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Tripeptide Inhibitors of *Yersinia* Protein-Tyrosine Phosphatase

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Abstract—The protein-tyrosine phosphatase (PTP) 'YopH' is a virulence factor of *Yersinia pestis*, the causative agent of plague. Potential use of *Yersinia* as a bioterrorism agent renders YopH inhibitors of therapeutic importance. Previously, we had examined the inhibitory potencies of a variety of phosphotyrosyl (pTyr) mimetics against the human PTP1B enzyme by displaying them in the EGFR-derived hexapeptide sequence, 'Ac-Asp-Ala-Asp-Glu-Xxx-Leu-amide', where Xxx = pTyr mimetic. The poor inhibitory potencies of certain of these pTyr mimetics were attributed to restricted orientation within the PTP1B catalytic pocket incurred by extensive peripheral interaction of the hexapeptide platform. Utilizing the smaller tripeptide platform, 'Fmoc-Glu-Xxx-Leu-amide' we demonstrate herein that several of the low affinity hexapeptide-expressed pTyr mimetics exhibit high PTP1B affinity within the context of the tripeptide platform. Of particular note, the mono-anionic 4-(carboxydifluoromethyl)Phe residue exhibits affinity equivalent to the di-anionic F_2Pmp residue, which had previously been among the most potent PTP-binding motifs. Against YopH, it was found that all tripeptides having Glu residues with an unprotected side chain carboxyl were inactive. Alternatively, in their Glu-OBn ester forms, several of the tripeptides exhibited good YopH affinity with the mono-anionic peptide, Fmoc-Glu(OBn)-Xxx-Leu-amide, where Xxx = 4-(carboxymethyloxy)Phe providing an IC_{50} value of 2.8 μM. One concern with such inhibitors is that they may potentially function by non-specific mechanisms. Studies with representative inhibitors, while failing to provide evidence of a non-specific promiscuous mode of inhibition, did indicate that non-classical inhibition may be involved.

Plague is a disease caused by *Yersinia pestis*, a gram-negative bacillus that spreads via lymphatics to lymph nodes and then to the bloodstream from which it involves all organs. Upon infection, common syndromes include bubonic, pneumonic, and septicemic plague.[1,2] *Yersinia* has been identified as a potential organism for use in bioterrorism (with pneumonic being the most likely to be used for such purposes). The pathogenicity of *Yersinia* is absolutely dependent on the activity of a bacterial virulence factor called YopH, a protein-tyrosine phosphatase (PTP) that disrupts host signal transduction processes.[3] Accordingly, inhibitors of YopH could be useful as anti-bioterrorism agents.

Included among the few reports of YopH inhibitors are RNA aptamers that bind near the active site[4] and α-ketocarboxylic acid-containing aromatics.[5,6] In our own efforts to develop PTP inhibitors, we have taken advantage of the fact that key elements of PTP substrate recognition are often provided by phosphotyrosyl (pTyr) residues.[7–9] Accordingly, we had previously prepared a number of novel pTyr mimetics that we examined in PTP1B assays after displaying within the context of an 'Ac-Asp-Ala-Asp-Glu-Xxx-Leu-amide' sequence (Xxx = pTyr mimetic).[10–14] One unexpected finding from these studies was the very poor inhibitory potencies of several analogues including both mono- and di-anionic phosphate mimetics. This was explained as potentially reflecting limitations placed on functional group orientation within the catalytic pocket due to extensive protein–ligand interactions incurred by the hexapeptide platform.[14] In an effort to develop peptide leads for inhibition of YopH, we examined a similar set of pTyr mimetics, however the truncated tripeptide 'Fmoc-Glu-Xxx-Leu-amide' was utilized as a display platform. The results of this work are reported herein.

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Peptide Synthesis

Syntheses of tripeptides were accomplished using Rink amide resin[15] under standard Fmoc-based solid-phase protocols. For incorporation of pTyr-mimicking residues, the previously reported protected amino acid analogues **1**,[16] **2**,[13] **3**,[17] **4**,[12] **5**,[17] **6**,[14] **7**,[13] **8**,[18] **9**,[14] **10**,[19] and **11**[13] were employed (Fig. 1). Final global deprotection and cleavage from the resin was accomplished in the presence of TFA. For peptide **13**, pretreatment with pyridinium hydrogen fluoride in THF:NMP (1:1) for 1 h was utilized prior to TFA treatment. Use of tetrabutylammonium fluoride (1 M in THF) resulted in concomitant *N*-Fmoc cleavage. For peptide **20**, *O*-deacetylation was accomplished using *n*-butylamine prior to TFA-mediated cleavage from the resin. In the case of peptide **19**, *N*-Fmoc-L-F_2Pmp-OH (**8**) was coupled as its free phosphonic acid using benzotriazole-1-yloxy-tris(dimethylamino)phosphonium hexafluoro-phosphate (BOP) *N,N*-diisopropylamine and HOBt in NMP. Synthesis of **22c** was accomplished using Cbz-Glu(O^{t}Bu)-OH. For the preparation of N-terminal phenethyl-containing **22d**, piperidine-mediated *N*-Fmoc deprotection was followed by capping using a solution chloroformate generated by in situ reaction of phenethyl alcohol and phosgene in the presence of *N*-methylmorpholine (NMM).[20] Final tripeptide-products were purified to homogeneity by preparative HPLC.[21]

