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Non-thiol Farnesyltransferase Inhibitors: FTase-Inhibition and Cellular Activity of Benzophenone-based Bisubstrate Analogue Farnesyltransferase Inhibitors

Some 5-acylaminoacylamino-benzophenone derivatives were designed as bisubstrate analogue farnesyltransferase inhibitors. These compounds turned out to be only weakly active against farnesyltransferase, but displayed an antiproliferative effect rendering them suitable for further development as a novel type of cytostatic agents.

Keywords: Non-thiol farnesyltransferase inhibitors; Structure/Activity relationships; Bisubstrate analogs; MCF-7 breast cancer cells; Antiproliferative agents

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Introduction

Inhibition of farnesyltransferase has become a major strategy for the development of novel potential anti-cancer drugs [1–8]. Farnesyltransferase catalyzes the transfer of a farnesyl residue from farnesylpyrophosphate to the thiol of a cysteine side chain of proteins bearing the CAAX-tetrapeptide sequence (C: cysteine, A: aliphatic amino acid, X: serine or methionine) C-terminally [9, 10]. Farnesylation is a prerequisite for the transforming activity of oncogenic Ras, which is found in approximately 30 % of all cancers in humans. However, there is accumulating evidence that prevention of Ras farnesylation may not be the crucial cellular event responsible for the antiproliferative effect of farnesyltransferase inhibitors [5, 8]. Disregarding the unresolved mechanism of action of farnesyltransferase inhibitors, the efficacy of these compounds and their low toxicity has been demonstrated [5, 8, 11].

Most inhibitors described in literature are peptidomimetics resembling the CAAX-tetrapeptide recognition sequence of farnesylated proteins. The majority of these CAAX-peptidomimetics exhibits a free thiol group [1-8] which coordinates the enzyme-bound zinc ion as demonstrated for the native peptide substrate [12]. However, free thiols are associated with several adverse drug effects [13] and, therefore, the development of farnesyltransferase inhibitors is clearly directed towards the so-called non-thiol farnesyltransferase inhibitors.

We have described a series of benzophenone-based bisubstrate analogue farnesyltransferase inhibitors (e.g. **1**) (Figure 1), compounds displaying molecular features of both, the peptidic and the prenylic substrate [14]. In this type of compounds, the 2-acylaminobenzophenone moiety represents the peptidomimetic and the fatty acid the farnesylmimetic substructure. Here, we have replaced the aliphatic farnesyl surrogate by aromatic acrylic acid derivatives. In an earlier study, we identified such aromatic acrylic acids as farnesyl mimetics [15]. The compounds were evaluated for their farnesyltransferase inhibitory activity and their effect on the growth of MCF-7 breast cancer cells. In addition, two compounds were evaluated in the NCIs cancer cell line screen.

Chemistry

The compounds were prepared from the appropriate 2-acylamino-5-aminobenzophenones [16] (**10**) either by acylation of the 5-amino function by the appropriate *N*-acylamino acid [17, 18] activated as mixed anhydride or by acylation of 2-(tolylacetyl)amino-5-aminobenzophenone [16] (**10 b**) by the appropriate *N*-Boc protected amino acid and subsequent removal of the protective group followed by acylation with 4-phenylcinnamic acid chloride (Scheme 1).

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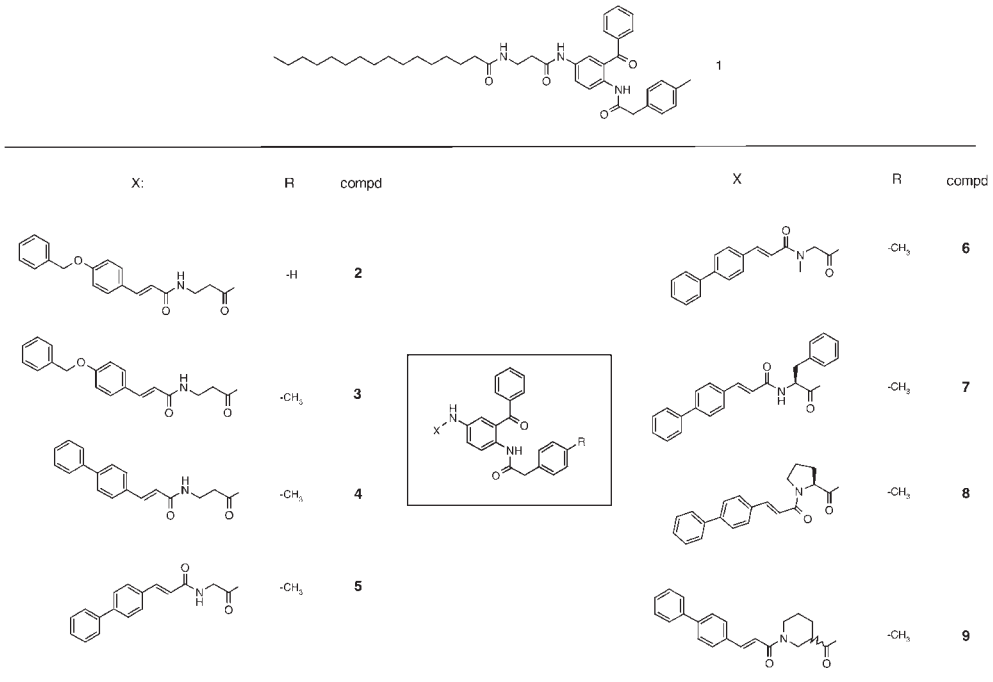
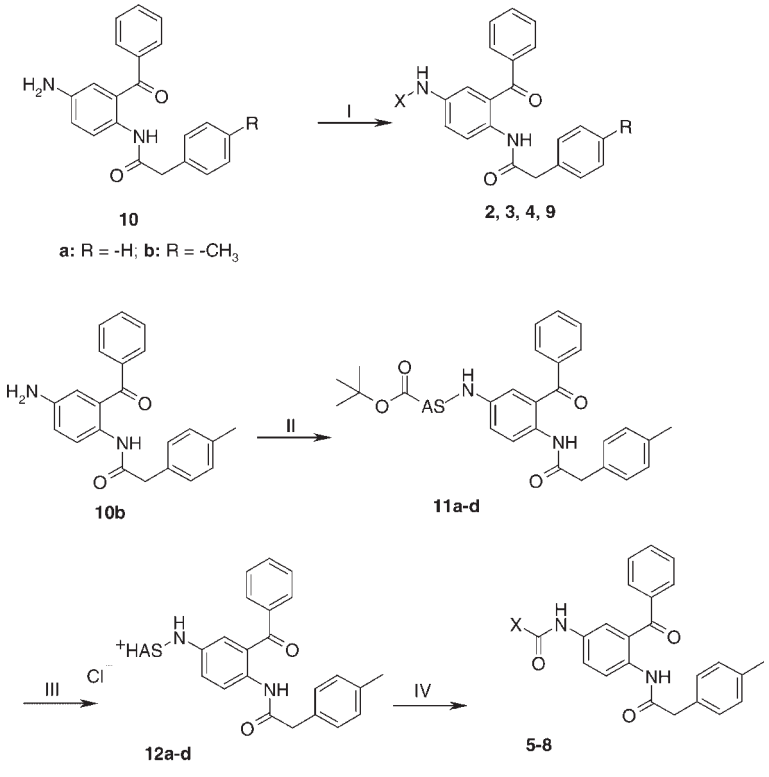


