

Formation of sulfur and nitrogen cross-linked macromolecules under aqueous conditions

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Received 19 February 2007; accepted in revised form 8 June 2007; available online 13 July 2007

Abstract

Polysulfides and ammonia are abundant in young depositional environments and play an important role in the formation of macromolecular structures such as protokerogen and humics. In this work, we study the co-incorporation of polysulfides and ammonia into simple carbonyl model compounds, octanal and *trans* 2-octenal, in order to study their effect on the formation of a cross-linked macromolecule and suggest a feasible mechanism. The reactions, performed in aqueous solutions at ambient temperature and pH ~6 to 9, simulate formation of S and N cross-linked polymers in the natural environment. The complex S and N containing polymer was studied by ¹⁵N enrichment coupled to 2D NMR (¹H, ¹³C, ¹⁵N) techniques and chemical degradation of S–S bonds followed by deuterium labeling and GC–MS analyses. In addition, molecular modeling techniques were used to provide theoretical interpretations and important insights at the molecular level. The results indicate that polysulfide out competes ammonia in the formation of Michael adducts while ammonia is equally competitive with polysulfides when the reaction is addition to the carbonyl position. The co-incorporation of ammonia and polysulfides into carbonyls rapidly forms N and S cross-linked polymers. The effects of ammonia and amines on the polymerization processes are by two means: (i) reaction with carbonyls through an imine functionality to form oligomers and polymers and (ii) catalysis of sulfur nucleophiles onto carbonyls by transfer of a proton which enhances the rate of polymerization. A similar catalytic effect is observed when glycine is used instead of ammonia. This mechanism is especially important under basic to neutral conditions like those that prevail in marine environments. The results show that ammonia and glycine or possibly other amino acids and/or peptides are intimately involved with sulfur nucleophiles throughout the polymerization processes that occur at low temperatures and thus are suggested as key reactants in diagenetic formation of protokerogen and humics.

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1. INTRODUCTION

Sulfur and nitrogen nucleophiles play an important role in the formation and preservation of sedimentary organic matter (OM). Intermolecular cross-linking mostly occurs through heteroatom-containing functional groups that form

macromolecular structures that are thought to be protected against biological degradation (Sinninghe Damste and de Leeuw, 1990). Sulfides and polysulfides are abundant in environments where sulfate reducing bacteria (SRB) are prevalent and sulfate reduction occurs readily (Mossmann et al., 1991; Luther et al., 2001; Neretin et al., 2003). Ammonia (or ammonium) from the degradation of amino acids is abundant in the same environments, because depositional environments that support SRB activity usually have a high OM productivity, low levels of oxygen, and therefore high amounts of OM (Jorgensen, 1982; Canfield, 1989; Aizenshtat et al., 1999). Ammonia and reduced sulfur nucleophiles often

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coexist in the environment (Aizenshtat et al., 1983; Bregant et al., 1990; Schulz et al., 1994; Passier et al., 1998; Cotner et al., 2004) and compete for the same electrophiles during the formation of geo-macromolecules. Polysulfide anions are the most active reduced sulfur species and are suggested to be the most important species in diagenetic sulfur incorporation into organic matter (Aizenshtat et al., 1983; Francois, 1987; Lalonde et al., 1987; Mossmann et al., 1991; Vairavamurthy et al., 1992; Krein, 1993; Loch et al., 2002). The most reactive electrophiles toward polysulfide and ammonia are carbonyls (preferably α,β -unsaturated carbonyls) as determined by theoretical calculations, laboratory experiments and analysis of geo-macromolecules (Lalonde et al., 1987; Kohnen et al., 1993; Krein and Aizenshtat, 1994; Schouten et al., 1994; Adam et al., 2000; Filley et al., 2002; Amrani and Aizenshtat, 2004a,b,c; Schaefer et al., 2006). Such carbonyl compounds in soil and marine sediments are formed by biosynthesis, biodegradation, photooxidation and mineral catalysis of alcohols, double bonds, fatty acids, and other functionalized lipids that are abundant in recent sediments (Grossi et al., 2000; Marchand and Rontani, 2001; Rontani et al., 2005). Other important sources for carbonyls are sugars and polysugars. Their important role in OM polymerization and preservation processes is increasingly becoming recognized (Van Kaam-Peters et al., 1998; Ciglonecki et al., 2000; van Dongen et al., 2003).

The complexity of polymers that form from reactions with ammonia and carbonyls under aqueous conditions, and their low synthetic use has led to limited studies on this subject (Asinger et al., 1959; Lalonde, 1990). Moreover, unlike polymers that are cross-linked by sulfur or oxygen, there are no suitable chemical degradation methods for selective cleavage of the C–N bond which might allow for assembly of structural models regarding the polymer make-up. Therefore, a significant part of nitrogen functionality in recently formed macromolecular organic matter is poorly characterized. In a previous study that used ammonium polysulfide as a sulfurizing agent, we reported a consistent increase in nitrogen of the polymer formed during the course of reaction (Amrani and Aizenshtat, 2004b). The synergetic influence and mechanism of these *N*-nucleophiles in the formation of sulfur cross-linked polymers is not clear. The presence of ammonia or amine probably changes the sulfurization mechanism possibly through an imine intermediate (Asinger et al., 1959; Magnusson, 1959, 1962; Amrani and Aizenshtat, 2004b).

In the present study, we focus on co-incorporation of polysulfides and ammonia into simple carbonyl model compounds, octanal and *trans* 2-octenal, in order to study their effect on the formation of polysulfide cross-linked macromolecules and suggest a feasible mechanism. Reactions, performed in aqueous solutions, ambient temperature and at pH ~6 to 9, simulate formation of S and N cross-linked polymers in the natural environment. The resulting complex S and N containing polymer is examined by ^{15}N enrichment coupled to 2D NMR (^1H , ^{13}C , ^{15}N) techniques and chemical degradation (MeLi/MeI) with deuterium labeling followed by GC–MS analysis. In addition, we apply molecular modeling techniques to provide theoretical interpretations and important insights at the molecular level.

2. EXPERIMENTAL SECTION

2.1. Chemicals

The following chemicals were used in the experiments: Octanal (99%), *trans* 2-octenal (94%), CH_3Li (1.6 M in diethylether), CD_3Li (0.5 M as a complex with LiI in diethylether), CH_3I (99%), CD_3I (99.5%), CH_2Cl_2 (>99.5%), *n*-hexane (>99.5%), diethylether (>99% redistilled and dried over sodium), $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$ (99.99%), $(\text{NH}_4)_2\text{S}$ (20 wt% in water), elemental sulfur (99.98%), all purchased from Aldrich.

2.2. Reaction between polysulfides and aldehydes

Elemental sulfur (0.94 g, 29 mmol) was dissolved in 10 ml of ammonium sulfide solution (20% w/w in water, Merck) or into a 1:1 stoichiometric ratio of sulfide to elemental sulfur i.e. $(\text{NH}_4)_2\text{S}_2$ for more details about the composition of this polysulfide solution see (Amrani et al., 2006). The pH was buffered by the ammonium solution to 8.5–9, while the Na_2S_2 solution was prepared by dissolving 6.96 g $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$ (29 mmol) in 10 ml distilled water, adding 0.94 g of elemental sulfur (29 mmol) and reducing the pH to 9 by adding concentrated HCl. In some cases we used octane amine (0.4 mmol) or glycine (9 mmol) with Na_2S_2 to replace ammonium as the nitrogen source. The organic substrate (octanal or 2-octenal) was added (0.4–0.8 mmol) to the polysulfide solution and the vessel was sealed under nitrogen atmosphere. At the end of each reaction (usually 1 week), the aqueous solution was extracted three times with CH_2Cl_2 . The organic extract was dried over anhydrous MgSO_4 and filtered. The solvent was removed by evaporation at ambient temperature to yield a solid polymer, described henceforth as organic matter, or OM.

2.3. Chemical degradation of the residual OM by $\text{CH}_3\text{Li}/\text{CH}_3\text{I}$ or $\text{CD}_3\text{Li}/\text{CD}_3\text{I}$

Selective cleavage of organic polysulfide (S–S) bonds was performed in order to characterize sulfurized OM (Eliel et al., 1976; Kohnen et al., 1991). To 50–100 mg of OM in dry diethyl ether, an excess of methyl lithium (MeLi) was introduced and the mixture stirred at ambient temperature. After 5 min, an excess of methyl iodide (MeI) was added and after an additional period of 15 min the reaction was quenched with water, extracted 3 times with hexane, and the hexane extract was dried over anhydrous MgSO_4 , and filtered. The products were analyzed by GC–MS as described below. The normal product yield for this method is in the range of 50–80%. In several experiments we used $\text{CD}_3\text{Li}/\text{CD}_3\text{I}$ following the above procedure in order to label methylated products. In this case 3 mass units have been added to each inserted methyl.

2.4. Nuclear magnetic spectroscopy

2D HSQC (Heteronuclear Single Quantum Coherence) NMR spectroscopy (Reynolds and Enriquez, 2002) and HMBC (Heteronuclear Multiple Bond Correlation) were utilized in conjunction with ^{15}N isotopic enrichment. The

2D ^1H - ^{15}N HSQC NMR spectroscopy observes protons directly bonded to nitrogen and is sensitive to covalent bonds adjacent to the observed nitrogen-proton pairs. HMBC NMR spectroscopy observes protons that are 2 bonds away from nitrogen within molecules and therefore can detect tertiary amines or secondary imines that HSQC cannot detect. In addition, ^1H - ^{13}C HSQC experiments were also performed on these samples. Samples dissolved in CDCl_3 were transferred into 3 mm tubes. ^1H and ^{13}C chemical shifts were calibrated against the internal standard tetramethylsilane (TMS). ^{15}N chemical shifts were referenced indirectly using the δ notation (Wishart et al., 1995) in which liquid NH_3 is set as 0 ppm. The 2D HSQC NMR spectra were obtained on a Bruker DMX-600 spectrometer with a 5 mm TXI (triple-resonance inverse) probe (^1H , ^{13}C , and ^{15}N) equipped with a xyz -gradient coil. The HSQC spectra (32 scans, with the FID time domain F2 having a digital acquisition of 2048 data points and the time domain F1 having 256 data points) were acquired using a relaxation delay of 1.5 s with phase sensitive mode (States-TPPI). All liquid-state NMR experiments were performed at 298 K. The acquired NMR data were then processed by XWIN-NMR, version 3.1 (Bruker Biospin Corporation, Billerica, MA). Zero-filling and window multiplication were applied prior to Fourier transformation. For the 2D ^1H - ^{15}N HMBC, the delay for evaluation of long range coupling was set to 50 ms. The matrix size was 1024 for ^1H , 128 for ^{15}N , and the number of scans was 512.

