THE SEPARATION AND PURIFICATION OF NEW COMPACT CONDENSED HETEROCYCLIC SYSTEMS WITH THIAZOLIC RING BY HPLC

Cristina Rădulescu*, C.M. Tărăbăşanu, Ana Maria-Hossu*, Ionica Ionită* and V. Magearu *****

abstract: In this present study two original compact condensed heterocyclic systems with thiazolic ring, 2-aminothiazolo[4,5-b]chinoxalin-6-carboxylic acid and 2-aminothiazolo[5,4-b]chinoxalin-7-carboxylic acid, in mixture, were synthesized. They have never been quoted in the specific literature and never been separated by classic methods of separation. The high performance liquid chromatography, HPLC, was applied in this case to lead at the separation and purification of the aminothiazolo-chinoxalines mixture. The chromatograph Jasco 800 was used in this work and if can be used for both analytical and purification purposes. An important stage choosing the chromatographically column; finally several tests were performed in order to purify and separate the heterocyclic systems from the mixture. The gel permeation columns were chosen NUCLEOGEN® 4000-7 DEAE type. They were considered to be the most appropriate because they allow retention times long enough for an efficient separation and do not present the colmation phenomenon for the heterocyclic systems. IR, UV-VIS, NMR spectroscopy and elemental analysis characterized the structure of synthesized heterocyclic systems.

Introduction

The obtaining of mixed condensed aromatic ring systems was the object of numerous studies in the last years, this due to the special characteristics which these structures confer to the dyestuffs.

The stability of the compact condensed substances having thiazolic ring are the characteristic of every aromatic ring. Thus, the special biological activities of the thiazolic ring determined scientists to study and to apply them in many areas and there are series of precursors of heterocyclic azo dyes deriving from thiazolo-chinoxalines.

The direct substitution of thiazolic heterocyclic system has formed the object of many studies, because the compact condensed substances with thiazolo ring was used for synthesis of cationic dyes.

* University ''Valahia''of Târgovişte, Faculty of Science, Department of Chemistry, 18-22 Unirii Bdvl., Târgoviste, Romania, mail: radulescucristiana@yahoo.com
** ''Polytechnica'' University of Bucharest, Faculty of Industrial Chemistry, 149, the Victory Way, Bucharest, Romania
*** University of Bucharest, Faculty of Chemistry, Elisabeta Queen Bdvl, no. 2-14, 70346, Bucharest, Romania

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The structures, which we have obtained, have beside the thiazolil ring a chinoxalinic ring unit condensed with the first.

Experimental part

The heterocyclic systems 2-aminothiazolo[4.5-b]chinoxalin-6-carboxylic and 2-amino-thiazolo[5.4-b]chinoxalin-7-carboxylic acids were obtained by next stage, in accordance with reactions presented in figure no 1.

1) In synthesis were used the 4-aminobenzoic acid premium matter. After this acid was treated with NaOH 20% solution, was obtained the sodium salt of 4-aminobenzoic acid, **2**. The protection of the amino group, $-NH_2$, was made by acetylating with acetic acid when resulted the N-acetyl-4-aminobenzoic acid, **3,** white powder.

2) Nitration of N-acetyl-4-aminobenzoic acid with nitrating acid was made by usual methods found in the literature.[1] The compound **4** was hydrolyzed in medium HCl 32%, to recovery the amino group, resulting the 5-nitro-4-aminobenzoic acid, **5,** orange powder, witch was purified by recrystallization of ethyl alcohol.

3) Reduction the compound **5** at **6** using method Béchamps in accordance with literature [1] and the 4,5-diaminobenzoic acid was purified by recrystallization of ethyl alcohol.

4) The 2,3-dihidroxichinoxalin-6-carboxilic acid, **7,** was obtained by condensation of compound **6** with oxalic acid in water acidified with sulphuric acid 98%, in accordance with the literature [2]. The purification of this product was made by recrystallization of ethyl alcohol, when resulted yellow dark powder.

5) The nucleophile substitution reaction at groups –OH (compound **7**) with –Cl (compound **8**) was made by chlorination with PCl_5 and $POCl_3$ at reflux, in 8 hours. Finally the reaction product was transferred over cool water and ice when the yellow 2,3-dichlorochinoxalin-6-carboxilic acid, precipitated immediately.

