Open access • Journal Article • DOI:10.1039/C7OB01403A

Carboxylate isosteres for caspase inhibitors: the acylsulfonamide case revisited

Yves Adriaenssens, D. Jiménez Fernández, L Vande Walle, Filipe Elvas ...+5 more authors

Institutions: University of Antwerp, Ghent University

Published on: 13 Sep 2017 - Organic and Biomolecular Chemistry (The Royal Society of Chemistry)

Topics: Carboxylate

Carboxylate isosteres for caspase inhibitors : the acylsulfonamide case revisited

CARBOXYLATE ISOSTERES FOR CASPASE INHIBITORS: THE ACYLSULFONAMIDE CASE REVISITED

Y. Adriaenssens[a], D. Jiménez Fernández[b,d], L. Vande Walle[b,d], F. Elvas[a], J. Joossens[a], A. Lambeir[c] K. Augustyns[a], M. Lamkanfi[b,d], P. Van der Veken*[a]

[a] *Laboratory of Medicinal Chemistry, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium E-mail: pieter.vanderveken@uantwerpen.be*

[b] *Center for Inflammation Research, VIB, Technologiepark 927, B-9052 Ghent, Belgium*

[c] *Laboratory of Medical Biochemistry, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium*

[d] *Department of Internal Medicine, Ghent University, Technologiepark 927, B-9052 Ghent, Belgium*

Abstract

As part of an ongoing effort to discover inhibitors of caspase-1 with an optimized selectivity and biopharmaceutical profile, acylsulfonamides were explored as carboxylate isosteres in caspase inhibitors. Acylsulfonamide analogues of the clinically investigated caspase-1 inhibitor VRT-043198 and of the *pan*-caspase inhibitor Z-VAD-CHO were synthesized. The isostere-containing analogues with an aldehyde warhead had inhibitory potencies comparable to the carboxylate references. In addition, the conformational and tautomeric characteristics of these molecules were determined using ^{1}H- and ^{13}C-based NMR. The propensity of acylsulfonamides with an aldehyde warhead to occur in a ring-closed conformation at physiological pH, significantly increases sensitivity to hydrolysis of the acylsulfonamide moiety, yielding the parent carboxylate containing inhibitors. These results indicate that the acylsulfonamide analogues of the aldehyde-based inhibitor VRT-043198 might have potential as a novel prodrug type for the latter. Finally, inhibition of caspase 1 and 11 mediated inflammation in mouse macrophages were found to correlate with the compounds' potencies in the enzymatic assays.

Key words

caspases, acylsulfonamide, isostere, VX-765, VRT-043198, NCGC00183434, inhibitor, warhead

Introduction

The caspases are a family of aspartate selective cysteine proteases, of which orthologues occur in a wide range of species. Human caspases are typically classified into two subfamilies on the basis of their sequence homologies and biological functions.[1, 2] Members of the first subfamily ("death caspases") have key roles in the initiation (caspase-8, -9, -10) and execution (caspase-3, -6, -7) of apoptosis (programmed cell death). The second subfamily consists of the inflammatory caspases. This subfamily controls inflammatory and host defense responses during infection and in the context of autoimmune and –inflammatory diseases by a threefold mode of action; 1) by modulating the maturation and secretion of inflammatory cytokines interleukin (IL)-1ß and IL-18, 2) by inducing a lytic cell death mode termed pyroptosis and 3) by releasing additional alarmin molecules that contribute to pathogen eradication.[3, 4] Caspase-1 (interleukin-1β-converting enzyme, ICE) is by far the best studied member, while the other human inflammatory caspases (caspase-4 and -5) have so far received less attention. Dysregulated caspase-1 is involved in the pathogenesis of many inflammatory diseases such as atherosclerosis, Alzheimer's disease, type-2 diabetes, gout and rare auto-inflammatory disorders.[5] Caspase-1 is therefore considered as a target for influencing these pathological processes through inhibitor-mediated blocking of protease activity. So far, no caspase inhibitors have been approved as drugs by FDA or EMA. Only two inhibitors of caspase-1 have found their way into clinical trials; VX-765 (**1**, belnacasan) and VX-740 (pralnacasan). The latter however was withdrawn from clinical trials due to liver toxicity in long-term animal studies.[6] VX-765 is an orally absorbed ethyl hemiacetal prodrug that is rapidly converted into VRT-043198 (**2**) under the action of plasma and liver esterases (**Figure 1**). VRT-043198, like other aspartylaldehyde-based caspase inhibitors, is known to occur mainly as an acyl hemiacetal and, to a lesser extent, in a hydrated form in aqueous solution.[7] All these forms equilibrate with the ring-opened, aldehyde-containing carboxylate form that binds to caspase-1. This drug has been tested for the treatment of chronic epilepsy and psoriasis,[8-10] and more recently in HIV-1 infected individuals and in patients with rheumatoid arthritis.[11-13]

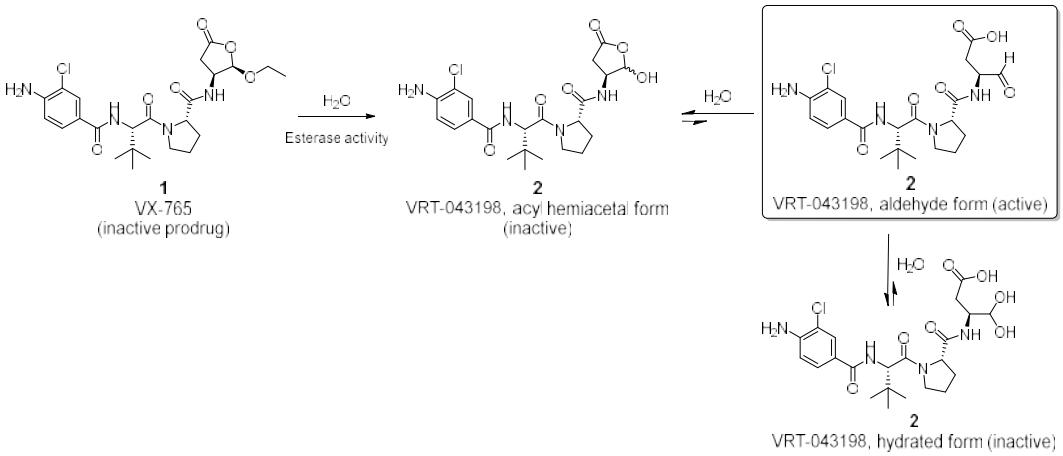


Figure 1. Structures of prodrug VX-765 and its active metabolite VRT-043198.

All caspases use the P1-aspartate residue in their physiological peptide substrates as a main molecular recognition feature. Consequently, most reported caspase inhibitors also incorporate a carboxylate function as an affinity-enhancing structural element. The close phylogenetic relationship between caspases makes selective caspase targeting a huge obstacle in drug discovery and biological research.[14] In the past, several strategies have been explored to overcome this lack of selectivity. Vickers *et al.* have demonstrated that significant selectivity can be obtained via the incorporation of unnatural amino acids in peptide substrates and inhibitors.[15-17] In the same way, Ganesan *et al.* achieved reasonable selectivity towards caspase-3, using aza-peptide epoxide inhibitors that interact with the S1' subsite of the enzyme.[18] Another potential approach to gain selectivity could consist of isosterically modifying the carboxylate function that caspases use for substrate and inhibitor recognition. Replacement of this carboxylate by another acidic group with distinctive size or electronic properties could reasonably be expected to have differing impacts on the recognition process by individual members of the caspase family. Isosteric replacement could also provide biopharmaceutical advantages for caspase-targeting compounds: the carboxylate's "hard" ionic character at physiological pH and its potential for toxic metabolite formation have convincingly been demonstrated to critically discount on cellular permeability and ADME-Tox properties of compounds.[19, 20] Illustratory thereof, most caspase inhibitors are used as ester-type prodrugs in preclinical and clinical settings. So far, the number of reports on isosteric carboxylate replacement in caspase-targeting compounds has been limited, and existing reports have mainly focused on improvement of physicochemical and/or biopharmaceutical properties of inhibitors. Prasad *et al.* replaced the carboxylate function in aspartate-derived α-(arylacyl)oxymethyl ketones with a tetrazole moiety. These analogues however lacked notable target affinity.[21] Similarly, Boxer *et al.* investigated a tetrazole analogue of VRT-043198. In spite of improved resistance to degradation, its potency for all caspases dropped at least 50-

fold compared to the carboxylate analogue.[22] In a third case, Okamoto *et al.* found that the use of an *N*-acyl hydroxamate isostere resulted in a dramatic decrease of caspase-1 inhibitory potency. An *N*-acylsulfonamide evaluated by the same authors, resulted in similar potency as the carboxylate-containing compound. Moreover, a fourfold increase of inhibitory activity was reported in cellular assays and a drastically improved cell permeability compared to the parent molecule.[23] Notwithstanding these promising results and the number of successful applications of acylsulfonamide isosteres in other domains of drug discovery, the full potential of this functional group for caspase research has not been further investigated.[24-27] Likewise, no data on caspase selectivity, physico-chemical properties and *in vitro* pharmacokinetic profile are available for this compound type. Part of our ongoing effort to discover inhibitors of caspase-1 with an optimized selectivity and biopharmaceutical profile, we decided to investigate acylsulfonamide-containing analogues of **2** (VRT-043198) (**Figure 2, entry 1**). Taking into account the spatial confinement of the caspase P1-pocket, only analogues **3** and **4** with a small (methyl, ethyl) sulfonyl substituent were planned. In addition, **2**'s carbonitrile analogue (**5**, NCGC00183434) and the acylsulfonamide derivative thereof (**6**) were synthesized (**Figure 2, entry 2**).[22] Compared to the aldehyde warhead, the milder electrophilicity of the carbonitrile function could in itself contribute to a potentially improved selectivity and biopharmaceutical profile for **5** and **6**.[6, 28] As an illustration, several compounds with a carbonitrile warhead, including the cysteine protease inhibitor odanacatib, have been successfully approved as drugs by the FDA during recent years.[29-31] Third, we also opted to include *pan*-caspase inhibitor **7** (Z-VAD-CHO) and its acylsulfonamide analogue **8** in the study. (**Figure 2, entry 3**) The lack of selectivity of parent molecule **7** seemed interesting as an additional reference point to benchmark the acylsulfonamide's potential to increase selectivity for individual caspase family members.[32]

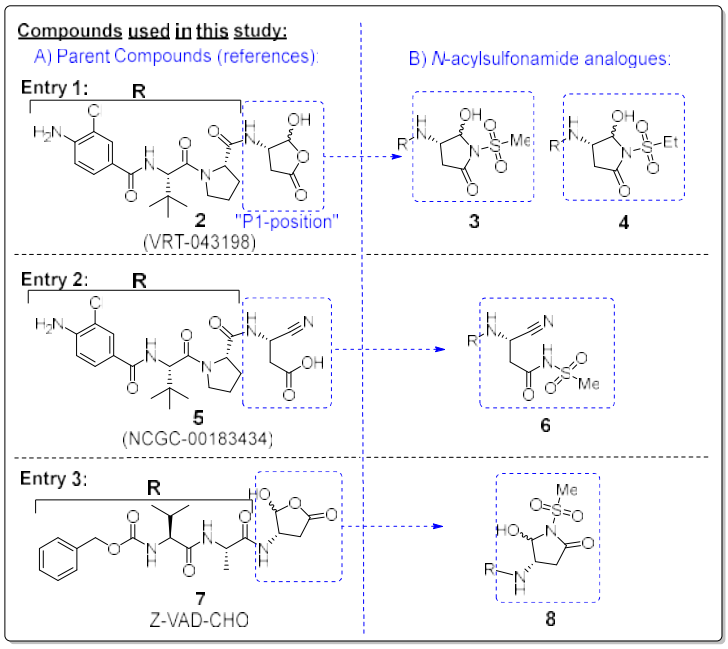


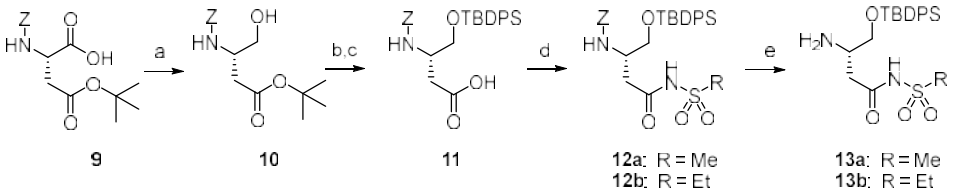
Figure 2. Overview of compounds used in this study. Aldehyde-containing inhibitors (**2-4** and **7-8**) are represented as the corresponding acyl hemiacetal or acyl hemiaminal tautomers (*vide infra*).

