# Catalytic promiscuity of glycopeptide *N*-methyltransferases enables bio-orthogonal labeling of biosynthetic intermediates

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## Electronical Supplementary Information

**Content:**

**General Methods**

All chemicals and solvents were obtained from commercial suppliers (Sigma-Aldrich; VWR) and used without further purification.

HPLC analysis and purifications were carried out using a High Performance Liquid Chromatograph/Mass Spectrometer LCMS-2020 (ESI, operating both in positive and negative mode) equipped with a SPD-M20A Prominence Photo Diode Array Detector in preparative mode and a SPD-20A Prominence Dual Wavelength UV Detector in analytical mode, all from Shimadzu. For analytical analyses the solvent delivery module LC-20AD was used; for preparative purifications two LC-20AP units were used. Analytical separations were performed on Waters XBridge BEH300 $C_{18}$ columns (5 or 10 µm, 4.6 x 250 mm). Preparative separations were performed on a Waters XBridge BEH300 Prep $C_{18}$ column (5 µm, 19 x 150 mm) at a flow rate of 20 mL/min. The solvents used were HPLC-grade acetonitrile + 0.1% formic acid (solvent A) and water + 0.1% formic acid (solvent B). UHPLC-MS measurements were performed on a Shimadzu Nexera X2 LCMS 8050 system (triple quadrupole ESI, operating both in positive and negative mode) using an Acquity UPLC peptide BEH C18 column (130 Å, 1.7 µm, 2.1 x 100 mm) and MS-grade acetonitrile + 0.1% formic acid (solvent A) and water + 0.1% formic acid (solvent B) as solvents. Electrospray Ionization Mass Spectrometry (ESI-MS) and ESI-MS/MS measurements were performed with HPLC-purified samples on a Bruker maXis ultra-high resolution time-of-flight (TOF) mass spectrometer. Samples were analysed in positive mode and fragmented by collision induced dissociation (CID). NMR studies were performed at the Institute for Pharmacy and Molecular Biotechnology of the University of Heidelberg using a Varian 500 MHz NMR spectrometer.

The synthesis of the teicoplanin-like heptapeptide-Coenzyme A conjugate **3-CoA** has been previously reported.[1,2] The teicoplanin producing P450 enzymes $OxyB_{tei}$ (Protein ID: Q70AY8),[3] and $OxyA_{tei}$ (Protein ID: Q6ZZI8)[4] were also generated as previously described. Cloning, expression and purification for the PCP-X di-domain protein as a GB1 (IgG binding B1 domain of *Streptococcus*)[5]-fusion protein with an N-terminal hexahistidine tag and a C-terminal Strep-II-tag was performed according to references.[6] Redox partner proteins were obtained from Dr. Stephen Bell (Adelaide, Australia).[7]

**Cloning of methyltransferases $Mtfa_{dbv}$ and $Mtfa_{pek}$**

Genes were PCR amplified from genomic DNA using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, USA). The primers MtfA WAC4229 NdeI F (5' - **TTTCATATG**AGTGATCAGCTGGAGCACG -3') and MtfA WAC4229 HindIII R (5' - **TTTAAGCTT**CATGCGGGACCGGTCTTC -3') were used to amplify *$mtfa_{pek}$* from the pekiskomycin producer *S. malachitospinus* WAC4229 [11]. The oligonucleotides MtfA dbv HindIII R (5' - **TTTAAGCTT**CTAATGCGCGTCTTCCAC -3') and MtfA dbv NdeI F (5' - **AAACATATG**ATAAGCAAAGCAATGCATG -3′) were employed to amplify *$mtfa_{dbv}$* from the A4096 producer *Actinomadura sp.* ATCC 39727. PCR products and the vector pET28a (Novagen, USA) were digested with *Nde* I and *Hind* III and ligated together with T4 DNA ligase. Ligation reactions were transformed into E.coli TOP10 (Thermo Fisher Scientific, USA). Error-free constructs were validated by Sanger sequencing using standard T7 promoter and terminator primers.

**Expression and Purification of methyltransferases $Mtfa_{dbv}$ and $Mtfa_{pek}$**

The methyltransferase proteins were expressed in the E. coli BL21Gold (DE3) strain (Agilent, Waldbronn, Germany) with an N-terminal hexahistidine tag. 2L LB medium (+50 mg/L kanamycin) were inoculated with an overnight starter culture of transformed E.coli BL21(DE3) and grown at 37 °C until OD 600 reached 0.6. After cooling down to 20 °C protein expression was induced by adding 100 mM IPTG and the cell culture was incubated at 20 °C overnight. Cells were harvested by centrifugation at 4 °C (5000 x g), resuspended in lysis buffer (50 mM Tris pH 8.0, 50 mM NaCl, 10 mM imidazole) including protease inhibitor cocktail (Sigma-Aldrich, München, Germany) and lysed by passing tree times through a fluidizer (Microfluidics, Newton, USA). After centrifugation (50000 x g, 4 °C, 25 min) the soluble fraction was incubated for 1h at 4°C with 4 mL of Ni-NTA resin pre-equilibrated with 2 x 10 column bed volumes (CV) of wash buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10 mM imidazole). After separating the resin from the supernatant by centrifugation at 1000g it was transferred into a column and washed with 10 CV of wash buffer. Proteins were eluted with 3 CV of elution buffer (50 mM Tris pH 8.0, 300 mM NaCl, 300 mM imidazole). The elution fraction was further purified by size exclusion chromatography on a Superose-12 column (GE Healthcare, München, Germany) connected to an Äkta FPLC using exchange buffer (20 mM Tris.HCl pH 7.4, 20 mM NaCl, 5 mM DTE). Elution fractions were analysed by SDS-PAGE, appropriate fractions pooled and concentrated using centrifugal concentrators with a 10,000 MW cut-off (Sartorius Stedim Biotech, Göttingen, Germany). Aliquots were flash frozen in liquid nitrogen and stored at -80 °C.

Proteins were obtained in a yield of 15.8 mg (0.5 µmol) /L culture for $Mtfa_{dbv}$ and 32 mg (1 µmol) /L culture for $Mtfa_{pek}$.

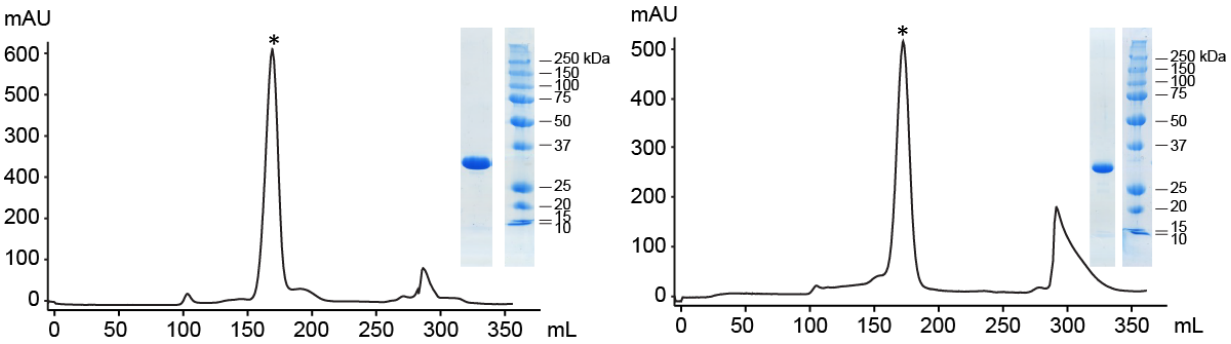



**Fig S1**. Gel filtration chromatograms and electrophoretic analysis (10 % SDS-PAGE gel) of purified $Mtfa_{dbv}$ (left side, marked with a star) and $Mtfa_{pek}$ (right side, marked with a star). As marker "Precision Puls Protein All Blue Standards (BioRad) was used; staining was performed using Instant Blue (Expedeon).

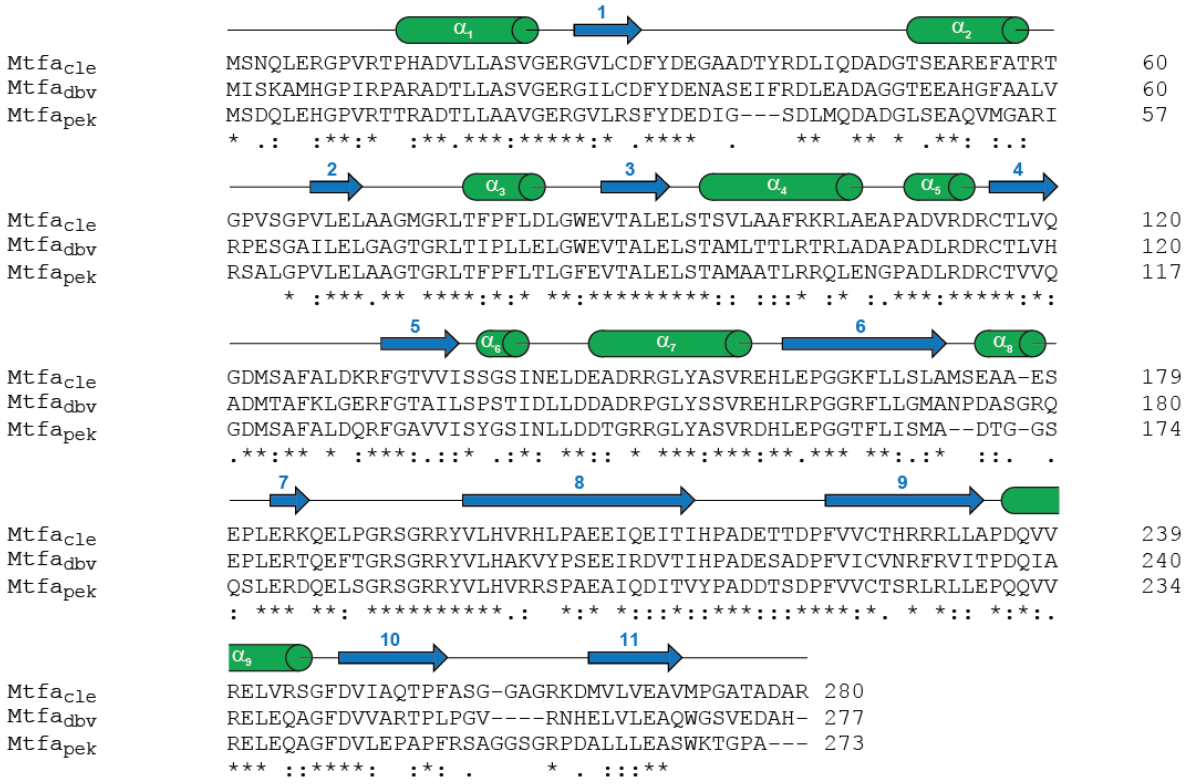



**Fig. S2:** Alignment of $Mtfa_{dbv}$ and $Mtfa_{pek}$ with the already characterised $Mtfa_{cle}$ from the chloroeremomycin producing *A. orientalis*. Secondary structure elements are taken from $Mtfa_{cle}$ and shown above the alignment. The sequences display 46 % identity. Alignment was performed with ClustalOmega.[8]

**Synthesis of compounds**

Teicoplanin aglycone **1** was obtained as describe in literature.[9] Briefly, teicoplanin (45 mg, SantaCruz, Heidelberg, Germany) was sonicated in a mixture of conc. HCl (1.12 mL) and acetic acid (10.2 mL) until a clear solution was obtained. This mixture was heated to 80 °C under vigorous shaking. After 2 h ice-cold diethyl ether (35 mL) was added and a white solid precipitated. After keeping the mixture at -24 °C overnight, the solid was separated and purified using preparative HPLC. After lyophilisation of the respective HPLC fractions teicoplanin aglycone **1** was obtained as white foam. Stock solutions (1 mM) were prepared in 20 % DMSO and stored at -24 °C.

AdoPropen **2b** and AdoViBenz **2c** were synthesized according to literature:[10,11] S-adenosyl-L-homocysteine (20 mg, 52 µmol) was dissolved in 3 mL of a 1:1 mixture of formic and acetic acid and cooled to 0 °C. 3-Bromo-1-propene (264 µL, 3.1 mmol) or 4-vinylbenzylchloride (533 µL, 3.7 mmol) was slowly added to the stirred solution. The reaction mixture was allowed to warm up to room temperature and stirred for 4 days. By adding the reaction mixture into 10 mL cooled water the reaction was quenched and the water phase was extracted with 5 mL diethyl ether for four times. After lyophilisation the crude product was purified by preparative reversed-phase HPLC using following gradient: 0 -15 min up to 10 % solvent A. The obtained diastereomeric mixture was concentrated by lyophilisation. Stock solutions (10 mM) in water were aliquoted and stored at −80 °C before use.

