Conjugates with an Antimicrobial Effect Based on New Derivatives of Mercaptobenzoxazole Esterified onto Poly(maleic anhydride-*alt*-vinyl acetate)

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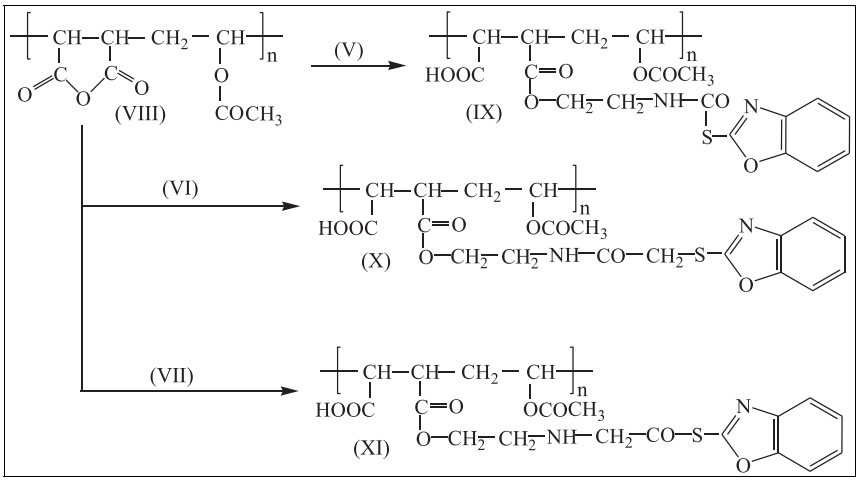

New compounds with biological activity based on hydroxy-amino derivatives of benzoxazolyl-2-mercaptoformic acid, benzoxazolyl-2-mercaptoacetic acid, and chloracetyl-2-mercaptobenzoxazole have been synthesized. The chemical bonding of these compounds to poly(maleic anhydride-*alt*-vinyl acetate), through esterification, leads in obtaining conjugates of polymer biologically active compound type, tests indicating a sustained release of the active chemical, with time (between 5 and 6 h). Reaction products were characterized through elemental and spectral analysis (FTIR and ^{1}H NMR). Toxicology and antimicrobial activity tests recommend compounds with small molecule, as well as their conjugates as therapeutical candidates (antimicrobial inhibitors) for pharmacological application.

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INTRODUCTION

The controlled release of a drug, as a concept, was first proposed by Dr. Judah Folkman in 1964, when he observed that a silicone capsule can be implanted in the human body and that it can release, with a constant speed, the active matter [1]. In this case, a tank-type system was used. This is the beginning of the polymer–drug system with sustained/controlled release research, which has seen explosive growth over the last five decades.

According to the method of obtaining them, systems with controlled release can be classified as follows:

a) *physical systems*, when the active matter is physically incorporated in a matrix (polymeric or not)
b) *chemical systems*, when the active matter is tied to the polymer through chemical hydrolyzable bounds [2].

By accomplishing these methods, we attempt to increase the effectiveness [3] (by the sustained and controlled release of the drug with time, avoiding toxic or under-therapeutical concentration levels), the transportation, and protection of the medicine until it reaches the target organ, increasing the compliance of the patient to the treatment.

The benzoxazole and more and more of its derivatives belong to the heterocyclic compounds category, with a well-known biological activity, for which scientific literature mentions antifungal (3-[2-[(2-fluorophenoxy) methyl]benzoxazol-4-yloxy]-*N*-(pyridin-3-ylmethyl) propan-1-amine and other similar structured derivatives [4], 2-*tert*-butyl-4-[(3*S*)-3-(dimethylamino) pyrrolidin-1-yl]-5-phenyl-1,3-benzoxazole-7-carbonitrile [5]), antimicrobial, tuberculostatic (derivatives of 5,7-di-*tert*-butylbenzoxazoles [6]), and antitumoral activity (6-[3-benzoxazol-2-ylthio) propoxy)-*N*-(3-chloro-4-fluoro-phenyl)-7-methoxy-quinazolin-4-amine [7], (6-[3-benzoxazol-2-ylthio)propoxy)-*N*-(4-bromo-2-ethylphenyl)-7-methoxy-quinazolin 4- amine [7], and conjugates of benzoxazole-pyrrolo[2,1-*c*][1,4] benzodiazepine [8]).

Also, it is used in treating cognitive dysfunctions, disorders of the central nervous system [9], Alzheimer's [9],

schizophrenia [9] (derivatives of benzoxazolyl-2[benzyl-1-carboxylated piperazine] [9]), HIV [10,11], and inflammatory [10] and neurodegenerative [10] diseases (derivatives of *N*-aryl and *N*-alkyl-2-aminobenzoxazole [10], 2-(1,2-difluoro-2-metoxy-2-phenyl-ethyl) benzoxazole [11], and 2-(benzoxazole-2-yl)-2,2 difluoro-1- pyridine-3-ylethyl-3-(trifluoromethyl)benzoate [11] and derivatives of 6, 8-dichloro[1,2,4]triazolo[3,4-b][1,3]benzoxazol-3(2H)-thione that also have antioxidative and anthelmintic activity, respectively) [12].

A first objective of this study is the synthesis and characterization, in terms of the biological activity, of new derivatives from benzoxazole.

Its second goal is the synthesis of conjugates through chemical binding of some of the obtained derivatives, with the purpose of obtaining systems capable of sustained/controlled release of a biologically active matter. To achieve this objective, we had to select a macromolecular compound, which is reactive in moderate temperature conditions, without the need for catalysis. Selection was stopped over vinyl acetate and maleic anhydride copolymer. A priority is that it is a biocompatible [13], nontoxic compound, and it has a high reactivity imprinted by the anhydrous cycle. It has biological activity *per se* namely antitumoral activity, and also, it is involved in the transformation of the pyruvic acid into lactic acid [14].

The chemical bond of some of the synthesized benzoxazole derivatives on the mentioned copolymer was obtained by esterification of the macromolecular support to the anhydrous cycle by the terminal hydroxyl group of the derived.

