Supplementary Information for:

Tandem Wittig-Diels Alder diversification of genetically encoded peptide libraries

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Contents

Abbreviations

DIPEA	*N*,*N*-Diisopropylethylamine
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
dsDNA	Double-stranded DNA
ESI	Electrospray ionization
h	Hour
HBTU	2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HPLC	High-performance liquid chromatography
MeCN	Acetonitrile
min	Minute
MOPS	3-Morpholinopropane-1-sulfonic acid
MQ	Milli-Q
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RP	Reversed phase
rpm	Revolutions per minute
RT	Room temperature
s	Second
TFA	Trifluoroacetic acid
OPA	Oxaphosphetane

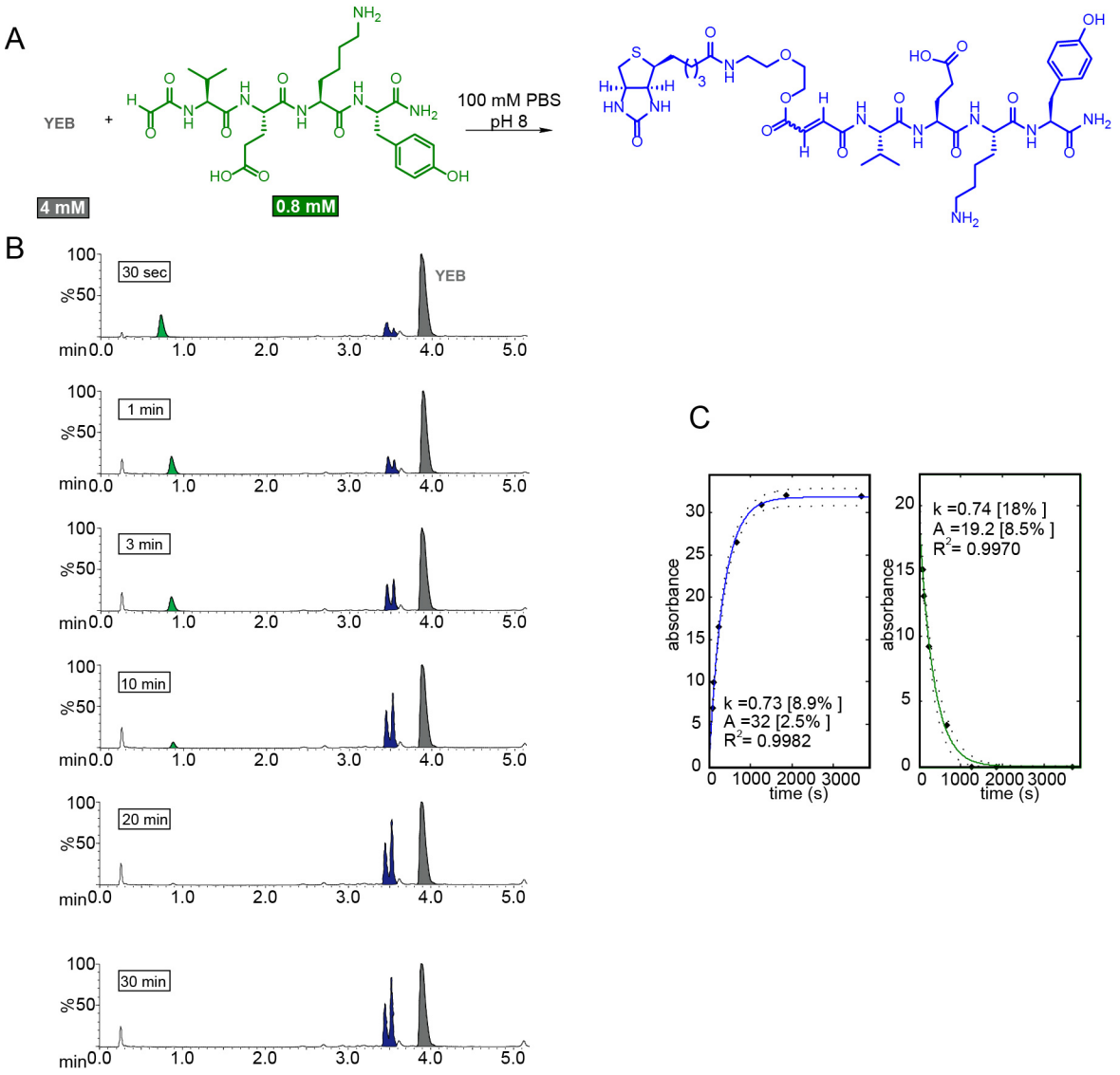


Fig. S1 Example of LCMS traces used to obtain rate constants with SVEKY. A) Scheme of Wittig reaction on the model peptide SVEKY. B) Example of LCMS traces from which the area % were extracted for kinetic traces. C) Curve fitting was performed to pseudo first order kinetics using Matlab (see page S24).

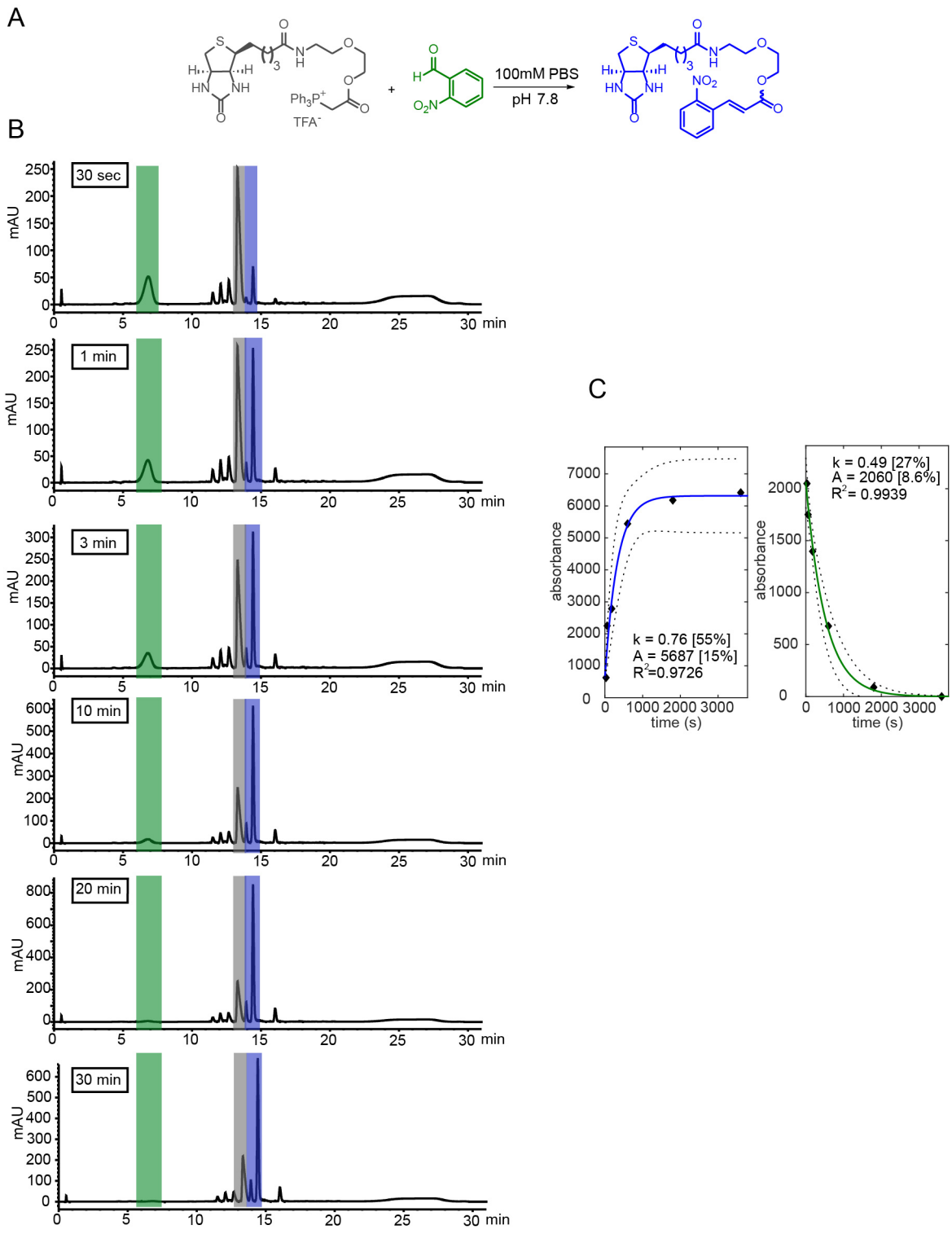


Fig. S2 Example of HPLC traces used to obtain rate constants with 2-nitrobenzaldehyde. A) Scheme of Wittig reaction on model aldehyde 2-nitrobenzaldehyde. B) Example of HPLC trace

from which absolute areas were extracted in order to obtain kinetic traces. C) Curve fitting was performed to pseudo first order kinetics using Matlab (see page S24).

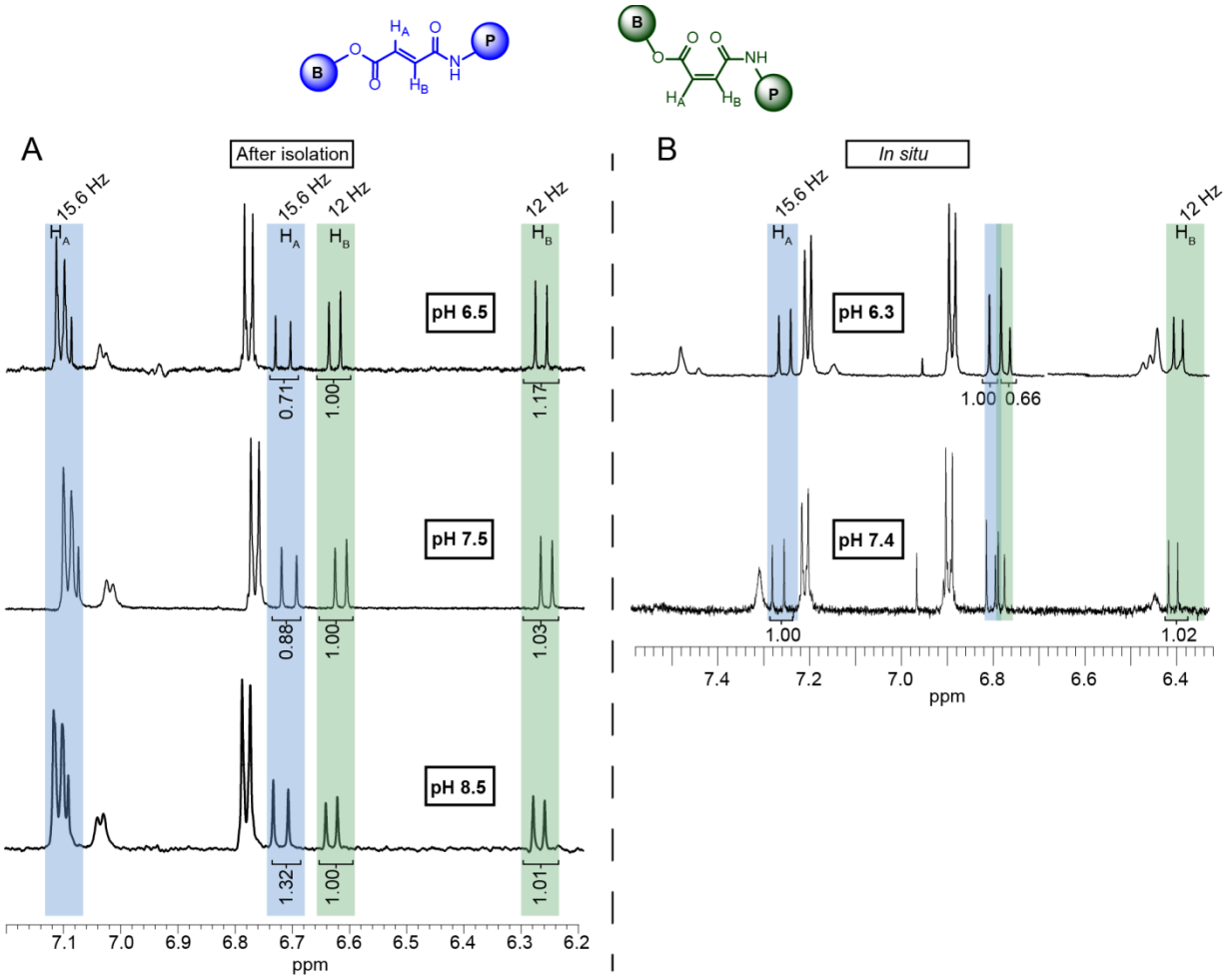


Fig. S3 E/Z ratio for Wittig reaction with o-VEKY model peptide at different pH values. A) For measurements performed after isolation of Wittig product, the reactions were run at the indicated pH values. The Wittig adduct was subsequently purified by HPLC, dissolved in D_2O and the E/Z ratio was measured by integration of ^1H NMR spectrum. B) To confirm that the work-up and purification were not affecting E/Z ratios,[1] the reactions were run again in buffers at the pH values shown. At the endpoint of the reaction, 10% D_2O was added and the crude E/Z ratio was measured. We avoided performing the reaction directly in D_2O directly to prevent H to D exchange in YEB ylide.

