Site-specific conjugation of peptides and proteins via re-bridging of disulfide bonds using the thiol-yne coupling reaction

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Example 9a and 9b


Under an atmosphere of argon, a two necked bound-bottom flask was purged with Terlipressin acetate (50.17 mg, 37.23 µmol, sequence: H-Gly-Gly-Gly-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH₂, cyclic disulfide) and dissolved in 1.1 ml water. TCEP-HCl (16.01 mg, 55.85 µmol) was added and the mixture was degassed three times and stirred at room temperature for 2h. Hept-6-ynoic acid (4.70 mg, 37.23 µmol) dissolved in 100 µl methanol and LAP (0.55 mg, 1.86 µmol, prepared according to Gong et al., Chem Commun (Camb) 2013, 49, 7950-2) dissolved in 150 µl water were added. A UV-LED pen (Omnicure LX400, 365 nm, igb-tech GmbH, Germany) was mounted into the neck of the two necked round-bottom flask (sealed with Teflon, distance of 40 mm to the surface of the reaction mixture). The reaction mixture was irradiated with UV light (365 nm) for 1h. After adding additional LAP (0.55 mg, 1.86 µmol) the mixture was again irradiate with UV light (365 nm) for 1h. The isomeric products were isolated via preparative HPLC (column: Waters X-Bridge BEH130 Prep C18 10 µm OBD, 19 x 250 mm; mobile phase A: water with 0.05% TFA, mobile phase B: acetonitrile with 0.05% TFA; gradient: 0.0 min 5% B → 40 min 40% B).

Isomer 1:
LC/MS: \( R_t = 3.53 \) min; MS (ESpos): \( m/z = 1355 \ [M+H]^+ \).

Isomer 2:
Yield: 1.3 mg (3%).

LC/MS: \( R_t = 3.57 \) min; MS (ESpos): \( m/z = 1355 \ [M+H]^+ \).
\(^{13}\)C-NMR (125.78 MHz, D\(_2\)O, δ (1,4-Dioxan) = 67.4 ppm): δ [ppm] = 183.1 (S), 182.1 (S), 178.5 (S), 175.2 (3S), 174.7 (S), 174.0 (S), 173.9 (S), 172.7 (S), 172.5 (S), 171.5 (S), 170.9, 168.7, 155.2 (S), 137.0 (S), 131.4 (S), 130.0 (S), 129.7 (S), 128.8 (S), 128.2 (S), 116.3 (S), 61.7 (D), 57.1 (D), 55.6 (D), 55.3 (D), 54.5 (D), 53.4 (D), 52.6 (D), 50.7 (D), 48.8 (T), 45.7 (D), 43.2 (T), 43.0 (T), 42.9 (T), 41.3 (T), 40.1 (T), 38.8 (T), 37.0 (T), 36.6 (T), 36.4 (2T), 34.0 (T), 33.6 (T), 31.9 (2T), 30.9 (T), 30.1 (T), 27.0 (T), 26.9 (T), 26.8 (T), 25.6 (T), 25.5 (T), 22.9 (T).

The \(^1\)H-NMR of isomer 2 is shown in Figure 1.

**Example 12a and 12b**

**12a:** (6R,9S,12S,15S,18S,21R)-21-amino-9-(2-amino-2-oxoethyl)-12-(3-amino-3-oxopropyl)-15-benzyl-2-(4-carboxybutyl)-18-(4-hydroxybenzyl)-8,11,14,17,20-pentaoxo-1,4-dithia-7,10,13,16,19-pentaazacyclodocosane-6-carboxylic acid

**12b:** (6R,9S,12S,15S,18S,21R)-21-amino-9-(2-amino-2-oxoethyl)-12-(3-amino-3-oxopropyl)-15-benzyl-3-(4-carboxybutyl)-18-(4-hydroxybenzyl)-8,11,14,17,20-pentaoxo-1,4-dithia-7,10,13,16,19-pentaazacyclodocosane-6-carboxylic acid

**12a:**

![Chemical Structure 12a](image)

