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Synthesis of *N*-Hydroxyacetaminophen, a Postulated Toxic Metabolite of Acetaminophen, and Its Phenolic Sulfate Conjugate

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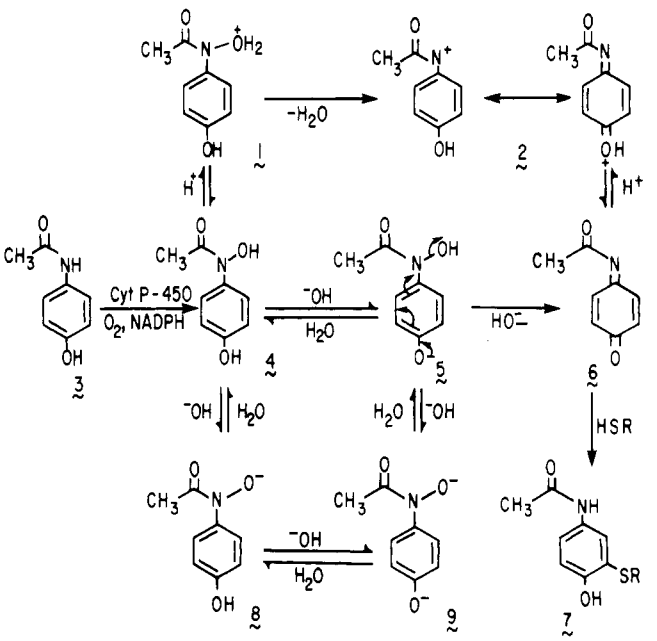
The synthesis of *N*-hydroxyacetaminophen (*N*-acetyl-*N*-hydroxy-*p*-aminophenol, 4), a postulated toxic metabolite of acetaminophen (*N*-acetyl-*p*-aminophenol, 3), and its phenolic sulfate conjugate (potassium *N*-acetyl-*N*-hydroxy-*p*-aminophenyl sulfate) (13) is described. Potassium *p*-nitrophenyl sulfate was reduced to the hydroxylamine, acetylated, and treated with sulfatase to yield *N*-hydroxyacetaminophen. The structures assigned are supported by the spectral data (IR, UV, MS, ^{1}H NMR, and ^{13}C NMR). *N*-Hydroxyacetaminophen was found to be moderately unstable at physiological pH and temperature, whereas its phenolic sulfate conjugate was stable.

Acetaminophen (*N*-acetyl-*p*-aminophenol, 3) is a widely used mild analgesic. It is largely metabolized to glucuronide and sulfate conjugates which are readily excreted by the kidney and are considered nontoxic. The possibility that a small fraction of the administered dose of acetaminophen might also be metabolized to a toxic metabolite arose from the high incidence of severe hepatotoxicity resulting from suicidal overdosage in man.[1] The probable mechanism of toxicity has emerged from studies in animals in which it has been shown that acetaminophen is converted to a metabolite which depletes hepatic glutathione and then covalently binds to tissue macromolecules.[2] Depletion of hepatic glutathione and the subsequent covalent binding of radio-labeled acetaminophen to hepatic protein are enhanced by pretreatment with agents known to stimulate the Cyt P-450 mixed function oxidase system and decreased by inhibitors of drug metabolism.[2] The pathway that has been postulated to account for the covalent binding is shown in Scheme I and involves the formation of *N*-hydroxyacetaminophen (*N*-acetyl-*N*-hydroxy-*p*-aminophenol, 4) or its dehydration product, *N*-acetyl-*p*-benzoquinone imine (6), which then reacts with the sulfhydryl group of glutathione (7) or other cellular nucleophiles. More recently it has been proposed that the same biochemical mechanism may underly acetaminophen-induced acute renal necrosis as well as the nephropathy of chronic analgesic abuse.[3,4] Although *N*-hydroxyacetaminophen is a key compound in the proposed mechanism of toxicity, it has not been synthesized. Many questions involving either hepatic or renal toxicity require the availability of *N*-hydroxyacetaminophen for toxico-

