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Synthesis and bradykinin inhibitory activity of novel non-peptide compounds, and evaluation of in vivo analgesic activity

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ABSTRACT

A series of novel non-peptide diamide compounds was synthesized and evaluated as antibradykinin agents by utilizing guinea-pig ileum smooth muscle. Among the final compounds, (*Z*)-4-(4-(bis(4-fluorophenyl)methyl)piperazin-1-yl)-4-oxo-*N*-(4-phenylbutan-2-yl)but-2-enamide showed most favorable bradykinin inhibitory activity and demonstrated analgesic efficacies in the rat models of inflammatory and neuropathic pain.

1. Introduction

Bradykinin (H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH), an endogenous nonapeptide generated by limited proteolysis of kininogen in tissues and body fluids, elicits numerous responses including vasodilation, edema, smooth muscle contraction, inflammation, trauma, burns, shock, and allergy.[1–3] It induces pain by direct activation of nociceptive nerve terminals, by sensitizing other fibers to become nociceptors, and by stimulating the release of other substances such as neurokinin A, substance P, prostanoids, and cytokines involved in nociception.[4–7] Compelling evidence has shown that bradykinin participates in a number of hyperalgesic and inflammatory preclinical models.[8,9] The growing knowledge of the biological role of kinins, in particular in pain and inflammation, has sustained the development of potent and selective bradykinin receptor modulators as potential therapeutics.[10]

Two types of bradykinin receptors B1 and B2 have been identified: the B1 receptors are not present in tissues under normal conditions but are induced during prolonged insults such as chronic inflammation, and the B2 receptors constitutively expressed in the nervous system and other tissues involve in the transmission of nociceptive and acute inflammatory pain.[11,12] In addition, the activation of the B1 receptor showing a small portion of normal dorsal root ganglion (DRG) neurons exaggerated heat-evoked current in DRG neurons with a more sustained effect than the B2 receptor.[13] Taken together, these results suggested that the B2 receptors played a key role in nociceptive and acute types of pain, whereas the B1 receptors might be involved in the chronic types of pain.[14–16]

The formalin test, a commonly used method of inflammatory pain, produces a biphasic behavioral response. The short-lived first response is thought to be produced by direct activation of nociceptive neurons by formalin and the second phase is thought to be a part of an inflammatory response to tissue injury. Several studies in mice and in rats have shown that bradykinin receptor antagonists greatly inhibit the second phase and at higher doses reduce the first phase of pain behavior.[17–27]

Injury to a nociceptive pathway can also result in an exaggeration of pain. Peripheral and central nerve injury leads to chronic neuropathic pain characterized by spontaneous burning pain, allodynia and hyperalgesia. The B1 and B2 receptor gene expression is up-regulated in animal models of neuropathic pain.[28,29] In addition, the novel induction of B1 receptor expression after peripheral nerve injury is particularly meaningful due to that the B1 receptor is not readily desensitized after agonist application, and thus these changes can be maintained chronic types of pain.[30,31]

Icatibant (H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg-OH), used as a positive control, is a peptide B2 receptor selective antagonist. Although icatibant has shown highly potent and long-acting bradykinin antagonism both in vitro and in vivo studies,[32,33] it has a limited therapeutic use due to its poor oral bioavailability. In our search for the new bradykinin antagonists, we previously reported the bradykinin antagonistic effects of a series of non-peptide

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compounds with piperazine, three amide bonds and a lipophilic ring system in each molecule.[34] In this study, we have designed and synthesized new compounds modified the linker from previous series having two amide bonds with various substituents in each side. Four amines (**b1**–**3** and **b6**) were selected from the previous series of iminodiacetic acid triamide derivatives for their relatively high bradykinin inhibitory activities.[34] Compound **33**, which has shown the most potent inhibitory activity (87% inhibition at 1 μM), was selected for further studies of its in vivo analgesic capabilities using the rat models of inflammatory and neuropathic pain.

2. Results and discussion

2.1. Chemistry

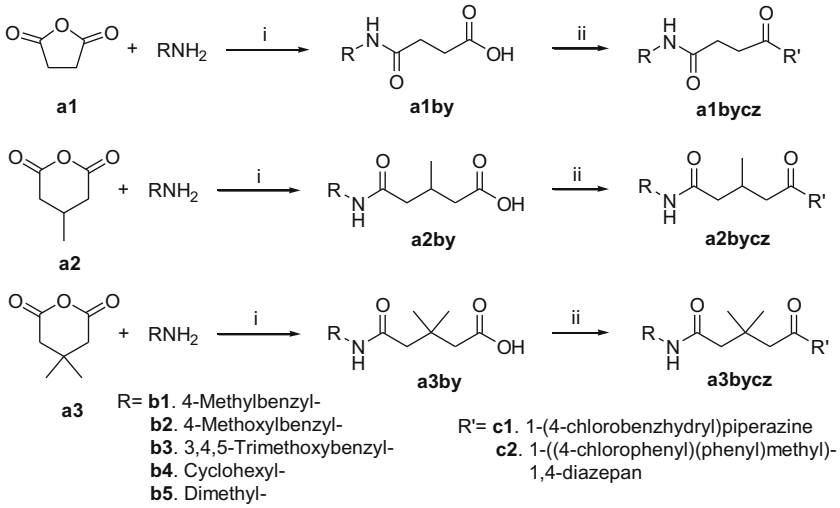
The combinatorial synthesis of the 33 diamide compounds was accomplished by coupling reactions (Schemes 1 and 2). The anhydrides such as succinic anhydride (**a1**), 3-methylglutaric anhydride (**a2**), and 3,3-dimethylglutaric anhydride (**a3**), or maleic acid (**a4**), followed by treatment with various amines (**b1**–**6**) afforded the monoamides **axby**. Each product was treated with 1-(4-chlorobenzhydryl)piperazine (**c1**), 1-((4-chlorophenyl)(phenyl)methyl)-1,4-diazepan (**c2**),[35] or 1-(bis(4-fluorophenyl)methyl)piperazine (**c3**) and PyBOP to afford diamides **axbycz** which were effectively purified by acid and base extractions to remove reaction by-products, the unreacted starting materials and reagents. Each individual compound synthesized was characterized by ^{1}H NMR, ^{13}C NMR, and high resolution MS. The structures of different linkers and amines in both ends are illustrated in Table 1.

2.2. Bradykinin inhibitory activity

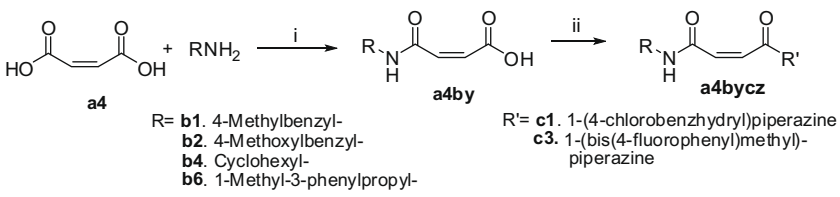
Each compound was tested for their bradykinin-induced contractilities on guinea-pig ileum smooth muscle at 1 μM concentration. As shown in Table 2, the inhibitory activity of synthesized compounds to maximum contractility of bradykinin was 28–87% (n = 4–13). By the modification of the linkers (**a**), compounds with succinamide (**a1** series), 3-methylpentanediamide (**a2** series), or maleamide linker (**a4** series) showed higher bradykinin inhibitory activity than compounds with 3,3-dimethylpentanediamide linker (**a3** series). In terms of amines (**b**), substituted benzyl or cyclohexyl groups showed comparable inhibitory activity, while the dimethyl amine group (**13**) showed decreased inhibitory activity. Amines **c1**–**3** exhibited similar inhibitory activity except for 1-((4-chlorophenyl)(phenyl)methyl)-1,4-diazepan (**c2**) in compounds with 3-methylpentanediamide linker (**a2**) that showed an increased inhibitory activity compared with 1-(4-chlorobenzhydryl)piperazine (**c1**) congeners. Among the synthesized compounds, five compounds **6**, **14**, **17**, **30**, and **33** showed potent inhibitory activity (>80%). The effect of compound **33** as analgesics was tested in rodent models of inflammatory and neuropathic pain.

2.3. Effect of compound 33 on formalin-induced pain

As illustrated in Figure 1, the rats treated with the mixture of formalin and vehicle showed significant increase of the time spent in licking, shaking or biting the affected paw during both the early and late phases. However, **33** (60 μg, sc) injected with formalin significantly reduced the response duration in the both phases [$^{*}P$ <0.05 vs vehicle group (Student's unpaired t-test)]. The response duration of pain behaviors in the vehicle and **33** groups in the early phase was 47.05 ± 8.53 and 14.82 ± 7.23 s, respectively. In the late phase, the time spent in licking, shaking or biting the paw in each group was 334.21 ± 51.10 and 129.31 ± 48.11 s, respectively. These results indicate that compound **33** is effective in relieving both nociceptive and inflammatory pain when treated locally.



Scheme 1. Reagents and conditions: (i) DMF, rt, 20 h; (ii) **c1** or **c2**, PyBOP, DIEA, DMF, rt, 16 h.



Scheme 2. Reagents and conditions: (i) EDCI, DMF, rt, 17 h; (ii) **c1** or **c3**, PyBOP, DIEA, DMF, rt, 16 h.

