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Characterization of vitamin B_{12} compounds in biofertilizers containing purple photosynthetic bacteria

Tomohiro Bito[1], Noriharu Ohishi[2], Shigeo Takenaka[3], Yukinori Yabuta[1,2], Emi Miyamoto[4], Eiji Nishihara[1,2] and Fumio Watanabe[1,2,*]

[1]Division of Applied Bioresources Chemistry, United Graduate School of Agricultural Sciences, Tottori University, Tottori, Japan, [2]School of Agricultural, Biological and Environmental Sciences, Tottori University, Tottori, Japan, [3]Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka 598-8531, Japan, [4]Department of Health and Nutrition, Nagasaki International University, Sasebo, Japan

ABSTRACT

There is currently no data available on the vitamin B_{12} content of biofertilizers containing purple photosynthetic bacteria or whether these biofertilizers contain "true B_{12}" or inactive corrinoid compounds (or both). Therefore, we tested the vitamin B_{12} content of three commercially available biofertilizers containing purple photosynthetic bacteria. No or traces of vitamin B_{12} were found in two of the biofertilizers (A and B) tested, while the third (C) contained a considerable amount (53.5 μg /L). To evaluate whether biofertilizer C actually contained vitamin B_{12} or other corrinoids inactive in humans, the extracted compounds were purified using an immunoaffinity column and then identified as vitamin B_{12} (main) and/or inactive corrinoids (minor, Factor III) using liquid chromatography electrospray ionization mass spectrometry (LC/ESI-MS/MS).

KEYWORDS: biofertilizer, cobalamin, lettuce, purple photosynthetic bacteria, vitamin B_{12}

INTRODUCTION

Vitamin B_{12} (B_{12}) or cobalamin is synthesized only by certain bacteria [1]. B_{12} synthesized by bacteria is concentrated mainly in the bodies of higher predatory organisms in the natural food chain. Foods derived from animals (i.e., meat, milk, egg, fish, and shellfish), but not plants are considered to be the major dietary sources of B_{12} [2]. Thus, strict vegetarians have a greater risk of developing a B_{12} deficiency compared to non-vegetarians [3]. The major symptoms of B_{12} deficiency are neuropathy and megaloblastic anemia [4]. Therefore, there is a need to identify plant foods that contain high levels of B_{12} to prevent vegetarians from developing a B_{12} deficiency.

Mozafar [5] demonstrated that the addition of an organic fertilizer, cow manure, significantly increased the B_{12} content (17.8 ng/g dry weight) in spinach leaves. However, our unpublished works indicate that most organic fertilizers, especially those made with animal manure, contain considerable amounts of inactive corrinoid compounds, which have also been reported to be present in human feces and account for more than 98% of total corrinoid compounds [6].

Biofertilizers are products containing living cells of beneficial microorganisms, which can accelerate and improve plant growth by providing nutritionally important elements (e.g., nitrogen and phosphorus) [7]. Although cyanobacteria are responsible for biological nitrogen (N_2) fixation in

*Corresponding author
watanabe@muses.tottori-u.ac.jp

flooded rice fields [8], they contain large amounts of pseudovitamin B_{12} (pseudo-B_{12}) that is inactive in humans [9]. N_2-fixing purple photosynthetic bacteria were reported to have beneficial effects on plant growth [10] and the ability to synthesize B_{12} *de novo* [11]. If biofertilizers containing purple photosynthetic bacteria contain a substantial amount of "true B_{12}," these fertilizers would be useful for enriching B_{12} in plants. However, there is no information on the B_{12} content of biofertilizers containing purple photosynthetic bacteria or whether these biofertilizers contain "true B_{12}" or inactive corrinoid compounds (or both).

In this study, we determined the B_{12} content of three biofertilizers containing purple photosynthetic bacteria and characterized the B_{12} compounds found in these biofertilizers using liquid chromatography electrospray ionization mass spectrometry (LC/ESI-MS/MS).