2.5. GC-MS analysis

Capillary GC-MS was performed on a Hewlett Packard 6890 series GC system connected to a Pegasus II time of flight spectrometer (Leco Corporation, MI). The GC column was a 30 m \times 0.25 mm (i.d.) fused silica capillary column with 5% methyl silicon bonded phase SUPELCO DB-5 (film thickness: 0.25 μm). The samples were injected in the split mode (1:20). The column was run with constant flow of He (1.5 ml/min) and the temperature was ramped from 50 to 200 $^\circ\text{C}$ at 15 $^\circ\text{C}/\text{min}$ and then to 300 $^\circ\text{C}$ at 25 $^\circ\text{C}/\text{min}$ where upon the temperature was held for 3 min. The ionization mode on the mass spectrometer was electron impact at 70 eV. Compounds were identified from comparisons of their mass spectra to a NIST database of mass spectra and from fragmentation patterns.

2.6. Molecular modeling

Since all compounds studied are organic molecules involving only C, N, O, S, and H atoms, the AM1 (Austin Method 1) approach as implemented in the SPARTAN package was used. Such semi-empirical method has been proven to generate reasonable molecular geometry and energy calculations for organic molecules as their parameters have been well defined. Gas phase and water solvated molecular geometry of all stable and intermediate configurations has been fully optimized and the molecular energies are calculated. All transition states have been confirmed for the unique imaginary vibrational mode and for all stable configurations with real vibrational motions. The forma-

tion energies, defined as the difference of the molecular energies, have also been computed. To examine the charge redistribution during reactions, the Mulliken charges at selected atoms were calculated. To ensure more accurate calculated transition barriers, we employed high-level Density Functional Theory calculation at the DFT/B3LYP/6-31G** levels. The water solvation effect was also considered using the continuum media model with a dielectric constant of 80.35 and a probe radius of 1.4 Å .

3. RESULTS

3.1. Molecular modeling

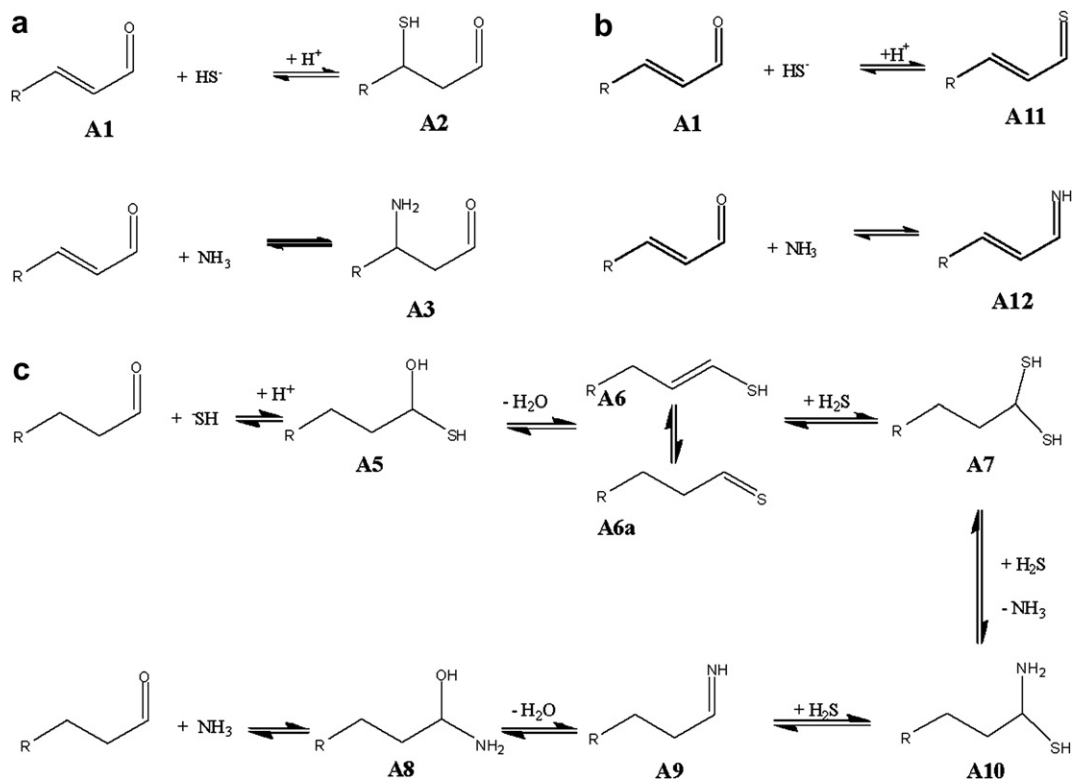
Molecular modeling provides theoretical interpretations and important insights at the molecular level. For simplicity we employ HS^- as a surrogate for polysulfides. We first calculate the energy differences of the Michael adducts from reaction between α,β -unsaturated aldehyde and HS^- or NH_3 (Scheme 1a). The results show that the Michael adduct of the reaction with HS^- has lower energy by about 4 kcal (Table 1). This suggests that the reaction with HS^- is thermodynamically favorable by at least one order of magnitude over the reaction with NH_3 . Michael addition, a 1,4-addition, is favorable over the 1,2-addition of α,β unsaturated aldehydes by ~ 18 and 12 kcal for HS^- and NH_3 nucleophiles, respectively (Scheme 1b and Table 1).

The nucleophilic addition of HS^- or NH_3 into the saturated carbonyl carbon analog has smaller energy differences than the Michael addition (in the case of the α,β -unsaturated aldehydes), but still favors the HS^- addition in the first step (see Scheme 1c and Table 1). However, the formation of imine is favorable over the thioaldehyde because of the smaller intermediate energy differences. The most likely transition barrier or rate determining step is the water elimination. To examine closely the difference between the rate determining steps of the HS^- and NH_3 pathways, several transition states were calculated and the transition barriers are listed in Table 2. To ensure more accurately calculated transition barriers, we employ high-level Density Functional Theory (DFT) calculation at the DFT/B3LYP/6-31G** levels. The results show that the involvement of ammonia in the elimination of water significantly reduces the transition barrier energy. These results are discussed below in Section 4.

3.2. Reactions with *trans* 2-octenal

3.2.1. NMR studies of the polysulfide cross-linked polymer (PCLM)

The reaction between *trans* 2-octenal and $(\text{NH}_4)_2\text{S}_x$ yielded a solid yellow polymer that was partly soluble in CH_2Cl_2 or ethyl ether. The ^1H NMR shows that the conjugated double bond (6.15 and 6.9 ppm) and the aldehyde (9.55 ppm) peaks of the precursor disappear (not shown). The ^1H - ^{13}C HSQC spectrum (Fig. 1a) shows a broad and intense cross peak around 3.1(50) ppm that relates to the *gem*-disulfide bonds (*gem* means that two identical atoms are bound to the same carbon). The peaks around 4-5(60-80) ppm can be related to $(\text{CH})_x$ of hydroxyl/amine



Scheme 1. (a) The Michael addition (1,4) of NH_3 and HS^- into α,β unsaturated aldehydes. (b) 1,2-addition into α,β unsaturated aldehydes. (c) Intermediates of the 1,2-addition into aldehydes by NH_3 and HS^- in the formation of *gem*-dithiol. See Table 1 for the energies of these structures.

Table 1

Energies of selected compounds and the formation energies of 1,2- and 1,4-(Michael) additions between HS^- and NH_3 nucleophiles and model aldehydes^a

Compound	Energy (kcal/mol)	Formation energy (kcal/mol)
H_2S	1.32	
NH_3	-7.84	
H_2O	-64.67	
A1	-29.82	
A2	-52.63	-24.13
A3	-58.50	-20.84
A4	-57.59	
A5	-76.11	-19.84
A6	-0.19	-7.27
A6a	4.76	-3.64
A7	-49.34	-20.84
A8	-82.55	-17.12
A9	-8.62	-7.86
A10	-26.39	-19.09
A11	-29.90	-6.27
A12	18.41	-8.62

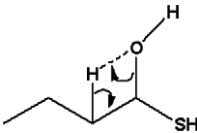
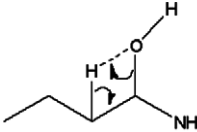
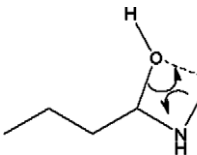
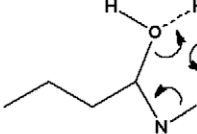
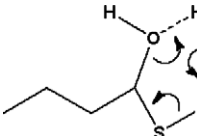
^a See Scheme 1 for the structures.

groups produced from incomplete reaction with polysulfides. The two downfield peaks at 4.8(108) and 6.1(130) ppm represent conjugated double bonds to amine (enamine). The small peak at 7.8(167) ppm is an imine peak ($\text{H}-\text{C}=\text{N}$). There is no aldehyde peak [around 9.5(200)], which suggests that the aldehyde was fully consumed in the reaction.

In order to investigate the type of nitrogen bonding in our PCLM, we reacted *trans* 2-octenal with $^{15}\text{NH}_4\text{Cl}$ and with Na_2S_x at pH = 9. The PCLM was then analyzed by $^1\text{H}-^{15}\text{N}$ HSQC and $^1\text{H}-^{15}\text{N}$ HMBC. The $^1\text{H}-^{15}\text{N}$ HSQC, which correlates protons directly bound to nitrogens, shows some intense peaks at 0.8–2.6(38–55) ppm (not shown). The peak at 1.1(38) ppm is a terminal amine ($\text{R}-\text{NH}_2$) while the other peaks belong to the R_2NH structure. There are some small peaks in the more downfield region, 6.1–8.1(108–130) ppm that are probably associated with Maillard and melanoidine type structures or terminal amide groups (Knicker et al., 1996).

