

## ORIGINAL ARTICLE

# Anti-*Helicobacter pylori* activity of some newly synthesized derivatives of xanthone

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A series of 20 xanthone derivatives was synthesized and evaluated for anti-*Helicobacter pylori* (*H. pylori*) activity. Qualitative and quantitative *in vitro* tests using the Kirby–Bauer method (agar disc-diffusion method) were performed. The tested compounds were screened against clarithromycin- and/or metronidazole-resistant strains of *H. pylori*. As a reference, Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacterial strains were examined. On the basis of microbiological assays, xanthones can be considered as potential anti-*H. pylori* agents. They displayed significant activity against the examined strains, which was higher against the bacteria resistant to metronidazole than clarithromycin. The lowest MIC values ranging up to 20 mg l<sup>-1</sup> were observed for the following compounds: 3, 4, 8, 9, 12, 19 (against the metronidazole-resistant strains) and the compound 10 (against the clarithromycin-resistant strain). These preliminary results for screening of xanthone derivatives form a part of an ongoing study of the structure–activity relationships of a large group of compounds. Microbiological assays will be conducted afterwards to determine the mechanism of xanthones' action against *H. pylori*.

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## INTRODUCTION

Xanthone derivatives comprise an interesting group of compounds that, depending on the type of substituent and its localization in one of the xanthone's rings, exhibit diversified biological activity. Biological properties of synthetic xanthone derivatives have been investigated since 1968, when Da Re *et al.*<sup>1</sup> described, for the first time, central stimulating and analeptic activities of synthetic aminoalkyl xanthone derivatives. Since then, xanthone skeleton has been widely modified as it is a potential pharmacophoric structure. Literature reports confirmed broad spectrum of activity of both natural and synthetic derivatives of xanthone (including antimalarial,<sup>2</sup> antituberculous,<sup>3</sup> antibacterial<sup>4</sup> and antifungal<sup>5</sup> activity).

Searching for biologically active structures in the group of xanthone derivatives remains one of the main directions of the research carried out at our Department of Bioorganic Chemistry. We synthesized and evaluated a number of xanthone derivatives that exhibited microbiological activity, especially against *Mycobacterium tuberculosis*<sup>6</sup> or pathogenic bacteria and fungi.<sup>7</sup> We have not determined anti-*Helicobacter pylori* (*H. pylori*) activity of the synthesized xanthone derivatives so far, and according to our literature survey, only naturally occurring xanthenes or flavones isolated from *Garcinia fusca*,<sup>8</sup> *Artocarpus obtusus*,<sup>9</sup> *Glycyrrhiza glabra*<sup>10</sup> and *Cratoxylum arborescens* showing anti-*H. pylori* activity were described. Chimenti *et al.*<sup>11</sup> of La Sapienza University of Rome also reported anti-*H. pylori* activity of synthesized *N*-substituted derivatives of 2-oxo-2H-1-benzopyran-3-carboxamides which showed significant

structural similarity to the xanthone scaffold. These results encouraged us to synthesize xanthone derivatives with potential anti-*H. pylori* activity.

*H. pylori* is a Gram-negative, spiral-shaped bacterium that inhabits gastric mucosa of about 50% of human population worldwide. *H. pylori* is the main pathogenic factor of type B chronic gastritis. Moreover, *H. pylori* infections are associated with peptic ulcer disease or dyspepsia. Since the year 1994, the International Agency for Research on Cancer of the World Health Organization (IARC/WHO) concluded that *H. pylori* has been a class I carcinogen, that has an important role in mucosa-associated lymphoid tissue lymphoma and gastric cancer.<sup>12–18</sup> Furthermore, many studies revealed a relation between *H. pylori* infections and extra-gastric diseases like iron-deficiency anemia, idiopathic thrombocytopenic purpura, cardiovascular disorders, neurological diseases, as well as obesity, diabetes mellitus, asthma or diseases of oral mucosa.<sup>12,19–21</sup>

Guidelines for the management of *H. pylori* infection and treatment regimens are regularly issued by the European Helicobacter Study Group. Standard treatment protocol includes a combination of three kinds of drugs: a proton pump inhibitor, a cytoprotectant drug containing bismuth salts and an antibiotic or chemotherapeutic (clarithromycin, amoxicillin, metronidazole, tetracycline, levofloxacin, rifabutin). Despite 10–14 days of triple or quadruple therapy, a considerable increase of *H. pylori* resistance to the antimicrobial agents could be observed. Resistance to antimicrobials, especially to

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clarithromycin, is a major therapeutic problem because the standard treatment regimens become less effective.<sup>12,22</sup>

Discovery of novel antimicrobial compounds appears to be a good solution to overcome therapeutic problems resulting from increasing resistance of *H. pylori* strains to antibiotics and chemotherapeutics. The use of new drugs is therefore an alternative to the common antibiotics/chemotherapeutics and give chance to achieve a successful eradication therapy. Xanthenes are well-known for their antimicrobial and antifungal activity, as well as anti-*H. pylori* activity.<sup>8,23</sup>

Herein we report the *in vitro* activity of some new structures against *H. pylori* strains. Selected compounds were varied in order to find potential structure–activity relationships. Taking into account the structures of previously evaluated flavonoids and *N*-substituted derivatives of 2-oxo-2H-1-benzopyran-3-carboxamides,<sup>10</sup> our structures, *inter alia*, contained: halogen atoms, methoxyl, hydroxyl or ester groups, unsaturated bonds and additional aromatic rings.

## RESULTS

### Antibacterial activity

**Results of the qualitative testing.** The *in vitro* antibacterial activity of the 20 synthesized compounds against 12 representative strains of bacteria: *H. pylori* ATCC 43504, *H. pylori* ATCC 700684, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and 7 clinical *H. pylori* strains was evaluated by disc-diffusion method. Antibacterial activity of the tested compounds was estimated by measuring the diameters of the inhibition zones (mm). The obtained results for new compounds and the reference compounds were presented in Table 1. We used the interpretative criteria described above, that means zones with diameters <8 mm indicated no antibacterial activity, zones with diameters of ≥8 mm indicated antibacterial activity and zones with diameters of ≥30 mm indicated strong antibacterial activity.

The results presented in Table 1 suggest that all the tested compounds exhibited an inhibitory effect on the growth of *H. pylori* strains.

Diameters of the growth inhibition zones for the reference *H. pylori* strain ATCC 43504, resistant to metronidazole, ranged from 11 mm (compound 5) to 54 mm (compound 2). Strong activity (inhibition diameter ≥30 mm) was displayed by nine compounds: 1, 1A, 2, 4, 6, 10, 11, 13 and 19.

Diameters of the growth inhibition zones for the reference *H. pylori* strain ATCC 700684, resistant to clarithromycin, ranged from 19 mm (compound 18) to 38 mm (compound 10). Eleven compounds: 1, 2, 4, 6, 10, 11, 13, 14, 16 and 19 exhibited strong activity.

The strongest activity against clinical *H. pylori* strains resistant to metronidazole was shown by the compounds 2 and 10, with the diameters of inhibition zones ranging up to 39 mm or more, whereas compound 5 presented the weakest activity.

Compounds exhibiting the strongest activity against clinical *H. pylori* strains resistant to clarithromycin were 2, 10 and 11 with diameter of inhibition zone ranging up to 34 mm or more, whereas compounds 7, 18 showed the weakest activity. It was shown that twelve compounds had strong antibacterial activity against these clarithromycin-resistant strains.

Double-resistant *H. pylori* strains were susceptible to all tested xanthenes, however, the strongest activity was exhibited by compound 2 with the inhibition zone diameter of up to 50 mm for strain 143/207, and compound 10 with the diameter size of 56 mm for strain 126/181. The weakest activity against double-resistant strains was displayed by compound 5, with diameter of the growth inhibition zone ranging from 16 to 23 mm.

