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Carbon Tetrachloride Dechlorination and Metal Chelating Properties of Pyridine-2,6-bis(thiocarboxylic acid) Produced from *Pseudomonas stutzeri* Strain KC

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INTRODUCTION

Carbon tetrachloride (CCl_4) is a toxic, carcinogenic compound that has been used as a solvent and degreaser for various purposes, including the manufacture of nuclear weapons (1). Due to improper disposal, CCl_4 is present at many sites as a contaminant of groundwater, posing a potential threat to ecosystems and to human health. Biological transformations of CCl_4 have been studied for many years, both to understand the toxicology of CCl_4 and to identify methods for its destruction in the environment. It is not surprising that the types of transformation identified include reduction of CCl_4 as an initial step; CCl_4 is an oxidized species, the carbon atom having the same formal oxidation state as CO_2. Biological mediators of the reductive transformation of CCl_4 include the tetrapyrroles heme, factor F_{430}, and cobalamins (2-5). Products arising from reactions of CCl_4 and these cofactors can be explained by the various fates of the trichloromethyl radical ($\cdot CCl_3$) generated by one-eletron reduction of CCl_4, the product spectra depending largely on the chemical reductants used to regenerate the reduced form of the cofactor and their reactivity with trichloromethyl radical (6-8). The iron-limited cultures of *Pseudomonas stutzeri* strain KC transform CCl_4 to CO_2 and non-volatile products, while producing only trace amounts of $CHCl_3$ and showing no activity toward $CHCl_3$, chlorinated ethanes, or fluorotrichloromethane (9, 10). We have purified compounds whose CCl_4 transformation activity is consistent with that of live cultures of strain KC with regard to its substrate range and the physiological conditions of its production. Analytical spectroscopy and comparisons with synthetic material have confirmed its structure as pyridine-2,6-bis(thiocarboxylic acid). Studies of the effects of transition metals on the CCl_4 dehalogenation activity of cultures of strain KC have indicated a positive role for copper, and negative roles for cobalt, iron, and possibly banadium (9, 10, 11). These studies did not resolve whether these effects were mediated through regulation of biosynthesis of the active agent of through direct interaction with it. The identification of PDTC and our ability to synthesize it in high purity have allowed us to study its reaction with CCl_4 in simple mixtures. We have used this simple system to examine the role of transition metals in PDTC-promoted CCl_4 transformation. We have also characterized the products of CCl_4 dechlorination reactions in order to resolve the type of mechanism leading to activation of the carbon-chlorine bond. These data allowed us elucidate the mechanism of transformation of CCl_4 by cultures of bacteria producing PDTC, and should be useful for prediction the outcome of remediation efforts using such bacteria.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Pseudomonas stuzeri strain KC was originally obtained from C. Criddle (Stanford University). The control strain incapable of CCl_4 dehalogenation was *P. stutzeri* ATCC 17588. DRM growth medium was used to cultivate strain KC for production of active compounds. The medium, which consists of (per liter) 6 g K_2HPO_4, 2 g Na acetate, 1 g NH_4Cl, and 0.5 g $NaNO_3$, was adjusted to pH 7.7-7.9 and sterilized by autoclaving. $MgSO_4$, $Ca(NO_3)_2$, and were added separately from sterile stock solutions to give 1mM, 0.1mM, and 5nM final concentrations, respectively. Actively growing, 1-liter DRM cultures grown in 2-liter Erlenmeyer flasks were used to inoculate large-volume cultures (20-L) grown in polyethylene carboys at ambient temperature (22-24°C) for 4 days with constant aeration by filtered (0.2-μm pore size) compressed air supplied through a 4-mm (i.d.) stainless steel tube inserted to the bottom of the vessel. ^{13}C- and ^{15}N-enriched growth substrates were purchased from Cambridge Isotope Laboratories (Andover, MA).

