Supplementary Information for:

Rapid Biocompatible Macrocyclization of Peptides with Decafluoro-diphenylsulfone

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Abbreviations:

LCMS: Liquid chromatography Mass Spectrometry
HPLC: High Performance Liquid Chromatography
EDT: Ethanedithiol
TIPS: Triisopropylsilane
TFA: Trifluoroacetic acid
DFS: Decafluoro-diphenylsulfone
OFS: Octafluoro-diphenylsulfone (conjugate)
PBS: Phosphate Buffered Saline
DMF: Dimethylformamide
BIA: Biotin-PEG2-iodoacetamide
TCEP: Tris(2-carboxyethyl)phosphine)
r.t.: retention time
TLC: Thin Layer Chromatography
BIA: biotin iodoacetamide
DBMX: Dibromo-*m*-xylene
DMMX: Dimethyl-*m*-xylene

General information

Chemical reagents and solvents were purchased from Sigma-Aldrich or Fisher Scientific unless noted otherwise. All model perfluoro-arenes were purchased from Sigma-Aldrich or Alfa Aesar. Pentafluorophenyl sulfide was purchased from Matrix scientific and decafluoro-diphenylsulfone **(DFS)** was synthesized as described below. Reagents for peptide synthesis were purchased from ChemPep; model peptides were synthesized using standard Fmoc solid phase synthesis as described below, whereas all peptide hormones were purchased from Bachem.

Reactions were monitored by TLC which was carried out on silica gel 60 F_{254} (Merck) plates and visualized by UV-light (λ=254nm) and/or by spraying potassium permanganate, anisaldehyde followed by heating. Flash column chromatography was performed using silica gel 60 (40-63 μm) using ISCO Teledyne Combiflash Rf instrument. The subsequent evaporation of solvents *in vacuo* was performed using IKA RV10 rotary evaporator.

Proton (^{1}H NMR) and Carbon decoupled fluorine (^{13}C {^{19}F} NMR) nuclear magnetic resonance spectra were recorded on an Agilent/Varian VNMRS two channel 500 MHz or Agilent/Varian Inova two-channel 400MHz spectrometer. The chemical shifts are given in part per million (ppm) on the delta scale. The solvent peak was used as reference values. For ^{1}H NMR: $CDCl_3$= 7.24 ppm and for ^{13}C NMR: $CDCl_3$= 77.16 ppm. The following abbreviations have been used: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; b, broad; d, doublet of doublets; ddd, doublet of doublet of doublets; td, triplet of doublets.

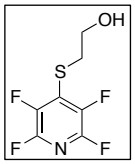
All ^{19}F NMR kinetics studies were performed in an Agilent/Varian DD2 MR two channel 400 MHz NMR spectrometer (detailed procedure below). UV kinetics was recorder on SpectraMax plate reader (detailed procedure below).

High resolution ESI mass spectra were recorded by Agilent Technologies 6220 oaTOF. High resolution EI mass spectra were recorded by Kratos Analytical MS 50 instrument. LCMS data was obtained on Agilent Technologies 6130 LCMS. A gradient of solvent A (MQ water) and solvent B (MeCN/H_2O 95/5) was run at a flow rate of 0.5 mL/min (0-4 min 5% B; 4-5 min 5%→60% B; 5-5.50 min 60%→100% B; 5.50-7.50 100% B, 7.50-11 min 100%→5% B) and for peptides conjugate DFS a gradient of solvent A (MQ water, 0.1% (v/v) formic acid) and solvent B (MeCN. 0.1% (v/v) formic acid) were run at a flow rate of 0.5 ml/min (0-4 min 2% B; 4-5 min 2→60% B; 5-5.50 min 60→95% B; 5.50-8.50 min 95% B; 8.50-12 min 95%→2% B). Analytical and preparative HPLC was conducted using Waters 1525 Binary pump equipped with a Waters Symmetryprep 19×50 mm C18 Columns and Waters 2489 UV detector. Removal of aqueous solvents was performed using Labconco Freezone 2.5w system.

General procedure for S_NAr reaction between βME and perfluoroarenes

To a solution of β-mercaptoethanol (1 eq.) in MeCN (2.34 mL), we added perfluoroarene (10 eq.) as a solid or liquid and further added aqueous solution of Tris buffer (0.660 mL, 50 mM, pH 8.5). This solution was stirred at r.t. until disappearance of the starting material, as monitored on TLC. The solvent was then evaporated under reduced pressure and the residue was dissolved in DCM and washed with water. The organic layer was concentrated *in vacuo*. The residue was purified on normal-phase flash chromatography column (80:20 pentane/Et_2O as eluent).

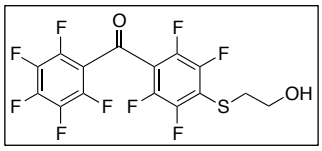
2-((perfluoropyridin-4-yl)thio)ethanol:



Colorless oil (0.066 g, 100% yield).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.86 (t, 2H, J= 5.81 Hz), 3.35 (t, 2H, J= 5.81 Hz), 1.98 (s, 1H). **^{13}C {^{19}F} NMR ($CDCl_3$, 125 MHz):** δ 143.39, 141.20, 130.71, 61.39 (t, C coupled with CH_2), 35.93 (t, C coupled with CH_2). **^{19}F NMR ($CDCl_3$, 376 MHz):** δ (-91.02)—(-90.84) (m, 2F), (-137.83)—(-137.66) (m, 2F), **HRMS-EI:** *m/z* calcd for $C_7H_5F_4NOS$ 227.0027 found 227.0027, 0.0 ppm.

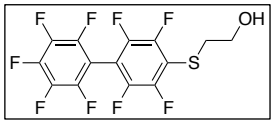
(perfluorophenyl)(2,3,5,6-tetrafluoro-4-((2-hydroxyethyl)thio)phenyl)methanone:



Yellow solid (0.107 g, 100% yield).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.82 (t, 2H, J= 5.80 Hz), 3.25 (t, 2H, J= 5.80 Hz), 1.95 (br, 1H). **^{13}C {^{19}F} NMR ($CDCl_3$, 125 MHz):** δ 176.30, 146.65, 145.05, 144.33, 143.99, 137.69, 120.56, 117.51, 114.24, 61.30 (t, C coupled with CH_2), 37.25 (t, C coupled with CH_2). **^{19}F NMR ($CDCl_3$, 376 MHz):** δ (-132.30)—(-132.41) (m, 2F), -140.97 (dquintet, 2F, J= 19.66 and 6.09 Hz), (-141.57)—(-141.68) (m, 2F), -145.81 (tt, 1F, J= 20.76 and 4.98 Hz), (-159.31)—(-159.47) (m, 2F). **HRMS-EI:** *m/z* calcd for $C_{15}H_5F_9O_2S$ 419.9866 found 419.9865, 0.3 ppm.

2-((perfluoro-[1,1'-biphenyl]-4-yl)thio)ethanol:

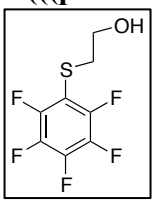


Yellow oil (0.010 g, 60% yield).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.79 (t, 2H, J= 5.82 Hz), 3.21 (t, 2H, J= 5.81 Hz), 1.81 (br, 1H).

^{13}C {^{19}F} NMR ($CDCl_3$, 100.68 Hz): δ 147.13, 144.48, 144.08, 142.49, 137.88, 116.62, 106.19, 102.14, 61.11 (t, C coupled with CH_2), 37.66 (t, C coupled with CH_2). **^{19}F NMR ($CDCl_3$, 376 MHz):** δ (-132.41)—(-132.51) (m, 2F), (-137.16)—(-137.27) (m, 2F), (-137.48)—(-137.63) (m, 2F), -149.90 (tt, 1F, J= 20.92 and 3.04 Hz), (-160.32)—(-160.47) (m, 2F). **HRMS-EI:** *m/z* calcd for $C_{14}H_5F_9OS$ 391.9917 found 391.9924, -1.8 ppm.

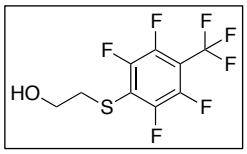
2-(((perfluorophenyl)thio)ethanol:



Colorless oil (0.010 g, 30% yield).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.70 (q, 2H, J= 5.90 Hz), 3.04 (t, 2H, J= 5.78 Hz), 2.00 (t, 1H, J= 6.23 Hz). **^{13}C {^{19}F} NMR ($CDCl_3$, 100.58 MHz):** δ 147.77, 141.63, 137.72, 108.11, 60.84 (t, C coupled with H_2), 38.17 (t, C coupled with H_2). **^{19}F NMR ($CDCl_3$, 376 MHz):** δ -143.16 (dd, 2F, J= 8.32 and 22.19 Hz), -155.35 (t, 1F, J= 20.82 Hz), -161.63 (dq, 2F, J= 10.71 and 21.11 Hz). **HRMS-EI:** *m/z* calcd for $C_8H_5F_5OS$ 243.9981 found 243.9982, -0.3 ppm.

2-((2,3,5,6-tetrafluoro-4-(trifluoromethyl)phenyl)thio)ethanol:

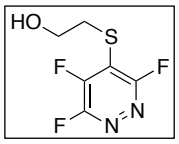


Colorless oil (0.006 g, 30% yield).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.77 (t, 2H, J= 5.7 Hz), 3.20 (t, 2H, J= 5.84 Hz), 2.37 (s, 1H).

^{13}C {^{19}F} NMR ($CDCl_3$, 100.58 MHz): δ 146.94, 144.01, 120.65, 119.79, 108.98, 61.23 (t, C coupled with CH_2), 37.10 (t, C coupled with CH_2). **^{19}F NMR ($CDCl_3$, 468.65 MHz):** δ -56.26 (t, 3F, J= 21.77 Hz), -131.97 (td, 2F, J= 7.82 and 8.44 Hz), (-139.97)—(-14.17) (m, 2F). **HRMS-EI:** *m/z* calcd for $C_9H_5F_7OS$ 293.9949 found 293.9952, -1.0 ppm.

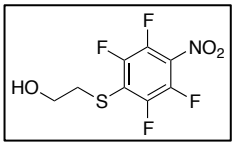
2-((3,5,6-trifluoropyridazin-4-yl)thio)ethanol:



Pale yellow oil (0.034 g, 76% yield).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.93 (t, 2H, J= 5.71 Hz), 3.40 (t, 2H, J= 5.71 Hz), 2.11 (s, 1H). **^{13}C {^{19}F} NMR ($CDCl_3$, 100.58 MHz):** δ 162.78, 155.56, 149.66, 121.23, 61.75 (t, C coupled with H_2), 35.41(t, C coupled with H_2). **^{19}F NMR ($CDCl_3$, 400 MHz):** δ -73.58 (dd, 1F, J=20.38 and 30.76 Hz), -98.44 (dd, 1F, J= 26.11 and 30.71 Hz), -122.13 (dd, 1F, J= 20.36 and 26.00 Hz). **HRMS-EI:** *m/z* calcd for $C_6H_5F_3N_2OS$ 210.0074 found 210.0072, 0.9 ppm.

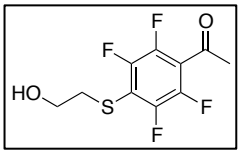
2-((2,3,5,6-tetrafluoro-4-nitrophenyl)thio)ethanol:



Yellow oil (0.418 g, 66% yield).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.82 (t, 2H, J= 5.76 Hz), 3.24 (t, 2H, J= 5.77 Hz), 1.95 (br, 1H). **^{13}C {^{19}F} NMR ($CDCl_3$, 100.58 MHz):** δ 146.65, 140.14, 129.36, 120.63 (t, C coupled with H_2), 61.46 (t, C coupled with H_2), 37.20 (t, C coupled with H_2). **^{19}F NMR ($CDCl_3$, 400 MHz):** δ (-130.21)—(-130.33) (m, 2F), (-146.16)—(-146.27) (m, 2F). **HRMS-EI:** *m/z* calcd for $C_8H_5F_4NO_3S$ 270.9926 found 270.9932, -2.3 ppm.

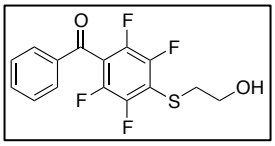
1-(2,3,5,6-tetrafluoro-4-((2-hydroxyethyl)thio)phenyl)ethanone:



White solid (0.417 g, 65% yield).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.76 (q, 2H, J= 5.89 Hz), 3.17 (t, 2h, J= 5.80 Hz), 2.62 (s, 3H), 1.99 (t, 1H, J= 6.13 Hz). **^{13}C {^{19}F} NMR ($CDCl_3$, 100.58 MHz):** δ 191.74, 146.92, 143.81, 119.17, 117.48, 61.10 (t, C coupled with H_2), 37.44 (t, C coupled with H_2), 32.36 (q, C coupled with H_3). **^{19}F NMR ($CDCl_3$, 400 MHz):** δ -132.57 (q, 2F, J= 11.46 Hz), -141.12 (dd, 2F, J= 12.19 and 23.42 Hz). **HRMS-EI:** *m/z* calcd for $C_{10}H_8F_4O_2S$ 268.0181 found 268.0184, -1.2 ppm.

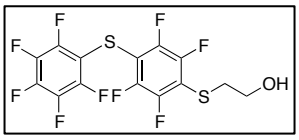
phenyl(2,3,5,6-tetrafluoro-4-((2-hydroxyethyl)thio)phenyl)methanone:



Colorless oil (0.104 g, 57% yield).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.86 (d, 2H, J= 7.26 Hz), 7.68 (t, 1H, J= 7.44 Hz), 7.52 (t, 2H, J= 7.71 Hz), 3.78 (t, 2H, J= 5.72 Hz), 3.19 (t, 2H, J= 5.82 Hz), 2.12 (br, 1H). **^{13}C {^{19}F} NMR ($CDCl_3$, 100.58 MHz):** δ 185.85, 146.96, 143.27, 135.85, 134.17, 130.15 (d, C coupled with CH), 128.50 (d, C coupled with CH), 118.85, 116.28, 61.09 (t, C coupled with CH_2), 37.67 (t, C coupled with CH_2). **^{13}C {^{19}F} NMR ($CDCl_3$, 100.58 MHz):** δ -131.99 (q, 2F, J= 11.76 Hz), -139.94 (q, 2F, J= 11.73 Hz). **HRMS-EI:** *m/z* calcd for $C_{15}H_{10}F_4O_2S$ 330.0337 found 330.0336, 0.3 ppm.

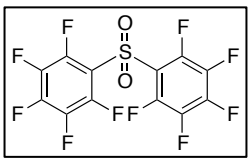
2-((2,3,5,6-tetrafluoro-4-((perfluorophenyl)thio)phenyl)thio)ethanol:



White solid (0.092 g, 40%).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.73 (q, 2H, J= 5.93 Hz), 3.14 (t, 2H, J= 5.85 Hz), 1.93 (t, 1H, J= 6.18 Hz). **^{13}C {^{19}F} NMR ($CDCl_3$, 100.58 MHz):** δ 147.39, 147.05, 146.59, 142.58, 137.81, 116.12, 111.17, 105.76, 61.07 (t, C coupled with H_2), 37.68 (t, C coupled with H_2). **^{19}F NMR ($CDCl_3$, 400 MHz):** δ (-131.48)—(-131.57) (m, 2F), -132.09 (q, 2F, J= 12.13 Hz), -132.92 (ddt, 2F, J= 4.40 and 9.02 and 22.42 Hz), -149.60 (tt, 1F, J= 3.46 and 20.88 Hz), (-159.67)—(-159.81) (m, 2F). **HRMS-EI:** *m/z* calcd for $C_{14}H_5F_9OS_2$ 423.9638 found 423.9640, -0.6 ppm.

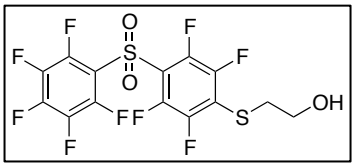
6,6'-sulfonylbis(1,2,3,4,5-pentafluorobenzene) (DFS) [1]



According to the procedure described in the reference, a solution of H_5IO_6 (7.467 g, 32.76 mmol) in acetonitrile (17 mL) was stirred vigorously at room temperature for 30 min. To this solution, we added CrO_3 (0.246 g, 2.46 mmol) and stirred at room temperature until appearance a clear orange solution. A solution of sulfide (1.000 g, 2.73 mmol) in CH_3CN (5 mL) was then added to the reaction mixture. The reaction was put under reflux and monitored by TLC for 1 h. The mixture was then filtered and the filter cake was washed with CH_3CN. The filtrate was concentrated under reduced pressure. The residue was re-dissolved in ethyl acetate, washed with saturated aqueous Na_2SO_3 solution and brine, and dried over $MgSO_4$. The solvent was removed *in vacuo*. The final product was sufficiently pure by ^{1}H NMR and ^{19}F NMR and required no further purification. The product was obtained as a yellow solid (0.878 g, 81% yield).

^{13}C {^{19}F} NMR ($CDCl_3$, 100.58 MHz): δ 145.77, 145.27, 138.01, 116.58. **^{19}F NMR ($CDCl_3$, 400 MHz):** δ -135.08- -135.15 (m, 4F), -140.44 (tt, 2F, J= 8.37 and 21.12 Hz), (-156.80)—(-156.97) (m, 4F). **HRMS-EI:** *m/z* calcd for $C_{12}F_{10}O_2S$ 397.9459 found 397.9460, -0.2 ppm.

2-((2,3,5,6-tetrafluoro-4-((perfluorophenyl)sulfonyl)phenyl)thio)ethanol



White solid (0.042 g, 36% yield).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.82 (t, 2H, J= 5.78 Hz), 3.26 (t, 2H, J= 5.78 Hz), 1.76 (br, 1H). **^{13}C {^{19}F} NMR ($CDCl_3$, 100.58 MHz):** δ 146.47, 145.66, 145.29, 144.10, 137.98, 124.65, 118.62, 116.65, 61.51 (t, C coupled with CH_2), 36.82 (t, C coupled with CH_2). **^{19}F NMR ($CDCl_3$, 400 MHz):** δ -131.03 (td, 2F, J= 18.50 and 10.20 Hz), -135.20 (dqt, 2F, J= 18.99, 9.33 and 4.67 Hz), (-136.84)—(-136.99) (m, 2F), -140.98 (tt, 1F, J= 21.09 and 8.44 Hz), (-157.10)—(-157.27) (m, 2F). **HRMS-EI:** *m/z* calcd for $C_{14}H_5F_9O_3S_2$ 455.9536 found 455.9542, -1.2 ppm.

General procedure for peptide synthesis

Peptides were prepared on resin using standard solid phase amide coupling. Rink amide AM resin (300 mg, 0.96 mmol/g, 0.31 mmol) was weighed into a PolyPrep® chromatography column. The column

was set up on a manifold vacuum and equipped with a three-way stopcock that allows draining of the solvent by vacuum flitration and agitation of the resin by nitrogen gas as described by Verdine and co-workers.[2] DCM (3 mL) was added to the dried resin for swelling. After 15 min, the solvent was drained by vacuum filtration. The resin was washed with DMF (4 mL) and then deprotected with 20% (v/v) piperidine in DMF (4 mL) for 1 min. The deprotection was repeated for another 10 min using fresh 20% (v/v) piperdidine in DMF (4 mL). The resin was then washed with DMF (4 x 4 mL). Fmoc-protected amino acid (1.2 mmol, 4 eq.) dissolved in DMF (4 mL) were added to the resin. After 30 seconds of agitation, the reagents were removed by vacuum filtration and the resin was washed with DMF (4 x 4 mL). The Fmoc-deprotection, amide coupling, and washing steps were repeated consecutively to elongate the sequence up to the N-terminal residue. After Fmoc-deprotection the resin was washed with DMF (5 x 4 mL), followed by DCM (5 x 4 mL). The resin was left on the manifold for 10 min to dry on vacuum. A cleavage cocktail (4 mL) containing TFA/H_2O/TIPS/Phenol/EDT, 94/2.5/5/5/2.5 (v/v/v/w/v), was added to the dried resin. The column was left on the rocker for 2 h to cleave the peptide. The flow through from the column was collected and the resin was rinsed with TFA (1 mL). The combined cleavage mixture was added drop-wise to cold diethyl ether (40 mL) in a 50 mL polypropylene centrifuge tube (Falcon, Thermo Fisher). The mixture was incubated on ice for 30 min. The precipitate was separated by centrifugation (5 min, 3000 rpm). Supernatant was decanted and the precipitates were washed with cold diethyl ether (40 mL). The centrifugation and washing steps were repeated for two more cycles. The precipitates were first air-dried and then dried *in vacuo* overnight.

