Antimicrobial Effect of Monomers and Polymers with Azole Moieties

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Received 15 July 2002; accepted 27 January 2003

ABSTRACT: Five monomers with azole moieties were synthesized and their antimicrobial activities were investigated. The antimicrobial activity of the monomers was evaluated by the halo zone test method. The results strongly depended on the chemical structure of the group attached to the azole moieties. Polymers with (benzimidazol-2-yl)thio groups and with (5-methyl-1,3,4-thiadiazol-2-yl)thio groups were synthesized. The shake flask test showed that the two polymers possessed excellent antimicrobial activity. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 90: 2933–2937, 2003

Key words: bioengineering; biomaterials; functionalized polymers

INTRODUCTION

Some azole compounds constitute versatile and valuable sources of antimicrobial agents. They appear to transcend the chemotherapeutic boundaries of other antiparasitic drugs with a spectrum of activity that includes the majority of fungi, as well as many bacteria, protozoa, and even helminthic species.

Paulus made a good review on toxophoric groups and their mechanism of activity.[1,2] Denyer[3] tabulated and classified various biocides according to their target regions in microbial cells.

Polymers with antimicrobial activity are often required for food packaging, sanitary, or medical applications. Those materials are usually prepared by the formulation of polymers with side groups having antimicrobial activity, which can be cleaved from the polymer matrix.

Kanazawa et al.[4–6] synthesized several polymeric phosphonium salts by way of radical polymerization,[7] surface grafting,[8] or condensation polymerization.[9] All of them exhibited high antibacterial activity.

Polymers with pendant biguanide groups for antibacterial agents[10] or *N*-sulfenyl derivatives[11] for antifungal agents were prepared via radical polymerization. The minimum growth inhibition concentration of the polymeric *N*-sulfenyl derivatives was 4 times higher than that of the corresponding monomers.[11]

Pentachlorophenyl acrylate was synthesized and copolymerized with both vinyl acetate and ethyl acrylate. The resulting polymer retarded or prevented growth of *Aspergillus* sp., *Pseudomonas* sp., *Alternaria* sp., and *Aureobasidium pullulans*.[12]

Some polymers containing an antimicrobial pharmacophore can be prepared by the chemical anchoring of the pharmacophore to polymers. The polymers having phenolic hydroxy moieties were prepared by the reaction of amine-functionalized copolymers with *p*-hydroxybenzoic acid, 2,4-dihydroxybenzoic acid, and 3,4,5-trihydroxybenzoic acid. The antibacterial activity of the polymers increased in the order of the increasing number of hydroxy groups.[13]

Methyl 2-benzimidazolecarbamate (carbendazim) is known to inhibit the growth of fungi very effectively. Park et al. introduced a 2-benzimidazolecarbamoyl moiety to poly(ethylene-*co*-vinyl alcohol), imbuing the polymer with antifungal activity.[14]

In this report, vinyl or acryl monomers with some azole moieties were synthesized. Some azole moieties were either polymerized or chemically bound to a polymer matrix. The antimicrobial activity of the monomers and the modified polymers was examined.

EXPERIMENTAL

Materials

Bis(2-methylindenlyl)zirconium dichloride [$(2\text{-MeInd})_2\text{-}ZrCl_2$] was prepared and recrystallized by precipitation in CH_2Cl_2 after reacting lithium salt of 2-methylindene

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Contract grant sponsor: KOSEF; contract grant number: R01-2002-000-00146-0.

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TABLE I
Characteristics of Polymers

Sample	Oxidizer	Yield (g)	T_m[a] (°C)	T_c[a] (°C)	ΔH_f[a] (J/g)	Epoxidation (%)	M_w ($\times 10^{-3}$)	M_w/M_n
PEOD	—	—	108.7	93.3	33.6	—	19.4	5.7
PEOD[b]	Peracetic acid	1.63	112.4	98.5	9.4	57	24.2	5.7

[a] Obtained from the second scan DSC thermograms.
[b] After the epoxidation.

with $ZrCl_4 \cdot THF$ in a toluene medium. Methyl aluminoxane (MAO; Akzo Chemical Co., modified methyl aluminoxane (MMAO)-4 type, 11.6 wt % Al in toluene and MMAO-3A type, 8.4 wt % Al in toluene) was used without further purification. The purity of the ethylene gas was more than 99.5%, and a trace of impurities was removed by passing the gas successively through $CaCl_2$ and $CaSO_4$.