The HMBC experiment has been performed in order to correlate protons that are two bonds away from nitrogen and therefore enables the identification of secondary imine or tertiary amine compounds. The 1.1–2.2(47–76) ppm region is the most intense and represents the $(\text{CH})_x$ to amine correlation (Fig. 1b). The most interesting peaks are far downfield in the ^{15}N chemical shift range (332–343 ppm) and represent the imine (Schiff base) functionality (Creemers et al., 1999). However, the ^1H shifts of correlated peaks appear at widely differing positions, 1.8–2.5 and 7.8–7.9 ppm. If we examine the structure of an alkylated imine ($\text{R}-\text{HC}=\text{N}-\text{CH}_2-\text{R}$) it is clear that there are at least two types of protons that could correlate with the imine nitrogen. The more unshielded one (7.8–7.9 ppm) is $\text{R}-\text{HC}=\text{N}-\text{CH}_2\text{R}$ while the more shielded (1.8–2.5) is $\text{R}-\text{HC}=\text{N}-\text{CH}_2\text{R}$. *Cis-trans* configurations of the $\text{C}=\text{N}$ may be responsible for the existence of two peaks. Alterna-

Table 2
Calculated transition barriers of the selected transition state structures during the elimination of water

Transition state	Configuration	E_0 (Hartree)	ZPE (kcal/mol)	δG ($T = 23$ °C) (kcal/mol)	E_{sol} (kcal/mol)	ΔG^a (kcal/mol)	Transition barrier ^b (kcal/mol)
	R-SH TS-SH	-631.870867 -631.757931	87.789 84.328	-18.489 -18.162	-4.266 -4.8951	-396439.94 -396372.83	67.11
	R-NH ₃ TS-NH ₃	-289.037835 -288.943175	99.362 97.856	-17.891 -17.632	-8.9402 -15.8594	-181301.46 -181250.22	51.23
	TS-NH32	-288.952448	96.471	-17.746	-10.1701	-181251.85	49.60
	R-NH ₃ -NH ₃ TS-NH ₃ -NH ₃	-345.610957 -345.550714	124.235 128.117	-20.874 -18.909	-12.8755 -11.9756	-216783.67 -216739.12	44.55
	R-SH-NH ₃ TS-SH-NH ₃	-688.434040 -688.404344	112.301 116.791	-21.009 -19.737	-9.4596 -10.7828	-431917.07 -431893.99	23.07

^a Gibbs' free energy ΔG is calculated from $\Delta G = E_0 + ZPE + \delta G + E_{sol}$, where E_0 is the total electronic energy at $T = 0$ K, ZPE is the Quantum mechanics zero-point energy correction, δG is the Gibb's free energy change from $T = 0$ to 298.15 K, and E_{sol} is the solvation energy.

^b The transition barrier is defined as $\Delta\Delta G = \Delta G(TS) - \Delta(R)$, where $\Delta G(TS)$ and $\Delta(R)$ are the calculated Gibbs' free energies of the transition state and reactant configurations.

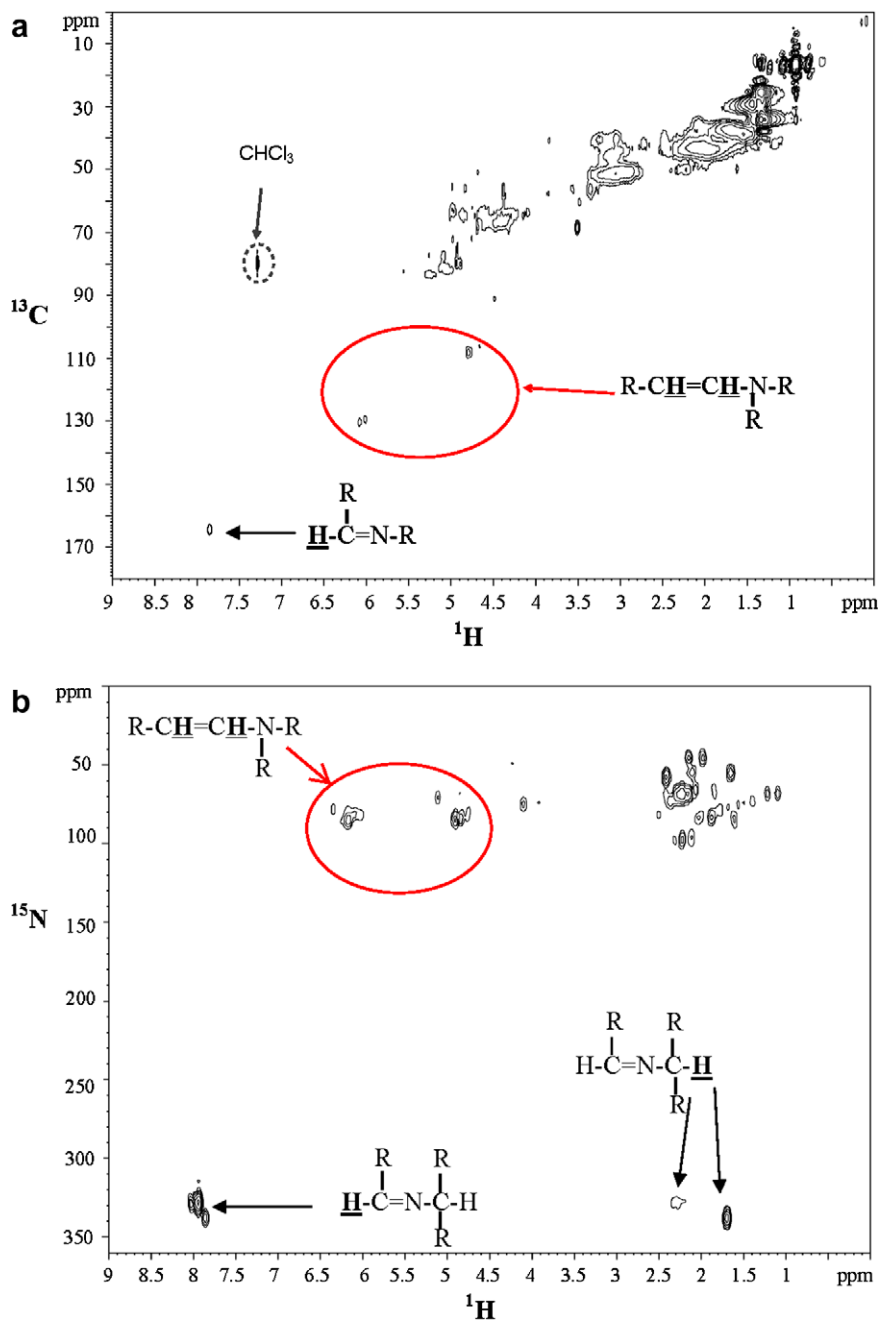


Fig. 1. NMR spectra for the reaction products of $(\text{NH}_4)_2\text{S}_x$ and 2-octenal. (a) HSQC ^1H - ^{13}C spectrum. (b) HMBC ^1H - ^{15}N spectrum.

tively, imine functionality may be found in various chemical environments within the polymer and induce chemical shift dispersion.

3.2.2. Chemical degradation followed by GC-MS studies

MeLi/MeI treatment of the PCLM cleaves S-S bonds and adds a methyl group to each sulfur moiety, enabling analysis of methylated monomers by GC-MS (Eliel et al., 1976; Kohnen et al., 1991). However, MeLi/MeI treatment methylates carbonyl and imine groups (Grignard type mechanism) as well as thiol and amine groups. The interpretation of the results requires consideration for this. The results of this treatment yielded several sulfur

and nitrogen containing products as presented in the GC chromatograms shown in Fig. 2. The first eluting sulfur containing product is compound **1** with $m/z = 206$. The second is its methylated derivative, compound **2**, with $m/z = 220$ and containing two methylthioether groups (see mass spectra in Fig. 3). Both of these compounds are in a very low abundance. Compound **3** with three methylthioether groups has a similar structure to those reported previously for other α,β unsaturated aldehydes reacted with $(\text{NH}_4)_2\text{S}_x$ (Krein and Aizenshtat, 1994; Amrani and Aizenshtat, 2004b) that showed sulfurization occurring in C3 and C1. The two later eluting compounds (Compounds **4** and **5**) show the presence of at least 4

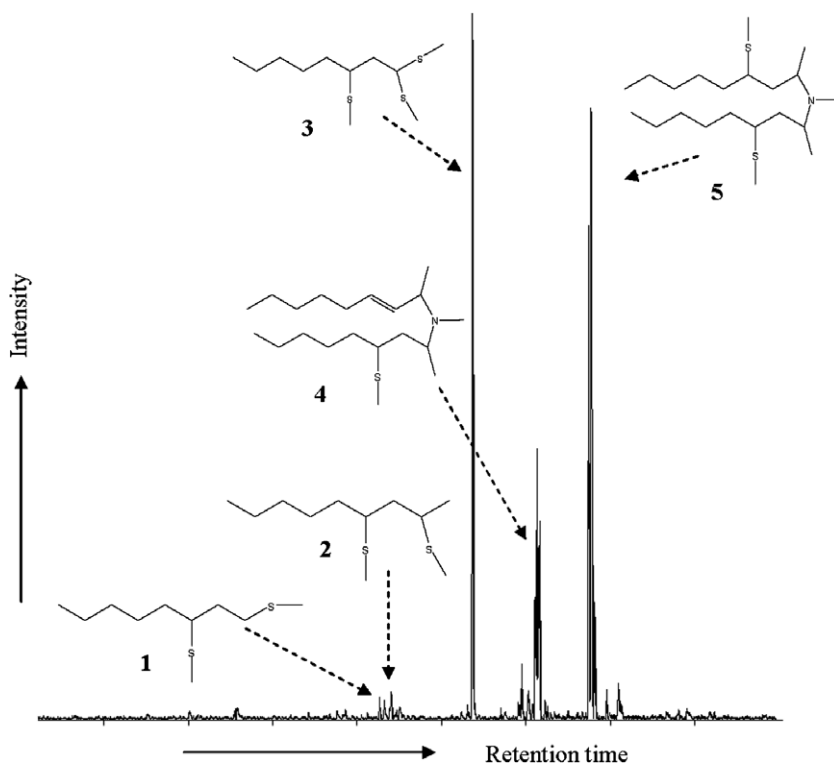


Fig. 2. Total ion current (TIC) chromatogram of the reaction products after MeLi/MeI treatment of the PCLM produced by the reaction of *trans* 2-octenal and aqueous $(\text{NH}_4)_2\text{S}_x$ solution at ambient temperature for 1 week.

isomers and their fragmentation patterns (Fig. 3) suggest the existence of both sulfur and nitrogen. In order to identify these products, we combined two methods; ^{15}N enrichment of the PCLM followed by $\text{CH}_3\text{Li}/\text{CH}_3\text{I}$ treatment, and $\text{CD}_3\text{Li}/\text{CD}_3\text{I}$ chemical degradation of the non ^{15}N enriched PCLM. ^{15}N enrichment adds 1 mass unit to any fragments that contains nitrogen while the $\text{CD}_3\text{Li}/\text{CD}_3\text{I}$ adds 3 mass units to any inserted methyl groups. $\text{CD}_3\text{Li}/\text{CD}_3\text{I}$ treatment labels the S–S positions as well as imine carbon ($\text{C}=\text{N}$) and NH positions. The MS data for the products of these treatments is presented in Table 3. The ^{15}N mass spectrum clearly shows that there is only one nitrogen atom in compounds 4 and 5 and no nitrogen in compounds 1, 2, and 3.

