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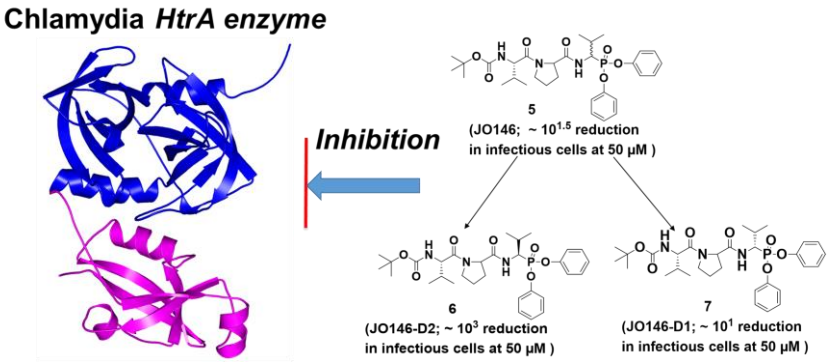

Stereochemical Basis for the Anti-Chlamydial Activity of the Phosphonate Protease Inhibitor JO146

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1. Introduction

Chlamydia is the world's most common sexually transmitted bacterial infection. The largely asymptomatic infection often leads to complications such as ectopic pregnancy, blindness, infertility, and pneumonia,[1-4] particularly in areas where there is limited access to quality healthcare. The ever increasing global prevalence of this infection[5] is a result (at least in part) of the inadequacies of present management options and necessitates the adoption of better strategies. Central to this need is the development of drugs with good resistance profiles.

Chlamydia are obligate intracellular bacterial pathogens, and here we focus on *Chlamydia (C.) trachomatis*. The *Chlamydia* high temperature requirement A (CtHtrA) protease is a multimeric, multidomain serine protease that is crucial for the survival and virulence of the *Chlamydia* pathogen.[6] Thus, it is an attractive target for small molecule therapeutics. JO146 [Boc-Val-Pro-Val^{P}(OPh)$_2$], a mixture of two diastereomers with *R*-/*S*-Val at P_1, was previously identified as a covalent inhibitor of CtHtrA (IC_{50} = 12.5 μM) in a screen against a library of 1090 serine protease inhibitors. The N-terminal Boc group is retained as a hydrophobic P4 mimic and is necessary for activity. Further evaluation in different biological models revealed its selective toxicity against human and koala pathogenic *Chlamydia* species,[6-7] presenting the molecule as a viable lead towards the development of anti-chlamydial drugs with organism specificity and a different mode of action from current antibiotics.

It is well known that diastereomers interact differently with biological systems and often produce different biological effects.[8-9] As a result, it has become mandatory to have diastereomers

properly investigated for potential therapeutic and toxicological variation in the course of drug development.[10] In this study, we report the synthesis of JO146, **5**, and the isolation and characterization of its two diastereomers JO146-D2, **6** and JO146-D1, **7** (Figure 1). Of the diastereomers, JO146-D2 **6** displayed the best inhibitory activity against human neutrophil elastase (HNE), and *C. trachomatis* cell culture, providing strong evidence that it is the diastereomer with the physiologically relevant valine residue. For peptidyl phosphonate compounds, the physiologically relevant L-amino acid at P_1 has an *R*-configuration (instead of the usual *S*-configuration) due to a substitution of a phosphonate group for the carbonyl, causing a reversal of the direction in assignment priority. ^{1}H NMR spectroscopic analysis of the change in chemical shift for protons of the P_2-proline and P_3-valine residue on **6** vs **7** indicated shielding/deshielding effects that may enable differentiation of diastereomeric mixtures of α-aminoalkylphosphonate diaryl/diphenyl esters in the future.

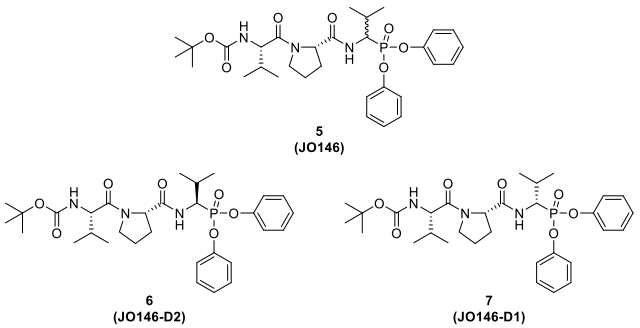


Figure 1. Structures of JO146 **5** and the isolated diastereomers **6** and **7**.