Protein Expression and Purification

PTP1B[22] and the *Yersinia* PTPase (YopH)[23] were expressed in *E. coli* BL21 (DE3) cells and purified according to the previously published procedures.

Enzyme Inhibition Assays

All assays were done at 30 °C and pH 7.0 using *p*-nitrophenyl phosphate (*p*NPP) (Fluka) as the substrate in 100 μL reaction volumes in 96-well microplates (Corning). The buffer used was 50 mM 3,3-dimethyl glutarate, 1 mM EDTA, with the ionic strength adjusted to 150 mM with sodium chloride. Assays were initiated by adding an appropriate amount of enzyme and quenched with 25 μL 5 M sodium hydroxide. The absorbance was read at 405 nm using a SpectraMax 340 plate reader from Molecular Devices. For each compound, the IC_{50} determination was done with the *p*NPP concentration fixed at the experimentally determined K_m value for each enzyme. Six to eight concentrations bracketing the IC_{50} value were used for each compound. The IC_{50} value was determined by plotting the relative pNPP activity versus inhibitor concentration and fitting to eq 1 using GraFit.

$$V_i = V/\left[1 + ([I]/IC_{50})^S\right] + B \quad (1)$$

In this case, V_i is the reaction velocity when the inhibitor concentration is [I], B is background, V is the velocity in the absence of inhibitor, V_0 minus the value of B, and s is the slope factor.

Results of Enzyme Inhibition Assays

Development of YopH inhibitors is of particular interest due to the key role this enzyme plays in the pathogenesis of the potential bioterrorism agent, *Yersinia pestis*. Our approach toward inhibitor design is predicated on our previous studies directed at development of PTP1B inhibitors that examined a variety of pTyr mimetics displayed within the context of a common hexapeptide platform.[10–14,24] The current study differs from these earlier efforts in that the tripeptide sequence 'Glu-Xxx-Leu-amide' (where Xxx = pTyr mimetic) was used rather than the full EGFr-derived hexapeptide sequence 'Asp-Ala-Asp-Glu-Xxx-Leu.' We had previously speculated that use of the latter extended sequence may potentially limit the orientation of pTyr-mimicking residues within the catalytic site, and thereby provide deceptively low potencies for pTyr mimetics.[14]

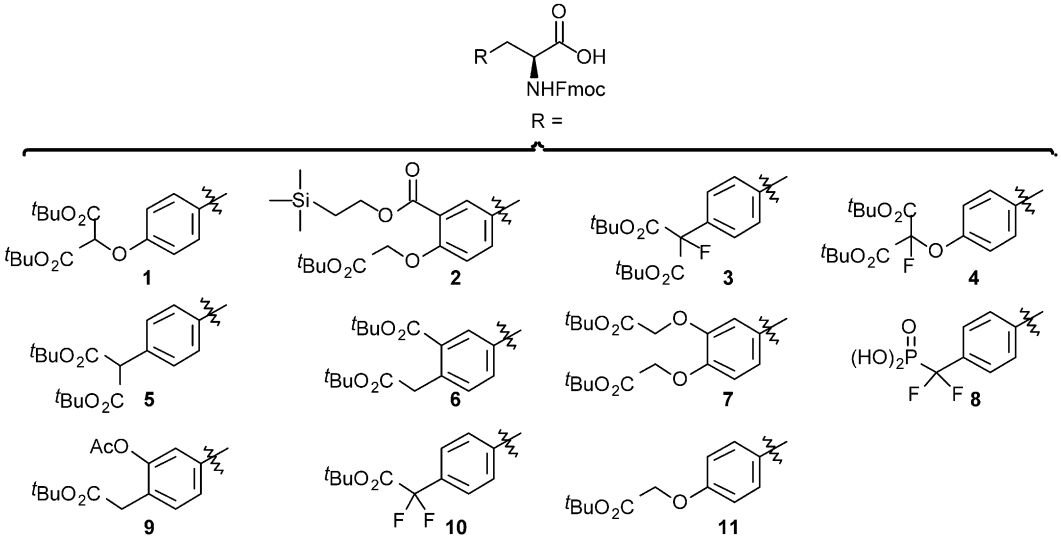


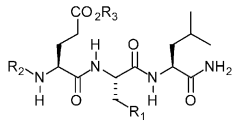
Figure 1. Structures of pTyr mimetic reagents used for solid-phase synthesis of tripeptide products.

