Electronic Supplementary Material (ESI) for Chemical Science.

An Active Site Titration Reagent for α-Amylases

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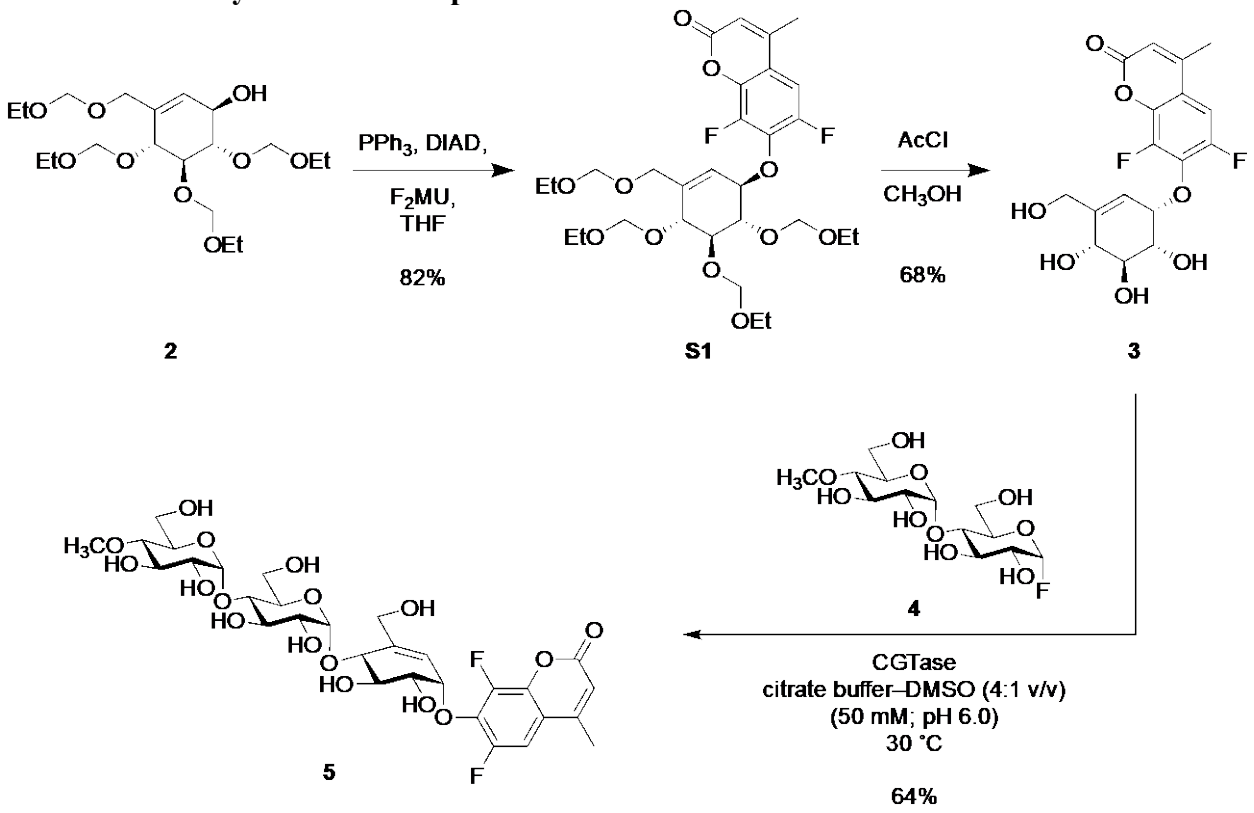
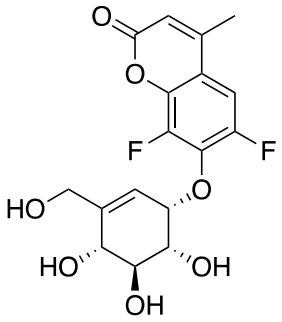
1. Synthesis. General Methods:

All reagents were purchased from commercial sources and used without further purification. 2-Chloro-4-nitrophenol α-maltotrioside (CNP-G3) was purchased from Carbosynth. Cyclodextrin glucanotransferase (CGTase) was a gift from Amano Enzyme, Inc. *Effusibacillus pohliae* amylase (EPA), *Aspergillus oryzae* amylase (AOA), *Rhizomucor pusillus* amylase (RPA), [1] and amyloglucosidase (AMG) were gifts from Novozymes. Porcine pancreatic amylase (PPA) was purchased from Sigma Aldrich Chemical Company; human pancreatic α-amylase (HPA) was isolated as described previously.[2] Compounds **2** and **4** were prepared via literature methods.[3,4] 2,6-Difluorocoumarin is referred to as F_2MU. Diisopropyl azodicarboxylate is referred to as DIAD. Petroleum ether is referred to as pet. ether. Dry THF was distilled from Na/benzophenone under N_2. Dry CH_3OH was distilled from KOH under N_2. Unless otherwise stated, all reactions were carried out under an argon atmosphere and were monitored by TLC on silica gel 60 F_{254} (0.25 mm, Merck). Spots were visualized by UV light and/or by charring with 1% $Ce(SO_4)_2 \cdot 4H_2O$ and 5% $(NH_4)Mo_7O_{24} \cdot 4H_2O$ in 10% H_2SO_4. In the processing of reaction mixtures, solutions of organic solvents were washed with equal volumes of aqueous solutions. Solvents were evaporated *in vacuo* at temperatures ≤ 40 ºC. Column chromatography was performed on silica gel 60 (Silica Flash, 40–63 μm, 60 Å). ^{1}H NMR spectra were recorded at 400 MHz and chemical shifts are referenced to HOD (D_2O, 4.79 ppm). ^{13}C NMR spectra were recorded at 101 MHz, and chemical shifts referenced to external acetone (31.07 ppm, D_2O). ^{19}F NMR spectra were recorded at 377 MHz, and chemical shifts referenced to external $CFCl_3$ (0.00 ppm, CD_3OD). All NMR assignments were made by appropriate 2D NMR experiments. Electrospray ionization mass spectra (ESI-MS) were recorded on a Waters/Micromass ion-trap spectrometer with samples dissolved in CH_2Cl_2, CH_3OH, or H_2O.

Scheme for the Synthesis of Compound 5:

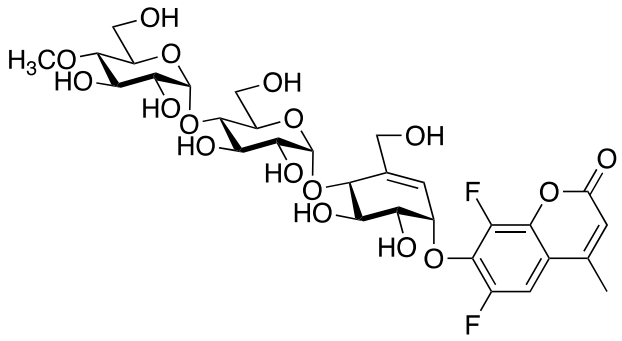


Synthetic Procedures:



7-*O*-[(1*S*,2*S*,3*S*,4*R*)-2,3,4-tris-*O*-(hydroxy)-5-hydroxymethyl-cyclo-5-ene]-6,8-difluoro-4-methylcoumarin (3). To a solution of PPh_3 (957 mg, 3.65 mmol) in dry THF (15.0 mL), was added DIAD (0.40 mL, 2.03 mmol) dropwise at 0 ºC, and the resulting mixture was stirred for 15 min at 0 ºC. A solution of **2**[3] (268 mg; 0.66 mmol) and F_2Mu (183 mg, 0.86 mmol) in THF (10.0 mL) was added dropwise to the DIAD–PPh_3 solution at 0 ºC, and the resulting mixture was stirred for 3 h at 4 ºC, then a solution of CH_3OH–H_2O (2.5 mL, 1:1 v/v) was added. The mixture was

