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Synthesis of a pH-Sensitive Spin-Labeled Cyclohexylcarbodiimide Derivative for Probing Protonation Reactions in Proton-Pumping Enzymes

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Abstract: The synthesis of a pH-sensitive spin-labeled carbodiimide, N-cyclohexyl-N'-(1-oxyl-2,2,3,5,5-pentamethylimidazolidine-4-yl)-methylcarbodiimide (**1**) is described. The compound is an analog of dicyclohexylcarbodiimide and reacts specifically with the membrane sector of the proton-pumping F_1F_o-type ATP synthase. This reaction of **1** with the ATP synthase (and perhaps other proton-pumping enzymes) should enable studies of protonation reactions in the enzyme using ESR spectroscopy. The preparation of another potentially useful pH-sensitive spin-label, 4-isothiocyanatomethyl-2,2,3,5,5-pentamethylimidazolidine-1-oxyl (**8**) is also described.

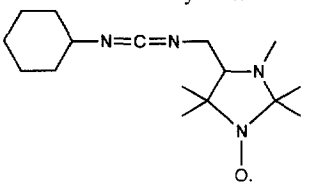
Carbodiimide-derivatives can be useful general reagents for the chemical modification of aspartic, glutamic and C-terminal carboxyl residues in proteins. Stable N-acyl-urea products can be obtained after rearrangement of the O-acyl-isourea when the environment of the derivatized amino acid does not contain a second nucleophile capable of attacking the primary O-acyl-isourea reaction product.[1] This is often the case with membrane proteins and within the hydrophobic interior of soluble proteins. Beechey and coworkers observed in 1967 that dicyclohexylcarbodiimide (DCCD) exerts an inhibitory effect on the membrane bound ATP synthase activities of mitochondria.[2] These original observations on the inhibitory effects of DCCD have been greatly extended in the last three decades[3]. It is now known that DCCD can react specifically with a membrane-embedded carboxyl residue of the proteolipid subunit of the ATP synthase F_o sector (aspartic acid-61 of the subunit c protein in the *Escherichia coli* F_o) and that this reaction leads to inhibition of oxidative phosphorylation and ATP-driven proton-pumping in the synthase, and to inhibition of proton translocation through F_1-depleted F_o-containing membranes. These chemical inhibition studies and recent genetic / mutational investigations of the ATP synthase[4] have led to speculative models for the mechanism of proton translocation through the F_o sector including the role that Asp-61 may play.

The first spin-labeled carbodiimide was reported by Kumarev and Knorre in 1970.[5] In 1973, Azzi and coworkers reported the synthesis of a stable nitroxyl-radical derivative of dicyclohexylcarbodiimide, N-cyclohexyl-N'-(1-oxyl-2,2,6,6-tetramethylpiperidine-4-yl)-carbodiimide (NCCD), that was used as an ESR probe of the mitochondrial ATP synthase.[6] More recently, NCCD and a spin-labeled maleimide were used by Girvin and Fillingame[7] in the elucidation of a high resolution structural model of subunit c by the technique of spin label difference 2D NMR spectroscopy.

In the 1980s, several members of the imidazolidine family of stable nitroxyl spin labels were synthesized that show pH-sensitivity in their ESR spectra depending on the protonation state of the

imidazolidine-N^3-nitrogen.[8-12] These pH-sensitive imidazolidine spin labels have been used as probes of the surface potential and polarity of membranes and proteins[12] and of intracellular and intravesicular pH.[8] Experiments using pH-sensitive spin-labels to monitor the intraliposomal pH changes that occur during transmembrane proton transport have also been reported.[13]

We thought it of interest to synthesize a pH-sensitive imidazolidine carbodiimide derivative, N-cyclohexyl-N'-(1-oxyl-2,2,3,5,5-pentamethylimidazolidine-4-yl)-methylcarbodiimide (**1**) to investigate the environment of the DCCD reactive Asp61 residue of the ATP synthase subunit c protein. Such a compound might also be of interest in investigations of other carbodiimide-reactive proteins, including other proton-pumps such as cytochrome c oxidase,[1,14] and the cytochrome bc_1- and cytochrome bf complexes.[15,16] In this paper we present the chemical synthesis of **1** that was completed with good overall yield as well as initial biochemical studies of the effects of reaction of **1** with the *E. coli* ATP synthase.

