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Design of miscellaneous prodrugs of the potent cardiovascular agent N^{ω}-hydroxy-L-arginine: Synthetic approaches and *in vitro* characterization

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A. Summary of available data for the synthesized compounds (Table S1)

	^{1}H	^{13}C	HPLC/ LRMS	HRMS	EA	%*ee*
2b	x	x	x	-	x	-
4a	x	x	-	-	-	-
4b	x	x	-	-	-	-
5	x	x	x	-	x	-
6b	x	x	x	-	-	-
7a	x	x	x	-	x	-
7b	x	x	x	-	x	-
7c	x	-	x	-	-	-
8b	x	x	x	x	x	x
8c	x	x	-	x	x	x
8d	x	x	-	x	x	-
9a	x	x	x	-	x	x
9b	x	x	-	x	x	x
9c	x	x	-	x	-	-
10	x	x	x	x	x	-
11	x	x	x	-	x	-
12	x	x	x	-	-	-
13	x	x	-	x	-	-
18						
19a	x	x	-	-	-	-
19b	x	x	-	-	-	-

B. Synthetic protocols, full characterization of all compounds

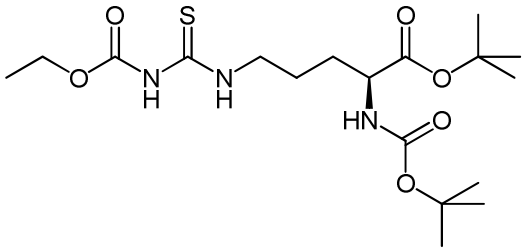
General, Reagents and Material.

Melting points are uncorrected. ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were obtained on a Bruker ARX 300 spectrometer at 300 K. Chemical shifts (δ values) are reported in ppm relative to TMS, 3-(trimethylsilyl)-1-propanesulfonic acid-d_6 sodium salt (TPS) as an internal standard, or alternatively, relative to the residual solvent signal. All coupling constants (*J* values) were obtained by first order analysis of the multiplets. Low resolution mass spectra were recorded on a Bruker-Esquire-LC with an electrospray ionization (ESI). Recordings of high mass resolution spectra were conducted at the Institute of Analytical Chemistry, University of Leipzig, Germany on a Bruker 7.4 Tesla FTICR mass spectrometer BioApex II equipped with an ESI-ion source (Agilent); substances were dissolved in 60% acetonitrile in water and introduced by flow injection analysis. Elemental analyses were performed on a CHNS analysator (HEKAtech GmbH) and were within ±0.4 %. Reactions were monitored by TLC on precoated silica gel plates (SiO_2 60, F_{254}). All compounds could be visualized by ninhydrine spray and heating at 120 °C. Purification of synthesized compounds was performed by column chromatography using silica gel (particle size 40–63 µm). Flash chromatography on a RP-18 Redi*Sep*® column (43 g) was performed with a CombiFlash®RETRIEVE system. All starting materials were commercially available and used without further purification. N^{α}-Boc-L-ornithine *tert*-butylester hydrochloride (**1**) was purchased from Bachem. CbzNCS was prepared according to literature procedures.[1-3] All solvents were distilled and dried according to standard procedures.

N^{α}-(*t*-Butyloxycarbonyl)-N^{ω}-benzyloxycarbonyl-L-thiocitrulline *t*-Butylester (2a)[1, 3]

Has been prepared in analogy to the thiourea **2b** described below. Described in the literature. Yield: 93%.

N^{α}-(*t*-Butyloxycarbonyl)-N^{ω}-ethoxycarbonyl-L-thiocitrulline *t*-Butylester (2b)



N^{α}-(*t*-Butyloxycarbonyl)-L-ornithine-*t*-butylester (2.02 g, 7.0 mmol) are dissolved in 250 mL of dry CH_2Cl_2. The solution is chilled on ice and ethoxycarbonylisothiocyanate (917 mg, 7.0 mmol), dissolved in 15 mL of dry CH_2Cl_2, are added dropwise for about 30 min. The reaction mixture is stirred for 2 h at room temperature, concentrated to one third of the original volume and washed with 25 mL 1 % HCl, water and brine. The organic phase is dried with Na_2SO_4 and concentrated in vacuo. Thiourea 2b is at this point already pure by >95 % (TLC). The product was dissolved in a small amount of eluent (Cy/EtOAc, 4:1), charcoal added and purified on a short silica gel column (ca. 20 g SiO_2). Yield: 2.76 g of a colorless oil (94 %) that solidifies upon standing in the refridgerator; Mp. 81 °C; TLC: R_f = 0.31 (Cy/EtOAc, 4:1).

^{1}H-NMR ($CDCl_3$):

δ/ppm = 1.31 (t, 3H, ^{3}J = 7.1 Hz, CH_2-C<u>H</u>$_3$), 1.45, 1.47 (2 × s, 9H, C(CH_3)$_3$), 1.61-1.90 (m, 4H, β,γ-CH_2), 3.66 (pseudo q, 2H, N-CH_2), 4.22 (q, 2H, ^{3}J = 7.1 Hz, C<u>H</u>$_2$-CH_3), 5.08 (m, 1H, α-CH), 8.06, 9.70 (2 × br s, 1H, NH).

^{13}C-NMR ($CDCl_3$):

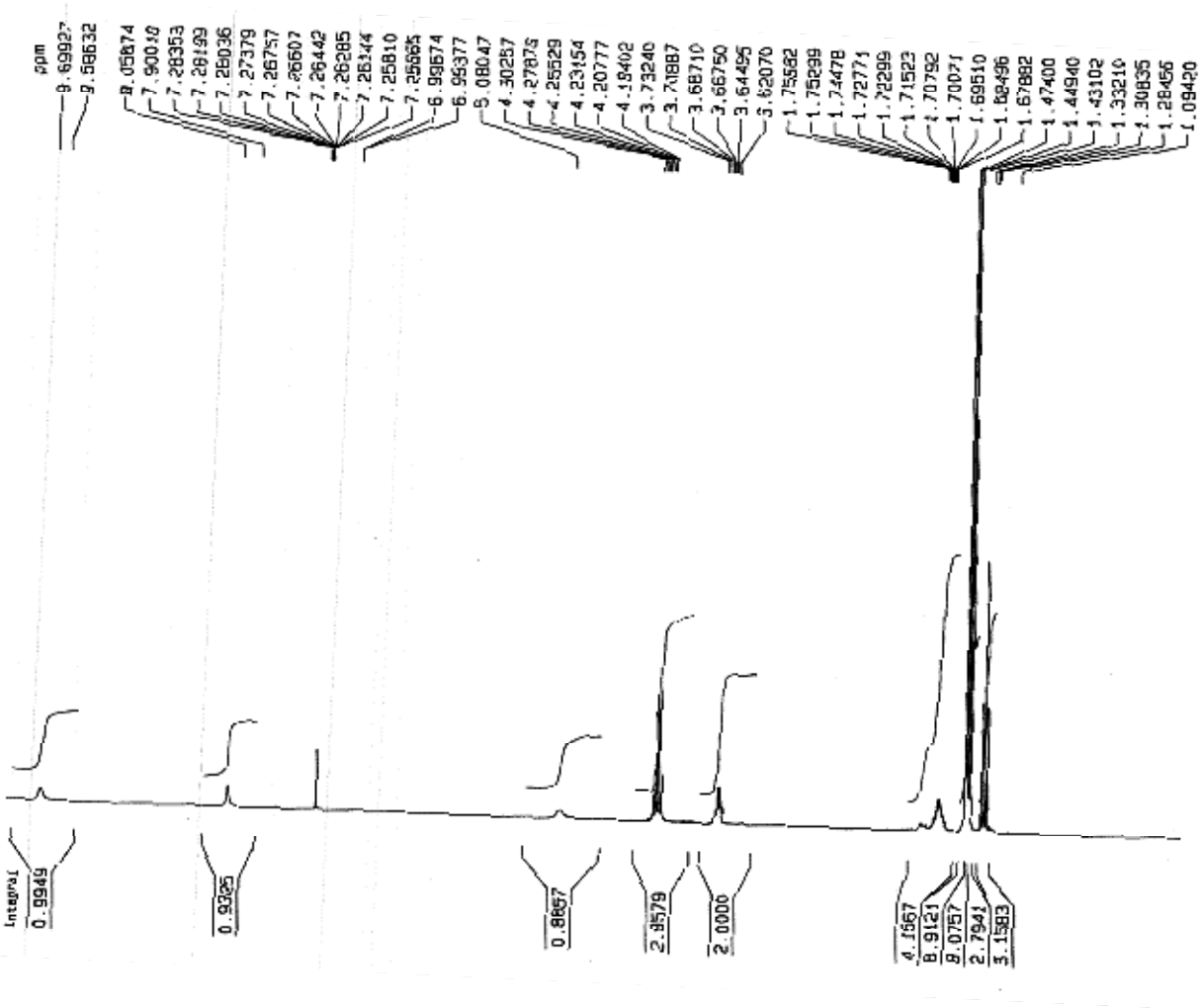
δ/ppm = 14.9 (CH_2-<u>C</u>H_3), 24.9 (γ-CH_2), 28.7, 29.0 (2 × C(<u>C</u>H_3)$_3$), 31.0 (β-CH_2), 45.8 (N-CH_2), 54.3 (α-CH), 63.4 (O-CH_2), 82.8 (<u>C</u>(CH_3)$_3$), 153.4 (CO-Eoc), 156.2 (CO-Boc), 172.5 (COO^{t}Bu), 180.1 (C=S).

MS (ESI):

m/z = 442 $[M + Na]^+$, 420 $[M + H]^+$, 308 $[M – 2 \times C_4H_8 + H]^+$, 264 $[M - 2 \times C_4H_8 - CO_2 + H]^+$.

$C_{18}H_{33}N_3O_6S$ (419.54)

Calc.	C 51.53	H 7.93	N 10.02	S 7.64
Found	C 51.68	H 7.97	N 10.06	S 7.62



^{1}H NMR ($CDCl_3$, 300 MHz) of N^{α}-(*t*-Butyloxycarbonyl)-N^{ω}-ethoxycarbonyl-L-thiocitrullin-*t*-butylester (**2b**).

Aminooxyacetic acid Methylester (4a)[4-6]

A solution of 3.0 g of aminooxyacetic acid semihydrochloride (27.4 mmol) in 22 mL of the MeOH (HPLC quality) is chilled to 0 °C. HCl gas is bubbled through this solution and left at room temperature for 24 h. The mixture is concentrated in a vacuum, chilled to 0 °C and 10 mL of ice-cold water added. The aqueous solution is brought to pH 8-9 with sodium carbonate and extracted twice with diethyl ether. The combined organic phases are dried (Na_2SO_4) and the crude product purified by distillation to yield a colorless liquid. Yield: 2.0 g (70 %).

^{1}H-NMR (DMSO-d_6):

δ/ppm = 3.66 (s, 3H, O-CH_3), 4.16 (s, 2H, O-CH_2), 6.30 (br s, 2H, NH_2).

^{13}C-NMR (DMSO-d_6):

δ/ppm = 51.2 (O-CH_3), 72.0 (O-CH_2), 170.8 (CO).

Aminooxyacetic acid Ethylester (4b)[6, 7]

This compound has been prepared in analogy to the methylester **4a**.

Yield: 2.5 g of a colorless liquid (76 %).

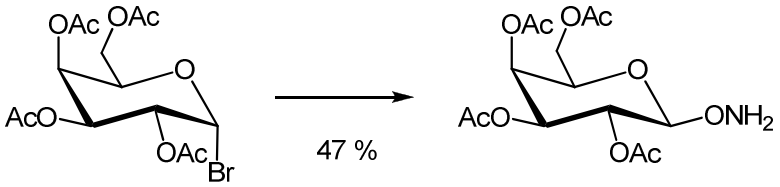
^{1}H-NMR (DMSO-d_6):

δ/ppm = 1.21 (t, ^3J = 7.1 Hz, 3H, CH_2-C$\underline{H}_3$), 4.13 (q, ^3J = 7.1 Hz, 2H, C$\underline{H}_2$-CH_3), 4.14 (s, 2H, O-CH_2), 6.29 (br s, 2H, NH_2).

^{13}C-NMR (DMSO-d_6):

δ/ppm = 14.0 (CH_2-$\underline{C}H_3$), 59.9 ($\underline{C}H_2$-CH_3), 72.1 (O-CH_2), 170.3 (CO).

1-Aminooxy-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranose (5) [8,9, 10]



The protected precursor of **5** (*N*-[(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranos-1-yl)oxy]phthalimide) was prepared according to modified literature procedures.[8, 9]

To a solution of 1.85 g of 1-bromo-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranose (4.5 mmol), 3.68 g *N*-hydroxyphthalimide (22.6 mmol) and 1.52 g tetrabutylammonium hydrogensulfate (4.5 mmol) in 36 mL of CH_2Cl_2 are added 36 mL of 1 M NaOH (water) and stirred for 4 h at room temperature. 40 mL of CH_2Cl_2 are added and the organic phase washed 6 times with water and twice with brine (20 mL each). The organic phase is dried with Na_2SO_4 and concentrated in a vacuum. Further purification was carried out by two chromatographic work ups on silica gel. The first column was short, using max. 50 g of SiO_2 and Cy/EtOAc(1:3, R_f = 0.73) as the eluent. The second column consisted of ca. 100 g of SiO_2, using toluene/EtOAc (1:1) as the eluent. Yield: 1.55 g of a white crystalline foam (70 %) (lit. 80 %[9], 55 %[8]); TLC: R_f = 0.54 (toluene/EtOAc, 1:1).

^{1}H-NMR ($CDCl_3$):

δ/ppm = 1.97, 1.99, 2.17, 2.20 (4 × s, 3H, $COCH_3$), 3.93 (td, ^3J = 6.8, 1.0 Hz, 1H, 5'-CH), 4.19 (m, 2H, 6'-CH_2), 4.99 (d, ^3J = 8.2 Hz, 1H, 1'-CH), 5.11 (dd, ^3J = 10.4, 3.4 Hz, 1H, 3'-CH), 5.40 (dd, ^3J = 3.4, 1.0 Hz, 1H, 4'-CH), 5.45 (dd, ^3J = 10.4, 8.2 Hz, 1H, 2'-CH), 7.75-7.86 (m, 4H, ArH).

^{13}C-NMR ($CDCl_3$):

δ/ppm = 21.16, 21.18, 21.3, 21.4 (CO$\underline{C}H_3$), 61.5 (6'-CH_2), 67.0, 67.7, 71.3, 72.1 (2',3',4',5'-CH), 106.9 (1'-CH), 124.5 (4,5-CH), 129.4 (2a,6a-C), 135.4 (3,6-CH), 163.2 (2 × CO), 170.4, 170.6, 170.86, 170.89 ($\underline{C}OCH_3$).

MS (ESI):

m/z = 516 $[M + Na]^+$, 511 $[M + NH_4]^+$, 331 $[C_{14}H_{19}O_9]^+$.

$C_{22}H_{23}NO_{12}$ (493.42)

Calc.	C 53.55	H 4.70	N 2.84
Found	C 53.94	H 4.81	N 2.99

Deprotection of this phthalimide-precursor was carried out according to a modified protocol of Renaudet et al.:[9]

A solution of 1.43 g of the phthalimide-protected precursor (2.90 mmol) are dissolved in 28 mL of CH_2Cl_2/MeOH (1:1). To this solution 458 µL of methylhydrazine (8.70 mmol) are added followed by 25 min stirring at room temperature. The reaction is quenched by addition of 20 mL of a saturated $NaHCO_3$ solution. The aqueous phase is extracted 3 times with 30 mL CH_2Cl_2. The combined organic phases are washed with brine, dried with Na_2SO_4 and concentrated on a rotary evaporator. Further purification was performed by flash chromatography on SiO2 using cyclohexane/EtOAc (1:3) as the eluent. Yield: 1.94 g of a colorless foam (67 %); TLC: R_f = 0,43 (Cy/EtOAc, 1:3).

^{1}H-NMR ($CDCl_3$):

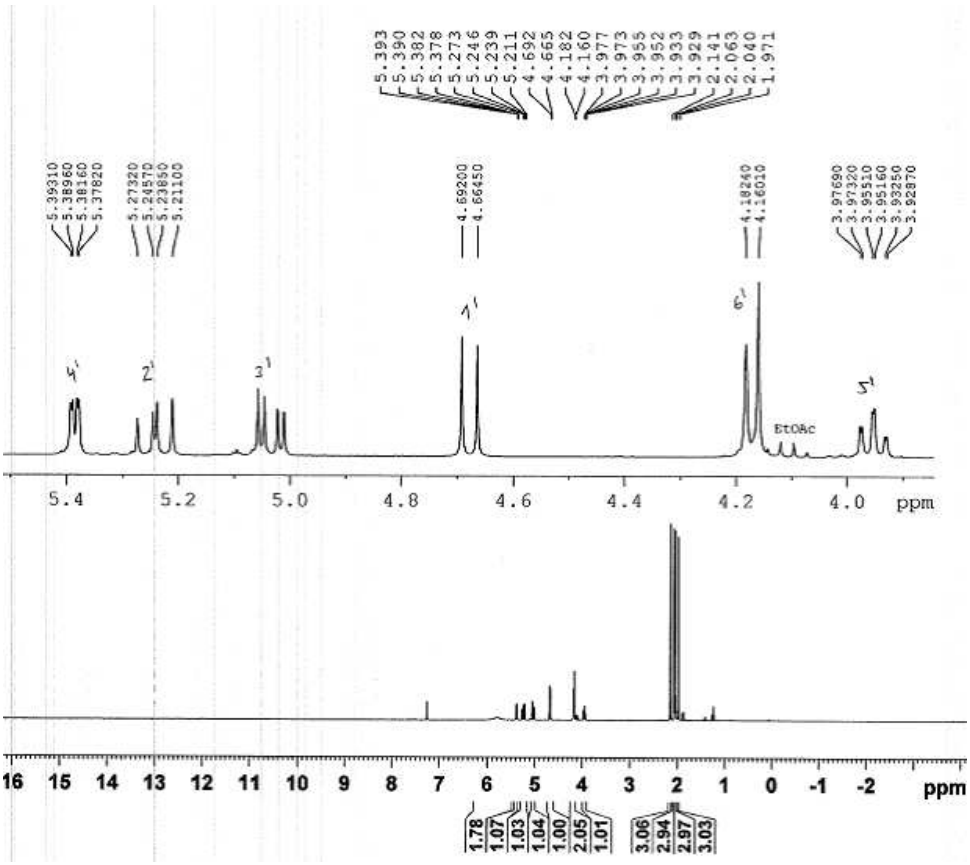
δ/ppm = 1.97, 2.04, 2.06, 2.14 (4 × s, 3H, $COCH_3$), 3.95 (td, ^3J = 6.7, 1.1 Hz, 1H, 5'-CH), 4.17 (d, ^3J = 6.7 Hz, 2H, 6'-CH_2), 4.68 (d, ^3J = 8.3 Hz, 1H, 1'-CH), 5.03 (dd, ^3J = 10.4, 3.4 Hz, 1H, 3'-CH), 5.24 (dd, ^3J = 10.4, 8.3 Hz, 1H, 2'-CH), 5.38 (dd, ^3J = 3.4, 1.0 Hz, 1H, 4'-CH), 5.74 (br s, 2H, NH_2).

