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Synthesis of Profluorescent Strigolactone Probes for Biochemical Studies

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1 **Chapter's title: Profluorescent strigolactone probes for biochemical studies**

2

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9 **Running head: Profluorescent strigolactone probes**

12 **i. Summary/Abstract**

13 In this chapter, we will describe a method we set up to synthesize two profluorescent strigolactone (SL) mimic
14 probes (GC240 and GC242) and the optimized protocols developed to study the enzymatic properties of various
15 strigolactone receptors. The Arabidopsis AtD14 SL receptor is used here as a model for this purpose.

17 **ii. Key Words**

18 Strigolactones, Bioactive profluorescent probes, Fluorescence, α/β Hydrolase, Receptor, Enzymatic properties

20 **1. Introduction**

21 In this chapter, we present a method for characterizing strigolactone (SL) α/β hydrolase receptors containing the
22 Ser, His and Asp catalytic triad located in the hydrophobic active site. The protocols involving direct hydrolyses
23 of SLs have several drawbacks: large consumption of SL receptors and substrates, long reaction time for
24 enzymatic hydrolysis and tedious HPLC analysis (1). In order to develop a more convenient assay, we designed
25 specific profluorescent probes exhibiting high bioactivity, increased stability *versus* hydrolysis and better
26 detection sensitivity. Organic fluorophores are widely used as signal reporter for many applications because they
27 generally exhibit high sensitivity of detection (1-100 nM) depending on their brilliance (2, 3). Especially,
28 measurement of enzyme activity in the biological sciences (4) has seen significant progress in recent years with
29 the design of enzyme-triggerable off-on probes with high signal/background ratio (e.g., profluorophores) (5, 6).
30 Contrary to classical fluorescent probes, also developed in SL research (7), they exhibit a unique selectivity due
31 to the release of the signal following a specific event. In our case, we present here probes that inhibit shoot-
32 branching *in planta* (8) (see 1), but also release a fluorescent signal following their enzymatic hydrolysis. We
33 used this smart strategy to design several bioactive profluorescent SL mimics, the most effective of which are
34 described here (GC series, Fig. 1a). These compounds allowed us to detect the *in vitro* α - β /hydrolase activity of
35 RMS3 (8), the pea homolog of the SL receptor AtD14/D14/DAD2 and to highlight its velocity in the first few
36 minutes of the reaction (9) (Fig. 1b). Noteworthy, a similar probe has also been developed by Tsuchiya et al (10).
37 Here, we determine the kinetic constants of our probes using AtD14 protein to characterize the hydrolase activity
38 of this SL receptor. These probes allow developing a simple bioassay for a potential high-throughput chemical
39 screening for the discovery of putative SL receptor agonists and/or antagonists (8).

40 **2. Materials**

41 **2.1. General experimental procedures for the synthesis of profluorescent SL probes**

- 42 1. Run all non-aqueous reactions under an inert atmosphere (argon), by using standard techniques for
43 manipulating air-sensitive compounds.
- 44 2. Store all glassware in the oven and/or it was flame-dried prior to use.
- 45 3. Obtain anhydrous solvents by filtration through drying columns or from commercial suppliers.
- 46 4. Perform analytical thin-layer chromatographies (TLC) on plates precoated with silica gel layers.
47 Visualize compounds by one or more of the following methods: (1) illumination with a short
48 wavelength UV lamp (i.e., $\lambda = 254$ nm), (2) spray with a 1% (w/v) KMnO_4 solution in H_2O .
- 49 5. Perform flash column chromatography using 40-63 mesh silica.

50 **2.2 (\pm)-GC240: 6,8-difluoro-4-methyl-7-[(4-methyl-5-oxo-2,5-dihydrofuran-2-yl)oxy]-2H-chromen-2-one**

- 51 1. Add to a solution of 5-bromo-3-methylfuran-2(5H)-one (26 mg, 150 μmol) prepared according to Wolff
52 et al. (11) in CH_3CN (1 mL) 6,8-difluoro-7-hydroxy-4-methyl-2H-chromen-2-one (DiFMU) (see 2 and
53 3) (16 mg, 75 μmol) and anhydrous *N,N*-diisopropylethylamine (DIEA) (52 μL , 300 μmol) (see 4).
- 54 2. Stir the resulting mixture at room temperature for 12 h and check for completion by TLC
55 (Heptane/EtOAc 1:1).
- 56 3. Remove the solvent under vacuum.
- 57 4. Purify the resulting residue on a silica gel column (Heptane/EtOAc 6:4) giving (\pm)-GC240 as a white
58 solid (22 mg, 71 μmol , 95 %).
- 59 5. Obtain the following characterizations for (\pm)-GC240 (see 5). R_f (heptane/EtOAc, 1:1, v/v) = 0.41.
60 M.p.: 191 $^\circ\text{C}$. $^1\text{H-NMR}$ (300 MHz, CDCl_3): δ 2.00-2.02 (t, $J = 1.4$ Hz, 3H), 2.40-2.41 (d, $J = 1.6$ Hz,
61 3H), 6.35 (s, 1H), 6.40-6.41 (t, $J = 1.5$ Hz, 1H), 7.09-7.10 (t, $J = 1.7$ Hz, 1H), 7.15-7.19 (dd, $J_1 = 10.3$
62 Hz, $J_2 = 2.3$ Hz, 1H) (Fig. 2a). $^{13}\text{C-NMR}$ (75 MHz, CDCl_3): δ 10.8 (CH_3), 18.9 (CH_3), 101.1-101.2
63 (CH , t, $J = 3.0$ Hz), 105.8-106.3 (CH , dd, $J_1 = 21.8$ Hz, $J_2 = 3.8$ Hz), 116.1 (CH), 117.3-117.4 (C, d, $J =$
64 8.7 Hz), 135.4 (C), 141.4 (CH), 142.0-142.1 (C, d, $J = 5.0$ Hz), 145.3-145.4 (C, d, $J = 3.8$ Hz), 150.0-
65 150.1 (C, d, $J = 3.3$ Hz), 151.1 (C), 153.4-153.5 (C, d, $J = 2.2$ Hz), 158.7 (C), 170.6 (C). IR ν_{max} (film,
66 cm^{-1}): 701, 731, 753, 815, 832, 870, 883, 938, 955, 987, 1041, 1102, 1165, 1202, 1266, 1300, 1318,
67 1340, 1373, 1406, 1454, 1508, 1574, 1633, 1741, 1776, 2344, 2925, 3083. HRMS (ESI): m/z calc. for
68 $\text{C}_{15}\text{H}_{11}\text{F}_2\text{O}_5$ $[\text{M} + \text{H}]^+$: 309.0575, found: 309.0581.
- 69 6. Prepare a (\pm)-GC240 stock solution at 10 mM in DMSO (see 6).

