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Original article

Synthesis, antimicrobial activity and QSARs of new benzoxazine-3-ones

S. Alper-Hayta [a], E. Akı-Sener [a,*], B. Tekiner-Gulbas [a], I. Yıldız [a], O. Temiz-Arpacı [a], I. Yalcın [a], N. Altanlar [b]

[a] *Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Degol Street, TR-06100 Tandogan, Ankara, Turkey*
[b] *Ankara University, Faculty of Pharmacy, Department of Microbiology, TR-06100 Tandogan, Ankara, Turkey*

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Abstract

New ethyl 3,4-dihydro-3-oxo-4,6,7-trisubstituted-2*H*-1,4-benzoxazine-2-acetate derivatives were synthesized and their structures were elucidated by IR, ^{1}H NMR and mass spectral data. Antimicrobial activity of the compounds was investigated by using the method of twofold serial dilution technique against different Gram-positive, Gram-negative bacteria and some *Candida* species in comparison to standard drugs. Microbiological results indicated that the synthesized compounds possessed a broad spectrum of activity having MIC values of 6.25–100 μg/ml against the tested microorganisms. The QSAR analysis of a set of these compounds tested for growth inhibitory activity against *Candida krusei* was performed by using the computer-assisted multiple regression procedure. The activity contributions for substituent effects of these compounds were determined from the correlation equation for predictions of the lead optimization.

Keywords: 1,4-Benzoxazine-3-one; Antibacterial activity; Antifungal activity; QSAR

1. Introduction

The usage of most antimicrobial agents is limited, not only by the rapidly developing drug resistance, but also by the unsatisfactory status of present treatments of bacterial and fungal infections and drug side effects [1–4]. Therefore, the development of new and different antimicrobial drugs is a very important objective and much of the research program efforts are directed towards the design of new agents.

A group of 1,4-benzoxazine-3-ones was isolated from maize, wheat and rye several years ago [5–9]. 2,4-Dihydroxy-1,4-benzoxazine-3-one, DIBOA (Fig. 1) and its methoxy derivative DIMBOA (Fig. 1) have been shown to inhibit germination of spores of the phytopathogenic fungi [10]. They play an important role in the chemical defence of cereals against deleterious pests such as insects, pathogenic fungi and bacteria [11–14]. These molecules are present naturally in the plants as glucosides from which the aglycones are released rapidly by enzymatic hydrolysis after physical and biological injury of the plants and they exhibit antifungal, antibacterial and insecticide properties [15,16]. Only a limited number of compounds containing 1,4-benzoxazine ring system have been studied for their chemotherapeutic activity. Ofloxacin (Fig. 1) is one of the antimicrobial agents possessing the 1,4-benzoxazine ring system in its structure. All these observations prompted us to investigate this heterocyclic system to ascertain if it would offer any advantage over the other known clinically used antimicrobial drugs [17,18].

The common method for preparing 1,4-benzoxazine ring having ethyl acetate group at position 2 is by heating aminophenols with ethyl 2,3-dibromopropanoate and potassium carbonate in acetone [19].

Michael addition method is used for preparing 3,4-dihydro-3-oxo-2*H*-1,4-benzoxazine-2-acetates. Reaction of various *o*-aminophenols with maleic anhydride refluxed in ethanol in the presence of triethylamino or ether or benzene gives 1,4-benzoxazine-3-ones with ethyl acetate group at position 2 in

* Corresponding author. Tel.: +90 312 2239253; fax: +90 312 2236940.
E-mail address: sener@pharmacy.ankara.edu.tr (E. Akı-Sener).

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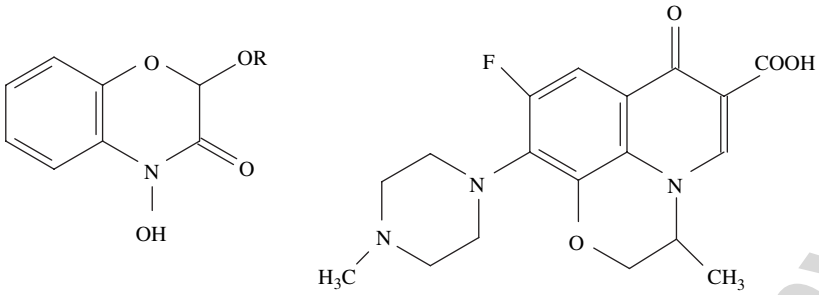


1 DIBOA, R=H
2 DIMBOA, R=OCH_3

3 OFLOXACIN

Fig. 1. Biologically active 1,4-benzoxazines.

%30 yield [20–22]. Another similar method is heating *o*-aminophenol with fumaric acid chloride monoethylester. This reaction gives the fumaramides which upon treatment with potassium carbonate in ethanol produced 3-oxo-2*H*-1,4-benzoxazine-2-acetates in %26–90 yield [22,23].

Alkylation of 2*H*-1,4-benzoxazine-3-one derivatives occurs at the ring nitrogen. Using alkyl halides and sodium hydride as bases is the most common method for alkylation [24,25].

