Template-Constrained Cyclic Sulfopeptide HIV-1 Entry Inhibitors

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Supporting Information

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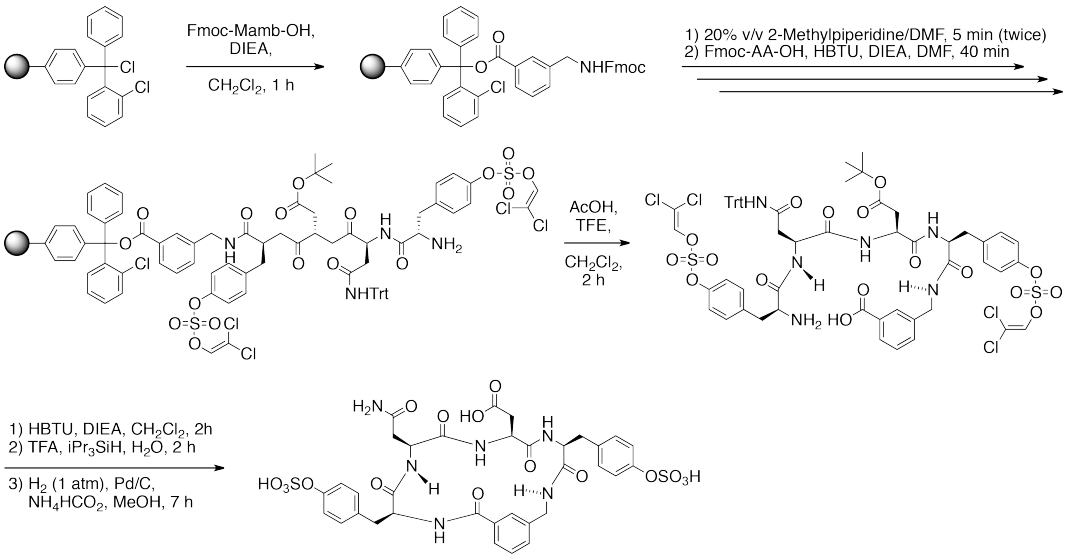
Scheme S1. Synthesis of peptide 3.



Scheme S2. Full library of peptides screened.