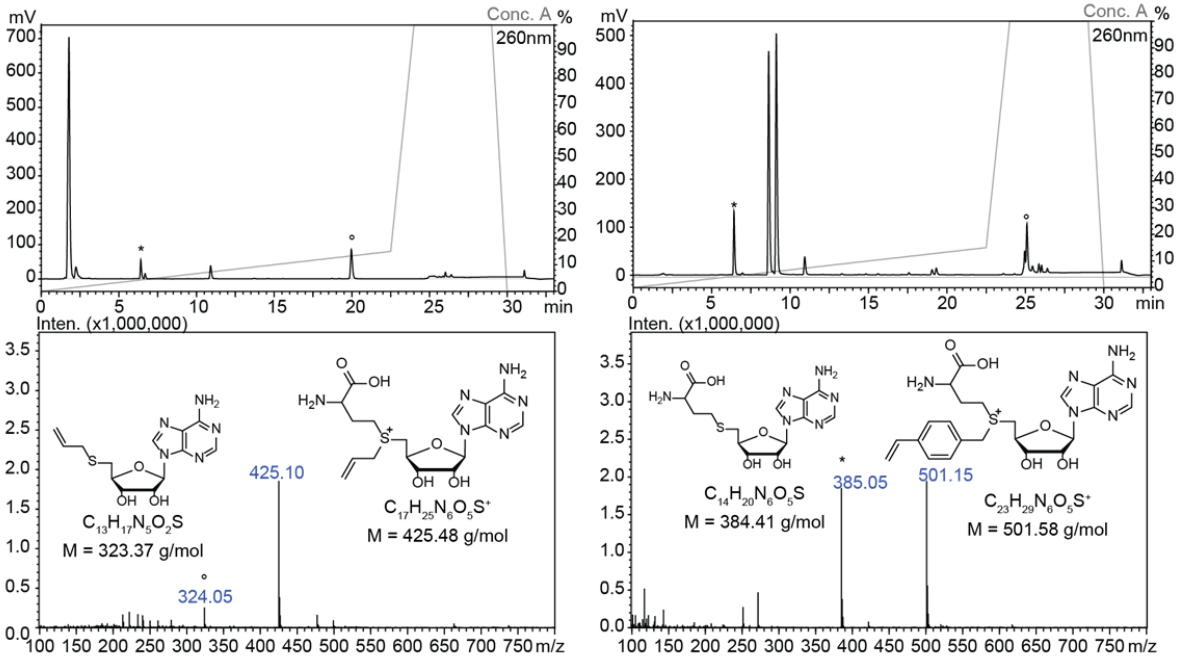
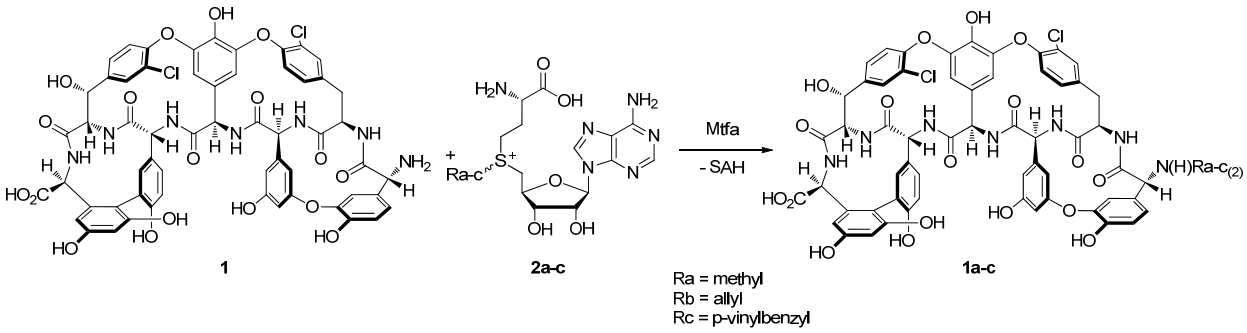



**Fig. S3**: Reversed phase HPLC chromatograms and MS spectra of purified **2b** (left side) and **2c** (right side) monitored at λ = 260 nm. Due to degradation of the cofactor traces of S-adenosylhomocysteine (*) and the respective allyl- or benzvinyl-thioadenosine can be detected (°). Gradient: 0 – 22.5 min 0% - up to 15% solvent A; flow rate 1.0 mL/min; Waters Cortecs C18+column, 2.7µm, 4.6 x 150 mm.

**Methyltransferase *in vitro* activity assay with teicoplanin aglycone**




**Scheme S1**: Reaction scheme of Mtase-catalysed transalkylation reactions.

Methyltransferase catalysed transfer reactions were performed in 50 mM Hepes pH 7.4, 50 mM NaCl buffer: for that teicoplanin aglycone (50 µM) was used together with $Mtfa_{dbv}$ or $Mtfa_{pek}$ (5 µM) and the reaction was started by the addition of S-adenosylmethionine **2a** (500 µM) or cofactor analogues AdoPropen **2b** and AdoViBenz **2c** (500 µM). The mixture was incubated for 1 h at 30 °C under gentle shaking (350 rpm). The reaction was stopped upon addition of an equal volume of ice-cold methanol and stored at -24 °C for 30 min. After removal of precipitated protein by centrifugation at 15000 rpm (10 min), the supernatant was concentrated under vacuum and analysed using UHPLC-MS (gradient: 0 – 0.5 min 5% solvent A, 0.5 – 0.7 min up to 30% solvent A, 0.7 – 6 min up to 80% solvent A; flow rate 0.5 mL/min).

Large scale transalkylation assays for antibiotic activity and NMR studies were performed in a total volume of 1 mL 50 mM Hepes pH 7.4, 50 mM NaCl buffer containing 500 µM teicoplanin aglycone 1, $Mtfa_{dbv}$ or $Mtfa_{pek}$ (10 µM) and cofactor analogues AdoPropen **2b** and AdoViBenz **2c** (750 µM). The reaction was incubated overnight at 30°C under gentle shaking (350 rpm). The reaction was stopped upon addition of an equal volume of ice-cold methanol and stored at -24 °C for 30 min. After removal of precipitated protein by centrifugation, the supernatant was concentrated under vacuum and purified using analytical HPLC (gradient: 0 – 4 min 5% solvent A, 4 – 5 min, up to 20% solvent A, 5 – 25 min up to 75% solvent A; flow rate 1 mL/min).

**Loading of teicoplanin-like-CoA peptide (3-CoA) onto apo-PCP-$X_{tei}$**

The peptide-CoA conjugate was loaded onto the PCP-domain of the teicoplanin PCP-$X_{tei}$ di-domain protein catalyzed by the Sfp variant R4-4[12] by incubating a mixture of 60 µM PCP-$X_{tei}$, 6 µM Sfp, 120 µM **3**-CoA, 50 mM Hepes pH 7.0, 50 mM NaCl, 10 mM $MgCl_2$ for 1 h at 30 °C. Afterwards, excess of free peptide was removed by a dilution-concentration procedure with low salt buffer (50 mM Hepes pH 7.0 for P450 activity assay or 50 mM Hepes pH 7.4 for methyltransferase activity assay, 50 mM NaCl, 4 x 1:5 dilution) using centrifugal filter units with a 10,000 MW cut-off (Merck Millipore, Darmstadt, Germany). The generated *peptidyl*-PCP-$X_{tei}$ construct was immediately used for further enzymatic *in vitro* activity assays.

**Coupled P450 / methyltransferase *in vitro* activity assay**

In vitro oxidation experiments were performed in 50 mM Hepes, pH 7.0, 50 mM NaCl buffer at 30 °C. Under standard turnover conditions 2 µM of $OxyB_{tei}$ or $OxyB_{tei}$ and $OxyA_{tei}$ were used together with 50 µM *peptidyl*-PCP-$X_{tei}$ immediately after the PCP loading reaction. Palustrisredoxin B variant A105V (PuxB) together with palustrisredoxin reductase (PuR) were used as a redox system[7] in 5:1:50 (PuxB:PuR:*peptidyl*-PCP-X) ratio. The turnover reaction was started upon addition of 2 mM NADH (Gerbu, Biotechnik, Heidelberg, Germany). For NADH regeneration 0.33% glucose and 9 U/mL glucose dehydrogenase (Sorachim, Lausanne, Switzerland) were included in the reaction mixture. The turnover reaction was incubated for 1h with gentle shaking (350 rpm).

For the subsequent methyltransferase assay either protocol a) or b) was used.

Protocol a) Methyltransferase assay with PCP-$X_{tei}$-bound peptides as substrates

To stop the oxidation reaction in the cytochrome P450 turnover mixture NADH and glucose were removed by a dilution-concentration procedure with buffer (50 mM Hepes pH 7.4, 50 mM NaCl, 3 x 1:5 dilution) using centrifugal filter units with a 10,000 MW cut-off (Merck Millipore, Darmstadt, Germany). Following that, the mixture was separated into triplicates and *peptidyl*-PCP-$X_{tei}$ (50 µM) was incubated with methyltransferase $Mtfa_{dbv}$ or $Mtfa_{pek}$ (5 µM) and S-adenosylmethionine **2a** (500 µM) for 1 h at 30 °C (350 rpm). Control reactions were performed without methyltransferase or co-substrate. For workup, peptides were released from the PCP adding methylamine (40 % solution in $H_2O$, 15 min incubation at RT), resulting in peptide methylamides. For the following purification procedure ice-cold diluted formic acid was added to neutralise the turnover mixture, which was

then purified via solid phase extraction (Strata-X polymeric reversed phase 3mg/mL, Phenomenex, Aschaffenburg, Germany): 1. loading sample using gravity flow, 2. washing column with 5 % methanol in water, 3. elution of peptide with 100 % methanol + 0.1 % formic acid. The elution fraction was concentrated under vacuum and analysed by analytical UHPLC-MS using single ion monitoring in negative mode. After integration of the signals obtained for the different peptide species the Mtfa activities were calculated based on the percentage of alkylated peptide relative to the respective substrate obtained from the P450 turnover.

Protocol b) Methyltransferase assay with PCP-X-cleaved peptides as substrates

The cytochrome P450 turnover mixture was treated with methylamine to cleave the peptide from the PCP-$X_{tei}$ di-domain protein as described in protocol a). After neutralisation with diluted formic acid and purification via solid phase extraction the elution fraction was concentrated under vacuum and dissolved in 50 mM Hepes pH 7.4, 50 mM NaCl (equal volume as for cytochrome P450 turnover assay). The mixture was split into triplicates and $Mtfa_{dbv}$ or $Mtfa_{pek}$ (5 µM) and co-substrate derivatives **2a-c** (500 µM) were added. Control reactions were performed without methyltransferase. After incubation for 1 h at 30 °C (350 rpm) the reaction was quenched with an equal volume of ice-cold methanol and stored at -24 °C for 30 min. Precipitated protein was removed by centrifugation and the supernatant was concentrated under vacuum followed by UHPLC-MS analysis. Data analysis was performed as described in protocol a).

For enzymatic modifications with co-substrate **2a** and **2b** the following gradient was used: 0 – 0.5 min 5% solvent A, 0.5 – 0.7 min up to 20% solvent A, 0.7 – 6 min up to 70% solvent A; flow rate 0.5 mL/min. For reactions with co-substrate **2c**: 0 – 0.5 min 5% solvent A, 0.5 – 0.7 min up to 20% solvent A, 0.7 – 7 min up to 90% solvent A; flow rate 0.5 mL/min.

**Enzymatic modification of teicoplanin aglycone followed by inverse electron-demand Diels-Alder reaction**

Teicoplanin aglycone **1** (500 µM) was incubated together with $Mtfa_{pek}$ (50 µM) and AdoViBenz **2c** (600 µM) in 50 mM Hepes, pH 7.4, 50 mM NaCl buffer for 2 h at 30 °C (350 rpm). After methanol precipitation and removal of precipitated protein by centrifugation the supernatant was purified using analytical HPLC to separate 4-vinylbenzyl N-terminally modified teicoplanin aglycone **1c** from unreacted cofactor species. After removal of the HPLC solvent **1c** was dissolved in 50 mM Hepes, pH 7.4/ acetonitrile (1:1) and incubated with 6-methyl-tetrazine-5-TAMRA **6** (1 mM of 10 mM stock solution in DMSO, Jena Bioscience, Jena, Germany) or tetrazine-PEG4-biotin **7** (1 mM of 50 mM stock solution in DMSO, Jena Bioscience, Jena, Germany) at 37 °C for 24 h excluding light exposure (strong shaking at 2000 rpm). The reaction was analysed using analytical HPLC and labelled teicoplanin aglycones **8** and **9** were characterised using MS fragmentation (see Fig. S10-S12).

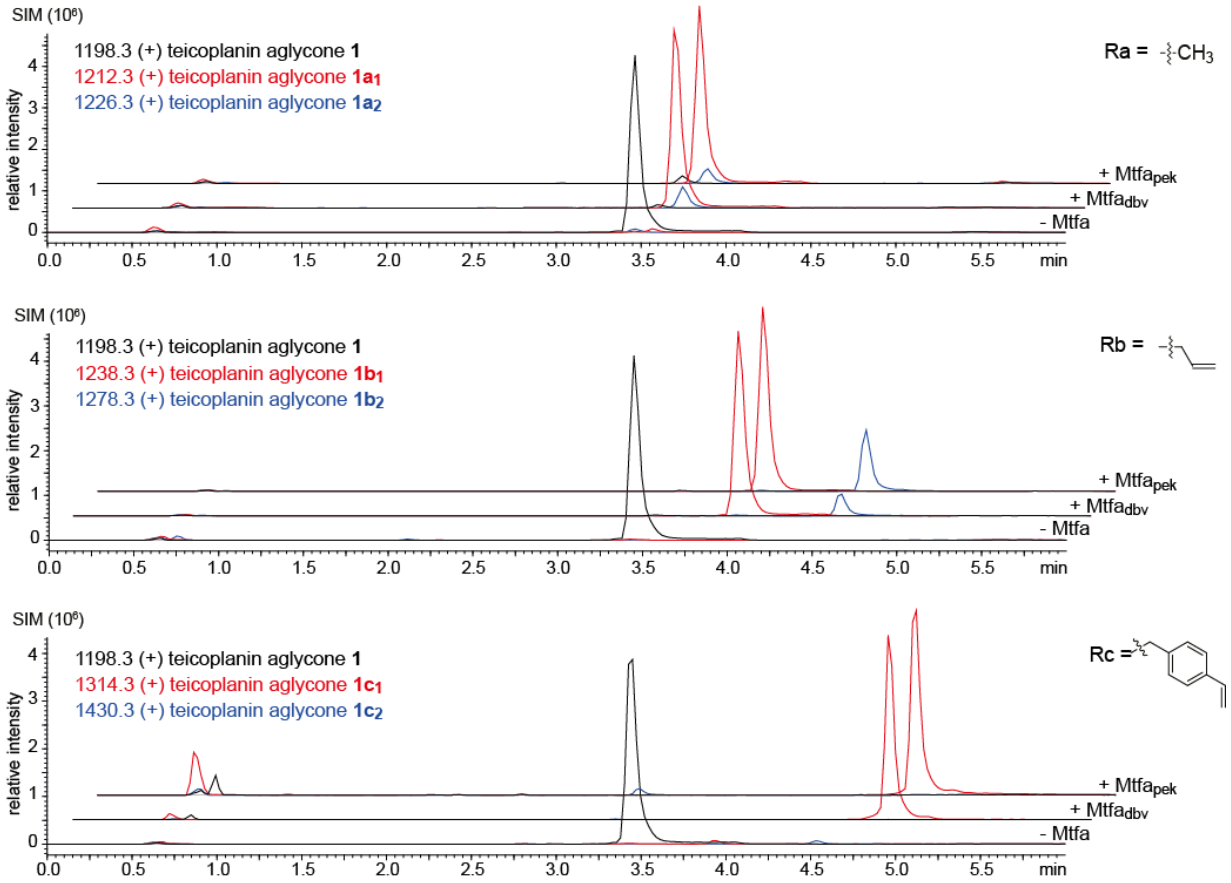



**Fig. S4:** Representative UHPLC-MS chromatograms of methyltransferase turnover reactions with teicoplanin aglycone **1** and different co-substrates **2a-c**. Chromatograms were recorded using single monitoring for unmodified teicoplanin aglycone (black line), mono- (red line) and bisalkylated aglycone (blue line) in positive mode.