6) The 2,3-dichlorochinoxalin-6-carboxilic acid was treated with ammonium sulphocyanate in DMF, at reflux 10 hours; the molar ratio compound **8**:NH_4SCN = 1:1. The sulphocyanine derivatives of chinoxaline **9** and **10** were prepared and purified by recrystallization of ethyl alcohol and by HPLC. These methods proved the presence of a mixture of two compounds with were used in the next step [4,9].

7) 2-amino-3-sulphocyanochinoxalin-6-carboxilic and 2-sulphocyano-3-aminochinoxalin-6-carboxilic acids were obtained by treating compounds **9** and **10** with NH_3 in CH_3OH at 90-100^{0}C, under pressure, in stainless steel reactor; products **11** and **12** (dark yellow powder) was treated with ammonia alcoholic solution, 20 hours, in reactor at 60-70 at; finally the mixture, compounds **11** and **12**, was purified with ethyl alcohol for remove the resins.

   The classic chromatographic resolution methods indicate the presence of impurities that could not by remove totally of the mixture of the two carboxylic acids. Separation of compounds **11** and **12** of the mixture was possible by HPLC. The next step of the reaction, the *NA* was possible on chemically cleans compounds (yeld~51,5 %) [4,9].

8) Compounds **11** and **12**, in mixture, were refluxed 12 hours in HCl 5N and finally mixture filtered hot; the filtrate (contain chlorhidrats the compounds **10** and **11**) is treated with NH_3 25% when pH=7,5. 2-aminothiazolo[4,5-b]chinoxalin-6-carboxilic acid, **13**, and 2-aminothiazolo[5,4-b]chinoxalin-7-carboxilic acid **14,** in mixture, were purified by recrystallization of ethyl alcohol (yield ~35%) [4,9].

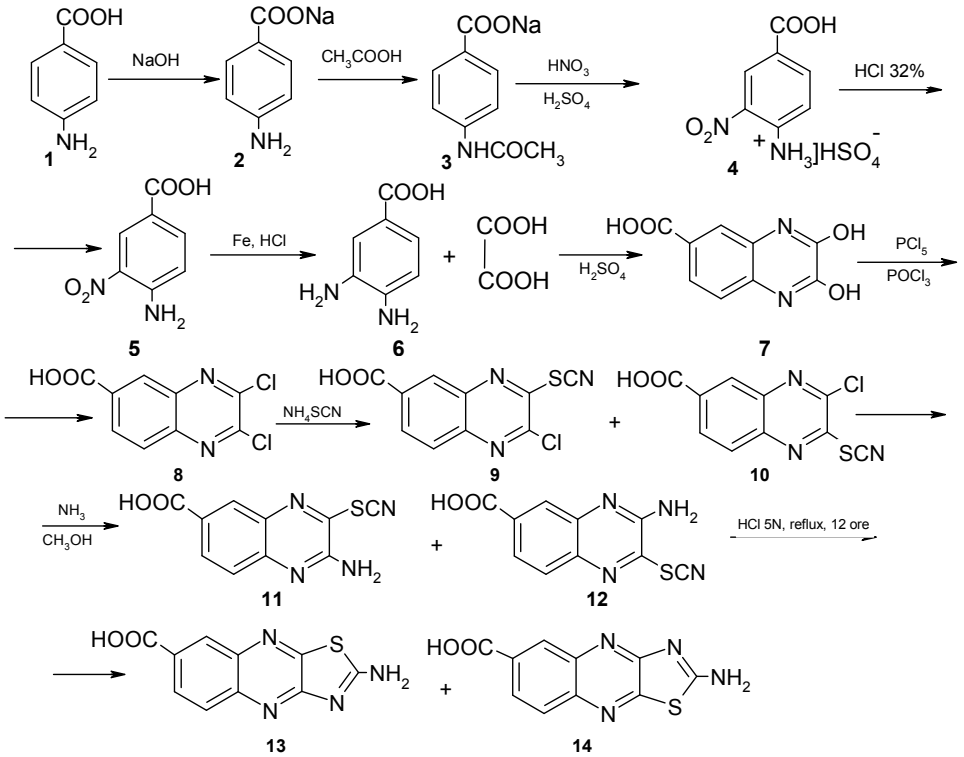

Fig. 1. *Synthesis of 2-aminothiazolo[4,5-b] chinoxalin-6-carboxylic acid and 2-aminotiazolo [5,4-b]chinoxalin-7-carboxylic acid*

Purification methods, the melting points and the yields of synthesis of compounds **5-14** were presented in Table 1.

