Eur. J. Med. Chem. 35 (2000) 721−726

S0223523400001628/FLA

Preliminary communication

Non-thiol farnesyltransferase inhibitors: the concept of benzophenone-based bisubstrate analogue farnesyltransferase inhibitors

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Received 3 January 2000; revised 28 February 2000; accepted 6 March 2000

Abstract – Replacement of the thiol in a benzophenone-based CAAX-peptidomimetic farnesyltransferase inhibitor by a carboxylic acid moiety resulted in a marked drop in inhibitory potency. Transformation of these carboxylic acid derivatives into bisubstrate analogues by addition of a lipophilic alkyl chain, which should be able to occupy considerable portions of the farnesyl binding region in the farnesyltransferase's active site, resulted in a regain of the inhibitory activity. These bisubstrate analogues represent new lead structures for non-thiol farnesyltransferase inhibitors. © 2000 Éditions scientifiques et médicales Elsevier SAS

farnesyltransferase / non-thiol farnesyltransferase inhibitors / bisubstrate analogues / benzophenones

1. Introduction

Cancer is caused by a stepwise accumulation of mutations that affect growth control, differentiation and cell survival [1]. Ras proteins play a central role in the signal transduction cascades controlling these processes [2, 3]. Mutated forms of Ras, which are constitutively active, are found in approximately 30% of all human cancers. Several post-transformational modifications take place before Ras obtains its full biological activity. The crucial step is the transfer of a farnesyl residue from farnesylpyrophosphate to the thiol of a cysteine side chain of the C-terminal CAAX-tetrapeptide sequence catalysed by the enzyme farnesyltransferase [4]. Therefore, inhibition of farnesyltransferase has received considerable interest in past years [5–7] as a strategy for the development of novel potential cancer therapeutics. However, there is accumulating evidence that Ras may not be the only substrate of the farnesyltransferase involved in oncogenesis [8, 9]. Regardless of the unresolved issue of the mechanism by which farnesyltransferase inhibitors exert their antiproliferative effects, these compounds have demonstrated their efficacy combined with a surprisingly low toxicity and therefore are regarded as a major emerging strategy in cancer therapy.

We have developed 2-acylaminobenzophenones as novel types of CAAX-peptidomimetic farnesyltransferase inhibitors [10]. Compared to a native CAAX-tetrapeptide in our lead structure **1** (IC_{50} = 0.08 µM) the entire terminal tripeptide has been replaced by a non-peptidic structure (*figure 1a, b*). In this initial approach, the terminal cysteine with its thiol crucial for complexing the essential zinc has been preserved. However, thiols are an undesired feature in potential drugs because of their sensitivity towards oxidation. More importantly, free thiols are a source of adverse drug effects as has been seen with the angiotensin converting enzyme inhibitor captoprile [11]. Therefore, current development is clearly directed towards the so called non-thiol farnesyltransferase inhibitors.

With the angiotensin converting enzyme inhibitors the thiol has successfully been replaced by a carboxylic acid moiety. We intended to explore whether this strategy might also work with our farnesyltransferase inhibitors (*figure 1c*).