**12b:**

![Chemical Structure 12b](image)
Under an atmosphere of argon, a two necked bound-bottom flask was purged with Pressinoic acid (15.33 mg, 19.78 µmol, sequence: H-Cys-Tyr-Phe-Gln-Asn-Cys-OH, cyclic disulfide) and dissolved in 500 µl water with 0.1% acetic acid. TCEP-HCl (8.7 mg, 30.35 µmol) was added and the mixture was degassed three times and stirred at room temperature for 2h. Water with 0.1% acetic acid (200 µl) and Acetonitrile (500 µl) were added. Hept-6-ynoic acid (2.5 mg, 19.82 µmol) dissolved in 100 µl water with 0.1% acetic acid was added. Then LAP (5.8 mg, 19.72 µmol, prepared according to Gong et al., Chem Commun (Camb) 2013, 49, 7950-2) was dissolved in 500 µl water with 0.1% acetic acid and 50 µl of this solution were added. A UV-LED pen (Omnicure LX400, 365 nm, igb-tech GmbH, Germany) was mounted into the neck of the two necked round-bottom flask (sealed with Teflon, distance of 40 mm to the surface of the reaction mixture). The reaction mixture was irradiated with UV light (365 nm) for 1h. The addition LAP solution (50 µl) and subsequent irradiation with UV light (365 nm) for 1h was repeated two times. The isomeric products were isolated via preparative HPLC (column: Waters X-Bridge BEH130 Prep C18 10 µm OBD, 19 x 250 mm; mobile phase A: water with 0.1% TFA, mobile phase B: acetonitrile with 0.08% TFA; gradient: 0.0 min 5% B → 3 min 5% B → 33 min 40% B).

**Fraction 1:**
Yield: 1.4 mg (8%).

LC/MS: R<sub>i</sub> = 1.25 min; MS (ESpos): m/z = 903.4 [M+H]<sup>+</sup>.

The <sup>1</sup>H-NMR of fraction 1 is shown in Figure 2.

**Fraction 2:**
Yield: 0.6 mg (3% d. Th.).
LC/MS: \( R_t = 1.25 \) min; MS (ESpos): \( m/z = 903.4 \) [M+H]\(^+\) und \( R_t = 1.27 \) min; MS (ESpos): \( m/z = 903.3 \) [M+H]\(^+\).

HRMS: for \( C_{40}H_{55}O_{12}N_{8}S_{2} \) [M+H]\(^+\) calculated: 903.3375 found: 903.3371.

**Fraction 3:**

Yield: 0.7 mg (4%).

LC/MS: \( R_t = 1.27 \) min; MS (ESpos): \( m/z = 903.3 \) [M+H]\(^+\).

The \(^1\)H-NMR of fraction 3 is shown in Figure 3.

**Example 20a and 20b**

**20a:** 4-\{4-[[6R,9S,12S,15S,18S,21R]-21-\]\

**20b:** 4-\{4-[[6R,9S,12S,15S,18S,21R]-21-\]\

**20a:**

![Molecule Image]
Under an atmosphere of argon, a two necked bound-bottom flask was purged with Terlipressin acetate (21.54 mg, 15.98 µmol, sequence: H-Gly-Gly-Gly-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH₂, cyclic disulfide) and dissolved in 800 µl DPBS buffer. TCEP-HCl (6.5 mg, 22.68) was added and the mixture was degassed three times and stirred at room temperature for 2h. 4-(Hex-5-yn-1-yl)-4-methylmorpholin-4-ium iodide (4.9 mg, 15.84 µmol) dissolved in 500 µl DPBS was added. Then LAP (4.7 mg, 15.98 µmol, prepared according to Gong et al., Chem Commun (Camb) 2013, 49, 7950-2) was dissolved in 500 µl DPBS and 50 µl of this solution were added. A UV-LED pen (Omnicure LX400, 365 nm, igb-tech GmbH, Germany) was mounted into the neck of the two necked round-bottom flask (sealed with Teflon, distance of 40 mm to the surface of the reaction mixture). The reaction mixture was irradiated with UV light (365 nm) for 1h. The addition LAP solution (50 µl) and irradiation with UV light (365 nm) for 1h was repeated two times. The isomeric products were isolated via preparative HPLC (column: Phenomenex Kinetex Prep 5 µm C18 100 Å AXIA Packed LC Column, 21.2 x 100 mm; mobile phase A: water with 0.1% TFA, mobile phase B: acetonitrile with 0.1% TFA; gradient: 0.0 min 5% B → 3 min 5% B → 63 min 40% B → 64.30 min 95% B → 69.30 min 95% B).

