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Methylenediphosphonotetrathioate: Synthesis, Characterization, and Chemical Properties

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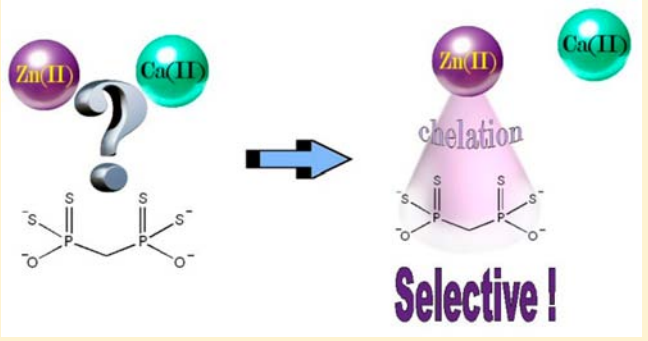
Supporting Information

ABSTRACT: Metal chelators are potential therapeutic agents for treating diseases such as Wilson's and Alzheimer's where the pathology involves an excess of metal-ions (Cu(II) and Zn(II)/Cu(II)/Fe(II/III), respectively). In addition to the high affinity of the metal-ion to the chelators, metal selectivity of the chelators is essential to achieve the therapeutic goal, that is, the successful removal of excess of harmful metal-ions in a physiological extracellular medium rich in alkali and alkali earth metal-ions. For this purpose, we synthesized a novel chelator, methylenediphosphonotetrathioate (MDPT) which is the tetrathio analogue of methylenediphosphonic acid (MDP). MDPT was synthesized from bis-methylene-(phosphonicdichloride) in a 3-step synthesis and a 31% overall yield. MDPT formed a stable complex with Zn(II) (log K = 10.84), which is 10^7 times more stable than the corresponding Ca(II) complex. Moreover, the MDPT-Zn(II) complex was 50-fold more stable than the MDP-Zn(II) complex. In addition, MDPT was found to inhibit the Cu(I)-catalyzed Fenton reaction (IC_{50} 26 μM) 2.5 times more potently than a Fe(II)-catalyzed Fenton reaction, and 2.5 times more potently than EDTA (IC_{50} 64 μM) in the Cu(I)/H_2O_2 system, as monitored by electron spin resonance (ESR). Furthermore, MDPT was found to be relatively stable in both acidic (pD 1.9, $t^1/_2$ = 71.5 h) and basic media (pD 12.4, $t^1/_2$ = 81 h) as monitored by $^{31}P/^1H$ NMR. However, MDPT was not stable in air because of intramolecular oxidation and disulfide formation (33% oxidation after 27 h). In conclusion, MDPT was found to be a water-soluble chelator showing a clear preference to soft/borderline metal-ions and a remarkable selectivity to those metal-ions vs Ca(II) ions. The relative sensitivity of MDPT to oxidation may limit its use; however, the application of MDPT in acidic or basic media will increase its lifetime.



INTRODUCTION

Neurodegenerative diseases such as Alzheimer's and Wilson's disease, as well as heavy metal poisoning are triggered by an excess of soft/borderline metal-ions such as Cu(II), Zn(II), Hg(II), Pb(II). and Cd(II).[1] Metal chelators have been suggested as a treatment for these diseases. For instance, deferiprone[2] and 5′-nucleoside-phosphorthioate derivatives[3] have been suggested to interfere with amyloid beta oligomerization in Alzheimer's disease, and penicillamine, as a copper-ion chelator, has been proposed for the treatment of Wilson's disease.[4] Dimercaptosuccinic acid and dimercaprol are used to treat heavy metal poisoning by metal-chelation and extraction through the urine.[5]

Because of the high extracellular physiological concentration of alkali and alkali earth metal ions (e.g., sodium ion: 138−142 mM,[6] and calcium ion: 160 mM[7]), chelators exhibiting high metal ion selectivity are required to achieve specific metal-ion chelation in a way which does not alter metal homeostasis and metalloprotein activity.[8] For instance, methylene diphosphonic acid (MDP), **1**, and its derivatives are used as therapeutic agents for treating bone diseases. Specifically, MDP derivatives bind Ca(II) ions, adsorb to the bone, and interfere with bone resorption.[9] However, although MDP binds Ca(II) ions with a relatively high affinity[10,11] MDP also binds Mg(II),[11] Cu(II),[12] and other ions,[13−15] thus making MDP derivatives nonselective chelators in a physiological environment. Here, we aimed to improve the selectivity of MDP to soft/borderline metal-ions by replacing the MDP oxygen atoms with sulfur atoms, which are softer ligands, to obtain methylenediphosphonotetrathioate (MDPT), **2** (Figure 1).

Recently, we reported on the synthesis of *O*,*O*′-diester-methylenediphosphonotetrathioate derivatives, **3**, and the characteristics of their metal complexes.[16] Derivatives **3**

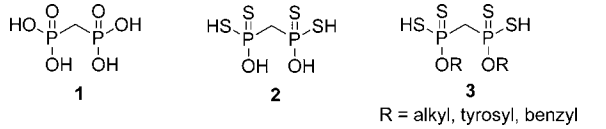


Figure 1. Structures of MDP **1**, MDTP **2**, and *O*,*O*′-diester-methylenediphosphonotetrathioate derivatives **3**.

