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(54) Title: COMBINATORIAL LIBRARY OF BIFUNCTIONAL MOLECULES

(57) Abstract: The invention discloses a novel combinatorial library comprising a plurality of bifunctional molecules having the formula CF - PF wherein CF is a moiety of a specific chemical or biological activity and PF is a second moiety imparting the molecule tunable physical properties. Structural modifications in the second moiety vary the physical properties of the bifunctional molecule without significantly modifying the chemical or biological activity, for obtaining optimized effect of the chemical or biological activity at the environment where the chemical or biological activity is to be expressed. Optionally the second moiety may further imparts the molecule a second specific chemical or biological activity.



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COMBINATORIAL LIBRARY OF BIFUNCTIONAL MOLECULES

The present invention relates and claims priority from US provisional patent application
5 serial number 60/310,360 titled BIFUNCTIONAL MOLECULES, filed 6 August 2001.

FIELD OF THE INVENTION

The present invention relates to chemical combinatorial libraries in general and
10 to a combinatorial library of bifunctional molecules in particular.

BACKGROUND OF THE INVENTION

The field of combinatorial chemistry has developed rapidly over the last decade,
15 motivated by the search for new molecules for use in various medical and agricultural
applications. The concept of bifunctional molecules is also well known. However, the
term "bifunctional" usually describes two distinctly different chemical entities combined
together. An example for this type of bifunctionality are targeted molecules, where one
entity selectively binds to a specific site while the other entity, e.g., a drug, has the
20 desirable chemical or biological activity to be expressed at this particular site. In a typical
library of bifunctional compounds, variation in one of the functional groups is meant to
cause a chemical variation.

The present invention discloses a unique class of bifunctional compounds where
two functions, different by nature are presented. The first is a specific chemical or
25 biological activity which is kept unchanged, while the second has a sole physical
function for tuning the physical properties of the molecule.

Throughout the application various publications are referenced by superscript
numerals. Full list of the cited publications may be found at the end of the specification
preceding the claims.

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SUMMARY OF THE INVENTION

A general aspect of the present invention is a combinatorial library comprising of a plurality of bifunctional molecules wherein each member of the library comprises a first moiety being of a specific chemical function and a second moiety being of specific physical properties. In a library in accordance with the present invention, members in the library differ from each other by variations either in the first moiety, in the second moiety or in both. Variations among members cause changes in two vectors. When the chemical function is fixed, the physical function may be changed and vice versa, i.e., when the physical function is fixed the chemical function may be changed. In accordance with the present invention, the second moiety optionally further imparts the molecule a second chemical function.

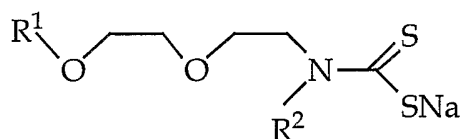
Another aspect of the present invention is a combinatorial library comprising of a plurality of bifunctional molecules of the structure CF – PF wherein, CF is a moiety of a first specific chemical or biological activity; and PF is a second moiety imparting the molecule certain physical properties and wherein structural modifications in the second moiety vary the physical properties of the bifunctional molecule without significantly modifying the first chemical or biological activity for obtaining optimized effect of the first chemical or biological activity at the environment where the chemical or biological activity is to be expressed. The varied physical properties are preferably one or combination of the following: lipophilic solubility, hydrophilic solubility, lipophilic-hydrophilic partition, surface activity, transport properties and hydrolysis rate.

In accordance with the present invention, the first moiety can be any bioactive or agroactive group such as a drug, a cosmetic, a peptide, a hormone, a UV responsive molecule, a light responsive molecule, an ultrasound responsive molecule, a microwave responsive molecule, an NMR or EPR responsive molecules, an olfactory molecule, a taste-responsive molecule, an oligonucleotide, a nucleic acid, a protein etc. The second moiety can be an amphiphilic group, more preferably an oligoether group. Optionally, the second moiety further imparts the molecule a second specific chemical or biological activity which is significantly unchanged when the physical properties of the bifunctional molecule are varied.

In accordance with a preferred embodiment of the present invention the first moiety is a metal chelator, more preferably the first moiety is a disubstituted dithiocarbamic acid or a salt thereof and the second moiety is an amphiphilic group,

more preferably an oligoether group.

In accordance with another preferred embodiment the bifunctional molecules are of the formula:



- 5 where R¹ and R² are independently a straight or branched, substituted or known substituted alkyl of 1 to 20 carbons, more preferably R¹ is selected from the group consisting of ethyl and butyl and R² is an alkyl of more than 6 carbons, most preferably R² is selected from the group consisting of hexyl, octyl, decyl and dodecyl

10 Yet according to another preferred embodiment of the present invention the second moiety is a herbicide molecule, more preferably a paraquat molecule and the first moiety is disubstituted dithiocarbamic acid or a salt thereof.

A third aspect of the present invention is a bifunctional molecule comprising a first moiety and a second moiety, the first moiety imparts said molecule a first specific chemical activity and the second moiety imparts the molecule specific physical
15 properties and optionally a second specific chemical activity, wherein structural modifications of said second moiety tune the physical properties of said bifunctional molecule without significantly modifying said first and optionally second chemical activities for obtaining optimized effect of said first and optionally second chemical activities at the environment where said chemical activities are to be expressed.

20

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be understood and appreciated more fully from the following detailed description taken in conjunction with the drawings in which:

- 5 Figure 1: Scheme 2 gives the general scheme of the synthesis of dithiocarbamates and their methyl esters.

$R_1 = \text{Et or Bu}$; $R_2 = \text{Et, Bu, Hex, Octyl, Decyl or Dodecyl}$

Figure 2: Rates of decomposition of Na, K and Li salt (equi-molar solutions).

- 10 Figure 3: Formation of Cu(DTC)_2 complex upon addition of Cu^{2+} ions to the Fe(DTC)_3 complex. Spectra: Fe(DTC)_3 (0.5 mM in 75% EtOH) - thin line; Cu(DTC)_2 (0.1 mM in 75% EtOH) - dotted line; the final mixture, containing both Cu(DTC)_2 and Fe(DTC)_3 complexes - thick line.

- 15 Figure 4: The stepwise transformation of Fe(DTC)_3 to Cu(DTC)_2 . Spectrum A: solution containing about 50 μM Fe(DTC)_3 (and an excess of Fe^{3+}) after addition of CuSO_4 , time of incubation = 0 min. Spectrum B: the same solution after 18 minutes of incubation. The intervening lines were from scans of approximately 3-minute intervals.

- 20 Figure 5: Inhibition of the dismutation activity of CuZnSOD by amphiphilic dithiocarbamates (DTCs). The activity of SOD was measured after preincubation of the enzyme with different amounts of DTCs for 2.5 h, as described in the Experimental section. Data are presented as percent of control without chelators. The curves marked with the same letter were not significantly different from each other, which was supported by linear regression analysis (at 95% confidence interval), as per Experiment.

Figure 6: Recovery of SOD dismutation activity after incubation of inactivated by Et_2DTC enzyme with copper ions

- 25 Figure 7: Inhibition of *peroxides* activity of SOD by different DTCs. EPR spectra of the $\text{DMPO}^{\bullet}\text{OH}$ adduct formed in solutions containing H_2O_2 and CuZnSOD, as described in the Experimental. 1 mM SOD was kept at room temperature in carbonate buffer with pH 10.2 (spectrum A), SOD was preincubated in the same buffer with 25

mM Et₂DTC (spectrum B), 25 mM Et-Hex-DTC (spectrum C), 25 mM Et-Oct-DTC (spectrum D) and 25 mM Bu-Oct-DTC (spectrum E).

Figure 8: Differential effects of dithiocarbamates on the rates of copper removal from SOD. Measurements were made spectrometrically at 0.1 mM SOD and 0.5 - 3 mM DTC in 50 mM carbonate buffer pH = 10.2 for the linear plot of the first 30 seconds of complex formation. Mean values were calculated on the basis of two to four independent experiments. An ANOVA test was performed for the statistical analysis (see Experimental). Means with the same letter are not significantly different (at $\alpha = 0.05$).

Figure 9: Equilibrium surface tensions vs concentration of DTCs.

Figure 10: Lack of disruption of liposome membranes by DTCs.

Figure 11: Full substitution of calcein from the calcein-copper complex by DTC. The fluorescent spectra of compounds in Tris-saline buffer: 0.6 μ M calcein solution - (Cal); 0.6 μ M Cal-Cu complex - (Cal-Cu); and 0.6 μ M Cal-Cu in the presence of 3.5 μ M Et₂DTC (one minute after addition of DTC) - (Cal-Cu + DTC).

Figure 12: Scheme 3 is a schematic description of the experiment on the influence of DTC-Cu complex on the stability of liposomes

Figure 13: Detection of both calcein and calcein-copper complex in the inner volume of vesicles after the second gel filtration. The fluorescent spectra of the liposomes before and after treatment with DTC, after the second gel filtration and after Triton X-100 and EDTA addition are shown. The fluorescent signals: 1 - after the first gel filtration; 2 - after the treatment with less than stoichiometric amount of DTC; 3 - after the second gel filtration (sample was diluted on the column); 4 - after the addition of EDTA and Triton X-100. Numbers on the fluorescent spectra correspond to the numbers of the steps of the experiment, presented in the Scheme 3

Figure 14: Substitution kinetics of Cu²⁺ by DTC from calcein-copper complex in buffer solution and in liposomes (appearance of free calcein ligand upon addition of equi-molar concentrations of DTC). DTC are Et₂DTC, Et-Hex-DTC and Bu-Oct-DTC.

Figure 15: Scheme 4 synthesis of PQ-DTC

Figure 16: Scheme 5 general formula for inner salt structure

Figure 17: Scheme 6 structural formula of the monosubstituted MPQ-DTC (compound 7) is an inner salt

Figure 18: UV absorption spectra of PQ-DTC, the precursor of PQ-DTC--- compound 5, Et₂DTC and the spectrum difference between PQ-DTC and compound 5.

- 5 Figure 19: Chelation of copper by PQ-DTC. Changes in the spectrum of 0.1 mM PQ-DTC solution upon addition of different amounts of Cu²⁺ ions (as CuSO₄ solution), formation of PQ-DTC band at 435 nm.

- 10 Figure 20: Chelation of iron by PQ-DTC. Changes in the spectrum of 0.1 mM PQ-DTC solution upon addition of different amounts of iron ions (as FeSO₄ solution), no complexation was observed.

Figures. 21A-D: Variation in UV-visible absorption spectra with time for 5×10⁻⁴ M PQ-DTC in different solvents.

Figure 22: The stability of PQ-DTC in different pH

- 15 Figure 23: Inhibition of the dismutation activity of CuZnSOD by Et₂DTC, PQ-DTC and compound 5.

Figure 24: The weak herbicidal activity of PQ-DTC in vivo system.

DETAILED DESCRIPTION OF THE INVENTION

The present invention discloses a unique class of bifunctional compounds. Normally, "bifunctional" describes two distinctly different chemical entities. In a library of bifunctional compounds, variation in one of the functional groups is meant to cause a chemical variation. In the present case, we have two functions, different by nature. The first is a specific chemical or biological activity which is unchanged and has the sole chemical function of binding metal ions). The second has a sole physical function of trying to change the physical properties of the molecule. Thus, we present a bifunctional molecule of the general type:

chem. function — physical function

Or

CF — PF

In the following, two examples of combinatorial libraries in accordance with the present invention are demonstrated and discussed in detail. In the first example (part A), CF was chosen to be a metal chelator of the dithiocarbamic acid (DTC) class, of known selectivity to Cu(II) ions. The PF was chosen as an oligoether group. Such a group, because it is an amphiphile, allows the change of solubility, surface activity, and transport properties without affecting other chemical properties, such as the property of the CF group. Thus, the first library (part A in the following) comprises a plurality of metal chelator – amphiphile bifunctional molecules, more precisely of disubstituted diethyldithiocarbamate (DTC) – oligoether, wherein the chemical function (CF) is the chemical ability to bind specific metal ions. The physical function (PF) is introduced in the form of an amphiphile which allows changing the physical properties of the molecule, in this case the micellar properties such as surface tension. The second library (part II in the following) is of a herbicide – metal chelator bifunctional molecules, more precisely of paraquat – DTC. In this case the amphiphile group of the first library is replaced by paraquat, which is not only an amphiphile but in addition imparts the bifunctional molecules a second chemical activity of the herbicide type.

The two libraries presented here were studied as special cases for obtaining controlled inhibition of the enzyme Cu/Zn superoxide dismutase (SOD), one of the most important enzymes of the antioxidant defense system of aerobic organisms, and of great

importance for medical and agricultural applications. However, it will be appreciated by persons skilled in the art that the two libraries described in the following, are given for the sake of clarifying and demonstrating and that other similar libraries can be constructed along the same lines without departing from the scope of the present invention. In particular, the present invention can be implemented for constructing libraries of various bioactive molecules. For example, the amphiphile physical function (PF) can be combined with a chemical function (CF) of any bioactive group or agroactive group such as a drug, a cosmetic, a peptide, a hormone, UV or light responsive molecules, an ultrasound responsive molecule, a microwave responsive molecule, an NMR or EPR responsive molecules, an olfactory molecule a taste-responsive molecule, a oligonucleotide, a nucleic acid, a protein, etc. Said groups can be defined as Interactive Functions (IF) having affinity interaction or long term interaction, such as host-guest interaction or agonist-antagonist interaction, with a specific site or molecule in the media where said molecules are to be expressed. By constructing such IF-amphiphile libraries, it will be possible to monitor the physical properties of large sections of bioactive molecules of great importance in human and animal care and in agricultural applications. Furthermore, instead of with amphiphilic moiety, said groups can be combined with a moiety having a controlled hydrolysis, and in particular a DTC (see Table 3 below) in applications such as drug delivery, where said moiety of controlled hydrolysis serves for allowing slow and/or controlled release of the other moiety.

A. Amphiphilic dithiocarbamates

Introduction

In the fight to produce ever-increasing quantities of essential food staples such as wheat, rice, and various other grains, farmers apply herbicides to combat over-abundant weeds. The sturdy weeds respond by developing a protection system based on antioxidant enzymes, such as superoxide dismutase (SOD). The activity of this enzyme must be considered in the broad biological phenomena of oxidative stress.

Oxidative stress resulting from the excessive production of reactive oxygen species (ROS) is an important phenomenon in many biological systems.¹ Aerobic organisms had to evolve an antioxidant defense system to exist. This system may be comprised of (a) low molecular weight compounds that scavenge oxidants and/or (b) antioxidant enzymes that either scavenge oxidants or produce or regenerate scavengers.²

Superoxide dismutase (SOD), one of the most important antioxidant enzymes, is found in all aerobic organisms studied. Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide anion ($O_2^{\bullet-}$) to O_2 and H_2O_2 .^{3,4} Despite this important function of SOD, this enzyme may not be tolerated in excess, because some disorders are associated with excessive SOD activity coupled with an inability to detoxify peroxide.

CuZnSOD (EC 1.15.1.1) in the presence of high concentrations of H_2O_2 exhibits the peroxidase activity utilizing hydrogen peroxide as a substrate.⁵ The molecular mechanism of this reaction is currently under active discussion.⁶ It is known that increased levels of CuZnSOD can stimulate lipid peroxidation⁷ and induce cell death⁸. It has been hypothesized that overexpression of CuZnSOD may be connected to neurodegenerative diseases.^{9,10}

It was found that high SOD activity is partially responsible for the failure of herbicide action on some weeds. In other words, such weeds are capable of overpowering the oxidative stress induced by the herbicides¹¹. This led to the idea that inhibitors of SOD can allow the killing of weed species at lower doses of photooxidant-generating herbicides.¹² This concept of combining herbicides and SOD inhibitors to reduce the load of herbicide use is the basis for the present work.

One way to inhibit CuZnSOD is to inactivate its copper cofactor with copper-specific chelators. In the following, combinatorial libraries of bifunctional copper-specific metal chelators are proposed as inhibitors of superoxide dismutase and, hence, as augmentors (or synergists, as we propose to call them) to the action of herbicides.

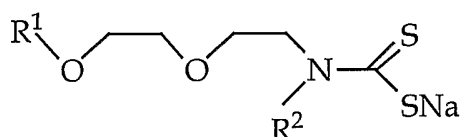
The rationale for this proposal is that Cu/Zn SOD has a catalytically active copper atom on each of two identical subunits of a dimer.¹³ Chelators such as diethyldithiocarbamate can remove copper from superoxide dismutase, inhibiting the catalytic activity of this enzyme.¹⁴ Zinc does not directly participate in the catalytic process¹⁵ and is therefore of less interest as a target for complexation. The inhibition of superoxide dismutation activity of SOD due to the complexation of Cu^{2+} in the active site of SOD by diethyl-dithiocarbamate is well known.¹⁶

In accordance with the above, Part A describes a limited "sublibrary" comprising bifunctional metal chelators wherein the first chemical function is the metal chelator DTC (sodium diethyldithiocarbamate (Et_2DTC)), which has the sole chemical function

of specific metal binding and the second function is an amphiphile group (more precisely, an oligoether), having a sole physical function: changing the physical properties of the molecule. Thus, in accordance with the general structure of the bifunctional molecules of the present invention:



the structure of “CF—PF bifunctional molecule” described in this part is a DTC group attached to a variable hydrophobic alkyl group (R^2) and a variable hydrophilic oligoether group ending with R^1 having the general formula (Scheme 1):



10 In the following sections, novel copper chelators with variable amphiphilic properties: disubstituted dithiocarbamates (DTCs, $R^1(CH_2CH_2O)_2NR^2CS_2Na$) with one alkyl (R^2 = hexyl, octyl, decyl or dodecyl) and one oligoether ($R^1(CH_2CH_2O)_2$, where R^1 = Et or Bu) substituents, are disclosed and discussed. The synthesis of the amphiphilic copper ion of Scheme 1 and their specific balance of lipophilic-hydrophilic properties are presented.. We then explore all the key-properties required for their success as herbicide synergists, namely, suitable partition coefficients, effective transport properties (through lipid bilayer models) and, mainly, effective inhibition of the enzyme SOD. The octanol-water distribution ratios of the dithiocarbamates (partition measurements by the Shake-Flask method) and their penetration through the liposome bilayer were measured to predict their transport behavior through biological membranes. The comparative copper binding constants and the stepwise transformation of $Fe(DTC)_3$ to $Cu(DTC)_2$ were measured and show the selectivity of the ligands for copper over iron. Differential effects of dithiocarbamates on the rates of copper removal from SOD are shown.

25 The influence of DTCs on SOD superoxide dismutation activity was measured by the cytochrome C/xanthine/xanthine oxidase assay. The SOD dismutation activity was recovered after incubation of inactivated SOD with copper. Inhibition of peroxidase activity of SOD by different DTCs was determined using EPR spectra of the $DMPO \cdot OH$ adduct formed in solutions containing H_2O_2 and $CuZnSOD$ and in the presence of the spin trap compound. Part A also describes the preparation of phospholipid vesicles incorporating copper ion and a fluorescent chelator, as well as the use of a sensitive fluorescent method for determining the rate of transport of the metal chelating

amphiphiles into the liposomes.

The conclusions are that the addition of an oligoether chain of up to eight aliphatic carbon atoms to the structure of dithiocarbamates leads to an increase in the hydrophobicity (relative to Et₂DTC) of more than 1000 fold, but only a 2.3 fold decrease in the ability to inhibit SOD dismutation activity. However, the rates of decomposition of Na, K and Li salts are tremendously enhanced. These unexpectedly high rates of hydrolysis may be due to high interfacial activity as may be deduced from the preliminary interfacial tension and CMC data. DTCs have close similarity in their ability to transport through bilayer membranes when the rates of substitution of Cu²⁺ by DTCs from calcein-copper complex in buffer solution and in liposomes are compared.

Structural attenuation leads to a balance in desired properties. Library members with decyl and dodecyl groups are poor inhibitors of SOD dismutase activity. Amphiphilic dithiocarbamates (hexyl and octyl substituted) reduce the peroxidase activity of SOD.

Table 1 lists the bifunctional molecules that were studied:

Table 1 Structures of Amphiphilic Dithiocarbamates

$$\begin{array}{c}
 \text{R}_1 \\
 \diagdown \\
 \text{N} - \text{C} = \text{S} \\
 \diagup \quad \quad \quad \diagdown \\
 \text{R}_2 \quad \quad \quad \text{SNa}
 \end{array}$$

compound	R ₁	R ₂
Et-Et-DTC	Et(O(CH ₂) ₂) ₂ -	Et
Et-Bu-DTC	Et(O(CH ₂) ₂) ₂ -	Bu
Et-Hex-DTC	Et(O(CH ₂) ₂) ₂ -	Hex
Et-Oct-DTC	Et(O(CH ₂) ₂) ₂ -	Octyl
Et-Dodec-DTC	Et(O(CH ₂) ₂) ₂ -	Dodecyl
Bu-Oct-DTC	Bu(O(CH ₂) ₂) ₂ -	Octyl
Bu-Dec-DTC	Bu(O(CH ₂) ₂) ₂ -	Decyl
Bu-Dodec-DTC	Bu(O(CH ₂) ₂) ₂ -	Dodecyl
Bu ₂ DTC	Bu	Bu

Results and Discussion

1. Synthesis

N-Alkyl-*N*-(2-(2-ethoxyethoxy)ethyl)dithiocarbamates or *N*-Alkyl-*N*-(2-(2-ethoxyethoxy)butyl)dithiocarbamates were synthesized according to the Scheme shown in Scheme 2 in Fig. 1. Synthesis of glycol(alkyl)dithiocarbamates can be conventionally divided into two steps: step one is the synthesis of secondary amines; step two is the synthesis of dithiocarbamates.

The method used for the preparation of 2,3-dioxypropyl(alkyl)amines (reaction of 2,3-dioxypropylchloride with 8-10 times excess of alkylamine)¹⁷ was adapted for the synthesis of alkyl(ethoxyethoxyalkyl)amines. Relatively pure secondary amines in good yields were prepared from ethoxyethoxyethylchloride or ethoxyethoxybutylchloride using 10-fold excess of primary amines. The main problem in this alkylation step was the formation of aminehydrochlorides, which hampered the distillation process, blocking the distillation apparatus and reducing the yield of secondary amines. This problem appeared only during the synthesis of more lipophilic amines with the long alkyl chains. Addition of sodium carbonate to the reaction mixture during synthesis and washing the chloroform-amine solution with a saturated aqueous solution of sodium hydrocarbonate (the common procedure in the synthesis of secondary amines by alkylation of primary amines) did not solve the problem. Finally, the amine hydrochloride was decomposed by prolonged stirring in concentrated NaOH solution (2M) with further washing with water.

The synthesis of sodium dithiocarbamates¹⁸ is performed by reaction of a secondary amine with CS₂ in the presence of NaOH. Et-Oct-DTC and Bu-Oct-DTC were synthesized from the corresponding amines by the reaction with NaOH and carbon disulfide in water. These compounds were then purified by washing with cooled diethyl ether. More lipophilic dithiocarbamates (Bu-Dec-DTC and Bu-Dodec-DTC) were prepared from the reaction of the corresponding secondary amines in tetrahydrofuran with sodium hydride and an excess of carbon disulfide. The increasing lipophilicity of the dithiocarbamic salts, due to the increasing size of the aliphatic substituents on nitrogen, produced problems in the process of purification of the DTCs after synthesis. More lipophilic dithiocarbamates were less soluble in water and had a good solubility in Et₂O. Therefore, these compounds were purified by several consecutive recrystallizations from cooled hexane solutions. All dithiocarbamates were characterized by ¹H NMR. The dithiocarbamate salts were converted to the corresponding methyl esters by alkylation

with methyl iodide prior to analytical characterization by TLC, NMR, mass spectra or elemental analysis.

Physical properties of dithiocarbamates

The partition coefficient (P_{ow}) and the distribution ratio (D_{ow}) between octanol
 5 and water are the most commonly used expressions for the hydrophobicities of compounds.²⁰ The degree of octanol/water partitioning is useful in predicting the *in vivo* transport properties of any compound through hydrophobic barriers (cell membranes skin or leaf cuticles).

The octanol/water partition measurements of the DTCs are presented in Table.2.

10

Table 2 Distribution ratios D_{ow} for different dithiocarbamates, measured by the Shake-Flask Method

Compound	D_{ow}^a
Et ₂ DTC	less than 0.02 ^b
Et-Et-DTC	(0.02-0.03)
Et-Bu-DTC	0.05
Et-Hex-DTC	0.14
Bu ₂ DTC	0.21
Et-Oct-DTC	1.88
Bu-Oct-DTC	21.45

^a Distribution ratios $D_{ow} = C_{oct}/C_w$, where C_{oct} and C_w are concentrations of dithiocarbamic acids in octanol and in water phases respectively.