^{13}C-NMR ($CDCl_3$):

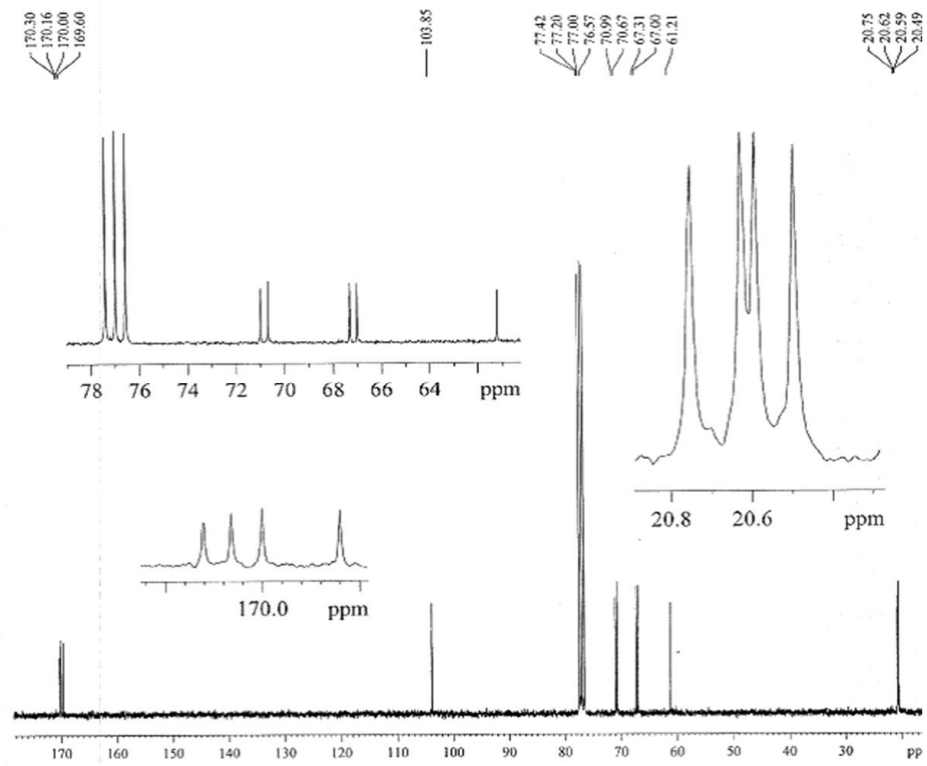
δ/ppm = 20.7, 20.75, 20.79, 20.9 (4 × $CO\underline{C}H_3$), 61.4 (6'-CH_2), 67.2, 67.5, 70.8, 71.2 (2',3',4',5'-CH), 104.0 (1'-CH), 169.8, 170.2, 170.3, 170.5 ($\underline{C}OCH_3$).

MS (ESI):

m/z = 726 $[2 \times M]^+$, 386 $[M + Na]^+$, 364 $[M + H]^+$, 331 $[C_{14}H_{19}O_9]^+$.



^{1}H NMR ($CDCl_3$, 300 MHz) of 1-Aminooxy-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranose (**5**)



^{13}C NMR ($CDCl_3$, 75 MHz) of 1-Aminooxy-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranose (**5**)

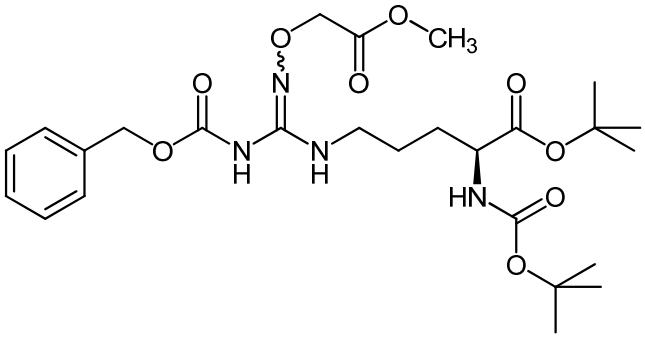
General procedure for the synthesis of fully protected NOHA derivatives 6 and 7:

A literature protocol for the preparation of carbamoylguanidines **6** and **7** was used for the herein presented protected NOHA derivatives.[1-3] 1.5 Equivalents of DIPEA, *O*-substituted hydroxylamine (**3-5**) and EDCI were reacted with 0.5 mmol thiourea **2a,b** in 10 mL of dry CH_2Cl_2. For hydroxylamines that were applied as hydrochlorides (= methoxylamine hydrochloride, **3**) 3 equivalents of DIPEA were used. Unless noted otherwise, reactions were complete after stirring overnight. The organic phase was diluted with 10 mL of CH_2Cl_2 and washed with small amounts of 1 % aqueous HCl, water and brine. The resulting oils were purified by column chromatography on silica gel.

***N*$^{\omega}$-Benzyloxycarbonyl-*N*$^{\alpha}$-*tert*-butyloxycarbonyl-*N*$^{\omega'}$-methoxy-L-arginine *tert*-Butylester (6a)[3]**

Described in the literature. Yield: 95%.

***N*$^{\omega}$-Benzyloxycarbonyl-*N*$^{\alpha}$-(*t*-butyloxycarbonyl)-*N*$^{\omega'}$-(methoxycarbonyl)methoxy-L-arginine *t*-Butylester (6b)**



Eluent: Cy/EtOAc (3:2), R_f = 0.48; yield: 254 mg (92 %) of a colorless oil.

^{1}H-NMR ($CDCl_3$):

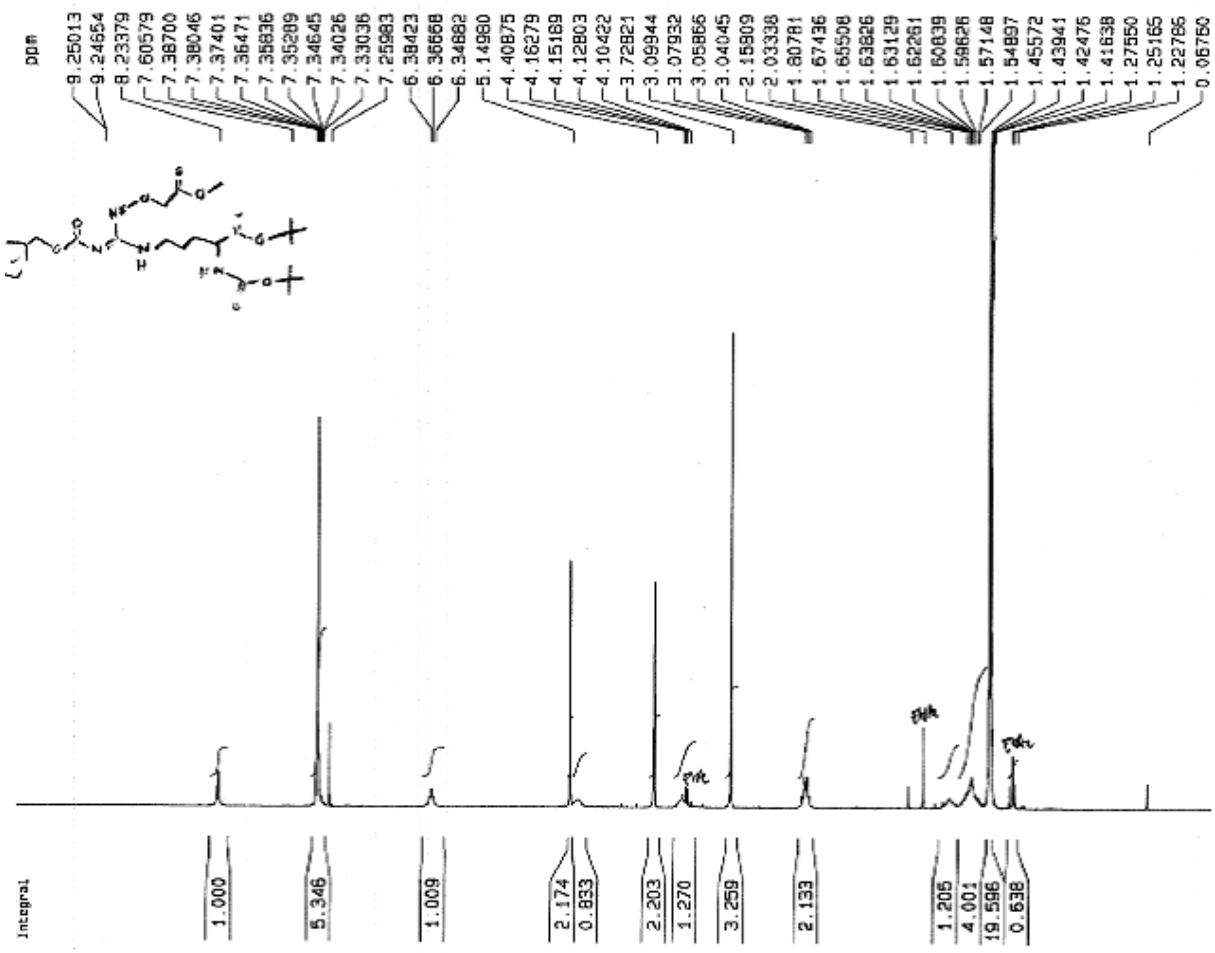
δ/ppm = 1.44, 1.46 (2 × s, 9H, $C(CH_3)_3$), 1.50-1.87 (m, 4H, β,γ-CH_2), 3.07 (m, 2H, N-CH_2), 3.73 (s, 3H, O-CH_3), 4.16 (m, 1H, α-CH), 4.41 (s, 2H, O-CH_2), 5.08 (m, 1H, NH), 5.15 (s, 2H, CH_2-Cbz), 6.37 (br t, ^3J = 5.3 Hz, 1H, NH), 7.30-7.39 (m, 5H, ArH), 8.23 (br s, 1H, NH).

^{13}C-NMR ($CDCl_3$):

δ/ppm = 25.5 (γ-CH_2), 28.7 (β-CH_2), 29.0, 30.9 (2 × C($\underline{C}H_3)_3$), 41.2 (N-CH_2), 52.4 (O-CH_3), 54.5 (α-CH), 68.3 (CH_2-Cbz), 71.2 (O-CH_2), 80.3, 82.5 (2 × $\underline{C}(CH_3)_3$), 129.0, 129.28, 129.34 (ArCH), 135.9 (ArC), 150.8 (C=N), 153.7 (CO-Cbz), 156.0 (CO-Boc), 171.7, 172.4 (COO^{t}Bu, COOMe).

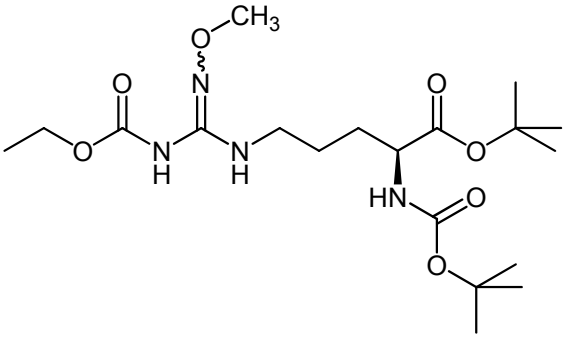
MS (ESI):

m/z = 575 $[M + Na]^+$, 553 $[M + H]^+$, 497 $[M – C_4H_8 + H]^+$.



^{1}H NMR ($CDCl_3$, 300 MHz) of N^{ω}-Benzyloxycarbonyl-N^{α}-(*t*-butyloxycarbonyl)-$N^{\omega'}$-(methoxycarbonyl)methoxy-L-arginine *t*-Butylester (**6b**)

N^{α}-(*t*-Butyloxycarbonyl)-N^{ω}-ethoxycarbonyl-$N^{\omega'}$-methoxy-L-arginine *t*-Butylester (7a)



Eluent: CH_2Cl_2/MeOH (98:2), R_f = 0.26; yield: 203 mg (94 %) of a colorless oil.

^{1}H-NMR ($CDCl_3$):

δ/ppm = 1.27 (t, ^3J = 7.1 Hz, 3H, CH_2-C<u>H</u>$_3$), 1.43, 1.45 (2 × s, 9H, $C(CH_3)_3$), 1.56-1.89 (m, 4H, β,γ-CH_2), 3.09 (m, 2H, N-CH_2), 3.66 (s, 3H, O-CH_3), 4.16 (br q, ^3J = 7.1 Hz, 3H, C<u>H</u>$_2$-CH_3, α-CH), 5.10, 6.26 (2 × br m, 1H, NH), 7.80 (br s, 1H, NH).

^{13}C-NMR ($CDCl_3$):

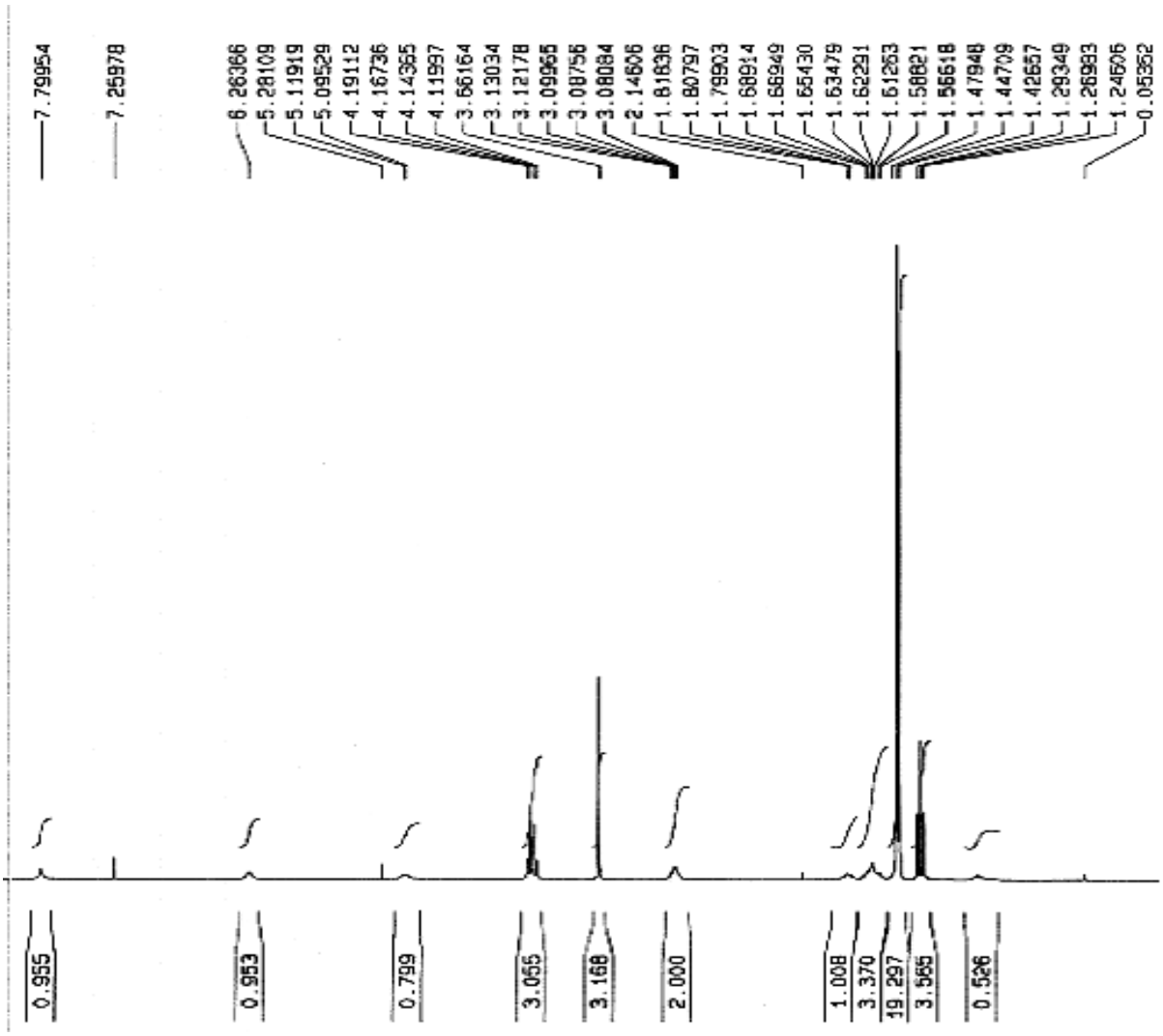
δ/ppm = 14.9 (CH_2-<u>C</u>H$_3$), 25.6 (γ-CH_2), 29.7 (β-CH_2), 29.0, 30.9 (2 × C(<u>C</u>H$_3$)$_3$), 41.2 (N-CH_2), 54.5 (α-CH), 62.0 (<u>C</u>H$_2$-CH_3), 62.6 (O-CH_3), 80.2, 82.5, (2 × <u>C</u>(CH$_3$)$_3$), 149.0 (C=N), 153.8 (CO-Eoc), 156.0 (CO-Boc), 172.4 (COO^{t}Bu).

MS (ESI):

m/z = 455 $[M + Na]^+$, 433 $[M + H]^+$, 377 $[M – C_4H_8 + H]^+$.

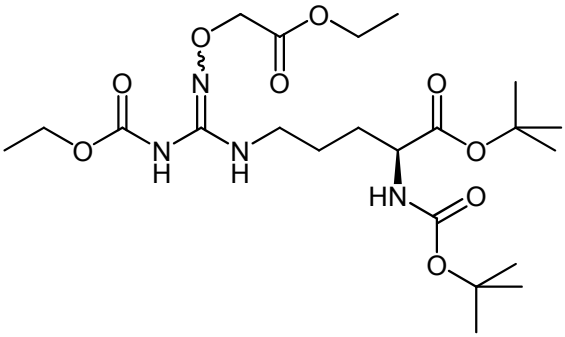
$C_{19}H_{36}N_4O_7$ (432.52)

Calc.	C 52.76	H 8.39	N 12.95
Found	C 53.65	H 8.57	N 13.24



^{1}H NMR ($CDCl_3$, 300 MHz) of N^{α}-(*t*-Butyloxycarbonyl)-N^{ω}-ethoxycarbonyl-$N^{\omega'}$-methoxy-L-arginine *t*-Butylester (**7a**)

N^{α}-(*t*-Butyloxycarbonyl)-N^{ω}-ethoxycarbonyl-$N^{\omega'}$-(ethoxycarbonyl)methoxy-L-arginine *t*-Butylester (7b)



Eluent: Cy/EtOAc (3:2), R_f = 0.51; yield: 250 mg (99 %) of a colorless oil.

^{1}H-NMR ($CDCl_3$):

δ/ppm = 1.26 (t, ^3J = 7.1 Hz, 3H, CH_2-C$\underline{H}_3$), 1.27 (t, ^3J = 7.1 Hz, 3H, CH_2-C$\underline{H}_3$), 1.42, 1.44 (2 × s, 9H, $C(CH_3)_3$), 1.51-1.85 (m, 4H, β,γ-CH_2), 3.06 (m, 2H, N-CH_2), 4.16 (q, ^3J = 7.11 Hz, 2H, C$\underline{H}_2$-CH_3), 4.19 (q, ^3J = 7.17 Hz, 2H, C$\underline{H}_2$-CH_3), 4.39 (s, 2H, O-CH_2), 5.08 (m, 1H, NH), 6.40 (br t, 1H, NH), 8.19 (br s, 1H, NH).