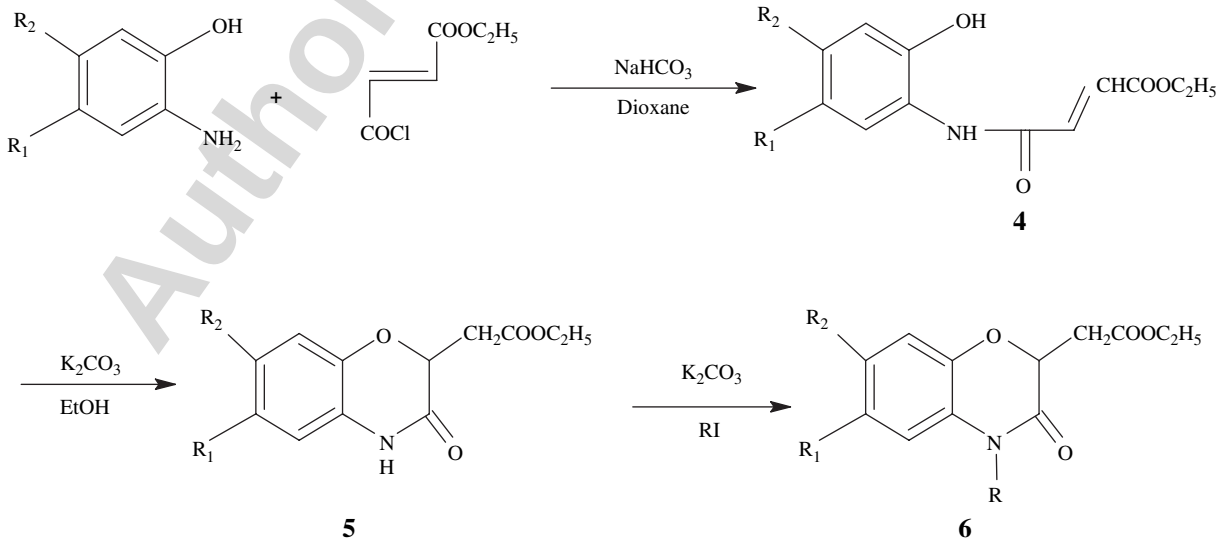
In this research, some novel ethyl 3,4-dihydro-3-oxo-6 and/or 7-disubstituted-2*H*-1,4-benzoxazine-2-acetate compounds and their *N*-alkylated derivatives were synthesized in order to interpret their antifungal activity against different *Candida* species and antibacterial activity against different Gram-positive and Gram-negative bacteria. The target of this research was to observe differences between the derivatives with free NH and *N*-alkylated ones towards antimicrobial activity. The alkylated derivatives were synthesized to avoid the behaviour of free NH as a hydrogen bond donor. 2D-QSAR analysis was also aimed to explain clearly the relation of physicochemical parameters with the activity. QSAR analysis of these compounds was performed by multiple regression analysis in order to predict the lead optimization for antifungal activity against *Candida krusei*.

2. Chemistry

Monoethyl fumaryl chloride was added to a mixture of appropriate *o*-aminophenol and sodium bicarbonate in dioxane. The prepared phenylcarbamoyl acrylate derivatives **4** were then stirred with potassium carbonate in ethanol at room temperature to form compound **5**. Alkylated compounds **6** were prepared by stirring compound **5** in acetone with methyliodide and/or ethyliodide and potassium carbonate (Scheme 1).

3. Results and discussion

A series of ethyl 3,4-dihydro-3-oxo-4,6,7-trisubstituted-2*H*-1,4-benzoxazine-2-acetate derivatives (**7**–**23**) have been synthesized by using a three-step procedure as shown in Scheme 1. All of the derivatives were supported by spectral data. The IR and ^{1}H NMR spectra are in agreement with the proposed



Scheme 1. Synthesis of 1,4-benzoxazine derivatives.

structures. Physical and spectral data of the compounds are reported in Section 4. In order to determine the antimicrobial activity of the synthesized compounds (**7**–**23**), three Gram-positive, two Gram-negative bacteria and three *Candida* species were screened using the twofold serial dilution technique [26]. All biological results of the compounds are given in Table 1.

Compounds showed antimicrobial activity against *Staphylococcus aureus* and *Streptococcus faecalis* at MIC values of 25–50 μg/ml. Compounds **11** and **13** were found to be more active than the others at an MIC value of 12.5 μg/ml against *Bacillus subtilis*. The synthesized compounds showed antimicrobial activity with MIC values between 25 and 100 μg/ml against *Escherichia coli* which exhibited lower potencies than the compared control drugs. Compound **7** was found to be the most active derivative against *Pseudomonas aeruginosa* at an MIC value of 12.5 μg/ml among the tested compounds, and showed the same potency with Gentamycin.

