Synthesis of Profluorescent Strigolactone Probes for Biochemical Studies

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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.

16

17 **ii. Key Words**

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

19

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**

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

1. Run all non-aqueous reactions under an inert atmosphere (argon), by using standard techniques for manipulating air-sensitive compounds.

2. Store all glassware in the oven and/or it was flame-dried prior to use.

3. Obtain anhydrous solvents by filtration through drying columns or from commercial suppliers.

4. Perform analytical thin-layer chromatographies (TLC) on plates precoated with silica gel layers. Visualize compounds by one or more of the following methods: (1) illumination with a short wavelength UV lamp (i.e., $\lambda = 254$ nm), (2) spray with a 1% (w/v) KMnO$_4$ solution in H$_2$O.

5. Perform flash column chromatography using 40-63 mesh silica.

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

1. Add to a solution of 5-bromo-3-methylfuran-2(5*H*)-one (26 mg, 150 µmol) prepared according to Wolff et al. (*11*) in CH$_3$CN (1 mL) 6,8-difluoro-7-hydroxy-4-methyl-2*H*-chromen-2-one (DiFMU) (*see* 2 and 3) (16 mg, 75 µmol) and anhydrous *N,N*-diisopropylethylamine (DIEA) (52 µL, 300 µmol) (*see* 4).

2. Stir the resulting mixture at room temperature for 12 h and check for completion by TLC (Heptane/EtOAc 1:1).

3. Remove the solvent under vacuum.

4. Purify the resulting residue on a silica gel column (Heptane/EtOAc 6:4) giving (±)-GC240 as a white solid (22 mg, 71 µmol, 95 %).

5. Obtain the following characterizations for (±)-GC240 (*see* 5). R$_f$ (heptane/EtOAc, 1:1, v/v) = 0.41. M.p.: 191 °C. ^{1}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, 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 Hz, *J$_2$* = 2.3 Hz, 1H) (Fig. 2a). ^{13}C-NMR (75 MHz, CDCl$_3$): δ 10.8 (CH$_3$), 18.9 (CH$_3$), 101.1-101.2 (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* = 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), 150.0-150.1 (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, cm^{-1}): 701, 731, 753, 815, 832, 870, 883, 938, 955, 987, 1041, 1102, 1165, 1202, 1266, 1300, 1318, 1340, 1373, 1406, 1454, 1508, 1574, 1633, 1741, 1776, 2344, 2925, 3083. HRMS (ESI): *m/z* calc. for C$_{15}$H$_{11}$F$_2$O$_5$ [M + H]$^+$: 309.0575, found: 309.0581.

6. Prepare a (±)-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(5*H*)-one (29 mg, 200 µmol) synthetized according to

74   Canévet et al. (*12*) in CH_3CN (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 Et_2O 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. ^{1}H- NMR (300 MHz, $CDCl_3$): 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). [13]C-

81   NMR (75 MHz, $CDCl_3$): δ 8.7 (CH_3), 11.6 (CH_3), 18.9 (CH_3), 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 $C_{16}H_{13}F_2O_5$ $[M + H]^+$: 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 *Arabidopsi*s 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.

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).

3.1 Standard profluorescent enzymatic assay

106 All 100 µL 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× 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 µM solution of probes to be tested ((±)-GC242 or (±)-GC240). Add 9.6 µL 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 µL of probe solution into line A

121   wells (column 1 to 4).

122   2.3 Set up 0.2 mL of 80 µM solution of DiFMU in enzyme assay buffer for calibration curve. Add 1.6

123   µL 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 µL 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.

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.   <u>Prepare Plate B with the protein</u>

134   3.1   Create a 20 ng/µL (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 ((±)-GC242) or slowly increased ((±)-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 µM/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 (^{1}H; ^{13}C NMR) were recorded respectively at [300; 75] MHz on a Bruker

186   DPX 300 spectrometer. For the ^{1}H spectra, data are reported as follows: chemical shift, multiplicity (s = singlet,

187   d = doublet, t = triplet, q = quartet, m = multiplet, bs = broad singulet, 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 °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(*5H*)-one part is as

195   bioactive as GC240 with 4-methylfuran-2(*5H*)-one part and shown better stability toward nonspecific hydrolysis.

