (0.2 eq.) from *Thermoanaerobacterium thermosaccharolyticum* [2]. The absorbance at 340 nm was followed every

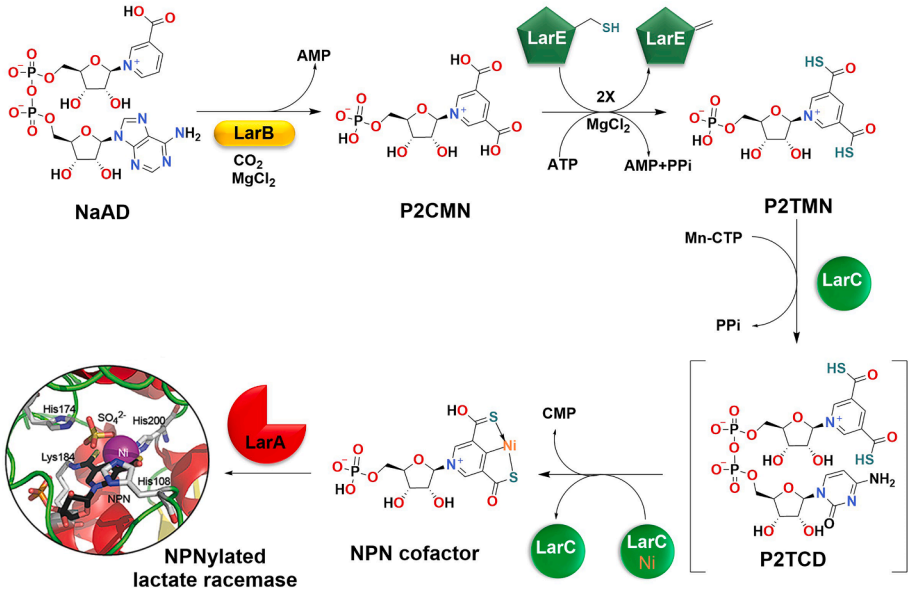

Fig. 1. NPN biosynthetic pathway and structure of the lactate racemase catalytic site (PDB code: 5HUQ) with bound NPN (see text for details). The catalytic residues and the residues required for NPN binding are indicated in stick model. (Modifications highlighted in yellow in the Figure). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

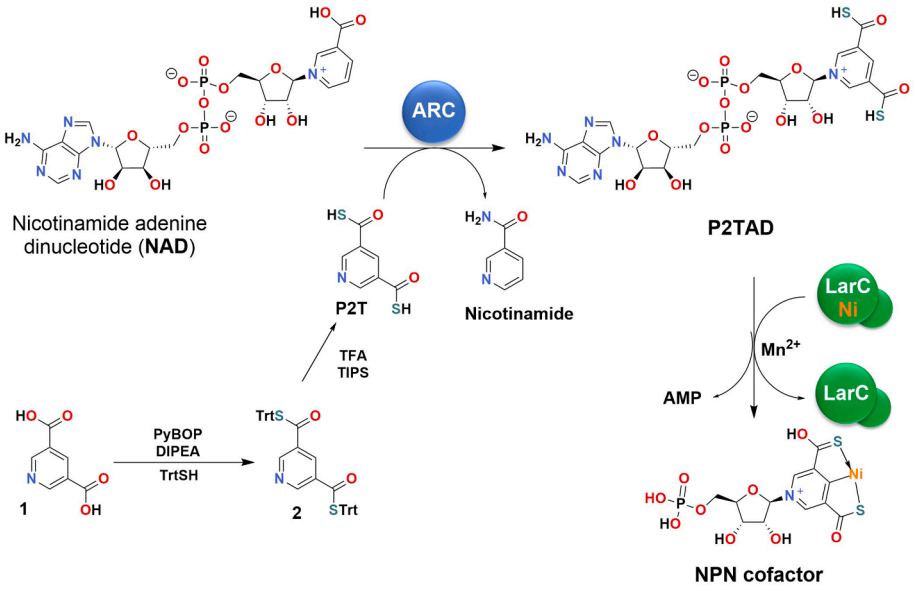

Fig. 2. New chemoenzymatic route towards NPN biosynthesis. Modification highlighted in the Chemdraw figure.

minute for 10 min on an Infinite Pro 200 (Tecan Trading AG).

