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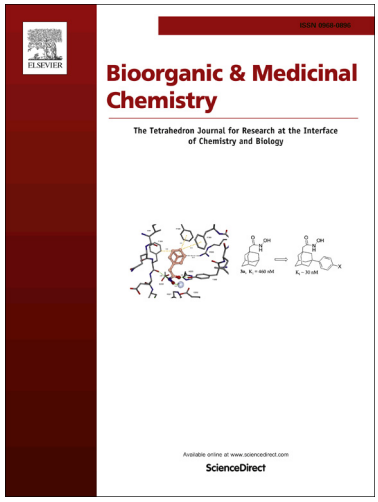

Developing new chemical tools for DNA methyltransferase 1 (DNMT 1): a small-molecule activity-based probe and novel tetrazole-containing inhibitors

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Graphical Abstract

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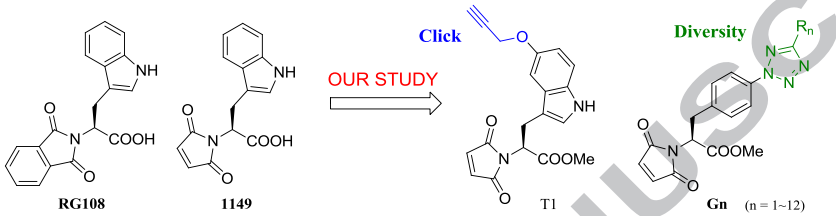

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ABSTRACT

DNA methylation is an important epigenetic modification catalyzed by DNA methyltransferases (DNMTs). Abnormal expression of endogenous DNMTs in human causes alterations in the genome methylation patterns which subsequently lead to the development of cancers. Thus detection of endogenous DNMT activities and efficient inhibition of DNMTs have important therapeutic significance. In this work, a small molecule activity-based probe (ABP) of DNA methyltransferase 1 (DNMT1), **T1**, was developed. The probe was a clickable analog of tryptophan and was able to covalently label endogenous DNMT1 and inhibit its enzymatic activity more effectively than previously known DNMT1 inhibitors (**RG108** and its maleimide analogue **1149**). In addition, we also discovered a new type of small molecule DNMT inhibitors based on tetrazole-containing compounds which were analogs of **1149**. Amongst these compounds, which we called **Gn**, one of them (**G6**) possessed reasonable inhibitory activity against DNMT1 in both *in vitro* enzymatic assays and cell growth proliferation experiments. Both **T1** and **G6** showed effective labeling of endogenous DNMT1 from mammalian cells by using *in vitro* competitive pull-down and live-cell bioimaging experiments.

1. Introduction

DNA methylation is an epigenetic modification that leads to the addition of a methyl group at the C-5 position of cytosine residues of DNA without interfering with usual base pairings. The process prevents gene expression, stabilizes DNA-protein complexes and promotes normal DNA expression.[1-3] In mammalian genes, the principal targets of DNA methylation are CpG sequences, ~60-90% of which have been methylated. However, there are some unmethylated regions called "CpG islands" which occur predominantly in the promoters of mammalian genes. Hypermethylation of CpG islands is regarded as a common character of cancer cells because it will cause the silencing of tumor suppressor genes. Failure in maintaining normal DNA methylation patterns is associated with mis-expression of certain proteins, which may lead to cancers or other diseases. The abnormal DNA methylation has become an interesting and promising cancer therapeutic target. [4-6]

DNA methylation is catalyzed by DNA methyltransferases (DNMTs). This enzyme family mainly contains two categories, including the maintain-type DNMT1 and the *de novo*-type DNMT3.[1,7-11] DNMT1 is the most abundant enzyme of human DNMTs expressed in somatic cells, which has high affinity for hemi-methylated DNA and helps to maintain the methylation patterns of DNA. DNMT3 is present in small quantities in somatic cells and has no differential affinity between unmethylated and hemi-methylated CpG sites, whose main role is in *de novo* methylation. The catalytic mechanism of DNMTs has been studied extensively (shown in Fig. 1A).[12] During the methylation processes, DNMTs first bind to the C-6 position of cytosine residues of DNA, then transfer a methyl group from S-adenosyl methionine (SAM; a methyl group donor) to the C-5 of cytosine, followed by the concomitant release of S-adenosyl-L-homocysteine (SAH). Subsequently, the proton at the C-5 position is removed and the free enzyme is released via β-elimination to generate the methylated cytosine.

Since the alteration in genome methylation and mis-DNA methylation are caused by the mis-expression of DNMTs, based on the catalytic mechanism of these enzymes, many small molecule DNMT inhibitors have been developed, including nucleoside analogues (5-Azacytidine and Zebularine), non-nucleoside analogues (Procaine, Procainamide, Hydralazine, Curcumin and **RG108**) and other SAH analogues.[5,11,13] However, their relatively poor inhibitory activities towards DNMTs coupled with significant cytotoxicity make such compounds

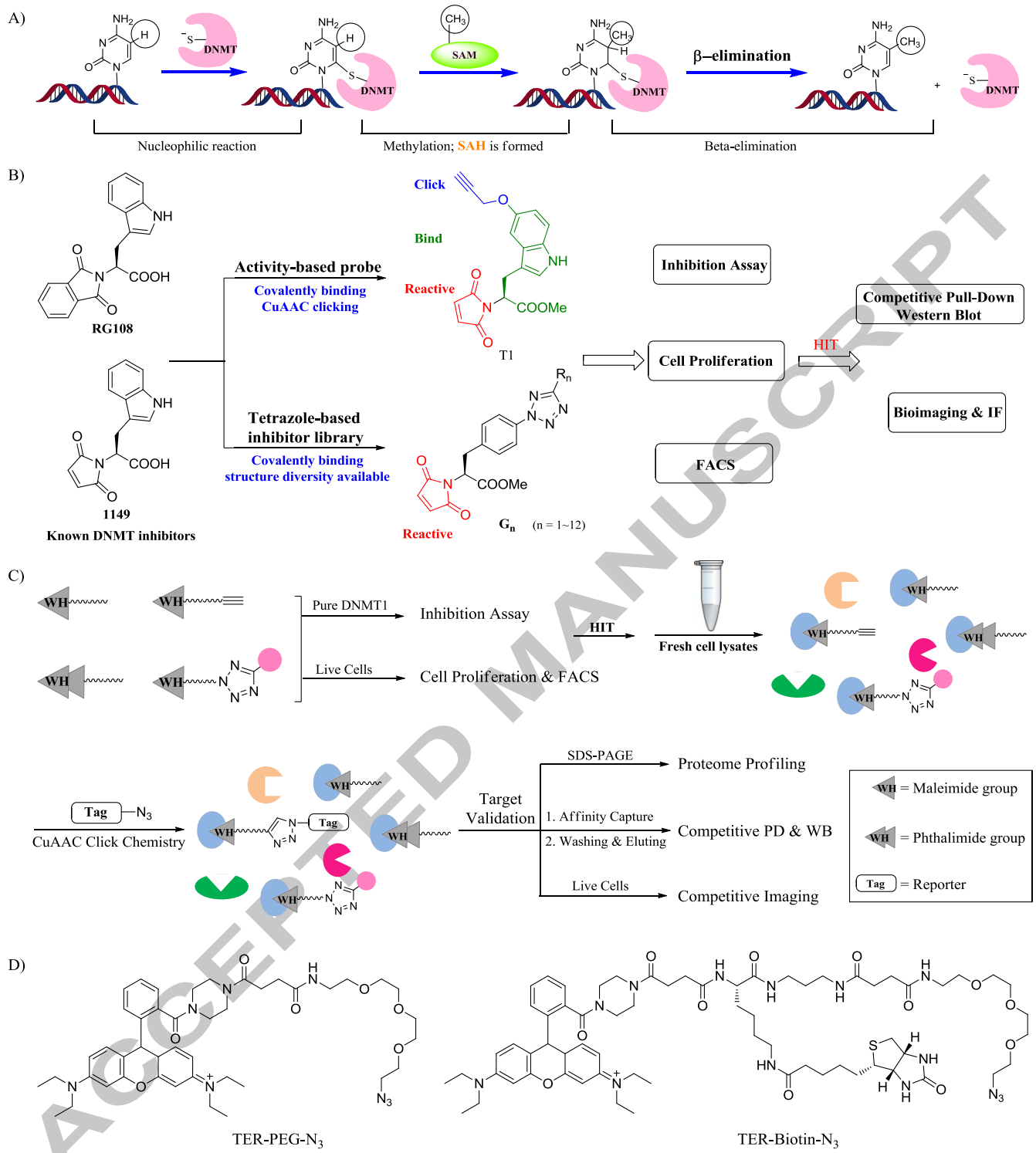
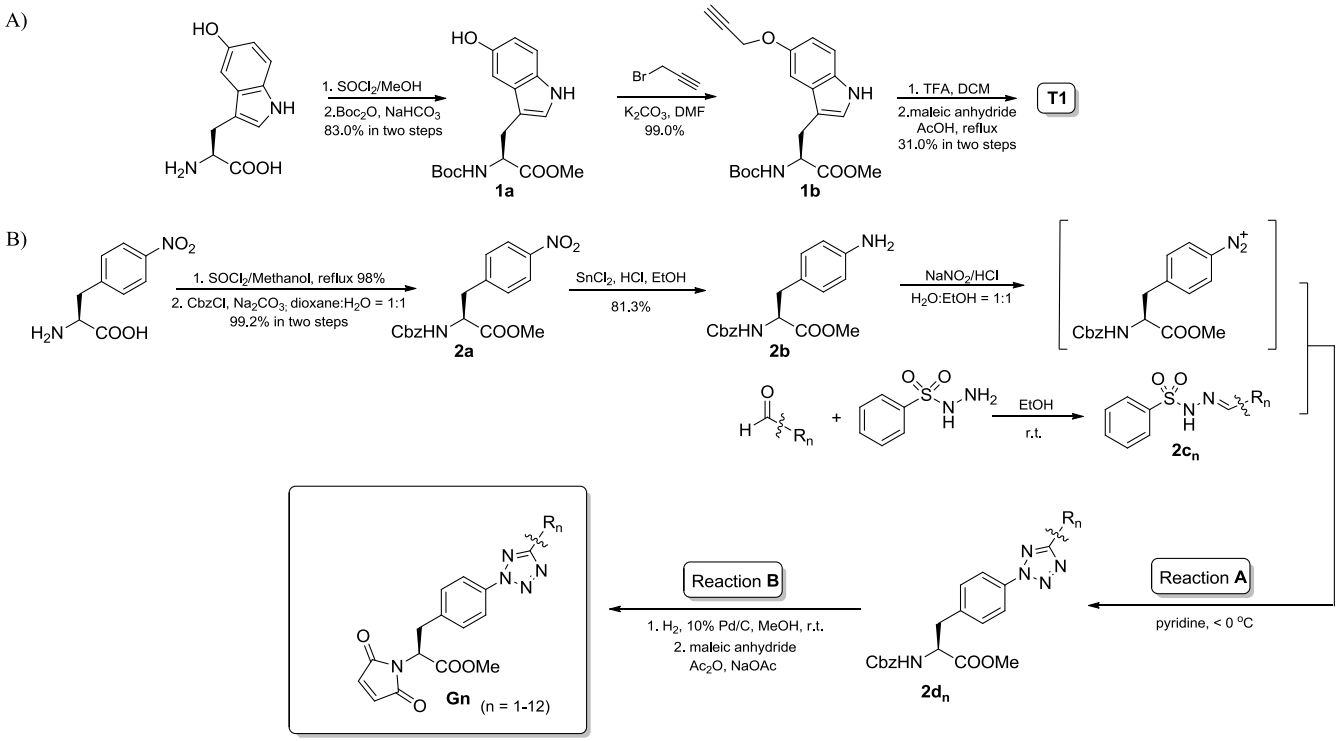