70 **2.3 (±)-GC242: 6,8-difluoro-4-methyl-7-[(3,4-dimethyl-5-oxo-2,5-dihydrofuran-2-yl)oxy]-2H-chromen-2-one**
71 (see 7)

- 72 1. Add sequentially DiFMU (see 2) (21 mg, 100 μmol) and anhydrous DIEA (70 μL, 400 μmol) (see 4) to
73 a solution of 5-chloro-3,4-dimethylfuran-2(5H)-one (29 mg, 200 μmol) synthesized according to
74 Canévet et al. (12) in CH₃CN (1 mL).
- 75 2. Stir the resulting mixture at room temperature for 12 h until the desired product precipitates.
- 76 3. Filtrate off the suspension, rinse with Et₂O to give pure racemic (±)-GC242 (see 8) as a white solid (31
77 mg, 96 μmol, 96 %).
- 78 4. Obtain the following characterizations for (±)-GC242 (see 5). R_f(heptane/EtOAc, 1:1, v/v) = 0.30. M.p.
79 181 °C. ¹H-NMR (300 MHz, CDCl₃): δ 1.92-1.93 (t, *J* = 1.2 Hz, 3H), 2.19 (s, 3H), 2.42-2.43 (d, *J* =
80 1.4 Hz, 3H), 6.17 (s, 1H), 6.36 (s, 1H), 7.18-7.22 (dd, *J*₁ = 10.3 Hz, *J*₂ = 2.3 Hz, 1H) (Fig. 2b). ¹³C-
81 NMR (75 MHz, CDCl₃): δ 8.7 (CH₃), 11.6 (CH₃), 18.9 (CH₃), 98.4 (C), 103.1-103.2 (CH, t, *J* = 3.1
82 Hz), 106.0-106.3 (CH, dd, *J*₁ = 21.8 Hz, *J*₂ = 3.8 Hz), 116.1 (CH), 127.7 (C), 141.9-142.0 (C, d, *J* = 5.0
83 Hz), 145.3-145.4 (C, d, *J* = 6.5 Hz), 150.0-150.1 (C, d, *J* = 3.2 Hz), 151.1-151.2 (C, d, *J* = 2.8 Hz),
84 153.2 (C), 153.4 (C), 158.7 (C), 171.2 (C). IR ν_{max} (film, cm⁻¹): 703, 731, 755, 841, 886, 943, 994,
85 1041, 1086, 1110, 1168, 1205, 1271, 1299, 1314, 1364, 1412, 1454, 1507, 1571, 1630, 1735, 1789.
86 HRMS (ESI): *m/z* calc. for C₁₆H₁₃F₂O₅ [M + H]⁺: 323.0731, found: 323.0721.
- 87 5. Prepare a (±)-GC242 stock solution at 10 mM in DMSO (see 6).

88 **2.4 Other chemicals and buffer**

- 89 1. Prepare a DiFMU stock solution at 10 mM in DMSO (see 2).
- 90 2. Enzyme assay buffer: PBS (100 mM phosphate, pH 6.8, 150 mM NaCl) (see 9).
- 91 3. Milli-Q water.
- 92 4. DMSO.

93 **2.5 Arabidopsis strigolactone receptor protein (AtD14).**

94 AtD14 protein was expressed according to de Saint-Germain et al. (8) (see 10).

95 **2.6 Enzymatic assay**

- 96 1. Eppendorf Safe-Lock Tubes™ of 1.5-mL capacity.
- 97 2. Falcon tubes of 15-mL capacity.
- 98 3. Polystyrene 96 well-plates black flat bottom.
- 99 4. Pipetting robot (VIAFLO 96 Integra) (Fig. 3a).

- 100 5. Multi-mode microplate reader (TECAN SPARK) (Fig. 3b).
- 101 6. GraphPad Prism Software.

102 3. Methods

103 The enzymatic activity is determined by measuring the release of fluorescent DiFMU after hydrolysis of
104 profluorescent probes by the protein of interest (here AtD14).

105 3.1 Standard profluorescent enzymatic assay

106 All 100 μ L enzyme reactions should be carried out in 96-well plates flat bottom black polystyrene (*see* 10. The
107 expression and purification of numerous AtD14 mutant proteins and also RMS3 and mutant proteins have been
108 done following the same method.

109 11). Always perform three technical replicates per condition and at least two independent experiments.

- 110 1. Start up a microplate reader and equilibrate for 30 min at 25 °C. Setup a program for reading 40 wells
111 (or 96 well if you designed a full plate) and set the excitation wavelength to 365 nm and emission
112 wavelength to 460 nm (or use appropriate filters) (*see* 12). Set the spectrophotometer to automatic gain.
113 Set up a cycle to measure 90 \times every 20 seconds (30 min total) (*see* 13). During the incubation and
114 between measurements, the reaction mixture is shaken. Prepare two distinct plates: Plate A with
115 reaction mixture and Plate B with the purified enzyme (Fig. 4).
- 116 2. Prepare Plate A with the substrate:
 - 117 2.1 Set up 1.2 mL of 80 μ M solution of probes to be tested ((\pm)-GC242 or (\pm)-GC240). Add 9.6 μ L of
118 10 mM probes stock solution into 1.1904 mL of enzyme assay buffer (*see* **Materials**). The solution
119 contains 0.8% DMSO (*see* 14).
 - 120 2.2 According to the pipetting scheme displayed in Fig. 4, pipet 50 μ L of probe solution into line A
121 wells (column 1 to 4).
 - 122 2.3 Set up 0.2 mL of 80 μ M solution of DiFMU in enzyme assay buffer for calibration curve. Add 1.6
123 μ L of 10 mM probes stock solution into 0.1984 mL of enzyme assay buffer. The solution contains
124 0.8% DMSO.
 - 125 2.4 According to the pipetting scheme displayed in Fig. 4, pipet 100 μ L of DiFMU solution into line A
126 column 5 well.
 - 127 2.5 Set up 10 mL of 0.8% DMSO solution in enzyme assay buffer to maintain constant DMSO
128 concentration in assay wells.