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Scheme 1. Synthesis of MDPT, 2

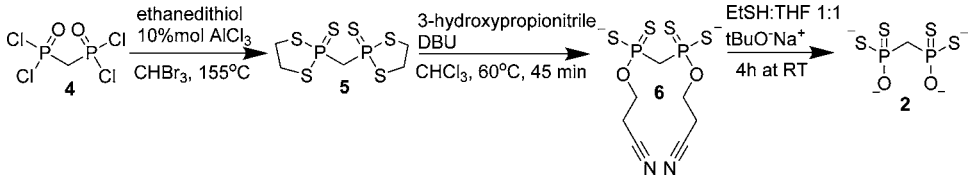

Table 1. Negative Logarithms of the Acidity Constants of Bisphosphonate Species As Determined by Potentiometric pH Titrations in an Aqueous Solution at 24°C

	$pK_a^H{}_{LH_4}$	$pK_a^H{}_{LH_3}$	$pK_a^H{}_{LH_2}$	$pK_a^H{}_{LH}$	background electrolyte
MDP[10]		2.75 ± 0.01	7.10 ± 0.01	10.75 ± 0.01	0.1 M $(CH_3)_4N(NO_3)$
MDP[11]	1.08	2.65	6.86	10.09	0.1 M $NaClO_4$
MDP[13]	2.19	3.26	7.00	9.97	0.1 M KNO_3
MDP[14]		2.71 ± 0.036	6.91 ± 0.044	10.17 ± 0.026	0.15 M NaCl
MDP		3.07 ± 0.03	6.86 ± 0.01	9.94 ± 0.01	0.1 M $NaNO_3$
MDPT		2.70 ± 0.01	4.78 ± 0.02	7.99 ± 0.01	0.1 M $NaNO_3$

metal-ion complexation was assayed by FT-IR for Hg(II) and Pb(II) complexes, ^{1}H NMR for Zn(II) complex, UV−vis for Ni(II) complex, and electron spin resonance (ESR) for determining the modulation of OH radical formation in the Cu(I)/Fe(II)-H_2O_2 system by derivatives **3**. However, all those complexes are not water-soluble.

In this Article, we describe our quest for a water-soluble and biocompatible metal-ion chelator showing high-affinity and selectivity to borderline/soft metal ions. Specifically, we report on the synthesis of methylenediphosphonotetrathioic acid (MDPT), **2**, which is the tetrathio analogue of MDP, and its water-soluble complexes. In addition, we report on the determination of the acidity constants of MDPT vs MDP and the stability constants of Zn(II) and Ca(II) complex of MDPT vs MDP, as well as species distribution analyzed under physiological pH. Moreover, we evaluated MDPT as an antioxidant in a Cu(I)/Fe(II) induced Fenton reaction, compared to MDP, as determined by ESR. Finally, we determined the pH-dependent stability under acidic and basic pH and the tendency to air-oxidation.

RESULTS AND DISCUSSION

Synthesis. Recently, we reported on the synthesis of methylene-bis(1,3,2-dithiaphospholane-2-sulfide), **5**, from bis-methylene(phosphonicdichloride), **4**, treated with 1,2-ethanedithiol and 10 mol % $AlCl_3$ in $CHBr_3$.[16] When compound **5** was further reacted with primary alcohols in the presence of DBU,[17] derivatives **3** were obtained. Here, we targeted the preparation of the water-soluble parent molecule, MDPT, **2** (Scheme 1). For this purpose, compound **5** was reacted with 3-hydroxypropionitrile in the presence of DBU at 60 °C to give compound **6** (^{31}P NMR: 104.3 ppm (compound **6**) vs 90.5 ppm (compound **5**)) in a 75% yield. Diester **6** was treated with various bases including NH_4OH, NaOH, and DBU to allow β-elimination and MDPT formation. However, in the presence of DBU, MDPT decomposed, and in 15% NH_4OH, the thiol group in MDPT reacted with the eliminated product, acrylonitrile, to form thio-ester. To overcome this obstacle, we used $tBuO^-Na^+$ in tetrahydrofuran (THF) and scavenged the acrylonitrile with ethanethiol. MDPT precipitated together with EtSNa salt from the reaction mixture. By acidifying the aqueous solution of MDPT and EtSNa with DOWEX MAC-3, a weakly acidic resin, and water evaporation, pure MDPT, **2**, was obtained, without any need for further purification, in a 95% yield (^{31}P NMR: 77 ppm).

Potentiometric Titrations. The chemical characterization of MDPT involved studying acid−base equilibria of MDPT as compared to MDP, and determining the stability/selectivity of the corresponding Zn(II) and Ca(II) complexes by potentiometric titrations. According to Hard−Soft Acid−Base (HSAB) theory,[18] we hypothesized that the MDPT-Zn(II) complex will have a higher log K value as compared to the MDP-Zn(II) complex. Furthermore, we targeted a significant Zn(II) vs Ca(II)-ion selectivity of MDPT, to allow specific Zn(II) binding in a Ca(II)-rich physiological medium. Hence, we compared the stability of MDPT-Zn(II) to the MDPT-Ca(II) complex to determine metal-ion selectivity. Furthermore, we compared those data with the corresponding log K values of MDP-Zn(II) and MDP-Ca(II) complexes to assess the preference of MDPT to borderline metal-ions.

Acidity Constants of MDP and Methylenediphosphonodithioic Acid (MDPT). Three protonation equilibria of MDP and MDPT are expected to occur in the pH range of 2.5−11, and they are described in eqs 1−3:

$$H_3(MDPT)^- \rightleftarrows H(MDPT)^{2-} + H^+ \quad (1a)$$

$$K^H_{H_3(MDPT)} = [H_2(MDPT)^{2-}][H^+]/[H_3(MDPT)^-] \quad (1b)$$

$$H_2(MDPT)^{2-} \rightleftarrows H(MDPT)^{3-} + H^+ \quad (2a)$$

$$K^H_{H_2(MDPT)} = [H(MDPT)^{3-}][H^+]/[H_2(MDPT)^{2-}] \quad (2b)$$

$$H(MDPT)^{3-} \rightleftarrows (MDPT)^{4-} + H^+ \quad (3a)$$

$$K^H_{H(MDPT)} = [MDPT^{4-}][H^+]/[H(MDPT)^{3-}] \quad (3b)$$

The first acidity constant of MDPT (eqs 1a, 1b) was lower than that of MDP by only ~0.4 log units (2.71 vs 3.07), whereas the second and the third acidity constants (eqs 2a, 2b, 3a, 3b) of MDPT (4.78 and 7.99) were lower by approximately 2 log units (Table 1). These data are reminiscent of pK_a values of dithiophosphate (3.90 and 8.37).[19] The acidity constants of dithiophosphate were lower than those of inorganic phosphate (7.20 and 12.33).[19] Since sulfur atoms are larger and more polarizable than oxygen atoms, they stabilize the negative charge better and hence, increase acidity. The same