CCl_4 transformation assays

CCl_4 transformation assays were performed in 2 ml of 35 mM potassium phosphate buffer (KH_2PO_4/KOH, pH 7.7) prepared with glass-distilled deionized water and stored in an anaerobic chamber with 1 g of Chelex 100 chelating resin (Sigma Chemical Co., St. Louis, MO) per 100 ml. Reactions were carried out anaerobically in an anaerobic chamber (Forma Scientific, atmosphere N_2:H_2:CO_2, 85:10:5). Reaction mixtures (2 ml) were prepared in the anaerobic chamber in 20-ml-headspace autosampler vials. Hydrogen sulfide was from Aldrich (Milwaukee, WI). Sodium sulfide (Na_2Sx9H_2O) was from EM Science (Gibbstown, NJ). Titanium(III) citrate was prepared in the anaerobic chamber from 20% Ti(III)Cl_3/HCl solution (Fisher), trisodium citrate, and sodium carbonate to give a final pH of 7.7 and a final concentration of 0.5 M Ti. CCl_4 (Omnisolv, Merck) was added from a methanol stock solution (approx. 0.8%, vol/vol). $^{13}CCl_4$ was from Cambridge Isotope Laboratories (Andover, MA) and $^{14}CCl_4$ was from DuPont NEN, (Wilmington, DE). Additions of CCl_4 were made with a 25-μl gas-tight syringe (Hamilton, Reno), butyl rubber stopper (West Co., Phoenixville, PA) and an aluminum crimp seal. Reactions were incubated at 25°C in an inverted position.

Purification of active compounds

Bacterial cells were removed from large-scale cultures by filtration through a hollow fiber cartridge (H5P100-43, 100kDa cutoff) using an Amicon ultrafiltration system. The cell-free filtrate was adjusted to pH 2.0 with concentrated HCl and allowed to stand at 4°C overnight to allow formation of a precipitate. The precipitate was collected by centrifugation, (9400 × g, 20min, 4°C; Beckman JA-10 rotor). After freeze-drying, this preparation gave a yellow-white powder which was redissolved in 1% ammonium

hydroxide, and the remaining insoluble material was removed by filtration through a 30-mm-diameter, 0.45-μm (pore size) filter (Millex-25; Millipore, Bedford, MA). This solution was then applied to a 3 × 60 cm column of Bio-Gel P-2 (Bio-Rad Laboratories, Hercules, CA) previously equilibrated and then eluted with deionized water. Fractions were collected using a Frac-100 fraction collector (Pharmacia, Uppsala, Sweden). Those containing CCl_4 transformation activity were pooled and concentrated by freeze-drying. Purity was assessed by HPLC using a Columbus C_8 column (15 × 0.46 cm; Phenomenex, Torrance, CA) and 0.2% formic acid: acetonitrile (85:15) at a flow rate of 1 ml/min.

Synthesis of PDTC and its metal complexes

PDTC was synthesized by the method of Hildebrand et al.(12). Aqueous solutions of $PDTCH_2$ (5 mM) were prepared by dissolving in anaerobic 35 mM potassium phosphate buffer (pH 7.7) and filtration through 0.2-μ (pore size) membranes. The Cu-Cl and Cu-Br complexes were synthesized as the tetrabutylammonium salts, as described for the Pd-Br:PDTC complex (13). Elemental analysis of the Cu-Br complex gave(theoretical in parentheses): C 47.5% (47.37); H 6.62% (6.74); N 4.81% (4.80); S 11.17% (11.00).

Analytical methods

The purity of synthetic metal:PDTC preparations and identification of species present in reation mixtures was assessed by negative or positive electrospray inoization tandem mass spectrometry (Quattro II, Micromass Ltd., UK). Purity was assessed with 0.1-5.0 mM solutions of CuPDTC in DMF (N,N`-dimethylformamide; Sigma). Concentrated reactions used for mass spectral identification of products were 2 mM Cu:PDTC and approximately 20 μl CCl_4 per ml of reaction volume in DMF:H_2O, 1:1.

Chloride was measured using a Dionex 2010i ion chromatograph equipped with as AS4a column (Dionex), Na_2CO_3/$NaHCO_3$ eluent at 2 ml/min, and using suppressed conductivity detection.

Electrospray ionization tandem MS was performed using a Micromass Quattro II instrument operated in the negative ion detection mode. Samples were directly injected in methanol:water (1:1 by volume) solution by a syringe pump at a rate of 0.3 ml/h. Electron-impact ionization spectra were obtained using a Hewlett Packard 5989A spectrometer.

