Supporting Information

General Methods. Fmoc-*p*-azido-L-phenylalanine was purchased from Bachem, whereas *p*-azido-L-phenylalanine was prepared according to previous reports.[1,2] Sec B protein containing *p*-azido-L-phenylalanine (N_3-SecB) was prepared via unnatural protein translation as described previously.[3] Organic solvents, as well as hydroxyl precursors for unsymmetrical phosphites were purchased from Acros Organics. D-(+)-Biotin was purchased from ABCR. Tetrazole solution (0.45 M in MeCN) was purchased from Fluka. All buffers were treated with Chelax 100 (Sigma) before use. SDS-PAGE gels (15 %) were run at 180 V. Transfer of proteins into PVDF membrane (Immobilon-P, Millipore) was carried at 0.8 A×cm^2 on a Biorad Trans-Blot SD Semi-Dry Transfer Cell. For blocking, Roti-Block (10×, Roth) was diluted with TBS. StAv-HRP was purchased from Roche (1/1000). Chemiluminescence detection was performed utilizing the Chemiluminescence-peroxidase Substrate Kit (Sigma-Aldrich) which was visualized on a Biozym MultiImage Light Cabinet (Alpha Innotech Coorperation). MALDI-MS was performed on an AB SCIEX TOF/TOF 5800 (Applied Biosystems Deutschland GmbH, Darmstadt, Germany) equipped with a Nd:YAG laser (355 nm). Samples were prepared with alpha-cyano-4-hydroxycinnamic acid matrix and measured in the linear mode. The spectrum obtained was the mean of 5000 laser shots. EI-MS was recorded on a MAT 711 (Varian) with the ionisation energy of 80 eV. LC-UV and LC-MS spectra were recorded on an Agilent 6210 TOF LC/MS system (Agilent Technologies, Santa Clara, CA). Spray voltage was set to 4 kV, and drying gas flow rate was set to 25 psi. Separation of samples was performed on a single Luna 5 μC18(2) 100 A column (5 μm, 4.6×150 mm) at a flow rate of 0.6 mL/min, utilizing gradients of MeCN in H_2O contained 1 % AcOH. Preparative HPLC purification was performed on a JASCO LC-2000 Plus system using a Kromasil RP18 column (25×250 mm) at a flow rate of 16 mL/min, utilizing gradients of MeCN in H_2O contained 0.1 % TFA. ^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR spectra were

recorded on a Jeol ECX/400, a Bruker 500 AMC, or a Bruker AMC 700 in CD_3CN, MeOD or D_2O. The chemical shifts are reported in ppm relatively to the residual solvent peak.

Specification of the model azido protein (N_3-SecB).

MSEQNNTEMT FQIQRIYTKD ISFEAPNAPH VFQKDWQPEV KLDLDTASSQ LADDVYEVVL RVTVTASLGE ETAFLCEVQQ GGIFSIAGIE GTQMAHCLGA YCPNILFPYA RECITSMVSR GTFPQLNLAP VNFDALFMNY LQQQAGEGTE EHQDA**X**GHHH HHH ; **X** = Phe(*p*-N_3)

Amino acid count: A = 14; C = 4; D = 8; E = 13; F =9; G = 10; H = 9; I = 8; K = 3; L = 12; M = 5; N = 7; P = 7; Q = 14; R = 4; S = 8; T = 11; V = 10; W = 1; **X** = 1; Y = 5.

Estimated pI = 4.9 (4.4 without HisTag); estimated charge at pH 8.2 = -16.[4]

Synthesis of unsymmetrical phosphites 4a-f.

Phosphites 4a-c and 4e-f. Dimethyl diisopropylphosphoramidite **8** (one equivalent),[5] was added to a mixture of an alcohol (0.2 mL, 5.5 mmol of ethanol for **4a**; 0.3 mL, 2.28 mmol of decanol for **4b**; 0.4 mL, 1.87 mmol of 2-propanol for **4c**; 230 mg, 2.44 mmol of phenol for **4e;** and 0.3 mL, 2.9 mmol of benzyl alcohol for **4f**) and tetrazole in MeCN (0.7-1 eq of 0.45 mM solution). The reaction was stirred for 1-2 h at RT, and diethyl ether (30 mL) was added. The resulting solid was filtered off. The residual solution was concentrated (a special care needs to be applied during evaporation of MeCN from mixtures containing phosphites **4a** and **4c**, as these compounds are highly volatile) to yield a residue, which was partially redissolved in diethyl ether, and then filtered off. Upon removal of ether, unsymmetrical phosphites **4a-c, e, f** were obtained as colorless liquids possessing characteristic odour in 80-98 % yield (see Table 1).

Biotinyl dimethyl phosphite 4d. Biotin (1.0 g, 4.08 mmol) was suspended in THF (15 mL) and $LiAlH_4$ (185 mg) was added. The mixture was heated to 45 °C for 2 h followed by addition of the second portion of $LiAlH_4$ (500 mg). The reaction was stirred at 45 °C until

completion (TLC, SiO_2, 20 % MeOH in acetone). Water was added and the mixture was concentrated. The residue was extracted with EtOH. The solvent was evaporated and the product was taken up in DMF and evaporated twice to remove traces of ethanol. The pure product was obtained as a white solid (795 mg, 84 %). The spectroscopical data was in accordance to the literature.[6] To a solution of thus prepared biotinol (145 mg, 0.63 mmol) and tetrazole (2.1 mL, 0.45 M in MeCN) in DMF (2 mL) was added dimethyldiisopropylphosphoramidite **8** (194 µL, 0.945 mmol) under inert conditions. The reaction mixture was stirred at RT for 90 min. The mixture was evaporated to dryness in high vacuum. It was possible at this point to dissolve the mixture in DMSO and freeze it, so that it could be used as a stock solution without decomposition for at least two weeks. For isolation, the residue was treated with ethyl acetate and filtered. The filtrate was concentrated and the crude product was purified via column chromatography (AlO_3, EtOAc, 5-20 % MeOH) to yield a white fluffy solid (96 mg, 49 %, mp=90 °C). The procedure was performed during one day; the purified product could be stored at -14 °C for at least 3 months.

