Supporting Information for:

New Substrates and Enzyme Assays for the Detection of Mucopolysaccharidosis III (Sanfilippo Syndrome) Types A, B, C and D by Tandem Mass Spectrometry

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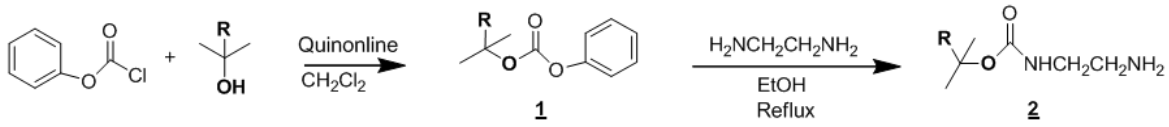
Synthetic procedures, spectroscopic data, Schemes S1-S4, Figures S1-S8.

Synthesis of the MPS III type A,B,C and D Substrates, Products and Internal Standards



Scheme S1. The diamine linkers seen in Figure S1 were synthesized using the following procedure.

Preparation of Compound 1 Type A:

2-methyl-2-butanol (423 mg, 4.80 mmol, 1 eq) was combined with quinoline (0.567 mL, 4.80 mmol, 1 eq) in 1 mL of anhydrous dichloromethane and stirred under nitrogen at room temperature. Phenyl chloroformate (752 mg, 4.80 mmol, 1 eq) was added dropwise to the reaction mixture over the course of 2 to 3 hours. The reaction mixture was stirred for 24 hours. The solvent was removed under vacuum. The product was loaded onto silica gel and eluted with hexane to give **Compound 1** (587 mg, 59%): R_f 0.22 (hexane).

Compound 1 Type A: ^{1}H NMR (300 MHz, $CDCl_3$):): δ7.50-7.10 (m, 5H, H-5), δ1.85 (q, 2H, $CC\mathbf{H}_2CH_3$), δ1.51 (s, 6H, 2x $CC\mathbf{H}_3$), δ0.96 (t, 3H, $CCH_2C\mathbf{H}_3$).

Compound 1 Type C: ^{1}H NMR (300 MHz, $CDCl_3$):): δ7.50-7.10 (m, 5H, H-5), δ1.85 (q, 2H, $CC\mathbf{H}_2CH_2CH_2CH_3$), δ1.55 (s, 6H, 2x $CC\mathbf{H}_3$), δ1.38 (m, 4H, $CCH_2C\mathbf{H}_2C\mathbf{H}_2CH_3$), δ0.95 (t, 3H, $CCH_2CH_2CH_2C\mathbf{H}_3$).

Compound 1 Type D: ^{1}H NMR (300 MHz, $CDCl_3$):): δ7.50-7.10 (m, 5H, H-5), δ1.85 (q, 2H, $CC\mathbf{H}_2CH_2CH_3$), δ1.56 (s, 6H, 2x $CC\mathbf{H}_3$), δ1.48 (m, 2H, $CCH_2C\mathbf{H}_2CH_3$), δ0.99 (t, 3H, $CCH_2CH_2C\mathbf{H}_3$).

Chains:

Preparation of Compound 2 Type A:

Ethylenediamine (30 mg, 0.499 mmol, 1 eq) was added to a solution containing **Compound 1 Type A** (135 mg, 0.499 mmol, 1.3 eq) in 2 mL of absolute ethanol. The reaction mixture was refluxed for 24 hours. The mixture was cooled to room temperature, and the solvent was removed under vacuum. 3 mL of H_2O was added to the residue. 1 M hydrochloric acid was added dropwise to the solution until the pH was adjusted to 3. The solution was then extracted three times with 50 mL of dichloromethane. 1 M sodium hydroxide was added to the solution until the pH was greater than 10, after which it was extracted twice with 50 mL of dichloromethane. The organic layer was dried over Na_2SO_4 and concentrated under vacuum to yield **Compound 2** (30 %) R_f 0.065 (CH_2Cl_2:MeOH, 9:1).

Compound 2 Type A: ^{1}H NMR (300 MHz, $CDCl_3$):): δ3.20 (m, 2H, $H_2NCH_2C\mathbf{H}_2NHCO_2$), δ2.81 (t, 2H, $H_2NC\mathbf{H}_2CH_2NHCO_2$), δ1.80 (q, 2H, $CC\mathbf{H}_2CH_3$), δ1.41 (s, 6H, 2x $CC\mathbf{H}_3$), δ0.90 (t, 3H, $CCH_2C\mathbf{H}_3$).

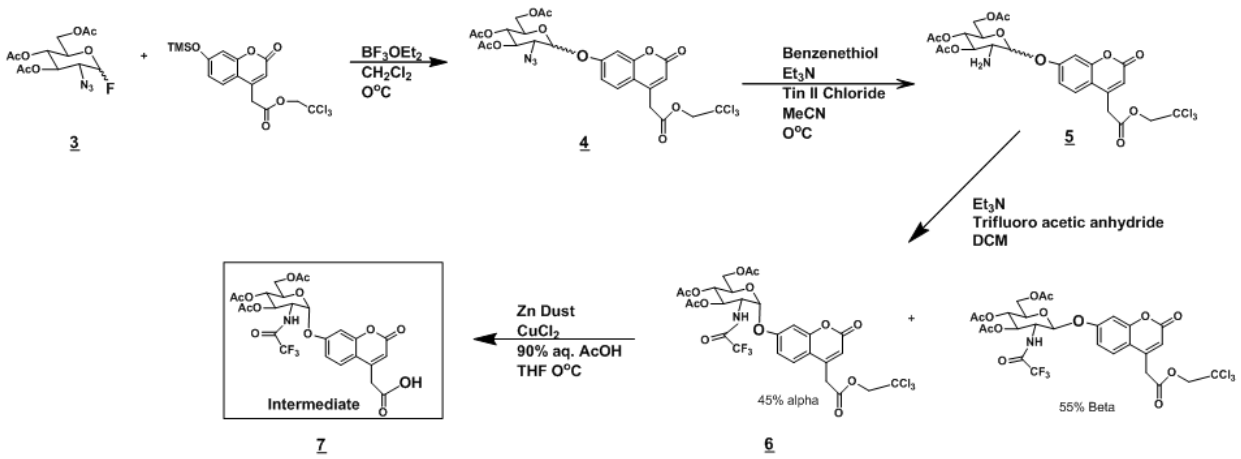
Compound 2 Type A-IS: ^{1}H NMR (300 MHz, $CDCl_3$):): δ1.80 (q, 2H, $CC\mathbf{H}_2CH_3$), δ1.41 (s, 6H, 2x $CC\mathbf{H}_3$), δ0.90 (t, 3H, $CCH_2C\mathbf{H}_3$).

Compound 2 Type C: ^{1}H NMR (300 MHz, $CDCl_3$):): δ3.17 (m, 2H, $H_2NCH_2C\mathbf{H}_2NHCO_2$), δ2.80 (t, 2H, $H_2NC\mathbf{H}_2CH_2NHCO_2$), δ1.73 (q, 2H, $CC\mathbf{H}_2CH_2CH_2CH_3$), δ1.43 (s, 6H, 2x $CC\mathbf{H}_3$), δ1.30 (m, 4H, $CCH_2C\mathbf{H}_2C\mathbf{H}_2CH_3$), δ0.91 (t, 3H, $CCH_2CH_2CH_2C\mathbf{H}_3$).

Compound 2 Type C-IS: ^{1}H NMR (300 MHz, $CDCl_3$):): δ1.73 (q, 2H, $CC\mathbf{H}_2CH_2CH_2CH_3$), δ1.43 (s, 6H, 2x $CC\mathbf{H}_3$), δ1.30 (m, 4H, $CCH_2C\mathbf{H}_2C\mathbf{H}_2CH_3$), δ0.91 (t, 3H, $CCH_2CH_2CH_2C\mathbf{H}_3$).