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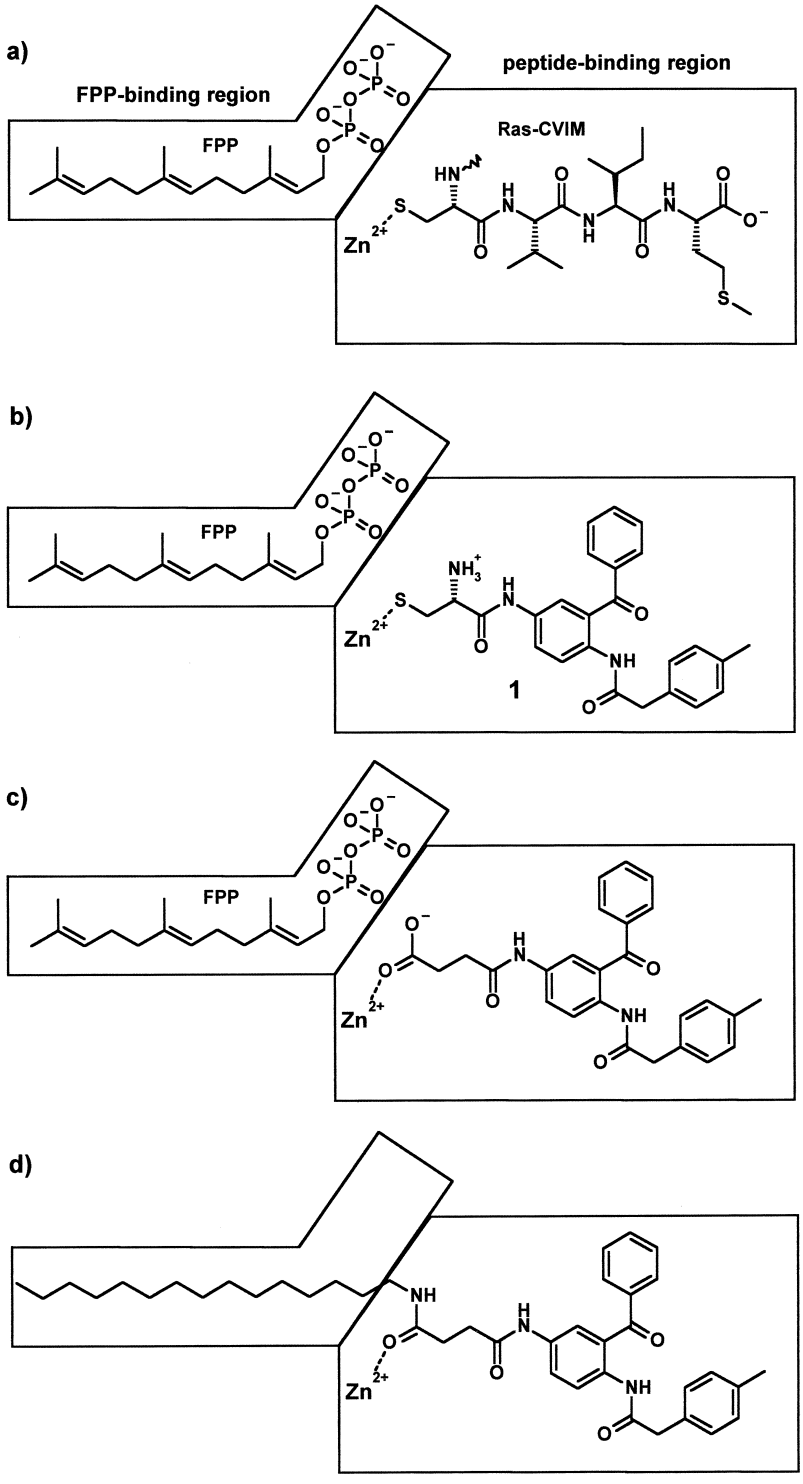

Figure 1. Schematic representation of the farnesyltransferase's active site. Presumable alignment of: **a)** the natural substrates FPP and CAAX-tetrapeptide; **b)** the cysteinyl benzophenone inhibitor **1**; **c)** a carboxylic acid benzophenone inhibitor; **d)** a bisubstrate benzophenone inhibitor.

