Supporting material

Deuterium labeled peptides give insights into the directionality of class III lantibiotic synthetase LabKC.

Bartlomiej Krawczyk‡, Paul Ensle‡, Wolfgang M. Müller, Roderich D. Süssmuth*

1.Experimental procedures

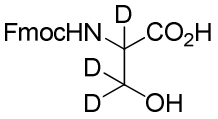
1.1 Enzyme expression, purification and activity assays.

LabKC-his$_6$ was heterologously expressed in *E. coli* and purified using IMAC and SEC as described previously (Müller et. al., Biochemistry 2011). The reaction mixture (30-50 µl) was composed as following: 20 mM tricin buffer (pH=7.4), 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM GTP, 0.6 µM LabKC-his$_6$, 9 µM precursor peptide. Reaction was carried at 28 °C and quenched by addition of methanol (1:1 vol). For elimination experiments with phosphorylated peptides, GTP was excluded from the mixture and the reaction was quenched after 20min. After centrifugation samples were directly analyzed by HPLC-ESI-MS.

In experiments intended for further MS/MS analysis, reaction was quenched by addition of EDTA (final conc. 40 mM) and the leader peptide was proteolytically removed using trypsin (New England Biolabs). After 4-8h of incubation at 28 °C, methanol was added (1:1 vol.), samples were centrifuged and analyzed. Due to the presence of two Arg in the leader peptide hydrolysis might occure at two different positions: -1 and -5 (highlighted): MASILELQNLDVEHA**R**GEN**R**SDWSLWECASTGSLFACA. For most of the peptides cleavage at Arg(-1) was observed (yielding peptide: SDWSLWECASTGSLFACA), however when the first Ser was modified (phosphorylation/dehydration), cleavage was observed at Arg(-5) yielding the product with the leader peptide overhang (GNERSDWSLWECASTGSLFACA).

1.2 Peptide synthesis and purification

2-(((9H-Fluoren-9-yl)methoxy)carbonyl-(2,3,3-D)-L-serine:



Chemical Formula: $C_{18}H_{14}D_3NO_5$
Exact Mass: 330.12950

A solution of 9-fluorenylmethyl succinimidyl carbonate (1.638 g, 4.86 mmol, 1.05 eq) in dioxane (10 ml) was cooled to 0 °C in ice bath. A solution of (2,3,3-D)-L-serine (0.50 g, 4.63 mmol, 1.0 eq) and sodium carbonate (0.54 g, 5.09 mmol, 1.1 eq) in water (6 ml) was added dropwise. After addition, the ice bath was removed and the solution was stirred for 16 h. The dioxane was evaporated and water (10 ml) was added. The solution was acidified with 5% aqueous HCl to pH 2. The aqueous layer was extracted with EtOAc (10 x 50 ml). The combined organic layer were washed with brine (100 ml) and concentrated in rotary evaporator. After drying in high vacuo, the residue was recrystallized from chloroform to give the product as a white solid (1.42 g, 93%).

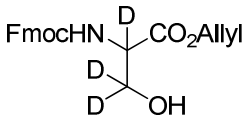
R_f = 0.56 (EtOAc/nBuOH/THF/H_2O 2:2:1:1)

^{1}H NMR (400 Mhz, DMSO-d_6): δ = 12.64 (bs, 1H), 7.90-7.88 (d, 2H, J_1 = 7.52 Hz), 7.76-7.73 (dd, J_1 = 7.25 Hz, J_2 = 4.03 Hz), 7.47 (s, 1H), 7.43-7.40 (t, 2H, J_1 = 7.12 Hz), 7.35-7.31 (t, 2H, J_1 = 7.39 Hz), 4.95 (s, 1H), 4.29-4.20 (m, 3H)

^{13}C NMR (100 Mhz, DMSO-d_6): δ = 172.16, 156.08, 143.85, 140.72, 127.65, 127.11, 125.36, 120.12, 65.73, 46.60

HRMS (ESI+) m/z calculated for $C_{18}H_{15}D_3NO_5^+$ ($[M+H]^+$): 331.13678, found 331.13689; $C_{18}H_{14}D_3NO_5Na^+$ ($[M+Na]^+$): 353.11872, found 353.11855

2-(((9H-Fluoren-9-yl)methoxy)carbonyl-(2,3,3-D)-L-serine-allylester:

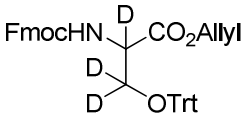


Chemical Formula: $C_{21}H_{18}D_3NO_5$
Exact Mass: 370.16080

To a solution of Fmoc-(2,3,3-D)-L-serine (1.40 g, 4.24 mmol, 1.0 eq) in dry DMF (25 ml), potassium carbonate (0.70 g, 5.06 mmol, 1.2 eq) and allyl bromide (0.81 ml, 9.25 mmol, 2.2 eq) were added. The mixture was stirred at room temperature for 15 h. DMF was removed in vacuo. The residue was resolved in water and EtOAc and the layers were separated. The aqueous layer was extracted with EtOAc (5 x 40 ml) and the combined organic layer were washed with brine (2 x 50 ml). The organic solvent was removed under high vacuum and the residue was dried in high vacuo for 24 h. The product was used for the next reaction without further purification

R_f = 0.11 (Hex/EtOAc 3:1)

2-(((9H-Fluoren-9-yl)methoxy)carbonyl-(2,3,3-D)-3-trityloxy-L-serine-allylester:



Chemical Formula: $C_{40}H_{32}D_3NO_5$
Exact Mass: 612.27035

Fmoc-(2,3,3-D)-L-serine-allylester was dissolved in dry DCM (40 ml) and DIPEA (2.4 ml,13.88 mmol, 3.0 eq) was added. A solution of triphenylmethane chloride (2.58 g, 9.25 mmol, 2.0 eq) in dry DCM (10 ml) was added dropwise. The solution was stirred for 16 h. The solvent was removed in vacuo. Purification with flash chromatography on silica gel (Hex/EtOAc 7:1) gave the product as a white solid (1.86 g, 72% over two steps).

