Synthesis of DOTA-conjugated multivalent cyclic-RGD peptide dendrimers via 1,3-dipolar cycloaddition and their biological evaluation: implications for tumor targeting and tumor imaging purposes*

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Experimental Section

Instruments and methods

Peptides were synthesized on an ABI 433A automatic Peptide Synthesizer using the FastMoc solid phase peptide synthesis protocols. Microwave-assisted reactions were carried out in a Biotage microwave reactor. Analytical HPLC runs were carried out on a Shimadzu HPLC system and preparative HPLC runs were performed on a Gilson HPLC workstation. Analytical HPLC runs were performed on Alltech ProSephe C4 or C8 and Adsorbosphere XL C18 columns (250 × 4.6 mm, pore size 300 Å, particle size: 5 μm) or on a Merck LiChroCART CN column (250 × 4.6 mm, pore size 100 Å, particle size: 5 μm) at a flow rate of 1.0 mL/min using a linear gradient of buffer B (0 – 100% in 25 min) in buffer A (buffer A: 0.1% TFA in H2O, buffer B: 0.1% TFA in CH3CN/H2O 95:5 v/v).

Preparative HPLC runs were performed on an Alltech ProSephe C4 or C8 column (250 × 22 mm, pore size 300 Å, particle size: 10 μm), and semi-prep HPLC runs were performed on an Alltech Adsorbosphere XL C18 column (250 × 10 mm, pore size 300 Å, particle size: 10 μm) or on a Merck LiChroCART CN column (250 × 10 mm, pore size 100 Å, particle size: 10 μm) at a flow rate of 10.0 mL/min (semi-prep HPLC: 4.0 mL/min) using a linear gradient of buffer B (0 – 100% in 50 min) in buffer A (buffer A: 0.1% TFA in H2O, buffer B: 0.1% TFA in CH3CN/H2O 95:5 v/v). Liquid chromatography electrospray ionization mass spectrometry was measured on a Shimadzu LCMS-QP8000 single quadrupole bench-top mass spectrometer operating in a positive ionization mode. LC/MS/MS) runs were performed on a Finnigan LCQ Deca XP MAX LC/MS equipped with a Shimadzu 10A VP analytical HPLC system. The samples were dissolved in 10% formic acid in CH3CN/H2O 1:1 v/v and analyzed using a Phenomenex Gemini C18 column (150 × 4.6 mm, particle size: 3 μm, pore size: 110 Å) at a flow rate of 1.0 mL/min using a linear gradient of 100% buffer A (0.1% TFA in H2O/CH3CN 95:5 v/v) to 100% buffer B (0.1% TFA in CH3CN/H2O 95:5 v/v) in 50 min. MALDI-TOF analysis was performed on a Kratos Axima CFR apparatus with bradykinin(1-7)

