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Facile isolation of α -ribazole from vitamin B₁₂ hydrolysates using boronate affinity chromatography

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Abstract

Alpha-ribazole (α -R) is a unique riboside found in the nucleotide loop of coenzyme B₁₂ (CoB₁₂). α -R is not an intermediate of the *de novo* biosynthetic pathway of coenzyme B₁₂, but some bacteria of the phylum *Firmicutes* have evolved a two-protein system (transporter, kinase) that scavenges α -R from the environment and converts it to the pathway intermediate α -RP. Since α -R is not commercially available, one must either synthesize α -R, or isolate it from hydrolysates of vitamin B₁₂ (cyano-B₁₂, CNB₁₂), so the function of the above-mentioned proteins can be studied. Here we report a facile protocol for the isolation of α -R from CNB₁₂ hydrolysates. CNB₁₂ dissolved in NaOH (5 M) was heated to 85°C for 75 min, then cooled to 4°C for 30 min. The solution was neutralized with HCl (5 M), and the hydrolysate was diluted with an equal volume of ammonium acetate (0.3 M, pH 8.8). Alkaline phosphatase was added and the mixture was incubated at 37°C for 16 h. After incubation, the sample was loaded onto a boronate affinity resin column, washed with ammonium sulfate (0.3 M, pH 8.8), water (to remove residual corrinoids) and finally with formic acid (0.1 M) to release (α -R). Formic acid was removed by lyophilization, and the final yield of α -R was 85% from the theoretically recoverable amount. Methods for quantifying the concentration of α -R are reported.

Keywords

α -ribazole isolation; B₁₂ biosynthesis precursors; boronate affinity chromatography; late steps of coenzyme B₁₂ biosynthesis

1. Introduction

Coenzyme B₁₂ (CoB₁₂, Fig. 1A) is the largest coenzyme known, only some microbes synthesize it, and its synthesis requires ~30 enzymes [reviewed in [1]]. The enzyme that catalyzes the penultimate step of the pathway attaches two activated intermediates, one is alpha-ribazole-5'-phosphate (α -R-5'P, α -RP; Fig. 1A, bottom right), the other one is adenosyl-cobinamide-GDP (structure not shown); the product of this reaction is CoB₁₂-P. The last enzyme of the pathway removes the 5'-phosphate group of α -RP yielding the biologically active coenzyme (Fig. 1A).

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Most CoB₁₂-synthesizing organisms synthesize α -RP from 5,6-dimethylbenzimidazole (DMB) (Fig. 1B). Notably, some *Firmicutes* lack the NaMN:DMB phosphoribosyltransferase (CobT) enzyme, and instead have evolved a DMB-riboside (α -R, bottom left, Fig. 1A; UV-Visible spectrum, Fig. 1C) transporter (*i.e.*, CblT), and a kinase (*i.e.*, CblS) to convert α -R into α -RP [2, 3]. At present, in-depth biochemical studies of the CblT and CblS enzymes are limited by the fact that α -R is not commercially available.

Protocols for the chemical synthesis of α -R and the alkaline release of α -R from vitamin B₁₂ have been reported [4, 5]. For our initial studies of CblT and CblS enzymes [2] we followed a protocol that hydrolyzed CNB₁₂ under alkaline conditions to release α -R, and used reverse-phase liquid chromatography to resolve α -R from the mixture. The low yield of α -R generated by this method was likely due to the hydrophilic nature of α -R and corrinoids, a property that makes it difficult to separate these compounds by reverse-phase chromatography. Hence, we developed a protocol for the isolation of mg amounts of α -R from hydrolysates of CNB₁₂ using boronate affinity chromatography (BAC). We took advantage of the rapid and reversible formation of cyclic esters between boronic acids and *cis*-diol containing biomolecules to improve the yield of α -R. Under alkaline conditions, boronic acids and *cis*-diols form cyclic esters, and these interactions can be reversed by medium acidification. BAC has been extensively used for the analysis of complex mixtures containing saccharides, ribosylated metabolites, nucleosides and nucleotides [6–9]. On average, the protocol described herein yielded 3.5 mg of α -R per 25 mg of CNB₁₂ hydrolyzed.