The tested compounds exhibited high anti-*H. pylori* activity, whereas their activity against methicillin-resistant *S. aureus* and *E. coli* strains was lower. The latter strains were susceptible only to some of the tested compounds and their inhibition zone diameters were smaller.

Our results showed that among twenty tested xanthone derivatives, fourteen exhibited activity against *S. aureus* ATCC 25922, compound 14 gave the largest zone of growth inhibition with a diameter of 17 mm.

Methicillin-resistant *S. aureus* was also susceptible to fourteen compounds, but the strongest activity was shown by the compounds 1 and 2, with zone of growth inhibition diameters of up to 23 mm.

*E. coli* ATCC 25922 was resistant to the majority of tested derivatives, however, this strain displayed susceptibility to five of them: 1, 2, 4, 13 and 14. The largest growth inhibition zone was produced by the compound 2, with a diameter of 10 mm.

To sum up, qualitative testing showed that all *H. pylori* strains were susceptible to newly synthesized xanthone derivatives, whereas only few of them were active against other than *H. pylori* strains.

The strongest anti-*H. pylori* activity was exhibited by compounds for which the diameters of the growth inhibition zones were the largest: 1, 2, 4, 10, 11 and 13.

On the basis of the result of the screening qualitative method (zone inhibition method) we have chosen the fourteen most active compounds (1, 2, 3, 4, 6, 8, 9, 10, 11, 12, 13, 14, 16 and 19; Table 1) and performed quantitative testing (means determination of minimum inhibitory concentration (MIC) values).

**Results of the quantitative testing.** Fourteen samples of xanthone derivatives were tested quantitatively in order to determine the MIC value of each compound. These were the following compounds: 1, 2, 3, 4, 6, 8, 9, 10, 11, 12, 13, 14, 16, 19.

The compounds were studied with the use of two bacterial strains: reference *H. pylori* strain ATCC 43504 (resistant to metronidazole) and clinical *H. pylori* strain 132/194 (resistant to clarithromycin).

The obtained results were interpreted according to the following criteria:

- zone of growth inhibition <8 mm—compound showed no antibacterial activity;
- zone of growth inhibition ≥8 mm—compound showed antibacterial activity.

The MIC value was considered to be the lowest concentration of the examined compound that inhibited the growth of bacteria (that is, the last dilution that exhibited antibacterial activity—zone of growth inhibition with a diameter ≥8 mm).

The highest concentrations of all fourteen compounds exhibited antimicrobial activity: I (5 mg ml<sup>-1</sup>), II (1 mg ml<sup>-1</sup>) and III (500 mg l<sup>-1</sup>) against both examined *H. pylori* strains.

The results presented in Figure 1 suggest that there were some differences between the profile of susceptibility of *H. pylori* strains (expressed as the MIC values of each compound) to the tested xanthone derivatives. We observed that the MIC values for the *H. pylori* strain ATCC 43504 ranged from 5 to 150 mg l<sup>-1</sup>, whereas for the *H. pylori* strain 132/194 it ranged from 10 to 500 mg l<sup>-1</sup>.

Compound 3 showed the strongest activity against the reference *H. pylori* strain ATCC 43504, that is, the MIC value was the lowest one (5 mg l<sup>-1</sup>). The weakest activity was exhibited by compound 16 with the MIC value of 150 mg l<sup>-1</sup>.

**Table 1** Chemical structures of newly synthesized xanthenes and the results of qualitative testing: diameter means of the zones of growth inhibition caused by the tested compounds in 1% concentration

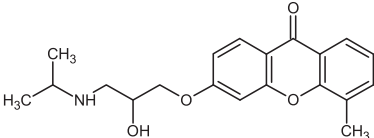
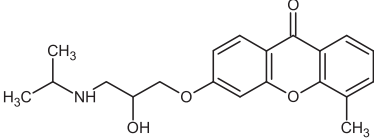
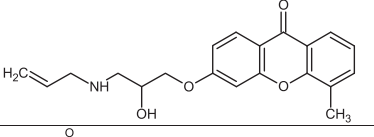
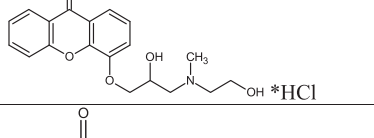
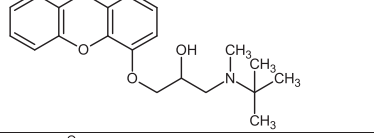
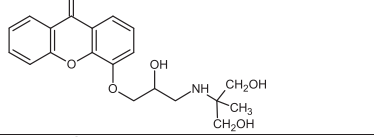
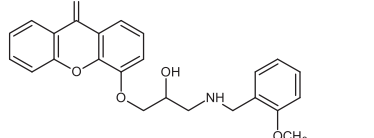
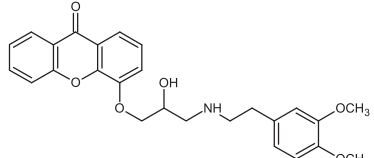
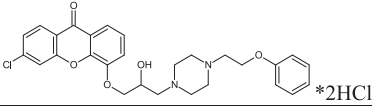
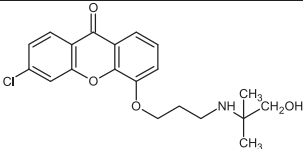
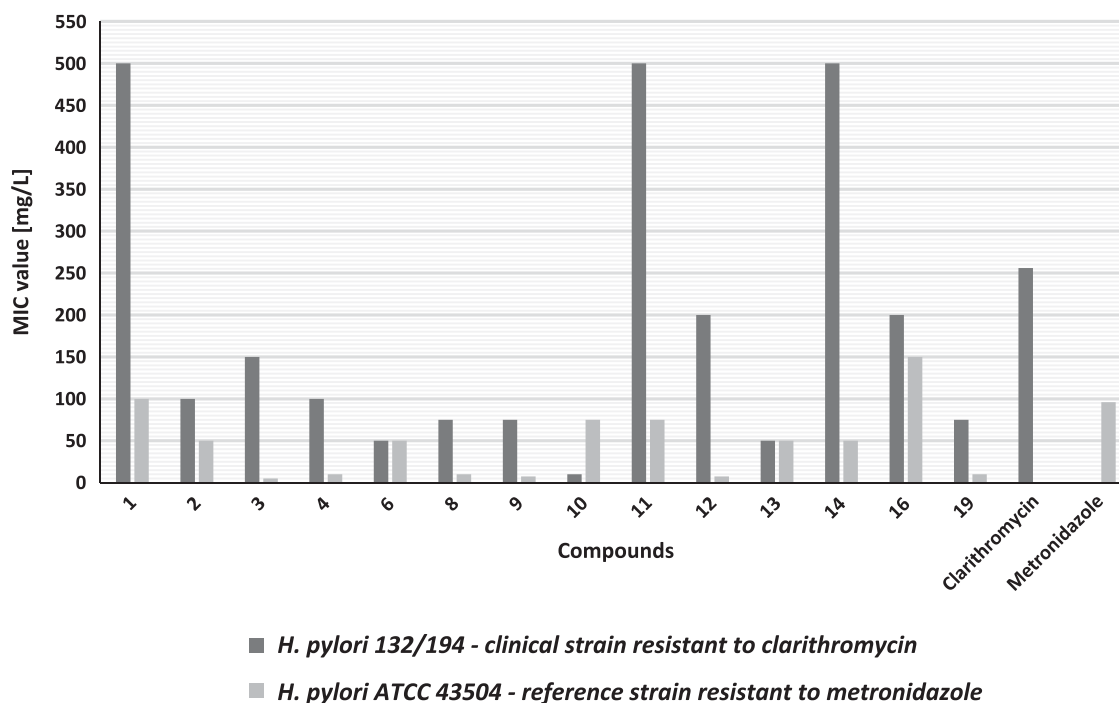
Compounds	Chemical structures	Zone of growth inhibition [mm]											
		Strains other than <i>H. pylori</i>			Clarithromycin resistant <i>H. pylori</i> strains			Metronidazole resistant <i>H. pylori</i> strains				Double resistant <i>H. pylori</i> strains	
		<i>S. aureus</i> ATCC 25923 (reference strain)	<i>S. aureus</i> MRSA 14,002 (reference strain)	<i>E. coli</i> ATCC 25922 (reference strain)	ATCC 700684 (reference strain)	HP 132/194 (clinical strain)	HP 115/168 (clinical strain)	ATCC 43504 (reference strain)	HP 125/180 (clinical strain)	HP 139/202 (clinical strain)	HP 143/207 (clinical strain)	HP 126/181 (clinical strain)	HP 106/154 (clinical strain)
Ref. 1	Metronidazole	-	-	-	34	30	36	32	13	18	20	24	16
Ref. 2	Clarithromycin	23	-	13	12	19	25	30	32	30	30	30	10
1		13	23	8	36	40	40	42	40	44	44	40	39
1 A	 *HCl	11	18	-	32	34	26	35	36	46	29	40	33
2		15	23	10	34	42	46	54	46	52	58	50	47
3	 *HCl	-	-	-	28	30	30	21	28	38	36	28	26
4		12	15	9	35	42	38	41	36	48	42	48	39
5		-	-	-	24	22	17	11	17	26	22	23	16
6		12	11	-	34	32	31	36	40	40	32	34	31
7	 *HCl	-	-	-	20	16	25	19	20	20	22	20	21

