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The synthesis and kinetic evaluation of aryl α-aminophosphonates as novel inhibitors of *T. cruzi* trans-sialidase

Zexin Chen[1], Patricia Marcé[2], Ricardo Resende[2], Pedro M. Alzari[3], A. Carlos Frasch[4], Jean M.H. van den Elsen[1], Susan J. Crennell[1], Andrew G. Watts[2*]

[1] Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK.

[2] Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, UK.

[3] Institut Pasteur, Unité de Microbiologie Structurale, CNRS URA 3528 & Université Paris Diderot, 25 rue de Dr. Roux, 75724, Paris, France.

[4] Instituto de Investigaciones Biotecnológicas, Universidad de General San Martin, Casilla de Correo 30, 1650 San Martin, Argentina.

[*] Corresponding author

Email: a.watts@bath.ac.uk

Phone: +44 (0)1225 386788

Abbreviations:

TcTS, *Trypanosoma cruzi* trans-sialidase; CAZy, Carbohydrate-Active EnZymes database; DANA, *N*-Acetyl-2,3-dehydro-2-deoxyneuraminic acid; GH, glycoside hydrolase; MuNANA, 2'-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid;

Abstract

The trans-sialidase protein expressed by *Trypanosoma cruzi* is an important enzyme in the life cycle of this human pathogenic parasite and is considered a promising target for the development of new drug treatments against Chagas' disease. Here we describe α-amino phosphonates as a novel class of inhibitor of *T. cruzi* trans-sialidase. Molecular modelling studies were initially used to predict the active-site binding affinities for a series of amino phosphonates, which were subsequently synthesised and their IC_{50}s determined *in vitro*. The measured inhibitory activities show some correlation with the predictions from molecular modelling, with 1-napthyl derivatives found to be the most potent inhibitors having IC_{50}s in the low micromolar range. Interestingly, kinetic analysis of the mode of inhibition demonstrated that the α-aminophosphonates tested here operate in a non-competitive manner.

1. Introduction

Trans-sialidase (TcTS) is a crucial enzyme in the life cycle of the protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas' disease [1 - 3]. *T. cruzi* is unable to synthesise sialic acid *de novo*, so TcTS functions to sequester sialic acid from host cell surfaces and transfer it to mucins on the surface of the parasite [4, 5]. The process of surface sialylation plays an important role in the pathogenicity of *T. cruzi*, by facilitating its invasion into host cells and helping to mask itself from the host immune system [6 - 9]. For these reasons, TcTs is considered a promising drugable target, with considerable effort being put into the discovery of trans-sialidase inhibitors as potential therapeutics for the treatment of Chagas' disease [10 - 11].

The 3-D structure and enzymatic mechanism of TcTS have been studied extensively. TcTS belongs to the glycoside hydrolase family GH33 (CaZY) [12], which contains most of the prokaryotic and some eukaryotic sialidases. Despite its inclusion in a glycoside hydrolase family, the predominant function of TcTS is trans-glycosidation, where α-2,3-linked sialic acid residues are preferentially transferred between galactose residues on glycoconjugates [13 - 15]. This trans-glycosidation reaction has been demonstrated to operate through a double-displacement mechanism utilising a tyrosine residue as the catalytic nucleophile and displays classical ping-pong reaction kinetics [16 - 18].

The catalytic domain of TcTS comprises a six-bladed beta propeller topology, also observed with viral, bacterial and human sialidases [19]. Similarly, the active site of TcTS is highly conserved amongst members of the sialidase super family (GH33, 34 and 83) comprising eight strictly conserved amino acids which span three distinct binding motifs, the sialic acid, carboxylate and lactose binding sites (**Fig. 1a**) [17, 19]. Studies into the design of inhibitors of TcTS have investigated derivatives targeted towards all three of these binding motifs, either individually, or in combination [10]. Compounds based on derivatives of sialic acid have been found generally to be very poor inhibitors of TcTS. DANA (**1**) (**Fig. 1b**) is a low micromolar inhibitor of influenza neuraminidases, however was found to be only a weak inhibitor of TcTS (K_i = 12.3 mM) [20]. Compounds binding to the lactose site have shown more promise as inhibitors, such as the pentasaccharide Gal$p$(β1→2)[Gal$p$(β1→3)]Gal$p$(β1→6) [Gal$f$(β1→4)]GlcNAc-4)]GlcNAcol with an IC_{50} of 0.61 mM towards TcTS [21]. Also, the reduced disaccharide lactitol (**2**) (**Fig. 1b**) was shown to inhibit TcTS with a K_M = 0.26 mM and was also shown to reduce infection of mammalian cells [22]. Compounds incorporating a phosphonate group such as **3** (IC_{50} = 5 mM) [23] and **4** (K_i = 7.3 mM) [24] have also been investigated in attempts to improve affinity to the carboxylate binding Arg triad, but have been found to be only weak inhibitors. Alternatively, the most potent inhibitors of TcTS found to date are not carbohydrate based at all, but instead contain multiple aromatic rings such as the anthraquinone (**5**) [25] and chalcone (**6**) [26] with K_i = 0.89 μM and IC_{50} = 0.9 μM, respectively (**Fig. 1b**). Interestingly, compounds **4** and **5,** which contain aromatic rings, were both found to inhibit TcTS in a non-competitive manner.

Here we describe the design, synthesis and kinetic evaluation of a series of aryl α-aminophosphonates as inhibitors of TcTS. These compounds were designed to target all three binding motifs of the trans-sialidase (sialic acid, carboxylate and lactose), but incorporate substituted aromatic residues to occupy the carbohydrate binding regions, which simplifies their chemical synthesis significantly.
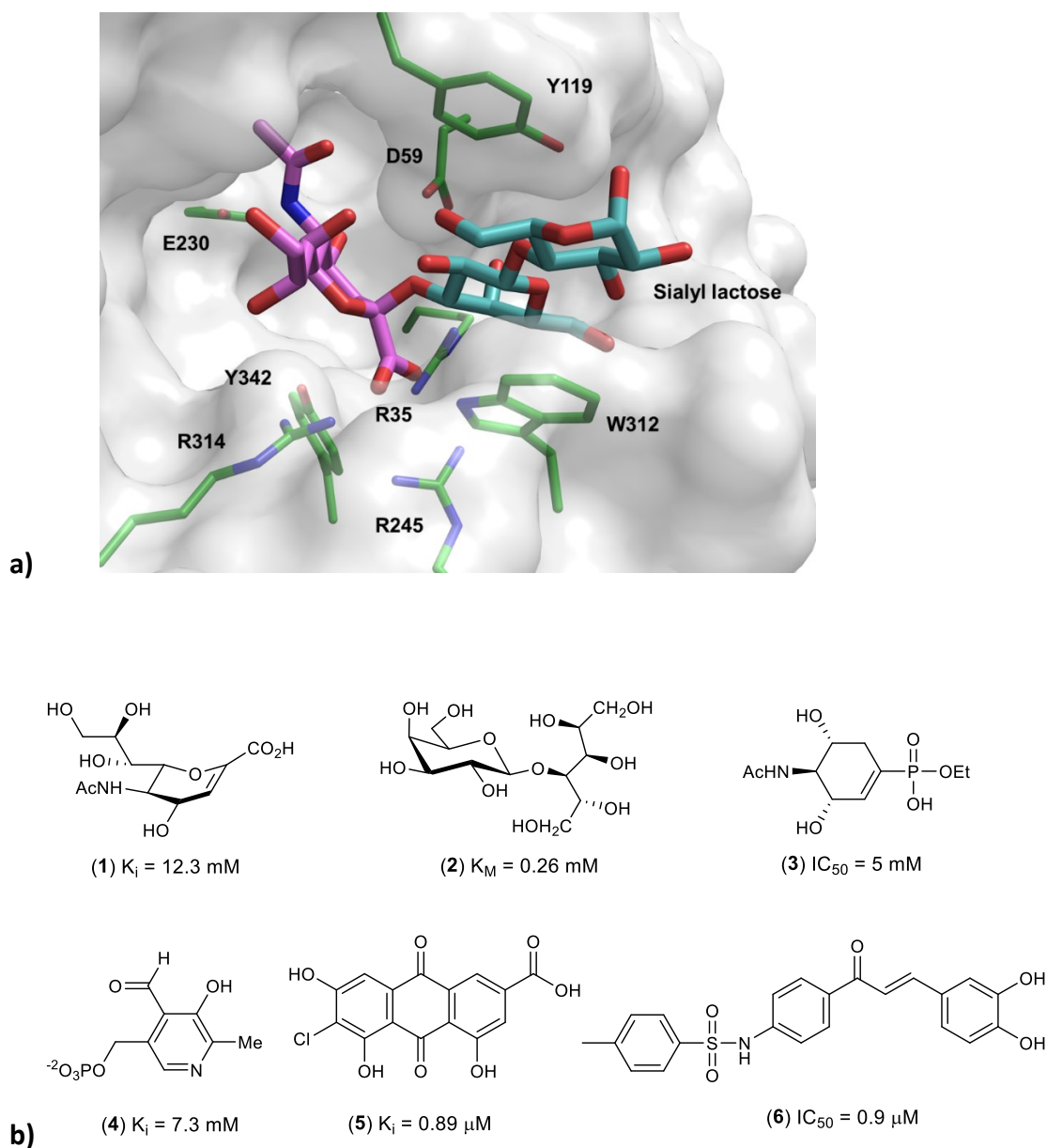
Fig. 1. a) Active site of TcTS with sialyl lactose bound (PDB; 1S0I) showing the strictly conserved amino acids that define the sialic acid, carboxylate and lactose binding regions. b) Structures of known inhibitors of TcTS and their reported inhibitory activities.