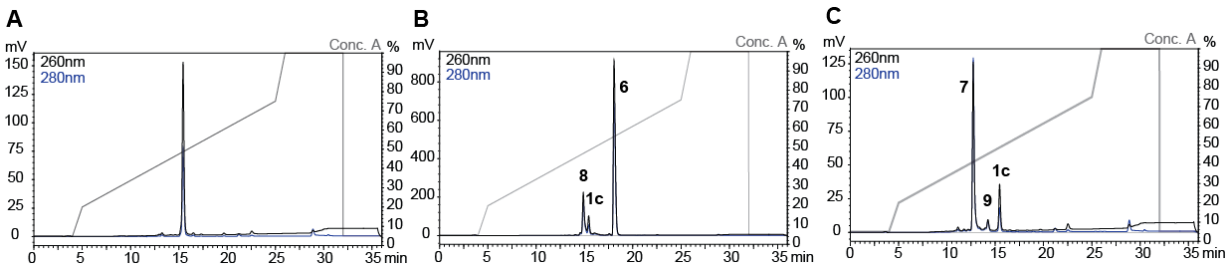



**Fig. S5**: HPLC chromatograms of inverse electron demanding Diels Alder reactions monitored at λ = 260 and 280 nm. A) HPLC trace of purified teicoplanin aglycone derivative **1c**; B) HPLC trace of reaction from **1c** with tetrazine-TAMRA derivative **6** yielding 80 % of product **8** after 18h reaction time; C) HPLC trace of reaction from **1c** with tetrazine-PEG derivative **7** to product **9.**

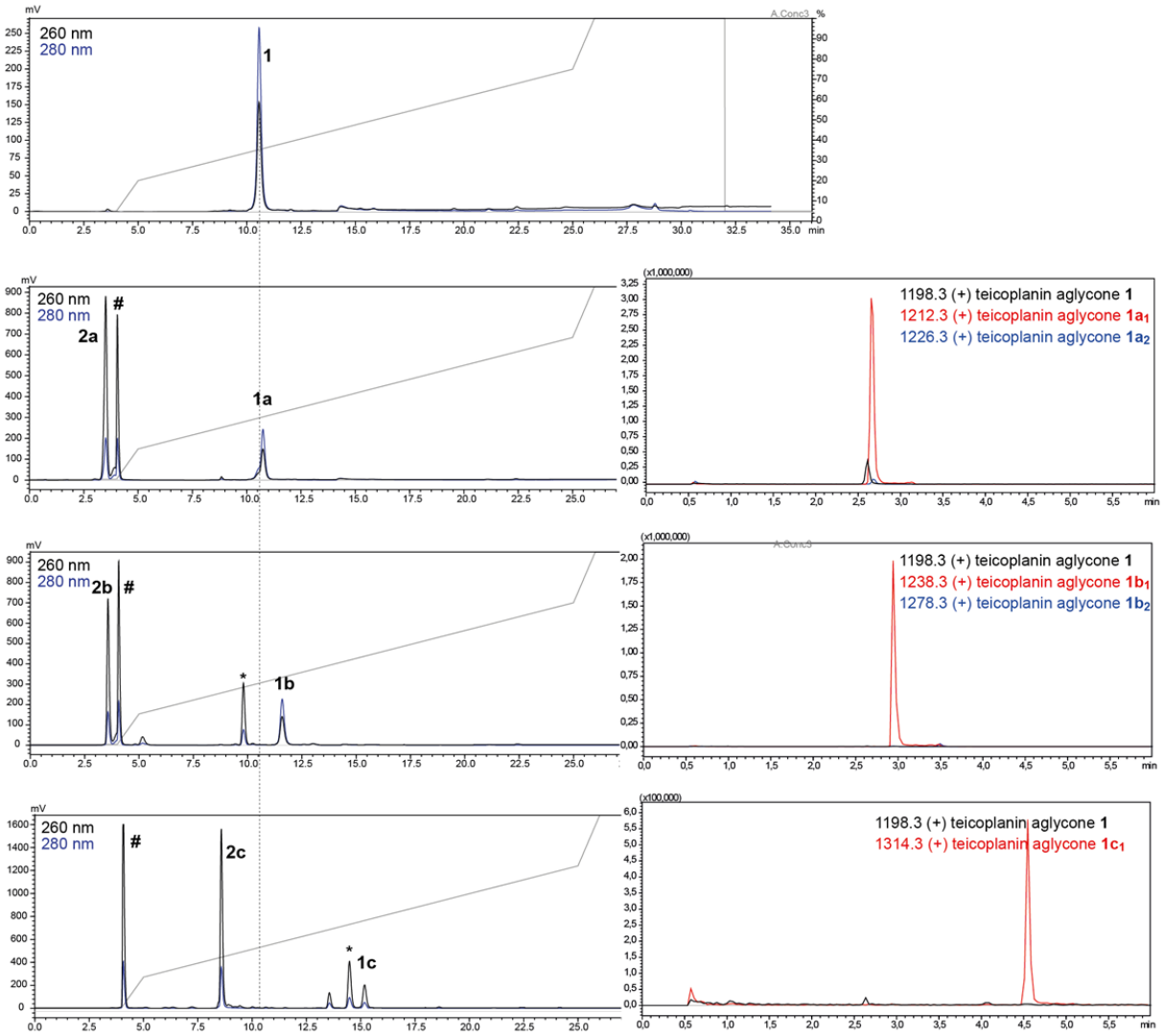
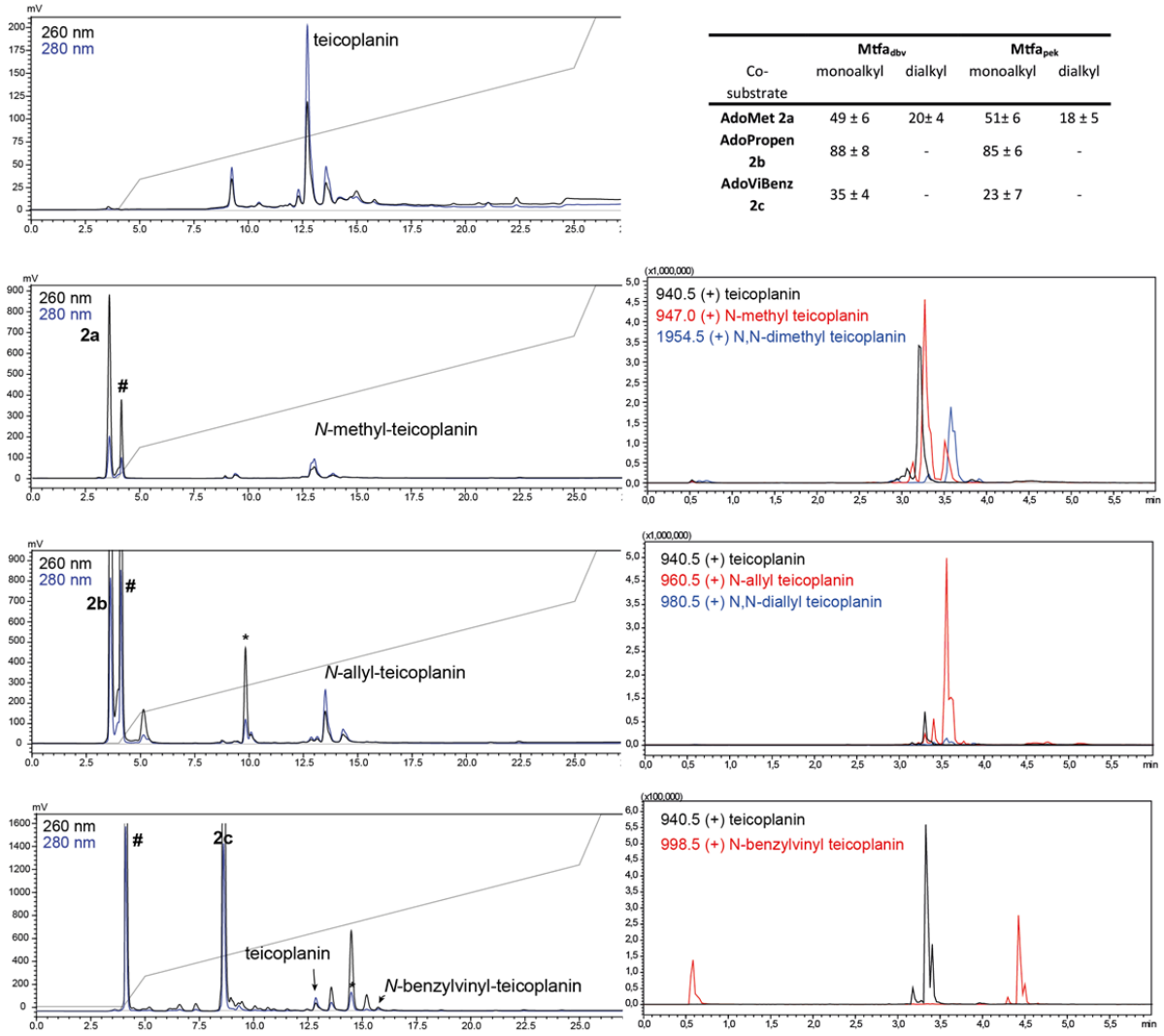


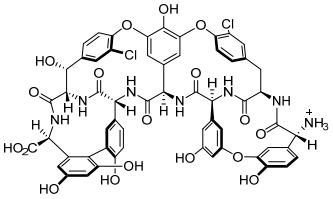

**Fig. S6:** $Mtfa_{wac}$ turnover reactions with teicoplanin aglycone **1** and different co-substrates **2a-c** analysed with analytical UV/VIS HPLC (left side, monitored at 260 and 280 nm) and UHPLC-MS (right side, monitored using single ion detection in positive mode). UV/VIS-HPLC monitoring confirms full conversion of teicoplanin aglycone 1 as shown by UHPLC-MS analysis. Peaks representing co-substrates **2a-c**, co-substrate product of Mtase reaction S-adenosylhomocysteine (#) and co-substrate degradation products allyl-thioadenosine (for **2b**, *) or vinylbenzyl-thioadenosine (**2c**, *) are marked.



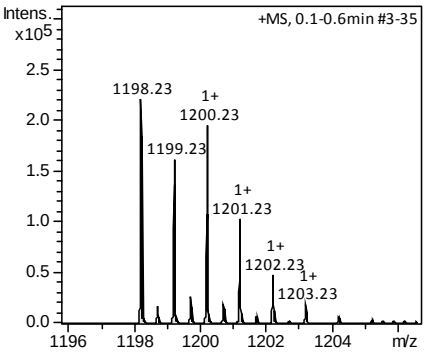

| Co-substrate | $Mtfa_{dbv}$ monoalkyl | $Mtfa_{dbv}$ dialkyl | $Mtfa_{pek}$ monoalkyl | $Mtfa_{pek}$ dialkyl |
|---|---|---|---|---|
| **AdoMet 2a** | 49 ± 6 | 20± 4 | 51± 6 | 18 ± 5 |
| **AdoPropen 2b** | 88 ± 8 | - | 85 ± 6 | - |
| **AdoViBenz 2c** | 35 ± 4 | - | 23 ± 7 | - |

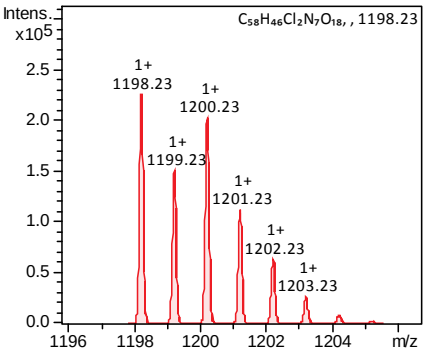
**Fig. S7:** $Mtfa_{dbv}$ turnover reactions with teicoplanin and different co-substrates **2a-c** analysed with analytical UV/VIS HPLC (left side, monitored at 260 and 280 nm) and UHPLC-MS (right side, monitored using single ion detection of doubly charged teicoplanin species in positive mode). Turnover yields determined in triplicates for $Mtfa_{dbv}$ and $Mtfa_{pek}$ are listed. Peaks representing co-substrates **2a-c**, co-substrate product of Mtase reaction S-adenosylhomocysteine (#) and co-substrate degradation products allyl-thioadenosine (for **2b**, *) or vinylbenzyl-thioadenosine (**2c**, *) are marked. Turnover reactions were performed with commercial teicoplanin (Sigma-Aldrich, Germany) using the same conditions as for teicoplain aglycone **1** (50 mM Hepes, pH 7.4, 50 mM NaCl, 50 µM teicoplanin, 5 µM $Mtfa_{dbv}$ or $Mtfa_{pek}$, 500 µM co-substrate **2a-c**, 1h at 30°C).