Table 1
The structures of synthesized compounds

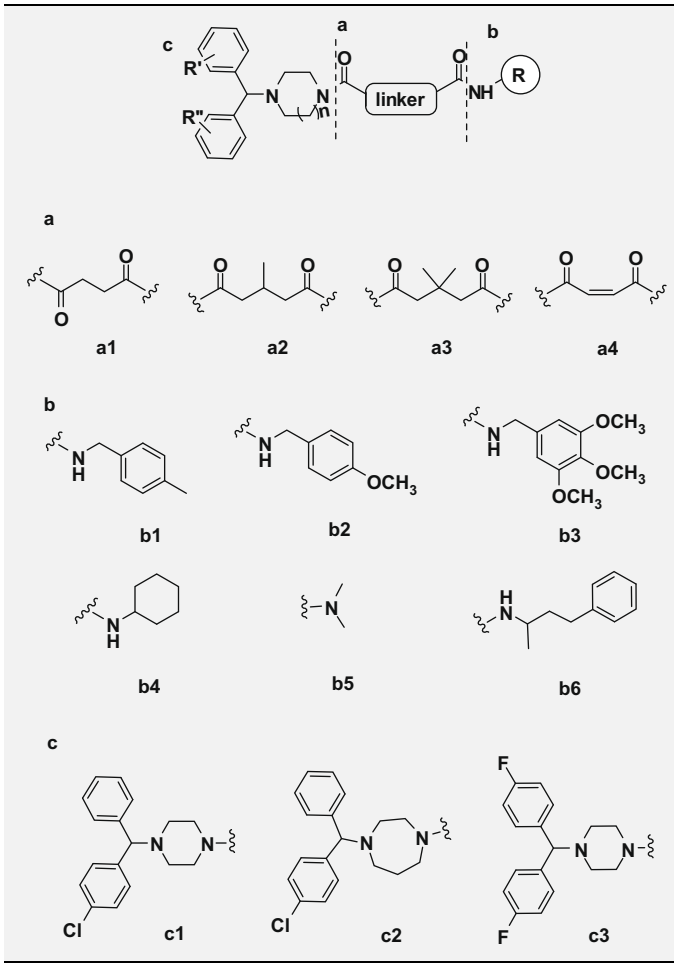


Table 2
Bradykinin inhibitory activity of synthesized compounds at 1 μM concentration in the guinea-pig ileum

Compd #	Structure	% Inhibition
1	**a1b1c1**	67.91 ± 2.51
2	**a1b2c1**	57.25 ± 2.19
3	**a1b3c1**	65.19 ± 3.68
4	**a1b4c1**	79.03 ± 2.39
5	**a1b1c2**	67.65 ± 9.64
6	**a1b2c2**	80.86 ± 1.79
7	**a1b3c2**	72.62 ± 1.68
8	**a1b4c2**	71.68 ± 3.32
9	**a2b1c1**	64.80 ± 3.62
10	**a2b2c1**	64.88 ± 4.15
11	**a2b3c1**	53.10 ± 4.57
12	**a2b4c1**	60.84 ± 5.70
13	**a2b5c1**	35.97 ± 2.24
14	**a2b1c2**	81.35 ± 3.62
15	**a2b2c2**	78.28 ± 2.34
16	**a2b3c2**	69.70 ± 2.84
17	**a2b4c2**	80.27 ± 2.93
18	**a3b1c1**	32.92 ± 5.58
19	**a3b2c1**	31.59 ± 2.73
20	**a3b3c1**	49.15 ± 3.01
21	**a3b4c1**	28.01 ± 6.11
22	**a4b1c1**	78.53 ± 2.96
23	**a4b2c1**	74.09 ± 5.16
24	**a4b4c1**	57.37 ± 5.38
25	**a4b6c1**	77.54 ± 2.92
26	**a4b1c2**	79.44 ± 8.83
27	**a4b2c2**	75.01 ± 6.28
28	**a4b4c2**	66.01 ± 3.12
29	**a4b6c2**	79.73 ± 5.81
30	**a4b1c3**	84.55 ± 1.28
31	**a4b2c3**	79.59 ± 2.07
32	**a4b4c3**	65.43 ± 6.23
33	**a4b6c3**	86.61 ± 1.20
Icatibant		100.00 ± 0.00

2.4. Effect of compound 33 on neuropathic pain

Before nerve injury, animals rarely exhibited tail withdrawal responses to von Frey filaments, cold (4 °C) and warm (40 °C) water stimuli (Fig. 2). However, 2 weeks after nerve injury, rats showed significant decrease of tail withdrawal threshold and latencies in response to mechanical and, cold and warm water stimuli, respectively [P <0.05 vs presurgical value (Pre), Paired t-test]. We regarded these increased sensitivities as the signs of mechanical, cold and warm allodynia, and these neuropathic animals were subjected to the treatment of **33**. As shown in Figure 2, compound **33** (60 mg, ip) administered significantly suppressed the cold and warm, but not mechanical, allodynia until 5 hr after the injection unlike vehicle group (*P <0.05 vs baseline, one-way repeated measured ANOVA followed by Bonferroni's t-test). This discrepancy between the two efficacies may be due to that the spinal ascending pathways involved in the processing of tactile allodynia is segregated from that of cold and warm allodynia.[36–38] These results indicate that compound **33** has an analgesic effect against cold and warm allodynia induced by peripheral nerve injury.

3. Conclusion

A series of non-peptide diamide compounds were designed and synthesized with different linkers and evaluated their bradykinin inhibitory activity using guinea-pig smooth muscle. The synthetic method for the preparation of the diamide compounds was simple with mild conditions. Most of the compounds synthesized showed inhibitory activity on bradykinin-induced contraction at 1 μM concentration in the guinea-pig ileum. The most potent compound **33** among the series demonstrated analgesic efficacies to formalin-induced nociceptive and inflammatory pain, and cold and warm allodynia following peripheral nerve injury.

4. Experimental

4.1. Materials and methods

Melting points were measured on an electrothermal digital melting point (Buchi, Germany) without calibration. ^{1}H NMR and ^{13}C NMR spectra were recorded on Varian NMR AS and Varian Unity Inova 400 and 500 MHz NMR spectrometers. Chemical shifts were reported in parts per million (δ) units relative to the solvent peak. The ^{1}H NMR data were reported as peak multiplicities: s for singlet; d for doublet; t for triplet; and m for multiplet. Coupling constants were recorded in hertz. Mass spectral data were obtained from the Korea Basic Science Institute (Daegu) on a Jeol JMS 700 high resolution mass spectrometer. Reagents were of commercial grade and were purchased from Sigma–Aldrich Co., Merck, Ducksan Pure Chemical Co.

4.2. General procedure for substituted monoamide derivatives (a1by–a3by)

Anhydrides **a1**–**3** (2 mmol) were dissolved in 3 mL of dimethylformamide (DMF). Amines **b1**–**5** (2 mmol) were added and stirred at room temperature for 20 h. Twenty millilitres of 10% HCl was put into the reaction solution, and extracted with 30 mL of EtOAc.

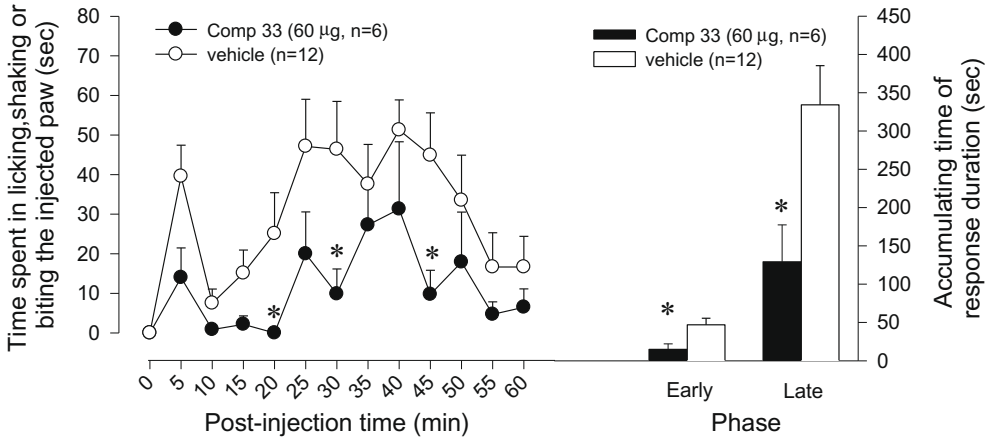


Figure 1. The effect of subcutaneous injection of compound **33** (60 μg) on formalin-induced pain response. Values represent time (s) spent paw licking or biting in each 5 min interval following subcutaneous injection of formalin (5%, 50 μl). In the bar graph, values represent licking or biting during the early (0–10 min) and late (10–60 min) phases after formalin injection. Data represent means ± SEM. Significance of differences was analyzed by Student's unpaired *t*-test. **P* <0.05.

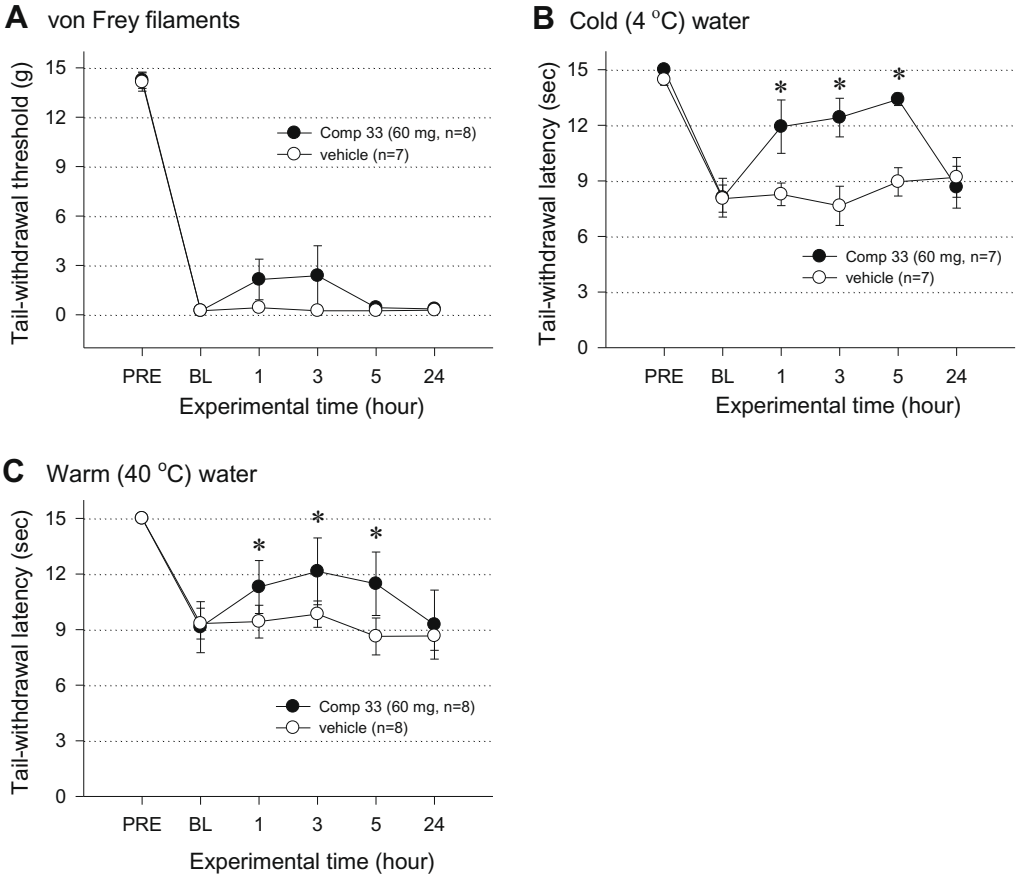


Figure 2. Effect of compound **33** on mechanical (A), cold (B), and warm (C) allodynia induced by rat tail nerve injury (TNI). 'PRE' represents the 1 day prior to nerve injury. At 2 weeks after nerve injury, rats were given baseline (BL) test and then were subjected to the injection of compound **33** (60 mg, ip) or vehicle (Methyl pyrrolidone/Tween80/Saline = 1:1:8). Behavioral tests were re-performed 1, 3, 5, and 24 h after the injection. **P* <0.05 versus BL (one-way repeated measured ANOVA followed by Bonferroni's *t*-test).

