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Selective Inhibition of Type-I Geranylgeranyltransferase In Vitro and in Whole Cells by CAAL Peptidomimetics

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Abstract—In this paper we describe the synthesis of a family of CAAL peptidomimetics as GGTase-I inhibitors. These inhibitors lack the central dipeptide AA in the key CAAL carboxy terminal sequence of geranylgeranylated proteins and are more selective for GGTase-I over FTase. In whole cells, these compounds are very potent inhibitors of the processing of the geranylgeranylated protein Rap1A without affecting the farnesylated protein H-Ras. One derivative, GGTI-298, inhibited cell division by blocking cells in the G_1 phase of the cell cycle.

Introduction

The study of protein isoprenylation has revealed that either a C_{15} farnesyl or C_{20} geranylgeranyl group can be covalently attached to proteins through the thiol group of a cysteine side chain near the carboxylate terminus.[1] Several small G-proteins require this prenylation for proper cellular localization and regulation of signal transduction. Enzymes that catalyze protein prenylation are farnesyltransferase (FTase), type-I geranylgeranyltransferase (GGTase-I), and type-II geranylgeranyltransferase (GGTase-II).[2–4] FTase catalyzes the farnesylation of proteins with a CAAX sequence at their carboxylate terminus where C is cysteine, A is an aliphatic amino acid and X is methionine, serine, glutamine, alanine, or cysteine.[1] Like FTase, GGTase-I catalyzes the geranylgeranylation of proteins terminating with CAAX sequences where X is restricted to leucine, isoleucine or to a lesser extent, phenylalanine.[5] In contrast to FTase and GGTase-I, GGTase-II modifies proteins with CC or CXC sequences at the carboxylate terminus.[6]

So far, the major effort in designing prenyltransferase inhibitors has been targeted to FTase, an enzyme that catalyzes the farnesylation of oncogenic Ras, in the hope of developing new classes of anti-tumor agents.[7] However, the majority of prenylated proteins are geranylgeranylated.[8] In cells, the number of geranylgeranylated proteins is at least 5 to 10 times more than farnesylated proteins[1]. Proteins modified by GGTase-I include Rap1A (CLLL), Rap1B (CQLL), Rac1 (CLLL), Rac2 (CSLL), G25K (CCIF), and RhoA (CLVL).[9] Recently, it was shown that the geranylgeranylated proteins Rho, Rac, and Cdc42 played an essential role in cell cycle progression and mitosis.[10] Therefore, it is important to obtain specific GGTase-I inhibitors and apply them as probes to study the effects of protein geranylgeranylation in normal and oncogenic cell growth.

The design of GGTase-I inhibitors can be approached by mimicking the protein substrate at the C-terminal sequence (CAAL) or by mimicking the isoprenyl substrate geranylgeranylpyrophosphate (GGPP). GGPP analogs have been reported recently to inhibit geranylgeranylation of Rap in cell cultures.[11] In this paper we report the design of a novel series of peptidomimetic inhibitors of GGTase-I and show them to be effective at selectively blocking Rap1A processing in whole cells.

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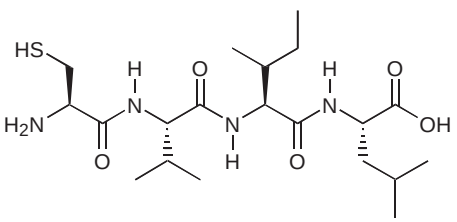
Synthesis and Biological Assay

Peptidomimetics **2a** and **2b** were synthesized using a similar approach to that described previously.[12] The coupling of 4-N-Boc-aminobenzoic acid with L-leucine methyl ester gave the 4-N-Boc-aminobenzoylleucine methyl ester. Deprotection of the Boc group and subsequent reductive amination with N-Boc-S-trityl-L-cysteinal gave the fully protected derivatives which were then deprotected to give **2a** and **2b**. Compounds **3a** and **3b** were synthesized from 4-nitro-2-phenylbenzoic acid.[13] The coupling of this carboxylic acid with L-leucine methyl ester in the presence of EDCI and HOBT provided the corresponding amide. The nitro group was reduced by hydrogenation and the amine was reacted with N-Boc-S-trityl-L-cysteinal in the presence of sodium cyanoborohydride. The fully protected compounds were deprotected by first treatment with aqueous LiOH/THF and then trifluoroacetic acid and triethylsilane. The crude product was purified by preparative HPLC (C-18 column, linear gradient within forty minutes from 5% acetonitrile to 60% acetonitrile in aqueous solution containing 0.1% trifluroacetic acid) to give **3a**. Compound **3b** was prepared by deprotection of the fully protected compound using trifluoroacetic acid and triethylsilane. Compounds **4a** and **4b** were prepared using an analogous route except that the 4-nitro-2-(1-naphthyl)benzoic acid was used as the spacer. The proton and carbon NMR data of **4a** and **4b** showed two isomers due to the restricted rotation of the aryl–aryl bond on the NMR time scale. Similarly, compounds **5** and **6** were prepared by using, respectively, the methyl esters of isoleucine and norleucine in place of leucine methyl ester. The purity of all final compounds was checked by analytical HPLC and structures were consistent with ^{1}H NMR, ^{13}C NMR, high resolution mass spectra and microanalytical data.