15 ^b Partitioning of Et₂DTC was measured at water/octanol volume ratio 100 to 1. The accuracy of this measurement is low because of the low solubility of Et₂DTC in octanol

20 The results show that increasing length of the aliphatic substituent (R^2) and the aliphatic part of the oligoether chain (R^1) results in an increase in the octanol/water distribution ratios (Table 2).

The distribution ratios of the compounds Et-Dodec-DTC, Bu-Dec-DTC, and Bu-Dodec-DTC were not measured, due to technical difficulties arising from their high lipophilicity, relatively low stability, and high surfactant activities. Rough estimations show that they are more lipophilic than Bu-Oct-DTC, with $D_{ow} > 50$.

Hydrolytic stability of sodium salts of dithiocarbamic acids

Sodium dithiocarbamates decompose to amines and carbon disulfide²¹ in a pH-dependent reaction, occurring in the course of several hours for Et₂DTC at neutral pH values. At higher pH values (10 to 12) the stability of this compound is in the range of weeks. We were therefore surprised to observe, even at high pH values, unusually fast rates of decomposition for the amphiphilic DTCs. We surmised that some enhanced hydrolysis might be due to an alkali metal complexation by the oligoglycol side chains (a crown ether effect) or due to a micellar effect. The semi-quantitative data in Table 8 below show that the rates of decomposition of amphiphilic dithiocarbamates may be linked to their lipophilicity and perhaps to their surface tension properties. More accurate experiments to understand this phenomena are planned.

The stability of sodium salts of dithiocarbamic acids with varying substituents

The hydrolytic stabilities of the various dithiocarbamic acids (as sodium salts) were determined to address the first possibility that of a catalytic enhancement effect. The stabilities if the DTCs were measured spectrophotometrically in 0.1 mM NaOH solution at an initial pH of 10 at about 260 nm (the λ_{max} was changed slightly for different compounds), and in borate buffer at pH 8.0, 9.2 and 10.5. The time of "half maximal" decrease of UV absorbance of dithiocarbamic acids was taken as the time of 50% decomposition of these compounds (t_{50}). Data on decomposition of dithiocarbamic acids are presented in Table 3.

Table 3 Time of 50% decomposition of a series of DTC

compound	pH 8.0	pH 9.2	pH $\approx$ 10 ^a	pH 10.5
Et ₂ DTC	3.8 days ^b	> 35 days ^b	> 10 days	
Et-Hex-DTC			> 10 days	
Et-Oct-DTC			3.4 days	
Et-Dodec-DTC	< 10 min	1.6 hours	4.1 hours	2 days
Bu-Oct-DTC	10.6 hours	-	2.3 days	13 days
Bu-Dec-DTC	-	-	9.6 hours	-
Bu-Dodec-DTC	-	-	50 min	10.6 hours

^a These measurements lasted only 10 days

^b These values were taken from the paper.²²

The absorbance peak of CS₂ (the product of DTC decomposition) at $\lambda = 205$ nm increases quickly for rapidly decomposing compounds, such as Et-Dodec-DTC, Bu-Dec-DTC, and Bu-Dodec-DTC. The CS₂ spectrum overlaps with the absorbance spectrum of the dithiocarbamic ligand, rendering the UV absorbance measurements imprecise.

Another method (e.g., iodometric titration) must be applied for an absolutely accurate measurement of DTC concentrations. The data in Table 3 show that chemical instability increases for long aliphatic substituents. The rates of decomposition of amphiphilic dithiocarbamates are positively correlated with the lipophilicity of the compounds.

10 **Influence of different cations on the stability of the dithiocarbamates**

It was necessary to address the possibility that the (CH₂CH₂O)₂- group exerts an influence on the hydrolytic decomposition of the dithiocarbamic salts. To that end, the sodium, potassium and lithium salts of the octyl-2-(2-ethoxyethoxy)ethyl dithiocarbamic acid were synthesized by the reaction of the corresponding amine with CS₂ in water in the presence of NaOH, KOH, and LiOH, respectively (see experimental part).

The decomposition of Et-Oct-DTC (Na, K, and Li salts) was studied in water at pH 8.4 by UV/VIS spectrophotometer. Absorbance at $\lambda = 260$ nm, 286 nm decreased and that of the peak at $\lambda = 205$ nm increased with time. The rates of decomposition of Na, K and Li salts are shown in Figure 2 and Table 4

Table 4 Influence of the cation on the time of half decomposition of Et-Oct-DTC

cation	t ₅₀ , min
Na	2200
K	2450
Li	2200

The times of half decomposition of Na, K, and Li salts of octyl-2-(2-ethoxyethoxy) ethyl dithiocarbamic acid are presented in Table 4. They are not significantly different from each other.

The stability of Et-Oct-DTC is independent of the cation present in the dithiocarbamic salt. Thus, a "crown ether effect" may not be ruled out for the DTCs with

the short-chain oligoglycol substituent (up to two $\text{CH}_2\text{CH}_2\text{O}$ groups) and requires synthesis of longer-chain oligoether substituents.

Metal binding properties

5 a) Stability constants of $\text{Cu}(\text{DTC})_2$ complexes

The comparative stability constants of Et-Et-DTC, Et-Bu-DTC, Et-Hex-DTC, and Bu_2DTC were determined spectrophotometrically by the competition method described by Janssen.^{23, 24} This method is based on the competition of dithiocarbamate ligands with 8-hydroxyquinoline ligands for copper. Its application to amphiphilic DTCs is described in detail in literature.²⁵ The competition (K_C) values presented in this article show the relative competitive stabilities of $\text{Cu}(\text{DTC})_2$ complexes over $\text{Cu}(\text{hydroxyquinoline})_2$ (Table 5). The higher the K_C values are, the stronger the copper-dithiocarbamate complex is.

15 Table 5 K_C values for different dithiocarbamates, determined in 75% aqueous *tert*-butanol²⁵

Compound	K_C
Et-Et-DTC	0.34×10^2
Et-Bu-DTC	0.89×10^2
Et-Hex-DTC	1.07×10^2
Bu_2DTC	33.11×10^2

The overall stability constant of $\text{Cu}(\text{DTC})_2$ complex β_2 is equal to $K_C \times \beta'_2$, where β'_2 is the stability constant of $\text{Cu}(\text{hydroxyquinoline})_2$ complex. Thus, β'_2 (determined in the same solvent as K_C) is required for obtaining an overall stability constant of $\text{Cu}(\text{DTC})_2$. We did not measure β'_2 in water-*tert*-butanol system, but for the very similar water-ethanol system, $\beta'_2 = 1.6 \times 10^{26}$, which is rather high (i.e., 8-hydroxyquinoline forms a strong complex with copper). K_C for all the dithiocarbamates mentioned in Table 5 is larger than 1, therefore, we conclude that these DTCs are good complexing agents for copper.

b) Selectivity of DTC for copper over iron

Diethyldithiocarbamate has higher selectivity for copper over iron.²⁶ The comparative stabilities of $\text{Cu}(\text{Et-Hex-DTC})_2$ complex over $\text{Fe}(\text{Et-Hex-DTC})_3$ complex were determined to prove that the selectivity for copper over iron, typical to diethyldithiocarbamate, also holds true for all the amphiphilic DTCs in this work. For this purpose, $\text{Fe}(\text{DTC})_3$ complex was prepared, and the ability of Cu^{2+} ion to substitute Fe^{3+} from this complex was tested.

In the above experiment, ~100% replacement of Fe^{3+} from $\text{Fe}(\text{DTC})_3$ complex by Cu^{2+} was observed (Figure 3). The absorbance (at 434 nm) of the final solution is practically equal to the calculated absorbance of a theoretical final solution at 100% Cu-Fe exchange (with the composition: $\text{Cu}(\text{DTC})_2$ (0.1 mM), $\text{Fe}(\text{DTC})_3$ (0.43 mM), and Fe^{3+} (70 μM)).

The Cu-Fe exchange was also performed in water at pH = 2.8 to dissolve all the reaction components. This is a semi-quantitative experiment, as some amount of free DTC ligand could decompose during the complexation with iron. Both $\text{Fe}(\text{DTC})_3$ and $\text{Cu}(\text{DTC})_2$ complexes are stable at acidic pH, as ascertained in separate experiments (there were no changes in the absorbance of $\text{Fe}(\text{DTC})_3$ and of $\text{Cu}(\text{DTC})_2$ complexes at 343 nm and 438 nm, respectively, for 30 minutes at pH = 2.8).

The stepwise transformation of $\text{Fe}(\text{DTC})_3$ complex to $\text{Cu}(\text{DTC})_2$ complex, presented in Figure 4, clearly demonstrates the selectivity of the amphiphilic DTC for copper over iron.

Inhibition of superoxide dismutation (SOD) activity *in-vitro* by dithiocarbamates using xanthine/xanthine oxidase/cytochrome C system

The influence of dithiocarbamates with variable lipophilicities on the ability of SOD to superoxide radical generated by xanthine/xanthine oxidase was measured by cytochrome C reduction.²⁷ The amphiphilic dithiocarbamates, despite their steric bulkiness, are quite good inhibitors of superoxide dismutase (except Bu-Dec-DTC and Bu-Dodec-DTC) (Figure 5). The smallest ligand, Et_2DTC , works only slightly better than the bulky dithiocarbamates.

The ligands, Et-Hex-DTC, Bu-Oct-DTC, and Et-Oct-DTC (with the distribution ratios $D_{\text{ow}} = 0.14, 21.45, \text{ and } 1.88$, respectively) showed quite similar dismutation

activity, as supported by linear regression analysis ($\alpha = 0.05$, see experimental part). Et₂DTC ($D_{ow} < 0.02$) with a steppe slope and Bu-Dec-DTC ($D_{ow} > 50$) with its much smaller slope, were significantly different from the others (Et-Hex-DTC, Bu-Oct-DTC, and Et-Oct-DTC). The data for Bu-Dodec-DTC do not fit the model $\log(y) = \beta_0 + \beta_1 X$,
5 because Bu-Dodec-DTC starts to inhibit SOD activity at much higher [DTC]/[SOD] molar ratios than the other compounds. The DTC/SOD molar ratio leading to 50% decrease of SOD activity during preincubation (A_{50}) ranges from 3.4 for Et₂DTC to 7.9 for Et-Oct-DTC and Bu-Oct-DTC.

The inactivation of superoxide dismutase by sodium diethyldithiocarbamate is
10 time-dependent, and previous researchers reported variations from part of an hour to several hours.²⁸⁻³¹ After preliminary experiments, we chose 2.5 hour preincubation time of SOD with dithiocarbamates. This time is sufficient for completion of the inhibition reaction and was standard for all the compounds in this series.

The catalytic activity of SOD is pH independent in the range pH 5 to 9 and
15 decreases at more alkaline values.³² We used alkaline solutions (pH 10.2) for preincubation of the dithiocarbamates with SOD to reduce the decomposition of DTCs. pH 7.8 was used for measuring the SOD activity. The high pH could lead to some decrease of SOD activity. It was checked and found that this decrease of activity is mainly reversible by lowering the pH.³² Nevertheless, to correct for the influence of the
20 changes in pH, all measured SOD activity after incubation with DTCs was normalized against the activity of native SOD kept at pH 10 buffer for the same 2.5 hour preincubation period.

In preliminary control experiments, it was demonstrated that the DTCs did not directly inhibit the superoxide generating activity of xanthine oxidase or influence the
25 cytochrome C reduction at the concentrations used.

Excess Cu²⁺ ions added to inactivated SOD almost fully restored the dismutation activity of the enzyme (Figure 6).

Thus, the inhibition of superoxide dismutase by dithiocarbamates is the result of copper binding and not due to denaturation of the enzyme in water/organic media.
30

Effect of dithiocarbamates on CuZnSOD *peroxidase* activity (in-vitro) using spin-trapping and EPR spectroscopy

The ability of dithiocarbamates to inhibit the peroxidase function of SOD was

studied using spin-trapping and EPR spectroscopy. 5,5'-Dimethyl-1-pyrroline *N*-oxide (DMPO) was used as a spin trap. It produces a DMPO-•OH adduct from the •OH generated by CuZnSOD and H₂O₂.^{33, 34} Superoxide dismutase was preincubated in Et₂DTC or with amphiphilic dithiocarbamates for 2.5 hours at a 25:1 [DTC]/[SOD] molar ratio. This excessive amount of the chelators almost completely inhibited the SOD dismutation activity (see Figure 5). The generation of DMPO-•OH was also drastically decreased by preincubation of superoxide dismutase with Et₂DTC, Et-Hex-DTC, and Et-Oct-DTC (Figure 7).

Bu-Oct-DTC was less effective than other dithiocarbamates, for reasons not clear at this time. The Cu(DTC)₂ complexes did not produce paramagnetic DMPO adducts in the presence of H₂O₂ (data not shown), implying that no interfering signals from the Cu-DTC complexes were formed during the experiment.

Abstraction of copper from SOD: The rates of dithiocarbamate complexation of superoxide dismutase-bound copper

The efficiency of the copper chelators to abstract copper from the enzyme and to inhibit its activity was determined by measuring the kinetics of the formation of copper-dithiocarbamate complexes. The results are presented in Figure 8.

From the DTCs described in this paper, three copper-chelators that showed a good ability to inhibit SOD dismutation activity and one (Bu-Dodec-DTC) with much lower inhibition ability were chosen for the kinetic work (Figure 5). The rates of formation of Cu(Et-Hex-DTC)₂, Cu(Et₂DTC)₂, and Cu(Bu-Oct-DTC)₂ (Figure 8) were not significantly different, which was supported by analysis of variances (ANOVA, see Experimental). At higher concentrations of the dithiocarbamate ligands (Figure 8), the rate of Cu(Bu-Dodec-DTC)₂ formation was lower, compared with the other complexes.

Colored copper-diethyldithiocarbamate complex forms after the incubation of Et₂DTC with CuZnSOD.^{16b,35} The insoluble dark brown precipitates were sedimented from SOD by centrifugation.³⁵

An experiment analogous to that described in reference³⁵ was performed (see experimental part) to ascertain that the amphiphilic chelators could complex copper from SOD in a similar manner to that of diethyldithiocarbamate. Centrifugation at 39,000×g was sufficient for sedimenting the Cu-diethyldithiocarbamate complex (consistent with

reference³⁶), but 245,000×g was needed to sediment the Cu-Bu-Oct-DTC complex. The analyses of amounts of copper by ICP spectroscopy, both in the precipitate and in the supernatant, showed that the removal of Cu-Et₂DTC and Cu-Bu-Oct-DTC complexes from the copper-deficient protein was essentially complete. There was no precipitation of the Cu free enzyme during the centrifugation.

The surface-activity properties of amphiphilic dithiocarbamates: their influence on the stability of phospholipid bilayer membranes

Determination of CMCs

One of the controlling factors for the evaluation of surfactant interfacial performances is the determination of its critical micellar concentration (CMC). CMCs of amphiphilic dithiocarbamates (DTCs) were assessed from the Wilhelmy plate surface tension measurements, as previously described for other surface-active molecules.^{37a-37c} Figure 9 shows the equilibrium surface tensions measured for the different DTCs. Results of the CMC measurements are collected in Table 6.

Table 6 Surface activity data

Name of Compound	CMC (M)	Minimum Surface Tension in mN/m
Bu-Oct-DTC	$6 \cdot 10^{-7}$	52
Et-Hex-DTC	$2 \cdot 10^{-4}$	49
Et-Oct-DTC	$8 \cdot 10^{-7}$	49
Et-Dodec-DTC	$3 \cdot 10^{-6}$	44
Bu-Dec-DTC	$6 \cdot 10^{-5}$	35
Bu-Dodec-DTC	$7 \cdot 10^{-5}$	30

From the data summarized in Table 6, it is apparent that the absorption effectiveness, e.g., the maximum reduction in surface tension of the buffer, necessitates a butyl group in the R₁ position and a dodecyl group in the R₂ position. When the decyl group is replaced by dodecyl, the surface tension is lowered by 5 mN/m, but both compounds display their CMCs at almost the same concentration. These results coincide

with those recently observed with doubly modified carboxy-methylcellulose derivatives, which showed that the highest decay in surface tension took place when one of the CH_2COOH of cellulose was substituted by hexadecylamine (C_{16}) and the second CH_2COOH was substituted by octylamine (C_8). When octylamine was replaced by a shorter hydrocarbon butylamine - C_4 , the decay in surface tensions decreased^{37d}. Evidently, a narrower ethyl group in the R_1 position appeared to be insufficient to yield a substantial diminution in surface tension. The strange behavior of Et-Hex-DTC cannot be explained at this stage. However, in agreement with generally observed trends, the CMCs of DTCs bearing shorter groups both in the R_1 and R_2 positions resulted in CMCs appearing at lower solution concentrations. One possible explanation for this behavior is that DTCs bearing shorter groups form micelles at low solution concentrations, because short groups interact between themselves easier without being hindered.

Influence of amphiphilic dithiocarbamates on the membrane bilayer – using liposome model

In the design of metal chelators for *in vivo* use, the possible interrelation with the biological membranes is an essential factor to be considered. The possibility that the amphiphilic dithiocarbamates may cause some leakage of the phospholipid membrane of the cell cannot be excluded. To study such possible interaction, phospholipid vesicles (liposomes) were selected as a model for a biomembrane. The leakage and fusion of liposomes in the presence of the DTC and their copper complexes were carefully studied (see below).

The behavior of lipophilic, ionized compounds in lipid membrane-water systems is certainly different from that in octanol/water.^{38, 39} Therefore, some experiments were performed to determine the rate of penetration of amphiphilic DTC through cell membranes. In this case, the ability of dithiocarbamates to traverse the bilayer of phosphatidylcholine liposomes and to complex with copper ions incorporated into the inner volume of a liposome served as a model for the transport of DTCs into living cells.

In the first experiments, liposomes containing aqueous Cu^{2+} (as CuSO_4) in the internal volume were treated with DTCs on the external side of the liposome. The rates of formation of Cu-DTC chromophore complexes were followed at $\lambda_{\text{max}} = 440 \text{ nm}$. The application of UV/VIS spectrometry posed a problem, as the concentrations of encapsulated CuSO_4 needed to be high, which caused leakage of the liposomes. To

reduce the encapsulated CuSO_4 concentration, a thousand-fold higher-sensitivity fluorescent technique was introduced. The fluorescent method is based on a calcein-copper complex entrapped inside the liposome. Calcein is a water-soluble low-molecular-weight dye, which forms a non-fluorescent complex with Cu^{2+} ions.⁴⁰ The incoming DTC has a higher affinity for Cu^{2+} than calcein and removes copper from the calcein-Cu complex. This increases fluorescence and, thus, allows detecting the Cu-DTC complex formation (see below).

a) The effect of amphiphilic dithiocarbamates on the stability of membrane phospholipid bilayers

Small unilamellar vesicles (SUV) prepared from a phospholipid-cholesterol mixtures were used. The two amphiphilic dithiocarbamates (Et-Hex-DTC and Bu-Oct-DTC) show the highest ability to inhibit SOD activity was chosen. The ability of free DTC ligands and their DTC-copper complexes to cause leakage in liposomes was studied and compared with the well-known diethyldithiocarbamate. The liposomes were encapsulated with calcein at a self-quenching concentration,⁴¹ and the fluorescence emanating upon addition of varying concentrations of DTCs was measured.

The test is based on the fact that the fluorescence of polar-fluorescent dyes (e.g., calcein) is quenched at high concentrations. Various processes resulting in a dilution of the dyes produce an increase in the fluorescence, thereby providing a facile method for monitoring these events. Calcein entrapped in lipid vesicles at high concentrations displays very low fluorescence intensity because of self-quenching. If the liposomes lose integrity, the probe is released from the vesicles. The local concentration of calcein decreases and the amplitude of the fluorescent signal increases due to the dilution of calcein in the bulk-surrounding phase.

No significant fluorescence was observed when diethyldithiocarbamate or amphiphilic DTC was added to the vesicles. The addition of Triton X-100 (final concentration 0.5%) to the mixture was necessary for the destruction of the liposomes and for a drastic increase in fluorescence (Figure 10).

These results show that the addition of Et_2DTC solution up to 1 mM, and/or solutions of amphiphilic dithiocarbamates up to 0.1 mM concentration, do not cause disruption of the vesicles and leakage of liposomes.

The gradual formation of Cu-DTC complex inside the vesicles may cause vesicle eruption from the inside. The leakage of calcein-copper from the inside of SUV after

treatment with DTC was examined. When DTC is stoichiometrically added to the calcein-copper, or in excess, it fully substituted calcein (Figure 11).

The following careful procedure was developed. Egg lecithin/cholesterol small unilamellar liposomes (SUV) were prepared, containing calcein-copper complex in the internal volume. Compounds traverse the liposome membranes and substitute the copper ions from the calcein-copper complex inside the liposomes. When less than a stoichiometric amount of DTC ligand is used, the reaction of the ligand exchange inside the liposomes proceeds only partially. The vesicles then contain three components: DTC-copper complex formed; calcein-copper complex remained; and some free calcein was displaced by DTC from its copper complex. After reaction with DTC, the liposomes are returned to the gel filtration column (the second gel filtration procedure) to separate them from any released calcein or calcein-copper that would react with EDTA. The DTC-copper complex is lipophilic enough to penetrate through the bilayer to the external solution. If this penetration process does not cause leakage (or the penetration does not happen) and the vesicles remain intact, two things happen. The free calcein forms, and the calcein-copper complex remains in the internal volume of the liposomes after the second gel filtration (both calcein and calcein-copper do not traverse the liposome bilayer). Hence, complete lysis of the liposomes with Triton X-100 and subsequent addition of the DTC (or another ligand that can compete with calcein for copper, e.g., EDTA) will result in the liberation of the additional portion of free calcein ligand. This is noted by the increase of the fluorescence.

Scheme 3 in Fig. 2 shows the schematic representation of this experiment.

The presence of free calcein after the second gel filtration is shown in Figure 13 (spectrum 3 shows higher fluorescence than the spectrum 1).

Nevertheless, spectrum 3 lies lower than spectrum 2 because the liposome sample was 1.7 times diluted on the column. Figure 13 also demonstrates the presence of calcein-copper in the inner volume of liposomes after the second gel filtration. Actually, when the liposomes after the second column were broken by Triton X-100, the fluorescent signal increased as the result of the appearance of free calcein, due to the reaction of calcein-copper with EDTA (EDTA plays the same role as DTC, complexing Cu^{2+}) (spectrum 4).

Detection of both calcein and calcein-copper complex in the inner volume of vesicles after the second gel filtration means that the appearance of a Cu-DTC complex

does not influence the integrity of the liposomes.

b) Rates of penetration of dithiocarbamates through the liposome bilayer

The behavior of lipophilic, ionized compounds in lipid membrane-water systems will differ from these compounds behavior in octanol/water.³⁸ Therefore, we considered it useful to look at the transport properties of the amphiphilic DTCs in an anisotropic water/lipid membrane system, adopting a fluorescent method used for assessing chelation of intracellular iron, described in reference.⁴²

The rates of formation of Cu-DTC complexes were followed fluorimetrically by the appearance of free calcein ligand.

The penetration of dithiocarbamates (Et-Hex-DTC, Bu-Oct-DTC and Et₂DTC) through the liposome bilayer was measured by comparing the rate of Cal-Cu + DTC <— > Cal + DTC-Cu ligand exchange reaction in liposomes and in buffer solution (Figure 13). Octanol-water distribution ratios of Et-Hex-DTC and Bu-Oct-DTC are 0.14 and 21.45, respectively, and the distribution ratio of Et₂DTC is less than 0.02 (see Table 2).

The initial rates of substitution of calcein by DTC from calcein-copper complex in the liposomes (Figure 14) were calculated and compared with the rates in buffer solution (Table 7).

Table 7 Initial rates of substitution of calcein by DTC from calcein-copper complex in buffer solutions and in liposomes

compound	rates in liposomes, nM/s	rates in buffer, nM/s	buff/lip ratio*
Et ₂ DTC	12.6	20.5	1.63
Et-Hex-DTC	16.8	26	1.55
Bu-Oct-DTC	7.8	11.08	1.38

*- the ratio of rates in the buffer to the rates in the liposome solution

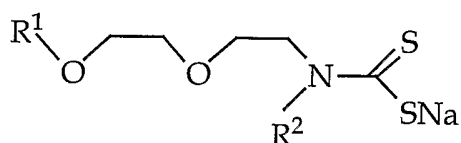
The crossing of a liposomal bilayer reduces the initial rates of the ligand exchange reaction only by a factor of 1.4-1.6 (Table 7). The measured rates consist of the addition of two steps: the penetration of the DTC through the membrane bilayer and the following ligand exchange reaction inside the liposomes. The close numbers for the penetration rates for the measured DTCs suggests that the liposome bilayer is an easy barrier for the dithiocarbamate ligands and that the ligand exchange reaction is the limiting step.