Figure 1. Stuctures of inhibitors **1–9**.



Scheme 1. (I) N-4-benzyloxycinnamoyl-β-alanine or N-4-phenylcinnamoy-β-alanine or N-4-phenylcinnamoylpiperidine-3-carboxylic acid, isobutyl chloroformate, N-methylmorpholine, DMF, –15 °C, 5 min., then add **10**, DMF, –15 °C to RT, overnight; (II) N-Boc-glycine or N-Boc-N-methyl-glycine or N-Boc-phenylalanine or N-Boc-proline, isobutyl chloroformate, N-methylmorpholine, DMF, –15 °C, 5 min., then add **10b**, DMF, –15 °C to RT, overnight; (III) HCl 4 M in dioxane, RT, 1 h; (IV) 4-phenylcinnammic acid chloride, N-methylmorpholine, DCM, 0 °C → RT, 4 h.

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Farnesyltransferase inhibition assay

The inhibitory activity was determined using the fluorescence enhancement assay as described by Pompliano [20]. The assay employs yeast farnesyltransferase (FTase) fused to Glutathione *S*-transferase at the N-terminus of the β-subunit [21]. Farnesylpyrophosphate and the dansylated pentapeptide Ds-GlyCysValLeuSer were used as substrates. Upon farnesylation of the cysteine thiol, the dansyl residue is placed into a lipophilic environment. The resulting enhancement of fluorescence at 505 nm is used to monitor the enzyme reaction.

Cell culture assays

The influence of the FTase inhibitors on the proliferation of breast cancer cells was evaluated on the MCF-7 cell line. MCF-7 cells were treated with the compounds **2**–**9** in three concentrations (1, 5, 10 μM) 48 h after seeding. In this experiment, the drug- (and vehicle (DMSO)) containing culture media were left unchanged throughout the incubation period of about 244 h. The results are summarized in Figure 2. In these plots of T/C_{corr} vs. time of incubation, time zero indicates the time of drug addition. T/C_{corr} was calculated according to Equation 1

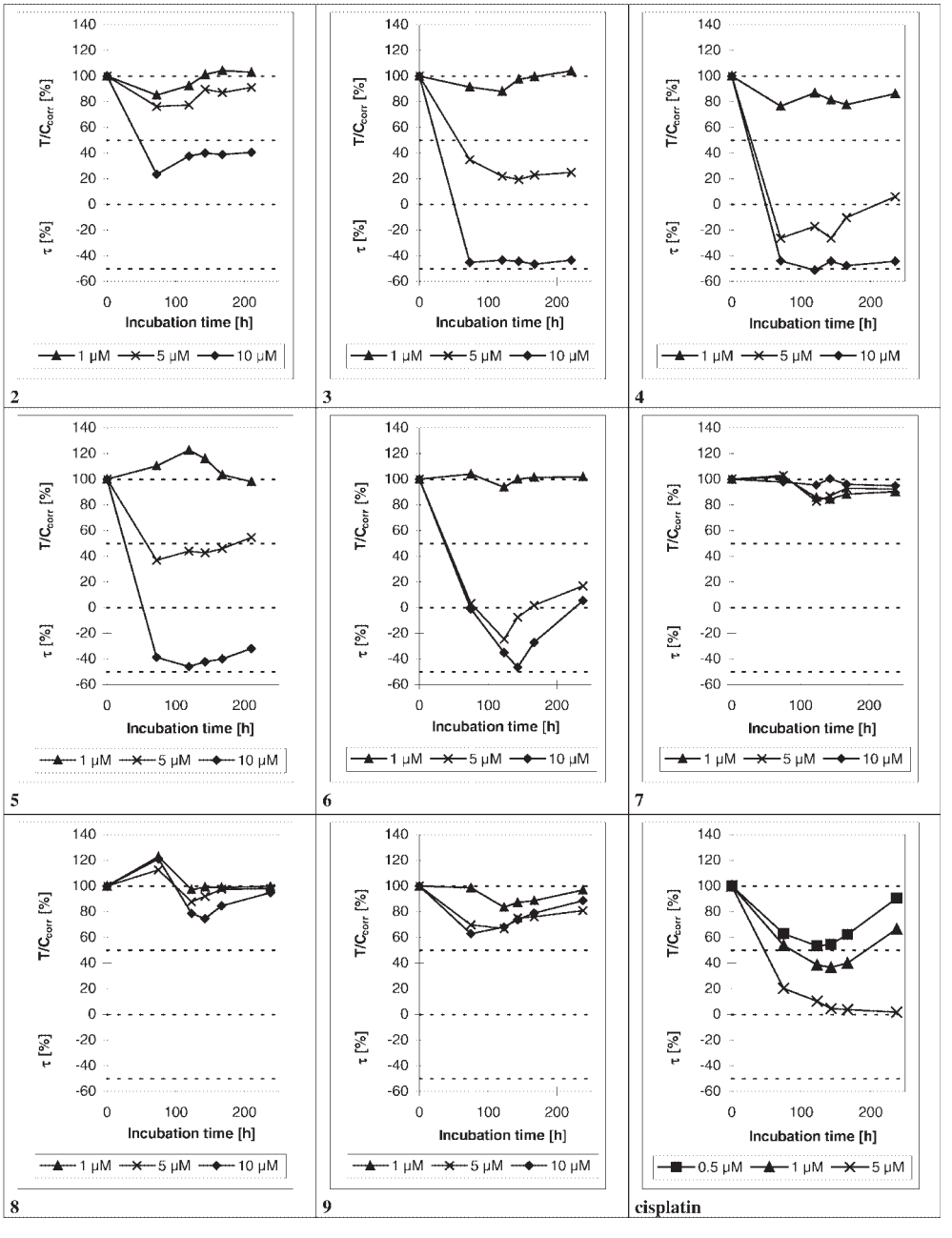

Figure 2. Activity of compounds **2–9** against MCF-7 breast cancer cells.

