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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 α - and γ -dipeptides. The key step was the esterification of Z-Glu-Gly-OtBu 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 (=carbobenzyloxy, 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 γ -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 μm) and Merck Silica Gel 60 F₂₅₄ sheets, respectively.
64 TLCs were visualised under UV light ($\lambda = 254 \text{ nm}$ or 365 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 μm column (Phenomenex, 250 \times 4.6 mm) served as stationary phase. A binary gradient system of 0.1%
69 CF₃COOH/water (solvent A) and 0.1% CF₃COOH/CH₃CN (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 ¹H and ¹³C 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 λ

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-Carbobenzoxyglutamic 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]); ¹H NMR (400 MHz, CDCl₃), δ
99 (ppm): 1.94 (qd, ²J=13.1 Hz, ³J=5.6 Hz, 1H, C_β-HH), 2.45-2.59 (m, 1H, C_β-HH), 2.90 (ddd, ²J=18.9 Hz,
100 ³J=13.0 Hz, ³J=6.2 Hz, 1H, C_γ-HH), 3.06 (ddd, ²J=18.7 Hz, ³J=5.6 Hz, ³J=2.2 Hz, 1H, C_γ-HH), 4.38-4.52 (m,
101 1H, C_α-H), 5.15 (s, 2H, CH₂O), 5.57 (br s, 1H, NH), 7.30-7.42 (m, 5H, Ph-H); ¹³C NMR (100 MHz, CDCl₃),
102 δ (ppm): 23.72 (C_β), 29.83 (C_γ), 51.48 (C_α), 67.77 (CH₂O), 128.39, 128.64, 128.80, 135.80, (C_{arom}), 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 C₁₃H₁₃NO₅, 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_f 0.35 (*n*-hexane/ethyl acetate/acetic acid 1:4:0.01); Mp 96-103 °C (lit 106- 107 °C [20]); ¹H
120 NMR (400 MHz, CDCl₃), δ (ppm): 1.45 (s, 9H, (CH₃)₃C), 1.88 – 2.01 (m, 1H, C_β-HH), 2.09 – 2.20 (m, 1H,
121 C_β-HH), 2.41 – 2.59 (m, 2H, C_γ-H₂), 3.83 – 4.00 (m, 2H, ^{Gly}C_α-H₂), 4.41 – 4.49 (m, 1H, ^{Glu}C_α-H), 5.05-5.17 (m,
122 2H, CH₂O), 5.88 (d, ³J=8.6 Hz, 1H, Glu-NH), 7.10-7.16 (br s, 1H, Gly-NH), 7.27-7.39 (m, 5H, Ph-H); ¹³C
123 NMR (100 MHz, CDCl₃), δ (ppm): 28.16 (3×CH₃), 28.28 (C_β), 29.91 (C_γ) 42.25 (^{Gly}C_α), 53.85 (^{Glu}C_α), 67.44
124 (CH₂O), 82.75 ((CH₃)₃C), 128.20, 128.37, 128.68, 136.14 (4×C_{arom}), 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 C₁₉H₂₆N₂O₇:
126 calcd. C: 57.86%, H: 6.64%, N: 7.10%, found: C: 58.97%, H: 6.79%, N: 6.94%.

127 2.4. N²-((Benzyloxy)carbonyl)-N⁵-(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); ^1H NMR (400 MHz, CDCl_3) δ (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, CH_2O), 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}C NMR (100 MHz, CDCl_3) δ (ppm): 28.12
137 ($3\times\text{CH}_3$), 28.50 (C_β), 32.13 (C_γ), 42.35 ($^{\text{Gly}}\text{C}_\alpha$), 53.48 ($^{\text{Glu}}\text{C}_\alpha$), 67.24 (CH_2O), 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 N_2 atmosphere. DIPEA (282 μL , 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 CH_2Cl_2 (15 mL), transferred to a
146 separatory funnel and washed with 1 M HCl (1 \times 3 mL), sat. NaHCO_3 (2 \times 3 mL), H_2O (1 \times 3 mL) and brine
147 (1 \times 1 mL). The organic layer was dried over Na_2SO_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 $^\circ\text{C}$; ^1H NMR
158 (400 MHz, CDCl_3) δ (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 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, CHHO), 5.15 (d, $^2J=12.9$ Hz, 1H, 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}C NMR
164 (100 MHz, CDCl_3) δ (ppm): 18.88 (CH_3 of coumarin), 28.20 ($3\times\text{CH}_3$), 28.30 (C_β), 30.52 (C_γ), 42.18 (C_α of
165 Gly), 53.92 (C_α of Glu), 67.43 (CH_2O), 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%.

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 CH_2Cl_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 N_2 stream under ambient pressure. The oily residue was taken up into CH_2Cl_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 Na₂SO₄ and evaporated *in vacuo* to obtain 112 mg of a
182 white solid. Since an impurity was detectable in the ¹H 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_f 0.01 (*n*-hexane/ethyl acetate 2:8); Mp 167-171 °C; ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm):
188 1.88 – 1.98 (m, 1H, C_β-HH), 2.02 – 2.14 (m, 1H, C_β-HH), 2.44 (d, ⁴J=1.3 Hz, 3H, CH₃-coumarin), 2.68 – 2.75
189 (m, 2H, C_γ-H₂), 3.73 (dd, ²J=17.5, ³J=5.6 Hz, 1H, C_α-HH of Gly), 3.83 (dd, ²J=17.5, ³J=6.0 Hz, 1H, C_α-HH of
190 Gly), 4.15-4.21 (m, 1H, C_α-H of Glu), 5.02 (d, ²J=12.6 Hz, 1H, CHHO), 5.07 (d, ²J=12.6 Hz, 1H, CHHO),
191 6.39 (d, ⁴J=1.3 Hz, 1H, H-3 of coumarin), 7.18 (dd, ³J=8.7, 2.3 Hz, 1H, H-6 of coumarin), 7.26 (d, ⁴J=2.3 Hz,
192 1H, H-8 of coumarin), 7.28 – 7.39 (m, 5H, Ph-H), 7.54 (d, ³J=8.3 Hz, 1H, Glu-NH), 7.81 (d, ³J=8.7 Hz, 1H,
193 H-5 of coumarin), 8.29 (t, ³J=5.8 Hz, 1H, Gly-NH). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 18.11 (CH₃ of
194 coumarin), 26.98 (C_β), 30.00 (C_γ), 40.66 (C_α of Gly), 53.49 (C_α of Glu), 65.51 (CH₂O), 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+NH₄]⁺:
199 514.1825, found: 514.1821 (100%), [2M+NH₄]⁺: calculated: 1010.3307, found: 1010.3313 (57.2%),
200 [3M+NH₄]⁺: calculated: 1506.4789, found: 1506.4799 (80.52%). Elemental analysis: C₂₅H₂₄N₂O₉·H₂O
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. NaHCO₃ (2×10 mL)
211 and brine (1×10 mL), dried over Na₂SO₄ 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; ¹H NMR (400 MHz, CDCl₃): δ=1.41 (s, 9H, 3×CH₃ of
214 Boc), 2.90 (s, 6H, 2×CH₃ of dansyl), 3.23–3.15 (m, 4H, 2×CH₂ of piperazine), 3.45–3.39 (m, 2H, CH₂ of
215 piperazine), 3.69–3.62 (m, 2H, CH₂ of piperazine), 3.86 (d, ³J=4.5 Hz, 2H, CH₂ Gly), 5.36 (br s, NH),
216 7.21 (d, ³J=7.5 Hz, 1H, H_{Dansyl}), 7.55 (dd, ³J=8.6, 7.6 Hz, 2H, 2×H_{Dansyl}), 8.20 (dd, ³J=7.3 Hz, ⁴J=1.2 Hz, 1H,
217 H_{Dansyl}), 8.37 (d, ³J=8.7 Hz, 1H, H_{Dansyl}), 8.59 (d, ³J=8.5 Hz, 1H, H_{Dansyl}); ¹³C NMR (100 MHz, CDCl₃):
218 δ=28.45 (3×CH₃ Boc), 41.68 (CH₂ of piperazine), 42.28 (CH₂ of Gly), 44.24 (CH₂ of piperazine), 45.48
219 (CH₂ of piperazine), 45.63 (CH₂ of piperazine), 45.65 (2×CH₃ dansyl), 80.03 (C_{quartär} of Boc), 115.67,
220 119.65, 123.45, 128.45, 130.16 (C_{quart} of dansyl), 130.44 (C_{quart} of dansyl), 131.03, 131.16, 132.30 (C_{quart} of
221 dansyl), 151.68 (C_{quart} of dansyl), 155.87 (CO of Boc), 167.07; MS (ESI+) 477.3 ([M+H]⁺). ¹H and ¹³C
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 CH₂Cl₂ (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. NaHCO₃ (20 mL) and extracted with CH₂Cl₂
229 (5×5 mL). The organic phase was dried over Na₂SO₄ and subsequently evaporated to dryness to

230 obtain **6b** (131 mg, 100%) as a light-green solid; ^1H NMR (400 MHz, DMSO- d_6): δ =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, CH_2 Gly),
232 7.27 (d, 3J =7.5 Hz, 1H, H_{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, H_{Dansyl}),
233 8.30 (d, 3J =8.7 Hz, 1H, H_{Dansyl}), 8.53 (d, 3J =8.5 Hz, 1H, H_{Dansyl}), signal for NH_2 not detectable; ^{13}C NMR
234 (100 MHz, DMSO- d_6): δ =40.89 (CH_2 of piperazine), 42.53 (CH_2 Gly), 43.39 (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 (C_{quart} of dansyl), 129.62
236 (C_{quart} of dansyl), 130.06, 130.39, 132.48 (C_{quart} of dansyl), 151.42 (C_{quart} of dansyl), 171.55 (CO); MS
237 (ESI+) 377.2 ($[\text{M}+\text{H}]^+$) ^1H and ^{13}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 μL , 0.60 mmol) in CH_2Cl_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. NaHCO_3 (2×10 mL), brine (1×10 mL); dried over
245 Na_2SO_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; ^1H NMR (400 MHz, CDCl_3): δ =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, CH_2 of piperazine), 3.72–3.66 (m, 2H, CH_2
248 of piperazine), 4.08 (d, 3J =4.2 Hz, 2H, 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, H_{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, N_aH of Gly), 7.31 (d, 3J =7.6 Hz, 1H, H_{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, H_{Dansyl}), 8.48 (d, 3J =8.7 Hz, 1H, H_{Dansyl}), 8.62 (d, 3J =8.6 Hz, 1H, H_{Dansyl}); ^{13}C
252 NMR (100 MHz, CDCl_3): δ =41.38 (CH_2 of Gly), 41.86 (CH_2 of piperazine), 44.36 (CH_2 of piperazine),
253 45.41 (CH_2 of piperazine), 45.59 (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 (C_{quart} of dansyl), 130.01 ($\text{CH}=\text{CH}_2$), 130.43 (C_{quart} of dansyl), 130.57,
255 131.26, 132.59 (C_{quart} of dansyl), 149.58 (C_{quart} of dansyl), 165.92, 166.56. ^1H and ^{13}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 ± 20 nm) and emission (450 ± 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 μL)
263 contained 190 μL aqueous solution and 10 μL DMSO (5% v/v). The following three buffer systems
264 were used: assay buffer I (100 mM MES, 3 mM CaCl_2 , 50 μM EDTA, adjusted to pH 6.5 with 1 M
265 NaOH), assay buffer II (100 mM MOPS, 3 mM CaCl_2 , 50 μM EDTA, adjusted to pH 8.0 with 1 M
266 NaOH) and enzyme buffer (100 mM MOPS, 3 mM 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 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.

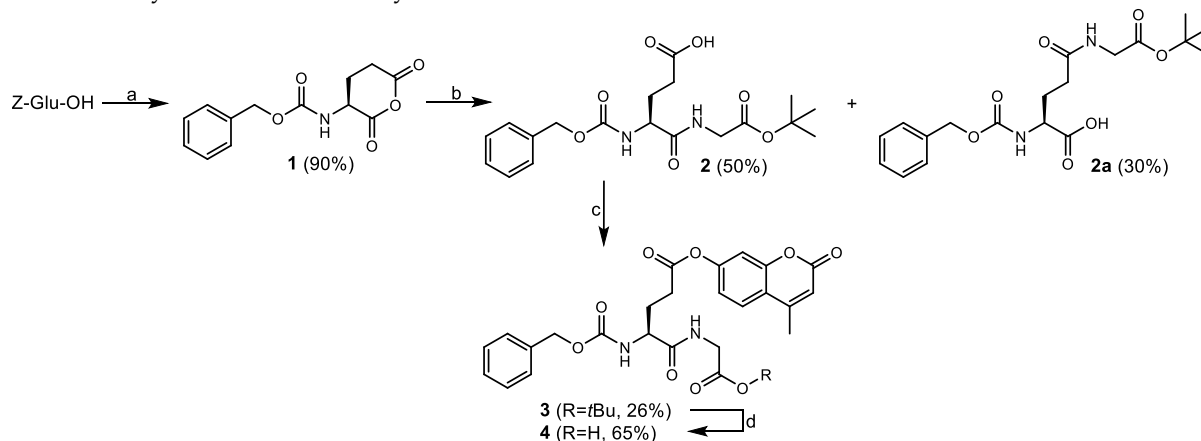
298 Concerning the kinetic characterisation of the biogenic amines, the two sets of initial rates
299 ($v_{0\text{total}}=f([\text{amine}])$ and $v_{0\text{control}}=f([\text{amine}])$) were also globally analysed using the model of total and
300 nonspecific binding (instead of simple subtraction of $v_{0\text{control}}$ 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 α - and γ -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^{α} -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 ^1H 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

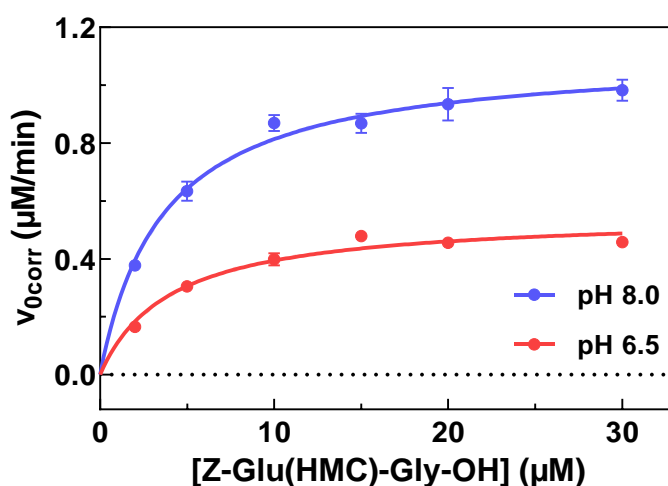
325 of the free carboxylic acid, the obtained material was recrystallised to furnish highly pure compound
 326 **3**. In the final step, *tert*-butyl ester **3** was cleaved by treatment with trifluoroacetic acid. During work-
 327 up, excessive TFA was efficiently removed by acidic aqueous washing to 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, CHCl₃, room temperature; c) HATU, DIPEA,
 334 4-methylumbelliferone, DMF, room temperature; d) TFA, CH₂Cl₂, 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.