2.3. Experimental procedures

2.3.1. Synthesis of 3,5-pyridinethiocarboxylic acid, 3,5-trityl thioester [2,11,12]

Pyridine-3,5-dicarboxylic acid [1] (500 mg, 2.99 mmol), PyBOP (3.50 g, 2.25 eq., 6.73 mmol) and triphenylmethanethiol (1.86 g, 2.25 eq., 6.73 mmol) were dissolved in anhydrous DMF (15 mL) at room temperature under argon. The reaction mixture was cooled to −15 °C, and DIPEA (2.35 mL, 4.5 eq., 13.46 mmol) was added dropwise under argon. The reaction mixture was allowed to warm to room temperature and then stirred overnight at room temperature. The solvent was then evaporated under reduced pressure. The crude product was washed with chilled EtOH, then it was filtered and the solid was purified by chromatography (silica gel, DCM/MeOH 100/0 then 80/20). The fractions containing the product were collected and washed once with chilled EtOH, then it was filtrated and the solid was dried under reduced pressure to give **2** (715 mg, 35 %).

^{1}H NMR ($CDCl_3$, 500 MHz): δ = 9.20 (d, 2H, H2 and H6), 8.60 (t, 1H, J = 5.0, 2.5 Hz, H4), 7.27–7.33 (m, 31H, H_{Ar}). **^{13}C NMR ($CDCl_3$, 125 MHz)**: δ = 187.4 ($C_{C=O}$), 151.5 (C2 and C6), 143.2 (C_{qAr}), 133.9 (C4), 133.2 (C3 and C5), 130.0 (C_{Ar}), 129.5 (C_{Ar}), 128.1 (C_{Ar}), 128.0 (C_{Ar}), 127.7 (C_{Ar}), 127.6 (C_{Ar}), 127.4 (C_{Ar}). **ESI-HRMS** $[M + H]^+$ m/z = 684.2032 (calculated for $C_{45}H_{34}NO_2S_2^+$: 684.2025). **IR (cm^{-1})**: ν = 1675, 1574, 1486, 1444, 1425, 1319, 1240, 1185, 1133, 1022, 955, 911, 887, 838, 752, 740, 732.

2.3.2. Synthesis of 3,5-pyridinethiocarboxylic acid (P2T, 3) [12]

To a flask containing compound **2** (1 g, 1.46 mmol) was added, at the same time, TFA (21 mL, 190 eq., 277 mmol) and TIPS (1.05 mL, 3.5 eq., 5.11 mmol) at room temperature under argon. The reaction mixture was stirred 45 min at room temperature. A monitoring by TLC (eluent: DCM, Rf of starting material: 0.33) showed the disappearance starting material. The solvent was removed under *vacuum* and the solid was washed 2 times with chilled Et_2O (2x10 mL), 3 times with cyclohexane (3x10 mL) and 2 times with pentane (3x10 mL). Then, it was dried under *vacuum* to afford **P2T** (274 mg, 60 %).

^{1}H NMR (D_2O, 500 MHz): δ = 9.51 (br s, 1H, H4), 9.35 (br s, 2H, H2 and H6). **^{13}C NMR (D_2O, 125 MHz)**: δ = 206.2 (C═O), 142.2 (*C*2 and C6), 141.4 (C4), 140.7 (C3 and C5). **^{19}F NMR (D_2O, 471 MHz)**: δ = -75.5 **ESI-HRMS** $[M + Na]^+$ m/z = 199.9832 (calculated for $C_7H_6NO_2S_2^+$: 199.9834). **IR (cm^{-1})**: ν = 3372, 2980, 2462, 1620, 1576, 1501, 1447, 1388, 1353, 1241, 1120, 1069, 1013, 941, 907, 836, 771, 712.