2-Mercaptobenzimidazole (Aldrich), triethylamine (Aldrich), 3-bromo-1-propanol (Aldrich), and allyl bromide (Aldrich) were reagent grade and were used as received. Toluene was refluxed for over 8 h in the presence of sodium and benzophenone and used after the second distillation. 1,7-Octadiene (Aldrich) and 1,4-dioxane (Junsei) were purified by vacuum distillation.

Copolymerization of ethylene/1,7-octadiene

The copolymerization reactions were carried out in a 500-mL glass reactor with a magnetic stirrer. One hundred milliliters of toluene was used as a reaction medium. Ethylene gas was first introduced at 1 atm and 1,7-octadiene was added at different concentrations. The copolymerization was initiated by the addition of MAO and $(2\text{-MeInd})_2ZrCl_2$ catalyst (5.9×10^{-5} *M*). The ratio of the concentration of the cocatalyst to that of the catatlyst ([Al]/[Zr]) was maintained at 3000. The reaction time was 1 h, and the reaction was terminated by adding methanolic HCl. The products were washed 3 times with plenty of methanol and dried in a vacuum oven (30°C) until constant weight was attained. The characteristics of the resulting poly(ethylene-*co*-1,7-octadiene) (PEOD) are demonstrated in Table I.

Instrumentation

The molecular weight and its distribution were measured using gel permeation chromatography (model 150C, Waters, Milford, MA) with 1,2,4-trichlorobenzene as an eluent at 1.0 mL/min and 135°C. A column with a porosity of 10 μm (Styragel® HT6E, HT5, HT3) was employed with polystyrene (Showadenko SL-105) as a standard.

Polymeric biocides were characterized by ^{1}H-NMR spectra recorded at 110°C on a Fourier transform NMR spectrometer (AC-250, Bruker Instruments). Ten milligrams of the copolymer was dissolved in 0.5 mL of 1,2-dichlorobenzene-d_4 (20% w/v) and was subjected to the ^{1}H-NMR measurements.

The thermal properties of the polymers were determined by differential scanning calorimetry (DSC, Perkin–Elmer DSC 7). The thermal history of the products was removed by scanning to 200°C with a heating rate of 20°C/min (first scan). After cooling the sample at a rate of −5°C/min to room temperature, it was reheated at 20°C/min to 200°C and the second scan DSC thermograms were obtained.

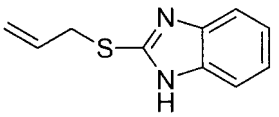
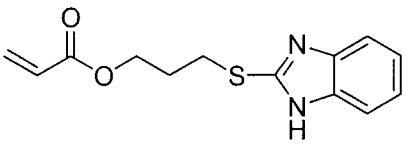
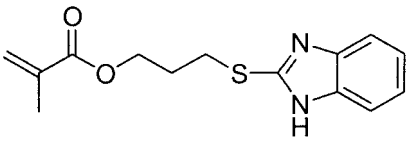
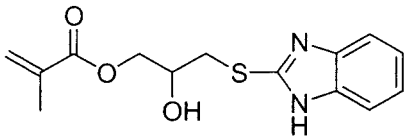
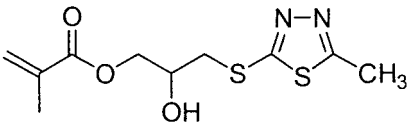
Synthesis of 2-allylthiobenzimidazole (AZ-1)