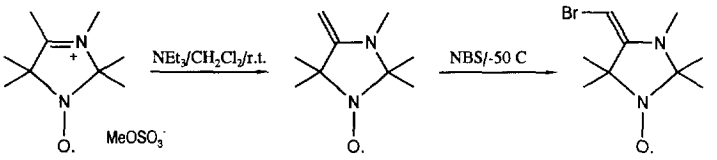


1

Results and Discussion

Synthesis of N-Cyclohexyl-N'-(1-oxyl-2,2,3,5,5-pentamethylimidazolidine-4-yl)-methylcarbodiimide (1)

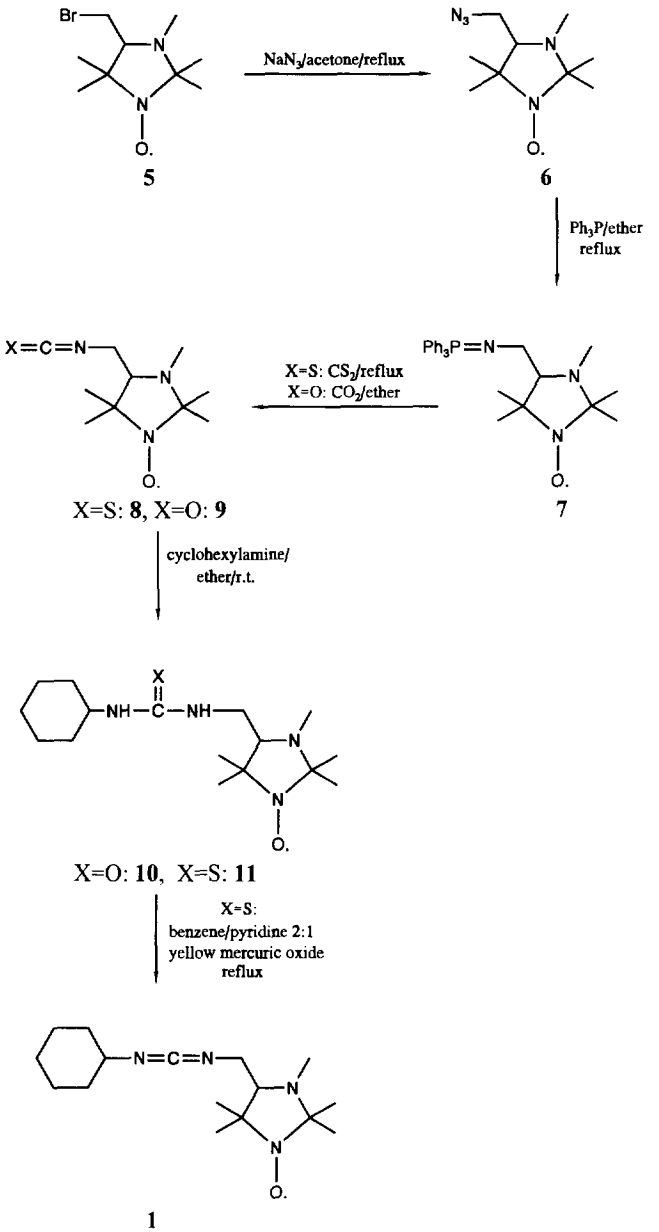
Compound **1** was obtained in a 14-step synthesis starting with 2-methyl-2-butene. Compound **2** was synthesized as described by Volodarsky and coworkers.[11,17] The published procedures for the synthesis of **4** used either dioxane dibromide in ether[18] or bromine in ether[11] to brominate the enamine **3**. We found that the reaction of **3** with N-bromosuccinimide[19-21] (NBS) in methylene chloride at low temperature in analogy to ref. 21 was a convenient alternative. The monobromoenamine **4** was obtained from **2** in a one-pot reaction. The resulting compound **4** was reduced to **5** as previously described.[11,18]



2 **3** **4**

To introduce the needed exocyclic N-functional group, bromine was substituted with azide by refluxing **5** in a solution of sodium azide in acetone/water to form **6**. Staudinger's syntheses of iminophosphoranes by the reaction of triphenylphosphine with organic azides[22,23] have been shown to be suitable for various types of nitroxyls.[24] The iminophosphorane **7** was synthesized by heating **6** with equimolar amounts of triphenylphosphine in absolute ether. Heating **7** in carbon disulfide gave high yields of isothiocyanate **8**. Alternatively, when a stream of dry CO_2 was passed into a suspension of **7** in dry ether, the isocyanate **9** was formed. Compound **9** was not isolated in pure form. Both **8** and **9** reacted with cyclohexylamine to give the

respective urea- **10** and thiourea- **11** derivatives. As described in references,[5,6,24] the desulfuration of **11** could be accomplished with yellow mercuric oxide in dry benzene/pyridine (2:1) to yield the carbodiimide **1**:



Reaction of 1 with the Membrane-Bound F_0 Sector of the ATP Synthase

In order to test the specificity of reaction of **1** with the ATP synthase F_0 sector, two types of biochemical assays were used. In the first experiments, the inhibition by **1** of passive proton translocation through F_0 was tested. F_1-depleted *Escherichia coli* cell membrane vesicles were prepared as described[25] and reacted for one hour at 30° C with 200 μM DCCD or **1**. Transmembrane proton gradient formation was observed indirectly using acridine orange fluorescence quenching assays as described.[26] Table 1 shows the effects of carbodiimide reaction on passive proton translocation through the F_0 sector. The F_1-depleted, F_0-containing membrane vesicles used here catalyze the rapid, passive translocation of protons through the membrane. Oxidation of NADH and concomitant proton-pumping by the respiratory chain contained within these membranes was predicted to result in the generation of a pH gradient across the membrane only when F_0-catalyzed passive proton translocation was inhibited. In the untreated sample shown in Table 1, no proton gradient formation was observed as expected, since protons could be freely translocated back through the membrane through the F_0 proton pores. In contrast to this result, the reaction of the F_1-depleted membrane vesicles with **1** or DCCD inhibited the translocation of protons through F_0 and allowed the generation of a net proton gradient upon NADH oxidation. These results demonstrate that **1** reacted specifically with the F_0 sector of the ATP synthase and inhibited passive proton flux through F_0 in a manner similar to that of DCCD.

Table 1. Effects of Carbodiimide Reaction on Proton Translocation through F_0

Carbodiimide[1]	NADH-Driven Acridine Orange Fluorescence Quenching (%)[2]
none	0
DCCD	100
1	68

[1] The carbodiimides were added from stock solutions made with acetonitrile. Acetonitrile alone had no effect. Results are averaged values from independent duplicate experiments.

[2] The degree of quenching of the DCCD-treated membrane sample was set to 100%.

Table 2. Effects of Carbodiimide Reaction on Proton Translocation through the ATP Synthase

Carbodiimide	ATP-Driven Acridine Orange Fluorescence Quenching (%)[1]
none	100
DCCD	0
1	44

[1] Quenching of the non-treated sample was arbitrarily set to 100%. The results presented are from averaged independent duplicate experiments.

In order to test whether active proton transport through the intact F_1F_0-ATP synthase was also inhibited by reaction with **1**, F_1-stripped membranes were treated with **1** or DCCD as above. Before addition of the

treated membranes to the acridine orange test, purified F_1-ATPase was reconstituted to the F_o sectors.[26] In these experiments, reaction of the carbodiimides with F_o is expected to inhibit ATP-driven proton transport catalyzed by the ATP synthase. Table 2 clearly shows that reaction of F_o with **1** or DCCD specifically inhibited ATP-driven active proton transport through the ATP synthase. As expected, NADH-driven proton transport catalyzed by the respiratory chain was not inhibited in these samples (data not shown).

The Effect of pH on the ESR-Spectral Characteristics of the N-Cyclohexyl-N'-(1-oxyl-2,2,3,5,5-pentamethyl-imidazolidine-4-yl)-methylurea (10)

As described previously by Khramtsov and Weiner,[29] the protonation of the N^3-nitrogen of several imidazolidine nitroxyl compounds was observable by ESR spectroscopic methods as the superposition of two triplet ESR spectra with different A and g-factor tensors (one derived from protonated imidazolidine and one from the unprotonated form). When we attempted to measure the effect of protonation of the imidazolidine ring of **1** in ESR experiments, we observed in aqueous solutions at low pH a decomposition of **1** including the loss of free radical signal by an as yet unknown mechanism. It was therefore not possible to study the effects of protonation of **1** directly. In other experiments, ESR spectra recorded on samples of membrane bound F_o that were previously reacted with **1** under the conditions given in Tables 1 and 2 showed no indication of such decomposition, suggesting that after reaction with F_o, the presumptive N-acylurea derivative[1,3] of **1** became stable. These results suggested that it would be of interest to synthesize the urea-derivative of **1** (compound **10**) as an analogous compound to the expected product of reaction of **1** and the F_o protein, so that the effects on the ESR spectra of protonation of the imidazolidine ring could be measured.