The organic layer was washed with 20 mL of 10% HCl, and then washed with 20 mL of a saturated NaCl solution twice. The organic layer was collected, dried over anhydrous $MgSO_4$, and filtered under reduced pressure. The organic solvent in the filtrate was removed under reduced pressure. The residue was recrystallized from EtOAc and methanol to afford desired compounds.

4.2.1. 3-(4-Methylbenzylcarbamoyl)propanoic acid (a1b1)

White solid, yield: 77%; mp 147–149 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.18–7.14 (m, 4CH), 5.90 (br s, NH), 4.42 (s, CH_2), 2.74 (t, J = 6.0 Hz, CH_2), 2.55 (t, J = 6.0 Hz, CH_2) 2.34 (s, CH_3); HR-FABMS calcd for $C_{12}H_{16}NO_3$ (M^++1): 222.1130, found: 222.1127.

4.2.2. 3-(4-Methoxybenzylcarbamoyl)propanoic acid (a1b2)

Light yellow solid, yield: 56%; mp 158–160 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.24–7.19 (m, 2CH), 6.99–6.89 (m, 2CH), 5.87 (br s, NH), 4.40 (s, CH_2), 3.80 (s, OCH_3), 2.73 (t, J = 6.4 Hz, CH_2), 2.56 (t, J = 6.4 Hz, CH_2); HR-FABMS calcd for $C_{12}H_{16}NO_4$ (M^++1): 238.1079, found: 238.1076.

4.2.3. 3-(3,4,5-Trimethoxybenzylcarbamoyl)propanoic acid (a1b3)

Light yellow solid, yield: 18%; mp 122–125 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.49 (s, 2CH), 4.38 (s, CH_2), 3.85 (s, 3OCH_3), 2.74 (t, J = 6.8 Hz, CH_2), 2.55 (t, J = 6.8 Hz, CH_2); HR-FABMS calcd for $C_{14}H_{20}NO_6$ (M^++1): 298.1291, found: 298.1286.

4.2.4. 3-(Cyclohexylcarbamoyl)propanoic acid (a1b4)

White solid, yield: 60%; mp 170–173 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.58 (s, NH), 3.81–3.74 (m, CH), 2.69 (t, J = 6.4 Hz, CH_2), 2.51 (t, J = 6.4 Hz, CH_2), 1.95–1.90 (m, CH_2), 1.75–1.60 (m, 2CH_2), 1.42–1.13 (m, 2CH_2); HR-FABMS calcd for $C_{10}H_{17}NO_3Na$ (M^++Na): 222.1106, found: 222.1103.

4.2.5. 4-(4-Methylbenzylcarbamoyl)-3-methylbutanoic acid (a2b1)

White solid, yield: 96%; mp 133–134 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.16 (s, 4CH), 5.90 (s, NH), 4.41 (d, J = 6.0 Hz, CH_2), 2.43–2.46 (m, CH), 2.40 (t, J = 7.2 Hz, CH_2), 2.34 (s, CH_3), 2.28 (t, J = 6.4 Hz, CH_2), 1.08 (d, J = 6.4 Hz, CH_3); HR-FABMS calcd for $C_{14}H_{20}NO_3$ (M^++1): 250.1443, found: 250.1444.

4.2.6. 4-(4-Methoxybenzylcarbamoyl)-3-methylbutanoic acid (a2b2)

White solid, yield: 74%; mp 89–90 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.20 (d, J = 9.2 Hz, 2CH), 6.87 (d, J = 9.2 Hz, 2CH), 5.94 (s, NH), 4.38 (d, J = 5.6 Hz, CH_2), 3.80 (s, OCH_3), 2.36–2.48 (m, CH), 2.32 (dd, J = 6.4 Hz, CH_2), 2.23 (dd, J = 6.4 Hz, CH_2), 1.06 (d, J = 6.4 Hz, CH_3); HR-FABMS calcd for $C_{14}H_{20}NO_4$ (M^++1): 266.1392, found: 266.1382.

4.2.7. 4-(3,4,5-Trimethoxybenzylcarbamoyl)-3-methylbutanoic acid (a2b3)

White solid, yield: 28%; mp 118–119 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.50 (s, 2CH), 4.39 (d, J = 5.6 Hz, CH_2), 3.85 (s, 3OCH_3), 2.41–2.48 (m, CH), 2.45 (d, J = 4.4 Hz, CH_2), 2.31 (d, J = 5.6 Hz, CH_2), 1.10 (d, J = 6.8 Hz, CH_3); HR-FABMS calcd for $C_{16}H_{24}NO_6$ (M^++1): 326.1604, found: 326.1606.

4.2.8. 4-(Cyclohexylcarbamoyl)-3-methylbutanoic acid (a2b4)

White solid, yield: 69%; mp 133–134 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.56 (s, NH), 3.73–3.84 (m, CH), 2.35–2.45 (m, CH), 2.30; 2.27 (dd, J = 5.6; 6.0 Hz, CH_2), 2.24; 2.21 (dd, J = 7.2 Hz, CH_2), 1.93 (d, J = 12.0 Hz, CH_2), 1.72 (d, J = 12.4 Hz, CH_2), 1.63 (d, J = 12.8 Hz, CH_2), 1.41; 1.34 (dd, J = 12.0 Hz, CH_2), 1.16 (t, J = 11.6 Hz, CH_2), 1.08 (d, J = 6.4 Hz, CH_3); HR-FABMS calcd for $C_{12}H_{22}NO_3$ (M^++1): 228.1600, found: 228.1601.

4.2.9. 4-(Dimethylcarbamoyl)-3-methylbutanoic acid (a2b5)

White solid, yield: 2%; mp 116–117 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.05 (s, CH_3), 2.98 (s, CH_3), 2.35–2.49 (m, CH), 2.33 2.31 (dd, J = 5.6 Hz, CH_2), 2.27; 2.23 (dd, J = 7.6; 7.2 Hz, CH_2), 1.08 (d, J = 6.0 Hz, CH_3); HR-FABMS calcd for $C_8H_{15}NO_3Na$ (M^++Na): 196.0950, found: 196.0949.

4.2.10. 4-(4-Methylbenzylcarbamoyl)-3,3-dimethylbutanoic acid (a3b1)

White solid, yield: 79%; mp 85–86 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 12.09 (s, OH), 8.32 (t, J = 5.8 Hz, NH), 7.10–7.15 (m, 4CH), 4.21 (d, J = 6.0 Hz, CH_2), 2.28 (s, CH_2), 2.27 (s, CH_3), 2.19 (s, CH_2), 1.02 (s, 2CH_3); HR-FABMS calcd for $C_{15}H_{22}NO_3$: (M^++1): 264.1600, found: 264.1602.

4.2.11. 4-(4-Methoxybenzylcarbamoyl)-3,3-dimethylbutanoic acid (a3b2)

White solid, yield: 85%; mp 102–103 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 12.05 (s, OH), 8.32 (t, J = 5.8 Hz, NH), 7.17 (d, J = 8.0 Hz, 2CH), 6.87 (d, J = 8.0 Hz, 2CH), 4.18 (d, J = 5.6 Hz, CH_2), 3.72 (s, OCH_3), 2.28 (s, 2CH_2), 1.04 (s, CH_3), 1.02 (s, CH_3); HR-FABMS calcd for $C_{15}H_{22}NO_4$ (M^++1): 280.1549, found: 280.1542.

4.2.12. 4-(3,4,5-Trimethoxybenzylcarbamoyl)-3,3-dimethylbutanoic acid (a3b3)

Light yellow solid, yield: 54%; mp 118–119 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 12.07 (s, OH), 8.33 (t, J = 5.8 Hz, NH), 6.56 (d, J = 8.0 Hz, 2CH), 4.21 (d, J = 6.0 Hz, CH_2), 3.74 (s, 2OCH_3), 3.62 (s, OCH_3), 2.29 (s, CH_2), 2.21 (s, CH_2), 1.05 (s, 2CH_3); HR-FABMS calcd for $C_{17}H_{26}NO_6$: (M^++1): 340.1760, found: 340.1757.

4.2.13. 4-(Cyclohexylcarbamoyl)-3,3-dimethylbutanoic acid (a3b4)

White solid, yield: 67%; mp 77–78 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 12.07 (s, OH), 7.76 (d, J = 8.0 Hz, NH), 3.53 (s, CH), 2.25 (s, CH_2), 2.09 (s, CH_2), 1.52–1.72 (m, 2CH_2), 1.04–1.30 (m, 3CH_2), 1.05 (s, CH_3), 1.01 (s, CH_3); HR-FABMS calcd for $C_{13}H_{24}NO_3$ (M^++1): 242.1756, found: 242.1752.

4.3. General procedure for (*Z*)-3-(substituted)acrylic acid derivatives (a4by)

Maleic acid (1 mmol) was dissolved in 3 mL of DMF. Then, 1 mmol of 1-(3-dimethylaminopropyl)-3-ethyl-carbodiamide HCl (EDCI) was added and stirred at room temperature for 1 hour. After 1 hour, amine (**b1**–**2**, **b4** or **b6)** was added and stirred at room temperature for 16 h. Thirty millilitres of 10% HCl was put into the reaction solution, and extracted with 50 mL of EtOAc. The organic layer was washed with 30 mL of 10% HCl, and then washed with 20 mL of a saturated NaCl solution twice. The organic layer was collected, dried over anhydrous $MgSO_4$, and filtered under reduced pressure. The organic solvent in the filtrate was removed under reduced pressure. The residue was recrystallized from EtOAc and methanol to obtain desired compounds.