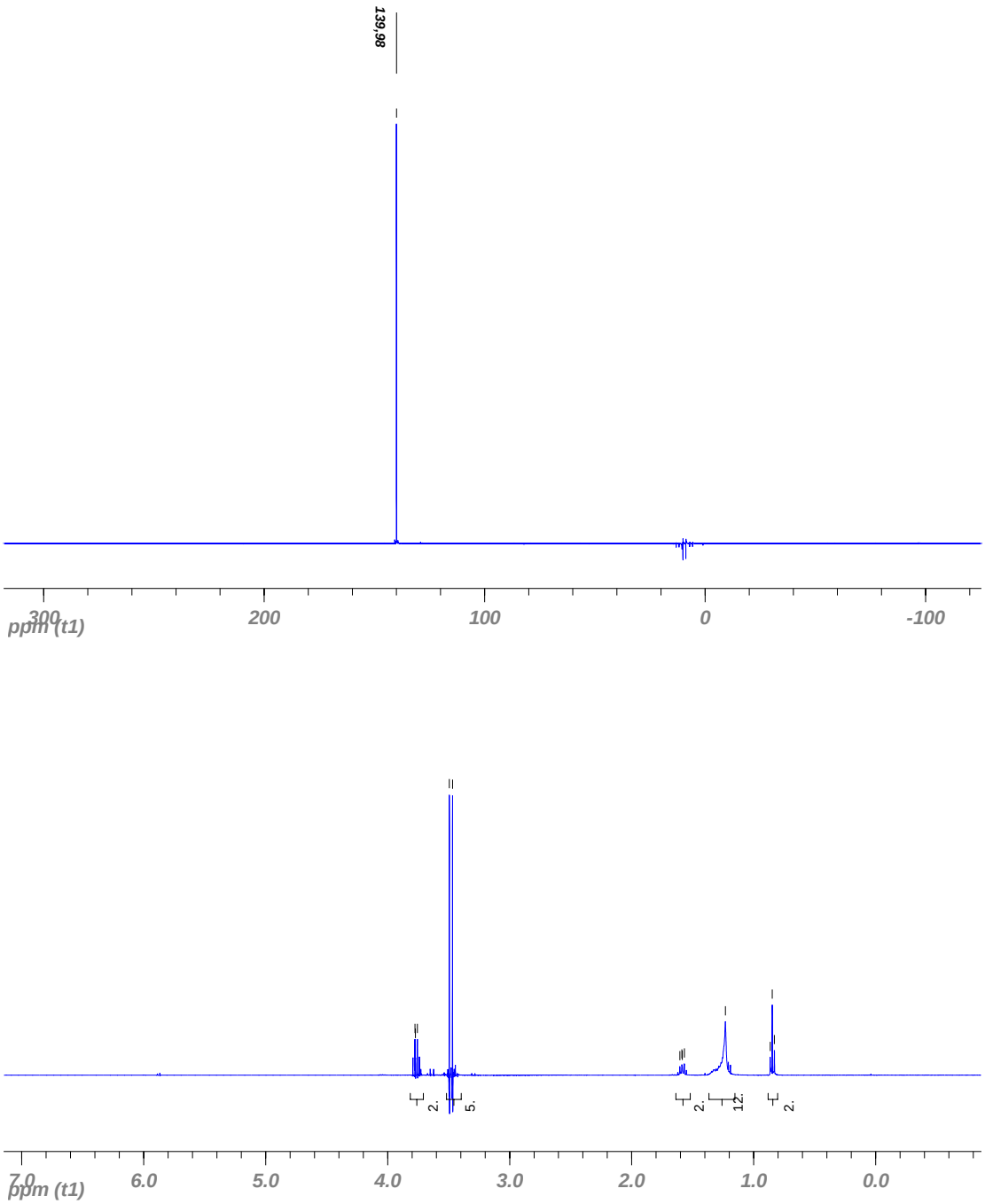
Spectroscopical Data: structures of all phosphites were confirmed by ^{1}H-, ^{31}P-, ^{13}C-NMR and HRMS, since literature values were incomplete. For new phosphites, **4b** and **4d**, 1H- and 31P-NMR were inserted. Known compounds were in consistency with literature values, given as follows:

Ethyl dimethyl phosphite **4a**[7]: ^{1}H-NMR δ (400 MHz, *CNCD*$_3$)= 1.18 (1 H, ddd, *J* = 14.28, 7.18, 0.52 Hz), 3.43 (6 H, dd, *J* = 10.51, 0.54 Hz), 3.77-3.83 (2 H, m); ^{31}P-NMR (162 MHz, *CDCl*$_3$) δ = 140.0; ^{13}C-NMR δ (101 MHz, *CD*$_3$*CN*) = 16.3 (d, *J* = 5.00 Hz, CH_3), 48.33 (d, *J* = 9.60 Hz, $POCH_3$), 58.05 (d, *J* = 13.16 Hz, $POCH_2$); EI-MS: 138.0 (35 %, $[M]^+$), 110 (40 %, $[HOP(OMe)_2]^+$); HR-EI-MS: m/z = 138.0444 $[M]^+$, calcd.: 138.0446

