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Depyrimidination of Synthetic Poly(uridylic acid) Analogue

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ABSTRACT: A poly(uridylic acid) analogue, poly{[1′-(β-uracil-1-yl)-5′-deoxy-D-*erythro*-pent-4′-enofuranose]-*alt*-[maleic acid]} (**3**), was synthesized by the alternating copolymerization of nucleoside derivative **1** and maleic anhydride and subsequent hydrolysis. *N*-glycosidic bonds of the polymer were hydrolyzed spontaneously to liberate uracil from the polymer backbone in a buffer solution (pH 7.4) at room temperature. The depyrimidination rate constant of the polymer at pH 7.4 at 80 °C was 8.2×10^{-5} s^{-1}, which was 10^4 times higher than that of the depyrimidination of DNA (1.2×10^{-9} s^{-1}) under the same condition. The activation energy for the depyrimidination was 16 kcal/mol, which was about half of that for the relevant nucleoside reactions. The increase in the depyrimidination rate was attributable to the high potential energy of the polymer caused by the crowded environment around the bases, so that the polymer was more susceptible to the hydrolysis. Because natural nucleic acids often have compact structures with a crowded environment around the bases by an intricate chain folding, the pyrimidination also may have been accelerated in a similar manner in the biological system. © 2000 John Wiley & Sons, Inc. J Polym Sci A: Polym Chem 38: 423–429, 2000
Keywords: depyrimidination; poly(uridylic acid) analogue; polynucleotide analogue

INTRODUCTION

The spontaneous depurination or depyrimidination of DNA, that is, the release of purine (adenine, guanine, and hypoxanthine) or pyrimidine (cytosine and thymine) bases from nucleic acids by hydrolysis of the *N*-glycosidic bond, gives rise to alterations of the cell genome.[1,2] From the rates of depurination measured *in vitro* with purified DNA at a temperature above 65 °C, the rate constant under biological conditions was calculated to be 3×10^{-11} s^{-1}; that is, a mammalian cell containing 3.86×10^9 nucleotides spontaneously loses 10,000 bases from its genome in 24 h.[3] The depyrimidination of denatured DNA *in vitro* at 80 °C and pH 7.4 also was found to be 1.2×10^{-9} s^{-1}. The spontaneous depurination or depyrimidination results uniquely from a thermal disruption of the *N*-glycosidic bonds. The apurinic sites resulting from depurination or depyrimidination are quite stable,[5] and cells have evolved mechanisms to repair these lesions.[6] Unrepaired apurinic sites have been shown to have two biological consequences, lethality[7,8] and base-substitution errors.[8]

Recently we reported the depurination of a synthetic poly(inosinic acid) analogue.[9] Its rate constant was 10^5 times higher than that of the depurination of DNA occurring in biological sys-

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Table I. Copolymerization Data of **1** with Maleic Anhydride with AIBN at 95°C for 24 h

Monomer **1**	MA[a]	DMF	Yield (%)	M_n[b]	PD[c]
0.4 g (1.28 mmol)	0.25 g (2.56 mmol)	0.3 mL	54	1,150	4.29
0.4 g (1.28 mmol)	0.25 g (2.56 mmol)	none	62	14,900	3.87

[a] Maleic anhydride.
[b] Number-average molecular weight of the hydrolyzed polymer (**3**) as measured by gel permeation chromatography with poly(ethylene glycol) standards.
[c] Polydispersity.

tems; this was attributed to the high potential energy of the polymer caused by the crowded environment around the bases. As depyrimidination is known to occur much more slowly than depurination,[3,4] it is of interest to investigate whether it takes place in a similar fashion on the same polymer backbone containing pyrimidine bases.

