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A para-amino substituent on the D-ring of green tea polyphenol epigallocatechin-3-gallate as a novel proteasome inhibitor and cancer cell apoptosis inducer

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Abstract—Analogs of (−)-EGCG containing a para-amino group on the D-ring in place of the hydroxyl groups have been synthesized and their proteasome inhibitory activities were studied. We found that, the *O*-acetylated (−)-EGCG analogs possessing a *p*-NH_2 or *p*-NHBoc (Boc; *tert*-butoxycarbonyl) D-ring (**5** and **7**) act as novel tumor cellular proteasome inhibitors and apoptosis inducers with potency similar to natural (−)-EGCG and similar to (−)-EGCG peracetate. These data suggest that the acetylated amino-GTP analogs have the potential to be developed into novel anticancer agents.

1. Introduction

Green tea, produced from *Camellia sinensis*, is a highly consumed beverage around the world and regular drinking of green tea has been claimed to reduce incidence of a variety of cancers.[1–3] Although a number of green tea polyphenols (GTPs) have been identified in green tea,[4] (−)-epigallocatechin-3-gallate [**1**, (−)-EGCG] is the most abundant constituent and is considered to be the most biologically active among the GTPs. A number of epidemiological and biological studies involving (−)-EGCG have been reported in the last decade and have shown that (−)-EGCG can reduce or inhibit tumor growth in breast,[5–7] lung,[8] and urinary[9–11] and GI tracts.[12]

The eukaryotic proteasome is a large multi-catalytic, multi-subunit protease complex possessing at least three distinct activities, which are associated with three different β subunits, chymotrypsin-like (with β5 subunit), trypsin-like (with β2 subunit), and peptidyl-gluthamyl peptide-hydrolyzing-like (PGPH- or caspase-like; with β1 subunit).[13–15] Inhibition of the chymotrypsin-like, but not the trypsin-like, activity of the proteasome has been found to be associated with induction of tumor cell apoptosis.[16–18] Inhibition of the proteasome prevents ubiquitin-targeted proteolysis which can affect multiple signaling cascades within the cell. Since this disruption of normal homeostatic mechanisms can lead to cell death, the discovery of new proteasome inhibitors with little or no toxicity is highly desirable in anticancer therapy.[19,20]

We have previously reported that (−)-EGCG inhibits the chymotrypsin-like activity of the proteasome in vitro (IC_{50} 0.1–0.2 μM) and in intact tumor cells (1–10 μM).[21,22] Enantiomerically synthesized (+)-EGCG and other synthetic analogs of green tea catechins with an ester bond have also been shown to inhibit the proteasomal chymotrypsin-like activity,[22,23] leading to accumulation of proteasome target proteins (such as IκB-α, p27, and Bax) and apoptosis in human cancer cell lines, as measured by activation of caspases and cleavage of poly(ADP-ribose) polymerase (PARP).[24] Furthermore,

Keywords: Green tea polyphenols; Epigallocatechin-3-gallate; para-Aminobenzoic acid; Pro-drug; Proteasome; Apoptosis.
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in silico docking studies have indicated that (−)-EGCG predictably binds to the N-terminal threonine (Thr) of the proteasomal chymotrypsin active site. The orientation of (−)-EGCG bound to the proteasome is suitable for nucleophilic attack by the hydroxyl group of Thr 1 to the carbonyl carbon of (−)-EGCG, thus inhibiting the proteasomal chymotrypsin-like activity (Fig. 1).[25] Furthermore, the hydroxyl groups of the (−)-EGCG D-ring were found to form hydrogen-bonds with Gly_{47} or Ser_{131} of the proteasome, thus contributing to the binding stability of (−)-EGCG to the proteasome (Fig. 1). In support of this model, compound **2** (Fig. 2), which contains only one *p*-hydroxy group on the D-ring, was found to be a much weaker proteasome inhibitor than (−)-EGCG (Table 1).[26] Since an amino group is capable of forming hydrogen bonds both as a donor and an acceptor, we are therefore interested in studying the replacement of the hydroxyl group in the D-ring by an amino group in an attempt to increase the potency of compound **2**.

