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A combinatorial approach towards the synthesis of non-hydrolysable triazole–iduronic acid hybrid inhibitors of human α-L-iduronidase: discovery of enzyme stabilizers for the potential treatment of MPSI†

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Preparation of substituent-diverse, triazole–iduronic acid hybrid molecules by click reaction of an azido iduronic acid derivative with randomly chosen alkynes is described. Library members were screened for their ability to inhibit α-L-iduronidase, and hit molecules and analogues were then investigated for their ability to stabilize rh-α-IDUA in a thermal denaturation study. This work resulted in the discovery of the first small molecules that can be used to stabilize exogenous rh-α-IDUA protein *in vitro*.

α-L-Iduronidase (IDUA; EC 3.2.1.76) is a human lysosomal enzyme that catalyzes the hydrolysis of the terminal α-L-iduronic acid (IdoA) residue in glycosaminoglycans, such as dermatan sulfate and heparan sulfate (Fig. 1).[1] Deficiencies in the activity of this enzyme result in the accumulation of glycosaminoglycans in the lysosomes, leading to mucopolysaccharidosis type I (MPS I). MPSI disease can be treated by enzyme replacement therapy (ERT),[2] wherein recombinant human α-IDUA (rh-α-IDUA) is injected into the patient to reduce accumulated substrates, and alleviate clinical symptoms.[3] But rh-α-IDUA is highly expensive and inherently unstable.[1–3] Accordingly, methods to enhance the stability of this protein drug are urgently needed. One means of enzyme stabilization is the use of reversible inhibitors, which can protect a protein drug from denaturation by binding to it.[4–6]

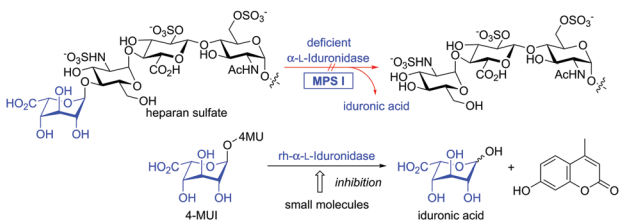

Fig. 1 Natural and fluorogenic substrates of IDUA.

Because preparation of IdoA-typed molecules such as 4-methylumbelliferyl α-L-iduronide (4-MUI, **1**), the fluorogenic substrate of IDUA, is tedious,[7] most IDUA inhibitors except 5F-IdoAF (**2**, see in Fig. 1) possess other structurally diverse skeletons, and display only weak to moderate inhibitory activity.[8,9] Interestingly, **2** acts as an enzyme inactivator because it forms a covalent glycosyl-enzyme intermediate, followed by regeneration of free IDUA.[9]

In previous work, we used natural product-inspired, combinatorial chemistry to discover novel inhibitors or chemical chaperones of sugar-processing enzymes. A straightforward coupling reaction such as amide bond formation (with randomly selected carboxylic acids) was employed to prepare diverse molecules.[10,11] However, our attempts to use the same strategy in this work were thwarted at an early stage when our proposed amine synthesis (reduction of the protected azido iduronic acid intermediate) did not work well (see the ESI†).[12] Accordingly, conjugation of the azido intermediate directly *via* a click reaction, a type of 1,3-dipolar cycloaddition, was applied.[13] Triazole-containing, sugar-based molecules have been previously used to generate bioactive molecules for the study of enzyme inhibitors and receptor recognition.[13] However, to the best of our knowledge, they have not been applied to discover new, non-hydrolysable, and reversible IDUA stabilizers.

The initial designed molecules (types I and II), inspired by IdoA and 4-MUI (**1**), are depicted in Fig. 2. Structurally, they possess the conformation and configuration of IdoA. The sugar and triazole moieties are linked by an alpha linkage in type I, and a beta linkage in type II; and their corresponding precursors are iduronyl azides **3** and **4**, respectively.