(Experimental). In addition, compounds **4** and **5** have been assayed against the full panel of cancer cell lines of the NCI [22].

Results and discussion

In contrast to the benzophenone-based bisubstrate analogue farnesyltransferase inhibitors (e.g. **1**) described earlier [14], compounds **2–9**, carrying an aromatic farnesylmimetic instead of an aliphatic one, exhibited only weak farnesyltransferase inhibitory activity. The IC_{50}-values are ranging between 10 and 36 μM (Table 1). Although the effects are weak, some structure/activity relationships (SAR) can be delineated. Compound **3**, carrying a tolylacetic acid at the 2-amino group of the benzophenone scaffold, was more active than the phenylacetic acid carrying inhibitor **2**. The same SAR has also been observed with benzophenone-CAAX-peptidomimetics [16]. In addition, deviation from β-alanine as linker between the peptidomimetic and the farnesylmimetic substructures resulted in a decreased activity in most cases, a result, which has also been obtained with bisubstrate analogues of different structure [18].

Table 1. Farnesyltransferase inhibitory activity of compounds **1–9**.

Compd.	IC_{50} (μM)	Compd.	IC_{50} (μM)
1 [14]	0.4 ± 0.033	**6**	10 ± 6
2	22 ± 8	**7**	32 ± 9
3	14 ± 5	**8**	25 ± 7
4	9.3 ± 4	**9**	30 ± 11
5	36 ± 10		

However, some of these compounds displayed concentration-dependent cytotoxic – or at higher concentrations – even cytocidal effects against MCF-7 breast cancer cells. The compounds **4** and **6** were more active than the antitumor drug cisplatin (see Figure 2).

Again, a clear dependency of the antiproliferative effect on the structure of the compounds was visible. In analogy to the activity enhancing effect of the methyl group at the arylacetic acid substructure with respect to farnesyltransferase inhibition this residue also enhanced antiproliferative activity (Figure 2; **3** vs. **2**). Direct comparison of the inhibitors **3** and **4** revealed some superiority of the 4-phenylcinnamoyl-substituted compound **4** over the 4-benzyloxycinnamoyl derivative **3**. Therefore, this study was continued with phenylcinnamoyl derivatives. The replacement of β-alanine by glycine (**5**) resulted in a decreased antiproliferative activity when assayed in a concentration of 5 μM, while the activity remained nearly constant in the higher concentration of 10 μM. Virtually inactive compounds (**7–9**) were obtained when β-alanine was replaced by phenylalanine, proline, or piperidine-3-carboxylic acid. The use of *N*-methylglycine in the central position resulted in compound **6**. The time activity curve of **6** presented in Figure 2 reveals high cytocidal effects in the 5 μM and 10 μM concentration after 100–150 h of incubation (10 μM, $\tau = -46$ % after 148 h; 5 μM $\tau = -25$ % after 128 h). However, the cells develop resistance against **6** because the cytocidal effects are lost towards the end of the test. On the other hand, **3**, **4,** and **5** preserved their strong antiproliferative potency during the time of incubation, so **4** and **5** were selected for further evaluation against 60 different cancer cell lines in the NCI's drug screen (Table 2).

GI_{50}-values are predominantly in the low micromolar range with little differences between the two compounds. No superiority of one inhibitor over the other is thereby visible. A total growth inhibition (TGI) is generally observed at similar concentrations while those required reducing the cell number by 50 % (LC_{50}) are in some cases significantly higher.

Although these compounds have initially been designed as bisubstrate analogue farnesyltransferase inhibitors and some parallels being between antiproliferative and farnesyltransferase inhibitory activity, there are two facts arguing strongly for an antiproliferative mechanism different from farnesyltransferase inhibition. First, antiproliferative activity is generally observed at lower concentrations than farnesyltransferase inhibition and second, compound **5** displays considerable antiproliferative activity while its farnesyltransferase inhibition (IC_{50} = 36 μM) is the weakest of the whole series.

Therefore, we consider these compounds as a new type of antiproliferative agents with a mechanism yet to be determined. Early structure activity relationships can be deduced from this series, which suggest that a further development may be worthwhile.

Acknowledgements

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Table 2. Activity of compounds **4** and **5** in NCI's drug screen. GI_{50}: 50 % growth inhibition; TGI: total growth inhibition (0 % growth); LC_{50}: 50 % reduction of cell number.