RESULTS

Using a chemical assay of CCl_4 transformation activity, we monitored purification of compounds produced by iron-limited cultures of strain KC. Initial experiments determined that activity was reversibly inhibited by acidification to pH 2. Acidification produced a clouding of the culture supernatant; if the lost even when the pH was found to contain the activity when dissolved in 1% ammonium hydroxide, so the active compound could be purified from the bulk medium components, which remained soluble at pH 2. Material collected by this procedure from large-volume cultures was further purified by size-exclusion

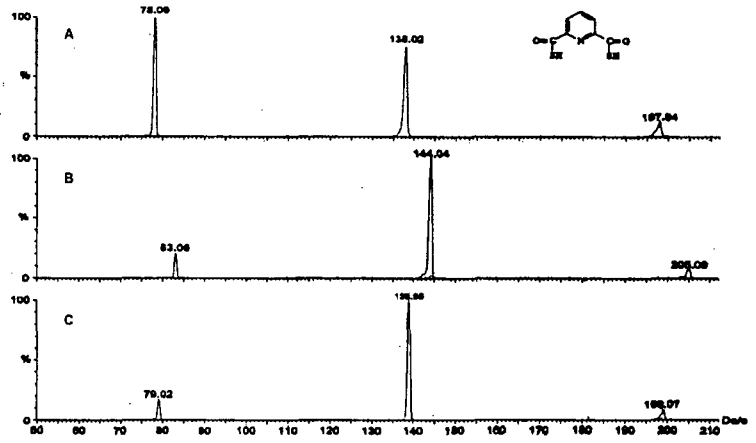

Fig. 1. Electrospray negative MS/MS of biologically produced pyridine-2,6-bis(thiocarboxylate). (A) $^{12}C/^{14}N$ daughters of native 198 Da compound (199-proton); argon used as collision gas for secondary ionization, (B) $^{13}C/^{14}N$ daughters of 205 Da compound (206-proton); proof of seven carbon atoms in structure, (C) $^{12}C/^{15}N$ daughters of 199 Da compound (200-proton); proof of one nitrogen atom in structure.

chromatography on a Bio-Gel P2 column with deionized water used as the eluent. Activity was found in two discrete peaks. The earlier and more active peak, with UV absorbance maxima at 270 and 335 nm, was designated the A fraction. Samples of the A fraction were determined to be ≥ 99% pure by reversed phase HPLC and capillary electrophoresis. Electrospray tandem MS using negative ion detection mode gave an apparent molecular weight of 198 Da $(199\text{-H})^-$ for the A fraction. The molecular ion showed a propensity to lose fragments totaling 60 and 120 Da (Fig. 1A). To determine the number of carbon and nitrogen atoms present in the molecular ion and lost fragments, preparations were made from cultures of strain KC grown in media containing either 1,2- ^{13}C acetate or $^{15}NH_4SO_4$. These gave molecular ions corresponding to the incorporation of seven carbon atoms and one nitrogen atom from the substrates (Fig. 1B, C). Particle beam electron-impact MS operated in positive ion detection mode gave the following m/z (%): 199(5.56); $[M]^+$, 166(28.81); $[M\text{-}^{\bullet}SH]^+$, 139(100); $[M\text{-}^{\bullet}COS]^+$, 138(36.20); $[M\text{-}^{\bullet}COSH]^+$, 79(1.49); $[^{\bullet}pyridine]^+$, 78(31.28); $[^{\bullet}pyridine\text{-}H]^+$, 77(58.57); $[^{\bullet}pyridine]^+$. The spectroscopic data were consistent with a symmetrically substituted pyridine with two thiocarboxylate groups in 2,6 position, accounting for the loss of neutral fragments of m/z 60 and in good agreement with data for pyridine-2,6-bis (thiocarboxylic acid) (PDTC, CAS 69945-42-2) (14). PDTC was synthesized by the method of Hildebrand *et al.* (11), and electrospray/negative ion MS confirmed its identified as a bacterial metabolite accumulated by an isolate of *Pseudomonas putida* grown under iron limitation (12). It has been characterized with regard to its complexes with a variety of transition metal ions (13, 15, 16). Addition of metal salts to PDTC purified from strain KC cultures or synthetic material and analysis by electrospray MS gave spectra consistent with the expected 2:1 complexes for iron, with evidence for both ferrous and