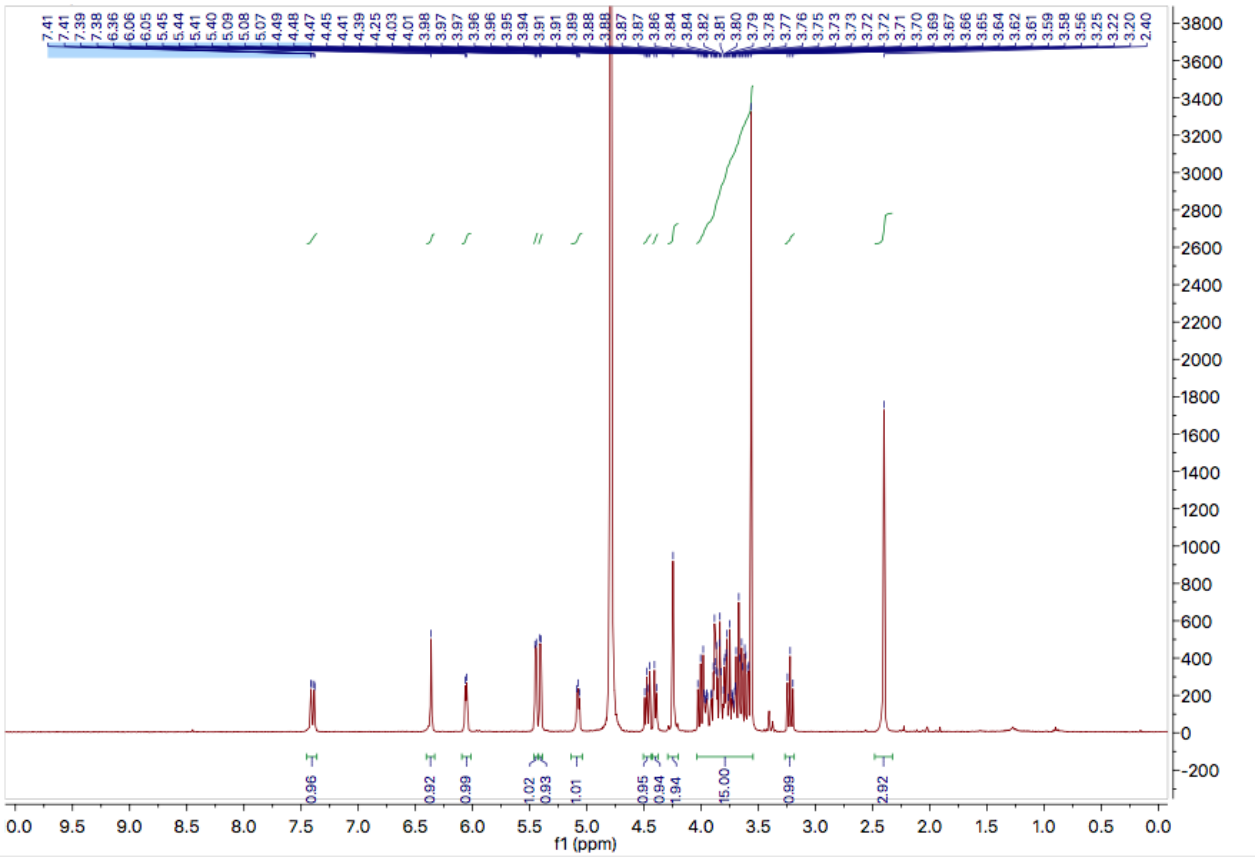
concentrated, redissolved in EtOAc (30.0 mL), washed twice with a solution of 1.0 M $NaOH_{(aq)}$ and then once with brine. The organic layer was then dried with Na_2SO_4, filtered, concentrated, and purified by silica gel column chromatography (3:1→2:1 pet. ether–EtOAc) to yield the protected cyclitol intermediate **S1** (323 mg, 82%) as a yellow oil. The characterization data matches what has been reported previously.[5] To a solution of this protected cyclitol intermediate **S1** (317 mg, 0.53 mmol) in dry CH_3OH (42.0 mL), was then added acetyl chloride (0.17 mL) dropwise at 0 ºC, and the resulting mixture was stirred for 50 h at 4 ºC. The mixture was then warmed to rt, neutralized by the addition of Et_3N, concentrated, and purified by silica gel column chromatography (100% EtOAc→20% CH_3OH in EtOAc) and lyophilized to yield **3** (133 mg, 68%), as a fluffy, white solid. The characterization data match what has been reported previously.[5]



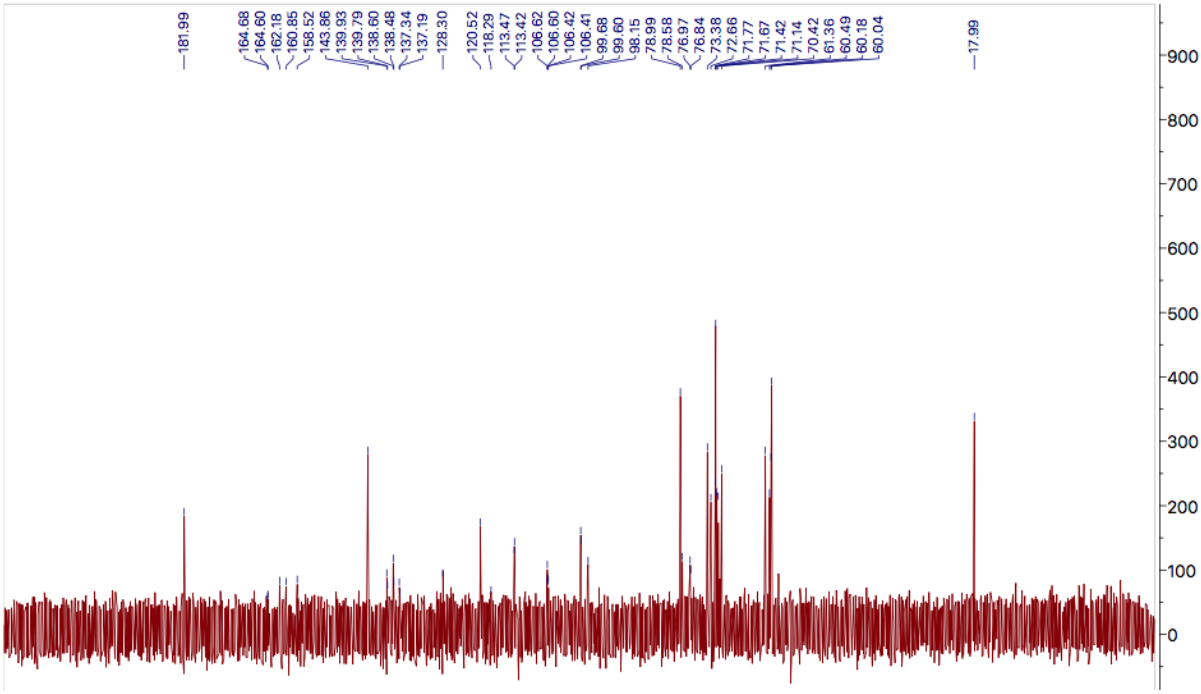
7-*O*-[(1*S*,2*S*,3*S*,4*R*)-2,3,4-tris-*O*-(hydroxy)-5-hydroxymethyl-cyclo-5-ene]-6,8-difluorocoumarin 4'-*O*-methyl-α-D-glucopyranosyl-(1→4)-α-D-glucopyranoside (5). To a solution of 4'-*O*-methyl maltosyl α-fluoride **4** (25 mg, 70 μmol)[4] in sodium citrate buffer (1.50 mL, 50 mM, pH 6.0), was added **3** (34 mg, 92 μmol) in DMSO (400 μL). CGTase (200 μL, 50 ng/μL) was added, and the mixture was incubated for 16 h at 30 ºC, until TLC showed complete consumption of 4'-*O*-methyl maltosyl α-fluoride **4**. The reaction mixture was placed in a centrifugal filter device (10,000 MWCO, NanoSep), spun down to remove CGTase, and concentrated to dryness by lyophilization. The crude oil was purified by silica gel column

chromatography (100% EtOAc→9:1 EtOAc–CH_3OH→7:2:1 EtOAc–CH_3OH–H_2O) and lyophilized to yield **5** (32 mg, 64%), as a fluffy, white solid. R_f 0.31 (7:2:1 EtOAc–CH_3OH–H_2O); ^{1}H NMR (400 MHz, D_2O, δ_H) 7.40 (dd, *J* = 11.2, 1.9 Hz, 1 H), 6.36 (s, 1 H), 6.06 (d, *J* = 4.9 Hz, 1 H), 5.45 (d, *J* = 4.0 Hz, 1 H), 5.41 (d, *J* = 3.9 Hz, 1 H), 5.11–5.05 (m, 1 H), 4.47 (dd, *J* = 10.1, 7.3 Hz, 1 H), 4.40 (d, *J* = 7.3 Hz, 1 H), 4.25 (s, 2 H), 4.05–3.53 (m, 15 H), 3.22 (t, *J* = 9.6 Hz, 1 H), 2.40 (d, *J* = 1.1 Hz, 3 H); ^{13}C NMR (101 MHz, D_2O, δ_C) 182.0, 164.64 (d, *J* = 8.5 Hz), 161.52 (d, *J* = 134.4 Hz), 158.5, 143.9, 139.20 (dd, *J* = 131.4, 14.2 Hz), 137.26 (d, *J* = 15.6 Hz), 128.3, 120.5, 118.3, 113.45 (d, *J* = 5.0 Hz), 106.51 (dd, *J* = 19.5, 1.5 Hz), 99.7, 99.6, 98.2, 79.0, 78.6, 76.8, 73.4, 72.7, 71.8, 71.7, 71.4, 71.1, 70.4, 61.4, 60.5, 60.2, 60.0, 18.0; ^{19}F NMR (377 MHz, D_2O, δ_F) –131.6 (d, *J* = 5.0 Hz), –147.8 (d, *J* = 5.0 Hz); HRMS–ESI–TOF calcd for $[M+Na]^+$ $C_{30}H_{38}O_{17}F_2Na$: 708.2077. Found 708.2073.