The current study examined tripeptide inhibitors in both YopH and PTP1B systems in order to allow comparison with the original PTP1B hexapeptide data, and thereby provide some indication of effects of display platform on pTyr mimetic inhibitory potency. Besides shorter sequence length, some peptides in our current study also differ from our earlier hexapeptides in having an N-terminal Fmoc group (rather than an *N*-acetyl group). This was based on the work of Hamilton who demonstrated its potential utility in PTP-directed peptides.[25] Another distinction is that unlike our former hexapeptides, which contained Glu residues having unprotected side chain carboxyl groups, certain of the current tripeptides have Glu residues derivatized as their O-Bn esters.

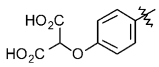
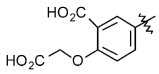
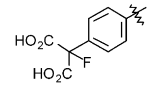
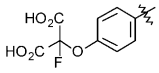
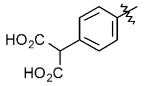
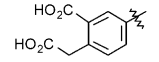
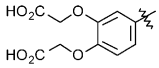
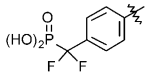
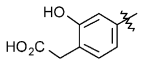
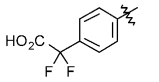
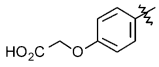
As shown in Table 1, peptides **12**, **13**, and **15** that contain dicarboxy-based pTyr mimetics as well as the F_2Pmp-containing **19**, showed potent inhibition of PTP1B in agreement with previous results obtained using the hexapeptide platform, where inhibition values of 10,[11] 19,[14] 1[12] and 0.2 μM[10] were reported, respectively. Surprisingly, dicarboxy pTyr mimetic-containing tripeptides **14**, **16**, and **17** that previously showed PTP1B inhibition values of 430, 1500, and 3700 μM in hexapeptide platforms,[14] all exhibited sub-micromolar PTP1B IC_{50} values in the current assay. Additionally, dicarboxy pTyr mimetic-containing **18** also provided good PTP1B inhibition in the context of a tripeptide platform, while an IC_{50} value of 800 μM was observed with the hexapeptide.[13] Finally, monocarboxy pTyr mimetics all showed good PTP1B inhibitory potency in tripeptides **20**, **21**, and **22**, while giving extremely poor PTP1B inhibition when incorporated into the hexapeptide platform (showing hexapeptide IC_{50} values of 13,000, 650, and 1200 μM, respectively[14]). Of particular note, the mono-anionic carboxydifluoromethyl-containing pTyr mimetic (in **21a**) provided PTP1B inhibitory potency equivalent to that observed for the di-anionic F_2Pmp-containing **19b**. These results show the potential PTP-directed utility not only of 4-(carboxydifluoromethyl)Phe[19] but also of a number of other pTyr mimetics that had previously been considered of limited promise based on results from hexapeptide studies.

Although discrepancies in PTP1B inhibitory potencies for pTyr mimetics resulting from peptide display platform are important observations in their own right, the focus of the current study is application of these pTyr mimetics to YopH-directed inhibitors. As shown in Table 1, a close concordance of YopH and PTP1B inhibitory potencies was found for tripeptides containing Glu residues derivatized as their OBn esters ('b' series peptides). While the corresponding peptides having Glu residues with unprotected carboxyl side chains ('a' series) also showed good PTP1B inhibitory potencies, these peptides uniformly exhibited poor YopH inhibitory potencies. Therefore, the OBn ester is a key component of YopH recognition for these tripeptides. The lack of YopH/PTP1B selectivity for Glu(OBn)-containing peptides is not necessarily a source of concern, since in physiological contexts inhibition of PTP1B may not be dissadvantageous. The more general issue of selectivity against other PTPs was not a subject for investigation within the current study.

