A Semi-synthetic Epitope for Kinase Substrates

Supplementary Methods

All chemicals and reagents were obtained from Sigma-Aldrich unless otherwise stated.

Synthesis of *p*-nitrobenzylmesylate (PNBM). *p*-nitrobenzyl alcohol (1.0 g, 6.5 mmol) in THF (10 mL) was treated with triethylamine (1.8 mL, 13 mmol), cooled to –10°C and treated dropwise with methanesulfonyl chloride (0.61 mL, 7.8 mmol). After stirring for 30 min (a precipitate formed during this time) 10% HCl (25 mL) was added. An additional 10 mL of water was added and the mixture was extracted twice with ethyl acetate. The combined organic layers were dried over Na_2SO_4 (anhyd), filtered and concentrated to afford an off-white solid in 93% yield. Analytical data was consistent with the literature[1]. ^{1}H NMR (400 MHz, DMSO-D6) δ 8.25 (d, 2H), 7.68 (d, 2H), 5.38 (s, 2H), 3.25 (s, 3H).

Hapten Synthesis (See **Supplementary Figure 5a**)
Dibenzyloxyphosphorothioate 2. 3-(Boc-amino)-1-propanol (203 mg, 1.2 mmol) was dissolved in 10.2 mL anhydrous dioxane and N,N-diisopropyl dibenzyl phosphoramidite (500 mg, 1.45 mmol) was added, followed by 1-H-tetrazole (122 mg, 1.7 mmol). After 1.5 hour phenylacetyl disulfide (807 mg, 2.9 mmol) was added and the mixture was stirred for 2 hours and then stored at –20°C overnight. The solvent was removed under reduced pressure and the residue was partitioned with ethyl acetate (50 mL) and water (50 mL). The organic layer was washed with brine, dried with Na_2SO_4, and concentrated to a light yellow oil. After flash chromatography (10-20% ethyl acetate, 90-80% hexanes) a clear viscous liquid was obtained (409 mg, 78% yield). ^{1}H NMR (400 MHz, DMSO-D6) δ 7.32 (m, 10H), 5.02 (d, 4H), 3.96 (m, 2H), 2.91 (q, 2H), 1.65 (p, 2H), 1.32 (s, 9H). ESI-MS (*m/z*): $[M+H]^+$ calcd for $C_{22}H_{30}NO_5PS$, 452.16; found, 452.13.

Hapten 4. A mixture of dibenzyloxyphosphorothioate **2** (100 mg, 0.22 mmol), *m*-cresol (0.46 mL, 4.4 mmol), and thiophenol (0.45 mL, 4.4 mmol) was cooled to 0 °C and treated with trifluoroacetic acid (2.95 mL) and bromotrimethylsilane (0.57 mL, 4.4 mmol). The mixture was stirred at 0°C for 1.5 hour and then diluted with 2 mL toluene and concentrated under reduced pressure. An additional 2 mL of toluene was added and the mixture was concentrated again. The residue was partitioned between 5 mL H_2O and 5 mL pentane. The pentane was discarded and the aqueous layer washed with an additional 5 mL of pentane. *p*-nitrobenzyl bromide (95 mg, 0.442 mmol) was dissolved in 1 mL of ethanol and added to the aqueous layer containing **3**. The pH was adjusted to 8.5 with saturated Na_2CO_3, and allowed to stir at RT for 2 hours. After extraction with 5 mL pentane the aqueous layer was filtered and separated by high performance liquid chromatography (HPLC) (^{18}C prep column, 60 min gradient, H_2O-CH_3CN 95:5 to H_2O-CH_3CN 40:60, 250 nm). The fractions containing the hapten (25 min) were collected and lyophilized to afford a white powder (15 mg, 22% yield over two steps). ^{1}H NMR (400 MHz, DMSO-D6) δ 8.10 (m, 2H), 7.57 (m, 2H), 3.86 (d, 2H), 3.62 (m, 2H), 2.76 (t, 2H), 1.65 (p, 2H). ESI-MS (*m/z*): $[M+H]^+$ calcd for $C_{10}H_{15}N_2O_5PS$, 307.04; found, 307.07.

Hapten-KLH. Hapten **4** (5 mg, 0.016 mmol) was added to 1 mL of a 5 mg/mL solution of succinylated keyhole limpet hemocyanin solution (KLH). *N*-Ethyl-*N*'-(3-dimethylaminopropyl)carbodiimide HCl (EDCI) (50 mg, 0.26 mmol) and N-hydroxysulfosuccinimide (Sulfo-NHS) (Pierce) (57 mg, 0.261 mmol) were added. The pH was adjusted to 8 with 1 M NaOH and the coupling proceeded overnight at RT (RT). The mixture was purified on a PD-10 column (Amersham Biosciences), using PBS as the eluent. Protein concentration was determined by Bradford assay (1.3 mg/mL), and the number of hapten molecules/ KLH (260 hapten molecules/KLH molecule) was determined by standard curves of hapten and KLH absorbances at 280 nm. A Hapten-BSA conjugate was prepared using this same procedure (100 hapten molecules/KLH molecule).

Succinylated Hapten 5. Hapten **4** (9.2 mg, 0.03 mmol) was dissolved in 0.4 mL N,N-diethylformamide (DMF) then diisopropyl ethylamine (DIPEA) (10 µL, 0.06 mmol) and succinic anhydride (3.6 mg, 0.036 mmol) were added. The reaction proceeded for 16 hours at RT. The solvent was removed under reduced pressure and the resulting oil was dissolved in 2 mL 1:4, acetonitrile:H_20 and separated by HPLC (^{18}C prep column, 60 min gradient, H_2O-CH_3CN 95:5 to H_2O-CH_3CN 40:60, 250 nm), using the same solvent and gradient as described for hapten **4**; the fractions containing the product (30 min) were lyophilized to afford a clear oil (11 mg, 90% yield). ESI-MS (*m/z*): $[M+H]^+$ calcd for $C_{14}H_{19}N_2O_8PS$, 407.06; found, 407.09.

