SYNTHESIS AND CHARACTERIZATION OF NEW HETEROCYCLIC COMPOUNDS WITH POTENTIAL ANTITUBERCULOSIS ACTIVITY AND THEIR IMMOBILIZATION ON POLYMER SUPPORTS

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New biologically active compounds, obtained by synthesizing new hydrazides derived from benzoxazolyl-2-mercaptoformic acid, respectively benzoxazolyl-2-mercaptoacetic acid, have been chemically immobilized onto poly(maleic anhydride-*alt*-vinyl acetate) supports, forming drug-polymer conjugates with controlled delivery. The reaction products were characterized by elemental and spectral analyses (FT-IR, ^{1}H-NMR). Toxicological and tuberculostatic activity tests recommend certain reaction products as therapeutic candidates with pharmacological application.

Keywords: 2-mercapto-benzoxazole derivatives, hydrazides, drug–polymer conjugates, tuberculosis inhibitors

INTRODUCTION

The main aim of this paper was to prepare biologically active principles – polymer systems with drug delivery characteristics. A copolymer of maleic anhydride with vinyl acetate has been selected as the macromolecular compound, due to its ability to open the anhydride ring by reaction with bioactive compounds containing amino groups. Copolymers of maleic anhydride, especially with vinyl acetate,[1-3] are raising increasing interest, due to their numerous applications in industry, medicine and pharmacy. Literature describes this polymeric support as usually associated through chemical bonding, with low molecular weight compounds with potential biological activity.

Benzoxazole and its derivatives are heterocyclic compounds with a high therapeutic efficiency. Literature mentions the antibacterial and antifungal activity of this class of compounds as being far more efficient than the active substances already available on the market.[4,5] The antitumoral,[1,5-9] tuberculostatic,[10] antiinflamatory,[11] HIV-1 reverse transcriptase inhibitory[5,11,12] and imagistic fluorescent[13-15] activity should be also mentioned for benzoxazole derivatives.

The present research was oriented towards decyclization of the highly reactive anhydride ring from poly(maleic anhydride-*alt*-vinyl acetate) by reaction with alkaline heterocyclic compounds. Therefore, new bioactive structures with 2-mercaptobenzoxazole derivatives as active principles have been obtained, to be further immobilized onto the copolymeric support.

EXPERIMENTAL

Materials and methods

All reagents were used as purchased (Aldrich, Fluka, Merk, S.C. Chemical Company S.A.).

FT-IR spectra were registered on a FT-IR spectrophotometer (ATR) Brucker Tensor-27; ^{1}H-NMR analysis was performed on a Brucker ARX 400 spectrometer (5 mm QNP probe; 1H/13C/31P/19F) and elemental analysis employed an Exeter Analytical CE 440 elemental analyser. The melting points of the obtained compounds were determined with a Mel-

Temp melting point module, provided with digital thermometer.

Ethyl ester of benzoxazolyl-2-mercaptoformic acid (II)

In a reaction flask provided with a refluxing cooler, 100 mL absolute ethyl alcohol were introduced and then 0.01 mol metallic sodium was carefully added. The mixture was stirred until the total quantity of sodium reacted. To the formed sodium ethoxide solution, 0.1 mol 2-mercaptobenzoxazole was added in small portions, under stirring and gentle heating on a water bath for homogenisation. 0.11 mol ethyl chloroformate were added in small doses to the hot alcoholic solution of the 2-mercaptobenzoxazole sodium derivative (I), and the mixture was left under heating and stirring for another hour, when sodium chloride was formed. Sodium chloride was then removed by filtration under vacuum and, after cooling to room temperature, the obtained solution was poured dropwise, under stirring, into cold iced water, when a voluminous precipitate was separated, filtered under vacuum and dried. The product was purified by recrystallization from boiling ethyl alcohol.

Cream-coloured solid (57.41 g; yield, %: 87.2), melting point: 85-87 °C. FT-IR; ν_{max}cm^{-1}: 2850, 3300 (CH), 1730 (C=O), 745 (-C-S-). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 1.6 (t, 3H, CH_3); 4.4-4.5 (d, 2H, CH_2); 7.40 (d, 2H Ar CH); 7.70 (d, 2H, Ar CH). Anal. calcd. for $C_{10}H_9NO_3S$ (%): C, 53.81; H, 4.03; N, 6.27; S, 14.39; Found: C, 54.20; H, 4.47; N, 6.69; S, 14.76.