In this study, we synthesized a poly(uridylic acid) analogue and found that a spontaneous depyrimidination occurred whose rate constant was 8.2×10^{-5} s^{-1} at 80 °C and pH 7.4, corresponding to a rate acceleration of 10^4 compared to that of a heat-induced reaction of DNA. This result was quite surprising because the reaction did not occur in the corresponding monomeric systems or nucleosides. The potential energy of the polymer probably was increased because of the crowded environment around the bases, the polymer being more susceptible to hydrolysis. Similar steric effects could be assumed to exert themselves on the depyrimidination of natural nucleic acids. Here we report on the depyrimidination of synthetic poly(uridylic acid) analogues.

EXPERIMENTAL SECTION

Materials

Uridine, triphenyl phosphine, iodine, and 1,8-diazabicyclo[5,4,0]undec-7-ene were used as received. Maleic anhydride (MA) and azoisobutyronitrile (AIBN) were recrystallized from benzene and methanol, respectively. Acetic anhydride and dimethylformamide (DMF) were distilled before use. Monomer **1**, 1′-β-uracil-1-yl-2′,3′-di-*O*-acetyl-5′-deoxy-D-*erythro*-pent-4-enofuranose, was synthesized according to the literature (mp = 158–160 °C).[10]

^{1}H NMR ($CDCl_3$, δ): 2.12, 2.16 (ss, 6H, $2CH_3CO$), 4.49 (d, 1H, $H_{5'a}$, J = 3 Hz), 4.72 (d, 1H, $H_{5'b}$, J = 3 Hz), 5.38 (t, 1H, $H_{2'}$, J = 5 Hz), 5.84 (d, 1H, H_5, J = 8 Hz).

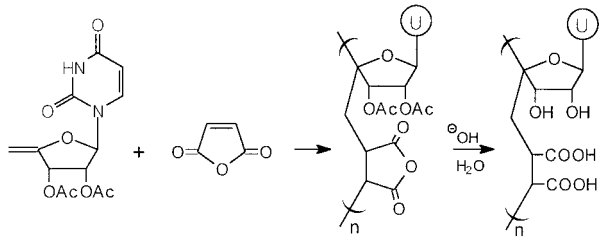
Copolymerization

Monomer **1** (0.4 g, 1.28 mmol) and MA (0.25 g, 2.56 mmol) were copolymerized in a DMF solution or in bulk with AIBN (0.063 g, 1 mol % of total amount of monomers) at 95 °C for 24 h. The polymerization products were dissolved in DMF and precipitated in ethyl ether to give poly[(1′-β-uracil-1-yl-2′,3′-di-*O*-acetyl-5′-deoxy-D-*erythro*-pent-4-enofuranose)-*alt*-(maleic anhydride)] (**2**). As the polymer of a higher molecular weight was obtained by the bulk polymerization in a high yield (Table I), it was used in this investigation hereafter.

^{1}H NMR (DMSO-d_6, δ): 1.0–2.4 (8H, CH_3CO, —CH_2—), 2.6–3.2 (2H, succinyl groups), 5.2–6.2 (4H, $H_{1'}$, $H_{2'}$, $H_{3'}$, H_5), 7.2–7.8 (1H, H_6), 11.2–11.7 (1H, NH). ^{13}C NMR (DMSO-d_6, δ): 19.5 (—CH_3), 35.4 ($C_{5'}$), 37.9 ($C_{6'}$, $C_{7'}$), 72.8 ($C_{2'}$, $C_{3'}$), 85.8 ($C_{4'}$), 90.4 ($C_{1'}$), 103.9 (C_5), 145.1 (C_6), 153.2 (C_2), 165.9 (C_4), 172.3 (acetyl C═O), 179.2 (anhydride C═O). IR (KBr): 3250, 1860, 1786, 1759, 1699, 1245, 1109, 947 cm^{-1}.