2. Materials and Methods

2.1 Reagents

Immobilized Boronic Acid Gel® (#20244) was obtained from Thermo Fisher Scientific. Unless otherwise indicated, chemicals were obtained from Millipore Sigma. Alkaline phosphatase (ALP) derived from bovine intestinal mucosa was obtained from Millipore Sigma and contains >10 diethanolamine (DEA) units per mg of solid.

2.2 Bacterial strains

All bacterial strains and plasmids used in this study are listed in Supplemental Table 1 (Suppl. 1.6).

2.3 α -Ribazole synthesis

2.3.1 Vitamin B₁₂ preparation and hydrolysis—Vitamin B₁₂ (CNB₁₂; 50 mg; 73.8 μ mol) was re-suspended in 5 mL of water that was deionized using a MilliQ Water Purification System (Millipore). After transferring the solution into a screw-capped 250-mL Erlenmeyer flask, either 5 mL of NaOH (5 M) or water was added to the mixture. The resulting solution was placed in a water bath (85–90°C) for 75 min. After cooling for 30 min at 4°C, the solution was neutralized with HCl (5 M) prior to dephosphorylation by alkaline phosphatase (ALP). To determine the amount of α -R release, a 5- μ L sample was taken at each step; samples were diluted 100-fold prior to measuring fluorescence at 312 nm (Suppl. 1.2).

2.3.2 De-phosphorylation of α -R-3'-P—To convert α -R-3'-P to its riboside, ALP was added to the solution. Fifteen mL of a solution containing $[(\text{NH}_4)\text{Ac}]$ (0.3 M), MgCl_2 (2 mM, pH 8.8) was added to the hydrolysate. ALP was added in excess (>1000 U; 1U = amount of enzyme that hydrolyses 1 μmol of nitrophenyl-phosphate per minute at 37°C) and the solution was incubated at 37°C for at least 16 h. The resulting solution was centrifuged at $3,220 \times g$ for 20 min and the supernatant was filtered using a syringe filter (0.45 μm polyethersulfone membrane; VWR).

2.3.3. Boronate affinity chromatography (BAC)—After ALP treatment, α -R was isolated by passing the solution over a boronate column as described [10] with some modifications. Briefly, a 1.5-mL column of Immobilized Boronic Acid Gel® (Thermo Fisher Scientific; 110 μmol AMP binding capacity/mL of resin) was equilibrated with $(\text{NH}_4)\text{Ac}$ (0.3 M, pH 8.8). The hydrolysate mixture was passed over the column, and the column was washed with $(\text{NH}_4)\text{Ac}$ (0.3 M, pH 8.8) until the eluate was clear. The column appeared slightly pink likely due to non-specific binding of a corrinoid. The column was washed with 1.5 mL of MilliQ water to remove $(\text{NH}_4)\text{Ac}$. Bound α -R was desorbed with formic acid (0.1 M; 6-mL). The column was regenerated between cycles with five column volumes of formic acid (0.1 M) and five column volumes of $(\text{NH}_4)\text{Ac}$ (0.3 M, pH 8.8). As per the manufacturer's instructions, the resin can be used for up to 10 cycles but should be discarded if it remains colored after regeneration.

2.3.4. Product concentration—The eluent (3 mL) was shell-frozen in a 50-mL conical tube (VWR) over a mixture of ethanol and dry ice. Parafilm was used to seal the end of the tube and punctured 5-6 times before lyophilization that resulted in a white crystalline powder. The dry product was re-suspended on 200 μL dimethyl sulfoxide (DMSO), and the α -R final concentration and yield were determined by measuring fluorescence (Suppl. 1.2) or absorbance (Suppl. 1.3.2). The final product was analyzed by MALDI-TOF mass spectrometry to confirm its identity. The biological activity of the product was assessed by bioassay (Suppl. 1.5).