Results and discussion

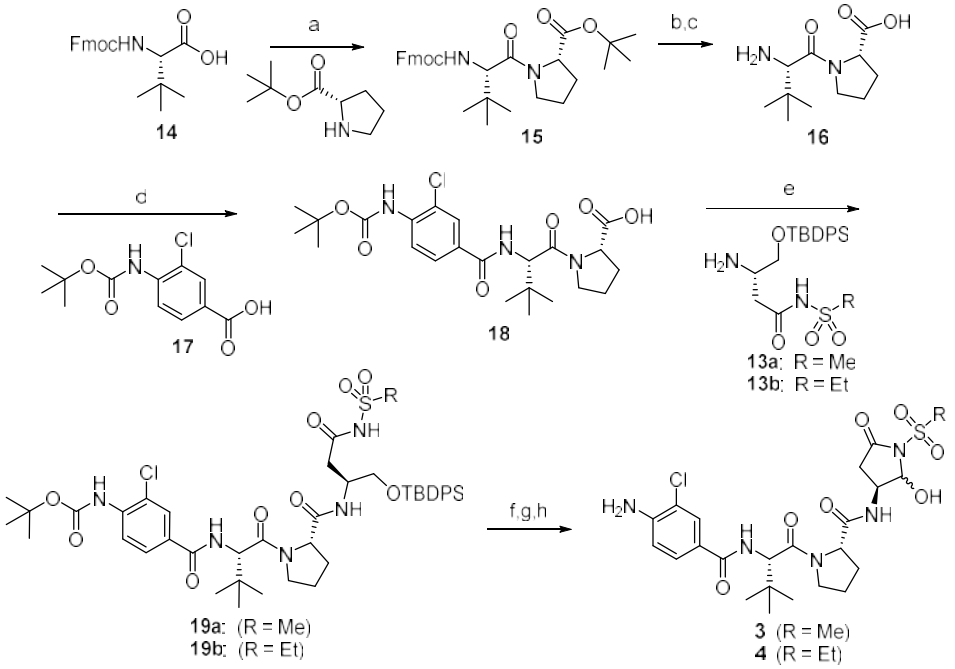
Design and synthesis

The synthetic strategy for aldehyde-based acylsulfonamide analogues of VRT-043198 (**3** and **4**) consisted of separately synthesizing advanced precursors of the required P1-building blocks (**Scheme 1**), after which coupling to the acylated peptide tail of VRT-043198 and oxidative installation of the aldehyde group, was expected to deliver the final products (**Scheme 2**). First, side-chain protected Z-Asp(OtBu)-OH was activated with isobutyl chloroformate and the adduct was cleanly reduced with $NaBH_4$ to yield **10**. The resulting primary alcohol was protected with a *tert*-butyldiphenylsilyl (TBDPS) protecting group, which proved to be stable under the conditions of the subsequent acidolytic deprotection of the side-chain carboxylate, affording intermediate **11**. In the latter, an isosteric acylsulfonamide group was installed through coupling of the free carboxylate with either methane- or ethanesulfonamide using carbonyl diimidazole (CDI) as the coupling reagent to produce **12a** and its homologue **12b**, respectively.[33] Hydrogenolytic removal of the Z-protecting group, delivered the advanced P1-building block precursors. Standard peptide synthesis procedures were then used for the preparation of dipeptide **16**. *N*-acylation of the latter with *N*-Boc-4-amino-3-chlorobenzoic acid (**11**) provided the protected peptide tail **18**. Protection of the aminobenzoate building block was considered advisory due to concerns of potential aniline incompatibility with the oxidizing agent used in the penultimate step (*vide infra*). Next, P1-

building blocks **13a** and **13b** were coupled with the protected peptide tail and the TBDPS function in intermediates **19a** and **19b** was cleaved off with tetra-*n*-butylammonium fluoride (TBAF). The resulting primary alcohol was then oxidized with Dess-Martin periodinane (DMP) to yield the corresponding aldehydes. The final acidolytic deprotection delivered final products **3** and **4**.

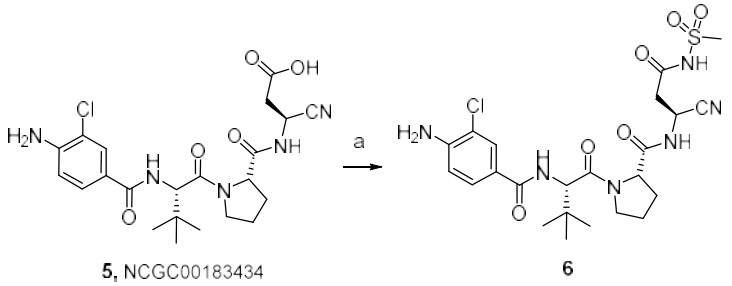


Scheme 1. *Reagents and conditions:* a) isobutyl chloroformate, 4-methylmorpholine, $NaBH_4$ (H_2O), DME, -10 °C, 1 h, 68%; b) TBDPSCl, imidazole, DMAP, DCM, RT, overnight; c) TFA, DCM, RT, 30 min, 46 % over 2 steps; d) CDI, RSO_2NH_2, DBU, DCM, RT, overnight, 100 %. e) H_2, Pd, MeOH, RT, overnight, 95 %.



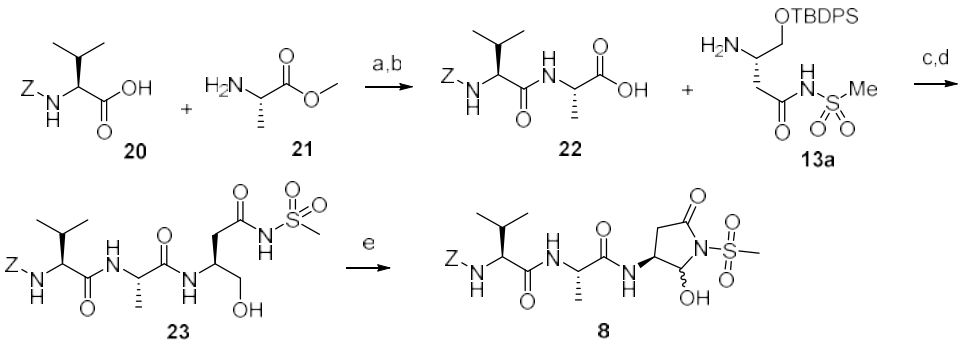
Scheme 2. *Reagents and conditions:* a) EDC.HCl, HOBt, DMF, RT, 8 h, >99 %; b) TFA, DCM, RT, 1 h; c) Et_2NH, THF, RT, 2 h , >99 % over 2 steps; d) EDC.HCl, HOBt, DIPEA, DMF, RT, 2 h, 51 %; e) EDC.HCl, HOBt, DIPEA, DMF, RT, 3 h, 40 %; f) TBAF, THF, RT, overnight, 73 %; g) Dess-Martin, DCM, RT, 2 h, 89 %. h) TFA, DCM, RT, 30 min, 72 %.

References VRT-043198 (**2**), NCGC00183434 (**5**) and Z-VAD-CHO (**7**) were also synthesized during the study. Analytical data for the prepared compounds were identical to data reported by Boxer *et al*. (for **2** and **5**) and by Graybill *et al.* (for **7**) [22,34] Synthesis of the carbonitrile-containing acylsulfonamide **6** was straightforward, starting from **5** using EDC as the coupling reagent (**Scheme 3**).



Scheme 3. *Reagents and conditions*: a) $CH_3SO_2NH_2$, EDC.HCl, DMAP, DCM, RT, overnight, 81 %.

To obtain acylsulfonamide analogue **8** of Z-VAD-CHO (**Scheme 4**), the dipeptide precursor Z-VA-OH (**22**) was synthesized from Z-protected valine (**20**) and alanine methyl ester (**21**), followed by deprotection in basic aqueous media. Next, **22** was coupled to P1-building block **13a** and transformed into final compound **8** after TBDPS-deprotection and oxidation to produce the aldehyde warhead.



Scheme 4. *Reagents and conditions:* a) EDC.HCl, HOBt, DIPEA, DCM, RT, overnight, 52 %; b) LiOH, H_2O, MeOH, RT, 2 h, 54 %; c) isobutyl chloroformate, 4-methylmorpholine, THF, -20 °C, 3 h; d) TBAF, THF, RT, overnight, 83 % over 2 steps; e) Dess-Martin, DCM, RT, 2 h, 39 %.

Inhibitory potency

The prepared set of compounds was first evaluated for their inhibitory potency against a panel of human caspases using published protocols.[35] The panel was composed of all inflammatory caspases, supplemented with the most relevant representatives of the initiator and executioner subgroups. Reference inhibitors VRT-043198 (**2**) and Z-VAD-CHO (**7**) displayed IC_{50} values that were in good agreement with data published by other groups (**Tables 1 and 2**).[22, 36, 37] Reference **5** (NCGC00183434) however, showed slight differences in comparison with published results (*vide infra*).

In the aldehyde series, potencies of the acylsulfonamide-containing inhibitors (**3-4, 8**) were generally similar to those of VRT-043198 and Z-VAD-CHO. These data indeed indicate that the acylsulfonamide group is a promising carboxylate surrogate for caspase ligands. Noteworthy however, this type of isosteric replacement did not turn out to be a proficient instrument to increase selectivity for a specific caspase. This is directly illustrated by

comparison of the caspase-1 selectivity indices (SIs) for **2** (VRT-043198) and its isostere-containing analogues **3** and **4**, which are roughly comparable for each enzyme (**Table 1**). A very limited but consistent increase in caspase-8 and caspase-9 affinity of **3** and **4** seems nonetheless present. However, this effect is absent for the acylsulfonamide analogue **8** of Z-VAD-CHO (**7**). It therefore remains to be demonstrated that introduction of an acylsulfonamide isostere in reported caspase-8 or caspase-9 inhibitors would be a good strategy to obtain more selective compounds. Finally, it deserves mentioning that no significant differences were detected between the methyl- and ethyl acylsulfonamide analogues.

Looking at the evaluation data for carbonitriles **5** and **6**, three observations can be made. First, the presence of a carbonitrile warhead leads to a pronounced decrease in caspase potency, potentially directly related to the lower electrophilicity of this warhead type. As an exception to that trend, the affinity for human caspase-1 of reference **5** (NCGC-00183434) remains grossly conserved, making this one of the most selective caspase-1 inhibitors known to date. Second, as mentioned above, a noticeable difference is observed with the experiments from Boxer *et al*.[22] This group reported a ninefold improvement in potency for human caspase-1 upon changing the warhead from an aldehyde to a carbonitrile group. Moreover, their selectivity indices appear to be generally lower than ours. A potential cause is most likely the use of different substrates in the potency assays. While our potency assays were run with five optimized substrates for the enzymes in the panel, Boxer *et al.* used two general caspase substrates in their experiments.[38, 39] Furthermore, the acylsulfonamide-containing carbonitrile **6** displayed a further decrease in caspase potency. Although we have no definitive rationalization for this finding, it is not inconceivable that introduction of the isosteric replacement significantly impacts on the parts of conformational space that are accessible to the inhibitor. This could preclude optimal positioning of the warhead to efficiently engage in covalent bond formation with the caspases' catalytic cysteine residue.

Table 1. IC_{50} values (in nM) and selectivity indices with respect to h casp-1 for inhibitors **2-8** in a human caspase panel[a]

Human caspases[(a)]												
		Inflammatory caspases					Apoptotic caspases					
Compound	Warhead	casp-1	casp-4		casp-5		casp-3		casp-8		casp-9	
		IC_{50} (nM)	IC_{50} (nM)	SI[(b)]	IC_{50} (nM)	SI	IC_{50} (nM)	SI	IC_{50} (nM)	SI	IC_{50} (nM)	SI
2 (VRT-043198)	**-CHO**	10.5 ± 0.41	62.1 ± 4.1	5.9	190 ± 60	18.1	10 910 ± 1 151	1039	59.6 ± 4.2	5.7	110 ± 29	10.4
3	**-CHO**	20.6 ± 1.8	123 ± 48	5.9	289 ± 118	14	>25 000	>1214	38.9 ± 4.6	1.9	80 ± 14	3.9
4	**-CHO**	22.1 ± 1.2	101 ± 15	4.6	214 ± 59	9.7	>25 000	>1132	51.9 ± 3.4	2.3	113 ± 15	5.1
5 (NCGC-00183434)	**-CN**	34.9 ± 7.4	1 272 ± 253	36.4	850 ± 91	24.3	>100 000	>2865	4 184 ± 853	118.9	2 846 ± 225	81.5
6	**-CN**	5 264 ± 866	>100 000	>19	>50 000	>9.5	>100 000	>19	>100 000	>19	>25 000	>4.7
7 (Z-VAD-CHO)	**-CHO**	685 ± 70	>50 000	>73	2 813 ± 180	4.1	>100 000	>146	6 309 ± 1 297	9.2	405 ± 35	0.6
8	**-CHO**	1 966 ± 382	>50 000	25.4	7 840 ± 2 038	4	>100 000	>51	13 464 ± 2 928	6.85	>25 000	>12.7

[(a)]Substrates tested were Ac-WEHD-AMC for casp-1,-4,-5; Ac-DEVD-AMC for casp-3; Ac-IETD-AMC for casp-8 and Ac-LEHD-AMC for casp-9. [(b)]Selectivity Indices (SIs) were calculated as (IC_{50}(caspase-x)/IC_{50}(caspase-1))

Since caspase-1 inhibitors **2-6** were evaluated in a mouse-derived model of cellular inflammation (*vide infra*), these compounds were also evaluated against a panel of murine inflammatory caspases (**Table 2**). Similar to the human caspase panel, acylsulfonamides **3** and **4** show comparable potencies as for the reference inhibitor VRT-043198 (**2**). However, in contrast to the human inflammatory panel, the selectivity has been largely favored for murine caspase-1, which is surprising since caspase-11 is considered the murine homolog of human caspase-4 and -5. While carbonitrile-containing reference inhibitor **5** (NCGC-00183434) shows a moderate potency decrease, its selectivity towards murine caspase-1 is even more distinct. Similar to the human caspase panel in Table 1, the acylsulfonamide analogue **6** does not present promising inhibitory potencies.

Table 2. IC_{50} values (in nM) and selectivity indices with respect to m casp-1 for inhibitors **2-6** in a murine caspase panel[a]

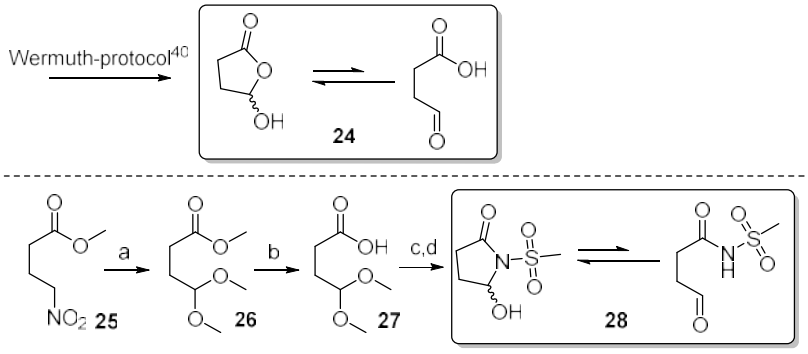
Murine inflammatory caspases[(a)]						
Cmp	Warhead	casp-1	casp-11		casp-12	
		IC_{50} (nM)	IC_{50} (nM)	SI[(b)]	IC_{50} (nM)	SI
2 (VRT-043198)	**-CHO**	5.47 ± 0.28	5 604 ± 767	1024	>100 000	>18 281
3	**-CHO**	15.5 ± 1.8	11 409 ± 4 598	736	>100 000	>6 451
4	**-CHO**	7.0 ± 1.2	7 991 ± 1 312	1142	>100 000	>14 285
5 (NCGC-00183434)	**-CN**	145 ± 20	>100 000	>689.7	>100 000	>689.7
6	**-CN**	6 503 ± 623	>100 000	>15.4	>100 000	>15.4

[(a)]Substrates tested were Ac-WEHD-AMC for m casp-1 and -11 and Ac-ATAD-AFC for m casp-12. [(b)]Selectivity indices were calculated as (IC_{50}(caspase-x)/IC_{50}(caspase-1))

The IC_{50}-values in **Tables 1** and **2** clearly indicate that the acylsulfonamide function could serve as a carboxylate surrogate in caspase inhibitors drug discovery. Nonetheless, gaining insight in the inhibitors' conformational behavior seemed essential for further understanding of the potency data. Analogous to the well-known intramolecular acyl hemiacetal formation in **2** and **7**, acylsulfonamides could theoretically engage in intramolecular cyclization by addition of the acylsulfonamide onto the warhead function. Furthermore, this reaction could be reversible or (practically) irreversible under ambient conditions. Since cyclic products would lack both the acidity required for caspase recognition and the electrophilic warhead function, ring-closed products can reasonably be expected to lack enzyme affinity, similar to the situation with carboxylate-based compounds. The following factors were therefore important to address for the acylsulfonamides: 1) their tendency to form cyclic tautomers, and, if so, 2) the relative stabilities of the open and ring-closed forms. 3) Finally, to assess whether equilibration between both forms is possible under ambient conditions, the reversibility and activation energy of the tautomerization process would be interesting to probe. No literature data were present that address these issues.