The in vitro inhibition assays of GGTase-I and FTase were carried out by measuring the [^{3}H]-GGPP and [^{3}H]-FPP incorporated into H-Ras-CVLL and H-Ras-CVLS, respectively, using the same procedure described before (selected data are shown in Figure 2).[12] The in vivo inhibition of geranylgeranylation and farnesylation was determined based on the level of inhibition of Rap1A and H-Ras processing, respectively.[14] Briefly, cells were treated with various concentrations of peptidomimetics and the cell lysates were separated on 12.5% SDS-PAGE. The separated proteins were transferred to nitrocellulose and immunoblotted using an anti-Ras antibody (Y-13-258) or an anti-Rap1A antibody (SC-65). Antibody reactions were visualized using either peroxidase-conjugated goat anti-rat IgG or goat anti-rabbit IgG and an enhanced chemiluminescene detection system.

Results and Discussion

It has been shown that the simple tetrapeptide Cys-Val-Ile-Leu **1** has a similar potency in inhibiting FTase (IC_{50} = 17 μM) and GGTase-I (IC_{50} = 13 μM), and that



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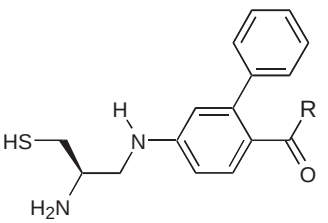
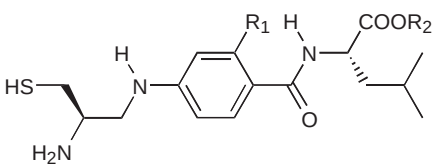


5: R = L-isoleucine: GGTI-2115.

6: R = L-norleucine: GGTI-2117.



2a: R_1 = H, R_2 = H: GGTI-279.

2b: R_1 = H, R_2 = CH_3: GGTI-280.

3a: R_1 = phenyl, R_2 = H: GGTI-287.

3b: R_1 = phenyl, R_2 = CH_3: GGTI-286.

4a: R_1 = 1-naphthyl, R_2 = H: GGTI-297.

4b: R_1 = 1-naphthyl, R_2 = CH_3: GGTI-298.

Figure 1. Synthesized peptidomimetics as GGTase-I inhibitors.

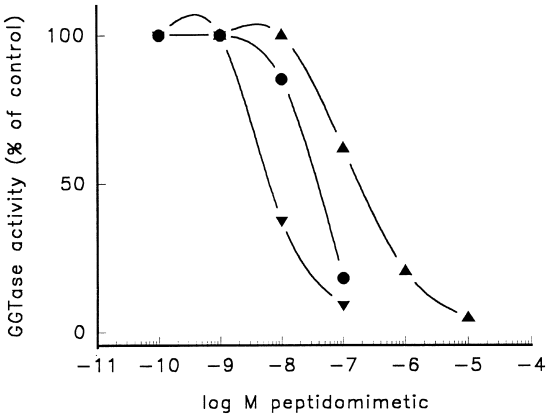


Figure 2. GGTase-I inhibition studies based on ability of peptidomimetics to inhibit the transfer of [^{3}H]geranylgeranyl to Ha-Ras-CVLL (▲, **2a**; ▼, **3a**; ●, **4a**; ◆, **5**).[12]

reduction of the cysteine amide bond in **1** can increase the selectivity for GGTase-I ($IC_{50} = 1.9$ nM) over FTase ($IC_{50} = 550$ nM)[15]. In our earlier studies of FTase inhibitors, we proposed that the central dipeptide AA in the tetrapeptide CAAX might contribute to the hydrophobic interaction in the enzyme substrate binding site and the amide bond might play no important role[16]. Since GGTase-I is closely related to FTase and the two enzymes share the same α subunit, we applied a similar strategy to the design of GGTase-I inhibitors. The leucine residue in tetrapeptide **1** was retained to maintain the selectivity for GGTase-I. The dipeptide AA in **1** was replaced with a series of aromatic hydrophobic spacers and the cysteine amide bond was reduced to an amine (compounds **2**–**4**). Since CAAX tetrapeptides with an isoleucine at the X position are also preferred substrates for GGTase-I, compounds **5** and **6** were prepared where an isoleucine and a norleucine replaced the leucine residue. The structures of the synthesized inhibitors are shown in Figure 1.

We first determined the ability of peptidomimetics **2**–**6** to inhibit the incorporation of GGPP and FPP into the geranylgeranylated protein H-Ras-CVLL (Figure 2) and the farnesylated protein H-Ras-CVLS, respectively. Table 1 shows that compounds GGTI-279, GGTI-287 and GGTI-297 are more potent inhibitors of GGTase-I than FTase, with selectivities between three- and four-fold. Interestingly, when the leucine residue in GGTI-287 was replaced with an isoleucine or norleucine, the selectivity for GGTase-I over FTase decreased. The IC_{50} values for GGTI-279 are 135 nM for GGTase-I and 418 nM for FTase. Hydrophobic functionalization of the aromatic spacer improved the inhibition potency significantly. Peptidomimetic GGTI-287 has IC_{50} values of 7.3 nM and 21 nM for GGTase-I and FTase, respectively. When compared to tetrapeptide **1**, the inhibition potency of the CAAX peptidomimetics was improved between 100- and 2600-fold.