The stability experiments showed that neither amphiphilic DTC nor their copper complexes cause leakage of the phospholipid bilayer membranes.

Compounds Et-Hex-DTC, Bu-Oct-DTC, and Et₂DTC (with significant variance in the distribution ratios in the octanol-water system) exhibited similar abilities to
5 penetrate lipid bilayers in the time scale limited by the calcein-Cu-DTC ligand exchange reaction. This can be explained by the existence of the membrane-water interface,³⁸ which distinguishes between the lipid membrane-water and the octanol-water systems. Contrary to the bulk octanol media, the bilayers do not have to be electrically neutral. Electrostatic interactions at the membrane-water interface should be taken into account,
10 especially in partitioning ionized molecules (such as dithiocarbamic acids). Liposomes can accommodate charged molecules, sometimes to a large extent.³⁹ The difference between the two models of biological membranes is exemplified by the distribution of ionized pentachlorophenol, which is several hundred times greater for egg-PC membranes compared with octanol.³⁸

15 **Conclusive Summary**

The purpose of the development of a "CF-PF bifunctional chelators" concept (of which the DTC amphiphiles described here are the first example) is to aid herbicides in their action by using an auxiliary synergistic molecule--a copper-specific chelator SOD inhibitor. The field experiments described in a previous publication showed only very
20 limited improvements when one chelator was added to the herbicide in a mixture. By varying the oligoether component, it was possible to introduce large variations in the physical properties of the sub-library members without significant changes in the chemical properties of the DTC functionality. The overall results obtained are best presented by means of the tables. For the sake of clarity, we have condensed key features
25 from Tables 1-7 into Table 8. Of course, all the data in the figures could not be condensed, and, thus, must be referred separately.

Table 8 Structure, octanol/water distribution ratios, inhibition of SOD dismutation activity and stabilities of dithiocarbamates

Compound	R^1	R^2	D_{ow}	CMC, M	A_{50}^a	t_{50}/h^b
Et ₂ DTC	Et instead of $R^1(OCH_2CH_2)_2-$	Et	less than 0.02	-	3.4	> 240
Et-Hex-DTC	Et	Hexyl	0.14	2×10^{-4}	5.5	> 240
Et-Oct-DTC	Et	Octyl	1.88	8×10^{-7}	7.9	82
Bu-Oct-DTC	Bu	Octyl	21.45	6×10^{-7}	7.9	55
Bu-Dec-DTC	Bu	Decyl	> 50	6×10^{-5}	47.0	10
Bu-Dodec-DT	Bu	Dodecyl	> 50	7×10^{-5}	> 50	<1

^a A_{50} are DTC/SOD molar ratios leading to 50% decrease of SOD activity after a 2.5

5 hours of preincubation of SOD with DTCs. A_{50} are DTC/SOD molar ratios leading to 50% decrease of SOD activity after a 2.5 hours of preincubation of SOD with DTCs.

^b t_{50} is the time of 50% decomposition of DTCs at pH 10.

The main conclusions are:

1. Very limited structural variations on the small oligoether chain (two oxyethylene units, with R^1 changing from Ethyl to Butyl) and somewhat wider variations on the Alkyl group (with R^2 changing from Hexyl to Dodecyl) provide very broad changes in the physical properties (e.g., partition coefficients between octanol and water and surface tension properties).
2. The large variations in the FP component did not cause significant changes in the chemical specificity of the DTC chelator group toward its favorite ions Cu and Fe. The amphiphilic DTC still binds Cu preferentially.
3. Metal selectivity, rates of Cu(II) abstraction from the enzyme, and Fenton reaction. All three vectors concern the chemical reactivity of the CF-PF bifunctional molecule.
- 20 The determination of Kc values has shown the close proximity in the values for all the DTCs, despite the large variations in the structural and physical properties. The selectivity of Et-Hex-DTC toward Cu(II) against Fe(III) is retained. We surmise that

this will be true for other members in the sublibrary. The kinetic data for Et-Hex-DTC in the Fenton reaction, in the presence of the favorite Cu(II) ion, shows that Et-Hex-DTC is antioxidant, but acts as prooxidant in the case of the less-favored Fe(III) ion. This means that presence of free DTCs in a cell will have a strong effect on the Fenton-type reactions taking place within the cell and on the effective concentration of free hydroxyl radicals.

The interaction between the enzyme SOD and DTCs: Et₂DTC, Et-Hex-DTC, and Bu-Oct-DTC show similar rates of Cu(II) abstraction from the enzyme, and the most bulky chelator (Bu-Dodec-DTC) is less active in Cu(II) removal.

Similarly, these three chelators inhibit the dismutation activity of SOD, and the more bulky DTCs (Bu-Dec-DTC and Bu-Dodec-DTC) are much less effective, even at DTC/SOD molar ratio of 50 : 1. It was also shown that excess Cu(II) in the system causes full reactivation of the enzyme. In other words, insufficient supply of Cu(II) ions or an excess supply of DTC ligands of optimal size (not exceeding Et-Hex-DTC) will cause full enzyme inhibition. In the case of excess supply of DTC, the ligand also inhibits the excess production of OH[•] radicals (the so-called peroxidase activity of SOD).

4. Transport properties and overall effectiveness of DTCs as SOD inhibitors and radical scavengers or suppressors.

The rates of transport of DTCs through a lipid bilayer membrane (liposome), measured by the fluorescent method, showed very high values. The transport is complete within 15-20 seconds, almost regardless of the bulkiness of the DTC ligand. Of course, the overall effectiveness of the DTC transport is obtained by multiplication of the D_{ow} values with the rates values; inasmuch the last values remain practically unvaried, the effective concentration of the DTC ligands is governed mainly by the solubility factor (i.e., D_{ow} values). This conclusion is yet to be supported by experimental measurements.

Overall analysis of results

An overall analysis of the results discussed so far must correlate synthetic-structural-physical properties of the amphiphilic DTCs with their possible availability to act as inhibitors of SOD and as synergists to the action of herbicides. The limitation of this task is that the experimental results include only a limited sublibrary of DTCs. First, it is possible to expand on the sublibrary by adding DTCs based on higher oxyethylene

glycoles. Second, it is possible to incorporate the herbicide as part of the DTC. This is subject to further synthetic work.

The overall analysis of the results may be presented as a hypothetical scheme in four phases.

5 Phase A presents the effects concerning the transport of the DTCs. The transport is affected by (a) rate of hydrolytic decomposition of the DTCs, which is a function of their structure and pH (b) rate of transport via the cell membrane and (c) solubilization in the membrane, as indicated by D_{ow} .

10 Phase B presents the events taking place following the transport step, inside the cell, with a very complex equilibrium among the ligand DTC, the transition metal ions, Cu(II) and Fe(II), and the enzyme SOD (other metaloenzymes are ignored at this time). More complications arise from the side reactions, such as Fenton reaction, in which H_2O_2 is producing $OH^\bullet$ radicals. As known, the Fenton reaction is effected by the DTCs.

15 Phase C discusses the aspects of enzyme inhibition. The conclusion is that the less bulky DTCs totally inhibit the enzyme. The effective inhibition of SOD by Et_2DTC , $Et-Hex-DTC$, $Et-Oct-DTC$, and $Bu-Oct-DTC$ required at least 10-50 molar excess of these compounds. Then the DTCs are free to interact with free Fe(II) in the cell and produce $OH^\bullet$ radicals. In this way, they should synergies the herbicide. Finally, at Phase D (infinitesimal time $t \rightarrow \infty$ varying from one DTC to another) the DTCs are fully
20 decomposed, and a system D, free of DTC, is restored.

Experimental

1. Synthesis

Chemicals and instrumentation for synthesis of DTC

The following chemicals were used for syntheses: thionylchloride (E. Merck, for synthesis); octylamine (Fluka, AG); decylamine (Fluka, AG); dodecylamine (Fluka, AG); dibutylamine (Fluka, AG); potassium hydroxide (E. Merck, GR, for analysis); sodium hydroxide (E. Merck, GR, for analysis); sodium hydroxide volumetric standard (1.018 N solution in water, Aldrich); sodium carbonate (E. Merck, for analysis); ethyl alcohol absolute (BIO-LAB, A.R.); carbon disulfide (Fluka, puriss.); tetrahydrofuran (BIO-LAB, A.R., and then distilled under LiAlH_4 and passed through an Al_2O_3 column); sodium hydride suspension (80% in paraffin oil, E. Merck, for synthesis); methyl iodide (E. Merck, for synthesis); calcium chloride anhydrous (Fluka, AG); N, N-dimethylformamide (Aldrich), lithium hydroxide, monohydrate (99%, BDH Chemicals Ltd.); potassium hydroxide (Frutarom Ltd., chemically pure).

Proton NMR spectra were measured on a Bruker WH-270, a Bruker DPX-250, or a Bruker AMX-400 NMR spectrometer. Either all chemical shifts are reported in δ units downfield from tetramethylsilane as an internal standard, or the H_2O signal was used as a reference. The following abbreviations are used: s-singlet, d-doublet, t-triplet, q-quartet, and m-multiple. Deuterated CDCl_3 (Aldrich) and D_2O (E. Merck) used for NMR were of 99.8% isotope purity. Flash column chromatography separations were performed on silica gel Merck 60 (230-400 mesh ASTM). UV/VIS spectra were measured on a Hewlett-Packard 8450A diode array spectrophotometer. TLC was performed on E. Merck Kieselgel 60 F254 plates. Staining of TLC plates was done by (a) basic aqueous 1% KMnO_4 and (b) 0.3% ninhydrin in EtOH_{abs} . Tetrahydrofuran was distilled under LiAlH_4 and passed through an Al_2O_3 column. High Resolution mass spectra (DI, EI-MS) were measured on PGS-70B Finnigan-Mat instrument at the Chemical Faculty of the Israel Institute of Technology (Technion), Haifa.

Syntheses and analyses

Compounds Et-Et-DTC, Et-Bu-DTC, and Et-Hex-DTC were synthesized according to literature⁴⁷. Compounds are called I-Et-DTC, I-Bu-DTC, and I-Hex-DTC, respectively, in this paper.

Dibutyl dithiocarbamic acid was synthesized from dibutylamine in water according to reference.¹⁹ Synthesis of 2-(2-ethoxyethoxy)ethyl chloride was described in

literature.⁴⁷

2-(2-Ethoxyethoxy)butyl chloride

A solution of SOCl_2 (48 g, 0.4 moles, 30% excess) in chloroform (30 ml) was added dropwise at the rate of 1 drop/sec in nitrogen flow, for 1 hr, to a cooled, stirred solution of diethylene glycol monobutyl ether (49.7 g, 0.3 moles) in chloroform (50 ml) and DMF (4 ml). The gases were trapped into the stirred NaOH solution (4 M, 300 ml). The mixture was stirred for 1 hr at room temperature followed by 2.5 h at 50°C. After the removal of chloroform by distillation, the residue was distilled twice at low pressure (87°C, 1 mm Hg) to yield 42.6 g (70.8 % yield) of colorless liquid. TLC: $R_f = 0.47$ (CHCl_3 , staining with KMnO_4). ^1H NMR: δ (CHCl_3 , 250 MHz; Me_4Si): 0.92 (3H, t, $\text{CH}_3\text{CH}_2\text{CH}_2$), 1.30-1.43 (2H, m, $\text{CH}_3\text{CH}_2\text{CH}_2$), 1.52-1.62 (2H, m, $\text{CH}_3\text{CH}_2\text{CH}_2$), 3.47 (2H, t, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2$), 3.57-3.74 (6H, m, $\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{Cl}$), 3.77 (2H, t, CH_2Cl).

n-Octyl (2-(2-ethoxyethoxy)butyl)amine

n-Octylamine (65 ml, 50.6 g, 0.39 mole, 10 times excess) was placed in a 250 ml three-necked flask, fitted with a magnetic stirrer, dropping funnel, and reflux condenser with the nitrogen outlet. 2-(2-Ethoxyethoxy)butyl chloride (7.08 g, 39 mmole) was added dropwise at room temperature. The reaction mixture was heated in an oil bath at 110°C for 40 h and cooled at room temperature overnight. A gel-like substance formed. NaOH solution (4M, 40 ml) was added and stirring continued at room temperature overnight. Hexane (50 ml) was then added, and the organic and water phases were separated. The organic phase was washed with water (80 ml) twice, then with NaOH solution (2M, 30 ml), and dried over KOH overnight. Hexane was removed followed by distillation of the excess of *n*-octylamine (80°C/10 mm Hg). The product was distilled twice under vacuum (105-110 °C/ 0.05 mm Hg) to yield 9.3 g (87%) of colorless liquid. TLC: $R_f = 0.81$ (CHCl_3 : MeOH : NH_3 (25% aq) 9:1:0.1 staining with ninhydrin. ^1H NMR: δ (CHCl_3 , 250 MHz; Me_4Si): 0.84-0.94 (6H, m, two CH_3CH_2), 1.2-1.38 (12H, m, $\text{CH}_3(\text{CH}_2)_5$ and $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 1.38-1.6 (4H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$ and $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 1.73 (1H, br s, NH), 2.59 (2H, t, $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$), 2.78 (2H, t, $\text{OCH}_2\text{CH}_2\text{N}$), 3.46 (2H, t, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 3.6 (6H, m, $\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{N}$).

n-Octyl (2-(2-ethoxyethoxy)ethyl)amine

Synthesis was as described for *n*-octyl (2-(2-ethoxyethoxy)butyl)amine, except that 2-(2-ethoxyethoxy)ethyl chloride was used instead of 2-(2-ethoxyethoxy)butyl chloride. TLC: $R_f = 0.82$ ($\text{CHCl}_3 : \text{MeOH} : \text{NH}_3(25\% \text{ aq})$ 9 : 1 : 0.1 staining with ninhydrin). ^1H NMR: δ (CHCl_3 , 400 MHz; Me_4Si): 0.87 (3H, t, $\text{CH}_3\text{CH}_2\text{CH}_2$), 1.21 (3H, t, $\text{CH}_3\text{CH}_2\text{O}$), 1.2-1.38 (10H, m, $\text{CH}_3(\text{CH}_2)_5$), 1.4-1.6 (2H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$), 2.52 (1H, br s, NH), 2.62 (2H, t, $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$), 2.81 (2H, t, $\text{OCH}_2\text{CH}_2\text{N}$), 3.53 (2H, q, $\text{CH}_3\text{CH}_2\text{O}$), 3.56-3.64 (6H, m, $\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{N}$).

A small amount of the amine (~1 g) was further purified by flash chromatography (chromatographic separations were performed on silica gel Merck 60 (230-400 mesh ASTM)). The eluent used for this was $\text{CH}_2\text{Cl}_2 : \text{CH}_3\text{OH} : \text{NH}_3 = 9.8 : 0.1 : 0.1$. The eluent was evaporated from the product by rotavapor and then in vacuum (1 mm Hg). The purity of the product was ~100% and it was used for synthesis of sodium, potassium, and lithium salts for experiments on hydrolytic stability of compounds. TLC: $R_f = 0.78$ ($\text{CHCl}_3 : \text{CH}_3\text{OH} : \text{NH}_3 = 19 : 1 : 0.2$, staining by ninhydrine).

15 *n*-Decyl (2-(2-ethoxyethoxy)butyl)amine

Synthesis was as described for *n*-octyl (2-(2-ethoxyethoxy)butyl)amine except that *n*-decylamine was used instead of *n*-octylamine. TLC: $R_f = 0.8$ ($\text{CHCl}_3 : \text{MeOH} : \text{NH}_3(25\% \text{ aq})$ 9 : 1 : 0.1 staining with ninhydrin) ^1H NMR : δ (CHCl_3 , 250 MHz; Me_4Si): 0.85-0.94 (6H, m, two CH_3CH_2), 1.2-1.4 (16H, m, $\text{CH}_3(\text{CH}_2)_7$ and $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 1.4-1.63 (4H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$ and $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 1.79 (1H, br s, NH), 2.59 (2H, t, $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$), 2.78 (2H, t, $\text{OCH}_2\text{CH}_2\text{N}$), 3.46 (2H, t, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 3.6 (6H, m, $\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{N}$).

n-Dodecyl (2-(2-ethoxyethoxy)butyl)amine

Synthesis was as described for *n*-octyl (2-(2-ethoxyethoxy)butyl)amine, except that *n*-dodecylamine was used instead of *n*-octylamine. TLC: $R_f = 0.73$ ($\text{CHCl}_3 : \text{MeOH} : \text{NH}_3(25\% \text{ aq})$ 9 : 1 : 0.1, staining with ninhydrin). ^1H NMR δ (CHCl_3 , 270 MHz; Me_4Si): 0.85-0.94 (6H, m, two CH_3CH_2), 1.2-1.4 (20H, m, $\text{CH}_3(\text{CH}_2)_9$ and $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 1.42-1.62 (4H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$ and $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 1.71 (1H, br s, NH), 2.59 (2H, t, $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$), 2.78 (2H, t, $\text{OCH}_2\text{CH}_2\text{N}$), 3.46 (2H, t, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 3.6 (6H, m, $\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{N}$).

n-Octyl (2-(2-ethoxyethoxy)ethyl)dithiocarbamic acid, sodium salt (compound Et-Oct-

DTC)

n-Octyl (2-(2-ethoxyethoxy)ethyl)amine (0.99 g, 4 mmole) and NaOH (4 ml of 1 M water solution, 4 mmole) were placed in a 25 ml round-bottom flask, fitted with a magnetic stirrer and an ice bath. CS₂ (0.265 ml, 4.4 mmole) was added to the rapidly stirred, cooled mixture. Five minutes after addition of carbon disulfide the ice bath was removed, and the mixture was stirred for 3 hr at room temperature. The solution was lyophilized for 1.5 days. The yellow powder obtained was washed with ice-cooled ether and dried under vacuum. The yield was 0.92 g (67%) of white powder. ¹H NMR: δ (CHCl₃, 250 MHz; Me₄Si): 4.28 (2H, t, J 5.4, OCH₂CH₂N); 4.01 (2H, t, J 7.8, CH₂CH₂CH₂N); 3.82 (2H, t, J 5.7, OCH₂CH₂N); 3.65-3.54 (4H, m, OCH₂CH₂O); 3.53 (2H, q, J 7, CH₃CH₂O); 1.70 (2H, br m, CH₂CH₂CH₂N); 1.27 (10H, br s, CH₃(CH₂)₅); 0.85-0.93 (6H, m, CH₃CH₂O and CH₃(CH₂)₅).

n-Octyl (2-(2-ethoxyethoxy)butyl)dithiocarbamic acid, sodium salt (compound Bu-Oct-DTC)

Synthesis was as described for compound Et-Oct-DTC, except that *n*-octyl (2-(2-ethoxyethoxy)butyl)amine was used instead of *n*-octyl (2-(2-ethoxyethoxy)ethyl)amine. ¹H NMR: δ (CHCl₃, 400 MHz; Me₄Si): 4.31 (2H, t, J 5.6, OCH₂CH₂N); 4.02 (2H, t, J 7.9, CH₂CH₂CH₂N); 3.82 (2H, t, J 5.7, OCH₂CH₂N); 3.66-3.58 (4H, m, OCH₂CH₂O); 3.51 (2H, t, J 6.9, CH₃CH₂CH₂CH₂O); 1.70 (2H, br m, CH₂CH₂CH₂N); 1.59 (2H, m, CH₃CH₂CH₂CH₂O); 1.35 (2H, m, CH₃CH₂CH₂CH₂O); 1.26 (10H, br s, CH₃(CH₂)₅); 0.85-0.93 (6H, m, CH₃CH₂CH₂CH₂O and CH₃(CH₂)₅).

Synthesis of n-octyl 2-(2-ethoxyethoxy) ethyl dithiocarbamic acid, potassium salt

n-Octyl 2-(2-ethoxyethoxy) ethylamine (purified by flash chromatography, 0.109 g, 0.444 mmole) and KOH (4.53 ml, 0.098 M, 0.444 mmole) were placed in a flask with magnetic stirrer in an ice bath. Carbon disulfide (0.22 ml, 3.552 mmole) was added rapidly to the cooled, stirred solution. The procedure for the preparation of *n*-octyl 2-(2-ethoxyethoxy) ethyl dithiocarbamic acid, sodium salt was then followed. The product (104.4 mg) was white amorphous powder. Yield = 95.8%. ¹H NMR (250 MHz): δ (CDCl₃) = 0.85-0.90 t; 1.19-1.28 m; 1.64-1.65 m; 2.60-2.67 t; 2.82-2.86 t; 3.52-3.65 m; 3.77-3.82 t; 4.03-4.09 t; 4.38-4.42 t.

Synthesis of n-octyl 2-(2-ethoxyethoxy) ethyl dithiocarbamic acid, lithium salt

n-Octyl 2-(2-ethoxyethoxy) ethyl amine (purified by flash chromatography, 0.116

g, 0.472 mmole) and LiOH (5.26 ml, 0.0897 N, 0.472 mmole) were placed in a flask with magnetic stirrer in an ice bath. Carbon disulfide (0.23 ml, 3.774 mmole) was added rapidly to the cooled, stirred solution. The same procedure as for the preparation of *n*-octyl 2-(2-ethoxyethoxy) ethyl dithiocarbamic acid, sodium salt was used. The product
 5 (115.7 mg) was yellowish solid, yield = 99.74%. ¹H NMR (250 MHz): δ (CDCl₃) = 0.85-0.90 t; 1.18-1.28 m; 1.57-1.69 m; 2.77-2.81 broad peak; 2.99-3.05 broad peak; 3.51-3.73 m; 3.76-3.90 t; 3.92-4.08 t; 4.12-4.30 t.

n-Dodecyl (2-(2-ethoxyethoxy)butyl)dithiocarbamic acid, sodium salt (compound Bu-Dodec-DTC)

10 *n*-Dodecyl (2-(2-ethoxyethoxy)butyl)amine (2.93 g, 8.89 mmole), 20 ml of THF and 80% NaH (0.259 g, 8.63 mmole of pure compound) were placed in a 50 ml round-bottom flask, equipped with a magnetic stirrer and ice bath, and connected to the Ar system. CS₂ (1.5 ml, 24.9 mmole) was added to the rapidly stirred, cooled mixture. Ten
 15 minutes after addition of carbon disulfide, the ice bath was removed, and the mixture was stirred for 2.5 h at room temperature. Solution was filtered in Ar atmosphere through the sinter glass filter N° 3 with celite (under vacuum) and washed with THF (10 ml). THF was evaporated under vacuum (25°C / 1 mm Hg), and the residue (yellow oil) was dried over P₂O₅ overnight. The product was then recrystallized several times from the cooling hexane solution. The product was dried over P₂O₅ overnight. The yield was
 20 1.48 g (40%) of white powder. ¹H NMR: δ (CHCl₃, 400 MHz; Me₄Si): 4.34 (2H, t, J 5.68, OCH₂CH₂N); 4.02 (2H, t, J 7.9, CH₂CH₂CH₂N); 3.82 (2H, t, J 5.6, OCH₂CH₂N); 3.66-3.58 (4H, m, OCH₂CH₂O); 3.59 (2H, t, J 7, CH₃CH₂CH₂CH₂O); 1.70 (2H, br m, CH₂CH₂CH₂N); 1.60 (2H, m, CH₃CH₂CH₂CH₂O); 1.36 (2H, m, CH₃CH₂CH₂CH₂O); 1.25 (18H, br s, CH₃(CH₂)₉); 0.90 (3H, t, J 7.4, CH₃CH₂CH₂CH₂O); 0.88 (3H, t, J 6.8, CH₃(CH₂)₉).
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n-Decyl (2-(2-ethoxyethoxy)butyl)dithiocarbamic acid, sodium salt (compound Bu-Dec-DTC)

Synthesis was as described for compound Bu-Dodec-DTC, except that *n*-decyl (2-(2-ethoxyethoxy)butyl)amine was used instead of *n*-dodecyl (2-(2-ethoxyethoxy)butyl)amine. ¹H NMR: δ (CHCl₃, 400 MHz; Me₄Si): 4.45 (2H, t, J 5.04, OCH₂CH₂N); 4.02 (2H, t, J 7.9, CH₂CH₂CH₂N); 3.80 (2H, t, J 5.2, OCH₂CH₂N); 3.70-
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3.62 (4H, m, $\text{OCH}_2\text{CH}_2\text{O}$); 3.58 (2H, t, J 7, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$); 1.71 (2H, br m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$); 1.64 (2H, m, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$); 1.35 (2H, m, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$); 1.25 (14H, br s, $\text{CH}_3(\text{CH}_2)_7$); 0.85-0.93 (6H, m, two CH_3).

n-Octyl (2-(2-ethoxyethoxy)butyl)dithiocarbamic acid, methyl ester

- 5 *n-Octyl (2-(2-ethoxyethoxy)butyl)amine sodium salt* (0.292 g, 0.79 mmole) and 5 ml of absolute ethanol were placed in a 25 ml round-bottom flask equipped with a magnetic stirrer and an ice bath. CH_3I (49 μl , 0.79 mmole) was added to the rapidly stirred cooled mixture. The ice bath was then removed, and the mixture was stirred overnight at room temperature. The ethanol was removed under vacuum. The residue
- 10 was rinsed with hexane (5 ml), after which the hexane solution was filtered, and hexane was removed under vacuum. The product was cleaned on the preparative TLC (Et_2O : Hex = 3 : 7, direct inspection with UV light, desorbition with chloroform). Chloroform was evaporated under vacuum (1 mm Hg) to yield 188 mg (66%) of yellow oil. TLC: R_f = 0.66 (Et_2O : Hex = 3 : 7, direct inspection with UV light and staining by KMnO_4). ^1H
- 15 NMR: δ (CHCl_3 , 400 MHz; Me_4Si): 4.2-3.8 (6H, m, $\text{OCH}_2\text{CH}_2\text{NCH}_2$); 3.61-3.56 (4H, m, $\text{OCH}_2\text{CH}_2\text{O}$); 3.46 (2H, t, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$); 2.63 (3H, s, SCH_3); 1.71 (2H, br m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$); 1.59-1.55 (2H, m, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$); 1.39-1.35 (2H, m, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$); 1.31 (10H, m, $\text{CH}_3(\text{CH}_2)_5$); 0.94-0.86 (6H, m, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$ and $\text{CH}_3(\text{CH}_2)_5$). EI-MS m/z 363.2315 (M^+ , calc. 363.2265 for $\text{C}_{18}\text{H}_{37}\text{O}_2\text{NS}_2$).