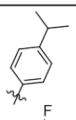
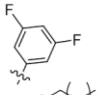
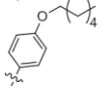
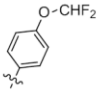
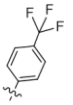
Figure. 1. A) Schematic showing DNA methyltransferases (DNMTs) catalyze DNA methylation. SAM acts as the methyl group donor; B) Overall strategy of our probe (**T1**) and the inhibitor library (**Gn**) derived from known DNMT1 inhibitors **1149** and **RG108**; C) Workflow of ABPP and the corresponding competitive ABPP technology used in the current study; D) Structures of the two different azide reporters used in the present study.

unsuitable as potential therapeutic agents. New potent and highly selective DNMT inhibitors are urgently needed.

Activity-based protein profiling (ABPP) technology has become an important method for cell-based proteome profiling. Recently, its corresponding probes, activity-based probes (ABPs) and affinity-based probes (A*f*BPs) have also become promising chemical tools that enable reliable and comprehensive identification of potential "on" and "off" cellular targets of drug candidates.[14-18] With such probes, proteome-wide assessment of bioactive compounds and their cellular mechanism-of-action can now be investigated at the earliest stages of drug development, thus potentially providing a better success rate in subsequent clinical trials.[15] These so-called "*in situ* drug profiling" strategies have in recent years been successfully applied to a number of biologically important drug candidates, including the FDA-approved anti-obesity drug Orlistat,[19] several anti-cancer small molecule kinase inhibitors,[20-24] and the newly developed epigenetic protein-protein interaction (PPI) inhibitor JQ-1.[25]

Herein, we extend this approach to the study of DNMTs (Fig. 1B-D). Inspired by two known DNMT1 inhibitors **1149** and **RG108**,[26-28] two types of activity-based probes **T1** and **Gn** were designed and synthesized (Fig. 1B). To design these compounds as potential covalent DNMT inhibitors, the maleic moiety in **1149** was strategically retained, which should result in covalent interaction between the compound and the active-site cysteine residue in DNMT1. In addition, a terminal alkyne and a tetrazole moiety were introduced into **T1** and **Gn**, respectively, which would serve as click handles for reaction with azide- and alkene-containing reporters under copper (I)-catalyzed azide-alkyne [3+2] cycloaddition (CuAAC) conditions,[29-32] and light-induced tetrazole-alkene-1,3-dipolar cycloaddition (LITAC) conditions, respectively (Fig. 1C & 1D).[33-35] In the case of **Gn**, we envisaged that the tetrazole moiety not only served as a well-known "photo-click" handle, it could also be an effective aromatic mimetic to introduce compound diversity (i.e. **G1** to **G12**; see Table 1). The tryptophan analogue probe **T1** was designed via conversion of a known DNMT1 inhibitor, **1149**. The chemical structures of the two previously reported, azide-containing reporters are shown in Figure 1D (TER-PEG-N_3 & TER-Biotin-N_3), and would be used to "click" to **T1**-labeled mammalian proteomes for subsequent in-gel fluorescence scanning, pull-down (PD)/Western blotting (WB) and bioimaging experiments.



R_n	Structure	Reaction A /%	Reaction B /%	R_n	Structure	Reaction A /%	Reaction B /%
R_1		85.5	34.5	R_7		74.3	43.9
R_2		88.4	42.8	R_8		68.8	17.0
R_3		83.7	48.1	R_9		32.7	22.0
R_4		37.3	35.1	R_{10}		26.5	26.6
R_5		23.5	9.0	R_{11}		35.1	28.5
R_6		56.9	51.4	R_{12}		76.8	17.9

Scheme 1. Chemical synthesis of A) **T1** and B) **Gn** library

2. Results and discussion

2.1. Chemistry

2.1.1 Synthesis of activity-based probe (T1)

Probe **T1** was a derivative of **1149**, containing an additional small alkyne handle at the 5-position of the tryptophan indole ring. The probe was synthesized in five steps from the commercially available *L*-5-hydroxytryptophan as shown in Scheme 1A. Briefly, both the carboxylic acid and the primary amine group in *L*-5-hydroxytryptophan were first protected with a methyl and a *tert*-butyloxycarbonyl group, respectively. The resulted compound **1a** was reacted with propargyl bromide under basic conditions to generate compound **1b** (99.0% yield). Deprotection of the Boc group by TFA gave the free primary amine which was further reacted with maleic anhydride under refluxing conditions to afford the final probe **T1** in 31.0% yield (two steps).

2.1.2 Synthesis of tetrazole-based library (Gn)

The 12 tetrazole-based compounds **Gn** (n = 1-12) were synthesized in several steps as shown in Scheme 1B. Starting from the commercially available 4-nitro-3-phenyl-*L*-alanine, the carboxylic acid moiety was protected by a methyl group, followed by amine protection to give the corresponding Cbz-protected methyl ester, **2a**. Subsequently, the nitro group in **2a** was reduced by tin (II) chloride under acidic conditions to give **2b** in 81.3% yield. Next, the diazonium salt was prepared by slowly adding a cold $NaNO_2$ solution into a cold solution of **2b** (dissolved in 1:1 ethanol/water). Independently, 12 commercially available common aldehydes, $\mathbf{N_{1\text{-}12}}$, were each individually mixed with benzenesulfonyl hydrazide in ethanol and stirred at room temperature. Upon solvent evaporation, the corresponding $\mathbf{2c_n}$ (n = 1-12) were obtained. Subsequently, they were re-dissolved in pyridine and cooled in an ice/NaCl bath to -10 ~ -20 °C for 20 min, then used to react with the above-described, freshly prepared diazonium salt (Reaction **A**). The resulting mixtures were allowed to warm to room temperature, thus affording the corresponding tetrazole compounds $\mathbf{2d_n}$ in acceptable yields (24-89%). Subsequently, the Cbz group in each compound was removed by H_2/Pd-C treatment to afford compound $\mathbf{2e_n}$. Finally, further reaction of the primary amine in each compound with maleic anhydride in acetic anhydride/sodium acetate under refluxing conditions delivered the desired products **Gn** in acceptable yields (9-52% over two steps). The 12 analogues of **Gn** (n= 1-12) were so chosen based on their structural diversity and the commercial availability of their aldehyde precursors, thus delivering a variety of diverse aromatic groups linked to the core inhibitor structure via the centrally located tetrazole moiety (see summary Table in Scheme 1B).

2.2. Biological evaluation

With all compounds in hand, we next confirmed the biological properties of these two types of probes by side-by-side comparison with known DNMT inhibitors. As earlier mentioned, for the 12 **Gn** compounds, they were initially designed as covalent modifiers of DNMT1 (via the maleimide moiety). Upon covalent reaction with endogenous DNMT1, we had planned to subsequently carry out LITAC reaction with a suitable reporter tag to detect the probe-bound DNMT1 by taking advantage of the presumably bioorthogonal nature of the photo-induced tetrazole-alkene ligation reaction. To our great surprise, we found this reaction was not as "bioorthogonal" as expected (Fig. S1);[33-35] by taking **G6** as a model substrate, and UV-irradiated it for 2 min following published conditions,[36] we found the compound rapidly reacted with a variety of biological nucleophiles including acids, amines, thiols and others, yielding highly fluorescent products which were distinctly different from the nitrile imine