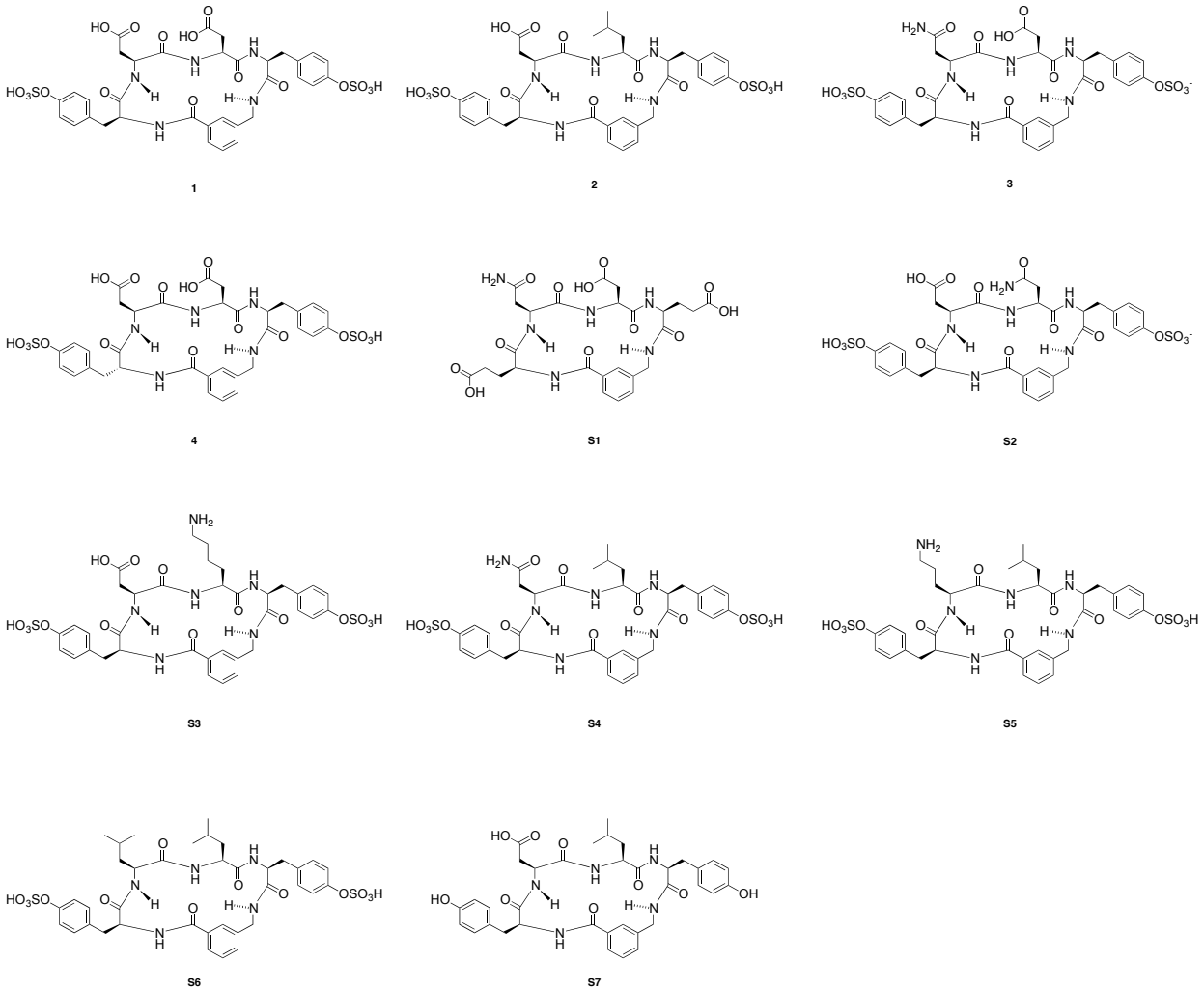
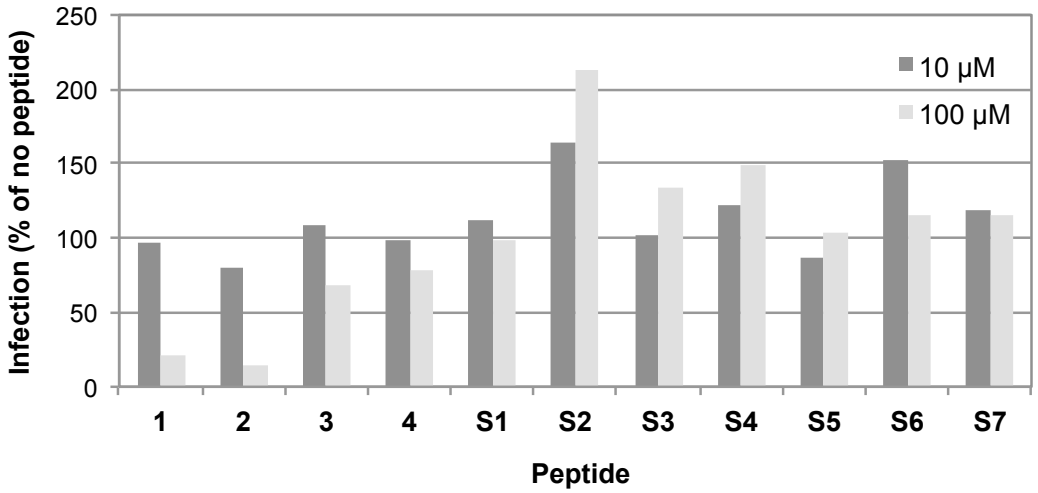


Table S1. Electrospray mass spectrometry data for the cyclic peptides.

Peptide	Sequence	ESI–MS (*m*/*z*)		
		ion	calcd.	found
1	cyclo(Tys-Asp-Asp-Tys-Mamb)	$[M + AcO^- - H]^{2-}$	453.6	453.8
2	cyclo(Tys-Asp-Leu-Tys-Mamb)	$[M + AcO^- - H]^{2-}$	452.6	452.2
3	cyclo(Tys-Asn-Asp-Tys-Mamb)	$[M + AcO^- - H]^{2-}$	452.6	452.2
4	cyclo(D-Tys-Asp-Leu-Asp-Mamb)	$[M + AcO^- - H]^{2-}$	453.6	453.8

Figure S1. HIV pseudotype infection is inhibited by cyclic peptides in a dose-dependent manner.

