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Synthesis and activity of tryptophan sulfonamide derivatives as novel non-hydroxamate TNF-α converting enzyme (TACE) inhibitors

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ABSTRACT

A novel series of non-hydroxamate tryptophan sulfonamide derivatives containing a butynyloxy P1′ moiety was identified as inhibitors of TNF-α converting enzyme (TACE). The structure–activity relationship of the series was examined via substitution on the tryptophan indole ring. Of the compounds investigated, 2-(4-(but-2-ynyloxy)phenylsulfonamido)-3-(1-(4-methoxybenzyl)-1*H*-indol-3-yl)propanoic acid (**12p**) has the best in vitro potency against isolated TACE enzyme with an IC_{50} of 80 nM. Compound **12p** also shows good selectivity over MMP-1, -13, -14.

1. Introduction

TNF-α (tumor necrosis factor-α) is a major immunomodulatory and pro-inflammatory cytokine and is responsible for the initiation of protective inflammatory reactions.[1] Also, TNF-α has been implicated in several autoimmune disorders including rheumatoid arthritis (RA),[2] Crohn's disease, and psoriasis.[3] Biological agents that modulate TNF levels, such as etanercept (Enbrel®),[4] a soluble TNF-α receptor, and infliximab (Remicade®),[5] and adalimumab (Humira®),[6] monoclonal TNF-α antibodies, have been proven to be clinically efficacious in treating these diseases. The efficacy of these biological agents has led to the search for novel, orally active, and selective small molecule inhibitors of TACE for the treatment of these diseases. TACE (TNF-α converting enzyme) is a zinc endopeptidase and a member of the ADAM (a disintegrin and metalloprotease-containing enzyme) family of proteases (ADAM17). TACE is the primary enzyme responsible for cleaving 26 kDa membrane-bound TNF-α, pro-TNF-α, to generate 17 kDa soluble TNF-α.[7] The potential for inhibitors of TACE to treat RA by affecting levels of soluble TNF has made the development of orally active small molecule TACE inhibitors the subject of intense interest for the last decade.

Many TACE inhibitors have been derived from the modification of matrix metalloprotease (MMP) inhibitor scaffolds because of the structural similarities between the active sites of MMPs and TACE, and many of these inhibitors show limited selectivity for TACE over MMPs.[8] While TACE inhibitors with significant activity against some MMPs may provide an advantage in treating RA, since various MMPs are over-expressed in RA synovial tissue and contribute to joint destruction,[9] most broad spectrum MMP inhibitors have suffered from dose-limiting toxicity in clinical trials.[10] More recently, a number of inhibitors of TACE with excellent selectivity over MMPs have been reported.[11] While most of these inhibitors have incorporated a hydroxamic acid moiety as a bidentate chelator of the TACE active site zinc, certain liabilities of hydroxamates, such as poor pharmacokinetics (PK) due to hydrolysis, reduction and O-glucuronidation of this functional group, have led to the search for alternative zinc chelators.[12] In the past several years, non-hydroxamate TACE inhibitors have been reported[13] (Fig. 1) wherein the ubiquitous hydroxamate zinc binding group (ZBG) has been replaced by other ZBGs such as a pyrimidinetrione (**1**, **2**), hydantoin (**3**), triazolone (**4**), imidazolone (**5**) and triazolethione (**6**). Herein, we report the synthesis and SAR of a series of carboxylic acid TACE inhibitors derived from tryptophan.

2. Results and discussion

2.1. Chemistry

Structure-based design at Wyeth previously led to the discovery of sulfonamide[14] and sulfone hydroxamate[11a–d,15] TACE inhib-

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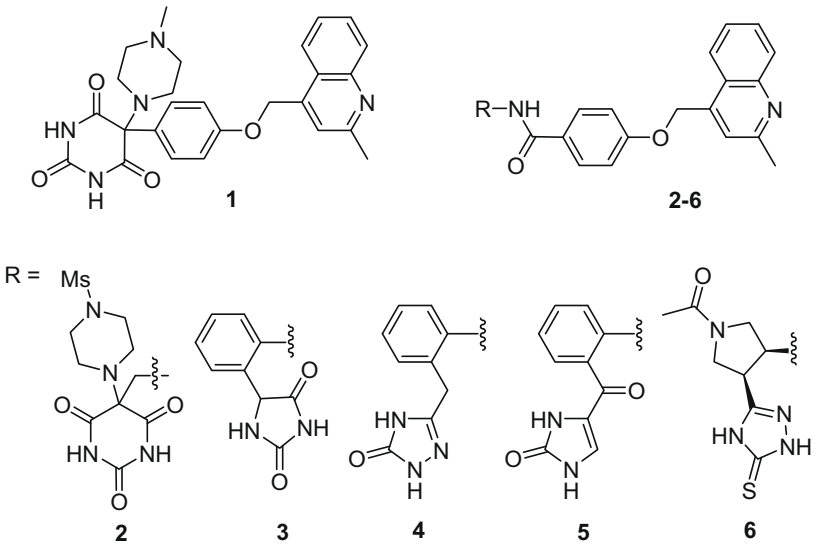


Figure 1. Reported non-hydroxamate TACE inhibitors.

itors bearing a novel butynyloxy P1′ group. The butynyloxy P1′ group provides varying levels of selectivity for TACE, and cellular activity, depending on the scaffold employed. In order to search for sulfonamide carboxylate TACE inhibitors, 4-butynyloxybenzenesulfonyl chloride **7**[14b] was reacted with 73 commercially available amino acids (Fig. 2). The resulting sulfonamide carboxylates were tested in a TACE FRET assay and the D and L tryptophan sulfonamide derivatives, **8** and **9**, were identified as the only single digit micromolar inhibitors. Interestingly, D-isomer **9** is three-fold more potent than L-isomer **8**, with IC_{50}s of 2.5 μM and 8.2 μM, respectively.

In an effort to further explore the SAR of the tryptophan sulfonamides, analogs of **8** were prepared via two synthetic routes depending on the availability of starting materials and targeted molecules. In the first route, commercially available tryptophans substituted on the phenyl ring of the indole group were functionalized as shown in Scheme 1. Thus, mono-substituted tryptophan **10** reacts with 4-butynyloxybenzenesulfonyl chloride **7**[14b] in the presence of triethylamine to give tryptophan sulfonamides **11a–n** in high yield (88–93%). N-Alkylation of **9** with alkyl halides in the presence of sodium hydride gives the desired tryptophan sulfonamides **12a–q** in good yield (70–85% yield).

