Full Paper

Synthesis and Biochemical Characterization of New Phenothiazines and Related Drugs as MDR Reversal Agents

Matthias Schmidt[1], Marlen Teitge[1], Marianela E. Castillo[1], Tobias Brandt[2], Bodo Dobner[2], and Andreas Langner[2]

[1] Department of Medicinal Chemistry, Institute of Pharmacy, Martin-Luther-University Halle-Wittenberg, Halle (Saale), Germany
[2] Department of Biochemical Pharmacy, Institute of Pharmacy, Martin-Luther-University Halle-Wittenberg, Halle (Saale), Germany

Chemotherapy is one of the most important methods in the treatment of cancer. However, development of drug resistance during chemotherapy is the leading cause of treatment failure and decreased survival in cancer patients. Multidrug resistance (MDR) is one of the extensively studied forms of drug resistance for more than 30 years. The members of ATP-binding cassette protein family are responsible for multidrug resistance with P-glycoprotein as most representative transporter. To overcome multidrug resistance, pharmacological modulation of the transporters by efflux pump inhibitors seem to be the first choice, but preclinical studies did not lead to clinical applications. Therefore, a systematical research for pharmacophor structures is a promising strategy to increase the efficacy of those drugs still influencing multidrug resistance. In this study a range of phenothiazine derivatives was synthesizied with systematical variation of three molecule domains. The biochemical determination of multidrug resistance reversal activity was achieved with the crystalviolet assay on LLC-PK1/MDR1 cells. The results will be discussed considering of hypotheses in the literature directed to new structure-acitivity relationships to overcome drug resistance in the future.

Keywords: Chemotherapy / Crystalviolet assay / LLC-PK1/MDR1 cells / P-Glycoprotein / Structure-acitivity relationships

Received: June 12, 2008; accepted: July 15, 2008

DOI 10.1002/ardp.200800115

Introduction

Multidrug resistance (MDR) is one of the most important reasons of failure in cancer chemotherapy. Responsible for stagnation of the cytotoxic process is an evident drug efflux mainly caused by the transmembrane transport P-glycoprotein (p-gp). P-gp is a membrane-integrated transport protein from the family of ATP-binding casette (ABC) proteins. The direct structure-function relationships of p-gp are presently unknown. A range of clinically used drugs, *e. g.* calcium channel blockers, anti arrhythmics, neuroleptics, and antidepressants are known to be able to reverse MDR [1]. But the original pharmacological effect of these substances turns into an unrequested side effect. The last decades are characterized by the development of the second (*e. g.* valspodar) and third (e. g. elacridar and tariquidar) generation of potential modulators but, actually, there is no drug with MDR indication in clinical application at all [2]. An optimal chemosensitizer for overcoming of multidrug resistance should inhibit the transport protein, *e. g.* p-gp, without noteworthy side effects and tolerable cytotoxicity [3]. Series of phenothiazines, thioxanthenes, and structurally related compounds which are representative for the well studied class of neuroleptics were investigated by CoMFA (Com-

Correspondence: Matthias Schmidt, Department of Medicinal Chemistry, Institute of Pharmacy, Martin-Luther-University Halle-Wittenberg, 06120 Halle (Saale), Wolfgang-Langenbeck-Str. 4, Germany.
E-mail: matthias.schmidt@pharmazie.uni-halle.de
Fax: 0049 345 552-7018

Abbreviations: ATP-binding casette (ABC); modulator quotient (MQ); multidrug resistance (MDR); p-glycoprotein (p-gp)

parative Molecular Field Analysis) and CoMSIA (Comparative Molecular Similarity Indices Analysis). The dominant role of the hydrophobic and hydrogen-bond acceptor fields for MDR reversing activity of the investigated compounds was demonstrated [4, 5]. The structural regions responsible for differences in anti-MDR activity were analyzed with respect to their hydrophobic, hydrogen-bond acceptor, and steric nature [6].

A key role of p-gp modulators is its high lipophilicity and the presence of two or more aromatic rings. Furthermore, at physiological pH, they have a positive charge caused by the basic part of the molecule resulting in an amphiphilic character [7]. Tertiary amine structures show a better efficiency compared to primary or secondary. Additionally, the integration of tertiary amines into cyclic structures, *e. g.* piperazine and piperidine derivatives, is beneficial [8]. Pearce *et al*. postulated two aromatic domains and a basic nitrogen atom, connected by an aliphatic linker as essential structural feature of MDR modulators [9]. Hait and Aftab published a model for a phenothiazine binding site on p-gp [10]. Thereby, the lipophilic aromatic core structure of phenothiazines interacts with two phenylalanine residues by π-electron interactions. The N-containing protonable part of the molecule interacts with a hydrophilic binding domain consisting of three glutamic acid residues. Not only the presence of aromatic ring systems, but also their steric orientation plays an important role in the modulatoric potential of the compounds. Suzuki *et al*. found the angle of 90 – 105° between the aromatics to be optimal, and the distance between the protonable nitrogen and the center of the hydrophobic domaine should be at least 5 Å [11]. Seelig postulated the existance of two or three electron-donor groups with a defined steric distance as essential characteristic. Simultaneously, the strength of the binding to p-gp correlates with the number and strength of the electron-donor groups [12]. *De facto*, the transmembranal domains 4 – 6 and 11 – 12 possess different amino acids with electron-acceptor groups [13]. Most of these transmembranal domains are exactly the areas responsible for substrate binding and transport [14]. Based on the analysis of various biological test systems, Ekins *et al*. developed a pharmacophor model consisting of an H-bond acceptor, an aromatic ring, and two hydrophobic molecule areas [15]. All models described overlap only in parts, permiting the hypothesis that different substrate binding sites (3 – 4) exist in P-glycoprotein. A general pharmacophor model was created by Pajeva *et al.* [16] based on investigations to the affinity of modulators to the verapamil binding site [17]. It contains two lipophilic domains, three H-bond acceptors, and a H-bond donor in stericly defined orientation. The binding site can be divided in various domains undergoing H-bond interactions and hydrophobic interactions in different ways. Another pharmacophor model was developed for Hoechst 33342 [18] containing five aromatic centers, four points for H-bond acceptors and three points for H-bond donors. The nitrogen can act as a donor or acceptor depending on protonation. Generally, the results of all published models allow to hazard the guess that p-gp has multiple binding sites and can bind and release substrates in multiple pathways [19].

As result of the research concerning calmodulin antagonists of first generation modulators, a range of drugs (*e. g.* trifluoperazine, chlorpromazine, trifluopromazine, flupenthixol) reversed MDR significantly at concentrations ranging from 1 to 10 μM [20, 21, 4]. In continuation of our work to new potential modulators of MDR [22], we synthesized new phenothiazines and related drugs followed by biochemical characterization to contribute to a better understanding of multidrug-resistance phenomenon.

Chemistry

Phenothiazines and structurally related drugs are known to possess antidopaminergic, anticalmodulin, and antitumor properties. Miller *et al*. also demonstrated that these compounds potentiate the clinical activity of cytostatic drugs in carcinoma patients [23]. Several studies have been performed to correlate the *in-vitro* MDR reversal activity and the structure of this class of compounds. The results obtained show the role of hydrophobicity as a space-oriented molecular property to explain the relationship between structure and activity [24, 25].

Among the class of MDR-reversing agents, phenothiazines and related compounds are known to sensitize MDR by interacting with ABC-transporters like p-gp. There exists a range of general structure-acitivity relationships [26]. Zamora *et al.* emphasized the importance of aromatic rings in the hydrophobic moiety of the drugs and also the relative ring position [7]. The compounds with phenyl rings deviating from the plane show a higher activity than planar ring systems. In investigations to chemosensitizers against chloroquine-resistant *Plasmodium falciparum*, a range of analogous compounds was designed and synthesized. Various aromatic amine ring systems, cyclic or noncyclic amino groups, and hydrophilic linkers were examined [27]. Variations in the "butterfly" angle between the two aromatic rings and series of *N*-10 alkyl amides of phenothiazines were looked at in relation to their potency and selectivity toward cholinesterase inhibition [28].

For the synthesis of phenothiazines, three general methods are known. Bernthsen primarily described the reaction of diphenylamine derivatives with sulfur in the presence of iodine as catalyst [29]. Phenothiazines with substituents in position 2, 3, and 4 can be achieved via the analogous {2-[(2-chlorophenyl)thio]phenyl}amine derivatives [30]. An often used method for preparation of 2-substituted compounds comprises the reaction of 2-aminobenzenethiol with 2-chloro-1-nitrobenzene derivatives followed by acetylation and Smiles rearrangement [31, 32].

Various phenothiazines with functionalities at the ring system in combination with modification of the *N*-alkyl side chain were already synthesized by Golinski *et al*. [33]. These phenothiazines were evaluated for their ability to inhibit the calmodulin-mediated inhibition of phosphodiesterase. The synthesis of the 2-phenothiazinyl ketones was achieved by Friedel–Crafts method starting from 10-acetylphenothiazine [34].

Due to the structural similarity to the class of phenothiazines, a range of alternative structures were investigated regarding their MDR-reversing activity. This core structures are well-known as components of drugs with different indications – not yet in connection with MDR. Analysis of general structure-activity relationships let assume a good MDR reversing effect for the structures: 5*H*-dibenzo[*b*,*f*]azepine, 10,11-dihydro-5*H*-dibenzo[*b*,*f*]azepine, 6,7-dihydro-5*H*-dibenzo[c,e]azepine, 1,2,3,9-tetramethoxy-6,7-dihydro-5*H*-dibenzo[*c*,*e*]azepine, 9*H*-carbazol, 5,5-diphenylimidazolidin-2,4-dione. Synthesis of 6,7-dihydro-5*H*-dibenzo[*c*,*e*]azepine was achieved by the modified method of Hawkins and Fu [35] by direct reaction of 2,2′-bis(bromomethyl)-1.1′-biphenyl with the sodium salt of the trifluoroacetamid. After cyclization, the alkaline hydrolysis of the amide led to the expected compound. All further structures were commercially available.

One possibility for the insertion of the linkers is the reaction in strongly alkaline medium created by the addition of sodiumhydrid [36]. Under these conditions, the deprotonation of the nitrogen from weakly NH-acid compounds, e. g. R and S (abbreviations see Table 1), also succeeded. The reaction was arranged with DMSO as solvent under argon atmosphere resulting in a methylsulfinylcarbanion [37]. Addition of the secondary amine occurred after formation of the Na-dimsyl followed by deprotonation and finally adding the alkylhalogenides.

The last reaction step in our synthetic scheme is the N-alkylation of the basic residue with the alkylated core structures as intermediates leading to the desired final compounds. All final products are characterized by the essential molecular structure consisting of lipophilic, aromatic domain, linker, and basic moiety. All final compounds were purified by flash chromatography and converted into the maleic acid salts to have a water-soluble form for biochemical tests. Table 1 shows the synthesized final compounds. Group R^1 represents the variable basic moiety, R^2–R^5 substituents at the core structure, and n means the length of the linker. Some of the synthesized derivatives have been described elsewhere. In this study, all the final compounds were fully characterized by ^{1}H-NMR, ESI-MS, and elemental analysis.

Bioevaluation

Preliminary for *in-vitro* cell tests of the synthesized compounds, the crystalviolet assay was performed on LLC-PK1 cells. This method determines the viability of cells. Consequently, it is possible to quantify the antiproliferative effect of the cytostatic drug, and the cytostatic drug in combination with the test substance which is a parameter of the MDR-reversal activity of the test substances. The cytotoxicity of the compounds can also be analyzed by incubation of cells with different concentrations of the test substances and determination of the number of surviving cells. The crystalviolet test is based on the assessment of the crystalviolet-dye uptake by living cells. The absorption of the dye determined at a wavelength of 620 nm correlates with the number of living cells [38]. The tests were performed at a porcine kidney epithelial cell line (LLC-PK1) [39]. In this cell line, human P-glycoprotein was overexpressed to warrant a high content of the transporter (LLC-MDR1) which was approved by western blot analysis [40]. LLC-MDR1 cells (0.5×10^4 cells/mL) were seeded in 96-well microtiter plates and preincubated for 5 h at 37°C and 5% CO_2. For the resistant cells posses a resistance against vincristine, we determined an IC_{50} value for vincristine of 2.12 μM with a quite high standard deviation especially in the high concentration interval which is founded by the resistance phenomenon. According to clinical practices, we decided to use a constant molar concentration of the cytotoxic drug vincristine of 640 nM as standard. Therefore, cells were incubated with vincristine (640 nM), vincristine (640 nM) + references (0.1–400 μM), and finally, vincristine (640 nM) + test substance (0.1–400 μM) for 68 h at 37°C. The test for cytotoxicity was carried out treating of cells only with the test substance in decreasing concentrations. Every experiment was performed 3–5 times at different days.

For the evaluation of this test, the IC values were determined by the GraphPadPrism and Origin 7G program.

To determine the modulator potential of the investigated substances, different values were calculated

Table 1. Scheme of the final compounds with activity.

