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Synthesis of 1,3,4-thiadiazole derivatives as aminopeptidase N inhibitors

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Received May 26, 2008, accepted June 30, 2008

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Pharmazie 64: 67–70 (2009) *doi: 10.1691/ph.2009.8630*

Aminopeptidase N (APN) is a zinc-dependent ectopeptidase which plays an important role in the invasion of metastatic tumors. In this study, we report the synthesis and *in vitro* enzyme inhibition assay of 1,3,4-thiadiazole scaffold compounds. These new compounds have potent inhibitory activities toward APN with IC_{50} values in the micromolar range.

1. Introduction

Aminopeptidase N (APN), also known as CD13, is a widespread ectopeptidase in mammalian tissues including the central nervous system, kidney, intestine and lung which preferentially releases neutral and basic amino acids from the N-terminal end of peptides (Riemann et al. 1999a). APN has been reported to play an important role in the invasion of metastatic tumors *in vitro* (Saiki et al. 1993; Menrad et al. 1993). Observations show enhanced APN levels in tumor cells including melanoma (Fujii et al. 1995), renal, pancreas (Ikeda et al. 2003), colon (Hashida et al. 2002), prostate (Ishii et al. 2001), gastric (Carl-McGrath et al. 2004), and thyroid cancers (Kehlen et al. 2003). Tumor-infiltrating T cells in lung and renal cancers are CD13-positive (Riemann et al. 1994b). APN activity is elevated in plasma and effusions of cancer patients (van Hensbergen et al. 2002). APN activity on neutrophils from patients affected by a rare adrenal gland tumor, adrenal pheochromocytoma, is significantly increased as compared with healthy controls (Balog et al. 2003). APN is overexpressed in acute and chronic myeloid leukemias (Boldt et al. 1994; Tatsumi et al. 2002) and in anaplastic large cell lymphomas (Popnikolov et al. 2000; Dunphy et al. 2000). Overexpression of APN in T lymphocytes or neutrophils occurs in several inflammatory diseases (chronic pain, various forms of joint effusions, rheumatoid arthritis, multiple sclerosis, systemic sclerosis, systemic lupus erythematosus, polymyositis/dermatomyosytis, pulmonary sarcoidosis) (Dan et al. 2003; Abe et al. 1998). All these findings make this enzyme an interesting target for possible therapeutic applications, which require the development of potent and selective inhibitors. APN inhibition by bestatin leads to a loss of motility. Likewise, CD13 antibodies inhibit cell growth and cell motility (Antczak et al. 2001; Lohn et al. 2002).

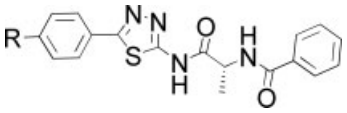
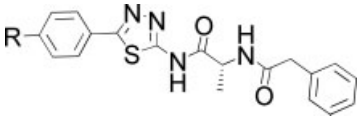
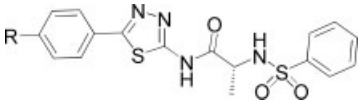
Natural products including bestatin, actinonin (Grujić et al. 2002), MA-387A and B (Bauvois et al. 2006) and amastatin (Harding et al. 1987) have been found to act as inhibitors of APN. Synthetic APN inhibitors have been developed from analogues of amino acids such as aminohydroxamates, aminoboronic acids (Shenvi et al. 1986), β-aminothiols (Fournié-Zaluski et al. 1992), aminoaldehydes (Andersson et al. 1982) and aminophosphonic acids. Among them, only β-aminothiols are both selective and relatively potent inhibitors.

Inhibitors containing zinc-binding groups (ZBGs) should be designed to inhibit the activity of APN. This class of inhibitors can interact with the zinc ions of zinc-dependent metalloenzymes and sequentially inhibit the metastatic spread of tumors and block the processes of tumor neovascularization (Becker et al. 1995). There are two hydrophobic domains beside the catalytic activity center of APN, called pockets S_1 and S_1'. The hydrophobic thiadiazole and aromatic ring may interact with pockets S_1 and S_1'. APN exhibits a broad specificity for peptides with a N-terminal neutral or basic amino acid such as alanine, arginine or leucine. If we link thiadiazole with alanine and the aromatic ring, the resulting 1,3,4-thiadiazole scaffold compounds should be capable of inhibiting the enzymatic activity of APN. So different 1,3,4-thiadiazole derivatives were designed and synthesized (Levy et al. 1942; Song et al. 1992).