An example for the isotopic labeling approach is presented in Fig. 4 for compound 5. The characteristic peaks at m/z 131 and 173 for the non ^{15}N enriched sample (Fig. 4a) shows no enrichment in ^{15}N because these fragments are identical in the ^{15}N enriched PCLM (Fig. 4b). However, in Fig. 4c we see enrichment from m/z 131 to 134 and from 173 to 179 that suggests insertion of one and two methyl groups, respectively. The characteristic peaks at m/z 202 and 230 (Fig. 4a) contain nitrogen because the ^{15}N enriched polymer yields m/z 203 and 231 (Fig. 4b). The spectra of $\text{CD}_3\text{Li}/\text{CD}_3\text{I}$ treatment on the PCLM gave rise to m/z 211 and 242 which suggest addition of 3 and 4 methyls, respectively. The combined data from the three mass spectra and the NMR enable us to ascertain the structures.

The ^1H – ^{13}C HSQC spectrum of PCLM after $\text{CH}_3\text{Li}/\text{CH}_3\text{I}$ treatment supports the suggested structures (Fig. 5). The cross peak at 1.1(20) ppm is the added methyl alpha to the nitrogen while peaks at 2–2.3(34) ppm are methyls

directly connected to N. The cross peaks at 1.9–2.2(16) are methylated S. The double bond peaks of structure 4 at 5.4–5.5(134–137) prove that this double bond is not an artifact of the GC although it could be an artifact of the MeLi/MeI treatment. The reason for the formation of this double bond is not clear. One possibility is 1,2-addition of ammonia to the carbonyl carbon instead of 1,4-addition of polysulfide or ammonia (Adam et al., 2000). However, 1,2-addition of weak base such as ammonia (or polysulfide) are very unlikely and the Gibbs free energy for the 1,2 adduct after elimination of water is higher by ~ 12 kcal then the corresponding Michael adduct (entry A12, Table 1). Moreover, the ^1H – ^{13}C NMR of the PCLM before chemical degradation does not support the 1,2-addition. A more probable scenario is elimination of the SCH_3 group during the MeLi/MeI treatment. Compound 4a and its resonance structure (differs by ~ 0.5 kcal) are the most probable precursors for compound 4 before MeLi/MeI treatment (Scheme 2). The cross peaks at 4.8(108) and 6.1(130) in the HSQC of the untreated polymer (Fig. 1a) support structure 4a and suggest enamine functionality. The condensation reaction between ammonia and aldehyde is known to form some unstable compounds (Dayagi and Degani, 1970) that later can degrade into 4a type structures as shown in Scheme 3.

These results show that the incorporation of ammonia into 2-octenal in the presence of polysulfide is restricted to the carbonyl position. We could not detect any Michael adducts containing nitrogen in these reactions.

Reactions with Na_2S_x (in the absence of ammonia) were performed for comparison. The only product after MeLi/

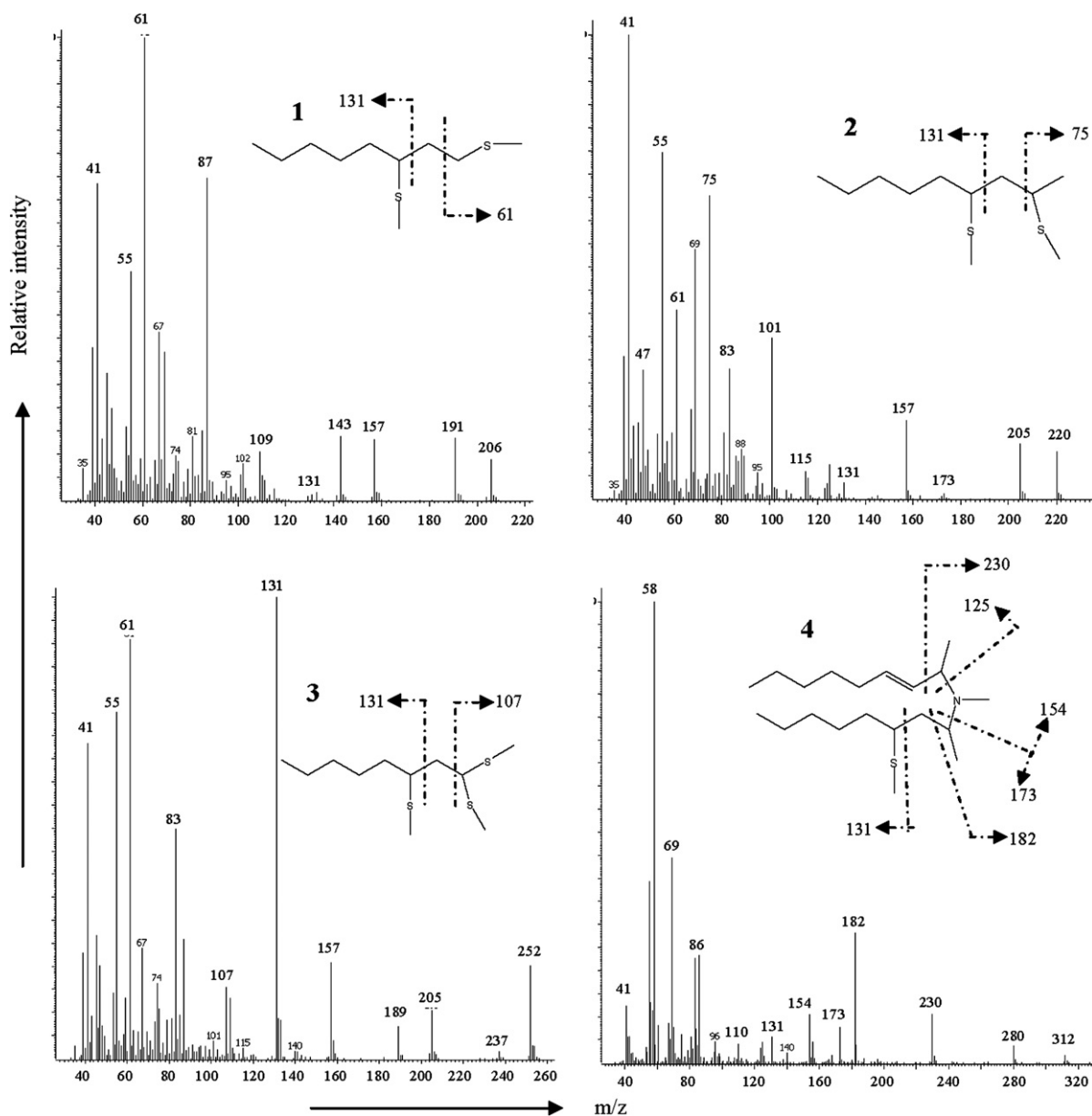


Fig. 3. Mass spectra of some of the MeLi/MeI products from the reaction as of *trans* 2-octenal and aqueous $(\text{NH}_4)_2\text{S}_x$ solution at ambient temperature for 1 week.

MeI was compound **6**. This agrees with previous experiments of α,β -unsaturated aldehydes in aqueous media (Amrani and Aizenshtat, 2004b). After products were extracted from the aqueous solution and analyzed, they were introduced into $(\text{NH}_4)_2\text{S}_x$ solution for overnight reaction. The reaction yields the same products as described in Fig. 2, demonstrating the importance of ammonia in this reaction, and that ammonia adds solely to the carbonyl carbon (and does not form Michael adducts) in the presence of polysulfides.

Like ammonia, octane amine and glycine were able to activate the C1 carbon for the formation of *gem* dithiols. In both cases we detected relatively large amounts of the product (compound **3**) after MeLi/MeI treatment and full conversion of 2-octenal. In the case of glycine, we could

not detect nitrogen containing compounds after organic extraction of the products. The reaction with octane amine yielded more nitrogen compounds than the reaction with ammonium polysulfide. This is probably because octane amine dissolves in the organic phase and access to the reactive sites in the aldehyde molecule is facilitated.

We could not observe major differences in the reaction products when the reaction between 2-octenal and $(\text{NH}_4)_2\text{S}_x$ took place at three distinct pH's: 6, 7.5, and 8.5–9. The only difference was the lower yield for the lower pH experiments, giving rise to the intermediate structure **6** while the nitrogen product distributions remained similar. However, the reaction with 2-octenal and Na_2S_x at pH 6 was different, and yielded compound **3**, whereas, at the higher pH's, the only product that could be detected was **6**.

Table 3

Characteristic peaks for mass spectral analyses on the MeLi/MeI chemical degradation on polysulfide cross-linked macromolecule (PCLM) from the reaction between $(\text{NH}_4)_2\text{S}_x$ and 2-octenal or octanal^a