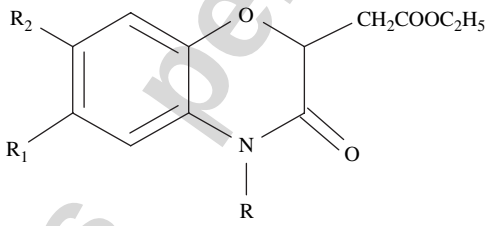
Compounds **7**–**23** were also tested against *Candida albicans*, *Candida krusei*, and *Candida glabrata* for their antimycotic activity, and they indicated significant antimycotic activity displaying MIC values between 6.25 and 50 μg/ml. Although compounds **13**, **14**, **15**, **17** and **19** showed activity having an MIC value of 12.5 μg/ml against *C. albicans*, they exhibited lower potencies than the compared control drugs. Compounds **8** and **12** were found to be the most active derivatives against *C. krusei* and *C. glabrata* at an MIC value of 6.25 μg/ml among the other compounds whereas compound **10** showed the same activity against *C. krusei*.

According to structure–activity relationships (SAR), it can be concluded that compounds with a free NH (**7**–**13**) are generally more active than the *N*-alkylated ones (**14**–**23**) against selected microorganisms and an electron-withdrawing substituent at position 6 of benzoxazine moderately favours the inhibition of the growth. Additionally, a nitro group at position 7 of the fused ring slightly helped to inhibit the growth of the microorganisms.

Furthermore, the QSARs of these molecules were analysed by multiple regression analysis (MRA) in order to predict the lead optimization in this set of compounds. From the data as seen in Table 2, the following QSAR was developed:

$$\log 1/C = +0.0499(\pm 0.039)B_{1R} + 0.169(\pm 0.053)\sigma_{R_1} + 0.0569(\pm 0.020)I_{xR_2} + 3.950 \quad (1)$$

Table 1
The *in vitro* antimicrobial activity of the synthesized compounds and the control drugs



Comp. no.	R	R_1	R_2	Fungi			Gram-positive			Gram-negative	
				C. albicans	*C. krusei*	*C. glabrata*	*S. aureus*	*St. faecalis*	*B. subtilis*	*E. coli*	*P. aeruginosa*
7	H	H	H	25	12.5	12.5	50	50	25	50	12.5
8	H	CH_3	H	25	6.25	6.25	50	50	25	25	25
9	H	Cl	H	25	12.5	12.5	50	50	25	50	50
10	H	$COOC_2H_5$	H	25	6.25	12.5	50	50	25	50	25
11	H	H	NO_2	25	12.5	12.5	25	25	12.5	25	50
12	H	H	NH_2	50	6.25	6.25	25	50	25	50	25
13	H	Cl	NO_2	12.5	12.5	12.5	25	25	12.5	25	25
14	CH_3	H	H	12.5	12.5	12.5	50	50	25	50	25
15	CH_3	CH_3	H	12.5	12.5	12.5	50	50	25	25	25
16	CH_3	Cl	H	25	50	25	50	50	50	50	25
17	CH_3	$COOC_2H_5$	H	12.5	12.5	12.5	50	50	25	50	25
18	CH_3	Cl	NO_2	25	12.5	12.5	50	50	50	25	25
19	CH_3	H	NO_2	12.5	12.5	12.5	50	50	50	50	25
20	C_2H_5	H	H	25	25	25	25	–	25	100	–
21	C_2H_5	Cl	H	25	25	25	25	–	50	50	–
22	C_2H_5	H	NO_2	25	50	25	50	–	50	50	–
23	C_2H_5	Cl	NO_2	25	25	25	25	–	100	100	–
Oxiconazole	–	–	–	6.25	–	–	–	–	–	–	–
Haloprogin	–	–	–	3.12	–	–	–	–	–	–	–
Miconazole	–	–	–	3.12	1.56	3.12	–	–	–	–	–
Ciprofloxacin	–	–	–	–	–	–	3.125	–	1.56	3.125	0.78
Gentamycin	–	–	–	–	–	–	3.125	12.5	1.56	12.5	12.5

Table 2
The parameters used in Eq. (1)

Comp. no.	I_{xR_2}	σ_{R_1}	B_{1R}	Obs. log 1/*C*	Calc. log 1/*C*	Residuals
7	0	0.00	1.00	3.973	4.000	−0.027
8	0	−0.17	1.00	3.998	3.972	0.026
9	0	0.23	1.00	4.032	4.039	−0.007
10	0	0.45	1.00	4.089	4.076	0.013
11	1	0.00	1.00	4.049	4.057	−0.008
12[a]	–	–	–	–	–	–
13	1	0.23	1.00	4.099	4.096	0.003
14	0	0.00	1.52	3.998	4.026	−0.028
15	0	−0.17	1.52	4.022	3.997	0.025
16	0	0.23	1.52	4.054	4.065	−0.011
17	0	0.45	1.52	4.106	4.102	0.004
18	1	0.23	1.52	4.118	4.122	−0.004
19	1	0.00	1.52	4.070	4.083	−0.013
20	0	0.00	1.52	4.022	4.026	−0.004
21	0	0.23	1.52	4.075	4.065	0.010
22	1	0.00	1.52	4.091	4.083	0.008
23	1	0.23	1.52	4.136	4.122	0.014

[a] This compound is outlier in QSAR analysis.

