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A Strategy for the Synthesis of Sulfated Peptides

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A. General Methods

All reactions were run in flame-dried glassware under an inert atmosphere of nitrogen or argon and monitored by thin-layer chromatography (TLC). Reaction temperatures were monitored via external bath temperature. All materials obtained from commercial suppliers were used as received, unless otherwise noted. Anhydrous reaction solvents were distilled as follows: tetrahydrofuran and benzene from sodium/benzophenone ketyl; methylene chloride, triethylamine and pyridine from calcium hydride. DMF was rendered amine-free by treatment with Dowex 50WX8-200 cation exchange resin, H^+ form, 1g/L. *N*-Chlorosuccinimide was recrystallized from benzene and residual solvent was removed under high vacuum (< 0.1 torr). *N*-Bromosuccinimide was recrystallized from water and the crystalline material dried over phosphorus pentoxide, under high vacuum (< 0.1 torr). *N*-Iodosuccinimide was recrystallized from dioxane/carbon tetrachloride, dried under high vacuum and stored in the dark. Tetrabutyl ammonium azide was prepared by the method of Brändström *et al.* (A. Brändström; B. Lamm; I. Palmertz, *Acta Chem. Scand. B* **1974,** *28,* 699-701.) followed by azeotropic removal of residual moisture with pyridine and storage over phosphorus pentoxide.

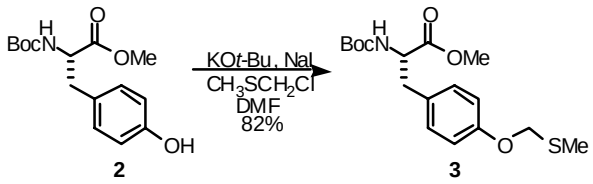
High pressure liquid chromatography (HPLC) was performed on a Spectra-Physics UV2000 instrument using ultraviolet absorption at 220 nm and/or 254 nm for analyte detection. Samples were eluted on reverse phase C18 columns from Alltech (Econosil L = 250mm, ID = 22 mm 10 μ particle size) or Vydac (L = 220mm, ID = 5 mm, 10 μ particle size). Infrared spectra were recorded on a Mattson Polaris FT-IR spectrometer and are reported in wavenumbers (cm^{-1}). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC-300 spectrometer or Varian Unity 500 spectrometer, and are referenced to internal standards ($CHCl_3$: ^{1}H: δ 7.24, ^{13}C: δ 77.0; CH_3OH: ^{1}H: δ 3.31, ^{13}C: 49.15). ^{1}H-^{1}H couplings are interpreted as first order. Peak multiplicity is reported as: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), broad (br), and apparent (ap).
Analytical thin layer chromatography (TLC) was carried out on E. Merck (Darmstadt) TLC plates pre-coated with silica gel 60 F_{254} (250 μ layer thickness). Analyte visualization was accomplished using a UV lamp, and charring with at least one of the following solutions: a *p*-anisaldehyde stain (18 mL *p*-anisaldehyde, 7.5 mL glacial acetic acid, 25 mL conc. H_2SO_4, 675 mL absolute EtOH), ninhydrin solution (200 mg ninhydrin, 95 mL *n*-butanol, 5 mL 10% AcOH), potassium permanganate solution (3g $KmnO_4$, 20g K_2CO_3, 5 mL 5% aqueous NaOH, 300 mL H_2O). Flash chromatography was performed on Scientific Adsorbents Incorporated silica gel (32-63 μM, 60 Å pore size) using distilled reagent grade hexanes and ACS grade ethyl acetate, methanol and chloroform. The term, "concentrated *in vacuo*" refers to the removal of solvents and other volatile materials using a rotary evaporator at water aspirator pressure (< 20 torr),

followed by residual solvent removal at high vacuum (< 0.1 torr). The term, “high vacuum,” refers to vacuum achieved by a mechanical belt-drive oil pump.

All yields are reported as the average of three independent trials with the exception of the transformation of compound **6** to **1** (two trials) and peptide syntheses (one trial each). This averaging accounts for the discrepancy in yields reported in the below, representative procedures and in the text.