2. Investigations, results and discussion

We prepared analogues substituting the phenyl ring in position 5 of the 1,3,4-thiadiazole moiety with various halogens (**1b–d, 2b–d, 3b–d**), electron-withdrawing groups (**1e, 2e, 3e**), and moderately electron-donating groups (**1f–g, 2f–g, 3f–g**) and assessed the effects that these changes had on APN and MMP-2 inhibitory activity. All the inhibition results are summarized in the Table. The inhibition results showed that most of the target compounds display excellent potency toward APN with IC_{50} values lying in a micromolar

Table: ***In vitro*** **enzyme assay results for compounds 1–3 and bestatin**

Structure	Compounds	R	IC_{50}[a]/μM
 **1a–g**	**1a**	−H	170.1 ± 12.8
	1b	−F	190.5 ± 16.3
	1c	−Cl	287.5 ± 12.8
	1d	−Br	308.9 ± 17.6
	1e	$-NO_2$	317.8 ± 118.3
	1f	$-CH_3$	117.8 ± 17.5
	1g	$-OCH_3$	69.8 ± 7.1
 **2a–g**	**2a**	−H	141.6 ± 12.9
	2b	−F	169.0 ± 14.6
	2c	−Cl	205.8 ± 17.9
	2d	−Br	296.6 ± 18.6
	2e	$-NO_2$	309.1 ± 10.8
	2f	$-CH_3$	107.0 ± 17.5
	2g	$-OCH_3$	39.9 ± 4.5
 **3a–g**	**3a**	−H	373.2 ± 14.3
	3b	−F	115.3 ± 12.4
	3c	−Cl	96.4 ± 3.2
	3d	−Br	107.3 ± 18.1
	3e	$-NO_2$	167.8 ± 12.6
	3f	$-CH_3$	337.8 ± 15.9
	3g	$-OCH_3$	48.8 ± 8.0
Bestatin			8.5 ± 0.6

[a] Values are means of three experiments, standard deviation is given

level. Comparing phenylamide and benzylamide analogues (**1a–g, 2a–g**), halogens and electron-withdrawing groups attached to the 4-position of the phenyl ring decreased the potency against APN. However, The electron-donating group (**1f–g, 2f–g**) improved the inhibitory activity against APN. Substitution by electron-donating substituents produces an electron-sufficient phenyl ring, which may improve aryl-aryl stacking interactions with aromatic side chains in the active site of APN. The more active compound **2g** was introduced the flexible benzylamide which may improve the interaction with the active site of APN. Halogens, electron-withdrawing groups and electron-donating groups of benzylsulfonamide analogues all increased the potency against APN. In addition, all the target compounds show inactivity toward MMP-2 which is a zinc-dependent endopeptidase associated with the tumorigenic process. MMP-2 is a zinc-dependent endopeptidase that can cut the peptide to parts from the specific amino acid residue of the peptide. However, APN is a membrane-bound zinc exopeptidase that catalyzes the removal of the NH-terminal amino acid from the peptide. Due to the structural differences between MMP-2 and APN, there were different structural requirements for their respective inhibitors. So, most of compounds show more inhibitory activities on APN than on MMPs due to the recognition of APN.

In conclusion, we have developed a new series of potent APN inhibitors. Most of the compounds have potent inhibitory activities toward APN and could be used as lead compounds for the development of low molecular-weight peptidomimetic APN inhibitors. The critical point in designing selective inhibitors is to optimize the recognition of APN.

3. Experimental

3.1. Chemistry

Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. All the solvents except DMF have been distilled before use. All reactions were monitored by TLC on 0.25 mm silica gel plates (60GF-254) and visualized with UV light. Column chromatography was performed on silica gel (200–300 mesh). ESI-MS were determined on an Aglient-1100 series LC/MSD trap spectrometer. IR were recorded on a FTIR-8400 spectrometer. Melting points were determined on a electrothermal melting point apparatus and are uncorrected. ^{1}H NMR spectra were obtained on a Bruker-400. The chemical shifts are expressed in δ values (parts per million) relative to tetramethylsilane (TMS) as internal standard. Significant ^{1}H NMR data are reported in the following order: multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet) number of protons.