Scheme I



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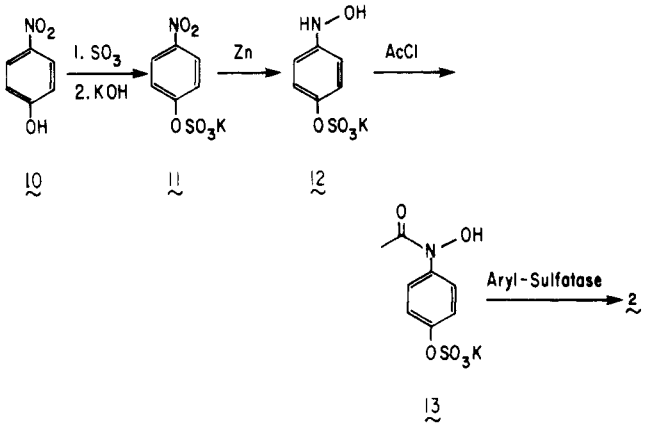
Scheme II



logical studies, e.g., the detailed examination of the reactions between 4 and cellular nucleophiles or the renal mechanisms by which 4 might become concentrated within the tubular fluid and parenchyma of the kidney to produce papillary lesions.

In this paper, we are reporting the first synthesis[5] of 4, a sulfate conjugate of 2 (potassium *N*-acetyl-*N*-hydroxy-*p*-aminophenyl sulfate, 13), and an analogue of 2 (*N*-acetyl-*p*-methoxyphenylhydroxylamine, 16). We are also reporting the stability of 4 and 13 in the range of physiological pH and temperature.

Syntheses. Potassium *N*-acetyl-*N*-hydroxy-*p*-aminophenyl sulfate was synthesized since it is a likely phenolic sulfate conjugate of 4. As illustrated in Scheme II, potassium *p*-nitrophenyl sulfate (11) was prepared from *p*-nitrophenol (10) by the method of Burkhardt and Wood[6] and then reduced to the hydroxylamine 12 by the method of Kamm,[7] which was then acetylated with acetyl chloride by the method of Smissman and Corbett[8] to 13. The product (13) was purified by column chromatography using Sephadex G10 as an absorbent and as a molecular sieve. The yield of 13 from 11 varied from 25 to 45%.

The synthetic route to *N*-hydroxyacetaminophen (4) is also outlined in Scheme II. An aqueous solution of 13 was added to a buffered solution of aryl sulfatase. When back titration with dilute base indicated that all of 13 had been converted to 4, 4 was extracted from the aqueous solution with ether. Initially, the sulfatase was Sigma Type VI[9] of which one unit hydrolyzes 1 μmol of *p*-nitrophenyl sulfate per minute at pH 7.1 and 37 °C. However, very low yields of 4 (<1%) were obtained, presumably due to the pH. The use of a different sulfatase, Sigma Type H-2[9] of which one unit hydrolyzes 1 μmol of *p*-nitrocatechol sulfate per hour at pH 5.0 and 37 °C, increased the yields and decreased the cost of enzyme. The amount of 4 produced per unit of sulfatase was increased by precipitating with barium chloride the sulfate ions which were generated.

N-Acetyl-*p*-methoxyphenylhydroxylamine (16) was synthesized for spectral comparison with 4. *p*-Nitroanisole (14) was reduced to *p*-methoxyphenylhydroxylamine (15) by the method of Rising[10] which was then acetylated with acetyl chloride to 16 by the method of Smissman and Corbett.[8]

The structures assigned to 4, 13, and 16 are supported by the spectral data presented in the Experimental Section. The glycolic amide and phenolic isomers of the above compounds would have an amide II band in the IR spectra and different ^{13}C NMR chemical shifts.[11] The presence of a sulfated phenol in 13 is supported by the IR absorption bands (7.81–8.20 and 9.35–9.62 μ).[12] The mass spectra of 4 and 16[13] are consistent with the patterns reported for other *N*-aryl-*N*-hydroxyacetamides,[14,15] i.e., relatively weak molecular ions and ions at M − 16, M − 59, and M − 88; however, a satisfactory mass spectrum of 13 was not obtained using electron impact or field desorption.