For HPLC purification, crude peptide powder was dissolved in MeCN and water; addition of acetic acid was necessary in some cases to dissolve the peptide. The solution was injected into a semi-preparative RP-HPLC system equipped with C18 column (Waters Symmetryprep 19 × 50 mm). A gradient of solvent A (MQ water, 0.1% (v/v) TFA) and solvent B (MeCN, 0.1% (v/v) TFA) was run at a flow rate of 12 mL/min (0-2 min 2% A; 2-18 min 2→50% B). The fractions corresponding to the main peak were collected. CH_3CN was removed by evaporation under reduced pressure. The aqueous solution was lyophilized to yield the peptide as white powder. Identity and purity of the peptides, as confirmed by LCMS are described on the subsequent pages.

Synthesis and characterization of peptide-OFS conjugates

To a solution of peptide (10 mmol) in 2 mL MeCN and 2 ml deionized water, we added 1eq of DFS in MeCN. The pH of the reaction was increased to 8.5 by adding 50 mM Tris buffer. This solution was incubated at room temperature for 10 min, then any precipitates formed during the course of the reaction were re-dissolved by adding more MeCN up to 60-70%. Reaction mixture was injected on

semi-preparative RP-HPLC system equipped with C18 column (Waters Symmetry Prep, 19 ×50 mm, 5 μm). A gradient of solvent A (MQ water, 0.1% (v/v) TFA) and solvent B (MeCN, 0.1% (v/v) TFA) was run at a flow rate of 12 mL/min (starting with 2% B for 2 minutes, followed by 2% → 50% B in 16 min; 50% → 100% in 3 min, 100% B for 3 min and then 100% → 2% in 2 min). The peak corresponding to peptide-DFS conjugate was collected. MeCN was removed by evaporation under reduced pressure. The aqueous solution was lyophilized to yield the peptide as white powder. Identity and purity of the peptides, as confirmed by LC-MS are described on the subsequent pages.

Peptide hormones and DFS were synthesized similarly with minor modification (MeCN was used as co-solvent and reaction was conducted in PBS, pH 7.4) Example of the preparative synthesis is described in Supporting Figure S16.

Table S1. Summary of characterization of synthetic peptides and their OFS-conjugates.

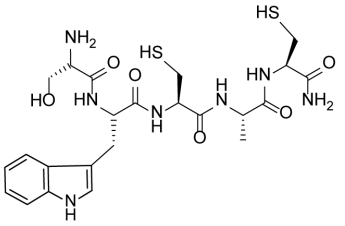
Sequence	**Net formula (M+H)**	**Calculated mass**	**Observed mass**	**Further Info** (page number)
H_2N-SWCAC-$CONH_2$	$C_{23}H_{34}N_7O_6S_2$	568.2006	568.2006	S10: LCMS S80: ^{1}H NMR
SWCAC-OFS conjugate	$C_{25}H_{32}F_8N_7O_8S_3$	926.1341	926.1340	S11: LCMS S80: ^{1}H NMR
H_2N-SWCRC-$CONH_2$	$C_{26}H_{41}N_{10}O_6S_2$	653.2646	653.2652	S10: LCMS
SWCRC-OFS conjugate	$C_{38}H_{40}F_8N_{10}O_8S_3$	1011.1981	1011.1979	S11: LCMS
H_2N-SWCSC-$CONH_2$	$C_{23}H_{34}N_7O_7S_2$	584.1955	584.196	S11: LCMS
SWCSC-OFS conjugate	$C_{35}H_{32}F_8N_7O_9S_3$	942.1290	942.1295	S12: LCMS
H_2N-SWCDC-$CONH_2$	$C_{24}H_{34}N_7O_8S_2$	612.1904	612.1914	S10: LCMS
SWCDC-OFS conjugate	$C_{36}H_{32}F_8N_7O_{10}S_3$	970.1240	970.124	S12: LCMS
H_2N-SWCDYRC-$CONH_2$	$C_{39}H_{54}N_{12}O_{11}S_2$	931.3549	931.3554	S11: LCMS
SWCDYRC-OFS conjugate	$C_{51}H_{52}F_8N_{12}O_{13}S_3$	1289.2884	1289.2883	S13: LCMS

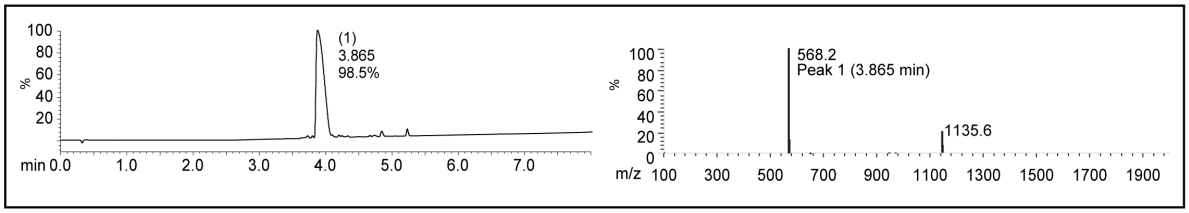
Table S2. Summary of peptide-hormones. Molecular weights of disulfide- and OFS-stapled peptides are shown.

Peptide name and sequence	**S-S Calc. (M+H)**	**OFS conjugate Calc. / measur. (M+H)**		**Other**
Oxytocin CYIQNCPLG-NH_2	1008.2	1368.4	1368.3 MALDI	S40: HPLC, LCMS
Urotensin II ETPDCFWKYCV	1389.6	1749.8	1749.7 MALDI	S42: HPLC
Calcitonin (salmon) CSNLSTCVLGKLSQELHKLQTYPRTNT-GSGTP-NH_2	3432.9	3793.1	3793.0 MALDI	S41: HPLC
Melanin-Concentrating Hormone DFDMLRCMLGRVYRPCWQV	2387.9	2748.0	2747.8 MALDI	S41: HPLC
Somatostatin-14 AGCKNFFWKTFTSC	1638.9	1999.1	1998.9 MALDI	
Atrial Natriuretic Factor (1-28) SLRRSSCFGGRMDRIGAQSGLGCNSFRY	3081.5	3441.7	3441.6 MALDI	S42: HPLC

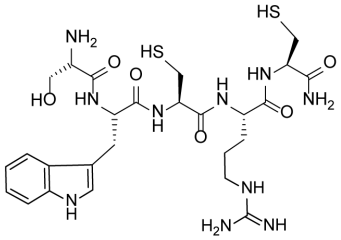
LCMS characterization of the peptides and peptide-OFS conjugates. All LCMS traces were recorded at λ= 220 nm.

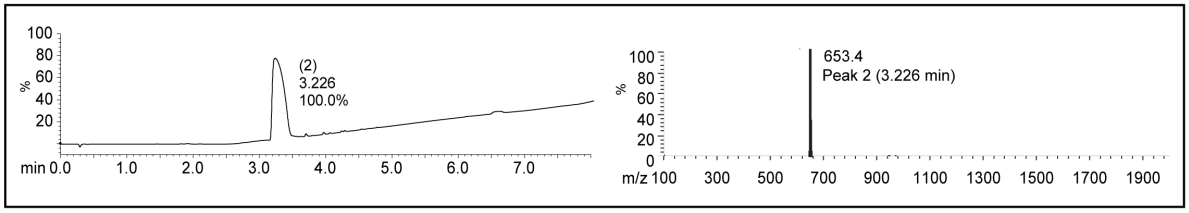
SWCAC



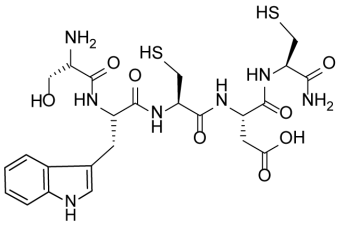


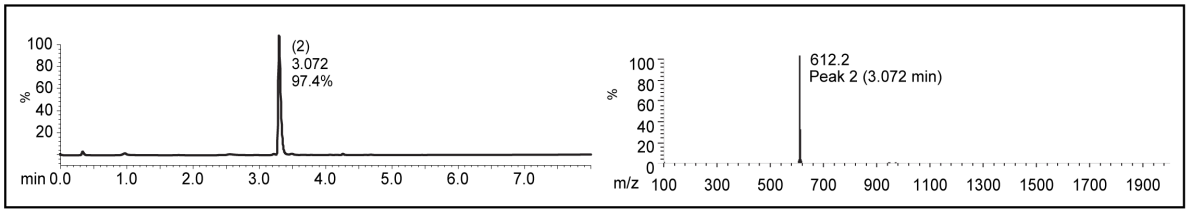
SWCRC





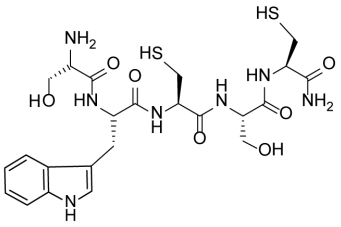
SWCDC

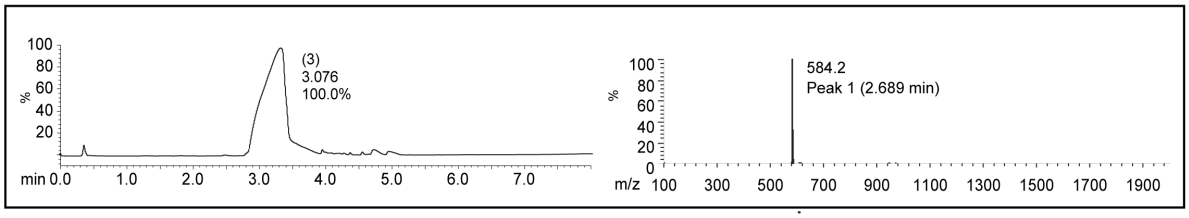




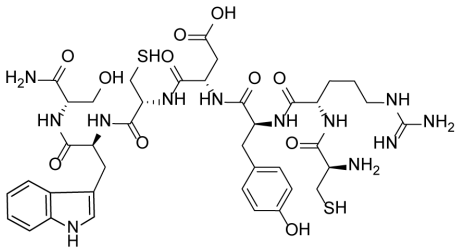
LCMS characterization of the peptides and peptide-OFS conjugates (continued). All LCMS traces are recorded at λ= 220 nm.

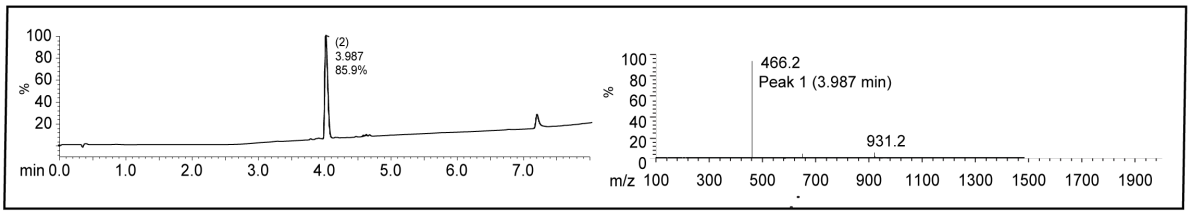
SWCSC





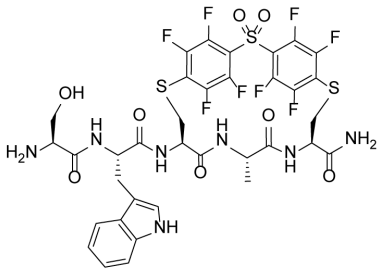
SWCDYRC

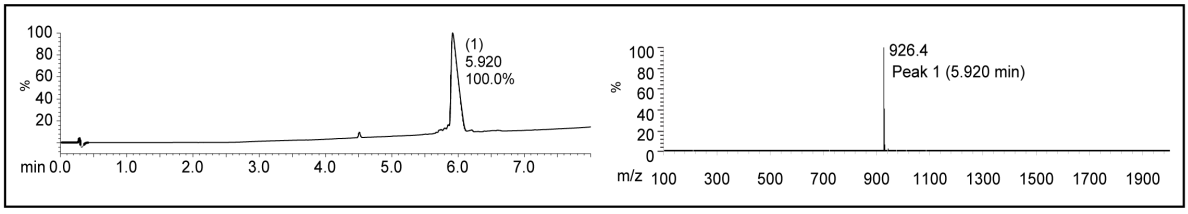




SWCAC-DFS conjugate
Isolated yield: 61%

HRMS
Calculated Mass for $C_{35}H_{32}F_8N_7O_8S_3$ (M+H)
Calculated = 926.1341
Observed = 926.1340
Difference = -0.14 ppm

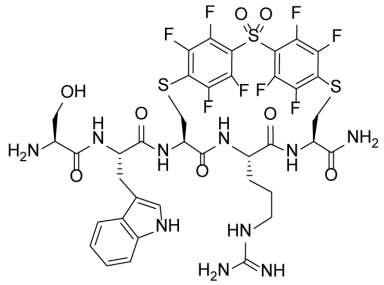


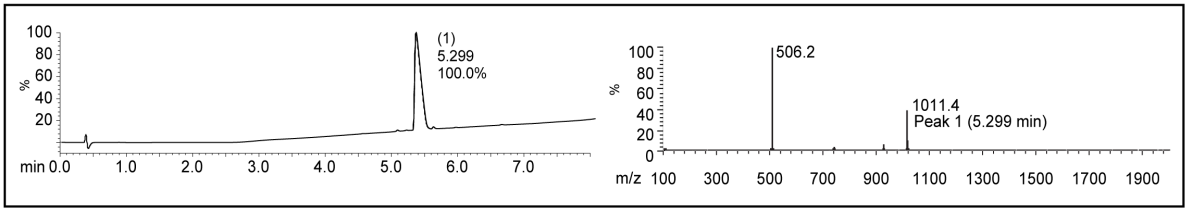


LCMS characterization of the peptides and peptide-OFS conjugates (continued). All LCMS traces are recorded at λ= 220 nm.

SWCRC-DFS conjugate
Isolated yield: 80%

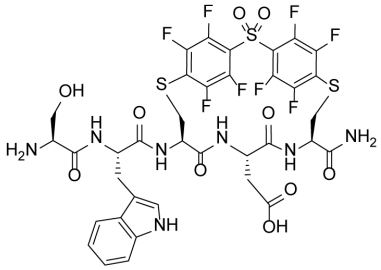
HRMS
Calculated Mass for $C_{38}H_{40}F_8N_{10}O_8S_3$
Calculated = 1011.1981
Observed = 1011.1979
Difference = -0.2 ppm

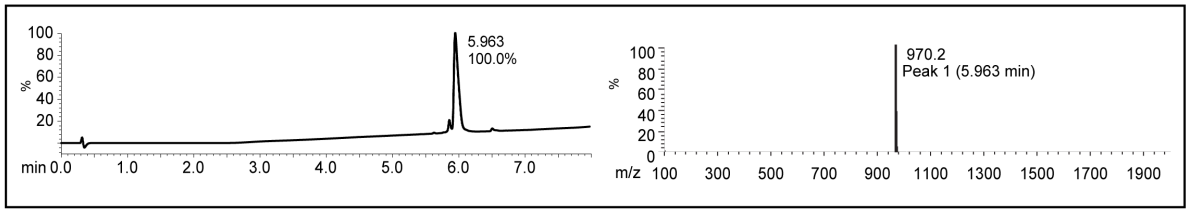




SWCDC-DFS conjugate
Isolated yield: 68%

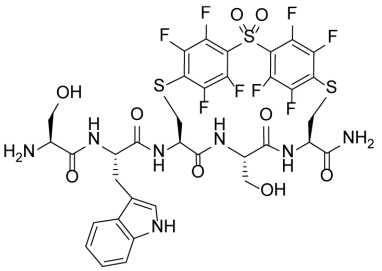
HRMS
Calculated Mass for $C_{36}H_{32}F_8N_7O_{10}S_3$ (M+H)
Calculated = 970.1240
Observed = 970.1240
Difference = 0.03 ppm

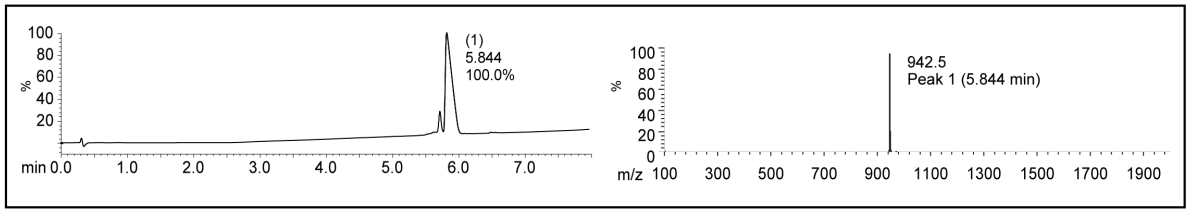




SWCSC-DFS conjugate
Isolated yield: 70%

HRMS
Calculated Mass for $C_{35}H_{32}F_8N_7O_9S_3$
Calculated = 942.1290
Observed = 942.1295
Difference = 0.45 ppm

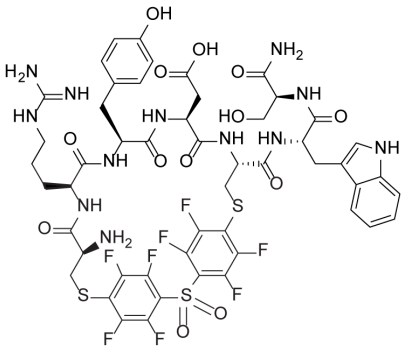


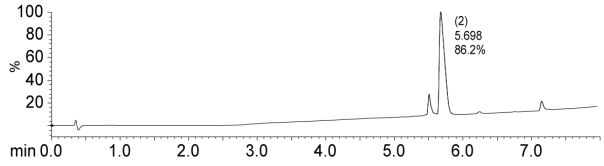


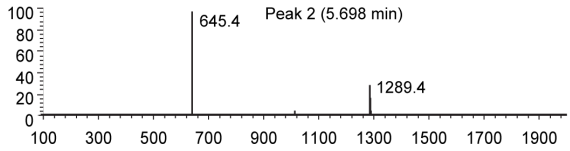
LCMS characterization of the peptides and peptide-OFS conjugates (continued). All LCMS traces are recorded at λ= 220 nm.

SWCDYRC-DFS conjugate
Isolated yield: 60%

HRMS
Calculated Mass for $C_{51}H_{52}F_8N_{12}O_{13}S_3$
Calculated = 1289.2884
Observed = 1289.2883
Difference = -0.06 ppm







General Procedure for ^{19}F NMR kinetics:

β-mercaptoethanol (10 mM in MeCN) was added to MeCN:Tris buffer (50 mM, pH 8.5) in an NMR tube. To this solution, we added perfluoroarene (139 mM in MeCN) and the tube was mixed for 3-5 seconds by shaking and placed in NMR instrument immediately (total ratio of MeCN:H_2O, 78%).^{19}F NMR spectra were recorded every 13 seconds for 1 hour. We adjusted relaxation time (d1)=0.1, number of scans=4 and time of acquisition=3. For slow-reacting perfluoroarenes, reaction was monitored at 5 min intervals over the course of 18-48 hours.

The integration values were extracted for all peaks (product and reactant). In some cases, such as perfluoropyridine described in Figure S2, all peaks could be resolved for starting material and product concurrently. In other cases, reactant and product peaks overlapped for some F-nuclei. In these cases, such as those described in Figure S3, only peaks sufficiently separated from the neighboring peaks were used for the analysis.

For slow kinetics, we fit the initial rate in the linear range (usually 1000-2000 seconds) to obtain the rate in $ppm^{-1}s^{-1}$ and used relative stoichiometry in the reaction to extrapolate the value of k in $M^{-1}s^{-1}$. Examples of traces and calculations are provided in Figures S2-S3. For rapid kinetics, which reached significant convergence over the course of the reaction, we fit the integration using first order kinetics equation $I_t=I_0+I(1+e^{-kCt})$ for product and $I_t=I_0+Ie^{-kCt}$ for reactant where I_t is integration observed at time t, I_0 integration at time 0, I integration due to appearance of the product, k is rate constant in $M^{-1}s^{-1}$, t is

time in seconds, and C is the concentration of the excess reagent in M. Note that we allowed $I_0 \neq 0$ for the product because delay during the mixing and setting up of the NMR experiment ranges from 1 to 3 minutes and could not be measured precisely. This type of fit is also justified because for the true first order process, the value of k is invariant of the starting time (i.e., any fraction of the first order kinetics curve can be fit to yield the same k value).

General procedure for UV kinetics:

We placed 2-10 μL of peptide solution (0.4-4.0 mM in H_2O) in 12 wells of quartz-bottom 96-well plate and added 200 μL of fluoroarene solution (at least 3 eq. excess) in 50 mM Tris-MeCN solution using 12-channel pipette. In the last solution we varied the pH and ratio (v/v) of H_2O:MeCN. Reading by SpectraMax plate reader was initiated within 15-30 seconds after the addition of the reagent; the instrument monitored the absorbance at 280, 310 or 320 nm every 10-15 seconds for 10-30 minutes.