An alternative route, used for the synthesis of di- and tri-substituted tryptophan sulfonamide derivatives **19a–w**, is shown in Scheme 2. Indoles **13** react with oxime **14**,[16] prepared from ethyl 3-bromo-2-oxopropanoate, in the presence of sodium carbonate at room temperature to give indole-oxime **15** as a 3:1 mixture of regioisomers. Reduction of **15** with zinc powder in acetic acid then provides tryptophanyl esters **16**. Sulfonylation of **16** in the presence of triethylamine at room temperature gives benzenesulfonamide ester **17** (55–70% yield for three steps), which is then

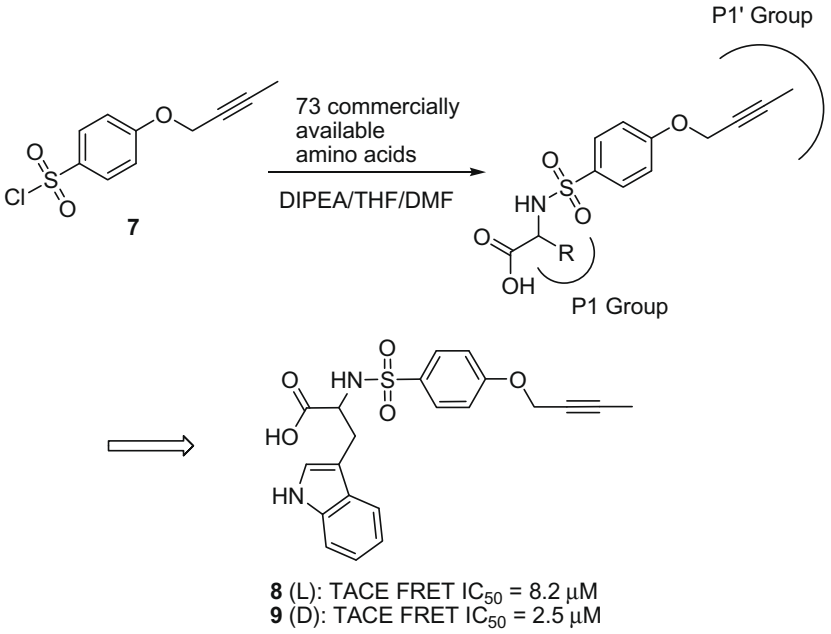
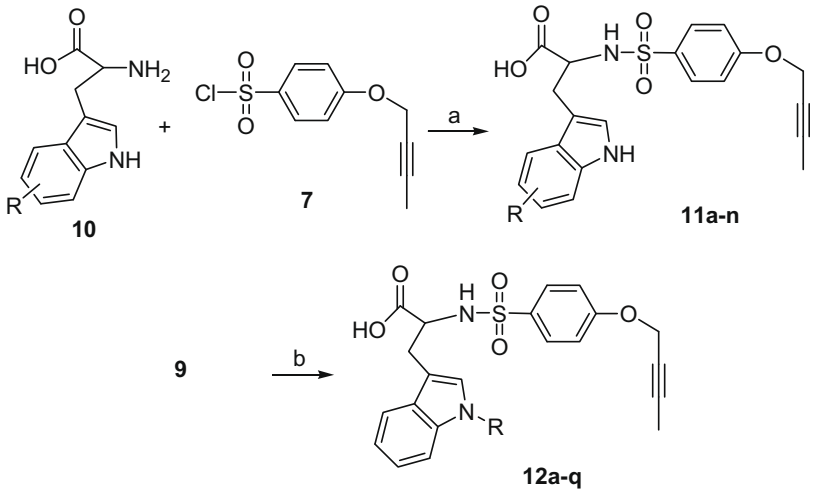


Figure 2. Screening the sulfonamides prepared by the reaction of 73 commercially available amino acids with 4-butynyloxybenzenesulfonyl chloride **7**.

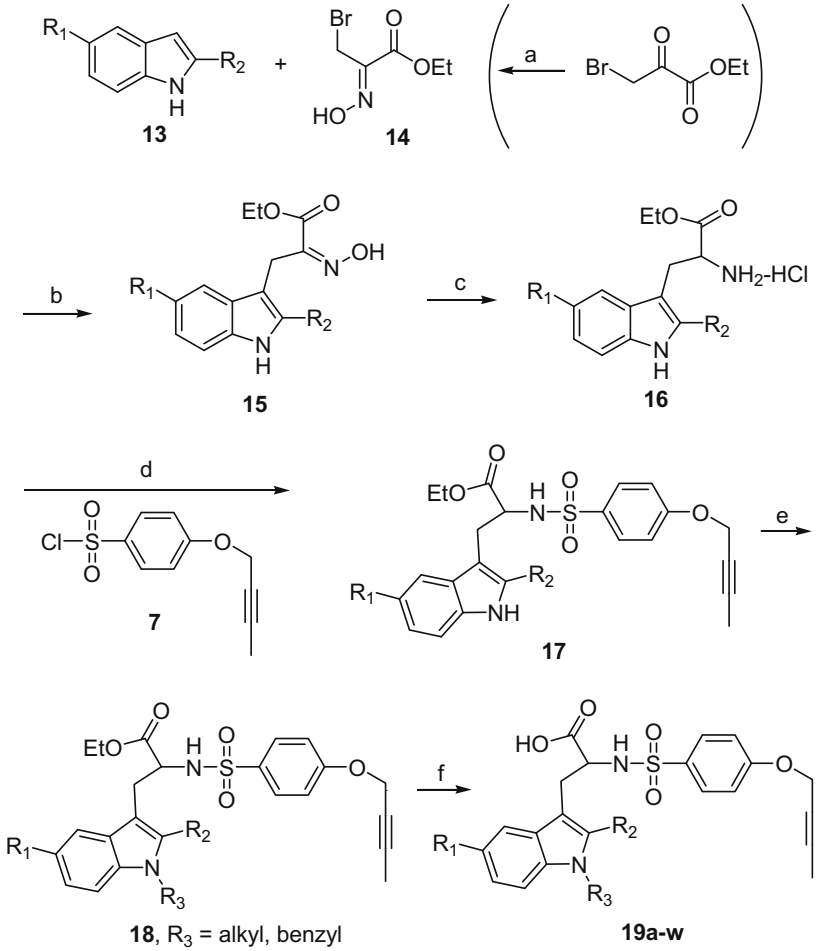


Scheme 1. Preparation of **11a–n** and **12a–q**. Reagents and conditions: (a) triethylamine, rt, 20 h, H_2O–dioxane, 88–93%; (b) NaH, 0 °C, 10 h, DMF, R_2X (X = I, Br, Cl) 70–85%.

reacted with alkyl or benzyl halides in the presence of NaH at 0 °C to give N-substituted tryptophanyl esters **18** in good to moderate yields (65–85%). Finally, esters **18** are hydrolyzed with 2 N sodium hydroxide solution in MeOH–THF at room temperature to give the desired tryptophan sulfonamide derivatives **19a–w** in high yield (90–96% yield).

