On the Biosynthesis of the Benzimidazole Bases Occurring in Corrinoids

Synthesis of \(6',7'-\text{Dimethylnaphtho-(2',3')-imidazole-4,5}\) and \(6',7'-\text{Dimethylnaphtho-(2',3')-imidazole-4,5-yl-Cobamide}\)

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A hypothesis by BERNHAUER on the biosynthesis of the benzimidazole bases of corrinoids implies the possible existence of a corrinoid containing \(6',7'-\text{dimethylnaphtho-(2',3')-imidazole-4,5}\) (DMNIA). In order to facilitate the detection of this corrinoid in natural sources by means of a reference compound, DMNIA was synthesized and added to cultures of Pseudomonas aeruginosa. Thus the new vitamin B\(_12\)-analog DMNIA-cobamide was formed. The DMNIA-cobamide was degraded and its base isolated. This base had the same UV-spectrum and electrophoretic behaviour as the product prepared by chemical synthesis. On degradation of DMNIA-cobamide with cerous hydroxide DMNIA-C-ribofuranoside is formed. Perchloric acid treatment of DMNIA-cobamide yields DMNIA-C-ribofuranoside 3'-phosphate. The microbiological activity of DMNIA-cobamide cyanide tested with the vitamin B\(_12\)-requiring Lactobacillus leichmannii ATCC 7830 was equal to the activity of vitamin B\(_12\), with the Escherichia coli-mutant 113-3 it exhibited about 50% of the growth-activity of vitamin B\(_12\).

DMNIA-cobamide coenzyme shows approximately 10% of the coenzymatic activity of coenzyme B\(_12\) in the propanediol dehydrase reaction.

Corrinoids produced by microorganisms under aerobic conditions usually contain a base of the benzimidazole type, whereas most of the corrinoids produced under anaerobic conditions contain a purine base.

The benzimidazole bases (1–4) of corrinoids found in activated sludge are shown in Fig. 1.

![Fig. 1. Bases of the benzimidazole-type occurring in corrinoids found in activated sludge (1–4) together with DMNIA](image)

The fact that these bases show certain similarities led BERNHAUER to put forward the following hypothesis: It can be thought that all these bases have derived from 4,5-dimethylimidazole (6) by condensation of the two methyl groups with an "activated" diketo compound:

\[
\begin{align*}
\text{"activated" methylglyoxal} & \rightarrow 6 \rightarrow 2 \\
\text{"activated" diacetyl} & \rightarrow 6 \rightarrow 3 \\
\text{"activated" glyoxal} & \rightarrow 3 \rightarrow 4
\end{align*}
\]

Moreover BERNHAUER pointed out that, if this hypothesis proved true, one should expect to find also a corrinoid with 5 as base among the corrinoids of activated sludge. As activated sludge still contains several unidentified corrinoids an authentic sample of the up to now hypothetical DMNIA-cobamide as a reference compound would greatly facilitate the finding of DMNIA-cobamide in this material. Since the synthesis of 5 was not yet described, many efforts were made to synthesize 5, but without success. In this paper the synthesis of 5 is described as well as the biochemical preparation of DMNIA-cobamide using Propionibacterium shermanii.

Investigations in order to find DMNIA-cobamide in activated sludge using DMNIA-cobamide synthesized a described here as reference compound are presently undertaken by W. FRIEDRICH, Hamburg.

Materials and Methods

Synthesis of DMNIA

2,3-Dimethoxy-6,7-dimethylnaphthalene was prepared as described by HAWORTH et al. with the exception

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that the 2,3-dimethyl-6,7-dimethoxy-1,2,3,4-tetrahydro-
naphthalene was dehydrogenated in an open tube for 2
hours at 270° with a Pd/C-catalyst as described for
tetrahydrod napthalene rather than with selenium.