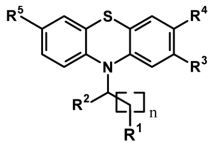
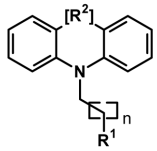
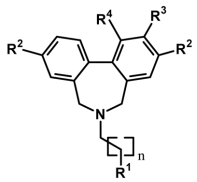
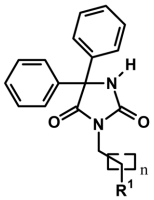
Compound	n	R^1	R^2	R^3	R^4	R^5	$IC_{50mod.}$ (μM ± SD)	$pIC_{50mod.}$	$IC_{50tox.}$	$pIC_{50tox.}$ (μM ± SD)	MQ
											
P3MP	2	MP	H	H	H	H	1.96 ± 0.47	5.71	8.91 ± 1.24	5.05	4.54
P4MP	3	MP	H	H	H	H	1.09 ± 0.19	5.96	4.44 ± 1.03	5.35	4.09
P5MP	4	MP	H	H	H	H	1.27 ± 0.25	5.90	7.83 ± 0.49	5.11	6.17
P6MP	5	MP	H	H	H	H	2.26 ± 0.42	5.65	16.55 ± 2.53	4.78	7.32
P7MP	6	MP	H	H	H	H	3.15 ± 0.67	5.50	17.72 ± 0.71	4.75	5.62
P8MP	7	MP	H	H	H	H	2.52 ± 0.21	5.60	7.57 ± 0.91	5.12	3,01
P10MP	9	MP	H	H	H	H	4.87 ± 1.27	5.31	12.00 ± 3.81	4.92	2.46
P11MP	10	MP	H	H	H	H	7.56 ± 1.27	5.12	23.57 ± 2.02	4.63	3.12
P12MP	11	MP	H	H	H	H	6.25 ± 1.10	5.20	15.21 ± 3.26	4.82	2.43
P3acMP	2	MP	=O	H	H	H	7.64 ± 0.67	5.12	119.67 ± 8.21	3.92	15.67
P2O2MP	1	-$O(CH_2)_2$MP	H	H	H	H	4.32 ± 1.06	5.37	17.58 ± 1.88	4.76	4.07
2MeP3MP	2	MP	H	Me	H	H	1.73 ± 0.19	5.76	14.87 ± 0.26	4.83	8.60
2AcP3MP	2	MP	H	Ac	H	H	1.50 ± 0.06	5.82	25.41 ± 5.94	4.60	16.97
2Prop(ac)P3MP	2	MP	H	Prop(ac)	H	H	0.49 ± 0.08	6.31	13.18 ± 2.06	4.88	26.89
2Bu(ac)P3MP	2	MP	H	Bu(ac)	H	H	0.72 ± 0.02	6.14	15.77 ± 1.30	4.80	21.88
2BzlP3MP	2	MP	H	Bzl	H	H	0.22 ± 0.04	6.66	6.31 ± 0.39	5.20	28,79
3MeOP3MP	2	MP	H	H	MeO	H	0.91 ± 0.22	6.04	14.25 ± 0.62	4.85	15.71
3EtOP3MP	2	MP	H	H	EtO	H	0.46 ± 0.18	6.34	7.64 ± 0.39	5.12	16.53
DtBuP3MP	2	MP	H	H	tBu	tBu	1.24 ± 0.39	5.91	6.13 ± 1.05	5.21	4.95
[a]BnP3MP	2	MP	H	[a]Bn	H	H	0.40 ± 0.14	6.40	7.91 ± 1.08	5.10	19.93
[c]BnP3MP	2	MP	H	H	[c]Bn	H	0.41 ± 0.13	6.39	8.33 ± 1.85	5.08	20,31
2MeOP4MP	3	MP	H	MeO	H	H	4.40 ± 0.73	5.36	22.65 ± 3.12	4.65	5.15
2MeSP4MP	3	MP	H	MeS	H	H	3.07 ± 0.35	5.51	15.73 ± 0.75	4.80	5.12
2AcP4MP	3	MP	H	Ac	H	H	1.88 ± 0.26	5.73	22.47 ± 5.58	4.65	11.92
2Prop(ac)P4MP	3	MP	H	Prop(ac)	H	H	0.76 ± 0.11	6.12	11.53 ± 3.40	4.94	15.16
2Bu(ac)P4MP	3	MP	H	Bu(ac)	H	H	0.36 ± 0.01	6.44	7.32 ± 0.18	5.14	20.19
2BzlP4MP	3	MP	H	Bzl	H	H	0.20 ± 0.01	6.70	7.66 ± 0.58	5.12	38.37
3MeP4MP	3	MP	H	H	Me	H	9.82 ± 1.42	5.01	47.43 ± 3.05	4.32	4.83
3BuP4MP	3	MP	H	H	Bu	H	5.17 ± 0.97	5.29	14.66 ± 0.66	4.83	2.84
3iBuOP4MP	3	MP	H	H	iBuO	H	5.99 ± 0.55	5.22	21.78 ± 2.98	4.66	3.63
3PhP4MP	3	MP	H	H	Ph	H	3.06 ± 0.36	6.51	13.32 ± 1.21	4.88	4.35
tBuP4MP	3	MP	H	H	tBu	H	5.42 ± 1.55	5.27	21.44 ± 3.16	4.67	3.96
DtBuP4MP	3	MP	H	H	tBu	tBu	1.97 ± 0.40	5.71	5.42 ± 0.58	5.27	2.75
[a]BnP4MP	3	MP	H	[a]Bn	H	H	1.17 ± 0.17	5.93	10.32 ± 2.33	4.99	8.85
[c]BnP4MP	3	MP	H	H	[c]Bn	H	1.89 ± 0.39	5.72	10.24 ± 2.05	4.99	5.40
2BzlP6MP	5	MP	H	Bzl	H	H	0.51 ± 0.04	6.29	6.21 ± 0.43	5.21	12.18
2Prop(ac)P8MP	7	MP	H	Prop(ac)	H	H	0.62 ± 0.14	6.21	8.01 ± 0.74	5.10	12.92
2Bu(ac)P8MP	7	MP	H	Bu(ac)	H	H	2.29 ± 0.46	5.64	27.74 ± 1.55	4.56	12.10
2BzlP8MP	7	MP	H	Bzl	H	H	1.22 ± 0.13	5.91	8.43 ± 0.62	5.07	6.92
P4DPh1P	3	DPh1P	H	H	H	H	1.99 ± 0.14	5.70	24.09 ± 2.04	4.62	12.14
P4DPh2P	3	DPh2P	H	H	H	H	1.68 ± 0.17	5.78	24.04 ± 1.68	4.62	14.28
P4DPh3P	3	DPh3P	H	H	H	H	1.72 ± 0.20	5.76	22.82 ± 2.44	4.64	13.26
P4DPh4P	3	DPh4P	H	H	H	H	0.58 ± 0.07	6.24	9.17 ± 1.06	5.04	15.79
P4DPh5P	3	DPh5P	H	H	H	H	0.70 ± 0.08	6.16	10.55 ± 0.88	4.98	15.16
P4DPh1A	3	DPh1A	H	H	H	H	5.32 ± 0.24	5.27	308 ± 18.21	3.51	8.73
P4DPh2A	3	DPh2A	H	H	H	H	2.62 ± 0.26	5.58	19.28 ± 2.72	4.72	7.36
P4DPh3A	3	DPh3A	H	H	H	H	2.39 ± 0.89	5.62	12.58 ± 1.19	4.90	5.24
P4Cl-Ph.Ph1P	3	Cl-Ph.Ph1P	H	H	H	H	5.25 ± 0.65	5.28	43.33 ± 1.44	4.36	8.26
P4DFPh1P	3	DFPh1P	H	H	H	H	4.77 ± 0.31	5.32	57.07 ± 2.91	4.24	11.96
P4DPhOH1PIP	3	DPhOH1PIP	H	H	H	H	28.22 ± 3.71	4.55	62.41 ± 2.95	4.21	2.21

Table 1. Continued.

Compound	n	R^1	R^2	R^3	R^4	R^5	$IC_{50mod.}$ (μM ± SD)	$pIC_{50mod.}$	$IC_{50tox.}$	$pIC_{50tox.}$ (μM ± SD)	MQ
											
MTC4MP	3	MP	–	–	–	–	4.53 ± 0.44	5.34	14.81 ± 0.44	4.83	3.27
MTC4DPP	3	DPh5P	–	–	–	–	0.63 ± 0.06	6.20	13.64 ± 0.47	4.87	21.70
Ib4MP	3	MP	CH_2-CH_2	–	–	–	41.44 ± 5.48	4.38	361.47 ± 7.89	3.44	8.72
Ib4DPP	3	DPh5P	CH_2-CH_2	–	–	–	1.09 ± 0.11	5.96	15.13 ± 2.74	4.82	13.88
Is4MP	3	MP	CH=CH	–	–	–	1.12 ± 0.23	5.95	15.65 ± 1.29	4.81	13.9
Is4DPP	3	DPh5P	CH=CH	–	–	–	5.96 ± 1.08	5.23	53.83 ± 8.49	4.27	9.03
											
R4DPP	3	DPh5P	H	H	H	–	2.85 ± 0.25	5.55	8.38 ± 1.47	5.08	2.94
S4DPP	3	DPh5P	OCH_3	OCH_3	OCH_3	–	4.59 ± 0.49	5.34	15.03 ± 1.38	4.82	3.27
											
MTP4MP	3	MP	–	–	–	–	45.48 ± 3.27	4.34	130.93 ± 8.45	3.88	2.88
MTP4DPP	3	DPh5P	–	–	–	–	1.99 ± 0.15	5.70	6.61 ± 0.89	5.18	3.32
trifluoperazine	–	–	–	–	–	–	1.20 ± 0.48	5.92	9.41 ± 1.23	5.03	7.84
flupentixol	–	–	–	–	–	–	0.45 ± 0.12	6.35	9.67 ± 0.56	5.02	21.71
fluphenazine	–	–	–	–	–	–	1.22 ± 0.13	5.91	10.58 ± 1.11	4.98	8.68
propafenone	–	–	–	–	–	–	1.30 ± 0.27	5.89	25.10 ± 2.4	4.60	19.28
verapamil		–	–	–	–	–	1.30 ± 0.24	5.89	65.03 ± 10.33	4.19	50.00

Abbreviations: MP = methylpiperazine; DPh1P = diphenylmethylpiperazine; DPh2P = diphenylethylpiperazine; DPh3P = diphenylpropylpiperazine; DPh4P = diphenylbutylpiperazine; DPh5P = diphenylpentylpiperazine, DPh1A = diphenylmethylamine; DPh2A = diphenylethylamine; DPh3A = diphenylpropylamine; Cl-Ph,Ph1P = (4-chlorophenyl)phenylmethylpiperazine; DFPh1P = bis(4-fluorophenyl)methylpiperazine; DPhOH1PIP = 4-[hydroxy(diphenyl)methyl]piperidine; Prop(ac) = propionyl residue; Bu(ac) = butyryl residue; $IC_{50mod.}$ = molar concentration (μM) of the modulator that inhibits the growth of cells by 50% in presence of a constant concentration of 640 nM vincristine, $IC_{50tox.}$ = molar inhibitory concentration (μM) of the modulator without vincristine; MQ = modulator quotient.

(Table 1). $IC_{50tox.}$ represents the molar concentration of a substance with a viability of the cells of 50%. It is a quantity of the toxicity of the investigated compound. $IC_{50mod.}$ represents the molar concentration of the substance with a viability of 50% in the presence of a constant concentration of cytostatic drugs. During the experiments, we chose 640 nM vincristine sulfate which was well tolerated by the LLC-MDR1 cells. The modulator quotient MQ is the ratio of $IC_{50mod.}$ to $IC_{50tox.}$ and is deemed to be the measurement of the modulator activity of substances depending on their toxicity.

$$MQ = IC_{50tox.} / IC_{50mod.}$$

It was assumed that an inhibition of the transport by modulators leads to an increase of the toxic effect of cyto-

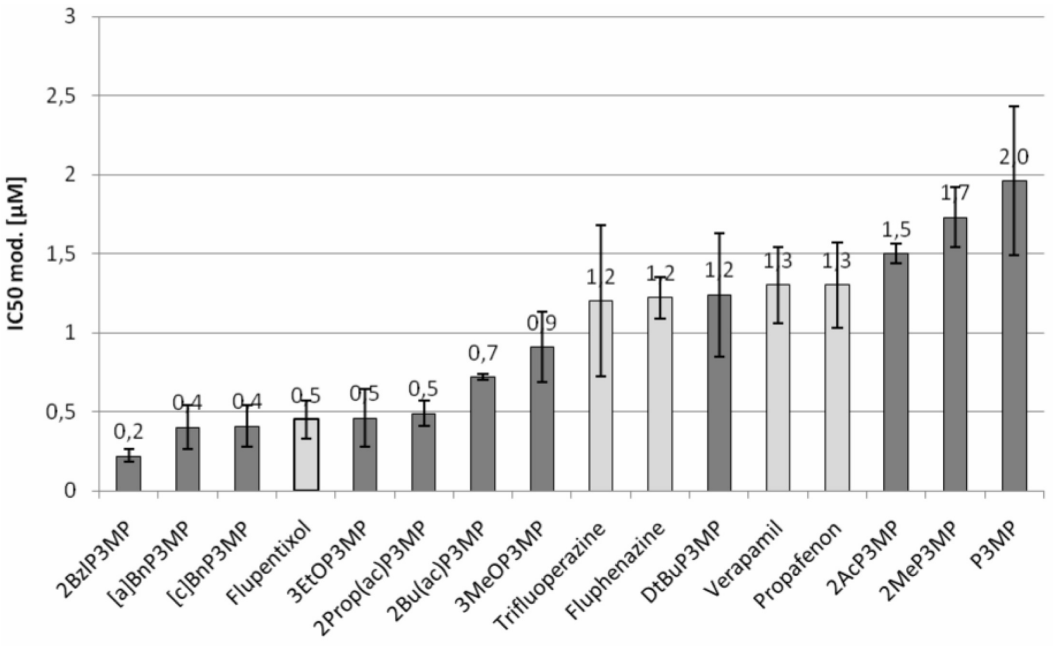


Figure 1. Comparison of $IC_{50mod.}$ values (dark-colored) with variable phenothiazine core structures, constant linker length (C3) and 4-methylpiperazine residue (control substances light-colored).

static drug and, consequently, to a decrease of the viability of cells.