2.2. Biological assays

The prepared compounds **11a–n**, **12a–q** and **19a–w** were tested in a FRET assay using the catalytic domain of TACE[17] and selected analogs were then profiled for selectivity against MMP-1, MMP-2, MMP-13 and MMP-14. Also, selected compounds were evaluated



Scheme 2. Preparation of **19a–w**. Reagents and conditions: (a) $(NH_2OH)_2$–H_2SO_4, rt, 24 h, 90%; (b) Na_2CO_3, CH_2Cl_2, rt, 24 h; (c) (i) Zn, AcOH, rt, 12 h; (ii) HCl (d) TEA, rt, 20 h, dioxane–H_2O, 55–70% in three steps; (e) 2 equiv NaH, R_3X (X = Br or Cl), 0 °C, 10 h, DMF, 65–85%; (f) NaOH, rt, 12 h, THF–MeOH, 90–96%.

for their ability to inhibit LPS-stimulated TNF production in Raw cells.[17]

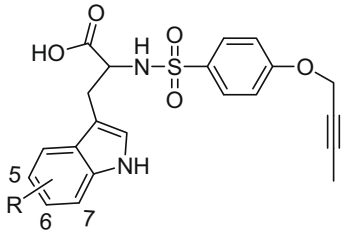
2.3. Structure–activity relationships

As shown in Table 1, racemic tryptophan analogs mono-substituted at the 5- or 6-position of the indole (**11a**, **11d**, **11g**, **11j**–**n**) exhibit better TACE enzyme activity than initial lead compound **9**, with the exception of the 6-fluoro derivative **11m**. The 5-methyl and 5-methoxy substituents provide the most activity. As for enantiomers **8** and **9**, chiral separation of **11a**, **11d**, and **11g**, provided one enantiomer (**11c**, **11f**, **11i**) that was greater than 10-fold more potent than the other enantiomer (**11b**, **11e**, **11h**).

The activity of a series of N-substituted indolyl carboxylic acids is shown in Table 2. Thus, most *N*-alkyl and *N*-benzyl substituents are tolerated, showing activity similar to or better than **9**, with the notable exception of the *ortho*-trifluoromethylbenzyl analog **12j**. In particular, compound **12p** bearing a *p*-methoxybenzyl group is by far the most active compound in this series with an IC_{50} of 0.08 μM, 31-fold more potent than initial lead **9**. Also, as expected, L isomer **12q** is 15-fold less active than D isomer **12p**.

A variety of racemic tryptophan derivatives di- and tri-substituted on the indole moiety were also prepared and evaluated in vitro, as shown in Table 3. Of the substituents surveyed at the 5-position (R_1) only the carboxylic acid group was not tolerated (**19a**–**c**). Comparison of the di-substituted analog **19f** with the corresponding mono-substituted compounds **11a** and **12p** indicates that 2,5-disubstitution diminishes activity relative to the parent mono-substituted analogs. Furthermore, no analog di-substituted at R_1 and R_3 has an IC_{50} less than 0.89 μM. However, tri-substituted analogs with activity less than 0.40 μM can be obtained. Thus, a significant effect on activity is seen with substitution at the 2-position (R_2), that increases potency more than four-fold (**19j** vs **19k**, **19l** vs **19m**, and **19p** vs **19q**). When R_1 and R_2 are held constant as methoxy and methyl, respectively, the best activity is seen from the R_3 benzyl derivative **19i**, 3.5-fold more potent than the corresponding R_3 methyl derivative **19h**. Substituents on the R_3 benzyl group are tolerated (**19n**, **19o**, **19q**–**w**) but do not improve binding activity relative to **19i**.

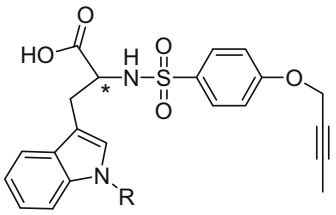
Table 1
Inhibition of mono-substituted indole sulfonamide carboxylates in TACE assay



Compound	R	TACEIC$_{50}$ (μM)
8	H, (L)	8.20
9	H, (D)	2.53
11a	5-Me (racemic)	0.28
11b	5-Me (enantiomer-1)	27%[a]
11c	5-Me (enantiomer-2)[b]	0.14
11d	5-MeO (racemic)	0.36
11e	5-MeO (enantiomer-1)	10.1
11f	5-MeO (enantiomer-2)	0.14
11g	5-BnO (racemic)	0.75
11h	5-BnO (enantiomer-1)	6.98
11i	5-BnO (enantiomer-2)	0.37
11j	5-F (racemic)	1.56
11k	5-OH (racemic)	1.61
11l	5-Br (racemic)	0.63
11m	6-F (racemic)	2.96
11n	6-Me (racemic)	1.57

[a] % Inhibition at 1 μM.
[b] D isomer based on X-ray complex structure with TACE.

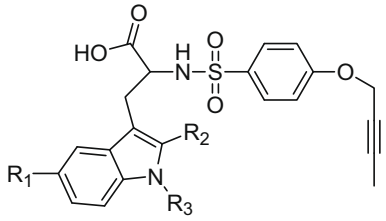
Table 2
Inhibition of N-substituted indole sulfonamide carboxylates in TACE assay



Compound	R	Stereochem.	TACE IC$_{50}$ (μM)
12a	BOC	D	3.67
12b	Me	D	1.43
12c	*n*-Pentyl	D	2.31
12d	Cyclobutylmethyl	D	1.71
12e	4-PhO-Bu	D	1.25
12f	Bn[b]	D	2.15
12g	*o*-Ph-Bn	D	0.97
12h	*p*-Allyloxy-Bn	D	0.54
12i	3,5-Dimethoxy-Bn	D	0.98
12j	*o*-CF_3-Bn	D	2.4%[a]
12k	*m*-CF_3-Bn	D	2.30
12l	*p*-CF_3-Bn	D	4.40
12m	*p*-Me-Bn	D	3.59
12n	*p*-F-Bn	D	1.90
12o	*p*-Cl-Bn	D	3.45
12p	*p*-MeO-Bn	D	0.08
12q	*p*-MeO-Bn	L	1.18

[a] % Inhibition at 1 μM.
[b] Bn = benzyl.

