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

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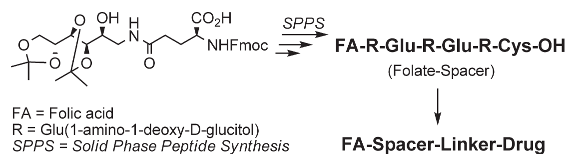

To better regulate the biodistribution of the vinblastine−folate conjugate, EC145, a new folate−spacer that incorporates 1-amino-1-deoxy-D-glucitol-γ-glutamate subunits into a peptidic backbone, was synthesized. Synthesis of Fmoc-3,4;5,6-di-*O*-isopropylidene-1-amino-1-deoxy-D-glucitol-γ-glutamate **20**, suitable for Fmoc-strategy solid-phase peptide synthesis (SPPS), was achieved in four steps from δ-gluconolactone. Addition of alternating glutamic acid and **20** moieties onto a cysteine-loaded resin, followed by the addition of folate, deprotection, and cleavage, resulted in the isolation of the new folate−spacer: Pte-γGlu-(Glu(1-amino-1-deoxy-D-glucitol)-Glu)$_2$-Glu(1-amino-1-deoxy-D-glucitol)-Cys-OH (**21**). The addition of **21** to an appropriately modified desacetylvinblastine hydrazide (DAVLBH) resulted in a conjugate (**25**) with an improved therapeutic index. Treatment of **25** with DTT in neutral buffer at room temperature demonstrated that free DAVLBH would be released under the reductive environment of the internalized endosome.

Introduction

Receptor-targeted chemotherapy has emerged as one of the major approaches in modern drug discovery as it can potentially satisfy the selective delivery criteria for toxic agents to pathologic cells. In a previous publication,[1] we reported the design and synthesis of a folate-targeted desacetylvinblastine hydrazide (DAVLBH) conjugate, EC145, which is currently in phase 2 clinical trials. In EC145, the anticancer drug vinblastine was modified and attached to folic acid (FA) via a highly charged water-soluble peptide-based spacer unit (**1**, Figure 1) and a self-immolative linker system containing a reducible disulfide bond. Once administered, the conjugate EC145 targets cancer cells which overexpress folate receptor (FR) and releases the base drug after internalization.[2] Receptor-targeted delivery reduces collateral toxicity, resulting in improved efficacy. The dose-limiting toxicity for EC145 was found to be related to ileus, albeit in heavily pretreated patients.[3] Because of the low to undetectable levels of the folate receptor (FR) expressed in hepatic tissue,[4] it was suspected that non-FR related liver clearance, with subsequent metabolic release of free DAVLBH, may have been (in part) responsible for producing the observed gastrointestinal toxicity. Support for our hypothesis came from (i) our ability to block the liver uptake of folate conjugates with coinjected bromosulfophthalein (an organic anion transporter inhibitor) and (ii) the detection of free DAVLBH in the bile of rats that had been intravenously dosed with EC145.

Work by Suzuki et al. on alkylglycoside-derivatized arginine vasopressin (AVP) suggested a method to modify the biodistribution.[5] When AVP is modified, particularly by specific glucopyranosyl, mannopyranosyl, and 2-deoxyglucopyranosyl

(1) Vlahov, I. R.; Santhapuram, H. K.; Kleindl, P. J.; Howard, S. J.; Stanford, K. M.; Leamon, C. P. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5093.

(2) Leamon, C. P.; Reddy, J. A. *Adv. Drug Delivery Rev.* **2004**, *56*, 1127. Reddy, J. A.; Dorton, R.; Westrick, E.; Dawson, A.; Smith, T.; Xu, L. C.; Vetzel, M.; Kleindl, P. J.; Vlahov, I. R.; Leamon, C. P. *Cancer Res.* **2007**, *67*, 4434. Yang, J.; Chen, H.; Cheng, J.-X.; Vlahov, I. R.; Low, P. S. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 13872.

(3) Li, J.; Sausville, E. A.; Klein, P. J.; Morgenstern, D.; Leamon, C. P.; Messmann, R. A.; LoRusso, P. *J. Clin. Pharm.* **2009**, *49*, 1467.

(4) Parker, N.; Turk, M. J.; Westrick, E.; Lewis, J. D.; Low, P. S.; Leamon, C. P. *Anal. Biochem.* **2005**, *338*, 284.

(5) Reviewed in: Molema, G.; Meijer, D. K. F. *Drug Targeting: Organ Specific Strategies*; Wiley-VCH: New York, 2001; Vol. 12, p 126. Suzuki, K.; Susaki, H.; Okuno, S.; Yamada, H.; Watanabe, H. K.; Sugiyama, Y. *J. Pharmacol. Exp. Ther.* **1999**, *288*, 888.