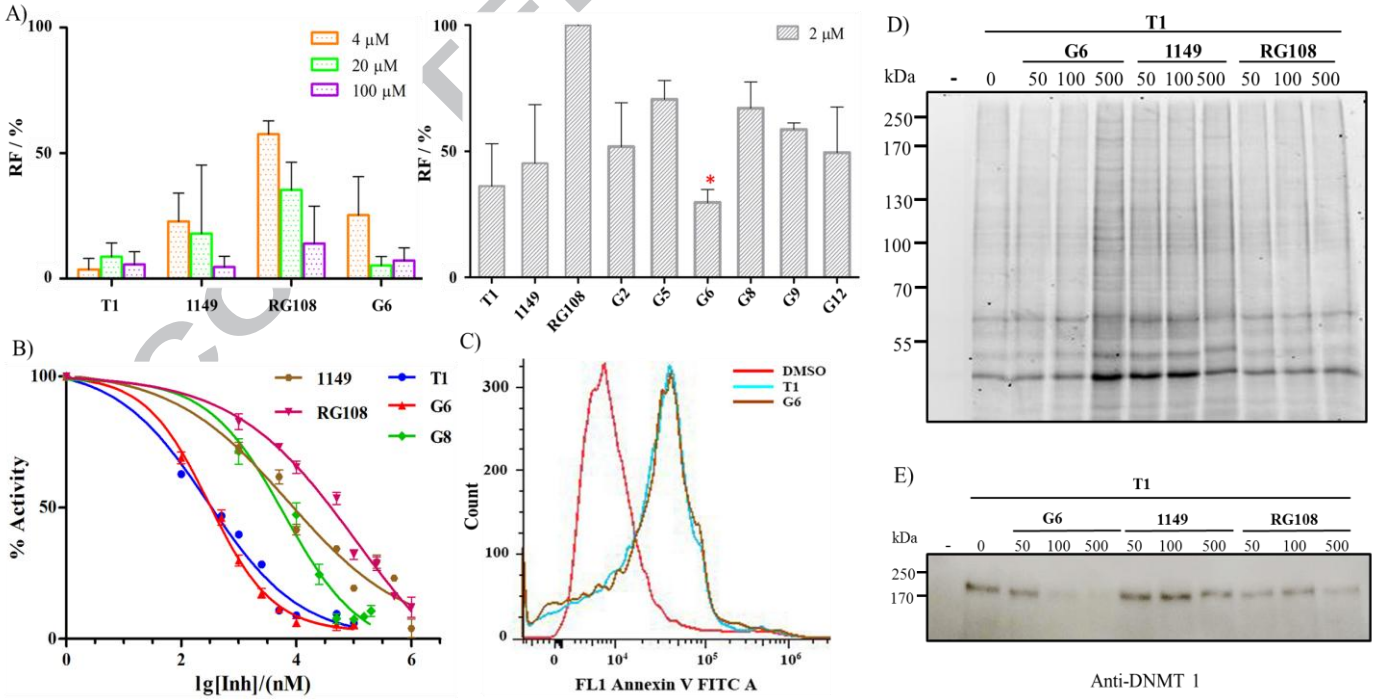


Figure. 2. A) Left: dose-dependent inhibition assay of **T1**, **Gn** as well as the positive controls (**RG108** and **1149**) against recombinant DNMT1; Right: Inhibition assay of different inhibitors at 2 μM; B) GI_{50} plot of several inhibitors against MCF-7 cells; C) Fluorescence-activated cell sorting (FACS) results of MCF-7 cells treated with 10 μM probes (**T1**, **G6**, **1149** and **RG108**); D) Protein profiling results of MCF-7 cells treated with **T1** (10 μM)/competitors (**G6**, **1149** & **RG108**; 50, 100 & 500 μM); minus sign (-): cells treated with DMSO (negative control); E) competitive PD/WB results of **T1** against DNMT1. The size of DNMT1 is around 183 kDa. MCF-7 cells were treated with **T1** (10 μM) and competitors including **G6**, **1149** & **RG108** (50, 100 & 500 μM); the minus sign (-) means cells were treated with DMSO (negative control).

Table 1. GI_{50} values of all the probes/inhibitors

Probe	GI_{50}/μM	Probe	GI_{50}/μM
T1	0.29 ± 0.07	**G6**	0.30 ± 0.06
1149	7.31 ± 0.06	**G7**	1.10 ± 0.06
RG108	127.90 ± 0.07	**G8**	6.15 ± 0.07
G1	0.38 ± 0.06	**G9**	1.86 ± 0.05
G2	1.16 ± 0.07	**10**	0.73 ± 0.07
G3	0.48 ± 0.06	**G11**	1.77 ± 0.06
G4	0.87 ± 0.06	**G12**	0.78 ± 0.05
G5	1.08 ± 0.06	-	-

intermediate initially released from UV irradiation. Upon further checking in the literature, we found several reports had previously documented that the nitrile imine generated from a UV-irradiated tetrazole indeed readily react with different nucleophiles.[37,38] Thus, **Gn** were not employed as activity-based probes in our subsequent experiments. They were nevertheless found to act as novel covalent inhibitors of DNMT1 from our present study (Table 1, Fig. 2 & Fig. 3). Overall, we assessed **T1** and **Gn**'s ability to inhibit recombinantly purified DNMT1 in an *in vitro* enzyme inhibition assay. We subsequently evaluated their effect in inhibition of mammalian cell growth in an XTT anti-proliferation assay. Finally, we carried out *in vitro* cell-based proteome profiling experiments with **T1** and competitive ABPP with **Gn** followed by cellular bioimaging.

2.2.1 Inhibition against DNMT1

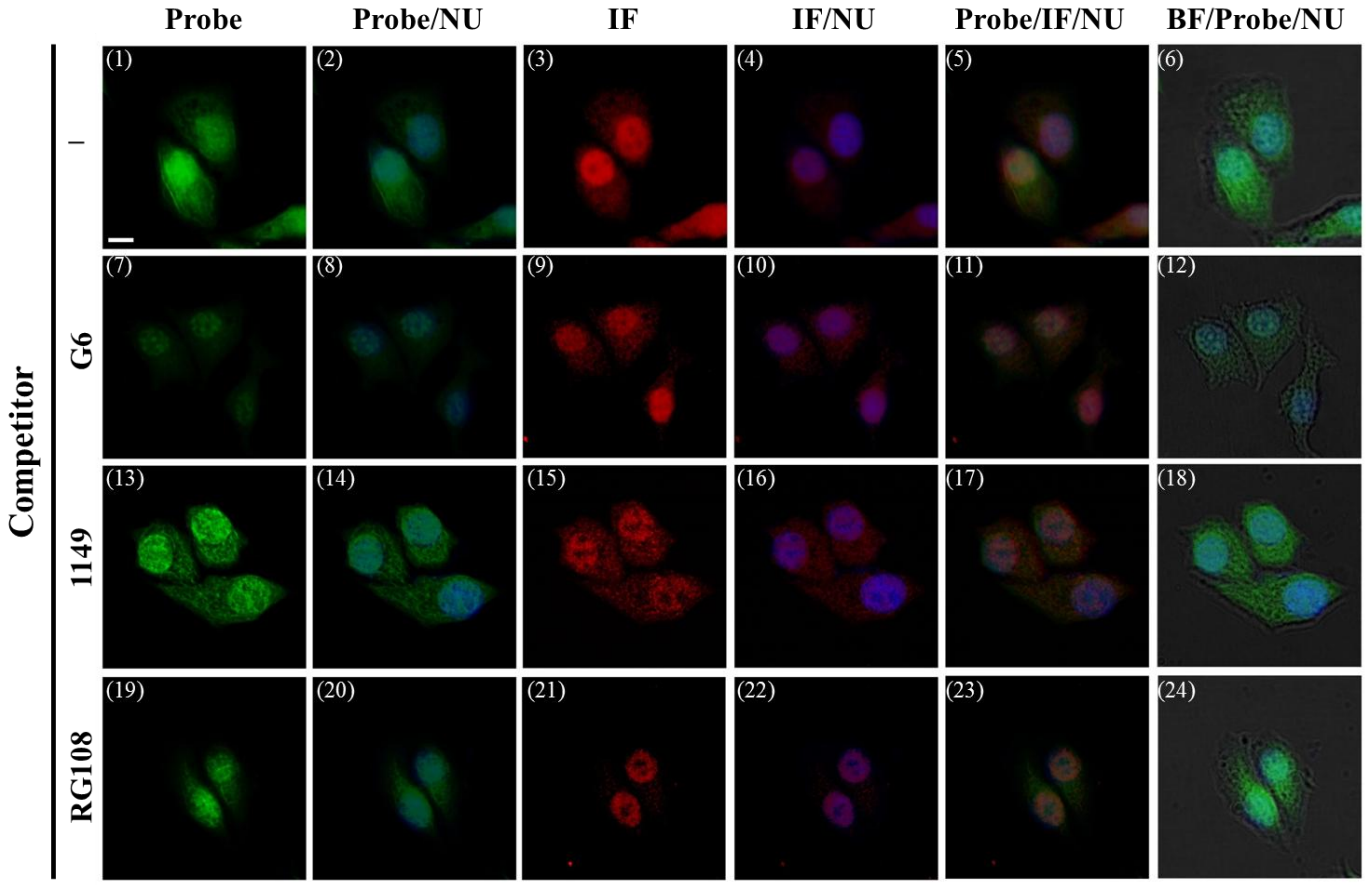
First, **T1** and **Gn** library were evaluated for their inhibition efficiency against pure DNMT1 using DNMT Activity/Inhibition Assay Ultra Kit (Fluorometric, EpiQuik™). Generally, with an increase in the inhibitor concentrations, a concomitant decrease in the enzyme activity was observed until a saturation point was reached (Fig. 2A, left). For **T1**, when compared to **RG108** or **1149**, it appeared that introduction of the terminal alkyne in 5-hydroxytrptophan did not have significant impact toward the inhibition of DNMT1. Interestingly, all tetrazole-based inhibitors, **Gn** (n = 1-12), appeared to possess reasonable inhibitory property against DNMT1 (Fig. 2A, right); among them, **G6** possessed the highest inhibitory activity against DNMT1 and inhibited the enzyme even more effectively than the two known DNMT1 inhibitors, **RG108** and **1149**. This thus provides the first direct evidence that the tetrazole moiety in these compounds indeed served as a good mimic to the indole ring of tryptophan and could effectively bind to the DNMT1 active site via non-covalent interactions. Encouraged by these preliminary results and due to high cost of the DNMT1 enzymatic assay, we subsequently carried out cell-based assays, ABPP and cell-based imaging experiments to further assess selected compounds.