MATERIALS AND METHODS

Materials

B_{12} was obtained from Sigma (St. Louis, MO, USA) and 5-hydroxybenzimidasolyl cyanocobamide (Factor III) was provided by Dr. Stüpperich, Ulm University, Germany. The B_{12} assay medium for *Lactobacillus delbrueckii* subspecies *lactis* (formerly *L. leichmannii*) ATCC7830 was obtained from Nissui (Tokyo, Japan). Silica gel 60 thin-layer chromatography (TLC) aluminum sheets were obtained from Merck (Darmstadt, Germany). A Shimadzu (Kyoto, Japan) UV-visible spectrophotometer (UV-2550) was used to measure the turbidity of *L. delbrueckii* test cultures with the microbiological B_{12} assay method. Biofertilizers containing purple photosynthetic bacteria were purchased at local markets in Japan. All other reagents used were of the highest purity commercially available.

Extraction and assay of vitamin B_{12}

Each biofertilizer (1 L) was added to the same volume of 5 mmol/L acetate buffer (pH 4.5) containing 0.01% (w/v) potassium cyanide (KCN). Total B_{12} was extracted by boiling this in a draught chamber (Dalton Co., Tokyo, Japan) for 30 min. B_{12} was assayed at least five times independently by the microbiological method with *L. delbrueckii* ATCC 7830. Since *L. delbrueckii* ATCC 7830 can utilize both deoxyribosides and deoxyribonucleotides (known as an alkali-resistant factor) as well as B_{12} [12], the amount of B_{12} was calculated by subtracting the values of the alkali-resistant factor from the total B_{12} values.

Bioautography of vitamin B_{12} compounds with vitamin B_{12}-dependent *Escherichia coli* 215

Bioautography of the B_{12} compounds was done according to the method of Tanioka *et al.* [13]. After an aliquot of each B_{12} extract of the biofertilizers was concentrated and partially purified with a Sep-Pak Plus C18 cartridge (Waters Corp.), concentrated (100 times) extracts, authentic B_{12}, and pseudo-B_{12} (10 μg/L each) were spotted on a silica gel 60 TLC sheet and developed with 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) in the dark at 25°C. Once the TLC sheet was dried, agar containing basal medium and precultured *E. coli* 215 was overlaid and then incubated at 30°C for 20 h. After being sprayed with a methanol solution of 2,3,5-triphenyltetrazolium salt on the gel plate, B_{12} compounds were stained as red in color indicating *E. coli* growth.

LC/ESI-MS/MS analysis

The selected biofertilizer extracts were put on Sep-Pak ®Vac 20cc (5 g) C18 Cartridges (Waters Corp.), which had been washed with 75% (v/v) ethanol and then equilibrated with distilled water. After the C18 Cartridge was washed with 30 mL of distilled water, B_{12} compounds were eluted with 30 mL of 75% (v/v) ethanol. The eluate was evaporated to dryness under reduced pressure. The residual fraction was dissolved with 5 mL of distilled water and centrifuged at 10000 g for 10 min to remove insoluble materials. The supernatant fraction was loaded onto an immunoaffinity column [EASI-EXTRACT® B_{12} Immunoaffinity Column (P80) R-Biopharm AG, Darmstadt, Germany] and then B_{12} compounds were purified according to the manufacture's recommended protocol.

The purified samples, authentic B_{12}, and Factor III were dissolved in 0.1% (v/v) acetic acid and filtered with a Nanosep MF centrifuge device (0.4 μm, Pall Corp., Tokyo, JAPAN) to remove small particles. An aliquot (2 μL) of the filtrate was then analyzed

