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Oxime-based linker libraries as a general approach for the rapid generation and screening of multidentate inhibitors

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Abstract

The described oxime-based library protocol provides detailed procedures for the linkage of aminooxy functionality with aldehyde building blocks that result in the generation of libraries of multidentate inhibitors. Synthesis of inhibitors for protein tyrosine phosphatases (PTPs) and antagonists directed against the human tumor susceptibility gene 101 (Tsg101) are shown as examples. Three steps are involved: a) the design and synthesis of aminooxy platforms; b) tethering with aldehydes to form oxime-based linkages with sufficient purity; and c) direct *in vitro* biological evaluation of oxime products without purification. Each coupling reaction is a) performed in capped microtubes at room temperature; b) diluted for inhibitory evaluation and c) screened with targets in microplates to provide IC₅₀ or K_d values. The synthesis of the aminooxy platforms takes 3–5 days; tethering with the aldehydes takes 24 h; and inhibition assay of enzymes and protein-protein interactions (PPIs) takes 30 min and 2 h respectively.

Keywords

Oxime; aminooxy platform; multidentate inhibitors; PTP; YopH; PPIs; Tsg101; click chemistry

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INTRODUCTION

Development of multidentate small molecule ligands that can bind to multiple sites on proteins is an important and evolving area for the discovery of potent therapeutic agents. In the case of protein tyrosine phosphatases (PTPs), the active sites often share high sequence homology and it is generally thought that inhibitors targeting only the catalytic pockets may lack selectivity. However, crystal structures of PTPs show that protein surface topology proximal to the active sites may provide features that are unique to each PTP and that residues in these peripheral sites may be important for unique substrate recognition for each PTPs.^{1–6} Therefore, multidentate agents targeting the active sites as well as sites outside the catalytic pocket may exhibit enhanced selectivity. In addition, tethering of components to provide multidentate ligands that simultaneously bind both within the active site and in peripheral areas outside the catalytic pocket should lead to inhibitors that are more potent than those that bind only within the active catalytic pocket. Protein-protein interactions (PPIs) have also been considered to be difficult targets due to the extended nature of their contact surfaces. However, X-ray studies often show the presence of discrete pockets within these contact areas, which form by the plasticity of the interface upon binding with different ligands.^{7–9} This feature allows for the development of high affinity multidentate ligands that can target these interface subpockets.

Ligand tethering strategies are widely used for the expedited development of multivalent agents. One approach uses Cu(I)-catalyzed “click chemistry” to accelerate the synthesis and discovery of drug-like molecules targeting enzymes.¹⁰ The term click chemistry was introduced by K. Barry Sharpless¹¹ to describe a class of reactions that includes the Huisgen 1,3-dipolar cycloaddition of alkynes to azides to form 1,4-disubstituted-1,2,3-triazoles.¹² These reactions are highly versatile due to the mild reaction conditions required and good chemoselectivity. To this date, this form of click chemistry has been successfully used in drug discovery, where it has been employed to assemble and generate a large number of small molecule inhibitors and to successfully screen them against various enzymes.^{13–23} However, a limitation associated with this approach is that the azide-containing starting materials may either be expensive or of limited commercial availability, thereby requiring their high-throughput synthesis.^{14–19,24} In addition, the reactions typically require catalysis by Cu(I)^{13,25–28} which is either dissolved in organic solvents or prepared *in situ* from the reduction of Cu(II) using ascorbic acid^{29,30} in mixtures of H₂O and *tert*-butanol. This can necessitate purification of the reaction products prior to biological evaluation. The azide-alkyne click chemistry, although easy to execute, results in reaction products with moderate yields (~30–80%).^{13,15,18} It can also be limited by the availability of azide starting materials and a requirement for product purification. This can present significant limitation for the rapid generation of multivalent inhibitors in sufficient purity for direct biological evaluation. Oxime-based ligation provides an alternate form of click chemistry that does not require high cost or synthesis of library building blocks, and that results in the generation of reaction products with sufficient purity and biocompatibility for direct biological evaluation. This results in expedited tethering and *in situ* screening of multidentate ligands.

We have recently used an alternate methodology that uses an oxime-based tethering approach for the rapid generation and screening of multivalent ligands without the need for purification. A distinctive feature of this type of ligation is its use of aminoxy-containing handles that are reacted with aldehydes to form stable oxime bonds (Fig. 1a). Oxime tethering is an attractive and efficient approach that uses inexpensive commercially available aldehyde starting materials. Oxime bond formation does not require high reaction temperatures, it can be performed under aqueous conditions, it is chemospecific and sufficiently stable to allow for initial biological evaluation without purification. Each tethering reaction is carried out by incubating an aminoxy-containing platform with alkyl

and aryl aldehyde fragments in the presence of AcOH in a molar ratio of 1:1:2–5. Pre-calculated molar concentrations of the reactants allow serial dilution of final oxime products. Reactions are complete overnight at room temperature (20–23 °C) in typically 90% or greater yields as shown by random HPLC analysis for both alkyl and aryl tethering. The oxime bond is very stable and its cleavage occurs only under acidic reflux condition.³¹ Thus oxime products in DMSO solutions can be stored indefinitely at 2–8 °C (small molecules) and –20 °C (peptidic oximes). The reaction products can be transferred without purification to titertubes or 96-well microplates and diluted for evaluation against biological targets at different ligand concentrations. We have applied this rapid oxime ligation approach to the development of non-peptidic multidentate protein tyrosine phosphatases (PTPs) inhibitors, where the binding interactions span regions both inside and outside of the conserved catalytic pocket.^{32–34} We have also used this method to optimize peptide antagonist targeting interactions between human tumor susceptibility gene 101 (Tsg101) and HIV-1 Gag in an effort to develop virus budding inhibitors.^{35–37} Therefore, discovery of potent inhibitors through oxime library diversification is applicable to targets that have multiple binding pockets (catalytic and secondary pockets/peripheral sites), such as kinases and phosphatases and enzymes with extended sites, such as proteases. The approach is also applicable for generating oxime-based peptides that can serve as effective binding antagonists inhibiting protein-protein interactions (PPIs).

General approach

The general steps taken to develop enzyme inhibitors using oxime library diversification are: a) identification of both core and peripheral sites of the enzyme; b) identification of lead compounds that interact with the active site and the strategic introduction of aminooxy handles; c) oxime-based tethering by reacting library of aldehydes with the aminooxy platform and d) screening against a target enzyme for the identification of potential inhibitors.

Non-peptidic multidentate PTP inhibitors

PTPs are a class of enzymes that reverse the actions of protein tyrosine kinases (PTKs). They exert important regulatory functions, with deregulation having been linked to a number of diseases, including cancer, diabetes and osteoporosis.^{38–40} Our lab has been interested in “YopH”, a highly active PTP protein injected into host cells during infection by *Yersinia pestis* (*Y. pestis*: a gram negative bacteria that serves as causative agent of pneumonic, septicemic and bubonic plague). The potential use of *Y. pestis* for bioterrorism has led to an urgent need to develop potent and selective YopH inhibitors.

We have recently reported the development of an aminooxy inhibitor platform **1** (Fig. 2a) that was derived from substrate screening.³⁴ The crystal structure of **1** in complex with YopH (PDB: 2Y2F, Fig. 2a) showed that the difluoromethyl phosphonate (DFMP) mimetic of **1** is tightly bound within the catalytic pocket through hydrogen bonds to the signature motif phosphate binding loop “P-loop”⁴¹ and the invariant tryptophan, proline, aspartic acid - loop “WPD-loop”.⁴² These interactions are similar to those involved by a native phosphate group. A crucial feature revealed by the structure involved the conserved water molecule (Wa43), which lies outside the catalytic pocket and acts as a hydrogen bonding bridge between the aminooxy group and D231. This suggested that the platform could be used for inhibitor design by oxime tethering with one part of the molecule binding to a site outside the catalytic cleft. The R groups from RCHO building block can be easily be assembled onto the aminooxy functionality via oxime bond formation to yield additional binding interactions with residues at peripheral sites.

The furanyl-based oxime **2** (Fig. 2b) was strategically designed using ICM Chemist Pro[®] software. Docking of the inhibitor onto YopH showed that in addition to oxime nitrogen atom, the furanyl oxygen hydrogen bonds with the D231 residue through the Wa43 molecule. Based on these results a focused furanyl-oxime library was synthesized by a parallel reaction of the aminooxy platform with furanyl aldehydes. Without purification, the resultant products were directly screened in an *in vitro* YopH assay. Inhibitory potencies (IC₅₀ values) were obtained spectrophotometrically using *p*-nitrophenylphosphate (*p*NPP) as the chromogenic substrate. Inhibitor **3** was found to have IC₅₀ value with a 6-fold improvement relative to the parent oxime **2**. *In silico* docking studies of inhibitor **3** showed multiple interactions of the carboxyl group with R230 and D231.