^{1}H NMR Spectrum of Compound 5:



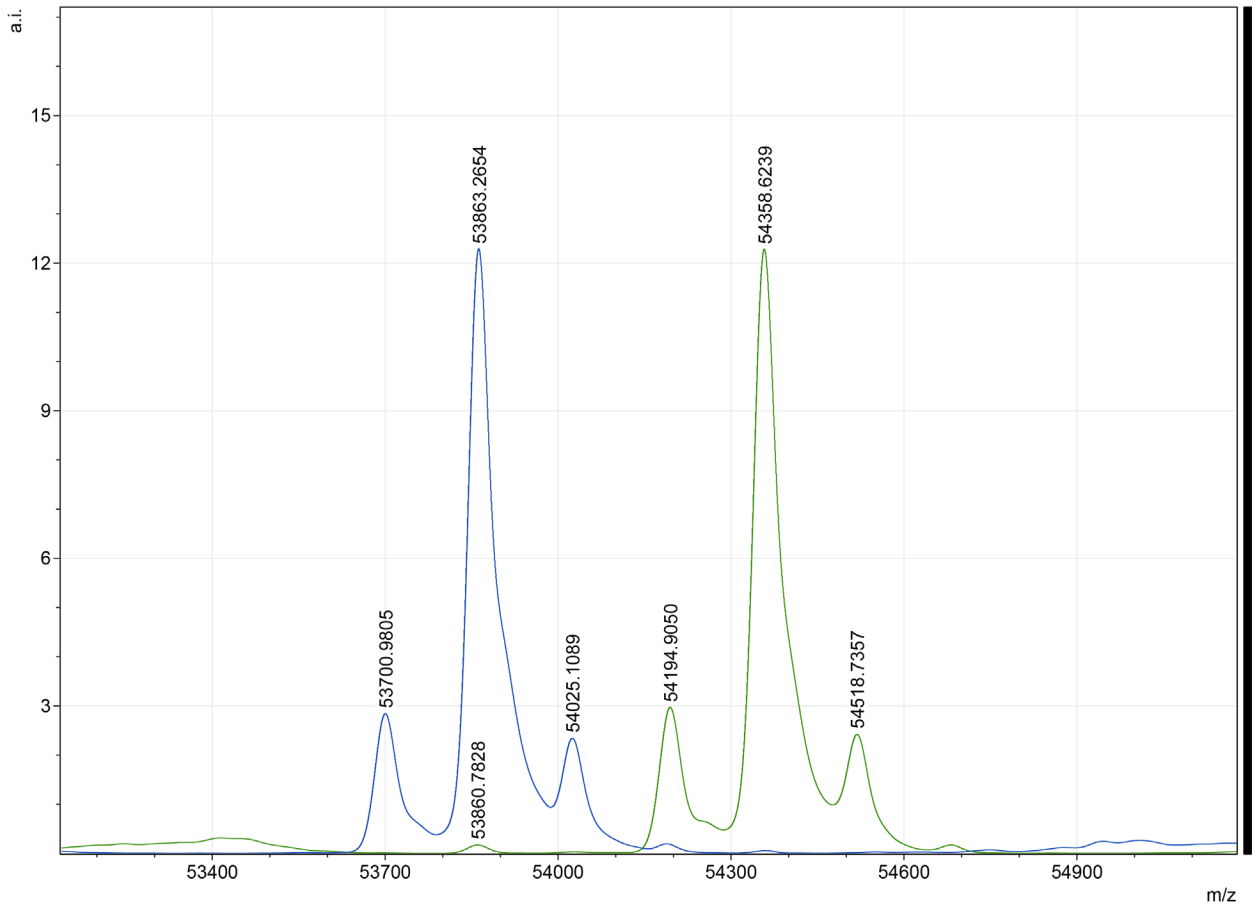
^{13}C NMR Spectrum of Compound 5:



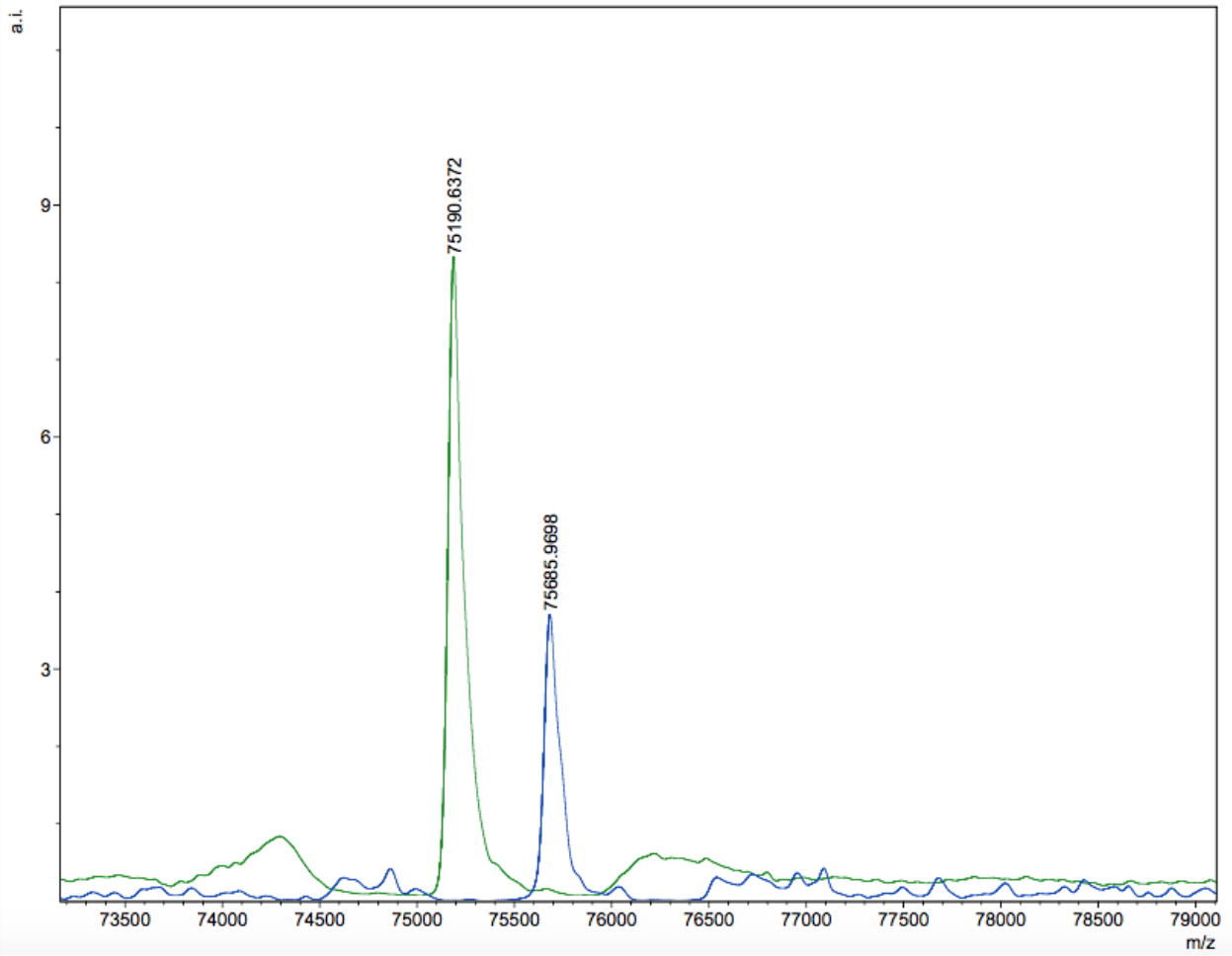
2. Intact-MS Experiments.

Each amylase was diluted to 100 nM in its respective buffer (Table S1) containing either 0 μM **5** (wild-type amylase control) or 500 μM **5**. Samples were then incubated for 30 min after which the reaction mixtures were placed in a centrifugal filter device (10,000 MWCO, NanoSep) and spun down to concentrate protein away from excess reagent. Each protein was immediately dissolved in 500 μL of a 30% acetonitrile, 0.1% formic acid solution, and this sample (5 μL) was injected onto a liquid chromatography system (Agilent 1200 HPLC equipped with an Agilent Zorbax 300SB-C8 column) and eluted using a gradient of 5%→95% acetonitrile (with 0.1% formic acid) for analysis by coupled ESI-MS (Agilent 6550 qTOF mass spectrometer). The protein elution peak was integrated, and UniDec 3.1.0[6] was used to deconvolve from the multiple charged species, followed by plotting with mMass 5.5.0.[7] The spectra are shown below.