The data was processed using MatLab to fit the reading to pseudo first order kinetic process: $A_t = A_0 + A(1-e^{-kCt})$, where A_t is absorbance at time t, A_0 is initial absorbance, A is change in absorbance due to the formation of the product, k rate constant and C is the initial concentration of fluoroarene. The numbers on the graphs in Figures S9, S11-S15 are fit value and [percent confidence of the fit], defined as: 100% x (95% upper bound of the fit – 95% lower bound of the fit) / fit value. MatLab script is provided below.

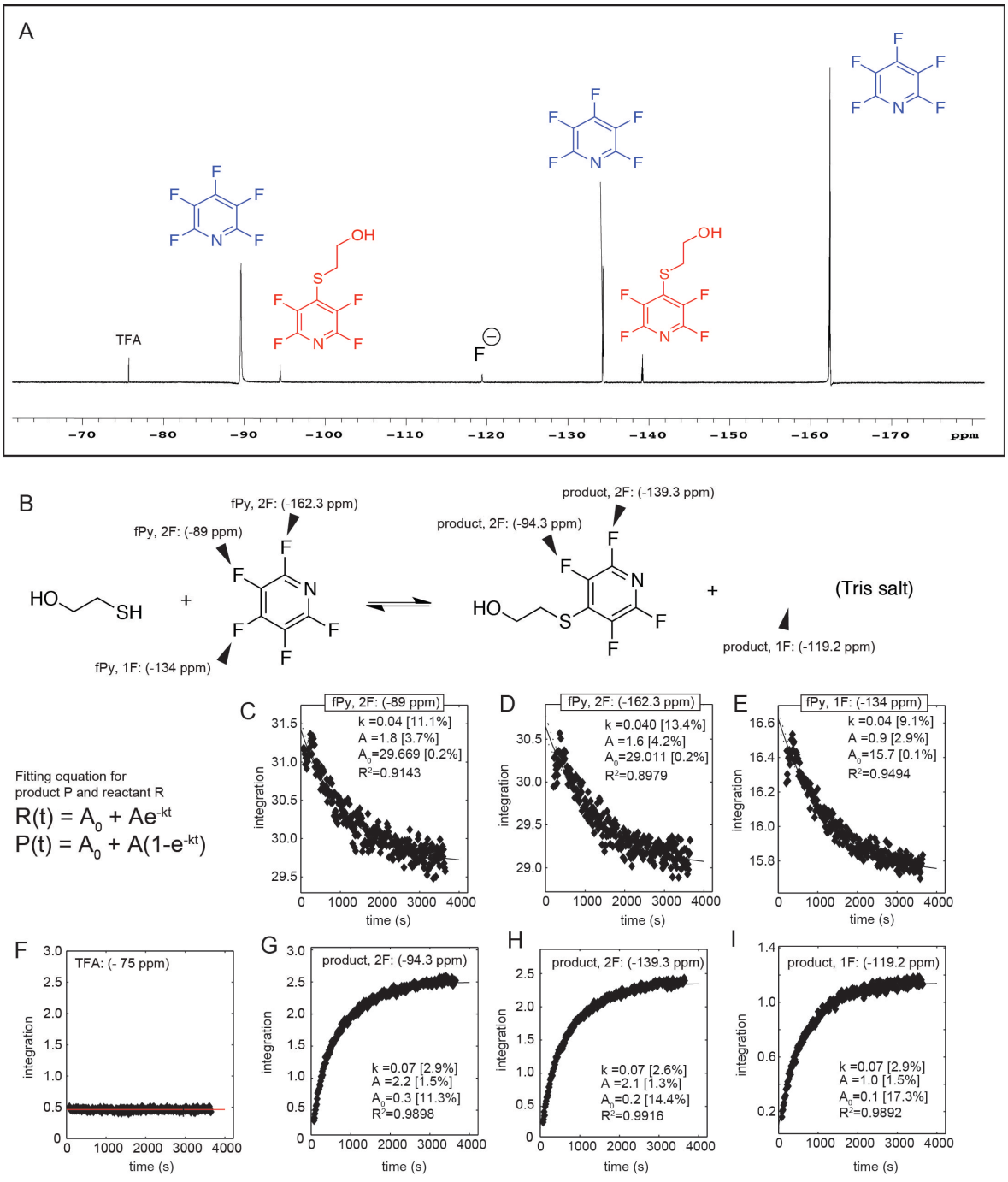
Supporting Script S1. MatLab Script used to fit the UV-kinetics data.

```
clear all;
close all;
% file should be in present working directory; change dir if necessary
dir = '';
% name of the raw .txt file produced by SpectraMax plate reader
name = 'SK-III-43-2nd experiment';
conc = 0.2*0.001*[1 1 1 1 1 1 1 1 1 1 1 1]; % M concentrations of excess reagent in each well
delay=18;  % seconds before the first read
time=13; % seconds between the reads

fh = fopen(fullfile(dir,[name '.txt']),'r+');
j=0;
record=0;

% number of wells analyzed simultaneously (between 1 and 12)
Nexp = 12;

% extract the values form the txt file
 AllVar = textscan(fh,'%s');
 for i=1:numel(AllVar{1})
   if strcmp(AllVar{1}{i},'0:00')
      record = 1;
   end
   if strcmp(AllVar{1}{i},'~End')
      record = 0;
```

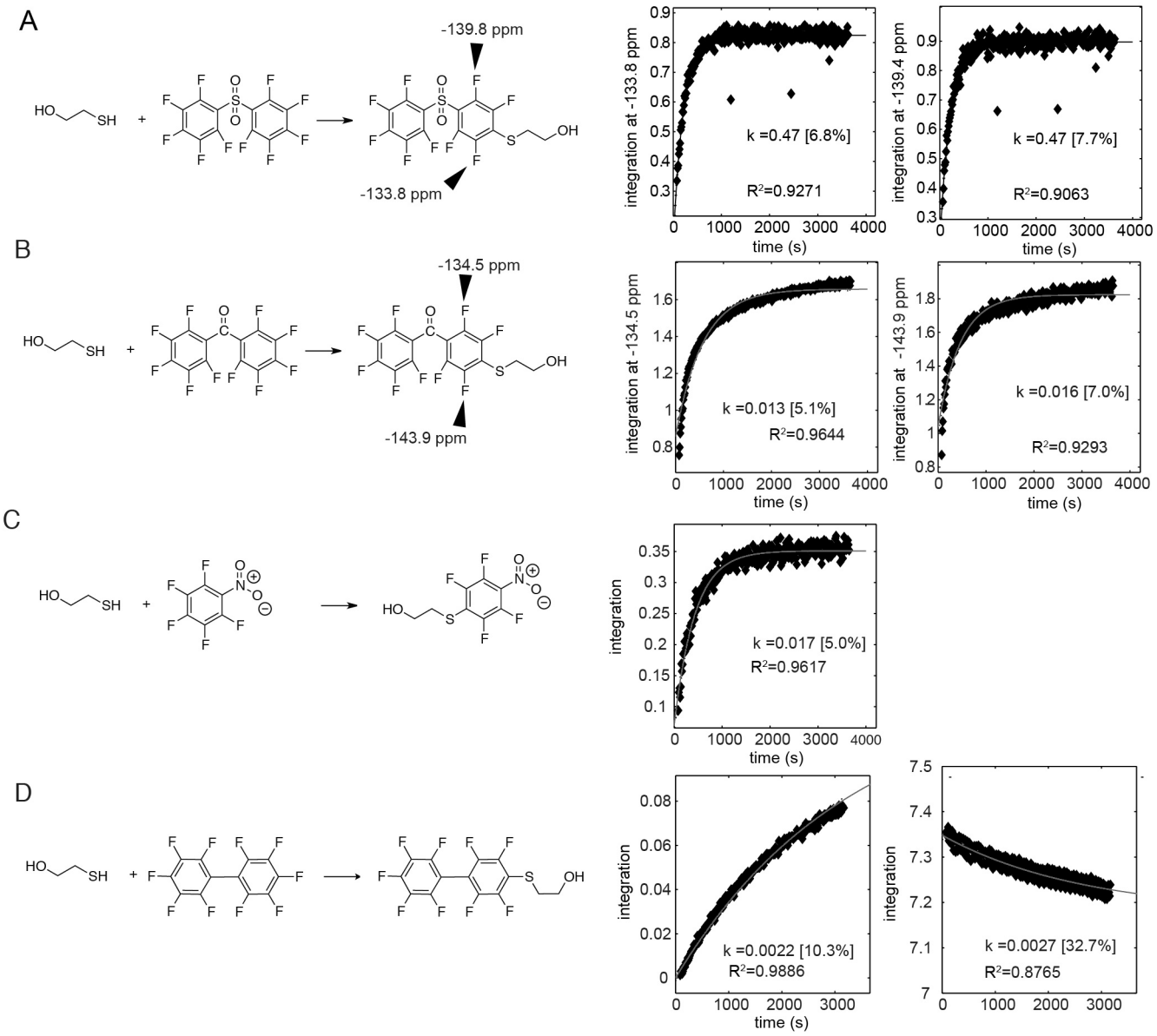
```
    end
    if record
        j=j+1;
        if mod(j-1,(Nexp+2))==0
            A(j)=time*round((j-1)/(Nexp+2)) + delay;
        else
            A(j) = str2num(AllVar{1}{i});
        end
    end
end

out = reshape(A,(Nexp+2),numel(A)/(Nexp+2));
figure(2)
imagesc(out(3:end,:));

% place the data into the structura that will be used for fitting
raw.data = out(3:end,:);
raw.time = out(1,:);
raw.forfit = [];
raw.conc  = conc';

% preview the data on a plot
for i=1:size(raw.data,2)
    raw.forfit = [raw.forfit raw.data(:,i) ];
end

%% part where fit is done

h=figure(100);
scrsz = get(0,'ScreenSize');
set(100,'Position',[scrsz(3)/3 scrsz(4)/2 scrsz(3)/3 scrsz(4)/1.5])
out=[];

for col=1:Nexp

%%%%%%%%%% define the data for fitting here %%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
x= raw.time;
y= raw.forfit(col,:);
t1= 5;
%%%%% make your best guess about k1 and k2 parameters %%%%%%%%%%%%%%%%%%%%%

A0 = 0;
A  = 1;
k  = 0.001;
%%%%%%%%%%%%%%%%%%%%%%%%    End of Data section   %%%%%%%%%%%%%%%%%%%%%%%%%%

%%%%% define the fit options   %%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
s = fitoptions('Method','NonlinearLeastSquares',...
               'Lower',[0,   0, 0 ],...
               'Upper',[2,   100, 0.5],...
               'Startpoint',[A k A0],...
               'TolFun', 1e-10 );

%%%%% define the fit equation (see Pcap.m file)  %%%%%%%%%%%%%%%%%%%%%%%%%%
ft = fittype( 'A0 + A*(1-exp(-k*x))','options',s );


%%%%% fit the data  %%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
[c2,gof2,output] = fit(x',y',ft);

%%%%% find the 95% confidence bounds and confidence interval  %%%%%%%%%%%%

CON = confint(c2); % confidence interval
x2=0:0.1:max(x);
p22 = predint(c2,x2,0.95,'functional','on');
```

```
%%%%% plot the raw data as black diamonds %%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
subplot(4,3,col);
plot(x,y,'dk',...
        'MarkerEdgeColor','k',...
        'MarkerFaceColor','k',...
        'MarkerSize',5);
hold on;

%%%%% plot the fit data as black lines and 95% confidence bounds as dash %%

plot( c2,'r');
plot(x2,p22,'k:');
legend off;
drawnow;

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%
%%%%%%%%%%%%%%%  define the axis, re-scale them, label them  %%%%%%%%%%%%%%

xmax = max(x)*1.05;
if ~isnan(p22) & ~isnan(conc(col))
    ymax = max(max(p22))*1.05;
    ymin = min(min(p22));
else
    ymax = max(max(raw.forfit))*1.05;
    ymin = min(min(raw.forfit));
end

xlim([0 xmax])
ylim([ymin ymax])
xlabel(['time (s)']);
ylabel(['absorbance']);

%  display the results of the fit on the plot
raw.fitk(col) = c2.k / raw.conc(col);
STD(1) = 100*abs(CON(1,1) - CON(2,1))/2 / c2.A;
STD(2) = 100*abs(CON(1,3) - CON(2,3))/2 / c2.k;
STD(3) = 100*abs(CON(1,2) - CON(2,2))/2 / c2.A0;

TL{1} = [' k =' num2str(raw.fitk(col),'%0.3f') ...
        ' [' num2str(STD(2),'%0.1f')  '%]'];
TL{2} = [' A =' num2str(c2.A,'%0.3f') ...
         ' [' num2str(STD(1),'%0.1f')  '%]'];
TL = [' A^0=' num2str(c2.A0,'%0.3f') ...
         ' [' num2str(STD(3),'%0.1f')  '%]'];
TL{4} = [' R^2=' num2str(gof2.rsquare,'%0.4f') ];

if ~isnan(p22) & ~isnan(conc(col))
    text(0.4*xmax,0.25*(ymax-ymin)+ymin, char(TL));
else
    text(0.4*xmax,0.25*(ymax-ymin)+ymin, 'control well');
end
text(-0.1*xmax,1.1*(ymax-ymin)+ymin, char(64+col),'FontSize',18);

hold off;

end

%% save the output matrix and save the JPEG and EPS files of the plots
save(fullfile(dir,[name '.mat']),'raw');
set(gcf,'PaperPositionMode','auto')
print(h, '-djpeg', fullfile(dir,[name '-' num2str(delay) 's-delay.jpeg']));
print(h, '-deps', fullfile(dir,[name '-' num2str(delay) 's-delay.eps']));
```

>>> End of Script >>>



Supporting Figure S1. (A-B) Examples of the ^{19}F NMR kinetic trace for perfluoropyridine. Arrows indicate the fluorine monitored in the reaction and their chemical shifts. (C-E) describes integration of the reactant and (G-I) integration of the product. (F) Integration of the reference compound (trifluoroacetic acid).



Supporting Figure S2. Examples of the NMR kinetic trace for reaction of βME with four perfluoroarenes (excess reagent, 140 mM) in 80-90% acetonitrile and 10-20% aqueous Tris buffer, pH=8.5. We used only the integration of the peaks that were well-resolved and can be reliably quantified over time. In (D) integration of both starting material and product can be fit independently to yield similar rate constants.

Calculating the rate constants using NMR and initial rates.

Integration and concentration are linearly dependent: $_0 = aI_0$ or $a = _0/I_0$ where I_0 is initial integration, $_0$ initial concentration of perfluoroarene and "a" is the conversion factor. The latter factor allows calculating the concentration C_t at any time point from integration I_t at that time point as:

$C_t = aI_t = I_t \, _0/I_0$

Definition of the initial rate: $Rate_0 = k \, _0[\beta ME]_0 = k(_0)^2 n$, where n is the ratio: $[\beta ME]_0/_0$

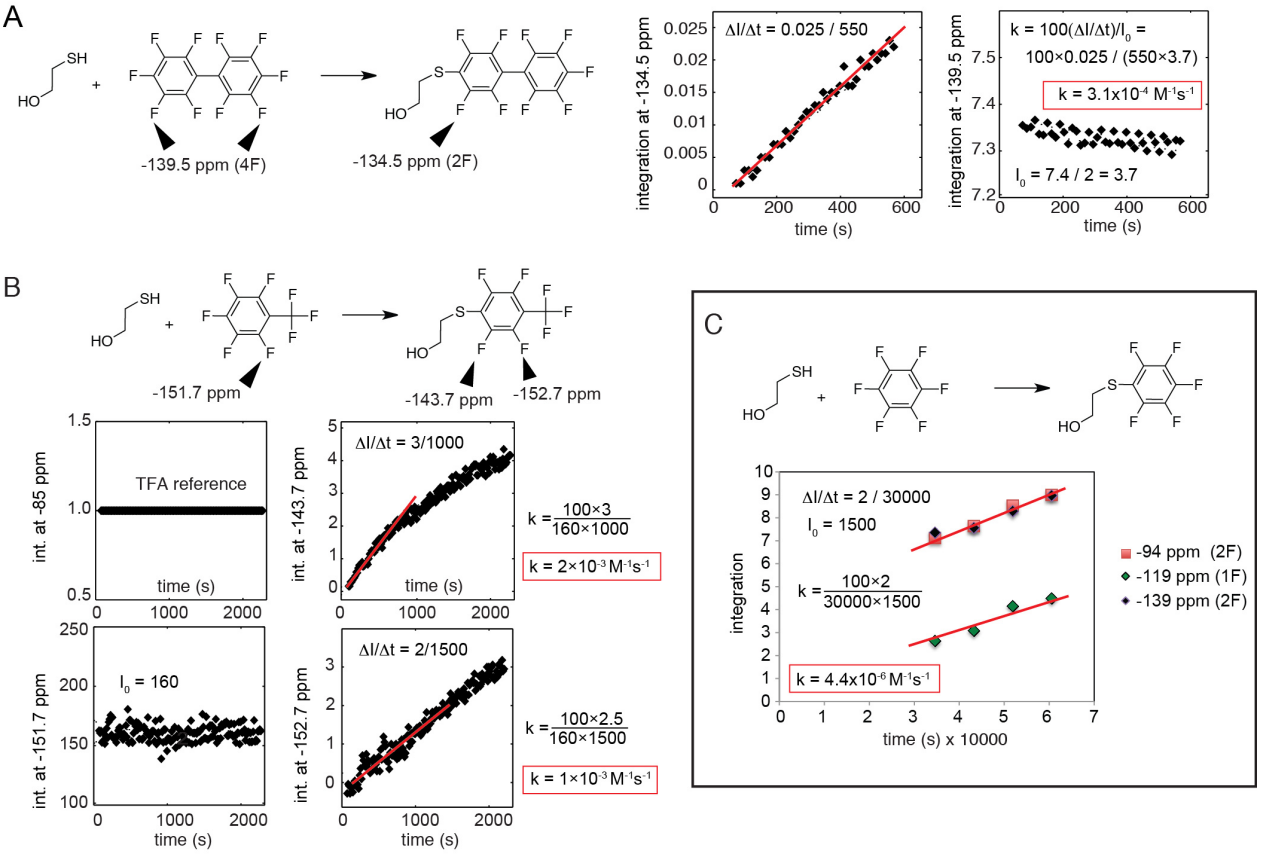
Rate of change in integration in the linear region of the plot $(\Delta I_t/\Delta t)$ is:

$$\Delta[Prod]/\Delta t = (\Delta I_t/\Delta t) \, _0/I_0 = k(_0)^2 n,$$

From the last equation, we can express "k":

$$k = (\Delta I_t/\Delta t) \, _0/(I_0 \, (_0)^2 n) = (\Delta I_t/\Delta t) / (I_0 n_0) = (\Delta I_t/\Delta t) / (I_0(1/14)*0.14) = 100 \, (\Delta I_t/\Delta t) / I_0$$

In the last expression, we used the typical initial concentration, $_0 = 0.14$ M and n=(1/14)



Supporting Figure S3. Examples of measurements of rate constants in slow S_NAr reactions. (A) In reaction of biphenyl, we calculated the $(\Delta I_t/\Delta t)$ and I_0, then used the expression derived above to calculate the rate constant. (B) Reaction with perfluorotoluene illustrates that calculation of rate can be influenced by close proximity of reactant peak to product peak: the value of k calculated from integration of well-separated peak at 143.7 ppm differs from that calculated by integration of peak at 152.7 ppm. Note the noisy integration values at 152.7 ppm. In (A-B) reactions were recorded continuously every 14 seconds. (C) Slow S_NAr reaction with perfluorobenzene required acquisition over the course of 10 hours with each point acquired at ~2 hour intervals.

Calculation of pKa values.

Calculation of pKa values was conducted by method suggested by Muckerman *at al*.[3] Gaussian 09 with default parameters was used for all calculations.[4] Structures were generated in Pymol and their geometries were optimized using B3LYP/6-311+G(d,p), 5d DFT functional and basis set. Hessians were obtained to confirm convergence and obtain vibrational free energy correction values, G_{vib}, in the gas phase. The relationship between calculated free energy differences between protonated and neutral forms was established using linear regression analysis for training set of analogs with known experimental pKa values in DMSO. The best correlation for this class of compounds (adjusted R^2=0.98) was obtained using gas phase free energies without correcting for solvation effects.