The UV-VIS electronic spectra [3] were performed with SECOMAN S 750 apparatus in quartz cells (1cm) for ethyl alcohol of c ~ $2 \cdot 10^{-5}$ M of the compounds synthesized. The absorption bands characterized by maximum wave length are presented in same table.

The high performance liquid chromatography, was applied in this case to lead at separation and purification of 2-aminothiazolo[4.5-b]chinoxalin-6-carboxilic and 2-aminothiazolo [5.4-b]chinoxalin-7-carboxylic acids in mixture and was achieved about the usual technique; the chromatograph JASCO 800 was used and the scheme is presented in Fig. 2.

This apparatus is composed of solvent tank, S, which the tree solvent were introduced, the degasifier on-line Waters used for degasification of solvent because can appeared gap of solvent of liquid route. The solvent system introduced in degasifier was passed in ternary system of pump which created pressure of 1 – 400 atm. This pump is connected at

controller which prescribes the analysis parameter: pressure, flow, the gradient of pressure, the gradient of solvent concentration. Next apparatus is injection cock which introduced the probe in chromatographically column, where the mixture was separated; the compounds were eluted separate.

Table 1. The purification methods and the characteristics of UV-VIS spectra of compounds 5-14

Cmpd	Chromatography method	Melting points, ^{0}C	Yield, %	λ_{max} , nm (Absorbtevity)
5	*Substratum*: silica gel F_{254} (Merck) on aluminium layer. *Eluent*: ethyl alcohol : benzene = 90:10 (vol/vol); R_f = 0.74	>250	81.5	209.4 (0.671) 248.8 (1.734) 380.2 (0.369)
6	*Substratum*: silica gel F_{254} (Merck) on aluminium layer. *Eluent*: ethyl alcohol : benzene= 90:10 (vol/vol); R_f = 0.69	240-241	62	213.6 (0.603) 249.0 (1.274) 272.9 (0.938) 378.1 (0.326
7	*Substratum*: silica gel F_{254} (Merck) on aluminium layer. *Eluent*: acetic acid : benzene = 90:10 (vol/vol) ;R_f =0.54	>250	71	248.8 (1.194) 378.1 (0.248) 394.5 (0.320)
8	*Substratum*: silica gel F_{254} (Merck) on aluminium layer. *Eluent*: acetic acid : benzene = 90:10 (vol/vol) ;R_f = 0.46	157-159	62.5	207.6 (1.572) 222.0 (1.725) 248.6 (1.567) 317.9 (1.359)
9,10	*Substratum*: silica gel F_{254} (Merck) on aluminium layer. *Eluent:* acetic acid : ethyl alcohol= 90:10 (vol/vol); R_f = 0.89; 0.79	>250	67	209.0 (0.987) 247.1 (1.023) 331.0 (0.074)
11,12	*Substratum*: silica gel F_{254} (Merck) on aluminium layer. *Eluent*: acetic acid : pyridine = 90:10 (vol/vol); R_f = 0.73; 0.62	>250	51.5	211.2 (0.867) 248.6 (1.102) 287.3 (0.765) 330.4 (0.043
13,14	*Substratum*: silica gel F_{254} (Merck) on aluminium layer. *Eluent*: acetic acid : ethyl alcohol = 90:10 (vol/vol); R_f = 0.59; 0.43	>250	34.2	212.7 (0.190) 244.3 (0.133) 327.5 (0.093) 378.3 (0.032)