Compound **10** was found to be stable in the pH range tested (pH 7 to 1) and showed pH-dependent changes in its ESR spectral properties. The effect of protonation of **10** was observed as a splitting of the high field line in the X-band ESR spectra into two resolved components. The hyperfine coupling constant A-tensor (a_N) of the fully unprotonated form of **10** at pH 7 was observed to be 15.8 Gauss and a_N of the fully protonated **10** at pH 1 was 14.6 Gauss. This is in good agreement with the values for various other imidazolidine nitroxyl radicals evaluated previously.[29] As related additional indicators of these protonation effects, we observed a change in the high field to middle field peak height ratio (H_F/M_F) of **10** as the pH was changed. The H_F/M_F of 0.80 measured at pH 7 (10 mM phosphate) was found to substantially decrease to a value of 0.65 when the pH was lowered to 2 (also in 10 mM phosphate) and then to increase back to a H_F/M_F value of 0.81 when the pH was further decreased to pH 1 (10 mM phosphate). These pH-dependent effects on the H_F/M_F ratios are due to the superposition of the spectra of the protonated and unprotonated forms of **10** that display different A- and g-factor tensors and the resulting peak broadening of the high field signal. These effects of pH on the ESR spectral properties of the urea derivative **10** strongly support the contention that **1** after reaction with a protein

carboxyl residue to the expected N-acylurea will be a potentially useful pH-sensitive ESR probe for enzymes such as the ATP synthase.

CONCLUSIONS

In the first part of this paper, we presented the synthesis of a pH-sensitive spin-labeled affinity probe, **1**. Results of biochemical tests of the inhibition of passive proton translocation through F_o and active proton transport by the ATP synthase after reaction of **1** with the F_o sector of the ATP synthase presented in the second part of this paper, indicate that **1** specifically reacts with the ATP synthase. In the last experimental section of this paper we reported the pH-dependent changes we observed in an analog of the likely reaction product between **1** and a protein carboxyl group, the cyclohexylimidazolidine methylurea **10**. These results taken together suggest that **1** may indeed be a potentially useful pH-sensitive spin label for investigating the ATP synthase and perhaps other proton-pumping enzymes. Preliminary ESR studies of the ATP synthase modified by **1** are currently underway.

EXPERIMENTAL METHODS

General Methods

All chemicals used were of the highest quality available. If needed, solvents were dried and purified according to standard methods.[28] Column chromatography was performed with ICN SiliTech (0.063-0.2 mm) silica gel. TLC was performed on Macherey-Nagel Polygram SIL G/UV_{254} precoated silica gel plates (8 x 4 x 0.025 cm). Melting points were determined using a copper block; the values were not corrected. IR spectroscopy: The spectra were recorded using Perkin-Elmer 881 infrared spectrometers or a Bruker FT-IR IFS 48 spectrometer. Spectra were recorded on samples that were prepared in potassium bromide, as films or in solution. Mass spectra: Spectra were determined using a Finnigan MAT 90 mass spectrometer. Elementary analyses: All C,H,N analyses were performed on a Perkin-Elmer Analyser 240. ESR spectroscopy: All spectra were recorded using a Bruker ESP 300 E spectrometer operating in the X-band mode with 100 kHz modulation frequency and 1.0 G modulation amplitude using a TE_{102} resonator. The ESR spectra of the samples (except for **4** and **9**) were recorded in aqueous solutions. The concentrations of the radicals were 10^{-4} M. All samples exhibited ESR spectra typical for ^{14}N-nitroxyl radicals with hyperfine splitting of 15.8 G at pH 7. Fluorescence was measured with a Perkin-Elmer LS-5 luminescence spectrometer.