These results demonstrated the success of the hydrophobic spacer strategy in our peptidomimetic design. Table 1 also showed that the leucine residue in our peptidomimetics is critical for selectivity for GGTase-I. However, this selectivity is not as high as for our previously reported FTase inhibitors. When the leucine residue in GGTI-287 was replaced with a methionine, the compound (FTI-276) became an FTase selective inhibitor, with IC_{50} values for FTase and GGTase-I of 0.5 and 50 nM, respectively.[13] Nonetheless, the selectivity for GGTase-I inhibition over FTase is still improved when compared to the parent tetrapeptide **1**. The methyl esters GGTI-280 and GGTI-286 showed a 50-fold decrease in GGTase-I inhibition potency when compared to their free carboxylic acid forms GGTI-279 and GGTI-287. This result suggested that a free carboxylic acid is required for the potent inhibition of GGTase-I. Interestingly, GGTI-280 and GGTI-286 showed the reverse selectivity in the in vitro studies, suggesting that the requirement of a free carboxylate is more stringent for GGTase-I as compared to FTase. However, in whole cells the methyl ester derivatives were more selective for GGTase-I over FTase.

We next determined the ability of these peptidomimetics to inhibit the processing of Rap1A and H-Ras in whole cells. Treatment of NIH-3T3 cells that overexpress an activated H-Ras oncoprotein with GGTI-286 resulted in inhibition of processing of the exclusively

Table 1. Inhibition of GGTase-I and FTase in vitro by CAAX peptidomimetics

Peptidomimetics	GGTase-I inhibition IC_{50}(nM) ± SEM(n)	FTase inhibition IC_{50}(nM) ± SEM(n)
2a GGTI-279	135 ± 36(4)	418 ± 184(4)
2b GGTI-280	6733 ± 2311(3)	897 ± 183(3)
3a GGTI-287	7.3 ± 2.6(5)	21 ± 11(4)
3b GGTI-286	240 ± 65(3)	183 ± 104(3)
4a GGTI-297	56 ± 20(10)	203 ± 71(6)
5 GGTI-2115	32 ± 6(3)	56 ± 23(3)
6 GGTI-2117	41 ± 10(3)	47 ± 13(3)

geranylgeranylated protein Rap1A with an IC_{50} value of 2 μM.[14] In contrast, inhibition of the processing of the exclusively farnesylated protein H-Ras was less pronounced with an IC_{50} of 10 μM. Substitution of the phenyl group in GGTI-286 by a naphthyl group, as in GGTI-298, increased the selectivity towards GGTase-I over FTase in whole cells. GGTI-298 inhibited the processing of Rap1A with an IC_{50} of 3 μM and had no effect on the processing of H-Ras at concentrations as high as 15 μM. At 20 μM of GGTI-298, H-Ras processing was inhibited by 10%. Higher concentrations of GGTI-298 were toxic to cells. Even though GGTI-287 is more potent in vitro than GGTI-297, in whole cells the corresponding methyl ester derivatives GGTI-286 and GGTI-298 are equipotent suggesting that GGTI-298 is more cell permeable or a better substrate for intracellular esterases. Furthermore, in whole cells GGTI-298 is more selective than GGTI-286 even though in vitro the corresponding free carboxylate derivatives, GGTI-297 and GGTI-287 have similar selectivities (Table 1). In addition, GGTI-298 and GGTI-286 are not selective for GGTase-I over FTase in vitro. These data coupled with the selectivity results in whole cells suggest that the methyl groups are cleaved in the cytosol to the active free carboxylate forms.

Recently, we have investigated the effects of inhibiting protein geranylgeranylation but not farnesylation on receptor tyrosine kinase signaling and cell cycle progression. We found that treatment of NIH-3T3 cells with GGTI-298 (10 μM) inhibited PDGF- and EGF-stimulated tyrosine phosphorylation of their receptor tyrosine kinases.[17] Furthermore, GGTI-298 was also extremely effective at blocking cells in the G_1 phase of the cell cycle at concentrations that had little effect on protein farnesylation in whole cells.[18] These results clearly demonstrate the usefulness of CAAL peptidomimetics as powerful tools for determining the role of geranylgeranylated proteins in early events in growth factor signal transduction as well as later events in the proliferation process, such as cell cycle progression.