129 2.6 According to the pipetting scheme displayed in Fig. 4, pipet 50 μL 0.8% DMSO solution in enzyme
130 assay buffer of into line B to line H (all columns).

131 2.7 Perform a serial dilution by consecutively transferring 50 μL from line A to line H with good
132 mixing. Trash the 50 μL left over of the line H. You can use a multi-channel pipette, 12 channels.

133 3. Prepare Plate B with the protein

134 3.1 Create a 20 $\text{ng}/\mu\text{L}$ (0.660 μM) protein solution, diluting with enzyme assay buffer (*see* 15).

135 3.2 According to the plate scheme displayed in Fig. 4, add 50 μL of the protein solution in the samples
136 wells and 50 μL of buffer in the control wells.

137 4. Add simultaneously in all wells the content (50 μL) of Plate A (substrate) in Plate B (protein) using 96
138 tips robot) (*see* 16) and mix well by up and down pipetting.

139 5. Immediately introduce the plate B in the spectrophotometer (*see* 17).

140 6. Record fluorescence over time (at least 30 min).

141 7. Copy the raw data from the spectrophotometer into a spreadsheet.

142 **3.2 Analyzing data**

143 1. Perform the analysis in a spreadsheet.

144 2. If necessary subtract the no-substrate control values from the corresponding experimental samples (*see*
145 18).

146 3. Generate a calibration curve using fluorescence data from the DiFMU serial dilution (column 5). The
147 data is plotted as fluorescence at 460 nm *versus* μmoles of DiFMU.

148 4. Determine the slope as the fluorescence units per μmole of DiFMU.

149 5. Use the slope value to calculate the quantity of DiFMU in μM produced by the protein for each time
150 point.

151 6. Plot independently the data of the three samples replicates (y: μM DiFMU, x: time in min). A
152 logarithmic curve should be displayed (*see* Fig. 5). Dependent on the kind of substrate in use, an initial
153 increase (Initial phase = pre-steady state) within the first 3 min may be observed, after which the signal
154 plateaued ((\pm)-GC242) or slowly increased ((\pm)-GC240) (slow phase). This second phase is ascribed to
155 the turnover reaction, when the D-ring is slowly released (Fig. 5) (*see* 19).

156 7. Identify the linear range of the initial phase, and compute the slope, v_0 (= initial velocity in $\mu\text{M}/\text{min}$) for
157 each substrate concentration.

158 8. Generate a table value with the initial velocities, v_0 , at various concentrations of profluorescent probes.

159 9. Use them to obtain the kinetic parameters of the enzyme (V_{max} , $K_{1/2}$ and k_{cat}) from the Michaelis–
160 Menten curve using the GraphPad Prism software.

161 9.1 Create an XY table selecting “Enzyme kinetics—Michaelis–Menten” as sample data and paste the
162 triplicates of the enzyme activities obtained for each substrate concentration.

163 9.2 Perform a “Nonlinear regression” analysis by selecting “Enzyme Kinetics—Substrate *versus*
164 Velocity” and “Michaelis–Menten equation. Enzyme initial reaction rate, v_0 , at various probes
165 concentrations were fitted to the equation $v_0 = \frac{k_{cat} \cdot [E_{tot}] \cdot [S]}{K_{1/2} + [S]}$, where v_0 is the initial reaction velocity,
166 k_{cat} the rate of the slowest step, E_{tot} the total enzyme concentration, $[S]$ the concentration of the
167 probes, and $K_{1/2}$ is the probes concentration that gives half maximal velocity (see 20), in order to
168 determine the pre-steady state enzymatic constant.

169 9.3 As a result of the analysis, a regression curve is superimposed on the graph (Fig. 6) and a table with
170 the values of V_{max} , $K_{1/2}$ and k_{cat} together with the statistical parameters is retrieved.

171 4. Notes

172 1. The shoot-branching inhibition of GC240 and GC242 in pea was demonstrated using direct bud application,
173 root feeding using hydroponic treatment and feeding to the vascular stream of shoots as described in Chapter
174 ”Methods for phenotyping shoot branching and testing SL bioactivity for shoot branching in Arabidopsis and
175 pea” by Rameau et al.

176 2. DiFMU was prepared by using the multistep synthetic procedure described by Hedberg et al. (13) or is also
177 commercially available.

178 3. Our first profluorescent probes were easily synthesized as a racemic mixture by nucleophilic substitution of 5-
179 bromo-4-methylfuran-2(5*H*)-one and 3,4-dimethylfuran-2(5*H*)-one with 7-hydroxycoumarine as fluorescent
180 core. These probes were demonstrated to be as bioactive as GR24 in pea (Note 1) for the bud outgrowth
181 inhibition. However, the similar bioactivity in pea and the better fluorescent properties of DiFMU (λ_{ex} DiFMU >
182 λ_{ex} 7-hydroxycoumarine and λ_{em} DiFMU > λ_{em} 7-hydroxycoumarine) prompted us to choose the DiFMU analogs
183 (GC240, GC242) for our *in vitro* studies.

184 4. This anhydrous reagent is commercially available.

185 5. Nuclear magnetic resonance spectra (^1H , ^{13}C NMR) were recorded respectively at [300; 75] MHz on a Bruker
186 DPX 300 spectrometer. For the ^1H spectra, data are reported as follows: chemical shift, multiplicity (s = singlet,

187 d = doublet, t = triplet, q = quartet, m = multiplet, bs = broad singlet, coupling constant in Hz and integration.
188 IR spectra are reported in reciprocal centimeters (cm^{-1}). Mass spectra (MS) and high-resolution mass spectra
189 (HRMS) were determined by electrospray ionization (ESI) coupled to a time-of-flight analyzer (Waters LCT
190 Premier XE).