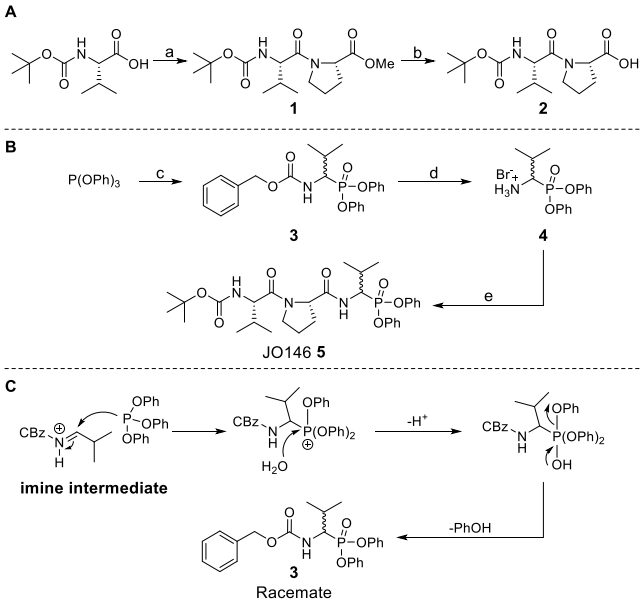
2. Results

2.1. Synthesis

The synthesis of JO146 **5** was carried out over five steps (Scheme 1A and Scheme 1B). Boc-Val-Pro-OH **2** was synthesized via saponification of Boc-Val-Pro-OMe **1**, which was formed by HBTU-promoted peptide coupling of Boc-Val-OH to Pro-OMe (Scheme 1A). Concurrently, racemic Val-phosphonate **4** (isolated as the HBr salt) was synthesized in two steps via an α-amidoalkylation-type reaction, followed by deprotection of the benzylcarbamate (CBz) group (Scheme 1B). Finally, HBTU-promoted coupling of **4** with **2** provided JO146 **5** (Scheme 1B) as a mixture of JO146-D1:JO146-D2 (46:54, See Table S1). The two diastereomers were isolated via silica gel column chromatography JO146-D2 (*R*-P_1-Val) **6** and JO146-D1 (*S*-P_1-Val) **7**, in sufficient amounts and purity (≥90%, Table S1 and HPLC traces in supporting information) for biological testing. The formation of the racemic CBz-protected Val-phosphonate **3** with *R/S* configuration at the residue, which becomes the P_1-Val, occurs during the α-amidoalkylation-type reaction. The condensation of isobutyraldehyde with benzylcarbamate in acetic acid generates an imine intermediate that permits both frontside and backside attack by the incoming nucleophilic triphenyl phosphite ($P(OPh)_3$),[11] thereby generating an enantiomeric mixture (Scheme 1C).

Analysis of 2D NMR experiments run on JO146 **5** (see supporting information), allowed for the complete assignment of the P_1 to P_3 residue protons on **6** and **7** in their respective 1D NMR spectra. The doublet in the ^{13}C NMR spectra centered at δ 51.3 and δ 51.1 for **6** and **7** respectively, had a ^{13}C-^{31}P coupling constant (*J*) of ~155 Hz, and was assigned as the P_1-valine alpha (α) carbon. Using the chemical shift of the P_1-valine α carbon and proton, we were able to assign the beta (β) and gamma (γ) protons of the P_1-valine, and subsequently, the protons of the P_3-valine. This was important, as it enabled us to establish the influence of stereochemistry at the P_1-valine on the proton chemical shifts of the P_2-proline and P_3-valine residues (*vide infra*).

Scheme 1. Synthesis of JO146 **5** (A and B) and mechanism of racemate formation (C).[a]



[a]Reagents and conditions: (a) Proline methyl ester, HBTU, DIEA, DMF, 25 °C, 20 h, 57%; (b) LiOH.H_2O, THF, H_2O, 25 °C, 2 h, 90%; (c) Benzyl carbamate, isobutyraldehyde, 80-90 °C, 2 h, 72%; (d) 33% HBr/AcOH, 25 °C, 20 h, 75%; (e) **2**, HBTU, DIEA, DMF, 25 °C, 20 h, 90%.

2.2. Protease activity

Next, a preliminary assessment of the bioactivity of the diastereomers **6** and **7** alongside JO146 **5** was carried out. The initial protease inhibition assays were performed against human neutrophil elastase (HNE), a human serine protease with similar substrate specificity as CtHtrA, and one that has been well studied as a target for phosphonate inhibitors.[12-14] The substrate specificity of HNE is largely dependent on the structural makeup of its S_1 substrate-binding pocket, and has been reported to consist mainly of hydrophobic residues[15] that enable it to bind preferentially to substrates with small hydrophobic amino acids such as valine and alanine at P_1.[16] The substrate specificity of CtHtrA is similar to this as it also preferentially cleaves substrates with small hydrophobic residues including Val, Pro, Ala and Ile at P_1.[17-18] As a result, activity against HNE would normally provide a good prediction for potential anti-CtHtrA activity. We therefore used HNE as a frame of reference. An *in vitro* off-target assessment of the inhibitors was also conducted using the abundant digestive serine proteases trypsin and chymotrypsin.

The results obtained from the HNE inhibition assay revealed JO146-D2 **6** to be ~2.5 – fold more potent than JO146-D1 **7** (Table 1). No significant protease inhibition of trypsin or chymotrypsin was observed for either isomer (Table 1, IC_{50} > 500 μM). Based on previous X-ray crystallography studies,[19-21] and those reported by Winiarski *et al.*,[14] which confirmed that the P_1-(*R*)-diastereomers of phosphonate inhibitors are better inactivators of HNE-type serine proteases, our HNE inhibition studies in conjunction with our molecular docking and cell-based *C. trachomatis* data (*vide infra*), suggest that JO146-D2 **6** is the isomer containing the (*R*)-valine at P_1.In an attempt to unequivocally confirm the stereochemical assignment of **6** and **7**, 2D-NOESY experiments were carried out (Figures S23 and S24). However, no distinct NOE correlations between the α-proton of

the P_1-valine and the neighbouring α-proton on the P_2-proline were observed. Any distinct NOE correlations between the β- and γ-protons of the P_1-valine and the P_2-P_3 residues were not evident, and as a result, stereochemical differentiation of both diastereomers could not be achieved using this technique. However, a comparison of the ^{1}H NMR spectra of **6, 7** and the *N*-Boc protected tripeptide *S,S,S*-Boc-Val-Pro-Val-OMe **8** with a C-terminal L-valine residue (Figures S14, S17 and S25), revealed close similarity between JO146-D2 **6** and **8**. While peaks corresponding to the stereogenic P_1-Val-αH, and P_2-Pro-αH resonated at the same approximate frequency (δ 4.78 – 4.69) in **7,** both peaks are clearly separated in **6** (δ 4.77 – 4.69 and δ 4.46) and **8** (δ 4.64 – 4.54 and δ 4.42), suggesting similar stereochemistry at P_1 for both of these structures. This further supports **6** as having the P_1-levorotatory valine (*R*-configuration) isomer.

