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Biosynthesis of coenzyme F430 in methanogenic bacteria

Identification of 15,17³-seco-F430-17³-acid as an intermediate

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Coenzyme F430 is a hydroporphinoid nickel complex present in all methanogenic bacteria. It is part of the enzyme system which catalyzes methane formation from methyl-coenzyme M. We describe here that under certain conditions a second nickel porphinoid accumulates in methanogenic bacteria. The compound was identified at 15,17³-seco-F430-17³-acid. The structural assignment rests on ^{14}C-labelling experiments, fast-atom-bombardment mass spectra, ^{1}H-NMR spectra of the corresponding hexamethyl ester, and ultraviolet/visible spectral comparison with model compounds. In cell extracts and in intact cells of methanogenic bactera, 15,17³-seco-F430-17³-acid was converted to F430. These findings indicate that the new nickel-containing porphinoid is an intermediate in the biosynthesis of coenzyme F430.

Coenzyme F430 [1] is a yellow non-fluorescent nickel porphinoid found in all methanogenic bacteria [2–5]. Its structure has been recently determined [6–9]. As shown in Fig. 1, coenzyme F430 **1** possesses a highly saturated ligand system with a chromophore not previously encountered among natural tetrapyrroles. It may be considered a tetrahydro derivative of a corphin [6, 10], combining structural elements of both porphyrins and corrins. The isolated imine double bond, the lactam ring attached to ring B, and the six-membered carbocyclic ring, built from the propionate side chain in ring D, further distinguish coenzyme F430 from other natural prophinoids. Suggestions that the parent coenzyme is larger, containing coenzyme M and a lumazine derivative covalently bound to the porphinoid ligand skeleton, in the meantime, have been ruled out [7, 11, 12].

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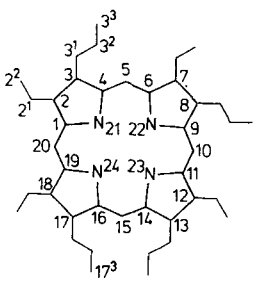
Dedicated to Professor Dr H.G. Wood on the occasion of his 80th birthday.

Abbreviations. F430, coenzyme F430; F430M, pentamethyl ester of coenzyme F430; NiX, nickel-containing material finally identified as 15,17³-seco-F430-17³-acid; NiXM, methanolysis product of NiX; coenzyme M, 2-mercaptoethane sulfonate; methyl-coenzyme M, 2-(methylthio)-ethane sulfonate; δAla, 5-aminolevulinic acid; FAB-MS, fast atom bombardment mass spectroscopy; t_R, retention time.

Enzyme. Methyl-coenzyme M reductase (EC 1.8.–.–).

Note. The numbering of the F430-porphinoid ligand skeleton follows IUPAC-IUB Recommendations for the nomenclature of *tetrapyrroles* [*Eur. J. Biochem. 108*, 1–30 (1980)].



As a cofactor of methyl-coenzyme M reductase [13, 14], to which it is tightly but not covalently bound, coenzyme F430 is involved in the reduction of methyl-coenzyme M to methane and coenzyme M [15, 16], a reaction which in the cell is coupled with the phosphorylation of ADP [17, 18]. There are indications that nickel in coenzyme F430 may undergo redox changes during catalysis [19, 20].

Coenzyme F430 is present in the cells also in a non-protein-bound, free form [7, 11, 21]. Free F430 has been shown to be the precursor of bound F430 in the biosynthesis of methyl-coenzyme M reductase [21]. The free and the enzyme-bound form (the latter determined after dissociation from the protein) have identical structures [7, 11].

The biosynthesis of coenzyme F430 has been partially unravelled. The pathway starts from glutamate [22], which via glutamyl-tRNA and glutamate 1-semialdehyde is converted to 5-aminolevulinic acid (δAla) [23]. From there the biosynthesis proceeds along the well established pathway to uroporphyrinogen III [24], the common precursor of all natural tetrapyrroles [25]. Analogous ot the biosynthesis of vitamin B_{12} and siroheme [25], uroporphyrinogen III is then methylated at positions 2 and 7 by *S*-adenosylmethionine [6, 26] in a sequence of reactions which, according to the available evidence, lead to 15,23-dihydro-sirohydrochlorin [9, 26–28]. The subsequent steps converting dihydro-sirohydrochlorin to coenzyme F430 and the intermediates involved are still unknown.

In this communication we report the isolation of a nickel-containing biosynthetic precursor of coenzyme F430 which was found to be a 15,17³-seco derivative of F430, possessing a free propionate side chain in ring D in place of the six-membered carbocyclic ring (**3** Fig. 1).

MATERIALS AND METHODS

Materials

$^{63}NiCl_2$ was from Amersham Buchler (Braunschweig, FRG). 5-Amino[4-^{14}C]levulinic acid and L-[*methyl*-^{3}H]methionine were from New England Nuclear (Dreieich, FRG).

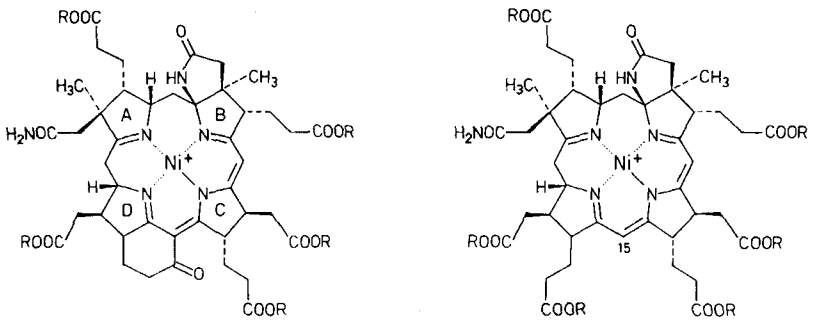


Fig. 1. *Structure of coenzyme F430* **1**, *15,17³-seco-F430-17³-acid* **3**, *and the corresponding penta- and hexamethyl esters* **2** *and* **4**, *respectively*

QAE-Sephadex A-25 was from Pharmacia Fine Chemicals (Uppsala, Sweden). *Methanobacterium thermoautotrophicum* strain Marburg (DSM 2133), *Methanobrevibacter arboriphilus* AZ (DSM 744) and *Methanospirillum hungatei* GP1 (DSM 1101) were from the *Deutsche Sammlung für Mikroorganismen* (Göttingen, FRG). Infusion bottles (125 ml, 500 ml, and 1 l) with steel screw caps were from Müller & Krempel (Bülach, Switzerland), Para-rubber stoppers (40–5 shore A) from Maag Technik (Dübendorf, Switzerland).