191 6. GC probes can be stored as dry solid or as mother solutions in dry DMSO (100 mM, 10 mM or 2 mM) and
192 kept at $-18\text{ }^{\circ}\text{C}$. GC probes are insoluble in acetone, a common solvent used for SLs. Do not keep in water or
193 alcohol solutions as for natural SLs, analogs and mimics.

194 7. As expected according to our previous results (14, 15) GC242 with 3,4-dimethylfuran-2(5*H*)-one part is as
195 bioactive as GC240 with 4-methylfuran-2(5*H*)-one part and shown better stability toward nonspecific hydrolysis.

196 8. Resolution of the (\pm)-GC242 racemic mixture in the the 2 enantiomers can be done using a chiral analytical
197 HPLC column with a 2695 HPLC Alliance from Waters coupled with a 2995 PDA Detector (Waters) and a
198 WFCIII collector (Waters). A CHIRALPAK® IA (4.6×250mm, 5 μm) column was used with the eluent 55%
199 heptane/ 45% EtOH/ CH_2Cl_2 9/1 with 1 mL/min as flow rate at $35\text{ }^{\circ}\text{C}$. Injections (each time (15 μL)) of the
200 racemic mixture in CH_2Cl_2 (10 mg/mL) were performed and each peak was collected. After purification, the
201 solvent was evaporated to concentrate each enantiomer and the 2 fractions were re-injected with a concentration
202 of 1 mg/mL to check the purity. We obtained (+)-GC242 (retention time 13 min) 97.5% $[[\alpha]_{\text{D}}^{20}: +100$ (*c* 0.2 in
203 CHCl_3)] for the first peak and for the second peak (–)-GC242 (retention time 14.7 min) 99.5% of purity $[[\alpha]_{\text{D}}^{20}$:
204 -95 (*c* 0.5 in CHCl_3)] (8).

205 9. Our protocol uses phosphate buffer pH 6.8 because as observed with GR24, we detected a significant amount
206 of non-enzymatic cleavage of probes at pH > 7. We also established that the enzymatic activity of AtD14 is
207 maintained at pH 5.5 but disappeared at pH 4.5.

208 10. The expression and purification of numerous AtD14 mutant proteins and also RMS3 and mutant proteins
209 have been done following the same method.

210 11. Use black microplates with flat and black bottom and avoid lid. Black microplates have low background
211 fluorescence and minimize light scattering.

212 12. Before starting this protocol, ensure that you are familiar with the measurement modes of your plate reader.
213 If your plate reader does not have monochromator, you will need to know what filters your instrument has for

214 measuring DiFMU fluorescence: excitation (355 nm is recommended) and emission (460 nm is recommended)
215 filters.

216 13. The recording time should be determined empirically. If the reaction is slow (not found to date with SL
217 receptors), you should have to record during 1, 2 or 3 hours. If the reaction is too fast you should use another
218 device to record fluorescence as stopped-flow spectrophotometer.

219 14. The final highest concentration of probes is divided by 2-fold after protein addition (40 μM). The probe
220 concentrations need to be adjusted to observe an increase of enzymatic activity throughout the time and to
221 saturate the initial velocity. The range of probe concentrations used to calculate the kinetics of the enzyme is:
222 0.3125, 0.625, 1.25, 2.5, 5, 10, 20 and 40 μM .

223 15. The final concentration of protein is divided by 2 after substrate addition (330 nM). The amount of
224 recombinant enzyme added to the reaction mixture should be determined empirically. It can be increase if you
225 don't see any signal (10-fold 100-fold even 1000-fold). If the reaction is too fast you can decrease the amount of
226 enzyme.

227 16. If robot is not available to you, use a multichannel pipette at 50 μL settings. Start with the blanks and then
228 quickly proceed with the sample wells. Take care not to create any bubbles. Score the lag between the first
229 addition and the first measurement. This lag should be added in the data curve.

230 17. A lag of 20 s is observed between the substrate addition and the first measurement. Do not forget to adjust
231 the kinetics with this lag. If there is a lag (especially by manual pipetting) you can miss the initial phase.

232 18. The no-protein control fluorescence value should not increase during the half-hour assay. But if you notice a
233 significant hydrolysis you have to subtract the values to the sample corresponding well.

234 19. The hydrolysis of (\pm)-GC240 by AtD14 is too fast to determine initial velocity.

235 20. $K_{1/2}$ is equivalent to K_m , but here we quantify pre-steady state parameter therefore we cannot assimilate the
236 dissociation constant to Michaelis constant. For this reason we call it $K_{1/2}$.