A. Intact-MS spectra for *A. oryzae* amylase (AOA) with 0 μM **5** (blue) and with 500 μM **5** (green).



B. Intact-MS spectra for *E. pohliae* amylase (EPA) with 0 µM **5** (green) and with 500 µM **5** (blue).



C. Intact-MS spectra for human pancreatic amylase (HPA) with 0 μM **5** (blue) and with 500 μM **5** (orange)

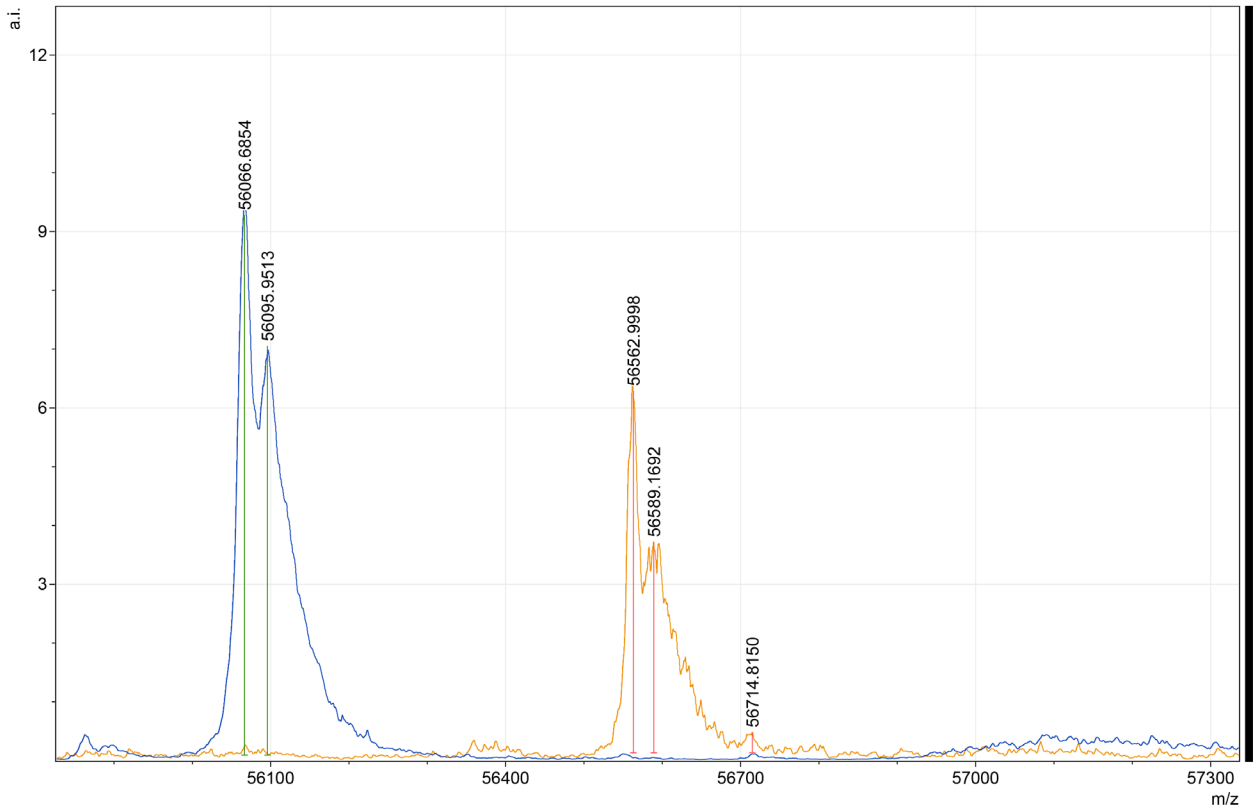


Figure S1. Intact protein mass spectra for A.) AOE, B) EPA, and C) HPA.

3. Enzyme Kinetics.

Enzyme kinetic experiments were performed as described below using the conditions outlined in Table S1. All experiments were performed in buffer with DMSO content (v/v) <5%.

Enzyme	Buffer	pH	T (ºC)
E. pohliae amylase (EPA)	50 mM NaOAc, 1 mM $CaCl_2$	6.0	25
A. oryzae amylase (AOA)	50 mM NaOAc, 1 mM $CaCl_2$	6.0	25
R. pusillus amylase (RPA)	50 mM NaOAc, 1 mM $CaCl_2$	6.0	25
Human Pancreatic Amylase (HPA)	50 mM NaP_i, 100 mM NaCl	7.0	30
Porcine Pancreatic Amylase (PPA)	50 mM NaP_i, 100 mM NaCl	7.0	30

Time-based Inactivation of Amylases.

Inactivator **5** (1 mM) was incubated with each enzyme under conditions listed in Table S1. At appropriate time-points, aliquots of this enzyme-inactivator reaction mixture were removed and diluted into a cuvette containing CNP-G3 (20 mM; >5 x K_m) that had been preincubated in the same buffer and temperature conditions (Table S1), such that the final volume was 200 µL. The initial rate of CNP-G3 hydrolysis for each time-point aliquot was determined by monitoring the change in absorbance of the sample at 355 nm with respect to time on a Cary 4000 UV/Visible spectrophotometer. At the same time points, aliquots of enzyme without **5** treated in the same conditions were also removed, diluted into a cuvette containing CNP-G3 (20 mM; >5 x K_m) to a final volume of 200 µL, and the initial rate of CNP-G3 hydrolysis was also determined by monitoring the change in absorbance of the sample at 355 nm with respect to time. The rates of CNP-G3 hydrolysis for each enzyme (with and without 1 mM **5**) were then compared, giving the remaining % activity over the time course of the experiment (Figure S2).

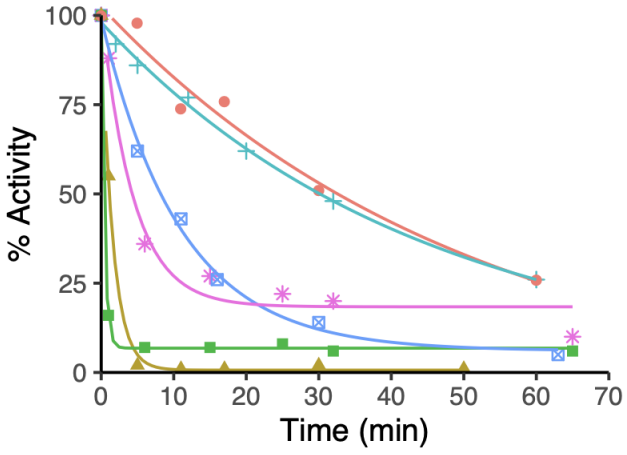
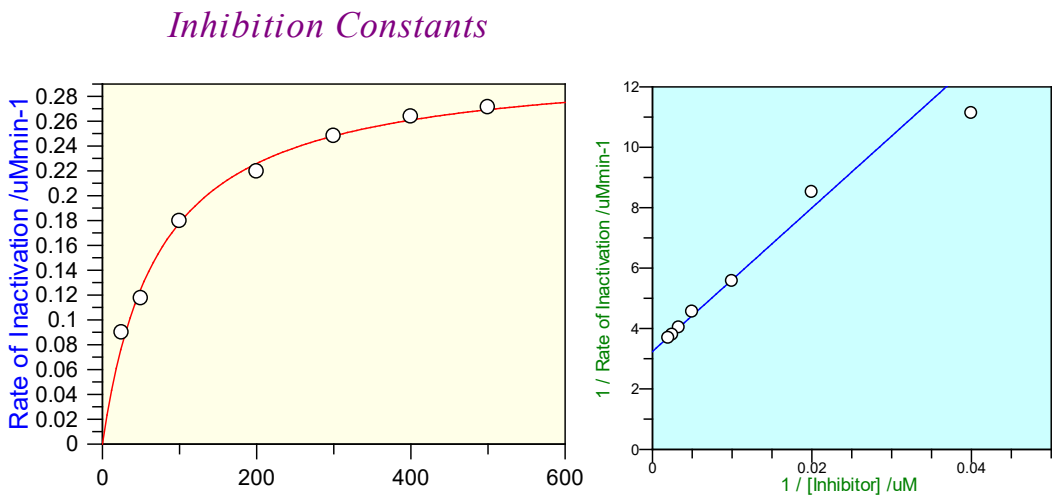


Figure S2. Percent activity remaining of various amylases with compound **5** (1 mM). Orange Circle: HPA. Blue Cross: AMG. Pink Star: RPA. Blue Box with X: PPA. Green Square: EPA. Yellow Triangle: AOA.

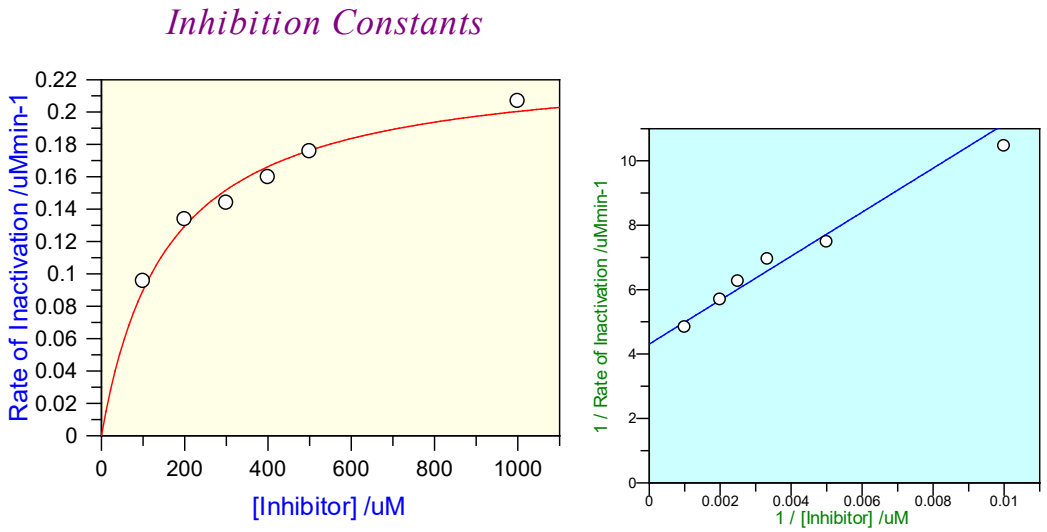
Determination of K_i, k_{on}, and k_{off} for the various amylases.

All experiments were carried out on a Cary UV/Visible spectrophotometer with each enzyme in the buffer and at the temperature listed in Table S1. The data were analyzed using the program GraFit 7.0 (Erithacus software). Varying concentrations of compound **5** were pre-incubated in buffer at 25 °C or 30 °C, based on the optimal conditions of the enzyme to be measured (Table S1), and the reaction then was initiated by the addition of enzyme. The release of F_2Mu was monitored by absorbance at 370 nm (or at 380 nm for HPA and PPA). Re-plots of the observed initial rates for the formation of the cyclitol–enzyme intermediate vs [**5**] were fit to the Michaelis-Menten equation to provide k_{on} and K_i values. Rates of cyclitol release (k_{off}) from the active site were determined at saturating concentrations of **5**, with the observed rate, V_{off}, divided by [E_0] to provide k_{off}.