In conclusion, this method allows fast quantification of the interaction of drugs with p-gp and has the potential to serve as a high-throughput screening to detect compounds prone to p-gp-mediated transport.

Results and discussion

Based on the results of the cell experiments and besides general structure-activity relationships, conclusions should also be drawn on the effectiveness of separate structures of the compounds by systematic variation and combination of molecule domains (lipophilic core structure, linker, basic residue). In the following schemata, the determined values of selected compounds will be illustrated, each with two identical, constant, and one variable molecule domain. The $IC_{50mod.}$ values of the respective substances will be compared. We used these values, and not MQ, because the drawback of this factor is that the quotient of high scores can sham a good MQ-value with very low toxicity and minor modulator acitivity. Therefore, in analysis and discussion, the $IC_{50mod.}$ was chosen as parameter to make conclusions about structure-activity-relationships.

Figure 1 shows the $IC_{50mod.}$ values with variable phenothiazine core structures, constant linker length (C3), and 4-methylpiperazine residue based on the structure of trifluperazine as variance comparison. It was shown that the benzoyl, propionyl, and butyryl residues in the 2-position are the most effective substituents. Good results were obtained with compounds containing a condensed benzene ring in [*a*] and [*c*] position, or ethoxy and methoxy groups in the 3-position. Less effective were substances with voluminous *tert.*-butyl-residues or hydrophobic alkyl residues in positions 3 and 7.

One of the main problems of MDR is the fact that the original pharmacological effect of drugs, *e. g.* phenothiazines (blockade of dopamine, muscarinic or histamine receptors) can limit the use as MDR modulator. We tried to weaken the neuroleptic effects by modifying the length of the linker. First step was the elongation of the linker to four carbon atoms. As a result of this test, compounds with a C4-linker also show the same tendency in substitution resulting in substance **2BzlP4MP** with the lowest $IC_{50mod.}$ value (0.20 µM) of all synthesized compounds. Generally, it should be noted that the extension of the linker leads at most to a marginal reduction of the modulator activity of the compounds tolerable by expectant loss of side effects (Fig. 2).

Figure 3 shows the influence of different linkers and alkyl chains on the modulator activity of phenothiazine derivatives with constant core structure and a 4-methylpiperazine residue. Introduction of a carbonyl or ethylene group leads to reduction of the activity as well as the

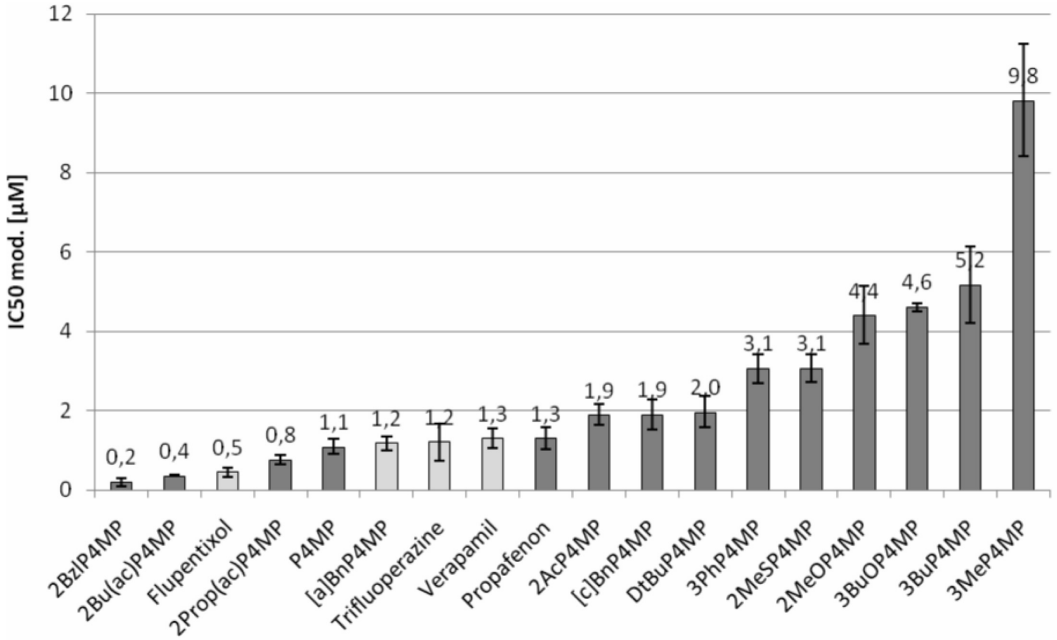


Figure 2. Comparison of $IC_{50mod.}$ values (dark-colored) with variable phenothiazine core structures, constant linker length (C4) and 4-methylpiperazine residue (control substances light-colored).

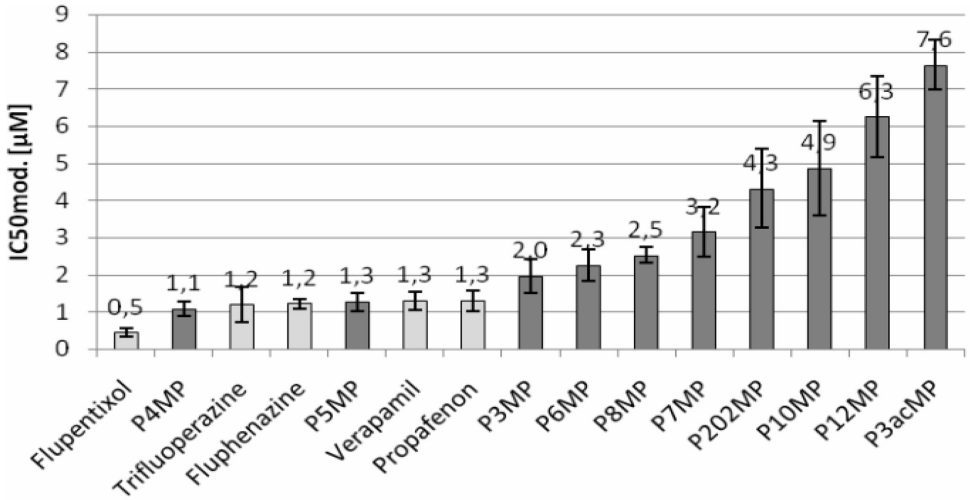


Figure 3. Comparison of $IC_{50mod.}$ values (dark-colored) with variable linker, constant phenothiazine core structure and 4-methylpiperazine residue (control substances light-colored).

elongation of the alkyl chain to about six carbon atoms. Linker length between C3 and C5 do not seem to differ significantly in their activity considering the SD-values.

Figure 4 shows the $IC_{50mod.}$ values of compounds with variable basic residue and a constant 10-butyl-phenothiazine residue. As a result of these investigations, the diphenylalkylpiperazine residues with butyl and pentyl chains show better activity than the 4-methylpiperazine residue as standard. Contraction of the linker between diphenyl residue and piperazine structure (C1 – C3), introduction of a hydroxyl group or H-bond donator groups (Cl-Ph, Ph1P and DFPh1P), and loss of an N-containing ring system (DPh1A, DPh2A, DPh3A) lead to reduction of basicity of the piperazine and piperidine structures, respectively, other basic moieties. Generally, the combination of N-containing ring systems with high basicity and a defined distance between piperazine and the hydrophobic domains is favorably for modulator activity.

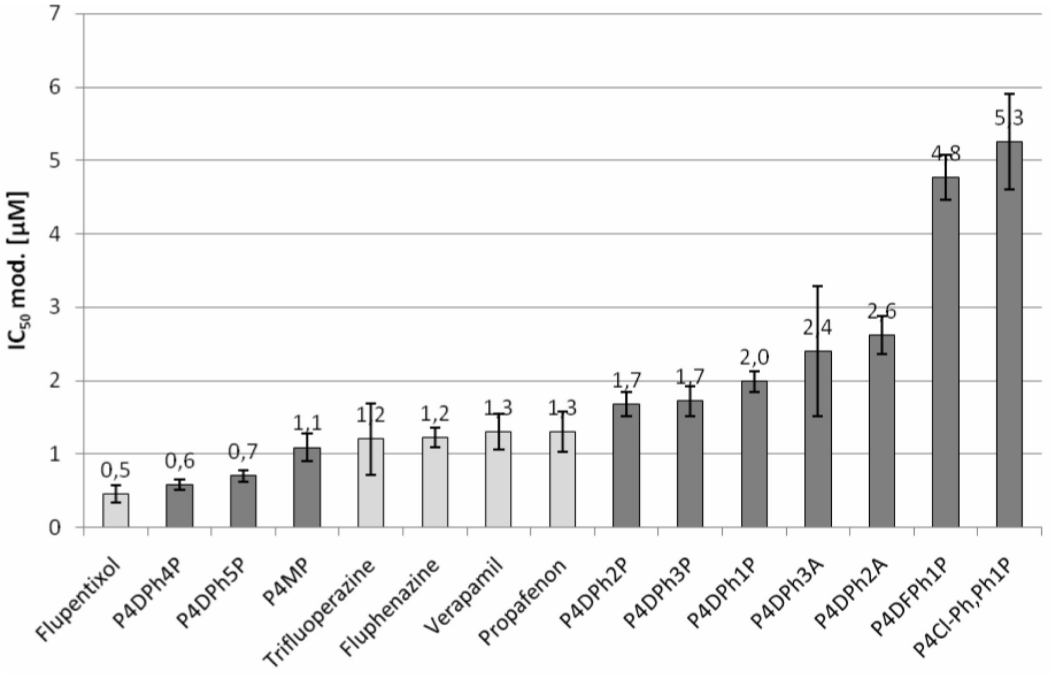


Figure 4. Comparison of $IC_{50mod.}$ values (dark-colored) with variable basic residue and constant 10-butyl-phenothiazine residue (control substances light-colored).

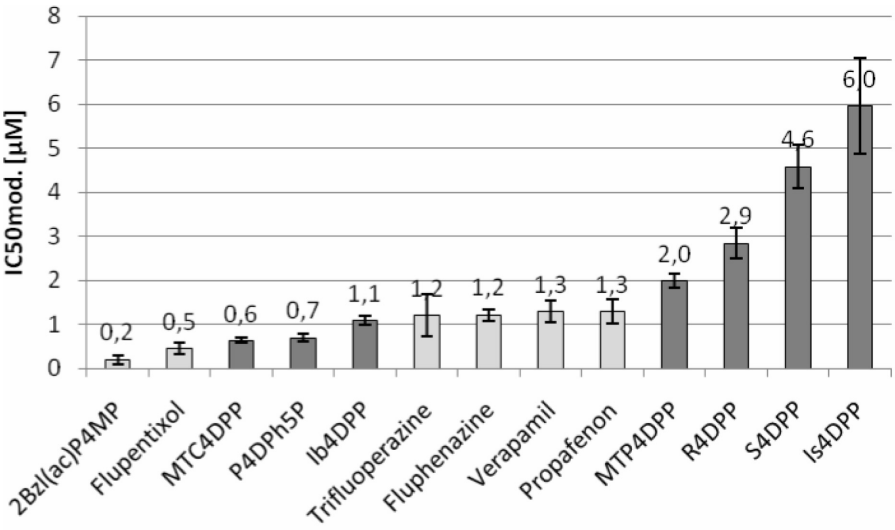


Figure 5. Comparison of $IC_{50mod.}$ values (dark-colored) with variable non phenothiazine core structures and constant diphenylpentylpiperazine residue with C4 linker (control substances light-colored).

Comparison of the other hydrophobic core structures was realized using substances with constant C4 linker and diphenylpentylpiperazine residue as most efficient basic moiety resulting from the analysis (Fig. 4). Generally, most of the tested compounds have a lower potential to reverse MDR in this assay. Only the carbazole derivative **MTC4DPP** and the 5*H*-dibenzo[*b*,*f*]azepine derivative **Ib4DPP** show $IC_{50mod.}$ values comparable with the best phenothazine derivatives (Fig. 5). These results are astonishing because carbazole is a planar tricyclic ringsystem (180°) and 5*H*-dibenzo[*b*,*f*]azepine also differs with an angle of 120° from the hypothesis of Suzuki *et al.*

In this comparison, as in the other comparisons, the IC_{50mod} values of clinical drugs with known modulatoric

effect on p-gp were used as controll substances. The best modulator activity was determined for the thioxanthene derivative *cis*-flupentixol. The phenothiazine derivatives trifluoperazine and fluphenazine, the calcium antagonist verapamil, and the anti-arrhythmic propafenon show approximately identical values. Considering the toxicity, the most potent efflux-pump inhibitor is verapamil as result of evaluation of the modulator quotient values (MQ).