2. Results and Discussion

2.1. Molecular docking studies

Molecular docking simulations were performed using AutoDock Vina [27] to investigate potential binding modes of a series of aryl α-aminophosphonates in the active site of TcTS and to predict the impact of substitutions to the aromatic rings of groups **X-** and **Y-**, designed to bind to the sialic acid and lactose binding sites, respectively (**Table 1**). The

crystallised structure of TcTS chosen to perform the docking studies on was 1S0I (PDB code) [17] which is a complex (soaked) of trans-sialidase with the substrate α-(2,3)-sialyl lactose. It was considered important to use a structure representing the Michaelis complex, where residue Tyr119 has rotated into the 'closed' binding conformation, in order to evaluate potential π-π stacking interactions in the lactose binding site. Additionally, as the α-carbon of the proposed aminophosphonates is chiral, docking studies were performed on both the (R)- and (S)- isomers of each derivative to evaluate the preferred stereochemistry.

Table 1.
Predicted binding energies (kcal/mol) of the (R)- and (S)- isomers of α-aminophosphonates **7a-7k** using AutoDock Vina [27].

7 a-e   7 f-k
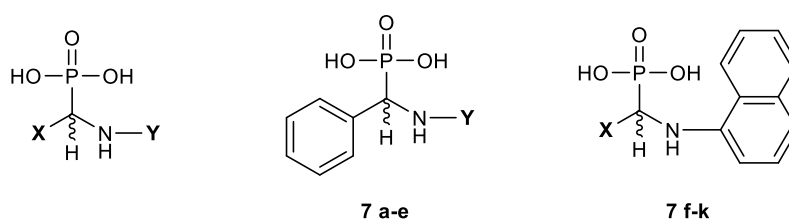
Compound	X	Y	Predicted affinity (kcal/mol)	
			(R)-isomer	(S)-isomer
7a		phenyl	-4.5	-3.8
7b		1-napthyl	-8.6	-7.0
7c	phenyl	2-napthyl	-6.8	-5.6
7d		2-biaryl	-4.8	-3.6
7e		3-biaryl	-4.6	-3.7
7f	4-methoxy phenyl		-8.9	-7.4
7g	4-chloro phenyl		-8.8	-7.7
7h	4-trifluoromethyl phenyl	1-napthyl	-7.5	-6.6
7i	4-N-acetamido phenyl		-9.6	-8.1
7j	4-nitro phenyl		-9.8	-8.0
7k	3,5-di-trifluoromethyl phenyl		-8.2	-7.6

Initially, **X** was fixed as a phenyl group (sterically conservative in comparison to sialic acid) and a series of **Y** = aryl derivatives screened for their predicted binding in the lactose site. The phenyl, naphthyl and bi-aryl **Y** groups were chosen to investigate the importance towards binding of number of aromatic groups, planarity and regiochemistry of attachment. For all derivatives at **Y** modelled (**7a-7e**), docking studies predict the (R)-isomer to form

slightly more favourable binding interactions with TcTS (**Table 1**). The 1-napthyl derivative (*R*)-**7b** was predicted to bind most tightly (-8.6 kcal/mol), indicating that a planar group consisting of two aromatic residues is preferred. The predicted binding position of (*R*)-**7b** is presented in **Fig. 2a**, showing the phosphonate group occupying the carboxylate binding pocket (Arg triad), while the phenyl group (**X**) occupies the sialic acid binding site and the napthyl group (**Y**) occupies the lactose binding site, capable of forming π-stacking interactions between Tyr119 and Trp312.

Based on the initial docking results, **Y** was then fixed as a 1-napthyl group and the **X** position screened using a series of modified phenyl derivatives for predicted binding to the sialic acid site. Again, the predicted binding energies for all modelled derivatives **7a-7f** slightly favoured the (*R*)- isomer. Encouragingly, the 4-*N* modified phenyl derivatives (*R*)-**7d** and (*R*)-**7e**, which more resemble 4-N-acetyl neuraminic acid, were predicted to bind with the highest affinities of -9.6 and -9.8 kcal/mol, respectively (**Table 1**). An overlay of the predicted binding position of compounds (*R*)-**7a-7f** is shown in **Fig. 2b**. It can be seen that all derivatives, except for **7h** (Fig. 2b: green sticks), are predicted to bind in a very similar position, with **7h** predicted to have the poorest binding affinity.
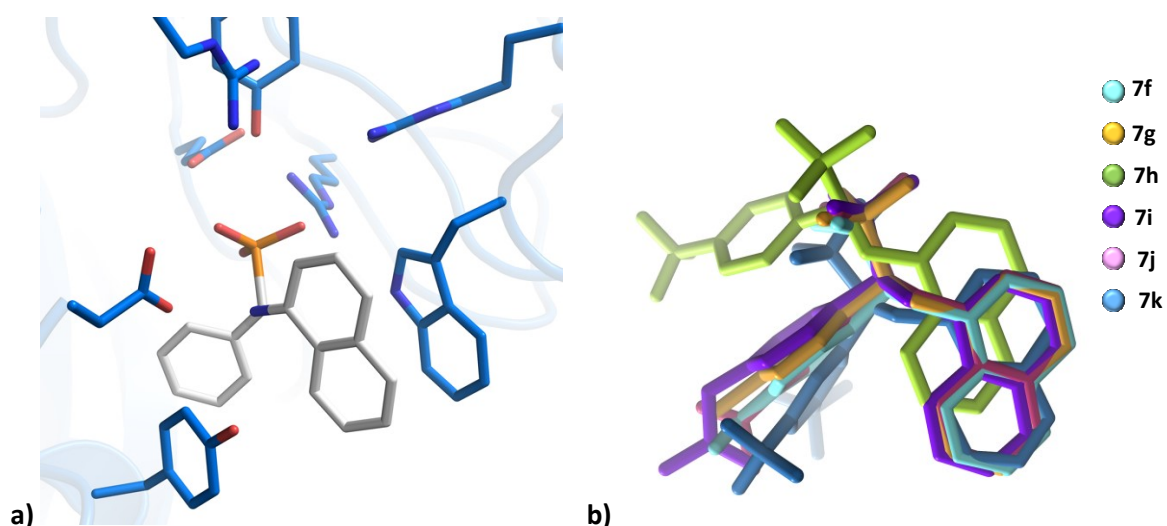

Fig. 2. a) Predicted binding position of (*R*)-isomer of compound **7b** (grey sticks) in the TcTS active site (side-chains in blue sticks). b) Overlay of predicted binding positions for (*R*)-isomer of compounds **7f-7k** in TcTS.

