Synthesis of 4-Aza-5,6-dimethylbenzimidazole and Biosynthetic Preparation of 4- and 7-Aza-5,6-dimethylbenzimidazolylcobamide

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4-Aza-5,6-dimethylbenzimidazole, Vitamin B12-Analogs, *Propionibacterium shermanii*, *Eubacterium limosum*, Vitamin B12-Activity

We report on the preparation of 4-aza-5,6-dimethylbenzimidazolylcobamide and 5,6-dimethyl-7-azabenzimidazolylcobamide. These vitamin B12-analogs were required as reference compounds for comparison with a corrinoid previously isolated in small amounts from *Eubacterium limosum* grown in the presence of 4(5)-aminoimidazole.

4(7)-Aza-5,6-dimethylbenzimidazole was synthesized from N-1-benzyl-4-nitroimidazole which was reduced to N-1-benzyl-4-aminoimidazole and condensed with 1-dimethylamino-2-methylbutan-3-one to yield N-1-benzyl-4-aza-5,6-dimethylbenzimidazole. The benzyl group of this compound was split off by catalytic hydrogenation to form 4(7)-aza-5,6-dimethylbenzimidazole.

4(7)-Aza-5,6-dimethylbenzimidazole was transformed by a growing culture of *Propionibacterium shermanii* into 4-aza-5,6-dimethylbenzimidazolylcobamide and 5,6-dimethyl-7-azabenzimidazolylcobamide. Both vitamin B12-analogs were almost as active as Vitamin B12 in a growth test with the vitamin B12-dependent *Escherichia coli*-mutant DSM 4261.

Introduction

Recently we found (Endres *et al.*, 1995) that the anaerobic vitamin B12-producing microorganism *E. limosum* transforms 4(5)-aminoimidazole into 7-azabenzimidazolylcobamide and 5,6-dimethyl-7-azabenzimidazolylcobamide. Since these vitamin B12 analogs were only formed in microgram quantities, it was desirable to prepare them at least in milligram amounts for further studies.

4(7)-Azabenzimidazole is commercially available. On addition of this base to cultures of *P. shermanii* or *E. limosum* 4-azabenzimidazolylcobamide and 7-azabenzimidazolylcobamide were readily prepared (Endres *et al.*, 1995). By contrast 4(7)-aza-5,6-dimethylbenzimidazole is not a commercial product. In the synthesis of this base described in the literature (Dornow *et al.*, 1958) the pyridine structure is formed first, followed by the imidazole structure.

Since Ramsden and his group (Lythgoe and Ramsden, 1994) showed that 5-unsubstituted 4-aminoimidazoles are well suited for reactions in position 5, we tried to synthesize 4(7)-aza-5,6-dimethylbenzimidazole from N-1-benzyl-4-aminoimidazole forming the pyridine ring as last step.

Here we report on this synthesis, and on the biosynthetic preparation of 4-aza-5,6-dimethylbenzimidazolylcobamide and 5,6-dimethyl-7-azabenzimidazolylcobamide by a *P.shermanii*-culture grown in the presence of 4(7)-aza-5,6-dimethylbenzimidazole. In addition we tested the growth activity of 4-azabenzimidazolylcobamide, 4-aza-5,6-dimethylbenzimidazolylcobamide, 7-azabenzimidazolylcobamide and 5,6-dimethyl-7-azabenzimidazolylcobamide with a vitamin B12-dependent *E. coli*-mutant.

Results and Discussion

We prepared 4(7)-aza-5,6-dimethylbenzimidazole (**5**) by a new synthetic method from N-1-benzyl-4-nitroimidazole (**1**) via N-1-benzyl-4-aminoimidazole (**3**) and N-1-benzyl-4-aza-5,6-dimethylbenzimidazole (**4**) (Fig. 1). N-1-Benzyl-4-nitroimidazole was synthesized from 4(5)-nitroimidazole and benzyl chloride according to a variation (see experimental section) of a procedure described in the literature (Iradyan *et al.*, 1978). Theoretically the benzylation of 4(5)-nitroimidazole could lead