Table 1 (Continued)

Compounds	Chemical structures	Zone of growth inhibition [mm]											
		Strains other than <i>H. pylori</i>			Clarithromycin resistant <i>H. pylori</i> strains			Metronidazole resistant <i>H. pylori</i> strains				Double resistant <i>H. pylori</i> strains	
		<i>S. aureus</i> ATCC 25923 (reference strain)	<i>S. aureus</i> MRSA 14-002 (reference strain)	<i>E. coli</i> ATCC 25922 (reference strain)	ATCC 700684 (reference strain)	HP 132/194 (clinical strain)	HP 115/168 (clinical strain)	ATCC 43504 (reference strain)	HP 125/180 (clinical strain)	HP 139/202 (clinical strain)	HP 143/207 (clinical strain)	HP 126/181 (clinical strain)	HP 106/154 (clinical strain)
8		-	-	-	27	29	34	28	32	38	32	38	31
9		-	-	-	25	30	33	23	35	32	36	36	34
10		9	11	-	38	48	44	39	50	54	50	56	45
11		10	16	-	34	40	40	37	40	40	48	45	40
12		12	13	-	26	22	25	25	32	28	23	25	21
13		16	16	9	35	44	46	50	42	40	50	26	30
14		17	15	9	34	36	38	28	32	32	33	41	35
15		12	15	-	23	19	24	15	18	31	26	20	19
16		16	16	-	30	32	28	24	28	36	40	26	26
17		-	-	-	22	24	27	24	27	26	24	25	24

Table 1 (Continued)

Compounds	Chemical structures	Zone of growth inhibition [mm]											
		Strains other than <i>H. pylori</i>			Clarithromycin resistant <i>H. pylori</i> strains			Metronidazole resistant <i>H. pylori</i> strains			Double resistant <i>H. pylori</i> strains		
		<i>S. aureus</i> ATCC 25923 (reference strain)	<i>S. aureus</i> MRSA 14,002 (reference strain)	<i>E. coli</i> ATCC 25922 (reference strain)	ATCC 700684 (reference strain)	HP 132/194 (clinical strain)	HP 115/168 (clinical strain)	ATCC 43504 (reference strain)	HP 125/180 (clinical strain)	HP 139/202 (clinical strain)	HP 143/207 (clinical strain)	HP 126/181 (clinical strain)	HP 106/154 (clinical strain)
18		9	9	-	19	22	25	22	29	30	25	27	25
19		13	12	-	31	36	34	36	42	32	40	24	30



**Figure 1** Activity of fourteen newly synthesized xanthone compounds and reference compounds (clarithromycin, metronidazole) expressed by MIC values against clinical *H. pylori* strain 132/194 and reference *H. pylori* strain ATCC 43504.

Three compounds (1, 11, 14) exhibited high MIC values (up to  $500 \text{ mg l}^{-1}$ ) against the clinical *H. pylori* strain 132/194. Against the same isolate, compound 10 exhibited the strongest activity and produced the lowest MIC values ( $10 \text{ mg l}^{-1}$ ).

## DISCUSSION

The aim of this study was to evaluate anti-*H. pylori* activity of the new compounds—derivatives of xanthone. In particular, the present

study includes: design, synthesis, physicochemical analysis and microbiological evaluation of the chosen structures.

From these results it can be concluded that among xanthone derivatives new potential anti-*H. pylori* structures can be found. Our compounds were much more effective than the reference compound (metronidazole or clarithromycin), when bacterial strain was resistant to this drug. Surprisingly, the largest zones of growth inhibition in qualitative evaluation did not guarantee the lowest MIC values in the quantitative studies. The tested substances manifested

higher antibacterial activity against the reference *H. pylori* strain resistant to metronidazole, than to the clinical *H. pylori* 132/194 strain resistant to clarithromycin. This result is consistent with the average MIC values of both strains. The average MIC value of the tested compound for *H. pylori* ATCC 43504 was lower than the average MIC value for *H. pylori* 132/194 and were determined to be 46 and 185 mg l<sup>-1</sup>, respectively. Only compound **10** showed higher activity against *H. pylori* strains resistant to clarithromycin than against the metronidazole-resistant one.

The lowest MIC value was observed with compound **3** on the reference *H. pylori* strain ATCC 43504 and was estimated to be 5 mg l<sup>-1</sup>, whereas the clinical *H. pylori* strain showed MIC value of about 150 mg l<sup>-1</sup>. The clinical and reference strains differ by their profiles and mechanisms of resistance. This can be the cause of differences between their susceptibility to the same compound.

The analysis of structure–activity relationship revealed that presence of two hydroxylic groups in the amine moiety strongly contributed to the reduction in anti-*H. pylori* activity. Until now, hydroxylic groups were considered to be a factor enhancing anti-*H. pylori* activity, but these groups were connected directly to the xanthone scaffold (not to the side chain).<sup>8</sup> It suggests that activity of the tested compounds is determined more by the structure and spherical configuration than the hydrophilic character.

We also observed that the compounds containing *tert*-butylamine substructure were more active than those containing *iso*-propylamine group.

However, we did not observe the structure–activity relationship between HCl salts and free-base forms. To ascertain that we performed comparative analysis of the same structure in different forms (base and salt)—compounds **1** and **1A**—we obtained similar zones of growth inhibition for these two forms as shown in Table 1.

It can be concluded that xanthone derivatives are potential anti-*H. pylori* agents. Their activity vary depending on place and type of substitution in the xanthone core. More-detailed findings concerning the structure–activity relationship can be obtained from microbiological analysis of a wider group of structures. Currently, we screen other xanthone derivatives in order to explain their mechanism of anti-*H. pylori* action.