A first hint at the tautomeric behavior of the acylsulfonamides was obtained from the NMR-spectra of the molecules. In the ^{13}C spectra of **3**, **4** and **8** (that combine an acylsulfonamide and aldehyde function) in MeOD-d_4 or DMSO-d_6, no aldehyde carbons were detected. Instead, in each case one specific signal around 87 ppm was present that could not be assigned to a carbon of the corresponding peptidomimetic/peptidic tail, therefore suggesting that these acylsulfonamide-containing inhibitors adapt other tautomeric forms. A similar pattern was observed in the corresponding carboxylate-based reference compounds **2** and **7** (data not shown). In the case of carbonitrile **6** however, no cyclized form was observed (100 % abundancy of the specific acidic proton) (data in supporting information). To gain further insight into the tautomeric preferences for molecules containing a 1,2-carbaldehyde-acylsulfonamide functionalization, an additional NMR study was carried out using fragment-sized probes **24** and **28** (**Scheme 5**). Choosing these compounds has the advantage of avoiding any compound-specific biases in the experiments (*e.g.*, interference with cyclization by the peptide tail of the inhibitors). In addition, interpretation and integration of NMR spectra is easier for fragments than in the inhibitor series: there, cyclization creates an additional stereocenter, leading to diastereomerization and resulting in peak splitting. In case of overlaps with other peaks in the spectra, this would complicate peak integration. Finally, the higher aqueous solubility of the fragments (compared to the full-length inhibitors) allows the use of D_2O as an NMR solvent, which is relevant to mimick the physiological conditions in which the inhibitors are normally used. The first fragment, succinic semialdehyde (**24**), was prepared

according to a procedure by Wermuth.[40] To obtain the isosteric acylsulfonamide fragment **29**, the terminal nitro group in **25** was first transformed into an acetal via a modified Nef protocol.[41] After deprotection of the methyl ester, coupling with methanesulfonamide was performed. Lastly, acidic hydrolysis of the acetal led to fragment **28**.



Scheme 5. *Reagents and conditions:* a) NaOMe, MeOH, 0 °C, 30 min, followed by H_2SO_4, MeOH, 0 °C, 45 min, 81 %; b) 1 M NaOH (aq), MeOH, RT, overnight, 90 %; c) $CH_3SO_2NH_2$, DMAP, EDC.HCl, overnight, RT; d) TFA, H_2O, RT, 18 h, 32 % over 2 steps.

Both fragments were analyzed with ^{1}H- and ^{13}C-NMR spectroscopy in protic and aprotic solvents. Identification of the individual tautomers/derivatives occurring in solution was done based on the chemical shifts of the circled hydrogen and carbon atoms in **Figure 3**. In aprotic solvents, such as dimethyl sulfoxide or chloroform, reference **24** was found to be present in a 2:1 aldehyde:cyclic hemiacetal equilibrium (**Table 3**). In methanol and water, only the acyclic methyl hemiacetal (**29a**) and the hydrated aldehyde (**29b**), respectively, were observed. In case of acylsulfonamide fragment **28** however, the equilibrium was totally shifted to the cyclic acyl hemiaminal form in $CDCl_3$ and D_2O (**Table 4**). Only in MeOD-*d*$_4$ a small fraction (12 %) was observed in the acyclic methyl hemiacetal tautomer (**30a**). In none of these cases an aldehyde proton/carbon was detected.

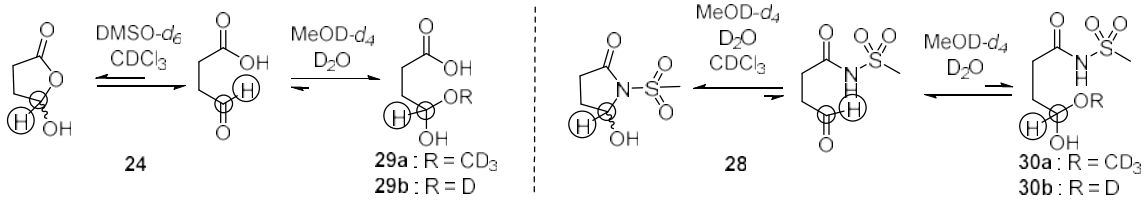


Figure 3. Overview of tautomers and derivatives of **24** and **28** observed in the ^{1}H and ^{13}C NMR study. Chemical shifts of circled atoms were used as diagnostic for structure assignment.

Table 3. ^{1}H and ^{13}C NMR chemical shifts of aldehyde hydrogen and carbon atoms of **24** (circled in Figure 3) in different solvents.

Solvent	δ (^{1}H NMR)		δ (^{13}C NMR)	Occurrence	Ratio
MeOD-*d*$_4$	4.58	t	97.34	methyl hemiacetal	100 %

D_2O	4.99	t	90.00	Hydrate	100 %
DMSO-d_6	9.67	s	202.52	Aldehyde	66 %
	5.80	m	99.79	cyclic acyl hemiacetal	33 %
$CDCl_3$	9.83	s	199.96	Aldehyde	66 %
	5.95	m	98.91	cyclic acyl hemiacetal	33 %

Table 4. ^{1}H and ^{13}C NMR chemical shifts of aldehyde hydrogen and carbon atoms of **28** (circled in Figure 3) in different solvents.

Solvent	δ (^{1}H NMR)		δ (^{13}C NMR)	Occurrence	Ratio
MeOD-d_4	4.58	t	98.55	methyl hemiacetal	12 %
	5.82	dd	84.81	cyclic acyl hemiaminal	88 %
D_2O	5.93	dd	84.90	cyclic acyl hemiaminal	100 %
$CDCl_3$	5.91	dd	83.38	cyclic acyl hemiaminal	100 %

The data in **Table 3** and **4** seem to be in general agreement with the preliminary observations for peptide-based inhibitors in this study. As a confirmation thereof, carboxylate-based inhibitors (**2**,**7**) and the acylsulfonamide-containing inhibitors (**3**, **8**) were additionally studied using ^{1}H-NMR in D_2O (spectra are provided as Supporting Information). Overall, highly comparable tautomeric structure distributions were observed in D_2O for the fragment-sized references and full-length inhibitors. Notably nonetheless, the cyclic acyl hemiaminals of inhibitors **3** and **8** were found to appear in the spectrum as two diastereomers: separate signals were detected with chemical shifts in the range of the values of **Table 4**. Finally, the limited aqueous solubility of these inhibitors implied that sufficiently high compound concentrations to allow additional ^{13}C-NMR experiments could not be attained.

Notably in water, the relative stabilities of the aldehyde tautomers of both **24** and **28** are lower than that of the corresponding hydrate and the cyclic acyl hemiaminal, respectively: this is directly reflected by the product distributions in solution. The absence of a hydrated derivative for **28** furthermore seems to indicate that the cyclized acyl hemiaminal tautomer is particularly stable in water. This observation could be relevant for interpreting caspase binding data for inhibitors containing fragment **28**, since strong intramolecular stabilization generally implies a significant penalty for the thermodynamic target affinity of compounds. More specifically, thermodynamic affinity correlates with the overall Gibbs free energy difference (ΔG) for the target binding process, starting with unbound inhibitor and target caspase, and ending with the formed [inhibitor-target] complex. Intramolecular stabilization (lowering the inhibitor's initial Gibbs free energy), will therefore reduce the overall ΔG-value (ΔG_1) compared to the situation where a ring-opened form of the inhibitor would be mainly present in solution (ΔG_2) (**Figure 4**). Theoretically, an inhibitor with improved affinity could be obtained by

selecting a suitable warhead functionality that does not engage in intramolecular adduct formation with the acylsulfonamide. The potency data obtained for carbonitrile-based compound **6** nonetheless demonstrate that suitable warhead identification is not a trivial task in caspase inhibitor design.

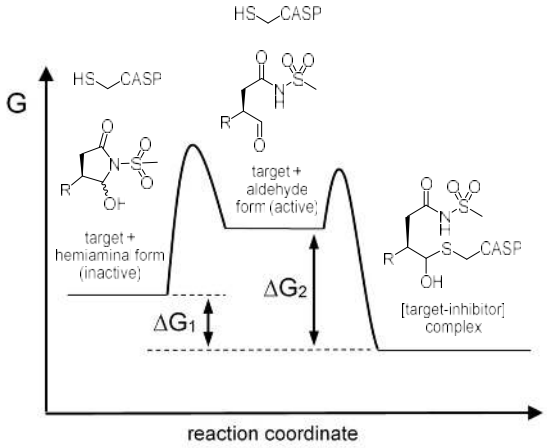


Figure 4. Hypothetical reaction diagram for formation of enzyme-inhibitor complex with inhibitors containing fragment **28**.

Finally, we used an experimental approach to qualitatively probe the dynamics of the equilibrium between the stable acyl hemiaminal tautomer and the ring-opened form of the inhibitor that is required for target binding. If the activation energy barrier to tautomerization would be significantly higher than in the carboxylate case, the acylsulfonamide inhibitors would be characterized by a very slow target binding process. This in turn would call for extending the pre-incubation time between enzyme and inhibitor in the enzymatic assays in order to obtain realistic potency data. As a proxy for equilibrium kinetics, the rate of hydrazone formation of **28** with phenylhydrazine in aqueous media was investigated (**Figure 5**). Hydrazone formation requires the presence of a reactive aldehyde function, implying tautomerization from the acyl hemiaminal to the ring-opened tautomer. As a reference, the same reaction with **24** was taken.

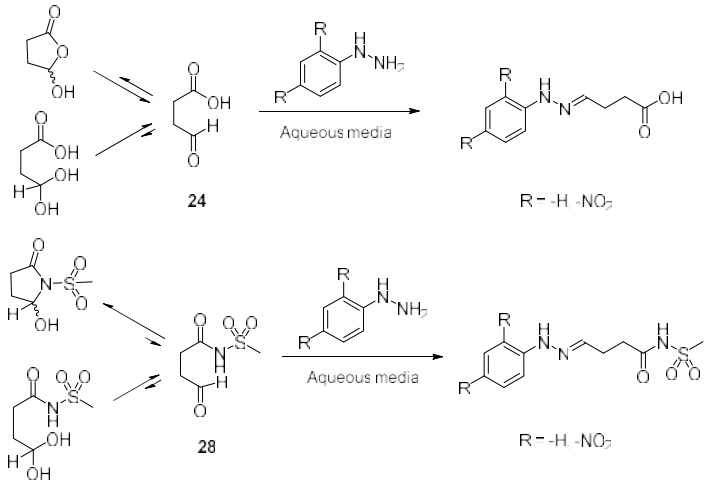


Figure 5. Determination of the equilibrium kinetics of **24** and **28** by hydrazone formation. Quantification of the corresponding hydrazone was carried out with LC-MS.

In both cases, full conversion of **24** and **28** into the corresponding hydrazones was observed after 1-2 minutes (quantification was carried out with LC-MS). Prolongation of the reaction time did not provide a concentration increase of the hydrazone. Furthermore, experiments with the less reactive (2,4-dinitrophenyl)hydrazine, used in anticipation of slowing down the conversion, had an identical outcome. These findings indicate that the tautomerization process is sufficiently fast to not interfere with the potency assay protocols used.

Stability measurements

The chemical stability of all studied compounds in buffered aqueous conditions (pH 4 and 7.4) was quantified over a period of 24 hours (**Figure 6)**. All reference and acylsulfonamide inhibitors displayed satisfactory chemical stability at pH 4. At physiological pH however, acylsulfonamides **3, 4** and **8** (possessing an aldehyde warhead) degraded relatively quickly, with half-lives around 1 hour. Identical results were obtained in metabolic stability tests with human and murine liver microsomes, indicating that eventual specific metabolization processes for these compounds are subordinate to the non-enzymatic breakdown process taking place at physiological pH. Closer inspection learned that in all conditions, a clean hydrolysis of **3**, **4** and **8** into the corresponding carboxylates **2** and **7** was the only degradation pathway present (data in supporting information). Noteworthy, the nitrile-containing acylsulfonamide **6** was found to be stable under all conditions.

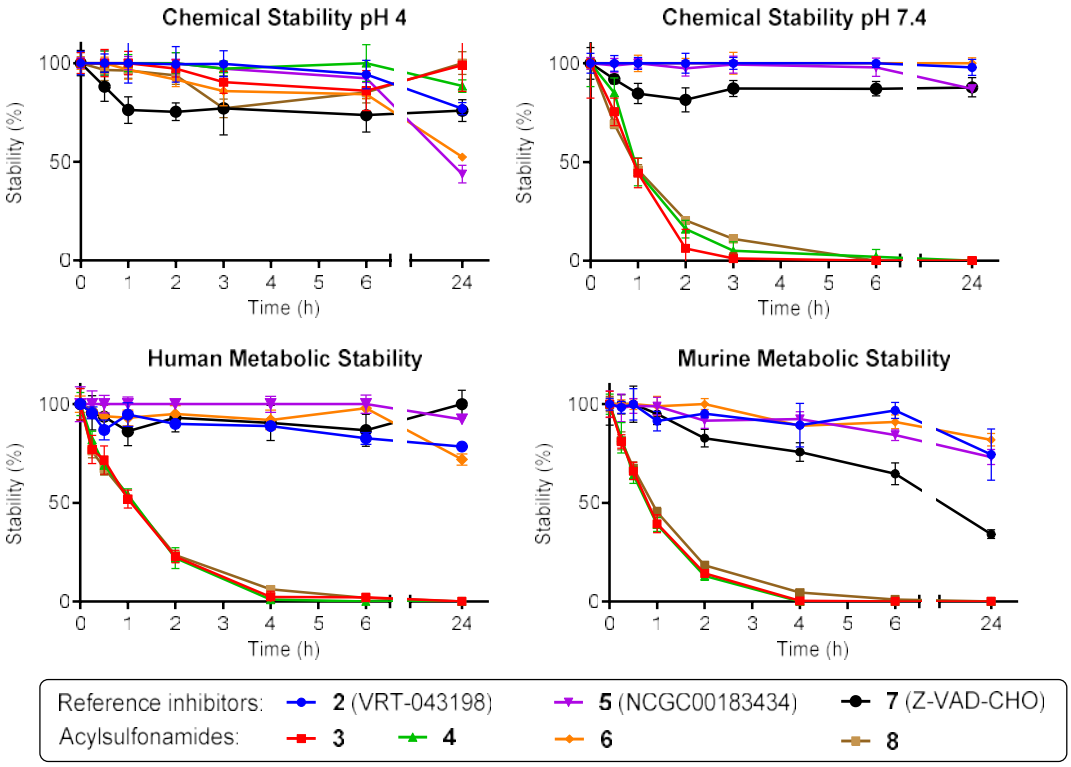


Figure 6. Stability profiles of all inhibitors. Measurements were carried out for 24 hours.