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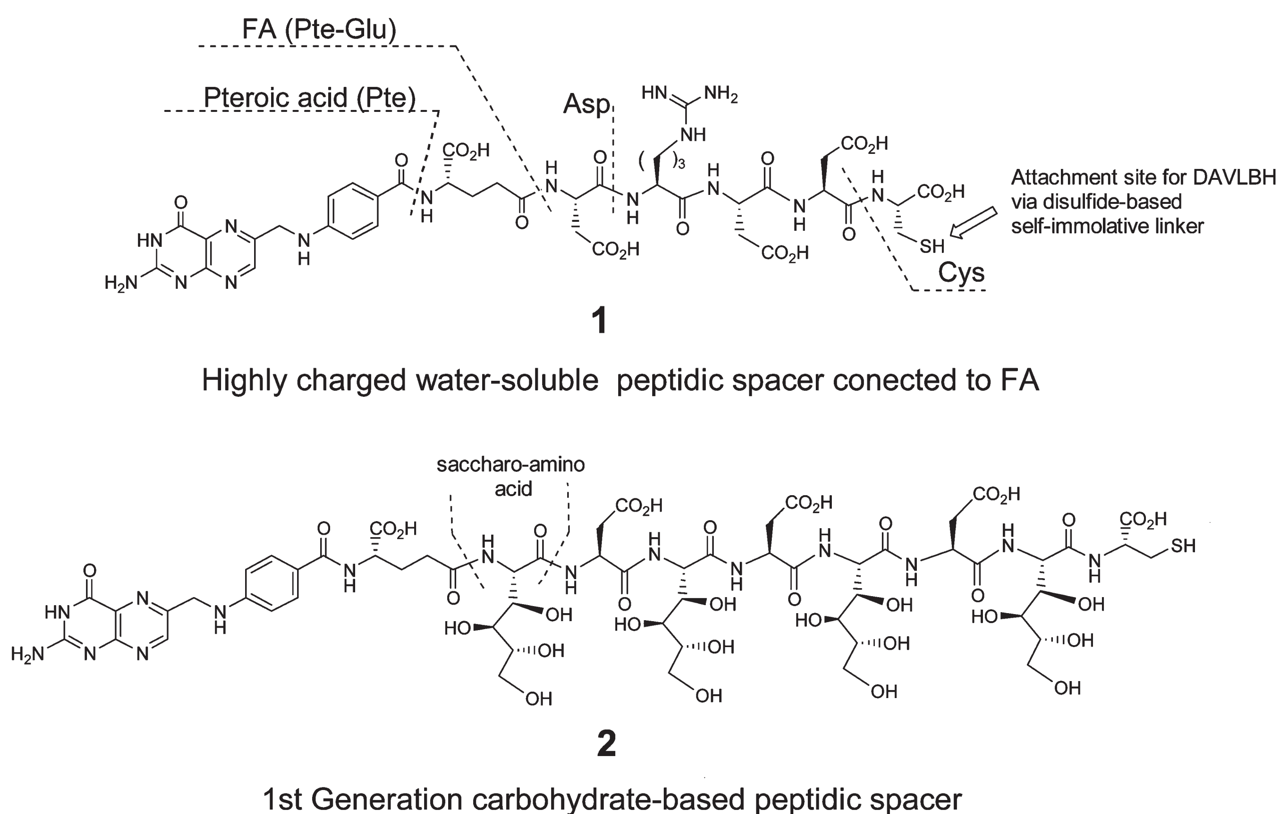
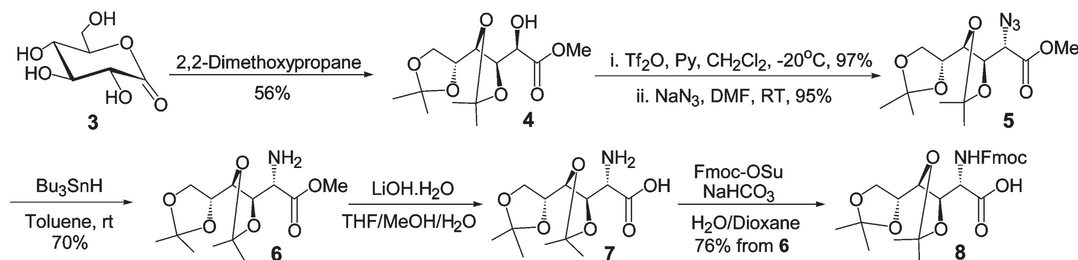


FIGURE 1. FA−spacer units.

SCHEME 1. Synthesis of 3,4;5,6-Di-*O*-isopropylidene-2-deoxy-2-(Fmoc-amino)-D-mannonic Acid



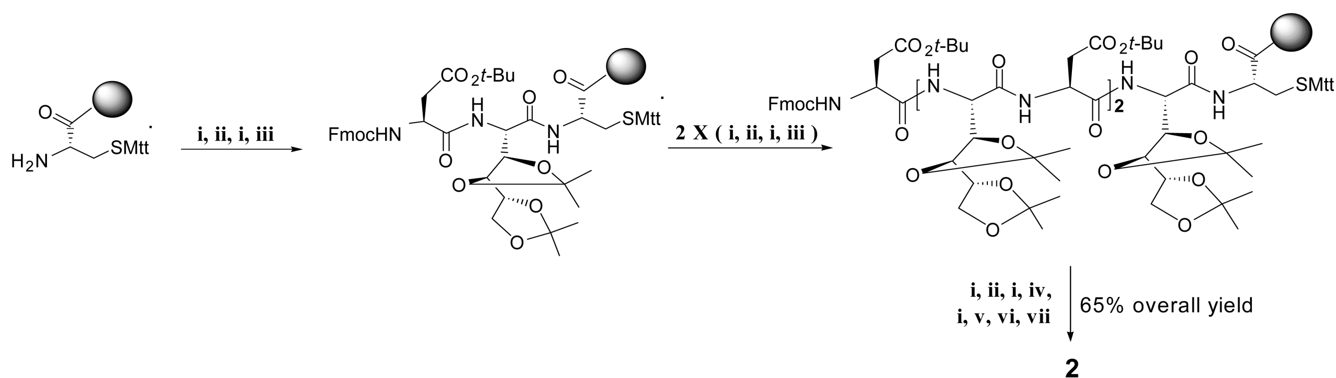
moieties, a significant shift in the partitioning of the pro-drug toward the kidneys is observed. Folate conjugates are known to have high kidney uptake with little associated toxicity.[4] A folate conjugate with carbohydrate character seemed to be a promising synthetic target. Such an approach could modulate the gastrointestinal toxicity.