Affinity Column Containing Succinylated Hapten 5. An affinity purification column was prepared as follows: diaminodipropyl amine gel (2 mL, 16-20 µmol/mL, Pierce) was placed in a small fritted column. Buffer was allowed to drain off and the remaining solid was washed with PBS (6 mL). Succinylated hapten **5** (10 mg, 0.025 mmol) in conjugation buffer (2 mL, 0.1 M MES, 0.9% NaCl, pH= 7.4) was added to the washed column, followed by EDCI (60 mg, 0.312 mmol) and Sulfo-NHS (60 mg, 0.274 mmol) in conjugation buffer (0.5 mL). The ends of the column were capped and the column was placed on a rotator overnight at RT. The column was drained, washed with 10 mL of 1 M NaCl, and stored at 4°C in PBS with 0.05% sodium azide until use.

Purification of Polyclonal IgG and IgY. The affinity column was washed with PBS (10 mL), and 5 mL of total immune IgY (Aves Labs) or total immune IgG (Epitomics) was added. The column was drained until the anti-sera had fully entered the beads. Unbound antibodies were washed from the column with 150 mL PBS and bound antibodies were eluted with acidic elution buffer (0.1 M glycine, pH=2.8), followed by basic elution buffer (0.1 M triethylamine pH 11). Individual fractions (1 mL) were immediately neutralized with 50 µl 1M TRIS, pH= 9.5 or 50 µl 1M TRIS, pH= 3.5. Fractions were pooled, concentrated, and exchanged into PBS with an Amicon centrifugal 10,000 MWCO filter as per the manufacturer's instructions. Absorbance at 280 nm was used to determine the concentration (1 Au ~ 0.75 mg/mL): α-hapten-IgY (3.7 mg/mL, 900 µL recovered) and α-hapten-IgG (5.1 mg/mL, 500 µL recovered).

Synthesis of Thioether Control (See **Supplementary Figure 5b**)

6. N, N'-bis-Boc-L-cysteine (Novabiochem) (750 mg, 1.7 mmol) was dissolved in 8.5 mL of dichloromethane. NHS (469 mg, 4.1 mmol), EDCI (782 mg, 4.1 mmol), and methylamine (4.25 mL of 2M in MeOH, 8.5 mmol) were added and the mixture was stirred overnight at RT. 50 mL of ethyl acetate was added and the mixture was washed with 50 mL of saturated sodium carbonate, followed by 50 mL of 10% citric acid. The organic layer was dried with Na_2SO_4 and concentrated under reduced pressure to afford 630 mg of white powder. The major product was the bisamide **6**: ESI-MS (*m/z*): $[M+H]^+$ calcd for $C_{18}H_{34}N_4O_6S_2$, 467.19; found, 467.1. A small amount of the monoamide was also formed: ESI-MS (*m/z*): $[M+H]^+$ calcd for $C_{17}H_{31}N_3O_7S_2$, 454.16; found, 454.1. This mixture was deprotected without further purification.

7. Crude **6** was dissolved in 5 mL TFA, 4.8 mL dichloromethane, and 200 µL water. The mixture was stirred at RT, and appearance of the free amine was monitored by TLC and ninhydrin staining. After two hours 30 mL toluene was added and the mixture was concentrated under reduced pressure. 580 mg of crude product was obtained: ESI-MS (*m/z*): $[M+H]^+$ calcd for $C_8H_{18}N_4O_2S_2$, 267.09; found, 267.2.

8. Crude **7 (**290 mg, 0.58 mmol) was dissolved in 5.8 mL of dichloromethane and then DIPEA (300 µL, 2.3 mmol) was added. Another 1.5 mL of DMF was added to further solubilize **7**, followed by succinic anhydride (139 mg, 1.4 mmol). The reaction proceeded overnight at RT, disappearance of the amine was monitored by TLC and ninhydrin staining. Ethylene diamine (0.5 mL, 7.5 mmol) was added to quench excess succinic anhydride. After stirring for one hour, ethyl acetate (50 mL) and 1 M HCl (50 mL) were added. The aqueous layer was lyophilized and purified with HPLC (^{18}C prep column, 60 min gradient, H_2O-CH_3CN 0.1% TFA 95:5 to H_2O-CH_3CN 0.1% TFA 40:60, 215 nm). ESI-MS (*m/z*): $[M+H]^+$ calcd for $C_{16}H_{26}N_4O_8S_2$, 467.12; found, 467.1.

9. 8 (27 mg, 0.058 mmol) was dissolved in 1.2 mL H_2O and the pH was adjusted to 7.5 with saturated Na_2CO_3. Tris(2-carboxyethyl)phosphine HCl (TCEP) (33 mg, 0.12 mmol) was added and reduction proceeded at RT for 2 hours. *p*-nitrobenzyl mesylate (PNBM) (37 mg, 0.16 mmol), dissolved in 1.0 mL DMF, was added and the reaction proceeded overnight at RT. The reaction mixture was washed with 5 mL of pentane and concentrated under reduced pressure. HPLC purification was performed as described for **8**. ESI-MS (*m/z*): $[M+H]^+$ calcd for $C_{15}H_{19}N_3O_6S$, 370.10; found, 370.1.

Thioether-BSA

9 was coupled to BSA as described for hapten-KLH. The coupling ratio was 10 molecules of **9**/BSA molecule).

ELISA

96 well plates (Nunc Immunosorb) were coated overnight at 4º C with 2.5 µg/mL of BSA, BSA-hapten, or BSA-thioether in 50 µL/well of PBS. The wells blocked with 200 µL of 1% BSA in PBST (PBS with 0.05% Tween 20) for 1 hour at RT and washed twice with 200 µL PBST. 50 µL of each hybridoma supernatant was added and allowed to bind overnight at 4º C. The wells were washed as above and 100 µL/well of goat α-rabbit-IgG

–HRP (Promega), diluted 1:2,000 in 0.25% BSA PBST was added. After 45 minutes at RT the plates were developed as described.[8]

HeLa lysate labeling
HeLa-S3 cell pellets ($0.75x10^9$ cells) were obtained from the National Cell Culture Center (NCCC). After thawing, 10 mL of RIPA lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 1.0% NP-40, 0.1% SDS, with protease inhibitors (Roche, complete)) was added and the cells were rotated at 4ºC for 15 minutes. After centrifugation at 20,000 g for 10 minutes the supernatant was decanted and used for kinase assays. Alternatively the lysate could be stored at -80ºC. For assays of wild type cellular kinases, HeLa lysate (15 mg/mL in RIPA lysis buffer) was diluted 1:1 with 10 mM HEPES pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$ in the presence or absence of 1 mM ATPγS. After 30 minutes at RT PNMB in DMSO (final concentration was 2.5 mM PNBM, 5% DMSO) or DMSO was added. Alkylation proceeded for 2 hours at RT, and the lysates were analyzed by western blotting.