B. Detailed Synthetic Procedures

Preparation of BocTyr(MTM)OMe:

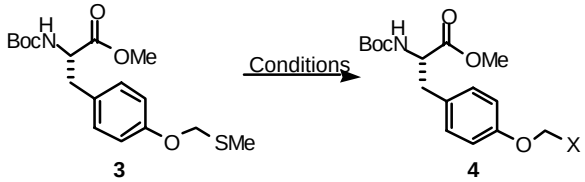


2 **3**

To a solution of BocTyrOMe (4.05 g, 13.7 mmol) and sodium iodide (206 mg, 1.37 mmol) in DMF (30 mL) chilled via an external ice bath was added a THF (15 mL) solution of potassium *t*-butoxide (1.73 g, 15.1 mmol). To the resultant phenoxide (clear green solution), chloromethyl methyl sulfide (1.33 mL, 15.1 mmol) was added slowly. The reaction was allowed to warm gradually to room temperature. After 4.5 h, the reaction was cloudy and TLC analysis (4:1 hexanes/EtOAc) indicated complete consumption of starting material. The reaction mixture was diluted with EtOAc (60 mL) and washed with H_2O (1 x 45 mL), aqueous citric acid solution (5%, 1 x 45 mL) and brine (1 x 45 mL). The aqueous washing were pooled and washed with EtOAc (2 x 60 mL). The

combined organic extracts were pooled and dried over $MgSO_4$. The $MgSO_4$ was removed by filtration and volatiles removed *in vacuo.* The residue was purified by flash column chromatography (silica, gradient elution 4:1 hexanes/EtOAc to 2:1 hexanes/EtOAc) to afford a clear syrup on concentration (3.99 g, 81%). $\mathbf{R_f}$ = .44 (4:1, hexanes/EtOAc); **IR** (Neat): 3368, 1744, 1714, 1510 cm^{-1}; **^{1}H NMR** (300 MHz, $CDCl_3$): δ 7.02-6.81(AA^1BB^1, J = 9.5, 4H), 5.06 (s, 2H), 4.99 (d, J = 5.4, 1H), 4.49 (q, J = 5.8, 1H), 3.66 (s, 3H), 2.18 (s, 3H), 1.36 (s, 9H); **^{13}C NMR** (75 MHz, $CDCl_3$): δ 172.2, 156.0, 130.3, 129.1, 116.0, 72.3, 54.4, 52.2, 37.5, 28.3, 14.4; **LRMS** (ESI): *m/z* 378 [$M+Na^+$ calc'd for $C_{17}H_{25}NO_5S$ 378.1]

The selective activation of *O,S*-acetal **3** required considerable optimization. The precedented conditions for



3 **4**

this transformation were unsuitable for our target.

Scheme 2. Activation of *O,S*-Acetal for azidomethylene installation.

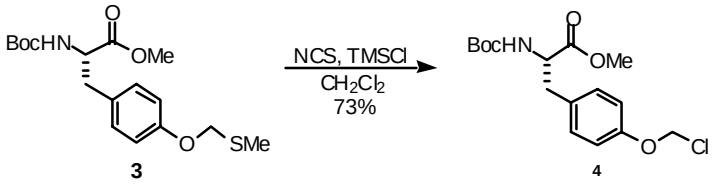
Table 1. Activation of *O,S*-acetal under various conditions

entry	activator	solvent	additive	X	yield (%)
1	NCS	CH_3CN	--	Cl	30
2	NCS	CH_2Cl_2	--	Cl	44
3	NCS	CH_2Cl_2	NaBr	Br	48
4	NBS	CH_3CN	--	Br	22
5	NBS	CH_2Cl_2	--	Br	38
6	NBS	CH_2Cl_2	HF•pyr	--	no rxn
7	NBS	THF	HF•pyr	--	no rxn
8	NIS	CH_2Cl_2	--	--	decomp.
9	SO_2Cl_2	CH_2Cl_2	pyr.	--	decomp.
10	NCS	CH_2Cl_2	$TMSN_3$	--	decomp.