Polymer Reactions

2 (250 mg) was dissolved in 30 mL of 0.1 N NaOH and stirred at room temperature for 24 h. The reaction mixture was dialyzed through a cellulose membrane (Spectrum Medical Ind. Inc. MWCO-1000, Rancho Dominiquez, CA) with a constant flow of distilled water for 24 h. The polymer remaining in the membrane tube was freeze-dried to give poly[(1′-β-uracil-1-yl-5′-deoxy-D-*erythro*-pent-4-enofuranose)-*alt*-(maleic acid)] (**3**; yield = 79%). The uracilyl groups on the polymer chain were liberated during the hydrolysis. To measure the content of uracil remaining on the polymer, the hydrolyzed solution prior to dialysis was subjected to analysis by high-performance liquid chromatography (HPLC). Seventy-seven percent of the uracil remained on polymer **3**, which was used for the investigation of depyrimidination.



Scheme 1.

The number-average molecular weight was 14,900 with a polydispersity of 3.87 by gel permeation chromatography with poly(ethylene glycol) standards.

^{1}H NMR (D_2O, δ): 1.0–1.6 (2H, —CH_3—), 2.90 (2H, succinyl groups), 3.5 (1H, $H_{3'}$), 4.20 (1H, $H_{2'}$), 5.60–6.30 (2H, $H_{1'}$, H_5), 7.6–8.10 (1H, H_6). ^{13}C NMR (D_2O, δ): 40.8 ($C_{5'}$), 45.3 ($C_{6'}$, $C_{7'}$), 76.2 ($C_{2'}$, $C_{3'}$), 91.2 ($C_{1'}$,$C_{4'}$), 105.4 (C_5), 146.1 (C_6), 155.2 (C_2), 168.9 (C_4), 187.1 (acid C═O). IR (KBr): 3432, 3253, 1699, 1591, 1396, 562 cm^{-1}.

Measurement of Depyrimidination

The polymers (2.4 mg) were dissolved in buffer solutions (20 mL) at an ionic strength of 0.02 (KCl) that were kept at definite temperatures (±0.1 °C) controlled by a thermostat. The reaction mixture was analyzed by HPLC on an ultrahydrogel column in different reaction times. The buffer materials for different pHs were citrate (pH 4–6), Tris buffer (pH 7–9), and carbonate (pH 10–11).

^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Gemini 200 (Palo Alto, CA) spectrometer. IR spectra were obtained with a Nicolet (Madison, WI) Magna-IRTM 550 spectrometer. Fluorescence spectra were recorded on a Kontron Instrument SFM25 fluorescence spectrophotometer. Circular dichroism (CD) curves were measured on a Jasco J-710 (Tokyo, Japan) spectropolarimeter.

RESULTS AND DISCUSSION

Radical copolymerization of **1** with MA resulted in the alternating copolymer **2** (Scheme 1) because neither **1** nor MA was homopolymerized under the same reaction conditions, and the copolymerization of vinyl ethers with MA is known to give alternating copolymers.[9,11] The anhydride groups incorporated into polymer **2** were 50.5 mol % by titration with sodium methoxide.[12] The polymerization results are given in Table I. The ^{1}H NMR spectrum of polymer **2** is shown in Figure 1. The peaks of the double-bond protons (δ = 4.52 and 5.09) in the dihydrofuran ring of monomer **1** disappeared. The simple hydrolysis of polymer **2** in 0.1 N aqueous NaOH resulted in polymer **3.** We monitored the completion of the reaction by following the disappearance of the carbonyl IR peaks at 1860 and 1786 cm^{-1} of the anhydride groups and the proton signal at δ = 1.0 − 2.4 ppm for the acetyl groups in the ^{1}H NMR spectrum of polymer **2**. Because 23% of the uracil on the poly-