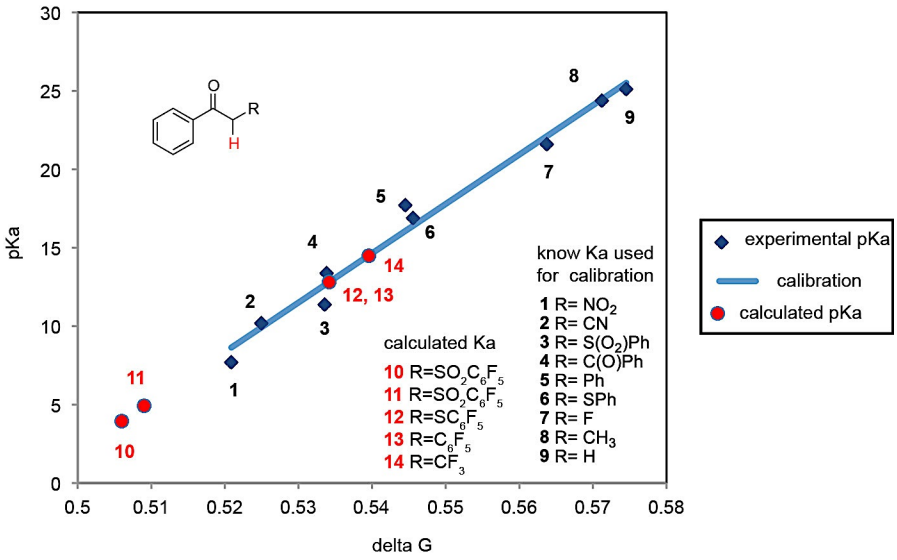
Calculation of the pKa of an unknown compound was calculated of energy difference between neutral and ionized form of the compound (ΔG).[5]

$$BH^+ - H^+ \longrightarrow B$$

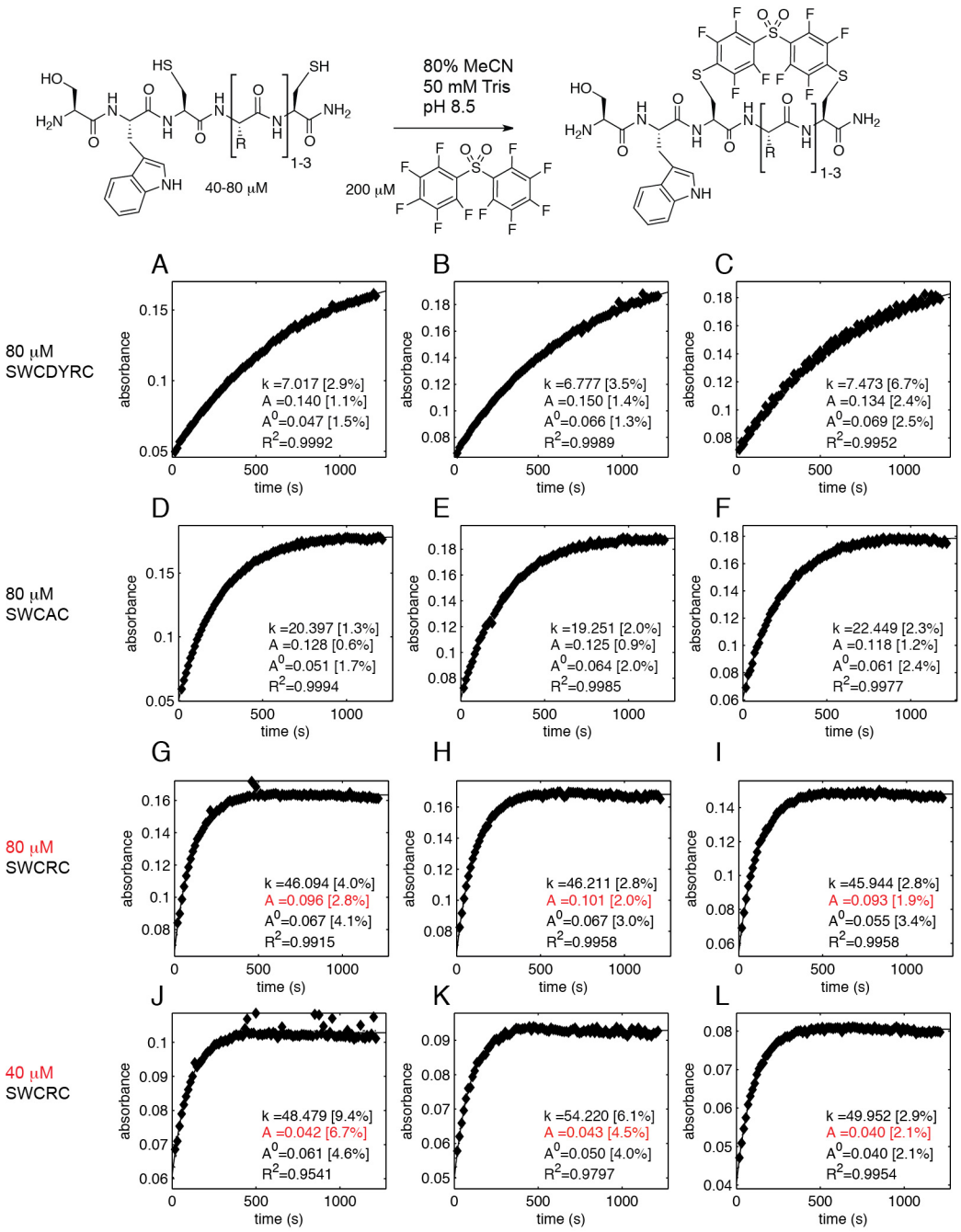
$$\Delta G = [E_{el}(B) + G_{vib}(B)] - [E_{el}(BH^+) + G_{vib}(BH^+)]$$

$$pK_a = a + b\Delta G$$

Where ΔG is the proton dissociation energy, E_{el} is electronic energy of the compound in the gas phase, G_{vib} is thermodynamic free energy correction from normal mode analysis.

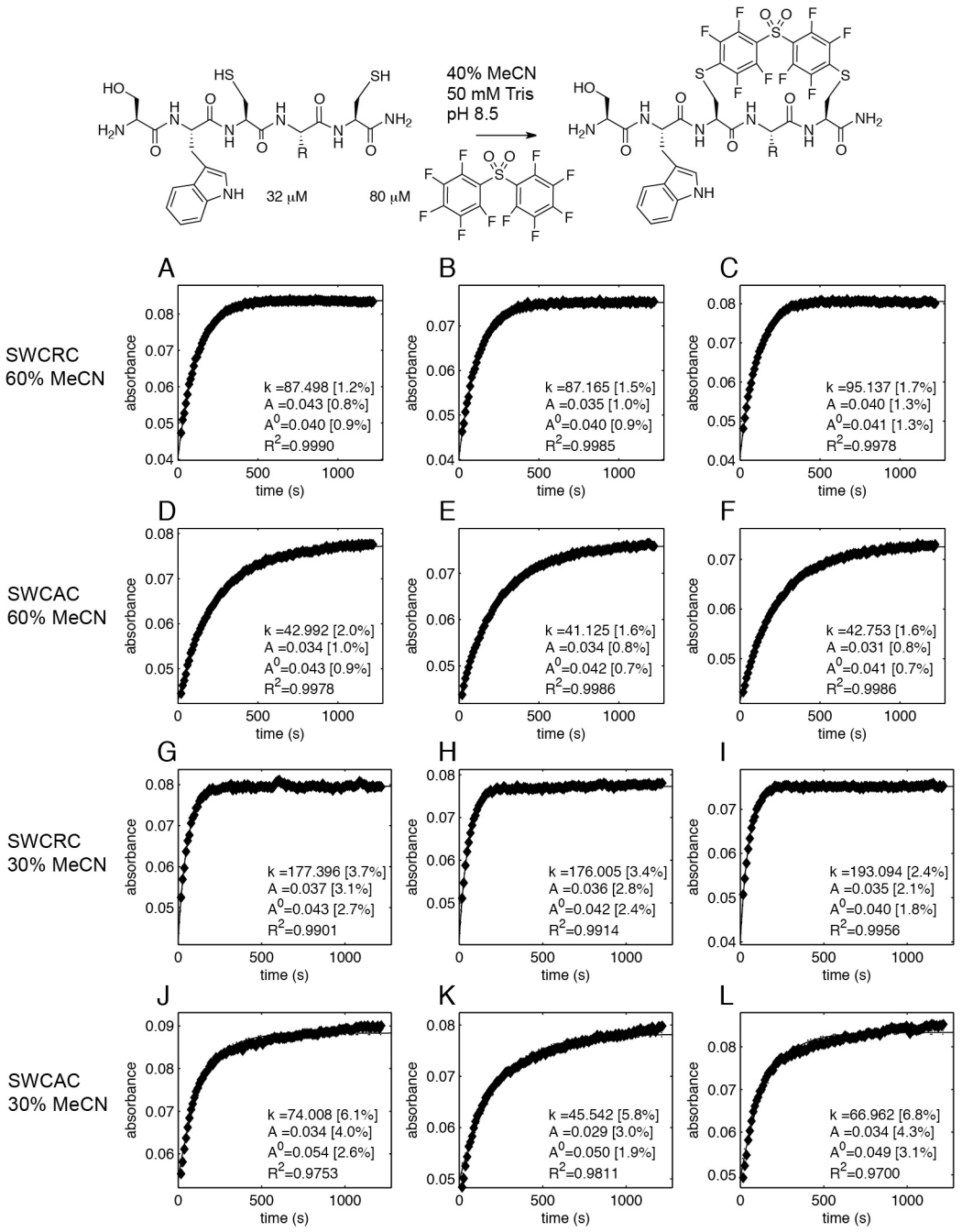


Supporting Figure S4. Correlation between experimental pKa values in DMSO and calculated *ab initio* free energy differences ΔG between protonated and deprotonates states for a series of substituted acetophenone C-H acids.

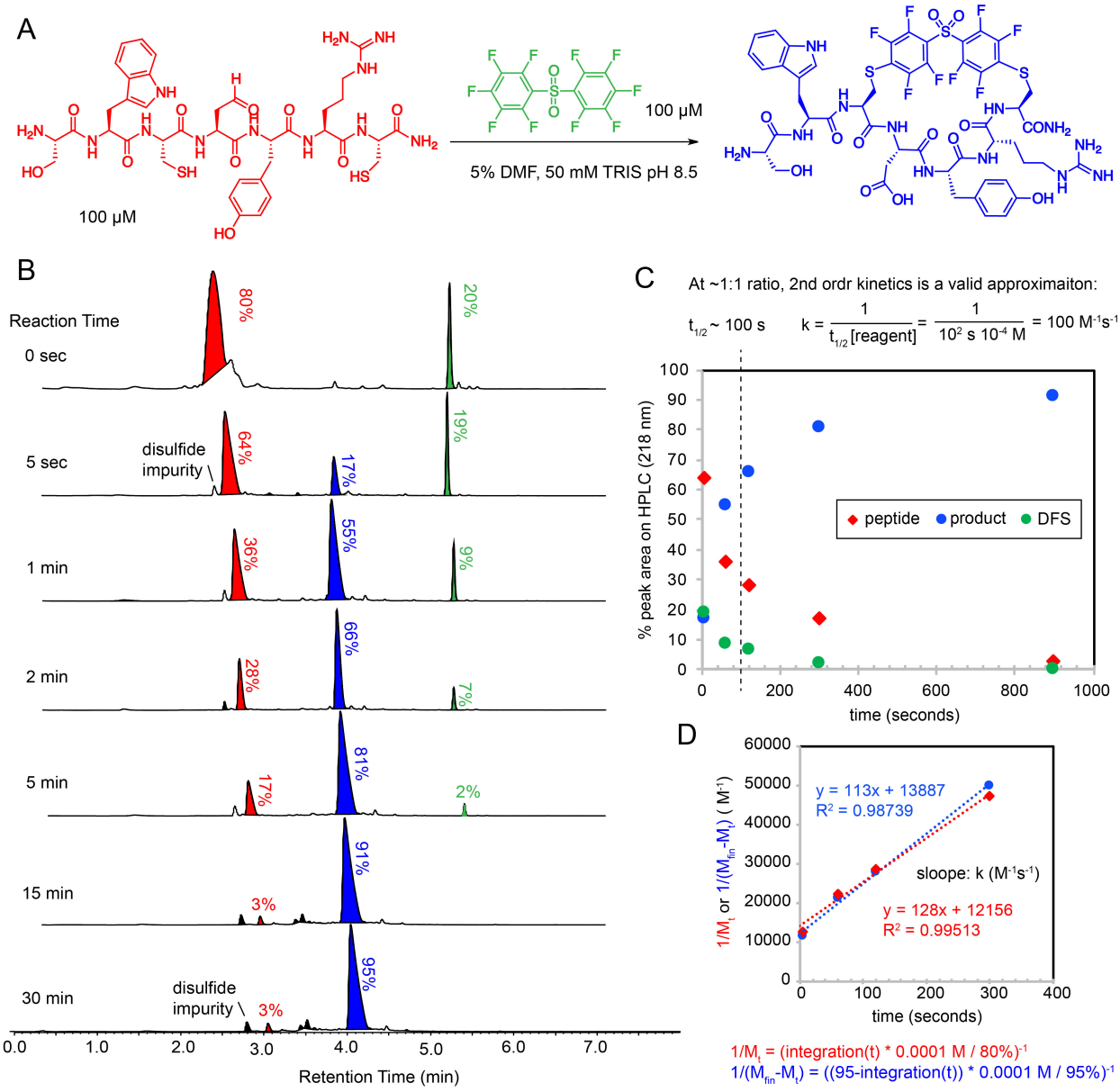


Supporting Figure S5. UV measurement of kinetics between **DFS** (20 μM) and peptides in 8:2 (v/v) ratio of MeCN and 50 mM Tris, pH 8.5. We placed 2 μL of peptide solution (4 mM in H_2O) in wells of quartz-bottom 96-well plate, added 100 μL of **DFS** solution, and monitored the absorbance at 320 nm every 10 seconds. The data was fit to using MatLab (see **Supporting Script S1**) to pseudo-first order kinetic process: $A_t = A_0 + A(1-e^{-kCt})$, where A_t is absorbance at time t, A_0 is initial absorbance, A is change in absorbance due to formation of the product, k rate constant, and C is the initial concentration of DFS. The numbers are fit value and [percent confidence of the fit].

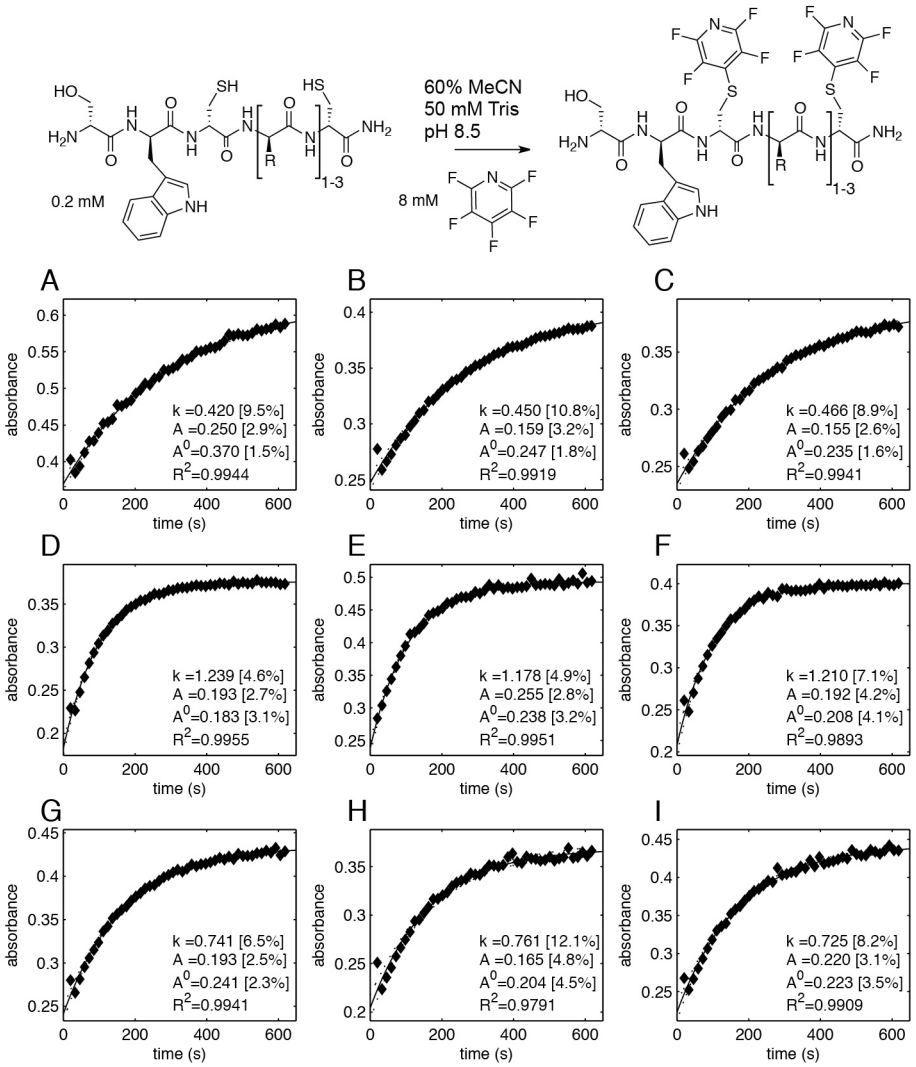
Absorbance correlates with the amount of the formed product because fit value of A was proportional to concentrations of the peptide: compare (G-H) and (J-H).



Supporting Figure S6. UV measurement of kinetics between **DFS** (80 μM) and peptide SWCRC or SWCAC at (A-F) 6:4 (v/v) or (G-L) 3:7 (v/v) ratio of MeCN and 50 mM Tris, pH 8.5. We placed 8 μL of peptide solution (0.4 mM in H_2O) in wells of quartz-bottom 96-well plate, added 200 μL of **DFS** solution, 80 μM in MeCN:Tris-buffer. We monitored the absorbance at 320 nm every 10 seconds and processed the data using MatLab (**Supporting Script S1**) as described in the legend for the Supporting Figure S5.

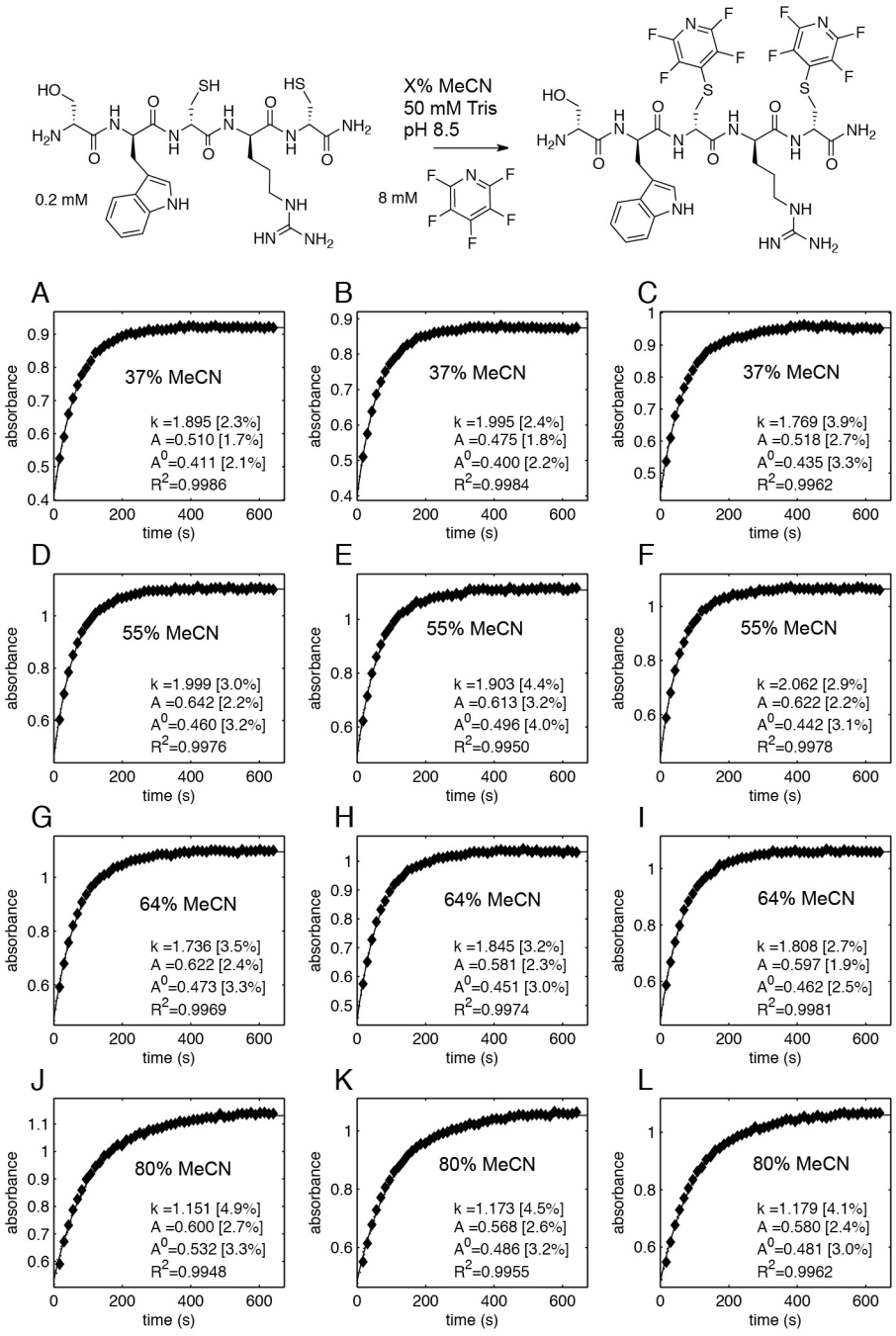


Supporting Figure S7. (A) Reaction between SWCDYRC (100 µM) and **DFS** (100 µM) in 5% DMF: Tris pH 8.5. (B) The reaction was monitored by LCMS for 30 min (only LC traces are shown). MS confirmed the identity of each product; peaks corresponding to peptide, **DFS** and product are color coded. Minor impurity present in the starting material—cyclic disulfide of the SWCDYRC peptide—served as a convenient internal reference that did not react with **DFS**. Peptide and **DFS** are completely consumed by 15 minutes yielding a single product. Residual 3% of the unreacted peptide is the result of the 3% variation in the calculation of stoichiometry and weighing error. (C) Plotting the conversion as a function of time estimated the half-life of the reaction to be 100 s, from which the rate constant can be estimated to be 100 $M^{-1}s^{-1}$. An alternative fitting of the data to derive the rate constant as a slope of linear plot of inverse concentrations of starting material ($1/M_t$) or product: $1/(M_{fin}-M_t)$. These concentrations were derived from the integrations using initial concentration of the reagents (0.0001 M) and initial (80%) or final (95%) integrations as shown in the equation. Estimates or rate constant are 113-128 $M^{-1}s^{-1}$.