Analytical methods

HPLC. NiX: reverse-phase 12 × 250-mm column, Techsil C_{18}, (10 μm); flow rate 1.2 ml/min; HP-1040A diode array detection system. Solvent A: 0.01 M aqueous NaH_2PO_4/Na_2HPO_4 pH 6.0; solvent B: methanol/H_2O 4:1. For elution the following gradient was applied: 0–10 min 100% A, and 10–50 min 0–30% B.

NiXM (system A): 12 × 140-mm column, Chromsilgel (5 μm); 2.2 ml/min; detection at 430 nm; solvent system: CH_2Cl_2/CH_3CN/iPrOH/0.01 M aqueous $LiClO_4$ (500:80:70:9).

NiXM (system B): 9 × 250-mm column, Lichrosorb Si100-5 μm; 2.2 ml/min; detection at 270 nm; CH_2Cl_2/CH_3CN/iPrOH/0.02 M aqueous $LiClO_4$ (500:100:25:4).

TLC. Merck TLC plates silica gel 60, 0.25 mm; before use, the plates were developed with methanol to remove impurities and reactivated at 120 °C.

FAB MS [29]. Measured on a Kratos AEI MS-50, fitted with an M-scan FAB system; matrix: glycerol/SeO_2 for NiX, *m*-nitrobenzyl alcohol for NiXM.

Growth of bacteria

M. arboriphilus was grown in 400-ml culture volumes in a 500-ml glass fermenter at 37 °C and pH 7 on H_2 and CO_2 as energy source. The fermenter was filled with 360 ml basal medium of the following composition: KH_2PO_4, 50 mM, NH_4Cl, 40 mM, Difco yeast extract, 1 g/l; nitrilotriacetate, 0.45 mM, $MgCl_2$, 0.2 mM, $FeCl_2$, 50 μM; $CoCl_2$, 1 μM, Na_2MoO_4, 1 μM; $NiCl_2$, as indicated; and resazurin, 20 μM. The fermenter with the basal medium was autoclaved for 30 min at 120 °C. Then 12 ml sterile 1 M Na_2CO_3 was added and the medium gassed with 80% H_2/20% CO_2/0.2% H_2S for 15 min at a rate of 200 ml/min via a microfilter candle, porosity 3 (Schott, Mainz, FRG). After inoculation (40 ml inoculum) the culture was continuously stirred with a Teflon-coated stirring paddle at 500 rpm and gassed with 80% H_2/20% CO_2/0.2% H_2S at a rate of 250 ml/min. The bacteria grew exponentially with a doubling time of 4 h to a cell concentration of 6 g (wet weight) per l ($\Delta A_{578} = 3$) at which the culture was cooled down to 4 °C and then anaerobically harvested by centrifugation. The cell pellet (2.4 g) was anaerobically suspended in 50 ml growth medium, from which the yeast extract was omitted, and samples of the suspension were directly used in the experiments for NiX accumulation.

M. thermoautotrophicum was grown in 400-ml culture volumes in a 500-ml glass fermenter at 65 °C on H_2 and CO_2 as sole carbon and energy source essentially as described by Schönheit et al. [30]. Where indicated $NiCl_2$ was omitted from the medium. The cells were anaerobically harvested at a cell concentration of 6.6 g (wet weight) per l ($\Delta A_{578} = 3.3$) and the cell pellet (3 g) used for the preparation of cell extracts.

M. hungatei was grown in 200-ml culture volumes in 500-ml infusion bottles at 37 °C on H_2 and CO_2 as energy source and acetate plus CO_2 as carbon sources essentially as described by Balch et al. [31]. The cells grew to a final cell concentration of 2 g (wet weight)/l within 5–6 days.

Preparation of cell extracts for the conversion of NiX to F430

M. thermoautotrophicum was grown for three or four generations on medium to which no $NiCl_2$ was added. Extracts of such grown cells proved more active in catalyzing the conversion of NiX to F430 than extracts from cells not grown under conditions of nickel limitation.

Cells of such grown *M. thermoautotrophicum* (3 g wet weight) were anaerobically suspended at 4 °C in 14 ml 10 mM potassium phosphate buffer pH 6.8 containing 60 mM $NaHCO_3$, 10 mM $MgCl_2$ and per ml: 0.02 mg deoxyribonuclease, 0.002 mg coenzyme M, 0.5 mg penicillin G, 0.05 mg δAla, and 0.05 mg L-methionine. The suspension was anaerobically passed twice through a French pressure cell at 137 MPa (20000 lb/in²). After centrifugation for 30 min at 37000 × *g* the supernatant was transferred into a 125-ml infusion bottle with 80% H_2/20% CO_2 at 150 kPa as gas phase. After addition of NiX (^{63}NiX or [^{14}C]NiX, 30–50 nmol) and $^{58}NiCl_2$ (5 nmol) the bottle was placed in a water bath shaker at 55 °C and incubated in the dark for 24 h under continuous shaking at 50 rpm. After 24 h the incubation mixture was cooled down to 0 °C and 0.5 ml 2 M $HClO_4$ was added. Precipitated protein was removed by centrifugation. The supernatant was adjusted to pH 9.5 with $KHCO_3$ and KOH as described below and was then analyzed for F430 and NiX.

Extraction of F430 and of NiX from cells

Cells (0.4 g wet weight) were suspended in 5 ml H_2O containing 0.5% sodium deoxycholate and 1 mg deoxyribonuclease and stirred for 30 min at 0°C. Then 100 µl 2 M $HClO_4$ was added by which the pH was lowered to 2. After further stirring for 30 min at 0°C precipitated material was removed by centrifugation (20 min, $16000 \times g$). The yellow supernatant was separated from the pellet which was re-extracted with 5 ml 10 mM $HClO_4$ (30 min, 0°C). The combined supernatants (approximately 10 ml) were neutralized by the addition of solid $KHCO_3$, adjusted to pH 9.5 with 5 M KOH, and then stored at −20°C. After thawing, the $KClO_4$ crystals were removed by filtration (0.2 µm pore size). The filtrate was diluted 1:2 with H_2O for chromatography on QAE-Sephadex A-25.