$n = 16$, $R^2 = 0.942$, $s = 0.018$, $F = 31.471 > F_{\text{tab}(3,\ 12,\ 0.05)} = 3.49$, $Q^2 = 0.790$, $S_{\text{PRESS}} = 0.024$.

Eq. (1) was found to be the best fit for the predictions according to the examined validation test results. As can be deduced from Table 2, the goodness of fit of equation is significant, possessing a high R^2 (0.942) and a small s (0.018) with an overall F test value of 31.471 at a significant level of $p < 0.05$. The correlation coefficients which are given in Table 3 revealed that there was no colinearity between the independent variables used in Eq. (1). The parameters used in this QSAR analysis together with the observed, calculated and residual values are given in Table 2.

QSAR analysis revealed that the substitution of positions R and R_1 was more significant than the position R_2 for the tested antifungal activity against *C. krusei*. The QSAR was developed based on the σ values of the substituents at position 6, where *C* is the minimum concentration of the compound that inhibited the growth of *C. krusei*. From this relationship, we see that an electron-withdrawing substituent at position 6 of benzoxazine favours inhibition of growth. The positive coefficients of B_1 parameter indicated that the width of the substituent at position 4 was conducive for the activity. Therefore, the compounds having an N–H instead of *N*-alkylated of 1,4-benzoxazine moiety were found to be more active than the others.

In conclusion, it was found that the 2D-QSAR study confirmed the SAR study for antimycotic activity against *C. krusei*. We could conclude that the width substituent on position R is important for the antifungal activity against *C. krusei*. These observations could guide us to design further new lead antifungal compounds.

Table 3
Correlation matrix of variables used in Eq. (1)

	X-1	*X*-2	*X*-3
X-1	1.000	0.001	0.004
X-2		1.000	0.000
X-3			1.000

$X - 1 = I_{xR_2}$, $X - 2 = \sigma_{R_1}$, $X - 3 = B_{1R}$.

4. Experimental procedures

4.1. Chemistry

Silicagel HF_{254} chromatoplates (0.3 mm) were used for TLC. The solvent systems were chloroform:methanol (15:0.5) and ethyl acetate:*n*-hexane (20:10) for compounds **7–23**. Melting points were taken on a Buchi SMP 20 capillary apparatus and are uncorrected. IR spectra were recorded by FT/IR-420 in KBr discs. ^{1}H NMR spectra were obtained with a Bruker GmbH D PX-400 MHz spectrometer in chloroform; tetramethylsilane (TMS) was used as an internal standard. Elemental analyses were carried out with a Perkin Elmer model 240-C apparatus. The results (C, H, N) were within ±0.4% of the calculated values.

4.2. General procedure for compounds 7–23

Monoethyl fumaric acid (10 mmol) and thionyl chloride (5 mmol) were stirred for 3 h at 80 °C in benzene (15 ml). At the end of the reaction, excess thionyl chloride was evaporated. Obtained monoethyl fumaryl chloride was added dropwise to a suspension of appropriate *o*-aminophenol (10 mmol) and $NaHCO_3$ (10 mmol) in dioxane. The reaction mixture was stirred for 24 h at room temperature, then poured into water and extracted with ethyl acetate (15 ml). The extract was washed with water, dried and evaporated, respectively. The residue was recrystallised from EtOH to give **4** ethyl 3-[(4- and/or 5-substituted-2-hydroxyphenyl)carbamoyl]acrylate derivatives. A suspension of these compounds (10 mmol) and K_2CO_3 (5 mmol) in EtOH (70 ml) was stirred for 3 h at room temperature. The reaction mixture was poured into water and extracted with ethyl acetate (15 ml). The extracts were washed with water, dried and evaporated, respectively [22,23]. The residue was recrystallised from EtOH.

The general synthetic procedure as seen above was employed to prepare compounds **14–23** involving the reaction of methyliodide or ethyliodide (15 mmol) and ethyl 3,4-dihydro-6-and/or 7-substituted-3-oxo-2*H*-1,4-benzoxazine-2-acetate derivatives (10 mmol) **5**, by heating in the presence of potassium carbonate (15 mmol) in acetone (100 ml) for 4 h at 40 °C. After removal of the solvent, the residue was extracted with ethyl acetate (15 ml). The extracts were washed with water, then dried and evaporated, respectively [24,25]. The residue was recrystallised from petroleum ether.

For the preparation of compounds **10** and **17**, first of all, mixture of 3-amino-4-hydroxy benzoic acid (10 mmol), sulphuric acid (0.5 ml) and absolute ethanol (30 ml) was cooled in an ice bath and dry HCl gas was passed through it. When the mixture was saturated with HCl (g), it was stirred at room temperature for 5 h. At the end of the reaction, the precipitates were collected by filtration, washed with water and dried *in vacuo* to give ethyl 3-amino-4-hydroxy benzoate

crystals [27]. Then the general synthetic procedure as seen above was employed. Quantitative reduction of nitro group in compound **11** was achieved by catalytic hydrogenation to make compound **12**. Compound **11** (3.5 mmol) in EtOH (75 ml) was subjected to hydrogenation using 40 psi of H_2 and 10% Pd–C (40 mg) until uptake of H_2 ceased. The catalyst was filtered on a bed of celite, washed with EtOH, and the filtrate was concentrated *in vacuo* to give compound **12** [28].