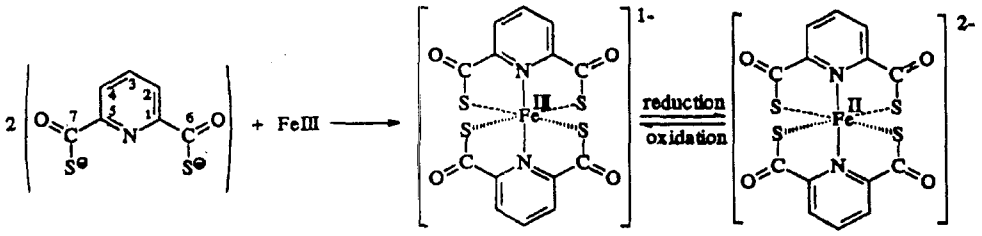

Fig. 2. Structure of *Pseudomonas stutzeri* strain KC carbon tetrachloride-dehalogenating factor pyridine-2,6-bis(thiocarboxylate) and its iron complexes. Structures were originally described as iron-binding agents for *Pseudomonas putida* by Budzikiewicz's group. Molecular weight signals of 450 Da and 225 Da were observed for ferric and ferrous complexes, respectively. The apparent mass of the ferrous complex was half that of the ferric complex, because the ferrous complex carried two negative charges (m/z^{-1} *vs.* m/z^{-2}).

ferric forms, as noted earlier by others (12, 13), as well as cobalt, and a 1:1 complex with copper which includes a chloride ion (not shown). Dithionite-reduced iron complexes of synthetic PDTC, purified active fraction from strain KC cultures, and crude culture filtrates all gave identical visible absorption spectra (max. 687 nm; $\varepsilon_{687} = 8.435 \times 10^3$). A non-reduced ferric complex solution in water showed maxima at 446, 600, and 736 nm. The molar absorption coefficient was $\varepsilon_{600} = 2.3731 \times 10^3$. Structures of PDTC and its iron complexes are shown in Fig. 2. In assays using Ti[III], synthetic PDTC showed the same activity as material purified from strain KC culture supernatant (CCl_4 degradation, but no activity toward $CHCl_3$, $CFCl_3$, 1,1,1-trichloroacetic acid). Quantitation of PDTC in cultures by the spectrophotometric method of Budzikiewicz (14) showed a yield of 10-30 μg/ml, depending on the amount of iron in the medium. Maximal production of PDTC was observed with no added iron; 10 μM iron caused about 70% reduction in PDTC produced. At >25 μM iron PDTC was below our detection limit by the method of Budzikiewicz. To determine whether a metal ion was necessary for CCl_4 dechlorination by PDTC, and to describe the effects of metal ions known to alter the CCl_4 dechlorination activity of strain KC cultures, experiments were performed with PDTC in the free acid form ($PDTCH_2$) and in mixtures with transition metal ions. Experiments using bacterial cells in a bioassay showed that $PDTCH_2$ and complexes formed by mixing with $CuCl_2$, $FeCl_3$, and $NiCl_2$ all afforded CCl_4 transformation by the otherwise inactive bacterial strain used (Fig. 3). Inclusion of $CoSO_4$ inhibited the activity. Cell-free assays were also performed to resolve direct effects of the metals on the PDTC/CCl_4 reaction and effects on cells. These experiments were performed in the buffer used in the culture medium (potassium phosphate, pH 7.7) to approximate the situation created by PDTC secretion by bacteria. Without reducing agents, $CuCl_2$/PDTC mixtures showed CCl_4 dechlorination activity with a stoichiometry of approximately 1.8 moles CCl_4/mole PDTC (Table 1). Ti(III) citrate had a similar effect on CCl_4 disappearance; the rate of dechlorination by Cu:PDTC and the stoichiometric yield were increased (Table 1). Iron showed more