As a rationale for the selective hydrolysis of the aldehyde containing inhibitors, their ability to engage in cyclic acyl hemiaminal formation seems highly likely. Functionalization of the acylsulfonamide NH (*e.g.*, by alkylation, or in this case, via acyl hemiaminal formation) is well known to destabilize the functional group and to make it more sensitive to nucleophilic attack. This principle is, for example, applied in the reversed Kenner safety-catch linker used in solid-phase peptide synthesis. (**Figure 7**).[42] Nonetheless, to the best of our knowledge we are the first to report instability for this type of inhibitors.

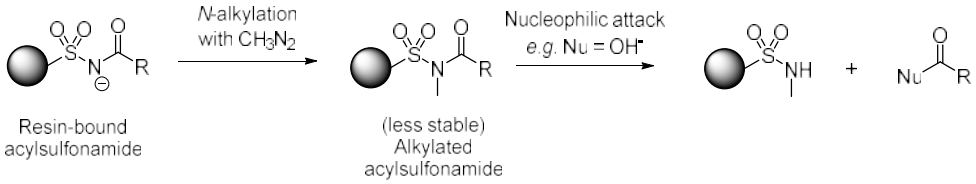


Figure 7. Principle of the reversed Kenner safety-catch linker. Figure was derived from ref [42].

Regardless, the stability issue is highly relevant, since it implies that IC_{50} values for **3**, **4** and **8** (**Tables 1** and **2**) could be flawed, and rather represent the affinities of the corresponding carboxylate-containing parent compounds **2** and **7** (formed upon hydrolysis). Close inspection however demonstrated that the results obtained for **3**, **4** and **8** correctly

reflected enzyme potencies of the acylsulfonamides. More specifically, all experiments were terminated within a timeframe of 35 minutes following dilution of the inhibitors from freshly prepared DMSO stocks. In addition, all inhibitors were found not to degrade in DMSO. Although **3**, **4** and **8** can be expected to partially hydrolyze (up to 30% at the end point) during the 35 minutes of the assay, the progress curves for these compounds clearly showed a constant degree of inhibition throughout the measurement. Hypothetically speaking, significant progress curve flattening or steepening would be visible in case the starting acylsulfonamides would have different potencies than the carboxylate analogues into which they are gradually transformed. Nonetheless, curve profiles clearly indicate that the intrinsic IC_{50}-values for **3**, **4** and **8** are comparable to those for **2** and **7**.

Since the acylsulfonamide inhibitors with an aldehyde warhead combine 1) stability at lower pH, 2) higher reported gastro-intestinal permeability than the corresponding carboxylates and 3) gradual conversion into the latter upon elevation of the pH, they can be considered potential prodrug candidates. In this view, the acylsulfonamides would start releasing the carboxylate inhibitors once in circulation, after diffusing through the gastro-intestinal epithelium. Esters, as in VX-765, are the most commonly used prodrugs for pharmacophoric carboxylate functions. However, inter-individual differences in plasma/liver esterase activity can lead to unpredictable bioconversion of esters. Likewise, low levels of plasma esterase activity are known to result in incomplete bioconversion and lower than predicted bioavailability.[43] The non-enzymatic hydrolysis at physiological pH observed for the acylsulfonamide function in **3**, **4** and **8** could therefore constitute an efficient alternative for the use of ester prodrugs. Several acylsulfonamide prodrugs have been reported in literature, including the recently approved prostacyclin receptor agonist selexipag.[44-49] Generally lacking intramolecular cyclization/derivatization potential, these compounds also mostly rely on slow, metabolic activation of the acylsulfonamide function to release a physiologically active carboxylate or sulfonamide drug.

Biological assays

Finally, VRT-043198 (**2**), its carbonitrile analogue **5** and the acylsulfonamide derivatives of both (**3, 4** and **6**) were investigated as inhibitors of caspase-1 and caspase-11 mediated inflammatory responses in mouse macrophages. As described earlier, combined stimulation of mouse macrophages with lipopolysaccharide (LPS) and the ionophore nigericin results in caspase-1-dependent pyroptosis and secretion of mature IL-1ß and IL-18 through the 'canonical Nlrp3 inflammasome'. In contrast cytosolic delivery of LPS provokes caspase-11-mediated pyroptosis, with secretion of IL-1ß and IL-18 requiring both caspases 1 and 11 in the 'non-canonical' Nlrp3 inflammasome pathway.[4,50] Both cell death (quantified based on lactate

dehydrogenase (LDH) release in the medium) and IL-1β and -18 levels were monitored as read-outs of the experiments (**Figure 8** and **9**). All inhibitors were evaluated at 50 μM concentration. In line with published results, the highly potent but poorly selective irreversible fluoromethylketone (fmk) inhibitors Z-VAD-fmk and Z-WEHD-fmk served as positive controls.

VRT-043198 (**2**) and its direct acylsulfonamide analogues (**3** and **4**) reduced cell death counts and secreted IL-1β and -18 levels with comparable potency in both assay types. As can be anticipated by the significantly higher caspase-1 affinity (compared to caspase-11 affinity) of all compounds, the strongest effect was seen on interleukin maturation levels, while the impact on cell death was considerably smaller. These observations roughly reflect the comparable potencies of the inhibitors in the IC_{50}-assays. At the same time, the data suggest that the acylsulfonamide containing inhibitors do not benefit from a significantly higher cellular permeability at this concentration. Furthermore, **3** and **4** can be expected to hydrolyze to a significant extent during the experiment (read-out takes place 105 minutes after inhibitor dosing). Notwithstanding this, the eventual presence of a permeability advantage for the acylsulfonamides would still be anticipated to translate in significantly higher cellular potencies: hydrolysis taking place after compound permeation can reasonably be expected to lead to entrapment and accumulation of a potent inhibitor in the cytoplasm. This finding contrasts with earlier data by Okamoto *et al.,* who reported cellular permeabilities that were an order of magnitude higher for an acylsulfonamide inhibitor compared to a carboxylate reference.[23] Noteworthy, Okamoto *et al.* also did not report stability data for their acylsulfonamide inhibitor. Structural considerations however, would also lead to the conclusion that this literature compound has similar limited stability as **3** and **4**.

In case of the carbonitrile-based inhibitors, carboxylate **5** showed a less pronounced effect on caspase-1 and -11 dependent cell death and interleukin levels compared to reference inhibitor **2**. Again, this is reflected by the relative potencies of both compounds in the IC_{50}-assays. Finally, acylsulfonamide **6** does not cause a significant decrease of the inflammatory response. Also for this molecule, the results in the cellular assay roughly correspond to the IC_{50} values for murine caspases-1 and -11.

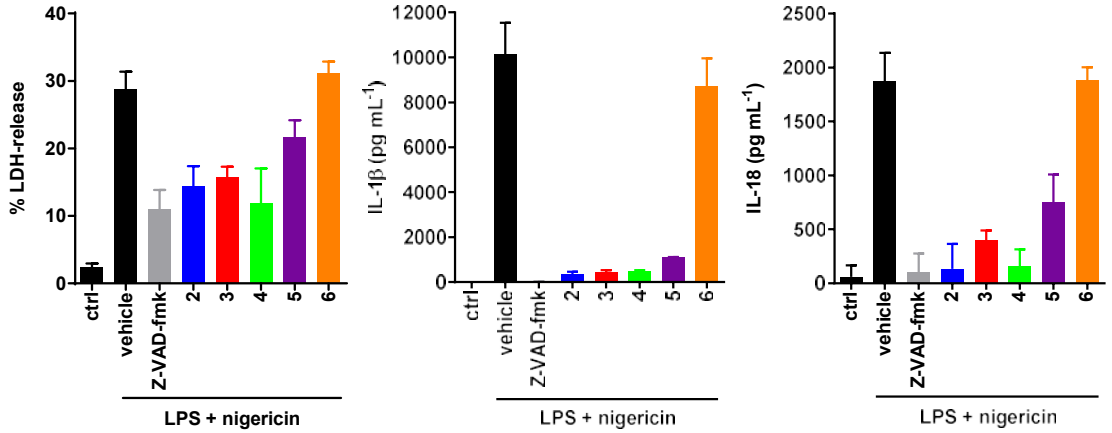


Figure 8. Canonical Nlrp3 inflammasome-mediated pyroptosis and secreted IL-1β and -18 levels of stimulated macrophages after treatment with inhibitors **2-6**. The inhibitor concentration used was 50 µM for all compounds. The blank experiment ("ctrl") corresponds to cells that were left untreated. "Vehicle" represents cells that were only treated with the inhibitor vehicle, DMSO, prior to nigericin challenge. "Z-VAD-fmk" represents a positive control experiment. Data represent mean ± s.d. of one out of three biological replicates, with three technical replicates each.

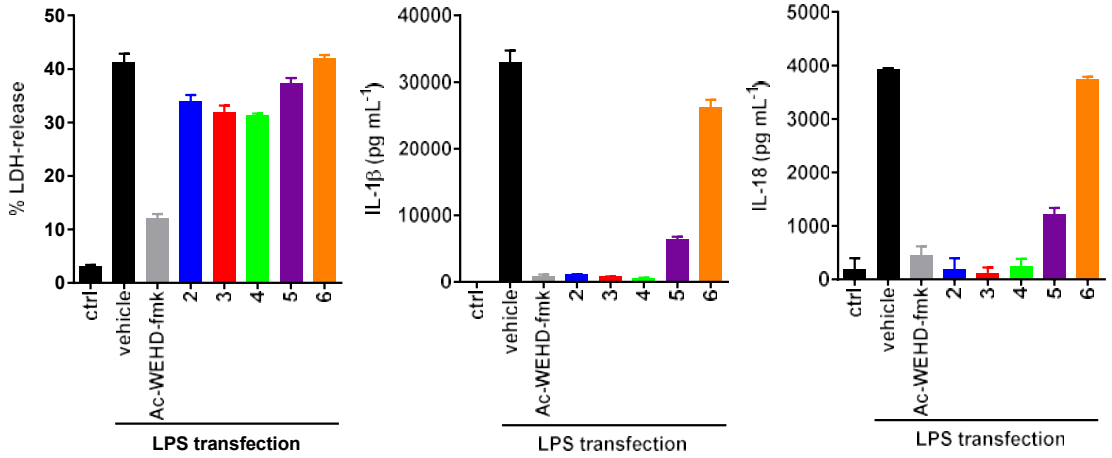


Figure 9. Non-canonical Nlrp3 inflammasome-mediated pyroptosis and secreted IL-1β and -18 levels of stimulated macrophages after treatment with inhibitors **2-6**. The inhibitor concentration used was 50 µM for all compounds. The blank experiment ("ctrl") corresponds to cells that were left untreated. "Vehicle" represents cells that were only treated with the inhibitor vehicle, DMSO prior to LPS transfection. "Ac-WEHD-fmk" represents a positive control experiment. Data represent mean ± s.d. of one out of three biological replicates, with three technical replicates each.

Conclusions

Acylsulfonamides were explored as carboxylate isosteres in caspase inhibitors. Analogues of the clinically investigated caspase-1 inhibitor VRT-043198 and of the *pan*-caspase inhibitor Z-VAD-CHO were synthesized. The isostere-containing analogues with an aldehyde warhead were found to display inhibitory potencies comparable to the carboxylate reference. In addition, a ^{1}H- and ^{13}C-based NMR study was carried out to study conformational and tautomeric characteristics of these compounds. The propensity of acylsulfonamides with

an aldehyde warhead to occur in a ring-closed conformation at physiological pH, was found to significantly increase hydrolysis of the acylsulfonamide moiety, yielding the parent carboxylate containing inhibitors. These results indicate that the acylsulfonamide analogues of the aldehyde-based inhibitor VRT-043198 might have potential as a novel type of prodrug for the latter. Finally, caspase-1 and -11 mediated cellular inflammation experiments were carried out. Obtained data were found to be in line with the potency data in the enzymatic assays. At the inhibitor concentrations used with the stimulated macrophages, a clear cellular permeability advantage was absent for the acylsulfonamides. Nonetheless, in depth evaluation of the gastro-intestinal absorption profile of these molecules could be interesting to further assess their prodrug potential.

Conflicts of Interest

There are no conflicts of interest to declare.

Acknowledgements

The Laboratory of Medicinal Chemistry is a partner of the Antwerp Drug Discovery Network (www.addn.be). The NMR and purification equipment were financed by the Hercules Foundation. We also acknowledge Filip Lemière (Biomolecular and Analytical Mass Spectrometry, University of Antwerp) for recording HRMS spectra and Sophie Lyssens for the stability profiling. Work in Mohamed Lamkanfi's laboratory was supported by Grants from the Ghent University Concerted Research Actions (BOF14/GOA/013), and the Fund for Scientific Research-Flanders (FWO, G030212N). Lieselotte Vande Walle is a postdoctoral fellow with FWO.