2.2. Chemistry

Each of the α-aminophosphonates modelled in the docking experiments were then synthesised chemically to facilitate their biochemical evaluation as inhibitors of TcTS. One of the most convenient methods for the synthesis of α-aminophosphonates is reported by Naydenova *et al.* [28] which uses a one-pot, solvent-free modification of the original Kabachnik-Fields reaction [29, 30]. Here, the relevant **X**-benzaldehyde and **Y**-amine derivatives were heated by microwave irradiation in dimethylphosphite to generate the respective α-aminophosphonate dimethyl esters **8a-8k** (**Scheme 1**). Treatment of the protected phosphonates **8a-8k** with trimethylsilyl bromide then gave the target compounds **7a-7k**, which were isolated as racemates in generally good to excellent yields over the two steps (**Table 2**). Considerable attempts were made to isolate the products **8a-8k** as pure enantiomers using methods such as co-crystallisation with chiral resolving agents and chiral HPLC. As none of these methods were successful in providing pure enantiomers (or even any enantiomeric enrichment), the kinetic evaluations were performed on the racemates.
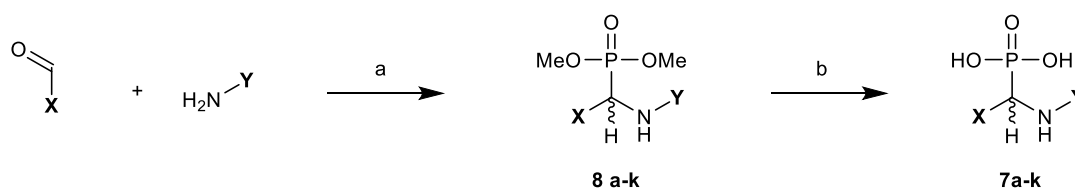
Scheme 1. Reagents and conditions: (a) Microwave (90W, time specified in Table 2), dimethylphosphite (10 equiv. w/v). (b) TMSBr (equiv. specified in Table 2), CH_2Cl_2/DMF, 0°C, 30 minutes. **X** and **Y** are defined in Table 1.

Table 2

Specific reaction conditions and product yields for the synthesis of compounds **7a-7k**.

Compound	Time (min)	Yield of **8** (%)	TMSBr (equiv.)	Yield of **7** (%)
7a	5	75	5	94
7b	5	85	3	82
7c	5	70	5	94
7d	7	84	7.5	86
7e	5	76	4.5	74
7f	7	66	5	49
7g	5	87	4.5	79
7h	7	55	5	64
7i	8	72	2.5	94
7j	6	72	4.5	59
7k	7	84	3.5	40

2.3 Trans-sialidase inhibition studies

The inhibitory activities of the newly synthesised α-aminophosphonates **7a-7k** were then evaluated by determining their IC_{50} values towards TcTS following methods described previously [31]. The assay employed uses 2'-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MuNANA) as the substrate to measure the hydrolytic activity of TcTS by detection of the fluorescent product of hydrolysis, 4-methylumbelliferone [19]. The IC_{50}s determined for compounds **7a-7k** are presented in **Table 3**.

For compounds **7a-7e**, where the **Y** group was screened with the **X** group fixed as phenyl, some broad agreement was observed between the predicted binding energies and the IC_{50} values measured. The two napthyl derivatives **7b** and **7c** were predicted to bind most favourably with TcTS (-8.6 and -6.8 kcal/mol, respectively) for this set of compounds and both were indeed found to have the two lowest IC_{50}s (0.47 and 0.51 mM, respectively). However, docking studies predicted essentially no difference between the binding energies of the two bi-aryl derivatives **7d** and **7e**, while the inhibition studies demonstrate a 10-fold difference between the observed IC_{50}s of 0.63 mM and 5.58 mM, respectively.

Table 3

Inhibitory values determined for compounds **7a**-**7k** against TcTS.

Compound	IC$_{50}$ (mM)[a]	Std. error (mM)	K$_i$ (mM)	Mode of inhibition
7a	>7.5 [b]	>20		
7b	0.47	±0.09	0.43	Non-competitive
7c	0.51	±0.09		
7d	0.63	±0.04		
7e	5.58	±2.37		
7f	0.27	±0.03		
7g	0.71	±0.05		
7h	0.29	±0.01		
7i	0.23	±0.03	0.19	Non-competitive
7j	0.21	±0.04	0.14	Non-competitive
7k	2.35	±0.83		

[a] Data are expressed as the mean average of three (n=3) independent experiments. [b] Saturating concentrations could not be reached owing to solubility.

For the second set of compounds **7f-7k**, where **Y** was fixed as a 1-napthyl group and derivatives screened for **X**, there was again mixed agreement between predicted binding energies and the observed IC$_{50}$s. In general, compounds substituted at the 4-position of the pheny group (**7f-7j**) were all found to have comparable IC$_{50}$ values (0.21 – 0.71 mM), while the 3,5-modified derivative **7k** was found to be a significantly poorer inhibitor with an IC$_{50}$ of 2.35 mM. Notably, the 4-trifluoromethyl derivative **7h** was predicted to be the poorest binder in docking studies, but its IC$_{50}$ = 0.29 mM was found to be almost equivalent to the best inhibitors.

To determine whether α-aminophosphonates display any selectivity for inhibition of TcTS over other family GH33 sialidases (which includes the human sialidases), we determined the IC$_{50}$s of the most potent inhibitor, compound **7j**, against the two bacterial sialidases *Clostridium perfringens* neuraminidase (CpNA) and *Staphylococcus pneumoniae* neuraminidase A (SpNA) (Supplementary Data). Compound **7j** was found to be a poor inhibitor of both enzymes, with IC$_{50}$s of 2.39 mM and 1.41 mM against SpNA and CpNA, respectively. As such, **7j** displays 7 to 12 fold better inhibition against TcTS (IC$_{50}$ 0.21 mM) than the bacterial sialidases suggesting that α-aminophosphonates possess some degree of selectivity for trans-sialidase.

In summary, the IC$_{50}$ values determined here suggest that α-aminophosphonates are effective inhibitors of TcTS and possess some selectivity for this enzyme over other family GH33 sialidases. Furthermore, derivatives consisting of **Y** groups containing at least two aromatic residues in a planar conformation and **X** groups modified at the C-4 position, possess the most potent inhibitory activities towards trans-sialidase.