Panel/Cell Line	Compd. 5 GI_{50}	TGI	LC_{50}	Compd. 4 GI_{50}	TGI	LC_{50}
Leukemia						
CCRF-CEM	4.78E-06	1.71E-05	4.94E-05	2.16E-06	8.84E-06	9.71E-05
HL-60 (TB)	7.44E-06	2.07E-05	4.82E-05	1.69E-05	5.44E-05	>1.00E-04
K-562	3.33E-06	1.03E-05	3.21E-05	1.11E-05	>1.00E-04	>1.00E-04
MOLT-4	3.50E-06	1.53E-05	3.92E-05	4.17E-06	9.16E-06	>1.00E-04
RPMI-8226	1.04E-05	2.92E-05	8.22E-05	2.40E-06	1.47E-05	8.02E-05
SR	1.84E-06	4.9cE-06	1.67E-05			
Non-Small Cell Lung Cancer						
A549/ATCC	2.03E-06	5.37E-06	2.09E-05	1.77E-06	4.32E-06	1.16E-05
EKVX	3.47E-06	1.58E-05	4.71E-05	1.32E-06	3.58E-06	9.71E-06
HOP-62	2.56E-06	6.95E-06	2.67E-05	2.11E-06	4.15E-06	8.18E-06
HOP-92	4.17E-06	1.54E-05	4.45E-05			
NCI-H226	2.79E-06	8.39E-06	3.49E-05	2.42E-06	6.15E-06	2.42E-05
NCI-H23	2.06E-06	4.71E-06	1.21E-05	1.85E-06	4.20E-06	9.50E-06
NCI-H322M	5.21E-06	2.46E-05	>1.00E-04	3.47E-06	1.77E-05	>1.00E-04
NCI-H460	2.86E-06	1.11E-05	3.72E-05	1.88E-06	4.60E-06	1.70E-05
NCI-H522	5.13E-06	1.84E-05	4.73E-05			
Colon Cancer						
COLO 205	6.93E-06	3.20E-05	>1.00E-04	1.75E-06	4.53E-06	1.39E-05
HCC-2998	1.68E-06	4.98E-06	1.84E-05	2.37E-08	1.61E-06	8.20E-06
HCT-116	1.37E-06	2.65E-06	5.15E-06	1.12E-06	2.96E-06	7.82E-06
HCT-15	3.69E-06	1.32E-05	3.63E-05	2.71E-06	9.53E-06	4.04E-05
HT29	1.58E-06	2.99E-06	5.65E-06	5.06E-06	2.03E-05	6.63E-05
KM12	3.57E-06	1.45E-05	4.49E-05	2.93E-06	1.46E-05	>1.00E-04
SW-620	4.75E-06	1.95E-05	8.73E-05	1.57E-06	3.44E-06	7.50E-06
CNS Cancer						
SF-268	2.46E-06	7.51E-06	3.29E-05	2.62E-06	9.47E-06	7.41E-05
SF-295	2.22E-06	4.86E-06	1.19E-05	1.36E-06	3.05E-06	6.84E-06
SF-539	2.70E-06	8.85E-06	3.69E-05	1.68E-06	3.67E-06	8.03E-06
SNB-19	4.57E-06	2.03E-05	7.08E-05	3.06E-06	1.60E-05	8.55E-05
SNB-75				1.93E-06	6.59E-06	3.26E-05
U251	2.77E-06	8.67E-06	3.24E-05	1.85E-06	4.35E-06	1.07E-05
Melanoma						
LOX IMVI	3.69E-06	1.34E-05	3.88E-05	2.03E-06	4.84E-06	1.45E-05
MALME-3M	2.99E-06	1.23E-05	7.73E-05	3.39E-06	1.15E-05	6.06E-05
M14	4.81E-06	1.73E-05	5.31E-05	2.57E-06	7.30E-06	3.13E-05
SK-MEL-2	1.39E-05	3.53E-05	8.99E-05	1.89E-06	5.79E-06	2.25E-05
SK-MEL-28	1.17E-05	6.66E-05	>1.00E-04	3.04E-06	9.00E-06	3.95E-05
SK-MEL-5	1.69E-06	6.32E-06	2.60E-05	2.10E-06	5.14E-06	1.60E-05
UACC-257	6.33E-06	2.27E-05	6.35E-05	2.05E-06	4.71E-06	1.23E-05
UACC-62	1.31E-05	3.08E-05	7.27E-05	2.17E-06	4.87E-06	1.30E-05

Table 2. (continued)

Panel/Cell Line	Compd. 5 GI_{50}	TGI	LC_{50}	Compd. 4 GI_{50}	TGI	LC_{50}
Ovarian Cancer						
IGROV1	8.25E-06	2.17E-05	5.11E-05	9.75E-07	2.16E-06	4.72E-06
OVCAR-3	4.97E-06	1.91E-05	4.93E-05	1.88E-06	4.32E-06	9.89E-06
OVCAR-4				1.48E-06	3.41E-06	7.82E-06
OVCAR-5	4.48E-06	2.31E-05	>1.00E-04	2.09E-06	5.77E-06	2.79E-05
OVCAR-8	1.59E-06	3.27E-06	6.73E-06	8.72E-07	2.21E-06	5.12E-06
SK-OV-3	1.07E-05	2.74E-05	6.98E-05	1.85E-06	4.36E-06	1.07E-05
Renal Cancer						
786-0	2.27E-06	5.47E-06	1.88E-05	1.60E-06	3.34E-06	6.97E-06
A498	9.89E-07	8.73E-06	3.27E-05	2.31E-06	5.31E-06	1.74E-05
ACHN	4.79E-06	1.62E-05	4.17E-05	3.08E-06	1.09E-05	4.17E-05
CAKI-1	4.89E-06	2.04E-05	6.05E-05	3.29E-06	1.13E-05	7.09E-05
RXF 393	2.38E-06	6.60E-06	2.36E-05	1.46E-06	3.52E-06	8.50E-06
SN12C	2.74E-06	9.93E-06	3.69E-05	1.92E-06	5.51E-06	2.62E-05
TK-10	2.08E-06	5.95E-06	2.56E-05	2.78E-06	8.72E-06	>1.00E-04
UO-31	1.18E-05	2.47E-05	5.18E-05	3.67E-06	1.37E-05	3.71E-05
Prostate Cancer						
PC-3	1.98E-06	4.07E-06	8.37E-06	1.46E-06	3.48E-06	8.25E-06
DU-145	2.49E-06	7.54E-06	3.59E-05	3.93E-06	>1.00E-04	>1.00E-04
Breast Cancer						
MCF7	1.81E-06	3.69E-06	7.51E-06	1.72E-06	4.92E-06	3.09E-05
NCI/ADR-RES	9.71E-06	2.98E-05	9.02E-05	5.41E-06	>1.00E-04	>1.00E-04
MDA-MB-231/ATCC	2.62E-06	9.21E-06	3.40E-05	1.87E-06	5.64E-06	2.45E-05
HS 578T	4.71E-06	2.11E-05	9.11E-05	1.73E-06	3.74E-06	8.07E-06
MDA-MB-435	3.67E-06	1.61E-05	4.76E-05	3.27E-06	1.08E-05	4.32E-05
MDA-N	4.74E-06	2.09E-05	7.99E-05	2.50E-06	6.61E-06	2.53E-05
BT-549	1.09E-05	2.40E-05	5.27E-05	1.46E-06	3.11E-06	6.64E-06
T-47D	1.76E-06	3.89E-06	8.62E-06	2.85E-06	1.80E-05	>1.00E-04

Experimental

General

^{1}H-NMR spectra were recorded on a Jeol JMN-GX-400 and a Jeol JMN-LA-500 spectrometer (Jeol USA Inc., Peabody, MA, USA). Mass spectra were obtained with a Vacuum Generators VG 7070 H (Vacuum Generators, Manchester, UK) using a Vector 1 data acquisition system from Teknivent (Teknivent Corp., Maryland Heights, MO, USA) or an AutoSpec mass spectrometer from Micromass (Micromass, Manchester, UK). IR spectra were recorded on a Nicolet 510P FT-IR-spectrometer (Thermo Nicolet Corp., Madison, WI, USA). Microanalyses were obtained from a CH analyzer according to Dr. Salzer from Labormatic and from a Hewlett Packard CHN-analyzer type 185 (Hewlett Packard, Palo Alto, CA, USA). Melting points were obtained with a Leitz microscope (Leitz, Wetzlar, Germany) and are uncorrected. Column chromatography was carried out using silica gel 60 (0.062–0.200 mm) from Merck (Darmstadt, Germany).