Experimental

Chemistry

Reagents were obtained from Sigma-Aldrich, Acros, Fluorochem or Apollo Scientific and were used without further purification. Synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR and mass spectrometry. ^{1}H NMR and ^{13}C NMR spectra were recorded with a 400 MHz Bruker Avance DRX 400 spectrometer. and analyzed by use of MestReNova analytical chemistry software. Purities were determined with a Waters Acquity UPLC system coupled to a Waters TUV detector. ESI source and a Waters Acquity Qda Mass Detector. ADME measurements (UPLC-MS/MS) were carried out on a Waters Acquity UPLC system coupled to a Waters TUV detector, ESI source and a Waters Acquity TQ Mass Detector. A Waters

Acquity UPLC BEH C_{18} 1.7 µm 2.1 x 50 mm column was used, set at a temperature of 40 °C. Solvent A: water with 0.1% formic acid, solvent B: acetonitrile with 0.1% formic acid. HRMS involved 10 µL injection of a 10 µM sample solution using the CapLC system (Waters) and electrospray using a standard electrospray source. Positive ion mode accurate mass spectra were acquired using a Q-TOF II instrument (Waters). The mass spectrometer was calibrated prior to use with a 0.2% H_3PO_4 solution. The spectra were lock mass-corrected using the known mass of the nearest H_3PO_4 cluster. Where necessary, flash purification was performed with a Biotage ISOLERA One flash system equipped with an internal variable dual-wavelength diode array detector (200–400 nm).

(*S*)-*tert*-Butyl 3-(((benzyloxy)carbonyl)amino)-4-hydroxybutanoate (10)

To a cold (-5°C) solution of Z-Asp-(OtBu)-OH (**9**, 2 g. 6.19 mmol) in anhydrous DME (50 mL), were successively added triethylamine (0.95 mL, 6.80 mmol) and isobutylchloroformate (1.05 mL, 8.04 mmol). After 5 min the reaction mixture was filtered. and the precipitate was washed with additional DME (2 x 20 mL). The filtrate and the washings were combined and cooled to -5°C. A solution of Sodiumborohydride (702 mg. 18.56 mmol) in water (10 mL) was added in several portions. producing a strong evolution of gas, which ceased rapidly. After 5 min, additional water (60 mL) was added to the mixture and stirring was continued for 1 h. The mixture was extracted with EtOAc (2 x 60 mL). The organic layers were combined, dried (Na_2SO_4) and concentrated *in vacuo*. A transparent oil was obtained (1.80 g, 94 %). ^{1}H NMR (400 MHz. $CDCl_3$) δ 1.43 (s, 9H). 2.52 (m, 2H), 3.15 (s, 1H), 3.66 (d, *J* = 4.8 Hz, 2H), 3.98 - 4.12 (m, 1H), 5.09 (m, 2H), 5.70 (d, *J* = 8.2 Hz, 1H), 7.34 ppm (m, 5H); ^{13}C NMR (101 MHz. $CDCl_3$) δ 28.01, 37.25, 50.0, 64.19, 66.79, 81.33, 128.00-128.20 (2 signals), 128.50, 136.39, 156.32, 171.06 ppm; UPLC-MS *m/z*: 310.4 $[M+H]^+$.

(*S*)-3-(((Benzyloxy)carbonyl)amino)-4-((*tert*-butyldiphenylsilyl)oxy)butanoic acid (11)

tert-Butyl chlorodiphenylsilane (1.720 ml, 6.61 mmol), imidazole (0.614 g, 9.02 mmol) and 4-dimethylaminopyridine (0.110 g, 0.902 mmol) was added to a solution of **10** (1.86 g, 6.01 mmol) in DCM (25 mL) at 0 °C. A white precipitate formed. The solution was allowed to warm to room temperature and was stirred overnight under nitrogen atmosphere. The reaction mixture was quenched with water (50 mL). The mixture was extracted with DCM (2 x 50 mL). The combined organic layers were washed with saturated ammonium chloride solution (30 mL) and brine (30 mL), dried (Na_2SO_4) and concentrated *in vacuo*. Flash chromatography was performed (Hex:EtOAc) to give protected alcohol (3.8 g) R_f = 0.25 (1:1 Hex:EtOAc, visualization by UV); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.11 (m, 19H (impured)), 1.44 (s, 9H), 2.56 - 2.67 (m, 2H), 3.75 (m, 2H), 4.16 (m, 1H), 5.12 (s, 2H), 5.33 (d, *J* = 8.5 Hz, 1H), 7.42 (m, 17H), 7.63 - 7.69 (m, 4H), 7.73 - 7.78 ppm (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 26.60, 28.05, 31.61, 37.14, 49.64,

64.87, 66.66, 81.02, 127.62 - 127.90 (m), 128.05, 128.49, 129.53 - 129.93 (m), 133.06, 133.17, 134.82, 135.29, 135.52, 135.54, 136.58, 155.69, 170.65 ppm. Deprotection of the tert-butyl ester function was subsequently carried out. To this end, 3.2 g of the intermediate was dissolved in DCM (20 mL) and stirred at room temperature. Slowly TFA (20 mL) was added. After 30 min the reaction was stopped. DCM (200 mL) and H_2O (200 mL) were added to the mixture. Extraction was done. The aqueous phase was washed with DCM (200 mL). The organic layers were combined, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified using flash chromatography (hept:EtOAc + 0.5% FA) to give compound **11** (1.1 g, 46 % over steps). R_f = 0.67 (1:1 Hex:EtOAc, visualization by UV); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.08 (s, 8H), 1.10 (s, 3H), 2.74 (d, *J* = 5.7 Hz, 2H), 3.76 (s, 2H), 4.15 (m, impured, 1H), 5.11 (s, 2H), 5.35 (m, impured, 3H), 7.30 - 7.48 (m, 12H), 7.60 - 7.67 (m, 3H), 7.72 - 7.77 ppm (m, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 26.57, 26.85, 35.51, 49.20, 49.36, 64.75, 66.95, 127.74, 127.83, 128.20, 128.55, 129.68, 129.92, 132.80, 132.86, 134.81, 135.13, 135.53, 153.55, 155.90, 176.22 ppm; UPLC-MS *m/z*: 492.4 $[M+H]^+$.

Benzyl (*S*)-(1-((*tert*-butyldiphenylsilyl)oxy)-4-(methylsulfonamido)-4-oxobutan-2-yl)carbamate (12a)

Carbonyldiimidazole (0.660 g, 4.07 mmol) was added to a solution of **5** (1g, 2.034 mmol) in THF (10 mL) under an atmosphere of nitrogen. The reaction mixture was stirred for 3 h at room temperature. Methanesulfonamide (0.387 g, 4.07 mmol) was added followed by the addition of 1,8-diazabicyclo[5.4.0]undec-7-ene (0.608 mL, 4.07 mmol). The resulting mixture was stirred at room temperature overnight. The reaction mixture was diluted with EtOAc, washed with 1 N HCl, water, brine, dried (Na_2SO_4) and concentrated *in vacuo* to give a white foam (1.24 g, quant.). R_f = 0.56 (1:1 EtOAc:Hex + 0.1% FA, visualization by UV); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.08 (s, 9H), 2.66 (m, 2H), 3.16 (s, 3H), 3.74 (d, *J* = 3.7 Hz, 2H), 5.10 (d, *J* = 2.0 Hz, 2H), 5.37 (s, 1H), 7.30 - 7.49 (m, 12H), 7.58 - 7.68 (m, 4H), 9.48 ppm (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 19.28, 26.90, 41.23, 64.95, 67.26, 127.97, 128.14, 128.33, 128.61, 130.12, 132.51, 132.62, 135.49, 135.52, 136.00, 156.52, 169.85 ppm; UPLC-MS *m/z*: 567.6 $[M-H]^-$.

Benzyl (*S*)-(1-((*tert*-butyldiphenylsilyl)oxy)-4-(ethylsulfonamido)-4-oxobutan-2-yl)carbamate (12b)

The title compound was prepared from **11** (1 g, 2.034 mmol) and ethanesulfonamide (0.444 g, 4.07 mmol) in the same manner as described for the synthesis of **12a**. Comound **12b** was obtained as a white foam (0.96 g, 81 %). R_f = 0.56 (1:1 EtOAc:Hex + 0.1% FA, visualization by UV); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.08 (s, 9H), 1.20 - 1.37 (m, 3H), 2.69 (m, 2H), 3.36 (q, *J* = 7.4 Hz, 2H), 3.67 - 3.80 (m, 2H), 4.15 (m, 2H), 5.09 (s, 2H), 5.35 - 5.48 (d, *J* = 6.14 Hz, 1H), 7.30 - 7.49 (m, 11H), 7.59 - 7.68 (m, 4H), 9.46 ppm (s, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 7.63,

19.28, 26.90, 38.83, 47.80, 49.70, 64.93, 67.18, 127.93, 127.94, 128.08, 128.27, 128.58, 130.04, 130.07, 132.61, 132.74, 135.49, 135.52, 136.05, 156.41, 170.04 ppm; UPLC-MS *m/z*: 583.6 $[M+H]^+$.

(*S*)-3-Amino-4-((*tert*-butyldiphenylsilyl)oxy)-*N*-(ethylsulfonyl)butanamide (13b)

The title compound was prepared from **12b** (1.07 g, 1.836 mmol) in the same manner as described for the synthesis of **13a**. Amine **13b** was obtained (0.86 g, quant.). UPLC-MS *m/z*: 449.3 $[M+H]^+$. Because of the suspected instability due to this compound's free amine function, the crude material was immediately used in the next step (peptide coupling to intermediate **18**).

(*S*)-3-Amino-4-((*tert*-butyldiphenylsilyl)oxy)-*N*-(methylsulfonyl)butanamide (13a)

10% Palladium on carbon (49 mg, 0.459 mmol) was added to a solution of **6** (870 mg, 1.530 mmol) in dry MeOH (40 mL). The solution was flushed with nitrogen and the mixture was stirred under several balloons of hydrogen gas for 18 h. The reaction mixture was filtered through Celite and eluted with EtOAc and MeOH. The filtrate was concentrated *in vacuo*. Amine **13a** was obtained (630 mg, 95 %). UPLC-MS *m/z*: 435.2 $[M+H]^+$. Because of the suspected instability due to this compound's free amine function, the crude material was immediately used in the next step (peptide coupling to intermediate **18**).

(*S*)-*tert*-Butyl 1-((*S*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3,3-dimethylbutanoyl)pyrrolidine-2-carboxylate (15)

A solution of *N,N*-di-isopropylethylamine (2.96 mL, 16.98 mmol) and H-Pro-OtBu·HCl (3.05 g, 14.68 mmol) in DMF (15 mL) was added to a solution of Fmoc-Tle-OH (5 g, 14.15 mmol), EDC·HCl (3.25 g, 16.98 mmol) and 1-hydroxybenzotriazole hydrate (2.60 g, 16.98 mmol) in DMF (35 mL). The mixture was stirred at room temperature overnight. The reaction mixture was diluted with EtOAc (250 mL), washed with 1 M HCl (200 mL), 5 % aqueous $NaHCO_3$ (200 mL) and brine (150 mL). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. Dipeptide **15** was obtained (7.3 g, 100 %). R_f = 0.61 (1:1 Hex:EtOAc, visualization by UV); UPLC-MS *m/z*: 507.3 $[M+H]^+$.

Diethylammonium (*S*)-1-((*S*)-2-amino-3,3-dimethylbutanoyl)pyrrolidine-2-carboxylate (16)

Dipeptide **15** was dissolved in DCM (25 mL) and TFA (25 mL) was added. The reaction mixture was stirred for 1 h and concentrated *in vacuo*, after addition of toluene (50 mL). Diethyl ether

was added to the crude and concentrated to dryness. The outcome was dissolved in THF (20 mL) and diethylamine (20.58 mL, 200 mmol) was added. The reaction mixture was left stirring for 1.5 h at room temperature. The volatiles were removed *in vacuo*, EtOAc was added and concentrated again. Water + 10 % ACN (50 mL) was added and extraction with heptane (3 x 100 mL) was done. The aqueous layer was concentrated *in vacuo*. Compound **16** was obtained in the form of a salt with diethylamine (2.9 g, quant.). ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.95 (s, 3H), 0.97 (s, 2H), 1.15 (t, *J* = 7.3 Hz, 3H), 1.56 - 1.75 (m, 1H), 1.75 - 1.95 (m, 1H), 1.98 - 2.18 (m, 1H), 2.88 (q, *J* = 7.3 Hz, 2H), 3.21 - 3.31 (m, 1H), 3.37 - 3.55 (m, 1H), 3.65 - 3.75 (m, 1H), 4.17 - 4.34 ppm (m, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 11.60, 22.81, 25.17, 26.61, 26.73, 29.39, 31.52, 34.03, 35.30, 41.63, 46.39, 47.91, 58.98, 59.39, 59.94, 62.70, 171.31, 174.58, 175.15 ppm; UPLC-MS *m/z*: 229.2 $[M+H]^+$.

(*S*)-1-((*S*)-2-(4-((*tert*-Butoxycarbonyl)amino)-3-chlorobenzamido)-3,3-dimethylbutanoyl)pyrrolidine-2-carboxylic acid (18)

A solution of **16** (salt with diethylamine, 719 mg, 2.385 mmol) and *N,N*-di-iso-propylethylamine (0.415 mL, 2.385 mmol) in DMF (15 mL) was added to a preactivated (30 min) solution of 4-(Boc-amino)-3-chlorobenzoic acid (**17**) (540 mg, 1.988 mmol), EDC·HCl (457 mg, 2.385 mmol) and 1-hydroxybenzotriazole hydrate (365 mg, 2.385 mmol) in DMF (8 mL). The mixture was stirred overnight at room temperature. EtOAc (100 mL) was added and extraction was done with 1 M HCl (3 x 50 mL), brine (50 mL), dried (Na_2SO_4) and concentrated *in vacuo*. Flash chromatography (DCM:MeOH) was done to afford compound **18** (850 mg, 51 %) with a purity of 57% (by UV). R_f = 0.30 (95:5 DCM:MeOH, visualization by UV); UPLC-MS *m/z*: 480.1 $[M-H]^-$.

***tert*-Butyl (4-(((*S*)-1-((*S*)-2-(((*S*)-1-((*tert*-butyldiphenylsilyl)oxy)-4-(methylsulfonamido)-4-oxobutan-2-yl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamoyl)-2-chlorophenyl)carbamate (19a)**

To a solution of **18** (400 mg, 0.830 mmol) in DMF (10 mL) was added **13a** (433 mg, 0.996 mmol), *N,N*-di-iso-propylethylamine (0.361 mL, 2.075 mmol), 1-hydroxybenzotriazole hydrate (153 mg, 0.996 mmol) and EDC·HCl (191 mg, 0.996 mmol). The reaction mixture was stirred for 3 h at room temperature. EtOAc (70 mL) was added and extraction was done with 1 M HCl (2 x 60 mL), water (70 mL) and brine (70 mL). The organic layer was concentrated *in vacuo* and flash chromatography (Hex:EtOAc) was done. Compound **19a** was obtained (300 mg, 40 %). R_f = 0.40 (1:3 Hex:EtOAc, visualization by UV); UPLC-MS *m/z*: 895.8 $[M-H]^-$.