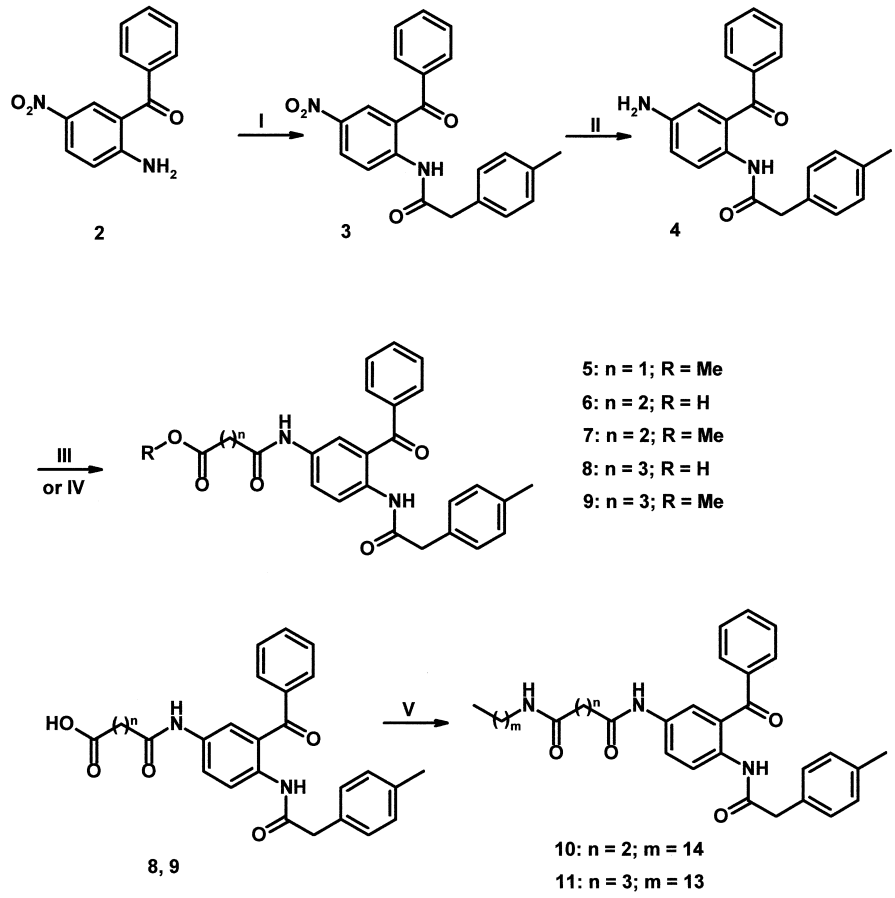

Figure 2. Synthesis of compounds **5–11**: (I) $H_3C–C_6H_4–COCl$, toluene, reflux; (II) $SnCl_2·2H_2O$, EtOAc, reflux; (III) succinic or glutaric acid anhydride, toluene/dioxane, reflux; (IV) $MeOOC–(CH_2)_n–COCl$, toluene/dioxane, reflux; (V) $H_3C–(CH_2)_m–NH_2$, PyBOP, DIPEA, DMF, RT.

2. Chemistry

Compound **4** was prepared according to a procedure published previously [10]. It was acylated by appropriate carboxylic acid chlorides and anhydrides, respectively, in an inert solvent. The long chain alkyl amide derivatives **10** and **11** were prepared from the carboxylic acid derivatives **6** and **8** using the PyBOP methodology (*figure 2*).

3. Pharmacology

The inhibitory activity of the potential synthetic inhibitors was determined using the fluorescence enhancement assay as described by Pompliano [12]. The assay employed yeast farnesyltransferase (FTase) fused to glutathione *S*-transferase at the N-terminus of the β-subunit [13]. Yeast farnesyltransferase is a close homologue and functionally similar to the human enzyme; it is widely used for the evaluation of farnesyltransferase inhibitors. Farnesylpyrophosphate and the dansylated pentapeptide Ds-GlyCysValLeuSer were used as substrates. Upon farnesylation of the cysteine thiol the dansyl residue is placed in a lipophilic environment which, upon excitation at 340 nm, results in an enhancement of fluorescence at 505 nm which is used to monitor the enzyme reaction.

4. Results and discussion

The carboxylic acid derivatives **5–9** of our lead structure turned out to be only very weak inhibitors of the farnesyltransferase (*table I*), thereby it made no differ-

Table I. Inhibitory activity of compounds **5–11**.

Compound	n	m	R	$IC_{50} \pm SD$ (μM)
5	1	–	Me	130 ± 35
6	2	–	H	> 200
7	2	–	Me	130 ± 33
8	3	–	H	120 ± 42
9	3	–	Me	180 ± 45
10	2	14	–	7.8 ± 0.4
11	3	13	–	1.0 ± 0.1

ence whether there was a free carboxyl group or the corresponding methyl ester situated at the terminus of the molecules. Obviously, we lost a lot of binding energy upon the replacement of the thiol by a carboxyl group. Addition of a lipophilic moiety to the terminal carboxyl group, which presumably occupies considerable portions of that region in the farnesyltransferases active site which nomally harbours the farnesyl residue, should result in a regain of at least some of the lost affinity (*figure 1d*). The additional hydrophobic interactions would enhance the overall binding energy. Thus, we would transform our peptidomimetic inhibitors into the so-called bisubstrate analogues, compounds which display molecular features of both the peptidic and the prenylic substrate.