Results and Discussion

In this paper, we report a novel, chemistry-based approach to substantially decrease the hepatobiliary route of clearance of free DAVLBH without affecting the conjugate's targeted antitumor activity. Thus, selective placement of structurally optimized carbohydrate segments in the spacer region resulted in conjugates that were equipotent but less toxic than EC145. Our first generation carbohydrate-containing FA−spacer unit **2** was designed to be bifunctional, containing alternately repeating acidic (Asp) and saccharo-amino acids, thus providing high water-solubility of the final drug conjugate under physiological conditions. This unit was assembled using standard fluorenylmethyloxycarbonyl-based solid-phase peptide synthesis (Fmoc SPPS) techniques. The structural design of the saccharo-amino acid was of crucial importance to the successful execution of a simple and high-yielding SPPS protocol. The selected approach relied on a ring-opened carbohydrate chain rather than on cyclic pyranoside or furanoside units. Pyranosides and furanosides are of limited usefulness for a simplified SPPS-based protocol because they require inherently tedious protecting/deprotecting strategies, utilize sensitive glycosyl donors, and generate diastereomeric glycosidic linkages. Therefore, we designed and synthesized uniformly protected 2-amino-2-deoxyglyconic acid derivatives that are orthogonally stable to SPPS. Thus, treatment of commercially available and inexpensive D-glucono-1,5-lactone **3** (Scheme 1) with 2,2-dimethoxypropane in acetone under mild acidic conditions resulted in the well-known methyl 3,4;5,6-di-*O*-isopropylidene-D-gluconate **4**.[6] According to Csuk and Vasella,[7]

(6) Redeling, H.; de Rouville, E.; Chittenden, G. *Recl. Trav. Chim. Pays-Bas* **1987**, *106*, 461.

SCHEME 2. Synthesis of First Generation Carbohydrate-Based FA−Spacer Unit 2[a]



[a]Reagents and conditions: (i) 20% piperidine, DMF; (ii) **8**, PyBop, DIPEA, DMF; (iii) Fmoc-Asp(O-*t*-Bu)-OH, PyBop, DIPEA, DMF; (iv) Fmoc-Glu-O-*t*-Bu, PyBop, DIPEA, DMF; (v) N^{10}-TFA-Pte-OH, PyBop, DIPEA, DMSO, DMF; (vi) 2% NH_2NH_2 in DMF; (vii) TFA, water, $HSCH_2CH_2SH$, *i*-Pr_3SiH.

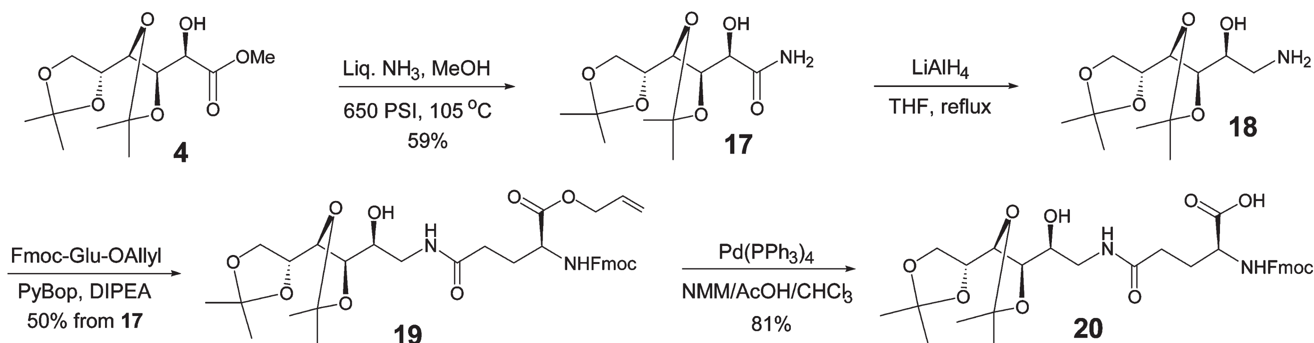
SCHEME 3. Optimizing the Carbohydrate-Based FA−Spacer Unit

1st Generation:
3 Major Problems!
1. Aspartimide Formation
R = *t*-Bu
2. Epimerization
B⁻
On the resin during SPPS
H⁺
During removal of the spacer from the resin
R' = H
3. "Peeling"
α-Asp peptide
β-Asp peptide
Epimerization Site
No Aspartimide Formation
(one C-atom more)
2nd Generation:
Solutions!
No "Peeling"
(No 5- or 6-membered ring possible)
No Epimerization possible

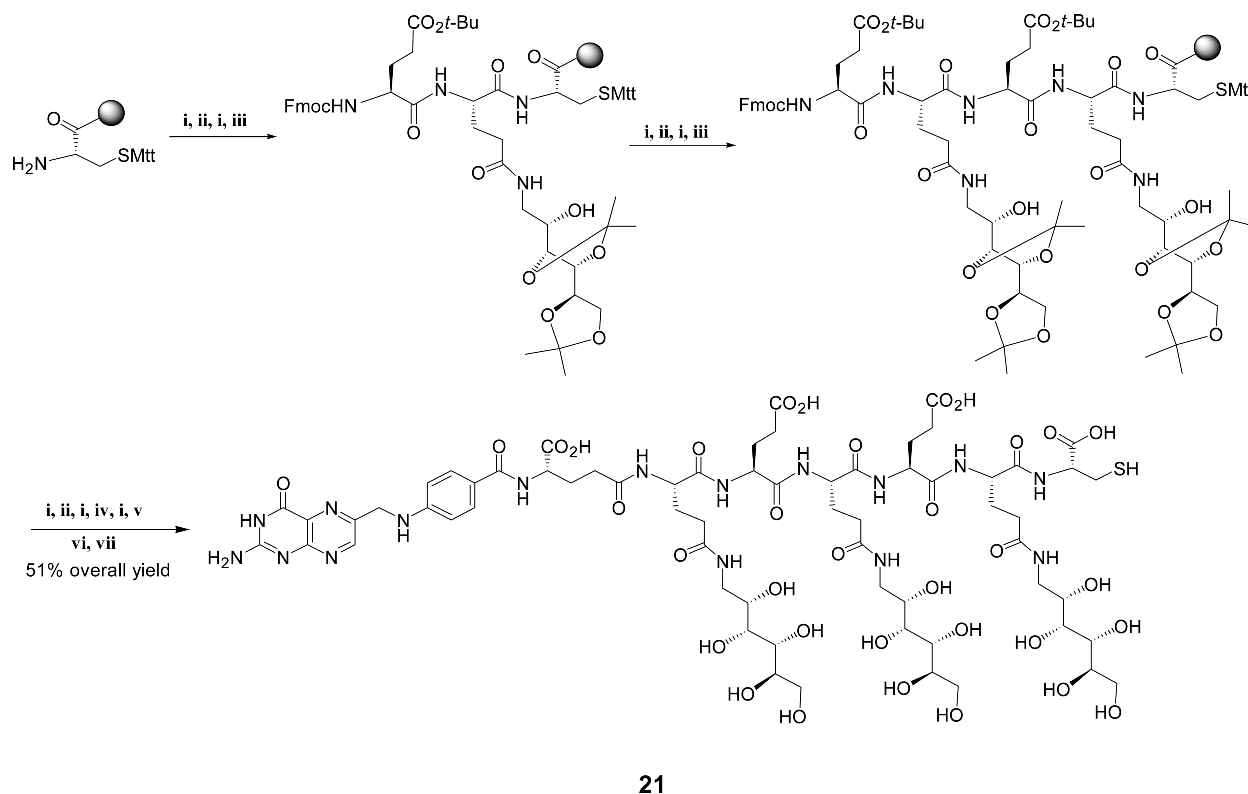
this compound was transformed via triflate into *manno*-azide **5**. Reduction with Bu_3SnH[8] followed by hydrolysis of the methyl ester[9] and introduction of an Fmoc group resulted in protected saccharo-amino acid derivative **8**.

