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Original article

Synthesis and evaluation of vinyl sulfones as caspase-3 inhibitors. A structure–activity study

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ABSTRACT

The first structure–activity relationship study of vinyl sulfones as caspase-3 inhibitors is reported. A series of 12 vinyl sulfones was synthesized and evaluated for two downstream caspases (caspases-3 and -7). Dipeptidyl derivatives were significantly superior to their counterparts containing only Asp at P_1, as caspase-3 inhibitors. Fmoc-Val-Asp-*trans*-CH=CH–SO_2Me was the most potent inhibitor of caspase-3 in the series, with a IC_{50} of 29 μM and a second-order rate constant of inactivation, k_{inact}/K_i, of 1.5 M^{-1} s^{-1}. Computational studies suggest that the second amino acid occupies position S_3 of the enzyme. In addition, Fmoc-Val-Asp-*trans*-CH=CH–SO_2Ph was inactive for caspase-7 for the tested concentrations.

1. Introduction

Caspases are a family of cysteine endoproteases that are involved in cytokine maturation and apoptosis [1–3]. Excessive neuronal apoptosis leads to a variety of diseases such as stroke, Alzheimer's disease, Huntington's disease and Parkinson's disease [4,5]. In consequence, caspases are recognized as novel therapeutic targets for central nervous diseases in which cell death occurs mainly by an apoptosis mechanism.

One type of irreversible cysteine proteases inhibitors that has received special attention in the last few years are the ones based on Michael acceptor scaffolds. This class of inhibitors includes vinyl sulfones, which have been developed as highly potent inhibitors of many clan CA cysteine proteases including papain, cathepsins B, L, S, and K, calpains, and cruzain [6]. In contrast, there are almost no reports of vinyl sulfones as inhibitors of cysteine proteases from the clan CD, to which caspases belong to (e.g. VSB-C11, Fig. 1) [7]. The lack of adequate activity–structure relationships regarding caspase inhibition by vinyl sulfones has precluded the optimization of this scaffold.

One of the most striking features of caspases is their stringency for Asp at the P_1 residue. For example, the DEVD and LETD sequences are optimal sequences for caspase-3 and caspase-8, respectively [1]. However, a truncated sequence (e.g. AD or VD) is generally enough to obtain selective and potent inhibitors of caspases by coupling a reactive functionality to these recognition moieties (e.g. activated ketones) [8,9]. With this in mind, vinyl sulfones **3a–d** and **6a–h** (Scheme 1) containing Asp at P_1 were designed to bind in the S_1 and S_2 subsites of the enzyme.

2. Results and discussion

2.1. Chemistry

Vinyl sulfones **3a–d** derivatized with N-protected aspartic acid were prepared by Horner–Wadsworth–Emmons condensation reaction. Aldehydes **1a–b** were obtained from the appropriate *N*-Cbz and *N*-Fmoc aspartic acids using Weinreb chemistry [10]. Acid deprotection with TFA afforded compounds **3a–d** with yields of 32–56% from the correspondent aldehydes (Scheme 1). To improve the potency of the inhibitors, we decided to extend the recognition structure by incorporating a second amino acid. The side chains at the R^2 position were chosen based upon literature precedent, which suggest that Ala and Val are preferred at the S_2 subsite. In order to compare the effect on caspase-3 inhibition, a third amino acid, Ile, was also used. The first approach to obtain the dipeptidyl vinyl sulfones evolved the deprotection of the nitrogen atom of the vinyl sulfones **2**, in order to couple the second

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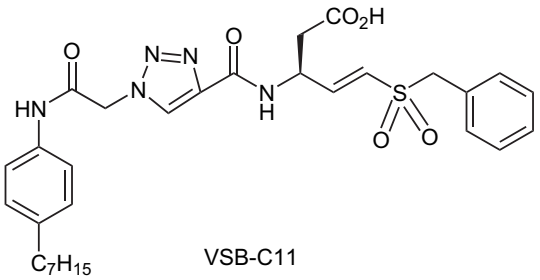


Fig. 1. Vinyl sulfone caspase inhibitor.

amino acid. However, deprotection of the Cbz group, in the presence of H_2/Pd, did not result. Instead, the double bond of the Michael acceptor was reduced.

Deprotection of the Fmoc group using several different basic conditions (piperidine, triethylamine, DBU) was also not successful leading to yields of 4% of unprotected product. We then decided to use as starting material the dipeptidyl aldehydes **4a**–**d** synthesized from the correspondent dipeptide acids [11] using Weinreb chemistry [10]. Deprotection of vinyl sulfones **5a**–**h** with TFA, afforded vinyl sulfones **6a**–**h** with yields of 34–75% from the correspondent aldehydes (Scheme 1).

