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Occurrence of Biologically Inactive Corrinoid Compounds in Canned Edible Apple Snails (Escargots)

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Abstract

In this study, we characterized and quantified vitamin B_{12} in canned apple snails, escargots, (boiled plain) using a microbiological assay based on *Lactobacillus delbrueckii* ATCC 7830. Vitamin B_{12} contents of canned escargots (boiled plain) were varied from approximately 0.8 μg/100g weight to approximately 5.5 μg/100g weight (mean values, 2.2 μg/100g weight). We identified vitamin B_{12} compounds from escargots using liquid chromatography-electrospray ionization/tandem mass spectrometry. We found that escargots contained true vitamin B_{12} and two inactive corrinoids, which were identified as factor IIIm (or methoxymensimidazolyl cyanocobamide), and factor S (or 2-methylmercaptoadenyl cyanocobamide). These results indicate that canned escargots (boiled plain) are not good sources of vitamin B_{12} for humans.

Keywords

Apple Snails, Escargots, Canned Products, Factor S, Factor IIIm, Vitamin B_{12}

1. Introduction

Various species of edible land snails are consumed as apple snails (or escargots) worldwide. Escargots are high-

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ly appreciated in France, Italy, Germany, and Austria. “Snails in Garlic-Herb Butter” is a standard menu item in restaurants and bistros [1]. Nowadays, they are grown and provided by snail forming. However, import of any living snails is restricted in Japan and other countries because snail pests attack crops such as leafy vegetables and fruits. Therefore, snails are usually imported as canned products [1].

Vitamin B_{12} is synthesized only by certain bacteria and concentrated mainly in the bodies of higher predatory animals in the natural food chain. The usual dietary sources of vitamin B_{12} are animal food products (*i.e.*, meat, milk, egg, fish, and shellfish) [2]. Shellfish that siphon large quantities of vitamin B_{12}-synthesizing bacteria from seawater and freshwater is excellent sources of vitamin B_{12} [2] [3]. However, these vitamin B_{12}-synthesizing bacteria can also synthesize other corrinoids with a different base moiety in the lower ligand of the molecule [4]. An edible sea snail, whelks *Buccinum middendorffi*, contained high amount of vitamin B_{12} contents (10.5 - 21.4 μg/100g wet weight), whereas abalone *Haliotis diversicolor aquatilis* had extremely low vitamin B_{12} contents (0.3 μg/100g wet weight). In abalone, vitamin B_{12} and pseudovitamin B_{12} (an inactive corrinoid) were observed to be major and minor corrinoid compounds, respectively [5]. However, little information is available on the vitamin B_{12} contents of apple snails (or escargots) and on whether these contain vitamin B_{12} or pseudovitamin B_{12}, which is biologically inactive in humans.

Here, we describe determination of the vitamin B_{12} contents of canned escargots (boiled plain) and characterization of their vitamin B_{12} compounds to evaluate whether they are good sources of vitamin B_{12}.

2. Materials and Methods

2.1. Materials

Vitamin B_{12} was obtained from Sigma (St Louis, Missouri, USA). A vitamin B_{12} assay medium based on *Lactobacillus delbrueckii* subspecies *lactis* (formerly *L. leichmannii*) ATCC 7830 was obtained from Nissui (Tokyo, Japan). Silica gel 60 thin layer chromatography (TLC) aluminum sheets were obtained from Merck (Darmstadt, Germany). Canned escargots (boiled plain) were purchased from local markets in Japan.

2.2. Extraction and Assay of Vitamin B_{12} in Canned Escargot

After broth of canned escargots (boiled plain) was removed, all solid content (escargot whole body) was homogenized using a mixer (TML160; Tescom & Co., Ltd., Tokyo, Japan). An aliquot (2.0 g) of the homogenate was used as the sample. The vitamin B_{12} compounds were extracted from the sample by boiling at pH 4.5 and then assayed using a microbiological method based on *L. delbrueckii* ATCC 7830, according to a previously described method [4]. *L. delbrueckii* ATCC 7830 can utilize deoxyribosides and deoxyribonucleotides (known to be an alkali-resistant factor) as well as vitamin B_{12}. Thus, the correct vitamin B_{12} values were calculated by subtracting the values for the alkali-resistant factor from the total vitamin B_{12} values.

