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Hybrid molecules of estrone: New compounds with potential antibacterial, antifungal, and antiproliferative activities

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Dedicated to Prof. Roland Mayer (Dresden) on the occasion of his 80th birthday.

Abstract—New hybrid molecules of estrone were synthesized as compounds indicating promising biological activity (antibacterial, antimycobacterial, antifungal, and antiproliferative). The prepared molecules contained various heterocyclic units (pyridine, benzylsulfanyl derivatives of pyridine or derivatives of tetrazole) linked to estrone by *n*-heptyl bridges. The compounds with charge on molecule (the hybrid pyridinium or benzylsulfanylpyridinium salts) exhibited significant biological activity (antibacterial, antimycobacterial, antifungal, and antiproliferative). On the other hand, the compounds not in the form of salts (ω-(1-phenyl-5-tetrazolylthio)heptylethers of estrone) were inactive. The antimycobacterial activities of three different series of tetrazole derivatives (i.e., the hybrid molecules with estrone, tetrazole-5-thiols, and 5-benzylsulfanyl-1-phenyltetrazoles) with the same substituents on phenyl ring were compared. Amongst them, the 5-benzylsulfanyl-1-phenyltetrazoles were the most potent.

1. Introduction

The recent reappearance of tuberculosis and other infectious diseases represents a serious problem worldwide.[1] In the past few years, we were trying to find new groups of compounds active against these diseases. Now it has been demonstrated that the combination of a steroid unit, which is able to interact with cell membranes, and another antimicrobially potent functionality may result in an increase of activity.[2]

Previously, we have described that alkylsulfanyl group bound to an electron deficient carbon of heterocyclic system presents a pharmacophore responsible for a significant antibacterial activity (e.g., in some benzylsulfanyl derivatives of pyridine,[3] benzimidazole,[4] other benzazoles[5] or in some derivatives of tetrazole[6–8]).

It has been found before that ω-pyridiniumalkylethers of steroidal phenols[2] exhibit good biological activity (antibacterial). The aim of the present study was to prepare new hybrid molecules where the pyridine was replaced by a different heterocyclic moiety that itself exhibited some biological effect. The studied hybrid molecules contained in their structure a steroidal unit (3-hydroxy-estra-1,3,5(10)-triene, estrone) and various heterocyclic compounds (namely: pyridine, benzylsulfanyl derivatives of pyridine or derivatives of tetrazole-5-thiols) linked by an alkyl bridge. Hybrid molecules (**3**,

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Scheme 2) were prepared as ammonium bromides. The activities of the obtained hybrid molecules were further compared to the activities of their heterocyclic precursors.

2. Results and discussion

2.1. Chemistry

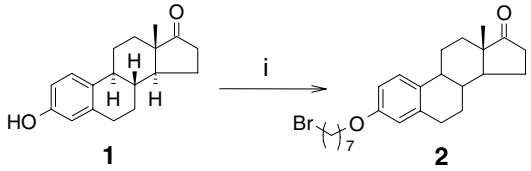
Compound **1**, a commercially available pharmaceutical (estrone), reacted with ω,ω′-dibromoheptane in the presence of potassium carbonate to give (ω-bromoheptoxy)-estra-1,3,5(10)-triene-17-one **2** (Scheme 1) as a key intermediate for the following reactions with (a) pyridine, (b) 4-benzylsulfanyl pyridine, (c) substituted 4-benzylsulfanyl pyridines, and (d) derivatives of 1-phenyltetrazole-5-thiols (Scheme 2). Reaction of compound **2** with pyridine or benzylsulfanyl derivatives of pyridine **7a**–**7c** (Scheme 3) in boiling acetonitrile resulted in the desired ω-pyridinium salts of the heptylethers of the 3-hydroxy-estra-1,3,5(10)-trienes (**3a**–**3d**, Scheme 2). In the case of the derivative **3b**, the reaction was carried out also in autoclave (3 atm, 100 °C) using methanol as a solvent. Nevertheless, no product was achieved under these conditions.

Reaction of the intermediate **2** with compounds **5a**–**5d** (Scheme 3) in boiling anhydrous THF and anhydrous TEA gave the corresponding derivatives of 3-[7′-(1-phenyl-5-tetrazolylthio)heptyloxy]-estra-1,3,5(10)-triene-17-one **4a**–**4d** (Scheme 2). Attempts to replace TEA by sodium ethanolate were unsuccessful; many by-products were isolated under these drastic conditions.

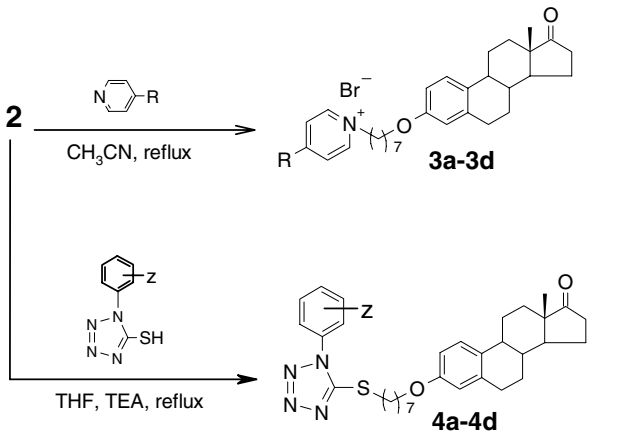
2.2. Biology

The prepared compounds **3** and **5** were screened in vitro for antibacterial, antimycobacterial, antifungal, antiproliferative, and cytotoxic activities. For the purpose to find out which part of the molecule contributes to the activity, the both parts of the hybrid molecules were subjected to evaluation assay, that is, the steroid unit **2** and heterocyclic moiety **5** and **7**. For the sake of comparison of the activity of various tetrazole derivatives, the previously prepared 5-benzylsulfanyl-1-phenyltetrazole derivatives[6–8] were also included. All tested phenyltetrazole derivatives (**4**–**6**) have the same substituents on the phenyl ring.

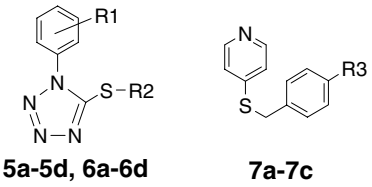
It has been reported that some antimycobacterial compounds are often antifungal as well.[9,10] Therefore, the hybrid molecules **3** and **4** together with the 5-benzylsulfanyl-1-phenyltetrazoles **6** and precursor **2** were evaluated for their antifungal activity.