**A**



$C_{58}H_{46}Cl_2N_7O_{18}^+$
Exact Mass: 1198,23

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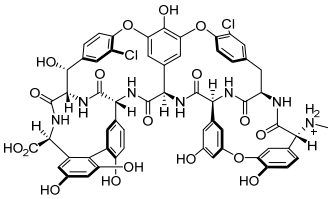
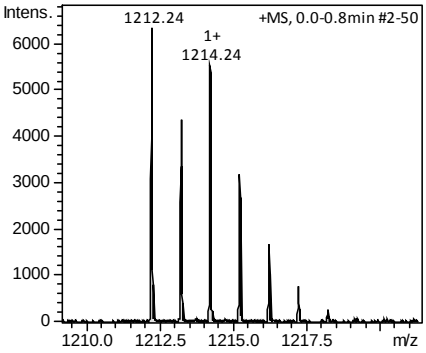
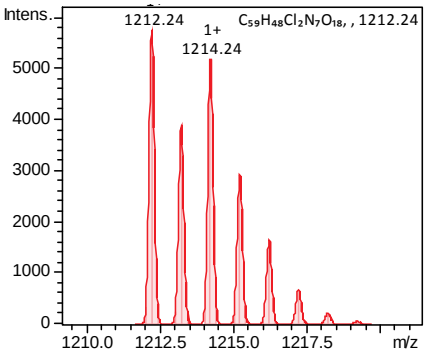
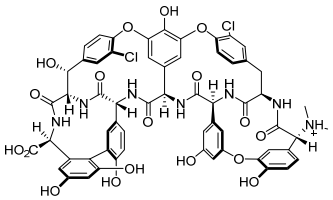


$C_{59}H_{48}Cl_2N_7O_{18}^+$
Exact Mass: 1212,24

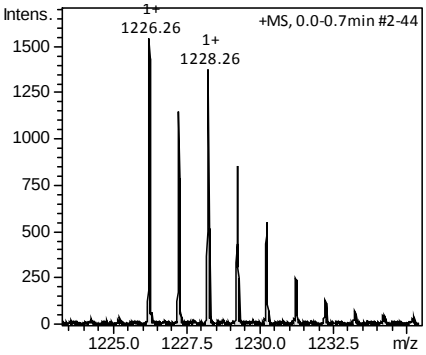


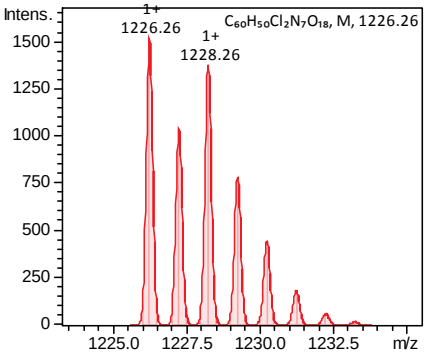




**C**

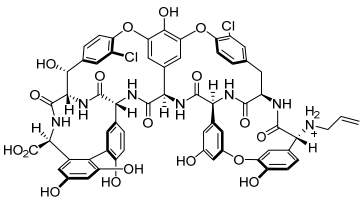


$C_{60}H_{50}Cl_2N_7O_{18}^+$
Exact Mass: 1226,26




**D**



$C_{61}H_{50}Cl_2N_7O_{18}^+$
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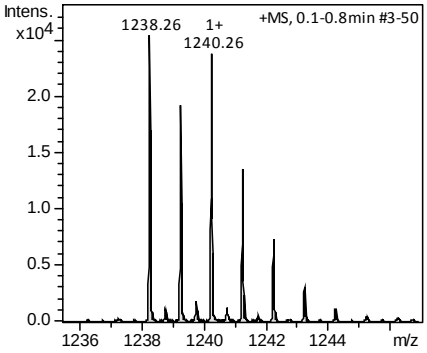
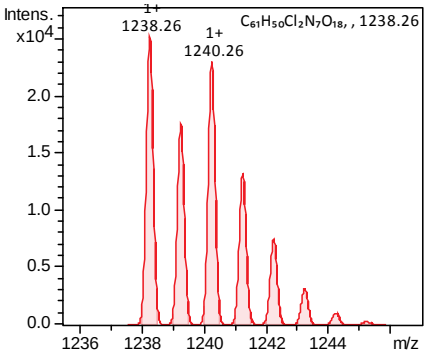


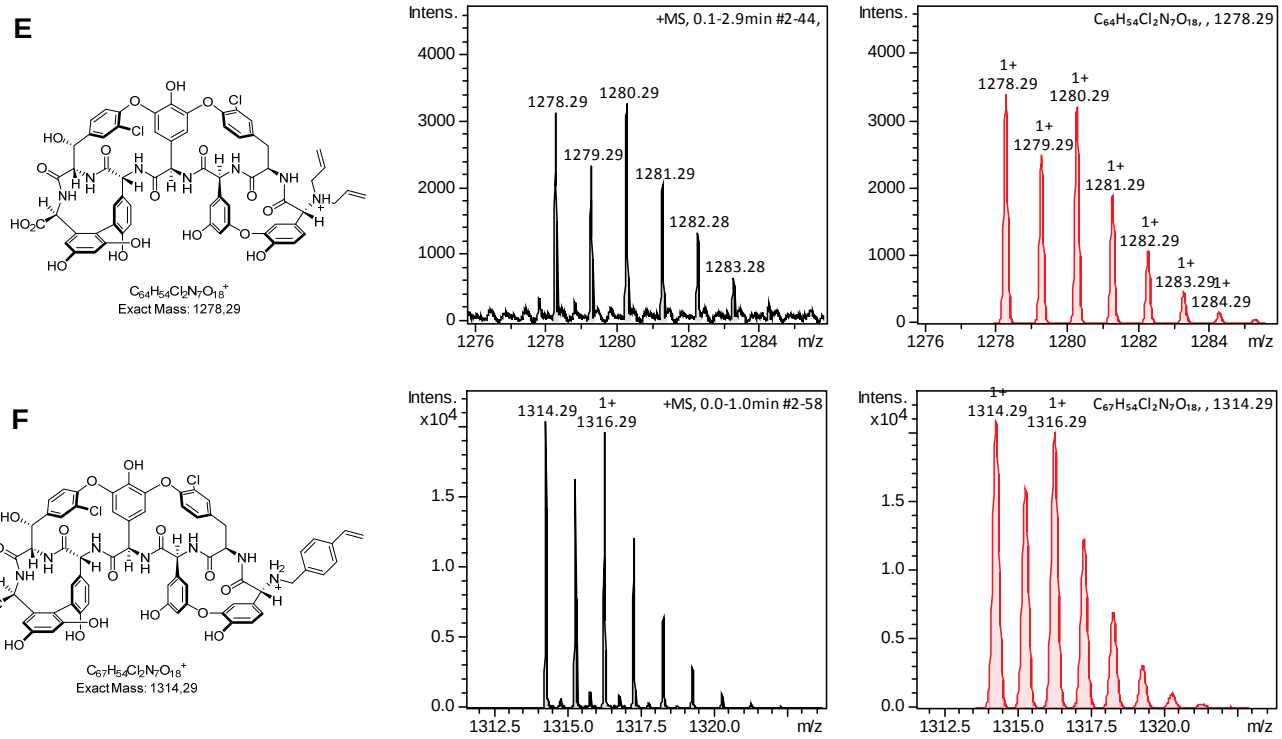
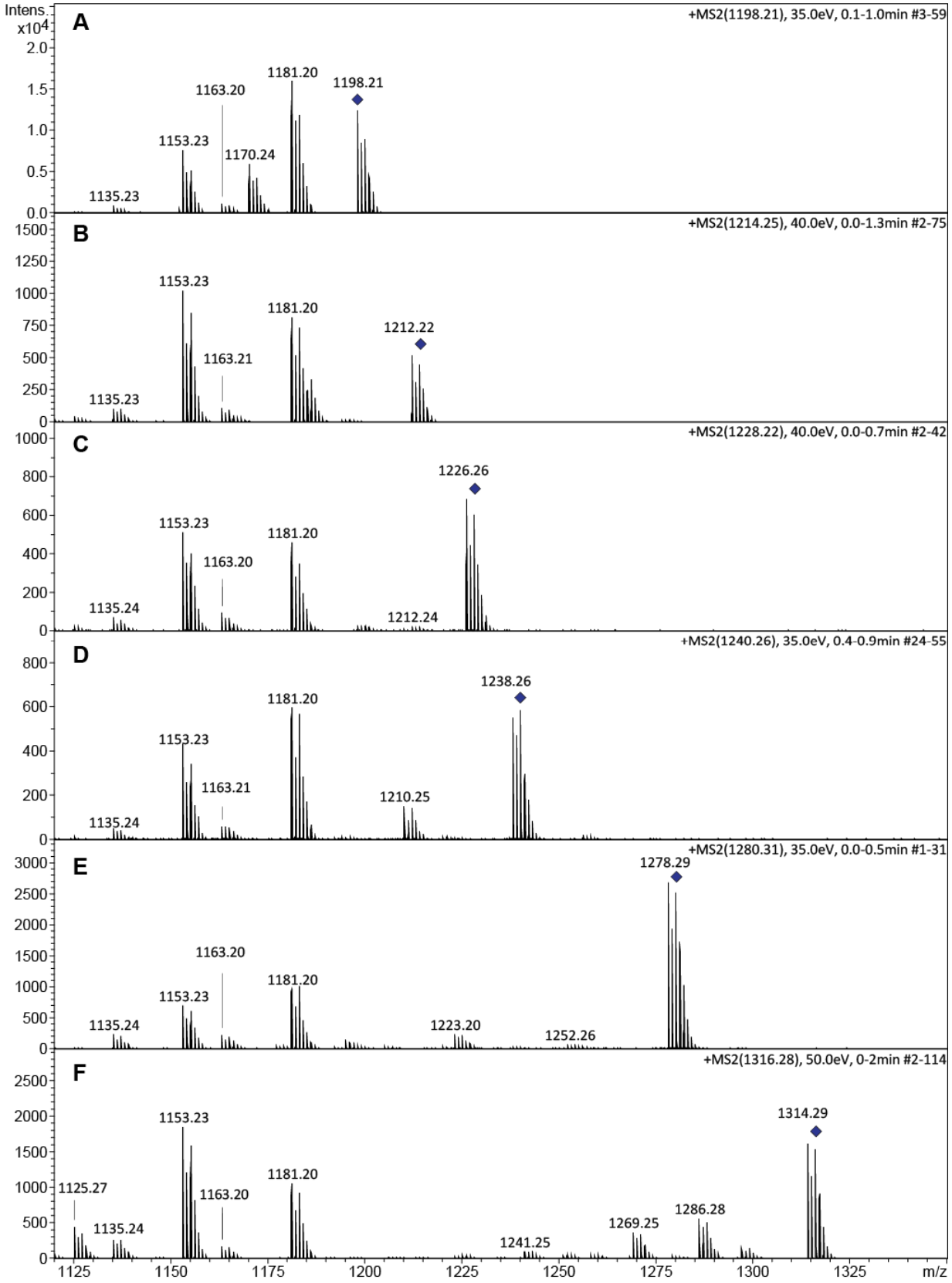



**Fig. S8**: ESI-TOF analysis of teicoplanin aglycone derivatives **1** (A), **1a$_1$** (B), **1a$_2$** (C), **1b$_1$** (D), **1b$_2$** (E), **1c** (F). MS spectra in the middle represent the measured MS profile showing the characteristic isotope distribution for teicoplanin derivatives. On the right side calculated MS profiles are shown. Measured and calculated spectra were obtained using Bruker Compass MS Software DataAnalysis 4.1.


Intens.
x10^4
A
+MS2(1198.21), 35.0eV, 0.1-1.0min #3-59
1181.20
1198.21
1163.20
1153.23
1170.24
1135.23
B
+MS2(1214.25), 40.0eV, 0.0-1.3min #2-75
1153.23
1181.20
1212.22
1163.21
1135.23
C
+MS2(1228.22), 40.0eV, 0.0-0.7min #2-42
1226.26
1153.23
1181.20
1163.20
1135.24
1212.24
D
+MS2(1240.26), 35.0eV, 0.4-0.9min #24-55
1238.26
1181.20
1153.23
1163.21
1210.25
1135.24
E
+MS2(1280.31), 35.0eV, 0.0-0.5min #1-31
1278.29
1163.20
1181.20
1153.23
1135.24
1223.20
1252.26
F
+MS2(1316.28), 50.0eV, 0-2min #2-114
1314.29
1153.23
1181.20
1125.27
1163.20
1286.28
1135.24
1269.25
1241.25
1125
1150
1175
1200
1225
1250
1275
1300
1325
m/z

**G**

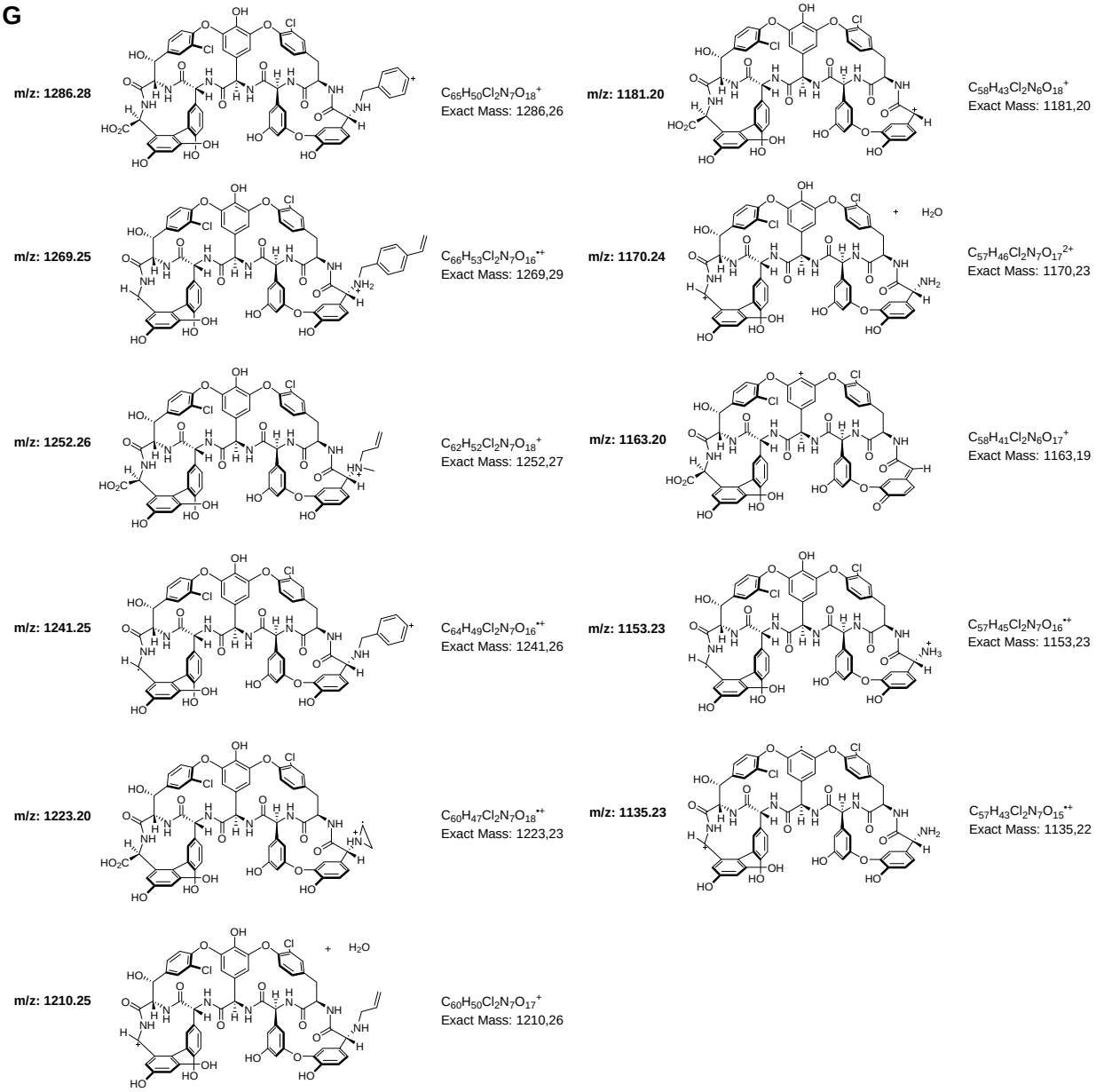



**Fig. S9:** ESI-MS/MS analysis of teicoplanin aglycone derivatives **1** (A), **1a$_1$** (B), **1a$_2$** (C), **1b$_1$** (D), **1b$_2$** (E), **1c** (F). Data were obtained in positive mode. G) Proposed structures for characteristic fragments observed during tandem mass spectrometry.

