High-throughput synthesis of azide libraries suitable for direct "click" chemistry and *in situ* screening†

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A key challenge in current drug discovery is the development of high-throughput (HT) amenable chemical reactions that allow rapid synthesis of diverse chemical libraries of enzyme inhibitors. The Cu(I)-catalyzed, 1,3-dipolar cycloaddition between an azide and an alkyne, better known as "click chemistry", is one such method that has received the most attention in recent years. Despite its popularity, there is still a lack of robust and efficient chemical strategies that give access to diverse libraries of azide-containing building blocks (key components in click chemistry). We report herein a highly robust and efficient strategy for high-throughput synthesis of a 325-member azide library. The method is highlighted by its simplicity and product purity. The utility of the library is demonstrated with the subsequent "click" synthesis of the corresponding bidentate inhibitors against PTP1B.

Introduction

The field of high-throughput enzymology (or "Catalomics" as we call it) necessitates the development of highly efficient and robust synthetic methods that enable rapid generation of enzyme probes and inhibitors.[1] The Cu(I)-catalyzed, 1,3-dipolar cycloaddition between an azide and an alkyne, better known as "click chemistry", is one such method that has received the most attention in recent years.[2] The salient features of this reaction include its high efficiency, chemoselectivity, modularity and biocompatibility, which allow direct *in situ* screening of the products formed without the need of tedious purification steps.[3] Consequently, many research groups have successfully adopted this platform for the high-throughput screening and discovery of inhibitors against numerous enzymes, including HIV-1 protease,[4a,b] sulfotransferases,[4c] fucosyltransferases,[4d] matrix metalloproteases,[4e] acetylcholinesterase,[4f] and others.[4g–i] For example, we recently used click chemistry to discover a potent, cell-permeable inhibitor against PTP1B by assembling together an alkyne-containing isoxazole "warhead" (a bioisostere of phosphotyrosine) and an azide-modified aryl group that binds to the secondary binding site in the enzyme (Fig. 1).[4g] PTP1B has previously been identified as a key target for diseases such as diabetes, obesity and cancer, and our inhibitor design was based on the elegant work reported by Zhang *et al* and researchers from Abbott laboratories.[5]

When compared with other existing drug discovery approaches, our method (as well as other published "click"-based enzyme screening methods),[4] though advantageous, remains to be truly high-throughput due to the lack of a robust and efficient chemical strategy that gives access to diverse libraries of azide-containing building blocks. The standard azidation method calls for the use of NaN_3 (a highly toxic chemical) which, while efficient, necessitates the compounds to be individually synthesized and purified using solution phase chemistry, therefore is time-consuming and low-throughput.[4] Recently, improved methods for azide synthesis have shown some compatibility with click chemistry.[6] For example, Fokin *et al* reported the *in situ* generation of azides from alkyl and aryl halides followed by direct "click" chemistry.[6a] Other methods include the *in situ* generation of azides from alcohols,[6b] the direct conversion of amines to azides by employing the diazotransfer reaction using TfN_3 or imidazole-1-sulfonyl azide hydrochloride,[6c–e] the use of TPP/DDQ/*n*-Bu_4NN_3 reagents to convert alcohols directly to azides,[6f] and the microwave-assisted azidation of tosylates.[6g,h] All of them, again due to the use of NaN_3 (or equivalents), are not suitable for direct *in situ* screening with enzymes, unless additional purification steps (either before or after click chemistry) are carried out. Herein, we report a truly high-throughput amenable, solid-phase strategy for the synthesis of high-quality azides which, without purification, could be used for "click assembly" and direct *in situ* screening of enzyme inhibitors (Scheme 1, top). With this new method, the whole process

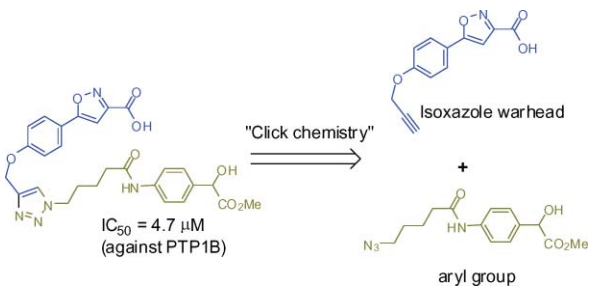

Fig. 1 PTP1B inhibitor assembled by "click chemistry".[4g]

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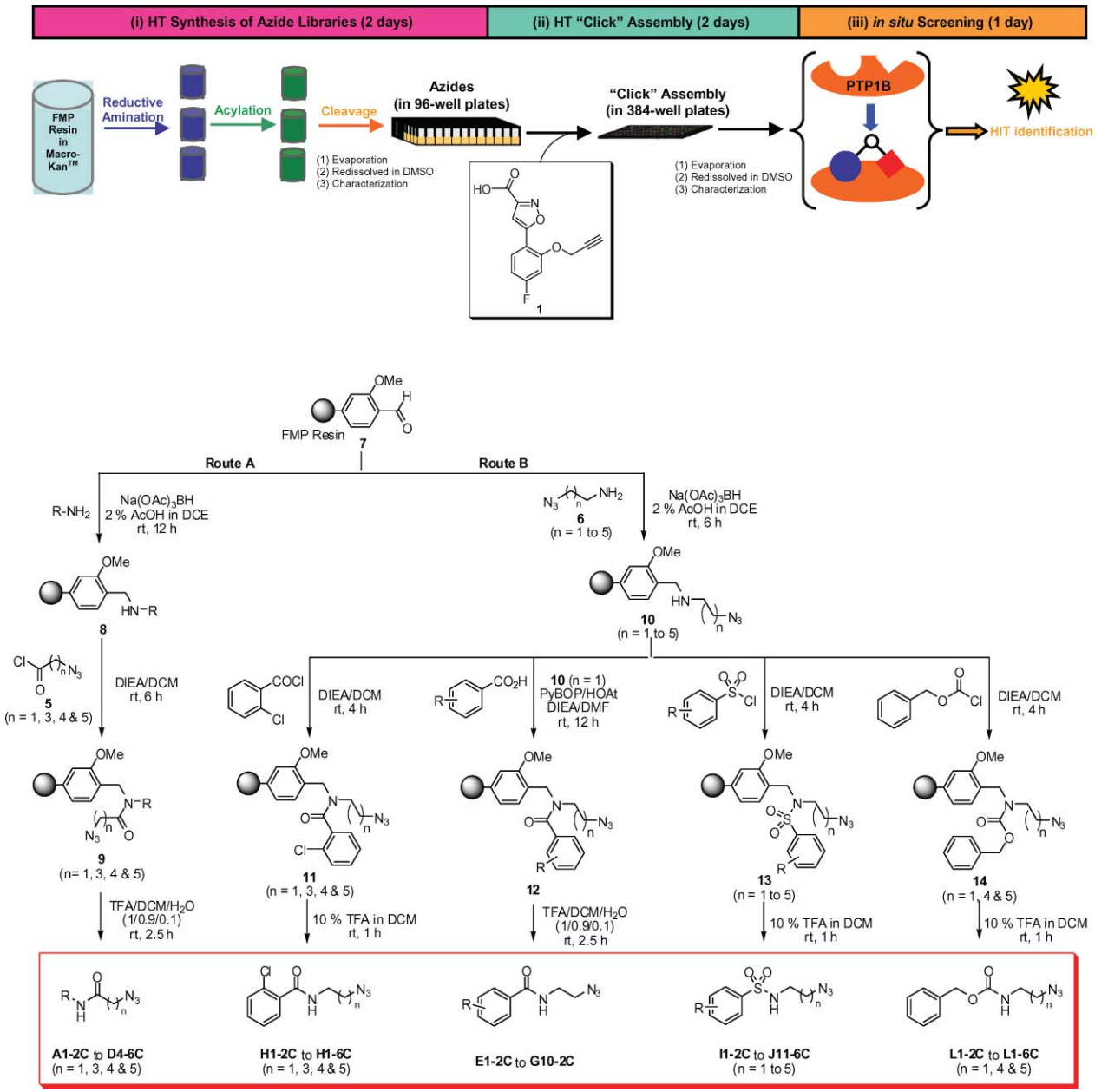

