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# Solution-Phase Synthesis of the Fluorogenic TGase 2 Acyl Donor Z-Glu(HMC)-Gly-OH and its Use for Inhibitor and Amine Substrate Characterisation

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**Abstract:** A reliable solution-phase synthesis of the water-soluble dipeptidic fluorogenic transglutaminase substrate Z-Glu(HMC)-Gly-OH is presented. The route started from Z-Glu-OH, which was converted into the corresponding cyclic anhydride. This building block was transformed into the regioisomeric  $\alpha$ - and  $\gamma$ -dipeptides. The key step was the esterification of Z-Glu-Gly-*Ot*Bu with 4-methylumbelliferone. The final substrate compound was obtained in an acceptable yield and excellent purity without the need of purification by RP-HPLC. The advantage of this acyl donor substrate for the kinetic characterisation of inhibitors and amine-type acyl acceptor substrates is demonstrated by evaluating commercially available or literature-known irreversible inhibitors and the biogenic amines serotonin, histamine and dopamine, respectively.

**Keywords:** fluorogenic enzyme substrates; side-chain esterified peptides; aryl esters; peptide synthesis; enzyme kinetics; biogenic amines

## 1. Introduction

Transglutaminase 2 is a multifunctional enzyme whose distinct physiological roles are incompletely understood [1, 2]. Its eponymous and best-characterised function represents the catalysis of the acyl transfer between glutamine residues and a variety of primary amines [3]. To assess the significance of this enzymatic activity in cells, substrates (especially low molecular weight polyamines) [4, 5] and inhibitors [6] were applied amongst other biochemical techniques [7, 8]. The prerequisite for an accurate interpretation of potential effects mediated by those TGase 2-targeting molecules is the knowledge of their kinetic properties towards the isolated protein and also towards

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### Abbreviations

d...doublet, DIPEA...*N,N*-diisopropylethylamine, EDTA...*N,N'*-ethylenediamine tetraacetic acid, GDH...glutamate dehydrogenase, hTGase 2...human TGase 2; HMC...7-hydroxy-4-methylcoumarin (=4-methylumbelliferone), HATU...(1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate, HPLC...high performance liquid chromatography, Hsp...heat shock protein, LC...liquid chromatography, m...multiplet, MES...2-(*N*-morpholino)ethanesulfonic acid, MOPS...(3-(*N*-morpholino)propanesulfonic acid), MS...mass spectrometry, NMM...*N*-methylmorpholine, NMR...nuclear magnetic resonance, Np...4-nitrophenyl, PyBOP...benzotriazol-1-yl-oxypyrrolidinophosphonium hexafluorophosphate, q...quadruplet, RP...reversed phase, s...singlet, t...triplet, TCEP...tris(2-carboxyethyl)phosphine, TLC...thin layer chromatography, UPLC...Ultra Performance Liquid Chromatography, Z...benzyloxycarbonyl (=carbobenzyoxy, Cbz); all other abbreviations are in accordance to the recommendations of the IUPAC-IUB commission on biochemical nomenclature, see Eur. J. Biochem. 15 (1970) 203-208 and Eur. J. Biochem. 138 (1984) 9-37

32 the other members of the transglutaminase family. The other main function of TGase 2 is dealing  
33 with the binding of GDP and GTP and catalysis of hydrolysis of the latter [9]. Both functions are  
34 mutually exclusive as they are associated with distinct conformational states of the enzyme molecule  
35 [10]. Therefore, occupation of the guanine nucleotide binding site of TGase 2 can be analysed via  
36 detection of its acyltransferase activity [11, 12], which indicates the value of robust assay methods  
37 that allow for the monitoring of this activity [13]. A rigorous kinetic assessment of the enzyme's  
38 acyltransferase activity is enabled by the dipeptidic coumarinyl ester Z-Glu(HMC)-Gly-OH as  
39 fluorogenic substrate. In addition to its suitable kinetic and spectral properties, the compound's  
40 utility is owed to its propitious water solubility [14]. Favourably, this compound can be also used for  
41 the assay of other transglutaminase isoforms [15]. The synthesis of peptides containing glutamate  
42 coumarinyl esters was achieved by solid-phase peptide synthesis using the chlorotrityl linker for  
43 attachment to the solid support. The ester moiety in the glutamate side-chain was installed after  
44 deblocking the allyl ester-protected  $\gamma$ -carboxylic acid function prior to acidic cleavage from the resin  
45 [14]. Although this synthesis strategy is advantageous for obtaining various peptidic substrates, the  
46 product scale is limited by the use of the costly polymeric resin and the need of preparative RP-HPLC  
47 for purification. Efficient solution-phase methods have been described by Chung *et al.* [16] and  
48 Leblanc *et al.* [17] for the synthesis of the corresponding chromogenic dipeptidic substrate Z-  
49 Glu(ONp)-Gly-OH, which bears a nitrophenyl group attached to the identical peptidic scaffold. This  
50 pathway was adopted by us for an efficient access to Z-Glu(HMC)-Gly-OH, which will be reported  
51 herein together with its kinetic evaluation towards TGase 2-catalysed hydrolysis. In this context, it  
52 was envisaged to re-prove the fluorogenic substrate's previously established value for TGase 2  
53 inhibitor and substrate characterisation exemplarily for commercially available irreversible inhibitors  
54 for which robust second-order inactivation constants  $k_{\text{inact}}/K_{\text{I}}$  have not been reported so far.  
55 Furthermore, kinetic parameters for selected physiologically relevant amine substrates were  
56 determined by taking advantage of the title compound.

57

## 58 2. Materials and Methods

### 59 2.1. General

60 All starting materials, reagents and solvents were commercially obtained and used without  
61 further purification. Compound 5, *N*-monodansylpiperazine and *N*-acryloxysuccinimide were  
62 synthesised as previously published [15]. Chromatographic separations and TLC detections were  
63 carried out with Merck Silica Gel 60 (63–200  $\mu\text{m}$ ) and Merck Silica Gel 60 F<sub>254</sub> sheets, respectively.  
64 TLCs were visualised under UV light ( $\lambda = 254 \text{ nm}$  or  $365 \text{ nm}$ ). Analytical RP-HPLC was carried out  
65 with a system consisting of a Merck Hitachi L7100 gradient pump combined with a Jasco DG2080  
66 four-line degasser with UV detection with a Merck Hitachi L7450 diode array detector. The system  
67 was operated with D-700 HSM software and use of a Merck Hitachi D7000 interface. A Luna C18 5  
68  $\mu\text{m}$  column (Phenomenex, 250 $\times$ 4.6 mm) served as stationary phase. A binary gradient system of 0.1%  
69 CF<sub>3</sub>COOH/water (solvent A) and 0.1% CF<sub>3</sub>COOH/CH<sub>3</sub>CN (solvent B) at a flow rate of 1 mL/min  
70 served as the eluent. The following elution programme was run to separate the components: 0–3 min  
71 80% A, 3–45 min gradient to 70% B, 45–46 min gradient to 95% B, 46–50 min 95% B, 50–55 min gradient  
72 back to 80% A, 55–60 min 80% A. For purification of compound 7, preparative HPLC were performed  
73 on a Varian Prepstar system equipped with a UV detector (Prostar, Varian). A Microsorb C18 60–8  
74 column (Varian Dynamax 250 mm  $\times$  21.4 mm) was used as the stationary phase. The same binary  
75 gradient system as for the analytical RP-HPLC was applied using an appropriate gradient from low  
76 to high percentage of B with a slope of 1% per min.

77 Melting points were determined on a Galen III Boetius apparatus from Cambridge Instruments.  
78 NMR spectra were recorded on a Varian Unity 400 MHz or an Agilent DD2 600 MHz spectrometer  
79 equipped with ProbeOne probe. Chemical shifts of the <sup>1</sup>H and <sup>13</sup>C spectra were reported in parts per  
80 million (ppm) referenced to the (residual) solvent resonances relative to tetramethylsilane. UPLC-MS  
81 was performed on a Waters ACQUITY UPLC I-Class system including an ACQUITY UPLC PDA e $\lambda$

82 setector coupled to a Xevo TQ-S mass spectrometer High-resolution mass spectra were obtained on  
83 an Agilent UHD Accurate-Mass Q-TOF LC MS G6538A mass spectrometer operated in combination  
84 with coupled with the Agilent 1260 Infinity II HPLC system. Elemental microanalysis was performed  
85 on a Euro EA 3000 Elemental Analyzer (Euro Vector Instruments & Software).  
86

### 87 2.2. Benzyl (S)-(2,6-dioxotetrahydro-2H-pyran-3-yl)carbamate (1, N-Carbobenzoxylglutamic anhydride)

88 Z-Glu-OH (2.5 g, 8.89 mmol) was suspended in acetic anhydride (20 mL) and the temperature was  
89 slowly raised to 55 °C (oil bath temperature), whereupon a clear solution was formed. From the time of  
90 complete dissolution, stirring was continued for additional 10 min. The solution was concentrated  
91 under high vacuum (rotary evaporator equipped with hybrid vacuum pump) to obtain a nearly  
92 colourless oil. Ether (30 mL) was added to the crude product and ethyl acetate (5-10 mL) was added  
93 until a homogeneous solution was obtained upon heating. The solution was transferred to an  
94 Erlenmeyer flask and cyclohexane (15-20 mL) was added until the solution became turbid. The mixture  
95 was kept at -20 °C over night, whereupon an oil separated. Crystallisation was initiated by scratching  
96 with a glass rod. More cyclohexane (10 mL) was added and the mixture was kept again at -20 °C over  
97 night. The crystalline material was collected by vacuum filtration and dried *in vacuo* to obtain **1** (2.1 g,  
98 0.80 mmol, 90%) as a white powder; Mp 82-86 °C (lit 86-88 °C [18]); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>), δ  
99 (ppm): 1.94 (qd, <sup>2</sup>J=13.1 Hz, <sup>3</sup>J=5.6 Hz, 1H, C<sub>β</sub>-HH), 2.45-2.59 (m, 1H, C<sub>β</sub>-HH), 2.90 (ddd, <sup>2</sup>J=18.9 Hz,  
100 <sup>3</sup>J=13.0 Hz, <sup>3</sup>J=6.2 Hz, 1H, C<sub>γ</sub>-HH), 3.06 (ddd, <sup>2</sup>J=18.7 Hz, <sup>3</sup>J=5.6 Hz, <sup>3</sup>J=2.2 Hz, 1H, C<sub>γ</sub>-HH), 4.38-4.52 (m,  
101 1H, C<sub>α</sub>-H), 5.15 (s, 2H, CH<sub>2</sub>O), 5.57 (br s, 1H, NH), 7.30-7.42 (m, 5H, Ph-H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>),  
102 δ (ppm): 23.72 (C<sub>β</sub>), 29.83 (C<sub>γ</sub>), 51.48 (C<sub>α</sub>), 67.77 (CH<sub>2</sub>O), 128.39, 128.64, 128.80, 135.80, (C<sub>arom</sub>), 155.81,  
103 OCONH), 164.53, 166.65 (2×C=O anhydride). NMR data are in agreement to those reported in [17].  
104 Elemental analysis C<sub>13</sub>H<sub>13</sub>NO<sub>5</sub>, calcd. C: 59.31%, H: 4.98%, N: 5.32%, found: C: 58.33%, H: 5.15%, N:  
105 5.16%.

### 106 2.3. (S)-4-(((Benzyloxy)carbonyl)amino)-5-((2-(tert-butoxy)-2-oxoethyl)amino)-5-oxopentanoic acid (2, Z- 107 Glu-Gly-OtBu)

108 To a solution of compound **1** (0.5 g, 1.90 mmol) in chloroform (20 mL) was added glycine *tert*-butyl  
109 ester hydrochloride (H-Gly-OtBu×HCl; 0.32 g, 1.90 mmol) as a solid followed by triethyl amine (0.19 g,  
110 0.26 mL, 1.90 mmol). The resulting solution was stirred for 30 min at room temperature. The solvent  
111 was removed *in vacuo* and the obtained crude product mixture was adsorbed on silica gel and loaded  
112 onto a silica column. Product **2** was obtained by elution with ether/ethyl acetate/acetic acid 1:1:0.01. As  
113 the UV absorption of both products is rather low, visualisation of compound spots during monitoring  
114 of the chromatographic purification by TLC analysis of distinct fractions was done by using Hanessian's  
115 stain [19]. The product-containing fraction were combined and brought to dryness *in vacuo*. The  
116 obtained oily residue was crystallised by dissolving in a minimum amount of ethyl acetate and  
117 subsequent addition of a larger volume of cyclohexane. The oil which separated from the solvent  
118 mixture solidified at -20 °C. The solid was collected by vacuum filtration to obtain 373 mg (50 %) of  
119 compound **2**. R<sub>f</sub> 0.35 (*n*-hexane/ethyl acetate/acetic acid 1:4:0.01); Mp 96-103 °C (lit 106- 107 °C [20]); <sup>1</sup>H  
120 NMR (400 MHz, CDCl<sub>3</sub>), δ (ppm): 1.45 (s, 9H, (CH<sub>3</sub>)<sub>3</sub>C), 1.88 – 2.01 (m, 1H, C<sub>β</sub>-HH), 2.09 – 2.20 (m, 1H,  
121 C<sub>β</sub>-HH), 2.41 – 2.59 (m, 2H, C<sub>γ</sub>-H<sub>2</sub>), 3.83 – 4.00 (m, 2H, <sup>Gly</sup>C<sub>α</sub>-H<sub>2</sub>), 4.41 – 4.49 (m, 1H, <sup>Glu</sup>C<sub>α</sub>-H), 5.05-5.17 (m,  
122 2H, CH<sub>2</sub>O), 5.88 (d, <sup>3</sup>J=8.6 Hz, 1H, Glu-NH), 7.10-7.16 (br s, 1H, Gly-NH), 7.27-7.39 (m, 5H, Ph-H); <sup>13</sup>C  
123 NMR (100 MHz, CDCl<sub>3</sub>), δ (ppm): 28.16 (3×CH<sub>3</sub>), 28.28 (C<sub>β</sub>), 29.91 (C<sub>γ</sub>) 42.25 (<sup>Gly</sup>C<sub>α</sub>), 53.85 (<sup>Glu</sup>C<sub>α</sub>), 67.44  
124 (CH<sub>2</sub>O), 82.75 ((CH<sub>3</sub>)<sub>3</sub>C), 128.20, 128.37, 128.68, 136.14 (4×C<sub>arom</sub>), 156.70 (OCONH), 169.06, 171.93,  
125 176.71 (3×C=O). NMR data are in agreement to those reported in [17]. Elemental analysis C<sub>19</sub>H<sub>26</sub>N<sub>2</sub>O<sub>7</sub>:  
126 calcd. C: 57.86%, H: 6.64%, N: 7.10%, found: C: 58.97%, H: 6.79%, N: 6.94%.

### 127 2.4. N<sup>2</sup>-((Benzyloxy)carbonyl)-N<sup>5</sup>-(2-(tert-butoxy)-2-oxoethyl)-L-glutamine (2a)

128 Compound **2a** was obtained by further elution of the chromatographic column described above  
129 with ether/ethyl acetate/acetic acid 1:4:0.01. The product-containing fractions were combined and

130 brought to dryness *in vacuo*. The obtained oily residue was washed with cyclohexane under  
131 ultrasonification to remove residual acetic acid, which was followed by washing with *n*-pentane. The  
132 oil was dried under very low pressure (< 0.1 mbar) to obtain 223 mg (30%) of compound **2a** as a white  
133 foam.  $R_f$  0.17 (*n*-hexane/ethyl acetate/acetic acid 1:4:0.01);  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  (ppm): 1.45 (s,  
134 9H,  $(\text{CH}_3)_3\text{C}$ ), 1.99 – 2.09 (m, 1H,  $\text{C}_\beta\text{-HH}$ ), 2.15 – 2.27 (m,  $\text{C}_\beta\text{-HH}$ , 1H), 2.30 – 2.49 (m, 2H,  $\text{C}_\gamma\text{-H}_2$ ), 3.84 –  
135 3.96 (m, 2H,  $^{\text{Gly}}\text{C}_\alpha\text{-H}_2$ ), 4.32 – 4.43 (m, 1H,  $^{\text{Glu}}\text{C}_\alpha\text{-H}$ ), 5.08 (s, 2H,  $\text{CH}_2\text{O}$ ), 6.02 (d,  $^3J=7.5$  Hz, 1H, Glu-NH),  
136 6.77 (t,  $^3J=4.9$  Hz, 1H, Gly-NH), 7.27 – 7.35 (m, 5H, Ph-H),  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  (ppm): 28.12  
137 ( $3\times\text{CH}_3$ ), 28.50 ( $\text{C}_\beta$ ), 32.13 ( $\text{C}_\gamma$ ), 42.35 ( $^{\text{Gly}}\text{C}_\alpha$ ), 53.48 ( $^{\text{Glu}}\text{C}_\alpha$ ), 67.24 ( $\text{CH}_2\text{O}$ ), 82.91 ( $(\text{CH}_3)_3\text{C}$ ), 128.19, 128.31,  
138 128.65, 136.28 ( $4\times\text{C}_{\text{arom}}$ ), 156.61 (OCONH), 169.39, 173.61, 174.34 ( $3\times\text{C}=\text{O}$ ). Elemental analysis  
139  $\text{C}_{19}\text{H}_{26}\text{N}_2\text{O}_7$ : calcd. C: 57.86%, H: 6.64%, N: 7.10%, found: C: 57.36%, H: 6.74%, N: 6.58%.