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Table 2. Logarithms of the Stability Constants of M(H;L), M(OH;L), M(2OH;L), and M(L) Complexes, Where L = Ligand (bisphosphonate) and M = Metal (Zn, Ca) As Determined by Potentiometric pH-Titrations in an Aqueous Solution, Together with the Negative Logarithms of the Acidity Constants of the Corresponding M(H;L) Complexes (24 °C; I = 0.1 M)

M(II)	L	log $K^{M}_{M(L)}$	log $K^{M}_{M(H;L)}$	log $K^{M}_{M(OH;L)}$	log $K^{M}_{M(2OH;L)}$	p$K^{H}_{aM(H;L)}$	background electrolyte
Ca(II)	MDP[10]	5.97 ± 0.06	2.89 ± 0.08				0.1 M $(CH_3)_4N(NO_3)$
Ca(II)	MDP	5.05 ± 0.02	2.96 ± 0.02	7.85 ± 0.05		7.85 ± 0.01	0.1 M $NaNO_3$
Zn(II)	MDP	9.17 ± 0.01	5.01 ± 0.01	14.09 ± 0.01	17.64 ± 0.03	5.78 ± 0.01	0.1 M $NaNO_3$
Ca(II)	MDPT	3.86 ± 0.02		6.87 ± 0.06			0.1 M $NaNO_3$
Zn(II)	MDPT	10.84 ± 0.05	6.05 ± 0.06	16.21 ± 0.18	19.97 ± 0.34	3.21 ± 0.05	0.1 M $NaNO_3$

phenomenon was observed for acid−base equilibria in phosphorothioic acid,[20] methyl thiophosphate, AMP-α-S, UMP-α-S,[21] and other nucleoside phosphorothioate derivatives, as compared to the corresponding phosphate compounds.[22] The effect of the number of sulfur atoms on pK_a values is not additive because the replacement of only one oxygen atom with sulfur, as in phosphorus acid and nucleoside phosphorothioate derivatives, decreased the pK_a values by more than 1 order of magnitude ($\Delta pK_a \cong 1.4$).[21]

Stability Constants of Zn(II)/Ca(II)-MDP and Zn(II)/Ca(II)/MDPT Complexes. The formation of 1:1 complexes of Zn(II) and Ca(II) with MDPT and MDP is described in eqs 4 and 5.

$$(H{\cdot}MDPT)^{3-} + M^{2+} \rightleftarrows M(H{\cdot}MDPT)^{-} \quad (4a)$$

$$K^{M}_{M(H{\cdot}MDPT)} = [M(H{\cdot}MDPT)^{-}]/[M^{2+}][(H{\cdot}MDPT)^{3-}] \quad (4b)$$

$$(MDPT)^{4-} + M^{2+} \rightleftarrows M(MDPT)^{2-} \quad (5a)$$

$$K^{M}_{M(MDPT)} = [M(MDPT)^{2-}]/[M^{2+}][(MDPT)^{4-}] \quad (5b)$$

The deprotonation of the complex is represented in eq 6a.

$$M(H{\cdot}MDPT)^{-} \rightleftarrows H^{+} + M(MDPT)^{2-} \quad (6a)$$

$$K^{H}_{M(H{\cdot}MDPT)} = [H^{+}][M(MDPT)^{2-}]/[(M(H{\cdot}MDPT)^{-}] \quad (6b)$$

The acidity constant of the complex is calculated in eq 7[23]

$$pK^{H}_{M(H{\cdot}MDPT)} = pK^{H}_{H(MDPT)} + \log K^{M}_{M(H{\cdot}MDPT)} - \log K^{M}_{M(MDPT)} \quad (7)$$

The stability constants of hydroxo complexes are calculated based on the following equations[24]

$$M^{2+} + (MDPT)^{4-} + rH \rightleftarrows [M^{2+}(MDPT)H_r]^{2-(r-)} \quad (8a)$$

$$\beta_{M(MDPT)rH} = [M^{2+}(MDPT)H_r]^{2-(r-)}/[M^{2+}][MDPT^{4-}][H^{+}]^{r} \quad (8b)$$

A negative value of r indicates the presence of hydroxide in the complex.

If we define H_{-1} as OH^{-}, and n as the number of hydroxide ions, and use the convention: $[OH^{-}] = K_w[H^{+}]^{-1}$ we get eq 9[24]:

$$[M(MDPT)(H_{-1})_n] = \beta[M^{2+}][MDPT^{4-}]\left(\frac{[OH^{-}]}{K_w}\right)^{n} \quad (9a)$$

$$\log K^{M}_{M(MDPT)(OH)_n} = \log \beta_{M(MDPT)(OH)_n} - n\log K_w \quad (9b)$$

The stability constants of complexes of Ca(II) and Zn(II) with both MDP and MDPT were determined by pH-titrations of 1:1 ligand:M(II) mixtures (Table 2). The stability constant of the Ca(II)-MDP complex was determined before[10,11,25] whereas the stability constant value of the Zn(II)-MDP complex has not been reported to date.