2.4 Mode of inhibition studies

Having demonstrated that α-aminophosphonates, as a class, are effective inhibitors of TcTS, the mode of inhibition of the parent 1-napthyl compound **7b** and the two most potent compounds **7i** and **7j** were then investigated. Lineweaver-Burk plots were produced by plotting residual enzyme activity against substrate concentration for a series of inhibitor concentrations. The regression lines for each inhibitor concentration converge on the X-axis demonstrating that all three compounds display non-competitive inhibition kinetics towards TcTS (**Fig. 3**). The K$_i$ values for **7b**, **7i** and **7j** were determined to be 430 μM, 190 μM and 140 μM, respectively (**Table 3**). It is interesting to note that a number of inhibitors containing multiple aromatic rings, including **5**, have been shown previously by other groups to display non-competitive inhibition kinetics towards TcTS [25].
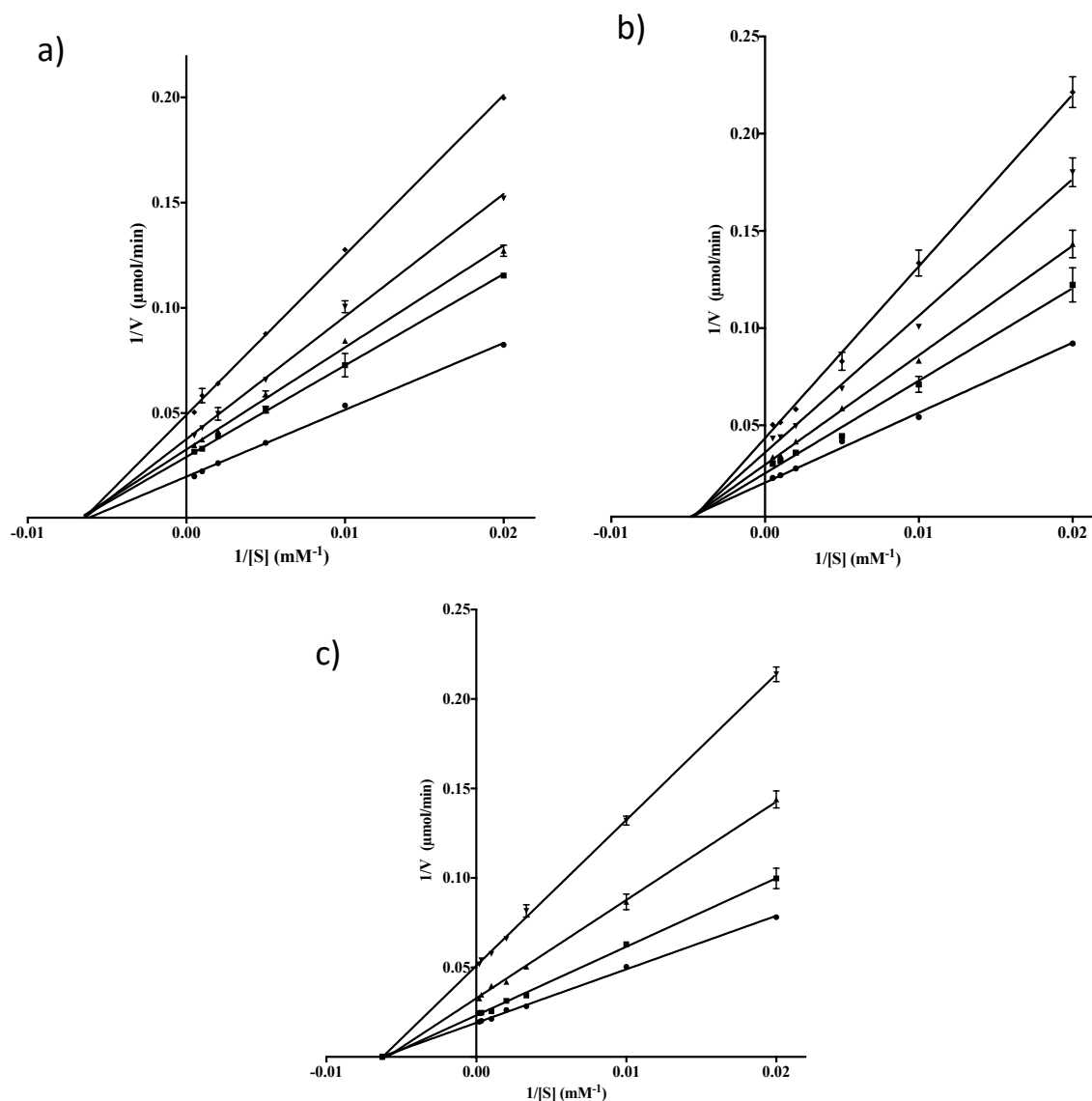
Fig. 3. Plots of Lineweaver-Burk kinetics performed using [MuNANA] = 2.0, 1.0, 0.5, 0.2, 0.1, 0.05 and 0.01 mM for: a) Compound **7b** at 0 (●), 0.05 (■), 0.10 (▲), 0.20 (▼) and 0.40 (◆) mM. b) Compound **7i** at 0 (●), 0.02 (■), 0.05 (▲), 0.10 (▼) and 0.15 (◆) mM. c) Compound **7j** at 0 (●), 0.05 (■), 0.10 (▲) and 0.20 (▼) mM.

3. Conclusions

We have successfully synthesised a series of eleven aryl α-aminophosphonates in two chemical steps in good to excellent overall yields. Several of these derivatives were demonstrated to inhibit TcTS in the low micromolar range, comparable to some of the most potent carbohydrate-based inhibitors of this enzyme. Furthermore, we have shown that these compounds display non-competitive inhibition kinetics towards TcTS, consistent with several other compounds containing multiple aromatic residues. Together, these results

demonstrate that α-aminophosphonates are a promising class of compounds for further development towards more potent inhibitors of *T. cruzi* trans-sialidase.

4. Experimental protocols

4.1. General methods and materials

Chemical reagents were purchased from Sigma-Aldrich unless specifically stated. Anhydrous solvents were purchased from Sigma-Aldrich and used without further purification. Purified *Clostridium perfringens* neuraminidase (CpNA) was purchased from Sigma-Aldrich. All other solvents were purchased from Fisher Scientific. Analytical thin layer chromatography (TLC) was carried out on Merck aluminium backed TLC plates silica gel 60 F254 (0.25 mm thickness), viewed using UV light of wavelength 254 nm or stained with potassium permanganate solution or *para*-anisaldehyde stain. Silica gel chromatography was performed on silica gel 60 Å (200-400 mesh) from Sigma-Aldrich. Preparative reverse phase chromatography (C-18) was performed using a VersaFlash hand held column (23 x 110 mm) from Supelco. Melting points were obtained using a Thermo Fisher IA9000 digital melting point apparatus. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded using Bruker Advance III 400 MHz spectrometer with acquisition frequencies of 400 for ^{1}H; 101 MHz for ^{13}C; and 162 MHz for ^{31}P. Deuterated solvents were purchased from Cambridge Isotope Laboratories. The NMR chemical shifts δ were recorded in parts per million (ppm) with reference to tetramethylsilane for ^{1}H and ^{13}C and phosphoric acid for ^{31}P NMR spectroscopy. High resolution mass spectrometry was performed using a BrukerMicrOTOF electrospray ionisation mass spectrometer.

4.2 Molecular Docking

A structure of TcTS obtained by X-ray diffraction with the ligand α(2,3)-sialyl lactose bound (PDB code: 1S0I) was used for all docking studies. A model of the structure was prepared using AutoDock tools, which involved removing the bound ligand, mutating residue A59D, adding all hydrogens to the model, setting the lactose binding site residues Y119 and W312 to be flexible, and limiting the region of docking to the catalytic site. 3-D models of α-phosphonate ligands were built using Phenix eLBOW [32] or CCP4 JLigand [33]. Molecular docking experiments were run using AutoDock Vina [27] to screen for potential binding positions and to calculate the predicted binding energy as the docking score.

4.3 General procedure for the synthesis of protected α-aminophosphonates **8a-8k**.

A mixture of the aldehyde (4.7 mmol, 1 eq.) and the amine (4.7 mmol, 1 eq.) were dissolved in dimethylphosphite (10 equiv. w/v) and the resultant solution subjected to microwave heating (90 W) for the specified time (Table 2). The reaction mixture was then

diluted with dichloromethane, and the organic phase washed sequentially with saturated sodium bicarbonate solution and brine. The organic phases were then combined and dried over anhydrous magnesium sulphate, filtered and the filtrate concentrated. The resultant residue was then purified by column chromatography (35% EtOAc/petroleum ether) to give the protected α-aminophosphonates **8a-8k**.

4.3.1 (±) Dimethyl-[α-(phenyl)(phenylamino)methyl] phosphonate (*8a*)

The title compound was prepared from benzaldehyde and aniline. White solid; yield 75%; ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.50 – 7.40 (m, 2H, Ar), 7.35 – 7.27 (m, 2H, Ar), 7.27 – 7.18 (m, 1H, Ar), 7.12 – 7.01 (m, 2H, Ar), 6.66 (dd, *J* = 7.3, 1.1 Hz, 1H, Ar), 6.61 – 6.52 (m, 2H, Ar), 4.81-4.70 (m, 2H, NH, P-CH), 3.71 (d, *J* = 10.6 Hz, 3H, OMe), 3.43 (d, *J* = 10.6 Hz, 3H, OMe).^{13}C NMR (100.6 MHz, CDCl$_3$): δ 146.16, 136.12, 129.13, 128.65, 127.98, 127.76, 118.48, 113.85, 55.68 (d, *J* = 151.3 Hz), 53.75, 53.69. ^{31}P NMR (161.93 MHz, CDCl$_3$): δ 24.96. HRMS (ESI): Calculated for C$_{15}$H$_{18}$NO$_3$PNa (M+Na)$^+$: m/z= 314.0922, found m/z = 314.0907.