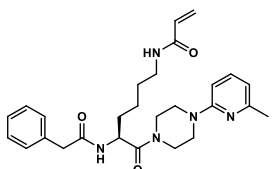
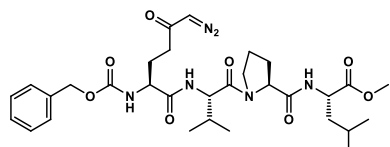
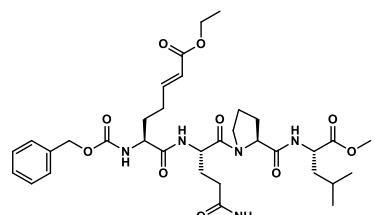
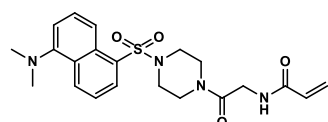
343

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 μ M TCEP, 3 μ 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

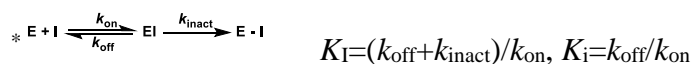
pH value	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)
6.5	4.05 (0.23)	0.30 (0.01)	74,100
8.0	3.93 (0.48)	0.61 (0.03)	155,000

350 Rate constant for spontaneous hydrolysis: k_{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 α,β -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_{inact}/K_i has been reported for the latter. The
362 results of the inhibitor characterisation are included in Table 2.364 **Table 2:** Second-order inactivation constants (k_{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_{inact}/K_i ($\text{M}^{-1}\text{s}^{-1}$) ^{a*}	K_i (μM) ^{a*}	IC_{50} (nM)
5		4,880 (20) ^[15]	5.73 (0.77) ^[15]	310 ^{[15]b} 14 (8.2) ^{[26]c}
Z006		191,000 (3,000)	0.096 (0.01)	20 ^{[27]c}
Z013		51,500 (5,800)	0.45 (0.02)	200 ^{[28]d}
1-155 (7)		12,700 (700) 4,962 ^{[21]f}	1.43 (0.01)	6.1 (0.4) ^{[21]e}

^aData shown are mean values (\pm SEM) of two separate experiments, each performed in duplicate.

^bTGase 2-catalysed incorporation of R-I-Cad in DMC (transamidation) at pH 8.0 (5 min preincubation period of enzyme and inhibitor). ^cTGase 2-catalysed incorporation of Boc-K-NH(CH₂)₂NH-Dns in DMC (transamidation) at pH 7.4 (30 min preincubation period of enzyme and inhibitor). ^dIsopeptidase-assay using the substrate Abz-NE(CAD-DNP)EQVSPLTLLK-OH (A101, Zedira). ^eTGase 2-catalysed incorporation of *N*-(biotinyl)cadaverine in immobilised DMC (transamidation) at pH 8.5 (30 min preincubation period of enzyme and inhibitor). ^fGDH-coupled assay (deamidation) at pH 7.2.



366

367

Schaertl *et al.* have determined an IC₅₀ value of 20 nM for Z006 together with various other

368 compounds by employing a fluorescence-based transamidase-detecting assay using *N,N*-

369 dimethylcasein as acyl donor and *N*-monodansylcadaverin as acyl acceptor substrate, respectively

370 [27]. In a later study, *N*^ε-acryloyllysine-based irreversible inhibitor **5** has been synthesised, which was

371 investigated in the same assay [26]. The IC₅₀ value of **5** was 14 nM under these conditions, which

372 suggests that it should be equipotent to Z006. However, the k_{inact}/K_i value of Z006 determined herein

373 (191 000 M⁻¹s⁻¹) is 37-fold greater than that of **5**. Even though the applied assay methods detect

374 different TGase 2-catalysed reactions (transamidation in Schaertl *et al.* *vs.* hydrolysis herein) and the

375 pH values are different (6.5 *vs.* 7.4), the potencies of Z006 and **5** should not be influenced to such

376 extent. In agreement with the value of k_{inact}/K_i determined for Z006, Khosla's group reported

377 inactivation constants of 48 000 M⁻¹s⁻¹ [29] and 139 000 M⁻¹s⁻¹ [30] for structurally related peptidic

378 diazomethyl ketones, which have been determined using the GDH-coupled assay. The higher

379 reactivity of Z006 towards TGase 2 in comparison to **5** is reasonable, as diazomethyl ketones are

380 inherently more reactive towards thiols than acrylamides. Moreover, as the peptidic structures of

381 Z006 and Z013 are based on gliadin peptides, which are natural substrates of TGase 2, they can

382 probably be better accommodated by the active site, which in turn results in stronger non-covalent

383 interactions. This is reflected in the low K_i value of 96 nM for Z006. The similar IC₅₀ values of Z006

384 and **5** and the drastically differing inactivation constants can be rationalised when the assay

385 conditions are considered. The IC₅₀ values were determined using a TGase 2 concentration of 20 nM

386 and a preincubation time of 30 min [27]. Therefore, the IC₅₀ limit, which corresponds to half of the

387 employed active enzyme concentration ($[E]_{\tau/2}$) [31, 32], might have been reached for both

388 compounds. In this context, it should be pointed out that the IC₅₀ value of an irreversible inhibitor in

389 general will always be equal to $[E]_{\tau/2}$, provided that **[inhibitor] ≥ [enzyme]/2** and enough incubation

390 time is given. The other investigated peptidic inhibitor Z013 exhibits a k_{inact}/K_i of 51 500 M⁻¹s⁻¹, which

391 is significantly more potent than **5**. As for Z006, the higher inhibitory potency of Z013 can be

392 attributed to the more reactive electrophilic warhead and the peptidic structure, which corresponds

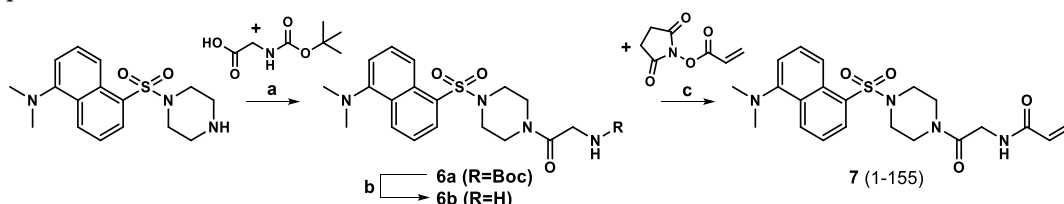
393 to that of Z006, apart from one amino acid side chain. An IC₅₀ value of 200 nM has been reported for

394 Z013; however, no information on enzyme concentration and preincubation time were provided [28].

395 Again, the comparison to the kinetic parameter k_{inact}/K_i indicates that IC₅₀ values are not suitable for

396 evaluating the potency of irreversible inhibitors, unless identical conditions and carefully adjusted

397 preincubation times are used [33].



398

399

Scheme 2: Synthesis of inhibitor 1-155

400

Reagents and conditions: **a**) PyBOP, DIPEA, DMF, 3 h; **b**) TFA/CH₂Cl₂ (1:1, v/v), 2 h; **c**) TEA, CH₂Cl₂, 2 h.

401

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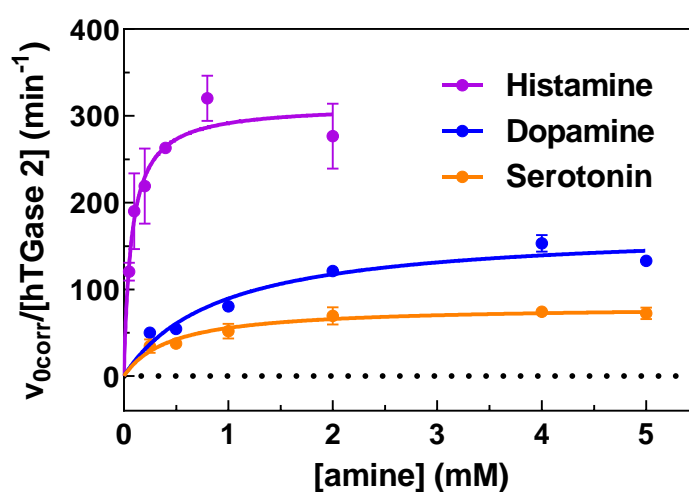
The synthesis of 1-155 (compound **7**) started with the amide bond coupling between Boc-Gly-

403 OH and *N*-monodansylpiperazine to **6a** using PyBOP and DIPEA, in difference to the published

404 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_{2A} receptors in rat cortical cells raises the cytosolic 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 μ M Z-Glu(HMC)-Gly-OH (4), 500 μ M TCEP, 0.6, 2 and 3 μ 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^{app} (mM)	$k_{\text{cat}}^{\text{app}}$ (s^{-1})	k_{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 ($E_{\text{T}}=6.15$ 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-NH₂ 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 μ 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_{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^{app} of 80 μ 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 $^{14}\text{N}/^{15}\text{N}$ 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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510

511 **References**

512

513 [1] N. Agnihotri, K. Mehta, Transglutaminase-2: Evolution from pedestrian protein to a promising therapeutic
514 target, *Amino Acids*, 49 (2017) 425-439.

515 [2] R.L. Eckert, Transglutaminase 2 takes center stage as a cancer cell survival factor and therapy target, *Mol.*
516 *Carcinog.*, 58 (2019) 837-853.

517 [3] J.E. Folk, J.S. Finlayson, The ϵ -(γ -glutamyl)lysine crosslink and the catalytic role of transglutaminases, *Adv.*
518 *Protein Chem.*, 31 (1977) 1-133.

519 [4] R. van Geel, M.F. Debets, D.W. Lowik, G.J. Pruijn, W.C. Boelens, Detection of transglutaminase activity using
520 click chemistry, *Amino Acids*, 43 (2012) 1251-1263.

521 [5] S. Hauser, R. Wodtke, C. Tondera, J. Wodtke, A.T. Neffe, J. Hampe, A. Lendlein, R. Löser, J. Pietzsch,
522 Characterization of tissue transglutaminase as a potential biomarker for tissue response toward biomaterials,
523 *ACS Biomater. Sci. Eng.*, 5 (2019) 5979-5989.

524 [6] K. Jambrovics, I.P. Uray, Z. Keresztessy, J.W. Keillor, L. Fesus, Z. Balajthy, Transglutaminase 2 programs
525 differentiating acute promyelocytic leukemia cells in all-trans retinoic acid treatment to inflammatory stage
526 through NF- κ B activation, *Haematologica*, 104 (2019) 505-515.

- 527 [7] M.S. Pavlyukov, N.V. Antipova, M.V. Balashova, M.I. Shakhparonov, Detection of transglutaminase 2
528 conformational changes in living cell, *Biochem. Biophys. Res. Commun.*, 421 (2012) 773-779.
- 529 [8] N.S. Caron, L.N. Munsie, J.W. Keillor, R. Truant, Using FLIM-FRET to measure conformational changes of
530 transglutaminase type 2 in live cells, *PLoS One*, 7 (2012) e44159.
- 531 [9] S. Liu, R.A. Cerione, J. Clardy, Structural basis for the guanine nucleotide-binding activity of tissue
532 transglutaminase and its regulation of transamidation activity, *Proc. Natl. Acad. Sci. USA*, 99 (2002) 2743-2747.
- 533 [10] W.P. Katt, M.A. Antonyak, R.A. Cerione, Opening up about tissue transglutaminase: When conformation
534 matters more than enzymatic activity, *Med One*, 3 (2018).
- 535 [11] A. Case, R.L. Stein, Kinetic analysis of the interaction of tissue transglutaminase with a nonpeptidic slow-
536 binding inhibitor, *Biochemistry*, 46 (2007) 1106-1115.
- 537 [12] K. Thangaraju, B. Biri, G. Schlosser, B. Kiss, L. Nyitray, L. Fesus, R. Kiraly, Real-time kinetic method to
538 monitor isopeptidase activity of transglutaminase 2 on protein substrate, *Anal. Biochem.*, 505 (2016) 36-42.
- 539 [13] M. Pietsch, R. Wodtke, J. Pietzsch, R. Löser, Tissue transglutaminase: An emerging target for therapy and
540 imaging, *Bioorg. Med. Chem. Lett.*, 23 (2013) 6528-6543.
- 541 [14] R. Wodtke, G. Schramm, J. Pietzsch, M. Pietsch, R. Löser, Synthesis and kinetic characterisation of water-
542 soluble fluorogenic acyl donors for transglutaminase 2, *ChemBioChem*, 17 (2016) 1263-1281.
- 543 [15] R. Wodtke, C. Hauser, G. Ruiz-Gómez, E. Jäckel, D. Bauer, M. Lohse, A. Wong, J. Pufe, F.-A. Ludwig, S.
544 Fischer, S. Hauser, D. Greif, M.T. Pisabarro, J. Pietzsch, M. Pietsch, R. Löser, *N^ε*-Acryloyllysine piperazides as
545 irreversible inhibitors of transglutaminase 2: Synthesis, structure-activity relationships, and pharmacokinetic
546 profiling, *J. Med. Chem.*, 61 (2018) 4528-4560.
- 547 [16] S.I. Chung, R.I. Shrager, J.E. Folk, Mechanism of action of guinea pig liver transglutaminase. VII. Chemical
548 and stereochemical aspects of substrate binding and catalysis, *J. Biol. Chem.*, 245 (1970) 6424-6435.
- 549 [17] A. Leblanc, C. Gravel, J. Labelle, J.W. Keillor, Kinetic studies of guinea pig liver transglutaminase reveal a
550 general-base-catalyzed deacylation mechanism, *Biochemistry*, 40 (2001) 8335-8342.
- 551 [18] N.J. Manesis, M. Goodman, Synthesis of a novel class of peptides: Dilactam-bridged tetrapeptides, *J. Org.*
552 *Chem.*, 52 (1987) 5331-5341.
- 553 [19] D.S. Pedersen, Let's talk about TLCs. Part 2 - hanessian's stain, 2006.
554 <http://curlyarrow.blogspot.com/2006/11/lets-talk-about-tlcs-part-2-hanessians.html>; accessed on 10/12/2019.
- 555 [20] E. Klieger, E. Schröder, Synthese von α -Glutamyl-Peptiden mit Carbobenzyloxy-L-glutaminsäure- α -
556 phenylester, *Liebigs Ann. Chem.*, 661 (1963) 193-201.
- 557 [21] E. Badarau, Z. Wang, D.L. Rathbone, A. Costanzi, T. Thibault, C.E. Murdoch, S. El Alaoui, M. Bartkeviciute,
558 M. Griffin, Development of potent and selective tissue transglutaminase inhibitors: Their effect on TG2 function
559 and application in pathological conditions, *Chem. Biol.*, 22 (2015) 1347-1361.
- 560 [22] M. Bergmann, L. Zervas, Über ein allgemeines Verfahren der Peptid-Synthese, *Ber. Dtsch. Chem. Ges.*, 65
561 (1932) 1192-1201.
- 562 [23] J.D. Twibanire, T.B. Grindley, Efficient and controllably selective preparation of esters using uronium-based
563 coupling agents, *Org. Lett.*, 13 (2011) 2988-2991.
- 564 [24] C. Hauser, R. Wodtke, R. Löser, M. Pietsch, A fluorescence anisotropy-based assay for determining the
565 activity of tissue transglutaminase, *Amino Acids*, 49 (2017) 567-583.
- 566 [25] M. Griffin, D. Rathbone, B.L. Eduard, Acylpiperazines as inhibitors of transglutaminase and their use in
567 medicine. WIPO Patent WO 2014/057266 A1, April 17, 2014.
- 568 [26] J. Wityak, M.E. Prime, F.A. Brookfield, S.M. Courtney, S. Erfan, S. Johnsen, P.D. Johnson, M. Li, R.W.
569 Marston, L. Reed, D. Vaidya, S. Schaertl, A. Pedret-Dunn, M. Beconi, D. Macdonald, I. Muñoz-Sanjuan, C.

