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Oxime-based click chemistry in the development of 3-isoxazolecarboxylic acid-containing inhibitors of *Yersinia pestis* protein tyrosine phosphatase, YopH

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Abstract

Pathogenicity of *Yersinia pestis* (*Y. pestis*) relies on several effector proteins, including YopH, a protein-tyrosine phosphatase. Previously, we screened a library of analogues based on the ubiquitous PTP substrate, p-nitrophenylphosphate (pNPP) and found that incorporation of a 3-phenyl substituent (6-nitro-[1,1'-biphenyl]-3-yl dihydrogen phosphate (**1**)) enhanced affinity. The current study reports the conversion of **1** from a substrate to inhibitor by replacing the hydrolysable phosphoryl group with a 3-isoxazolecarboxylic acid moiety and by introduction of an aminooxy group and subsequent diversification using oxime-based click chemistry. As reported herein, this approach led to the identification of non-promiscuous low micromolar affinity bidentate YopH inhibitors.

Keywords

Substrate screening; Aminooxy platform; Oxime-based click chemistry; YopH inhibitors; *In silico* docking studies

Introduction

Yersinia pestis (*Y. pestis*) has played important roles in human history by serving as the causative agent for pneumonic and bubonic plague.^[1, 2] Recently, *Y. pestis* has gained attention as a potential biological warfare or bioterrorism agent and this has engendered renewed interest in the development of anti-plague therapeutics. For pathogenicity *Y. pestis* employs a Type III secretion system (T3SS) to inject into host cells a variety of “Yop” proteins that include YopH, a highly active protein-tyrosine phosphatase (PTP).^[3] Inappropriate dephosphorylation by YopH can interfere with normal cellular function and lead to pathogenesis, and accordingly, YopH inhibitors could potentially provide a basis for new anti-plague therapeutics.

PTPs share a common mechanism of action, which involves substrate recognition by a conserved (H/V)CX5R(S/T) signature motif that forms the heart of the catalytic cleft. Catalysis occurs in two steps by initial transfer of the phosphoryl group to the active-site Cys residue and subsequent release of dephosphorylated substrate and hydrolysis of the phosphoprotein thioester intermediate to liberate inorganic phosphate and regenerate the free enzyme. The phosphotyrosyl (pTyr) phenylphosphate functionality plays a defining role in

substrate recognition. One approach to inhibitor development is to identify high affinity substrates, which can subsequently be converted to inhibitors by replacement of the hydrolysable phosphoryl group with non-hydrolysable mimetics. Identification of substrates as platforms for inhibitor development (a known approach^[4–7] that has recently been termed, “substrate activity screening” (SAS)^[8]) has the potential advantage of overcoming “false positives” that can arise from inhibition by promiscuous mechanisms.^[9, 10]

As an application of SAS we recently screened YopH against a library of analogues based on the ubiquitous PTP substrate, *p*-nitrophenylphosphate (pNPP, K_M of 600 μM) and found that incorporation of a 3-phenyl substituent resulted in enhanced affinity (6-nitro-[1,1'-biphenyl]-3-yl dihydrogen phosphate (**1**, Figure 1), K_M = 80 μM).^[11] The current study reports the conversion of **1** from a substrate to a series of inhibitors (**3**) by replacing the hydrolysable phosphoryl group with a 3-isoxazolecarboxylic acid moiety and by introduction of an aminooxy handle and subsequent diversification of this platform (**2**) using oxime-based click chemistry (Figure 1). As reported herein, this approach led to the identification of non-promiscuous low micromolar affinity bidentate YopH inhibitors.

Results and Discussion

Conversion of substrate **1** to inhibitor platform **2**

Substrate **1** was modified by replacement of its phosphoryl group with the non-hydrolysable phosphate mimetic, 3-isoxazolecarboxylic acid.^[12, 13] Further modification by removal of the nitro group and introduction of an aminooxy handle yielded platform **2**, which served as the starting point for preparing a library of oxime-based bidentate inhibitors **3**. The synthesis of **3** was accomplished in five steps starting from ((3-bromophenyl)ethynyl)trimethylsilane (**4**, Scheme 1). Removal of the TMS group (potassium carbonate in MeOH) and treatment of the resulting alkyne with 2-chloro-2-(hydroxyimino)-acetate gave the 5-bromophenyl-3-isoxazole derivative **5** in good yield.^[14] Suzuki coupling to provide the corresponding benzylic alcohol **6** and Mitsunobu reaction with *N*-hydroxyphthalimide gave **7**. Treatment with hydrazine hydrate followed by ester hydrolysis provided the aminooxy-containing platform **2**. The synthesis of oxime **3** was accomplished by reacting **2** with a library of aldehydes using AcOH as a catalyst.