Scheme 1. Synthesis of the intermediate **2**. Reagents and conditions: (i) $Br(CH_2)_7Br$, K_2CO_3, anhydrous acetone, reflux, 17 h.



Scheme 2. Syntheses of hybrid molecules of estrone **3a**–**3d** and **4a**–**4d**. Reagents: R: H (**3a**), benzylsulfanyl (**3b**), 4-chlorobenzylsulfanyl (**3c**), 4-methylbenzylsulfanyl (**3d**). Z: 4-CH_3 (**4a**), H (**4b**), 4-Cl (**4c**), 3,4-Cl_2 (**4d**).



Scheme 3. Derivatives of 1-phenyltetrazolyl-5-thiols, 5-benzylsulfanyl-1-phenyltetrazoles, and 4-benzylsulfanylpyridines. R1, 4-CH_3; R2, H (**5a**); R1, H; R2, H (**5b**); R1, 4-Cl; R2, H (**5c**); R1, 3,4-Cl_2; R2, H (**5d**); R1, 4-CH_3; R2, $C_6H_5CH_2$ (**6a**); R1, H; R2, $C_6H_5CH_2$ (**6b**); R1, 4-Cl; R2, $C_6H_5CH_2$ (**6c**); R1, 3,4-Cl_2; R2, $C_6H_5CH_2$ (**6d**); R3, H (**7a**); R3, 4-Cl (**7b**); R3, 4-CH_3 (**7c**).

2.2.1. Antibacterial and antimycobacterial activities. Antibacterial activity of some of the studied compounds was evaluated in vitro against the following strains: *Staphylococcus aureus* (SG 511), *S. aureus* (MRSA 994/93), *S. aureus* (MRSA 134/94), *Micrococcus luteus* (ATCC 10240), *Enterococcus faecalis* (1528), *Mycobacterium smegmatis* (SG 987), *M. aurum* (SB 66), *M. vaccae* (10670), *M. fortuitum* Borstel. The activities expressed as minimum inhibitory concentrations (MIC, μg/mL) are presented in Table 1. The derivatives of tetrazole **4**–**6** exhibited no activity in the preliminary screening (data not shown).

The antimycobacterial activities were studied against tuberculous and non-tuberculous mycobacterial strains: *Mycobacterium tuberculosis* CNCTC My 331/88, *M. kansasii* CNCTC My 235/80, *M. avium* CNCTC My 330/88, and a clinical isolate of *M. kansasii* 6 509/96. The results are presented in Table 2, MICs are expressed in μmol/L.

The antibacterial assessment revealed that the prepared compounds possess mostly only a moderate or slight activity. Mycobacterial strains were more sensitive to evaluated compounds. The most active compounds

Table 1. In vitro antibacterial activities; MIC (μg/mL)

Strain/compound	I	II	III	IV	V	VI	VII	VIII	IX
3a	3.12	6.25	25	0.8	12.5	3.12	6.25	3.12	6.25
3b	3.12	3.12	3.12	0.4	6.25	1.56	3.12	1.56	6.25
3c	3.12	3.12	6.25	0.8	1.56	6.25	3.12	1.56	6.25
3d	1.56	3.12	3.12	0.8	3.12	6.25	3.12	1.56	6.25
7a	100	100	>100	100	100	100	25	50	50
7b	50	100	>100	50	100	25	12.5	25	12.5
7c	50	100	100	100	100	50	25	25	12.5
CPB	0.8	1.56	3.12	0.4	1.56	6.25	6.25	1.56	12.5
DMSO/MeOH	>100	>100	>100	>100	100	100	100	100	100
CIP	0.2	>100	25	12.5	0.8	0.78	0.1	0.78	0.4

I, *S. aureus* (SG 511); II, *S. aureus* (MRSA 994/93); III, *S. aureus* (MRSA 134/94); IV, *M. luteus* (ATCC 10240); V, *E. faecalis* (1528); VI, *M. smegmatis* (SG 987); VII, *M. aurum* (SB 66); VIII, *M. vaccae* (10670); IX, *M. fortuitum* Borstel.
CPB, *N*-cetylpyridinium bromide; CIP, ciprofloxacin; solvent, DMSO.

Table 2. In vitro antimycobacterial activities; MIC (μmol/L)

Strain	*M. tuberculosis*		*M. avium*		*M. kansasii*					
	My 331/88		My 330/88		My 235/80			6509/96[a]		
Days	14	21	14	21	7	14	21	7	14	21
2	32	n	16	32	62.5	n	n	62.5	62.5	n
3a	16	32	32	32	16	32	32	16	32	32
3b	4	8	2	2	8	8	8	8	8	16
3c	16	16	8	8	16	16	16	16	16	16
3d	4	8	8	8	16	16	16	16	16	16
4a	500	*x*	*x*	n	n	*x*	*x*	n	n	n
4b	*x*	*x*	n	n	n	*x*	*x*	n	n	*x*
4c	n	n	n	n	n	n	n	n	n	n
4d	n	*x*	n	n	n	*x*	*x*	n	n	*x*
5a	500	*x*	500	*x*	500	*x*	*x*	500	500	*x*
5b	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*
5c	500	*x*	*x*	*x*	500	*x*	*x*	250	500	*x*
5d	250	250	*x*	*x*	500	500	500	250	500	500
6a	32	125	62.5	n	16	32	250	16	32	250
6b	16	62.5	32	125	16	32	125	16	32	125
6c	16	n	62.5	n	16	32	n	16	16	n
6d	16	32	62.5	125	8	16	32	16	32	62.5
7a	125	125	125	125	62.5	125	125	62.5	125	125
7b	32	62.5	62.5	62.5	32	62.5	62.5	32	62.5	62.5
7c	62.5	62.5	62.5	125	32	62.5	125	32	62.5	125
INH	0.5	1	*y*	*y*	*y*	*y*	*y*	4	4	8

[a] Clinical isolate; n, not evaluated because of the precipitation (either growth of bacteria or crystallization of the compound); *x*, >500; *y*, >250; INH, isoniazide.

from evaluated set were the hybrid molecules **3**. The hybrid molecules **3** showed significant activity in contrast to the compounds **4** that showed practically no activity. The antibacterial and antimycobacterial activities are probably connected with the presence of a charge on nitrogen atom in the molecule. The comparison of the antimycobacterial activities of hybrid molecules **3** with heterocyclic moiety **7** and steroidal moiety **2** confirmed our hypothesis that steroidal part may result in an increase of the activity. Hydrophobic steroid unit interacts with cell membranes, leading to an enhancement of the concentration of the effective molecule in the inside of the cell. But the way that the steroid unit is connected to the heterocyclic moiety is very important (see the different activity of compounds **3** and **4**).