^{13}C-NMR ($CDCl_3$):

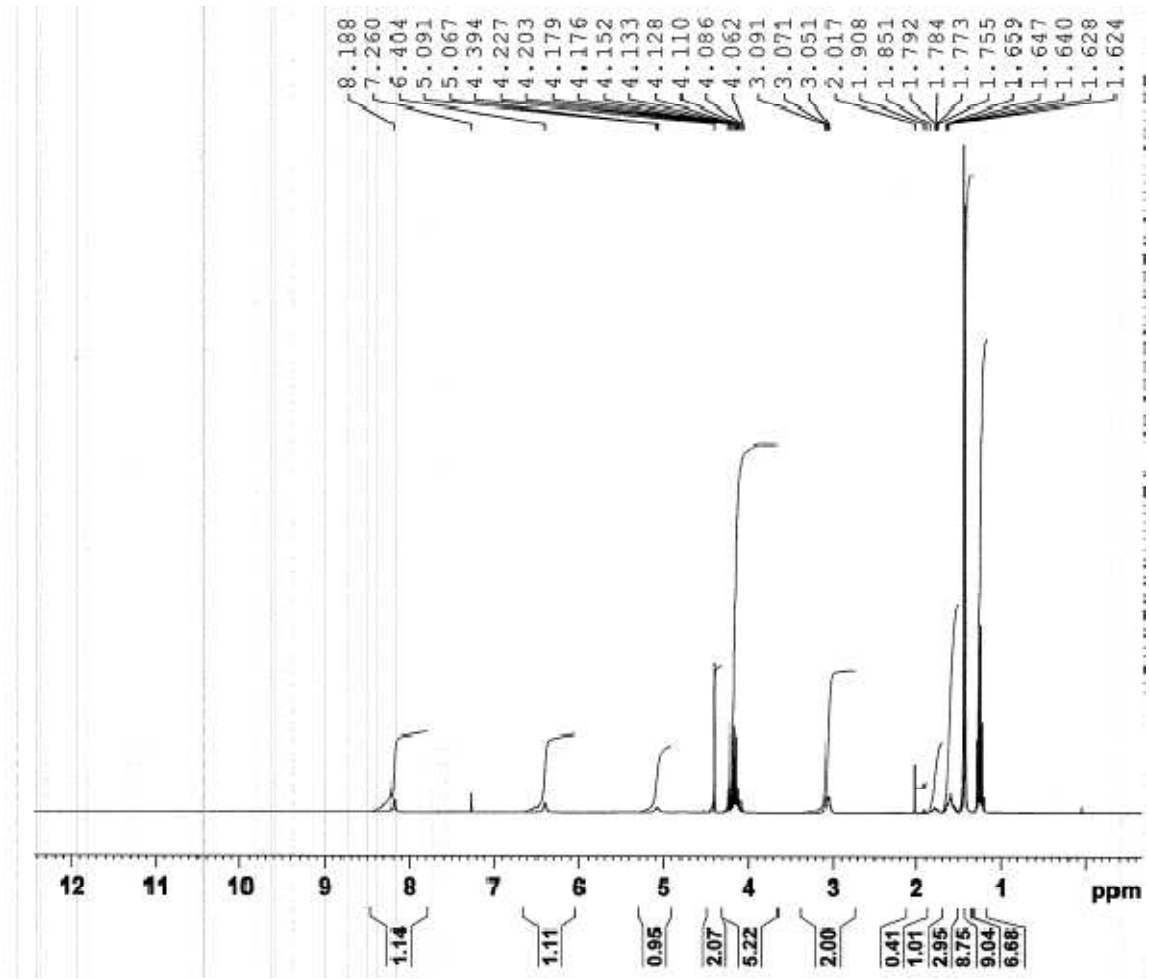
δ/ppm = 14.1, 14.2 (2 × CH_2-$\underline{C}H_3$), 24.8 (γ-CH_2), 27.9, 28.3 (2 × $C(\underline{C}H_3)_3$), 30.2 (β-CH_2), 40.5 (N-CH_2), 53.7 (α-CH), 60.8, 61.9 (2 × $\underline{C}H_2$-CH_3), 70.7 (O-CH_2), 79.5, 81.8 (2 × $\underline{C}(CH_3)_3$), 150.4 (C=N), 153.2 (CO-Eoc), 155.3 (CO-Boc), 170.7, 171.7 (COOEt, COO^{t}Bu).

MS (ESI):

m/z = 527 $[M + Na]^+$, 505 $[M + H]^+$, 449 $[M – C_4H_8]^+$.

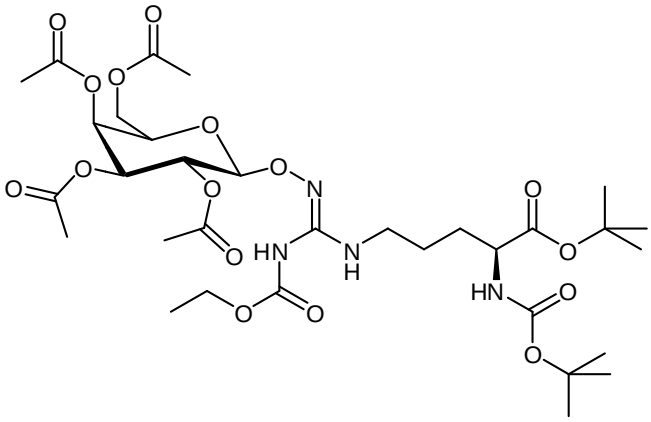
$C_{22}H_{40}N_4O_9$ (504.59)

Calc.	C 52.37	H 7.99	N 11.10
Found	C 53.09	H 7.90	N 11.44



^{1}H NMR ($CDCl_3$, 300 MHz) of N^{α}-(*t*-Butyloxycarbonyl)-N^{ω}-ethoxycarbonyl-$N^{\omega'}$-(ethoxycarbonyl)methoxy-L-arginine *t*-Butylester (**7b**)

N^{α}-(*t*-Butyloxycarbonyl)-N^{ω}-ethoxycarbonyl-$N^{\omega'}$-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranos-1-yl)oxy-L-arginine *t*-Butylester (7c)



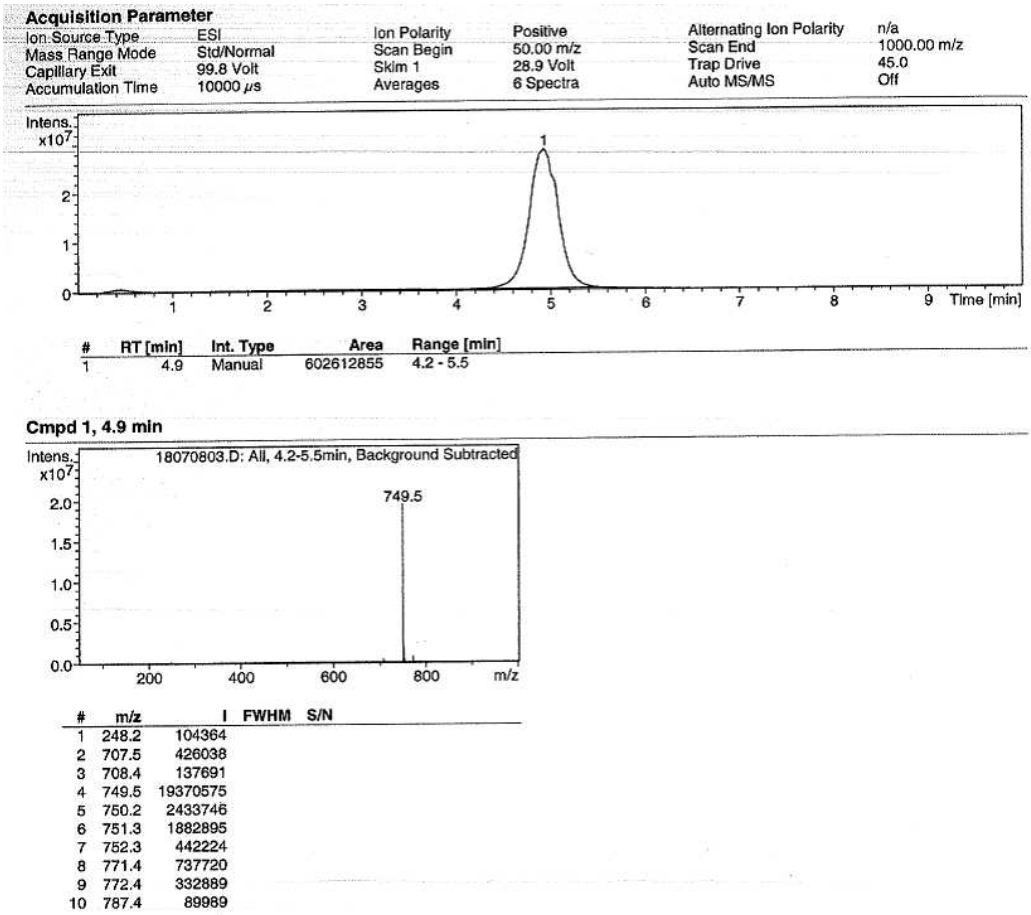
Eluent: CH_2Cl_2/MeOH (97:3), R_f = 0.35; yield: 262 mg (70 %) of a colorless oil.

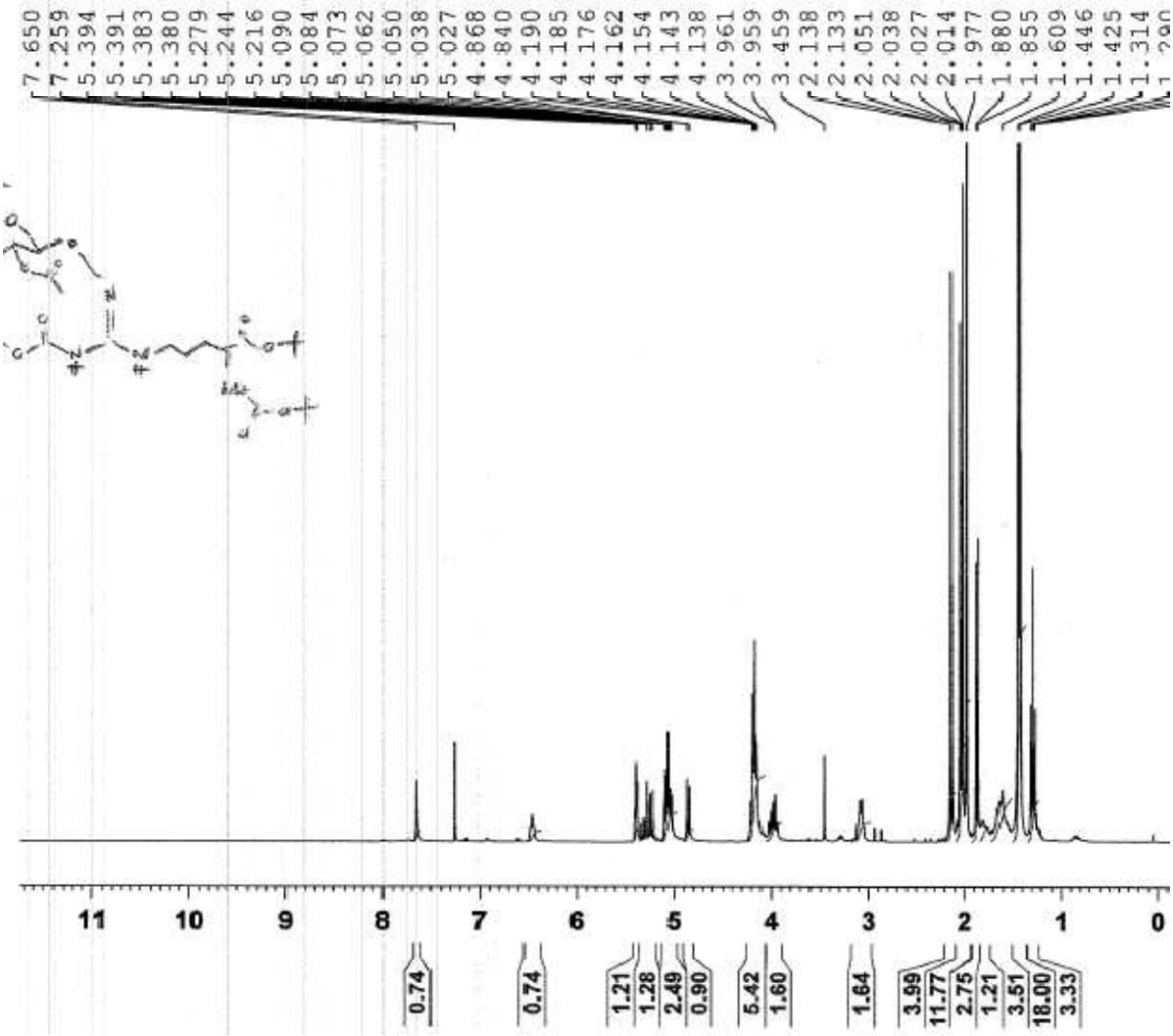
^{1}H-NMR ($CDCl_3$):

δ/ppm = 1.29 (t, ^3J = 7.1 Hz, 3H, CH_2-C<u>H</u>$_3$), 1.43, 1.45 (2 × s, 9H, $C(CH_3)_3$), 1.52-1.86 (m, 4H, β,γ-CH_2), 1.98, 2.02, 2.05, 2.13 (4 × $COCH_3$), 3.08 (m, 2H, N-CH_2), 3.96 (br t, ^3J = 6.8 Hz, 1H, 5'-CH), 4.11-4.21 (m, 5H, α-CH, C<u>H</u>$_2$-CH_3, 3'-CH, NH), 4.85 (d, ^3J = 8.3 Hz, 1H, 1'-CH), 5.06 (m, 2H, 6'-CH_2), 5.25 (dd, ^3J = 10.4, 8.3 Hz, 1H, 2'-CH), 5.39 (dd, ^3J = 3.5, 1.0 Hz, 1H, 4'-CH), 6.47 (br t, 1H, NH), 7.65 (br s, 1H, NH).

MS (ESI):

m/z = 772 $[M + Na]^+$, 750 $[M + H]^+$, 707 $[M – C_2H_2O + H]^+$.



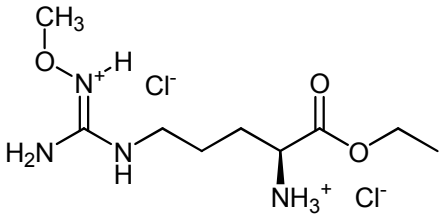


^{1}H NMR ($CDCl_3$, 300 MHz) of N^{α}-(*t*-Butyloxycarbonyl)-N^{ω}-ethoxycarbonyl-$N^{\omega'}$-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranos-1-yl)oxy-L-arginine *t*-Butylester (**7c**)

N^{ω}-Methoxy-L-arginine Bis(trifluoroacetate) (8a)[3]

Described in the literature. Yield: 99%.

N^{ω}-Methoxy-L-arginine Ethylester Dihydrochloride (8b)



For esterification 238 mg of the free amino acid precursor **8a** (0.55 mmol) were dissolved in 5 mL of absolute ethanol under an argon atmosphere. This solution was stirred for 30 min at -10 °C before gently bubbling HCl gas through for 5-10 min. For completion of the reaction, it is stirred for one hour at 0 °C and left in the refridgerator for 36 h. The mixture is carefully concentrated under reduced pressure and lyophilized to obtain a hygroscopic amorphous solid, that liquifies upon contact with air.

Yield: 168 mg (99 %) of a colorless oil; R_f = 0.18 (*i*-propanol/H_2O/AcOH, 8:1:1)

^{1}H-NMR (DMSO-d_6):

δ/ppm = 1.24 (t, ^3J = 7.2 Hz, 3H, CH_2-C$\underline{H}_3$), 1.47-1.90 (m, 4H, β,γ-CH_2), 3.22 (m, 2H, N-CH_2), 3.64 (s, 3H, O-CH_3), 3.99 (m, 1H, α-CH), 4.21 (q, ^3J = 7.2 Hz, 2H, C$\underline{H}_2$-CH_3), 8.04 (br s, 2H, NH_2), 8.31 (br t, 1H, NH), 8.72 (br s, 3H, NH_3^+), 11.33 (br s, 1H, NH^+).

^{13}C-NMR (DMSO-d_6):

δ/ppm = 13.9 (CH_2-$\underline{C}H_3$), 24.0 (γ-CH_2), 27.0 (β-CH_2), 40.0 (N-CH_2), 51.4 (α-CH), 61.7 ($\underline{C}H_2$-CH_3), 64.4 (O-CH_3), 157.2 (C=N), 169.2 (CO).

HRMS (m/z):

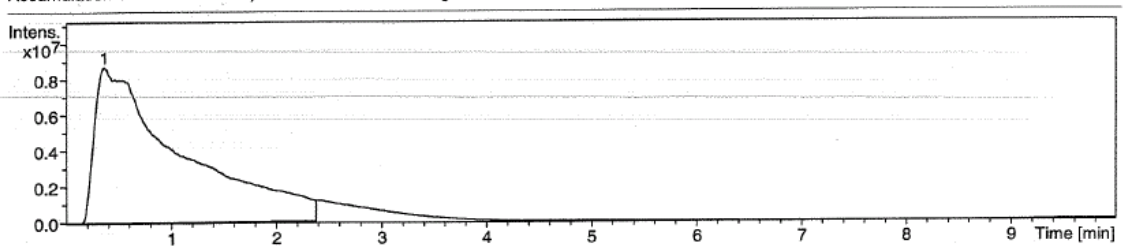
Calculated for $C_9H_{21}N_4O_3$ $[M + H]^+$ = 233.16082, found: 233.16064.