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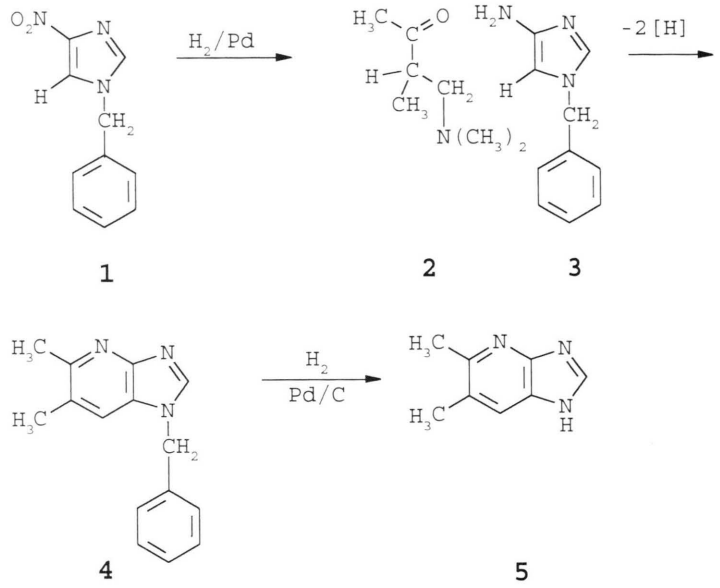


Fig. 1. Scheme of the chemical synthesis of 4(7)-aza-5,6-dimethylbenzimidazole (**5**).
(**1**) = N-1-benzyl-4-nitroimidazole, (**2**) = 1-dimethylamino-2-methylbutan-3-one, (**3**) = N-1-benzyl-4-aminoimidazole, (**4**) = N-1-benzyl-4-aza-5,6-dimethylbenzimidazole.

to N-1-benzyl-4-nitroimidazole or to N-1-benzyl-5-nitroimidazole or to a mixture of both. However, N-1-benzyl-4-nitroimidazole prepared according to our procedure was exclusively the 4-nitro-compound. This was demonstrated by ^{1}H NMR measurements (NOE experiments, data not shown).

4(7)-Aza-5,6-dimethylbenzimidazole was transformed by the aerotolerant anaerobe *P. shermanii* into a mixture of 4-aza-5,6-dimethylbenzimidazolylcobamide (**7**) and 5,6-dimethyl-7-azabenzimidazolylcobamide (**9**) which was separated by HPLC (Fig. 2). According to the mass spectrum both corrinoids had the same molecular mass of 1356. The 4-position of the aza nitrogen in the base moiety of 4-aza-5,6-dimethylbenzimidazolylcobamide was determined by a ^{1}H-NOE-spectrum of its dicyano form in ^2H_2O. Irradiation at the frequency of the 1'-H-proton of the ribose (6.17 ppm) evoked the signals of the protons at C-2 (8.31 ppm) and C-7 (7.66 ppm) of the base.

Finally the isolated vitamin B12-analogs were tested for their vitamin B12 activity with the vitamin B12-requiring *E. coli*-mutant DSM 4261. As shown in Table I the two dimethylazabenzimidazolylcobamides **7** and **9** are almost as active as vitamin B12 whereas the azadimethylbenzimidazolylcobamides **6** and **8** are much less active. The values of single determinations are scattered over a relatively wide range. This is due to the high sensitivity of the test (Skeggs, 1966).

Table I. Vitamin B12 activity of 4(7)-azabenzimidazolylcobamides and 4(7)-aza-5,6-dimethylbenzimidazolylcobamides tested with the Vitamin B12-requiring *Escherichia coli* DSM 4261 mutant.

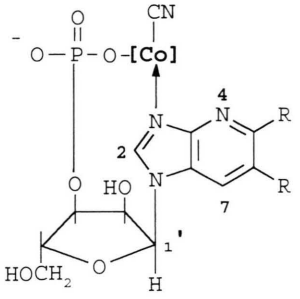
Corrinoid	Activity (%) (Cyanocobalamin=100%) Single determinations				Mean value
4-Azabenzimidazolylcobamide (**6**)	48	45	39	36	42
7-Azabenzimidazolylcobamide (**8**)	29	30	36		32
4-Aza-5,6-dimethylbenzimidazolylcobamide (**7**)	102	91	89		94
5,6-Dimethyl-7-azabenzimidazolylcobamide (**9**)	89	95	71		85

It would be interesting to test now the influence of these corrinoids on eukaryotic cells in culture, and their Co-5'-desoxyadenosyl derivatives (coenzym form) on vitamin B12-dependent reactions.

Materials and Methods

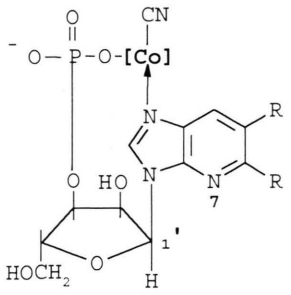
Solvent for TLC: A: chloroform/ethanol/acetic acid = 85/15/2 (by vol.). Solvent for descending paper chromatography: B: butan-2-ol/water/acetic acid/1% aqueous HCN = 70/28/1/1 (by vol.).