Table 1. Protease Inhibition Data[a] for Compounds **5**, **6** & **7** against Human Neutrophil Elastase (HNE), Trypsin and Chymotrypsin

Compound	HNE IC_{50} (μM)	Chymotrypsin IC_{50} (μM)	Trypsin IC_{50} (μM)
5	0.83 ± 0.01	> 500	> 500
6	0.67 ± 0.01	> 500	> 500
7	1.65 ± 0.11	> 500	> 500

[a]Data are presented as the mean ± SEM of triplicate experiments.

2.3. Molecular modelling

To give an indication of the binding mode to CtHtrA which would be the desired target in the cell culture assays, diastereomers **6** and **7** were docked (GOLD v5.2 https://www.ccdc.cam.ac.uk/solutions/csd-discovery/components/gold/) into a homology model of CtHtrA based on the crystal structure of *Escherichia coli* DegP (PDB ID: 3OTP).[22-23] The ligands were prepared and docked as phosphonic acids without the aryl portion as formation of the covalent bond is accompanied by the loss of both phenoxy groups as observed in several crystal structures of phosphonate based inhibitors e.g. PDB IDs 4MVN, 3UFA, 1P12, 1CGH and 1MAX.[24-27] The highest ranked binding mode and interactions for both diastereomers were viewed and analyzed. Similar interactions were observed in both cases (Figure 2); individual residues of the inhibitors occupy the corresponding enzyme S_1-S_4 sub-pockets. JO146-D2 **6** was however found to be more stereochemically favored, interacting with the S_1 specificity pocket by virtue of its (*R*)-configuration, which enables the isopropyl group of valine to make significant van der Waals interactions with I242 of the pocket.[28] JO146-D1 **7** on the other hand had a weakened interaction at this point as the (*S*)-configuration orients the isopropyl group away from I242 (Figure 2). This structural difference is thought to be responsible for the observed difference in *in vitro* potency against HNE.

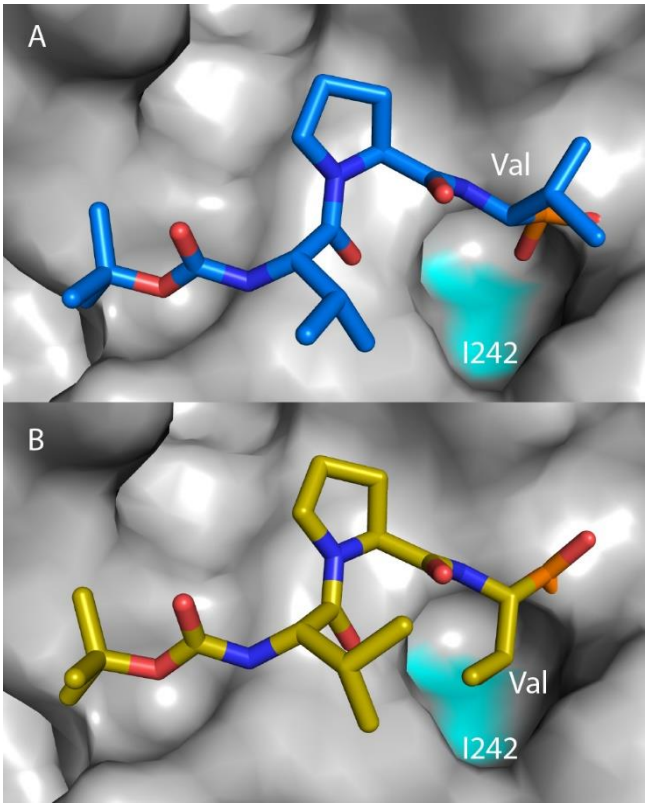


Figure 2. Binding poses of **7**, JO146-D1 (A) and **6**, JO146-D2 (B) docked into a homology model of CtHtrA (solid surface) showing the different orientations of the respective P_1 isopropyl group relative to the S_1 pocket binding I242 residue (cyan).

2.4. Cellular activity

Based on our HNE inhibition data and the docking experiments, we hypothesized that the stereochemical difference between the diastereomers **6** and **7** would affect the cellular activity of the inhibitors against *C. trachomatis*. JO146 **5** has been previously identified to be most effective when added to *C. trachomatis* cell culture at 16 h post infection (PI)[6] which corresponds to the mid-replicative phase of the *C. trachomatis* developmental cycle. When 10 μM of JO146 **5** was added at this time point, it was highly effective against *Chlamydia* cells with about 1.5 log loss of infectious progeny (inclusion forming units; IFU/mL). In the present study, treatment of the cell culture with 50 μM concentration of **5, 6** and **7** at 16 h PI, resulted in appreciable loss of infectious progeny that ranged between 10^1 and 10^3 relative to the negative DMSO control (Figure 3). JO146-D2 **6** was most effective with over 1000 – fold reduction in IFU/mL relative to DMSO, and approx. 100 – fold difference in activity compared to JO146-D1 **7** and JO146 **5** which had approximately 10^1 and $10^{1.5}$ reduction in IFU/mL, respectively. At a concentration of 10 μM, **7** had no detectable anti-chlamydial activity while **6** still produced a significant 10^1 reduction in IFU/mL (Figure 3).

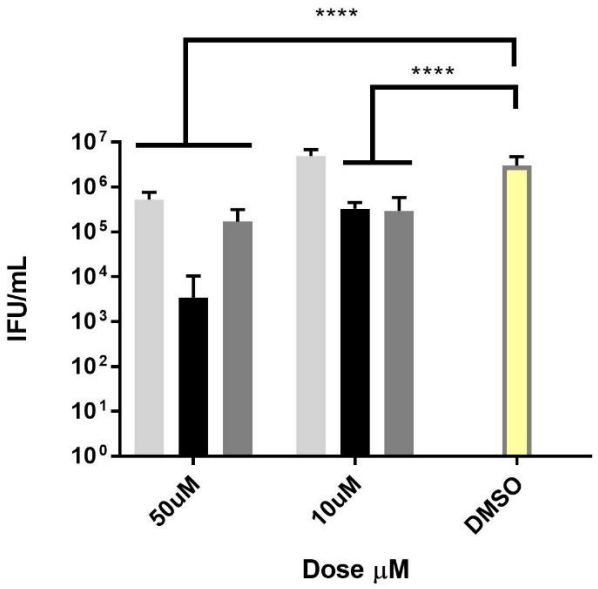


Figure 3. Inhibition of *C. trachomatis* by JO146 **5** and its diastereomers **6** and **7** during mid-replicative phase of the pathogen's developmental cycle. Infectious yield counts (IFU/mL) were determined upon completion of developmental cycle. Black bars are JO146-D2 **6**, light gray bars are JO146-D1 **7** and dark gray bars are the JO146 **5**. Error bars indicate SEM obtained from experimental replicates (minimum of 5). ****P < 0.0001