- 570 Dominguez, SAR development of lysine-based irreversible inhibitors of transglutaminase 2 for huntington's
571 disease, *ACS Med. Chem. Lett.*, 3 (2012) 1024-1028.
- 572 [27] S. Schaertl, M. Prime, J. Wityak, C. Dominguez, I. Muñoz-Sanjuan, R.E. Pacifici, S. Courtney, A. Scheel, D.
573 Macdonald, A profiling platform for the characterization of transglutaminase 2 (TG2) inhibitors, *J. Biomol.*
574 *Screen.*, 15 (2010) 478-487.
- 575 [28] Zedira - Z-MA-QPL-OMe, [https://zedira.com/Mechanism-of-TG-inhibitors/Michael-acceptor-](https://zedira.com/Mechanism-of-TG-inhibitors/Michael-acceptor-peptidomimetics/Z-MA-QPL-OMe_Z013)
576 [peptidomimetics/Z-MA-QPL-OMe_Z013](https://zedira.com/Mechanism-of-TG-inhibitors/Michael-acceptor-peptidomimetics/Z-MA-QPL-OMe_Z013); accessed on 10/12/2019.
- 577 [29] F. Hausch, T. Halttunen, M. Mäki, C. Khosla, Design, synthesis, and evaluation of gluten peptide analogs as
578 selective inhibitors of human tissue transglutaminase, *Chem. Biol.*, 10 (2003) 225-231.
- 579 [30] D.M. Pinkas, P. Strop, A.T. Brunger, C. Khosla, Transglutaminase 2 undergoes a large conformational
580 change upon activation, *PLoS Biol.*, 5 (2007) e327.
- 581 [31] J.W. Williams, J.F. Morrison, The kinetics of reversible tight-binding inhibition, *Methods Enzymol.*, 63 (1979)
582 437-467.
- 583 [32] P.F. Cook, W.W. Cleland, *Enzyme kinetics and mechanism*, Garland Science Publishing, London, New York,
584 2007, p. 203.
- 585 [33] R.A. Copeland, *Evaluation of enzyme inhibitors in drug discovery*, 2nd ed., John Wiley & Sons, Hoboken, NJ,
586 2013, p. 350.
- 587 [34] B. van der Wildt, A.A. Lammertsma, B. Drukarch, A.D. Windhorst, Strategies towards in vivo imaging of
588 active transglutaminase type 2 using positron emission tomography, *Amino Acids*, 49 (2017) 585-595.
- 589 [35] D.D. Clarke, M.J. Mycek, A. Neidle, H. Waelsch, The incorporation of amines into protein, *Arch. Biochem.*
590 *Biophys.*, 79 (1959) 338-354.
- 591 [36] D.J. Walther, S. Stahlberg, J. Vowinckel, Novel roles for biogenic monoamines: From monoamines in
592 transglutaminase-mediated post-translational protein modification to monoaminylation deregulation diseases,
593 *FEBS J.*, 278 (2011) 4740-4755.
- 594 [37] N.A. Muma, Z. Mi, Serotonylation and transamidation of other monoamines, *ACS Chem. Neurosci.*, 6 (2015)
595 961-969.
- 596 [38] T.S. Lai, C.J. Lin, C.S. Greenberg, Role of tissue transglutaminase-2 (TG2)-mediated aminylation in biological
597 processes, *Amino Acids*, 49 (2017) 501-515.
- 598 [39] N.A. Muma, Transglutaminase in receptor and neurotransmitter-regulated functions, *Med One*, 3 (2018).
- 599 [40] D.J. Walther, J.-U. Peter, S. Winter, M. Höltje, N. Paulmann, M. Grohmann, J. Vowinckel, V. Alamo-
600 Bethencourt, C.S. Wilhelm, G. Ahnert-Hilger, M. Bader, Serotonylation of small GTPases is a signal transduction
601 pathway that triggers platelet α -granule release, *Cell*, 115 (2003) 851-862.
- 602 [41] Y. Dai, N.L. Dudek, T.B. Patel, N.A. Muma, Transglutaminase-catalyzed transamidation: A novel
603 mechanism for Rac1 activation by 5-HT_{2a} receptor stimulation, *J. Pharmacol. Exp Ther.*, 326 (2008) 153-162.
- 604 [42] Y. Dai, N.L. Dudek, Q. Li, N.A. Muma, Phospholipase C, Ca²⁺, and calmodulin signaling are required for 5-
605 HT_{2a} receptor-mediated transamidation of Rac1 by transglutaminase, *Psychopharmacology (Berlin)*, 213 (2011)
606 403-412.
- 607 [43] J.C. Lin, C.C. Chou, Z. Tu, L.F. Yeh, S.C. Wu, K.H. Khoo, C.H. Lin, Characterization of protein serotonylation
608 via bioorthogonal labeling and enrichment, *J. Proteome Res.*, 13 (2014) 3523-3529.
- 609 [44] L.A. Farrelly, R.E. Thompson, S. Zhao, A.E. Lepack, Y. Lyu, N.V. Bhanu, B. Zhang, Y.E. Loh, A.
610 Ramakrishnan, K.C. Vadodaria, K.J. Heard, G. Erikson, T. Nakadai, R.M. Bastle, B.J. Lukasak, H. Zebroski, 3rd,
611 N. Alenina, M. Bader, O. Berton, R.G. Roeder, H. Molina, F.H. Gage, L. Shen, B.A. Garcia, H. Li, T.W. Muir, I.

- 612 Maze, Histone serotonylation is a permissive modification that enhances TFIID binding to H3K4me3, *Nature*,
613 567 (2019) 535-539.
- 614 [45] J. Vowinckel, S. Stahlberg, N. Paulmann, K. Bluemlein, M. Grohmann, M. Ralser, D.J. Walther,
615 Histaminylation of glutamine residues is a novel posttranslational modification implicated in G-protein
616 signaling, *FEBS Lett.*, 586 (2012) 3819-3824.
- 617 [46] T.S. Lai, C.S. Greenberg, Histaminylation of fibrinogen by tissue transglutaminase-2 (TGM-2): Potential role
618 in modulating inflammation, *Amino Acids*, 45 (2013) 857-864.
- 619 [47] R. Hummerich, J.O. Thumfart, P. Findeisen, D. Bartsch, P. Schloss, Transglutaminase-mediated
620 transamidation of serotonin, dopamine and noradrenaline to fibronectin: Evidence for a general mechanism of
621 monoaminylation, *FEBS Lett.*, 586 (2012) 3421-3428.
- 622 [48] R. Hummerich, V. Costina, P. Findeisen, P. Schloss, Monoaminylation of fibrinogen and glia-derived
623 proteins: Indication for similar mechanisms in posttranslational protein modification in blood and brain, *ACS*
624 *Chem. Neurosci.*, 6 (2015) 1130-1136.
- 625 [49] S.W. Qiao, J. Piper, G. Haraldsen, I. Oynebraten, B. Fleckenstein, O. Molberg, C. Khosla, L.M. Sollid, Tissue
626 transglutaminase-mediated formation and cleavage of histamine-gliadin complexes: Biological effects and
627 implications for celiac disease, *J. Immunol.*, 174 (2005) 1657-1663.
- 628 [50] P.I. Nagy, Competing intramolecular vs. Intermolecular hydrogen bonds in solution, *Int. J. Mol. Sci.*, 15
629 (2014) 19562-19633.
- 630 [51] J.W. Keillor, C.M. Clouthier, K.Y. Apperley, A. Akbar, A. Mulani, Acyl transfer mechanisms of tissue
631 transglutaminase, *Bioorg. Chem.*, 57 (2014) 186-197.
- 632 [52] M. Bader, Serotonylation: Serotonin signaling and epigenetics, *Front. Mol. Neurosci.*, 12 (2019) 288.
- 633 [53] J. Stammaes, B. Fleckenstein, L.M. Sollid, The propensity for deamidation and transamidation of peptides by
634 transglutaminase 2 is dependent on substrate affinity and reaction conditions, *Biochim. Biophys. Acta*, 1784
635 (2008) 1804-1811.
- 636 [54] E.A. Wells, M.A. Anderson, T.N. Zeczycki, (15)(V/K) kinetic isotope effect and steady-state kinetic analysis
637 for the transglutaminase 2 catalyzed deamidation and transamidation reactions, *Arch. Biochem. Biophys.*, 643
638 (2018) 57-61.
- 639