A critical issue concerning the potential application of (−)-EGCG as an anticancer agent is its known low bioavailability which is thought to be partly due to the poor stability of (−)-EGCG in alkaline or neutral solutions.[27,28] Because the pH values of intestine and body fluids are neutral or slightly alkaline, (−)-EGCG is potentially unstable inside the human body.[27] Additionally, in vivo metabolic transformations of (−)-EGCG by glucuronidation, sulfonation or methylation into various metabolites may also contribute to its reduced bioavailability.[29]

Recently, we suggested that (−)-EGCG peracetate (**3**, Pro-E), a synthetic derivative of (−)-EGCG, can act as a pro-drug.[27] Pro-E is also converted under cellular conditions by esterases to (−)-EGCG with enhanced bioavailability in vivo.[30] Consistently, even though Pro-E has no inhibitory effect against a purified 20S proteasome, it nevertheless showed much higher potency than (−)-EGCG to inhibit proliferation and transforming activity and to induce apoptosis in human prostate, breast, leukemic, and simian virus 40-transformed cells.[31] Recently, in a related study, we showed that Pro-E can be converted to (−)-EGCG in human breast cancer MDA-MB-231 cell cultures and xenografts, leading to a higher intracellular concentration (>2.4-fold) of (−)-EGCG than those cells treated with same dose of (−)-EGCG.[32] Pro-E proved to be more efficacious in inhibiting breast cancer tumor growth in mice than (−)-EGCG.[32]

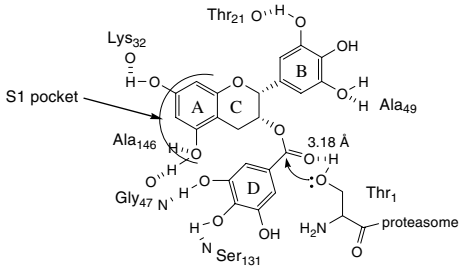


Figure 1. Docking model of (−)-EGCG in the β5-subunit of 20S proteasome. The number is distance in Å for the H-bond indicated.

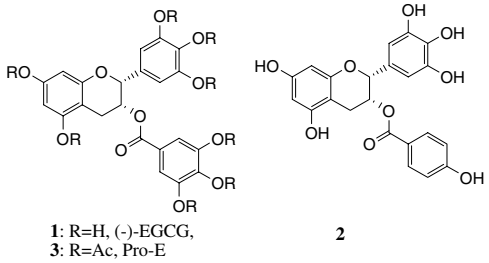


Figure 2. Structures of selected GTPs and analogs.

Table 1. Inhibition of chymotrypsin-like activity of purified 20S proteasome[a]

Compound	IC_{50} (μM)
(−)-EGCG	0.2 ± 0.1
3 (Pro-E)	na[c]
2	40.4 ± 0.17[b]
4	0.84 ± 0.06
5	na[c]
6	3.85 ± 0.33
7	na[c]

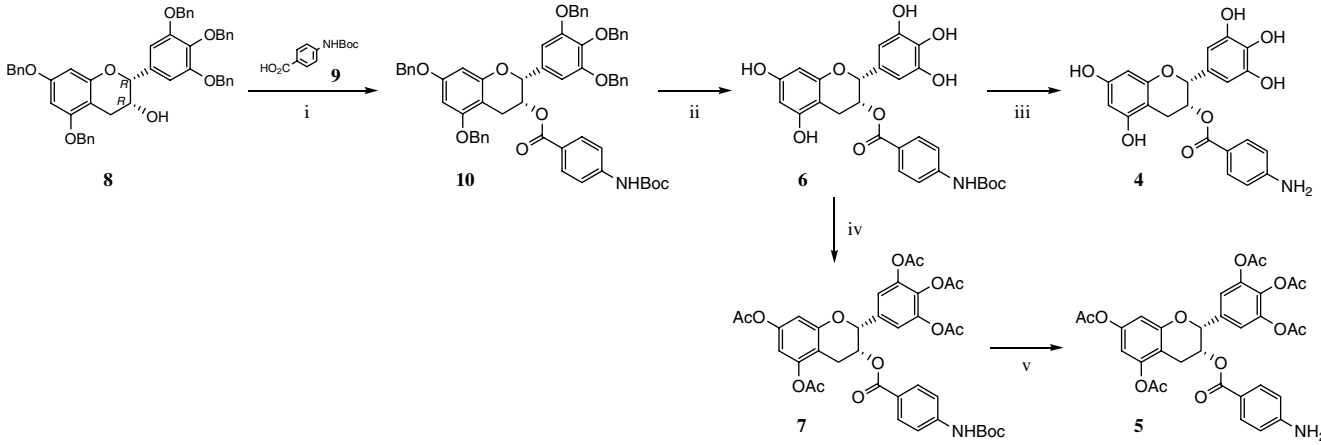
[a] Results obtained from 3 independent experiments performed in triplicate.
[b] Ref. 26.
[c] na indicates that the inhibitory activity of the purified proteasome at 50 μM was <20%.