Conclusions

The aim of this study was the systematical modification of phenothiazine derivatives in order to find new structures suitable as MDR-reversal agents. Generally, the majority of our newly synthezised compounds has shown significant modulatoric activity on p-gp. The *in-vitro* tests were realized with the crystalviolet assay on LLC-PK1/MDR1 cells to determine IC_{50} values of the modulatoric activity, toxicity, and modulator quotient, respectively.

In particular, the influence of substituents in positions 2, 3, and 7 at the phenothiazine core structure was investigated with the result that the 2-benzoyl residue is the most effective group. Investigations of various linker lengths and structures revealed that the butyl chain is the optimal linker with the benefit of side effect reversal. Amongst compounds with different basic moieties, the diphenylpentylpiperazine residue is the most promising group.

The data generated in this project enable suggestions to be made for further structure-activity relationships. Future studies will be directed to design compounds with optimal combination of several molecule domains, and to computer-aided methods involving new, more efficient QSAR methods, which will hopefully lead to the directed prediction of simplified, even more active and less toxic substances for further development.

This work was supported by research funds of "Kultusministerium des Landes Sachsen-Anhalt".

The authors have declared no conflict of interest.

Experimental section

Materials and general methods

Mass spectra (MS) were recorded on a Finnigan MAT-710C spectrometer (Thermo Electron Corporation, Bremen, Germany). Gas chromatography-mass spectra (GC-MS) were recorded on a Hewlett-Packard HP 5890 II / MS: 5971 A (Hewlett-Packard, Palo Alto, CA, USA). Elemental analyses were performed on a CHNS-932 microanalyzer (LECO-Corporation, St. Joseph, MI, USA). ^{1}H-NMR spectra were obtained with a Varian Gemini 2000 spectrometer (Varian Inc., Palo Alto, CA, USA) operating at 400 MHz; all values are reported in ppm (δ) downfield from solvent. Polarimetric measurements were accomplished by an Eloptron/Polartronic E (Fa. Schmid + Haensch GmbH & Co, Germany). Flash chromatography was performed on silica gel (Kieselgel 60, 40–63 mesh; Merck, Darmstadt, Germany). TLC was carried out on silica gel plates (E. Merck 60 F_{254}); zones were detected visually by ultraviolet irradiation (254 nm). All reagents were used as purchased unless otherwise stated. Solvents were dried, according to standard procedures. All reactions were carried out under an atmosphere of dry argon. All chemicals were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany).

Chemistry

5,7-Dihydro-6H-dibenz[c,e]azepin R

Synthesis of **R** was achieved by a modified method of Hawkins and Fu [26]. Experimental data corresponds with the literature [41].

25: Yield 51%; Anal. $C_{14}H_{13}N$ requires: C, 86.12; H, 6.71; N 7.17; found: C, 86.19; H, 6.78; N, 7.08; MS (ES+) *m/z*: 195.3 [M + H].

General procedure for the final compounds

10 mmol of the compounds core structure and 10 mmol sodium hydrid were dissolved in 25 mL DMSO and stirred for 1 h at RT under argon atmosphere. To the mixture, 10 mmol dibromoalkane was added and stirred for 12 h at RT. Once the reaction was finished, the mixture was hydrolyzed with water. The organic layer was diluted with 100 mL AcOEt, washed twice with 30 mL saturated sodium bicarbonate solution, dried (Na_2SO_4) and evaporated. The crude residue was analyzed by MS (ES+). The intermediates were used without futher purification.

Method A: 10 mmol of the hydrophobic structure / linker was dissolved in 30 mmol of the basic moiety and heated for 3 h at 120°C. Then, 50 mL water and 50 mL AcOEt were given to the mixture. The organic layer was separated, washed with water, dried (Na_2SO_4), and evaporated *in vacuo*. The residue was purified by flash chromatography ($CHCl_3$ / MeOH 9 : 1) to get the product as base which was converted into the maleic acid salts.

Method B: 10 mmol sodium hydrid was dissolved in 30 mL DMSO and stirred for 1 h at room temperature. 10 mmol of the basic moiety was given to the mixture and stirred for another hour at room temperature. Then 10 mmol of the hydrophobic structure / linker was given to the mixture and stirred for 12 h at room temperature. Once the reaction was finished, it was hydrolyzed with water. The mixture was extracted with AcOEt. The organic layer was dried (Na_2SO_4) and evaporated *in vacuo*. The residue was purified by flash chromatography ($CHCl_3$ / MeOH 9 : 1) to get the product as base which was converted into the maleic acid salts.

General method for synthesis of the maleic acid salts

To a solution of the free base in Et_2O was dropped a saturated solution of maleic acid in Et_2O. Once the precipitation was finished, the salt was filtered off *in vacuo* and recrystallized from MeCN to get the salts as piperidine hydrogen maleate and piperazine bis-(hydrogen maleate).

10-[3-(4-Methylpiperazino)propyl]phenothiazine bis-(hydrogen maleate) P3MP

Yield 67%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.93 – 2.03 (qd, 2H, -$CH_2CH_2CH_2$-), 2.66 – 2.75 (m, 9H, -CH_3, -NCH_2-), 3.03 (m, 4H, -$CH_2CH_2CH_2$-, -NCH_2-), 4.04 (t, 2H, -$CH_2CH_2CH_2$-), 6.27 (s, 4H, maleate), 6.79 – 7.02 (m, 4H, aromat.), 7.10-7.22 (m, 4H, aromat.); Anal. $C_{28}H_{33}N_3O_8S$ requires: C, 58.83; H, 5.82; N, 7.35; S 5,61; found: C, 58.62; H, 5.89; N, 7.38; S, 5.52; MS (ES+) *m/z*: 340.5 [M + H].

10-[4-(4-Methylpiperazino)butyl]phenothiazine bis-(hydrogene maleate) P4MP

Yield 69%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.69 – 1.88 (m, 4H, -$CH_2CH_2CH_2CH_2$-), 2.63 – 2.92 (m, 13H, -CH_3, -NCH_2-), 4.03 (t, 2H, -CH_2-$(CH_2)_3$-), 6.27 (s, 4H, maleate), 6.83 – 7.01 (m, 4H, aromat.), 7.11 – 7.23 (m, 4H, aromat.); Anal. $C_{29}H_{35}N_3O_8S$ requires: C, 59.47; H, 6.02; N, 7.17; S, 5.47; found C, 59.71; H, 6.17; N, 7.24; S, 5.53; MS (ES+) *m/z*: 354.5 [M + H].

10-[5-(4-Methylpiperazino)pentyl]phenothiazine bis-(hydrogen maleate) P5MP

Yield 57%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.52 – 1.85 (m, 6H, -$CH_2(CH_2)_3CH_2$-), 2.61 – 2.91 (m, 13H, -CH_3, -NCH_2-), 3.95 (t, 2H, -$CH_2(CH_2)_4$-), 6.27 (s, 4H, maleate), 6.87 – 6.98 (m, 4H, aromat.), 7.08 – 7.21 (m, 4H, aromat.); Anal. $C_{30}H_{37}N_3O_8S$ requires: C, 60.09; H, 6.22; N, 7.01; S, 5.35; found: C, 60.18; H, 6.32; N, 7.12; S, 5.27; MS (ES+) *m/z*: 368.5 [M + H].

10-[6-(4-Methylpiperazino)hexyl]phenothiazine bis-(hydrogen maleate) P6MP

Yield 58%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.54 – 1.89 (m, 8H, -$CH_2(CH_2)_4$-), 2.60-2.93 (m, 13H, -CH_3, -NCH_2-), 3.99 (t, 2H, -$CH_2(CH_2)_5$-), 6.31 (s, 4H, maleate), 6.75 – 6.99 (m, 4H, aromat.), 7.07 – 7.24 (m, 4H, aromat.); Anal. $C_{31}H_{39}N_3O_8S$ requires: C, 60.67; H, 6.41; N, 6.85; S, 5.22; found: C, 60.58; H, 6.37; N, 6.92; S, 5.29; MS (ES+) *m/z*: 382.6 [M + H].

10-[7-(4-Methylpiperazino)heptyl]phenothiazine bis-(hydrogen maleate) P7MP

Yield 53%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.55 – 1.92 (m, 10H, -$CH_2(CH_2)_5$-), 2.59 – 2.91 (m, 13H, -CH_3, -NCH_2-), 3.95 (t, 2H, -$CH_2(CH_2)_6$-), 6.33 (s, 4H, maleate), 6.76 – 6.98 (m, 4H, aromat.), 7.04 – 7.24 (m, 4H, aromat.); Anal. $C_{32}H_{41}N_3O_8S$ requires: C, 61.23; H, 6.58; N, 6.69; S, 5.11; found: C, 61.31; H, 6.61; N, 6.62; S, 5.07; MS (ES+) *m/z*: 396.6 [M + H].

10-[8-(4-Methylpiperazino)octyl]phenothiazine bis-(hydrogen maleate) P8MP

Yield 56%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.30 – 1.89 (m, 12H, -$CH_2(CH_2)_6CH_2$-), 2.60 – 2.96 (m, 13H, -CH_3, -NCH_2-), 3.93 (t, 2H, -$CH_2(CH_2)_6CH_2$-), 6.27 (s, 4H, maleate), 6.89 – 6.95 (m, 4H, aromat.), 7.06 – 7.15 (m, 4H, aromat.); Anal. $C_{33}H_{43}N_3O_8S$ requires: C, 61.76; H, 6.75; N, 6.85; S, 5.00; found: C, 61.68; H, 6.81; N, 6.81; S, 4.93; MS (ES+) *m/z*: 410.6 [M + H].

10-[10-(4-Methylpiperazino)decyl]phenothiazine bis-(hydrogene maleate) P10MP

Yield 55%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.22 – 2.13 (m, 16H, -$CH_2(CH_2)_8CH_2$-), 2.62 (s, 3H, -CH_3) 3.00 – 3.20 (m, 10H, -NCH_2-), 4.21 (t, 2H, -$CH_2(CH_2)_8CH_2$-), 6.26 (s, 4H, maleate), 7.23 – 7.89 (m, 8H, aromat.); Anal. $C_{35}H_{47}N_3O_8S$ requires: C, 62.76; H, 7.07; N, 6.27; S, 4.79; found: C, 62.68; H, 7.14; N, 6.24; S, 4.63; MS (ES+) *m/z*: 438.7 [M + H].

10-[11-(4-Methylpiperazino)undecyl]phenothiazine bis-(hydrogen maleate) P11MP

Yield 48%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.25 – 2.11 (m, 18H, -$CH_2(CH_2)_9CH_2$-), 2.61 (s, 3H, -CH_3) 3.02 – 3.18 (m, 10H, -NCH_2-), 4.18 (t, 2H, -$CH_2(CH_2)_9CH_2$-), 6.24 (s, 4H, maleate), 7.18 – 7.85 (m, 8H, aromat.); Anal. $C_{36}H_{49}N_3O_8S$ requires: C, 63.23; H, 7.22; N, 6.14; S, 4.69; found: C, 63.18; H, 7.15; N, 6.22; S, 4.56; MS (ES+) *m/z*: 452.7 [M + H].

10-[12-(4-Methylpiperazino)dodecyl]phenothiazine bis-(hydrogen maleate) P12MP

Yield 45%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.22 – 2.12 (m, 20H, -$CH_2(CH_2)_{10}CH_2$-), 2.58 (s, 3H, -CH_3) 3.01 – 3.23 (m, 10H, -NCH_2-), 4.19 (t, 2H, -$CH_2(CH_2)_{10}CH_2$-), 6.24 (s, 4H, maleate), 7.05 – 7.81 (m, 8H, aromat.); Anal. $C_{37}H_{51}N_3O_8S$ requires: C, 63.68; H, 7.37; N, 6.02; S, 4.59; found: C, 63.57; H, 7.21; N, 6.11; S, 4.53; MS (ES+) *m/z*: 466.7 [M + H].

12-[3-(4-Methylpiperazino)propyl]benzo[a]phenothiazine bis-(hydrogene maleate) Bn[a]P3MP

Yield 53%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.81 (m, 2H, -$CH_2CH_2CH_2$-), 2.15 (s, 3H, -CH_3), 2.58 – 2.77 (m, 10H, -NCH_2-), 4.07 (m, 2H, -$CH_2CH_2CH_2$-), 6.26 (s, 4H, maleate), 6.97 – 7.76 (m, 9H, aromat.), 8.04 (d, 1H, C1 aromat.); Anal. $C_{32}H_{35}N_3O_8S$ requires: C, 61.82; H, 5.67; N, 6.76; S, 5.16; found: C, 61.75; H, 5.75; N, 6.68; S, 5.09; MS (ES+) *m/z*: 390.6 [M + H].