In most cases, the desired product was accompanied by a significant amount of the hydrolysis product **(2)**. Activation

with *N*-chlorosuccinimide (Table 1, entries 1, 2) yielded marginal consumption of starting material, and some hydrolysis accompanied the desired product. Activation with *N*-bromosuccinimide resulted in greater consumption of starting material. However, the bromomethylene ether was prone to degradation to compound **2** upon flash silica gel chromatography, reducing the yield and complicating the purification of this intermediate. Because the corresponding iodide, produced by the influence of *N*-iodosuccinimide was more susceptible to hydrolysis, we were unable to isolate this intermediate. We attempted to drive the activation reaction to completion by the addition of nucleophilic trapping agents such as sodium bromide (Table 1, entry 3). In this case greater conversion was observed. Consumption of the starting material, however, was not complete and we were unable to isolate the labile bromide compound in satisfactory purity or yield. We turned to nucleophilic trapping to produce more stable derivatives such as the fluoromethylene derivative or the azidomethylene product. Attempts to generate the fluoride (Table 1, entries 6, 7) were unsuccessful. (C. Unverzagt, H. Kunz, *J. Prakt. Chem./Chem.-Ztg.* **1992,** *334,* 570-578.) Attempts to install the azidomethylene ether in a “one-pot” reaction by trapping of the chloride intermediate using azide sources (e.g., Table 1, entry 10) yielded only facile cleavage of the *O,S*-acetal to the free phenol derivative. This reaction was accompanied by the evolution of a gas, and we attribute this result to reduction of the nascent azidomethylene group by methane thiolate present in the reaction mixture. The reduced intermediate would, of course, expel the phenolate by the designed deprotection pathway. Lewis acid activation of NCS results in complete consumption of the *O,S*-acetal and produces a product of suitable stability for isolation and characterization.

Preparation of BocTyr(CH_2)Cl:

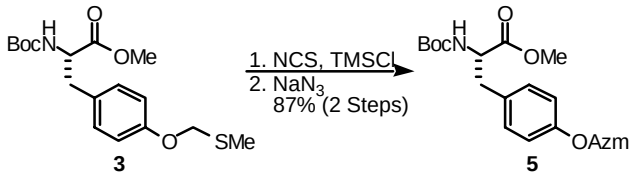


3 **4**

The *O,S*-acetal **3** (287 mg, 0.81 mmol) was dissolved in dichloromethane (3.0 mL), solid NCS (119 mg, 0.89 mmol) was added. The reaction was allowed to stir for 2.5 h, then trimethylsilyl chloride (0.11 mL, 0.89 mmol) was added. After an additional 2 hours, the crude reaction mixture was loaded directly on to a flash silica gel column. Elution 5:1 hexanes/EtOAc and drying *in vacuo* provided the title compound as clear oil in pure form for characterization. The product was purified by crystallization to yield white plates (202 mg, 73%). The mass balance was recovered as BocTyrOMe **2** after elution with EtOAc.

R_f = .40 (4:1, hexanes/EtOAc); **^{1}H NMR** (300 MHz, $CDCl_3$): δ 7.10-6.98 (AA^1BB^1, J = 8.8 Hz, 4 H), 5.84 (s, 2H), 4.98 (d, J = 7.7 Hz, 1H), 4.53 (q, J = 7.8 Hz, 1H), 3.68 (s, 3H), 3.05-2.95 (m, 2H), 1.38 (s, 9H); **^{13}C NMR** (75 MHz, $CDCl_3$): δ 172.1, 154.6, 130.4, 116.1, 54.3, 52.1, 37.4, 28.1; **LRMS** (MALDI, α-cyano-4-hydroxycinnamic acid matrix, positive ion mode): *m/z* 368.2, 366.1, 318.2 [calc'd $M+Na^+$ for $C_{16}H_{22}ClNO_5$ 366.11; $M+Na^+$ for $C_{15}H_{21}NO_5$ 318.13]

Preparation of BocTyr(Azm)OMe:

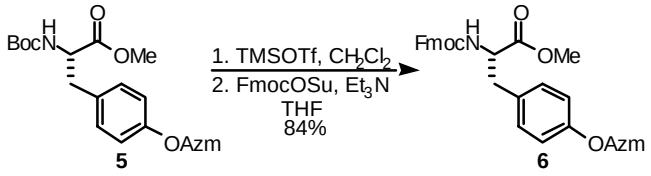


3 **5**

The *O,S*-acetal **3** (4.28 g) was dissolved in CH_2Cl_2 (35 mL) and solid *N*-chlorosuccinimide (1.76 g) was added. The reaction was allowed to stir at room temperature for 4 h. Trimethylsilyl chloride (1.68 mL) was then added slowly. After an additional 6 h, the reaction was diluted with $CHCl_3$ (30 mL) and saturated $NaHCO_3$ solution (60 mL) was added. The organic layer was separated and the aqueous fraction was extracted with $CHCl_3$ (2 x 60 mL). The combined organic extracts were concentrated via rotary evaporation and the residue dissolved in DMF (15 mL). Sodium azide (1.2 g, 18.5 mmol) was dissolved in H_2O (15 mL) and added to the solution of crude tyrosyl chloride. This reaction was allowed to stir for 5 h at room temperature. The reaction was then diluted with saturated $NaHCO_3$ solution (15 mL) and washed with EtOAc (3 x 30 mL). The combined organic extracts were dried ($MgSO_4$) concentrated *in vacuo,* and the residue subjected to flash column chromatography (silica, gradient elution 4:1 hexanes/EtOAc to 2:1 hexanes/EtOAc). After removal of volatiles, azidomethylene **2.67** was isolated as a clear oil (3.64 g, 87%). $\mathbf{R_f}$ = .41 (4:1 hexanes/EtOAc); **IR** (Neat): 2132, 2110 cm^{-1}; ^{1}**H NMR** (300 MHz, $CDCl_3$): δ 7.08-6.90 (AA^1BB^1, J = 8.5 Hz, 4 H), 5.13 (s, 2H), 4.95 (d, J = 6.7 Hz, 1H), 4.55 (d, J =6.7 Hz 1H), 3.71 (s, 3H), 3.0 (m, 2H), 1.41 (s, 9H); ^{13}**C NMR** (75 MHz, $CDCl_3$): δ172.1, 155.4, 130.3,

115.8, 79.6, 54.3, 51.9, 37.2, 28.0; **LRMS** (FAB): *m/z* 373.1 [M+Na$^+$ calc'd for $C_{16}H_{22}N_4O_5$ 373.2]

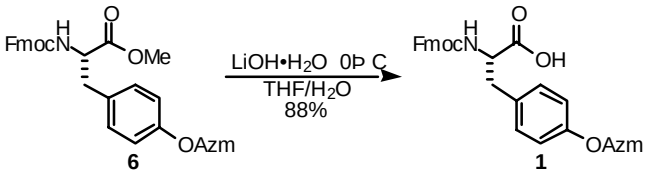
Preparation of FmocTyr(Azm)OMe:



Boc protected compound **5** (769 mg, 2.19 mmol) was dissolved in CH_2Cl_2 and cooled with an external ice bath. TMSOTf (0.79 mL, 4.4 mmol) was added dropwise. TLC analysis (4:1 Hexanes/EtOAc) indicated complete consumption of starting material after less than 10 min. 5% aqueous Na_2CO_3 was added (15 mL), followed by EtOAc (15 mL). The organic layer was separated and the aqueous phase extracted with EtOAc (3 x 15 mL). Volatiles were removed *in vacuo* and the residue was taken up in THF (7 mL). Triethylamine (0.91 mL, 6.6 mmol) was added, followed by solid FmocOSu (814 mg, 2.4 mmol). The reaction was left to stir for 3.5 h during which time a white precipitate formed. The reaction was diluted with $CHCl_3$ (15 mL) and washed with H_2O, 5% citric acid solution, and brine (15 mL each). The combined aqueous washings were extracted with $CHCl_3$ (4 x 15 mL). The pooled organic extracts were dried over $MgSO_4$ and filtered. The solvent was reduced to ca. 5 mL via rotary evaporation and loaded directly on a silica gel column. Flash chromatography (2:1 hexanes/EtOAc) yielded the title compound as a crystalline white solid (870 mg, 84%).

R_f = .36 (2:1 hexanes/EtOAc); **^{1}H NMR** (300 MHz, $CDCl_3$): δ 7.76-7.74 (d, J = 7.7 Hz, 2H, 7.57-7.53 (m, 2H), 7.41-7.36 (t, J = 7.1 Hz, 2H), 7.36-7.23 (t, J = 7.3 Hz, 2H), 7.02-6.88 (AA^1BB^1, J = 7.4 Hz, 4H), 5.28 (d, J = 8.1 Hz, 1H), 5.09 (s, 2H), 4.63 (dd, J = 7.0, J = 10.6, 1H), 4.44 (dd, J = 7.0, J = 10.6, 1H), 4.16 (t, J = 7.0, 1H), 3.71 (s, 3H), 3.13-2.99 (m, 2H); **^{13}C NMR** (75 MHz, $CDCl_3$): δ 171.7, 155.6, 143.7, 141.2, 130.4, 129.8, 127.6, 126.9, 126.8, 124.9, 119.9, 115.9, 79.6, 66.7, 54.7, 52.2, 47.0, 37.7; **LRMS** (ESI): *m/z* 495.1 [M+Na^+ calc'd for $C_{26}H_{24}N_4O_5$ 495.16]