Regarding the tetrazole derivatives **4**–**6**, 5-benzylsulfanyl derivatives of 1-phenyltetrazoles **6** exhibited the best activity; on the other hand, tetrazole-5-thiols **5** and hybrid molecules **4** were practically inactive. The efficacy of compounds **6** corresponded to our hypothesis described previously that alkylsulfanyl group bound to an electron deficient carbon presents a pharmacophore responsible for antimycobacterial activity.[3–5]

The antibacterial activity of the hybrid molecules seems to be further related to the length of the alkyl chain between the steroidal unit and the heterocyclic nitrogen. The antibacterial activity of compound **3a** lied between those of similar homologues with the connective chain length of 6 and 8 carbons that were prepared previously.[2] This finding corresponded well with the theory about the relationship between the alkyl chain length and antibacterial activity of the hybrid molecules.

2.2.2. Antifungal activity. Antifungal activity of compounds **2**, **3**, **4** and **6** was evaluated against following fungal strains: *Candida albicans* ATCC 44859, *Candida*

Table 3. In vitro antifungal activities; MIC (μmol/L)

Strain	I		II		III		IV		V		VI		VII		VIII	
Time (h)	24	48	24	48	24	48	24	48	24	48	24	48	24	48	72	120
2	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
3a	3.9	3.9	15.6	15.6	15.6	31.3	7.8	15.6	3.9	7.8	3.9	62.5	>250	>250	31.3	62.5
3b	3.9	3.9	15.6	15.6	7.8	7.8	15.6	15.6	3.9	7.8	15.6	31	31	31	7.8	7.8
3c	7.8	7.8	15.6	15.6	7.8	15.6	15.6	15.6	7.8	7.8	15.6	31	62.5	62.5	7.8	15.6
3d	n	3.9	n	7.8	n	7.8	n	7.8	n	7.8	n	7.8	n	15.6	n	7.8
4a–4d	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	250	>250	>250	>250	>250	>250
6a	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	62.5	250	>250	>250	31.3	62.5
6b	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	31.3	62.5
6c	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	62.5	62.5	>250	>250	31.3	62.5
6d	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	250	>250	>250	>250	125	125
FLU	0.8	1.6	1.6	>250	52.2	104.5	13.1	52.2	3.3	6.5	>250	>250	>250	>250	26.1	52.2
AMB	0.02	0.07	0.07	0.07	0.14	0.14	0.04	0.14	1.08	2.16	0.27	0.27	1.08	2.16	1.08	1.08

I, *C. albicans* (ATCC 44859); II, *C. tropicalis* (156); III, *C. krusei* (E28); IV, *C. glabrata* (20/I); V, *T. beigelii* (1188); VI, *A. fumigatus* (231); VII, *Absidia corymbifera* (272); VIII, *T. mentagrophytes* (445).
FLU, fluconazole; AMB, amphotericin B; n, not evaluated.

tropicalis 156, *Candida krusei* E28, *Candida glabrata* 20/I, *Trichosporon beigelii* 1188, *Aspergillus fumigatus* 231, *Absidia corymbifera* 272, and *Trichophyton mentagrophytes* 445. The MICs (μmol/L) are presented in Table 3.

As shown in Table 3, significant antifungal activity was observed only in hybrid compounds carrying the charge on molecule **3**. The hybrid compounds of tetrazole **4** were inactive and the 5-benzyl-1-phenyltetrazoles **6** exhibited only slight activity against *T. mentagrophytes*. This finding (the relationship of biological activity and the charge on the molecule) is well consistent with that found for the antibacterial activity.

2.2.3. Antiproliferative and cytotoxic activities. The compounds **2**–**7** were assayed against cell lines K-562 and L-929 for their antiproliferative effects (GI_{50}: concentration which inhibited cell growth by 50%), and against HeLa for their cytotoxic effects (CC_{50} and CC_{10}: cytotoxic concentration which contains a specific destructive action by 50% and 10%, respectively; used particularly in referring to the lysis of cells), see Table 4. The cells were incubated with 10 concentrations of the tested compounds. The antiproliferative and cytotoxic assays have been described previously.[11]

Table 4. Antiproliferative and cytotoxic effects

	Antiproliferative activity		Cytotoxicity
	L-929[a] GI_{50} (μg/mL)	K-562[b] GI_{50} (μg/mL)	HeLa[c] CC_{50} (μg/mL) CC_{10} (μg/mL)
2	49.9	13.1	>50 (16.3)
3a	16.7	5.3	17.5 (5.1)
3b	4.0	2.3	3.1 (1.2)
3c	2.8	1.0	4.1 (1.9)
3d	3.5	3.0	1.9 (1.0)
4a	>50	>50	>50 (28.7)
4b	>50	>50	>50 (20.3)
4c	>50	>50	>50 (50)
4d	>50	>50	>50 (50)
5a	>50	42.8	>50 (33)
5b	>50	>50	>50 (40)
5c	>50	42.6	48.9 (26.4)
5d	33.4	31	33.3 (18.1)
6a	12.5	24.3	19.7 (4)
6b	22.5	36.1	41.6 (8.7)
6c	16.7	>50	50 (5)
6d	19.1	>50	48.6 (9.9)
7a	34.3	>50	23.6 (14.0)
7b	21.2	19.8	16.3 (8.6)
7c	24.3	37.5	21.4 (13.3)

One to 10 μg/mL, strong activity; >10 μg/mL, weak activity.
[a] Mouse fibroblast cells.
[b] Human leukemia.
[c] Human cervical carcinoma; solvent, DMSO.

3. Conclusion

A series of new hybrid molecules of estrone with various heterocycles was prepared. The heterocyclic moiety involved: pyridine, 4-benzylsulfanylpyridine derivatives, and 1-phenyltetrazole-5-thiol derivatives. Their biological activity against various bacterial, mycobacterial, and fungal strains as well as the antiproliferative and cytotoxic activities were evaluated. For the comparison, the precursors of the studied hybrid molecules were screened too.

It was observed that the presence of a charge on the molecule was important for all the studied activities (the most active compounds were prepared as salts). The best potency in the prepared group was found in the ω-pyridinium derivatives of estrone **3**.