(7) Csuk, R.; Vasella, A. *Helv. Chem. Acta* **1988**, *71*, 609.
(8) Vlahov, I. R.; Vlahova, P. I.; Schmidt, R. R. *Tetrahedron Lett*. **1992**, *33*, 7503.
(9) Lee, S. G.; Park, K. H.; Yoon, Y. *J. Heterocycl. Chem*. **1998**, *35*, 711.

SCHEME 4. Synthesis of Fmoc-3,4;5,6-di-*O*-isopropylidene-1-amino-1-deoxy-D-glucitol-γ-glutamate



SCHEME 5. Synthesis of the Second Generation Carbohydrate-Based Folate−Spacer Unit[a]



[a]Reagents and conditions: (i) 20% piperidine, DMF; (ii) **20**, PyBop, DIPEA, DMF; (iii) Fmoc-Glu(O-*t*-Bu)-OH, PyBop, DIPEA, DMF; (iv) Fmoc-Glu-O-*t*-Bu, PyBop, DIPEA, DMF; (v) N^{10}-TFA-Pte-OH, PyBop, DIPEA, DMSO, DMF; (vi) 2% NH_2NH_2 in DMF; (vii) TFA, water, $HSCH_2CH_2SH$, *i*-Pr_3SiH.

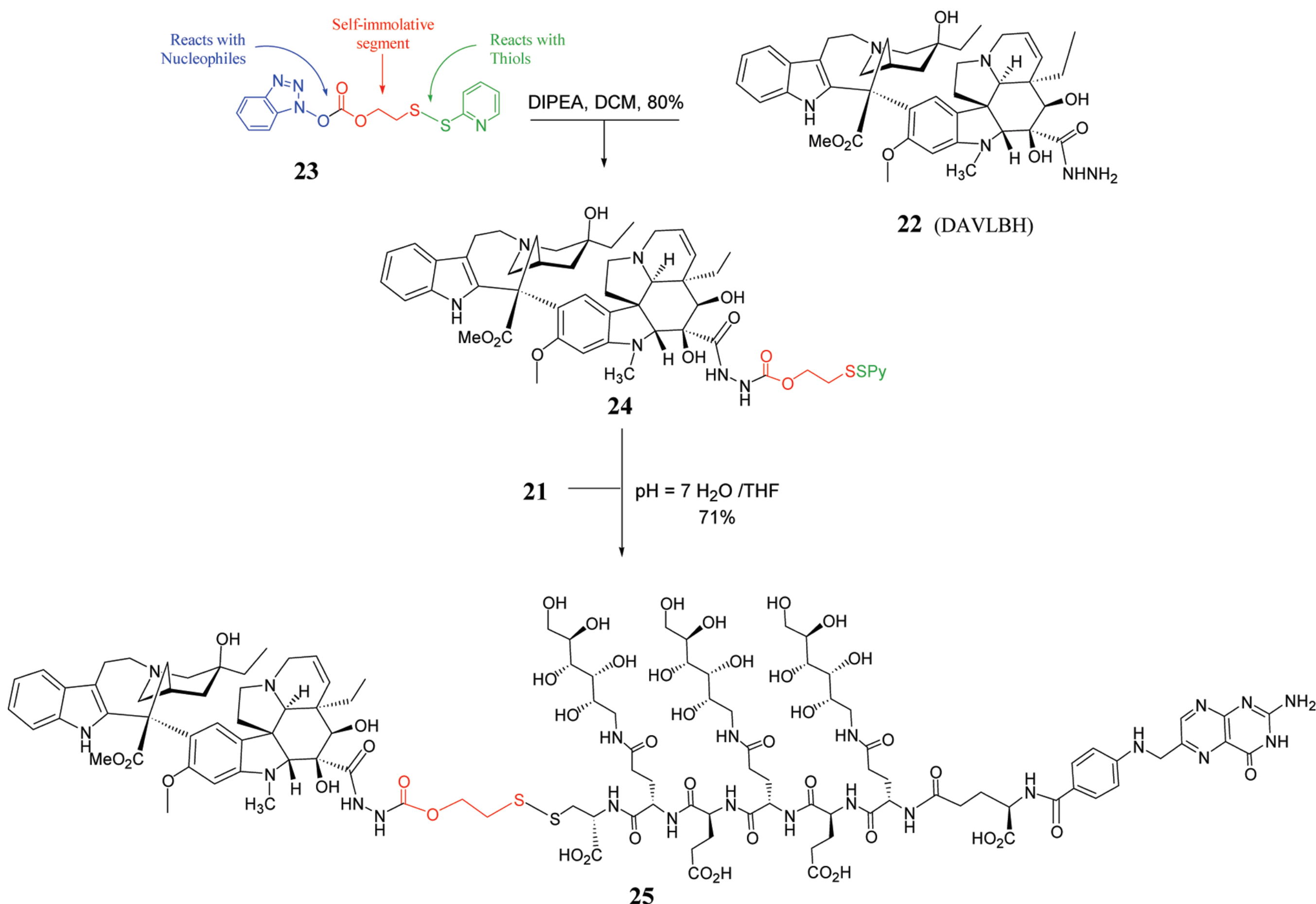
The newly designed base- and acid-sensitive compound **8** allowed for the assembly of the FA−spacer unit **2** using standard Fmoc SPPS on a Wang-resin polymeric support (Scheme 2). Pteroic acid[10] served as the *N*-terminus, whereas the thiol group of cysteine (Cys) was selected as the attachment site for the self-immolative linker system.