Farnesyltransferase has the uncommon feature of displaying a high affinity to its reaction products, the farnesylated proteins. After the transfer of the farnesyl residue to the cysteine thiol, the farnesylated proteins remain tightly bound to the enzyme. They are only released in a displacement reaction upon binding of another molecule of farnesylpyrophosphate [14]. Bisubstrate inhibitors can also be regarded as analogues of these farnesylated proteins, e.g. as product analogues and therefore are an interesting class of compounds to study.

The two amide derivatives **10** and **11** inhibited farnesyltransferase in the low micromolar range. In the case of **11**, this represents a more than 100-fold improvement in the inhibitory activity of the bisubstrate analogue over the corresponding carboxylic acid non-thiol CAAX-peptidomimetic **8**.

5. Conclusion

We have demonstrated that the transformation of the CAAX-peptidomimetic farnesyltransferase inhibitors carrying a carboxylic acid moiety in place of the original thiol into bisubstrate inhibitors enhances their inhibitory activity by two orders of magnitude. These bisubstrate analogues represent a lead structure for a novel type of non-thiol farnesyltransferase inhibitors whose potential is currently exploited. The results will be reported in due course.

6. Experimental

6.1. Chemistry

^{1}H- and ^{13}C-NMR spectra were recorded on a Jeol JMN-GX-400 and a Jeol JMN-LA-500 spectrometer. Mass spectra were obtained with a Vacuum Generators VG 7070 H using a Vector 1 data acquisition system from Tecnivent or an AutoSpec mass spectrometer from Micromass. IR spectra were recorded on a Nicolet 510P FT-IR-spectrometer. Microanalyses were obtained from a CH analyser according to Dr Salzer from Labormatic and from a Hewlett Packard CHN-analyser type 185. Column chromatography was carried out using silica gel 60 (0.062–0.200 mm) from Merck.

6.1.1. General procedure for compounds **5–9**

To a solution of **4** in hot toluene (50–80 mL) the appropriate carboxylic acid chloride or anhydride dissolved in dioxane (10 mL) was added. The resulting mixture was refluxed for 2 h. After cooling, most of the solvent was removed in vacuo. The resulting solution was kept at 8 °C until crystallization occurred.

6.1.1.1. N-(2-Benzoyl-4-nitrophenyl)-2-(4-methylphenyl)acetic acid amide **3**

To a solution of 2-amino-5-nitrobenzophenone (**2**) (1.2 g, 5 mmol) in hot toluene (50 mL), 2-(4-methylphenyl)acetic acid chloride (0.843 g, 5 mmol) was added. The mixture was refluxed for 2 h. Then most of the solvent was removed in vacuo. The product crystallized upon cooling and was recrystallized from EtOH. Yield: 1.75 g (93%). ^{1}H-NMR ($CDCl_3$): δ = 2.33 (s, 3H), 3.74 (s, 2H), 7.17 (m, 2H), 7.24 (m, 2H), 7.51 (m, 2H), 7.65 (m, 3H), 8.37 (m, 1H), 8.41 (m, 1H), 8.88 (d, *J* = 9 Hz, 1H), 11.05 (s, 1H).

6.1.1.2. N-(4-Amino-2-benzoylphenyl)-2-(4-methylphenyl)acetic acid amide **4**

To a solution of **3** (1.75 g, 4.7 mmol) in hot EtOAc (80 mL) $SnCl_2 \cdot 2H_2O$ (5.35 g) was added. The mixture was refluxed for 2 h and then poured into water (300 mL). The pH of the resulting mixture was adjusted to pH 8 by addition of sat. $NaHCO_3$-solution. The mixture was extracted with EtOAc (3 × 150 mL). The combined organic extracts were washed with brine, dried with $MgSO_4$ and evaporated to dryness. The resulting solid was recrystallized from EtOH. Yield: 1.053 g (65%). ^{1}H-

NMR ($CDCl_3$): δ = 2.25 (s, 3H), 3.55 (s, 2H), 6.66 (d, *J* = 3 Hz, 1H), 6.78 (m, 1H), 7.07 (m, 2H), 7.13 (m, 2H), 7.38 (m, 2H), 7.51 (m, 1H), 7.61 (m, 2H), 8.18 (d, *J* = 9 Hz, 1H), 9.97 (s, 1H).

