Supporting Information
for

Carbohydrate-Based Synthetic Approach to Control Toxicity Profiles of Folate-Drug Conjugates


Endocyte Inc., 3000 Kent Ave, Suite A1-100, West Lafayette, IN 47906, USA

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Experimental Section

Reagents & Instrumentation:

Anhydrous solvents including CH$_2$Cl$_2$, CHCl$_3$, DMF, DMSO, pyridine, EtOAc, petroleum ether and other reagents were obtained commercially and were used as received. Moisture and oxygen sensitive reactions were carried out under argon with standard syringe/septa techniques unless otherwise noted. Thin layer chromatography (TLC) was performed on silica gel TLC plates (EMD Silica Gel 60 F$_{254}$, 5 × 10 cm). $^1$H and $^{13}$C NMR spectra were recorded on 300 MHz and 800 MHz spectrometers and are referenced to residual CHCl$_3$ and DMSO. Analytical and preparative HPLC were carried out using a Waters X-Bridge C18, 5 µm, 3.0×50 mm column and Waters Novapak C18 or XTerra C18, 7 µm, 19×300 mm respectively. 10 mM Ammonium acetate, pH = 5.0, 2.0 mM sodium phosphate pH = 7.0, and acetonitrile were used as the mobile phases for the HPLC chromatography.

Synthesis of methyl 3,4;5,6-di-O-isopropylidene-D-gluconate 4:

\[
\text{C}_{13}\text{H}_{22}\text{O}_7\quad \text{Exact Mass: 290.14 } \text{Mol. Wt.: 290.31}
\]

In a dry 250 mL round bottom flask, under argon δ-gluconolactone (4.14 g, 23.24 mmol) was suspended in acetone-methanol (1:1, 50 mL). To this suspension, dimethoxypropane (17.15 mL, 139.44 mmol) followed by catalytic amount of $p$-toulenesulfonic acid (200 mg) were added. This solution was stirred at room temperature for 16 h. TLC (50% EtOAc in petroleum ether) showed that all of the starting material had been consumed and product had been formed. Acetone-methanol was removed under reduced pressure. The residue of the reaction was dissolved in EtOAc and washed with water. The organic layer was washed with brine, dried over Na$_2$SO$_4$, and concentrated to dryness. This material was then loaded onto a SiO$_2$ column and chromatographed (30% EtOAc in petroleum ether) to yield pure methyl 3,4,5,6-di-O-isopropylidene-D-gluconate (3.8 g, 56%) and regio-isomer methyl 2,3;5,6-di-O-isopropylidene-D-gluconate (0.71 g, 10%).

Synthesis of methyl 3,4;5,6-di-O-isopropylidene-2-azido-2-deoxy-D-mannate 5:

\[
\text{C}_{13}\text{H}_{21}\text{N}_3\text{O}_6\quad \text{Exact Mass: 315.14 } \text{Mol. Wt.: 315.32}
\]

In a dry 100 mL round bottom flask, under argon methyl 3,4,5,6-di-O-isopropylidene-D-gluconate (3.9 g, 13.43 mmol) was dissolved in methylene chloride (40
mL) and cooled to -20 °C to -25 °C. To this solution pyridine (3.26 mL, 40.29 mmol) followed by triflic anhydride (3.39 mL, 20.15 mmol) were added. This white turbid solution was stirred at -20 °C for 1 h. TLC (25% EtOAc in petroleum ether) showed that all of the starting material had been consumed and product had been formed. The reaction mixture was poured into crushed-ice and extracted with diethyl ether. The organic layer was washed with water, brine, dried over Na₂SO₄, and concentrated to yield methyl 3,4;5,6-di-O-isopropylidene-2-amino-2-deoxy-D-mannate (5.5 g, 97%). This material was dissolved in DMF (20 mL). To this solution NaN₃ (0.93 g, 14.32 mmol) was added. This solution was stirred at room temperature for 1 h. TLC (8% EtOAc in petroleum ether, triple run) showed that all of the starting material had been consumed and product had been formed. DMF was removed under reduced pressure. The reaction mixture was diluted with brine and extracted with EtOAc. The organic layer was washed with water, brine, dried over Na₂SO₄, and concentrated to dryness. This crude material was then loaded onto a SiO₂ column and chromatographed (20% EtOAc in petroleum ether) to yield pure methyl 3,4;5,6-di-O-isopropylidene-2-amino-2-deoxy-D-mannate (3.8 g, 93%).