2.3.3. Synthesis of P2TAD (4)

For the synthesis of **P2TAD** (**4**), the dithiocarboxylic acid pyridine (120 mg, 3.0 eq., 0.6 mmol) was solubilized in 200 mL distilled water and the pH was adjusted to 4 with 1 M NaOH. To this solution, solid NAD^+ (133 mg, 0.2 mmol) was added, followed by *Aplysia* cyclase enzyme (0.5 mg) overexpressed and purified from *Pichia pastoris* cells from 1 l batch culture as described [13] and the reaction was incubated for 2 h at 37 °C as described. [14] The reaction was monitored with the D-/L-Lactic Acid (D-/L-Lactate) (Rapid) Assay Kit (Megazyme) without NAD^+ and analyzed on an Infinite Pro 200 (Tecan) multiplate reader in order to assay the NAD^+ left after the reaction. The pH was adjusted to 8, and the crude reaction mixture was purified by anion exchange chromatography with a Hi-Load 26/10 Q Sepharose column (Fisher Scientific) on an Akta purifier FPLC system with a gradient of 10–1000 mM NaCl in 20 mM Tris-HCl buffer pH 8 and the product eluted at around 600 mM NaCl. The fractions with the **P2TAD** (**4**) were detected by **NPN** synthesis by LarC followed by lactate racemase reaction assayed with the D-/L-Lactic Acid (D-/L-Lactate) (Rapid) Assay Kit (Megazyme) and analyzed on an Infinite Pro 200 (Tecan) multiplate reader as described [10]. The crude product was purified by HPLC (conditions: semi-prep column RP-C18 (Agilent Zorbax SB-C18 4.6 × 250 mm 5 μM), flow 1 mL/min, solvents: acetonitrile/buffer (%) (acetate/triethylammonium 50 mM, pH = 6.8) 0/100 to 50/50 in 30 min, t_R = 13.635 min) to lead to **P2TAD** (10.4 mg, 0.014 mmol, 7 %).

^{1}H NMR (D_2O, 500 MHz): δ = 9.46 (br s, 1H, H_{Ar}), 9.29 (s, 2H, H_{Ar}), 8.39 (s, 1H, H_{Ar}), 8.09 (s, 1H, H_{Ar}), 5.94 (d, 1H, J = 5.0 Hz, H1 or H1′), 5.89 (d, 1H, J = 5.0 Hz, H1′ or H1), 4.65 (t, 1H, J = 15.0 Hz, 5.0 Hz, H3 or H3′), 4.45 (t, 1H, J = 5.0 Hz, 2.5 Hz, H3′ or H3), 4.41–4.37 (m, 3H, H4 or H4′ and H5 or H5′), 4.31 (br s, 1H, H2 or H2′), 4.28–4.23 (m, 3H, H4′ or H4 and H5′ or H5), 4.21–4.16 (m, 1H, H2′ or H2). **^{31}P NMR (D_2O, 202 MHz)**: δ = -10.8 (d, J = 20.2 Hz), −11.1 (d, J = 20.2 Hz). **ESI-HRMS** $[M-2H]^{2-}$: m/z = 369.0093 (calculated for $C_{22}H_{25}N_6O_{15}P_2S_2^{2-}$: 369.0114).

3. Results and discussion

The first step towards the chemoenzymatic preparation of the NPN cofactor is the synthesis of 3,5-pyridinedithiocarboxylic acid (P2T). Its

synthesis was realized in two steps with the final on 1 g scale and 60 % yield [11,12] (see in Supporting Information (SI)). More classical methods to introduce the sulfur atom (using the Lawesson's reagent or by nucleophilic or basic deprotection of bisthioesters) were found troublesome and very low yielding. The second step is the enzymatic exchange of the nicotinamide group of nicotinamide adenine dinucleotide (NAD^+) with P2T in order to generate pyridinium-3,5-bisthiocarboxylic acid adenine dinucleotide (P2TAD) (Fig. 2). This was realized with *Aplysia californica* ADP-ribosyl cyclase (ARC), an enzyme which is known to catalyze the exchange of the nicotinamide group of NADP or NAD^+ with nicotinic acid derivatives with substituents at either position 4 or 5 in order to produce the corresponding NaADP or NaAD analogues [14]. A variety of nicotinic acid derivatives have been exchanged for nicotinamide [14,15], but the carboxylic group of nicotinic acid was never modified. The fact that P2T is a substrate of ARC shows that the carboxylic group of nicotinic acid can be modified as well, and still be a substrate of ARC. The P2TAD molecule **4** produced by ARC was then purified by anion exchange chromatography followed by preparative high-performance liquid chromatography (HPLC) at a 10-milligram scale allowing its characterization by NMR. In the ^1H NMR spectrum, the characteristic resonances of the adenine, pyridinium and the two anomeric protons could be distinctly observed. Moreover, the ^{31}P NMR spectrum showed two characteristic doublets consistent with a pyrophosphate moiety ($J = 20.2$ Hz). High resolution mass spectrometry data were consistent with the proposed structure. Analytical HPLC of the crude reaction mixture before semi-preparative HPLC purification indicated a proportion of P2TAD of 36 % (Fig. S16). The low isolated yield of pure P2TAD **4** (7 %) can be explained by the instability of the molecule in solution. For instance, the molecule fully decomposed in D_2O over a period of 12 h at room temperature. It is thus likely that some decomposition also occurred during the purification steps.