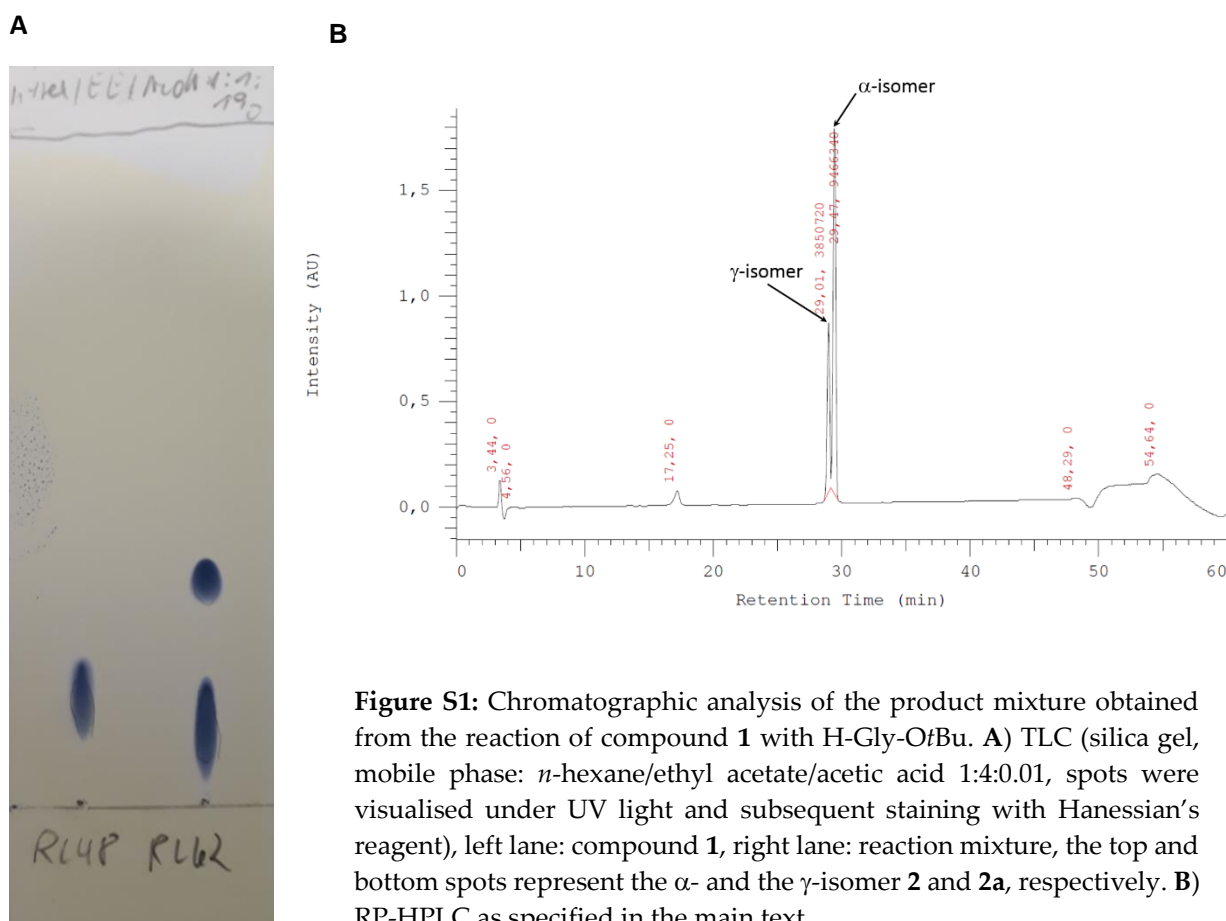
Supporting Information

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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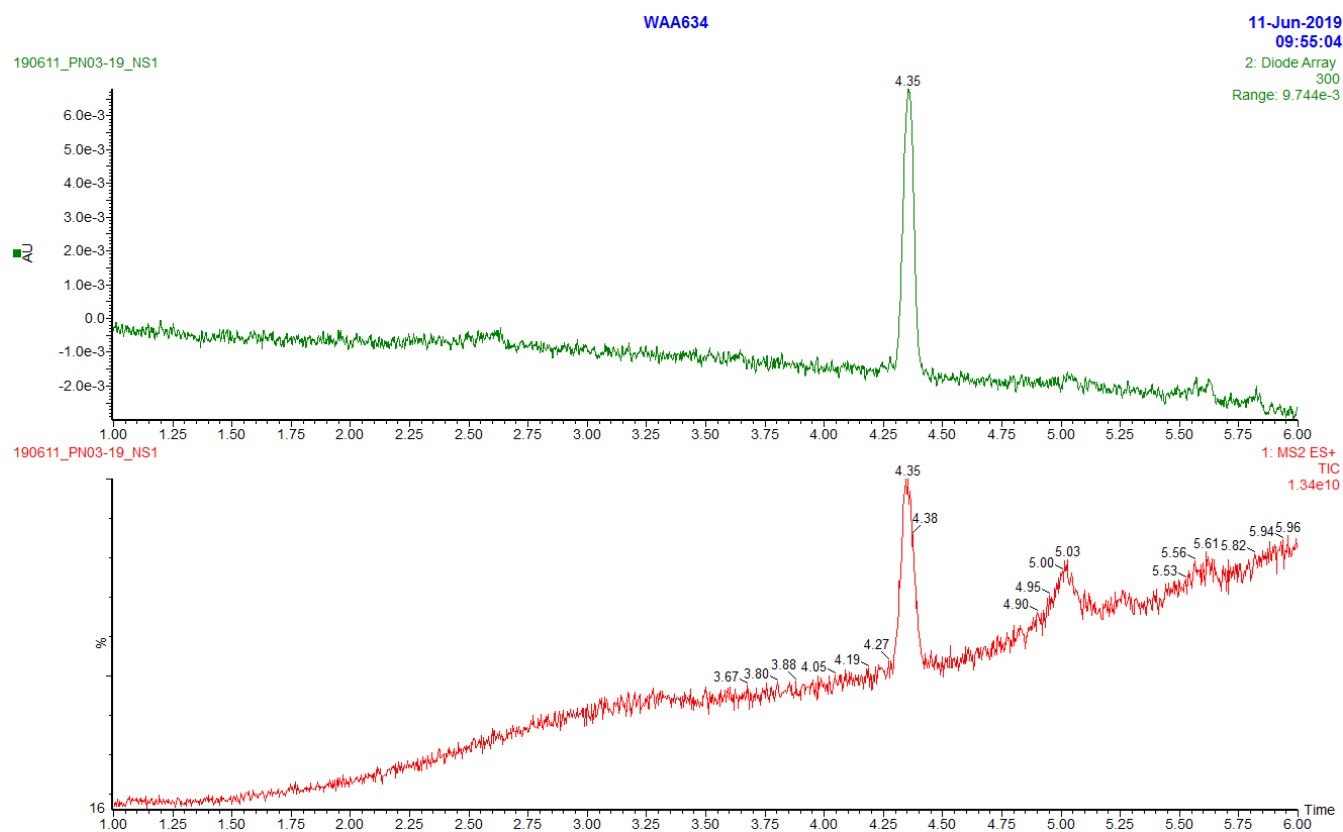


Figure S2: UPLC-MS analysis of Z-Glu(HMC)-Gly-OH (**4**). The upper panel shows the UV trace at 300 nm of the photo diode array (PDA) detector. The lower panel shows the total ion current as detected by the ESI-MS detector. For details of UPLC-MS analysis see Scheme S1.

