# Supplementary Material (ESI) for Molecular BioSystems
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**Materials and Methods:**

**Chemicals**: Diphenylmethyl ester of 7-Amino 3 chloromethyl 3 cephem-4-carboxylic acid (ACLH.HCl) was a gift from Otsuka Chemical Co. Ltd, Japan. All other materials were obtained from Sigma-Aldrich, Fisher Scientific and Pierce Endogen Rockford, IL. Commercially available reagents were used without further purification, unless noted otherwise. The solvents were dried according to protocols mentioned in literature. All other chemicals were reagent grade or better. Synthesized compounds were characterized using $^{1}$H NMR (ECX-400 -JEOL Acorn NMR, Livermore, CA) and ESI-MS spectrometric analysis was performed at the Mass Spectrometric Facility of Stanford University, CA. Reverse Phase HPLC was performed on Waters 600 equipped with a PDA detector. The TEM- 1β lactamase (Bla) constitutively expressing CHO-K1-Bla cells, tissue culture reagents such as L-glutamine, non essential amino acids, fetal bovine serum, phenol red free minimum essential medium (MEM) and penicillin/streptomycin and the Lytic BLAzer-FRET B/G Homogenous kit were purchased from Invitrogen, Carlsbad, CA. Pure TEM-1 β lactamase (Bla) was obtained from Biologics Process Development, Inc., Poway, CA. The 96- and 384-well black and white poly-lysine coated plates were purchased from Becton Dickinson, Franklin Lakes, NJ. Other reagents such as CHOK1 cells, Enzyme Acceptor (EA) enzyme core assay buffer, Chemiluminescent (CL) substrate and Enzyme donor dilution buffer (EDDB) were from in-house repository. The Enzyme Donor (ED) peptide was synthesized by American Peptide, Sunnyvale, CA.

(Ac-CSLAVVLQRRDWENPGVTQLNRLAAHPPFASWRNSEEARTDCPSQQL-OH)

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**HPLC purification**: Analytical and Semi-preparative HPLC was performed on Zorbax 300 A SB bonded phase C-18 column (250X10mm) at a flow rate of 4 ml /min. An eluting system consisting of C (0.1% trifluoroacetic acid in water) and D (0.1% trifluoroacetic acid in acetonitrile) was used under a linear gradient to elute the product, which was monitored by UV-VIS absorbance at 260 nm and 280 nm, respectively. In general the linear gradient of 25C-45%D in 25 min was employed for elution of the final product.

**UV-VIS, Fluorescence and Chemiluminescent read out measurements**: UV-visible spectra was recorded on Agilent 8452 Diode array spectrophotometer for quantitation of enzyme donor conjugate. The fluorescence and chemiluminescent kinetic readouts were performed on Victor 3.0 and Packard Lumicount instruments, respectively.