***tert*-Butyl (4-(((*S*)-1-((*S*)-2-(((*S*)-1-((*tert*-butyldiphenylsilyl)oxy)-4-(ethylsulfonamido)-4-oxobutan-2-yl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamoyl)-2-chlorophenyl)carbamate (19b)**

The title compound was prepared from **18** (400 mg, 0.830 mmol) and **13b** (559 mg, 1.245 mmol) in the same manner as described for the synthesis of **19a**. Compound **19b** was obtained (370 mg, 40 %) with a purity of 86 % (UV). R_f = 0.56 (1:1 EtOAc:Hex + 0.1 %); UPLC-MS *m/z*: 910.1 $[M-H]^-$.

(*S*)-1-((*S*)-2-(4-Amino-3-chlorobenzamido)-3,3-dimethylbutanoyl)-*N*-((*S*)-4-(methylsulfonamido)-1,4-dioxobutan-2-yl)pyrrolidine-2-carboxamide (3)

Compound **19a** was dissolved in THF (6 mL). Tetrabutylammonium fluoride (1 M in THF, 0.501 mL, 0.501 mmol) was added and the reaction mixture was left stirring overnight. Additional TBAF (1M, 250 µL) was added to the solution and after stirring 24 h the reaction was finished. The reaction mixture was diluted with EtOAc (50 mL) and extracted with 0.5 M HCl (2 x 40 mL) and brine (40 mL). The organic layer was dried (Na_2SO_4) and concentrated *in vacuo*. Flash chromatography (EtOAc:MeOH) was done to afford the alcohol (170 mg, 73%). R_f = 0.50 (90:10 EtOAc:MeOH, visualization by UV); UPLC-MS *m/z*: 658.1 $[M-H]^-$. The alcohol was dissolved in dry DCM (5 mL) and DMF (2 mL) and Dess-Martin periodinane (142 mg, 0.335 mmol) was added. The reaction mixture was stirred for 7 h at room temperature. EtOAc (20 mL) was added and extraction was done with saturated NH_4Cl (20 mL) and 1 M HCl (20 mL). The organic layer was concentrated *in vacuo*. Flash chromatography (DCM:MeOH) was done to afford the aldehyde (150 mg, 89%). R_f = 0.29 (95:5 DCM:MeOH, visualization by UV); UPLC-MS *m/z*: 656.1 $[M-H]^-$. The aldehyde (120 mg, 0.182 mmol) was dissolved in DCM (2 mL) and TFA (0.4 mL, 3.04 mmol) was added. The reaction mixture was left stirring for 30 min. DCM (10 mL) and toluene (10 mL) were added and the mixture was concentrated *in vacuo*. Reversed phase flash chromatography (water:acetonitrile) was done and the final crude was lyophilized to afford final compound **3** as a white powder (70 mg, 69%). R_f = 0.19 (100 % EtOAc, visualization by UV); ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.95 (s, 2H), 1.02 (s, 8H), 1.65 - 2.15 (m, 5H), 2.32 (d, *J* = 18.0 Hz, 1H), 3.13 (dd, *J* = 18.1, 7.4 Hz, 1H), 3.17 - 3.28 (m, 3H), 3.55 – 3.67 (m, 1H), 3.74 - 3.84 (m, 1H), 3.93 (t, *J* = 6.8 Hz, 1H), 4.22 (t, *J* = 7.0 Hz, 1H), 4.33 - 4.45 (m, 1H), 4.68 (d, *J* = 8.8 Hz, 1H), 5.29 (d, *J* = 6.6 Hz, 1H), 5.91 (s, 2H), 6.76 (d, *J* = 8.5 Hz, 1H), 7.23 (d, *J* = 6.6 Hz, 2H), 7.56 - 7.70 (m, 2H), 7.81 (d, *J* = 2.0 Hz, 1H), 8.53 ppm (d, *J* = 6.2 Hz, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 24.81, 26.68, 29.54, 31.34, 34.92, 34.99, 35.99, 41.36, 47.90, 50.89, 57.39, 59.44, 87.37, 114.03, 116.01, 121.70, 127.79, 129.00, 147.61, 165.44, 169.40, 172.12, 172.74 ppm; HRMS (ESI): calcd for $C_{23}H_{33}N_5O_7SCl^+$ $[M+H]^+$: 558.1784, found: 558.1766.

(*S*)-1-((*S*)-2-(4-Amino-3-chlorobenzamido)-3,3-dimethylbutanoyl)-*N*-((*S*)-4-(ethylsulfonamido)-1,4-dioxobutan-2-yl)pyrrolidine-2-carboxamide (4)

The title compound was prepared from **19b** (370 mg, 0.405 mmol) in the same manner as described for the synthesis of **3**. Firstly, intermediate alcohol was obtained (210 mg, 77 %); R_f = 0.36 (95:5 EtOAc:MeOH, visualization by UV); UPLC-MS *m/z*: 672.1 $[M-H]^-$. Secondly, intermediate aldehyde was obtained (170 mg, 81 %); R_f = 0.33 (95:5 EtOAc:MeOH, visualization by UV); UPLC-MS *m/z*: 670.1 $[M-H]^-$. Finally, **4** was obtained as a white powder (72 mg, 71 %). R_f = 0.32 (100 % EtOAc, visualization by UV); ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.02 (s, 9H), 1.24 (t, *J* = 7.4 Hz, 3H), 1.63 - 1.76 (m, 1H), 1.76 - 2.15 (m, 4H), 2.33 (d, *J* = 18.0 Hz, 1H), 3.10 - 3.21 (m, 1H), 3.27 - 3.47 (m, under water peak), 3.56 - 3.68 (m, 1H), 3.74 - 3.85 (m, 1H), 3.89 - 3.96 (m, 0.6H), 4.23 (dd, *J* = 8.0, 6.1 Hz, 0.6H), 4.35 - 4.44 (m, 0.5H), 4.64 - 4.73 (m, 1H), 5.28 (d, *J* = 6.4 Hz, 0.6H), 5.89 (d, *J* = 7.1 Hz, 1H), 6.76 (d, *J* = 8.5 Hz, 1H), 7.14 - 7.25 (m, 0.6H), 7.54 - 7.68 (m, 1.6H), 7.81 ppm (d, *J* = 2.0 Hz, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 7.1, 24.77, 26.64, 29.52, 34.93, 35.69, 47.41, 47.71, 51.18, 57.38, 59.31, 87.22, 114.02, 116.01, 121.74, 127.74, 128.97, 147.59, 165.4, 169.37, 172.11, 172.66 ppm; HRMS (ESI): calcd for $C_{24}H_{35}N_5O_7SCl^+$ $[M+H]^+$: 572.1940, found: 572.1936.

(*S*)-1-((*S*)-2-(4-Amino-3-chlorobenzamido)-3,3-dimethylbutanoyl)-*N*-((*S*)-1-cyano-3-(methylsulfonamido)-3-oxopropyl)pyrrolidine-2-carboxamide (6)

Methanesulfonamide (19.90 mg, 0.209 mmol), 4-dimethylaminopyridine (19.17 mg, 0.157 mmol) and EDC·HCl (30.1 mg, 0.157 mmol) were added to a solution of **5** (50 mg, 0.105 mmol) in DCM (2.5 mL) and DMF (0.5 mL) under an atmosphere of nitrogen. The reaction mixture was stirred for 1 day at room temperature. The solution was diluted with EtOAc (20 mL), washed with 1 M HCl (15 mL), water (20 mL), brine (20 mL), dried (Na_2SO_4) and concentrated *in vacuo*. The crude was lyophilized to afford final compound **6** as a white powder (52 mg, 90 %). R_f = 0.31 (100 % EtOAc, visualization by UV); ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.02 (s, 9H), 1.68 - 2.01 (m, 3H), 2.01 - 2.16 (m, 1H), 2.77 - 3.01 (m, 2H), 3.21 (m, 3H), 3.56 - 3.70 (m, 1H), 3.70 - 3.83 (m, 1H), 4.27 (dd, *J* = 8.2, 5.6 Hz, 1H), 4.67 (d, *J* = 8.7 Hz, 1H), 4.93 (q, *J* = 7.2 Hz, 1H), 5.92 (s, 2H), 6.76 (d, *J* = 8.5 Hz, 1H), 7.59 (dd, *J* = 8.5, 2.0 Hz, 1H), 7.68 (d, *J* = 8.7 Hz, 1H), 7.81 (d, *J* = 2.0 Hz, 1H), 8.79 (d, *J* = 7.5 Hz, 1H), 12.02 ppm (s, 1H); ^{13}C NMR (101 MHz, MeOD-d_4) δ 26.20, 27.11, 30.52, 36.61, 37.97, 39.12, 41.46, 49.88, 59.33, 59.43, 61.51, 115.49, 118.68, 118.81, 123.30, 123.33, 128.36, 130.09, 149.38, 168.87, 168.94, 170.01, 172.23, 173.81 ppm; HRMS (ESI): calcd for $C_{23}H_{32}N_6O_6SCl^+$ $[M+H]^+$: 555.1787, found: 555.1758.

(*S*)-2-((*S*)-2-(((Benzyloxy)carbonyl)amino)-3-methylbutanamido)propanoic acid (22)

N-Carbobenzyloxy-L-valine (4 g, 15.92 mmol), 1-hydroxybenzotriazole hydrate (2.68 g, 17.51 mmol), EDC.HCl (3.36 g, 17.51 mmol) were dissolved in dry DCM (80 mL) at 0 °C and left stirring 20 min at the same temperature. L-Alanine methyl ester hydrochloride (2.444 g, 17.51 mmol) and *N,N*-di-iso-propylethylamine (6.93 ml, 39.8 mmol) were added to the mixture. The reaction mixture was allowed to warm up to room temperature and was left stirring overnight. The reaction mixture was diluted with DCM (50 mL), quenched with water (100 mL), extracted and washed with saturated $NaHCO_3$ (2 x 100 mL), 0.5 M HCl (100 mL) and brine (100 mL). The organic layer was dried (Na_2SO_4) and concentrated *in vacuo*. A back-extraction was done with the first aqueous phase. The organic phase was then extracted with the same washing solvents and combined with the previous crude after concentrating *in vacuo*. The resulting crude was dissolved in MeOH (42 mL) and a solution of LiOH (600 mg, 25.06 mmol) in water (14 mL) was. The reaction mixture was left stirring overnight at room temperature, followed by quenching with a 10% aqueous solution of citric acid (180 mL). This was left stirring for 15 min and the formed precipitate was filtered. Flash chromatography (hept:EtOAc) of the crude was performed to afford **22** (1.47 g, 28%). R_f = 0.27 (1:1 EtOAc:Hex + 0.1 % FA, visualization by PMA); ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.84 (d, *J* = 6.7 Hz, 3H), 0.89 (d, *J* = 6.8 Hz, 3H), 1.27 (d, *J* = 7.3 Hz, 3H), 1.93 - 1.94 (m, 1H), 3.90 (dd, *J* = 8.9, 7.0 Hz, 1H), 4.19 (p, *J* = 7.1 Hz, 1H), 5.03 (s, 2H), 7.24 (d, *J* = 9.1 Hz, 1H), 7.27-7.41 (M, 5H), 8.20 (d, *J* = 6.9 Hz, 1H), 12.49 ppm (s, 1H); UPLC-MS *m/z*: 323.1 $[M+H]^+$.

Benzyl ((*S*)-1-(((*S*)-1-(((*S*)-1-hydroxy-4-(methylsulfonamido)-4-oxobutan-2-yl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (23)

4-Methylmorpholine (0.101 ml, 0.920 mmol) was added to a solution of acid **22** (270 mg, 0.837 mmol) in THF (15 mL), followed by addition of isobutyl chloroformate (0.119 ml, 0.920 mmol) at -20 °C. The reaction mixture was stirred at -20 ° C for 20 min, followed by addition of amine **13a** (400 mg, 0.920 mmol). The resulting mixture was stirred at -20 °C for 3 h, quenched with 1M HCl (60 mL) and extracted with EtOAc (2 x 60 mL). The combined organic layers were washed with water (60 mL), brine (50 mL), dried (Na_2SO_4) and concentrated *in vacuo*. The crude was dissolved in dry THF (10 mL), tetrabutylammonium fluoride (1 M in THF, 5.68 ml, 5.68 mmol) was added and the reaction mixture was left stirring overnight. The reaction mixture was diluted with EtOAc (100 mL) and extracted with water (100 mL). The aqueous phase was washed with EtOAc (100 mL). The combined organic layers were dried (Na_2SO_4), concentrated *in vacuo* and purified by flash chromatography (EtOAc:MeOH) to afford alcohol **23** (350 mg, 83 %). R_f = 0.07 (EtOAc + 0.1 % FA, visualization by UV/PMA); ^{1}H NMR (400 MHz, MeOD-d_4) δ 0.98 (dd, *J* = 14.4, 6.8 Hz, 6H), 1.38 (d, *J* = 7.1 Hz, 3H), 2.05 - 2.16 (m, 1H), 2.48 - 2.68 (m, 2H), 3.22 (s, 3H), 3.54 - 3.67 (m, 2H), 3.91 - 3.98 (m, 1H), 4.25 (p, *J* = 5.8 Hz, 1H), 4.34 (q, *J* = 7.1 Hz, 1H), 5.08 - 5.20 (m, 2H), 7.17 (d, *J* = 7.2 Hz, 0.2H), 7.29 - 7.43 ppm (m, 5H); ^{13}C

NMR (101 MHz, MeOD-*d*$_4$) δ 16.63, 17.06, 18.35, 30.36, 37.23, 39.88, 49.19, 60.94, 62.57, 66.50, 127.52, 127.66, 128.09, 136.71, 157.55, 170.95, 172.71, 173.14 ppm; UPLC-MS *m/z*: 501.3 $[M+H]^+$.