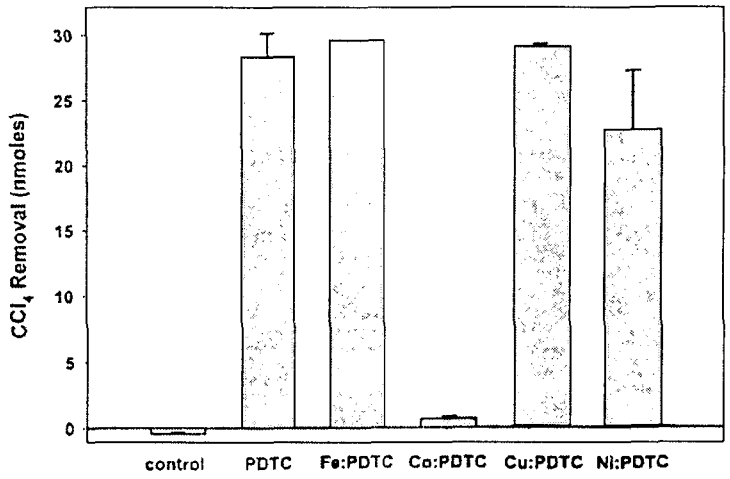

Fig. 3. CCl_4 transformation by cells of *P. stutzeri* ATCC 17588 in the presence or absence of PDTC and added transition metals. Cells were grown overnight in defined medium (DRM) and triplicate 1-ml aliquots were used with additions of 50 μmoles of PDTC, 25μmoles of the indicated metals, and 30nmoles of CCl_4. CCl_4 removal was calculated as the amount of CCl_4 remaining in cell suspensions subtracted from that remaining in sterile medium.

inhibition of $PDTCH_2$ dechlorination than $CoSO_4$ under these conditions (Table 1). Only the Cu:PDTC complex was active in all cases, i. e., with no reductant, with H_2S or Ti(III)citrate, or in the bioassay (Fig. 3, Table 1). The structure of a complex between PDTC and cooper has not been described. The Cu(II)-CI:PDTC complex gave a 1:1 stoichiometry using negative ion electrospray MS(Fig. 4). End product analyses were used to compare reactions using synthetic PDTC to products observed previously with bacterial cultures and to allow explanation of reaction pathway. Mass balance determinations using $^{14}CCl_4$ radiotracer were used initially and showed that Cu:PDTC without added reducing agent gave a product profile consisting of approximately 65% CO_2, 15% non-volatile material , and 20% volatile organic products(Fig. 5). The ^{13}C-enrichment of CS_2 and COS was not detectably different from the $^{13}CCl_4$ substrates, indicating no significant COS contribution from cleavage of unlabeled monothiocarboxylate groups of PDTC. Reactions performed in an air headspace with air-equilibrated buffer yielded much less carbon disulfide (15.3% anaerobically , 0.4% aerobically). Addition of Na_2S yielded much CS_2 (7.5 mole% without Na_2S; Table 1). Less COS was seen when Na_2S was added, but was not quantitated. Cu/PDTC reactions gave levels of chloroform below reliable detection in experiments without added reductant; however, addition of sulfide led to readily detectable amounts of chloroform(Table 1). Addition of Ti(III) citrate also increased the chloroform yield (Table 1). Mass spectra showed that the concentration of CU:PDTC decreased, and that Cu:dipicolinate, and an ion attributed to the partial hydrolysis product pyridine-2-carboxlic-6-thiocarboxylic acid appeared (Fig. 6). Some of the reaction products (CS_2, CO_2) are predicted to occur through a radical substitution mechanism (17). Experiments were conducted to

Table 1. CCl_4 transformation by PDTC with and without transition metals and chemical reducing agents

				No reductant			0.5 mM Na_2S			Ti(III) citrate		
	CCl_4/mole	$\%CHCl_3$	$\%CS_2$	CCl_4/mole	$\%CHCl_3$	$\%CS_2$	CCl_4/mole	$\%CHCl_3$	$\%CS_2$
$PDTCH_2$	0.2±0.3	n.f.	n.f.	≥4	4.5±0.3	50.4±2.9	3.1±0.1	5.1±0.8	6.3±0.5
Cu:PDTC	2.2±0.2			≥4					
		<0.1	7.5±0.8		0.9±0.05	63.3±5.2	≥3.7	0.2±0.1	4.5±0.5
	(1.85±0.1)*			(6.71±0.1*)					
Fe:PDTC	0.3±0.3	n.f.	n.f.	≥4	5.2±0.6	53.5±4.6	2.4±0.4	7.8±0.9	8.7±1.0
Co:PDTC	<0.1	n.f.	n.f.	0.3±0.2	<0.7	3.9±8.9	1.8±0.2	34.2±0.3	n.f.
Ni:PDTC	<0.1	n.d.	n.d.	≥4	1.6±0.3	39.6±2.7	3.0±0.1	2.4±0.8	3.2±0.9