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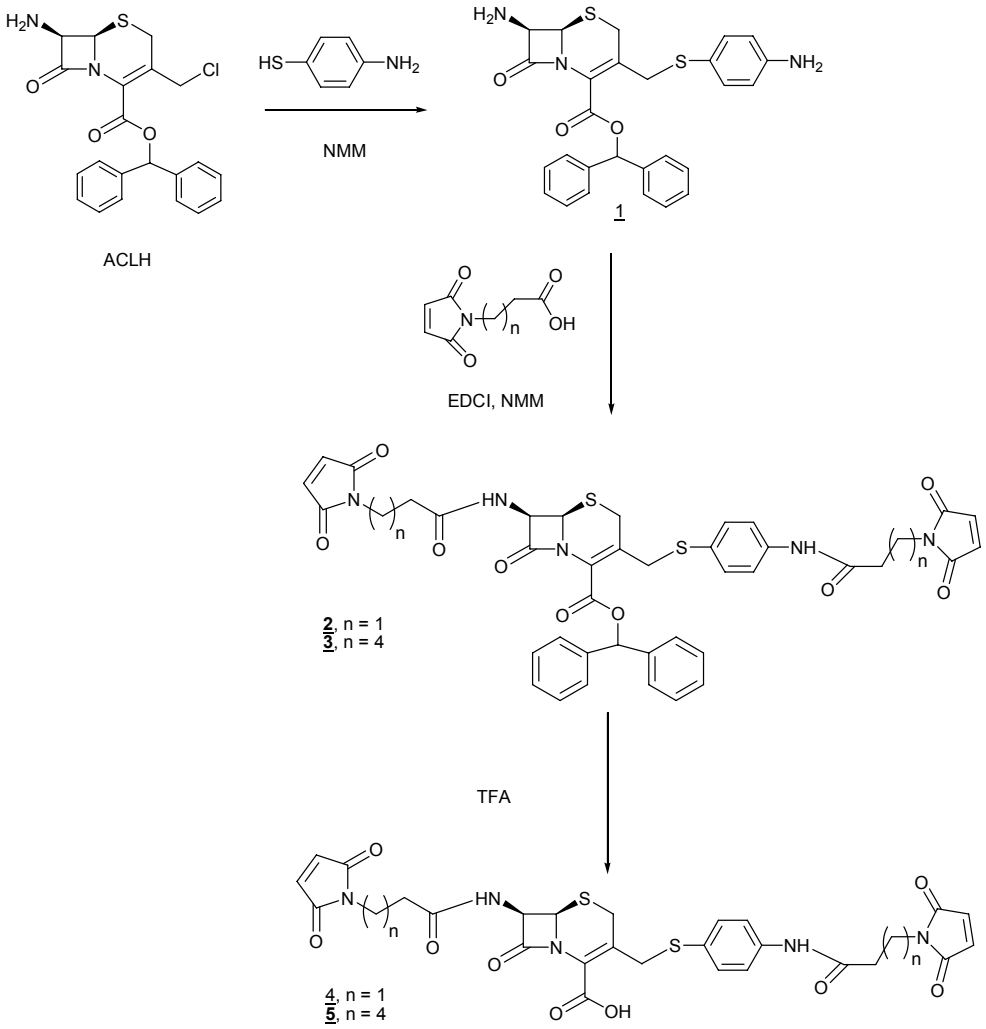
## Synthesis and characterization of 1, 2 & 3

Scheme 1 below represents the synthesis of lactam for cyclization to ED



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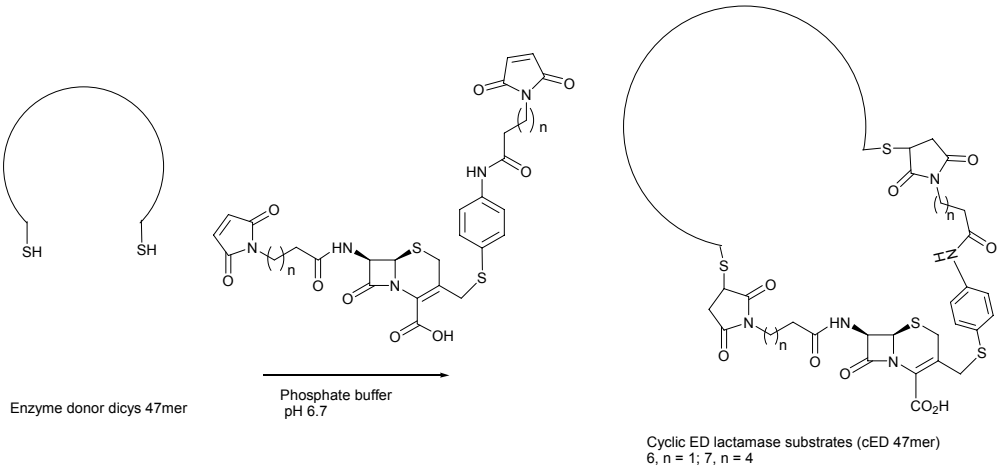
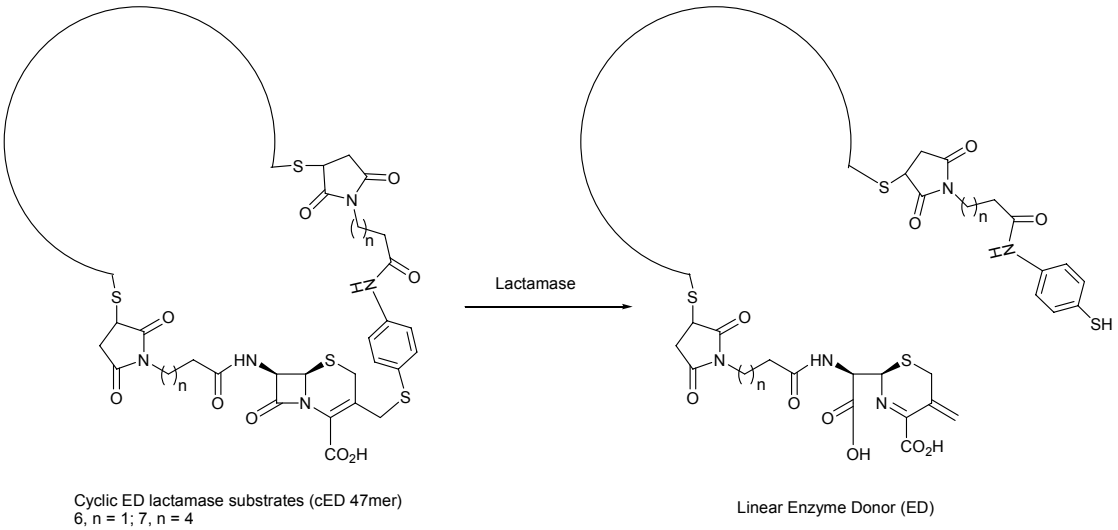
Scheme 2 below represents the Cyclization of ED with cephem derivatives **4** & **5**