using LCMS-IT-TOF coupled with an Ultra-Fast LC system (Shimadzu, Kyoto, JAPAN). The purified sample was injected in an InertSustain column (3 μm, 2.0 x 100 mm, GL Science, Tokyo, JAPAN) and equilibrated with 85% solvent A [0.1% (v/v) acetic acid] and 15% solvent B (100% methanol) at 40°C. B_{12} compounds were eluted with a linear gradient of methanol (15% solvent B for 0-5 min, 15%-90% solvent B for 5-11 min, and 90%-15% solvent B for 11-15 min) at a flow rate of 0.2 mL/min. ESI conditions were determined by injecting authentic Factor III or B_{12} into the MS detector to achieve optimum parameters to detect parent and daughter ions of the B_{12} compound. ESI-MS was operated in positive ion mode with argon as the collision gas. The identification of Factor III (m/z 672.272) and B_{12} (m/z 678.292) representing $[M+2H]^{2+}$ was confirmed by comparison of the observed molecular ions and the retention times.

RESULTS AND DISCUSSION

The amount of B_{12} in three commercially available biofertilizers containing purple photosynthetic bacteria was determined using a microbiological method. No or traces of B_{12} were found in two of the biofertilizers (A and B) tested while the third (C) contained a considerable amount (53.5 μg /L) of the vitamin.

The B_{12} compounds found in biofertilizer C were analyzed with the *E. coli* 215 bioautogram after being separated with silica gel 60 TLC (Fig. 1). A concentrated extract of biofertilizer C was identified as a clear single spot, with a R_f value identical to that of authentic B_{12} but not to that of pseudo-B_{12}, which is inactive in humans. For biofertilizers A and B, no or an indistinct spot was detected. B_{12} compounds were purified from the extracts of biofertilizer C with an immunoaffinity column and then identified by LC/ESI-MS/MS (Fig. 2). As described previously [14], authentic B_{12} ($C_{63}H_{88}CoN_{14}P$; monoisotopic mass 1354.5674) was eluted as a peak with a retention time of 7.30 min. MS results of authentic B_{12} indicated a major divalent ion of m/z 678.2937 $[M+2H]^{2+}$, and isotope distribution data supported the determination that B_{12} predominantly formed a divalent ion under the LC/ESI-MS conditions. MS/MS spectrum of B_{12} indicated that the ion of m/z 359.0981 was predominantly formed due to the nucleotide moiety of the molecule.

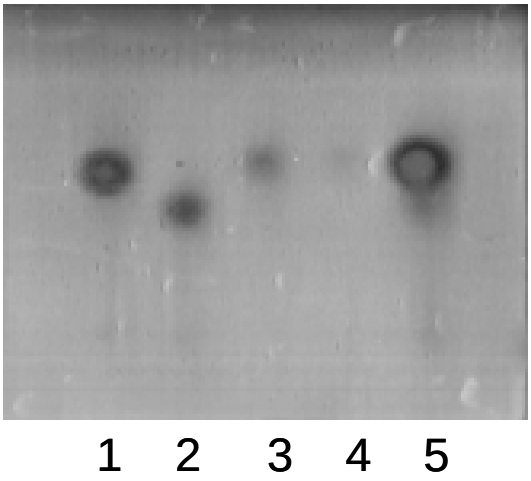

Fig. 1. *Escherichia coli* 215 bioautogram analysis of B_{12} compounds found in commercially available biofertilizers containing photosynthetic purple bacteria. 1) Authentic B_{12}, 2) authentic pseudo-B_{12}, 3) biofertilizer A concentrated extract (5 μL), 4) biofertilizer B concentrated extract (5 μL), and 5) biofertilizer C concentrated extract (2 μL). Data represent typical bioautograms of three independent experiments.

In the case of authentic Factor III ($C_{61}H_{84}CoN_{14}P$; monoisotopic mass 1342.5310), this corrinoid was eluted as a peak with a retention time of 7.14 min. MS of authentic Factor III had a major divalent ion at m/z 672.2715 $[M+2H]^{2+}$ (Fig. 2A and B). MS/MS spectrum of Factor III indicated that the ion of m/z 347.0636 was predominantly formed due to the nucleotide moiety (Fig. 2C).