2.2.2 Cell proliferation

Inhibition of DNMT1 induces cell death and apoptosis of human cancer cells that harbor abnormal active DNMTs, such as MCF-7 breast cancer cell line.[1] In the XTT cell proliferation assay (Fig. 2B, S2 & Table 1), we observed the corresponding dose-dependent inhibition of **T1** and **Gn** towards MCF-7. GI_{50} value is the concentration of the anti-cancer drug that inhibits the growth of cancer cells by 50%. In this study, GI_{50} values of selected compounds, together with those of the two known inhibitors (**RG108** and **1149**) were subsequently determined and summarized in Table 1. According to the results, it was obvious that **T1** showed better inhibition than both **1149** and **RG108**. The results once again confirmed that the tetrazole-based compounds had reasonable GI_{50} against MCF-7. **G6**, the smallest-size tetrazole amongst the 12 compounds, showed the best inhibition efficiency which was consistent with the earlier *in vitro* DNMT1 enzyme inhibition results. The good inhibition potency of **T1** and **G6** against MCF-7 was further determined by Fluorescence-Activated Cell Sorting (FACS) experiments (Fig. 2C); by using a BD Pharmingen ™ FITC Annexin V Apoptosis Detection Kit I to stain the inhibitor-treated cells with FITC Annexin V (detecting apoptosis) and propidium iodide (PI; detecting cell death), the fluorescence signals were quantified. Based on the data histogram, it was observed that after treating live MCF-7 cells with inhibitors at a concentration of 10 μM, both **T1** and **G6** showed a noticeable increase in the number of Annexin V labelled cells (Fig. 2C). From the statistics values, **T1** caused 61~62% cell apoptosis and death; the value was higher than that of the known DNMT1 inhibitor **RG108**. These results thus indicate that both **T1** and **G6** indeed were relatively potent and novel DNMT1 inhibitors in causing mammalian cancer cell death and apoptosis.

2.2.3 Target profiling with T1 and competitors

T1 showed better potency against DNMT1 than both **RG108** **1149**, and as a covalent modifier of the enzyme, its terminal alkyne could serve as a clickable tag for subsequent cell-based proteome profiling and target identification.[15,19] To do so, we first obtained the optimized **T1**-proteome labeling conditions: 100 μg of freshly prepared MCF-7 cell lysates were incubated with 10 μM of **T1** for 1 h at room temperature, followed by 2 h of CuAAC click chemistry with previously reported protocols (using TER-PEG-N_3 or TER-Biotin-N_3).[19] Subsequently, the same conditions were used for dose-dependent, competitive ABPP by using lysates of mammalian cells pretreated with either **G6**, **1149** or **RG108** (Fig. 2D); in-gel fluorescence scanning results showed some distinctly fluorescent bands at < 70 kDa, but not at ~183 kDa, where the labeled endogenous DNMT1 was expected to appear. This indicates **T1** was not able to directly profile the presence of endogenous DNMT1 under such experimental conditions, presumably due to the low endogenous expression level of the enzyme in mammalian cells and insufficient detection sensitivity of the probe. We therefore carried out profiling experiments by using an alternative strategy - the competitive ABPP followed by click chemistry with TER-Biotin-N_3, then protein enrichment by pull-down (PD) followed by DNMT1 visualization with Western blotting (WB) analysis (Fig. 2E); successful detection of the endogenous DNMT1 (~183 KDa) in **T1**-labeled proteomes, but not in the unlabeled proteome clearly showed that **T1** indeed was a novel activity-based probe of DNMT1 capable of covalent labeling of endogenous DNMT1 from complex mammalian protein lysates, albeit with a low sensitivity (thus rendering the labeled target detected only upon enrichment and signal amplification). When the same labeling/PD/WB experiments were carried out on lysates of mammalian cells pre-treated with increasing concentrations of **G6**, **1149** or **RG108**, we observed concomitant decreases in the signals of the labeled DNMT1, indicating all three inhibitors effectively competed for binding to the active site of DNMT1 even under complex cellular environments. The success of competitive ABPP also confirmed that the covalent labeling of DNMT1 by **T1** was



indeed activity-based, making it the first known ABP of DNMT1 to our knowledge.

that **T1** was able to bind to endogenous DNMT1 in live mammalian cells.

Figure. 3. Competitive fluorescence imaging of MCF-7 cells with **T1**. A total of four imaging channels were employed, including bright-field (BF), probe-treated channel (upon click chemistry with TER-PEG-N_3), immunofluorescence (IF) and Hoechst-stained channel (NU). Panels 1-6: cells were treated with 5 μM of **T1**; Panels 7-12: cells were pretreated with **G6** (100 μM); Panels 13-18: cells were pretreated with **1149** (100 μM); Panels 19-24: cells were pretreated with **RG108** (100 μM). Scale Bar: 10 μm.

2.2.4 Bioimaging

Bioimaging is another interesting way to report endogenous enzyme-drug interactions, including drug uptake amount and target localizations.[39-48] In recent years, small molecule-based activity-based probes have been used to successfully image endogenous cellular targets.[24,25,44,45-48] Since **T1** appeared to possess better cellular activities than both **RG108** and **1149**, and it was also a more potent DNMT1 inhibitor, we reasoned that it might serve as a potential DNMT1 imaging probe as well. To confirm this hypothesis, we carried out cellular imaging experiments (Fig. 3). MCF-7 cells were first treated with an optimized amount of **T1** (5 μM) for 30 min in a cell culture incubator. Subsequently, cells were fixed with 3.7% formaldehyde, permeabilized by 0.1% Triton X-100, and clicked with TER-PEG-N_3 by following previously optimized protocols.[19,24] The same cells were further treated with anti-DNMT1 antibodies by following standard immunofluorescence (IF) protocols, then stained with Hoechst to visualize their nuclei. Finally, cells were imaged by fluorescence microscopy under four different imaging channels (Fig. 3); no fluorescence was detected in cells treated with DMSO alone (Fig S6; panels 5-8). Strong **T1** fluorescence signals were detected throughout the nuclei of the cells (green), which co-localized reasonably well with signals obtained from the immunofluorescence with anit-DNMT1 antibodies (IF; red; panels 1-6), clearly indicating

A competitive imaging experiment was developed to test whether our newly developed, tetrazole-based DNMT1 inhibitors, e.g. **G6**, were indeed able to bind to the same DNMT1 active site in live mammalian cells as **T1**, thus directly competing with **T1** for binding to the enzyme. Live MCF-7 mammalian cells were pre-treated with either **G6**, **RG108** or **1149** (100 μM) for 1 h prior to incubation with **T1** as earlier described, then imaged (Fig. 3; panels 7-24); a significant decrease in the fluorescence signals was detected in **T1**-labeled cells pre-treated with **G6**. Interestingly, neither RG108 nor 1149 were as effective as G6 in competitively decreasing the fluorescence signals of labeled cells, once again indicating **G6** was a better DNMT1 inhibitor than both **RG108** and **1148** under native cellular environments.

3. Conclusion

We have successfully developed two types of novel chemical tools for the study of DNMT1 – an small molecule, activity-based probe (**T1**) and twelve tetrazole-containing small molecule covalent inhibitors (**Gn**; n = 1-12). We found **T1** could serve as an effective activity-based profile for cell-based profiling of endogenous DNMT1 expressed in mammalian cells, albeit with relatively low detection sensitivity. Further improvement will clearly be needed on this probe in order to make such profiling experiments a routine endeavor for future chemical and cellular studies of DNMT1 biology. As tetrazole-derived tryptophan

analogs, **G6** was found to be a novel small molecule inhibitor of DNMT1, with better inhibitory activities than well-known DNMT1 inhibitors such as **RG108** and **1148** under both *in vitro* and cellular settings. **G6** was initially designed to serve not only as a covalent inhibitor, but also a potential activity-based probe of DNMT1. Unfortunately, throughout our preliminary studies, we found the so-called "photo-click" reaction involving tetrzoles was not bioorthogonal as previously reported,[33-36] and the UV-irradiated tetrozole intermediate (nitrile imine) appeared to react with many common biological nucleophiles.[37,38] More extensive studies are underway to better understand such reactions and results will be reported in due course. We caution however, the bioorthogonality of "photo-click" chemistry needs to be carefully scrutinized before it is used in other chemical biological applications. Nevertheless, based on our in vitro DNMT1 inhibition assay, XTT anti-cell proliferation assay, FACS, in situ competitive APBB as well as cellular imaging results, we could confirmed that **G6** was indeed a reasonable DNMT1 inhibitor and may be further developed into a useful chemical tool for the study of DNMT1 biology.

4. Experimental

All chemicals were purchased from commercial vendors and used without further purification, unless otherwise noted. *N*,*N*-Dimethylformamide (DMF) and Dichloromethane (CH_2Cl_2, DCM) were distilled over CaH_2. All non-aqueous reactions were carried out under nitrogen atmosphere in oven-dried glassware. Reaction progress was monitored by TLC on pre-coated silica plates (Merck 60 F_{254}, 250 μm thicknesses) and spots were visualized by ceric ammonium molybdate, ninhydrin, basic $KMnO_4$, UV light or iodine. Flash column chromatography was carried out using Merck silica gel (0.040-0.063). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker model Advance 300 MHz or DPX-500 MHz NMR spectrometer. Chemical shifts are reported in parts per million relative to internal standard tetramethylsilane ($Si(CH_3)_4$ = 0.00 ppm) or residual solvent peaks ($CDCl_3$ = 7.26 ppm, MeOD = 3.31 ppm). ^{1}H NMR coupling constants (*J*) are reported in Hertz (Hz) and multiplicity is indicated as follows: s (singlet), d (doublet), t (triplet), m (multiplet), dd (doublet of doublet), dt (doublet of triplet). MS spectra were recorded on a Finnigan LCQ mass spectrometer, a Shimadzu LC-IT-TOF spectrometer or a Shimadzu LC-ESI spectrometer. Analytical HPLC was carried out on Shimadzu LC-IT-TOF or LC-ESI systems equipped with an autosampler, using reverse-phase Phenomenex Luna 5 μm C18 100 Å 50 × 3.0 mm columns. Preparative HPLC was carried out on Gilson preparative HPLC system using Trilution software and reverse-phase Phenomenex Luna 5 μm C18 (2) 100 Å 50 × 30.00 mm column. 0.1% TFA/H_2O and 0.1% TFA/acetonitrile were used as eluents for all HPLC experiments. The flow rate was 0.6 mL/min for analytical HPLC and 8 mL/min for preparative HPLC. UV-vis absorption and fluorescence spectra were measured by using a Shimadzu UV-vis spectrophotometer and a Perkin Elmer LS50 spectrofluorometer, respectively. All the measurements were performed at room temperature.