Immunoprecipitation of Histone H1 with Hybridoma Supernatants.
100-200 µL of each hybridoma cell culture supernatant (Epitomics) was bound to 50 µL of a 50% slurry of rProtG agarose (Invitrogen) in a total volume of 500 µL RIPA. Antibodies bound to the beads for 2 hours at 4°C. The supernatant was removed and 500 µL of RIPA containing 0.4 mg/mL BSA and 2 µg affinity tagged H1 (prepared as described[8]) was added. Immunoprecipitations proceeded overnight at 4°C. The beads were washed three times with 1.0 mL RIPA and eluted with SDS-PAGE sample buffer (DTT was omitted to keep antibody molecules intact). The immunoprecipitates were analyzed by polyclonal α-hapten-IgG western blotting.

Purification of Rabbit Monoclonal Antibody (RmAb 51-8)
Concentrated tissue culture supernatant (Epitomics) was purified with an Immunopure IgG Purification Kit (Pierce), according to the manufacturers' instructions. The eluted fractions were pooled, concentrated, and exchanged into PBS using a 10,000 MWCO centrifugal filter (Amicon), according to the manufacturer's instructions. Absorbance at 280 nm was used to determine the concentration (1 Au ~ 0.75 mg/mL): RmAb 51-8 α-hapten-IgG (5.0 mg/mL, 750 µL recovered).

Synthesis of Thiophosphorylated Peptide (Ac-DSES*PSQK)
FMOC-L-Lys(Boc)-Wang Resin (120 mg) (Novabiochem) was swollen with DMF (EM Science, peptide synthesis grade) in a 3 mL syringe vessel (Isolute®). Synthesis was performed using standard FMOC solid phase peptide synthesis, with diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBT) (Novabiochem) activation of amino acids. The amino acids were protected as follows FMOC-Asp(Boc)-OH, FMOC-Glu(Boc)-OH, FMOC-Ser(OtBu)-OH, FMOC-Gln(trt)-OH. The N-terminus was acetylated using the same coupling procedure, by substituting the amino acid with acetic acid. For example, 0.48 mmol of FMOC-Ser-OH and 74 mg HOBT were dissolved in 1.2 mL DMF and then activated with 70 µL DIC for 10 minutes. The mixture was added to the washed resin and reacted at room temperature for 3 hours and then washed with DMF. The FMOC protecting group was removed after each coupling

by treatment with 20% piperidine in DMF for 20 minutes. As described[2], the serine to be thiophosphorylated was incorporated as the unprotected FMOC-Ser-OH. After the solid phase synthesis was complete the resin was washed (three times with DMF, three times with dichloromethane, three times with methanol), dried overnight in a vacuum desiccator, and flushed with argon. A solution containing 1-H-tetrazole (1.4 mmol, 100 mg (obtained from concentration of a 3% solution in acetonitrile)), dibenzyl N,N-diisopropylphosphoramidite (0.47 mmol, 167 μL), and anhydrous DMF (1.6 mL) were added to the dried resin and allowed to react for 1.0 hour at RT with rotation. The resin was then washed (three times with DMF, three times with acetonitrile) and reacted with phenylacetyl disulfide (0.66 mmol, 204 mg) in acetonitrile (1.6 mL) for two hours at RT with rotation. The resin was washed (three times with DMF, three times with dichloromethane, three times with methanol, three times with dichloromethane) and treated with a cleavage cocktail (67% trifluoroacetic acid (TFA), 11.5% trimethylsilyl bromide, 10.4% thiophenol, and 10.4% m-cresol) for 2 hours at RT. After *in vacuo* concentration and filtration the peptide was HPLC purified (^{18}C prep column, 60 min gradient, H_2O-CH_3CN 98:2 to H_2O-CH_3CN 60:40, 215 nm) and lyophilized to afford a white powder. ESI-MS (*m/z*): $[M+H]^+$ calcd for $C_{36}H_{60}N_{10}O_{20}PS$, 1015.34; found, 1015.10.

Alkylation and Analytical HPLC of Thiophosphorylated Peptide (See **Supplementary Figure 3)**
Peptide (Ac-DSES*PSQK, 750 μM) in 10 mM HEPES pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$ was treated with DMSO or PNMB in DMSO (final concentration 2.5 mM PNBM, 5% DMSO) for 1 hour at RT. The reaction was diluted and a portion was analyzed by HPLC (^{18}C analytical column, 60 min gradient, H_2O-CH_3CN 98:2 to H_2O-CH_3CN 70:30, 215 nm). Mass spectrometric analysis of the peak eluting at 22 minutes was performed on a QSTAR XL (Applied Biosystems) as described for c-Jun-GST.

Synthesis of A*TPγS (see **Supplementary Figure 6a**)
N-6-benzyladenosine (**10, R=benzyl**) is commercially available (Acros Organics).
N-6-phenethyladenosine (10, R=phenethyl)
6-chloropurine ribonucleoside (3.5 mmol) was refluxed overnight in ethanol (21 mL) containing phenethylamine (21 mmol) as described[3]. After evaporation *in vacuo*, 150 mL of ethanol was added and the mixture was crystallized at 4°C for 5 hours. The crystals were filtered, washed with ice cold ethanol, and obtained in 51% yield. Higher yields could be obtained by performing another round of crystallization on the remaining liquid. ESI-MS (*m/z*): $[M+H]^+$ calcd for $C_{18}H_{21}N_5O_4$, 372.16; found, 372.22.