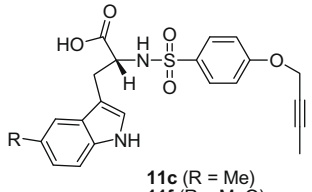
Table 3
Inhibition of di- or tri-substituted indole sulfonamide carboxylates in TACE assay



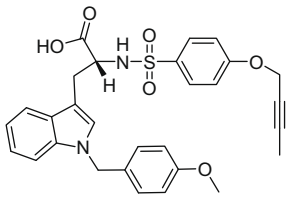
Compound	R_1	R_2	R_3	TACE IC$_{50}$ (μM)
19a	CO_2H	H	Me	>100[b]
19b	CO_2H	H	Et	>100[b]
19c	CO_2H	H	Bn[a]	>100[b]
19d	MeO	H	2-Methylpropyl	1.23
19e	Cl	Me	2-Methylpropyl	0.30
19f	Me	H	*p*-MeO-Bn	0.89
19g	Cl	Me	*p*-MeO-Bn	0.19
19h	MeO	Me	Me	0.90
19i	MeO	Me	Bn	0.25
19j	MeO	H	3,4-Methylenedioxy-Bn	1.50
19k	MeO	Me	3,4-Methylenedioxy-Bn	0.33
19l	MeO	H	*m*-MeO-Bn	2.24
19m	MeO	Me	*m*-MeO-Bn	0.28
19n	MeO	Me	*o*-CF_3-Bn	0.50
19o	MeO	Me	*p*-CF_3-Bn	0.49
19p	MeO	H	*o*-F-Bn	2.24
19q	MeO	Me	*o*-F-Bn	0.30
19r	MeO	Me	*m*-F-Bn	0.34
19s	MeO	Me	*p*-F-Bn	0.32
19t	MeO	Me	*p*-Cl-Bn	0.29
19u	Cl	Me	*p*-Cl-Bn	0.33
19v	MeO	Me	*m*-CN-Bn	0.41
19w	MeO	Me	*p*-CN-Bn	0.47

[a] Bn = benzyl.
[b] Not active @ 100 μM concentration.

Table 4
IC_{50}s (μM) of selected tryptophan derivatives **11c**, **11f**, **11i**, and **12p** in MMP and Raw cells assays



11c (R = Me)
11f (R = MeO)
11i (R = BnO)



12p

Compound	MMP-1	MMP-2	MMP-13	MMP-14	TACE	Raw cells
11c	>50	0.27	1.81	6.82	0.14	7.8
11f	>50	1.27	13.6	20.7	0.14	57
11i	>50	3.27	4.44	19.7	0.37	
12p	>50	1.44	4.56	13.0	0.08	>100

2.4. Selectivity and cellular activity

Four tryptophan carboxylate compounds (**11c**, **11f**, **11i**, and **12p**) that exhibited good TACE activity were selected for selectivity profiling against MMP-1, MMP-2, MMP-13 and MMP-14. As shown in Table 4, all four compounds show greater than 100-fold selectivity against MMP-1, more than 50-fold selectivity against MMP-14, and greater than 10-fold selectivity against MMP-13. Selectivity over MMP-2 is the most difficult to achieve, with only compound **12p** exceeding 10-fold. Compounds, **11c**, **11f**, and **12p**, were also assayed for their ability to inhibit LPS-induced TNF production in Raw cells. Unfortunately, none of these derivatives display submicromolar levels of cell activity, perhaps due to permeability issues stemming from the carboxylate moiety.

2.5. X-ray co-complex structure

As shown in Figure 3, an X-ray structure of compound **11c** bound to TACE was successfully obtained at a resolution of 2.1 Å.[18] As expected, the carboxylate of **11c** is ligated to the active site zinc ion that is also coordinated to three histidine residues, His405, His409 and His415. The 5-methyl indole moiety is involved in hydrophobic interactions with Ala351 and Val353 and may participate in a π interaction with His 409. As with the other butynyloxy phenyl sulfonamide inhibitors previously disclosed, the butynyloxy P1′ group rests in the narrow hydrophobic channel connecting the S1′ and S3′ pockets of TACE. Based on this crystal structure of **11c** it is assumed that the eutamers of the separated enantiomeric pairs shown in Table 1 are also derived from the D amino acids, as the enzyme would not be able to efficiently accommodate the indole group of the corresponding L isomers.

3. Summary and conclusion

In summary, we have identified a novel series of tryptophan sulfonamides as non-hydroxamate inhibitors of TACE and explored the effect of substitution around indole core on TACE activity. It was found that 5-methyl or 5-methoxy substituents at the 5-position of the indole and *N*-*p*-methoxybenzyl indole derivatives significantly increase inhibitory activity by providing additional hydrophobic interactions and/or π interactions with neighboring amino acid residues in the TACE active site, resulting in IC_{50}s of less than

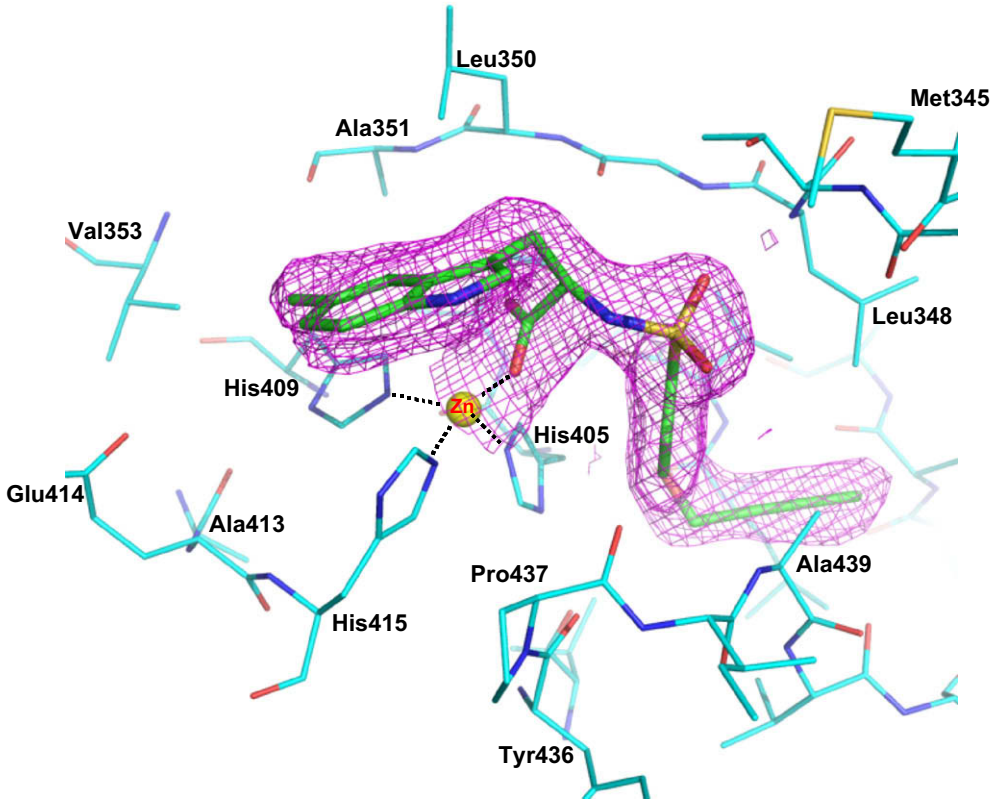


Figure 3. An X-ray co-complex structure of **11c** bound to TACE enzyme (resolution of 2.1 Å). Three histidine residues and a carboxylate coordinate to the active site zinc ion (yellow sphere). The butynyloxy P1′ group is located between the S1′ and S3′ pockets of TACE.