*7-[3-(4-Methylpiperazino)propyl]benzo[c]phenothiazine bis-(hydrogen maleate) Bn[c]***P3MP**

Yield 57%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.82 (m, 2H, -$CH_2CH_2CH_2$-), 2.28 (s, 3H, -CH_3), 2.52 – 2.80 (m, 10H, -NCH_2-), 4.05 (m, 2H, -$CH_2CH_2CH_2$-), 6.25 (s, 4H, maleate), 6.96 – 7.84 (m, 9H, aromat.), 8.05 (d, 1H, C6 aromat.); Anal. $C_{32}H_{35}N_3O_8S$ requires: C, 61.82; H, 5.67; N, 6.76; S, 5.16; found: C, 61.78; H, 5.71; N, 6.69; S, 5.11; MS (ES+) *m/z*: 390.6 [M + H].

3,7-Di-tert-butyl-10-[3-(4-methylpiperazino)propyl]-phenothiazine bis-(hydrogen maleate) DTBuP3MP

Yield 52%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.36 (s, 18H, -CCH_3), 1.94 – 1.98 (m, 2H, -$CH_2CH_2CH_2$-), 2.53 – 3.19 (m, 13H, -CH_3, -NCH_2), 3.59 (t, 2H, -$CH_2CH_2CH_2$-), 6.26 (s, 4H, maleate), 7.09 – 7.82 (m, 6H, aromat.); Anal. $C_{36}H_{49}N_3O_8S$ requires: C, 63.23; H, 7.22; N, 6.14; S, 4.69; found: C, 63.32; H, 7.27; N, 6.07; S, 4.63; MS (ES+) *m/z*: 452.7 [M + H].

10-[3-(4-Methylpiperazino)propanoyl]phenothiazine bis-(hydrogen maleate) P3acMP

To a solution of 25 mmol phenothiazine dissolved in 25 mL toluene 30 mmol 3-chloropropanoyl chloride was given dropwise.

The mixture was refluxed for 5 h. When the reaction was finished the mixture was evaporated and recrystallized from EtOH. The intermediate was processed according to Method A.

Yield 72%; ^{1}H-NMR (CD_3OD) δ [ppm]: 2.25 (t, 2H, -COC*H*$_2$-), 2.39 (s, 3H, -C*H*$_3$), 3.13 – 3.71 (m, 10H, -NC*H*$_2$-), 6.24 (s, 4H, maleate), 7.16 – 7.34 (m, 4H, aromat.), 7.40 – 7.52 (m, 4H, aromat.); Anal. $C_{28}H_{31}N_3O_9S$ requires: C, 57.43; H, 5.34; N, 7.18; S, 5.47; found: C, 57.37; H, 5.39; N, 7.08; S, 5.36; MS (ES+) *m/z*: 354.5 [M + H].

10-[(4-Methylpiperazino)ethoxyethyl]phenothiazine bis-(hydrogen maleate) P2O2MP

Yield 79%; ^{1}H-NMR (CD_3OD) δ [ppm]: = 2.67 (s, 3H, -C*H*$_3$), 2.78 – 2.96 (m, 10H, -OCH$_2$C*H*$_2$-, -NC*H*$_2$-), 3.63 (t, 2H, -OC*H*$_2$CH$_2$-), 3.79 (t, 2H, -CH$_2$C*H*$_2$O-), 4.14 (t, 2H, -C*H*$_2$CH$_2$O-), 6.27 (s, 4H, maleate), 6.90 – 7.01 (m, 4H, aromat.), 7.10 – 7.23 (m, 4H, aromat.); Anal. $C_{29}H_{35}N_3O_9S$ requires: C, 57.89; H, 5.86; N, 6.98; S, 5.33; found: C, 57.68; H, 5.75; N, 6.82; S, 5.18; MS (ES+) *m/z*: 370.5 [M + H].

10-[4-(4-Diphenylmethyl)piperazino)butyl]phenothiazine bis-(hydrogen maleate) P4DPh1P

Yield 42%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.61 – 1.78 (m, 4H, -CH$_2$C*H*$_2$C*H*$_2$CH$_2$-), 2.73 – 3.12 (m, 12H, -NC*H*$_2$-), 3.98 (t, 2H, -C*H*$_2$CH$_2$CH$_2$CH$_2$-), 4.21 (s, 1H, -C*H*-), 6.25 (s, 4H, maleate), 6.83 – 7.01 (m, 4H, aromat.), 7.15 – 7.29 (m, 14H, aromat.); Anal. $C_{41}H_{43}N_3O_8S$ requires: C, 66.74; H, 5.87; N, 5.69; S, 4.34; found: C, 66.82; H, 5.81; N, 5.75; S, 4.28; MS (ES+) *m/z*: 506.7 [M + H].

10-[4-(4-(2,2-Diphenylethyl)piperazino)butyl]-phenothiazine bis-(hydrogen maleate) P4DPh2P

Yield 47%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.62 – 1.80 (m, 4H, -CH$_2$C*H*$_2$C*H*$_2$CH$_2$-), 2.74 – 3.10 (m, 12H, -NC*H*$_2$-), 3.99 (t, 2H, -C*H*$_2$CH$_2$CH$_2$CH$_2$-), 4.22 (t, 1H, -C*H*-), 6.27 (s, 4H, maleate), 6.89 – 7.00 (m, 4H, aromat.), 7.10 – 7.27 (m, 14H, aromat.); Anal. $C_{42}H_{45}N_3O_8S$ requires: C, 67.09; H, 6.03; N, 5.59; S, 4.26; found: C, 67.16; H, 6.12; N, 5.47; S, 4.18; MS (ES+) *m/z*: 520.7 [M + H].

10-[4-(4-(3,3-Diphenylpropyl)piperazino)butyl]-phenothiazine bis-(hydrogen maleate) P4DPh3P

Yield 54%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.52 – 1.58 (m, 2H, -CH$_2$CH$_2$C*H*$_2$CH$_2$-*P*), 1.78 – 1.85 (m, 2H, -CH$_2$C*H*$_2$CH$_2$CH$_2$-*P*), 2.04 – 2.26 (m, 4H, -C*H*$_2$C*H*$_2$CH-), 2.58 – 2.83 (m, 10H, -NC*H*$_2$-), 3.71 (t, 1H, -C*H*-), 3.98 (t, 2H, -C*H*$_2$CH$_2$CH$_2$CH$_2$-*P*), 6.25 (s, 4H, maleate), 6.87 – 7.28 (m, 18H, aromat.); Anal. $C_{43}H_{47}N_3O_8S$ requires: C, 67.43; H, 6.19; N, 5.49; S, 4.19; found: C, 67.31; H, 6.31; N, 5.42; S, 4.12; MS (ES+) *m/z*: 534.8 [M + H].

10-[4-(4-(4,4-Diphenylbutyl)piperazino)butyl]-phenothiazine bis-(hydrogen maleate) P4DPh4P

Yield 57%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.52-1.59 (m, 2H, -CH$_2$CH$_2$C*H*$_2$CH$_2$-*P*), 1.70 – 1.75 (m, 2H, -CH$_2$C*H*$_2$CH$_2$CH-), 1.83 – 1.85 (m, 2H, -CH$_2$C*H*$_2$CH$_2$CH$_2$-*P*), 2.08 – 2.16 (m, 2H, -CH$_2$CH$_2$C*H*$_2$CH-), 2.62 – 2.86 (m, 12H, -NC*H*$_2$-), 3.46 (t, 1H, -C*H*-), 3.96 (t, 2H, -C*H*$_2$CH$_2$CH$_2$CH$_2$-*P*), 6.27 (s, 4H, maleate), 6.96 – 7.31 (m, 18H, aromat.); Anal. $C_{44}H_{49}N_3O_8S$ requires: C, 67.76; H, 6.33; N, 5.39; S, 4.11; found: C, 67.65; H, 6.27; N, 5.28; S, 4.02; MS (ES+) *m/z*: 548.8 [M + H].

10-[4-(4-(5,5-Diphenylpentyl)piperazino)butyl]-phenothiazine bis-(hydrogen maleate) P4DPh5P

Yield 54%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.38 – 2.05 (m, 10H, -C*H*$_2$-), 2.61 – 3.12 (m, 12H, -NC*H*$_2$-), 3.37 (t, 2H, -C*H*$_2$CH$_2$CH$_2$CH$_2$-*P*), 3.78 (t, 1H, -C*H*-), 6.24 (s, 4H, maleate), 6.92 – 7.33 (m, 18H, aromat.); Anal. $C_{45}H_{51}N_3O_8S$ requires: C, 68.07; H, 6.47; N, 5.29; S, 4.04; found: C, 67.95; H, 6.37; N, 5.21; S, 4.01; MS (ES+) *m/z*: 562.8 [M + H].

10-[4-(Diphenylmethylamino)butyl]phenothiazine hydrogen maleate P4DPh1A

Yield 18%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.76 – 1.83 (m, 4H, -C*H*$_2$-), 2.95 – 3.03 (t, 2H, -CH$_2$CH$_2$CH$_2$C*H*$_2$NH-), 3.83 (t, 2H, -C*H*$_2$CH$_2$CH$_2$CH$_2$NH-), 4.18 – 4.26 (t, 1H, -C*H*-), 6.24 (s, 2H, maleate), 6.91 – 7.22 (m, 18H, aromat.); Anal. $C_{33}H_{32}N_2O_4S$ requires: C, 71.72; H, 5.84; N, 5.07; S, 5.80; found: C, 71.76; H, 5.81; N, 5.03; S, 5.75; MS (ES+) *m/z*: 437.6 [M + H].

10-[4-(2,2-Diphenylethylamino)butyl]phenothiazine hydrogen maleate P4DPh2A

Yield 24%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.78 – 1.82 (m, 4H, -C*H*$_2$-), 2.96 – 3.04 (t, 2H, -CH$_2$CH$_2$CH$_2$C*H*$_2$NH-), 3.59 – 3.63 (d, 2H, -NHC*H*$_2$-), 3.97 – 4.04 (t, 2H, -C*H*$_2$CH$_2$CH$_2$CH$_2$NH-), 4.21 – 4.29 (t, 1H, -C*H*-), 6.23 (s, 2H, maleate), 6.88 – 7.39 (m, 18H, aromat.); Anal. $C_{34}H_{34}N_2O_4S$ requires: C, 72.06; H, 6.05; N, 4.94; S, 5.66; found: C, 72.09; H, 6.03; N, 4.93; S, 5.45; MS (ES+) *m/z*: 451.6 [M + H].

10-[4-(3,3-Diphenylpropylamino)butyl]phenothiazine hydrogen maleate P4DPh3A

Yield 36%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.72 – 1.87 (m, 4H, -C*H*$_2$-), 2.26 – 2.38 (q, 2H, -NHCH$_2$C*H*$_2$-), 2.79 – 2.95 (m, 4H, -C*H*$_2$NHC*H*$_2$-), 3.91 – 4.01 (m, 3H, -NC*H*$_2$-, -C*H*-), 6.23 (s, 2H, maleate), 6.88 – 7.33 (m, 18H, aromat.); Anal. $C_{35}H_{36}N_2O_4S$ requires: C, 72.39; H, 6.25; N, 4.82; S, 5.52; found: C, 72.42; H, 6.23; N, 4.79; S, 5.49; MS (ES+) *m/z*: 465.7 [M + H].

rac-10-[4-(4-(4-chlorophenyl)phenylmethyl)piperazino)-butyl]phenothiazine bis-(hydrogene maleate) P4PhpClPh1P

Yield 39%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.62 – 1.79 (m, 4H, -CH$_2$C*H*$_2$C*H*$_2$CH$_2$-), 2.71 – 3.17 (m, 12H, -NC*H*$_2$-), 4.01 (t, 2H, -C*H*$_2$CH$_2$CH$_2$CH$_2$-), 4.26 (s, 1H, -C*H*-), 6.24 (s, 4H, maleate), 6.81 – 7.03 (m, 8H, aromat.), 7.19 – 7.45 (m, 9H, aromat.); Anal. $C_{41}H_{42}ClN_3O_8S$ requires: C, 63.76; H, 5.48; Cl, 4.59; N, 5.44; S, 5.94; found: C, 63.81; H, 5.45; Cl, 4.54; N, 5.41; S, 5.89; MS (ES+) *m/z*: 541.2 [M + H].

10-[4-(4-(Bis-(4-fluorophenyl)methyl)piperazino)butyl]-phenothiazine bis-(hydrogene maleate) P4DpFPh1P

Yield 43%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.58 – 1.82 (m, 4H, -CH$_2$C*H*$_2$C*H*$_2$CH$_2$-), 2.69 – 3.12 (m, 12H, -NC*H*$_2$-), 3.98 (t, 2H, -C*H*$_2$CH$_2$CH$_2$CH$_2$-), 4.27 (s, 1H, -C*H*-), 6.25 (s, 4H, maleate), 6.85 – 7.07 (m, 8H, aromat.), 7.25 – 7.78 (m, 8H, aromat.); Anal. $C_{41}H_{41}F_2N_3O_8S$ requires: C, 63.64; H, 5.34; F, 4.91; N, 7.76; S, 5.92; found: C, 63.58; H, 5.41; F, 4.84; N, 7.68; S, 5.88; MS (ES+) *m/z*: 542.7 [M + H].