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244 5. References

- 245 1. Hamiaux C, Drummond RSM, Janssen BJ, Ledger SE, Cooney JM, Newcomb RD, Snowden KC
246 (2012) DAD2 Is an alpha/beta Hydrolase Likely to Be Involved in the Perception of the Plant
247 Branching Hormone, Strigolactone. *Curr Biol* 22: 2032-2036. doi:10.1016/j.cub.2012.08.007
- 248 2. Sun WC, Gee KR, Haugland RP (1998) Synthesis of novel fluorinated coumarins: Excellent UV-light
249 excitable fluorescent dyes. *Bioorg Med Chem Lett* 8: 3107-3110. doi:10.1016/s0960-894x(98)00578-2
- 250 3. Shi W, Ma H (2012) Spectroscopic probes with changeable pi-conjugated systems. *Chem Commun* 48:
251 8732-8744. doi:10.1039/c2cc33366j
- 252 4. Chen X, Sun M, Ma H (2006) Progress in Spectroscopic Probes with Cleavable Active Bonds. *Curr Org*
253 *Chem* 10: 477-489. doi:10.2174/138527206776055312
- 254 5. Bouteiller C, Clavé G, Bernardin A, Chipon B, Massonneau M, Renard P-Y, Romieu A (2007) Novel
255 water-soluble near-infrared cyanine dyes: Synthesis, spectral properties, and use in the preparation of
256 internally quenched fluorescent probes. *Bioconjugate Chem* 18: 1303-1317. doi:10.1021/bc0700281
- 257 6. Lai KS, Ho N-H, Cheng JD, Tung C-H (2007) Selective fluorescence probes for dipeptidyl peptidase
258 activity - Fibroblast activation protein and dipeptidyl peptidase IV. *Bioconjugate Chem* 18: 1246-1250.
259 doi:10.1021/bc0603586
- 260 7. Van Overtveldt M, Braem L, Struk S, Kaczmarek AM, Boyer F-D, Van Deun R, Gevaert K,
261 Goormachtig S, Heugebaert TSA, Stevens CV (2019) Design and visualization of second-generation
262 cyanoisindole-based fluorescent strigolactone analogs. *Plant J* 98: 165-180. doi:10.1111/tpj.14197
- 263 8. de Saint Germain A, Clavé G, Badet-Denisot M-A, Pillot J-P, Cornu D, Le Caer J-P, Burger M,
264 Pelissier F, Retailleau P, Turnbull C, Bonhomme S, Chory J, Rameau C, Boyer F-D (2016) An histidine
265 covalent receptor and butenolide complex mediates strigolactone perception. *Nat Chem Biol* 12: 787-
266 794. doi:10.1038/nchembio.2147
- 267 9. Waters MT, Gutjahr C, Bennett T, Nelson DC (2017) Strigolactone Signaling and Evolution. *Annu Rev*
268 *Plant Biol* 68: 291-322. doi:10.1146/annurev-arplant-042916-040925
- 269 10. Tsuchiya Y, Yoshimura M, Sato Y, Kuwata K, Toh S, Holbrook-Smith D, Zhang H, McCourt P, Itami
270 K, Kinoshita T, Hagihara S (2015) Probing strigolactone receptors in *Striga hermonthica* with
271 fluorescence. *Science* 349: 864-868. doi:10.1126/science.aab3831
- 272 11. Wolff S, Hoffmann HMR (1988) Aflatoxins revisited - convergent synthesis of the ABC-moiety.
273 *Synthesis*: 760-763. doi:10.1055/s-1988-27700
- 274 12. Canévet JC, Graff Y (1978) Réactions de Friedel-Crafts de dérivés aromatiques sur des composés
275 dicarbonylés-1,4 éthyléniques-2,3.II Alkylations par quelques hydroxy-5 ou chloro-5 dihydro-2,5
276 furannones-2. Nouvelle méthode de synthèse des acides 1H-indèncarboxyliques-1. *Tetrahedron* 34:
277 1935-1942. doi:10.1016/0040-4020(78)80100-8
- 278 13. Hedberg C, Dekker FJ, Rusch M, Renner S, Wetzel S, Vartak N, Gerding-Reimers C, Bon RS,
279 Bastiaens PI, Waldmann H (2011) Development of highly potent inhibitors of the Ras-targeting human
280 acyl protein thioesterases based on substrate similarity design. *Angew Chem Int Ed* 50: 9832-9837.
281 doi:10.1002/anie.201102965
- 282 14. Boyer F-D, de Saint Germain A, Pillot J-P, Pouvreau J-B, Chen VX, Ramos S, Stévenin A, Simier P,
283 Delavault P, Beau J-M, Rameau C (2012) Structure-Activity Relationship Studies of Strigolactone-
284 Related Molecules for Branching Inhibition in Garden Pea: Molecule Design for Shoot Branching.
285 *Plant Physiol* 159: 1524-1544. doi:10.1104/pp.112.195826
- 286 15. Boyer F-D, de Saint Germain A, Pouvreau J-B, Clavé G, Pillot J-P, Roux A, Rasmussen A, Depuydt S,
287 Laussergues D, Frei dit Frey N, Heugebaert TSA, Stevens CV, Geelen D, Goormachtig S, Rameau C
288 (2014) New Strigolactone Analogs as Plant Hormones with Low Activities in the Rhizosphere. *Mol*
289 *Plant* 7: 675-690. doi:10.1093/mp/sst163

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291 6. List of figures

292 Fig. 1 – (a) Profluorescent probes, GC series and GR24 SL synthetic analog. (b) Principle of profluorescent
293 probes allowing the enzymatic activity detection of AtD14 in aqueous media.

294 Fig. 2 – ¹H NMR spectrum of (±)-GC240 (a) and (±)-GC242 (b).

295 Fig. 3 – Pictures of the equipment used for the enzymatic assay. (a) An INTEGRA Viaflo 96 pipetting robot and
296 (b) a multi-mode plate reader Spark from TECAN.

297 Fig. 4 – Experimental design in a 96-well plate format. **Plate A.** Green triangle indicates decreasing final
298 substrate concentration from top to bottom. Green circles (columns 1 to 3) represent standard enzymatic
299 reactions in triplicates per condition. Green circles (column 4) indicate no-protein control. Red circles (column
300 5) indicates DiFMU calibration curve. **Plate B.** Blue circles (columns 1 to 3) represent wells containing protein
301 and corresponding standard enzymatic reactions in triplicates per condition. Grey circles (columns 4 and 5)
302 represent wells containing buffer and corresponding to non-protein control and calibration curve.

303 Fig. 5 – Enzymatic kinetics for AtD14 proteins incubated with (±)-GC242 or (±)-GC240. Progress curves during
304 probes hydrolysis, monitored (λ_{em} 460 nm) at 25 °C. Protein catalyzed hydrolysis with 330 nM of protein and 5
305 μ M of probes.

306 Fig. 6 – Hyperbolic plot of presteady state kinetics reaction velocity with (±)-GC242. Initial velocity was
307 determined with profluorescent probes concentration from 0.3125 μ M to 40 μ M and AtD14 at 330 nM. Error
308 bars represent SE of the mean of three replicates.