Ethyl ester of benzoxazolyl-2-mercaptoacetic acid (III)

The ethyl ester of benzoxazolyl-2-mercaptoacetic acid (III) was synthesized similarly to compound (II), using 100 mL absolute ethylic alcohol, 0.1 mol metallic sodium, 0.1 mol 2-mercaptobenzoxazole and 0.11 mol ethyl chloroacetate.

Grey-coloured solid (19.1 g; yield, %: 80.6), melting point: 47-48 °C. FT-IR; (ν_{max} cm^{-1}): 2933, 3000 (CH), 1728 (C=O), 744 (-CH_2-S-). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 1.20 (t, 3H, CH_3), 3.4-3.7 (d, 2H, CH_2), 4.50 (S, 2H, CH_2S), 7.30 (d, 2H Ar CH), 7.60 (d, 2H, Ar CH). Anal. calcd. for $C_{11}H_{11}NO_3S$ (%): C, 55.69; H, 4.64; N, 5.90; S, 13.5; Found: C, 56.10; H, 5.13; N, 6.37; S, 13.90.

Benzoxazolyl-2-mercaptoformic acid hydrazide (IV)

0.01 mol ethyl ester of benzoxazolyl-2-mercaptoformic acid (II) was suspended into 10 mL absolute ethyl alcohol in a reaction flask. 0.06 mol hydrazine hydrate (98%) was added, under stirring and gentle heating. After 15 min, a clear solution was obtained, subsequently left at room temperature for another 6 h for completing the reaction, when a voluminous precipitate was separated, filtered and dried under vacuum. The product was purified by recrystallization from boiling distilled water.

White-coloured solid (1.92 g; yield, %: 92), melting point: 168-171 °C. FT-IR; (ν_{max} cm^{-1}): 3289 (NH), 1659 (CO-NH), 614 (-C-S-), 1508 (C=N). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 4.50 (s, 2H, NH_2), 7.20-7.40 (d, 2H, Ar CH), 7.50-7.9 (d, 2H, Ar CH), 9.5 (s, 1H, NH). Anal. calcd. for $C_8H_7N_3O_2S$ (%): C, 45.93; H, 3.34; N, 20.09; S, 15.31; Found: C, 46.20; H, 3.65; N, 20.55; S, 15.43.

Benzoxazolyl-2-mercaptoacetic acid hydrazide (V)

Benzoxazolyl-2-mercaptoacetic acid hydrazide (V) was synthesized similarly to compound (IV), using 0.01 mol ethyl ester of benzoxazolyl-2-mercaptoacetic acid (III), 0.06 mol hydrazine hydrate (98%) and 10 mL absolute ethyl alcohol.

White-coloured solid (1.62 g; yield, %: 72.65), melting point: 173-175 °C. FT-IR; ν_{max} cm^{-1}: 3202, 3304 (NH), 2994, 3037 (CH), 1645 (-CO-NH-), 1503 (C=N). ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 4.10 (s, 2H, NH_2), 4.37 (s, 2H, CH_2-S), 7.32-7.48 (d, 2H, Ar CH), 7.60-8.0 (d, 2H, Ar CH), 9.48 (s, 1H, NH). Anal. calcd. for $C_9H_9N_3O_2S$ (%): C, 48.43; H, 4.03; N, 18.83; S, 14.34; Found: C, 48.82; H, 4.22; N, 19.27; S, 14.67.

Poly(maleic anhydride-alt-vinyl acetate) copolymer (PMAVA) (VI)

Poly(maleic anhydride-alt-vinyl acetate) copolymer (PMAVA) (VI) was synthesized by maleic anhydride copolymerization with vinyl acetate at 80 °C, in the presence of benzoyl peroxide.[1] The copolymer was obtained as a white solid of 100000 g mol^{-1} molecular weight.

Benzoxazolyl-2-mercaptoformil hydrazide PMAVA (VII)

In a reaction flask provided with a refluxing cooler, 0.0025 mol poly(maleic anhydride-*alt*-vinyl acetate) copolymer, 20 mL anhydrous dioxane and 0.0025 mol benzoxazolyl-2-mercaptoformic acid hydrazide (IV) were poured. The reaction mixture was heated on a water bath (95-100 °C) for 90 min, when a homogenous solution was formed. After removing the dioxane excess by distillation under reduced pressure, the product was washed several times with anhydrous ether, and then filtered and dried under vacuum at 50 °C for 4 h.