With the knowledge that *O*-acetyl protected GTPs could be cytotoxic against tumor, but not normal cells,[31,32] we have continued to search for more potent anticancer agents. We have previously studied the biological activities of some EGCG analogs with modifications of ABC-ring moiety and gallate (D-ring) moiety.[25–27,31,32] Here we report structure–activity relationship (SAR) analysis with newly designed analog compounds, which possess a para-amino substituent on the D-ring (compound **4**), as well as the *O*-acetyl derivatives **5**, and their corresponding *N*-*tert*-butoxycarbonyl (Boc) derivatives **6** and **7**.

2. Results and discussion

2.1. Enantioselective syntheses of amino-GTP analogs 4–7

The syntheses of compounds **4**–**7** were achieved as outlined in Scheme 1. We have previously reported on the total synthesis of (−)-EGCG in which (−)-(2*R*, 3*R*)-5,7-bis(benzyloxy)-2-[3,4,5-tris(benzyloxy)phenyl]chroman-3-ol (**8**) was prepared as the key intermediate.[33] Esterification of **8** with boc-4-aminobenzoic acid (**9**) afforded **10** with excellent yield. Catalytic hydrogenation of **10** removed the benzyl protecting group readily to give **6**. Deprotection of Boc group with trifluoroacetic acid furnished **4**. On the other hand, acetylation of **6** with Ac_2O and pyridine gave **7** which on subsequent deprotection of Boc group gave **5**.



Scheme 1. Synthetic route of amino GTP analogs. Reagents and conditions: (i) DCC, DMAP, CH_2Cl_2, rt, 20 h; (ii) $Pd(OH)_2$, H_2, THF/MeOH, rt, 1 h; (iii) $CHCl_3$/TFA, rt, 30 min; (iv) Ac_2O, pyridine, rt, 30 min; (v) $CHCl_3$/TFA, rt, 6.5 h.

2.2. Inhibition of chymotrypsin-like activity of a purified 20S proteasome

We examined these synthetic amino-GTPs, both unprotected (**4** and **6**) and *O*-acetyl protected (**5** and **7**), for their SAR in comparison with natural (−)-EGCG (**1**), **2**, and Pro-E (**3**) against the chymotrypsin-like activity of a purified 20S proteasome (Table 1). Compound **4** exhibited reasonably strong proteasome inhibition (IC_{50} value, 0.84 μM). Even though the activity of **4** is not as high as (−)-EGCG (IC_{50}value, 0.20 μM,[34]) it is much more active than compound **2** of which a hydroxyl group is in place of the amino group in compound **4** (Fig. 2 vs Scheme 1). This suggests that an amino substituent is more effective than a hydroxyl in enhancing proteasome inhibition.

On the other hand, compound **6**, with a *p*-NHBoc on the D-ring, is less active (IC_{50} = 3.85 μM) than **4** but still much more active than compound **2**. We also examined the *O*-acetyl protected analogs **5** and **7** to evaluate their proteasome-inhibitory activities. As expected, the *O*-acetyl protected analogs were not potent in inhibiting the chymotrypsin-like activity of the purified 20S proteasome due to the lack of cellular esterases for their conversion into de-acetyl and activated forms.[30,32,34] These acetylated analogs displayed exceptionally weak inhibitory activity against the purified 20S proteasome [less than 20% inhibition at 50 μM concentration (Table 1)]. This finding again demonstrates that *O*-acetyl analogs are inactive in a purified enzyme system.

2.3. Inhibition of proteasome activity in intact tumor cells

In order to determine whether the synthetic amino-GTP analogs exhibit proteasomal inhibition in whole cells, leukemia Raji B cells were treated with 25 μM of each amino-GTP analog for 4 and 24 h (Fig. 3). Inhibition of intact tumor cell proteasome activity was assayed in whole cell extracts using a chymotrypsin-like activity assay. Compound **6** was unable to inhibit the chymotrypsin-like activity in whole cells after 4- and 24-h incubation and compound **4** exhibited minimal proteasome activity at the same time points, while (−)-EGCG induced only 4% and 10% inhibition at 4 and 24 h, respectively (Fig. 3). These findings suggest that **4** and **6** are unstable within the cellular environment, similar to (−)-EGCG. However, the *O*-acetylated analogs **5** and **7** exhibited far greater potency against the proteasomal chymotrypsin-like activity, 39% and 65% inhibition, respectively, after 24 h.