140 2.5. 4-Methyl-2-oxo-2H-chromen-7-yl (S)-4-(((benzyloxy)carbonyl)amino)-5-((2-(tert-butoxy)-2-  
141 oxoethyl)amino)-5-oxopentanoate (**3**, Z-Glu(HMC)-Gly-OtBu)

142 Compound **2** (0.373 g, 0.95 mmol) and HATU (0.361 g, 0.95 mmol) were dissolved in DMF (3 mL)  
143 under a  $\text{N}_2$  atmosphere. DIPEA (282  $\mu\text{L}$ , 1.88 mmol) and a solution of 4-methylumbelliferone (0.168 g,  
144 0.95 mmol) in DMF (1 mL) were added to this mixture. The resulting yellow solution was stirred for 2  
145 h at room temperature. Subsequently, the solution was diluted with  $\text{CH}_2\text{Cl}_2$  (15 mL), transferred to a  
146 separatory funnel and washed with 1 M HCl (1 $\times$ 3 mL), sat.  $\text{NaHCO}_3$  (2 $\times$ 3 mL),  $\text{H}_2\text{O}$  (1 $\times$ 3 mL) and brine  
147 (1 $\times$ 1 mL). The organic layer was dried over  $\text{Na}_2\text{SO}_4$  and evaporated *in vacuo* (the remaining DMF was  
148 removed using a rotary evaporator equipped with hybrid pump for very low pressure) to obtain a  
149 yellow viscous oil. The crude product was purified by column chromatography on silica gel using a  
150 mixture of *n*-hexane and ethyl acetate of increasing polarity (ratio starting from 6:4 over 1:1 to 4:6; the  
151 final ratio was adjusted 20 fractions after unconverted 4-methylumbelliferone eluted). The product-  
152 containing fractions were collected and evaporated *in vacuo* to obtain 243 mg of compound **3** as an off-  
153 white solid. As the product still contained 4-methylumbelliferone as trace impurity, the material was  
154 recrystallised by adding ethyl acetate (3.2 mL) to a refluxing suspension of the product in cyclohexane  
155 (4 mL). Upon keeping the solution at 4  $^\circ\text{C}$  overnight, a precipitate was formed, which was collected by  
156 vacuum filtration, washed with cyclohexane and *n*-hexane and dried *in vacuo* to obtain compound **3**  
157 (134 mg, 26%) as a white granulous solid.  $R_f$  0.16 (*n*-hexane/ethyl acetate 1:1); Mp 61-63  $^\circ\text{C}$ ;  $^1\text{H}$  NMR  
158 (400 MHz,  $\text{CDCl}_3$ )  $\delta$  (ppm): 1.47 (s, 9H,  $(\text{CH}_3)_3\text{C}$ ), 2.02 – 2.14 (m, 1H,  $\text{C}_\beta\text{-HH}$ ), 2.26-2.38 (m, 1H,  $\text{C}_\beta\text{-HH}$ ),  
159 2.43 (d,  $^4J=1.3$  Hz, 3H,  $\text{CH}_3\text{-coumarin}$ ), 2.67-2.90 (m, 2H,  $\text{C}_\gamma\text{-H}_2$ ), 3.90 (dd,  $^2J=18.2$  Hz,  $^3J=5.1$  Hz, 1H,  $\text{C}_\alpha\text{-}$   
160  $\text{HH}$  of Gly), 3.98 (dd,  $^2J=18.3$  Hz,  $^3J=5.4$  Hz, 1H,  $\text{C}_\alpha\text{-HH}$  of Gly), 4.37 – 4.44 (m, 1H,  $\text{C}_\alpha\text{-H}$  of Glu) 5.11 (d,  
161  $^2J=12.3$  Hz, 1H,  $\text{CHHO}$ ), 5.15 (d,  $^2J=12.9$  Hz, 1H,  $\text{CHHO}$ ), 5.58 (d,  $^3J=8.0$  Hz, 1H, Glu-NH), 6.27 (q,  $^4J=1.3$   
162 Hz, 1H, H-3 of coumarin), 6.51 (br s, 1H, Gly-NH) 7.07 – 7.11 (m, 1H, H-6 of coumarin), 7.13 (d,  $^4J=1.8$   
163 Hz, 1H, H-8 of coumarin), 7.28 – 7.38 (m, 5H, Ph-H), 7.57 (d,  $^3J=8.6$  Hz, 1H, H-5 of coumarin).  $^{13}\text{C}$  NMR  
164 (100 MHz,  $\text{CDCl}_3$ )  $\delta$  (ppm): 18.88 ( $\text{CH}_3$  of coumarin), 28.20 ( $3\times\text{CH}_3$ ), 28.30 ( $\text{C}_\beta$ ), 30.52 ( $\text{C}_\gamma$ ), 42.18 ( $\text{C}_\alpha$  of  
165 Gly), 53.92 ( $\text{C}_\alpha$  of Glu), 67.43 ( $\text{CH}_2\text{O}$ ), 82.75 ( $(\text{CH}_3)_3\text{C}$ ), 110.65 (C8 of coumarin), 114.76 (C3 of  
166 coumarin), 118.09, 118.27 (C4a and C6 of coumarin), 125.53 (C5 of coumarin), 128.30, 128.42, 128.73  
167 ( $3\times\text{CH}$  of phenyl), 136.18 (C1 of phenyl), 151.99, 153.10, 154.34 (C4, C7 and C8a of coumarin), 156.37  
168 (OCONH), 160.61 (C2 of coumarin), 168.62, 171.00, 171.54 ( $3\times\text{C}=\text{O}$ ). HR-MS (ESI $^+$ ):  $[\text{M}+\text{NH}_4]^+$   
169 calculated: 570.2451, found: 570.2445 (90%),  $[\text{2M}+\text{NH}_4]^+$  calculated: 1122.4559, found: 1122.4565  
170 (100%). Elemental analysis  $\text{C}_{29}\text{H}_{32}\text{N}_2\text{O}_9$ : calcd. C: 63.04%, H: 5.84%, N: 5.07%, found: C: 62.48%, H:  
171 5.82%, N: 4.73%.

172

173 2.6. (S)-2-(((Benzyloxy)carbonyl)amino)-5-((4-methyl-2-oxo-2H-chromen-7-yl)oxy)-5-oxopentanoylglycine  
174 (**4**, Z-Glu(HMC)-Gly-OH)

175 To a solution of compound **3** (130 mg, 0.24 mmol) in  $\text{CH}_2\text{Cl}_2$  (0.5 mL) was added slowly  
176 trifluoroacetic acid (4 mL) under ice cooling. After stirring for 2 h under ice cooling the solvents were  
177 removed under a  $\text{N}_2$  stream under ambient pressure. The oily residue was taken up into  $\text{CH}_2\text{Cl}_2$  (20  
178 mL) and washed with 0.1 M HCl (5 mL). As addition of HCl resulted in the formation of an emulsion,  
179 brine (3 mL) was added, whereupon a white precipitate was formed, which was filtered off and

180 redissolved in ethyl acetate (15 mL). The aqueous layer was separated from the combined biphasic  
181 mixture and the organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated *in vacuo* to obtain 112 mg of a  
182 white solid. Since an impurity was detectable in the <sup>1</sup>H NMR spectrum, the crude product was  
183 recrystallised. To this end, the solid was dissolved in a mixture of ethyl acetate (4 mL) and acetonitrile  
184 (2 mL) under heating. Cyclohexane (6 mL) was added and the resulting solution was kept at 4 °C for 30  
185 min, whereupon a gelatinous precipitate formed. The material was collected by vacuum filtration,  
186 washed with cyclohexane and *n*-pentane and dried *in vacuo* to obtain compound **4** (77 mg, 65%) as a  
187 white solid. R<sub>f</sub> 0.01 (*n*-hexane/ethyl acetate 2:8); Mp 167-171 °C; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ (ppm):  
188 1.88 – 1.98 (m, 1H, C<sub>β</sub>-HH), 2.02 – 2.14 (m, 1H, C<sub>β</sub>-HH), 2.44 (d, <sup>4</sup>J=1.3 Hz, 3H, CH<sub>3</sub>-coumarin), 2.68 – 2.75  
189 (m, 2H, C<sub>γ</sub>-H<sub>2</sub>), 3.73 (dd, <sup>2</sup>J=17.5, <sup>3</sup>J=5.6 Hz, 1H, C<sub>α</sub>-HH of Gly), 3.83 (dd, <sup>2</sup>J=17.5, <sup>3</sup>J=6.0 Hz, 1H, C<sub>α</sub>-HH of  
190 Gly), 4.15-4.21 (m, 1H, C<sub>α</sub>-H of Glu), 5.02 (d, <sup>2</sup>J=12.6 Hz, 1H, CHHO), 5.07 (d, <sup>2</sup>J=12.6 Hz, 1H, CHHO),  
191 6.39 (d, <sup>4</sup>J=1.3 Hz, 1H, H-3 of coumarin), 7.18 (dd, <sup>3</sup>J=8.7, 2.3 Hz, 1H, H-6 of coumarin), 7.26 (d, <sup>4</sup>J=2.3 Hz,  
192 1H, H-8 of coumarin), 7.28 – 7.39 (m, 5H, Ph-H), 7.54 (d, <sup>3</sup>J=8.3 Hz, 1H, Glu-NH), 7.81 (d, <sup>3</sup>J=8.7 Hz, 1H,  
193 H-5 of coumarin), 8.29 (t, <sup>3</sup>J=5.8 Hz, 1H, Gly-NH). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ (ppm): 18.11 (CH<sub>3</sub> of  
194 coumarin), 26.98 (C<sub>β</sub>), 30.00 (C<sub>γ</sub>), 40.66 (C<sub>α</sub> of Gly), 53.49 (C<sub>α</sub> of Glu), 65.51 (CH<sub>2</sub>O), 110.04 (C8 of  
195 coumarin), 113.69 (C3 of coumarin), 117.47 (C4a of coumarin), 118.34 (C6 of coumarin), 126.32 (C5 of  
196 coumarin), 127.66, 127.74, 128.27 (3×CH of phenyl), 136.88 (C1 of phenyl), 152.82, 152.90, 153.48 (C4,  
197 C7 and C8a of coumarin), 155.92 (OCONH), 159.55 (C2 of coumarin), 170.82, 171.03, 171.51 (3×C=O).  
198 NMR data are in agreement to those reported in [14]. HR-MS (ESI+): m/z calculated for [M+NH<sub>4</sub>]<sup>+</sup>:  
199 514.1825, found: 514.1821 (100%), [2M+NH<sub>4</sub>]<sup>+</sup>: calculated: 1010.3307, found: 1010.3313 (57.2%),  
200 [3M+NH<sub>4</sub>]<sup>+</sup>: calculated: 1506.4789, found: 1506.4799 (80.52%). Elemental analysis: C<sub>25</sub>H<sub>24</sub>N<sub>2</sub>O<sub>9</sub>·H<sub>2</sub>O  
201 calcd. C: 58.36%, H: 5.09%, N: 5.45%, found: C: 58.36%, H: 4.92%, N: 5.23%. Chromatograms for  
202 UPLC-MS analysis of Z-Glu(HMC)-Gly-OH (**4**) are shown in Figure S2 (Supporting Information).  
203

204 2.7. *tert*-Butyl (2-(4-((5-(dimethylamino)naphthalen-1-yl)sulfonyl)piperazin-1-yl)-2-oxoethyl)carbamate (**6a**,  
205 *N*-Boc-glycine-4-dansylpiperazide)

206 To a solution of Boc-glycine (98.8 mg, 0.56 mmol) and DIPEA (196.5 μL, 0.12 mmol) in DMF  
207 (5 mL) was added PyBOP (293 mg, 0.56 mmol). The solution was stirred for 5 min at room  
208 temperature and, subsequently, *N*-monodansylpiperazine (90 mg, 0.28 mmol) was added. After  
209 stirring for 3 h, the solvent of the reaction mixture was removed *in vacuo* and the obtained residue  
210 was dissolved in ethyl acetate (15 mL). The organic phase was washed with sat. NaHCO<sub>3</sub> (2×10 mL)  
211 and brine (1×10 mL), dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The obtained crude product was  
212 subjected to column chromatography (silica; eluent: petroleum ether/ethyl acetate (25 → 50%)) to  
213 obtain **6a** (133 mg, 100%) as a light-green solid; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ=1.41 (s, 9H, 3×CH<sub>3</sub> of  
214 Boc), 2.90 (s, 6H, 2×CH<sub>3</sub> of dansyl), 3.23–3.15 (m, 4H, 2×CH<sub>2</sub> of piperazine), 3.45–3.39 (m, 2H, CH<sub>2</sub> of  
215 piperazine), 3.69–3.62 (m, 2H, CH<sub>2</sub> of piperazine), 3.86 (d, <sup>3</sup>J=4.5 Hz, 2H, CH<sub>2</sub> Gly), 5.36 (br s, NH),  
216 7.21 (d, <sup>3</sup>J=7.5 Hz, 1H, H<sub>Dansyl</sub>), 7.55 (dd, <sup>3</sup>J=8.6, 7.6 Hz, 2H, 2×H<sub>Dansyl</sub>), 8.20 (dd, <sup>3</sup>J=7.3 Hz, <sup>4</sup>J=1.2 Hz, 1H,  
217 H<sub>Dansyl</sub>), 8.37 (d, <sup>3</sup>J=8.7 Hz, 1H, H<sub>Dansyl</sub>), 8.59 (d, <sup>3</sup>J=8.5 Hz, 1H, H<sub>Dansyl</sub>); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  
218 δ=28.45 (3×CH<sub>3</sub> Boc), 41.68 (CH<sub>2</sub> of piperazine), 42.28 (CH<sub>2</sub> of Gly), 44.24 (CH<sub>2</sub> of piperazine), 45.48  
219 (CH<sub>2</sub> of piperazine), 45.63 (CH<sub>2</sub> of piperazine), 45.65 (2×CH<sub>3</sub> dansyl), 80.03 (C<sub>quartär</sub> of Boc), 115.67,  
220 119.65, 123.45, 128.45, 130.16 (C<sub>quart</sub> of dansyl), 130.44 (C<sub>quart</sub> of dansyl), 131.03, 131.16, 132.30 (C<sub>quart</sub> of  
221 dansyl), 151.68 (C<sub>quart</sub> of dansyl), 155.87 (CO of Boc), 167.07; MS (ESI+) 477.3 ([M+H]<sup>+</sup>). <sup>1</sup>H and <sup>13</sup>C  
222 NMR spectra in agreement to published data [21].  
223

224 2.8. 2-Amino-1-(4-((5-(dimethylamino)naphthalen-1-yl)sulfonyl)piperazin-1-yl)ethan-1-one (**6b**, Glycine-4-  
225 dansylpiperazide)

226 Compound **6a** (167 mg, 0.35 mmol) was dissolved in a mixture of TFA and CH<sub>2</sub>Cl<sub>2</sub> (10 mL; 1:1,  
227 v/v) and stirred for 2 h at room temperature. The volatiles were removed at ambient pressure in a  
228 nitrogen stream. The residue was dissolved in sat. NaHCO<sub>3</sub> (20 mL) and extracted with CH<sub>2</sub>Cl<sub>2</sub>  
229 (5×5 mL). The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub> and subsequently evaporated to dryness to

230 obtain **6b** (131 mg, 100%) as a light-green solid;  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$ =2.83 (s, 6H,  $2\times\text{CH}_3$   
231 of dansyl), 3.12–3.05 (m, 4H,  $2\times\text{CH}_2$  of piperazine), 3.53–3.20 (m, 6H,  $2\times\text{CH}_2$  of piperazine,  $\text{CH}_2$  Gly),  
232 7.27 (d,  $^3J$ =7.5 Hz, 1H,  $\text{H}_{\text{Dansyl}}$ ), 7.70–7.59 (m, 2H,  $2\times\text{H}_{\text{Dansyl}}$ ), 8.13 (dd,  $^3J$ =7.4 Hz,  $^4J$ =1.2 Hz, 1H,  $\text{H}_{\text{Dansyl}}$ ),  
233 8.30 (d,  $^3J$ =8.7 Hz, 1H,  $\text{H}_{\text{Dansyl}}$ ), 8.53 (d,  $^3J$ =8.5 Hz, 1H,  $\text{H}_{\text{Dansyl}}$ ), signal for  $\text{NH}_2$  not detectable;  $^{13}\text{C}$  NMR  
234 (100 MHz, DMSO- $d_6$ ):  $\delta$ =40.89 ( $\text{CH}_2$  of piperazine), 42.53 ( $\text{CH}_2$  Gly), 43.39 ( $\text{CH}_2$  of piperazine), 45.04  
235 ( $2\times\text{CH}_3$  of dansyl), 45.37 ( $2\times\text{CH}_2$  of piperazine), 115.33, 118.86, 123.70, 129.20 ( $\text{C}_{\text{quart}}$  of dansyl), 129.62  
236 ( $\text{C}_{\text{quart}}$  of dansyl), 130.06, 130.39, 132.48 ( $\text{C}_{\text{quart}}$  of dansyl), 151.42 ( $\text{C}_{\text{quart}}$  of dansyl), 171.55 (CO); MS  
237 (ESI+) 377.2 ( $[\text{M}+\text{H}]^+$ )  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra are in agreement to published data [21].  
238

239 2.9. *N*-(2-(4-((5-(Dimethylamino)naphthalen-1-yl)sulfonyl)piperazin-1-yl)-2-oxoethyl)acrylamide (7, *N*-  
240 Acryloylglucine-4-dansylpiperazide)

241 To a solution of compound **6b** (112 mg, 0.30 mmol) and TEA (82.5  $\mu\text{L}$ , 0.60 mmol) in  $\text{CH}_2\text{Cl}_2$   
242 (10 mL) was added *N*-acryloxysuccinimide (50.3 mg, 0.30 mmol) as solid. After 1 h, the same amount  
243 of *N*-acryloxysuccinimide was added again and the mixture was stirred for additional 1 h at room  
244 temperature. The solution was washed with sat.  $\text{NaHCO}_3$  ( $2\times 10$  mL), brine ( $1\times 10$  mL); dried over  
245  $\text{Na}_2\text{SO}_4$  and evaporated to dryness. The crude product was purified by preparative RP-HPLC to  
246 obtain **7** (10.9 mg, 7%) as a white solid;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$ =3.01 (s, 6H,  $2\times\text{CH}_3$  of dansyl),  
247 3.27–3.18 (m, 4H,  $2\times\text{CH}_2$  of piperazine), 3.52–3.45 (m, 2H,  $\text{CH}_2$  of piperazine), 3.72–3.66 (m, 2H,  $\text{CH}_2$   
248 of piperazine), 4.08 (d,  $^3J$ =4.2 Hz, 2H,  $\text{CH}_2$  Gly), 5.68 (dd,  $^3J$ =10.2 Hz,  $^2J$ =1.4 Hz, 1H,  $\text{CH}=\text{CHH}$ ), 8.62 (d,  
249  $^3J$ =8.6 Hz, 1H,  $\text{H}_{\text{Dansyl}}$ ), 6.15 (dd,  $^3J$ =17.0, 10.2 Hz, 1H,  $\text{CH}=\text{CH}_2$ ), 6.28 (dd,  $^3J$ =17.0 Hz,  $^2J$ =1.4 Hz, 1H,  
250  $\text{CH}=\text{CHH}$ ), 6.74 (bs, 1H,  $\text{N}_a\text{H}$  of Gly), 7.31 (d,  $^3J$ =7.6 Hz, 1H,  $\text{H}_{\text{Dansyl}}$ ), 7.64–7.56 (m, 2H,  $2\times\text{H}_{\text{Dansyl}}$ ), 8.24  
251 (dd,  $^3J$ =7.4 Hz,  $^4J$ =1.1 Hz, 1H,  $\text{H}_{\text{Dansyl}}$ ), 8.48 (d,  $^3J$ =8.7 Hz, 1H,  $\text{H}_{\text{Dansyl}}$ ), 8.62 (d,  $^3J$ =8.6 Hz, 1H,  $\text{H}_{\text{Dansyl}}$ );  $^{13}\text{C}$   
252 NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$ =41.38 ( $\text{CH}_2$  of Gly), 41.86 ( $\text{CH}_2$  of piperazine), 44.36 ( $\text{CH}_2$  of piperazine),  
253 45.41 ( $\text{CH}_2$  of piperazine), 45.59 ( $\text{CH}_2$  of piperazine), 45.84 ( $2\times\text{CH}_3$  of dansyl), 116.22, 121.07, 124.15,  
254 127.71 ( $\text{CH}=\text{CH}_2$ ), 128.30, 129.57 ( $\text{C}_{\text{quart}}$  of dansyl), 130.01 ( $\text{CH}=\text{CH}_2$ ), 130.43 ( $\text{C}_{\text{quart}}$  of dansyl), 130.57,  
255 131.26, 132.59 ( $\text{C}_{\text{quart}}$  of dansyl), 149.58 ( $\text{C}_{\text{quart}}$  of dansyl), 165.92, 166.56.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra in  
256 agreement to published data [21].