3.1.1. General procedure for the preparation of 1,3,4-thiadiazole derivatives. Synthesis of (2R)-N-[5-phenyl-1,3,4-thiadiazol-2-yl]-2-[(benzoyl)-amino]-propanamide (**1a**)

N,N-Dicyclohexylcarbodiimide (6.2 mmol) was added to a cooled solution of *N*-benzoyl- D-alanine (6.0 mmol) and *N*-hydroxystinimide (6.0 mmol) in freshly distillation dioxane (30 ml). The reaction mixture was stirred overnight at room temperature. The insoluble material was filtered off and washed with cold dioxane. 5-Phenyl-1,3,4-thiadiazol-2-Amino (5.6 mmol) was added to the filtrate and the reaction mixture was stirred for 48 h at room temperature. The solvent was removed under reduced pressure. The residual was dissolved in EtOAc and the insoluble material was filtered off. The filtrate was washed successively with saturated Na_2CO_3 solution (20 ml, × 3), water (20 ml, × 1), 0.1 M HCl (20 ml, × 3) and water (20 ml, × 1). The organic layer was evaporated *in vacuo*, the residual was recrystallized from methanol/acetonitrile (5 : 1) and dried to afford the target compound as white solid 1.27 g, yield: 64.5%; m.p.: 220–222 °C; IR (KBr, σ/cm^{-1}): 3436, 3243 (υ_{NH}), 1700, 1654 ($\upsilon_{C=O}$), 689 (υ_{C-S}), 1638 ($\upsilon_{C=N}$); ^{1}H NMR (DMSO-d$_6$): δ 1.49–1.51 (d, J = 7.08 Hz, 3H), 4.69–4.76 (m, 1H), 7.48–7.59 (m, 6H), 7.88–7.95 (t, J = 12.8Hz, 4H), 8.82–8.84 (d, J = 6.04 Hz, 1H), 12.86 (s, 1H); ESI-MS: m/z $[M + H]^+$ 353.3.

3.1.2. (2R)-N-[5-(4-Fluorophenyl)-1,3,4- thiadiazol-2-yl]-2-[(benzoyl)amino]- propanamide (**1b**)

Yield: 60.2%; m.p.: 170–171 °C; IR (KBr, σ/cm^{-1}): 3275, 3166 (υ_{NH}), 1701, 1655 ($\upsilon_{C=O}$), 694 (υ_{C-S}), 1635 ($\upsilon_{C=N}$); ^{1}H NMR (DMSO-d$_6$): δ 1.47–1.49 (d, J = 7.12 Hz, 3H), 4.68–4.72 (t, J = 6.76 Hz, 1H), 7.36–7.40 (t, J = 8.74 Hz, 2H), 7.48–7.51 (t, J = 7.36 Hz, 2H), 7.55–7.58 (t, J = 6.94 Hz, 1H), 7.91–7.93 (d, J = 8.04 Hz, 2H), 7.98–8.02 (t, J = 6.90 Hz, 2H), 8.84–8.85 (d, J = 5.84 Hz, 1H), 12.89 (s, 1H); ESI-MS: m/z $[M + H]^+$ 371.1.

3.1.3. (2R)-N-[5-(4-Chlorophenyl)-1,3,4-thiadiazol-2-yl]-2-[(benzoyl)amino]-propanamide (**1c**)

Yield: 69.8%; m.p.: 216–217 °C; IR (KBr, σ/cm^{-1}): 3333, 3159 (υ_{NH}), 1698,1643 ($\upsilon_{C=O}$), 691 (υ_{C-S}), 1630 ($\upsilon_{C=N}$); ^{1}H NMR (DMSO-d$_6$): δ 1.48–1.50 (d, J = 7.12 Hz, 3H), 4.69–4.73 (t, J = 6.72 Hz, 1H), 7.47–7.60 (m, 5H), 7.92–7.97 (t, J = 9.28 Hz, 4H), 8.87–8.88 (d, J = 5.96 Hz, 1H), 12.95 (s, 1H); ESI-MS: m/z $[M + H]^+$ 387.1.