TLC aluminium sheets silica gel 60 F_{254} (Merck) were used for analytical purposes. Semipreparative purification of N-1-benzyl-4-aza-5,6-dimethylbenzimidazole and 4(7)-aza-5,6-dimethylbenzimi-



6: R = H

7: R = CH_3



8: R = H

9: R = CH_3

Fig. 2. Structure of the vitamin B12 analogs (monocyano form) 4-azabenzimidazolylcobamide (**6**) or 4-aza-5,6-dimethylbenzimidazolylcobamide (**7**) respectively 7-azabenzimidazolylcobamide (**8**) or 5,6-dimethyl-7-azabenzimidazolylcobamide (**9**). [Co] = corrin ring. For nomenclature of corrinoids see IUPAC – IUB (Commission on Biochemical Nomenclature (CBN), 1973).

dazole was carried out on pre-coated TLC plates silica gel 60 F_{254} 20 x 20 cm, 2 mm (Merck). N-1-Benzyl-4-nitroimidazole, N-1-benzyl-4-aza-5,6-dimethylbenzimidazole and 4(7)-aza-5,6-dimethylbenzimidazole were detected on TLC under ultraviolet light of 254 nm.

The concentration of corrinoids was measured as described before (Endres *et al.*, 1995). ^{1}H NMR spectra were recorded as published previously (Endres *et al.*, 1995). Molecular masses of corrinoids were determined with methanolic solutions by electrospray mass spectroscopy in a Finnigan TSQ 700 spectrometer.

N-1-Benzyl-4-nitroimidazole

2.26 g (20 mmol) 4-nitroimidazole was suspended in 60 ml triethylamine, heated under reflux and magnetic stirring. 5 ml (43.5 mmol) benzyl chloride was added dropwise within 1–2 h. Heating was continued for another 4 h. The solvent was decanted from the honey-colored precipitate and discarded. The product was dissolved in 30 ml ethanol, diluted with 70 ml water, brought to boiling in the presence of decolorizing carbon and filtered. On cooling white to lightly yellow crystals separated. Yield 3.45 g (17 mmol, 85%). Mp. 75–78 °C (after recrystallization, 76 °C according to Cosar *et al.*, 1966). R_f in solvent A: 4-nitroimidazole 0.47; N-1-benzyl-4-nitroimidazole 0.88.

N-1-Benzyl-4-aza-5,6-dimethylbenzimidazole

2.03 g (10 mmol) N-1-benzyl-4-nitroimidazole, dissolved in 50 ml ethanol, was hydrogenated with 1 g Pd/$BaSO_4$ (5% Pd, Sigma, Deisenhofen, Germany) for 6 h at 8 atm hydrogen and 40 °C. The catalyst was removed by centrifugation, the solvent evaporated, the residue dissolved in 30 ml ethanol/acetic acid (5 : 1, by vol.), 1.42 g (11 mmol) 1-dimethylamino-2-methylbutan-3-one (Becker *et al.*, 1996) added, and heated under reflux for 5 h. The solvent was evaporated, the residue dissolved in 4 M HCl, extracted three times with methylene chloride, and the organic phase discarded. The aqueous phase was adjusted to pH 11–12 with 4 M NaOH, and the N-1-benzyl-4-aza-5,6-dimethylbenzimidazole extracted with three portions of methylene chloride. The solvent was evaporated, and the product distilled at reduced pressure to the cooling device of a sublimation apparatus at 180–190 °C. Yield 970 mg (4.09 mmol, 41%).

The crude product was recrystallized from water/ethanol = 95/5 in the presence of charcoal. Yield 130 mg, mp. 160–163 °C. R_f in solvent A: 0.76. A better yield of pure product was obtained by preparative TLC (50 mg, dissolved in ethanol, per plate) with solvent A. The main band was eluted from the silicagel with ethanol/conc. aqueous ammonia = 100/5 (vol/vol), the solvent evaporated, and N-1-benzyl-4-aza-5,6-dimethylbenzimidazole extracted from an aqueous alkaline solution as described above.

MS (70 eV); *m/z* (%): 237 (36) [M^+], 236 (26) [M^+-H], 222 (6) [M^+-CH_3], 160 (4), 119 (2), 101 (4), 91 (100), 65 (34), 28 (10).

^{1}H NMR (CD_3OD): d = 2.18 (s, CH_3 at C-6 and C-5), 5.59 (s, 1'-H), 6.88 (m) and 7.16 (m) (phenyl-Hs), 8.06 (s, 7-H), 8.26 (s, 2-H).