$C_9H_{20}N_4O_3 \cdot 2.0\ HCl \cdot 0.7\ H_2O$ (317.82)

Calc.	C 34.01	H 7.42	N 17.63
Found	C 33.65	H 7.66	N 18.20

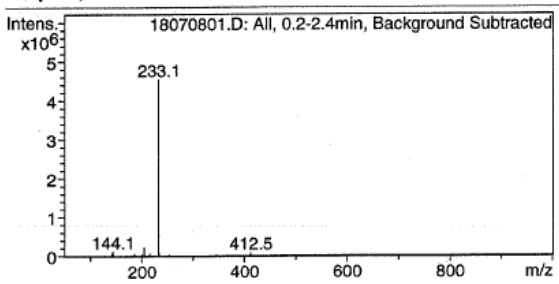
Acquisition Parameter

Ion Source Type	ESI	Ion Polarity	Positive	Alternating Ion Polarity	n/a
Mass Range Mode	Std/Normal	Scan Begin	50.00 m/z	Scan End	1000.00 m/z
Capillary Exit	84.4 Volt	Skim 1	17.0 Volt	Trap Drive	28.1
Accumulation Time	10000 µs	Averages	6 Spectra	Auto MS/MS	Off

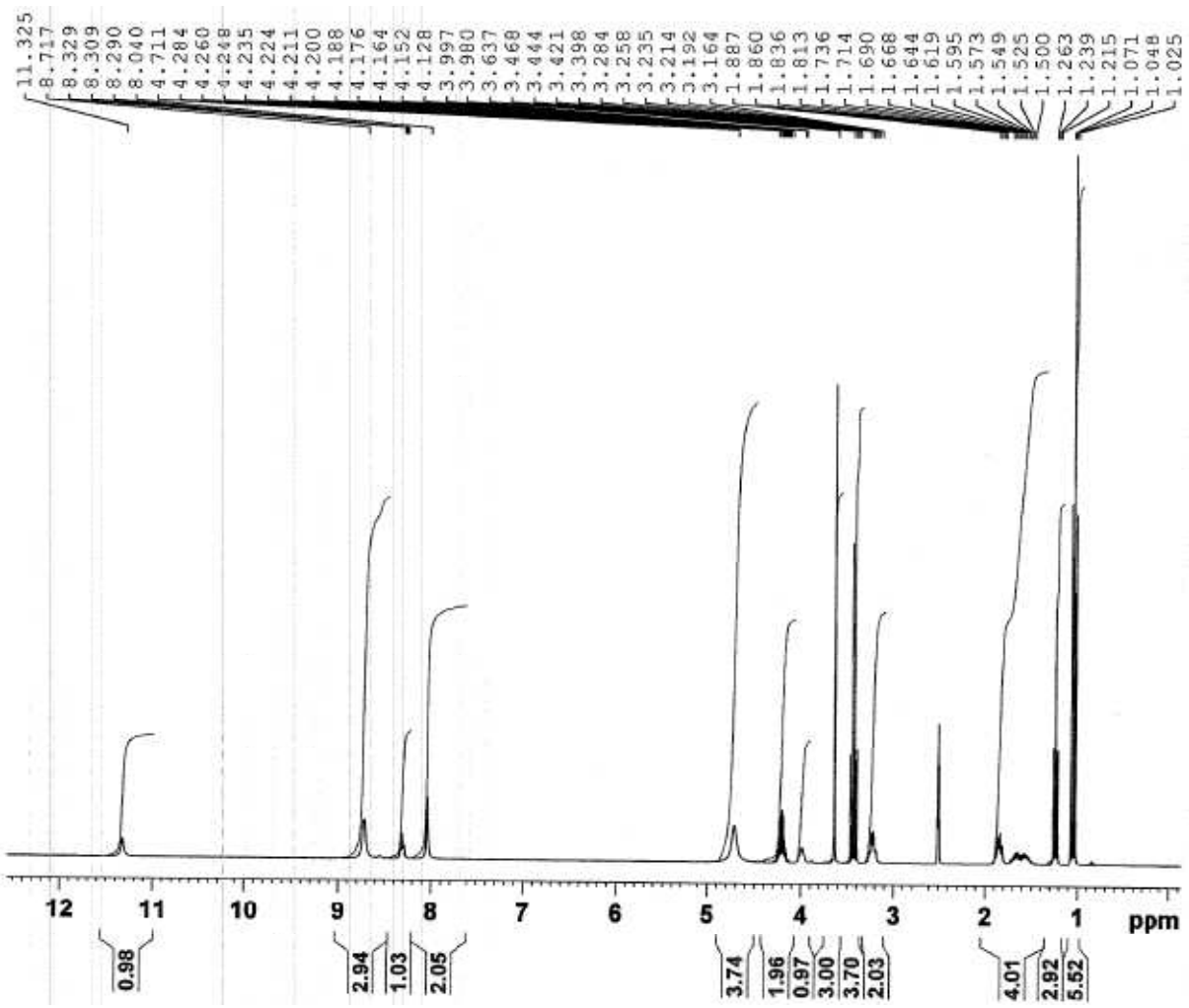


#	RT [min]	Int. Type	Area	Range [min]
1	0.4	Manual	495375719	0.2 - 2.4

Cmpd 1, 0.4 min

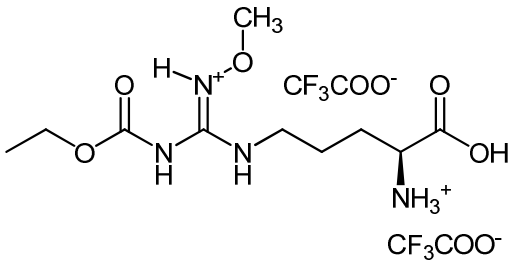


#	m/z	I	FWHM	S/N
1	142.1	53519		
2	144.1	110014		
3	186.1	51493		
4	201.2	38296		
5	205.2	215323		
6	216.2	53739		
7	233.1	4524705		
8	233.9	602562		
9	235.1	66294		
10	412.5	80645		



^{1}H NMR (DMSO-d_6, 300 MHz) of N^{ω}-Methoxy-L-arginine Ethylester Dihydrochloride (**8b**). NMR sample contained residual amounts of EtOH.

N^{ω}-Ethoxycarbonyl-$N^{\omega'}$-methoxy-L-arginine Bis(trifluoroacetate) (8c)



200 mg of the fully protected precursor **7a** (0,46 mmol) are dissolved in 5 mL of TFA at 0 °C, stirred at that temperature for 30 min and then 3 h at room temperature. TFA is removed in a vacuum (not exceeding 20 °C) and the residue is taken up in a minimum amount of water. The crude product is purified by flash chromatography on a RP-18 column (0.1 % TFA in water). Ninhydrin-positive fractions are combined and concentrated on a rotary evaporator (not exceeding 30 °C) to a volume of ca. 10 mL, and were finally lyophilized.

Yield: 225 mg (97 %) of a colorless oil; R_f = 0.44 (*i*-propanol/H_2O/AcOH, 8:1:1)

^{1}H-NMR (DMSO-d_6):

δ/ppm = 1.22 (t, ^3J = 7.1 Hz, 3H, CH_2-C<u>H</u>$_3$), 1.50-1.87 (m, 4H, β,γ-CH_2), 3.12 (br t, 2H, N-CH_2), 3.62 (s, 3H, O-CH_3), 3.90 (m, 1H, α-CH), 4.13 (q, ^3J = 7.1 Hz, 2H, C<u>H</u>$_2$-CH_3), 7.35 (br s, 1H, NH), 8.28 (br s, 3H, NH_3^+).

^{13}C-NMR (DMSO-d_6):

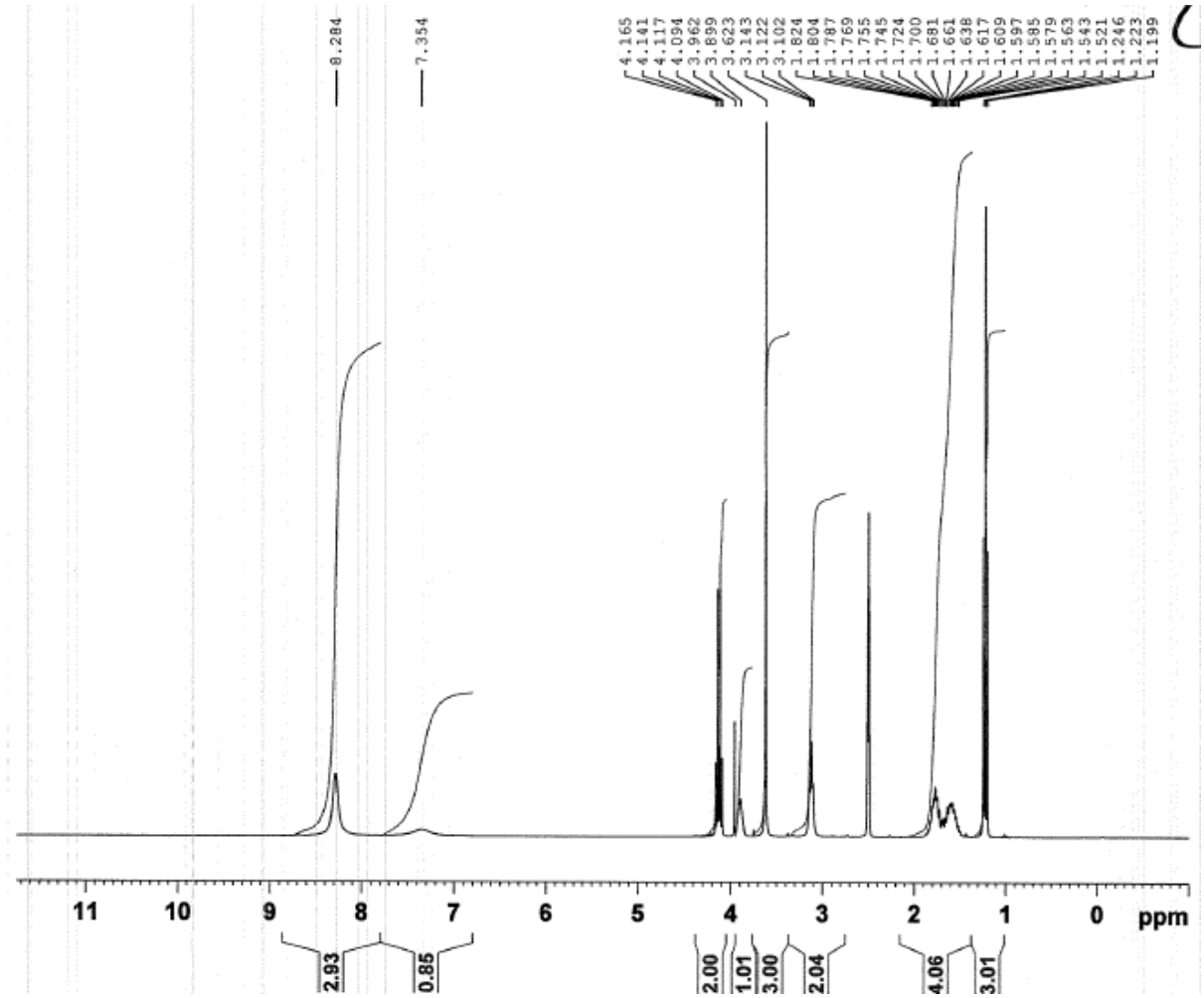
δ/ppm = 14.1 (CH_2-<u>C</u>H_3), 24.6 (γ-CH_2), 27.3 (β-CH_2), 40.6 (N-CH_2), 51.7 (α-CH), 61.6 (<u>C</u>H_2-CH_3), 62.1 (O-CH_3), 149.0 (C=N), 153.5 (CO-Eoc), 170.9 (CO).

HRMS (m/z):

Calculated for $C_{10}H_{21}N_4O_5$ $[M + H]^+$ = 277.15065, found: 277.15049.

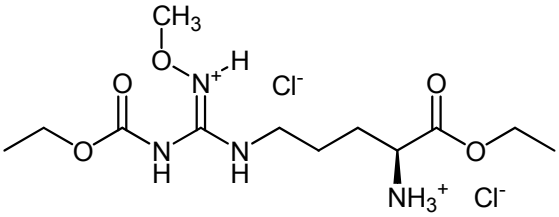
$C_{10}H_{20}N_4O_5 \cdot 2.0\ CF_3COOH \cdot 0.4\ H_2O$ (513.56)

Calc.	C 32.74	H 4.87	N 10.91
Found	C 32.44	H 4.69	N 10.53



^{1}H NMR (DMSO-d_6, 300 MHz) of N^{ω}-Ethoxycarbonyl-$N^{\omega'}$-methoxy-L-arginine Bis(trifluoroacetate) (**8c**)

N^{ω}-Ethoxycarbonyl-$N^{\omega'}$-methoxy-L-arginine Ethylester Dihydrochloride (8d)



The esterification of the free amino acid **8c** was carried out as described for **8b**. Yield: 150 mg (99 %) of a colorless oil, starting from 200 mg (0.397 mmol) of **8c**.

^{1}H-NMR (DMSO-d_6):

δ/ppm = 1.23, 1.25 (2 × t, ^{3}J = 7.0 Hz, 3H, CH_2-C$\underline{H}_3$), 1.55-1.88 (m, 4H, β,γ-CH_2), 3.32 (br t, 2H, N-CH_2), 3.71 (s, 3H, O-CH_3), 3.95 (m, 1H, α-CH), 4.19 (m, 4H, 2 × C$\underline{H}_2$-CH_3), 8.80 (br s, 4H, NH_3^+, NH).

^{13}C-NMR (DMSO-d_6):

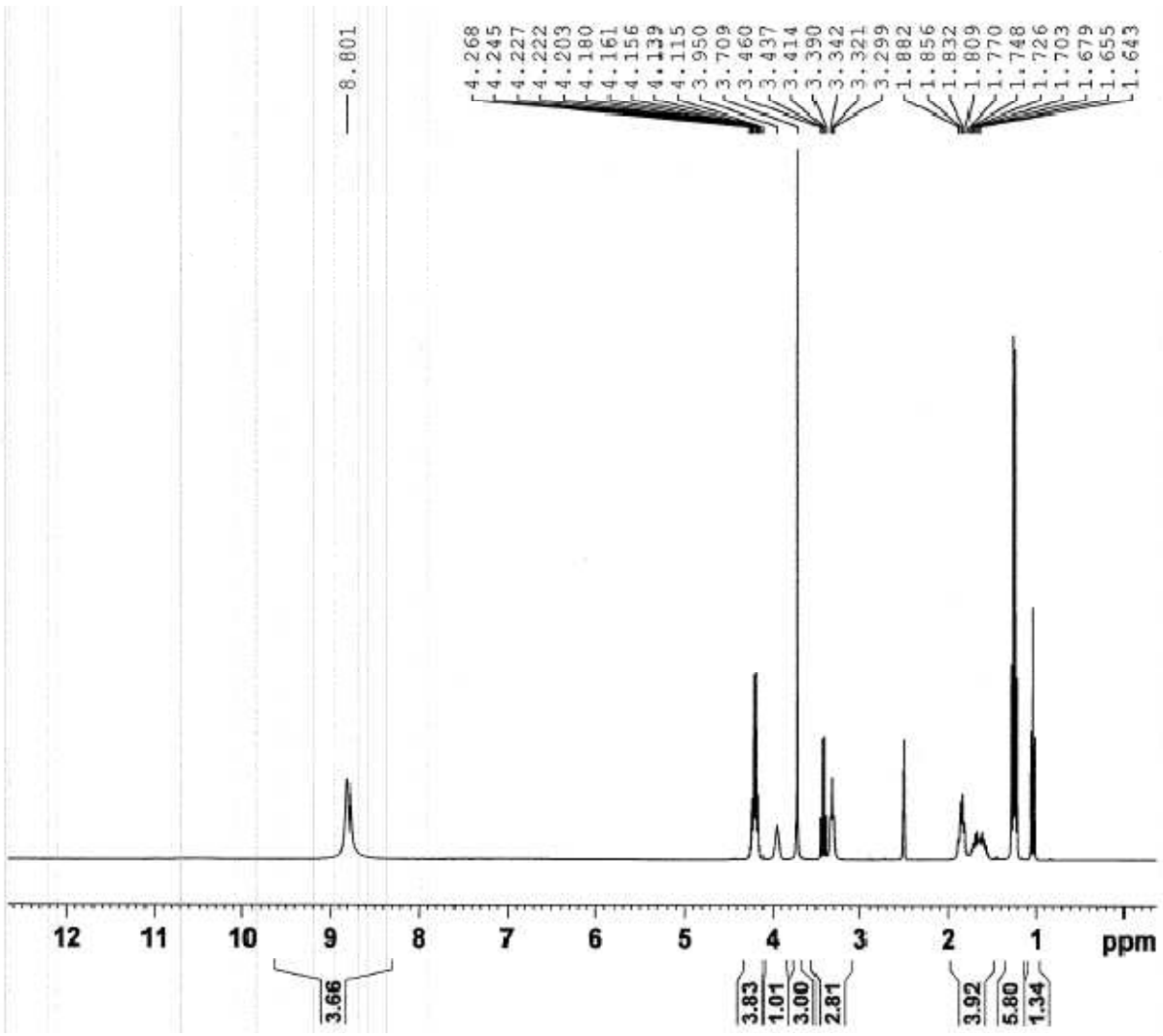
δ/ppm = 13.9, 14.0 (2 × CH_2-$\underline{C}H_3$), 24.0 (γ-CH_2), 26.9 (β-CH_2), 41.1 (N-CH_2), 51.4 (α-CH_2), 61.7, 62.5 (2 × $\underline{C}H_2$-CH_3), 63.9 (O-CH_3), 150.7 (C=N), 152.6 (CO-Eoc), 169.2 (COOEt).

HRMS (m/z):

Calculated for $C_{12}H_{25}N_4O_5$ $[M + H]^+$ = 305.18195, found: 305.18176.

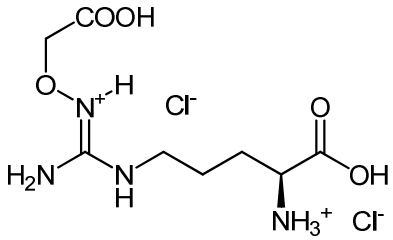
$C_{12}H_{24}N_4O_5 \cdot 2.0\ HCl \cdot 0.6\ H_2O$ (388.08)

Calc.	C 37.14	H 7.06	N 14.44
Found	C 36.67	H 7.59	N 15.00



^{1}H NMR (DMSO-d_6, 300 MHz) of N^{ω}-Ethoxycarbonyl-$N^{\omega'}$-methoxy-L-arginine Ethylester Dihydrochloride (**8d**). NMR sample contained residual amounts of EtOH.

N^{ω}-Carboxymethoxy-L-arginine Dihydrochloride (9a)



The fully protected precursor **6b** (270 mg, 0.489 mmol) are stirred for 4 h at 50-60 °C in 5 mL aqueous 6N HCl. The mixture is concentrated to dryness, taken up with ca. 1-2 mL water and subjected to flash chromatography on a RP-18 column (eluent = 0.1 % TFA in water). Ninhydrin-positive fractions are combined, concentrated to a few residual milliliters and lyophilized. Yield: 150 mg of a fine, amorphous solid (96 %); R_f = 0.53 (*i*-propanol/H_2O/AcOH, 6:3:1)

^{1}H-NMR (D_2O):

δ/ppm = 1.76-2.18 (m, 4H, β,γ-CH_2), 3.41 (br t, ^3J = 6.7 Hz, 2H, N-CH_2), 4.17 (br t, ^3J = 6.2 Hz, α-CH), 4.63 (s, 2H, O-CH_2).

^{13}C-NMR (D_2O, TPS):

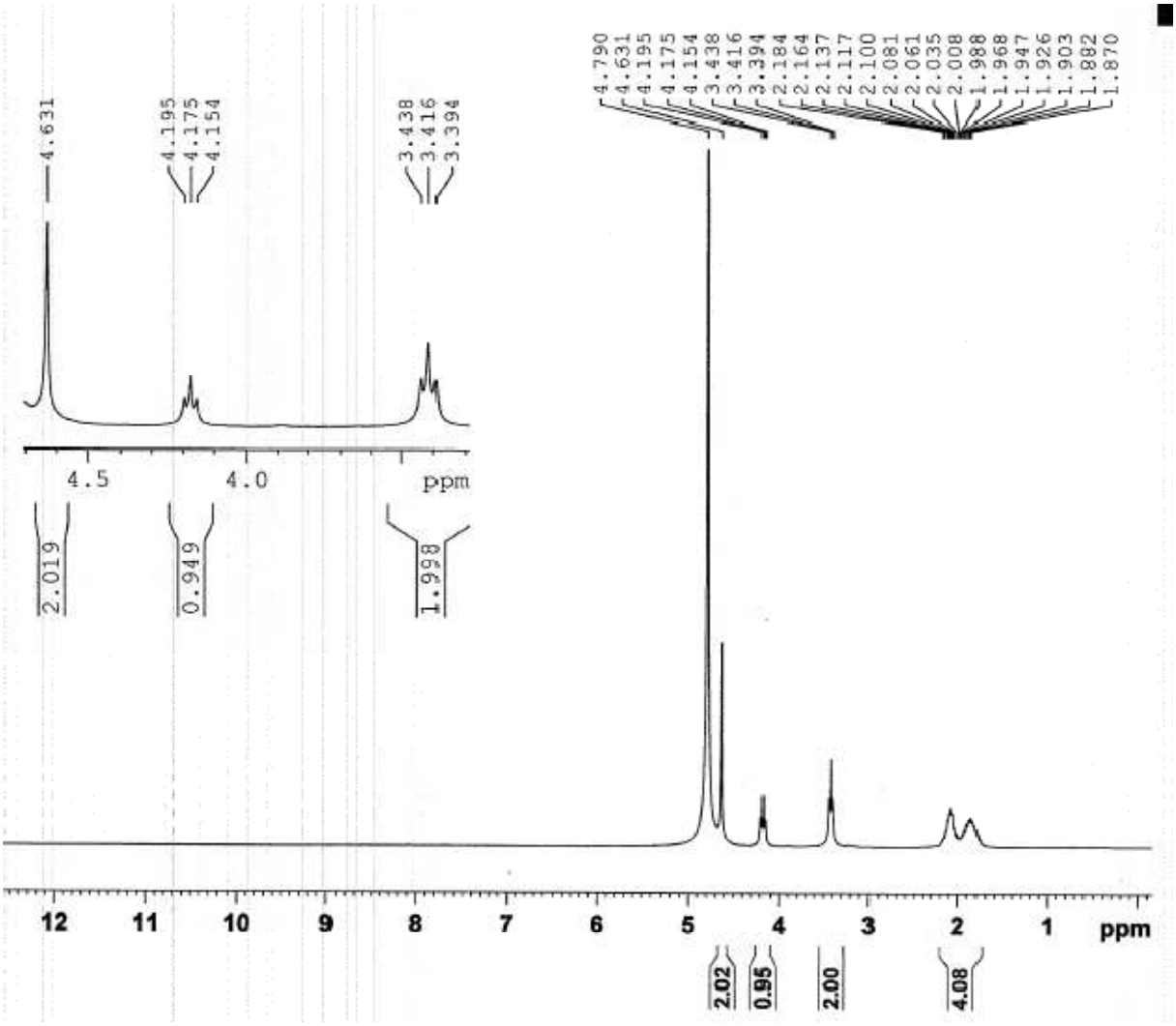
δ/ppm = 26.3 (γ-CH_2), 29.6 (β-CH_2), 43.2 (N-CH_2), 55.3 (α-CH), 75.5 (O-CH_2), 161.0 (C=N), 174.6, 175.3 (2 × CO).