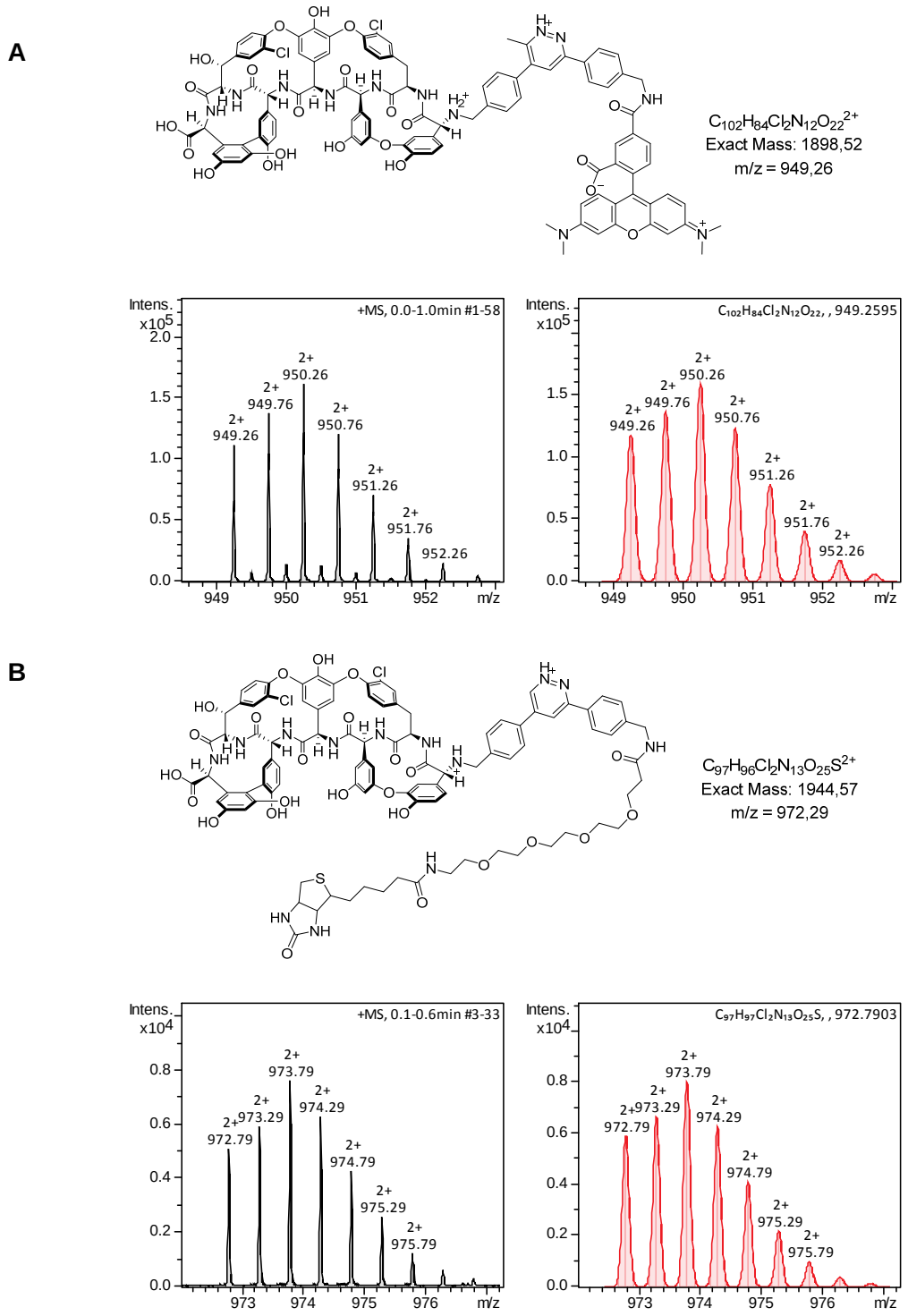



**Fig S10**: ESI-TOF analysis of labeled teicoplanin aglycone derivatives **8** (A) and **9** (B). Left: measured spectra showing the characteristic isotope distribution for teicoplanin derivatives; on the right side calculated MS profiles are shown. Measured and calculated spectra were obtained using Bruker Compass MS Software DataAnalysis 4.1.

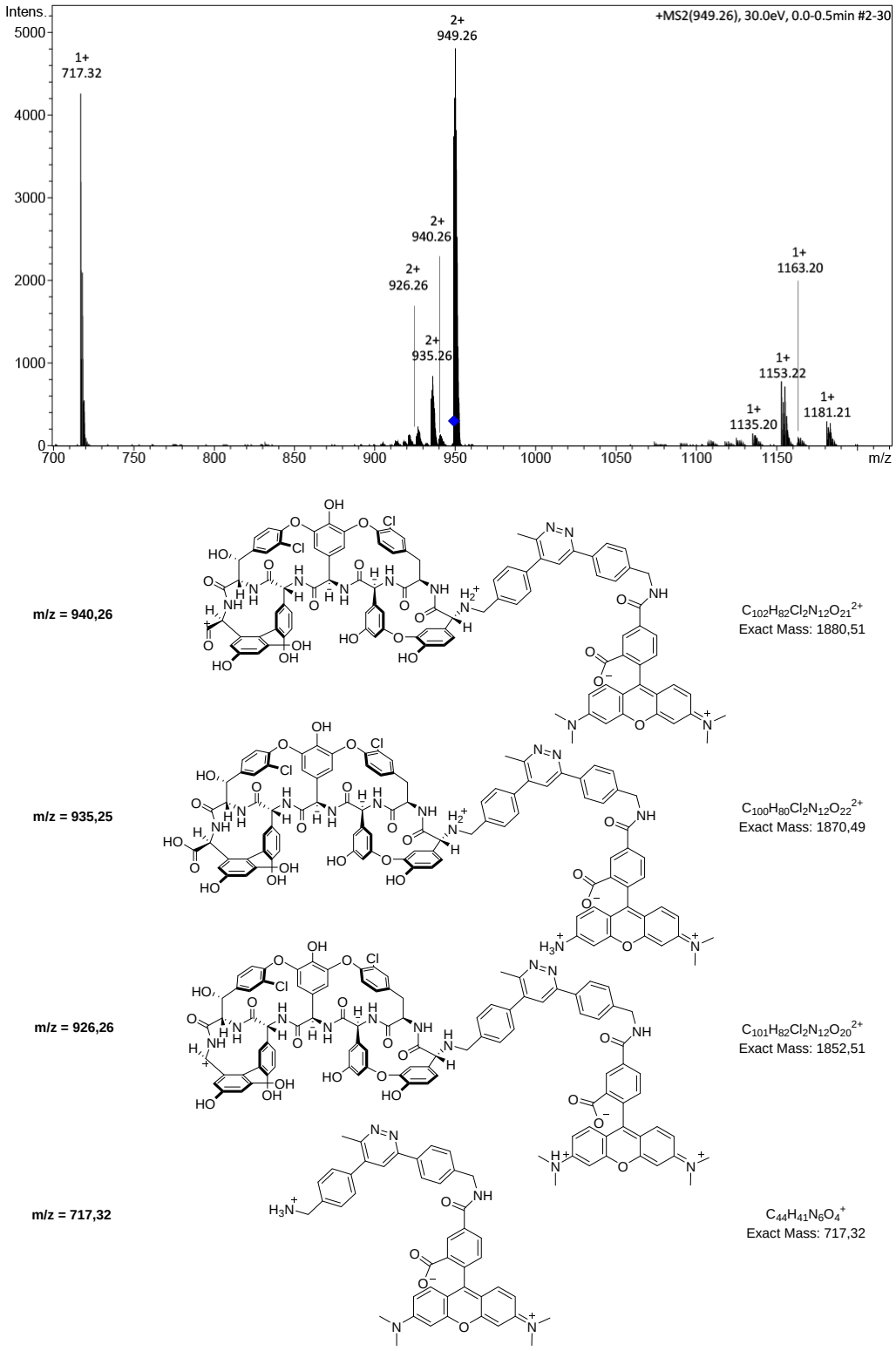



**Fig S11**: ESI-MS/MS analysis of labeled teicoplanin aglycone derivative **8** (positive mode) with proposed structures for characteristic fragments observed during tandem mass spectrometry.

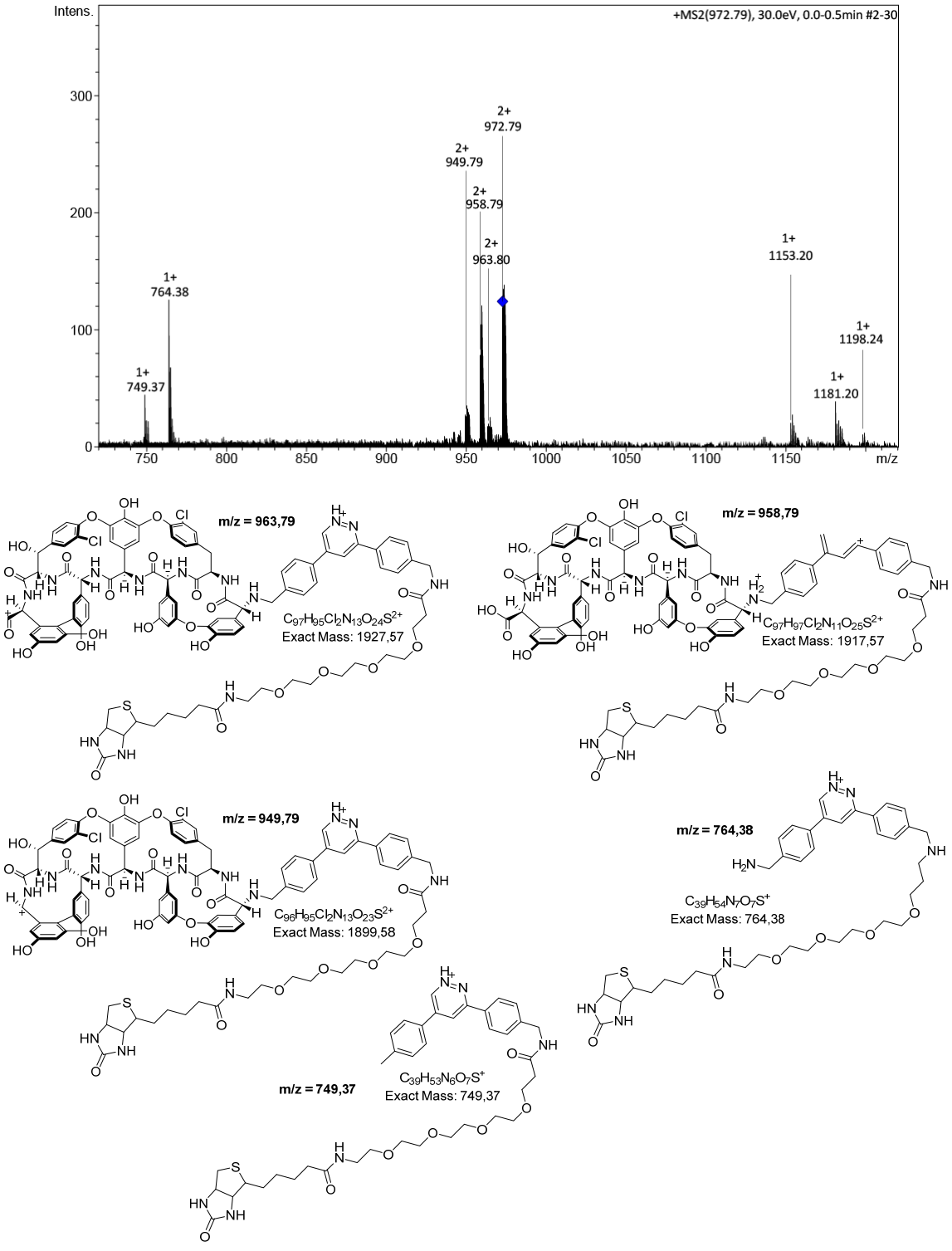
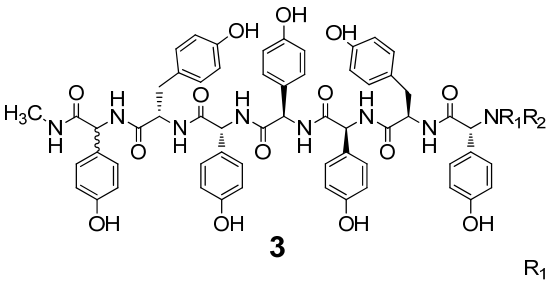



**Fig S12**: ESI-MS/MS analysis of labeled teicoplanin aglycone derivative **9** (positive mode) with proposed structures for characteristic fragments observed during tandem mass spectrometry.