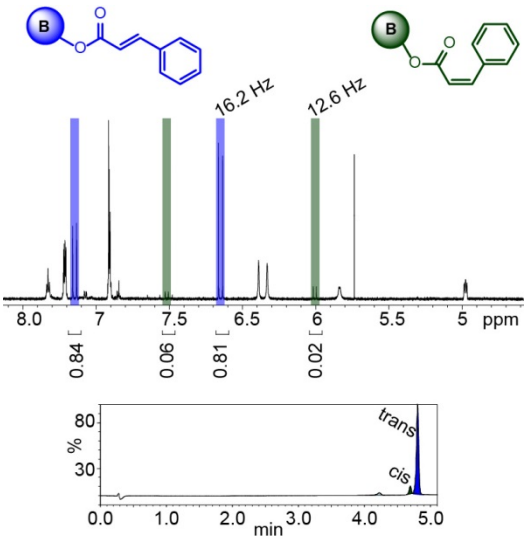


Fig. S4 E/Z ratio for Wittig reaction with benzaldehyde. The reaction was run in 100 mM PBS at pH 7.5. Following the reaction we added 10% D_2O was added and measured the crude E/Z ratio by ^1H NMR integration. We avoided performing the reaction in D_2O to prevent H to D exchange in the YEB ylide Shown above is the *in situ* NMR measurement and the after purification LCMS trace (254 nm).

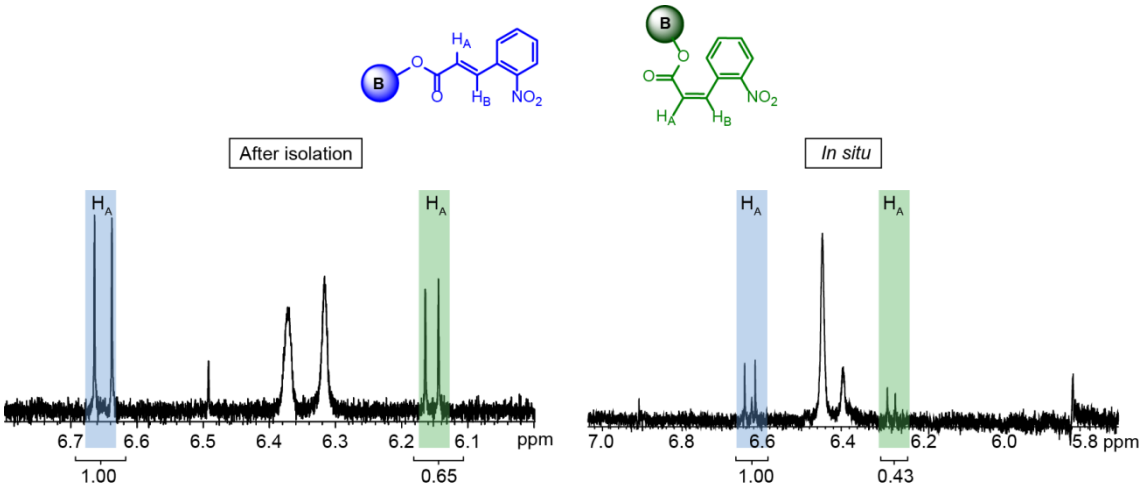


Fig. S5 E/Z selectivity of 2-nitrobenzaldehyde Wittig reaction. A) 1H NMR-expanded spectrum of Wittig adduct in D_2O after HPLC purification. B) ^1H NMR-expanded spectrum of the crude mixture in 10% D_2O performed to confirm that the work-up and purification were not affecting E/Z ratios.[1]

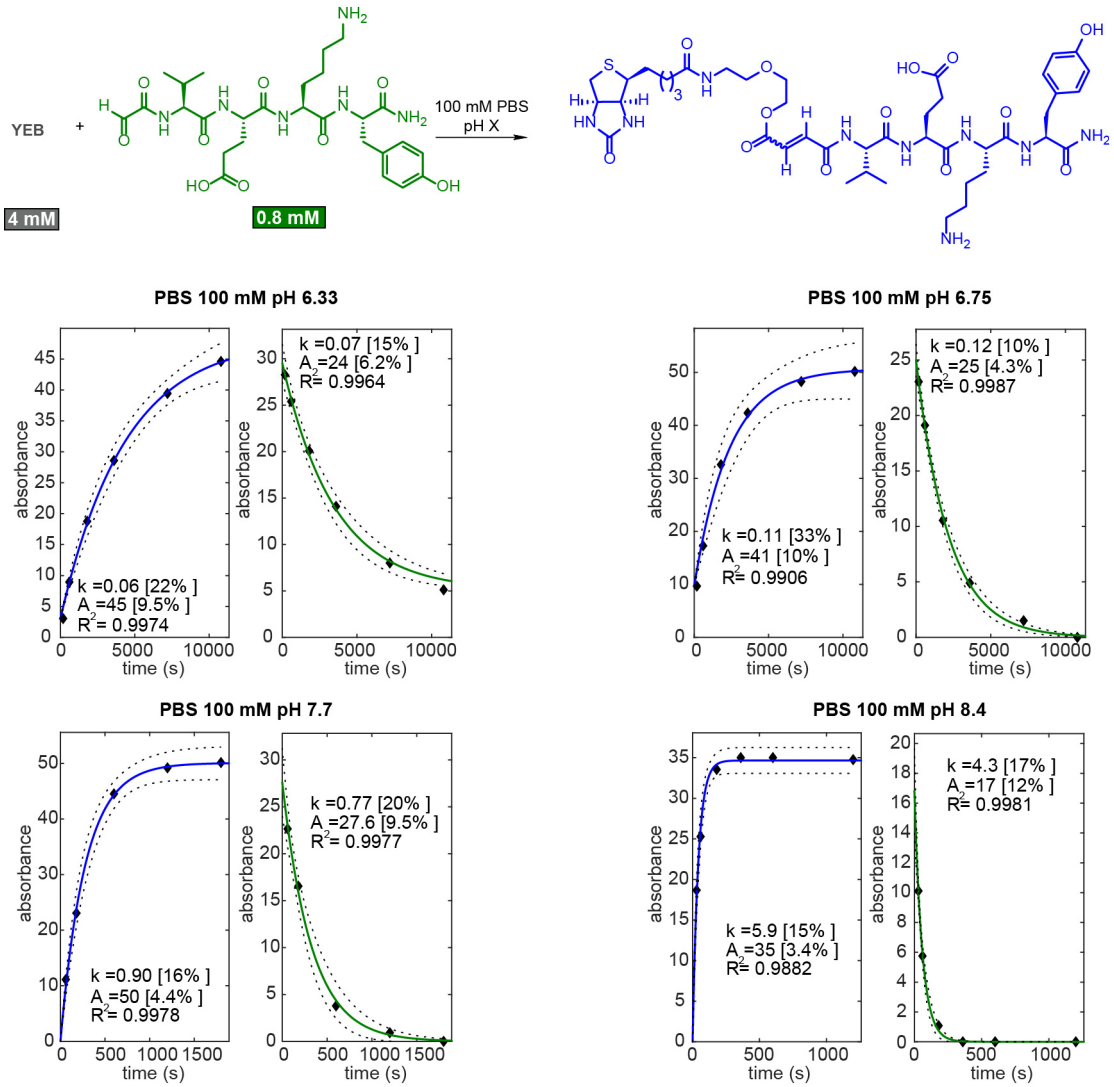


Fig. S6 Kinetic traces for Wittig reaction on model peptide o-VEKY at different pH values

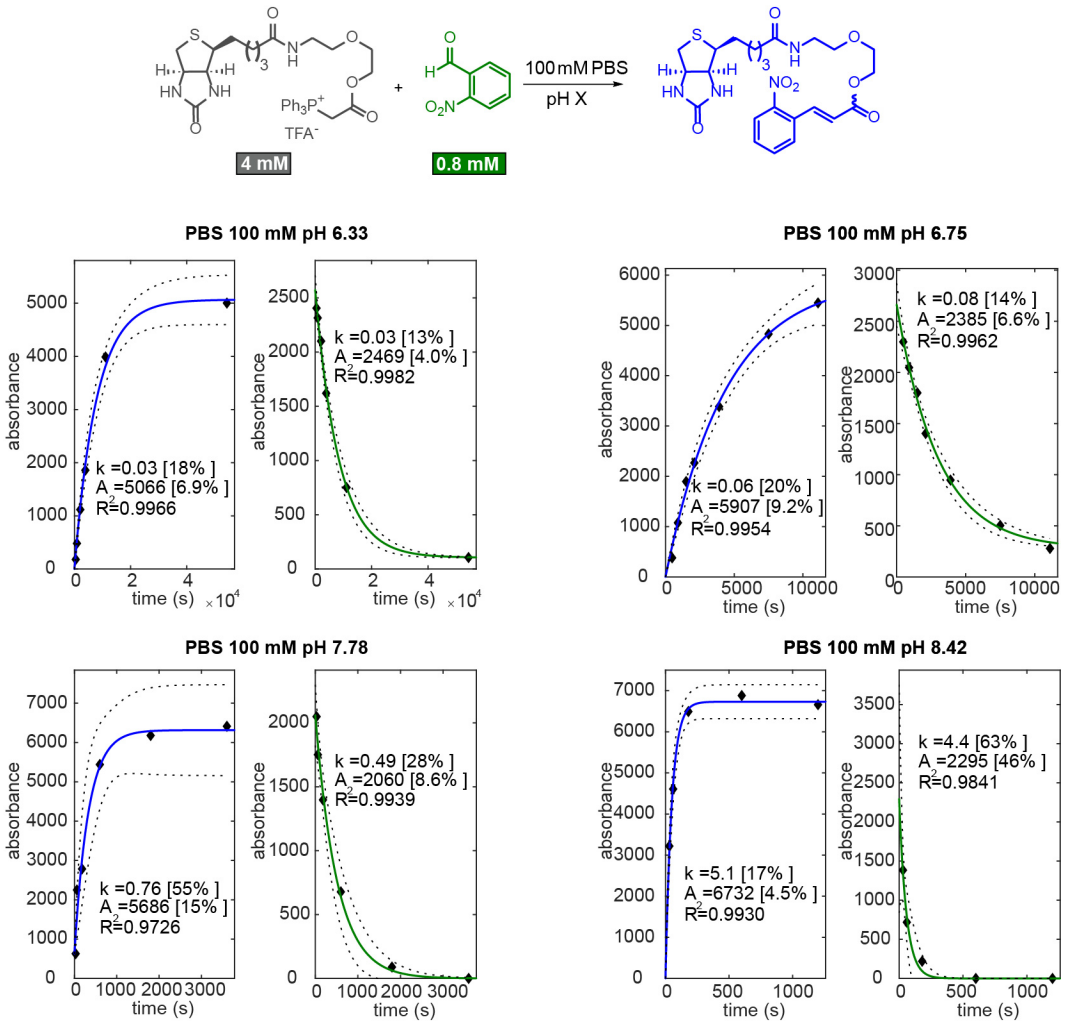


Fig. S7 Kinetic traces for Wittig reaction on 2-nitrobenzaldehyde at different pH values

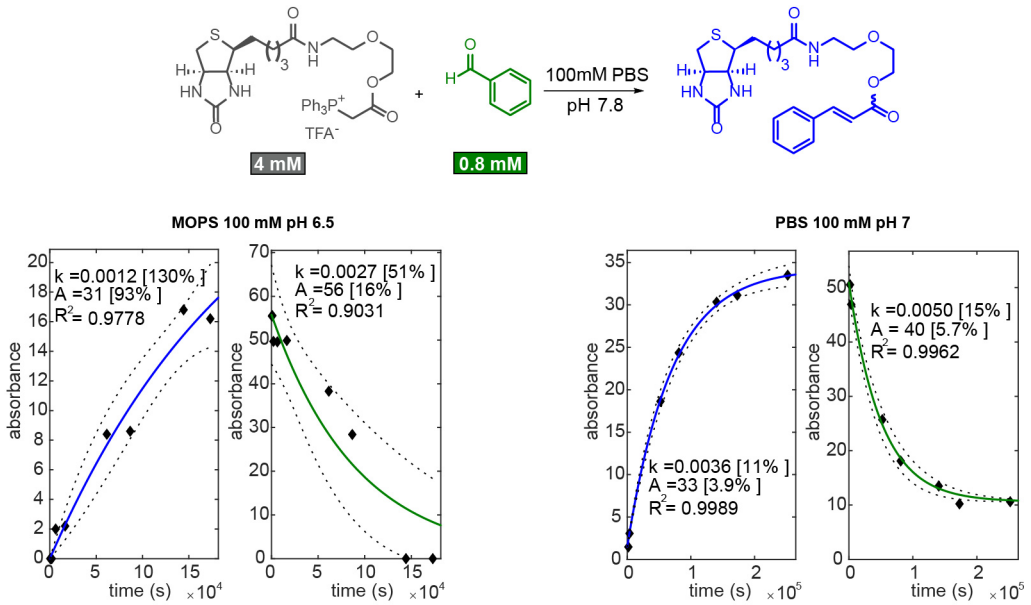


Fig. S8 Kinetic traces for Wittig on benzaldehyde at different pH values. Acquisition of kinetic traces at pH > 7 was complicated due to the rate of ester hydrolysis being equal or higher than the rate of the Wittig reaction.