2.3. TLC-Bioautography Assay Using Vitamin B_{12}-Dependent *Escherichia coli* 215

A bioautography assay to detect corrinoid compounds was performed as previously described [6]. The vitamin B_{12} extract (20 mL) prepared as described above was partially purified and concentrated using a Sep-Pak Plus®C18 cartridge (Waters Corp., Milford, MA) that was washed with 5 mL of 75% (v/v) ethanol and equilibrated with 5 mL of distilled water. The C18 cartridge was washed with 5 mL of distilled water and vitamin B_{12} compounds were eluted using 2 mL of 75% (v/v) ethanol. The eluate was evaporated in a centrifugal concentrator (Integrated Speed Vac® System ISS110; Savant Instruments Inc., NY, USA), and the residual fraction was dissolved in 1.0 mL of distilled water. Concentrated extracts (1 μL) and vitamin B_{12} and pseudovitamin B_{12} (each 0.1 mg/L) were spotted onto the silica gel 60 TLC plates and developed in the dark using 2-propanol/NH_4OH (28%)/water (7:1:2 v/v) at room temperature (25˚C). The TLC plate was dried and overlaid with agar-containing basal medium and precultured *E. coli* 215, followed by incubation at 30˚C for 20 h. The gel plate was subsequently sprayed with methanol solution containing 2,3,5-triphenyltetrazolium salt, and vitamin B_{12} compounds were visualized as red, which indicated *E. coli* growth.

2.4. Identification of Vitamin B_{12} Compounds by LC/ESI-MS/MS

Each vitamin B_{12} extract (40 mL) was partially purified and concentrated using a Sep-Pak® Plus C18 cartridge

(Waters Corp.) as described above. The eluate was evaporated to dryness under reduced pressure and the residual fraction was dissolved in 3 mL distilled water and centrifuged at 10,000 × g for 10 min to remove insoluble material. The supernatant fraction was loaded onto an immunoaffinity column [EASI-EXTRACT®B_{12} Immunoaffinity Column (P80) R-Biopharm AG, Darmstadt, Germany], and vitamin B_{12} compounds were purified according to the manufacturer's protocol. The vitamin B_{12} compounds were purified by passage through the immunoaffinity column, dissolved in 0.1% (v/v) acetic acid, and filtered through a Nanosep MF centrifuge device (0.4 μm; Pall Corp., Tokyo, Japan) to remove small particles. An aliquot (2 μL) of the filtrate was analyzed using a LC/MS IT-TOF (ion trap-time-of-flight) system coupled to an Ultra-Fast LC system (Shimadzu, Kyoto, Japan). Each purified corrinoid was injected into an Inert-Sustain column (3 μm, 2.0 × 100 mm; GL Science, Tokyo, Japan) and equilibrated with 100% solvent A [0.1% (v/v) acetic acid] and 0% solvent B (100% methanol) at 40˚C. Corrinoids were eluted using 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). The flow rate was 0.2 mL/min. ESI conditions were determined by injecting the corrinoids into the MS detector, thereby identifying the optimum parameters for detecting parent and daughter ions of vitamin B_{12} compounds. The ESI-MS system was operated in the positive ion mode, and argon was used as the collision gas. Vitamin B_{12} (*m/z* 678.2914), factor IIIm or methoxymensimidazolyl cyanocobamide (*m/z* 679.7834), and factor S or 2-methylmercaptoadenyl cyanocobamide (*m/z* 695.7657) and as $[M + 2H]^{2+}$ were confirmed by comparing the observed molecular ions and retention times.

3. Results and Discussion

3.1. Vitamin B_{12} Contents of Canned Escargots (Boiled Plain)

We analyzed the vitamin B_{12} contents of canned escargots (boiled plain) that are available in Japan using the *L. delbrueckii* ATCC 7830 microbiological assay method (**Table 1**). Vitamin B_{12} contents of canned escargots (boiled plain) were varied from approximately 0.8 μg/100g weight to approximately 5.5 μg/100g weight. Mean values (2.2 μg/100g weight) were approximately 3.6-times greater than the value (0.6 μg/100g weight) of the Japanese food composition database [7], but is very lower than values (17.4 - 39.4 μg/100g weight) of canned clams (boiled plain) [8].