White-coloured solid (0.61 g; yield, %: 62.41), melting point: 167-171 °C. FT-IR; (ν_{max} cm^{-1}): 3004 (NH), 2962, 2976 (CN), 1723 (C=O), 571 (-C-S-), 1495 (CO-NH), 1505 (C=N). ^{1}H-RMN (DMSO-d_6, 400 MHz) δ (ppm): 4.15 (t, 3H, CH_3), 5.2 (d, 2H, CH_2), 5.55 (s, 1H, NH), 7.4-7.5 (d, 2H, Ar CH), 7.7-7.8 (d, 2H, Ar CH), 9.45 (s, 1H, NH), 10.4 (s, 1H, COOH). Anal. calcd. for $C_{16}H_{15}N_3O_7S$ (%): N, 10.68; Found: N, 7.09.

Benzoxazolyl-2-mercaptoacetil hydrazide PMAVA (VIII)

Benzoxazolyl-2-mercaptoacetil hydrazide PMAVA (VIII) was synthesized similarly to compound (VII), using 0.0025 mol poly (maleic anhydride-*alt*-vinyl acetate) copolymer, 30 mL anhydrous dioxane and 0.0025 mol benzoxazolyl-2-mercaptoacetic acid hydrazide (V).

White-coloured solid (0.25 g; yield, %: 25.6), melting point: 175-180 °C. FT-IR; (ν_{max} cm^{-1}): 3373, 3327 (NH), 2970 (CH), 1625, 1705 (C=O), 1490 (CO-NH), 1505 (C=N), 748 (-CH_2-S-). ^{1}H-RMN (DMSO-d_6, 400MHz) δ (ppm): 4.3 (t, 3H, CH_3), 4.4 (s, 2H, CH_2-S), 5.0 (d, 2H, CH_2), 5.7 (s, 1H, NH), 7.5-7.6 (d, 2H, Ar CH), 7.8-8.0 (d, 2H, Ar CH), 9.6 (s, 1H, NH), 10.54 (s, 1H, COOH). Anal. calcd. for $C_{17}H_{17}N_3O_7S$ (%): N, 10.31; Found: 5.01.

RESULTS AND DISCUSSION

Chemistry

The utilization of 2-mercaptobenzoxazole (I) through a series of chemical reactions (Fig. 1) gave the low molecular weight compounds (II) and (III). First, a 2-mercaptobenzoxazol sodium derivative was prepared by reaction with sodium ethoxide under heating, followed by its reaction with ethyl chloroformate/chloroacetate, when the ethyl ester of benzoxazolyl-2-mercaptoformic acid (II), respectively, the ethyl ester of benzoxazolyl-2-mercaptoacetic acid (III) were formed.

This method is advantageous, as the separation stage of the 2-mercaptobenzoxazole sodium derivative in solid state from the reaction mixture is eliminated.

The chemical structure of compounds II and III was confirmed by elemental and spectral analyses (FT-IR, ^{1}H-NMR). FT-IR spectra presented absorption bands for C=O (ester) at 1728-1730 cm^{-1}, and for the C-S group, respectively, at 744-745 cm^{-1}. Absorption bands determined by vibrations of the aromatic ring appeared at 3340-3440 cm^{-1}, and of the C=N bond from oxazolone heterocycle at 850-1100 cm^{-1}.

^{1}H-NMR spectra certified the presence of structural elements characteristic of every compound. Therefore, methyl groups have been identified at proper values in the aliphatic area. Proton signals for CH_2 appeared at 3.4-3.7 ppm and, for the aromatic ring, at 7.3-7.7 ppm.

Literature provides thorough information on compounds with hydrazide-like structures and on their biological activity, such as tuberculostatic,[16-18] antiviral,[19] anticonvulsive,[20] antitumoral[21] and anti-inflammatory.[22]

Considering the data above, benzoxazolyl-2-mercaptoformic acid hydrazide (IV), respectively benzoxazolyl-2-mercaptoacetic acid hydrazide (V), have been synthesized by treating esters (II) and (III) with hydrazine hydrate (98%) in anhydrous ethylic alcohol, at room temperature (Fig. 2).

The chemical structures of compounds (IV) and (V) were demonstrated by the results of elemental and spectral (FT-IR, ^{1}H-RMN) analyses.