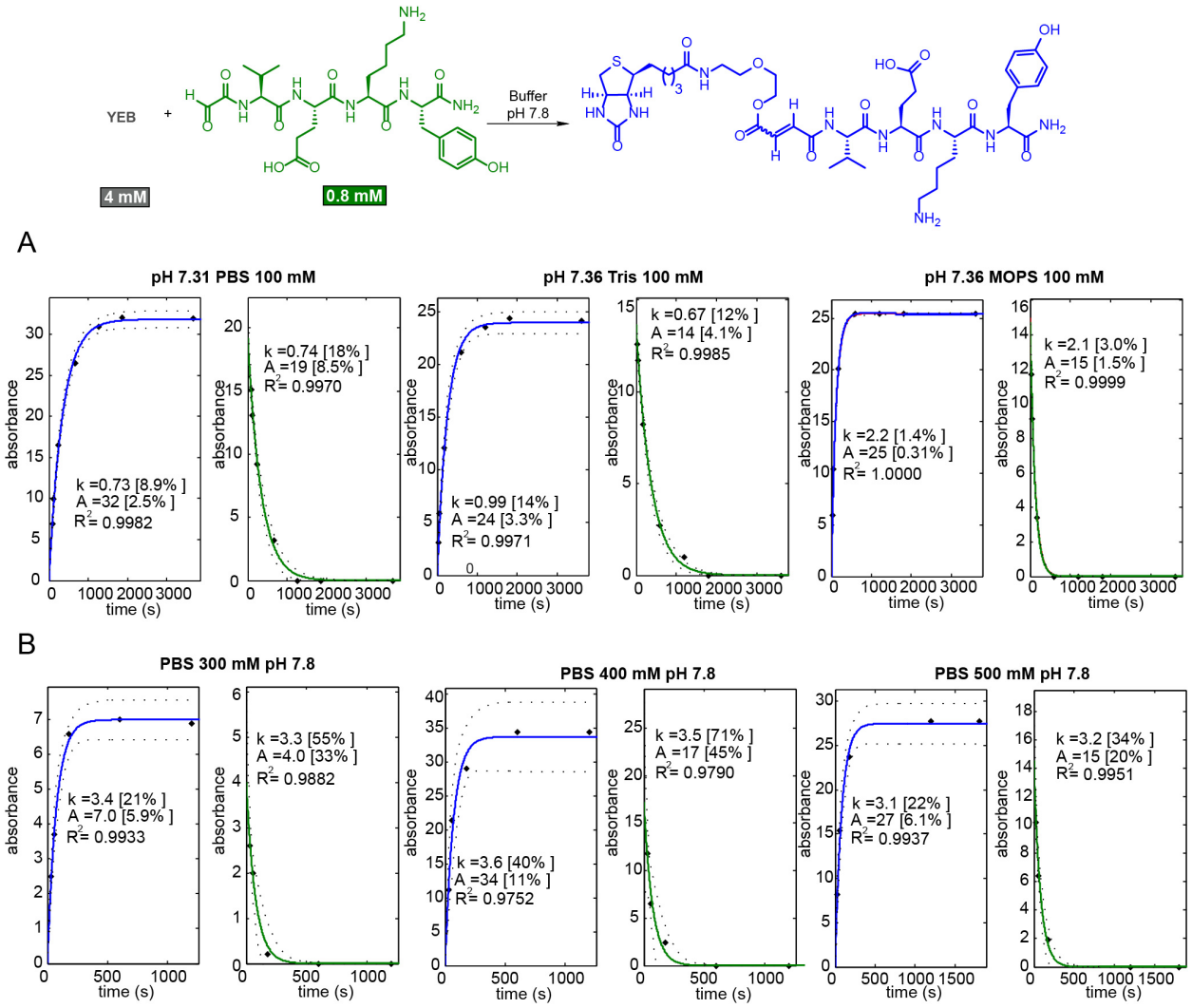


Fig. S9 Rate dependence of Wittig reaction for the model sequence o-VEKY A) in different buffers B) at different concentrations of buffer.

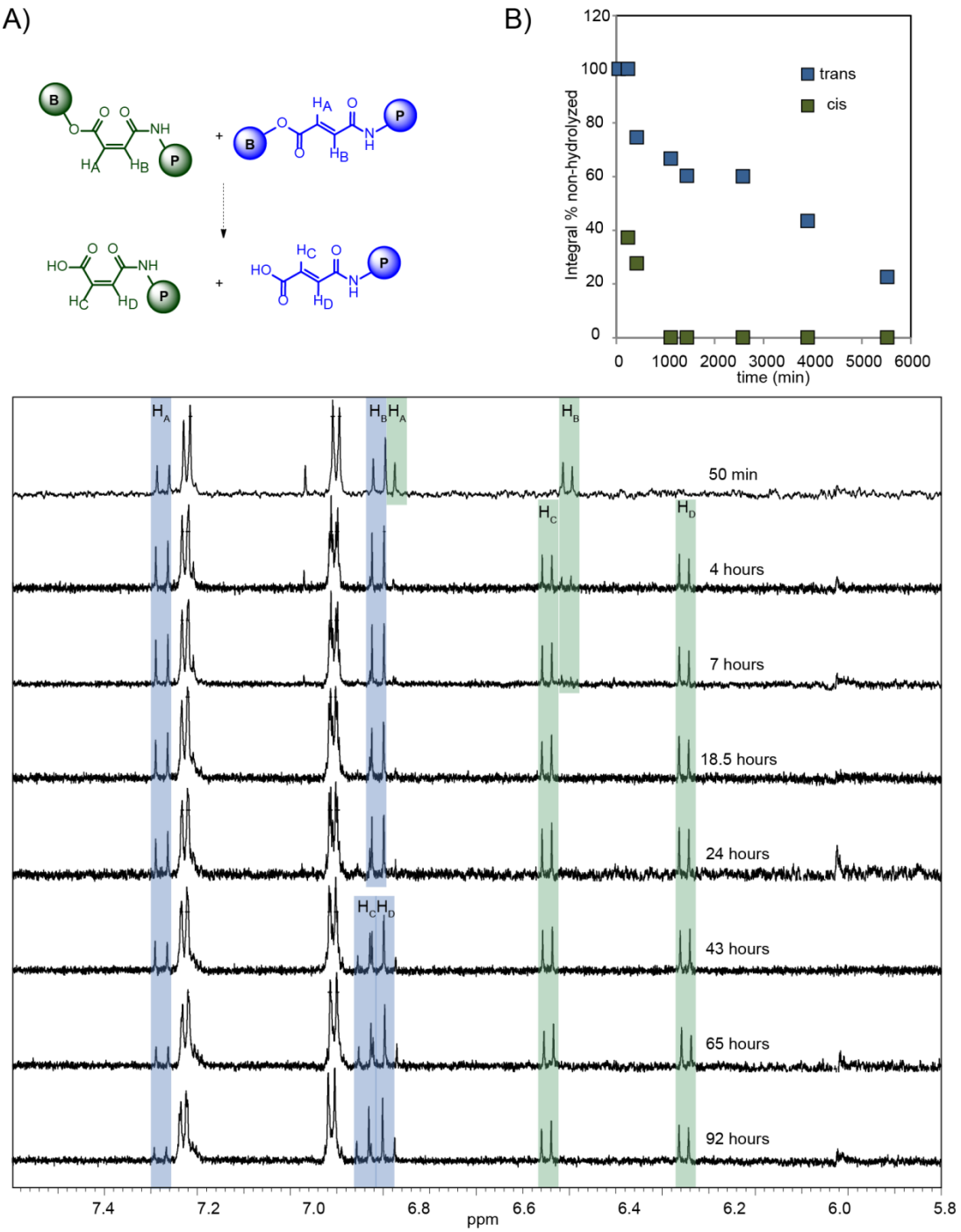
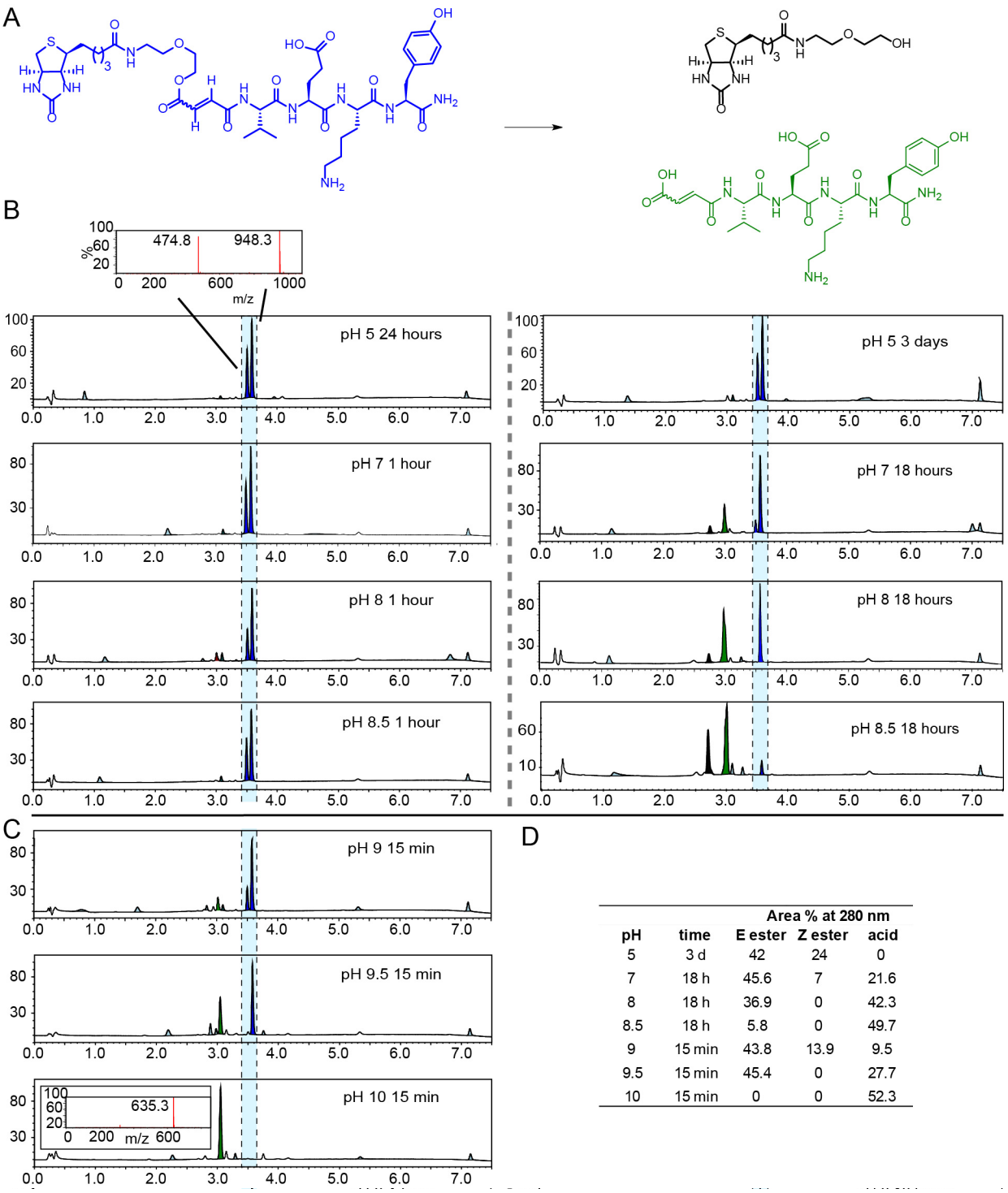


Fig. S10 Rate of hydrolysis for *cis* and *trans* isomers of YEB-VEKY as measured by ^{1}H NMR at pH 6.5.