Scheme 1 (top) Workflow of our strategy. (bottom) Traceless solid-phase synthesis of azide libraries.

of inhibitor discovery can be now completed in less than one week.

Results and discussion

Chemistry

As shown in Scheme 1, our strategy made use of the commercially available PL-FMP resin on which an immobilized 4-formyl-3-methoxyphenoxy moiety efficiently captured a variety of primary amines *via* reductive amination and converted them to the corresponding secondary amines which were subsequently acylated.[7] Following TFA cleavage, the desired products were obtained in uniformly high quality. There are numerous advantages in this method: (1) it is a traceless solid-phase strategy, allowing the use of as-is starting materials (*i.e.* same as solution-phase methods) as building blocks and is easily amenable to high-throughput synthesis (*i.e.* IRORI(tm) split-and-pool sorting technology[8]) of a large number of azides. We routinely make several hundreds of azides in milligram scales in 2–3 days; (2) it is highly robust, capable of making a variety of azides from different functionalized building blocks (*i.e.* amines, acids, sulfonyl chloride, and chloroformates) with suitable linkers; (3) it gives rise to products with extremely high purity, thus allowing them to be used directly for "click" assembly followed by *in situ* enzyme screening.

To demonstrate our strategy, a 325-member azide library was synthesized as shown in Scheme 1 (bottom). For comparison, 9 azides were synthesized using standard solution-phase method.[4g] Depending on the choice of building blocks and linkers, either route **A** or **B** was taken. In route **A**, 40 different commercially

available aromatic amine building blocks were treated with the PL-FMP aldehyde resin, **7**, in the presence of $Na(OAc)_3BH$/2% glacial acetic acid, to give the corresponding secondary amines, **8**. The reductive amination reaction proceeded smoothly with a variety of aromatic amines bearing different substituents such as -F, -Cl, -OR, -SCH_3, -CO_2R and -R. Benzyl, naphthyl and anthracenyl amines too underwent reductive amination smoothly. Subsequently, treatment with azidoalkynoyl chlorides, **5**, furnishing the N-acylated products, **9**. Other coupling conditions attempted (*e.g.* DIC/EDC/HATU/PyBOP) failed to give the desired products in sufficient purity. In route **B**, we first carried out reductive amination with azide-functionalized amine linkers **6** to give **10**. Subsequently, the resulting resins were treated with different commercially available building blocks, including acid chlorides, aryl acids, sulfonyl chlorides and chloroformates, giving **11**, **12**, **13** and **14** respectively. We found that coupling conditions using acid chlorides, sulfonyl chlorides and chloroformates (giving **11**, **13** & **14** respectively) were excellent as expected. With aryl acid building blocks, however, the PyBOP/HOAt coupling was the method of choice, providing complete coupling to give **12**. Other coupling conditions were tried but failed to give satisfactory results. We also found building blocks bearing different functionalities such as -F, -Cl, -Br, -R, -OH, -OCH_3, -CF_3, -CN, -OH, -NO_2 and -vinyl were tolerated. Finally, cleavage of the resins with optimized TFA cocktails gave the desired products, which, upon concentration *in vacuo*, analysis and characterizations by LC-MS/NMR, showed uniformly high purity (> 95% in most cases). In total, 198 amide-bearing azides (*i.e.* **A1-2C** to **D4-6C**, **E1-2C** to **G10-2C**, **H1-2C** to **H1-6C**), 115 different sulfonamide-containing azides (*i.e.* **I1-2C** to **J11-6C**), and 3 carbamate-derivatized azides (*i.e.* **L1-2C** to **L1-6C**) were successfully prepared. Stock solutions of these azides were prepared in uniform concentration with DMSO in 96-deep well plates and used directly, without any purification, for subsequent *in situ* click assembly followed by enzymatic screening.

Inhibitor screening

To make potential cell-permeable, bidentate inhibitors against PTP1B, we subsequently "click"-assembled the above 325-member azide library with the alkyne-modified isoxazole warhead, **1**, as shown in Scheme 1. The choice of this warhead was guided by our previous results and computational modeling.[4g] The synthesis of the warhead is essentially the same as previously reported. Optimizations of the click chemistry conditions were essential in order for the products to be generated in quantitative yield. With $CuSO_4$/sodium ascorbate catalysts and H_2O/*t*-BuOH as cosolvents, we were able to carry out the reactions in high throughput and small scale (100 μL/reaction volume) using 384-deep well plates. At the end of the reaction, (~2 days), products were evaporated *in vacuo*, redissolved in DMSO, and directly characterized (by LCMS and NMR); most were found to be of sufficient purity (> 95% excluding excess starting materials in some cases; see ESI) and used for direct *in situ* enzymatic screening. The inhibition potency of the 325-member "click" products against PTP1B was determined using a standard fluorescence microplate-based assay as previously reported.[4g] From the preliminary results, eight representative "hits" were identified (Fig. 2). They were further purified, characterized and their IC_{50} values were determined (Table 1). Of particular interest is **B11-2C-W2**, which has an IC_{50} of 11.1 μM, nearly as potent as the best "click" inhibitor previously identified (shown in Fig. 1). This thus confirms the utility of our azide library and its potential applications with other click chemistry-based strategies. We are currently testing the *in vivo* activity of this new target compound and its analogs.