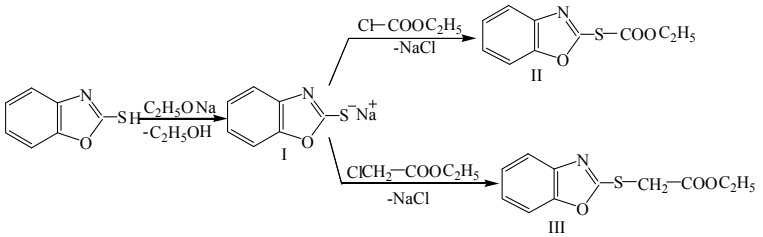
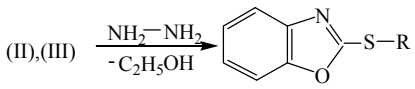


Figure 1: Synthesis of ethyl ester of benzoxazolyl-2-mercaptoformic acid (II) and ethyl ester benzoxazolyl-2-mercaptoacetic acid (III)



(IV): R = -CO-NH-NH_2; (V): R = -CH_2-CO-NH-NH_2

Figure 2: Synthesis of benzoxazolyl-2-mercaptoformic acid hydrazide (IV) and benzoxazolyl-2-mercaptoacetic acid hydrazide (V)

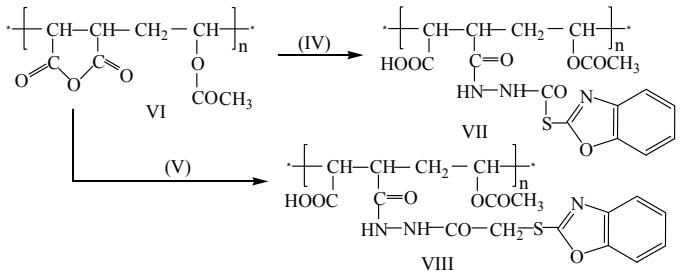


Figure 3: Synthesis of benzoxazolyl-2-mercaptoformyl hydrazide PMAVA (VII) and benzoxazolyl-2-mercaptoacetyl hydrazide PMAVA (VIII)

Table 1
Yields of hydrazides (IV, V) coupled onto the copolymer support

Drug-polymer conjugate	Coupling yield (% weight)	Active principle coupled onto polymeric support (mol/mol)
VII	62.41	0.34
VIII	48.59	0.298

Thus, the FT-IR spectra of the two synthesized hydrazides showed at 3202-3304 cm^{-1} the characteristic band for the NH group, at 1645-1659 cm^{-1} the band corresponding to the carbonyl group, and at 2994-3037 cm^{-1} – absorption bands specific of the CH groups in the aromatic ring. The spectra also presented an absorption band at 1503-1508 cm^{-1}, attributed to the C=N bond from the oxazolone heterocycle.

NMR spectra showed 4 signals at 7.2-8 ppm, given by the four hydrogen atoms from the aromatic ring, at 4.37 ppm for CH_2 protons and at 9.48-9.5 ppm for NH proton as a singlet; also, hydrazide NH_2 protons were observed at 4.1-4.5 ppm. As, generally, benzoxazole derivatives possess important biological properties, the research has been directed towards coupling hydrazides (IV) and (V) to the poly(maleic anhydride-*alt*-vinyl acetate) polymer (VI), to obtain active principle-polymer systems with lower toxicity and increased pharmacological efficiency, mainly favoured by the polymer support.

Hydrazides (IV) and (V) can open the anhydride ring of copolymer (VI), due to their high alkalinity, forming benzoxazolyl-2-mercaptoformyl hydrazide PMAVA (VII), respectively benzoxazolyl-2-mercaptoacetyl hydrazide PMAVA (VIII) (Fig. 3).

Spectral and elemental analysis confirmed the chemical structure of the coupling products (VII), (VIII). FT-IR spectra showed absorption bands for NH at 3004-3373 cm^{-1}, for CO-NH (amide) at 1490-1495 cm^{-1} and for the frequency of the valence vibrations of the C=O (ester) at 1625-1723 cm^{-1}; also, the absorption bands at 2962-2976 cm^{-1} were attributed to CH in the aromatic ring, at 1505 cm^{-1} to C=N (oxazolone heterocycles) and at 571 cm^{-1} and 748 cm^{-1} – to the CS, respectively CH_2-S groups.

NMR spectra presented signals specific for aromatic rings at 7.4-8 ppm. CH_2 protons were evidenced at 4.4 ppm, CH_3 protons (ester group) at 4.15-4.3 ppm and NH protons at 5.2-5.9 and 9.45-9.6 ppm. At 10.4-10.54 ppm, the signals were assigned to COOH protons, proving decyclization of the PMAVA anhydride ring.