Benzyl ((*S*)-3-methyl-1-(((*S*)-1-(((S)-4-(methylsulfonamido)-1,4-dioxobutan-2-yl)amino)-1-oxopropan-2-yl)amino)-1-oxobutan-2-yl)carbamate (8)

Alcohol **23** was dissolved in dry DCM (5 mL) and DMF (1.5 mL) and Dess-Martin periodinane (99 mg, 0.234 mmol) was added. This reaction mixture was stirred at room temperature for 2 h. EtOAc (20 mL) was added and extraction was done with saturated NH_4Cl (20 mL) and 1M HCl (20 mL). The organic layer was concentrated *in vacuo*. Flash chromatography (EtOAc:MeOH) was done and the final crude was lyophilized to afford the final compound **8** as a white powder (35 mg, 39 %). R_f = 0.22 (95:5 DCM:MeOH, visualization by UV/PMA); ^{1}H NMR (400 MHz, MeOD-*d*$_4$) δ 0.90 - 1.03 (m, 6H), 1.38 (t, *J* = 6.7 Hz, 3H), 2.06 (m, 1H), 2.42 (d, *J* = 18.2 Hz, 0.6H), 2.70 - 2.77 (m, 0.7H), 3.15 (dd, *J* = 18.2, 7.5 Hz, 0.6H), 3.26 (s, 1.8H), 3.27 (s, 1.2H), 3.82 - 4.03 (m, 1H), 4.16 (d, *J* = 7.3 Hz, 0.6H), 4.38 (dq, *J* = 46.9, 8.9 Hz, 2H), 4.48 - 4.58 (m, 0.5H), 5.06 - 5.19 (m, 2H), 5.53 (s, 0.6H), 5.76 (d, *J* = 5.0 Hz, 0.4H), 7.10 - 7.21 (m, 0.6H), 7.28 - 7.42 (m, 5H), 8.24 ppm (t, *J* = 5.6 Hz, 0.5H); ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 0.85 (dd, *J* = 16.8, 6.8 Hz, 6H), 1.22 (t, *J* = 6.2 Hz, 3H), 1.96 (td, *J* = 13.6, 6.9 Hz, 1H), 2.31 (d, *J* = 17.9 Hz, 0.7H), 2.57 - 2.73 (m, 0.7H), 3.14 (dd, *J* = 18.1, 7.3 Hz, 0.7H), 3.24 (s, 2H), 3.27 (s, 1H), 3.79 - 4.04 (m, 2H), 4.16 - 4.28 (m, 0.8H), 4.28 - 4.47 (m, 0.7H), 4.97 - 5.10 (m, 2H), 5.29 (d, *J* = 5.6 Hz, 0.6H), 5.53 (s, 0.3H), 7.18 (d, *J* = 6.3 Hz, 0.3H), 7.22 - 7.48 (m, 6H), 7.99 - 8.14 (m, 1.5H), 8.47 ppm (d, *J* = 6.0 Hz, 0.7H); ^{13}C NMR (101 MHz, MeOD-*d*$_4$) δ 16.44, 16.74, 17.14, 17.27, 18.27, 18.38, 30.34, 30.56, 34.48, 35.35, 40.51, 40.64, 46.54, 48.73, 49.10, 51.08, 60.65, 61.07, 66.39, 66.49, 81.68, 87.62, 127.40, 127.44, 127.65, 128.12, 136.76, 171.99, 172.66, 172.73, 172.93, 173.53, 173.71 ppm; ^{13}C NMR (101 MHz, DMSO-*d*$_6$) δ 18.08, 18.27, 19.16, 30.29, 31.30, 34.38, 35.83, 41.33, 41.41, 46.24, 48.02, 50.69, 59.95, 65.38, 81.49, 87.37, 127.63, 127.76, 128.33, 137.05, 156.17, 170.81, 171.45, 172.52 ppm; HRMS (ESI): calcd for $C_{21}H_{31}N_4O_8S^+$ $[M+H]^+$: 499.1857, found: 499.1870.

5-Hydroxydihydrofuran-2(3H)-one or 4-oxobutanoic acid (24)

Compound **24** was prepared according to a procedure by Wermuth.[40] Additional characterization was done by ^{1}H and ^{13}C NMR in different solvents (spectra included in the supporting information): ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 1.88 (m, 0.5H), 2.26 - 2.38 (m, 0.6H), 2.46 - 2.53 (m, under solvent), 2.66 (t, *J* = 6.5 Hz, 2H), 5.79 (q, *J* = 5.7 Hz, 0.5H), 7.50 (d, *J* = 6.0 Hz, 0.5H), 9.67 (s, 1H), 12.21 (s, 1H); ^{13}C NMR (101 MHz, DMSO-*d*$_6$) δ 26.84, 27.80, 30.02, 38.50, 99.79, 174.04, 176.89, 202.52. ^{1}H NMR (400 MHz, MeOD-*d*$_4$) δ 1.85 (dd, *J* = 7.5, 5.5 Hz, 2H), 2.40 (t, *J* = 7.5 Hz, 2H), 4.58 (t, *J* = 5.5 Hz, 1H); ^{13}C NMR (101 MHz, MeOD-

d$_4$) δ 28.77, 31.62, 97.34, 175.78; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.09 – 2.28 (m, 0.6H), 2.28 – 2.60 (m, 1.5H), 2.71 (m, 2.5H), 2.82 (m, 2H), 5.95 (s, 0.5H), 9.83 (s, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 26.30, 27.02, 29.46, 38.23, 98.91, 177.08, 177.75, 199.96; ^{1}H NMR (400 MHz, D_2O) δ 1.81 (td, *J* = 7.5, 5.7 Hz, 2H), 2.39 (t, *J* = 7.5 Hz, 2H), 4.99 (t, *J* = 5.6 Hz, 1H); ^{13}C NMR (101 MHz, D_2O) δ 29.25, 32.13, 90.00, 177.95.

Methyl 4,4-dimethoxybutanoate (26)

To a solution of sodium methoxide (29.4 g, 136 mmol) in MeOH (90 mL) was added methyl 4-nitrobutanoate (**25**, 8.70 mL, 68.0 mmol) at 0 °C over 30 min through an additional funnel. The resulting suspension was added dropwise via another addition funnel, over 15 min, to a round-bottom flask equipped with methanol (100 mL) and conc. sulfuric acid (21.06 mL, 394 mmol, 96% solution in water), held at 0 °C. Stirring continued for 30 min. The reaction mixture was poured into DCM (250 mL) and water (250 mL). Extraction was done and the aqueous layer was washed with DCM (100 mL). The organic layers were combined, washed with 1 % aqueous NaOH (100 mL), ice water (100 mL) and brine (100 mL), dried (Na_2SO_4) and concentrated *in vacuo* to afford the acetal **26** (8.96 g, 81 %) as a slightly yellow liquid. R_f = 0.25 (4:1 Hex:EtOAc, visualization by $KMnO_4$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.91 (td, *J* = 5.6, 7.5 Hz, 8H), 2.37 (t, *J* = 7.5 Hz, 8H), 3.31 (s, 6H), 3.66 (s, 3H), 4.38 ppm (t, *J* = 5.6 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 27.92, 29.19, 51.74, 53.27, 103.71, 173.82 ppm. Due to the low molecular weight of this compound, no ESI-mass spectra could be recorded.

4,4-Dimethoxybutanoic acid (27)

To a solution of methyl 4,4-dimethoxybutanoate (**26**, 5 g, 30.8 mmol) in MeOH (45 mL) was added dropwise 1 M NaOH (35.5 mL, 35.5 mmol) at 0 °C. After stirring overnight at room temperature, the mixture was acidified to pH = 2 by addition of 1 M HCl at 0 °C. The mixture was extracted with EtOAc (3 × 100 mL). The combined organic layers were washed with brine (100 mL), dried (Na_2SO_4) and concentrated *in vacuo* to afford the acid **27** (4.11 g, 90 %). R_f = 0.24 (1:1 Hex:EtOAc, visualization by $KMnO_4$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.93 (td, *J* = 7.4, 5.6 Hz, 2H), 2.43 (t, *J* = 7.4 Hz, 2H), 3.33 (s, 6H), 4.42 ppm (t, *J* = 5.6 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 26.87, 28.82, 53.4, 56.83, 105.32, 176.82 ppm. Due to the low molecular weight of this compound, no ESI-mass spectra could be recorded.

5-Hydroxy-1-(methylsulfonyl)pyrrolidin-2-one (28)

Methanesulfonamide (1.118 g, 11.76 mmol), 4-dimethylaminopyridine (1.436 g, 11.76 mmol) and EDC.HCl (2.253 g, 11.76 mmol) were added to a solution of 4,4-dimethoxybutanoic acid (**27**, 1 g, 5.88 mmol) in DCM (50 mL) under an atmosphere of nitrogen and the reaction mixture was stirred overnight at room temperature. The mixture was diluted with water (100 mL) and acidified with 1 M citric acid till pH 2 was obtained. The aqueous layer was extracted with EtOAc (2 x 100 mL). The organic layers were combined, washed with brine (100 mL), dried (Na_2SO_4) and concentrated *in vacuo*. Flash chromatography (hept:EtOAc) was done. The outcome was dissolved in $CHCl_3$ (10 mL), set at 0°C and a mixture of 1:1 water:TFA (5 mL) was added. The reaction mixture was stirred for 90 min at the same temperature, followed by stirring for 18 h at room temperature. The mixture was co-evaporated with toluene. Flash chromatography (hept:EtOAc) was performed to obtain final compound **28** (340 mg, 32 %). R_f = 0.24 (3:1 EtOAc:Hex, visualization by $KMnO_4$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.03 - 2.14 (m, 1H), 2.32 - 2.44 (m, 1H), 2.47 - 2.57 (m, 1H), 2.76 - 2.89 (m, 1H), 3.30 (s, 3H), 4.92 (s, 1H), 5.91 ppm (dd, *J* = 6.3, 1.6 Hz, 1H); ^{1}H NMR (400 MHz, D_2O) δ 2.02 - 2.11 (m, 1H), 2.43 - 2.57 (m, 1H), 2.59 - 2.69 (m, 1H), 2.85 - 2.96 (m, 1H), 3.38 (s, 3H), 5.93 ppm (dd, *J* = 6.2, 1.0 Hz, 1H); ^{1}H NMR (400 MHz, MeOD-*d*$_4$) δ 1.78 - 1.92 (m, 0.5H), 1.92 - 2.02 (m, 1H), 2.39 (m, 1.5H), 2.53 (m, 1H), 2.81 (m, 1H), 3.25 (s, 0.3H), 3.26 (s, 2.7H), 4.58 (t, *J* = 5.5 Hz, partly under waterpeak), 5.82 (dd, *J* = 5.9, 0.7 Hz, 1H), 6.51 ppm (s, 0.25H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 27.22, 30.02, 42.01, 83.38, 174.22 ppm; ^{13}C NMR (101 MHz, D_2O) δ 28.16, 30.74, 42.07, 84.90, 178.34 ppm; ^{13}C NMR (101 MHz, MeOD-*d*$_4$) δ 28.88, 30.01, 30.74, 32.73, 41.86, 43.24, 84.81, 98.55, 176.46, 177.04 ppm; UPLC-MS *m/z*: 178,1 $[M-H]^-$.

Phenylhydrazine kinetic study

Colorless 2-phenylhydrazin-1-ium chloride (84 mg, 0.581 mmol) and sodium acetate (130 mg, 1.585 mmol) were dissolved in water (3 mL). The aldehyde (0,490 mmol), dissolved in a 1 mL of ethanol (free from aldehydes and ketones), was added. The mixture was shaken until a clear solution was obtained (1-2 min). Follow-up was directly done by UPLC-UV-MS and showed full conversion into (E)-4-(2-phenylhydrazono)butanoic acid in the case of **24** (UPLC-MS *m/z* 191.1 $[M-H]^-$, t_R = 1.45 min) and (E)-*N*-(methylsulfonyl)-4-(2-phenylhydrazono)butanamide (UPLC-MS *m/z* 268.1 $[M-H]^-$, t_R = 1.43 min) in the case of **28**. The reaction of **28** with (2,4-dinitrophenyl)hydrazine was also set up; a clear solution was prepared by warming a solution of 2,4-dinitro-phenylhydrazine, 1 mL of concentrated hydrochloric acid (37%) and 9 ml of ethanol. 10 mg of **28** was added to 400 µL of the previous solution, followed by 1.5 mL EtOH. The mixture was shaken until the solution became clear (1-5 min). Full conversion of **28** into (E)-4-(2-(2,4-dinitrophenyl)hydrazono)-*N*-(methylsulfonyl)butanamide was detected by UPLC-UV-MS (UPLC-MS *m/z* 358.1 $[M-H]^-$, t_R=1.55 min). The UPLC-UV-MS system consisted of a Waters Acquity UPLC system coupled to a Waters TUV detector, ESI source and a Waters

Acquity Qda Mass Detector. A Waters Acquity UPLC BEH C_{18} 1.7 µm 2.1 x 50 mm column was used, set at a temperature of 40 °C. Solvent A: water with 0.1% formic acid, solvent B: acetonitrile with 0.1% formic acid. The gradient used started with a flow rate of 0.7 mL/min at 95% A, 5% B for 0.15 min then in 1.85 min from 95% A, 5% B to 100% B, isocratically at the same percentage for 0.25 min and finally 0.75 min (0.350 mL/min), 95% A, 5% B. The wavelength for UV detection was 254 nm.

Biochemical evaluation: IC_{50} determination

In a 96 well plate, caspases and inhibitors were incubated for 15 minutes at 37 °C before substrate hydrolysis was monitored at 37 °C for 20 minutes in 2-minute intervals. Assays were run in two technical duplicates and all experiments were performed in at least two independent repeats. Total volume per well was 50 µL. A positive control for caspase activity has been included in all of the assays (caspase + substrate + DMSO). Substrates were Ac-WEHD-AMC for m casp-11 and h casp-1,-4,-5; Ac-ATAD-AFC for m casp-12; Ac-DEVD-AMC for h casp-3; Ac-IETD-AMC for h casp-8 and Ac-LEHD-AMC for h casp-9. All the assays have been performed at 100 µM substrate concentration. IC_{50}-values were calculated using at least 7 inhibitor concentrations giving between 10 and 90% inhibition. IC_{50}-values were obtained by fitting the data using the 4-parameter logistics function with background correction. Buffer composition was 20 mM PIPES, 100 mM NaCl, 1 mM EDTA, 0.1% (w/v) CHAPS, 10% sucrose and adjusted till pH 7.5. DTT was added just before filling the wells to obtain a 10 mM final concentration. FLx800, a fluorescent microplate reader (Bio-Tek) was used to measure fluorescence after caspase activation. Excitation and emission wavelengths for AMC and AFC substrates are 360 nm (AMC), 400 nm (AFC) and 460 nm (AMC), 528 nm (AFC) respectively.