Table I. Decomposition of 4 and 13 at 37 °C as a Function of pH

Compd (2 mM)	pH[a]	$t_{1/2}$, min[b]	Compd (2 mM)	pH[a]	$t_{1/2}$, min[b]
4	1.5	1100	13	1.5	910
	5.0	*c*		5.0	*c*
	6.6	150		7.0	*c*
	7.0	66		8.5	13000[d]
	7.2	21		12.7	*c*
	8.0	9.8			
	12.7	*c*			

[a] Buffer is 100 mM (Na_2HPO_4 for pH 6.6, 7.2, 8.0, and and 8.5; AcONa for pH 5.0; HCl for pH 1.5; and NaOH for pH 12.7). [b] The slope was obtained from a linear regression analysis of the experimental points obtained in the first 3 half-lives or in the course of the experiment (6 h). [c] The slope was not significantly different from zero at $p < 0.05$. [d] The decomposition was followed for 24 h.

The stability of 4 and 13 in the range of physiological pH was determined by following their decomposition with a ferric chloride assay.[16] Aliquots of a solution of 4 or 13 in buffer were periodically withdrawn and assayed for the amount of *N*-hydroxy compound present. Two factors interfered with the assays and were appropriately controlled. First, the decomposition of 4 and 13 led to products which increased the absorbance at the wavelength monitored. Second, if 4 was present in the aliquot assay, the complex formed with ferric chloride was unstable, in contrast to 13 and 16, and the absorbance decreased with time.

The rate of decomposition of 4 and 13 (Table I) was pseudo-first-order. In an acidic solution, 4 and 13 slowly decomposed. In the range of physiological pH, 13 was stable but the rate of decomposition of 4 increased as the pH increased. Both 4 and 13 were stable in alkaline solutions.

Discussion

At low pH, *N*-hydroxyacetaminophen and *N*-acetyl-*N*-hydroxy-*p*-aminophenyl sulfate probably decompose by the same mechanism proposed for other *N*-acetyl-*N*-arylhydroxylamines,[17,18] i.e., decomposition to a nitrenium ion (top of Scheme I). Unlike other *N*-acetyl-*N*-arylhydroxylamines, *N*-hydroxyacetaminophen is unstable in neutral solutions. A possible mechanism is shown in Scheme I. As the pH is increased from 6 to 8, the proportion of *N*-hydroxyacetaminophen present as the phenolate anion (5) increases and, correspondingly, the rate of dehydration to 6 increases. At pH 12.7, *N*-hydroxyacetaminophen is present as the dianion 9 and is stable.

The stability characteristics of *N*-hydroxyacetaminophen have several implications. In aqueous solution and at the pH of blood, *N*-hydroxyacetaminophen is far more stable than previously postulated,[15] with a half-life of slightly less than 20 min. However, the stability of *N*-hydroxyacetaminophen is strongly pH dependent. Over the physiological range for urine (pH 5–8) the half-life varies from infinity to 10 min. Although the stability has not been determined under in vivo conditions, the above characteristics of *N*-hydroxyacetaminophen raise the possibility that the compound might be formed in one organ and then disseminated by the blood to react at a distant site. In support of this, in preliminary experiments Calder[5] has shown that, after intraperitoneal injection, *N*-hydroxyacetaminophen depletes tissue glutathione in

both liver and kidney. In the case of the kidney, *N*-hydroxyacetaminophen might be formed in the cortex, diffuse into the tubular fluid, and then react with tissue proteins at more distal sites of the nephron within the papilla. Although *N*-hydroxyacetaminophen is more stable than previously postulated, it is nevertheless significantly labile under physiological conditions of pH and temperature. This poses an analytical problem for its detection in biological fluids.

Experimental Section

Melting points were determined with a Thomas-Hoover capillary apparatus and are uncorrected. The following instruments were used for spectral measurements: NMR, JEOL JNM-FX 60Q; MS (low resolution), Finnigan Model 4023; IR, Perkin-Elmer 137; UV, Cary 118; and VIS, Gilford 300-N. NMR spectra were obtained in solutions of dimethyl-d_6 sulfoxide with tetramethylsilane as internal standard. Chemical shifts are reported in parts per million relative to the internal standard. Mass spectra were obtained at an ionizing potential of 70 eV by direct insertion. Elemental analyses were performed by Micro-Tech Laboratories, Skokie, Ill., and agree with the calculated values within ±0.4%.

The $FeCl_3$ used was U.S.P. grade. Evaporations were performed under reduced pressure at room temperature unless otherwise stated. Liquid chromatography was performed as follows. Degassed solvent was pumped with a peristaltic pump through a three-way valve attached to a column with flow adapters and to a syringe pump for sample injection. The column effluent was monitored at 254 nm and then collected with a fraction collector.

p-Nitrophenyl sulfate, potassium salt (11) was synthesized by the method of Burkhardt and Wood[6] and recrystallized twice from H_2O affording yellow crystals: mp 250–253 °C; 78% (lit.[6] mp not given, 80%).