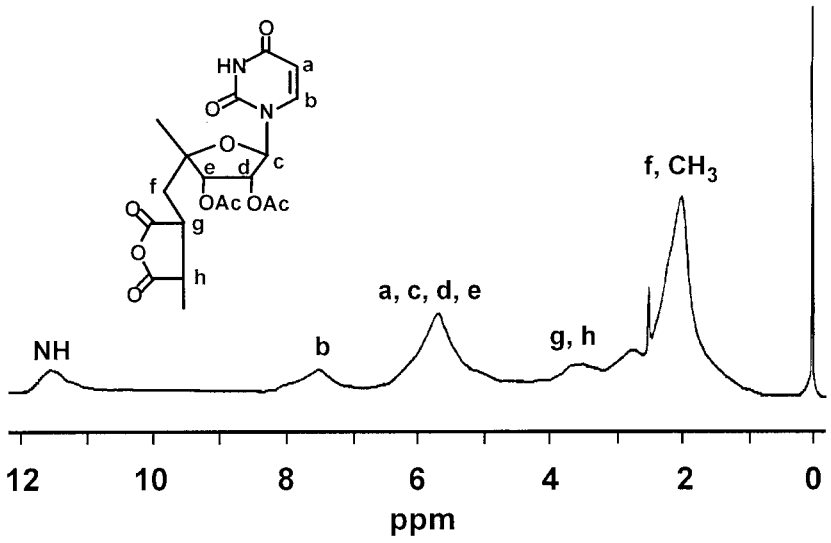
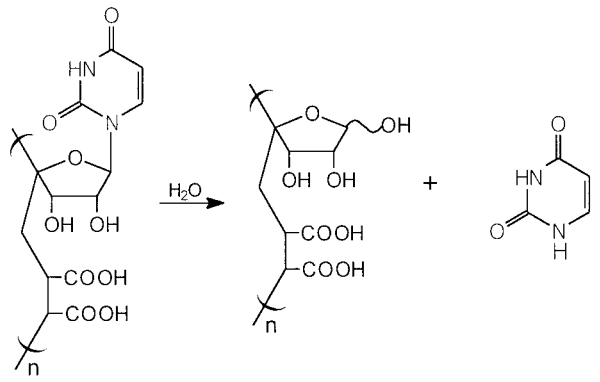


Figure 1. ^{1}H NMR spectrum of polymer **2** in DMSO-d_6.



Scheme 2.

mer was eliminated during the hydrolysis and purification, polymer **3**, used for further investigation, contained 77% uracil.

Because polymer **3** was an alternating copolymer of nucleoside derivatives with 1,2-dicarboxytrimethylene and contained hydroxyl groups on the 2′ position of the furanosyl rings, its structure was analogous to poly(uridylic acid). The polymer was soluble in water. The chiral atoms on C1′, C2′, and C3′ of uridine were intact during the synthesis of the monomer, the copolymerization, and the hydrolysis, and polymer **3** was optically active, which allowed the used of CD for the investigation of the polymer conformations in aqueous solutions.

When polymer **3** was dissolved in a buffer solution (pH 7.4) above 30 °C, the *N*-glycosidic bonds of the polymer were hydrolyzed spontaneously to liberate uracil from the polymer backbone (Scheme 2), which was measured by HPLC. The rates of depyrimidination at different temperatures are shown in Figure 2. The reaction at the beginning was very fast at higher temperatures (95 and 80 °C) and slow at a lower temperature (37 °C). When about 50% of the uracil was eliminated, no more depyrimidination occurred.

To determine the rate constants, the logarithmic concentrations of the uracil remaining on the polymer chain were plotted against time (Fig. 3), which obeyed the first-order kinetics at the initial stages of the reactions. The initial rate constants at different temperatures are given in Table II. The rate constant at 80 °C was 10^4 times higher than that of the heat-induced depyrimidination of DNA under the same conditions.[4]

The initial rate constants of the depyrimidination, measured in buffer solutions of different pHs at 60 °C, are plotted in Figure 4. The rate constants increased with increasing pH when the pH