4.3.1. (*Z*)-3-(4-Methylbenzylcarbamoyl)acrylic acid (a4b1)

White solid, yield: 73%; mp 143–144 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.923–6.945 (m, 4CH), 6.325 (d, J = 12.8 Hz, CH), 6.077 (d, J = 12.8 Hz, CH), 4.225 (s, CH_2), 2.350 (s, CH_3); HR-EIMS calcd for $C_{12}H_{13}NO_3$: (M^+): 219.0895, found: 219.0895.

4.3.2. (*Z*)-3-(4-Methoxybenzylcarbamoyl)acrylic acid (a4b2)

White solid, yield: 72%; mp 140–142 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.766–6.800 (m, 2CH), 6.553–6.588(m, 2CH), 6.402 (d, J = 12.8 Hz, CH), 6.051 (d, J = 12.8 Hz, CH), 4.395 (s, CH_2), 3.810 (s, CH_3); HR-EIMS calcd for $C_{12}H_{13}NO_4$: (M^+): 235.0845, found: 235.0843.

4.3.3. (*Z*)-3-(Cyclohexylcarbamoyl)acrylic acid (a4b4)

White solid, yield: 34%; mp 150–151 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.357 (d, J = 12.8 Hz, CH), 6.197 (d, J = 12.8 Hz, CH), 3.812–3.906 (m, CH), 2.046–1.959 (m, CH_2), 1.800–1.747 (m, CH_2), 1.705–1.635 (m, 1/2CH_2), 1.458–1.348 (m, CH_2), 1.292–1.156 (m, CH_2, 1/2CH_2); HR-EIMS calcd for $C_{10}H_{15}NO_3$: (M^+): 197.1052, found: 197.1055.

4.3.4. (*Z*)-3-(4-Phenylbutan-2-yl-carbamoyl)acrylic acid (a4b6)

White solid, yield: 91%; mp 113–115 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.08–7.21 (m, 5CH), 6.75 (d, J = 12.8 Hz, CH), 6.510 (d, J = 12.8 Hz, CH), 3.762–3.796 (m, CH), 2.602–2.630 (m, CH_2), 1.838–1.402 (m, CH_2), 1.302 (d, J = 6.8 Hz, CH_3); HR-EIMS calcd for $C_{14}H_{17}NO_3$: (M^+): 247.1208, found: 247.1205.

4.4. General procedure for substituted diamide derivatives (1–27)

Monoamide derivatives **axby** (0.5 mmol), 0.55 mmol of amines **c1**–**3**, and benzotriazol-1-yloxytripyrrolidino phosphonium hexafluorophosphate (PyBOP, 0.55 mmol) were dissolved in 3 mL of DMF. Then, 1.0 mmol of *N*,*N*-diisopropylethylamine (DIEA) was added and stirred at room temperature for 16 h. Twenty millilitres of 10% HCl was put into the reaction solution, and extracted with 30 mL of EtOAc. The organic layer was washed with 20 mL of 10% HCl, 20 mL of a saturated $NaHCO_3$ solution twice and with 20 mL of a saturated NaCl solution twice. The organic layer was collected, dried over anhydrous $MgSO_4$, and filtered. The organic solvent in the filtrate was removed under reduced pressure. The residue was purified by column chromatography with EtOAc and methanol (20:1) to afford desired compounds.

4.4.1. 4-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-*N*-(4-methylbenzyl)-4-oxobutanamide (1)

Light yellow solid, yield: 60%; mp 75–78 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.37–7.34 (m, 4CH), 7.32–7.13 (m, 5CH), 7.15–7.11 (t, J = 4.4 Hz, CH_2), 3.46 (t, J = 4.4 Hz, CH_2), 2.64 (t, J = 6.4 Hz, CH_2), 2.53 (t, J = 6.4 Hz, CH_2), 2.36–2.32 (m, $2CH_2$), 2.31(s, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.17, 170.26, 141.39, 140.61, 136.63, 135.38, 132.68, 129.05, 128.98, 128.68, 128.62, 127.62, 127.50, 127.30, 74.99, 51.61, 51.25, 45.23, 43.07, 31.10, 28.37, 20.92; HR-FABMS calcd for $C_{29}H_{33}ClN_3O_2$: (M^++1): 490.2261, found: 490.2249.

4.4.2. 4-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-*N*-(4-methoxybenzyl)-4-oxobutanamide (2)

Light yellow solid, yield: 44%; mp 119–121 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.36–7.32 (m, 4CH), 7.29–7.19 (m, 5CH), 7.17–7.15 (m, $2CH_2$), 6.82–6.78 (m, 2CH), 4.31(s, CH_2), 4.19 (s, CH), 3.74 (s, OCH_3), 3.59–3.50 (m, CH_2), 3.44–3.31 (m, CH_2), 2.61 (t, J = 6.4 Hz, CH_2), 2.94 (t, J = 6.4 Hz, CH_2), 2.34–2.33 (m, CH_2), 2.30–2.28 (m, CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.15, 170.21, 158.85, 141.50, 140.70, 130.55, 129.08, 128.98, 128.80, 128.73, 127.74, 127.40, 113.97, 75.14, 55.25, 51.76, 51.42, 45.37, 42.97, 31.38, 28.58; HR-FABMS calcd for $C_{29}H_{33}ClN_3O_3$: (M^++1): 506.2210, found: 506.2196.

4.4.3. 4-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-4-oxo-*N*-(3,4,5-trimethoxybenzyl)butanamide (3)

White solid, yield: 20%; mp 71–73 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.37–7.31 (m, 4CH), 7.30–7.25 (m, 5CH), 6.50(s, 2CH), 4.35(s, CH_2), 4.21 (s, CH), 3.84 (s, $3OCH_3$), 3.56–3.46 (m, $2CH_2$), 2.66 (t, J = 6.4 Hz, CH_2), 2.54 (t, J = 6.4 Hz, CH_2), 2.38–2.27 (m, $2CH_2$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 172.62, 170.45, 153.56, 137.31, 134.48, 129.35, 129.01, 128.00, 106.97, 104.78, 75.40, 61.04, 56.52, 56.35, 51.99, 46.56, 43.96, 31.62, 26.67; HR-FABMS calcd for $C_{31}H_{37}ClN_3O_5$: (M^++1): 566.2422, found: 566.2415.

4.4.4. 4-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-*N*-cyclohexyl-4-oxobutanamide (4)

Yellow solid, yield: 52%; mp 59–61 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.36–7.34 (m, 4CH), 7.30–7.19 (m, 5CH), 5.34 (s, NH), 4.21 (s, CH), 3.60–3.55 (m, CH_2), 3.49–3.46 (m, CH_2), 2.61 (t, J = 6.8 Hz, CH_2), 2.46 (t, J = 6.8 Hz, CH_2), 2.35–2.33 (m, $2CH_2$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.29, 170.31, 141.38, 140.60, 131.33, 129.79, 129.00, 128.68, 128.62, 127.64, 127.29, 75.04, 51.71, 51.35, 48.00, 45.34, 32.86, 31.52, 28.58, 25.44, 24.68; HR-FABMS calcd for $C_{27}H_{35}ClN_3O_2$: (M^++1): 468.2418, found: 468.2407.

4.4.5. 4-(4-((4-Chlorophenyl)(phenyl)methyl)-1,4-diazepan-1-yl)-*N*-(4-methylbenzyl)-4-oxobutanamide (5)

Light yellow solid, yield: 58%; mp 72–74 °C; ^{1}H NMR ($CDCl_3$, 400 MHz); δ 7.36–7.34 (m,4CH), 7.23–7.18 (m, 5CH), 7.17–7.11 (m, 4CH), 6.34 (s, NH), 4.55 (s, CH), 4.39 (s, CH_2), 3.66–3.45 (m, $2CH_2$), 2.72–2.66 (m, $2CH_2$), 2.67–2.53 (m, 4CH), 2.32 (s, CH_3), 1.82–1.73 (m, CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.33, 171.55, 171.45, 142.48, 141.60, 136.77, 135.45, 132.63, 132.55, 129.17, 129.03, 128.95, 128.71, 128.65, 128.60, 127.67, 127.57, 127.26, 127.19, 74.21, 74.02, 54.27, 53.63, 53.24, 52.64, 48.65, 46.41, 46.37, 44.58, 43.19, 31.43, 28.92, 28.75, 28.50, 27.50, 21.02; HR-FABMS calcd for $C_{30}H_{35}ClN_3O_2$: (M^++1): 504.2418, found: 504.2393.

4.4.6. 4-(4-((4-Chlorophenyl)(phenyl)methyl)-1,4-diazepan-1-yl)-*N*-(4-methoxybenzyl)-4-oxobutanamide (6)

Light yellow solid, yield: 21%; mp 57–60 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.36–7.31 (m, 4CH), 7.29–7.20 (m, 5CH), 7.19–7.17 (m, 2CH), 6.84–6.81 (m, 2CH), 6.85 (s, NH), 4.66 (d, J = 6.8 Hz, CH), 4.35 (d, J = 5.6 Hz, CH_2), 3.77 (s, OCH_3), 3.63–3.55 (m, CH_2), 3.54–3.41 (m, CH_2), 2.70–2.52 (m, $4CH_2$), 1.81–1.71 (m, CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.31, 171.52, 171.44, 158.80, 142.41, 141.63, 132.64, 132.55, 129.04, 128.95, 128.72, 128.66, 128.60, 127.68, 127.60, 127.27, 127.18, 113.93, 74.22, 74.02, 55.23, 54.29, 53.66, 53.26, 52.66, 48. 68, 46.43, 44.61, 42.93, 31.47, 28.96, 28.80, 28.54, 27.52; HR-FABMS calcd for $C_{30}H_{35}ClN_3O_3$: (M^++1): 520.2367, found: 520.2346.