Experimental

Nuclear magnetic resonance spectra were acquired using Bruker AM-300 series spectrometers (300 MHz for ^1H, 75 MHz for ^{13}C). Elemental analysis were performed by Atlantic Microlabs, Inc., Norcross, GA. Optical rotations $[\alpha]^{25}_D$ were measured using a Perkin–Elmer 241 polarimeter. All synthesized final compounds (amine hydrochloride or amine trifluoroacetate salts) were checked for purity by analytical high pressure liquid chromatography which was performed using a Rainin HP controller and a Rainin UV-C detector with a Rainin 250×4.6 mm, 5 μm Microsorb C18 column. Preparative HPLC was performed on a Waters 600E controller and a Waters 490E multi-wavelength UV detector with a 25×10 cm Delta-Pak C-18 300 Å cartridge column inside a Waters 25×10 cm Radial Compression Module. Solvents consisting of 20 to 80% acetonitrile and 0.1% TFA in water were used with a flow rate of 15 mL/min in 40 min.

***N*-4-[2(*R*)-Amino-3-mercaptopropyl]aminobenzoyl]-(L)-leucine hydrochloride (2a).** N-Boc-4-aminobenzoic acid (from Boc protection of 4-aminobenzoic acid) was coupled with L-leucine methyl ester to give N-Boc-4-aminobenzoyl-leucine methyl ester (90%). mp 185–186 °C. ^1H NMR ($CDCl_3$) δ 7.72 (d, J=8.7 Hz, 2H), 7.43 (d, J=8.7 Hz, 2H), 6.68 (s, 1H, Boc amide), 6.45 (d, J=8.2 Hz, 1H, amide), 4.83 (ddd, J=5.0, 8.2 Hz, 1H, Leu α H), 3.76 (s, 3H, OCH_3), 1.63–1.77 (m, 3H, Leu), 1.52 (s, 9H, Boc), 0.97 (dd, J=6.0 Hz, 6H).

The above compound was deprotected to remove the Boc group by gaseous hydrochloride in methanol and the resulting hydrochloride salt was treated with N-Boc-S-trityl-L-cysteinal (1.0 equiv) in the presence of sodium cyanoborohydride (1.5 equiv) to give a reductive amination product (68%). mp 86 °C (decomp). ^1H NMR ($CDCl_3$) δ 7.62 (d, J=8.6 Hz, 2H, aryl), 7.40–7.44 (m, 6H, trityl), 7.20–7.32 (m, 9H, trityl), 6.47 (d, J=8.7 Hz, 2H, aryl), 6.31 (d, J=8.2 Hz, 1H, amide), 4.84 (ddd, J=5.0, 8.2 Hz, 1H, Leu α H), 4.57 (br d, J=7.5 Hz, 1H, Boc amide), 4.15 (br, 1H, NH), 3.81 (m, 1H, Cys α H), 3.75 (s, 3H, OCH_3), 3.10 (t, J=5.9 Hz, 2H, CH_2N), 2.46 (d, J=5.2 Hz, 2H, CH_2S), 1.68–1.75 (m, 2H, Leu), 1.63 (t, J=6.8 Hz, 1H, Leu), 1.43 (s, 9H, Boc), 0.96 (dd, J=6.0 Hz, 6H); Anal. calcd for $C_{41}H_{49}O_5N_3S{\cdot}0.4CH_2Cl_2$: C 68.15, H 6.83, N 5.76; Found C 68.16, H 6.96, N 5.81.

The fully protected compound from above was first treated with aqueous LiOH-THF and then deprotected by TFA in the presence of triethylsilane. The resulting TFA salt was converted to a hydrochloride salt by first dissolving the salt in 1.7 N HCl in acetic acid and then adding 3 N HCl in ether to precipitate the product (58%). mp 145 °C (foaming). $[\alpha]^{25}_D$ −60.7 (*c* 0.4, MeOH). ^1H NMR (CD_3OD) δ 7.74 (d, J=8.5 Hz, 2H, aryl), 6.77 (d, J=8.7 Hz, 2H, aryl), 4.63 (dd, J=4.5, 8.5 Hz, 1H, Leu α H), 3.43–3.59 (m, 3H, Cys α H, CH_2N), 2.94 (dd, J=4.6, 14.5 Hz, 1H, CH_2SH), 2.83 (dd, J=5.4, 14.5 Hz, 1H, CH_2SH), 1.64–1.82 (m, 3H, Leu), 0.96 (dd, J=6.1 Hz, 6H); LRMS (EI) for $C_{16}H_{25}O_3N_3S$ 339 (M^+, 15), 263 (80), 133 (100); HRMS (EI) calcd 339.1616, obsd 339.1602.

***N*-4-[2(*R*)-Amino-3-mercaptopropyl]aminobenzoyl-(L)-leucine methyl ester hydrochloride (2b).** This compound was prepared by first treating the fully protected reduc-

tive amination product (406 mg) with mercuric chloride and then bubbling hydrogen sulfide to precipitate the mercuric sulfide. Final product was isolated as a hydrochloride salt (168 mg, 68%). mp 105 °C (foaming). ^{1}H NMR (CD_3OD) δ 7.71 (d, J = 8.7 Hz, 2H, aryl), 6.75 (d, J = 8.7 Hz, 2H, aryl), 4.64 (dd, J = 4.7, 8.8 Hz, 1H, Leu α H), 3.71 (s, 3H, OCH_3), 3.42–3.58 (m, 3H, Cys α H, CH_2N), 2.95 (dd, J = 4.5, 14.5 Hz, 1H, CH_2SH), 2.81 (dd, J = 5.3, 14.5 Hz, 1H, CH_2SH), 1.65–1.81 (m, 3H, Leu), 0.96 (dd, J = 6.1 Hz, 6H); LRMS (EI) for $C_{17}H_{27}N_3O_3S$ 353 (M^+, 20), 277 (90), 133 (100); HRMS (EI) calcd 353.1773, obsd 353.1781. Anal. calcd for $C_{17}H_{27}O_3N_3S$·1.5HCl: C 50.03, H 6.98, N 10.30, S 7.84; Found: C 49.73, H 7.16, N 10.27, S 7.72.