After cleavage from the solid support, the LC−MS profile of the reaction mixture displayed a major peak and several satellite peaks with very close retention times. All possessed molecular masses identical with that expected for structure **2**. We also observed an additional peak which mass indicated was the expected structure **2** lacking the *C*-terminal Cys. These results were attributed to three major problems related to the molecular architecture of FA−spacer unit **2**. Aspartimide formation (Scheme 3, route 1, **9**) is a frequently encountered side reaction affecting Asp residues during Fmoc SPPS.[11] In the presence of a strong base such as piperidine, used to remove the Fmoc group, the α-amide nitrogen atom attacks the carbonyl group of the *β*-carboxy side chain resulting in ring closure with loss of the ester protecting group. Aspartimides of type **9** are susceptible to base-catalyzed epimerization to **10**, and both of these forms easily experience ring-opening with predominant formation

(10) Xu, L.; Vlahov, I. R.; Leamon, C. P.; Santhapuram, H. K. R.; Li, C. U.S. Patent 009153, **2006**.

(11) Nicolas, E.; et al. *Tetrahedron Lett*. **1989**, *30*, 497.

SCHEME 6. **Application of Optimized Carbohydrate-Based Spacer for the Synthesis of a Releasable Folate−DAVLBH Conjugate**



of β-aspartyl peptide chain **11** in the presence of water. Compound **2** and all epimerized and β-rearranged counter partners possess identical masses and elute closely or coelute with the desired α-isomer on HPLC. In our first generation spacer design, the peptide sequence possessed multiple sites for potential aspartimide formation making this problem even more serious. Therefore, we exchanged all aspartic acid residues for glutamic acids. To our surprise, there were still peaks in the LC−MS with identical masses and close retention times. That they exhibited different fragmentation patterns in the mass-spectrum brought us to the indirect conclusion that, most likely, we were dealing not only with rotameric forms but also with some other sort of closely related structural isomers. In compound **2** or in its isopropylidene-protected precursor, the protons attached to C-2-atoms in the saccharo-amino acid moieties in **2** are sufficiently acidic to readily undergo epimerization (Scheme 3, route 2, **14**) under the strong basic and/or acidic reaction conditions of SPPS protocols.[12] "Peeling" of the *C*-terminal Cys (Scheme 3, route 3) is a side reaction initiated by 5- and/or 6-membered ring closure, resulting in energetically stabilized lactone forms (e.g., **15**).[13] Apparently, in our first-generation design of the saccharo-amino acid we ignored this not so obvious problem and suitably positioned a hydroxyl group in the carbohydrate chain that attacked the carboxy amide bond of *C*-terminal Cys and formed lactones.

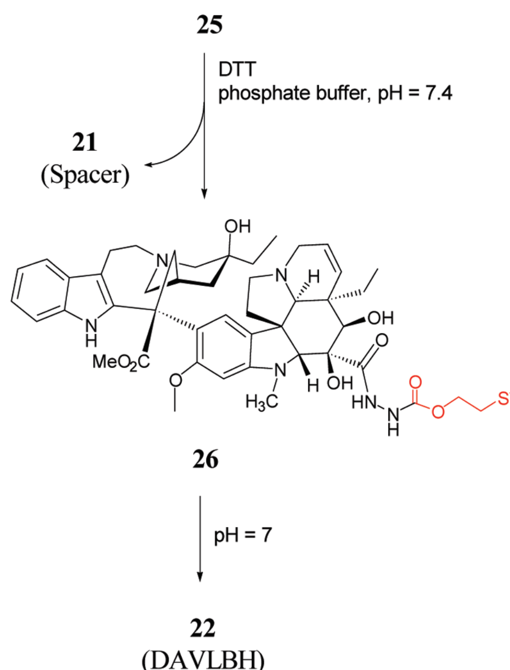
To address and avoid epimerization and "peeling" issues, we redesigned the structure of our saccharo-amino acid by connecting via an amide bond two epimerization-inert modules, namely glutamic acid and glucamine. Each possess C−H bonds that are not inherently prone to changes of configuration. Also, in the new FA−spacer unit **16**, all hydroxyl groups in the glucamine module are more than seven atoms away from the amide bond connecting the saccharo-amino acid and the *C*-terminal Cys, thus suppressing lactone formation and the resulting "peeling". Diisopropylidene- and Fmoc-protected saccharo-amino acid **20** is orthogonally stable to the reaction conditions used in classical Fmoc SPPS protocols. This compound is easily accessible on a large scale using simple synthetic manipulations (Scheme 4). In brief, treatment of **4** with ammonia and reduction of the resulting amide provided the desired glucamine derivative **18** in high yield. Coupling the amine group in **18** with α-carboxy-protected glutamic acid and consecutive allyl ester removal furnished protected amino acid **20**.