**Synthesis of methyl 3,4;5,6-di-O-isopropylidene-2-amino-2-deoxy-D-mannate 6:**

![Chemical Structure](image)

C₁₃H₂₉NO₆
Exact Mass: 289.15
Mol. Wt.: 289.32

In a dry 100 mL round bottom flask, methyl 3,4;5,6-di-O-isopropylidene-2-amino-2-deoxy-D-mannate (1.96 g, 6.22 mmol) was dissolved in toluene (30 mL). To this solution Bu₃SnH (5.43 g, 18.67 mmol) was added. The resulting clear solution was stirred at room temperature for 24 h. TLC (10% methanol in methylene chloride) showed that all of the starting material had been consumed and product had been formed. Toluene was removed under reduced pressure and crude material was then loaded onto a SiO₂ column and chromatographed (2% methanol in methylene chloride) to yield pure methyl 3,4;5,6-di-O-isopropylidene-2-amino-2-deoxy-D-mannate (1.26 g, 70%).

**Synthesis of 3,4;5,6-di-O-isopropylidene-2-deoxy-2-(Fmoc-amino)-D-mannonic acid 8:**

![Chemical Structure](image)

C₂₇H₃₁NO₈
Exact Mass: 497.20
Mol. Wt.: 497.54

In a dry 100 mL round bottom flask, methyl 3,4;5,6-di-O-isopropylidene-2-amino-2-deoxy-D-mannate (1.24 g, 4.29 mmol) was dissolved in THF/MeOH (20 mL/5 mL). To this solution LiOH·H₂O (215.8 mg, 5.14 mmol) in water (5 mL) was added. This light yellow solution was stirred at room temperature for 2 h. TLC (10% methanol in methylene chloride) showed that all of the starting material had been consumed and product had been formed. THF/MeOH was removed under reduced pressure. The aqueous phase was re-suspended in sat. NaHCO₃ (10 mL). To this suspension Fmoc-
OSu (1.74 g, 5.14 mmol) in 1,4-dioxane (10 mL) was added. This heterogeneous solution was stirred at room temperature for 18 h. TLC (10% methanol in methylene chloride) showed that most of the starting material had been consumed and product had been formed. Dioxane was removed under reduced pressure. The aqueous layer was extracted with diethyl ether to remove the less polar impurities. Then the aqueous layer was acidified to pH 6 using 0.2N HCl, and re-extracted with EtOAc. The EtOAc layer was washed with brine, dried over Na₂SO₄, and concentrated to yield 3,4;5,6-di-O-isopropylidene-2-deoxy-2-(Fmoc-amino)-D-mannonic acid (1.6 g, 76%). This material was used in the next reaction without further purification.

**Synthesis of Pte-γGlu-Mann-Asp-Mann-Asp-Mann-Cys-OH 2**
(abbreviation “Mann” refers to completely deprotected mannonic acid 8 (peptide chain form)):

![Chemical Structure Image]

**Table: Reagents for peptide synthesis**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>mmol</th>
<th>equivalent</th>
<th>MW (g/mol)</th>
<th>Amount</th>
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<td>3,4;5,6-di-O-isopropylidene-2-deoxy-2-(Fmoc-amino)-D-mannonic acid</td>
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<td>Fmoc-Asp(Or-Bu)-OH</td>
<td>0.4</td>
<td>2</td>
<td>411.5</td>
<td>0.165 g</td>
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<tr>
<td>3,4;5,6-di-O-isopropylidene-2-deoxy-2-(Fmoc-amino)-D-mannonic acid</td>
<td>0.25</td>
<td>1.25</td>
<td>497.54</td>
<td>0.124 g</td>
</tr>
<tr>
<td>Fmoc-Asp(Or-Bu)-OH</td>
<td>0.4</td>
<td>2</td>
<td>411.5</td>
<td>0.165 g</td>
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<tr>
<td>3,4;5,6-di-O-isopropylidene-2-deoxy-2-(Fmoc-amino)-D-mannonic acid</td>
<td>0.25</td>
<td>1.25</td>
<td>497.54</td>
<td>0.124 g</td>
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mannonic acid |  |  |  |  |
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<tr>
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<tr>
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<td>DIPEA</td>
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<td>4</td>
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<td>(d = 0.742)</td>
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<tr>
<td>PyBOP</td>
<td>0.4</td>
<td>2</td>
<td>520</td>
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**Coupling steps:**