Figure S3: ^1H NMR Spectrum of compound **1**

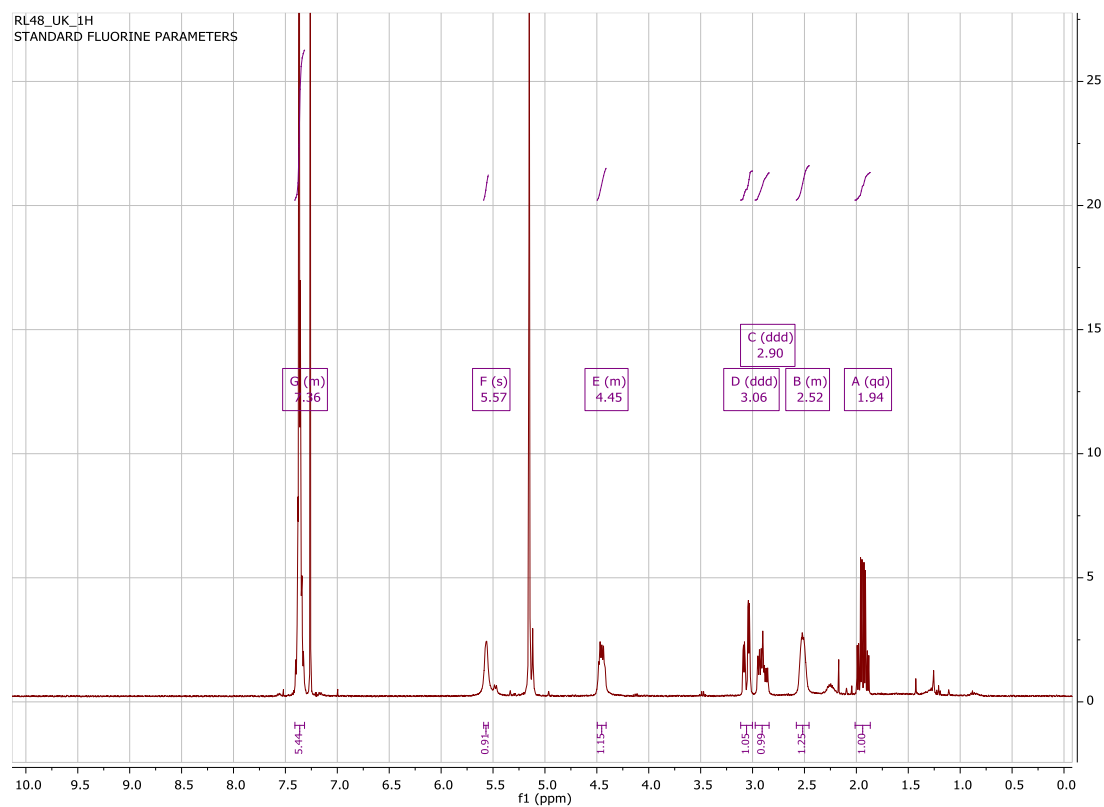


Figure S4: ^{13}C NMR Spectrum of compound **1**

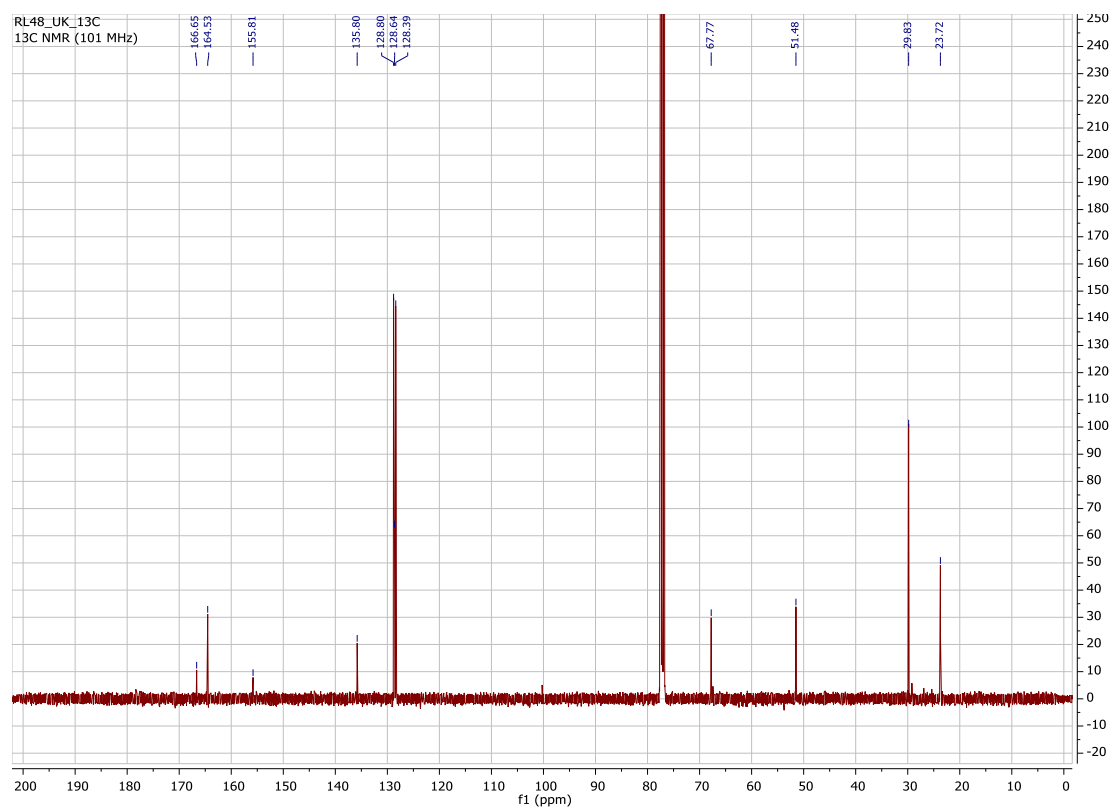


Figure S5: ^1H , ^{13}C HSQC Spectrum of compound 1

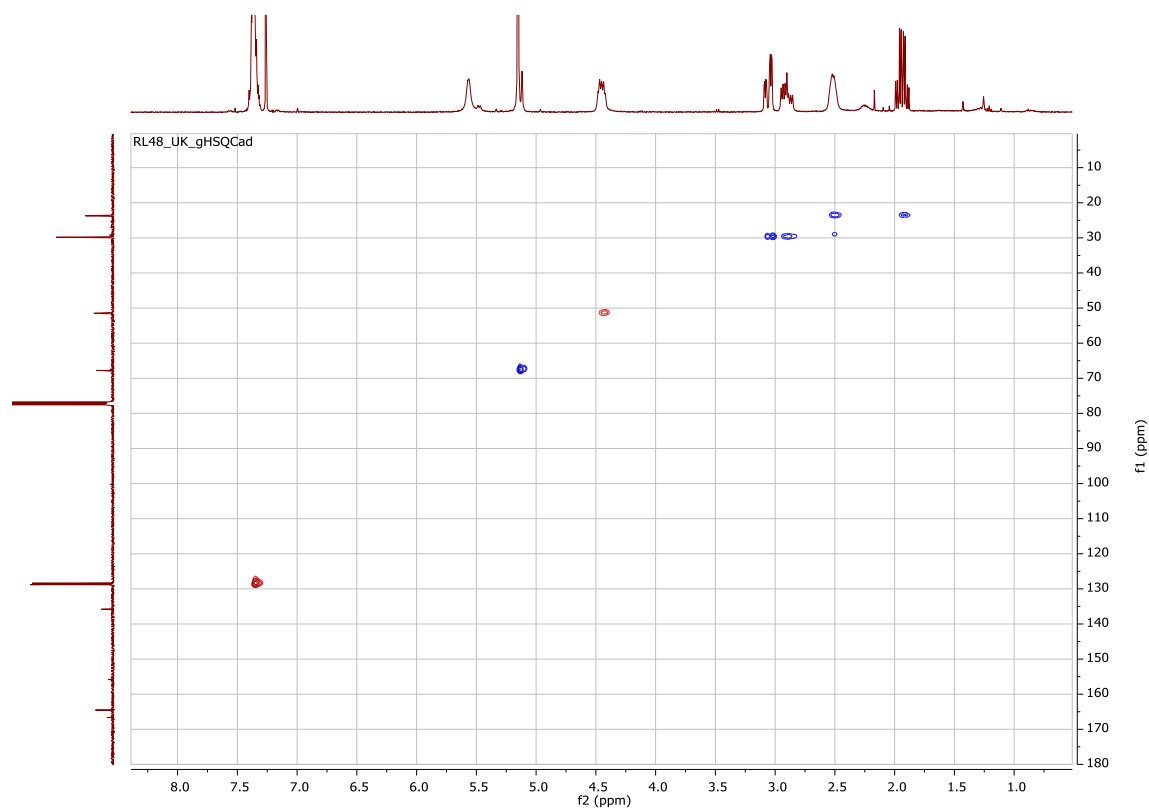


Figure S6: ^1H NMR Spectrum of compound 2

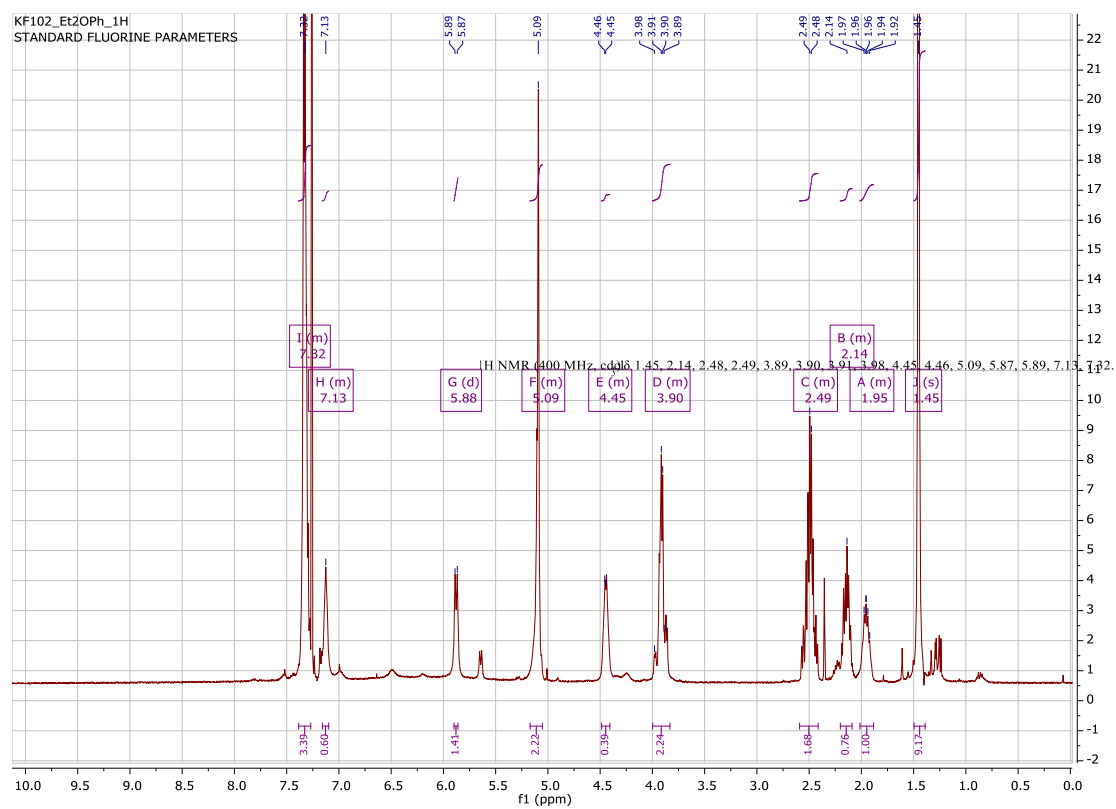


Figure S7: ^{13}C NMR Spectrum of compound 2

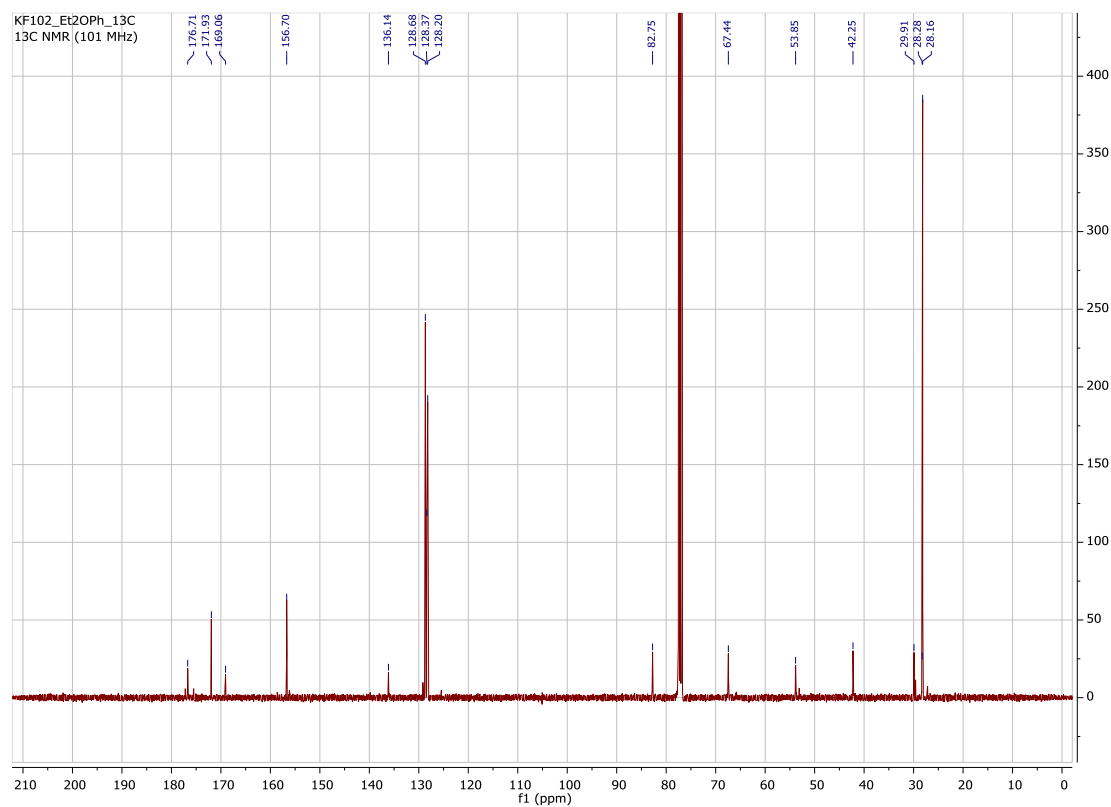


Figure S8: ^1H , ^{13}C HSQC Spectrum of compound 2

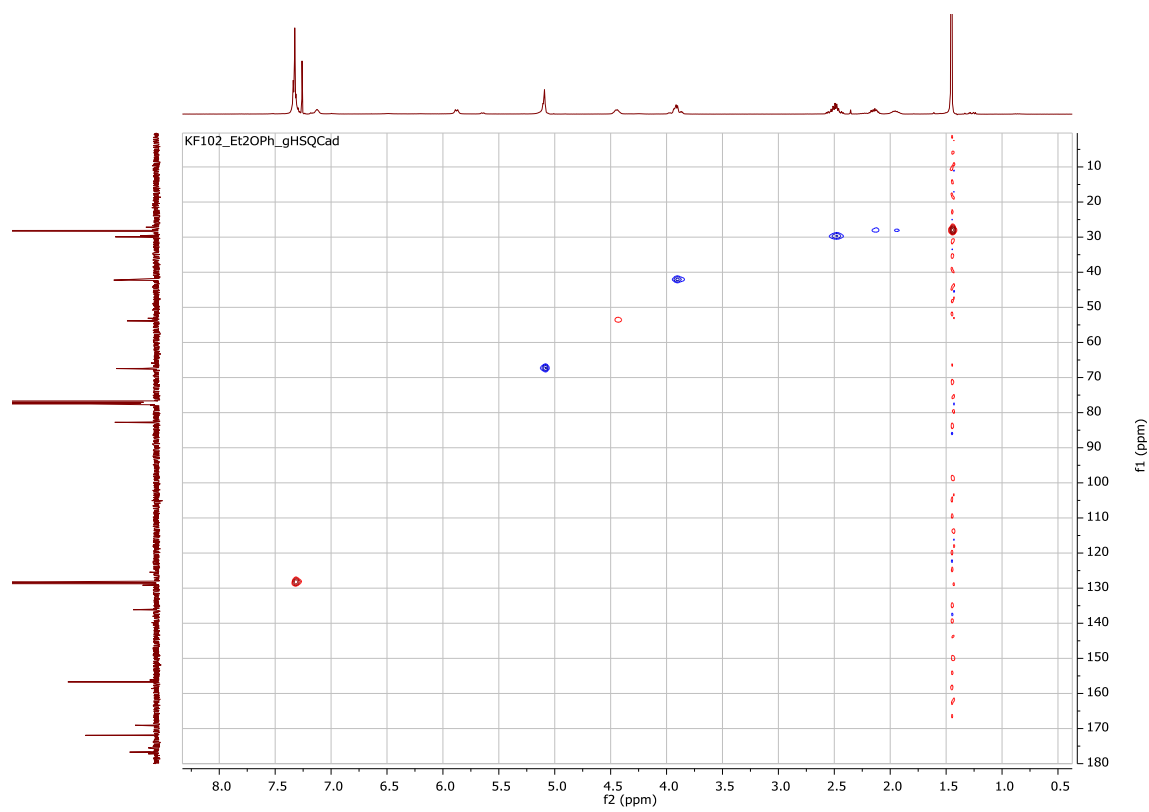


Figure S9: ^1H NMR Spectrum of compound 2a

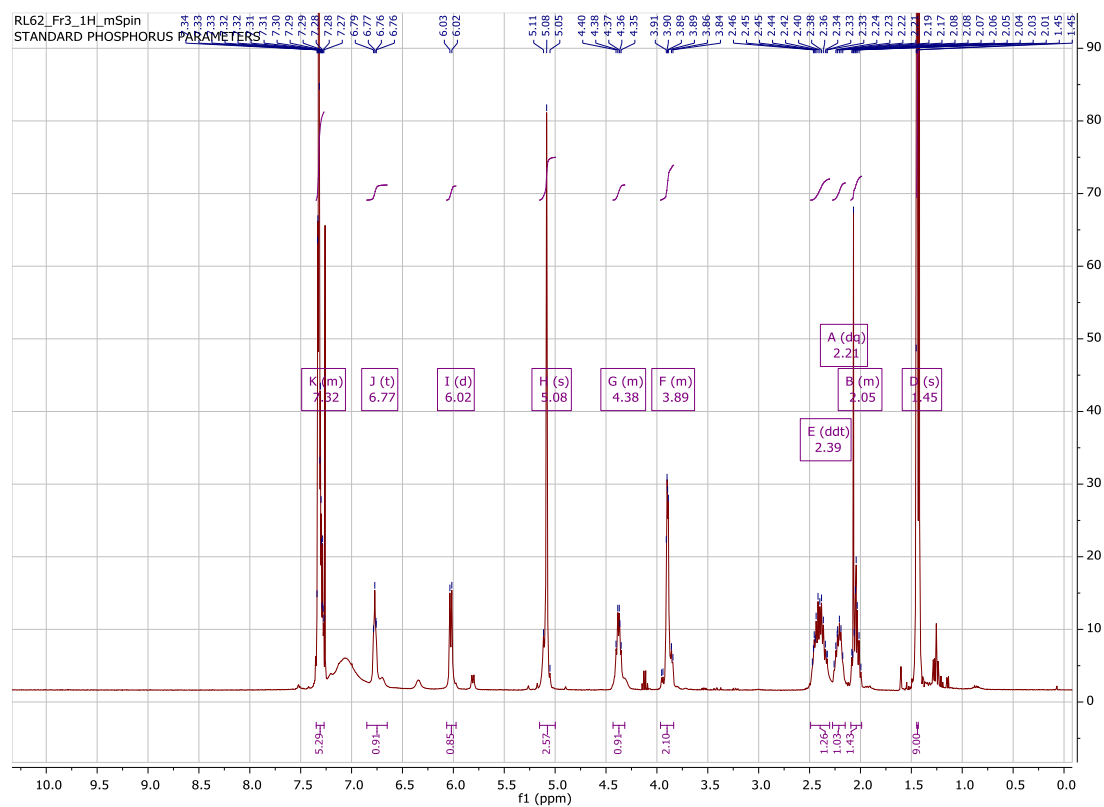