The Zn(II)-MDP stability constant was found to be ~10^4-times more stable than the Ca(II)-MDP complex (log K 9.17 vs 5.05). A similar phenomenon was observed on the corresponding ternary Zn-hydroxo complexes. The Zn(II)-MDP-(OH) complex was found to be even more stable, by more than 6 log units, as compared to the Ca(II)-MDP-(OH) complex (log K 14.09 vs 7.85). The pK_a of MDP (eqs 3a, 3b) was decreased by 2 orders of magnitude (pK_a 7.85), upon the formation of the Ca(II)-complex, whereas for the Zn(II)-complex the pK_a was decreased by 4 orders of magnitude (pK_a 5.78). This finding indicates that Zn(II) forms a more stable complex with MDP than Ca(II). These results are supported by the known stability constants of diphosphate monoesters (e.g., n-butyl diphosphate and phenyl diphosphate), and pyrimidine nucleoside 5′-diphosphates (e.g., CDP, UDP, and dTDP) with Zn(II), Ca(II), showing that these diphosphate monoesters form more stable complexes with Zn(II) than with Ca(II) by more than 1 order of magnitude.[26] Because of the replacement of four oxygen atoms in MDP with sulfur, the stability constant of the Zn(II)-MDPT complex increased by ~1.7 log units, as compared to that of Zn(II)-MDP (log K 10.84 vs 9.17). Yet, the stability constant of Ca(II)-MDPT was decreased by approximately 1 order of magnitude, as compared to the Ca(II)-MDP complex (log K 3.86 vs 5.05). A similar decrease was reported for the complex of [thiophosphonato)-methyl] phosphonic acid with Mg(II),[11] where the stability constant of this complex decreased by approximately 1 log unit, as compared to the MDP-Mg(II) complex.

MDPT formed a 10^7-fold more stable complex with Zn(II) than with Ca(II), whereas the Zn(II)-hydroxo complex with MDPT was more stable by ~9.5 orders of magnitude, as compared to the Ca(II)-hydroxo complex. Moreover, the acidity constant of MDPT (eqs 3a, 3b) decreased upon the formation of the Zn(II)-complex by approximately 5 log units, whereas in the Zn(II)-MDP complex, the decrease of the acidity constant was 4 orders of magnitude. The high affinity of Zn(II) to MDPT vs MDP was expected, according to the HSAB theory.[18] Zinc is a borderline metal ion which prefers to coordinate with large and polarizable atoms such as sulfur. In addition, Zn(II) forms more hydroxo species in the formation of complexes with MDP and MDPT, as compared to Ca(II). Zn-MDPT$(OH)_2$ was found to be more stable than Zn-MDP$(OH)_2$ (log K 19.97 vs 17.64).

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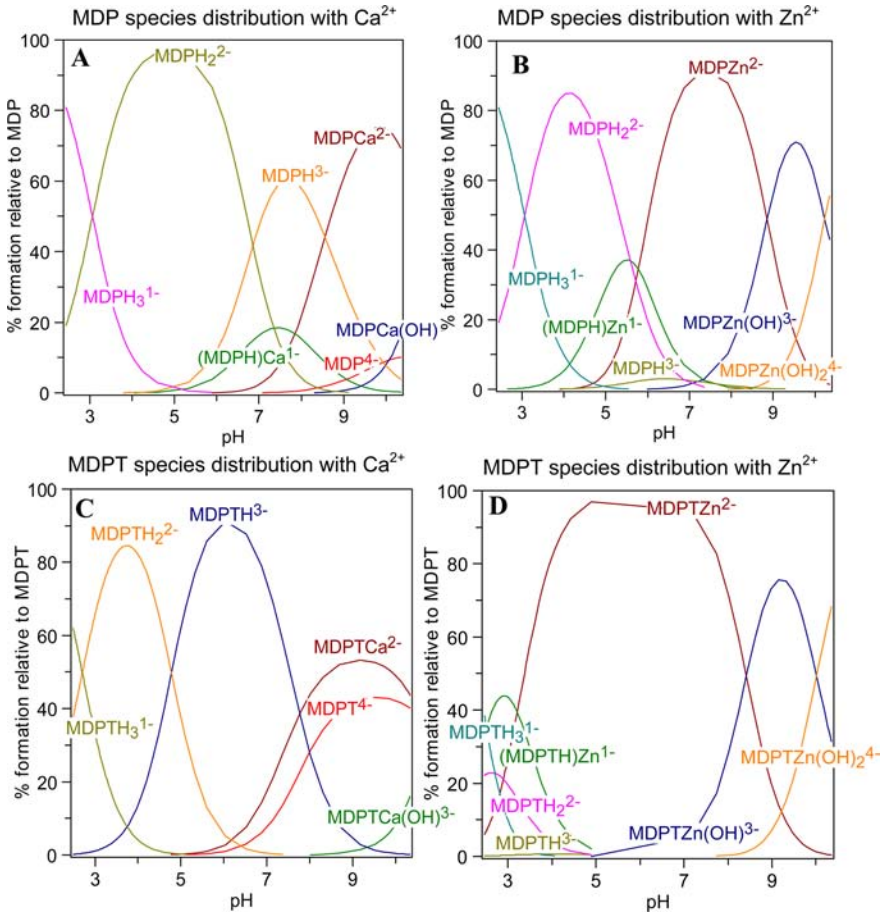

Figure 2. Simulation of pH-titration of 1:1 MDP and MDPT complexes with Ca(II) and Zn(II). MDP species distribution with (A) Ca(II) and (B) Zn(II). MDPT species distribution with (C) Ca(II) and (D) Zn(II).

To determine the major species in titration solutions, and species distribution, we performed titration simulations using the Hyss program[27] (Figure 2). According to Figures 2A and 2B, under physiological pH (7.4), the major species in the presence of Ca(II) is $MDPH^{3-}$ (~58%), and in the presence of Zn(II) $MDPZn^{2-}$ (~91%), whereas the minor species are $MDPCa^{2-}$ (~7%) and $MDPH^{3-}$ (~2%) respectively. Namely, MDP forms a much more stable complex with Zn(II) than with Ca(II) under neutral pH. MDP forms a complex with Ca(II) only under basic pH (9–10) where $MDPCa^{2-}$ is the major species (up to 75%). This finding is consistent with previous reports.[10] For MDPT, the major species under physiological pH in the presence of Ca(II) is $MDPTH^{3-}$ (~57%, Figure 2C), and in the presence of Zn(II) the major species is $MDPTZn^{2-}$ (~90%, Figure 2D), respectively, whereas, the minor species are $MDPT^{4-}$ (~14%) and $MDPTZn(OH)^{3-}$ (~10%). $MDPTZn^{2-}$ is the major species in a wide pH range 3.3–8.3 (Figure 2D), whereas, $MDPZn^{2-}$ is the major species in a relative narrow pH range 5.9–8.8 (Figure 2B). Furthermore, from pH 8, free MDPT is at least 30% of all species, and does not form a complex with Ca(II) (Figure 2C), whereas at pH ~9.5, the maximum complexation with Ca(II) occurs. Like MDP, MDPT forms a complex with Ca(II) under basic pH (8–10). However, MDP is a better Ca(II)-chelator. In the presence of Ca(II) (Figure 2A), up to 10% of free MDP does not form a complex. These results clearly demostrate Zn(II) vs Ca(II) ion selectivity of MDPT.