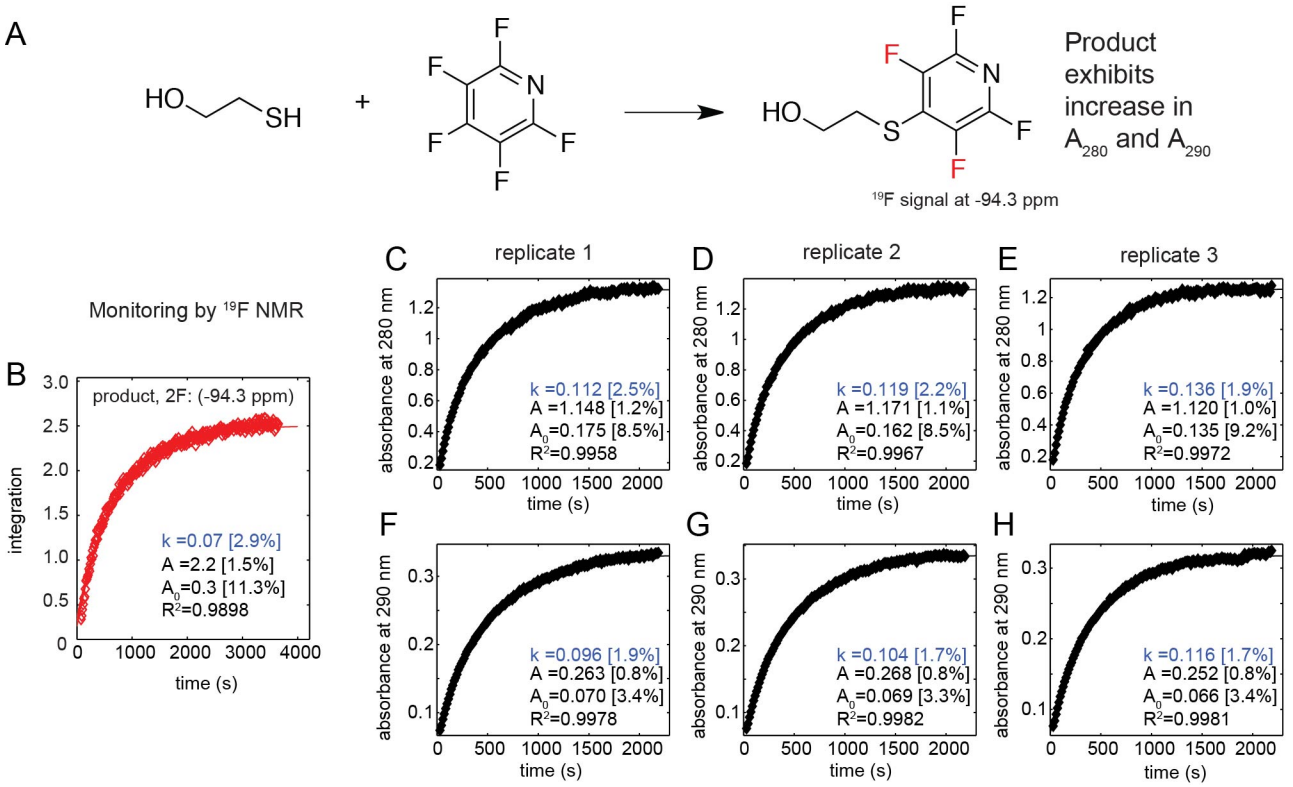
Supporting Figure S8. Example of the UV measurement of kinetics between pentafluoropyridine and three different peptides: (A-C) SWCDYRC; (D-F) SWCRC; (G-I) SWCAC. We placed 5 μL of peptide solution (4 mM in H_2O) in wells of quartz-bottom 96-well plate, added 100 μL of fPy solution (8 mM in 50 mM Tris, pH 8.5 in 4:6 (v/v) H_2O:MeCN) and monitored the absorbance at 280 nm every 13 second.

The data was processed using MatLab (**Supporting Script S1**) to fit the reading to pseudo-first order kinetic process: $A_t = A_0 + A(1-e^{-kCt})$, where A_t is absorbance at time t, A_0 is initial absorbance, A is change in absorbance due to formation of the product, k rate constant, and C is the initial concentration of the excess reagent (here, fPy). We fit three parameters simultaneously. The values represent the fit value and percent confidence of the fit defined as: 100% x (95% upper bound – 95% lower bound) / fit value.

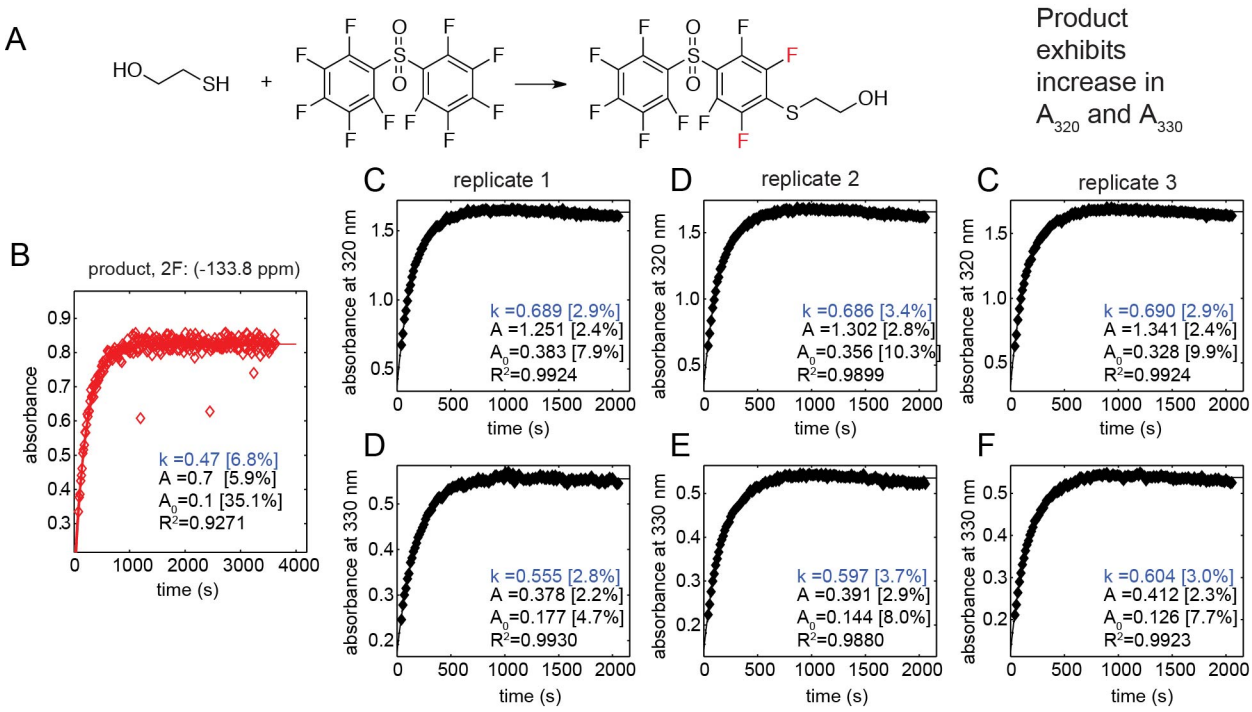


Supporting Figure S9. Example of the UV measurement of kinetics between pentafluoropyridine and peptide SWCRC in solvents of different water-MeCN content: (A-C) 37% MeCN; (D-F) 55% MeCN; (G-I) 64% MeCN; (J-L) 80% MeCN. We placed 10 μL of peptide solution (4 mM in H_2O) in wells of quartz-bottom 96-well plate, added 100 μL of fPy solution (8 mM in 50 mM Tris, pH 8.5 in H_2O:MeCN at indicated ratio), and monitored the absorbance at 280 nm every 13 second.

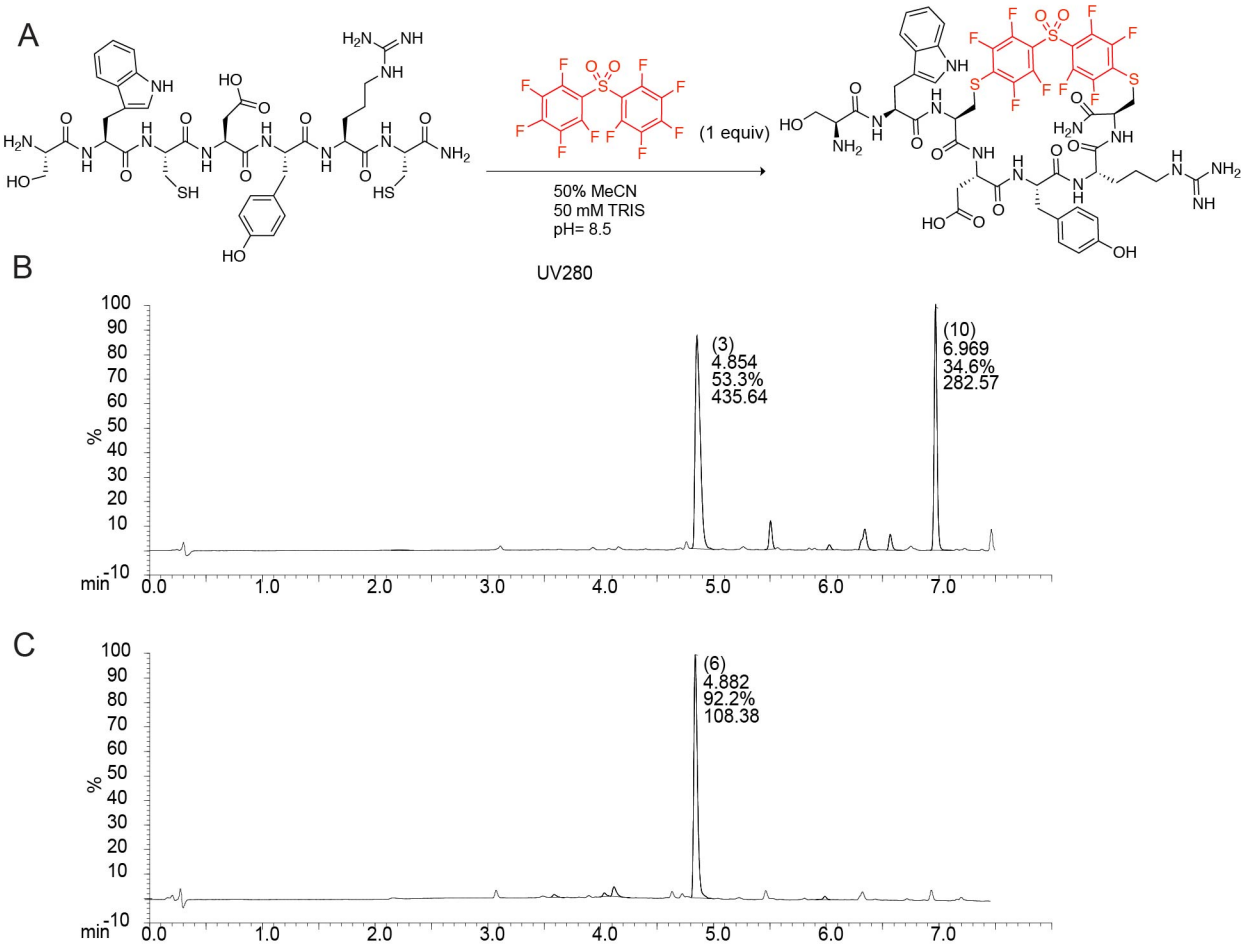
The data was processed using MatLab (**Supporting Script S1**) to fit the reading to pseudo-first order kinetic process: $A_t = A_0 + A(1\text{-}e^{-kCt})$, where A_t is absorbance at time t, A_0 is initial absorbance, A is change in absorbance due to formation of the product, k rate constant, and C is the initial concentration of fPz. The numbers are fit value and [percent confidence of the fit].



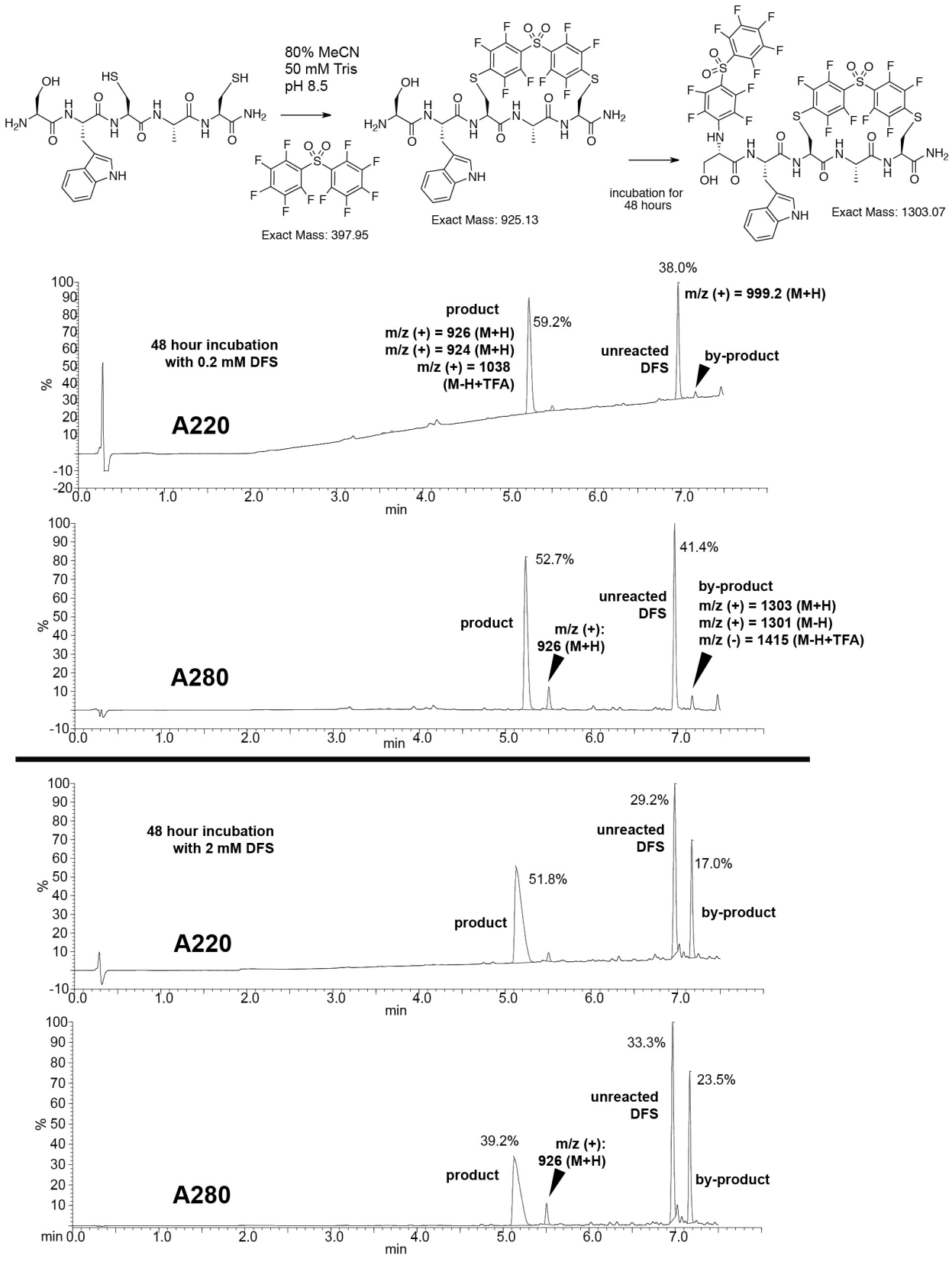
Supporting Figure S10. Reaction rates between pentafluoropyridine and βME measured by UV are similar to those measured by NMR. ^{19}F NMR and UV kinetics were acquired using the same stock solutions. Concentration of excess reagent (here, pentafluoropyridine) is 20 mM.



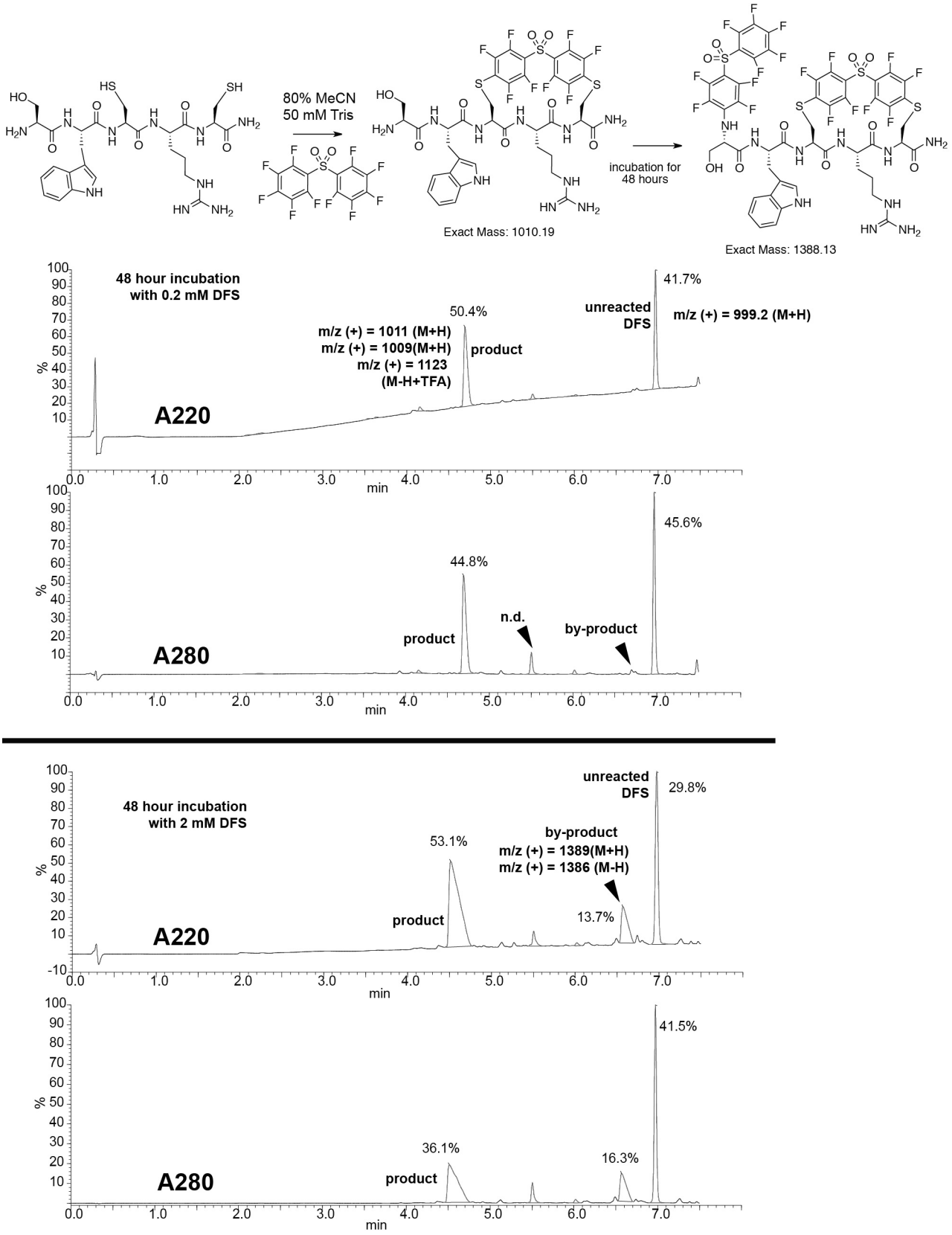
Supporting Figure S11. Reaction rates between **DFS** and βME measured by UV are similar to those measured by NMR. ^{19}F NMR and UV kinetics were acquired using the same stock solutions. Concentration of excess reagent (here, **DFS**) is 10 mM.