Compound	Nonenriched PCLM	¹⁵ N enriched PCLM	D enriched PCLM
1	206(M ⁺ , 9%), 191(M-CH ₃ , 14), 157(14), 143(191-CH ₃ SH, 14), 131(1), 87(70), 61(100)	206(M ⁺ , 9%), 191(M-CH ₃ , 14), 157(14), 143(14), 131(1), 87(70), 61(100)	212(M ⁺ , 9%), 194(M-CD ₃ , 12), 160(6), 143(194-CD ₃ SH, 12), 134(2), 90(35), 64(100)
2	220(M ⁺ , 12%), 173(M-CH ₃ S, 1), 157(17), 125(173-CH ₃ SH, 7), 131(4), 75(66)	220(M ⁺ , 12%), 173(M-CH ₃ S, 1), 157(17), 125(173-CH ₃ SH, 7), 131(4), 75(66)	229(M ⁺ , 13%), 179(M-CD ₃ S, 1), 160(19), 125(173-CD ₃ SH, 9), 134(4), 81(100)
3	252(M ⁺ , 10%), 205(M-CH ₃ S, 7), 157(205-CH ₃ SH, 17), 131(95) 109(157-CH ₃ SH, 14), 107(16), 61(100)	252(M ⁺ , 10%), 205(M-CH ₃ S, 7), 157(205-CH ₃ SH, 17), 131(95) 109(157-CH ₃ SH, 14), 107(16), 61(100)	261(M ⁺ , 20%), 211(M-CD ₃ S, 10), 160(211-CD ₃ SH, 22), 134(100) 109(160-CD ₃ SH, 12), 113(19), 64(85)
4	327(M ⁺ , <1%) 312(M-CH ₃ , <1%), 280(M-CH ₃ S, 2), 230(M-C ₇ H ₁₃ , 11), 182(10), 154(4), 131(2), 125(2), 58(100)	313(M-CH ₃ , 1%), 281(M-CH ₃ S, 2), 231(M-C ₇ H ₁₃ , <1) 183(10), 155(4), 131(2), 125(2), 59(100)	321(M-CH ₃ , 6%), 289(M-CD ₃ S, 2), 242(8), 191(27), 160(5), 134(5), 128(2), 64(100)
5	375(M ⁺ , <1%) 360(M-CH ₃ , 1), 328(M-CH ₃ S, 7), 230(67), 182(2), 173(37), 131(10), 125(10), 86(100)	376(M ⁺ , <1%), 361(M-CH ₃ , 1), 329(M-CH ₃ S, 9), 231(88), 183(2), 173(44), 131(11), 125(12), 87(100)	390(M ⁺ , <1%) 372(M-CD ₃ , 2), 340(M-CD ₃ S, 6), 242(74), 191(2), 179(32), 134(8), 128(8), 95(100)
6	190(M ⁺ , 16%), 142(M-CH ₃ SH 17), 131(13), 71(100), 45(77)		
7	160(M ⁺ , 13%), 145(M-CH ₃ , 14), 112(M-CH ₃ SH, 4), 75(9), 61(71), 48(26), 41(100)	160(M ⁺ , 13%), 145(M-CH ₃ , 14), 112(M-CH ₃ SH, 4), 75(9), 61(71), 48(26), 41(100)	163(M ⁺ , 17%), 145(M-CD ₃ , 17), 112(M-CD ₃ SH, 4), 78(10), 61(88), 51(37), 41(100)
8	174(M ⁺ , 9%), 159(M-CH ₃ , 3), 126(M-CH ₃ SH, 5), 97(12), 75(85), 41(100)	174(M ⁺ , 9%), 159(M-CH ₃ , 3), 126(M-CH ₃ SH, 5), 97(12), 75(85), 41(100)	180(M ⁺ , 19%), 162(M-CD ₃ , 5), 129(M-CD ₃ SH, 8), 100(8), 81(100), 41(37)
9	206(M ⁺ , 11%), 159(M-CH ₃ S, 58), 107(6), 87(17), 69(100), 61(80)	206(M ⁺ , 11%), 159(M-CH ₃ S, 58), 107(6), 87(17), 69(100), 61(80)	212(M ⁺ , 12%), 162(M-CD ₃ S, 66), 113(9), 90(19), 69(100), 64(84)
10	267(M ⁺ , <1%), 252(M-CH ₃ 1) 196(6), 168(12), 154(75), 84(100)	268(M ⁺ , <1%), 253(M-CH ₃ 1), 197(7), 169(12), 155(74), 85(100)	273(M ⁺ , <1%), 255(M-CD ₃ 1) 202(7), 174(8), 157(85), 90(100)
11	283(M ⁺ , <1%), 268(M-CH ₃ 5), 184(45), 156(2), 58(100)	284(M ⁺ , <1%), 269(M-CH ₃ 5), 185(48), 157(2), 59(100)	292(M ⁺ , <1%), 274(M-CD ₃ 5), 193(60), 162(2), 64(100)
12	363(M ⁺ , 2%), 348(M-CH ₃ >1) 292(19), 264(17), 236(16), 43(100)	364(M ⁺ , 2%), 349(M-CH ₃ >1) 293(16), 265(15), 237(14), 43(100)	366(M ⁺ , 2%), 348(M-CH ₃ >1) 295(15), 267(14), 236(12), 43(100)

^a There are three sets of data for each experiment: MeLi/MeI on PCLM, MeLi/MeI on ¹⁵N enriched PCLM and D enriched MeLi/MeI on PCLM.

When polysulfides are not present, ammonia reacts in both positions at basic pH to produce N cross-linked polymers. Under slightly acidic conditions (pH 6) we could not observe the Michael adduct with 2-octenal while octenal reacted rapidly via imine formation.

3.3. Reaction with octanal

The reaction with octanal and polysulfides required significantly longer time to complete than 2-octenal. The products could be divided into sulfur containing products and nitrogen containing products. Unlike the reaction with 2-octenal, we could not identify any products that contain both nitrogen and sulfur.

3.3.1. NMR studies of PCLM

The ¹H NMR data show the aldehyde peak at 9.8 ppm completely disappears and new peaks form between 3.5 and 5.3 ppm when polymerization takes place. The ¹H-¹³C HSQC peaks at 1.6–1.9(40) ppm relate the β proton to the gem-disulfide (Fig. 6a). The group of peaks around 4–5(60–80) ppm can be related to (CH)_x of some hydroxyl/amine groups in gem positions with respect to the polysulfide bond. Some of these ¹H shifts (e.g. 4.2 and 4.4 ppm)

are also observed in the ¹H-¹⁵N HMBC experiment (Fig. 6b) and suggest nitrogen bonding. We did not observe peaks in the more downfield region of the ¹H-¹³C HSQC spectra associated with double bonds or imine functionality as in the case of 2-octenal (Fig. 1a). However, the ¹H-¹⁵N HMBC spectrum (Fig. 6b) shows very distinctive zones for the ¹⁵N chemical shifts: 50–100 ppm that represent several kinds of amines and a peak at 330–350 ppm that is related to imine (Schiff base) functionality. The fact that there are no peaks at lower than 50 ppm suggests a low amount of terminal amines (R-NH₂), indicating mostly secondary and tertiary amines. The ¹H peaks are distributed from 0.8 to 6.15 ppm in this zone and suggest the presence of a variety of hydrogens adjacent to N. The 0.8–2.35 ppm region is attributed to aliphatic and saturated molecules while the 4.15–6.15 ppm region relates to a double bond conjugated to amine. The more downfield ¹⁵N chemical shifts (80–95 ppm) are probably double bond conjugated gem-aminos that are similar to guanidine functionality.

3.3.2. Chemical degradation followed by GC-MS studies

The GC chromatogram for the MeLi/MeI products of the octanal reaction with $(\text{NH}_4)_2\text{S}_x$ is very complex, contains many products, and most of them contain nitrogen

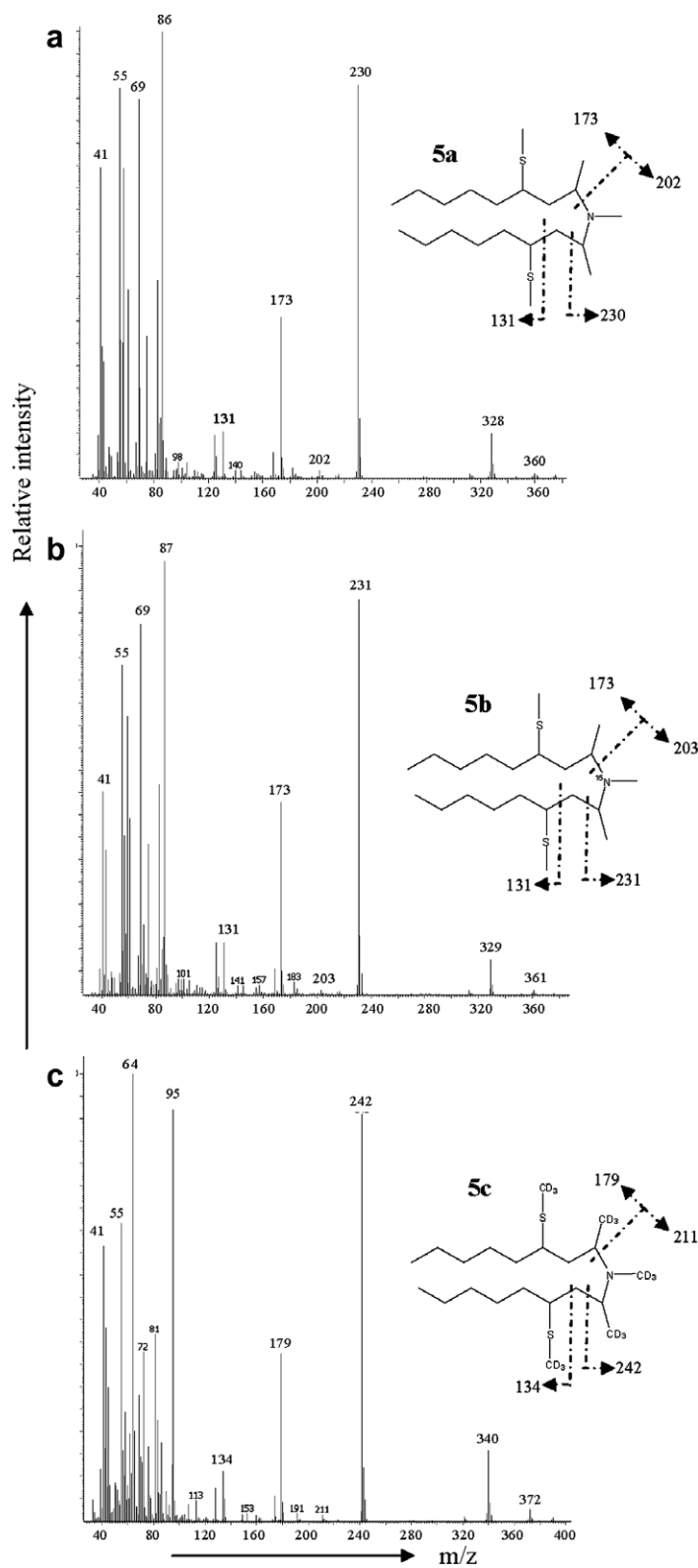


Fig. 4. An example (compound 5) for the method used in this study to identify the MeLi/MeI products as described in Fig. 2, using combined ¹⁵N enrichment of the PCLM and deuterated MeLi/MeI. (a) Treated with CH₃Li/CH₃I on nonisotopically enriched PCLM. (b) ¹⁵N enriched PCLM treated with CH₃Li/CH₃I. (c) PCLM treated with CD₃Li/CD₃I.