3. Discussion

While peptidyl α-aminoalkylphosphonate diaryl/diphenyl esters have been extensively studied as serine protease inhibitors, the absolute configurations of isomers obtained in previous studies were determined by X-ray crystallography .[19-20, 24] We did not obtain the absolute stereochemistry using these techniques, but our inhibition data, supported by previous HNE activity assays by Winiarski *et al.*[14] in which they identified the P_1-(*R*)-diastereomers for similar P_1-substituted tripeptide phosphonates as better inactivators of HNE compared to their P_1-(*S*)-counterparts, suggests that JO146-D2 **6** and JO146-D1 **7** contain the (*R*)-L-Val and (*S*)-D-Val at P_1, respectively. The better inhibition of HNE and CtHtrA by **6** is also supported by our molecular docking study (Figure 2). Upon careful inspection of the ^{1}H and ^{13}C NMR spectra for JO146 **5**, **6**, and **7**, we identified some key differences in the respective chemical shifts for the P_2-Pro and P_3-Val residues (Table 2). The most notable change was for the protons on the P_3-Val residue, which were relatively more shielded in the ^{1}H NMR spectrum of **7**. Also of note, and to the best of our knowledge, the ^{13}C NMR spectra of small peptidyl α-aminoalkylphosphonate diaryl/diphenyl esters has not been fully reported. Interestingly, the ^{13}C NMR for **6** and **7**, both contain eight doublets in the aromatic region (due to ^{13}C-^{31}P coupling), indicating that the two phenyl groups are not in an identical chemical space; i.e. they are diastereotopic, and as a result magnetically non-equivalent. While preliminary, the interpretation of the ^{1}H and ^{13}C NMR spectra suggests that the bulky P_1-valine residue and the P_2-proline residue could be playing a role in the observed $\Delta\delta^{RS}$ for P_2-Pro and P_3-Val on **6** and **7**. The observed chemical shift differences provide spatial information that may be useful for determining the absolute configurations of both molecules. The general application of this phenomenon could be further investigated as a potential method for rapid stereochemical identification of future inhibitors in the diphenyl/diaryl phosphonate series. By substituting the proline residue at P_2 with a non-cyclic amino acid and utilizing a less bulky P_1-substituent (e.g. alanine), it may be possible to determine if the shielding/deshielding effect at P_3 is due to the conformational restriction imposed by the residues at P_1 and P_2.

Table 2. Observed chemical shift (δ) differences for protons on residues P_2 and P_3 of JO146-D2 **6** and JO146-D1 **7**.

Residue/Proton	(*R*)-6 (ppm)	(*S*)-7 (ppm)	$\Delta\delta^{RS}$ (ppm)
P_2-Pro-αH	4.46 (dd)	4.74 (m)[a]	-0.28
P_2-Pro-δH	3.64 (m)[a]	3.71 (m)[a]	-0.07
	3.61(m)[a]	3.48 (m)[a]	+0.13
P_3-Val-αH	4.30 (dd)	4.24 (dd)	+0.06
P_3-Val-βH	2.01 (m)[a]	1.82 (m)[a]	+0.19
2 × P_3-Val-γH's	0.99 (d)	0.86 (d)	+0.13
	0.93 (d)	0.83 (d)	+0.10

[a]Centre of multiplet (and not range) reported.

4. Conclusion

In conclusion, significantly higher anti-chlamydial activity was observed for the P_1-Val levorotatory JO146-D2 **6** over the P_1-Val dextrorotary JO146-D1 **7**. Therefore, future inhibition assays in the series should be conducted with phosphonates having the physiologically relevant (*R*)-stereochemistry at the P_1 residue. Our data also suggest that phosphonate ester inhibitors have selectivity for HNE and the structurally related CtHtrA over abundantly available serine proteases (e.g. trypsin and chymotrypsin), an important feature as we develop our inhibitors for future *in vivo* pre-clinical studies.

5. Experimental section

5.1. General

All solvents and reagents were of reagent grade and used without further purification except where stated. All reactions were conducted in standard glassware and in air or under nitrogen gas unless otherwise stated. Organic solvent extracts were dried with $MgSO_4$ and subjected to rotary evaporation, and finally dried at 10^{-1} mbar using a high vacuum pump.

Analytical thin layer chromatography (TLC) was performed on Merck TLC aluminium plates coated with 0.2 mm silica gel 60 F_{254}. Spots were generally detected by UV and/or permanganate staining. Flash column chromatography was performed using silica gel 60 (0.040 – 0.063 mm, 200 – 400 mesh) with all solvent systems expressed as volume to volume (v/v) ratios. Solids were recrystallized from a minimum amount of hot solvent and collected by vacuum filtration.

High performance liquid chromatography (HPLC) was carried out on an Agilent HPLC. Samples of 20 µL volume were injected onto a Gemini 5 µm C18 110 Å column (250 by 4.6 mm, Phenomenex, New Zealand). The compounds were eluted using solvent A: 0.1% trifluoroacetic acid (TFA) in water and solvent B: 0.1% TFA in acetonitrile (ACN) in a linear gradient manner. At a flow rate of 1.0 mL/min, compounds were detected at the wavelengths of 254/210 nm.