Scheme 3 below represents the cleavage of cyclic to linear ED with lactamase



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**Preparation of 1**: ACLH. HCl (500mg, 1.1 mmole) was suspended in 10 ml DMF followed by N- methyl morpholine NMM (150µl) to neutralize the HCl salt and get a clear solution. 4-aminothiophenol (150mg, 1.5 mmole) was then added to the above solution and the reaction stirred at ambient temperature for 4-5 h. The reaction was monitored on TLC. The DMF was concentrated off *in vacuo* and the concentrated oil was made into slurry using silica gel (230-400 mesh). It was purified by flash chromatography on a silica gel (230 –400 mesh) using MeOH/$CH_2Cl_2$ gradient of 2-15% to afford 315 mg (62.6%) of title compound. $^1$H NMR (400 MHz, DMSOd6): δ 8.8 (s, 2H), 7.61-7.25 (m, 10H), 6.97 (d, J= 8.6 Hz, 2H), 6.75 (s, 1H), 6.46 (d, J= 8.6 Hz, 2H), 4.99 (d, J=5.2 Hz, 1H), 4.82 (d, J= 4.80 Hz, 1H), 3.84 (d, J= 12.8 Hz, 1H), 3.62 (d, J= 13.6 Hz, 1H), 3.48 (d, J=17.6 Hz, 1H), 3.44 (d, J=10.0 Hz, 1H); ESI-MS: M+H 504 (Found); 503 (calc).

**Preparation of 2**: A solution of N- Maleoyl-β-alanine (0.30g, 1.78 mmole,) and 1-Hydroxybenzotriazole (0.25g, 1.78mmole, 6 equiv.) in 1.5 ml of anhydrous DMF was cooled at 0°C. EDCI.HCl (0.34g, 1.78 mmole,) freshly prepared in DMF (200µl) and neutralized with NMM (100µl) was then added. To this activated solution, **1** (150 mg, 0.29 mmole) in anhydrous DMF (1.5 ml) was added and the reaction mixture stirred at ambient temperature overnight. The solvent was concentrated under reduced pressure on a rotary evaporator and the residual oil suspended in water. The suspension was extracted with ethyl acetate (50ml x3) and the organic phase washed with 5% citric acid (10 ml x 2), water (10 ml x 2) and 5% $NaHCO_3$ (10 ml x 2) and finally with water till neutral pH and dried over anhydrous sodium sulfate. The solvent was removed and the residual oil

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triturated with hexane to afford 260 mg (32.2%) of crude product. The crude product was subjected to flash silica gel column chromatography. The compound was eluted at 0-3 % MeOH/ $CH_2Cl_2$ solvent gradient to afford 170 mg (21%) of pure product **2.** $^1$H NMR (400 MHz, $CDCl_3$): 7.41-7.26 (m, 11H), 7.25 (d, J= 8.8 Hz, 2H), 6.88 (s, 2H), 6.75 (s, 2H), 6.70 (d, J=8.4 Hz, 2H), 6.65 (s, 2H), 5.17 (d, J=4.0 Hz, 1H), 4.87 (d, J=4.0 Hz, 1H), 3.90 (d, J=18.4, 1H), 3.85 (m, 4H), 3.50 (d, J=17.6 Hz, 1H), 2.75 (m, 4H); ESI-MS: M+(Na+)= 828 (Found); 805 (calc).