N-6- benzyl-ADP (11, R=benzyl) and N-6-phenethyl-ADP (11, R=phenethyl)
Formation of the intermediate dichlorophosphate was performed essentially as described[4], with slight modifications. 2 mmol of **10** (R=phenethyl or R=benzyl) was dissolved in 5 mL of triethylphosphate (TEP) and cooled to 0°C. $POCl_3$ (4 mmol) was added dropwise and the mixture was stirred at 0°C for two hours. To form the diphosphates[5] a mixture of 10 mmol of H_3PO_4 (solid) and 10 mmol 1,8-diazabicyclo[5.4.0]undec-7-en (DBU) in 5 mL of TEP was added. Formation of a light yellow solid was instantaneous and after two minutes the reaction was quenched with 30

mL of 0.1M triethylammonium bicarbonate (TEAB). After stirring at for 30 minutes at RT the reaction was filtered with a PTFE syringe filter (0.45 μm, Acrodisk). Aliquots of the reaction were purified by strong anion exchange chromatography on an Acta FPLC (Amersham Biosciences). Two HiPrep 16/10 QFF anion exchange columns (Amersham Biosciences) were set up in series and the products were separated using an 80 min gradient (100% 0.1 M TEAB to 50:50 0.1M TEAB: 2M TEAB, 280 nm (see **Supplementary Fig. 6b**)). TEAB was prepared by bubbling CO_2 into a 2 M solution of triethylamine. The fractions containing the diphosphate (33-43 min) were pooled and lyophilized*, yields for the ADP analogs ranged from 25-30%. For N-6-benzyl-ADP, ESI-MS (*m/z*): $[M-H]^-$ calcd for $C_{17}H_{21}N_5O_{10}P_2$, 516.08; found, 516.10. For N-6-phenethyl-ADP, ESI-MS (*m/z*): $[M-H]^-$ calcd for $C_{18}H_{23}N_5O_{10}P_2$, 530.08; found 530.10. Significant amounts of the AMP and ATP analogs are also obtained in this synthesis.

Disodium S-2-Carbamoylethyl Phosphorothioate (12).
This procedure is adapted from Cook[6], with the substitution of trisodium thiophosphate for trilithium thiophosphate. A solution of trisodium thiophosphate (18.6 mmol) in 28 mL of water was treated with a solution of 3-chloropropionamide (28 mmol) in 5.6 mL of DMF for 48 hours at RT. The reaction was then filtered and 100 mL of ethanol was added to the filtrate. Upon addition a white precipitate formed, to increase the size of the crystals an additional 100 ml of ethanol was added and the mixture was chilled at 4ºC overnight. The precipitate was then filtered, washed with ethanol, and dried *in vacuo* to afford disodium S-2-carbamoylethyl phosphorothioate (**12**) in 86% yield. ^1H NMR (400 MHz, MeOH-D4 with D_2O) δ 2.94 (m, 2H), 2.63 (t, 2H). ESI-MS (*m/z*): $[M-H]^-$ calcd for $C_4H_8NO_4PS$, 183.99; found, 184.00.

13.
This procedure is adapted from Goody et al[7]. Disodium S-2-carbamoylethyl phosphorothioate (**12**) (2.0 mmol) was dissolved in 10 mL of methanol:H_20 (1:1) and applied to a column containing DOWEX® 501-X8 ion exchange resin (5 mL, pyridinium form**). The pyridinium salt of **12** was eluted with another 10 mL of methanol:H_20 (1:1). Solvent was removed *in vacuo* and 20 mL of methanol was added to the residue, which was then converted to the mono(tri-*n*-octylammonium) salt by addition of tri-*n*-octylamine (2 mmol). Again the solvent was removed *in vacuo* and the residue was dried by repeated evaporation (twice) of 10 mL aliquots of dry DMF. The residue was dissolved in dioxane (14 mL) and diphenyl phosphorochloridate (0.6 mL) was added, followed by tri-*n*-butylamine (0.92 mL). The reaction proceeded at RT for 2 hours, with stirring. The solvent was removed under reduced pressure and ether (20 mL) was added. After the residue had dissolved warm petroleum ether (40 mL) was added and the mixture was chilled at 4°C for 30 minutes. An oily residue separated and the supernatant was decanted. The residue was then dissolved in 10 mL of dioxane and the solvent was removed under reduced pressure. At this point the reaction was split and put on a high vacuum line for one hour and then stored overnight at -20ºC. The reaction product was not characterized, and 100% yield was assumed.

N-6-benzyl-ATPγS (14, R=benzyl) and N-6-phenethyl-ATPγS (14, R=phenethyl)
Either N-6- benzyl-ADP (**11**, R=benzyl) (0.3 mmol) or N-6-phenethyl-ADP (**11**, R=phenethyl) (0.3 mmol) as the triethylammonium (TEA) salts, were dissolved in 5 mL methanol:H_20 (1:1). It was assumed that there were three TEA molecules per ADP analog. ADP analogs were converted to the pyridinium salts using the same procedure as described above for compound **12,** except that 5 mL of methanol:H_20, 1:1 was used as the eluent. After removal of solvent *in vacuo*, methanol (12 mL), tri-*n*-octylamine (0.3 mmol), and tri-*n*-butylamine (0.3 mmol) were added. After solution had occurred the solvent was removed and the residue dried by repeated (three times) evaporation of 5 mL aliquots of pyridine. The residue was then dissolved in 3.3 mL of pyridine and added to 0.6 mmol of **13**, the reaction was stirred at RT for 2 hours during which time a precipitate formed. Solvent was removed, 20 mL of 0.2 M NaOH was added, and the reaction was then heated to 100°C for 10 minutes. The pH was adjusted to pH 7 with DOWEX® 501-X8 ion exchange resin (pyridinium form) and 0.4 mL β-mercaptoethanol was added. The crude reaction could be stored at -20°C until purification. The reaction was then filtered and purified as described for the ADP analogs (see **Supplementary Figure 6c**). The fractions containing ATPγS analogs were pooled and lyophilized*. The compounds were obtained in 6-8% yield and were analyzed using mass spectrometry. For N-6-benzyl-ATPγS, ESI-MS (*m/z*): $[M-H]^-$ calcd for $C_{17}H_{22}N_5O_{12}P_3S$, 612.02; found 611.95. For N-6-phenethyl-ATPγS, ESI-MS (*m/z*): $[M-H]^-$ calcd for $C_{18}H_{24}N_5O_{10}P_2$, 626.02; found 625.90. Absorbance at 280 nm, as compared to the N-6-modified adenosines, was used to obtain accurate concentrations. Degradation was observed after freeze thaw cycles, so small aliquots were stored at -80°C.

*A second round of lyophilization was performed to obtain a white solid and to remove excess TEAB.
** DOWEX® 501-X8 ion exchange resin (approximately 20 mL of dry resin) was swollen with 20 mL of methanol:H_20:pyridine (50:45:5). Two fritted columns were loaded with 5 mL of the swollen resin and washed with 20 mL of methanol:H_20 (1:1), these columns were used to exchange the counter-ions on **11** and **12.** The additional resin was kept for neutralization of the thiophosphorylation reaction after deprotection.