10-[4-(4-(Diphenylhydroxymethyl)piperidin-1-yl)butyl]phenothiazine P4DPhMeOH1P

Yield 32%; ^{1}H-NMR (CD_3OD) δ [ppm]: = 1.63 – 1.93 (m, 9H, -$CH_2CH_2CH_2CH_2$-, -C*H*-, -CH_2-), 2.15 (s, 1H, -O*H*), 2.67 – 2.93 (m, 6H, -NCH_2-), 3.60 (t, 2H, -$CH_2CH_2CH_2CH_2$-), 7.12-7.54 (m, 18H, aromat.); Anal. $C_{34}H_{36}N_2OS$ requires: C, 78.42; H, 6.97; N, 5.38; S, 6.16; found: C, 78.51; H, 5.42; N, 5.35; S, 6.12; MS (ES+) *m/z*: 521.7 [M + H].

5-(4-[4-Methylpiperazinyl]butyl)-10,11-dihydro-5H-dibenz[b,f]azepine bis-(hydrogene maleate) Ib4MP

Yield 83%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.61 – 1.66 (m, 4H, -CH_2-CH_2-CH_2-CH_2-), 2.62 (s, 3H, -CH_3), 2.73 – 2.81 (m, 10H, -$N_{pip.}CH_2$-), 3.11 – 3.12 (m, 2H, -$N_{az.}CH_2$-), 3.50 – 3.67 (m, 4H, -$C_{az.}H_2$-), 6.27 (s, 4H, maleate), 7.25 – 7.33 (m, 8H, aromat.); Anal. $C_{31}H_{39}N_3O_8$ requires: C, 64.01; H, 6.76; N, 7.2; found: C, 64.35; H, 7.03; N,7.53; MS (ES+) *m/z*: 350.5 [M + H].

5-(4-[4-(5,5-Diphenylpentyl)piperazinyl]butyl)-10,11-dihydro-5H-dibenz[b,f]azepine bis-(hydrogene oxalate) trihydrate Ib4DPP

Yield 65%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.34 – 2.15 (m, 10H, 2[-CH_2-CH_2-CH_2-CH_2-], -CH_2-CH-), 3.04 – 3.26 (m, 12H, -$N_{pip.}CH_2$-), 3.64 – 3.70 (m, 4H, -$C_{az.}H_2$-), 3.92 (t, 1H, -C*H*-), 4.13-4.18 (m, 2H, -$N_{az.}CH_2$-), 7.07 – 7.26 (m, 18H, aromat.); Anal. $C_{43}H_{57}N_3O_{11}$ requires: C, 65.22; H, 7.25; N, 5.31; found: C, 65.21; H, 7.05; N, 5.57; MS (ES+) *m/z*: 558.2 [M + H].

5-(4-[4-(5,5-Diphenylpentyl)piperazinyl]butyl)-5H-dibenz[b,f]azepine bis-(hydrogen oxalate) dihydrate Is4DPP

Yield 68%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.24 – 2.15 (m, 10H, 2[-CH_2-CH_2-CH_2-CH_2-], -CH_2-CH-), 3.29 – 3.31 (m, 15H, -$N_{pip.}CH_2$-, -$N_{az.}CH_2$-), 3.92 (t, 1H, -C*H*-), 6.70 (s, 2H, =C*H*-), 7.21 – 7.80 (m, 18H, aromat.); Anal. $C_{43}H_{53}N_3O_{10}$ requires: C, 66.91; H, 6.92; N, 5.44; found: C, 66.78; H, 6.89; N, 5.40; MS (ES+) *m/z*: 556.8 [M + H].

5-(4-[4-Methylpiperazinyl]butyl)-5H-dibenz[b,f]azepine bis-(hydrogen maleate) Is4MP

Yield 92%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.54 – 1.57 (m, 4H, -CH_2-CH_2-CH_2-CH_2-), 2.17 – 2.69 (m, 13H, -$N_{pip.}CH_2$-, -CH_3), 3.71 (t, 2H, -$N_{az.}CH_2$-), 6.26 (s, 4H, maleate), 6.70 (s, 2H, =C*H*-), 6.98 – 7.78 (m, 8H, aromat.); Anal. $C_{31}H_{37}N_3O_8$ requires: C, 64.24; H, 6.43; N, 7.25; found: C, 64.29; H, 6.52; N, 7.21; MS (ES+) *m/z*: 348.5 [M + H].

9-(4-[4-Methylpiperazinyl]butyl)-9H-carbazole bis-(hydrogen oxalate) hydrate MTC4MP

Yield 86%; ^{1}H-NMR ($CDCl_3$) δ [ppm]: 1.53 – 1.61 (m, 2H, -CH_2-), 1.86 – 1.93 (m, 2H, -CH_2-), 2.27 (s, 3H, -N-CH_3), 2.33 – 2.43 (m, 10H, -$N_{pip.}CH_2$-), 4.31 (t, 2H, -$N_{carb.}CH_2$-), 7.18 – 8.09 (m, 8H, aromat.); Anal. $C_{25}H_{33}N_3O_9$ requires: C, 57.80; H, 6.40; N, 8.09; found: C, 57.79; H, 6.42; N, 8.05; MS (ES+) *m/z*: 322.5 [M + H].

9-[4-(4-[5,5-Diphenylpentyl]piperazinyl)butyl]-9H-carbazole bis-(hydrogen oxalate) dihydrate MTC4DPP

Yield 78%; ^{1}H-NMR ($CDCl_3$) δ [ppm]: 1.43 – 1.60 (m, 6H, 3[-CH_2-]), 1.85 – 1.98 (m, 4H, -CH_2-), 2.19 – 2.56 (m, 12H, -$N_{pip.}CH_2$-), 4.08 – 4.13 (m, 1H, -CH-), 4.29 – 4.33 (t, 2H, $N_{carb.}CH_2$-), 7.12 – 8.09 (m, 18H, aromat.); Anal. $C_{39}H_{49}N_3O_{10}$ requires: C, 65.07; H, 6.86; N, 5.84; found: C, 65.12; H, 6.91; N, 5.82; MS (ES+) *m/z*: 530.8 [M + H].

3-[4-(4-Methylpiperazinyl)butyl]-5,5-diphenyl-imidazolidine-2,4-dion bis-(hydrogen maleate) MTP4MP

Yield 92%; ^{1}H-NMR ($CDCl_3$) δ [ppm]: 1.38 – 1.68 (m, 4H, -CH_2-), 2.23 (s, 3H, -CH_3), 2.30 – 2.60 (m, 10H, -$N_{pip.}CH_2$-), 3.56-3.60 (m, 2H, -$N_{imi.}CH_2$-), 6.26 (s, 4H, maleate), 7.29 – 7.38 (m, 10H, aromat.); Anal. $C_{32}H_{38}N_4O_{10}$ requires: C, 60.18; H, 6.00; N, 8.77; found: C, 60.22; H, 6.04; N, 8.79; MS (ES+) *m/z*: 407.5 [M + H].

3-(4-(4-(5,5-Diphenylpentyl)piperazinyl)butyl)-5,5-diphenylimidazolidine-2,4-dion bis-(hydrogen maleate) MTP4DPP

Yield 81%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.33 – 2.14 (m, 10H, -CH_2-), 2.63 – 3.14 (m, 12H, -$N_{pip.}CH_2$-), 3.60 (t, 2H, -$N_{imi.}CH_2$-), 3.91 (t, 1H, -C*H*-), 6.27 (s, 4H, maleate), 7.12 – 7.43 (m, 20H, aromat.); Anal. $C_{48}H_{54}N_4O_{10}$ requires: C, 68.07; H, 6.43; N, 6.61; found: C, 68.32; H, 6.48; N, 6.70; MS (ES+) *m/z*: 615.8 [M + H].

6-(4-[4-(5,5-Diphenylpentyl)piperazinyl]butyl)-6,7-dihydro-5H-dibenz[c,e]azepine bis-(hydrogen oxalate) dihydrate R4DPP

Yield 58%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.27 – 1.64 (m, 8H, -CH_2-]), 2.05 – 2.11 (m, 2H, -CH_2-CH-), 2.56-2.60 (t, 2H, -$N_{az.}CH_2$-CH_2-), 3.23 – 3.30 (m, 12H, -$N_{pip.}CH_2$-), 3.32 – 3.51 (dd, 4H, -$CH_2N_{az.}CH_2$-), 3.89 (t, 1H, -C*H*-), 7.09 – 7.63 (m, 18H, aromat.); Anal. $C_{43}H_{55}N_3O_{10}$ requires: C, 67.08; H, 6.68; N, 5.46; found: C, 67.02; H, 6.74; N, 5.39; MS (ES+) *m/z*: 558.8 [M + H].

6-(4-[4-(4,4-Diphenylpentyl)piperazinyl]butyl)-1,2,3,9-tetramethoxy-6,7-dihydro-5H-dibenz[c,e]azepine bis-(hydrogen maleate) S4DPP

Yield 46%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.47 – 1.51 (m, 8H, -CH_2-), 2.07-2.13 (m, 12H, -$N_{pip.}CH_2$-), 2.61 – 2.66 (m, 2H, -$N_{az.}CH_2$-CH_2-), 3.18 – 3.20 (m, 3H, -OCH_3-), 3.73 (t, 1H, -C*H*-), 3.85 – 3.97 (m, 13H, -OCH_3-, -$CH_2N_{az.}CH_2$-]), 6.27 (s, 2H, maleate), 7.10 – 7.27 (m, 14H, aromat.); Anal. $C_{50}H_{61}N_3O_{12}$ requires: C, 67.02; H, 6.86; N, 4.69; found: C, 66.92; H, 6.99; N, 4.58; MS (ES+) *m/z*: 664.9 [M + H].

2-Acetyl-10-[3-(4-methylpiperazino)propyl]phenothiazin 2AcP3MP

Yield 63%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.94 – 2.01 (qd, 2H, -$CH_2CH_2CH_2$-), 2.58 (s, 3H, -COCH_3), 2.79 (s, 3H, -NCH_3), 2.89 – 3.17 (m, 10H, -NCH_2-), 4.03 (t, 2H, -$N_{PTA}CH_2$-), 6.27 (s, 4H, maleate), 6.85 – 7.07 (m, 4H, aromat.), 7.28 – 7.65 (m, 3H, aromat.); Anal. $C_{30}H_{35}N_3O_9S$ requires: C, 58.72; H, 5.75; N, 6.85; S 5.22; found: C, 58.65; H, 5.86; N, 6.81; S, 5.15; MS (ES+) *m/z*: 382.5 [M + H].

2-Propionyl-10-[3-(4-methylpiperazino)propyl]-phenothiazine bis-(hydrogen maleate) 2Prop(ac)P3MP

Yield 57%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.31 (t, 3H, -CH_2CH_3), 1.93 – 2.03 (qd, 2H, -$CH_2CH_2CH_2$-), 2.84 – 3.31 (m, 15H, -NCH_2-, -NCH_3, -COCH_2-), 4.00 (t, 2H, -$N_{PTA}CH_2$-), 6.26 (s, 4H, maleate), 6.88 – 7.04 (m, 4H, aromat.), 7.17 – 7.54 (m, 3H, aromat.); Anal. $C_{31}H_{37}N_3O_9S$ requires: C, 59.32; H, 5.94; N, 6.69; S 5.11; found: C, 59.41; H, 5.98; N, 6.61; S, 5.05; MS (ES+) *m/z*: 396.6 [M + H].

2-Butyryl-10-[3-(4-methylpiperazino)propyl]-phenothiazine bis-(hydrogen maleate) (P-4) 2Bu(ac)P3MP

Yield 60%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.17 (t, 3H, -CH_2CH_3), 1.62 – 1.75 (m, 2H, -CH_2CH_3), 2.01 – 2.07 (qd, 2H, -$CH_2CH_2CH_2$-), 2.84 – 3.27 (m, 15H, -NCH_2-, -NCH_3, -$COCH_2$-), 3.97 (t, 2H, -$N_{PTA}CH_2$-), 6.26 (s, 4H, maleate), 6.98 – 7.81 (m, 7H, aromat.); Anal. $C_{32}H_{39}N_3O_9S$ requires: C, 59.89; H, 6.13; N, 6.55; S 5.00; found: C, 59.82; H, 6.18; N, 6.49; S, 4.95; MS (ES+) *m/z*: 410.6 [M + H].

2-Benzoyl-10-[3-(4-methylpiperazino)propyl]-phenothiazine bis-(hydrogen maleate) 2BzlP3MP

Yield 52%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.93 – 1.97 (m, 2H, -CH_2-), 2.63 – 2.81 (m, 13H, -CH_3, -NCH_2), 3.99 (t, 2H, -$N_{PTA}CH_2$-), 6.26 (s, 4H, maleate), 6.88 – 7.72 (m, 12H, aromat.); Anal. $C_{35}H_{37}N_3O_9S$ requires: C, 62.21; H, 5.52; N, 6.22; S, 4.74; found: C, 62.28; H, 5.59; N, 6.13; S, 4.62; MS (ES+) *m/z*: 444.6 [M + H].