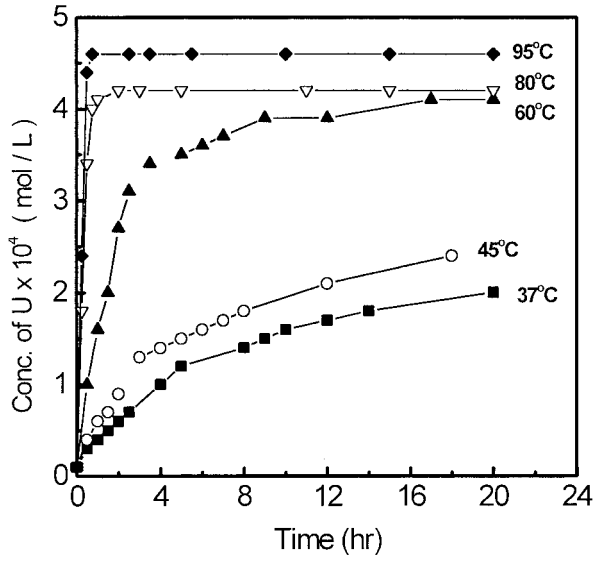


Figure 2. Released uracil as a function of time at pH 7.4 and different temperatures.

was less than 5, no significant changes were observed in the pH range of 5 to 9.5, and the constants increased again at pHs higher than 9.5. This was quite different from the acid-catalyzed hydrolysis of deoxyribonucleosides.[13,14] Consequently, the mechanism of the depyrimidination of polymer **3** was different from that of the acid-catalyzed hydrolysis of nucleosides.

As the pH increased, we expected carboxyl groups of **3** to be converted into carboxylate

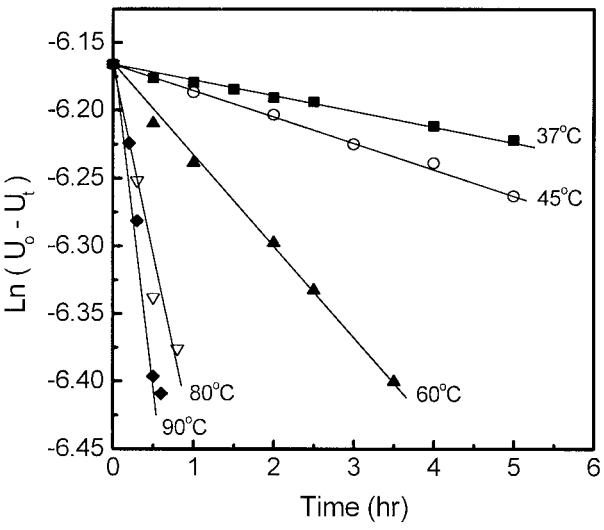


Figure 3. Logarithmic concentrations of the uracil remaining on the polymer chain [$Ln(U_o - U_t)$] vs the time at pH 7.4 and different temperatures.

Table II. Depyrimidination Rate Constants at pH 7.4 and Different Temperatures

Temperature (°C)	$k \times 10^5$ (s^{-1})	$k \times 10^9$ (s^{-1})[a]
37	0.23	—
45	0.56	—
60	2.28	—
80	8.22	1.2
95	12.30	23.0

[a] Depyrimidination rate constants of denatured DNA *in vitro*.[4]

groups at pH 4.75 and ^{3}NH imide groups to be converted into imide anions at pH 9.65, the pKa of ^{3}NH of 1N-alkylated uracil derivatives (Scheme 3).[15] Accordingly, electrostatic repulsions between the anions would increase drastically at pH 4.75 as well as 9.65, give the polymer more extended conformations, as observed in the polyelectrolytes, exerting more strain on the polymer chains, and make the hydrolysis easier. The evidence of the conformational change of polymer **3** at different pHs was obtained in the CD curves (Fig. 5), which showed quite different maximum wave lengths and intensities for the peaks and troughs at pH 3.5, 7.4, and 11.