4.3.2 (±) Dimethyl-[α-(phenyl)(napthalen-1-ylamino)methyl] phosphonate (*8b*)

The title compound was prepared from benzaldehyde and 1-aminonapthalene. White solid; yield 85%; ^{1}H NMR (400 MHz, CDCl$_3$): δ 8.06-8.03 (m, 1H, Ar), 7.79- 7.75 (m, 1H, Ar), 7.56 – 7.45 (m, 4H, Ar), 7.38 – 7.19 (m, 5H, Ar), 6.49 – 6.37 (m, 1H, Ar), 5.49 (s, 1H, NH), 5.01 (d, *J* = 19.8 Hz, 1H, P-CH), 3.80 (d, *J* = 10.6 Hz, 3H, OMe), 3.53 (d, *J* = 10.6 Hz, 3H, OMe). ^{13}C NMR (101 MHz, CDCl$_3$) δ 141.05, 135.25, 134.20, 128.72, 128.62, 128.07, 127.58, 126.13, 125.85, 125.11, 123.88, 119.97, 118.69, 106.54, 55.87 (d, *J* = 151.0 Hz), 53.85, 53.81. ^{31}P NMR (162 MHz, CDCl$_3$): δ 24.94. HRMS (ESI) calculated for C$_{19}$H$_{21}$NO$_3$P [M+H]$^+$: 342.1259. Found: 342.1252.

4.3.3 (±) Dimethyl-[α-(phenyl)(napthalen-2-ylamino)methyl] phosphonate (*8c*)

The title compound was prepared from benzaldehyde and 2-aminonapthalene. White solid; yield 70%; ^{1}H NMR (400 MHz, CDCl$_3$): δ 8.03-8.01 (m, 1H, Ar), 7.78-7.76 (m, 1H, Ar), 7.53-7.45 (m, 4H, Ar), 7.35-7.22 (m, 4H, Ar), 7.12-7.14 (m, 1H, Ar), 6.42-6.40 (m, 1H, Ar), 5.46 (s, 1H, NH), 4.97 (d, *J* = 21.5 Hz, 1H, P-CH), 3.78 (d, *J* = 10.8 Hz, 3H, OMe), 3.52 (d, *J* = 10.4, 3H, OMe). ^{13}C NMR (101 MHz, CDCl$_3$) δ 141.12, 140.97, 135.24, 135.21, 134.20, 128.79, 128.76, 128.66, 128.14, 128.12, 127.63, 127.57, 126.18, 125.91, 125.75, 125.16, 123.87, 120.02, 118.72, 106.55, 56.33 (d, *J* = 149.6 Hz), 54.03, 53.96, 53.89. ^{31}P NMR (162 MHz, CDCl$_3$): δ 24.90. HRMS (ESI) calculated for C$_{19}$H$_{21}$NO$_3$P [M+H]$^+$: 342.1259. Found: 342.1250.

4.3.4 (±) Dimethyl-[α-(phenyl)([1,1'-biphenyl]-2-ylamino)methyl] phosphonate (*8d*)

The title compound was prepared from benzaldehyde and 1,1'-biphenyl-2-amine. White solid; yield 76%; ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.52 – 7.42 (m, 4H, Ar), 7.42 – 7.35 (m, 3H, Ar), 7.35 – 7.29 (m, 2H, Ar), 7.28 – 7.24 (m, 1H, Ar), 7.13 – 7.05 (m, 2H, Ar), 6.80 – 6.75 (m, 1H, Ar), 6.55 – 6.50 (m, 1H, Ar), 4.97 (s, 1H, NH), 4.78 (d, *J* = 24.2 Hz, 1H, P-CH), 3.62 (d, *J* = 10.6 Hz, 3H, OMe), 3.47 (d, *J* = 10.6 Hz, 3H, OMe). ^{13}C NMR (100.6 MHz, CDCl$_3$): δ 143.14, 138.88, 135.61,

130.25, 129.18, 128.92, 128.78, 128.62, 127.96, 127.62, 127.50, 118.28, 111.89, 56.13 (d, J = 150.8 Hz), 53.69, 53.52. ^{31}P NMR (162 MHz, CDCl$_3$): δ 24.51. HRMS (ESI) calculated for C$_{21}$H$_{22}$NO$_3$PNa [M+Na]$^+$: 390.1235. Found: 390.1247.

4.3.5 (±) Dimethyl-[α-(phenyl)([1,1'-biphenyl]-3-ylamino)methyl] phosphonate (*8e*)

The title compound was prepared from benzaldehyde and 1,1'-biphenyl-3-amino. White solid; yield 84%; ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.49 – 7.42 (m, 2H, Ar), 7.42 – 7.37 (m, 2H, Ar), 7.36 – 7.27 (m, 4H, Ar), 7.26 – 7.20 (m, 2H, Ar), 7.11 (t, J = 7.8 Hz, 1H, Ar), 6.87 (dd, J = 7.6, 1.7 Hz, 1H, Ar), 6.79 (t, J = 2.0 Hz, 1H, Ar), 6.52 (dd, J = 8.2, 2.4 Hz, 1H, Ar), 5.22 (s, 1H, NH), 4.75 (br d, J = 25.0 Hz, 1H, P-CH), 3.74 (d, J = 10.7 Hz, 3H, OMe), 3.43 (d, J = 10.6 Hz, 3H, OMe). ^{13}C NMR (101 MHz, CDCl$_3$) δ 146.42, 143.45, 142.02, 135.56, 129.49, 128.70, 128.52, 128.03, 127.79, 127.73, 127.11, 126.96, 117.57, 112.81, 112.67, 55.71 (d, J = 151.4 Hz), 53.76, 53.69. ^{31}P NMR (162 MHz, CDCl$_3$): δ 24.88. HRMS (ESI) calculated for C$_{21}$H$_{23}$NO$_3$P [M+H]$^+$: 368.1416. Found: 368.1416.

4.3.6 (±) Dimethyl-[α-(4-methoxyphenyl)(napthalen-1-ylamino)methyl] phosphonate (*8f*)

The title compound was prepared from 4-methoxybenzaldehyde and 1-aminonapthalene. White solid; yield 66%; ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.03-8.01 (m, 1H, Ar), 7.80-7.77 (m, 1H, Ar), 7.53-7.42 (m, 4H, Ar), 7.26-7.23 (m, 2H, Ar), 7.20-7.16 (m ,1H, Ar), 6.88 – 6.82 (m, 2H, Ar), 5.90 (br s, 1H, NH), 4.93 (d, J = 24.6 Hz, 1H, P-CH), 3.80 (s, 3H, Ar-OMe), 3.79 (d, J = 10.5 Hz, 3H, OMe), 3.55 (d, J = 10.5 Hz, 3H, OMe). ^{13}C NMR (101 MHz, CDCl$_3$) δ 157.3, 144.4, 134.21, 128.73, 128.68, 128.59, 126.12, 125.80, 125.06, 123.93, 119.96, 118.65, 114.21, 114.18, 106.62, 55.51 (d, J = 150.3 Hz), 53.80, 53.69, 34.52. ^{31}P NMR (162 MHz, CDCl$_3$): δ 25.12. HRMS (ESI) Calculated for C$_{20}$H$_{23}$NO$_4$P [M+H]$^+$: 372.1364. Found 372.1359.