4-Bromomethylene-2,2,3,5,5-pentamethylimidazolidine-1-oxyl (4)

7.00 g (24.9 mmol) of **2** was suspended in 250 ml dry methylene chloride. Upon slow addition of dry triethylamine (15 ml), a yellow solution of **3** was obtained. This solution was cooled to -50°C whereupon 4.40 g (24.9 mmol) N-bromosuccinimide in 250 ml dry methylene chloride was added dropwise over a period of 1 h. The mixture was then stirred for an additional hour. During that time, the temperature was allowed to rise to room temperature. The solvent was then evaporated in vacuo and the residue was subsequently extracted with five 10 ml portions of hexane. The combined extracts were kept overnight at -20°C. The precipitate was filtered and dried in vacuo to yield 4.50 g (73%) of bright-orange colored crystals (from hexane) that decomposed at 72-74° C without melting (Lit[11,18]: m.p. 87-89°C). $C_9H_{16}BrN_2O$ calc (%): C 43.57; H 6.50; N 11.28. (248.13) found: C 43.41; H 6.36; N 11.32 %. IR (KBr): $\nu = 1616\ cm^{-1}$ (C=C) (Lit.[11,18]: $\nu = 1620\ cm^{-1}$). Mass spectrum (EI): m/e (relative intensity) 247, 249 (both M^+, 1% each).

4-Bromomethyl-2,2,3,5,5-pentamethylimidazolidine-1-oxyl (5)

Compound **5** was prepared from **4** exactly as described.[11,18]

4-Azidomethyl-2,2,3,5,5-pentamethylimidazolidine-1-oxyl (6)

1.00 g (4 mmol) of **5** and 0.52 g (8 mmol) sodium azide were dissolved in a mixture of 20 ml of acetone and 2 ml of water. This solution was refluxed for 4 h. Upon cooling, the acetone was removed in vacuo. 10 ml of water was then added to the residue. The water phase was extracted 3 times with ether and the combined ether extracts were dried over $MgSO_4$. The ether was evaporated and the residue was purified by chromatography on a silica gel column eluted with an ether/hexane 1:1 mixture. Yield: 0.55 g (65%), yellow crystals. The sample used for analysis was crystallized from hexane at -20°C; m.p. 35-36°C. $C_9H_{18}N_5O$ calc (%): C 50.93; H 8.55; N 32.98. (212.24) found: C 51.06; H 8.34; N 33.31 %. IR (CCl_4): $\nu = 2101\ cm^{-1}$ (N_3). Mass spectrum (CI): m/e (relative intensity) 212 (M+, 100%). ESR (in 10 mM phosphate, pH 7): $H_F/M_F = 0.90$; linewidth = 1.41 Gauss.

4-Triphenylphosphiniminomethyl-2,2,3,5,5-pentamethylimidazolidine-1-oxyl (7)

0.58 g (2.73 mmol) of **6** was dissolved in 5 ml dry ether. 0.72 g (2.73 mmol) triphenylphosphine was added to this solution and the mixture was refluxed for 1 h. The precipitate was filtered off and washed with dry ether until no more triphenylphosphine was found in the ether phase (as controlled by thin-layer

chromatography). The solid was dried in vacuo yielding 0.88 g (65%) of a pale yellow powder which was found to be analytically pure; m.p. 166-168°C. $C_{27}H_{33}N_3OP$ calc. (%): C 72.62; H 7.45; N 9.41. (446.56) found: C 72.66; H 7.35; N 9.40 %. IR (KBr): ν = 1170 cm^{-1} (N=P). Mass spectrum (EI): m/e (relative intensity) 446 (M^+, 5%). ESR (in 10 mM phosphate, pH 7): H_F/M_F = 0.76; linewidth = 1.52 Gauss.

4-Isothiocyanatomethyl-2,2,3,5,5-pentamethylimidazolidine-1-oxyl (8)

Carbon disulfide (5 ml) was added to 2.30 g (5.15 mmol) of **7** and the mixture was refluxed for 90 min. The carbon disulfide was then evaporated in vacuo and the residue was extracted with 10 ml of ether in order to separate most of the triphenylphosphine sulfide. The ether was then removed in vacuo. The residue was dissolved in chloroform and chromatographed on silica gel using chloroform as eluent. Yield: 1.04 g (92%) of an orange colored oil that slowly crystallized at -20°C. The sample proved to be analytically pure; m.p. 40-41°C. $C_{10}H_{18}N_3OS$ calc (%): C 52.62; H 7.95; N 18.40. (228.25) found: C 52.73; H 7.75; N 18.31 %. IR (KBr): ν = 2170, 2090 cm^{-1} (N=C=S). Mass spectrum (EI): m/e (relative intensity) 228 (M^+, 14%). ESR (in 10 mM phosphate, pH 7): H_F/M_F = 0.90; linewidth = 1.39 Gauss.