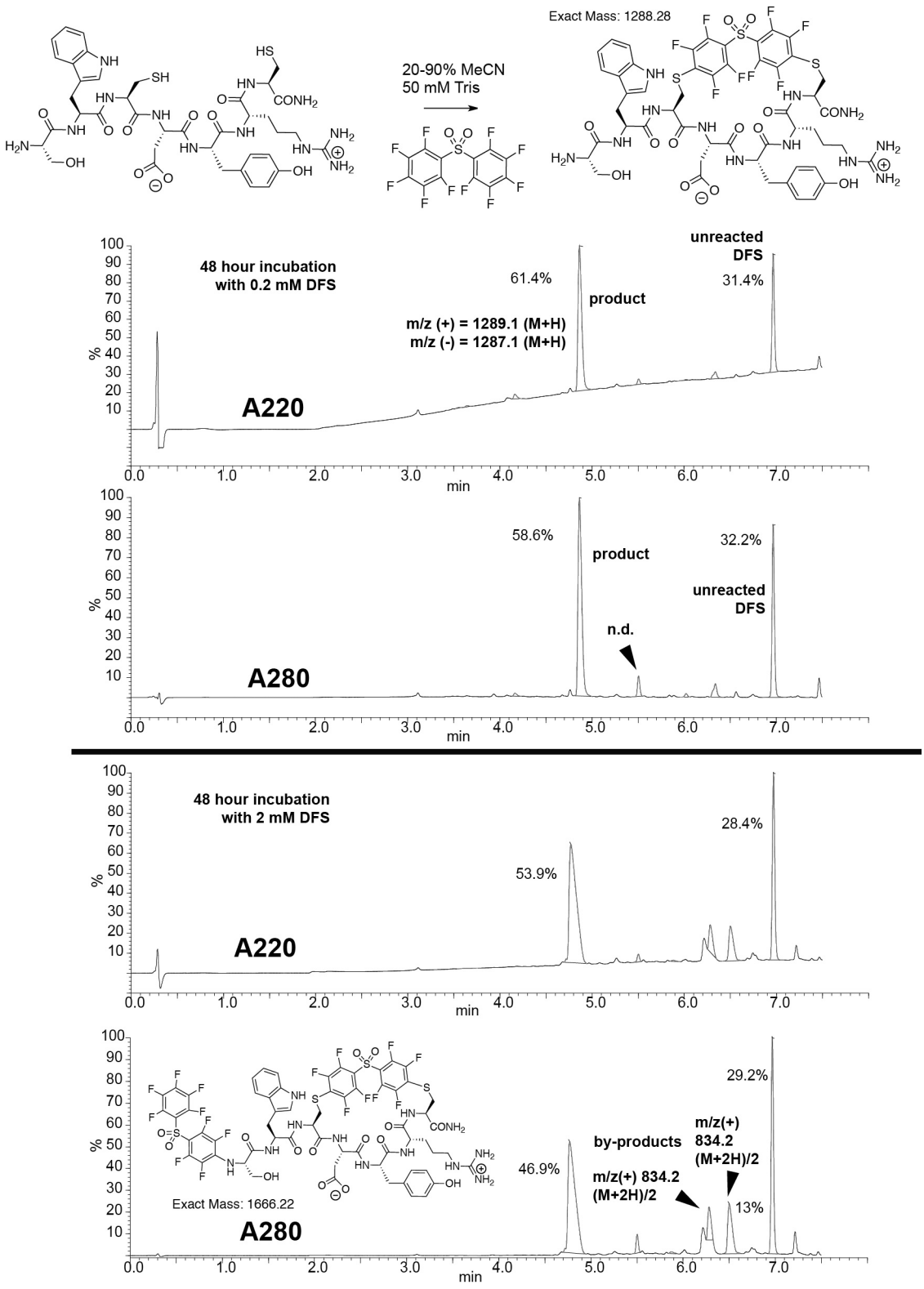
Supporting Figure S12. Low solubility of **DFS** in aqueous solution revealed by LCMS analysis of the composition of the reaction between peptide SWCDYRC and excess of **DFS**. (A) Reaction scheme. Reactions mixture was generated by combining the solutions of peptide in water, aqueous Tris buffer (pH 8.5), a stock solution of **DFS** in MeCN, and a pre-calculated amount of MeCN to yield a desired volume fraction of MeCN (20% or 90%) and a desired final concentration of **DFS** (0.2 mM). Reactions were incubated overnight and analyzed by LCMS. (B) Analysis of the reaction, performed in the solvent that contains 90% MeCN. Peak at 4.8 min corresponds to the product (SWCDYRC-DFS conjugate), whereas peak at t=7 min corresponds to unreacted **DFS**. (C) In solution that contains 20% MeCN, unreacted **DFS** is virtually undetectable in solution due to its low solubility. Based on the integration of the peak at t=7 min we estimate that true concentration of dissolved **DFS** in this reaction was <0.001 mM (the rest of DFS precipitated out of the solution, and thus, was not detected on the LC trace). Note that this reaction still proceeds to completion to yield the SWCDYRC-**OFS** conjugate. Both LC traces were recorded at 280 nm. MS validated the identified of the product and unreacted **DFS**.



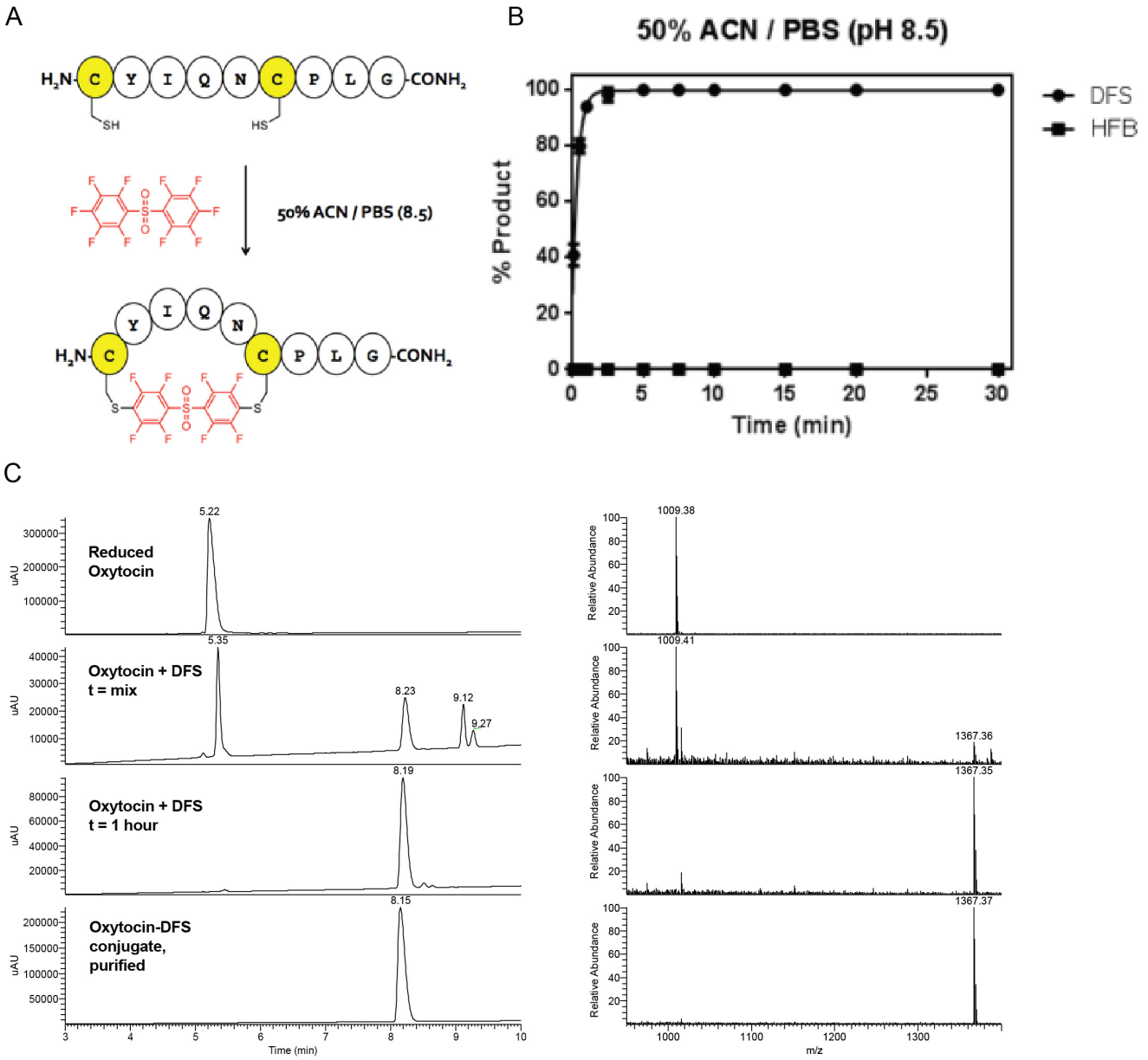
Supporting Figure S13. Analysis of the reaction products and by-products over long-term incubation by LCMS. Example 1: SWCAC peptide.



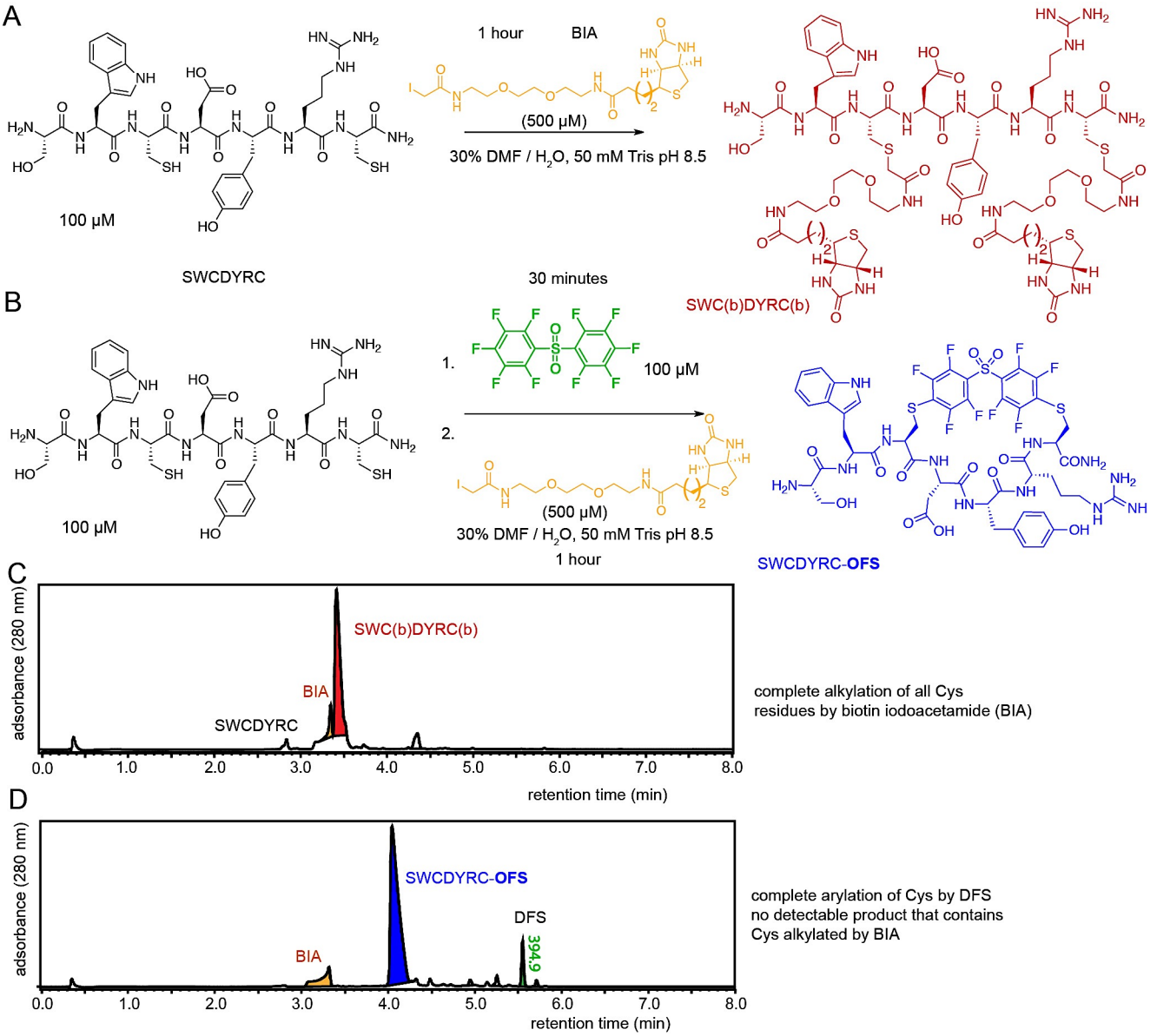
Supporting Figure S14. Analysis of the reaction products and by-products over long-term incubation by LCMS. Example 2: SWCRC peptide.



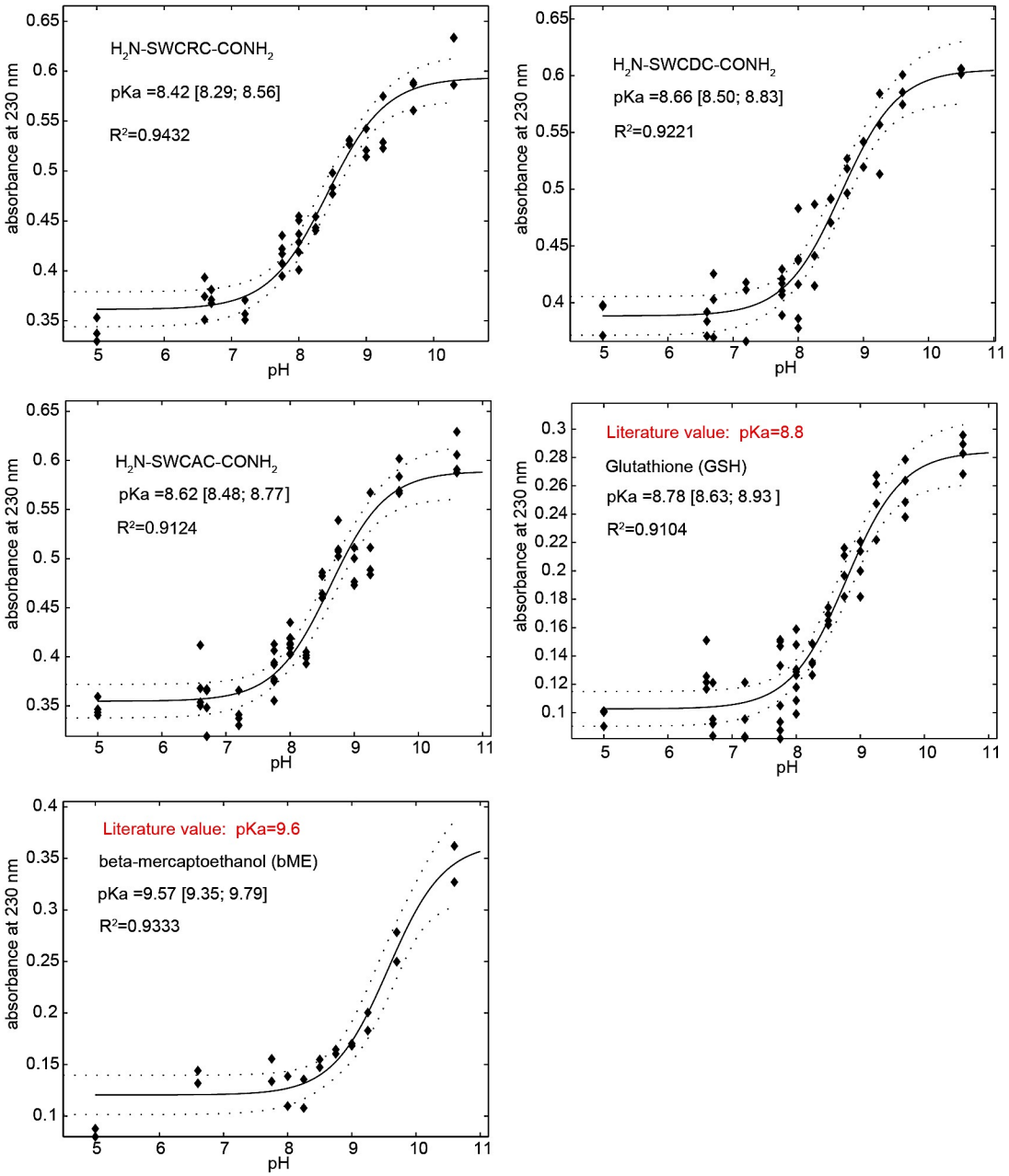
Supporting Figure S15. Analysis of the reaction products and by-products over long-term incubation by LCMS. Example 3: SWCDYRC peptide.