Having synthesized **20**, a new FA−spacer unit, which was not saddled with the "peeling" and isomerization issues of **2**, could be assembled. Rather than synthesizing **16**, a simplified

(12) Subsequent HPLC chromatography using a Waters Atlantis 3.0 mm × 50 mm column allowed for the resolution of one of isomers (derived from either aspartimide formation or epimerization) of **2**. The spectrum is shown in the Supporting Information.

(13) An LC−MS spectrum of a monosaccharo analogue of **2**, exemplifying the "peeling" seen with this type of compound, is shown in the Supporting Information.

SCHEME 7. Chemical Release of 22 from 25 via Reduction with DTT



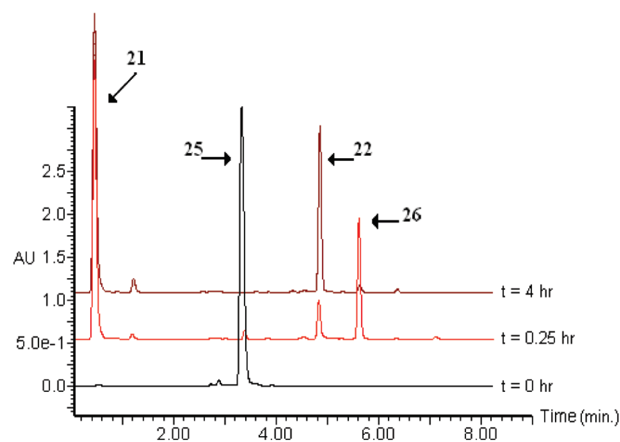


FIGURE 2. HPLC profile (280 nm) of the treatment of **25** with DTT at $t = 0$, 0.25, and 4 h.

version (**21**) would be synthesized. The new FA−spacer unit would preserve the *N*- and *C*-termini of **2**, while replacing the components of the core peptidic backbone with alternating glutamic acid and saccharo-amino acid **20** moieties. FA−spacer unit **21** was prepared using standard Fmoc SPPS on a Wang-resin polymeric support as shown in Scheme 5. After cleavage from the resin, LC−MS on the crude reaction mixture of **21** displayed a single peak of the desired mass. **21** was purified by preparative RP-HPLC, and its structure was confirmed by ^{1}H and ^{13}C NMR and LC−MS [ESI $(M + H)^+$ 1679] analysis.

Our drug, DAVLBH **22**, was prepared from commercially available vinblastine (VLB) sulfate following a literature procedure.[14] Activated carbonate **23**[1] served as heterobifunctional cross-linker in the assembly of the final conjugate. Generally, **23** has been found to react under mild conditions with many *N*- and *O*-nucleophiles and in our hands has been shown to be a convenient and universal tool for the incorporation of reductively labile disulfide linkages into a wide variety of drug conjugates.

As shown in Scheme 6, DAVLBH was treated with the activated carbonate **23** and diisopropylethylamine (DIPEA) in dichloromethane to yield 2-(vinblastinyl)hydrazinecarboxylic acid 2-pyridyldithioethyl ester **24**. After chromatographic purification on silica gel, **24** was isolated in 80% yield. Treatment of a suspension of FA−spacer **21** in H_2O under argon with 0.1 N $NaHCO_3$ resulted in a clear yellow solution at pH > 6.8. To this mixture was added at once under extensive stirring a solution of **24** in THF. According to the HPLC profile, the reaction was completed in 15 min. HPLC purification gave pure conjugate **25**, and recorded LC−MS (ESI) and ^{1}H and ^{13}C NMR signals were in agreement with the expected structure. In brief, the ^{1}H NMR spectrum (800 MHz, DMSO-d_6) contained 11 aromatic signals in the range from 6.4 to 8.8 ppm (five from the folate moiety and six from the desacetylvinblastine moiety). The signals for the two olefinic protons in DAVLBH moiety appeared at 5.53 ppm (d) and 5.69 ppm (m).

Release of the drug from conjugate **25** was also studied (Scheme 7). A 1 mM solution of **25** in phosphate buffer (pH = 7.4) was treated with 40 equiv of dithiothreitol (DTT) at room temperature. The HPLC profile (UV detection at 280 nm) showed complete cleavage of the disulfide bond with concomitant release of the FA−spacer **21** within 15 min (Figure 2). At this early time point, the DAVLBH exists as its free form (**22**) with the remnants of the linker still attached (**26**). Over the next 4 h, the remaining linker is released leaving only **21** and **22** in solution.

Following positive in vitro and in vivo results and toxicological evaluation, compound **25**, also known as EC0489, was selected as a clinical candidate. The results of the pharmacological, toxicological, and phase 1 investigations are being prepared for publication and will be reported soon in an appropriate scientific journal.

Experimental Section

Synthesis of 3,4;5,6-Di-*O*-isopropylidene-1-amino-1-deoxy-(Fmoc-Glu-Oallyl)-D-glucitol 19. Fmoc-Glu-OAll (2.17 g, 1 equiv), PyBOP (2.88 g, 1 equiv), and DIPEA (1.83 mL, 2 equiv) were added to a solution of **18** (1.40 g, 5.3 mmol) in dry DMF (6 mL), and the reaction mixture was stirred at rt under Ar for 2 h. The solution was diluted with EtOAc (50 mL) and washed with brine (10 mL × 3), and the organic layer separated, dried ($MgSO_4$), filtered, and concentrated to give a residue, which was purified by a flash column (silica gel, 60% EtOAc/petroleum ether) to afford **19** (1.72 g, 50%) as a solid. ^{1}H NMR (300 MHz, CD_3OD): δ 7.79 (d, $J = 7.2$ Hz, 2H), 7.67 (t, $J = 6.9$ Hz, 2H), 7.39 (t, $J = 6.9$ Hz, 2H), 7.31 (t, $J = 7.2$ Hz, 2H), 6.02−5.82 (m, 1H), 5.32 (d, $J = 17.4$ Hz, 1H), 5.21 (m, 1H), 4.62 (d, $J = 5.4$ Hz, 2H), 4.46−4.20 (m, 2H), 4.15−4.00 (m, 2H), 4.00−3.83 (m, 3H), 3.79 (m, 1H), 3.40−3.33 (m, 2H), 2.32 (t, $J = 6.9$ Hz, 2H), 2.26−1.88 (m, 2H), 1.38 (s, 3H), 1.36 (s, 3H), 1.34 (s, 3H), 1.27 (s, 3H). ^{13}C NMR (75.5 MHz, CD_3OD): δ 175.2, 173.4, 158.8, 145.5, 145.3, 142.8, 133.4, 129.0, 128.3, 126.5, 126.4, 121.1, 118.8, 111.0, 110.9, 82.4, 78.8, 78.5, 69.7, 68.7, 68.2, 67.0, 55.2, 44.7, 33.4, 28.7, 27.6, 27.2, 27.1, 25.6. HRMS (ESI): $(M + Na)^+$ calcd for $C_{35}H_{44}N_2O_{10}$ 675.2894, found 675.2899.