General procedure A: amide formation using acids activated as mixed anhydrides.

The appropriate acid was dissolved in a sufficient amount of dry DMF in a flame dried flask under Ar atmosphere. After addition of *N*-methylmorpholine [NMM] (0.25 ml per mmol acid) the solution was cooled to –15 °C and isobutyl chloroformate (0.13 ml per mmol acid) was added. A solution of the amine component (1 equivalent) in dry DMF was added after 5 min. In case the amine component was employed as a hydrochloride, additional NMM (0.25 mL per mmol) was added. The mixture

was allowed to warm up to room temperature overnight, and then poured into brine (400–800 mL). In case of solid precipitate formation, this was collected by suction and thoroughly washed with water. Otherwise, the aqueous mixture was extracted with ethyl acetate (3 × 100 mL) and the combined organic extracts were washed successively with 2 N citric acid, sat. $NaHCO_3$-solution and brine, and dried with $MgSO_4$. The residue obtained after removal of the solvent was purified by recrystallisation or flash chromatography.

General procedure B: amide formation using acids chlorides

Carboxylic acids were dissolved in dichloromethane and 0.2 mL oxalychloride per mmol acid was added. The mixture was stirred at room temperature for 2 h and the volatiles were evaporated *in vacuo*. The residue obtained was dissolved in toluene or dioxane (approx. 10 mL) and added to a solution of the appropriate aromatic amine in hot toluene (approx. 50 mL). The mixtures were heated under reflux for 2 h. Then, the solvent was removed *in vacuo* to give the crude products.

General procedure C: N-Boc-deprotection and acylation using acid chlorides

The appropriate Boc-protected derivative **11** was stirred in a 4 N solution of HCl(g) in dioxane for 1 h. After the volatiles were removed *in vacuo*, the residue **12** was used without further purification for the reaction with phenylcinnamic acid chloride according to general procedure B.

N-[2-[3-Benzoyl-4-(phenylacetylamino)phenylcarbamoyl]ethyl]-3-(4-benzyloxyphenyl)-acrylamide (**2**)

From 3-[3-(4-benzyloxyphenyl)acryloylamino]propionic acid (440 mg, 1.35 mmol) according to general procedure A. Yield: 800 mg (93 %). Mp 138 °C. – IR (KBr): ν = 3304, 3064, 3032, 2962, 1654, 1601, 1560, 1509 cm^{-1}. – ^1H NMR ($CDCl_3$): δ = 2.54 (t, *J* = 6 Hz, 2 H), 3.55 (t, *J* = 6 Hz, 2 H), 3.64 (s, 2 H), 4.99 (s, 2 H), 6.13 (d, *J* = 16 Hz, 1 H), 6.43 (m, 1 H), 6.85 (m, 2 H), 7.28–7.41 (m, 16 H), 7.45 (m, 1 H), 7.59 (m, 2 H), 7.75 (m, 1 H), 8.43 (m, 1 H), 8.58 (s, 1 H), 10.45 (s, 1 H). – MS (EI): *m/z* 91 (100), 366 (27), 253 (27), 311 (22), 212 (17), 266 (15), 637 (0.2) [M^+]. Anal. calcd. for $C_{40}H_{35}N_3O_5$: C, 75.34; H, 5.53; N, 6.59; found: C, 75.13; H, 5.70; N, 6.22.

N-[2-[3-Benzoyl-4-(p-tolylacetylamino)phenylcarbamoyl]-ethyl]-3-(4-benzyloxy-phenyl)acrylamide (**3**)

From 3-[3-(4-benzyloxyphenyl)acryloylamino]propionic acid (327 mg, 1 mmol) according to general procedure A. Yield: 443 mg (68 %). Mp 137 °C. – IR (KBr): ν = 3296, 3061, 2923, 1656, 1602, 1539, 1510, 1251, 1174 cm^{-1}. – ^1H NMR ($CDCl_3$): δ = 2.26 (s, 3 H), 2.54 (m, 2 H), 3.58 (m, 2 H), 3.60 (s, 2 H), 5.00 (s, 2 H), 6.13 (d, *J* = 15 Hz, 1 H), 6.26 (m, 1 H), 6.86 (m, 2 H), 7.10 (m, 2 H), 7.16 (m, 2 H), 7.21–7.43 (m, 11 H), 7.59 (m, 3 H), 7.75 (s, 1 H), 8.29 (s, 1 H), 8.43 (m, 1 H), 10.39 (s, 1 H). – MS (EI): *m/z* 91 (100), 105 (14), 253 (12), 649 (0.7) [M^+]. Anal. calcd. for $C_{41}H_{37}N_3O_5$: C, 75.56; H, 5.72; N, 6.45; found: C, 75.35; H, 5.59; N, 6.39.

N-[2-[3-Benzoyl-4-(p-tolylacetylamino)phenylcarbamoyl]-ethyl]-3-biphenyl-4-yl-acrylamide (**4**)

From 3-(3-biphenyl-4-yl-acryloylamino)propionic acid (307 mg, 1 mmol) according to general procedure A. Yield: 385 mg (62 %). Mp 181 °C. – IR (KBr): ν = 3270, 3030, 2921, 1653, 1612, 1537, 1510 cm^{-1}. – ^1H NMR ($CDCl_3$: δ = 2.25 (s, 3 H), 2.54 (t, *J* = 6 Hz, 2 H), 3.57 (s, 2 H), 3.58 (t, *J* = 6 Hz, 2 H), 6.30 (d, *J* = 16 Hz, 1 H), 6.55 (m, 1 H), 7.08 (m, 2 H), 7.14 (m, 2 H), 7.28 (m, 1 H), 7.30–7.44 (m, 6 H), 7.45–7.51 (m, 6 H), 7.55–7.61 (m, 3 H), 7.77 (m, 1 H), 8.40 (m, 1 H), 8.60 (s, 1 H), 10.38 (s, 1 H). – MS (EI): *m/z* 223 (100), 266 (75), 212 (64), 207 (63), 178 (54), 379 (51), 621 (18) [M^+]. Anal. calcd. for $C_{40}H_{35}N_3O_4$: C, 77.27; H, 5.67; N, 6.76; found: C, 76.95; H, 5.67; N, 6.48.