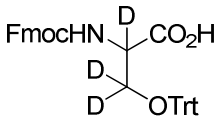
R_f = 0.49 (Hex/EtOAc 3:1)

^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.01 (s, 1H), 7.89-7.88 (d, 2H, J_1 = 7.52 Hz), 7.74-7.72 (dd, 2H, J_1 = 7.66 Hz, J_2 = 3.90 Hz), 7.42-7.35 (m, 8H), 7.32-7.23 (m, 11H), 5.90-5.80 (m, 1H), 5.29-5.25 (dd, 1H, J_1 = 17.26 Hz, J_2 = 1.54 Hz), 5.18-5.15 (dd, 1H, J_1 = 10.54 Hz, J_2 = 1.28 Hz), 4.58-4.57 (d, 2H, J_1 = 5.37 Hz), 4.28-4.20 (m, 3H)

^{13}C NMR (100 MHz, DMSO-d_6): δ = 170.03, 155.94, 143.72, 143.34, 140.67, 132.11, 128.23, 127.86, 127.64, 127.08, 125.28, 120.10, 117.90, 86.08, 65.94, 65.07, 46.57,

HRMS (ESI+) m/z calculated for $C_{40}H_{32}D_3NNaO_5^+$ ([M+Na]$^+$): 635.25957, found 635.25901

2-(((9H-Fluoren-9-yl)methoxy)carbonyl-(2,3,3-D)-3-trityloxy-L-serine:



Chemical Formula: $C_{37}H_{28}D_3NO_5$
Exact Mass: 572.23905

Fmoc-(2,3,3-D)-L-serine(Trt)-allylester (1.844 g, 3.01 mmol, 1.0 eq) was dissolved in dry THF (15 ml) under argon and covered with aluminium foil. After addition of tetrakis(triphenylphosphin)-palladium (0.175 g, 0.15 mmol, 0.05 eq) to the stirred solution, morpholin (0.275 ml, 3.16 mmol, 1.05 eq) dissolved in THF (12 ml) was added dropwise. After 90 min, the reaction mixture was poured into EtOAc (200 ml) and the organic layer was washed with brine (2 x 50 ml). The aqueous layer was extracted with EtOAc (5 x 20 ml). The solvent was removed with rotary evaporator to give the product (1.912 g, purity >90%), which was used for peptide synthesis without further purification.

R_f= 0.45($CHCl_3$/MeOH 9:1)

^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.90-7.88 (d, 2H, J_1 = 7.52 Hz), 7.78 (s, 1H), 7.76-7.73 (dd, 2H, J_1 = 7.46 Hz, J_2 = 1.68 Hz), 7.58 (bs, 1H), 7.42-7.37 (m, 8H), 7.31-7.27 (m, 11H), 4.29-4.22 (m, 3H)

^{13}C NMR (100 MHz, DMSO-d_6): δ = 171.60, 155.89, 143.75, 143.52, 140.67, 128.24, 127.81, 127.62, 127.02, 125.29, 120.08, 91.97, 65.87, 46.59

HRMS (ESI+) m/z calculated for $C_{37}H_{28}D_3NNaO_5^+$ ([M+Na]$^+$): 595.22827, found 595.22651

HRMS (ESI-) m/z calculated for $C_{37}H_{27}D_3NO_5^-$ ([M-H]$^-$): 571.23178, found 571.23149

Peptide synthesis and purification
Fmoc-protected amino acids were purchased from Orpegen (Heidelberg, Germany). Isotope labeled (2,3,3-D)-L-serine was purchased from Euriso-top (Saint-Aubin Cedex, France). The coupling reagents were obtained from Iris Biotech (Marktredwitz, Germany) and Merck (Darmstadt, Germany). The TCP resin was bought from Intavis (Reutlingen, Germany). DMF (99.8%) was purchased from VWR (Darmstadt, Germany). The chemicals for peptide cleavage were obtained from Sigma-Aldrich (Schnelldorf, Germany) and ABCR (Karlsruhe, Germany). Automated solid phase Fmoc-synthesis was performed on a Prelude parallel synthesizer by Protein Technologies (Tucson, AZ, USA).

Synthesis protocol for automated solid-phase peptide synthesis

Automated solid-phase peptide synthesis was performed in 25 µmol scale.

Loading: To a 10 ml syringe reactor with frit and cap were added 1 g of tritylchloride (TCP) resin (1.56 mmol/g) and 7 ml dry DCM. The resin was pre-swollen for 10 min and the solvent was drained by vacuo. A mixture of the amino acid (0.6 mmol) and 3 equivalents of DIPEA dissolved in 5 ml dry DCM was added to the resin. The syringe was agitated for 30 min at room temperature. The solution was removed and the resin was washed (2x 5 ml DMF, 2x 5 ml DCM). Capping of non-reacted functional groups of the resin was performed with DCM, methanol and DIPEA 80:15:5 (2x 10ml, 10 min). After washing (5x 5 ml DMF), Fmoc-removal was achieved with DMF/piperidine (20 %, 5 ml, 1x 2 min, 1x 20 min). After final washing (2x 5 ml DMF, 1x 5 ml methanol, 3x 5 ml DCM), the resin was dried in vacuo.

Coupling of Fmoc/tBu-protected amino acids: To 50 mg of the resin (~ 0.5 mmol/g), a 250 mM solution of the amino acid in DMF (10 eq relative to resin loading) was added. After addition of a 500 mM solution of DIPEA in DMF (10 eq) and a 250 mM solution of TBTU in DMF (10 eq), the reaction solution was mixed for 15 min. A second coupling was performed for 30 min. For couplings subsequent to the 10th amino acid, a third coupling was performed with 45 min coupling time. Finally, the resin was washed with DMF (3x 2.5 ml), methanol (2x 2.5 ml) and DMF (3x 2.5 ml).