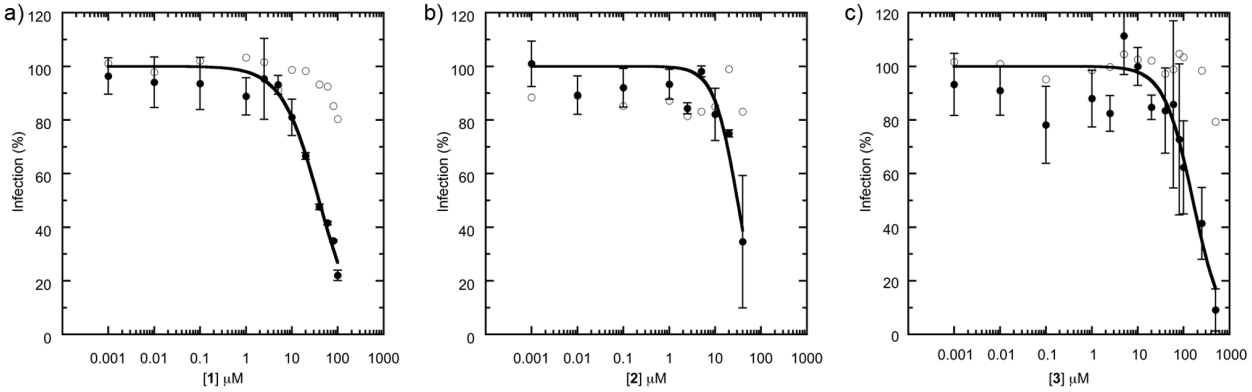
TA1 pseudotypes were pre-incubated with cyclic peptides at 10 µM or 100 µM before spinoculation onto NP2.CD4.CCR5 cells. Results are normalized to infection in the absence of cyclic peptide, and are the mean of three independent experiments, +/- SEM.



Further consideration of the data presented in Figure S1 and Scheme S2 suggest that the interaction targeted by the cyclic sulfopeptides is quite specific. Each of the active peptides 1–3 has an overall high content of acidic amino acids. However, the nature of the acidic side chains is important as shown by peptide S1 which has Glu residues in place of the *O*-sulfonated tyrosine residues of 3. The high degree of sequence specificity can be seen when the Asn and Asp residues of the active peptide 3 are swapped to yield the inactive peptide S2. Remarkably, an Asp residue at the $i + 2$ position of peptide 2 results in an inactive peptide yet increases the activity of peptides 1 and 3 compared to 2. Thus, when Asp is in the $i + 2$ position of the cyclic peptides, acidic (3) and nonpolar (5) residues appear equally well tolerated at the $i + 3$ position, but not neutral polar (S2) or basic/cationic (S3) residues. Alternatively, when Asp is in the $i + 3$ position of the cyclic peptides, neutral polar (3) and acidic/anionic (1) residues are preferred at the $i + 2$ position. Altering the conformation of peptide 1 from type I to type II' in peptide 4 by inverting the stereochemistry of the $i + 1$ position ablated activity against TA1 despite our expectation to

the contrary. Our initial design paradigm does not fully account for these differences in activity. The active peptides may bind to gp120 via alternative orientations not shown in Figure 1.

Figure S2. Dose-response profiles for peptides a) 1 (IC_{50} = 38 ± 3 μM), b), 2 (IC_{50} = 31 ± 5 μM), and c) 3 (IC_{50} = 159 ± 30 μM). Viral pseudotypes were pre-incubated for 30 min with the cyclic peptide before spinoculation onto NP2.CD4.CCR5 cells. Results are normalized to infection in the absence of cyclic peptide, and are the mean of two independent experiments; error bars designate ± the standard error of the mean. The solid line indicates the best fit to the data from which we determined the reported IC_{50} values. Data shown as open circles are for VSVG.



Experimental Section

Materials. Fmoc-Tyr(OSO_3DCV)-OH was prepared according to a literature procedure.[1] Fmoc-Asp(OtBu)-OH, Fmoc-Leu-OH, and 2-chlorotrityl resin (1.01 mmol/g loading) were used as received from EMD/Novabiochem. Fmoc-Mamb-OH was used as received from AnaSpec. Anhydrous dichloromethane (dry CH_2Cl_2), anhydrous dimethylformamide (dry DMF), 2-methylpiperidine, 2,2,2-trifluoroethanol (TFE), NH_4HCO_2, 10 wt% Pd/C, and acetic anhydride (Ac_2O) were used as received from Aldrich. Diisopropylethylamine (DIEA) was used as received from Chem-Impex International. *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HBTU) was used as received from GL Biosciences. Methanol (MeOH), isopropanol (^{i}PrOH), dimethylformamide (DMF), dichloromethane (DCM), acetonitrile (MeCN), ethyl acetate (EtOAc), chloroform ($CHCl_3$), NaCl, ammonium acetate (NH_4OAc), Na_2SO_4 and glacial acetic acid (AcOH) were used as received from Fisher. Trifluoroacetic acid (TFA) was used as received from Halocarbon.