## MATERIALS AND METHODS

### Chemistry

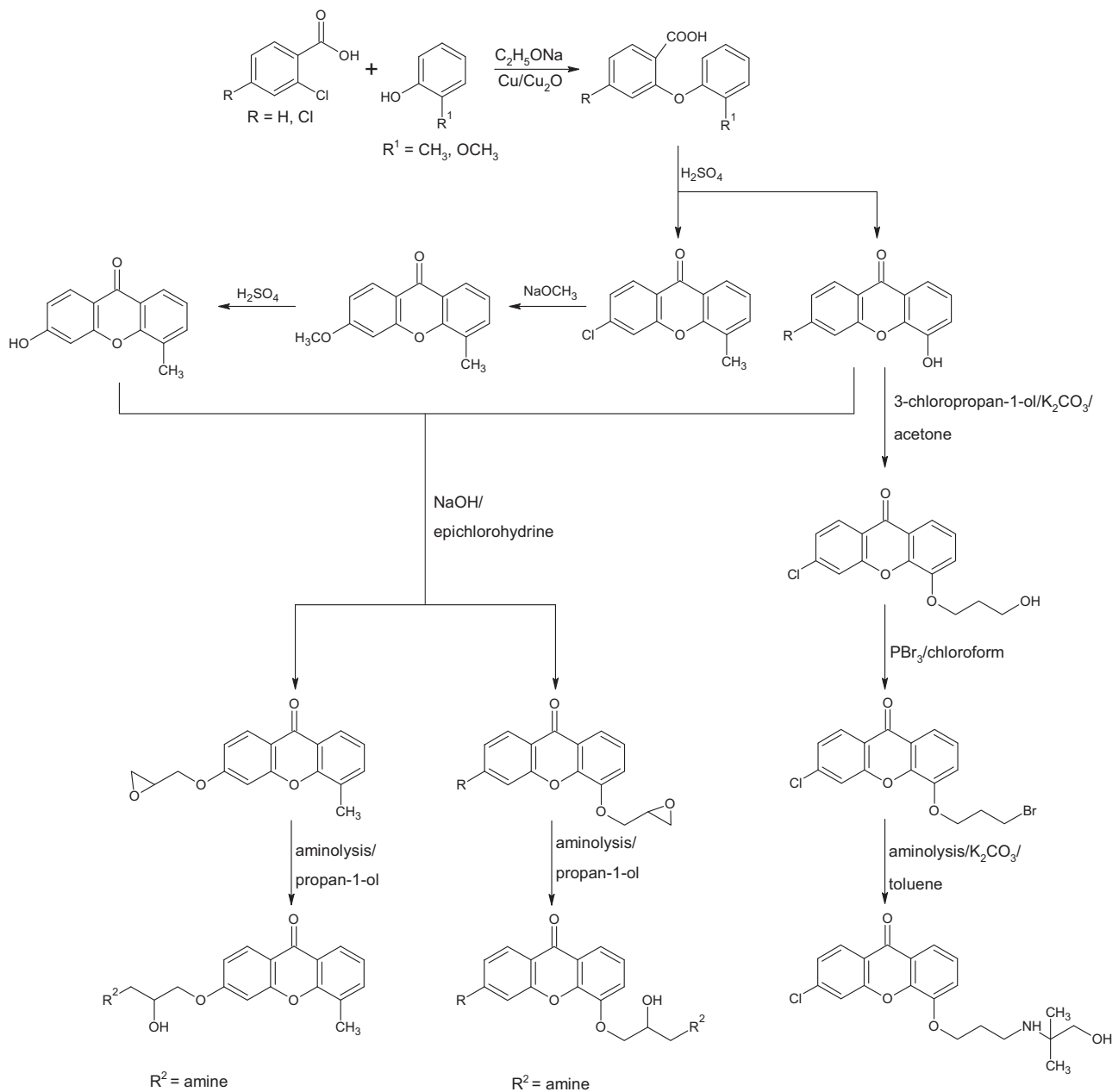
Reagents: 2-amino-2-methylpropan-1,3-diol, 2-amino-2-methylpropan-1-ol, 2,3-dimethoxyphenethylamine, ethyl 1-piperazinecarboxylate, 2-methoxybenzylamine, 2-(methylamino)ethanol, benzylpiperazine, epichlorohydrin and phenoxyethylpiperazine were purchased from Sigma-Aldrich Chemie (Steinheim, Germany), whereas the reagents: allylamine, *t*-butylamine, 2-chlorobenzoic acid, 3-chloro-1-propanol, 2,4-dichlorobenzoic acid, 1-(2-fluorophenyl)piperazine, 2-methoxyphenol, 2-methoxyphenylpiperazine, *N-tert*-butylmethylamine, phosphorus tribromide and *i*-propylamine were provided by Lancaster Synthesis (Frankfurt am Main, Germany). Solvents were commercially available and of reagent grade.

Melting points (m.p.) are uncorrected and were determined using a Büchi SMP-20 apparatus (Büchi Labortechnik, Flawil, Switzerland). The <sup>1</sup>H NMR were recorded on a Bruker AMX spectrometer (Bruker, Karlsruhe, Germany) with 500.13 MHz or Varian Mercury-VX 300 NMR spectrometer (Varian, Palo Alto, CA, USA) using signal from DMSO-*d*<sub>6</sub> and CHCl<sub>3</sub> in CDCl<sub>3</sub> as an internal standard. The results are presented in the following format: chemical shift  $\delta$  (p.p.m.), multiplicity, *J*-values in Hz, number of protons and proton's position. Multiplicities are abbreviated as follows: s (singlet), bs (broad singlet), d (doublet), dd (doublet of doublets), ddd (double doublet of doublets), dddd (double doublet of doublets), td (triplets of doublets),

t (triplet), qu (quintet) and m (multiplet). Elemental analyses were performed on an Elementar Vario EL III (Elementar Analysensysteme, Hanau, Germany). For MS analyses, samples were prepared in acetonitrile/water (10/90 v/v) mixture. The LC/MS system consisted of a Waters Acquity UPLC, coupled to a Waters TQD mass spectrometer (Waters, Milford, MA, USA, electrospray ionization mode ESI-tandem quadrupole). All the analyses were carried out using an Acquity UPLC BEH C18, 1.7  $\mu$ m, 2.1  $\times$  100 mm column. A flow rate of 0.3 ml min<sup>-1</sup> and a gradient of (5–95)% B over 10 min and then 100% B over 2 min was used. Eluent A: water/0.1% HCO<sub>2</sub>H; eluent B: acetonitrile/0.1% HCO<sub>2</sub>H. LC/MS data were obtained by scanning the first quadrupole in 0.5 s in a mass range from 50 to 1000 Da; 8 scans were summed up to produce the final spectrum. The purity of obtained compounds was confirmed by the TLC, carried out on pre-coated plates (silica gel, 60F-254, Merck, Darmstadt, Germany) using the solvent systems mentioned below. The obtained corresponding spots were visualized under UV light.