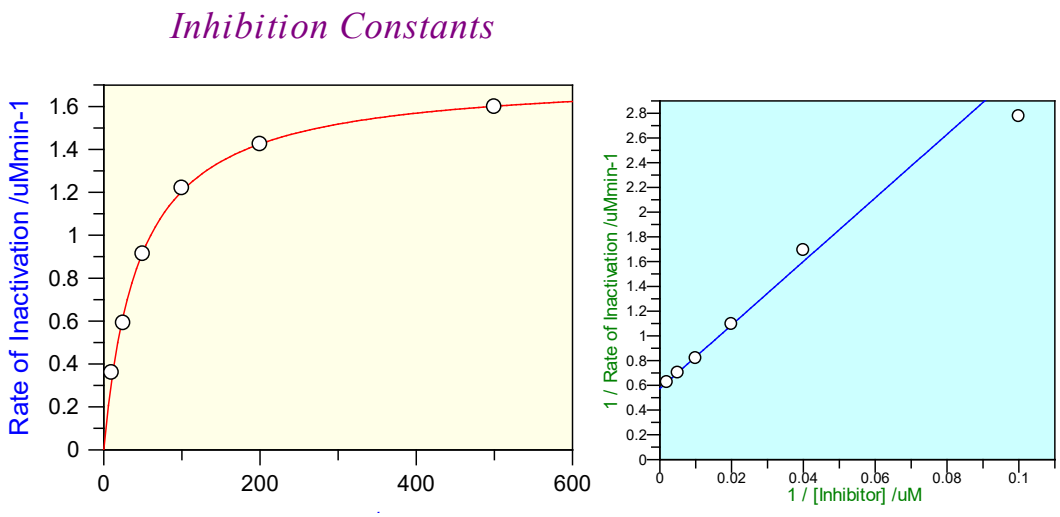
A. oryzae amylase (AOA):



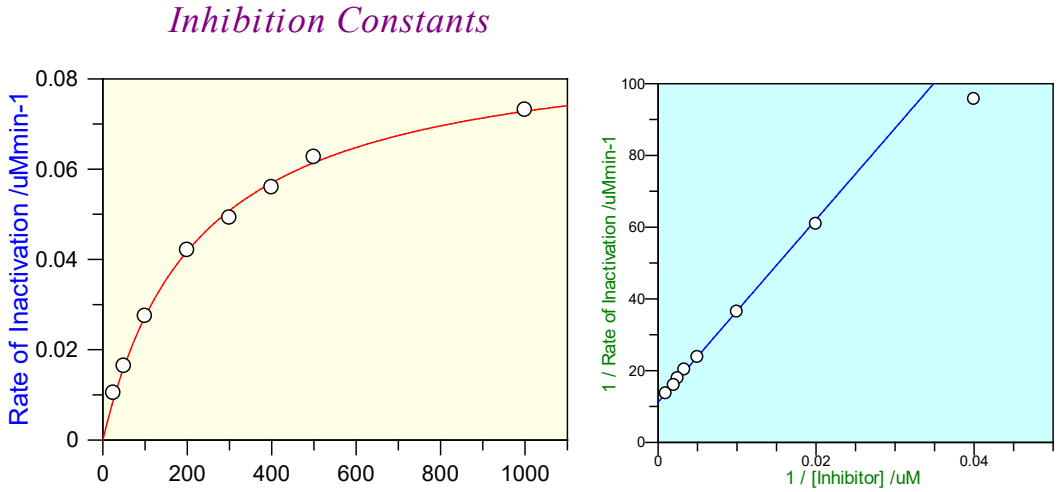
R. pusillus amylase (RPA):



E. pohliae amylase (EPA):



PPA:



HPA:

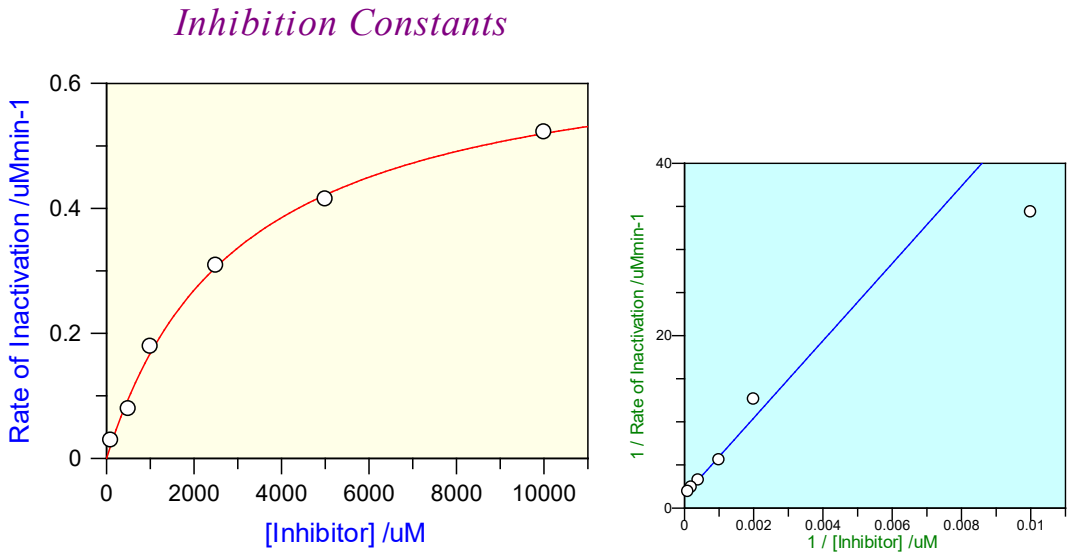


Figure S3. Michaelis-Menten plots to determine K_i and V_{max} of several amylases via the plotting of initial rates with titration reagent **5**.

Enzyme	**K_i** (μM)	**V_{max}** (min^{-1})
A. oryzae amylase (AOA)	73.39	0.309
R. pusillus amylase (RPA)	158.5	0.232
E. pohliae amylase (EPA)	44.84	1.745
PPA	227.5	0.089
HPA	3038	0.678

Table S2. Michaelis-Menten parameters (K_i and V_{max}) of several amylases determined via plotting of initial rates with titration reagent **5**.

4. Fluorescence Calibration Curve of [F_2Mu].

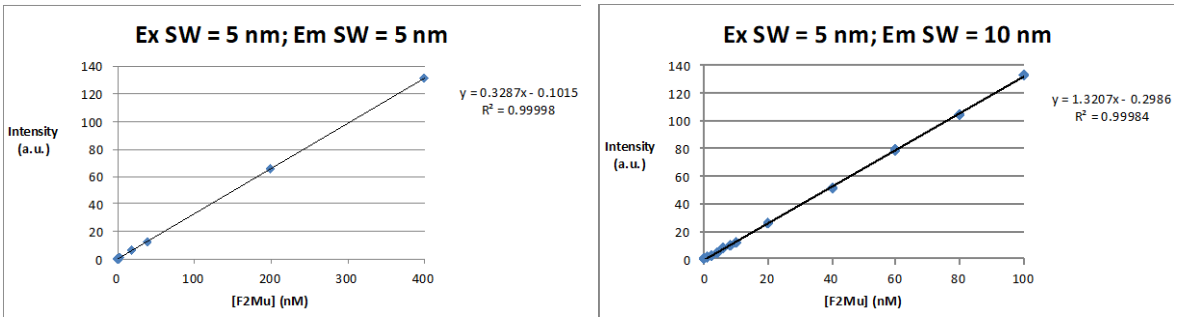


Figure S4. Calibration Curves for F_2Mu: Excitation Slit Width = 5 nm, Emission Slit Width = 5 nm (left); Excitation Slit Width = 5 nm, Emission Slit Width = 10 nm (right).

The calibration curves were prepared by a 1000x dilution of a standard of F_2MU of known concentration (1 mM in DMSO) to a concentration of 1 μM in 50 mM NaOAc, 1 mM $CaCl_2$, pH 6.0 buffer. This 1 μM sample was then diluted in series down to 10 nM. This series of dilutions, and a blank sample containing 0 nM F_2MU, were then scanned (λ_{ex} = 343 nm, λ_{em} = 451 nm) at 25 °C in sets of triplicates. The max intensity was then recorded and plotted vs. the known concentration of F_2MU to give the corresponding plots at each set of slit widths described above.

5. Titrations of α-Glucosidases.

Compound **3** was dissolved in DMSO to provide a 50 mM stock, and diluted in distilled H_2O to a working concentration of 1 mM. A model enzyme, Brewer's yeast glucosidase (Type VI, Cat. No. G6136, Millipore-Sigma), was used directly from lyophilized powder diluted in water (~1 mg/100 µL) and used immediately or stored on ice. Cuvettes were primed with 350 µL of buffer (50 mM NaPi, 100 mM NaCl, pH = 7.0) and 30 µL of compound **3** (final concentration of 75 µM). Fluorescence intensity was monitored (λ_{ex} = 343 nm, λ_{em} = 451 nm) for approximately 8 minutes at 25 °C. Readings were then briefly paused and the reaction was initiated by addition of α-glucosidase (20 µL) to each cuvette with mixing. Fluorescence intensity monitoring continued for an additional 50 min. This was repeated with 2 further dilutions of α-glucosidase to ensure response indeed scaled with amount of glucosidase. Final volume of 400 µL in each cuvette was maintained by increasing the volume of buffer.