The purified sample was eluted as several total ion peaks, indicating that impurities still existed (Fig. 3A). The ion peaks of m/z 672.27 and 678.29 due to Factor III and B_{12}, respectively, were also found and their retention times were identical to those of authentic Factor III and B_{12}. MS at retention times of 7.14 and 7.35 min showed the formation of both Factor III and B_{12} divalent ions of m/z 672.2715 (Fig. 3B) and 678.2996 (Fig. 3C), respectively. MS/MS spectrum of each ion peak was identical to that of authentic Factor III and B_{12} (Fig. 3D and E). These results indicated that biofertilizer C contained both B_{12} (main) and Factor III (minor).

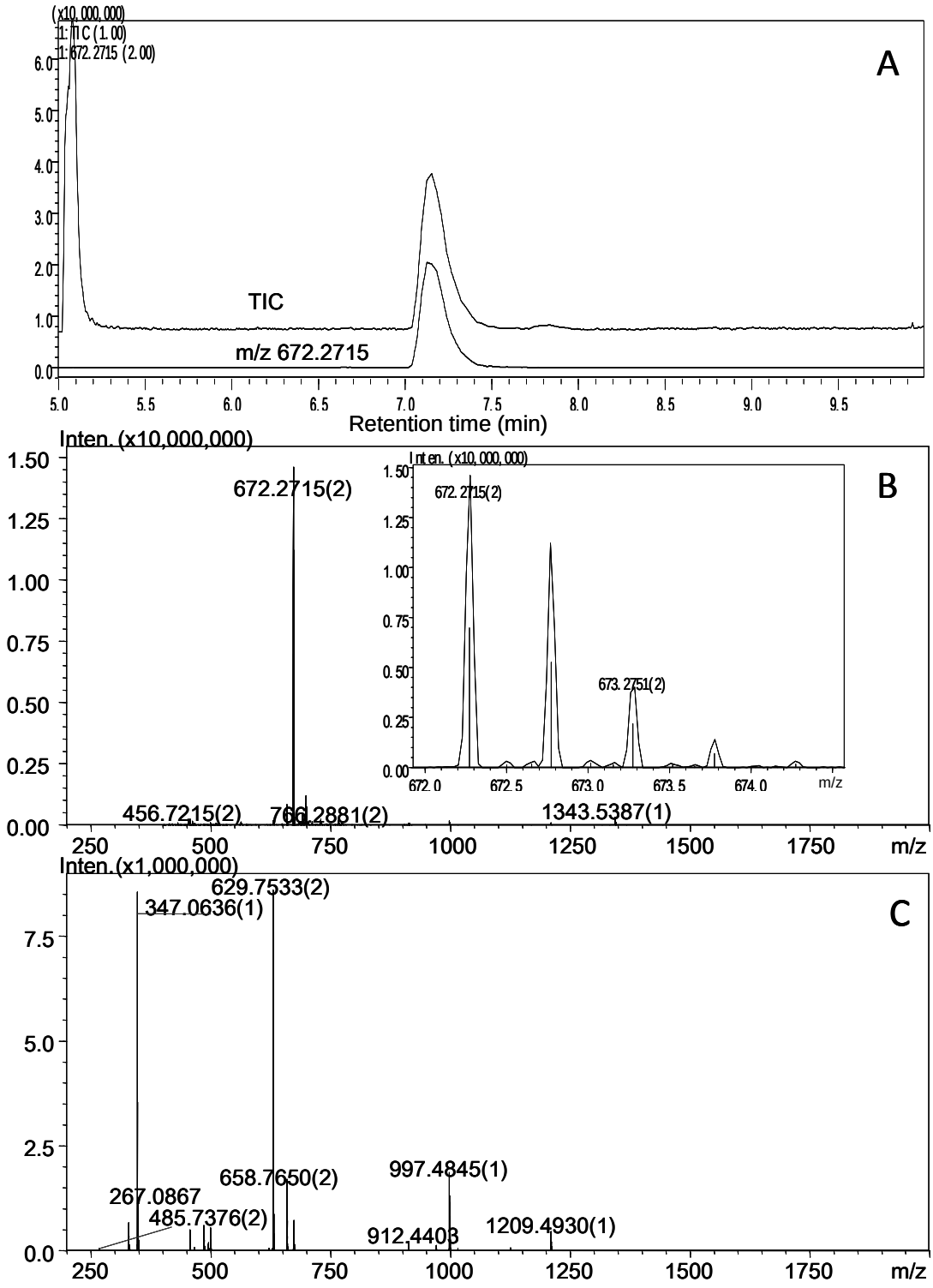

Fig. 2. Liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS/MS) chromatograms of authentic Factor III. Factor III was analyzed with LCMS-IT-TOF (Shimadzu) as described in the text. The total ion chromatogram (TIC) of authentic Factor III is shown in panel A. The mass spectrum of authentic Factor III at 7.14 min is shown in panel B; the magnified spectrum from m/z 672 to 675 is inserted in the panel. The MS/MS spectrum for the peak of m/z 672.2715 from authentic Factor III is shown in panel C.