Potassium *N*-Acetyl-*N*-hydroxy-*p*-aminophenyl Sulfate (13). To a vigorously stirred solution of potassium *p*-nitrophenyl sulfate (**11**) (11.3 g, 41 mmol) and NH_4Cl (2.7 g, 83 mmol) in 100 mL of H_2O at 45 °C, Zn dust (5.49 g, 83 mmol) was added in very small portions over the course of 20 min. The mixture was then vigorously stirred for an additional 30 min. The reaction is exothermic. The temperature of the mixture rose to 65 °C and was then maintained at 65 °C. The mixture was filtered and the yellow filtrate was lyophilized to a yellow powder. The yellow powder was added to a flask containing 250 mL of ether and a slurry of $NaHCO_3$ (6.7 g, 80 mmol)–H_2O (25 mL) at −10 °C. The mixture was vigorously stirred as AcCl (2.6 g, 33 mmol) in 100 mL of anhydrous ether was added dropwise over 60 min. The solution was stirred for an additional 15 min and then 100 mL of H_2O was added. The ethereal layer was decanted off and the aqueous layer stirred under reduced pressure to remove the remaining ether. The solution was left overnight at 4 °C, filtered, and lyophilized to a powder.

The crude product was purified by gel filtration using the following general procedures unless otherwise noted: (1) pH adjustments were made with 1 M AcOH or 1 M KOH; (2) fractions were redissolved in a minimum of H_2O and then adjusted to the pH of the eluent before application to the column; and (3) fractions collected were adjusted to pH 7 and then lyophilized. To prevent column overloading and band spreading the redissolved crude product was chromatographed in portions. The chromatographic sequence was as follows: (1) one-half of the sample per run was applied to a column of Sephadex G10 (2.6 × 40 cm) and eluted with 25 mM $KHCO_3$, pH 10; (2) one-third of the sample per run was applied to a column of Sephadex G10 (2.6 × 40 cm) and eluted with 25 mM $KHCO_3$, pH 10, and the collected fractions were adjusted to pH 5, stirred, and then adjusted to 7; (3) one-half of the sample per run was applied to a column of Sephadex G10 (2.6 × 40 cm) and eluted with H_2O; and (4) the sample was twice applied to a column of Sephadex G10 (2.6 × 100 cm) and eluted with H_2O. The product **13** was obtained as a beige powder, 5.1 g (44%). An analytically pure sample was obtained by flocculating **13** from H_2O with EtOH: mp 139–140 °C dec.

Pertinent spectral data for **13** are as follows: UV (90% EtOH) λ_{max} 252 nm (ϵ 11 600), (0.1 M AcOH) λ_{max} 244 nm (7300), and (0.1 M KOH) λ_{max} 216, 274 nm (7860, 3500); IR (KBr) 2.97, 3.54, 6.06, 6.67, 7.26, 7.74, 7.90, 8.14, 9.44, 11.39, 11.84, and 13.66 μ; ^{1}H NMR 2.14 (3 H, s) and 7.05–7.57 (4 H, AA'BB'); ^{13}C NMR 22.1, 119.9, 121.9, 137.0, 150.3, and 168.8. Anal. ($C_8H_8KNO_6S$) C, H, N, S.

***N*-Hydroxyacetaminophen (4).** A solution of **13** (1.1 g, 4.0 mmol) in 18 mL of H_2O was added in 3.0-mL aliquots to a stirred solution of sulfatase (Sigma Type H2,[9] 15000 U) in 200 mL of 20 mM AcONa, pH 5.0, at 32 °C. After the initial addition of an aliquot of **13**, the succeeding aliquots of **13** were added after the pH, which had decreased to 4.92, was adjusted to 5.00 with equal amounts of 0.20 M NaOH and 0.20 M $BaCl_2$. When the pH remained constant, the solution of sulfatase and **4** was cooled to 25 °C and then extracted with 15% i-$C_5H_{11}OH$–Et_2O (4 × 600 mL). The combined extracts were washed with 50 mL of NaH_2PO_4 (25 g/50 mL) and evaporated to a brown powder which was extracted with anhydrous ether (2 × 250 mL). The combined extracts were evaporated to dryness to give 0.53 g (87%) of **4**. An analytically pure sample was prepared by recrystallizing **4** from THF–AcOEt: mp 125–127 °C dec.