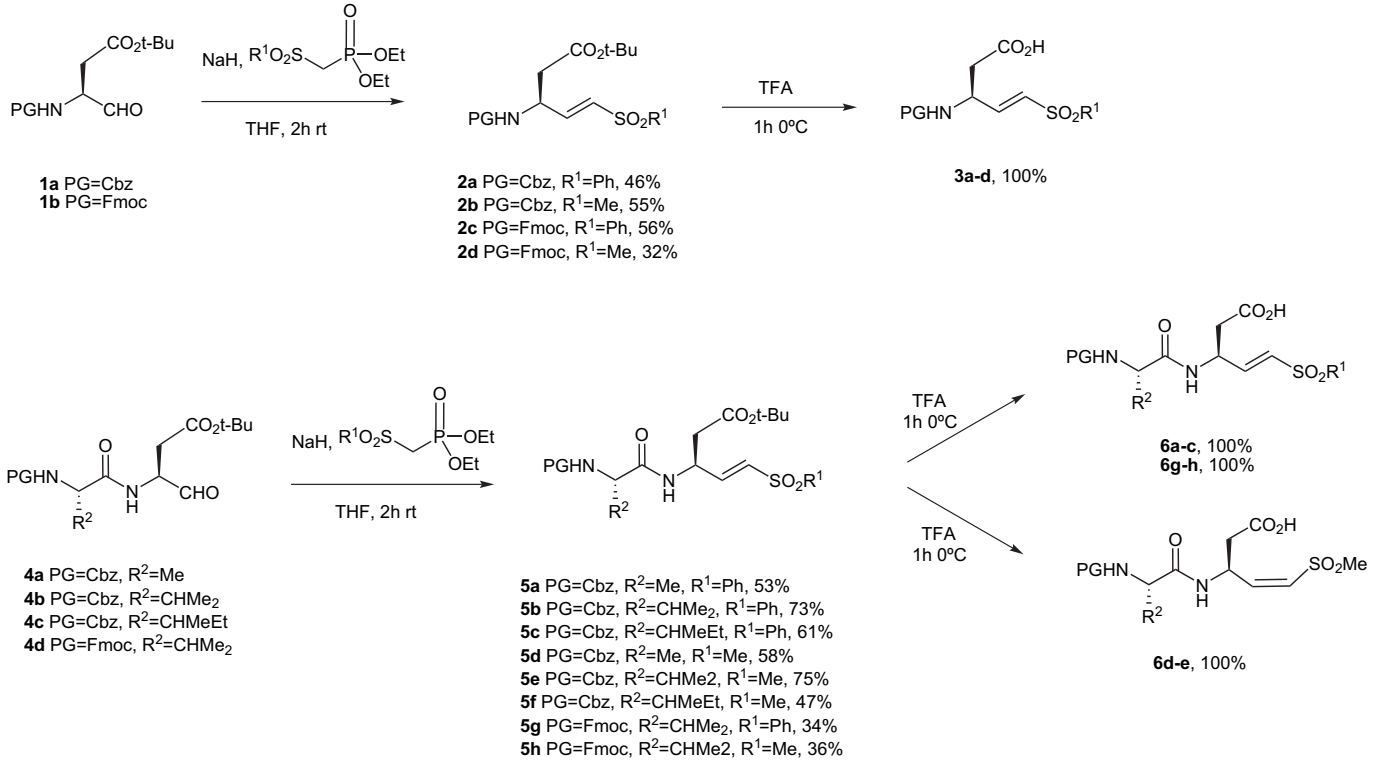
All of the proposed structures were established by NMR (^{1}H, ^{13}C, COSY and HMQC), IR, and MS. The stereochemistry around the double bond was established using the corresponding ^{1}H NMR coupling constant. A double doublet (J = 4.0, 15.0 Hz) and a doublet (J = 15.0 Hz) or double doublet (J = 1.5, 15.0 Hz) at δ 6.5–7.0 ppm are observed for the vinyl sulfones **3a**–**d**, **6a**–**c** and **6f**–**h** confirming the presence of *E* isomer. Surprisingly, deprotection with TFA of *N*-Cbz methyl vinyl sulfones **5e**–**f** led to isomerization of the double bond, as vinyl sulfones **6e**–**f** show a singlet for 2 protons at δ 6.8 ppm. Interestingly, deprotection of the Cbz-Ala and Cbz-Val methyl vinyl sulfones **5d**–**e** led only to the formation of the *Z* isomer, while Cbz-Ile methyl vinyl sulfone **5f** led to a mixture of *Z*/*E* isomers in a 1:3 ratio.

2.2. Biological activity

The vinyl sulfones synthesized were examined for their ability to inhibit the activity of recombinant human effector caspases-3 and -7. The IC_{50} values were determined for compounds **3a**–**d** and **6a**–**h** using a fluorimetric assay. The tetrapeptidyl aldehyde Ac-DEVD-CHO was used as positive control. All vinyl sulfones displayed IC_{50} values in the μM range against caspase-3 (Table 1). These values are in the same range of caspase-7 inhibition observed for the vinyl sulfone VSB-C11 [7]. In contrast, compounds **3** and **6** showed to be inactive against caspase-7, thus indicating that our vinyl sulfones are selective for caspase-3.