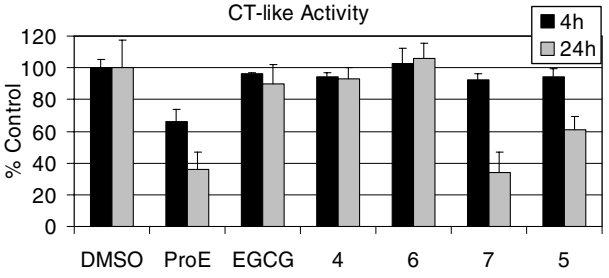


Figure 3. *O*-Acetylated amino-GTP analogs are potent proteasome inhibitors in cultured tumor cells. Leukemia Raji B cells were treated with 25 μM of each analog for 4 and 24 h, harvested and analyzed for the chymotrypsin-like activity, as described in Section 4.

Inhibition of proteasomal activity should lead to accumulation of ubiquitinated proteins and proteasome target proteins, such as IκB-α, p27, and Bax.[16,17] Both **4** and **6** slightly increased levels of ubiquitinated proteins in a time-dependent manner, although they had little effect on levels of IκB-α, p27, and Bax proteins (Fig. 4). In contrast, **5** and **7** were more potent with respect to accumulation of ubiquitinated proteins and proteasome target proteins, after both 4-h and 24-h treatment (Fig. 4). Consistent with the level of proteasome inhibition observed in whole cells (Fig. 3), **7** induced higher levels of ubiquitinated proteins than **5** at both 4 and 24 h (Fig. 4). After 4-h treatment with **7**, IκB-α, p27, and Bax were accumulated by 1.4-, 2.1-, and 3.5-fold, respectively. In contrast, a treatment with **5** did not accumulate IκB-α and accumulated only p27 and Bax by 2.0- and 3.3-fold, respectively, after 4 h (Fig. 4).

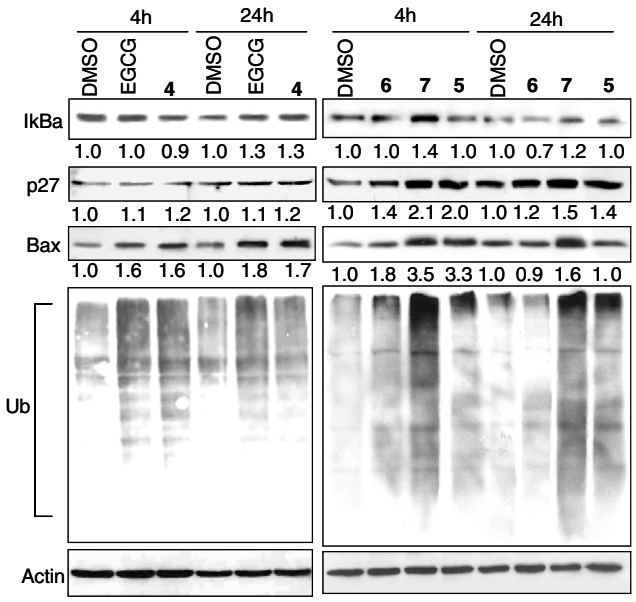


Figure 4. *O*-Acetylated amino-GTP analogs accumulate proteasome target proteins in cultured tumor cells. Western blot analysis using specific antibodies to Ubiquitin, Bax, p27, IκB-α, and actin.

2.4. Induction of apoptotic cell death

It has been shown that inhibition of the proteasomal chymotrypsin-like activity is associated with induction of apoptosis in a wide variety of cancer cells.[16,17] We therefore determined the cytotoxicity of the *O*-acetylated and un-acetylated forms of the amino-GTP analogs by Trypan blue dye exclusion analysis. Leukemia Raji B cells were treated with each pair of the acetylated and un-acetylated analogs at 25 μM for 4- and 24-h, followed by Trypan blue analysis (Fig. 5a). Blue cells and cells with apoptosis-associated morphological changes (shrunken, blebbing, *etc.*) were scored as dead cells. We found that the un-acetylated amino-GTP analogs (**4** and **6**) induced no more than 10% cell death at up to 24 h (Fig. 5a) supporting the idea that they are either unstable or inactive within the cell.[30,32,34] In contrast, the acetylated analogs (**5** and **7**) were much more potent in inducing the cell death (Fig. 5a). Cell death was increased by 3.5- and 5-fold in cells treated with the acetyl protected amino-GTP analogs **5** and **7**, respectively, after 24-h incubation compared to cells treated with (−)-EGCG (Fig. 5a). To determine whether the observed cell death (Fig. 5a) was representative of apoptosis, aliquots of the cell extracts from the same experiment were utilized for measurement of caspase-3 activation (Fig. 5b) and poly(ADP-Ribose) polymerase (PARP) cleavage (Fig. 5c). After 24-h treatment, **5** and **7** induced a 4.7- and 5.5-fold increase in caspase-3 activity, respectively (Fig. 5b). Additionally Western blot analysis revealed that apoptosis-specific PARP cleavage was observed in cells treated with analogs **5** and **7** after 24 h (Fig. 5c). In contrast, the PARP cleavage was not observed in cells treated with the un-acetylated amino-GTP analogs **4** and **6** (Fig. 5c). These results indicate that the acetyl protected analogs are more potent than the un-acetylated analogs in inhibiting proteasome