Cell Culture and Plasmids
The WT and AS Erk2 mouse embryonic fibroblasts (MEFs) were prepared from 13.5-day old embryos of *Erk1*$^{-/-}$ *Erk2*$^{+/+}$ and *Erk1*$^{-/-}$ *Erk2* $^{AS/AS}$ mice respectively. The MEFs were grown in Dulbecco's modified Eagle medium (DMEM, high glucose with L-glutamine), supplemented with 10% fetal bovine serum, 0.292 mg/mL L-glutamine, 1 mM sodium pyruvate, 55 μM 2- mercaptoethanol, 1X MEM non-essential amino acids (Gibco), 100 μg/mL streptomycin, and 100 units/mL penicillin, at 37°C, 5% CO_2. Cells at or before pass 5 were used in the experiments. COS-7 cells were purchased from American Type Culture Collection (ATCC), and grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.1 mg/mL sodium pyruvate, 100 μg/mL streptomycin, and 100 units/mL penicillin. The cells were maintained at 37°C, 10% $C0_2$. pCDNA3-FLAG-*JNK1* M108G, L168A, (JNK1 AS kinase) has been described previously[9]. Rat PKCδ was aligned with other protein kinases and the gatekeeper amino acid was identified as methionine 425 in *PKCδ*. Flag-*PKCδ*

was generated by performing PCR with the following 5' primer (5'-CGCGGATCCATGGACTATAAGGACGATGATGACAA AGCACCGTTCCTGC GCATCTCCTTC-3') and 3' primer (5'-CCGGAATTCCTATTC CAGGAATTGCTCA TATTTGG -3') using pRSV-rat *PKCδ* as the template. The PCR product was digested with *Bam*HI and *Eco*RI, and ligated into pcDNA3 vector (Invitrogen). The *PKCδ* AS mutant (M425A) was generated with 5' primer (5'-GGACCACC TCTTCTTTGTGGCCGAGTTCCTCAATGGGGGCG-3') and 3' primer (5'-CGCCCCCATTGAGGAACTCGGCCACAAAGAAGAGGTGGTCC-3') using QuickChange site-directed mutagenesis kit (Stratagene). The coding sequence was sequenced to confirm error free PCR and the M425A mutation. Yeast *CDC5* (YMR001C) cloned into pFastBacHT-A (Invitrogen) (pFastBacHT A-*CDC5*) was a kind gift of David Morgan (University of California, San Francisco). pFastBacHT A-*CDC5* L158G, encoding the AS Cdc5 kinase[10], was constructed from pFastBacHT A-*CDC5* by QuikChange site-directed mutagenesis (Stratagene).

Kinase Expression and Purification

PKCδ wild type (WT) and analog specific (AS)

FLAG- *PKCδ* (WT or AS) plasmids (90 μg) were transfected into COS-7 cells (4x150 cm plates per plasmid) using Superfect (Quiagen) according to the manufacturer's instructions. After 72 hours the cells were washed 1X with PBS and then FLAG lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X-100, phosphatase inhibitor cocktail 1, and complete protease inhibitor cocktail (Roche)) was added. After 20 minutes of incubation on a rocker at 4°C the cells were collected and centrifuged at 12,000g for 10 min. The supernatants were added to anti-FLAG M2-agarose gel and incubated at 4°C for three hours. The beads were transferred to a small column and washed extensively with ice cold lysis buffer, followed by wash buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, phosphatase inhibitor cocktail 1, and complete protease inhibitor cocktail (Roche)). The immunoprecipitated PKCδ was eluted into storage buffer containing FLAG peptide (20 mM HEPES pH 7.4, 0.1 mM EGTA, 25% glycerol, 0.03% Triton X-100, 150 ng/μL FLAG peptide). Concentrations of WT and AS PKCδ were determined by comparison with pure PKCδ ((Panvera) using Sypro Ruby (Molecular Probes). Approximately 150 μL of purified kinase was obtained: WT = 94 ng/μL, AS = 25 ng/ μL. The kinases were aliquoted and stored at -80°C until further use.

JNK1 (WT and AS)

FLAG-*JNK1* WT plasmid (70 μg) was transfected into COS-7 cells (3x150 mm plates) using Superfect (Qiagen) according to the manufacturer's instructions. FLAG-*JNK1* AS plasmid (225 μg) was transfected into COS-7 cells (7x150 mm plates) using Superfect (Qiagen) according to the manufacturer's instructions. After 48 hours the medium was removed and the cells were exposed to 60 J/m^2 ultraviolet light (using the tissue culture hood UV lamp) for 45 seconds to activate JNK1. The medium was replaced and the cells were incubated at 37ºC, 5% CO_2 for another 30 minutes. After washing 1X with PBS, FLAG lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X-100, phosphatase inhibitor cocktail 1, phosphatase inhibitor cocktail 2, and complete protease inhibitor cocktail (Roche)) was added. Cells were incubated for 20 on a rocker at 4°C and then collected and centrifuged at 12,000 g for 10 min. Supernatants were

added to anti-FLAG M2-Agarose gel and incubated for three hours at 4°C. The beads were transferred to a small column and washed extensively with ice cold lysis buffer. Immunoprecipitated JNK1 was eluted into storage buffer containing FLAG peptide (20 mM HEPES pH 7.4, 0.1 mM EGTA, 25% glycerol, 0.03% Triton X-100, phosphatase inhibitor cocktail 1, phosphatase inhibitor cocktail 2, complete protease inhibitor cocktail (Roche), and 150 ng/μL FLAG peptide). Western blotting against dually phosphorylated JNK1 (α–pT183, α–pY185 IgG, Cell Signaling), was used to quantitate the amount of activated kinase, as compared to a JNK1 standard (Upstate). Yield: 100 μL of wild-type JNK1 (5 ng/μL) and 200 μL of AS JNK1 (5 ng/μL). The kinases were aliquoted and stored at -80°C until further use.