Inspection of the data in Table 1, allows the following observations to be made:

1. For inhibitors containing only the P_1 residue (i.e. Asp), the presence of a more bulky protective group, such as Fmoc seems to improve the inhibitory activity against caspase-3 (e.g. Fmoc vinyl sulfones **3c** and **3d** versus their Cbz-protected counterparts **3a** and **3b**). The same trend was observed for the dipeptidyl vinyl sulfones (vinyl sulfones **6g**–**h** versus compounds **6a**–**f**).
2. The presence of a P_2 amino acid residue, i.e. vinyl sulfones **6a**–**h**, improves, but not dramatically, the inhibitory activity against caspase-3, when compared to the compounds in which the P_2 residue is lacking, i.e. **3a**–**d**. For example, the inhibitory activity of the *N*-Cbz-Ile-Asp vinyl sulfones **6c** and **6f** are *ca* 2- and 4-fold higher than that of its truncated counterpart, the *N*-Cbz-Asp vinyl sulfone **3a** and **3b**, respectively, while the



Scheme 1. Synthesis of vinyl sulfones **3a**–**d** and **6a**–**h**.

Table 1
Inhibition of human caspases-3 and -7 by vinyl sulfones [PG-N-X-CH=CHSO$_2$R].

Compound	PG	X	R	IC$_{50}$ (μM)	
				Caspase-3	Caspase-7
3a	Cbz	Asp	Ph	453.0 ± 78.4	ND
3b	Cbz	Asp	Me	434.6 ± 92.3	ND
3c	Fmoc	Asp	Ph	228.0 ± 64.4	>200
3d	Fmoc	Asp	Me	163.2 ± 41.1	>200
6a	Cbz	Ala–Asp	Ph	343.0 ± 65.6	ND
6b	Cbz	Val–Asp	Ph	387.9 ± 4.1	ND
6c	Cbz	Ile–Asp	Ph	185.4 ± 17.5	>200
6d	Cbz	Ala–Asp	Me	655.8 ± 204.6	ND
6e	Cbz	Val–Asp	Me	133.4 ± 27.5	>200
6f	Cbz	Ile–Asp	Me	94.6 ± 29.5	>200
6g	Fmoc	Val–Asp	Ph	69.1 ± 8.1	>200
6h	Fmoc	Val–Asp	Me	28.8 ± 3.5	>200
Ac-DEVD-CHO	–	–	–	1.6 ± 0.2 nM	59.1 ± 6.0 nM

ND = Not done.

N-Fmoc dipeptidyl vinyl sulfones **6g** and **6h** are *ca* 3- and 6-fold more active than their *N*-Fmoc-Asp vinyl sulfone **3c** and **3d** counterparts, respectively.

3. The order of inhibitory activity against caspase-3 depends on the nature of the P$_2$ residue and varies in the order Ile > Val > Ala.
4. Except for compound **6d**, all the dipeptidyl methyl vinyl sulfones present better activity against caspase-3 when compared to the related phenyl vinyl sulfones.

The inhibitory activity of compound 6h towards caspase-3 was also determined using the progress curve method [12]. The pseudo-first-order rate constants, k_{obs}, varied linearly with inhibitor concentration, yielding a second-order rate constant of inactivation, k_{inact}/K_i, of 1.51 M^{-1} s^{-1} for **6h**. Also, the addition of excess substrate 30 min after **6h** addition did not result in an increase in activity (Fig. 2), indicating that vinyl sulfone **6h** acts irreversibly.

The value of k_{inact}/K_i obtained for **6h** is *ca* 100–5000 times smaller than those reported for other dipeptidyl Michael acceptors containing vinyl amide or ester scaffolds. For example, the aza-peptides Cbz-Val-AAsp-*trans*-CH=CH–CONHCH$_2$Ph and Cbz-Val-AAsp-*trans*-CH=CH–CO$_2$Et inhibit caspase-3 with second-order rate constants of 185 and 8000 M^{-1} s^{-1}, respectively [13]. This result is quite surprising as vinyl sulfones are *ca*. 2–10 times more reactive towards nucleophiles than vinyl esters [14,15]. However, the poor to modest inhibition of caspases displayed by vinyl sulfones **3** and **6** is line to that reported for VSB-C11 (Fig. 1), and may reflect a lower intrinsinc reactivity of vinyl sulfones containing Asp at P$_1$ when compared to related systems based on other amino acids.

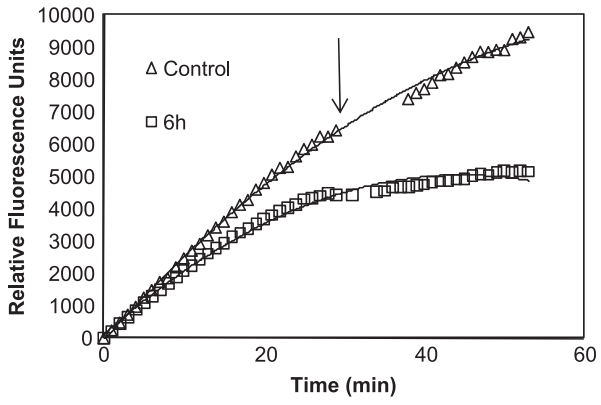


Fig. 2. Progress curves from competition experiments with **6h**. Standard caspase-3 enzyme assays were run in the presence or absence of 6h (300 μM), and the activity was determined as a function of assay time. Substrate was added at 10 μM, an excess of substrate (100 μM) was added 30 min after the assay was initiated, in order to determine whether the inhibition was reversible. The figure shown is a representative example of two experiments.