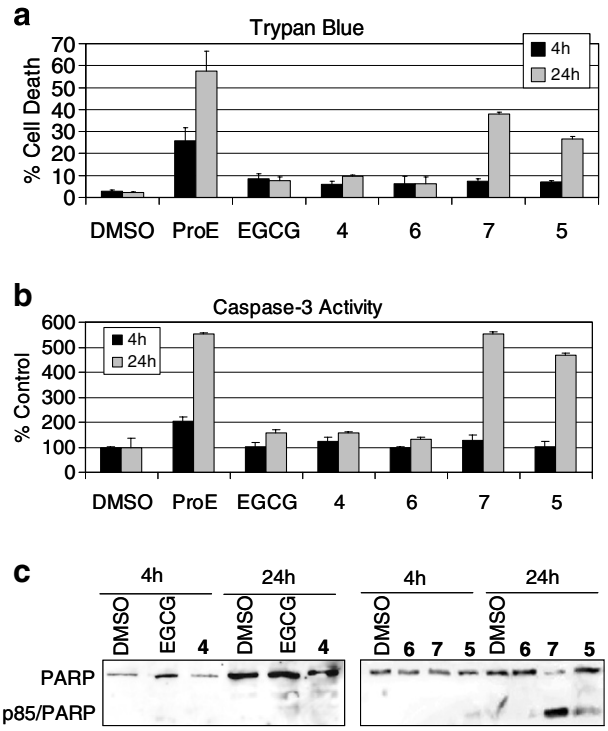


Figure 5. *O*-Acetylated amino-GTP analogs induce time-dependent cell death in cultured tumor cells. Leukemia Raji B cells were treated with the solvent (DMSO) or 25 μM of the indicated analog for 4 or 24 h, followed by Trypan blue dye exclusion assay. The data represented are the mean number of dead cells over total cell population ±SD after treatment (a). A cell-free caspase-3/-7 activity assay was performed as described in Section 4. Caspase-3/-7 activity is represented as a percentage of the solvent (DMSO) control (b). Western blot analysis was done using specific antibodies to PARP (c).

activity and inducing apoptotic cell death in a time-dependent manner. These results are consistent with the hypothesis that compounds **5** and **7** are behaving as pro-drugs of the un-acetylated analogs **4** and **6**, in the same manner as Pro-E serving as the pro-drug for EGCG.[27,32]

3. Conclusions

In conclusion, we have demonstrated that a para-amino substituent on the D-ring of green tea polyphenols exhibits proteasome-inhibitory activity with potency similar to that of (−)-EGCG and ProE. Furthermore, the *O*-acetylated amino-GTP analogs appear to behave as prodrugs and the mechanism of action involves targeting the proteasome in tumor cells thereby inducing cell death. Although the para-amino compounds are not inherently more potent than ProE, the presence of the para-amino substituent could increase the bioavailability of these compounds by limiting biotransformation reactions to the hydroxyls, such as glucuronidation, sulfonation, and methylation.[29] Since the para-aminobenzoic acid moiety is a normal constituent of folic acid and generally presumed to be innocuous,[35] the study of these para-amino substituted green tea polyphenols may offer the possibility of discovering novel anticancer drugs.

4. Experimental

4.1. Reagents

Fetal Bovine Serum was purchased from Tissue Culture Biologicals (Tulare, CA). RPMI 1640, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad,CA). Dimethylsulfoxide (DMSO) and (−)-EGCG were purchased from Sigma (St. Louis, MO). Suc-Leu-Leu-Val-Tyr-AMC (a proteasomal chymotrypsin-like substrate) and Ac-DEVD-AMC (a caspase-3 substrate) were obtained from Biomol (Plymouth Meeting, PA). Purified 20S proteasome from rabbit was acquired from Boston Biochem (Cambridge, MA). Monoclonal antibodies to Bax (H280) and Ubiquitin (P4D1), polyclonal antibodies to IκB-α (C15) and Actin (C11), and anti-goat, anti-rabbit, and anti-mouse IgG-horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody to p27 (554069) was from BD Biosciences (San Diego, CA). The polyclonal antibody to PARP was purchased from Biosource (Camarillo, CA).