3-Methoxy-10-[3-(4-methylpiperazino)propyl]-phenothiazine bis-(hydrogen maleate) 3MeOP3MP

Yield 30%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.98 (qd, 2H, -CH_2-CH_2-CH_2-), 2.63 – 2.91 (m, 13H, -$N_{pip.}CH_2$-, -CH_3), 3.73 (s, 3H, -OCH_3), 3.91 – 3.97 (t, 2H, -$N_{PTA}CH_2$-), 6.27 (s, 4H, maleate), 6.74 – 6.79 (m, 2H, aromat.), 6.86 – 6.97 (m, 3H, aromat.), 7.09 – 7.22 (m, 2H, aromat.); Anal. $C_{29}H_{35}N_3O_9S$ requires: C, 57.89; H, 5.86; N, 6.98; S, 5.33; found: C, 57.92; H, 5.96; N, 6.85; S, 5.27; MS (ES+) *m/z*: 602.7 [M + H].

3-Ethoxy-10-[3-(4-methylpiperazino)propyl]phenothiazine bis-(hydrogen maleate) 3EtOP3MP

Yield 54%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.33 (t, 3H, -OCH_2CH_3), 1.91 – 2.01 (qd, 2H, -CH_2-CH_2-CH_2-), 2.67 – 2.74 (m, 9H, -$N_{pip.}CH_2$-, -CH_3), 2.92 – 3.06 (m, 4H, -$N_{pip.}CH_2$-), 3.90 – 4.01 (m, 4H, -OCH_2CH_3, -$N_{PTA}CH_2$-), 6.26 (s, 4H, maleate), 6.71 – 7.21 (m, 7H, aromat.); Anal. $C_{30}H_{37}N_3O_9S$ requires: C, 58.52; H, 6.06; N, 6.82; S, 5.21; found: C, 58.36; H, 5.79; N, 6.91; S, 5.38; MS (ES+) *m/z*: 616.7 [M + H].

2-Methyl-10-[3-(4-methylpiperazino)propyl]phenothiazine bis-(hydrogen maleate) 2MeP3MP

Yield 61%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.95 – 2.04 (qd, 2H, -$CH_2CH_2CH_2$-), 2.22 (s, 3H, Ar-CH_3), 2.86 (s, 3H, -NCH_3), 3.02 – 3.28 (m, 10H, -NCH_2-), 4.03 (t, 2H, -$N_{PTA}CH_2$-), 6.27 (s, 4H, maleate), 6.98 – 7.58 (m, 7H, aromat.); Anal. $C_{29}H_{35}N_3O_8S$ requires: C, 59.47; H, 6.02; N, 7.17; S 5,47; found: C, 59.42; H, 6.09; N, 7.11; S, 5.42; MS (ES+) *m/z*: 354.5 [M + H].

3-Methyl-10-[4-(4-methylpiperazino)butyl]phenothiazine bis-(hydrogen maleate) 3MeP4MP

Yield 64%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.67 – 1.96 (m, 4H, -6.2.3.29 $CH_2CH_2CH_2CH_2$-), 2.18 (s, 3H, Ar-CH_3), 2.67 – 3.24 (m, 13H, -CH_3, -NCH_2-), 4.03 (t, 2H, -$N_{PTA}CH_2$-), 6.26 (s, 4H, maleate), 6.67 – 7.25 (m, 7H, aromat.); Anal. $C_{30}H_{37}N_3O_8S$ requires: C, 60.09; H, 6.22; N, 7.01; S, 5.35; found: C, 59.99; H, 6.17; N, 6.98; S, 5.31; MS (ES+) *m/z*: 368.6 [M + H].

3-Butyl-10-[4-(4-methylpiperazino)butyl]phenothiazine bis-(hydrogen maleate) 3BuP4MP

Yield 58%; ^{1}H-NMR (CD_3OD) δ [ppm]: 0.91 (t, 3H, -CH_2CH_3), 1.32 – 1.98 (m, 8H, -CH_2CH_2-), 2.37 – 2.46 (m, 2H, Ar-CH_2-), 2.74 – 3.25 (m, 13H, -CH_3, -NCH_2-), 4.01 (t, 2H, -$N_{PTA}CH_2$-), 6.27 (s, 4H, maleate), 6.68 – 7.27 (m, 7H, aromat.); Anal. $C_{33}H_{43}N_3O_8S$ requires: C, 61.76; H, 6.75; N, 6.55; S, 5.00; found: C, 61.71; H, 6.81; N, 6.48; S, 4.97; MS (ES+) *m/z*: 410.6 [M + H].

2-Methylthio-10-[4-(4-methylpiperazino)butyl]-phenothiazine bis-(hydrogen maleate) 2MeSP4MP

Yield 56%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.65 – 1.97 (m, 4H, -$CH_2CH_2CH_2CH_2$-), 2.32 (s, 3H, Ar-SCH_3), 2.84 – 3.32 (m, 13H, -CH_3, -NCH_2-), 4.00 (t, 2H, -$N_{PTA}CH_2$-), 6.27 (s, 4H, maleate), 6.68 – 7.67 (m, 7H, aromat.); Anal. $C_{30}H_{37}N_3O_8S_2$ requires: C, 57.04; H, 5.90; N, 6.65; S, 10.15; found: C, 56.99; H, 5.97; N, 6.58; S, 10.03; MS (ES+) *m/z*: 400.6 [M + H].

2-Methoxy-10-[4-(4-methylpiperazino)butyl]-phenothiazine bis-(hydrogen maleate) 2MeOP4MP

Yield 62%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.68 – 1.99 (m, 4H, -$CH_2CH_2CH_2CH_2$-), 2.81 – 3.27 m, 13H, -CH_3, -NCH_2-), 3.78 (s, 3H, -OCH_3), 4.02 (t, 2H, -$N_{PTA}CH_2$-), 6.26 (s, 4H, maleate), 6.58 – 7.37 (m, 7H, aromat.); Anal. $C_{30}H_{37}N_3O_9S$ requires: C, 58.52; H, 6.06; N, 6.82; S, 5.21; found: C, 58.49; H, 6.17; N, 6.85; S, 5.13; MS (ES+) *m/z*: 384.6 [M + H].

3-Butoxy-10-[4-(4-methylpiperazino)butyl]phenothiazine bis-(hydrogen maleate) 3BuOP4MP

Yield 53%; ^{1}H-NMR (CD_3OD) δ [ppm]: 0.96 (t, 3H, -CH_2CH_3), 1.43 – 2.02 (m, 8H, -CH_2CH_2-), 2.77 – 3.32 (m, 13H, -NCH_3, -NCH_2-), 3.96 – 4.04 (m, 4H, -OCH_2-, -$N_{PTA}CH_2$-), 6.26 (s, 4H, maleate), 6.72 – 7.26 (m, 7H, aromat.); Anal. $C_{33}H_{43}N_3O_9S$ requires: C, 60.26; H, 6.59; N, 6.39; S, 4.87; found: C, 60.17; H, 6.68; N, 6.34; S, 4.76; MS (ES+) *m/z*: 426.6 [M + H].

3-Isobutoxy-10-[4-(4-methylpiperazino)butyl]-phenothiazine bis-(hydrogen maleate) 3isoBuOP4MP

Yield 57%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.12 (d, 6H, -$CH(CH_3)_2$), 1.64 – 1.96 (m, 4H, -CH_2CH_2-), 2.24 – 2.36 (m, 1H, -CH-), 2.84 – 3.36 (m, 13H, -NCH_3, -NCH_2-), 3.76 – 3.78 (m, 2H, -OCH_2-), 4.01 (t, 2H, -$N_{PTA}CH_2$-), 6.27 (s, 4H, maleate), 6.74 – 7.42 (m, 7H, aromat.); Anal. $C_{33}H_{43}N_3O_9S$ requires: C, 60.26; H, 6.59; N, 6.39; S, 4.87; found: C, 60.15; H, 6.46; N, 6.31; S, 4.79; MS (ES+) *m/z*: 426.6 [M + H].

3-Phenyl-10-[4-(4-methylpiperazino)butyl]phenothiazine bis-(hydrogen maleate) 3PhP4MP

Yield 59%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.72 – 1.98 (m, 4H, -$CH_2CH_2CH_2CH_2$-), 2.76 – 2.92 (m, 13H, -CH_3, -NCH_2-), 4.02 (t, 2H, -$N_{PTA}CH_2$-), 6.27 (s, 4H, maleate), 6.64 – 7.58 (m, 12H, aromat.); Anal. $C_{35}H_{39}N_3O_8S$ requires: C, 63.52; H, 5.94; N, 6.35; S, 4.84; found: C, 63.47; H, 5.97; N, 6.27; S, 4.73; MS (ES+) *m/z*: 430.6 [M + H].

10-[4-(4-Methylpiperazino)butyl]benzo[c]phenothiazine bis-(hydrogen maleate) Benz[c]P4MP

Yield 63%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.67 – 1.85 (m, 4H, -$CH_2CH_2CH_2CH_2$-), 2.61 – 2.97 (m, 13H, -CH_3, -N-CH_2-), 4.02 (t, 2H,

-CH_2-$(CH_2)_3$-), 6.27 (s, 4H, maleate), 6.92 (d, 2H, aromat.), 7.12 – 7.21 (m, 3H, aromat.), 7.32 (t, 1H, aromat.), 7.46 (t, 1H, aromat.), 7.63 (d, 1H, aromat.), 7.71 (d,1H, aromat.), 8.10 (d, 1H, aromat.); Anal. $C_{33}H_{37}N_3O_8S$ requires: C, 62.35; H, 5.87; N, 6.61; S, 5.04; found: C, 62.31; H, 5.91; N, 6.52; S, 5.03; MS (ES+) *m/z*: 404.6 [M + H].

10-[4-(4-Methylpiperazino)butyl]benzo[a]phenothiazine bis-(hydrogen maleate) Benz[a]P4MP

Yield 47%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.66 – 1.92 (m, 4H, -$CH_2CH_2CH_2CH_2$-), 2.74 – 3.14 (m, 13H, -CH_3, -NCH_2-), 4.02 (t, 2H, -$N_{PTA}CH_2$-), 6.26 (s, 4H, maleate), 6.98 (d, 2H, aromat.), 7.08 – 7.16 (m, 3H, aromat.), 7.36 (t, 1H, aromat.), 7.52 (t, 1H, aromat.), 7.64 (d, 1H, aromat.), 7.78 (d,1H, aromat.), 8.14 (d, 1H, aromat.); Anal. $C_{33}H_{37}N_3O_8S$ requires: C, 62.35; H, 5.87; N, 6.61; S, 5.04; found: C, 62.42; H, 5.85; N, 6.49; S, 5.01; MS (ES+) *m/z*: 404.6 [M + H].

3,7-Di-tert-butyl-10-[4-(4-methylpiperazino)butyl]-phenothiazine bis-(hydrogen oxalate) 3,7DTBuP4MP

Yield 48%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.38 (s, 18H, -CCH_3), 1.82 – 2.15 (m, 4H, -CH_2-), 2.53 – 3.34 (m, 13H, -CH_3, -$N_{pip.}CH_2$), 3.58 (t, 2H, -$N_{PTA}CH_2$-), 6.89 – 7.62 (m, 6H, aromat.); Anal. $C_{33}H_{47}N_3O_8S$ requires: C, 61.37; H, 7.34; N, 6.51; S, 4.96; found: C, 61.24; H, 7.38; N, 6.37; S, 4.87; MS (ES+) *m/z*: 466.7 [M + H].

3-tert-Butyl-10-[4-(4-methylpiperazino)butyl]phenothiazin bis-(hydrogen maleate) 3TBuP4MP

Yield 52%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.32 (s, 9H, -CCH_3), 1.76 – 2.04 (m, 4H, -CH_2-), 2.78 – 3.32 (m, 13H, -CH_3, -$N_{pip.}CH_2$), 3.60 (t, 2H, -$N_{PTA}CH_2$-), 6.86 – 7.32 (m, 6H, aromat.); Anal. $C_{33}H_{43}N_3O_8S$ requires: C, 61.76; H, 6.75; N, 6.55; S, 5.00; found: C, 61.64; H, 6.81; N, 6.47; S, 4.97; MS (ES+) *m/z*: 410.6 [M + H].

2-Acetyl-10-[4-(4-methylpiperazino)butyl]phenothiazine bis-(hydrogen maleate) 2AcP4MP

ield 63%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.68 – 2.00 (m, 4H, -CH_2-), 2.54 (s, 3H, -$COCH_3$), 2.76 (s, 3H, -NCH_3), 2.92 – 3.24 (m, 10H, -NCH_2-), 4.01 (t, 2H, -$N_{PTA}CH_2$-), 6.26 (s, 4H, maleate), 6.84 – 7.14 (m, 4H, aromat.), 7.26 – 7.48 (m, 3H, aromat.); Anal. $C_{31}H_{37}N_3O_9S$ requires: C, 59.32; H, 5.94; N, 6.69; S 5.11; found: C, 59.26; H, 5.98; N, 6.61; S, 5.07; MS (ES+) *m/z*: 396.6 [M + H].