20 *n-Octyl (2-(2-ethoxyethoxy)ethyl)dithiocarbamic acid, methyl ester*

- Synthesis was as described for methyl ester of *n-octyl (2-(2-ethoxyethoxy)butyl)dithiocarbamic acid*, except that the sodium salt of *n-octyl (2-(2-ethoxyethoxy)ethyl)amine* was used instead of the sodium salt of *n-octyl (2-(2-ethoxyethoxy)butyl)amine*, and the preparative TLC was made with chloroform. TLC:
- 25 R_f = 0.42 (CHCl_3 , direct inspection with UV light and staining by KMnO_4). ^1H NMR: δ (CHCl_3 , 250 MHz; Me_4Si): 4.2-3.7 (6H, m, $\text{OCH}_2\text{CH}_2\text{NCH}_2$); 3.62-3.50 (6H, m, $\text{CH}_3\text{CH}_2\text{OCH}_2\text{CH}_2\text{O}$); 2.63 (3H, s, SCH_3); 1.71 (2H, br m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$); 1.31-1.2 (13H, m, $\text{CH}_3(\text{CH}_2)_5$ and $\text{CH}_3\text{CH}_2\text{O}$); 0.89 (3H, t, $\text{CH}_3(\text{CH}_2)_5$). EI-MS m/z 335.1916 (M^+ , calc. 335.1953 for $\text{C}_{16}\text{H}_{33}\text{O}_2\text{NS}_2$)

30 *n-Decyl (2-(2-ethoxyethoxy)butyl)dithiocarbamic acid, methyl ester*

Synthesis was as described for methyl ester of *n-octyl (2-(2-*

- ethoxyethoxy)butyl)dithiocarbamic acid, except that the sodium salt of *n*-decyl (2-(2-ethoxyethoxy)butyl)amine was used instead of the sodium salt of *n*-octyl (2-(2-ethoxyethoxy)butyl)amine. TLC: $R_f = 0.7$ (Et₂O : Hex = 3 : 7, direct inspection with UV light and staining by KMnO₄). ¹H NMR: δ (CHCl₃, 400 MHz; Me₄Si): 4.2-3.7 (6H, m, OCH₂CH₂NCH₂); 3.61-3.54 (4H, m, OCH₂CH₂O); 3.45 (2H, t, CH₃CH₂CH₂CH₂O); 2.62 (3H, s, SCH₃); 1.70 (2H, br m, CH₂CH₂CH₂N); 1.61-1.52 (2H, m, CH₃CH₂CH₂CH₂O); 1.40-1.32 (2H, m, CH₃CH₂CH₂CH₂O); 1.25 (14H, m, CH₃(CH₂)₇); 0.93-0.86 (6H, m, CH₃CH₂CH₂CH₂O and CH₃(CH₂)₇). EI-MS m/z 391.2585 (M⁺, calc. 391.2579 for C₂₀H₄₁O₂NS₂)
- 10 *n*-Dodecyl (2-(2-ethoxyethoxy)butyl)dithiocarbamic acid, methyl ester
- Synthesis was as described for methyl ester of *n*-octyl (2-(2-ethoxyethoxy)ethyl)dithiocarbamic acid, except that the sodium salt of *n*-dodecyl (2-(2-ethoxyethoxy)butyl)amine was used instead of the sodium salt of *n*-octyl (2-(2-ethoxyethoxy)ethyl)amine. TLC: $R_f = 0.57$ (Et₂O : Hex = 2 : 8, direct inspection with UV light and staining by KMnO₄). ¹H NMR: δ (CHCl₃, 400 MHz; Me₄Si): 4.2-3.7 (6H, m, OCH₂CH₂NCH₂); 3.61-3.54 (4H, m, OCH₂CH₂O); 3.46 (2H, t, CH₃CH₂CH₂CH₂O); 2.63 (3H, s, SCH₃); 1.70 (2H, br m, CH₂CH₂CH₂N); 1.60-1.55 (2H, m, CH₃CH₂CH₂CH₂O); 1.39-1.34 (2H, m, CH₃CH₂CH₂CH₂O); 1.26 (18H, m, CH₃(CH₂)₉); 0.94-0.86 (6H, m, CH₃CH₂CH₂CH₂O and CH₃(CH₂)₉). EI-MS m/z 419.2902 (M⁺, calc. 419.2892 for C₂₂H₄₅O₂NS₂)

Influence of different cations on the stability of the dithiocarbamates

- The stability of Na, K, and Li salts were measured in the phosphate buffer (K₂HPO₄ : KH₂PO₄ = 7 : 0.195, pH = 8.38). Absorbance at $\lambda = 260$ nm and 286 nm (the peaks of dithiocarbamic salts) and at $\lambda = 205$ nm (the peak of the decomposition product) were measured. The time of "half maximum" decrease of UV absorbance of 10 mM dithiocarbamates was taken as the half time of decomposition of these compounds.

Lipophilicity of dithiocarbamates

- 1-Octanol (Riedel-deHaan, synthesis grade (250-270 nm abs. < 0.06) and glass distilled deionized water were used as the partitioning solutions. A Hewlett-Packard 8450A diode array spectrophotometer was used for the quantitative determination of dithiocarbamates in the standard solutions and in the partitioned solutions. Partitioning

by the shake-flask method was performed according to Leo²⁰, with 10 mM NaOH added to the aqueous phase to prevent the decomposition of dithiocarbamates. The aqueous phase stock solutions were shaken with an excess of octanol to presaturate them and were then allowed to stand overnight before use. The octanol stock solutions were also presaturated with NaOH and allowed to settle overnight. The experiments were performed in 10-ml stoppered centrifuge tubes. The tubes were inverted gently for 5 min and then, to assure complete phase separation, they were centrifuged for 20 min at 1,000-2,000 g. The aqueous and organic phases were removed separately and analyzed by UV spectrophotometer.

10 Selectivity of DTC for copper over iron

Iron (III) sulfate pentahydrate, copper (II) sulfate pentahydrate, and iron (III) chloride hexahydrate were purchased from Aldrich. Analytical ethanol (Frutarom) was used.

The Cu-Fe exchange experiment in 75% ethanol proceeded as follows: $\text{Fe}_2(\text{SO}_4)_3$ (0.25 mM) and DTC solutions (1.5 mM) were incubated for 10 minutes to obtain $\text{Fe}(\text{DTC})_3$ complex. Then CuSO_4 was added ($\text{Cu}^{2+} : \text{Fe}^{3+}$ was equal to 1 : 5), the mixture was incubated for additional 15 minutes to reach equilibrium, and the UV/VIS spectra were measured. Ethanol (75% in water) was used as a solvent, because all the components of the reactions are soluble in this solvent.

The Cu-Fe exchange in water at pH = 2.8 proceeded as follows: DTC (Et-Hex-DTC, 0.15 mM) was added to FeCl_3 water solution (1 mM, pH = 2.8) to obtain 0.5 mM $\text{Fe}(\text{DTC})_3$ and an excess of Fe^{3+} . The solution was incubated for 10 minutes. CuSO_4 (75 μM in water) was then added, and the kinetics of metal exchange were measured spectrophotometrically.

25 Inhibition of SOD by competitive complexation of copper

Chemicals and instrument

Human recombinant CuZnSOD, which was kindly provided by Biotechnology General, Weizmann Science Park, Nes-Ziona, Israel; diethyldithiocarbamic acid sodium salt, trihydrate (sodium diethyldithiocarbamate, Et_2DTC) (Aldrich, A.C.S. reagent); xanthine (Aldrich); cytochrome C (Sigma, from bovine heart); xanthine oxidase (E. Merck); dimethyl sulfoxide (DMSO) (E. Merck, for synthesis); hydrogen peroxide (30%, E. Merck, for synthesis); 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) (Aldrich, and then

activated charcoal purified); 50 mM phosphate buffer (pH 7.8) was prepared from potassium dihydrogen phosphate and di-sodium hydrogen phosphate anhydrous (both from E. Merck, analysis grade); 50 mM carbonate buffer (pH 10.2) was prepared from sodium carbonate anhydrous (E. Merck, for analysis) and sodium hydrogen carbonate (Aldrich, A.C.S. reagent). A stock 10 mM solution of Cu^{2+} was prepared from copper (II) sulfate pentahydrate (E. Merck, GR, for analysis). UV/VIS spectra were measured on a Hewlett-Packard 8450A diode array spectrophotometer. The EPR spectra were measured in a Bruker ER 200 D-SRC spectrometer in a flat cell. Copper concentration in solutions was measured on ICP-AES (inductively coupled plasma atomic emission spectrometer) "Spectroflame" instrument at the Faculty of Agriculture of the Hebrew University of Jerusalem, Rehovot campus.

Dismutation activity of superoxide dismutase

The superoxide dismutation activity of SOD was measured by monitoring the inhibition of the reduction of ferricytochrome C by superoxide generated by a xanthine/xanthine oxidase reaction, as described in (27). All DTCs were preincubated with 1 mM SOD at pH 10.2 for 2.5 hours and room temperature in 50 mM carbonate buffer (pH 10.2) at molar ratios of DTC/SOD 2:1 to 50:1. Enzyme-dithiocarbamate solutions were diluted after the preincubation with 50 mM potassium phosphate buffer (pH 7.8). The SOD activity reaction mixture contained 3 nM enzyme. SOD, preincubated for 2.5 hours at pH 10.2 without a DTC, was used as a control.

The copper-deficient SOD was reconstituted to an active form by the addition of Cu^{2+} (as CuSO_4) to a 0.1 μM solution of inactivated enzyme and incubated for three hours or overnight. Overnight incubation was sufficient to restore more than 90% of the initial activity. Copper ions were added in sufficient excess to replace the copper removed from SOD and to complex remaining free dithiocarbamate. Final concentrations of Cu^{2+} were 3 μM , 1.8 μM , and 1 μM for molar ratios of DTC/SOD 50 : 1, 25 : 1 and 10 : 1, correspondingly.

Separation of Cu-DTC complexes from SOD

The Cu-DTC complexes were first formed by incubation of 1.5 mM SOD with 10 mM DTC in buffer (pH 10.2) for 24 hours. The complexes were then 10-fold diluted with the same buffer and sedimented from the protein by centrifugation at 39,000 $\times g$ for Et_2DTC or 245,000 $\times g$ for Bu-Oct-DTC. The amounts of copper in the precipitate (after

solubilization in nitric acid) and in the enzyme (supernatant) were measured by inductively coupled plasma (ICP) spectroscopy. Protein was assayed with Coomassie Brilliant Blue.⁴³

EPR measurement of peroxidase activity of SOD

5 Superoxide dismutase was inactivated by preincubation of 1 mM SOD with 25 mM DTC (Et₂DTC, Et-Hex-DTC, Et-Oct-DTC, or Bu-Oct-DTC) in 50 mM carbonate buffer (pH 10.2) for 2.5 hours at room temperature. The solutions were then diluted with phosphate buffer (pH 7.8) to 5 μ M SOD.

10 Cu(Et₂DTC)₂ and Cu(Et-Hex-DTC)₂ (10 μ M) in 10% DMSO/phosphate buffer (pH 7.8) with 12.5 times excess of dithiocarbamate ligands were prepared as follows: CuSO₄ (20 μ l of 10 mM), 2 ml of DMSO, and then 50 μ l of 50 mM solution of dithiocarbamate ligand in buffer (pH 10.2) were put into the 20 ml volumetric flask. Volume was adjusted with 50 mM phosphate buffer (pH 7.8). The concentrations of the resulting complexes were confirmed by UV spectroscopy in 10 ml path length UV
15 cuvettes at $\lambda = 450$ nm (for Cu(Et₂DTC)₂) or $\lambda = 440$ nm (for Cu(Et-Hex-DTC)₂).

20 The peroxidase activity of superoxide dismutase refers to the "bound" hydroxyl radical: $\bullet$ OH produced by SOD and H₂O₂ remains bound to the copper atom at the active site. The peroxidase activity was measured by spin-trapping technique and electron paramagnetic resonance (EPR) spectroscopy.^{33, 34} The spin-trapping technique converts
transient free radicals to stable free radicals. A relatively long-lived spin adduct DMPO/ $\bullet$ OH is formed from SOD, hydrogen peroxide, and a diamagnetic spin trap DMPO. The identity of the free radical was determined on the basis of hyperfine coupling (hfc) constants of the spin adduct by EPR spectroscopy.

25 EPR solutions for measurements of the influence of Cu(DTC)₂ complexes on the peroxidase activity of SOD in the presence of H₂O₂ contained 45 mM DMPO, 5 μ M Cu(DTC)₂, and 40 mM H₂O₂ in 50 mM phosphate buffer (pH 7.8). The solutions of SOD inactivated by DTCs contained 45 mM DMPO, 2.5 μ M SOD (and 62.5 μ M DTC for spectra B, C, D and E (Figure 7) and 30 mM H₂O₂ in 50 mM phosphate buffer at pH 7.8.

30 The EPR signals were measured in 100- μ l flat cell at room temperature. Spectral acquisition began 3 minutes after initiation of the reaction by the addition of hydrogen peroxide. The spectrometer settings were as follows: receiver gain, 4×10^5 ; modulation

amplitude, 1 G; time constant, 1.25 sec; sweep time, 200 sec; center field, 3500 G; sweep width, 100 G; microwave power, 20 mW.

Rates of formation of dithiocarbamate complexes with copper of SOD

The kinetics of SOD copper chelation by the different dithiocarbamates were measured spectrophotometrically by the appearance of colored Cu-DTC complexes ($\lambda_{\text{max}} = 440 \text{ nm}$ for amphiphilic DTCs and $\lambda_{\text{max}} = 450 \text{ nm}$ for Et_2DTC). Measurements were made with 0.1 mM SOD and 0.5 - 3 mM DTC in 50 mM carbonate buffer pH = 10.2. The rates of appearance of the absorption bands at 440-450 nm were measured during the first 30 seconds of complex formation.

Statistical analysis

The slopes (β_1) of the linear regression curves were measured to compare the influence of dithiocarbamates on the superoxide dismutation activity of SOD. The linear model that best fit the data (all R^2 were between 0.87 and 0.95) was $\log(y) = \beta_0 + \beta_1 x$, when y is SOD activity (% of control) and x is $[\text{DTC}]/[\text{SOD}]$ molar ratios. The changes of the logarithm of SOD activity with increase of 1 unit in $[\text{DTC}]/[\text{SOD}]$ molar ratio were represented by β_1 . The point where $[\text{DTC}]/[\text{SOD}]$ molar ratio is 50 was not used for calculations, as the SOD activity was completely inhibited by some compounds. The logarithms of the SOD activities could not then be calculated. Regression curves were constructed on the basis of average values (the same values that were used for the construction of the curves: SOD activity (% of control) versus $[\text{DTC}]/[\text{SOD}]$ molar ratio, Figure6), as the duplicates were dependent values. The residual plots showed that the underlying assumptions for the regressions were met, and therefore no further transformations were needed. 95% confidence intervals were calculated for the slopes, and overlapping of these confidence intervals means that the corresponding slopes are not significantly different.

The rates of copper-dithiocarbamate complex formation were analyzed by analysis of variances (ANOVA). When the ANOVA analysis showed significant differences, they were followed by Fisher's least significant difference procedure. Mean values were calculated on the basis of two to four independent experiments. Means that followed the same letter are not significantly different (at $\alpha = 0.05$, where α is the significance level).

The StatView computer program (Abacus Concepts, Inc.) was used for all the

statistical calculations.

Determination of CMC

CMCs of amphiphilic dithiocarbamates were determined from the Wilhelmy plate surface tension measurements. The Wilhelmy plate experiments were performed with a Krüss digital instrument K10T using small volume (15 ml) glass measurement cells with a glass side arm incorporated in their walls. The measurement cell filled with a NaCl-NaOH buffer at pH 12, was mounted on a magnetic stirrer of the Krüss apparatus. First, the surface of the buffer solution was swept with blotting silk paper and its surface tension value (γ_0) was recorded. Then, keeping in contact the roughened platinum Wilhelmy plate in contact with the buffer solution, a DTC solution was injected into the buffer subphase through the side arm of the measurement cell. A Teflon stir-bar inserted inside the buffer solution was slowly rotated for 2 min. The measurement was taken without detaching the plate from the interface and the data were continuously recorded as a function of time for each studied DTC concentration until equilibrium was reached.

The precision of the force transducer of the apparatus was ± 0.1 mN/m. The reported values are mean values of three measurements at room temperature, and the discrepancy between the results never exceeded $\pm 2\%$ of the mean. The almost identical profiles of $\gamma = f(t)$ relationships obtained for three independently conducted runs, at any given DTC bulk concentration, provided evidence of homogenization of DTC solutions during stirring and that the absorbed films were not disturbed by stirring. NaOH (about 10^{-2} M) in the buffer solution was used to prevent possible decomposition of dithiocarbamates during the experiment.

Influence of DTCs on the membrane bilayer

Egg phosphatidylcholine (from fresh egg yolk, dissolved in chloroform, Sigma); cholesterol (extra pure) (Merck, Darmstadt, Germany); calcein (Sigma); and Triton X-100 (t-octylphenoxypolyethoxyethanol)(Sigma) were used.

Stock solutions of calcein (60 mM) were prepared by dilution of calcein in 110 mM Na_2CO_3 solution. A stock solution of Cu^{2+} was prepared from copper (II) sulfate pentahydrate (E. Merck, GR, for analysis). Tris-saline buffer (10 mM Tris/HCl, 150 mM NaCl, pH = 7.4) was used in all experiments.

Calcein-copper complex (Cal-Cu) was prepared as follows: stock Cu^{2+} solution and stock calcein solution were added to tris-saline buffer. The resulting calcein- Cu^{2+}

complex solution contained 60 μM Cal-Cu and 5 μM of free Cu^{2+} ions (totally 65 μM of Cu^{2+}). The presence of a small excess (8% in our case) of free copper ions is necessary to achieve more than 90% inhibition of calcein fluorescence. Fluorescent spectra were measured on SLM-AMINCO model 8100 spectrofluorimeter in the quartz cell, equipped with magnetic stirrer.

Preparation of phospholipid vesicles

Small unilamellar vesicles (SUV) were prepared by sonication of multilamellar vesicles using a probe sonicator. The following procedure was used (100): egg phosphatidylcholine (PC) and cholesterol (in 1:1 molar ratio) was dissolved in chloroform/methanol mixture (2:1 v/v) to obtain 10 mg/ml solution of phospholipid. The solvents were then evaporated under a stream of nitrogen to yield a dry thin lipid film on the wall of a glass vial (5 mg lipid / 2 cm^3 vial). Then, the film was dried under high vacuum for 2-3 hours.

SUV containing calcein-copper complex were prepared as follows: a solution of calcein-copper complex (60 μM in tris-saline buffer, 1 ml) were added to the vial with the thin film of lipid to obtain 5 mg/ml PC solution, and the mixture was shaken on a vortex for 10 minutes. To form unilamellar liposomes, the multilamellar vesicles thus formed were sonicated until the solution was transparent.

SUV containing the self-quenching concentration of calcein were prepared the same way as the SUV containing calcein-copper complex, but the stock solution of calcein (60 mM) was added instead of calcein-copper.

Gel filtration SUV were separated from the non-encapsulated reagents by gel filtration⁴⁴ on Sephadex G-50 (20 ml bed volume) pre-equilibrated with tris-saline buffer (tris - 10 mM, NaCl - 150 mM, pH = 7.8). Purified vesicles were eluted in the void volume and were monitored by absorption at 340 nm.

Measurements of liposome stability

a) Leakage caused by the presence of free DTC ligand

A fluorescent technique⁴⁵ was used for checking the leakage and fusion of the liposomes in the presence of DTC. The liposomes were encapsulated with calcein at self-quenching concentration (60 mM in tris-saline buffer) to obtain 5 mg/ml PC. The fraction of SUV after gel filtration (fraction with the higher absorbance at 340 nm) was then diluted more than 100 times to keep absorbance at 496 nm not higher than 0.2. The fluorescence of this liposome solution and the fluorescence upon addition of varying

concentrations of DTCs was measured (excitation wavelength was 495 nm and emission wavelength - 515 nm). Triton X-100 (final concentration 0.5%) was added at the end of each experiment to destroy the vesicles and to get the full value of fluorescence.

b) Leakage caused by the formation of DTC-Cu complex

5 Calcein-copper containing liposomes collected after the gel filtration column were first checked for their integrity (a calcein-Co – EDTA assay for monitoring vesicle fusion⁴⁶ was used as a basis). EDTA ligand liberates calcein from calcein-copper complex and does not penetrate the liposome bilayer. EDTA solution (60 mM final concentration) was added to the liposomes after the gel filtration with subsequent
10 breakage of liposomes with Triton X-100 (0.5%) to determine the assay end-point. The fluorescence was measured at 512 nm (excitation λ = 484 nm).

The experiment on determining the leakage, possibly induced by the formation of DTC-Cu complex, proceeded as follows:

DTC (0.3 μ M final concentration) was added to the Sephadex purified liposomes
15 and after 5 minutes the fluorescence was measured (excitation 484 nm, emission range 500-550 nm). Then, the sample was returned to the same gel filtration column (second gel filtration). EDTA (67 mM final concentration) and Triton X-100 (0.3 % final concentration) were added to the liposomes after the second gel filtration, and after 5 minutes the fluorescence was measured.

20 **Rates of penetration through liposome bilayer**

Kinetics of ligand exchange reaction $\text{Cal-Cu} + \text{DTC} \rightleftharpoons \text{Cal} + \text{DTC-Cu}$ was determined both in the liposomes and in buffer solution.

Calcein-copper containing liposomes, eluted from the column and diluted twice (to obtain 1.2 mg/ml PC), or buffer solution containing calcein-copper complex (0.08
25 μ M) was used for the determination of initial rates of appearance of free calcein ligand (excitation wavelength = 484 nm, emission wavelength = 512 nm). The fluorescence was measured under constant mixing. The measurements were monitored each 0.5 seconds for a few seconds before adding DTC (baseline) and until 6 minutes after adding it to the vesicle mixture (2 mM final concentration of DTC). Triton X-100 (0.1% final
30 concentration) was added at the end of experiments containing liposomes to break the vesicles and get the full value of fluorescence.

A control experiment with EDTA was made to check the integrity of liposomes after the gel filtration.

B. Bifunctional herbicide and metal chelator combination

Introduction

Diquaternary salts of 2,2' and 4,4'-bipyridyls are well known herbicides.⁴⁸ The mode of action of bipyridylum herbicides,⁴⁹⁻⁵¹ is related to one-electron metabolic reduction of paraquat (PQ^{2+}) by replacing naturally occurring ferredoxin to form the corresponding radical $PQ^{\cdot+}$ and instantaneous reoxidation to produce very reactive oxygen species (ROS). In plant leaves,⁵² the reduction is due to photoinduced electron transport from chlorophyll through the photosystems and it is generally believed that the phytotoxic activity of paraquat is due to membrane lipoxidation⁵³, causing membrane structure disintegration with water loss, leading to wilting. In fact, paraquat exerts its phytotoxic effect by accepting (iron-sulfur centers, replacing ferredoxin of photosystem I (PSI).⁵⁴ The electrons are transferred to oxygen (O_2) to produce different and very reactive toxic oxygen species, such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$).