Preparation of FmocTyr(Azm)OH:



Methyl ester **2.71** (339 mg, 0.72 mmol) was dissolved in THF 7 mL and cooled to 0 •C with an external ice bath. LiOH•H_2O (60 mg, 1.4 mmol) was dissolved in H_2O (7 mL) and added dropwise over 10 minutes to the chilled solution of methyl ester. After an additional 25 min the starting material was completely consumed as judged by analytical TLC (2:1 hexanes/EtOAc). The pH was then adjusted to ca. 3 by adding .3 M aqueous HCl. The cloudy aqueous solution was extracted with EtOAc (4 x 15 mL). The organic extracts were pooled, dried over $MgSO_4$, filtered and concentrated *in vacuo*. Flash silica gel chromatography (10% MeOH/$CHCl_3$) provides the title compound as a white solid after drying *in vacuo* (292 mg, 89%). It should be noted that saponification of carbamate protected α-amino acid esters can result in racemization. As determined by synthesis of diastereomeric

dipeptides, below, this step did not result in significant racemization. See, main text, footnote 16 for other examples using this saponification protocol.

^{1}H NMR (300 MHz, $CDCl_3$): δ 10.05 (br s, 1H), 7.78 (d, J = 7.7 Hz, 2H), 7.59-7.52 (m, 2H), 7.41 (t, J =7.4 Hz, 2H), 7.32 (t, J = 7.4 Hz, 2H), 7.09-6.90 (AA^1BB^1, J = 6.9 Hz, 4H), 6.20 (m, minor rotamer NH), 5.43 (d, J = 8.1 Hz, major rotamer NH), 5.08 (s, 2H), 4.67 (q, J = 6.6, 1H), 4.52-4.46 (m, 1H), 4.40-4.34 (m, 1H), 4.23-4.17 (m, 1H), 3.23-3.05 (m, major rotamer βH), 2.98-2.80 (m, minor rotamer βH);**^{13}C NMR** (75 MHz, $CDCl_3$): δ 155.4, 147.8, 143.3, 140.9, 130.2, 127.4, 126.7, 126, 124.6, 79.3, 66.7, 46.7, 36.5; **LRMS** (ESI): *m/z* 457.1, 235.1 [MH calc'd for $C_{25}H_{22}O_5N_4$ 457.15; MH calc'd for $C_{25}H_{22}O_5N_4$ – $C_{15}H_{11}O_2$ 235.1].

Attachment of the *C*-terminal amino acid to 2-chlorotrityl chloride resin:

Amino acid is suspended in dichloromethane (DCM, 10 mL per gram of resin) and dimethylformamide (DMF) is added dropwise until the amino acid dissolves. 1.1 equivalents of diisopropylethylamine should be used relative to the total mmoles amino acid plus mmoles chloride. The amino acid is added to the resin along with 1/3 the total amount of diisopropylethylamine. After stirring with a small stir bar for five minutes the rest of the base is added, and the mixture is stirred for 1 hour. After 1 hour, 1 mL of methanol is added to cap the resin. The resin slurry is then transferred to a fritted funnel and rinsed with DCM (3X), DMF (2X), iPrOH (2X), DMF (2X), iPrOH (2X), MeOH (2X), and Et_2O (2X). Solvent volume for all washes is 8 mL per gram of resin.

The stability of the carboxylate–chlorotrityl bond is enhanced by deblocking of the α-amino group. Thus, the Fmoc group is cleaved by rinsing of the resin with 10% piperidine/CH_2Cl_2 (2x), followed by 20% piperidine/DMF for 20 minutes. The resin is agitated via sparging with nitrogen gas during this reaction. At the conclusion of the Fmoc cleavage the resin is rinsed, DCM (3X), DMF (2X), iPrOH (2X), DMF (2X), iPrOH (2X), MeOH (2X), and Et_2O (2X). Solvent volume for all washes is 8 mL per gram of resin. The resin is then dried under high vacuum and stored at sub-zero temperatures. In general, superior loadings are achieved using this protocol relative to commercially available, pre-loaded resins.