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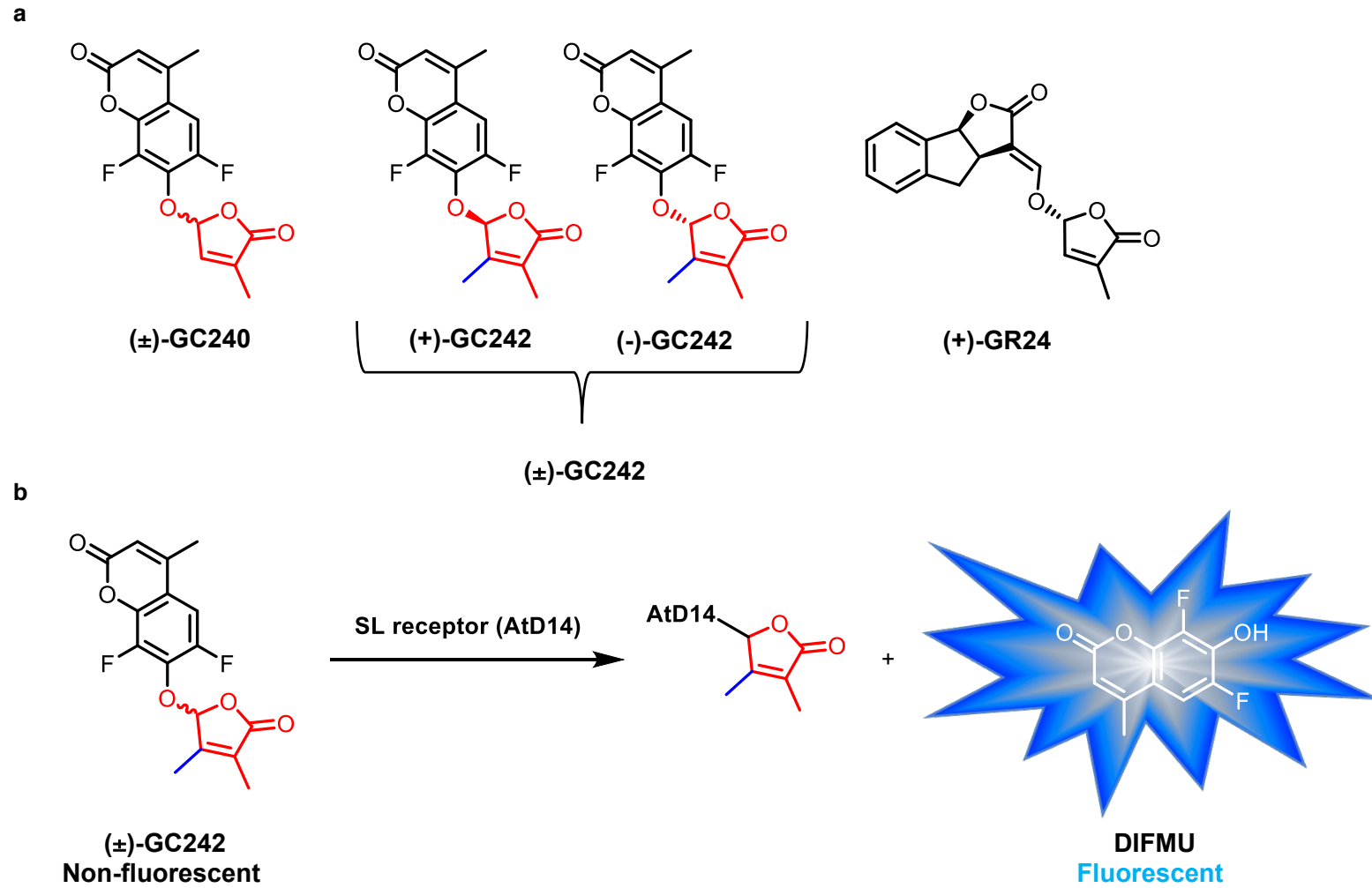