4.1. Synthesis of activity-based probe T1

4.1.1. (*S*)-methyl 2-((tert-butoxycarbonyl)amino)-3-(5-hydroxy-1H-indol-3-yl) Propanoate (1a)

Thionyl Chloride (2.25 mL) was added dropwise to *L*-5-Hydroxytryptophan (1 g, 4.54 mmol) in a methanol (22.5 mL) solution at 0 ^{o}C. The brown solution was refluxed for 4 h. When the reaction was completed, the solution was evaporated to remove the solvent and excessive $SOCl_2$ under reduced pressure. The dark green residue was dissolved in a minimal amount of hot methanol. Et_2O was added to obtain the dark green product precipitation as a solid. The crude product was then re-dissolved in dioxane-H_2O cosolvent (110 mL, dioxane:H_2O = 1:1). To the solution, a $NaHCO_3$ solution (sat.) was added at 0 ^{o}C to adjust the *p*H of the solution to 8. Then Boc_2O (0.99 g, 4.53 mmol) was added to the above solution at 0 ^{o}C, followed by stirring overnight at room temperature. The reaction mixture was extracted with EtOAc for three times, the organic layer was combined, washed with brine and dried over Na_2SO_4. Flash column chromatography (Hexane:EtOAc = 3:1) was used to afford the protected unnatural amino acid **1a** as a pale white solid (1.25 g, 83.0% in two steps); ^{1}H NMR (300 MHz, MeOD) δ 7.15 (d, *J* = 8.6 Hz, 1H), 7.00 (s, 1H), 6.90 (d, *J* = 2.2 Hz, 1H), 6.67 (dd, *J* = 8.6, 2.1 Hz, 1H), 4.39 (d, *J* = 6.7 Hz, 1H), 3.65 (s, 3H), 3.22-2.92 (m, 2H), 1.31 (d, *J* = 48.1 Hz, 9H); ^{13}C NMR (75 MHz, MeOD) δ 174.76, 157.51, 151.34, 132.95, 129.40, 125.19, 112.73, 112.51, 109.97, 103.41, 80.67, 55.98, 52.58, 28.65. MS (ESI): [M+H$^+$] calcd for $C_{17}H_{22}N_2O_5$ 335.15, found 335.20.

4.1.2. (*S*)-methyl 2-((tert-butoxycarbonyl)amino)-3-(5-(prop-2-yn-1-yloxy)-1H-indol-3-yl)propanoate (1b)

Compound **1a** (200 mg, 0.598 mmol) was dissolved in anhydrous DMF (2 mL). Then anhydrous potassium carbonate (124 mg, 0.897 mmol) was added to the solution. The obtained mixture was stirred at room temperature for 30 min. Propargyl bromide (80% in toluene, 0.1 mL, 0.897 mmol) was added to the reaction mixture, which was subsequently stirred at room temperature overnight. After the completion of the reaction, water was added to dilute the reaction solution. The crude product was extracted with EtOAc for three times, the organic layer was combined, washed with water and brine, dried over Na_2SO_4 and concentrated. Flash column chromatography (Hexane:EtOAc = 2:1) was used to generate the titled product **1b** as a yellow oil (220.35 mg, 99.0%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.28 (s, 1H), 7.23 (d, *J* = 8.8 Hz, 1H), 7.10 (d, *J* = 1.5 Hz, 1H), 6.99-6.87 (m, 2H), 5.28 (s, 1H), 4.72 (d, *J* = 2.2 Hz, 2H), 3.67 (s, 3H), 3.24 (s, 1H), 3.22 (s, 1H), 2.53 (s, 1H), 1.42 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.78, 155.12, 152.05, 131.90, 127.86, 123.81, 112.93, 111.93, 109.81, 102.93, 79.94, 79.18, 75.19, 57.05, 54.17, 52.22, 28.25. MS (ESI): [M+H$^+$] calcd for $C_{20}H_{24}N_2O_5$ 373.17, found 373.14.

4.1.3. (*S*)-methyl 2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-3-(5-(prop-2-yn-1-yloxy)-1H-indol-3-yl) propanoate (T1)

Compound **1b** (200 mg, 0.54 mmol) was dissolved in HPLC-grade DCM (2 mL). To the solution, TFA (2 mL) was added dropwise. The reaction mixture was stirred at room temperature for 1 h. After the reaction was completed, the volatile was removed under reduced pressure. The residue was diluted with water. After adjusting the *p*H value of the solution to 7~8 with saturated $NaHCO_3$ solution, then the product was extracted with EtOAc. The organic phase was combined, washed with brine, dried over Na_2SO_4 and concentrated. The product 1b-amine can be directly used in the next step without further purification.

Maleic anhydride (19.8 mg, 0.202 mmol) was dissolved in glacial acetic acid (0.5 mL) at room temperature. To the solution, a solution of compound **1b**-amine (50 mg, 0.184 mmol) in glacial acetic acid (0.5 mL) was added dropwise. The reaction mixture was refluxed for 4 h. After the reaction completion, the reaction was cooled to room temperature and water was added to quench the reaction. Saturated sodium bicarbonate solution was

employed to neutralize the reaction solution. The crude product was extracted with EtOAc for three times, the organic layer was combined, washed with water and brine, dried over Na_2SO_4 and concentrated. Flash column chromatography (DCM:MeOH = 95:5) was used to afford the titled product **T1** as a yellowish solid (20 mg, 31.0% in two steps): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.22 (d, *J* = 8.8 Hz, 1H), 7.08 (d, *J* = 2.3 Hz, 1H), 6.98 (d, *J* = 2.3 Hz, 1H), 6.89 (dd, *J* = 8.8, 2.4 Hz, 1H), 6.57 (s, 2H), 5.04 (dd, *J* = 9.2, 7.0 Hz, 1H), 4.73 (d, *J* = 2.4 Hz, 2H), 3.80 (s, 3H), 3.64-3.54 (m, 2H), 2.54 (t, *J* = 2.4 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.04, 169.65, 152.26, 134.18, 131.89, 127.63, 123.64, 113.39, 112.06, 111.01, 102.63, 79.34, 75.35, 57.14, 53.00, 52.63, 24.79. HRMS: [M+Na^+] calcd for $C_{19}H_{16}N_2O_5$ 375.0951, found 375.0965.

4.2. Synthesis of tetrazole-based compounds (Gn)

4.2.1. (*S*)-methyl 2-amino-3-(4-nitrophenyl)propanoate (2a)

Thionyl chloride (12 mL) was added dropwise to a solution of 4-nitro-3-phenyl-*L*-alanine (10 g, 47.58 mmol) in HPLC-grade methanol (150 mL) at 0 ^{o}C. The mixture was refluxed for 2 h. When the reaction was completed, the reaction mixture was cooled to room temperature and removed the solvent under reduced pressure. The residue was re-dissolved in water and $NaHCO_3$ was added to adjust the *p*H of the solution to 7~8. The mixture was extracted with DCM for three times. The organic phase was combined, washed with water and brine. Finally the organic phase was dried with Na_2SO_4 and concentrated *in vacuo* to afford the methylated amino acid as yellow oil (10.46 g, 98.1%). The product was used in the next step without further purification.

The methylated amino acid (1.933 g, 8.62 mmol) was dissolved in a dioxane/H_2O co-solvent (50 mL, dioxane:H_2O = 1:1). To the solution, a $NaHCO_3$ solution (sat.) was added at 0 ^{o}C to adjust the *p*H of the solution to around 8. To this solution, a solution of benzyl chloroformate (1.765 g, 10.34 mmol) in dioxane was added dropwise at 0 ^{o}C. After addition, the reaction mixture was allowed to warm to room temperature, followed by stirring overnight. After the reaction was completed, EtOAc was used to extract the crude product for three times, the organic layer was collected and combined, washed with diluted HCl solution, saturated $NaHCO_3$ solution, water and brine, dried over Na_2SO_4 and concentrated. The crude product was purified by flash column chromatography (Hexane:EtOAc = 3.5:1) to yield the protected unnatural amino acid **2a** as a white solid (3.06 g, 99.2%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.07 (d, *J* = 8.3 Hz, 2H), 7.37-7.27 (m, 5H), 7.24 (s, 2H), 5.51 (s, 1H), 5.06 (dd, *J* = 30.8, 12.2 Hz, 2H), 4.68 (dd, *J* = 13.3, 6.6 Hz, 1H), 3.72 (s, 3H), 3.27 (dd, *J* = 13.8, 5.4 Hz, 1H), 3.10 (dd, *J* = 13.8, 6.8 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.38, 155.58, 147.08, 143.85, 136.09, 130.19, 128.51, 128.30, 128.09, 123.62, 67.06, 54.52, 52.60, 38.06. MS (ESI): [M+H^+] calcd for $C_{18}H_{18}N_2O_6$ 359.12, found 359.10.