Chemical Stability of MDPT. The chemical stability of MDPT was assayed in both acidic and basic media by $^{31}P/^{1}H$ NMR, and decomposition products were analyzed using ESI mass spectroscopy. In addition, oxidation and disulfide formation was assayed by bubbling air into a D_2O MDPT solution, and monitoring by $^{31}P/^{1}H$ NMR.

While thiophosphate compounds[28] and diester **3** formed a disulfide bond upon treatment with an oxidizing agent such as I_2 (unpublished results), MDPT underwent oxidation simply by bubbling air through an aqueous MDPT solution. The ^{1}H NMR spectrum after 0.5 h showed two signals (Figure 3): a triplet at 3.45 ppm corresponding to P-CH_2-P, and a minute

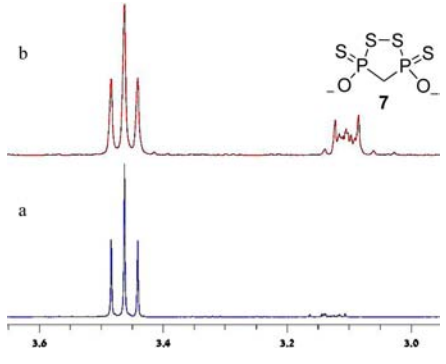

Figure 3. ^{1}H NMR (600 MHz) spectra of MDPT disulfide formation after air bubbling for (a) 0.5 h and (b) 27 h.

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second order signal at 3.1 ppm implying the formation of an intramolecularly oxidized product **7**. The new asymmetric centers that formed after the oxidation of MDPT are the cause of this complex signal. After 27 h at room temperature (RT), 33% of MDPT was oxidized to disulfide. However, after ~30 h, impurities were observed in addition to the oxidized product.

Next, we evaluated the stability of MDPT in an acidic medium. The reaction was conducted at pD 1.9 for 11 days, and was monitored by $^{31}P/^{1}H$ NMR. In the course of the experiment, new signals emerged in ^{31}P NMR spectra at 91.8 and 65.0 ppm (Figure 4), and in ^{1}H NMR spectra at 3.2 (t, J = 15 Hz) ppm. The compound was stable under pD 1.9 with only 8% decomposition for up to 48 h (Figure 4a), after 120 h, 77% of MDPT decomposed (Figure 4b), and by 264 h only 15% of MDPT remained (Figure 4d). MDPT was relatively stable under those drastic conditions with approximate half-life of about 71.5 h.

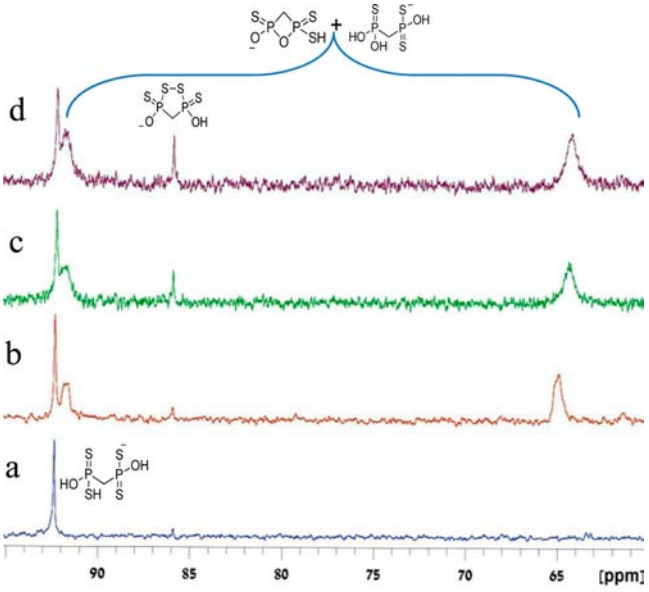

Figure 4. ^{31}P NMR (81 MHz) spectra of MDPT at pD 1.9 after (a) 48 h, (b) 120 h, (c) 192 h, and (d) 264 h.

Mass spectrum (ESI-QTOF negative) analysis of freeze-dried MDPT after 11 days at pD 1.9 revealed the following MDPT fragmentation products (Figure 5).

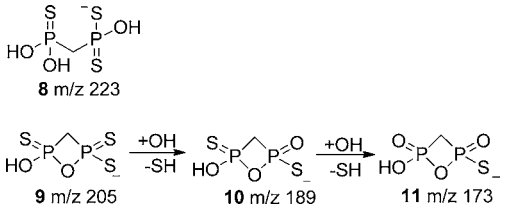

Figure 5. MDPT products after 11 days at pD 1.9, as observed by mass spectrum.