4(7)-Aza-5,6-dimethylbenzimidazole

500 mg (2.1 mmol) N-1-benzyl-4-aza-5,6-dimethylbenzimidazole was dissolved in 50 ml ethanol, 600 mg Pd/C (10% Pd) added, and the mixture stirred under 15 atm hydrogen at 120 °C for 72 h. The catalyst was centrifuged off, the solvent evaporated, and the residue dissolved in 5 ml ethanol. The solution was applied as a band onto 5 TLC-silica gel F_{254} plates (2 mm) and developed with solvent A. Thus 4-aza-5,6-dimethylbenzimidazole was separated from small amounts of faster moving N-1-benzyl-4-aza-5,6-dimethylbenzimidazole. R_f of N-1-benzyl-4-aza-5,6-dimethylbenzimidazole in solvent A: 0.76, of 4-aza-5,6-dimethylbenzimidazole 0.51. 4-Aza-5,6-dimethylbenzimidazole was eluted from the silica gel with ethanol/conc. aqueous ammonia = 100/5. On evaporation the acetate of 4-aza-5,6-dimethylbenzimidazole was obtained, due to the presence of acetic acid in solvent A. Yield 247 mg (1,68 mmol, 80%).

In order to obtain the free base of 4-aza-5,6-dimethylbenzimidazole the acetate was dissolved in 1 M HCl, and extracted three times with methylene chloride. The organic phase was discarded, the aqueous phase adjusted to pH 8.0 with 1 M NaOH, and extracted three times with methylene chloride. The solvent was evaporated and the residue dried in vacuo. Mp. 227–231 °C (after sublimation in vacuo; 220 °C according to Dornow *et al.*, 1958).

MS (70 eV): *m/z* (%): 147 (100) [M^+], 146 (26) [M^+-H], 132 (52) [M^+-CH_3], 119 (16) [M^+-H-HCN], 91 (6), 75 (8), 65 (17), 51 (12), 39 (12), 28 (10).

^{1}H NMR (CD_3OD): d = 2.42 and 2.56 (s, CH_3 at C-6 and CH_3 at C-5), 8.16 (s, 7-H), 8.29 (s, 2-H).

Biosynthetic preparation of 4-aza-5,6-dimethylbenzimidazolylcobamide and 5,6-dimethyl-7-azabenzimidazolylcobamide by the addition of 4-aza-5,6-dimethylbenzimidazole to a culture of P. shermanii

P. shermanii St 33 (similar to *Propionibacterium freudenreichii subsp. shermanii* DSM 4902) was grown as described earlier (Renz, 1971). 90 mg of 4(7)-aza-5,6-dimethylbenzimidazole (free base or acetate), dissolved in 3 ml 70% aqueous ethanol, was added to a 3-l culture 48 h after inoculation, and the culture grown for another 5 d. 143 g wet cells were obtained. The corrinoids were isolated from the bacteria as their monocyano derivatives as described (Renz *et al.*, 1993). R_{B12}-values (descending paper chromatography with solvent B, Schleicher & Schuell-paper No. 3469): 4-aza-5,6-dimethylbenzimidazolylcobamide 0.99; 5,6-dimethyl-7-azabenzimidazolylcobamide 0.73. Final purification of these corrinoids was achieved by HPLC on a LiChrospher 250–4 RP-18 (5 mm) column (Merck) with 0.1% aqueous acetic acid/methanol (75/25, v/v) as solvent (flow rate 1 ml/min, detection at 361 nm) (Endres *et al.*, 1995). Retention time (min): 4-aza-5,6-dimethylbenzimidazolylcobamide 15.1; 5,6-dimethyl-7-azabenzimidazolylcobamide 10.0; vitamin B12 (cyanocobalamin) 13.3. Yields: 4-aza-5,6-dimethylbenzimidazolylcobamide 5.14 mg; 5,6-dimethyl-7-azabenzimidazolyl-cobamide 2.15 mg. The molecular mass of both corrinoids was 1356, determined from the M + Na (1379) and M + K (1395) peaks.

Growth tests with Escherichia coli DSM 4261 on vitamin B12 activity

The vitamin B12-requiring *E. coli* mutant was obtained from DSM – Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). The tests were carried out as described (Mücke, 1957; Endres, 1996) in 4 ml of the minimal medium published for *Escherichia coli B* by Süßmuth (Süßmuth *et al.*, 1987). The growth activity was tested with the following concentrations of the vitamin B12 analogs (pmol/4 ml): 0; 0.005; 0.01; 0.02; 0.04; 0.08; 0.1;0.2; 0.4;0.8; 2. Triplicate assays were performed for each concentration. Concomittantly triplicate assays were carried out with vitamin B12 at the same concentrations. The test tubes were incubated for 15 h at 30 °C with shaking (80 min^{-1}). The absorbance was measured at 578 nm using the assay without corrinoid as reference.

The logarithm of the concentration (abscissa) was drawn in a diagram versus the absorbance (ordinate). The per cent activity was calculated from the curve of the vitamin B12 analog in comparison with the curve of vitamin B12 (Endres, 1996).

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