257 2.10. General assay procedure and analysis

258 All measurements were conducted at 30 °C over 900 s (interval of 8 or 10 s) using a Cytation 5  
259 Microplate Reader (BioTek Instruments) and black 96-well microplates with  $\mu\text{CLEAR}^{\text{®}}$  bottom  
260 (greiner bio-one). Fluorescence was detected in bottom read mode and predefined settings for  
261 excitation ( $360\pm 20$  nm) and emission ( $450\pm 20$  nm) of 7-HMC were chosen. Measurements at  $\text{pH}=8.0$   
262 and  $\text{pH}=6.5$  were conducted with a sensitivity of 45 and 50, respectively. The assay mixture (200  $\mu\text{L}$ )  
263 contained 190  $\mu\text{L}$  aqueous solution and 10  $\mu\text{L}$  DMSO (5% v/v). The following three buffer systems  
264 were used: assay buffer I (100 mM MES, 3 mM  $\text{CaCl}_2$ , 50  $\mu\text{M}$  EDTA, adjusted to  $\text{pH}$  6.5 with 1 M  
265 NaOH), assay buffer II (100 mM MOPS, 3 mM  $\text{CaCl}_2$ , 50  $\mu\text{M}$  EDTA, adjusted to  $\text{pH}$  8.0 with 1 M  
266 NaOH) and enzyme buffer (100 mM MOPS, 3 mM  $\text{CaCl}_2$ , 10 mM TCEP, 20% (v/v) glycerol). The  
267 buffers were stored at 0 °C for periods of up to 2 weeks and freshly prepared after that period. The  
268 concentration of the hTGase 2 stock solution was 1 mg/mL. All regression analyses were  
269 accomplished using GraphPad Prism (version 8.2.1, August 20, 2019). To provide values of mean and  
270 SEM, the respective regression analyses were separately accomplished for each experiment and the  
271 obtained fit values were collected and statistically analysed. hTGase 2 (T022) and inhibitors Z006 and  
272 Z013 were purchased from Zedira (Darmstadt, Germany).

273 Detailed descriptions of the assay procedures and the kinetic analyses for the characterisation of  
274 Z-Glu(HMC)-Gly-OH (**4**) towards enzymatic hydrolysis at  $\text{pH}$  6.5 and 8.0 as well as for the kinetic  
275 characterisation of amines and irreversible inhibitors are given in recent publications of our group  
276 [14, 15]. In brief, the recorded time courses of type  $(\text{RFU}-\text{RFU}_0)=f(t)$  for the enzymatic and  
277 spontaneous conversion were analysed by nonlinear (one-phase association) and linear regressions  
278 to the experimental data. Subsequently, initial rates  $v_{0\text{total}}$  (in the presence of enzyme) and  $v_{0\text{control}}$   
279 (spontaneous reaction in the absence of enzyme) were derived. Both data sets were globally analysed

280 by applying the model of total and nonspecific binding (implemented in GraphPad Prism) to obtain  
281 the kinetic parameters for the enzymatic reaction. According to that model, the following rule was  
282 defined:

$$283 \quad v_{0\text{total}}(\text{enzymatic} + \text{spontaneous}) = v_{0\text{corr}}(\text{enzymatic}) + v_{0\text{control}}(\text{spontaneous}) \quad (1)$$

284 where  $v_{0\text{corr}}$  represents the rates for the enzymatic conversions (without spontaneous reaction). The  
285 spontaneous and enzymatic conversions are mathematically defined within this model by the  
286 following equations:

$$287 \quad v_{0\text{control}} = k_{\text{obs}} * [S] \quad (2)$$

$$288 \quad v_{0\text{corr}} = \frac{v_{\text{max}} * [S]}{K_{\text{m}} + [S]} \quad (3)$$

289 Accordingly,  $v_{0\text{total}}$  is defined by the sum function of equations (2) and (3). Due to negligible  
290 spontaneous reaction of **4** at pH 6.5, the above mentioned rule simplifies as follows:

$$291 \quad v_{0\text{total}} = v_{0\text{corr}} \quad (4)$$

292 However, concerning the kinetic analyses the previously published procedures were slightly  
293 adjusted, as detailed below.

294 Concerning the enzymatic hydrolysis of Z-Glu(HMC)-Gly-OH (**4**) at pH 6.5, the recorded time  
295 courses of type  $(\text{RFU}-\text{RFU}_0)=f(t)$  were analysed by either nonlinear (one-phase association) or linear  
296 regression over the entire measurement period of 900 s (instead of only the first 300 s) to the  
297 experimental data depending on the shape of the curve.

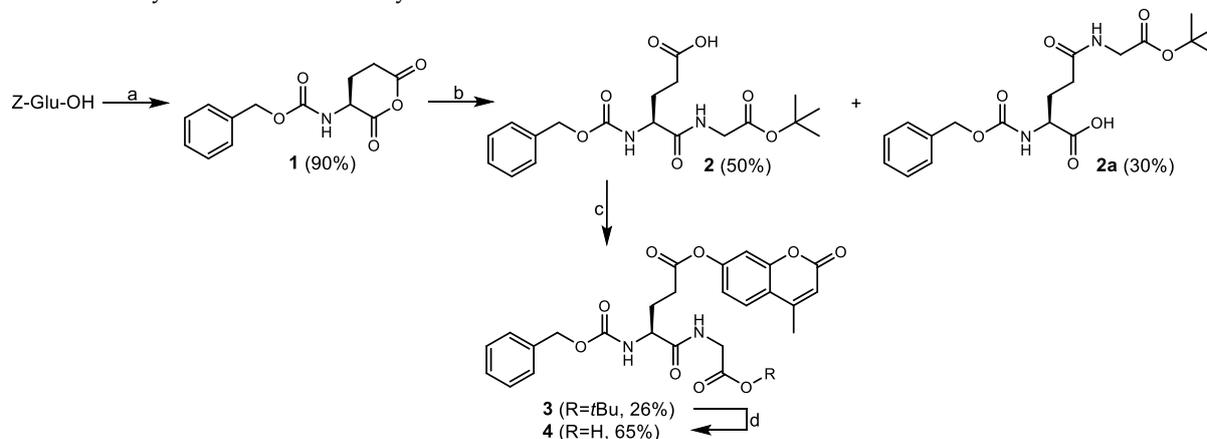
298 Concerning the kinetic characterisation of the biogenic amines, the two sets of initial rates  
299 ( $v_{0\text{total}}=f([\text{amine}])$  and  $v_{0\text{control}}=f([\text{amine}])$ ) were also globally analysed using the model of total and  
300 nonspecific binding (instead of simple subtraction of  $v_{0\text{control}}$  from  $v_{0\text{total}}$  values) as conducted for the  
301 analysis of the enzymatic hydrolyses).

302

### 303 **3. Results and Discussion**

304 The four-step synthesis started from commercially available Z-Glu-OH and the first two steps  
305 followed mainly the procedures published by Leblanc *et al.* [17] (Scheme 1). Treatment of Z-Glu-OH  
306 with acetic anhydride, which acts both as solvent and condensation agent, at elevated temperature  
307 led to the corresponding cyclic anhydride **1**. The oily crude product obtained by concentration of the  
308 reaction mixture could be crystallised by treatment with ether, ethyl acetate and cyclohexane (see  
309 Material and Methods section), which resulted in an almost quantitative yield of 90%. From a  
310 historical point of view, it is worth to mention that glutamic anhydride **1** has been used as building  
311 block for the synthesis of glutamate-containing peptides when peptide chemistry was still in its  
312 infancy [22]. Compound **1** was subjected to ring opening with glycine *tert*-butyl ester, which resulted  
313 in quantitative consumption of the anhydride within 30 min under the formation of the regioisomeric  
314  $\alpha$ - and  $\gamma$ -dipeptides **2** and **2a**. The ratio of both isomers was determined to be 7:3 in the favour to the  
315 desired product **2** by HPLC analysis (see Figure S1, Supporting Information). As reported by Leblanc  
316 *et al.*, both products were conveniently separated by chromatography on silica gel. By-product **2a** was  
317 characterised and could – in its  $N^{\alpha}$ -Fmoc-protected version – be a useful building block for solid-  
318 phase peptide synthesis as it represents an extended glutamate analogue. Dipeptide Z-Glu-Gly-O*t*Bu  
319 (**2**) was esterified with the fluorophore 4-methylumbelliferone by applying conditions that have been  
320 elaborated by Twibanire and Grindley for the efficient acylation of alcohols of varying reactivity  
321 using HATU as activating agent [23], which provided compound **3**. Column chromatographic  
322 purification of the crude product yielded the product, which contained an unknown impurity  
323 according to the  $^1\text{H}$  NMR spectrum beside trace amounts of unreacted 4-methylumbelliferone. As  
324 these impurities would be more difficult to remove after the final step considering the higher polarity

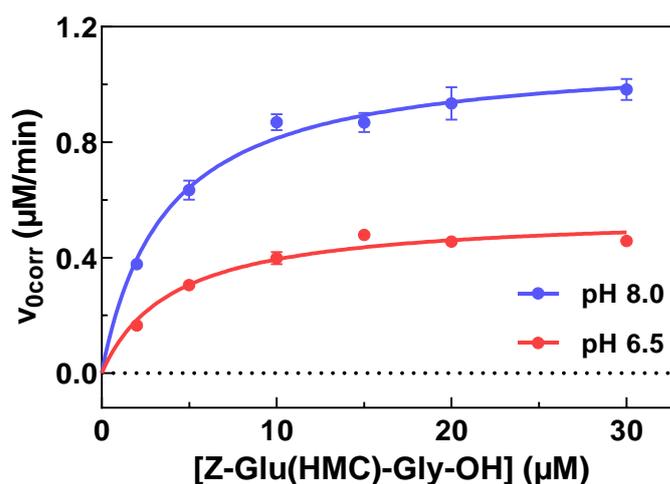
325 of the free carboxylic acid, the obtained material was recrystallised to furnish highly pure compound  
 326 **3**. In the final step, *tert*-butyl ester **3** was cleaved by treatment with trifluoroacetic acid. During work-  
 327 up, excessive TFA was efficiently removed by acidic aqueous washing to obtain the crude product in  
 328 crystalline state. This material was recrystallised from ethyl acetate/acetonitrile/cyclohexane to obtain  
 329 compound **4** as monohydrate, according to results of elementary microanalysis. Its purity has been  
 330 confirmed by RP-HPLC/MS analysis.



331  
 332 **Scheme 1:** Four-step synthesis of Z-Glu(HMC)-Gly-OH (**4**) starting from Z-Glu-OH. Reagents and conditions: a)  
 333 acetic anhydride, 55 °C, 10 min; b) H-Gly-OtBu·HCl, triethylamine, CHCl<sub>3</sub>, room temperature; c) HATU, DIPEA,  
 334 4-methylumbelliferone, DMF, room temperature; d) TFA, CH<sub>2</sub>Cl<sub>2</sub>, room temperature.

335

336 Compound **4** was investigated regarding the kinetics of hTGase 2-catalysed hydrolysis both at  
 337 pH 6.5 and pH 8.0. The results are shown in Figure 1 and Table 1 (see also Figure S19 in Supporting  
 338 Information). The obtained catalytic and Michaelis constants are in good agreement with the recently  
 339 published values [14]. The fact that the determined  $K_m$  values are slightly lower than those recently  
 340 published can be attributed to the better-defined composition of the crystalline compound material  
 341 obtained herein whereas previously obtained substrate preparations furnished amorphous and  
 342 slightly hygroscopic material after lyophilisation.



343

344 **Figure 1:** hTGase 2-catalysed hydrolysis of Z-Glu(HMC)-Gly-OH (**4**) at pH=8.0 and pH 6.5. Plots of  $v_{0corr}=f([4])$   
 345 for pH 8.0 and pH 6.5 with the nonlinear regressions (—) using Equation (3) (Michaelis-Menten equation,  
 346 Materials and Methods section). Data shown are mean values  $\pm$ SEM of 3 separate experiments, each performed  
 347 in duplicate. When not apparent, error bars are smaller than the symbols. Conditions: pH=8.0 or 6.5, 30 °C, 5%  
 348 DMSO, 500  $\mu$ M TCEP, 3  $\mu$ g/ml of hTGase 2.

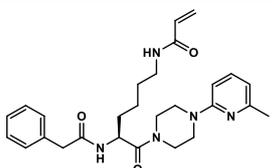
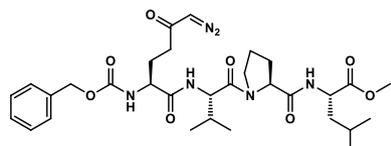
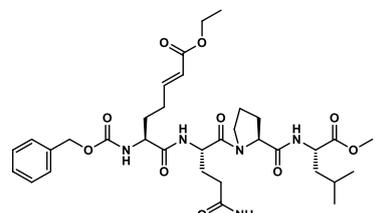
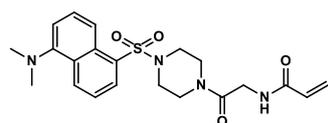
349 **Table 1:** Kinetic parameters for the hTGase 2-catalysed hydrolyses of Z-Glu(HMC)-Gly-OH (**4**) at pH 6.5 and 8.0

pH value	$K_m$ ( $\mu\text{M}$ )	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$k_{\text{cat}}/K_m$ ( $\text{M}^{-1}\text{s}^{-1}$ )
6.5	4.05 (0.23)	0.30 (0.01)	74,100
8.0	3.93 (0.48)	0.61 (0.03)	155,000

350 Rate constant for spontaneous hydrolysis:  $k_{\text{obs}}$  (TCEP, pH 8.0) =  $16.8 (0.5) \times 10^{-3} \text{ min}^{-1}$ 351 For details on calculation of the kinetic parameters see Materials and Methods Section. Data shown are mean  
352 values ( $\pm$ SEM) of three separate experiments, each performed in duplicate. Active concentrations of hTGase 2  
353 ( $E_T=30.8 \text{ nM}$ ) were determined by active site titration as recently described [24].354 Following the hTGase 2-catalysed hydrolysis at pH 6.5, the utility of substrate **4** for the kinetic  
355 characterisation of irreversible inhibitors was demonstrated/validated by investigating three  
356 inhibitors which were reported in the literature. The selected compounds comprised the  
357 commercially available peptidic inhibitors Z006 and Z013, which bear a diazomethyl ketone or an  
358  $\alpha,\beta$ -unsaturated carboxylic ester as electrophilic warhead, respectively. Furthermore, the nonpeptidic  
359 acrylamide 1-155 (compound **7**), which was recently published by Badarau et al. [21, 25], was  
360 synthesised according to Scheme 2 in orientation to the described procedure. In contrast to the former  
361 two compounds, a second-order inactivation constant  $k_{\text{inact}}/K_i$  has been reported for the latter. The  
362 results of the inhibitor characterisation are included in Table 2.

363

364 **Table 2:** Second-order inactivation constants ( $k_{\text{inact}}/K_i$ ) and  $K_i$  values of different literature-known irreversible  
365 inhibitors of hTGase 2 at pH 6.5 compared to their reported inhibitory parameters

compound	structure	$k_{\text{inact}}/K_i$ ( $\text{M}^{-1}\text{s}^{-1}$ ) <sup>a*</sup>	$K_i$ ( $\mu\text{M}$ ) <sup>a*</sup>	$\text{IC}_{50}$ (nM)
<b>5</b>		4,880 (20) <sup>[15]</sup>	5.73 (0.77) <sup>[15]</sup>	310 <sup>[15]b</sup> 14 (8.2) <sup>[26]c</sup>
Z006		191,000 (3,000)	0.096 (0.01)	20 <sup>[27]c</sup>
Z013		51,500 (5,800)	0.45 (0.02)	200 <sup>[28]d</sup>
1-155 ( <b>7</b> )		12,700 (700) 4,962 <sup>[21]f</sup>	1.43 (0.01)	6.1 (0.4) <sup>[21]e</sup>

<sup>a</sup>Data shown are mean values ( $\pm$ SEM) of two separate experiments, each performed in duplicate.

<sup>b</sup>TGase 2-catalysed incorporation of R-I-Cad in DMC (transamidation) at pH 8.0 (5 min preincubation period of enzyme and inhibitor). <sup>c</sup>TGase 2-catalysed incorporation of Boc-K-NH(CH<sub>2</sub>)<sub>2</sub>NH-Dns in DMC (transamidation) at pH 7.4 (30 min preincubation period of enzyme and inhibitor). <sup>d</sup>Isopeptidase-assay using the substrate Abz-NE(CAD-DNP)EQVSPLTLK-OH (A101, Zedira). <sup>e</sup>TGase 2-catalysed incorporation of *N*-(biotinyl)cadaverine in immobilised DMC (transamidation) at pH 8.5 (30 min preincubation period of enzyme and inhibitor). <sup>f</sup>GDH-coupled assay (deamidation) at pH 7.2.