MS (ESI):

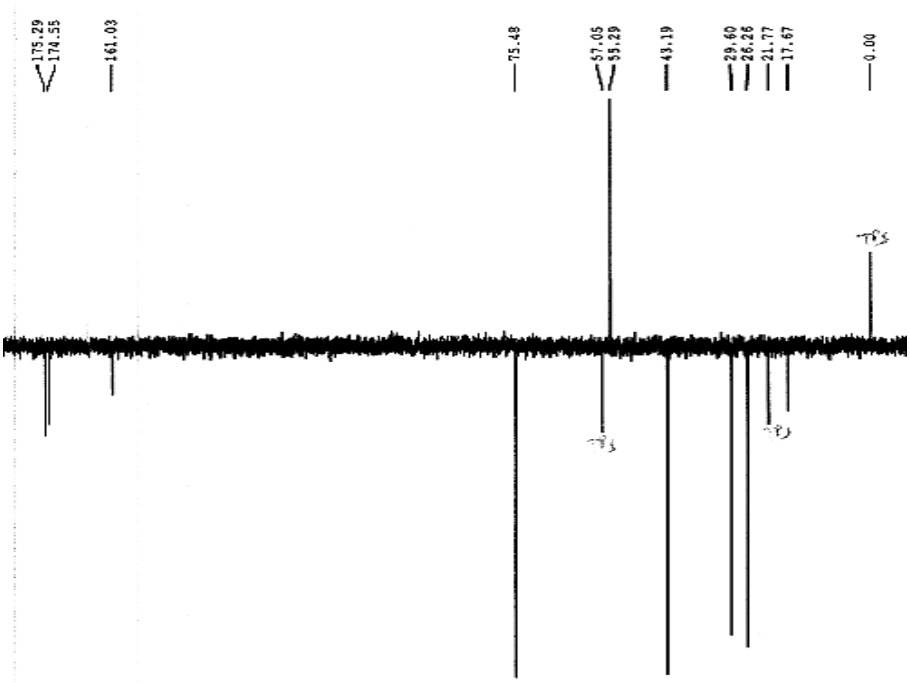
m/z = 249 $[M + H]^+$.

$C_8H_{16}N_4O_5 \cdot 2.0\ HCl \cdot 0.5\ H_2O$ (330.17)

Calc.	C 29.10	H 5.80	N 16.97
Found	C 29.00	H 5.99	N 17.16

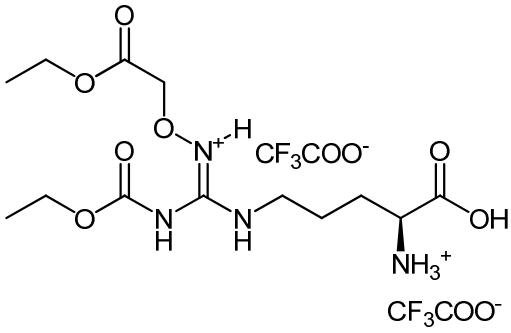


^{1}H NMR (D_2O, 300 MHz) of N^{ω}-Carboxymethoxy-L-arginine Dihydrochloride (**9a**).



^{13}C-NMR (D_2O, TPS, 75 MHz) of of N^{ω}-Carboxymethoxy-L-arginine Dihydrochloride (**9a**).

N^{ω}-Ethoxycarbonyl-$N^{\omega'}$-(ethoxycarbonyl)methoxy-L-arginine Bis(trifluoroacetate) (9b)



Deprotection of **7b** is carried out in analogy to the protocol for **8c**, but starting from 0.5 mmol (252 mg) starting material **7b**. The purification was performed by flash chromatography on a RP-18 column using a stepwise gradient (0.1 % TFA in water with MeOH from 5-30 %). Ninhydrin-positive fractions were combined, concentrated (not exceeding 30 °C) and lyophilized. Yield: 277 mg of a colorless oil (96 %); R_f = 0.62 (*i*-propanol/H_2O/AcOH, 8:1:1).

With DMSO-d_6 as the solvent **9b** appears as isomers in the ratio of ca. 8.6:1.4 (at 300 K, referred to the CH_2 singlet of the ethoxycarbonylmethoxy group). The stated ppm values refer to the main isomer.

^{1}H-NMR (DMSO-d_6):

δ/ppm = 1.19 (t, ^3J = 7.10 Hz, 3H, CH_2-C$\underline{H}_3$), 1.21 (t, ^3J = 7.08 Hz, 3H, CH_2-C$\underline{H}_3$), 1.48-1.87 (m, 4H, β,γ-CH_2), 3.02 (m, 2H, N-CH_2), 3.89 (m, 1H, α-CH), 4.10 (q, ^3J = 7.04 Hz, 2H, C$\underline{H}_2$-CH_3), 4.12 (q, ^3J = 7.13 Hz, 2H, C$\underline{H}_2$-CH_3), 4.37 (s, 2H, O-CH_2), 6.54 (br s, 1H, NH), 8.24 (br s, 3H, NH_3^+), 10.64 (br s, COOH).

^{13}C-NMR (DMSO-d_6):

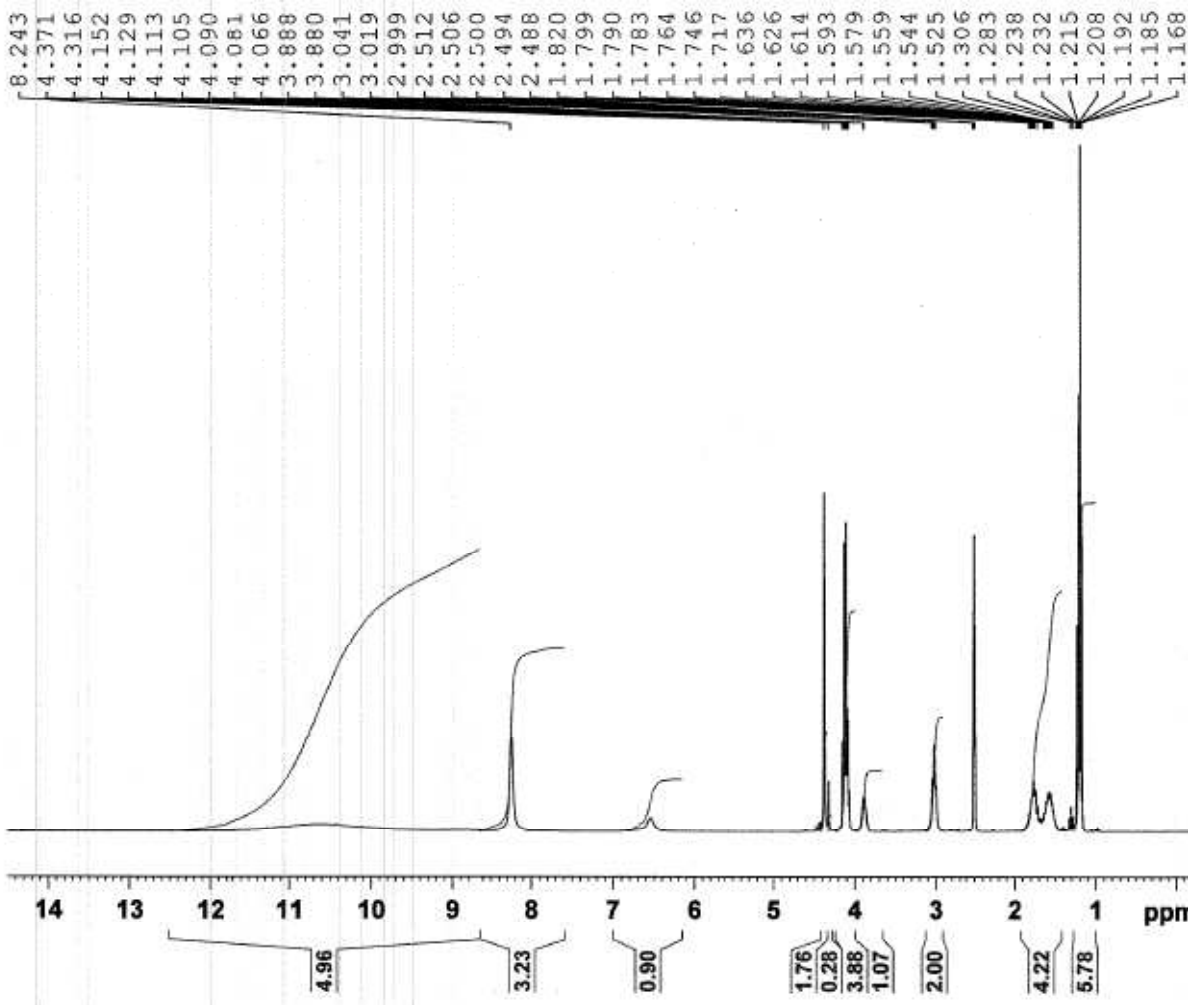
δ/ppm = 14.0, 14.2 (2 × CH_2-$\underline{C}H_3$), 27.4 (CH_2), 40.3 (N-CH_2), 51.8 (α-CH), 60.2, 61.3 (2 × $\underline{C}H_2$-CH_3), 70.3 (O-CH_2), 169.9, 171.0 (3 × CO).

HRMS (m/z):

Calculated for $C_{13}H_{25}N_4O_7$ $[M + H]^+$ = 349.17178, found: 349.17155.

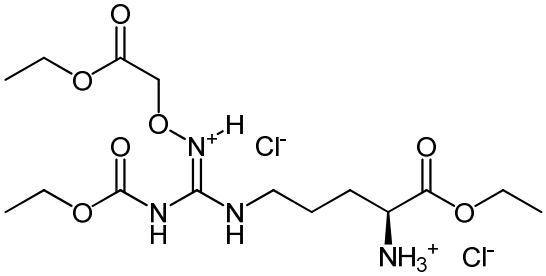
$C_{13}H_{24}N_4O_7 \cdot 3.2\ CF_3COOH \cdot 1.5\ H_2O$ (740.26)

Calc.	C 31.48	H 4.11	N 7.57
Found	C 31.52	H 4.24	N 7.40



^{1}H NMR (DMSO-d_6, 300 MHz) of N^{ω}-Ethoxycarbonyl-$N^{\omega'}$-(ethoxycarbonyl)methoxy-L-arginine Bis(trifluoroacetate) (**9b**)

N^{ω}-Ethoxycarbonyl-$N^{\omega'}$-(ethoxycarbonyl)methoxy-L-arginine Ethylester Dihydrochloride (9c)



The esterification of the free amino acid **9b** was carried out as described for **8b**. Yield: 222 mg (99 %) of a colorless oil, starting from 288 mg (0.5 mmol) of **9b**; R_f = 0,57 (*i*-propanol/H_2O/AcOH, 8:1:1).

^{1}H-NMR (DMSO-d_6):

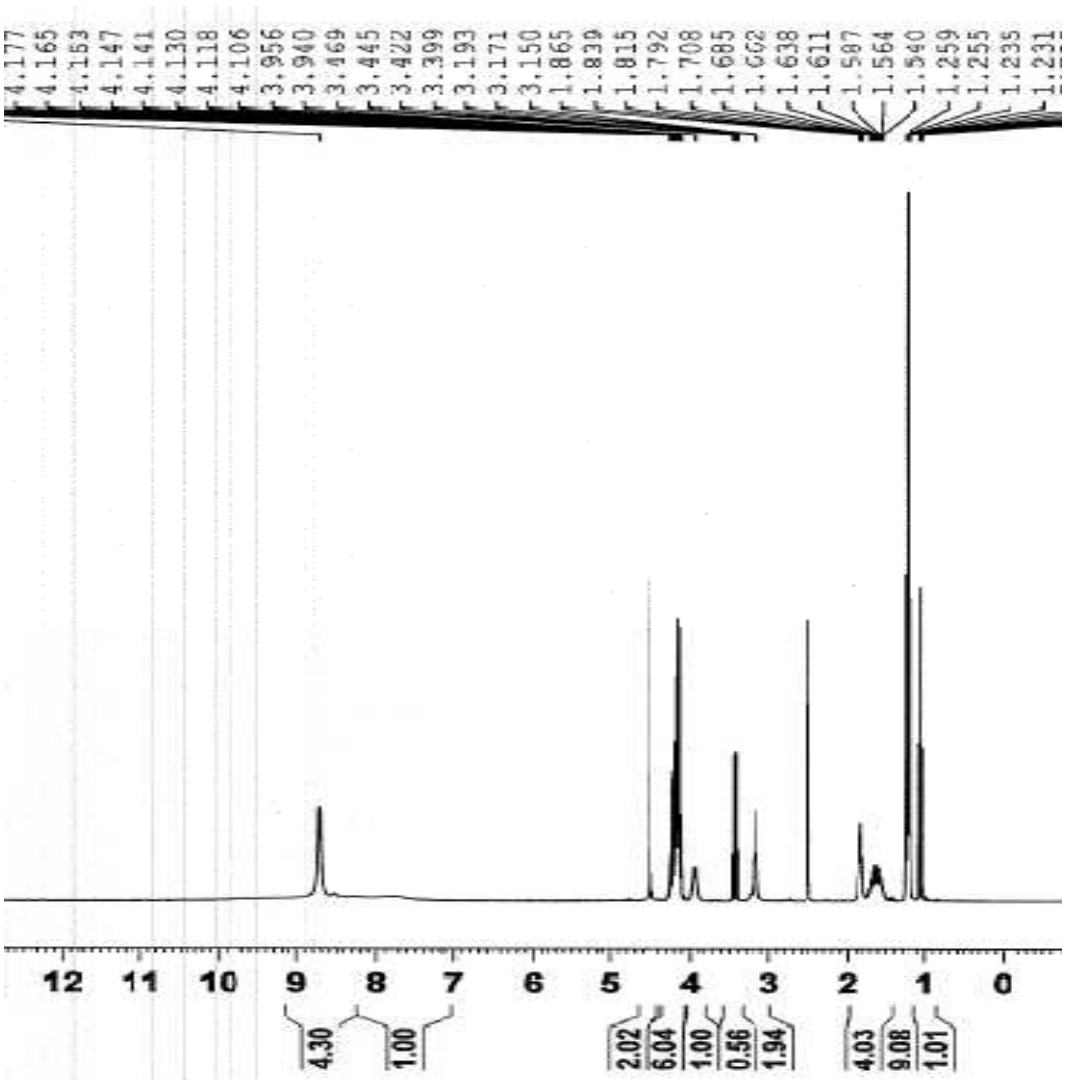
δ/ppm = 1.20 (t, ^3J = 7.16 Hz, 3H, CH_2-C$\underline{H}_3$), 1.23 (t, ^3J = 7.13 Hz, 3H, CH_2-C$\underline{H}_3$), 1.26 (t, ^3J = 7.10 Hz, 3H, CH_2-C$\underline{H}_3$), 1.51-1.87 (m, 4H, β,γ-CH_2), 3.17 (m, 2H, N-CH_2), 3.95 (m, 1H, α-CH), 4.14 (q, ^3J = 7.12 Hz, 2H, C$\underline{H}_2$-CH_3), 4.15 (q, ^3J = 7.10 Hz, 2H, C$\underline{H}_2$-CH_3), 4.17-4.25 (m, 2H, C$\underline{H}_2$-CH_3), 4.51 (s, 2H, O-CH_2), 7.80 (br s, 1H, NH), 8.55 (br s, 1H, NH), 8.72 (br s, 3H, NH_3^+).

^{13}C-NMR (DMSO-d_6):

δ/ppm = 13.9, 14.0, 14.1 (3 × CH_2-$\underline{C}H_3$), 24.2 (γ-CH_2), 27.2 (β-CH_2), 40.8 (N-CH_2), 51.5 (α-CH), 60.5, 61.7, 61.9 (3 × $\underline{C}H_2$-CH_3), 71.1 (O-CH_2), 150.3 (C=N), 153.0 (CO-Eoc), 169.1, 169.3 (2 × CO).

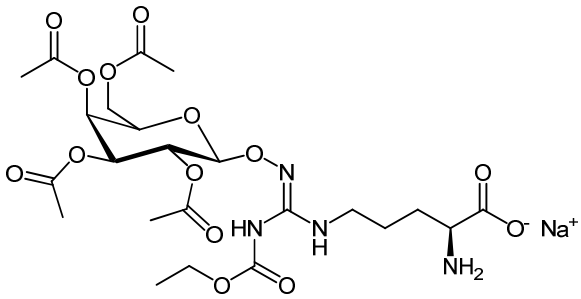
HRMS (m/z):

Calculated for $C_{15}H_{29}N_4O_7$ $[M + H]^+$ = 377.20308, found: 377.20287.



^{1}H NMR (DMSO-d_6, 300 MHz) of N^{ω}-Ethoxycarbonyl-$N^{\omega'}$-(ethoxycarbonyl)methoxy-L-arginine thylester Dihydrochloride (**9c**). NMR sample contained residual amounts of EtOH.

Sodium N^{ω}-Ethoxycarbonyl-$N^{\omega'}$-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranos-1-yl)oxy-L-argininate (10)



In a Schlenk flask 120 mg (0.16 mmol) of the fully protected, thoroughly dried precursor **7c** are dissolved in absolute Et_2O under an argon atmosphere. The solution is stirred for ca. 30 min at -15 °C, and HCl gas is carefully bubbled through the solution. The reaction mixture is left in the refridgerator overnight and then concentrated in a vacuum. The white-yellow solid residue is taken up with ca. 1-2 mL of 0.5 M aqueous $NaHCO_3$ and purified by flash chromatography on a RP-18 column (eluent = water, with a stepwise MeOH gradient of 10 %, 25 %, 50 %). The product containing fractions were combined, concentrated on a rotary evaporator (not exceeding 30 °C) and lyophilized. Yield: 74 mg of a fine, white powder (75 %).