4-Isocyanatomethyl-2,2,3,5,5-pentamethylimidazolidine-1-oxyl (9)

In an argon atmosphere, 0.45 g (1 mmol) of **7** was suspended in 10 ml of dry ether. A stream of dry CO_2 was then passed into the suspension for about 90 min. At the end of the 90 minute period, the ether was evaporated and 5 ml of dry pentane was added to the residue. The precipitated triphenylphosphine oxide was removed using a glass filter. Evaporation of the filtrate yielded 0.17 g of the crude isocyanate radical, m.p. 53-55, which was immediately used in the next reaction. IR (CCl_4): ν = 2260 cm^{-1} (N=C=O); Mass spectrum (EI): m/e (relative intensity) 212 (M^+, 11%).

N-Cyclohexyl-N'-(1-oxyl-2,2,3,5,5-pentamethylimidazolidine-4-yl)-methylurea (10)

28 mg of crude **9** was dissolved in 1 ml of dry ether. To this solution 99 mg (1 mmol) of cyclohexylamine was added. The urea **10** immediately began to precipitate. The reaction mixture was stirred at ambient temperature overnight. The urea was suction-filtered, washed twice with 1 ml of dry pentane and carefully dried in vacuo. Yield: 32 mg (74%, based on crude **9**) of a pale yellow powder, m.p. 194-195°C (dec.), was obtained that was found to be analytically pure. $C_{16}H_{31}N_4O_2$ calc (%): C 61.71; H 10.03; N 17.98. (311.42) found: C 61.80; H 10.03; N 17.77 %. IR (KBr): ν = 3338 cm^{-1} (NH), 1623, 1590 cm^{-1}

(CONH). Mass spectrum (EI): m/e (relative intensity) 311 (M^+, 2%). ESR (in 10 mM phosphate, pH 7): H_F/M_F = 0.80; linewidth = 1.47 Gauss; (in 10 mM phosphate, pH 1): H_F/M_F = 0.81; linewidth = 1.33 Gauss.

N-Cyclohexyl-N'-(1-oxyl-2,2,3,5,5-pentamethylimidazolidine-4-yl)-methylthiourea (11)

228 mg (1 mmol) of **8** was dissolved in 1 ml dry ether and 99 mg (1 mmol) cyclohexylamine in 1 ml dry ether was added. The solution was stirred for 18 h at room temperature after which the precipitation of the thiourea was complete. The yellow solid was filtered off, washed with several portions of pentane and dried in vacuo to give 311 mg (96%) of a yellow powder; m.p. 111°C. $C_{16}H_{31}N_4OS$ calc (%): C 58.68; H 9.54; N 17.10. (327.42) found: C 58.53; H 9.62; N 17.24 %. IR (KBr): ν = 3295 cm^{-1} (NH). Mass spectrum (EI): m/e (relative intensity) 327 (M^+, 13%). ESR (in 10 mM phosphate, pH 7): H_F/M_F = 0.79; linewidth = 1.48 Gauss.

N-Cyclohexyl-N'-(1-oxyl-2,2,3,5,5-pentamethylimidazolidine-4-yl)-methylcarbodiimide (1)

800 mg (3.7 mmol) yellow mercuric oxide was added to 164 mg (0.5 mmol) of **11** in 10 ml dry benzene/pyridine 2:1. The mixture was refluxed for 1 h. Upon cooling, the residue was removed by filtration and dried in vacuo. The resultant oil was chromatographed on silica gel using a mixture of dry chloroform/acetonitrile 9:1 as eluent. Yield: 109 mg (74%) of **1** was obtained as an analytically pure yellow oil. $C_{16}H_{29}N_4O$ calc (%): C 65.50; H 9.96; N 19.09. (293.41) found: C 65.21; H 10.08; N 18.98 %. IR (film): ν = 2127 cm^{-1} (N=C=N). Mass spectrum (EI): m/e (relative intensity) 293 (M^+, 6%). ESR (in 10 mM phosphate, pH 7): H_F/M_F = 0.85; linewidth = 1.43 Gauss.

Biochemical Methods

The growth of *Escherichia coli* K12 strain SWM1[27] and the preparation of F_1-depleted membrane vesicles was performed as described.[25] Indirect measurement of transmembrane proton gradient formation was performed by the acridine orange fluorescence quenching assays as described.[26]

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