**Preparation of 3**: A solution of ε- maleimidocaproic acid (0.53g, 0.0025 mmole,) and 1-Hydroxybenzotriazole (0.38g, 0.0025 mmole,) in 1.5 ml of anhydrous DMF was cooled at 0°C. EDCI.HCl (1.92g, 0.01 mmole,) freshly prepared in DMF (200µl) was neutralized with NMM (100µl). To this activated solution, **1** (0.25g, 0.0005mmole) in anhydrous DMF (1.5 ml) was added and the reaction mixture stirred at ambient temperature overnight. The solvent was concentrated under reduced pressure on a rotary evaporator and the residual oil suspended in water. The suspension was extracted with ethyl acetate (50 ml x3) and the organic phase washed with 5% citric acid (10 ml x 2), water (10 ml x 2) and 5% $NaHCO_3$ (10 ml x 2), and finally with water till neutral pH and dried over anhydrous sodium sulfate. The solvent was removed and the residual oil triturated with hexane to afford 260 mg (36 %) of crude product. The crude product was subjected to flash silica gel column chromatography. The compound was eluted at 0-3 % MeOH/ $CH_2Cl_2$ solvent gradient to afford 140 mg (19%) of pure product **3.** $^1$H NMR (400 MHz, $CDCl_3$): 7.41-7.26 (m, 11H), 7.25 (d, J= 8.8 Hz, 2H), 6.88 (s, 2H), 6.75 (s, 2H), 6.70 (d, J=8.4 Hz, 2H), 6.65 (s, 2H), 5.17 (d, J=4.0 Hz, 1H), 4.87 (d, J=4.0 Hz, 1H), 3.90 (d,

J=18.4, 1H), 3.50 (m, 4H), 3.50 (d, J=17.6 Hz, 1H), 1.75 (m, 16H); ESI-MS: M+=891 (Found); 891 (calc).

**Preparation of 4:** To a solution of **2** (6.7 mg, 0.0083 mmole) in 1mL of anhydrous dichloromethane ($CH_2Cl_2$) was added m-cresol (50 µl) and trifluoroacetic acid (150 µL) with cooling (ice bath). The mixture was stirred for 1h at 0°C, followed by anhydrous diethyl ether (1 mL) addition. The precipitate was collected and washed with ether (1mL x 5) to afford 1.2 mg (90%) of **4** as a yellow colored powder. The purity of the compound was assessed by RP-HPLC. ESI-MS: M+ 640 (Found); 640 (Calc.).

**Preparation of 5:** To a solution of **3** (12.0 mg, 0.0013 mmole) in 1mL of anhydrous dichloromethane ($CH_2Cl_2$) was added m-cresol (50 µl) and trifluoroacetic acid (150 µL) with cooling (ice bath). The mixture was stirred for 1h at 0°C, followed by anhydrous diethyl ether (1 mL) addition. The precipitate was collected and washed with ether (1mL x 5) and then purified by RP-HPLC to afford 5 mg (90%) of **5** as a yellow colored powder. The purity of the compound was assessed by RP-HPLC. ESI-MS: M+ 724 (Found); 724(Calc.).

**Reduction of Linear enzyme donor (ED):** Enzyme donor (ED) (5 mg) was dissolved in water (1mL) and treated with DTE (5 mg) overnight at ambient conditions. It was then subjected to RP-HPLC purification employing 25%C-45%D (Buffer C=0.1% TFA/H2O; Buffer D=0.1% TFA/CH3CN) gradient in 25 min to afford pure 2.5 mg (50%) linear ED

Ac-CSLAVVLQRRDWENPGVTQLNRLAAHPPFASWRNSEEARTDCPSQQL-OH

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having free sulfhydryls. This was then immediately employed in the next step for cyclization.