Recently, there is a growing interest in paraquat due to its high toxicity to living cells in a variety of biological systems ranging from microorganisms through mammals⁵⁵ to man.⁵⁶ The mechanism of paraquat cytotoxicity in several *in vitro* test-systems,⁵⁷⁻⁶¹ including *E. coli* bacterial cells⁶²⁻⁶⁷ is believed to involve the generation of active oxygen radicals and the production of oxidative stress. Though there is no correlation between paraquat itself and Parkinsonisms other bipyridilliums, that do not replace ferredoxin and do not accept electrons in plant cause Parkinson like symptoms in a model system. The toxicity of paraquat is being used for *in vivo* studies of the mechanism of neuronal cell death.⁷² Due to the severe toxicity of paraquat (the mortality rate is 80% or even higher when large amounts are injected or absorbed through stem⁵⁶), it is most difficult to save an acutely poisoned or suicidal person. Until now, no specific therapy of paraquat poisoning is available since there are neither antidotes nor inhibitors of paraquat. Thus clinical aspects of accidental poisoning by paraquat remains an unsolved problem. The search for less toxic, yet strong, herbicide is therefore very important and justified.

Basic research towards detoxification of paraquat generated ROS was done in a variety of biological systems. Indeed, several reports show that induction of endogenous superoxide dismutase and catalase provided a modicum of cellular protection.^{61, 64, 73-75}

Thioureas,⁷⁶ xanthine oxidase and xanthine oxidase inhibitors,⁷⁷ polyamines⁷⁸⁻⁸¹ were also effective at inhibiting its toxicity. Superoxide is a source of hydrogen peroxide for the Haber-Weiss and Fenton reactions.^{67, 82} Transition metals, such as iron and copper, are able to accelerate the conversion of H₂O₂ to hydroxyl radical (OH[•]),^{83, 84, 65]} and hence transition-metal specific chelators especially iron chelators can reduce or prevent biological deleterious effects of paraquat.⁸⁵ Toxicity investigations of combined paraquat with diethyldithiocarbamate (Et₂DTC) are unequivocal in in vitro systems⁸⁶. However, our own previous results⁸⁷ and reports from literature^{88,59} demonstrated that DTCs could potentiate the toxicity of paraquat.

Part A of the present application shows that amphiphilic dithiocarbamates (DTCs) strongly inhibit SOD, with a pronounced increased ability to cross lipophylic membrane barriers compared to DTC salts. The next step was to consider a bifunctional herbicide and DTC combination, so that quickly penetrating paraquat will carry the covalently linked DTC unit which will act as a strong copper-specific chelator and inhibit SOD increasing activity. Therefore, we designed and synthesized a bifunctional molecule (PQ-DTC), incorporating the well-known paraquat and metal chelator, presupposed to aid in the production of H₂O₂ from O₂^{•-} by inhibiting SOD.

The synthesis was scheduled according to Scheme 4 (Figure 15). The strong complexation with Cu(II) indicates that the bifunctional molecule may remove-copper from SOD. The lower inhibition of SOD activity leads to lower herbicidal activity in comparison with paraquat especially with the synergistic mixture of paraquat and Et₂DTC. These findings are in accordance with the notion that metal chelators could reduce or prevent the biological deleterious effects of paraquat. These findings contradict our anticipation of increased herbicidal activity by PQ-DTC, but lead to an important conclusion that the toxicity of paraquat can be mitigated, as in this analogous PQ molecule, by incorporating a copper specific ligand. The explanation for this mediation is that the inner salt form of PQ-DTC could relay higher stability to the paraquat moiety, resulting in lower measured radical activity.

Experimental Section

Materials

4,4'-Bipyridine, 1,5-dibromopentane and carbon disulfide (99.5%) were obtained from Fluka. Methyl iodide was purified by redistillation (Aldrich, for synthesis). Di-*tert*-butyl dicarbonate (97%) and xanthine oxidase were purchased from Merck.

Diethyldithiocarbamic acid sodium salt, trihydrate (Et_2DTC) and xanthine were from Aldrich. Cytochrome C (from bovine heart) was obtained from Sigma. Recombinant human CuZnSOD was kindly provided by Biotechnology General, Weizmann Science Park, Nes-Ziona, Israel. Other compounds and solvents used were analytical grade and were used without further purification (except ethyl ether, which was dried over calcium chloride). Phosphate buffer (50 mM pH 7.8) was prepared from potassium dihydrogen phosphate and disodium hydrogen phosphate anhydrous (both from E. Merck, analysis grade). Carbonate buffer (50 mM pH 9.5), was prepared from sodium carbonate anhydrous (E. Merck, for analysis) and sodium hydrogen carbonate (Aldrich, A.C.S. reagent). A solution of 0.5 M Cu^{2+} was made from copper (II) sulfate pentahydrate (E. Merck, GR, for analysis).

General Methods

UV/VIS spectra were recorded from a Hewlett-Packard 8450A diode array spectrophotometer. Proton NMR spectra were measured on a Bruker WH-270, or Bruker DPX-250 NMR spectrometers. TLC was performed on Kieselgel 60 F254, E. Merck plates. Electrospray Ionization mass spectra (ESI-MS) were detected on Micromass Platform LCZ 4000 spectrometer. Infrared spectra from 400-4000 cm^{-1} were obtained on a Nicolet 460 single beam Fourier Transform Infrared Spectrophotometer (FTIR) (KBr pellets).

Synthesis

Table 9 lists structures and ^1H NMR data (δ , ppm) for all synthesized compounds.

N-(5-bromopentyl)-4-(4'-pyridyl) pyridinium bromide (**1**)

The synthesis of this compound was performed similar to literature.⁸⁹ 4,4'-Bipyridine (476mg, 3mmol) was placed in a 25 ml round-bottom flask, equipped with a magnetic stirrer in an oil bath. 1,5-Dibromopentane (6 ml) was added dropwise with stirring at room temperature. The reaction mixture was heated to 60°C, and stirring was continued for 10 h until disappearance of the 4,4'-bipyridine, as detected by TLC ($\text{CH}_3\text{Cl}:\text{CH}_3\text{OH}:\text{NH}_3 = 7:3:1$). Upon cooling of the mixture, 25 ml ether were added to achieve more precipitate, which was then filtered from the solution (1,5-dibromopentane remained in the solution). The solid was collected and then dissolved in a minimal amount of methanol. Acetone (15 ml) was added to precipitate the yellow-colored by-products, then **1** was precipitated from the acetone solution by the addition of

10 ml dried ether. The dissolution in 1 ml methanol with further precipitation from 20 ml acetone was several times until only one spot was observed by UV light was observed on the TLC plate. Compound 1 was finally obtained as a white powder (650 mg, 55% yield). $R_f = 0.2$ (CHCl_3 : CH_3OH : $\text{NH}_3 = 7: 3: 1$) and 0.49 (CH_3OH : H_2O : $\text{CH}_3\text{COOH} = 7: 3: 2$). ESI-MS: $m/z = 306$ (positive mode).

N-[5-(*n*-Butyl)aminopentyl]-4-(4'-pyridyl)pyridinium bromide (2)

Compound 1 (616 mg, 1.6 mmol) and 16 ml ethanol were placed in a 25 ml round-bottom flask, equipped with a magnetic stirrer and reflux condenser with calcium chloride outlet. A solution of (1.6 ml, 16 mmol, butylamine at 10 times excess was added dropwise to the mixture. About 5 mg of NaI was added to the mixture as a catalyst. The solution was stirred at 60°C for 32 hours. After the completion of the reaction (disappearance of 1 which was followed by TLC as above), the mixture was concentrated (~1/3 volume) by vacuum evaporation, and the crude product was precipitated from the dry ethyl ether. The precipitate was thoroughly washed with 25 ml ether to remove traces of bipyridine decomposition. The solid was then filtered and dried in vacuo. The residue was dissolved in a minimum amount of methanol and precipitated from 20 ml acetone. The product (compound 2) was filtered washed three times, each with 40 ml fresh acetone and dried in vacuo yielding 530 mg (78% yield) of yellow powder. $R_f = 0.15$ (CH_3OH : H_2O : $\text{CH}_3\text{COOH} = 7: 3: 2$), direct inspection with UV light. ESI-MS: $m/z = 298$ (positive mode).

N-[5-(*n*-Butyl)-*N*-(*tert*-butoxycarbonyl)-aminopentyl]-4-(4'-pyridyl)pyridinium bromide (3)

Compound 2 (500 mg, 1.2 mmol) was partially dissolved in 40 ml dioxane in a 100 ml round-bottom flask, equipped with magnetic stirrer and ice bath. A solution of (140 mg, 1.32 mmol Na_2CO_3) in 10 ml water was added at cooling. Di-*tert*-butyl dicarbonate (365 μl , 1.6 mmol) was diluted in 5 ml dioxane and dripped into the mixture. The solution was left at room temperature overnight until the reaction was completed, as detected by TLC: CHCl_3 : CH_3OH : $\text{NH}_3 = 7: 3: 1$). The mixture obtained was filtered to remove the by-product (the white precipitate), and then dioxane was evaporated in vacuo. Compound 3 was a yellow waxy material (410 mg, 65% yield). $R_f = 0.2$ (CHCl_3 : CH_3OH : $\text{NH}_3 = 7: 3: 1$) and 0.49 (CH_3OH : H_2O : $\text{CH}_3\text{COOH} = 7: 3: 2$), by direct inspection with UV light.

N-Methyl-N'-[5-(n-butyl)-N-(tert-butoxycarbonyl)-aminopentyl]-4,4'-bipyridinium dibromide (4)

Compound **3** (410mg, 0.78mmol) and (20 ml) DMF were placed in a 50 ml round-bottom flask, equipped with magnetic stirrer. Methyl iodide (500 μ l, 7.8 mmol) dissolved in 2 ml DMF was added dropwise and the solution was allowed to stir 8 hours at room temperature until the starting materials disappeared, (as detected by TLC: CHCl₃: H₂O: CH₃COOH = 7: 3: 1). Ethyl ether (10 ml) was added into the mixture. The precipitate (compound **4**) was separated from the solution by filtration, then washed 3 times with 5 ml ethyl ether and dried in vacuo. A red powder was obtained (300 mg, 71% yield). R_f = 0.20 (CH₃OH: H₂O: CH₃COOH = 7: 3: 2), staining with UV.

n-Butyl [N-methyl-N'-(5-aminopentyl)-4,4'-bipyridinium] amine tri(trifluoroacetate) (5)

Trifluoroacetic acid (4 ml) was added to a cooled 1 ml aqueous solution of compound **4** (300 mg, 0.45 mmol). The solution was stirred at room temperature for 6 hours. The mixture was filtered and some solvent (~3 ml) was removed by vacuum. Ethyl ether (5 ml) was added for the precipitation of the crude product. The precipitate was filtered, dissolved in 5 ml water to get rid of water-insoluble impurities. Water was removed by lyophilization to yield 259. mg (65%) of compound **5** as a red solid. ESI-MS: m/z = 313 (positive mode).

n-Butyl [N-methyl-N'-(5-aminopentyl)-4,4'-bipyridinium difluoroacetate]dithiocarbamic acid inner salt (6)(PQ-DTC)

Compound **5** (118.4 mg, 0.18 mmol) dissolved in 3 ml of water, with 200 μ l, 1M NaOH in a 25 ml round-bottom flask equipped with the magnetic stirrer and an ice bath. Carbon disulfide (90 μ l, 1.1.5 mmol, 8.2 times excess) was added dropwise to the cooled mixture. After half-hour, the ice bath was removed and the mixture was stirred at room temperature for 4 hours. The excesses of carbon disulfide and water were removed by lyophilization. The progress of the reaction was followed by NMR to calculate the ratio between product **6** and unreacted compound **5**. The reaction was half complete after 4 h. Thus, the same process is needed to carry out the reaction until 4% of compound **5** remained (according to NMR). ESI-MS: m/z = 388 (M⁺⁺ - Na) (positive mode). IR: 1640 cm⁻¹ (absorption for the quarternized pyridinium salt) and 961 cm⁻¹ ($\nu_{C=S}$)

N-[5-(n-butyl)aminopentyl]-4-(4'-pyridyl)pyridinium bromide]dithiocarbamic acid inner salt (7)(monosubstituted PQ-DTC)(M PQ-DTC)

Compound **2** (110 mg, 0.3 mmol) was dissolved into 2 ml of water and 300 μ l of 1.2 M NaOH were added to adjust the pH to 9.3-9.7. Carbon disulfide (36 μ l, 2 times excess) was dripped into the mixture under ice-bath. After the addition, the ice-bath was removed and the reaction mixture was allowed to stir 2 h at room temperature. Product **7** was collected by filtration, washed with ether, then dried in vacuo to yield of 105 mg. (78%) $R_f = 0.10$ (CH₃OH: H₂O: CH₃COOH = 7: 3: 2). ESI-MS: $m/z = 396$ ($M^+ + Na$) and 769 (dimer of *monosubstituted PQ-DTC*) (positive mode). IR: 1640 cm^{-1} (absorption for the quarternized pyridinium salt) and 961 cm^{-1} ($\nu_{C=S}$)

Formation of PQ-DTC-Cu²⁺ complex (see Figure 19)

PQ-DTC (0.1 mM) was mixed with various concentrations of Cu²⁺ (as CuSO₄) in 50 mM phosphate buffer (pH = 7.8) and incubated for one hour before measurements. The formation of PQ-DTC-copper (II) complex was measured spectrophotometrically by following the appearance of colored complex ($\lambda_{max} = 435$ nm).

Hydrolytic stability test (Figure 22)

The stability of PQ-DTC and compound **7** (MPQ-DTC) was directly measured in D₂O and in deuterated DMSO directly in an NMR tube, respectively. ¹H NMR spectra were recorded daily for more than 20 days. The NMR tube was opened several times for two hours, to introduce fresh air.

Inhibition of SOD activity by PQ-DTC (Figure 23)

The activity of SOD was measured by monitoring the inhibition of the reduction of ferricytochrome C by superoxide generated by a xanthine/xanthine oxidase reaction as per ⁴⁴. The compounds, Et₂DTC, PQ-DTC and paraquat secondary amine (compound **5**) were preincubated at the molar ratio of compound /SOD of 10 : 1, 25 : 1 and 50 : 1, with 1 mM SOD at pH 7.8 (or at pH 9.5 for Et₂DTC) for 2.5 hours at room temperature, These solutions were then diluted with 50 μ l 50 mM potassium phosphate buffer (pH 7.8). The SOD active reaction mixture contained 3 nM enzyme-SOD, was preincubated for 2.5 hours at pH 7.8 and used as a control.

Herbicidal activity of PQ-DTC in vivo (Figure 24)

Herbicidal activities of PQ-DTC and paraquat (for comparison) were determined *in vivo* on *Spirodella oligorrhiza* sp. by the bleaching of chlorophyll 72 h after the addition of various concentrations PQ-DTC and paraquat, and incubation at 25°C at a light intensity

of $500 \mu\text{Ein s}^{-1} \text{m}^{-2}$. The quantification of chlorophyll was made by extraction of chlorophyll into 80% acetone. The absorption value was recorded at 645 nm and 663 nm, respectively, and chlorophyll content calculated according to Arnon.

5 Results and discussion

Synthesis

Our original approach to the synthesis of PQ-DTC-type chelators aimed for synthetic simplicity. Hence, we decided to try bisquaternization of 4,4'-bipyridine with dihalo or monohalo alkanes, followed by introduction of symmetrical bis-amino or mono-amino alkyl substituents. Consequently, we prepared a series of new bisquaternized 4,4'-bipyridium salts with different substituents. Appendix 1 describes the experimental conditions and the structures of these compounds. However, attempts to introduce the secondary amine function failed and led to dealkylation under the basic conditions^[45] Therefore, we needed to elaborate a more complicated scheme 4 (Figure 15) for this synthesis: starting with monoquaternized 4,4'-bipyridium salt (compound 1), following by reaction with butyl amine to compound 2, while protecting the secondary amine (in compound 3). Then quaternization of compound 3 by methyl iodide giving compound 4 and subsequent removal of the N-Boc group and transformation back to secondary amine (in compound 5); finally from compound 5 to the paraquat-DTC bifunctional molecule (PQ-DTC, compound 6).

A monosubstituted analogue of PQ-DTC, compound 7 (MPQ-DTC) was synthesized by the reaction of compound 2 with CS_2 in the presence of NaOH. Surprisingly, MPQ-DTC is relatively insoluble in water and precipitated during the reaction. The proposed inner salt structure could well explain this solubility behaviour.

25 UV spectra

PQ-DTC and Cu(II) complexation in water

The UV spectra of PQ-DTC, compound 5, and Et_2DTC in a pH 7.8 buffer solution are shown in Figure 18. Et_2DTC has two bands 256 nm, 283 nm. The 256 nm in Et_2DTC band is associated with polarization of the nitrogen conjugation, and the band at 283 nm is attributed to sulfur conjugation.^[46] In comparison, PQ-DTC (compound 6) and its precursor (compound 5) display absorption bands at 228 nm and 260 nm, and PQ-DTC has a shoulder around 283 nm. After subtraction of the spectrum of compound 5 from the spectrum of PQ-DTC (curve 4 in Figure 18) the spectrum is that of Et_2DTC .

This interpretation of the UV spectra supports the contention that PQ-DTC, is bifunctional consisting of PQ and DTC. Subunits.

The influence of Cu(II) on the UV spectrum of 0.1mM M PQ-DTC is shown in Figure 19. A band appears at 435 nm, as a result of the complexation between Cu(II) and dithiocarbamate group. Furthermore, the ratio of complex between Cu(II) and DTC unit present on PQ-DTC is found to be 1:2. PQ-DTC (like DTC itself) does not complex with iron at pH 7.8 buffer (see Figure 20). Therefore, PQ-DTC like DTC is a superior selective ligand toward Cu(II) over iron.

Production of PQ-DTC radical $[PQ-DTC]^+$ in polar organic solvents

The absorption spectra of 0.1mM M PQ-DTC in different polar solvents (CH_3OH , DMF, DMSO and CH_3CN) and the spectral time dependence are presented in Figures 21. All spectra have two pronounced absorption maxima around 410 nm and 575 nm, characteristic of 4,4'-bipyridium radical cations. Solutions of PQ-DTC ethanol and acetonitrile(CH_3CN) are gradually reduced with a dramatic color change from pale yellow to blue owing to the accumulation of PQ-DTC radical $[PQ-DTC]^+$. Dissolving PQ-DTC in DMF and DMSO results in immediate formation of blue-violet color. This PQ-DTC radical is stable and based on the spectral variations in time. However, in CH_3OH , we found the color disappears with time, indicating that PQ-DTC is reformed. , There is also spectral time-dependency and in CH_3CN , the primary peak is around 400 nm and a more intense second peak is at 575 nm. Both peaks increase simultaneously. The primary peak in DMSO, is at 412 nm and the second, a less intense peak is at 575 nm. Both of the shoulder at 412 nm and the 577 nm increase with time.

PQ-DTC in DMF exhibits the strongest absorption at 577 nm and the fastest rate to reach a constant radical concentration compared to the other three solvents. UV spectroscopy is the basis for most of the evidence of PQ radical-cation dimerization or association and there are many examples and references cited in ^[47]. Therefore, the spectral variation of PQ-DTC could be related to the form of association of the cation radical $[PQ-DTC]^+$. With either mono-cation dimers or di-cation dimers in different solvents.

The precursor of PQ-DTC (compound 5) was spectrally analyzed in the same solvents to try again and to understand the production of such stable radical from PQ-DTC,. No evidence for the presence of the blue-violet radical type of PQ was found with this intermediate. Neither from PQ-DTC nor compound 5. Generate any stable PQ

radical in water. These results point to two important directions: (1) the influence of the internal factor of the substituent R [$\sim(\text{CH}_2)_4\text{CH}_2\text{N}(\text{DTC})(\text{CH}_2)_3\text{CH}_3$] in compound 6, having a great influence in decreasing PQ-DTC the production of radical $[\text{PQ-DTC}]^+$ and (2) polar organic solvents promote the formation and association of PQ type cation radicals $[\text{PQ-DTC}]^+$ (3). We must take into account to interpret these phenomena. the structure of PQ-DTC. as Dam et al⁹³ claim that the stability and the intensive color of the radical ions results from the extensive delocalization of the electron added to the π -electron system of the bipyridium group upon reduction. If any of substituents affect the π -electron system, the redox potential will change. The value of the redox potential of a particular bipyridilium in organic solvents differs from that in aqueous solution, a topic well described.^{92,93} In the absence of an external electron donor, the dithiocarbamate group ($-\text{CS}_2^-$) in PQ-DTC acts to enhance the production of free radicals in polar organic solvents. The stability of the $[\text{PQ-DTC}]^+$ radical may be attributed to dimerization and to an intrinsic factor related to delocalization caused by electronegative atoms.

Stability and inner salt structure

PQ-DTC decomposes into compound 5 and CS_2 . Following the integration values of $\text{PQ-CH}_2\text{NHCH}_2\sim$ ($\delta = 2.86\text{ppm}$) and $\text{PQ-CH}_2\text{N}(\text{DTC})\text{CH}_2\sim$ ($\delta 3.70\sim 3.90\text{ppm}$), the percent decomposition of PQ-DTC can be roughly calculated. (Fig. 22) Decomposition increases with time and depends on pH. The decomposition rate increases rapidly at a more acidic pH. However, the decomposition of PQ-DTC is much slower than that of the family of amphiphilic dithiocarbamates (see Part A). The PQ unit in PQ-DTC, bearing two quaternary nitrogens at one end, is capable of forming inner salts with the $\sim\text{CS}_2^-$ group. Kloppe^[49] described a group of quaternary ammonium inner salts of the general formula $\text{Q}^+-(\text{CH}_2)_2\text{NRCS}_2^-$, where Q is $\text{N}(\text{alkyl})_3$ or pyridinium, and R is H or alkyl. An inner salt structure of the general formula is shown in Scheme 5 (Figure 16). Experimental results provide evidence to support this structure. Mass spectrum of PQ-DTC (in positive mode) is 388, supporting a molecular ion lacking a sodium counterion. In order to put this question to the test, we looked at a synthesized monoquarterized analog of PQ-DTC. Monosubstituted PQ-DTC (MPQ-DTC) is relatively insoluble in water and its mass spectrum shows the molecular weight of $m/e = 396 (\text{M}^+ + \text{Na})$ and a dimer of $m/e = 769$. Thus, monosubstituted MPQ-DTC (compound 7) is an inner salt as shown in Scheme 6 (Figure 17). The formation of inner salts could

be one of the reasons why PQ-DTC (compound **6**) is hydrolytically more stable than the family of amphiphilic dithiocarbamates.

Similarly, MPQ-DTC decomposes into compound **2** and CS₂. The stability of MPQ-DTC was checked by NMR with deuterated DMSO as the solvent. A new peak at δ 2.80ppm (MPQ~CH₂NHCH₂~) appeared at the expense of the peak at δ 4.00ppm ((MPQ~CH₂N(DTC)CH₂~). The decomposition is faster than PQ-DTC in water since 30% of MPQ-DTC was decomposed after 6 days.

From the dimeric structures of PQ-DTC and MPQ-DTC, we infer that the changes in the length of the spacer between the pyridinium unit and the DTC chelator will not change the stability of the dimer. A different chelator (Che) incapable of forming ionic associations with the quaternary nitrogen on the pyridine skeleton will be needed. Such new chelators are being developed.

Activity of PQ-DTC in vitro and in vivo systems

In order to analyze the possible modes of action of a bifunctional compound carrying a herbicidal active group, e.g., paraquat, and a metal binding group, e.g., DTC, the activity of each function should be examined separately. This means that the paraquat activity in PQ-DTC must be compared to paraquat activity in PQ itself and in PQ compounds related. Properties, such as (1) rates of PQ⁺ radical formation, radical stability and decay; (2) effect of substituent on the redox potential of PQ; (3) herbicidal activity; and finally (4) toxicity must be checked. The DTC activity in PQ-DTC must be compared to Et₂DTC activity in certain selected reactions.

Inhibition of SOD Activity in vitro

Superoxide was generated in a biological assay by xanthine/xanthine oxidase reaction and SOD was actively detected by the inhibition reduction of cytochrome C. Experimental result shows that PQ-DTC and its precursor compound **5** do not influence the cytochrome C reduction at the concentrations used. It means that PQ-DTC and compound **5** do not accept an electron from xanthine/xanthine oxidase reaction to produce [PQ-DTC]⁺, and then produce superoxide.

In order to test the DTC part of PQ-DTC, we have employed the inhibition effect of DTCs on the dismutase activity of CuZn-SOD. (Fig. 23. Compound **5** is totally inactive, while PQ-DTC (contains 4% of its precursor compound **5**) has some ability to inhibit SOD, but it is lower than the activity of Et₂DTC at all DTC/SOD ratios.