Decanyl dimethyl phosphite **4b**: ^{1}H-NMR δ (400 MHz, *CDCl*$_3$) = 3.76 (ddd, *J* = 11.38, 7.06, 5.68 Hz, 2H), 3.48 (d, *J* = 10.50 Hz, 6H), 1.63-1.54 (m, 2H), 1.33-1.21 (m, 14H), 0.85 (t, *J* =

6.91, 6.91 Hz, 3H); ^{31}P-NMR δ (162 MHz, *CDCl$_3$*) = 139.98; ^{13}C-NMR δ (101 MHz, *CD$_3$CN*) = 62.26 (d, *J* = 12.20 Hz, CH_2), 48.33 (d, *J* = 9.45 Hz, CH_3), 31.00 (d, *J* = 5.02 Hz,CH_2), 31.75 (s, CH_2), 29.42 (CH_2), 29.40 (CH_2), 29.17 (s, CH_2), 29.08 (s,CH_2), 25.63 (s, CH_2), 22.50 (s, CH_2), 13.52 (s, CH_3); HR-ESI-MS: m/z = 273.1337 $[M+H]^+$, calcd.: 273.1590.

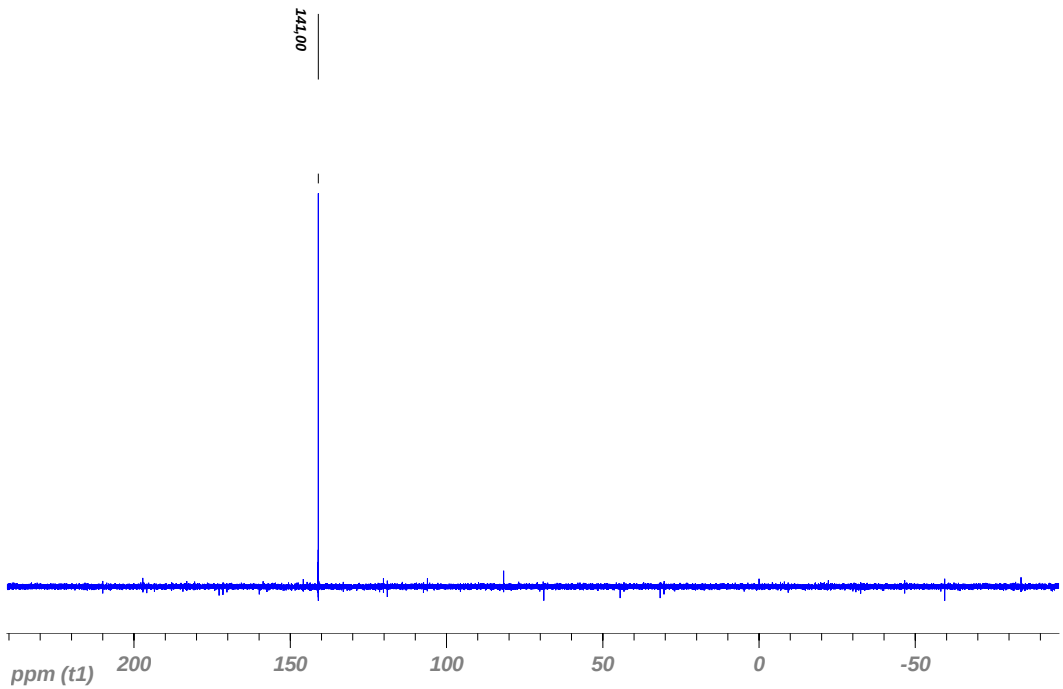
^{31}P-NMR (top) and ^{1}H-NMR (bottom) spectra for **4b**:



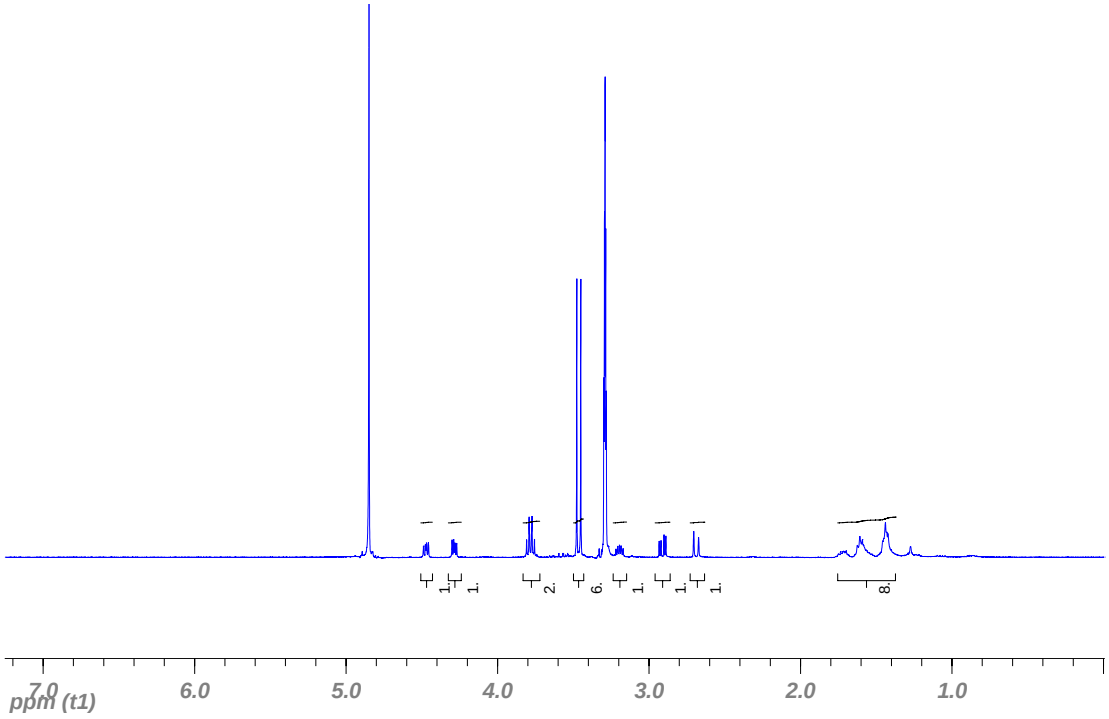
Dimethyl *iso*-propyl phosphite **4c**[8]**:** ^{1}H NMR ∂ (400 MHz, $CDCl_3$) = 1.19 (6 H, d, *J* = 6.18 Hz), 3.42 (6 H, dd, *J* = 10.37, 0.65 Hz), 4.26-4.37 (1 H, m); ^{31}P NMR ∂ (162 MHz, *CDCl*$_3$ ppm 140.17; ^{13}C NMR ∂ (101 MHz, *CDCl*$_3$) 23.83 (d, *J* = 3.81 Hz, OCH(**C**H_3)$_2$), 48.10 (d, *J* = 8.80 Hz, P(O**C**H_3)$_2$), 66.43 (d, *J* = 15.90 Hz, PO**C**H(CH_3)); EI-MS: 151.9 (20 %, $[M]^+$), 109.9 (50 %, $[HOP(OMe)_2]^+$); HR-EI-MS: m/z = 152.0603 $[M]^+$, calcd.: 152.0602.

Biotinyl dimethyl phosphite **4d**: ^{1}H-NMR: ∂ (400 MHz, MeOD): 1.37-1.50 (4 H, m) 1.54-1.60 (3 H, m), 1.75-1.68 (1 H m), 2.69 (1 H, d, J = 12.75 Hz), 2.91 (1 H, dd, J = 12.74, 4.99 Hz), 3.19 (1 H, ddd, J = 19.11, 5.41, 4.88 Hz), 3.46 (3 H, d, J = 10.43 Hz), 3.78 (2 H, td, J = 7.54, 6.45 Hz), 4.28 (1 H, dd, J = 7.89, 4.49 Hz), 4.47 (1 H, ddd, J = 7.96, 4.93, 0.71 Hz); ^{31}P-NMR: ∂ (162 MHz, MeOD): 141.0 (1 P, s); ^{13}C-NMR: ∂ (100 MHz, MeOD) ppm 25.6 (CH_2), 28,5 (CH_2), 28,7; (CH_2), 30.8 (d, J = 4.93 Hz, CH_2), 39.7 (CH**C**H_2S), 47.9 (d, J = 9.7 Hz, P(O**C**H_3)$_2$), 55.8 (N**C**HCHR-S), 60.3 (CH) , 62.0 (d, J = 12.1 Hz, PO**C**H_2), 62.1 (CH) 164.8 ((RNH)$_2$**C**O); HR-ESI-MS: m/z = 345.1009 $[M+Na]^+$, calcd.: 345.1009.

^{31}P-NMR spectrum for **4d**:



^{1}H-NMR spectrum for **4d**:

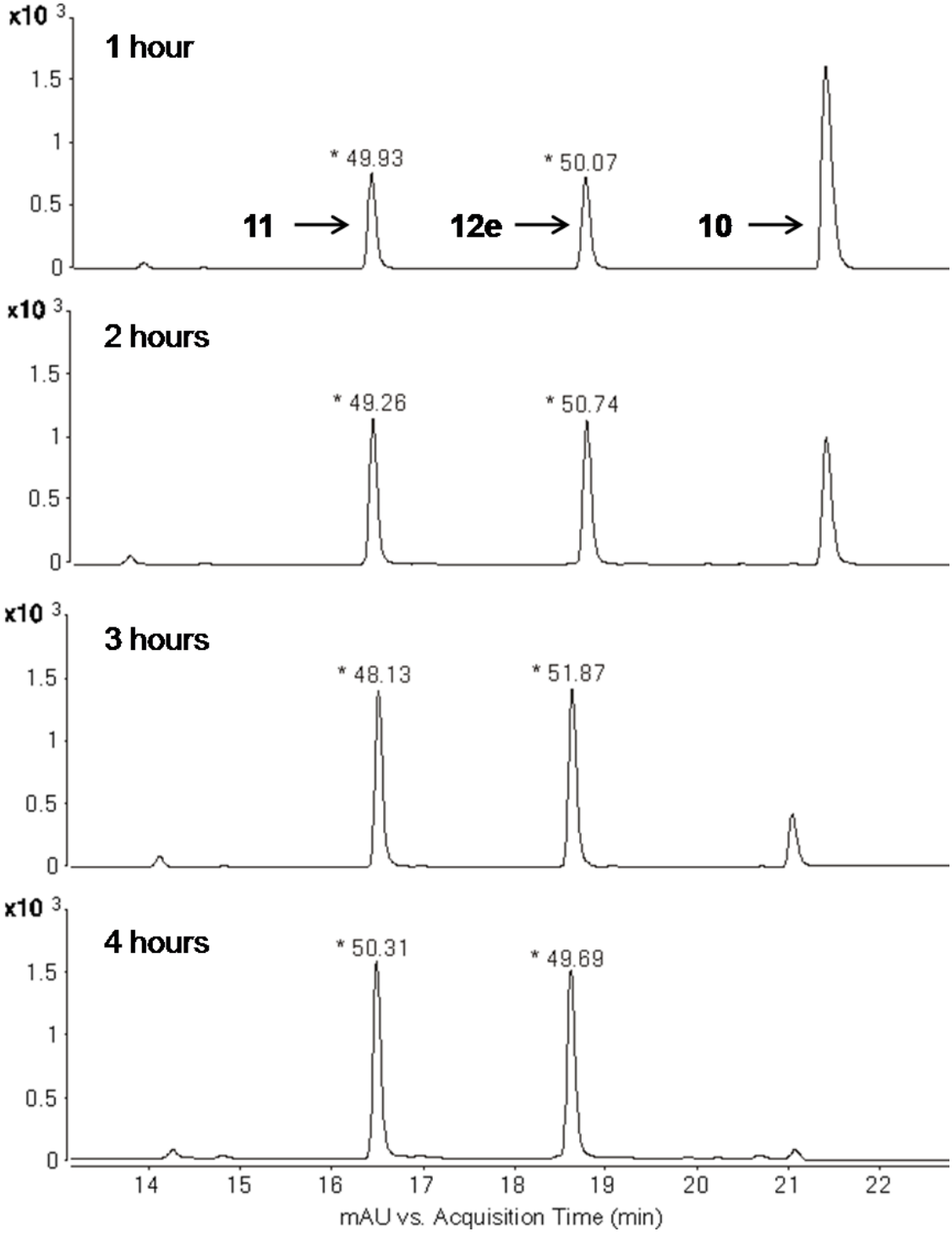


Benzyl dimethyl phosphite **4e**[9]: ^{1}H NMR (400 MHz, *CDCl*$_3$ δ = 4.87 (d, *J* = 8.12 Hz, 2H), 3.52 (d, *J* = 10.69 Hz, 6H), 7.35 (m, *J* = 4.71, 1.62 Hz, 5H); ^{31}P NMR δ (162 MHz, *CDCl*$_3$ = 140.16 ; ^{13}C NMR δ (101 MHz, *CD*$_3$*CN* = 63.84 (d, *J* = 11.79 Hz,CH_2), 48.75 (d, *J* = 9.99 Hz, CH_3), 138.73 (d, *J* = 5.75 Hz, C), 128.518 (CH), 127.541 (CH), 117.361 (C); EI-MS: 200.1 (30 %, $[M]^+$), 90.8 (100 %, $[C_7H_7]^+$); HR-EI-MS: m/z = 200.0601 $[M]^+$, calcd.: 200.0602.

Dimethyl phenyl phosphite **4f**[9]: ^{1}H NMR δ (500 MHz, *CDCl3)* 7.35-7.31 (m, 2H), 7.13-7.08 (m, 3H), 3.68 (d, *J* = 10.25 Hz, 6H); ^{31}P NMR δ (202 MHz, *CDCl*$_3$= 134.73 (d, *J* = 101.40 Hz,1 C); ^{13}C NMR δ (101 MHz, *CD*$_{3C}$*N)* = 152.51 (d, *J* = 6.42 Hz, C), 129.89 (s, CH), 123.72 (d, *J* = 1.31 Hz, C), 120.27 (d, *J* = 7.63 Hz, CH), 48.92 (d, *J* = 7.25 Hz,CH_3);; EI-MS: 186 (40 %, $[M]^+$), 155 (15 %, $[M\text{-}OMe]^+$); HR-EI-MS: m/z = 186.0446 $[M]^+$, calcd.: 186.0446.

Modification of Fmoc-*p*-azido-L-phenylalanine with unsymmetrical phosphites. To a solution of Fmoc[Phe(*p*-N_3)]OH **10** (0.43 mg, 1.0 μmol) in DMSO (70 μL), tris buffer (10 μL,

1 M, pH 8.2) and a solution of unsymmetrical phosphite **4** in DMSO (3.0 μmol in 20 μL) were added. The mixture was vortex-mixed vigorously for 1 min, and then incubated at 28 °C for 6 h. Following the incubation, reaction mixtures were immediately analyzed by RP-HPLC (injection volume: 10 μL) connected in line to a UV detector and an ESI-TOF detector. Identities of products were supported by MS data, which were obtained in good agreement with the calculated values (HR-ESI-MS for **11**: m/z = 511.1648 $[M+H]^+$, calcd.: 511.1629; for **12a**: m/z = 525.1757 $[M+H]^+$, calcd.: 525.1785; for **12b**: m/z = 637.2996 $[M+H]^+$, calcd.: 637.3037; for **12c**: m/z = 539.1947 $[M+H]^+$, calcd.: 539.1942; for **12d**: m/z = 709.2473 $[M+H]^+$, calcd.: 709.2455; for **12e**: m/z = 573.1751 $[M+H]^+$, calcd.: 573.1785; for **12f**: m/z = 587.1934 $[M+H]^+$, calcd.: 587.1942). The fractions of products **11** and **12a-f** in the post reaction mixtures were calculated based on the Fmoc absorbance at 300 nm measured for the corresponding HPLC peaks. In all reactions, the consumption of substrate **10** was 95-100 %, based on the LC-UV analysis. The constant ratio between products **11** and **12** during the course of the transformation was confirmed in the reaction between **10** with **4e**, in which case aliquots were withdrawn from the reaction mixture after 1, 2, 3 and 4 h; and they were analyzed by LC-UV (SI Figure 1).