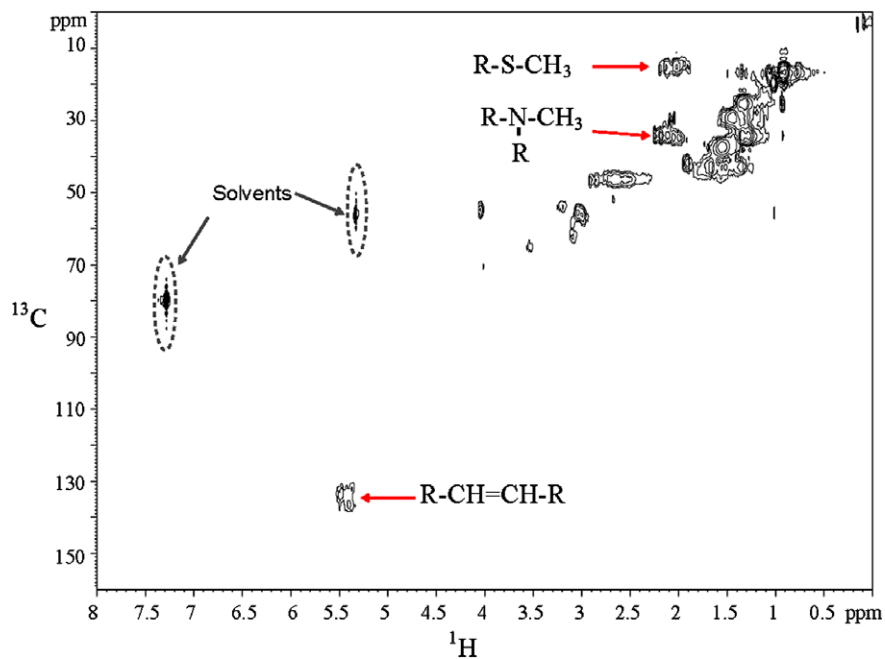
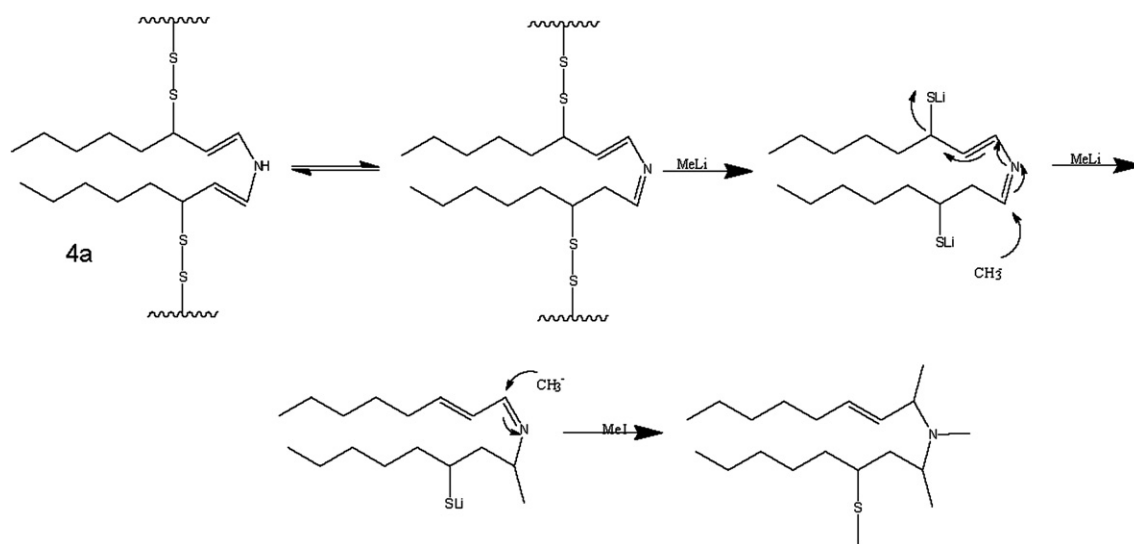
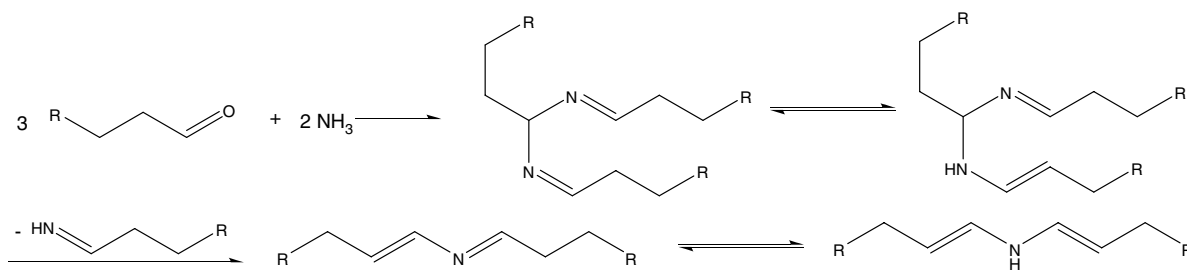


Fig. 5. HSQC ^1H - ^{13}C spectrum after MeLi/MeI treatment of the PCLM formed by the reaction of *trans* 2-octenal and aqueous $(\text{NH}_4)_2\text{S}_x$ solution at ambient temperature for 1 week.



Scheme 2. Suggested pathway for the formation of compound 4 during MeLi/MeI chemical degradation.



Scheme 3. Condensation reaction of ammonia and aldehydes (modified after Dayagi and Degani, 1970).

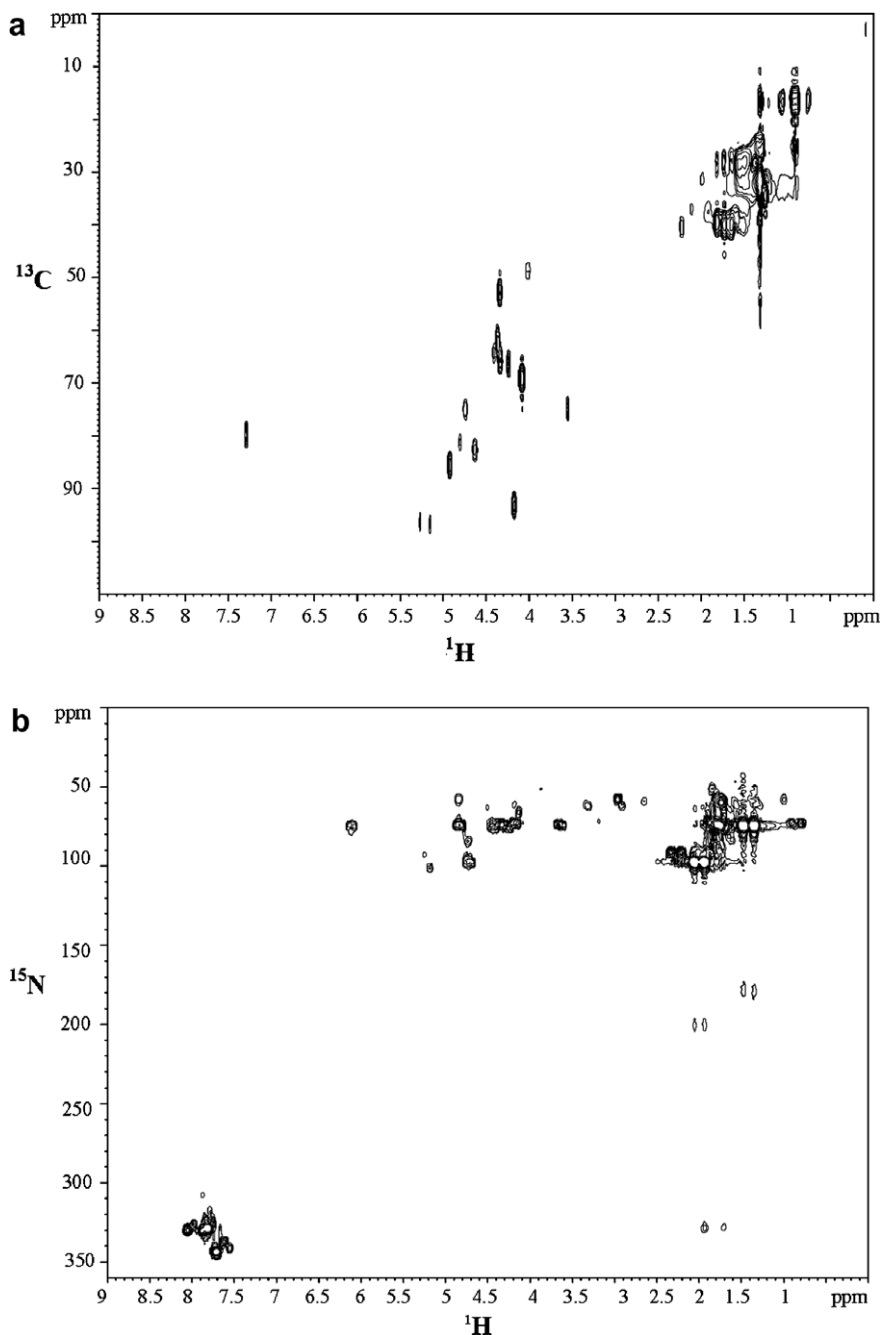


Fig. 6. NMR spectra for the reaction products between $(\text{NH}_4)_2\text{S}_x$ and octanal (a) HSQC ^1H - ^{13}C spectrum. (b) HMBC ^1H - ^{15}N spectrum.

(Fig. 7). Reaction conversion was about 80% and the unreacted octanal converted into 2-nonanol after the MeLi/MeI treatment. Compounds **7**, **8**, and **9** are sulfurized products of the nucleophilic addition to the carbonyl carbon (see mass spectra at Fig. 8). Compound **7** may form through the reductive sulfurization pathway (Adam et al., 2000) while compound **8** represents thioaldehyde that is an intermediate to both structures **7** and **9**. Compounds **10**, **11**, and **12** contain nitrogen (Fig. 8 and Table 3). The mass fragmentation of compound **10** suggests that the imine functionality survived the MeLi/MeI treatment. This is also supported by the ^1H - ^{13}C HSQC spectrum that shows

peaks in the imine region (not presented). Compound **11** is a dimer bonded through nitrogen. As in the case of 2-octenal, the original structure before MeLi/MeI treatment is an imine functionality. Compound **12** is a trimer with two conjugated double bonds to the amine. The methylation on the carbon that bonded to the amine suggests imine functionality before the chemical degradation treatment. The other minor peaks are some unidentified nitrogen containing compounds as evident from their fragmentation pattern and comparison to the ^{15}N enriched PCLM.