3.2. Identification of Corrinoid Compounds from Canned Escargots (Boiled Plain) Using the *E. coli* 215 Bioautography

The corrinoids observed in all escargot samples were analyzed using an *E. coli* 215 bioautogram after separation by silica gel 60 TLC. The corrinoids observed in all escargot samples produced clear spots which had the identical Rf value of authentic vitamin B_{12}, although sample A and B showed another sport, which was not identical to that of pseudovitamin B_{12} (**Figure 1**).

3.3. LC/ESI-MS/MS Analysis

To evaluate escargot vitamin B_{12} compounds, each vitamin B_{12} extract was purified using a vitamin B_{12} immunoaffinity column and analyzed by LC/ESI-MS/MS. Authentic vitamin B_{12} was eluted as peak with a retention

Table 1. Vitamin B_{12} contents of various canned escargots (boiled plain).

	Amount of vitamin B_{12} content		Production area
	(μg/100g wet weight)	(μg/whole body)	
Sample A (n = 3)	5.5 ± 1.0	0.4 ± 0.1	Indonesia
Sample B (n = 3)	1.8 ± 0.5	0.1 ± 0.0	Indonesia
Sample C (n = 3)	0.8 ± 0.4	0.1 ± 0.0	Indonesia
Sample D (n = 3)	1.7 ± 0.4	0.1 ± 0.0	France
Sample E (n = 3)	1.4 ± 0.4	0.1 ± 0.0	France
Mean ± SD	2.2 ± 1.9	0.2 ± 0.1	

Total vitamin B_{12} compounds were extracted from an aliquot (2.0 g) of each sample homogenate by boiling at pH 4.5 in the presence of 4.0×10^{-4}% KCN and assayed using the *Lactobacillus delbrueckii* ATCC 7830 microbiological assay. The vitamin B_{12} assay was performed in triplicate.

time of 7.50 min (**Figure 2(a)**). Mass spectrum of authentic B_{12} indicated that a doubly charged ion with an *m/z* of 678.2883 $[M + 2H]^{2+}$ was prominent (**Figure 2(b)**). The exact mass calculated from its formula ($C_{63}H_{88}CoN_{14}O_{14}P$) was 1354.5674, and the isotope distribution data showed that vitamin B_{12} was the major doubly charged ion under the LC/ESI-MS conditions used in our assay. The MS/MS spectrum of authentic vitamin B_{12} indicated that the dominant ion at *m/z* 359.0984 was attributable to the nucleotide moiety. The vitamin B_{12} compounds purified from escargots were eluted as three ion peaks with *m/z* 679.7834, *m/z* 678.2914, and *m/z* 695.7657 at retention times of 7.35 min, 7.50 min, and 7.65 min, respectively (**Figure 3(a)**). The mass spectrum of the ion peak

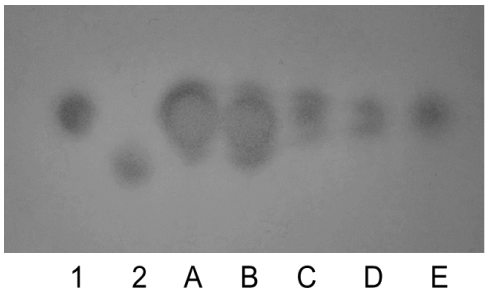

Figure 1. *Escherichia coli* 215 bioautogram analysis of vitamin B_{12} compounds detected in various canned ecargots (boiled plain). Authentic vitamin B_{12} (1) and pseudovitamin B_{12} (2), and extracts of canned escargots (boiled plain) sample A to sample E (A - E). Data presented are typical bioautograms from three independent experiments.