6.1.1.3. 3-[N-[3-[3-Benzoyl-4-[[2-(4-methylphenyl)acetyl]amino]phenylamino]carbamoyl]acetic acid methyl ester **5**

From **4** (0.688 g, 2 mmol) and malonic acid methylester chloride (0.24 mL, 2.2 mmol). Purification: recrystallization from toluene. Yield: 0.46 g (50%). M.p. 104 °C. IR (KBr): ν = 3 295, 2 955, 1 740, 1 690, 1 660, 1 560 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ = 2.22 (s, 3H), 3.32 (s, 2H), 3.40 (s, 2H), 3.62 (s, 3H), 6.95 (m, 2H), 7.01 (m, 2H), 7.45 (m, 2H), 7.54 (m, 1H), 7.62 (m, 4H), 7.73 (m, 1H), 10.0 (s, 1H), 10.22 (s, 1H). ^{13}C-NMR ($CDCl_3$): δ = 16.7, 38.4, 39.5, 48.0, 116.5, 118.4, 120.4, 124.3, 124.9, 125.0, 125.6, 127.1, 128.1, 128.3, 128.7, 131.1, 131.3, 133.6, 160.1, 164.0, 165.2, 191.1. Anal. $C_{26}H_{24}N_2O_5$ (C, H, N).

6.1.1.4. 3-[N-[3-[3-Benzoyl-4-[[2-(4-methylphenyl)acetyl]amino]-phenylamino]carbamoyl]-propionic acid **6**

From **4** (0.688 g, 2 mmol) and succinic acid anhydride (0.200 g, 2 mmol). Purification: recrystallization from toluene. Yield: 0.880 g (99%). M.p. 158 °C. IR (KBr): ν = 3 330, 2 900–2 600, 1 725, 1 655, 1 560 cm^{-1}. ^{1}H-NMR (DMSO-d_6): δ = 2.19 (s, 3H), 2.43 (m, 4H), 3.51 (s, 2H), 6.93 (m, 2H), 6.98 (m, 2H), 7.42 (m, 2H), 7.50 (m, 1H), 7.59 (m, 4H), 7.70 (m, 1H), 9.90 (s, 1H), 9.93 (s, 1H). ^{13}C-NMR (DMSO-d_6): δ = 20.4, 28.6, 30.9, 42.1, 120.0, 121.9, 124.0, 128.0, 128.6, 128.7, 129.3, 131.3, 132.1, 132.4, 135.2, 135.3, 137.0, 168.8, 170.0, 173.4, 194.9. MS (EI): m/z (%) = 444 (5, M^+), 321 (45), 294 (100), 105 (74). Anal. $C_{26}H_{24}N_2O_5$ (C, H, N).

6.1.1.5. 3-[N-[3-[3-Benzoyl-4-[[2-(4-methylphenyl)acetyl]amino]phenylamino]carbamoyl]-propionic acid methyl ester **7**

From **4** (0.344 g, 1 mmol) and succinic acid methylester chloride (0.20 mL, 1 mmol). Purification: recrystallization from toluene. Yield: 0.348 g (76%). M.p. 144 °C. IR (KBr): ν = 3 375, 2 950, 2 930, 1 735, 1 715, 1 690, 1 635, 1 550 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ = 2.33 (s, 3H), 2.57 (m, 2H), 2.69 (m, 2H), 3.65 (s, 3H), 3.66 (s, 2H), 7.15 (m, 2H), 7.22 (m, 2H), 7.48 (m, 3H), 7.57 (m, 1H), 7.67 (m, 2H), 7.75 (m, 1H), 7.80 (m, 1H), 8.46 (m, 1H), 10.44 (s, 1H). ^{13}C-NMR ($CDCl_3$): δ = 21.1, 29.1, 31.8, 45.0, 52.0, 122.4, 124.2, 124.4, 125.1, 128.3, 129.3, 129.6, 130.0, 131.2, 132.4, 132.7, 135.2, 136.1, 137.0, 138.0, 169.7, 170.3, 173.6, 198.5. MS (EI): m/z (%) = 458 (37, M^+), 321 (55), 294 (100), 212 (47), 105 (100). Anal. $C_{27}H_{26}N_2O_5$ (C, H, N).