(14) Barnett, C. J.; Cullinan, G. J.; Gerzon, K.; Hoying, R. C.; Jones, W. E.; Newlon, W. M.; Poore, G. A.; Robison, R. L.; Sweeney, M. J.; Todd, G. C. *J. Med. Chem.* **1978**, *21*, 88.

Synthesis of 3,4;5,6-Di-*O*-isopropylidene-1-amino-1-deoxy-(Fmoc-Glu-OH)-D-glucitol 20. $Pd(Ph_3)_4$ (300 mg, 0.1 equiv) was added to a solution of **19** (1.72 g, 2.81 mmol) in NMM/AcOH/$CHCl_3$ (2 mL/4 mL/74 mL). The resulting yellow solution was stirred at rt under Ar for 1 h, to which was added a second portion of $Pd(Ph_3)_4$ (300 mg, 0.1 equiv). After being stirred for an additional 1 h, the reaction mixuture was washed with 1 N HCl (50 mL × 3) and brine (50 mL), and the organic layer separated, dried ($MgSO_4$), filtered, and concentrated to give a yellow foamy solid, which was subjected to chromatography (silica gel, 1% MeOH/$CHCl_3$ followed by 3.5% MeOH/$CHCl_3$) to give **20** (1.3 g, 81%) as a solid: ^{1}H NMR (300 MHz, CD_3OD): δ 7.79 (d, J = 7.2 Hz, 2H), 7.66 (t, J = 6.9 Hz, 2H), 7.38 (t, J = 7.2 Hz, 2H), 7.30 (t, J = 7.2 Hz, 2H), 4.45−4.15 (m, 4H), 4.15−4.00 (m, 2H), 4.00−3.85 (m, 3H), 3.73 (m, 1H), 3.40−3.35 (m, 2H), 2.29 (t, J = 7.5 Hz, 2H), 2.25−2.05 (m, 2H), 1.38 (s, 3H), 1.35 (s, 3H), 1.34 (s, 3H), 1.26 (s, 3H); ^{13}C NMR (75.5 MHz, CD_3OD): δ 175.4, 158.8, 145.5, 145.3, 142.7, 128.9, 128.3, 126.5, 126.4, 121.1, 111.0, 110.9, 82.3, 78.8, 78.5, 69.7, 68.7, 68.2, 55.0, 44.7, 33.6, 29.0, 27.6, 27.2, 27.1, 25.6; HRMS (ESI) $(M + Na)^+$ calcd for $C_{32}H_{40}N_2O_{10}$ 635.2581, found 635.2573.

Synthesis of Pte-γGlu-(Glu(1-amino-1-deoxy-D-glucitol)-Glu)$_2$-Glu(1-amino-1-deoxy-D-glucitol)-Cys-OH 21. H-Cys(4-methoxytrityl)-2-chlorotrityl-resin (0.17 g, 0.10 mmol) was loaded into a peptide synthesis vessel and washed with *i*-PrOH (3 × 10 mL), followed by DMF (3 × 10 mL). To the vessel was then introduced a solution of **20** (82 mg, 0.13 mmol) in DMF, *i*-Pr_2NEt (2 equiv), and PyBOP (1 equiv). The resulting solution was bubbled with Ar for 1 h, the coupling solution was drained, and the resin washed with DMF (3 × 10 mL) and *i*-PrOH (3 × 10 mL). Kaiser tests were preformed to assess reaction completion. Fmoc deprotection was carried out using 20% piperidine in DMF (3 × 10 mL). This procedure was repeated to complete all coupling steps (1.9 equiv of Fmoc-Glu(O-*t*-Bu)-OH and Fmoc-Glu-O-*t*-Bu and 1.6 equiv of N^{10}TFA-pteroic acid were used on each of their respective coupling steps). After the pteroic acid coupling, the resin was washed with 2% hydrazine in DMF (3 × for 5 min each) to remove the trifluoroacetyl protecting group. The resin was washed with DMF (3 × 10 mL) and MeOH (10 mL) and dried under reduced pressure. The peptide was cleaved from the resin in the peptide synthesis vessel using a cleavage mixture consisting of 92.5% CF_3CO_2H, 2.5% H_2O, 2.5% triisopropylsilane, and 2.5% ethanedithiol. Twenty-five milliliters of the cleavage mixture was added to the peptide synthesis vessel, and the reaction was bubbled under Ar for 10 min. The resin was treated with two additional 15 mL quantities of the cleavage mixture for 5 min each. The cleavage mixture was concentrated to ca. 5 mL, and ethyl ether was added to induce precipitation. The precipitate was collected by centrifugation, washed with ethyl ether three times, and dried under high vacuum, resulting in the recovery of ca. 100 mg of crude material. One-half of the material was purified by preparative HPLC (mobile phase: A = 10 mM ammonium acetate pH = 5, B = ACN; method: 0% B to 20% B in 25 min at 15 mL/min). The pure fractions were pooled and freeze-dried, furnishing spacer **21** (43 mg, 51%). ^{1}H NMR (800 MHz, DMSO-d_6/D_2O): δ 8.6 (s, 1H), 7.6 (d, J = 8 Hz, 2H), 6.62 (d, J = 8 Hz, 2H), 4.47 (s, 2H), 4.26−4.08 (m, 7H), 3.61(m, 3H), 3.53(m, 6H), 3.46(m, 3H), 3.37(m, 6H), 3.21(m, 3H), 3.03(m, 3H), 2.80 (dd, 1H), 2.72 (dd, 1H), 2.3−2.08 (m, 12H), 1.95−1.6 (m, 12H). ^{13}C NMR (125 MHz, DMSO-d_6/D_2O): δ 175.2, 175.0, 174.9, 173.4, 173.3, 173.2, 173.0, 172.6, 172.1, 172.0, 171.3, 167.2, 162.2, 156.2, 154.3, 151.2, 149.5, 149.3, 129.5, 128.1, 121.8, 112.0, 72.2, 71.82, 71.78, 70.1, 63.6, 56.2, 53.2, 53.0, 52.8, 42.4, 32.3, 30.8, 28.3, 28.2, 27.6, 27.4, 27.2, 26.8. LCMS (ESI): $(M + H)^+$ = calcd for $C_{65}H_{98}N_{16}O_{34}S$, 1679.6, found 1680.0.