N-[[3-Benzoyl-4-(p-tolylacetylamino)phenylcarbamoyl]-methyl]-3-biphenyl-4-yl-acrylamide (**5**)

From 4-phenylcinnamic acid (224 mg, 1 mmol) according to general procedure B. Yield: 476 mg (78 %). Mp 223 °C. – IR (KBr): ν = 3406, 3276, 3059, 2925, 1695, 1654, 1608, 1560, 1509 cm^{-1}. ^1H NMR (DMSO-d_6): δ = 2.23 (s, 3 H), 3.34 (s, 2 H), 3.99 (s, 2 H), 6.77 (d, *J* = 16 Hz, 1 H), 6.97 (m, 2 H), 7.02 (m, 2 H), 7.20 (m, 1 H), 7.34–7.37 (m, 2 H), 7.44–7.49 (m, 4 H), 7.55–7.71 (m, 9 H), 7.75 (m, 2 H), 8.32 (m, 1 H), 9.99 (s, 1 H), 10.10 (s, 1 H). – MS (EI): *m/z* 325 (100), 212 (54), 207 (44), 458 (32), 344 (25), 607 (8) [M^+]. Anal. calcd for $C_{39}H_{33}N_3O_4$: C, 77.08; H, 5.47; N, 6.92; found: C, 77.36; H, 5.75; N, 6.66.

N-[[3-Benzoyl-4-(p-tolylacetylamino)phenylcarbamoyl]-methyl]-3-biphenyl-4-yl-N-methylacrylamide (**6**)

From **11 b** (773 mg, 1.5 mmol) according to general procedure C. Yield: 457 mg (49 %). Mp 202 °C. – IR (KBr): ν = 3277, 3056, 3032, 2923, 1689, 1646, 1599, 1559, 1508 cm^{-1}. – ^1H NMR ($CDCl_3$): δ 2.32 (s, 3 H), 3.31 (s, 3 H), 3.67 (s, 2 H), 4.17 (s, 2 H), 6.88 (d, *J* = 16 Hz, 1 H), 7.15 (m, 2 H), 7.25 (m, 2 H), 7.37 (m, 1H), 7.42–7.49 (m, 4 H), 7.53–7.62 (m, 8 H), 7.66–7.73 (m, 3 H), 7.85 (m, 1 H), 8.47 (m, 1 H), 9.07 (s, 1 H), 10.48 (s, 1 H). – MS (EI): *m/z* 212 (100), 344 (86), 207 (59), 278 (55), 309 (52), 621 (2) [M^+]. Anal. calcd. for $C_{40}H_{35}N_3O_4$: C, 77.27; H, 5.67; N, 6.76; found: C, 77.48; H, 5.53; N, 6.72.

(S)-N-[1-[3-Benzoyl-4-(p-tolylacetylamino)phenylcarbamoyl]-2-phenylethyl]-3-biphenyl-4-yl-acrylamide (**7**)

From **11 c** (591 mg, 1 mmol) according to general procedure C. Yield: 420 mg (60 %). Mp 211 °C. – IR (KBr): ν = 3260, 3018, 2890, 1649, 1620, 1514 cm^{-1}. – ^1H NMR ($CDCl_3$): δ 2.23 (s, 3 H), 3.00 (m, 2 H), 3.60 (s, 2 H), 4.91 (dd, *J* = 7/14 Hz, 1 H), 6.37 (d, *J* = 16 Hz, 1 H), 7.06–7.13 (m, 7 H), 7.16–7.30 (m, 7 H), 7.35 (m, 5 H), 7.39–7.47 (m, 7 H), 7.77 (s, 1 H), 8.43 (m, 1 H), 8.82 (s, 1 H), 10.05 (s, 1 H). – MS (EI): *m/z* 476 (100), 362 (91), 167 (85), 679 (76), 494 (73), 262 (63), 697 (9) [M^+]. Anal. calcd. for $C_{46}H_{39}N_3O_4$: C, 79.17; H, 5.63; N, 6.02; found: C, 78.94; H, 5.58; N, 6.20.

(S)-N-[3-Benzoyl-4-(p-tolyl-acetylamino)phenyl]-1-(3-biphenyl-4-yl-acryloyl)-pyrrolidine-2-carboxylic acid amide (**8**)

From **11 d** (537 mg, 1 mmol) according to general procedure C. Yield: 101 mg (16 %). Mp 135 C. – IR (KBr): ν = 3277, 2876, 1700, 1647, 1594, 1560, 1507 cm^{-1}. – ^1H NMR ($CDCl_3$): δ = 1.77 (m, 1 H), 2.00 (m, 1 H), 2.09 (m, 1 H), 2.26 (s, 3 H), 2.52 (m, 1 H), 3.57 (m, 1 H), 3.59 (s, 2 H), 3.72 (m, 1 H), 4.81 (m, 1 H), 6.70 (d, *J* = 16 Hz, 1 H), 7.09 (m, 2 H), 7.15–7.20 (m, 2 H), 7.31 (m, 1 H), 7.39 (m, 4 H), 7.49–7.57 (m, 8 H), 7.63 (m, 2 H), 7.69 (m, 1 H), 7.79 (m, 1 H), 8.41 (m, 1 H), 9.94 (s, 1 H), 10.33 (s, 1 H). – MS (EI): *m/z* 207 (100), 344 (24), 304 (24), 647 (5) [M^+]. Anal. calcd. for $C_{42}H_{37}N_3O_4$: C, 77.88; H, 5.76; N, 6.49; found: C, 77.95; H, 5.74; N, 6.38.