Peptide antagonists targeting interactions between human tumor susceptibility gene 101 (Tsg101) and HIV-1 Gag

A second example of our oxime-based ligation concerns our development of PPI antagonists directed against the human tumor susceptibility gene 101 (Tsg101). Tsg101 is a subunit protein of mammalian endosomal sorting complexes required for transport-I (ESCRT-I) related to the cellular vascular protein sorting (Vps) pathway.⁴³ In HIV-infected cells, viral release requires direct interaction of the ubiquitin E2 variant (UEV) domain of Tsg101 with HIV-1 Gag protein.⁴⁴ Structural analysis of the TSG101-Gag complex has shown the importance of a highly conserved motif “Pro-Thr-Ala-Pro” (“PTAP”), which is a proline-rich sequence located in the Gag p6 region. Interaction of Tsg101 with Gag P6 represents a target for the development of competitive PPI inhibitors as potential new antiviral therapies.^{45–48}

The nonamer PTAP-containing sequence “P¹E²P³T⁴A⁵P⁶P⁷E⁸E⁹” has been shown by NMR solution studies^{46,49} to bind to Tsg101 with modest affinity ($K_d \sim 50\text{--}60\text{ }\mu\text{M}$).³⁶ Based on the lead 9-mer sequence we synthesized a series of Tsg101-binding peptides using post solid-phase oxime formation that involved the reaction of a set of aminooxy-containing peptides with libraries of aldehydes.^{35–37} The aminooxy-containing amino acids **4–7** shown in Fig. 1b were used to synthesize the peptide libraries **8a–l** by replacement of the respective amino acids, Pro with either **6** or **7**; Thr with **4** and Glu with **5**.³⁶ The peptides **8a–l** were synthesized by solid-phase peptide synthesis (SPPS)^{50,51} from **4–7** and other commercially available peptides and purified using HPLC. The resulting purified aminooxy-containing peptides **8a–l** were then subjected to oxime-based library diversification by reacting each peptide with a series of commercially available aldehydes **a–l** (Fig. 3). The peptide oximes (>90% pure) were used directly in binding studies with Tsg101. As a result of this study the 3,4-dimethoxybenzyl oxime-containing peptide **9** was found to exhibit 15–20 fold binding enhancement ($K_d = 3.3\text{ }\mu\text{M}$, Fig. 4) as compared to the wild-type parent peptide ($K_d \sim 50\text{--}60\text{ }\mu\text{M}$). The protein interactions of the 3,4-dimethoxybenzyl groups in the crystal structure³⁷ allowed the identification of a new binding region that most probably would not have been revealed using coded amino acids.

Overview of the procedure

The current protocol describes the synthesis of aminooxy building blocks **1** for YopH (Fig. 5; steps **1–18**) and **8d** for Tsg101 (Fig. 6 and 7; steps **19–74**) inhibitor libraries. Amino acid **18** is not commercially available and is therefore synthesized using solution phase synthesis (steps **19–40**) and coupled using standard SPPS conditions to incorporate it onto peptide **8d** (steps **41–74**). Detailed procedures for the generation of oxime derivatives (steps **75–77** and **78–80**), as well as dilution and screening of the resulting oxime derivatives using 96/384-well plates (Fig. 8 and 9, steps **81–87** and **88–106**) and inhibitory determination (IC₅₀ steps **107–116** and K_d steps **117–128**) are also presented here. The optimized reaction conditions for oxime ligation utilized DMSO as solvent and AcOH as catalyst at room temperature

overnight with reaction products being of sufficient purity for direct biological evaluation. Specific molar concentrations of each starting material (aminoxyl: RCHO: AcOH; 1:1:2–5) strategically provides reaction products in appropriate molar concentrations for dilution prior to biological screening. The molar concentration of catalytic AcOH depends on the nature of the aminooxy platform, where 2 and 5 equivalents of AcOH have been used for small molecule and peptidic aminooxyls functionality, respectively. A parallel independent assay of one more replicate is performed for each compound **3** and **9**. The identified inhibitors can then be synthesized in high scale, purified and re-examined against their intended targets to verify the reproducibility of the initially observed inhibition. The rapid generation and *in-situ* screening of oxime-based tethered constructs described herein can be applied for the rapid design and development of other enzyme or PPI-targeted multidentate inhibitors.

MATERIALS

REAGENTS

Critical: All procedures should be conducted in a fume hood. The solid and liquid waste products generated should be disposed of appropriately, as defined locally. The solvents used in the reactions should be of the highest quality available. When an anhydrous solvent is to be used, this is indicated clearly in the PROCEDURE. Anhydrous solvents are dried according to the methods indicated, or are purchased in anhydrous form as indicated. The solvents used in the extraction and workup phases should be of reagent quality or better.

Caution: Wear gloves and goggles, and use fumehood as some of these chemicals are especially dangerous and these have been indicated.

Reagents and materials for the synthesis of **1** and **18**

- Zinc (Aldrich, cat. no. 209988)
- Diethyl (bromodifluoromethyl)-phosphonate (Aldrich, cat. no. 411361)
- Copper bromide (Aldrich, cat. no. 254185)
- 4-(Hydroxymethyl)phenylboronic acid (Aldrich, cat. no. 512338)
- Tetrakis(triphenylphosphine)palladium(0) (Aldrich, cat. no. 216666)
- Potassium carbonate (Aldrich, cat. no. 209619)
- *N*-Hydroxyphthalimide (Aldrich, cat. no. H53704)
- Triphenylphosphine (Aldrich, cat. no. T84409)
- Diisopropyl azodicarboxylate, DIAD (Aldrich, cat. no. 225541) **! CAUTION** Hazardous
- Hydrazine hydrate (Aldrich, cat. no. 225819) **! CAUTION** Toxic, corrosive and hazardous
- Bromotrimethylsilane (Aldrich, cat. no. 194409) **! CAUTION** Corrosive
- (*R*)-4-Hydroxy-*L*-proline (Aldrich, cat. no. H54409)
- Benzyl chloroformate, CbzCl (Aldrich, cat. no. 119938) **! CAUTION** Corrosive
- Sodium bicarbonate (Aldrich, cat. no. S6014)
- Benzyl bromide (Aldrich, cat. no. B17905)

- Triethylamine (Aldrich, cat. no. T0886)
- *p*-Nitrobenzoic acid (Aldrich, cat. no. 461091) **! CAUTION** Corrosive
- Diethyl azodicarboxylate solution (Aldrich, cat. no. 563110) **! CAUTION** Hazardous
- Sodium azide (Aldrich, cat. no. S2002) **! CAUTION** Toxic
- 15-Crown-5 (Aldrich, cat. no. 188832)
- Di-*tert*-Butyl dicarbonate (Aldrich, cat. no. 199133) **! CAUTION** Toxic
- Palladium on activated charcoal (Aldrich, cat. no. 75990)
- *N*-(9*H*-Fluoren-2-ylmethoxycarbonyloxy)succinimide, Fmoc-5-Ava-OH (Acros, cat. no. 24310)
- Magnesium sulfate (Aldrich, cat. no. M7506)
- Silica gel 60 Å (Silicycle, cat. no. F10030B)
- Celite (Aldrich, cat. no. 06858)
- White septum stopper (ChemGlass, cat. no. 3022-28)
- Phenex filter membranes (Phenomenex, cat. no. AFO-0501)

Solvents

- *N,N*-Dimethylformamide (Aldrich, cat. no. 227056)
- Ethanol, EtOH (Pharmco-AAPER, cat. no. 111ACS200)
- Toluene (Aldrich, cat. no. 244511) **! CAUTION** Hazardous
- Tetrahydrofuran (Aldrich, cat. no. 401757)
- Dichloromethane (Aldrich, cat. no. 270997) **! CAUTION** Hazardous
- Methanol, MeOH (Aldrich, cat. no. 322415) **! CAUTION** Hazardous and toxic
- Acetonitrile (Aldrich, cat. no. 271004)
- Dimethyl sulfoxide (Aldrich, cat. no. D8418)
- Acetic acid, AcOH (Aldrich, cat. no. 320099) **! CAUTION** Corrosive
- Ethylacetate, EtOAc (FisherSci, cat. no. E145-20)
- Hexanes (FisherSci, cat. no. H292-20)
- Diethylether, Et₂O (FisherSci, cat. no. E138-1)

Reagents for the synthesis of aminooxy platform 8d

- Pierce disposable plastic columns (FisherSci, cat. no. PI-29924)
- NovaSyn®TGR resin (Novabiochem, cat. no. 01-64-0060)
- Fmoc-Ala-OH (ChemImpex, cat. no. 02369)
- Fmoc-Thr(*t*Bu)-OH (ChemImpex, cat. no. 02460)
- Fmoc-Glu(*Ot*Bu)-OH (ChemImpex, cat. no. 02413)
- 1-Hydroxybenzotriazole (Aldrich, cat. no. 711489)
- *N, N'*-Diisopropylcarbodiimide (Aldrich, cat. no. D125407)