**Cyclization of enzyme donor (ED) with 4 to make cED 6:** To the above freshly purified linear ED (2.8mL, 50 μM), 100 mM sodium phosphate buffer pH 8.0 (1 mL) was added to adjust the pH between 6.7 to 7.0. To this water (1mL) and DMF (500 μL) was added to make the volume upto 6.5mL. To this solution equimolar concentration of compound **4** in DMF (200 μl) was added drop-wise under vigorous stirring for 10 min. The reaction was left at ambient conditions for 15 min and then a second aliquot of equimolar concentration of compound **4** was added so as to ensure complete reaction with the sulfhydryls. The reaction mixture was left at ambient conditions for 3 h and then the progress of the reaction monitored by RP-HPLC. The cyclic ED lactamase substrate was purified to homogeneity by RP-HPLC and the pure fractions collected were lyophilized and resuspended in water. The concentration of the cyclic enzyme donor lactamase substrate assessed by UV-VIS spectroscopy at 280 nm (~15% unoptimized yield). The molecular weight of the cyclic enzyme donor was corroborated by ESI-MS: M+H 6031 (Found); 6030 (calc.).

**Cyclization of enzyme donor (ED) with 5 make cED 7:** To the above freshly purified linear enzyme donor (4mL, 65 μM), 100 mM sodium phosphate buffer pH 8.0 (1 mL) was added to adjust the pH between 6.7 to 7.0. To this water (1mL) and DMF (500 uL) was added to make the volume upto 6.5mL. To this solution equimolar concentration of compound **5** in DMF (200 ul) was added drop-wise under vigorous stirring for 10 min.

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The reaction was left at ambient conditions for 15 min and then a second aliquot of equimolar concentration of compound **5** was added so as to ensure complete reaction with the sulfhydryls. The reaction mixture was left at ambient conditions for 3 h and then the progress of the reaction monitored by RP-HPLC. The cyclic enzyme donor lactamase substrate was purified to homogeneity by RP-HPLC and the pure fractions collected were lyophilized and resuspended in water. The concentration of the cyclic enzyme donor lactamase substrate assessed by UV-VIS spectroscopy at 280 nm (~30% unoptimized yield). The molecular weight of the cyclic ED was corroborated by ESI-MS: M+ 6114 (Found); 6114 (calc.).

**Enzyme Fragment Complementation (EFC) activity and enzyme kinetics:**

**Assay Principle:** The Enzyme Fragment Complementation (EFC) assay is based on the linearization and activation of a complementation inactive enzyme donor peptide by lactamase induced cleavage of a cephem substrate bridge. Substituted cephem derivatives were incorporated in the linear enzyme donor peptide to get the cyclic peptide without altering the inability to complement with enzyme acceptor (EA). The cyclic conjugates after incubation with enzyme acceptor have approximately 2 % of the catalytic activity of the equivalent concentration of linear enzyme donor (ED). Addition of the TEM-1β lactamase (Bla) enzyme results in linearization of the enzyme donor conjugate, which is then free to complement with enzyme acceptor. Since linear enzyme donor (ED) complements with enzyme acceptor (EA) approximately 50 fold more efficiently than circular enzyme donor (ED), an increase in catalytic activity is observed.

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**Enzyme fragment complementation (EFC) assay:** For determination of EFC activity, 10 µl of 50 nM cyclic enzyme donor lactamase substrate (cED **6** & cED **7**) or 10 µl of 50 nM linear enzyme donor (cysteines capped with maleimide and HPLC purified cleaved linear ED) was added to 20 µl enzyme donor dilution buffer in a 384-well white plate. Subsequently, 10 µl enzyme acceptor (EA) followed by 10 µl of chemiluminescence substrate in enzyme acceptor core assay buffer were added and further incubated at room temperature for 60 min. The signal was read on Lumicount (Packard) with integration time of 1s per well and PMT set to 1100V. Fig 1 represents the EFC activity for cyclic enzyme donor lactamase (cED) **6** and cyclic enzyme donor lactamase (cED) **7** with respect to linear enzyme donor (enzyme donor cysteines capped and HPLC purified cleaved linear ED).

**Protocol for Lactamase cleavage**: The assay comprises of two basic steps:

1) hydrolysis of the lactam in the cyclic enzyme donor lactamase substrate (cED) by the TEM-1β lactamase (Bla) to yield a linear enzyme donor (ED) and

2) detection of the linear enzyme donor (ED) by enzyme acceptor (EA) and β-galactosidase chemiluminescent substrate.