Reaction with Na_2S_x and octanal was performed under the same conditions in order to compare polysulfide reactiv-

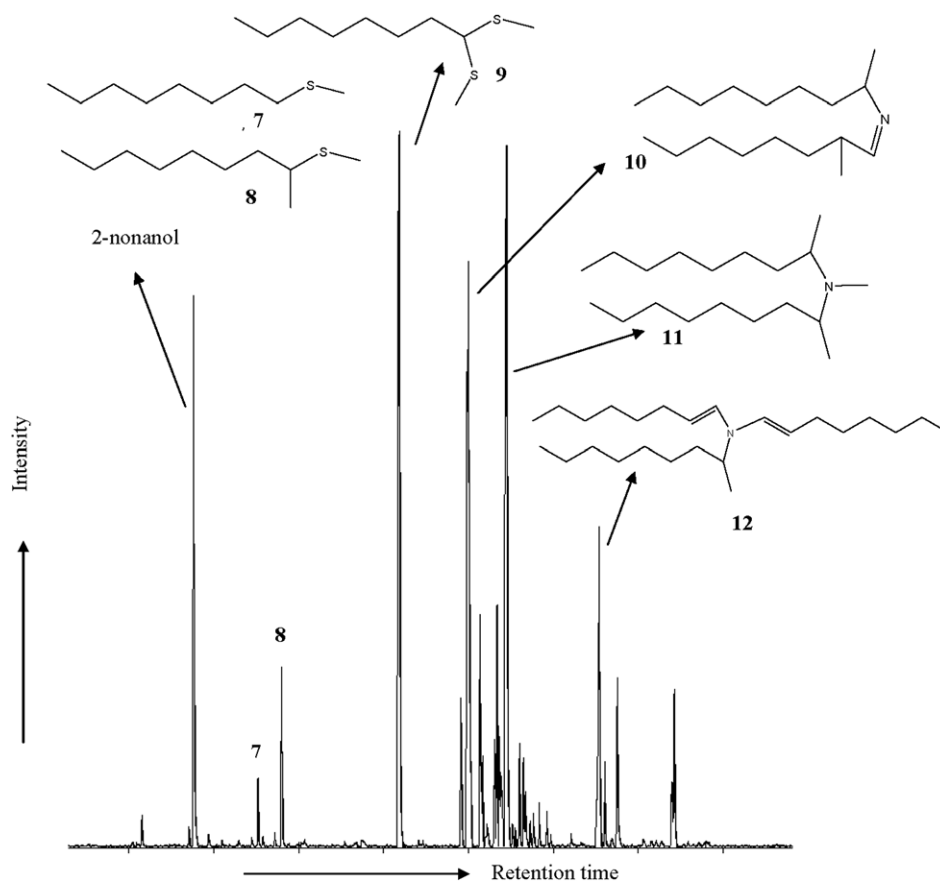


Fig. 7. TIC chromatogram of the reaction products from the MeLi/MeI treatment of the polysulfide cross-linked macromolecule (PCLM) produced by the reaction of octanal and aqueous $(\text{NH}_4)_2\text{S}_x$ solution at ambient temperature for 1 week.

ity with and without the presence of nitrogen compounds. After one week of reaction only 3% conversion of the aldehyde was observed in the reaction with Na_2S_x , compared with $\sim 80\%$ conversion for the reaction with $(\text{NH}_4)_2\text{S}_x$ over the same period of time. The only products that could be identified were compounds **8** and **9** in much lower abundance than in the reaction with $(\text{NH}_4)_2\text{S}_x$.

4. DISCUSSION

4.1. The competition between ammonia and polysulfides

Ammonia and (poly)sulfides are expected to compete for the same electrophilic positions. There are two possible positions for nucleophilic attack on 2-octenal, C1 and C3, while octanal has only the C1 position. Polysulfides (and sulfides) being much stronger nucleophiles than ammonia are expected to out compete ammonia in both positions if the mechanism depends solely on nucleophilicity strength. Our experimental and molecular modeling results show that at C3 (Michael addition) polysulfide does indeed out compete ammonia. However, ammonia is shown to compete well in the carbonyl position (C1) in both 2-octenal and octanal. Our results also show that the reaction of ammonia with aldehyde involves the formation of imine through nucleophilic attack on the carbonyl carbon and elimination of water. The formed imine is very reactive and can react

with another aldehyde, imine or enamine molecule. Secondary imines can further react with another molecule of aldehyde to form tertiary amines however; steric effects may hinder this reaction as suggested by our results that show mostly secondary imines. Another known route for the reaction of ammonia with aldehyde is condensation of two molecules of ammonia with three molecules of aldehyde to form *gem* di-imine (Dayagi and Degani, 1970). This product is not stable and decomposes to yield several other nitrogen containing products such as a conjugated imine (Scheme 3).

The Gibbs free energies for the reaction between alkyl aldehyde and NH_3 or HS^- were calculated. The two intermediate structures after NH_3 and HS^- attack on the carbonyl have an energy difference of ~ 2.6 kcal (Table 1) which is smaller than in the Michael addition but still favors the S adduct. However, it seems that the energy differences between the intermediates favor imine formation over thioaldehyde (Table 1). The ability of ammonia to compete with polysulfide nucleophiles at the carbonyl position is probably because the ammonia/ammonium can supply (or transfer) a proton and stabilize the transition state. Support for that mechanism is obtained from experiments at different pH. The reaction between $(\text{NH}_4)_2\text{S}_x$ and 2-octenal is not affected significantly by changes in pH from 6 to 9. At basic conditions, ammonia ($\text{p}K_a = 9.2$) is more abundant and therefore more available for nucleophilic attack on

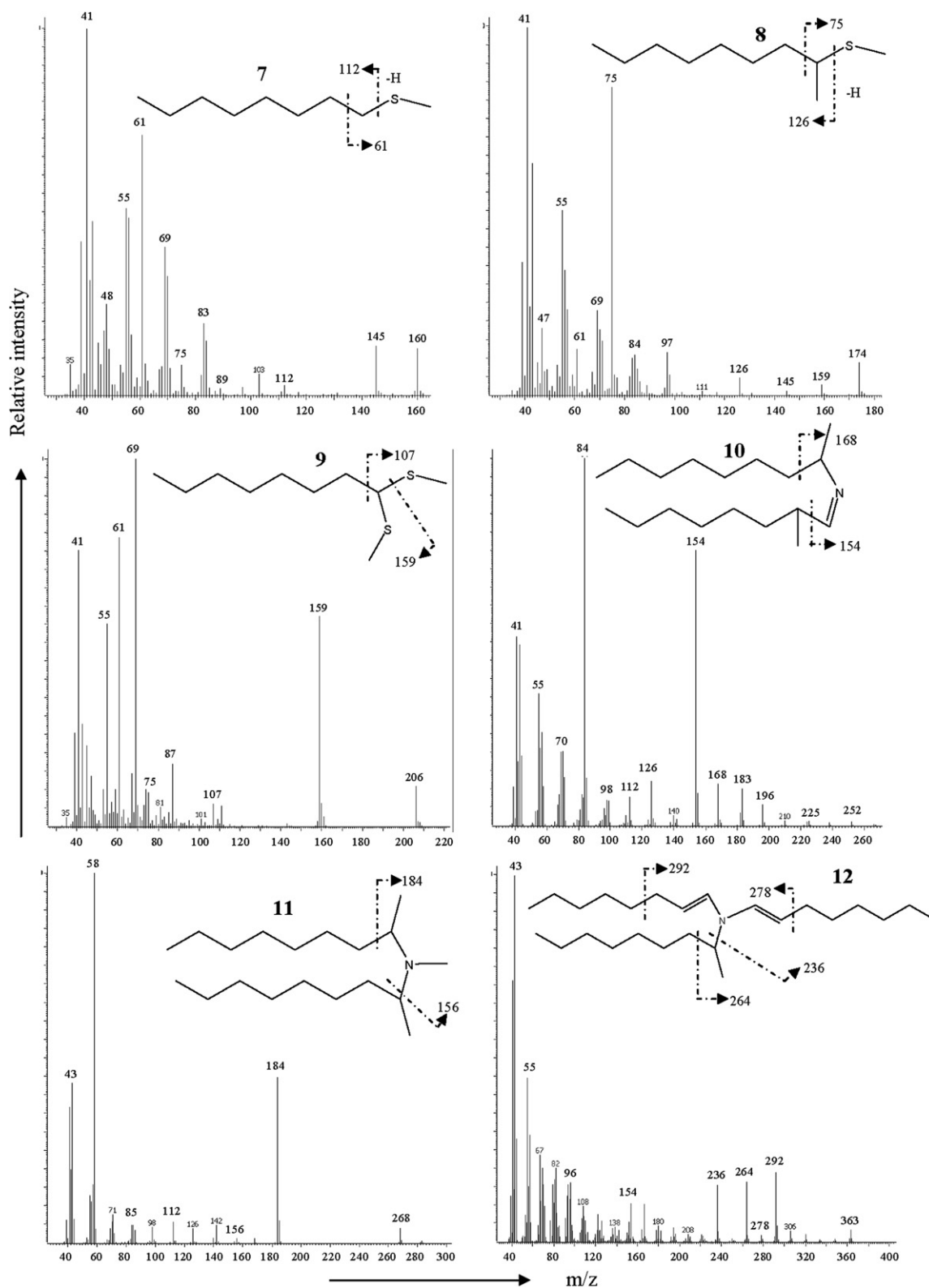


Fig. 8. Mass spectra of some of the MeLi/MeI products from the reaction of octanal and aqueous $(\text{NH}_4)_2\text{S}_x$ solution at ambient temperature for 1 week.

the carbonyl which is the first stage in the formation of imine product. On the other hand, at pH 6 ammonia is predominantly (>99.9%) in the protonated state (ammonium),

which is not a nucleophile. However, at lower pH values protons can stabilize the transition state of imine formation, therefore this reaction is still favorable (Jencks,

1959; Cordes and Jencks, 1963). This may be the reason why ammonia can compete so well with polysulfide on the C1 carbonyl position.

In a previous study of ammonium polysulfide, enrichment of isoprenoidal unsaturated aldehydes, we detected smaller amounts of high mass compounds eluting after the *gem*-disulfide product (Amrani and Aizenshtat, 2004b). We considered these groups of compounds to be dimers or oligomers and explained their presence as products of incomplete degradation by the MeLi/MeI treatment. Reevaluation of that data showed a very similar peak pattern as the reaction with 2-octenal. The first group of compounds after the elution of the *gem*-disulfide compound is analogous to compound 4 and the second group of peaks is analogous to compound 5. The lower amount of these nitrogen containing compounds as compared with the reaction with 2-octenal may be attributed to the isoprenoid structure that may limit the formation of a dimer in this position (steric effects).