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(monoisotopic [M + H]+ 757.399), human ACTH(18-39) (monoisotopic [M + H]+ 2465.198) and bovine insulin oxidized B chain (monoisotopic [M + H]+ 3494.651) as external references and α-cyano-4-hydroxycinnamic acid or sinapinic acid as matrices. 1H NMR spectra were recorded on a Varian G-300 (300 MHz) spectrometer and chemical shifts are given in ppm (δ) relative to TMS. 13C NMR spectra were recorded on a Varian G-300 (75.5 MHz) spectrometer and chemical shifts are given in ppm relative to CDCl3 (77.0 ppm). The 13C NMR spectra were recorded using the attached proton test (APT) sequence. 1H NMR spectra in H2O/D2O 9:1 v/v were recorded on a Varian Inova-500 (500 MHz) spectrometer and chemical shifts are given in ppm (δ) relative to 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (0.00 ppm). Peak assignments are based on DQF-COSY, TOCSY (mixing times: 20 or 60 ms) and ROESY (mixing times: 150 or 250 ms) spectra. HSOQ and HMBC spectra were measured on a Varian Inova-500 spectrometer and chemical shifts are given in ppm (δ) relative to 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (0.00 ppm). Fourier transform infrared spectra (FTIR) were measured on a Bio-Rad FTS-25 spectrophotometer. Melting points were measured on a Büchi Schmelzpunktbestimmungsapparatur and are uncorrected. Elemental analyses were done by Kolbe Mikroanalytisches Labor (Milheim/Ruhr, Germany). Rf values were determined by thin layer chromatography (TLC) on Merck precoated silica gel 60F254 plates. Spots were visualized by UV-quenching, ninhydrin or Cl2/TDM. 1 The 2-chlorotritryl chloride resin (Hecheng Science & Technology Company) was used in all solid phase syntheses. The coupling reagents 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and benzotriazol-1-yloxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) were obtained from Biosolve. N-hydroxybenzotriazole (HOBt) was from Advanced ChemTech and N9,9-fluorenlymethylxycarbonyl (Fmoc) amino acids were obtained from MultiSynTech. The side-chain protecting groups were chosen as tert-butyl for aspartic acid, tert-butyloxycarbonyl (Boc) for lysine and 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf) for arginine. Peptide-grade tert-butanol (tBuOH), dichloromethane,
N,N-dimethylformamide (DMF), 1,1,1,3,3,3-hexafluoropropanol (HFIP) tert-butyl methylether (MTBE), N-methylpyrrolidone (NMP), and trifluoroacetic acid (TFA) and HPLC-grade acetonitrile were purchased from Biosolve. 2-(4,7,10-tris(2-tert-butoxy-2-oxoethyl)-1,4,7,10-tetraazaazacyclodec-1-yl) acetic acid (DOTA(O'Bu)₃) was purchased from Macrocylics. Piperidine, N,N-diisopropylethylamine (DIPEA), CuSO₄ and sodium ascorbate were obtained from Acros Organics. Triisopropylsilane (TIS) and HPLC-grade TFA were obtained from Merck. Triflic anhydride and propargylbromide were purchased from Aldrich.

**Syntheses**

**3-(prop-2-ynyloxy) methyl benzoate (2):** 3-hydroxymethylbenzoate² (2.35 g, 15.5 mmol) was dissolved in dry DMF (25 mL) and anhydrous K₂CO₃ (2.63 g, 19.8 mmol, 1.3 equiv) was added. To this suspension, a solution of propargylbromide in toluene (2 mL, 17.9 mmol, 1.15 equiv) was added dropwise. The reaction mixture was stirred for 16 h at room temperature. Then, DMF was removed by evaporation and the residue was redissolved in EtOAc (75 mL) and the organic phase was washed with H₂O (3 x 25 mL.), 1N KHSO₄ (3 x 25 mL.) and brine (3 x 25 mL), dried (Na₂SO₄) and evaporated *in vacuo*. Propargyl ether 2 was obtained as a pale brownish waxy solid in 95% yield (2.80 g).

\[ R(\text{EtOAc/hexane 4:1 v/v): 0.69; } ^1\text{H-NMR (CDCl}_3) \delta 2.56 (t (J 2.47 Hz), 2H, CH), 3.90 (s, 3H, OCH)\]

4.72 (d (J 2.47 Hz), 4H, -O-CH₂), 7.16 (m, 1H, aron H), 7.35 (d (J 7.81 Hz), 1H, aron H), 7.62 (s, 2H, aron H), 7.66 (d (J 7.81 Hz), 1H, aron H); \[ ^13\text{C-NMR (CDCl}_3) \delta 52.0, 55.8, 76.0, 75.8, 115.1, 120.0, 122.7, 131.4, 157.3; \] MS analysis: calcd for C₁₁H₁₀O₃ 190.20, found ES-MS 191.38 [M + H]⁺.