The synthetic route of the tested compounds and starting materials is outlined in Scheme 1. The compounds mentioned above were obtained by means of a multistage synthesis. In the first stage, the xanthone skeleton was synthesized. This synthesis included Ullmann's condensation<sup>24,25</sup> of appropriate chlorobenzoic acid and phenol derivative. The reaction was performed for few hours—in oil—at 195–200 °C, in the presence of Cu/Cu<sub>2</sub>O catalyst. After condensation, intermediate product underwent cyclization in the presence of concentrated H<sub>2</sub>SO<sub>4</sub>. In case of 2-methoxyphenol derivatives, concomitant demethylation occurred. It was noticed that the longer the mixture was heated, the more effective demethylation was achieved. The optimal refluxing time was 2 days. Then the reacting mixture was poured into crushed ice, filtered and washed to neutral reaction. Unreacted acid was washed out with NaHCO<sub>3</sub> aqueous solution. The detailed methodology and physicochemical properties of 4-hydroxy-9*H*-xanthen-9-one, 3-chloro-5-hydroxy-9*H*-xanthen-9-one and 3-chloro-5-methyl-9*H*-xanthen-9-one are described in<sup>26–28</sup> 3-Chloro-5-methyl-9*H*-xanthen-9-one underwent methanolysis. Sodium methoxide was prepared by gradual dissolution of sodium in an excess of methanol (with cooling). Then equimolar amount of 3-chloro-5-methyl-9*H*-xanthen-9-one was added and the mixture was heated for about 48 h. After distillation to dryness, water and H<sub>2</sub>SO<sub>4</sub> were added to the residue. The precipitate was filtered and dried. 3-Methoxy-5-methyl-9*H*-xanthen-9-one was subjected to demethylation using a tenfold excess of condensed H<sub>2</sub>SO<sub>4</sub> to obtain 3-hydroxy-5-methyl-9*H*-xanthen-9-one. This newly synthesized compound was recrystallized from ethanol and its physicochemical properties are described below. At this point the synthesis diverged into two separate pathways, one promoting the bromopropoxy derivative of xanthone and the other the oxirane products. 5-(3-Bromopropoxy)-3-chloro-9*H*-xanthen-9-one was synthesized by the reaction of 3-chloro-5-hydroxy-9*H*-xanthen-9-one with 3-chloropropanol in acetone, in the presence of a large excess of K<sub>2</sub>CO<sub>3</sub>. Next, the solvent was distilled off and the unreacted hydroxyxanthone was elutriated using NaOH solution. The intermediate product was brominated using PBr<sub>3</sub> in CHCl<sub>3</sub>. Subsequently, CHCl<sub>3</sub> was distilled off and the unreacted intermediate product was washed out with NaHCO<sub>3</sub> aqueous solution. The crude product was recrystallized from *n*-hexane/toluene (1:6). The physicochemical properties of 5-(3-bromopropoxy)-3-chloro-9*H*-xanthen-9-one were described formerly.<sup>29</sup> The oxirane derivatives of xanthone were obtained in the reaction of appropriate hydroxyxanthone with epichlorohydrin according to the procedures described earlier.<sup>30</sup> Epichlorohydrin was used in double excess. Reacting mixture was stirred overnight at the room temperature. Next, the precipitate was filtered and the unreacted substrates were washed out with 10% NaOH solution. Physicochemical properties of 4-((oxiran-2-yl)methoxy)-9*H*-xanthen-9-one were described by Marona *et al.*<sup>26</sup> in 2008, and the characteristic of 3-chloro-5-((oxiran-2-yl)methoxy)-9*H*-xanthen-9-one was published by Marona *et al.*<sup>27</sup> in 2009. 5-Methyl-3-((oxiran-2-yl)methoxy)-9*H*-xanthen-9-one was a new compound, recrystallized from ethanol and its physicochemical properties are summarized below.

Compounds **1–19** were obtained by amination of the parent compounds with appropriate amines in *n*-propanol (**1–18**) or toluene in the presence of K<sub>2</sub>CO<sub>3</sub> (**19**), as described previously.<sup>13,16,31</sup> After aminolysis, the solvents were distilled off. The resulting amines were dissolved in diluted HCl, cleaned with charcoal and precipitated using NaOH solution. Some of the bases were converted into hydrochloride salts using an excess of ethanol saturated



Scheme 1 Synthesis of the xanthone derivatives.

with HCl. The crude products were recrystallized from acetone/ethanol (1:3). Most of the synthesized compounds are new and their physicochemical properties are summarized below. Some structures were evaluated previously for their cardiovascular activity and their physicochemical properties were described elsewhere (compounds **5**, **14**, **15**—Marona *et al.*,<sup>27</sup> compound **8**, **10**—Marona *et al.*<sup>32</sup> and compounds **17**, **18**—Szkaradek *et al.*<sup>29</sup>). Compounds **3**, **4**, **11** are taken under consideration for publication in *Bioorganic and Medicinal Chemistry* according to their additional anticancer activity. Compounds **1** and **1A** possessed the same structure but differed by the form: **1** is free base, whereas **1A** is a HCl salt. These compounds were evaluated to compare influence of the form of the compound on its microbiological activity. Structures of the tested compounds are presented in Table 1.

3-Hydroxy-5-methyl-9H-xanthen-9-one was obtained as a white solid (yield 50%), m.p. 288–290 °C. *Anal* calcd for C<sub>14</sub>H<sub>10</sub>O<sub>3</sub>: C, 74.33; H, 4.46; found: C,

74.15; H, 4.44. <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz): δ (p.p.m.) 10.93 (s, 1H, -OH), 8.01 (dd, *J* = 7.6, *J* = 2.7, 1H, H-1), 7.97 (dd, *J* = 8.0, *J* = 1.5, 1H, H-8), 7.66 (dd, *J* = 8.0, *J* = 1.5, 1H, H-6), 7.31 (t, *J* = 8.0, 1H, H-7), 6.92–6.87 (m, 2H, H-2, H-4), 2.48 (s, 3H, -CH<sub>3</sub>). LC-MS: calcd for [M+H]<sup>+</sup>: C<sub>14</sub>H<sub>11</sub>O<sub>3</sub> *m/z*: 227.06, found 227.12. *R*<sub>F</sub> = 0.70 (toluene/acetone (5:3)).

5-Methyl-3-((oxiran-2-yl)methoxy)-9H-xanthen-9-one was obtained as a white solid (yield 55%), m.p. 153–154 °C. *Anal* calcd for C<sub>17</sub>H<sub>14</sub>O<sub>4</sub>: C, 72.33; H, 5.00; found: C, 72.44; H, 5.11. <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz): δ (p.p.m.) 8.07 (d, *J* = 9.0, 1H, H-1), 7.98 (dd, *J* = 7.6, *J* = 1.8, 1H, H-8), 7.68 (d, *J* = 7.6, 1H, H-6), 7.33 (t, *J* = 7.6, 1H, H-7), 7.19 (d, *J* = 2.3, 1H, H-4), 7.06 (dd, *J* = 9.0, *J* = 2.3, 1H, H-2), 4.56 (dd, *J* = 11.5, *J* = 2.3, 1H, Ar-O-CHH-), 4.01 (dd, *J* = 11.5, *J* = 6.9, 1H, Ar-O-CHH-), 3.39 (dddd, *J* = 6.9, *J* = 4.7, *J* = 2.7, *J* = 2.3, 1H, =CH-), 2.88 (t, *J* = 4.7, 1H, -CHH-), 2.74 (dd, *J* = 4.7, *J* = 2.7, 1H, -CHH-), 2.50 (s, 1H, -CH<sub>3</sub>). LC-MS: calcd for [M+H]<sup>+</sup>: C<sub>17</sub>H<sub>15</sub>O<sub>4</sub> *m/z*: 283.29, found 283.04. *R*<sub>F</sub> = 0.80 (toluene/acetone (5:3)).

3-(2-Hydroxy-3-(propan-2-ylamino)propoxy)-5-methyl-9H-xanthen-9-one (compound 1) was obtained as a white solid (yield 65%), m.p. 126–127 °C. *Anal* calcd for  $C_{20}H_{24}NO_4Cl$ : C, 63.57; H, 6.40; N, 3.71. Found: C, 63.52; H, 6.70; N, 3.65.  $^1H$  NMR ( $CDCl_3$ , 300 MHz):  $\delta$  (p.p.m.) 8.25 (dd,  $J = 6.8$ ,  $J = 2.6$ , 1H, H-1), 8.17 (dd,  $J = 8.0$ ,  $J = 1.8$ , 1H, H-8), 7.54 (dd,  $J = 7.2$ ,  $J = 1.8$ , 1H, H-6), 7.29-7.23 (m, 3H, H-4, H-2, H-7), 7.00-6.95 (m, 2H, -OH, -NH-), 4.14-4.03 (m, 3H, Ar-O-CH<sub>2</sub>-, =CH-OH), 2.94 (dd,  $J = 12.1$ ,  $J = 3.8$ , 1H, -CHH-NH-), 2.85 (qu,  $J = 6.2$ , 1H, =CH-), 2.76 (dd,  $J = 12.2$ ,  $J = 7.6$ , 1H, -CHH-NH-), 2.55 (s, 3H, -CH<sub>3</sub>), 1.11 (d,  $J = 6.2$ , 6H, (-CH<sub>3</sub>)<sub>2</sub>).  $R_F = 0.16$  (methanol).

