Biodegradation **14:** 65–72, 2003.

Heteroaromatic monothiocarboxylic acids from *Pseudomonas* spp.

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Accepted 3 January 2003

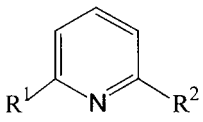
Key words: bacterial metabolites, Pseudomonas, thiocarboxylic acids

Abstract

Pyridine derivatives substituted with monothiocarboxylic acid groups are the unique metabolites of certain *Pseudomonas* species. Pyridine-2,6-di-(monothiocarboxylic acid) **1a** was found during a screening program for antibiotically active bacterial metabolites due to its ability to complex Fe^{3+}. The structure of this complex, its redox behavior and the biogenesis of the ligand molecule were studied in detail. This lead to the discovery of a new class of natural products, viz. acylsulfenic acid derivatives. Interest in **1a** was revived shortly when complexes with other metals were studied as models for sulfur-containing enzymes. It could also be shown that a quinoline monothiocarboxylic acid derivative acted as an alternative siderophore for *Pseudomonas fluorescens*. But a real renaissance was observed only when the role of **1a** in the degradation of CCl_4 by *Pseudomonas stutzeri* became evident.

Introduction

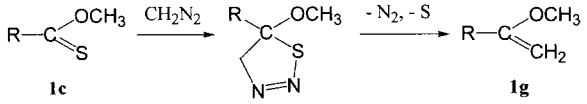
In 1978 it was observed that the culture medium of a bacterial strain later on identified as *Pseudomonas putida* turned dark blue upon addition of a ferric citrate solution (Ockels et al. 1978). This started an investigation which led to the discovery of a new class of natural products and some interesting chemistry with partially unexpected results. The compound giving the blue iron complex was identified as pyridine-2,6-di-(monothiocarboxylic acid) (**1a**). The metal complexes of **1a** were studied for a wile as models for sulfur-containing enzymes, but then the interest faded, and it was revived only recently in context with studies on the degradation of CCl_4 by *Pseudomonas stutzeri* (Lee et al. 1999; Sepúlveda-Torres 2001; Lewis et al. 2000; Cortese et al. 2002).



	R^1	R^2
1a	COSH	COSH
1b:	$COSCH_3$	$COSCH_3$
1c	$COSCH_3$	$CSOCH_3$
1d	$COSCH_3$	$COOCH_3$
1e	$COSCH_3$	$CONH_2$
1f	$COSCH_3$	$COCH_3$
1g	$COSCH_3$	$C(OCH_3)=CH_2$
1h	COCl	COCl
1i	COOH	COOH
1j	COOH	COSH

Pyridine-2,6-di-(monothiocarboxylic acid)

The blue compound obtained from the bacterial culture medium turned brown in the presence of air and could be transformed again into its blue form by addition of $Na_2S_2O_4$. After its isolation by chromatography it resisted structure elucidation by the various spectroscopic methods. However treatment of a methanolic solution with diazomethane resulted *i.a.* in a mixture of products (cf. below) from which gas chromatographically pyridine-2,6-di-(thiocarboxylic acid)



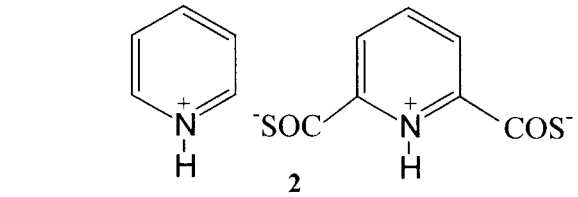
Scheme 1. Reaction of thiocarboxylate esters with diazomethane.

di-S-methyl ester **1b** could be obtained. The structure of this derivative was established from its spectroscopic characteristics (mass spectrometry and NMR) and confirmed by synthesis (see below). Also the free acid was obtained synthetically and it could be transformed into the original iron complex (Ockels et al. 1978).