**3,5-bis-(prop-2-ynyloxy) methyl benzoate (3):** This compound was synthesized as described for 2 on a 130 mmol scale (3,5-dihydroxymethylbenzoate², 21.4 g) in dry DMF (250 mL) in the presence of anhydrous K₂CO₃ (45 g, 330 mmol, 2.5 equiv) and a solution of propargylbromide in toluene (35 mL).
314 mmol, 2.5 equiv). Diallyl ether 3 was obtained as off-white crystals in 81% yield (25.2 g). M.p.: 110 °C; Rf(EtOAc/hexane 4:1 v/v): 0.76; Rf(CH2Cl2/MeOH 98:2 v/v): 0.87; Rf(CH3Cl/MeOH/ACOH 95:20:3 v/v): 0.83; 1H-NMR (CDCl3) δ 2.55 (t (J 2.47 Hz), 2H, CH), 3.91 (s, 3H, OCH3), 4.72 (d (J 2.47 Hz), 4H, -O-CH2), 6.81 (t (J 2.20 Hz), 1H, aron H4), 7.29 (d (J 2.20 Hz), 2H, aron H2/H6), 13C-NMR (CDCl3) δ 52.4, 56.0, 76.0, 77.9, 107.5, 108.8, 132.0, 157.8, 158.4; MS analysis: calcd for C14H22O4 244.24, found ES-MS 244.99 [M + H]+; Elemental analysis: calcd for C14H22O4 C 68.83, H 4.95, found C 68.76, H 4.95.

3-(prop-2-ynloxy) benzoic acid (4): Compound 2 (1.0 g, 5.24 mmol) was dissolved in dioxane/MeOH (50 mL, 14.5 v/v) and 4N NaOH (2 mL, 1.5 equiv) was added in one portion. The obtained reaction mixture was stirred for 5 h at room temperature. Then, the reaction mixture was neutralized by the addition of 1N HCl and the solvents were removed by evaporation. The residue was redissolved in EtOAc (50 mL) and the organic phase was washed with 1N KHSO4 (3 × 20 mL) and brine (3 × 20 mL), dried (Na2SO4) and evaporated in vacuo. The residual solid was obtained in 97% yield (900 mg) and used without further purification in the next synthesis step. M.p.: 126-131 °C; Rf(EtOAc/hexane 7:3 v/v): 0.65; 1H NMR (CDCl3) δ: 2.55 (s, 1H, CH), 4.77 (s, 2H, -O-CH2), 7.25 (m, 1H, aron H2), 7.42 (t, 1H, aron H5), 7.72 (m, 1H, aron H3), 7.77 (d, 1H, aron H1); 13C NMR (CDCl3) δ: 56.0, 76.0, 78.0, 115.6, 121.2, 123.5, 129.6, 130.6, 157.5, 171.9; Elemental analysis: calcd for C10H8O3 C 68.18, H 4.58, found C 67.87, H 4.70.

3,5-bis-(prop-2-ynloxy) benzoic acid (5): Methyl ester 3 (5.66 g, 23.2 mmol) was saponified as described for compound 4. The acid 5 was obtained in 96% yield (5.13 g) and used without further purification in the next synthesis step. M.p.: 171-174 °C; Rf(CH2Cl2/MeOH 9:1 v/v): 0.26; 1H-NMR (DMSO-d6) δ: 3.59 (broad s, 2H, CH), 4.85 (d (J 2.20 Hz), 4H, -O-CH2), 6.86 (t (J 2.47 Hz), 1H, aron 56
H4), 7.17 (d (J 2.47 Hz), 2H, arom H2/H6); 13C-NMR (DMSO-d6) δ: 55.8, 78.6, 78.9, 107.0, 108.4, 132.9, 158.2, 166.8; MS analysis: calculated for C16H12O4 230.22, found ES-MS 231.01 [M + H]+; Elemental analysis: calculated for C16H12O4 C 67.82, H 4.38, found C 67.56, H 4.11.