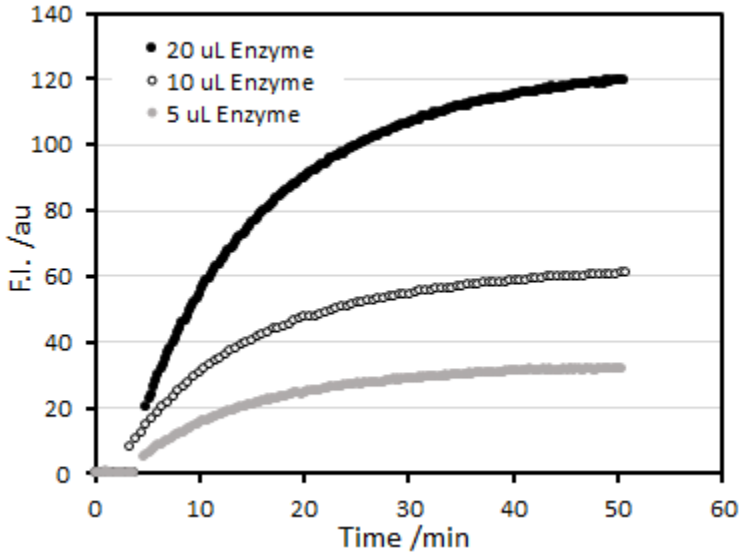


Figure S5. Titration of an α-glucosidase from Brewer's Yeast with compound **3** (75 µM).

Titrations of Amylases.

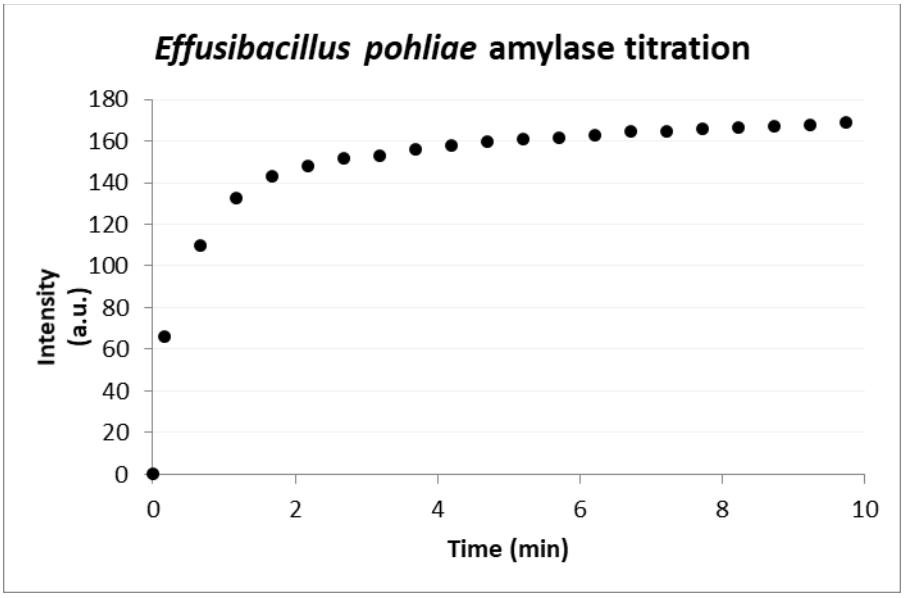
All experiments were carried out in buffer (depending on the enzyme measured; Table S1) with a Cary Eclipse fluorescence spectrophotometer at 20 °C. In a cuvette, the enzyme was pre-incubated in buffer (360 μL total) for 2 min, and the reaction was initiated by the addition of 1 mM titration reagent **5** (40 μL). At the same time, two more cuvettes were prepared in this manner to get triplicate measurements. Also at the same time, 1 mM titration reagent **5** (40 μL) was added to buffer (360 μL) in a separate cuvette to give $F_{background}$ (the background fluorescent signal of F_2Mu present in solution). Fluorescence was then monitored over time for all four prepared cuvettes. These measurements gave initial burst phases (F_{final}), as well as a linear steady-state line that corresponds to the slow turnover phases (k_{off}). After subtraction of $F_{background}$ from F_{final} (Equation S1), this steady-state line was extrapolated back to the y-intercept (t=0) to determine the burst ($F_{response}$) for each titration. F_2Mu release was calculated based on the calibration curve of F_2Mu (Figure S3). The concentration of the enzyme was then calculated according to Equation 1 in the main text, and the enzymes' corresponding rate constants (Table 2).

Equation S1: $F_{response} = F_{final} - F_{background}$

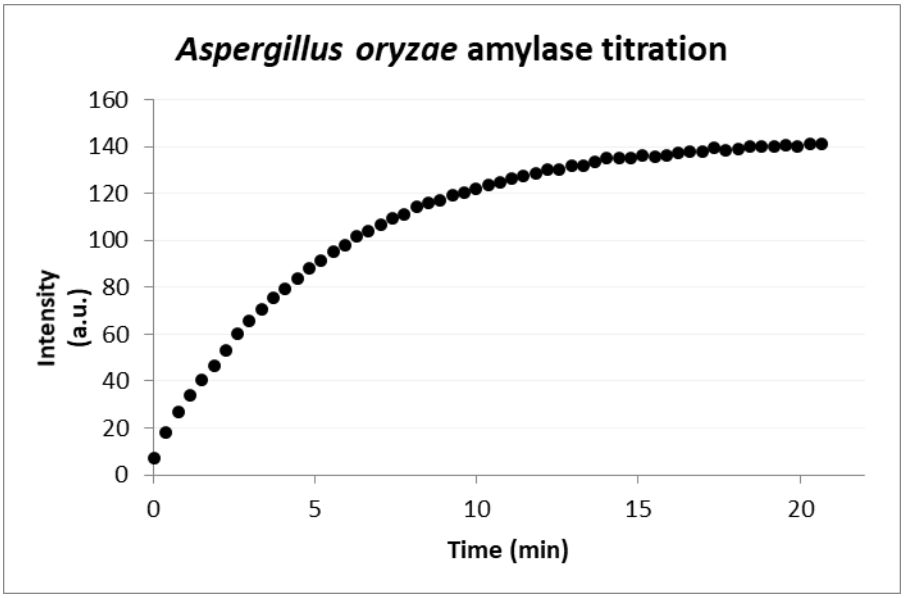
Equation S2: $F_{background} = 0.903(\text{Intensity/min})*t + F_{initial(t=0)}$

Burst Plots of Amylase Titrations.

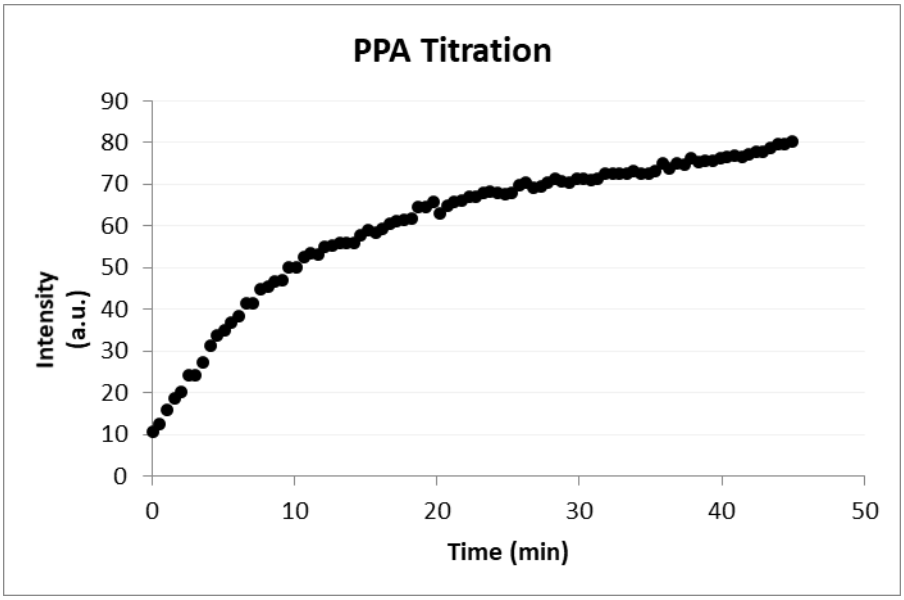
A. *E. pohliae* amylase (EPA):