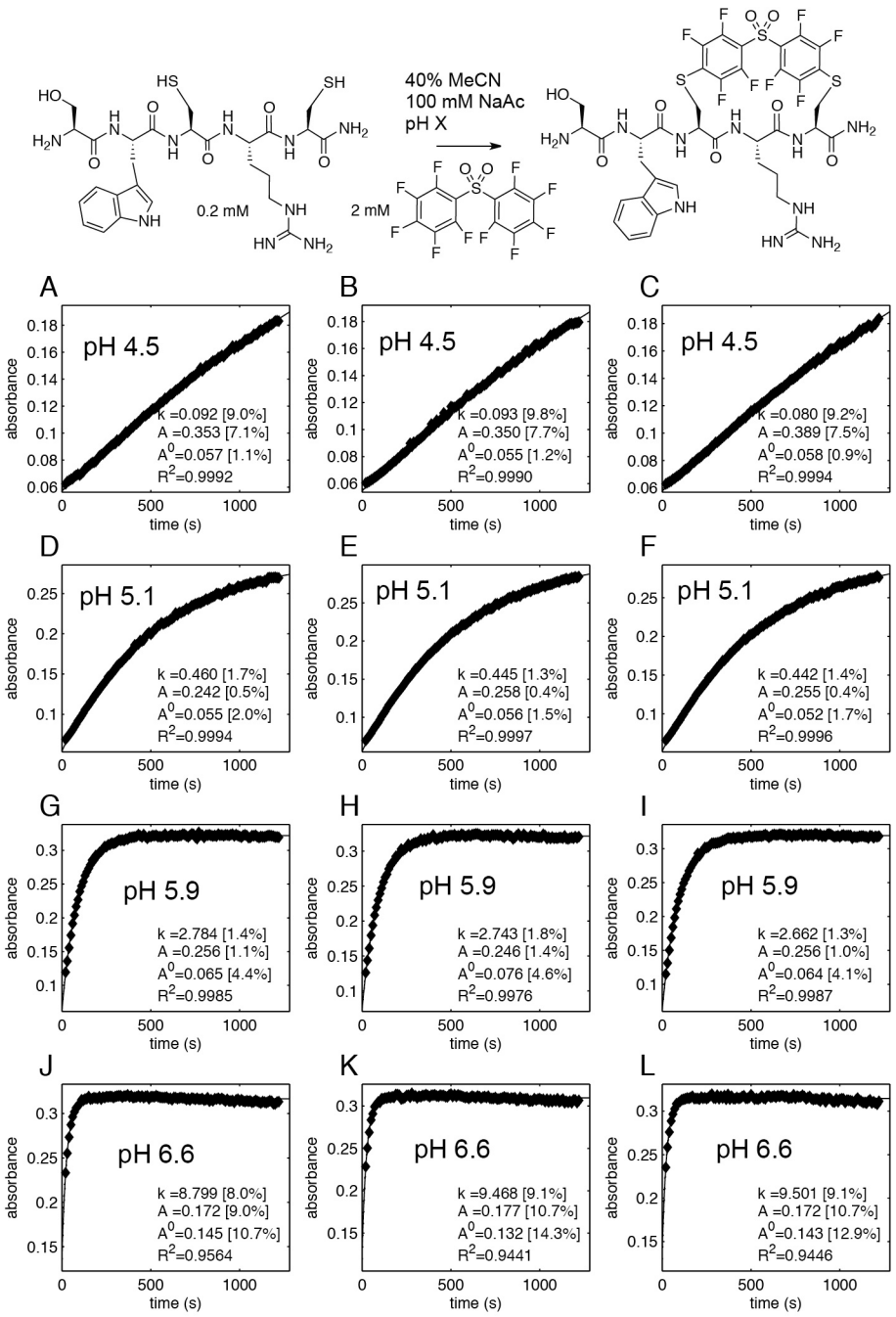
Supporting Figure S16. (A-B) Kinetic trace of reaction between oxytocin (0.1 mM) and **DFS** (1 mM) or hexafluorobenzene (**HFB**, 1 mM) at pH=8.5 acquired using HPLC-LCMS. At specific times, the reaction was quenched with TFA and injected on HPLC. Data is average from two independent experiments. Rate constant k = 37.2 $M^{-1}s^{-1}$. Note that **HFB** is completely unreactive in these conditions. (C) Preparative synthesis of oxytocin-octafluorosulfone (OFS) macrocycle. 10 mg oxytocin + 5 eq **DFS** in 50% MeCN / PBS (7.4) for 1 h. Traces at T=mix, t=30 sec, t=1 hour, and after purification. Traces on the right are total MS signature of the LC trace from 3 to 10 minutes



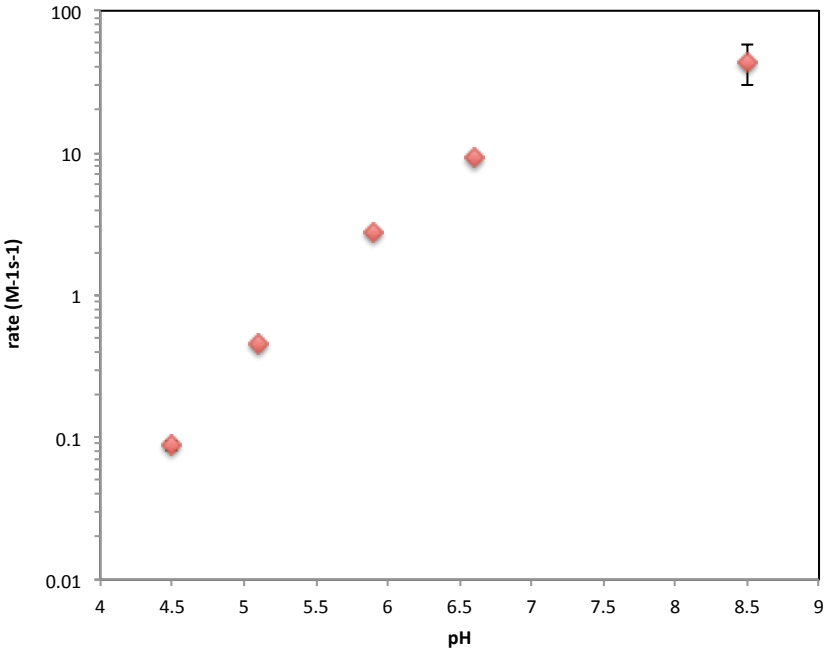
Supporting Figure S17. (A) Reaction between SWCDYRC peptide (100 µM) and biotin iodoacetamide (BIA, 500 µM) in aqueous Tris buffer (50 mM, pH 8.5) supplemented with 30% DMF for 1 hour. (B) The reaction between SWCDYRC (100 µM) and **DFS** (120 µM) in 30% DMF for 30 min, followed by addition of BIA (500 µM) and additional incubation for 1 h. (C-D) LCMS analysis of reactions shows complete alkylation of Cys residues by BIA (A) and complete conversion to octafluorosulfone (OFS) containing macrocycle (denoted as SWCDYRC-**OFS**). Although the product has been incubated with BIA for 1 hour, we observed no alkylation of the product by BIA confirming that **DFS** reacted with two Cys residues. Related experiment showing the time course of arylation of this peptide by **DFS** in 5% DMF and location of the starting material is available in **Figure S7**.



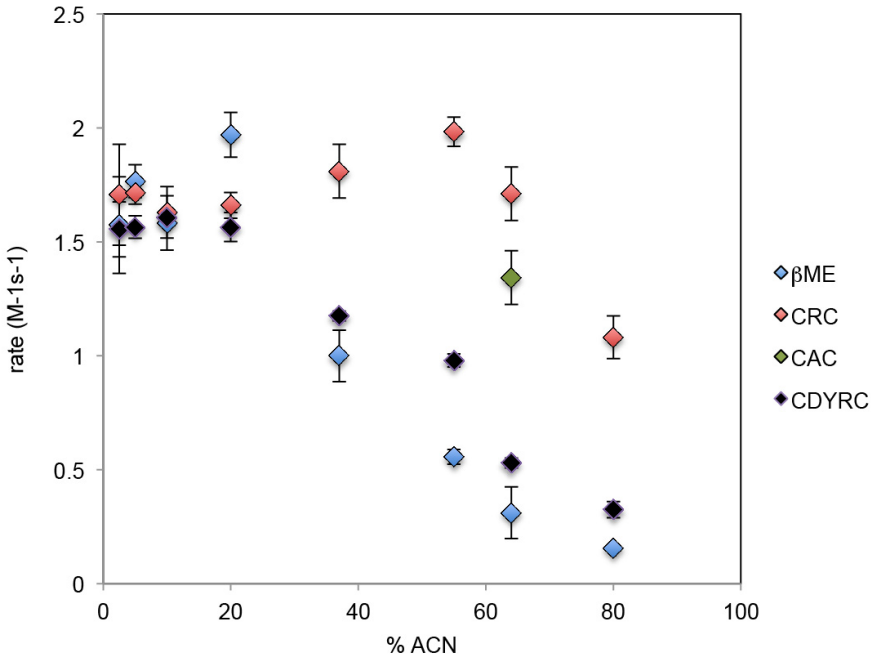
Supporting Figure S18. The pKa of thiol group in different peptides measured by a method similar to that described by Woycechowsky and Raines.[6] A stock of peptides (2 μL, 5 mM) was added to 100 μL of 50 mM Tris- or acetate-buffered solutions of pre-measured pH. Absorbance at 230 nm for each pH was recorded in replicates of 4 using SpetraMax plate reader and quartz-bottom 96-well plate. The data was fit to Henderson-Hasselbach equation in MatLab. Value represents the average fit, and values in brackets are the 95% confidence bounds of the fit. Glutathione and βME were used as reference, and the pKa values measured for these compounds closely resembled the literature values.



Supporting Figure S19. UV measurement of kinetics between **DFS** (2 mM) and peptide SWCRC at four different pH values. We placed 5 µL of peptide solution (4 mM in H_2O) in wells of quartz-bottom 96-well plate, added 100 µL of **DFS** solution, 2 mM in 100 mM Na-acetate, pH 4.5, 5.1, 5.9, or 6.6 and mixed MeCN in with 6:4 (v/v) ratio. We monitored the absorbance at 320 nm every 10 seconds and processed the data using MatLab (**Supporting Script S1**) as described in the legend for the Supporting Figure S5.



Supporting Figure S20. Kinetics of reaction between DFS (2 mM) and peptide SWCRC in 40%:60% (v/v) water:MeCN and different pH values. Value at pH 8.5 was measured as described in Supporting Figure S6. Values at pH 6.6 and below were measured as described in Supporting Figure S19 with one exception: data depicted here described solutions that contained 60% MeCN.

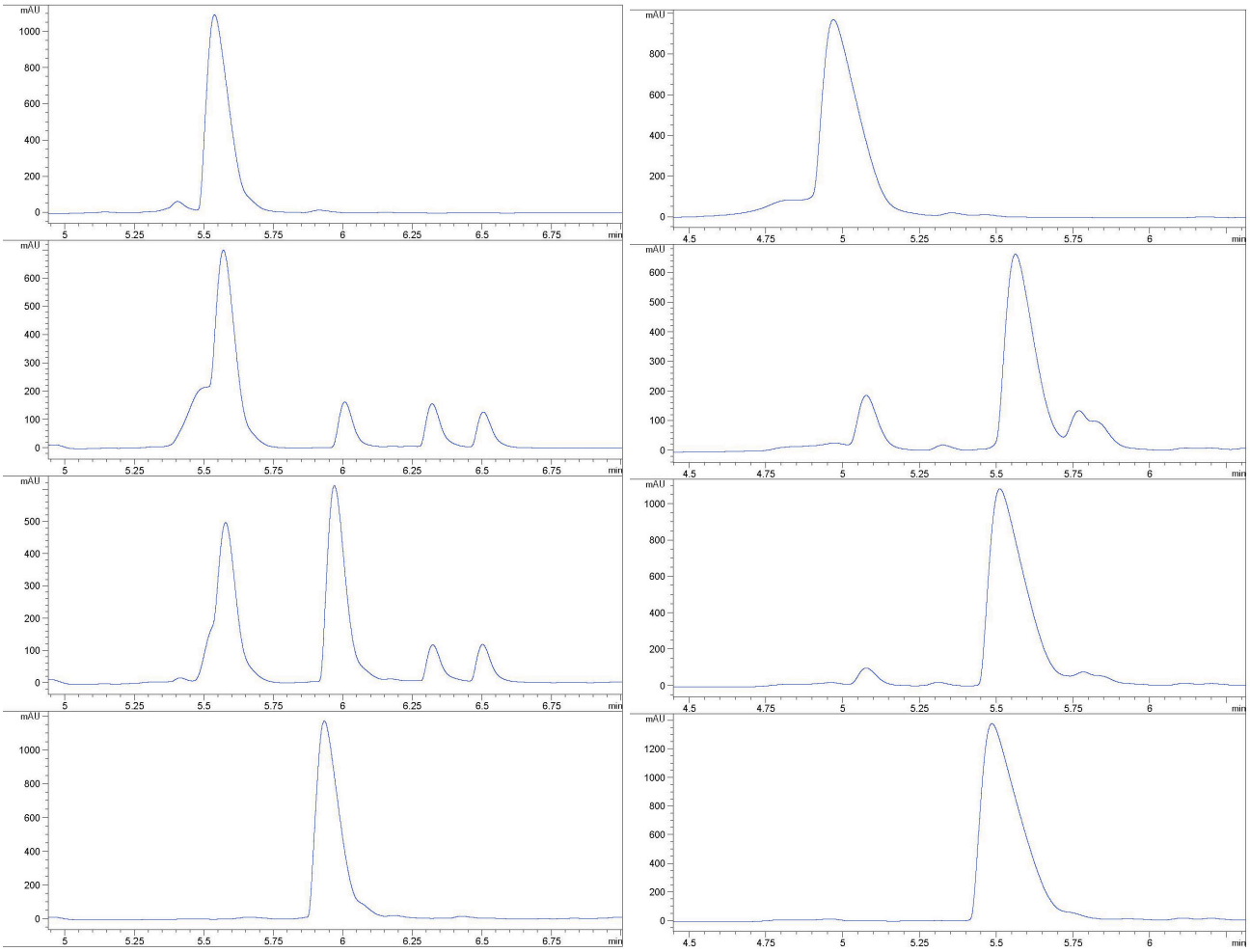


Supporting Figure S21. Summary of kinetics between perfluoropyridine various and βME or peptides, water:MeCN Tris buffer pH 8.5 values are indicated. See Supporting Figures S5, S6, and S10 for example of raw traces and specific procedures.

General procedure: We placed 5 µL of peptide of βME solution (4 mM in H_2O) in wells of quartz-bottom 96-well plate, added 100 µL of perfluoropyridine solution (8 mM in 50 mM Tris, pH 8.5 in **X:Y** (v/v) H_2O:MeCN) and monitored the absorbance at 280 nm every 13 seconds.

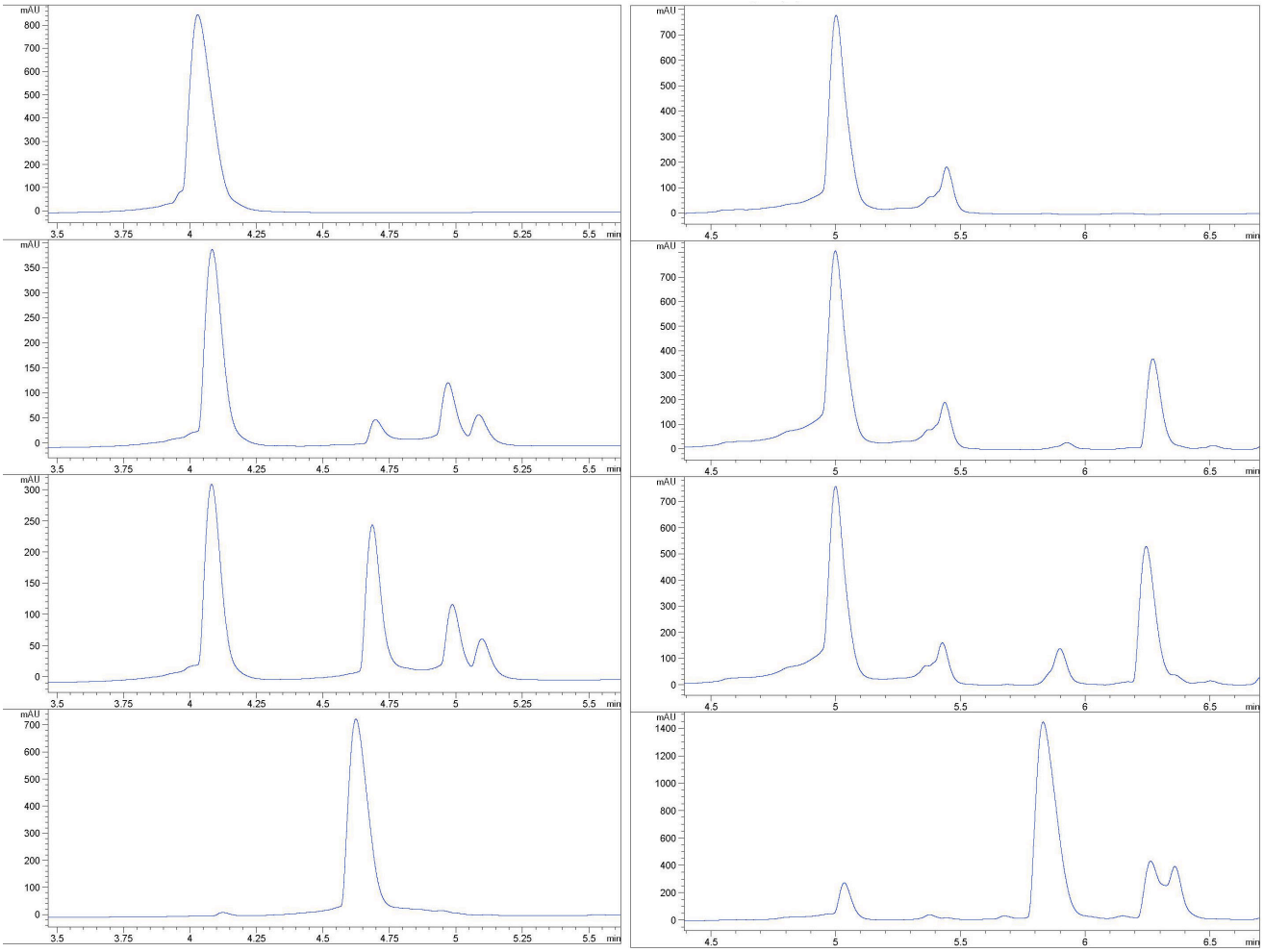
The data was processed using MatLab (**Supporting Script S1**) to fit the reading to pseudo-first order kinetic process: $A_t = A_0 + A(1\text{-}e^{-kCt})$, where A_t is absorbance at time t, A_0 is initial absorbance, A is change in absorbance due to formation of the product, k rate constant, and C is the initial concentration of the excess reagent (here, perfluoropyridine). We fit three parameters simultaneously. The values represent the fit value and percent confidence of the fit defined as: 100% x (95% upper bound – 95% lower bound) / fit value.

(A) Melanin-concentrating hormone + DFS (B) Salmon calcitonin + DFS



Supporting Figure S22. (A-B) Kinetic analysis of reaction between MCH or salmon calcitonin (0.1 mM) and **DFS** (1 mM) at pH=8.5 acquired using HPLC. This data was used in Main Text Figure 3C. A small aliquot of the reaction mixture was removed and immediately quenched by TFA for analysis by HPLC. Shown are four different time points (top-to-bottom): prior to addition of **DFS**, after mixing (~5-10 seconds), 30 seconds, and 5 minutes. Both reactions proceed to complete conversion in 5 minutes. All LC-traces were recorded at 215 nm.

(A) Atrial natriuretic factor + DFS (B) Urotensin II+ DFS



Supporting Figure S23. (A-B) Kinetic analysis of reaction between ANF (1-28) or urotensin II (0.1 mM) and **DFS** (1 mM) at pH=8.5 acquired using HPLC. This data was used in Main Text Figure 3C. A small aliquot of the reaction mixture was removed and immediately quenched by TFA for analysis by HPLC. Shown are four different time points (top-to-bottom): prior to addition of **DFS**, after mixing (~5-10 seconds), 30 seconds, and 5 minutes. Both reactions proceed to nearly complete conversion in 5 minutes. All LC-traces were recorded at 215 nm.

General procedure for modification of phage with DFS and quantification of the yield

To a solution of 10^{11} pfu/mL of phage in 50 mM Tris buffer (pH 8.5), we added 2 μL of 100 mM TCEP and incubated this solution at r.t. for 30 min. To modify the phage with biotinylating agent, we added 5 μL of the above solution to 91 μL of MeCN/Tris buffer (50 mM, pH = 8.5), followed by 4 μL of 100 mM Biotin iodoacetamie (BIA). We incubated the reaction at r.t. for 30 min, diluted 10^5 fold to the concentration of ~10^4 pfu/mL with LB medium and determined the biotinylation of phage by **biotin capture** as following: First, we titered diluted phage solution to determine the number of biotinylated and non-biotinylated phage in this solution ("titer before"). We then incubated the diluted solution of phage (100 μL) with suspension of streptavidin coated magnetic beads (20 μL, Promega) for 15 minutes and captured the beads using a magnetic stand. Titering of the supernatant yielded the amount of non-biotinylated phage ("titer after"). The % yield of biotinylation was determined as:

Yield = [("titer before" - "titer after") / "titer before"] x 100%

To modify the phage with DFS, we combined 5 μL of the TCEP-reduced phage solution with to 85 μL of a 70/30 mixture of MeCN/Tris buffer (50 mM, pH = 8.5) and 10 μL of DFS solution (100 mM in MeCN). The reaction was incubated at r.t. for 30 min and the number of unreacted thiols was determined using BIA-labeling and biotin-capture. Briefly, we added 4 μL of BIA to the reaction mixture, incubated it for 30 min, diluted 10^5 fold to the concentration of ~10^4 pfu/mL with LB medium, and determined the yield of biotinylation of phage by biotin capture as described above

Modification of phage libraries (New England Biolabs, Ph.D.™-C7C) by DFS in 5% DMF.

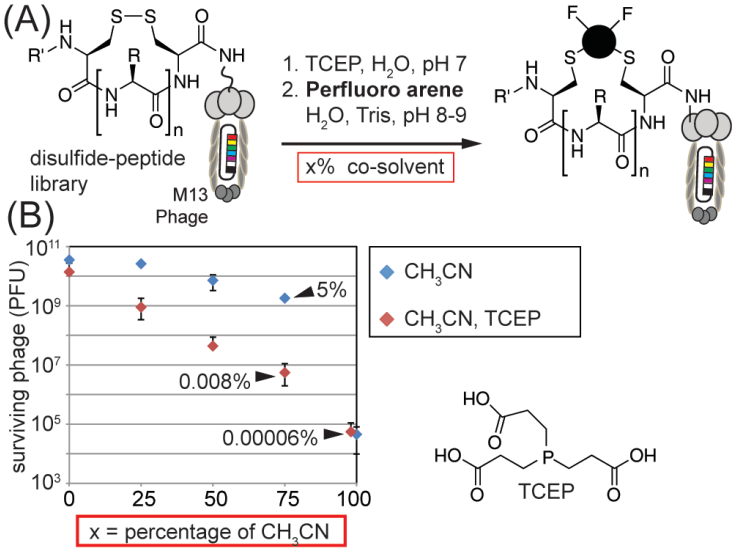
First, we combined phage library stock* with reducing agent and incubated for 30 minutes.