2,3-Dihydroxy-6,7-dimethylnaphthalene

900 mg of 2,3-dimethoxy-6,7-dimethylnaphthalene
was heated under reflux with a mixture of 10 ml of
acetic acid and 10 ml of 48% hydrobromic acid
for 2 hours. After addition of 100 ml of water the pH
was adjusted to about 10–12 with a 50% solution of
sodium hydroxide. The solution was extracted twice
with diethyl ether to remove unreacted starting mate-
rial. The aqueous phase was adjusted to pH 3–4 with
conc. hydrochloric acid and extracted twice with di-
ethyl ether. The ether was evaporated to dryness. The
residue was recrystallized from 100 ml of 10% aqueous
acetic acid. 400 mg of 2,3-dihydroxy-6,7-dimethyl-
naphthalene was obtained. From the mother liquor
another 30 mg were isolated. 430 mg of crude 2,3-di-
hydroxy-6,7-dimethylnaphthalene was dissolved in 5 ml
of diethyl ether and 4 g of Kieselgel (Merck, Darm-
dstadt, Germany, No. 7754, 0.05–0.2 mm, 70–325
mesh) were added. The dry adsorbate was applied to a
column (2.5 × 15 cm) of Kieselgel, previously sus-
pended in petroleum ether (bp 60–80°). On elution
with petroleum ether/diethyl ether = 8/2 v/v and col-
lection of 10 ml-fractions 2,3-dihydroxy-6,7-dime-
thylnaphthalene was found in fractions 35 to 54. After
evaporation of the solvent 380 mg (48% yield) of pro-
duct was obtained, mp 214–215°. Anal. calc. for
C_{12}H_{12}O_{2}: C 76.57; H 6.43. Found: C 76.39; H 6.46.

2,3-Diamino-6,7-dimethylnaphthalene

A suspension of 280 mg of 2,3-dihydroxy-6,7-dime-
thylnaphthalene in 10 ml of conc. aqueous ammornia
was kept at 240° for 12 hours in a heavy walled
sealed tube. After addition of 5 ml of 1 N sodium
hydroxide the reaction mixture was extracted 10 times
with 15 ml-portions of diethyl ether. The combined ether
extracts were evaporated to dryness and the residue
recrystallized from 50 ml of 40% aqueous etha-
nol. Light yellow needles, 150 mg (54% yield), mp
224–225°. Anal. calc. for C_{12}H_{14}N_{2}: C 77.38; H 7.57; N 15.04. Found: C 76.98; H 7.36; N 15.44.

6,7'-Dimethyl naphtho-(2',3')-imidazole-4,5 8

100 mg of 2,3-diamino-6,7-dimethylnaphthalene was
heated under reflux with 5 ml of formic acid (98–
100%) for 4 hours. The formic acid was removed under
reduced pressure and the residue boiled with 15 ml of
water in the presence of 5 mg of charcoal and filtered.
The filtrate was neutralized with ammonia. Recrystal-
lization of the precipitate from 10 ml of 30 per cent
aqueous ethanol yielded 80 mg of product. From the
mother liquor another 15 mg were obtained (total
yield 90%). In order to remove some minor impurities
DMNIA was recrystallized several times from 0.6 N
hydrochloric acid and was then reconverted to the base
with ammonia. Mp 263–265° under partial decompo-
sition. Anal. calc. for C_{13}H_{12}N_{2}: C 79.59; H 6.12; N
14.29. Found: C 79.12; H 6.39; N 13.95. Molar ab-
sorptivity: base £328um = 9920; hydrochloride £330nm
= 11540.

Biochemical Preparation of DMNIA-cobamide

Propionibacterium shermanii St 33 was grown ac-
cording to a modification of the procedure described by
BERNAUER et al. 10. Two days after inoculation of a
8 liter-culture, grown in the presence of 12 mg of
CoSO_{4}·7 H_{2}O per liter, 40 mg of DMNIA dissolved in
5 ml of 70% aqueous ethanol was added. After
another 4 days the cells were harvested (wet weight
300 g), and the corrinoids released from the bacteria
on treatment with KCN 3. The corrinoids were ab-
 sorbed on a column (4 × 15 cm) of Amberlite XAD-2,
the resin was extensively washed with water, and the
corrinoids were eluted with methanol/water = 8/2
v/v 11. The corrinoid-solution was evaporated to dry-
ess, the residue dissolved in water, and applied to a
column (2 × 25 cm) of Dowex 2 X4 in the formate-
form. Neutral and basic corrinoids were eluted with
water, the volume of the resulting solution was reduced
to a few ml in vacuo and this solution applied to a
column (4 × 20 cm) of CM-cellulose in the H^+ form.
The neutral corrinoids were eluted with water and passed through a column (28 × 3 cm) of Sephadex G 10
with water as solvent. Finally the neutral corrinoids
were separated by chromatography on Whatman 3 MM- paper with sec. butanol/acetic acid/water/HCN =
70/1/30/0.05 as solvent. The main band is DMNIA-
cobamide cyanide migrating with an R_{B} value of 1.5.
This compound was eluted from the paper with water.
From a conc. aqueous solution DMNIA-cobamide cry-
stallizes in the form of rectangular plates projecting
into pointed ends along one side of the longitudinal
Once DMNIA-cobamide is a dry solid, it dissolves much
more slowly in water than vitamin B_{12}.