Herbicidal activity of PQ-DTC *in vivo*

The PQ-DTC herbicidal activity *in vivo* was evaluated by the loss of chlorophyll from the *Spirodella oligorrhiza* and was compared with the herbicidal activity of paraquat. (Fig.24). The PQ-DTC has (about two orders of magnitude) less herbicidal activity than to paraquat on a molar equivalence basis.

The results of the preliminary experiments described above show that the PQ-DTC could inhibit the activity of SOD *in vitro* (xanthine/xanthine oxidase/cytochrome C assay) to some extent. The radical production activity of the PQ-DTC is too low to permeabilize the cell membranes and kill the plants *in vivo*.

Finally, some interesting conclusions come from this work:

- (1) The DTC part in PQ-DTC retains quite significant ability to inhibit SOD activity.
- (2) The herbicidal activity of the PQ part in PQ-DTC is almost completely suppressed (>99%).
- (3) The DTC part is still able to strongly complex Cu^{2+} ions but its affinity to Fe^{3+} is weakened by the positive charges in PQ-DTC. The reduced activity of PQ-DTC *in vitro* could be anticipated from the longer lifetimes of PQ^+ radical in organic solvents.

The great reduction in herbicidal activity of PQ-DTC can be interpreted as stemming from the close interaction between the PQ unit and the DTC unit, through the formation of ionic associations (shown in Schemes 5 and 6, in Figures 16 and 17). Structural changes, such as removing the secondary amine position along the aliphatic chain may not affect such interactions and increases the herbicidal activity of paraquat. The only solution would be to change the DTC chelator to a Cu chelator with less ionic properties.

Conclusions

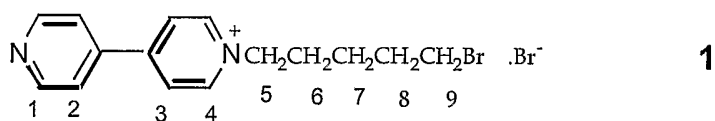
Bifunctional-(paraquat) dithiocarbamate (DTC) chelator groups were reconstructed into one molecule. The structural existence of both functional groups in PQ-DTC was verified by spectral methods and by formation of a 2 : 1 PQ-DTC/Cu complex, but the herbicide activity was almost totally lost. The PQ function did produce stable free radicals $[\text{PQ-DTC}]^+$ in organic polar solvents (DMF, DMSO, CH_3OH and CH_3CN) but not in water, yet shows almost total herbicidal inactivity. The explanation lies in the structural form of an inner salt of PQ-DTC (Scheme 5, Figure 16) inferred from spectral data and absence of sodium in the analysis of compound 6. Further support is gained from similar conclusions about the ionic dimeric form of MPQ-DTC (scheme

6, Figure 17). The extension of this work to other PQ-DTC compounds with variable spacer groups, and to other paraquat chelators (PQ-Che) of attaching some synergistic entity to any rapidly penetrating herbicide is of great interest. The synergistic entity may be an inhibition of the degradation of the herbicide in the target plant (Gressel) It may allow designing herbicides with controllable properties (toxicity, herbicidal activity). This may also lead to new directions towards drug-like molecules with controlled activity and controlled degradation of themselves or of the toxic metabolites they generate.

Paraquat is a potent oxygen radical generating compound in aerobic systems, especially plants but including all aerobic types. Its effects are often partially offset by Cu/Zn superoxide dismutase (SOD), which detoxifies superoxide. Cu/Zn SOD can be inhibited by copper specific chelators. This is a first example of a bifunctional-herbicide chelator (paraquat), and a dithiocarbamate (DTC)-copper chelator, that was constructed into one molecule (PQ-DTC). The aim was to see if the PQ-DTC combination will cause a synergistic action that will produce more free radicals to stimulate and enhance the inhibition of superoxide dismutase (SOD) more than paraquat and dithiocarbamate alone. The synthesis was scheduled according to Scheme 4 (Figure 15). The structural existence of both individual functions in PQ-DTC was verified by spectral methods and by formation of a 2 : 1 PQ-DTC/Cu complex. Experimental evidence shows that PQ-DTC has a remarkably reduced activity of its photooxidative herbicide function. The structure of inner salt (Scheme 5) leads to a higher stability of PQ-DTC compared to other members in the family of dithiocarbamate chelators (DTCs). Further support of inner salt structure proposed for PQ-DTC is gained from similar conclusions about the ionic dimeric form for monosubstituted paraquat-DTC (MPQ-DTC) (Scheme 6, Figure 17). Spectroscopic analysis suggests that PQ-DTC produces stable free radical $[PQ-DTC]^+$ in organic polar solvents (DMF, DMSO, CH_3OH and CH_3CN), but not in water, and yet shows almost total herbicidal inactivity. PQ-DTC decomposes (Figure 22) slowly into compound **5** and CS_2 by following the integration values of $PQ-CH_2NHCH_2-$ ($\delta = 2.86ppm$) and $PQ-CH_2N(DTC)CH_2-$ ($\delta 3.70\sim 3.90ppm$). The results presented here indicate that PQ-DTC does not work well as synergistic type herbicide, but lead to insight how to design other paraquat chelators (PQ-Che) or even general herbicide chelator (Her-Che) with 'tunable properties' such as toxicity and herbicidal activity.

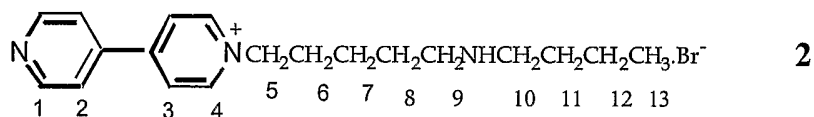
Table 9:

¹H NMR Data (chemical shift, ppm) for products. s-singlet, d-double, t-triplet, q-quartet, m-multiplet



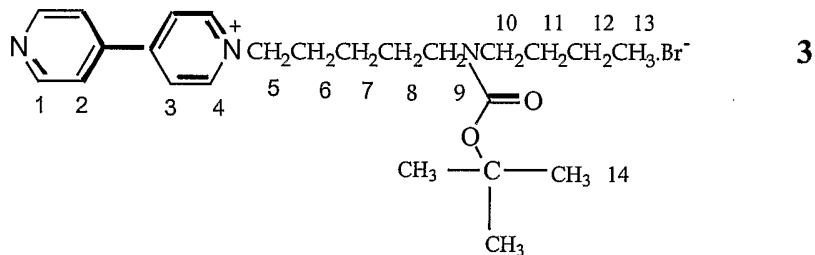
¹H NMR in D₂O

1	2	3	4	5	6	7
7.72, 2H, d	8.24, 2H, d	8.61, 2H, d	8.80, 2H, d	4.60, 2H, t	1.94, 2H, m	1.33-1.42, 2H, m
8	9	H ₂ O				
1.76, 2H, m	3.34, 2H, t	4.63, s				



¹H NMR in D₂O

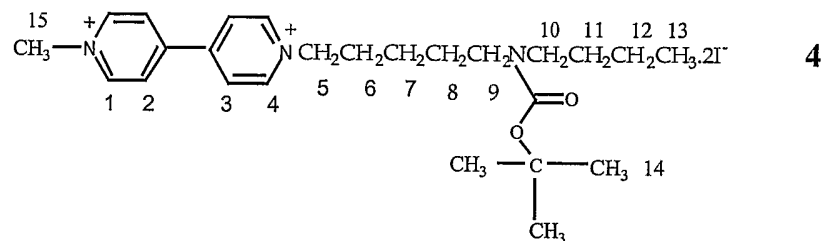
1	2	3	4	5	6	7+12
7.74, 2H, d	8.23, 2H, d	8.55, 2H, d	8.78, 2H, d	4.55, 2H, t	1.93, 2H, m	1.12-1.32, 4H, m
8+11	9+10	13	H ₂ O			
1.4-1.6, 4H, m	2.88, 4H, m	0.76, 3H, t	4.63, s			



¹H NMR in D₂O

1	2	3	4	5	6	7, 8, 12,
7.78, 2H, d	8.35, 2H, d	8.65, 2H, d	8.85, 2H, d	4.56, 2H, t	1.97, 2H, m	1.05-1.31, 6H, m
9+10	11	13	14	H ₂ O		
2.95-3.15, 4H, m	1.45, 2H, m	0.73, 3H, t	1.23, 9H, s	4.63, s		

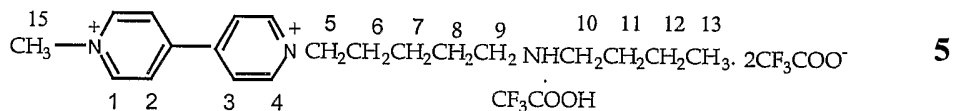
Table 9 (continue)

¹H NMR in D₂O

1	2+3	4	5+H ₂ O	6	7+8+12
8.95, 2H, d	8.38, 4H, m	8.89, 2H, d	4.63, m	1.95, 2H, m	1.04-1.13, 6H, m
9+10	11	13	14	15	
2.84-3.05, 4H, m	1.47, 2H, m	0.71, 3H, t	1.23, 9H, s	4.33, 3H, s	

¹H NMR in DMSO

1	2+3	4	5	6	7+8+11+12+14
9.36, 2H, d	8.68, 4H, m	9.20, 2H, d	4.67, 2H, t	2.00, 2H, m	1.2-1.6, 17H, m
9+10	13	15			
3.01, 4H, m	0.88, 3H, t	4.40, 3H, s			

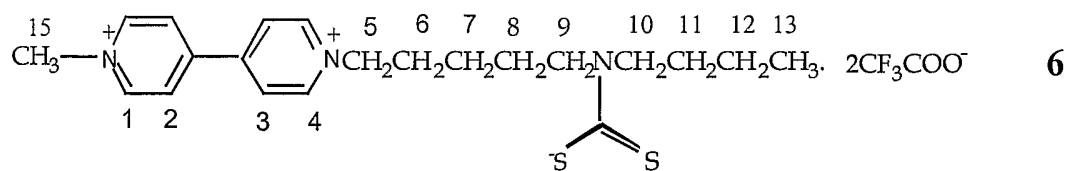
¹H NMR in D₂O

1	2+3	4	5+H ₂ O	6	
8.94, 2H, d	8.36, 4H, m	8.86, 2H, d	4.56-4.65, m	1.95, 2H, m	
	7+8+11+12	9+10	13	15	
	1.18-1.61, 8H, m	2.87, 4H, m	0.74, 3H, t	4.33, 3H, s	

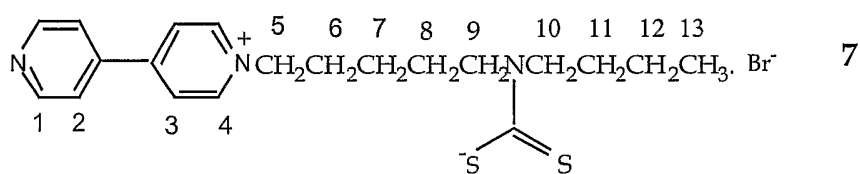
¹H NMR in DMSO

1	2+3	4	5	6	8+11	7+12
9.40, 2H, d	8.77, 4H, m	9.28, 2H, d	4.66, 2H, t	1.91, 2H, m	1.46-1.72, 4H, m	1.20-1.46, 4H, m
9+10	13	15				
2.86, 4H, m	0.89, 3H, t	4.44, 3H, s				

Table 9 (continue)

¹ H NMR in D₂O

1	2+3	4	5+H ₂ O	6	7+12
8.93, 2H, d	8.40, 4H, m	8.88, 2H, d	4.57-4.65, m	1.98, 2H, m	1.09-1.24, 4H, m
8	9+10	11	13	15	
1.47, 2H, m	3.70-3.90, 4H, m	1.62, 2H, m	0.72, 3H, t	4.34, 3H, s	

¹ H NMR in DMSO

1	2	3	4	5	6
8.05, 2H, d	8.63, 2H, m	8.87, 2H, m	9.30, 2H, m	4.69, 2H, t	2.00, 2H, m
7+12	8+11	9+10	13		
1.16-1.31, 4H, m	1.50-1.70, 4H, m	4.00, 4H, m	0.87, 3H, t		

Appendix 1

Table 10 lists ^1H NMR data (δ , ppm) for following compounds.

N-(Decanyl)-4-(4'-pyridyl)pyridinium bromide (1)

The synthesis was based on ⁸⁹ 1.6 g (10 mmol) of 4, 4'-bipyridine was dissolved into 10 ml DMF. A ml solution (5 M) 1-bromodecan in 5 ml DMF was added dropwise. The mixture was heated for 17 hours at 85°C. The precipitate was filtered, then 20ml ether was added to the filtrate for precipitation product. The white product (1.30g, 69% yield) was collected, filtered and washed with ether, then dried under vacuum. $R_f = 0.39$ (CH₃OH: H₂O: CH₃COOH = 7: 3: 2).

N-Decanyl-N'-(5-bromopentyl)-4,4'-bipyridinium dibromide (2)

Compound **1** (197 mg, 0.5 mmol) and a large excess (8.0 ml) 1, 5-dibromopentane were allowed to stand on oil bath at 80°C. The reaction was followed by TLC (CH₃Cl: H₂O: CH₃COOH = 7: 3: 1) until **1** was completely disappeared. The mixture was cooled, then ether was added. The crude product **2** collected, filtered and washed with ether for a few of times. It was dried in vacuo yielding yellow product (200 mg, 64% yield). $R_f = 0.22$ (CH₃OH: H₂O: CH₃COOH = 7: 3: 2).

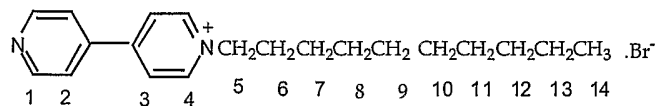
4,4'-Di(5-bromopentyl)dipyridinium dibromide (3)

313mg (2mmol) of 4, 4'-bipyridine were dissolved in 5 ml of DMF. A 10 times excess of 1, 5-dibromopentane (5.4 ml) was dropped from funnel with stirring. The mixture was heated on oil bath at 60°C for 18 hours. The precipitate was collected and washed with ether. Drying and removal of the solvent gave **3** (1.07 g, 87% yield). $R_f = 0.19$ (CH₃OH: H₂O: CH₃COOH = 7: 3: 2).

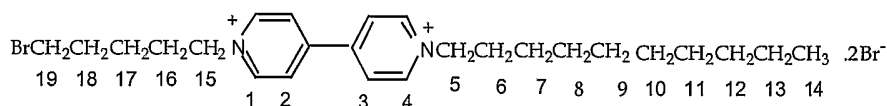
N-Methyl-N'-(5-bromopentyl)-4,4'-bipyridinium bromide iodide (4)

N-methyl-4-(4-pyridyl)pyridinium iodide (60 mg, 0.2 mmol) was dissolved into 25 ml acetonitrile. A solution of 1,5-dibromopentane (1.4 ml) in 5 ml of acetonitrile was added dropwise with stirring. The mixture was refluxed for 12 hours. After cooling, The crude product was filtrated out from the solvent, then washed with CH₃CN, drying in vacuo to give 34 mg of pure product **4** (32% yield) $R_f = 0.20$ (CH₃OH: H₂O: CH₃COOH = 7: 3: 2).

Table 10:

¹H NMR Data (chemical shift, ppm) for products. s-singlet, d-double, t-triplet, m- multiplet**1**¹H NMR in D₂O

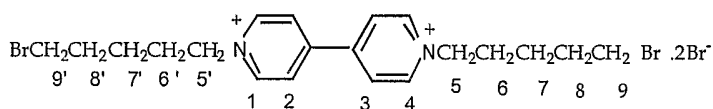
1	2	3	4	5	6	7-13
7.73, 2H, d	8.27, 2H, d	8.59, 2H, d	8.78, 2H, d	4.54, 2H, t	1.80, 2H, m	1.0-1.16, 14H, m
14	H ₂ O					
0.70, 3H, t	4.63, s					

**2**¹H NMR in D₂O

1+4	2+3	5+15+H ₂ O	19	6+16	17	18
8.96, 4H, m	8.42, 4H, d	4.53-4.65, m	3.3, 2H, t	1.80-2.0, 4H, m	1.70-1.90, 2H, m	1.20, 2H, m
7-13	14					
1.00-1.18, 14H, m	0.67, 3H, t					

¹H NMR in DMSO

1+4	2+3	5+15	19	6+16+17	18	9-13
9.42, 4H, d	8.84, 4H, d	4.72, 4H, m	3.56, 2H, t	1.89-2.02, 6H, m	1.40, 2H, m	1.15-1.25, 14H, m
14						
0.83, 2H, t						

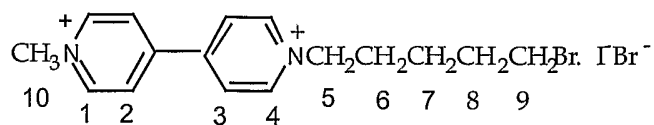
**3**¹H NMR in D₂O

1+4	2+3	5+5'+H ₂ O	9+9'	6+6'	8+8'	7+7'
8.98, 4H, m	8.40, 4H, d	4.60-4.65, m	3.35, 4H, m	1.98, 4H, m	1.78, 4H, m	1.40, 4H, m

¹H NMR in DMSO

1+4	2+3	5+5'	9+9'	6+6'	8+8'	7+7'
9.50, 4H, m	8.90, 4H, m	4.79, 4H, m	3.58, 4H, m	2.05, 4H, m	1.90, 4H, m	1.47, 4H, m

Table 10 (continue)

**4**¹H NMR in D₂O

1	2+3	4	5+H ₂ O	6	7	8
8.87, 2H, d	8.35, 4H, m	8.94, 2H, d	4.57-4.64, m	1.95, 2H, m	1.36, 2H, m	1.76, 2H, m
9	10					
3.30, 2H, t	4.32, 3H, s					

¹H NMR in DMSO

1	2+3	4	5	6+8	7
9.41, 2H, d	8.80, 4H, m	9.28, 2H, d	4.65, 2H, t	1.83-2.00, 4H, m	1.42, 2H, m
9	10				
3.57, 2H, t	4.72, 3H, s				

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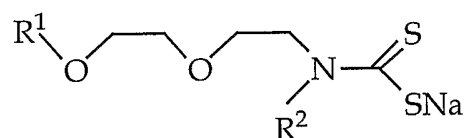
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CLAIMS

1. A combinatorial library comprising a plurality of bifunctional molecules having the formula CF - PF wherein,
CF is a first moiety of a first specific chemical or biological activity; and
PF is a second moiety imparting the molecule tunable physical properties;
and wherein structural modifications in the second moiety vary and tune the physical properties of the bifunctional molecule without significantly modifying the first chemical or biological activity for obtaining optimized effect of the first chemical or biological activity at the environment where the chemical or biological activity is to be expressed.
2. The combinatorial library of claim 1 wherein the second moiety further imparts the molecule a second specific chemical or biological activity and wherein the structural modifications in the second moiety vary the physical properties of the bifunctional molecule without significantly modifying the second chemical or biological activity.
3. The combinatorial library of claim 1 wherein the second moiety is an amphiphilic group.
4. The combinatorial library of claim 1 wherein the second moiety is an oligoether group.
5. The combinatorial library of claim 1 wherein the physical properties are one or combination of the following: lipophilic solubility, hydrophilic solubility, lipophilic-hydrophilic partition, surface activity, transport properties and hydrolysis rate.
6. The combinatorial library of claim 1 wherein the first moiety is a metal chelator.
7. The combinatorial library of claim 1 wherein the first moiety is a disubstituted

dithiocarbamic acid or a salt thereof.

8. The combinatorial library of claim 1 wherein the first moiety is disubstituted dithiocarbamic acid or a salt thereof and the second moiety is an oligoether group.
9. The combinatorial library of claim 1 wherein the bifunctional molecule is of the formula:



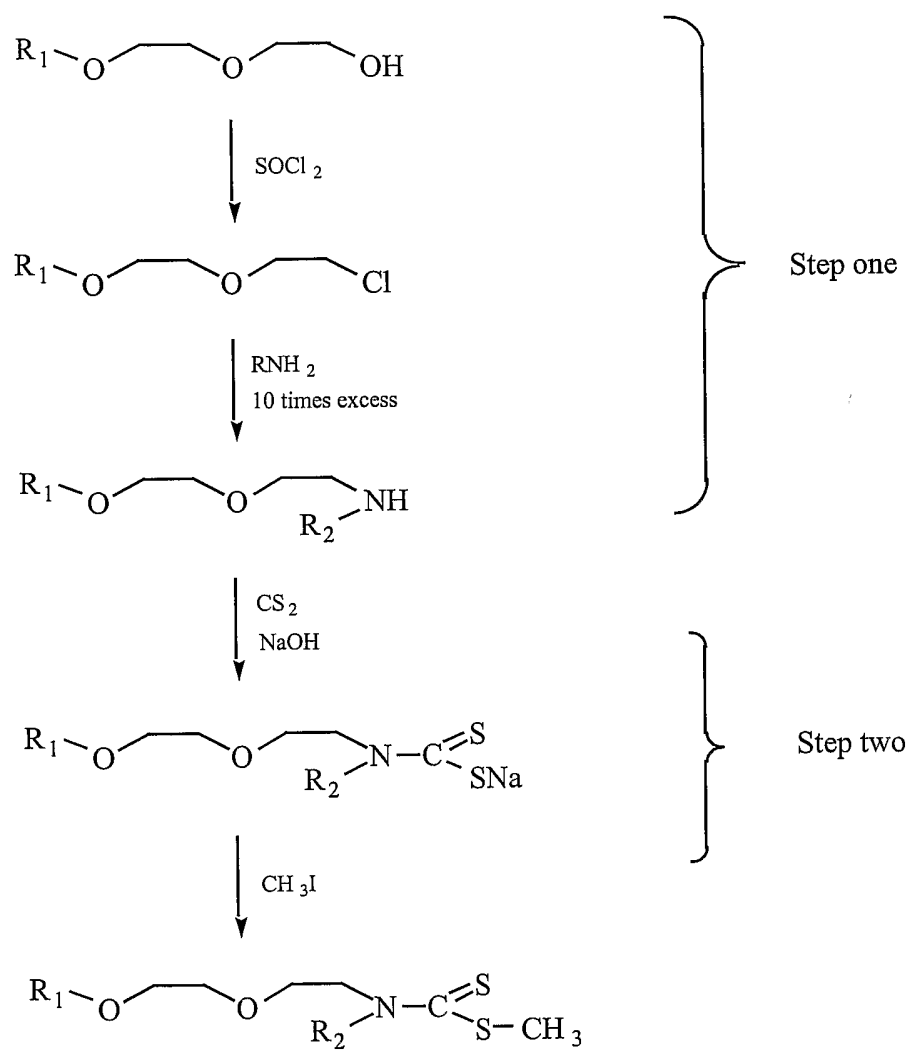
where R¹ and R² are independently a normal or branched, substituted or unsubstituted alkyl of 1 to 20 carbons.