- 5 μL of library stock solution (3x10^{12} pfu/mL, total number of clones: 1.5x10^{10} pfu)
- 10 μL of 10x Tris buffer (500 mM solution of Tris, pH 8.5)
- 2 μL of TCEP stock solution (100 mM in water)
- 83 μL of water

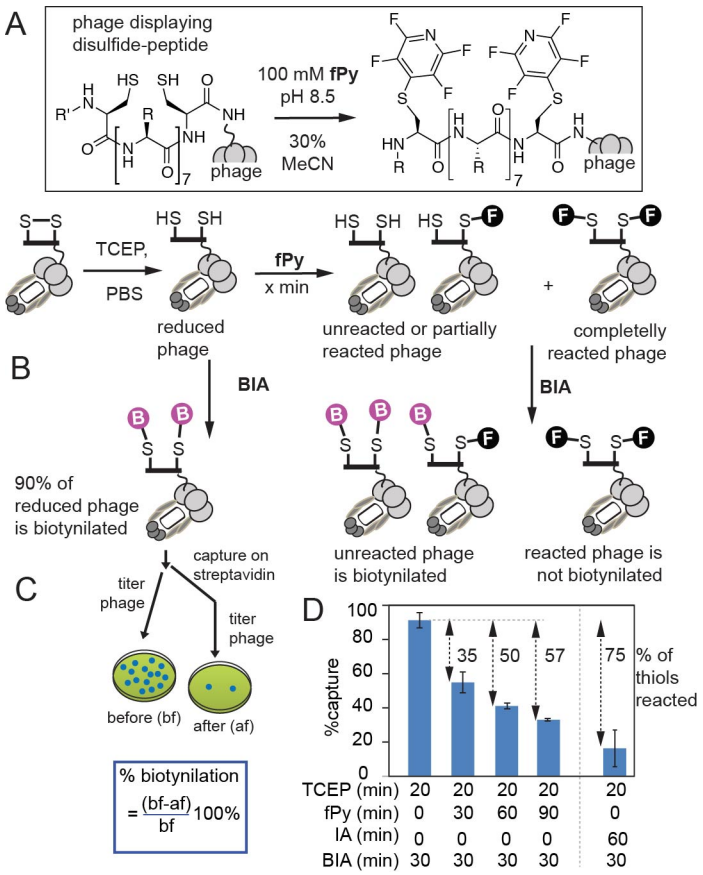
Total **100 μL of "reduced library"**

We modified 90 μL of the "reduced library" with **DFS**. Reagents were mixed in the indicated order and incubated for 30 minutes or 2 hours: • 90 μL of the "reduced library" • 335 μL water • 50 μL of 10x Tris buffer • 22.5 μL of DMF • 2.5 μL of **DFS** stock (100 mM in DMF) Total: **500 μL of the "OFS-library"**** We then used 50 μL of the "**OFS**-library" mixture to estimate the number of unreacted thiols: • 50 μL of the "**OFS**-library" • 39 μL water • 10 μL of 10x Tris buffer • 1 μL of BIA stock (100 mM in DMF) After 30 min incubation, we determined the biotinylation by biotin capture as described above	We used 10 μL of the "reduced library" mixture to estimate the number of phage with reduced thiols • 10 μL of the "reduced library" • 76 μL water • 10 μL of 10x Tris buffer • 1 μL of BIA stock (100 mM in DMF) After 30 min incubation, we diluted the phage 10^5 fold to the concentration of ~10^4 pfu/mL with LB medium, and determined the yield of biotinylation of phage by biotin capture as described above

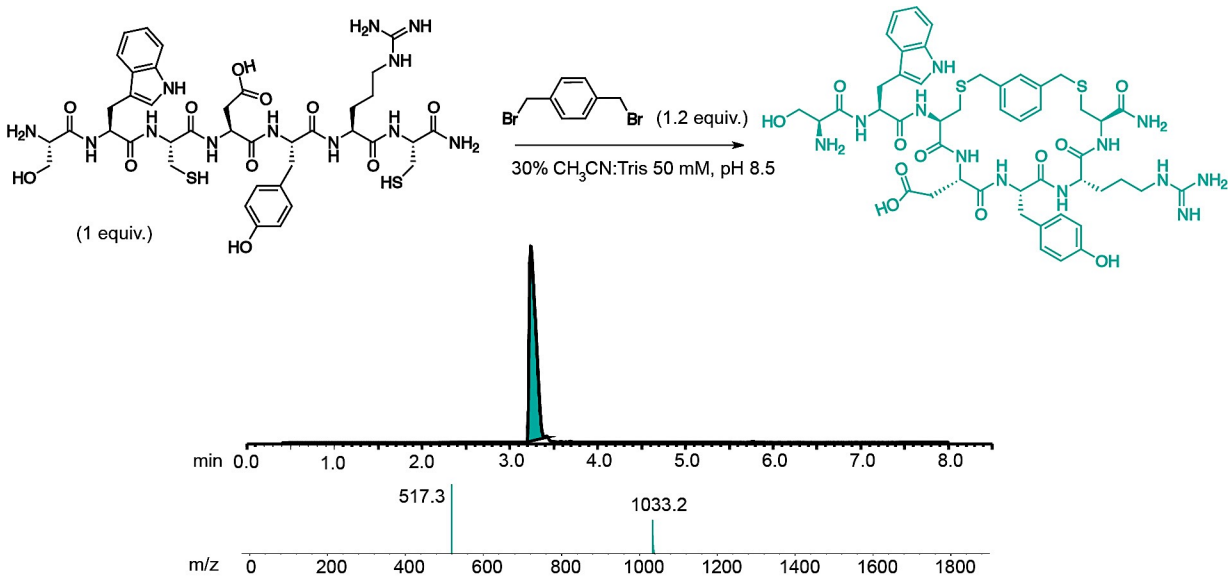
* Commercial library kit contains 0.1 mL of 10^{13} pfu/mL of phage in glycerol. For optimization of DFS-modification, we recommend using re-amplified a stock of this library. If library will be used for panning, the original stock should be used.
** OFS-modified library, ~10^{10} pfu/mL, can be purified by dialysis and used for standard phage display selection.



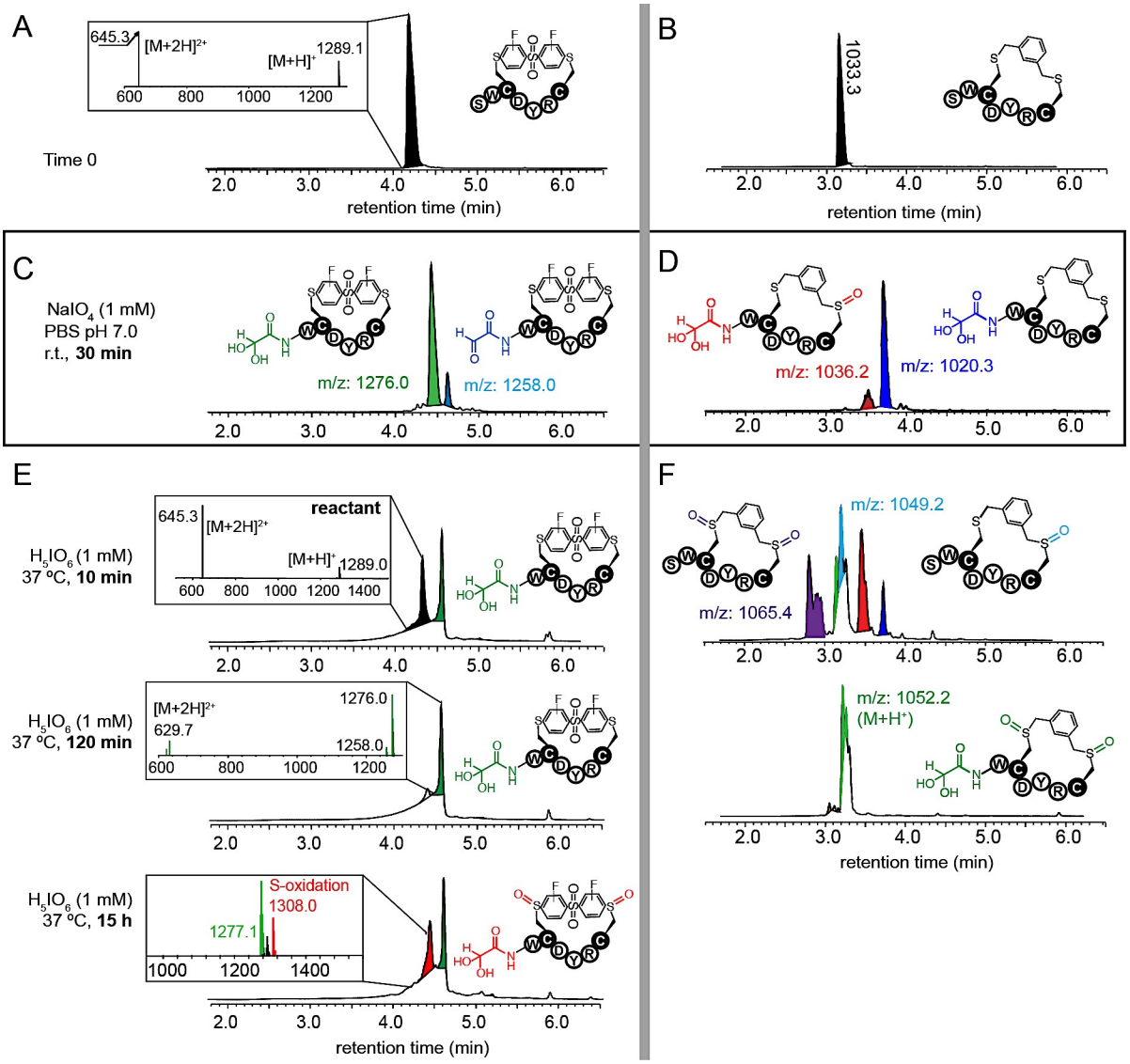
Supporting Figure S24. (A) To permit arylation of Cys-residues, the phage has to be exposed to a reducing agent, such as TCEP,[7, 8] followed by the reaction with the electrophile in an organic solvent. Most fAr are water-insoluble and require organic co-solvent. (B) M13 phage tolerates MeCN as co-solvent at up to 50% MeCN at pH=8-9 for 3 hours. Increasing the fraction of MeCN resulted in decreased phage viability. The S_NAr substrates suitable for modification of phage, thus, have to be active in aqueous buffers that contain ≤50% MeCN. Reduction and arylation cannot be performed in "one pot" because the mixture of CH_3CN and 0.5 mM TCEP is toxic and TCEP has to be diluted or removed by dialysis or dilution to mitigate this toxicity. For example, our typical modification protocol (see previous page) used 10-fold dilution step after reduction to decrease the amount of TCEP present at the arylation step.



Supporting Figure S25. (A) Measuring the reactivity of phage with perfluoropyridine (fPy). (B) Reduction of the phage displayed peptide-disulfides produces in free thiols, which can be alkylated by fPy. To check the number of reduced thiols, we exposed phage to BIA. (C) We then measured the number of biotinylated phage using capture by streptavidin-coated magnetic beads and titering before and after capture. (D) Over 90% of the TCEP-reduced phage can be biotinylated by BIA. In contrast, only 55% of phage can be biotinylated by BIA, when TCEP-reduced phage is pulsed by fPy for 30 min. Increasing the length of the "pulse" with fPy to 60 and 90 minutes, decreased the number of thiols accessible to biotinylation. Disappearance of thiols has a half-life of ~60 min, which corresponds to pseudo-first order rate constant of $k = 1.6 \times 10^{-4}$ s^{-1}. At 100 mM concentration of fPy, it corresponds to the second order rate constant of $k = 1.6 \times 10^{-3}$ M^{-1} s^{-1}. Iodoacetamide (IA, 10 mM) reacts with phage significantly faster, and it alkylates >75% of the thiols in 30 min.



Supporting Figure S26. The reaction between SWCDYRC and dibromo-*meta*-xylene (**DBMX**) in 30% CH_3CN:Tris 50 mM, pH 8.5, for 1 hour yields complete conversion to SWCDYRC-**DMMX**

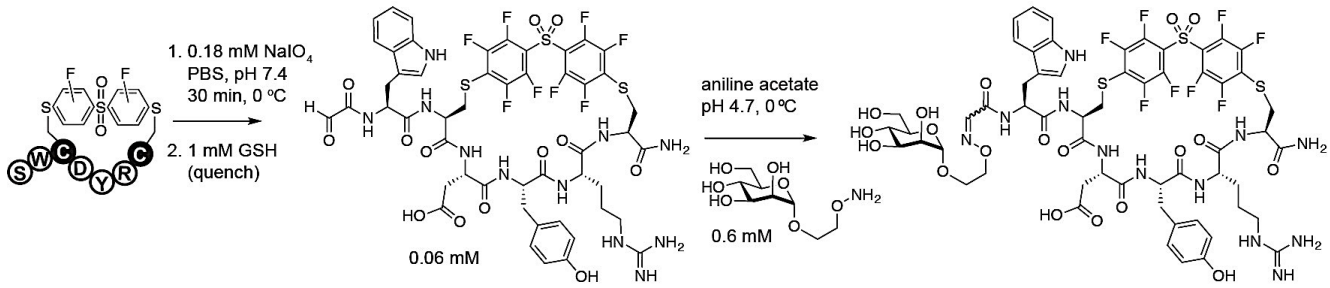


Supporting Figure S27. Oxidation of peptide conjugates (100 μM) with 1 mM $NaIO_4$ in PBS pH 7.0 at room temperature or 1 mM H_5IO_6 at 37 ºC monitored by LCMS. LC traces from LCMS analysis are color-coded. Where necessary we show major peaks observed in the MS spectrum confirming the identity of the products.

A) Purity of the SWCDYRC-**OFS** before oxidation. Due to space limitation, we use an abbreviated structure of octafluorosulfone. B) Purity of the conjugate of SWCDYRC and xylene before oxidation. C) Treatment of SWCDYRC-**OFS** with 1 mM $NaIO_4$ quantitatively converted N-terminal Ser to N-terminal aldehyde in 30 minutes. We did not detect any oxidation of aryl-sulfides after 30 minutes or even after 16 hours of incubation with 1 mM $NaIO_4$ at room temperature (data not shown). D) Unlike SWCDYRC-**OFS** conjugate, which is stable to oxidation, the conjugate of SWCDYRC and xylene is susceptible to unwanted oxidation. 30 min treatment with $NaIO_4$ (1 mM) in PBS pH 7.0 at room temperature oxidized 20% of the sulfide to sulfoxide.

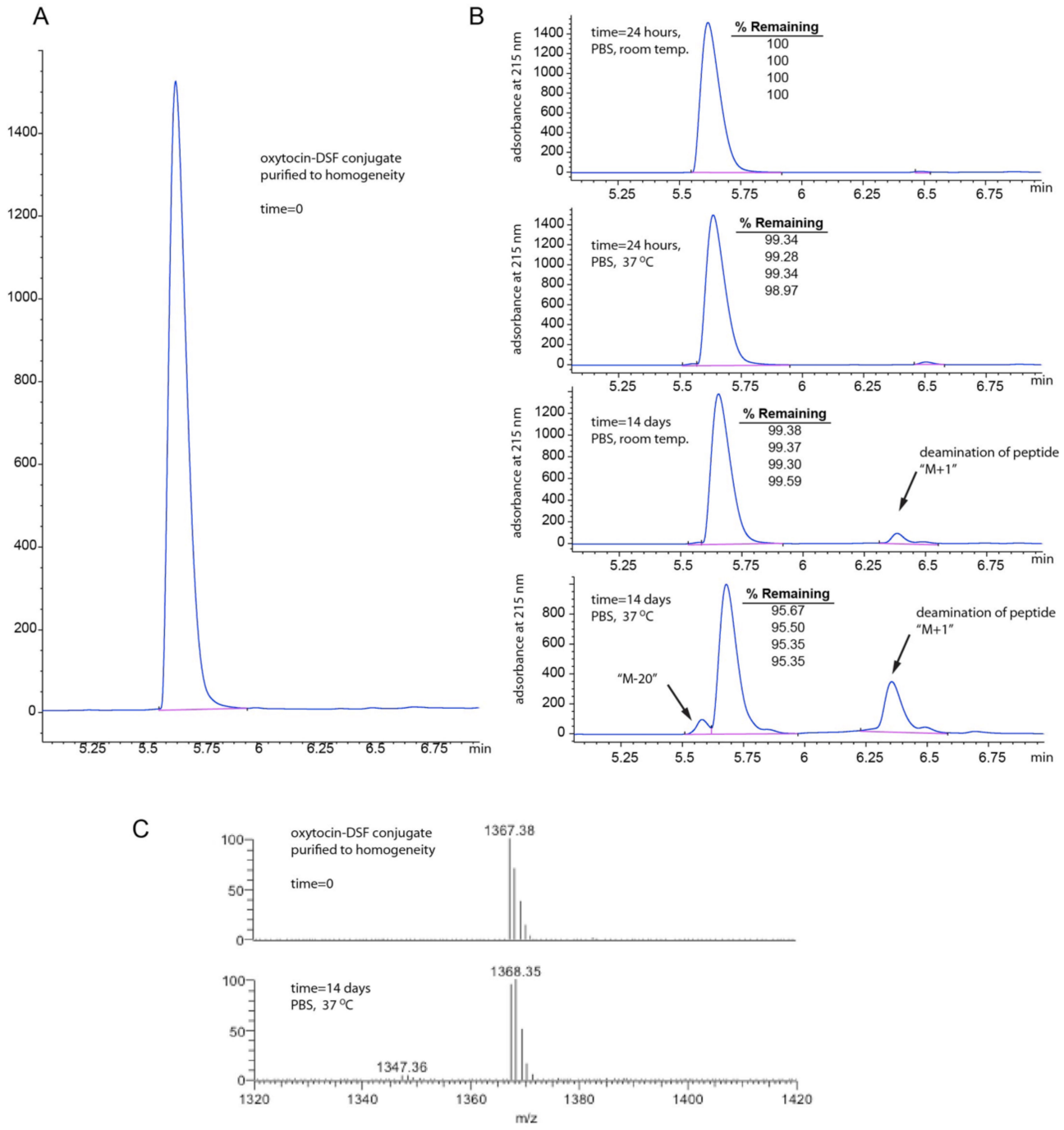
E) SWCDYRC-**OFS** conjugate is stable to treatment with 1 mM H_5IO_6 at 37 ºC. Less than ~50% of the **OFS**-sulfides were converted to sulfoxides after 15 h of incubation wit H_5IO_6. F) Even brief, 10 min treatment of the conjugate of SWCDYRC and xylene with 1 mM H_5IO_6 at 37ºC yields concomitant oxidation of both N-terminal Ser and sulfide. Among five distinct products, two are the conjugates in which sulfide has been oxidized before N-terminal Ser has been converted to aldehyde.

General protocol for oxidation of serine and oxime ligation[9]



A solution of peptide 0.12 mM SWCDYRC-**OFS** (e.g., see Figure S15) in 30% aqueous DMF was oxidized with $NaIO_4$ (0.18 mM final concentration) in 10X PBS,* pH 7.4 at 0 ºC. The oxidation reaction was quenched after 2 h by adding 1 mM glutathione (GSH) at 0 ºC for 15 min. A portion of the oxidized product was modified with $H_2NO\text{-}OC_2H_4$-mannose by combining 10 µl (0.06 mM) of Ald-WCDYRC-**OFS** and 10 µL (0.6 mM) solution of $H_2NO\text{-}OC_2H_4$-mannose in anilinium acetate (100 mM, pH 4.5). The pH of the reaction was adjusted to pH 4.5 by adding 4 µL of glacial acetic acid. The reaction mixture was kept at 0 ºC for 2 h and then was injected the reaction mixture into the LCMS (see main text Figure 6).

* 10X PBS is 1.37 M NaCl, 120 mM Na_2HPO_4, 27 mM KCl, pH 7.4



Supporting Figure S28. LCMS analyses of purified oxytocin-**OFS** macorcycle (A) stability over the period of 24 hours and 14 days at room temperature and 37 °C in phosphate-buffered saline (PBS) pH 7.4. (B) The numbers next to the peak represent results of measurements from four independent experiments. Even after 2 weeks at elevated temperature, 95% of the oxytocin-**OFS** conjugate is stable. (C) Total MS-traces of the oxytocin before and after 14 days of incubation at 37 °C. Major degradation product has mass M+1 (where M is the mass of oxytocin-**OFS** conjugate). This mass, most likely, corresponds to deamination of the C-terminus or a side chain. Minor side product at 5.6 min observed only after 14 days at 37 ºC has the mass of M-20. The identity of this by-product is unknown.

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