		Area % at 280 nm		
pH	time	E ester	Z ester	acid
5	3 d	42	24	0
7	18 h	45.6	7	21.6
8	18 h	36.9	0	42.3
8.5	18 h	5.8	0	49.7
9	15 min	43.8	13.9	9.5
9.5	15 min	45.4	0	27.7
10	15 min	0	0	52.3

Fig. S11 Hydrolysis of YEB-VEKY at different pH values. A) Hydrolysis products for YEB-VEKY. B) pH values when product has a half-life >8 hours. C) pH values where product has a half-life <8 hours. D) Area percentages obtained by LCMS at 280 nm for Wittig products and after hydrolysis products.

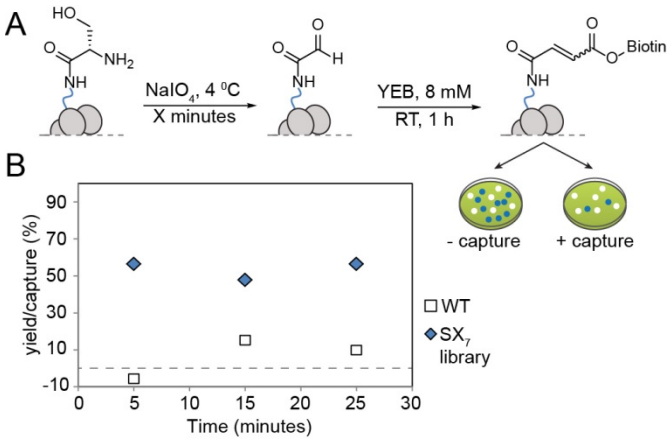


Fig. S12 A) Diagram of performed reactions. B) Determination of conversion (%) for Wittig reaction as a function of oxidation time using biotin pull-down assay.

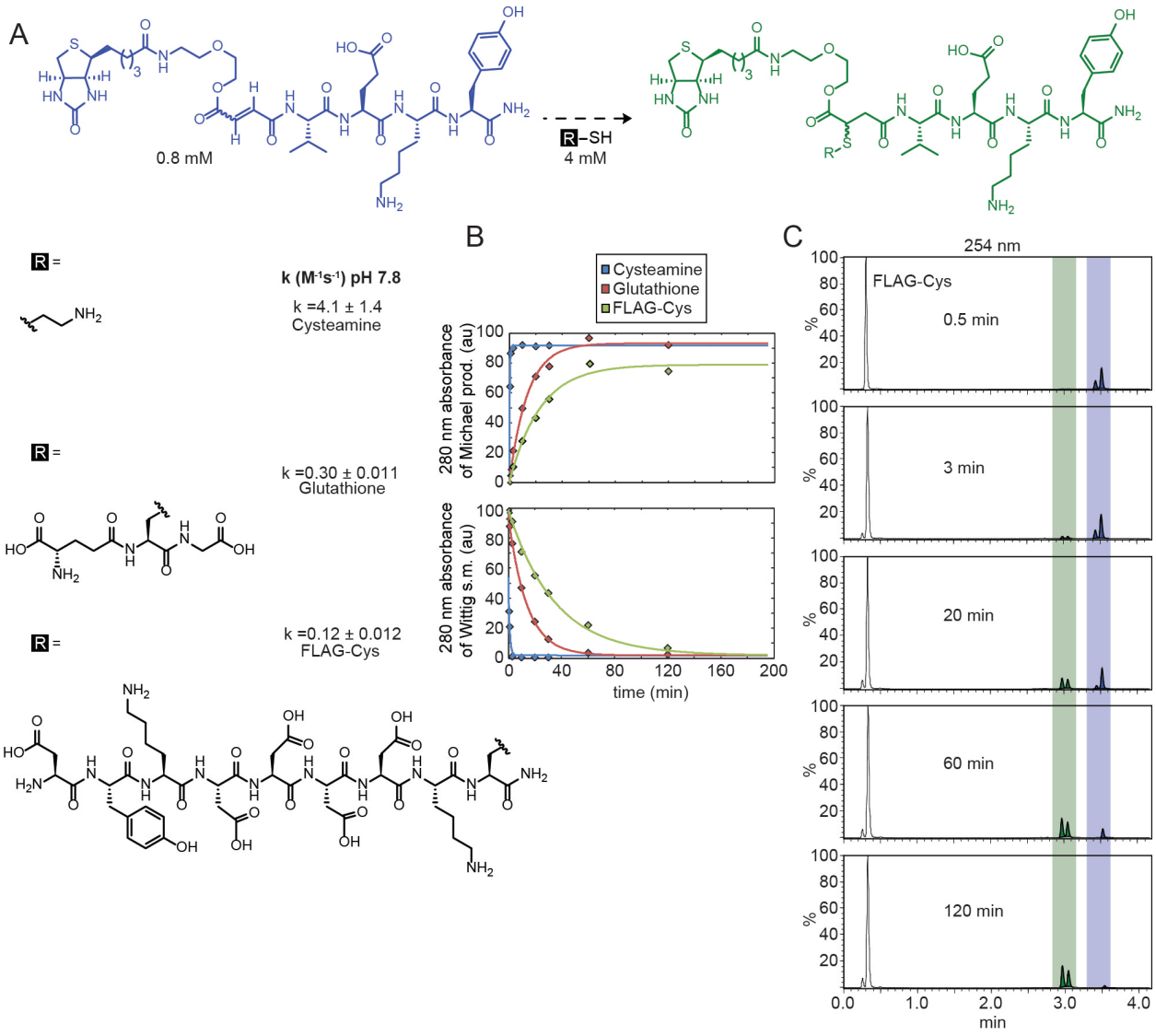


Fig. S13 (A) Michael addition on YEB-VEKY. B) Kinetic traces for the tested three thiols. (C) LCMS traces (254 nm) for Michael addition using FLAG-Cys peptide. In the tested conditions, the *cis* isomer reacts faster than *trans* isomer.

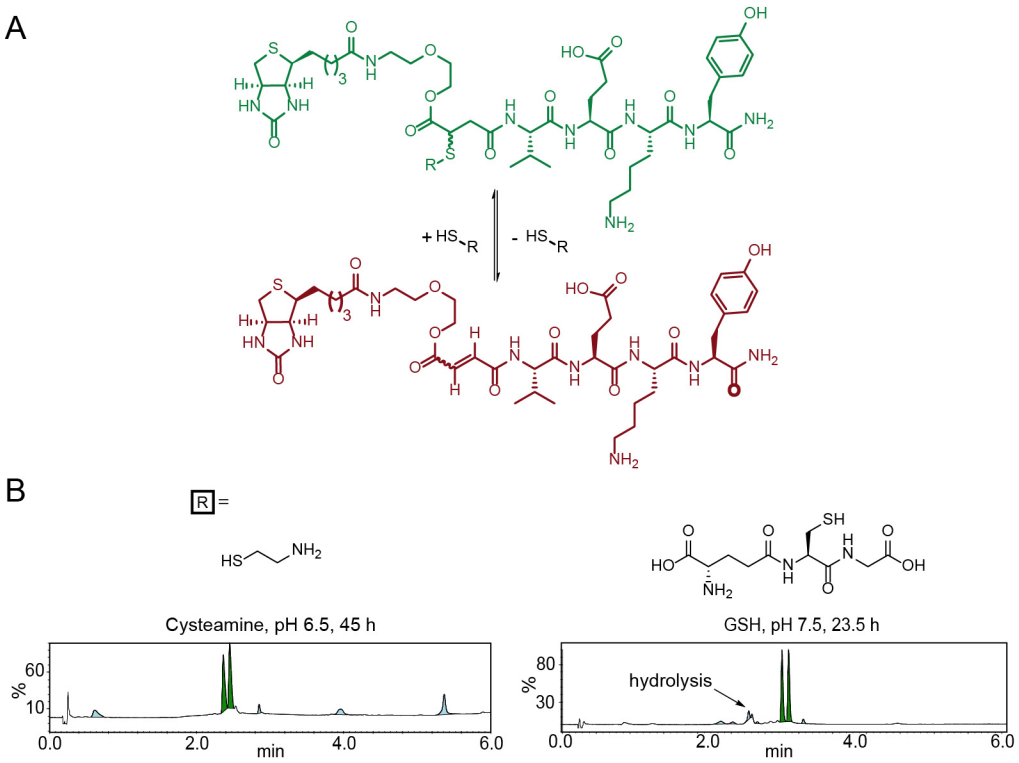


Fig. S14 Retro-Michael tests on isolated Michael addition adducts. A solution (1 mM) of o-VEKY-Michael cysteamine adduct was prepared in (MOPS pH 6.5 100 mM). An aliquot (2 µL) of the solution was injected into the UPLC machine to determine the feasibility of retro-Michael reactivity. As no retro-Michael product was detected, higher pH (PBS 100 mM pH 7.5) solution of o-VEKY-Michael GSH adduct was prepared due to lower pKa of GSH when compared to cysteamine. No retro-Michael product was detected.

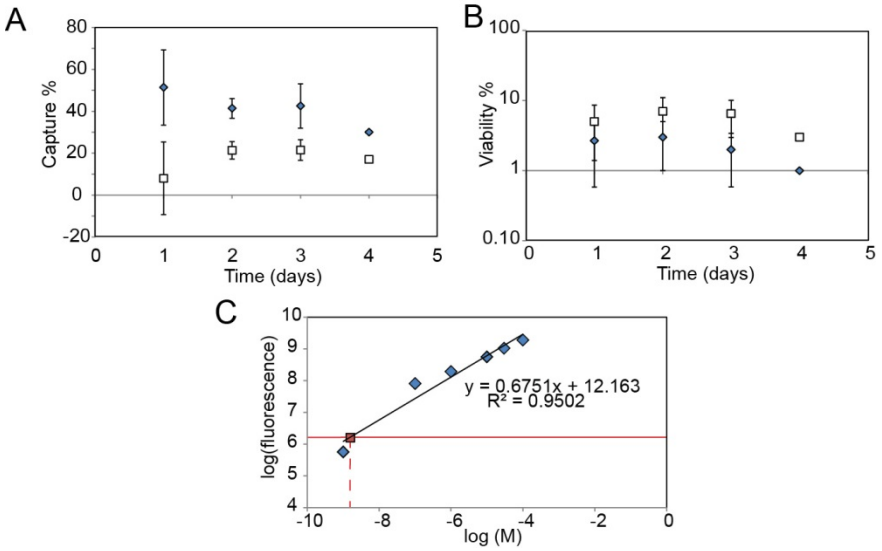


Fig S15 Storage and purification studies of biotinylated phage. A) Percentage of biotinylation of phage library vs. time when stored at pH 5. B) Viability of phage library vs. time at pH 5 following 24 hours of dialysis. C) Fluorescein calibration curve showing that final concentration (red line) is in the single digit nM range (1.5 nM in this specific study).