2-Propionyl-10-[4-(4-methylpiperazino)butyl]-phenothiazine bis-(hydrogen maleate) 2Prop(ac)P4MP

Yield 54%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.28 (t, 3H, -CH_2CH_3), 1.67 – 2.02 (m, 4H, -CH_2-), 2.78 – 3.34 (m, 15H, -NCH_2-, -NCH_3, -$COCH_2$-), 4.03 (t, 2H, -$N_{PTA}CH_2$-), 6.27 (s, 4H, maleate), 6.84 – 7.08 (m, 4H, aromat.), 7.18 – 7.46 (m, 3H, aromat.); Anal. $C_{32}H_{39}N_3O_9S$ requires: C, 59.89; H, 6.13; N, 6.55; S 5.00; found: C, 59.83; H, 6.19; N, 6.52; S, 4.98; MS (ES+) *m/z*: 410.6 [M + H].

2-Butyryl-10-[4-(4-methylpiperazino)butyl]phenothiazine bis-(hydrogen maleate) 2Bu(ac)P4MP

Yield 56%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.06 (t, 3H, -CH_2CH_3), 1.62 – 2.06 (m, 6H, -CH_2CH_3, -CH_2-), 2.81 – 3.24 (m, 15H, -NCH_2-, -NCH_3, -$COCH_2$-), 3.99 (t, 2H, -$N_{PTA}CH_2$-), 6.26 (s, 4H, maleate), 6.86 – 7.68 (m, 7H, aromat.); Anal. $C_{33}H_{41}N_3O_9S$ requires: C, 60.44; H, 6.30; N, 6.41; S 4.89; found: C, 60.32; H, 6.35; N, 6.38; S, 4.83; MS (ES+) *m/z*: 424.6 [M + H].

2-Benzoyl-10-[4-(4-methylpiperazino)butyl]phenothiazine bis-(hydrogen maleate) 2BzlP4MP

Yield 45%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.35 – 1.38 (m, 2H, -CH_2-), 1.93 – 1.97 (m, 2H, -CH_2-), 2.64 – 2.83 (m, 13H, -CH_3, -$N_{pip.}CH_2$), 4.00 (t, 2H, -$N_{PTA}CH_2$-), 6.26 (s, 4H, maleate), 6.82 – 8.01 (m, 12H, aromat.); Anal. $C_{36}H_{39}N_3O_9S$ requires: C, 62.69; H, 5.70; N, 6.09; S, 4.65; found: C, 62.54; H, 5.81; N, 6.21; S, 4.53; MS (ES+) *m/z*: 458.6 [M + H].

2-Propionyl-10-[8-(4-methylpiperazino)octyl]-phenothiazine bis-(hydrogen maleate) 2Prop(ac)P8MP

Yield 53%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.24 – 1.98 (m, 15H, -CH_2CH_3, -$CH_2(CH_2)_6CH_2$-), 2.74 – 3.32 (m, 15H, -NCH_2-, -NCH_3, -$COCH_2$-), 4.01 (t, 2H, -$N_{PTA}CH_2$-), 6.26 (s, 4H, maleate), 6.86 – 7.04 (m, 4H, aromat.), 7.16 – 7.48 (m, 3H, aromat.); Anal. $C_{36}H_{47}N_3O_9S$ requires: C, 61.96; H, 6.79; N, 6.02; S 4.59; found: C, 61.88; H, 6.84; N, 5.98; S, 4.51; MS (ES+) *m/z*: 466.7 [M + H].

2-Butyryl-10-[8-(4-methylpiperazino)octyl]phenothiazine bis-(hydrogen maleate) 2Bu(ac)P8MP

Yield 57%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.04 (t, 3H, -CH_2CH_3), 1.28 – 2.06 (m, 14H, -CH_2CH_3, -$CH_2(CH_2)_6CH_2$-), 2.78 – 3.26 (m, 15H, -NCH_2-, -NCH_3, -$COCH_2$-), 3.96 (t, 2H, -$N_{PTA}CH_2$-), 6.27 (s, 4H, maleate), 6.86 – 7.63 (m, 7H, aromat.); Anal. $C_{37}H_{49}N_3O_9S$ requires: C, 62.43; H, 6.94; N, 5.90; S 4.50; found: C, 62.35; H, 6.98; N, 5.86; S, 4.43; MS (ES+) *m/z*: 480.7 [M + H].

2-Benzoyl-10-[6-(4-methylpiperazino)hexyl]-phenothiazine bis-(hydrogen maleate) 2BzlP6MP

Yield 37%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.38 – 1.87 (m, 8H, -CH_2-), 2.61 – 2.88 (m, 13H, -CH_3, -$N_{pip.}CH_2$), 3.99 (t, 2H, -$N_{PTA}CH_2$-), 6.28 (s, 4H, maleate), 6.78 – 7.98 (m, 12H, aromat.); Anal. $C_{38}H_{43}N_3O_9S$ requires: C, 63.58; H, 6.04; N, 5.85; S, 4.47; found: C, 63.52; H, 6.11; N, 5.72; S, 4.41; MS (ES+) *m/z*: 486.7 [M + H].

2-Benzoyl-10-[8-(4-methylpiperazino)octyl]phenothiazine bis-(hydrogen maleate) 2BzlP8MP

Yield 32%; ^{1}H-NMR (CD_3OD) δ [ppm]: 1.28 – 1.97 (m, 12H, -CH_2-), 2.62 – 2.87 (m, 13H, -CH_3, -$N_{pip.}CH_2$), 3.99 (t, 2H, -$N_{PTA}CH_2$-), 6.27 (s, 4H, maleate), 6.82 – 8.03 (m, 12H, aromat.); Anal. $C_{40}H_{47}N_3O_9S$ requires: C, 64.41; H, 6.35; N, 5.63; S, 4.30; found: C, 64.45; H, 6.41; N, 5.57; S, 4.21; MS (ES+) *m/z*: 514.7 [M + H].

Biological evaluation

Cell culture and drugs

LLC-PK1 cell line, which was obtained from American Type Culture Collection (ATCC), Rockville, MD, USA (passage 36) was kept under standard culture conditions (Dulbecco's medium 199, 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin) at 37°C in the presence of 5% CO_2. Medium for LLC-MDR cells aditionally contained 640 nM vincristine sulfate to abide p-gp expression. Cells were subcultured by trypsinization every week and the medium was replaced twice a week. The resistant lines were obtained by stepwise selection in 10 nM vincristine containing medium. Cell culture reagents were obtained from Gibco BRL (Invitrogen, Karlsruhe, Germany). Vincristine was obtained from Universitätsapotheke Klinikum Kröllwitz (Halle). References were obtained from Sigma-Aldrich Chemie GmbH (Germany).

Crystalviolet assay

Cells were seeded in 96-well plates (Millipore, Eschborn, Germany) at a density of 5×10^3 cells per well. After incubation at 37°C and 5% CO_2 for 4 h, cells were left unloaded (control, 0.1% DMSO) or treated with vincristine (1 μM); vincristine (1 μM) + trifluoperazine (0.1 – 400 μM) and vincristine + test substance (0.1 – 400 μM) for 68 h at 37°C. After the completion of drug exposure, the supernatant of dead cells was removed. Residue with living cells was denaturated with 100 μL MeOH for 10 min, fixed, and washed. Then, cells were dyed with 100 μL of 0.1% aqueous solution of crystalviolet for 10 min. After washing, the absorbed dye was dissolved with 0.1 M ethanolic solution of sodium citrate. The absorbance was measured at λ = 620 nm on a Polarstar Galaxy plate reader (BMG LabTechnologies GmbH, Offenburg, Germany). The percentage of viable cells was calculated to get IC_{50}-values.

References

[1] C. Avendano, J. Menéndez, *Curr. Med. Chem.* **2002**, *9*, 159 – 193.

[2] R. Krishna, L. D. Mayer, *Eur. J. Pharm. Sci.* **2000**, *11*, 265 – 283.

[3] J. Robert, C. Jarry, *J. Med. Chem.* **2003**, *23*, 4805 – 4817.

[4] J. M. Ford, E. P. Briggeman, I. Pastan, M. Gottesman, W. N. Hait, *Cancer Res.* **1990**, *50*, 1748 – 1756.

[5] I. Pajeva, M. Wiese, *J. Med. Chem.* **1998**, *41*, 1815 – 1826.

[6] J. M. Ford, W. C. Prozialeck, W. N. Hait, *Mol. Pharmacol.* **1989**, *35*, 105 – 115.

[7] J. M. Zamora, H. L. Pearce, W. T. Beck, *Mol. Pharmacol.* **1988**, *33*, 454465.

[8] M. Wiese, I. K. Pajeva, *Current Med. Chem.* **2001**, *8*, 685 – 713.

[9] H. Pearce, A. Safa, N. Bach, M. Winter, *et al.*, *Proc. Natl. Acad. Sci. U. S. A* **1989**, 5128 – 5132.

[10] W. Hait, D. Aftab, *Biochem. Pharmacol.* **1992**, *43*, 103 – 107.

[11] T. Suzuki, N. Fukazawa, K. San-nohe, W. Sato, *et al.*, *J. Med. Chem.* **1997**, 2047 – 2052.

[12] A. Seelig, *Eur. J. Biochem.* **1997**, 252 – 261.

[13] A. Seelig, E. Landwojtowicz, *Eur. J. Pharm. Sci.* **2000**, 31 – 40.

[14] T. W. Loo, D. M. Clarke, *J. Membrane Biol.* **2005**, 173 – 185.

[15] S. Ekins, R. B. Kim, B. F. Leake, A. H. Dantzig, *et al.*, *Mol. Pharmacol.* **2002**, 974 – 981.

[16] I. K. Pajeva, M. Wiese, *J. Med. Chem.* **2002**, *45*, 5671 – 5686.

[17] S. Döppenschmitt, H. Spahn-Langguth, C. Regårdh, P. Langguth, *Pharm. Res.* **1998**, 1001 – 1006.

[18] I. K. Pajeva, C. Globisch, M. Wiese, *J. Med. Chem.* **2004**, *47*, 2523 – 2533.

[19] C. Globisch, I. Pajewa, M. Wiese, *Chem.Med.Chem.* **2008**, *3*, 280 – 295.

[20] T. Tsuruo, H. Iida, S. Tsukagoshi, Y. Sakurai, *Cancer Res.* **1982**, *42*, 4730 – 4733.

[21] R. Ganapathi, D. Grabowski, R. Turinic, R. Valenzuela, *Eur. J. Cancer Clin. Oncol.* **1984**, *20*, 799 – 806.

[22] M. Schmidt, J. Ungvári, J. Glöde, B. Dobner, A. Langner, *Bioorg. Med. Chem*. **2007**, *15*, 2283 – 2297.

[23] R. L. Miller, R. M. Bukowski, G. T Budd, *J. Clin. Oncol.* **1988**, *6*, 880 – 888.

[24] A. Ramu, N. Ramu, *Cancer Chemother. Pharmacol.* **1992**, *30*, 165 – 173.

[25] I. Pajeva, M. Wiese, *Quant. Struct.-Act. Relat.* **1997**, *16*, 1 – 10.

[26] G. Klopman, L. M. Shi, A. Ramu, *Mol. Pharmacol.* **1997**, *52*, 323 – 334.

[27] J. Guan, D. E. Kyle, L. Gerena, Q. Zhang, *et al.*, *J. Med. Chem.* **2002**, *45*, 2741 – 2748.

[28] S. Darvesh, R. S. McDonald, A. Penwell, S. Conrad, *et al.*, *Bioorg. Med. Chem*. **2005**, *13*, 211 – 222.

[29] A. Bernthsen, *Ber. Dtsch. Chem. Ges.* **1883**, *16*, 2896 – 2904.

[30] Rhône-Poulenc, *Belg. P. 556475*, **C. 1960**, 16863.

[31] H. L. Yale, *JACS,* **1955**, *77*, 2270 – 2272.

[32] W. J. Evans, S. Smiles, *J. Chem. Soc.* **1935**, *181*, 1263 – 1265.

[33] M. Golinski, P. J. DelaLuz, R. Floresca, T. J. Delcamp, *et al.*, *Bioconjugate Chem.* **1995**, *6*, 549 – 557.

[34] S. P. Massie, I. Cooke, W. A. Hills, *J. Org. Chem.* **1956**, *21*, 1006 – 1008.

[35] J. M. Hawkins, G. C. Fu, *J. Org. Chem.* **1986**, *51*, 2820 – 2822.

[36] L. Adams, C. Gray, R. Williams, *Tetrahedron Letters* **2004**, *45*, 4489 – 4493.

[37] O. Kimura, T. Osawa, K. Mochida, Jpn. Kokai Tokkyo Koho, **1989**, 7 – 11.

[38] D. A. Flick, G. E. Gifford, *J. Immunol. Methods* **1984**, 167 – 175.

[39] Y. Tanigawara, N. Okamura, M. Hirai, M. Yasuhara, *et al.*, *J. Pharmacol. Exp. Ther.* **1992**, *263*, 840 – 845.

[40] A. H. Schinkel, E. Wagenaar, L. van Deemter, C. A. Mol, P. Borst, *J. Clin. Invest.* **1995**, 1698 – 1705.

[41] P. Scafato, G. Cunsolo, S. Labano, C. Rosini, *Tetrahedron* **2004**, *40*, 8801 – 8806.