Pertinent spectral data for **4** are as follows: UV (EtOH) λ_{max} 254 nm (ϵ 8500), (0.02 M AcONa, pH 5.02) λ_{max} 233 nm (8700); IR (KBr) 3.11, 3.47, 6.09, 7.87, 8.14, 10.09, and 11.90 μ; ^{1}H NMR 2.09 (3 H, s), 6.66–7.38 (4 H, AA'BB'), 9.46 (1 H, s), and 10.33 (1 H, s); ^{13}C NMR 21.8 114.8, 124.1, 133.4, 155.1, and 168.4; MS m/e (rel intensity) 167 (M^+, 6), 151 (2), 150 (3), 149 (9), 135 (2), 134 (1), 125 (26), 111 (0.5), 108 (38), 100 (8), 81 (19), 79 (13), 52 (69), and 43 (100); calcd mol wt m/e 167.05824, found mol wt (MS) m/e 167.05770. Anal. ($C_8H_9NO_3$) C, H, N.

***p*-Methoxyphenylhydroxylamine (15)** was freshly prepared by the method of Rising[10] yielding white flakes, mp decomposes on melting, 42% (lit.[10] mp not given, 50%).

***N*-Acetyl-*p*-methoxyphenylhydroxylamine (16).** To a vigorously stirred mixture of **15** (22.2 g, 160 mmol), $NaHCO_3$ (14.8 g, 176 mmol), H_2O (150 mL), and ether (1250 mL) at 0 °C, AcCl (13.8 g, 176 mmol) in 220 mL of anhydrous ether was added dropwise over 75 min. The mixture was slowly warmed to 25 °C and the aqueous layer removed by aspiration. The ethereal solution was washed with H_2O (100 mL) and then extracted with 1 M NaOH (4 × 200 mL). The combined alkaline extracts were neutralized with NaH_2PO_4(s) and extracted with ether (4 × 150 mL). The combined ether extracts were washed with H_2O (50 mL) and then brine (100 mL), dried ($MgSO_4$), and evaporated in vacuo to yield 7.29 g (25%) of **16** as a tan powder. Recrystallization from ether gave 6.11 g (21%) of **16** as beige crystals, mp 48–49 °C.

Pertinent spectral data for **16** are as follows: UV (0.1 M HCl) λ_{max} 235 nm (ϵ 10 300), (0.1 M Na_2HPO_4, pH 7.0) λ_{max} 235 nm (10 300); IR (KBr) 3.50, 6.22, 6.67, 7.21, 7.72, 7.99, 9.70, and 12.08 μ; ^{1}H NMR 2.16 (3 H, s), 3.74 (3 H, s), 6.84–7.58 (4 H, AA'BB'), and 10.50 (1 H, s); ^{13}C NMR 22.0, 55.1, 113.5, 122.2, 123.1, 134.8, 156.6, and 169.0; MS m/e (rel intensity) 181 (M^+, 7), 165 (1), 164 (1), 149 (3), 139 (37), 134 (5), 122 (100), 108 (29), 106 (22), 95 (40), 93 (5), 80 (25), 78 (38), 52 (21), 43 (68), and 42 (69). Anal. ($C_9H_{11}NO_3$) C, H, N.

Aqueous Stability of 4 and 13. A 2.0 mM solution of **4** or **13** was prepared by adding 2.0 mL of a 25 mM solution of **4** or **13** to 20 mL of buffer at 37 °C, diluting with additional buffer to 25.0 mL, and thermostating at 37 °C. Aliquots (1.0 mL) were removed periodically and added to 2.0 mL of 10% $FeCl_3$–0.7 M HCl and to 2.0 mL of 0.7 M HCl. The assay solutions were immediately vortexed and the absorbance was determined at 540 nm. The decomposition of **4** and **13** was followed to completion or for 6 h. The 100 mM buffers used were NaOH (pH 12.7), Na_2HPO_4 (pH 8.5, 8.0, 7.2, 7.0, and 6.6), AcONa (pH 5.0), and HCl (pH 1.5). The stock solutions were prepared in EtOH (**4**) and in H_2O (**13**). The absorbance at 540 nm due to **4** or **13** was determined by subtracting the absorbance of the $FeCl_3$ blank (1.0 mL of buffer and 2.0 mL of 10% $FeCl_3$–0.7 M HCl) and of the acid sample blank (1.0 mL of sample and 2.0 mL of 0.7 M HCl) from the absorbance of the $FeCl_3$ sample (1.0 mL of sample and 2.0 mL of 10% $FeCl_3$–0.7 M HCl) and of the acid blank (1.0 mL of buffer and 2.0 mL of 0.7 M HCl).