DMNIA-cobamide coenzyme

(All operations were done in dim light)

The bacteria grown as described above were sus-
pended in 0.05 M Na-phosphate buffer pH 7.5 and kept
in an autoclave at 120° for 15 minutes. After
cooling the suspension was centrifuged (10 min.,
10,000 g). The clear supernatant liquid, containing the
coenzyme was applied to a column of Amberlite XAD-2.
The column was extensively washed with water, and
then the coenzyme eluted with methanol/water = 8/2.
After evaporation to dryness the coenzyme was dis-
solved in a few ml of water, applied to a column of
Dowex-2-X4 in the acetate form, and eluted with water.
The coenzyme was further purified by descending paper
chromatography on Whatman 3 MM-paper with sec.
butanol/acetic acid/water = 70/1/30. The coenzyme
was eluted from the paper, and submitted to paper
electrophoresis in 0.5 M acetic acid. The yellow band
which migrated to the cathode was eluted with water. The coenzyme solution was finally filtered through Sephadex G-10, and then used for the dioldehydrase assay.

Cerous hydroxide degradation

This was carried out with salt-free cerous hydroxide according to the method of FRIEDRICH et al. 12. The resulting mixture of corrinoids and nucleoside was applied to a column of CM-cellulose in the H\(^+\)-form. Traces of intact DMNIA-cobamide were eluted with water, cobinamide was eluted with 0.1% aqueous hydrogen cyanide, and, after removal of excess of hydrogen cyanide from the column by washing with water, the nucleoside was eluted with 0.1 N HCl.

Degradation of DMNIA-cobamide with 6 N HCl

500 µg of DMNIA-cobamide, dissolved in 1 ml of 6 N HCl, was kept at 150° for 6 hours in a sealed tube. The solution was evaporated to dryness, the solid dissolved in ethanol, and placed as a strip onto a thin layer plate (20 x 20 cm) covered with silica gel without fluorescent indicator. Solvent: diethyl ether/acetone/conc. NH\(_3\) = 75/25/0.5. The corrinoids remain at the origin. The UV-fluorescent region was eluted with warm acetone, filtered, and evaporated to dryness. The residue, dissolved in ethanol, was placed on Schleicher and Schuell-paper 2043a, and was subjected to electrophoresis in 0.5 M acetic acid together with authentic DMNIA as marker. The band migrating as fast as the DMNIA-marker was eluted and used for spectrophotometric analysis.

Degradation of DMNIA-cobamide with perchloric acid

This was carried out as described by DELLWEG et al. 14.

Dioldehydrase Assay

Dioldehydrase was a gift of Pierrel S.p.A., Milan, Italy. Coenzyme B\(_{12}\) was purchased from Calbiochem. The assay was carried out as described in a manual of Pierrel S.p.A., which is according to the method of ABELES et al. 15, 16.

Microbiological Tests were carried out with Lactobacillus leichmannii ATCC 7830 as described by HOFFMANN et al. 17, using Difco's Bacto B\(_{12}\) Assay Medium, and with the E. coli-mutant 113-3 as described by DAVIS et al. 18.

Results

DMNIA

The synthesis of DMNIA was achieved by a rather straightforward procedure (see Material and Methods). As starting material 2,3-dimethoxy-6,7-dimethylnaphtalene was used, which was synthesized as described by HAWORTH et al. 5. Since the synthesis of 2,3-dimethoxy-6,7-dimethylnaphtalene involves six steps and is rather tedious, another synthetic route was developed 8, starting with the inexpensive 2,3-dimethylnaphthalene, and leading in four steps to DMNIA.