*4.2.1. 3,4-Dihydro-3-oxo-2H-1,4-benzoxazine-2-acetate (**7**)*

$C_{12}H_{13}NO_4$ – m.p.: 106–107 °C [22]; yield (%): 80; ^{1}H NMR (ppm): 1.25 (t, 3H, $CH_2COOCH_2CH_3$, $J = 7.1$), 3.00 (dd, 2H, $CH_2COOCH_2CH_3$), 4.20 (q, 2H, $CH_2COOCH_2CH_3$, $J = 7.2$), 5.00 (q, 1H, *CH*), 6.80–7.10 (m, 4H, *aromatic H*), 8.50 (s, 1H, *NH*); IR (cm^{-1}): 3230–2860, 1740, 1695, 1625, 1520, 1480–1280, 1240–1030, 950–585; MS (70 eV) *m/z*: 235 (M+), 161 (100%).

*4.2.2. 3,4-Dihydro-3-oxo-6-methyl-2H-1,4-benzoxazine-2-acetate (**8**)*

$C_{13}H_{15}NO_4$ – m.p.: 115–116 °C [22]; yield (%): 36; ^{1}H NMR (ppm): 1.25 (t, 3H, $CH_2COOCH_2CH_3$, $J = 7.1$), 2.25 (s, 3H, CH_3), 2.80–3.00 (dd, 2H, $CH_2COOCH_2CH_3$), 4.20 (q, 2H, $CH_2COOCH_2CH_3$, $J = 7.1$), 5.00 (q, 1H, *CH*), 6.50 (d, 1H, 5-*H*, $J_{5,7} = 1.1$), 6.75–6.85 (m, 2H, 7-*H*/8-*H*), 8.10 (s, 1H, *NH*); IR (cm^{-1}): 3220–2890, 1750, 1690, 1618, 1430–1500, 1460–1330, 1300–1035, 990–590; MS (70 eV) *m/z*: 249 (M+), 42 (100%).

*4.2.3. 3,4-Dihydro-3-oxo-6-chloro-2H-1,4-benzoxazine-2-acetate (**9**)*

$C_{12}H_{12}NO_4Cl$ – m.p.: 143–145 °C [22]; yield (%): 50; ^{1}H NMR (ppm): 1.25 (t, 3H, $CH_2COOCH_2CH_3$, $J = 7.1$), 3.00 (dd, 2H, $CH_2COOCH_2CH_3$), 4.20 (q, 2H, $CH_2COOCH_2CH_3$, $J = 7.1$), 4.95 (q, 1H, *CH*), 6.75–6.95 (m, 3H, *aromatic H*), 8.30 (s, 1H, *NH*); IR (cm^{-1}): 3200–2895, 1740–1700, 1615, 1510, 1430–1330, 1300–1030, 990–590; MS (70 eV) *m/z*: 269 (M+), 195 (100%).

*4.2.4. 3,4-Dihydro-3-oxo-6-ethoxycarbonyl-2H-1,4-benzoxazine-2-acetate (**10**)*

$C_{15}H_{17}NO_6$ – m.p.: 125–130 °C; yield (%): 60; ^{1}H NMR (ppm): 1.15 (t, 3H, $CH_2COOCH_2CH_3$, $J = 7.1$), 1.30 (t, 3H, $COOCH_2CH_3$, $J = 7.1$), 3.00 (dd, 2H, $CH_2COOCH_2CH_3$), 4.30 (q, 2H, $CH_2COOCH_2CH_3$, $J = 7.1$), 4.90 (q, 2H, $COOCH_2CH_3$, $J = 7.1$), 5.10 (q, 1H, *CH*), 6.70–7.70 (q, 3H, *aromatic H*, $J_{8,5} = 8.5$, $J_0 = 2.0$, $J_{7,5} = 8.5$), 8.70 (s, 1H, *NH*); IR (cm^{-1}): 3200–2850, 1687, 1615, 1493, 1376, 1298–1132, 989–420; MS (70 eV) *m/z*: 308 (M+), 45 (100%).

*4.2.5. 3,4-Dihydro-3-oxo-7-nitro-2H-1,4-benzoxazine-2-acetate (**11**)*

$C_{12}H_{12}N_2O_6$ – m.p.: 172 °C [23]; yield (%): 39; ^{1}H NMR (ppm): 1.25 (t, 3H, $CH_2COOCH_2CH_3$, $J = 7.1$), 3.00 (dd, 2H, $CH_2COOCH_2CH_3$), 4.20 (q, 2H, $CH_2COOCH_2CH_3$), 5.00 (q, 1H, *CH*), 6.80 (d, 1H, 5-*H*, $J_m = 8.7$), 7.80 (m, 2H, 6-*H*/8-*H*), 8.30 (s, 1H, *NH*); IR (cm^{-1}): 3200–2900, 1740–1690, 1613, 1550–1510, 1440–1320, 1260–1030, 990–730; MS (70 eV) *m/z*: 280 (M+), 42 (100%).