0.15 μM. A stereochemical preference was found for derivatives of D tryptophan over the analogous L stereoisomers. Of the compounds in this series assayed for selectivity over various MMPs, 50- to 100-fold selectivity was achieved over MMP-1 and MMP-14, while lesser selectivity was seen for MMP-13 and MMP-2. Based on these data, the tryptophan sulfonamide scaffold is deemed a useful lead for novel non-hydroxamate TACE inhibitiors.

4. Experimental

4.1. Chemistry

All reactions were performed under N_2 atmosphere using anhydrous solvents. Purification of products was carried out by flash chromatography using EM Silica Gel 60 (230–400 mesh). Semi-preparative HPLC was used under these conditions: A = 0.02% TFA in water, B = 0.02% TFA in acetonitrile, 10–95% B in 8 min, 34 mL/min, 50 °C, 215 nm detection, Waters Xterra™ 20 × 50 mm column. ^{1}H NMR spectra were recorded at 300 MHz. Tetramethylsilane (δ = 0 ppm) was used as an internal standard, and $CDCl_3$, acetone-d_6 or DMSO-d_6 were used as solvents. Chiral separation was performed by preparative chiral HPLC using CHIRALPACK-AD (250 × 20 mm) and the chiral purity of the different enantiomers was determined by chiral HPLC using CHIRALPACK-AD (250 × 4.6 mm).

4.1.1. 2-(4-But-2-ynyloxy-benzenesulfonyl-amino)-3-(5-methyl-*1H*-indol-3-yl)-propionic acid (11a)

Triethylamine (0.38 mL, 2.75 mmol) was added to a solution of 5-methyl-DL-tryptophan (0.20 g, 0.92 mmol) and 4-but-2-ynyloxy-benzenesulfonyl chloride (0.25 g, 1.01 mmol) in H_2O–dioxane (1.6 mL:2.4 mL) at room temperature. The reaction mixture was stirred for 20 h at room temperature. The mixture was then concentrated and acidified with HCl. The mixture was extracted with ethyl acetate (3 × 10 mL). The organic solution was washed with H_2O and brine, dried over Na_2SO_4, and concentrated. The residue was purified by RP-HPLC to give 0.32 g (81%) of the title compound as a pale yellow solid. Mp = 45–46 °C; ^{1}H NMR (DMSO-d_6): 400 MHz δ 12.54 (br s, 1H), 10.64 (s, 1H), 8.05 (d, J = 8.0 Hz, 1H), 7.53 (m, 2H), 7.17 (d, J = 8.0 Hz, 1H), 7.00 (m, 2H), 6.95 (m, 2H), 6.86 (dd, J = 8.0, 4.0 Hz, 1H), 4.78 (q, J = 2.4 Hz, 2H), 3.81 (q, J = 7.6 Hz, 1H), 3.00 (dd, J = 14.4, 6.8 Hz, 1H), 2.79 (dd, J = 14.4, 7.8 Hz, 1H), 2.33 (s, 3H), 1.82 (t, J = 2.4 Hz, 3H); HRMS: calcd for $C_{22}H_{23}N_2O_5S$ (ESI+, $[M+H]^{1+}$), 427.1322; found 427.1327; LC–MS m/z (ESI) $[M+H]^{1+}$ 427, retention time = 2.69 min.

4.1.2. Chiral separation of 11a to give 11b and 11c

Preparative HPLC of **11a** using Chiralpak-OD (20 × 250 mm) and 15% isopropyl alcohol in heptane (0.02% TFA) as eluant (instrument: JAI-908, flow rate: 19 mL/min) gave D and L enantiomers as white solids. Analytical chiral HPLC (Chiralcel-OD, 4.6 × 250 mm, ethanol/heptane (containing 0.02% TFA) = 13:87, 1.0 mL/min, 225 nm) retention time (T_R): 23.0 min (1st peak: **11b**) and 26.2 min (2nd peak: **11c**). The absolute stereochemistry of **11c** was determined by X-ray crystallography.

4.1.3. 2-(4-But-2-ynyloxy-benzenesulfonyl-amino)-3-(5-methoxy-*1H*-indol-3-yl)-propionic acid (11d)

The title compound was prepared using the same procedure for the synthesis of **11a** except using 5-methoxy-DL-tryptophan instead of 5-methyl-DL-tryptophan. ^{1}H NMR (DMSO-d_6): 400 MHz δ 12.55 (br s, 1H), 10.63 (s, 1H), 8.06 (d, J = 8.0 Hz, 1H), 7.53 (dd, J = 8.0, 4.0 Hz, 2H), 7.18 (d, J = 8.0 Hz, 1H), 7.02 (d, J = 2.0 Hz, 1H), 6.92 (d, J = 8.0 Hz, 2H), 6.82 (d, J = 2.0 Hz, 1H), 6.68 (dd, J = 8.0, 2.0 Hz, 1H), 4.78 (q, J = 2.0 Hz, 2H), 3.87 (q, J = 7.6 Hz, 1H), 3.72 (s, 3H), 3.00 (dd, J = 14.4, 6.4 Hz, 1H), 2.82 (dd, J = 14.4, 7.6 Hz, 1H), 1.83 (t, J = 2.0 Hz, 3H); HRMS: calcd for $C_{22}H_{24}N_2O_6S$ (ESI+, $[M+H]^{1+}$), 443.1271; found 443.1265; LC–MS m/z (ESI) [M+H] 443, retention time = 2.50 min.

4.1.4. Chiral separation of 11d to give 11e and 11f

Preparative HPLC of **11d** using Chiralpak-OJ (20 × 250 mm) and 15% ethanol in heptane (0.1% TFA) as eluant (flow rate: 7 mL/min) gave D and L enantiomers as white solids. Analytical chiral HPLC (Chiralcel-OJ, 4.6 × 250 mm, ethanol/heptane (containing 0.02% TFA) = 85:15, 1.0 mL/min, 215 nm) retention time (T_R): 4.86 min (1st peak: **11e**) and 6.32 min (2nd peak: **11f**). The absolute stereochemistry of the two enantiomers was not determined.