Data are from triplicate 2-ml reaction in 35mM potassium phosphate (pH 7.7) containing approximately 200 nmoles CCl_4, 52 nmoles PDTC, and 26 nmoles
of the respective transition metals, and incubated for 72 hours at 25°C. Determinations were made by headspace GC. Control experiments containing the
respective transition metal salts and reductants showed losses of less than 22 moles CCl_4.
n.f. = not found
* Values in parentheses were calculated from chloride determinations of reactions containing excess CCl_4.

explore the possibility of radical intermediates of the CU:PDTC/CCl_4 reaction. The identification of possible radical intermediates included the use of the stable free radical 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) in reaction mixtures. Positive electrospray ioniztion MS showed the presence of 2,2,6,6-

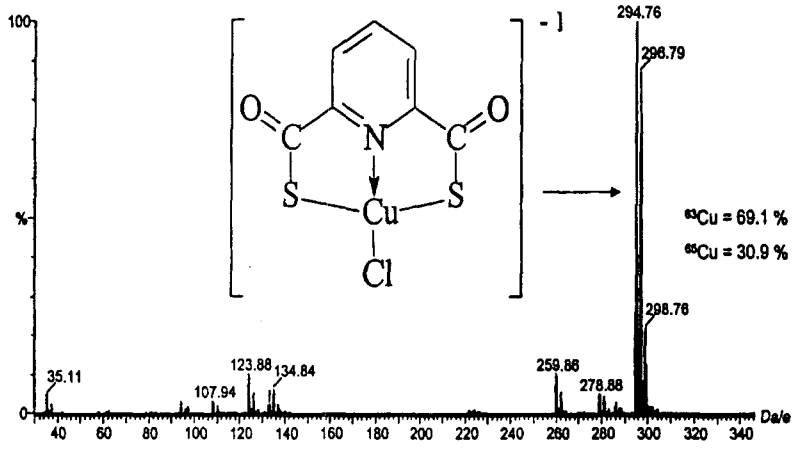

Fig. 4. Negative ion electrospray mass spectrum and structure of the Cu^{II}(PDTC) complex. The spectrum was obtained from 50% DMF in water. The large +2 peak in the spectrum is the result of the natural distribution of copper and chloride isotopes.

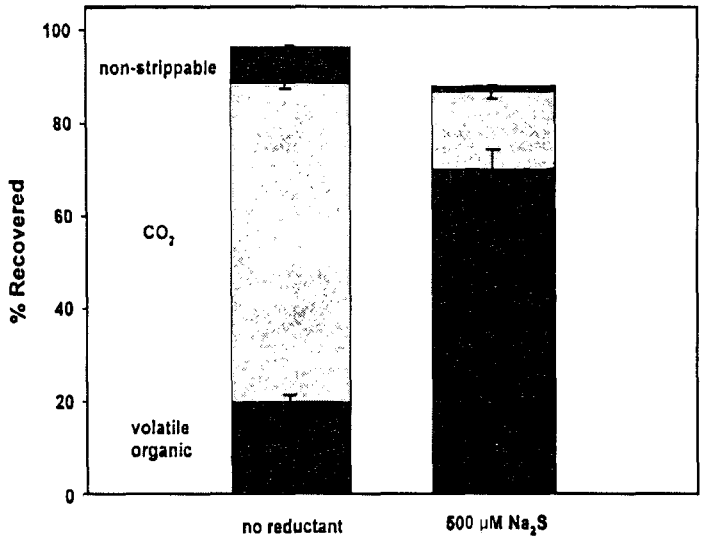

Fig. 5. Fractionation of radiotracer from $^{14}CCl_4$ (50nmoles) after reaction with Cu:PDTC (100 nmoles) in borate buffer (pH 8.1). Reactions were acidified with HCl before purging volatiles with N_2 through a trapping manifold.