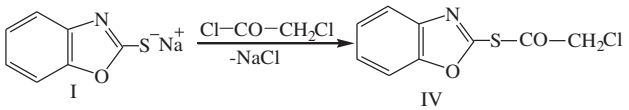
RESULTS AND DISCUSSION

Chemistry. The esters (**II**) and (**III**) are prepared by condensation of 2-mercapto-benzoxazole and ethyl chloroformate/chloracetate, in the presence of sodium ethoxide (Scheme 1).

The advantage of the method is that it eliminates the separation phase of the sodium salt of solid 2-mercapto-benzoxazole.

To obtain the compound (**IV**), we isolate the sodium salt (**I**) of 2-mercapto-benzoxazole that is treated with monochloroacetic acid chloride and leads to 2-mercapto-benzoxazole chloracetyl (**IV**) (Scheme 2).

Scheme 2. Synthesis of 2-mercapto-benzoxazole chloracetyl (**IV**).

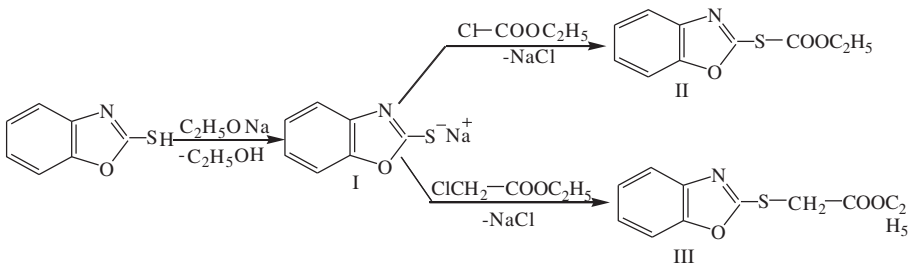


Confirmation of compound structure (**II**, **III**, and **IV**) was carried out by elemental and spectral analysis (FTIR, ^{1}H NMR). FTIR spectrum presents at 1728 cm^{-1} (compound **III**), 1730 cm^{-1} (compound **II**), a characteristic bandwidth for ester C═O group, and at 744 cm^{-1}(compound **III**), 745 cm^{-1} (compound **II**), and 787 cm^{-1} (compound **IV**) a specific bandwidth for C-S group. The absorption band for vibrations of aromatic ring appears at 3300 cm^{-1} (compound **II**), 3000 cm^{-1} (compound **III**), and 2944 cm^{-1} (compound **IV**). C═N bond vibrations, from the oxazolic heterocycle, were identified at 811 cm^{-1} (compound **II**), 3000 cm^{-1} (compound **III**), and 2944 cm^{-1} (compound **IV**). ^{1}H NMR spectra certify the presence of characteristic structural elements of each compound. At adequate values, methyl groups are identified in the aliphatic area. Signal of the CH_2 protons appear at 3.4–3.7 ppm, whereas those of the aromatic protons occur at 7.3–7.7 ppm.

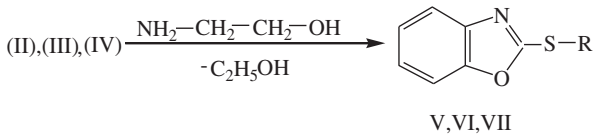
Scientific literature contains data regarding biological importance of the compounds that include monoethanolamine in their molecule. They constitute the objective of numerous studies on the anti-inflammatory [15] and anticancer [15] properties. They are also used in treating cardiovascular disease [16], venous malformations [16], benign vascular lesions [17], esophageal varicose [17], hemangiomas [17], sublingual varicose [17], *Shigella flexneri* [18] (Gram negative bacteria that causes diarrhea), pulmonary cancer [19], and improve metabolism [20].

Considering these facts, we synthesized [(β-hydroxy-ethyl-amino)-formyl]-2-mercapto-benzoxazole (**V**) and [(β-hydroxy-ethyl-amino)-acetyl]-2-mercapto-benzoxazole (**VI**) by treating the esters (**II**) and (**III**) with monoethanolamine in anhydrous dioxane on reflux. Similarly, we obtained [(β-hydroxy-ethyl-amino)-carboxymethyl]-2-mercapto-benzoxazole (**VII**) by treating 2-mercapto-benzoxazole chloracetyl (**IV**) with monoethanolamine (Scheme 3).

Scheme 1. Synthesis of ethyl ester of the 2-mercapto-benzoxazol-yl-formic acid (**II**) and ethyl ester of the 2-mercapto-benzoxazol-yl-acetic acid (**III**).



Scheme 3. Synthesis of β-hydroxy-ethyl benzoxazol-yl-2-mercaptoformic (**IV**), benzoxazolyl-2-mercaptoacetic acid amides (**V**), and 2-mercapto-benzoxazole chloracetyl.



V,VI,VII

V - R = - CO - NH - CH_2 – CH_2 - OH;

VI - R = - CH_2 – CO – NH - CH_2 – CH_2 - OH;

VII – R = - CO - CH_2 –NH - CH_2 – CH_2 - OH;

The structure of compounds **V**, **VI**, and **VII** was tested through the results of the elemental and spectral analysis (FTIR and ^{1}H NMR).

FTIR spectra of the three synthesized compounds show at 1069 cm^{-1} (compound **VII**), 1079 cm^{-1} (compound **VI**), and 1089 cm^{-1} (compound **V**), lines characteristic of the C-NH group, at 1644 cm^{-1} (compound **VI**), 1649 cm^{-1} (compound **VII**), and 1616 cm^{-1} (compound **V**), lines characteristic of the carbonyl group, and at 3223 cm^{-1} (compound **VII**), 3250 cm^{-1} (compound **V**), and 3375 cm^{-1} (compound **VI**) specific absorption bands of CH groups in aromatic ring. The compounds still show a band at 1459 cm^{-1} (compound **VII**), 1450 cm^{-1} (compound **VI**), and 1471 cm^{-1} (compound **V**) because of its C=N connection from the oxazole heterocycle. The OH specific group line appears at 3513 cm^{-1} (compound **VII**), 3608 cm^{-1} (compound **VI**), and at 3611 cm^{-1} (compound **V**).

The ^{1}H NMR spectra show four signals at 7.28–7.70 ppm assigned to the four hydrogen atoms of the benzene nucleus; at 3.21–3.75 ppm, we observe signals for the protons from the CH_2 group and the NH protons as a singlet form at 8.36–8.65. The protons from OH group show signals at 4.65–4.72 ppm.