The elemental composition was established by means of nitrogen analysis. The found nitrogen content, of 7.09%, *vs.* the calculated value of 10.68% for compound VII, and of 5.01%, respectively, *vs.* the calculated value of 10.31% for compound VIII, indicated that hydrazides were coupled onto the polymeric support with a yield of 62.36% (VII), respectively 48.59% (VIII). The active principles/copolymer molar ratios were also calculated based on nitrogen analysis data (Table 1).

Biological activity

Toxicity tests. The toxicity of compounds IV, V, VII and VIII was assessed by determining the

DL_{50} on groups of 6 male Wistar white mice of approximately 20±2g. The mice were kept under observation for 7 days, at constant temperature (22±1 °C), while receiving usual nourishment; they were weighed every two days at the same hour, the underweight mice being eliminated from the study groups. The tested compounds were administered intraperitoneally as suspensions in Tween80, and mice mortality was registered after 24 hours, 48 hours and 7 days. The lethal dose DL_{50} (Table 2) for every compound was established by the Spearman-Karber[23] arithmetic method.

Compared to isonicotinic hydrazide (commercial anti-tuberculosis agent), the found DL_{50} values for the synthesized compounds showed lower toxicity, which could recommend them for laboratory screening. Compounds (VII) and (VIII), with the hydrazide coupled to the polymer support, showed the lowest toxicity.

Anti-tuberculosis activity. Compounds IV, V, VII and VIII were tested for their *in vitro* biological activity, using the serial dilution technique, and *Youmans* medium with bovine serum as liquid environment, inoculated with *M. tuberculosis*, var. *hominis* (strain $H_{37}R_V$), in a concentration of 10^{-2} mg/mL. The biologically active compounds were previously dissolved in dimethylsulphoxide, due to their low solubility in saline solutions. Solutions formed of 100 μg compound dissolved in 1 mL solvent (DMSO/phosphate buffer solution pH 7 of ¼ (v/v)) were used in concentrations of 5, 10, 20, 30 and 40 μg compound/mL culture environment, the results being registered (Table 3) 6 and 15 days after inoculation.

The results on the biological activity showed that benzoxazolyl-2-mercaptoformyl hydrazide PMAVA (VII) presented the lowest minimum inhibitory concentration (10 μg/mL), similar to isonicotinic hydrazide (commercial tuberculostatic agent), followed by benzoxazolyl-2-mercaptoacetyl hydrazide PMAVA (VIII), with MIC = 15 μg/mL. It can be assumed that the biological activity of hydrazides increased due to their coupling onto the polymer support.

Table 2
DL_{50} (mg/kg body weight) for compounds IV, V, VII, VIII

Compound	DL_{50} (mg/kg body)			
	24 hours	48 hours	7 days	Average
IV	1270	1270	1210	1250
V	1155	1155	1050	1120
VII	1480	1480	1430	1463
VIII	1390	1390	1320	1366
Isonicotinic hydrazide				176

Table 3
Tuberculostatic activity of compounds IV, V, VII and VIII against *M. tuberculosis*, *vs.* isonicotinic hydrazide

Compound	Active principle concentration in culture medium (μg/mL)										MIC* (μg/mL)
	5		10		20		30		40		
	6 days	15 days	6 days	15 days	6 days	15 days	6 days	15 days	6 days	15 days	
IV	++	++	+	+	-	-	-	-	-	-	20
V	++	++	++	++	++	-	-	-	-	-	25
VII	++	+	-	-	-	-	-	-	-	-	10
VIII	++	++	+	+	-	-	-	-	-	-	15
Isonicotinic hydrazide	-	-	-	-	-	-	-	-	-	-	10

- no microbial growth; + medium microbial growth; ++ high microbial growth
* MIC = minimum inhibitory concentration

CONCLUSIONS

The ethyl ester of benzoxazolyl-2-mercaptoformic acid, respectively, the ethyl ester of benzoxazolyl-2-mercaptoacetic acid, based on which the corresponding hydrazides were obtained by reaction with hydrazine hydrate, were synthesised. The newly synthesized hydrazides were immobilized onto the poly(maleic

anhydride-*alt*-vinyl acetate) copolymer support, forming new active principle-polymer systems.

The chemical structures of the new compounds (II-V, VII and VIII) were confirmed by elemental and spectral analyses (FT-IR, ^{1}H-NMR).

The toxicity of compounds IV, V, VII and VIII was evaluated by determining DL_{50}; the immobilization of active principles (IV, V) on the polymer support reduced their toxicity.

Also, the *in vitro* biological activity of hydrazides and conjugates against *M. tuberculosis* was evaluated, the conjugates presenting similar minimum inhibitory concentration as isonicotinic hydrazide.

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