(RS)-N-[3-Benzoyl-4-(p-tolyl-acetylamino)phenyl]-1-(3-biphenyl-4-yl-acryloyl)-piperidine-3-carboxylic acid amide (**9**)

From **10 b** (334 mg, 1 mmol) according to general procedure B. Yield: 330 mg (50 %). Mp 149°C. – IR (KBr): ν = 3430, 3277, 3058, 2938, 2860, 1653, 1640, 1560, 1507 cm^{-1}. – ^1H NMR ($CDCl_3$): δ = 1.56 (m, 2 H), 1.81 (m, 1 H), 2.21 (m, 1 H), 2.26 (s, 3 H), 2.56 (m, 1 H), 3.54 (m, 1 H), 3.61 (s, 2 H), 3.63 (m, 1 H), 3.72 (m, 1 H), 4.03 (m, 1 H), 6.77 (d, *J* = 16 Hz, 1 H), 7.08 (m, 3 H), 7.17 (m, 2 H), 7.30 (m, 1 H), 7.37–7.43 (m, 7 H), 7.47 (m,

2 H), 7.49–7.55 (m, 2 H), 7.65 (m, 3 H), 7.91 (m, 1 H), 8.45 (m, 1 H), 9.23 (s, 1 H), 10.44 (s, 1 H). – MS (EI): *m/z* 149 (100), 212 (79), 105 (78), 344 (73), 244 (55), 661 (16) [M^+]. Anal. calcd. for $C_{43}H_{39}N_3O_4$: C, 78.04; H, 5.94; N, 6.35; found: C, 77.70; H, 5.75; N, 6.41.

N-[[3-Benzoyl-4-(p-tolyl-acetylamino)phenylcarbamoyl]-methyl]-N-methylcarbamic acid tert-butyl ester (**11 b**)

From Boc-sarcosine (378 mg, 2 mmol) according to general procedure A. Yield: 592 mg (57 %). – ^{1}H NMR ($CDCl_3$): δ 1.37 (s, 9 H), 2.26 (s, 3 H), 2.89 (s, 3 H), 3.61 (s, 2 H), 3.82 (s, 2 H), 7.09 (m, 2 H), 7.17 (m, 2 H), 7.40 (m, 2 H), 7.44 (m, 1 H), 7.51 (m, 1 H), 7.58–7.63 (m, 3 H), 7.74 (s, 1 H), 8.44 (m, 1 H), 10.43 (s, 1 H).

N-[1-[3-Benzoyl-4-(p-tolylacetylamino)phenylcarbamoyl]-2-phenylethyl]carbamic acid tert- butylester (**11 c**)

From Boc-phenylalanine (530 mg, 2 mmol) according to general procedure A. Yield: 763 mg (65 %). – ^{1}H NMR ($CDCl_3$): δ 1.35 (s, 9 H), 2.33 (s, 3 H), 3.05 (d, J = 7 Hz, 2 H), 3.66 (s, 2 H), 3.69 (t, J = 7 Hz, 1 H), 7.14–7.25 (m, 7 H), 7.34 (m, 1 H), 7.46 (m, 3 H), 7.58 (m, 1 H), 7.66 (m, 4 H), 7.92 (s, 1 H), 8.43 (m, 1 H), 10.48 (s, 1 H).

N-[3-Benzoyl-4-(p-tolylacetylamino)phenyl]-N$^{\alpha}$-tert.-butyl-oxycarbonylprolineamide (**11 d**)

From Boc-proline (430 mg, 2 mmol) accorrding to general procedure A. Yield: 688 mg (64 %). – ^{1}H NMR ($CDCl_3$): δ 1.36 (s, 9 H), 1.60 (m, 1 H), 1.83 (m, 1 H), 2.26 (s, 3 H), 2.28 (m, 1 H), 2.48 (m, 1 H), 3.30 (m, 1 H), 3.60 (s, 2 H), 3.81 (m, 1 H), 4.36 (m, 1 H), 7.10 (m, 3 H), 7.18 (m, 2 H), 7.39 (m, 2 H), 7.49 (m, 2 H), 7.62 (m, 2 H), 7.70 (m, 1 H), 8.42 (s, 1 H), 10.38 (s, 1 H).

1-(3-Biphenyl-4-yl-acryloyl)-piperidine-3-carboxylic acid

4-Phenylcinnamic acid (450 mg, 2 mmol) was converted to the acid chloride following general procedure B and added, dissolved in acetone (20 mL), to the solution of nipecotic acid (260 mg, 2 mmol) in water (40 mL) and K2CO3 (860 mg). After 1 h at 0 °C and 1 h at room temperature, ice water was added and the pH was adjusted to 1 by addition of conc. HCl. The resulting solid was washed with water and dried. Yield: 380 mg (57 %). – ^{1}H NMR (DMSO-d_6): δ 1.40 (m, 1 H), 1.65 (m, 2 H), 1.95 (m 1 H), 3.10 (m, 1 H), 3.48 (m, 1 H), 3.93 (m, 1 H), 4.07 (m, 1 H), 4.45 (m, 1 H), 7.24 (d, J = 16 Hz, 1 H), 7.34 (m, 1 H), 7.43 (m, 2 H), 7.47 (d, J = 16 Hz, 1 H), 7.66 (m, 4 H), 7.74 (m, 2 H), 12.23 (s, 1 H).

Enzyme preparation

Yeast farnesyltransferase was used as a fusion protein to Glutathione S-transferase at the N-terminus of the b-subunit. Farnesyltransferase was expressed in *Escherichia coli* DH5α grown in LB media containing ampicillin and chloramphenicol for co-expression of pGEX-DPR1 and pBC-RAM2 for farnesyltransferase production [21]. The enzyme was purified by standard procedures with glutathione-agarose beads for selective binding of the target protein.

Farnesyltransferase assay

The assay was conducted as described [20]. Farnesylpyrophosphate (FPP) was obtained as a solution of the ammonium salt in methanol-10 mM aqueous NH_4Cl (7:3) from Sigma-Aldrich (Sigma-Aldrich Deutschland, Taufkirchen, Germany). Dansyl-GlyCysValLeuSer (Ds-GCVLS) was custom synthesized by ZMBH, Heidelberg, Germany. The assay mixture (100 mL volume) contained 50 mM Tris/HCl pH 7.4, 5 mM $MgCl_2$, 10 μM $ZnCl_2$, 5 mM dithiothreitol (DTT), 7 μM Ds-GCVLS, 20 μM FPP and 5 nmol (approx.) yeast GST-farnesyltransferase and 1 % of various concentrations of the test compounds dissolved in dimethylsulfoxide (DMSO). The progress of the enzyme reaction was followed by monitoring the enhancement of the fluorescence emission at 505 nm (excitation 340 nm). The reaction was started by addition of the enzyme and run in a Quartz cuvette thermostated at 30 °C. Fluorescence emission was recorded with a Perkin Elmer LS50B spectrometer (Perkin Elmer, Shelton, CT, USA). IC_{50} values (concentrations resulting in 50 % inhibition) were calculated from initial velocity of three independent measurements of four to five different concentrations of the respective inhibitor.