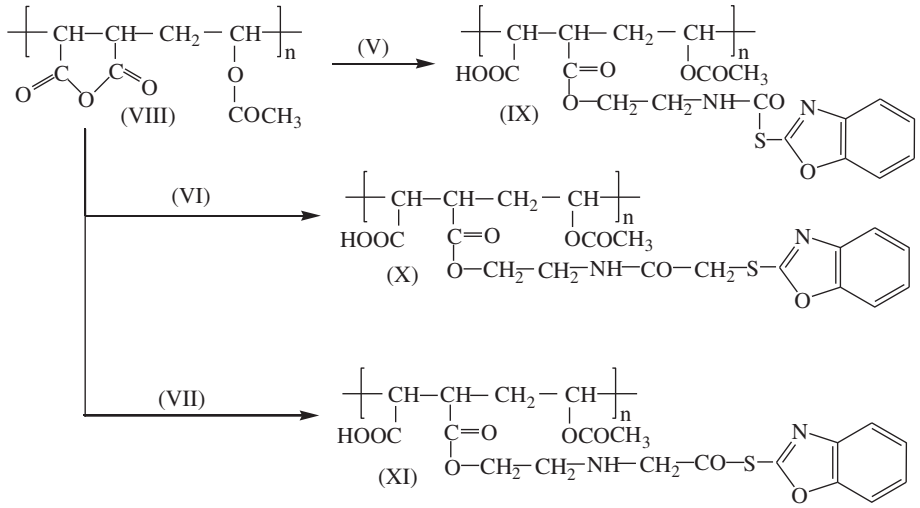
Starting from the premise that the benzoxazole derivatives have important biological properties, our research was directed towards compounds **V**, **VI**, and **VII** immobilization by chemical binding (esterification) on the maleic anhydride-*alt*-vinyl acetate copolymer (**VIII**). Our purpose was obtaining a number of polymer-active matter systems with a low level of toxicity, better and longer pharmacological effects because of the polymeric support, and a longer release as proven by other studies [21–23].

The nucleophilic character of compounds **V**, **VI**, and **VII** is quite pronounced, and therefore, they can determine the decyclization of the anhydride from copolymer (**VIII**), leading to [(β-ethyl-amino-formyl)-2-mercapto-benzoxazole-yl]-poly(maleic anhydride-*alt*-vinyl acetate)-ester (IX), [(β-ethyl-amino-acetyl)-2-mercapto-benzoxazole-yl]-poly(maleic anhydride-*alt*-vinyl acetate)-ester (X), and [(β-ethyl-amino-carboxymethyl)-2-mercapto-benzoxazole-yl]-poly(maleic anhydride-*alt*-vinyl acetate)-ester (XI), respectively (Scheme 4).

Spectral and elemental analysis (determination of nitrogen amount) data confirm the structure of the products **IX**, **X**, and **XI**.

In the FTIR spectrum, the OH group characteristic absorption appears at 3114 cm^{-1} (compound **XI**), 3318 cm^{-1} (compound **X**), and 3344 cm^{-1} (compound **IX**). The amidic CO-NH group stands out by the absorption band from 1460 cm^{-1} (compound **XI**), 1485 cm^{-1} (compound **X**), 1512 cm^{-1} (compound **IX**), and 1726 cm^{-1} (compound **IX**); at 1717 cm^{-1} (compound **X**) and 1703 (compound XI) appear absorption bands characteristic to the C=O esteric group. At 2922 cm^{-1} (compound **X**), 2980 cm^{-1}

Scheme 4. Synthesis of [(β-ethyl-amino-formyl)-2-mercapto-benzoxazole-yl]-PMAVA ester and [(β-ethyl-amino-acetil)-2-mercapto-benzoxazole-yl]-PMAVA ester.



(compound **XI**), and 3114 cm^{-1} (compound **IX**) absorption bands similar to the CH groups from the aromatic ring appeared.

The C═N group from the oxazole heterocycle causes some absorption bands around 1079 cm^{-1} (compound **X**), 1143 cm^{-1} (compound **IX**), and 1238 cm^{-1} (compound **XI**), whereas the C-S group is associated with 753 cm^{-1} (compound **XI**), 805 cm^{-1} (compound **X**), 865 cm^{-1} (compound **IX**) and the CH_2-S group with 744 cm^{-1} (only in compound **X**).

At 7.32–7.63 ppm, the ^{1}H NMR spectra contain signals specific to the aromatic ring. The protons from the CH_2 aliphatic group slightly stand out at 4.72 ppm. At 1.23 ppm, the ester CH_3 group protons appear, whereas the NHCO proton appears at 9.8 ppm. The 12.4–12.53 ppm range is associated only with the COOH protons that show that the decycling took place. An example of ^{1}H NMR spectrum is shown in the third image for the **X** conjugate (Fig. 1).

Among the ^{1}H NMR spectra of the (**IX**, **X**, **XI**) conjugates, we have shown the molar ratio between the active (**V**, **VI**, **VII**) biological compounds coupled with the maleic anhydride of the copolymer (Table 1), taking into consideration the integrals for the COOH protons (formed after the opening of the anhydric cycle of the copolymer) and the aromatic ring protons.

Comparing the result of the study on the nitrogen atom from the conjugates (**IX** 5.21%; **X** 5.76%; **XI** 5.78) with the theoretical one and taking into consideration the fact that the biological compound opened the anhydric cycles, these values (**IX** 6.63%; **X** 6.42%; **XI** 6.42%) allow the evaluation of the bonding, 78.66%, 85.98%, and 90.03%, respectively.

Table 1

The efficiency of the bonding and the molar ratio of the compounds **IX**, **X**, and **XI** in the active polymer-principle conjugates obtained.

Polymer-active matter conjugates—active biological compound	Bonding efficiency[a] (%)	The active biological compound/copolymer in conjugate ratio (mol/mol)[b]
IX	78.7	0.70
X	86.0	0.77
XI	90.0	0.80

[a]Presented as the ratio of the amount of benzoxazolic derivative linked to the support and the theoretical one (all anhydric rings having reacted).
[b]Calculated from the ^{1}H NMR.