3. Results and Discussion

3.1. CNB₁₂ hydrolysis conditions

In other described protocols, alkaline hydrolysis of CNB₁₂ is performed at 100°C for 50 min [2]. We tested whether heating at a lower (85°C) or higher temperature (121°C at 124.1 kPa) would make a difference in the yield of α -R. Six samples of CNB₁₂ were prepared using ultrapure water (10 mg mL^{-1}) in screw-capped 250-mL Erlenmeyer flasks (2.3.1). The initial CNB₁₂ concentration was needed to determine the theoretical α -R yield (Suppl. 1.1). From 100 mg of CNB₁₂, we calculated a maximum yield of ~ 21 mg of α -R.

To assess the impact of NaOH on α -R release, an equal volume (5 mL) of water or NaOH was added to each flask containing dissolved CNB₁₂ (50 mg; ~ 7.4 mM). Three sets of hydrolysis mixtures, each with two replicates, were incubated at different temperatures: i) room temperature (25°C , benchtop), ii) 85°C (water bath), and iii) 121°C (45-min autoclave cycle, 124.1 kPa). The samples that were not autoclaved were incubated for 75 min at the stated temperature, followed by a 30-min cooling time at 4°C . The period of incubation (75

min) was chosen to match the duration of a 45-min autoclave cycle. Using fluorescence and the calculated quantum yield for α -R (Suppl. 1.2), the amount of α -R released was compared to an expected yield of ~10 mg. In the absence of NaOH, the amount of α -R released was negligible regardless of the method of heating used (data not shown).

The results for α -R release under alkaline conditions under the different conditions are shown in Table 1. An average of 0.3 mg of α -R (*i. e.*, 2.5% of the theoretical yield) was detected following treatment at room temperature (25°C). Growth was detected by the corrinoid scavenging assay (Suppl. 1.4), indicating incomplete CNB₁₂ hydrolysis.

At 85°C, 8.2 mg of α -R (*i. e.*, 79% of the theoretical yield) was detected while 8.5 mg of α -R (*i. e.*, 82% of the theoretical yield) was detected in the autoclaved samples. The B₁₂ indicator strain did not grow when a sample of the solution of CNB₁₂ that was heated at 85°C was added to the culture medium. We observed a similar response when a sample of the CNB₁₂ solution that was autoclaved was added to the culture medium (Suppl. 1.4). We interpreted these results to mean that the indicator strain could not repair the damage done to CNB₁₂ under the conditions used. Because autoclave hydrolysate products could not be removed from the boronate resin, autoclave treatment was discontinued for the remainder of the study.

3.2 No loss of α -R was detected in an ALP-treated CNB₁₂ solution

Alkaline hydrolysis of CNB₁₂ resulted in a mixture of the riboside α -R and α -R-3'-P [5]. To increase the concentration of α -R, ALP was used to dephosphorylate α -R-3'-P (2.3.2). After overnight incubation at 37°C, samples were clarified by centrifugation (15 min, 3000 $\times g$) and filtration. Quantification of free α -R by fluorescence (Suppl. 1.2) indicated no change following ALP treatment.

3.2.1. Resolving α -R from other hydrolysis products—A method previously used by our laboratory relied on reverse-phase chromatography (Lichroprep® RP-18, Merck) to resolve products using a methanol gradient [2]. That procedure generated large volumes (0.5 L), with α -R distributed in 20% of the fractions collected. In addition, α -R eluted off the column together with an unidentified corrinoid. To expedite the resolution of α -R from other hydrolysis products, we switched to boronate affinity chromatography (2.3.3). The specificity of boronate for *cis*-diol-containing compounds has been used for decades to separate nucleotides, nucleosides, catechols, carbohydrates, glycoproteins, etc [11–13]. Interactive compounds (in our case α -R) bind to the resin at alkaline pH and compounds are released from the column by acidification. Here, we used a described batch BAC method to rapidly decrease the pH from 8.8 to 3 [10].