- Bromo-tris-pyrrolidino-phosphonium hexafluorophosphate, PyBroP (Chem-Impex, cat. no. 02275)
- *N,N*-Diethylisopropylsilane (Aldrich, cat. no. 550043) **! CAUTION** Corrosive
- Piperidine (Aldrich, cat. no. 104094) **! CAUTION** Corrosive and toxic
- Fmoc-5-Ava-OH (Aldrich, cat. no. 04066)
- Fluorescein isothiocyanate (Aldrich, cat. no. F3651)
- Trifluoroacetic acid, TFA (Aldrich, cat. no. T6508) **! CAUTION** Corrosive
- Triisopropylsilane (Aldrich, cat. no. 233781)

EQUIPMENT

- Common synthetic organic chemistry glassware
- High pressure liquid chromatography (HPLC) instrument (Waters Prep-LC 4000)
- Preparative HPLC column (Phenomenex Gemini 10 μ m, 110 Å C18 columns (250 mm $\times$ 21.20 mm, 10 μ m))
- Analytical HPLC column (Phenomenex Gemini 5 μ m, 110 Å C18 columns (250 mm $\times$ 4.60 mm, 5 μ m))
- Vacuum pump (FisherSci Maxima C Plus)
- Rotary evaporator (Bushi B-480)
- Thin layer chromatography, TLC (Analtech cat. no. 21521, 10 cm $\times$ 2.5 cm for each plate)

REAGENT SETUP

Critical: Prepare fresh for each of the following reagents

0.1% Aqueous TFA (HPLC mobile phase) Add 4.0 l Millipore pure H₂O in 4.0 l bottle. Measure 4.0 ml TFA and add it into the bottle and shake.

0.1% TFA acetonitrile (HPLC mobile phase) Add 4.0 ml TFA into a bottle containing 4.0 l acetonitrile and shake.

24 mM stock of aminoxy 1 Add 0.024 mmol in 1.0 ml DMSO

24 mM stock aldehyde (YopH oximes) Add 0.024 mmol in 1.0 ml DMSO

48 mM AcOH (YopH oximes) Add 0.048 mmol in 1.0 ml DMSO

15 mM stock of aminoxy 8d Add 0.015 mmol in 1.0 ml DMSO

15 mM aldehyde (Tsg101 oximes) Add 0.015 mmol in 1.0 ml DMSO

70 mM AcOH (Tsg101 oximes) Add 0.07 mmol in 1.0 ml DMSO

EQUIPMENT SETUP

HPLC Final aminoxy products (**1** and **8d**) and the identified potent inhibitors (**3** and **9**) can be purified by HPLC using a Waters Prep-LC 4000 system and Phenomenex Gemini 10 μ m, 110 Å C18 columns (250 mm $\times$ 21.20 mm, 10 μ m) at a flow rate of 10 ml/min (preparative HPLC) with a mobile phase of A = 0.1% aqueous TFA and B = 0.1% TFA in acetonitrile. In

our laboratory, typical gradients are from 10% B to 100% B over 40 min for **1** and **3** (UV detection at 210, 254, and 280 nm); and 0% B to 100% B over 30 min followed by 100% B for 10 min for **8d** and **9** (UV detection at 225 nm, 254, and 280 nm). The purity of final tested products (**3** and **9**) can be determined by analytical HPLC using a Waters Prep-LC 4000 system and Phenomenex Gemini 5 μ m, 110 Å C18 columns (250 mm–4.60 mm, 5 μ m) at a flow rate of 1 ml/min with a mobile phase of A = 0.1% aqueous TFA and B = 0.1% TFA in acetonitrile. In our laboratory, typical gradients are from 10% B to 100% B over 40 min for **3** (UV detection at 210, 254, and 280 nm); and 5% B to 100% B over 30 min followed by 100% B for 10 min for **9** (UV detection at 225 nm, 254, and 280 nm).

SPPS column: It is important to perform SPPS (steps 41–74) in Pierce disposable columns with agitation using a Barnstead/ThermoLyne Labquake tube shaker.

PROCEDURE

▲ CRITICAL Run all reactions for the synthesis of **1** and **18** under an inert atmosphere (argon) unless otherwise indicated and use round bottomed flasks that are oven-dried, equipped with magnetic stirring bars, and capped with rubber septum stoppers, unless otherwise indicated. Organic solvents are dried over MgSO₄ for 10–15 min. Organic solvents are removed under vacuum using a rotary evaporator unless otherwise indicated.

Synthesis of aminooxy platform **1** (Refer Fig. 5) • **TIMING 5 d**

- 1** Add diethyl (bromodifluoromethyl)phosphonate (3.4 ml, 19.4 mmol) in 2 ml of DMF dropwise to a suspended solution of Zn dust (1.27 g, 19.4 mmol) in 3 ml of DMF while maintaining the reaction temperature between 50–60 °C.
! CAUTION The reaction is exothermic add dropwise over 30 min. Cool to room temperature (20–23 °C) and stir the reaction mixture for 3 h.
- 2** Add CuBr (2.79 g, 19.4 mmol) in one portion, and stir the mixture for 30 min at room temperature.
- 3** Add a solution of bromo-3-iodobenzene (2.00 g, 7.1 mmol) in 1 ml of DMF dropwise. Stir for 24 h at room temperature.
- 4** Add H₂O (10 ml) and Et₂O (10 ml), and pass the mixture through Celite. Separate the layers using a separatory funnel, and extract the aqueous layer with Et₂O (3 × 20 ml).
- 5** Dry the organic extracts over MgSO₄, filter, and remove the solvent.
- 6** Purify the crude material via silica gel column chromatography (9:1 to 2:1 hexanes/EtOAc) to give diethyl ((3-bromophenyl)difluoromethyl)phosphonate **10** (TLC (hexanes:EtOAc 2:1 v/v) R_f = 0.40).

? TROUBLESHOOTING

- 7** Add **10** (500.0 mg, 1.46 mmol), 4-(hydroxymethyl)phenylboronic acid (332.0 mg, 2.19 mmol), and Pd(PPh₃) (84.0 mg, 0.07 mmol) in 5 ml of a saturated solution of K₂CO₃, 2 ml of EtOH, and 5 ml of toluene to a round bottomed flask, place condenser with running H₂O and stir by heating the reaction mixture at 70 °C for 12 h.
- 8** Add H₂O (20 ml), then extract the aqueous layer with EtOAc (3 × 20 ml), dry the organic extracts over MgSO₄, filter, and remove the solvent.

- 9 Purify the crude material via silica gel column chromatography (1.5:1 to 1:3 hexanes/EtOAc) to give diethyl (difluoro(4'-(hydroxymethyl)-[1,1'-biphenyl]-3-yl)methyl)phosphonate **11** (TLC (hexanes:EtOAc 1:3 v/v) R_f = 0.35).

? TROUBLESHOOTING

- 10 Add diisopropyl azodicarboxylate (DIAD) (0.13 ml, 0.65 mmol) to a mixture of **11** (184.0 mg, 0.50 mmol), *N*-hydroxyphthalimide (98.1 mg, 0.60 mmol), and PPh_3 (170.2 mg, 0.65 mmol) in anhydrous THF (5 ml). Stir the reaction mixture at room temperature for 12 h.
- 11 Partition the reaction mixture (H_2O /EtOAc, 1:1, 20 ml), and dry the organic layer over $MgSO_4$, filter and remove the solvent.
- 12 Add 50% aqueous hydrazine hydrate (80 μ l, 1.30 mmol) to a solution of the resultant crude product (168 mg, 0.33 mmol) in EtOH (5 ml). Stir the mixture at room temperature for 4 h.
- 13 Remove the resulting precipitate by filtration, and remove solvent from the filtrate.
- 14 Purify the crude product by silica column chromatography (50–100% EtOAc in Hexanes) to give diethyl ((4'-((aminooxy)methyl)-[1,1'-biphenyl]-3-yl)difluoromethyl)phosphonate **12** (TLC (100% EtOAc) R_f = 0.45).

? TROUBLESHOOTING

- 15 Add bromotrimethylsilane (0.13 ml, 0.93 mmol) to a solution of **12** (100 mg, 0.26 mmol) in anhydrous CH_2Cl_2 (5 ml). Stir the reaction mixture vigorously at room temperature for 3 h.
- 16 Remove solvent.
- 17 Dissolve the resulting crude material in H_2O (2 ml) and MeOH (2 ml) and filter using a syringe filter.
- 18 Purify by preparative HPLC using the method as described in the Equipment Setup section (retention time 15.598 min). Lyophilization provides **1** as a white solid.

■ **PAUSE POINT** The resultant aminooxy **1** can be stored for extended period at $-20^\circ C$.