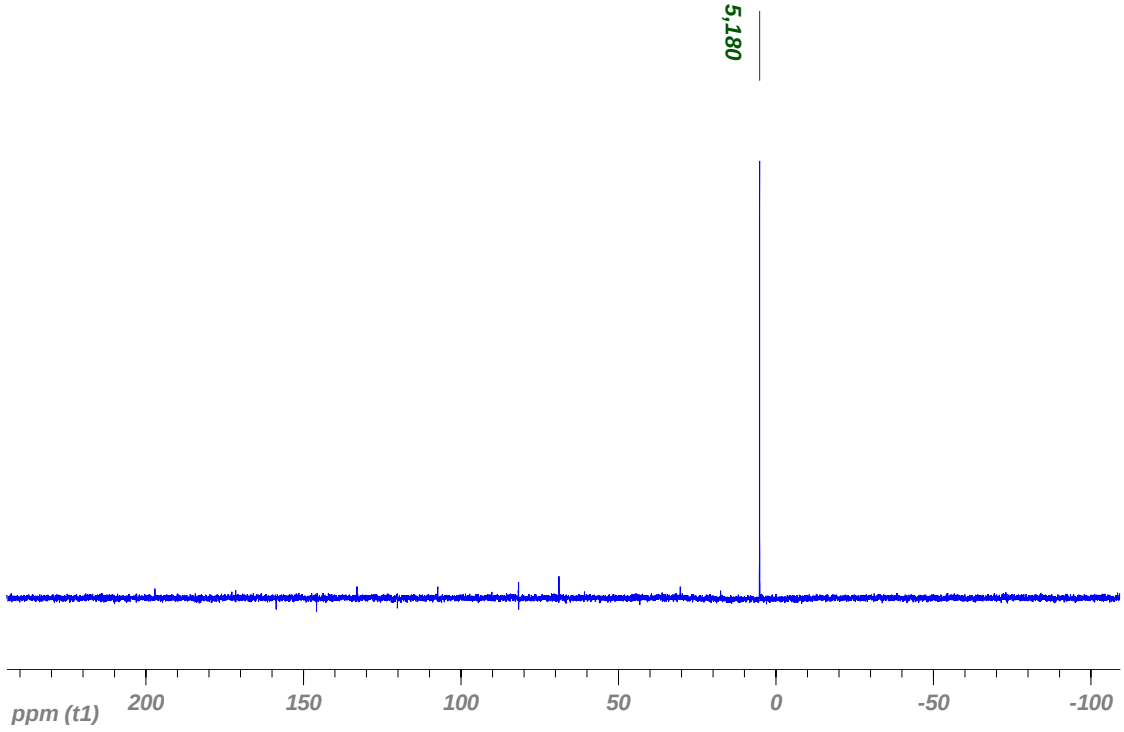


SI Figure 1. LC-UV_{300nm} based monitoring of the reaction between **10** and **4e** to yield **11** and **12e**. Numbers above peaks indicate fractions of phosphoramidate products; 1, 2, 3 and 4 h after the reaction was initiated.

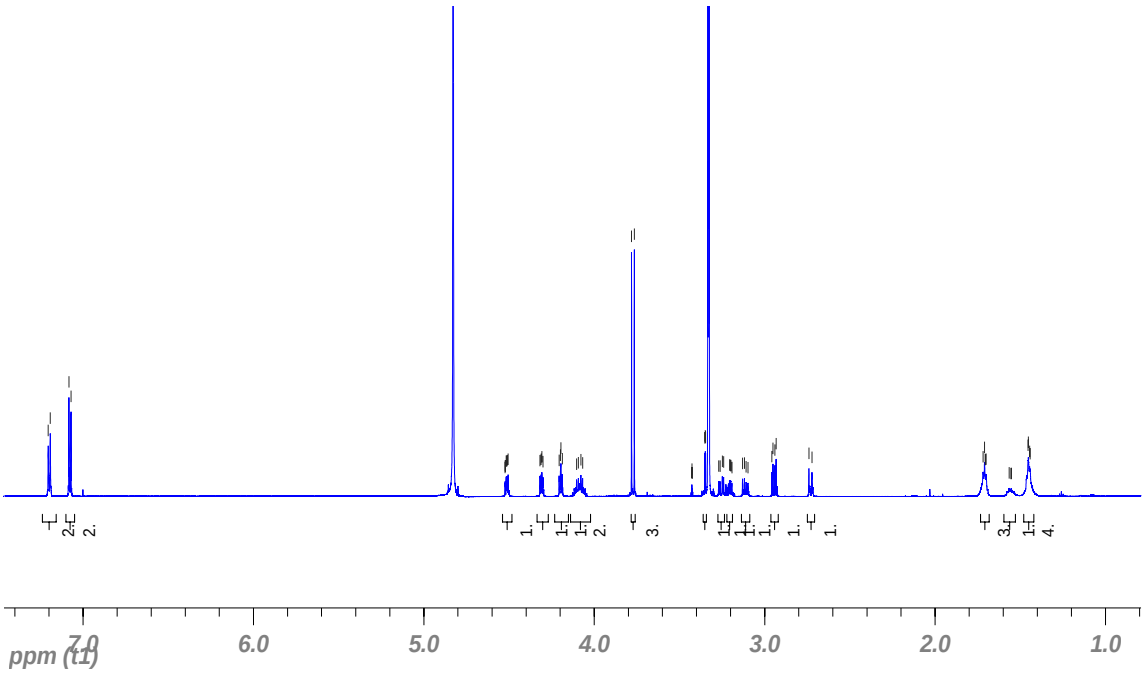
Biotinylation of *p*-azido-L-phenylalanine with phosphite 4d. To a solution of H[Phe(*p*-N_3)]OH[1, 2] **13** (3.3 mg, 15.9 μmol) in tris buffer (800 μL, 1 M, pH 8.2), phosphite **4d** (8.7 mg, 28.6 μmol) was added. The mixture was vortex-mixed vigorously for 3 min, and then