**Fraction 1:**

Yield: 1.5 mg (4%).

LC/MS: Rᵣ = 6.49 min; purity 65%; MS (ESpos): m/z = 705.8397 [M+H]^{2+}.

HRMS: for C_{63}H_{97}O_{16}N_{17}S_{2} [M+H]^{2+} calculated: 705.8365, found: 705.8361.
The $^1$H-NMR of fraction 1 is shown in Figure 4.

**Fraction 2:**
Yield: 2.2 mg (8%).

LC/MS: $R_t = 6.48$ min; purity 87%; MS (ESpos): $m/z = 705.8401$ [M+H]$^{2+}$.

HRMS: for C$_{63}$H$_{97}$O$_{16}$N$_{17}$S$_2$ [M+H]$^{2+}$ calculated: 705.8365, found: 705.8377.

The $^1$H-NMR of fraction 2 is shown in Figure 5.

**Example 21a and 21b**

21a:
(2S)-1-([(6R,9S,12S,15S,18S,21R)-21-]

{[(aminoacetyl)amino]acetyl}amino)acetyl]amino}\-9-(2-amino-2-oxoethyl)-12-(3-
amino-3-oxopropyl)-15-benzyl-18-(4-hydroxybenzyl)-8,11,14,17,20-pentaaxo-2-(27-oxo-
2,5,8,11,14,17,20,23-oxaaxa-26-aziacontan-30-yl)-1,4-dithia-7,10,13,16,19-
pentaazacyclodocaxan-6-yl]carbonyl}-N-{(2S)-6-amino-1-[2-amino-2-oxoethyl]amino]-1-
oxohexan-2-yl}pyrrolidine-2-carboxamide

21b:
(2S)-1-([(6R,9S,12S,15S,18S,21R)-21-]

{[(aminoacetyl)amino]acetyl}amino)acetyl]amino]\-9-(2-amino-2-oxoethyl)-12-(3-
amino-3-oxopropyl)-15-benzyl-18-(4-hydroxybenzyl)-8,11,14,17,20-pentaaxo-3-(27-oxo-
2,5,8,11,14,17,20,23-oxaaxa-26-aziacontan-30-yl)-1,4-dithia-7,10,13,16,19-
pentaazacyclodocaxan-6-yl]carbonyl}-N-{(2S)-6-amino-1-[2-amino-2-oxoethyl]amino]-1-
oxohexan-2-yl}pyrrolidine-2-carboxamide

21a:
Under an atmosphere of argon, a two necked bound-bottom flask was purged with Terlipressin acetate (20.64 mg, 15.32 µmol, sequence: H-Gly-Gly-Gly-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH$_2$, cyclic disulfide) and dissolved in 500 µl DPBS buffer. TCEP-HCl (13.30 mg, 46.39) was added and the mixture was degassed three times and stirred at room temperature for 2h. N-(2,5,8,11,14,17,20,23-Octaoxapentacosan-25-yl)hex-5-ynamide (7.20 mg, 15.15 µmol) dissolved in 150 µl DPBS was added. LAP (4.40 mg, 14.95 µmol, prepared according to Gong et al., *Chem Commun (Camb)* 2013, 49, 7950-2) was dissolved in 500 µl DPBS and 50 µl of this solution were added. A UV-LED pen (Omnicure LX400, 365 nm, igb-tech GmbH, Germany) was mounted into the neck of the
two necked round-bottom flask (sealed with Teflon, distance of 40 mm to the surface of the reaction mixture). The reaction mixture was irradiated with UV light (365 nm) for 1h. The addition of LAP solution (50 µl) and irradiation with UV light (365 nm) for 1h was repeated two times. The isomeric products were isolated via preparative HPLC (column: Waters X-Bridge BEH130 Prep C18 10 µm OBD, 19 x 250 mm; mobile phase A: water with 0.1% TFA, mobile phase B: acetonitrile with 0.1% TFA; gradient: 0.0 min 5% B → 3 min 5% B → 43 min 40% B → 44.30 min 95% B → 49.30 min 95% B).