Compound 2 Type D: ^{1}H NMR (300 MHz, $CDCl_3$):): δ3.14 (m, 2H, $H_2NCH_2C\mathbf{H}_2NHCO_2$), δ2.77 (t, 2H, $H_2NC\mathbf{H}_2CH_2NHCO_2$), δ1.67 (q, 2H, $CC\mathbf{H}_2CH_2CH_3$), δ1.41 (s, 6H, 2x $CC\mathbf{H}_3$), δ1.32 (m, 2H, $CCH_2C\mathbf{H}_2CH_3$), δ0.90 (t, 3H, $CCH_2CH_2C\mathbf{H}_3$).

Compound 2 Type D-IS: ^{1}H NMR (300 MHz, $CDCl_3$):): δ1.67 (q, 2H, $CC\mathbf{H}_2CH_2CH_3$), δ1.41 (s, 6H, 2x $CC\mathbf{H}_3$), δ1.32 (m, 2H, $CCH_2C\mathbf{H}_2CH_3$), δ0.90 (t, 3H, $CCH_2CH_2C\mathbf{H}_3$).



Scheme S2. Synthetic scheme for MPS III typ A,B,C and D substrates and internal standards.

Preparation of Compound 4: $LiClO_4/SiO_2$ was prepared by mixing 0.4g of $LiClO_4$ and 0.8g of silica gel in 10 mL of dry ether. The mixture was allowed to stir at room temperature for 20 minutes then the solvent was removed by rotary evaporation and the solid was further dried under high vacuum. Dichloromethane was dried over P_2O_5 and distilled under dry N_2. The first and last fractions were discarded while the middle fraction was collected on a bed of dry 3A molecular sieves. The dry coumarin[S1] (555 mg, 1.26 eq) and $LiClO_4$ / SiO_2 (200 mg) were placed in the freshly distilled dichloromethane (~10 mL) . (Note: distillation is necessary due to the stabilizer present in dichloromethane which causes low yields) under nitrogen atmosphere and 1,1,1,3,3,3 hexamethyldisilizane (659 µL, 2.52 eq) was added dropwise. The reaction mixture was allowed to stir at room temperature for 30 minutes and became a dark yellow color. After 30 minutes the solution was diluted with more freshly distilled dichloromethane (~20 mL) and was quickly filtered through cotton into a round bottom flask containing **Compound 3** (421 mg, 1 eq)[S2,S3]. The solvent was quickly removed by rotary evaporation (room temperature water bath), then the residue was allowed to dry further under high vacuum for ~10 min. The residue was placed under a nitrogen atmosphere. The residue was dissolved in freshly distilled dichloromethane (~20 mL) and was cooled in an ice bath. BF_3 etherate (154 µL) was added dropwise, and the solution was allowed to warm to room temperature and continued to stir for 2 hours. The solution was then concentrated by rotary evaporation. The mixture was dissolved in acetic anhydride (10 mL) and BF_3 etherate (100 µL) was added to re-acetylate any hydroxyls that had been unintentionally deprotected during the coupling reaction. The solution was allowed

to stir for 20 minutes then was diluted with 200 mL dichloromethane. The organic layer was washed with saturated sodium bicarbonate solution, brine and finally water. The dichloromethane was then dried with magnesium sulfate, filtered and the solvent was removed by rotary evaporation. The compound was purified by flash chromatography using a hexane/ ethyl acetate gradient 0 to 100 % ethyl acetate. The compound was isolated as a light yellow syrup 68% yield, 40% alpha, 60% beta. Rf 0.54 in $CHCl_3$/MeOH 3%.

S1. Blanchard, S.; Sadilek, M.; Scott, C. R.; Turecek, F.; Gelb, M. H. *Clin. Chem.* **2008**, *54*, 2067-2070.

S2. Tailler, D.; Jacquinet, J. C.; Noirot, A. M.; Beau, J. M. *J. Chem. Soc. Perkin Trans. 1* **1992**, 3163-3164.

S3. Dasgupta, F.; Masada, R. I. *Carbohydrate Res.* **2002**, *337*, 1055-1058.

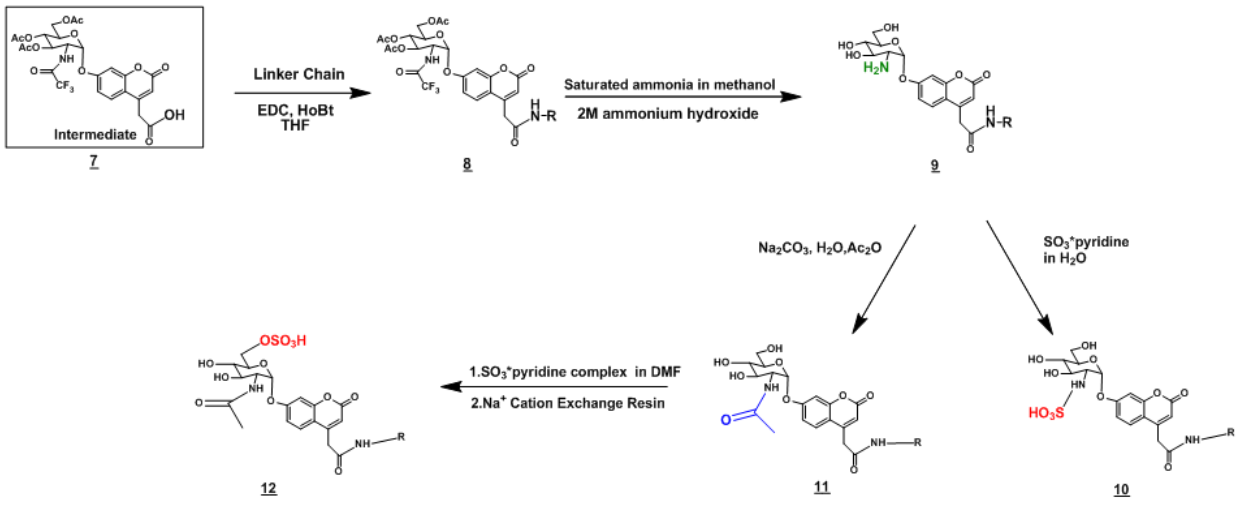
Compound 4: ^{1}H NMR (300 MHz, $CDCl_3$): δ7.54 (d,0.4H, **H**-5), δ7.50 (d, 0.6H, **H**-5), δ7.12-6.88 (m, 2H, **H**-8, **H**-6), δ6.31 (s,1H, **H**-3), δ5.68-5.56 (m,1H, **H**-1α', **H**-3α'), δ5.14-4.94 (m,2.2H, **H**-1β' ,**H**-3β', **H**-4'), **H**-1β) δ4.73(s, 2H, **H**- trichloroethyl), δ4.3-3.98 (m, 3H, **H**-5'**,** **H**-6'), δ3.88 (s,2H, C**H**$_2$CO$_2$), δ3.79 (dd, 0.6H, **H**-2β'), δ3.57 (dd, 0.4H, **H**-2α'), δ2.14-2.06 (3s, 9H, **OAc**).

Preparation of Compound 6: Compound 4 (2.8g, 1 eq) was dissolved in 28 mL of dry acetonitrile under nitrogen and was cooled in an ice bath. To this solution was added a ice-cold solution of tin II chloride (0.19g, 0.20 eq), triethylamine (1.27 g, 3 eq) and thiophenol (1.85 g, 4 eq) in 13 mL of dry acetonitrile which caused the solution to turn yellow. The solution was allowed to stir on ice for 1 hour or until no starting material was visible on TLC. The sample was then concentrated by rotary evaporation, diluted with dichloromethane then washed with cold 0.5 M NaOH, then cold water. Mass spectrometric analysis showed *m/z* 638 and 640 confirming that the reaction had worked. The organic layer was then dried by rotary evaporation, and the crude product (**Compound 5**) was used for the next step without further purification. The crude product was dried under vacuum and placed under a nitrogen atmosphere. The compound was dissolved in dry 28 mL of dichloromethane and cooled in an ice bath. Triethylamine (1.01g, 2 eq) was first added dropwise, followed by dropwise addition of trifluoroacetic anhydride (1.58 g, 1.5 eq). The sample was allowed to stir on ice for 16 hours. The solvent was removed by rotary evaporation, and the compound was purified using flash chromatography with a hexane/ethyl

acetate gradient 0 to 100% ethyl acetate. In order to achieve adequate separation on silica it is important to limit the injection width and to not overload the column. 62.5% yield over 2 steps. 25% alpha over 2 steps. R_f 0.4 for alpha, 0.3 for beta anomer in $CHCl_3$/MeOH 3%.