With D_2O as the solvent **10** appears as isomers in the ratio of ca. 6:4 (at 300 K, referred to the CH_3 triplet of the ethoxycarbonyl group). The stated ppm values refer to the main isomer.

^{1}H-NMR (D_2O, TPS):

δ/ppm = 1.28 (t, ^3J = 7.1 Hz, 3H, CH_2-C$\underline{H}_3$), 1.52-1.97 (m, 4H, β,γ-CH_2), 2.02, 2.08, 2.12, 2.23 (4 × s, 3H, $COCH_3$), 3.16 (br t, ^3J = 6.8 Hz, 2H, 6'-CH_2), 3.75 (t, ^3J = 6.3 Hz, 1H, 5'-CH), 4.15-4.30 (m, 5H, C$\underline{H}_2$-CH_3, α-CH, N-CH_2), 5.07 (d, ^3J = 8.0 Hz, 1H, 1'-CH), 5.21-5.33 (m, 2H, 2',3'-CH), 5.47 (m, 1H, 4'-CH).

^{13}C-NMR (D_2O, TPS):

δ/ppm = 16.2 (CH_2-$\underline{C}H_3$), 22.7, 22.8, 22.9, (4 × CO$\underline{C}H_3$), 26.6 (γ-CH_2), 30.6 (β-CH_2), 42.7 (N-CH_2), 57.2 (α-CH), 64.6 (6'-CH_2), 65.8 ($\underline{C}H_2$-CH_3), 70.6, 70.7, 73.4, 74.1 (2',3',4',5'-CH), 104.1 (1'-CH), 155.6 (C=N), 156.8 (CO-Eoc), 175.4, 175.8, 176.2, 177.1 (4 × $\underline{C}OCH_3$).

MS (ESI):

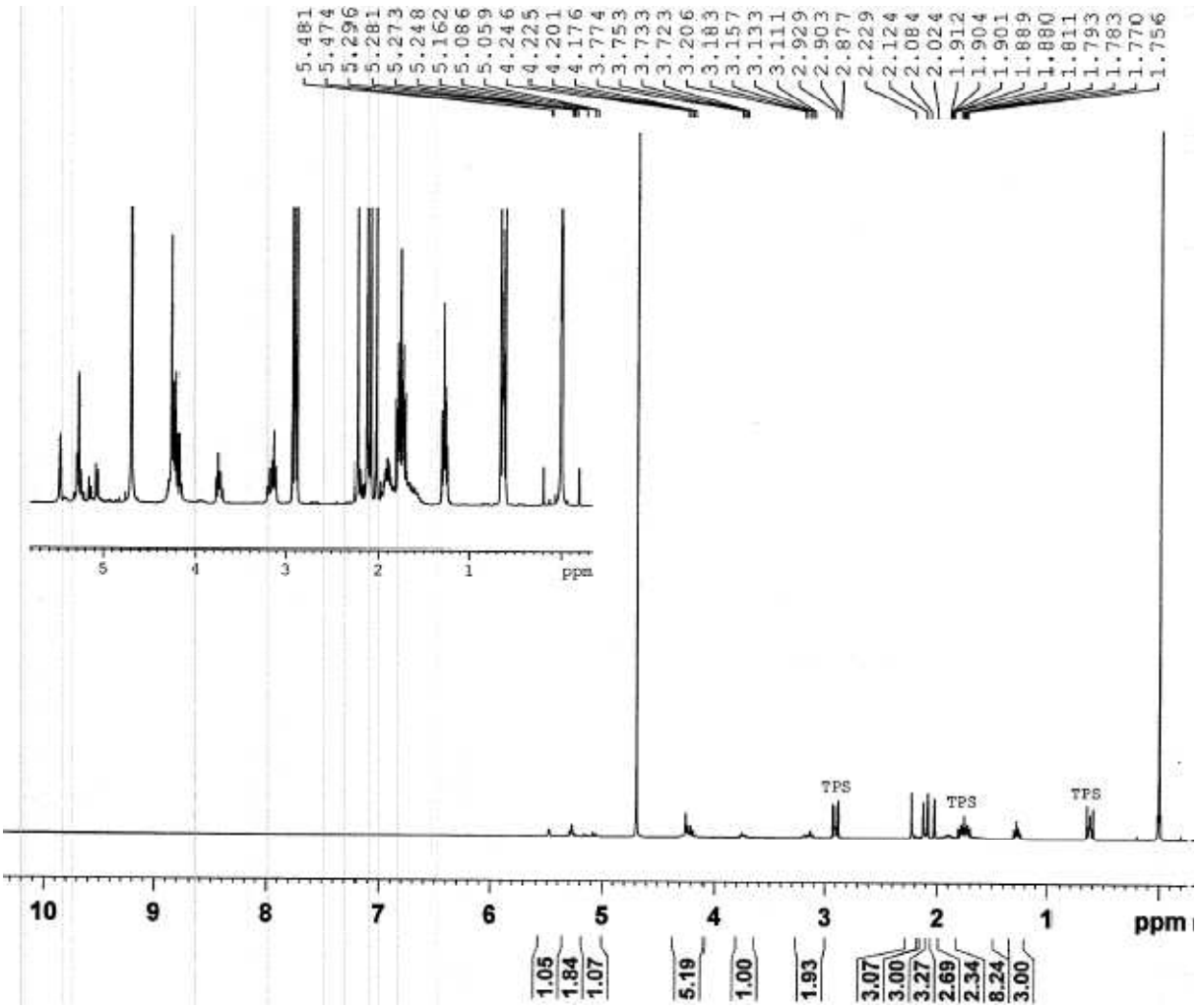
m/z = 615 $[M + Na]^+$, 593 $[M + H]^+$, 551 $[M – C_2H_2O + H]^+$, 331 $[C_{14}H_{19}O_9]^+$.

HRMS (m/z):

Calculated for $C_{23}H_{33}N_4O_{14}Na$ $[M + Na]^+$ = 615.21202, found: 615.21164

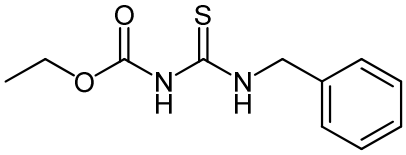
$C_{23}H_{35}N_4NaO_{14}$ (614.53)

Calc.	C 44.95	H 5.74	N 9.12
Found	C 45.08	H 6.21	N 8.77



^{1}H NMR (D_2O, TPS, 300 MHz) of Sodium N^{ω}-Ethoxycarbonyl-$N^{\omega'}$-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranos-1-yl)oxy-L-argininate (**10**).

***N*-Benzyl-*N*'-ethoxycarbonylthiourea (11)**[2]



Thiourea **11** was prepared in analogy to thiourea **2b** (see above), starting from 7.7 mmol of benzylamine and ethoxycarbonylisothiocyanate, respectively. For purification a short column of ca. 10 g SiO_2 was used, and CH_2Cl_2/MeOH (9:1) as the eluent. Yield: 1.76 g of a white solid (96 %); Mp.: 103 °C; TLC: R_f = 0.43 (Cy/EtOAc, 4:1)

^{1}H-NMR ($CDCl_3$):

δ/ppm = 1.30 (t, ^3J = 7.1 Hz, 3H, CH_2-C<u>H</u>$_3$), 4.20 (q, ^3J = 7.1 Hz, 2H, O-CH_2), 4.86 (d, ^3J = 6.0 Hz, 2H, N-CH_2), 7.26-7.37 (m, 5H, ArH), 8.23, 9.95 (2 × s, 1H, NH).

^{13}C-NMR ($CDCl_3$):

δ/ppm = 14.8 (CH_2-<u>C</u>H$_3$), 50.3 (N-CH_2), 63.4 (O-CH_2), 128.8, 129.2, 129.5 (ArCH), 137.0 (ArC), 153.4 (CO), 180.1 (C=S).

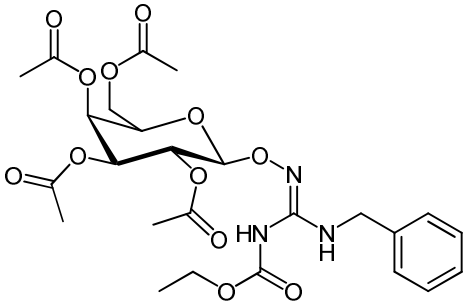
MS (ESI):

m/z = 261 $[M + Na]^+$, 239 $[M + H]^+$, 91 $[C_7H_7]^+$.

$C_{11}H_{14}N_2O_2S$ (238.31)

Calc.	C 55.44	H 5.92	N 11.76	S 13.46
Found	C 55.69	H 6.06	N 11.71	S 13.30

_N_-Benzyl-_N_'-ethoxycarbonyl-_N_''-[(2,3,4,6-tetra-_O_-acetyl-β-D-galactopyranos-1-yl)oxy]guanidine (12)



119 mg of thiourea **11** (0.5 mmol), 218 mg of the protected 1-aminooxygalactose **5** (0.6 mmol) and 104.5 µl DIPEA (0.6 mmol) are dissolved in 5 mL of dry CH_2Cl_2. The mixture is cooled to 0 °C, after which 115 mg of EDCI (0.6 mmol) is added. This mixture is stirred for 48 h at room temperature. For work up, 10 mL of CH_2Cl_2 are added and the organic phase is washed with 1 % HCl, water and brine (5 mL each). The organic phase is dried with Na_2SO_4 and concentrated in a vacuum. The crude product is purified by column chromatography on SiO_2 using Cy/EtOAc (1:1) as the eluent. Yield: 255 mg of a colorless oil (90 %); TLC: R_f = 0.54 (Cy/EtOAc, 1:1).

^{1}H-NMR ($CDCl_3$):

δ/ppm = 1.30 (t, ^3J = 7.1 Hz, 3H, CH_2-C<u>H</u>$_3$), 1.99, 2.02, 2.06, 2.15 (4 × s, 3H, $COCH_3$), 3.98 (br t, ^3J = 6.7 Hz, 1H, 5'-CH), 4.10-4.30 (m, 6H, 6'-CH_2, N-CH_2, C<u>H</u>$_2$-CH_3), 4.90 (d, ^3J = 8.3 Hz, 1H, 1'-CH), 5.08 (m, 1H, 3'-CH), 5.29 (br t, ^3J = 9.6 Hz, 1H, 2'-CH), 5.41 (m, 1H, 4'-CH), 6.79 (br t, 1H, NH), 7.22-7.35 (m, 5H, ArH), 7.72 (br s, 1H, NH).

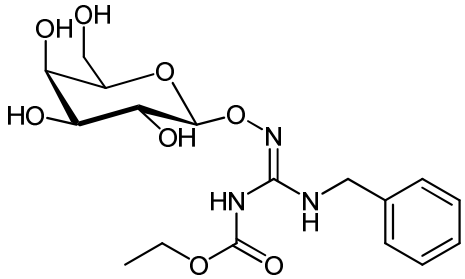
^{13}C-NMR ($CDCl_3$):

δ/ppm = 14.3 (CH_2-<u>C</u>H$_3$), 20.6, 20.7, 20.9 (4 × CO<u>C</u>H$_3$), 45.1 (N-CH_2), 61.1 (6'-CH_2), 62.2 (<u>C</u>H$_2$-CH_3), 67.0, 68.1, 70.7, 71.0 (2',3',4',5'-CH), 102.7 (1'-CH), 127.4, 127.8, 128.6 (ArCH), 138.3 (ArC), 150.4 (C=N), 153.2 (CO-Eoc), 170.0, 170.2, 170.3 (4 × <u>C</u>OCH$_3$).

MS (ESI):

m/z = 590 $[M + Na]^+$, 568 $[M + H]^+$, 526 $[M – C_2H_2O + H]^+$, 331 $[C_{14}H_{19}O_9]^+$.

_N_-Benzyl- _N_'-ethoxycarbonyl-_N_''-[(β-D-galactopyranos-1-yl)oxy]guanidine (13)



150 mg of the protected precursor **12** (0.26 mmol) are dissolved in 5 mL MeOH and gently stirred with ca. 30 mg of strong anion exchanger resin (Merck Ionenaustauscher III, OH^-) over night. The solution is filtered, washed with small amounts of MeOH and concentrated on a rotary evaporator. The product is further purified by flash chromatography on SiO_2 using a step-wise gradient of EtOAc/MeOH (9:1 to 8:2). Yield: 84 mg of a fine white powder (80 %); TLC: R_f = 0.66 (EtOAc/MeOH, 8:2).

In MeOH-d_4 as the NMR solvent the substance occurs as isomers at a ratio of ca. 8:2 (300 K, referred to the triplett of the ethyloxycarbonyl methyl-group). In DMSO-d_6 this ratio is ca. 6:4. The stated ppm values refer to the main isomer.

^{1}H-NMR (MeOH-d_4):

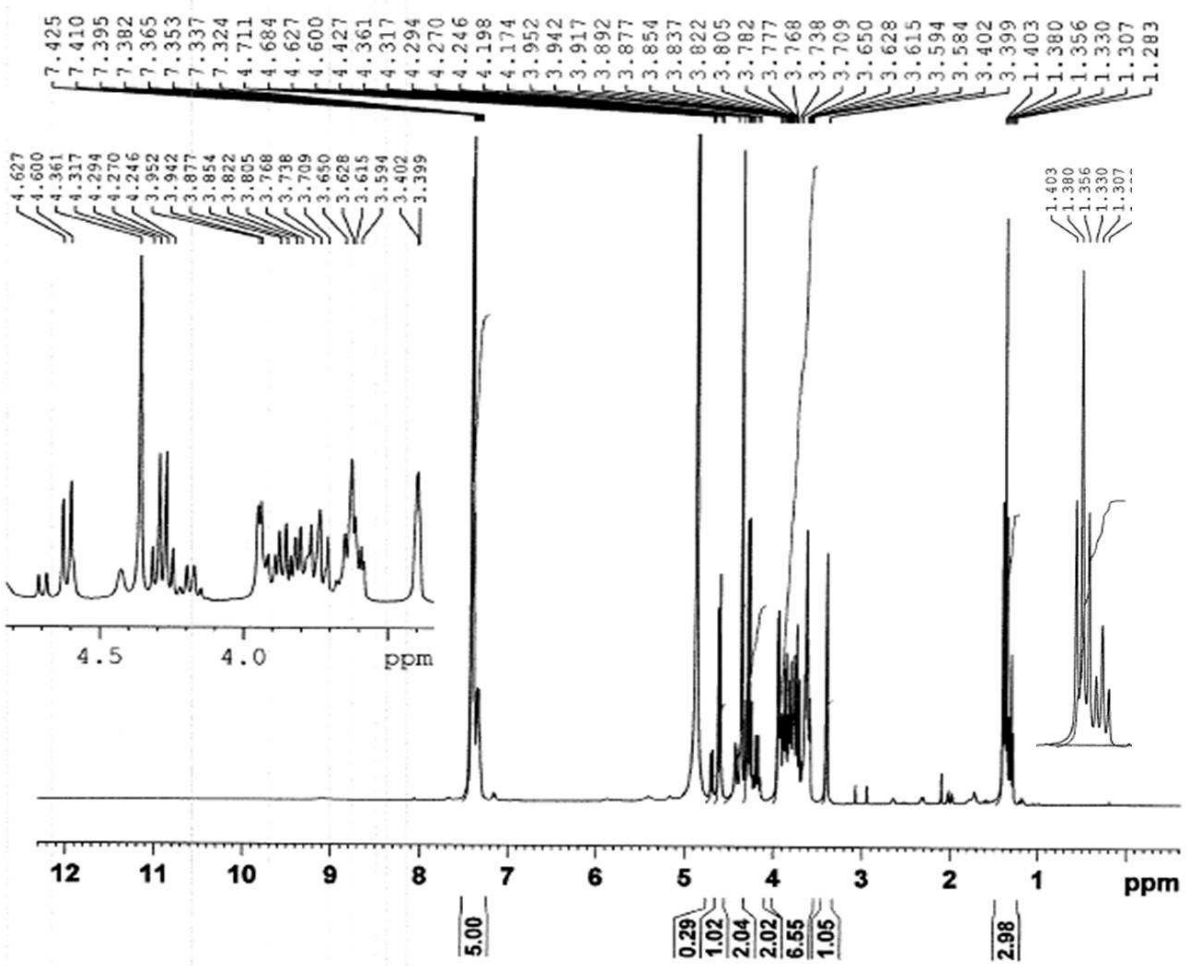
δ/ppm = 1.38 (t, ^3J = 7.1 Hz, 3H, CH_2-C<u>H</u>$_3$), 3.58-3.67 (m, 2H, 6'-CH_2), 3.71-3.96 (m, 4H, 2',3',4',5'-CH), 4.28 (q, ^3J = 7.1 Hz, 2H, C<u>H</u>$_2$-CH_3), 4.36 (br s, 2H, N-CH_2), 4.61 (d, ^3J = 8.1 Hz, 1H, 1'-CH), 7.31-7.44 (m, 5H, ArH).

^{13}C-NMR (MeOH-d_4):

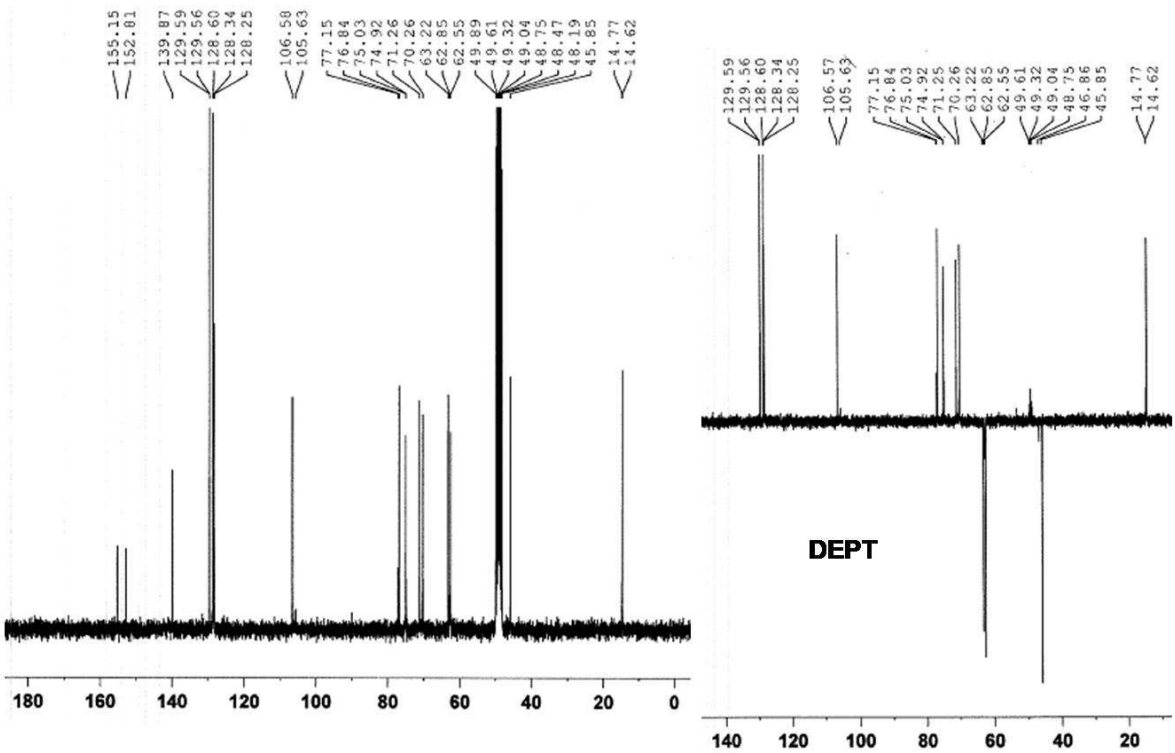
δ/ppm = 14.6 (CH_2-<u>C</u>H$_3$), 45.8 (N-CH_2), 62.5 (6'-CH_2), 63.2 (<u>C</u>H$_2$-CH_3), 70.2, 71.2, 75.0, 76.8 (2',3',4',5'-CH), 106.6 (1'-CH), 128.3, 128.6, 129.6 (ArCH), 139.8 (ArC), 152.8 (C=N), 155.1 (CO).