Peptide Synthesis: Synthesis using FmocTyr(Azm)OH for the introduction of sulfotyrosine residues was carried out on 25 μM scale using an automated synthesizer (Applied Biosystems Model 432A “Synergy”). Standard techniques were used. (G.B. Fields, R.L. Noble, *Int. J. Pept. Prot. Res.* **1990,** *35,* 161-214.) The first amino acid was attached as above or the pre-loaded resins were purchased from Advanced Chemtech (Louisville, Kentucky). Carboxylic and alcoholic side chains were protected with benzyl groups.

Each synthesis cycle is initiated with the cleavage of the Fmoc group from the α-amino group, using 20% piperidine in DMF. Three equivalents (75 μmol)of the amino acid to be coupled is dissolved in DMF and added to the resin cartridge with HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3 tetramethyluronium hexafluorophosphate) and HOBt (*N*-hydroxybenzotriazole). The reaction cartridge is subjected to continuous flow conditions during each reaction. Following the coupling of the final amino acid, the

peptide–resin cartridge is removed from the synthesizer. All subsequent manipulations of the peptide–resin are performed manually. Subsequent reaction mixtures are agitated by the “double syringe” method. Briefly, a luer lock syringe is attached to each end of the peptide synthesis cartridge and the syringes are moved reciprocally and in tandem to agitate the reaction.

Fmoc-cleavage: The resin-bound peptide is flushed with 5% piperidine/CH_2Cl_2 followed by treatment with 20% piperidine/DMF for 20 min. The piperidine solution is removed, and the resin rinsed [DCM (3X), DMF (2X), iPrOH (2X), DMF (2X)].

Acetylation: The terminal amino group is acetylated prior to further synthetic manipulations. The peptide synthesis cartridge (PSC) is flushed with inert gas and rinsed with DMF (1mL), 2:1 pyridine/Ac_2O (1 mL) is then added and the acetylation reaction agitated via the double syringe method. After three hours the resin is rinsed with 2:1 pyridine/Ac_2O (1 mL), and alternately with DMF, MeOH, CH_2Cl_2, Et_2O (3 x 1 mL each). Amine capping is confirmed by the Kaiser test.

General protocol for azidomethylene cleavage of resin-bound peptides: To a conical flask charged with anhydrous $SnCl_2$ (57 mg 0.3 mmol) is added THF (2 mL), PhSH (104µL, 1.2 mmol) and Et_3N (170µL, ca.1.2 mmol). This mixture is stirred briefly under Ar. The dry resin (.025 mmol peptide) is flushed with THF (3 x 0.5 mL) and 1 mL of the reducing cocktail is added to the PSC. The reaction is agitated via the double syringe method. After ca. 5 minutes the reducing mixture is removed from the PSC and the resin rinsed with THF. The remainder of the reducing cocktail is added and the resin is agitated for ca. 5 minutes. The reducing

cocktail is then removed and the resin washed alternately with moist THF:Et_3N (9:1, 4 x 1 mL), CH_2Cl_2 (4 x 1 mL), MeOH (4 x 1 mL) and DMF (4 x 1 mL).

Sulfation: The PSC is flushed with 4:1 DMF/pyridine (1 mL). DMF•SO_3 (115 mg, 0.75 mmol) is dissolved in 4:1 DMF/pyridine and the resultant solution added to the PSC. The sulfation reaction is agitated by the double syringe method. After eight hours the resin is rinsed with 4:1 DMF/ pyridine (1 mL) and the sulfation repeated with fresh DMF•SO_3 for an additional eight hours. The resin is then washed alternately with 4:1 DMF/pyridine and methanol (3 x 1 mL), then DMF, CH_2Cl_2, Et_2O (3 x 1mL each).

AcYA: This dipeptide was synthesized manually using the double syringe method. Fmoc-L-Ala was attached to 2-Clt resin according to the standard procedure. Fmoc cleavage was followed by coupling of FmocTyr(Azm) (PyBop, DIPEA) according to the method of Castro et. al. Fmoc cleavage and acetylation was followed by cleavage from the support. HPLC purification [Vydac C18, gradient elution: A = .1% TFA/H_2O, B: CH_3CN/.1% TFA 0–20% B/30min t_2 = 26.76 Excision of the peaks and weighing on an analytical balance revealed relative peak size of 92.3:7.7 (84.6% ee). The synthesis of this dipeptide was repeated using Carpino's amide bond forming conditions (HATU, HOAt, collidine). All other steps were performed in the same way. This synthesis yielded material with a relative peak size of at least 95:5 (>90% de). The observed diastereomeric excess is consistent with that observed by Carpino and coworkers using the conditions described above. See, L.A. Carpino, D. Ionescu, A. El-Faham, *J. Org. Chem.* **1996,** *61,* 2460-2465.