Oxime-based library diversification

The syntheses of oxime-containing inhibitors (**3**) were performed in DMSO by reacting the aminooxy-containing platform **2** (24 mM) with appropriate aldehydes and AcOH in the ratio (1:1:2). The resulting crude oxime reaction mixtures were of sufficient purity (> 90%) for direct biological evaluation. Inhibitory IC_{50} values were determined spectrophotometrically in an in vitro YopH assay using pNPP as substrate.^[15] A library of 65 commercially available aldehydes was employed with structural diversity ranging from simple linear and branched alkyls to phenyl and phenylmethyls and heterocycles.^[16] Inhibitory potencies (IC_{50} values) for the resulting compounds ranged from 3 μM to 115 μM , with aromatic-containing analogues exhibiting greater inhibitory potency than the linear and branched alkyl-containing analogues. The most potent inhibitors (**3a** – **3d**) showed IC_{50} values of approximately 10 μM (Table 1). Addition of a 5-carboxyl group to the indolyl portion of oxime **3d** resulted in an approximate 3 - fold enhancement of inhibitory potency (**3e**; IC_{50} = 3.1 μM , Table 1). When assays were conducted in the presence and absence of 0.01% TX-100^[11, 17, 18] it was found that the inhibitory potency of **3e** was independent of detergent at the concentration tested. This provided strong evidence that YopH inhibition by **3e** does not arise through promiscuous mechanisms. Kinetic analysis of **3e** indicated inhibition by a competitive mechanism with a K_i value of $3.9 \pm 0.3 \mu\text{M}$ (Figure 4).

Potential role of nitro functionality

The conversion of substrate **1** to the lead platform **2** was done with removal of a nitro group, since nitro functionality can impart undesirable biological properties,^[19] and the original function of this moiety as a chromophoric agent was no longer necessary. However, it was unclear what effects deletion of the nitro group had on the binding affinities of the final ligands (**3a** – **3d**). In order to clarify this issue, 4-nitro functionality was introduced into the inhibitors. This involved the synthesis of the aminooxy-containing platform **15** (Scheme 2). The synthesis of **15** started by the Suzuki coupling of 3-bromo-4-nitrophenol (**8**) with (4-(hydroxymethyl)phenyl)boronic acid to give the biphenyl product **9**. Reaction of **9** with a mild triflating reagent (*N*-phenyl-bis(trifluoromethanesulfonimide))^[20] provided **10** in quantitative yield. Sonogashira coupling of **10** with TMS-acetylene afforded **11** followed by removal of the TMS-group to give the alkyne **12**. Treatment of **12** with 2-chloro-2-(hydroxyimino)-acetate gave the 3-isoxazolecarboxylic acid ester **13**, which provided phthalimide **14** under Mitsunobu conditions. Deprotection by treatment with hydrazine hydrate followed by ethyl ester hydrolysis gave the nitro-containing aminooxy platform **15**. Synthesis of the nitro-containing oxime-containing inhibitors (**16a** – **16e**) was then performed in DMSO as before and YopH inhibitory potencies were determined for the resulting oximes without purification. The similarity of IC₅₀ values for compounds with (Table 2) and without (Table 1) nitro groups indicated that this functionality apparently had little effect on binding affinity.

Potential YopH binding modes of synthetic ligands

In order to identify potential YopH binding modes, *in silico* docking studies were performed^[21, 22] starting from our earlier X-ray crystal structure of YopH in complex with the peptide Ac-Asp-Ala-Asp-Glu-F₂Pmp-Leu-amide ((PDB 1QZ0),^[23, 24] where “F₂Pmp” represents the non-hydrolyzable pTyr mimetic, phosphonodifluoromethylphenylalanine.^[25, 26] The portion of the peptide bound within the catalytic pocket was isolated and the phosphonodifluoromethyl group was replaced with a 3-isoxazolecarboxylic acid moiety. The resulting 5-phenyl-3-isoxazolecarboxylic acid structure was re-docked alternatively in the presence and absence of a catalytically-conserved H₂O molecule.^[27] Inclusion of the conserved H₂O resulted in additional bridging interactions with Q357 and Q450 (Figure 2) that were not possible in the absence of the H₂O. These additional interactions were reflected in more favourable calculated binding scores in subsequent docking studies of fully elaborated oxime-containing inhibitors.

Potential YopH interactions with **3d** (Figure 3a) and **3e** (Figure 3b) were examined. The phenyl ring originating from the *meta*-position of the 5-phenyl-3-isoxazolecarboxylic acid group was found to bind within a hydrophobic channel lying between residues I232 and Q446, which is identical to the region occupied by the Leu side chain in the original Ac-Asp-Ala-Asp-Glu-F₂Pmp-Leu-amide peptide (PDB 1QZ0). The most favourable binding poses for both inhibitors involved insertion of their tethered indolyl rings into the previously identified pockets P1 and P2 (Figure 3).^[28–30] The higher binding affinity of **3e** relative to **3d** could be explained by bonding interactions of its indolyl 5-carboxy group with residues in the base of each pocket (the guanidinium group of R473 in Pocket 1 and the amide nitrogen of G442 in the base of pocket 2).