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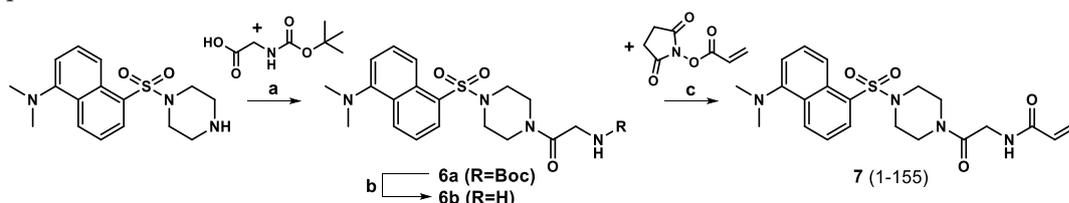
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Schaertl *et al.* have determined an IC<sub>50</sub> value of 20 nM for Z006 together with various other compounds by employing a fluorescence-based transamidase-detecting assay using *N,N*-dimethylcasein as acyl donor and *N*-monodansylcadaverin as acyl acceptor substrate, respectively [27]. In a later study, *N*<sup>ε</sup>-acryloyllysine-based irreversible inhibitor **5** has been synthesised, which was investigated in the same assay [26]. The IC<sub>50</sub> value of **5** was 14 nM under these conditions, which suggests that it should be equipotent to Z006. However, the *k*<sub>inact</sub>/*K*<sub>I</sub> value of Z006 determined herein (191 000 M<sup>-1</sup>s<sup>-1</sup>) is 37-fold greater than that of **5**. Even though the applied assay methods detect different TGase 2-catalysed reactions (transamidation in Schaertl *et al.* vs. hydrolysis herein) and the pH values are different (6.5 vs. 7.4), the potencies of Z006 and **5** should not be influenced to such extent. In agreement with the value of *k*<sub>inact</sub>/*K*<sub>I</sub> determined for Z006, Khosla's group reported inactivation constants of 48 000 M<sup>-1</sup>s<sup>-1</sup> [29] and 139 000 M<sup>-1</sup>s<sup>-1</sup> [30] for structurally related peptidic diazomethyl ketones, which have been determined using the GDH-coupled assay. The higher reactivity of Z006 towards TGase 2 in comparison to **5** is reasonable, as diazomethyl ketones are inherently more reactive towards thiols than acrylamides. Moreover, as the peptidic structures of Z006 and Z013 are based on gliadin peptides, which are natural substrates of TGase 2, they can probably be better accommodated by the active site, which in turn results in stronger non-covalent interactions. This is reflected in the low *K*<sub>I</sub> value of 96 nM for Z006. The similar IC<sub>50</sub> values of Z006 and **5** and the drastically differing inactivation constants can be rationalised when the assay conditions are considered. The IC<sub>50</sub> values were determined using a TGase 2 concentration of 20 nM and a preincubation time of 30 min [27]. Therefore, the IC<sub>50</sub> limit, which corresponds to half of the employed active enzyme concentration ([E]<sub>T</sub>/2) [31, 32], might have been reached for both compounds. In this context, it should be pointed out that the IC<sub>50</sub> value of an irreversible inhibitor in general will always be equal to [E]<sub>T</sub>/2, provided that [inhibitor] ≥ [enzyme]/2 and enough incubation time is given. The other investigated peptidic inhibitor Z013 exhibits a *k*<sub>inact</sub>/*K*<sub>I</sub> of 51 500 M<sup>-1</sup>s<sup>-1</sup>, which is significantly more potent than **5**. As for Z006, the higher inhibitory potency of Z013 can be attributed to the more reactive electrophilic warhead and the peptidic structure, which corresponds to that of Z006, apart from one amino acid side chain. An IC<sub>50</sub> value of 200 nM has been reported for Z013; however, no information on enzyme concentration and preincubation time were provided [28]. Again, the comparison to the kinetic parameter *k*<sub>inact</sub>/*K*<sub>I</sub> indicates that IC<sub>50</sub> values are not suitable for evaluating the potency of irreversible inhibitors, unless identical conditions and carefully adjusted preincubation times are used [33].



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#### Scheme 2: Synthesis of inhibitor 1-155

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Reagents and conditions: **a**) PyBOP, DIPEA, DMF, 3 h; **b**) TFA/CH<sub>2</sub>Cl<sub>2</sub> (1:1, v/v), 2 h; **c**) TEA, CH<sub>2</sub>Cl<sub>2</sub>, 2 h.

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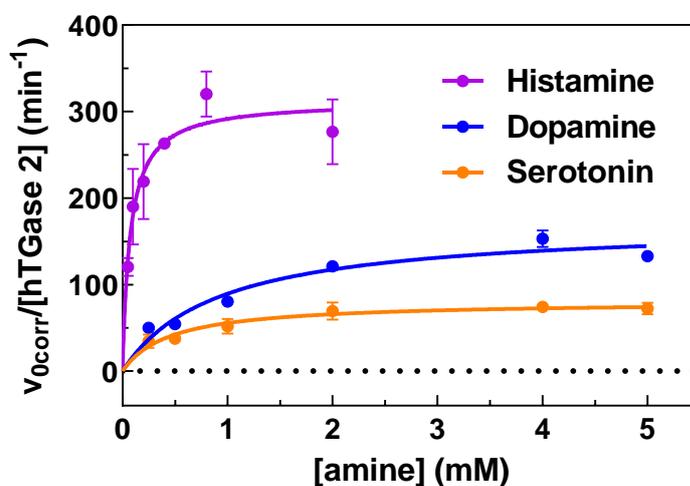
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The synthesis of 1-155 (compound **7**) started with the amide bond coupling between Boc-Gly-OH and *N*-monodansylpiperazine to **6a** using PyBOP and DIPEA, in difference to the published procedure, which used EDC, HOBT and NMM as coupling reagents (Scheme 2) [21]. After TFA-

405 mediated Boc cleavage the acryloyl moiety was introduced by reacting **6b** with *N*-  
406 acryloxysuccinimide. The last step proceeded in low yield and should better be performed with  
407 acryloyl chloride as acylating agent. A  $k_{\text{inact}}/K_{\text{I}}$  of  $12\,700\text{ M}^{-1}\text{s}^{-1}$  was determined for 1-155. This value is  
408 approximately 2.5-fold higher than both that for the *N* $^{\epsilon}$ -acryloyllysine **5** and that of 1-155 determined  
409 by Badarau *et al.* using the GDH-coupled assay. However, taking into account that the two assay  
410 methods work at different pH values (6.5 *vs.* 7.2) the results can be considered comparable. The higher  
411 efficiency of 1-155 compared to **5** as determined in the fluorimetric assay is remarkable. This is  
412 probably the result of well-balanced conformational flexibility/rigidity and targeting of interaction  
413 partners in the enzymes binding site for 1-155. Considering its smaller size (30 *vs.* 35 non-hydrogen  
414 atoms), binding of 1-155 might be entropically more favoured than that of **5**.

415 In addition to the kinetic characterisation of inhibitors, a further asset of the fluorogenic substrate  
416 **4** is the convenient investigation of amine-based acyl acceptor substrates, which are interesting for  
417 the development of TGase 2-directed imaging agents [34]. Moreover, their substrate properties are of  
418 general interest. Therefore, we decided to determine exemplarily the kinetic parameters of histamine,  
419 serotonin and dopamine as common biogenic amines of the primary arylethylamine type. These  
420 compounds are known as classical neurotransmitters and the former two are important mediators in  
421 non-neuronal cells and tissues as well. They have been known for 60 years to be substrates of TGase  
422 2 [35]. However, the physiological significance of TGase-mediated aminylation as an important post-  
423 translational modification has been recognised only in the recent decades [36-39]. In particular, the  
424 TGase 2-catalysed serotonylation of small GTPases such as RhoA and Rab4 in thrombocytes has been  
425 shown to be critical for blood haemostasis. Worth of note, serotonylation of Gln63 in RhoA renders  
426 its GTPase activity permanently active [40]. A similar mechanism has been identified for the  
427 constitutive activation of Rac1 in the central nervous system [41, 42]. In particular, activation of the  
428  $G_{q/11}$ -coupled 5-HT<sub>2A</sub> receptors in rat cortical cells raises the cytosolic  $\text{Ca}^{2+}$  level, which in turn  
429 activates TGase 2 for catalysing the serotonylation of Rac1 [42]. Apart from small GTPases, other  
430 proteins have been identified as substrates for TGase 2-mediated serotonylation [43] and, most  
431 recently, the transamidation of Gln5 of histone H3 to serotonin has been discovered as a transcription-  
432 permissive modification [44]. Similar to serotonylation, TGase 2-mediated histaminylation leads to  
433 the constitutive activation of small and heterotrimeric G proteins [45]. Histamine transfer to  
434 extracellular proteins such as fibrinogen has been suggested as a mechanism for attenuating the pro-  
435 inflammatory effects of this endogenous mediator [46]. Furthermore, fibronectin, another  
436 extracellular TGase 2 substrate, has been shown to undergo monoaminylation, including  
437 dopaminylation [47, 48]. Despite this overwhelming body of evidence for the biological relevance of  
438 TGase 2-catalysed protein acyl transfer to biogenic monoamines, exact kinetic data on their substrate  
439 properties are scarce or have not been determined at all.



441 **Figure 2.** hTGase 2-catalysed incorporation of different biogenic amines into compound Z-Glu(HMC)-Gly-OH  
 442 (4). Plots of  $v_{0\text{corr}}/[\text{hTGase 2}] = f([\text{amine}])$  with nonlinear regressions (—) using Equation (3) (Michaelis-Menten  
 443 equation, Materials and Methods section). Data shown are mean values  $\pm$ SEM of two separate experiments, each  
 444 performed in duplicate. When not apparent, error bars are smaller than the symbols. Conditions: pH=8.0 (100  
 445 mM MOPS was used, which ensures that the pH value is maintained over the entire range of amine  
 446 concentrations), 30 °C, 5% DMSO, 100  $\mu$ M Z-Glu(HMC)-Gly-OH (4), 500  $\mu$ M TCEP, 0.6, 2 and 3  $\mu$ g/mL of  
 447 hTGase 2 for histamine, dopamine and serotonin, respectively.

448 **Table 3.** Kinetic parameters of different biogenic amines as acyl acceptors for hTGase 2 at pH=8.0

biogenic amine	$K_m^{\text{app}}$ (mM)	$k_{\text{cat}}^{\text{app}}$ ( $\text{s}^{-1}$ )	$k_{\text{cat}}/K_m$ ( $\text{M}^{-1}\text{s}^{-1}$ )
histamine	0.08 (0.01)	5.35 (0.58)	66,900
dopamine	0.96 (0.06)	2.90 (0.04)	3,000
serotonin	0.50 (0.17)	1.35 (0.04)	2,700

449 For details on calculation of the kinetic parameters see Experimental section. Data shown are mean values  
 450 ( $\pm$ SEM) of two separate experiments, each performed in duplicate. Active concentrations of hTGase 2  
 451 ( $E_T=6.15$  nM for histamine, 20.5 nM for dopamine and 30.8 nM for serotonin) were determined by active site  
 452 titration as recently described [24].

453 The results on the kinetic characterisation of histamine, serotonin and dopamine are shown in  
 454 Figure 2 and the calculated parameters are included in Table 3 (see also Figure S20 in Supporting  
 455 Information). The substrate concentration range and the employed enzyme concentration were  
 456 adjusted for each substrate in preliminary tests. Notably, no substrate inhibition occurred for any of  
 457 the amine substrates. The formation of the corresponding secondary amide products upon TGase 2-  
 458 catalysed substrate conversion was confirmed by LC-MS/MS analysis (see Figures S21-S26 in  
 459 Supporting Information). In agreement with previously published data using the GDH-coupled  
 460 assay and the gluten-derived heptapeptide Ac-PQPQLPF-NH<sub>2</sub> as acyl donor, histamine is a very  
 461 efficient acyl acceptor substrate for TGase 2 [49]. In contrast, the second-order performance constants  
 462 for both serotonin and dopamine are approximately 20-fold lower. In the GDH-assay mentioned  
 463 before, which employs TGase 2 at a concentration as high as 50  $\mu$ g/mL (640 nM), conversion of  
 464 serotonin was not detectable. This enzyme concentration is approximately 20-fold higher than that  
 465 applied herein for the analysis of serotonin. This demonstrates the value of activated fluorogenic acyl  
 466 donors such as compound 4 as even poor acyl acceptor substrates can be analysed and the  
 467 consumption of valuable enzyme material is minimised. According to its  $k_{\text{cat}}/K_m$ , dopamine is a  
 468 slightly more efficient amine substrate for TGase 2 than serotonin, even though the apparent  
 469 Michaelis constant is lower for serotonin. Considering that all three studied biogenic amines share  
 470 the 2-aminoethyl chain and an (hetero)aromatic core, the distinct substrate properties of histamine  
 471 are remarkable. One could argue that its imidazole ring could assist in the deacylation step of the  
 472 catalytic cycle by acting as general base in the proton transfer from the amine nitrogen, as  
 473 intramolecular hydrogen bonds have been identified in both the neutral and side-chain protonated  
 474 form of histamine [50]. Thus, the imidazole ring of the substrate would fulfil the same function during  
 475 attack of the neutral amino group on the thioester intermediate as the imidazole of active-site His335  
 476 [51]. However, its  $K_m^{\text{app}}$  of 80  $\mu$ M, which is significantly lower than the corresponding values for  
 477 dopamine and serotonin, suggests specific interactions within the enzyme's active site. Furthermore,  
 478 it is surprising that serotonin is the least efficient substrate of the investigated amines even though  
 479 the most findings on physiologically relevant protein monoamination have been obtained for this  
 480 biogenic amine [52]. In this context, it is worth to consider that beside availability of the particular  
 481 monoamine substrate, which can vary from cell type and depends on the subcellular localisation, the

482 sequence in which the Gln substrate residue is embedded should also influence the substrate  
483 specificity towards the amine-based acyl acceptor substrate. This is evidenced by investigations on  
484 the sequence dependence of TGase 2-catalysed modification of Hsp20, which occurs at two of its five  
485 Gln residues, Gln31 and Gln66. Interestingly, concerning the differentiation between the acyl  
486 acceptor the outcome is different for both residues as Gln31 is exclusively transamidated whereas  
487 Gln66 is exclusively deamidated. However, when the isolated undecapeptides of the sequences in  
488 which both residues are embedded are considered, Gln31 is not converted at all whereas Gln66  
489 undergoes both transamidation and deamidation [53]. This indicates that the results on the substrate  
490 properties of the biogenic monoamines obtained with Z-Glu(HMC)-Gly-OH (**4**) as acyl donor  
491 substrate should be extrapolated with care towards large protein substrates. Therefore, we would  
492 like to define the elucidation of the molecular mechanisms that lead to the coupling between acyl  
493 donor and acyl acceptor substrate specificity as a future challenge for TGase research. In this context,  
494 the influence of the acceptor nucleophile on the mechanism of the acylation step in TGase 2 catalysis  
495 has been established very recently on the basis of <sup>14</sup>N/<sup>15</sup>N kinetic isotope effects [54].  
496

#### 497 4. Conclusions

498 A reliable solution phase synthesis of the title substrate compound **4** has been established, which  
499 will support its use in transglutaminase research. Its value for the evaluation of TGase 2 inhibitors  
500 based on meaningful kinetic parameters has been demonstrated. The compound's utility for the  
501 characterisation of amine substrates as exemplified by the biogenic amines histamine, serotonin and  
502 dopamine has been shown, which allows for the first time their comparison on a robust kinetic basis.

503 **Supporting Information:** Supplementary data associated with this article (chromatograms for synthesis, NMR  
504 spectra for compounds 1-4, kinetic data, identification of reaction products for biogenic amines) can be found in  
505 the online version at doi:

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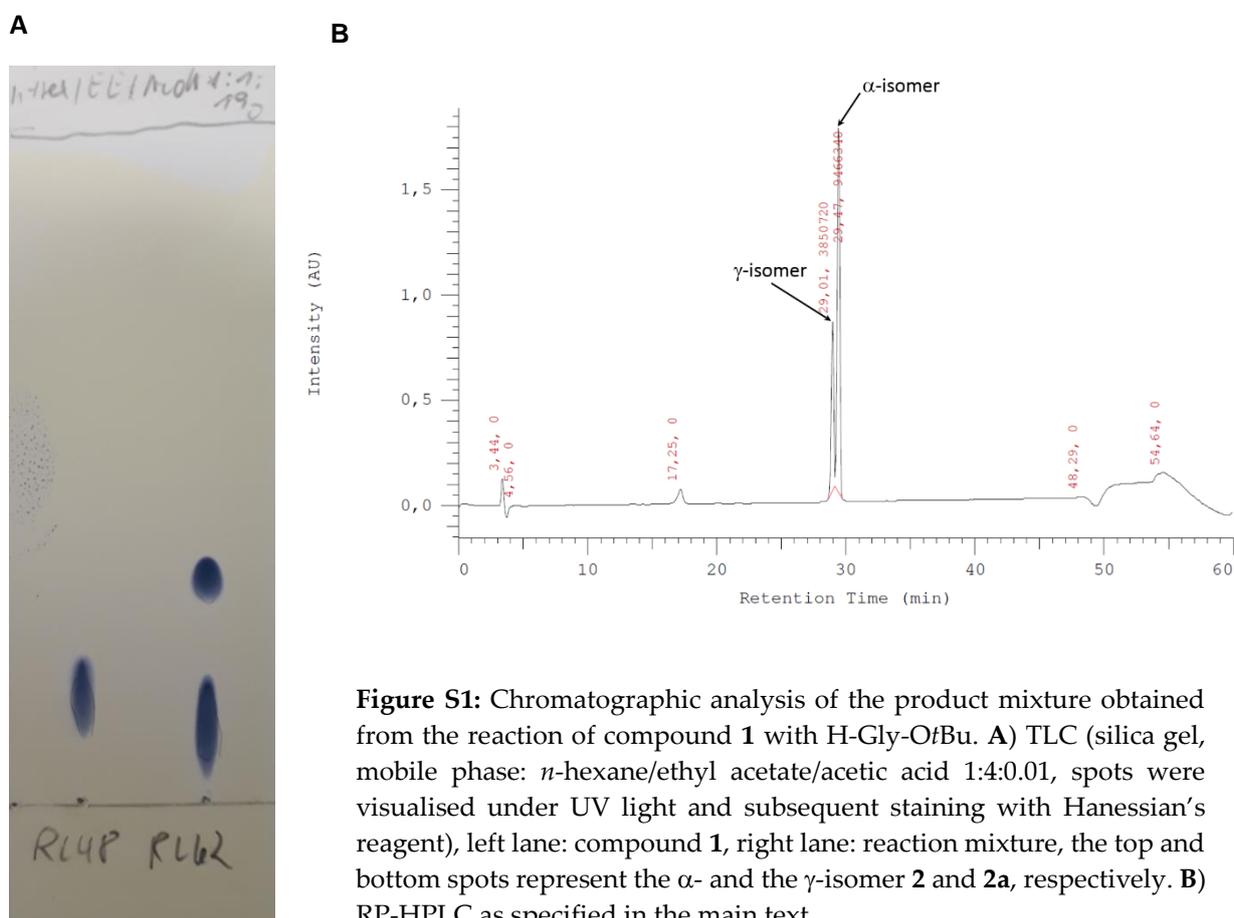
## Supporting Information