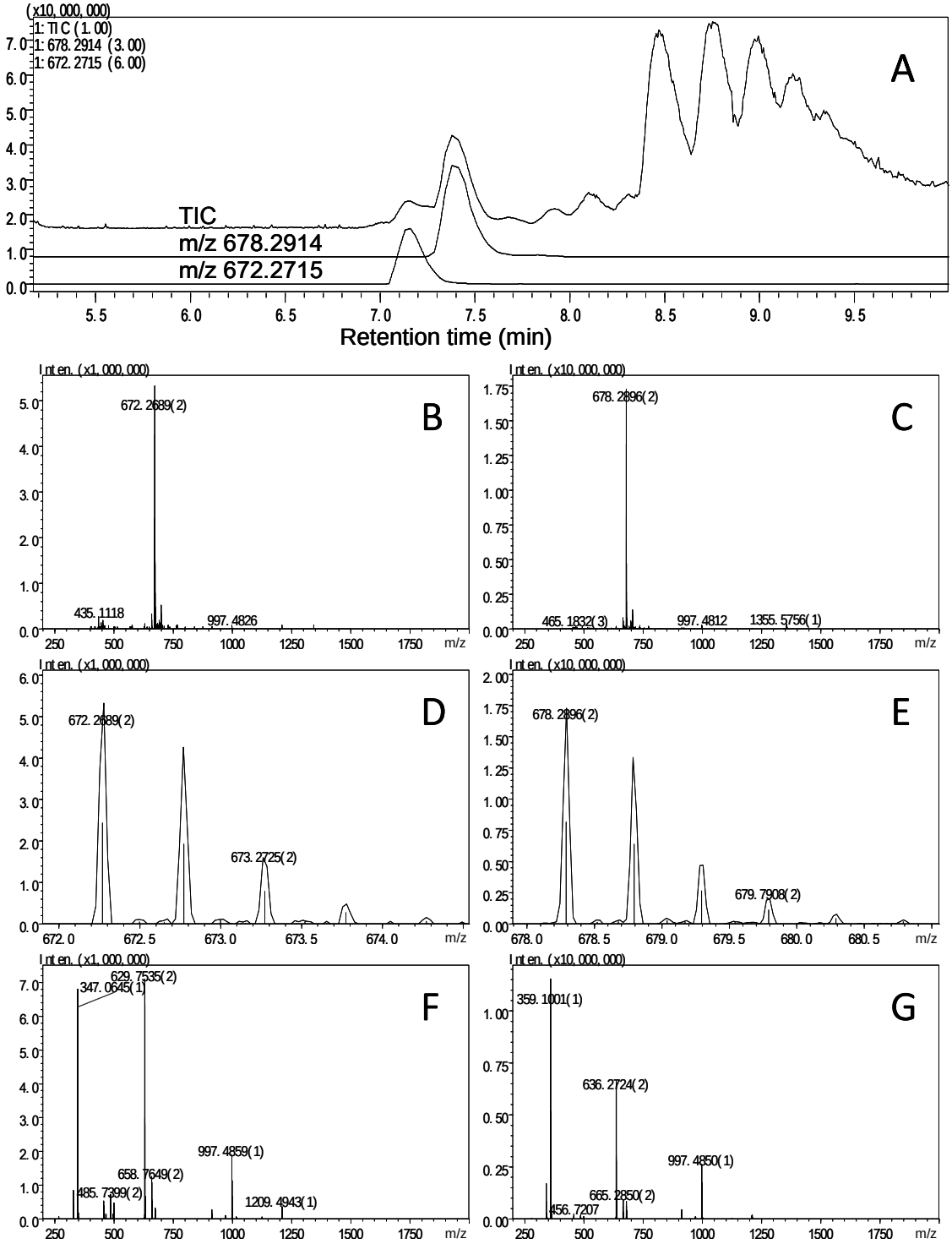

Fig. 3. LC/ESI-MS/MS chromatograms of purified B_{12} compounds from biofertilizer C. TIC and reconstructed chromatograms of m/z 678.29 and 672.27 of the purified B_{12} compound are shown in panel A. The mass spectra of the purified B_{12} compound at 7.14 and 7.30 min are shown in panels B and C, respectively; the magnified spectra from m/z 672 to 675 and m/z 678 to 681 are shown in panels D and E, respectively. The MS/MS spectra for the peak of m/z 672.27 and 678.29 from the purified B_{12} compound are shown in panels F and G, respectively.

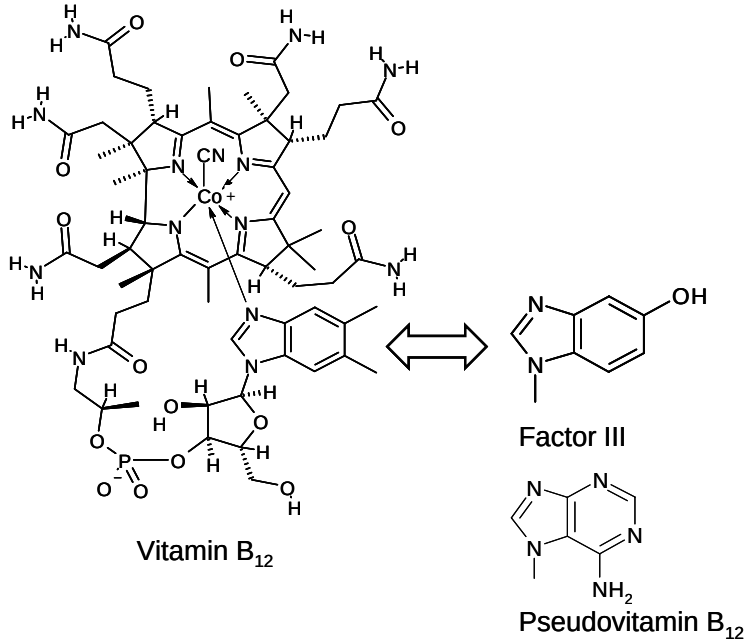

Fig. 4. Corrinoid compounds (vitamin B_{12} and Factor III) found in biofertilizer C containing purple photosynthetic bacteria. Partial structures of Factor III and pseudo-B_{12} showing only that portion of the molecule that differs from B_{12}.

Since the N_2-fixing photosynthetic purple bacterium *Rhodobacter capsulatus*, used as a biofertilizer, has the ability to synthesize B_{12} *de novo* [11], Factor III may be derived from other concomitant bacteria in commercial biofertilizer C (Fig. 4).

Despite the detection of B_{12} in one of the biofertilizers tested, in our preliminary experiments, B_{12} was not detected in the lettuce leaves grown with and without biofertilizer C treatment of the soil and leaves once a week for three weeks according to the manufacturer's recommended protocol. These results indicated that commercially available biofertilizers containing purple photosynthetic bacteria are not suitable for B_{12}-enrichment of plants due to the low B_{12} content.

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