Synthesis of Pte-γGlu-(Glu(1-amino-1-deoxy-D-glucitol)-Glu)$_2$-Glu(1-amino-1-deoxy-D-glucitol)-Cys(S-ethyl-3-(4-desacetylvinblastinyl)hydrazinecarboxylate) 25. In a polypropylene centrifuge bottle, folate linker **21** (26 mg, 0.015 mmol) was dissolved in 2.5 mL of Ar-sparged water. In another flask, a saturated $NaHCO_3$ solution was Ar sparged for 10 min. The pH of the linker solution was carefully adjusted, with argon bubbling, to 6.9 using the $NaHCO_3$ solution. Vinblastine hydrazide derivative **24** (15 mg, 1.0 equiv) in 2.5 mL of tetrahydrofuran (THF) was added quickly to the above solution. The resulting clear solution was stirred under argon. Progress of the reaction was monitored by analytical HPLC (2 mM sodium phosphate buffer, pH = 7.0 and acetonitrile). After 20 min, 2 mM phosphate buffer (pH = 7, 12 mL) was added to the reaction. The resulting cloudy solution was filtered, and the filtrate was injected on the prep-HPLC (mobile phase: A = 2 mM sodium phosphate pH = 7, B = ACN; method: 1% B to 50% B in 25 min at 26 mL/min). Pure fractions were pooled and freeze-dried resulting in the recovery of **25** as a fluffy yellow powder (27.5 mg, 71%). ^{1}H NMR (800 MHz, DMSO-d_6/D_2O): δ 8.60(s, 1H), 7.59 (d, J = 8.8 Hz, 2H), 7.36 (d, J = 8 Hz, 1H), 7.21 (d, J = 8 Hz, 1H), 7.0 (t, J = 8 Hz, 1H), 6.93 (t, J = 8 Hz, 1H), 6.77 (d, J = 8.8 Hz, 2H), 6.36 (s, 1H), 6.17 (s, 1H), 5.69 (bd, J = 8 Hz, 1H), 5.52 (d, J = 10.4 Hz, 1H), 4.47 (s, 2H), 4.19 (m, 2H), 4.13−4.05 (m, 6H), 3.96 (m, 1H), 3.76 (s, 1H), 3.68 (s, 3H), 3.64−3.62 (m, 4H), 3.55 (m, 6H), 3.5 (s, 3H), 3.48 (m, 3H), 3.40 (m, 8H), 3.21 (m, 6H), 3.11−3.00 (m, 5H), 2..93 (m, 1H), 2.88 (m, 1H), 2.72 (s, 3H), 2.70 (d, J = 7.2, 1H), 2.60 (m, 1H), 2.41 (bs, 1H), 2.34 (bd, J = 10.4 Hz, 1H), 2.24 (s, 1H), 2.15−2.05 (m, 12H), 1.97−1.78 (m, 14H), 1.53 (m, 2H), 1.31 (d, J = 11.2, 1H), 1.21−1.17 (m, 4H), 0.76 (t, J = 7.2 Hz, 3H), 0.70 (t, J = 7.2 Hz, 3H), 0.65 (m, 1H). ^{13}C NMR (125 MHz, DMSO-d_6/D_2O): δ 177.7, 177.4, 175.9, 175.6, 174.2, 173.5, 173.3, 173.2, 172.7, 172.6, 172.3, 172.2, 172.1, 171.2, 166.5, 158.0, 156.6, 156.1, 155.1, 152.9, 151.0, 149.4, 149.2, 135.6, 131.8, 131.7, 129.2, 129.1, 128.1, 124.0, 123.7, 123.0, 122.4, 122.1, 119.9, 119.0, 118.5, 116.3, 112.1, 111.8, 93.5, 83.2, 80.9, 73.9, 72.2, 71.9, 71.8, 70.0, 69.9, 68.0, 65.8, 63.6, 63.3, 62.9, 56.6, 55.6, 54.5, 54.3, 53.9, 53.7, 53.3, 52.9, 52.6, 50.5, 49.7, 46.9, 46.2, 45.4, 42.5, 38.7, 38.4, 37.8, 35.3, 34.7, 33.6, 33.5, 33.2, 32.3, 32.2, 30.6, 29.7, 28.9, 28.7, 28.0, 27.7, 25.7, 8.7, 7.6. LCMS (ESI): $(M + H)^+$ = calcd for $C_{111}H_{158}N_{22}O_{43}S_2$ 2551.1, found 2551.8.

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Supporting Information Available: Experimental procedures and spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.