Compound 6: ^{1}H NMR (300 MHz, $(CD_3)_2CO$)):): δ7.77 (d,0.4H, **H**-5), δ7.22 (m, 2H, **H**-8, **H**-6), δ6.44 (s,1H, **H**-3), δ5.97 (d,1H, *J* 3.3 Hz, **H**-1α'), δ5.57 (t,1H, **H**-3'), δ5.20 (t, 1H, **H**-4'), δ4.92(s, 2H, trichloroethyl), δ4.64(m, 1H, **H**-5'), δ4.32-4.05(m, 5H, **H**-2', **H**-6', C**H**$_2$CO$_2$), δ2.01-1.96 (3s, 9H, **OAc**).

Preparation of Compound 7: Compound 6 (77 mg, 1 eq) was dissolved in 3.6 mL tetrahydrofuran and cooled in an ice bath. Aqueous acetic acid (90 %, 0.48 mL) , copper II chloride (14.5 mg, 1 eq) and zinc dust (71 mg, 10 eq) were then added. Ten more equivalents of zinc dust were added after both 15 and 24 hours. After the solution had stirred for 39 hours the mixture was filtered through cellite, and the liquid was concentrated under vacuum. The compound was dissolved in dichloromethane, and the organic layer was washed two times with water and once with brine. The organic layer was dried with sodium sulfate. The compound was purified by flash chromatography on silica using a dichloromethane/ethyl acetate gradient with both solvents containing 1 % acetic acid. Yield 82%.



Scheme S3. Conversion of the intermediate (**7**) into the desired substrates and internal standards.

Preparation of Compound 8: Compound 7 (6.2 mg, 1 eq) was dissolved in 1 mL of anhydrous tetrahydrofuran and cooled in an ice bath under N_2. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (1.7 mg, 1.1 eq) and hydroxybenzotriazole (1.5 mg, 1.1 eq) were then added, and the soultion was allowed to stir on ice for 30 minutes. The diamine chain (1.64 mg, 1 eq) dissolved in 100 µL of dry dimethylforamide was then added dropwise causing the solution to turn yellow. The reaction was removed from the ice and allowed to stir over night at room temperature. The solvent was removed by rotary evaporation, and the compound was dissolved in ethyl acetate. The organic layer was washed with 1 M HCl, water and finally brine. The organic layer was then dried using sodium sulfate and the compound was purified by flash chromatography dichloromethane/MeOH 0 to 10 %. Yield 81%.

Compound 8 Type A: Rf = 0.71, CH_2Cl_2/MeOH 10%. ^{1}H NMR (300 MHz, $CDCl_3$):): δ7.65 (d, 1H, **H**-5), δ7.13-7.04 (m, 2H, **H**-8, **H**-6), δ6.30 (s, 1H, **H**-3), δ5.71 (d, 1H, *J* 3.3 Hz, **H**-1α'), δ5.51 (t, 1H, **H**-3'), δ5.27 (t, 1H, **H**-4'), δ4.94(m, 1H, **H**-5'), δ4.52-4.24(m, 3H, **H**-2', **H**-6'), δ3.67 (s, 2H, C**H**$_2$CO), δ 3.37 (t, 2H, NHC**H**$_2$), δ3.24(t, 2H, C**H**$_2$NH), δ2.10-2.02 (3s, 9H, **OAc**), δ1.77 (m, 2H, C**H**$_2$CH$_3$), δ1.43 (s, 6H, BOC), δ0.89 (t, 3H, CH_2C**H**$_3$).

Compound 8 Type A-IS: Rf = 0.71, CH_2Cl_2/MeOH 10%. ^{1}H NMR (300 MHz, $CDCl_3$):): δ7.65 (d, 1H, **H**-5), δ7.13-7.04 (m, 2H, **H**-8, **H**-6), δ6.30 (s, 1H, **H**-3), δ5.71 (d, 1H, *J* 3.3 Hz, **H**-1α'), δ5.51 (t, 1H, **H**-3'), δ5.27 (t, 1H, **H**-4'), δ4.94(m, 1H, **H**-5'), δ4.52-4.24(m, 3H, **H**-2', **H**-6'), δ3.67 (s, 2H, C**H**$_2$CO), δ2.10-2.02 (3s, 9H, **OAc**), δ1.77 (m, 2H, C**H**$_2$CH$_3$), δ1.43 (s, 6H, BOC), δ0.89 (t, 3H, CH_2C**H**$_3$).

Compound 8 Type B: Rf = 0.58, CH_2Cl_2/MeOH 10%. ^{1}H NMR (300 MHz, $CDCl_3$):): δ7.65 (d, 1H, **H**-5), δ7.13-7.04 (m, 2H, **H**-8, **H**-6), δ6.30 (s, 1H, **H**-3), δ5.71 (d, 1H, *J* 3.3 Hz, **H**-1α'), δ5.51 (t, 1H, **H**-3'), δ5.27 (t, 1H, **H**-4'), δ4.94(m, 1H, **H**-5'), δ4.52-4.24(m, 3H, **H**-2', **H**-6'), δ3.67 (s, 2H, C**H**$_2$CO), δ 3.37 (t, 2H, NHC**H**$_2$), δ3.24(t, 2H, C**H**$_2$NH), δ2.10-2.02 (3s, 9H, **OAc**), δ1.43 (s, 9H, BOC),.

Compound 8 Type C: Rf = 0.87, CH_2Cl_2/MeOH 10% ^{1}H NMR (300 MHz, $CDCl_3$):): δ7.65 (d, 1H, **H**-5), δ7.13-7.04 (m, 2H, **H**-8, **H**-6), δ6.30 (s, 1H, **H**-3), δ5.71 (d, 1H, *J* 3.3 Hz, **H**-1α'), δ5.51 (t, 1H, **H**-3'), δ5.27 (t, 1H, **H**-4'), δ4.94(m, 1H, **H**-5'), δ4.52-4.24(m, 3H, **H**-2', **H**-6'), δ3.67 (s, 2H, C**H**$_2$CO), δ 3.37 (t, 2H, NHC**H**$_2$), δ3.24(t, 2H, C**H**$_2$NH), δ2.10-2.02 (3s, 9H, **OAc**), δ1.73 (m, 2H, C**H**$_2$CH$_2$CH$_2$CH3), δ1.43 (s, 6H, BOC), δ1.31 (m, 4H, CH_2C**H**$_2$C**H**$_2$CH$_3$) δ0.91 (t, 3H, CH_2C**H**$_3$).

Compound 8 Type C-IS: Rf = 0.87, CH_2Cl_2/MeOH 10% ^{1}H NMR (300 MHz, $CDCl_3$):): δ7.65 (d, 1H, **H**-5), δ7.13-7.04 (m, 2H, **H**-8, **H**-6), δ6.30 (s, 1H, **H**-3), δ5.71 (d, 1H, *J* 3.3 Hz, **H**-1α'), δ5.51 (t, 1H, **H**-3'), δ5.27 (t, 1H, **H**-4'), δ4.94(m, 1H, **H**-5'), δ4.52-4.24(m, 3H, **H**-2', **H**-6'),δ3.67 (s, 2H, C**H**$_2$CO), δ2.10-2.02 (3s, 9H, **OAc**), δ1.73 (m, 2H, C**H**$_2$CH$_2$CH$_2$CH3), δ1.43 (s, 6H, BOC), δ1.31 (m, 4H, CH_2C**H**$_2$C**H**$_2$$CH_3$) δ0.91 (t, 3H, $CH_2CH_2CH_2$C**H**$_3$).