Allyl bromide (8.06 g, 66.6 mmol) was added dropwise into a solution of 2-mercaptobenzimidazole (10.0 g, 66.6 mmol) in 1,4-dioxane (50 mL) at 5°C. After refluxing for 4 h, the reaction mixture was cooled and the solid was filtered. The solid was treated with 0.10*N* NaOH and the mixture was extracted with ethyl acetate (50 mL ×3). The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated to give a solid (11.6 g, 91%; Table II). ^{1}H-NMR ($CDCl_3$, 200 MHz, δ): 3.95 (d, 2H, CH_2), 5.12–5.36 (m, 2H, C═CH_2), 5.92–6.12 (m, 1H, C—CH═C), 7.16–7.23 (m, 2H, aromatic), 7.43–7.57 (m, 2H, aromatic); IR (cm^{-1}): 2963, 1632, 1511, 1441, 1272, 1234.

Synthesis of 3-(1H-benzimidazol-2-yl)thiopropane-1-ol

A mixture of 2-mercaptobenzimidazole (1.00 g, 5.98 mmol), 1,4-dioxane (5 mL), triethylamine (0.665 g, 6.58 mmol), and 3-bromo-1-propanol (0.91 g, 6.57 mmol) was refluxed overnight. The organic layer was washed with water and brine, dried over $MgSO_4$, and concentrated *in vacuo*. The oily residue was purified by column chromatography on silica gel (EtOAc/methylene chloride = 1:19) to give of 3-(1H-benzimidazol-2-yl)-thiopropane-1-ol (0.38 g, 31.7%) as a colorless oil. ^{1}H-NMR (DMSO-d_6, 200 MHz, δ): 1.85 (m, 2H), 3.49 (t, 2H), 3.53 (t, 2H), 7.41–7.46 (m, 2H), 7.63–7.69 (m, 2H); ^{13}C-NMR (DMSO-d_6, 60 MHz, δ): 27.8, 30.2, 57.1, 111.5, 123.4, 131.2, 149.4; IR (cm^{-1}): 3259, 1616, 1514, 1451, 1204, 1062.

TABLE II
Structure of Monomeric Biocide

Monomeric Biocides	Structure
AZ-1	
AZ-2	
AZ-3	
AZ-4	
AZ-5	

Synthesis of 3-(1H-benzimidazol-2-yl)thiopropyl acrylate (AZ-2)

Acryloyl chloride (1.13 g, 12.4 mmol) was added dropwise to a mixture of 3-(1H-benzimidazol-2-yl)thiopropane-1-ol (2.00 g, 8.88 mmol), DMF (20 mL), and triethylamine (2.24 g, 22.2 mmol) at 0°C. After stirring overnight at 50°C, the organic layer was washed with water and brine, dried over $MgSO_4$, and concentrated *in vacuo*. The oily residue was purified by column chromatography on silica gel (EtOAc/methylene chloride = 1:19) to give AZ-2 (0.98 g, 42.8%) as a colorless oil (Table II). ^{1}H-NMR ($CDCl_3$, 200 MHz, δ): 2.05–2.20 (m, 2H), 3.38 (t, 2H), 4.25 (t, 2H), 5.75–5.80 (d, H), 5.97–6.11 (m, 1H), 6.31–6.41 (d, 1H), 7.15–7.20 (m, 2H), 7.47–7.53 (m, 2H); ^{13}C-NMR ($CDCl_3$, 60 MHz, δ): 28.6, 29.1, 62.7, 114.0, 122.3, 128.0, 131.1, 139.2, 149.9, 166.2.

Synthesis of 3-(1H-benzimidazol-2-yl)thiopropyl methacrylate (AZ-3)

The AZ-3 was prepared as a white solid by the same method described above for AZ-2 (Table II). ^{1}H-NMR ($CDCl_3$, 200 MHz, δ): 1.92 (s, 3H, CH_3), 2.09–2.22 (m, 2H, CH_2), 3.40 (t, 2H, CH_2), 4.27 (t, 2H, CH_2), 5.54 (s, 1H, C═CH_2), 6.09 (s, 1H, C═CH_2), 7.14–7.25 (m, 2H, aromatic), 7.50 (bs, 2H, aromatic), 10.80 (bs, 1H, imidazole—NH); ^{13}C-NMR ($CDCl_3$, 60 MHz, δ): 18.2, 28.6, 29.2, 62.9, 122.2, 125.9, 136.0, 149.8, 167.5.