In Fig. 2 the 60 MHz-proton magnetic resonance spectrum of DMNIA in hexadeuterodimethylsulfoxide is shown. Because the protons at C-1' and C-4', as well as the protons at C-5' and C-8' are in para position to each other, only 2 signals arising from this 4 protons should be expected. These 2 signals are indeed found at 7.7 and 8.0 ppm. The third signal (at 8.4 ppm) is due to the proton at
C-2. Because of a rapid exchange the proton bound to the nitrogen cannot be seen in the spectrum. Fig. 3 shows the UV-spectrum of DMNIA. In Table 1 the electrophoretic mobility of DMNIA at pH 2.5, the mobilities of some other imidazole bases occurring in vitamin B\textsubscript{12}-analogues, and the mobility of cobinamide are listed.

<table>
<thead>
<tr>
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<th>cm\textsuperscript{2}/V \times sec \times 10\textsuperscript{5}</th>
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<tbody>
<tr>
<td>Benzimidazole</td>
<td>2.51</td>
</tr>
<tr>
<td>5,6-Dimethylbenzimidazole</td>
<td>2.21</td>
</tr>
<tr>
<td>Naphtho-(2',3')imidazole-4,5</td>
<td>1.84</td>
</tr>
<tr>
<td>DMNIA</td>
<td>0.72</td>
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<tr>
<td>Cobinamide</td>
<td>0.78</td>
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Table I. Electrophoretic mobility of DMNIA, of some other imidazole bases, and of cobinamide at pH 2.5 \(a\).

\(a\) 0.5 M acetic acid; Schleicher and Schuell-paper 2043 a.

DMNIA-cobamide

Propionibacterium shermanii, grown anaerobically in the presence of cobaltous sulfate, easily converts added DMNIA into DMNIA-cobamide, which constitutes about 95 percent of the corrinoids isolated (besides vitamin B\textsubscript{12}, cobinamide and a monocarboxylic acid of DMNIA-cobamide). Fig. 4 shows the absorption spectrum of DMNIA-cobamide cyanide. The characteristic shoulder at approximately 325 nm is due to the presence of DMNIA in the molecule. The DMNIA-part of this corrinoid makes the molecule more hydrophobic than vitamin B\textsubscript{12}, which can be seen by the fact that it migrates 1.5 times as fast as vitamin B\textsubscript{12} on descending paper chromatography (see Materials and Methods). The electrophoretic behaviour of DMNIA-cobamide is identical with that of vitamin B\textsubscript{12}.

<table>
<thead>
<tr>
<th>DMNIA</th>
<th>Products of DMNIA-cobamide degradation</th>
<th>DMNIA-(\alpha)-D-ribofuranoside (c)</th>
<th>DMNIA-(\alpha)-D-ribofuranoside-3'-phosphate (d)</th>
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<tr>
<td>free base (e)</td>
<td>hydrochloride (f)</td>
<td>free base hydrochloride</td>
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Table II. Maxima (in nm) of the UV-absorption spectrum of the chemically synthesized DMNIA, and of the base, the nucleoside, and the nucleotide isolated from DMNIA-cobamide \(a\).

\(a\) Spectra recorded with a M4QIII Zeiss-spectrophotometer. \(b\) After degradation of DMNIA-cobamide with 6 N HCl. \(c\) After cerous hydroxide degradation of DMNIA-cobamide. \(d\) After degradation of DMNIA-cobamide with perchloric acid.

\(e\) Ethanol solution with 1 drop of conc. ammonia added. \(f\) Ethanol solution containing 0.01 N HCl. \(g\) sh = shoulder.

The base isolated after HCl-degradation of DMNIA-cobamide has a UV-spectrum and electrophoretic behaviour identical with that of the base added to the fermentation.

Table II shows the maxima of the UV-spectrum of chemically synthesized DMNIA together with the UV-maxima of the base, the nucleoside and the nucleotide isolated from DMNIA-cobamide after degradation with HCl, cerous hydroxide, and perchloric acid respectively. In microbiological tests with Lactobacillus leichmannii ATCC 7830 DMNIA-cobamide is as active as vitamin B\textsubscript{12}, in tests with E. coli 113-3 DMNIA-cobamide has only 50% of the activity of vitamin B\textsubscript{12}.

In Fig. 5 the absorption spectrum of DMNIA-cobamide coenzyme is shown. The coenzymatic activity of DMNIA-cobamide coenzyme was tested in the cobamide coenzyme dependent dioldehydrase reaction. Fig. 6 shows the time-course of pro-

![Fig. 4. UV-absorption spectrum of a 3 \times 10^{-5} M aqueous solution of DMNIA-cobamide cyanide.](image-url)
BIOSYNTHESIS OF BENZIMIDAZOLE BASES

Fig. 5. UV-absorption spectrum of DMNIA-cobamide coenzyme (1.95 x 10^{-5} m) in 2% acetic acid, pH 2.8 (solid line) and in aqueous solution, pH 6.0 (broken line).