On the basis of these results, we also determined the active molar compound and copolymer; in the resulted conjugate, its value indicating to which extent the copolymer anhydric rings participated to the reaction. Both theoretical and experimental results using ^{1}H NMR methods led us to the same results.

The efficiency of the bonding has to be observed. It is manifested through the high quantity of active product in the conjugate of the copolymer anhydric cycle on one hand and on the other of the benzoxazole derivatives (by the -OH group introduced in the hydroxylamine reaction).

Biological activities

Toxicity. To administer them to a living organism, the obtained conjugates, with potential biological activity,

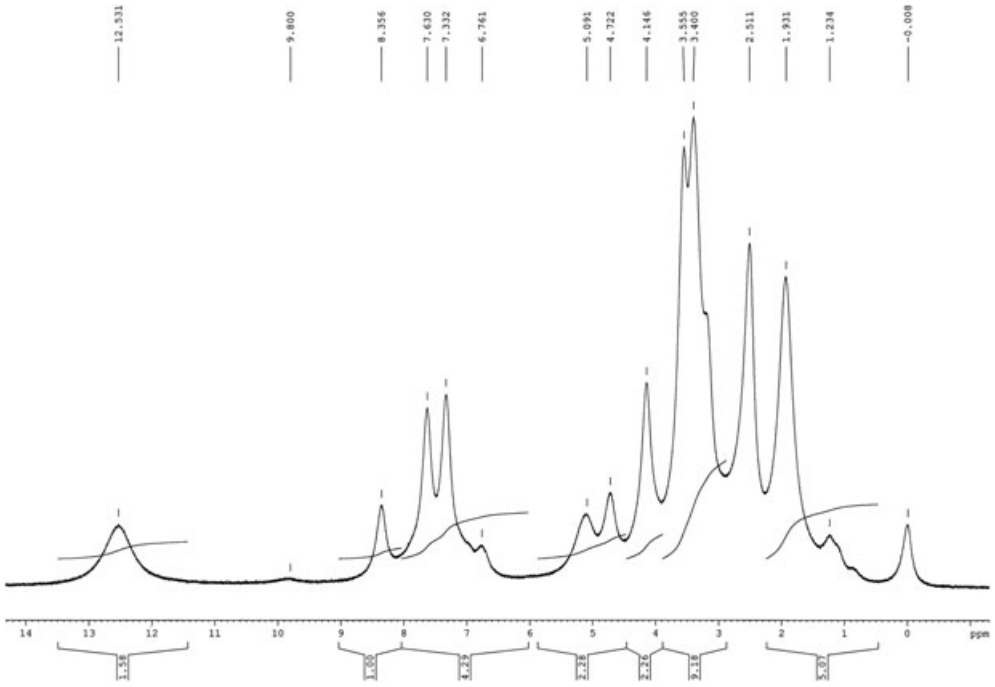


Figure 1. ^{1}H NMR spectrum of [(β-ethyl-amino-acetyl)-2-mercapto-benzoxazole-yl]-poly(maleic anhydride-*alt*-vinyl acetate)-ester (**X**).

must meet a number of constraints, among which the biocompatibility and the lack of toxicity.

Toxicity is frequently measured through LD_{50}, calculated by using the Spearman–Karber [24] arithmetic method; the results are presented in Table 2.

The resulted conjugates (**IX**, **X**, **XI**) show a lower toxicity level than the synthetic compounds, and they are not hazardous to the organism.

The final results allow us to appreciate that the polymeric support is characterized by its own biological activity and a very good biocompatibility that can reduce the toxicity level of the conjugate, the synthetic products to the lab screening being recommended.

The antimicrobial activity. The antimicrobial activity tests carried out on four bacterial cultures show that the germs are resistant to small concentrations of the conjugates. The inhibition area diameter is sensitive to the 2 mg/mL concentration.

The tested products present the ability to inhibit some of our strains (*Staphylococcus aureus* and *Escherichia coli*), making them "sensitive". The *Enterococcus faecalis* and *Salmonella enteritidis* strains were not inhibited by these compounds. The compound activity is similar to the sulphaphenazole (5 mg/mL on the culture environment), noting that we used smaller concentration levels for the **V–VII** and **IX–XI** compounds. The results are shown in Table 3; Figures 2(a), (b) and 3(a), (b) show the Petri dishes sown with the *E. coli* (a) *and S. aureus* (b) strains.

Figure 2(a), (b) demonstrates the inhibition areas for [(β-hydroxy-ethyl-amino)-acetyl]-2-mercapto-benzoxazole (**VI**), and Figure 3(a), (b) shows the conjugate [(β-ethyl-amino-acetyl)-2-mercapto-benzoxazole-yl]-poly(maleic anhydride-*alt*-vinyl acetate)-ester (X).

Table 2

LD_{50} values (mg/kg body) for the **V**, **VI**, **VII**, **IX**, **X**, and **XI** compounds.

Compound	LD_{50} (mg/kg body)			
	24 h	48 h	7 days	Average
V	8575	8575	8500	8550
VI	8975	8975	8750	8900
VII	4905	4905	4530	4780
IX	9030	9030	8940	9000
X	9330	9330	9240	9300
XI	4950	4950	4860	4920

The release of the active principle from the macromolecular support. In the case of the basic hydrolysis (at pH = 9.64), we saw evidence of altered pH, and consequently, the release of active principle took place rapidly, the pH reaching a stable value in approximately 30–45 min because of the ester break between the polymer and principle and also the reaction of the side chain of the polymer where there is an ester linkage. In the case of a side test made only from copolymer, we saw a relatively rapid pH decrease that gets to a constant value in approximately 60 min.

Given that our initial objective was not achieved (the sustained release of the active principle on a larger scale of time), we abandoned the systematic study of the release of the active principle in this pH conditions.

We continued by monitoring the kinetics of release for the **IX** and **XI** compounds, within the acid hydrolysis environment, similar to the stomach and observed that the pH increased until a constant value that is much slower (up to 5–6 h).

The variations of the pH of the system with time are due to the partial consumption of the acid from the reaction environment through the hydrolysis of the ester groups and the release of its active compound.