The NP2.CD4.CCR5 cell line was cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 60μg/ml penicillin and 100μg/ml streptomycin (complete DMEM), plus 0.3mg/ml G418 and 1μg/ml puromycin. The NL4-3-based luciferase vector, pNL-luc (E-, Vpr+), and R3A, TA1, YU2, BaL, JRFL, pre-5.2, R3, and VSVG envelope expression plasmids have been previously described.[2]

Techniques. Peptides were purified on a Varian ProStar 210 binary HPLC equipped with a variable wavelength UV detector monitoring at 254 nm and Hamilton PRP-1 column (7 μm particle size; 250 mm x 10 mm). Fractions were collected manually and lyophilized. Analytical

HPLC was performed on an Agilent 1100 Series LC system equipped with a Hamilton PRP-1 column (7 μm particle size; 250 mm x 4.6 mm) and monitored at 210 nm. For all HPLC, the eluents were Solvent A = 20 mM NH_4OAc (aq) and Solvent B = 85:15 MeCN/20 mM NH_4OAc (aq). Electrospray ionization mass spectrometry (ESI-MS) was performed by direct injection of samples on an Applied Biosystems 3200 Q Trap LC/MS/MS system.

Synthesis of H-Tyr(OSO_3DCV)-Asp(O^{t}Bu)-Asp(O^{t}Bu)-Tyr(OSO_3DCV)-Mamb-OH. Reactions on solid-phase were performed in a 10-mL peptide synthesis vessel with gentle agitation by a Mistral Multi-Mixer under ambient temperature and atmosphere. Liquids were removed from the peptide synthesis vessel by vacuum filtration, except where noted otherwise. A stock solution of 2.230 g HBTU in 16 mL dry DMF was used for all of the peptide coupling reactions. To swell the resin, 117 mg 2-chlorotrityl resin and 2 mL dry CH_2Cl_2 were mixed for 1 h. To the resin, 1.2 mL of a solution containing 262.4 mg Fmoc-Mamb-OH and 344 mL DIEA in 5.0 mL dry CH_2Cl_2 was added.[3] After agitating the mixture for 1.5 h, the liquids were removed and the resin was washed sequentially with 2 mL DMF twice, 2 mL CH_2Cl_2 twice and 2 mL DMF. The resin was suspended in 1.5 mL 20% v/v 2-methylpiperidine/DMF and agitated for 5 min. The liquids were removed and the resin was washed sequentially with 2 mL DMF twice, 2 mL CH_2Cl_2 twice and 2 mL DMF. A solution of 205.4 mg Fmoc-Tyr(OSO_3DCV)-OH and 126 mL DIEA in 0.9 mL HBTU/DMF stock solution was allowed to stand for 30-60 s prior to addition to the resin. After agitating the mixture for 40 min, the liquids were removed and the resin was washed sequentially with 2 mL DMF twice, 2 mL CH_2Cl_2 twice and 2 mL DMF. The resin was suspended in 1.5 mL 20% v/v 2-methylpiperidine/DMF and agitated for 5 min. The liquids were removed and the resin was washed sequentially with 2 mL DMF twice, 2 mL CH_2Cl_2 twice and 2 mL DMF. A solution of 155.9 mg Fmoc-Asp(O^{t}Bu)-OH and 126 mL DIEA in 0.9 mL HBTU/DMF stock solution was allowed to stand for 30-60 s prior to addition to the resin. After agitating the mixture for 40 min, the liquids were removed and the resin was washed sequentially with 2 mL DMF twice, 2 mL CH_2Cl_2 twice and 2 mL DMF. The resin and 1.5 mL Ac_2O/DIEA/DMF solution (0.5:0.5:9 v/v/v) were agitated for 5 min. The liquids were removed and the resin was washed sequentially with 2 mL DMF twice, 2 mL CH_2Cl_2 twice and 2 mL DMF. The resin was suspended in 1.5 mL 20% v/v 2-methylpiperidine/DMF and agitated for 5 min. The liquids were removed and the resin was washed sequentially with 2 mL DMF twice, 2 mL CH_2Cl_2 twice and 2 mL DMF. A solution containing 532.8 mg Fmoc-Asp(O^{t}Bu)-OH and 443 mL DIEA in 3.1 mL HBTU/DMF stock solution was allowed to stand for 30-60 s. Of this stock solution, 1.5 mL was added to the reaction vessel.[4] After agitating the mixture for 40 min, the liquids were removed and the resin was washed sequentially with 2 mL DMF twice, 2 mL CH_2Cl_2 twice and 2 mL DMF. The resin and 1.5 mL Ac_2O/DIEA/DMF solution (0.5:0.5:9 v/v/v) were agitated for 5 min. The liquids were removed and the resin was washed sequentially with 2 mL DMF twice, 2 mL CH_2Cl_2 twice and 2 mL DMF. The resin was suspended in 1.5 mL 20% v/v 2-methylpiperidine/DMF and agitated for 5 min. The liquids were removed and the resin was washed sequentially with 2 mL DMF twice, 2 mL CH_2Cl_2 twice and 2 mL DMF. A solution of 249.0 mg Fmoc-Tyr(OSO_3DCV)-OH and 126 mL DIEA in 0.9 mL HBTU/DMF stock solution was allowed to stand for 30-60 s prior to addition to the resin. After agitating the mixture for 40 min, the liquids were removed and the resin was washed sequentially with 2 mL DMF twice, 2 mL CH_2Cl_2 twice and 2 mL DMF. The resin was suspended in 1.5 mL 20% v/v 2-methylpiperidine/DMF and agitated for 5 min. The liquids were removed and the resin was washed sequentially with 2 mL DMF twice, 2 mL CH_2Cl_2 twice, 2 mL MeOH twice, and 2 mL