4.2. Chemical synthesis

The synthesis was accomplished according to Scheme 1. All reactions were performed under an atmosphere of N_2, and glassware was dried completely in an oven at 110 °C prior to use. Tetrahydrofuran (THF) was dried by distillation over sodium benzophenone, and dry dichloromethane (CH_2Cl_2), dimethylformamide (DMF), and toluene were obtained by distillation from CaH_2. Unless otherwise stated, solvents or reagents were used as received without further purification. (−)-(2*R*, 3*R*)-5,7-bis(benzyloxy)-2-[3,4,5-tris(benzyloxy)benzyl]-3-chroman-3-ol (**8**) was prepared by following reported procedure.[33]

4.3. (−)-(2*R*, 3*R*)-5,7-Bis(benzyloxy)-2-[3,4,5-tris(benzyloxy)phenyl]chroman-3-yl 4-*N*-(*tert*-butoxycarbonyl)-aminobenzoate (10)

To a solution of 4-*N*-Boc-aminobenzoic acid (**9**, 166 mg, 698 μmol) in CH_2Cl_2 (1.00 mL) was added *N*,*N*′-dicyclohexylcarbodiimide (217 mg, 1.05 mmol). The mixture was stirred at room temperature for 10 min, cooled to 0 °C. 4-Dimethylaminopyridine (21.4 mg, 175 μmol) was added to the solution and the mixture was stirred for 5 min. A solution of (−)-(2*R*, 3*R*)-5,7-bis(benzyloxy)-2-[3,4,5-tris(benzyloxy)phenyl]-chroman-3-ol (**8**, 264 mg, 349 μmol) in CH_2Cl_2 (2.50 mL) was added dropwise at 0 °C and the mixture was stirred at room temperature overnight. The solvent was evaporated in vacuo and the resulting oil was purified by flash SiO_2 column chromatography (hexane/EtOAc, 4:1) to give 308 mg (90%) of the title compound as a pale yellow amorphous solid: $[\alpha]_D^{20} = -60°$ (*c* 1.05, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ7.91 (d, $J = 8.6$ Hz, 2H), 7.48–7.17 (m, 27H), 6.78 (s, 2H), 6.61 (s, 1H), 6.34 (br s, 1H), 6.29 (br s, 1H), 5.66 (br s, 1H), 5.08–4.91 (m, 8H), 4.76 (d, $J =$ 11.9, 2H), 3.14–3.04 (m, 2H), 1.51 (s, 9H); ^{13}C NMR ($CDCl_3$) δ165.95, 159.72, 158.92, 156.51, 153.74, 152.87, 143.95, 139.14, 138.73, 137.94, 137.82, 137.76, 134.28, 132.10, 129.57, 129.49, 129.33, 129.02, 128.99, 128.86, 128.70, 128.66, 128.57, 128.42, 128.16, 125.02, 118.17, 107.53, 101.91, 95.67, 94.88, 82.14, 78.82, 76.04, 72.10, 71.11, 70.92, 69.04, 29.21, 27.07; HRMS *m*/*z* calculated for $C_{62}H_{57}O_{10}Na$ (M+Na) 998.3880, found 998.3845.

4.4. (−)-(2*R*, 3*R*)-5,7-Dihydroxy-2-(3,4,5-trihydroxyphenyl)-chroman-3-yl 4′-(*tert*-butoxycarbonyl)-aminobenzoate (6)

To a solution of (−)-(2*R*, 3*R*)-5,7-bis(benzyloxy)-2-[3,4,5-tris(benzyloxy)phenyl]chroman-3-yl 4′-*N*-(*tert*-butoxy-carbonyl)aminobenzoate (**10**, 101 mg, 104 μmol) in THF (10.0 mL) and MeOH (10.0 mL) was added palladium hydroxide on carbon powder [20% Pd (100 mg)]. The mixture was stirred under a H_2 atmosphere at room temperature for 1 h, filtered, and eluted with MeOH and the eluate was evaporated in vacuo. The obtained colorless oil (66.8 mg) was purified by flash SiO_2 column chromatography (AcOEt/hexane, 2:1) to give 45.3 mg (83%) of the title compound as a pale yellow amorphous solid: $[\alpha]_D^{20} = -88°$ (*c* 0.14, MeOH); ^{1}H NMR ($CDCl_3$) (complexity due to rotamers from the amide function) δ 7.81 (d, $J = 9.5$ Hz, $1/6 \times 2$H), 7.78 (d, $J = 9.5$ Hz, $5/6 \times 2$H), 7.45 (d, $J = 9.5$ Hz, $1/6 \times 2$H), 7.43 (d, $J = 9.5$ Hz, $5/6 \times 2$H), 6.54 (s, $1/6 \times 2$H), 6.52 (s, $5/6 \times 2$H), 6.05 (d, $J = 2.4$ Hz, $1/6 \times 1$H), 5.99–5.97 (m, 1H), 5.96 (d, $J = 2.4$ Hz, $5/6 \times 1$H), 5.54 (br s, $1/6 \times 1$H), 5.52 (br s, $5/6 \times 1$H), 5.10-4.68 (m, 5H), 5.02 (s, $1/6 \times 1$H), 5.00 (s, $5/6 \times 1$H), 3.03 (dd, $J = 17.0$, 4.4 Hz, $1/6 \times 1$H), 3.00 (dd, $J = 17.0$, 4.4 Hz, $5/6 \times 1$H), 2.91 (dd, $J = 17.0$, 2.0 Hz, $1/6 \times 1$H), 2.89 (dd, $J = 17.0$, 2.0 Hz, $5/6 \times 1$H), 1.51 (s, $1/6 \times 9$H), 1.49 (s, $5/6 \times 9$H); ^{13}C NMR ($CDCl_3$) δ 168.06, 158.74, 158.65, 158.01, 147.55, 146.23, 134.54, 132.60, 131.61, 125.71, 119.36, 107.56, 107.51, 100.13, 97.40, 97.32, 96.66, 96.58), 82.20, 79.37, 79.28, 71.31, 19.43, 29.40, 27.50; HRMS *m*/*z* calculated for $C_{27}H_{27}NO_{10}Na$ (M+Na) 548.1533, found 548.1537.