Separation and quantitative determination of F430 and NiX

The $HClO_4$ extract (pH 9.5) diluted 1:2 with H_2O was applied to a QAE-Sephadex A-25 column (1×10 cm) previously equilibrated with 50 mM glycine/KOH pH 9.5. The column was washed with 80 ml 0.1 M NaCl in 50 mM glycine/KOH pH 9.5 and then F430 and NiX eluted with 0.3 M NaCl in 50 mM glycine/KOH pH 9.5. The fractions containing F430 (10 – 30 ml) and those containing NiX (36 – 70 ml) were pooled and the concentration of F430 and of NiX was determined photometrically at 430 nm and 427 nm, respectively, using $\varepsilon_{430} = 23000\ cm^{-1}\ M^{-1}$ for F430 [2, 6] and $\varepsilon_{427} = 23000\ cm^{-1}\ M^{-1}$ for NiX (see Results). When F430 and NiX were labelled with ^{63}Ni the concentrations were calculated via the radioactivity and specific radioactivity of the $^{63}NiCl_2$ used in the synthesis of these compounds.

Partial purification of NiX

The pooled fractions containing NiX were diluted 1:4 with H_2O and re-applied to a QAE-Sephadex A-25 column (1×10 cm) equilibrated with 50 mM glycine/KOH pH 9.5. The column was washed with 80 ml 0.1 M NaCl in glycine/KOH pH 9.5 and NiX eluted with 0.3 M NaCl in glycine/KOH pH 9.5. The fractions containing NiX (36 – 70 ml) were pooled, diluted 1:4 with H_2O, and re-applied to a QAE-Sephadex A-25 column (1×10 cm) equilibrated with 50 mM Tris/HCl pH 7. The column was washed with 80 ml 1 mM HCl. Then NiX was eluted with 2.5 mM HCl in 90 – 100 ml [32]. The pooled fractions were lyophilized and stored under N_2 at −20°C.

Methanolysis of NiX

A sample of partially purified NiX (≈ 1.4 µmol) was dissolved in anhydrous methanol (4 ml), in a glass ampoule with teflon stopcock. After addition of anhydrous 4-toluene sulfonic acid (44 mg, 0.25 mmol), the solution was degassed at 133 mPa applying three freeze-thaw cycles. The ampoule was sealed under vacuum and heated in an oil bath to 50°C for 4 h (cf. [6]). The reaction mixture was cooled to room temperature, diluted with dichloromethane (5 ml) and extracted with 0.1 M aqueous $NaClO_4$ (3×5 ml). The organic layer was filtered through cotton, concentrated and the resulting brown residue applied to a TLC plate (silica gel 20×20 cm; impregnated with a methanolic solution of $NaClO_4$ at 1 g/100 ml and reactivated at 120°C, as described in [6]). The plate was developed four times with CH_2Cl_2/hexane/methanol (16:1:1). The silica gel of the two overlapping main bands, $R_F = 0.15$ (yellow) and 0.17 (reddish brown), separated from several minor bands between $R_F = 0.19 - 0.98$, was triturated with $KClO_4$ and extracted with CH_2Cl_2/methanol (5:1). After evaporation of the solvent, the residue was taken up in dichloromethane and extracted with a small amount of water to remove inorganic salts. The resulting product was purified by preparative HPLC (system A). The main fraction ($t_R = 18$ min) which had been separated from a minor fraction ($t_R = 14$ min) was again subjected to HPLC (system B) to remove a reddish impurity ($t_R = 33$ min). The main fraction ($t_R = 39$ min) which showed the same absorption spectrum as NiX, was concentrated and applied to a small TLC plate (10×1 cm). After chromatography with a solution of $NaClO_4$ in ethyl acetate (0.5 g/100 ml) the yellow product was isolated from the plate as described above. After drying at 23°C and 133 mPa, analytically pure hexamethyl ester of 15,17^3-seco-F430-17^3-acid was isolated as an amorphous yellow solid (≈ 0.6 mg, 40% yield).

RESULTS

When factor F430 was isolated from ^{63}Ni-grown cells of *Methanobrevibacter arboriphilus* or of *Methanobacterium thermoautotrophicum* it was occasionally observed that a second ^{63}Ni-containing low-molecular mass compound was present in the extracts. The compound, designated NiX, eluted with 0.3 M NaCl (pH 9.5) after F430 from the QAE-Sephadex A-25 column used as first step in the purification procedure for F430 (for conditions see Fig. 2). The amounts (deduced via the specific radioactivity of ^{63}Ni) were, however, too low (< 1 nmol/g wet weight cells) to allow an identification. We therefore looked for conditions under which NiX accumulated in the cells using as working hypothesis that NiX could be an intermediate in F430 synthesis and thus should be synthesized from 5-aminolevulinate (δAla).

Conditions for NiX accumulation

It was found that cell suspensions of *M. arboriphilus* synthesized NiX and F430 when incubated under 80% H_2 and 20% CO_2 at 37°C in the presence of $NiCl_2$ and δAla. The amount of NiX and F430 formed was dependent on how the cells had been grown, on the $NiCl_2$ and δAla concentrations and on the incubation time.

Cells of *M. arboriphilus* depleted in nickel supply proved most active in synthesizing NiX. Such cells were obtained by growing the bacteria in a medium without added nickel using a 10% inoculum which had been grown in a medium with 5 µmol $NiCl_2$/l.