The modelling in Figure 3 orients the 5-phenyl-3-isoxazolecarboxylic acid group within the catalytic cleft, while the 5-carboxyindolyl group interacts in a secondary Pocket. It is worth noting that 5-carboxyindolyl-containing motifs have been incorporated into PTP inhibitors as phenylphosphate mimetics designed to interact within the catalytic clefts.^[31–33] This could potentially indicate that (in the specific case of **3e**) alternate binding modes may be possible in which the 5-carboxyindolyl group rather than the 5-phenyl-3-isoxazolecarboxylic

acid group is bound within the catalytic cleft. In fact modelling of such “reversed” orientations does show that they are possible. However, the resulting binding interactions are significantly less favourable than the orientations shown in Figure 3, and they most probably do not contribute significantly to overall binding affinity^[34] as well as the dual specificity phosphatases.^[35]

Examination of Specificity of 3e

In order to ascertain some measure of enzyme specificity, the inhibitory activity of **3e** was measured against a panel of phosphatases. These included the classical tyrosine-specific phosphatases PTP1B and LAR,^[36] DUSP14, DUSP22 and the Variola phosphatase VH1 (Table 3). In these assays relative to YopH, **3e** showed good selectivity against LAR (45 – fold) and VH1 (> 400 – fold), and less selectivity against other the other phosphatases examined (from 4 – fold to 8 – fold).

Conclusion

The focus of the current study was to develop new inhibitors directed against YopH by the replacement of the phosphoryl group in previously identified high affinity substrate **1** with 3-isoxazolecarboxylic acid moiety and appendage of an aminooxy handle to yield a lead inhibitor scaffold that was optimized using oxime-based library diversification. This protocol resulted in the identification of bivalent analogue that showed low micromolar, non-promiscuous inhibition against YopH, with moderate to good enzyme selectivity. Although the specific target of the study was YopH, the approach may be more broadly applicable for the development of inhibitors directed against other phosphatases.

Experimental Section

General

The following reagents used for YopH enzyme assays were obtained from Sigma-Aldrich: *p*NPP tablets; 30% BSA solution (protease free); 1.0 M HEPES solution (pH 7.0 – 7.6) and dithiotreitol (DTT). Aqueous EDTA (0.5 M, pH 8.0) was obtained from Invitrogen and 96-well clear bottom plates were purchased from FisherSci (Costar 3603). All reactions were carried out under argon unless otherwise stated. All solvents were anhydrous and obtained from Sigma-Aldrich. High pressure liquid chromatography (HPLC) was performed using a Waters Prep-LC 4000 system and Phenomenex Gemini 10 μ , 110 Å C₁₈ columns (250 $\times$ 21.20 mm 10 micron) at a flow rate of 10 mL/minute (prep. HPLC) and 1 mL/minute (analytical HPLC) with a mobile phase of A = 0.1% aqueous TFA and B = 0.1% TFA in aqueous acetonitrile. Typical gradients were from 10% B to 100% B over 40 minutes with UV monitoring at 220 nm, 254 nm and 280 nm. NMR spectra were recorded using a Varian 400 MHz spectrometer. Unit mass resolution LC-MS were obtained on synthetic intermediates and high resolution mass spectra (HRMS) were obtained for final products (University of California at Riverside Mass Spectral Facility). Optical densities were measured with Biotek Synergy 2 spectrophotometer at λ_{abs} 405 nm using absolute readout for determination of IC₅₀ values.

Recombinant proteins

The PTPse domain of YopH (residues 164–468) was expressed in *Escherichia coli* according to the previously published procedure.^[3, 24] as were the variola major H1^[37] and human DUSP-14 dual specificity phosphatases.^[38] Human DUSP-22, PTPase1B and LAR catalytic domains were expressed and purified using generic methodology.^[39]

General syntheses of oximes 3 and 16

A solution of 72 mM aminoxy platform (15 μ L DMSO) and a solution of 72 mM aldehyde (15 μ L DMSO) were placed in 1.5 mL microtube with cap. To this mixture was added 144 mM AcOH (15 μ L DMSO). The reaction mixture was then gently agitated overnight at RT and the resultant oximes (24 mM) were directly evaluated in vitro against YopH without any further purification.

Determination of YopH IC₅₀ values

Total reactions volumes of 100 μ L/well of reaction volume were used in 96 well plates. Buffer was prepared as above. To each well was added 79 μ L of assay buffer, 0.25% BSA (5 μ L) followed by 5 μ L of inhibitors in DMSO at dilutions of 1200, 480, 192, 77, 31, 25, 12, 5, 2, 0.8, 0.4 and 0 μ M. To the reaction mixtures was then added 5 μ L of YopH in buffer (25 μ g/mL) followed by 6 μ L of 10 mM *p*NPP buffer each plate was agitated gently at 25° C for 15 – 20 minutes. Hydrolysis of the substrate was immediately measured. IC₅₀ values were determined by fitting the data with sigmoidal curve generated using the Boltzman equation. A parallel independent assay was performed with 0.01% TritonX-100. Inhibition constants for **3a–e** and **16a–e** are provided in Table 1 and 2. Data curves are provided in the Supporting Information.

Determination of K_i value

Total reactions volumes of 100 μ L/well of reaction volume were used in 96 well plates. Buffer was prepared as above. To each well was added 65 μ L of assay buffer, 0.25% BSA (5 μ L) followed by 5 μ L of inhibitors in DMSO at dilutions 8, 4, 2 μ M. To each of the fixed inhibitor concentration was then added 20 μ L of *p*NPP with dilutions ranging from 2000 to 3.9 μ M (*K_M* *p*NPP against YopH with no inhibitor is 600 μ M). The reaction was then started by the addition of 5 μ L YopH and was immediately monitored at 405 nm. Data were then fitted for the determination of *K_i* and mechanism of inhibition.