4.3.7 (±) Dimethyl-[α-(4-chlorophenyl)(napthalen-1-ylamino)methyl] phosphonate (*8g*)

The title compound was prepared from 4-chlorobenzaldehyde and 1-aminonapthalene. White solid; yield 87%; ^{1}H-NMR (400 MHz, CDCl$_3$): δ 8.02 - 7.99 (m, 1H, Ar), 7.81 - 7.77 (m, 1H, Ar), 7.55 - 7.44 (m, 4H, Ar), 7.33 - 7.30 (m, 2H, Ar), 7.27 – 7.24 (m, 1H, Ar), 7.19-7.15 (m, 1H, Ar), 6.38 – 6.35 (m, 1H, Ar), 5.42 (s, 1H, NH), 4.98 (d, J = 24 Hz, 1H, P-CH), 3.82 (d, J = 10.8 Hz, 3H, OMe), 3.62 (d, J = 10.8 Hz, 3H, OMe). ^{13}C NMR (101 MHz, CDCl$_3$): δ 140.83, 140.69, 134.21, 133.93, 133.89, 128.96, 128.93, 128.85, 128.68, 126.04, 125.96, 125.84, 123.85, 119.87, 119.02, 106.59, 56.09 (d, J = 150.3 Hz, P-CH), 54.59, 54.08. ^{31}P NMR (162 MHz, CDCl$_3$): δ 24.10. HRMS (ESI) Calculated for C$_{19}$H$_{19}$ClNNaO$_3$P [M+Na]$^+$: 398.0688. Found 398.0674.

4.3.8 (±) Dimethyl-[α-(4-(trifluoromethyl)phenyl)(napthalen-1-ylamino)methyl] phosphonate (*8h*)

The title compound was prepared from 4-trifluoromethylbenzaldehyde and 1-aminonapthalene. White solid; yield 56%; ^{1}H NMR (400 MHz, CDCl$_3$): δ 8.01 (dd, J = 7.7, 1.7 Hz, 1H, Ar), 7.79 (dd, J = 7.5, 2.0 Hz, 1H, Ar), 7.70 – 7.44 (m, 6H, Ar), 7.26 (d, J = 8.2 Hz, 1H, Ar), 7.15 (dd, J = 8.2, 7.6 Hz, 1H, Ar), 6.42 – 6.18 (m, 1H, Ar), 5.45 (s, 1H, NH), 5.02 (d, J = 24.4

Hz, 1H, P-CH), 3.81 (d, J = 10.7 Hz, 3H, OMe), 3.61 (d, J = 10.7 Hz, 3H, OMe). ^{13}C NMR (101 MHz, CDCl$_3$): δ 141.85, 140.66, 140.16, 139.28, 135.37, 132.46, 128.88, 128.48, 127.92, 126.43, 126.08, 125.65, 125.27, 124.36, 122.36, 120.07, 117.04, 58.31 (d, J = 153 Hz, P-CH), 53.97, 53.67. ^{31}P NMR (162 MHz, CDCl$_3$): δ 23.95. HRMS (ESI) Calculated for C$_{20}$H$_{19}$F$_3$NNaO$_3$P [M+Na]$^+$: 432.0952. Found 432.0942.

4.3.9 (±) Dimethyl-[α-(4-N-acetamidophenyl)(napthalen-1-ylamino)methyl] phosphonate (8i)
The title compound was prepared from 4-*N*-acetamidobenzaldehyde and 1-aminonapthalene. White solid; yield 72%; ^{1}H NMR (400MHz, CDCl$_3$): δ 8.00 (d, J = 6.4Hz, 1H, Ar), 7.83-7.71 (m, 1H, Ar), 7.53 - 7.71 (m, 6H, Ar), 7.25 (d, J = 8.0 Hz, 1H, Ar), 7.18 - 7.14 (m, 1H, Ar), 6.41 (d, J = 6.0 Hz, 1H, Ar), 5.39 (s, 1H, NH), 4.97(d, J = 20 Hz, 1H, P-CH), 3.81 (d, J = 10.4Hz, 3H, OMe), 3.57 (d, J = 9.6Hz, 3H, OMe), 2.15(s, 3H, NAc). ^{13}C NMR (101 MHz, CDCl$_3$): δ 163.63, 141.15, 141.06, 138.28, 134.37, 130.65, 128.85, 128.38, 128.07, 126.33, 126.07, 125.37, 124.06, 123.33, 120.05, 119.04, 55.55 (d, J = 151 Hz, P-CH), 54.23, 54.07, 24.63. ^{31}P-NMR (162 MHz, CDCl$_3$): δ 25.57. HRMS (ESI) Calculated for C$_{21}$H$_{23}$N$_2$NaO$_4$P [M+H]$^+$: 421.1293. Found 421.1269.

4.3.10 (±) Dimethyl-[α-(4-nitrophenyl)(napthalen-1-ylamino)methyl] phosphonate (8j)
The title compound was prepared from 4-nitrobenzaldehyde and 1-aminonapthalene. Yellow solid; yield 72%; ^{1}H NMR (400 MHz, CDCl$_3$): δ 8.24 – 8.10 (m, 2H, Ar), 8.05 – 7.92 (m, 1H, Ar), 7.79 (dd, J = 7.6, 1.7 Hz, 1H, Ar), 7.74 – 7.64 (m, 2H, Ar), 7.59 – 7.44 (m, 2H, Ar), 7.30 – 7.20 (m, 1H, Ar), 7.18 – 7.09 (m, 1H, Ar), 6.35 – 6.16 (m, 1H, Ar), 5.45 (s, 1H, NH), 5.06 (d, J = 24.8 Hz, 1H, P-CH), 3.82 (d, J = 10.8 Hz, 3H, OMe), 3.66 (d, J = 10.8 Hz, 3H, OMe). ^{13}C NMR (101 MHz, CDCl$_3$): δ 143.63, 143.28, 140.06, 134.32, 128.77, 128.43, 128.38, 126.16, 125.91, 125.49, 123.92, 123.88, 123.84, 119.75, 119.53, 106.60, 55.41 (d, J = 151 Hz, P-CH) 54.21, 53.93. ^{31}P NMR (162 MHz, CDCl$_3$): δ 23.26. HRMS (ESI) Calculated for C$_{19}$H$_{20}$N$_2$O$_5$P [M+H]$^+$: 387.1109. Found 387.1099.

4.3.11 (±) Dimethyl-[α-(3,5-bis(trifluoromethyl)phenyl)(napthalen-1-ylamino)methyl] phosphonate (8k)
The title compound was prepared from 3,5-bis(trifluoromethyl)benzaldehyde and 1-aminonapthalene. White solid; yield 84%. ^{1}H NMR (400 MHz, CDCl$_3$): δ 8.12 - 8.04 (m, 3H, Ar), 7.83 (d, J = 8.2 Hz, 2H, Ar), 7.58-7.50 (m, 2H, Ar), 7.32 (d, J = 8.4 Hz, 1H, Ar), 7.21 (m, 1H, Ar), 6.29 (d, J = 5.6 Hz, 1H, Ar), 5.48 (s, 1H, NH), 5.08 (d, J = 24.4 Hz, 1H, P-CH), 3.85 (d, J = 10.8 Hz, 3H, OMe), 3.69 (d, J = 10.8 Hz, 3H, OMe). ^{13}C NMR (101 MHz, CDCl$_3$): δ 142.65, 140.15, 140.06, 137.18, 133.27, 131.56, 128.95, 127.87, 126.23, 126.17, 125.37, 125.07, 124.76, 122.36, 121.83, 120.05, 116.04, 56.35 (d, J = 153.3 Hz, P-CH), 54.07, 53.97. ^{31}P NMR (162 MHz, CDCl$_3$): δ 21.06. HRMS (ESI) Calculated for C$_{21}$H$_{19}$F$_6$NO$_3$P [M+H]$^+$: 478.1006. Found 478.1003.

4.4 General procedure for the synthesis of α-aminophosphonates *7a-7k*.

TMSBr (equiv. specified in Table 3) was added dropwise to a solution of the dimethyl α-aminophosphonate (100 mg, 1.0 equiv.) in anhydrous CH_2Cl_2/DMF (2:1, 3 mL) at 0°C and the mixture held at this temperature for 30 minutes. The solution was then allowed to warm to room temperature and concentrated *in vacuo*. The resultant residue was then diluted with water (2.5 mL), neutralized (aq. 1M NaOH) and purified by C-18 reverse phase chromatography (20-40 % MeOH/H_2O) to give the title phosphonic acid.