Compound 8 Type D: Rf = 0.82, CH_2Cl_2/MeOH 10%. ^{1}H NMR (300 MHz, $CDCl_3$):): δ7.65 (d, 1H, **H**-5), δ7.13-7.04 (m, 2H, **H**-8, **H**-6), δ6.30 (s, 1H, **H**-3), δ5.71 (d, 1H, *J* 3.3 Hz, **H**-1α'), δ5.51 (t, 1H, **H**-3'), δ5.27 (t, 1H, **H**-4'), δ4.94(m, 1H, **H**-5'), δ4.52-4.24(m, 3H, **H**-2', **H**-6'), δ3.67 (s, 2H, C**H**$_2$CO), δ 3.37 (t, 2H, NHC**H**$_2$), δ3.24(t, 2H, C**H**$_2$NH), δ2.10-2.02 (3s, 9H, **OAc**), δ1.69 (m, 2H, C**H**$_2$CH2CH3), δ1.42 (s, 6H, BOC), δ1.31 (m, 2H, CH_2C**H**$_2$$CH_3$) δ0.91 (t, 3H, CH_2CH_2C**H**$_3$).

Compound 8 Type D-IS: Rf = 0.82, CH_2Cl_2/MeOH 10%. ^{1}H NMR (300 MHz, $CDCl_3$):): δ7.65 (d, 1H, **H**-5), δ7.13-7.04 (m, 2H, **H**-8, **H**-6), δ6.30 (s, 1H, **H**-3), δ5.71 (d, 1H, *J* 3.3 Hz, **H**-1α'), δ5.51 (t, 1H, **H**-3'), δ5.27 (t, 1H, **H**-4'), δ4.94(m, 1H, **H**-5'), δ4.52-4.24(m, 3H, **H**-2', **H**-6'), δ3.67 (s, 2H, C**H**$_2$CO), δ2.10-2.02 (3s, 9H, **OAc**), δ1.69 (m, 2H, C**H**$_2$CH2CH3), δ1.42 (s, 6H, BOC), δ1.31 (m, 2H, CH_2C**H**$_2$$CH_3$) δ0.91 (t, 3H, CH_2CH_2C**H**$_3$).

Preparation of Compound 9: Compound 8 (36.5 mg) was dissolved in 2 mL of 7 N NH_3 in methanol with 0.75 mL of 2 M NH_4OH. The vial was tightly sealed, wrapped with parafilm and was then allowed to stir overnight at room temperature. The next day the solvent was removed by rotary evaporation, and the residue was dissolved in pH 3 formic acid buffer. The compound was purified by HPLC (YMC S5 ODS column (20x100 mm, Waters Inc.)UV detection at 254 nm. Gradient 10 mL/ minute H_2O/MeOH, 100 % H_2O 0 to 5 minutes, gradient 0 to 100 % MeOH from 5 to 40 minutes, then 100 % MeOH from 40 to 45 minutes. Yield 59%.

Compound 9 Type A: Eluted at 22.5 minutes. ^{1}H NMR (300 MHz, D_2O):): δ7.72 (d, 1H, **H**-5), δ7.27 (d, 1H, **H**-8), δ7.20 (dd, 1H, **H**-6), δ6.39 (s,1H, **H**-3), δ5.95 (d,1H, **H**-1'), δ5.51 (t,1H, **H**-3'), δ5.27 (t, 1H, **H**-4'), δ4.94(m, 1H, **H**-5'), δ4.52-4.24(m, 6H, **H**-2, **H**3, **H**4, **H**5, **H**6), δ3.77 (s, 2H, C**H**$_2$CO), δ3.32(t, 2H, NHC**H**$_2$) δ3.17(t, 2H, C**H**$_2$NH), δ1.69 (q, 2H, C**H**$_2$$CH_3$), δ1.32 (s, 6H, BOC), δ0.78 (t, 3H, CH_2C**H**$_3$)

Compound 9 Type A-IS: Eluted at 22.5 minutes. ^{1}H NMR (300 MHz, D_2O):): δ7.72 (d, 1H, **H**-5), δ7.27 (d, 1H, **H**-8), δ7.20 (dd, 1H, **H**-6), δ6.39 (s,1H, **H**-3), δ5.95 (d,1H, **H**-1'), δ5.51 (t,1H, **H**-3'), δ5.27 (t, 1H, **H**-4'), δ4.94(m, 1H, **H**-5'), δ4.52-4.24(m, 6H, **H**-2, **H**3, **H**4, **H**5, **H**6), δ3.77 (s, 2H, C**H**$_2$CO), δ1.69 (q, 2H, C**H**$_2$$CH_3$), δ1.32 (s, 6H, BOC), δ0.78 (t, 3H, CH_2C**H**$_3$)

Compound 9 Type B: Eluted at 19.7 minutes. ^{1}H NMR (300 MHz, D_2O):): δ7.72 (d, 1H, **H**-5), δ7.27 (d, 1H, **H**-8), δ7.20 (dd, 1H, **H**-6), δ6.39 (s,1H, **H**-3), δ5.95 (d,1H, **H**-1'), δ5.51 (t,1H, **H**-3'), δ5.27 (t, 1H, **H**-4'), δ4.94(m, 1H, **H**-5'), δ4.52-4.24(m, 6H, **H**-2, **H**3, **H**4, **H**5, **H**6)δ3.77 (s, 2H, C**H**$_2$CO), δ3.32(t, 2H, NHC**H**$_2$) δ3.17(t, 2H, C**H**$_2$NH), δ1.21 (s, 9H, BOC)

Compound 9 Type C: Eluted at 28.4 minutes. ^{1}H NMR (300 MHz, D_2O):): δ7.72 (d, 1H, **H**-5), δ7.27 (d, 1H, **H**-8), δ7.20 (dd, 1H, **H**-6), δ6.39 (s,1H, **H**-3), δ5.95 (d,1H, **H**-1'), δ5.51 (t,1H, **H**-3'), δ5.27 (t, 1H, **H**-4'), δ4.94(m, 1H, **H**-5'), δ4.52-4.24(m, 6H, **H**-2, **H**3, **H**4, **H**5, **H**6)δ3.77 (s, 2H, C**H**$_2$CO), δ3.32(t, 2H, NHC**H**$_2$) δ3.17(t, 2H, C**H**$_2$NH), δ1.71 (q, 2H, C**H**$_2$CH$_2$CH$_2$CH$_3$), δ1.36 (s, 6H, BOC), δ1.28 (s, 4H, CH$_2$C**H**$_2$C**H**$_2$CH$_3$), δ0.84 (t, 3H, CH$_2$CH$_2$CH$_2$C**H**$_3$)

Compound 9 Type C-IS: Eluted at 28.4 minutes. ^{1}H NMR (300 MHz, D_2O):): δ7.72 (d, 1H, **H**-5), δ7.27 (d, 1H, **H**-8), δ7.20 (dd, 1H, **H**-6), δ6.39 (s,1H, **H**-3), δ5.95 (d,1H, **H**-1'), δ5.51 (t,1H, **H**-3'), δ5.27 (t, 1H, **H**-4'), δ4.94(m, 1H, **H**-5'), δ4.52-4.24(m, 6H, **H**-2, **H**3, **H**4, **H**5, **H**6), δ3.77 (s, 2H, C**H**$_2$CO), δ1.71 (q, 2H, C**H**$_2$CH$_2$CH$_2$CH$_3$), δ1.36 (s, 6H, BOC), δ1.28 (s, 4H, CH$_2$C**H**$_2$C**H**$_2$CH$_3$), δ0.84 (t, 3H, CH$_2$CH$_2$CH$_2$C**H**$_3$)

Compound 9 Type D: Eluted at 26.2 minutes. ^{1}H NMR (300 MHz, D_2O):): δ7.72 (d, 1H, **H**-5), δ7.27 (d, 1H, **H**-8), δ7.20 (dd, 1H, **H**-6), δ6.39 (s,1H, **H**-3), δ5.95 (d,1H, **H**-1'), δ5.51 (t,1H, **H**-3'), δ5.27 (t, 1H, **H**-4'), δ4.94(m, 1H, **H**-5'), δ4.52-4.24(m, 6H, **H**-2, **H**3, **H**4, **H**5, **H**6), δ3.77 (s, 2H, C**H**$_2$CO), δ3.32(t, 2H, NHC**H**$_2$) δ3.17(t, 2H, C**H**$_2$NH), δ1.66 (q, 2H, C**H**$_2$CH$_2$CH$_3$), δ1.36 (s, 6H, BOC), δ1.28 (s, 4H, CH$_2$C**H**$_2$CH$_3$), δ0.85 (t, 3H, CH$_2$CH$_2$C**H**3)