Pyridine-2,6-di-(monothiocarboxylic acid) **1a** is produced mainly during the exponential phase of bacterial growth. Optimal conditions are ca 25 °C, a pH of about 8 (phosphate buffer) and sufficient aeration. It can be obtained by acidification of the cultural broth and chromatographic work-up, its dimethyl ester **1b** by treatment of an isopropanol extract with diazomethane. Gas chromatographic analysis of the extract demonstrated the presence of a number of related compounds (**1c–1g**) (Budzikiewicz et al. 1983). Use of CD_2N_2 showed that esters were formed during the treatment with diazomethane and not originally present. Formation of the methylene compound **1g** has been explained (Beiner et al. 1973) by addition of CH_2N_2 to the C=S-group of an O-methyl ester with subsequent elimination of N_2 and S (Scheme 1). The ketone **1f** can then be formed by cleavage of the enol ether **1g**. Also here the deuterated analogs are observed when CD_2N_2 is used. **1a** is partially hydrolized giving (**1j** and **1i**) (Cortese et al. 2002). In addition, oxidative formation of disulfide bridges (2 – CO–SH → –CO–S–S–CO–) has been observed with all monothioacid derivatives discussed in this review.

For practical purposes it is easier to synthesize **1a** by treating pyridine-2,6-dicarboxylic acid chloride (**1h**) with H_2S in dry pyridine. In this way the orange 1:1 adduct of **1a** and pyridine is formed (**2**) which can be decomposed with acid; **1a** can then be extracted with CH_2Cl_2 (white crystals, mp 97–99°) (Hildebrand et al. 1983). For biogenetic studies also the synthesis of [3–^{2}H]– (**4**) and of [4–^{2}H]– analogs was developed (Hildebrand et al. 1984b). The mass spectrum of **1a** obtained by electron ionization (EI) is straightforward. M^+. (*m/z* 199) shows losses of ·SH, COS (100%), ·COSH and further decomposition products of these ions. Collision induced fragmentation after electrospray of $[M–H]^-$ results in the loss of twice COS (*m/z* 138 and 78) (Hildebrand et al. 1983; Lee et al. 1999). The EI spectra of pyridine carboxylic acid and thiocarboxylic acid methyl esters have been studied in detail (Budzikiewicz et al. 1981). Especially –$COSCH_3$ groups tend to show extensive rearrangement reactions. They can however be readily distinguished from the –$CSOCH_3$ isomers which show abundant fragments due to the loss of CH_2O (*ortho*) and CH_3O· (*meta* and *para*).

The protonation constants (pK) of **1a** were determined as 5.48, 2.58 and (estimated) 1.3 (Stolworthy et al. 2001).



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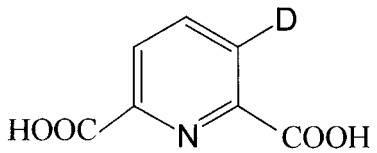

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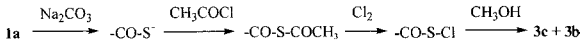
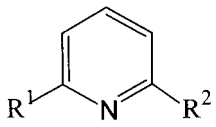
(Alkoxythio)carbonyl derivatives of pyridine (acylsulfenic acid esters)

Chromatographic work-up of the culture broth of several *Pseudomonas putida* strains after methylation yielded in addition to **1b** and the various artefacts mentioned above compounds which contained the hitherto not described –(C=O)–S–OCH_3 group. From the fact that by treatment with diazoethane the corresponding ethoxy compounds are obtained it can be concluded that in culture the free acids are formed. The main component is **3a** with one (CO)–S–OCH_3 and one CO–SCH_3 group, accompanied by variants with one CO–OCH_3 group (**3b**) and with two (CO)–S–OCH_3 groups (**3c**). The presence of this novel grouping in **3a** was deduced from spectral data. Fragmentation after EI showed losses of ·OCH_3, ·$SOCH_3$ and ·$COSOCH_3$ from M^{+}·, an NMR signal at 3.87 ppm demonstrated the presence of an OCH_3 group, and IR showed two carbonyl bands (Hildebrand et al. 1985a, b). The (Alkoxythio)carbonyl structure was confirmed by synthesis (Hildebrand et al. 1986) according to Scheme 2. In addition to **3c** some **3b** is formed due a competing



Scheme 2. Synthesis of acylsulfenic acid esters.

nucleophilic attack of CH_3OH at the carbonyl group. **3c** and **3b** can be separated by chromatography.