^{1}H NMR (300 MHz, CD_3OD): δ 8.35 (d, J = 6.5 Hz, amide NH), 7.10-6.69 (AB q, J = 6.4 Hz, 4H), 4.58 (dd, J = 4.3, J = 5.0, 1H), 4.43-4.36 (m, 1H), 3.11-2.73 (m, 2H), 1.90 (s, 3H), 1.41 (d, J = 7.5, 3H).

AcY-(D)-A: HPLC purification [Vydac C18, gradient elution: A = .1% TFA/H_2O, B: CH_3CN/.1% TFA 0–20% B/30min t_2 = 21.4 min. Excision and weighing of the peaks gave a ratio of 92.4:7.6 (84.8% ee). The synthesis of this dipeptide was repeated, as above, using Carpino's amide bond forming conditions (HATU, HOAt, collidine). This synthesis yielded material with a relative peak size of greater than 95:5 (>90% de).

AcY_sEY_sLDY_sDF: The peptide was synthesized on .025 mmol scale by standard coupling procedures. Commercially available pre-loaded resin (.5 mmol/g) was used. The The *N*-terminal Fmoc group was cleaved and the amino group acetylated. The azidomethylene group was cleaved in the usual way. Sulfation was performed as described above. Cleavage and lyophilization affords 28 mg of a white solid. HPLC purification [Alltech Econosil C18, one major peak: t_2 = 22.76 min, gradient system: CH_3CN/.1M aq. NH_4OAc 5%⟶75% in 40 min, 8 mL/min] affords 8 mg (27% based on resin loading, minus resin for characterization) of a flocculent white solid. **IR** (KBr): 1244 br, str, 1050 br, str; **LRMS** (MALDI, α-cyano-4-hydroxycinnamic acid matrix, negative ion mode): *m/z* 1138.3 [calc'd M-3SO_4+NH_4^+ 1138.48].

AcYEYLDYDF: A fraction of the phenol-deprotected material (10 mg resin) from the synthesis of AcY_sEY_sLDY_sDF above was cleaved (yield 6 mg), dissolved in MeOH/H_2O (2 mL) and subjected to hydrogenation over Pearlman's catalyst (10 mg) for 12 h under an H_2 filled balloon. Filtration through pre-rinsed Celite (MeOH/H_2O, 1:1 eluant) afforded 3 mg crude material after lyophilization. **LRMS** (FAB α-cyano-4-

hydroxycinnamic acid matrix, positive ion mode): *m/z* 1215.4 [calc'd MH + $2Na^+$ 1215.44].

AcYEY$_s$LDYDF: The solid phase synthesis was performed according to the general procedures described above. Cleavage from the resin gave 17 mg of crude peptide. This material was subjected to hydrogenation over Pearlman's catalyst (20 mg) for 12h under an H_2 filled balloon. Filtration through pre-rinsed Celite (H_2O eluant). This material was subjected to HPLC (Alltech Econosil C18) gave three major peaks, two of which appeared to be deletion peptides (by MALDI-MS, we were unable to assign a structure based on the mass spectra, however the peptides appeared to be sulfated, as judged by HPLC retention time). The longest retained peptide (t_r = 33.48 min, gradient system: CH_3CN/.1M aq. NH_4OAc 5%→75% in 40 min, 8 mL/min] pooling of this HPLC fraction and lyophilization afforded the desired peptide as a fluffy white solid (4.6 mg, 5.2 %) **LRMS** (MALDI, α-cyano-4-hydroxycinnamic acid matrix, negative ion mode): *m/z* 1170.4 [calc'd M-SO_4^-+NH_4 1169.42]; (MALDI, 2,4,6-trihydroxyacetophenone, negative ion mode): *m/z* 1191.6 [calc'd M – SO_3 + Na^+ for $C_{57}H_{68}N_8O_{22}S$ 1191.41] **IR** (KBr): 1256 br, str, 1049 br, str.