Figure 4a shows the variation with time of pH during the hydrolysis of conjugate **IX** and Figure 4b for the compound XI. On the basis of the pH variation curve, we calculated the number of ester bonds and thus the quantity (mol and mg, compared with the analyzed conjugate quantity) of released drug.

Figure 5 shows the variation of the quantity of active compound released because of hydrolysis in acid conditions

Table 3

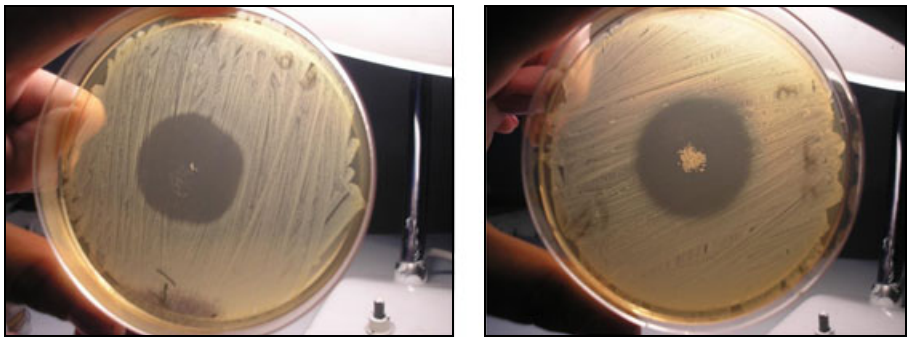
The diameter of the inhibition area for compounds **V–VII**, **IX–XI**, and **XII**–sulphaphenazole.

Compound	Diameter of the inhibition area (mm)							
	Staphylococcus aureus		*Escherichia coli*		*Enterococcus faecalis*		*Salmonella enteritidis*	
V	16	+	17	+	5	–	9	–
VI	17	+	18	+	4	–	8	–
VII	16	+	15	+	6	–	10	–
IX	19	+	18	+	6	–	11	–
X	21	+	20	+	4	–	10	–
XI	17	+	19	+	7	–	11	–
XII	23	+	19	+	8	–	18	+

+Antimicrobial activity.
–Lack of antimicrobial activity.

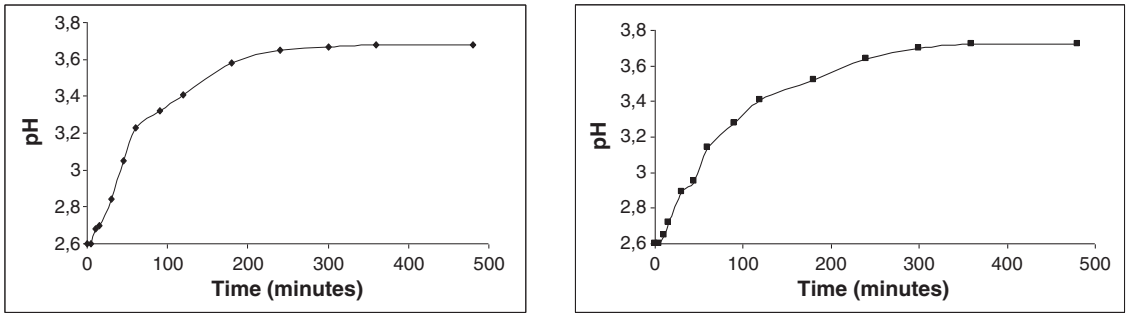
(a) (b)

Figure 2. Photography of a Petri dish sown with the *Escherichia coli* (a) and *Staphylococcus aureus* (b) strains probing the antimicrobial activity of compound **VI**.



(a) (b)

Figure 3. Photography of a Petri dish sown with the *Escherichia coli* (a) and *Staphylococcus aureus* (b) strains probing the antimicrobial activity of compound **X**.



a b

Figure 4. The variation as a function of time of pH during the hydrolysis of conjugate **IX** (a) and **XI** (b) in an acid hydrolysis environment (initial pH = 2.60, conjugate subjected to hydrolysis = 0.1211 mg (a) and 0.1213 mg (b)).

(mg active matter/g conjugate), (a) the efficiency of the release (b) for two of the obtained active polymer principle.

The effectiveness of the release after 6 h is 43.7% (compound **IX**) and 36.0% (compound **XI**); the peak was reached in approximately 5–6 h, similar to the time food stays in the body.

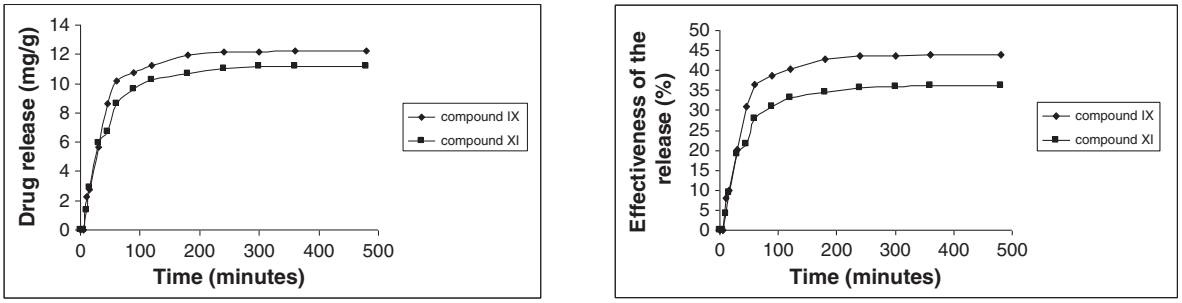
CONCLUSIONS

Ethyl esters of fatty 2-mercapto-benzoxazol-yl-formic acid,2-mercapto-benzoxazol-yl-acetic acid, and 2-mercapto-benzoxazole chloracetyl were synthesized. β-Ethyl-amino-formyl, β-ethyl-amino-acetyl, and β-hydroxy-ethyl-amino-carboxy-methyl of 2-mercapto-benzoxazole derivatives were obtained.

The new synthesized compounds were immobilized on maleic anhydride-*alt*-vinyl acetate copolymer support, resulting in new polymer-active principles systems.

The structure of the new compounds synthesized (**II–VII**, **IX–XI**) was confirmed by elemental and spectral analysis (FTIR and ^{1}H NMR).