The bacteria were harvested while still in the exponential growth phase at a cell concentration of 6 g (wet weight)/l. 1 g of these cells (wet weight) was suspended in 25 ml growth medium containing 10 mM δAla, 10 mM L-methionine, and 10 µM $NiCl_2$ and the suspension was anaerobically incubated at 37°C in a 1-l infusion bottle which was continuously shaken at 50 rpm. The gas phase was 80% H_2 and 20% CO_2 at 200 kPa pressure. During incubation the nickel concentration in the medium (due to Ni uptake by the cells) and the gas pressure (due to CH_4 formation) rapidly decreased. Therefore, after 2, 4, 6, and 18 h of incubation each time 0.5 µmol of $NiCl_2$ was added and the gas phase was set back to 200 kPa pressure with 80% H_2/20% CO_2. After a total of 48 h incubation the 1 g cells had synthesized between 100 – 200 nmol NiX and 500 – 700 nmol F430. Before the inbucation the cells had

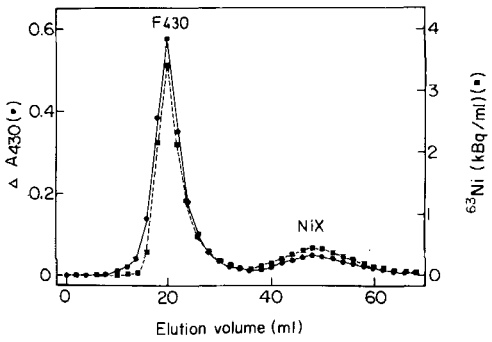


Fig. 2. *Separation of F430 and of NiX by chromatography on QAE-Sephadex A-25.* Cells (1 g wet weight) of *Methanobrevibacter arboriphilus* were suspended in 25 ml growth medium containing 10 mM δAla, 10 mM L-methionine, and 10 μM $^{63}NiCl_2$ (200 kBq/μmol) and incubated at 37°C for 48 h under 80% H_2/20% CO_2 at 200 kPa pressure. No further $^{63}NiCl_2$ was added during the incubation in this experiment. Perchloric acid extracts from the cells were adjusted to pH 9.5 and applied to a QAE-Sephadex A-25 column (1 × 10 cm) equilibrated with 50 mM glycine/KOH pH 9.5. The column was washed with 80 ml 0.1 M NaCl in 50 mM glycine/KOH pH 9.5 and then F430 and NiX eluted with 0.3 M NaCl in 50 mM glycine/KOH pH 9.5

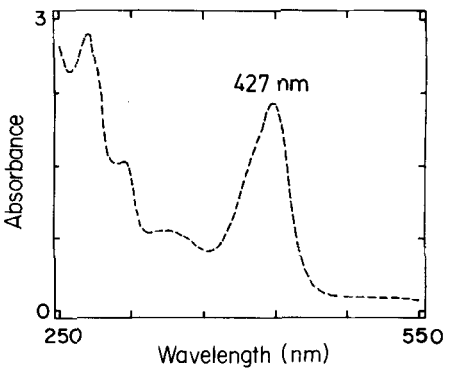


Fig. 3. *Ultraviolet/visible absorption spectrum of partially purified NiX in 2.5 mM HCl.* The concentration of NiX was 91 μM (ε_{427} = 23000 cm^{-1} M^{-1})

contained less than 1 nmol NiX and 50 nmol F430 per g (wet weight).

Cell suspensions of *M. thermoautotrophicum* also synthesized NiX and F430 when incubated in the presence of $NiCl_2$ and δAla. The amounts of NiX formed were, however, much lower than in *M. arboriphilus*.

Purification and some properties of NiX

Cells (1 g wet weight) of *M. arboriphilus*, which had been incubated for 48 h in the presence of $^{63}NiCl_2$, δAla (10 mM), and methionine (10 mM) were extracted for F430 and NiX as described in Materials and Methods. The extract (pH 9.5) was applied to a QAE-Sephadex A-25 column (1 × 10 cm) equilibrated with 50 mM glycine/KOH pH 9.5. F430 and NiX were adsorbed to the anion-exchange gel under these conditions. Both compounds were eluted with 0.3 M NaCl, NiX being retained longer than F430 (Fig. 2). The NiX-containing fractions had a yellow colour with an absorption maximum at 427 nm. The $A_{427}/^{63}Ni$ ratio was almost the same in all fractions, indicating that the yellow colour was derived from NiX.

The frist chromatography on QAE-Sephadex A-25 did not lead to a complete separation of F430 and NiX due to an overlap of the elution peaks. This separation step was therefore repeated once. The NiX-containing fraction were then re-applied to a third QAE-Sephadex A-25 column. After extensive washing of the column with 1 mM HCl, NiX was eluted with 2.5 mM HCl. (Since NiX and F430 coeluted under these conditions a prior complete separation of the two compounds by chromatography at pH 9.5 was essential.) The absorption spectrum of NiX-containing fractions is shown in Fig. 3. The solutions were not fluorescent.

The concentration of NiX in the solution was calculated from the radioactivity per ml and from the specific radioactivity of $^{63}NiCl_2$ used in the synthesis of NiX. From the absorbance difference at 427 nm and the concentration of ^{63}NiX, a molar absorption coefficient ε_{427} of 23000 cm^{-1} M^{-1} was obtained.

The purification procedure described above was performed at 4°C under strictly anaerobic conditions. In contact with air at room temperature NiX preparations slowly turned reddish. In this case, a mixture of NiX and two additional components was obtained which could be separated by reverse-phase HPLC [32]. The component which eluted first (t_R = 36.2 min) showed essentially the same absorption spectrum as NiX (t_R = 39.3 min). The third component (t_R = 40.1 min) was reddish and exhibited an absorption sepctrum with main bands at 290, 480, and 510 nm, reminiscent of 12,13-didehydro-F430 (F560) [8]. These data suggest that under these conditions NiX is partially epimerized and dehydrogenated in ring C, as has been observed for F430 [8].

To determine how tightly Ni is bound in NiX the exchange between ^{63}NiX and $^{58}NiCl_2$ at pH 1, pH 7, and pH 10 was studied. An exchange of Ni isotopes was not observed at any of the conditions tested.

Identification of NiX as an Ni-porphinoid derived from dihydrosirohydrochlorin

Cell suspensions of *M. arboriphilus* only synthesized NiX in considerable amounts when the medium was supplemented with δAla. The rate of NiX synthesis increased with increasing δAla up to a concentration of 10 mM. These findings indicated that NiX is an Ni-porphinoid.