^{i}PrOH twice. To the resin, 2 mL AcOH/TFE/DCM (2:2:6 v/v/v) was added. After agitating the mixture for 40 min, the liquids were removed by positive pressure displacement using N_2. The resin was washed with 2 mL AcOH/TFE/DCM (2:2:6 v/v/v) and the liquids were removed by positive pressure displacement using N_2. Volatiles from the combined filtrate and washing were removed under reduced pressure. The residue was partitioned between 3 mL $CHCl_3$ and 3 mL H_2O. The organic layer was separated, but an emulsion persisted in the aqueous phase. Saturated NaCl (aq) solution and EtOAc were added to the aqueous emulsion until the two phases separated. The organic layer was separated and combined with the $CHCl_3$ solution. The combined organic layers were dried over Na_2SO_4. The liquids were drawn from the solids by pipet and evaporated to dryness under reduced pressure. The residue was lyophilized from MeCN/H_2O (~4:1 v/v) to provide 139.8 mg crude linear peptide. ESI-MS (*m*/*z*): +1171.2, +1169.2, +1173.2, +1172.2, +1170.2, +1174.2, +1175.2.

Synthesis of cyclo(Tyr(OSO$_3$DCV)-Asp-Asp-Tyr(OSO$_3$DCV)-Mamb). In a 20-mL vial equipped with a magnetic stir bar, 139.8 mg crude H-Tyr(OSO$_3$DCV)-Asp(O^{t}Bu)-Asp(O^{t}Bu)-Tyr(OSO$_3$DCV)-Mamb-OH and 47.1 mg HBTU were combined with 62.5 mL DIEA and 6.0 mL dry CH_2Cl_2. The reaction mixture was stirred for 2 h. Volatiles were removed from the reaction mixture under reduced pressure. The residue was dissolved in 2 mL TFA and stirred for 2 h. Volatiles were removed from the reaction mixture under reduced pressure. The residue was lyophilized from MeCN/H_2O (~4:1 v/v). ESI-MS (*m*/*z*): +1040.9, +1038.9, +1042.9, +1041.8, +1039.9, +1043.9.

Synthesis of cyclo(Tyr(OSO$_3$H)-Asp-Asp-Tyr(OSO$_3$H)-Mamb). In a 20-mL vial equipped with a magnetic stir bar, crude cyclo(Tyr(OSO$_3$DCV)-Asp-Asp-Tyr(OSO$_3$DCV)-Mamb) and 137.6 mg NH_4HCO_2 were dissolved in 12.4 mL MeOH. To the solution, 70.8 mg 10 wt% Pd/C was added as a solid. The vessel was capped with a rubber septum and a H_2 atmosphere maintained using a balloon. After stirring for 7 h, the reaction mixture was passed through a 0.45 mm syringe filter to remove any solids. Volatiles were removed from the reaction mixture under reduced pressure. The residue was lyophilized from MeCN/H_2O (~2:1 v/v) to yield 199.9 mg crude product. Cyclo(Tyr(OSO$_3$H)-Asp-Asp-Tyr(OSO$_3$H)-Mamb) was isolated by gradient elution from a PRP-1 reversed phase HPLC column with H_2O/MeCN mobile phase containing 20 mM NH_4OAc as buffer. ESI-MS (*m*/*z*): -453.8, -454.2, -454.7, -455.2, -455.7.