Synthesis of 2-hydroxy-3-(3H-benzimidazol-2-yl)thiopropyl methacrylate (AZ-4)

To a solution of 2-mercaptobenzimidazole (3.0 g, 20.0 mmol) in 1,4-dioxane (50 mL) was added glycidyl methacrylate (GMA, 3.69 g, 30.0 mmol). After stirring for 5 h at 90°C, the reaction mixture was cooled to room temperature. Then the mixture was concentrated in *vacuo* and recrystallized in methylene chloride. A solid was filtered and dried *in vacuo* to give AZ-4 (5.73 g, 98.1%) as a white solid (Table II). ^{1}H-NMR ($CDCl_3$, 200 MHz, δ): 1.89 (s, 3H, CH_3), 2.25 (s, 3H), 2.71 (s, 3H), 3.25–3.44 (m, 2H), 4.08–4.35 (m, 3H), 5.52 (s, 1H), 6.08 (s, 1H), 6.92–7.48 (m, 4H); IR (cm^{-1}): 3114, 2912, 1739, 1724, 1294, 1174, 1019.

Synthesis of 2-hydroxy-3-(5-methyl-1,3,4-thiadiazol-2-yl)thiopropyl methacrylate (AZ-5)

AZ-5 was prepared from 2-mercapto-5-methyl-1,3,4-thiadiazole using the same method as AZ-4 (Table II). ^{1}H-NMR ($CDCl_3$, 200 MHz, δ): 1.93 (s, 3H, CH_3), 2.70 (s, 3H, CH_3), 3.41–3.58 (m, 2H), 4.25 (d, 2H), 5.58 (s, 1H, C═CH_2), 6.12 (s, 1H, C═CH_2); ^{13}C-NMR ($CDCl_3$, 60 MHz, δ): 15.5, 18.2, 37.3, 66.7, 68.9, 126.1, 135.7, 165.4, 165.9, 167.1; IR (cm^{-1}): 3441, 2951, 1724, 1643, 1382, 1301, 1173.

Epoxidation of PEOD

The EOD copolymer (2 g) was dissolved in 1,2-dichloroethane/1,2,4-trichlorobenzene (50 mL, 5:1 mixture) and then peracetic acid (3 mL) was added. The epoxidation was carried out at 27°C for 48 h. The product was precipitated into methanol and dried *in vacuo.*

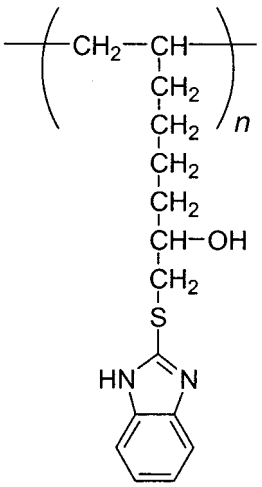
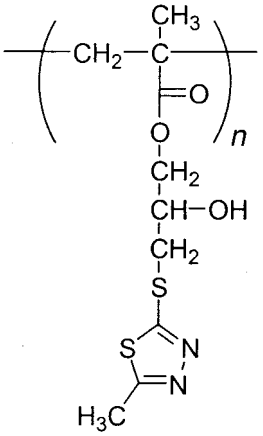
Synthesis of PEOD with thiobenzimidazoyl groups (PZ-1)

The epoxided PEOD (2 g) was dissolved in 1,2-dichloroethane (50 mL) and then 2-mercaptobenzimidazole (0.5 g) was added. The reaction was carried out at 100°C for 48 h. The product was precipitated into methanol and dried *in vacuo* followed by Soxhlet extraction with boiling acetone for 1 day to remove unreacted 2-mercaptobenzimidazole (Table III).

Synthesis of poly(2-hydroxy-3-(5-methyl-1,3,4-thiadiazol-2-yl)thiopropyl methacrylate) (PZ-2)

Solution polymerization of AZ-5 was performed in the presence of benzoyl peroxide (1 wt % based on AZ-5) using 1,4-dioxane as a medium at 80°C for 24 h. The mixture was precipitated with an excess of methanol, filtrated, and dried *in vacuo* (Table III).