To obtain evidence to confirm this, cells of *M. arboriphilus* were incubated in the presence of [^{14}C]δAla (10 mM, 17 kBq/μmol), $^{58}NiCl_2$ (10 μM), and L-methionine (10 mM) as described under *Conditions for NiX accumulation*. After 48 h NiX and F430 were isolated. A co-chromatography of ^{14}C with both NiX and F430 was observed. The specific radioactivity of purified NiX was 145 kBq/μmol and that of purified F430 was 140 kBq/μmol (corrected for the F430 content of the cells before incubation). Both compounds had approximately 8 times the specific radioactivity of [^{14}C]δAla. This finding can be taken as evidence that NiX is a prophinoid since only porphinoids are synthesized from 8 mol δAla/mol.

Dihydrosirohydrochlorin [28], which is assumed to be an intermediate in F430 biosynthesis [9, 26, 27], contains two methyl groups derived from *S*-adenosylmethionine. Cells of *M. arboriphilus* were therefore incubated in the presence of L-[*methyl*-^{3}H]methionine (10 mM, 5.4 kBq/μmol), δAla (10 mM) and $^{58}NiCl_2$ (10 μM) as described under *Conditions for NiX accumulation*. After 48 h NiX and F430 were isolated. A co-chromatography of ^{3}H with both NiX and F430 was

observed. The specific radioactivity of NiX (2 kBq/μmol) and of F430 (1.95 kBq/μmol) were almost identical suggesting that NiX, like F430, is biosynthetically derived from dihydrosirohydrochlorin.

The synthesis of NiX and of F430 in cell suspension was not dependent on the presence of extracellular methionine. Evidently, the intracellular *S*-adenosylmethionine pool was large enough to sustain the synthesis of the two porphinoids during the time of incubation. This explains why the specific radioactivity of NiX (2 kBq/μmol) and of F430 (1.95 kBq/μmol) was significantly lower than that of L-[*methyl*-^{3}H]-methione (5.4 kBq/μmol).

Conversion of NiX to F430

Three types of experiments were performed to determine whether NiX is an intermediate in F430 biosynthesis.

a) Experiments with M. arboriphilus. NiX is not taken up by intact cells of *M. arboriphilus*. We therefore attempted to demonstrate that intracellularly synthesized ^{63}NiX is converted to ^{63}Ni-F430. Cells of *M. arboriphilus* (2.2 g wet weight; nickel-depleted as described under *Conditions for NiX accumulation*) were suspended in 50 ml growth medium containing δAla (20 mM), methionine (20 mM), and $^{63}NiCl_2$ (50 nmol; 93.5 kBq). The gas phase was 80% H_2/20% CO_2 at 200 kPa pressure. After 4 h of incubation at 37°C, in which the cells had taken up 70 kBq $^{63}NiCl_2$ (35 nmol), $^{58}NiCl_2$ (5 μmol) was added (by which the specific radioactivity was decreased over 100-fold) and the cells were incubated for another 44 h. At the times indicated the cells were analyzed for ^{63}NiX and ^{63}Ni-F430. The amounts, which were determined spectrophotometrically, are given in brackets. After the first 4 h of incubation (before the addition of $^{58}NiCl_2$) the cells contained 28 kBq NiX (15 nmol) and 8.5 kBq F430 (170 nmol); 4 h after the addition of $^{58}NiCl_2$ the amounts were 30 kBq NiX (200 nmol) and 20 kBq F430 (200 nmol); after 17 h the amounts were 17 kBq NiX (140 nmol) and 38 kBq F430 (270 nmol), and after 44 h they were 17 kBq NiX (460 nmol) and 52.5 kBq F430 (490 nmol). Thus, during incubation of the cells in the presence of $^{58}NiCl_2$, the ^{63}NiX content decreased while that of ^{63}Ni-F430 increased, indicating that ^{63}NiX was converted to ^{63}Ni-F430. However, since more ^{63}Ni-F430 was formed than ^{63}NiX had disappeared the interpretation of the chase experiment is not unambiguous. It is pointed out that in the chase experiment, after the addition of $^{58}NiCl_2$ (5 μmol), the NiX content of the cells increased from 15 nmol to 460 nmol (in 2.2 g wet weight cells). The specific radioactivity of NiX dropped from 1870 kBq/μmol to 38 kBq/μmol. It is, therefore, not possible to calculate exactly the rate of NiX conversion to F430 from the radioactivity data. They allow one, however, to estimate that the rate of NiX conversion to F430 and the rate of F430 formation were of the same order of magnitude which is a prerequisite for NiX being an intermediate in F430 synthesis.

b) Experiments with M. thermoautotrophicum. NiX is not taken up either by intact cells of *M. thermoautotrophicum*. It was therefore tested whether cell extracts of *M. thermoautotrophicum* catalyzed the conversion of NiX to F430. This was to be expected since Mucha et la. [27] had shown that extracts of this organism (strain H) mediate the formation of F430 from sirohydrochlorin. Cell extracts of *M. thermoautotrophicum* were therfore supplemented with either ^{63}NiX or [^{14}C]NiX (synthesized from [^{14}C]δAla) and incubated for 24 h at 55°C. The gas phase was 80% H_2/20% CO_2 at 150 kPa pressure. After 24 h the extracts were analyzed for

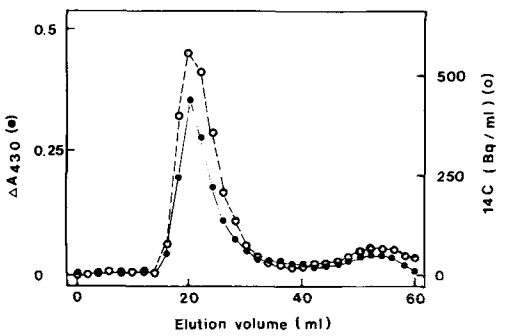


Fig. 4. *Conversion of NiX into F430 in cell extracts of* Methanobacterium thermoautotrophicum. Cell extracts of *M. thermoautotrophicum* (14 ml; 23 mg protein/ml) was supplemented with 46 nmol [^{14}C]NiX (144 kBq/μmol), incubated for 24 h at 55°C under 80% H_2/20% CO_2 and then analyzed for [^{14}C]NiX and [^{14}C]F430 by chromatography on QAE-Sephadex A-25 (for conditions see legend to Fig. 2). More than 60% of the NiX was converted to F430 under the experimented conditions

^{63}Ni-F430 or [^{14}C]F430. A typcial experiment is presented in Fig. 4. During incubation up to 60% of the added [^{14}C]NiX was converted to [^{14}C]F430.

c) Experiment with M. hungatei. Growing cells of *M. hungatei* were found to slowly take up NiX. After growth in the presence of ^{63}NiX in the medium (0.32 nmol/200 ml; 1870 kBq/μmol) 20% of the ^{63}NiX had been taken up by the cells from which 50% was converted to F430. ^{63}Ni was not released from ^{63}NiX under the experimental conditions.