Fraction 1:
Yield: 6.20 mg (23%)

LC/MS: R<sub>t</sub> = 7.41 min; purity (99%); MS (ESpos): m/z = 853.9101 [M+2H]<sup>2+</sup>.

HRMS: for C<sub>75</sub>H<sub>121</sub>O<sub>24</sub>N<sub>17</sub>S<sub>2</sub> [M+2H]<sup>2+</sup> calculated: 853.9100, found: 853.9100.

The <sup>1</sup>H and <sup>13</sup>C-NMR of fraction 1 are shown in figures 6 and 7.

Fraction 2:
Yield: 1.70 mg (7%).

LC/MS: R<sub>t</sub> = 4.09 min; MS (ESpos): m/z = 853.9149 [M+2H]<sup>2+</sup>.

HRMS: for C<sub>75</sub>H<sub>121</sub>O<sub>24</sub>N<sub>17</sub>S<sub>2</sub> [M+2H]<sup>2+</sup> calculated: 853.9100, found: 853.9095.

**Example 22a and 22b**


Under an atmosphere of argon, a two necked bound-bottom flask was purged with Terlipressin acetate (10.16 mg, 7.54 µmol, sequence: H-Gly-Gly-Gly-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH₂, cyclic disulfide) and dissolved in 300 µl DPBS buffer. TCEP-HCl (3.2 mg, 11.17 µmol) was added and the mixture was degassed three times and stirred at room temperature for 2h. (2S)-2-[(t-Butoxycarbonyl)amino]pent-4-ynoic acid (1.6 mg, 7.54 µmol) dissolved in 100 µl DPBS was added. Then LAP (3.2 mg, 10.87 µmol, prepared according to Gong et al., Chem Commun (Camb) 2013, 49, 7950-2) was dissolved in 500 µl DPBS and 50 µl of this solution were added. A UV-LED pen (Omnicure LX400, 365 nm, igb-tech GmbH, Germany) was mounted into the neck of the two necked round-bottom flask (sealed with Teflon, distance of 40 mm to the surface of the reaction mixture). The reaction mixture was irradiated with UV light (365 nm) for 1h.
The addition LAP solution (50 µl) and irradiation with UV light (365 nm) for 1h was repeated two times. The isomeric products were isolated via preparative HPLC (column: Phenomenex Kinetex Prep 5 µm C18 100 Å AXIA Packed LC Column, 21.2 x 100 mm; mobile phase A: water with 0.1% TFA, mobile phase B: acetonitrile with 0.1% TFA; gradient: 0.0 min 5% B → 3 min 5% B → 63 min 40% B → 64.30 min 95% B → 69.30 min 95% B).

Fraction 1:
Yield: 0.4 mg (4%).

LC/MS: R<sub>t</sub> = 7.11 min; MS (ESpos): m/z = 721.8130 [M+2H]<sup>2+</sup>.

HRMS: for C<sub>62</sub>H<sub>93</sub>O<sub>19</sub>N<sub>17</sub>S<sub>2</sub>[M+2H]<sup>2+</sup> calculated: 721.8132, found: 721.8130.

Das <sup>1</sup>H-NMR of fraction 1 is shown in Figure 8.

Fraction 2:
Yield: 1.6 mg (15% d. Th.).

LC/MS: R<sub>t</sub> = 7.36 min; purity 94%; MS (ESpos): m/z = 1442.6134 [M+H]<sup>+</sup>.

HRMS: for C<sub>62</sub>H<sub>93</sub>O<sub>19</sub>N<sub>17</sub>S<sub>2</sub>[M+2H]<sup>2+</sup> calculated: 721.8132, found: 721.8132.

The <sup>1</sup>H-NMR-Spektrum of fraction 2 is shown in Figure 9.

Fraction 3:
Ausbeute: 0.3 mg (3% d. Th.).

LC/MS: R<sub>t</sub> = 7.34 min; purity 89%; MS (ESpos): m/z = 721.8122 [M+2H]<sup>2+</sup>.

HRMS: for C<sub>62</sub>H<sub>92</sub>O<sub>19</sub>N<sub>17</sub>S<sub>2</sub>[M+H]<sup>+</sup> calculated: 1442.6191, found: 1442.6200.