To obtain the activation energy (Ea) of the depyrimidination, the Arrhenius relationship was plotted. The Ea was found to be 16.0 kcal/mol, which was about 15 kcal/mol lower than that for the hydrolysis of pyrimidine nucleosides (31–34

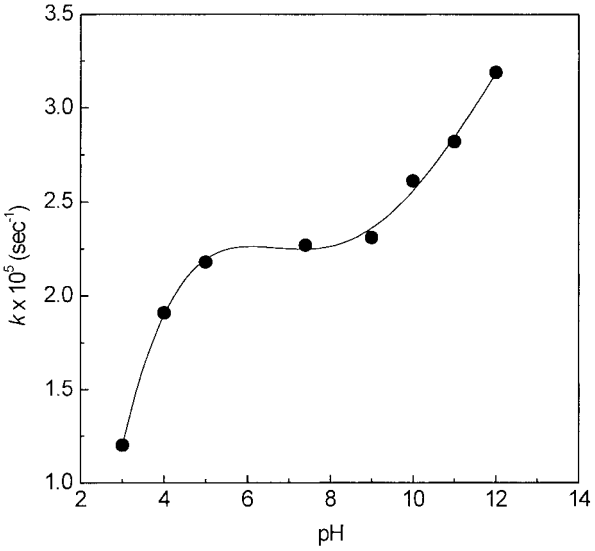
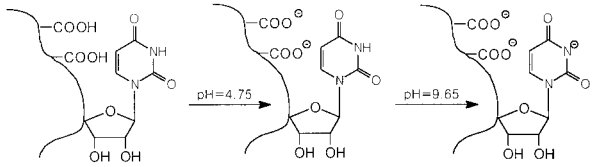


Figure 4. Rate constants as a function of pH at 60 °C.



Scheme 3.

kcal/mol).[16,17] This result was ascribed to the crowded environment around the uracil groups, where the ground state potential energy of the *N*-glycosidic bond in polymer **3** was elevated more than that of the transition state.

The fully extended distance between the adjacent riboses (from C4′ to C4″) of polymer **3** was calculated to be 4.96 Å, which was much shorter than that (6.19 Å) of nucleic acids. Using a simple energy minimization calculation,[18] we attempted to estimate the conformations of polymer **3** containing five repeating units. One of the probable conformations of the lowest energy for the polymer is shown in Figure 6. The uracilyl groups were stacked one upon the other with small dihedral angles between them. This conformation was supported by the fact that the uracilyl groups on the polymer chains formed excimers in an aqueous solution (Fig. 7). Excimers are observed in

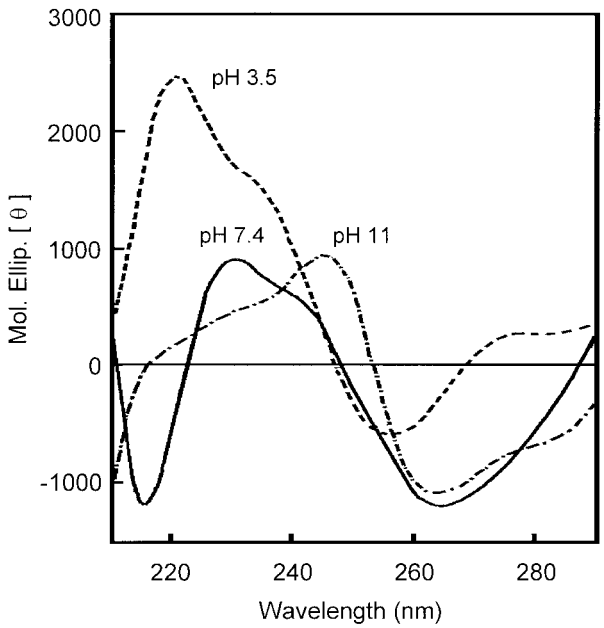


Figure 5. CD curves of polymer **3** at different pHs ([polymer] = 1×10^{-4} residue mol/L). The buffer materials were citrate (pH 3.5), Tris buffer (pH 7.4), and carbonate (pH 11).