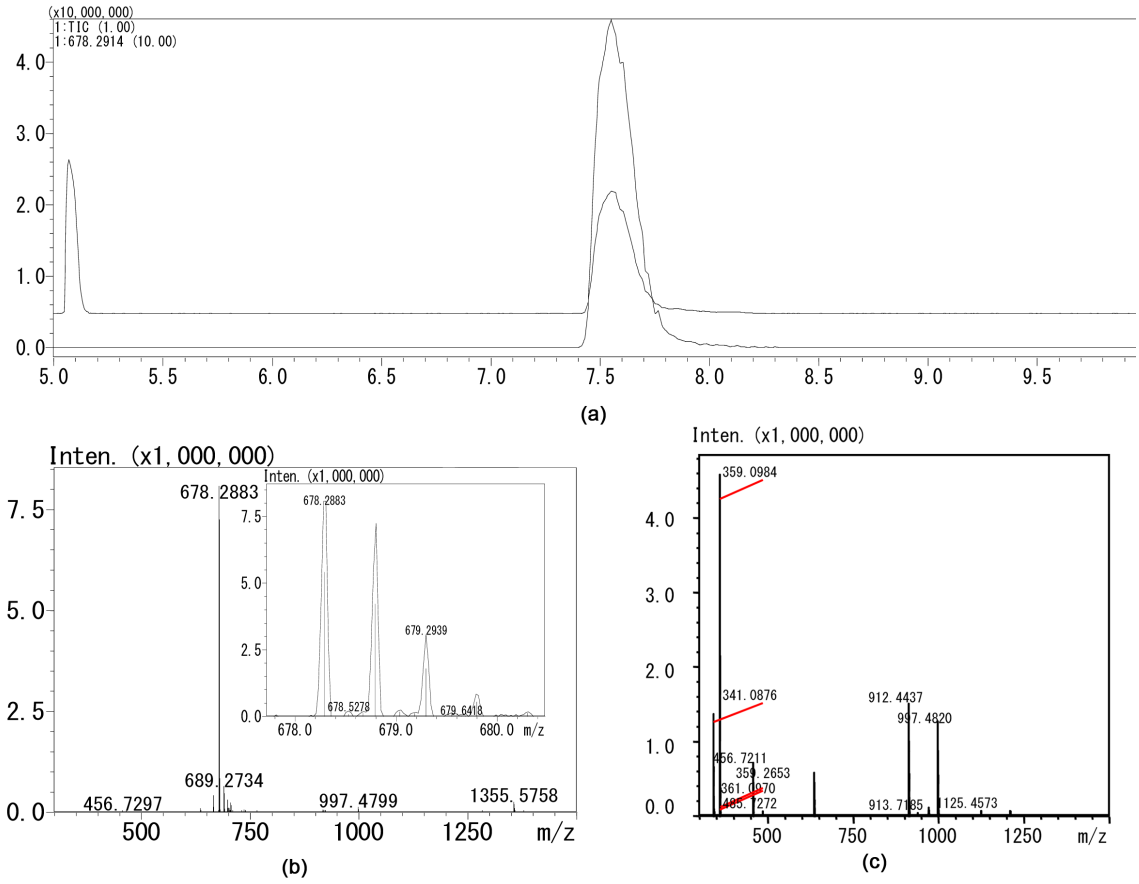

Figure 2. LC/ESI-MS/MS chromatogram of authentic vitamin B_{12}. Vitamin B_{12} was analyzed with LCMS-IT-TOF (Shimadzu) as described in the text. The total ion chromatogram (TIC) of authentic vitamin B_{12} is shown in panel (a). The mass spectrum of an ion peak from vitamin B_{12} is shown in panel (b). The magnified mass spectrum from *m/z* 678 to 680 in vitamin B_{12} is shown as an insert. The MS/MS spectrum of the peak of vitamin B_{12} is shown in panel (c).