***N*-4-[2(*R*)-Amino-3-mercaptopropyl]amino-2-phenylbenzoyl-(L)-leucine trifluoroacetate (3a, GGTI-287).** The coupling of 4-nitro-2-phenylbenzoic acid with L-leucine methyl ester hydrochloride by using EDCI and HOBT gave 4-nitro-2-phenylbenzoyl-leucine methyl ester (96%). mp 101–102 °C. ^{1}H NMR ($CDCl_3$) δ 8.24–8.26 (m, 2H), 7.86 (d, J = 8.7 Hz, 1H), 7.41–7.46 (m, 5H), 5.71 (d, J = 7.4 Hz, 1H, amide), 4.57 (ddd, J = 5.0, 7.4 Hz, 1H, Leu α H), 3.67 (s, 3H, OCH_3), 1.37–1.46 (m, 1H, Leu), 1.08–1.25 (m, 2H, Leu), 0.78 (dd, J = 5.0 Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 172.6, 167.1, 148.4, 141.2, 140.7, 137.8, 130.0, 128.9, 128.7, 128.5, 125.1, 122.2, 52.3, 51.0, 41.1, 24.4, 22.6, 21.7.

Reduction of the above nitro compound followed by reductive amination with N-Boc-S-trityl-L-cysteinal gave N-[4-[2(*R*)-*tert*-butoxycarbonylamino-3-(triphenylmethyl)-thiopropyl]amino-2-phenylbenzoyl]-leucine methyl ester (61.3%). mp 82 °C (decomp). ^{1}H NMR ($CDCl_3$) δ 7.68 (d, J = 8.6 Hz, 1H), 7.33–7.41 (m, 11H), 7.17–7.29 (m, 9H), 6.50 (d, J = 8.6 Hz, 1H, aryl), 6.31 (s, 1H, aryl), 5.43 (d, J = 7.8 Hz, 1H, amide), 4.60 (d, J = 6.1 Hz, 1H, Boc amide), 4.47 (ddd, J = 5.0, 8.2 Hz, 1H, Leu α H), 4.19 (br t, 1H, NH), 3.77 (br m, 1H, Cys α H), 3.62 (s, 3H, OCH_3), 3.09 (t, J = 5.9 Hz, 2H, CH_2N), 2.45 (br m, 2H, CH_2S), 1.40 (s, 9H, Boc), 1.27–1.33 (m, 1H, Leu), 1.03–1.18(m, 2H, Leu), 0.75 (dd, J = 6.0 Hz, 6H); ^{13}CNMR ($CDCl_3$) δ 173.2, 168.2, 155.6, 149.4, 144.4, 141.7, 141.2, 131.4, 129.5, 128.8, 128.5, 127.9, 127.6, 126.8, 122.7, 113.6, 111.3, 79.6, 67.1, 51.9, 50.9, 49.5, 47.1, 41.2, 34.3, 28.3, 24.4, 22.7, 21.8; Anal. calcd for $C_{47}H_{53}O_5N_3S$·0.6CH_2Cl_2: C 69.48, H 6.59, N 5.11, S 3.89; Found C 69.45, H 6.73, N 5.26, S 3.87.

The above fully protected compound was first deprotected by aqueous LiOH-THF then by TFA. The crude TFA salt was purified by preparative HPLC to give GGTI-287 (56%). mp 115 °C (decomp). $[\alpha]^{25}_D$ −1.8 (*c* 0.3, MeOH). ^{1}H NMR (CD_3OD) δ 7.42 (d, J = 8.5 Hz, 1H, aryl), 7.29–7.38 (m, 5H, phenyl), 6.73 (d, J = 8.5 Hz, 1H, aryl), 6.66 (s, 1H, aryl), 4.32 (dd, J = 4.3, 5.9 Hz, 1H, Leu α H), 3.41–3.57 (m, 3H, CH_2N, Cys α H), 2.94 (dd, J = 4.3, 14.5 Hz, 1H, CH_2SH), 2.78 (dd, J = 5.2, 14.5 Hz, 1H, CH_2SH), 1.45 (t, J = 6.7 Hz, 2H, Leu), 1.17–1.26 (m, 1H, Leu), 0.78–0.83 (t, J = 8.5 Hz, 6H); ^{13}CNMR (CD_3OD) δ 176.0, 173.0, 150.8, 143.4, 142.4, 131.1, 129.7, 129.4, 128.4, 125.9, 115.3, 111.9, 53.8, 52.5, 44.9, 41.3, 25.6, 25.2, 23.4, 21.7; Anal. calcd for $C_{22}H_{29}O_3N_3S$·CF_3COOH·H_2O: C 52.65, H 5.85, N 7.67, S 5.85; Found C 52.60, H 5.90, N 7.62, S 5.79.