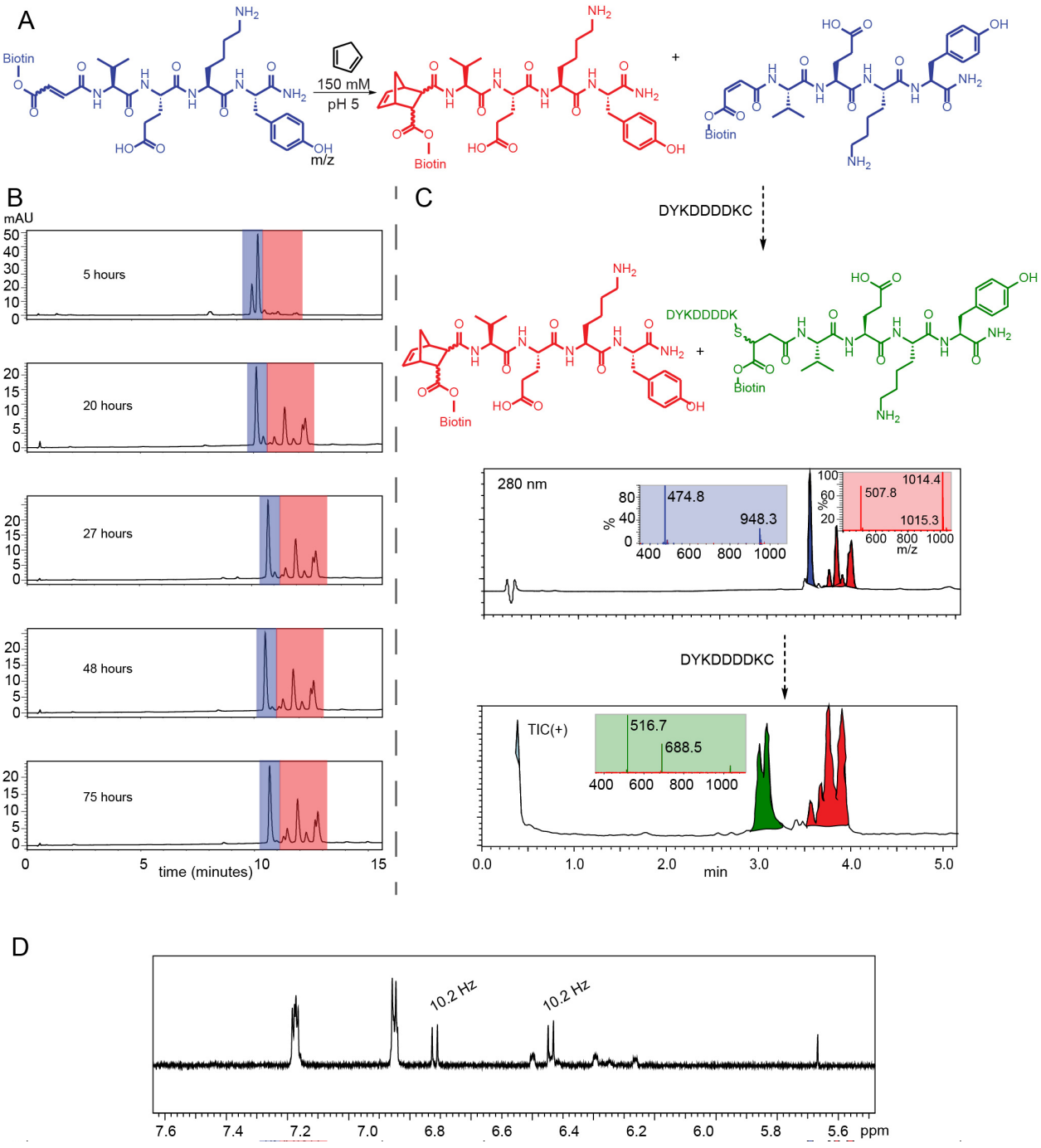


Fig. S16 Diels-Alder on YEB-VEKY and identification of the non-reactive isomer. A) Scheme illustrating the Diels-Alder reaction on YEB-VEKY. B) Monitoring of Diels-Alder reaction at different time intervals on YEB-VEKY shows reactivity of one of the isomers only. C) Michael addition reaction performed on the crude mixture following Diels-Alder reaction on YEB-VEKY. LCMS traces of the reaction mixtures show: i) Non-reacted dienophile YEB-VEKY + Diels-Alder adduct before addition of DYKDDDDKC, ii) Michael addition adduct + Diels-Alder

adduct after addition of DYKDDDDKC (TIC+ is shown for post-reaction LCMS due to excess thiol diminishing the *relative* absorbance of products). D) ^{1}H-NMR identification of Z olefin as remaining starting material after Diels-Alder reaction. The Diels-Alder adducts appear as four separate peaks, likely due to *trans* endo/exo mixture and traces of *cis* endo/exo mixture (smaller two peaks). We considered characterization of the norbornene-like moieties by ^{1}H-NMR, however such characterization is complicated due to overlap of the characteristic norbornene signals with the aliphatic proton signals of the biotin linker and peptide.

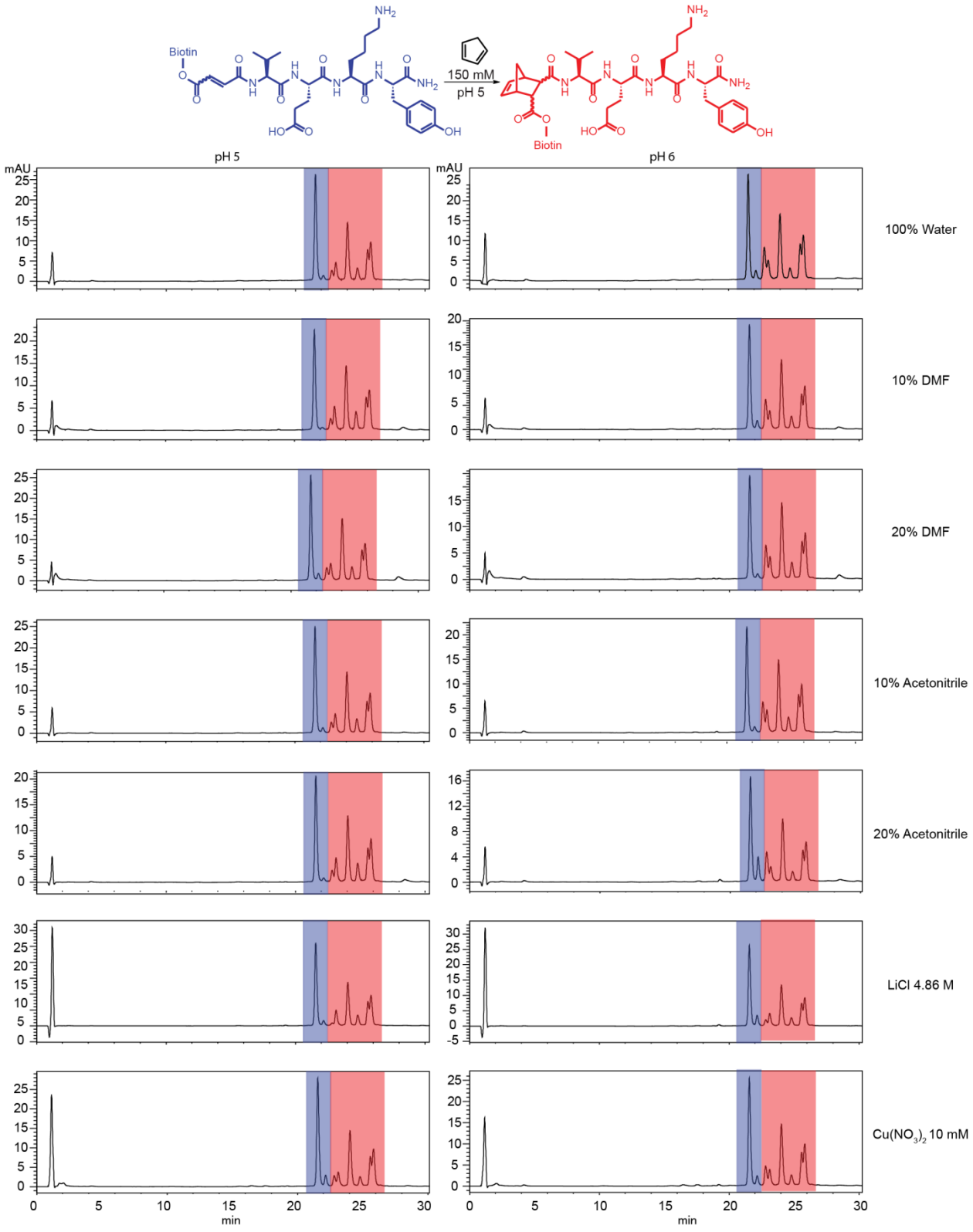


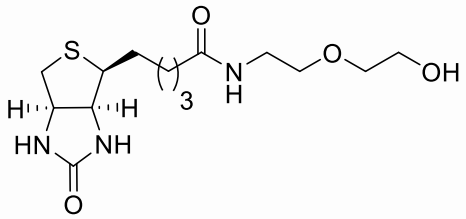
Fig. S17. Diels-Alder reaction on YEB-VEKY (0.8 mM) in different reaction conditions.

Materials, methods and general information

PBS 10X (0.1 M) buffer was prepared by mixing NaCl (80 g), KCl (2 g), Na_2HPO_4 (14.4 g), KH_2PO_4 (2.4 g) in 1 L of milliQ water (pH is adjusted to 7.4 or the needed values); Tris buffers were prepared as a solution of 50 mM Tris and adjusted with NaOH and HCl to the required pH values. MOPS buffer was prepared as a solution of 100 mM MOPS and adjusted to pH 6.5. All solutions used for phage work were prepared with milliQ water. The peptide (SVEKY) was synthesized using the Prelude X peptide synthesizer (Gyros Protein Technologies) using 200 mg Rink Amide AM resin. (Triphenylphosphoranylidene)ketene (Bestmann ylide, #15596-07-3) was purchased from TCI America. D-(+)-biotin (#58-85-5) was purchased from AK Scientific Inc. Carbonyldiimidazole (CDI, #530-62-1) was purchased from ChemPep. 2-(2'-aminoethoxy) ethanol was purchased from Sigma Aldrich. THF was dried with Na/Benzophenone and distilled. Product purification was accomplished with automated chromatography machine (CombiFlash® Rf, Teledyne Isco, Inc.) and preparative HPLC (Waters 2489). ^{1}H and ^{13}C NMR spectra were acquired on a 600 MHz four channel Agilent VNMRS spectrometer, equipped with a z gradient HCN probe and using VNMRJ 4.2A as the acquisition software. When specified, samples were dissolved/recorded in 90% H_2O/10% D_2O mixtures and suppression of the H_2O signal was performed using either presaturation or excitation sculpting.[2] Chemical shifts are reported in ppm and J couplins in Hz. The following abbreviations classify the multiplet peaks in the ^{1}H NMR: s = singlet, d = doublet, m = multiplet or unresolved. HRMS (ESI) spectra were recorded on Agilent 6220 oaTOF mass spectrometer using either positive or negative ionization mode. Characterization of reaction crude was performed with UPLC-MS using a C18 column (Phenomenex Kinetex 1.7 μm EVO C18, 2.1×50 mm) running with a gradient of water/acetonitrile with 0.1% formic acid from 98/2 at 0 min to 40/60 at 5 min under a flow rate of 0.5 mL/min. HPLC kinetics were performed in an Agilent 1100 Series using a Thermo Scientific C18 column (Hypersil GOLD, 3 μm, 50×2.1mm). The phage library (SX_7) was supplied by New England Biolabs and prepared as detailed in our previous publication.[3] Phage displaying SVEK sequence was a generous gift of Chris Noren and Beth Pascal (New England Biolabs).