AcY$_{Bn}$E$_{Bn}$YLD$_{Bn}$Y$_{Bn}$D$_{Bn}$F: After azidomethylene deprotection of the above peptide-resin a small portion was cleaved (8 mg resin) to yield ca. 2.5 mg of intermediate crude peptide. **LRMS** (FAB, α-cyano-4-hydroxycinnamic acid matrix, positive ion mode): *m/z* 1642.6 [calc'd MH + Na^+ 1642.69] Other lower molecular weight peaks were observed but not assignable.

Cleavage of sulfated peptides from chlorotrityl resin: The resin is dried under high vacuum for two hours before the cleavage reaction is attempted. Dichloromethane/trifluoroethanol/acetic acid cleavage solution (7:2:1 v:v:v, 10 mL per gram of resin) is cooled to

0 °C and added to a flask containing dried resin in an ice bath. The mixture is stirred for 1.5 hours at 0 •C. During this time the temperature does not exceed 5 °C. At the end of the reaction time the free peptide is filtered into a flask. The resin is then washed with the same volume of cleavage solution (at 0 °C) used in the reaction. Both washes are combined and most of the solvent is evaporated on a rotary evaporator (water bath less than 10 °C). Ether (40 mL) is added to the residue, the peptide is pelleted on the centrifuge and the ether is decanted. This procedure is repeated for another ether wash (40 mL) and for an ethyl acetate/ ether wash (1.5:1 v:v, 25 mL total). The peptide pellet is redissolved in methanol, transferred to a flask, and evaporated to an oil (rotary evaporator water bath less than 10 °C). The oil is redissolved in methanol and evaporated to remove acetic acid. The oil is then lyophilized twice from MQ water to remove any traces of acetic acid. After removal of acetic acid, the crude peptide is stored at -25•C until HPLC purification.

Crystallographic Experimental Section

Data Collection

A colorless crystal with approximate dimensions 0.09 x 0.02 x 0.02 mm^3 was selected under oil under ambient conditions and attached to the tip of a nylon loop. The crystal was mounted in a stream of cold nitrogen at 170(2) K and centered in the X-ray beam by using a video camera.

The crystal evaluation and data collection were performed at the a Bruker Kappa CCD-6000 diffractometer with Ag K_α (λ = 0.5594 Å) synchrotron radiation and the diffractometer to crystal distance of 4.937 cm.

The initial cell constants were obtained from one series of ω scans consisting of 120 frames collected at

intervals of 0.3° in a 36° range about ω with the exposure time of 3 seconds per frame. The reflections were successfully indexed by an automated indexing routine built in the SMART program. The final cell constants were calculated from a set of 5910 strong reflections from the actual data collection.

The data were collected by using the single run collection routine. The reciprocal space was surveyed to the extent of a full sphere to a resolution of 0.80 Å. A total of 26228 data were harvested by collecting three sets of frames with 0.3° scans in ω with an exposure time 3 sec per frame. These highly redundant datasets were corrected for Lorentz and polarization effects. The absorption correction was based on fitting a function to the empirical transmission surface as sampled by multiple equivalent measurements (see: Blessing, R.H. *Acta Cryst*. **1995**, *A51*, 33-38).

Structure Solution and Refinement

The systematic absences in the diffraction data were uniquely consistent for the space group $P2_12_12_1$ that yielded chemically reasonable and computationally stable results of refinement. All software and sources of the scattering factors are contained in the SHELXTL (version 5.1) program library (G. Sheldrick, Bruker Analytical X-Ray Systems, Madison, WI).

A successful solution by the direct methods provided most non-hydrogen atoms from the *E*-map. The remaining non-hydrogen atoms were located in an alternating series of least-squares cycles and difference Fourier maps. All non-hydrogen atoms were refined with anisotropic displacement coefficients. All hydrogen atoms were included in the structure factor calculation at idealized positions and were

allowed to ride on the neighboring atoms with relative isotropic displacement coefficients.

The final least-squares refinement of 317 parameters against 2803 data resulted in residuals *R* (based on F^2 for $I \bullet 2\sigma$) and *wR* (based on F^2 for all data) of 0.0667 and 0.2167, respectively. The final difference Fourier map was featureless.

The ORTEP diagram on the following page is drawn with 50% probability ellipsoids.