Compound 9 Type D-IS: Eluted at 26.2 minutes. ^{1}H NMR (300 MHz, D_2O):): δ7.72 (d, 1H, **H**-5), δ7.27 (d, 1H, **H**-8), δ7.20 (dd, 1H, **H**-6), δ6.39 (s,1H, **H**-3), δ5.95 (d,1H, **H**-1'), δ5.51 (t,1H, **H**-3'), δ5.27 (t, 1H, **H**-4'), δ4.94(m, 1H, **H**-5'), δ4.52-4.24(m, 6H, **H**-2, **H**3, **H**4, **H**5, **H**6),δ3.77 (s, 2H, C**H**$_2$CO), δ1.66 (q, 2H, C**H**$_2$CH$_2$CH$_3$), δ1.36 (s, 6H, BOC), δ1.28 (s, 4H, CH$_2$C**H**$_2$CH$_3$), δ0.85 (t, 3H, CH$_2$CH$_2$C**H**3)

Preparation of Compound 10: Compound 9 Type A (1.7 mg, 1 eq) was dissolved in 1 mL of purified water (Millipore, Milli-Q 18 MΩ) and sodium carbonate (3.7 mg, 11.1 eq) was added. The solution was allowed to stir until the sodium carbonate fully dissolved, and sulfur trioxide pyridine complex (2.5 mg, 5 eq) was added, the solution had a pH around 8-9. After 1 hour the pH of the solution was checked using pH paper and the reaction had become ~6-7. The pH was brought back to ~8 by addition of 0.1 M NaOH with care taken to keep the pH from going above 9. The reaction was then allowed to stir overnight. The reaction had still not reached completion so 5 more equivalents of sulfur trioxide pyridine complex were added and the solution was

allowed to stir another 3-4 hours. The reaction was then acidified with aqueous pH 3 trifluoroacetic acid solution, and the compound was purified by HPLC (Column: YMC S5 ODS column (20x100 mm, Waters Inc.)) using 0.08% trifluoroacetic acid. Gradient 10 mL/minute H_2O/MeOH, 100 % H_2O 0 to 5 minutes, gradient 0 to 100 % MeOH from 5 to 40 minutes, then 100 % MeOH from 40 to 45 minutes , the desired compound eluted at 25.2 minutes. The compound was concentrated to ~3 mL in a vacuum centrifuge (Speed-Vac) and then was re-purified by HPLC exactly as above but the elution solvents lacked trifluoroacetic acid. The compound eluted at 33.6 minutes. The compound was then concentrated to ~1mL (Speed-Vac) and was washed through 300 mg of cation exchange resin (Dowex 50wx8, sodium form) with 7 mL H_2O and the solution was concentrated. Yield 52%.

Compound 10 Type A: ^{1}H NMR (300 MHz, D_2O):): δ7.75 (d, 1H, **H**-5), δ7.30 (m, 2H, **H**-6, **H**-8), δ6.42 (s,1H, **H**-3), δ6.07 (d,1H, **H**-1'), δ3.9-3.7 (m, 4H, **H**-3', **H**-5', **H**-6'), δ3.79(s, 2H, C**H**$_2$CO), δ3.64 (m, 1H, **H**-4'), δ3.25(t, 2H, NHC**H**$_2$) δ3.18(t, 2H, C**H**$_2$NH), δ1.73 (m, 2H, C**H**$_2$CH$_3$), δ1.36 (s, 6H, BOC), δ0.82 (t, 3H, CH$_2$C**H**$_3$).

Preparation of Compound 11: Compound 9 (1.37 mg, 1 eq) was dissolved in 1 mL of purified water then Na_2CO_3 (2.6 mg, 10 eq) was added. The solution was allowed to stir until it was fully dissolved (~ 5-10 min), then acetic anhydride (1.26 mg, 5 eq) was added. The solution was allowed to stir at room temperature for 4 hours. The solution was then acidified with pH 3 formic acid in water and it was purified by HPLC(Column: YMC S5 ODS column (20x100 mm, Waters Inc.)) , Gradient H_2O/MeOH 10 mL / minute, 100 % H2O 0 to 5 minutes, gradient 0 to 100 % MeOH from 5 to 40 minutes, then 100 % MeOH from 40 to 45 minutes . Yield 81.1 %.

Compound 11 Type B Substrate: Eluted at 22.6 minutes. ^{1}H NMR (300 MHz, MeOD):): δ7.71 (d, 1H, **H**-5), δ7.18 (m, 1H, **H**-6, **H**-8), δ6.31 (s,1H, **H**-3), δ5.65 (d,1H, **H**-1'), δ4.15-4.07 (dd, 1H, **H**-2'), δ3.93-3.46 (m, 7H, **H**-3', **H**-4', **H**-5', **H**-6', C**H**$_2$CO), δ under MeoH peak(t, 2H, NHC**H**$_2$) δ3.15(t, 2H, C**H**$_2$NH), δ2.00 (s, 3H, NAc), δ1.44 (s, 9H, BOC).

Compound 11 Type C Product: Eluted at 30 minutes. ^{1}H NMR (300 MHz, MeOD):): δ7.71 (d, 1H, **H**-5), δ7.18 (m, 1H, **H**-6, **H**-8), δ6.31 (s,1H, **H**-3), δ5.65 (d,1H, **H**-1'), δ4.15-4.07 (dd, 1H, **H**-2'), δ3.93-3.46 (m, 7H, **H**-3', **H**-4', **H**-5', **H**-6', C**H**$_2$CO), δ under MeoH peak(t, 2H, NHC**H**$_2$) δ3.15(t, 2H, C**H**$_2$NH), δ2.00 (s, 3H, NAc), δ1.75(m, 2H, C**H**$_2$CH$_2$CH$_2$CH$_3$), δ1.41(s, 6H, BOC), δ1.33(m, 4H, CH$_2$C**H**$_2$C**H**$_2$CH$_3$), δ0.92(m, 3H, CH$_2$CH$_2$CH$_2$C**H**$_3$)

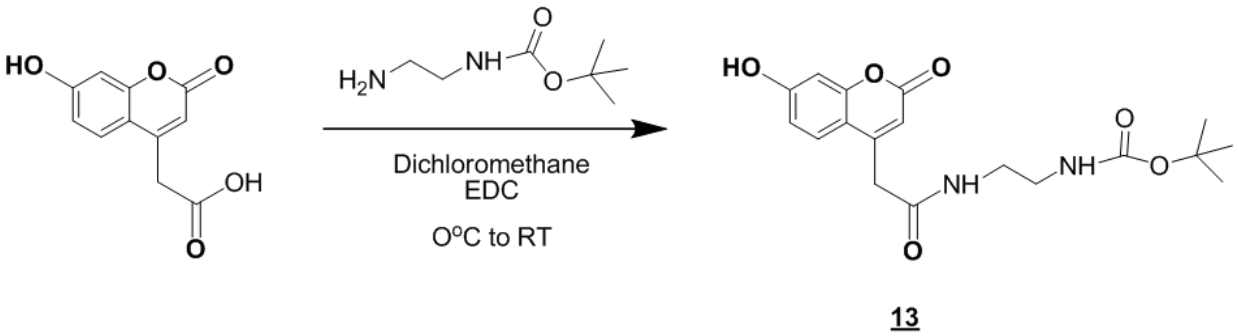
Compound 11 Type C Internal Standard: Eluted at 30 minutes. ^{1}H NMR (300 MHz, MeOD):): δ7.71 (d, 1H, **H**-5), δ7.18 (m, 1H, **H**-6, **H**-8), δ6.31 (s,1H, **H**-3), δ5.65 (d,1H, **H**-1'), δ4.15-4.07 (dd, 1H, **H**-2'), δ3.93-3.46 (m, 7H, **H**-3', **H**-4', **H**-5', **H**-6', C**H**$_2$CO), δ2.00 (s, 3H, NAc), δ1.75(m, 2H, C**H**$_2$CH$_2$CH$_2$CH$_3$), δ1.41(s, 6H, BOC), δ1.33(m, 4H, CH$_2$C**H**$_2$C**H**$_2$CH$_3$), δ0.92(m, 3H, CH$_2$CH$_2$CH$_2$C**H**$_3$)