The toxicity of the products (**V–VII**, **IX–XI**) was investigated obtaining LD_{50}. It was noted that the restraint on a polymeric support determines the loss of the biological active principles toxicity (**V–VII**).



a b

Figure 5. The variation of the quantity of active matter released because of hydrolysis in acid conditions (a) and the efficiency of the release of the active principle from the support (b) for compounds **IX** and **XI**.

The antimicrobial activity of synthesized compounds was researched and determined that some of them present a similar activity to the one of some reference sulphonamides.

The conjugates (**IX–XI**) present a more efficient bacteriostatic activity, probably because of the polymeric support influence.

Because of hydrolysis reaction, the release of the compounds, in an acid medium, is carried out in a 6 to 7-h interval.

EXPERIMENTAL

Materials. 2-Mercaptobenzoxazole was provided by Merck; monoethanolamine, monochloracetic acid chloride, metallic sodium, maleic anhydride, and ethyl acetate were supplied by Aldrich, dioxane by Fulka, ethanol anhydride and acetone delivered by S.C. Chemical Company S.A. Substances were used in the form provided by manufacturers, without additional purifying. Mueller Hinton agar culture medium used for antimicrobial tests was supplied by OXOID.

Methods of synthesis

Sodium salt of 2-mercaptobenzoxazole (I). In a volumetric flask, equipped with an ascendant cooler, 50 mL of anhydrous ethanol and 0.05 mol of metallic sodium are inserted, little by little, while stirring continuously. Over the hot sodium ethoxide solution, 0.05 mol of 2-mercapobenzoxazole is inserted; after which, the mixture is refluxated on the water bath for 90 min. Excess of ethanol was removed through low pressure distillation, and the isolated product was vacuum-filtered and dried.

Product properties: solid dark gray (16.73 g, yield %: 97.38), melting point: 299°C. IR; ν (cm^{-1}): 2950, 3150 (CH), 750 (CH_2-S-). ^{1}H NMR (DMSO-d_6, 400 MHz), δ (ppm): 7.60 (d, 2H, Ar CH); 7.75 (d, 2H, Ar CH). *Anal*. Calcd for C_7H_4NOSNa (%): C, 53.49; H, 2.56; N, 8.91; S, 20.39; Found: C, 52.23; H, 2.49; N, 8.98; S, 18.37.

Ethylic ester of benzoxazolyl-2-mercaptoformic acid (II). We introduce 100 mL of anhydrous ethanol in a flask with an upper condenser to which we add 0.1 mol of metallic sodium. Stir until all of the sodium reacts. Thus, we obtain a sodium ethoxide solution in which we introduce 0.1 mol of 2-mercaptobenzoxazol in small portions while stirring and slight warming, on water fountain for homogeneization.

We add 0.11 mol ethyl chloroformate to the hot alcoholic sodium derivative of 2-mercaptobenzoxazole (**I**) and continue heating for 60 min while we separate the sodium chloride. We filter it under vacuum to remove the sodium chloride. After cooling, we pour the resulted solution in thin stream in ice water while stirring.

An abundant precipitate was separated, which we filter under vacuum and dry. The crude product is purified by recrystallization from boiling ethyl alcohol.

Product properties: solid creamy (57.41 g, yield %: 87.2), melting point: 85–87°C. IR; ν (cm^{-1}): 3300 (CH), 1730 (C═O), 811 (C═N), 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, 53.80; H, 4.06; N, 6.27; S, 14.36; Found: C, 54.11; H, 4.17; N, 6.39; S, 14.54.

Ethyl ester of benzoxazol-yl-2-mercaptoacetic acid (III). We prepared similarly to compound **II**, starting from 100 mL of anhydrous ethyl alcohol, 0.1 mol metallic sodium, 0.1 mol 2-mercaptobenzoxazole, and 0.1 mol ethyl chloroacetate.

Product properties: solid gray (19.1 g, yield %: 80.6), melting point : 47–48°C. IR; ν (cm^{-1}): 3000 (CH), 1728 (C═O), 842 (C═N), 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, 55.68; H, 4.67; N, 5.90; S, 13.51; Found: C, 55.73; H, 4.83; N, 6.17; S, 13.70.

2-Mercapto-benzoxazole chloracetyl (IV). We use a flask with an upper water cooler to introduce 10 mL of acetone ethanol that contains 0.01 mol of sodium salt from 2-mercaptobenzoxazole. We add 0.01 mol of chloride of monochloroacetic acid while stirring. To perfect the reaction, we heat the reaction mixture at 65°C for about 90 min. We filter it under vacuum and then let the solution at room temperature crystallize the final product.

Product properties: solid creamy (1.78 g, yield %: 78.24), melting point: 115–118°C. IR; ν (cm^{-1}): 2944 (CH), 1728 (C═O), 824 (C═N), 733 (C-Cl), 787 (-C-S-). ^{1}H NMR (DMSO-d_6, 400 MHz), δ (ppm): 2.78–4.08 (d, 2H, CH_2); 7.20 (d, 2H Ar); 7.30 (d, 2H, Ar); *Anal*. Calcd for $C_9H_6NO_2SCl$ (%):C, 47.48; H, 2.65; N, 6.15; S, 14.08; Found: C, 47.29; H, 2.77; N, 6.26; S, 14.25.

UV spectrum in ethanol has two absorption maxima 255 nm and 298 nm (very intense).

[(β-Hydroxy-ethyl-amino)-formyl]-2-mercapto-benzoxazole (V). In a volumetric flask, equipped with an ascendent cooler, 0.01 mol of ethyl ester of the 2-mercapto-benzoxazolyl-formic acid that was previously dissolved in 10 mL of anhydrous dioxane is inserted, and then, we added 0.06 mol of monoethanolamine. The mixture is refluxed for 8 h on an oil bath. The reaction product is precipitated with water and purified by recrystallization from alcohol.

Product properties: white-gray solid (1.08 g, yield %: 45.27), melting point: 156/158°C. IR; ν (cm^{-1}): 3250 (CH), 3611 (OH), 1616 (C═O), 811 (-C-S-), 1089 (C-NH), 1471 (C═N). ^{1}H NMR (DMSO-d_6 400 MHz), δ (ppm): 3.21–3.30 (d, 2H, CH_2); 3.62–3.75 (d, 2H, CH_2); 4.65 (s,1H, OH); 7.28 (d, 2H Ar); 7.70 (d, 2H, Ar); 8.65 (s, 1H, NHCO). *Anal*. Calcd for $C_{10}H_{10}N_2O_3S$ (%): C, 50.41; H, 4.23; N, 11.75; S, 13.45; Found: C, 50.73; H, 4.81; N, 12.12; S, 13.74.