Synthesis of platform 18 (Refer Fig. 6) • TIMING 8 d

- 19 Add benzyl chloroformate (11.8 ml, 84.8 mmol) slowly to a mixture of (*R*)-4-hydroxy-*L*-proline (10.0 g, 76.0 mmol) and $NaHCO_3$ (12.8 g, 0.15 mol) in H_2O (150 ml) and THF (150 ml) at $0^\circ C$. Warm the reaction mixture to room temperature and stir for 12 h.
- 20 Remove organic solvent and extract the aqueous phase with Et_2O (2×100 ml).
- 21 Discard the Et_2O layer and acidify the aqueous phase to pH 2–3 by the addition of 1 N HCl and extract with EtOAc (3×100 ml). Wash the combined EtOAc extracts with brine (50 ml), dry over Na_2SO_4 and evaporate to provide Cbz-protected product.
- 22 Stir a portion of the resultant material (17.5 g, 66.0 mmol), benzyl bromide (8.7 ml, 73.0 mmol) and triethylamine (10.3 ml, 73.0 mmol) in THF (200 mL) at room temperature for 12 h.

- 23 Dilute with EtOAc (200 ml), wash with H₂O (2 × 50 ml) and brine (50 ml). Dry over Na₂SO₄, filter, remove solvent and purify by silica gel column chromatography (2:1 hexanes/EtOAc) to give (2*S*,4*R*)-dibenzyl 4-hydroxypyrrolidine-1,2-dicarboxylate **13** (TLC (hexanes:EtOAc 1:1 v/v) R_f = 0.30).
- 24 Add diethyl azodicarboxylate (DEAD) (7.06 g, 26.9 mmol) slowly to a solution of **13** (4.30 g, 12.2 mmol), *p*-nitrobenzoic acid (4.09 g, 24.46 mmol) and triphenylphosphine (7.06 g, 26.9 mmol) in THF (150 ml) at 0 °C. Warm the mixture to room temperature and stir for 12 h.
- 25 Dilute with EtOAc (200 ml), wash with H₂O (2 × 50 ml) and brine (50 ml). Dry over Na₂SO₄, then remove solvent and purify by silica gel column chromatography (4:1 hexanes/EtOAc) to give (2*S*,4*S*)-dibenzyl 4-((4-nitrobenzoyl)oxy)pyrrolidine-1,2-dicarboxylate **14** (TLC (hexanes:EtOAc 1:1 v/v) R_f = 0.70).
- 26 Stir a solution of **14** (6.00 g, 11.9 mmol), NaN₃ (3.10 g, 47.5 mmol) and 15-crown-5 (1.20 ml, 6.0 mmol) in MeOH (200 ml) at 40 °C for 2 days.
- 27 Dilute the mixture with H₂O (100 ml), remove MeOH, and extract the aqueous phase with EtOAc (2 × 100 ml).
- 28 Wash the combined EtOAc layer with brine (50 ml), dry over Na₂SO₄, filter and remove solvent. Purify by silica gel column chromatography (2:1 hexanes/EtOAc) to give (2*S*,4*S*)-dibenzyl 4-hydroxypyrrolidine-1,2-dicarboxylate **15** (TLC (hexanes:EtOAc 1:1 v/v) R_f = 0.30).
- 29 Add DEAD (40% in toluene, 11.4 ml, 24.9 mmol) slowly to a solution of **15** (4.00 g, 11.3 mmol), *N*-hydroxyphthalimide (3.67 g, 22.5 mmol) and triphenylphosphine (6.53 g, 24.9 mmol) in THF (150 ml) at 0 °C. Warm the mixture to room temperature and stir for 12 h.
- 30 Dilute with EtOAc (200 ml), wash with H₂O (2 × 50 ml) and brine (50 ml). Dry over Na₂SO₄, filter and remove solvent. Purify by silica gel column chromatography (4:1 hexanes/EtOAc) to give (2*S*,4*R*)-dibenzyl 4-((1,3-dioxoisindolin-2-yl)oxy)pyrrolidine-1,2-dicarboxylate **16** (TLC (hexanes:EtOAc 1:1 v/v) R_f = 0.55).
- 31 Add hydrazine hydrate (0.36 ml, 7.44 mmol) to a solution of **16** (3.10 g, 6.20 mmol) in MeOH (50 ml) at room temperature. Stir the solution for 2 h.
- 32 Remove MeOH and re-dissolve the residue in THF (50 ml).
- 33 Add triethylamine (1.74 ml, 12.4 mmol) and Boc₂O (2.70 g, 12.4 mmol) and stir the solution at room temperature for 12 h.
- 34 Dilute with EtOAc (200 ml), wash with H₂O (2 × 50 ml) and brine (50 ml). Dry over Na₂SO₄, filter and remove solvent. Purify by silica gel column chromatography (4:1 hexanes/EtOAc) to give (2*S*,4*R*)-dibenzyl 4-(((tert-butoxycarbonyl)amino)oxy)pyrrolidine-1,2-dicarboxylate **17** (TLC (hexanes:EtOAc 1:1 v/v) R_f = 0.45).
- 35 Stir a mixture of **17** (2.40 g, 5.10 mmol) and Pd/C (10% on activated carbon, 240 mg) in MeOH (50 ml) under H₂ (1 atm) at room temperature for 2 h.
- 36 Filter through Celite. Concentrate the filtrate to a white wax and dissolve in dioxane (20 ml) and H₂O (20 ml).

- 37 Add NaHCO₃ (0.94 g, 11.2 mmol) and Fmoc-OSu (1.89 g, 5.61 mmol) and stir the mixture at room temperature for 12 h.
- 38 Remove dioxane and wash the remaining aqueous phase with Et₂O (2 × 50 ml).
- 39 Discard the combined Et₂O layers and acidify the aqueous phase to pH 4 with 1 N HCl.
- 40 Extract with EtOAc (2 × 100 ml), and dry the combined EtOAc layer over Na₂SO₄, filter and remove solvent. Purify by silica gel column chromatography (9:1 CH₂Cl₂/MeOH) to give (2*S*,4*R*)-1-(((9H-fluoren-9-yl)methoxy)carbonyl)-4-(((tert-butoxycarbonyl)amino)oxy)pyrrolidine-2-carboxylic acid **18** (TLC (CH₂Cl₂:MeOH 10:1 v/v) R_f = 0.20).

Synthesis of aminoxy platform **8d** (Refer Fig. 7) • **TIMING 3 d**

- 41 Weigh 0.10 mmol of NovaSyn®TGR (loading 0.20–0.30 mmol/g) resin
- 42 Place the resin into a Pierce disposable plastic column.
- 43 Add 5 ml DMF, cap the column, place it on tube shaker and agitate for 20 min to swell the resin.
- 44 Attach the column to a vacuum manifold and wash the resin with DMF (3 × 5 ml). Remove column from the vacuum.
- 45 Add 3 ml of 20% piperidine/DMF solution to the column and place it on the shaker and agitate for 20 min.
- 46 Repeat step 44.
- 47 Place Fmoc-Glu(O*t*Bu)-OH (212.8 mg, 0.5 mmol), HOBT (67.6 mg, 0.5 mmol), DIC (63.1 mg, 0.5 mmol) and 3 ml DMF in the column and agitate on the shaker for 2 h.
- 48 Repeat steps 44–46 to give **19**.
- 49 Repeat steps 47 and 48 to give **20**.
- 50 Place Fmoc-Pro-OH (168.7 mg, 0.5 mmol), HOBT (67.6 mg, 0.5 mmol), DIC (63.1 mg, 0.5 mmol) and 3 ml DMF in the column and agitate on the shaker for 2 h.
- 51 Repeat step 44–46 to give **21**.
- 52 Place Fmoc-Pro-OH (168.7 mg, 0.5 mmol), PyBroP (233.1 mg, 0.5 mmol) and 3 ml DMF in the column and agitate on the shaker for 4 h.
- 53 Repeat step 44.
- ▲ **CRITICAL STEP** Use double coupling for secondary amines.
- 54 Repeat step 52 and then steps 44–46 to give **22**.
- ? **TROUBLESHOOTING**
- 55 Place Fmoc-Ala-OH (155.7 mg, 0.5 mmol), PyBroP (233.1 mg, 0.5 mmol) and 3 ml DMF in the column and agitate on the shaker for 4 h.
- 56 Repeat step 44.
- 57 Repeat step 55 and then 44–46 to give **23**.