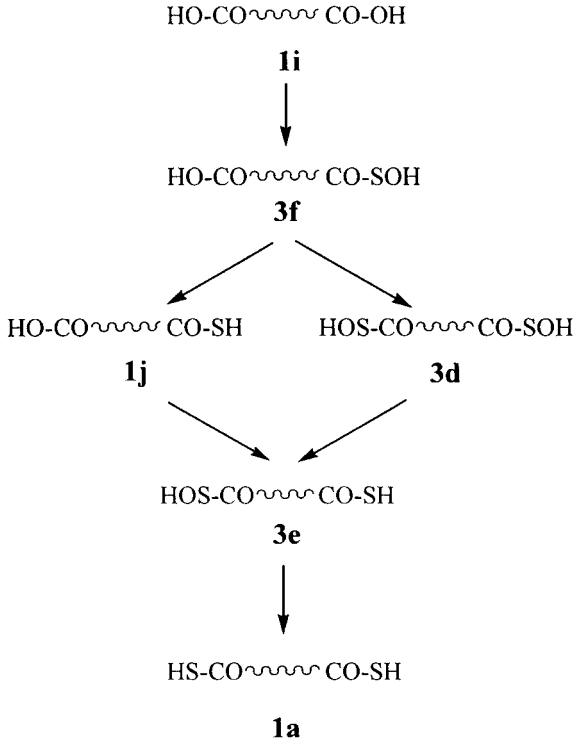


	R^1	R^2
3a	$CO\text{-}SCH_3$	$CO\text{-}SOCH_3$
3b	$CO\text{-}OCH_3$	$CO\text{-}SOCH_3$
3c	$CO\text{-}SOCH_3$	$CO\text{-}SOCH_3$
3d	CO-SOH	CO-SOH
3e	CO-SOH	CO-SH
3f	CO-SOH	CO-OH

Regarding the biosynthesis of pyridine-2,6-di-(monothiocarboxylic acid) **1a** some questions are still open. From the strain KC of *Pseudomonas stutzeri* several involved regulatory elements and genes could be identified (Lewis et al. 2000; Sepúlveda-Torres et al. 2002; Cortese et al. 2002) which, however, were not found in *Pseudomonas putida* DSMZ 3601, another producer of **1a**. It is suggested (Cortese et al. 2002) that *Pseudomonas stutzeri* may have acquired at least one of the genes by lateral transfer from mycobacteria. In a proposed biogenetic sequence (Sepúlveda-Torres et al. 2002) pyridine-2,6-dicarboxylic acid **1i**, a known bacterial metabolite (Gross 1970), is activated as its bis-adenosine monophosphate (AMP) derivative. The sulfur donor and its activation remained open.

Feeding experiments with the 3-deutero analog **4** (Hildebrand et al. 1984b) of **1a** could provide an answer and allow to suggest a new mechanism. **4** was added to the culture medium and the deuterium content of the various sulfur containing metabolites was determined by mass spectrometric analysis of the respective methyl esters. Since additional unlabelled **1i** is produced and metabolized during the bacterial growth, the deuterium content of any metabolite of **1i** should be the lower the farther down in the metabolic chain it is located. As can be seen from Scheme 3 the acylsulfenic acid species lie before the thioacids.

It is known e.g. from the fatty acid metabolism that –COOAMP can react with SH-groups (here possibly from cystein) to give a thioester (Michal 1999). The

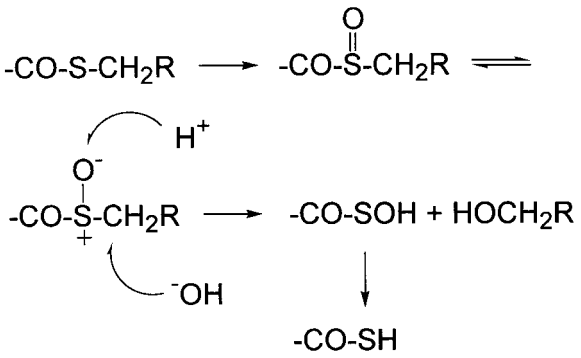


Scheme 3. Sequence of the metabolites of **1i** acccording to deuterium labelling studies.