Table 1 IC_{50} of selected "hits" against PTP1B

Inhibitor	IC_{50} (μM)	Inhibitor	IC_{50} (μM)
A5-2C-W2	634	B9-2C-W2	34.0
A6-2C-W2	159	B10-2C-W2	148
B7-2C-W2	55	B11-2C-W2	11.1
B8-2C-W2	70.8	C8-2C-W2	24.0

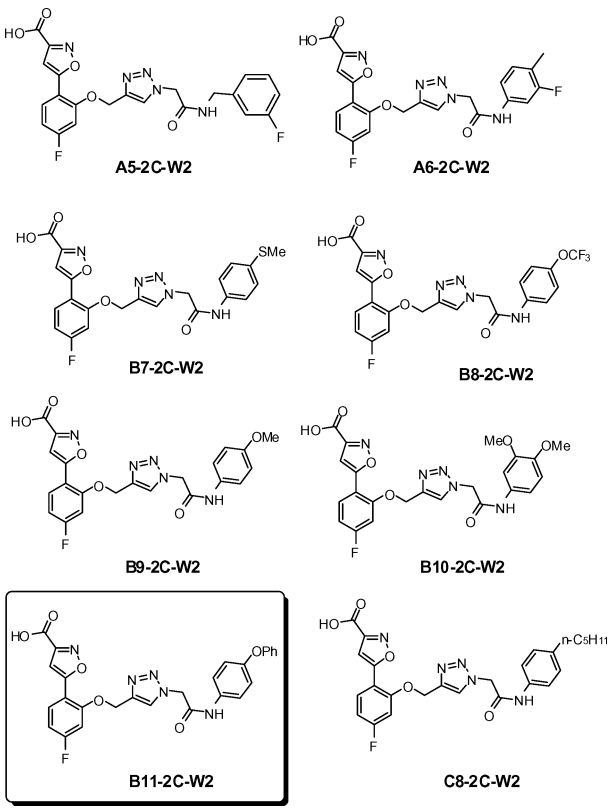

Fig. 2 Structure of eight representative hits.

Conclusions

In conclusion, we have developed a solid-phase strategy for the synthesis of high-quality azides which could be used for direct "click assembly" and *in situ* screening of enzyme inhibitors. With this new method, the whole process of inhibitor discovery, starting from the synthesis of building blocks to the identification of enzyme inhibitors, could now be completed in less than one week. The entire operation was performed in 96/384-well plates without any purification, therefore is truly high-throughput amenable. We foresee azide libraries synthesized using this approach will be equally useful for other classes of enzymes. Our present approach thus serves as a powerful chemical tool in click chemistry and in the emerging field of "Catalomics".[1]

Experimental

Material and methods

All chemicals were purchased from commercial vendors and used without further purification, unless otherwise noted. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker model Avance 300 MHz or DPX-300 MHz or DPX-500 MHz NMR spectrometer. Chemical shifts are reported in parts per million referenced with respect to residual solvent ($CHCl_3$ = 7.26 ppm and DMSO-d_6 = 2.50 ppm) or from internal standard tetramethylsilane (TMS = 0.00 ppm). The following abbreviations were used in reporting spectra: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets. HPLC grade solvents were used for all the reactions. Shimadzu LCMS-IT-TOF system equipped with auto-sampler (cat. no. LCMS-2010EV) was used for the analysis of the azide and the 1,2,3-triazole library. Sciclone ALH 3000 Liquid Handler Workstation (Caliper Life Sciences) and Multi-drop Combi dispenser (Thermo Scientific) were used to dispense and transfer reagents, solvents and reactants during the library synthesis. 384-deep well polypropylene plates (Genetix. cat. no. X7020) were used as reaction plates for the library synthesis.

Solid-phase synthesis of azides

Chemical synthesis of the alkyne warhead, **1**, is described in the ESI. The construction of the azide library was achieved by IRORI® split-and-pool directed sorting technology.[8] Full details of the synthesis are described in the ESI. Final products were released from the solid phase by our optimized TFA cleavage protocol (below). For complete structural information, ID and full characterizations of all azides, see Part 2 (in the ESI). Representative procedures for the solid-phase synthesis of resins **8** and **9**, followed TFA cleavage to give **A1-2C** to **D4-6C** (as shown in Scheme 1) are given below.

Synthesis of the reductive aminated resin (8). PL-FMP resin (200 × 150 mg, 0.9 mmol/g) was taken in 200 MacroKan™ reactors each containing a RF tag. The resin was swelled in 1,2-dichloroethane (250 mL) for about two hours after which the solvent was decanted. The 200 microreactors were then distributed equally into 40 different bottles of capacity 50 mL containing 2% acetic acid in 1,2-dichloroethane (30 mL). Amines (**A1**–**D4**) (5 eq) were added to the bottles, so that each bottle contains a unique amine. The reaction mixture was then incubated for about 3 hours after which sodium triacetoxyborohydride (6 eq) was added. After shaking for another 8 hours the solution was decanted and the reactors were combined and washed with DCM (200 mL × 5), MeOH (200 mL × 2) and THF (200 mL × 3) and dried to afford the resin **8**.

Synthesis of the N-acylated resin (9a–e). The 200 reductive aminated resins were separated into 5 sets (40 different reductive aminated resins × 5 sets). To the first set, DCM (100 mL), DIEA (10 eq) and acid chloride, **5a-e** (5 eq) were added and the reaction mixture was shaken for about 8 hours after which the solution was decanted and the resin was washed with DCM (100 mL × 3), MeOH (100 mL × 2) and THF (100 mL × 3) and dried to afford resin **9a-e**.

General protocol for cleavage and release of azides (A1-2C to D4-6C). Each dried resin was treated with a 1.5 ml solution containing TFA (50%), DCM (45%) and water (5%) and the mixture was shaken for 4 hours and transferred into 3 different 96-well plates and concentrated *in vacuo* to afford the azides below. Representative yield varied from 50% to 80%. The azides were re-dissolved in DMSO (1 mL) to give a stock solution (50 mM assuming 50% yield).

Characterizations of selected azides

Except for the azides cleaved from **9b** resin (with 3C linker; data not shown), almost all azides synthesized from the above protocols are of high purity (90–95%; see ESI Part 2 for detailed structures, ID and characterizations). Representative compounds were further characterizations, without any purification, by LCMS, ^{1}H and ^{13}C NMR, as shown below and in ESI.