Fmoc removal: DMF/piperidine (20 %, 2.5 ml) was added to the resin and mixed for 2.5 min. The procedure was repeated 4 times. For couplings subsequent to the 10th amino acid, Fmoc-cleavage was performed for 3 min with 5 repeats. The resin was washed with DMF (3x 2.5 ml), methanol (2x 2.5 ml) and DMF (3x 2.5 ml). After the final coupling cycle, the resin was washed with DCM (6x 2 ml).

Isotope labeled peptides: The deuterium labeled serines were coupled manually during automated synthesis. The amino acid (3 eq) and HATU (3 eq) were dissolved in DMF (1 ml) and DIPEA (4.5 eq) was added. The solution was added to the resin and mixed for 2 h. Couplings were repeated until Kaiser test showed complete conversion.

Phosphorylated peptides: The phosphorylated peptides were synthesized in the same manner. Automated synthesis was paused and the phosphorylated serine was coupled manually. Fmoc-Ser(PO(OBzl)OH)-OH (3 eq) and HATU (3 eq) were dissolved in DMF (1 ml) and DIPEA (4.5 eq) was added. The solution was added to the resin and mixed for 1 h. Couplings were repeated until Kaiser test showed complete conversion.

Global deprotection: The resin was transferred to a 5 ml syringe with frit and cap. After addition of the cleavage cocktail (TFA, H_2O, TES, DODT (3,6-dioxa-1,8-octane-dithiole) 92.5:2.5:2.5:2.5), the syringe was shaken for 3 h. The peptide was precipitated in ice cold diethyl ether and centrifuged. The supernatant was removed and the precipitate was washed with ethyl acetate twice. The peptide was resolved in 0.1% acetic acid and lyophilized.

Peptides purification: All peptides were purified prior usage by preparative RP-HPLC (Grom-Sil 120, ODS-5 ST, 10 µm, 20 x 250 mm; Grom, Rottenburg Hailfingen, Germany) coupled to an Agilent HPLC-1100-series (Agilent Technologies, Böblingen, Germany). Purity of peptides was confirmed by LC-MS. After solvent removal peptides were resuspended in 20 mM Tris buffer (pH=8.0) and the concentration was determined spectrophotometrically followed by reduction with 30 mM DTT.

1.3 HPLC-ESI-MS and MSMS analysis.

All HPLC-ESI-MS and MS/MS runs were performed using Agilent 1200 HPLC System (Agilent Technologies, Waldbronn, Germany) coupled to LTQ-Orbitrap XL (Thermo Fisher Scientific, Bremen, Germany). Chromatographic separation was achieved with reversed phase C18 column GromSil 120 ODS-5 ST (100 mm x 2.0 mm, Grace, Deerfield, IL, USA) with the mobile phase composed of water (A), and acetonitrile (B), supplemented with 0.1% formic acid. Gradient from 5% to 100% of B in 18 min was used. All LC-MS measurements were recorded in positive-ionization mode. Tandem MS/MS experiments were performed using the same setup and gradient. Fragmentation was performed in HCD cell using normalized collision energy of 24%, spectra were recorded in FTMS mode at resolution 7500.

2. Mass spectrometric data.

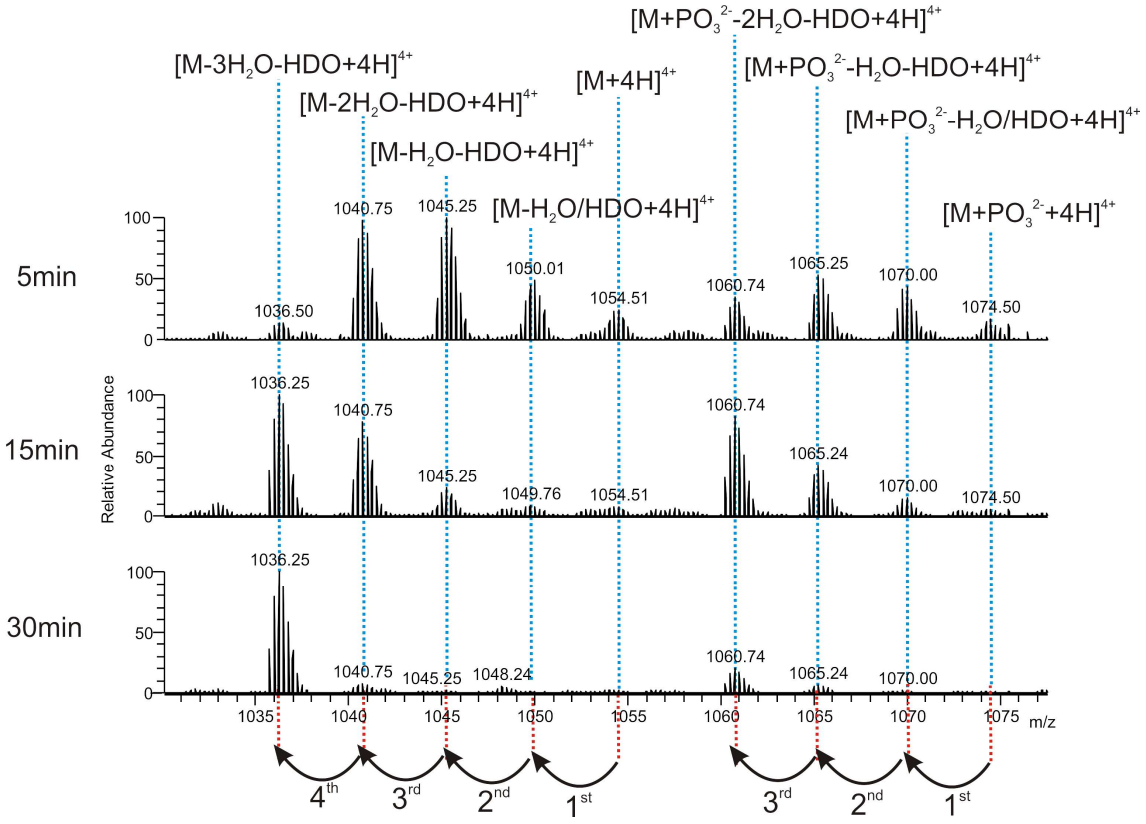


Figure S1. Time dependent substrate conversion by LabKC under typical reaction conditions. Both dehydration and phosphorylation intermediates have been assigned according to monoisotopic molecular masses. Arrows reflect the sequential dehydration.