Synthesized compounds

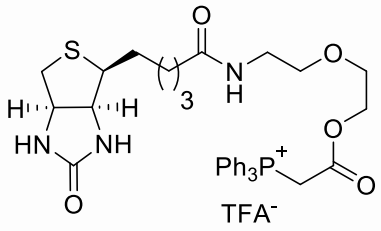
Synthesis of Biotin-PEG



D-(+)-biotin (1.0 equiv, 0.30 g) was dissolved in DMF (60 mL) at 55 °C. The solution was then allowed brought to room temperature. Carbonyldiimidazole (1.7 eq, 0.34 g.) was added in DMF (1.5 mL) to the the reaction mixture, which was stirred for 4 hours at room temperature. 2-(2'-aminoethoxy) ethanol (3.9 eq, 0.5 g, 480.4 μL.) in DMF (3.0 mL) was added to the reaction mixture which was stirred for another 16 hours. The solvent was removed using rotary evaporator and the crude residue was purified on silica gel (80 g) with a gradient of 0–7.5% methanol in DCM using CombiFlash® Rf to yield the product as a white solid (0.29 g, 72% yield). ^{1}H NMR (400 MHz, DMSO) δ = 7.80 (t, *J* = 5.6 Hz, 1 H), 6.41 (s, 1 H), 6.34 (s, 1 H),

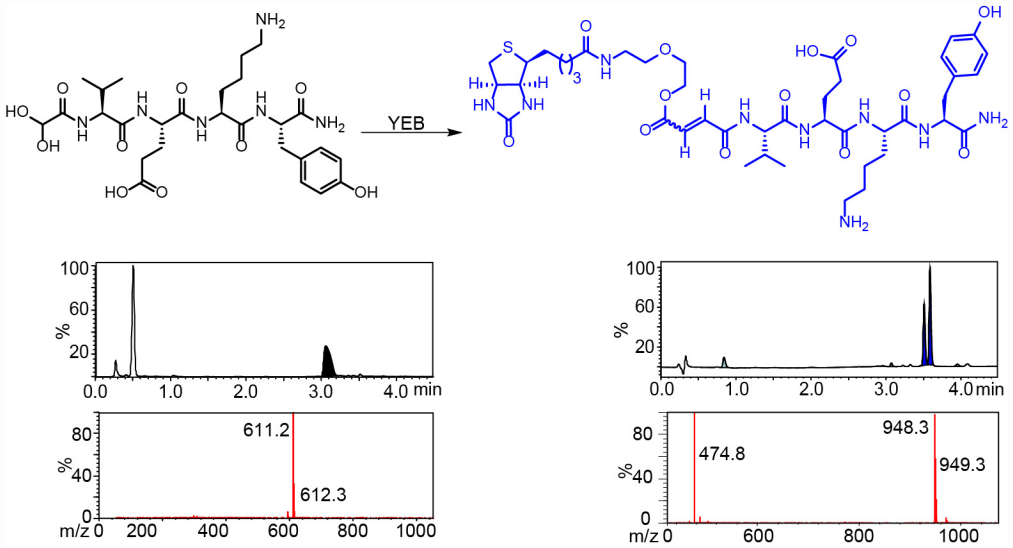
4.56 (t, J = 5.2 Hz, 1 H), 4.30 (dd, J = 7.6, 5.2 Hz, 1 H), 4.14-4.08 (m, 1 H), 3.51-3.47 (m, 2 H), 3.42-3.38 (m, 4 H), 3.21-3.16 (m, 2 H), 3.12-3.07 (m, 1 H), 2.82 (dd, J = 12.4, 4.8 Hz 1 H), 2.57 (d, J = 12.4, 1H), 2.06 (t, J = 7.4, 2H), 1.61-1.27 (m, 6H). ^{13}C NMR (400 MHz, DMSO) δ = 172, 163, 72, 69, 61, 60, 59, 55, 39, 38, 28, 25.

Synthesis of Ylide-Ester-Biotin (YEB)



Biotin-PEG (1 equiv, 0.10 mg,) was charged in a two-neck round bottom flask. The flask was evacuated and filled with N_2 three times. A mixture of dry THF (50 mL) and dry DMF (2 mL) were added into the flask and the solid was dissolved under reflux. Bestmann ylide (1.2 equiv, 0.11 g) was weighed and added to a vial which was then evacuated and filled with N_2 three times. Dry THF (5 mL) was used to dissolve and rinse resulting solution into the round bottom flask *via* cannula. The solution was refluxed for 4 hours, concentrated and purified by HPLC using an amide column (Waters XBridge BEH prep OBD Amide 5 μm 19×250 mm) running with a gradient of acetonitrile/water both with 0.1% TFA from 98/2 at 0 min to 50/50 at 30 min under a flow rate of 8 mL/min. The THF salt ylide was obtained as golden oil (140 mg, 62% yield). ^{1}H NMR (400 MHz, DMSO) δ = 7.88-7.69 (m, 16 H), 6.38 (b, 2 H), 5.38 (s, 1 H), 5.34 (s, 1 H), 4.27 (dd, J = 7.4, 4.6 Hz ,1 H), 4.15-4.11 (m, 3 H), 3.38-3.35 (m, 2 H), 3.26 (t, J = 6.2 Hz, 2 H), 3.14-3.06 (m, 3 H), 2.81 (dd, J = 12.6, 4.8 Hz, 1 H), 2.57 (d, J = 12.4 Hz, 1 H), 2.06 (t, J = 7.2 Hz 2 H), 1.62-1.26 (m, 6 H). ^{13}C NMR (400 MHz, DMSO) δ = 172.2, 164.6, 162.7, 135.1, 133.8, 133.7, 119.3, 118.5, 117.7, 68.9, 67.5, 65.4, 61.1, 59.2, 55.4, 38.2, 35.08, 29.6, 29.2, 28.1, 25.2.

Synthesis of o-VEKY-Wittig product



HPLC purified o-VEKY peptide (1.0 equiv, 2 mg) was dissolved in MOPS buffer (300 μL, pH 6.5, 100 mM). The solution was added into a 1.7 mL epi-tube containing YEB (2.0 eq, 4.9 mg). The mixture was incubated for 3 hours at RT, diluted to 1 mL with milliQ H_2O and purified by HPLC using a preparative C18 column (Waters SymmetryPrep C18 5 μm 19×50 mm) running a gradient of water/acetonitrile both with 0.1% TFA from 98/2 at 0 min to 50/50 at 30 min under a flow rate of 8 mL/min. The acetonitrile was removed using speed-vac and the sample was lyophilized overnight to yield the adduct (o-VEKY-Wiitg) as a white solid (1.2 mg, 38% yield).

Synthesis of o-VEKY Michael addition adducts

o-VEKY-Wittig (1 equiv, 1 mg) was dissolved in of 10X PBS (100 μL). Each thiol was added as a solution in 10X PBS (100 μL, 2 eq) and the reactions were incubated at RT for 3 hours. The mixtures were diluted to 1 mL with H_2O and purified by HPLC using a C18 column (Waters SymmetryPrep C18 5 μm 19×50 mm) running a gradient of water/acetonitrile both with 0.1% TFA from 98/2 at 0 min to 50/50 at 30 min under a flow rate of 8 mL/min. The acetonitrile was removed using speed-vac and the sample was lyophilized overnight to yield the Michael addition adduct as a white solid (0.7 mg), 63% yield for cysteamine; 1.2 mg, 54% yield for DYKDDDDKC).

Kinetic studies for Wittig reaction of aldehydes with YEB at different pH values

Aqueous YEB solution (5.00 equiv, 13.8 μL, 34.8 mM) was added to solutions of aldehydes in 100 mM buffer (1.00 equiv, 106 μL, 0.900 mM). The reactions were incubated at RT. At each time interval, an aliquot of the mixture (10 μL) was quenched by mixing with HCl (1 μL, 1 M). The quenched mixtures were then analyzed by UPLC-MS or HPLC to characterize the progress of the reaction. See Fig. S1-S3 for the spectra and Fig. S6-S8 for kinetic traces. All reactions were performed under pseudo-first-order condition. Fitting of the kinetic curve to the equation $A_t = 1 - e^{-k*[YEB]*t}$ yielded the second-order rate constant k, where A_t is the fraction of the product at time t and [YEB] is 0.004 M. MATLAB script used to fit the kinetic curve is outlined below.

```
clear all;      % clear all variables from the workplace
close all;      % close all opened windows
indir = '';     % define the directory which holds the file (present dir)

name = '';    % define the file name

path = fullfile(indir,name);    % define a full path: directory/name

raw = xlsread(path);     % read the raw data from Excel file

rows = 1:7;      % define in which rows your data is stored
%rows = 36:39;

delay = 0;   % introduce a fixed delay to improve the fit
x = raw(rows,1) - delay;  % extract the time

x = x*60;
```

```
column = [3 2];   % define in which columns the product and reactants are

% define two types of 1st order equations for product and reactant
equation = {'A*(1-exp(-k*x))',  'A*(exp(-k*x))'};

% define the concentration of excess reagent in Molar units
conc  = 0.004;

%%%%% make your best guess about k and A parameters %%%%%%%%%%%%%%%%%%%%
A  = 10;
k  = 0.01;

% run the same fit twice, plot the results in two subplots
for i = 1:2

y = raw(rows,column(i));  % extract the adsorbance from a current column

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

%%%%% define the fit equation and options %%%%%%%%%%%%%%%%%%%%%%%%%%
ft = fittype( equation{i},'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 %%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
figure(1);
subplot(1,2,i);

plot(x,y,'dk',...
         'MarkerEdgeColor','k',...
         'MarkerFaceColor','k',...
         'MarkerSize',5);
hold on;

%%%%% plot the fit data as red line 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;
ymax = max(max(p22))*1.05;

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

%  display the results of the fit on the plot

% extract the fit value of k, divide it by concentration to yield real k
FIRSTorderk = c2.k / conc;

% calculate the % standard deviations for k and A
STD(1) = 100*abs(CON(1,1) - CON(2,1))/2 / c2.A;
STD(2) = 100*abs(CON(1,2) - CON(2,2))/2 / c2.k;

% create a 3-line text string that will be displayed on the chart
TL{1} = [' k =' num2str(FIRSTorderk,'%0.2f') ...
         ' [' num2str(STD(2),'%0.2f')  '% ]'];
TL{2} = [' A =' num2str(c2.A,'%0.2f') ...
          ' [' num2str(STD(1),'%0.2f')  '% ]'];
TL{3} = [' R^2=' num2str(gof2.rsquare,'%0.4f') ];

% plate the text string on the chart into a predefined location
text(0.15*xmax,0.75*ymax, char(TL));

end
```

Kinetic studies for Wittig reaction of o-VEKY with YEB at different buffer concentrations

Aqueous YEB solutions (5.00 equiv, 13.8 μL, 34.8 mM) were added to solutions of aldehydes in PB buffer with pH 7.5 at different concentrations (1.00 equiv, 106 μL, 0.900 mM). The reactions were incubated at RT. At each time interval, an aliquot of the mixture (10 μL) was removed from the reaction and quenched by mixing with HCl (5 μL, 1 M). The quenched mixtures were then analyzed by HPLC to characterize the progress of the reaction. See Fig. S1-S3 for the spectra and Fig. S6-S8 for kinetic traces. All reactions were performed under pseudo-first-order condition. Fitting of the kinetic curve to the equation $A_t = 1 - e^{-k*[YEB]*t}$ yielded the second-order rate constant k, where A_t is the fraction of the product at time t and [YEB] is 0.004 M. MATLAB script used to fit the kinetic curve is outlined above.

E/Z selectivity determination following purification

o-VEKY (1 eq, 1 mg) was dissolved in 10X PBS (1 mL) at the indicated pH values (6.5, 7.5, 8.5) and mixed with YEB (2.2 equiv, 3.3 mg). The reaction mixture was incubated for 1.5 hours and

purified by HPLC with a C18 preparative column using a 30 minute gradient with 5-50% acetonitrile in water both with 0.1% TFA. The acetonitrile was removed using speed-vac for 40 minutes at 40 °C and the peptide derivative was obtained as white solid after lyophilizing the aqueous residue overnight. All of the obtained solid was dissolved in D_2O (750 μL) for NMR analysis.

***In situ* E/Z selectivity determination**

A solution of aldehyde (12.3 μL, 16.3 mM, 1.00 eq, 0.200 mmol) was mixed with 175.3 μL of 10X PBS at the specified pH values. A solution of YEB (37.4 μL, 26.7 mM, 5.00 eq, 1.00 mmol) was added. The mixture was incubated for 3 hours (pH 6.3) or 1.5 hours (pH 7.4) after which 25 μL of D_2O were added. Samples were transferred into 3 mm diameter NMR tubes and the ^1H NMR spectra were collected after solvent suppression was performed.

Hydrolysis monitoring of YEB-VEKY by NMR

Wittig product (1 mg) as an E/Z isomer mixture was dissolved in 1X PBS/D_2O (675/75 μL) and loaded into an NMR tube. Solvent suppression was performed and the ^1H NMR was monitored at the specified time intervals.

Hydrolysis monitoring of YEB-VEKY LCMS at different pH values

Solutions of Wittig product (1 mM) in the specified buffers (10 mM) were prepared. Aliquots (2 μL) were injected in the UPLC machine to monitor the hydrolysis rate at the specified time intervals.

Kinetic studies for Michael addition of YEB-VEKY with different thiols

A solution of YEB-VEKY product (82.8 μL, 1.00 mM, 1.00 eq) in MOPS (pH 6.5 100 mM) was mixed with an aliquot of the thiol stock solutions (7.2 μL, 50 mM, 5.0 eq). The reactions were incubated at RT. At each time interval, an aliquot of the mixture (5 μL) was quenched by mixing with HCl (1 μL, 1 M). The quenched mixtures were then analyzed by UPLC-MS to characterize the progress of the reaction (see Figure S14 for kinetic traces). All reactions were performed under pseudo-first-order condition. Fitting of the kinetic curve to the equation $A_t = 1 - e^{-k*[THIOL]*t}$ yielded the second-order rate constant k, where A_t is the fraction of the product at time t and [THIOL] is 0.004 M.