(A2-2C). 2-Azido-*N*-(3-fluoro-phenyl)-acetamide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 10.3 (s, 1H), 7.58 (d, J = 8 Hz, 1H), 7.39–7.29 (m, 1H), 7.09 (d, J = 50 Hz, 1H), 6.92 (m, 1H), 4.06 (s, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 167.1, 163.6, 131.0, 140.1, 115.5, 110.6, 106.6, 51.8. ESI-MS(TOF): m/z 195.060 $[M + H]^+$.

(B1-2C). 2-Azido-*N*-(2-phenoxy-phenyl)-acetamide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 9.71 (s, 1H), 7.39 (t, J = 8 Hz, 3H), 7.16–7.11 (m, 3H), 7.00 (d, J = 8 Hz, 2H), 6.89–6.87 (m, 1H), 4.06 (s, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 156.5, 129.9, 128.9, 125.3, 123.6, 123.5, 118.6, 118.5, 51.1. ESI-MS(TOF): m/z 269.098 $[M + H]^+$.

(B2-2C). 2-Azido-*N*-(3,4-dimethoxy-phenyl)-acetamide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 9.36 (s, 1H), 7.71 (s, 1H), 6.97 (d, J = 8.8 Hz, 1H), 6.66 (m, 1H), 4.14 (s, 2H), 3.79 (s, 3H), 3.69 (s, 3H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 166.9, 153.4, 143.9, 127.8, 112.4, 109.0, 108.49, 56.7, 55.8, 51.8. ESI-MS(TOF): m/z 237.091 $[M + H]^+$.

(B5-2C). 2-Azido-*N*-(4-pentyloxy-phenyl)-acetamide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 10.06 (s, 1H), 6.96 (s, 2H), 4.00 (s, 2H), 3.74 (s, 6H), 3.62 (s, 3H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 166.0, 152.7, 134.4, 133.6, 97.0, 60.05, 55.6, 51.2. ESI-MS(TOF): m/z 267.100 $[M + H]^+$.

(B12-2C). 2-Azido-*N*-(4-pentyl-phenyl)-acetamide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 10.10 (s, 1H), 7.28 (s, 1H), 7.22 (t, J = 8.2 Hz, 1H), 7.11 (d, J = 8.2 Hz, 1H), 6.66 (d, J = 8 Hz, 1H), 4.02 (s, 2H), 3.73 (s, 3H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 166.8, 160.0, 140.0, 130.1, 112.0, 109.6, 105.5, 55.4, 51.7. ESI-MS(TOF): m/z 207.080 $[M + H]^+$.

(C4-2C). 2-Azido-*N*-(3,4-dimethyl-phenyl)-acetamide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 9.96 (s, 1H), 7.36 (s, 1H), 7.29 (d, J = 8.2 Hz, 1H), 7.06 (d, J = 8.2 Hz, 1H), 3.99 (s, 2H), 2.19 (s, 3H), 2.16 (s, 3H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 165.8, 136.3, 136.0, 131.4, 129.6, 120.4, 116.7, 51.2, 19.5, 18.7. ESI-MS(TOF): m/z 205.101 $[M + H]^+$.

(C7-2C). 2-Azido-*N*-(4-ethyl-phenyl)-acetamide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 10.0 (s, 1H), 7.47 (d, J = 8.8 Hz, 2H), 7.15 (d, J = 8.2 Hz, 2H), 4.00 (s, 2H), 2.55 (m, 2H), 1.15 (t, J = 7.6 Hz, 3H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 166.4, 139.5,

136.6, 128.5, 119.8, 51.7, 28.0, 16.1. ESI-MS(TOF): m/z 205.103 $[M + H]^+$.

(C10-2C). 3-(2-Azido-acetylamino)-benzoic acid ethyl ester. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 10.36 (s, 1H), 8.24 (s, 1H), 7.85 (d, $J = 8.2$ Hz, 1H), 4.32 (m, $J = 7$ Hz, 2H), 4.07 (s, 2H), 1.32 (t, $J = 7$ Hz, 3H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 166.6, 165.4, 138.7, 130.4, 129.3, 124.2, 123.6, 119.6, 60.8, 51.2, 14.1.

(C11-2C). 2-Azido-*N*-(4-heptyl-phenyl)-acetamide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 10.03 (s, 1H), 7.46 (d, $J = 8.8$ Hz, 2H), 7.12 (d, $J = 8.8$ Hz, 2H), 4.01 (s, 2H), 2.51 (t, 2H), 1.52 (m, 2H), 1.24 (m, 8H), 0.85 (t, $J = 7$ Hz, 3H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 165.9, 137.6, 136.0, 128.4, 119.2, 51.2, 34.4, 31.1, 30.9, 28.5, 28.4, 22.0, 13.8. ESI-MS(TOF): m/z 275.179 $[M + H]^+$.

(D1-2C). *N*-Anthracen-1-yl-2-azido-acetamide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 10.40 (s, 1H), 8.48 (d, $J = 13.8$ Hz, 3H), 8.06 (t, $J = 9.4$ Hz, 3H), 7.56 (d, $J = 9.4$ Hz, 1H), 7.47 (m, 2H), 4.14 (s, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 167.1, 135.8, 132.2, 131.5, 129.3, 128.5, 128.1, 126.2, 125.6, 125.5, 121.2, 116.7, 114.5, 51.9. ESI-MS(TOF): m/z 277.099 $[M + H]^+$.

(A1-4C). 4-Azido-*N*-(4-fluoro-phenyl)-butyramide. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.94 (s, 1H), 7.57–7.54 (m, 2H), 7.09–7.06 (m, 2H), 3.35–3.33 (t, $J = 6.62$ Hz, 2H), 2.36–2.33 (m, 2H), 1.81–1.78 (m, 2H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 170.12, 158.75, 156.84, 135.56, 120.76(d), 115.20(d), 50.24, 33.07, 24.19.

(A6-4C). 4-Azido-*N*-(3-fluoro-4-methyl-phenyl)-butyramide. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 10.03 (s, 1H), 7.55–7.52 (m, 1H), 7.18–7.17 (m, 2H), 3.40–3.37 (t, $J = 6.625$ Hz, 2H), 2.51–2.50 (m, 2H), 2.16 (s, 3H), 1.86–1.81 (q, 2H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 170.37, 161.12, 159.21, 138.54(d), 131.25(d), 118.18(d), 114.53, 105.80, 5.21, 33.13, 24.52, 13.54.