Compound 11 Type D: Eluted at 27.7 minutes. ^{1}H NMR (300 MHz, MeOD):): δ7.71 (d, 1H, **H**-5), δ7.18 (m, 1H, **H**-6, **H**-8), δ6.31 (s,1H, **H**-3), δ5.65 (d,1H, **H**-1'), δ4.15-4.07 (dd, 1H, **H**-2'), δ3.93-3.46 (m, 7H, **H**-3', **H**-4', **H**-5', **H**-6', C**H**$_2$CO), δ under MeoH peak(t, 2H, NHC**H**$_2$) δ3.15(t, 2H, C**H**$_2$NH), δ2.00 (s, 3H, NAc), δ1.75(m, 2H, C**H**$_2$CH$_2$CH$_3$), δ1.41(s, 6H, BOC), δ1.33(m, 2H, CH$_2$C**H**$_2$CH$_3$), δ0.92(m, 3H, CH$_2$CH$_2$CH$_2$C**H**$_3$)

Compound 11 Type D Internal Standard: Eluted at 27.7 minutes. ^{1}H NMR (300 MHz, MeOD):): δ7.71 (d, 1H, **H**-5), δ7.18 (m, 1H, **H**-6, **H**-8), δ6.31 (s,1H, **H**-3), δ5.65 (d,1H, **H**-1'), δ4.15-4.07 (dd, 1H, **H**-2'), δ3.93-3.46 (m, 7H, **H**-3', **H**-4', **H**-5', **H**-6', C**H**$_2$CO), δ2.00 (s, 3H, NAc), δ1.75(m, 2H, C**H**$_2$CH$_2$CH$_3$), δ1.41(s, 6H, BOC), δ1.33(m, 2H, CH$_2$C**H**$_2$CH$_3$), δ0.92(m, 3H, CH$_2$CH$_2$CH$_2$C**H**$_3$)

Preparation of Compound 12 Type D: Compound 11 Type D (4.22 mg, 1 eq) was placed under nitrogen and 3Å molecular sieves (2 beads) were added. Dry dimethylforamide (0.5 mL) was added to dissolve the solid and the mixture was heated to 40 ^{o}C (bath temperature). SO_3-trimethylamine sulfate complex (5.94 mg, 6 eq) was dissolved in dry dimethylforamide, and was added dropwise to the reaction over an hour.. The solution was allowed to stir for a total of 4 hours after which it was quenched with methanol, and the solvent was removed (Speed-Vac). The compound was then dissolved in pH 3 formic acid water solution and the compound was purified by HPLC (Column: YMC S5 ODS column (20x100 mm, Waters Inc.)). Gradient H_2O/MeOH 10 mL / minute, 100 % H_2O 0 to 5 minutes, gradient 0 to 100 % MeOH from 5 to 40 minutes, then 100 % MeOH from 40 to 45 minutes. The eluted compound was then concentrated by speed vac and washed through Dowex 50wx8 Na^+ exchange gel. TLC showed some impurities so the compound was re-purified as above by HPLC. 43 % yield.

Compound 12: Eluted at 34.7 minutes. ^{1}H NMR (300 MHz, MeOD):): δ7.71 (d, 1H, **H**-5), δ7.18 (m, 1H, **H**-6, **H**-8), δ6.31 (s,1H, **H**-3), δ5.65 (d,1H, **H**-1'), δ4.20 (d, 2H, **H**-6'), δ4.15-4.07 (dd, 1H, **H**-2'), δ3.93-3.46 (m, 5H, **H**-3', **H**-4', **H**-5', C**H**$_2$CO), δXXXX(s, 2H, C**H**$_2$CO), δunder MeoH peak(t, 2H, NHC**H**$_2$) δ3.15(t, 2H, C**H**$_2$NH), δ2.17 (s, 3H, NAc), δ1.72(m, 2H, C**H**$_2$CH$_2$CH$_3$), δ1.41(s, 6H, BOC), δ1.31(m, 2H, CH$_2$C**H**$_2$CH$_3$), δ0.92(m, 3H, CH$_2$CH$_2$CH$_2$C**H**$_3$).



Scheme S4. Synthesis of the MPS III type B product and internal standard.

Preparation of Type B product and internal standard

7-Hydroxycoumarin-4-acetic acid (178 mg, 1 eq) and the diamine chain (152 mg, 1.1 eq) were combined in dry dichloromethane, and the solution was cooled in an ice bath. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (228 mg, 1.8 eq) was added, and the solution was allowed to stir overnight. The solvent was removed by rotovap and the compound was purified on a silica column using dichloromethane/MeOH (0 to 10%) to give a 74 % yield. R_f = 0.28 in dichloromethane/MeOH 10%.

Compound 13 Type B: ^{1}H NMR (300 MHz, MeOD):): δ7.58 (d, 1H, **H**-5), δ6.82 (dd, 1H, **H**-8), δ6.72 (d, 1H, **H**-6), δ6.19 (s, 1H, **H**-3), δ3.72 (s, 2H, C**H**$_2$CO), δ3.28(t, 2H, H$_2$NC**H**$_2$), δ3.15(t, 2H, C**H**$_2$NH), δ1.43 (s, 9H, BOC).

Compound 13 Type B-IS:^{1}H NMR (300 MHz, MeOD):): δ7.58 (d, 1H, **H**-5), δ6.82 (dd, 1H, **H**-8), δ6.72 (d, 1H, **H**-6), δ6.19 (s, 1H, **H**-3), δ3.72 (s, 2H, C**H**$_2$CO), δ3.28(t, 2H, H$_2$NC**H**$_2$), δ3.15(t, 2H, C**H**$_2$NH).

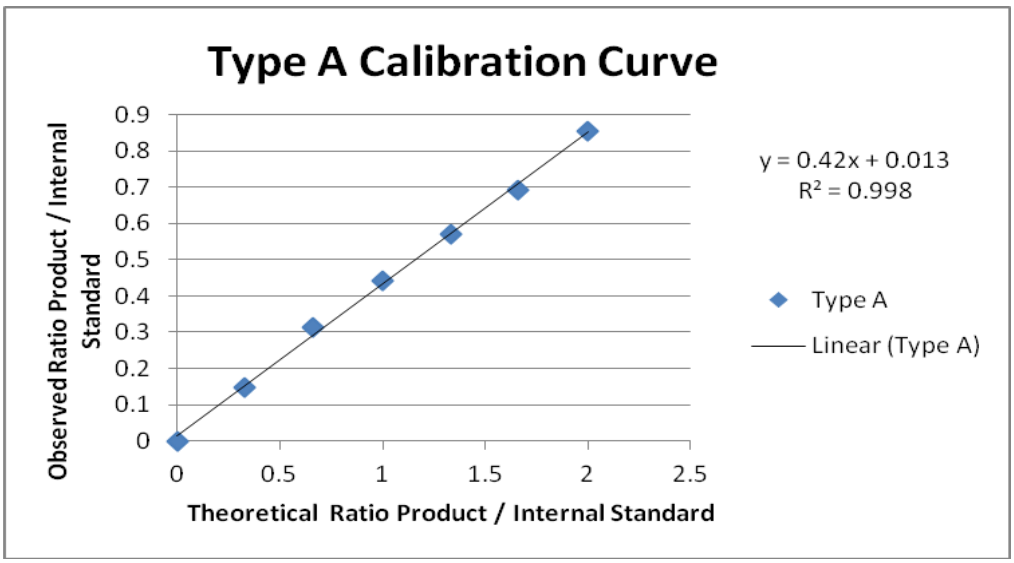


Figure S1. The observed ratio of product to internal standard plotted against the theoretical value for the MPS III type A product to internal standard ratio. This experiment was carried out with a set amount of internal standard and a varying amount of the product.