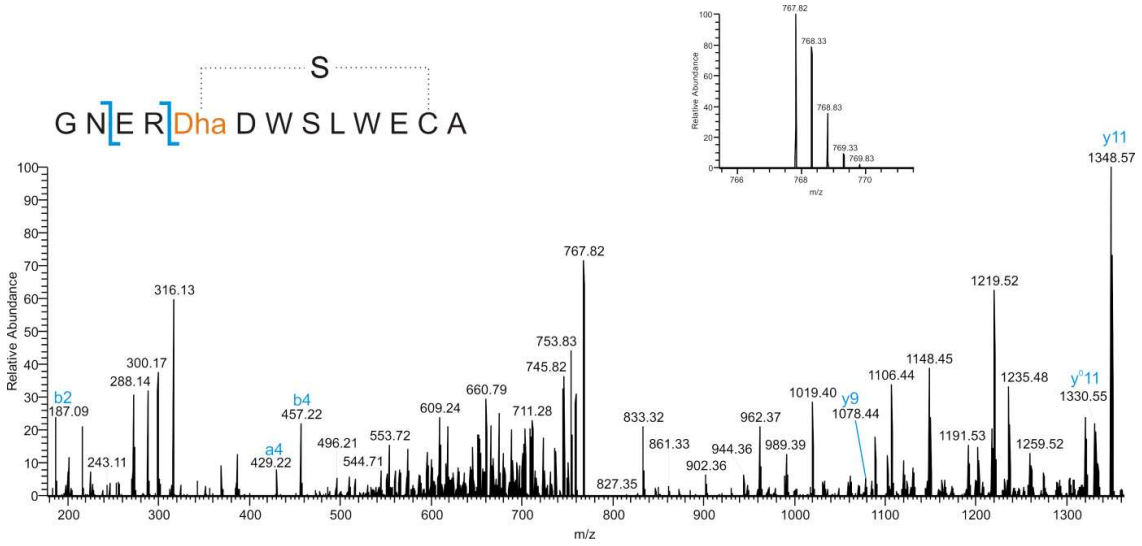


Figure S2. HPLC-ESI-MS/MS spectra of LabA2(1-9)^{pSer1} after elimination reaction and tryptic removal of leader peptide ([M+2H]=767.82). In the right upper corner HPLC-ESI-MS spectrum of intermediate after tryptic removal of the leader peptide.

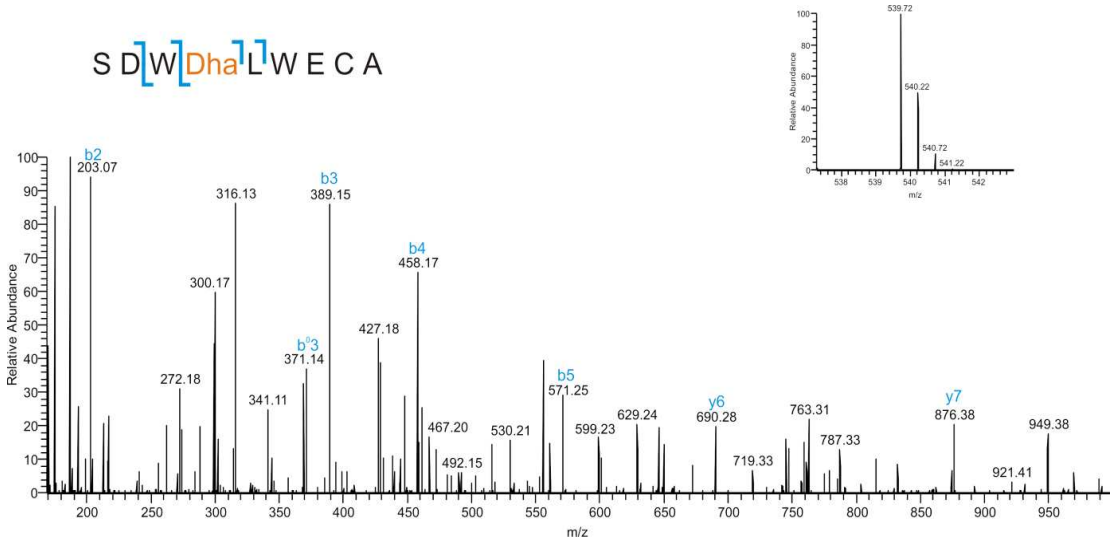


Figure S3. HPLC-ESI-MS/MS spectra of LabA2(1-9)^{pSer4} after elimination reaction and tryptic removal of leader peptide ([M+2H]=539.72). In the right upper corner HPLC-ESI-MS spectrum of intermediate after tryptic removal of the leader peptide.