usual hydrolytic cleavage results in the inversion of this reaction (attack of OH^- at the positive carbonyl-C and of H^+ at the sulfur) giving –COOH and HSR. Preceding oxidation to the S-oxide (cf. the oxidation of threonine to its sulfoxide (Brot & Weissbach 1991; Vogt 1995; Or-Rashid et al. 2001)) would allow an attack of H^+ at the sulfoxide oxygen and of OH^- at the CH_2 neighboring the positive sulfur (see Scheme 4). The cleavage products would then be an acylsulfenic acid and a hydroxy compound (serine from cystein). The last step would involve the reduction of the acylsulfenic to the thiocarboylic acid.

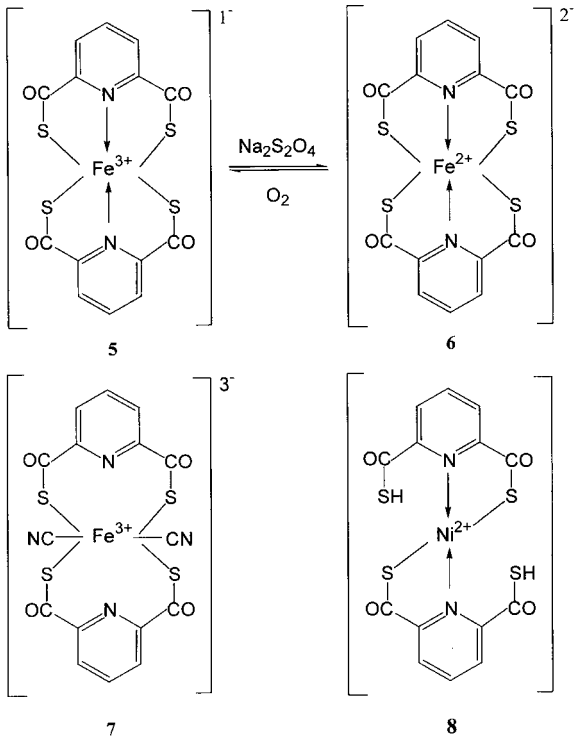
Metal complexes of pyridine-2,6-di-(monothiocarboxylic acid)

The complexes of Fe^{3+}, Co^{3+}, Ni^{3+} and Ni^{2+} with pyridine-2,6-di-(monothiocarboxylic acid) were investigated by X-ray analysis (for Fe^{2+} see below) (Hildebrand & Lex 1989; Hildebrand et al. 1984a; Krüger & Holm 1990). The trivalent metals form 1:2 complexes with **1a**, having one negative charge, the complexes of divalent metals having two negat-



Scheme 4. Proposed formation of –CO–SH from –CO–OH via –CO–SOH.

ive charges (see, e.g., **5** and **6**). The four sulfur and the two nitrogen atoms are the corner points of a slightly distorted octahedron and the two ligands are in a perpendicular position to each other and essentially planar. Deviations from the ideal geometry are due to orbital configurations of the central ion and differences in metal-sulfur and metal-nitrogen bond distances; interactions with the cations, e.g. by hydrogen bonds may play an additional role. The iron (both Fe^{3+}, **7**, and Fe^{2+}) complexes may also carry two additional cyanide ligands (Hildebrand et al. 1985c).