3.5-bis-(2,3,5-bis(prop-2-nyloxy)benzamido)ethoxy methyl benzoate (7): To a solution of 3,5-bis-(2-tert-butyloxy carbonylamino-ethoxy) methyl benzoate (6; 2.27 g, 5.0 mmol) in CH2Cl2 (25 mL), TFA (25 mL) was added to remove the Boc functionalities. After 1 h of stirring at room temperature, the volatiles were removed by evaporation and the residue was coevaporated with CH2Cl2 to remove any residual TFA. The obtained solid was used without further purification. Acid 5 (2.53 g, 11 mmol, 2.2 equiv) was dissolved in CH2Cl2 (100 mL) and the TFA-salt (dissolved in 50 mL CH2Cl2) followed by DIPEA (3.53 mL, 25 mmol, 5 equiv) were added. Finally, BOP (4.86 g, 11 mmol) was added and the obtained reaction mixture was stirred for 16 h at room temperature. Then, the solvent was removed in vacuo and the residue was redissolved in EtOAc (150 mL) and this solution was subsequently washed with H2O (3 × 75 mL), 1N KHSO4 (3 × 75 mL), H2O (3 × 75 mL), 5% NaHCO3 (3 × 75 mL) and brine (3 × 75 mL), dried (Na2SO4) and evaporated to dryness. The residue was crystallized from MeOH and was obtained as a white solid in 75% yield (2.54 g). M.p.: 113-124 °C; Rf(EtOAc/hexane 4:1 v/v): 0.49; Rf(CH2Cl2/MeOH 98:2 v/v): 0.13; Rf(CHCl3/MeOH/AcOH 95:20:3 v/v/v): 0.80; 1H NMR (DMSO-d6) δ: 3.58 (s, 4H, CH), 3.65 (m, 4H, -CH2-), 3.84 (s, 3H, OCH3), 4.19 (m, 4H, -CH2-), 4.85 (s, 8H, -O-CH2), 6.80 (s, 2H, arom H4'), 6.87 (s, 1H, arom H4), 7.12 (s, 2H, arom H2/H6), (s, 4H, arom H2/H6'), 8.67 (m, 2H, NH amide); 13C-NMR (CDCl3) δ: 40.4, 52.2, 56.9, 57.6, 77.0, 78.8, 106.3, 107.1, 107.6, 109.0 132.9, 137.4, 159.6, 160.3, 167.4, 168.1; MS analysis: calculated for C30H28N2O10 678.22, found ES-MS 679.40 [M + H]+, 701.45 [M + Na]+; MALDI-TOF 679.298 [M + H]+, 701.245 [M + Na]+; Elemental analysis: calculated for C30H28N2O10 C 67.25, H 5.05, N 4.13 found C 66.92, H 5.09, N 4.10.
3,5-bis-(2-(3,5-bis(prop-2-ynyloxy)benzamido)ethoxy benzoic acid (8): Methyl ester 7 (1.36 g, 2 mmol) was saponified as described for compound 4. Acid 8 was obtained as a white powder with nearly quantitative yield (1.33 g). M.p.: 163-168 °C; Rf(CH₂Cl₂/MeOH 9:1 v/v): 0.23; ¹H NMR (DMSO-d₆) δ: 3.58 (s, 4H, CH₂), 3.65 (m, 4H, -CH₂-), 4.19 (m, 4H, -CH₂-), 4.85 (s, 8H, -O-CH₂), 6.80 (s, 2H, arom H4'), 6.83 (s, 1H, arom H4), 7.11 (s, 2H, arom H2/H6), 7.15 (s, 4H, arom H2'/H6'), 8.66 (m, 2H, NH amide); ¹³C-NMR (DMSO-d₆) δ: 38.9, 55.8, 66.3, 78.4, 78.9, 105.0, 105.8, 106.8, 107.8, 132.9, 136.3, 158.2, 159.6, 165.8, 166.9; MS analysis: calculated for C₁₀H₁₉N₂O₁₆ 664.66, found ES-MS 665.75 [M + H]+, 687.60 [M + Na]+; Elemental analysis: calculated for C₁₀H₁₉N₂O₁₆ C 66.86, H 4.85, N 4.21 found C 66.76, H 4.72, N 4.11.

2-terr-butyloxy carbonylamino ethylamine (10): To a solution of 1,2-diaminoethane (13.4 mL, 200 mmol) in dioxane (100 mL) a solution of Boc₂O (5.46 g, 25 mmol) in dioxane (100 mL) was added dropwise over a period of 2 h. After the addition was complete, the obtained reaction mixture was stirred for 16 h at room temperature. Then, the solvent was removed by evaporation and the residue was suspended in H₂O (100 mL) and the white precipitate (bis-substitution product) was removed by filtration. The aqueous solution was extracted with CH₂Cl₂ (3 × 50 mL), and the collected organic layers were washed with brine (1 × 50 mL), dried (MgSO₄) and evaporated to dryness. Compound 10 was obtained as a yellowish oil with 56% yield (2.25 g). Rf(CH₂Cl₂/MeOH 94:6 v/v): 0.24 ¹H NMR (CDCl₃) δ: 1.45 (broad s, 11H, (CH₃), Boc/-NH₂), 2.79 (m, 2H, -CH₂-NH₂), 3.16 (m, 2H, -NH-CH₂-), 5.32 (m, 1H, NH urethane); ¹³C-NMR (CDCl₃) δ 28.2, 41.6, 43.1, 78.9, 156.2; MS analysis: calculated for C₁₀H₁₉N₂O₁₆ 160.12, found ES-MS 161.15 [M + H]+.