4.2.2. (S)-methyl 3-(4-aminophenyl)-2 (((benzyloxy)carbonyl) amino)propanoate (2b)

Compound **2a** (3.06 g, 8.54 mmol) was dissolved in a mixed solution of ethanol/H_2O (125 mL, EtOH:H_2O = 3:2). After that, 7.9 mL concentrated hydrochloric acid (37%) was added to the solution. Anhydrous tin (II) chloride (14.34 g, 75.62 mmol) was added and the resulting solution was refluxed at 70 ~ 80 ^{o}C for 50 min. When the reaction was completed, the reaction mixture was basified with $NaHCO_3$ to adjust the *p*H around 8 immediately. The precipitated $Sn(OH)_2$ was removed by filtration through a short pad of celite and washed with EtOAc for three times. The filtrate was collected and extracted with EtOAc. The organic phase was washed with water and brine, dried over Na_2SO_4 and concentrated. The crude product was further purified by flash column chromatography (Hexane:EtOAc = 3:1) to obtain the titled product **2b** as a golden yellow oil (2.28 g, 81.3%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40-7.28 (m, 5H), 6.88 (d, *J* = 8.3 Hz, 2H), 6.62 (d, *J* = 8.2 Hz, 2H), 5.22 (d, *J* = 7.3 Hz, 1H), 5.09 (d, *J* = 2.1 Hz, 2H), 4.59 (dd, *J* = 13.9, 5.8 Hz, 1H), 3.71 (s, 3H), 3.06-2.94 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.30, 155.76, 145.46, 136.28, 130.17, 128.57, 128.14, 128.07, 125.36, 115.42, 66.97, 55.06, 52.31, 37.40. MS (ESI): [M+H^+] calcd for $C_{18}H_{20}N_2O_4$ 329.14, found 329.12;

4.2.3. General procedure

4.2.3.1. Synthesis of tetrazole compound ($2d_n$)

Aldehyde **N** and $PhSO_2NHNH_2$ (1:1.2 equiv.) were dissolved in ethanol (6.8 mL/mmol) and the resulting solution was stirred at room temperature for 3 h. After the completion of the reaction, EtOH was removed under reduced pressure to give compound **$2c_n$**. The crude product was used in the next step directly without further purification.[36]

Compound **$2c_n$** (0.85 equiv.) was re-dissolved in pyridine (10.4 mL/mmol) and cooled to -10 ~ -20 ^{o}C in an ice/NaCl bath to derive **solution A**. Separately, to a solution of compound **2b** (1 equiv.) in mixed EtOH-H_2O (10.4 mL/mmol, EtOH:H_2O = 1:1) was added concentrated hydrochloric acid (0.48 mL/mmol), and the mixture was cooled to -5 ^{o}C with an ice/NaCl bath. Subsequently, a cold solution of $NaNO_2$ (1.5 equiv.) in water (1.74 mL/mmol) was added dropwise into to derive **solution B** (it is very important to keep the temperature below 0 ^{o}C during the entire addition process!).

Solution B was added to **solution A** slowly and kept the temperature below -5 ^{o}C at all the time. Upon the completion of addition, the reaction mixture was allowed to warm to room temperature. The solution was extracted with EtOAc/H_2O, washed with diluted HCl solution and brine, then dried with Na_2SO_4 and concentrated. The crude product was further purified via column chromatography (1% EtOAc in DCM) to afford the titled compound **$2d_n$**.

4.2.3.2. Synthesis of the target probe Gn (avoiding light during all the process)

To a solution of tetrazole amino acid derivatives **$2d_n$** (100 mg) in HPLC MeOH was added 10% Pd/C (20 mg). The reaction was sealed and air was removed by inletting nitrogen gas into the system. While air was totally removed, the reaction mixture was hydrogenated at room temperature (1 atm) overnight. And the catalyst was removed by filtration through a short pad of celite and washing with EtOAc. The solvent was removed under reduced pressure and the obtained product **$2e_n$** can be directly used in the next step.

To a solution of maleic anhydride in HPLC-grade $CHCl_3$ (0.8 mL/mmol), a solution of compound **$2e_n$** (1 equiv.) in HPLC $CHCl_3$ (0.2 mL/mmol) was added dropwise. The resulting mixture was stirred at room temperature for 2 h. After starting materials were completely reacted, the solvent was removed under reduced pressure. The residue was re-dissolved in acetic anhydride (1 mL/mmol) and sodium acetate (20 mg/mmol) was added. The mixture was refluxed for 2 h, cooled and quenched with water. The aqueous solution was extracted with EtOAc, washed with saturated $NaHCO_3$ solution, water and brine, dried over Na_2SO_4 and concentrated. The crude product **Gn** was purified by flash column chromatography.

4.2.4. (*S*)-methyl 2-(((benzyloxy)carbonyl)amino)-3-(4-(5-(4-isopropylphenyl)-2H-tetrazol-2-yl) phenyl) propanoate ($2d_1$)

A yellowish solid (130 mg, 85.5%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.17 (d, *J* = 8.2 Hz, 2H), 8.09 (d, *J* = 8.3 Hz, 2H), 7.41-7.27 (m, 9H), 5.31 (d, *J* = 7.7 Hz, 1H), 5.09 (t, *J* = 12.7 Hz, 2H), 4.75-4.69 (m, 1H), 3.75 (s, 3H), 3.27 (dd, *J* = 13.9, 5.6 Hz, 1H), 3.15 (dd, *J* = 13.9, 6.1 Hz, 1H), 3.03-2.95 (m, 1H), 1.31 (d, *J* = 6.9 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.61, 165.29, 155.56, 151.72, 137.64, 136.13, 135.98, 130.53, 128.56, 128.30, 128.17, 127.11, 127.08, 124.68, 119.98, 67.13, 54.71, 52.55, 38.02, 34.18, 23.84. MS (IT-TOF): [M+H$^+$] calcd for $C_{28}H_{29}N_5O_4$ 500.22, found 500.20.

4.2.5. (*S*)-methyl 2-(((benzyloxy)carbonyl)amino)-3-(4-(5-(4-fluorophenyl)-2H-tetrazol-2-yl)phenyl) propanoate ($2d_2$)

A yellowish solid (128 mg, 88.4%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.27-8.19 (m, 2H), 8.09 (d, *J* = 8.2 Hz, 2H), 7.36-7.27 (m, 6H), 7.25-7.18 (m, 3H), 5.29 (s, 1H), 5.09 (t, *J* = 11.8 Hz, 2H), 4.72 (s, 1H), 3.75 (s, 3H), 3.25 (s, 1H), 3.15 (d, *J* = 7.4 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.57, 165.23, 163.83 (d, *J* = 148.6 Hz), 155.53, 137.85, 136.11, 135.86, 130.58, 129.10 (d, *J* = 8.6 Hz), 128.55, 128.29, 128.16, 123.39, 119.95, 116.13 (d, *J* = 22.0 Hz). MS (IT-TOF): [M+H$^+$] calcd for $C_{25}H_{22}FN_5O_4$ 476.17, found 476.16.

4.2.6. (*S*)-methyl 2-(((benzyloxy)carbonyl)amino)-3-(4-(5-(naphthalen-1-yl)-2H-tetrazol-2-yl)phenyl) propanoate ($2d_3$)

A yellowish solid (208.1 mg, 83.7%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.02 (d, *J* = 8.5 Hz, 1H), 8.38 (d, *J* = 7.2 Hz, 1H), 8.18 (d, *J* = 8.0 Hz, 2H), 8.03 (d, *J* = 8.1 Hz, 1H), 7.96 (d, *J* = 8.0 Hz, 1H), 7.68-7.56 (m, 3H), 7.34 (d, *J* = 9.5 Hz, 7H), 5.33 (d, *J* = 7.8 Hz, 1H), 5.10 (t, *J* = 13.2 Hz, 2H), 4.74 (d, *J* = 6.9 Hz, 1H), 3.77 (s, 3H), 3.30 (dd, *J* = 13.6, 5.6 Hz, 1H), 3.18 (dd, *J* = 14.1, 6.0 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.61, 165.35, 155.56, 137.84, 136.13, 135.97, 134.01, 131.28, 130.64, 128.69, 128.67, 128.57, 128.32, 128.19, 127.44, 126.33, 125.78, 125.25, 123.92, 120.06, 67.14, 54.72, 52.58, 38.04. MS (IT-TOF): [M+H$^+$] calcd for $C_{29}H_{25}N_5O_4$ 508.19, found 508.18.

4.2.7. (*S*)-methyl 2-(((benzyloxy)carbonyl)amino)-3-(4-(5-(4-methoxyphenyl)-2H-tetrazol-2-yl) phenyl) propanoate ($2d_4$)

A yellowish solid (55.4 mg, 37.3%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.18 (d, *J* = 8.8 Hz, 2H), 8.08 (d, *J* = 8.4 Hz, 2H), 7.40-7.26 (m, 7H), 7.04 (d, *J* = 8.8 Hz, 2H), 5.30 (d, *J* = 8.2 Hz, 1H), 5.10 (q, *J* = 12.2 Hz, 2H), 4.72 (d, *J* = 7.6 Hz, 1H), 3.89 (s, 3H), 3.75 (s, 3H), 3.26 (dd, *J* = 13.7, 5.7 Hz, 1H), 3.15 (dd, *J* = 14.0, 6.1 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.60, 165.10, 161.51, 155.55, 137.58, 136.14, 135.99, 130.53, 128.60, 128.56, 128.30, 128.17, 119.93, 119.72, 114.39, 67.13, 55.41, 54.71, 52.54, 38.02. MS (IT-TOF): [M+H$^+$] calcd for $C_{26}H_{25}N_5O_5$ 488.19, found 488.18.

4.2.8. (*S*)-methyl 2-(((benzyloxy)carbonyl)amino)-3-(4-(5-(4-(dimethylamino)phenyl)-2H-tetrazol-2-yl)phenyl) propanoate ($2d_5$)

A yellowish solid (35.8 mg, 23.5%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.13-8.07 (m, 2H), 7.35-7.26 (m, 9H), 6.85 (s, 2H), 5.29 (d, *J* = 7.6 Hz, 1H), 5.11 (q, *J* = 12.2 Hz, 2H), 4.72 (d, *J* = 7.7 Hz, 1H), 3.75 (s, 3H), 3.28-3.23 (m, 1H), 3.18-3.13 (m, 1H), 3.07 (s, 6H). MS (IT-TOF): [M+H$^+$] calcd for $C_{27}H_{28}N_6O_4$ 501.22, found 501.19.

4.2.9. (*S*)-methyl 2-(((benzyloxy)carbonyl)amino)-3-(4-(5-phenyl-2H-tetrazol-2-yl)phenyl)propanoate ($2d_6$)

A yellowish solid (79.2 mg, 56.9%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.25 (dd, *J* = 7.8, 1.7 Hz, 2H), 8.10 (d, *J* = 8.4 Hz, 2H), 7.52 (dd, *J* = 5.7, 3.8 Hz, 3H), 7.38-7.27 (m, 7H), 5.31 (d, *J* = 7.7 Hz, 1H), 5.09 (t, *J* = 12.4 Hz, 2H), 4.73 (dd, *J* = 13.8, 6.0 Hz, 1H), 3.76 (s, 3H), 3.27 (dd, *J* = 13.9, 5.7 Hz, 1H), 3.16 (dd, *J* = 13.9, 6.2 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.60, 165.22, 155.58, 137.77, 135.94, 130.60, 130.57, 128.98, 128.56, 128.31, 128.17, 127.14, 127.07, 120.00, 67.14, 54.71, 52.56, 38.03. MS (IT-TOF): [M+H$^+$] calcd for $C_{25}H_{23}N_5O_4$ 458.18, found 458.17.