3.1.4. (2R)-N-[5-(4-Bromophenyl)-1,3,4-thiadiazol-2-yl]-2-[(benzoyl)amino]-propanamide (**1d**)

Yield: 42.5%; m.p.: 191–193 °C; IR (KBr, σ/cm^{-1}): 3328, 3166 (υ_{NH}), 1702, 1648 ($\upsilon_{C=O}$), 692 (υ_{C-S}); ^{1}H NMR (DMSO-d$_6$): δ 1.47–1.49 (d, J = 7.12 Hz, 3H), 4.69–4.72 (t, J = 6.78 Hz, 1H), 7.47–7.74 (m, 5H), 7.88–7.90 (d, J = 8.44 Hz, 2H), 7.92–7.93 (d, J = 7.44 Hz, 2H), 8.86–8.88 (d, J = 6.04 Hz, 1H), 12.95 (s, 1H); ESI-MS: m/z [M + H]$^+$ 432.9.

3.1.5. (2R)-N-[5-(4-Nitrorophenyl)-1,3,4-thiadiazol-2-yl]-2-[(benzoyl)amino]-propanamide (**1e**)

Yield: 20.7%; m.p.: 225–226 °C; IR (KBr, σ/cm^{-1}): 3415, 3333 (υ_{NH}), 1695, 1650 ($\upsilon_{C=O}$), 693 (υ_{C-S}), 1628 ($\upsilon_{C=N}$), 1517 (υas_{NO2}), 1347 (υs_{NO2}); ^{1}H NMR (DMSO-d$_6$): δ 1.49–1.50 (d, J = 7.20 Hz, 3H), 4.70–4.73 (t, J = 6.74 Hz, 1H), 7.48–7.52 (t, J = 7.44 Hz, 2H), 7.56–7.57 (d, J = 7.24 Hz, 1H), 7.92–7.95 (t, J = 6.46 Hz, 2H), 8.23–8.25 (d, J = 8.76 Hz, 2H), 8.35–8.38 (d, J = 8.80 Hz, 2H), 8.89–8.90 (d, J = 6.04 Hz, 1H), 13.10 (s, 1H); ESI-MS: m/z [M + H]$^+$ 398.1.

3.1.6. (2R)-N-[5-(4-Methylphenyl)-1,3,4-thiadiazol-2-yl]-2-[(benzoyl)amino]-propanamide (**1f**)

Yield: 29.2%; m.p.: 189–190 °C; IR (KBr, σ/cm^{-1}): 3301, 3165 (υ_{NH}), 1703, 1655 ($\upsilon_{C=O}$), 691 (υ_{C-S}); ^{1}H NMR (DMSO-d$_6$): δ 1.49–1.51 (d, J = 7.20 Hz, 3H), 2.41 (s, 3H), 4.71–4.74 (t, J = 6.84 Hz, 1H), 7.33–7.35 (d, J = 8.04 Hz, 2H), 7.48–7.59 (m, 3H), 7.82–7.84 (d, J = 8.12 Hz, 2H), 7.93–7.95 (t, J = 4.30 Hz, 2H), 8.82–8.84 (d, J = 6.32 Hz, 1H), 12.83 (s, 1H); ESI-MS: m/z [M + H]$^+$ 365.2.

3.1.7. (2R)-N-[5-(4-Methoxyphenyl)-1,3,4-thiadiazol-2-yl]-2-[(benzoyl)amino]-propanamide (**1g**)

Yield: 40.3%; m.p.: 155–156 °C; IR (KBr, σ/cm^{-1}): 3407, 3298 (υ_{NH}), 1699, 1660 ($\upsilon_{C=O}$), 692 (υ_{C-S}); ^{1}H NMR (DMSO-d$_6$): δ 1.47–1.49 (d, J = 7.12 Hz, 3H), 3.83 (s, 3H), 4.69 (s, 1H), 7.07–7.09 (d, J = 8.68 Hz, 2H), 7.48–7.51 (t, J = 7.36 Hz, 2H), 7.55–7.57 (d, J = 7.08 Hz, 1H), 7.86–7.89 (d, J = 8.68 Hz, 2H), 7.92–7.93 (d, J = 7.36 Hz, 2H), 8.84–8.86 (d, J = 6.08 Hz, 1H), 12.82 (s, 1H); ESI-MS: m/z [M + H]$^+$ 383.1.