4.1.5. 2-(4-But-2-ynyloxy-benzenesulfonyl-amino)-3-(5-benzyloxy-*1H*-indol-3-yl)-propionic acid (11g)

The title compound was prepared by using same procedure for the synthesis of **11a** except using 5-benzyloxy-DL-tryptophan instead of 5-methyl-DL-tryptophan. ^{1}H NMR (DMSO-d_6): 400 MHz δ 12.55 (br s, 1H), 10.64 (s, 1H), 8.05 (d, J = 8.0 Hz, 1H), 7.50–7.33 (m, 7H), 7.19 (d, J = 12.0 Hz, 1H), 7.03 (d, J = 4.0 Hz, 1H), 6.97 (d, J = 4.0 Hz, 1H), 6.87 (d, J = 8.0 Hz, 2H), 6.76 (dd, J = 8.0, 4.0 Hz, 1H), 5.04 (s, 2H), 4.74 (q, J = 2.4 Hz, 2H), 3.86 (q, J = 8.0 Hz, 1H), 3.00 (dd, J = 14.4, 6.4 Hz, 1H), 2.83 (dd, J = 14.4, 8.0 Hz, 1H), 1.82 (t, J = 2.4 Hz, 3H); HRMS: calcd for $C_{28}H_{27}N_2O_6S$ (ESI+, $[M+H]^{1+}$), 519.1584; found 519.1588; LC–MS m/z (ESI) [M+H] 519, retention time = 2.99 min.

4.1.6. Chiral separation of 11g to give 11h and 11i

Preparative HPLC of **11g** using Chiralpak-OJ (20 × 250 mm) and 15% ethanol in heptane (0.1% TFA) as eluant (flow rate: 13 mL/min) gave D and L enantiomers as white solids. Analytical chiral HPLC (Chiralcel-OJ, 4.6 × 250 mm, ethanol/heptane (containing 0.02% TFA) = 85:15, 1.0 mL/min, 215 nm) retention time (T_R): 6.76 min (1st peak: **11h**) and 13.1 min (2nd peak: **11i**). The stereochemistry of the two enantiomers was not determined.

4.1.7. 3-(5-Bromo-*1H*-indol-3-yl)-2-(4-but-2-ynyloxy-benzenesulfonylamino)-propionic acid (11l)

4.1.7.1. Synthesis of 3-bromo-2-hydroxyimino-propionic acid ethyl ester[16b]. A solution of hydroxylamine sulfate (5.00 g, 31 mmol) in water (30 mL) was added to a solution of ethylbrompyruvate (90%) (6.50 g, 30 mmol) in $CHCl_3$ (10 mL). The two-phase system was rapidly stirred for 24 h at room temperature. The mixture was extracted with $CHCl_3$ (3 × 30 mL). The combined extracts were dried over Na_2SO_4 and concentrated to give 7.00 g of the title compound as a white solid. ^{1}H NMR ($CDCl_3$): 300 MHz δ 10.08 (br s, 1H), 4.38 (q, J = 7.2 Hz, 2H), 4.27 (s, 2H), 1.39 (t, J = 7.2 Hz, 3H).

4.1.7.2. Synthesis of 2-hydroxyimino-3-(5-bromo-1*H*-indol-3-yl)-propionic acid ethyl ester. A solution of 3-bromo-2-hydroxyimino-propionic acid ethyl ester (3.21 g, 15.3 mmol) in CH_2Cl_2 (20 mL) was slowly added dropwise to a stirring mixture of 5-bromoindole (3.0 g, 15.3 mmol) and Na_2CO_3 (8.92 g, 84.2 mmol) in CH_2Cl_2 (30 mL) at room temperature. The mixture was stirred for 20 h, filtered through Celite and concentrated to give 4.94 g of the title compound. LC–MS m/z (ESI) $[M+H]^+$ calcd for $C_{13}H_{14}BrN_2O_3$ (Br^{79}) 325.2, found 325.2.

4.1.7.3. Synthesis of 2-amino-3-(5-bromo-1*H*-indol-3-yl)-propionic acid ethyl ester. Zn dust (3.30 g, 50.4 mmol) was added portionwise to a stirred solution of 2-hydroxyimino-3-(5-bromo-*1H*-indol-3-yl)-propionic acid ethyl ester (4.10 g, 12.61 mmol) in acetic acid (100 mL) over 30 min. After stirring overnight, the mixture was filtered through Celite and concentrated. The residue was dissolved in 1 N HCl and re-evaporated to give 4.30 g of the title com-

pound. LC–MS *m/z* (ESI) $[M-HCl+H]^+$ calcd for $C_{13}H_{15}BrN_2O_2$ (Br^{79}) 312.2, found 312.2.

4.1.7.4. Synthesis of 2-(4-but-2-ynyloxy-benzenesulfonylamino)-3-(5-bromo-1*H*-indol-3-yl)-propionic acid ethyl ester. Triethylamine (4.81 mL, 34.5 mmol) was added to a mixture of 2-amino-3-(5-bromo-*1H*-indol-3-yl)-propionic acid ethyl ester (4.00 g, 11.51 mmol) and 4-but-2-ynyloxy-benzenesulfonyl chloride (3.10 g, 12.66 mmol) in H_2O–dioxane (40 mL:60 mL) at room temperature. The reaction mixture was stirred for 20 h at room temperature. The mixture was concentrated and diluted with ethyl acetate. The organic solution was washed with H_2O and brine, dried over Na_2SO_4 concentrated. The residue was purified by flash column chromatography (silica, 40% ethyl acetate in hexanes) to give 3.67 g of the title compound as a pale yellow solid. ^{1}H NMR (DMSO-d_6): 400 MHz δ 11.05 (s, 1H), 8.31 (d, J = 8.8 Hz, 1H), 7.56 (d, J = 8.8 Hz, 2H), 7.45 (d, J = 1.6 Hz, 1H), 7.27 (d, J = 8.4 Hz, 1H), 7.14 (m, 2H), 6.99 (d, J = 8.8 Hz, 2H), 4.80 (q, J = 2.4 Hz, 2H), 3.88 (q, J = 7.6 Hz, 1H), 3.75 (q, J = 7.2 Hz, 2H), 3.00 (dd, J = 14.4, 7.2 Hz, 1H), 2.88 (dd, J = 14.4, 7.6 Hz, 1H), 1.82 (t, J = 2.4 Hz, 3H), 0.92 (t, J = 7.2 Hz, 3H); HRMS: calcd for $C_{23}H_{24}BrN_2O_5S$ (ESI+, Br^{79}, $[M+H]^{1+}$), 519.0584; found 519.0589.