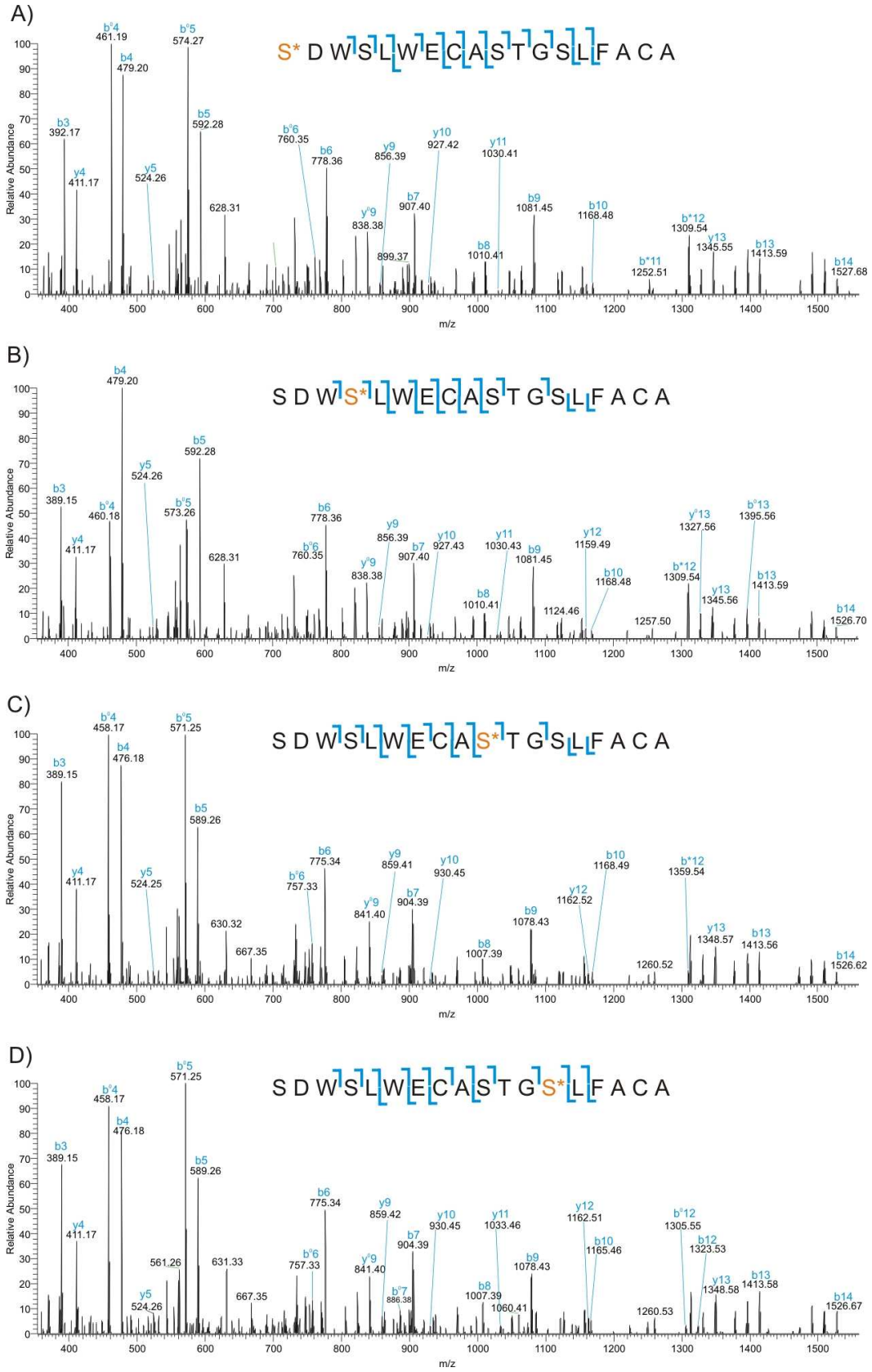


Figure S4. HPLC-ESI-MS/MS spectra of synthetic core peptides after tryptic removal of the leader peptide ([M+2H]=968.90), confirming the positions of labeled Ser residues (orange, marked with *). A) LabA2^{Ser1}*, B) LabA2^{Ser4}*, C) LabA2^{Ser10}*, D) LabA2^{Ser3}*