3-(2-Hydroxy-3-(prop-2-en-1-ylamino)propoxy)-5-methyl-9H-xanthen-9-one (compound 2) was obtained as a white solid (yield 68%), m.p. 94–96 °C. *Anal* calcd for  $C_{20}H_{21}O_4N$ : C, 70.78; H, 6.24; N, 4.13. Found: C, 70.61; H, 6.03; N, 4.08.  $^1H$  NMR ( $CDCl_3$ , 300 MHz):  $\delta$  (p.p.m.) 8.25 (dd,  $J = 6.4$ ,  $J = 2.8$ , 1H, H-1), 8.17 (dd,  $J = 8.0$ ,  $J = 1.8$ , 1H, H-8), 7.54 (dd,  $J = 8.0$ ,  $J = 1.8$ , 1H, H-6), 7.30-7.23 (m, 3H, H-4, H-4, H-7), 7.00-6.95 (m, 2H, -OH, -NH), 5.82-6.00 (m, 1H, -CH=), 5.22 (ddd,  $J = 16.9$ ,  $J = 3.3$ ,  $J = 1.3$ , 1H, =C<HH (*trans*)), 5.14 (ddd,  $J = 10.5$ ,  $J = 3.3$ ,  $J = 1.3$ , 1H, =C<HH (*cis*)), 4.17-4.09 (m, 3H, Ar-O-CH<sub>2</sub>-, =CH-OH), 3.35-3.30 (m, 2H, =CH-CH<sub>2</sub>-NH-), 2.97-2.76 (m, 2H, -CH<sub>2</sub>-), 2.55 (s, 3H, -CH<sub>3</sub>).  $R_F = 0.29$  (methanol/ethyl acetate (1/1)).

4-(3-(2-Methoxybenzylamino)-2-hydroxypropoxy)-9H-xanthen-9-one (compound 6) was obtained as a white solid (yield 75%), m.p. 138–140 °C.  $^1H$  NMR ( $CDCl_3$ , 300 MHz):  $\delta$  (p.p.m.) 8.33 (dd,  $J = 8.2$ ,  $J = 1.5$ , 1H, H-8), 7.93 (t,  $J = 4.6$ , 1H, H-2), 7.70 (ddd,  $J = 8.5$ ,  $J = 7.1$ ,  $J = 1.5$ , 1H, H-6), 7.45 (dd,  $J = 8.5$ ,  $J = 0.7$ , 1H, H-5), 7.38 (ddd,  $J = 8.2$ ,  $J = 7.1$ ,  $J = 0.7$ , 1H, H-7), 7.28 (d,  $J = 4.6$ , 2H, H-1, H-7), 7.26-7.22 (m, 2H, H-3', H-4'), 6.92 (td,  $J = 7.7$ ,  $J = 1.0$ , 1H, H-5'), 6.87 (d,  $J = 7.7$ , 1H, H-6'), 4.25-4.15 (m, 3H, -CH=, Ar-O-CH<sub>2</sub>-), 3.94-3.83 (m, 2H, -NH-CH<sub>2</sub>-Ar), 3.80 (s, 3H, -O-CH<sub>3</sub>), 3.00-2.85 (m, 2H, -CH<sub>2</sub>-NH-). Hydrochloride of compound 6: *Anal* calcd for  $C_{24}H_{24}O_5NCl$ : C, 65.23; H, 5.47; N, 3.17. Found: C, 64.62; H, 5.58; N, 3.15.  $R_F = 0.65$  (methanol:ethyl acetate (1:1)).

4-(3-(3,4-Methoxyphenyl)ethylamino)-2-hydroxypropoxy)-9H-xanthen-9-one hydrochloride (compound 7) was obtained as a white solid (yield 75%), m.p. 187–188 °C. *Anal* calcd for  $C_{26}H_{28}O_6NCl$ : C, 64.26; H, 5.81; N, 2.88. Found: C, 64.06; H, 6.19; N, 2.88.  $^1H$  NMR ( $DMSO-d_6$ , 300 MHz):  $\delta$  (p.p.m.) 8.65 (bs, 2H, -NHH<sup>+</sup>-), 8.19 (dd,  $J = 8.0$ ,  $J = 1.5$ , 1H, H-8), 7.88 (ddd,  $J = 8.6$ ,  $J = 6.9$ ,  $J = 1.5$ , 1H, H-6), 7.76 (dd,  $J = 8.0$ ,  $J = 1.5$ , 1H, H-1), 7.69 (dd,  $J = 8.6$ ,  $J = 1.0$ , 1H, H-5), 7.57 (dd,  $J = 8.0$ , 1.5 Hz, 1H, H-3), 7.49 (ddd,  $J = 8.0$ ,  $J = 6.9$ ,  $J = 1.0$ , 1H, H-7), 7.39 (t,  $J = 8.0$ , 1H, H-2), 6.86 (d,  $J = 8.2$ , 1H, H-5'), 6.84 (d,  $J = 2.1$ , 1H, H-2'), 6.75 (dd,  $J = 8.2$ ,  $J = 2.1$ , 1H, H-6'), 5.97 (bs, 1H, -OH), 4.36-4.28 (m, 1H, -CH=), 4.26-4.17 (m, 2H, Ar-O-CH<sub>2</sub>-), 3.72 (s, 3H, -O-CH<sub>3</sub>), 3.69 (s, 3H, -O-CH<sub>3</sub>), 3.27-3.10 (m, 4H, -CH<sub>2</sub>-NH-CH<sub>2</sub>-), 2.86-2.95 (m, 2H, -CH<sub>2</sub>-Ar).  $R_F = 0.15$  (methanol).

4-(3-(4-(2-Fluorophenyl)piperazin-1-yl)-2-hydroxypropoxy)-9H-xanthen-9-one dihydrochloride (compound 9) was obtained as a white solid (yield 70%), m.p. 224–226 °C. *Anal* calcd for  $C_{26}H_{27}O_4N_2Cl_2F$ : C, 59.89; H, 5.22; N, 5.38. Found: C, 59.51; H, 5.18; N, 5.17.  $^1H$  NMR ( $DMSO-d_6$ , 300 MHz):  $\delta$  (p.p.m.) 10.55 (bs, 1H-NH<sup>+</sup>-), 8.20 (dd,  $J = 8.0$ ,  $J = 1.8$ , 1H, H-8), 7.90 (ddd,  $J = 8.2$ ,  $J = 6.9$ ,  $J = 1.8$ , 1H, H-6), 7.76 (dd,  $J = 8.2$ ,  $J = 1.3$ , 1H, H-1, H-5), 7.60 (dd,  $J = 8.2$ ,  $J = 1.3$ , 1H, H-3), 7.50 (ddd,  $J = 8.0$ ,  $J = 6.9$ ,  $J = 1.3$ , 1H, H-7), 7.39 (t,  $J = 8.2$ , 1H, H-2), 7.00-7.21 (m, 4H, H-Ph), 6.02-6.16 (m, 1H, -OH), 4.57 (bs, 1H, -CH=), 4.23 (d,  $J = 4.6$ , 2H Ar-O-CH<sub>2</sub>-), 3.84-3.12 (m, 10H, (-N-CH<sub>2</sub>)<sub>5</sub>).  $R_F = 0.86$  (methanol/ethyl acetate (1/1)).