- 58** Place Fmoc-Thr(*t*Bu)-OH (198.8 mg, 0.5 mmol), HOBT (67.6 mg, 0.5 mmol), DIC (63.1 mg, 0.5 mmol) and 3 ml DMF in the column and agitate on the shaker for 2 h.
- 59** Repeat steps 44–46 to give **24**.
- 60** Place **18** (234.3 mg, 0.5 mmol), HOBT (67.6 mg, 0.5 mmol), DIC (63.1 mg, 0.5 mmol) and 3 ml DMF in the column and agitate on the shaker for 2 h.
- 61** Repeat steps 44–46 to give **25**.
- 62** Place Fmoc-Glu(O*t*Bu)-OH (212.8 mg, 0.5 mmol), 0.5 mmol PyBroP (233.1 mg, 0.5 mmol) and 3 ml DMF in the column and agitate on the shaker for 4 h.
- 63** Repeat step 44.
- 64** Repeat step 62 and then steps 44–46 to give **26**.
- 65** Repeat steps 50–51 to give **27**.
- 66** Place Fmoc-5-Ava-OH (169.7 mg, 0.5 mmol), HOBT (67.6 mg, 0.5 mmol), DIC (63.1 mg, 0.5 mmol), DIPEA (87 μ L, 0.5 mmol) and 3 ml DMF in the column and agitate on the shaker for 2 h.
- 67** Repeat step 44–46.
- 68** Place FITC (194.7 mg, 0.5 mmol), DIPEA (87 μ L, 0.5 mmol) and 3 ml DMF in the column and agitate on the shaker for 4 h.
- ▲ CRITICAL STEP** Run the FITC coupling reaction in the dark.
- 69** Attach the column to a vacuum manifold and wash the resin with DMF (5 ml $\times$ 3), MeOH (5 ml $\times$ 3), CH₂Cl₂ (5 ml $\times$ 3) and Et₂O (5 ml $\times$ 3). Remove from vacuum manifold.
- 70** Dry under high vacuum for 4 h to give **28**.
- 71** Cleave resultant resin by adding into the column a mixture of TFA/H₂O/TIS (5.0 ml, 9:5:5) agitate on the shaker for 2 h.
- 72** Attach the column to a vacuum manifold and clean Erlenmeyer flask. Collect the filtrate and wash the resin with TFA (3 $\times$ 5 ml).
- 73** Concentrate the filtrate using a rotary evaporator, precipitate crude with Et₂O and remove solvent.
- 74** Dissolve the resulting solid in 50% aqueous acetonitrile (5 ml) and purify by preparative HPLC to give **8d** as described in the Equipment Setup section.
- ▲ CRITICAL STEP** Aqueous acetonitrile contains 0.1% TFA.
- PAUSE POINT** The resultant peptide can be stored for extended period at –20 °C.

Oxime-based library diversification from aminooxy **1** • **TIMING 24 h**

- 75** Mix a solution of **1** (24 mM in DMSO, 30 μ L), aldehyde (24 mM in DMSO, 30 μ L), and AcOH (48 mM in DMSO, 30 μ L) in a 1.5 ml Sarstedt reaction tube and screw on the cap.
- 76** Agitate the mixture gently for 24 h at room temperature.
- 77** Use the resultant oxime solution **3** (8 mM in DMSO, 90 μ L) directly for inhibition assay.

■ **PAUSE POINT** The oxime solution can be stored extended long periods at a temperature of 2–8 °C.

Oxime-based library diversification from aminooxy **8d** • **TIMING 24 h**

78 Mix a solution of **8d** (15 mM in DMSO, 10 µl), aldehyde (15 mM in DMSO, 10 µl), and AcOH (70 mM in DMSO, 10 µl) in a 1.5 ml Sarstedt reaction tube and screw on the cap.

79 Agitate the mixture gently for 24 h at room temperature.

■ **PAUSE POINT** Perform HPLC analysis on random oxime products to examine completion of reaction. Products typically show >90% purity.

80 Use the resultant oxime solution **9** (5 mM in DMSO, 45 µL) directly for inhibition assays.

■ **PAUSE POINT** The oxime solution can be stored for extended periods at a temperature of –20 °C.

Dilution of YopH oxime inhibitors (Refer to Table 2 and Fig. 8) • **TIMING 15 min**

81 Place 12 micro test tubes (1.0 ml Titertube) in one row of a rack.

82 Place 25 µl of DMSO in each tube except for the first tube.

83 Place 38 µl of 8 mM oxime inhibitor **3** solution in the first tube.

84 Take 12.5 µl of inhibitor from the first tube and place it in the second tube containing 25 µl DMSO.

■ **PAUSE POINT** Mix well.

Final concentration = $(8 \text{ mM} \times 12.5 \text{ µl}) / 37.5 \text{ µl} = 2.67 \text{ mM}$ (or 8/3 mM)

85 Follow Table 2 to perform further dilutions listed for the first screen (wells A1–D12).

▲ **CRITICAL STEP** Do not add inhibitor to the last two tubes.

86 Place 12 micro test tubes (1.0 ml Titertube) in the second row of the rack.

87 Use the above dilution process for a second screen (wells E1–H12).

TROUBLESHOOTING

Troubleshooting advice can be found in Table 3.

Box.1 Screening of oxime inhibitors against YopH using 96-well plate (Refer to Fig. 8)

MATERIALS

REAGENTS

- 1.0 M *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid), HEPES solution, pH 7.0–7.6 (Aldrich, cat. no. H0887)
- *DL*-Dithiothreitol (Aldrich, cat. no. 43819)
- 0.5 M Ethylenediaminetetraacetic acid disodium salt (EDTA) solution (Aldrich, cat. no. E7889)
- Sodium chloride (Aldrich, cat. no. S7653)

- 30% Albumin solution from bovine serum (BSA), protease free (Aldrich, cat. no. A8577)
- *p*-Nitrophenyl phosphate disodium salt hexahydrate (*p*NPP) – 20 mg tablet (Aldrich, cat. no. N2765)
- YopH domain (residues 164–468) was expressed and purified in *Escherichia coli* according to a previously published procedure⁵²
- Millipore pure H₂O

EQUIPMENT

- 96-Well plates (Costar 96-well black clear-bottom plates; cat. no. 3603)
- Plate reader (Biotek Synergy 2)
- Well plate shaker (SynPep Corporation Shaker 30)
- 5–120 µl Single-channel Electronic pipette; Biohit (VWR, cat. no.14212-000)
- 10–300 µl Single-channel Electronic pipette; Biohit (VWR, cat. no.14212-002)
- 5–120 µl Multi-channel Electronic pipette; Biohit (VWR, cat. no. 47745-576)
- 10–300 µl Multi-channel Electronic pipette; Biohit (VWR, cat. no. 47745-578)
- Stand pipettor eLine charger F/4, Biohit (VWR, 14220-574)
- 5–350 µl, Nonsterile 10x96 tray Biohit pipette tips (FisherSci, cat. no. 21-166-10)
- Labcor pipettor solution basin, Biotix (VWR, cat. no. 21007-970)
- 0.6 ml General purpose microcentrifuge tubes (FisherSci, cat. no. 05-406-15)
- 1.5 ml General purpose microcentrifuge tubes (FisherSci, cat. no. 05-406-16)
- 1.0 ml Titertube micro test tubes (Biorad, cat. no. 223-9391)
- 1.5 ml Sarstedt screw cap microtube (FisherSci, cat. no. 50809238)
- 15 ml Conical polypropylene tube (Falcon, cat. no. 2097)

REAGENT SETUP

1.0 M *DL*-Dithiothreitol (DTT) 2.0 mmol in 2.0 ml Millipore pure H₂O. Use for HEPES buffer (see below). Distribute the remainder (1.0 ml) in 0.6 ml centrifuge tubes (25 µl/tube) and store at –78 °C.

HEPES buffer pH 7.2 Add 2.5 ml of 1.0 M HEPES solution (pH 7.0–7.6) to an Erlenmeyer flask. To this solution add 0.29 g NaCl, 1.0 ml of 1.0 M DTT solution, and 1.0 ml of 0.5 M EDTA solution. Bring to 100 ml by adding Millipore pure H₂O. **▲CRITICAL** Add 1 µl of fresh 1.0 M DTT solution per each 1.0 ml HEPES buffer solution immediately before each assay screen.

0.25% BSA solution Add 6.0 µl of 30% BSA solution in a 1.5 ml centrifuge tube and dilute with HEPES buffer pH 7.2 (714 µl).

10 mM *p*NPP solution Place 1 × 20 mg tablet of *p*NPP in a 15 ml conical culture tube and add 5.4 ml of HEPES buffer. **▲CRITICAL** *p*NPP hydrolyses when stored in solution for more than 2 weeks (–20 °C). Cover tube with aluminum foil at all times and keep on ice during experiments.

YopH Purified YopH domain (residues 164–468) was diluted to 1.0 mg/mL using HEPES buffer pH 7.2 and transfer aliquots of 25 µl of the enzyme solution to 0.6 ml centrifuge tubes and store at −78 °C.

EQUIPMENT SETUP

Biotek Synergy 2 plate reader setup Set the spectrometer to read 96-well plates to an absorbance of λ405 nm at a temperature of 25 °C.

Well plate shaker setup Set the shaker at a speed of 400 rpm at a temperature of 25 °C.

• TIMING 30 min

▲ **CRITICAL STEP** Use a total of 100 µl reaction volume per well.

- 1 Place 10 ml of HEPES buffer pH 7.2 in a Labcor pipettor solution basin.
▲ **CRITICAL STEP** Add a fresh solution of 1 µl DTT (1.0 M in H₂O) per each 1 ml solution of HEPES buffer before screening.
- 2 Using a multi-channel electronic pipette add 79 µl of HEPES buffer from the solution basin to each well.
- 3 Place 12 Biorad titertubes (1.0 ml Titertube) in one row on a rack.
- 4 Add 50 µl of 0.25% BSA to each of the titertubes.
- 5 Set the multi-channel electronic pipette to multi-pipetting format (8 × 5 µl).
- 6 Using the multi-channel pipette add 5 µl of 0.25% BSA on each row of the 96-well plate (Costar 3603).
- 7 Set the multi-channel electronic pipette to a single-pipetting format (5 µl).
- 8 Using the multi-channel pipette add 5 µl from the first titertube row of the diluted inhibitor to the first four rows of the 96-well plate (A1-D12 for screen 1).
- 9 Using the multi-channel pipette add 5 µl from the second titertube row of the diluted inhibitor to the last four rows of the 96-well plate (A1-H12 for screen 2).