TABLE III
Structure of Polymeric Biocides

PZ-1	PZ-2
	

Shake flask method

The number of bacterial cells in the bacteria culture suspension was about 5.0×10^5/mL. After their contact with polymer in diluted phosphate buffered saline, the suspension were incubated at 37°C for 24 h and the number of the bacterial cells was calculated by multiplying the number of colonies by the dilution factor.[15]

Halo zone test

Halo zone tests for microorganisms against the biocides were carried out according to the method of Bauer et al.[16] under strict adherence to National Committee for Clinical Laboratory Standards. The fungi (*Aspergillus fumigatus* IFO 30870 or *Penicillium pinophilum* ATCC 9644) were incubated on potato dextros agar slant medium at 28°C for 72 h. Spore suspension was prepared with 10 mL of sterile distilled water. The final concentrations of the spore suspension ranged from 10^3 to 10^5 spores/mL. The biocides dissolved in DMSO were spread on paper disks and exposed to UV light for 1 h. Agar plates were inoculated using a sterile cotton swab moistened with the spore suspension. A paper disk containing the biocidal agent was placed in the middle of the plate. The disks were pressed firmly on the agar surface. Then, all the test plates were incubated at 28°C for 72 h.

The bacteria (*Staphylococcus aureus* ATCC 6538P or *Pseuodomonas aeruginosa* ATCC 15522) were subcultured to nutrient agar and incubated overnight at 37°C. The bacteria (stationary growth phase) were inoculated in 50 mL of nutrient broth medium in a 250-mL Erlenmeyer flask and cultured for 5 h at 37°C. Then, the cells were suspended in the same medium to produce a suspension of 10^6 cfu/mL. Agar plates were streaked with a sterile swab moistened with the bacterial suspension. Biocides were dissolved in DMSO and spread on disks made of filter paper. The disks were exposed to UV for 1 h and then were aseptically applied to the surface of the agar plate. All the test plates were incubated overnight at 37°C. The susceptibility of microorganisms to the biocides was determined by the size of the growth of the inhibitory zone.

TABLE IV
Antifungal Activity of Monomers Measured by Halo Zone Test (mm)

		Concentration of Biocide (wt % in DMSO)				
Biocide	Strain	40%	20%	10%	5%	1%
AZ-1	*P. pinophilium*	0	0	0	0	0
	A. fumigatus	0	0	0	0	0
AZ-2	*P. pinophilium*	21	20	18	15	0
	A. fumigatus	21	21	21	21	0
AZ-3	*P. pinophilium*	16	16	14	15	0
	A. fumigatus	14	17	13	11	0
AZ-4	*P. pinophilium*	0	0	0	0	0
	A. fumigatus	0	0	0	0	0
AZ-5	*P. pinophilium*	40	33	0	0	0
	A. fumigatus	23	10	0	0	0

RESULTS AND DISCUSSION

Benzimidazole derivatives have been known to possess antibacterial and antifungal activity and are proposed to inhibit the cytochrome P-450 monooxygenase (lanosterol 14α-demethylase), a key enzyme in fungal ergosterol biosynthesis.[7,8]

The reaction of 2-mercaptobenzimidazole with allyl bromide gave AZ-1. The vinyl monomer containing the benzimidazole moiety had neither antifungal nor antibacterial activity, judging from the result of the halo zone test as demonstruted in Tables IV and V. When acryloyl chloride was treated with 2-mercapto-

TABLE V
Antibacterial Activity of Monomers Measured by Halo Zone Test (mm)