In Silico Studies—Docking of inhibitors **3d** and **3e** onto YopH was done with ICM Chemist Pro® software running on a MacIntosh computer (OSX v10.5.8) using default parameters and procedures.^[22] In summary, modelling started with the X-ray crystal structure of YopH in complex with the peptide Ac-Asp-Ala-Asp-Glu-F₂Pmp-Leu-amide (PDB 1QZ0),^[23, 24] where “F₂Pmp” represents the non-hydrolysable pTyr mimetic, phosphonodifluoromethylphenylalanine.^[25, 26] The “convert PDB” command was used to convert to native ICM format, with optimization of hydrogens. All H₂O molecules were removed from the enzyme with the exception of the catalytically-conserved H₂O (identified as “cw”). For creation of the ligand, the phosphonodifluoromethylphenyl portion of the peptide was first isolated, then the phosphonodifluoromethyl group was replaced by the 3-isoxazolecarboxylic acid moiety and the resulting 5-phenyl-3-isoxazolecarboxylic acid structure was re-docked using the “re-dock” option under the “Ligand” menu. All docking was performed using the standard “re-dock” command, which utilizes a rigid receptor protocol. Docking was performed both in the presence (Figure 2) and absence of the catalytically-conserved H₂O. Subsequent docking was conducted with retention of this H₂O. Binding scores for best poses were calculated automatically. To the docked 5-phenyl-3-isoxazolecarboxylic acid moiety was added the *meta*-phenyl ring and the resulting structure was re-docked. Further structural editing provided the final inhibitors, which were re-docked. The best binding poses (lowest binding scores) for **3d** and **3e** are shown in Figure 3.

Ethyl 5-(3-Bromophenyl)isoxazole-3-carboxylate (5): To a solution of ((3-bromophenyl)ethynyl)trimethylsilane (1.00 g, 3.95 mmol) in MeOH (5 mL) was added K₂CO₃ (2.90 g, 15.9 mmol) and the mixture was stirred at room temperature (1.5 h). Solvent

was removed and the residue was partitioned (EtOAc : H₂O), the organic phase was washed (H₂O) and dried (MgSO₄) and taken to dryness. Solvent was evaporated at reduced pressure and the resultant crude material was purified by silica gel chromatography (hexanes : EtOAc, 9 : 1) to yield intermediate 1-bromo-3-ethynylbenzene. A solution of this alkyne (0.54 g, 2.97 mmol) and ethylchloroximido acetate (0.76 g, 5.05 mmol) in anhydrous THF (5 mL) was purged with argon, then Et₃N (0.70 mL, 5.04 mmol) was via syringe over 30 minutes and the mixture was stirred at 50° C (overnight). After cooling to room temperature the mixture was partitioned (1 N HCl : EtOAc) and the aqueous phase was extracted (EtOAc) and the combined organic extract was dried and taken to dryness. Purification by silica column chromatography (hexanes : EtOAc, from 3 : 1 to 1 : 1) to provide **6** as a white amorphous solid (0.74 g, 84% yield). ¹H NMR (400 MHz, CDCl₃): δ = 7.95 (t, *J* = 2.0 Hz, 1H), 7.73 (m, 1H), 7.59 (m, 1H), 7.37 (t, *J* = 8.0 Hz, 1H), 6.95 (s, 1H), 4.48 (q, *J* = 7.2 Hz, 2H), 1.45 (t, *J* = 7.2 Hz, 3H). ¹³C NMR (400 MHz, CDCl₃): δ = 170.13 (1C), 159.87 (1C), 157.16 (1C), 133.82 (1C), 130.82 (1C), 128.94 (1C), 128.50 (1C), 124.57 (1C), 123.34 (1C), 100.87 (1C), 62.45 (1C), 14.29 (1C). ESI-MS (*m/z*): [M + Na]⁺ calcd. for C₁₂H₁₀BrNO₃, 318.0 and 320.0; found, 318.1 and 320.0.

Ethyl 5-(4'-(Hydroxymethyl)-[1,1'-biphenyl]-3-yl)isoxazole-3-carboxylate (6): A mixture of **5** (500 mg, 1.67 mmol), (4-(hydroxymethyl)phenyl)boronic acid (385 mg, 2.55 mmol), Pd(PPh₃)₄ (96 mg, 0.08 mmol), saturated aqueous K₂CO₃ (10 mL), toluene (10 mL) and EtOH (4 mL) was purged with argon and stirred at reflux (overnight). The reaction mixture was partitioned (H₂O : EtOAc) and the organic phase was dried, taken to dryness and the residue was purified by silica column chromatography (hexanes : EtOAc, from 3 : 1 to 1 : 1) to yield **6** as a white amorphous solid (372 mg, 69% yield). ¹H NMR (400 MHz, CDCl₃): δ = 8.01 (t, *J* = 2.0 Hz, 1H), 7.77 (m, 1H), 7.69 (m, 1H), 7.65 (m, 2H), 7.56 (t, *J* = 8.0 Hz, 1H), 7.48 (m, 2H), 6.98 (s, 1H), 4.77 (d, *J* = 2.8 Hz, 2H), 4.48 (q, *J* = 7.2 Hz, 2H), 1.45 (t, *J* = 7.2 Hz, 3H). ¹³C NMR (400 MHz, CDCl₃): δ = 171.77 (1C), 160.16 (1C), 157.14 (1C), 146.40 (1C), 142.07 (1C), 140.82 (1C), 139.43 (1C), 132.45 (1C), 130.46 (1C), 129.74 (1C), 127.73 (1C), 127.47 (1C), 127.27 (1C), 127.15 (1C), 124.78 (1C), 100.35 (1C), 64.71 (1C), 62.47 (1C), 14.33 (1C). ESI-MS (*m/z*): [M + Na]⁺ calcd. for C₁₉H₁₇NO₄, 346.1; found, 346.1.