(A8-4C). 4-Azido-*N*-(4-chloro-phenyl)-butyramide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 10.02 (s, 1H), 7.58–7.56 (d, $J = 8.85$ Hz, 2H), 7.30–7.28 (d, $J = 8.8$ Hz, 2H), 3.35–3.33 (t, $J = 6.95$ Hz, 2H), 2.37 (m, 2H), 1.82–1.77 (m, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 170.40, 138.09, 128.48, 126.49, 120.52, 50.22, 33.16, 24.12.

(A11-4C). 4-Azido-*N*-(4-chloro-2-methoxy-phenyl)-butyramide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 9.29 (s, 1H), 8.10 (s, 1H), 7.10–7.08 (m, 1H), 7.05–7.03 (m, 1H), 3.83 (s, 3H), 3.39–3.36 (t, $J = 6.9$ Hz, 2H), 2.54–2.49 (m, 2H), 1.86–1.80 (m, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 170.94, 148.05, 128.58, 123.74, 123.29, 120.84, 112.85, 112.35, 55.96, 50.21, 32.96, 24.26.

(B4-4C). 4-Azido-*N*-(2-methoxy-phenyl)-butyramide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 9.06 (s, 1H), 7.88–7.86 (m, 1H), 7.03–6.97 (m, 2H), 6.86–6.83 (t, 1H), 3.8 (s, 3H), 3.35–3.32 (t, $J = 6.92$ Hz, 2H), 2.46–2.41 (m, 2H), 1.80–1.77 (m, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 170.41, 149.64, 127.23, 124.26, 122.13, 120.09, 111.06, 55.55, 50.25, 32.94, 24.38.

(B7-4C). 4-Azido-*N*-(4-methylsulfanyl-phenyl)-butyramide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 9.89 (s, 1H), 7.51–7.50 (m, 2H), 7.17–1.16 (m, 2H), 3.35–3.32 (t, $J = 6.95$ Hz, 3H), 2.38 (m, 3H), 2.36–2.33 (t, 2H), 1.82–1.77 (q, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 170.14, 136.73, 131.42, 127.13, 119.71, 50.26, 33.14, 24.20, 15.57.

(B8-4C). 4-Azido-*N*-(4-trifluoromethoxy-phenyl)-butyramide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 10.09 (s, 1H), 7.66–7.64 (d, $J = 8.85$ Hz, 2H), 7.25–7.23 (d, $J = 8.2$ Hz, 2H), 3.36–3.33 (t, $J = 6.6$, 2H), 2.38–2.35 (m, 2H), 1.83–1.78 (m, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 170.49, 143.34, 138.37, 121.47, 120.30, 119.09, 50.24, 33.16, 24.15.

(B11-4C). 4-Azido-*N*-(4-phenoxy-phenyl)-butyramide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 9.92 (s, 1H), 7.57–7.56 (m, 2H), 7.32–7.29 (m, 2H), 7.05–7.02 (m, 1H), 6.94–6.90 (m, 4H), 3.36–3.33 (t, $J = 6.92$ Hz, 2H), 2.37–2.34 (t, $J = 7.25$ Hz, 2H), 1.83–1.78 (m, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 170.04, 157.38, 151.47, 135.13, 129.85, 122.83, 120.70, 119.37, 117.72, 50.26, 33.10, 24.26.

(B12-4C). 4-Azido-*N*-(4-methoxy-phenyl)-butyramide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 9.87 (s, 1H), 3.67 (s, 3H), 3.36–3.329 (m, 2H), 2.36–2.33 (t, $J = 7.25$ Hz, 2H), 1.82–1.77 (m, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 170.31, 159.44, 140.34, 129.36, 111.32, 108.42, 104.86, 54.86, 50.25, 33.23, 24.18.

(C6-4C). 4-Azido-*N*-(4-*tert*-butyl-phenyl)-butyramide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 9.80 (s, 1H), 7.45–7.44 (d, $J = 8.8$ Hz, 2H), 7.25–7.24 (d, $J = 8.2$ Hz, 2H), 3.35–3.32 (t, $J = 6.62$ Hz, 2H), 2.35–2.32 (t, $J = 7.57$ Hz, 2H), 1.82–1.76 (m, 2H), 1.2 (s, 9H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 170.00, 145.26, 136.58, 125.14, 118.84, 50.27, 33.89, 33.10, 31.13, 24.28.

(C7-4C). 4-Azido-*N*-(4-ethyl-phenyl)-butyramide. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.36–7.33 (d, $J = 8.7$ Hz, 2H), 6.85–6.82 (d, $J = 8.7$ Hz, 2H), 4.03–3.96 (m, 2H), 3.42–3.38 (t, $J = 6.27$ Hz, 2H), 2.47–2.43 (t, $J = 7.14$ Hz, 2H), 2.01–1.97 (t, $J = 6.79$ Hz, 2H), 1.41–1.37 (t, $J = 6.79$ Hz, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ 170.83, 142, 130, 122.30, 114.76, 63.68, 50.64, 33.91.

(A12-5C). 5-Azido-pentanoic acid (2-ethoxy-phenyl)-amide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 8.87 (s, 1H), 7.85 (m, 1H), 7.00–6.97 (m, 2H), 6.86–6.83 (m, 1H), 4.06–3.99 (m, 2H), 3.34–3.32 (m, 2H), 2.52–2.37 (m, 2H), 1.58 (m, 4H), 1.32–1.21 (m, 3H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 170.87, 148.98, 127.45, 124.33, 122.37, 120.07, 112.17, 63.82, 50.38, 44.75, 35.40, 27.74, 22.39, 14.52. ESI-MS(TOF): m/z 263.148 $[M + H]^+$.

(B3-5C). 5-Azido-pentanoic acid (4-ethoxy-phenyl)-amide. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.68 (s, 1H), 7.44–7.41 (d, $J = 15$ Hz, 2H), 6.81–6.78 (d, $J = 15.05$ Hz, 2H), 3.95–3.88 (m, 2H), 3.33–3.28 (d, $J = 10.67$ Hz, 2H), 2.27–2.23 (t, $J = 11.5$ Hz, 2H), 1.58–1.52 (m, 4H), 1.27–1.20 (m, 3H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 170.27, 154.24, 132.33, 120.55, 114.26, 63.01, 50.38, 35.55, 27.84, 22.43, 14.64. ESI-MS(TOF): m/z 263.148 $[M + H]^+$.