		Concentration of Biocide (wt % in DMSO)				
Biocide	Strain	40%	20%	10%	5%	1%
AZ-1	*P. aeruginosa*	0	0	0	0	0
	S. aureus	0	0	0	0	0
AZ-2	*P. aeruginosa*	17	16	15	14	0
	S. aureus	18	18	18	15	0
AZ-3	*P. aeruginosa*	0	0	0	0	0
	S. aureus	0	0	0	0	0
AZ-4	*P. aeruginosa*	17	16	13	0	0
	S. aureus	0	0	0	0	0
AZ-5	*P. aeruginosa*	13	11	0	0	0
	S. aureus	0	0	0	0	0

benzimidazole, the characteristic peaks of the acryloyl group were not found on the ^{1}H-NMR spectrum. The reaction of 2-mercaptobenzimidazole with 3-bromo-1-propanol gave 2-(3-hydroxypropyl)mercapto benzimidazole. The product was treated with acryloyl chloride or methacryloyl chloride to give acryl monomer (AZ-2), and in the halo zone test AZ-2 gave some positive effect against fungi, as well as bacteria. In contrast, the methacryl monomer (AZ-3) did not show antibacterial activity but exhibited some antifungal activity.

GMA was reacted with 2-mercaptobenzimidazole and 2-mercapto-5-methylthiadiazole to obtain the methacryl monomers AZ-4 and AZ-5, respectively. AZ-5 showed both antifungal and antibacterial activity whereas AZ-4 was active only against bacteria. Adding a methyl group at the Cα position of the acryl monomers did not show any considerable effect on the halo zone test results against fungi (AZ-2 compared with AZ-3), but the halo zone diameter against bacteria by AZ-2 was much larger than that of AZ-3. Introduction of a hydroxy group had a significant effect on the antifungal activity (AZ-3 and AZ-4).

The size of the inhibition zone in the halo zone test depends not only on the intrinsic antimicrobial activity but also on the diffusion rate of the antimicrobial agent.

AZ-2, AZ-3, and AZ-4 have almost the same molecular weight. Introduction of a methyl group (AZ-3) decreases the hydrophilicity of AZ-2, but introducing a hydroxy group (AZ-4) increases it. Because the molecular transport rate of AZ-2, AZ-3, and AZ-5 should not be very different from each other, the results in Table IV and Table V show that both the increase in the hydrophobicity and the increase in the hydrophilicity reduced the intrinsic antimicrobial activity of AZ-2. Russel[17] reported that hydrophilic molecules with a molecular weight of less than 600 can readily enter Gram-negative cells via the aqueous porins. Hydrophobic molecules, in contrast, diffuse across the membrane bilayer.

Polymerization of all the monomers was attempted. However, only the polymerization of AZ-5 was successful in giving a corresponding polymer (PZ-2). Because PZ-2 was not soluble in DMSO, the antibacterial activity of the polymer could not be compared with that of the corresponding monomer by the halo zone test. The shake flask test method was employed for the antibacterial activity of PZ-2 against *Escherichia coli*. As shown in Table VI, the viable cell of *E. coli* disappeared almost completely. It is interesting to observe that the shake flask test indicated PZ-2 to be an effective antibaterial agent while from the halo zone test reflected that the corresponding monomer (AZ-5) should be classified as a poorly active antibacterial agent. Therefore, it can be said that several test methods should be employed at the same time to have a definite conclusion on the activity of antimicrobial agents.

TABLE VI
Antibacterial Activity of PZ-1 and PZ-2 by Shake Flask Test

Bacteria	Sample	Bacteria/mL (24-h contact) (cfu)	Reduction (%)
S. aureus	Blank	1.23×10^{11}	—
	PZ-1	1.80×10^{6}	99.9
P. aeruginosa	Blank	1.52×10^{11}	—
	PZ-1	2.38×10^{8}	99.8
E. coli	Blank	8.60×10^{8}	—
	PZ-2	0	100

PEOD was epoxidized and then reacted with 2-mercaptobenzimidazole to give an imidazole polymer (PZ-1). The resulting polymer was hardly soluble in solvents and its antimicrobial activity was checked against *S. aureus* and *P. aeruginosa* by the shake flask test.

As was the case for PZ-2, PZ-1 effectively reduced the viable cell number of *S. aureus* and *P. aeruginsa* in spite of the fact that AZ-3 did not yield any clear zone in the halo zone test against the two bacteria.

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