*4.2.6. 3,4-Dihydro-3-oxo-7-amino-2H-1,4-benzoxazine-2-acetate (**12**)*

$C_{12}H_{14}N_2O_4$ – m.p.: 150 °C; yield (%): 35; ^{1}H NMR (ppm): 1.25 (t, 3H, $CH_2COOCH_2CH_3$, $J = 7.2$), 3.00 (dd, 2H, $CH_2COOCH_2CH_3$), 3.50 (s, 2H, NH_2), 4.20 (q, 2H, $CH_2COOCH_2CH_3$), 5.00 (q, 1H, *CH*), 6.20–6.50 (m, 3H, *aromatic H*, $J_m = 8.3$), 7.60 (s, 1H, *NH*); IR (cm^{-1}): 3400–2800, 1733–1682, 1523, 1375, 1278–1050, 855–439; MS (70 eV) *m/z*: 250 (M+), 83 (100%).

*4.2.7. 3,4-Dihydro-3-oxo-6-chloro-7-nitro-2H-1,4-benzoxazine-2-acetate (**13**)*

$C_{12}H_{11}N_2O_6Cl$ – m.p.: 162–165 °C [23]; yield (%): 50; ^{1}H NMR (ppm): 1.25 (t, 3H, $CH_2COOCH_2CH_3$, $J = 7.2$), 3.00 (dd, 2H, $CH_2COOCH_2CH_3$), 4.10 (q, 2H, $CH_2COOCH_2CH_3$, $J = 7.1$), 5.00 (q, 1H, *CH*), 6.90 (s, 1H, 5-*H*), 7.60 (s, 1H, 8-*H*), 8.40 (s, 1H, *NH*); IR (cm^{-1}): 3200–2900, 1740–1690, 1615, 1550–1515, 1440–1319, 1250–1000, 950–685; MS (70 eV) *m/z*: 314 (M+), 43 (100%).

*4.2.8. 3,4-Dihydro-3-oxo-4-methyl-2H-1,4-benzoxazine-2-acetate (**14**)*

$C_{13}H_{15}NO_4$ – m.p.: 35 °C; yield (%): 40; ^{1}H NMR (ppm): 1.25 (t, 3H, $CH_2COOCH_2CH_3$, $J = 7.1$), 3.00 (dd, 2H, $CH_2COOCH_2CH_3$), 3.40 (s, 3H, CH_3), 4.20 (q, 2H, $CH_2COOCH_2CH_3$, $J = 7.2$), 5.00 (q, 1H, *CH*), 7.10 (m, 4H, 5-*H*, 6-*H*, 7-*H*, 8-*H*); IR (cm^{-1}): 1744–1683; MS (70 eV) *m/z*: 249 (M+), 249 (100%).

*4.2.9. 3,4-Dihydro-3-oxo-4,6-dimethyl-2H-1,4-benzoxazine-2-acetate (**15**)*

$C_{14}H_{17}NO_4$ – m.p.: 58 °C; yield (%): 45; ^{1}H NMR (ppm): 1.30 (t, 3H, $CH_2COOCH_2CH_3$, $J = 7.1$), 2.30 (s, 3H, CH_3), 3.00 (dd, 2H, $CH_2COOCH_2CH_3$), 3.45 (s, 3H, CH_3), 4.25 (q, 2H, $CH_2COOCH_2CH_3$, $J = 7.1$), 5.00 (q, 1H, *CH*), 6.80 (m, 3H, 5-*H*, 7-*H*, 8-*H*); IR (cm^{-1}): 1743–1691; MS (70 eV) *m/z*: 263 (M+), 189 (100%).

*4.2.10. 3,4-Dihydro-3-oxo-4-methyl-6-chloro-2H-1,4-benzoxazine-2-acetate (**16**)*

$C_{13}H_{14}NO_4Cl$ – m.p.: 56 °C; yield (%): 41; ^{1}H NMR (ppm): 1.25 (t, 3H, $CH_2COOCH_2CH_3$, $J = 7.1$), 3.00 (dd, 2H, $CH_2COOCH_2CH_3$), 3.40 (s, 3H, CH_3), 4.20 (q, 2H, $CH_2COOCH_2CH_3$, $J = 7.1$), 5.00 (q, 1H, *CH*), 6.90 (m, 3H, 5-*H*, 7-*H*, 8-*H*); IR (cm^{-1}): 1741–1692; MS (70 eV) *m/z*: 283 (M+), 82 (100%).