In the mass spectrum, we did not observe any signal that can be correlated to MDPT (m/z 239), or fragmentation which can be attributed to MDPT. The combination of mass analysis with ^{31}P NMR data for MDPT subjected to acidic media for 11 days reveals that multiplets at 91.8 and 65 ppm are correlated to the asymmetric hydrolysis product **8** m/z 223, and asymmetric **9**, m/z 205. The latter compound is formed by an intramolecular nucleophilic attack and the loss of hydrogen sulfide. Moreover, four-membered ring heterocyclic compounds such as **9** were synthesized before, and the typical ^{31}P NMR signal at ~90 ppm we found here for **9** is in accordance with previous findings.[29]

At pD 12.4 ^{31}P NMR spectra after 48 h showed doublets at 73.3 (J = 22.5 Hz) and 46.8 (J = 22.5 Hz) ppm, indicating a probable hydrolysis of one thiol moiety. After 48 h at pD 12.4, 37% decomposition occurred (Figure 6b). After 84 h, 55% of MDPT remained, and a new signal characteristic of MDP emerged at 14.5 ppm (Figure 6d). After 156 h, MDPT could not be observed at 72.6 ppm, and instead a broad signal at 73.6 ppm was measured. The ^{1}H NMR spectrum exhibited at t = 0 a triplet at 3.53 (J = 13.1 Hz), corresponding to PCH_2P. After 48 h, the methylene signal could not be observed because of the exchange of the hydrogen atoms with deuterium, which implies the acidity of the phosphonate methylene group. MDTP was found to be relatively stable under basic conditions with $t^{1}/_{2}$ = 81 h.

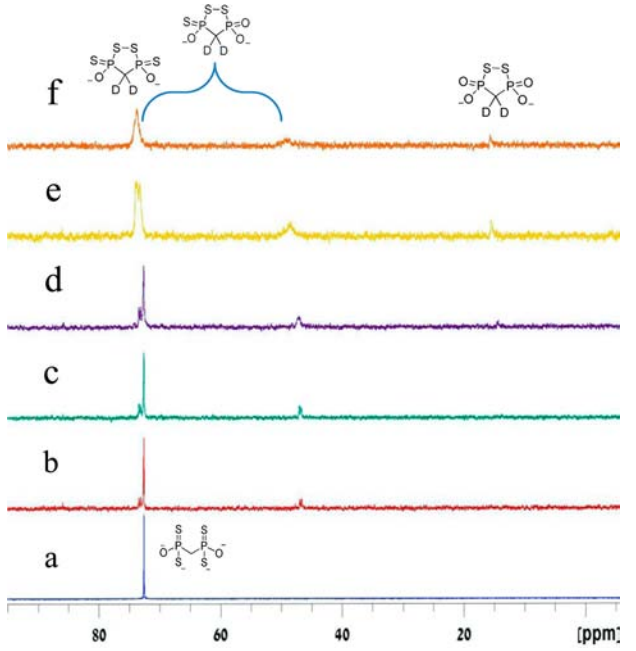

Figure 6. ^{31}P NMR (81 MHz) spectra of MDPT at pD 12.4 after (a) 0 h, (b) 48 h, (c) 60 h, (d) 72 h, (e) 132 h, and (f) 156 h.

Mass analysis of MDPT subjected to basic conditions (pD 12.4) for 6.5 days revealed two types of products (Figure 7).

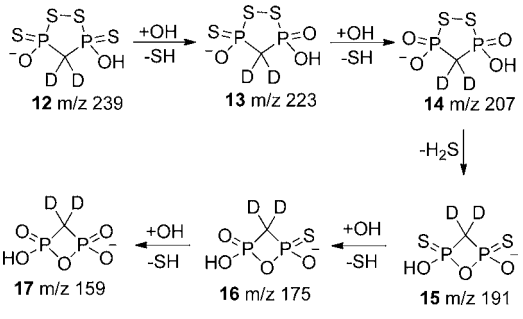

Figure 7. Mass spectrum fragmentation of MDPT after 6.5 days at pD 12.4.

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One product was a cyclic disulfide scaffold where hydrogen atoms were exchanged with deuterium (**12**−**14**). The other product set was due to a loss of hydrogen sulfide in the fragmentation process and the formation of a four-membered ring (**15**−**17**). The compilation of MS and $^{31}P/^{1}H$ NMR data implies that the broad signal at 73.6 ppm may be assigned to dithiophosphonate, where S^- is oxidized to disulfide, and CH_2 was transformed to CD_2 as in **12** (m/z 239). Hydrolysis of one sulfur atom as in **13** (m/z 223) is correlated to ^{31}P NMR signals at 73.6 ppm and 46.8 ppm. The signal at 15.5 ppm is broad ($LW_{1/2}$ = 29 Hz), and may indicate the formation of **14** (m/z 207).

The comparison of Figures 4 and 6 indicates that intramolecular oxidation and disulfide formation occurs preferentially at pD 1.9 rather than at pD 12.4. Basic pH relatively also stabilizes MDPT from oxidation as compared to neutral pH (pD 12.4: 37% oxidation after 48 h; pD 7.4: 33% oxidation after 27 h).

OH Radical Modulation Monitored by ESR. We found previously, that phosphorothioate compounds are highly efficient in reducing OH radical formation in the Fenton reaction.[16,30,31] OH radicals formed in the reaction were trapped by 5,5′-dimethyl-1-pyrroline-N-oxide (DMPO), and the amount of DMPO-OH adduct was then measured by ESR. The mechanisms of inhibition of the Fenton reaction could be attributed to metal-ion chelation as well as radical scavenging. Here, we evaluated indirectly Fe(II) and Cu(I)-binding to MDPT by measuring OH radical formation in Fe(II) and in a Cu(I)-induced Fenton reaction (Table 3). We anticipated that

Table 3. Modulation of OH Radical Formation in the Fenton Reaction by MDP and MDPT, Monitored by ESR

	IC_{50} [μM]	
compound	Fe(II)	Cu(I)
MDP	27 ± 4	88 ± 1
MDPT	66 ± 2	26 ± 2
EDTA	62 ± 1	64 ± 2
GSH	63 ± 5	216 ± 4

MDPT will reduce more significantly OH radical formation for the softer Lewis acid, Cu(I), than Fe(II). Indeed, while MDPT inhibited the Fenton reaction with IC_{50} of 66 μM for Fe(II), for Cu(I) MDPT exhibited IC_{50} of 26 μM. MDP exhibited opposite activity: a lower IC_{50} was obtained for Fe(II) (26 μM) than for Cu(I) (88 μM), indicating the reduced preference of MDP to soft metal-ions. Furthermore, MDTP was highly efficient in inhibiting OH radical formation in a Cu(I)/H_2O_2 system as compared to EDTA and an endogenous antioxidant glutathione (GSH), being 2.5- and 8-fold more potent, respectively.