196   8. Resolution of the (±)-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, 5μm) 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°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% [$[\alpha]_D^{20}$: +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 [$[\alpha]_D^{20}$:

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 (±)-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 $K_{1/2}$.

237   **4. Acknowledgement**

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6. List of figures

294   Fig. 2 – ^{1}H 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   µM 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 µM to 40 µM and AtD14 at 330 nM. Error

308   bars represent SE of the mean of three replicates.

309
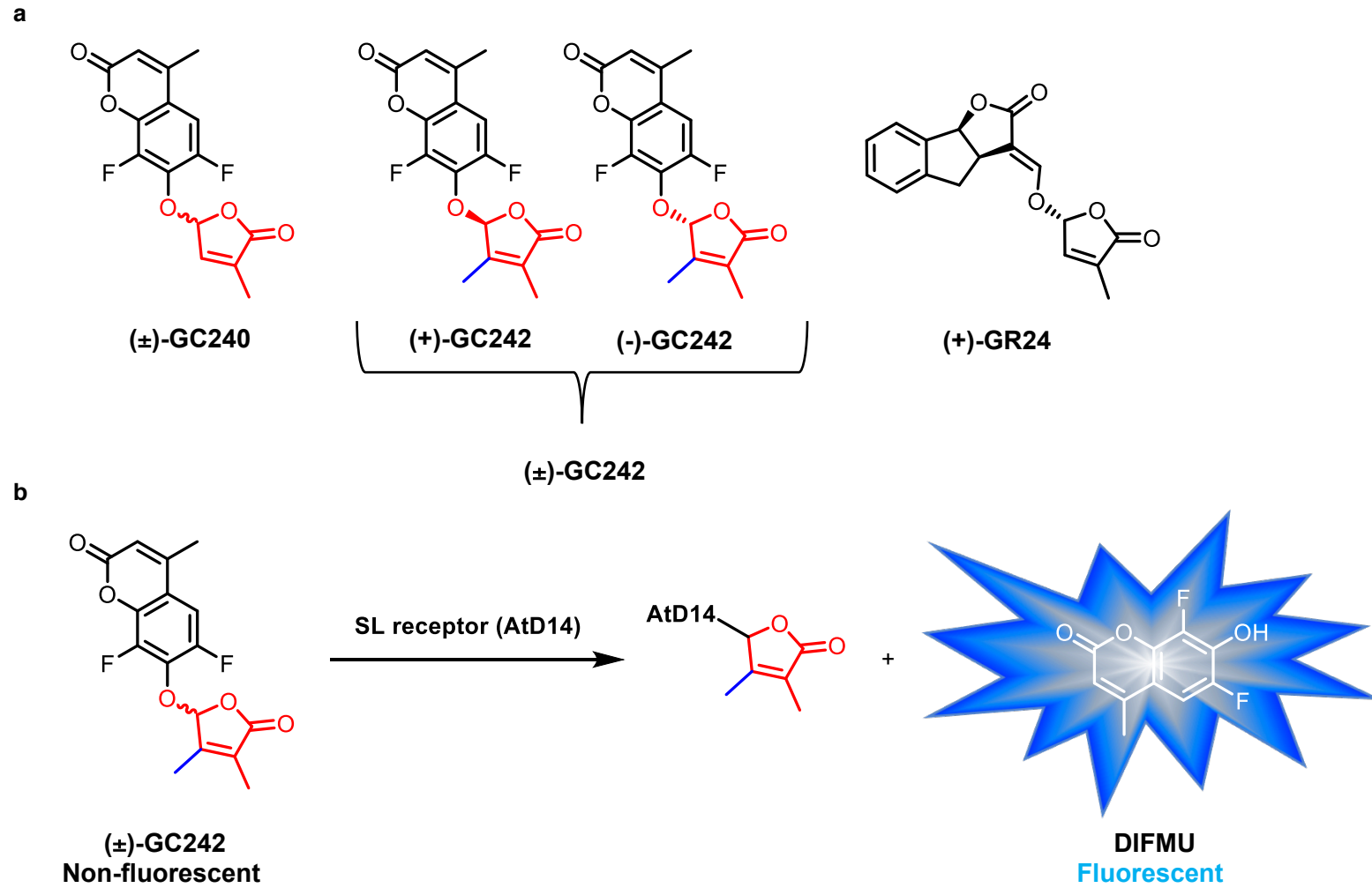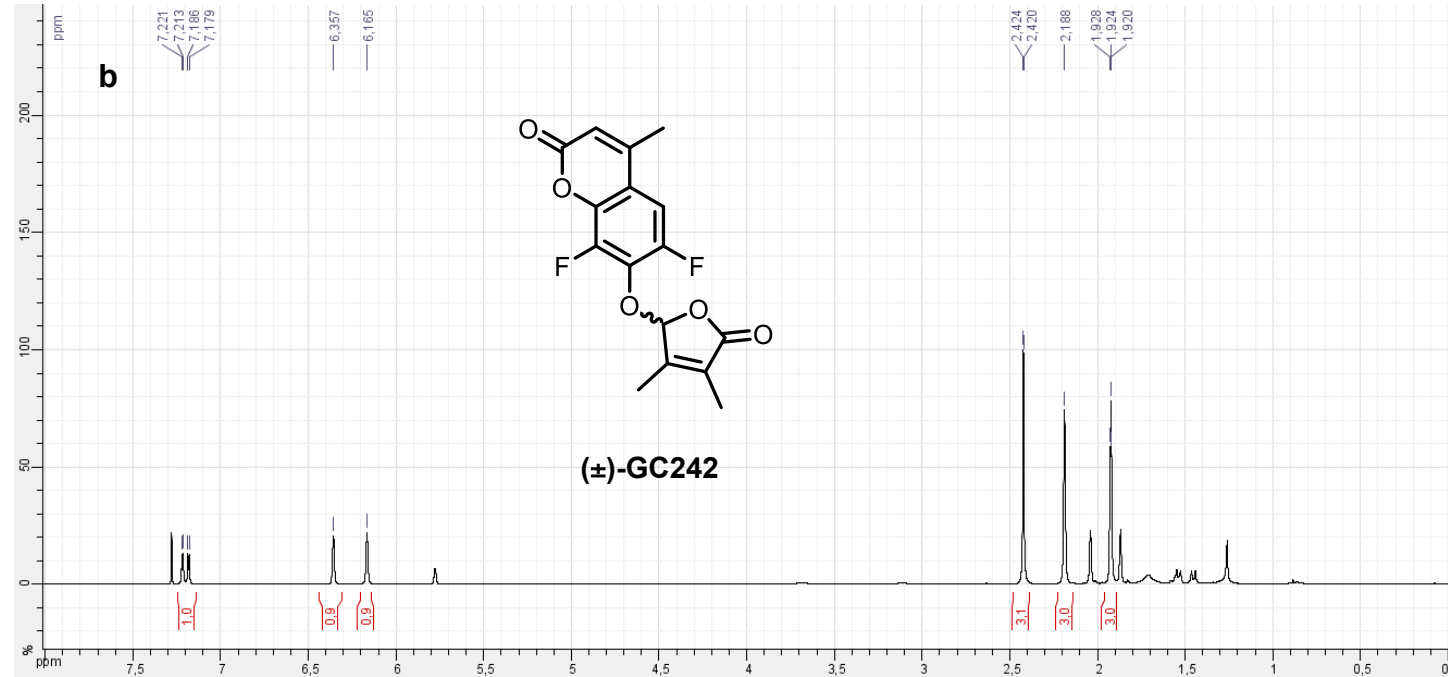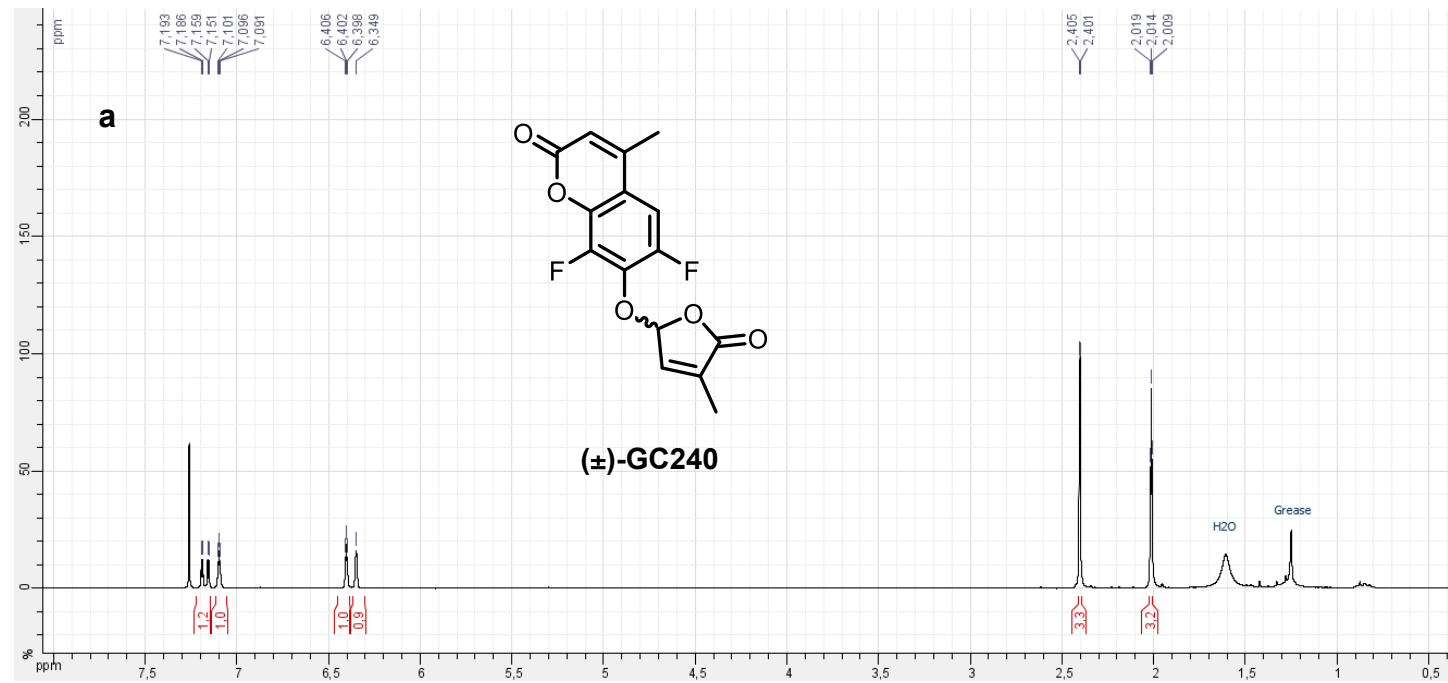
310

a

(±)-GC240   (+)-GC242   (-)-GC242   (+)-GR24

(±)-GC242

b

(±)-GC242
Non-fluorescent

SL receptor (AtD14)

AtD14

DIFMU
Fluorescent

Fig. 1

Fig. 2

a

b

Fig. 3

Plate A : Substrat

Probe solution at 80 µM

DiFMU solution at 80 µM

Transfer 100 µL into each well of line A

Transfer 100 µL into well A5

40 µM

50 µL

0.3125 µM

50 µL

Liquid waste

3 replicate samples

Control no protein

DiFMU standard

Plate B: Protein

Enzyme solution at 0.660 µM

Enzyme assay buffer

Transfer 50 µL into each well of columns 1 to 3

Transfer 50 µL into each well of column 4 and 5

3 replicate samples

Control no protein

DiFMU standard

Fig. 4

Fig. 5

Fig. 6