Chemical stability protocol

A 10 mM stock compound solution in DMSO was prepared. The stock solution was diluted to a 500 nM solution with the specific buffer. The mixture was gently shaken at 37 °C. At different time points (0 min - 30 min - 60 min - 120 min - 180 min - 360 min – 24 h) 100 µL was withdrawn which was analyzed with UPLC-MS/MS. Samples were prepared in duplicate and measured in triplicate. The percentage of parent compound remaining at each time point relative to the 0 min sample was then calculated from UPLC-MS/MS peak area ratios. A set of 5 dilutions (in the solution buffer) of the compound were prepared from a 10 µM solution to give final test concentrations between 31 nM and 500 nM. A calibration line was made to evaluate the

accuracy of the test method and to calculate the concentration. 10 mM PBS buffer was used for pH 7.4 and 10 mM acetic acid buffer for pH 4.0.

Metabolic stability protocol

Liver microsomes (20 mg protein/mL), NADPH regenerating system solutions A & B and 5 mM stock compound solution (100% DMSO) were prepared. The reaction mixture finally contained 713 µL purified water, 200 µL 0.5 M potassium phosphate pH 7.4, 50 µL NADPH regenerating system solution A (BD Biosciences Cat. No. 451220), 10 µL NADPH regenerating system solution B (BD Biosciences Cat. No. 451200) and 2 µL of the compound stock solution (10 µM final concentration). The reaction mixture was warmed to 37 °C for 5 minutes and the reaction was initiated by addition of 25 µL of liver microsomes (0.5 mg protein/mL final concentration). At different time points (0 min – 15 min – 30 min – 60 min – 120 min – 240 min – 360 min – 24h), 20 µL was withdrawn and 80 µL cold acetonitrile was added on ice for 10 minutes. Then the mixtures were centrifuged at 13 000 rpm for 5 min at 4 °C. The supernatant was further diluted in 90 % water/acetonitrile. At each time point, the compound was analyzed using UPLC-MS/MS. The calculation of parent percentage and the calibration line were the same as in the chemical stability measurements. Verapamil was used as a positive control. Samples were prepared in duplicate and measured in triplicate.

Macrophage differentiation, stimulation and evaluation

Bone marrow-derived macrophages (BMDMs) were prepared as described previously.[50] BMDMs were left untreated or stimulated with ultrapure LPS (0.5 µg ml^{-1}) (Invivogen) for 3 h. Cells were treated with the indicated inhibitors (50 µM, 45 min) prior to the addition of 20 µM nigericin (Sigma-Aldrich) for 1 h. In other setups, cells were left untreated or stimulated with Pam3CSK4 (1 µg ml^{-1}) (Invivogen) for 5 h. Cells were treated with the indicated inhibitors (50 µM, 45 min) prior to transfection with LPS (2 µg ml^{-1}) (Invivogen) for 7 h. Cytokine levels in culture medium were evaluated using Luminex technology (Bio-Rad). Cell death was measured using the LDH assay from Promega.

Abbreviations

ACN, acetonitrile; ADME; absorption-distribution-metabolism-excretion; AFC, 7-amino-4-trifluoromethylcoumarin; AMC, 7-amino-4-methylcoumarin; Boc, *tert*-butoxycarbonyl; CDI, 1,1'-carbonyldiimidazole; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCM, dichloromethane; DIPEA, *N,N*-diisopropylethylamine; DMAP, 4-dimethylaminopyridine; DME, dimethoxyethane; DMF, *N.N*-dimethylformamide; DMSO, dimethylsulfoxide; EDC·HCl, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride; EDTA, ethylenediaminetetraacetic acid; EtOAc, ethyl acetate; FA, formic acid; Hept, *n*-heptane; Hex, hexanes; HOBt, hydroxybenzotriazole; (HR)MS, (high resolution) mass spectrometry; IC_{50}, half maximal inhibitory concentration; IL, interleukin; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); PMA, phosphomolybdic Acid; RT, room temperature; TEA, trimethylamine; TFA. trifluoroacetic acid; THF, tetrahydrofuran; UPLC, ultra performance liquid chromatography; Z, carboxybenzyl.

References

1. U. Fischer, R. U. Janicke and K. Schulze-Osthoff, *Cell Death Differ.*, 2003, **10**, 76-100.
2. P. Fuentes-Prior and G. S. Salvesen, *Biochem. J.*, 2004, **384**, 201-232.
3. U. Fischer and K. Schulze-Osthoff, *Pharmacol. Rev.*, 2005, **57**, 187-215.
4. D. J. Fernandez and M. Lamkanfi, *Biol. Chem.*, 2015, **396**, 193-203.
5. S. Winkler and A. Rosen-Wolff, *Semin. Immunopathol.*, 2015, **37**, 419-427.
6. S. H. MacKenzie, J. L. Schipper and A. C. Clark, *Curr. Opin. Drug Discovery Dev.*, 2010, **13**, 568-576.
7. P. Galatsis, B. Caprathe, J. Gilmore, A. Thomas, K. Linn, S. Sheehan, W. Harter, C. Kostlan, E. Lunney, C. Stankovic, J. Rubin, K. Brady, H. Allen and R. Talanian, *Bioorg. Med. Chem. Lett.*, 2010, **20**, 5184-5190.
8. J. C. R. Randle, M. W. Harding, G. Ku, M. Schonharting and R. Kurrle, *Expert Opin. Invest. Drugs*, 2001, **10**, 1207-1209.
9. J. H. Stack, K. Beaumont, P. D. Larsen, K. S. Straley, G. W. Henkel, J. C. R. Randle and H. M. Hoffman, *J. Immunol.*, 2005, **175**, 2630-2634.
10. M. Maroso, S. Balosso, T. Ravizza, V. Iori, C. I. Wright, J. French and A. Vezzani, *Neurotherapeutics*, 2011, **8**, 304-315.
11. G. Doitsh, N. L. K. Galloway, X. Geng, Z. Y. Yang, K. M. Monroe, O. Zepeda, P. W. Hunt, H. Hatano, S. Sowinski, I. Munoz-Arias and W. C. Greene, *Nature*, 2014, **505**, 509-+.
12. N. L. K. Galloway, G. Doitsh, K. M. Monroe, Z. Y. Yang, I. Munoz-Arias, D. N. Levy and W. C. Greene, *Cell Rep.*, 2015, **12**, 1555-1563.
13. Y. Zhang and Y. Zheng, *Clin. Exp. Rheumatol.*, 2016, **34**, 111-118.
14. M. Poreba, A. Szalek, P. Kasperkiewicz, W. Rut, G. S. Salvesen and M. Drag, *Chem. Rev.*, 2015, **115**, 12546-12629.
15. C. J. Vickers, G. E. Gonzalez-Paez, K. M. Litwin, J. C. Umotoy, E. A. Coutsias and D. W. Wolan, *ACS Chem. Biol.*, 2014, **9**, 2194-2198.
16. C. J. Vickers, G. E. Gonzalez-Paez and D. W. Wolan, *ACS Chem. Biol.*, 2013, **8**, 1558-1566.
17. C. J. Vickers, G. E. Gonzalez-Paez and D. W. Wolan, *J. Am. Chem. Soc.*, 2013, **135**, 12869-12876.
18. R. Ganesan, S. Jelakovic, A. J. Campbell, Z. Z. Li, J. L. Asgian, J. C. Powers and M. G. Grutter, *Biochemistry*, 2006, **45**, 9059-9067.
19. N. A. Meanwell, *J. Med. Chem.*, 2011, **54**, 2529-2591.
20. C. Ballatore, D. M. Huryn and A. B. Smith, *ChemMedChem*, 2013, **8**, 385-395.
21. C. V. C. Prasad, C. P. Prouty, D. Hoyer, T. M. Ross, J. M. Salvino, M. Awad, T. L. Graybill, S. J. Schmidt, I. Kelly Osifo, R. E. Dolle, C. T. Helaszek, R. E. Miller and M. A. Ator, *Bioorg. Med. Chem. Lett.*, 1995, **5**, 315-318.
22. M. B. Boxer, A. M. Quinn, M. Shen, A. Jadhav, W. Leister, A. Simeonov, D. S. Auld and C. J. Thomas, *ChemMedChem*, 2010, **5**, 730-738.
23. Y. Okamoto, H. Anan, E. Nakai, K. Morihira, Y. Yonetoku, H. Kurihara, H. Sakashita, Y. Terai, M. Takeuchi, T. Shibanuma and Y. Isomura, *Chem. Pharm. Bull.*, 1999, **47**, 11-21.
24. Y. K. Yee, P. R. Bernstein, E. J. Adams, F. J. Brown, L. A. Cronk, K. C. Hebbel, E. P. Vacek, R. D. Krell and D. W. Snyder, *J. Med. Chem.*, 1990, **33**, 2437-2451.
25. D. E. Uehling, K. H. Donaldson, D. N. Deaton, C. E. Hyman, E. E. Sugg, D. G. Barrett, R. G. Hughes, B. Reitter, K. K. Adkison, M. E. Lancaster, F. Lee, R. Hart, M. A. Paulik, B. W. Sherman, T. True and C. Cowan, *J. Med. Chem.*, 2002, **45**, 567-583.
26. P. W. Glunz, X. J. Zhang, Y. Zou, I. Delucca, A. H. Nirschl, X. H. Cheng, C. A. Weigelt, D. L. Cheney, A. Z. Wei, R. Anumula, J. M. Luettgen, A. R. Rendina, M. Harpel, G. Luo, R. Knabb, P. C. Wong, R. R. Wexler and E. S. Priestley, *Bioorg. Med. Chem. Lett.*, 2013, **23**, 5244-5248.

27. N. F. Pelz, Z. G. Bian, B. Zhao, S. Shaw, J. C. Tarr, J. Belmar, C. Gregg, D. V. Camper, C. M. Goodwin, A. L. Arnold, J. L. Sensintaffar, A. Friberg, O. W. Rossanese, T. Lee, E. T. Olejniczak and S. W. Fesik, *J. Med. Chem.*, 2016, **59**, 2054-2066.
28. S. M. Zhenodarova, *Russ. Chem. Rev.*, 2010, **79**, 119-143.
29. F. F. Fleming, L. H. Yao, P. C. Ravikumar, L. Funk and B. C. Shook, *J. Med. Chem.*, 2010, **53**, 7902-7917.
30. L. J. Scott, *Drugs*, 2011, **71**, 611-624.
31. H. G. Bone, D. W. Dempster, J. A. Eisman, S. L. Greenspan, M. R. McClung, T. Nakamura, S. Papapoulos, W. J. Shih, A. Rybak-Feiglin, A. C. Santora, N. Verbruggen, A. T. Leung and A. Lombardi, *Osteoporosis Int.*, 2015, **26**, 699-712.
32. B. T. Fahr, T. O'Brien, P. Pham, N. D. Waal, S. Baskaran, B. C. Raimundo, J. W. Lam, M. M. Sopko, H. E. Purkey and M. J. Romanowski, *Bioorg. Med. Chem. Lett.*, 2006, **16**, 559-562.
33. *US Pat.,* US 2002/0137686 A1, 2002.
34. T. L. Graybill, R. E. Dolle, C. T. Helaszek, R. E. Miller and M. A. Ator, *Int. J. Pept. Protein Res.*, 1994, **44**, 173-182.
35. H. Roschitzki-Voser, T. Schroeder, E. D. Lenherr, F. Frolich, A. Schweizer, M. Donepudi, R. Ganesan, P. R. E. Mittl, A. Baici and M. G. Grutter, *Protein Expression Purif.*, 2012, **84**, 236-246.
36. W. Wannamaker, R. Davies, M. Namchuk, J. Pollard, P. Ford, G. Ku, C. Decker, P. Charifson, P. Weber, U. A. Germann, K. Kuida and J. C. Randle, *J. Pharmacol. Exp. Ther.*, 2007, **321**, 509-516.
37. D. S. Karanewsky, X. Bai, S. D. Linton, J. F. Krebs, J. Wu, B. Pham and K. J. Tomaselli, *Bioorg. Med. Chem. Lett.*, 1998, **8**, 2757-2762.
38. M. Poreba, A. Strozyk, G. S. Salvesen and M. Drag, *Cold Spring Harbor Perspect. Biol.*, 2013, **5**, a008680.
39. S. Roy, J. R. Sharom, C. Houde, T. P. Loisel, J. P. Vaillancourt, W. Shao, M. Saleh and D. W. Nicholson, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 4133-4138.
40. C. G. Wermuth, *J. Org. Chem.*, 1979, **44**, 2406-2408.
41. B. Simoneau and P. Brassard, *Tetrahedron*, 1988, **44**, 1015-1022.
42. D. Maclean, R. Hale and M. Y. Chen, *Org. Lett.*, 2001, **3**, 2977-2980.
43. K. Beaumont, K. Dack, L. Gardner, R. Webster and D. Smith, *Drug Metab. Rev.*, 2003, **35**, 58-58.
44. J. D. Larsen and H. Bundgaard, *Int. J. Pharm.*, 1987, **37**, 87-95.
45. J. J. Talley, S. R. Bertenshaw, D. L. Brown, J. S. Carter, M. J. Graneto, M. S. Kellogg, C. M. Koboldt, J. H. Yuan, Y. Y. Zhang and K. Seibert, *J. Med. Chem.*, 2000, **43**, 1661-1663.
46. J. D. Larsen, H. Bundgaard and V. H. L. Lee, *Int. J. Pharm.*, 1988, **47**, 103-110.
47. S. L. Huang, P. J. Connolly, R. H. Lin, S. Emanuel and S. A. Middleton, *Bioorg. Med. Chem. Lett.*, 2006, **16**, 3639-3641.
48. L. J. Scott, *Drugs*, 2016, **76**, 413-418.
49. T. Asaki, K. Kuwano, K. Morrison, J. Gatfield, T. Hamamoto and M. Clozel, *J. Med. Chem.*, 2015, **58**, 7128-7137.
50. L. Vande Walle, N. Van Opdenbosch, P. Jacques, A. Fossoul, E. Verheugen, P. Vogel, R. Beyaert, D. Elewaut, T. D. Kanneganti, G. van Loo and M. Lamkanfi, *Nature*, 2014, **512**, 69-73.