10. The combinatorial library of claim 9 wherein
 - R¹ is selected from the group consisting of ethyl and butyl; and
 - R² is selected is an alkyl of more than 6 carbon atoms.
11. The combinatorial library of claim 9 wherein
 - R¹ is selected from the group consisting of ethyl and butyl; and
 - R² is selected from the group consisting of hexyl, octyl, decyl or dodecyl
12. The combinatorial library of claim 1 wherein the second moiety is a herbicide molecule.
13. The combinatorial library of claim 1 wherein the second moiety is paraquat.
14. The combinatorial library of claim 1 wherein the first moiety is a metal chelator and the second moiety is herbicide.
15. The combinatorial library of claim 1 wherein said the first moiety is

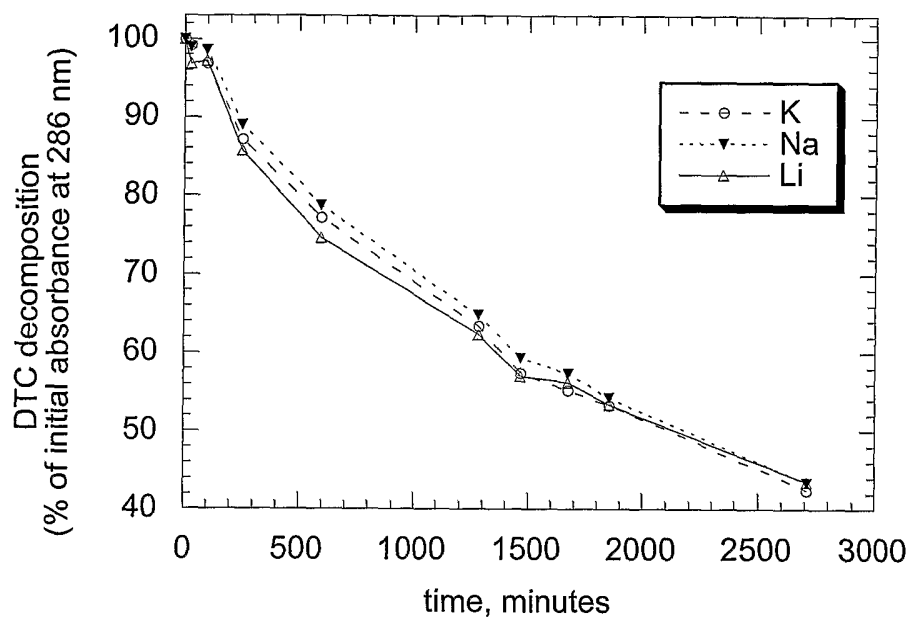
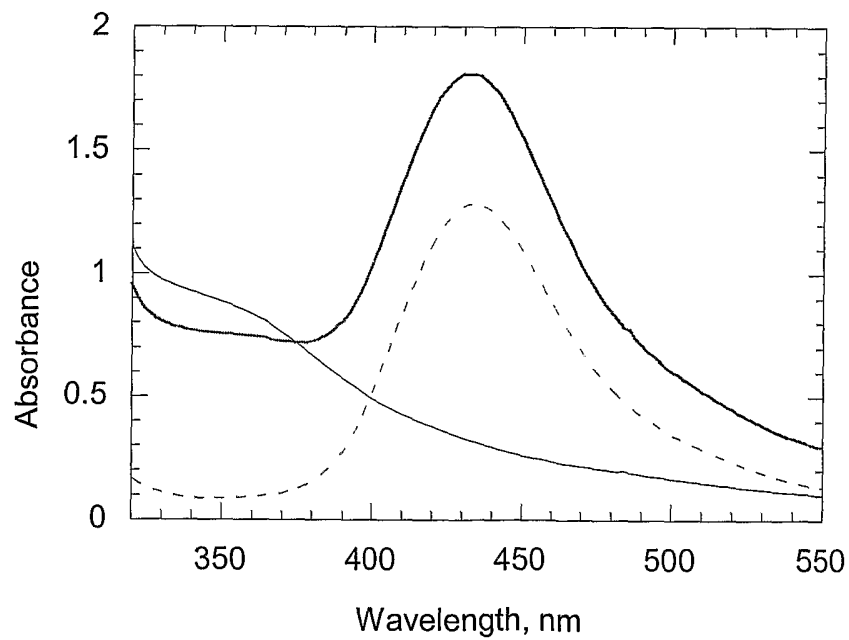
disubstituted dithiocarbamic acid or a salt thereof and the second moiety is herbicide.

16. The bifunctional molecule of claim 1 wherein the first moiety is a bioactive or agroactive group selected from the group consisting of a drug, a cosmetic, a peptide, a hormone, a UV responsive molecule, a light responsive molecule, an ultrasound responsive molecule, a microwave responsive molecule, an NMR or EPR responsive molecules, an olfactory molecule, a taste-responsive molecule, an oligonucleotide, a nucleic acid and a protein.
17. The bifunctional molecule of claim 15 wherein the second moiety is an amphophile.
18. The bifunctional molecule of claim 15 wherein the second moiety is disubstituted dithiocarbamic acid or a salt thereof.
19. A bifunctional molecule comprising a first moiety and a second moiety, the first moiety imparts said molecule a first specific chemical activity and the second moiety imparts the molecule specific physical properties and optionally a second specific chemical activity, wherein structural modifications of said second moiety tune the physical properties of said bifunctional molecule without significantly modifying said first and optionally second chemical activities for obtaining optimized effect of said first and optionally second chemical activities at the environment where said chemical activities are to be expressed.

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Scheme 2**FIGURE 1**

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**FIGURE 2****FIGURE 3**

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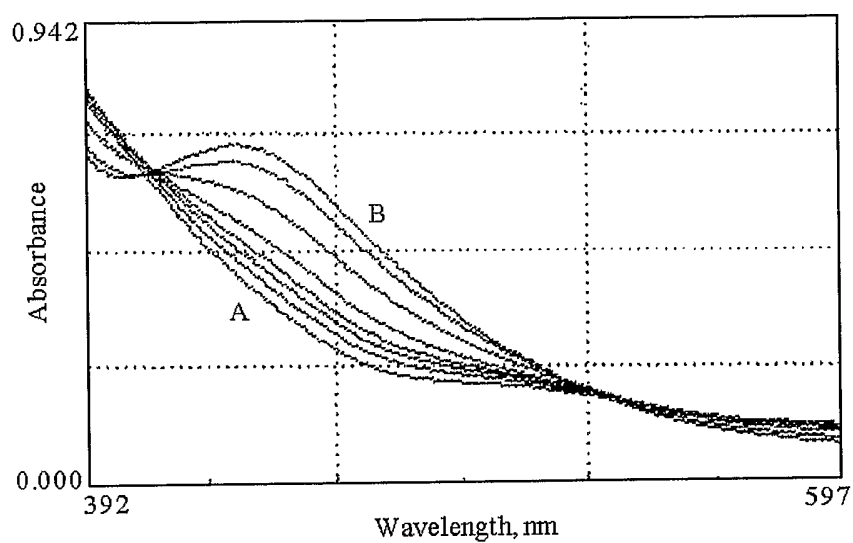


FIGURE 4

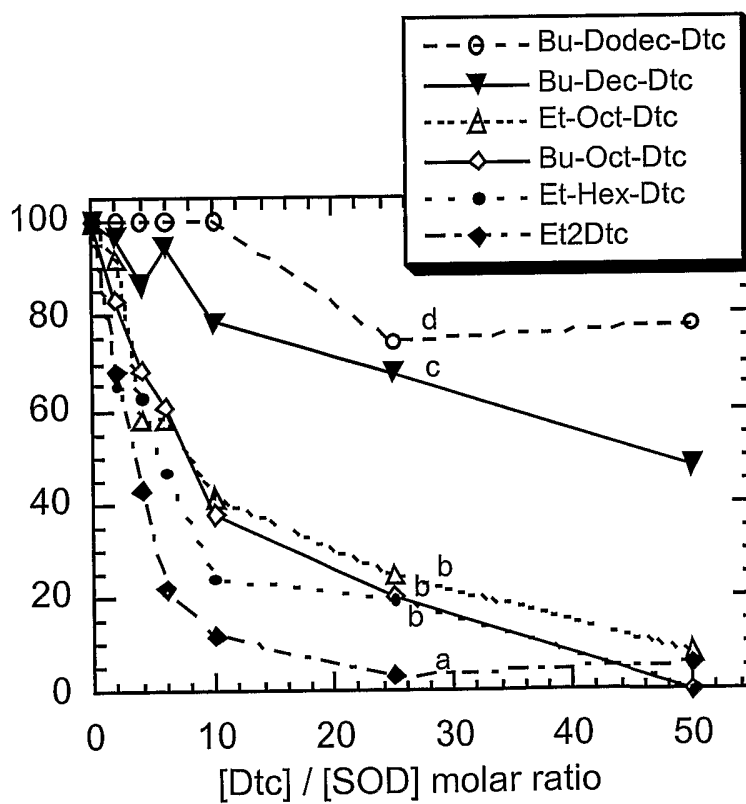


FIGURE 5

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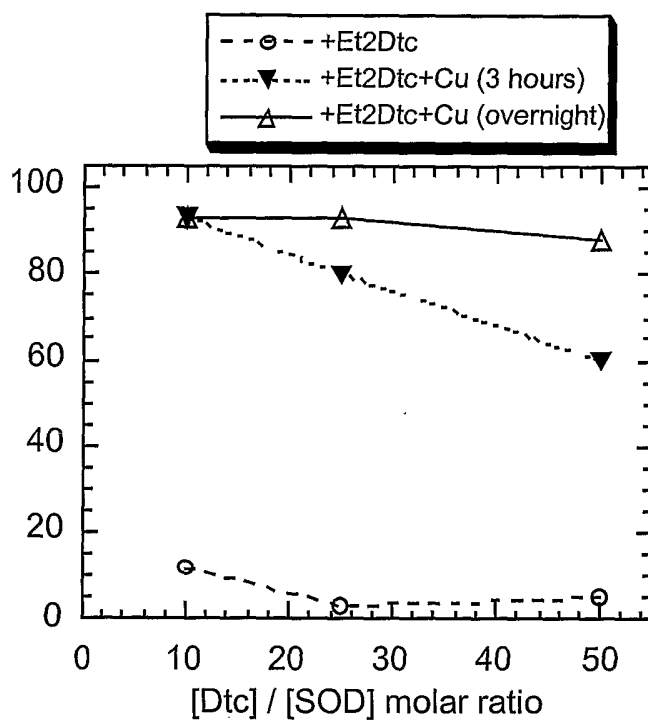


FIGURE 6

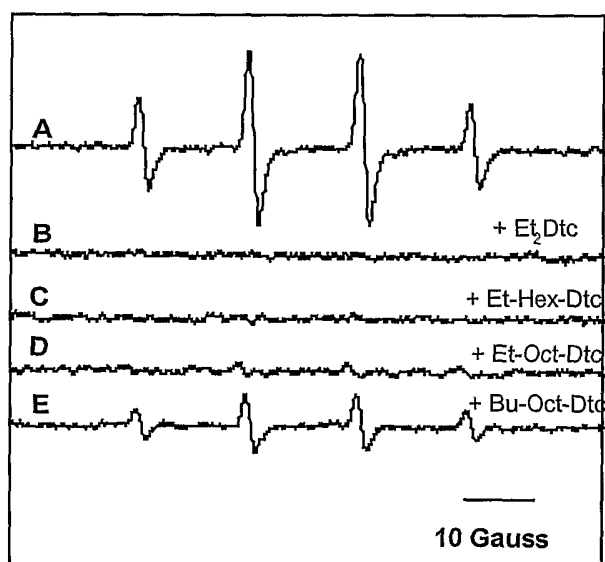


FIGURE 7

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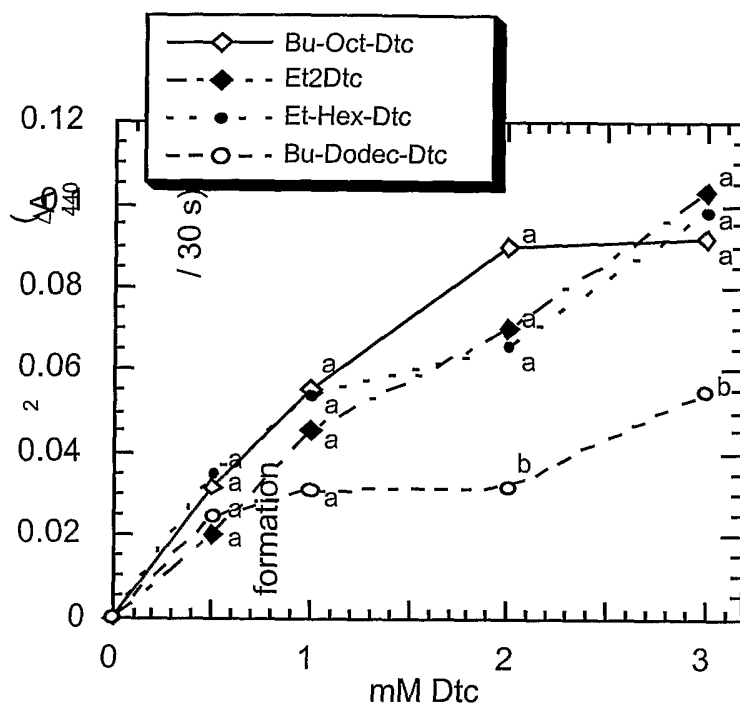


FIGURE 8

Equilibrium surface tensions versus solution concentration

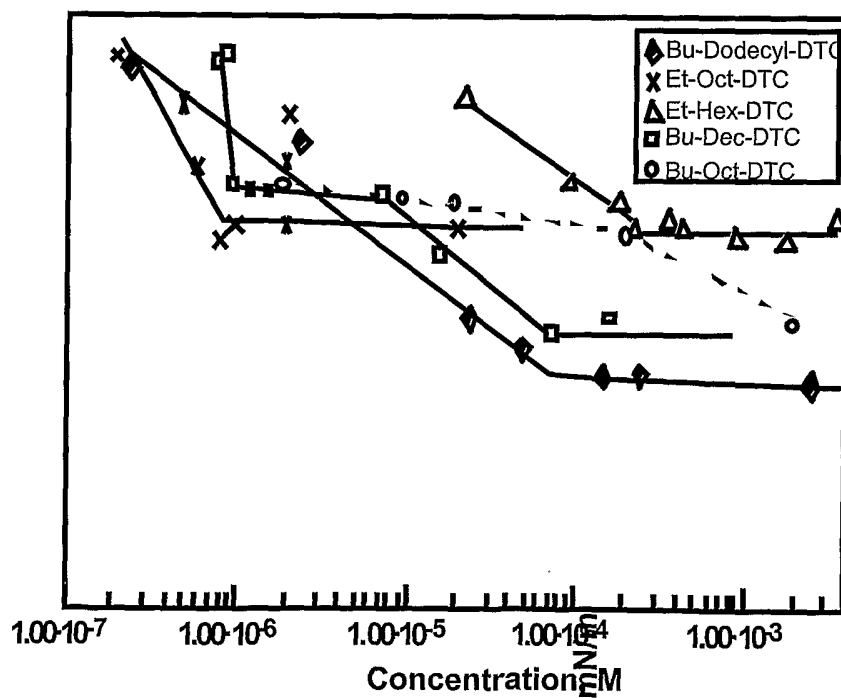


FIGURE 9

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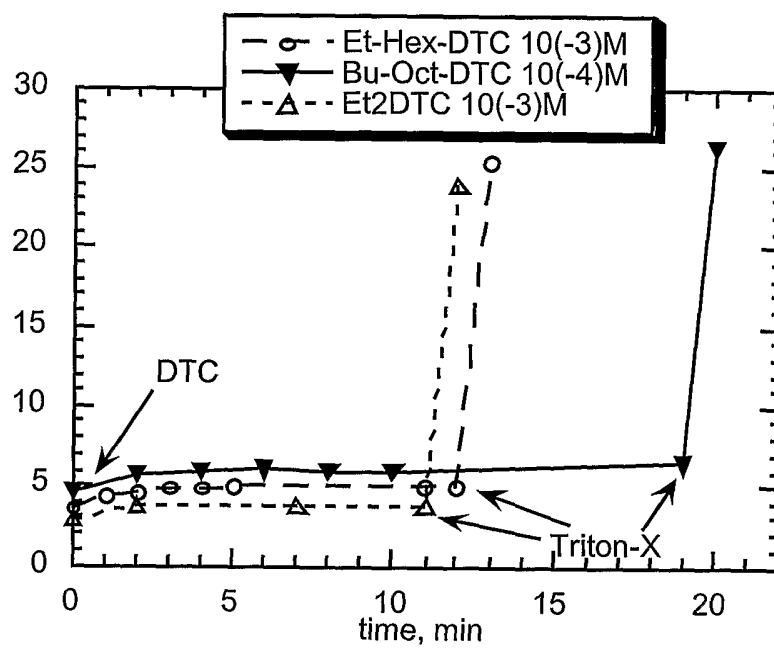


FIGURE 10

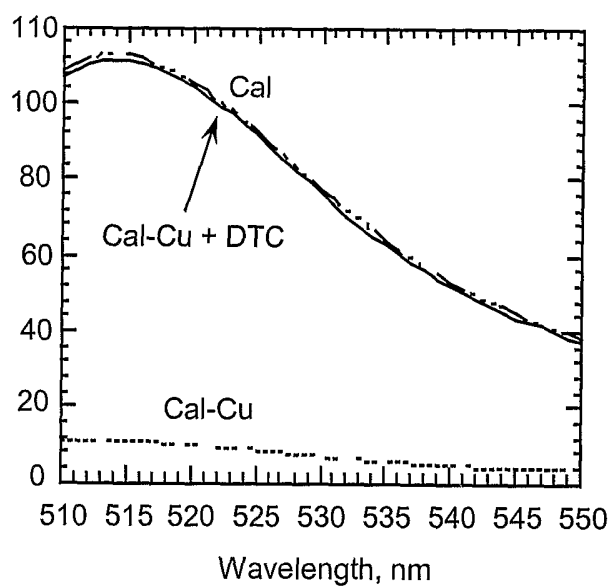


FIGURE 11

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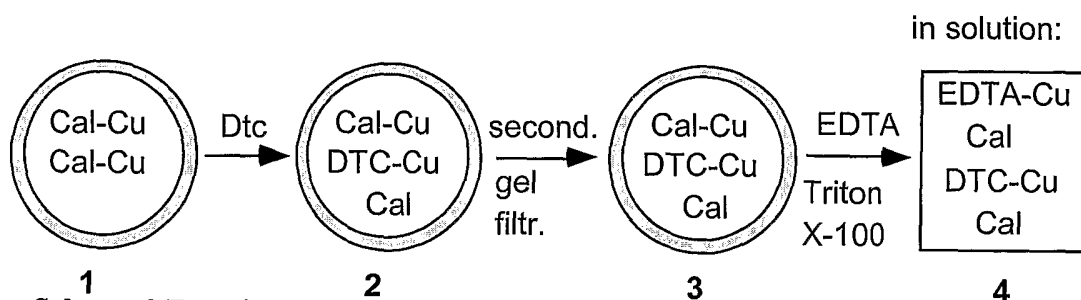


FIGURE 12

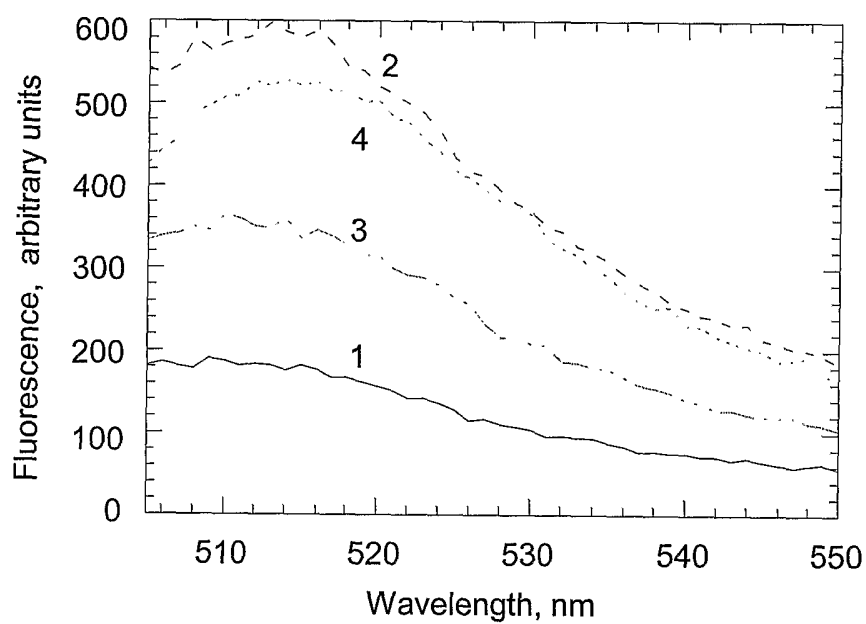


FIGURE 13

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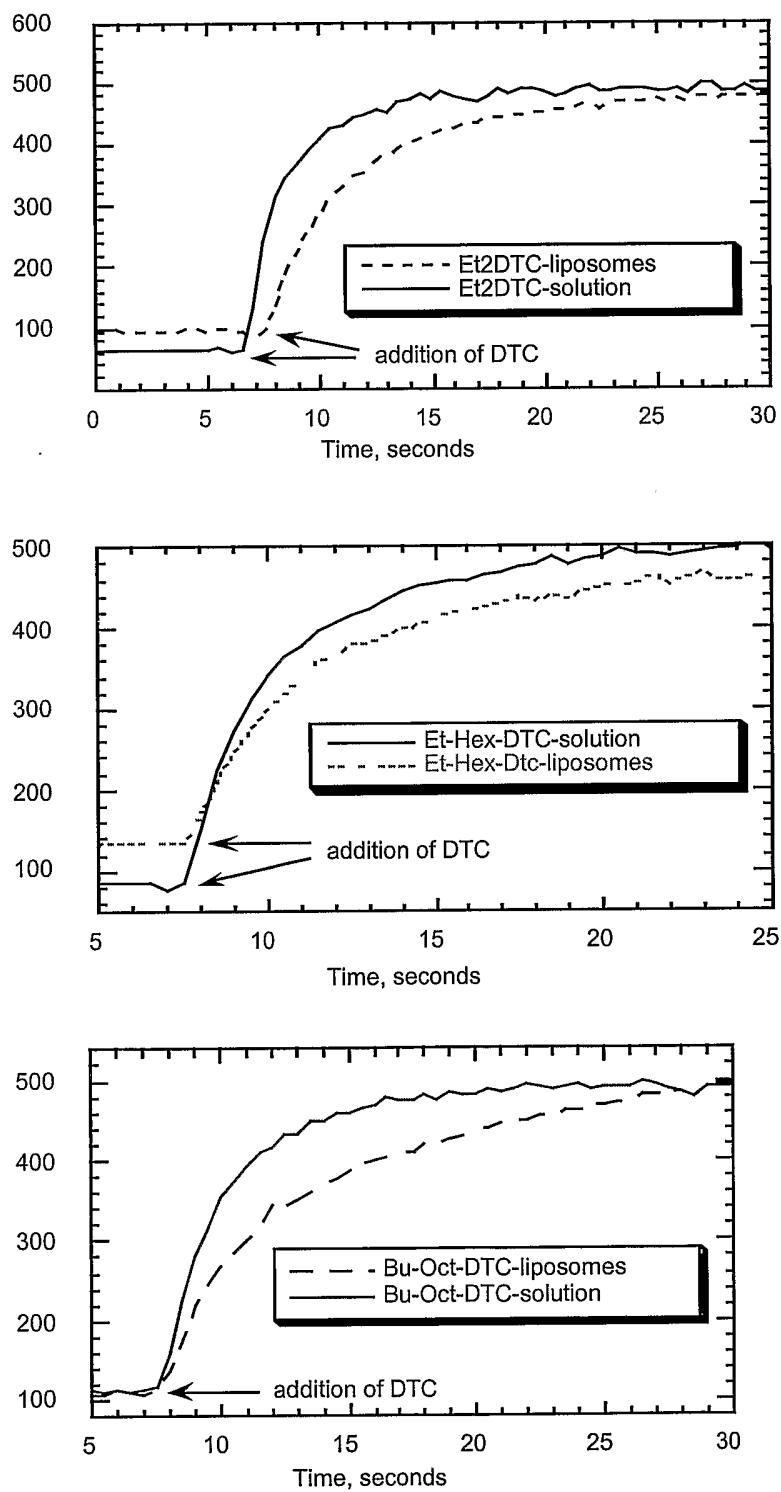