2.3. Docking studies

To get some insight to the binding mode of vinyl sulfones, compounds **3a**–**d** and **6a**–**h** were docked into the active site of caspase-3 using a covalent constraint as implemented in Gold 3.2 software [16] through a covalent bond between the vinyl β-carbon and the sulphur atom of the Cys285 thiol group of the enzyme. Each pose was ranked according to its ASP scoring function [17] result. The top ranking solutions were visually inspected and for each ligand the highest scoring pose was chosen as the binding pose. The aspartyl side chain at P$_1$ is involved in a series of polar interactions with the side chains of Arg179, Gln283 and Arg341 residues, i.e. similarly to the aldehyde Ac-DEVD-CHO inhibitor (Fig. 3). But quite surprisingly, all ligands **3a**–**d** and **6a**–**h** present the Cbz and Fmoc groups occupying partially the S$_2$ groove. In particular, the Fmoc-protected compounds **6g**–**h** present additional π–π stacking interactions with the benzyl ring of Phe381H (Fig. 3). Moreover, the *N*-terminal amino acid residue of **6a**–**h**, presents the corresponding side chain pointing towards Arg341 and Ser339, rather than towards the S$_2$ groove, as observed for Ac-DEVD-CHO. This modeling study indicates that the second residue is interacting with the S$_3$ pocket rather than with the S$_2$ site, which might explain, at least in part, the moderate activity displayed by vinyl sulfones **6a**–**h**.

3. Conclusion

In conclusion, vinyl sulfones containing Asp at P$_1$ were shown to be only moderate irreversible inhibitors of caspase-3, with IC$_{50}$ values in the μM range. Dipeptidyl vinyl sulfones displayed improved activity over their counterparts containing Asp as the

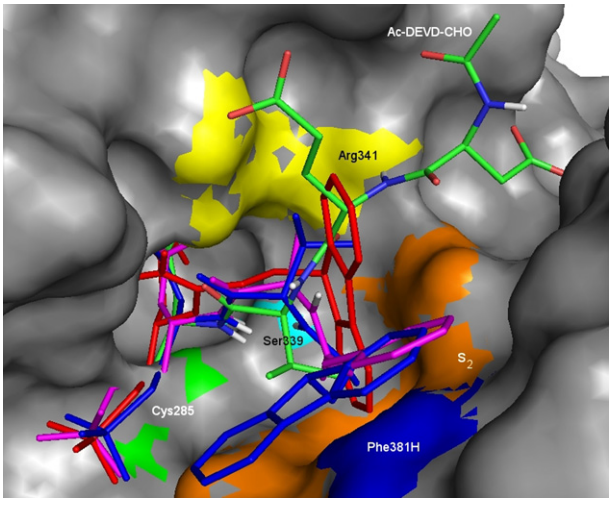


Fig. 3. Caspase-3 binding groove displaying the co-crystallized Ac-DEVD-CHO and inhibitors **3d** (red), **6d** (magenta) and **6h** (blue). Polar hydrogens are displayed for all molecules in white. The orange and blue region identifies the S$_2$ subsite, the yellow marks the location of the Arg341, the green marks the location of Cys285 and the cyan marks the location of Ser339. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

single amino acid residue, with this effect being particularly noticeably for the Fmoc-protected compounds. Importantly, methyl sulfones are preferred over phenyl sulfones, which agree with Goode's findings that phenyl ketone is less potent than aliphatic ketone caspase-3 inhibitors [18]. The most active compound, Fmoc-VD-VSMe, **6h**, presented an IC_{50} value of 29 μM. We extended these enzyme-inhibition studies by testing the ability of the most active vinyl sulfones **3** and **6** to block another downstream enzyme critical in the execution of apoptosis, caspase-7. Vinyl sulfones **3** and **6** were inactive for the tested concentrations. These results indicate that our vinyl sulfones are selective for caspase-3.

The docking studies indicate that the Asp vinyl sulfone core may be suitable for optimal enzyme binding. Docking studies suggest that there is space to improve the caspase-3 inhibitory activity by modifying the *N*-terminal protecting group and introducing a dipeptide sequence that can improve interaction with S_3 subsite. Further efforts aimed at improving compound potency are under way and will be reported in due course.