Viral Pseudotype Production and Infection Assays. Luciferase reporter pseudotype viruses were produced in 293T cells by cotransfection with pNL-luc (E-, Vpr+) and envelope expression plasmids.[2a,5] Viral titer was normalized by p24 value. For cell-based inhibition assays, 5 ng of virus (in a volume of 25 µL) was incubated with serial dilutions of the cyclic peptide (4x concentration in a volume of 25 µL) for 30-60 min at 37 °C. For inhibition assays in the presence of APL, MVC or TAK-779, NP2.CD4.CCR5 cells were pre-incubated with what concentration CCR5 antagonist for 30 min at 37 ºC before the addition of virus that had been separately incubated with cyclic peptide under the same conditions. Virus was spinoculated onto cells[6] and subsequently incubated at 37 °C for 3 h, at which time medium was replaced with fresh DMEMc with no drug or antibody. Cells were assayed for luciferase expression 3 d post-infection.[7] Monoclonal antibody 17b was obtained from the AIDS Research and Reference Reagent Program; CTC5 was obtained from R&D Systems (Minneapolis, MN), and 412d was the kind gift of Dr. James Robinson (Emory University).

ELISA Inhibition Assays. For gp120 enzyme-linked immunosorbent assay (ELISA), HIV-1 gp120 glycoproteins from strains R3A, TA1 and YU2 were expressed and purified by *Galanthus nivalis* lectin-agarose (Vector Laboratories, Burlingame, CA) affinity chromatography from 293T cells using recombinant vaccinia virus vectors.[8] gp120 was diluted to 1mg/ml in capture buffer (50 mM carbonate buffer, pH 9.6) and used to coat Immunolon HB plates O/N at 4C in a final volume of 100ul/well. After washing in PBS/0.05% Tween, the wells were blocked for 1.5 h with blocking buffer (PBS, 2% bovine serum albumin [BSA], 0.05% TWEEN 20), rinsed, and incubated for 1.5 h with 0.1ug soluble CD4 per well. After washing, cyclic peptide was added to wells at 40 or 400ng/ml for 1h. An equal volume of 17b or 412d antibody (at 0.01nM or 0.2nM respectively) was added directly to wells for 1 h at RT. The wells were washed with wash buffer (PBS, 0.05% TWEEN 20) and incubated 1 h with HRP-labeled secondary antibodies. After the wells were washed, they were incubated with 3,3',5,5'-tetramethylbenzidine for color development, which was stopped with 1 M ortho-phosphoric acid. Absorbance at an optical density at 450 nm (OD450) was read using an EMax microplate reader (Molecular Devices, Sunnyvale, CA).

References

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(4) The remaining solution was used for another reaction run in parallel.
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Cyclo(Tys-Asp-Asp-Tys-Mamb) (1)