▲ **CRITICAL STEP** The final concentration of the inhibitor in each well ($[I]_{\text{final}}$) can be calculated from the initial concentration of the inhibitor in the titertube ($[I]_{\text{initial}}$) using the formula below

$$[I]_{\text{final}} = \frac{([I]_{\text{initial}} \times V_{\text{initial}})}{V_{\text{final}}}$$

where V_{initial} = volume of inhibitor added to each well (5 µl)

V_{final} = total volume in each well (100 µl)

- 10 Dilute YopH stock solution (1.0 mg/ml, 25 µl) to 25 µg/ml by adding 975 µl of HEPES buffer pH 7.2.
- 11 Place 12 new titertubes (1.0 ml Titertube) in one row on a rack.

- 12 Add 50 µl of 25 µg/ml YopH solution to the first 11 titertubes.
- 13 Add 50 µl of HEPES buffer to the last titertube.
- ▲ CRITICAL STEP** The buffer in the last titertube is to be added to the last row in the 96-well plate (A12-H12). These wells are used only for substrate (*p*NPP) background readout.
- 14 Set the multi-channel electronic pipette to a multi-pipetting format (8 × 5 µl).
- 15 Using the multi-channel pipette add 5 µl of 25 µg/mL YopH on each row of the 96-well plate.
- 16 Read the background optical density (OD_{background}) using the Biotek Synergy 2 plate reader.
- ? TROUBLESHOOTING**
- 17 Place 12 new titertubes (1.0 ml Titertube) in one row on a rack.
- 18 Add 60 µl of 10 mM *p*NPP to each of the titertubes.
- ▲ CRITICAL STEP** The final *p*NPP concentration per well (0.6 mM) must be approximately equal to the substrate-YopH Michaelis-Menten constant ($K_M = 0.6$ mM).
- PAUSE POINT** Agitate the plate at room temperature for 10 min.
- 19 Read the reaction OD (OD_{reaction}) using the Biotek Synergy 2 plate reader.

Calculation of IC₅₀ value for YopH oxime-based inhibitors

- 20 Export OD values of the 96-well plate to Microsoft Excel.
- ▲ CRITICAL STEP** Export to two different excel files for each screen (wells A1-D12 and E1-H12).
- 21 Subtract OD_{background} values of each well from OD_{reaction} values of the same wells.
- 22 Average the resultant OD values for the same concentration (each column) to obtain OD_{average}.
- 23 Subtract the average OD value of *p*NPP (OD_{*p*NPP}; wells A12-D12 and E12-H12) from OD_{average} for each inhibitor concentration to obtain final OD values (OD_{final}).
- 24 Calculate the %inhibition of each inhibitor concentration using the formula below

$$\% \text{Inhibition} = \frac{(\text{OD}_{\text{final},0} - \text{OD}_{\text{final}})}{\text{OD}_{\text{final},0}} \times 100$$

where OD_{final,0} is the OD_{final} value at an inhibitor concentration = 0

- 25 Convert each inhibitor concentration to its log value (log[I]).
- 26 Transfer data points (log[I] and corresponding %inhibition) to a new workbook within Origin software v8.0951.

- 27 Plot a scatter graph using % Inhibition vs. log[I].
- 28 Calculate the IC₅₀ values by a non-linear curve fit using the Boltzmann equation.
- 29 Check reproducibility of IC₅₀ values for each screen.

? TROUBLESHOOTING

Box 2: Screening of oxime inhibitor against Tsg101 (Refer to Fig. 9)

MATERIALS

REAGENTS

- 1.0 M HEPES pH 7.5 (Invitrogen, cat. no. 15630)
- 5.0 M Sodium chloride (Quality Biological, cat. no. 351-036-101)
- 2-Mercaptoethanol (Sigma, cat. no. M-3148) **! CAUTION Toxic**
- Tris(2-carboxymethyl)phosphine hydrochloride (TCEP) (Pierce, cat. no. 20490)
- Tsg101 UEV domain with C-terminal His-Tag (hTsg101-UEV-His) was expressed in Escherichia coli according to a previously published procedure⁵³
- Millipore pure H₂O

EQUIPMENT

- 5–120 µl Single-channel Electronic pipette; Biohit (VWR, cat. no.14212-000)
- 10–300 µl Single-channel Electronic pipette; Biohit (VWR, cat. no.14212-002)
- 5–120 µl Multi-channel Electronic pipette; Biohit (VWR, cat. no. 47745-576)
- 10–300 µl Multi-channel Electronic pipette; Biohit (VWR, cat. no. 47745-578)
- Stand pipettor eLine charger F/4, Biohit (VWR, 14220-574)
- 5–350 µl, Nonsterile 10x96 tray Biohit pipette tips (FisherSci, cat. no. 21-166-10)
- Labcor pipettor solution basin, Biotix (VWR, cat. no. 21007-970)
- 1.5 ml Sarstedt screw cap microtube (FisherSci, cat. no. 50809238)
- 96-Well plates (Costar 96-well V bottom clear plates, cat. no. 3363)
- 384-Well plates (Costar 384-well low volume, Flat Bottom, low bind, black polystyrene plates, cat. no. 3820)
- Tecan Ultra Plate Reader (Tecan)

REAGENT SETUP

10x HEPES-buffered saline (HBS) Add 100 ml of 1.0 M Hepes, pH 7.5 and 300 ml of 5.0 M NaCl to a final volume of 1,000 ml of millipore pure H₂O.

500 mM TCEP Dissolve 143 mg of TCEP in 1.0 ml of millipore pure H₂O. Store this in 100 °l aliquots at 4 °C.

10 mM HEPES pH 7.5, 150 mM NaCl, 5 mM β-mercaptoethanol, 100 µM TCEP Take 2.0 ml of the 10x HBS stock, 7.0 µL of β-Mercaptoethanol, 4.0 µl of TCEP in a final volume of 20 ml millipore pure H₂O.

Tsg101 Prepare aliquots of 100 μ l of 450 μ M protein solution in 0.6 ml centrifuge tubes and store for a long time at -78°C .

▲ CRITICAL Prepare this buffer should the same day it will be used and then discard.

EQUIPMENT SETUP

Tecan Ultra plate reader setup Set the excitation wavelength to 485 nm and the emission wavelength to 535 nm. Measure the gain from the well that contains the unbound peptide oxime. Illuminate each well with 10 flashes and integrate for 40 μ s. Determine the G-factor from a standard solution of 1 nM fluorescein. Measure the plate at ambient temperature (25°C).

● TIMING 2 h

- 1 Take the fluorescein labeled peptide oxime (**9**, 5.0 mM in DMSO) and dilute to 5.0 μ M in 1x HBS.
- 2 From the 5.0 μ M stock (6 μ l) make a 50 nM solution by adding 595 μ l of 10 mM Hepes, pH 7.5, 150 mM NaCl, 100 μ M TCEP, 5 mM mercaptoethanol.
- 3 Aliquot oxime **9** (50 μ l of the 50 nM) across 11 wells of a row in the 96-well plate (Costar 3363). Place in each of the 7 remaining rows a different fluorescein labeled peptide oxime.
- 4 In the column 12 of the well (A12-H12) add 78 μ l of 50 nM of peptide oxime and then add 22 μ l of Tsg101 stock into this well for a final concentration of 100 μ M.
▲ CRITICAL STEP Mix well by pipetting up and down.
- 5 From the column 12 transfer using a multichannel pipette 50 μ l from the Tsg101–**9** mixture to the column 11 of the well (A11-H11) across the plate.
- 6 Continue to do this for each column across the entire plate. This results in a serial dilution of Tsg101 and a fixed concentration of 50 nM of the labeled peptidomimetic.
- 7 Seal the plate, cover with aluminum foil and allow the mixture to agitate and equilibrate for 10mins at room temperature.
- 8 Remove 40 μ l from each well with Tsg101–oxime mixture and place it into a 384-well plate (Costar 3820) for the measurement of anisotropy of the bound peptide oxime with Tsg101 (A_{bound}).
- 9 Transfer 40 μ l of a 50 nM solution of each free peptide oxime (without added Tsg101) to the 384-well plate for the measurement of anisotropy of the unbound/background peptide oxime (A_{free}).
- 10 Read the plate using the Tecan Ultra plate reader.
- 11 The fluorescence anisotropy, polarization and intensity values for each well are reported by the Tecan Ultra software.
- 12 The fluorescence anisotropy data are plotted against the concentration of Tsg101 using MatLab software and the binding curves fit using the following equation:

$$An(C) = \frac{((R \cdot An_{bound} - An_{free}) \cdot ((K_d + C + Lt) - [(K_d + C + Lt)^2 - 4Lt \cdot C]^{1/2}) / 2Lt) + An_{free}}{An_{free}}$$

where An(C) is the measured anisotropy as a function of concentration, Lt is the fluorescein-labeled peptidomimetic concentration, An_{free} is the anisotropy of unbound fluorescein-labeled peptidomimetic, An_{bound} is the anisotropy of the bound fluorescein-labeled peptidomimetic solution, R is the ratio of the fluorescence intensity of bound fluorescein-labeled peptidomimetic relative to free fluorescein-labeled peptidomimetic, C is the Tsg101 concentration, and K_d is the measured dissociation constant resulting from the fit.