Figure S10: ^{13}C NMR Spectrum of compound 2a

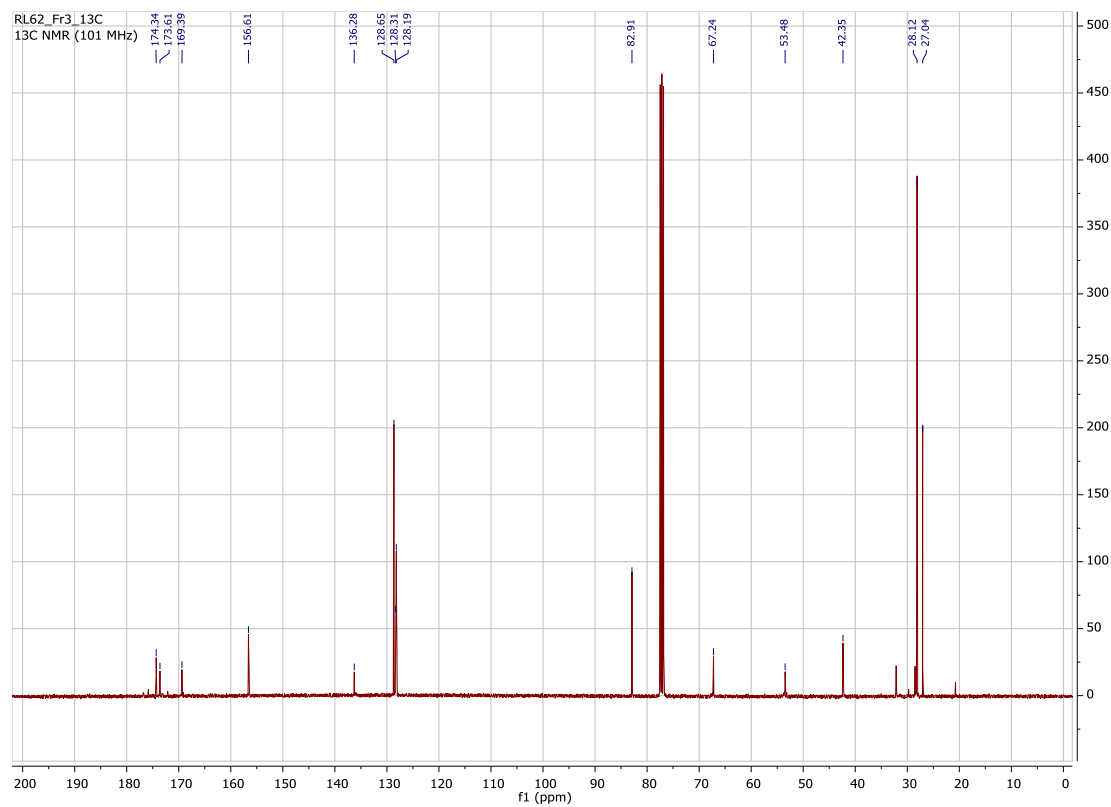


Figure S11: ^1H , ^{13}C HSQC Spectrum of compound 2a

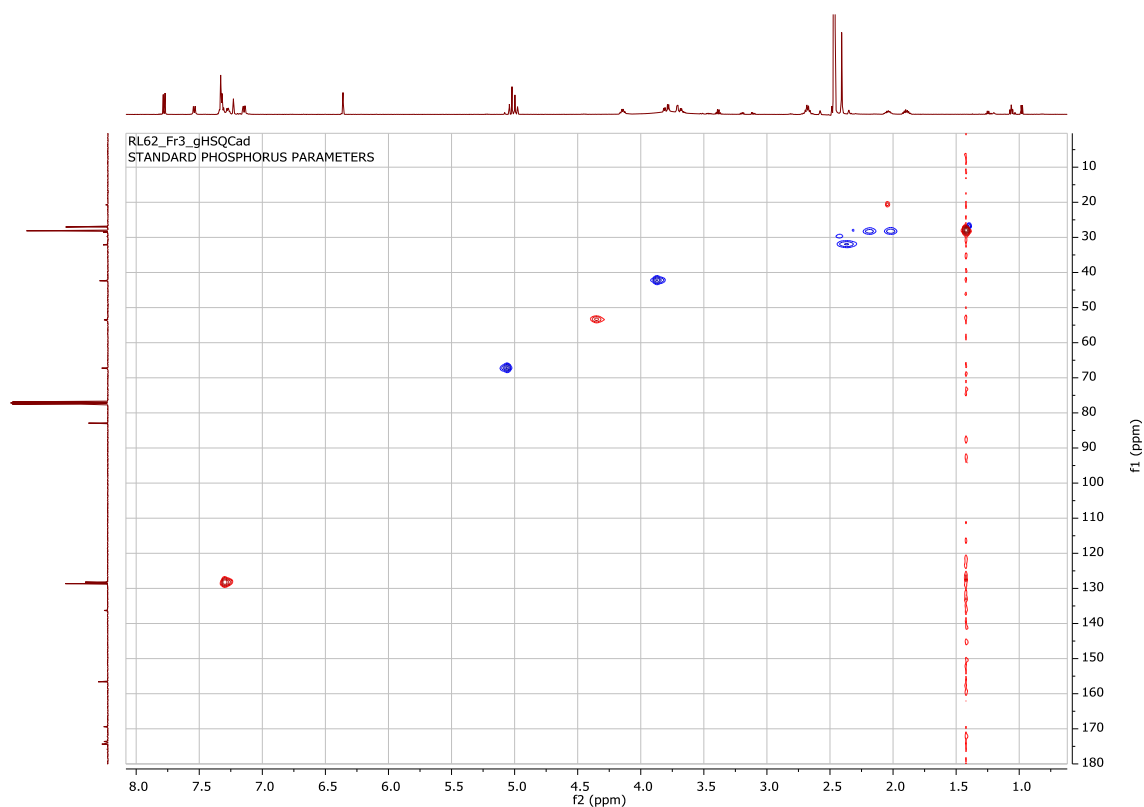


Figure S12: ^1H NMR Spectrum of compound 3

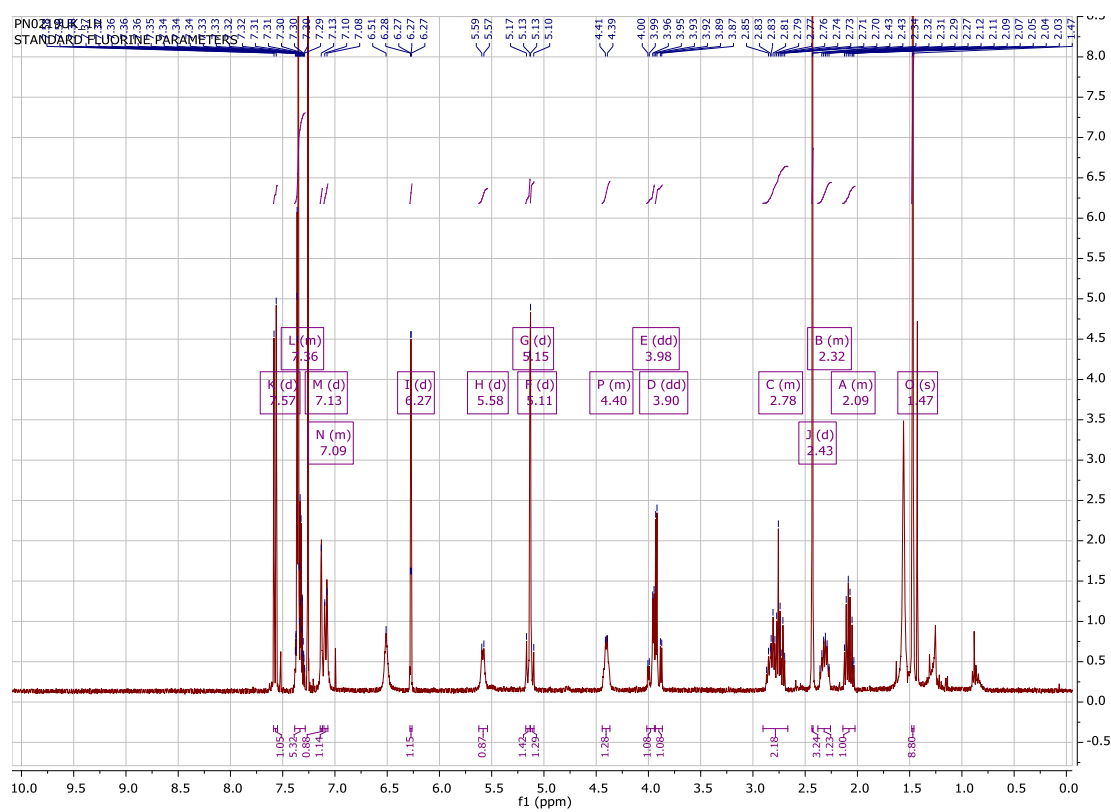


Figure S13: TOCSY Spectrum of compound 3

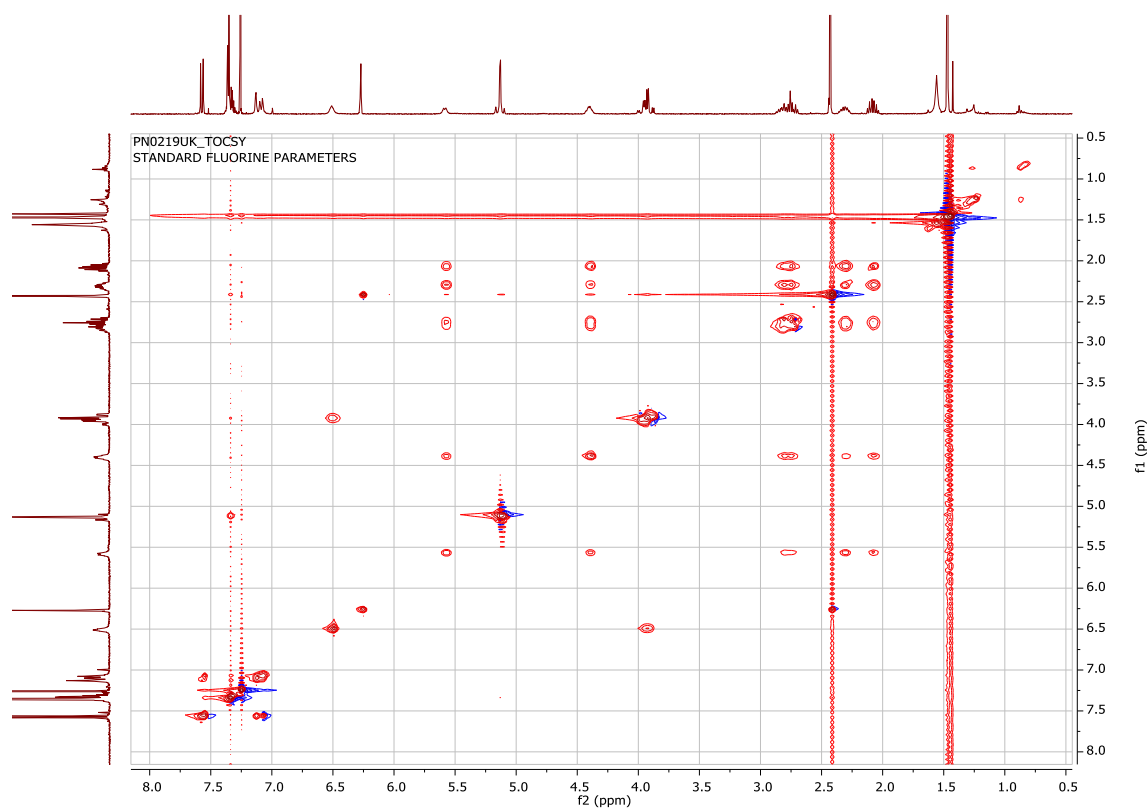


Figure S14: ¹³C NMR Spectrum of compound 3

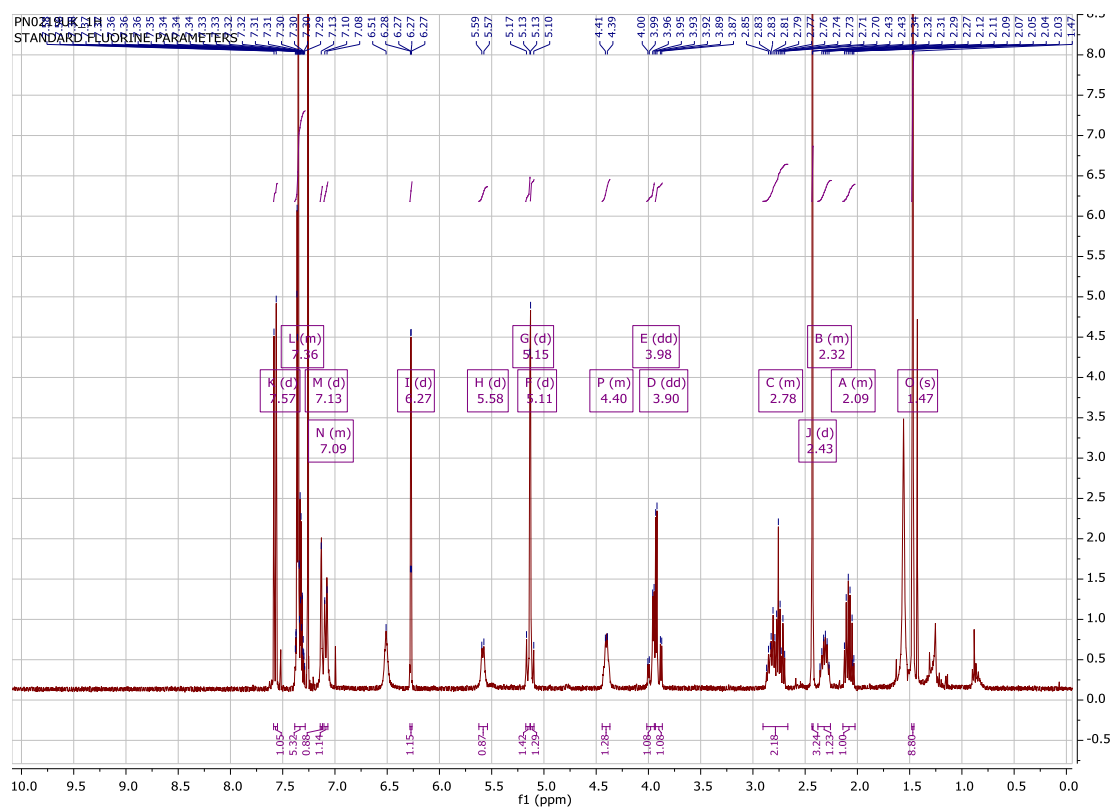


Figure S15: ^1H , ^{13}C HSQC Spectrum of compound 3

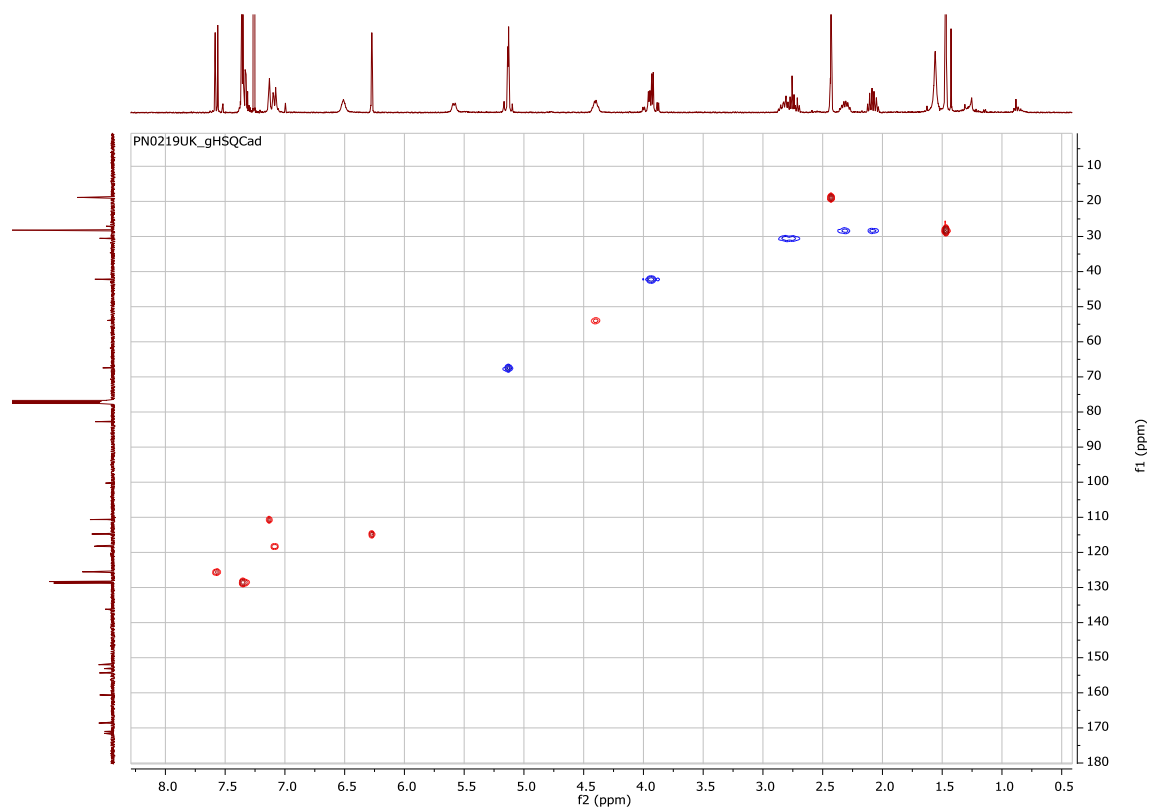


Figure S16: ^1H NMR Spectrum of compound 4 (Z-Glu(HMC)-Gly-OH)