4.2.10. (S)-methyl 2-(((benzyloxy)carbonyl)amino)-3-(4-(5-(3,5-difluorophenyl)-2H-tetrazol-2-yl) phenyl) propanoate ($2d_7$)

A yellowish solid (111.6 mg, 74.3%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.08 (d, *J* = 8.3 Hz, 2H), 7.84-7.73 (m, 2H), 7.33 (dd, *J* = 16.8, 7.3 Hz, 7H), 6.95 (tt, *J* = 8.8, 2.3 Hz, 1H), 5.32 (d, *J* = 6.4 Hz, 1H), 5.09 (t, *J* = 11.8 Hz, 2H), 4.73 (dd, *J* = 13.5, 6.1 Hz, 1H), 3.76 (s, 3H), 3.28 (dd, *J* = 13.9, 5.6 Hz, 1H), 3.16 (dd, *J* = 13.9, 6.2 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.56, 164.37 (d, *J* = 12.8 Hz), 163.45, 162.44, 155.54, 138.24, 135.69, 130.67, 128.57, 128.32, 128.18, 120.02, 110.20 (d, *J* = 7.0 Hz), 110.03 (d, *J* = 6.8 Hz), 105.95, 67.15, 54.69, 52.58, 38.04. MS (IT-TOF): [M+H$^+$] calcd for $C_{25}H_{21}F_2N_5O_4$ 494.16, found 494.14.

4.2.11. (*S*)-methyl 2-(((benzyloxy)carbonyl)amino)-3-(4-(5-(4-(hexyloxy)phenyl)-2H-tetrazol-2-yl) phenyl) propanoate ($2d_8$)

A yellowish solid (88.3 mg, 68.8%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.17 (d, *J* = 8.8 Hz, 2H), 8.08 (d, *J* = 8.4 Hz, 2H), 7.38-7.26 (m, 7H), 7.03 (d, *J* = 8.9 Hz, 2H), 5.29 (d, *J* = 7.9 Hz, 1H), 5.11 (q, *J* = 12.2 Hz, 2H), 4.72 (d, *J* = 7.7 Hz, 1H), 4.04 (t, *J* = 6.6 Hz, 2H), 3.75 (s, 3H), 3.26 (dd, *J* = 14.0, 5.7 Hz, 1H), 3.15 (dd, *J* = 13.9, 6.0 Hz, 1H), 1.86-1.78 (m, 2H), 1.53-1.25 (m, 6H), 0.92 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.58, 165.16, 161.12, 155.34, 136.00, 130.50, 128.55, 128.28, 128.15, 119.92, 119.43, 114.90, 68.19, 67.11, 54.70, 52.52, 37.88, 31.57, 29.17, 25.70, 22.59, 14.01. MS (IT-TOF): [M+H$^+$] calcd for $C_{31}H_{35}N_5O_5$ 558.26, found 558.23.

4.2.12. (S)-methyl 2-(((benzyloxy)carbonyl)amino)-3-(4-(5-(pyridin-4-yl)-2H-tetrazol-2-yl)phenyl)propanoate ($2d_9$)

A yellowish solid (39 mg, 32.7%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.83 (s, 2H), 8.12 (dd, *J* = 21.4, 6.5 Hz, 4H), 7.34 (dd, *J* = 7.5, 4.9 Hz, 7H), 5.33 (d, *J* = 7.8 Hz, 1H), 5.17-5.03 (m, 2H), 4.73 (dd, *J* = 13.7, 6.1 Hz, 1H), 3.76 (s, 3H), 3.29 (dd, *J* = 13.9, 5.6 Hz, 1H), 3.16 (dd, *J* = 13.9, 6.1 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.55, 163.12, 155.57, 150.41, 138.44, 136.13, 135.65, 130.72, 128.57, 128.33, 128.19, 121.10, 120.09, 67.16, 54.69, 52.60, 38.06. MS (IT-TOF): [M+H$^+$] calcd for $C_{24}H_{22}N_6O_4$ 459.17, found 459.14.

4.2.13. (S)-methyl 2-(((benzyloxy)carbonyl)amino)-3-(4-(5-(4-(difluoromethoxy)phenyl)-2H-tetrazol-2yl)phenyl)propanoate ($2d_{10}$)

A yellowish solid (34 mg, 26.5%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.26 (d, *J* = 8.7 Hz, 2H), 8.09 (d, *J* = 8.3 Hz, 2H), 7.38-7.26 (m, 9H), 6.60 (t, *J* = 73.4 Hz, 1H), 5.31 (d, *J* = 8.0 Hz, 1H), 5.14-5.07 (m, 2H), 4.72 (d, *J* = 7.5 Hz, 1H), 3.76 (s, 3H), 3.27 (dd, *J* = 13.9, 5.6 Hz, 1H), 3.15 (dd, *J* = 13.9, 6.2 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.58, 164.34, 155.55, 152.87, 137.92, 136.12, 135.86, 130.60, 128.76, 128.56, 128.31, 128.17, 124.37, 119.99, 119.80, 115.64, 67.14, 54.70, 52.55, 38.04. MS (IT-TOF): [M+H$^+$] calcd for $C_{26}H_{23}F_2N_5O_5$ 524.17, found 524.15.

4.2.14. (S)-methyl 2-(((benzyloxy)carbonyl)amino)-3-(4-(5-(4-(trifluoromethyl)phenyl)-2H-tetrazol-2-yl)phenyl)propanoate ($2d_{11}$)

A yellowish solid (48 mg, 35.1%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.38 (d, *J* = 8.2 Hz, 2H), 8.10 (d, *J* = 8.3 Hz, 2H), 7.80 (d, *J* = 8.3 Hz, 2H), 7.31 (dd, *J* = 24.4, 16.4 Hz, 7H), 5.31 (d, *J* = 7.6 Hz, 1H), 5.09 (dd, *J* = 15.8, 7.2 Hz, 2H), 4.73 (d, *J* = 6.0 Hz, 1H), 3.76 (s, 3H), 3.31-3.22 (m, 1H), 3.16 (dd, *J* = 13.8, 6.1 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.54, 164.02, 155.55, 138.12, 136.11, 135.77, 132.50, 132.24, 130.66, 128.57, 128.32, 128.18, 127.35, 126.01 (d, *J* = 3.9 Hz), 120.05, 67.15, 54.70, 52.58, 38.05. MS (IT-TOF): [M+H$^+$] calcd for $C_{26}H_{22}F_3N_5O_4$ 526.16, found 526.16.

4.2.15. (*S*)-methyl 2-(((benzyloxy)carbonyl)amino)-3-(4-(5-(3,5-dimethylphenyl)-2H-tetrazol-2-yl) phenyl) propanoate ($2d_{12}$)

A yellowish solid (97 mg, 76.8%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.10 (d, *J* = 8.3 Hz, 2H), 7.87 (s, 2H), 7.40-7.26 (m, 7H), 7.14 (s, 1H), 5.30 (d, *J* = 7.9 Hz, 1H), 5.11 (q, *J* = 12.2 Hz, 2H), 4.72 (dd, *J* = 13.5, 6.0 Hz, 1H), 3.75 (s, 3H), 3.27 (dd, *J* = 13.9, 5.6 Hz, 1H), 3.15 (dd, *J* = 13.9, 6.1 Hz, 1H), 2.42 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.60, 165.44, 155.31, 138.66, 137.68, 136.12, 135.98, 132.29, 130.54, 128.56, 128.30, 128.16, 126.86, 124.81, 119.99, 67.13, 54.71, 52.53, 38.03, 21.30 (d, *J* = 1.7 Hz). MS (IT-TOF): [M+H$^+$] calcd for $C_{27}H_{27}N_5O_4$ 486.21, found 486.19.

4.2.16. (S)-methyl 2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-3-(4-(5-(4-isopropylphenyl)-2H-tetrazol-2-yl)phenyl)propanoate (G1)

A yellowish solid (21 mg, 34.5%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.11 (dd, *J* = 19.3, 8.5 Hz, 4H), 7.36 (dd, *J* = 11.7, 8.4 Hz, 4H), 6.64 (s, 2H), 5.01 (dd, *J* = 11.1, 5.7 Hz, 1H), 3.80 (s, 3H), 3.57 (t, *J* = 8.3 Hz, 2H), 2.98 (dt, *J* = 13.8, 6.8 Hz, 1H), 1.30 (d, *J* = 6.9 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.75, 168.86, 165.27, 151.71, 138.36, 135.87, 134.18, 130.10, 127.09, 127.07, 124.63, 120.08, 77.29, 77.04, 76.78, 53.08, 52.84, 34.25, 34.18, 29.66, 23.84. HRMS: [M+Na$^+$] calcd for $C_{24}H_{23}N_5O_4$ 468.1642, found 468.1655.

4.2.17. (S)-methyl 2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-3-(4-(5-(4-fluorophenyl)-2H-tetrazol-2-yl)phenyl) propanoate (G2)

A yellowish solid (18.6mg, 42.8%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.23 (dd, *J* = 8.8, 5.4 Hz, 2H), 8.07 (d, *J* = 8.5 Hz, 2H), 7.35 (d, *J* = 8.5 Hz, 2H), 7.22 (dd, *J* = 16.4, 7.7 Hz, 3H), 6.64 (s, 2H), 5.01 (dd, *J* = 11.1, 5.7 Hz, 1H), 3.81 (s, 3H), 3.65-3.52 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.88, 168.97, 165.36, 163.96 (d, *J* = 146.3 Hz), 138.71, 135.88, 134.31, 130.29, 129.26, 123.49, 120.20, 116.28 (d, *J* = 22.5 Hz), 53.23, 52.96, 34.39. HRMS: [M+Na$^+$] calcd for $C_{21}H_{16}FN_5O_4$ 444.1079, found 444.1090.