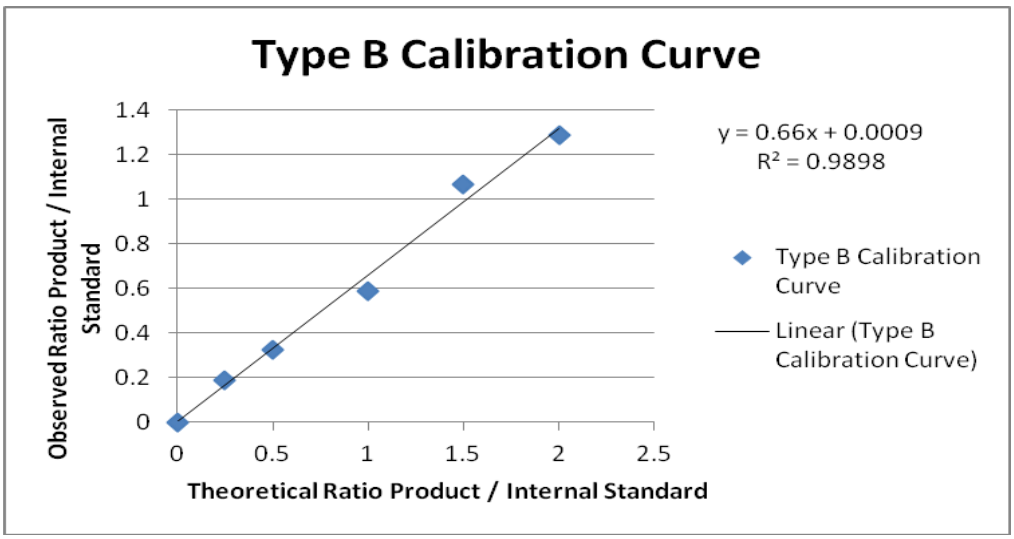


Figure S2. The observed ratio of product to internal standard plotted against the theoretical value for the MPS III type B product to internal standard. This experiment was carried out with a set amount of internal standard and a varying amount of the product.

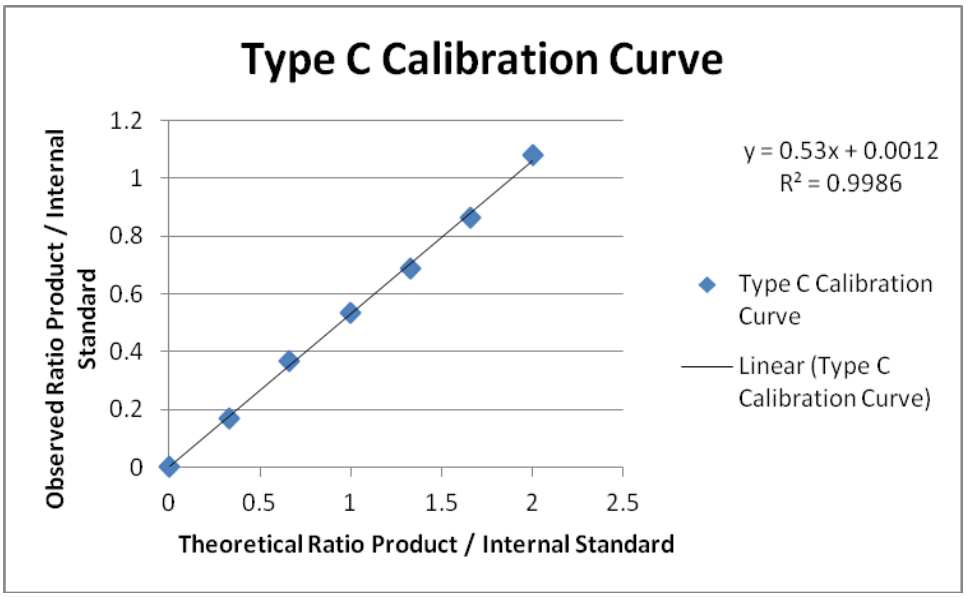


Figure S3. The observed ratio of product to internal standard potted against the theoretical value for the MPS III type A product and internal standard. This experiment was carried out with a set amount of internal standard and a varying amount of the product.

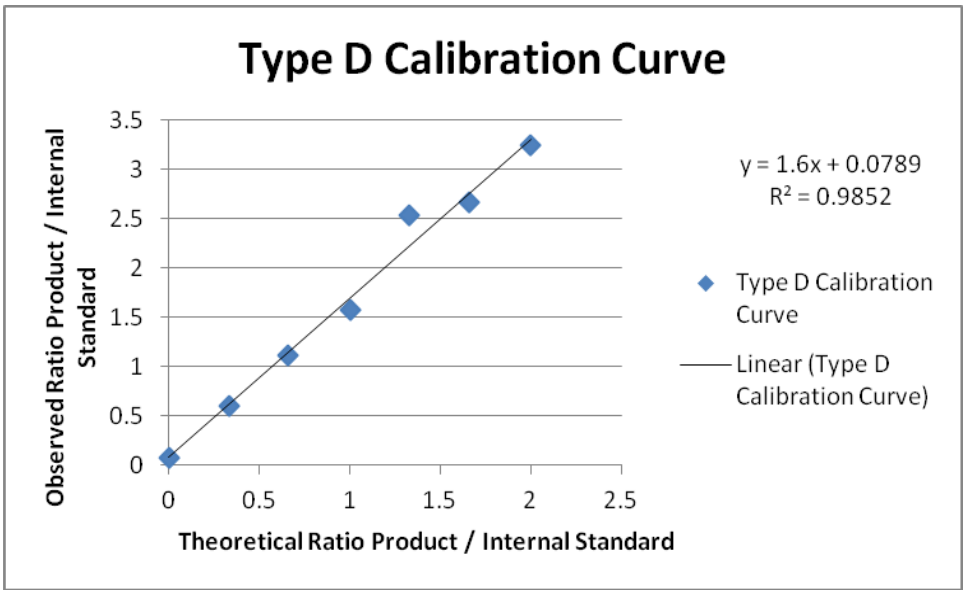


Figure S4. The observed ratio of product to internal standard plotted agaist the theoretical value for the MPS III type A product and internal standard. This experiment was carried out with a set amount of internal standard and a varying amount of the product.

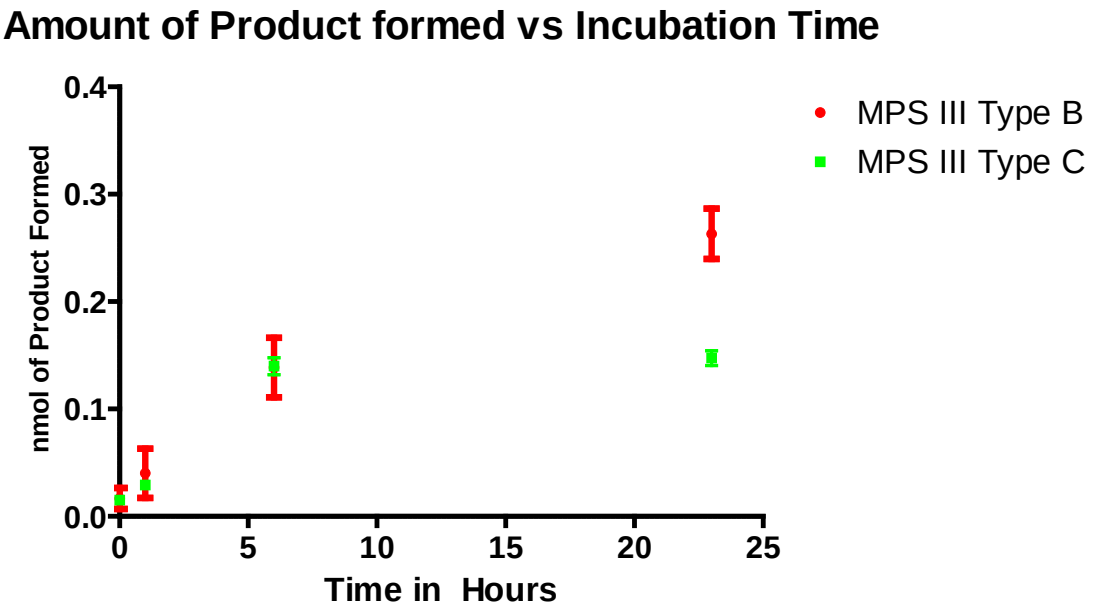


Figure S5. The amount of product formed versus the incubation time.