Troubleshooting advice can be found in Table 3.

ANTICIPATED RESULTS

Analytical data

Compound 10. Yield = 96%

¹H NMR (400 MHz, CDCl₃): δ 7.71 (s, 1H), 7.58 (m, 1H), 7.52 (m, 1H), 7.29 (m, 1H), 4.19 (m, 4H), 1.29 (m, 6H).

¹³C NMR (400 MHz, CDCl₃): δ 133.86 (1C), 129.99 (1C), 129.97 (1C), 129.85 (1C), 129.25 (1C), 129.97 (1C), 122.40 (1C), 64.92 (1C), 64.85 (1C), 16.29 (1C), 16.24 (1C).

APCI-MS (*m/z*): [M+H]⁺ calcd for C₁₁H₁₄BrF₂O₃P, 342.0 and 344.0; found, 343.0 and 345.0.

Compound 11. Yield = 57%

¹H NMR (400 MHz, CDCl₃): δ 7.83 (m, 1H), 7.79 (m, 1H), 7.58 (m, 3H), 7.52 (m, 1H), 7.43 (m, 2H), 4.71 (s, 2H), 4.21 (m, 4H), 2.42 (s, 1H), 1.31 (m, 6H).

¹³C NMR (400 MHz, CDCl₃): δ 141.15 (1C), 140.78 (1C), 139.12 (1C), 133.11 (1C), 132.98 (1C), 129.36 (1C), 128.90 (1C), 127.40 (2C), 127.16 (2C), 124.95 (1C), 124.73 (1C), 64.90 (1C), 64.83 (1C), 16.31 (1C), 16.26 (1C).

ESI-MS (*m/z*): [M+Na]⁺ calcd for C₁₈H₂₁F₂O₄P, 370.11; found, 393.20.

Compound 12. Yield = 79%

¹H NMR (400 MHz, CD₃OD): δ 7.81 (m, 2H), 7.59 (m, 4H), 7.46 (m, 2H), 4.73 (s, 2H), 4.20 (m, 4H), 1.30 (m, 6H).

¹³C NMR (400 MHz, CD₃OD): δ 141.17 (1C), 139.31 (1C), 137.26 (1C), 132.90 (1C), 132.76 (1C), 129.29 (1C), 129.27 (1C), 128.63 (2C), 128.23 (1C), 126.64 (2C), 124.63 (1C), 124.24 (1C), 76.98 (1C), 65.14 (1C), 65.07 (1C), 15.27 (1C), 15.21 (1C).

APCI-MS (*m/z*): [M+H]⁺ calcd for C₁₈H₂₂F₂NO₄P, 385.13; found, 386.10.

Aminoxy 1. Yield = 52%

Retention time = 15.598 min

^1H NMR (400 MHz, DMSO- d_6): δ 7.69-7.73 (m, 2H), 7.56 (m, 2H), 7.49 (m, 2H), 7.36 (m, 2H), 4.70 (s, 2H).

HRMS-ESI (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{14}\text{H}_{14}\text{NO}_4\text{F}_2\text{P}$, 330.0701; found, 330.0694.

In situ synthesis of inhibitor 3. Purity = 94% (Fig. 10a, left).

Retention time: 19.303 min (Fig. 10a, left).

HRMS-ESI (m/z): $[\text{M} - \text{H}]^-$ calcd for $\text{C}_{20}\text{H}_{16}\text{F}_2\text{NO}_7\text{P}$, 450.0560; found, 450.0562.

^1H NMR (400 MHz, CD_3OD): δ 8.07 (s, 1H), 7.77 (s, 1H), 7.67 (d, $J = 7.2$ Hz, 1H), 7.56-7.59 (m, 2H), 7.41-7.52 (m, 5H), 7.22, 7.16 (d, $J = 3.6$ Hz, 1H), 7.18, 6.76 (d, $J = 3.6$ Hz, 1H), 5.26, 5.17 (s, 2H).

^{13}C NMR (400 MHz, CD_3OD): δ 159.78 (1C), 150.55 (1C), 147.54 (1C), 145.59 (1C), 140.75 (1C), 139.89 (1C), 139.02 (1C), 136.89 (1C), 135.45 (1C), 128.55 (2C), 128.46 (1C), 128.44 (1C), 126.75 (1C), 126.66 (2C), 124.52 (1C), 118.78 (1C), 112.77 (1C), 76.01 (1C).

Compound 13. Yield = 96%

$[\alpha]_{\text{D}} -53.2$ (c 2.02, CHCl_3 , 20 °C)

^1H NMR (400 MHz, CDCl_3): δ 7.32-7.18 (m, 10H), 5.23-5.11 (m, 2H), 5.01-4.95 (m, 2H), 4.53 (m, 1H), 4.40 (m, 1H), 3.65-3.51 (m, 2H), 2.91 (br s, 1H), 2.26 (m, 1H), 2.02 (m, 1H).

Compound 14. Yield = 100%

$[\alpha]_{\text{D}} -63.8$ (c 1.36, CHCl_3 , 20 °C)

^1H NMR (400 MHz, CDCl_3): δ 8.16 (AB, $J_{\text{AB}} = 8.8$, 2.0 Hz, 2H), 8.03-7.97 (m, 2H), 7.38-7.28 (m, 5H), 7.22-7.15 (m, 5H), 5.58 (m, 1H), 5.25-5.02 (m, 4H), 4.73 (dd, $J = 9.2$, 1.6 Hz, 0.5H), 4.63 (dd, $J = 9.2$, 1.6 Hz, 0.5H), 3.92-3.85 (m, 2H), 2.66-2.46 (m, 2 H).

HRMS-ESI/APCI (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{27}\text{H}_{25}\text{N}_2\text{O}_8$, 505.1611; found, 505.1606.

Compound 15. Yield = 83%

$[\alpha]_{\text{D}} -17.1$ (c 2.56, CHCl_3 , 20 °C)

^1H NMR (400 MHz, CDCl_3): δ 7.35-7.24 (m, 10H), 5.30-5.00 (m, 4H), 4.49 (dd, $J = 9.6$, 1.6 Hz, 0.4H), 4.42 (dd, $J = 9.6$, 1.6 Hz, 0.6H), 4.34 (m, 1H), 3.72-3.57 (m, 2H), 3.27 (br s, 1H), 2.29 (m, 1H), 2.12 (m, 1H).

^{13}C NMR (100 MHz, CDCl_3): δ 174.12, 174.04, 154.98, 154.31, 136.36, 136.25, 135.23, 135.04, 128.58, 128.48, 128.44, 128.37, 128.27, 128.15, 128.08, 128.03, 127.90, 127.84, 70.97, 70.00, 67.46, 67.42, 67.38, 67.34, 67.25, 58.25, 57.88, 55.91, 55.57, 38.68, 37.80.

Compound 16. Yield = 95%

$[\alpha]_{\text{D}} -44.1$ (c 1.09, CHCl_3 , 20 °C)

^1H NMR (400 MHz, CDCl_3): δ 7.85-7.82 (m, 2H), 7.77-7.75 (m, 2H), 7.40-7.18 (m, 10H), 5.26-5.14 (m, 3 H), 4.99-4.96 (m, 2H), 4.78 (m, 1H), 4.08 (m, 1H), 3.75 (dd, J = 12.8, 4.0 Hz, 1H), 2.71 (m, 1H), 2.15 (m, 1H).

HRMS-ESI/APCI (m/z): $[\text{M}+\text{Na}]^+$ calcd for $\text{C}_{28}\text{H}_{24}\text{N}_2\text{NaO}_7$, 523.1481; found, 523.1473.

Compound 17. Yield = 81%

$[\alpha]_{\text{D}} -30.4$ (c 1.40, CHCl_3 , 20 °C)

^1H NMR (400 MHz, CDCl_3): δ 7.85 (s, 0.56H), 7.67 (s, 0.44H), 7.30-7.10 (m, 10H), 5.19-5.09 (m, 2H), 5.02-4.94 (m, 2H), 4.55-4.45 (m, 2H), 3.93 (d, J = 12.4 Hz, 0.56H), 3.82 (d, J = 12.4 Hz, 0.44H), 3.56 (m, 1H), 2.55 (m, 1H), 1.99 (m, 1H), 1.41 (s, 9H).