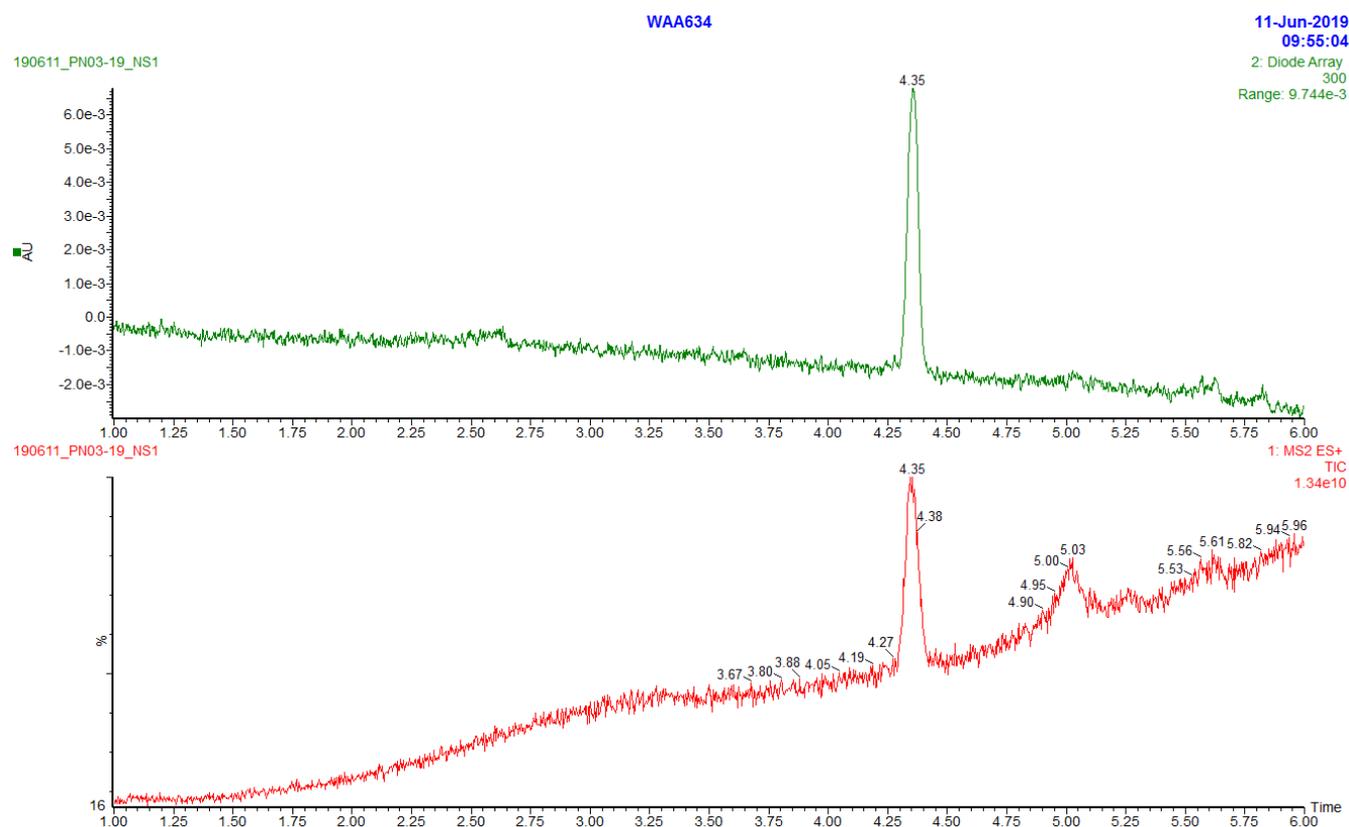
# Solution-Phase Synthesis of the Fluorogenic TGase 2 Acyl Donor Z-Glu(HMC)-Gly-OH and its Use for Inhibitor and Amine Substrate Characterisation

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**Figure S2:** UPLC-MS analysis of Z-Glu(HMC)-Gly-OH (**4**). The upper panel shows the UV trace at 300 nm of the photo diode array (PDA) detector. The lower panel shows the total ion current as detected by the ESI-MS detector. For details of UPLC-MS analysis see Scheme S1.