In an alcoholic solution (ethanol), UV spectrum presents two maxima of absorption: 255 nm and 298 nm (very intense).

[(β-Hydroxy-ethyl-amino)-acetyl]-2-mercapto-benzoxazole (VI). It was prepared similarly to compound **V**, from 0.01 mol of ethyl ester of the 2-mercaptobenzoxazolyl-acetic acid, 10 mL anhydrous dioxane, and 0.02 mol monoethanolamine.

Product properties: solid white (1.56 g, yield %: 62.27), melting point: 127/128°C; IR; ν (cm^{-1}): 3375 (CH, Ar), 2977 (CH), 3608 (OH), 805 (-C-S-), 1644 (C═O), 1079 (C-NH), 1450 (C═N).^{1}H NMR (DMSO-d_6, 400 MHz), δ (ppm): 3.18–3.35 (d, 2H, CH2); 3.42–3.43 (d, 2H, CH2); 4.15 (d, 2H, CH_2); 4.72 (s, 1H, OH); 7.33 (d, 2H Ar); 7.63 (d, 2H, Ar); 8.36 (s, 1H, NHCO). *Anal*. Calcd for $C_{11}H_{12}N_2O_3S$ (%): C, 52.36; H, 4.79; N, 11.10; S, 12.70; Found: C, 52.59; H, 4.98; N, 11.41; S, 12.92.

In an alcoholic solution (ethanol), UV spectrum presents two maxima of absorption: 247 nm (more intense) and 275 nm.

[(β-Hydroxy-ethyl-amino)-carboxymethyl]-2-mercapto-benzoxazole (VII). In a reaction flask, 0.06 mol monoethanolamine and 0.01 mol triethylamine were added on 0.01 mol 2-mercapto-benzoxazole chloracetyl (**IV**) in 10 mL anhydrous dioxane. The reaction mixture was heated under reflux for 3 h. The final product was precipitated after cooling. It was purified by recrystallization from boiling water.

Product properties: cream-colored solid (1.70 g, yield %: 67.69), melting point: 153–156°C; IR; ν (cm^{-1}): 3026 (CH), 3223 (CH, Ar), 3513 (OH), 824 (-C-S-), 1069 (C-NH), 1649 (C═O), 1459 (C═N). ^{1}H NMR (DMSO-d_6 400 MHz), δ (ppm): 3.37 (s, H, OH); 3.63 (d, 2H, CH_2); 4.44 (s, H, NH); 6.36–6.73 (d, 2H, CH_2); 6.9 (d, 2H, Ar); 7.3–7.4 (d, 2H, Ar). *Anal*. Calcd for $C_{11}H_{12}N_2O_3S$ (%): C, 52.36; H, 4.79; N, 11.10; S, 12.70; Found: C, 52.63; H, 5.03; N, 11.40; S, 12.95.

In an alcoholic solution (ethanol), UV spectrum presents two maxima of absorption: 242 nm (very intense) and 279 nm.

Poly (maleic anhydride-alt-vinyl acetate) copolymer (PMAVA) (VIII). It was synthesized by maleic anhydride copolymerization with vinyl acetate at 80°C in the presence of benzoyl peroxide [21]. The copolymer was obtained as a white solid of 10,000 g mol^{-1} molecular weight.

[(β-Ethyl-amino-formyl)-2-mercapto-benzoxazole-yl]-poly(maleic anhydride-alt-vinyl acetate)-ester (IX). We use a 50-mL flask, equipped with an ascendent cooler, to introduce 0.0025 mol maleic anhydride-*alt*-vinyl acetate copolymer predissolved in 10 mL of dioxane, and 0.0025 mol compound **V** was also dissolved in 10 mL of dioxane. The resulted solution is to be refluxed for 90 min. The dioxane excess will be distilled under low pressure.

The solid glue (**IX**) is filtered and dried under vacuum, and then, it is washed with anhydrous ethyl ether.

Product properties: solid white (0.76 g, yield %: 72.34), melting point: 177/182°C. IR; ν (cm^{-1}): 3114 (CH), 1726 (C═O), 1512 (CO-NH), 3344 (OH), 1143 (C═N), 865 (C-S). ^{1}H NMR (DMSO-d_6 400 MHz), δ (ppm): 1.20 (3H, CH_3), 4.70 (2H, CH_2), 4.25 (2H, CH_2), 7.25 (d, 2H, Ar), 7.60 (d, 2H, Ar), 8.8 (1H, NH), 12.13 (1H, COOH). *Anal*. Calcd for $C_{18}H_{18}N_2O_8S$ (%): N, 6.63; Found: N, 5.21.

[(β-Ethyl-amino-acetyl)-2-mercapto-benzoxazole-yl]-poly(maleic anhydride-alt-vinyl acetate)-ester (X). Compound **X** is synthesized using the same procedure that is used for compound **IX**, starting from 0.0025 mol of maleic anhydride-*alt*-vinyl acetate copolymer, 20 mL anhydrous dioxane, and 0.0025 mol compound **VI**.

Product properties: solid white (0.70 g, yield %: 64.81), melting point: 161/165°C. IR; ν (cm^{-1}): 3318, (OH), 1485, (CO-NH), 1079 (C═N), 744 (-CH_2-S-), 1717 (C═O), 1372 (C-O), 1140 (C-O), 805 (C-S), 2922 (CH).^{1}H NMR (DMSO-d_6, 400 MHz), δ (ppm): 1.23 (3H, CH_3), 4.04 (S, 2H, CH_2-S), 5.09 (2H, CH_2), 4.16 (2H, CH_2), 7.32 (d, 2H, Ar), 7.63 (d, 2H, Ar), 9.8 (1H, NH), 12.53 (1H, COOH) . *Anal*. Calcd for $C_{19}H_{20}N_2O_8S$ (%): N, 6.41; Found: N, 5.52.