4.2.18. (S)-methyl 2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-3-(4-(5-(naphthalen-1-yl)-2H-tetrazol-2-yl)phenyl) propanoate (G3)

A yellowish solid (29.2mg, 48.1%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.99 (d, *J* = 8.5 Hz, 1H), 8.36 (d, *J* = 7.2 Hz, 1H), 8.17 (d, *J* = 8.4 Hz, 2H), 8.02 (d, *J* = 8.2 Hz, 1H), 7.95 (d, *J* = 8.0 Hz, 1H), 7.67-7.56 (m, 3H), 7.38 (d, *J* = 8.4 Hz, 2H), 6.65 (s, 2H), 5.03 (dd, *J* = 11.5, 5.3 Hz, 1H), 3.81 (s, 3H), 3.67-3.51 (m, 2H).^{13}C NMR (125 MHz, $CDCl_3$) δ 169.90, 168.99, 165.46, 138.69, 135.98, 134.33, 134.11, 131.41, 130.72, 130.33, 128.82, 128.79, 127.58, 126.46, 125.87, 125.37, 124.00, 120.29, 53.23, 52.98, 34.42. HRMS: [M+Na$^+$] calcd for $C_{25}H_{19}N_5O_4$ 476.1329, found 476.1335.

4.2.19. (S)-methyl 2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-3-(4-(5-(4-methoxyphenyl)-2H-tetrazol-2-yl)phenyl) propanoate (G4)

A yellowish solid (21.5mg, 35.1%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.19-8.14 (m, 2H), 8.07 (d, *J* = 8.6 Hz, 2H), 7.33 (d, *J* = 8.5 Hz, 2H), 7.06-7.01 (m, 2H), 6.64 (s, 2H), 5.01 (dd, *J* = 11.5, 5.3 Hz, 1H), 3.89 (s, 3H), 3.81 (s, 3H), 3.61-3.53 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.73, 168.85, 165.07, 161.49, 130.08, 128.57, 120.03, 119.66, 114.37, 55.40, 53.06, 52.83, 34.23. HRMS: [M+Na$^+$] calcd for $C_{22}H_{19}N_5O_5$ 456.1278, found 456.1284.

4.2.20. (S)-methyl 3-(4-(5-(4-(dimethylamino)phenyl)-2H-tetrazol-2-yl)phenyl)-2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl) propanoate (G5)

A yellowish solid (1 mg, 9.4%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.15-8.02 (m, 4H), 7.32 (d, *J* = 8.5 Hz, 2H), 6.80 (d, *J* = 8.9 Hz, 2H), 6.63 (s, 2H), 5.01 (dd, *J* = 11.5, 5.4 Hz, 1H), 3.80 (s, 3H), 3.57 (dd, *J* = 16.5, 8.4 Hz, 2H), 3.05 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.74, 168.74, 165.65, 151.59, 137.98, 134.16, 130.01, 128.17, 119.98, 111.94, 53.05, 52.86, 40.24, 34.23. HRMS: [M+Na$^+$] calcd for $C_{23}H_{22}N_6O_4$ 469.1595, found 469.1611.

4.2.21. (S)-methyl 2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-3-(4-(5-phenyl-2H-tetrazol-2-yl)phenyl)propanoate (G6)

A yellowish solid (27.5 mg, 51.4%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.23 (dd, *J* = 7.7, 1.8 Hz, 1H), 8.09 (d, *J* = 8.5 Hz, 1H), 7.56-7.45 (m, 2H), 7.35 (d, *J* = 8.5 Hz, 1H), 6.64 (s, 1H), 5.01 (dd, *J* = 11.5, 5.3 Hz, 1H), 3.81 (s, 2H), 3.58 (ddd, *J* = 25.9, 14.4, 8.5 Hz, 1H), ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.88, 168.98, 165.33, 138.62, 135.96, 134.31, 130.73, 130.26, 129.10, 127.23, 127.18, 53.21, 52.97, 34.39. HRMS: [M+Na$^+$] calcd for $C_{21}H_{17}N_5O_4$ 426.1173, found 426.1181.

4.2.22. (S)-methyl 3-(4-(5-(3,5-difluorophenyl)-2H-tetrazol-2-yl)phenyl)-2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl) propanoate (G7)

A white solid (16.1 mg, 43.9%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.07 (d, *J* = 8.4 Hz, 2H), 7.76 (d, *J* = 6.0 Hz, 2H), 7.36 (d, *J* = 8.4 Hz, 2H), 6.99-6.90 (m, 1H), 6.64 (s, 2H), 5.01 (dd, *J* = 11.5, 5.3 Hz, 1H), 3.80 (s, 3H), 3.58 (ddd, *J* = 25.9, 14.4, 8.5 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.86, 168.93, 164.49 (d, *J* = 12.6 Hz), 163.57, 162.56, 139.08, 135.72, 134.32, 130.37, 120.27, 110.23 (dd, *J* = 20.8, 6.9 Hz), 106.07 (t, *J* = 25.4 Hz), 53.23, 52.94, 34.42. HRMS: [M+Na$^+$] calcd for $C_{21}H_{15}F_2N_5O_4$ 462.0984, found 462.0975.

4.2.23. (S)-methyl 2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-3-(4-(5-(4-(hexyloxy)phenyl)-2H-tetrazol-2-yl)phenyl) propanoate (G8)

A white solid (13.2 mg, 17.3%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.14 (d, *J* = 8.8 Hz, 2H), 8.07 (d, *J* = 8.5 Hz, 2H), 7.33 (d, *J* = 8.5 Hz, 2H), 7.02 (d, *J* = 8.8 Hz, 2H), 6.63 (s, 2H), 5.01 (dd, *J* = 11.5, 5.3 Hz, 1H), 4.03 (t, *J* = 6.6 Hz, 2H), 3.80 (s, 3H), 3.57 (ddd, *J* = 25.9, 14.4, 8.5 Hz, 2H), 1.86-1.76 (m, 2H), 1.53-1.45 (m, 2H), 1.36 (d, *J* = 3.7 Hz, 4H), 0.92 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.71, 168.83, 165.13, 161.10, 138.24, 135.86, 134.14, 130.06, 128.51, 120.00, 119.37, 114.87, 68.16, 53.03, 52.82, 34.22, 31.56, 29.15, 25.69, 22.58, 14.01. HRMS: [M+Na$^+$] calcd for $C_{27}H_{29}N_5O_5$ 526.2061, found 526.2080.

4.2.24. (S)-methyl 2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-3-(4-(5-(pyridin-4-yl)-2H-tetrazol-2-yl)phenyl) propanoate (G9)

A yellow solid (4.8 mg, 22.0%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.82 (s, 2H), 8.16 (d, *J* = 4.7 Hz, 2H), 8.10 (d, *J* = 8.4 Hz, 2H), 7.38 (d, *J* = 8.4 Hz, 2H), 6.65 (s, 2H), 5.01 (dd, *J* = 11.4, 5.3 Hz, 1H), 3.81 (s, 3H), 3.59 (dt, *J* = 14.2, 5.9 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.71, 168.74, 162.93, 149.89, 139.21, 135.52, 135.30, 134.18, 130.29, 121.21, 120.21, 53.08, 52.78, 34.29. HRMS: [M+Na$^+$] calcd for $C_{20}H_{16}N_6O_4$ 427.1125, found 427.1130.

4.2.25. (S)-methyl 2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-3-(4-(5-(4-(trifluoromethyl)phenyl)-2H-tetrazol-2-yl)phenyl) propanoate (G10)

A yellowish solid (2.6 mg, 16.6%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.24 (d, *J* = 8.8 Hz, 2H), 8.08 (d, *J* = 8.5 Hz, 2H), 7.35 (d, *J* = 8.5 Hz, 2H), 7.27 (d, *J* = 6.9 Hz, 2H), 6.78-6.40 (m, 3H), 5.01 (dd, *J* = 11.5, 5.3 Hz, 1H), 3.81 (s, 3H), 3.63-3.53 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.72, 168.80, 164.31, 152.85, 138.62, 135.73, 134.16, 130.15, 128.73, 124.32, 120.08, 119.76, 115.62. HRMS: [M+Na$^+$] calcd for $C_{22}H_{17}F_2N_5O_5$ 492.1090, found 492.1084.

4.2.26. (S)-methyl 3-(4-(5-(4-(difluoromethoxy)phenyl)-2H-tetrazol-2-yl)phenyl)-2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl) propanoate (G11)

A yellowish solid (7.9 mg, 28.5%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.36 (d, *J* = 8.1 Hz, 2H), 8.10 (d, *J* = 8.6 Hz, 2H), 7.79 (d, *J* = 8.2 Hz, 2H), 7.36 (d, *J* = 8.5 Hz, 2H), 6.64 (s, 2H), 5.01 (dd, *J* = 11.5, 5.3 Hz, 1H), 3.81 (s, 3H), 3.62-3.54 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.72, 168.78, 164.00, 138.87, 135.66, 134.17, 130.21, 127.32, 126.00, 125.97, 120.15, 53.07, 52.81, 34.28. HRMS: [M+Na$^+$] calcd for $C_{22}H_{16}F_3N_5O_4$ 494.1047, found 494.1033.

4.2.27. (S)-methyl 3-(4-(5-(3,5-dimethylphenyl)-2H-tetrazol-2-yl)phenyl)-2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl) propanoate (G12)

A white solid (11 mg, 17.9%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.08 (d, *J* = 8.0 Hz, 2H), 7.85 (s, 2H), 7.34 (d, *J* = 8.1 Hz, 2H), 7.13 (s, 1H), 6.64 (s, 2H), 5.01 (dd, *J* = 11.4, 5.2 Hz, 1H), 3.80 (s, 3H), 3.65-3.48 (m, 2H), 2.41 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.71, 168.83, 165.41, 138.63, 138.38, 135.84, 134.15, 132.26, 130.08, 126.80, 124.77, 120.08, 53.03, 52.82, 34.23, 21.26. HRMS: [M+Na$^+$] calcd for $C_{23}H_{21}N_5O_4$ 454.1486 [M+H$^+$], found 454.1477.

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Supplementary Material

Supplementary material that may be helpful in the review process should be prepared and provided as a separate electronic file. That file can then be transformed into PDF format and submitted along with the manuscript and graphic files to the appropriate editorial office.