Table 1. Evaluation of inhibitory potencies of synthetic tripeptides in YopH and PTP1B assays[a]



a R_3 = H; R_2 = Fmoc **d** R_3 = Bn; R_2 =
b R_3 = Bn; R_2 = Fmoc
c R_3 = Bn; R_2 = Cbz

R_1	No.	YopH (IC_{50} μM)	PTP1B (IC_{50} μM)
	**12a**	≫800	K_i = 29
	12b	82±16	116±20
	**13a**	>100	54.7±15.3
	13b	10.5±3.9	8.3±2.1
	**14a**	≫800	0.7
	14b	3.1±0.17	3.1±1.0
	**15b**	2.9±0.4	1.5±0.5
	**16a**	≫800	0.8
	**17a**	≫800	0.7
	17b	142±51	44±13
	**18b**	19.4±33	19.4±3.6
	**19b**	5.2±2.2	1.41±0.25
	**20a**	≫800	4
	**21a**	≫800	1.4
	**22b**	2.8±0.8	4.6±2
	22c	119±14	72±14
	22d	8.0±9.5	58.2±14.2

[a]PTP1B assays were conducted as previously reported in ref 26.

It was also of interest to examine effects of structural modifications to the N-terminal Fmoc group. Using peptide **22b** as a reference, both *N*-Cbz (**22c**) and *N*-phenylethoxycarbonyl (**22d**) were prepared as simplified, conformationally more flexible variants of the parent *N*-Fmoc group (Fig. 2). Both modifications resulted in significant reduction in binding affinity against YopH as well as PTP1B. The structural bases of Fmoc interactions with the YopH protein are currently being investigated using X-ray crystallography.

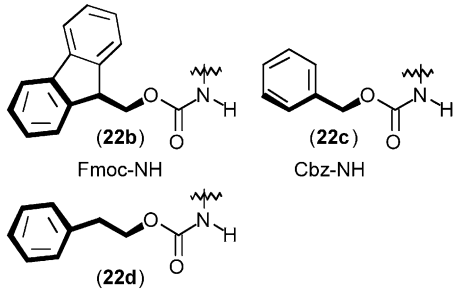


Figure 2. Comparison of N-terminal protecting groups for peptide **22**.

The Nature of Enzyme Inhibition

Recent reports have detailed a form of non-specific inhibition that involves disruption of enzyme function by a process of colloid formation.[27,28] In order to investigate whether this type of phenomena might underlie YopH inhibition by the current class of compounds, a representative compound was examined for its ability to form colloidal aggregates in solution. Dynamic light scattering measurements were carried out at room temperature in enzyme assay buffer, using a DynaPro-MS/X instrument (Protein Solutions, Inc.) with a 12 microliter quartz cuvette. This instrument is capable of detecting particles with a hydrodynamic radius as small as 0.5 nm. Attempts to detect dynamic light scattering (DLS) by analogue **14b** in assay buffer at 3 times the measured IC_{50} value (10 μM) failed to provide evidence of any aggregation under these conditions. A second DLS experiment was performed with 0.84 mg/mL YopH in the presence and absence of **14b** (ca. 50 μM). In neither case were any polydisperse aggregates observed. In both of these latter experiments, the hydrodynamic radius of YopH in solution exhibited a narrow, unimodal distribution consistent with that of a monomeric protein. These results failed to indicate colloid formation of either the inhibitor alone or in the presence of protein.

Concerns raised about potential non-classical inhibition prompted us to examine in detail kinetic aspects of YopH inhibition. Accordingly, we evaluated the concentration-dependence of **22b** during inhibition of YopH using the four-parameter eq 1. The inhibitor showed a steep IC_{50} curve, with a slope factor range from 3–5, depending on enzyme concentration. This potentially suggests that more than one molecule of **22b** is bound to YopH. In addition, observed IC_{50} values change with YopH concentration. For example, an IC_{50} of 4 μM was observed when 2.7 nM YopH was used in the assay, while an IC_{50} of 10 μM was observed when 13.3 nM YopH was used. Moreover, the inhibition profiles of **22b** do not conform to strictly competitive modes of inhibition. We also observed a time dependency for IC_{50} values. Preincubation of **22b** with YopH for 5 min decreased the IC_{50} from 3.2 to 1.5 μM. Finally, we also assessed the reversibility of YopH inhibition by **22b**. In this experiment, YopH (26.6 μM) was incubated with 200 μM of **22b** for 30 min. Subsequently, the mixture was diluted 5000-fold and assayed for enzyme activity. It was observed that treatment with **22b** caused a 40% loss of activity as compared with control. Collectively, the data suggests that **22b** may exhibit some of the properties associated with nonspecific promiscuous inhibitors. However, many of the observed effects (e.g., enzyme concentration dependence of IC_{50} values) are not as strong as previously noted for so-called 'promiscuous inhibitors.[27,28] When considered with the failure to detect aggregate formation by DLS experiments, it is possible that there may be additional binding modes, in addition to the active site interaction. Some of these additional modes of binding may explain the observed kinetic effects.

In conclusion, potent mono-anionic tripeptide inhibitors of YopH have been reported herein, which may potentially provide starting points for further development of inhibitors against this important enzyme.

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