Cdc5 (WT and AS)

Purified wild type 6xHis-Cdc5 was a kind gift of D. Randle and David Morgan (University of California, San Francisco). Purified analog specific 6xHis-Cdc5 was prepared as follows. Bacmid was produced from pFastBacHT A-*CDC5* L158G and transfected into Sf9 insect cells with Cellfectin using the Bac-to-Bac Baculovirus expression system (Invitrogen) according to the manufacturer's instructions. For AS Cdc5 expression, 1 L of Sf9 cells at $2x10^6$ cells/mL was infected with 30 mL of pass 3 baculovirus. Cells were harvested after 2 days at 27°C, and lysate was prepared by douncing in Cdc5 lysis buffer (CLB: 25mM HEPES pH 7.4, 300 mM NaCl, 10% glycerol, 5 mM NaF, 5 mM beta-glycerophosphate) with protease inhibitors (1 mM PMSF, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatin). The lysate was cleared by centrifugation at 7,649 x g for 15 min, followed by ultracentrifugation at 227,220 x g for 1 hr. The filtered supernatant was applied at 0.5 mL/min to a 1 mL HiTrap Chelating HP column (Amersham) chelated with $CoCl_2$. The column was washed with CLB, followed by CLB with 2 mM imidazole. 6xHis-Cdc5 L158G was eluted with CLB containing a gradient of imidazole from 100 mM to 200 mM. Peak 6xHis-Cdc5 L158G containing fractions were pooled, aliquoted and stored at -80°C until further use. The concentration of AS Cdc5 (150 ng/μL) was calculated from a Sypro Ruby (Molecular Probes) stained gel that contained known amounts of BSA.

***in vitro* Kinase Assays followed by PNBM alkylation**

PKCδ (WT and AS)

For Western Blot Analysis:

100 ng of kinase was added to a 30 μL reaction containing PKC kinase buffer (20 mM HEPES pH 7.4, 1 mM EGTA, 10 mM $MgCl_2$, 0.3% Triton X-100, 1 μM PMA, 500 μg/mL phosphatidyl serine (diluted 10X from a fresh 5 mg/mL aqueous stock), 2 μg Histone (Sigma, suitable for PKC assay), and 1 mM nucleotide (ATPγS, N-6-benzyl ATPγS, or N-6-phenethyl ATPγS)). The kinase reaction proceeded for 30 minutes at RT then 1.5 μL of 50 mM PNBM in DMSO was added (to afford a final concentration of 2.5 mM PNBM, 5% DMSO). After two hours at RT the samples were analyzed by western blotting.

For DELFIA Based Kinetic Measurements:

30 μL kinase reactions, in 96 well PCR plates, were carried out as above with the following exceptions: 10 μg of Histone was used in each reaction and PKCδ wild type and analog sensitive kinases were used at 4 nM and 8 nM respectively. The reactions

were initiated by addition of various concentrations of nucleotide (ATPγS or N-6-benzyl-ATPγS) and terminated after 30 minutes by addition of EDTA to a final concentration of 20 mM. Alkylation (2.5 mM PNBM, 5% DMSO) proceeded for 2 hours and the reactions were analyzed by DELFIA.

JNK1 (WT and AS)

For Western Blot Analysis:

10 ng of kinase was added to a 30 µL reaction containing 10 mM HEPES pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 2 µg GST-c-Jun or 2 µg GST-ATF-2 (Cell Signaling Technology) and 1 mM nucleotide (ATPγS, N-6-benzyl ATPγS, or N-6-phenethyl ATPγS). The kinase reaction proceeded for 30 minutes at RT then 1.5 µL of 50 mM PNBM in DMSO was added (to afford a final concentration of 2.5 mM PNBM, 5% DMSO). After two hours at RT the samples were analyzed by western blotting.

For DELFIA based Kinetic Measurements:

15 µL kinase reactions, in 96 well PCR plates, were performed as above with the following exceptions: 2.5 µg of GST-c-Jun was used in each reaction, JNK1 wild type and analog sensitive kinases were used at 5 nM. The reactions were initiated by addition of various concentrations of nucleotide (ATPγS or N-6-benzyl-ATPγS), and terminated after 30 minutes by addition of EDTA to a final concentration of 20 mM. Alkylation (2.5 mM PNBM, 5% DMSO) proceeded for 2 hours and the reactions were analyzed by DELFIA.

Preparation of c-Jun-GST for Mass Spectrometry

200 ng of JNK1 (Upstate) was added to 30 µL reactions containing 10 mM HEPES pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, and 2 µg c-Jun-GST (Cell Signaling Technology) in the presence or absence of 1 mM ATPγS. After 30 minutes PNBM was added to a final concentration of 2.5 mM PNBM, 5% DMSO. Alkylation proceeded for 2 hours at RT. Following SDS-PAGE and coomassie staining the c-Jun-GST bands were excised.

Cdc5 (WT and AS)

For Western Blot Analysis:

Cdc5 (6 ng of WT or 30 ng of AS) was added to a 30 µL reaction containing 25 mM HEPES pH 7.4, 50 mM NaCl, 15 mM $MnCl_2$, 10 µg α-casein (Sigma, dephosphorylated), 10 µg/mL BSA, 1mM 1,4-dithiothreitol (DTT), and 1 mM nucleotide (ATPγS, N-6-benzyl ATPγS, or N-6-phenethyl ATPγS). The kinase reaction proceeded for 30 minutes at RT and then 1.5 µL of 100 mM PNBM in DMSO was added, to afford a final concentration of 5 mM PNBM. Note the concentration of PNBM was increased to account for the fraction that could be consumed by DTT. After two hours at RT the samples were analyzed by western blotting.

For DELFIA based Kinetic Measurements:

30 µL kinase reactions, in 96 well PCR plates, were performed as above with the following exception: Cdc5 wild type and analog sensitive kinases were used at 0.3 nM and 1.5 nM respectively. Reactions were initiated by addition of various concentrations of nucleotide (ATPγS or N-6-phenethyl-ATPγS) and terminated after 20 minutes by addition of EDTA to a final concentration 30 mM. PNBM alkylation proceeded for 2 hours as described above for western blot analysis, and the reactions were analyzed by DELFIA.