When *M. hungatei* was grown in the presence of [^{63}Ni]F430 (140 nmol/200 ml; 342 kBq/μmol) 30% (= 51 nmol) was taken up by the cells (0.44 g wet weight). The specific radioactivity of the intracellular F430 was 316 kBq/μmol indicating that 80% of intracellular F430 was derived from F430 present in the medium. This is an interesting observation since it shows that *M. hungatei* possesses a transport system for F430 by which probably also NiX enters the cells.

Structural assignment of NiX

The results reported here so far demonstrate that NiX is a nickel porphinoid (deduced from labelling experiments with [^{14}C]δAla), which, presumedly, is derived from dihydrosirohydrochlorin (deduced from labelling experiments with L-[*methyl*-^{3}H]-methionine) and which can be converted to factor F430 in cell extracts and in growing cells of methanogenic bacteria. The finding that at pH 9.5 NiX binds tighter than F430 to QAE-Sephadex A-25 (Fig. 2) suggests that NiX has more free carboxyl groups than F430.

In the absorption spectrum, NiX exhibits a strong absorption band at 427 nm, similar to F430 (Fig. 5). Between 250 and 350 nm, however, the spectra of the two compounds clearly differ from each other. Among the series of model compounds prepared by Fässler et al. [33–36], a nickel complex **5** was found which, in the absorption spectrum, closely resembles NiX **3** (Fig. 5). The chromophore of complex **5** differs from that of F430 **2** and its model complex **6** mainly by the missing (electron-withdrawing) substituent at the *meso* position C–15. Accordingly, the distinct spectroscopical differences between complexes **5** and **6** can be attributed primarily to the cyano groups at C-15 in **6** which exerts a similar effect on the spectrum as the carbonyl groups of the carbocyclic ring in F430. The resemblance between the spectra of complex **5**

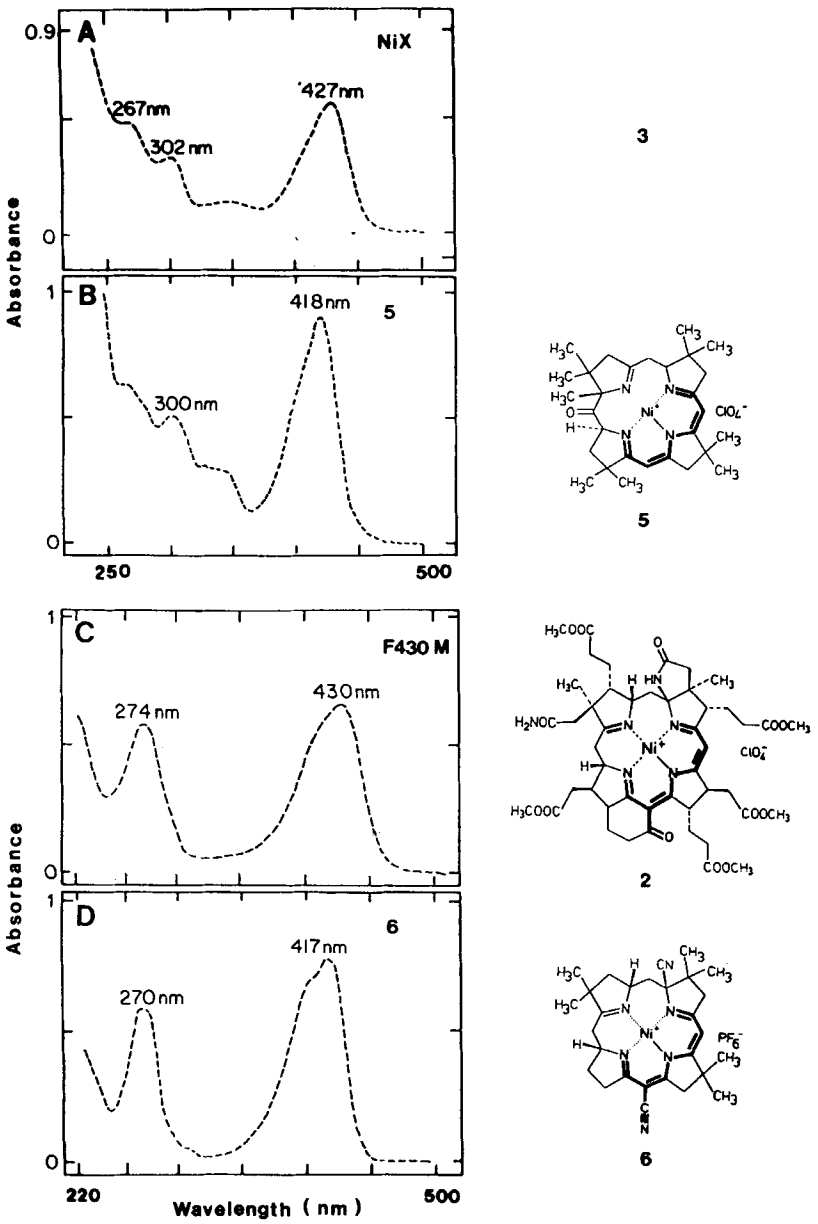


Fig. 5. *Ultraviolet/visible absorption spectra of NiX* **3** *(in aqueous 0.01 M phosphate buffer, pH 6), F430M* **2**, *and the model compounds* **5** *and* **6** *(all in methanol)*

and NiX **3** suggests that NiX might be a 15,17[3]-seco derivative of F430, lacking the six-membered carbocyclic ring.