Proton (^{1}H) and carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectra were acquired on Varian 400 or 500 MHz spectrometers. Samples were prepared in deuterated solvent, chloroform (δ 7.26, 77.16 ppm) with the respective ^{1}H and ^{13}C chemical shifts of the solvent shown in brackets. Chemical shifts (δ) are expressed in parts per million (ppm) and coupling constants (*J*) in Hertz (Hz), both measured against the internal standard.

Multiplicities are reported as either singlet (s), doublet (d), doublet of doublets (dd), triplet (t), quartet (q), multiplet (m) or broad signal (br). Compound **5** was fully characterized using two-dimensional NMR experiments, Homonuclear Correlation Spectroscopy (COSY), Heteronuclear Single Quantum Coherence (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC).

High resolution mass spectra were recorded on a Brucker microTOF-Q spectrometer with an electrospray ionization (ESI) source.

5.2. Synthetic Details

*5.2.1. Methyl(2S)-1-[(2S)-2-{[(tert-butoxy)carbonyl]amino}-3-methylbutanoyl]pyrrolidine-2-carboxylate (**1**)*

Compound **1** was synthesized using a modified literature procedure of solution-phase peptide coupling.[29] L-Proline methyl ester hydrochloride (0.605 g, 3.65 mmol) was dissolved in 5 mL dimethylformamide (DMF). To this was added Boc-Val-OH (0.833 g, 3.84 mmol), *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluoro-phosphate (HBTU) (1.662 g, 4.38 mmol) and *N,N*-Diisopropylethylamine (DIEA) (1.3 mL, 7.30 mmol). The reaction mixture was stirred under nitrogen overnight. The reaction was diluted with ethyl acetate (EtOAc; 30 mL) and an equal amount of sodium bicarbonate, washed with brine (6 × 30 mL), dried ($MgSO_4$) and concentrated. The crude product was chromatographed using flash silica gel column chromatography (Hexane/EtOAc, 7:3 v/v). This yielded **1** on vacuum concentration as a light yellow oil (0.723 g, 57%), which was spectroscopically identical to that previously reported.[29-30] ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.19 (d, *J* = 9.4 Hz, 1H, -NH), 4.54 – 4.48 (m, 1H, 1 × Pro-αH), 4.26 (dd, *J* = 9.3, 6.5 Hz, 1H, 1 × Val-αH), 3.80 – 3.73 (m, 1H, 1 × Pro-δH), 3.72 (s, 3H, OCH_3), 3.68 – 3.61 (m, 1H, 1 × Pro-δH), 2.25 – 2.14 (m, 1H, 1 × Pro-βH), 2.00 (m, 4H, 1 × Pro-βH, 2 × Pro-γH and 1 × Val-βH), 1.41 (s, 9H, ^{t}Butyl), 1.01 (dd, *J* = 6.8, 1.2 Hz, 3H, 3 × Val-γH), 0.92 (dd, *J* = 6.8, 1.2 Hz, 3H, 3 × Val-γH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.4, 171.2, 155.8, 79.4, 58.8, 56.8, 52.1, 47.1, 31.3, 29.0, 28.3, 25.0, 19.2, 17.3. ESI-MS (*m*/*z*): calcd for $C_{16}H_{28}N_2NaO_5$ (M + Na) *m*/*z* 351.1896 found 351.1877.

*5.2.2. (2S)-1-[(2S)-2-{[(tert-butoxy)carbonyl]amino}-3-methylbutanoyl]pyrrolidine-2-carboxylic acid (**2**)*

Compound **2** was produced by alkaline hydrolysis of **1** using a modified literature procedure.[31] **1** (1.372 g, 4.18 mmol) was dissolved in a 3:1 mixture of tetrahydrofuran (THF) and water (60 mL + 20 mL respectively). $LiOH.H_2O$ (0.878 g, 20.89 mmol) was added and the reaction mixture was stirred for 2 hours at room temperature. The reaction was quenched by acidification with 50 mL 0.5 M HCl, and the mixture was extracted with EtOAc (4 × 60 mL). The combined organic fraction was washed with brine (1 × 100 mL), dried over anhydrous $MgSO_4$ and the solvents evaporated under reduced pressure to yield a white solid (1.186 g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.37 (br s, 1H, carboxylic acid proton), 5.46 (d, *J* = 9.4 Hz, 1H, -NH), 4.55 (dd, *J* = 8.1, 4.8 Hz, 1H, 1 × Pro-αH), 4.26 (dd, *J* = 9.4, 6.8 Hz, 1H, 1 × Val-αH), 3.83 – 3.59 (m, 2H, 2 × Pro-δH), 2.25 – 1.86 (m, 5H, 2 × Pro-βH, 2 × Pro-γH and 1 × Val-βH,), 1.40 (s, 9H, ^{t}Butyl), 0.98 (d, *J* = 6.7 Hz, 3H, 3 × Val-γH), 0.91 (d, *J* = 6.7 Hz, 3H, 3 × Val-γH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.5, 172.4, 155.9, 79.6, 59.1, 57.0, 47.5, 31.2, 28.5, 28.3, 24.8, 19.2, 17.6. ESI-MS (*m*/*z*): calcd for $C_{15}H_{26}N_2NaO_5$ (M + Na) *m*/*z* 337.1739 found 337.1734.