HRMS-ESI/APCI (m/z): $[\text{M}+\text{Na}]^+$ calcd for $\text{C}_{25}\text{H}_{30}\text{N}_2\text{NaO}_7$, 493.1951, found, 493.1968.

Compound 18. Yield = 86%

$[\alpha]_{\text{D}} -41.2$ (c 0.97, CHCl_3 , 20 °C)

^1H NMR (400 MHz, CDCl_3): δ 7.76 (d, J = 7.6 Hz, 1H), 7.69 (d, J = 7.6 Hz, 1H), 7.59-7.51 (m, 2H), 7.41-7.25 (m, 4.35H), 7.18 (0.65H), 4.53-4.33 (m, 4H), 4.24 (t, J = 6.8 Hz, 0.65H), 4.13 (t, J = 6.8 Hz, 0.35H), 3.96 (d, J = 12.6 Hz, 0.35H), 3.85 (d, J = 12.6 Hz, 0.65H), 3.53 (m, 1H), 2.23 (m, 0.65H), 2.10 (m, 0.35H), 1.49 (s, 5.5H), 1.46 (s, 3.5H). HRMS-ESI/APCI (m/z): $[\text{M}+\text{Na}]^+$ calcd for $\text{C}_{25}\text{H}_{28}\text{N}_2\text{NaO}_7$, 491.1794; found, 491.1800.

Aminoxy 8d. Yield = 45%

Retention time = 20.479 min

ESI-MS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{68}\text{H}_{86}\text{N}_{13}\text{O}_{23}\text{S}$, 1484.6; found, 1484.6; $[\text{M}+2\text{H}]^{2+}$ calcd 742.0; found 742.0.

In situ synthesis of inhibitor 9. Purity = 98% (Fig. 10b, left).

Retention time: 21.636 min (Fig. 10b, left)

ESI-MS (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{77}\text{H}_{94}\text{N}_{13}\text{O}_{25}\text{S}$, 1632.6; found, 1632.6; $[\text{M}+2\text{H}]^{2+}$ calcd 828.0; found 828.0.

YopH inhibitors

The oximes resulting from the synthesis described in steps **75–77** are analyzed by HPLC to determine that they are of sufficient purity (Fig. 10a, left). In the example results shown, the products were directly diluted using DMSO to obtain 10 different concentrations (Fig. 8) and were transferred and evaluated with YopH in standard 96-well plates. From this oxime library, we were able to identify a potent inhibitor **3** with an IC_{50} value of $0.19 \mu\text{M} \pm 0.01$ (Fig. 10a, right). *p*NPP is a ubiquitous PTP substrate against YopH was used as a control to measure the specific activity for the enzyme. Substrate assays were performed under the same standard conditions as used for the inhibition assays. The Michaelis-Menten constant (K_{M}) for *p*NPP, which is a parameter used to measure the rate of substrate hydrolysis by the enzyme, was found to be 0.60 ± 0.05 mM. Two independent assays were run and the inhibitory activities were found to be reproducible. Compound **3** was also tested against a panel of PTPs and was found to be more than 2,000 fold selective except for PTP1B with 11.7 fold selectivity.³⁴

Antagonists for Tsg101 HIV-1 Gag interaction

The reactions described in steps **78–79** of the procedure can be completed in 24 h resulting in peptidic oximes with excellent purity (Fig. 10b, left) for direct Tsg101 fluorescence anisotropy binding assay. The wild type nonamer of sequence FITC-Ava-PEPTAPPEE ($K_d = 48.4 \mu\text{M}$) was used as a positive control. The most potent inhibitor **9** showed a K_d value of $3.3 \pm 0.1 \mu\text{M}$ (Fig. 10b, right) and the inhibitory activity was found to be reproducible with screening employing two independent assays.

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Figure 1.

Oxime-based tethering. a) Reaction scheme showing use of inexpensive aldehyde as building block and AcOH as catalyst. Members of the generated oxime library are of sufficient purity that they can be evaluated biologically without purification. b) Aminooxy residues used in the synthesis of peptides shown in Table 1. RT, room temperature.

a **Figure 2.**

a) Crystal structure of the aminooxy platform **1** with the catalytic domain of YopH (PDB: 2Y2F). b) Utilization of oxime-based tethering for the design of YopH inhibitor **3**. The binding mode shown is based on *in-silico* studies resulting in multiple interactions with residues outside the catalytic pocket.

Figure 3.
Structure of aldehydes used in post solid-phase peptide library diversification.

Figure 4.

Structure of peptide oxime **9**, which exhibited a 14.7 fold Tsg101 binding enhancement as compared to the wild type peptide nanomer FITC-Ava-PEPTAPPEE.

Figure 5.

Synthesis of aminooxy-containing **1**.³⁴ Reagents and conditions: (i) Zn, diethyl (bromodifluoromethyl)-phosphonate, CuBr, DMF; (ii) 4-(hydroxymethyl)phenylboronic acid, Pd(PPh₃)₄, sat. K₂CO₃, EtOH, PhMe; (iii) (a) *N*-hydroxyphthalimide, PPh₃, DIAD, THF; (b) NH₂NH₂·H₂O, EtOH; (iv) TMSBr, CH₂Cl₂.

Figure 6.

Synthesis of aminoxy-protected proline **18**³⁵. Reagents and conditions: (i) (a) CbzCl, NaHCO₃, H₂O, THF; (b) BnBr, Et₃N, THF; (ii) *p*-Nitrobenzoic acid, DEAD, PPh₃, THF; (iii) NaN₃, 15-crown-5, MeOH; (iv) *N*-hydroxyphthalimide, DEAD, PPh₃, THF; (v) (a) NH₂NH₂·H₂O, EtOH (b) Boc₂O, Et₃N, THF; (vi) (a) H₂, Pd/C, EtOH; (b) Fmoc-Osu, NaHCO₃, H₂O, dioxane.

Figure 7.

Synthesis of **8d**.³⁶ (i) (a) Amino acid, HOBt, DIC, DIEPA, DMF; (b) 20% piperidine in DMF; (ii) (a) Amino acid, PyBroP, DIEPA, DMF; (b) 20% piperidine in DMF; (iii) (a) Fmoc-Ava-OH, HOBt, DIC, DIPEA, DMF; (b) 20% piperidine in DMF; (c) Fluorescein isothiocyanate (FITC), DIPEA, DMF; (iv) TFA/H₂O/TIS.

Figure 8.
 Schematic representation of oxime-based synthesis, dilution, and inhibition assay against YopH.

Figure 9.
Schematic representation of oxime-based synthesis and inhibition assay against Tsg101.

Figure 10.

a) Analytical HPLC and IC_{50} curve for inhibitor **3**. b) Analytical HPLC and K_d curve for inhibitor **9**.

Table 1Aminooxy-containing parent peptides.³⁶

No	Peptide sequence	Aminooxy-Residue
8a	FITC-Ava- <u>P</u> EPTAPPEE	7
8b	FITC-Ava- <u>P</u> EPTAPPEE	6
8c	FITC-Ava- <u>P</u> EPTAPPEE	5
8d	FITC-Ava-PE <u>P</u> TAPPEE	7
8e	FITC-Ava-PE <u>P</u> TAPPEE	6
8f	FITC-Ava-PE <u>P</u> TAPPEE	4
8g	FITC-Ava-PEPTA <u>P</u> PEE	7
8h	FITC-Ava-PEPTA <u>P</u> PEE	6
8i	FITC-Ava-PEPTA <u>P</u> PEE	7
8j	FITC-Ava-PEPTA <u>P</u> PEE	6
8k	FITC-Ava-PEPTAP <u>P</u> EE	5
8l	FITC-Ava-PEPTAP <u>P</u> EE	5

Table 2

Dilution process for YopH oxime inhibitors.

Dilution of 8 mM oxime/ screen	Volume	
	Inhibitor (μL)	DMSO (μL)
8/1	38	
8/3	12.5	25
8/9	12.5	25
8/27	12.5	25
8/81	12.5	25
8/243	12.5	25
8/729	12.5	25
8/2187	12.5	25
8/6561	12.5	25
8/19683	12.5	25
0		25
0		25

Table 3

Troubleshooting table.

Step	Problem	Possible reasons	Possible solutions
6	Low yield of intermediate 10	Fast addition of reagents	Add (bromodifluoromethyl)phosphonate and bromo-3-iodobenzene slowly over the course of 30 min each.
9	Low yield of intermediate 11	Long reaction time and high temperature	Run reaction for 5 h at 50–55 °C.
14	Low yield of intermediate 12	Starting material not soluble in EtOH	Use dichloromethane instead of EtOH
54	Low yield of peptide bound resin 22	Steric hindrance Pro to Pro coupling	Use triple coupling
102	Inconsistent OD values of the same inhibitor concentration	Non-homogenous distribution of inhibitor and YopH	Agitate the 96-well plate for 1–2 min to mix well
116	Irreproducible IC ₅₀ values	Non-homogenous distribution of inhibitor, YopH, and pNPP	Agitate the 96-well plate to mix well