Example 24

24: (2S)-1-[[3R,6S,9S,12S,15S,18R)-18-
[{[[[aminoacetyl]amino]acetyl}amino]acetyl]amino}\-6-(2-amino-2-oxoethyl)-9-(3-
25 amino-3-oxopropyl]-12-benzyl-15-(4-hydroxybenzyl)-23-(hydroxymethyl)-5,8,11,14,17-
pentaoxohexacosahydro-20aH-cyclopropa[5,6]cycloocta[1,2-
Under an atmosphere of argon, a two necked bound-bottom flask was purged with Terlipressin acetate (26.24 mg, 19.47 µmol, sequence: H-Gly-Gly-Gly-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH₂, cyclic disulfide) and dissolved in 1.5 ml DPBS buffer. TCEP-HCl (8.4 mg, 29.31 µmol) was added and the mixture was degassed three times and stirred at room temperature for 2.5h. (1R,8S,9r)-bicyclo[6.1.0]non-4-yn-9-yl methanol dissolved in 200 µl methanol was added. Then LAP (5.7 mg, 19.38 µmol, prepared according to Gong et al., Chem Commun (Camb) 2013, 49, 7950-2) was dissolved in 500 µl DPBS and 50 µl of this solution were added. A UV-LED pen (Omnicure LX400, 365 nm, igb-tech GmbH, Germany) was mounted into the neck of the two necked round-bottom flask (sealed with Teflon, distance of 40 mm to the surface of the reaction mixture). The reaction mixture was irradiated with UV light (365 nm) for 1h. The addition LAP solution (50 µl) and irradiation with UV light (365 nm) for 1h was repeated two times. The isomeric products were isolated via preparative HPLC (column: Phenomenex Kinetex Prep 5 µm C18 100 Å AXIA Packed LC Column, 21.2 x 100 mm; mobile phase A: water with 0.1% TFA, mobile phase B: acetonitrile with 0.1% TFA; gradient: 0.0 min 5% B → 3 min 5% B → 63 min 40% B → 64.30 min 95% B → 69.30 min 95% B).

Fraction 1:

Yield: 3.1 mg (7.5%).

LC/MS: R_t = 6.92 min; purity 65%; MS (ESpos): m/z = 903.3 [M+H]^+. 
HRMS: for C_{62}H_{91}O_{16}N_{16}S_{2} [M+H]^+ calculated: 1379.6235, found: 1379.6244.

The ^1H-NMR-Spektrum of fraction 1 is shown in Figure 10.

**Example 29a and 29b**

C2-bridgeing thiol-yne reaction with an antibody FAB fragment:

Under an atmosphere of argon, a two necked bound-bottom flask was purged with a solution of the FAB fragment M14-G07 [described in Dittmer et al., US Pat. Appl. US 2014/0050743-A1, page 13, column 0105, 0106 und 0113ff] in PBS buffer (108.4 µl, c = 46.1 mg/ml, 0.107 mmol). TCEP-HCl (4.60 mg, 16.0 µmol) was dissolved in 400 µl. A small porting of the TCEP-HCl solution (4 µl) was added and the mixture was degassed three times and stirred at room temperature for 2.5h. A solution (4 µl) of Hept-6-ynoic acid (1.36 µl, 10.75 µmol) was methanol was added. Then LAP (3.1 mg, 10.53 µmol, prepared according to Gong et al., Chem Commun (Camb) 2013, 49, 7950-2) was dissolved in 500 µl DPBS and 1 µl of this solution were added. A UV-LED pen (Omnicure LX400, 365 nm, igb-tech GmbH, Germany) was mounted into the neck of the two necked round-bottom flask (sealed with Teflon, distance of 40 mm to the surface of the reaction mixture). The reaction mixture was cooled by an ice bath (5°C < T < 10°C) and irradiated with UV light (365 nm) for 1h. The addition LAP solution (1 µl) and irradiation with UV light (365 nm) for 1h was repeated three times. The mixture was diluted with 2400 µl DPBS buffer and filtered over a Sephadex® G-25M PD-10 column (GE Healthcare), which was prior, equilibrated with 5 ml DPBS buffer five times. The filtrate was transferred into a 15 ml centrifuge tube (Corning®) and centrifuged at 4000 rpm. After 5 min the supernatant was transferred into a centrifugal filter unit (Amicon® Ultra-4) and centrifuged for 15 min. The resulting concentrate was diluted with DPBS buffer to a total volume of 2.5 ml and transferred into a vial for storage.