Figure S3:  $^1\text{H}$  NMR Spectrum of compound 1

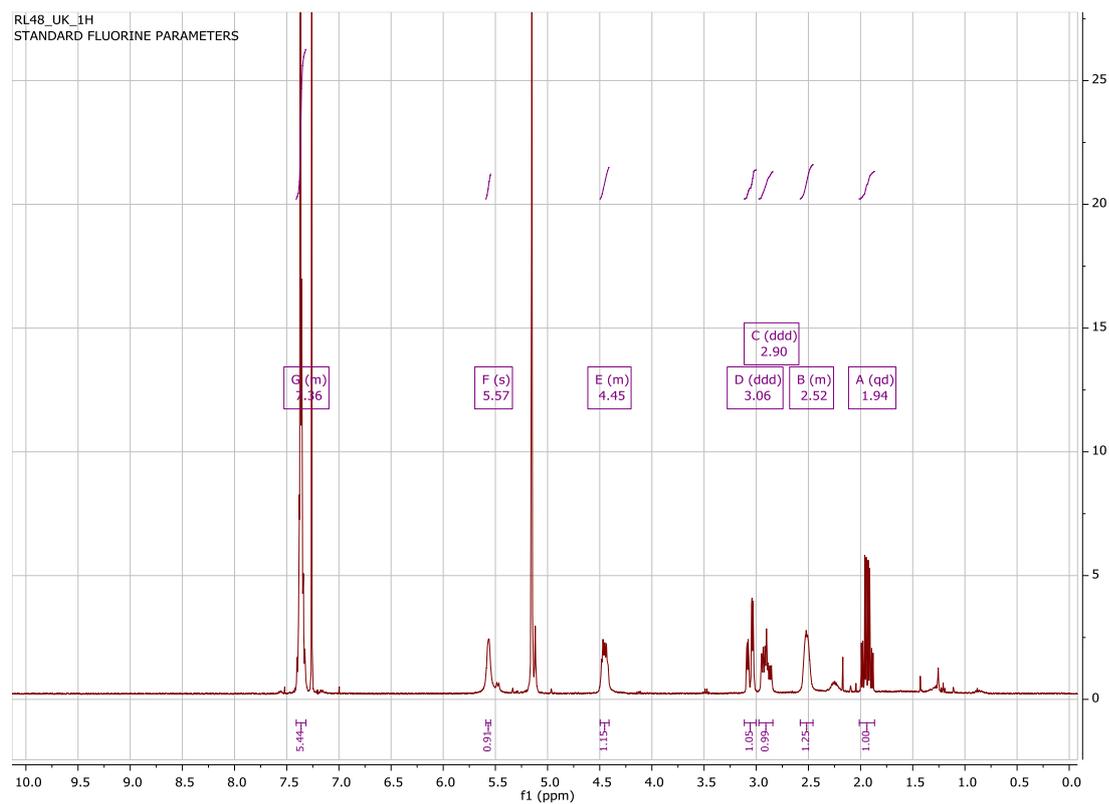


Figure S4:  $^{13}\text{C}$  NMR Spectrum of compound 1

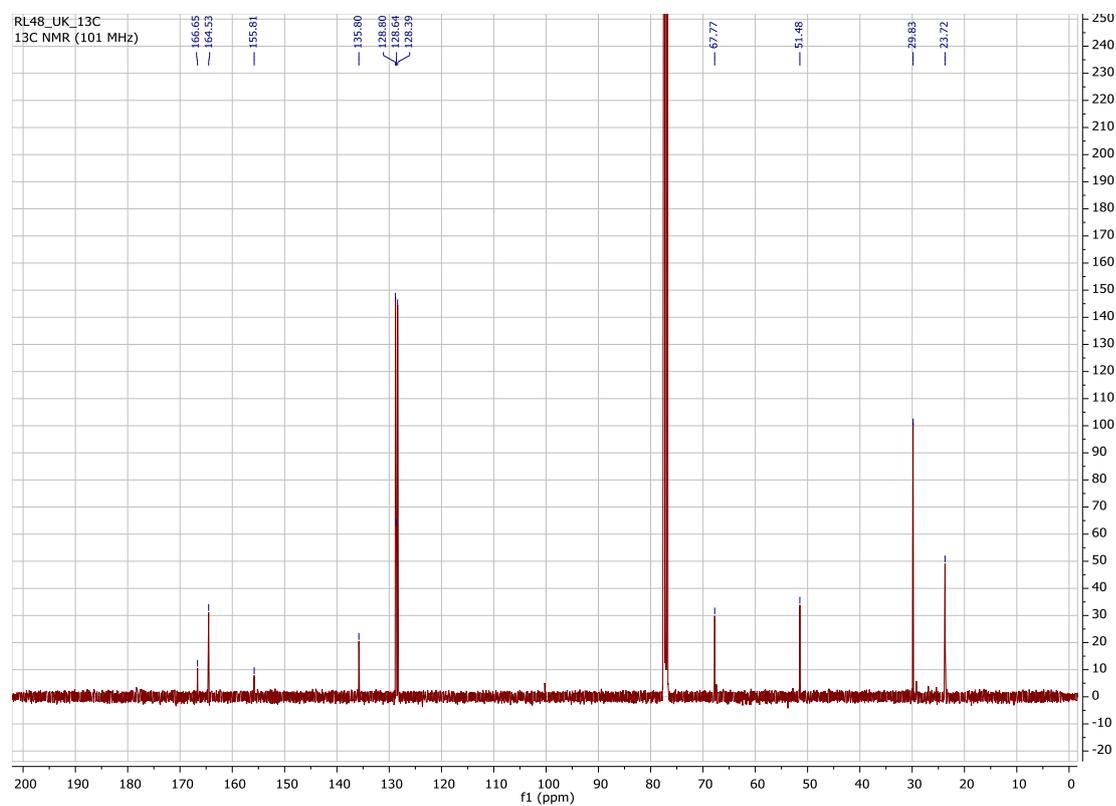


Figure S5:  $^1\text{H}$ ,  $^{13}\text{C}$  HSQC Spectrum of compound 1

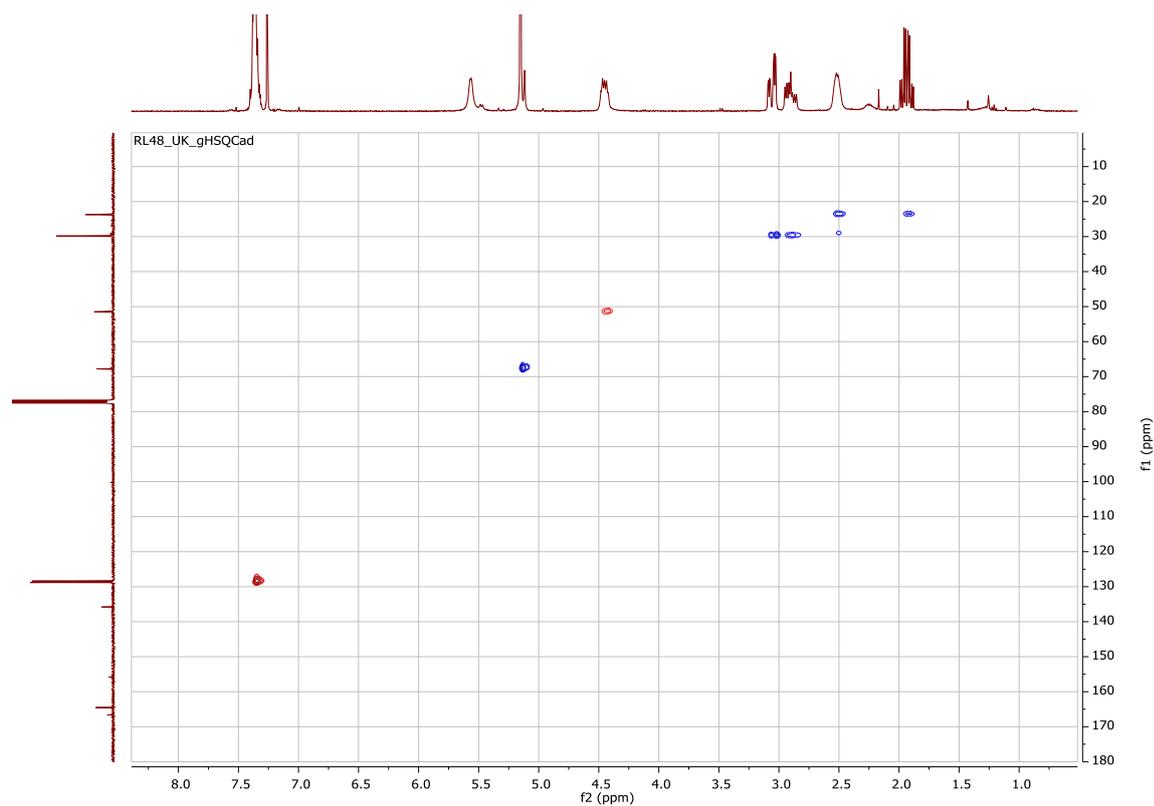


Figure S6:  $^1\text{H}$  NMR Spectrum of compound 2

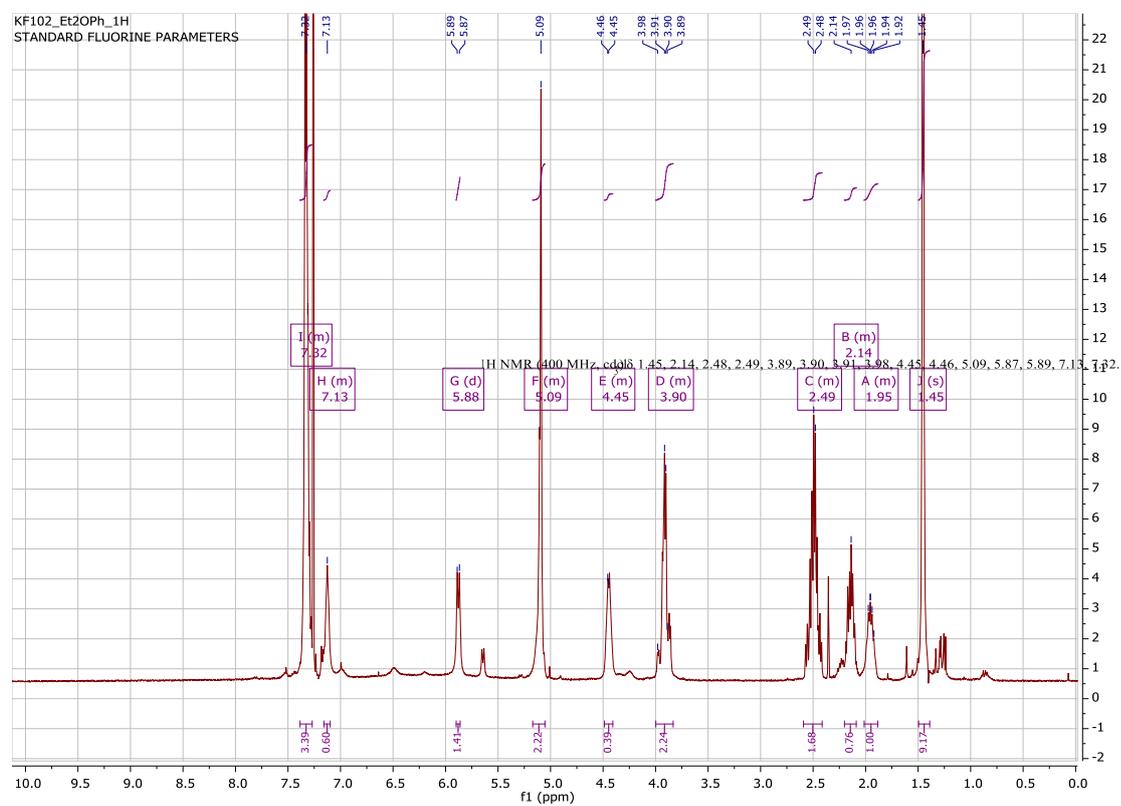


Figure S7:  $^{13}\text{C}$  NMR Spectrum of compound 2

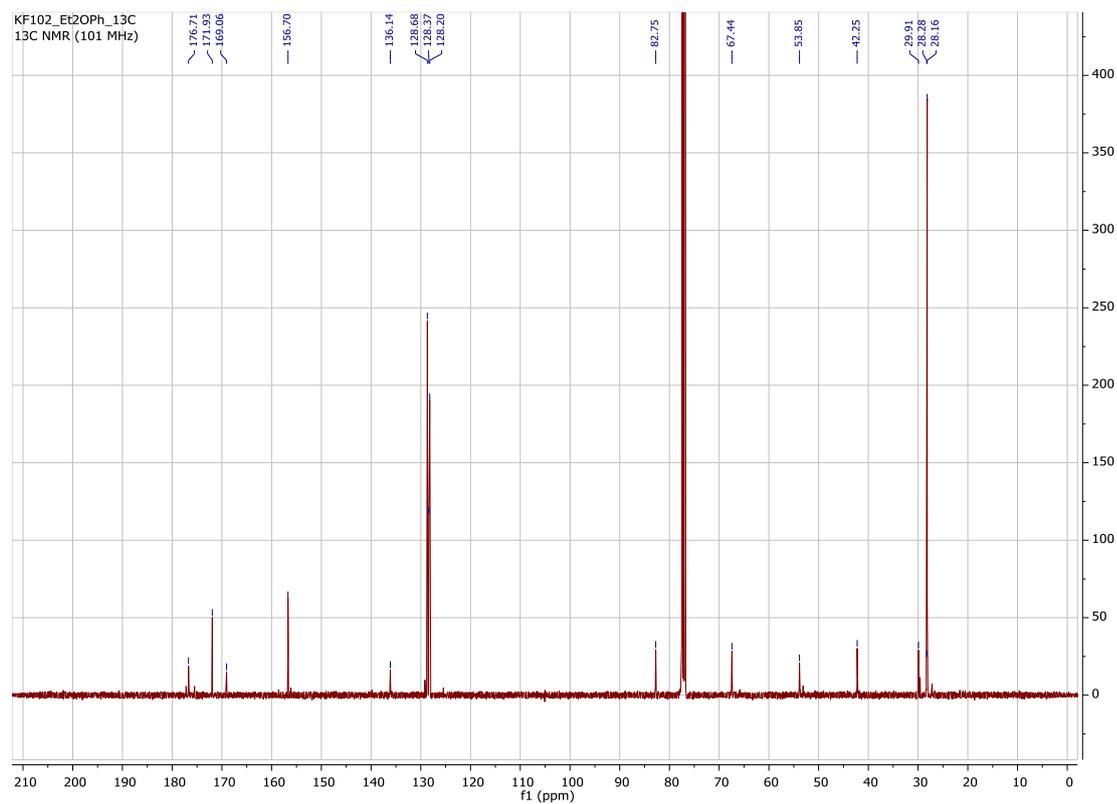


Figure S8:  $^1\text{H}$ ,  $^{13}\text{C}$  HSQC Spectrum of compound 2

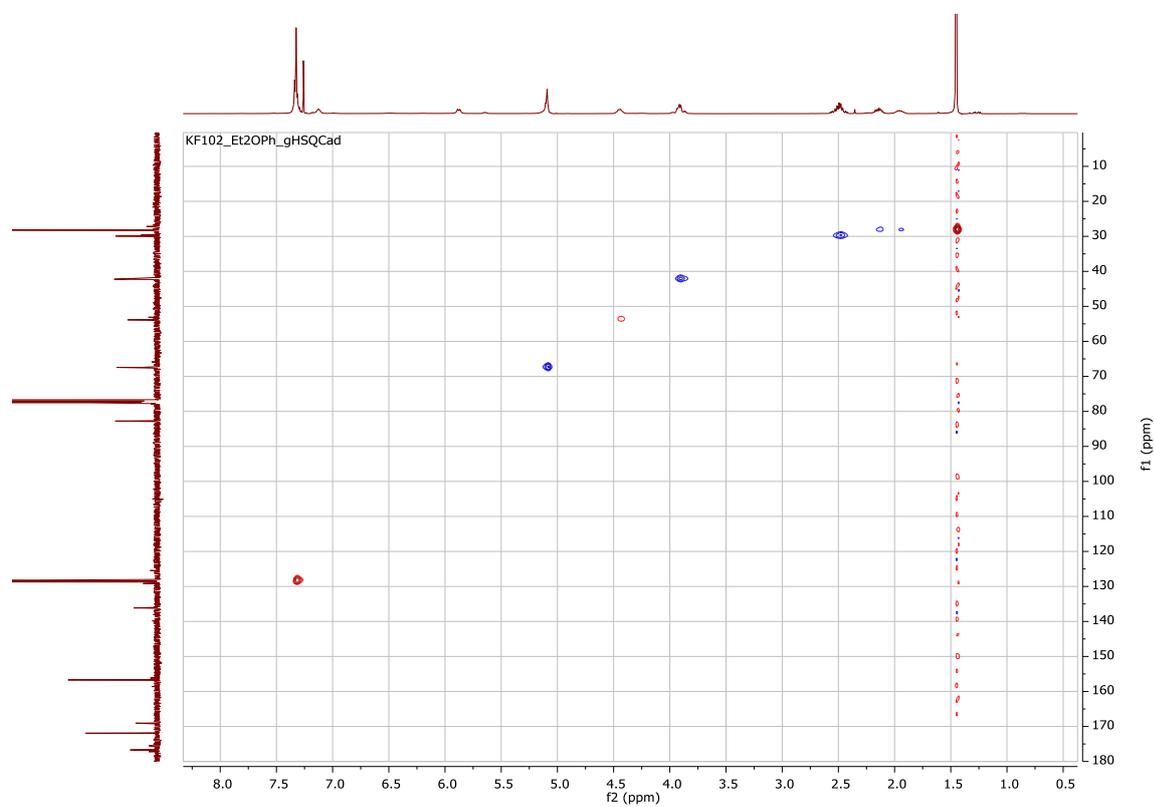


Figure S9:  $^1\text{H}$  NMR Spectrum of compound 2a

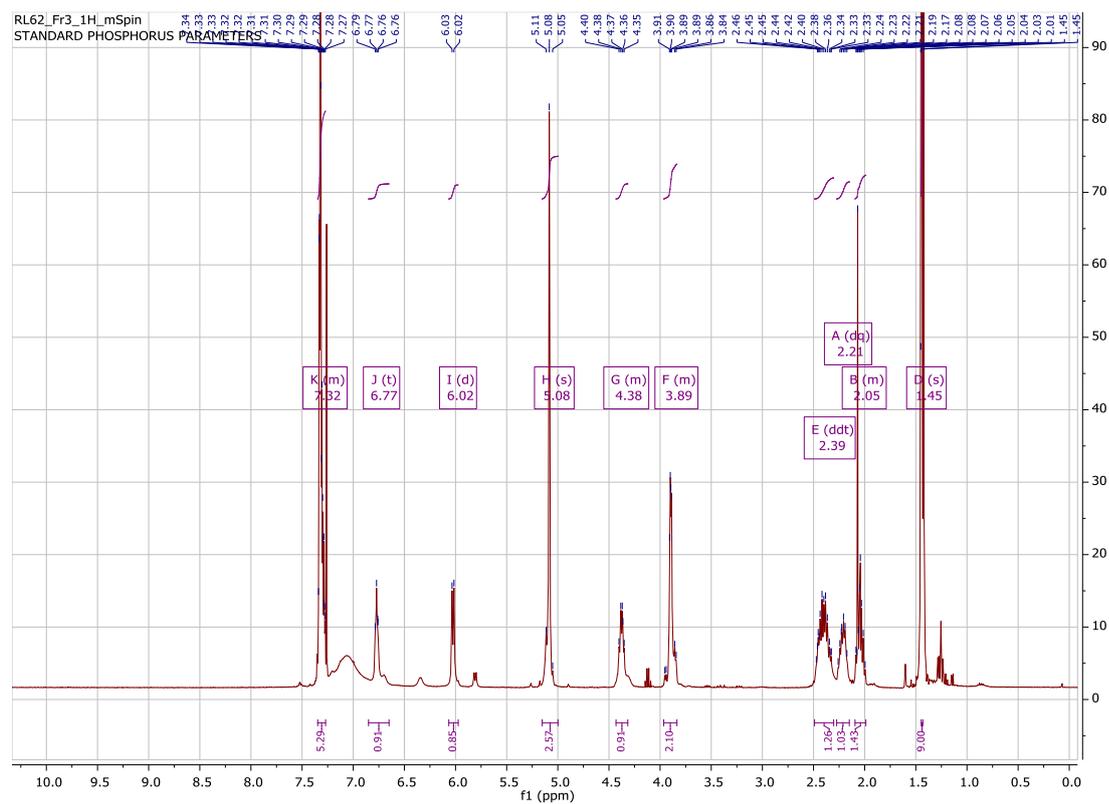


Figure S10:  $^{13}\text{C}$  NMR Spectrum of compound 2a

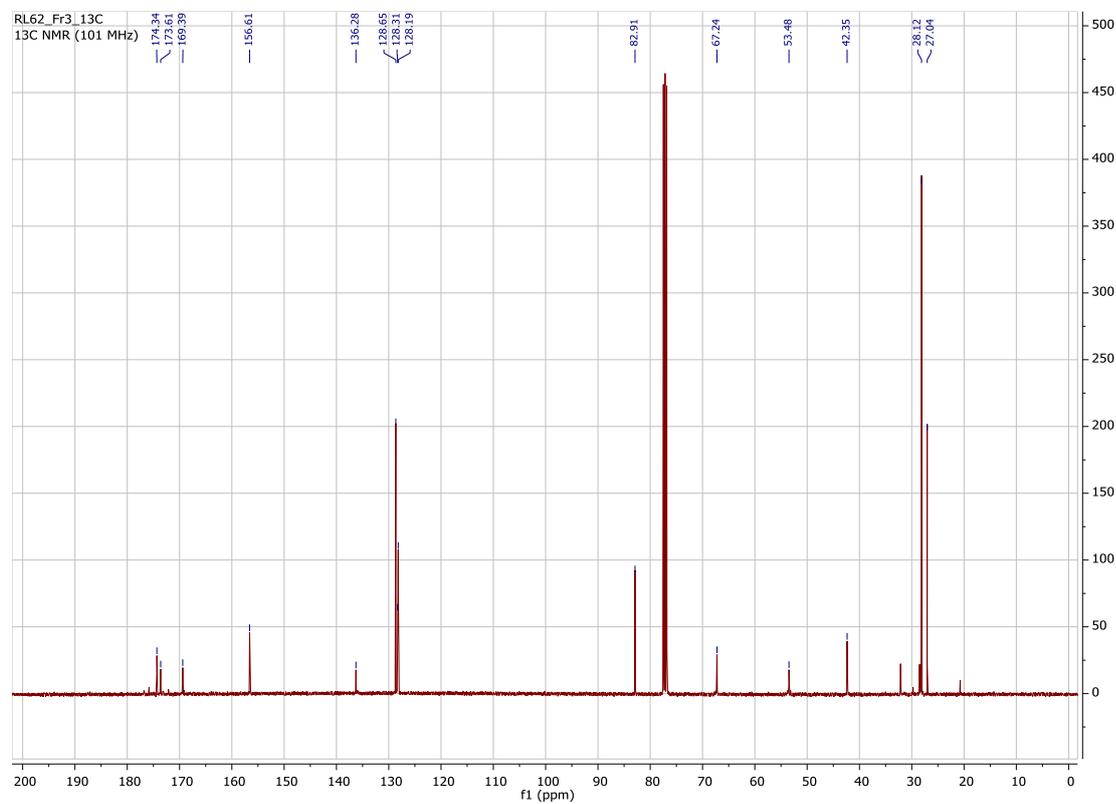


Figure S11:  $^1\text{H}$ ,  $^{13}\text{C}$  HSQC Spectrum of compound 2a

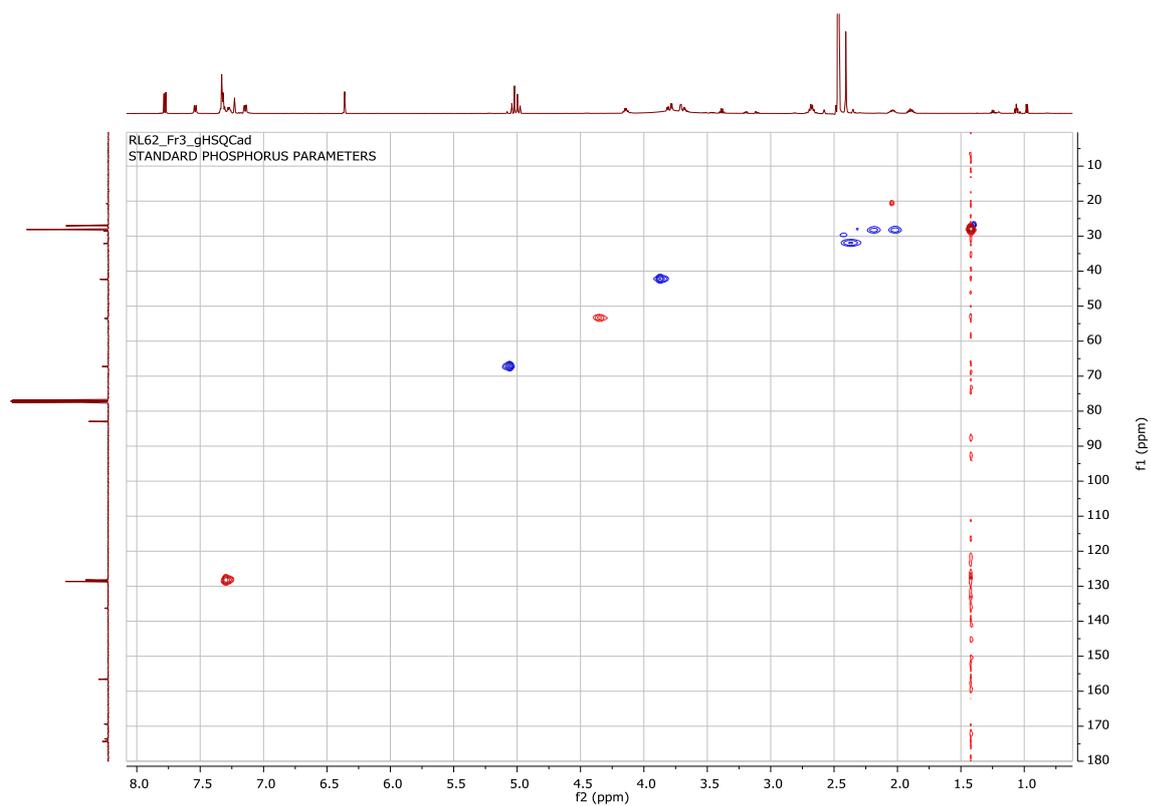


Figure S12:  $^1\text{H}$  NMR Spectrum of compound 3

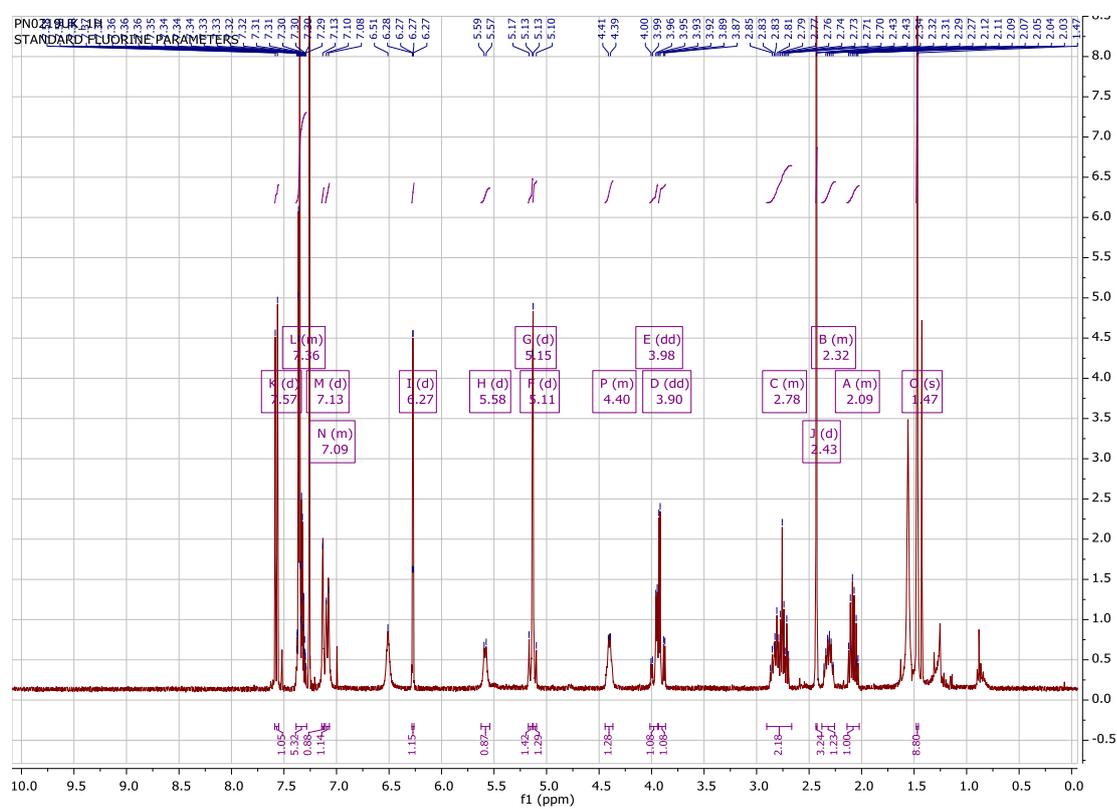


Figure S13: TOCSY Spectrum of compound 3

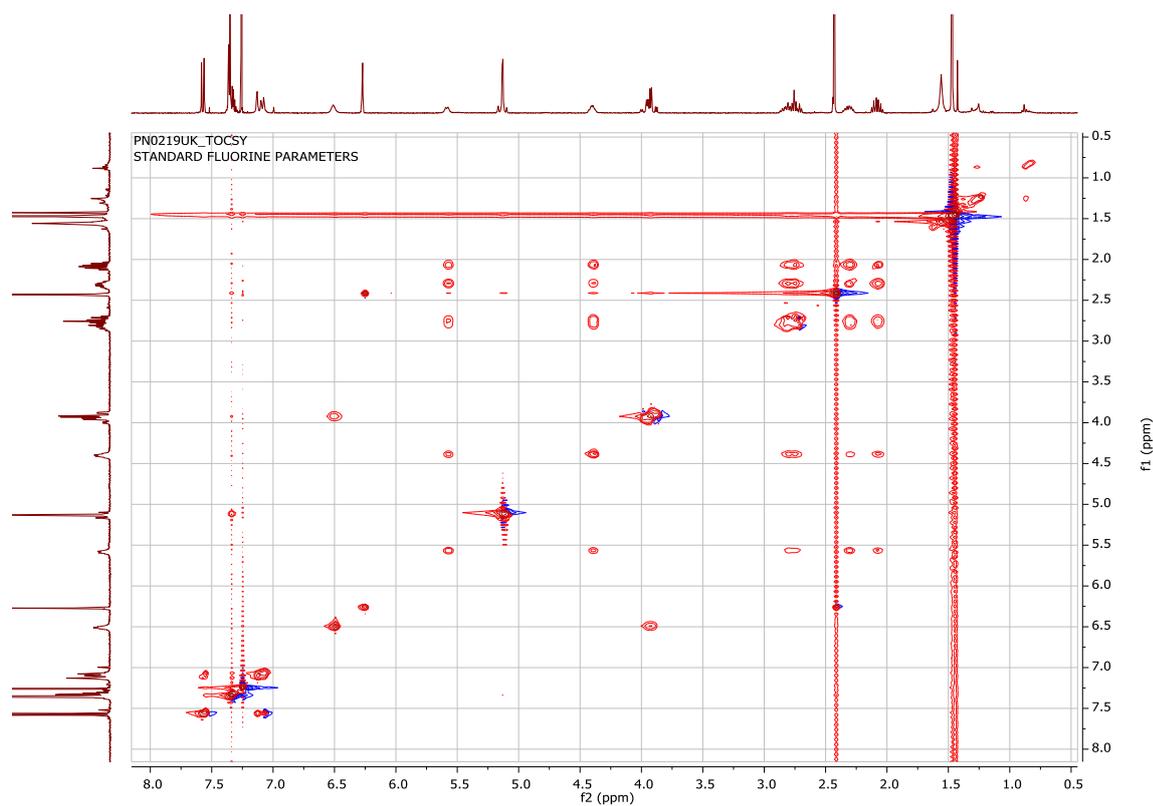


Figure S14: <sup>13</sup>C NMR Spectrum of compound 3

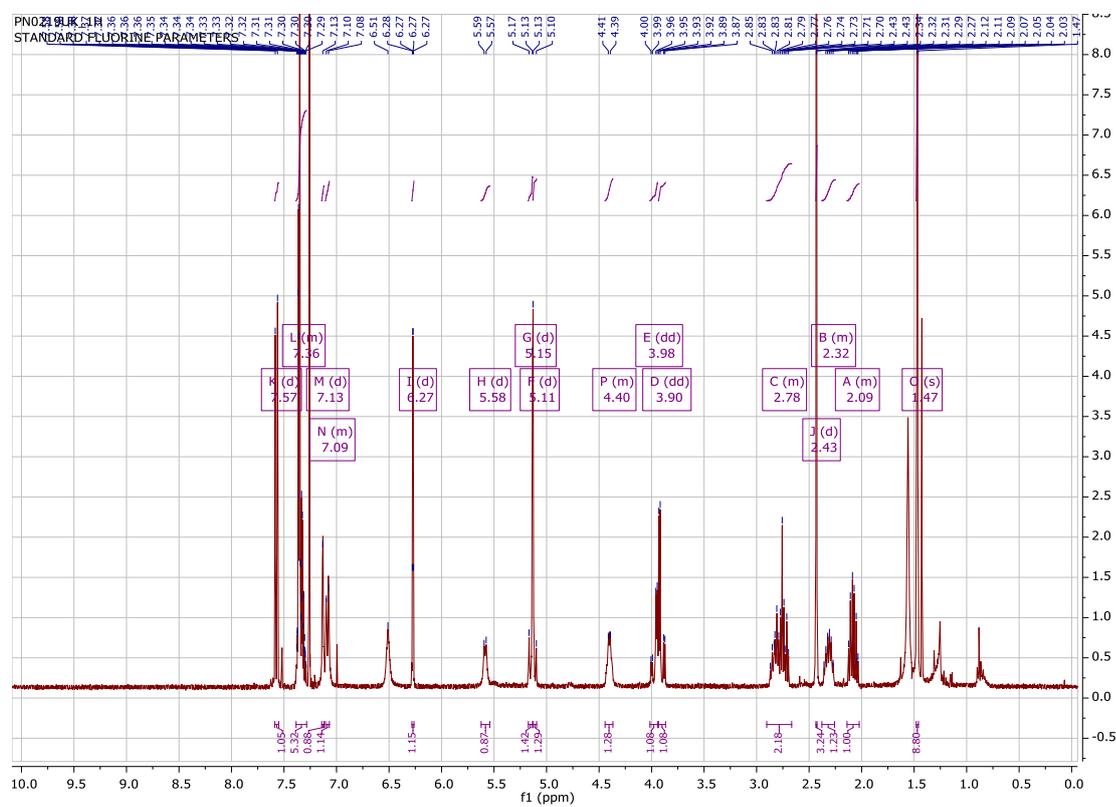


Figure S15:  $^1\text{H}$ ,  $^{13}\text{C}$  HSQC Spectrum of compound 3