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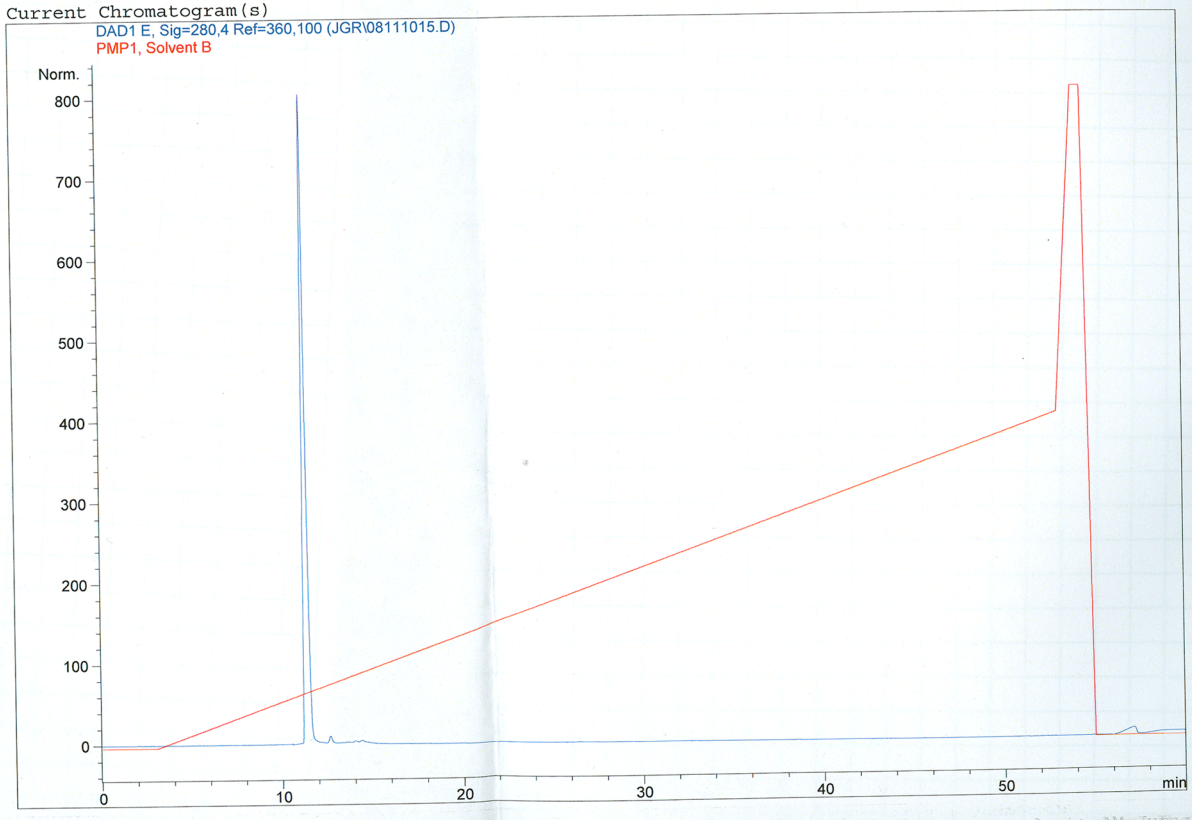

Cyclo(Tys-Asp-Leu-Tys-Mamb) (2)

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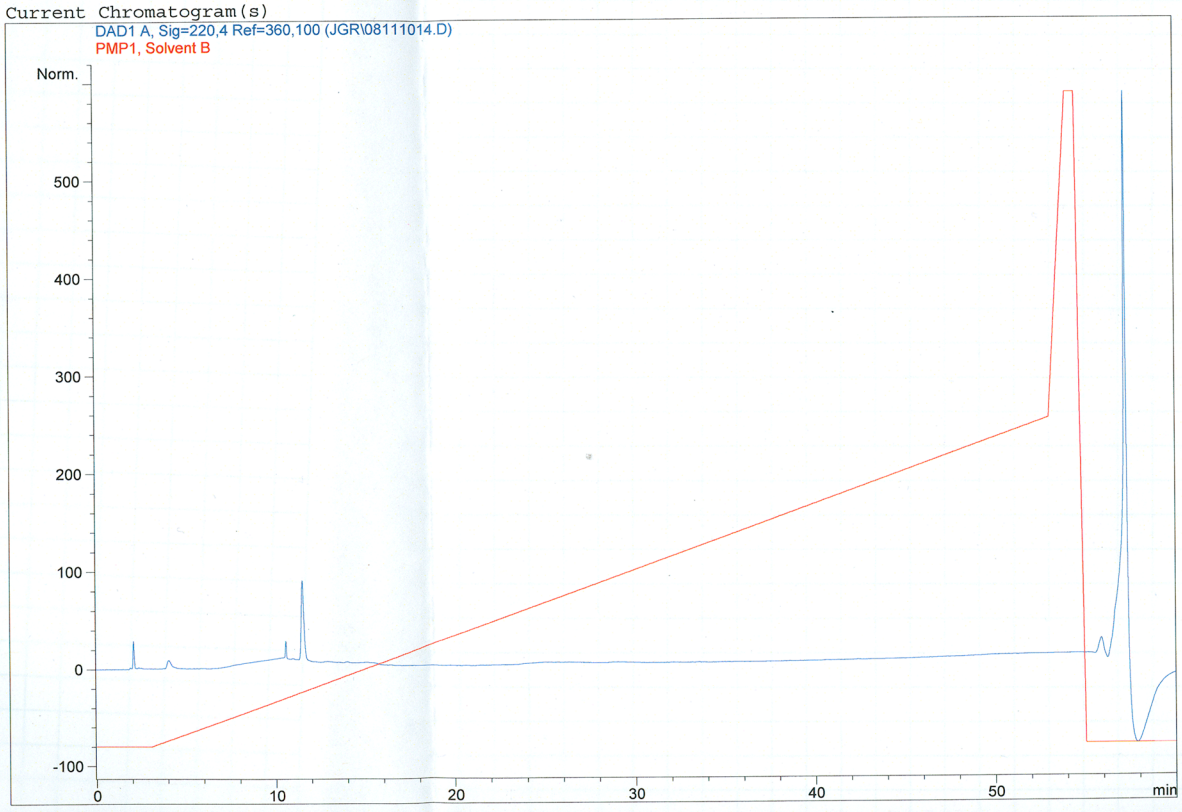