UV/vis Measurements of MDPT-Fe(II)/Cu(I) Complex. To further characterize MDPT-Cu(I)/Fe(II) complexes we performed UV/vis- monitored Cu(I)/Fe(II)-titrations of MDPT. To a 0.05 mM aqueous solution of MDPT, 5 μL of 5 mM $(NH_4)_2Fe(SO_4)_2$ aqueous solution or 5 mM $[Cu(CH_3CN)_4]PF_6$ in CH_3CN was added each time. After each addition the absorbance was measured at 272 and 254 nm, respectively (Figure 8). The signal at 254 nm indicates the formation of MDPT-Cu(I) complex. Changes of the 254 nm signal at Cu(I) amounts higher than 1 equiv could not be detected because of the broad signal of $[Cu(CH_3CN)_4]PF_6$ at 212 nm. Fe(II) also formed a complex with MDPT as indicated

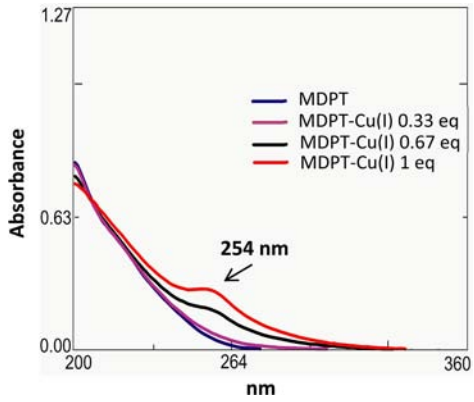

Figure 8. Titrations of **2** with Cu(I) as monitored by UV−vis spectra.

by the shoulder at 272 nm (Figure Supporting Information, Figure S1).

CONCLUSIONS

The replacement of four oxygen atoms in MDP with sulfur atoms, resulted in enhanced acidity of MDPT up to 100-fold. In addition, this modification increased the stability constant of MDPT with Zn(II) by ∼1.7 log units (log K = 10.84), and decreased the stability constant with Ca(II) by ∼1.2 log units as compared to the corresponding MDP complex. As a result, Zn(II)-MDPT was a more stable complex by a factor of 10^7 than the corresponding Ca(II)-complex. This finding demonstrates the dramatic metal-ion selectivity of this novel chelator. In contrast, Zn(II)-MDP is a more stable complex than Ca(II)-MDP by only 4 orders of magnitude. MDPT showed high selectivity in chelating a soft metal-ion, Cu(I), as compared to a borderline metal-ion, Fe(II), as demonstrated by the inhibition of a Cu(I)/Fe(II)-induced Fenton reaction. Here, the inhibition of the Cu(I)/H_2O_2 system was 2.5-fold more effective than that of the Fe(II)/H_2O_2 system. Moreover, although MDPT has a tendency to oxidize by air to disulfide (33% oxidation after 27 h) MDPT was relatively stable in acidic (pD 1.9) and basic media (pD 12.4), with an approximate half-life of 3 and 3.5 days, respectively. In summary, MDPT, exhibited a high affinity to Zn(II), and was found to be a tremendously selective chelator, effectively showing a complete preference to Zn(II) vs Ca(II) ions. Furthermore, MDPT discriminated between a soft metal-ion, Cu(I), and a borderline metal-ion, Fe(II). The relative sensitivity of MDPT to oxidation may limit its use; however, the application of MDPT in acidic or basic media will increase its lifetime.

EXPERIMENTAL SECTION

General Procedures. Reactions were performed in oven-dried flasks under Ar atmosphere. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) was purchased from Sigma-Aldrich and used without further purification. 3-Hydroxypropionitrile (Sigma-Aldrich) was distilled under reduced pressure, and $CHCl_3$ was distilled over P_2O_5. Flash chromatography (silica-gel and C_{18} reverse phase) was performed using a Biotage SP1 instrument. ^{1}H, ^{13}C, and ^{31}P NMR spectra were measured using Bruker AC-200 (200, 50, and 81 MHz for ^{1}H, ^{13}C, and ^{31}P NMR), and Bruker DMX-600 (600, 150, and 243 MHz for ^{1}H, ^{13}C, and ^{31}P NMR) machines. The concentration of the spin trap, DMPO, was determined by UV spectroscopy (ε_{228} nm = 8000 M^{-1}) after purification with active charcoal. Purified DMPO was stored at −18 °C subsequent to deaeration with an argon stream. The analysis of OH radicals produced in Cu(I) and Fe(II)-H_2O_2/tested compound systems was performed by a solution ESR spectroscopy using a Bruker

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ER 100d X-band spectrophotometer. Mass spectra analyses were performed on an ESI Q-TOF micro instrument (Waters, U.K.) and a high resolution MS-MALDI-TOF spectrometer with autoflex TOF/TOF instrument (Bruker, Germany). The pH titrations were carried out with a Metrohm 794 basic Titrino and Metrohm glass electrode, Viscotrode. The buffers (pH 4.00, 7.00, and 9.00)[32] used for calibration were purchased from Metrohm (Herisau, Switzerland). All potentiometric titrations were performed at 24 °C under an argon atmosphere. The titrations were performed at a high sensitivity of the electrode: Signal drift: 5 mV/min. 5 M NaOH, 2 M HNO_3, and potassium biphthalate were purchased from Merck (Darmstadt, Germany). $NaNO_3$ salt (background electrolyte), $Zn(NO_3)_2$, and $Ca(NO_3)_2$ standard solutions were purchased from Sigma (Steinheim, Germany). All solutions for the titrations were prepared with deionized water. The concentration of the titer of NaOH was determined with potassium biphthalate.