**3**

$R_1, R_2 = H$ : $C_{59}H_{58}N_8O_{14}$, $M_{exact}$: 1102.41

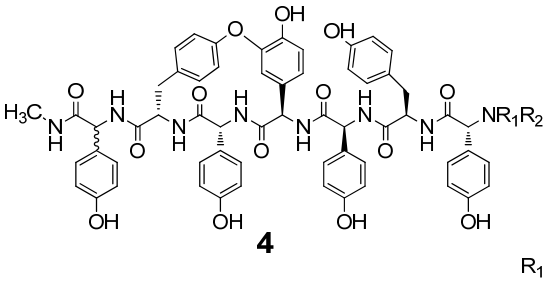
$R_1 = CH_3, R_2 = H$ : $C_{60}H_{60}N_8O_{14}$, $M_{exact}$: 1116.42

$R_1, R_2 = CH_3$ : $C_{61}H_{62}N_8O_{14}$, $M_{exact}$: 1130.44

$R_1$ = allyl, $R_2 = H$ : $C_{62}H_{62}N_8O_{14}$, $M_{exact}$: 1142.44

$R_1, R_2$ = allyl : $C_{65}H_{66}N_8O_{14}$, $M_{exact}$: 1182.47

$R_1$ = p-vinylbenzyl, $R_2 = H$ : $C_{68}H_{66}N_8O_{14}$, $M_{exact}$: 1218.47



**4**

$R_1, R_2 = H$ : $C_{59}H_{56}N_8O_{14}$, $M_{exact}$: 1100.39

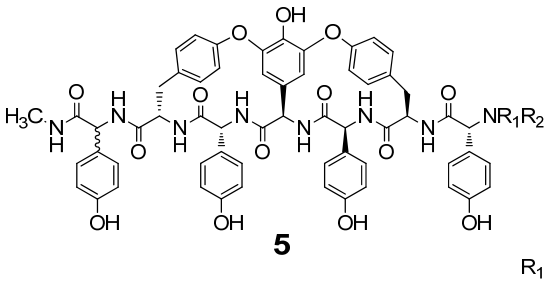
$R_1 = CH_3, R_2 = H$ : $C_{60}H_{58}N_8O_{14}$, $M_{exact}$: 1114.41

$R_1, R_2 = CH_3$ : $C_{61}H_{60}N_8O_{14}$, $M_{exact}$: 1128.42

$R_1$ = allyl, $R_2 = H$ : $C_{62}H_{60}N_8O_{14}$, $M_{exact}$: 1140.42

$R_1, R_2$ = allyl : $C_{65}H_{64}N_8O_{14}$, $M_{exact}$: 1180.45

$R_1$ = p-vinylbenzyl, $R_2 = H$ : $C_{68}H_{64}N_8O_{14}$, $M_{exact}$: 1216.45



**5**

$R_1, R_2 = H$ : $C_{59}H_{54}N_8O_{14}$, $M_{exact}$: 1098.38

$R_1 = CH_3, R_2 = H$ : $C_{60}H_{56}N_8O_{14}$, $M_{exact}$: 1112.39

$R_1, R_2 = CH_3$ : $C_{61}H_{58}N_8O_{14}$, $M_{exact}$: 1126.41

$R_1$ = allyl, $R_2 = H$ : $C_{62}H_{58}N_8O_{14}$, $M_{exact}$: 1138.41

$R_1, R_2$ = allyl : $C_{65}H_{62}N_8O_{14}$, $M_{exact}$: 1178.44

$R_1$ = p-vinylbenzyl, $R_2 = H$ : $C_{68}H_{62}N_8O_{14}$, $M_{exact}$: 1214.44

**Fig S13:** Overview about linear (**3**), monocyclic (**4**) and bicyclic (**5**) methylamide peptide derivatives obtained from coupled P450/methyltransferase *in vitro* activity studies with their corresponding exact masses.

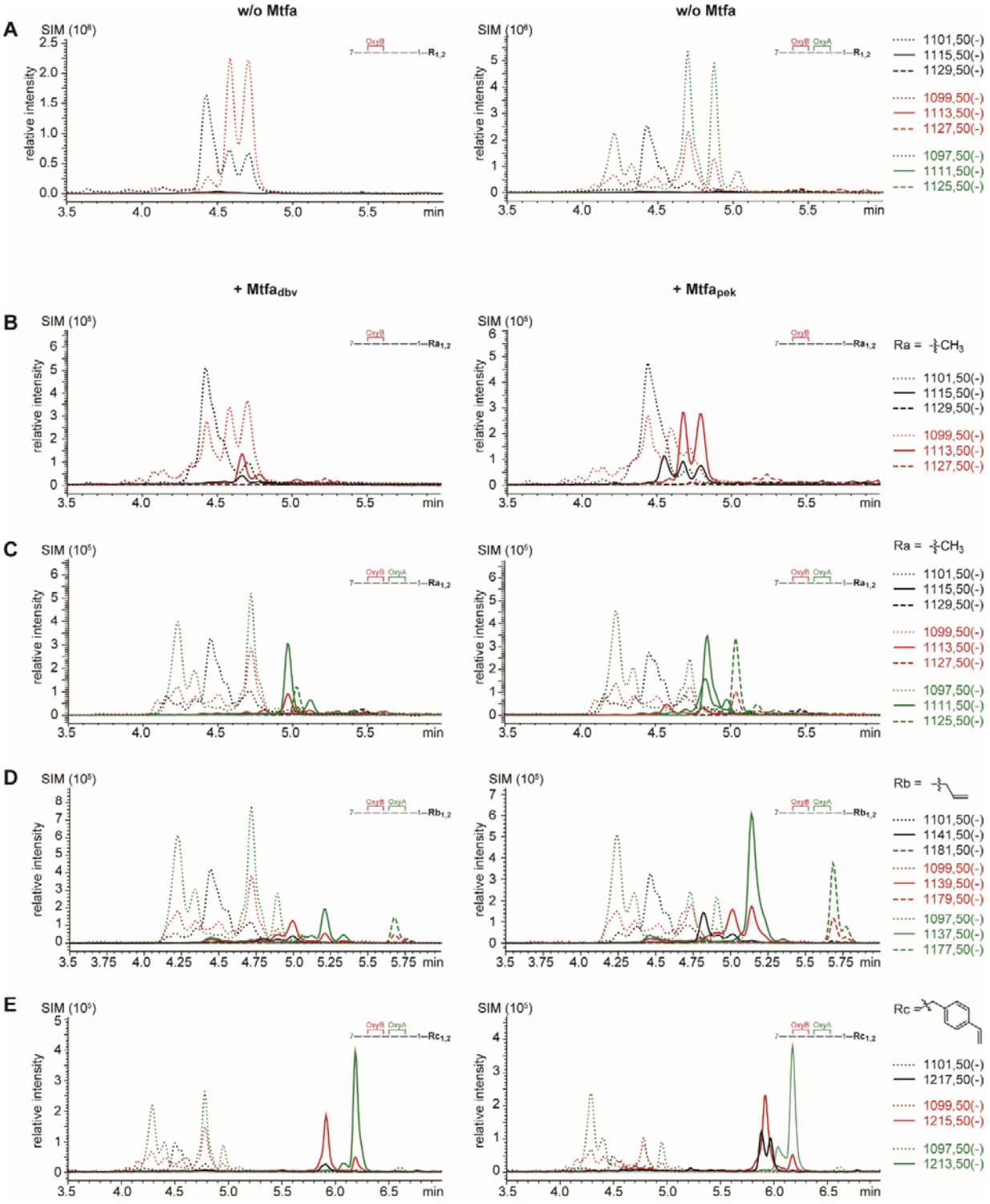
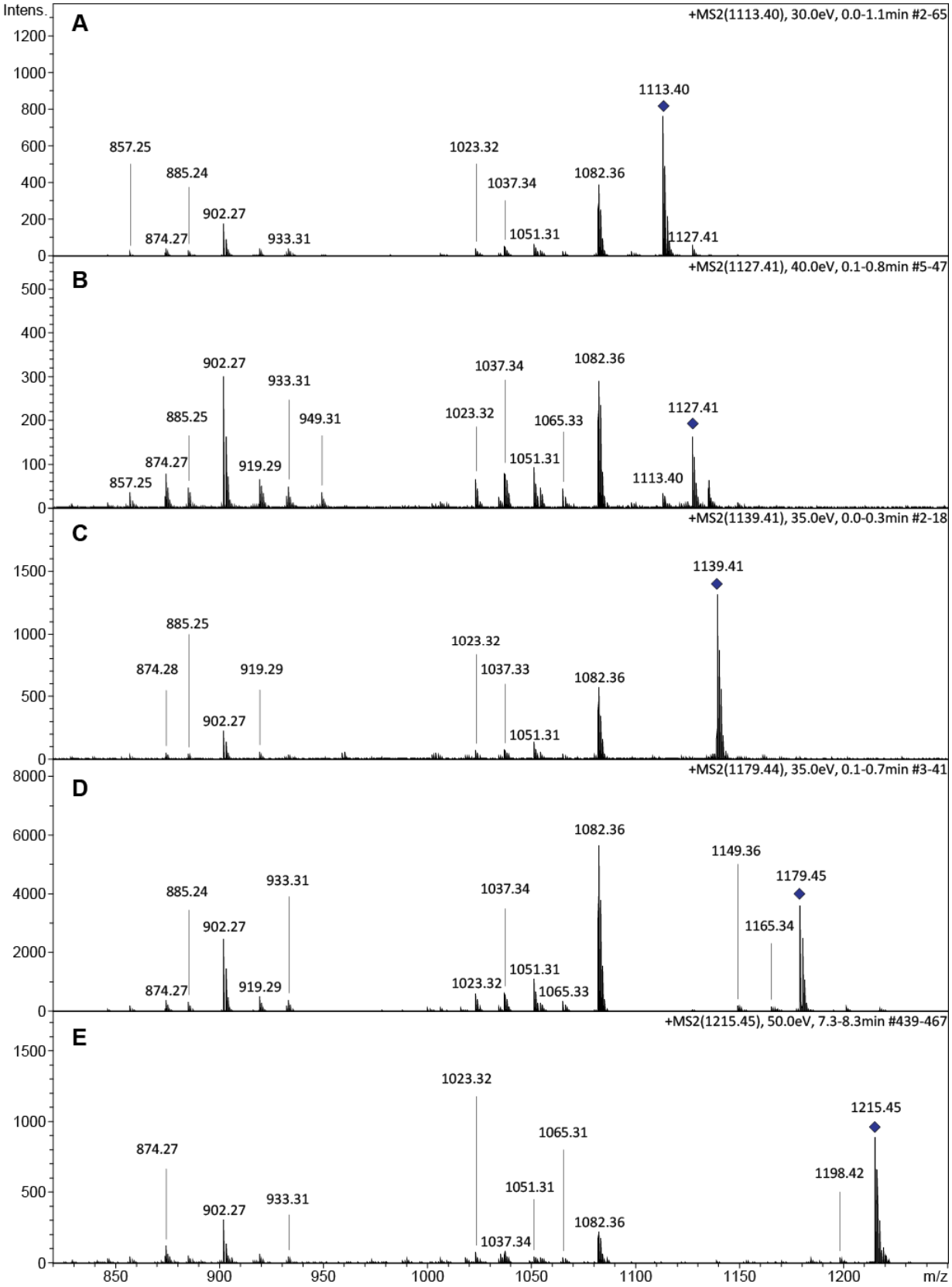



**Fig. S14:** Representative UHPLC-MS chromatograms for coupled P450/Mtase activity assays with different co-substrates using single ion monitoring in negative mode. A) Control reactions of the P450 activity assays just with OxyB$_{tei}$ (left side) or OxyB$_{tei}$ / OxyA$_{tei}$ (right side). B)-E) Representative chromatograms for coupled P450/Mtase activity assays with Mtfa$_{dbv}$ (left side)and Mtfa$_{pek}$ (right side) using solely OxyB$_{tei}$ (B) or OxyB$_{tei}$ / OxyA$_{tei}$ (C-E). As co-substrates AdoMet **2a** (B & C), AdoPropen **2b** (D) and AdoViBenz **2c** (E) were used.

Turnover reactions represent a mixture of linear (black lines), monocyclic (red lines) and bicyclic (green lines) peptide methylamide species. Traces for non-alkylated peptides are presented as dotted lines, mono-alkylated peptides as solid lines and bis-alkylated peptides as dashed lines.