4.5. (−)-(2*R*,3*R*)-5,7-Dihydroxy-2-(3,4,5-trihydroxyphenyl)-chroman-3-yl 4-aminobenzoate (4)

To a solution of (−)-(2*R*,3*R*)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)chroman-3-yl 4-(*tert*-butoxycarbonyl)-aminobenzoate (**6**, 20.6 mg, 39.2 μmol) in $CHCl_3$ (800 μL) was added trifluoroacetic acid (160 μL) and the mixture was stirred at room temperature for 30 min. The reaction mixture was directly evaporated in vacuo and 28.5 mg (>99%) of the title compound was obtained as a pale brown solid: $[\alpha]_D^{20} = -72°$ (*c* 0.19, MeOH); ^{1}H NMR ($CDCl_3$) δ7.67 (d, $J = 9.8$ Hz, 2H), 6.63 (d, $J = 9.8$ Hz, 2H), 6.54 (s, 2H), 6.00 (br s, 1H), 5.99 (br s, 1H), 5.51 (br s, 1H), 5.01 (s, 1H), 4.96–4.82 (m, 5H), 3.01 (dd, $J = 16.8$, 4.9 Hz, 1H), 2.89 (dd, $J = 16.8$, 3.3 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ168.97, 158.74, 158.68, 158.08, 151.05, 147.55, 134.57, 133.60, 133.51, 131.75, 119.52, 115.21, 115.09, 107.72, 107.61, 100.33, 97.35, 96.60, 79.48, 70.61, 27.58; HRMS *m*/*z* calculated for $C_{32}H_{29}NO_{13}$ 426.1189, found 426.1205.

4.6. (−)-(2*R*,3*R*)-5,7-Diacetoxy-2-(3,4,5-triacetoxyphenyl)-chroman-3-yl 4-(*tert*-butoxycarbonyl)-aminobenzoate (7)

To a solution of (−)-(2*R*, 3*R*)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)chroman-3-yl 4-(*tert*-butoxycarbonyl)-aminobenzoate (**6,** 94.0 mg, 179 μmol) in pyridine (1.00 mL) was added acetic anhydride (130 μL, 1.38 mmol) and the mixture was stirred at room temperature for 30 min. The solvent was evaporated in vacuo and the resulting yellow oil (113 mg) was purified by flash SiO_2 column chromatography (hexane/EtOAc, 1:1) to give 58.1 mg (59%) of the title compound as a colourless amorphous solid: $[\alpha]_D^{20} = -48°$ (*c* 1.11, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ7.78 (d, J = 8.5 Hz, 2H), 7.34 (d, J = 8.5 Hz, 2H), 7.27 (s, 2 H), 6.74 (d, J = 1.9 Hz, 1H), 6.68 (s, 1H), 6.59 (d, J = 1.9 Hz, 1H), 5.62–5.58 (m, 1H), 5.20 (s, 1H), 3.06 (br s, 2H), 2.30–2.27 (m, 6H), 2.26–2.23 (m, 9H), 1.50 (s, 9H); ^{13}C NMR ($CDCl_3$) δ169.93, 169.42, 168.55, 167.72, 166.40, 155.77, 153.00, 150.66, 144.31, 143.95, 136.58, 135.19, 132.15, 124.43, 119.71, 118.21, 110.73, 109.84, 108.96, 82.09, 77.54, 68.14, 29.20, 26.94, 22.06, 21.75, 21.58, 21.10; HRMS *m*/*z* calculated for $C_{37}H_{37}NO_{15}Na$ (M+Na) 758.2061, found 758.2068.