4.2. The synergetic effect between ammonia and polysulfides

In a previous study we have shown that the reaction rate between conjugated aldehydes and polysulfides in the presence of ammonia is very high and usually completed within 1 h. On the other hand, when ammonia is not present, the reaction rate decreases dramatically and is complete in a matter of days (Amrani and Aizenshtat, 2004b). Moreover, the sulfurized products are different and suggest a different mechanism of incorporation. Scheme 4 describes a possible mechanism for the formation of the PCLM in the presence of ammonia. Reactions with Na_2S_x yielded only the Michael adducts at C3, while reactions with $(\text{NH}_4)_2\text{S}_x$ were cross-linked in both C3 and C1 positions suggesting subsequent reaction on the remaining carbonyl carbon. Therefore, ammonia must play a role in the formation of *gem*-dithiols and may be considered catalytic to some extent by activation of the carbonyl carbon. Activation of C1 is also achieved if amines (octane amine or glycine) are used instead of ammonia. This is in agreement with previous studies that suggest imine as an intermediate in the formation of *gem*-dithiols during reaction of acetone and *n*-butyl amine (Asinger et al., 1959). Scheme 5 presents the energy profile for the imine pathway compared with the thioaldehyde pathway (no ammonia present). The imine pathway seems more favorable because of the smaller energy differences between the intermediates. If imine is indeed a very reactive intermediate in the formation of *gem*-disulfide, its stabilization in the polymer is quite surprising. It would be logical to assume that even after the dimerization step (Scheme 4), polysulfide can still use the imine as an intermediate in the formation of *gem*-disulfide in a similar way as described above. However, the direct identification of imine by NMR spectrometry analyses proves that imine is actually preserved in the PCLM at least in a laboratory time scale. One possible explanation for this is that the formation of the PCLM is very rapid and in a matter of minutes a nonsoluble solid polymer forms that “protects” the imine groups from the surrounding polysulfide anions. Scheme 6 shows the simpli-

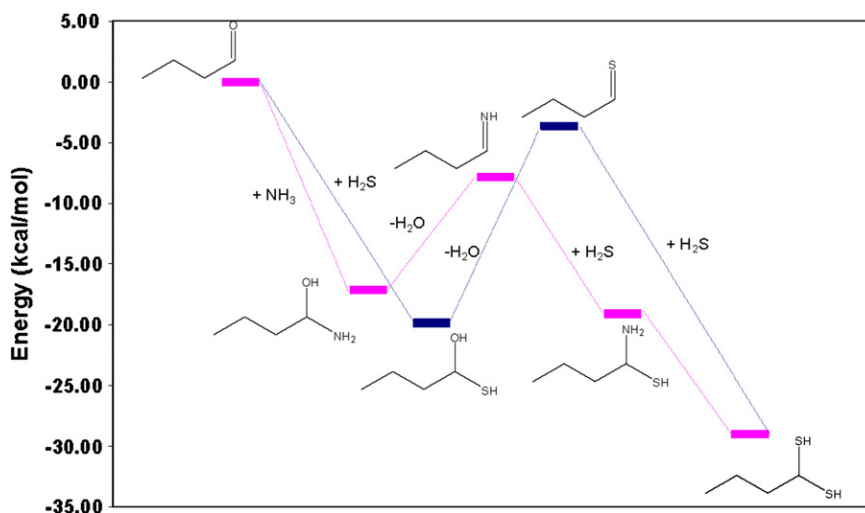
fied reconstructed polymer structure of the reaction between $(\text{NH}_4)_2\text{S}_x$ and 2-octenal.

Another possible explanation is that the imine is not an intermediate in the formation of sulfurized products but rather a competitive pathway. Our results show clearly that at least 50% of the available carbonyl positions are occupied by nitrogen, mostly as imine or enamine (see Figs. 2 and 7). Molecular modeling calculations do not demonstrate significant energetic benefit by going through the imine path (Table 1) though it is still a possible pathway because it does decrease the energy barrier. In both pathways the rate-determine steps are probably the elimination of water to form imine or thioaldehyde which have relatively high transition state energies in basic pH. Elimination of water requires a proton supply. Indeed, when we reacted 2-octenal with Na_2S_x under basic conditions no *gem*-disulfide formed. Repeating this reaction under acidic conditions or in the presence of ammonia/amine, as described in the results section, *gem*-disulfides were formed. This suggests that proton supply is the main factor in these reactions. We have calculated several transition state energies as presented in Table 2. These calculations point to an alternative pathway in which ammonia and amines act as a proton transfer agent between the hydroxyl and thiol in the intermediate structure (e) (Table 2). This has a much lower transition state energy than the other possible pathways. In this case ammonia and amines act as pure catalysts without participating in the reaction. An ionic pathway such as the ammonium/ammonia couple may also be possible and is currently under investigation.

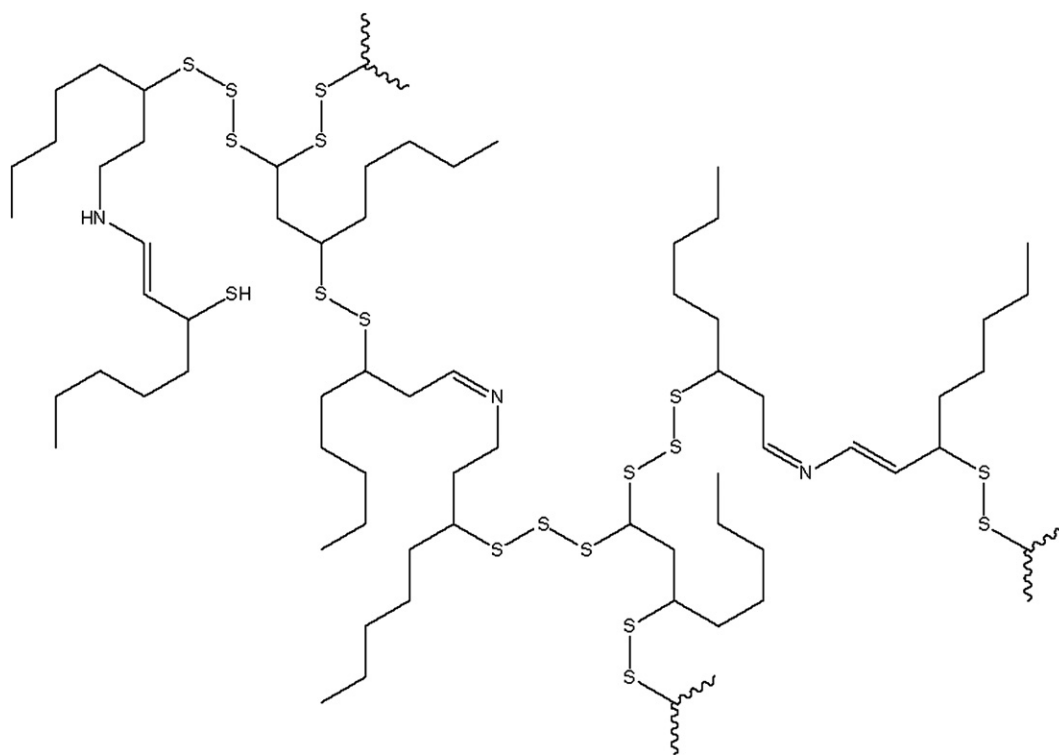
The reaction with glycine also appears to follow the proton transfer mechanism. We observed enhancement in the reaction rate and formation of *gem*-disulfide while no nitrogen containing products were detected. Unlike the reaction with ammonia or octane amine, any imine that may form by the reaction of glycine and carbonyl can be easily removed because of the soluble carboxyl group.

4.3. Enhancement in the polymerization rate in depositional environments

Depositional environments that support secondary sulfur enrichment of OM must have very high productivity because SRB consume large amounts of OM during the dissimilatory sulfate reduction process (e.g. Jorgensen, 1982; Canfield, 1989; Thamdrup and Canfield, 1996; Aizenshtat et al., 1999; Ferdelman et al., 1999). This dissimilatory sulfide is the main source for reduced inorganic sulfur species that react with the OM. In such an environment we are likely to find high amounts of ammonia/ amines from the mineralization by microorganisms. There is evidence for the presence of amino acids during the formation of sulfur rich kerogen (Riboulleau et al., 2002; del Rio et al., 2004). These authors reported on the preservation of amino acids (mainly glycine) by the sulfur cross-linked matrix of the kerogen. Such a process could occur through an encapsulation mechanism (Knicker and Hatcher, 1997; Zang et al., 2000). These reports show that peptides and amino acids are indeed present during sulfurization processes and therefore can activate the carbonyl



Scheme 5. Energy profile for the two reaction routes presented in Scheme 1c.

Scheme 6. A simplified reconstructed model for S and N cross-linked polymer from the reaction of 2-octenal and $(\text{NH}_4)_2\text{S}_x$.

proteinaceous material with OM (e.g. Nguyen and Harvey, 1998; Hsu and Hatcher, 2006). However, it is logical to assume that a significant amount of the organic nitrogen arrives from the reaction of ammonia and OM considering the relatively high concentrations and reactivity of ammonia in anaerobic marine sediments (Bregant et al., 1990; Schulz et al., 1994; Passier et al., 1998; Cotner et al., 2004; Knicker, 2004). Moreover, the ability of ammonia to form cross-linked bonds with OM, as demonstrated in the present study, could indicate another important route for the accumulation and preservation of organic nitrogen.

Unlike reduced sulfur species that have a renewable supply (the SRB activity) from the relatively large amounts of sulfate in marine waters, ammonia formation is mostly limited by the degradation of proteins. Therefore, we do not expect to observe an accumulation of nitrogen in OM as compared to the nitrogen content within the cycling inputs as in the case of organic sulfur. In fact, the amount of nitrogen in OM has been shown to decrease during the course of diagenesis and the formation of kerogen (Krein, 1993). However, in environments such as wetlands and some terrestrial zones, a net increase in the amount of nitrogen in

the preserved OM is possible. This is due to the initially low amount of nitrogen in some recalcitrant OM inputs. Amino acids, a major input of nitrogen to OM, are more easily degraded than OM inputs such as lignin, and aliphatic biopolymers which contain little or no nitrogen. Some preliminary results of experiments applying the same methods used in this study have shown nitrogen enrichment of aliphatic cuticular biopolymers (Turner et al., 2006).

5. CONCLUSIONS

This study presents possible pathways in which nitrogen and sulfur nucleophiles can rapidly form cross-linked polymers in aqueous systems. Ammonia and polysulfide that are abundant in many marine sediments, compete for the same electrophilic positions in OM. While polysulfides out compete ammonia in the formation of Michael adducts, ammonia has been shown to compete well at the carbonyl position. Similar effects are observed when amines (e.g. glycine) are used instead of ammonia. The effect of ammonia and amines on the polymerization processes is by two means: (i) reaction with carbonyls to form oligomers and polymers *via* imine formation and (ii) catalysis of the reaction of sulfur nucleophiles with carbonyls to enhance the rate of polymerization, probably through a proton transfer mechanism. The trace amounts of cyclic nitrogen found during low temperature reactions suggest that, like sulfur, the cyclization processes are mainly occurring during thermal maturation.

The wide pH range used in this study cover marine and soil (wetlands) environments. However, the mechanism presented in this study is especially important under basic to neutral conditions like those that prevail in marine environments. Ammonia and glycine or possibly other amino acids and/or peptides are intimately involved with sulfur nucleophiles throughout the polymerization processes that occur at low temperatures and thus are suggested as key reactants in diagenetic formation of protokerogen and humics.

ACKNOWLEDGMENTS

We thank Chunhua Yuan of the Campus Chemical Instrument Center at The Ohio State University (OSU) for assistance and helpful discussions on the NMR experiments. We also appreciate the insightful discussions with Christopher Hadad (OSU). This work was supported by the National Science Foundation (CHE 0089147). Comments by Zeev Aizenshtat, Jay Brandes, and an anonymous reviewer are gratefully acknowledged.

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Associate editor: Jay A. Brandes