4.1.7.5. Synthesis of 3-(5-bromo-1*H*-indol-3-yl)-2-(4-but-2-ynyloxy-benzenesulfonylamino)-propionic acid. 2-(4-But-2-ynyloxy-benzenesulfonylamino)-3-(5-bromo-*1H*-indol-3-yl)-propionic acid ethyl ester (0.100 g, 0.193 mmol) was dissolved in 0.5 mL tetrahydrofuran. To the solution were added MeOH (0.5 mL) and 1 N NaOH (0.4 mL, 0.4 mmol). The reaction mixture was placed in a shaker for 10 h at room temperature. The solution was acidified with 1 N HCl and extracted with ethyl acetate (3 × 3 mL). The organic solution was concentrated and the residue was purified by RP-HPLC to give 0.089 g (94%) of the title compound as a pale yellow solid. ^{1}H NMR (DMSO-d_6): 400 MHz δ 12.65 (br s, 1H), 11.01 (s, 1H), 8.08 (d, J = 8.8 Hz, 1H), 7.48 (m, 3H), 7.26 (d, J = 8.8 Hz, 1H), 7.13 (m, 2H), 6.89 (d, J = 9.2 Hz, 2H), 4.77 (q, J = 2.8 Hz, 2H), 3.82 (q, J = 8.4 Hz, 1H), 3.01 (dd J = 14.6, 6.4 Hz, 1H), 2.82 (dd, J = 14.6, 8.0 Hz, 1H), 1.83 (t, J = 2.4 Hz, 3H); HRMS: calcd for $C_{21}H_{20}BrN_2O_5S$ (ESI+, Br^{79}, $[M+H]^{1+}$), 491.0271; found 491.0287; LC–MS *m/z* (ESI) $[M-H]$ 489 (Br^{79}), retention time = 2.75 min.

4.1.8. 2-(4-But-2-ynyloxy-benzenesulfonyl-amino)-3-(1-cyclobutylmethyl-*1H*-indol-3-yl)-propionic acid (12d)

4.1.8.1. Synthesis of *N*-(*tert*-butoxycarbonyl)-1-cyclobutylmethyl-D-tryptophan. NaH (60% in mineral oil) (0.092 g, 2.30 mmol) was added to a solution of *N*-(*tert*-butoxycarbonyl)-D-tryptophan (0.200 g, 0.657 mmol) in anhydrous *N*,*N*-dimethylformamide (4 mL) at 0 °C. The reaction mixture was placed in a shaker for 20 min at 0 °C. (Bromomethyl)-cyclobutane (0.074 mL, 0.657 mmol) was added to the solution and allowed to react for 10 h at 0 °C. The reaction mixture was quenched with H_2O and acidified with 1 M $NaHSO_4$ until pH 3. The solution was extracted with CH_2Cl_2, washed with brine, dried over Na_2SO_4, and concentrated to give the title compound.

4.1.8.2. Synthesis of 1-cyclobutylmethyl-D-tryptophan. The dried crude product from Section 4.1.8.1 was dissolved in CH_2Cl_2-TFA (1 mL/1 mL) and the solution was stirred for 10 h at room temperature. The solution was concentrated and the resulting product was dissolved in CH_2Cl_2. The solution was washed with H_2O and brine, dried over Na_2SO_4, and concentrated to give the title compound.

4.1.8.3. Synthesis of 2-(4-but-2-ynyloxy-benzenesulfonylamino)-3-(1-cyclobutyllmethyl-1*H*-indol-3-yl)-propionic acid. Triethylamine (0.275 mL, 1.97 mmol) was added to a solution of crude 1-cyclobutylmethyl-D-tryptophan (0.657 mmol) and 4-but-2-ynyloxy-benzenesulfonyl chloride (0.177 g, 0.723 mmol) in H_2O–dioxane (2 mL:3 mL) at room temperature. The reaction mixture was stirred for 20 h at room temperature. The mixture was then concentrated and acidified with HCl. The mixture was extracted with ethyl acetate (3 × 10 mL). The organic solution was washed with H_2O and brine, dried over Na_2SO_4, and concentrated. The residue was purified by RP-HPLC to give 0.152 g (48% in three steps) of the title compound as a yellow solid. ^{1}H NMR (DMSO-d_6): 400 MHz δ 12.57 (br s, 1H), 8.07 (d, J = 8.4 Hz, 1H), 7.49 (d, J = 7.2 Hz, 2H), 7.37 (d, J = 8.0 Hz, 1H), 7.29 (d, J = 7.6 Hz, 1H), 7.08 (t, J = 7.4 Hz, 1H), 7.02 (s, 1H), 6.96–6.88 (m, 3H), 4.77 (q, J = 2.4 Hz, 2H), 4.04 (d, J = 6.8 Hz, 2H), 3.84 (q, J = 6.8 Hz, 1H), 3.02 (dd J = 14.4, 6.4 Hz, 1H), 2.82 (dd, J = 14.4, 8.4 Hz, 1H), 2.67 (p, J = 7.6 Hz, 1H), 1.91 (m, 2H), 1.83 (m, 5H), 1.75 (m, 2H); HRMS: calcd for $C_{26}H_{29}N_2O_5S$ (ESI+, $[M+H]^{1+}$), 481.1792; found 481.1786; LC–MS *m/z* (ESI) [M+H] 481, retention time = 3.22 min.

4.1.9. 2-(4-But-2-ynyloxy-benzenesulfonyl-amino)-3-[1-(2-trifluoromethyl-benzyl)-*1H*-indol-3-yl]-propionic acid (12j)

The title compound was prepared using the same procedure for the synthesis of **12d** except using 2-(trifluoromethyl)benzyl bromide instead of (bromomethyl)-cyclobutane. ^{1}H NMR (DMSO-d_6): 400 MHz δ 12.57 (br s, 1H), 8.13 (d, J = 8.4 Hz, 1H), 7.78 (d, J = 6.8 Hz, 1H), 7.57 (d, J = 8.8 Hz, 2H), 7.43 (m, 2H), 7.37 (d, J = 7.6 Hz, 1H), 7.19 (s, 1H), 7.12–6.99 (m, 3H), 6.93 (d, J = 8.8 Hz, 2H), 6.44 (d, J = 6.4 Hz, 1H), 5.51 (s, 2H), 4.77 (q, J = 2.0 Hz, 2H), 3.91 (q, J = 7.6 Hz, 1H), 3.08 (dd, J = 14.4, 7.2 Hz, 1H), 2.88 (dd, J = 14.4, 7.6 Hz, 1H), 1.82 (t, J = 2.4 Hz, 3H); HRMS: calcd for $C_{29}H_{26}F_3N_2O_5S$ (ESI+, $[M+H]^{1+}$), 571.1509; found 571.1505; LC–MS *m/z* (ESI) [M+H] 571, retention time = 3.37 min.