The lactamase reaction step comprises of 16µl of CHO-Bla (10,000K) cells in phosphate buffer, 4µl of cyclic enzyme donor (cED) lactamase substrate at 50 nM mixed with 2% CHAPS lysis buffer (1:1). The reaction mixture is then incubated at ambient temperature or 37C overnight. The detection step comprises of addition of 6µl enzyme acceptor (EA) (1-2µM) and 6µl chemiluminescent substrate reagent. The plate is then incubated at RT

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for 60 min and then read on a multi-well plate reader with an integration time of 1sec and PMT set at 1100V.

**Quantitation of β-lactamase in cell lysates**: Both CMV-Bla CHOK1 and wild type CHOK1 cells were suspended in phosphate buffer saline (pH 7.0) at a density of $5x10^5$ cell/ml. They were mixed at various ratios (0, 1, 2, 5, 10, 20, 50 &100% of CMV-Bla CHOK1 cells) and seeded in white multi-well plate overnight with the total number of cells maintained at 10K cells/well. These were incubated overnight @ 37°C in media. The media was aspirated, washed with PBS (x3) and 16µl of phosphate buffer saline was added to so as to maintain 10K cells per well. 4µl of (cyclic enzyme donor lactamase substrate) cED/Lysis buffer (1:1) was added to each well and incubated at 37°C overnight. To this 6µl of enzyme accept (EA) reagent and 6µl of chemiluminescent (CL) reagent was added and the plate read on a lumicount plate reader after 60 min. A standard curve with a serial dilution of the pure TEM-1β lactamase (Bla) enzyme was carried out utilizing the same protocol (with CHOk1 cell lysates as the matrix) in order to quantitate the exact levels of TEM-1β lactamase (Bla) produced in CMV-Bla- CHO K1 cells. The lytic blazer assay was performed with the same number of cells following the protocol recommended by the vendor. This assay can reliably detect 2.0% CHOK1-Bla cells in the background of the wild type cells which correspond to 3 pM of TEM-1β lactamase (Bla) as determined from the standard curve.

**Enzyme kinetics:** The kinetic experiment was carried out at room temperature using pure TEM-1 β lactamase (Bla) enzyme. To 10µl of PBS, 10 µl of a series of different

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concentrations of cyclic enzyme donor (cED) lactamase substrates (10, 25, 50, 100, 200, 300, 500 nM) were added a 10 µl of three dilutions (40, 120 and 370 pM) of TEM-1β lactamase (Bla) enzyme in a 384 well white plate. The cyclic enzyme donor (cED) lactamase substrate was incubated at 1,3,5,7,9,15 h at room temperature. This was followed by addition of 10 µl of enzyme acceptor (EA) and 10 µl of chemiluminescent (CL) substrate at the respective time points and the plate read at 60 min. The rate of increase in relative luminescence units (RLUs) upon β-galactosidase complementation was used to determine the kinetic properties of enzyme hydrolysis. The values of kinetic parameters (Km and kcat) were determined from double reciprocal plot of the hydrolysis rate (1/Velocity) versus substrate concentration (1/substrate) (Lineweaver-Burk Plot) and also by plot of velocity (V) versus substrate (S) concentration Michaelis Menten equation (data not shown).

**Functional TEM-1 β lactamase (Bla) assay:** We next tested the ability of cyclic enzyme donor (cED) lactamase substrate in detecting TEM-1 β lactamase (Bla) reporter enzyme produced upon cell stimulation in HEK293/CRE/Bla cells expressing G-Protein Coupled Dopamine 5 receptor (GPCR -D5R). HEK293/D5R/CRE/Bla cells ($6x10^4$) were plated in 100µl of DMEM supplemented with 10% FBS, glutamine (2mM) and penicillin/streptomycin (50µg/mL) in 96-well clear bottom plates and incubated overnight at 37°C. The cells were then washed (x2) with phenol red and serum free medium and starved overnight in the same medium. The cells were then stimulated with 10µM dopamine, respectively for 15h at 37°C in serum free medium. The unstimulated Hek293/D5R/CRE/Bla and HEK293 control cells had medium alone with vehicle control

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of 0.1% DMSO which matched the % of DMSO in stimulated cells. The medium was removed and the cells were assayed as follows: To the cells 30µl of cyclic enzyme donor lactamase substrate (50nM) and (phosphate buffer saline) PBS (1:1) was added and incubated at RT for 7h. To this 6µl of enzyme donor (EA) reagent and 6ul of chemiluminescent (CL) reagent was added and the plate read on a lumicount plate reader after 60 min with PMT set at 1100V.