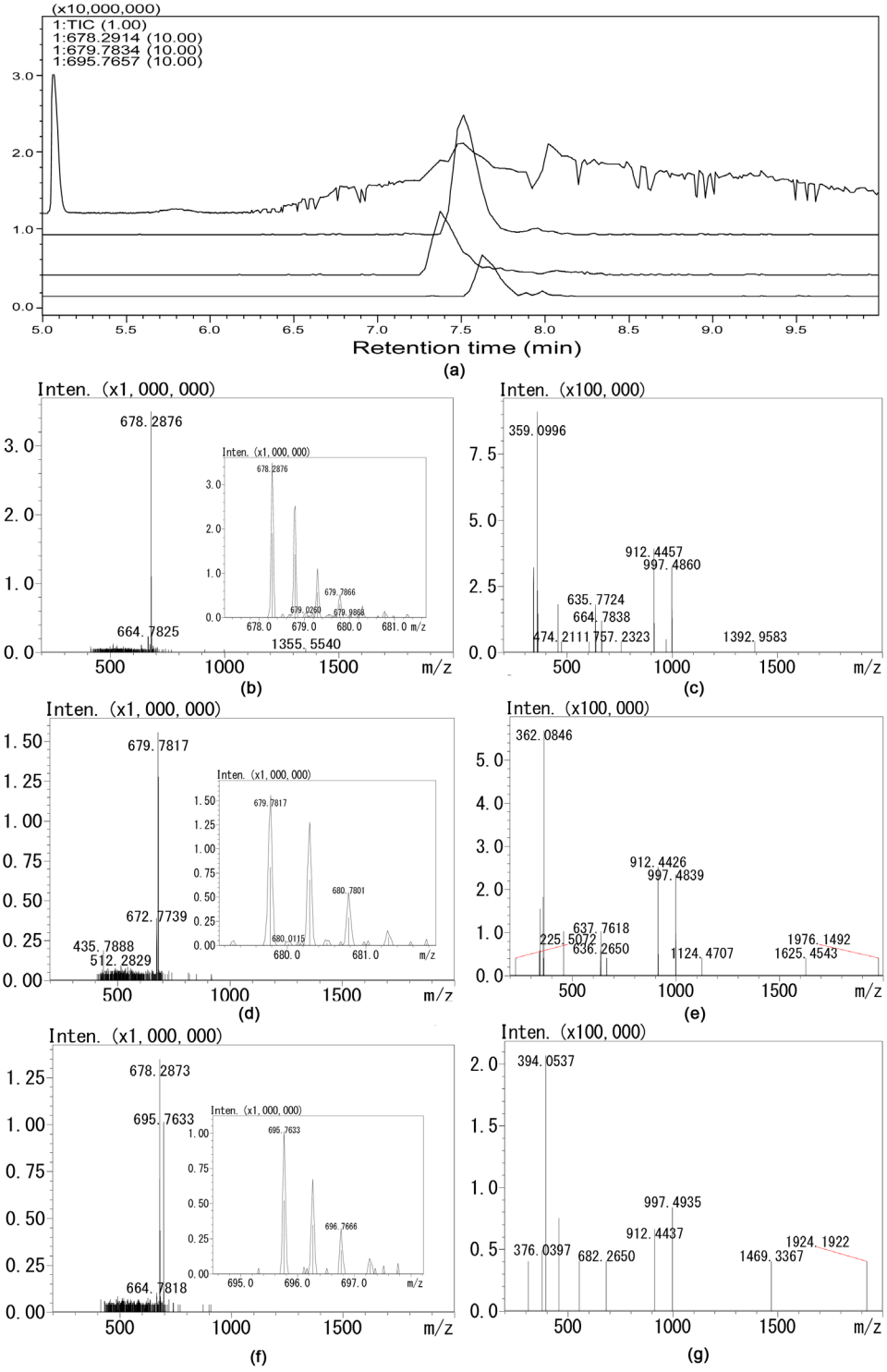

Figure 3. LC/ESI-MS/MS chromatograms of the vitamin B_{12} compounds purified from the escargot sample. Total ion chromatogram (TIC) and chromatograms for *m/z* 678.2914 (×10), 679.7834 (×10), and 695.7657 (×10) are shown in panels (a). The mass spectra of the ion peaks of the sample A at retention times of 7.50 min, 7.35 min and 7.65 min are shown in panels (b), (d) and (f), respectively. The magnified mass spectra from 768 to 680 in (b), from 672 to 674 in (d), and from 695 to 697 in (f), are shown as respective inserts. The MS/MS spectra for the peaks of the sample A at *m/z* 678.2876, 679.7817 and 695.7633 are shown in panels (c), (e) and (g), respectively.