4.7. (−)-(2*R*,3*R*)-5,7-Diacetoxy-2-(3,4,5-triacetoxyphenyl)-chroman-3-yl 4-aminobenzoate (5)

To a solution of (−)-(2*R*,3*R*)-5,7-diacetoxy-2-(3,4,5-triacetoxyphen)chroman-3-yl 4-(*tert*-butoxycarbonyl)-aminobenzoate (**7**, 60.0 mg, 81.6 μmol) in $CHCl_3$ (1.60 mL) was added trifluoroacetic acid (320 μL) and the mixture was stirred at room temperature for 6.5 h. The reaction was quenched with saturated aqueous $NaHCO_3$ at 0 °C and the mixture was extracted with $CHCl_3$ (3× 10.0 mL). The combined organic layers were dried (Na_2SO_4) and concentrated in vacuo. The obtained brown red amorphous solid (65.0 mg) was purified by flash SiO_2 column chromatography (1% triethylamine, EtOAc/hexane, 2:1 → EtOAc) to give 26.4 mg (51%) of the title compound as a yellow amorphous solid: $[\alpha]_D^{20} = -54°$ (*c* 1.32, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ7.57 (d, J = 8.7 Hz, 2H), 7.20 (s, 2H), 6.65 (br s, 1H), 6.50 (br s, 1H), 6.46 (d, J = 8.7 Hz, 2H), 5.51 (br s, 1H), 5.10 (s, 1H), 3.00–2.90 (m, 2H), 2.20 (s, 3H), 2.19 (s, 3H), 2.17 (s, 3H), 2.15 (s, 6H); ^{13}C NMR ($CDCl_3$) δ169.32, 168.80, 167.95, 167.13, 166.07, 155.18, 151.01, 150.03, 149.93, 143.62, 136.08, 134.52, 132.18, 119.16, 114.31, 110.26, 109.11, 108.26, 76.97, 66.89, 26.35, 21.40, 21.09, 20.91, 20.45; HRMS *m*/*z* calculated for $C_{32}H_{29}NO_{13}$ 635.1638, found 635.1643.

4.8. Cell culture

Human leukemia Raji B cells were cultured in RPMI supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell cultures were maintained in a 5% CO_2 atmosphere at 37 °C.

4.9. Cell extract preparation and Western blotting

Whole cell extracts were prepared as described previously.[36] Analysis of IκB-α, p27, Bax , PARP, and ubiquitinated proteins was performed using monoclonal or polyclonal antibodies, according to previously reported protocols.[21] Densitometry was quantified using Alpha-Ease FC software (Alpha Innotech Corporation, San Leandro, CA).

4.10. Inhibition of purified 20S proteasome activity by synthetic amino-GTP analogs

Measurement of the chymotrypsin-like activity of the 20S proteasome was performed by incubating 35 ng of purified rabbit 20S proteasome with 40 μM of fluorogenic peptide substrate, Suc-Leu-Leu-Val-Tyr-AMC, with or without a natural or synthetic GT.[37]

4.11. Inhibition of proteasome activity in intact tumor cells by synthetic amino-GTP analogs

Cells were treated with each compound at 25 μM for 4 or 24 h, harvested, and lysed as described previously.[16] Whole cell extracts (10 μg) were incubated with Suc-Leu-Leu-Val-Tyr-AMC (40 μM) fluorogenic substrate at 37 °C in 100 μL of assay buffer (50 mM Tris–HCL, pH 8) for 2.5 h. After incubation, production of hydrolyzed 7-amino-4-methylcoumarin (AMC) groups was measured using a Victor 3 Multilabel Counter with an excitation filter of 380 nm and an emission filter of 460 nm (Perkin-Elmer, Boston, MA, USA).

4.12. Induction of caspase-3 activity by synthetic amino-GTP analogs

Cells were treated with each compound at 25 μM for 4 or 24 h, harvested, and lysed as described previously.[21] Ac-DEVD-AMC (40 μM) was then incubated with the prepared cell lysates for 2.5 h and the caspase-3 activity was measured as described previously.[38]

4.13. Trypan blue assay and apoptotic morphology changes

The Trypan blue dye exclusion assay was used to ascertain cell death in Raji cells treated with either a natural or synthetic compound at 25 μM for 4 or 24 h. Cell morphology was assessed using phase-contrast microscopy as described previously.[22,31]

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