bichromophoric molecules, where the aromatic chromophores are separated by a three-atom linkage, and they form a complex in which two aromatic rings overlap in a sandwich-like arrangement.[19,20] When these geometrical requirements are satisfied for the pendant chromophores on the polymer chains, the polymer shows an excimer fluorescence as observed in poly(vinyl aromatic)s[20,21] and the polymers containing chromophoric pendant groups.[22] As shown in Figure 7, uridine showed a broad peak at 330 nm with a very low intensity, whereas polymer **3** gave a typical excimer fluorescence with a very high intensity at 430 nm; that is, it was redshifted relative to the emission band from uridine and was devoid of vibrational structures.

Because the hydrolysis of the *N*-glycosidic bond did not occur in the mixture of uridine and succinic acid under the same conditions, the depyrimidination can be explained by steric effects of the polymer structure. The environment of the bases was so crowded that a definite amount of them were forced sterically to be substituted by small groups (OH) to release the strain of the polymer chains, as observed in the case of steric acceleration in organic reactions.[23]

In conclusion, depyrimidination was observed in poly(uridylic acid) analogues. The *N*-glycosidic bonds on the polymer chains were hydrolyzed 10^4 times faster than those on the nucleosides because of steric assistance, something rarely found in polymer chemistry. The reaction took place in a way that released steric strain. Because most biological reactions with natural nucleic acids occur in a semisolid state, they often have compact structures with a crowded environment around the bases via an intricate chain folding, where we speculate that depyrimidination also may be accelerated in a similar manner.

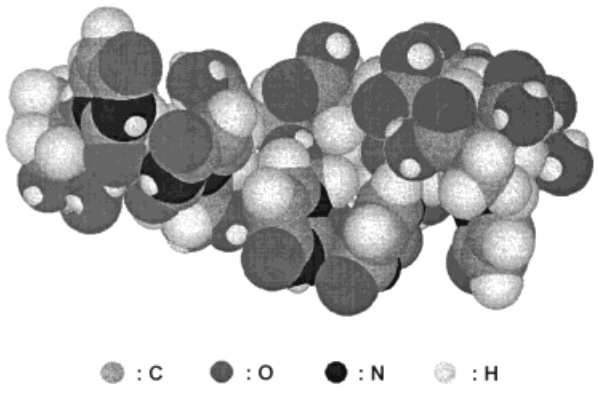


Figure 6. Space-filling model of polymer **3** with the minimal energy.

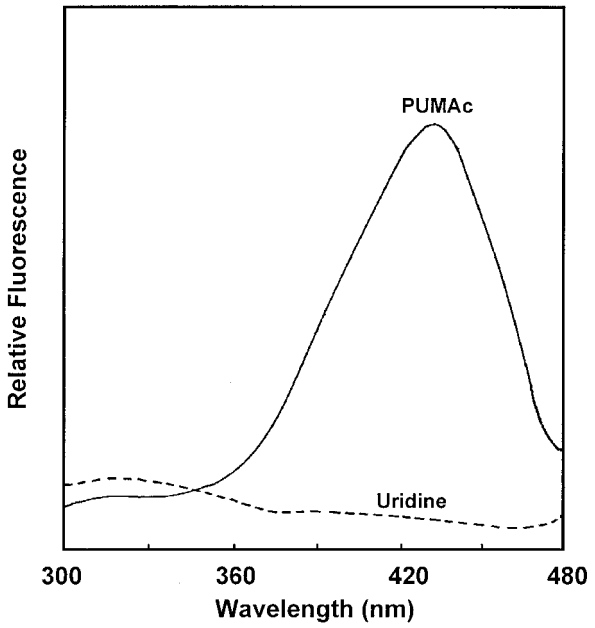


Figure 7. Fluorescence spectra of uridine and polymer **3** in H_2O. [uridine] = [polymer] = 1×10^{-5} residue mol/L.

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