3-Chloro-5-(2-hydroxy-3-(propan-2-ylamino)propoxy)-9H-xanthen-9-one (compound 12) was obtained as a white solid (yield 72%), m.p. 169–170 °C. *Anal* calcd for  $C_{19}H_{23}O_4NCl$ : C, 63.07; H, 5.57; N, 3.87. Found: C, 62.70; H, 5.79; N, 3.51.  $R_F = 0.23$  (methanol/ethyl acetate 1/1). Hydrochloride of the compound 12:  $^1H$  NMR ( $DMSO-d_6$ , 300 MHz)  $\delta$  (p.p.m.): 8.76 (bs, 2H, -NHH<sup>+</sup>), 8.18 (d,  $J = 8.4$ , 1H, H-8), 7.85 (d,  $J = 1.8$ , 1H, H-5), 7.74 (dd,  $J = 8.1$ ,  $J = 1.5$ , 1H, H-1), 7.59 (dd,  $J = 8.1$ ,  $J = 1.5$ , 1H, H-3), 7.54 (dd,  $J = 8.6$ ,  $J = 2.1$ , 1H, H-7), 7.41 (t,  $J = 7.8$ , 1H, H-2), 5.96 (bs, 1H, -OH), 4.34 (bs, 1H, =CH-OH), 4.24-4.22 (m, 2H, Ar-O-CH<sub>2</sub>-), 3.40-3.06 (m, 3H, -CH<sub>2</sub>-N-, -CH=), 1.28 (dd,  $J = 6.4$ ,  $J = 2.7$ , 6H, (-CH<sub>3</sub>)<sub>2</sub>).

3-Chloro-5-(2-hydroxy-3-((2-methylpropan-2-ylamino)propoxy)-9H-xanthen-9-one (compound 13) was obtained as a white solid (yield 70%), m.p. 154–155 °C. Hydrochloride of compound 13: *Anal* calcd for  $C_{20}H_{23}O_4NCl_2$ : C,

58.26; H, 5.62; N, 3.40. Found: C, 58.07; H, 6.13; N, 3.41.  $^1H$  NMR ( $DMSO-d_6$ , 300 MHz)  $\delta$  (p.p.m.): 8.50 (bs, 2H, -NHH<sup>+</sup>), 8.19 (d,  $J = 8.4$ , 1H, H-8), 7.80 (d,  $J = 1.8$ , 1H, H-5), 7.76 (dd,  $J = 8.1$ ,  $J = 1.5$ , 1H, H-1), 7.60 (dd,  $J = 8.0$ ,  $J = 1.2$ , 1H, H-3), 7.55 (dd,  $J = 8.7$ ,  $J = 2.1$ , 1H, H-7), 7.42 (t,  $J = 8.1$ , 1H, H-2), 5.92 (bs, 1H, -OH), 4.26 (bs, 3H, =CH-OH, Ar-O-CH<sub>2</sub>-), 3.40-3.06 (m, 2H, -CH<sub>2</sub>-N-), 1.32 (s, 9H, -(CH<sub>3</sub>)<sub>3</sub>).  $R_F = 0.16$  (methanol/ethyl acetate (1/1)).

3-Chloro-5-(2-hydroxy-3-(1-hydroxy-3-methylbutan-2-ylamino)propoxy)-9H-xanthen-9-one (compound 16) was obtained as a white solid (yield 70%), m.p. 133–135 °C. Hydrochloride of compound 16: *Anal* calcd for  $C_{21}H_{25}O_5NCl_2$ : C, 57.02; H, 5.70; N, 3.17. Found: C, 56.91; H, 6.06; N, 3.14.  $^1H$  NMR ( $DMSO-d_6$ , 300 MHz):  $\delta$  (p.p.m.) 8.77 (bs, 1H, -NHH<sup>+</sup>-), 8.37 (bs, 1H, -NHH<sup>+</sup>-), 8.18 (d,  $J = 8.5$ , 1H, H-8), 7.83 (d,  $J = 2.1$ , 1H, H-5), 7.75 (dd,  $J = 8.1$ ,  $J = 1.4$ , 1H, H-1), 7.59 (dd,  $J = 8.1$ ,  $J = 1.4$ , 1H, H-3), 7.54 (dd,  $J = 8.5$ ,  $J = 2.1$ , 1H, H-7), 7.41 (t,  $J = 8.1$ , 1H, H-2), 5.96 (d,  $J = 4.4$ , 1H, >CH-OH), 5.13 (bs, 1H, -CH<sub>2</sub>-OH), 4.30-4.19 (m, 3H, >CH-, Ar-O-CH<sub>2</sub>-), 3.61 (t,  $J = 6.3$ , 2H, -CH<sub>2</sub>-OH), 3.26 (bs, 1H, -CHH-NH-, overlap with signal from H<sub>2</sub>O in  $DMSO$ ), 3.07 (bs, 1H, -CHH-NH-), 1.89-1.77 (m, 2H, >CH-CH<), 1.34 (d,  $J = 1.3$ , 6H, (-CH<sub>3</sub>)<sub>2</sub>).  $R_F = 0.13$  (methanol).

3-Chloro-5-(3-(1-hydroxy-2-methylpropan-2-ylamino)propoxy)-9H-xanthen-9-one (compound 19) was obtained as a white solid (yield 60%), m.p. 151–153 °C. *Anal* calcd for  $C_{20}H_{23}O_4NCl_2$ : C, 58.26; H, 5.62; N, 3.40. Found: C, 58.25; H, 6.25; N, 3.34.  $^1H$  NMR ( $CDCl_3$ , 300 MHz):  $\delta$  (p.p.m.) 8.27 (d,  $J = 8.5$  Hz, 1H, H-8), 7.89 (dd,  $J = 7.4$ ,  $J = 2.1$ , 1H, H-1), 7.65 (d,  $J = 2.1$ , 1H, H-5), 7.36 (dd,  $J = 8.5$ ,  $J = 2.1$ , 1H, H-7), 7.29 (d,  $J = 7.4$ , 1H, H-3), 7.27 (m, 1H, H-2), 4.26 (t,  $J = 6.3$ , 2H, Ar-O-CH<sub>2</sub>-), 3.33 (s, 2H, -CH<sub>2</sub>-OH), 2.82 (t,  $J = 6.3$ , 2H, -CH<sub>2</sub>-NH-), 2.08 (qu,  $J = 6.3$ , 2H, -CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-), 1.50 (bs, 2H, -NH-, -OH), 1.11 (s, 6H, (-CH<sub>3</sub>)<sub>2</sub>).  $R_F = 0.07$  (methanol).

## Microbiology

The preliminary assay of antimicrobial activity of the 20 newly synthesized xanthone derivatives against selected representative bacterial strains were conducted. Antimicrobial activity was tested *in vitro* by the Kirby–Bauer method (agar disc-diffusion method).<sup>33,34</sup> This method was used for qualitative as well as quantitative testing.

The first step of the microbiological examination was the qualitative testing. The aim of this step was to select the compounds with the strongest anti-*H. pylori* activity. The activity of 19 compounds was tested to selected *H. pylori* strains. *S. aureus* and *E. coli* strains were used as controls.

The second step of the microbiological examination consisted of quantitative testing that aimed to assess the MIC value of the compound with the strongest anti-*H. pylori* activity.

Microbiological examination for the antibacterial activity of 20 xanthone derivatives against representative bacterial strains was carried out in the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Jagiellonian University Medical College in Krakow, Poland.

**Bacterial strains.** Twelve representative bacterial strains were used in the microbiological examination. Seven clinical *H. pylori* strains were included in the study. Strains were isolated from the endoscopic biopsy specimens of gastric mucosa from dyspeptic patients who applied to the Falck Medycyna Outpatient Clinic of Gastroenterology (Krakow, Poland). Isolation of *H. pylori* strains from biopsy samples as well as antibacterial susceptibility testing to clarithromycin and metronidazole with the use of strips impregnated with antimicrobial agents gradients (E-test, bioMérieux, Lyon, France) were performed as previously described by Karczewska et al.<sup>35</sup>

In addition, we used two *H. pylori* reference strains and single reference strains of *S. aureus* and *E. coli*.