Multiple peaks for one species can be ascribed to the substrate peptide **3**, which is used as a diastereomeric mixture (racemization of Hpg on position 7 can´t be prevented during synthesis).[4] For linear peptide methylamide **3** the diastereomers are not resolved yet but for monocyclic peptide methylamide **4** clearly two peaks can be distinguished (see A). For the bicyclic peptide **5** multiple peaks are observed which we ascribe to other peptide conformations such as cis-trans amide bond rotamers. Through the second P450 catalysed crosslink such rotamers are possibly locked. For one bicyclic species eluting at earlier retention times (4 -4.5 min) we observed generally no conversion through Mtases. We have previously observed the same behavior for P450 enzymes following $OxyA_{tei}$ in the crosslinking cascade (unpublished data), therefore this peak was excluded from calculations to determine the yield of alkylated bicyclic peptide methylamides. For quantitative analysis we assume that the different peptide species show similar ionization behavior (based on previous experiments performed with an internal standard).[2,4,6]


Intens.
A
+MS2(1113.40), 30.0eV, 0.0-1.1min #2-65
1113.40
857.25
1023.32
885.24
1037.34
1082.36
902.27
874.27
933.31
1051.31
1127.41
B
+MS2(1127.41), 40.0eV, 0.1-0.8min #5-47
902.27
933.31
1037.34
1082.36
885.25
949.31
1023.32
1065.33
1127.41
874.27
919.29
1051.31
857.25
1113.40
C
+MS2(1139.41), 35.0eV, 0.0-0.3min #2-18
1139.41
885.25
1023.32
874.28
919.29
1037.33
1082.36
902.27
1051.31
D
+MS2(1179.44), 35.0eV, 0.1-0.7min #3-41
1082.36
1149.36
933.31
1179.45
885.24
1037.34
902.27
1165.34
1051.31
874.27
919.29
1023.32
1065.33
E
+MS2(1215.45), 50.0eV, 7.3-8.3min #439-467
1023.32
1215.45
1065.31
874.27
1198.42
1051.31
933.31
902.27
1082.36
1037.34
850
900
950
1000
1050
1100
1150
1200
m/z

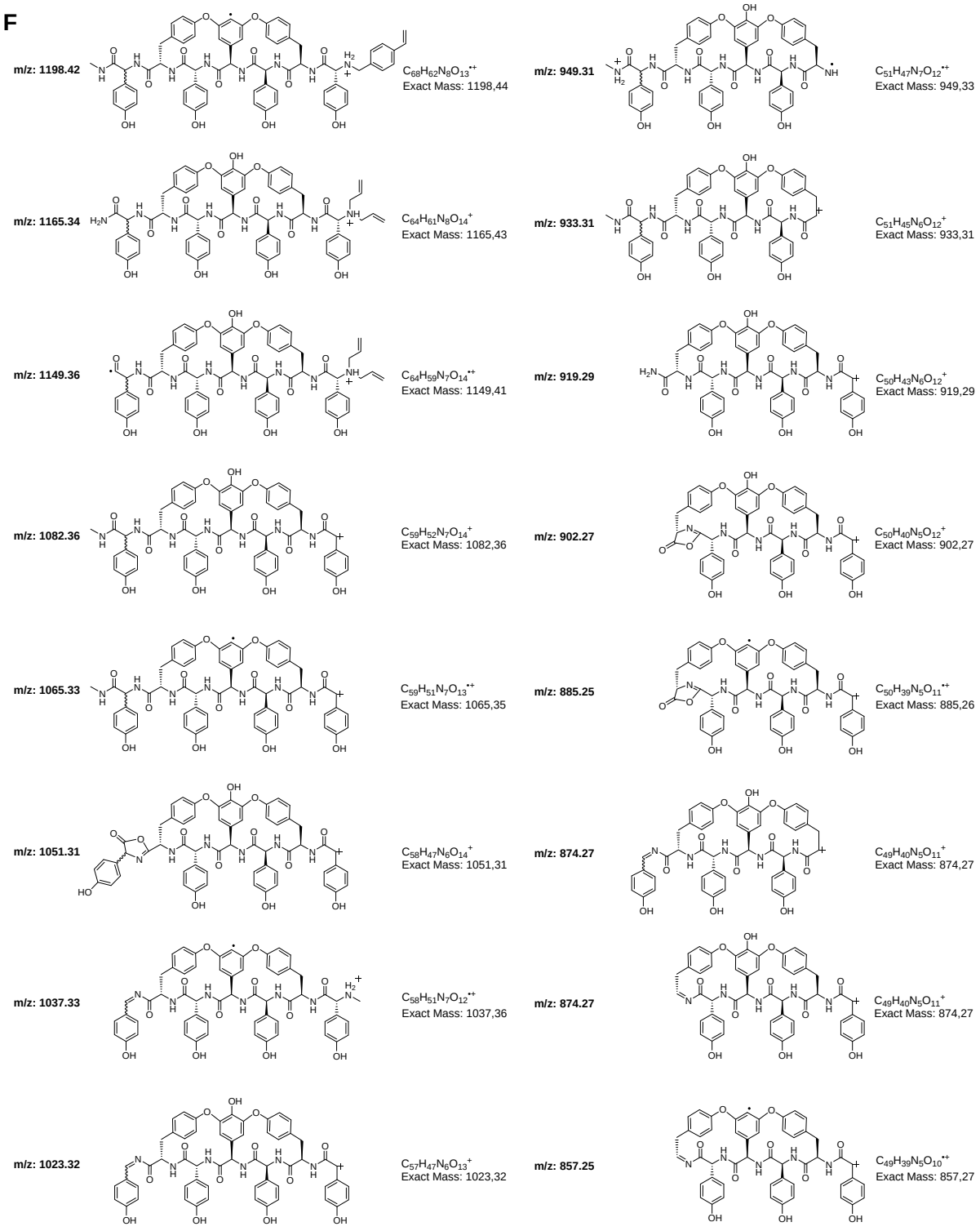



**Fig. S15**: ESI-MS/MS analysis of teicoplanin aglycone derivatives **5a$_1$** (A), **5a$_2$** (B), **5b$_1$** (C), **5b$_2$** (D), **5c** (E). Data were obtained in positive mode. G) Proposed structures for characteristic fragments observed during tandem mass spectrometry. Due to low amounts of monocyclic peptide species during turnover reactions, MS/MS analysis for these peptides was abandoned.

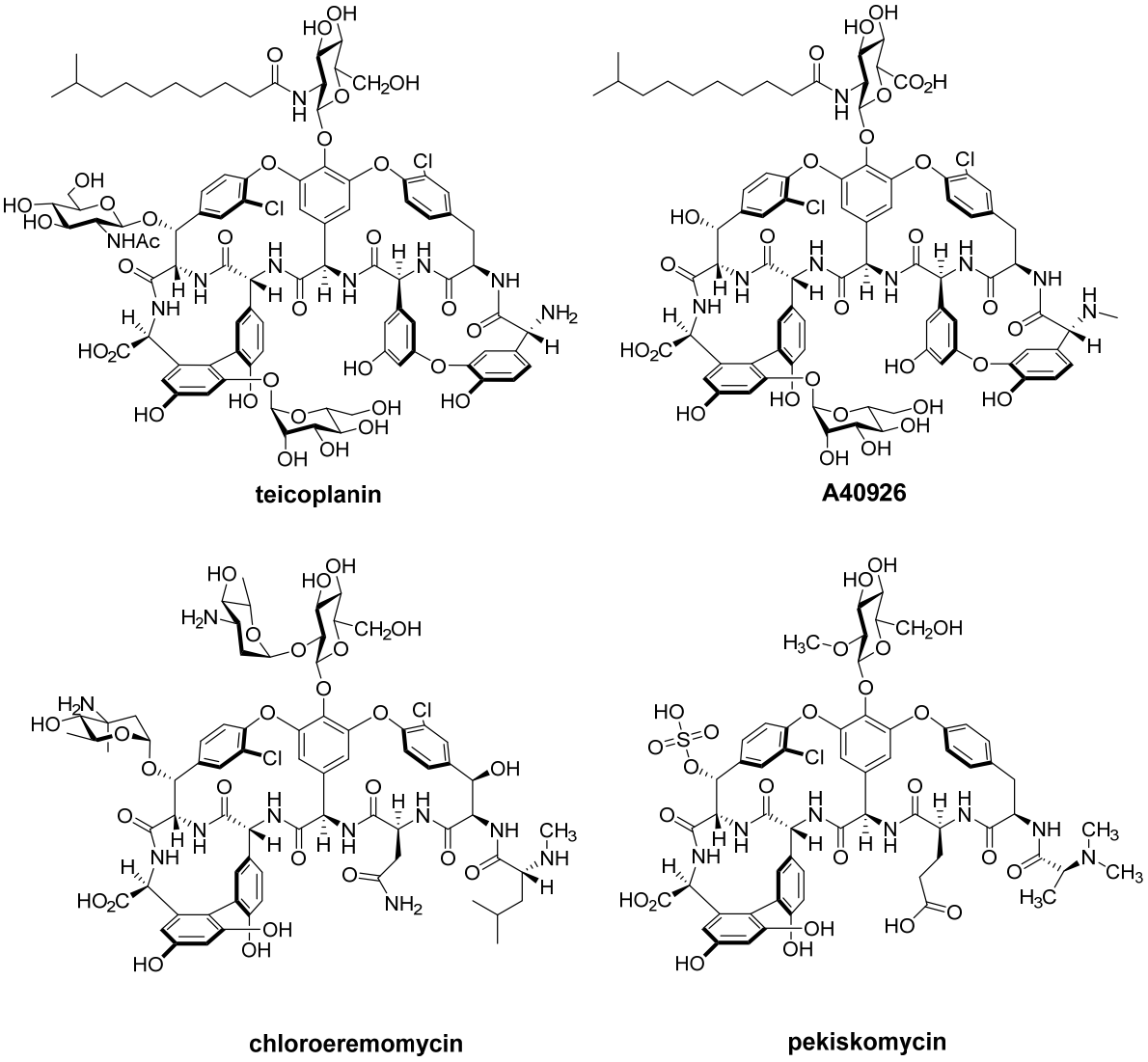



**Fig. S16:** Structures of the type-IV GPA teicoplanin and A40926 – the precursor of semisynthetic dalbavancin -- and the type-I GPAs chloroeremomycin and pekiskomycin.

**Table S1**. Minimal inhibitory concentration (MIC) of compounds **1**, **1b**, **1c**

| Compound No. | vancomycin | **1** | **1b** | **1c** |
|---|---|---|---|---|
| MIC in BHI (ug/ml) | | | | |
| *Enterococcus faecalis* (ATCC 29212) | 4 | 4 | 32 | 2 |
| *Enterococcus faecalis* VRE B (ATCC 51299) | 64 | 32 | >64 | 4 |
| MIC in MHB (ug/ml) | | | | |
| *Bacillus subtilis* 168 (ATCC 23857) | 0,25 | 0,5 | 8 | 1 |

MICs were determined in three independent experiments in duplicates for each strain and compound. *Enterococcus* MICs were determined using the broth microdilution method in BBL Brain Heart Infusion broth according to NCCLS protocols. *Bacillus* MICs were determined using the broth microdilution method in BBL Cation-adjusted Mueller Hinton broth according to NCCLS protocols.

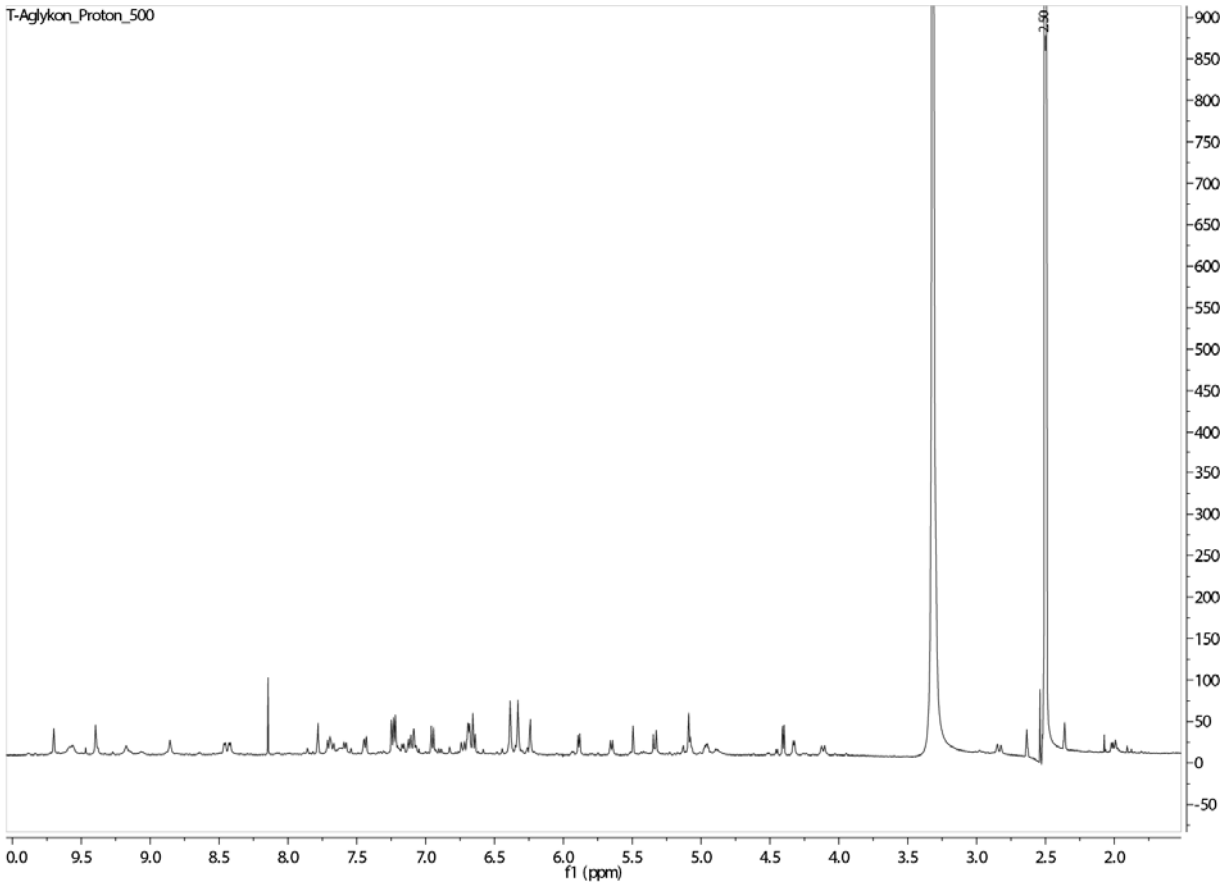



**Fig. S17**: $^{1}$H-NMR of teicoplanin aglycone **1**

$^{1}$H NMR (500 MHz, $^{1}$H-$^{1}$H-COESY, $^{1}$H-$^{1}$H-ROESY, DMSO-$d_6$) δ = 9.70 (s, 1H, OH), 9.56 (s, 1H, OH), 9.51 (s, 1H, OH), 9.39 (s, 1H, OH), 9.17 (s, 1H, OH), 8.85 (s, 1H, OH), 8.46 (d, *J* = 5.2 Hz, 1H, $Hpg_5$-NH), 8.41 (d, *J* = 6.0 Hz, 1H, $Dpg_7$-NH), 8.16 (s, 1H), 7.78 (d, *J* = 1.9 Hz, 1H, $Tyr_6$-Har), 7.71 – 7.67 (m, 2H, $Hpg_4$-NH, Har), 7.60 (d, *J* = 8.4 Hz, 1H, $Dpg_3$-NH), 7.55 (m, 1H, $Tyr_2$-NH), 7.44 (dd, *J* = 8.3, 2.0 Hz, 1H, Har), 7.25 (d, *J* = 3.2 Hz, 1H, Har), 7.24 (d, *J* = 3.3 Hz, 1H, Har),7.22 (d, *J* = 1.9 Hz, 1H, Har), 7.14 – 7.07 (m, 2H, Har), 6.94 (d, *J* = 8.3 Hz, 1H, Har), 6.73 (d, *J* = 11.8 Hz, 1H, $Tyr_6$-NH), 6.71 – 6.57 (m, 4H, Har), 6.38 (m, 2H, Har), 6.34 – 6.31 (m, 2H, Har), 6.24 (d, *J* = 2.3 Hz, 1H, Har), 5.89 (d, *J* = 6.6 Hz, 1H, β-OH), 5.66 (d, *J* = 8.2 Hz, 1H, $Dpg_3$- Hα), 5.50 (d, *J* = 2.1 Hz, 1H, $Hpg_4$-Har), 5.33 (d, *J* = 10.4 Hz, 1H, $Hpg_4$- Hα), 5.13 – 5.05 (m, 2H, $Tyr_6$-Hβ, $Hpg_4$-Har), 4.97 (m, 1H, $Tyr_2$-Hα), 4.77 (bs, 1H, $Hpg_1$-Hα), 4.40 (d, *J* = 5.8 Hz, 1H, $Dpg_7$-Hα), 4.33 (d, *J* = 5.5 Hz, 1H, $Hpg_5$-Hα), 4.12 (d, *J* = 11.8 Hz, 1H, $Tyr_6$-Hα), 3.35 (m, under water signal, 1H, $Tyr_2$-H$\beta_1$), 2.84 (m, 1H, $Tyr_2$-H$\beta_2$) ppm.