3.1.8. (2R)-N-[5-phenyl-1,3,4-thiadiazol-2-yl]-2-[(phenylacetyl)amino]-propanamide (**2a**)

Yield: 14.0%; m.p.: 245–247 °C; IR (KBr, σ/cm^{-1}): 3242, 3184 (υ_{NH}), 1712, 1646 ($\upsilon_{C=O}$), 688 (υ_{C-S}); ^{1}H NMR (DMSO-d$_6$): δ 1.36–1.39 (d, J = 7.12 Hz, 3H), 3.48–3.55 (t, J = 15.08 Hz, 2H), 4.49–4.56 (m, 1H), 7.20–7.24 (m, 1H), 7.27–7.32 (m, 4H), 7.53–7.54 (t, J = 2.78 Hz, 3H), 7.93–7.96 (m, 2H), 8.59–8.61 (d, J = 6.32 Hz, 1H), 12.80 (s, 1H); ESI-MS: m/z [M + H]$^+$ 367.3.

3.1.9. (2R)-N-[5-(4-Fluorophenyl)-1,3,4-thiadiazol-2-yl]-2-[(phenylacetyl)-amino]-propanamide (**2b**)

Yield: 41.8%; m.p.: 140–141 °C; IR (KBr, σ/cm^{-1}): 3397, 3309 (υ_{NH}), 1700, 1650 ($\upsilon_{C=O}$), 696 (υ_{C-S}); ^{1}H NMR (DMSO-d$_6$): δ 1.34–1.36 (d, J = 7.12 Hz, 3H), 3.50 (s, 2H), 4.48–4.51 (t, J = 6.94 Hz, 1H), 7.22–7.32 (m, 5H), 7.36–7.41 (t, J = 8.74 Hz, 2H), 7.99–8.02 (t, J = 6.95 Hz, 2H), 8.63–8.64 (d, J = 6.20 Hz, 1H), 12.84 (s, 1H); ESI-MS: m/z [M + Na]$^+$ 407.1.

3.1.10. (2R)-N-[5-(4-Chlorophenyl)-1,3,4-thiadiazol-2-yl]-2-[(phenylacetyl)-amino]-propanamide (**2c**)

Yield: 35.9%; m.p.: 120–121 °C; IR (KBr, σ/cm^{-1}): 3418, 3175 (υ_{NH}), 1703, 1650 ($\upsilon_{C=O}$), 695 (υ_{C-S}); ^{1}H NMR ($CDCl_3$): δ 1.55–1.57 (d, J = 7.40 Hz, 3H), 3.53–3.61 (dd, J_1 = 15.08 Hz, J_2 = 17.40 Hz, 2H), 4.80–4.87 (m, 1H), 7.09–7.15 (m, 5H), 7.48–7.50 (d, J = 8.48 Hz, 2H), 7.77–7.80 (dd, J_1 = 1.72 Hz, J_2 = 6.96 Hz, 2H), 7.89–7.91 (d, J = 6.88 Hz, 1H), 13.05 (s, 1H); ESI-MS: m/z [M + H]$^+$ 401.2.

3.1.11. (2R)-N-[5-(4-Bromophenyl)-1,3,4-thiadiazol-2-yl]-2-[(phenylacetyl)-amino]-propanamide (**2d**)

Yield: 26.6%; m.p.: 161–162 °C; IR (KBr, σ/cm^{-1}): 3366, 3262 (υ_{NH}), 1695, 1648 ($\upsilon_{C=O}$), 695 (υ_{C-S}); ^{1}H NMR (DMSO-d$_6$): δ 1.35–1.36 (d, J = 7.12 Hz, 3H), 3.50 (s, 2H), 4.48–4.52 (t, J = 6.84 Hz, 1H), 7.22–7.32 (m, 5H), 7.72–7.75 (d, J = 8.40 Hz, 2H), 7.88–7.91 (d, J = 8.40 Hz, 2H), 8.64–8.65 (d, J = 6.24 Hz, 1H), 12.88 (s, 1H); ESI-MS: m/z [M + H]$^+$446.9.