***in vitro* Kinase Assay of Erk2 (WT and AS)**

1.5 x10^6 WT or AS Erk2 MEFs cells were trypsinized, pelleted, and resuspended with DMEM to afford 10x10^6 cells/mL. To activate Erk2 the cells were stimulated with 20 ng/mL of phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin for 5 minutes at 37°C. Activated cells were lysed in 250 μl cell lysis buffer (1% NP-40, 5 mM EDTA, 25 mM HEPES, 150 mM NaCl supplemented with complete mini protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail sets I & II (Calbiochem)). Lysates were cleared by centrifugation at 14,000 g, 4°C for 10 minutes, then incubated with 15 μl immobilized anti-phospho-ERK1/2 mouse monoclonal antibody (Cell Signaling) per reaction at 4°C overnight. Immunoprecipitates were washed twice with 400 μl cell lysis buffer and once with 400 μl kinase buffer (Cell Signaling, 25 mM Tris-HCl (pH 7.5), 5 mM beta-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4, 10 mM $MgCl_2$) pre-chilled on ice. Immunoprecipitates were then incubated with 2 μg Elk-1 fusion protein (Cell Signaling) and 1 mM nucleotide (ATPγS, N-6-benzyl ATPγS or N-6-phenethyl ATPγS) in 30 μl of kinase buffer. The kinase reaction proceeded at 30 °C for 30 minutes with gentle rocking and then alkylated with 15 mM PNBM, 5 % DMSO for two hours at RT. The samples were resolved by 10% SDS-PAGE, transferred to PVDF membrane, and blotted with α-hapten-IgG.

For the following list of kinases the reaction conditions are described. Each reaction proceeded at RT for 30 minutes, and then 1.5 μL of 50 mM PNBM in DMSO was added (to afford a final concentration of 2.5 mM PNBM, 5% DMSO). Alkylation proceeded for two hours at RT and samples were analyzed by western blotting.

p38α

Kinase (10 ng) was added to a 30 μL reaction containing 10 mM HEPES pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, and 2 μg myelin basic protein in the presence or absence of 1 mM ATPγS.

ERK1 and ERK2

Kinase (25 ng; Upstate) was added to a 30 μL reaction containing 10 mM HEPES pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, and 2 μg myelin basic protein in the presence or absence of 1 mM ATPγS.

Akt1

Kinase (50 ng; Calbiochem) was added to a 30 μL reaction containing 10 mM HEPES pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, and 2 μg GSK3β-paramyosin fusion (Cell Signaling) in the presence or absence of 1 mM ATPγS.

Abl

Kinase[11] (500 ng) was added to a 30 μL reaction containing 25 mM HEPES pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 2 μg GST-Abl substrate (Stratagene), and 0.5 mg/mL BSA in the presence or absence of 1 mM ATPγS.

Src
Kinase[11] (500 ng) was added to a 30 µL reaction containing 25 mM HEPES pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 2 µg GST-Src substrate (Stratagene), and 0.5 mg/mL BSA in the presence or absence of 1 mM ATPγS.

CK1
Kinase (1000 units; New England Biolabs) was added to a 30 µL reaction containing 25 mM HEPES pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 2 µg casein (α- dephosphorylated), and 0.5 mg/mL BSA in the presence or absence of 1 mM ATPγS.

GSK3β
Kinase (1000 units; New England Biolabs) was added to a 30 µL reaction containing 25 mM HEPES pH 7.4, 10 mM $MgCl_2$, 2 µg myelin basic protein, and 0.5 mg/mL BSA in the presence or absence of 1 mM ATPγS

PKCε
Kinase (100 ng; Invitrogen) was added to a 30 µL reaction containing 20 mM HEPES pH 7.4, 1 mM EGTA, 10 mM $MgCl_2$, 0.3% Triton X-100, 1 µM PMA, 500 µg/mL phosphatidyl serine (diluted 10X from a fresh 5 mg/mL aqueous stock), and 2 µg histone (suitable for PKC assay) in the presence or absence of 1 mM ATPγS.

Western Blotting
Ten microliters of the SDS-PAGE sample buffer quenched kinase reactions were electrophoresed and transferred to nitrocellulose. The blots were blocked with 4% milk in TBST (20 mM Tris, 137 mM NaCl, 0.05% Tween 20) and then incubated overnight at 4º C with α-hapten-IgG (1:20,000 in 4% milk, TBST), α-hapten-IgY (1:15,000 in 5% BSA, TBST), or RmAb 51-8 (1:20,000 in 4% milk, TBST). For monoclonal screening experiments the hybridoma supernatants were diluted 1:100 in 5% BSA PBST and incubated overnight at 4º C. After washing with TBST the blot was incubated with secondary antibody (goat α-rabbit-IgG, Promega (1:15,000 in 5 % milk TBST)) for one hour at RT. The blots were imaged with film (Pierce) using chemiluminescence (West Pico, Pierce) or by camera (Alpha Innotech).

DELFIA
After PNBM alkylation the kinase reactions were diluted in coating buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3, pH 9.5) to concentrations that fell within the linear range of detection (as determined by coating concentration experiments, see **Supplementary Fig. 4a**). Ninety Six well plates (MaxiSorp, NUNC) were coated overnight (50 µL/well) with the following concentrations of each kinase substrate: 2.5 µg/mL of histone (PKCδ substrate), 2 µg/mL of c-Jun- GST (JNK1 substrate), and 2.5 µg/mL of α-casein (Cdc5 substrate). Each sample was coated in triplicate and each experiment was repeated at least three times. After removal of the coating buffer the plates were blocked (1% BSA

in TBST, 200 µL/well) for 1-2 hours at RT. Blocking buffer was removed, the wells were washed twice with 200 µL/well TBST wash buffer, and Eu^{3+} labeled polyclonal α-hapten-IgG[§] (1:400 in DELFIA assay buffer (Perkin Elmer)) was added. Binding proceeded overnight at 4ºC. The plates were washed 4X with 200 µL/well TBST followed by DELFIA enhancement solution (75 µL/well). After shaking for five minutes, time resolved fluorescence was measured with an Analyst HT plate reader (LJL Biosystems).

§ Eu^{3+} labeled α–hapten–IgG was prepared and quantified with a DELFIA Eu^{3+} labeling kit (Perkin Elmer). 100 µL of polyclonal α–hapten–IgG (10 mg/mL in PBS) was diluted to 4 mg/mL by adding 150 µL of 100 mM Na_2CO_3 pH 11. This solution was added to 0.2 mg of Perkin Elmer Eu^{3+} reagent, and incubated overnight at 4ºC. The antibody was purified with size exclusion chromatography, using PD-10 column matrix in a 1 mL column. Eu^{3+} labeled α–hapten–IgG was obtained at 0.95 mg/mL with 0.75 Eu^{3+} molecules/ IgG molecule.