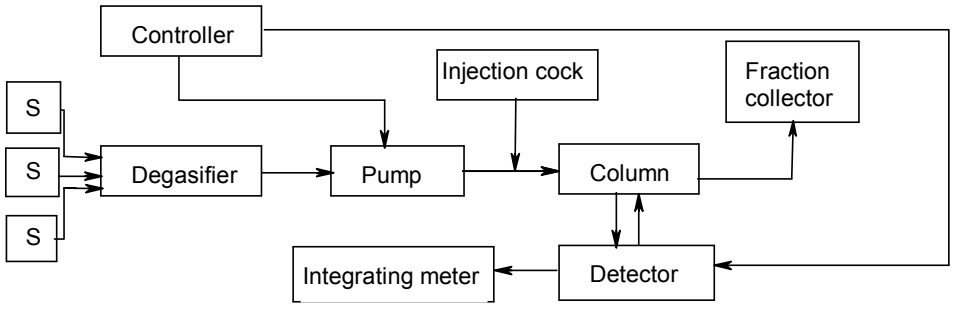

Fig. 2. *The scheme of chromatograph JASCO 800*

For this case were used the gel-permeable columns, Nucleosil® $5C_8$ and Nucleogen® 4000-7 DEAE, (Table 2) considered to be the best and the most appropriate because they allow retention times long enough for an efficient separation and do not present the colmation phenomenon for the heterocyclic systems. About chromatographically column the mixture separated passed in fraction collector by separation and purification or in detector for analytical analysis.

Table 2. The parameters of purification by HPLC of compounds 5-14

Cmpd	Column, flow	Elution analysis	Detection λ, nm
5	Nucleosil® $5C_8$ 1 mL/min.	15 mM octansulphonat of sodium, 5 mM ninhydryn in water : aceto-nitryl :methyl alcohol (92:3:5)	380.2
6	Nucleosil® $5C_8$ 1 mL/min.	15 mM octansulphonat of sodium, 5 mM ninhydrin in water : aceto-nitryl : CH_3OH (92:3:5)	378.1
7	Polygosil® 60-2540 C_{18} 0.8 mL/min.	1.015 mM heptan-sulphonat of sodium, 4 ml acetous acid conc., sol. methyl alcohol 4%	394.5
8	Polygosil® 60-2540 C_{18} 0.8 mL/min.	1.015 mM heptan-sulfonat de sodium, 4 ml acetous acid conc sol. methyl alcohol 4%	317.9
9,10	Nucleogen® 4000-7 DEAE, 2 mL/min	0.5 M NaCl, 6M urea, 25 mM phosphate of sodium	331.0
11,12	Nucleogen® 4000-7 DEAE 2 mL/min	0.5 M NaCl, 6M urea, 25 mM phosphate of sodium	330.4
13,14	Nucleogen® 4000-7 DEAE 2 mL/min	0.5 M NaCl, 6M urea, 25 mM phosphate of sodium	327.5

The chromatograph JASCO 800 are in endowment two detectors, one UV-VIS for λ=190-600 nm and one of fluorescence. In this case given up at integrating meter, because the retention times not present interesting, but wished to purify and separate the heterocyclic systems from the mixture for characterized this by NMR spectroscopy.

The parameters of purification and separation by HPLC are presented in Table 2.

The compounds **5-14** were analyzed by NMR spectroscopy using VARIAN GEMINI 300 BB apparatus, with frequency of registration in case ^{1}H-NMR is 300 MHz and in case ^{13}C-NMR the frequency is 75 Hz. The purified prove was soluble in DMSO or in deuterochloroform and signals was reported at TMS. We select compounds **13** and **14** to present in NMR spectra**.**

^{1}H-NMR spectra for 2-aminotiazolo[4.5-b]chinoxalin-6-carboxylic, **13,** and 2-aminotiazolo [5.4-b]chinoxalin-7-carboxylic acids, **14,** separated by HPLC, is identical and lead to the signals:

H^5 – *split singlet*, δ_H : 6.92 ppm with coupling constant in *meta* J(5.7) = 1.50 Hz.

H^7 – *split doublet*, δ_H :7.07 ppm, coupling constants *orto* J(7.8) = 8.00 Hz, *meta* J(7.5) = 1.50 Hz.

H^8 – *doublet*, δ_H : 6.32 ppm with the coupling constant *orto* J(8.7) = 8.00 Hz

The signals of 4.66 ppm, singlet, has been attributed the presence of amino group.

The proton of –COOH group present signal at 9.50 ppm, *singlet.*

The ^{13}C-NMR spectra (figure no. 4) present next signals for carbon atoms:

The carbon atoms C^5, C^7 and C^8 present signals of δ: 114.2; 118.6; 114.2 ppm.

The carbon atoms C^2, C^3, C^6, C^9 and C^{10}, δ: 163.0; 142.2; 126.4; 140.0; 135.2 ppm and the carbon of group -COOH has been attributed a signal 198,0 ppm (this value appear at all analyzed chinoxalinic compounds).

The signal, δ: 130.0 ppm, has been attributed at carbon atom on thiazolic ring.