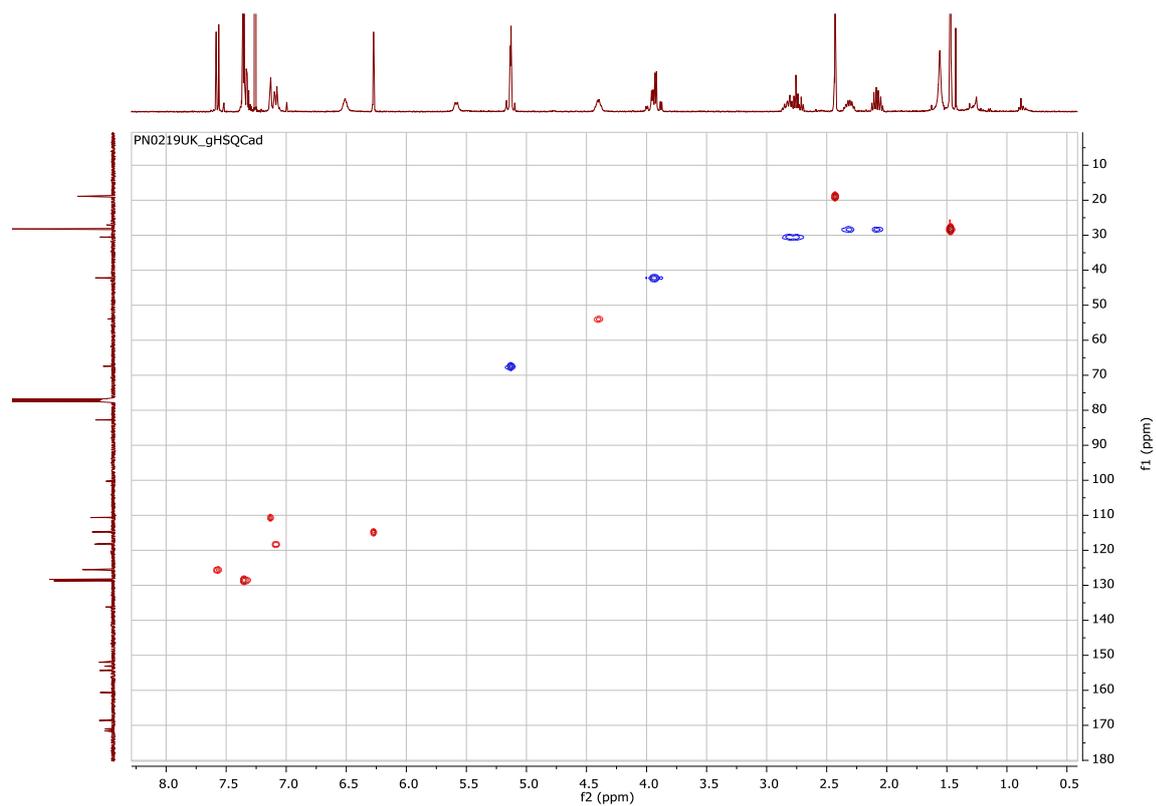
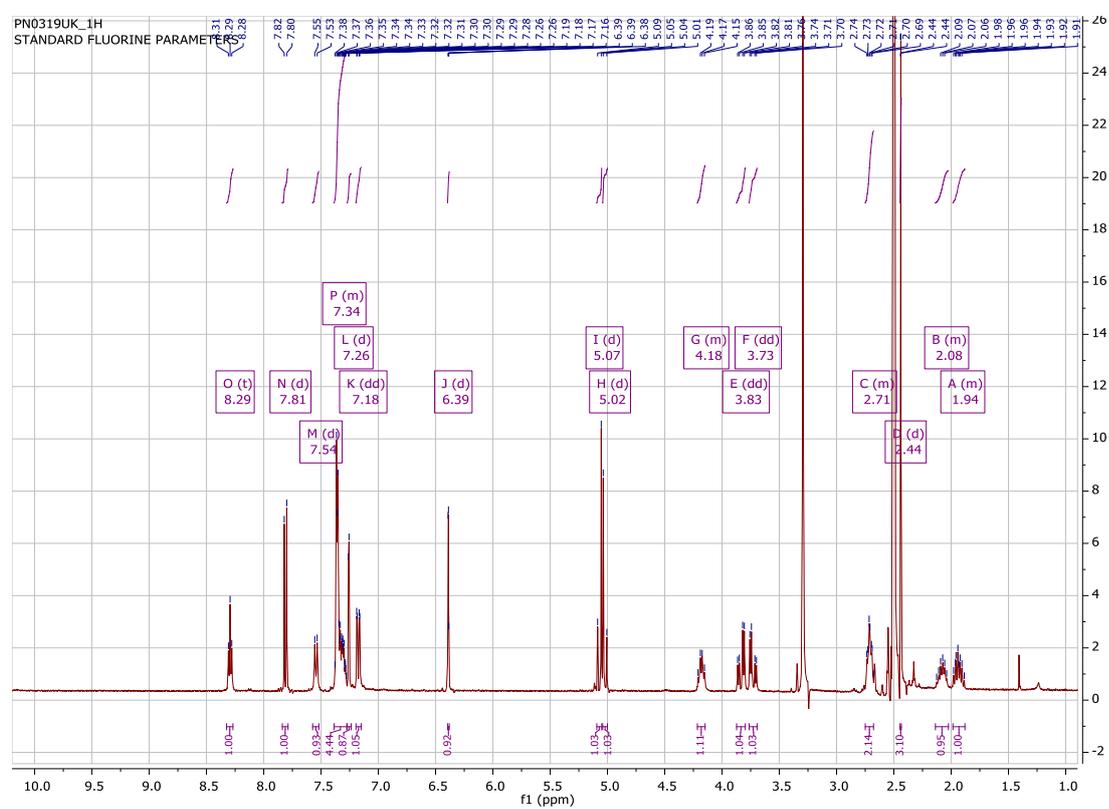
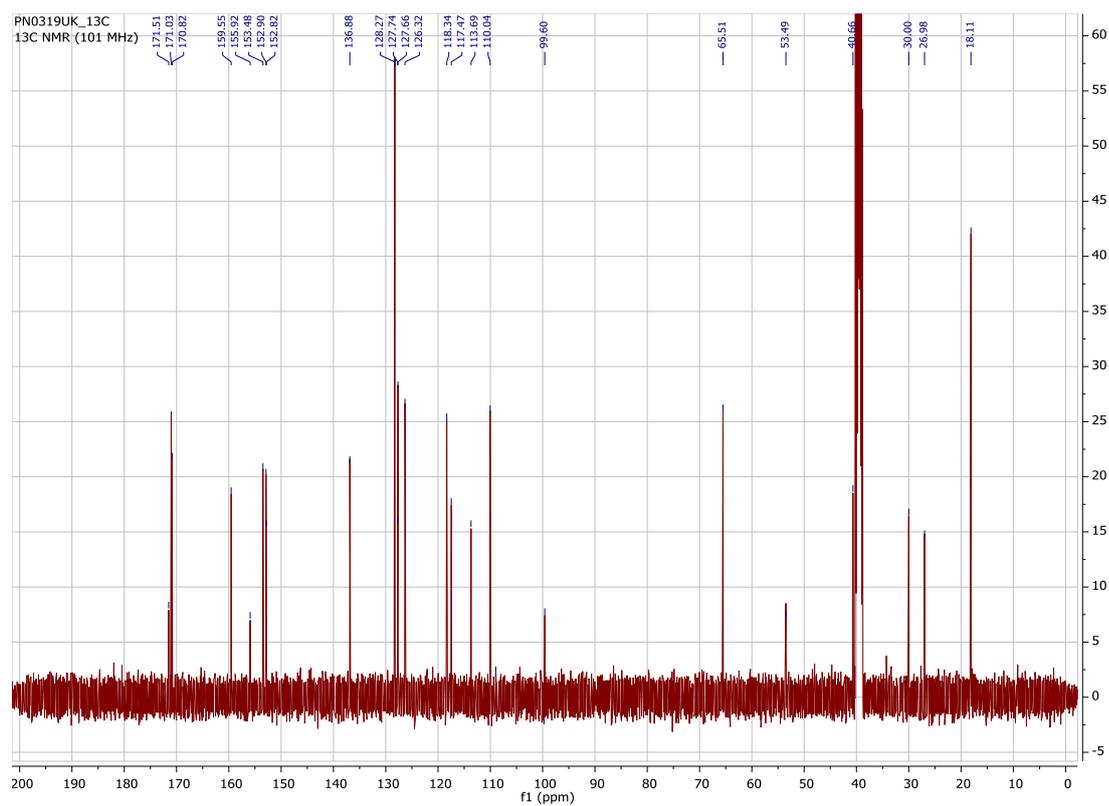


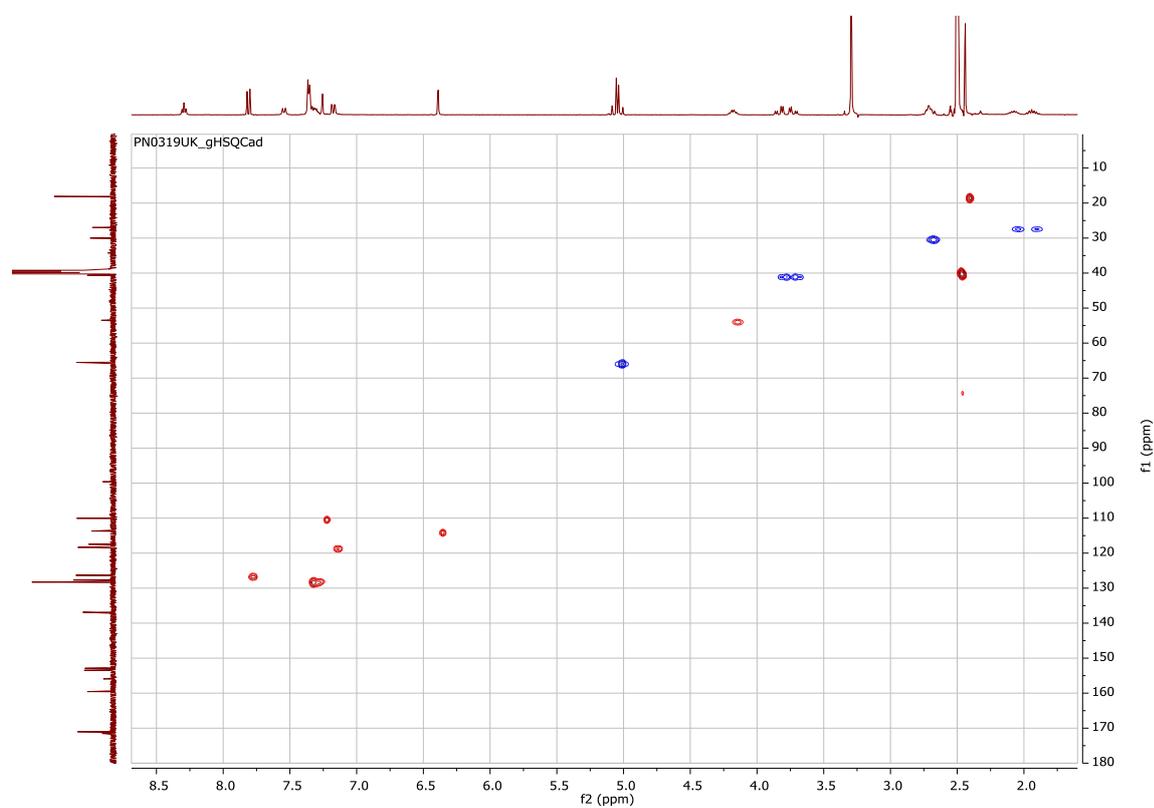
Figure S16:  $^1\text{H}$  NMR Spectrum of compound 4 (Z-Glu(HMC)-Gly-OH)

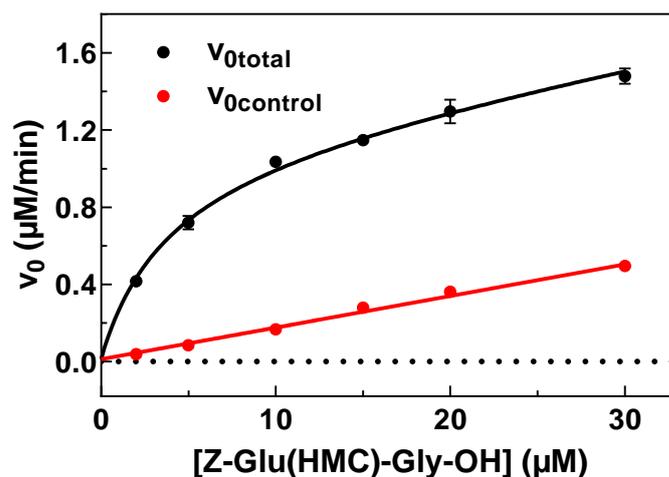


**Figure S17:**  $^{13}\text{C}$  NMR Spectrum of compound **4** (Z-Glu(HMC)-Gly-OH)



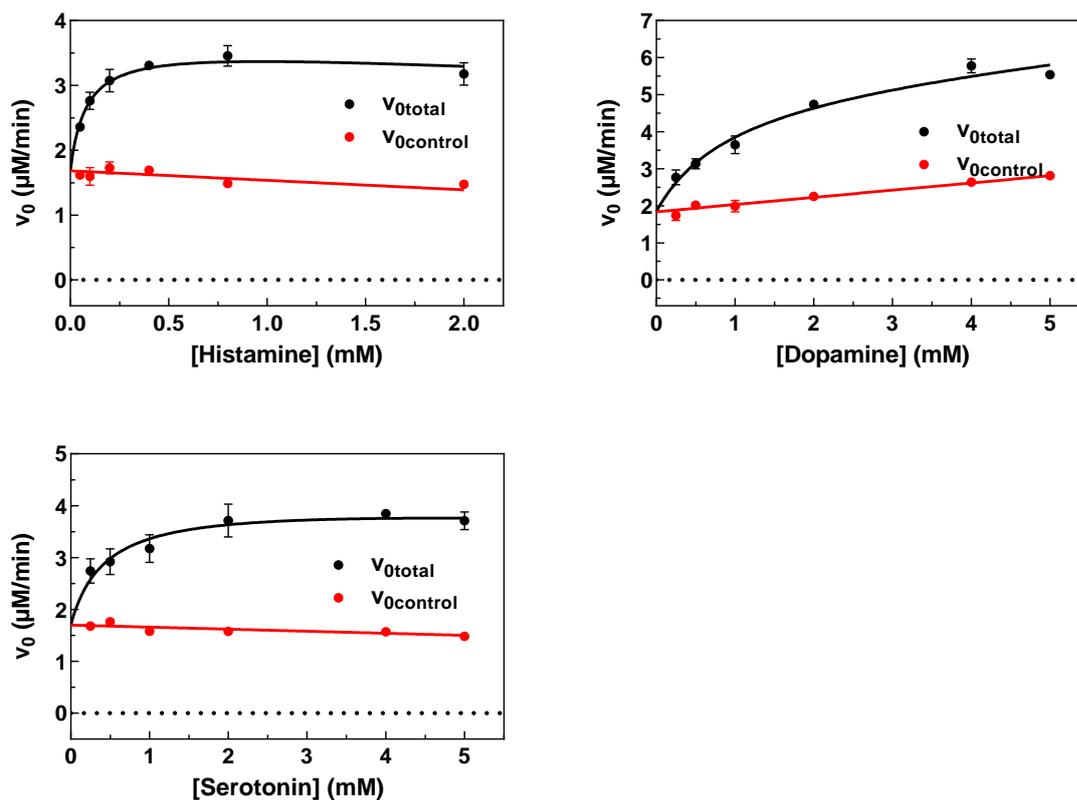
**Figure S18:**  $^1\text{H},^{13}\text{C}$  HSQC Spectrum of compound **4** (Z-Glu(HMC)-Gly-OH)





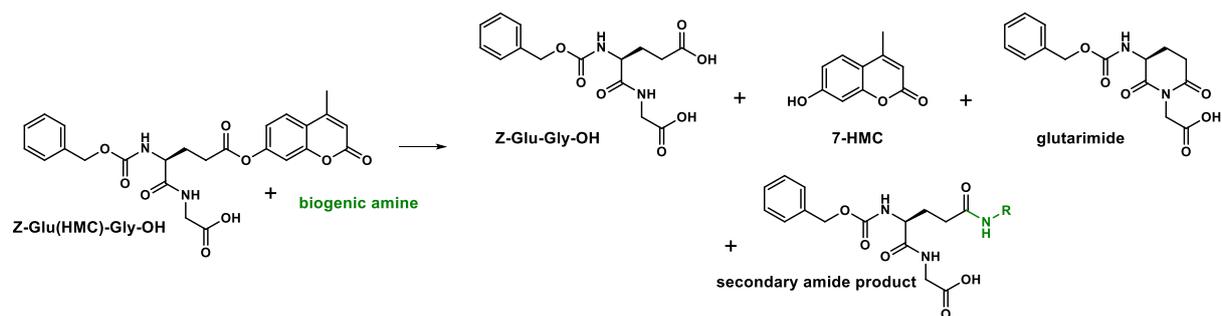
**Figure S19:** hTGase 2-catalysed hydrolysis of Z-Glu(HMC)-Gly-OH (**4**) at pH 8.0

Plots of  $v_{0total}=f([Z-Glu(HMC)-Gly-OH])$  and  $v_{0control}=f([Z-Glu(HMC)-Gly-OH])$  including the regression curves according to the global fit model of total and nonspecific binding as implemented in GraphPad Prism to determine the kinetic parameters of the enzymatic hydrolysis (see Materials and Methods section for a detailed description and see Figure 1 in the main article for the graph of  $v_{0corr}=f([Z-Glu(HMC)-Gly-OH])$ ). Data shown are mean values of three separate experiments each performed in duplicate. When not apparent, error bars are smaller than the symbols. Conditions: pH=8.0, 30 °C, 5% DMSO, 500 μM TCEP, 3 μg/mL hTGase 2.