Comparing the antibacterial, antimycobacterial, antiproliferative and cytotoxic activities of three different series of tetrazole derivatives (i.e., the hybrid molecules with estrone, tetrazole-5-thiols, and 5-benzylsulfanyl-1-phenyltetrazoles) with the same substituents it has been found that the 5-benzylsulfanyl-1-phenyltetrazoles were the most potent.

4. Experimental

4.1. General methods

All chemicals were purchased from Sigma–Aldrich (Schnelldorf, Germany). The derivatives of tetrazole

5a–**5d** and **6a**–**6d** were prepared previously in our laboratory.[6] Melting points were measured on a Koffler apparatus and are uncorrected. Elemental analyses were performed with a CHNS-932 (LECO) instrument. The samples for analysis and biological tests were dried over P_2O_5 at 61 °C and 66 Pa for 24 h. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DRX-400 spectrometer either in $CDCl_3$ or DMSO (250 or 400 MHz). MS spectra were measured on a Finnigan MAT SAQ 710 mass spectrometer. Solvents were purified, dried, and distilled according to conventional methods. All reactions were carried out under inert (argon) atmosphere. The reactions were monitored by TLC aluminum plates, silica gel 60 (Merck, Darmstadt, Germany), 0.2 mm, detection by UV (254 nm) or iodine. For the column chromatography, silica gel 60 (Lichroprep Si 60, 40–63 μm, Merck) was used; the preparative TLC was carried out using silica gel F_{254} (15–40 μm, Merck).

4.2. Syntheses

4.2.1. Procedure for the preparation of 3-(ω-bromoheptoxy)-estra-1,3,5(10)-triene-17-one (2). To a solution of the phenolic steroid (estrone **1**; 1 mmol) in anhydrous acetone (10 mL) containing freshly dried potassium carbonate (2 mmol), ω,ω′-dibromoheptane (4 mmol) was added. The reaction mixture was refluxed until completion (17 h), as determined by TLC. After cooling to room temperature, the potassium carbonate was filtered off and washed with acetone. Evaporation resulted in oily crude product, which was purified by column chromatography on silica gel with *n*-heptane/ethyl acetate (90:10) and by crystallization in methanol.

4.2.1.1. 3-(ω-Bromoheptoxy)-estra-1,3,5(10)-triene-17-one (2). White crystals (75% yield); mp 72–74 °C (MeOH); ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.83 (s, 3H, 18-H), 1.18–2.41 (m, 23H, 3× CH, 10× CH_2), 2.81 (m, 2H, 6-H), 3.33 (t, 2H, J = 6.8 Hz, 7′-H), 3.85 (t, 2H, J = 6.5 Hz, 1′-H), 6.56 (d, 1H, J = 2.4 Hz, 4-H), 6.63 (dd, 1H, J = 2.9 Hz, J = 8.5 Hz, 2-H), 7.11 (d, 1H, J = 8.6 Hz, 1-H). ^{13}C NMR (250 MHz, $CDCl_3$): δ 220.8, 157.1, 137.7, 131.9, 126.3, 114.5, 112.1, 67.7, 50.4, 48.0, 44.0, 38.4, 35.9, 33.9, 32.7, 31.6, 30.3, 29.6, 29.2, 28.5, 28.1, 26.6, 25.9, 21.6, 13.8. LMR MS (EI +Q1MS LMR UP LR): 446 ($M^{+\cdot}$, 62), 361 (4), 348 (1), 322 (2), 283 (2), 270 (54), 242 (9), 226 (9), 213 (17), 199 (7), 185 (52), 172 (32), 146 (46), 133 (25), 115 (20), 97 (29), 91 (16), 81 (10), 69 (15), 55 (100), 41 (48), 30 (2). IR (DIAMAND) υ_{max} (CO) 1734 cm^{-1}. Anal. Calcd for $C_{25}H_{35}BrO_2$ (447.46): C, 67.11; H, 7.88; Br, 17.86. Found: C, 67.32; H, 8.10; Br, 17.66.

4.2.2. General procedure for the preparation of compounds (3a–3d). A solution of compound **2** (0.2 mmol) and pyridine or benzylsulfanyl derivatives of pyridine (0.6 mmol) in anhydrous acetonitrile (10 mL) was refluxed for 24–29 h until complete conversion was achieved (TLC). The mixture was then concentrated in vacuo. The precipitate that separated out was filtered off, washed several times with cold *n*-heptane, and dried in vacuo. The compound **3a** was further purified by crystallization in methanol.

4.2.2.1. 1-[(17-Oxo-estra-1,3,5(10)-triene-3-yloxy)-heptyl]-pyridinium bromide (3a). Yellowish crystals (90% yield), reaction time: 24 h; mp 140–142 °C (MeOH); ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.88 (s, 3H, 18-H), 1.40–2.53 (m, 23H, 3× CH, 10× CH_2), 2.83–2.90 (m, 2H, 6-H), 3.88 (t, 2H, J = 6.3 Hz, –CH_2–O–), 5.02 (t, 2H, J = 7.4 Hz, –CH_2–N–), 6.60 (d, 1H, J = 2.9 Hz, 4-H), 6.66 (dd, 1H, J = 2.9 Hz, J = 8.5 Hz, 2-H), 7.16 (d, 1H, J = 8.6 Hz, 1-H), 8.11 (dd, 2H, J = 6.6 Hz, J = 7.5 Hz, 3′-H_{Py}, 5′-H_{Py}), 8.45–8.53 (m, 1H, 4′-H_{Py}), 9.50 (d, 2H, J = 5.6 Hz, 2′-H_{Py}, 6′-H_{Py}). ^{13}C NMR (250 MHz, $CDCl_3$): δ 220.8, 157.0, 145.1, 145.1, 145.1, 137.7, 131.9, 128.4, 128.4, 128.1, 126.3 114.5, 112.1, 67.6, 62.0, 50.4, 48.0, 44.0, 38.4, 35.8, 31.9, 31.6, 29.6, 29.1, 28.7, 26.5, 25.9, 25.8, 21.5, 13.8. LMR MS (CI +Q1MS LMR UP LR): 527 ($M^{+\cdot}$ H, 15), 447 (58), 446 (18), 368 (7), 270 (10), 130 (8), 80 (100). IR (DIAMAND) υ_{max} (CO) 1727 cm^{-1}. Anal. Calcd for $C_{30}H_{40}NO_2Br$ (526.56): C, 68.43; H, 7.66; N, 2.66; Br, 15.17. Found: C, 68.71; H, 7.88; N, 2.47; Br, 15.38.