*4.2.11. 3,4-Dihydro-3-oxo-4-methyl-6-ethoxycarbonyl-2H-1,4-benzoxazine-2-acetate (**17**)*

$C_{16}H_{19}NO_6$ – m.p.: 40 °C; yield (%): 35; ^{1}H NMR (ppm): 1.25 (t, 3H, $CH_2COOCH_2CH_3$, $J = 7.2$), 1.30 (t, 3H, $J = 7.1$), 3.00 (dd, 2H, $CH_2COOCH_2CH_3$), 3.45 (s, 3H, CH_3), 4.25 (q, 2H, $CH_2COOCH_2CH_3$, $J = 7.1$), 4.35 (q, 2H, $J = 7.1$), 5.00

(q, 1H, *CH*), 7–7.75 (m, 3H, 5-*H*, 7-*H*, 8-*H*); IR (cm^{-1}): 1711; MS (70 eV) *m/z*: 321 (M+), 247 (100%).

*4.2.12. 3,4-Dihydro-3-oxo-4-methyl-6-chloro-7-nitro-2H-1,4-benzoxazine-2-acetate (**18**)*

$C_{13}H_{13}N_2O_6Cl$ – m.p.: not detected; yield (%): 19; ^1H NMR (ppm): 1.30 (t, 3H, $CH_2COOCH_2CH_3$, $J = 7.1$), 3.00 (dd, 2H, $CH_2COOCH_2CH_3$), 3.45 (s, 3H, CH_3), 4.20 (q, 2H, $CH_2COOCH_2CH_3$), 5.00 (q, 1H, *CH*), 6.80 (s, 1H, 5-*H*), 7.30 (s, 1H, 8-*H*); IR (cm^{-1}): 1699; MS (70 eV) *m/z*: 328 (M+), 55 (100%).

*4.2.13. 3,4-Dihydro-3-oxo-4-methyl-7-nitro-2H-1,4-benzoxazine-2-acetate (**19**)*

$C_{13}H_{14}N_2O_6$ – m.p.: 95–100 °C; yield (%): 50; ^1H NMR (ppm): 1.35 (t, 3H, $CH_2COOCH_2CH_3$, $J = 7.1$), 3.10 (m, 2H, $CH_2COOCH_2CH_3$), 3.45 (s, 3H, CH_3), 4.30 (q, 2H, $CH_2COOCH_2CH_3$, $J = 7.1$), 5.10 (q, 1H, *CH*), 7.10 (d, 1H, 5-*H*, $J = 9.0$), 7.90 (d, 1H, 8-*H*, $J_0 = 2.5$), 8.10 (dd, 1H, 6-*H*, $J = 2.5$); IR (cm^{-1}): 1716–1598; MS (70 eV) *m/z*: 294 (M+), 220 (100%).

*4.2.14. 3,4-Dihydro-3-oxo-4-ethyl-2H-1,4-benzoxazine-2-acetate (**20**)*

$C_{14}H_{17}NO_4$ – m.p.: 40–45 °C; yield (%): 34; ^1H NMR (ppm): 1.10–1.40 (overlapped, 6H, CH_3), 3.00 (dd, 2H, $CH_2COOCH_2CH_3$), 3.90 (q, 2H, CH_2CH_3), 4.10 (q, 2H, $COOCH_2CH_3$), 4.90 (q, 1H, *CH*), 7.00 (m, 4H, 5-*H*, 6-*H*, 7-*H*, 8-*H*); IR (cm^{-1}): 1744–1683; MS (70 eV) *m/z*: 263 (M+), 263 (100%).

*4.2.15. 3,4-Dihydro-3-oxo-4-ethyl-6-chloro-2H-1,4-benzoxazine-2-acetate (**21**)*

$C_{14}H_{16}NO_4Cl$ – m.p.: 65 °C; yield (%): 46; ^1H NMR (ppm): 1.10–1.20 (overlapped, 6H, CH_3), 3.00 (dd, 2H, $CH_2COOCH_2CH_3$), 3.90 (q, 2H, CH_2CH_3), 4.20 (q, 2H, $COOCH_2CH_3$), 5.00 (q, 1H, *CH*), 6.90–7.00 (m, 3H, 5-*H*, 7-*H*, 8-*H*); IR (cm^{-1}): 1741–1692; MS (70 eV) *m/z*: 297 (M+), 297 (100%).

*4.2.16. 3,4-Dihydro-3-oxo-4-ethyl-7-nitro-2H-1,4-benzoxazine-2-acetate (**22**)*

$C_{14}H_{16}N_2O_6$ – m.p.: 100 °C; yield (%): 52; ^1H NMR (ppm): 1.10–1.30 (overlapped, 6H, CH_3), 3.00 (dd, 2H, $CH_2COOCH_2CH_3$), 3.90 (q, 2H, CH_2CH_3), 4.10 (q, 2H, $COOCH_2CH_3$), 4.90 (q, 1H, *CH*), 7.10 (d, 1H, 5-*H*), 7.90 (d, 1H, 8-*H*), 8.00 (dd, 1H, 6-*H*); IR (cm^{-1}): 1716–1598; MS (70 eV) *m/z*: 308 (M+), 97 (100%).