Fig. 1

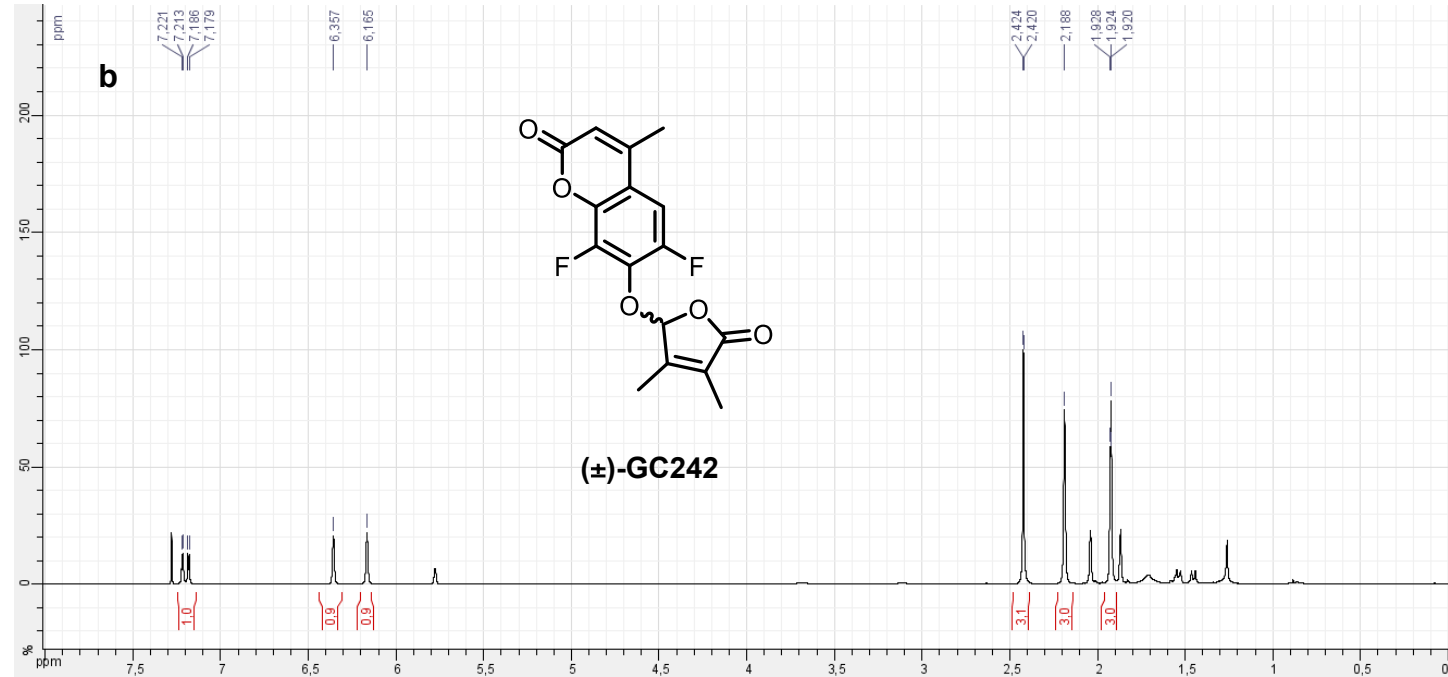
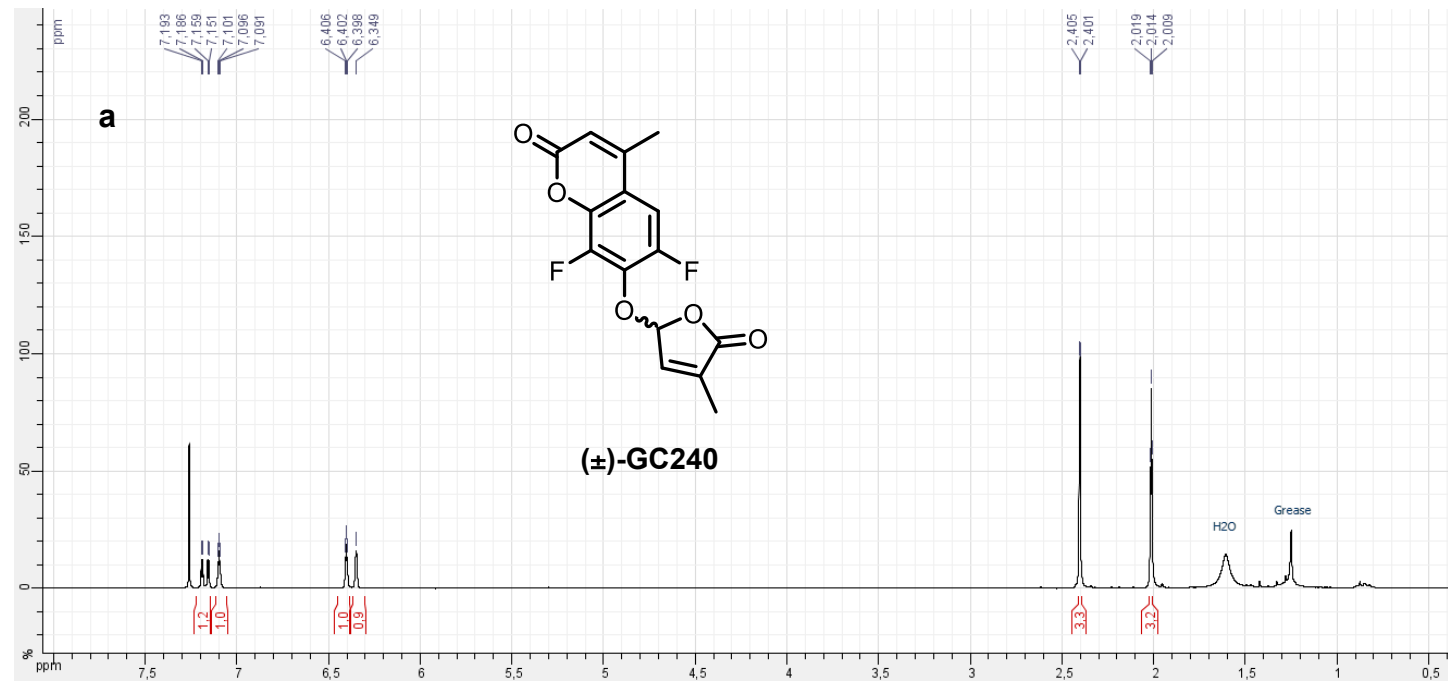