Coenyzme F430 with ^{58}Ni has an M_r of 905 [7]. The M_r of NiX, as determined by FAB MS, was found to be 923, Table 1), indicating that NiX is formally related to F430 by addition of one molecule of water. The intensities of the peaks between $m/z = 923$ and 927 are in accordance with the isotope distribution calculated for a compound with a molecular formula ($F430 + H_2O$). Based on these data, structure **3** (Fig. 1), which formally results from hydrolytic cleavage of the carbocyclic ring of F430, was proposed for NiX.

Structure **3** was finally confirmed by ^{1}H-NMR spectroscopy of the corresponding hexamethyl ester **4**. A sample of NiX was treated with 4-toluene sulfonic acid in methanol under the conditions previously used for the preparation of F430M from F430 [6]. After purification by preparative TLC

Table 1. *FAB-MS of NiX and its methanolysis product NiXM [29]*

NiX			NiXM		
m/z	relative intensity		*m/z*	relative intensity	
	measured	calculated		measured	calculated
	%			%	
921	33	–	1005	11	–
922	22	–	1006	12	–
923	100	100	1007	100	100
924	50	50	1008	66	57
925	45	54	1009	63	57
926	25	25	1010	26	28
927	18	13	1011	13	14

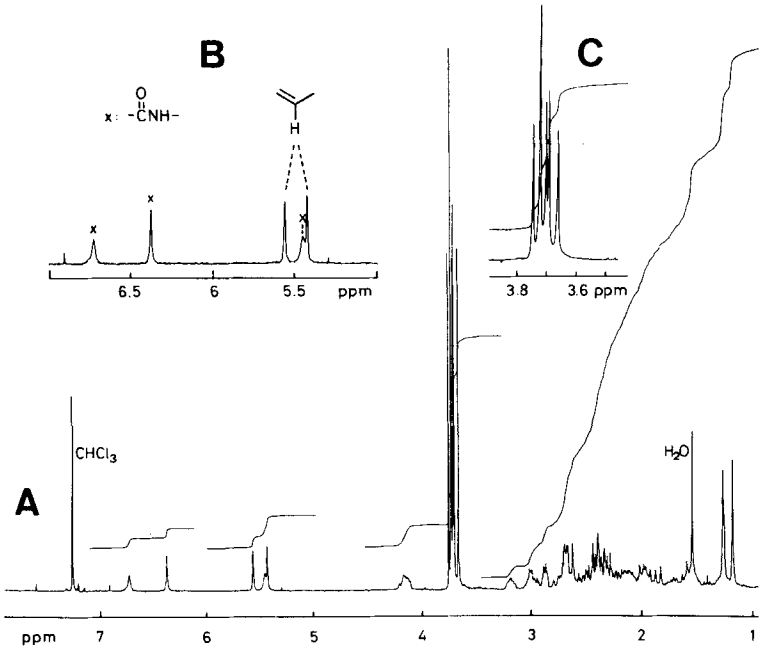


Fig. 6. *300-MHZ ^{1}H-NMR spectrum of NiXM (methanolysis product of NiX).* 1.5 mM solution in $CDCl_3$; chemical shift (δ) in ppm versus tetramethyl silane ($\delta = 0$). (A) Total spectrum; (B) enlarged section (5—7 ppm), (C) enlarged section with methoxycarbonyl signals

followed by HPLC, an analytically pure product, designated NiXM, was obtained which by absorption spectroscopy was indistinguishable from NiX. The ^{1}H-NMR spectrum of NiXM is shown in Fig. 6. The five singlets between 3.66 ppm and 3.75 ppm, which correspond to 18 protons, clearly demonstrate the presence of six methoxycarbonyl groups in NiXM. The two singlets at 5.44 ppm and 5.57 ppm which are readily assigned to the vinyl protons at positions C-10 and C-15 confirm that the propionate side chain in ring D does not form a carbocyclic ring as in F430. In accordance with the proposed structure, three concentration-dependent NH signals related to the acetamide side chain in ring A and to the lactam ring attached to ring B are found in the expected region between 5 and 7 ppm. The FAB mass spectrum (Table 1) with main peaks between $m/z = 1007$ (M^+-ClO_4^-/^{58}Ni) and 1011 provides additional confirmation for structure **4** of NiXM (Fig. 1).

DISCUSSION

We have shown that a nickel porphinoid related to coenzyme F430 can be isolated from methanogenic bacteria. Its structure has been assigned as 15,17^3-seco-F430-17^3-acid **3** (Fig. 1) which differs from F430 solely by an open propionate side chain in ring D in place of a carbocyclic ring. (Some structural details, notably the stereochemistry at the $C_{12}-C_{13}$ and $C_{17}-C_{18}$ positions [7, 8] remain to be ascertained to allow the proposal of the definitive structures **3** and **4** shown in Fig. 1.) In growing cells this compound is present in only very low concentrations but it accumulates when the cells are incubated in the presence of δAla under non-growth conditions. In cell extracts of *M. thermoautotrophicum* and in growing cells of *M. hungatei*, seco-F430 **3** is converted to F430 **1**. These findings suggest that seco-F430 **3** is a biosynthetic precursor of F430 **1**. They do not prove it, however, since seco-F430 **3** could be an isolation artefact which the cells are capable of converting to F430 **1**. For example, sirohydrochlorin is converted to vitamin B_{12} by *Propionibacterium shermanii* although 15,23-dihydrosirohydrochlorin rather than sirohydrochlorin is the actual intermediate in the biosynthesis of vitamin B_{12} [28]. Dihydrosirohydrochlorin is readily oxidized to sirohydrochlorin during purification but can be reduced back to the dihydro form by the cells [28].

The F430 seco-derivative **3** is on the same oxidation level as F430 **1**. Its conversion to F430 **1** proceeds via intramolecular acylation of the nucleophilic *meso* position C-15 [6], a type of process with many (non-enzymatic) precedents in porphyrin chemistry (see, e.g., the formation of anhydro-bonnelin [37]). Thus, from a chemical point of view, seco-F430 **3** can be regarded as a logical precursor of coenzyme F430. We therefore consider the possibility of seco-F430 **3** being an isolation artefact as not very likely.