Ethyl 5-(4'-(((1,3-Dioxoisindolin-2-yl)oxy)methyl)-[1,1'-biphenyl]-3-yl)isoxazole-3-carboxylate (7): To a mixture of **6** (350 mg, 1.08 mmol), *N*-hydroxyphthalimide (212 mg, 1.30 mmol), and PPh₃ (368 mg, 1.41 mmol) in anhydrous THF (10 mL) was added diisopropyl azodicarboxylate (DIAD) (0.28 mL, 1.41 mmol) and the mixture was stirred at room temperature (overnight). The mixture was partitioned (H₂O : CH₂Cl₂) and the organic extracts were dried, taken to dryness and the residue was purified by precipitation from EtOAc to give **7** as a white solid amorphous solid (463 mg, 91% yield). ¹H NMR (400 MHz, CDCl₃): δ = 8.02 (m, 1H), 7.84-7.62 (m, 10H), 7.57 (t, *J* = 8.0 Hz, 1H), 6.99 (s, 1H), 5.28 (s, 2H), 4.49 (q, *J* = 7.2 Hz, 2H), 1.45 (t, *J* = 7.2 Hz, 3H). ¹³C NMR (400 MHz, CDCl₃): δ = 171.69 (1C), 163.68 (1C), 160.15 (1C), 157.20 (1C), 141.82 (1C), 141.07 (1C), 134.66 (2C), 133.67 (1C), 130.63 (2C), 129.85 (1C), 129.72 (1C), 129.16 (1C), 129.06 (1C), 127.49 (2C), 127.45 (1C), 127.36 (1C), 125.16 (1C), 124.80 (1C), 123.72 (2C), 100.42 (1C), 79.60 (1C), 62.45 (1C), 14.36 (1C). ESI-MS (*m/z*): [M + Na]⁺ calcd. for C₂₇H₂₀N₂O₆, 491.1; found, 491.0.

5-(4'-((Aminooxy)methyl)-[1,1'-biphenyl]-3-yl)isoxazole-3-carboxylic Acid (2): To a solution of **7** (200 mg, 0.49 mmol) in EtOH (5 mL) was added 50% aqueous hydrazine hydrate (0.13 mL, 1.96 mmol) and the reaction mixture was stirred at room temperature (3 h). The resulting precipitate was removed by filtration and the filtrate was taken to dryness to yield a white solid, which was stirred with a mixture of THF (2 mL), H₂O (5 mL) and 1 N NaOH (2.0 mL) (1.5 h). Volatile organics were removed under reduced pressure and the

resulting aqueous phase was subjected directly to HPLC purification as described above in the General Synthetic methods (retention time = 17.7 minutes) to yield **2** with amorphous solid (79 mg, 52% yield; 100% purity by analytical HPLC). ¹H NMR (400 MHz, CD₃OD): δ = 8.87 (s, 1H), 8.26 (m, 1H), 7.96 (m, 1H), 7.87 (m, 3H), 7.66 (t, *J* = 8.0 Hz, 1H), 7.60 (s, 1H), 7.53 (m, 2H), 4.96 (s, 2H), 2.51 (t, *J* = 2.0 Hz, 2H). ¹³C NMR (400 MHz, CD₃OD): δ = 171.06 (1C), 161.22 (1C), 158.28 (1C), 140.96 (1C), 139.73 (1C), 134.87 (1C), 130.50 (1C), 129.98 (2C), 129.46 (1C), 127.47 (2C), 127.39 (1C), 125.25 (1C), 124.41 (1C), 101.85 (1C), 76.15 (1C). HRMS-ESI (*m/z*): [M + H]⁺ calcd for C₁₇H₁₅N₂O₄, 311.1026; found, 311.1025.