4.2.2.2. 4-Benzylsulfanyl-1-[(17-Oxo-estra-1,3,5(10)-triene-3-yloxy)-heptyl]- pyridinium bromide (3b). Gray crystals (55% yield), reaction time: 24 h; mp 135–137 °C; ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.89 (s, 3H, 18-H), 1.38–2.54 (m, 23H, 3× CH, 10 × CH_2), 2.85–2.88 (m, 2H, 6-H), 3.88 (t, 2H, J = 6.6 Hz, –CH_2–N–), 4.41 (s, 2H, –CH_2–S–), 4.74 (t, 2H, J = 7.3 Hz, –CH_2–O–), 6.61 (d, 1H, J = 2.6 Hz, 4-H), 6.69 (dd, 1H, J = 2.9 Hz, J = 8.6 Hz, 2-H), 7.16 (d, 1H, J = 8.5 Hz, 1H), 7.30–7.44 (m, 5H, H_{Bn}), 7.75 (d, 2H, J = 7.2 Hz, 3′-H_{Py},5′-H_{Py}), 9.04 (d, 2H, J = 6.9 Hz, 2′-H_{Py}, 6′-H_{Py}). ^{13}C NMR (250 MHz, $CDCl_3$): δ 220.9, 163.0, 157.0, 149.2, 149.0, 142.7, 137.7, 135.5, 132.7, 131.9, 128.9, 128.8, 128.7, 128.6, 126.3, 123.0, 114.5, 112.1, 67.6, 60.4, 50.4, 48.0, 44.0, 38.4, 36.4, 35.9, 35.7, 31.6, 31.5, 29.6, 29.1, 28.7, 26.5, 25.9, 25.8, 21.6, 13.9. ESI +VE +HMR BSCAN (LIN) UP PROF NRM: 568.3 (100); IR (DIAMAND) υ_{max}(CO) 1736 cm^{-1}. Anal. Calcd for $C_{37}H_{46}NO_2SBr$ (648.75): C, 68.50; H, 7.15; N, 2.16; S, 4.94; Br, 12.32. Found: C, 68.56; H, 6.80; N, 2.38; S, 5.31; Br, 12.02.

4.2.2.3. 4-(4-Chlorobenzylsulfanyl)-1-[(17-oxo-estra-1,3,5(10)-triene-3-yloxy)-heptyl]-pyridinium bromide (3c). Yellowish crystals (82% yield), reaction time: 24 h; mp 126–129 °C; ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.88 (s, 3H, 18-H), 1.31–2.54 (m, 23H, 3× CH, 10× CH_2), 2.82–2.89 (m, 2H, 6-H), 3.87 (t, 2H, J = 6.5 Hz, –CH_2–N–), 4.42 (s, 2H, –CH_2–S–), 4.71 (t, 2H, J = 7.3 Hz, –CH_2–O–), 6.60 (d, 1H, J = 2.7 Hz, 4-H), 6.66 (dd, 1H, J = 2.7 Hz, J = 8.5 Hz, 2-H), 7.15 (d, 1H, J = 8.9 Hz, 1-H), 7.28–7.41 (m, 4H, H_{Bn}), 7.80 (d, 2H, J = 6.9 Hz, 3′-H_{Py}, 5′-H_{Py}), 9.02 (d, .2H, J = 6.9 Hz, 2′-H_{Py}, 6′-H_{Py}). ^{13}C NMR (250 MHz, $CDCl_3$): δ 220.9, 162.6, 157.0, 149.3, 142.7, 137.7, 134.5, 134.1, 132.0, 131.5, 130.4, 130.0, 129.4, 129.0, 126.3, 123.2, 114.5, 112.1, 67.6, 60.5, 50.4, 48.0, 44.0, 38.3, 35.9, 35.7, 35.0, 31.6, 31.5, 29.6, 29.1, 28.7, 26.5, 26.0, 25.8, 21.6, 13.9. FAB +Q1MS LMR UP PROF: 603 (40), 600 (5), 547 (6), 505 (7), 480 (10), 479 (16), 447 (10), 420 (8), 398 (12), 360 (10), 348 (14), 334 (17),

332 (30), 307 (100), 289 (75), 276 (29), 262 (33), 249 (38), 238 (42), 236 (93), 202 (27). IR (DIAMAND) υ_{max}(CO) 1736 cm^{-1}. Anal. Calcd for $C_{37}H_{45}ClNO_2SBr$ (683.19): C, 65.05; H, 6.64; Cl, 5.19; N, 2.05; S, 4.69; Br, 11.70. Found: C, 64.92; H, 6.71; Cl, 5.29; N, 2.32; S, 4.53; Br, 11.65.

4.2.2.4. 4-(4-Methylbenzylsulfanyl)-1-[(17-oxo-estra-1,3,5(10)-triene-3-yloxy)-heptyl]-pyridinium bromide (3d). White crystals (42% yield), reaction time: 26 h; mp 108–111 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (s, 3H, 18-H), 1.34–2.53 (m, 26H, 3× CH, 10× CH_2, 1× $CH_{3–Bn}$), 2.84–2.90 (m, 2H, 6-H), 3.88 (t, 2H, J = 6.4 Hz, –CH_2–N–), 4.36 (s, 2H, –CH_2–S–), 4.73 (t, 2H, J = 7.3 Hz, –CH_2–O–), 6.61 (d, 1H, J = 2.5 Hz, 4-H), 6.66 (dd, 1H, J = 2.8 Hz, J = 8.5 Hz, 2-H), 7.15 (d, 1H, J = 7.7 Hz, 1-H), 7.24–7.30 (m, 4H, H_{Bn}), 7.73 (d, 2H, J = 7.0 Hz, 3′-H_{Py}, 5′-H_{Py}), 9.03 (d, 2H, J = 7.0 Hz, 2′-H_{Py}, 6′-H_{Py}). ^{13}C NMR (400 MHz, $CDCl_3$): δ 220.9, 163.2, 157.0, 142.7, 138.5, 137.7, 131.9, 129.9, 129.9, 129.6, 129.5, 129.5, 128.8, 128.6, 126.3, 123.0, 114.6, 112.2, 67.7, 60.4, 50.4, 48.0, 44.0, 38.4, 36.3, 35.9, 35.5, 31.6, 31.5, 29.6, 29.1, 28.7, 26.6, 26.0, 25.8, 21.6, 21.1, 13.9. ESI +VE +HMR BSCAN (LIN) UP PROF NRM: 582.3. IR (KBr) υ_{max}(CO) 1737 cm^{-1}. Anal. Calcd for $C_{38}H_{48}NO_2SBr$ (662.78): C, 68.87; H, 7.30; N, 2.11; S, 4.84; Br, 12.06. Found: C, 69.01; H, 7.12; N, 2.45; S, 5.19; Br, 11.98.