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Anionic Polymers and Biological Activities. Effects of Some New Polycarboxylic Acids on the Ascitic Sarcoma 180 of Mice

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Eleven new polymeric carboxylic acids with widely different solubilities in water have been synthesized. The activities of these polymers against the ascitic sarcoma 180 tumor of mice and their acute toxicities in mice have been compared with molecular parameters of the polymers such as molecular weights, charge densities, and abilities to complex calcium and magnesium ions. The maximum effectiveness of these polymers against ascitic sarcoma 180 of mice is greatest for those polymers having fewer carboxyl groups ionized at pH 7. Toxicities are lower for polymers having more carboxyl groups ionized at pH 7.

Synthetic polyelectrolytes often have drastic effects on biological systems.[1] Some polyacids are known to have antitumor[2] and antiviral[3] properties and the ability to induce the production of interferon in man and animals and the capacity to increase the immune response.[4] We hoped to increase these biological effects with new polymeric anions having structures that would optimize the chemical and physical properties of the polymers. Previous work has shown[2] that un-ionized water-soluble polymers such as poly(vinyl alcohol) have little effect on the growth rate of experimental tumors of animals, whereas polymers capable of ionizing at biological pH such as poly(acrylic acid) have significant antitumor activities. In this study we prepared polymers with varying numbers of carboxyl groups along the polymer chain in order to quantitate any relationship between the density of charges in the polymers and their antitumor activities. To accomplish this we prepared copolymers of (1) acrylic acid with isobutyl vinyl ether and (2) acrylic acid and itaconic acid or β-(*N*,*N*-dimethylamino)ethyl methacrylate. Since a possible mechanism of antitumor action of these polymers involves their interaction with calcium and magnesium ions, we also proposed to measure their abilities to complex calcium and magnesium ions and to correlate these properties with their antitumor activities.

Results and Discussion

Synthesis and Characterization of Polymers. Eleven new polymers containing carboxyl groups in their side chains have been synthesized and purified by methods given in the Experimental Section and in Table I. The five copolymers of acrylic acid and isobutyl vinyl ether presented special problems of synthesis. Acrylic acid is easily polymerized with free-radical initiators such as benzoyl peroxide. Pure isobutyl vinyl ether produces only oligomers with radical initiation[5] and requires the use of a cationic initiator such as aluminum acid sulfate to produce macromolecules. Therefore, a much greater portion of isobutyl vinyl ether was required in the reaction mixture in order to obtain the desired ratio of monomers in the copolymer; details are given in Table I.

Chemical compositions of the polymers, confirmed by chemical analysis and by NMR and infrared spectrometry, are shown in Table II. The NMR spectrum of poly(acrylic acid) (I) in D_2O shows three absorption peaks, a sharp singlet at δ 5.20 for the H–OD, a broad peak ca. δ 2.83 for the methine protons on the polymer chain, and a broad peak ca. δ 2.25 for the methylene proton on the polymer chain, all in agreement with the results of Sewell.[6] Integration of these peaks gave values of 1:1:2. The NMR spectrum of poly(isobutyl vinyl ether) (VII) in carbon tetrachloride shows a doublet at δ 0.91 with a coupling constant of 6 Hz. The absorption of other protons of VII all merge into two broad peaks around δ 3.41–3.09 and 1.69.

Polymer VIII, poly(itaconic acid), has an elemental analysis of C 40.66 and H 5.01, corresponding roughly to a formula of $C_5H_6O_4 \cdot 0.7H_2O$ for which the calculated values are C 42.08 and H 5.19. The NMR spectrum of VIII in D_2O shows the ratio of the H–OD to all other protons as 3.3:4.0; the corresponding ratio for the above formula is

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