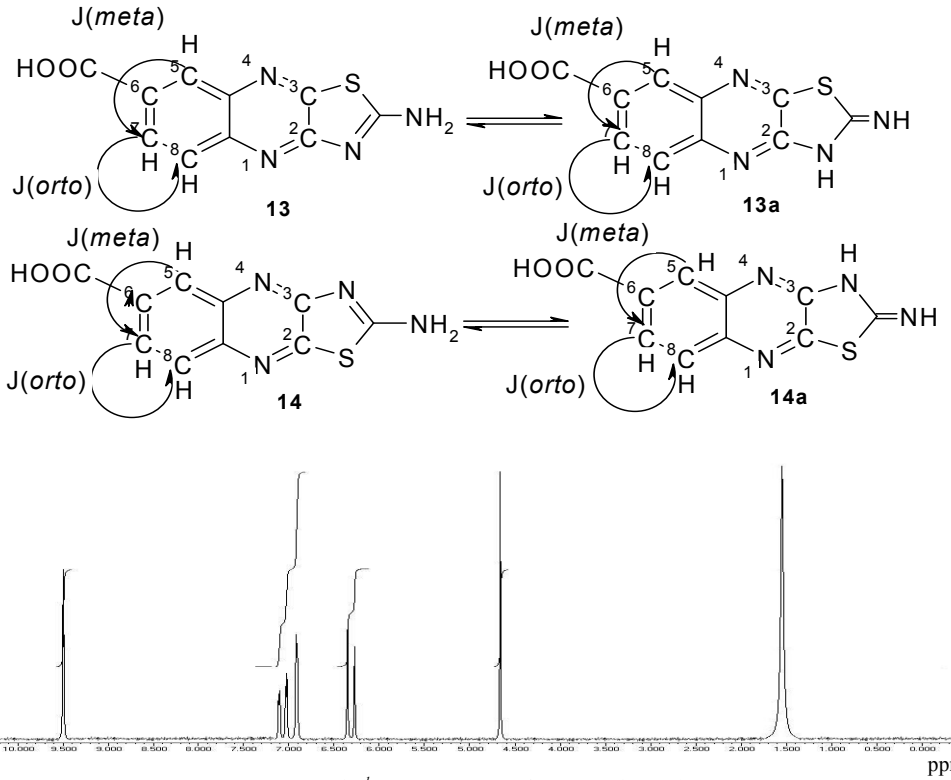

Fig. 3. *The ^{1}H-NMR spectra of compound 13(14)*

The IR spectra [5,7] were made including the synthesized compounds in KBr disks, absorption been measured with SPECORD 75 IR apparatus and the results were:

– The IR spectra* of compounds **13**, **14** confirm the proposed structures in accordance with the values found about the thiazolic ring: ν = 1610, 1055, 2460 cm^{-1}.

- The values of IR spectra for the group C=O and –OH, appear at different frequency, due to the internal conjugation p-π on the carboxyl group, frequency what appear of 1720 and 1430 cm^{-1}.
- The presence of the chinoxalinic ring appear at 1620, 1560, 1270 and 1350 cm^{-1} (depending on $\nu_{C=N}$ and ν_{C-N-}); this values were due to the conjugation with aromatic ring (the bands is sharps), lead at the values diminution $\nu_{C=N}$ and the intensity increase of valence vibration ν_{C-N}.
- The $–NH_2$ groups were observed, in IR spectra, about sharps bands of vibrations; the band more high correspond to asymmetrical valence vibration and this with the more small correspond of symmetrical valence vibration*(*the IR spectra of mixture is identically with those of the pure products).*

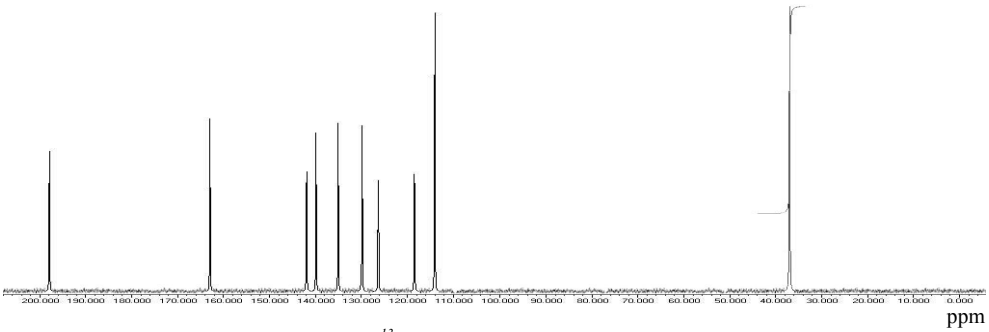

Fig. 4. *The ^{13}C-NMR spectra of compound 13(14)*

The elementary analysis results were presented in Table 3.