(B4-5C). 5-Azido-pentanoic acid (2-methoxy-phenyl)-amide. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.99 (s, 1H), 7.89–7.87 (d, $J = 12.85$, 1H), 7.04–6.96 (m, 2H), 6.87–6.81 (m, 1H), 3.77 (s, 3H), 3.33–3.29 (t, $J = 10.55$ Hz, 2H), 2.39–2.35 (t, $J = 11.1$ Hz, 2H), 1.61–1.51 (m, 4H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 170.99, 149.60, 127.30, 124.20, 122.06, 120.10, 111.04, 55.55, 50.35, 35.35, 27.77, 22.37. ESI-MS(TOF): m/z 249.133 $[M + H]^+$.

(B6-5C). 5-Azido-pentanoic acid (3-isopropoxy-phenyl)-amide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 9.78 (s, 1H), 7.24 (s, 1H), 7.13–7.08 (m, 2H), 6.54–6.51 (m, 1H), 4.51–4.43 (m, 1H), 3.3 (t, 2H), 2.30–2.26 (t, $J = 11.65$ Hz, 2H), 1.63–1.50 (m, 4H), 1.21

(s, 3H), 1.23 (s, 3H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 170.85, 157.64, 140.42, 129.34, 111.11, 110.12, 106.52, 69.06, 50.38, 35.74, 27.81, 22.23, 21.79. ESI-MS(TOF): m/z 277.163 $[M + H]^+$.

(B10-5C). 5-Azido-pentanoic acid (3,4-dimethoxy-phenyl)-amide. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.7 (s, 1H), 7.06–7.03 (d, J = 8.55 Hz, 1H), 6.83–6.80 (d, J = 8.52 Hz, 1H), 3.7 (s, 6H), 3.33–3.29 (t, J = 6.24 Hz, 2H), 2.28–2.24 (t, J = 6.73 Hz, 2H), 1.60–1.50 (m, 4H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 170.39, 148.50, 144.63, 132.96, 112.08, 110.94, 104.33, 55.71, 55.30, 50.40, 38.63, 35.65, 27.86, 22.32. ESI-MS(TOF): m/z 279.143 $[M + H]^+$.

(C2-5C). 5-Azido-pentanoic acid (5-chloro-2-phenoxy-phenyl)-amide. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.56 (s, 1H), 8.10 (s, 1H), 7.37–7.32 (m, 2H), 7.10–7.06 (m, 2H), 6.97–6.94 (d, J = 13.95 Hz, 2H), 6.87–6.84 (d, J = 14.5 Hz, 1H), 3.24 (t, 2H), 2.36–2.31 (t, J = 11.5 Hz, 2H), 1.52–1.41 (m, 4H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 171.67, 156.34, 145.81, 131.15, 129.89, 127.13, 124.11, 123.59, 122.71, 120.09, 118.30. ESI-MS(TOF): m/z 345.110 $[M + H]^+$.

(C5-5C). 5-Azido-pentanoic acid (3-ethyl-phenyl)-amide. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 9.77 (s, 1H), 7.40–7.31 (m, 2H), 7.16–7.07 (m, 1H), 6.83–6.77 (m, 1H), 3.33–3.27 (m, 2H), 2.52 (m, 2H), 2.30–2.22 (m, 2H), 1.55 (m, 4H), 1.14–1.05 (m, 3H).^{13}C-NMR (125 MHz, DMSO-d_6) δ 170.77, 144.12, 139.24, 128.47, 122.44, 118.39, 116.49, 50.39, 35.70, 28.23, 27.83, 22.29, 15.46. ESI-MS(TOF): m/z 247.153 $[M + H]^+$.

(C9-5C). 4-(5-Azido-pentanoylamino)-benzoic acid ethyl ester. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 10.19 (s, 1H), 7.87–7.84 (d, J = 12.35 Hz, 2H), 7.70–7.67 (d, J = 13.6 Hz, 2H), 4.26–4.22 (m, 2H), 3.33–3.29 (t, J = 10 Hz, 2H), 2.36–2.32 (t, J = 11.1 Hz, 2H), 1.61–1.51 (m, 4H), 1.28–1.22 (m, 3H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 171.48, 165.29, 143.56, 130.14, 123.95, 118.31, 60.32, 50.38, 35.78, 27.79, 22.10, 14.15. ESI-MS(TOF): m/z 291.144 $[M + H]^+$.

(A1-6C). 6-Azido-hexanoic acid (4-fluoro-phenyl)-amide. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.87 (s, 1H), 7.55 (m, 2H), 7.07 (m, 2H), 3.28 (t, J = 6.74 Hz, 2H), 2.25 (t, J = 7.23 Hz, 2H), 1.51 (m, 4H), 1.31 (m, 2H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 171.31, 159.74, 136.06, 121.13, 115.37, 50.89, 36.49, 28.40, 26.18, 24.97. ESI-MS(TOF): m/z 251.124 $[M + H]^+$.

(A8-6C). 6-Azido-hexanoic acid (4-chloro-phenyl)-amide. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.95 (s, 1H), 7.56 (d, J = 8.85 Hz, 2H), 7.30 (d, J = 8.88 Hz, 2H), 3.28 (t, J = 6.82 Hz, 2H), 2.27 (t, J = 7.30 Hz, 2H), 1.51 (m, 4H), 1.33 (m, 2H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 171.59, 138.62, 128.89, 126.81, 120.89, 50.88, 36.57, 28.39, 26.15, 24.90. ESI-MS(TOF): m/z 267.091 $[M + H]^+$.

(A11-6C). 6-Azido-hexanoic acid (4-chloro-2-methoxy-phenyl)-amide. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.15 (s, 1H), 8.06 (s, 1H), 7.10–6.95 (m, 2H), 3.79 (s, 3H), 3.28 (t, J = 6.80 Hz, 2H), 2.37 (t, J = 7.32 Hz, 2H), 1.52 (m, 4H), 1.31 (m, 2H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 172.10, 148.40, 129.09, 124.14, 123.54, 121.11, 112.76, 56.38, 50.91, 36.19, 28.39, 26.11, 24.99. ESI-MS(TOF): m/z 297.104 $[M + H]^+$.

(A12-6C). 6-Azido-hexanoic acid (2-ethoxy-phenyl)-amide. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.81 (s, 1H), 7.85 (d, 1H, J = 7.74 Hz), 7.03–6.95 (m, 2H), 6.86–6.79 (m, 1H), 4.03 (q, J = 6.9 Hz, 2H), 3.29 (t, J = 6.74 Hz, 2H), 2.34 (t, J = 7.32 Hz, 2H), 1.52 (m, 4H), 1.32 (m, 2H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 171.46, 149.31, 127.93, 124.64, 122.67, 120.47, 112.59, 64.22, 50.93, 36.31, 28.42, 26.12, 25.12, 14.93. ESI-MS(TOF): m/z 277.157 $[M + H]^+$.