Diels Alder Reaction on YEB-VEKY

A solution of YEB-VEKY (2 μL, 10.5 mM in water) was mixed with cyclopentadiene (0.25 μL) and the buffer, co-solvent and Lewis acid as described in Table 1. The mixtures were incubated for 48 hours at RT in a Labquake tube rotator (Barnstead/Thermolyne) after which an aliquot (2 μL) was diluted 10 fold with water and injected into HPLC to monitor the reaction. Another aliquot (2 μL) from one of the reactions was also injected into LCMS in order to obtain the mass values for the peaks.

Table S1. Screening of conditions for Diels Alder reaction between o-VEKY Wittig and cyclopentadiene

Entry	pH buffer	μL buffer	μL DMF	μL acetonitrile	μL LiCl	μL $Cu(NO_3)_2$
1	5	17.5	-	-	-	-
2	6	17.5	-	-	-	-
3	5	15.5	2.00	-	-	-
4	6	15.5	2.00	-	-	-
5	5	13.5	4.00	-	-	-
6	6	13.5	4.00	-	-	-
7	5	15.5	-	2.00	-	-
8	6	15.5	-	2.00	-	-
9	5	13.5	-	4.00	-	-
10	6	13.5	-	4.00	-	-
11	5	9.00	-	-	8.80	-
12	6	9.00	-	-	8.80	-
13	5	16.5	-	-	-	1.20
14	6	16.5	-	-	-	1.20

Phage modifications

Modification of SX_7 library and SVEK phage with YEB

A mixture of WT phage and SX_7 library or SVEK phage (1:1 ratio) in PBS buffer (pH 7.8, 100 mM) was prepared to yield a final titer of 2×10^{12} pfu mL^{-1}. To the mixture of phage (98 μL), $NaIO_4$ (1 μL, 6 mM solution in water) was added and the reaction was incubated on ice for 5 minutes. YEB (100 μL, 2 mM in water) was added and the reaction mixture was incubated at RT for 1h. The efficiency of the modification was quantified by biotin-capture assay as described previously.[4]

Kinetic studies of modification of SX_7 phage library with YEB

A mixture of phage SX_7 library and WT phage (1:1 ratio) in MOPS (pH 6.5, 100 mM) was prepared to yield a final titer of 2×10^{12} pfu mL^{-1}. To the mixture of phage (98 μL), $NaIO_4$ (1 μL, 6 mM solution in water) was added and the reaction was incubated on ice for 5 minutes. YEB (100 μL, 8 mM in water) was added to the reaction mixture, which was incubated at RT. At each time interval, a 2 μL aliquot was removed from the reaction and quenched by diluting 10^5 times. The efficiency of the modification was quantified by biotin-capture assay as described previously.[4] The studies—modification and quantification—were repeated three times on separate days to validate the reproducibility of the experiments. The reactions were performed under pseudo-first-order condition (i.e. modifying reagent was present in a large excess when compared to the concentration of peptide substrate displayed on phage).

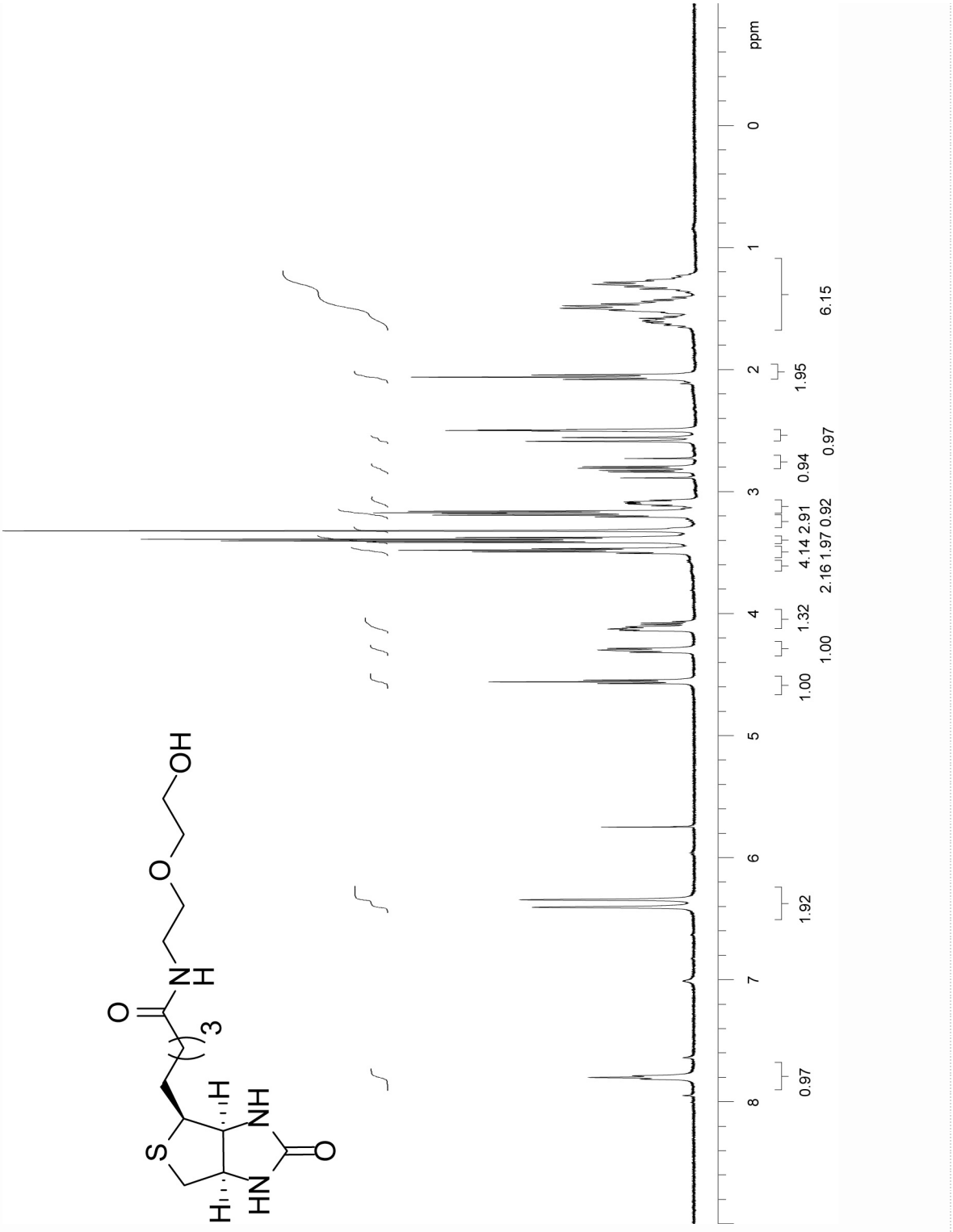
Modification of SVEK phage with YEB followed by reaction with DYKDDDDKC.

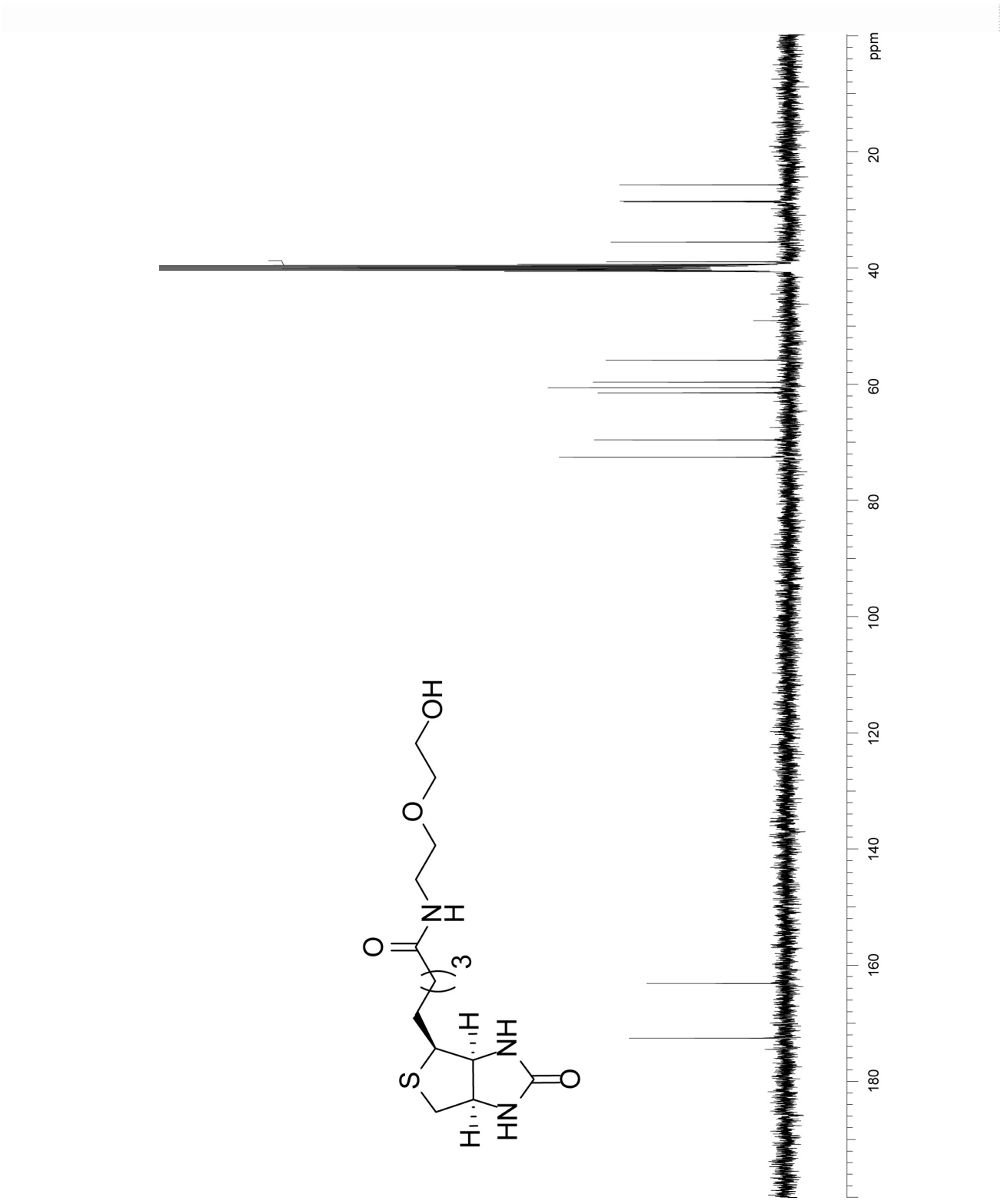
A mixture of SVEK phage and WT phage in MOPS buffer (pH 6.5, 100 mM) was prepared to yield a final titer of 2×10^{12} pfu mL^{-1}. To the mixture of phage (98 μL), YEB (0.1 mL, 8 mM in water) was added. The reaction mixture was incubated at RT for 3 h, injected into a dialysis cassette and dialyzed for 48 h against acetate buffer (10 mM at pH 5, at least 6 buffer exchanges). To an aliquot (10 μL) of purified, Wittig functionalized phage (1×10^{11} pfu mL^{-1}), a solution of DYKDDDDKC peptide was added (10 μL, 8 mM in PBS pH 8 100 mM). The reaction was incubated at RT for 2 hours. An aliquot (2 μL) was diluted 10^5 times at the time intervals described in Figure 3. The diluted phage solution (300 μL) was incubated with 30 μL of protein G beads for 1 hour at RT in a Labquake tube rotator. The protein G beads had been previously coated with anti-FLAG antibody (10 μg antibody in 300 μL 2% BSA, overnight in Labquake tube rotator at 4 °C). The efficiency of the modification was quantified by FLAG-capture assay, by using steps analogous to those of biotin-capture assay described previously.[4]

Modification of SVEK phage with YEB followed by reaction with cyclopentadiene and chase with DYKDDDDKC.