3.1.12. (2R)-N-[5-(4-Nitrorophenyl)-1,3,4-thiadiazol-2-yl]-2-[(phenylacetyl)-amino]-propanamide (**2e**)

Yield: 44.3%; m.p.: 218–220 °C; IR (KBr, σ/cm^{-1}): 3405, 3277 (υ_{NH}), 1699, 1651 ($\upsilon_{C=O}$), 690 (υ_{C-S}), 1521 (υas_{NO2}), 1344 (υs_{NO2}); ^{1}H NMR (DMSO-d$_6$): δ 1.34–1.36 (d, J = 7.12 Hz, 3H), 3.54 (s, 2H), 4.48–4.51 (t, J = 6.84 Hz, 1H), 7.22–7.32 (m, 5H), 7.33–7.35 (d, J = 7.96 Hz, 2H), 7.82–7.84 (d, J = 7.96 Hz, 2H), 8.62–8.64 (d, J = 6.28 Hz, 1H), 12.79 (s, 1H); ESI-MS: m/z [M + H]$^+$ 412.3.

3.1.13. (2R)-N-[5-(4-Methylphenyl)-1,3,4-thiadiazol-2-yl]-2-[(phenylacetyl)-amino]-propanamide (**2f**)

Yield: 32.1%; m.p.: 185–186 °C; IR (KBr, σ/cm^{-1}): 3421, 3301 (υ_{NH}), 1699, 1647 ($\upsilon_{C=O}$), 695 (υ_{C-S}), 1624 ($\upsilon_{C=N}$); ^{1}H NMR (DMSO-d$_6$): δ 1.34–1.36 (d, J = 7.12 Hz, 3H), 2.37 (s, 3H), 3.54 (s, 2H), 4.48–4.51 (t, J = 6.84 Hz, 1H), 7.22–7.32 (m, 5H), 7.33–7.35 (d, J = 7.96 Hz, 2H), 7.82–7.84 (d, J = 7.96 Hz, 2H), 8.62–8.64 (d, J = 6.28 Hz, 1H), 12.79 (s, 1H); ESI-MS: m/z [M + H]$^+$ 381.1.

3.1.14. (2R)-N-[5-(4-Methoxyphenyl)-1,3,4-thiadiazol-2-yl]-2-[(phenylacetyl)-amino]-propanamide (**2g**)

Yield: 38.8%; m.p.: 142–143 °C; IR (KBr, σ/cm^{-1}): 3329, 3165 (υ_{NH}), 1690, 1670 ($\upsilon_{C=O}$), 695 (υ_{C-S}); ^{1}H NMR (DMSO-d$_6$): δ 1.34–1.36 (d, J = 7.08 Hz, 3H), 3.46–3.53 (t, J = 15.36 Hz, 2H), 3.83 (s, 3H), 4.48–4.51 (t, J = 6.80 Hz, 1H), 7.07–7.09 (d, J = 8.68 Hz, 2H), 7.22–7.32 (m, 5H), 7.87–7.89 (d, J = 8.60 Hz, 2H), 8.61–8.63 (d, J = 6.24 Hz, 1H), 12.75 (s, 1H); ESI-MS: m/z [M + H]$^+$ 397.1.

3.1.15. (2R)-N-[5-phenyl-1,3,4- thiadiazol-2-yl]-2-[(phenylsulfonyl)amino]-propanamide (**3a**)

Yield: 25.8%; m.p.: 258–260 °C; IR (KBr, σ/cm^{-1}): 3447, 3175 (υ_{NH}), 1700 ($\upsilon_{C=O}$), 1321 ($\upsilon as_{O=S=O}$), 1147 ($\upsilon s_{O=S=O}$), 689 (υ_{C-S}); ^{1}H NMR (DMSO-d$_6$): δ 1.24–1.25 (d, J = 7.04 Hz, 3H), 4.16–4.23 (m, 1H), 7.51–7.56 (m, 6H), 7.79–7.82 (dd, J_1 = 1.36 Hz, J_2 = 7.72 Hz, 2H), 7.94–7.92 (m, 2H), 8.42–8.40 (d, J = 7.96Hz, 1H), 12.72 (s, 1H); ESI-MS: m/z [M + H]$^+$ 389.2.