In a peptide synthesis vessel the resin, the amino acid solution, DIPEA, and PyBOP were added. Argon was bubbled for 1hr. the resin was washed DMF and IPA (3 × 10 mL). 20% piperdine in DMF was then added for Fmoc deprotection (3 × for 10 min), before each subsequent amino acid coupling. This procedure was repeated until all seven coupling steps were repeated. The resin was then washed with 2% hydrazine in DMF (3 × 5 min) to cleave the TFA protecting group on pteroic acid.

**Cleavage step:**

Reagent:

92.5% (50 mL) TFA, 2.5% (1.34 mL) H\textsubscript{2}O, 2.5% (1.34 mL) triisopropylsilane, 2.5% (1.34 mL) ethanedithiol.

The cleavage reagent (25 mL) was added to the resin and bubbled for 20 min and drained into a clean vessel. The resin was washed 3 × with remaining reagent. The combined filtrates were concentrated under reduced pressure to a volume of 5 mL. Ethyl ether was then added to precipitate the crude peptide and the mixture centrifuged, and washed with ethyl ether to recover the crude product.

**HPLC Purification:**

Column : Waters NovaPak C\textsubscript{18} 300 × 19mm

Buffer A= 10mM ammonium acetate, pH 5

B= ACN

Method : 1%B to 20%B in 40 minutes at 15ml/min

**Total yield:** ~208 mg, 65%
Synthesis of 3,4;5,6-di-\(O\)-isopropylidene-\(D\)-gluconic amide 17:

Methyl 3,4;5,6-di-\(O\)-isopropylidene-\(D\)-gluconate (20 g) was dissolved in methanol (100 mL), and added to a high-pressure reaction vessel. The reactor was cooled with a dry ice/acetone bath, charged with 100 mL liquid ammonia, warmed up to room temperature, and heated to 160 °C/850 PSI for 2 hours. The reaction vessel was cooled to room temperature and the pressure released. Evaporation of the solvent gave a brownish syrup. To this was added a minimum amount of isopropyl alcohol and the mixture was refluxed to homogenize the mixture. The solution was cooled to -20 °C and the resulting solid was filtered to give 8.3 g of solid. The mother liquor was evaporated, and to the resulting residue, was added diethyl ether, and the mixture was refluxed until a homogeneous solution was achieved. The solution was then cooled to -20 °C and the resulting solid was filtered to give 4.0 g of product. The solid was combined and recrystallized in isopropyl alcohol to give 3,4;5,6-di-\(O\)-isopropylidene-\(D\)-gluconic amide (11.2 g, 59%) as a white powder.

Synthesis of 3,4;5,6-di-\(O\)-isopropylidene-1-amino-1-deoxy-\(D\)-glucitol 18:

In a dry 100 mL round bottom flask, under argon, LiAlH\(_4\) (450 mg, 11.86 mmol) was dissolved in THF (10 mL) and cooled to 0 °C. To this suspension 3,4;5,6-bisacetonide-\(D\)-gluconic amide (1.09 g, 3.96 mmol) in THF (30 mL) was added very slowly over 15 min. This mixture was refluxed for 5 h. TLC (10% MeOH in methylene chloride) showed that all of the starting material had been consumed and product had been formed. The reaction mixture was cooled to room temperature, and then cooled to ice-bath temperature. The reaction mixture was then diluted with diethyl ether (40 mL), followed by water (0.5 mL), 15 % aq. NaOH (0.5 mL), and then an additional 1.5 mL of water. The reaction mixture was warmed to room temperature and stirred for 30 min. MgSO\(_4\) was added and stirred for additional 15 min and filtered. The organic layer was concentrated to dryness to yield crude 3,4;5,6-bisacetonide-1-amino-1-deoxy-\(D\)-glucitol (1.0 g). This material was used in the next step without further purification.
$^1$H NMR Spectrum of methyl 3,4;5,6-di-$O$-isopropyldiene-$D$-gluconate 4
H NMR Spectrum of methyl 3,4,5,6-di-O-isopropylidene-2-azido-2-deoxy-D-mannate 5
$^1$H NMR Spectrum of methyl 3,4;5,6-di-$O$-isopropylidene-2-amino-2-deoxy-2-$D$-mannate 6
$^1$H NMR Spectrum of 3,4;5,6-di-O-isopropylidene-2-deoxy-2-(Fmoc-amino)-D-mannonic acid 8
$^1$H NMR Spectrum of Pte-$\gamma$Glu-Mann-Asp-Mann-Asp-Mann-Asp-Mann-Cys-OH
HPLC Chromatogram of Pte-γGlu-Mann-Asp-Mann-Asp-Mann-Asp-Mann-Cys-OH 2