Fig. 6. Time course of propionaldehyde formation by dioldehydrase, dependent on added coenzyme. Each assay mixture contained: potassium phosphate buffer, pH 8.0, 2 μamole, propanediol 13 μmole, dioldehydrase apoenzyme, 1 mg, B_{12}-coenzyme or DMNIA-cobamide coenzyme 10 picomole, or 5 picomole each when both coenzymes were added. Final volume 0.51 ml. Incubation at 37°. Determination of propionaldehyde as described by ABELES et al. Amount of propionaldehyde expressed in terms of OD 480 nm.

Discussion

As shown in this paper, P. shermanii utilizes added DMNIA to form the new B_{12}-analog DMNIA-cobamide. The answer to the question whether also DMNIA itself is produced by a certain species of microorganism has to await the investigation indicated above.

Recently it was found that riboflavin can function as precursor of the 5,6-dimethylbenzimidazole moiety of vitamin B_{12}. At the present stage of investigation it is not possible to decide if the benzimidazole bases of the other natural B_{12}-analogs (Fig. 1, 1, 2, 4) also originate from corresponding riboflavin analogs, since B_{12}-analogs with these bases, unlike vitamin B_{12} (3), can not yet be isolated from a single species of microorganism, but only from activated sludge containing numerous different microorganisms. Should it be possible to show that the benzimidazole bases indeed arise all from corresponding riboflavin analogs, the problem concerning the biosynthesis of the benzimidazole bases of corrinoids would have shifted to the problem how these riboflavin analogs are formed.

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1 Abbreviations: DMNIA = 6',7'-Dimethylnaphtho-(2',3')-imidazole-4,5; DMNIA-Cobamide = 6',7'-Dimethylnaphtho-(2',3')-imidazole-4,5-yl-Cobamide.
8 Recently DMNIA was also prepared by another way, see P. Renz, Z. Naturforsch. 26b, 701 [1971].
In this research we have investigated the high radio-protective ability of a mixture of tryptophan plus ethanol, on a Gamma irradiated water solution of purine, pyrimidine bases, DNA and even on irradiated seeds (*Lens Sculentum*).

From a group of non sulphydryl radical scavengers, tested: methanol, formaldehyde, acetaldehyde, formiate, acetate and ethanol (tryptophan); ethanol and tryptophan were selected because its high protector action on irradiated purine and pyrimidine bases. At molarities of the order of $10^{-4}$ for tryptophan-ethanol and bases and DNA the respective DRF values were: for guanine: 20.58; for adenine: 3.75; for thymine: 4.94; for cytosine: 5.52; for uracil: 5.43; and for DNA: 18.95. For irradiated seeds protected with tryptophan-ethanol ($1.5 \times 10^{-3}$ M; $2.6 \times 10^{-2}$ M) the DRF value was of 3.33 and radiation damage became evident only after a total dose of 2 x 10$^4$ rads.

A partial different mechanisms for radioprotection with ethanol and tryptophan is postulated; this is supported by the fact that when tryptophan molarities attains that of protected DNA, there is a rapid increase in its values of DRF, crossing up the ethanol protection curve. Actually tryptophan could act not only as a radical scavenger, but also, as localized radical quencher and/or energy trapper.

In the study of the radioprotective action of the mixture, the different mechanisms of tryptophan and ethanol protection, were evidenced.

### Materials and Methods

Adenine and uracil bases were purchased from Mann Research Lab. Inc.; cytosine from Sigma Chemical Co.; guanine* from Pabst Laboratories; thymine, L-tryptophan and DNA (ex. Salmon Sperm) from Calbiochem; methanol, ethanol, formaldehyde, acetaldehyde, acetic acid and sodium hydroxide, from E. Merck AG. and formic acid from Riedel de Haen AG.

Irradiation experiments were carried out in a 137 Cs source of 10$^6$ Ci at a dose rate of 5.376 to 8.922 rads/min.

Spectrophotometric determinations were done in a Cary 16, double beam spectrophotometer. The development of seeds, on moistened filter paper, were followed by measuring daily the growth of epicotyledons.

* Guanine was used as chlorhydrate.