4.4.7. 4-(4-((4-Chlorophenyl)(phenyl)methyl)-1,4-diazepan-1-yl)-4-oxo-*N*-(3,4,5-trimethoxybenzyl)butanamide (7)

Light yellow solid, yield: 41%; mp 83–85 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.37–7.33 (m, 4CH), 7.29–7.17 (m, 5CH), 6.52 (s, 2CH), 4.55 (d, J = 6.4 Hz, CH), 4.37 (d, J = 5.6 Hz, CH_2), 3.84 (s, $3OCH_3$), 3.65–3.62 (m, $1/2CH_2$), 3.59–3.55 (m, CH_2), 3.48–3.45 (m, $1/2CH_2$), 2.74–2.53 (m, $4CH_2$), 1.83–1.72 (m, CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.44, 171.47, 171.39, 153.34, 142.41, 141.64, 137.12, 134.27, 134.24, 132.70, 132.60, 129.07, 129.00, 128.70, 128.64, 127.71, 127.63, 127.32, 127.22, 104.62, 74.23, 74.05, 60.80, 56.11, 54.24, 53.72, 53.34, 52.69, 48.68, 46.46, 44.58, 43.75, 31.55, 28.96, 28.81, 28.56, 27.59; HR-FABMS calcd for $C_{32}H_{39}ClN_3O_5$: (M^++1): 580.2578, found: 580.2588.

4.4.8. 4-(4-((4-Chlorophenyl)(phenyl)methyl)-1,4-diazepan-1-yl)-*N*-cyclohexyl-4-oxobutanamide (8)

White solid, yield: 55%; mp 68–70 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.36–7.32 (m, 4CH), 7.29–7.18 (m, 5CH), 6.16 (s, NH), 4.56 (s, CH), 3.76–3.69 (m, CH), 3.65 (t, J = 5.6 Hz, $1/2CH_2$), 3.58 (t, J = 5.6 Hz, CH_2), 3.45 (t, J = 5.6 Hz, $1/2CH_2$), 2.69–2.49 (m, $4CH_2$), 1.89–1.85 (m, CH_2), 1.81–1.74 (m, CH_2), 1.71–1.65 (m, CH_2), 1.61–1.56 (m, $1/2CH_2$), 1.38–1.30 (m, CH_2), 1.19–1.06 (m, CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.62, 171.55, 142.44, 142.39, 141.66, 141.61, 132.68, 132.59, 129.06, 128.97, 128.74, 128.68, 128.61, 127.70, 127.62, 127.30, 127.21, 74.27, 74.13, 54.38, 53.75, 53.28, 52.73, 48.73, 48.04, 46.47, 46.41, 44.66, 33.00, 31.83, 29.20, 29.04, 28.64, 27.58, 25.54, 24.75; HR-FABMS calcd for $C_{28}H_{37}ClN_3O_2$: (M^++1): 482.2574, found: 482.2551.

4.4.9. 5-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-*N*-(4-methylbenzyl)-3-methyl-5-oxopentanamide (9)

Light yellow solid, yield: 65%; mp 62–63 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.35 (d, J = 8.0 Hz, 2CH), 7.29 (d, J = 7.2 Hz, 2CH), 7.26 (t, J = 8.2 Hz, 2CH), 7.22 (t, J = 7.3 Hz, CH), 7.16 (d, J = 8.0 Hz,

2CH), 7.11 (d, J = 7.2 Hz, 2CH), 6.29 (s, NH), 4.37 (d, J = 5.6 Hz, CH_2), 4.21 (s, CH), 3.59 (t, J = 5.6 Hz, CH_2), 3.48 (q, J = 5.6 Hz, CH_2), 2.43 (d, J = 6.8 Hz, CH_2), 2.34–2.39 (m, CH_2), 2.32 (s, CH_3), 2.28 (d, J = 6.0 Hz, CH_2), 2.25 (d, J = 6.4 Hz, CH_2), 2.13–2.23 (m, CH), 1.03 (d, J = 6.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.68, 170.49, 141.54, 135.44, 132.82, 129.28, 129.08, 128.80, 128.74, 127.73, 127.40, 75.19, 52.07, 51.58, 45.96, 43.20, 43.10, 41.65, 39.43, 28.49, 21.06, 20.28; HR-FABMS calcd for $C_{31}H_{37}ClN_3O_2$: (M^++1): 518.2574, found: 518.2584.

4.4.10. 5-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-*N*-(4-methoxybenzyl)-3-methyl-5-oxopentanamide (10)

White solid, yield: 63%; mp 61–62 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.35 (d, J = 8.4 Hz, 2CH), 7.29 (d, J = 7.6 Hz, 2CH), 7.26 (t, J = 8.2 Hz, 2CH), 7.23 (d, J = 7.6 Hz, CH), 7.20 (d, J = 8.8 Hz, 2CH), 6.84 (d, J = 8.0 Hz, 2CH), 6.31 (s, NH), 4.35 (d, J = 5.6 Hz, CH_2), 4.21 (s, CH), 3.79 (s, OCH_3), 3.59 (t, J = 5.0 Hz, CH_2), 3.48 (q, J = 5.2 Hz, CH_2), 2.42 (d, J = 7.2 Hz, CH_2), 2.34–2.38 (m, CH_2), 2.28 (d, J = 6.4 Hz, CH_2), 2.25 (d, J = 6.0 Hz, CH_2), 2.18–2.22 (m, CH), 1.03 (d, J = 6.8 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.66, 170.49, 158.87, 141.53, 140.74, 132.80, 130.61, 129.07, 128.79, 128.72, 127.72, 127.39, 113.99, 75.17, 55.25, 52.05, 51.56, 45.95, 43.07, 42.89, 41.64, 39.40, 28.48, 20.28; HR-FABMS calcd for $C_{31}H_{37}ClN_3O_3$: (M^++1): 534.2523, found: 534.2528.

4.4.11. 5-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-*N*-(3,4,5-trimethoxybenzyl)-3-methyl-5-oxopentanamide (11)

White solid, yield: 71%; mp 72–73 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.35 (d, J = 8.4 Hz, 2CH), 7.29 (d, J = 6.8 Hz, 2CH), 7.27 (t, J = 8.4 Hz, 2CH), 7.24 (d, J = 7.2 Hz, CH), 7.22 (d, J = 7.2 Hz, 2CH), 6.56 (s, NH), 6.51 (s, 2CH), 4.35 (d, J = 5.6 Hz, CH_2), 4.21 (s, CH), 3.83 (d, J = 4.8 Hz, 3OCH_3), 3.59 (t, J = 4.0 Hz, CH_2), 3.49 (q, J = 4.0 Hz, CH_2), 2.41 (d, J = 7.2 Hz, CH_2), 2.31–2.38 (m, CH_2), 2.29 (d, J = 6.0 Hz, CH_2), 2.25 (d, J = 8.4 Hz, CH_2), 2.14–2.20 (m, CH), 1.05 (d, J = 6.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.81, 170.45, 153.27, 140.45, 137.06, 134.36, 132.99, 129.12, 128.85, 128.79, 127.75, 127.54, 104.70, 75.28, 60.78, 56.05, 52.02, 51.55, 46.29, 46.24, 43.62, 43.00, 41.40, 39.30, 28.55, 26.41, 26.33, 20.36; HR-FABMS calcd for $C_{33}H_{41}ClN_3O_5$: (M^++1): 594.2735, found: 594.2757.

4.4.12. 5-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-*N*-cyclohexyl-3-methyl-5-oxopentanamide (12)

White solid, yield: 73%; mp 75–76 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.35 (d, J = 7.2 Hz, 2CH), 7.29 (d, J = 7.6 Hz, 2CH), 7.26 (t, J = 8.0 Hz, 2CH), 7.23 (d, J = 7.6 Hz, CH), 7.22 (d, J = 7.2 Hz, 2CH), 5.97 (s, NH), 4.21 (s, CH), 3.69–3.79 (m, CH), 3.61 (t, J = 4.8 Hz, CH_2), 3.51 (q, J = 4.8 Hz, CH_2), 2.43 (d, J = 6.8 Hz, CH_2), 2.31–2.39 (m, CH_2), 2.23 (d, J = 7.6 Hz, CH_2), 2.16–2.20 (m, CH), 2.12 (d, J = 6.0 Hz, CH_2), 1.88 (d, J = 8.8 Hz, CH_2), 1.69 (d, J = 12.0 Hz, CH_2), 1.59 (d, J = 12.4 Hz, CH_2), 1.35 (q, J = 12.7 Hz, CH_2), 1.14 (t, J = 11.4 Hz, CH_2), 1.02 (d, J = 6.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.75, 170.60, 141.53, 140.73, 132.81, 129.08, 128.79, 128.72, 127.72, 127.39, 75.20, 52.10, 51.60, 47.98, 46.01, 41.66, 39.29, 33.16, 33.13, 28.59, 25.51, 24.81, 20.15; HR-FABMS calcd for $C_{29}H_{39}ClN_3O_2$: (M^++1): 496.2731, found: 496.2724.

4.4.13. 5-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-*N*,*N*,3-trimethyl-5-oxopentanamide (13)

Light yellow gel, yield: 8%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.35 (d, J = 7.2 Hz, 2CH), 7.29 (d, J = 7.6 Hz, 2CH), 7.26 (t, J = 8.2 Hz, 2CH), 7.23 (d, J = 8.0 Hz, CH), 7.21 (d, J = 7.2 Hz, 2CH), 4.21 (s, CH), 3.60–3.71 (m, CH_2), 3.48–3.59 (m, CH_2), 3.01 (s, CH_3), 2.91 (s, CH_3), 2.57; 2.53 (dd, J = 5.2; 5.6 Hz, CH), 2.27–2.48 (m, 3CH_2), 2.16–2.26 (m, 1/2CH_2), 2.00–2.13 (m, 1/2CH_2), 1.03 (d, J = 6.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.77, 170.60, 141.44, 140.59, 132.83, 129.13, 128.79, 127.75, 75.11, 51.95, 51.52, 46.37, 46.32, 41.53, 40.13, 37.61, 27.58, 20.14; HR-FABMS calcd for $C_{25}H_{33}ClN_3O_2$: (M^++1): 442.2261, found: 442.2259.