Fig. 2



Fig. 3

Plate A : Substrat

Plate B: Protein

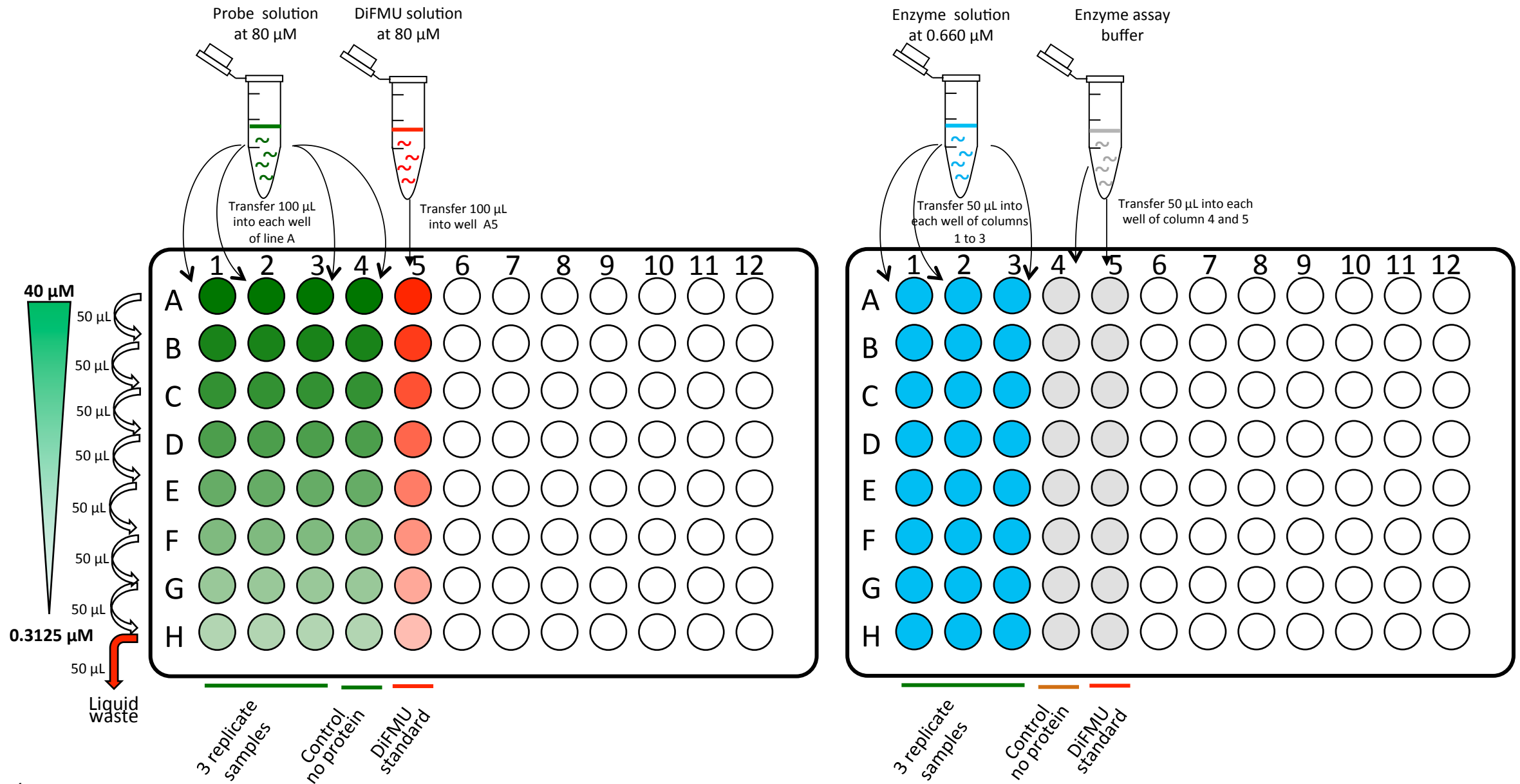


Fig. 4

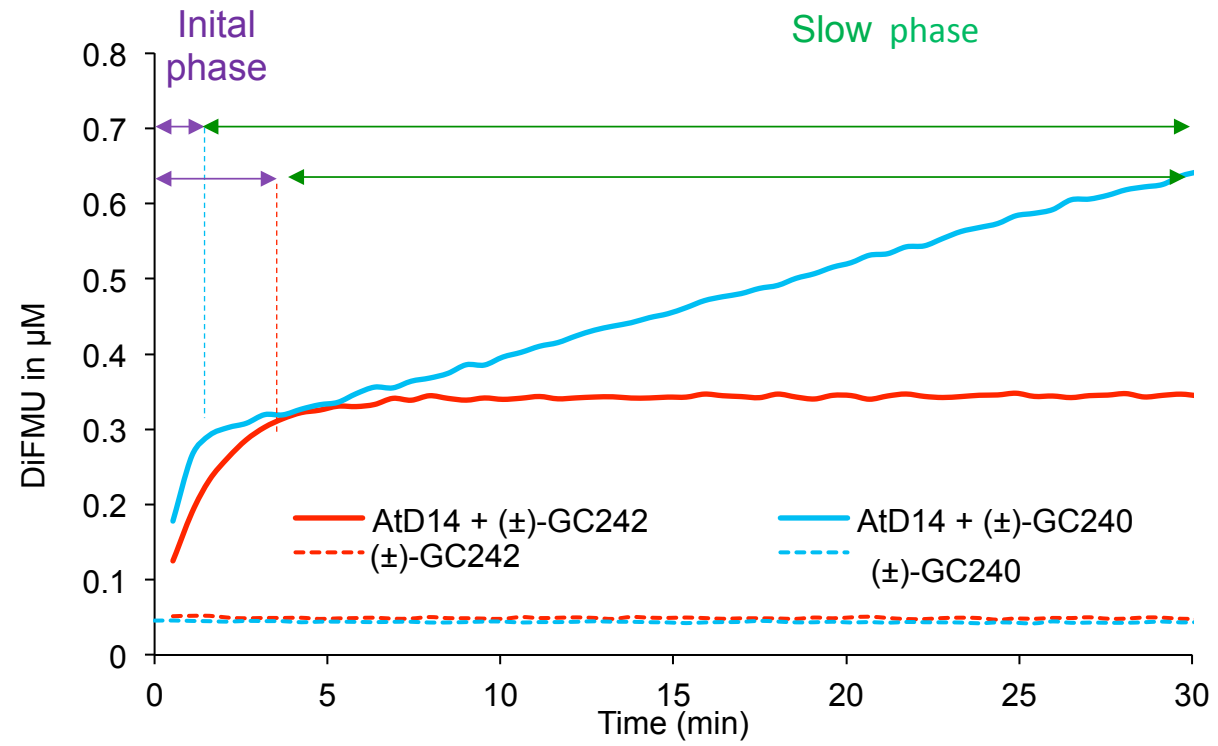


Fig. 5

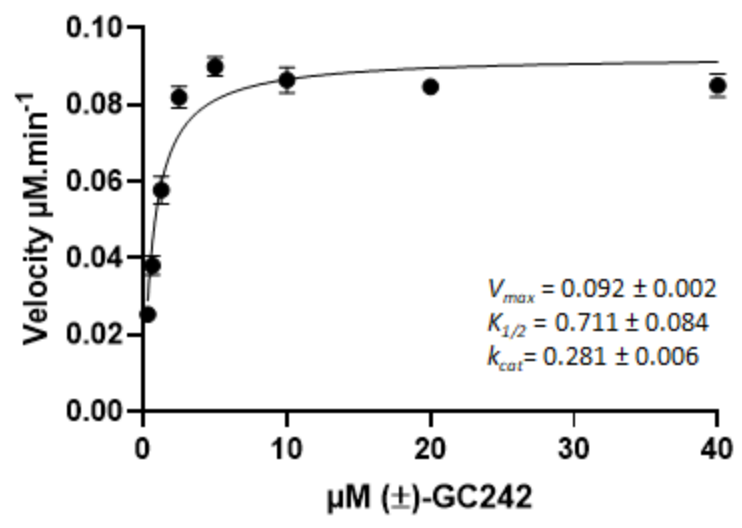


Fig. 6