In order to hydrolyze P2TAD into P2TMN, the substrate of LarC, we incubated P2TAD with LarB and observed the disappearance of P2TAD and the generation of P2TMN by Mass Spectroscopy (MS) (Fig. S1), confirming that LarB catalyzes the hydrolysis of the phosphoanhydride bond of P2TAD. Intriguingly, no adenosine monophosphate (AMP) was observed by MS (Fig. S1), suggesting that additional side-reactions involving AMP are catalyzed by LarB. Therefore, this chemo-enzymatic sequence (ARC/LarB/LarC) could already be used as a LarE-free alternative to the natural biosynthetic pathway.

To push further the simplification of the NPN cofactor synthesis, we reasoned that P2TAD **4** could be directly tested in the LarC reaction because it shares some structural similarity to P2TCD, the activated intermediate in LarC reaction (7) (depicted in Fig. 1). Rewardingly, LarC does catalyze the direct production of NPN from P2TAD without the need for CTP. As shown in Fig. 3A, malate racemase (Mar) activity of Mar2 [2] is used as a measure of NPN biosynthesis.

Although a CMP transfer is no longer needed starting from P2TAD **4**, we observed that Mn^{2+} is still required (Fig. 3A), which suggests that Mn^{2+} is used by LarC for at least one of the two reactions catalyzed during cyclometallation: the nickel insertion and the hydrolysis of the phosphoanhydride bond of P2T(A/C)D. The generation of AMP and P2TMN by LarC reaction with P2TAD **4** as substrate was confirmed by MS (Fig. S1) and demonstrates that P2TAD **4** is indeed hydrolyzed by LarC. Neither NPN nor the nickel-bound P2TAD was observed by MS, but this was not surprising, given the instability of the NPN cofactor in