4. Experimental protocols

4.1. Chemistry

All reagents and solvents were obtained from commercial suppliers and were used without further purification. Melting points were determined using a Kofler camera Bock monoscope M and are uncorrected. The infrared spectra were collected on a Nicolet Impact 400 FTIR infrared spectrophotometer. High resolution mass spectra (HMRS) were performed in Unidade de Espectrometria de Masas, Santiago de Compostela. Elemental analyses were carried out on a C. Erba Model 1106 (Elemental Analyzer for C, H and N) and the results are within ±0.4% of the theoretical values. Merck Silica Gel 60 F254 plates were used as analytical TLC; flash column chromatography was performed on Merck Silica Gel (200–400 mesh). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 Ultra-Shield (400 MHz). ^{1}H and ^{13}C chemical shifts are expressed in δ (ppm) referenced to the solvent used and the proton coupling constants (*J*) in hertz.

4.1.1. General procedure for the preparation of vinyl sulfones **2a–d** and **5a–h**

To a suspension of NaH 60% (1.8 mmol, 1.1 equiv) in THF (5 ml), at 0 °C, was added 1 eq. of the appropriate sulfone. The resulting solution was stirred at temperature room for 30 min. A solution of 1 eq. of the aldehyde in THF (16 ml) was added and stirred at room temperature for 2 h. The solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2. The organic solution was washed with brine, dried, and concentrated. The resulting residue was flash chromatographed and used directly for the next step.

4.1.2. General procedure for the preparation of vinyl sulfones **3a–d** and **6a–h**

Vinyl sulfones were treated with TFA at 0 °C for 1 h. TFA was removed under vacuum and the final products were recrystallized from ethyl acetate/hexane as white solids.

*4.1.2.1. CbzAspVSPh (**3a**).* Obtained in 100% yield. m.p. 81–82 °C. IR (cm^{-1}) 3349, 1721, 1528. ^{1}H NMR (400 Mhz, $CDCl_3$) δ 7.85 (2H, d, *J* = 8.0 Hz), 7.62 (t, 1H, *J* = 7.6 Hz), 7.52 (t, 2H, *J* = 7.6 Hz), 7.32 (m, 5H), 6.96 (dd, 1H, *J* = 15.2 Hz, *J* = 4.4 Hz, C*H*=CHSO$_2$Ph), 6.50 (d, 1H, *J* = 15.2 Hz, CH=C*H*SO$_2$Ph), 5.67 (d, 1H, *J* = 9.2 Hz), 5.06 (s, 2H), 4.80 (sl, 1H), 2.75 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.23, 156.01, 144.44, 139.45, 135.75, 133.93, 131.37, 129.55, 129.40, 128.99, 128.72, 128.53, 128.26, 127.79, 67.65, 47.99, 37.91. HRMS-EI: *m*/*z* calc $C_{19}H_{19}NO_6S$ (M^+) 389.0933, found 389.0930.

*4.1.2.2. CbzAspVSMe (**3b**).* Obtained in 90% yield. m.p. 106–107 °C. IR (cm^{-1}) 3327, 1784, 1695, 1525. ^{1}H NMR (400 Mhz, MeOD) δ 7.36 (m, 5H), 6.89 (dd, 1H, *J* = 15.1 Hz, *J* = 4.8 Hz, C*H*=CHSO$_2$Me), 6.67 (dd, 1H, *J* = 15.1 Hz, *J* = 1.59 Hz, CH=C*H*SO$_2$Me), 5.10 (s, 2H), 4.79–4.75 (m, 1H), 2.94 (s, 3H), 2.68 (m, 2H). ^{13}C NMR (100 MHz, MeOD) δ 173.40, 157.90, 147.30, 138.09, 131.48, 129.50, 129.06, 128.87, 67.76, 49.74, 42.70, 38.87. HRMS-EI: *m*/*z* calc $C_{14}H_{17}NO_6S$ (M^+) 327.0777, found 327.0783.

*4.1.2.3. FmocAspVSPh (**3c**).* Obtained in 100% yield. m.p. 97–98 °C. IR (cm^{-1}) 3409, 1716, 1641, 1532. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.44 (sl, 1H), 7.88 (d, 2H, *J* = 7.5 Hz), 7.84 (d, 2H, *J* = 7.3 Hz), 7.74–7.62 (m, 6H), 7.41 (t, 2H, *J* = 7.3 Hz), 7.29 (t, 2H, *J* = 7.3 Hz), 6.92 (dd, 1H, *J* = 15.4 Hz, *J* = 4.6 Hz, C*H*=CHSO$_2$Ph), 6.65 (d, 1H, *J* = 15.4 Hz, CH=C*H*SO$_2$Ph), 4.59 (sl, 1H), 4.34–4.26 (m, 2H), 4.19 (t, 1H, *J* = 6.7 Hz), 2.65 (dd, 1H, *J* = 16.1 Hz, *J* = 5.3 Hz), 2.51 (dd, 1H, *J* = 16.1Hz, *J* = 5.3 Hz). ^{13}C NMR (100 MHZ, DMSO-d_6) δ 171.35, 155.36, 146.22, 143.89, 143.69, 140.75, 133.76, 130.04, 129.66, 127.68, 127.21, 127.09, 125.17, 120.17, 65.51, 48.24, 46.67, 37. HRMS-EI: *m*/*z* calc $C_{26}H_{23}NO_6S$ (M^+) 477.1246, found 477.1244.