3.1.16. (2R)-N-[5-(4-Fluorophenyl)-1,3,4-thiadiazol-2-yl]-2-[(phenylsulfonyl)amino]-propanamide (**3b**)

Yield: 38.6%; m.p.: 199–200 °C; IR (KBr, σ/cm^{-1}): 3423, 3182 (υ_{NH}), 1696 ($\upsilon_{C=O}$), 1323 ($\upsilon as_{O=S=O}$), 1149 ($\upsilon s_{O=S=O}$), 690 (υ_{C-S}); ^{1}H NMR (DMSO-d$_6$): δ 1.21–1.23 (d, J = 7.04 Hz, 3H), 4.15–4.18 (t, J = 7.32 Hz, 1H), 7.36–7.41 (t, J = 8.76 Hz, 2H), 7.50–7.56 (dd, J_1 = 7.92 Hz, J_2 = 15.36 Hz, 3H), 7.77–7.79 (t, J = 3.94 Hz, 2H), 7.97–8.01 (dd, J_1 = 5.44 Hz, J_2 = 8.56 Hz, 2H), 8.43–8.45 (d, J = 7.96 Hz, 1H), 12.76 (s, 1H); ESI-MS: m/z [M + H]$^+$ 407.0.

3.1.17. (2R)-N-[5-(4-Chlorophenyl)-1,3,4-thiadiazol-2-yl]-2-[(phenylsulfonyl)-amino]-propanamide (**3c**)

Yield: 80.1%; m.p.: 226–228 °C; IR (KBr, σ/cm^{-1}): 3446, 3196 (υ_{NH}), 1703 ($\upsilon_{C=O}$), 1315 ($\upsilon as_{O=S=O}$), 1151 ($\upsilon s_{O=S=O}$), 691 (υ_{C-S}); ^{1}H NMR (DMSO-d$_6$): δ 1.21–1.23 (d, J = 7.08 Hz, 3H), 4.14–4.21 (m, 1H), 7.50–7.57 (m, 5H), 7.77–7.80 (dd, J_1 = 1.40 Hz, J_2 = 7.56 Hz, 2H), 7.91–7.93 (dd, J_1 = 1.84 Hz, J_2 = 6.80 Hz, 2H), 8.38–8.40 (d, J = 8.00 Hz, 1H), 12.74 (s, 1H); ESI-MS: m/z [M + H]$^+$ 423.1.

3.1.18. (2R)-N-[5-(4-Bromophenyl)-1,3,4-thiadiazol-2-yl]-2-[(phenylsulfonyl)-amino]-propanamide (**3d**)

Yield: 22.8%; m.p.: 240–241 °C; IR (KBr, σ/cm^{-1}): 3430, 3185 (υ_{NH}), 1708 ($\upsilon_{C=O}$), 1315 ($\upsilon as_{O=S=O}$), 1151 ($\upsilon s_{O=S=O}$), 691 (υ_{C-S}); ^{1}H NMR (DMSO-d$_6$): δ 1.22–1.23 (d, J = 6.96 Hz, 3H), 4.16–4.19 (t, J = 7.30 Hz, 1H), 7.50–7.55 (dd, J_1 = 7.04 Hz, J_2 = 14.44 Hz, 3H), 7.72–7.74 (d, J = 8.36 Hz, 2H), 7.78–7.80 (d, J = 6.64 Hz, 2H), 7.86–7.89 (d, J = 8.36 Hz, 2H), 8.44–8.46 (d, J = 7.96 Hz, 1H), 12.80 (s, 1H); ESI-MS: m/z [M + H]$^+$ 468.9.

3.1.19. (2R)-N-[5-(4-Nitrorophenyl)-1,3,4-thiadiazol-2-yl]-2-[(phenylsulfonyl)-amino]-propanamide (**3e**)

Yield: 46.8%; m.p.: 206–208 °C; IR (KBr, σ/cm^{-1}): 3428, 3198 (υ_{NH}), 1695 ($\upsilon_{C=O}$),1320 ($\upsilon as_{O=S=O}$), 1148 ($\upsilon s_{O=S=O}$), 691 (υ_{C-S}),1523 (υas_{NO2}), 1345 (υs_{NO2}); ^{1}H NMR (DMSO-d$_6$): δ 1.23–1.25 (d, J = 6.96 Hz, 3H), 4.17–4.20 (t, J = 7.32 Hz, 1H), 7.51–7.56 (dd, J_1 = 7.16 Hz, J_2 = 14.64 Hz, 3H), 7.78–7.80 (d, J = 6.68 Hz, 2H), 8.21–8.23 (d, J = 8.68 Hz, 2H), 8.36–8.38 (d, J = 8.68 Hz, 2H), 8.46–8.48 (d, J = 8.08 Hz, 1H), 12.95 (s, 1H); ESI-MS: m/z [M + H]$^+$ 434.3.