Azide **3** was prepared starting from D-glucuronolactone. Methanolysis of **3** followed by peracetylation gave a mixture of α- and β-anomers of **5** (Scheme 1).[14] Bromination of these at

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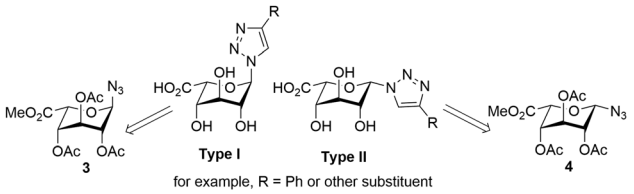


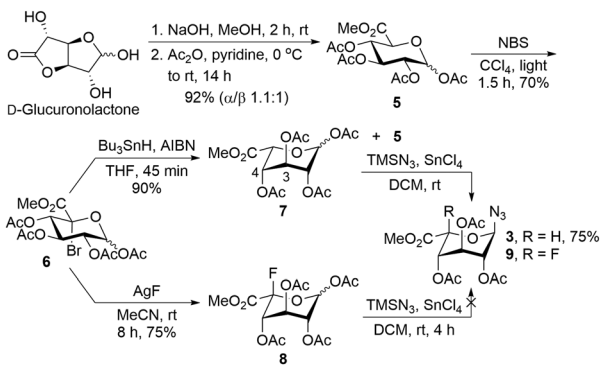
Fig. 2 Structures of desired iduronyl triazoles (types I and II) and their corresponding precursors **3** and **4**.

the C5 position under photo-induced radical conditions using *N*-bromosuccinimide gave **6** (70%).[15] Next, C5 brominated **6** was treated with tributyltin hydride and azobisisobutyronitrile (AIBN) in boiling THF, which gave a separable mixture of **7** and **5** (**7**/**5** = 1/2) in 90% yield.[16] Based on NMR analysis and literature reports, **7** was confirmed to be a $^{1}C_{4}$ conformer because of the smaller coupling constants of $J_{4,5}$ and $J_{4,3}$ (2.4 and 3.6 Hz, respectively) observed.[17] Treatment of **7** with $TMSN_3/SnCl_4$ gave the α-azide intermediate **3** in 75% yield.[18] Notably, the configuration at the anomeric center was confirmed by 2D (NOESY) NMR analysis (see the ESI†).

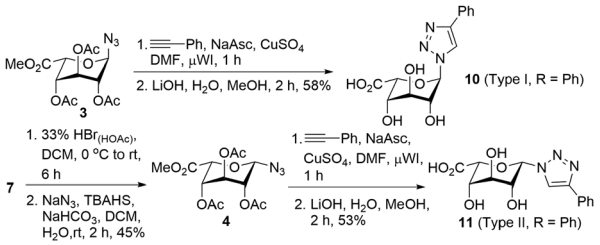
Additionally, we had planned to prepare azide **9** (bearing a fluoride at the C5 position) for comparison purposes (**3** *vs.* **9**). Although **6** could be converted to **8** through a nucleophilic halogen exchange (AgF) in good yield (75%), its subsequent conversion to **9** was not successful.[19] Presumably, the electron-withdrawing nature of the fluorine atom at C5 suppresses the anomeric effect of the proximal oxygen atom such that nucleophilic substitution at C1 cannot take place.

With azide **3** now in hand, the click reaction could be investigated. As shown in Scheme 2, treatment of azide **3** with phenylacetylene in the presence of sodium ascorbate (NaAsc) gave rise to a click reaction, which was followed by deprotection to afford the iduronyl triazole **10** (type I, R = Ph). Likewise, **11** (β-form) could also be prepared from **7**. Compound **7** was converted to an unstable α-anomeric bromide intermediate, followed by reaction with sodium azide to form the β-isomeric azide **4**. The anomeric configuration of **4** was confirmed by 2D (NOESY) NMR analysis (see the ESI†). Triazole **11** (type II, R = Ph) was obtained from **4** *via* click chemistry and Zemplén deacetylation.

Both model iduronyl triazoles **10** and **11** were evaluated in an IDUA inhibition study, and the results are shown in Table 1.



Scheme 1 Synthetic route for the preparation of azide **3**.



Scheme 2 Preparation of iduronyl triazoles **10** and **11** as model reactions.

Table 1 Inhibitory study of iduronyl triazoles **10** and **11** against IDUA

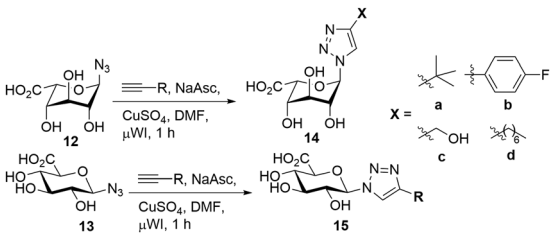
	%Inhibition	
Compound	1 mM	100 μM
10	83	55
11	42	15

Apparently, triazole **10** (α-linked) exhibited better activity and was a three-fold more potent inhibitor of IDUA than triazole **11** (β-linked), at 100 μM. This result led us choose **10** as the structural template and **3** as the starting point for the generation of a structurally diverse, triazole-based library.