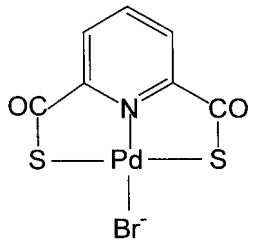
For some of the complexes ^{1}H-NMR data are available. They allow to say something about the orbital populations of the central metal ion (see Table 1). Low spin (according to Mössbauer data) d^5-Fe^{3+} is paramagnetic and consequently the pyridine protons are shifted downfield and broadened. The influence of the metal ion hardly reaches the pyridinium cation; its proton signals are only slightly broadened. Fe^{2+} and Co^{3+} are d_6-systems where the low-lying three d-orbitals are fully occupied. They are diamagnetic. In the Fe^{2+} in contrast to the Co^{3+} complex the β- and γ-signals of the pyridine ring almost coincide. Ni^{2+} at least in solution is paramagnetic. From this a sp^3 hybridization with two singly occupied d orbitals was deduced which would result in a tetrahedral configuration with two free thiocarboxylate groups (**8**). This would be in agreement with the easy oxidative polimerization of the Ni^{2+} complex under formation of –CO–S–S–CO– bridges as it is observed for all compounds having free –COSH groups (Hildebrand & Lex 1989). On the other hand an X-ray analysis of the crystalline complex (Krüger & Holm 1990) had shown a strained octahedral structure which would also be compatible with the paramagnetism of the complex (d^8 with three doubly and two singly occupied orbitals). The comparatively long mean metal-sulfur distance (2.42 Å) of the Ni^{2+} complex as compared with those of the Ni^{3+} (2.28 Å), Co^{3+} (2.26 Å) and Fe^{3+} complexes (2.28 Å) could explain the oxidative formation of the –S–S– bridges in solution. For IR, UV and EPR data of the complexes discussed above the original publications should be consulted. The stability constants (log K) for the Fe^{3+}, Co^{3+} and Ni^{2+} complexes were determined as 33.36, 33.93 and 33.28; that for Cu^{2+} is in the same order of magnitude (Stolworthy et al. 2001).

Pd^{2+} apparently forms essentially planar 1:1 complexes with pyridine-2,6-di-(monothiocarboxylic acid) where the forth position can be occupied by an anion (**9**) or a neutral. Also dimeric structures (**10**) were discussed. Only some IR data in addition to the X-ray analysis of **9** are reported (Espinet et al. 1994). Pd^{2+} complexes of 4-O-alkyl and 4-S-alkyl derivatives of pyridine-2,6-di-(monothiocarboxylic acid) were investigated for their ability to form liquid crystals (Espinet et al. 1999).

Table 1. [1]H-Chemical shifts of the pyridine-carbothioato ligands of various metal complexes

Metal	β-H	β-H	Cation	Solvent	Reference[1]
Co^{3+}	7.95, t	8.33, d	$(C_2H_5)_4N^+$	DMSO-d_6	a, b
Fe^{3+}	15.5 broad		pyridinium	DMSO-d_6/D2O 1:1	c
Fe^{2+}	7.85 broad s				
Ni^{2+}	65.8	20.0	$(C_2H_5)_4N^+$	DMSO-d_6	b
	66.1 broad	18.6 broad			a

[1]Reference a: Hildebrand & Lex (1989), b: Krüger & Holm (1990), c: Hildebrand et al. (1984a).



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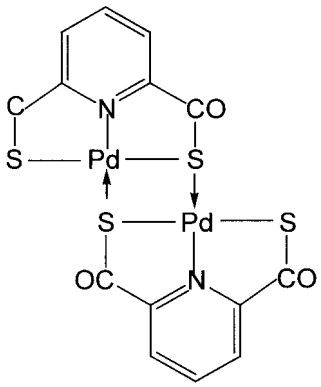

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Further metal complexes were obtained by addition of metal salts to a solution of **1a**. In some cases a color change was observed (Cd – light green, Cu – green, Fe – brown, Ni – green, Co – red, Pd – orange). Analysis by electrospray mass spectrometry and metal-to-ligand titration gave the following results: Mn^{2+}, Nd^{3+}, Sc^{3+} and UO_2^{2+} form 1:2 complexes as it had been shown for Co^{3+}, $Fe^{2+/3+}$ and $Ni^{2+/3+}$, while Au^{3+} and Cd^{2+} (as Pd^{2+} above) showed a 1:1 ratio; for Zn^{2+} both types were found (Sebat et al., 2001; Cortese et al. 2002).