*4.1.2.4. FmocAspVSMe(**3d**).* Obtained in 88% yield. m.p. 127–128 °C. IR (cm^{-1}) 3327, 1729, 1689, 1539. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.46 (sl, 1H), 7.90 (d, 2H, *J* = 7.3 Hz), 7.73–7.69 (m, 3H), 7.42 (t, 2H, *J* = 7.3 Hz), 7.34 (t, 2H, *J* = 7.3 Hz), 6.74 (dd, 1H, *J* = 14.9 Hz, *J* = 4.5 Hz, C*H*=CHSO$_2$Me), 6.65 (d, 1H, *J* = 16.1 Hz, CH=C*H*SO$_2$Me), 4.59 (sl, 1H), 4.33 (d, 2H, *J* = 7.0 Hz), 4.23 (t, 1H, *J* = 8.0 Hz), 3.00 (s, 3H), 2.64 (dd, 1H, *J* = 16.0 Hz, *J* = 6.0 Hz), 2.52 (dd, 1H, *J* = 16.0 Hz, *J* = 6.0 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.42, 155.40, 145.20, 143.90, 143.78, 140.78, 130.30, 127.71, 127.14, 125.23, 120.21, 65.61, 48.16, 46.72, 42.21, 36.99. Anal. Cald for $C_{21}H_{21}NO_6S$: C, 60.71; H, 5.09; N, 3.37; S 7.72. Found: C, 60.36; H, 5.39; N, 3.36; S 7.37.

*4.1.2.5. CbzAlaAspVSPh(**6a**).* Obtained in 95% yield. m.p. 102–103 °C. IR (cm^{-1}) 3340, 1723, 1675, 1525. ^{1}H NMR (400 MHz, MeOD) δ 7.89 (d, 2H, *J* = 7.7 Hz), 7.65–7.53 (m, 3H), 7.33 (m, 5H), 6.99 (dd, 1H, *J* = 15 Hz, *J* = 3.6 Hz, C*H*=CHSO$_2$Me), 6.89 (d, 1H, *J* = 15 Hz, CH=C*H*SO$_2$Me), 5.09–4.95 (m, 3H), 4.09–4.03 (m, 1H), 2.74 (dd, 1H, *J* = 16.9 Hz, *J* = 5.9 Hz), 2.61 (dd, 1H, *J* = 16.9 Hz, *J* = 8.1 Hz), 1.28 (d, 3H, *J* = 7.2 Hz). ^{13}C NMR (100 MHz, MeOD) δ 175.54, 173.45, 173.30, 158.19, 146.13, 141.71, 138.08, 134.75, 134.69, 132.54, 132.36, 130.54, 130.47, 129.47, 129.07, 129.00, 128.91, 128.65, 67.83, 67.70, 52.60, 52.13, 38.37, 28.25, 17.77. HRMS-EI: *m*/*z* calc $C_{22}H_{24}N_2O_7S$ (M^+) 460.1304, found 460.1307.

*4.1.2.6. CbzValAspVSPh (**6b**).* Obtained in 99% yield. m.p. 104–105 °C. IR (cm^{-1}) 3052, 1716, 1682, 1511. ^{1}H NMR (400 MHz, MeOD) δ 7.88 (d, 2H, *J* = 7.4 Hz), 7.67 (t, 1H, *J* = 7.4 Hz), 7.58 (t, 2H, *J* = 7.4 Hz), 7.36 (m, 5H), 6.97 (dd, 1H, *J* = 15.2 Hz, *J* = 4.5 Hz, C*H*=CHSO$_2$Ph), 6.70 (d, 1H, *J* = 14,9 Hz, CH=C*H*SO$_2$Me), 5.10–4.98 (m, 3H), 3.85 (d, 1H, *J* = 6.5 Hz), 2.72–2.70 (m, 2H), 2.06–1.97 (m, 1H), 0.89 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.84, 173.47, 158.73, 146.08, 141.47, 137.90, 134.84, 132.45, 130.58, 129.48, 129.24, 129.07, 128.94, 128.62, 67.89, 62.52, 38.40, 30.71, 19.67, 18.35. HRMS-EI: *m*/*z* calc $C_{24}H_{28}N_2O_7S$ (M^+) 488.1617, found 488.1615.