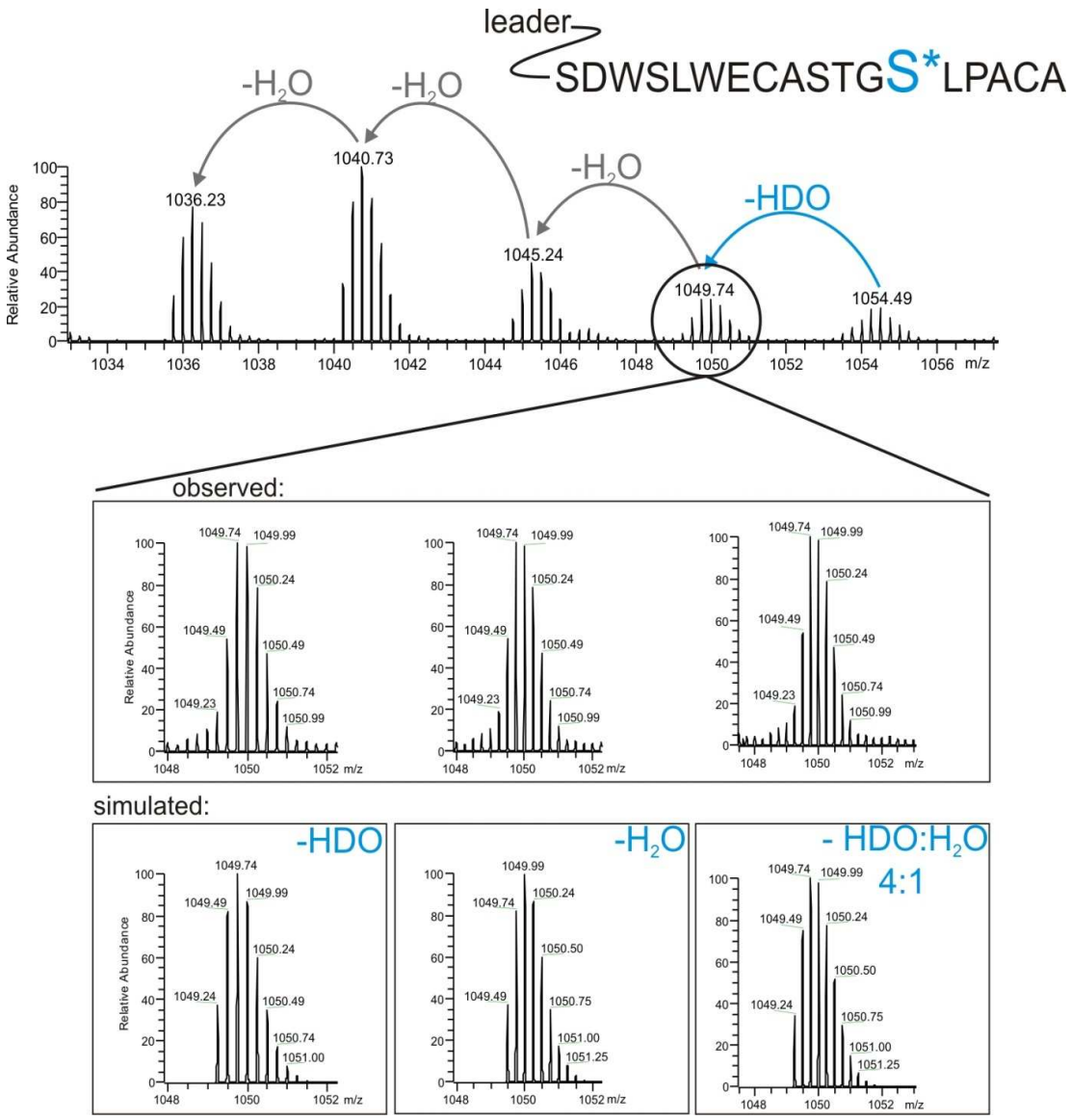


Figure S5. Simulated and observed (peptide LabA2^{Ser13}*) isotopic distributions for the first dehydration intermediate.

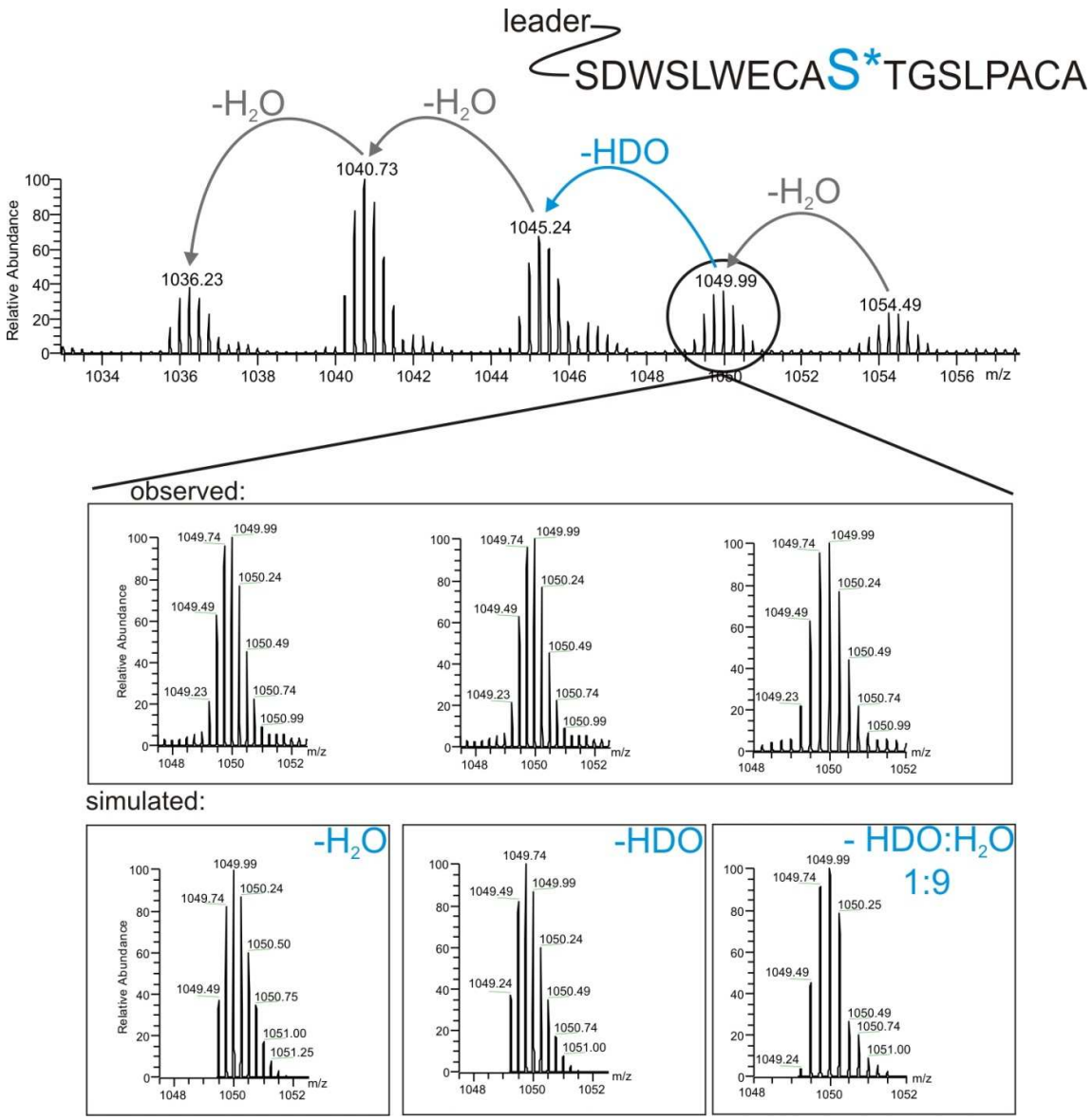


Figure S6. Simulated and observed (peptide LabA2^{Ser10}*) isotopic distributions for the first dehydration intermediate.