4'-(Hydroxymethyl)-6-nitro-[1,1'-biphenyl]-3-ol (9): Mixture of 3-bromo-4-nitrophenol (1.5 g, 6.88 mmol), (4-(hydroxymethyl)phenyl)boronic acid (1.5 g, 10.32 mmol) and Pd(PPh₃) (0.40 g, 0.34 mmol) in 10 mL satd solution of K₂CO₃, 4 mL EtOH and 10 mL toluene was purged with Argon and stirred at 70°C overnight. Water was added upon cooling and aqueous layer was extracted by EtOAc. Organic extract was dried over MgSO₄, filtered and solvent was removed. The crude material was purified via silica gel chromatography (CH₂Cl₂ : EtOAc 4:1) to give **9** as a yellow solid product (1.3 g, 76%). ¹H NMR (400 MHz, CDCl₃): δ = 7.88 (d, *J* = 9.2 Hz, 1H), 7.38 (d, *J* = 8.4 Hz, 2H), 7.26 (d, *J* = 8.4 Hz, 2H), 6.91 (dd, *J* = 2.4 Hz, *J* = 8.8 Hz, 1H), 6.80 (d, *J* = 4.0 Hz, 1H), 4.62 (s, 1H). ¹³C NMR (400 MHz, CDCl₃): δ = 161.59 (1C), 143.09 (1C), 140.12 (1C), 137.98 (1C), 129.52 (1C), 128.70 (1C), 128.28 (1C), 127.81 (1C), 119.28 (1C), 115.95 (1C), 115.58 (1C), 64.41 (1C). ESI-MS (*m/z*): [M - H]⁻ calcd. for C₁₃H₁₁NO₄, 244.1; found, 244.1.

4'-(hydroxymethyl)-6-nitro-[1,1'-biphenyl]-3-yl trifluoromethanesulfonate (10): To a solution of **9** (1.0 g, 4.08 mmol) and *N*-phenyl-bis(trifluoromethanesulfonamide) (1.6 g, 4.40 mmol) in 12 mL CH₃CN was added DIPEA (0.78 mL, 0.59 mmol) and the reaction mixture was stirred at room temperature for 2 h. TLC analysis showed completion of reaction and 10 mL EtOAc was added. The resultant mixture was washed with water, and organic layer was dried over MgSO₄, filtered and solvent removed. Purification via silica gel chromatography (1:1 Hex : EtOAc) gave **10** as a pure yellow oil product (1.49 g, 97%). ¹H NMR (400 MHz, CDCl₃): δ = 7.93 (d, *J* = 8.8 Hz, 1H), 7.43 (d, *J* = 8.4 Hz, 2H), 7.38 (dd, *J* = 2.4 Hz, *J* = 8.8 Hz, 1H), 7.33 (d, *J* = 2.8 Hz, 1H), 7.28 (d, *J* = 8.0 Hz, 2H), 4.74 (s, 2H). ¹³C NMR (400 MHz, CDCl₃): δ = 150.87 (1C), 148.09 (1C), 142.26 (1C), 139.12 (1C), 134.76 (1C), 129.70 (1C), 128.12 (1C), 127.58 (1C), 126.58 (1C), 124.97 (1C), 121.24 (1C), 121.58 (1C), 117.28 (1C), 64.89 (1C). ESI-MS (*m/z*): [M + Na]⁺ calcd. for C₁₄H₁₀F₃NO₆S, 400.0; found, 399.9.

(2'-Nitro-5'-((trimethylsilyl)ethynyl)-[1,1'-biphenyl]-4-yl)methanol (11): To a solution of **10** (1.5 g, 3.98 mmol), Pd(PPh₃)₂Cl₂ (0.28 g, 0.40 mmol), and CuI (0.15 g, 0.80 mmol) in 20 mL CH₃CN purged with Argon was added DIPEA (1.87 mL, 13.9 mmol) followed by dropwise addition of TMS-acetylene (2.25 mL, 15.9 mmol). The reaction mixture was stirred at room temperature for 2 days. Upon completion 15 mL EtOAc was added for dilution and was washed with satd solution of NH₄Cl. Organic layer was dried over MgSO₄, filtered and solvent removed. Purification via silica gel chromatography (Hex : EtOAc 2 : 1) gave **11** as a brown oil product (1.3 g, 98%). ¹H NMR (400 MHz, CDCl₃): δ = 7.54 (d, *J* = 8.0 Hz, 1H), 7.25 (m, 2H), 7.14 (d, *J* = 8.0 Hz, 2H), 7.02 (d, *J* = 6.4 Hz, 2H), 4.47 (s, 2H), 0.00 (s, 9H). ¹³C NMR (400 MHz, CDCl₃): δ = 148.05 (1C), 141.43 (1C), 136.63 (1C), 136.28 (1C), 135.62 (1C), 131.57 (1C), 129.83 (1C), 128.30 (1C), 128.06 (1C), 127.46 (1C), 124.59 (1C), 123.72 (1C), 102.83 (1C), 99.73 (1C), 65.12 (1C), 0.00 (3C). ESI-MS (*m/z*): [M + Na]⁺ calcd. for C₁₈H₁₉NO₃Si, 348.1; found, 348.1.