4.1.10. 2-(4-But-2-ynyloxy-benzenesulfonyl amino)-3-[1-(4-methoxy-benzyl)-5-methyl-*1H*-indol-3-yl]-propionic acid (19f)

NaH (60% in mineral oil) (0.033 g, 0.831 mmol) was added to a solution of 2-(4-but-2-ynyloxy-benzenesulfonylamino)-3-(5-methyl-*1H*-indol-3-yl)-propionic acid (**11a**) (0.100 g, 0.234 mmol) in anhydrous *N*,*N*-dimethylformamide (2 mL) at 0 °C. The reaction mixture was placed in a shaker for 20 min at 0 °C. 4-Methoxy-benzyl chloride (0.032 mL, 0.234 mmol) was added to the solution and allowed to react for 10 h at 0 °C. The reaction mixture was quenched with H_2O and acidified with HCl. The solution was extracted with EtOAc, washed with H_2O and brine, dried over Na_2SO_4, and concentrated. The residue was purified by RP-HPLC to give 0.086 g (67%) of the title compound as a pale yellow solid. ^{1}H NMR (DMSO-d_6): 400 MHz δ 12.58 (br s, 1H), 8.09 (d, J = 8.4 Hz, 1H), 7.53 (d, J = 11.6 Hz, 2H), 7.20 (d, J = 8.0 Hz, 1H), 7.09 (s, 1H), 7.07 (d, J = 8.4 Hz, 2H), 7.00 (s, 1H), 6.92–6.80 (m, 5H), 5.17 (s, 2H), 4.76 (q, J = 2.4 Hz, 2H), 3.82 (q, J = 8.0 Hz, 1H), 3.69 (s, 3H), 3.00 (dd J = 14.4, 6.8 Hz, 1H), 2.79 (dd, J = 14.4, 7.6 Hz, 1H), 2.32 (s, 3H), 1.82 (t, J = 2.4 Hz, 3H); HRMS: calcd for $C_{30}H_{31}N_2O_6S$ (ESI+, $[M+H]^{1+}$), 547.1897; found 547.1896; LC–MS *m/z* (ESI) $[M-H]$ 545, retention time = 2.76 min.

4.2. TACE FRET assay

4.2.1. Materials and methods

The proprietary pro-TNF-α substrate peptide Abz-LAQAVRSSSR-Dpa (AnaSpec, WARC-1) was prepared as a 2 mM stock solution in the TACE assay buffer (50 mM Tris–HCl, pH 7.4, 25 mM NaCl, 4% Glycerol, 0.005% Brij 35). The catalytic domain of the recombinant TACE protein was expressed and purified in-house. The TACE protein (1 μg/ml) was pre-treated with the inhibitors at various concentrations for 10 min at room temperature. The reaction was initiated by the addition of the substrate peptide. The increase in fluorescence was monitored at an excitation of 320 nM and an

emission of 420 nM over a period of 10 min. The initial rate (slope) of the reaction was determined using a fluorescence plate reader (Molecular Devices, Spectra Max Gemini XS).

4.2.2. Analysis of results

The basic measurement of the assay is the % of inhibition of the cleavage of the fluorogenic pro-TNF peptide by TACE or the IC_{50} determination by a fitting with the model-39 of LSW data analysis tool.[19]

4.3. Cell-based assay for inhibition of TNF-α secretion in Raw cells

4.3.1. Materials and methods

Raw 264.7 cells (ATCC Cat No. TIB-71) are maintained in DMEM medium containing 10% of serum, P/S, and glutamine. Cells are split twice a week by scraping and 1–10 or 20 dilutions. For testing compounds, cells are cultured to confluence and are seeded the day before the experiment in 24 well culture dishes at 0.5–1 million/ml/well. On the next morning, the medium from the overnight culture is replaced with the fresh growth medium. The compounds are added at various concentrations at the 0.2% of final DMSO concentrations (10 μl of a 20% DMSO solution containing testing compounds is added to each well). The cells are pre-incubated with compounds for 1 h. LPS (Sigma, L2262) is added to cells at a final concentration of 100 ng/ml (10 μl of a 10 μM LPS solution freshly diluted from a 1 mg/ml LPS stock solution in PBS is added to each well) and cells are further incubated for 4 h at 37 °C. 1 ml of the supernatant was collected, cells are spun down and the supernatants are frozen at −80 °C until use.

The TNF-α and other proinflammatory cytokines were detected by ELISA assay according to the manufacturer's instruction (Biosource International, Inc.) (For IL-1b measurement in Raw cells, one round of freeze and thaw of the cells was required).

4.3.2. Analysis of results

The basic measurement of the assay is the% of inhibition of TNF-α secretion and the IC_{50} determination by a fitting with the model-39 of LSW data analysis tool.

4.4. In vitro fluorescence assay of MMP-1 activity

4.4.1. Materials and methods

A continuous assay was used in which the substrate is a synthetic peptide containing a fluorescent group (7-methoxycoumarin; Mca) which is quenched by energy transfer to a 2,4-dinitrophenyl group. When the peptide was cleaved by MMP, a large increase in fluorescence was observed. The source of enzyme in the assay was the recombinant human catalytic domain of MMP-1 prepared at Wyeth-Research in Cambridge. The substrate used was Mca-PQGL-(3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-AR-OH (denoted as Wammp-5, custom synthesized by AnaSpec, Inc.). The assay buffer consisted of 50 mM Hepes (pH 7.4), 100 mM NaCl, 5 mM $CaCl_2$, and 0.005% Brij-35. Each well of black polystyrene 96-well plates contained a 200 μL reaction mixture consisting of assay buffer, purified MMP (final concentration of 25 ng/ml, prepared by diluting with the assay buffer), and varied concentrations of inhibitor (prepared by serially diluting a given inhibitor in DMSO in 96-well polypropylene plate). The plates were then incubated at 30 °C for 15 min. The enzymatic reactions were initiated by adding the substrate to a final concentration of 20 μM, and mixing 10 times with a pipette. The final DMSO concentration in the assay was 6.0%. The initial rate of the cleavage reaction was determined at 30 °C temperature with a fluorescence plate reader (excitation filter of 330 nm and emission filter of 395 nm) immediately after substrate addition.

4.4.2. Analysis of results

Plots of the inhibitor concentration versus the percent inhibition were fit to the following equation: $y = (a - d)/[1 + (x/c)^b] + d$, a general sigmoidal curve with Hill slope, a to d. x is the inhibitor concentration under test. y is the percent inhibition. a is the limiting response as x approaches zero. As x increases without bound, y tends toward its limit d. c is the inflection point (IC_{50}) for the curve. That is, y is halfway between the lower and upper asymptotes when $x = c$. b is the slope factor or Hill coefficient.[20]

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