H-Asp(O'Bu)-d-Phe-Lys(Boc)-Arg(Pbf)-Gly-O-2-chlorotrityl resin (17): The 2-chlorotrityl chloride resin was treated with SOCl₂/CH₂Cl₂ (1:1 v/v; 2 × 6 mL, 10 min) to convert it completely into the S8
chloride form prior loading of the first amino acid. To remove any residual SOCl₂, the resin was extensively washed with CH₂Cl₂ (6 × 10 mL, 10 min). Then, Fmoc-Gly-OH (430 mg, 1.44 mmol) was dissolved in CH₂Cl₂ (10 mL) and DIPEA (510 µL, 2.88 mmol) followed by the 2-chlorotrityl chloride resin (360 mg (1 mmol/g), 0.36 mmol) were added and the obtained slurry was gently swirled for 2 h at room temperature. The resin was subsequently washed with CH₂Cl₂/MeOH/DIPEA (17:2:1 v/v/v; 3 × 10 mL, 10 min) to cap any remaining linked tritylchloride, followed by CH₂Cl₂ (3 × 10 mL, 2 min), DMF (3 × 10 mL, 2 min) and CH₂Cl₂ (3 × 10 mL, 2 min). The loading of the resin, as calculated from an Fmoc determination, was 64% (0.64 mmol/g). The linear peptide sequence H-Asp(O’Bu)-d-Phe-Lys(Boc)-Arg(Pbf)-Gly-OH was synthesized according to the FastMoc solid phase peptide synthesis protocols⁴ and the final Fmoc-group was removed to obtain peptide resin 17.

cyclo(Arg-Gly-Asp-d-Phe-Lys) (18): Peptide resin 17 was treated twice with HFIP/CH₂Cl₂ (32 mL, 1:4 v/v) for 45 min each to cleave the protected peptide acid from the resin. After this, the resin was washed with CH₂Cl₂ (3 × 20 mL, 10 min) and all fractions were collected and evaporated to dryness. The crude protected peptide acid (MS analysis: calcld for C₄₀H₃₅N₄O₁₃S, 1030.24, found ES-MS 1031.55 [M + H]+) was obtained with 85% yield (202 mg, 0.20 mmol). This crude peptide was dissolved in CH₂Cl₂ (30 mL) and HOBT (30 mg, 0.20 mmol), BOP (41 mg, 0.20 mmol) followed by DIPEA (80 µL, 0.45 mmol, 2.25 equiv) were added and the obtained reaction mixture was stirred for 16 h at room temperature. Subsequently, the solvent was partly removed by evaporation and the oily residue was dissolved in CHCl₃ (50 mL) and washed with 1N KHSO₄ (3 × 20 mL), H₂O (3 × 20 mL) and brine (3 × 20 mL). After the final wash steps, the solvent was removed in vacuo a white solid was obtained. This compound was dissolved in TFA/H₂O/TIS (10 mL; 95:2.5:2.5 v/v/v) and stirred for 3 h to remove the side chain protecting groups. The crude cyclic peptide was isolated by precipitation with MTBE/hexane 1:1 v/v at −20°C. After centrifugation, the pellet was dissolved in tert-BuOH/H₂O 1:1
v/v, lyophilized and subsequently purified by HPLC (C8). Cyclic peptide 18 was obtained in 42% (51 mg) yield. $R_t$: 9.97 min (C4); $R_t$: 15.26 min (CN); MS analysis: calcd for $C_2H_2N_4O_5$, 603.31, found ES-MS 604.60 [M + H]$^+$. 