Clinical strains were as follows:

- three *H. pylori* strains resistant to metronidazole: HP 125/180, HP 139/202 and HP 143/207;
- two *H. pylori* strains resistant to clarithromycin: HP 115/168 and HP 132/194;
- two double-resistant *H. pylori* strains (that is, combined resistance to clarithromycin and metronidazole): HP 106/154 and HP 126/181.



**Table 2** Clarithromycin and metronidazole MIC values of twelve tested strains (clinical and reference *H. pylori* strains, reference *S. aureus* strains, reference *E. coli* strain)

Strains	MIC values of clarithromycin (mg l <sup>-1</sup> )	MIC values of metronidazole (mg l <sup>-1</sup> )
<i>Strains other than H. pylori</i>		
<i>S. aureus</i> ATCC 25923	0.5	>256
<i>S. aureus</i> MRSA 14.002	>256	>256
<i>E. coli</i> ATCC 25922	64	>256
<i>H. pylori</i> strains resistant to clarithromycin		
<i>H. pylori</i> ATCC 700684	32	0.094
HP 132/194	>256	0.5
HP 115/168	24	0.094
<i>H. pylori</i> strains resistant to metronidazole		
<i>H. pylori</i> ATCC 43504	0.032	96
HP 125/180	<0.016	48
HP 139/202	0.032	>256
HP 143/207	<0.016	>256
<i>Double-resistant H. pylori</i> strains		
HP 126/181	4	>256
HP 106/154	>256	>256

Abbreviation: MIC, minimal inhibitory concentration. Bold values represent the compounds revealing significant activity.

The resistance profiles of *H. pylori* strains were evaluated according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. The MIC values of clarithromycin and metronidazole were determined with the use of the strips impregnated with the antibiotic gradient (E-test, bioMérieux). The procedure of the examination was performed under the manufacturer's instructions. In accordance with the EUCAST guidelines, *H. pylori* strains were found as metronidazole or clarithromycin resistant when the MIC values for metronidazole and clarithromycin were >8 and >0.5 mg l<sup>-1</sup>, respectively.<sup>36</sup>

Moreover, the reference strains were used to the examination. We used the following strains from the American Type Culture Collection (ATCC):

- *H. pylori* strain resistant to metronidazole: ATCC 43504;
- *H. pylori* strain resistant to clarithromycin: ATCC 700684;
- *S. aureus* ATCC 25923;
- *E. coli* ATCC 25922.

One methicillin-resistant *S. aureus* 14.002 strain was also used. This strain originated from reference collection MIKROBANK of A National Reference Center for Microbial Susceptibility Testing in Poland.

Susceptibility of the tested strains to clarithromycin and metronidazole expressed by MIC values were presented in Table 2.

**Preparation of the compound samples.** Qualitative testing was performed with the use of 1% (10 000 mg l<sup>-1</sup>) solutions of the tested compounds (synthesized at the Department of Technology and Biotechnology of Drugs, Jagiellonian University Medical College) in dimethylsulfoxide (Merck).

The most active compounds were classified to the second step of microbiological examination, that is, quantitative testing. Twelve decreasing dilutions of the tested compounds in DMSO were prepared in order to perform

quantitative testing and to determine the MIC value of each compound. The dilutions were as follows:

- 5000 mg l<sup>-1</sup> (0.5%);
- 1000 mg l<sup>-1</sup> (0.1%);
- 500 mg l<sup>-1</sup> (0.05%);
- 200 mg l<sup>-1</sup> (0.02%);
- 150 mg l<sup>-1</sup> (0.015%);
- 100 mg l<sup>-1</sup> (0.01%);
- 75 mg l<sup>-1</sup> (0.0075%);
- 50 mg l<sup>-1</sup> (0.005%);
- 10 mg l<sup>-1</sup> (0.001%);
- 7.5 mg l<sup>-1</sup> (0.00075%);
- 5 mg l<sup>-1</sup> (0.0005%);
- 2.5 mg l<sup>-1</sup> (0.00025%).

**Susceptibility testing of bacterial strains.** Qualitative and quantitative assessment of the susceptibility of *H. pylori* strains to novel, potential antimicrobial agents was assessed by the Kirby–Bauer method (agar disk-diffusion method).

**Qualitative testing.** Qualitative testing was performed with the use of test discs impregnated with 1% (10 000 mg l<sup>-1</sup>) solutions of the compounds. In this part of study all 20 synthesized compounds were tested on all 12 bacterial strains. The analysis was conducted according to the procedure described below.

Bacterial suspensions were prepared from pure cultures in 0.85% aqueous NaCl solution (bioMérieux) in order to obtain an equivalent of 3.0 McFarland units (*H. pylori*) or 0.5 McFarland units (*S. aureus* and *E. coli*).

The suspensions of *H. pylori* were inoculated on the Schaedler agar with 5% sheep blood (bioMérieux) with sterile cotton swab. The suspensions of *S. aureus* or *E. coli* were applied onto Mueller–Hinton Agar. The inoculated agar was allowed to dry for 15 min. Test discs impregnated with the tested compounds were applied onto the inoculated agar.

The plates with *H. pylori* were incubated in microaerophilic atmosphere at 37 °C for 72 h, whereas the plates with *S. aureus* or *E. coli* were incubated in aerobic atmosphere at 37 °C for 24 h. After incubation, the diameters of complete growth inhibition zones were measured. Antibacterial activity was expressed as the mean of complete inhibition diameters (mm) produced by the tested compounds. A growth inhibition zone diameter of 8 mm was defined as the breakpoint to classify bacteria as susceptible to the tested compounds in adequate dilutions.

Qualitative testing was performed in order to choose the compounds with the strongest activity against *H. pylori*. The interpretative criteria were as follows:

- zone of growth inhibition <8 mm—compound showed no antibacterial activity;
- zone of growth inhibition ≥8 mm—compound showed antibacterial activity;
- zone of growth inhibition ≥30 mm—compound showed strong antibacterial activity.

We also conducted reference qualitative testing with the use of antibiotic. In order to compare antibacterial activity of the tested compounds to other antimicrobial agents and to confirm the efficacy of the chosen method, clarithromycin and metronidazole were used as a reference compounds.

**Qualitative testing.** Quantitative testing was conducted with the use of fourteen most active compounds (1, 2, 3, 4, 6, 8, 9, 10, 11, 12, 13, 14, 16 and 19), for which the stock solutions were diluted to prepare twelve decreasing solutions tested subsequently against the two selected *H. pylori* strains (ATCC 43504 and 132/194). We also applied the agar disc-diffusion method. Zone of growth inhibition <8 mm was considered as indicating no antibacterial activity, whereas an inhibition zone ≥8 mm was considered as indicating antibacterial activity. The MIC value was considered to be the lowest concentration of the examined compound that inhibited the growth of bacteria. Therefore, the last dilution at which antibacterial activity was expressed determined the MIC value (zone ≥8 mm).

We performed qualitative and quantitative tests (Kirby–Bauer method estimating MIC) to obtain values for clarithromycin and metronidazole against all clinical strains. Moreover we estimated the MIC values for clarithromycin and metronidazole using the strips impregnated with the antibiotic gradient (E-test, bioMérieux). The procedure of the examination was performed under the manufacturer's instructions. MIC values obtained using Kirby–Bauer method and E-test were the same, so we can conclude that the efficacy and accuracy of the chosen Kirby–Bauer method was confirmed. Table 2 shows the MIC values to antimicrobial agents of clinical *H. pylori* strains.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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