FIGURE 14

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Scheme 4

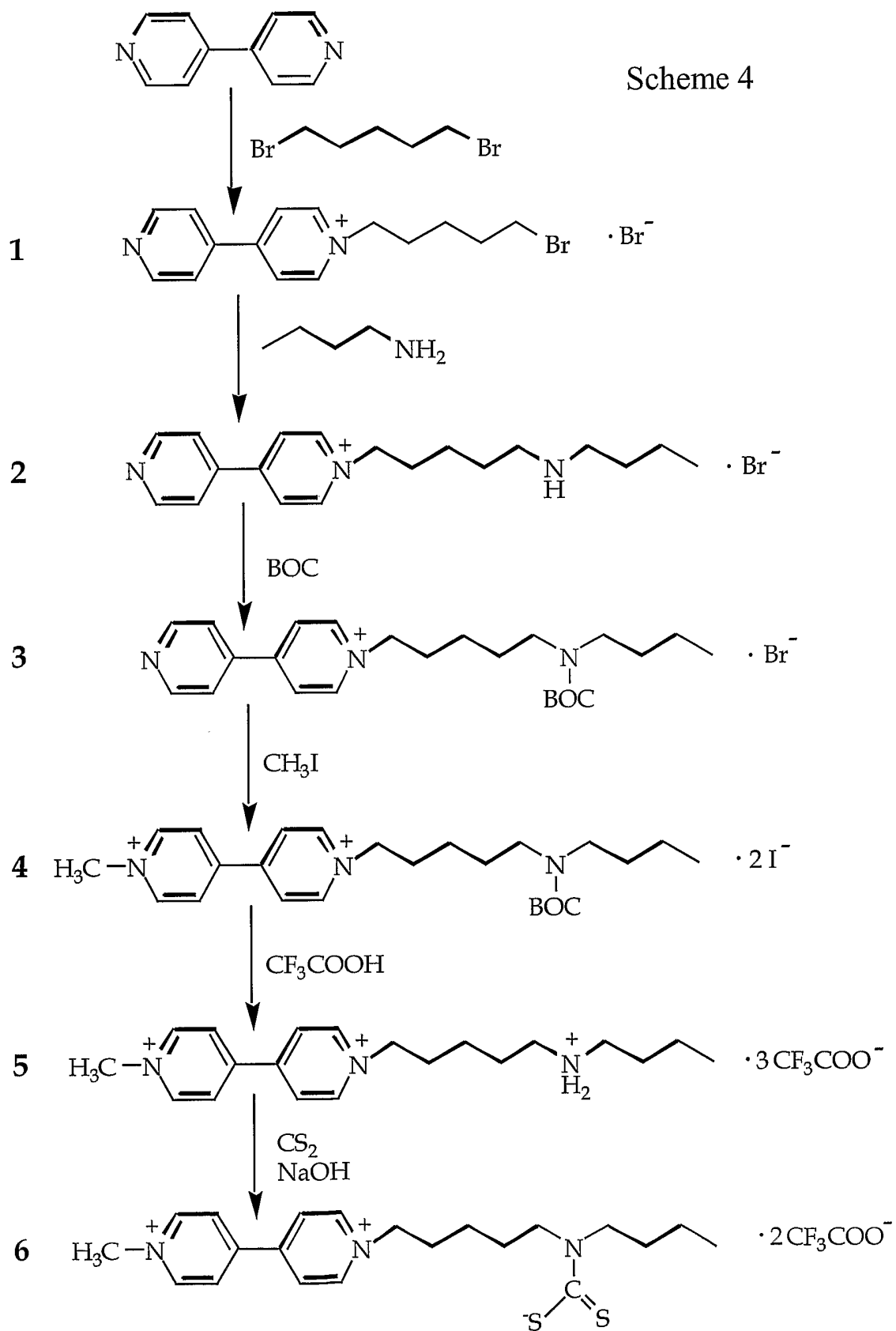


FIGURE 15

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Scheme 5

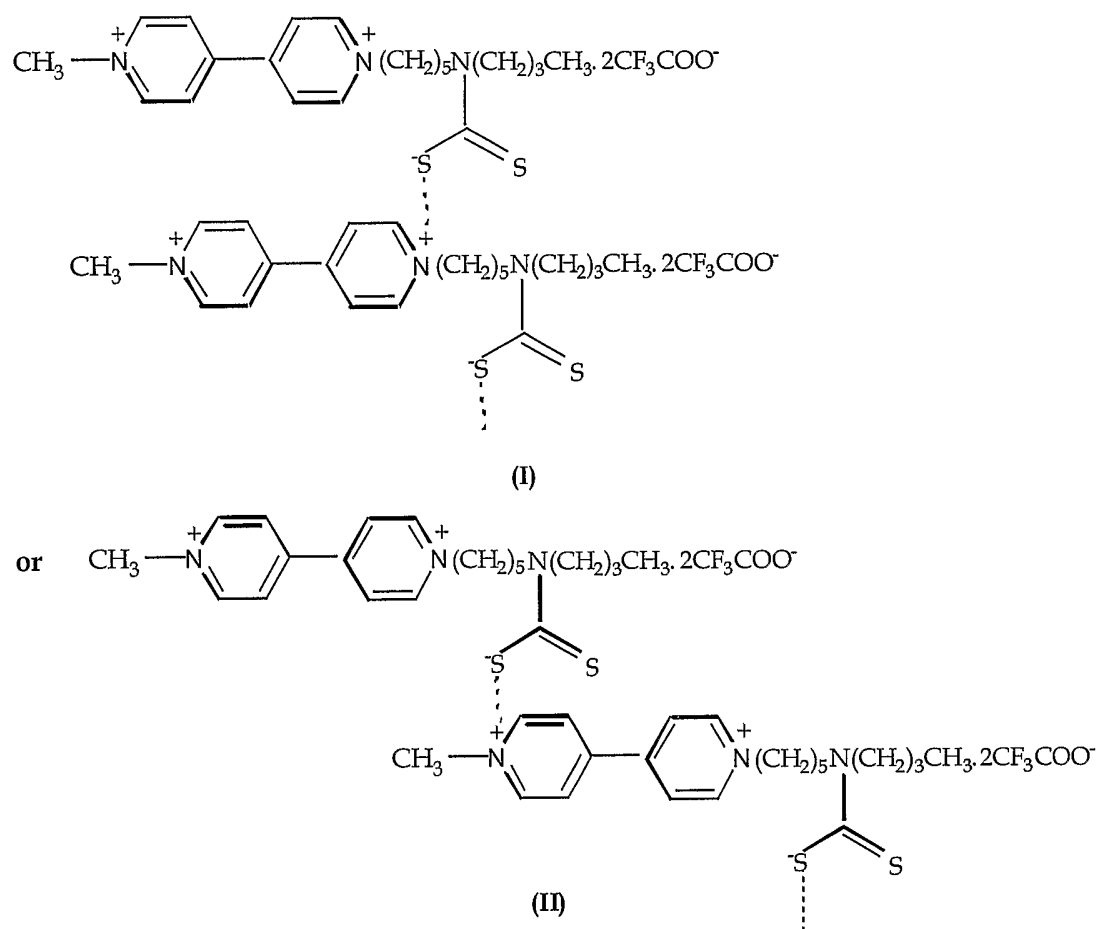


FIGURE 16

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Scheme 6

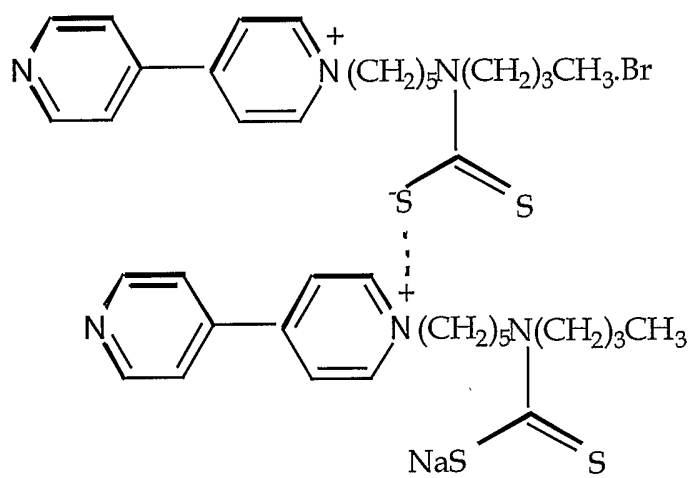


FIGURE 17

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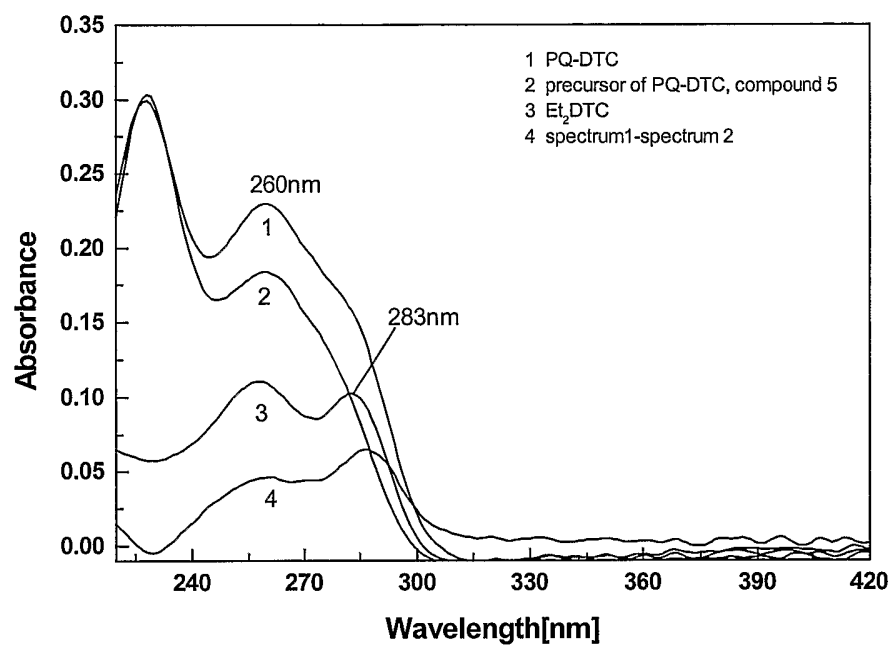


FIGURE 18

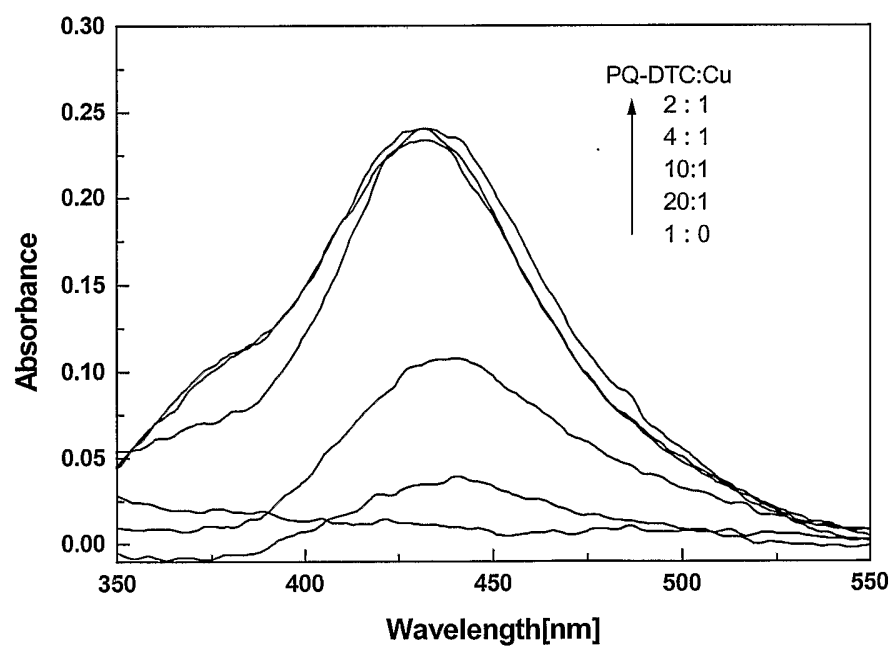


FIGURE 19

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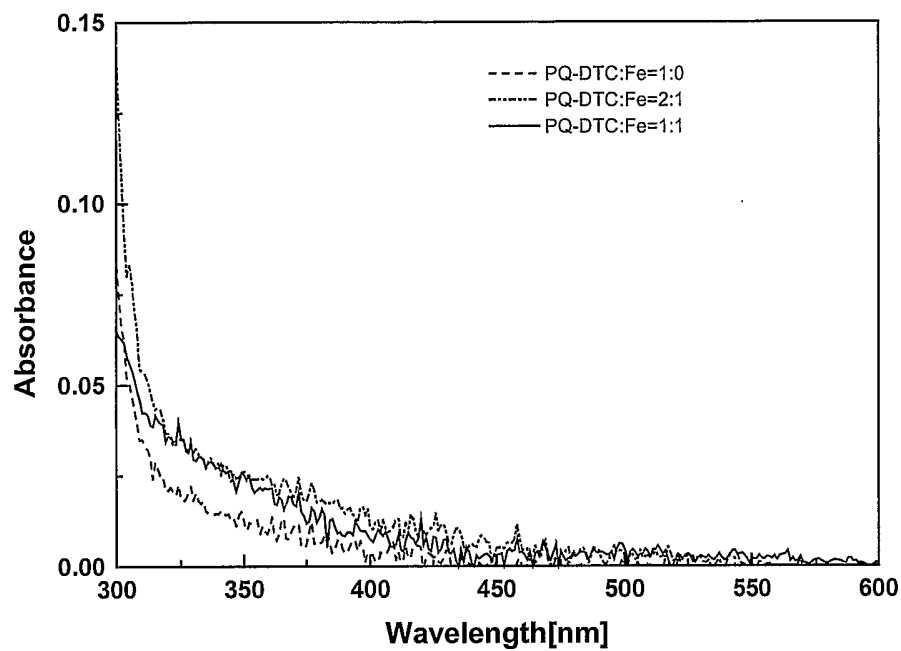


FIGURE 20

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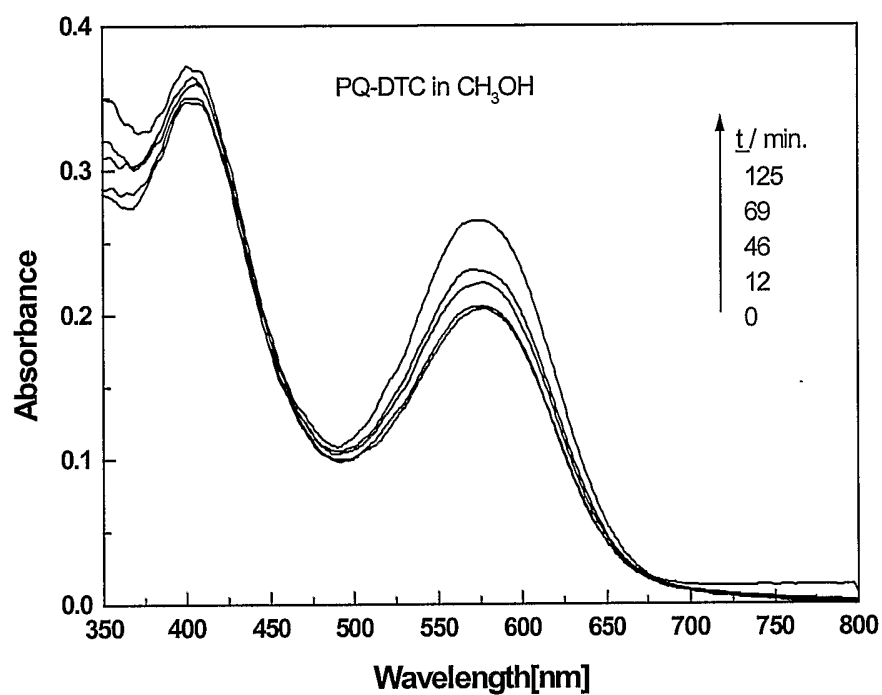


FIGURE 21A

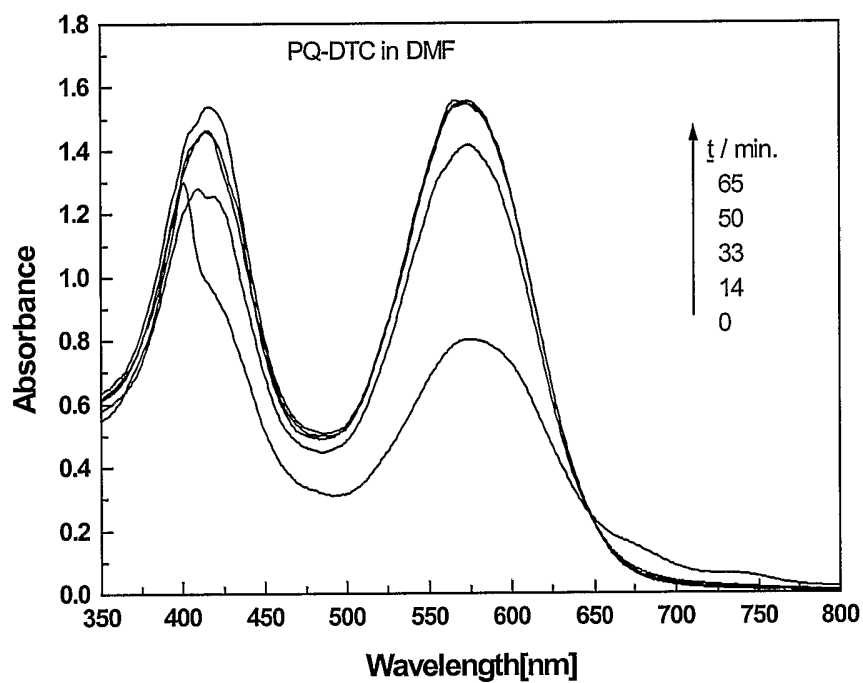


FIGURE 21B

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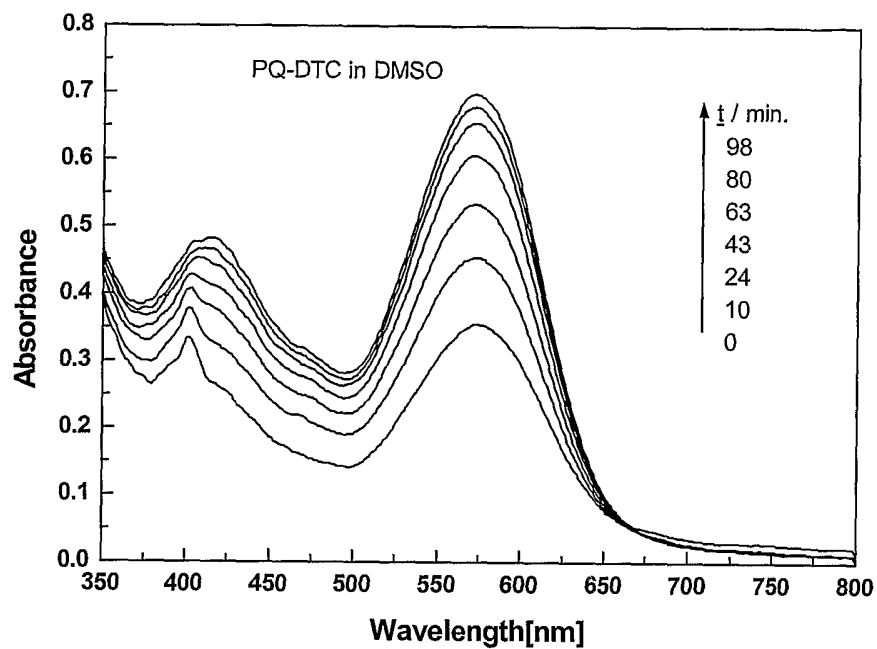


FIGURE 21C

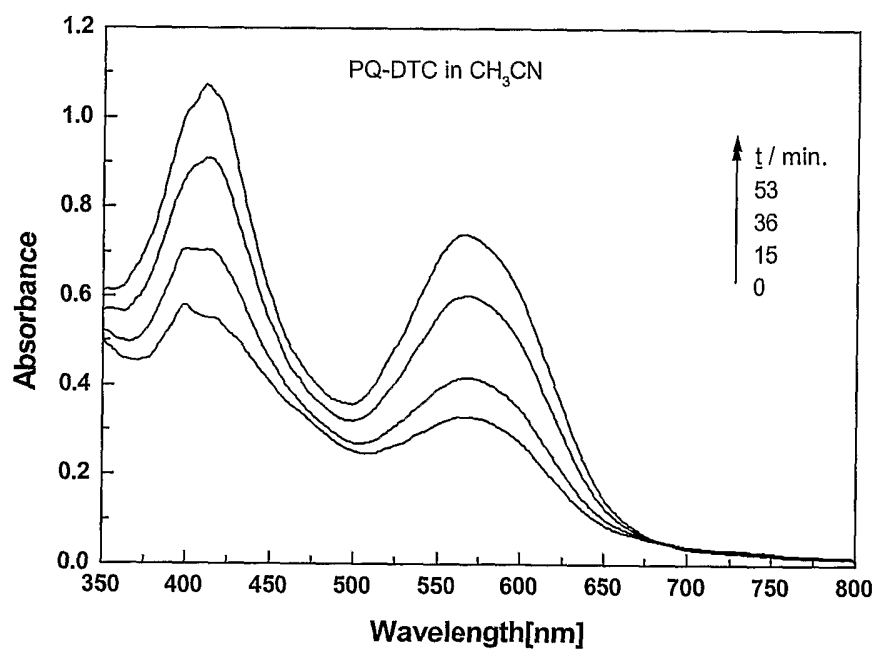


FIGURE 21D

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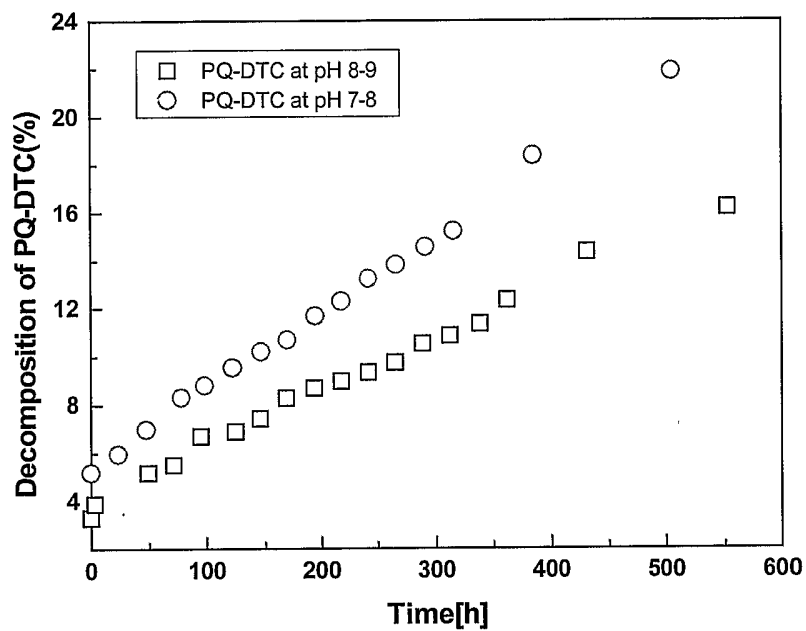


FIGURE 22

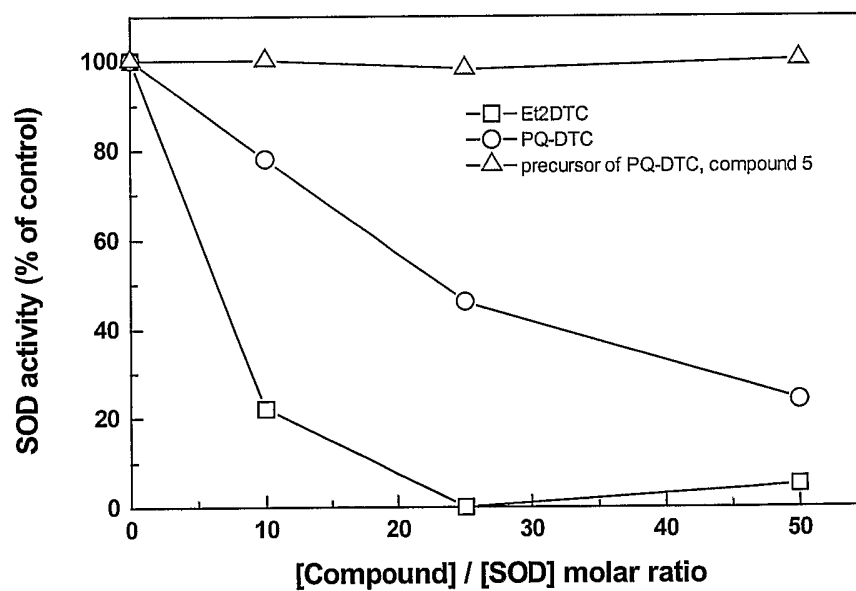


FIGURE 23

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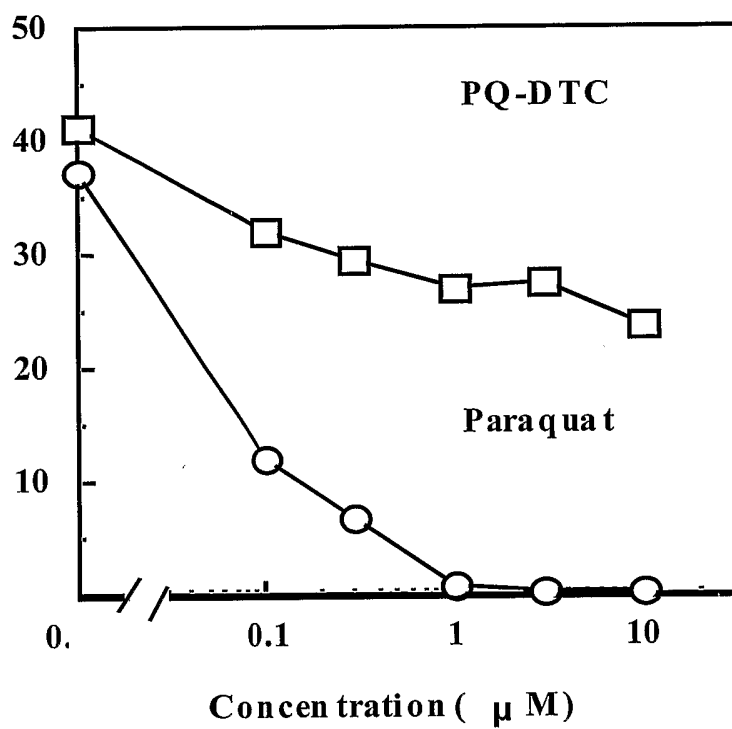


FIGURE 24