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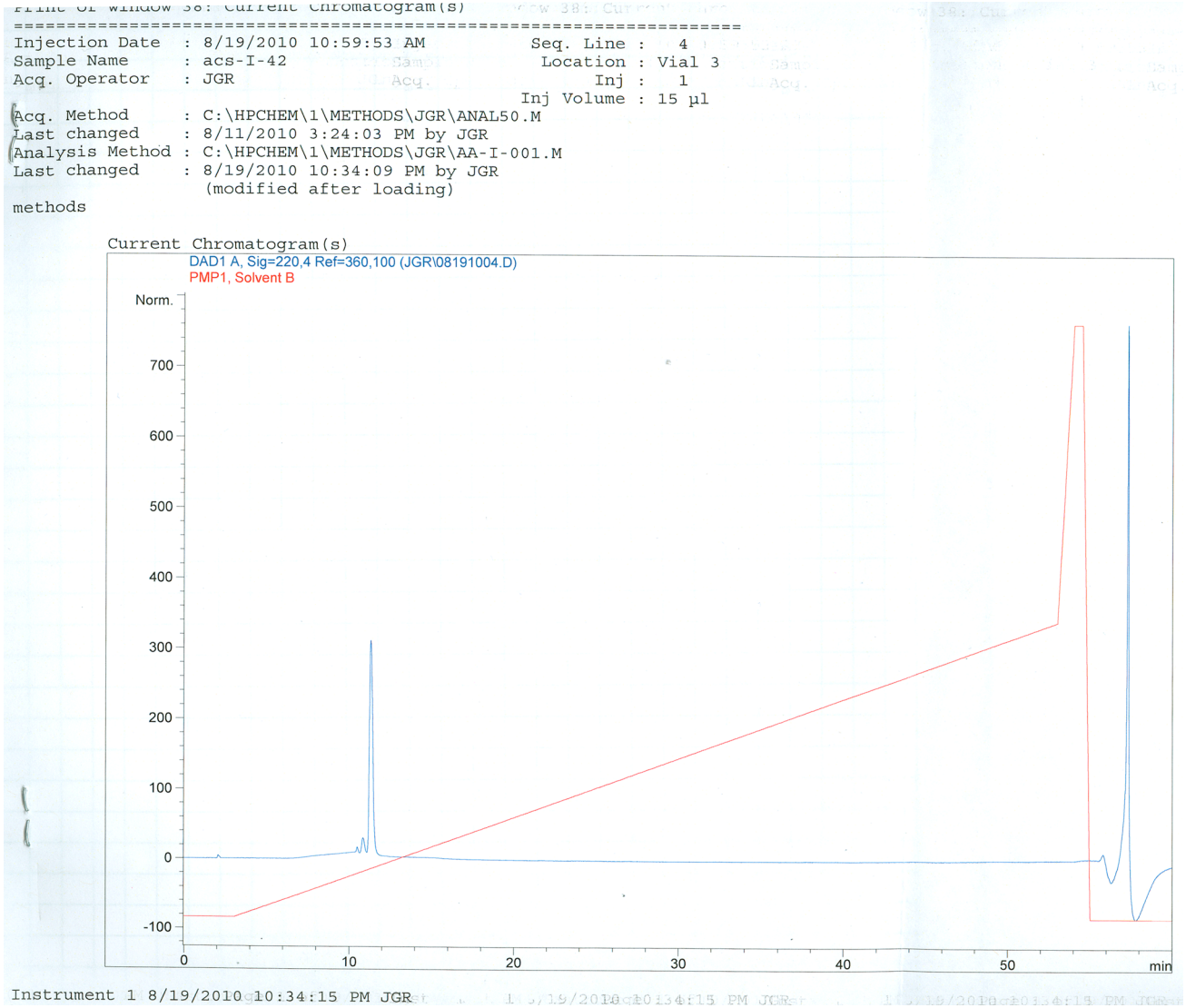

Cyclo(D-Tys-Asp-Leu-Tys-Mamb) (4)

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