(B3-6C). 6-Azido-hexanoic acid (4-ethoxy-phenyl)-amide. ^{1}H- NMR (300 MHz, DMSO-d_6) δ 9.64 (s, 1H), 7.42 (d, J = 9.03 Hz, 2H), 6.79 (d, J = 8.88 Hz, 2H), 3.92 (q, J = 7.08 Hz, 2H,), 3.28 (t, J = 6.82 Hz, 2H), 2.22 (t, J = 7.39 Hz, 2H), 1.52 (m, 4H), 1.26 (m, 2H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 170.86, 154.60, 132.79, 120.92, 114.66, 63.41, 50.90, 36.45, 28.41, 26.20, 25.07, 15.04. ESI-MS(TOF): m/z $[M + H]^+$ = 277.156.

(B4-6C). 6-Azido-hexanoic acid (2-methoxy-phenyl)-amide. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.97 (s, 1H), 7.88 (d, J = 7.71 Hz, 1H), 6.99 (m, 2H), 6.84 (m, 1H), 3.78 (s, 3H), 3.28 (t, J= 6.82 Hz, 2H), 2.34 (t, J = 7.23 Hz, 2H), 1.55 (m, 4H), 1.34 (m, 2H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 171.59, 149.96, 127.77, 124.53, 122.40, 120.51, 111.44, 55.97, 50.94, 36.24, 28.41, 26.16, 25.12. ESI-MS(TOF): m/z $[M + H]^+$ = 263.142.

(B5-6C). 6-Azido-hexanoic acid (3,4,5-trimethoxy-phenyl)-amide. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.74 (s, 1H), 6.94 (s, 2H), 3.68 (s, 6H), 3.58 (s, 3H), 3.29 (t, J = 6.73 Hz, 2H), 2.23 (t, J = 7.23 Hz, 2H), 1.53 (m, 4H), 1.31 (m, 2H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 171.29, 153.04, 135.86, 133.56, 97.13, 60.45, 56.02, 50.90, 36.65, 28.43, 26.19, 24.98. ESI-MS(TOF): m/z 323.160 $[M + H]^+$.

(B10-6C). 6-Azido-hexanoic acid (3,4-dimethoxy-phenyl)-amide. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.67 (s, 1H), 7.26 (s, 1H), 7.03 (d, J = 8.55 Hz, 1H), 6.82 (d, J = 8.70 Hz, 1H), 3.68 (s, 6H), 3.29 (t, J = 6.73 Hz, 2H), 2.23 (t, J = 7.30 Hz, 2H), 1.53 (m, 4H), 1.31 (m, 2H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 170.96, 148.88, 144.97, 133.40, 112.49, 111.28, 104.69, 56.12, 55.69, 50.90, 36.54, 28.42, 26.20, 25.03. ESI-MS: m/z 293.153 $[M + H]^+$.

(B11-6C). 6-Azido-hexanoic acid (4-phenoxy-phenyl)-amide. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 9.84 (s, 1H), 7.55 (d, J = 8.88 Hz, 2H), 7.31 (t, J = 7.56 Hz, 2H), 7.05 (t, J = 7.39 Hz, 1H), 6.92 (m, 4H), 3.28 (t, J = 6.80 Hz, 2H), 2.26 (t, J = 7.32 Hz, 2H), 1.54 (m, 4H), 1.34 (m, 2H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ 171.22, 157.82, 151.79, 135.66, 130.28, 123.23, 121.04, 119.81, 118.11, 50.90, 36.51, 28.41, 26.18, 25.03. ESI-MS(TOF): m/z 325.159 $[M + H]^+$.

Click chemistry

Alkyne **1** and a total of 325 azides (see ESI for complete details) were subject to Cu(I) catalyzed 1,3-dipolar cycloaddition which resulted in a 325 member library. The library was assembled in a 384 well plate. The conditions used for click chemistry were first optimized (ESI).

Alkyne solution (25 mM, 10 μL) was dispensed into each well of the 384-well plate using a Multi-drop Combi dispenser (Thermo Scientific) followed by the transfer of azide solutions (50 mM, 7 μL) from the 96-well storage plate to the first quadrant of the 384-well reaction plate using a Sciclone ALH 3000 Liquid Handler Workstation (Caliper Life Sciences). Similarly other azides were transferred from the 96-well storage plates to the rest of the three quadrants of the 384-reation plate. *t*-BuOH (43 μL) and catalyst mix (40 μL; containing a mixture of $CuSO_4$ solution (25 mM, 2 μL)

and Sodium ascorbate (50 mM, 5 μL) in water (33 μL)) were added to each well in the reaction plate using the bulk liquid dispenser. The 384-well plate was carefully sealed using a silicone based capmat and shaken for 2 days. The solvent was dried *in vacuo* and the resulting residue was redissolved in DMSO (100 μL/well). The 'click' products were analyzed by LC-MS to determine the quality and identity (see Part 3 of the ESI for complete data). The concentration of the product in each well was estimated to be 2.5 mM based on the assumption that all alkynes were reacted quantitatively. The compounds were stored in −20 °C. Selected products were further scaled-up, purified (by RP-HPLC) and fully characterized (by NMR). Subsequent biological screenings were carried out first with the direct "click" product (for preliminary screening), then with the HPLC-purified products (for detailed kinetic studies).

Characterizations of selected "click" inhibitors

Detailed synthesis and characterizations of all compounds and intermediates are reported in the ESI. Shown below are characterizations of selected inhibitors.

(A5-2C-W2). 5-(4-Fluoro-2-((1-(2-(3-fluorobenzylamino)-2-oxoethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)isoxazole-3-carboxylic acid. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 4.34 (m, 2H), 5.23 (s, 2H), 5.45 (s, 2H), 6.97–7.01 (m, 1H), 7.03 (s, 1H), 7.08–7.13 (m, 2H), 7.33–7.41 (m, 1H), 7.44–7.48 (dd, J_1 = 11.34 Hz, J_2 = 2.31 Hz, 1H), 7.93–7.99 (m, 1H), 8.28 (s, 1H), 8.88 (t, J = 5.76 Hz, 1H); ESI-MS (IT-TOF) m/z 470.1288 $[M + H]^+$.