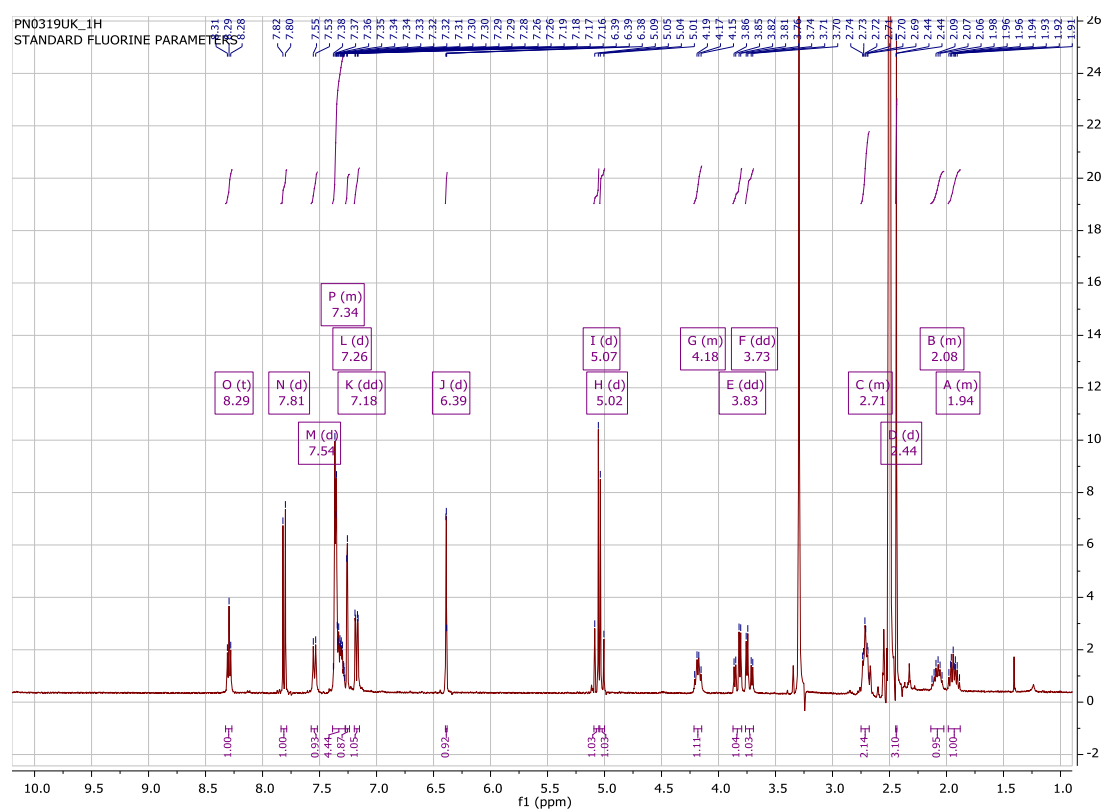


Figure S17: ^{13}C NMR Spectrum of compound **4** (Z-Glu(HMC)-Gly-OH)

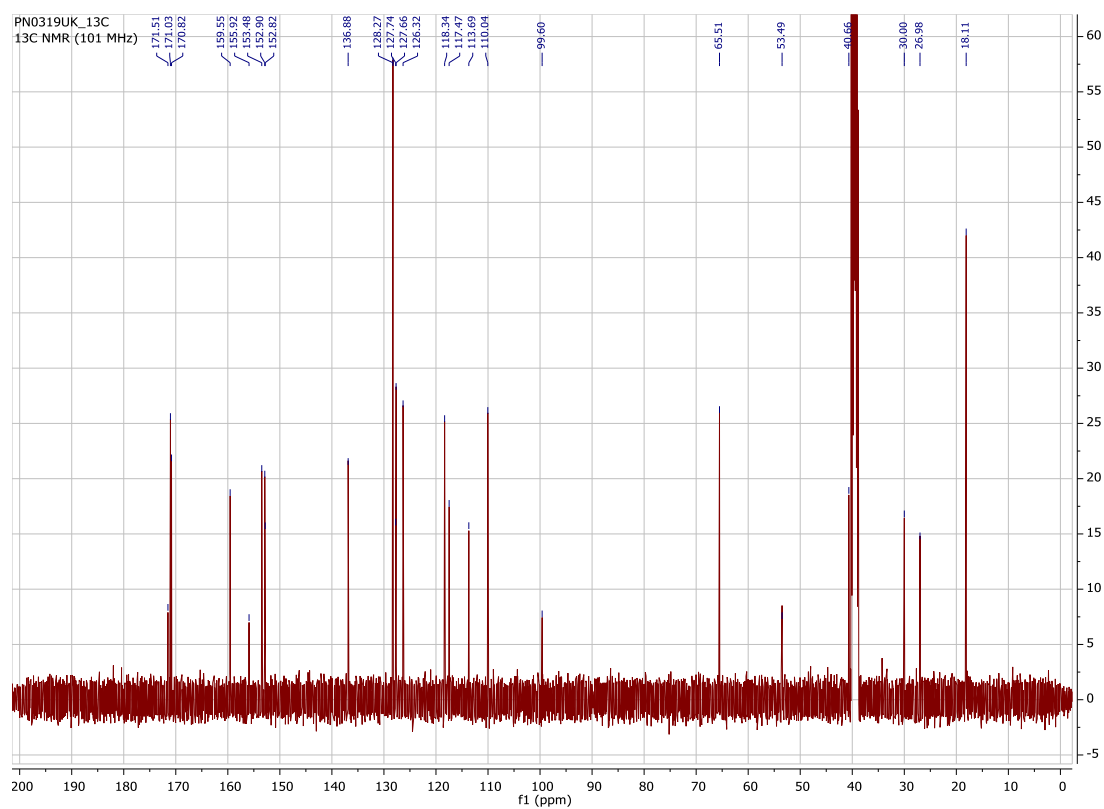
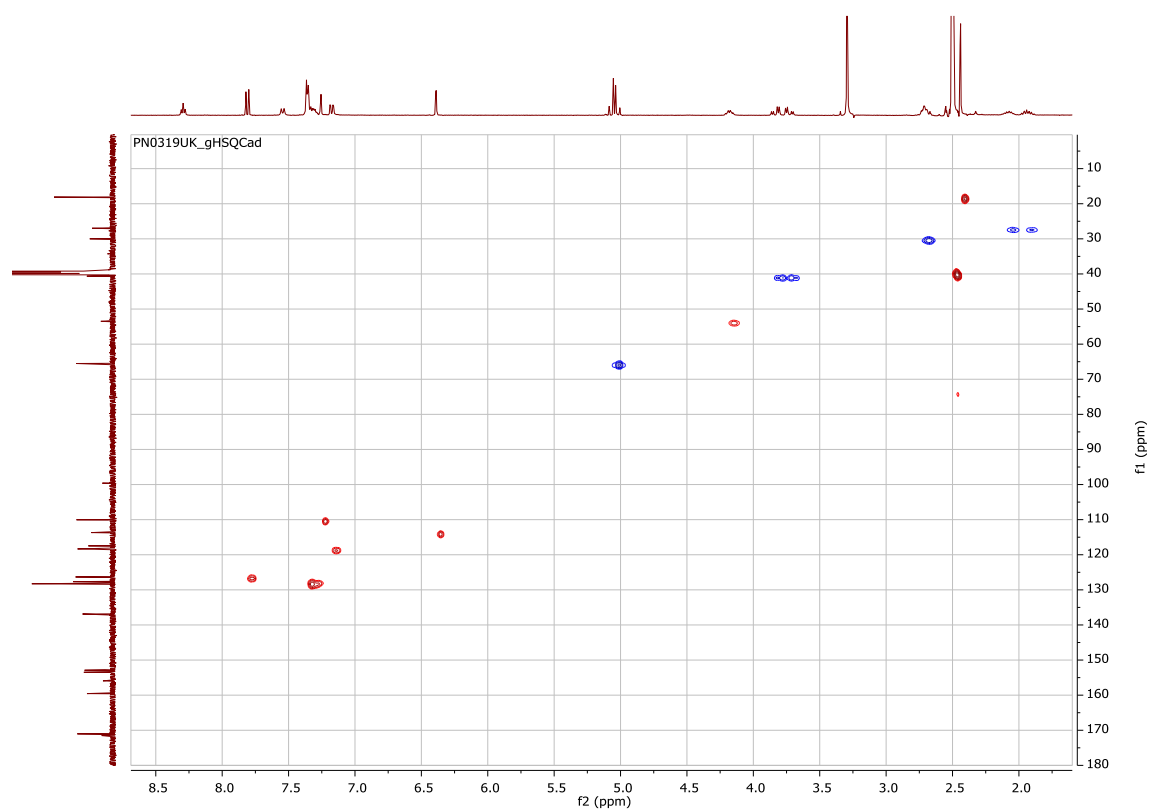


Figure S18: $^1\text{H},^{13}\text{C}$ HSQC Spectrum of compound **4** (Z-Glu(HMC)-Gly-OH)



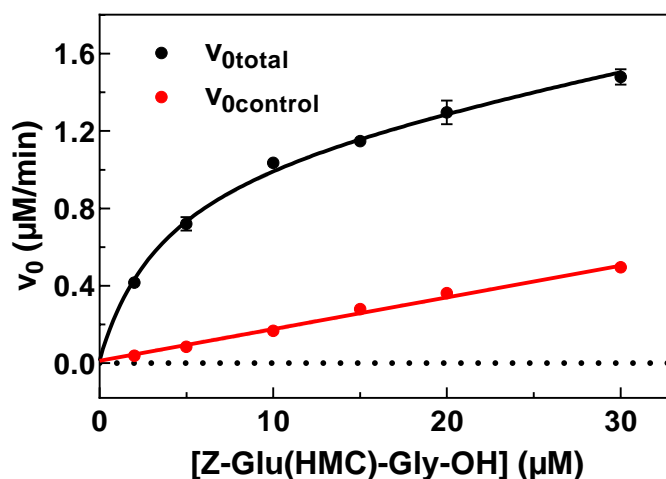


Figure S19: hTGase 2-catalysed hydrolysis of Z-Glu(HMC)-Gly-OH (**4**) at pH 8.0

Plots of $v_{0\text{total}}=f([\text{Z-Glu(HMC)-Gly-OH}])$ and $v_{0\text{control}}=f([\text{Z-Glu(HMC)-Gly-OH}])$ 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 $v_{0\text{corr}}=f([\text{Z-Glu(HMC)-Gly-OH}])$). 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 $\mu\text{g}/\text{mL}$ hTGase 2.

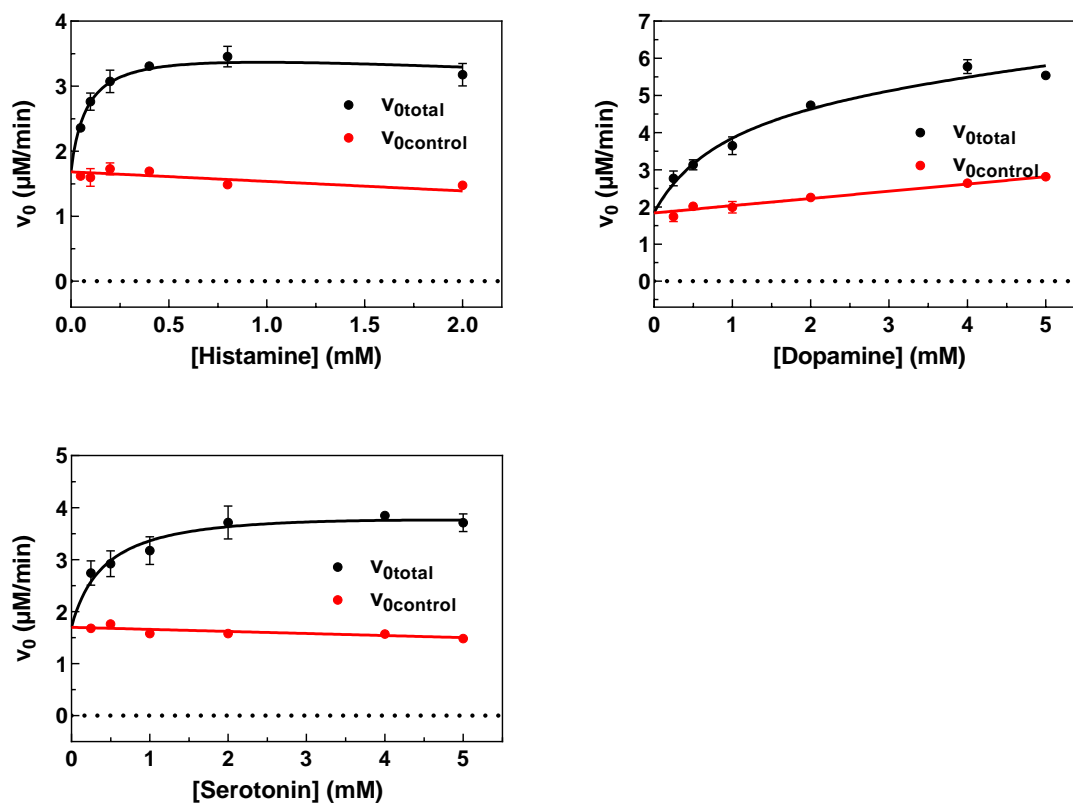
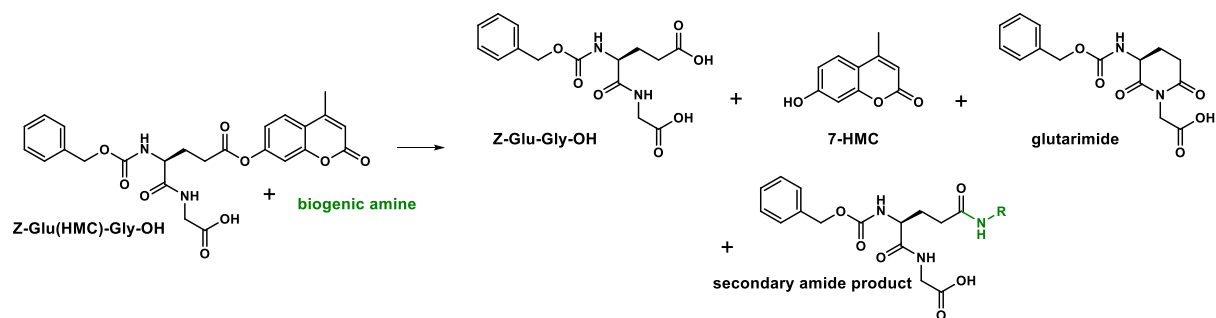