Redox systems

The redox system **5** ⇆ **6** started the investigation of the pyridine-2,6-di-(monothiocarboxylic acid) systems. Originally it had been assumed that both the reduced and the oxidized complex contained Fe^{2+} as the central ion (Ockels et al. 1978). This assumption was based mainly on the interpretation of Mössbauer data (isomeric shift against metallic Fe + 0.224 mm/s, quadrupole splitting 2.36 mm/s at 300 K) which however could be shown later on as inconclusive regarding the valency state of low-spin complexes. Two alternative explanations were considered, viz. (a) 2 $-COS^-$ ⇆ –COS–SCO–, a process observed for the uncomplexed ligand and (b) pyridine ⇆ dihydropyridine; (a) would probably lead to polymeric structures as in the case of the Ni^{2+} complex (see above) and (b) could be excluded, since repeated reduction with $NaBD_4$ and re-oxidation did not result in an incorporation of D. The final proof for the oxidized form as an Fe^{3+} complex came from an X-ray analysis (Hildebrand et al. 1984a).

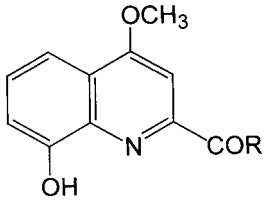
For several systems (the iron complexes **5** and **7**, the cobalt, copper and nickel complexes) the redox potentials were determined by cyclovoltametry. They lie in the range of metal enzymes.

Quinolobactin

8-hydroxy-4-methoxy-quinoline-2-monothiocarboxylic acid (**11a**) was isolated first from a *Pseudomonas fluorescens* strain, together with the carboxylic acid **11b** and its amide **11c**. As in the case of **1a** the latter two compounds are probably artifacts formed in the culture medium or during work-up (Neuenhaus et al. 1980); **11a** is readily hydrolyzed in aqueous solution. The disulfide oxidation product could also be isolated (unpublished). A synthesis of **11a** starts from 4,8-dihydroxyquiniline-2-carboxylic acid, exchange of

the 4-hydroxy group with chlorine by reaction with $POCl_3$ and subsequently with a methoxy group by reaction with CH_3ONa. Finally the carboxyl group is transformed to a thiocarboxyl group by reaction with H_2S and N,N′-carbonyldiimidazole (Neuenhaus et al. 1980).

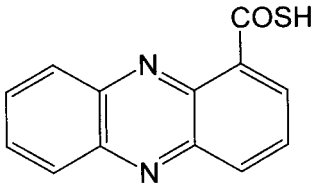
For many years **11a** was considered as "just another thioacid from a *Pseudomonas*". New interest in it arose when it could be shown the **11b** (named quinolobactin) could act as a siderophore in a mutant of *Pseudomonas fluorescens* ATCC 17400 which did not produce a pyoverdin, the main siderophore (Budzikiewicz 1997) of the wild type. It is probably taken up by a 75 kDa outer membrane receptor (Mossialos et al. 2000).



11a R = SH
11b R = OH
11c R = NH_2

Phenazine-1-monothiocarboxylic acid

Phenazine-1-carboxylic acid is one of the characteristic metabolites of many *Pseudomonas* strains (Budzikiewicz 1993). In view of the isolation of **1a** and **11a** it was of interest to search also for phenazine-1-monothiocarboxylic acid (**12a**). **12a** can readily be synthesized by treating the acid chloride with H_2S in an organic solvent. In this way a dark red compound is obtained which in aqueous solution is immediately hydrolyzed back to the carboxylic acid, and it is partially oxidized to the disulfide (Radermacher 1983). Thus, even if **12a** is formed by pseudomonads, it would hardly survive long enough for an isolation.



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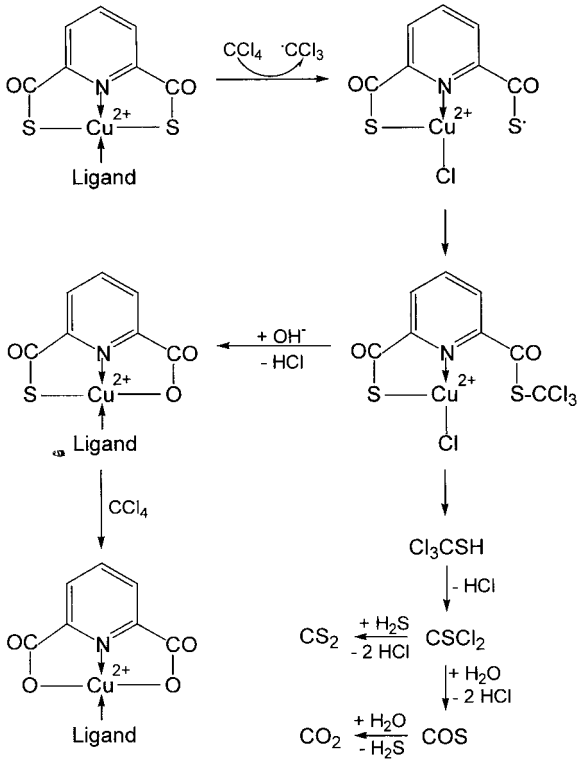
Pyridine-2,6-di-(monothiocarboxylic acid) in biodegradation