**Confirmation and quantification of covalently linked FAB fragments:**

The resulting product in DPBS buffer from the procedure described above was analyzed as follows:

The quantification of covalent was performed using RP chromatography of the reduced and denatured FAB fragment. To the sample solution (c = 1 mg/ml, 50 µl) was added guanidin hydrochloride (GuHCl) (28.6 mg) and a solution of dithiothreitol (DTT) (500 mM, 3 µl). The mixture was incubated 1h at 55°C and analyzed by analytical HPLC
(Agilent 1260, detection: 220 nm, column: Polymer Laboratories PLRP-S polymeric reversed phase 2.1 mm x 150 mm, particle size 8 µm, 1000 Å, flow rate: 1 ml/min, mobile phase A: water with 0.05% TFA, mobile phase B: acetonitrile with 0.05% TFA; gradient: 0.0 min 25% B → 3 min 25% B → 28 min 50% B).

The detected peaks were assigned by comparison of the retention time from the light chain (L0) and heavy chain /VH-CH1 = H0) of the non conjugated FAB fragments. The signal from the conjugated sample, which differs from the light chain and heavy chain signal was assigned as the covalently linked FAB-Fragments. The yield for the covalently linked FAB fragment was determined by integration of the signal. The resulting chromatograms and the yields are shown in Figure 11 and 12.

To confirm the covalent linkage of the FAB fragment, the denatured and reduced sample was analyzed on a desalting Grom-Sil 300 butyl 1-St column (particle size: 5 µm, column: 5 mm x 500 µm) via HPLC-ESI-TOF (Impact HD, Bruker Daltonik, flow rate: 5 µl/min, mobile phase A: water with 0.1% formic acid, mobile phase B: isopropanol/acetonitrile/water (v/v = 8:1:1) with 0.1% fromic acid; gradient: 0 min 22% B → 8 min 22% B → 10 min 24% B → 12 min 80% B → 18 min 95% B → 27 min 95% B → 30 min 22% B). The spectra received from the TIC (total ion chromatogram) signal were summated and the molecular weight of the different species was calculated based on the results from the MaxEnt deconvolution. The molecular weight of the covalently linked FAB fragment could be assigned.

The resulting spectrum is shown in Figure 13.
Figure 1:
$^1$H-NMR (500.13 MHz, D$_2$O) of isomer 2 from example 9a/b.

Figure 2:
$^1$H-NMR (500.13 MHz, D$_2$O) of fraction 1 from example 12a/12b.
Figure 3:
$^1$H-NMR (500.13 MHz, D$_2$O) of fraction 3 from example 12a/12b.

Figure 4:
$^1$H-NMR (500.13 MHz, D$_2$O) of fraction 1 from example 20a/20b.
Figure 5:
$^1$H-NMR (500.13 MHz, D$_2$O) of fraction 2 from example 20a/20b.

Figure 6:
$^1$H-NMR (500.13 MHz, D$_2$O) of fraction 1 from example 21a/b.
Figure 7: 
$^{13}$C-NMR (125.78 MHz, D$_2$O) of fraction 1 from example 21a/21b.

Figure 8: 
$^1$H-NMR (500.13 MHz, D$_2$O) of fraction 1 from example 22a/22b.
**Figure 9:**
$^1$H-NMR (500.13 MHz, D$_2$O) of fraction 2 from example **22a/22b**.

![Figure 9](image_url)

**Figure 10:**
$^1$H-NMR (500.13 MHz, D$_2$O) of fraction 1 from example **24**.

![Figure 10](image_url)
Figure 11:
RP-HPLC of the non-reduced FAB fragment.

% [covalent FAB] = area [covalent FAB] * 100% / Σ area [Total] = 39.8%

Figure 12:
RP-HPLC of the reduced FAB-Fragment.
Figure 13:
Mass spectrum of the de-natured and reduced sample analyzed via HPLC-ESI-TOF including full spectrum.