*5.2.3. Diphenyl [(1R,1S)-1-[(benzyloxy)amino]-2-methylpropyl]phosphonate (**3**)*

Carboxybenzyl (Cbz)-protected 1-Aminoalkyl-phosphonate diphenyl ester **3** was synthesized using a literature method.[32] Triphenyl phosphite (0.933 g, 3.007 mmol) was dissolved in glacial acetic acid (8 mL), and isobutyraldehyde (0.239 g, 3.308 mmol) and benzyl carbamate (0.500 g, 3.308 mmol) were added. The mixture was heated for 2 hours at 80-90 °C, and the solvent evaporated. The crude product was subjected to column chromatography (Hexane/EtOAc, 5:1 v/v) This yielded **3** as a colorless oil (0.950 g, 72%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 6.98 (m, 15H, 10 × Phenoxy-H & 5 × phenyl-H), 5.22 (dd, *J* = 10.9, 2.5 Hz, 1H, 1 × Val-NH), 5.15 – 5.01 (m, 2H, 2 × benzylic-H), 4.45 – 4.34 (m, 1H, 1 × Val-αH), 2.39 (m, 1H, 1 × Val-βH), 1.08 (dd, *J* = 6.8, 1.3 Hz, 6H, 6 × Val-γH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.2 (d, J_{C-P} = 7.1 Hz), 150.2 (d, J_{C-P} = 10.1 Hz), 149.9 (d, J_{C-P} = 9.6 Hz), 136.0, 129.7, 129.6, 128.5, 128.2, 128.1, 125.3, 125.1, 120.6 (d, J_{C-P} = 4.1 Hz), 120.4 (d, J_{C-P} = 4.1 Hz), 67.4, 53.3 (d, J_{C-P} = 161.6 Hz), 29.1 (d, J_{C-P} = 5.3 Hz), 20.3 (d, J_{C-P} = 14.1 Hz), 17.6 (d, J_{C-P} = 4.2 Hz). ESI-MS (*m*/*z*): calcd for $C_{24}H_{26}NNaO_5P$ (M + Na) *m*/*z* 462.1446 found 462.1400.

*5.2.4. Diphenyl [(1R,1S)-1-amino-2-methylpropyl]phosphonate (**4**)*

Compound **4** was obtained by dissolving compound **3** (0.425 g, 0.967 mmol) in 33% HBr/AcOH solution (3 mL). The reaction was carried out at room temperature for 2 hours. The volatile components of the mixture were removed under reduced pressure. The residue was dissolved in 1 mL methanol, and 80 mL ether was added. Compound **4** was left to crystallize at 4 °C to yield 0.28 g of the hydrobromide salt, a white solid. The amount of compound **4** thus obtained was 0.221 g, 75%. No further recrystallization was necessary. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.07 (s, 2H, 2 × NH$_2$), 7.35 – 7.04 (m, 10H, 10 × Phenoxy-H), 3.82 (brd, *J* = 14.2 Hz, 1H, 1 × Val-αH), 2.75 – 2.58 (m, 1H, Val-βH), 1.29 (dd, *J* = 6.8, 4.5 Hz, 6H, 6 × Val-γH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 149.42 (d, J_{C-P} = 9.6 Hz), 149.39 (d, J_{C-P} = 9.9 Hz), 129.7, 125.6, 125.5, 120.9 (d, J_{C-P} = 4.3 Hz), 120.6 (d, J_{C-P} = 4.5 Hz), 53.5 (d, J_{C-P} = 155 Hz), 28.4 (d, J_{C-P} = 2.0 Hz), 19.9 (d, J_{C-P} = 9.1 Hz), 19.1 (d, J_{C-P} = 4.0 Hz). ESI-MS (*m*/*z*): calcd for $C_{16}H_{20}NNaO_3P$ (M + Na) *m*/*z* 328.1078 found 328.1045.

*5.2.5. Tert-butylN-[(2S)-1-[(2S)-2-{[(1R,1S)-1-(diphenoxyphosphoryl)-2-methylpropyl]carbamoyl}pyrrolidin-1-yl]-3-methyl-1-oxobutan-2-yl]carbamate (**5**)*

Compound **2** (0.119 g, 0.570 mmol) was dissolved in 3 mL DMF. To this was added HBTU (0.173 g, 0.457 mmol) and DIEA (0.17 mL, 0.953 mmol) to generate the activated ester. After 10 minutes, compound **4** (0.22 g, 0.721 mmol) was added and the reaction mixture was stirred under nitrogen overnight. The reaction was diluted with EtOAc (25 mL) and an equal amount of sodium bicarbonate, washed with brine (6 × 25 mL), dried ($MgSO_4$) and concentrated *in vacuo*. The crude product was subjected to column chromatography (100% EtOAc). This yielded **5** as a mixture of diastereomers, as a colorless solid (0.205 g, 90%) which formed a white solid foam under reduced pressure. >99% purity for diastereomers by HPLC (Gemini 5 µm C18 110 Å, gradient elution, 40% acetonitrile/water with 0.1% TFA to 85% acetonitrile in 45 minutes; Retention time (t_r) = 33.690 and 34.713 min). ESI-MS (*m*/*z*): calcd for $C_{31}H_{44}N_3NaO_7P$ (M + Na) *m*/*z* 624.2815 found 624.2772. Full NMR characterization of the pure diastereomers **6** and **7** is provided below.

5.2.6. Separation of Diastereomers.

Single diastereomers of **5** were obtained by column chromatography on silica gel using $CHCl_3$/EtOAc, 4:1 v/v. Compounds **6** and **7** were thus obtained.

*5.2.6.1. Tert-butyl-N-[(2S)-1-[(2S)-2-{[(1R)-1-(diphenoxyphosphoryl)-2-methylpropyl]carbamoyl}pyrrolidin-1-yl]-3-methyl-1-oxobutan-2-yl]carbamate (**6**)*