4.4.14. 5-(4-((4-Chlorophenyl)(phenyl)methyl)-1,4-diazepan-1-yl)-*N*-(4-methylbenzyl)-3-methyl-5-oxopentanamide (14)

Light yellow solid, yield: 75%; mp 53–54 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.30–7.36 (m, 4CH), 7.23–7.29 (m, 3CH), 7.19–7.23 (m, 2CH), 7.17 (d, J = 8.4 Hz, 2CH), 7.12 (d, J = 8.0 Hz, 2CH), 6.42 (s, NH), 4.55 (d, J = 4.8 Hz, CH), 4.39 (d, J = 6.0 Hz, CH_2), 3.61–3.67 (m, 1/2CH_2), 3.49–3.60 (m, CH_2), 3.43–3.48 (m, 1/2CH_2), 2.53–2.68 (m, 2CH_2), 2.22–2.47 (m, 2CH_2), 2.13–2.22 (m, CH), 1.77 (q, J = 5.7 Hz, CH_2), 1.07 (d, J = 6.0 Hz, CH_3) 2.43 (d, J = 6.8 Hz, CH_2), 2.34–2.39 (m, CH_2), 2.32 (s, CH_3), 2.28 (d, J = 6.0 Hz, CH_2), 2.25 (d, J = 6.4 Hz, CH_2), 2.13–2.23 (m, CH), 1.03 (d, J = 6.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.88, 171.82, 171.61, 171.54, 142.26, 142.23, 141.50, 136.59, 135.49, 132.46, 132.37, 129.03, 128.89, 128.83, 128.54, 128.47, 127.50, 127.13, 127.05, 74.18, 74.01, 54.52, 53.48, 53.12, 52.36, 48.85, 46.55, 46.12, 44.40, 42.92, 39.45, 39.28, 28.69, 28.35, 27.31, 20.89, 20.18; HR-FABMS calcd for $C_{32}H_{39}ClN_3O_2$: (M^++1): 532.2731, found: 532.2720.

4.4.15. 5-(4-((4-Chlorophenyl)(phenyl)methyl)-1,4-diazepan-1-yl)-*N*-(4-methoxybenzyl)-3-methyl-5-oxopentanamide (15)

Brown solid, yield: 66%; mp 52–53 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.30–7.36 (m, 4CH), 7.24–7.29 (m, 3CH), 7.18–7.23 (m, 2CH), 6.84 (d, J = 6.8 Hz, 2CH), 6.43 (s, NH), 4.55 (d, J = 4.4 Hz, CH), 4.36 (d, J = 5.6 Hz, CH_2), 3.79 (s, OCH_3), 3.61–3.67 (m, 1/2CH_2), 3.53–3.58 (m, CH_2), 3.43–3.48 (m, 1/2CH_2), 2.54–2.68 (m, 2CH_2), 2.22–2.47 (m, 2CH_2), 2.13–2.21 (m, CH), 1.77 (q, J = 6.1 Hz, CH_2), 1.07 (d, J = 6.0 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.85, 171.80, 171.70, 171.65, 158.78, 142.39, 142.34, 141.59, 132.62, 132.53, 130.72, 129.00, 128.94, 128.69, 128.63, 128.58, 127.65, 127.58, 127.26, 127.17, 113.91, 74.34, 74.18, 55.20, 54.67, 53.66, 53.28, 52.50, 48.99, 46.69, 46.27, 44.53, 43.08, 42.80, 39.51, 39.32, 28.84, 28.55, 27.46, 20.35; HR-FABMS calcd for $C_{32}H_{39}ClN_3O_3$: (M^++1): 548.2680, found: 548.2690.

4.4.16. 5-(4-((4-Chlorophenyl)(phenyl)methyl)-1,4-diazepan-1-yl)-*N*-(3,4,5-trimethoxybenzyl)-3-methyl-5-oxopentanamide (16)

White solid, yield: 46%; mp 60–61 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.30–7.35 (m, 4CH), 7.25–7.29 (m, 3CH), 7.18–7.24 (m, 2CH), 6.66 (s, NH), 6.53 (s, 2CH), 4.55 (d, J = 4.0 Hz, CH), 4.37 (d, J=6.0 Hz, CH_2), 3.83 (d, J = 5.2 Hz, 3OCH_3), 3.61–3.67 (m, 1/2CH_2), 3.55–3.59 (m, CH_2), 3.46 (t, J = 4.6 Hz, 1/2CH_2), 2.53–2.69 (m, 2CH_2), 2.26–2.47 (m, 2CH_2), 2.16–2.22 (m, CH), 1.77 (q, J = 5.7 Hz, CH_2), 1.08 (d, J = 5.8 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.98, 171.94, 171.73, 171.69, 153.27, 142.39, 142.31, 141.60, 137.06, 134.43, 132.69, 132.61, 129.03, 128.95, 128.74, 128.67, 128.62, 127.67, 127.59, 127.31, 127.23, 104.69, 74.40, 74.26, 60.79, 56.04, 54.70, 53.73, 53.34, 52.51, 49.06, 46.76, 46.36, 44.56, 43.61, 43.08, 39.50, 39.33, 28.88, 28.74, 27.50, 20.46; HR-FABMS calcd for $C_{34}H_{43}ClN_3O_5$: (M^++1): 608.2891, found: 608.2901.

4.4.17. 5-(4-((4-Chlorophenyl)(phenyl)methyl)-1,4-diazepan-1-yl)-*N*-cyclohexyl-3-methyl-5-oxopentanamide (17)

White solid, yield: 60%; mp 62–63 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.31–7.35 (m, 4CH), 7.25–7.30 (m, 3CH), 7.18–7.24 (m, 2CH), 5.06 (s, NH), 4.55 (s, CH), 3.71–3.82 (m, CH), 3.63–3.70 (m, 1/2CH_2), 3.59 (t, J = 6.0 Hz, CH_2), 3.46–3.50 (m, 1/2CH_2), 2.61–2.68 (m, CH_2), 2.55–2.60 (m, CH_2), 2.35–2.47 (m, CH_2), 2.22–2.33 (m, CH), 2.09–2.15 (m, CH_2), 1.89 (d, J = 12.0 Hz, CH_2), 1.78 (q, J = 6.0 Hz, CH_2), 1.70 (d, J = 13.6 Hz, CH_2), 1.60 (d, J = 12.4 Hz,

CH_2), 1.36 (q, J = 12.5 Hz, CH_2), 1.15 (t, J = 10.2 Hz, CH_2), 1.05 (d, J = 5.8 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.79, 171.74, 170.96, 170.89, 142.34, 141.58, 132.60, 132.51, 129.00, 128.92, 128.67, 128.61, 128.56, 127.63, 127.55, 127.23, 127.15, 74.35, 74.19, 54.70, 53.67, 53.27, 52.50, 49.01, 47.94, 46.73, 46.27, 44.51, 43.23, 43.17, 39.41, 39.25, 33.05, 28.86, 28.61, 27.49, 25.47, 24.78, 20.18; HR-FABMS calcd for $C_{30}H_{41}ClN_3O_2$: (M^++1): 510.2887, found: 510.2864.

4.4.18. 5-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-*N*-(4-methylbenzyl)-3,3-dimethyl-5-oxopentanamide (18)

Light yellow solid, yield: 70%; mp 63–64 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.05 (t, J = 5.8 Hz, NH), 7.35 (d, J = 8.0 Hz, 4CH), 7.30 (d, J = 7.2 Hz, 3CH), 7.26 (t, J = 8.4 Hz, 2CH), 7.18 (d, J = 8.0 Hz, 2CH), 7.10 (d, J = 8.0 Hz, 2CH), 4.38 (d, J = 5.6 Hz, CH_2), 4.20 (s, CH), 3.56–3.66 (m, CH_2), 3.51 (t, J = 5.0 Hz, CH_2), 2.34–2.37 (m, $2CH_2$), 2.33 (s, CH_3), 2.32 (s, $2CH_2$), 1.02 (s, $2CH_3$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.31, 170.73, 141.44, 140.64, 136.52, 136.14, 132.88, 129.13, 129.03, 128.83, 128.76, 127.69, 127.66, 127.45, 75.16, 51.93, 51.56, 47.35, 46.77, 42.89, 41.65, 40.62, 34.42, 29.30, 21.07; HR-FABMS calcd for $C_{32}H_{39}ClN_3O_2$: (M^++1): 532.2731, found: 532.2719.

4.4.19. 5-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-*N*-(4-methoxybenzyl)-3,3-dimethyl-5-oxopentanamide (19)

White solid, yield: 63%; mp 64–65 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.06 (t, J = 5.6 Hz, NH), 7.35 (d, J = 6.4 Hz, 4CH), 7.29 (t, J = 8.1 Hz, 2CH), 7.26 (d, J = 8.8 Hz, 2CH), 7.21 (t, J = 8.8 Hz, CH), 6.83 (d, J = 8.8 Hz, 4CH), 4.35 (d, J = 6.0 Hz, CH_2), 4.20 (s, CH), 3.79 (s, OCH_3), 3.56–3.65 (m, CH_2), 3.51 (t, J = 5.0 Hz, CH_2), 2.33–2.39 (m, $2CH_2$), 2.32 (s, CH_2), 2.30 (s, CH_2), 1.05 (s, $2CH_3$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.23, 170.62, 158.52, 141.35, 140.58, 132.73, 131.29, 128.95, 128.89, 128.72, 128.66, 127.60, 127.34, 113.76, 75.02, 55.11, 51.81, 51.44, 47.22, 46.66, 42.46, 41.56, 40.50, 34.31, 29.17; HR-FABMS calcd for $C_{32}H_{39}ClN_3O_3$: (M^++1): 548.2680, found: 548.2684.

4.4.20. 5-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-*N*-(3,4,5-trimethoxybenzyl)-3,3-dimethyl-5-oxopentanamide (20)

White solid, yield: 46%; mp 67–68 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.37 (t, J = 5.8 Hz, NH), 7.35 (d, J = 8.4 Hz, 4CH), 7.29 (t, J = 7.6 Hz, 2CH), 7.26 (d, J = 8.8 Hz, 2CH), 7.21 (t, J = 7.2 Hz, CH), 6.83 (d, J = 8.8 Hz, 2CH), 4.37 (d, J = 6.0 Hz, CH_2), 4.20 (s, CH), 3.82 (s, $3OCH_3$), 3.56–3.67 (m, CH_2), 3.53 (t, J = 5.0 Hz, CH_2), 2.34–2.41 (m, $2CH_2$), 2.34 (s, CH_2), 2.30 (s, CH_2), 1.06 (s, $2CH_3$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.33, 170.75, 153.18, 141.40, 140.60, 136.83, 135.10, 132.90, 129.03, 128.84, 128.78, 127.68, 127.48, 104.62, 75.14, 60.80, 56.01, 51.95, 51.57, 47.33, 46.90, 43.33, 41.70, 40.45, 34.61, 29.33; HR-FABMS calcd for $C_{34}H_{43}ClN_3O_5$: (M^++1): 608.2891, found: 608.2861.