3.2.2. α -R concentration and yield quantification—Following elution from the resin, the α -R-containing fraction (confirmed by bioassay) was concentrated by lyophilization (2.3.4) and re-suspended in dimethyl sulfoxide (DMSO). MALDI-TOF mass spectrometry of the isolated product revealed a mass of 279.2 Da (Fig. S4), consistent with the theoretical mass of α -R (278.3 Da). We quantified the yield of α -R by fluorescence (Suppl. 1.2). Assuming diol equivalency between AMP and α -R, a 1.5-mL column of immobilized boronate was estimated to have an α -R capacity of 46 mg. From a freshly-prepared column

loaded with 8.2 mg of α -R, we recovered 6.2 mg of α -R, a recovery of 76%. The recovered α -R was active in our bioassay (Suppl. 1.5), supporting similar growth rates to previously isolated, equimolar concentrations of α -R (Fig. S5).

4. Conclusions

The boronate-affinity chromatography method for the isolation of α -R described herein (Fig. 2) is rapid, minimizes the amount of waste, and generates mg amounts of this coenzyme B₁₂ precursor. Two significant improvements of BAC over the reverse phase chromatography method described elsewhere [2] were a higher yield of α -R (36% higher; Table 2), and a 10-fold reduction in sample volume.

We anticipate this method to be adaptable for the isolation of different α -ribosides (other than α -R) present in cobamides synthesized by different microbes [14, 15]. This method could also be adapted for the identification of lower ligands in a population sample [16], as an alternative to reverse phase chromatography for the separation of cobamides [3].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- α -R is not commercially available and is needed for the analysis of a recently discovered α -R scavenging pathway
- A facile procedure is described for the production of α -R
- The procedure yields 85% of the theoretically recoverable amount of α -R from vitamin B₁₂
- The procedure reduces 90% of the waste generated by alternative methods

Figure 1. Structure of coenzyme B₁₂ (CoB₁₂), its precursors, and the known routes to α -R-5'-P
A (Top). Structure of CoB₁₂. The nucleotide loop (pink) containing 5,6-dimethylbenzimidazole (DMB) is tethered to the corrin ring by the aminopropanol (AP; blue) substituent. **A (bottom):** the DMB riboside α -R (bottom left) and ribotide α -R-5'-phosphate (α -RP; bottom right). **B.** The two known routes to α -RP. Abbreviations: cell membrane, CM; α -R transporter, CblT; α -R kinase, CblS; DMB:NaMN PRTase, CobT; nicotinate mononucleotide, NaMN; nicotinic acid, Na. **C.** The UV-visible spectrum of α -R dissolved in DMSO.

Figure 2. Schematic of the process for the isolation of $\alpha\text{-R}$ synthesis from CNB_{12} hydrolysates

The section corresponding to each step is found in parentheses. Corrinoïd refers to the inactive CNB_{12} derivative. Abbreviations: alkaline phosphatase, ALP; formic acid, HCOOH; ammonium acetate, (NH₄)Ac; α , refers to the configuration of the *N*-glycosidic bond.

Table 1
Comparison of the estimated amount of a-R released under different hydrolysis conditions.

Heating Treatment	Sample	Starting Material	a-R Expected (mg)	a-R Released (mg)	% Released
Room temperature (25 °C)	#1	CNB ₁₂ (50 mg)	10.4	0.3	2.7%
	#2	CNB ₁₂ (50 mg)	10.4	0.2	2.3%
	Average		10.4	0.3	2.5%
Water Bath (85 °C)	#1	CNB ₁₂ (50 mg)	10.4	8.4	80.8%
	#2	CNB ₁₂ (50 mg)	10.4	8.0	77.2%
	Average		10.4	8.2	79.0%
Autoclave (121 °C)	#1	CNB ₁₂ (50 mg)	10.4	8.5	82.1%
	#2	CNB ₁₂ (50 mg)	10.4	8.4	81.4%
	Average		10.4	8.5	81.8%

Table 2

A comparison to the previously published yield as determined by absorbance (Suppl. 1.3.2)

Comparison	Starting Material	α -R Expected(mg)	Yield (mg)	% Yield	% Yield, Adjusted ^I
Lichroprep® RP-C18/solid phase extraction [7]	CNB ₁₂ (100 mg)	20.5	8.0	39%	49%
Immobilized boronate/lyophilization	CNB ₁₂ (50 mg)	10.3	6.9	67%	85%

^I Adjusted to the maximum release of α -R from CNB₁₂ in a water bath (79%)