with *m/z* 678.2914 at a retention time of 7.50 min showed that the doubly charged ion was formed at *m/z* 678.2876 (**Figure 3(b)**). The MS/MS spectrum of the compound was identical to that of vitamin B_{12} (**Figure 3(c)**). The mass spectra of the ion peaks with retention times of 7.35 min and 7.65 min showed that the doubly charged ions were formed at *m/z* 679.7817 and 695.7633, respectively (**Figure 3(d)** and **Figure 3(f)**). The MS/MS spectra of the ion peaks of *m/z* 679.7817 and 695.7633 indicated that the dominant ions at *m/z* 362.0846 and *m/z* 394.0537, respectively, were attributable to each nucleotide moiety of these compounds; these spectral data coincided with the masses of nucleotide moieties of factor IIIm or methoxybenzimidazolyl cyanocobamide ($C_{62}H_{86}CoN_{14}O_{15}P$, 1356.5467) and factor S or 2-methylmercaptoadenyl cyanocobamide ($C_{60}H_{85}CoN_{17}O_{14}PS$, 1389.5252) (**Figure 4**). The results indicated that canned escargots (boiled plain) contained vitamin B_{12} and other two inactive corrinoid compounds, which are identified as factor IIIm and factor S. The similar results were obtained in the remaining samples B, C, D, and E. Relative contents of factor IIIm (34.4% ± 8.6%) and factor S (25.8% ± 6.9%) against vitamin B_{12} (100%) were shown in these samples from calculating height of respective three peaks at 360 nm.

3.4. Escargot as a Vitamin B_{12} Source

Vitamin B_{12} content (2.2 ± 1.9 μg/100g weight) of canned escargots (boiled plain) was very lower than values (17.4 - 39.4 μg/100g weight) of canned clams (boiled plain). Moreover, escargots contained two inactive corrinoid compounds, which were identified factor IIIm and factor S, as well as vitamin B_{12}. It remains unclear why these inactive corrinoids were present in escargots. Further detailed biochemical studies are required to elucidate the origins of these inactive corrinoid compounds. Escargots would be not suitable for use as a source of vitamin B_{12}.

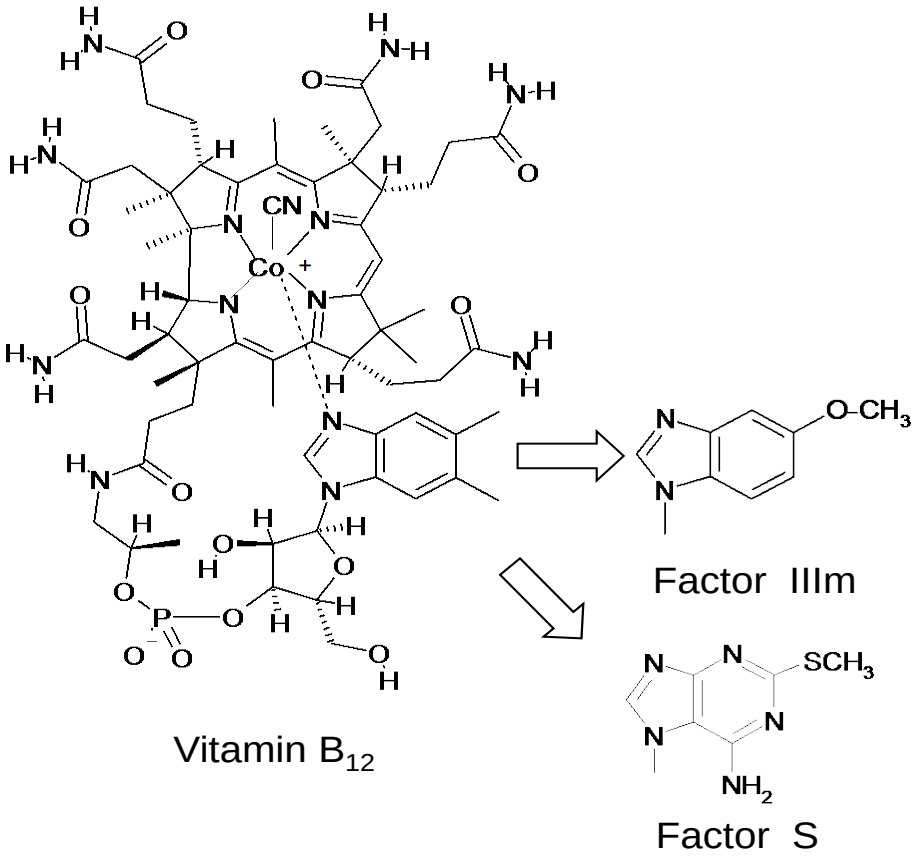

Figure 4. Structural formula of vitamin B_{12} and partial structures of corrinoid compounds identified from canned escargots (boiled plain). Partial structures of corrinoid compounds show only those portions of the molecule that differ from vitamin B_{12}.

Fund

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