3.1.20. (2R)-N-[5-(4-Methylphenyl)-1,3,4-thiadiazol-2-yl]-2-[(phenylsulfonyl)-amino]-propanamide (**3f**)

Yield: 31.5%; m.p.: 215–216 °C; IR (KBr, σ/cm^{-1}): 3436, 3164 (υ_{NH}), 1699 ($\upsilon_{C=O}$), 1315 ($\upsilon as_{O=S=O}$), 1151 ($\upsilon s_{O=S=O}$), 691 (υ_{C-S}); ^{1}H NMR (DMSO-d$_6$): δ 1.21–1.23 (d, J = 6.80 Hz, 3H), 2.37 (s, 3H), 4.15–4.19 (t, J = 7.12 Hz, 1H), 7.34–7.36 (d, J = 7.80 Hz, 2H), 7.52–7.56 (t, J = 6.98 Hz, 3H), 7.78–7.82 (t, J = 7.66 Hz, 4H), 8.41–8.43 (d, J = 7.72Hz, 1H), 12.70 (s, 1H); ESI-MS: m/z [M + H]$^+$ 403.0.

3.1.21. (2R)-N-[5-(4-Methoxyphenyl)-1,3,4-thiadiazol-2-yl]-2-[(phenylsulfonyl)amino]-propanamide (**3g**)

Yield: 10.9%; m.p.: 237–238 °C; IR (KBr, σ/cm^{-1}): 3445, 3205 (υ_{NH}), 1700 ($\upsilon_{C=O}$), 1304 ($\upsilon as_{O=S=O}$), 1151 ($\upsilon s_{O=S=O}$), 691 (υ_{C-S}); ^{1}H NMR (DMSO-d$_6$): δ 1.21–1.23 (d, J = 6.96 Hz, 3H), 3.83 (s, 3H), 4.16 (s, 1H), 7.08–7.10 (d, J = 8.64 Hz, 2H), 7.51–7.5 (dd, J_1 = 7.72 Hz, J_2 = 15.20 Hz, 3H), 7.78–7.79 (d, J = 6.76 Hz, 2H), 7.85–7.87 (d, J = 8.60 Hz, 2H), 8.41–8.43 (d, J = 7.80 Hz, 1H), 12.67 (s, 1H); ESI-MS: m/z $[M + H]^+$ 419.0.

3.2. Enzyme inhibition assay (in vitro)

The target compounds were evaluated for inhibitory activity toward APN and MMP-2. Bestatin was used as the positive control. The target compounds were assayed for the inhibitory activities against APN using L-leucine *p*-nitroanilide as the substrate (Lejczak et al. 1989). The compound and the enzyme were dissolved in phosphate sodium buffer (pH 7.2, 50 mM), and incubated at 37 °C for 30 min. The substrate was added and incubated at 37 °C for another 60 min. The resulting solutions were measured under 405 nm to gain OD_{405} values, which were then used to calculate the inhibitory rates by $[OD_{405}(100\%) - OD_{405}(\text{compound})]/[OD_{405}(100\%) - OD_{405}(\text{blank})] \cdot 100\%$. MMP assay was performed according to the literature (Baragi et al. 2000) in 96-well microtiter plates using succinylated gelatin as the substrate. The compound and the enzyme were dissolved in sodium borate buffer (pH 8.5, 50 mM), and incubated at 37 °C for 30 min. The substrate was added and incubated at 37 °C for another 60 min. Then 0.03% picrylsulfonic acid solution was added and incubated at 37 °C for an additional 20 min. The resulting solutions were measured under 450 nm to gain OD_{450} values, which were then used to calculate the inhibitory rates by $[OD_{450}(100\%) - OD_{450}(\text{compound})]/[OD_{450}(100\%) - OD_{450}(\text{blank})] \cdot 100\%$. There were three parallels in the tests. The IC_{50} value was calculated from a sigmoid curve fitted to the inhibitor-response data using Origin data analysis software.

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