(5'-Ethynyl-2'-nitro-[1,1'-biphenyl]-4-yl)methanol (12): Potassium carbonate (1.0 g, 7.37 mmol) was added to a solution of **11** in 10 mL MeOH and 5 mL CH₂Cl₂. The reaction mixture was stirred for 4h at room temperature. Solvent was evaporated and 10 mL water

and 10 mL EtOAc were added. The aqueous layer was extracted with EtOAc and the organic extract was dried over MgSO_4 , filtered and solvent removed. The crude product was purified via silica gel chromatography (Hex : EtOAc 2 : 1) to give **12** as a pale yellow solid product (0.64 g, 69 %). ^1H NMR (400 MHz, CDCl_3): δ = 7.79 (d, J = 8.0 Hz, 1H), 7.52 (m, 2H), 7.39 (d, J = 8.0 Hz, 2H), 7.26 (d, J = 6.4 Hz, 2H), 4.71 (s, 2H), 3.26 (s, 1H), 1.80 (s, 1H). ^{13}C NMR (400 MHz, CDCl_3): δ = 148.05 (1C), 141.28 (1C), 136.32 (1C), 135.71 (1C), 135.44 (1C), 131.55 (1C), 127.97 (2C), 127.18 (2C), 126.64 (1C), 124.28 (1C), 81.38 (1C). ESI-MS (m/z): $[\text{M} + \text{Na}]^+$ calcd. for $\text{C}_{15}\text{H}_{11}\text{NO}_3$, 246.1; found, 246.1.

Ethyl 5-(4'-(hydroxymethyl)-6-nitro-[1,1'-biphenyl]-3-yl)isoxazole-3-carboxylate (13): Mixture of **12** (445.3 mg, 1.76 mmol) and ethyl 2-chloro-2-(hydroxyimino)acetate (553.0 mg, 3.52 mmol) in 10 mL THF was purged with Argon. To this mixture triethylamine (0.49 mL, 3.52 mmol) was added slowly over 30 min and stirred at room temperature overnight. A mixture of 5 mL 1N HCl and 5 mL EtOAc was added. Organic layer was dried over MgSO_4 , filtered and solvent removed. The crude product was purified via silica gel chromatography (Hex : EtOAc 2 : 1) to give pale **13** as a yellow solid product (385.1 mg, 59%). ^1H NMR (400 MHz, CD_3CN): δ = 8.01 (m, 2H), 7.98 (m, 1H), 7.45 (m, 2H), 7.37 (m, 2H), 7.28 (s, 1H), 4.64 (d, J = 5.6 Hz, 2H), 4.10 (q, J = 7.2 Hz, 2H), 3.27 (t, J = 5.6 Hz, 1H), 1.37 (t, J = 7.2 Hz, 3H). ^{13}C NMR (400 MHz, CD_3CN): δ = 170.58 (1C), 160.85 (1C), 158.94 (1C), 144.31 (1C), 138.05 (1C), 136.48 (1C), 131.42 (1C), 130.81 (2C), 129.27 (2C), 128.40 (2C), 127.03 (1C), 126.55 (1C), 104.12 (1C), 64.63 (1C), 63.60 (1C), 14.75 (1C). ESI-MS (m/z): $[\text{M} + \text{Na}]^+$ calcd. for $\text{C}_{19}\text{H}_{16}\text{N}_2\text{O}_6$, 391.1; found, 391.0.

Ethyl 5-(4'-(((1,3-dioxoisindolin-2-yl)oxy)methyl)-6-nitro-[1,1'-biphenyl]-3-yl)isoxazole-3-carboxylate (14): Mixture of **13** (123.7 mg, 0.34 mmol), *N*-hydroxyphthalimide (65.7 mg, 0.40 mmol) and PPh_3 (115.0 mg, 0.44 mmol) was dissolved in anhydrous THF and was purged with Argon. Diisopropyl diazo-1,2-dicarboxylate (56.0 μL , 0.44 mmol) was slowly added to the above solution. The reaction mixture was stirred at room temperature overnight. The reaction mixture was cooled to 5–10 °C to precipitate the product and 10 mL water and 10 mL Et_2O were added. The aqueous layer was separated and the organic phase with the precipitate was filtered by washing with cold Et_2O . The filtrate was washed into a new flask by passing CH_2Cl_2 through the filter. Solvent was evaporated to give **14** as pure white solid product (104.0 mg, 60%) that was used without further purification. ^1H NMR (400 MHz, CDCl_3): δ = 7.97 (d, J = 8.4 Hz, 1H), 7.89 (dd, J = 8.4 Hz, J = 1.6 Hz, 1H), 7.83 (d, J = 1.6 Hz, 1H), 7.79–7.82 (m, 2H), 7.71–7.73 (m, 2H), 7.63 (d, J = 6.4 Hz, 2H), 7.35 (d, J = 6.4 Hz, 2H), 7.05 (s, 1H), 5.25 (s, 2H), 4.45 (q, J = 6.8 Hz, 2H), 1.42 (t, J = 6.8 Hz, 3H). ^{13}C NMR (400 MHz, CDCl_3): δ = 169.91 (1C), 163.41 (2C), 159.43 (1C), 157.74 (1C), 149.73 (1C), 137.25 (1C), 137.05 (1C), 134.46 (1C), 134.43 (1C), 130.17 (2C), 129.97 (1C), 129.25 (1C), 128.81 (1C), 128.03 (2C), 125.57 (1C), 125.22 (1C), 123.54 (4C), 102.22 (1C), 79.23 (1C), 62.48 (1C), 14.11 (1C). ESI-MS (m/z): $[\text{M} + \text{Na}]^+$ calcd. for $\text{C}_{27}\text{H}_{19}\text{N}_3\text{O}_8$, 536.1; found, 536.1.