*4.1.2.7. CbzIleAspVSPh (**6c**).* Obtained in 100% yield. m.p. 150–151 °C. IR (cm^{-1}) 3244, 1722, 1648, 1539. ^{1}H NMR (400 MHz, MeOD) δ 7.88 (d, 2H, *J* = 7.1 Hz), 7.67 (t, 1H, *J* = 7.1 Hz), 7.59 (t, 2H, *J* = 7.1 Hz), 7.34–7.32 (m, 5H), 6.97 (dd, 1H, *J* = 15.8 Hz, *J* = 5.5 Hz, C*H*=CHSO$_2$Ph), 6.69 (d, 1H, *J* = 15.8 Hz, CH=C*H*SO$_2$Ph), 5.10–4.96 (m, 3H), 3.90 (d, 1H, *J* = 7.1 Hz), 2.75–2.68 (m, 2H), 1.81–1.72 (m, 1H), 1.51–1.41 (m, 1H),

1.20–1.10 (m, 1H), 0.86 (m, 6H). ^{13}C NMR (100 MHz, MeOD) δ 173.85, 173.31, 158.74, 146.09, 134.80, 132.55, 130.58, 130.50, 129.50, 129.08, 128.99, 128.69, 67.87, 61.59, 47.96, 38.30, 38.06, 25.83, 15.98, 11.63. HRMS-EI: *m/z* calc $C_{25}H_{30}N_2O_7S$ (M^+) 502.1774, found 502.1782.

*4.1.2.8. CbzAlaAspVSMe (**6d**).* Obtained in 97% yield. m.p. 144–145 °C. IR (cm^{-1}) 3409, 1708, 1668, 1518. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.44 (sl, 1H), 8.27 (d, 1H, J = 8.3 Hz), 7.54 (d, 1H, J = 6.7 Hz), 7.35 (m, 5H), 6.77 (s, 2H), 5.02 (d, 2H, J = 6.4 Hz), 4.81 (dd, 1H, J = 15.6 Hz, J = 6.4 Hz), 4.01 (t, 1H, J = 7.0 Hz), 2.96 (s, 3H), 2.68 (dd, 1H, J = 16.2 Hz, J = 6.0 Hz), 2.49 (dd, 1H, J = 16.2 Hz, J = 6.0 Hz), 1.19 (d, 3H, J = 7.3 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.29, 171.50, 155.87, 145.18, 137.01, 130.38, 128.42, 127.88, 127.83, 65.45, 50.21, 46.19, 42.27, 37.50, 17.83. HRMS-EI: *m/z* calc $C_{17}H_{22}N_2O_7S$ (M^+) 398.1148, found 398.1151.

*4.1.2.9. CbzValAspVSMe (**6e**).* Obtained in 99% yield. m.p. 157–158 °C. IR (cm^{-1}) 3052, 1709, 1682, 1648, 1525. ^{1}H NMR (400 MHz, MeOD) δ 7.35 (m, 5H), 6.88 (s, 2H, C*H*=C*H*SO_2Me), 5.10–5.03 (m, 3H), 3.81 (d, 1H, J = 7.9 Hz), 2.77 (dd, 1H, J = 16.5 Hz, J = 5.4 Hz), 2.58 (dd, 1H, J = 16.5 Hz, J = 8.6 Hz), 2.03–1.95 (m, 1H), 0.98 (d, 3H, J = 4 Hz), 0.96 (d, 3H, J = 4 Hz). ^{13}C NMR (100 MHz, MeOD) δ 174.29, 173.20, 158.71, 146.62, 138.27, 131.88, 129.50, 128.97, 128.74, 67.58, 62.81, 48.37, 42.71, 38.47, 31.36, 19.71, 19.21. HRMS-EI: *m/z* calc $C_{19}H_{26}N_2O_7S$ (M^+) 426.1461, found 426.1463.

*4.1.2.10. CbzIleAspVSMe (**6f**).* Obtained in 99% yield. m.p. 125–127 °C. IR (cm^{-1}) 3327, 3258, 1723, 1648, 1539. *E* isomer: ^{1}H NMR (400 MHz, MeOD) δ 7.37–7.32 (m, 5H), 6.87 (dd, 1H, J = 15.4 Hz, J = 4.4 Hz, C*H*=C*H*SO_2Me), 6.71 (d, 1H, J = 15.4 Hz, CH=C*H*SO_2Me), 5.11–5.09 (m, 2H), 5.00–4.98 (m, 1H), 3.88 (d, 1H, J = 8.9 Hz), 2.88 (s, 3H), 2.75–2.55 (m, 2H), 1.84–1.76 (m, 1H), 1.58–1.49 (m, 1H), 1.23–1.16 (m, 1H), 0.95–0.89 (m, 6H). ^{13}C NMR (100 MHz, MeOD) δ 173.97, 173.35, 158.81, 146.62, 138.10, 131.96, 129.52, 128.95, 67.87, 61.68, 47.99, 42.77, 38.05, 37.41, 25.87, 16.05, 11.63. HMRS-EI: *m/z* calc $C_{20}H_{28}N_2O_7S$ (M^+) 440.1617, found 440.1610.