HRMS (m/z):

Calculated for $C_{17}H_{26}N_3O_8$ $[M + H]^+$ = 400.17144, found: 400.17147; calculated for $C_{17}H_{33}N_4O_{14}Na$ $[M + Na]^+$ = 422.15339, found: 422.15327.



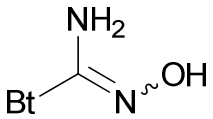
^{1}H NMR (MeOH-d_4, 300 MHz) of *N*-Benzyl- *N*'-ethoxycarbonyl-*N*''-[(β-D-galactopyranos-1-yl)oxy]guanidine (**13**).



^{13}C NMR spectra (MeOH-d_4, 300 MHz) of *N*-Benzyl- *N*'-ethoxycarbonyl-*N*''-[(β-D-galactopyranos-1-yl)oxy]guanidine (**13**).

C. Synthetic details for attempts towards *O*-acylated NOHA prodrugs

N'-Hydroxy-1*H*-1,2,3-benzotriazole-1-carboximidamide (18)



This compound was prepared according to a modified literature procedure.[11] In our hands, the literature protocol gave much lower yields than stated by the authors, and we envisioned a simpler work-up.

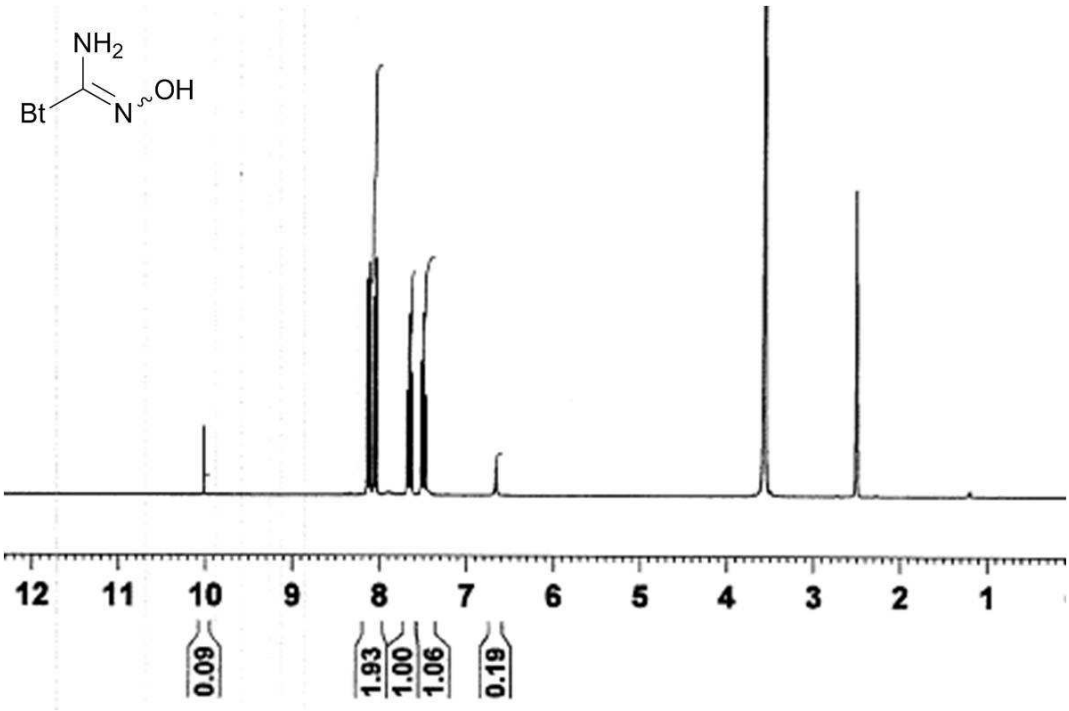
To a suspension of 2.63 g of di(1*H*-1,2,3-benzotriazol-1-yl)methanamine (10 mmol) in 100 mL of 1,4-dioxane were added 660 mg of free hydroxylamine base[12] (20 mmol). This mixture was stirred and heated for one hour at 55 °C. After cooling to room temperature the solvent is removed in a vacuum, the residue taken up with 30 mL of diethyl ether and the mixture stirred for ca. 30 min at room temperature. The precipitated crystals were removed by filtration. Yield: 1.4 g (79%).

^{1}H-NMR (DMSO-d_6):

δ/ppm = 6.56 (br s, 2H), 7.50 (ddd, 1H), 7.68 (ddd, 1H), 8.06 (dt, 1H), 8.10 (dt, 1H), 9.95 (s, 1H).

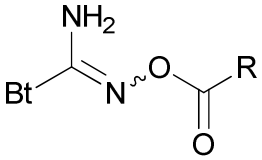
^{13}C-NMR (DMSO-d_6):

δ/ppm = 27.0, 114.0, 119.6, 125.5, 129.5, 130.7, 145.4, 150.8.



^{1}H NMR (DMSO-d_6, 300 MHz) of *N*'-Hydroxy-1*H*-1,2,3-benzotriazole-1-carboximidamide (**18**). After H/D-exchange.

_N_'-Acyloxy-1_H_-1,2,3-benzotriazole-1-carboximidamides (19)



100 mg of **18** (0.56 mmol) were dissolved in 5 mL of dioxane, 100 µL triethylamine and 0.56 mmol of the respective acid chloride added. The reaction mixture was stirred and heated for 1.5 hours at 55 °C. After removal of precipitated triethylammonium chloride 5 mL of diethyl ether were added to the residue. The precipitated crystals were filtered and dried in a vacuum over calcium chloride. Yields: 50-70 %, depending on the respective ester. Presumably, higher yields can be achieved by chromatographic work-up of the crude mixtures.

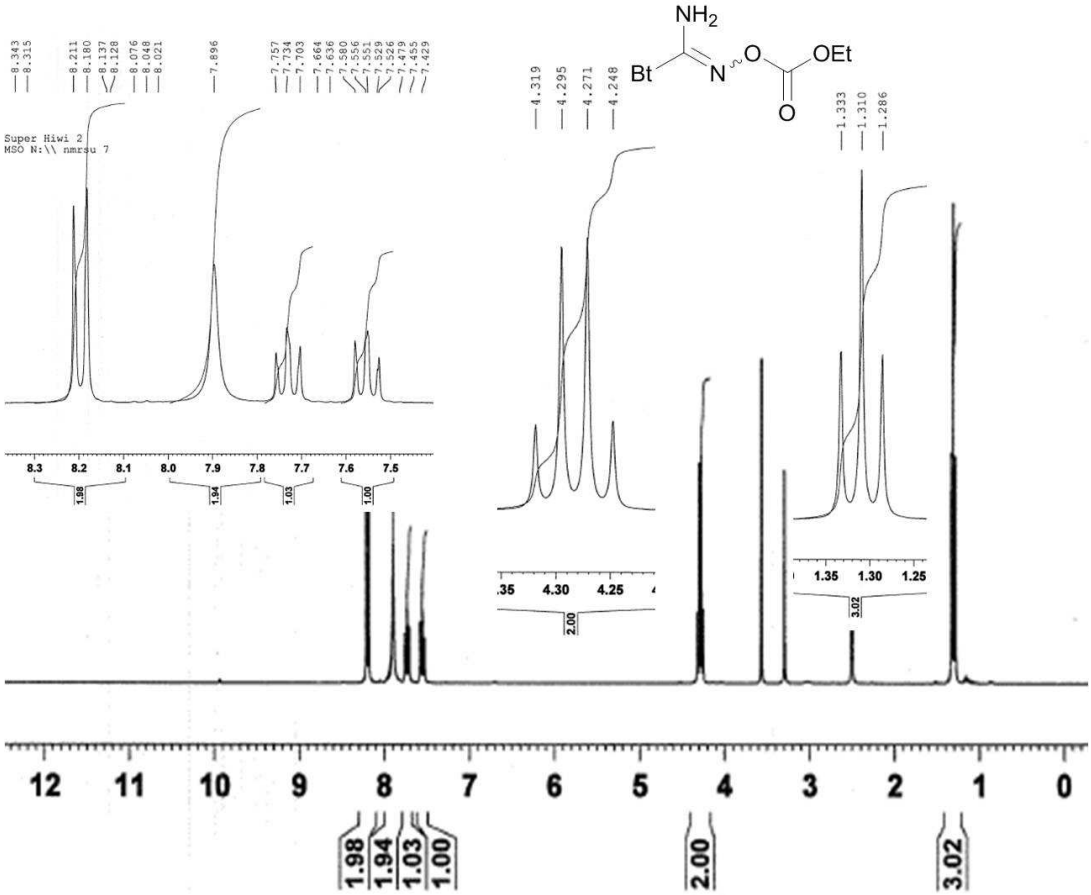
_N_'-(Ethoxycarbonyl)oxy-1_H_-1,2,3-benzotriazole-1-carboximidamide (**19a**)

^{1}H-NMR (DMSO-d_6):

δ/ppm = 1.31 (q, ^{3}J = 7.2 Hz, 2H), 4.28 (q, ^{3}J = 7.2 Hz, 2H), 7.55 (td, 1H), 7.73 (td, 1H), 7.90 (br s, 2H, NH_2), 8.20 (d, 2H).

^{13}C-NMR (DMSO-d_6):

δ/ppm = 14.1, 64.3, 113.7, 119.7, 125.6, 130.7, 145.4, 151.3, 153.1.



^{1}H NMR (DMSO-d_6, 300 MHz) of _N_'-(Ethoxycarbonyl)oxy-1_H_-1,2,3-benzotriazole-1-carboximidamide (**19a**).

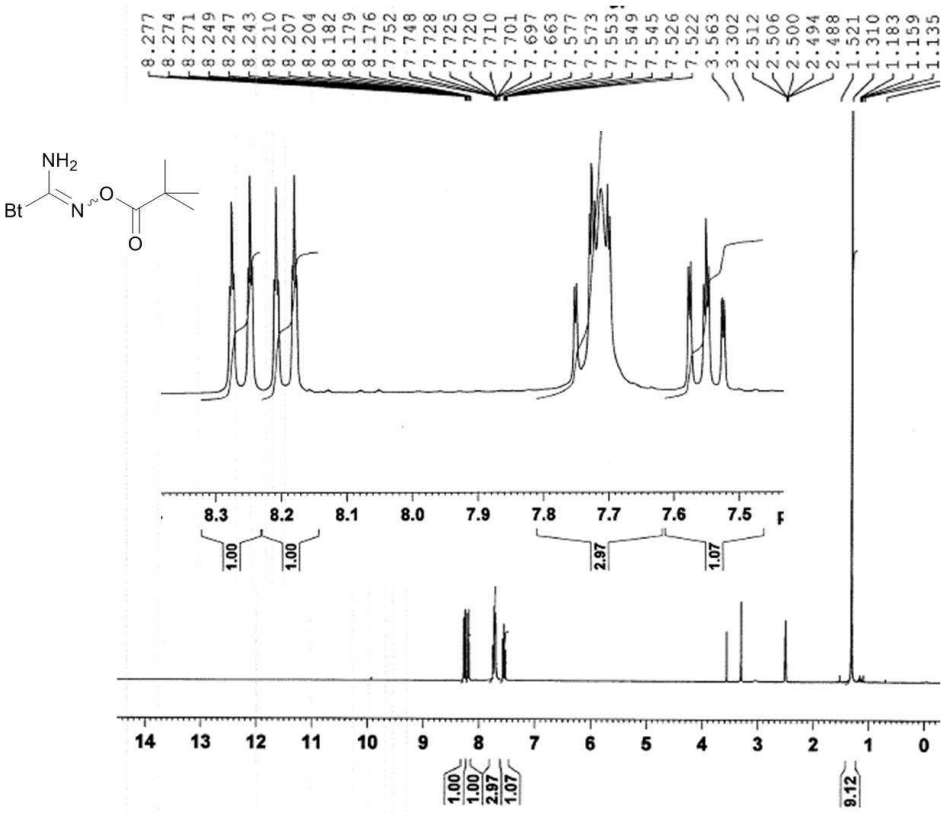
***N'*-Pivaloyloxy-1*H*-1,2,3-benzotriazole-1-carboximidamide (19b)**

^{1}H-NMR (DMSO-d_6):

δ/ppm = 1.31 (s, 9H, $C(CH_3)_3$), 7.55 (ddd, 1H), 7.71 (br s, 2H), 7.72 (ddd, 1H), 8.19 (dt, 1H), 8.26 (dt, 1H).

^{13}C-NMR (DMSO-d_6):

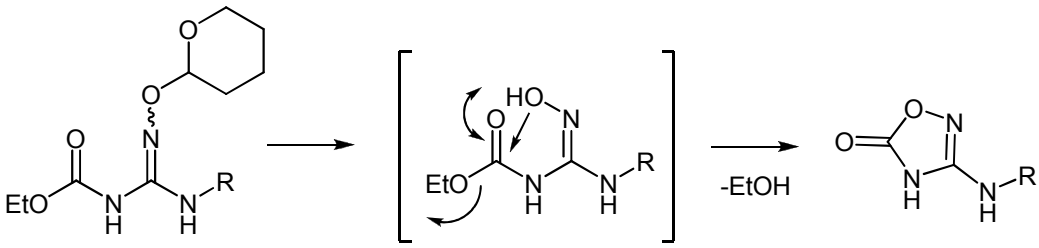
δ/ppm = 27.0, 114.0, 119.6, 125.5, 129.5, 130.7, 145.4, 150.8.

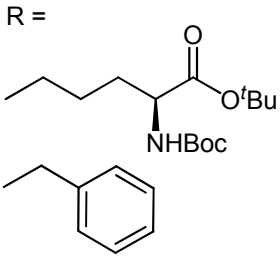


^{1}H NMR (DMSO-d_6, 300 MHz) of *N'*-Pivaloyloxy-1*H*-1,2,3-benzotriazole-1-carboximidamide (**19b**).

Towards the cyclic carbamate (1,2,4-oxadiazol-5-one) of NOHA

Attempts towards the synthesis of 1,2,4-oxadiazol-5-ones of NOHA included the preparation of *O*-THP-protected, N^{ω}-Eoc-substituted NOHA (and a simple benzyl-based model compound analog) in order to induce cyclization (EtOH elimination) after in situ chemoselective deprotection of the THP group (see figure below). Accordingly, formic acid, acetic acid and magnesium bromide were tested in different solvents (EtOH, DMF, DMSO, DCM, w/ and w/o small amounts of water) under conventional and microwave conditions to force the desired cyclization. But the desired product could not be isolated.

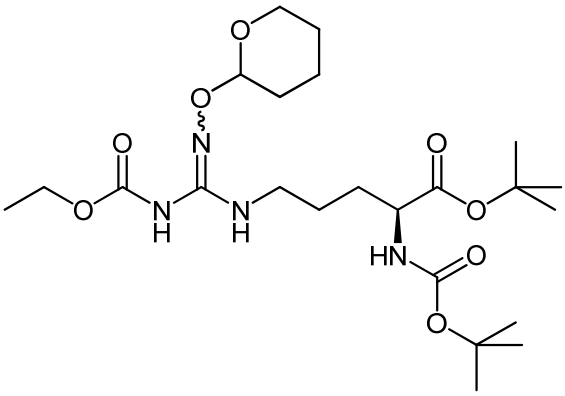




***O*-THP-protected *N*-Ethoxycarbonylhydroxyguanidines**

2.0 mmol of the respective thiourea precursor are dissolved in 20 mL of dry CH_2Cl_2, and 1.04 mL of DIPEA (3.0 mmol) as well as 351 mg of *O*-(Tetrahydro-2*H*-pyran-2-yl)hydroxylamine (3.0 mmol) are added. The solution is brought to 0 °C after which 574 mg EDCI (3,0 mmol) are added. The mixture is stirred over night, diluted with CH_2Cl_2 and washed with 1 % HCl (water), water and brine (10 mL each). The organic phase is dried with Na_2SO_4 and concentrated in vacuo. The resulting oil is purified by column chromatography on SiO_2.

***N*$^{\alpha}$-(*t*-Butyloxycarbonyl)-*N*$^{\omega}$-ethoxycarbonyl-*N*$^{\omega'}$-(tetrahydro-2*H*-pyran-1-yl)oxy-L-arginine *t*-Butylester**



Eluent: CH_2Cl_2/MeOH (98:2); yield: 935 mg of a colorless oil (93 %), TLC: R_f = 0.29 (toluene/EtOAc, 3:1)

^{1}H-NMR ($CDCl_3$):

δ/ppm = 1.30 (t, ^3J = 7.1 Hz, 3H, CH_2-C$\underline{H}_3$), 1.44, 1.46 (2 × s, 9H, $C(CH_3)_3$), 1.50-1.89 (m, 10H, β,γ-CH_2, 4',5',6'-CH_2), 3.14 (m, 2H, N-CH_2), 3.58, 3.91 (2 × m, 1H, 3'-CH_2), 4.18 (br q, ^3J = 7.1 Hz, 3H, C$\underline{H}_2$-CH_3, NH), 4.94 (m, 1H, 1'-CH), 5.06, 6.35, 7.80 (3 × br s, 1H, NH).

^{13}C-NMR ($CDCl_3$):

δ/ppm = 15.0 (CH_2-$\underline{C}H_3$), 21.5 (5'-CH_2), 25.63, 25.69 (γ-CH_2), 25.9 (4'-CH_2), 28.7, 29.0 ($C(\underline{C}H_3)_3$), 30.1 (6'-CH_2), 30.97, 31.02 (β-CH_2), 41.3 (N-CH_2), 54.5 (α-CH), 62.7 ($\underline{C}H_2$-CH_3), 64.7 (3'-CH_2), 80.5, 82.5 ($\underline{C}(CH_3)_3$), 102.1 (1'-CH).

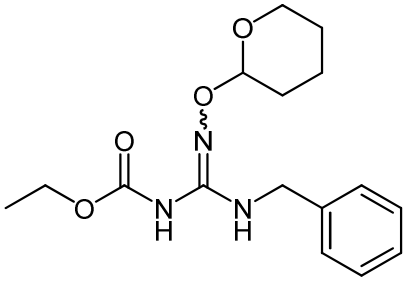
MS (ESI):

m/z = 525 $[M + Na]^+$, 503 $[M + H]^+$, 419 $[M – C_5H_8O + H]^+$.

$C_{23}H_{42}N_4O_8$ (502.60)

Calc.	C 54.96	H 8.42	N 11.15
Found	C 54.73	H 8.50	N 11.27

_N_-Benzyl-_N_'-ethoxycarbonyl-_N_''-[(tetrahydro-2_H_-pyran-1-yl)oxy]guanidine



Eluent: CH_2Cl_2/MeOH (98:2); yield: 580 mg of a white solid (90 %); TLC: R_f = 0.51 (toluene/EtOAc, 3:1); Mp.: 79 °C.