(A6-2C-W2). 5-(4-Fluoro-2-((1-(2-(3-fluoro-4-methylphenylamino)-2-oxoethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-isoxazole-3-carboxylic acid. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 2.17 (s, 3H), 5.36 (s, 2H), 5.47 (s, 2H), 6.98–7.01 (m, 1H), 7.03 (s, 1H), 7.17–7.24 (m, 2H), 7.45–7.52 (m, 2H), 7.96 (m, 1H), 8.33 (s, 1H), 10.59 (s, 1H); ESI-MS (IT-TOF) m/z 470.1295 $[M + H]^{+\bullet}$.

(B7-2C-W2). 5-(4-Fluoro-2-((1-(2-(4-(methylthio)phenylamino)-2-oxoethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-isoxazole-3-carboxylic acid. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 2.45 (s, 3H), 5.35 (s, 2H), 5.46 (s, 2H), 6.98–7.01 (m, 1H), 7.03 (s, 1H), 7.24 (d, J = 8.70 Hz, 2H), 7.49–7.55 (m, 3H), 7.93–7.99 (m, 1H), 8.33 (s, 1H), 10.48 (s, 1H); ESI-MS (IT-TOF) m/z 484.1053 $[M + H]^{+\bullet}$.

(B8-2C-W2). 5-(4-Fluoro-2-((1-(2-oxo-2-(4-(trifluoromethoxy)phenylamino)ethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-isoxazole-3-carboxylic acid. ^{1}H-NMR (300 MHz, MeOH-d_4) δ 5.39 (s, 2H), 5.44 (s, 2H), 6.88–6.95 (m, 1H), 7.05 (s, 1H), 7.23–7.25 (m, 2H), 7.28–7.29 (m,1H), 7.66–7.69 (m, 2H), 7.95–8.00 (m, 1H), 8.26 (s, 1H); ESI-MS (IT-TOF) m/z 522.1054 $[M + H]^+$.

(B9-2C-W2). 5-(4-Fluoro-2-((1-(2-(4-methoxyphenylamino)-2-oxoethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)isoxazole-3-carboxylic acid. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 3.75 (s, 3H), 5.33 (s, 2H), 5.46 (s, 2H), 6.88–6.91 (d, J = 8.88 Hz, 2H), 7.01–7.09 (m, 2H), 7.45–7.49 (m, 3H), 7.93–7.99 (m, 1H), 8.32 (s, 1H), 10.34 (s, 1H); ESI-MS (IT-TOF) m/z 468.1424 $[M + H]^+$.

(B10-2C-W2). 5-(2-((1-(2-(3,4-Dimethoxyphenylamino)-2-oxoethyl)-1H-1,2,3-triazol-4-yl)methoxy)-4-fluorophenyl)isoxazole-3-carboxylic acid. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 3.71 (s, 6H), 5.33 (s, 2H), 5.47 (s, 2H), 6.89–6.91 (d, J= 8.70 Hz, 1H), 7.02–7.05 (m, 3H), 7.29 (s, 1H), 7.45–7.50 (dd, J_1 = 2.31 Hz, J_2 = 11.34 Hz, 1H), 7.94–7.97 (m, 1H), 8.32 (s, 1H), 10.35 (s, 1H); ESI-MS (IT-TOF) m/z = $[M + H]^+$ 498.1454.

(B11-2C-W2). 5-(4-Fluoro-2-((1-(2-oxo-2-(4-phenoxyphenylamino)ethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)isoxazole-3-carboxylic acid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 5.36 (s, 2H), 5.47 (s, 2H), 6.95–7.13 (m, 7H), 7.33–39 (m, 2H), 7.45–7.50 (m, 3H), 7.54–7.58 (m, 2H), 7.97 (m, 1H), 8.33 (s, 1H), 10.51 (s, 1H); ESI-MS (IT-TOF) m/z 530.1579 $[M + H]^+$.

(C8-2C-W2). 5-(2-((1-(2-(4-Cyclohexylphenylamino)-2-oxoethyl)-1H-1,2,3-triazol-4-yl)methoxy)-4-fluorophenyl)isoxazole-3-carboxylic acid. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 0.82–0.87 (m, 4H), 1.22–1.32 (m, 5H), 1.48–1.58 (m, 2H), 5.34 (s, 2H), 5.47 (s, 2H), 6.98–7.01 (m, 1H), 7.05 (s, 1H), 7.14 (d, J = 8.37 Hz, 2H), 7.45–7.50 (m, 3H), 7.94–7.97 (m, 1H), 8.32 (s, 1H), 10.39 (s, 1H); ESI-MS (IT-TOF) m/z 508.2006 $[M + H]^+$.

Biological screenings

The PTP1B activity was determined by measuring the rate of hydrolysis of a commercially available, fluorogenic substrate, 6,8-difluoro-4-methylumbelliferyl phosphate (DIFMUP) (Invitrogen, USA).

The inhibition of phosphatases were screened using black polypropylene flat-bottom 384-well microtiter plates (Greiner, Germany) in a total reaction volume of 25 μL/well, monitored with a Tecan F200 fluorescence plate reader (Tecan, Germany), using the exciting and emission filters recommended by the vendor. In the preliminary screening with unpurified "click" products, A single-concentration assay format was adopted, in which a uniform concentration of inhibitors (ranging between 25–3 μM) was used, for quick identification of potential inhibitors as previously described.[4g] Controls were done with 1,3-dipolar ligation reactions carried out without either the alkyne or the azide component; they showed no inhibition against PTP1B (up to 260 μM inhibitor concentration). From the preliminary screening results, eight representative "click" products were identified, and accordingly scaled-up and further purified (by RP-HPLC) before being investigated by the measurement of their IC_{50} values against PTP1B. The IC_{50} values were obtained using dose-dependent reactions by varying the concentrations of the inhibitor, under the same enzyme and substrate concentration. Briefly, a two-fold dilution series of an inhibitor, from approximately 400 μM to 3.125 μM (final concentrations) was prepared. The reaction condition is as shown below.

PTB 1B (2.4 μg/mL) = 5 μL
DIFMUP (20 μM) = 5 μL
Inhibitor (varied) = 5 μL
Assay Buffer (1X) = 10 μL

[Assay buffer = 25 mM HEPES, 150 mM NaCl and 0.1 mg/mL BSA, pH = 7.5].

The enzymatic reactions were allowed to incubate at room temperature for 30 minutes before being initiated by the addition of DIFMUP. The enzymatic reactions were immediately monitored at λ_{ex} = 355 nm and λ_{em} = 460 nm for a period of 15 minutes. The IC_{50} was calculated by fitting the fluorescence outputs obtained using the Graphpad Prism software v.4.03 (GraphPad,

San Diego). Each IC_{50} plots were generated by 6 to 8 data points. Results are shown in Table 1 and ESI.

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