A colorless solid (0.03 g) which formed a white solid foam under reduced pressure. 96% purity by HPLC, the second diastereomer accounts for the remaining 4% (Gemini 5 µm C18 110 Å, gradient elution, 40% acetonitrile/water with 0.1% TFA to 85% acetonitrile in 45 min; t_r = 33.557 min), see full HPLC trace on page 17. Retention factor (R_f) = 0.34 ($CHCl_3$/EtOAc, 4:1, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33-7.14 (m, 11H, 10 × Phenoxy-H and 1 × P_1-Val-NH), 5.24 (d, *J* = 9.2 Hz, 1H, 1 × P_3-Val-carbamate-NH), 4.77 – 4.69 (m, 1H, 1 × P_1-Val-αH), 4.46 (dd, *J* = 8.4, 3.2 Hz, 1H, 1 × P_2-Pro-αH), 4.30 (dd, *J* = 9.6, 6.4 Hz, 1H, 1 × P_3-Val-αH), 3.77 – 3.51 (m, 1H, 1 × P_2-Pro-δH), 3.63-3.58 (m, 1H, 1 × P_2-Pro-δH), 2.44 – 2.34 (m, 1H, 1 × P_1-Val-βH), 2.24 – 2.09 (m, 2H, 2 × P_2-Pro-βH), 2.08 – 1.94 (m, 2H, 1 × P_3-Val-βH and 1 × P_2-Pro-γH), 1.88 – 1.79 (m, 1H, 1 × P_2-Pro-γH), 1.43 (s, 9H, ^{t}Butyl), 1.11 (d, *J* = 7.2 Hz, 3H, 3 × P_1-Val-γH), 1.04 (dd, *J* = 6.8, 1.2 Hz, 3H, 3 × P_1-Val-γH), 0.99 (d, *J* = 6.4 Hz, 3H, 3 × P_3-Val-γH), 0.93 (d, *J* = 6.8 Hz, 3H, 3 × P_3-Val-γH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.6, 171.2 (d, $J_{C\text{-}P}$ = 6.1 Hz), 155.8, 150.4 (d, $J_{C\text{-}P}$ = 10.1 Hz), 150.1 (d, $J_{C\text{-}P}$ = 10.1 Hz), 129.8 (d, $J_{C\text{-}P}$ = 1.0 Hz), 129.7 (d, $J_{C\text{-}P}$ = 0.6 Hz), 125.3 (d, $J_{C\text{-}P}$ = 1.3 Hz), 125.1 (d, $J_{C\text{-}P}$ = 0.9 Hz), 120.7 (d, $J_{C\text{-}P}$ = 4.1 Hz), 120.4 (d, $J_{C\text{-}P}$ = 4.4 Hz), 79.6, 60.0, 56.8, 51.3 (d, $J_{C\text{-}P}$ = 154.2 Hz), 47.7, 31.4, 29.3 (d, $J_{C\text{-}P}$ = 3.9 Hz), 28.3, 27.4, 25.3, 20.3 (d, $J_{C\text{-}P}$ = 13.6 Hz), 19.6, 18.0 (d, $J_{C\text{-}P}$ = 4.7 Hz), 17.4. ESI-MS (*m*/*z*): calcd for $C_{31}H_{44}N_3NaO_7P$ (M + Na) *m*/*z* 624.2815 found 624.2763.

*5.2.6.2. Tert-butyl-N-[(2S)-1-[(2S)-2-{[(1S)-1-(diphenoxyphosphoryl)-2-methylpropyl]carbamoyl}pyrrolidin-1-yl]-3-methyl-1-oxobutan-2-yl]carbamate (**7**)*

A colorless solid (0.015 g) which formed a white solid foam under reduced pressure. 90% purity by HPLC, the second diastereomer accounts for the remaining 10%. (Gemini 5 µm C18 110 Å, gradient elution, 40% acetonitrile/water with 0.1% TFA to 85% acetonitrile in 45 min; t_r = 34.496 min), see full HPLC trace on page 18. R_f =0.45 ($CHCl_3$/EtOAc, 4:1 v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (brd, *J* = 10.1 Hz, 1H, 1 × P_1-Val-NH), 7.31 – 7.26 (m, 5H, 5 × Phenoxy-H), 7.18 – 7.14 (m, 5H, 5 × Phenoxy-H), 5.17 (d, *J* = 9.6 Hz, 1H, P_3-Val-Carbamate-NH), 4.78 – 4.69 (m, 2H, 1 × P_1-Val-αH and 1 × P_2-Pro-αH), 4.24 (dd, *J* = 9.2, 6.0 Hz, 1H, 1 × P_3-Val-αH), 3.74 – 3.67 (m, 1H, 1 × P_2-Pro-δH), 3.50 – 3.45 (m, 1H, 1 × P_2-Pro-δH), 2.48-2.39 (m, 2H, 1× P_1-Val-βH and 1 ×P_2-Pro-βH), 2.00-1.91 (m, 3H, 1 × P_2-Pro-βH and 2 x P_2-Pro-γH), 1.86 – 1.77 (m, 1H, 1 × P_3-Val-βH), 1.42 (s, 9H, ^{t}Butyl), 1.13 (d, *J* = 6.8 Hz, 3H, 3 × P_1-Val-γH), 1.07 (dd, *J* = 6.8 Hz, 1.6 Hz, 3H, 3 × P_1-Val-γH), 0.86 (d, *J* = 6.8 Hz, 3H, 3 × P_3-γH), 0.83 (d, *J* = 6.8 Hz, 3H, 3 × P_3-Val-γH).^{13}C NMR (101 MHz, $CDCl_3$) δ 173.5, 171.2 (d, $J_{C\text{-}P}$ = 6 Hz), 155.8, 150.3 (d, $J_{C\text{-}P}$ = 10.0 Hz), 150.0 (d, $J_{C\text{-}P}$ = 9.2 Hz), 129.7 (d, $J_{C\text{-}P}$ = 0.9 Hz), 129.6 (d, $J_{C\text{-}P}$ = 0.6 Hz), 125.3 (d, $J_{C\text{-}P}$ = 1.1 Hz), 125.1 (d, $J_{C\text{-}P}$ = 0.8 Hz), 120.7 (d, $J_{C\text{-}P}$ = 4.4 Hz), 120.4 (d, $J_{C\text{-}P}$ = 4.3 Hz), 79.7, 60.3, 56.8, 51.1 (d, $J_{C\text{-}P}$ = 155.6 Hz), 47.7, 31.3, 29.3 (d, $J_{C\text{-}P}$ = 3.8 Hz), 28.3, 27.3, 25.0, 20.5 (d, $J_{C\text{-}P}$ = 13.9 Hz), 19.6, 17.9 (d, $J_{C\text{-}P}$ = 4.3 Hz), 17.3. ESI-MS (*m*/*z*): calcd for $C_{31}H_{44}N_3NaO_7P$ (M + Na) *m*/*z* 624.2815 found 624.2764.