Substrate Labeling in Digitonin Permeabilized MEFs

15 x10^6 *Erk1*$^{-/-}$ *Erk2*$^{+/+}$ or *Erk1*$^{-/-}$ *Erk2*$^{AS/AS}$ MEFs cells were trypsinized, pelleted, and resuspended in DMEM to afford 5x10^6 cells/mL. To activate Erk2 the cells were stimulated with 20 ng/mL of PMA and 1 µm ionomycin for 5 minutes at 37°C. Cells were transferred on ice and pelleted at 4°C. Permeabilization proceeded on ice for 5 min in a buffer containing 1X Dulbecco's phosphate buffered saline (DPBS), 1X kinase buffer (Cell Signaling), complete mini protease inhibitor cocktail (Roche), phosphatase inhibitor cocktails I and II (Calbiochem), and 50 µg/mL digitonin (Sigma). Cells were then pelleted at 4°C (200 xG) for 5 min and resuspended in the same buffer with the following exceptions: 100 µM N-6-phenethyl ATPγS and 1 mM GTP were included, and digitonin was omitted. The kinase reaction proceeded at 30 °C for 30 minutes with gentle rocking. Cells were then pelleted as above and lysed on ice for 15 min in 0.5 mL RIPA buffer containing 25 µM EDTA. Lysates were cleared by centrifugation at 14,000 g, 10 minutes 4°C. Proteins were alkylated with PNBM (8 mM PNBM, 2.5 % DMSO) for two hours at RT and frozen at -75°C. Ten µL of each sample was analyzed by western blot and the remainder was immunoprecipitated with α-hapten-IgG RmAb 51-8.

Immunoprecipitation with α-hapten-IgG (51-8 RmAb)

Samples labeled as above were thawed on ice and PNBM was removed as below. Note: PNBM is a potent inhibitor of immunoprecipitation and its' removal is absolutely necessary. Size exclusion chromatography has been the most effective method we have found to date. PD-10 columns (Amersham Biosciences) were equilibrated with RIPA containing protease inhibitors (Complete Protease Inbibitor Cocktail, Roche), 0.5 mL of alkylated proteins were applied to the column and eluted with RIPA containing protease inhibitors. Fractions (0.5 mL) were collected and the protein containing fractions (7-9), as determined by Bradford Assay (Promega), were pooled and pre-cleared with 100 µL of 50% rProtG agarose (Invitrogen) for 5 hours at 4 °C. Meanwhile 20 µg of 51-8 RmAb was bound to 100 µL of 50% rProtG agarose in 1.0 mL RIPA containing 0.5 mg/mL BSA for 5 hours at 4 °C. The beads were then removed from the lysates and the antibody coupled beads (washed 1X with 1.0 mL RIPA) were added to the precleared lysates (10 µg of antibody and 50 µL of 50 % beads per immunoprecipitation reaction).

Immunoprecipitation proceeded overnight at 4°C. The samples were then washed 4X with 1.0 mL RIPA and eluted by boiling in 30 µL SDS-PAGE sample buffer. Five µL of the immunoprecipitates were analyzed by western blot and the remaining 25 µL was analyzed by silver staining. The region of the gel from 200-60 KDa was cut into 1 mm slices (for both WT and AS immunoprecipitation reactions).

In Gel Digestion and Mass Spectrometry

All gel handling steps were performed in a laminar flow hood to minimize contamination. Methanol washed siliconized tubes (PGC Scientifics) and HPLC quality solvents were used. Gel slices were chopped into 1 mm cubes and washed twice with 200 µL aliquots of 25 mM ammonium bicarbonate, 50% acetonitrile. After desiccation (using vacuum centrifugation) the gel pieces were rehydrated with 20 µL of 5 ng/µL TPCK modified sequencing grade trypsin (Promega) and incubated at 37°C overnight. Peptides were extracted by addition of 30 µL of 5% formic acid in 50% acetonitrile. The samples were concentrated using vacuum centrifugation and analyzed by nano-liquid chromatography-electrospray ionization-quadrupole time of flight tandem mass spectrometry analysis as follows. Peptides were separated using a 75 µm × 15 cm reverse phase C-18 column (LC Packings) at a flow rate of 350 nL/min, running a 3–32% acetonitrile gradient in 0.1% formic acid on an Eksigent nano 1D HPLC (Eksigent) equipped with an auto sampler (Agilent Technologies). The liquid chromatography (LC) eluent was coupled to a microionspray source attached to a QSTAR XL mass spectrometer (MDS Sciex). Peptides were analyzed in positive ion mode. MS spectra were acquired for 1 s. For each MS spectrum, the most intense multiple charged peak was selected for generation of subsequent collision-induced dissociation (CID) mass spectra for 5 s. A dynamic exclusion window was applied which prevented the same m/z from being selected for 1 min after its acquisition. For sequencing of unmodified or PNBM modified cysteine containing peptides the CID collision energy was automatically adjusted based upon peptide charge and mass to charge (*m/z*) ratio. To attain quality CID spectra of thiophosphate ester containing peptides the CID collision energy was set to a value of 30 eV.

Equipment and Settings

Figure 1a and **1b** were created using Adobe Illustrator and ChemDraw Ultra.
Figure 1c was acquired on an Alpha Innotech (30 s exposure) and processed using Adobe Photoshop and Adobe Illustrator.
Figure 2a was created in Microsoft Word and Adobe Illustrator.
Figure 2b and **2c** data was acquired as described under the '**In Gel Digestion and Mass Spectrometry'** in this **Supplementary Methods** file. Spectra were exported from Analyst Software (MDS Sciex) into Adobe Illustrator.
Figure 3 western blot analysis was recorded on film and then scanned into Adobe Photoshop, labels were added in Adobe Illustrator.
Figure 4 western blot analysis was recorded on film and then scanned into Adobe Photoshop, labels were added in Adobe Illustrator. Chemical structures were exported from ChemDraw Ultra into Adobe Illustrator.
Figure 5a-c western blot analysis (film) and the silver stained gel were scanned into Adobe Photoshop, labels were added in Adobe Illustrator.

Figure 5d data was acquired as described under the '**In Gel Digestion and Mass Spectrometry'** in this **Supplementary Methods** file. Spectra were exported from Analyst Software (MDS Sciex) into Adobe Illustrator.

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