6.1.1.6. 3-[N-[3-[3-Benzoyl-4-[[2-(4-methylphenyl)acetyl]amino]-phenylamino]carbamoyl]-butyric acid **8**

From **4** (0.688 g, 2 mmol) and glutaric acid anhydride (0.228 g, 2 mmol). Purification: recrystallization from toluene. Yield: 0.740 g (81%). M.p. 124 °C. IR (KBr): ν = 3 285, 2 900–2 600, 1 735, 1 690, 1 660 cm^{-1}. ^{1}H-NMR (DMSO-d_6): δ = 1.76 (m, 2H), 2.22 (m, 5H), 2.28 (m, 2H), 3.31 (s, 2H), 6.95 (m, 2H), 7.01 (m, 2H), 7.45 (m, 2H), 7.51 (m, 1H), 7.62 (m, 4H), 7.75 (m, 1H), 9.91 (s, 1H), 9.93 (s, 1H), 11.91 (s, 1H). ^{13}C-NMR (DMSO-d_6): δ = 20.2, 20.4, 32.8, 35.2, 42.5, 120.1, 122.0, 124.0, 128.0, 128.6, 128.7, 129.3, 131.3, 132.1, 132.4, 135.2, 135.3, 137.0, 168.8, 170.6, 173.7, 194.3. MS (EI): m/z (%) = 458 (60, M^+), 326 (93), 212 (93), 105 (100). Anal. $C_{27}H_{26}N_2O_5$ (C, H, N).

6.1.1.7. 3-[N-[3-[3-Benzoyl-4-[[2-(4-methylphenyl)acetyl]amino]phenylamino]carbamoyl]-butyric acid methyl ester **9**

From **4** (0.344 g, 1 mmol) and glutaric acid methyl ester chloride (0.17 mL, 1 mmol). Purification: recrystallization from toluene. Yield: 0.310 g (65%). M.p. 106 °C. IR (KBr): ν = 3 300, 3 050, 2 950, 1 740, 1 660, 1 560 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ = 1.90 (m, 2H), 2.25 (s, 3H), 2.27 (m, 4H), 3.56 (s, 3H), 3.59 (s, 2H), 7.08 (m, 2H), 7.15 (m, 2H), 7.40 (m, 3H), 7.50 (m, 1H), 7.61 (m, 3H), 7.78 (m, 1H), 8.38 (m, 1H), 10.38 (s, 1H). ^{13}C-NMR ($CDCl_3$): δ = 20.5, 21.1, 32.9, 36.1, 45.0, 51.6, 122.4, 124.3, 124.4, 125.1, 128.3, 129.3, 129.6, 130.0, 131.1, 132.6, 132.7, 136.0, 137.0, 138.0, 170.3, 170.5, 173.7, 198.5. MS (EI): m/z (%) = 472 (50, M^+), 340 (70), 212 (81), 105 (83), 59 (100). Anal. $C_{28}H_{28}N_2O_5$ (C, H, N).

6.1.1.8. 3-[N-[3-[3-Benzoyl-4-[[2-(4-methylphenyl)acetyl]amino]phenylamino]-carbamoyl]-propionic acid N-pentadecyl amide **10**

To a solution of **6** (458 mg, 1 mmol), pentadecylamine (228 mg, 1 mmol) and PyBOP (520 mg 1 mmol) in dry DMF (20 mL) diisopropylethylamine (0.65 mL) was added. The mixture was stirred at room temperature overnight. Then the mixture was poured into brine (400 mL). The aqueous mixture was extracted with EtOAc (3 × 100 mL) and the combined organic extracts were washed successively with 2 N citric acid, sat. $NaHCO_3$-soln. and brine and dried with $MgSO_4$. The residue obtained after removal of the solvent was purified by flash chromatography on silica gel (EtOAc:n-Hexan 3:2). Yield: 0.50 g (76%). M.p. 105 °C. IR (KBr): ν = 3 300, 3 060, 2 925, 2 850, 1 640, 1 540 cm^{-1}. ^{1}H-NMR ($CDCl_3$):