4.4.21. 5-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-*N*-cyclohexyl-3,3-dimethyl-5-oxopentanamide (21)

White solid, yield: 9%; mp 77–78 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.65 (d, J = 8.4 Hz, NH), 7.35 (d, J = 8.4 Hz, 2CH), 7.29 (t, J = 7.4 Hz, 2CH), 7.25 (t, J = 8.0 Hz, CH), 7.22 (d, J = 6.8 Hz, 2CH), 7.21 (d, J = 6.4 Hz, 2CH), 4.21 (s, CH), 3.76 (s, CH), 3.60–3.70 (m, CH_2), 3.55 (t, J = 5.0 Hz, CH_2), 2.30–2.43 (m, $2CH_2$), 2.24 (s, CH_2), 2.17 (s, CH_2), 1.86 (d, J = 12.4 Hz, CH_2), 1.67 (d, J = 12.0 Hz, CH_2), 1.29–1.40 (m, CH_2), 1.10–1.28 (m, $2CH_2$), 1.05 (s, CH_3), 1.01 (s, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.84, 170.26, 141.45, 140.65, 132.92, 129.08, 128.87, 128.79, 127.74, 127.49, 75.22, 51.99, 51.63, 47.62, 47.47, 46.87, 41.72, 40.67, 34.38, 33.19, 29.34, 25.62, 24.85; HR-FABMS calcd for $C_{30}H_{41}ClN_3O_2$: (M^++1): 510.2887, found: 510.2874.

4.4.22. (*Z*)-4-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-*N*-(4-methylbenzyl)-4-oxobut-2-enamide (22)

Light yellow solid, yield: 81%; mp 76–78 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.330–7.360 (m, 5CH), 7.290–7.308 (m, 2CH), 7.189–7.234 (m, 2CH), 7.119–7.169 (m, 4CH), 6.328 (d, J = 12.8 Hz, CH), 6.076 (d, J = 12.8 Hz, CH), 4.425 (d, J = 5.6 Hz, CH_2), 4.204 (s, CH), 3.591–3.614 (m, CH_2), 3.441–3.467 (m, CH_2), 2.337 (s, CH_3), 2.310–2.232 (m, $2CH_2$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 165.71, 164.41, 141.40, 140.61, 136.94, 135.01, 132.86, 130.21, 130.03, 129.25, 129.03, 128.81, 128.74, 127.72, 127.69, 127.44, 74.98, 51.60, 51.06, 46.51, 43.17, 41.50, 21.09; HR-FABMS calcd for $C_{29}H_{31}O_2N_3Cl$: (M^++1): 488.2105, found: 488.2108.

4.4.23. (*Z*)-4-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-*N*-(4-methoxybenzyl)-4-oxobut-2-enamide (23)

Light yellow solid, yield: 80%; mp 77–78 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.330–7.360 (m, 5CH), 7.284–7.305 (m, 2CH), 7.196–7.250 (m, 4CH), 6.850–6.872 (m, 2CH), 6.322 (d, J = 12.8 Hz, CH), 6.072 (d, J = 12.8 Hz, CH), 4.396 (d, J = 6.0 Hz, CH_2), 4.186 (s, CH), 3.801 (s, CH_3), 3.578–3.619 (m, CH_2), 3.429–3.454 (m, CH_2), 2.290–2.329 (m, $2CH_2$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 165.79, 164.35, 158.86, 141.39, 140.61, 132.81, 130.17, 129.99, 129.11, 129.01, 128.78, 128.72, 127.66, 127.40, 113.95, 74.92, 55.23, 51.55, 51.02, 46.47, 42.84, 41.46; HR-FABMS calcd for $C_{29}H_{31}N_3O_3Cl$: (M^++1): 504.2054, found: 504.2058.

4.4.24. (*Z*)-4-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-*N*-cyclohexyl-4-oxobut-2-enamide (24)

White solid, yield: 69%; mp 152–154 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.208–7.351 (m, 9CH), 6.282 (d, J = 12.4 Hz, CH), 6.038 (d, J = 12.4 Hz, CH), 4.240 (s, CH), 3.775–3.796 (m, CH), 3.653–3.677 (m, CH_2), 3.457–3.481 (m, CH_2), 2.341–2.402 (m, $2CH_2$), 1.795–1.878 (m, CH_2), 1.666–1.718 (m, CH_2), 1.559–1.600 (m, $1/2CH_2$), 1.310–1.407 (m, CH_2), 1.142–1.248 (m, CH_2, $1/2CH_2$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 165.85, 163.69, 141.31, 140.52, 130.84, 129.39, 129.07, 128.81, 128.74, 127.73, 127.45, 74.98, 51.67, 51.16, 48.04, 46.57, 46.30, 32.76, 25.48, 24.55; HR-EIMS calcd for $C_{27}H_{32}N_3O_2Cl$: (M^+): 465.2183, found: 465.2184.

4.4.25. (*Z*)-4-(4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)-*N*-(4-phenylbutan-2-yl)-4-oxobut-2-enamide (25)

Light yellow solid, yield: 89%; mp 154–157 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.210–7.324 (m, 12CH), 7.176–7.196 (m, 2CH), 6.305 (d, J = 12.8 Hz, CH), 6.059 (d, J = 12.8 Hz, CH), 4.139 (s, CH), 4.026–4.063 (m, CH), 3.620–3.694 (m, CH_2), 3.463–3.489 (m, CH_2), 2.602–2.633 (m, CH_2), 2 .286–2.386 (m, $2CH_2$), 1.741–1.824 (m, CH_2), 1.184 (d, J = 6.8 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 165.75, 164.09, 141.80, 141.31, 140.50, 131.35, 129.05, 128.81, 128.75, 128.40, 128.36, 127.69, 127.45, 125.87, 74.98, 51.75, 51.17, 46.60, 45.23, 41.60, 38.49, 32.37, 20.84; HR-FABMS calcd for $C_{31}H_{35}N_3O_2Cl$: (M^++1): 516.2418, found: 516.2418.

4.4.26. (*Z*)-4-(4-((4-Chlorophenyl)(phenyl)methyl)-1,4-diazepan-1-yl)-*N*-(4-methylbenzyl)-4-oxobut-2-enamide (26)

Yellow solid, yield: 71%; mp 73–74 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.092–7.321 (m, 13CH), 6.350 (dd, J = 12.8 Hz, CH), 6.101 (dd, J = 13.2 Hz, CH), 4.535 (d, J = 4.4 Hz, CH), 4.437 (t, J = 5.4 Hz, CH_2), 3.443–3.579 (m, $2CH_2$), 2.510–2.642 (m, $2CH_2$), 2.303 (s, CH_3), 1.729–1.802 (m, CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.91, 164.62, 142.3, 141.50, 131.02, 129.78, 129.24, 128.98, 128.74, 127.61, 127.34, 74.12, 53.33, 52.91, 47.04, 46.38, 43.24, 26.35, 21.09; HR-EIMS calcd for $C_{30}H_{32}O_2N_3Cl$: (M^+): 501.2183, found: 501.2178.

4.4.27. (*Z*)-4-(4-((4-Chlorophenyl)(phenyl)methyl)-1,4-diazepan-1-yl)-*N*-(4-methoxylbenzyl)-4-oxobut-2-enamide (27)

Yellow solid, yield: 52%; mp 63–64 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.176–7.389 (m, 10CH), 6.812–6.856 (m, 2CH), 6.417 (dd, J = 12.8, 14.0 Hz, CH), 6.104 (t, J = 13.2 Hz, CH), 4.525 (s, CH), 4.401 (t, J = 5.4 Hz, CH), 3.785 (s, CH_3), 3.429–3.654 (m, $2CH_2$), 2.507–2.641 (m, $2CH_2$), 1.712–1.780 (m, CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.84, 164.56, 158.83, 142.31, 141.53, 131.37, 131.01, 129.73, 129.45, 129.07, 128.95, 128.70, 127.59, 127.28, 113.95, 74.03, 55.23, 54.17, 52.90, 47.29, 44.44, 42.90, 27.05; HR-EIMS calcd for $C_{30}H_{32}O_3N_3Cl$: (M^+): 517.2132, found: 517.2131.

4.4.28. (*Z*)-4-(4-((4-Chlorophenyl)(phenyl)methyl)-1,4-diazepan-1-yl)-*N*-cyclohexyl-4-oxobut-2-enamide (28)

Pale yellow solid, yield: 62%; mp 74–75 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.173–7.532 (m, 9CH), 6.812–6.856 (m, 2CH), 6.417 (dd, J = 12.8, 10.8 Hz, CH), 6.104 (dd, J = 9.2, 4.0 Hz, CH), 4.576 (d, J = 8.0 Hz, CH), 3.781–3.837 (m, CH), 3.438–3.718 (m, $2CH_2$), 2.553–2.697 (m, $2CH_2$), 1.191–1.948 (m, $6CH_2$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 167.34, 164.11, 142.53, 141.72, 132.99, 131.83, 129.37, 129.22, 129.03, 128.93, 127.93, 127.56, 74.49, 54.53, 53.07, 48.28, 47.49, 44.73, 31.82, 26.54, 25.77, 24.84; HR-EIMS calcd for $C_{28}H_{34}O_2N_3Cl$: (M^+): 479.2340, found: 479.2336.

4.4.29. (*Z*)-4-(4-((4-Chlorophenyl)(phenyl)methyl)-1,4-diazepan-1-yl)-*N*-(4-phenylbutan-2-yl)-4-oxobut-2-enamide (29)

Yellow oil, yield: 73%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.411–7.149 (m, 14CH), 6.342 (dd, J = 11.2, 13.2 Hz, CH), 6.100 (t, J = 13.0 Hz, CH), 4.505 (d, J = 8.8 Hz, CH), 4.040–4.057 (m, CH), 3.568–3.725 (m, $2CH_2$), 2.545–2.679 (m, $3CH_2$), 1.748–1.828 (m, $2CH_2$), 1.195 (dd, J = 1.6, 5.2 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 167.23, 164.47, 142.48, 142.19, 141.68, 132.93, 132.20, 129.22, 129.03, 128.61, 128.55, 127.85, 126.07, 74.44, 54.55, 52.99, 47.58, 45.46, 44.82, 38.76, 32.65, 27.43, 21.07; HR-EIMS calcd for $C_{32}H_{36}N_3O_2Cl$: (M^+): 529.2496, found: 529.2499.