4.2.3. General procedure for the preparation of compounds (4a–4d). A solution of 3-(ω-bromoheptoxy)-estra-1,3,5(10)-triene-17-one **2** (0.5 mmol) and the corresponding derivative of 1-phenyl-5-tetrazolylthiol **5a–5d** (0.75 mmol) in anhydrous THF (6 mL) and anhydrous TEA (0.75 mmol) was refluxed for 15–21 h until a complete conversion was achieved (TLC). Evaporation resulted in oily crude products, which were purified by column chromatography on silica gel with *n*-heptane/acetone (90:10).

4.2.3.1. 3-(7′-[1-(4-Methylphenyl)-5-tetrazolylthio]heptyloxy)-estra-1,3,5(10)-triene-17-one (4a). Yellow crystals (95% yield), reaction time: 21 h; mp 61–63 °C. ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.90 (s, 3H, 18-H), 1.24-2.56 (m, 26H, 3× CH, 10× CH_2, 1× $CH_{3–Ph}$), 2.85–2.93 (m, 2H, 6-H), 3.38 (t, 2H, J = 7.4 Hz, –CH_2–S–), 3.91 (t, 2H, J = 6.6 Hz, –CH_2–O–), 6.63 (d, 1H, J = 2.7 Hz, 4-H), 6.70 (dd, 1H, J = 2.8 Hz, J = 8.5 Hz, 2-H), 7.18 (d, 1H, J = 8.4 Hz, 1-H), 7.35 (d, 2H, J = 8.3 Hz, 3-H_{Ph}, 5-H_{Ph}), 7.41–7.47 (m, 2H, 2-H_{Ph}, 6-H_{Ph}). ^{13}C NMR (400 MHz, $CDCl_3$): δ 220.9, 157.0, 154.4, 140.4, 137.7, 131.9, 131.2, 130.3, 130.3, 126.3, 123.7, 123.7, 114.5, 112.1, 67.7, 50.4, 48.0, 44.0, 38.4, 35.8, 33.2, 31.6, 29.6, 29.2, 29.0, 28.7, 28.5, 26.5, 25.9, 25.9, 21.6, 21.3, 13.8. IR (DIAMAND) υ_{max}(CO) 1736 cm^{-1}. Anal. Calcd for $C_{33}H_{42}N_4O_2S$ (558.79): C, 70.93; H, 7.58; N, 10.03; S, 5.74. Found: C, 71.05; H, 7.43; N, 9.90; S, 5.72.

4.2.3.2. 3-(7′-[1-Phenyl-5-tetrazolylthio]heptyloxy)-estra-1,3,5(10)-triene-17-one (4b). White crystals (90% yield), reaction time: 19 h; mp 70.5–72.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.90 (s, 3H, 18-H), 1.25–2.54 (m, 23H, 3× CH, 10× CH_2), 2.86–2.91 (m, 2H, 6-H), 3.40 (t, 2H, J = 7.3 Hz, –CH_2–S–), 3.92 (t, 2H, J = 6.4 Hz, –CH_2–O–), 6.63 (d, 1H, J = 2.7 Hz, 4-H), 6.70 (dd, 1H, J = 2.9 Hz, J = 8.6 Hz, 2-H), 7.18 (d, 1H, J = 8.1 Hz, 1-H), 7.51–7.60 (m, 5H, H_{Ph}). ^{13}C NMR (400 MHz, $CDCl_3$): δ 220.8, 157.1, 154.5, 137.7, 131.9, 130.1, 129.8, 129.8, 126.3, 123.9, 123.9, 114.6, 112.1, 67.8, 50.5, 48.0, 44.0, 38.4, 35.9, 33.3, 31.9, 31.6, 29.7, 29.2, 29.0, 28.8, 28.6, 26.6, 25.9, 25.9, 21.6, 13.9. IR (DIAMAND) υ_{max}(CO) 1731 cm^{-1}. Anal. Calcd for $C_{32}H_{40}N_4O_2S$ (544.77): C, 70.55; H, 7.40; N, 10.28; S, 5.89. Found: C, 70.79; H, 7.44; N, 10.04; S, 5.69.

4.2.3.3. 3-(7′-[1-(4-Chlorophenyl)-5-tetrazolylthio]heptyloxy)-estra-1,3,5(10)-triene-17-one (4c). Light yellow crystals (91% yield), reaction time: 15 h; mp 69–71.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (s, 3H, 18-H), 1.28–2.53 (m, 23H, 3× CH, 10× CH_2), 2.86–2.89 (m, 2H, 6-H), 3.40 (t, 2H, J = 7.3 Hz, –CH_2–S–), 3.92 (t, 2H, J = 6.4 Hz, –CH_2–O–), 6.63 (d, 1H, J = 2.7 Hz, 4-H), 6.70 (dd, 1H, J = 2.9 Hz, J = 8.6 Hz, 2-H), 7.18 (d, 1H, J = 8.1 Hz, 1-H), 7.50–7.59 (m, 4H, H_{Ph}). ^{13}C NMR (400 MHz, $CDCl_3$): δ 220.8, 157.1, 154.5, 137.7, 136.2, 131.9, 130.0, 130.0, 126.3, 125.1, 125.1, 114.6, 112.1, 67.7, 50.5, 48.0, 44.0, 38.4, 35.9, 33.5, 31.9, 31.6, 29.7, 29.2, 29.0, 28.7, 28.5, 26.6, 26.0, 25.9, 22.7, 13.9. IR (DIAMAND) υ_{max}(CO) 1738 cm^{-1}. Anal. Calcd for $C_{32}H_{39}ClN_4O_2S$ (579.21): C, 66.36; H, 6.79; N, 9.67; Cl, 6.12; S, 5.54. Found: C, 66.76; H, 6.76; N, 9.37; Cl, 5.96; S, 5.17.