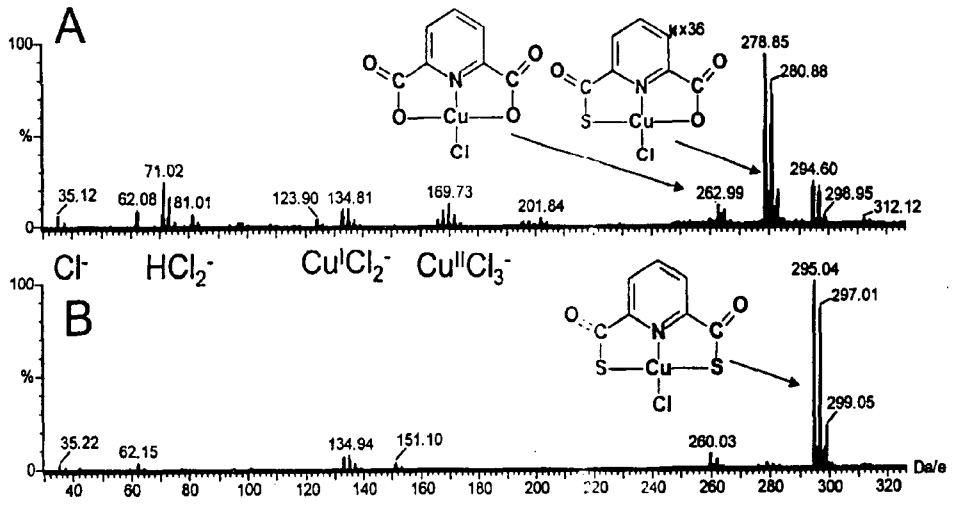

Fig. 6. Products of reaction between Cu:PDTC and CCl_4 detected by negative ion electrospray mass spectroscopy. Reactions were conducted in DMF:H_2O using 2 mM Cu:PDTC and excess CCl_4. **A.** Whole reaction mixture after 2 hours' incubation. **B.** No CCl_4 in solution.

tetramethylpiperidinium cation and a cation corresponding to a tetramethylpiperidine fragment(Fig. 7). Both of those species would be predicted from reactions in which a thiyl radical had condensed with the nitroxyl-containing compound and decomposed through a variety of routes, either spontaneously or in the MS ion source.

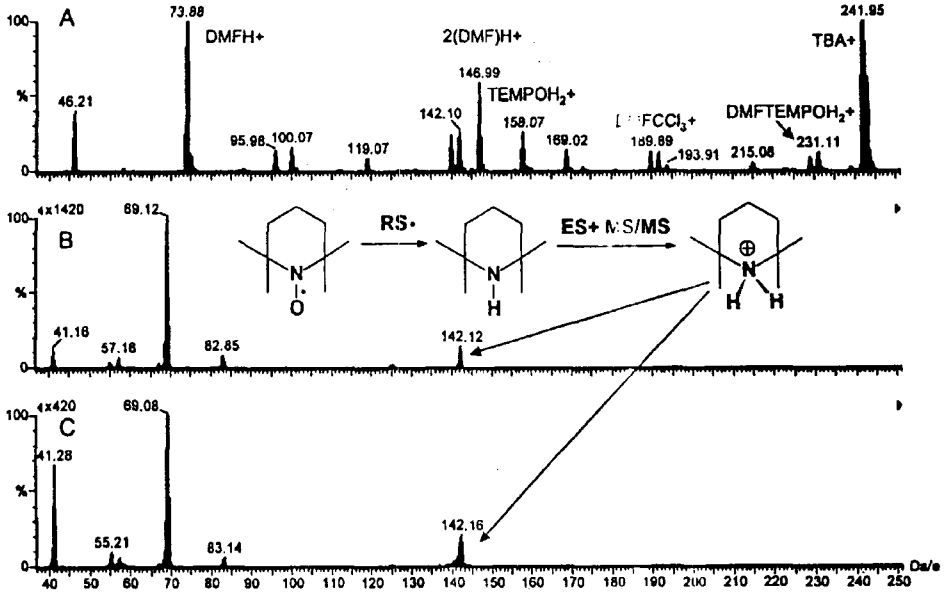

Fig. 7. Positive ion electrospray MS/MS of products of reaction between 2 mM Cu:PDTC and excess CCl4 in the presence of 2,2,6,6-tetramethylpiperidinyl oxide (TEMPO). **A,** ES+/MS spectrum of the reaction mixture in DMF:water (1:1, vol/vol). All ions except those at m/z 140 and m/z 142 were present in control incubations without CCl4. DMF:N,N-dimethylformamide; TBA+: tetrabutylammonium cation. **B,** ES+MS/MS daughter ion fragments from ion at m/z 142 from reaction in A. **C,** Daughter ion fragments from ion at m/z 142 from authentic 2,2,6,6-tetramethylpiperidine. For secondary ionization, argon gas was used.

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