*4.1.2.11. FmocValAspVSPh (**6g**).* Obtained in 91% yield. m.p. 108–109 °C. IR (cm^{-1}). 3314, 1740, 1666, 1516. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.56 (sl, 1H), 8.33 (d, 1H, J = 8 Hz), 7.93 (d, 2H, J = 8 Hz), 7.78 (m, 2H), 7.36 (m, 9H), 6.76 (dd, 1H, J = 16 Hz, J = 4 Hz, C*H*=C*H*SO_2Ph), 6.68 (d, 1H, J = 16 Hz, CH=C*H*SO_2Ph), 4.84 (sl, 1H), 4.32–4.25 (m, 3H), 3.88 (t, 1H, J = 4 Hz), 2,63 (m, 2H), 2.01 (sl, 1H), 0.88 (d, 6H, J = 8 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.34, 171.76, 160.73, 144.81, 140.94, 139.26, 136.77, 135.44, 133.05, 129.10, 128.65, 128.18, 127.43, 124.24, 122.82, 66.79, 59.11, 50.19, 48.05, 39.76, 30.48, 19.04. HRMS-ESI-TOF: *m/z* calc $C_{31}H_{32}N_2O_7SNa$ (M^+ + Na) 599.1825, found 599.1822.

*4.1.2.12. FmocValAspVSMe (**6h**).* Obtained in 93% yield. m.p. 93–94 °C. IR (cm^{-1}). 3291, 1746, 1700, 1660, 1522. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.53 (sl, 1H), 8.31 (d, 1H, J = 8 Hz), 7.90 (d, 2H, J = 8 Hz), 7.76 (m, 2H), 7.42–7.32 (m, 2H), 6.74 (dd, 1H, J = 16 Hz, J = 4 Hz, C*H*=C*H*SO_2Me), 6.66 (d, 1H, J = 16 Hz, CH=C*H*SO_2Me), 4.82 (sl, 1H), 4.30–4.22 (m, 3H), 3.85 (t, 1H, J = 4 Hz), 2.96 (s, 3H), 2.61 (m, 2H), 1.99 (sl, 1H), 0.85 (d, 6H, J = 8 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.54, 171.35, 160.78, 158.55, 144.86, 141.17, 139.26, 128.64, 127.43, 124.24, 122.84, 66.82, 59.11, 50.17, 48.06, 39.74, 37.99, 30.48, 19.04. HRMS-ESI-TOF: *m/z* calc $C_{26}H_{30}N_2O_7SNa$ (M^+ + Na) 537.1652, found 537.1666.

4.2. Pharmacology

4.2.1. Caspase-3 assays

Caspase-3 fluorimetric assay was used, which is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by caspase-3, resulting in the release of the fluorescent 7-amido-4-methylcoumarin (AMC) moiety. Briefly, 5 μL of appropriate dilution of caspase-3 (caspase-3, human, recombinant, Calbiochem) were added to 5 μL of the tested inhibitors at various concentrations. The reaction was initiated by the addition of 190 μL of substrate to a final concentration of 20 μM in assay buffer (20 mM HEPES, 2 mM EDTA, 0.1% CHAPS, and 5 mM DTT, pH 7.4). Liberation of AMC was monitored continuously at 37 °C using a Tecan infinite M200 (Tecan, Switzerland) 96-well plate reader (white plates from Greiner bio-one, Germany) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Inhibitors stock solutions were prepared in DMSO, and serial dilutions were made in DMSO. Controls were performed using enzyme alone, substrate alone, enzyme with DMSO and a positive control (Ac-DEVD-CHO, Calbiochem). IC_{50} values were determined by non-linear regression analysis. Progress curves, run in the absence and presence of compound, were used to determine the nature of inhibition. The reaction was initiated with 10 μM of substrate when the reaction attain a plateau, after 30 min, an excess substrate was added to final concentration 100 μM.

4.2.2. Caspase-7 assays

Caspase-7 kinetic assays were performed using the same conditions and the same substrate (Ac-DEVD-AMC, 2 mM stock solution in DMSO) of caspase-3 assays. The enzyme stock solution was 200 U/mL (final concentration in the well: 1 U) in the assay buffer.

4.3. Computational studies

Molecular docking studies were performed, using the GOLD software [16] (version 3.2) to predict the interactions and binding modes of all the synthesized inhibitors in the caspase-3 active site, and to evaluate their relative binding affinities. The 3D structure coordinates of caspase-3 were obtained after a search in the Protein Data Bank and electing the structure with code 1PAU with a resolution of 2.5 Å [19]. To prepare the enzyme for the docking studies, the Ac-DEVD-CHO inhibitor included in the 1PAU structure was removed, hydrogen atoms were added to caspase-3, crystallographic waters were removed and the protonation states were correctly assigned using the Protonate-3D tool within the Molecular Operating Environment (MOE) 2008.10 software package [20]. To evaluate and validate the effectiveness of our model system and the performance of the docking protocol, a set of studies was first performed using the co-crystallized inhibitor to reproduce the experimental binding pose. The best docking pose obtained was overlaid with the experimental crystal structure showing a minimal deviation of its atomic coordinates (RMSD = 0.82 Å). Since the validation study revealed that our model and software protocol could accurately reproduce the experimentally binding mode of the Ac-DEVD-CHO inhibitor, a series of synthesized vinyl sulfones were covalently docked into the caspase-3 binding site using the ASP fitness function [17].

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