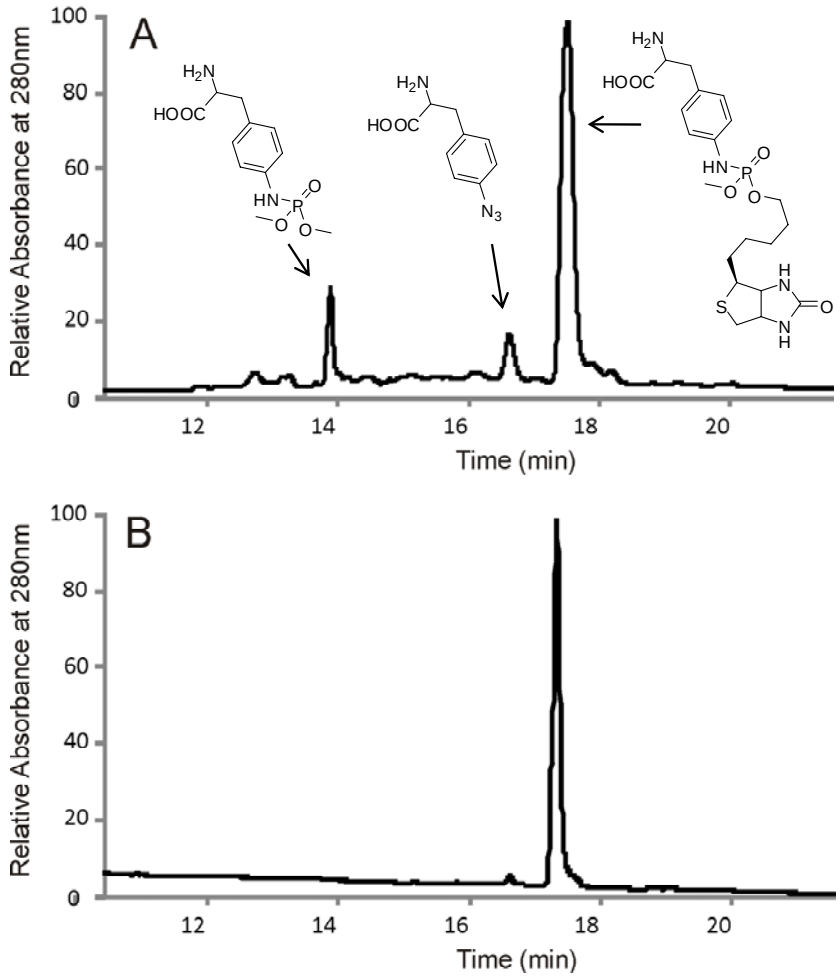
incubated at 25 °C for 16 h. The mixture was analyzed by LC-UV-MS to reveal that the biotinylated amino acid **14** was the major component (SI Figure 2). Compound **14** was isolated from the mixture via preparative HPLC, and lyophilized to give a white solid (3.1 mg, 40 %). ^{1}H-NMR: ∂ (700 MHz, MeOD) = 1.40-1.46 (4 H, m) 1.59-1.53 (1 H, m), 1.75-1.68 (3 H m), 2.73 (1 H, dd, J = 12.66, 3.96 Hz), 2.94 (1 H, ddd, J = 12.76, 5.10, 5.10 Hz), 3.11 (1 H, ddd, J = 14.57, 7.53, 3.30 H), 3.21 (1 H, ddd, J = 9.25, 5.48, 4.72 Hz), 3.26 (1 H, dd, J = 14.68, 5.51 Hz), 3.33-3.34 (1H, m), 3.77 (3 H, d, J = 11.47 Hz), 4.13-4.04 (2 H, m), 4.20 (1 H, ddd, J = 7.41, 5.55, 1.77 Hz), 4.31 (1 H, ddd, J = 7.67, 4.55, 3.33 Hz), 4.53-4.49 (1H, m), 7.11-7.04 (2 H, m), 7.20 (2 H, d, J = 8.40 Hz); ^{31}P-NMR: ∂ (280 MHz, MeOD) = 5.18 (1 P, s); ^{13}C-NMR: ∂ (176 MHz, MeOD) = 25.28 (CH_2), 28.32 (CH_2), 28.38 (CH_2), 29.58 (CH_2), 35.26 (CH**C**H_2S), 39.58 (**C**H_2Ar), 52.559 (d, J = 4.7 Hz, CH_3), 53.88 (R**C**HS), 55.74 (N**C**HCH_2-S, 60.28 (N**C**HCHR-S), 62.06 (H**C**NH_2), 66.74 (d, *J* = 5.47 Hz, CH_2), 118.05 (d, *J* = 7.48 Hz, CH), 127.12 (CH), 129.93 (C) 139.86 (C), 164.75 ((RNH)$_2$**C**O, 170.01 **(C**OOH); HR-ESI-MS for **14**: m/z = 487.1797 $[M+H]^+$, calcd.: 487.1775.