**SAMPLE INFORMATION**

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**Auto-Scaled Chromatogram**

**Peak Results**

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Report Method: Untitled
Mass Chromatogram of Pte-γGlu-Mann-Asp-Mann-Asp-Mann-Asp-Mann-Asp-Mann-Cys-OH 2
LCMS Chromatogram of Pte-γGlu-Mann-Asp-Mann-Asp-Mann-Asp-Mann-Cys-OH (2 + Aspartimide isomer)
HPLC Chromatogram of peeling of Cysteine from Pte-γGlu-Mann-Cys-OH
Mass Chromatogram of peeling of Cysteine from Pte-γGlu-Mann-Cys-OH
$^1$H NMR Spectrum of 3,4;5,6-di-O-isopropylidene-D-gluconic amide 17
H NMR Spectrum of 3,4,5,6-di-O-isopropylidene-1-amino-1-deoxy-D-glucitol 18
$^1$H NMR Spectrum of 3,4;5,6-di-$O$-isopropylidene-1-amino-1-deoxy-(Fmoc-Glu-Oallyl)-D-glucitol 19
$^{13}$C NMR Spectrum of 3,4;5,6-di-$O$-isopropylidene-1-amino-1-deoxy-1-(Fmoc-Glu-Oallyl)-D-glucitol 19
$^1$H NMR Spectrum of 3,4;5,6-di-$O$-isopropylidene-1-amino-1-deoxy-\((\text{Fmoc-Glu-OH})\)-D-glucitol 20
$^{13}$C NMR Spectrum of 3,4;5,6-di-O-isopropylidene-1-amino-1-deoxy-(Fmoc-Glu-OH)-D-glucitol 20
$^1$H NMR Spectrum of Pte-γGlu-Glu(1-amino-D-glucitol)-αGlu-Glu(1-amino-D-glucitol)-αGlu–Glu(1-amino-D-glucitol)-Cys-OH 21
$^{13}$C NMR Spectrum of Pte-$\gamma$Glu-Glu(1-amino-D-glucitol)-$\alpha$Glu-Glu(1-amino-D-glucitol)-$\alpha$Glu-Glu-HCys-OH
HPLC & LCMS Chromatogram of \( \text{Pte-} \gamma \text{Glu-Glu(1-amino-D-glucitol)-} \alpha \text{Glu-Glu(1-amino-D-glucitol)-} \alpha \text{Glu-Glu(1-amino-D-glucitol)} \text{-Cys-OH 21} \)
$^1$H NMR Spectrum of Pte-γGlu-Glu(1-amino-d-glucitol)-αGlu-Glu(1-amino-d-glucitol)-αGlu-Glu(1-amino-d-glucitol)-Cys(S-ethyl-3-(4-desacetylvinblastinyl)-hydrazinecarboxylate) 25
\(^{13}\text{C} \text{NMR Spectrum of Pte-} \gamma\text{Glu-Glu(1-amino-D-gluicitol)-}\alpha\text{Glu-Glu(1-amino-D-gluicitol)-}\alpha\text{Glu-Glu(1-amino-D-gluicitol)-Cys(S-ethyl-3-(4-desacetylvinblastinyl)-hydrazinecarboxylate)} \text{ 25}
HPLC & LCMS Chromatogram of Pte-γGlu-Glu(1-amino-D-glucitol)-αGlu-Glu(1-amino-D-glucitol)-αGlu–Glu(1-amino-D-glucitol)-Cys(S-ethyl-3-(4-desacetylvinblastinyl)-hydrazinecarboxylate) 25

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