*5.2.7. Methyl-(2S)-2-{[(2S)-1-[(2S)-2-{[(tert-butoxy)carbonyl]amino}-3-methylbutanoyl]pyrrolidin-2-yl]formamido}-3-methylbutanoate (**8**)*

Compound **2** (0.450 g, 1.43 mmol) was dissolved in 4 mL DMF. To this was added HBTU (0.619 g, 1.63 mmol) and DIEA (0.48 mL, 2.73 mmol) to generate the activated ester. After 10 minutes, L-Valine methyl ester hydrochloride (0.23 g, 1.37 mmol) was added and the reaction mixture was stirred under nitrogen overnight. The reaction was diluted with EtOAc (20 mL) and an equal amount of sodium bicarbonate, washed with brine (6 × 20 mL), dried ($MgSO_4$) and concentrated *in vacuo*. The crude product was subjected to column chromatography (Hexane/EtOAc, 2:3 v/v) yielding **8** as a white solid (0.26 g, 45%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.21 (d, *J* = 8.4 Hz, 1H, 1 × P_1-Val-NH), 5.25 (d, *J* = 9.3 Hz, 1H, 1 × P_3-Val-carbamate-NH), 4.64 – 4.54 (m, 1H, 1 × P_1-Val-αH), 4.42 (dd, *J* = 8.4, 5.1 Hz, 1H, 1 × P_2-Pro-αH), 4.29 (dd, *J* = 9.4, 6.2 Hz, 1H, 1 × P_3-Val-αH), 3.72 (s, 3H, OCH_3), 3.78 – 3.66 (m, 1H, 1 × P_2-Pro-δH), 3.64 – 3.54 (m, 1H, 1 × P_2-Pro-δH), 2.40 – 2.28 (m, 1H, 1 × P_1-Val-βH), 2.19 – 2.03 (m, 2H, 2 × P_2-Pro-βH), 2.01 – 1.91 (m, 2H, 1 × P_3-Val-βH and 1 × P_2-Pro-γH), 1.88 – 1.75 (m, 1H, 1 × P_2-Pro-γH), 1.42 (s, 9H, tButyl), 0.98 (d, *J* = 6.8 Hz, 3H, 3 × P_1-Val-γH), 0.94 – 0.87 (m, 9H, 3 × P_1-Val-γH and 6 × P_3-Val-γH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.58, 172.12, 170.92, 155.80, 79.56, 59.92, 57.54, 56.77, 52.04, 47.68, 31.43, 31.09, 28.32, 27.20, 25.15, 19.55, 18.94, 17.86, 17.40. ESI-MS (*m*/*z*): calcd for $C_{21}H_{37}N_3NaO_6$ (M + Na) *m*/*z* 450.2580 found 450.2540.

5.3. Protease Assays

The inhibition of human neutrophil elastase (HNE), trypsin and chymotrypsin by compounds **5**, **6** and **7** was also carried out. This was conducted by modifying an existing method.[33] The activity of the enzymes was measured at 37 °C over a period of 5 min using AAPV-pNA (Sigma-Aldrich M4765), Nα-Benzoyl-DL-R-pNA (Sigma-Aldrich B4875) and Suc-AAPF-pNA (Sigma-Aldrich S7388) as HNE, trypsin and chymotrypsin substrates respectively. Absorbance readings were measured at 405 nm and monitored at 15 sec intervals. The solubilization of the substrates which is critical for obtaining reliable results was achieved by dissolving 5 mg in 50 µL of dimethyl sulphoxide (DMSO) and making up to 5 mL with the assay buffer (0.10 M Tris-HCl, pH 8.1, 0.02 M $CaCl_2$). For Nα-Benzoyl-DL-R-pNA, 100% DMSO was required.

Inhibition by compounds **5**, **6** and **7** was assessed by incubating 50 µL of the enzyme solutions in the assay buffer with 1.5 µL of the inhibitors (dissolved in DMSO), all made up to 100 µL in the assay buffer. Incubation was carried out at 37 °C for 15 min before the addition of 50 µL of the substrate which initiated the reaction to give an intense yellow coloration for controls and wells without enzyme inhibition.

All dilutions were in triplicate and inhibition was measured as the percentage of enzyme activity remaining. Data analysis was conducted using GraphPad prism.

5.4. Cell-based Assays

In vitro C. trachomatis cell culture assays were conducted based on a previously reported method.[7] *C. trachomatis* D (D/UW-3/Cx) was obtained from the ATCC and routinely cultured in McCoy B cells on DMEM, 10% fetal calf serum (FCS) at 37 °C, 5% CO_2, 0.1 mg/mL streptomycin, and 0.05 mg/ml gentamycin. Inhibitor experiments were routinely conducted in 96-well plates seeded with 20,000 host cells per well 24 h prior to the *Chlamydia* infection. Infections were routinely conducted at a Multiplicity of Infection (MOI) of 0.3. The Inclusion Forming Units (IFU) were determined from cultures harvested at the completion of the developmental cycle during which inhibitor treatment was conducted. Briefly, JO146 (**5**) at 50 µM dose, the diastereomers (**6**

and **7**) at 50 µM and 10 µM doses alongside a DMSO control (JO146 solvent), were added at 16 h post infection (PI) and left in the cultures until completion of the developmental cycle. Harvested cultures were lysed by vigorous pipetting and serially diluted onto fresh monolayers, fixed and stained for enumeration of IFU/ml.

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Supplementary Material

HPLC spectra for JO146 and its diastereomers and NMR spectra are available in the Supplementary Material.