CCl_4 despite of its toxic and carcinogenic properties was used over the years as a non-inflammable solvent for e.g. degreasing, in fire extinguishers etc. Due to improper disposal it has become a contaminant of the soil and groundwater and together with other halogen compounds a threat to the ozone belt. In search for biodegrading microorganisms it was found that *Pseudomonas stutzeri* KC could transform CCl_4 into CO_2 (Criddle et al. 1990; Lewis & Crawford 1993; Dybas et al. 1995). By trapping studies $CSCl_2$ and $COCl_2$ were identified as intermediates (Lewis & Crawford 1995). Formation of $\cdot CCl_3$ radicals and of Cl^- was assumed to be the first step. $\cdot CCl_3$ could then react with O_2 or $RS\cdot$ giving $COCl_2$ and $CSCl_2$, while further (reductive) loss of Cl would lead to CCl_2 which could be hydrolyzed to HCOOH and finally oxidized to CO_2. In search for the active principle catalyzing the degradation of CCl_4 **1a** was isolated from cultures of *Pseudomonas stutzeri* KC and identified by mass spectrometry. *In vitro* experiments showed its activity in the presence of chemical reducing agents or actively metabolizing bacterial cultures otherwise not being able to degrade CCl_4 (Lee et al. 1999). It could be shown that the Cu^{2+} complex of **1a** is the active agent which by an one electron transfer starts the degradation chain (Scheme 5) (Lewis et al. 2001).



Scheme 5. Degradation of CCl_4 catalyzed by the Cu complex of **1a**.

Iron transport into the bacterial cells

Soil bacteria must make available Fe^{3+} bound in minerals mainly of the type of oxide hydrates. The prevalent idea has been that they bind whatever free Fe^{3+} is available and thus disturb the equilibrium between dissolved and bound Fe^{3+}. Additional amounts go into solution. They are removed again by the siderophores, and so on, resulting in weathering of minerals (Hersman et al. 1995). More recently it has been shown that reduction to Fe^{2+} plays an additional role for the iron supply of these microorganisms (Hersman et al. 1996, 2000). The action of extracellular iron reductases (Vartivarian & Cowart 1999) as well as that of low molecular weight reducing agents such as pyridine-2,6-di-(monothio)dicarboxylic acid (Hersman et al. 2000) has been invoked. It now could be shown that the Cu^{2+} complex mediates the Fe^{3+} reduction (Cortese et al. 2002).

Fe^{2+} salts are sufficiently soluble for an adequate iron supply. An open question is the transport into the cell. Concomitant siderophore formation with iron reduction is reported for *Pseudomonas mendocina* (Hersman et al. 1996, 2000), but no structural proposals are available. An answer at least for the fluorescent pseudomonads could also be the observation (Xiao & Kisaalita 1998) that various pyoverdins (Budzikiewicz 1997) can bind Fe^{2+} and induce its oxidization to Fe^{3+}. The Fe^{3+} complex is then transported into the bacterial cell. This aspect of the iron transport needs further investigation.

Conclusions

The importance of the monothiocarboxylic acid systems of pyridine and quinoline of pseudomonads has apparently been overlooked for many years. There were only hints in the literature that the redox systems of their iron complexes might play some role in the cell metabolism. Research has been concerned mainly with siderophores and antibiotically active metabolites and their importance in human health effects and on agriculture. Only recently it became evident that they could be of crucial importance in alternative ways of iron acquisition and in the detoxification of the bacterial habitat. It is to be hoped that these leads will be followed up in future.

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