4.4.1 (±) α-(phenyl)(phenylamino)methyl phosphonic acid (*7a*)

The title compound was prepared from **8a**. White solid; yield 94%. ^{1}H NMR (400 MHz, CD_3OD): δ 7.54 (d, *J* = 8.0 Hz, 2H, Ar), 7.25 - 7.20 (m, 2H, Ar), 7.12 - 7.09 (m, 1H, Ar), 7.03 - 6.89 (m, 2H, Ar), 6.60 - 6.44 (m, 3H, Ar), 4.49 (d, *J* = 21.6 Hz, 1H, P-CH). ^{13}C NMR (101 MHz, CD_3OD): δ 150.25, 144.04, 129.62, 129.15, 128.62, 126.46, 117.34, 114.74, 60.64 (d, *J* = 130 Hz). ^{31}P NMR (162 MHz, CD_3OD): δ 16.46. HRMS (ESI): Calculated for $C_{13}H_{13}NO_3P$ [M-H]⁻: 262.0633. Found 262.0633.

4.4.2 (±) α-(phenyl)(napthalen-1-ylamino)methyl phosphonic acid (*7b*)

The title compound was prepared from **8b**. White solid; yield 82%. ^{1}H NMR (400 MHz, CD_3OD): δ 8.32 (dd, *J* = 7.9, 1.5 Hz, 1H, Ar), 7.76 – 7.69 (m, 1H, Ar), 7.59 (dt, *J* = 8.0, 1.7 Hz, 2H, Ar), 7.52 – 7.35 (m, 2H, Ar), 7.24 (dd, *J* = 8.4, 6.9 Hz, 2H, Ar), 7.19 – 7.01 (m, 3H, Ar), 6.32 (dd, *J* = 6.6, 2.2 Hz, 1H, Ar), 4.64 (d, *J* = 21.3 Hz, 1H, P-CH). ^{13}C NMR (101 MHz, CD_3OD): δ 145.19, 143.55, 135.87, 129.13, 129.07, 128.60, 127.42, 126.46, 126.24, 125.27, 124.96, 122.25, 116.77, 106.31, 60.57 (d, *J* = 131 Hz). ^{31}P NMR (162 MHz, CD_3OD): δ 15.74. HRMS (ESI) calculated for $C_{17}H_{15}NO_3P$ [M-H]⁻: 312.0790. Found: 312.0796.

4.4.3 (±) α-(phenyl)(napthalen-2-ylamino)methyl phosphonic acid (*7c*)

The title compound was prepared from **8c**. White solid; yield 94%. ^{1}H NMR (400 MHz, CD_3OD): δ 7.61-7.48 (m, 4H, Ar), 7.36-7.34 (m, 1H, Ar), 7.24-7.21 (m, 3H, Ar), 7.14-7.03 (m, 3H, Ar), 6.49 (s, 1H, Ar), 4.60 (d, *J* = 21.1 Hz, 1H, P-CH). ^{13}C NMR (101 MHz, CD_3OD): δ 145.28, 145.17, 138.54, 134.52, 129.08, 128.42, 127.85, 127.81, 127.56, 127.29, 127.13, 126.62, 125.85, 122.55, 118.87, 105.93, 57.43 (d, *J* = 130 Hz). ^{31}P NMR (162 MHz, CD_3OD): δ 15.95. HRMS (ESI) calculated for $C_{17}H_{15}NO_3P$ [M-H]⁻: 312.0790. Found: 312.0776.

4.4.4 (±) α-(phenyl)([1,1'-biphenyl]-2-ylamino)methyl phosphonic acid (*7d*)

The title compound was prepared from **8d**. White solid; yield 86%. ^{1}H NMR (400 MHz, CD_3OD): δ 7.65 – 7.57 (m, 2H, Ar), 7.57 – 7.46 (m, 4H, Ar), 7.40 – 7.30 (m, 1H, Ar), 7.26 (t, *J* = 7.6 Hz, 2H, Ar), 7.12 (td, *J* = 7.2, 1.5 Hz, 1H, Ar), 7.02 – 6.90 (m, 2H, Ar), 6.60 (td, *J* = 7.4, 1.1 Hz, 1H, Ar), 6.50 (dd, *J* = 8.4, 1.0 Hz, 1H, Ar), 4.54 (d, *J* = 21.5 Hz, 1H, P-CH). ^{13}C NMR (101 MHz, CD_3OD) δ 150.62, 144.09, 143.43, 142.86, 130.06, 129.53, 129.23, 128.69, 127.86, 126.50, 116.19,

113.90, 113.38, 60.82 (d, J = 129.8 Hz). ^{31}P NMR (162 MHz, CD$_3$OD): δ 15.72. HRMS (ESI) calculated for C$_{19}$H$_{17}$NO$_3$P [M-H]$^-$: 338.0946. Found: 338.0962.

4.4.5 (±) α-(phenyl)([1,1'-biphenyl]-3-ylamino)methyl phosphonic acid (*7e*)

The title compound was prepared from **8e**. White solid; yield 74%. ^{1}H NMR (400 MHz, CD$_3$OD) δ 7.62 – 7.57 (m, 2H, Ar), 7.47 – 7.39 (m, 2H, Ar), 7.39 – 7.20 (m, 5H, Ar), 7.18 – 7.01 (m, 2H, Ar), 6.85 – 6.73 (m, 2H, Ar), 6.57 (dd, J = 8.2, 2.3 Hz, 1H, Ar), 4.51 (d, J = 21.5 Hz, 1H, P-CH). ^{13}C NMR (101 MHz, CD$_3$OD) δ 150.62, 144.09, 143.43, 142.86, 130.06, 129.53, 129.23, 128.69, 127.86, 126.50, 116.19, 113.90, 113.38, 60.82 (d, J = 129.8 Hz). ^{31}P NMR (162 MHz, CD$_3$OD): δ 16.28. HRMS (ESI) calculated for C$_{19}$H$_{17}$NO$_3$P [M-H]$^-$: 338.0946. Found: 338.0960.

4.4.6 (±) α-(4-methoxyphenyl)(napthalen-1-ylamino)methyl phosphonic acid (*7f*)

The title compound was prepared from **8f**. White solid; yield 49%. ^{1}H NMR (400 MHz, CD$_3$OD) δ 8.22-8.2 (m, 1H, Ar), 7.76-7.74 (m, 2H, Ar), 7.53-7.44 (m, 3H, Ar), 7.36-7.34 (m, 2H, Ar), 7.12-7.10 (m, 2H, Ar), 6.38-6.36 (m, 1H, Ar), 4.68 (d, J = 21.0 Hz, 1H, P-CH), 3.69 (s, 3H, Ph-OMe). ^{13}C NMR (101 MHz, CD$_3$OD): δ 157.08, 143.50, 143.20, 133.92, 128.73, 128.67, 128.27, 126.74, 126.18, 125.06, 123.06, 120.64, 116.32, 113.40, 105.40, 58.14 (d, J = 130.0 Hz), 55.21. ^{31}P NMR (162 MHz, CD$_3$OD): δ 15.52. HRMS (ESI) calculated for C$_{18}$H$_{17}$NO$_4$P [M-H]$^-$: 348.0906. Found: 348.0902.

4.4.7 (±) α-(4-chlorophenyl)(napthalen-1-ylamino)methyl phosphonic acid (*7g*)

The title compound was prepared from **8g**. White solid; yield 79%. ^{1}H-NMR (400 MHz, CD$_3$OD): δ 8.41 – 8.30 (m, 1H, Ar), 7.78 – 7.67 (m, 1H, Ar), 7.65 – 7.52 (m, 2H, Ar), 7.50 – 7.35 (m, 2H, Ar), 7.27 – 7.17 (m, 2H, Ar), 7.14 – 6.98 (m, 2H, Ar), 6.25 (dd, J = 7.5, 1.4 Hz, 1H, Ar), 4.65 (d, J = 21.1 Hz, 1H, P-CH). ^{13}C NMR (101 MHz, CD$_3$OD): δ 156.76, 147.94, 146.89, 144.98, 144.01, 132.43, 129.74, 129.50, 129.15, 126.64, 126.52, 125.37, 122.91, 122.40, 116.59, 106.32, 60.78 (d, J = 130 Hz). ^{31}P NMR (CD$_3$OD, 162 MHz): δ 14.89. HRMS (ESI) calculated for C$_{17}$H$_{14}$ClNO$_3$P [M-H]$^-$: 346.0400. Found: 346.0414.