Table 3. Elemental analysis of compounds 5-14

Compound	C	%	N	%	O	%	S	%
	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.
5	46.15	46.01	15.38	15.12	35.16	35.09	-	-
6	55.26	55.11	18.42	18.31	21.05	21.12	-	-
7	52.42	52.37	13.59	13.51	31.06	30.84	-	-
8	44.44	43.87	11.52	10.93	13.16	13.21	-	-
9(10)	45.19	44.91	15.81	15.65	12.05	12.11	12.05	12.09
11(12)	48.78	48.43	22.76	22.51	13.00	13.06	13.00	12.95
13(14)	48.78	48.40	22.76	22.51	13.00	13.06	13.00	12.95

The biological activities for these two synthesized compact condensed systems were investigated.

White mice of Swiss race weighing 20+/-2g and white rats of Wistar race weighing 140+/-20g were used in batches of 20 animals (10 males am=and 10 females) and the dose to be

tested. The solutions, 0.01% of heterocyclic systems were injected as water-solutions, in a unique dose [8,9].

The animals were supervised for 14 days and the modifications of their behaviour and the mortality have been registered. At the end of the testing period, there were performed biochemical determinations and anatomic-pathological exams of the rats in order to disclose possible damages of their principal internal organs. The conclusions:

1) For the maximum tolerated dose administrated p.o. and i.p., no particular clinical phenomena occurred and no mortality.
2) The biochemical tests applied did not show any modification comparing to the witnesses.
3) The anatomic-pathological exam of the rat at the end of the testing period does not show modifications of the principal internal organs for any dose of systems used.
4) The studies of acute toxicity applied on 2 species and using 2 ways of administration, with solutions of 0,01% heterocyclic systems proved no toxicity for both p.o. and i.p. administration. Therefore, the DL_{50} could not be calculated but it was determined the maximum dose.

The antimicrobial activity was tested to a stem of *Penicillium glaucum*. This type of mould is a superior one, is very wide spread in the exterior environment and is one of the most resistant to the action of external factors. After this mould has been isolated and obtained in pure culture, it was prepared a suspension of spores in sterile water with $4x10^6$ spores/ml, using the Thorna counter camera.[6,8,9]

After several dilutions in decimal system were made inseminations with the same inocul in Petri dishes with fluidized and cooled at around 45^0C Czapek medium, in the alternative of witness specimen without colouring agent added, used in relation to the growth medium, in a dose of 0.01 and 0.02%. The above samples were optimally maintained at 25^0C for 15 days. Afterward, the colonies developed were counted and the antifungal effect of the heterocyclic systems has been assessed.

The concentrations used to apply the colouring agent on fabrics were taken into account during the tests for the evaluation of their antimicrobial activity.

The results are presented in Table 4.

Table 4. Antimicrobial activity of 2-aminothiazolo[4,5-b]chinoxalin-6-carboxylic acid

Probe	Number colonies/plate	Inhibition of the growth, %	Observations
Witness	185	–	Colonies in diameter 2-4 mm, pigmented in light green, characteristic the stem of Penicillium glaucum
0.01%	136	26.08	More of ½ colonies have white color with yellow shadow.
0.02%	34	73.16	Number greatest of colonies, white colors, in comparative with the probe in which was utilized one doze de 0.01% synthesized compound organic

By doubling the concentration of 2-aminothiazolo [4,5-b]chinoxaline-6-carboxylic acid, the percent of the inhibiting effect over the growth of Penicillium stem increased by 2.5 times approximately. The antimicrobial effect of these dyes became obvious regarding also the dimensions of the colonies developed, they being much smaller (not reaching the maturity), by comparison with the tests performed with the other chemical compounds.

Conclusions

Were obtained new compounds by an original method, very profitable both technological and economical. The synthesized compounds were characterized by IR, UV-VIS and NMR spectroscopy, thin layer chromatography, HPLC, elementary analysis and decomposition points. The biological activities were demonstrated too for synthesized compact condensed heterocyclic systems.

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