B. *A. oryzae* amylase (AOA):



C. Porcine pancreatic amylase (PPA):



D. *R. pusillus* amylase (RPA):

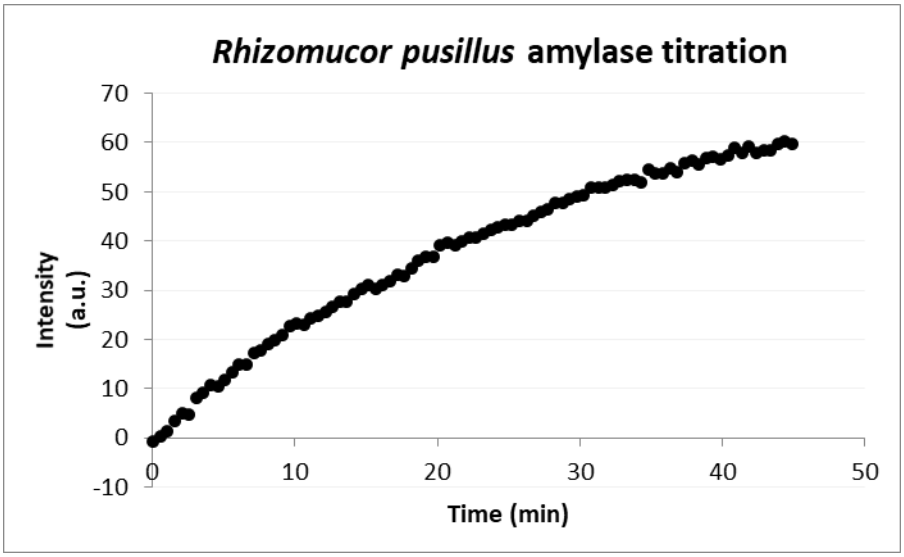
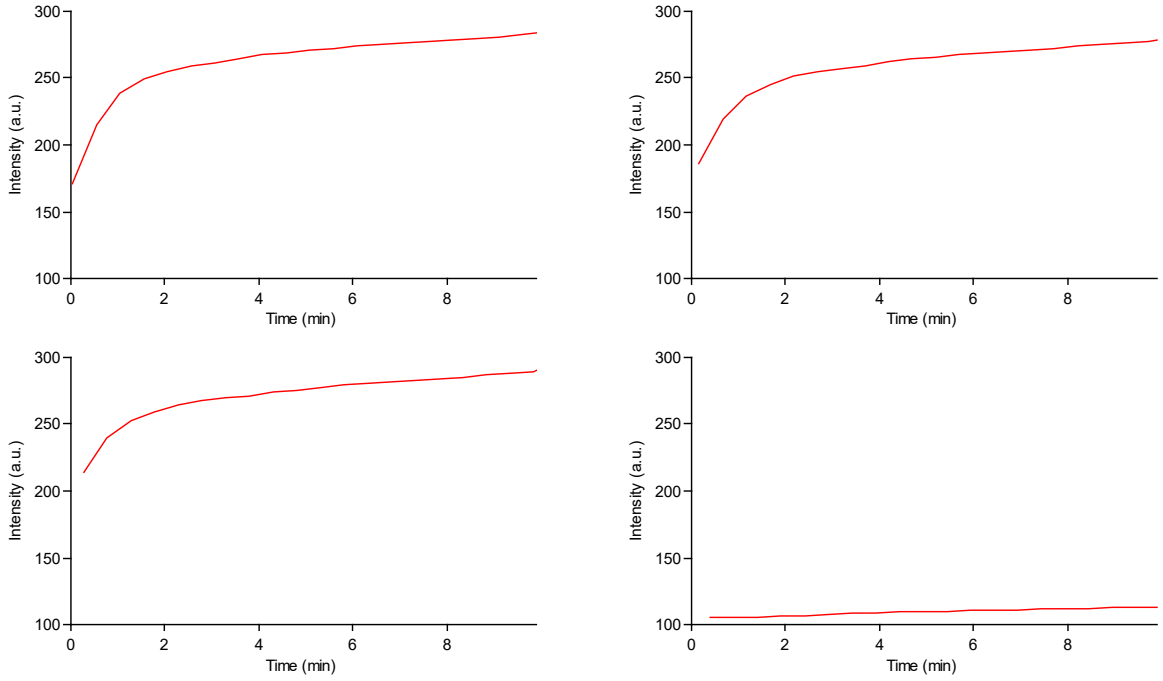


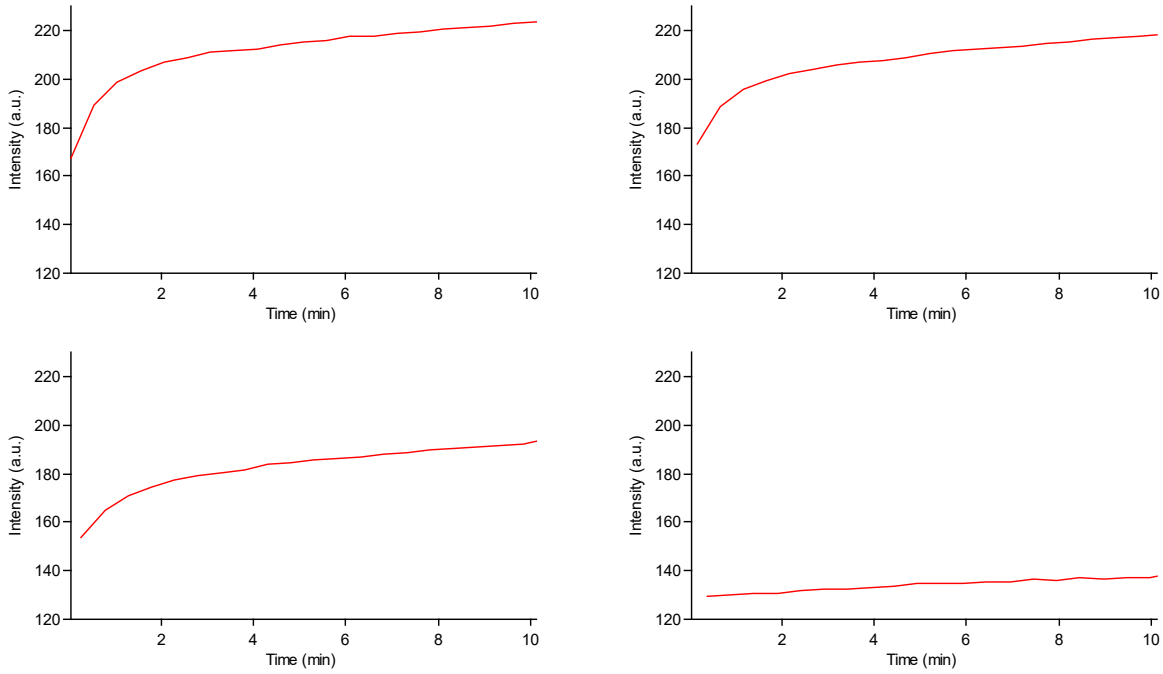
Fig S6: Plots of amylase titrations.

Raw Bursts of Titrations (serial 2x dilutions of *E. pohliae* amylase (EPA).

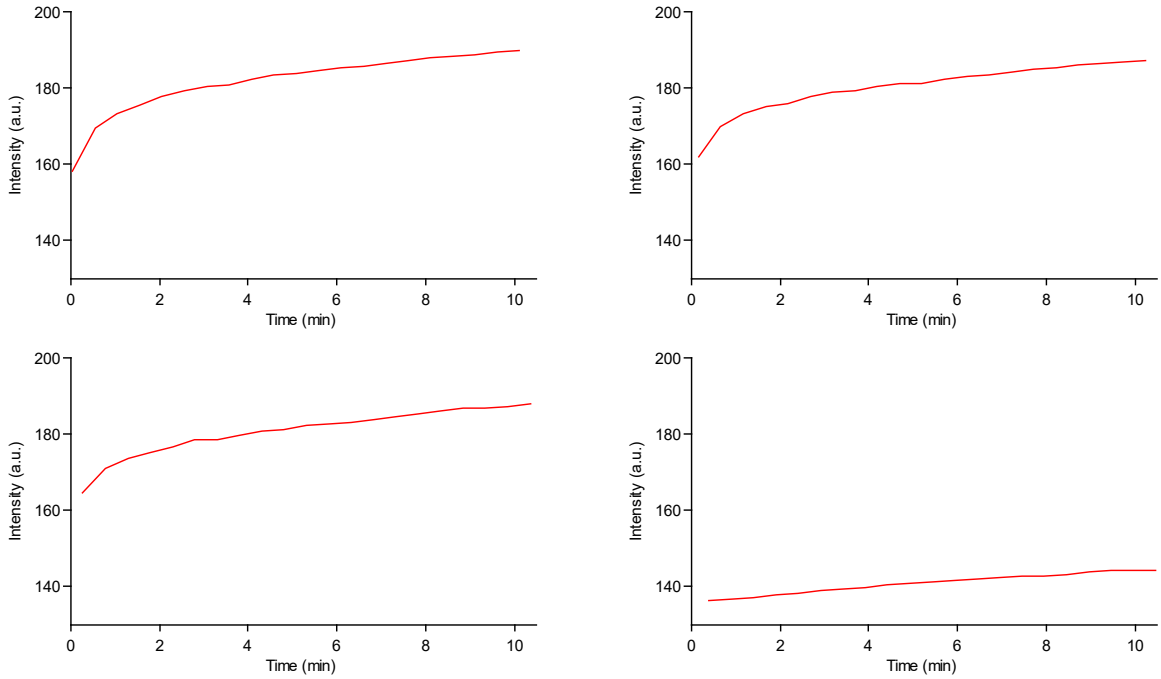
100 nM EPA:



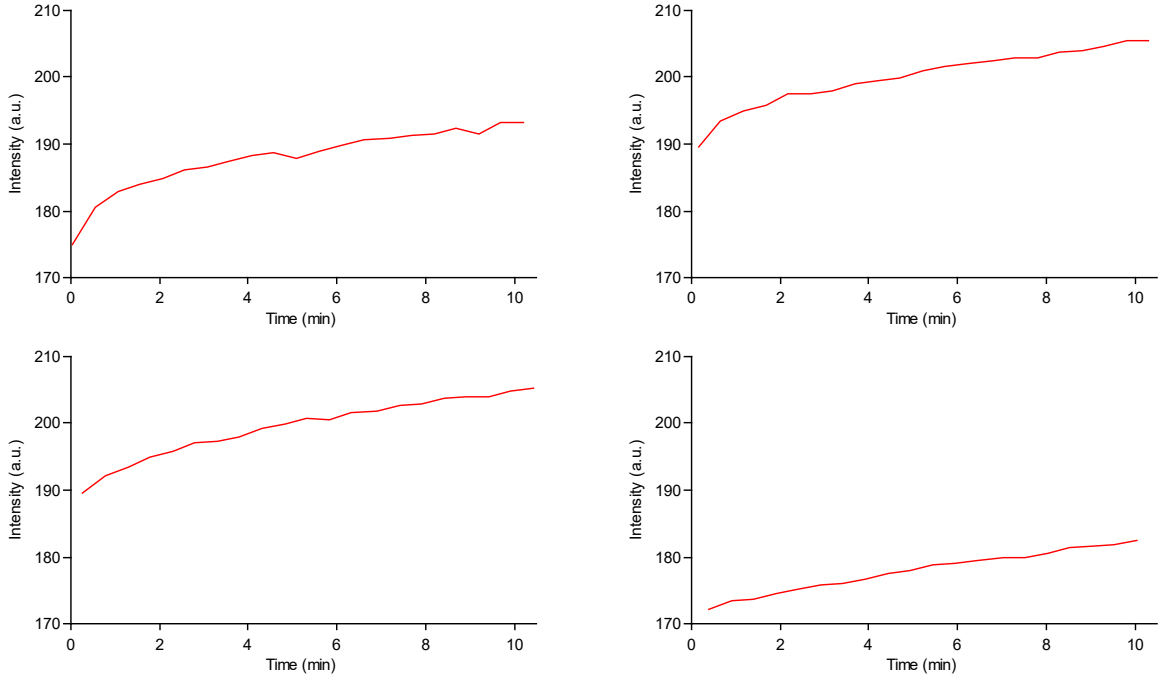
50 nM EPA:



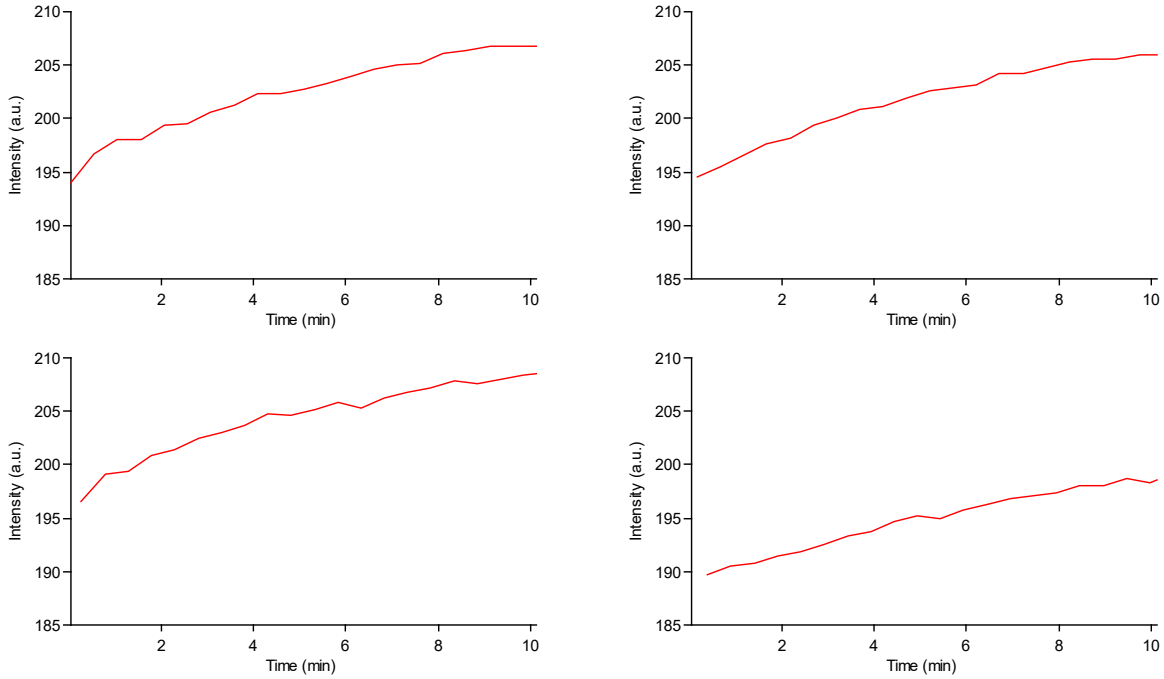
25 nM EPA:



12.5 nM EPA:



6.25 nM EPA:



3.1 nM EPA:

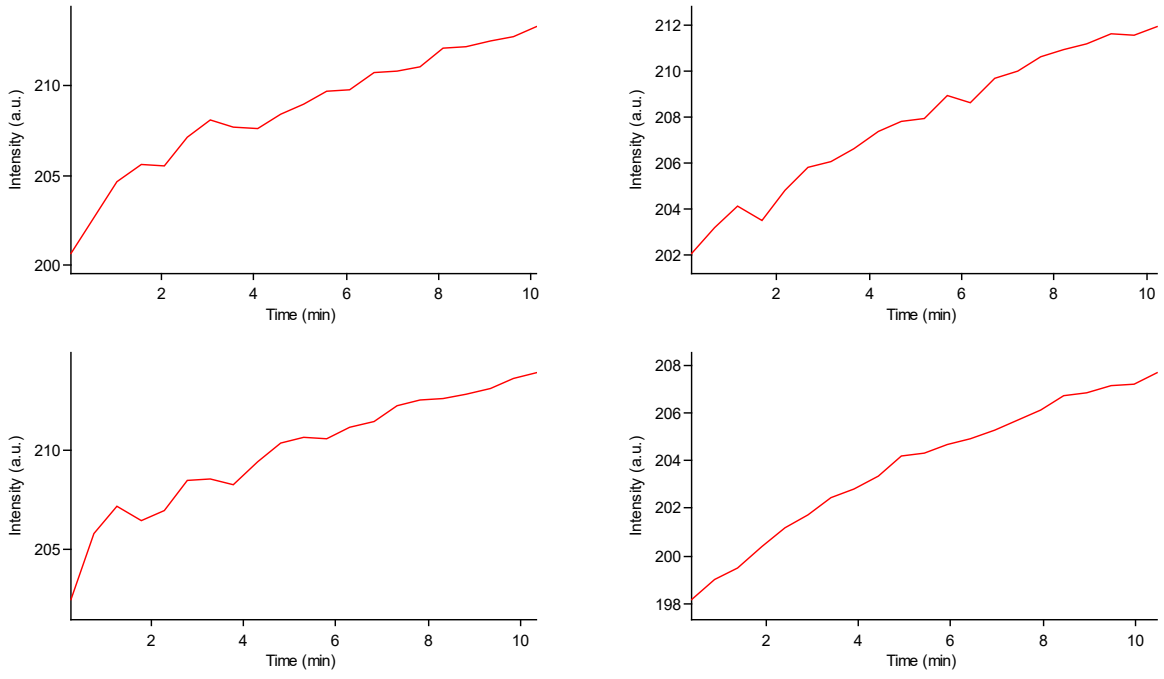


Figure S6. Raw fluorescence bursts for a series of 2 x dilutions of EPA (100 nM – 3.1 nM). Top left, top right, and bottom left plots are triplicates, and bottom right plot is hydrolysis of **5**.

References:

1 C. Roth, O. V. Moroz, J. P. Turkenburg, E. Blagova, J. Waterman, A. Ariza, L. Ming, S. Tianqi, C. Andersen, G. J. Davies and K. S. Wilson, *IJMS*, 2019, **20**, 4902–4915.
2 E. H. Rydberg, G. Sidhu, H. C. Vo, J. Hewitt, H. C. F. Cote, Y. Wang, S. Numao, R. T. A. MacGIllivray, C. M. Overall, G. D. Brayer and S. G. Withers, *Protein Sci.*, 1999, **8**, 635–643.
3 T. Shing, Y. Chen and W. Ng, *Synlett*, 2011, **9**, 1318–1320.
4 I. Damager, S. Numao, H. Chen, G. D. Brayer and S. G. Withers, *Carbohydr. Res.*, 2004, **339**, 1727–1737.
5 P. J. P. Adabala, S. S. K. Abadi, O. Akintola, S. Bhosale and A. J. Bennet, *J. Org. Chem.*, 2020, **85**, 3336–3348.
6 M. T. Marty, A. J. Baldwin, E. G. Marklund, G. K. A. Hochberg, Justin L. P. Benesch and C. V. Robinson, *Anal. Chem.*, 2015, 4370–4376.
7 T. H. J. Niedermeyer and M. Strohalm, *PLOS One*, 2012, **7**, e44913.