Figure S20: hTGase 2-catalysed incorporation of different biogenic amines into Z-Glu(HMC)-Gly-OH (**4**; $c=100 \mu\text{M}$) at pH 8.0

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 μM 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 μ L) were diluted with 1.25% HOAc/CH₃CN (800 μ 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 μ 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 λ -Detector coupled to a Xevo TQ-S mass spectrometer) and water (30 μ L) was added. A volume of 1-5 μ L of those solutions was injected into the system. A Waters Acquity BEH C18 column (2.1x100 mm, 1,7 μ m particle size) was used as stationary phase. A binary gradient system of water (containing 0.1% CH₃COOH) as solvent and CH₃CN/CH₃OH (1:1, containing 0.1% CH₃COOH) 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

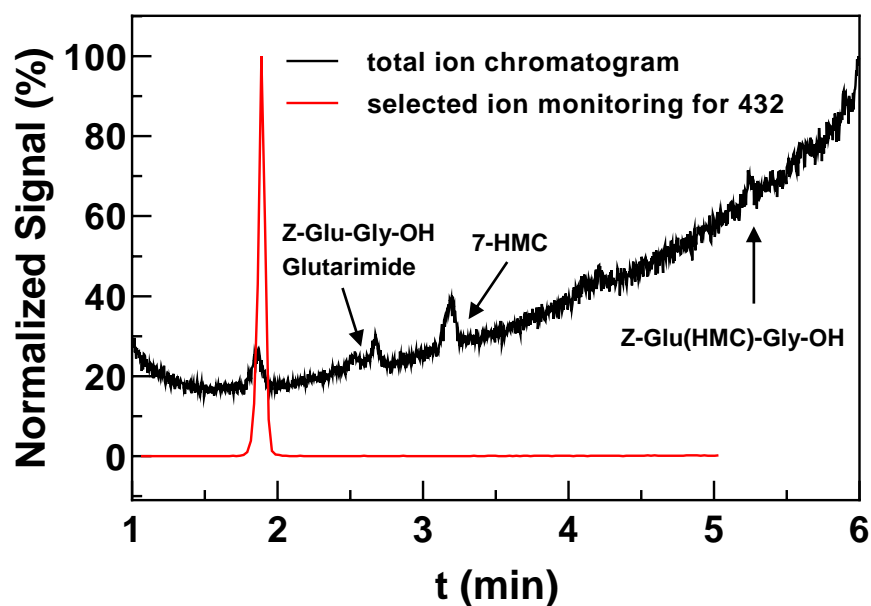


Figure S21: LC-MS analysis for the enzymatic aminolysis of **Z-Glu(HMC)-Gly-OH** with histamine. Conditions for the reaction mixture: pH 8.0, 30 °C, 5% DMSO, 500 μ M TCEP, 0.6 μ g/mL of hTGase 2, 2 mM of histamine, 100 μ M of **Z-Glu(HMC)-Gly-OH**, stop of the reaction after 1 h.

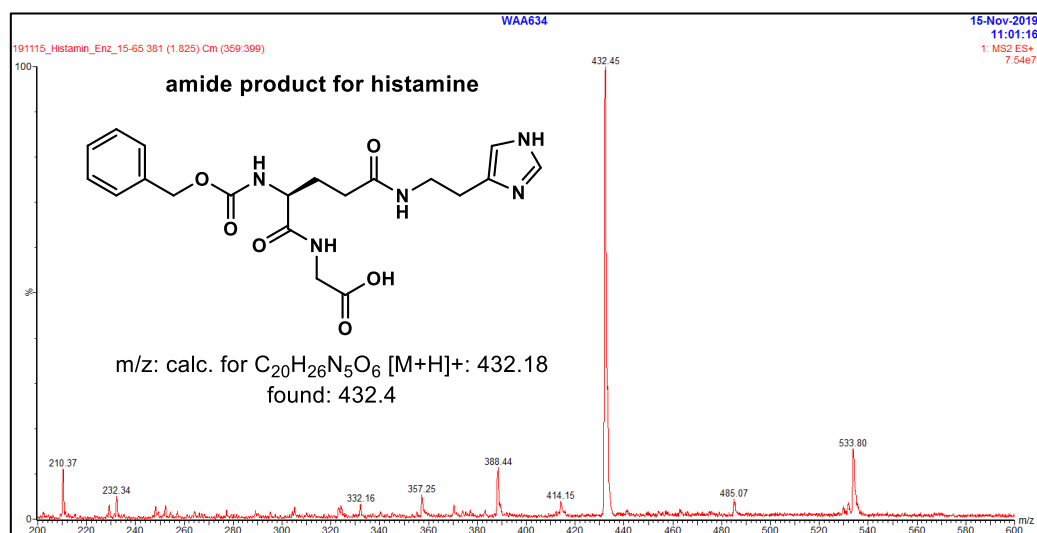


Figure S22: ESI(+) mass spectrum of the identified amide product for histamine.

Analysis for dopamine

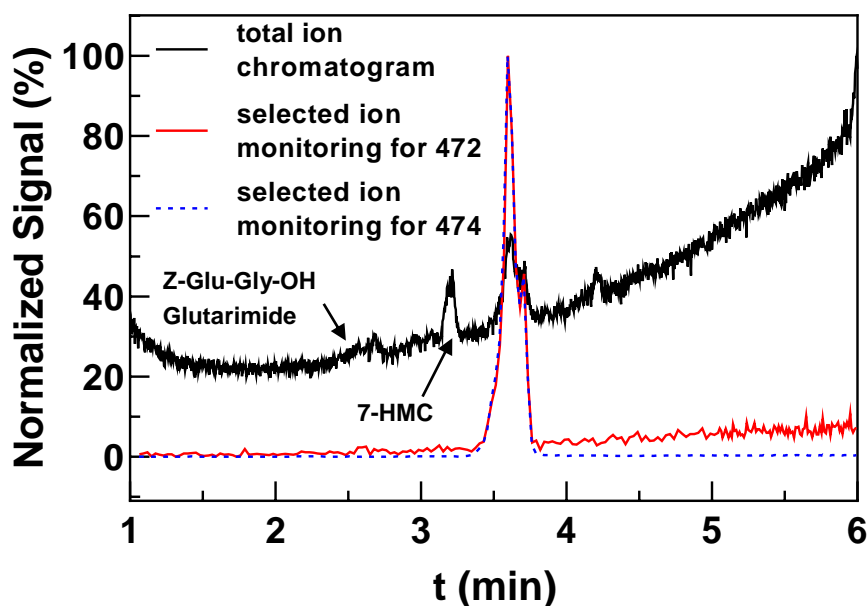


Figure S23: LC-MS analysis for the enzymatic aminolysis of **Z-Glu(HMC)-Gly-OH** with dopamine. Conditions for the reaction mixture: pH 8.0, 30 °C, 5% DMSO, 500 μ M TCEP, 2 μ g/mL of hTGase 2, 5 mM of dopamine, 100 μ M of **Z-Glu(HMC)-Gly-OH**, stop of the reaction after 1 h. The additional signal for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

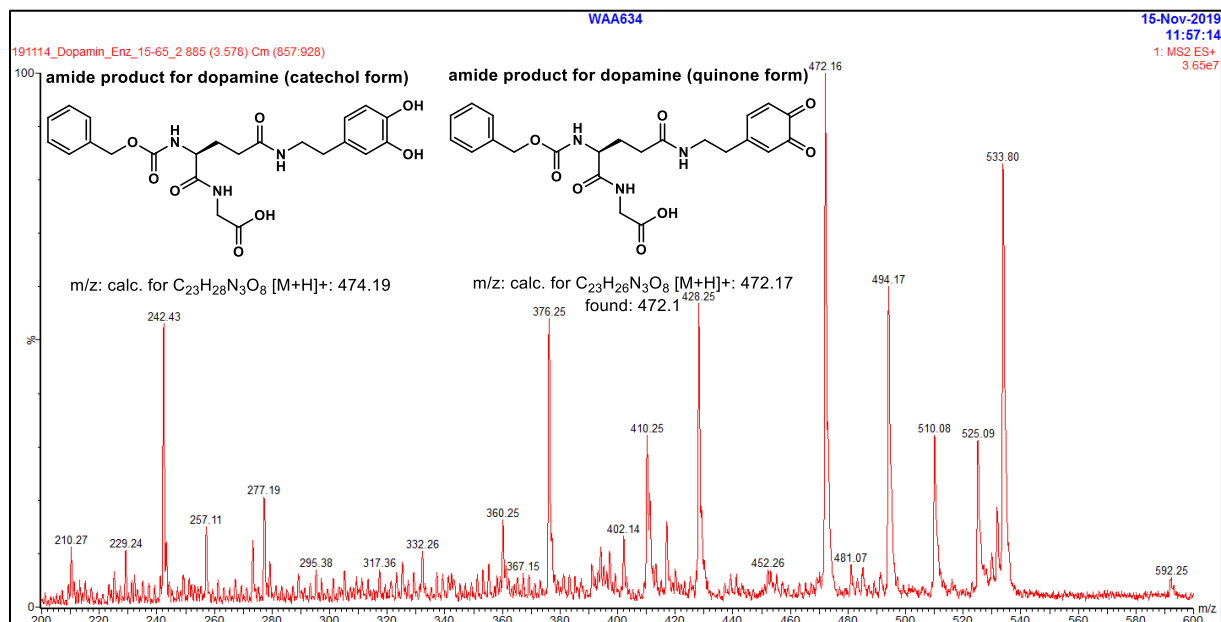


Figure S24: ESI(+) mass spectrum of the identified amide product for dopamine.

Analysis for serotonin

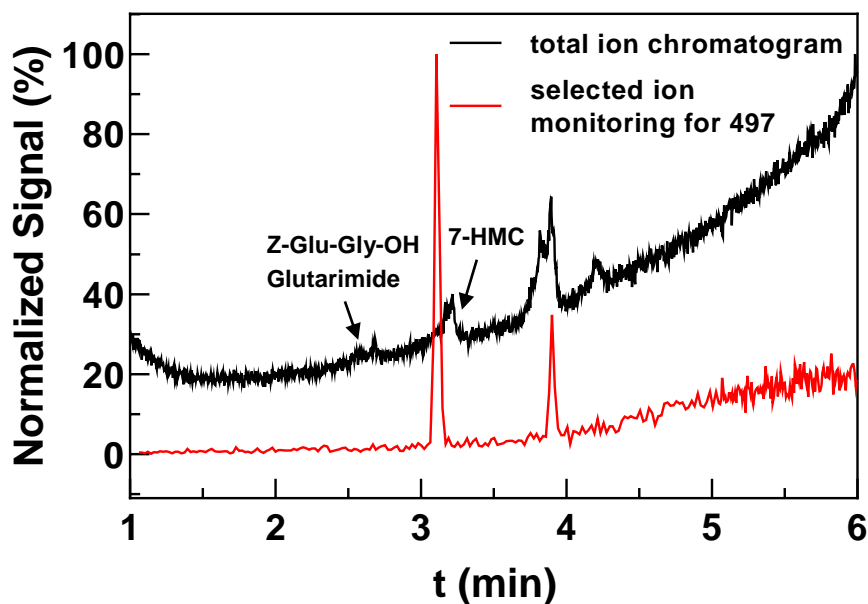


Figure S25: LC-MS analysis for the enzymatic aminolysis of **Z-Glu(HMC)-Gly-OH** with dopamine. Conditions for the reaction mixture: pH 8.0, 30 °C, 5% DMSO, 500 μ M TCEP, 3 μ g/mL of hTGase 2, 5 mM of serotonin, 100 μ M of **Z-Glu(HMC)-Gly-OH**, stop of the reaction after 1 h.

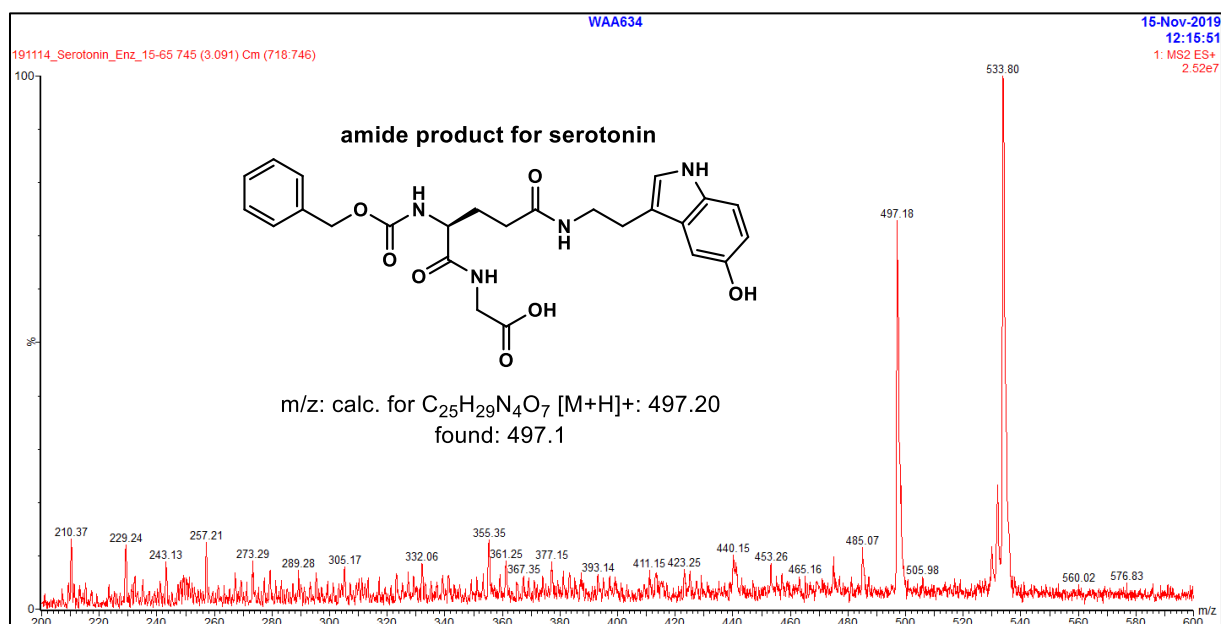


Figure S26: ESI(+) mass spectrum of the identified amide product for serotonin.

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.