[(β-Ethyl-amino-carboxymethyl)-2-mercapto-benzoxazole-yl]-poly (maleic anhydride-alt-vinyl acetate)-ester (XI). Compound **XI** is synthesized using the same method as for the compound **IX**, using 0.0025 mol of maleic anhydride-*alt*-vinyl acetate copolymer, 20 mL anhydrous dioxane, and 0.0025 mol compound **VII**.

Product properties: solid cream-colored (0.96 g, yield %: 88.46), melting point: 167/173°C). IR; ν (cm^{-1}): 3114 (OH), 2980 (CH), 1460 (CO-NH), 1238 (C═N), 753 (C-S-), 1703 (C═O). ^{1}H NMR (DMSO-d_6 400 MHz), δ (ppm): 1.10 (3H, CH_3), 6.35 (S, 2H, CH_2), 4.90 (2H, CH_2), 4.20 (2H, CH_2), 7.23 (d, 2H, Ar), 7.72 (d, 2H, Ar), 4.8 (1H, NH), 12.12 (1H, COOH). *Anal*. Calcd for $C_{19}H_{20}N_2O_8S$ (%): N, 6.41; Found: N, 5.78.

Methods of characterization

Structural characterization. FTIR spectroscopy was achieved using an FTIR (ATR) Brucker Tensor-27 spectrophotometer (Bruker Optik, Ettlinger, Germany). ^{1}H NMR spectra recordings were performed using a Brucker Avance DRX 400 spectrometer, equipped with 5 mm QNP ^{1}H/^{13}C/^{31}P/^{19}F samples, working with the Silicon Graphics Indigo interface.

Quantitative elemental analysis was assessed with Elemental Exeter Analytical CE 440 device (Exeter Analytical, Coventry, UK). The melting points were determined with Mel-Temp machine, which has a digital thermometer, and the pH determination was made with a Labcor Consort C_{831} pH meter (Cole-Parmer/Amex Export-Import SRL, Bucarest, Romania).

Determination of toxicity. White male mice (20 ± 2 g) were used to determine the toxicity of the compounds (**IV**, **V**, **VII**, **VIII**). The mice were kept under observation for 7 days, at constant temperature (22°C ± 1°C), receiving common nutrition. Their weighing was performed for 2 days at the same time, removing the ones that have decreased in weight. To determine the acute toxicity, six lots of mice were used. Tested substances were administered intraperitoneally, as a stabilized (by Tween 80) aqueous suspension, and mortality was recorded at 24 h, 48 h, and 7 days.

Determination of antimicrobial activity. Antimicrobial activity of the synthesized compounds was determined on *S. aureus* ATCC 25923 (bacil gram "+") cultures, *E. coli* ATCC 25922 (coc gram "−"), *E. faecalis* ATCC 51299 (coc gram "−"), and also *S. enteritidis* ATCC 13076 (bacil gram "−") cultures, in the serology laboratory of the Public Health Department, Iasi, Romania. For this, the capacity of inhibition of the compounds, on the mentioned cultures, was followed measuring the inhibition area using Kirby–Bauer [25] diffusimetric method.

According to international regulations, the classification in the category sensitive/resistant of bacterial strains (mentioned previously) is made depending on the inhibition area diameter as follows: sensitive ≥17 mm, intermediate sensitive = 13–16 mm, and resistant ≤12 mm.

The samples were dissolved in dimethylformamide (DMF) while ensuring a complete solubilization.

The germs were grown on Mueller Hinton agar. Their preparation must be proceeded as follows. Dissolve 38 g of dehydrated medium in 1 L of distilled water; for complete solubilization, you must reach boiling point and then sterilize by autoclaving at 121°C for 15 min.

Approximately 0.5 units McFarland of the strains for testing are sowed on Petri plates with a diameter of 9 cm in a 4–4.5 mm layer; after which, the test substance is applied.

The reading is performed after 18 h, and the average values will be heated at 37°C in the thermostat.

Different concentrations were used: 0.25, 0.5, 0.75, 1, and 2 mg/mL culture medium.

The activity of the synthesized compounds was compared with the activity of sulfenazole.

The way of working was identical, indicating that 5 mg of sulphaphenazole/mL culture medium was used.

The release of immobilized active principle from macromolecular support. In the case of chemically bonded polymer–drug systems, the release study can be accomplished by spectral methods (UV–vis), by chromatography (HPLC), or by pH modification with time, because of basic or acid hydrolysis, to which are exposed coupling by-products [3,26] (that stimulates the pH conditions from the digestive tract of the human body, stomach, and intestine, respectively).

Conjugates, presenting the same drug–polymer ester bound, were exposed to acid hydrolysis and basic hydrolysis.

By monitoring the pH variation with time, because of acid or base intake for breaking the esterified bounds, we can appreciate the quantity of product released through hydrolysis by calculating it.

In the case of hydrolysis in a basic environment, we used 0.12 g of compound (**IX**, **X**, **XI**) suspended in 20 mL NaOH solution, at 37°C. Although the pH value (pH = 9.64) is superior to the one in the large intestine, previous studies led to the conclusion that the order of the reaction remains constant [27,28] even though the speed of the hydrolysis reaction increases.

For all the analyzed conjugates, it was noted that the pH dropped very fast after the suspension in the basic solution of the conjugate, reaching a constant value after a few minutes.

The result indicates that this kind of product is not indicated for use in the treatment of bacterial infections of the large intestine because the release of the active compound is too fast. To be able to perform hydrolysis in acid medium, 0.12 g of compound were suspended in 25 mL HCl (8.17%) at 37°C. The quantitative evaluation of the bioactive product released by hydrolysis was carried out by pH monitoring of the medium in which the polymeric conjugate was suspended (type of pH meter: Labcor Consort C_{831}).

At the same time, control samples were performed consisting of placing the control copolymer in amounts equivalent to those analyzed in the hydrolysis environment.

The analysis was performed assuming that a part of the acid can be consumed by substituting groups of the polymer main chain.

However, it was found that within the hydrolysis environment used to analyze the conjugate, the solution in which the control polymer was added has not modified its pH during the experiment.

We conclude that the pH modification is due to the hydrolysis only in the case of the ester group of the control polymer and the active principle (in this way, we are able to calculate the produced active biological quantity released).

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