4.4.8 (±) α-(4-(trifluoromethyl)phenyl)(napthalen-1-ylamino)methyl phosphonic acid (*7h*)

The title compound was prepared from **8h**. White solid; yield 64%. ^{1}H NMR (400 MHz, CD$_3$OD): δ 8.40 (d, J = 8.2 Hz, 1H, Ar), 7.78 (d, J = 8.0 Hz, 2H, Ar), 7.76 – 7.68 (m, 1H, Ar), 7.57 – 7.37 (m, 4H, Ar), 7.13 – 6.97 (m, 2H, Ar), 6.23 (dd, J = 7.1, 1.7 Hz, 1H, Ar), 4.74 (d, J = 21.2 Hz, 1H, P-CH). ^{13}C NMR (101 MHz, CD$_3$OD): δ 149.08, 145.05, 135.919, 129.43, 129.39, 129.17, 128.59, 128.30, 127.63, 127.40, 126.37, 125.38, 125.26, 125.26, 125.08, 124.94, 122.29, 106.15, 61.44 (d, J = 130 Hz). ^{31}P NMR (162 MHz, CD$_3$OD): δ 14.50. HRMS (ESI) calculated for C$_{18}$H$_{14}$F$_3$NO$_3$P [M-H]$^-$: 380.0663. Found: 380.0679.

4.4.9 (±) α-(4-N-acetamidophenyl)(napthalen-1-ylamino)methyl phosphonic acid (7i)

The title compound was prepared from **8i**. White solid; yield 94%. ^{1}H NMR (400 MHz, CD$_3$OD): δ 8.34 (d, *J* = 8.4 Hz, 1H, Ar), 7.67 (d, *J* = 10.4 Hz, 1H, H-Ar), 7.54-7.51 (m, 2H, Ar), 7.41-7.33 (m, 4H, Ar), 7.04-6.95 (m, 2H, Ar), 6.26 (d, *J* = 7.6 Hz, 1H, Ar), 4.63 (d, *J* = 20.8 Hz, 1H, P-CH), 2.063 (s, 3H, Me). ^{13}C NMR (101 MHz, CD$_3$OD): δ 171.41, 145.16, 139.97, 137.05, 135.76, 129.19, 129.15, 129.04, 127.33, 126.17, 125.16, 122.17, 121.02, 116.70, 106.25, 60.77 (d, *J* = 129.6 Hz), 23.61. ^{31}P NMR (162 MHz, CD$_3$OD): δ 15.94. HRMS (ESI) calculated for C$_{19}$H$_{18}$N$_2$O$_4$P [M-H]$^-$: 369.1004. Found: 369.1016.

4.4.10 (±) α-(4-nitrophenyl)(napthalen-1-ylamino)methyl phosphonic acid (7j)

The title compound was prepared from **8j**. Yellow solid; yield 59%. ^{1}H NMR (400 MHz, CD$_3$OD): δ 8.40 (d, *J* = 8.3 Hz, 1H, Ar), 8.12 (d, *J* = 8.4 Hz, 2H, Ar), 7.82 (dd, *J* = 8.8, 2.0 Hz, 2H, Ar), 7.72 (d, *J* = 8.0 Hz, 1H, Ar), 7.53 – 7.39 (m, 2H, Ar), 7.07 (d, *J* = 4.3 Hz, 2H, Ar), 6.21 (t, *J* = 4.3 Hz, 1H, Ar), 4.81 (d, *J* = 21.3 Hz, 1H, P-CH). ^{13}C NMR (101 MHz, CD$_3$OD): δ 153.26, 147.42, 147.39, 144.81, 144.69, 135.93, 129.74, 129.70, 129.25, 127.34, 126.56, 125.27, 123.91, 122.20, 117.49, 106.22, 61.68 (d, *J* = 130 Hz). ^{31}P NMR (162 MHz, CD$_3$OD): δ 14.02. HRMS (ESI) calculated for C$_{17}$H$_{14}$N$_2$O$_5$P [M-H]$^-$: 357.0640. Found: 357.0635.

4.4.11 (±) α-(3,5-bis(trifluoromethyl)phenyl)(napthalen-1-ylamino)methyl phosphonic acid (7k)

The title compound was prepared from **8k**. White solid; yield 40%. ^{1}H NMR (400 MHz, CD$_3$OD): δ 8.39 (d, *J* = 8.2 Hz, 1H, Ar), 8.18 (s, 2H, Ar), 7.77 – 7.58 (m, 2H, Ar), 7.56 – 7.32 (m, 2H, Ar), 7.06 (d, *J* = 6.1 Hz, 2H, Ar), 6.14 (dd, *J* = 6.1, 2.7 Hz, 1H, Ar), 4.75 (d, *J* = 20.8 Hz, 1H, P-CH). ^{13}C NMR (101 MHz, CD$_3$OD): 148.35, 148.30, 144.79, 135.94, 131.82, 131.47, 131.15, 129.32, 129.20, 127.34, 126.75, 126.74, 126.52, 125.28, 124.09, 122.27, 122.07, 117.54, 105.93, 61.43 (d, *J* = 130 Hz). ^{31}P NMR (162 MHz, CD$_3$OD): δ 13.55. HRMS (ESI) calculated for C$_{19}$H$_{13}$F$_6$NO$_3$P [M-H]$^-$: 448.0537. Found: 448.0566.

4.5 Protein expression and purification

The plasmid pTrcHisA was used for expression of TcTS as described previously by Paris *et al.* [31]. This plasmid encodes for recombinant TcTS truncated to only contain the catalytic and lectin-like domains, with seven surface point mutations (N58F, S495K, V496G, E520K, D593G, I597D and H599R). These mutation were introduced to promote crystallisation and have been shown to have no impact on catalytic activity of TcTS [19]. Over expression was performed using *E. coli* strain BL21 (DE3) chemically competent cells (NEB).

4.6 IC$_{50}$ inhibition studies

The hydrolytic activity of TcTS was determined using a continuous fluorometric assay by measuring the rate of release of 4-methylumbelliferone from the substrate 2'-(4-methylumbelliferyl)-α-D-*N*-acteylneuraminic acid (MuNANA) as described previously [19]. Fluorescence was measured on a FLUOstar Omega (BMG labtech) using excitation and

emission filters at 360 and 460 nm, respectively. All assays were run in 20mM Tris-HCl and 50mM NaCl at pH 7.5, containing 0.01% Triton X100 at 35°C. TcTS (50 μg/mL) was pre-incubated with each compound over at least seven inhibitor concentrations (ranging from 1 μM to 15 mM) for 10 minutes at 35°C, then the assay initiated by the addition of MuNANA (200 μM) to give a final reaction volume of 100 μL. The fluorescence was analysed using MARs data analysis software (BMG labtech), where a linear region of the raw data curve was selected and the slope calculated (RFU/min) which represents hydrolytic activity of the enzyme. The IC_{50} values were determined as the concentration of inhibitor required to reduce hydrolytic activity of TcTS by 50% compared to the control (no inhibitor). All assays were repeated in three (n=3) independent experiments.

4.7 Mode of inhibition studies

The K_i determinations were performed by measuring the residual activity of TcTS in the presence of several different concentrations of MuNANA (0–400 μM) at specified inhibitor concentrations. Residual hydrolytic activity of TcTS was determined using the assay method described above. Nonlinear fitting was performed by GraphPad Prism software and K_m/V_{max} of different concentration of inhibitors was calculated. Further Ki and its standard error were calculated using competitive, non-competitive, uncompetitive and mixed inhibition models, the best fitting model were selected as the inhibition mode of the inhibitors. Lineweaver-Burk plots were generated from the reciprocals of both substrate concentration and activity at each inhibitor concentration to present the mode of inhibition.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/xxxxxxx

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