***N*-4-[2(*R*)-Amino-3-mercaptopropyl]amino-2-phenylbenzoyl-(L)-leucine methyl ester hydrochloride (3b, GGTI-286).** The fully protected precursor (described in the preparation of GGTI-287) was deprotected by TFA in the presence of triethylsilane. The resulting TFA salt was dissolved in 1.7 N gaseous HCl in acetic acid and then 3 N HCl in ether was added. The final compound was isolated as a hydrochloride salt. Purity (> 95%) was checked by analytical HPLC at both 220 nm and 285 nm. mp 105 °C (foaming). $[\alpha]^{25}_D$ −22.7 (*c* 0.1, MeOH). ^{1}H NMR (CD_3OD) δ 7.42 (d, J = 8.5 Hz, 1H, aryl), 7.31–7.38 (m, 5H, phenyl), 6.76 (d, J = 8.5 Hz, 1H, aryl), 6.68 (s, 1H, aryl), 4.33 (t, J = 7.8 Hz, 1H, Leu α H), 3.67 (s, 3H, OCH_3), 3.46–3.55 (m, 3H, Cys σ H, CH_2N), 2.95 (dd, J = 4.4, 14.5 Hz, 1H, CH_2S), 2.81 (dd, J = 5.1, 14.5 Hz, 1H, CH_2S), 1.44 (t, J = 7.6 Hz, 2H, Leu), 1.18–1.25 (m, 1H, Leu), 0.76–0.83 (dd, J = 5.1, 6.6 Hz, 6H, $2CH_3$); ^{13}C NMR (CD_3OD) δ 174.5, 173.2, 150.8, 143.5, 142.4, 131.1, 129.7, 129.4, 128.5, 125.7, 115.5, 112.1, 53.8, 52.7, 52.6, 44.9, 41.1, 25.6, 25.3, 23.3, 21.7; LRMS (EI) for $C_{23}H_{31}O_3N_3S$ 429 (M, 12), 353 (80), 285 (55), 209 (95), 180 (100); HRMS (EI) calcd 429.2086, obsd 429.2088.

***N*-4-[2(*R*)-Amino-3-mercaptopropyl]amino-2-naphthylbenzoyl-(L)-leucine hydrochloride (4a, GGTI-297).** Coupling of 4-nitro-2-naphthylbenzoic acid (prepared from the coupling of 2-bromo-4-nitrobenzoic acid methyl ester with 1-naphthylboronic acid and the sponification of the ester) with L-leucine methyl ester gave 4-nitro-2-naphthylbenzoyl-leucine methyl ester (93%). mp 144–145 °C. ^{1}H NMR ($CDCl_3$) δ 8.34–8.39 (m, 1H), 8.25 (s, 1H), 8.18 (d, J = 8.6 Hz, 0.5H), 8.02 (d, J = 8.6 Hz, 0.5H), 7.91–8.00 (m, 2H), 7.62 (t, J = 7.0 Hz, 0.5H), 7.48–7.58 (m, 3H), 7.41 (t, J = 7.0 Hz, 1.5H), 5.71 (d, J = 7.9 Hz, 0.5H, amide), 5.60 (d, J = 7.9 Hz, 0.5H, amide), 4.29 (m, 1H, Leu α H), 3.57 (s, 1.5H, OCH_3), 3.52 (s, 1.5H, OCH_3), 1.05–1.11 (m, 0.5H), 0.88–0.97 (m, 0.5H), 0.69–0.78 (m, 0.5H), 0.41–0.59 (m, 7H), 0.19–0.26 (m, 0.5H). The complex ^{1}H NMR is due to the restricted aryl–aryl bond rotation.

The above compound was first reduced to the amine by hydrogenation and then treated with N-Boc-S-trityl-L-cysteinal in the presence of $NaB(CN)H_3$ to give *N*-[4-[2(*R*)-*tert*-butoxycarbonylamino-3-(triphenylmethyl)-

thiopropyl]amino-2-naphthylbenzoyl]-leucine methyl ester (75%). mp 91 °C (decomp). ^{1}H NMR ($CDCl_3$) δ 7.85–8.00 (m, 3H), 7.47–7.67 (m, 4H), 7.39–7.43 (m, 7H), 7.14–7.37 (m, 9H), 6.61 (d, J = 8.6 Hz, 1H, aryl), 6.32 (s, 1H, aryl), 5.46 (d, J = 7.6 Hz, 0.6H, amide), 5.36 (d, J = 7.6 Hz, 0.4H, amide), 4.55 (d, J = 7.2 Hz, 1H, Boc amide), 4.20–4.27 (m, 2H, Leu α H, NH), 3.76 (br, 1H, OCH_3), 3.56 (s, 2H, OCH_3), 3.38 (br, 1H, Cys α H), 3.06 (t, J = 5.9 Hz, 2H, CH_2N), 2.43 (m, 2H, CH_2S), 1.36–1.43 (m, 9H, Boc), 0.81–1.03 (m, 1H), 0.55–0.67 (m, 2.8H), 0.36–0.45 (m, 4.7H), 0.00–0.09 (m, 0.6H); Anal. calcd for $C_{51}H_{55}O_5N_3S{\cdot}0.6CH_2Cl_2$: C 71.09, H 6.44, N 4.81; Found C 70.71, H 6.51, N 4.75.