^{1}H-NMR ($CDCl_3$):

δ/ppm = 1.30 (t, ^3J = 7.1 Hz, 3H, CH_2-C$\underline{H}_3$), 1.50-1.70 (m, 4H, O-CH_2-C$\underline{H}_2$-C$\underline{H}_2$), 1.71-1.90 (m, 2H, CH-C$\underline{H}_2$), 3.60, 3.95 (2 × m, 1H, O-CH_2), 4.18 (q, ^3J = 7.1 Hz, 2H, C$\underline{H}_2$-CH_3), 4.34 (m, 2H, N-CH_2), 5.03 (dd, ^3J = 5.9, 2.4 Hz, 1H, C$\underline{H}$-CH_2), 6.65 (br t, 1H, NH), 7.22-7.39 (m, 5H, ArH), 7.87 (br s, 1H, NH).

^{13}C-NMR ($CDCl_3$):

δ/ppm = 14.9 (CH_2-$\underline{C}H_3$), 21.5 (5'-CH_2), 25.9 (4'-CH_2), 30.1 (6'-CH_2), 45.8 (N-CH_2), 62.7 ($\underline{C}H_2$-CH_3), 64.7 (3'-CH_2), 102.0 (1'-CH), 127.9, 128.5, 129.2 (ArCH), 139.2 (ArC), 149.7 (C=N), 153.7 (CO).

MS (ESI):

m/z = 665 $[2 × M + Na]^+$, 322 $[M + H]^+$, 238 $[M – C_5H_8O + H]^+$.

$C_{16}H_{23}N_3O_4$ (321.37)

Calc.	C 59.80	H 7.21	N 13.08
Found	C 59.08	H 7.25	N 13.25

D. Enantiomeric purity.

General

Chromatography on chiral stationary phase was carried out using a Waters Breeze™ HPLC system: Waters 1525 binary pump, Waters 2487 dual wavelength absorbance detector, Waters 717 Plus autosampler; stationary phase: Crownpak Cr(+) column (250 × 4.6 mm) thermostated at 15 °C. For determination of %*ee* compounds were dissolved in *aqua bidest*. (1 mM) and 10 µL injected.

We did not synthesize D-enantiomers for this purpose but varied several chromatographic parameters that are known to affect the resolution of two enantiomers, i.e. column temperature, pH of eluent and flow-rate, and always observed highly symmetric peaks. In general, the separation factor (α) of similar amino acids on this chiral stationary phase is very high (we determined α = 2.83 for D,L-arginine, α = 2.59 for N^{δ}-methyl-D,L-arginine[13]). Thus, it appears very unlikely that substituents at the $N^{\delta\omega}$-position would dramatically impair the separation of the D,L-enantiomers. Essentially, based on the determined detection limit, we could determine %*ee* with a precision of ±0.1 % for many of the synthesized NOHA prodrugs.

The most highly substituted prodrugs (**8d**, **9c**) could not be analyzed using this chromatographic method as they were retained too long on the column, even with 10 % MeOH at 1.0 mL/min. Higher MeOH contents are not recommended for this stationary phase (manufacturer information).

O-Methylated NOHA prodrugs (8)

For the *O*-methylated NOHA prodrugs, we determined enantiomeric purities (L-enantiomers) of > 99,5 %*ee*. It is to be noted that for the analysis of the ethyl esters and carbamates, up to 2 % MeOH had to be added to the eluent. Otherwise, the compounds are retained for > 25 min on the column, resulting in peak broadening.

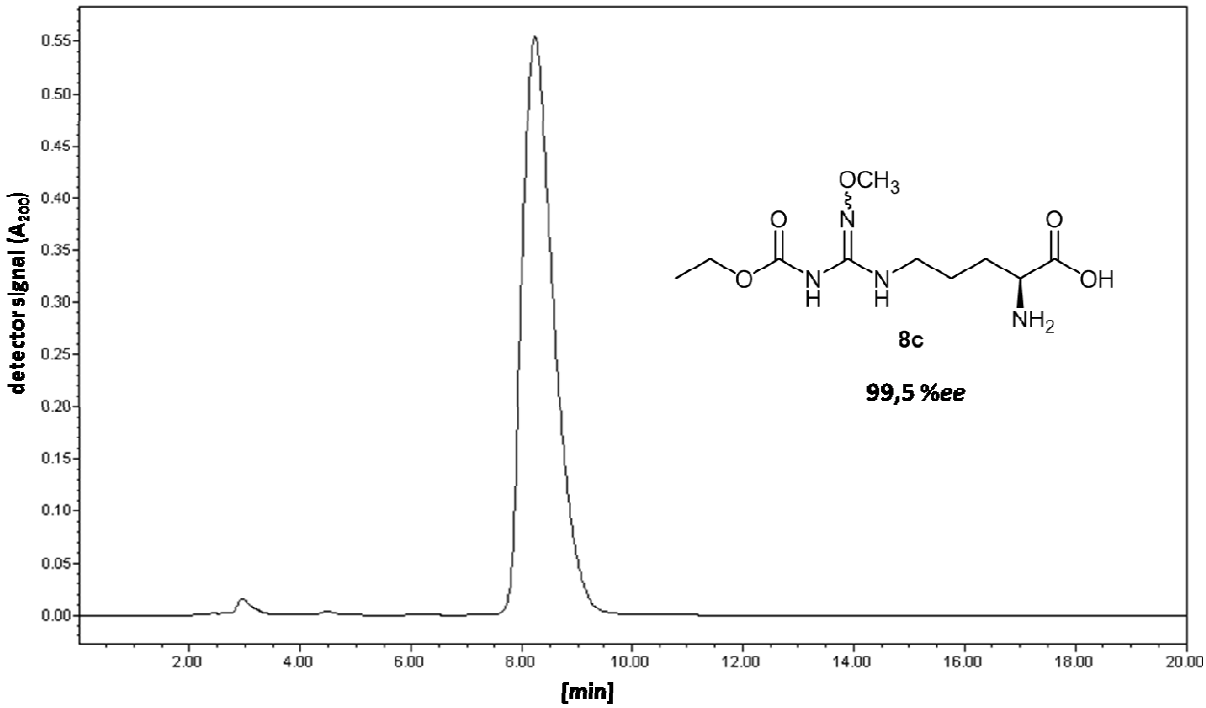


Figure S1: Representative chromatogram of N^{ω}-ethoxycarbonyl-$N^{\omega'}$-methoxy-L-arginine (**8c**) using a Crownpak Cr(+) column. Eluent: $HClO_4$ in water (*aqua bidest.*), pH 1.5, 2 % (v/v) MeOH; flow rate: 0.4 mL/min; detection: 200 nm (void volume: 3.1 min).

Table S2: Crownpak Cr(+) analytic of different N^{ω}-methoxy-L-arginine prodrugs (8)

compound	MeOH content[a] (%, v/v)	Flow rate (mL/min)	Retention time (min)	%*ee*
8a[3]	0	0.6	6.6 ± 0.1	99.9 ± 0.1
8b	2	0.4	9.1 ± 0.1	99.6 ± 0.1
8c	2	0.4	8.2 ± 0.2	99.5 ± 0.1

[a] in eluent = $HClO_4$ in water (*aqua bidest.*), pH 1.5

O-Carboxymethylated NOHA prodrugs (9)

For the *O*-carboxymethylated NOHA prodrugs, we determined enantiomeric purities (L-enantiomers) of > 99 %*ee*. Probably, the reaction conditions required for complete deprotection of **6b** to obtain the desired NOHA derivative **9a** already led to some racemization as 99.1 %*ee* were determined.

However, the determined 99.0 %*ee* for **9b** were surprising considering that identical synthetic conditions were applied as those described for the *O*-methyl series of NOHA prodrugs (**8**). One explanation may be that impurities of 0.4-0.5 % coeluted with the respective D-enantiomer, thereby falsifying this result.

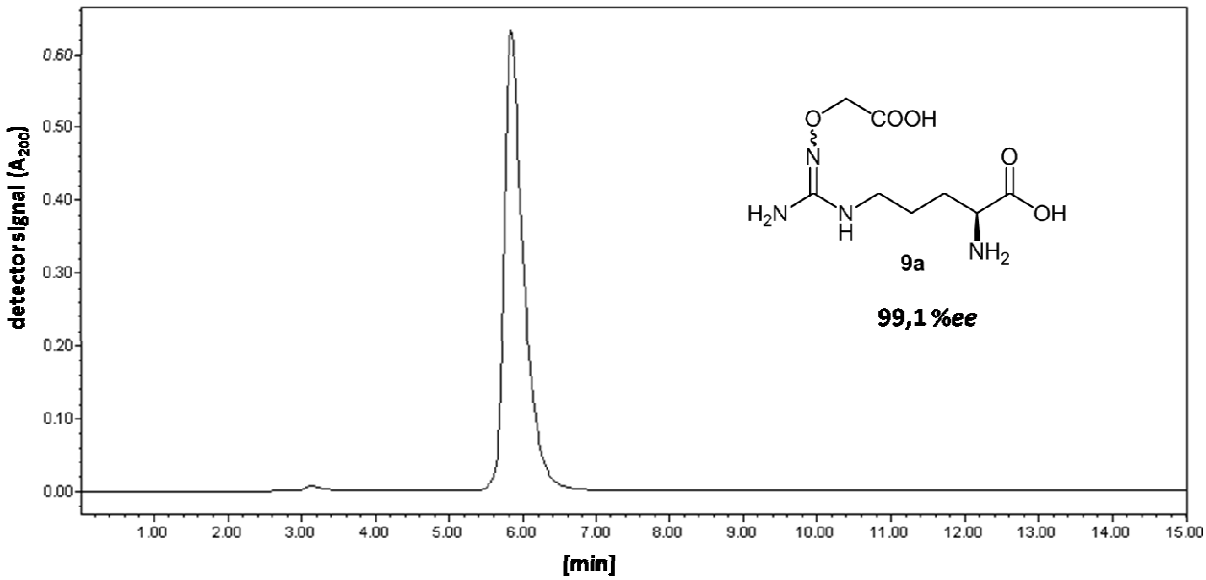


Figure S2: Representative chromatogram of N^{ω}-carboxymethoxy-L-arginine (**9a**) using a Crownpak Cr(+) column. Eluent: $HClO_4$ in water (*aqua bidest.*), pH 1.5, flow rate: 0.4 mL/min; detection: 200 nm (void volume: 3.1 min).

Table S3: Crownpak Cr(+) analytic of different N^{ω}-carboxymethoxy-L-arginine prodrugs

compound	MeOH content[a] (%, v/v)	Flow rate (mL/min)	Retention time (min)	%*ee*
9a	0	0.4	5.6 ± 0.2	99.1 ± 0.1
9b	10	1.0	8.8 ± 0.2	99.0 ± 0.1

[a] in eluent = $HClO_4$ in water (*aqua bidest.*), pH 1.5.

E. In vitro assays.

Material and methods

Commercially available materials were purchased from either Sigma Aldrich, Fluka, Roth or Merck. HPLC-grade methanol was obtained from Mallinckrodt Baker. D-Galactose concentration was analysed using the testkit "Lactose/Galactose" of Boehringer Mannheim.

Analytical reversed phase HPLC

Amino acids were derivatized with *o*-Pa and separated at 37 °C on a Nova-Pak RP_{18} 4 µm (4 x 150 mm, VDS Optilab) with a Phenomenex C18, 4 x 3 mm guard column. HPLC consisted of a Waters autosampler 717plus, Waters 600 Controller and a Waters 470 scanning fluorescence detector, set at λ_{ex}: 338 nm, λ_{em}: 425 nm. For derivatization the autosampler was set to mix 14 µL of *o*-Pa reagent with 10 µL of sample and allowed to react for two minutes at room temperature before injection. *o*-Pa reagent was prepared by dissolving 50 mg *o*-Pa in 9 mL of 0.2 M potassium borate buffer pH 9.4 and 53 µL of 2-mercaptoethanol. Eluent A: 10 mM potassium phosphate buffer (pH 4.7) / methanol (78 %/22 %); eluent B: methanol / aqua bidest. (80 %/20 %).

The following elution conditions were used: flow-rate was kept at 1 mL/min; 0 – 12 min isocratic with 90 % eluent A and 10 % eluent B; 12 – 25 min linear gradient to 100 % eluent B, 25 – 27 min, linear gradient to 90 % eluent A, 27 – 35 min reequilibration with 90 % eluent A and 10 % eluent B.

For *N*-benzyl-*N*'-ethoxycarbonyl-*N*''-[β-D-galactopyranos-1-yl)oxy]-guanidine (**13**) HPLC analytics were similar with the following alterations: Compounds were detected at 210 nm using a Waters 2487 dual wavelength absorbance detector. The elution was carried out isocratically at a flow rate of 1 mL/min with potassium phosphate (pH 4.7) / methanol (72 %/28 %).

Bioactivation of β-D-galactose conjugate 13.

Assay protocol:

A standard HPLC assay consisted of 0.5 U bovine β-galactosidase, 500 µM substrate, 1 mM $MgCl_2$ and 10 mM 2-mercaptoethanol in 200 µL of 50 mM potassium phosphate buffer pH 6.3. Incubations were performed at 37 °C. The reactions were stopped after 30 min by the addition of 200 µL ice-cold methanol. Samples were centrifuged (10000 rpm, 5 min) and analysed by HPLC as described above.

Additionally, this in vitro biotransformation was analysed via UV spectroscopy. The prodrug was incubated for 30 min as described above in a final volume of 300 µL. The reaction was stopped by adding 1 mL 100 mM potassium phosphate buffer pH 8.6 and 1.9 ml aqua bides. Absorbance A1 was measured at 340 nm. Subsequent, 1.2 U galactose-dehydrogenase and 1 mM NAD^+ were added, mixed and the formation of NADH was determined at 340 nm (A2). ΔA was used for the calculation of the Galactose concentration.

To demonstrate proof-of-principle that galactose conjugates of *N*-hydroxyguanidines are generally accepted as substrates by glycosidases and sufficiently bioactivated, the simple benzyl-based model compound **13** was incubated with bovine β-galactosidase and analyzed with two different methods (see figure S3). The quantification of **13** and its metabolite **14** by HPLC revealed a turnover rate of 15.7 $nmol \cdot min^{-1} \cdot mg^{-1}$ protein. A biochemical assay that quantifies released galactose showed a similar activity of 17.7 $nmol \cdot min^{-1} \cdot mg^{-1}$ protein. This experiment clearly demonstrates the substrate quality of **13** and suggests that other comparable conjugates are also efficiently catalyzed. In this regard, we surmise that the respective galactose conjugate of NOHA **10** would be bioactivated after partial or complete esterase-catalyzed cleavage of its *O*-acetyl groups.

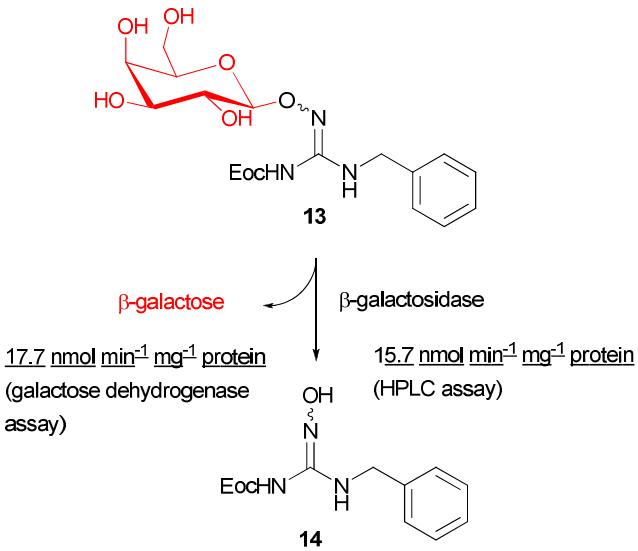


Figure S3. In vitro bioactivation of the *N*-hydroxyguanidine β-D-galactose conjugate **13** as a model compound.

Hydrolytic stability.

Compounds were dissolved in 10 mM potassium phosphate buffer pH 2, pH 7.4 and pH 9 to a final concentration of 250 µM. Samples were incubated at 37 °C and reactions analyzed immediately at the desired time points. Subsequent samples were analyzed by HPLC as described above.

For summarized data, see main article. Below you find a detailed view on degradation reactions of selected NOHA prodrugs at different pHs (figure S4).

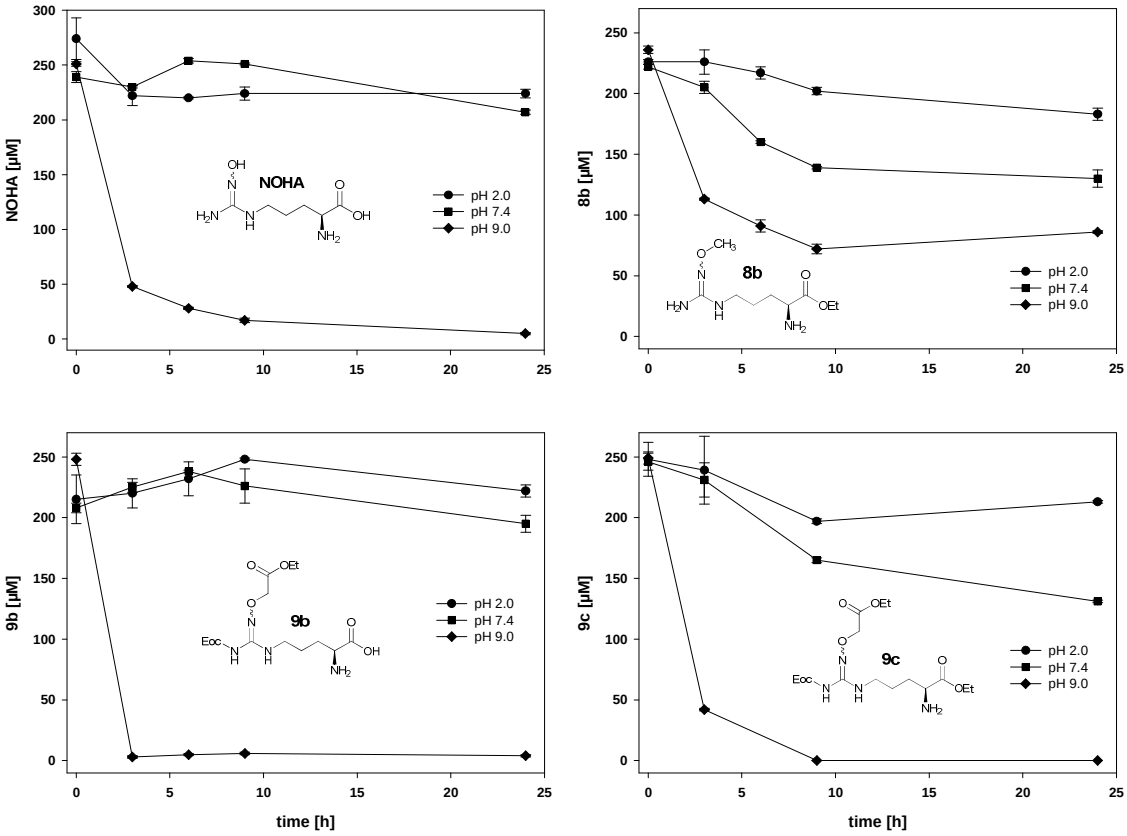


Figure S4: pH stability time curves: 250 µM compounds (**NOHA**, **8b**, **9b** and **9c**) in 10 mM potassium phosphate buffer pH 2, pH 7.4 and pH 9 after incubation at 37 °C for 0, 3, 6, 9 and 24 h (mean value of at least three independent experiments ± SD)

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