Next, to compare the influence on the enzyme activity of the sugar conformation and the orientation of the C5 carboxylic acid, α-L-IdoA configured **12** and β-D-GlcA configured **13** were synthesized by hydrolysis of the corresponding glycosyl azides **3** and **4**, respectively (see the ESI†). In our test reactions, **12** and **13** reacted with accessible terminal alkynes to give the corresponding **14a–d** and **15a–b**, respectively (Table 2). As expected, **14a–d** were found to be more potent inhibitors of IDUA than **15a–b** since the enzyme can recognize more substrate like molecules. It can also be seen from Table 2 that **14b**, bearing a substituted aryl moiety, displayed better activity than **14a**, **14c** and **14d** (with bulky or linear chains). Our results showed that both the sugar moiety and a glycan part form certain interactions with the enzyme.

These encouraging results prompted us to generate more structurally distinct iduronyl triazole-based molecules (type I).

Table 2 Chemical structures of iduronyl and glucuronyl triazoles and their inhibitory activities



Compound	%Inhibition[a]	Compound	%Inhibition[a]
12	46	**14d**	69
14a	58	**13**	NI[b]
14b	81	**15a**	NI[b]
14c	37	**15b**	17

[a] 1 mM. [b] NI refers to no inhibition (%inhibition <10%).

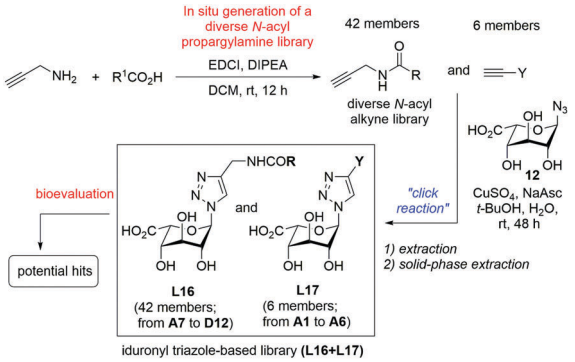


Fig. 3 Combinatorial approach to prepare an iduronyl triazole-based library (**L16** + **L17**) and its IDUA inhibition screening.

In addition to the six alkynes available in hand, a diverse library of alkynes was prepared by parallel conjugation of propargylamine with a 42-member carboxylic acid library to generate a structurally diverse *N*-acyl propargylamine-based library. As shown in Fig. 3, a combinatorial approach toward parallel synthesis of an iduronyl triazole-based library (**L16** + **L17**) could commence with a 48-membered alkyne library reacting with azide **12** *via* click reaction (see the ESI†). To improve the efficiency, all procedures were modified for compatibility with semi-automated instruments including a synthesizer, a modern multifunction liquid handler, and a multichannel concentrator.[20] After proper purification (solid-phase extraction),[21] a 48-membered library of diverse iduronyl triazoles with high purity and excellent regioselectivity was obtained (see the ESI†).

The IDUA inhibitory activities of all 48 members were tested at 100 μM and the results are shown in Fig. S2, ESI†. The activity is dependent on the structure of the *N*-substituent on the triazole ring. Generally, most iduronyl triazoles that contain an *N*-cinnamoyl substituent exhibit better inhibitory potency than a saturated *N*-acyl moiety. Among them, the inhibitory activity of **L16-C3** (renamed **18**, see in Fig. 4) bearing an (*E*)-2-methyl-3-phenylacryloyl group was particularly high (88% inhibition at 100 μM).

Based on the structure of **18**, three similar analogues **20–22** were designed and synthesized with structural differences at the substituted moiety. Besides, **19** (**L16-C3**), the saturated form of **18** was also synthesized for comparison purposes. The structures and inhibition results are shown in Fig. 4.

The ability of **18–22** to affect rh-α-IDUA stability was next investigated. The enzyme melting temperature (T_m) of rh-α-IDUA under various conditions was measured in a fluorescence-based

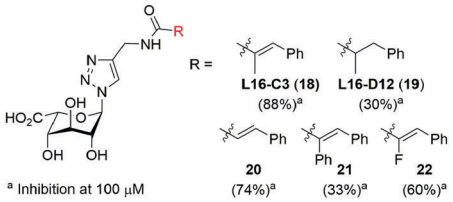


Fig. 4 Structures of **L16-C3** (**18**) and its analogues **20–22** and their IDUA inhibitory activities.