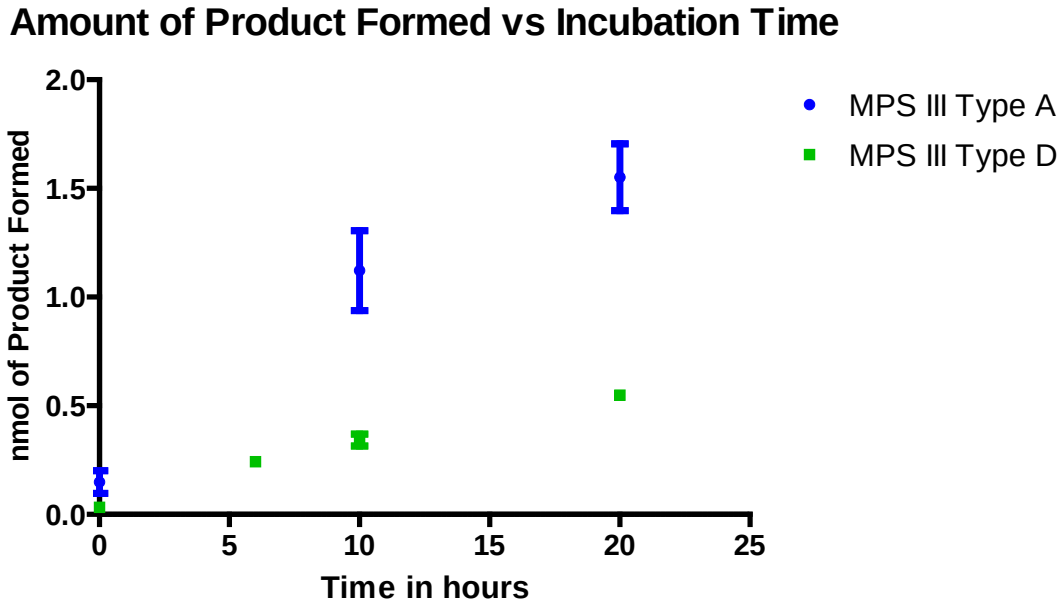


Figure S6. The amount of product formed versus the incubation time.

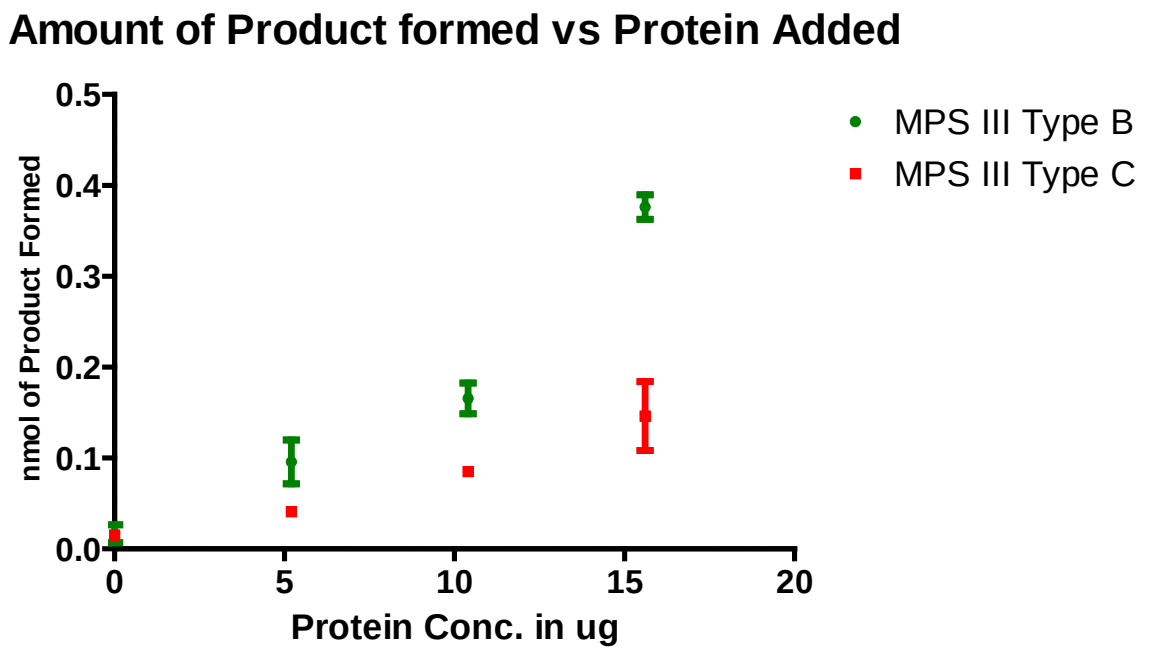


Figure S7. The amount of product formed versus the amount of protein added for the MPS III Type B and C assays during an 18 hour incubation.

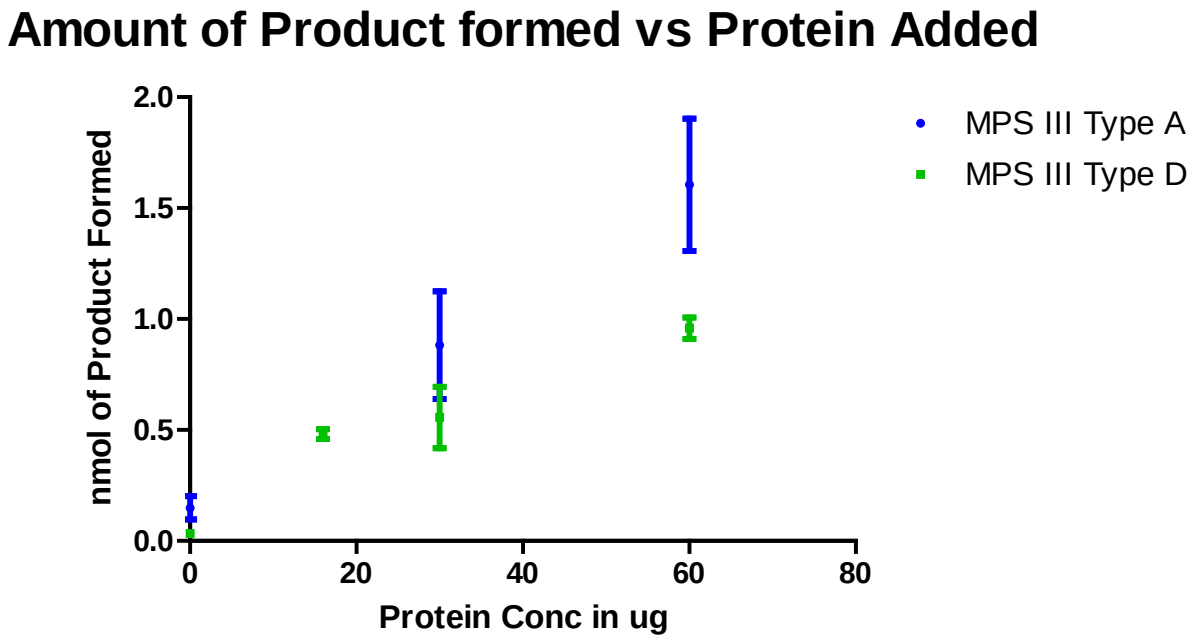


Figure S8. The amount of product formed versus the amount of protein added for the MPS III Type A and D assays during an 18 hour incubation.