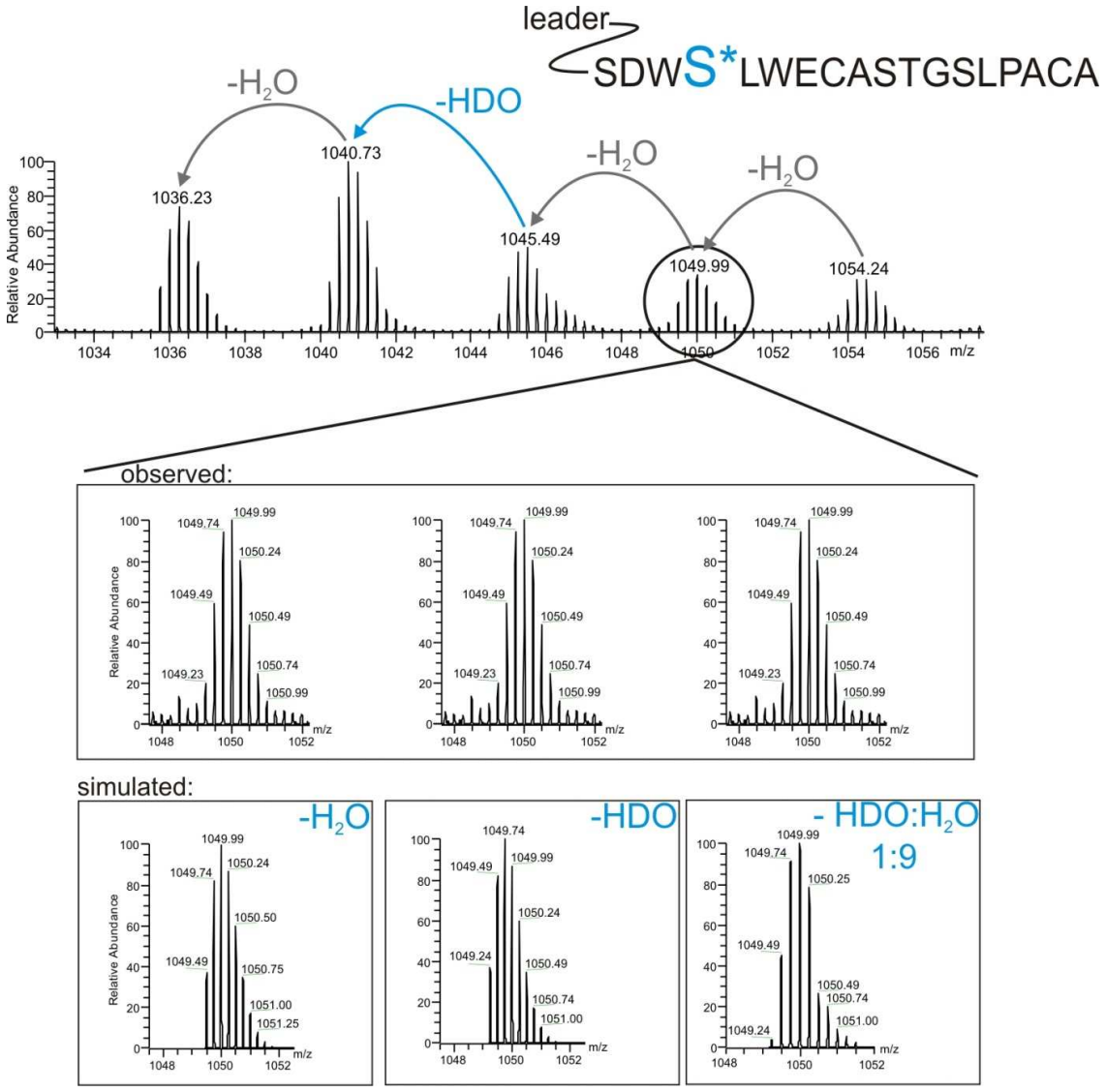


Figure S7. Simulated and observed (peptide LabA2^{Ser4}*) isotopic distributions for the first dehydration intermediate.