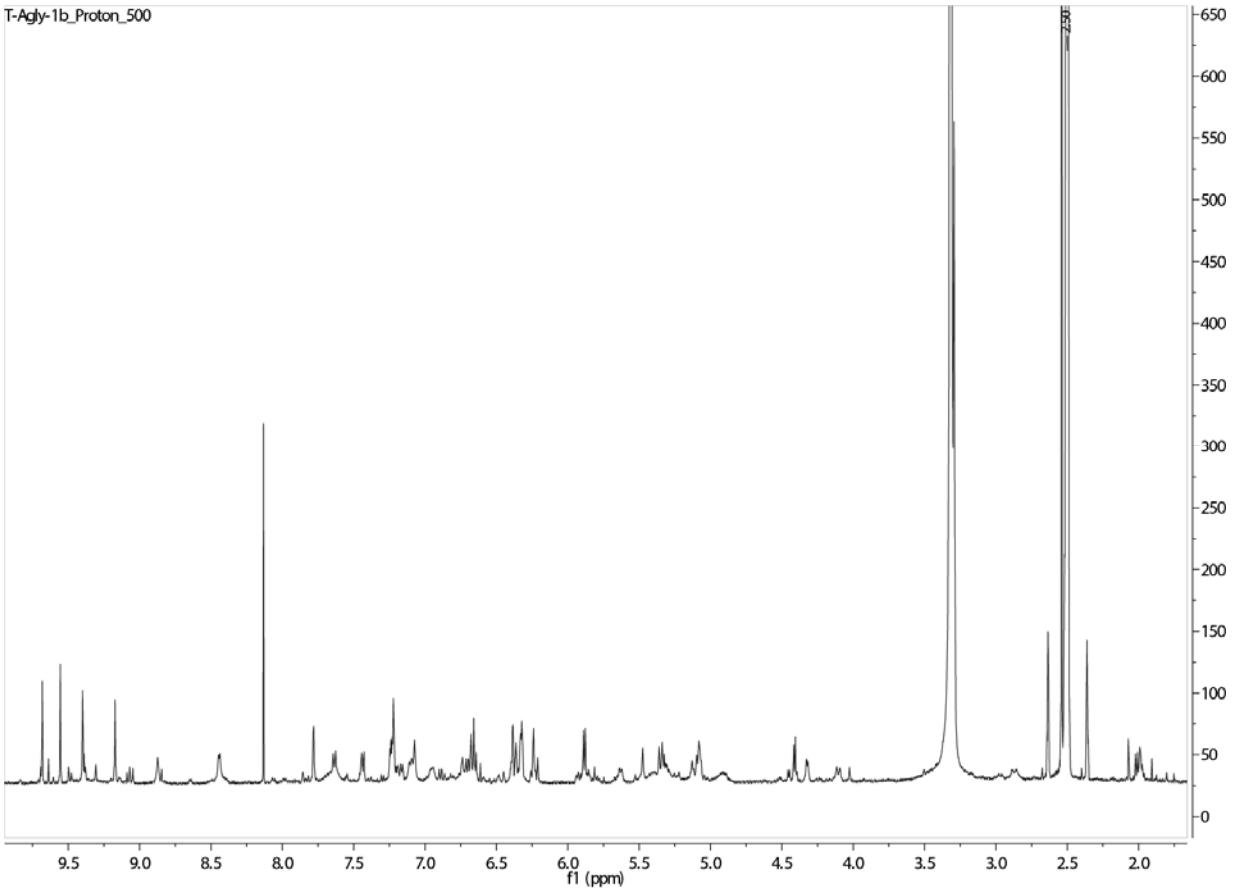



**Fig. S18**: $^{1}$H-NMR of teicoplanin aglycone derivative **1b**

$^{1}$H NMR (500 MHz, $^{1}$H-$^{1}$H-COESY, DMSO-$d_6$) δ 9.69 (d, *J* = 5.1 Hz, 1H, OH), 9.56 (s, 1H, OH), 9.42 – 9.37 (m, 2H, OH), 9.17 (s, 1H, OH), 8.87 (s, 1H, OH), 8.44 (m, 2H, Hpg$_5$-NH, Dpg$_7$-NH), 8.13 (s, 1H), 7.78 (d, *J* = 1.9 Hz, 1H, Tyr$_6$-Har), 7.70 – 7.61 (m, 3H, Hpg$_4$-NH, Dpg$_3$-NH, Har), 7.55 (m, 1H, Tyr$_2$-NH), 7.45 (dd, *J* = 8.4, 2.0 Hz, 1H, Har), 7.25 – 7.15 (m, 3H, Har), 7.12 – 7.06 (m, 2H, Har), 6.95 (m, 1H, Har), 6.91 – 6.85 (m, 1H), 6.77 – 6.61 (m, 3H, Har, Tyr$_6$-NH), 6.50 – 6.43 (m, 1H), 6.41 – 6.34 (m, 3H, Har), 6.27 – 6.21 (1H), 5.93 – 5.84 (m, 2H, allyl-H), 5.67 – 5.62 (m, 1H, Dpg$_3$- Hα), 5.47 (m, 1H, Hpg$_4$-Har), 5.39 – 5.20 (m, 4H, Hpg$_4$- Hα, allyl-H), 5.13 - 5.04 (m, 2H, Tyr$_6$-Hβ, Hpg$_4$-Har), 4.91 (m, 1H, Tyr$_2$-Hα), 4.41 (m, 1H, Dpg$_7$-Hα), 4.32 (d, *J* = 5.5 Hz, 1H, Hpg$_5$-Hα), 4.10 (d, *J* = 11.4 Hz, 1H, Tyr$_6$-Hα), 3.35 (m, under water signal, 1H, Tyr$_2$-Hβ$_1$), 2.87 (m, 1H, Tyr$_2$-Hβ$_2$) ppm.

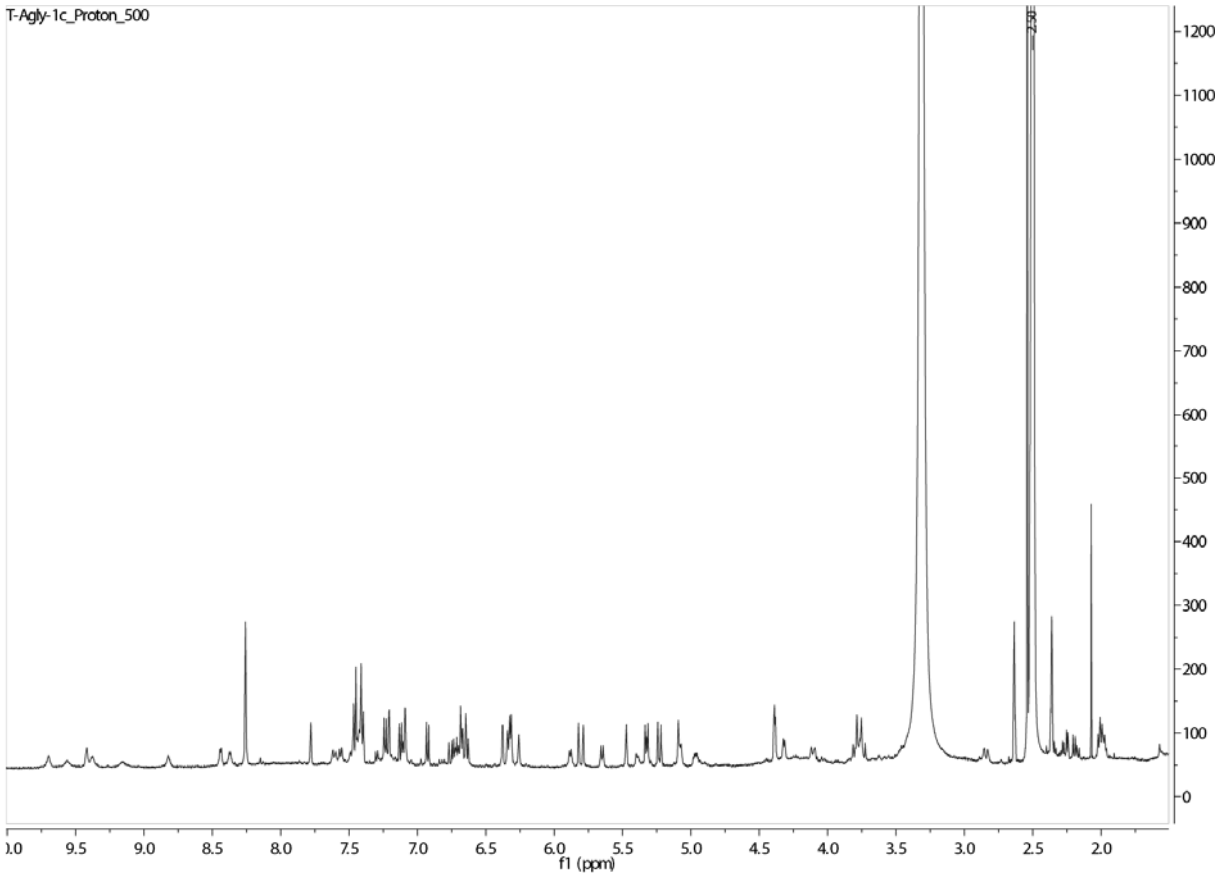



**Fig. S19**: $^{1}$H-NMR of teicoplanin aglycone derivative **1c**

$^{1}$H NMR (500 MHz, $^{1}$H-$^{1}$H-COESY, DMSO-$d_6$) δ 9.70 (s, 1H, OH), 9.56 (s, 1H, OH), 9.42 (s, 1H, OH), 9.38 (s, 1H, OH), 9.15 (s, 1H, OH), 8.82 (s, 1H, OH), 8.44 (d, *J* = 5.2 Hz, 1H, Hpg$_5$-NH) 8.37 (d, *J* = 5.8 Hz, 1H, Dpg$_7$-NH), 8.26 (s, 1H), 7.78 (d, *J* = 1.9 Hz, 1H, Tyr$_6$-Har), 7.61 (d, *J* = 10.3 Hz, 1H, Hpg$_4$-NH), 7.56 (d, *J* = 8.5 Hz, 1H, Dpg$_3$-NH), 7.49 – 7.40 (m, 6H, Tyr$_2$-NH, Har), 7.24 (d, *J* = 8.3 Hz, 1H, Har), 7.21 (d, *J* = 1.9 Hz, 1H, Har), 7.16 – 7.08 (m, 3H, Har), 6.93 (d, *J* = 8.3 Hz, 1H, Har), 6.74 (dd, *J* = 10.9, 17.6 Hz, Ar-C**H**=CH$_2$), 6.71 – 6.64 (m, 3H, Tyr$_6$-NH, Har) , 6.38 (d, *J* = 2.3 Hz, 1H, Har), 6.35 – 6.31 (m, 3H, Har), 6.26 (s, 1H, Har), 5.88 (d, *J* = 6.7 Hz, 1H, β-OH), 5.81 (dd, *J* = 17.6, 1.1 Hz, 1H, Ar-CH=C**H**$_2$-trans), 5.65 (d, *J* = 8.3 Hz, 1H, Dpg$_3$- Hα), 5.47 (d, *J* = 2.1 Hz, 1H, Hpg$_4$-Har), 5.35 – 5.32 (m, 1H, Hpg$_4$- Hα), 5.23 (dd, *J* = 10.9, 1.1 Hz, 1H, Ar-CH=C**H**$_2$-cis), 5.11 – 5.06 (m, 2H, Tyr$_6$-Hβ, Hpg$_4$-Har), 5.00 – 4.94 (m, 1H, Tyr$_2$-Hα), 4.42 – 4.38 (m, 1H, Dpg$_7$-Hα), 4.32 (d, *J* = 5.4 Hz, 1H, , Hpg$_5$-Hα), 4.11 (d, *J* = 11.8 Hz, 1H, Tyr$_6$-Hα), 3.86 – 3.70 (m, 2H, Hpg$_1$-Hα, Hpg$_1$-NH), 3.35 (m, under water signal, 1H, Tyr$_2$-Hβ$_1$), 2.84 (d, *J* = 13.9 Hz, 1H, Tyr$_2$-Hβ$_2$), 2.28 – 2.16 (m, NH-C**H**$_2$-Ar) ppm.

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