A chemically plausible scheme for the biosynthesis of coenzyme F430 is depicted in Fig. 7. All available evidence indicates that F430, siroheme, and vitamin B_{12} share the same biosynthetic pathway up to 15,23-dihydro-sirohydrochlorin **7** [28] as the last common intermediate [6, 9, 26, 27]. The subsequent steps, leading to seco-F430 **3**, must involve (not necessarily in that order): amidation of the acetate side chain in ring A and B, insertion of nickel, rearrangement of the π-system with concomitant reduction of two (C = C) double bonds, and formation of the lactam ring attached to ring B. In our tentative scheme, dihydro-sirohydrochlorin **7** is first converted to a nickel pyrrocorphinate **8a** or **8b** [10]. Protonation of the pyrrol ring then produces a cationic nickel corphinate which is easily reduced to the corresponding dihydrocorphinate **9** [33, 34]. Protonation at C-20 followed by a second reduction step, generates a tetrahydro-corphinate **10**. A reduction sequence of this type appears particularly attractive in view of the model studies by Fässler et al. [33—35], who demonstrated that nickel corphinates can be chemically reduced with remarkable selectivity to the corresponding tetrahydrocorphinates, possessing the same chromophore and the same relative configurations at C-4 and C-19 as the hypothetical F430 precursor **10**. Protonation of the terminal double

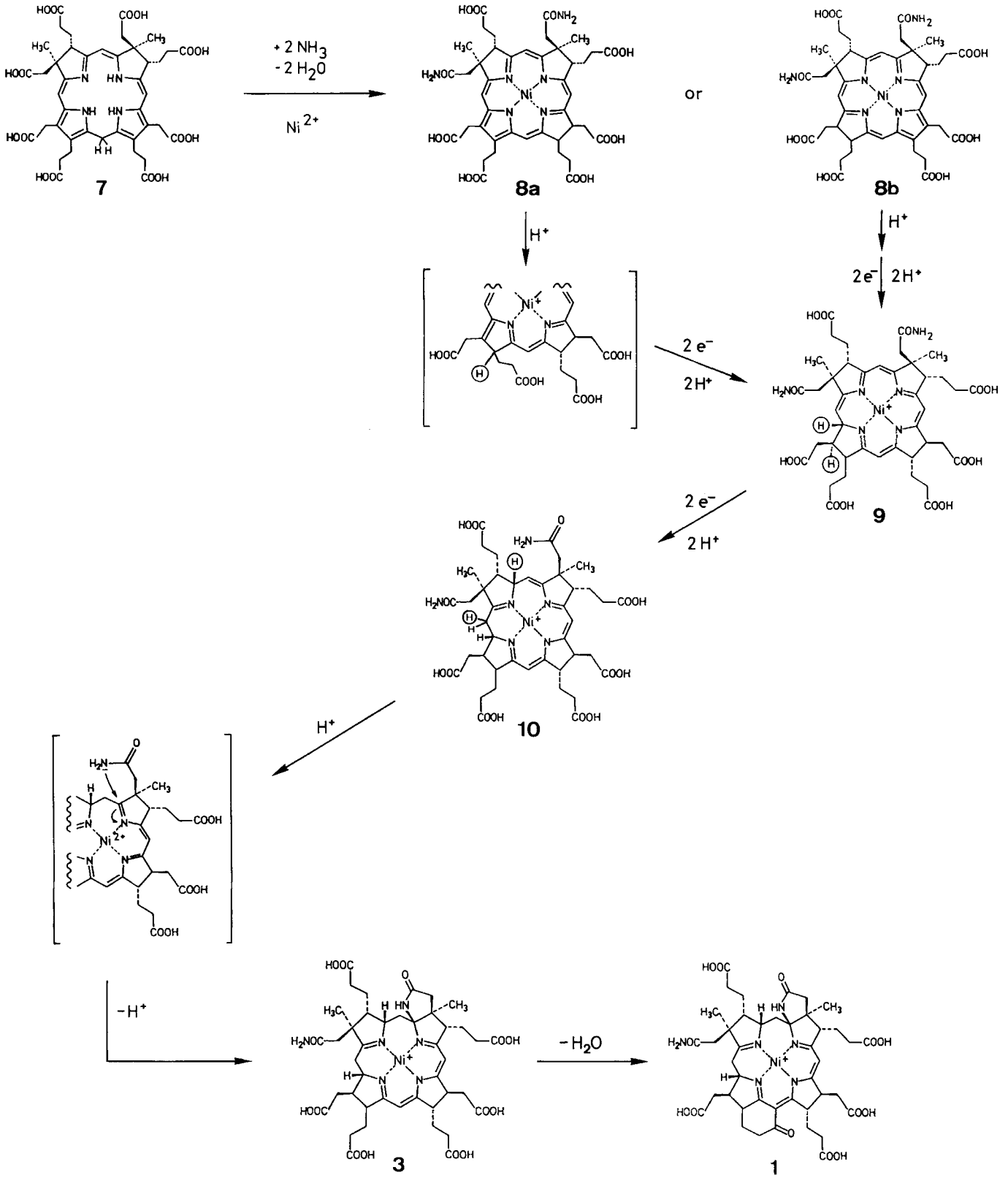


Fig. 7. *Proposed pathway for the biosynthesis of coenzyme F430* **1** *from 15,23-dihydrosirohydrochlorin 7.* With the exception of 15,17[3]-seco-F430 **3**, all other intermediates are hypothetical

bond at C-5, followed by lactam ring formation via nucleophilic addition to the resulting imine double bond at C-4, then leads to the F430 seco-precursor **3**. Analogous (*in vitro*) cyclizations at this position of the ligand system to form a lactam or a lacton ring, are known to occur with ease in related corrinoid compounds [38, 39].

Formation of the six-membered carbocyclic ring, a structural element of considerable consequence to the chemical

properties of the macrocyclic nickel complex [6, 8], is the last step in the biosynthesis of F430. This transformation requires selective activation of the propionate carboxyl group in ring D, in order to induce cyclization by electrophilic attack at the *meso*-position C-15. Accordingly, an activated form of the seco-intermediate **3**, e.g., a 17^3-carboxy-CoA adduct, is most likely the direct biosynthetic precursor of coenzyme F430.

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