δ = 0.80 (t, *J* = 7 Hz, 3H), 1.21 (m, 22H), 1.36 (m, 2H), 1.57 (m, 2H), 2.26 (s, 3H), 2.45 (m, 2H), 2.54 (m, 2H), 3.10 (m, 2H), 3.60 (s, 2H), 5.67 (m, 1H), 7.08 (m, 2H), 7.18 (m, 4H), 7.39 (m, 2H), 7.48 (m, 2H), 7.61 (m, 2H), 7.77 (m, 1H), 8.42 (m, 1H), 8.71(s, 1H), 10.41 (s, 1H). ^{13}C-NMR ($CDCl_3$): δ = 14.1, 21.1, 22.7, 26.9, 29.2, 29.3, 29.5, 29.56, 29.62, 29.65, 29.66, 29.67, 31.5, 31.9, 33.0, 39.8, 45.0, 122.3, 124.2, 125.1, 128.3, 129.3, 129.6, 130.0, 131.2, 132.6, 132.7, 136.1, 136.9, 138.1, 170.2, 170.6, 172.2, 198.6. MS (EI): m/z (%) = 653 (1, M^+), 294 (100), 293 (32), 212 (27), 105 (25). Anal. $C_{41}H_{55}N_3O_4$ (C, H, N).

6.1.1.9. 3-[N-[3-[3-Benzoyl-4-[[2-(4-methylphenyl)acetyl]amino]phenylamino]-carbamoyl]-butyric acid N-tetradecyl amide **11**

As described above from **8** (0.460 g, 1.0 mmol) and tetradecylamine (0.220 g, 1.0 mmol). Purification: flash-chromatography on silica gel (EtOAc:n-Hexan 3:2). Yield: 0.325 g (50%). M.p. 109 °C. IR (KBr): ν = 3 300, 3 060, 2 925, 2 855, 1 655, 1 550 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ = 0.81 (t, *J* = 7 Hz, 3H), 1.18 (m, 20H), 1.39 (m, 2H), 1.59 (m, 2H), 1.90 (m, 2H), 2.18 (m, 2H), 2.27 (s, 3H), 2.32 (m, 2H), 3.13 (m, 2H), 3.60 (s, 2H), 5.55 (m, 1H), 7.10 (m, 2H), 7.18 (m, 4H), 7.41 (m, 2H), 7.51 (m, 2H), 7.64 (m, 2H), 7.83 (m, 1H), 8.43 (m, 1H), 10.42 (s, 1H). ^{13}C-NMR ($CDCl_3$): δ = 14.1, 21.1, 22.7, 26.9, 29.2, 29.3, 29.5, 29.56, 29.6, 31.9, 35.0, 36.3, 39.7, 45.0, 122.3, 124.3, 125.1, 128.3, 129.3, 129.6, 130.0, 132.7, 132.8, 138.1, 170.2, 171.1, 172.7, 198.7. Anal. $C_{41}H_{55}N_3O_4$ (C, H, N).

6.2. Biological methods

6.2.1. Enzyme preparation

Yeast farnesyltransferase (FTase) fused to glutathione *S*-transferase at the N-terminus of the β-subunit. FTase was expressed in *Escherichia coli* DH5α grown in LB media containing ampicillin and chloramphenicol for co-expression of pGEX-DPR1 and pBC-RAM2 for FTase production [13]. The enzyme was purified by standard protocol using glutathione-agarose beads for selective binding of the target protein.

6.2.2. Farnesyltransferase assay

The assay was carried out as described [12]. FPP was obtained as ammonium salt solution in methanol: 10 mM aqueous NH_4Cl (7:3) from Sigma-Aldrich. Dansyl-GCVLS was custom synthesized by ZMBH, Heidelberg, Germany. The assay mixture (100 µL volume) contained 50 mM Tris/HCl pH 7.4, 5 mM $MgCl_2$, 10 µM, $ZnCl_2$, 5 mM DTT, 7 µM Ds-GCVLS, 20 µM FPP and approx. 5 nmol yeast GST-FTase [13] and 1% of various concentrations of the test compounds dissolved in DMSO. The progress of the enzyme reaction was followed by 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 thermostatted at 30 °C. Fluorescence emission was recorded with a Perkin Elmer LS50B spectrometer. IC_{50}s were calculated from initial velocity of three independent measurements of typically 4–5 inhibitor concentrations and expressed as mean ± SD.

Acknowledgements

The pGEX-DPR1 and pBC-RAM2 plasmids were kindly provided by Prof. F. Tamanoi (UCLA). M. Schlitzer wishes to thank Ms. K. Burk for technical assistance and the German Pharmaceutical Society for financial support. I. Sattler wishes to thank Prof. Dr S. Grabley and PD Dr R. Thiericke for generous support and Ms. S. Egner for excellent technical assistance.

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