Synthesis. *Disodium-O,O′-bis(2-cyanoethyl) Methylenediphosphonodithioate* ***6***. Compound **6** was synthesized according to our previously published synthetic pathway.[16] Briefly, 3-hydroxypropionitrile (0.13 g, 1.85 mmol), and **5** (0.1 g, 0.31 mmol) were heated to 60 °C in dry $CHCl_3$ (3 mL), followed by the addition of DBU (0.09 g, 0.62 mmol). After 45 min the reaction mixture was separated over silica gel, and then product **6** was purified on reverse-phase flash chromatography. The sodium salt was obtained by passing an aqueous mixture of the purified compound through DOWEX 50w-Na^+ form to obtain product **6** as a clear oil in a 75% yield (0.09 g). ^{1}H NMR (200 MHz, D_2O): δ 4.36−4.25 (m, 4H), 3.54 (t, J = 13.6 Hz, 2H), 2.97 (t, J = 6 Hz, 4H) ppm. ^{13}C NMR (50 MHz, D_2O): δ 119.6, 58.9, 56.8 (t, J = 63.4 Hz), 19.2 (t, J = 4.5 Hz) ppm. ^{31}P NMR (81 MHz, D_2O): δ 104.3 ppm. HRMS (MALDI) m/z calcd for $C_7H_{11}N_2O_2P_2S_4^-$ [M − H]$^-$: 344.917, found: 344.918.

Tetrasodium Methylenediphosphonodithioate ***2***. Compound **6** (120 mg, 0.31 mmol) was suspended in THF:EtSH 1:1 (4 mL) under Ar, and tBuONa (118 mg, 1.24 mmol) was added in three portions in the course of 2 h. The reaction mixture turned yellow with white precipitate, and the reaction commenced for 4 h. The solvent was then decanted, and the white solid was washed 3 times with THF. The solid was dissolved in water, and the solution was then evaporated to dryness. The residue was dissolved again in water and acidified with the addition of DOWEX MAC-3 until pH 3 was attained. The solution was evaporated to give a yellowish hygroscopic solid which was passed through DOWEX 50w-Na^+ form to obtain product **2** as the tetrasodium salt, as a yellowish solid in a 95% yield (97 mg). ^{1}H NMR (200 MHz, D_2O): δ 3.56 (t, J = 12.8 Hz, 2H) ppm. ^{13}C NMR (50 MHz, D_2O): δ 63.5 (t, J = 55.9 Hz) ppm. ^{31}P NMR (81 MHz, D_2O): δ 77 ppm. HRMS (MALDI) m/z calcd for $CH_3P_2S_4O_2$ [M-H]$^-$: 238.864, found: 238.865.

Potentiometric pH Titrations. All potentiometric titrations were performed under an argon atmosphere. Every titration proceeded for 30−45 min. The determination of the acidity constants, K^H_{HL}, K^H_{LH2}, and K^H_{LH3} (L -ligand), was made by the titration of 4.5 mL of aqueous 4 mL HNO_3 in $NaNO_3$ (from a 4.4 mM HNO_3/ $NaNO_3$ stock solution, I = 0.1 M) and 0.5 mL ligand (from a 4.5 mM MDP/MDPT stock solution). The acidity constants were calculated with HYPERQUAD software.[33] The determination of the stability constants, K^M_{ML}, K^M_{MLH}, $K^M_{ML(OH)}$, $K^M_{ML(OH)2}$, and p$K_a{}^H_{MLH}$ (M-metal) was achieved by the titration of 3−3.5 mL of aqueous HNO_3 in $NaNO_3$ (from a 4.4 mM HNO_3 stock solution, I = 0.1 M), 0−0.5 mL $NaNO_3$ (I = 0.1 M), 0.5 mM ligand (from a 4.5 mM ligand stock solution, I = 0.1 M), and 0.5 mL of $Zn(NO_3)_2$ or $Ca(NO_3)_2$ (from a 4.5 mM $Zn(NO_3)_2$ or $Ca(NO_3)_2$ stock solution, I = 0.1 M). The metal-ion:ligand ratio was 1:1. The pH range of all titrations was about 2.5−10.3. The end points of the MDP/MDPT-complex titrations were obtained by the second derivative method.[34] Each titration was repeated up to 4 times. The stability constants were calculated with the HYPERQUAD software. Both speciation and titration simulations were achieved with the HYSS2009 software.[27]

ESR Monitored OH Radical Assay. The ESR settings for OH radical detection were as follows: microwave frequency, 9.76 GHz; modulation frequency, 100 kHz; microwave power, 6.35 mW; modulation amplitude, 1.2 G; time constant, 655.36 ms; sweep time 83.89 s; and receiver gain 2 × 10^5 in experiments with Cu(I) and Fe(II).

One mM $Cu(CH_3CN)_4PF_6$ in acetonitrile (10 μL) or 1 mM $FeSO_4$ (10 μL) was added to 5−500 μM of a tested compound (10 μL) solution. All final solutions of $Cu(CH_3CN)_4PF_6$ contained 10% v/v acetonitrile. Afterward, 1 mM Tris buffer, pH 7.4 (10 μL) was added to the mixture. After mixing for 2 s, 100 mM DMPO (10 μL) were quickly added followed by the addition of 100 mM H_2O_2 (10 μL). The final sample pH values for the Cu(I) and Fe(II) systems ranged between 7.2 and 7.4. Each ESR measurement was performed 150 s after the addition of H_2O_2. All experiments were performed at room temperature in a final volume of 100 μL.

UV/vis Measurements of MDPT-Fe(II)/Cu(I) Complexes. To a 0.05 mM aqueous solution of MDPT (1490 μL), 5 μL of 5 mM $(NH_4)_2Fe(SO_4)_2$ in water or 5 mM $[Cu(CH_3CN)_4]PF_6$ in CH_3CN were added each titration point. After each addition the absorbance was measured at 272 and 254 nm respectively.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Further details are given about the titrations of methylenediphosphonotetrathioate (MDPT), **2** with Fe(II) as monitored by UV−vis spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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