The above fully protected compound was deprotected as described before. The final product GGTI-297 was isolated as a hydrochloride salt (77%). mp 160–162 °C (decomp). $[\alpha]^{25}_D$ +18.9 (*c* 0.8, MeOH). ^{1}H NMR (CD_3OD) δ 7.86–7.92 (m, 2H), 7.73 (d, J = 8.5 Hz, 0.6H), 7.35–7.67 (m, 5.4H), 6.89 (m, 1H), 6.67 (m, 1H), 4.06 (dd, J = 4.6, 5.5 Hz, 0.4H), 4.00 (dd, J = 4.6, 5.5 Hz, 0.6H), 3.71–3.82 (m, 0.6H), 3.44–3.58 (m, 2.4H), 2.73–2.96 (m, 2H), 1.14–1.29 (m, 0.5H), 0.97–1.10 (m, 1H), 0.69–0.78 (m, 1H), 0.59 (d, 1.2H), 0.44–0.49 (m, 2.8H), 0.31 (m, 1.9H), 0.19–0.25 (m, 0.5H); LRMS for $C_{26}H_{31}O_3N_3S$ 465 (M^+, 8), 389 (65), 259 (90), 246 (100); HRMS calcd 465.2086, obsd 465.2083.

***N*-4-[2(*R*)-Amino-3-mercaptopropyl]amino-2-naphthylbenzoyl-(L)-leucine methyl ester hydrochloride (4b, GGTI-298).** The fully protected product from reductive amination reaction was deprotected by TFA. The TFA salt was extracted with concentrated $NaHCO_3$ and methylene chloride. After evaporation of solvents, a waxy product was obtained (85%). ^{1}H NMR ($CDCl_3$) δ 7.98 (d, J = 8.5 Hz, 0.6H), 7.84–7.90 (m, 2.4H), 7.65 (d, J = 8.5 Hz, 0.4H), 7.43–7.58 (m, 3.6H), 7.34–7.39 (m, 1H), 6.72 (m, 1H), 6.45 (m, 1H), 5.46 (d, J = 7.8 Hz, 0.6H, amide), 5.40 (d, J = 7.7 Hz, 0.4H, amide), 4.64 (br, 1H, aryl NH), 4.23 (m, 1H, Leu α H), 3.54 (s, 2H, OCH_3), 3.30 (s, 1H, OCH_3), 3.25 (m, 1H, Cys α H), 2.97–3.06 (m, 2H, CH_2N), 2.67 (dd, J = 4.7, 13.1 Hz, 1H, CH_2S), 2.47 (dd, J = 6.5, 13.2 Hz, 1H, CH_2S), 1.45–1.65 (br, 2H, NH_2), 0.81–1.03 (m, 1H), 0.54–0.67 (m, 3H), 0.36–0.39 (m, 4.3H), 0.00–0.10 (m, 0.7H).

The above free amine was converted to a hydrochloride salt by first dissolving the amine in methylene chloride and then addition of 3 N gaseous HCl in ether to give compound GGTI-298 (72%). mp 103 °C (foaming). $[\alpha]^{25}_D$ +11.1 (*c* 0.4, MeOH). ^{1}H NMR (CD_3OD) δ 7.87–7.93 (m, 2H), 7.70 (d, J = 8.5 Hz, 0.6H), 7.62 (m, 1.2H), 7.45–7.59 (m, 3H), 7.34–7.42 (m, 1.2H), 6.88 (m, 1H), 6.67 (m, 1H), 4.11 (dd, J = 4.8, 5.4 Hz, 0.4H, Leu α H), 4.00 (dd, J = 4.6, 5.7 Hz, 0.6H, Leu α H), 3.71–3.78 (m, 0.6H), 3.46–3.59 (m, 5.4H, OCH_3, CH_2N, Cys α H), 2.93 (dd, J = 4.1, 14.8 Hz, 1H, CH_2S), 2.80 (dd, J = 4.3, 14.4 Hz, 1H, CH_2S), 0.96–1.26 (m, 1.5H), 0.72–0.82 (m, 1H), 0.59 (d, 1.2H), 0.55 (d, 2.8H), 0.34 (d, 1.9H), 0.23–0.32 (m, 0.6H); LRMS (EI) 479 (M^+, 15), 403 (50), 259 (100); HRMS (EI) calcd for $C_{27}H_{33}O_3N_3S$ 479.2243, obsd 479.2237. Anal. calcd for $C_{27}H_{33}O_3N_3S.1.8HCl$: C 59.48, H 6.38, N 7.71; Found C 59.15, H 6.59, N 7.54.