4.2.3.4. 3-(7′-[1-(3,4-Dichlorophenyl)-5-tetrazolylthio]heptyloxy)-estra-1,3,5(10)-triene-17-one (4d). White crystals (97% yield), reaction time: 16 h; mp 108–110 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.90 (s, 3H, 18-H), 1.24–2.54 (m, 23H, 3× CH, 10× CH_2), 2.86–2.91 (m, 2H, 6-H), 3.42 (t, 2H, J = 7.3 Hz, –CH_2–S–), 3.92 (t, 2H, J = 6.4 Hz, –CH_2–O–), 6.63 (d, 1H, J = 2.4 Hz, 4-H), 6.69 (dd, 1H, J = 2.6 Hz, J = 8.6 Hz, 2-H), 7.18 (d, 1H, J = 8.6 Hz, 1-H), 7.48 (dd, 1H, J = 2.5 Hz, J = 8.6 Hz, 5-H_{Ph}), 7.64 (d, 1H, J = 8.6 Hz, 6-H_{Ph}), 7.76 (d, 1H, J = 2.5 Hz, 2-H_{Ph}). ^{13}C NMR (400 MHz, $CDCl_3$): δ 220.8, 157.1, 154.6, 137.7, 134.6, 134.1, 132.8, 131.9, 131.5, 126.3, 125.6, 122.8, 114.6, 112.1, 67.7, 50.5, 48.0, 44.0, 38.4, 35.9, 33.6, 31.9, 31.6, 29.7, 29.2, 29.0, 28.7, 28.5, 26.6, 25.9, 25.9, 13.9. IR (KBr) υ_{max}(CO) 1739 cm^{-1}. Anal. Calcd for $C_{32}H_{38}Cl_2N_4O_2S$ (613.66): C, 62.63; H, 6.24; N, 9.13; Cl, 11.55; S, 5.23. Found: C, 62.66; H, 6.23; N, 9.23; Cl, 11.69; S, 4.91.

4.2.4. General procedure for the preparation of benzylsulfanyl derivatives of pyridine (7a–7c). Pyridine-4-thiol (5 mmol) in dry *N*,*N*-dimethylformamide (8 mL) was added to a solution of sodium (5 mmol) in dry methanol (2.5 mL). After 10 min of stirring at room temperature, the corresponding benzyl halide (5 mmol) was added in 2–3 portions and the resultant suspension was stirred for 3–8 h. The mixture was then poured into an ice bath and left to stand overnight. The solid was filtered off, washed with cold water (2× 30 mL), and air-dried. The crude products were purified by preparative TLC using *n*-hexane/acetone (2:1–6:1) followed by crystallization in aqueous ethanol or methanol.

4.2.4.1. 4-(Benzylsulfanyl)pyridine (7a). Light yellow crystals (56% yield); mp 54–57 °C (59–61 °C was reported[12]).

4.2.4.2. 4-(4-Chlorobenzylsulfanyl)pyridine (7b). Yellow crystals (25% yield); mp 70–71 °C (75–76.5 °C was reported[13]).

4.2.4.3. 4-(4-Methylbenzylsulfanyl)pyridine (7c). Light yellow crystals (40% yield); mp 75–77 °C (82–83 °C was reported[13]).

4.3. Biological assays

The prepared steroidal hybrid molecules together with the heterocyclic precursors were screened in vitro for antimicrobial, antimycobacterial, and antifungal activity. Some of the prepared derivatives were tested also for the antiproliferative and cytotoxic activities.

4.3.1. In vitro antibacterial activity including antimycobacterial. The antibacterial activity was evaluated in two workplaces:

(a) The activity against strains included in Table 1 was evaluated in the Hans-Knöll-Institut, Jena, Germany. The activity of the studied compounds was determined as the minimal inhibition concentration (MIC) according to NCCLS guidelines[14] using the microbroth dilution method. The cells were grown overnight at 37 °C in Mueller–Hinton broth (MHB, Difco). Fifty microliters of a compound solution of 400 μL/mL was serially diluted by factor two with MHB. The wells were then inoculated with 50 μL of bacteria to give a final concentration of 5×10^5 CFU/mL. After the microtiter plates were incubated at 37 °C for 24 h, the MIC (μg/mL) values were read with a Nepheloscan Ascent 1.4 automatic plate reader (Lab-systems, Vantaa, Finland) as the lowest dilution of antibiotic allowing no visible growth.

(b) The antimycobacterial activity against strains included in Table 2 was evaluated in the National Reference Laboratory for Mycobacterium kansasii, Regional Institute of Public Health, Ostrava, Czech Republic. The following strains, obtained from the Czech National Collection of Type Cultures (CNCTC), National Institute of Public Health, Prague, were used: *Mycobacterium tuberculosis* CNCTC My 331/88, *M. kansasii* CNCTC My 235/80, *M. avium* CNCTC My 330/88, and a clinical isolate of *M. kansasii* 6 509/ 96. Antimycobacterial activity of the compounds against these strains was determined in the Šula semisynthetic medium (Sevapharma, Prague). The Šula liquid medium (with bovine serum) is routinely used in the Czech Republic. Each strain was simultaneously inoculated into a petri dish containing the Löwenstein-Jensen medium for the control of sterility of the inoculum and its growth. The compounds were added to the medium as dimethylsulfoxide (DMSO) solutions. The final concentrations were 1000, 500, 250, 125, 62.5, 32, 16, 8, 4, 2, and 1 μmol/L. The MIC was determined after incubation at 37 °C for 7, 14, and 21 days. MIC (μmol/mL) was the lowest concentration of an antimycobacterially effective substance (on the above-stated concentration scale), at which inhibition of the growth of mycobacteria occurred.

4.3.2. Antifungal activity. The antifungal activity was determined in the Department of Biological and Medical Sciences, Faculty of Pharmacy, Charles University, Hradec Králové, Czech Republic.

4.3.2.1. Test compounds. Before testing, the compounds were dissolved in 100% DMSO so that the final concentration of DMSO in the test medium did not exceed 1% of the total solution composition. Twofold serial dilutions ranging from 250 to 0.49 μmol/L were tested for all compounds providing that a given compound was soluble in DMSO and no precipitation in the RPMI medium occurred. All solutions were prepared immediately before testing.