5-(4'-((Aminoxy)methyl)-6-nitro-[1,1'-biphenyl]-3-yl)isoxazole-3-carboxylic acid (15): To a solution of **14** (95.0 mg, 0.19 mmol) in CH_2Cl_2 (5 mL) was added 50% aqueous hydrazine hydrate (23.0 μL , 0.37 mmol) and the reaction mixture was stirred at room temperature (3 h). The solvent was removed and the resultant crude mixture was redissolved in EtOAc and filtered to remove solid byproduct. Solvent was removed under reduced pressure and the white solid product was stirred with a mixture of THF (2 mL), H_2O (5 mL) and 1 N NaOH (1.5 mL) (1.5 h). Volatile organics were removed under reduced pressure and the resulting aqueous phase was subjected directly to HPLC purification as described above in the General Synthetic methods (retention time = 19.5 minutes) to yield **15** as amorphous white solid product (33.1 mg, 49% yield; 99% purity by analytical HPLC). ^1H

NMR (400 MHz, DMSO- d_6): δ = 8.19 (m, 2H), 8.13 (m, 1H), 7.76 (s, 1H), 7.45-7.51 (m, 4H). MALDI-MS (m/z): $[M + Na]^+$ calcd. for $C_{17}H_{13}N_3O_6$, 378.0702; found, 378.0712.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Development of oxime-containing inhibitors **3** based on starting substrate **1**.

a)

b)

Figure 2.

Docking of 5-phenyl-3-isoxazolecarboxylic acid in the YopH catalytic pocket (a) Docking performed in the presence a catalytically-conserved H₂O molecule. (b) Overlay onto the docking pose of Panel A of the phosphonodifluoromethylphenyl group (shown in yellow) derived from the crystal structure of an F₂Pmp-containing peptide bound to YopH (PDB 1QZ0).

a)

b)

Figure 3. Predicted favoured YopH binding interactions for synthetic oxime-containing inhibitors **3d** (Panel a) and **3e** (Panel b) showing insertion of tethered aryl rings into well formed Pockets (1 and 2). The protein is rendered as an electrostatic surface with red indicating negative and blue positive charge.

Figure 4. Lineweaver-Burk plot for **3e** and inhibits YopH through a competitive mechanism. The inhibitor concentrations used were 2, 4, and 8 μM .

Scheme 1.

Synthetic procedure of platform **3**. Reagents and conditions: a) K_2CO_3 , MeOH, RT; b) $\text{EtO}_2\text{CC}(\text{Cl})=\text{NOH}$, Et_3N , THF, 50°C (84% yield); c) 4-hydroxymethylboronic acid, satd. K_2CO_3 , $\text{Pd}(\text{PPh}_3)_4$, EtOH, PhMe, 70°C (69% yield); d) *N*-hydroxyphthalimide, PPh_3 , DIAD, THF, RT (91% yield); e) $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}$, EtOH, RT f) 1 N NaOH, THF (52% yield); g) RCHO , AcOH, DMSO, RT (>90% yield).

Scheme 2.

Synthetic procedure of oxime **16**. Reagents and conditions: a) 4-hydroxymethylboronic acid, satd. K_2CO_3 , $Pd(PPh_3)_4$, EtOH, PhMe, 70° C (76% yield); b) $PhN(SO_2CF_3)_2$, DIPEA, CH_3CN , RT (97% yield); c) $Pd(PPh_3)_2Cl_2$, CuI, DIPEA, TMS-acetylene, CH_3CN , RT (98% yield); d) K_2CO_3 , MeOH, RT (69% yield); e) $EtO_2CC(Cl)=NOH$, Et_3N , THF, RT (59% yield); f) *N*-hydroxyphthalimide, PPh_3 , DIAD, THF, RT (60% yield); g) $NH_2NH_2 \cdot H_2O$, EtOH, RT h) 1 N NaOH, THF (49% yield); i) $RCHO$, AcOH, DMSO, RT (>90% yield).

Table 1In vitro YopH IC₅₀ values for selected inhibitors.

No.	R	IC ₅₀ ± S.E. (μM) ^[a]
3a		14.2 ± 0.2
3b		9.8 ± 0.2
3c		8.7 ± 0.3
3d		8.6 ± 0.3
3e		3.1 ± 0.2

^[a]IC₅₀ values were determined as indicated in the Experimental procedures.

Table 2In vitro YopH IC₅₀ values for inhibitors **16a–d**.

No.	R	IC ₅₀ ± S.E. (μM) ^[a]
16a		15.4 ± 0.8
16b		14.9 ± 0.5
16c		10.0 ± 0.4
16d		9.2 ± 0.2
16e		3.9 ± 1.4

^[a]IC₅₀ values were determined as indicated in the Experimental procedures.

Table 3

Inhibitory potencies of **3e** against a panel of phosphatases.

Phosphatase	IC ₅₀ (μM) ^[a]	Fold change
YopH	3.0	
PTP1B	13.7	4.7
LAR	134.9	45
DUSP-14	22.5	7.5
DUSP-22	24.5	8.2
Variola	> 1200	> 400

^[a] IC₅₀ values were determined as indicated in the Experimental procedures.

^[b] Relative to YopH