To an aliquot of purified, Wittig functionalized phage (1×10^{11} pfu mL^{-1}) in a total volume of 100 μL in acetate buffer (10 mM, pH 5), freshly cracked cyclopentadiene (1.3 μL) was added to reach a final concentration of 250 mM (cyclopentadiene was prepared by thermal cracking of dicyclopentadiene). The reaction was incubated at RT in a Labquake tube rotator for 48 h. An aliquot of the cyclopentadiene modified phage (10 μL), was then mixed with a solution of DYKDDDDKC peptide (10 μL, 4 mM in PBS pH 8 100 mM). The reaction was incubated at RT for 2 hours. An aliquot (2 μL) was diluted 10^5 times, and the phage dilution (300 μL) was incubated with 30 μL of protein G beads previously coated with anti-FLAG antibody (10 μg antibody in 300 μL 2% BSA in 1X PBS, overnight coating in Labquake tube rotator at 4 °C) for 1 hour at RT in a Labquake tube rotator. The efficiency of the modification was quantified by FLAG-capture assay, using steps analogous to those of biotin-capture assay described previously.[4] The same capture methodology was performed in the Wittig functionalized phage before cyclopentadiene treatment. Diels-Alder efficiency was calculated by subtraction of Michael addition efficiency before and after incubation with cyclopentadiene.

NMR spectra of synthesized compounds

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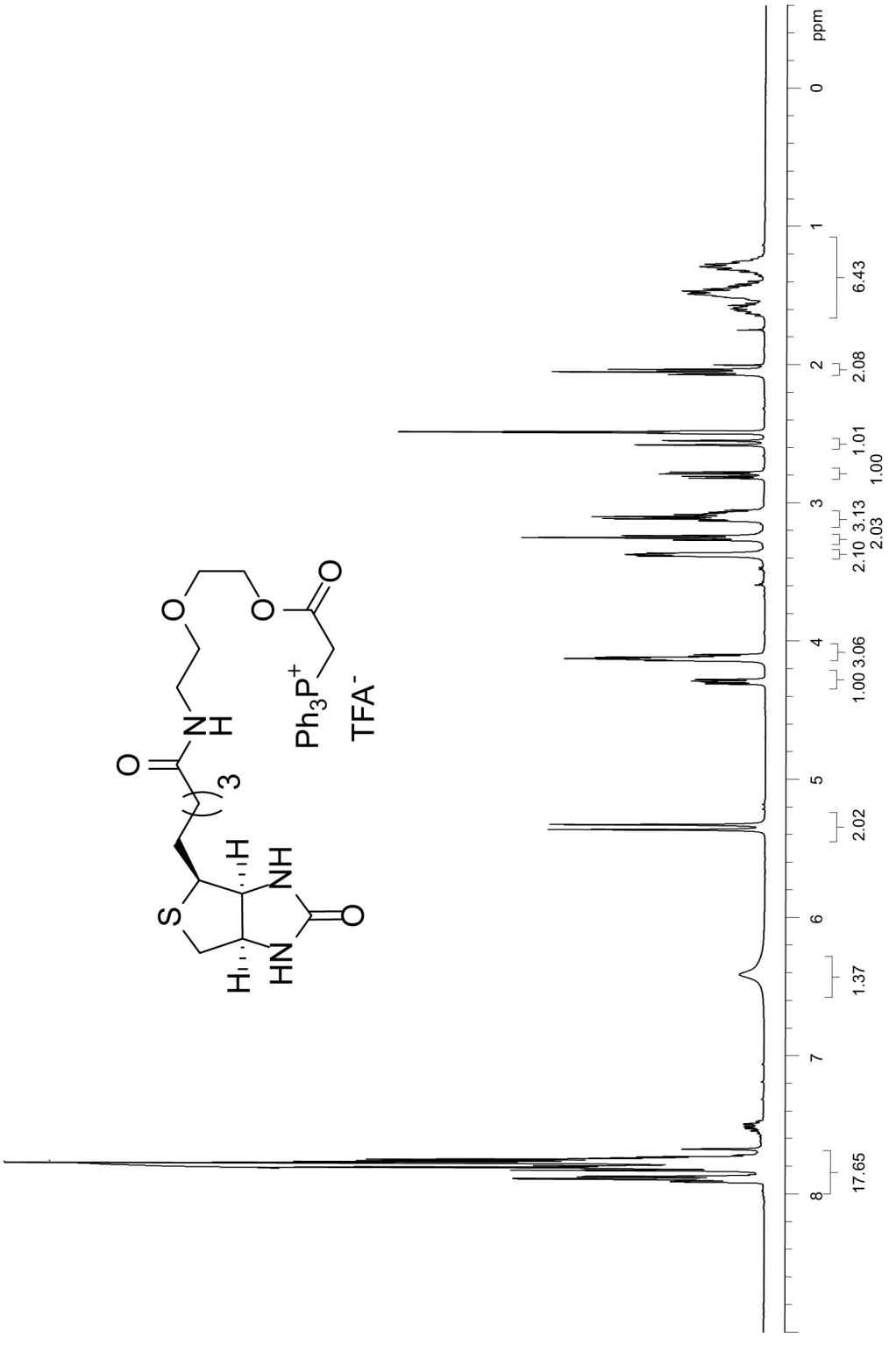
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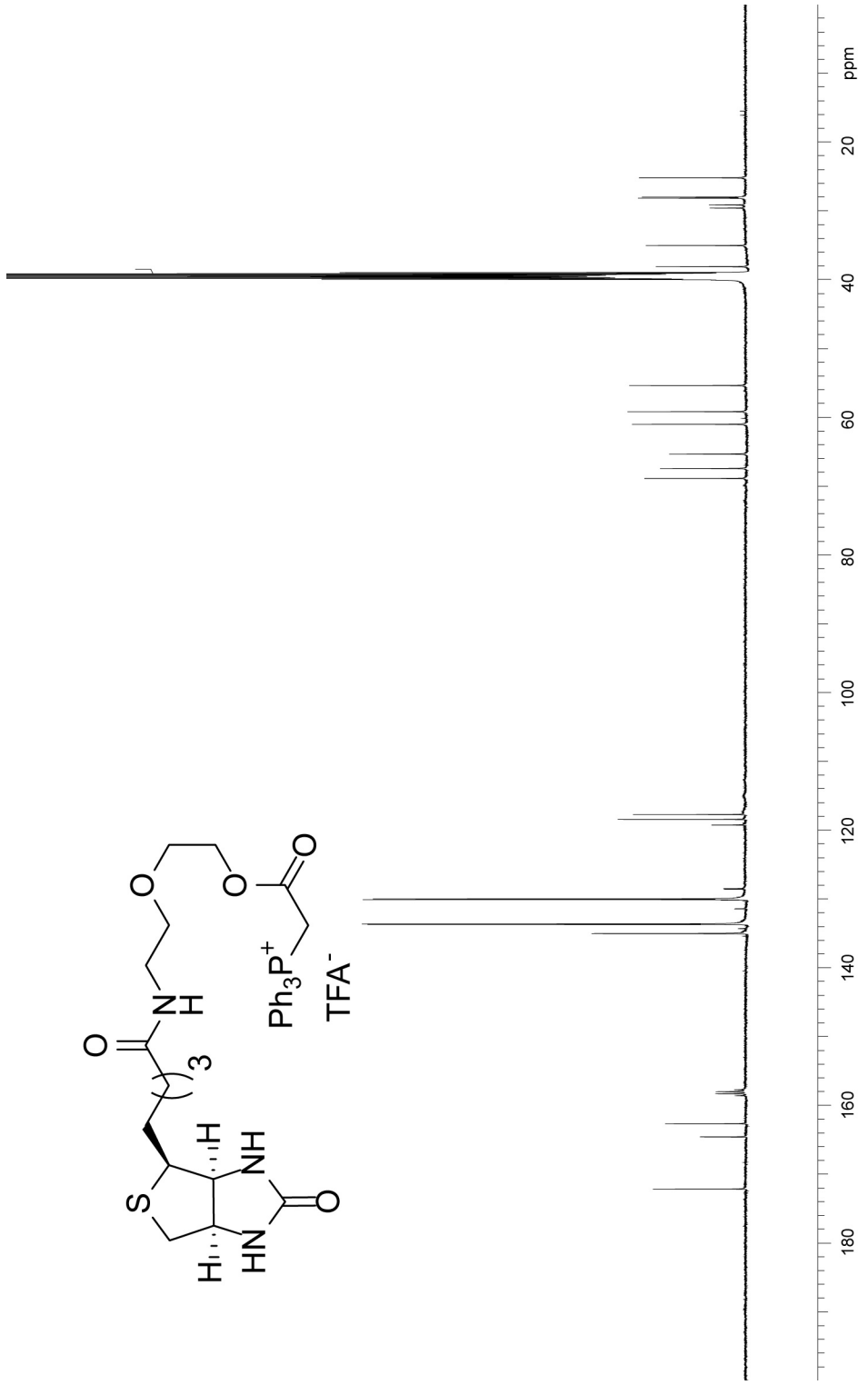
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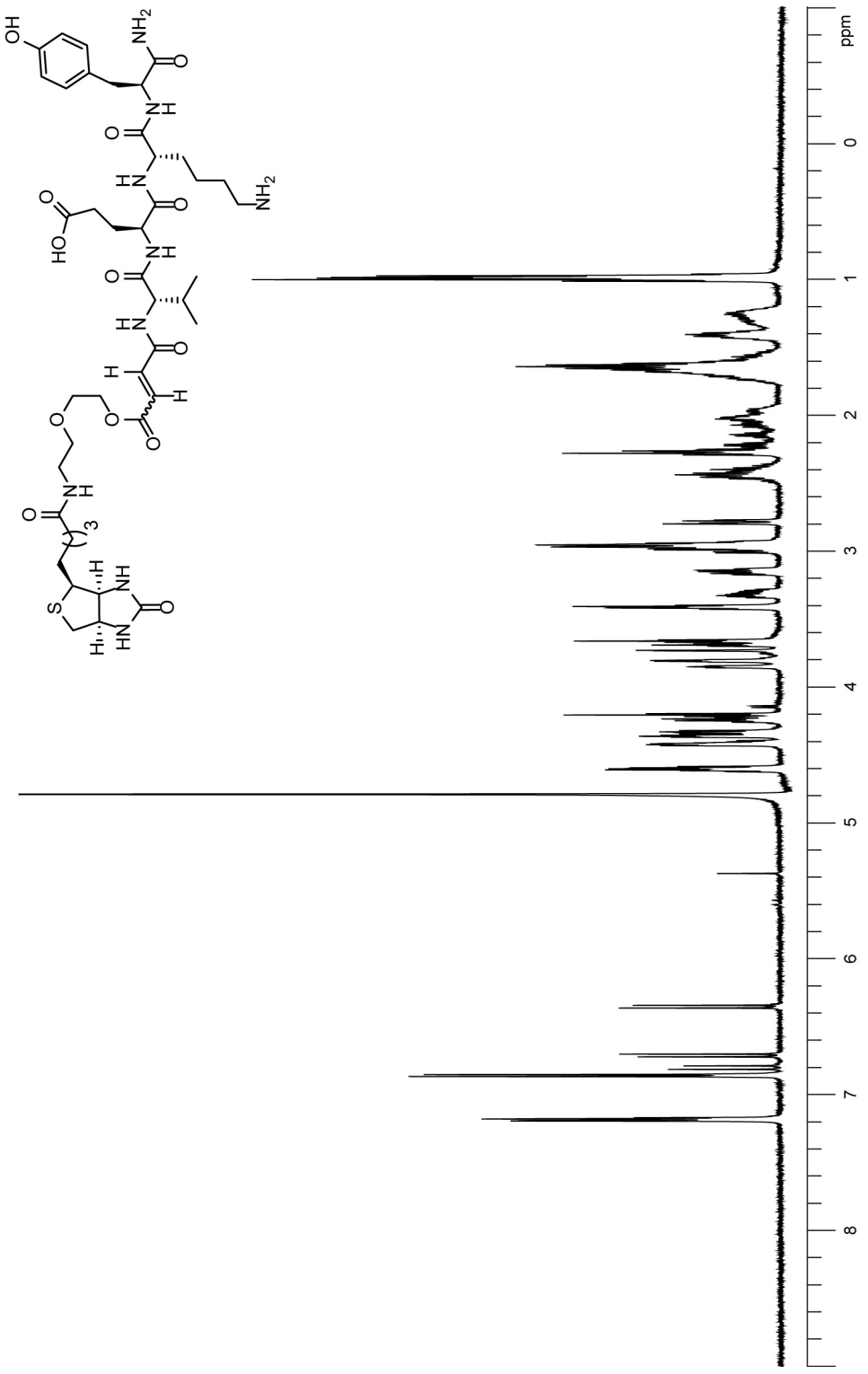
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Supporting information references

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