**Figure S20:** hTGase 2-catalysed incorporation of different biogenic amines into Z-Glu(HMC)-Gly-OH (**4**;  $c=100 \mu\text{M}$ ) at pH 8.0

Plots of  $v_{0\text{total}}=f([\text{amine}])$  and  $v_{0\text{control}}=f([\text{amine}])$  including the regression curves according to the global fit model of total and nonspecific binding as implemented in GraphPad Prism to determine the kinetic parameters of the enzymatic aminolysis (see Materials and Methods section for a detailed description and see Figure 2 in the main article for the graph of  $v_{0\text{corr}}=f([\text{amine}])$ ). Data shown are mean values of two independent experiments each performed in duplicate. When not apparent, error bars are smaller than the symbols. Conditions: pH=8.0 (100 mM MOPS was used, which ensures that the pH value is maintained over the entire range of amine concentrations), 30 °C, 5% DMSO, 500  $\mu\text{M}$  TCEP, 0.6, 2 and 3  $\mu\text{g}/\text{mL}$  of hTGase 2 for histamine, dopamine and serotonin, respectively. Substrate concentrations were adjusted within prior pilot experiments. Substrate inhibition did not occur at concentration as high as 5 mM such as for histamine. The high rates in the absence of amine ( $c=0$ ) are due to the TGase 2-catalysed and non-enzymatic hydrolysis of **4**.

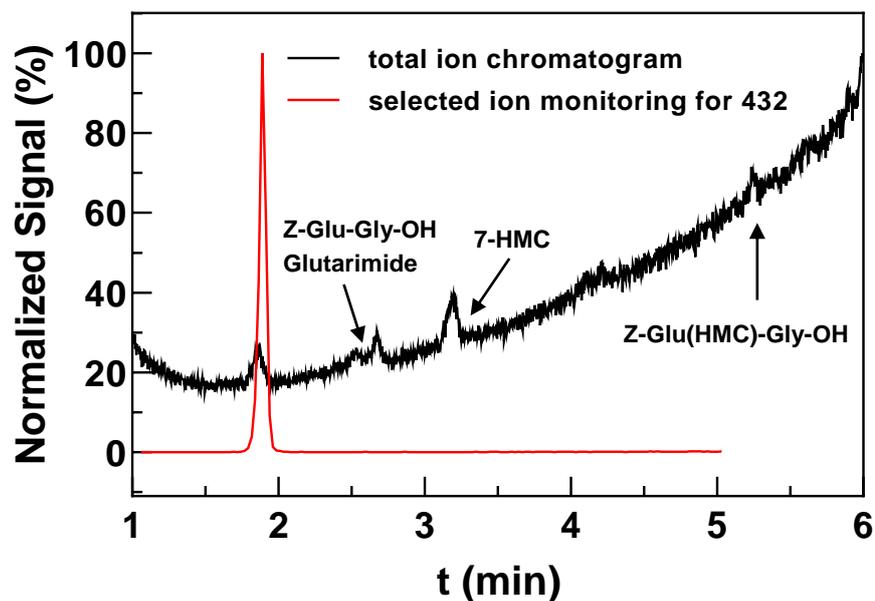


**Scheme S1:** Products of spontaneous and TGase 2-catalysed conversion of Z-Glu(HMC)-Gly-OH with biogenic amines.

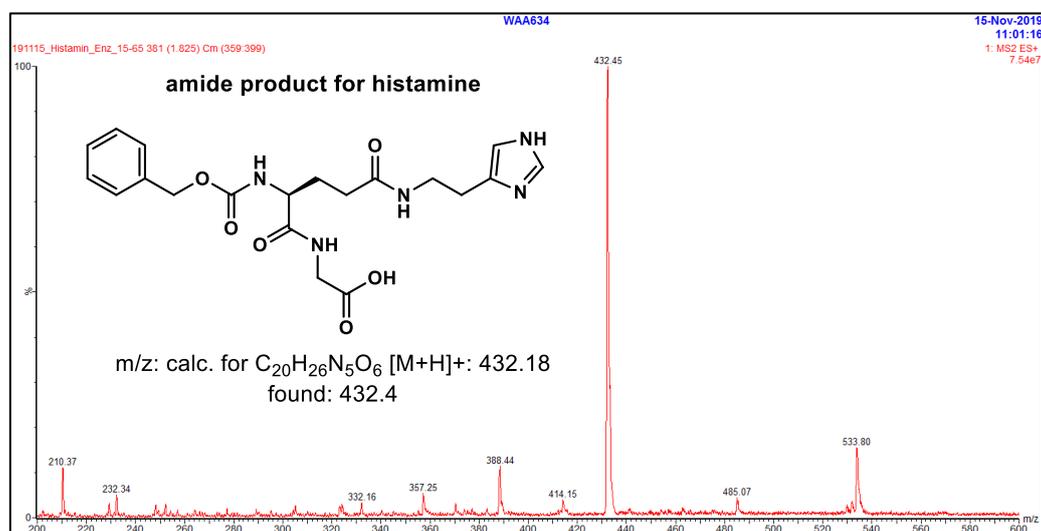
### General procedure for the LC-MS-analysis of the enzymatic aminolysis

The assay mixtures (200  $\mu$ L) were diluted with 1.25% HOAc/CH<sub>3</sub>CN (800  $\mu$ L, LC-MS grade) and were kept on ice for 30 min to fully precipitate the protein. Subsequently, the mixture was centrifuged at 4  $^{\circ}$ C for 10 min (12.700 rpm). An aliquot of the supernatant (90  $\mu$ L) was transferred to a sample vial for UPLC-DAD-MS analysis (system from Waters: ACQUITY UPLC I-Class System including a ACQUITY UPLC PDA e $\lambda$ -Detector coupled to a Xevo TQ-S mass spectrometer) and water (30  $\mu$ L) was added. A volume of 1-5  $\mu$ L of those solutions was injected into the system. A Waters Acquity BEH C18 column (2.1x100 mm, 1,7  $\mu$ m particle size) was used as stationary phase. A binary gradient system of water (containing 0.1% CH<sub>3</sub>COOH) as solvent and CH<sub>3</sub>CN/CH<sub>3</sub>OH (1:1, containing 0.1% CH<sub>3</sub>COOH) as solvent B at a flow rate of 0.4 mL/min served as the eluent (gradient 15-65% B, 5 min).

Analysis for histamine

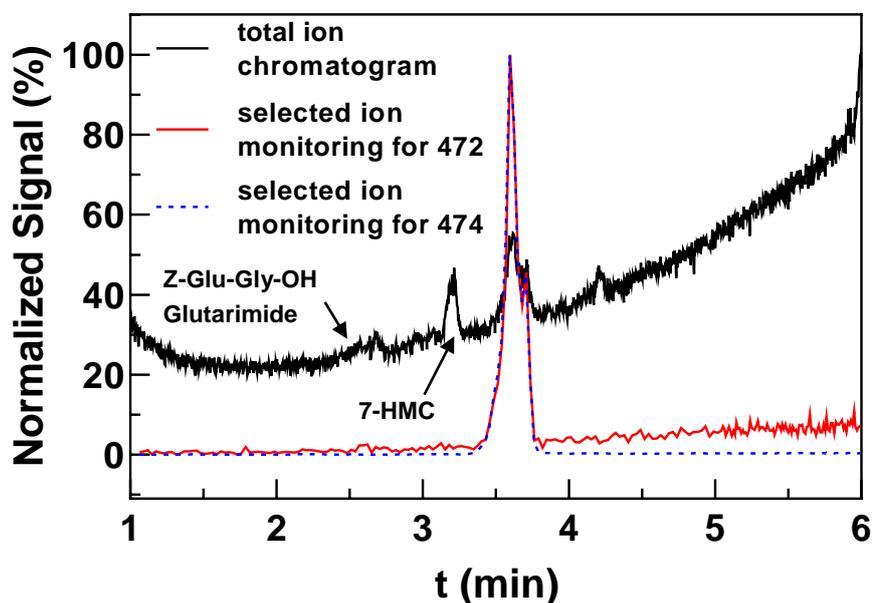


**Figure S21:** LC-MS analysis for the enzymatic aminolysis of **Z-Glu(HMC)-Gly-OH** with histamine. Conditions for the reaction mixture: pH 8.0, 30 °C, 5% DMSO, 500  $\mu$ M TCEP, 0.6  $\mu$ g/mL of hTGase 2, 2 mM of histamine, 100  $\mu$ M of **Z-Glu(HMC)-Gly-OH**, stop of the reaction after 1 h.

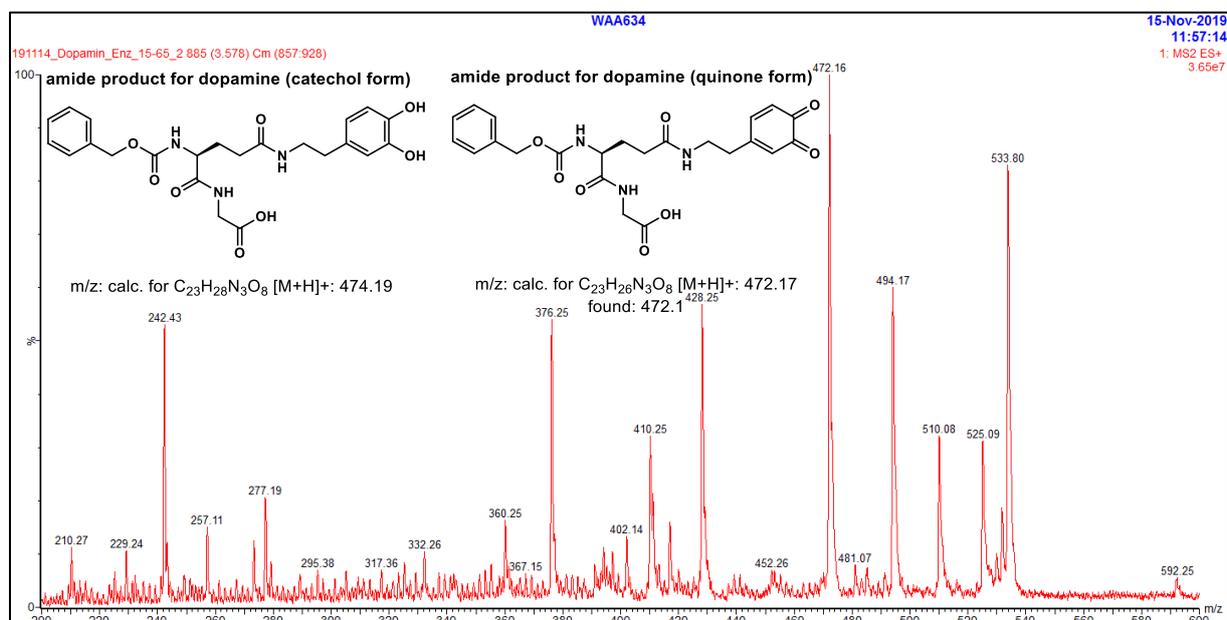


**Figure S22:** ESI(+) mass spectrum of the identified amide product for histamine.

## Analysis for dopamine

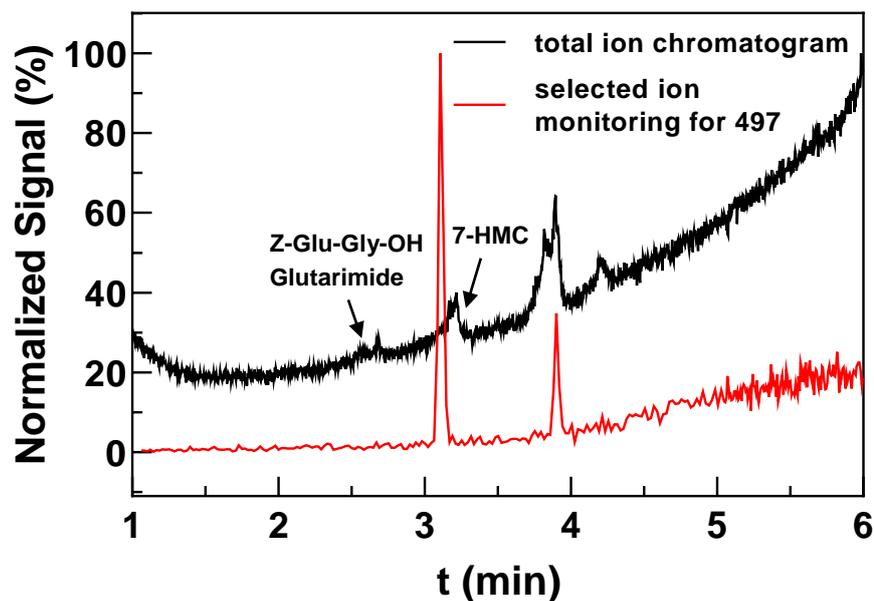


**Figure S23:** LC-MS analysis for the enzymatic aminolysis of **Z-Glu(HMC)-Gly-OH** with dopamine. Conditions for the reaction mixture: pH 8.0, 30 °C, 5% DMSO, 500  $\mu$ M TCEP, 2  $\mu$ g/mL of hTGase 2, 5 mM of dopamine, 100  $\mu$ M of **Z-Glu(HMC)-Gly-OH**, stop of the reaction after 1 h. The additional signal for  $m/z=472$  amu is attributed to partial oxidation of the aminolysis product in the presence oxygen [1].

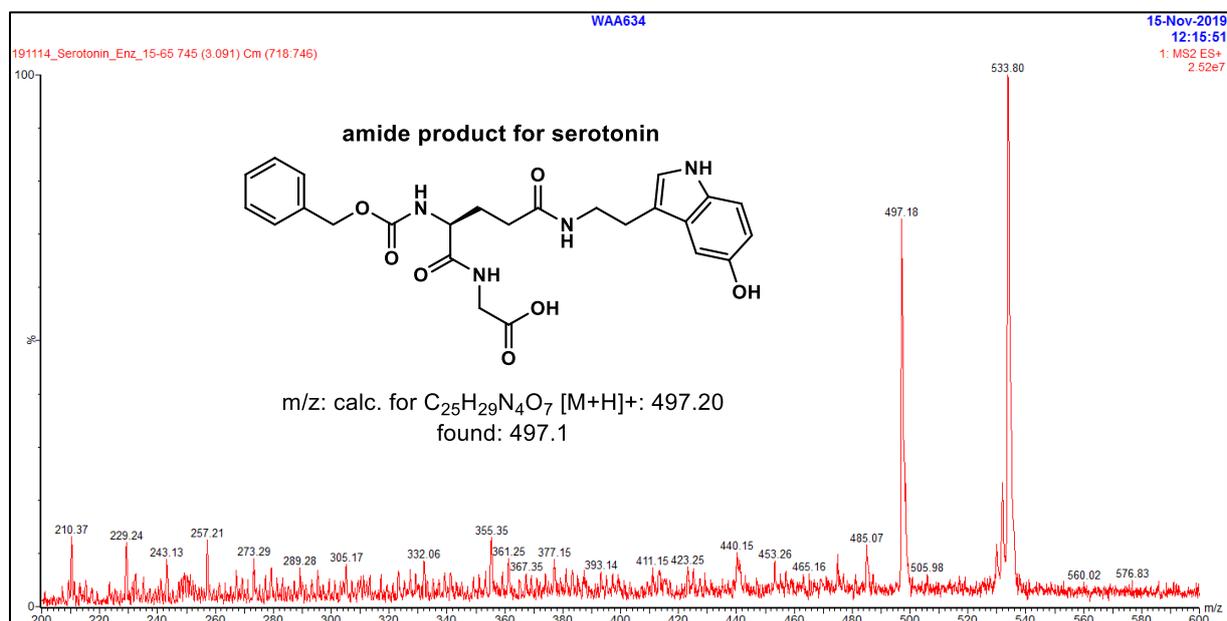


**Figure S24:** ESI(+) mass spectrum of the identified amide product for dopamine.

Analysis for serotonin



**Figure S25:** LC-MS analysis for the enzymatic aminolysis of **Z-Glu(HMC)-Gly-OH** with dopamine. Conditions for the reaction mixture: pH 8.0, 30 °C, 5% DMSO, 500  $\mu$ M TCEP, 3  $\mu$ g/mL of hTGase 2, 5 mM of serotonin, 100  $\mu$ M of **Z-Glu(HMC)-Gly-OH**, stop of the reaction after 1 h.



**Figure S26:** ESI(+) mass spectrum of the identified amide product for serotonin.

## **Supplemental references**

[1] J. Yang, M.A. Cohen Stuart, M. Kamperman, Jack of all trades: versatile catechol crosslinking mechanisms, *Chem. Soc. Rev.*, 43 (2014) 8271-8298.