*4.2.17. 3,4-Dihydro-3-oxo-4-ethyl-6-chloro-7-nitro-2H-1,4-benzoxazine-2-acetate (**23**)*

$C_{14}H_{15}N_2O_6Cl$ – m.p.: 75–80 °C; yield (%): 31; ^1H NMR (ppm): 1.10–1.35 (overlapped, 6H, CH_3), 3.00 (dd, 2H, $CH_2COOCH_2CH_3$), 4.00 (q, 2H, CH_2CH_3), 4.10 (q, 2H, $COOCH_2CH_3$), 5.00 (q, 1H, *CH*), 6.80 (s, 1H, 5-*H*), 7.30 (s, 1H, 8-*H*); IR (cm^{-1}): 1699; MS (70 eV) *m/z*: 342 (M+), 264 (100%).

4.3. Microbiology

For the antibacterial and antimycotic assays, the compounds were dissolved in absolute ethanol (0.8 mg/ml). Further dilutions of the compounds and standard drugs in the test medium were prepared at the required quantities of 400, 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 μg/ml concentrations with Mueller–Hinton broth and Sabouraud dextrose broth. The minimum inhibitory concentrations (MICs) were determined using the twofold serial dilution technique [26]. A control test was also performed containing inoculated broth supplemented with only ethanol at the same dilutions used in our experiments and found inactive in the culture medium. All the compounds were tested for their *in vitro* growth inhibitory activity against different bacteria and the yeasts *C. albicans ATCC 10145*, *C. krusei ATCC 6258*, *C. glabrata (isolated)*. Origin of bacterial strains are *S. aureus ATCC 25923*, *B. subtilis ATCC 6633* and *St. faecalis ATCC 10541* as Gram-positive and *E. coli ATCC 23556* and *P. aeruginosa ATCC 10145* as Gram-negative bacteria. ATCC strains of the microorganisms used in this study were obtained from the culture collection of Refik Saydam Health Institution of Health Ministry, Ankara, and maintained at the Microbiology Department of Faculty of Pharmacy of Ankara University.

Oxiconazole, Haloprogin, Ciprofloxacin, Gentamycin and Miconazole were used as control drugs. The data on the antifungal and antibacterial activities of the compounds and the control drugs as MIC, μg/ml, values are given in Table 1.

4.3.1. Antibacterial and antifungal assays

The cultures were obtained from Mueller–Hinton broth (Difco) for all the bacterial strains after 24 h of incubation at 37 ± 1 °C. *C. albicans*, *C. krusei* and *C. glabrata* were maintained in Sabouraud dextrose broth (Difco) after incubation for 24 h at 25 ± 1 °C. Testing was carried out in Mueller–Hinton broth and Sabouraud dextrose broth (Difco) at pH 7.4 and the twofold serial dilution technique was applied. The final inoculum size was 10^5 CFU/ml for the antibacterial assay and 10^4 CFU/ml for the antifungal assay. A set of tubes containing only inoculated broth were used as controls. For the antibacterial assay after incubation for 24 h at 37 ± 1 °C and after incubation for 48 h at 25 ± 1 °C for the antifungal assay, the last tube with no growth of microorganism and/or yeast was recorded to represent the MIC expressed in μg/ml. Every experiment in the antibacterial and antifungal assays was done in duplicate.

4.4. Data processing

Hansch analysis method which is an extra-thermodynamic approach in QSAR analysis was applied in order to determine the lead optimization due to the various physicochemical (electronic, steric and hydrophobic) parameters and structural indicator parameters [29,30].

Regression analysis and calculations were run on a PC using the BILIN statistical program package [31]. In equations, the figures in parentheses are the standard errors of the

regression coefficients. For a given equation, n is the number of compounds, R^2 denotes the square of the multiple correlation coefficients, F is the significance test and s represents the residual standard deviation.

On the other hand, in order to judge the predictive power as Q^2 and/or S_{PRESS} values of the performed QSAR model was also calculated by cross-validation technique which is a method to check the validity of regression models by eliminating each object leave-one-out technique [32].

4.5. Determination of parameters

In this study, the model is based on the *in vitro* activity of certain ethyl 3,4-dihydro-3-oxo-4,6,7-trisubstituted-2*H*-1,4-benzoxazine-2-acetate derivatives against *C. krusei*, where *C* is the MIC value expressed in molar concentration units (Table 1).

The variables used as descriptors in the analysis are electronic, steric and structural parameters. The structural indicator variable I_x expresses the replacement of H by nitro group at R_2 (position 7). I_x defined as 1 for NO_2, and as 0 for H.

The screened physicochemical parameters in this QSAR study are π for the hydrophobic effects, σ, F (field effect), R (resonance effect) as electronic influences and Verloop's STERIMOL parameters (L and B_1) for the steric interactions of the substituents R, R_1 and R_2. Values for all candidate physicochemical variables used in this QSAR study were taken from the table of Hansch and Leo [33]. The values of the descriptors used in the best equation are shown in Table 2.

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