**General procedure for the microwave-assisted click reaction:** the alkyne (1 equiv) and the azide (1.3 equiv per am) were dissolved in DMF/H$_2$O. To this solution, CuSO$_4$.5H$_2$O (0.05 equiv) and Na-ascorbate (0.50 equiv) were added. The reaction mixture was placed in a microwave reactor and irradiated during 10 – 30 min at 100°C. The cycloaddition was monitored on TLC and LC-MS for completion of the reaction.

**monovalent cyclo[RGDFK] peptide dendrimer (20):** Alkyne 2 (1.3 mg, 6.6 μmol) and azido peptide 19 (5.4 mg, 7.3 μmol, 1.1 equiv) were dissolved in DMF (500 μL) and 0.05 M Na-ascorbate (66 μL, 3.3 μmol, 0.50 equiv) followed by 6 mM CuSO$_4$.5H$_2$O (55 μL, 0.33 μmol, 0.05 equiv) were added. The reaction mixture was placed in the microwave reactor and irradiated for 30 min at 100°C. Then, the solvents were removed under reduced pressure and the residue was dissolved in tert- BuOH/H$_2$O 1:1 v/v and lyophilized and subsequently purified by semi-prep HPLC (C18). Yield: 3.1 mg (57%). $R_t$: 18.9 min (CN); MS analysis: calcd for $C_{18}H_{20}N_4O_6$, 819.366, found ES-MS 820.60 [M + H]$^+$, found MALDI-TOF 820.641 [M + H]$^+$, 842.620 [M + Na]$^+$. 

divalent cyclo[RGDFK] peptide dendrimer (21): For this synthesis, alkyne 3 (0.8 mg, 3.3 μmol) and azido peptide 19 (6.3 mg, 8.5 μmol, 1.3 equiv) were used. After purification by semi-prep HPLC compound 21 was obtained with 40% yield (1.9 mg). $R_t$: 19.0 min (C4); $R_t$: 20.30 min (CN); MS analysis: calcd for $C_{20}H_{16}N_2O_{18}$, 1502.680, found MALDI-TOF 1503.647 [M + H]$^+$. 

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tetravalent cyclo[RGDFK] peptide dendrimer (22): For this synthesis, alkyne 7 (1.10 mg, 1.6 μmol) and azido peptide 19 (6.3 mg, 8.5 μmol, 1.3 equiv) were used. After purification by semi-prep HPLC compound 22 was obtained with 14% yield (0.7 mg). tR: 21.2 min (CN); MS analysis: calc for C_{16}H_{19}N_{2}O_{3}, 3195.435 (M_{obs}), found MALDI-TOF 3196.573 [M + H]^{+}.

References and notes
HPLC Report Medicinal Chemistry

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Peak table for UV-VIS detector
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HPLC Report Medicinal Chemistry

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The molecular structure shown is 24 R = DOTA-Enh

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HPLC Report Medicinal Chemistry

Sample ID: AYR065-1-1
Filename: C:/Users/Anneloes/Data/060419/AYR065-1-1
Method file: C:/Users/Anneloes/Methods/ELSD.met
Detectors: PL-ELSD1000

Peak table for PL-ELSD detector

<table>
<thead>
<tr>
<th>Pk. #</th>
<th>Name</th>
<th>Retention Time</th>
<th>Area</th>
<th>Area Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>16.608</td>
<td>1982319</td>
<td>100.00</td>
</tr>
</tbody>
</table>

Totals | 1982319 | 100.00

einde

Σ15
Kratos PC Axima CFR V2.2.1: Mode reflectron, Power: 45, P.Ext. @ 819 (bin 89)

%Int.  5.0 mV[sum= 1288 mV] Profiles 1-260: (260 Tagged) Smooth Av 2 -Baseline 25

$\text{R}^R \text{O}$

$\text{O}$

$\text{N}^=\text{N}$

$\text{N}$

$\text{H}$

$\text{N}$

$\text{H}$

$\text{D}$

$20 \text{ R} = \text{OMe}$

Mass/Charge: 500 600 700 800 900 1000 1100 1200 1300 1400 1500