Cytotoxicity studies on the MCF-7 cell line

The human MCF-7 breast cancer cell line was obtained from the American Type Culture Collection (ATCC, Rockville, Md.; USA). Cell line banking and quality control were performed according to the seed stock concept reviewed by Hay [23]. The MCF-7 cells were maintained in L-glutamine containing Eagle's MEM (Sigma, München, Germany), supplemented with $NaHCO_3$ (2.2 g/L) sodium pyruvate (110 mg/L), gentamycin (50 mg/L; Sebio Walchsing, Germany), and 10% fetal calf serum (FCS; Gibco Eggenheim, Germany) using 75 cm^2 culture flasks (Falcon Plastics 3023) in a water-saturated atmosphere (95 % air/5 % CO_2) at 37 °C. The cell line was weekly passaged after treatment with trypsin (0.05 %) / ethylenediaminetetraacetic acid (0.02 %; EDTA; Boehringer, Mannheim, Germany). Mycoplasma contamination was routinely monitored, and only mycoplasma-free cultures were used.

In vitro chemosensitivity assay

The *in vitro* testing for antitumor activity was carried out on exponentially dividing human breast cancer cells according to a previously published microtiter assay [24, 25]. Briefly, in 96-well microtiter plates (Costar), 100 μL of a cell suspension at 500 cells/ml culture medium were plated into each well and incubated at 37 °C for 2–3 d in a humidified atmosphere (5 % CO_2). By addition of an adequate volume of a stock solution of the respective compound (solvent: DMSO) to the medium the desired test concentration was obtained (max. content of DMSO in the medium: 1 ppm). For each test concentration and for the control, which contained the corresponding amount of DMSO, 16 wells were used. After the proper incubation time the medium was removed, the cells were fixed with a glutardialdehyde solution and stored at 4 °C. Cell biomass was determined by a crystal violet staining technique [24, 25]. The effectiveness of the complexes is expressed as corrected T/C values according to the following equations:

$$\text{Cytotoxic effect: } T/C_{corr}\ [\%] = [(T\text{-}C_o)/(C\text{-}C_o)] \times 100 \qquad \text{Eq. (1)}$$

where T (test) and C (control) are the optical densities at 578 nm of the crystal violet extract of the cell lawn in the wells (i.e. the chromatin-bound crystal violet extracted with ethanol 70 %), and Co is the density of the cell extract immediately before treatment.

$$\text{Cytocidal effect: } \tau\ [\%] = [(T\text{-}C_o)/C_o] \times 100 \qquad \text{Eq. (2)}$$

For the automatic estimation of the optical density of the crystal violet extract in the wells a Microplate EL 309 Autoreader (BIO-TEK Instruments, Winooski, VT, USA) was used.

References

[1] D. M. Leonard, *J. Med. Chem.* **1997**, *40*, 2971.

[2] Y. S. Qian, M. Sebti, A. D. Hamilton, *Biopolymers* **1997**, *43*, 25.

[3] T. M. Williams, *Exp. Opin. Therp. Patents* **1998**, *8*, 553.

[4] T. M. Williams *Exp. Opin. Therp. Patents* **1999**, *9*, 1263.

[5] I. M. Bell, *Exp. Opin. Therp. Patents* **2000**, *10*, 1813.

[6] T. M. Williams, C. J. Dinsmore, *Adv. Med. Chem.* **1999**, *4*, 273.

[7] A. Wittinghofer, H. Waldmann, *Angew. Chem.* **2000**, *112*, 4360; *Angew. Chem. Int. Ed.* **2000**, *39*, 4192.

[8] E. K. Rowinsky, A. Patnaik, *Emerging Drugs* **2000**, *5*, 161.

[9] F. L. Zhang, P. J. Casey, *Annu. Rev. Biochem.* **1996**, *65*, 241.

[10] H.-W. Fu, P. J. Casey, *Rec. Prog. Hormon Res.* **1999**, *54*, 315.

[11] A. Oliff, *Biochim. Biophys. Acta* **1999**, *1423*, C19.

[12] C. L. Strickland,; W. T. Windsor, R. Syto, L. Wang, R. Bond, R. Wu, J. Schwartz, H. V. Le, L. S. Beese, P. C. Weber, *Biochemistry* **1998**, *37*, 16601.

[13] *Martindale The Extra Pharmacopeia*, 31st ed. (Ed.: J. E. F. Reynolds), Royal Pharmaceutical Society of Great Britain, London, **1996**; p. 821.

[14] M. Schlitzer, M. Böhm, I. Sattler, *Bioorg. Med. Chem.* **2002**, *10,* 615.

[15] M. Schlitzer, I. Sattler, *Bioorg. Med. Chem.* **1999**, *7*, 2391.

[16] J. Sakowski, M. Böhm, I. Sattler, H.-M. Dahse, M. Schlitzer, *J. Med. Chem.* **2001**, *44*, 2886.

[17] M. Schlitzer, M. Böhm, I. Sattler, H.-M. Dahse *Bioorg. Med. Chem.* **2000**, *8*, 1991.

[18] M. Schlitzer, I. Sattler, *Pharm. Pharmacol. Commun.* **2000**, *5*, 117.

[19] M. Fieser, L. F. Fieser, E .Toromanoff, Y. Hirata, H. Heymann, M. Tefft, S. Bhattacharya, *J. Am. Chem. Soc.* **1956**, *78*, 2825.

[20] D. L. Pompliano, R. P. Gomez, N. J. Anthony *J. Am. Chem. Soc.* **1992**, *114*, 7945.

[21] K. Del Villar, H. Mitsuzawa, W. Yang, I. Sattler, F. Tamanoi, *J. Biol. Chem.* **1997**, *272*, 680.

[22] M. R. Boyd, K. D. Paull, *Drug Dev. Rev.* **1995**, *34*, 91.

[23] R. Hay, *J. Anal. Biochem.* **1988**, *171*, 225.

[24] G. Bernhardt, H. Reile, H. Birnböck, T. Spruß, H. Schönenberger, *J. Cancer Res. Clin. Oncol.* **1992**, *118*, 35.

[25] H. Reile, H. Birnböck, G. Bernhardt, T. Spruß, H. Schönenberger, *Anal. Biochem.* **1990**, *187*, 262.