4.4.30. (*Z*)-4-(4-(Bis(4-fluorophenyl)methyl)piperazin-1-yl)-*N*-(4-methylbenzyl)-4-oxobut-2-enamide (30)

Light red solid, yield: 67%; mp 73–75 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.308–7.344 (m, 4CH), 7.114–7.184 (m, 4CH), 6.963–7.012 (m, 4CH), 6.319 (d, J = 12.8 Hz, CH), 6.070 (d, J = 12.8 Hz, CH), 4.421 (d, J = 6.0 Hz, CH_2), 4.213(s, CH), 3.589–3.614 (m, CH_2), 3.438–3.487 (m, CH_2), 2.332 (s, CH_3), 2.296–2.305 (m, 2 CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 165.70, 164.46, 163.17, 160.73, 137.52, 137.01, 130.38, 129.96, 129.30, 129.21, 129.13, 127.77, 115.73, 115.52, 74.11, 51.61, 51.07, 46.54, 43.24, 41.54, 21.12; HR-FABMS calcd for $C_{29}H_{30}O_2N_3F_2$: (M^++1): 490.2306, found: 490.2304.

4.4.31. (*Z*)-4-(4-(Bis(4-fluorophenyl)methyl)piperazin-1-yl)-*N*-(4-methoxybenzyl)-4-oxobut-2-enamide (31)

Light yellow solid, yield: 73%; mp 73–75 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.307–7.340 (m, 4CH), 7.176–7.220 (m, 2CH), 6.957–6.999 (m, 4CH), 6.837–6.858 (m, 2CH), 6.300 (d, J = 12.8 Hz, CH), 6.058 (d, J = 12.8 Hz, CH), 4.381 (d, J = 5.6 Hz, CH_2), 4.192 (s, CH), 3.787 (s, CH_3), 3.587 (br s, CH_2), 3.342 (br s, CH_2), 2.292 (br s, $2CH_2$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 166.01, 164.66, 163.15, 161.20, 159.18, 137.77, 130.51, 130.29, 129.43, 129.37, 115.95, 114.27, 74.31, 55.53, 51.84, 51.28, 46.77, 43.16, 41.75; HR-FABMS calcd for $C_{29}H_{30}N_3O_3F_2$: (M^++1): 506.2255, found: 506.2258.

4.4.32. (*Z*)-4-(4-(Bis(4-fluorophenyl)methyl)piperazin-1-yl)-*N*-cyclohexyl-4-oxobut-2-enamide (32)

Pale yellow solid, yield: 54%; mp 92–94 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.307–7.350 (m, 4CH), 6.961–7.009 (m, 4CH), 6.283 (d, J = 12.4 Hz, CH), 6.035 (d, J = 12.8 Hz, CH), 4.251 (s, CH), 3.779–3.799 (m, CH), 3.665 (t, J = 5.0 Hz, CH_2), 3.470 (t, J = 5.0 Hz, CH_2), 2.324–2.385 (m, $2CH_2$), 1.139–1.954 (m, $5CH_2$); ^{13}C NMR ($CDCl_3$. 100 MHz) δ 165.90, 163.67, 163.15, 160.70, 137.41, 130.73, 129.47, 129.23, 129.15, 115.69, 115.48, 74.05, 51.61, 51.09, 48.05, 46.55, 41.54, 32.76, 25.47, 24.55; HR-EIMS calcd for $C_{27}H_{31}N_3O_2F_2$: (M^+): 467.2384, found: 467.2383.

4.4.33. (*Z*)-4-(4-(Bis(4-fluorophenyl)methyl)piperazin-1-yl)-*N*-(4-phenylbutan-2-yl)-4-oxobut-2-enamide (33)

Light yellow solid, yield: 68%; mp 149–152 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.248–7.303 (m, 5CH), 7.176–7.196 (m, 4CH), 6.937∼6.996 (m, 4CH), 6.304 (d, J = 12.8 Hz, CH), 6.059 (d, J = 12.8 Hz, CH), 4.143 (s, CH), 4.027–4.064 (m, CH), 3.604∼3.686 (m, CH_2), 3.461–3.488 (m, CH_2), 2.601–2.661 (m, CH_2), 2.258–2.378 (m, $2CH_2$), 1.741–1.808 (m, CH_2), 1.183 (d, CH_3, J = 6.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 165.73, 164.06, 163.13, 160.69, 141.80, 137.42, 137.39, 131.34, 129.18, 129.10, 129.05, 128.36, 125.87, 115.68, 115.47, 74.05, 51.71, 51.11, 46.58, 45.23, 41.58, 38.50, 32.38, 20.54; HR-FABMS calcd for $C_{31}H_{34}N_3O_2F_2$: (M^++1): 518.2619, found: 518.2619.

4.5. Bradykinin-induced contractions of guinea-pig ileum

Bradykinin, captopril, indomethacin, dithiothreitol, atropine and Icatibant were obtained from Sigma (St. Louis, MO, USA). The composition of the Tyrode solution was as follows (in mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.15, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 5.6. The final DMSO concentration in bath was less than 0.1% and had no effect on the tissue's responsiveness to bradykinin. Male Hartley guinea-pigs weighing 275–500 g (Jaeil, Korea) were fasted overnight and decapitated. At a level 2 cm above the ileocecal junction, a section of ileum approximately 40 cm in length was removed and placed in warm (37 °C) Tyrode solution. Strips of muscle, 1.5–2 cm in length, were then mounted in a 50 mL bath containing Tyrode solution (37 °C) and aerated with 95%O_2/5%CO_2. Tissue contractions were recorded isometrically on a Grass model 76E polygraph. After an equilibration period of about 60 min a stable baseline tone was reached and two or three contractions were obtained in response to BK (0.1 μM), every 20 min interval, to assay sensitivity and reproducibility of the contractile response.[39,40] Only segments producing reproducible responses were used. The last control response was taken as 100% and subsequent results obtained with bradykinin antagonists expressed as a percentage of this. The segments were incubated with the bradykinin antagonists (1 μM) for 15 min before BK was added. To minimize degradation of bradykinin and to prevent responses due to neuronal activation or prostaglandin production, Tyrode solutions contained 1 μM each of captopril, atropine, dithiothreitol, and indomethacin. Moreover, to prevent histaminergic responses, 1 μM of dibenamine, which is an irreversible histamine H_1 blocker, was also added to the Tyrode solution.

4.6. Nociceptive and inflammatory pain

4.6.1. Formalin and compound 33 injection

To investigate the effect of **33** on both nociceptive and inflammatory pain, the formalin test was carried out. Each rat was placed in the observation chamber (20 × 20 × 20 cm) equipped with a mirror placed behind the chamber to allow an unobstructed view of the paw for a 30 min habituation period. Thereafter, animals were assigned to the two groups; the first group was subjected to the subcutaneous injection of the mixture of formalin (5%, 50 μl) and vehicle (Methyl pyrrolidone/Tween80/Saline = 1:1:8, 10 μl) under the plantar surface of the right hind paw using a 30-gauge syringe, the second group was treated with the mixture of formalin (5%, 50 μl) plus **33** (60 μg/10 μl) by the same way described above.

4.6.2. Behavioral testing for Formalin test

After the injection, animals were returned to the chamber immediately and the time spent in licking, shaking or biting the injected paw was recorded for every 5 min over a 60 min period. The early phase was defined as the first 10 min after formalin injection, and the late phase was the following 50 min.[41,42] The observations in this test were carried out by three investigators who were unaware of the treatment status.

4.7. Neuropathic pain

4.7.1. Tail nerve injury

Tail nerve injury (TNI) was based on the procedure previously described by Na et al.[43] Briefly, under enflurane anesthesia, animals were subjected to unilateral transection of the superior and inferior caudal trunks at the level between the S1 and the S2 spinal nerves. To prevent possible rejoining of the proximal and distal ends of the severed trunks, pieces of nerve about 2 mm in length were removed from the distal nerve ends. This surgery injured the S1 spinal nerve which innervates the tail.

4.7.2. Behavioral testing for neuropathic pain

Mechanical allodynia of rat tails was assessed by measuring the withdrawal thresholds in response to a series of calibrated von Frey filaments (3.92, 5.88, 9.80, 19.60, 39.20, 58.80, 78.40, and 147.00 mN, Stoeling, Wood Dale, IL, USA; equivalent to 0.4, 0.6, 1.0, 2.0, 4.0, 6.0, 8.0, and 15.0 g). The 50% withdrawal threshold was determined using the up-down method.[44] In brief, testing was initiated with a filament whose bending force was 19.60 mN in the middle of the series. When a withdrawal response was obtained, next weaker filament was used, whereas next stronger filament was administered when no response was obtained. Interpolation of the 50% threshold was carried out using the Dixon method.[45] A brisk tail withdrawal to von Frey filament application was regarded as a positive response. Testing for cold or warm allodynia was performed by measuring tail withdrawal latency to cold (4 °C) or warm (40 °C) water stimulation, respectively.[43] After immersing the tail into cold or warm water bath, the tail was continuously observed to find out whether it moved abruptly, and the latency of tail movement was measured within a cut-off time of 15 s. An abrupt tail movement within the cut-off time was considered as a positive withdrawal response, whereas a lack of tail movement until the cut-off time or slow tail movement within the cut-off time was considered to be not a positive response. The testing was repeated five times with 5 min intervals, and the average latency of tail response was calculated.

At 2 weeks after TNI, rats were given a baseline test and thereafter compound **33** (60 mg/kg) or vehicle (Methyl pyrrolidone/Tween80/Saline = 1:1:8) was administered intraperitoneally. Behavioral tests for mechanical, cold and warm allodynia were conducted at 1, 3, and 5 h after the injection. Behavioral testing was carried out by an investigator who was unaware of the treatment status of the rats.

4.8. Statistical analysis

All data are presented as mean ± SEM. Student's unpaired *t*-test, Repeated one-way ANOVA (Bonferroni *t*-test) and Paired *t*-test were used wherever appropriate. A difference of $P < 0.05$ was considered to be statistically significant.

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