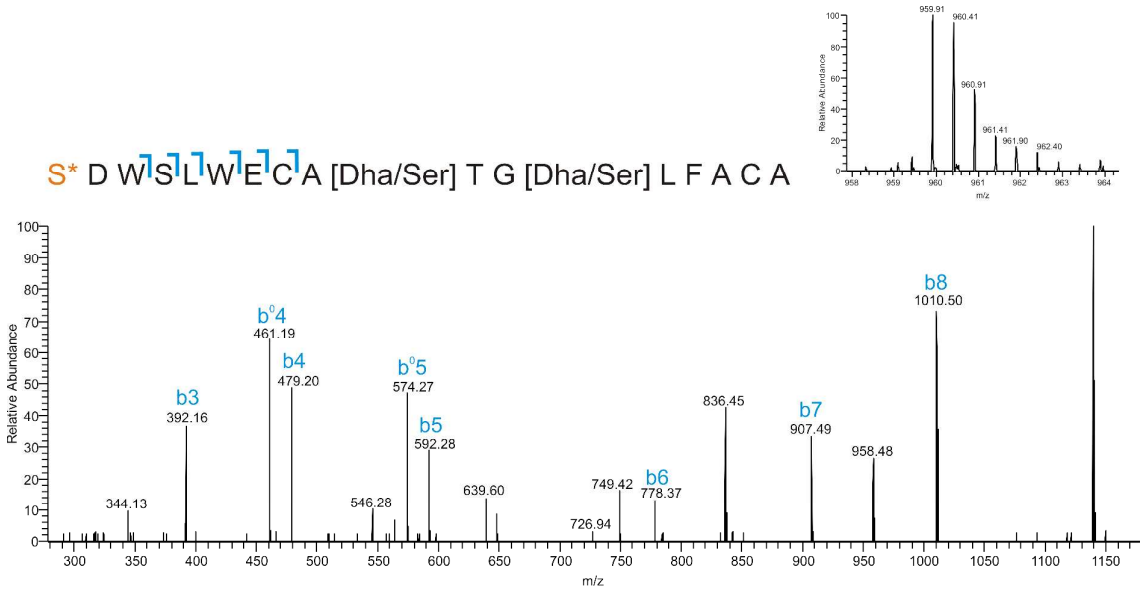


Figure S8. HPLC-ESI-MS/MS spectrum of singly dehydrated intermediate (after tryptic removal of the leader peptide, [M+2H]=959.91). Due to limited fragmentation the exact position of Dha could not be assigned (possible positions in square bracket). In the right upper corner HPLC-ESI-MS spectrum of intermediate after tryptic removal of the leader peptide.

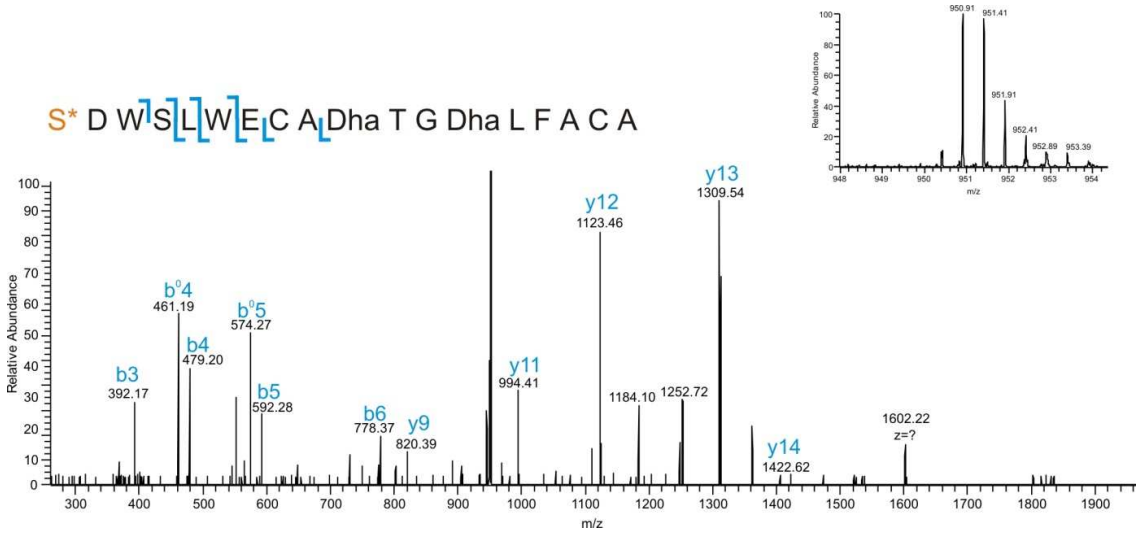


Figure S9. HPLC-ESI-MS/MS spectrum of doubly dehydrated intermediate (after tryptic removal of the leader peptide, [M+2H]=950.91). In the right upper corner HPLC-ESI-MS spectrum of intermediate after tryptic removal of leader peptide.

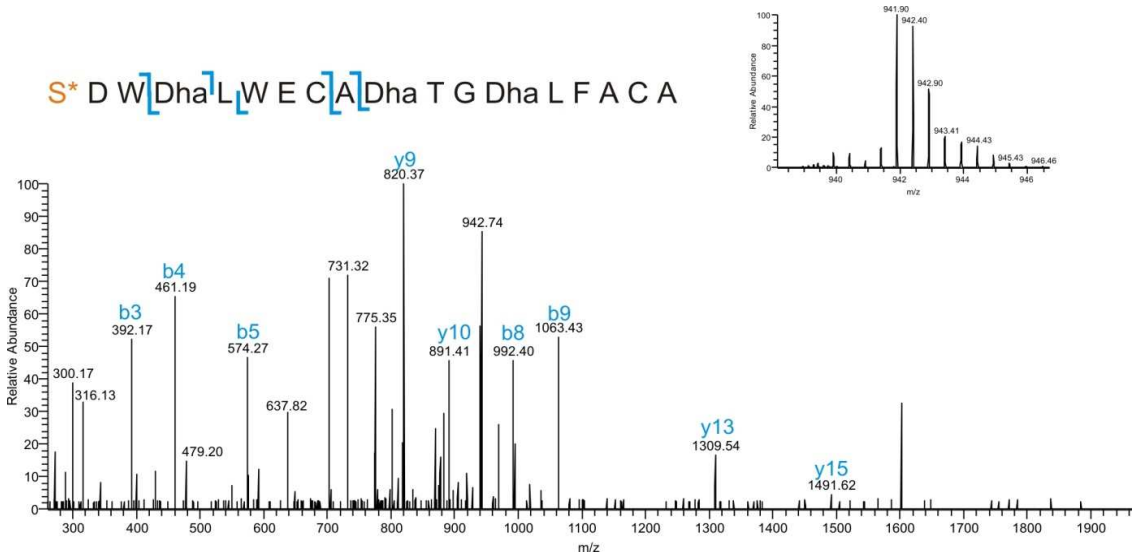


Figure S10. HPLC-ESI-MS/MS spectrum of singly dehydrated intermediate (after tryptic removal of the leader peptide, [M+2H]=941.90). In the right upper corner HPLC-ESI-MS spectrum of intermediate after tryptic removal of the leader peptide.