Table 3 Thermal shift study of small molecules toward rh-α-IDUA

	T_m (°C)					
Conditions[a]	**18**[b]	**19**	**20**[b]	**21**	**22**	**2**[c]
0 μM	56.2	56.2	56.2	56.2	56.2	56.2
250 μM	57.0	56.7	56.9	56.2	56.5	55.0
500 μM	58.3	56.7	57.7	56.8	57.8	55.6

[a] At pH 3.5. [b] The curves are shown in the ESI. [c] Reference compound.

thermal denaturation assay.[5,6,22] The T_m of rh-α-IDUA on its own was 56.2 °C. In contrast, the T_m of rh-α-IDUA increased to 58.3, 56.7, 57.7, 56.8 and 57.8 °C when incubated with 500 μM of **18–22**, respectively. The reference inhibitor **2** was also tested under the same conditions, but no significant increase in T_m was observed. Although a better inhibitor than **18–22**,[23] its covalent interaction with IDUA might have no benefit for the protection of enzyme during thermal denaturation. As shown in Table 3, these concentration-dependent T_m enhancement effects suggest these molecules protect rh-α-IDUA from denaturation. In contrast, glycerol (up to 40 mM, see the ESI†) did not show any ability to stabilize this enzyme.[24]

The ability of these molecules to protect the enzyme from heat-induced inactivation was also evaluated.[6,25] In a control experiment without treating molecules, the rh-α-IDUA activity was found to decrease below 5% of the initial value after 120 min of incubation at 48 °C. Upon treatment with **18** or **20**, this enzyme activity still remained around 60% at 500 μM (Fig. 5). These findings establish the utility of triazole–iduronic acid hybrid molecules as rh-α-IDUA stabilizers, the first demonstration of the feasibility of using small molecules to protect IDUA against denaturation.

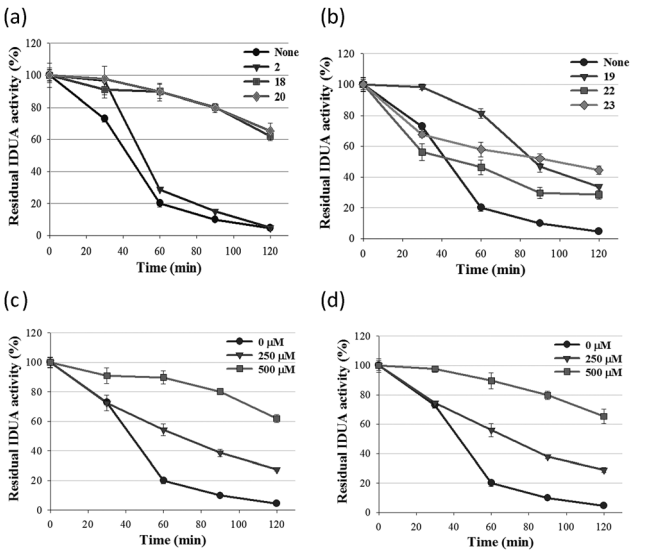


Fig. 5 A heat-induced denaturation study. (a and b) Treatment of 500 μM of molecules with rh-α-IDUA at pH 3.5 and 48 °C for the indicated amount of time. The enzyme was incubated at pH 3.5 with 4MU-α-iduronic acid. (c) Treatment with varied concentrations of **18**. (d) Treatment with varied concentrations of **20**.

In summary, a library of iduronyl triazole-based analogues has been efficiently synthesized using click reaction to conjugate an azido iduronic acid precursor with a wide range of structurally diverse alkynes. Library members were screened for their ability to inhibit IDUA, and hit molecules were then investigated for their ability to stabilize rh-α-IDUA in a thermal denaturation study. It was found that **18** and **20** could protect the enzyme from heat-induced inactivation. This is the first study to establish that small molecules can be used to stabilize exogenous rh-α-IDUA protein *in vitro*, and could potentially increase the lifetime of ERT when used in the treatment of MPSI disease. Our search for a more potent stabilizer of rh-α-IDUA for testing against MPSI patient cell lines by the synthesis of more diverse libraries is ongoing, and the results will be reported in due course.

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Conflicts of interest

There are no conflicts to declare.

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