4.3.2.2. Fungal organisms. A set of eight fungal strains of potentially pathogenic fungi for humans (*C. albicans* ATCC 44859, *C. tropicalis* 156, *C. krusei* E28, *C. glabrata* 20/I, *T. beigelii* 1188, *A. fumigatus* 231, *A. corymbifera* 272, *T. mentagrophytes* 445) was used for the antifungal screening tests by means of modified micro dilution broth method M27-A (NCCLS 1997).[14] They included clinical isolates from biological materials of patients with suspected or proven mycosis. The fungal isolates were stored in Skim Milk Medium (Becton–Dickinson) for 6–10 months at −40 °C before use. Prior to testing, each isolate was subcultured on Sabouraud dextrose agar (SDA, Difco) to ensure optimal growth characteristics and purity.

The isolates of yeasts had been grown for 1–4 days, the isolates of molds for 4–14 days, on SDA at room temperature. Yeast and conidia suspensions were prepared in sterile water supplemented with 0.01% of Tween 80. Each suspension of a yeast and a mold was diluted in the ratio of 1:50 in the RPMI 1640 medium to obtain the final inoculum, which ranged from 0.5×10^3–2.5×10^3 CFU/mL and 0.5×10^4–5×10^4 CFU/mL, respectively. The inoculum size was determined microscopically using the Bürker's chamber and verified by plating 100 μL of serial dilutions of each inoculum onto an SDA plate and incubation until growth became visible.

4.3.2.3. Antifungal assay. A stock solution of each antifungal compound was prepared in the RPMI 1640 medium with L-glutamine, but without sodium bicarbonate (Sevapharma, Prague). The RPMI medium was buffered to pH 7.0 ± 0.1 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma). Each well of microtiter plate with 200 μL of the medium was inoculated with 10 μL of the inoculum suspension, and the microtiter plates were incubated at 35 °C in a humid atmosphere. The MIC was determined visually as the lowest concentrations that showed 80% and higher growth inhibition as compared with the growth of the drug-free control wells. The MIC values were read after 24 and 48 h of incubation without agitation for yeasts,

A. fumigatus, *A. corymbifera*, and 72 and 120 h of incubation for *T. mentagrophytes*. Drug-free controls and quality control strains (*C. krusei* ATCC 6254, *C. parapsilosis* ATCC 22019) were included.

4.3.3. Antiproliferative and cytotoxic effect. Antiproliferative and cytotoxic activities were evaluated in the Hans-Knöll-Institut, Jena, Germany.

4.3.3.1. Antiproliferative and cytotoxicity assays.[11] The target compounds were assayed against cell lines K-562 and L-929 for their antiproliferative effects and against HeLa for their cytotoxic effects. The cells were incubated with 10 concentrations of the test compounds.

4.3.3.2. Cells and culture conditions. Cells of established suspended cell lines K-562 (DSM ACC 10) and adherent L-929 (DSM ACC 2) were cultured in RPMI medium (GIBCO BRL 42402-016), supplemented with 100 U/mL of penicillin G–sodium salt/100 μg/mL of streptomycin–sulfate (Gibco–BRL 15140-114), 10% of heat-inactivated fetal bovine serum (Gibco–BRL 10500-064), and L-glutamine (Gibco–BRL 25030-024) at 37 °C in high density polyethylene flasks (NUNC 156340). HeLa (DSM ACC 57) cells were grown in RPMI 1640 culture medium (Gibco–BRL 21875-034) supplemented with 100 U/mL of penicillin G-sodium salt/100 μg/mL of streptomycin–sulfate (Gibco–BRL 15140-114), 10% of heat-inactivated fetal bovine serum (Gibco–BRL 10500-064), and 10 mL/L of non-essential amino acid (Gibco–BRL 11140-035) at 37 °C in high density polyethylene flasks (NUNC 156340).

The adherent cells of L-929 and HeLa were harvested at the logarithmic growth phase after soft trypsinization, using 0.25% trypsin in HBSS containing 0.038% of EDTA (Gibco–BRL 25200-056).

For each antiproliferative assay with L-929 and K-562 approximately 10,000 cells were seeded with 0.1 mL of RPMI 1640 culture medium (Gibco–BRL 21875-034), containing 10% of heat-inactivated fetal bovine serum (Gibco–BRL 10500-064), 100 U/mL of penicillin G–sodium salt/100 μg/mL of streptomycin–sulfate (Gibco–BRL 15140-114), but without HEPES, into 96-well micro plates. The plates were previously prepared with 10 dilutions of test substances in 0.1 mL RPMI 1640 medium.

For cytotoxic assay with HeLa, approximately 10,000 cells were seeded with 0.1 mL RPMI 1640 culture medium per well of the 96-well micro plates. HeLa cells were preincubated for 48 h without the test substances. The solutions of the compounds of the corresponding concentrations were applied carefully on the monolayers of HeLa cells after the preincubation time.

The adherent cells of L-929 and HeLa were seeded into microplates NUNC 167008 and the suspended cells of K-562 were seeded into microplates NUNC 163320.

4.3.3.3. Incubation conditions. Cells of L-929, K-562, and HeLa were incubated for 72 h at 37 °C in a humidified atmosphere and 5% CO_2.

4.3.3.4. Methods of evaluation. Suspension cultures of K-562 in micro plates were analyzed by an electronic cell analyzer system CASY 1 (SCHÄRFE, Reutlingen, Germany) using an aperture of 150 μm. The software for data evaluation CASYSTAT (SCHÄRFE) offers fast graphical evaluation of the measurement parameters, for example, as diagrams of cell diameter distributions, overlays of different curves, and cell volume distributions. The 0.2 mL content of each well in the microplate was diluted 1:50 with CASYTON (NaCl, 7.93 g/L; Na_2 EDTA, 0.38 g/L; KCl ,0.4 g/L; NaH_2PO_4 monohydrate, 0.22 g/L; NaH_2PO_4 dihydrate, 2.45 g/L; NaF, 0.3 g/L; SCHÄRFE). Every count/milliliter was automatically calculated from the arithmetic mean of three successive counts of 0.4 mL each. From the dose response curves the GI_{50} values (concentration which inhibited cell growth by 50%) were calculated with CASYSTAT. The GI_{50} value was defined as being where the concentration–response curve intersected the 50% line, determined by means of the cell counts/milliliter, compared to control.

The monolayers of the adherent L-929 and HeLa cells were fixed by glutaraldehyde and stained with a 0.05% solution of methylene blue for 15 min. After gently washing, the stain was eluted by 0.2 mL of 0.33 N HCl in the wells. The optical densities were measured at 630 nm in a DYNATECH MR 7000 micro plate reader. Comparisons of the different values were performed with Microsoft EXCEL.

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