^{31}P-NMR spectrum for **14**:



^{1}H-NMR spectrum for **14**:

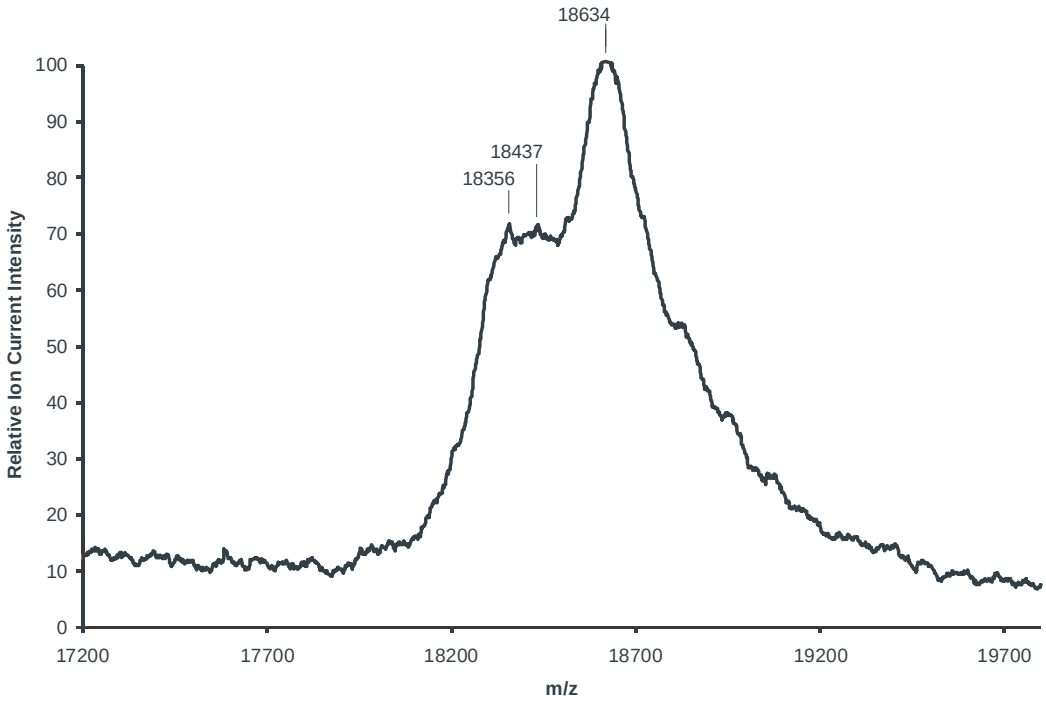




SI Figure 2. LC-UV based analysis of compound **14** in the mixture obtained upon incubation of **13** with **4d** for 16h (A) and after purification by preparative HPLC (B).

Biotinylation of protein 16 *via* Staudinger-phosphite reaction.

To a solution of N_3-Sec-B (40 μM) in phosphate buffer (10 μL, 100 mM, pH 8.0), was added a solution of phosphite **4d** (50 mM) in the buffer containing DMSO for the solubilisation of protein, which had precipitated upon storage (10 μL including 2-5 μL of DMSO), the samples were vortex-mixed and incubated overnight at 28 °C. As a control, a Sample with the N_3-SecB and biotinol in the buffer containing DMSO (10 μL including 5 μL of DMSO) was submitted to the same conditions. The samples were precipitated with cold acetone, decanted and the protein pellet was dissolved in sample buffer, followed by a short incubation (90 s) at 95 °C. The samples were analyzed via SDS-PAGE (15 %) and subsequent Western Blotting (semi dry, 28 mV, 50 min). The Western Blot was blocked with Roti-block solution over night and incubated for 1 h with streptavidin-horseradishperoxidase. The membrane was washed three times for 5 min with TBS. The luminescence reagents (2+1 mL Aldrich labeling Kit) were added according to the kit instructions and the luminescence image was taken. The membrane was then washed with water and TBS and stained with Ponceau S. For MALDI-MS analysis, upon the overnight incubation with **4d** the protein was purified with Ni-NTA magnetic beads (Qiagen) followed by ZipTip C18 (Millipore) chromatography. A mixture of compounds **16**, **17** and **18** has been observed, in which the biotinylated protein was the major component (SI Figure 3). Identities of products were supported by MS data, which were obtained in good agreement with the calculated values (MALDI-MS for **16**: m/z = 18356 $[M+H]^+$, calcd.: 18347; for **17**: m/z = 18437 $[M+H]^+$, calcd.: 18429; for **18**: m/z = 18634 $[M+H]^+$, calcd.: 18627.



SI Figure 3. MALDI-MS spectrum for the mixture obtained upon overnight incubation of protein **16** (0.4 nmol) with phosphite **4d** (0.5 μmol).

References

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