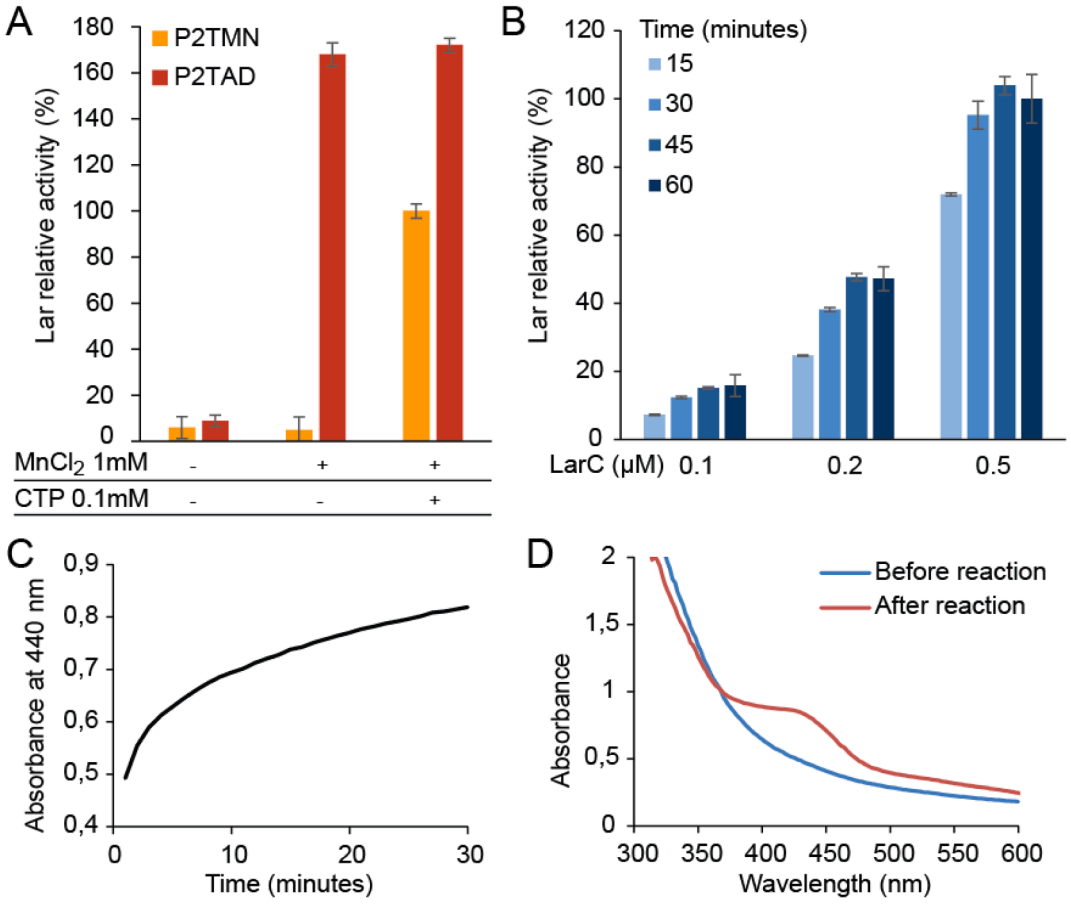

Fig. 3. P2TAD as substrate for LarC. **A**, NPN biosynthesis by LarC with P2TMN or P2TAD as substrate measured by Mar activity. The Mar activity with P2TMN was set to 100 %. **B**, Time-dependency of NPN bionsythesis by LarC at different LarC concentrations with P2TAD as substrate measured by Mar activity. The Mar activity with 0.5 µM LarC after 60 min was set to 100 %. The error bars indicate the standard deviation (n = 3). **C**, time-dependent increase of the absorbance at 440 nm during NPN biosynthesis with 300 µM LarC and 500 µM P2TAD. **D**, absorbance spectra of NPN biosynthesis before and after reaction.

an oxidizing environment [1]. Nevertheless, hydrolysis was not complete, as P2TAD **4** was still observable after overnight reaction with LarC (Fig. S1), suggesting that the hydrolysis reaction is coupled to the nickel insertion and does not take place independently at a high rate. In order to test whether LarC could catalyze multiple turnovers in presence of P2TAD **4**, we incubated increasing amounts of LarC in presence of an excess of P2TAD **4** and nickel and observed a strict dependency of NPN biosynthesis on LarC concentration, independently of the incubation time (Fig. 3B), indicating that LarC is still limited to one single turnover.

In order to further demonstrate the generation of NPN, a NPN biosynthesis reaction was conducted with 300 μM LarC and 500 μM P2TAD. The absorbance at 440 nm increased during the reaction (Fig. 3C), and the final absorbance showed a new absorbance peak at 440 nm, typical of the NPN cofactor [1]. The difference of absorbance at 440 nm, given the molar extinction coefficient of the NPN cofactor [16], would account for a generation of more or less 30 μM NPN, which was confirmed by the titration of Mar2 with decreasing concentrations of NPN (Fig. S2). This showed that only 6 % of P2TAD was converted into NPN, but LarC, and not P2TAD, is most probably the limiting factor in this reaction.

4. Conclusion

In conclusion, we optimized a chemo-enzymatic route for the synthesis of NPN, taking advantage of the promiscuous activity of both ARC on P2T **3** and LarC on P2TAD. The observation that P2TAD is a substrate of LarC confirms that LarC reaction mechanism proceeds through a dinucleotide intermediate, either the native cytidine dinucleotide or the synthetic adenine dinucleotide intermediate. The hydrolysis of this intermediate is crucial for LarC activity, as P2TMN cannot react with LarC in absence of CTP, and probably requires Mn^{2+} as Lewis acid for the activation of the hydrolytic water molecule. Our investigation also indicates that the kinetically limiting step of the LarC reaction is the dinucleotide hydrolysis and nickel insertion, and not CTP hydrolysis and generation of the P2TCD intermediate. Overall, this new chemo-enzymatic pathway made the synthesis of P2TAD **4** at a large scale possible, which allowed its characterization. It also simplified NPN biosynthesis by reducing the number of enzymes employed for its synthesis. In particular, LarB is not required anymore, as are the two molar equivalents of LarE. This new chemo-enzymatic route thus greatly facilitates the access to NPN. Furthermore, the fact that both ARC and LarC showed significant promiscuity opens interesting perspectives. For instance, by using different variants of P2T **3**, we might be able to generate different NPN analogs, that could serve as useful mechanistic probes for the study of NPN-dependent enzymes.

In summary, this new larger scale chemoenzymatic synthesis of P2TAD **4** should truly unlock the study of NPN-dependent enzymes and potentially allow their utilization in biotechnological processes.

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CRediT authorship contribution statement

Timothé Vucko: Writing – original draft, Methodology, Formal analysis. **Dmytro Strilets:** Data curation, Methodology, Validation. **Patrice Soumillion:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Benoît Desguin:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Stéphane P. Vincent:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2024.107879.

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