***N*-4-[2(*R*)-Amino-3-mercaptopropyl]amino-2-phenylbenzoyl-(L)-isoleucine trifluoroacetate (5).** This compound was prepared in the same way as the preparation of GGTI-287 except that the leucine was replaced with the isoleucine. The reductive amination gave the fully protected compound (62%). ^{1}H NMR ($CDCl_3$) δ 7.46 (d, J = 8.5 Hz, 1 H, aryl), 7.22–7.16 (m, 11 H), 7.10–6.97 (m, 9 H), 6.32 (d, J = 8.6 Hz, 1 H, aryl), 6.12 (s, 1 H, aryl), 5.42 (d, J = 8.1 Hz, 1 H, amide NH), 4.33 (m, 1 H, Boc-amide), 4.25 (dd, J = 4.9 Hz, 1 H, Ile-αH), 3.55 (br m, 1 H, cyst-αH), 3.38 (s, 3 H, OCH_3), 2.87 (d, J = 6.2 Hz, 2 H, CH_2N), 2.20 (br m, 3 H, NH and CH_2S), 1.23 (m, 1 H, Ile-CH), 1.15 (s, 9 H, Boc), 1.05 (m, 1 H, Ile), 0.92 (m, 1 H, Ile), 0.54 (m, 3 H, Ile-CH_3), 0.38 (d, J = 6.7 Hz, 3 H, Ile-CH_3). The deprotection of this compound gave the final product (47%). mp 101–105 °C. ^{1}H NMR (DMSO-d_6) δ 8.01 (br s, 3 H, NH_3), 7.61 (d, J = 8.6 Hz, 1 H, aryl), 7.32 (m, 6 H, phenyl and amide), 6.66 (d, J = 8.2 Hz, 1 H, aryl), 6.57 (s, 1 H, aryl), 6.27 (br s, 1 H, aryl NH), 4.07 (t, J = 3.3 Hz, 1 H, Ile-αH), 2.89–2.79 (m, 4 H, CH_2N and CH_2S), 1.74 (m, 1 H, Ile), 1.26 (m, 1 H, Ile), 0.89 (m, 1 H, Ile), 0.81 (m, 6 H, Ile); ^{13}C NMR (DMSO-d_6) δ 175.3, 170.9, 152.5, 147.4, 139.1, 125.4, 124.7, 123.5, 122.4, 121.4, 110.3, 109.3, 52.0, 51.4, 50.1, 28.7, 27.2, 21.4, 14.3, 13.7.

***N*-4-[2(*R*)-Amino-3-mercaptopropyl]amino-2-phenylbenzoyl-(L)-norleucine trifluoroacetate (6).** This compound was prepared in the same way as the preparation of GGTI-287 except that the leucine was replaced with the norleucine. The reductive amination gave the fully protected compound (58%). ^{1}H NMR ($CDCl_3$) δ 7.67 (d, J = 8.2 Hz, 1 H, aryl), 7.34–7.30 (m, 11 H, aryl), 7.28–7.22 (m, 9 H, aryl), 6.93 (d, J = 8.1 Hz, 1 H, aryl), 6.45 (s, 1 H, aryl), 5.59 (d, J = 8.4 Hz, 1 H, amide NH), 4.41 (br, 1 H, Boc-amide), 4.19 (ddd, J = 4.7 Hz, 8.1 Hz, 1 H, Nleu-α H), 3.37 (s, 3 H, OCH_3), 3.21 (br m, 2 H, cysteine α H and aryl NH), 3.01 (d, J = 6.2 Hz, 2 H, CH_2N), 2.71 (m, 2 H, CH_2S), 1.97–1.83 (m, 2 H, Nleu CH_2), 1.36–1.27 (m, 4 H, Nleu-CH_2), 0.97 (t, J = 8.0 Hz, 3 H, CH_3). The deprotection of this compound gave the final product (51%). mp 95–98 °C. ^{1}H NMR (DMSO-d_6) δ 12.37 (br s, 1 H, COOH), 8.15 (br s, 3 H, NH_3), 7.86 (d, J = 7.5 Hz, 1 H, aryl), 7.86–7.29 (m, 6 H, phenyl and amide), 6.67 (d, J = 8.4 Hz, 1 H, aryl), 6.58 (s, 1 H, aryl), 6.31 (br s, 1 H, aryl NH), 4.09 (ddd, J = 8.4 Hz, 3.9 Hz, 1 H, Nleu α H), 3.39 (br s, 2 H, CH_2N), 2.92 (m, 1 H, Cyst-αH), 2.78 (m, 2 H, CH_2SH), 1.50–1.59 (m, 2 H,

NLeu-CH_2), 1.20–1.08 (m, 4 H, NLeu-CH_2), 0.81 (t, $J = 7.5$ Hz, 3 H, Nleu-CH_3); ^{13}C NMR (DMSO-d_6) δ 177.0, 171.0, 148.8, 141.0, 138.7, 129.6, 128.2, 127.7, 126.7, 124.3, 113.4, 110.1, 51.9, 51.7, 43.1. 30.2, 27.2, 24.1, 21.4, 12.5.

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