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Antineoplastic Agents. 606. The Betulastatins

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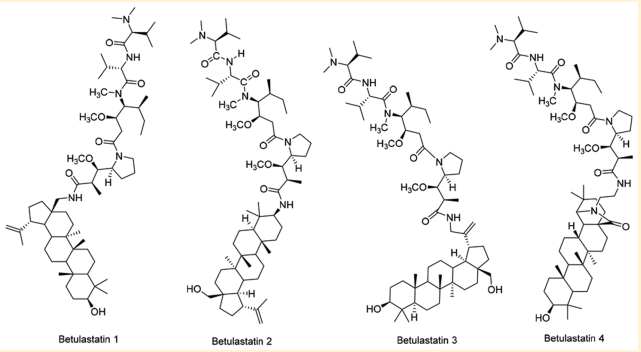
Supporting Information

ABSTRACT: The medicinal potential of the plant pentacyclic triterpene betulin has generated long-term interest focused on various SAR research avenues. The present approach was based on producing further analogues (chimeras) arising from a studied modification of betulin bonded to the Dov-Val-Dil-Dap unit of the powerful anticancer drug dolastatin 10, which provided betulastatins 1 (**7b**), 2 (**11b**), 3 (**16b**), and 4 (**18b**). Betulastatin 1, 2, and 4 exhibited modest levels of cancer cell growth inhibition against six cancer cell lines. Betulastatin 3 proved to be the most potent cancer cell growth inhibitor (GI_{50} 0.01 μg/mL) and seems worthy of further development, as the presumed mixture of anticancer mechanisms of action may prove to be useful.



Betulin, a pentacyclic triterpene, and its C-28 carboxylic acid derivative betulinic acid are well-known plant constituents especially in birch tree bark and in traditional medicine. Betulinic acid, owing to a variety of medicinal properties, and especially as a cancer cell growth inhibitor based on a mitochondrial mechanism of action, continues to be widely studied.[1,2] Some of these advances have, for example, been extended to a clinical trial against dysplastic nevus, a clinical precursor of melanoma. More broadly, betulinic acid has shown various levels of inhibition of growth in vitro against a variety of cancer types.[2] Earlier in investigations of betulin chemistry with respect to the discovery of useful anticancer drugs, we explored structural modifications that included a betulin/betulinic acid series[3] and, previously, α-apoallobetulin[4] and selected pentacyclic triterpenes including oleanolic and ursolic acids.[5] In the present extension of this work, we have examined a chimera approach by adding the Dov-Val-Dil-Dap[6a] sequence of the exceptionally potent peptide dolastatin 10[6b] to betulin structural modifications.

RESULTS AND DISCUSSION

To begin, the synthesis of **7b** was carried out as shown in Scheme 1. Betulin was acetylated, and the resulting diacetate selectively deprotected at C-28 in the presence of K_2CO_3 and aqueous MeOH to 3-*O*-acetylbetulin and then oxidized to aldehyde **1** with pyridinium dichromate.[7a−c] When the aldehyde **1** was reacted with hydroxylamine hydrochloride, the oxime **2**[8] was prepared and subsequently reduced to the corresponding amine **3**, using the reducing system sodium cyanoborohydride, 15% titanium chloride, and ammonium acetate.[9] The N-protected amino acid Boc-Dap (**4**)[10] was condensed with amine **3** using the organophosphorous coupling reagent diethylcyanophosphonate (DEPC) to give, following purification, amide **5**. Removal of the Boc protecting group using trifluoroacetic acid (TFA) followed by coupling with a Dov-Val-Dil.TFA (**6**) amino acid fragment[11] using DEPC gave peptide **7a** upon separation employing silica gel chromatography. Deacetylation of **7a** in the presence of tetrahydrofuran THF−MeOH (3:2) and 2 N NaOH gave the betulin peptide chimera **7b**, designated betulastatin 1.

A similar synthetic approach was followed for the synthesis of peptide **11b**, as shown in Scheme 2. The derivative 28-*O*-acetylbetulin[12] was employed to prepare oxime **8**, which, upon reduction, gave the 3α/β amine mixture **9a**/**9b**. Separation of these amine isomers was achieved by silica gel chromatography, and **9b** was found to be the major product of the reaction. The C-3 betulin peptide chimera **11a** was then synthesized using **9b**, by employing the coupling sequence described previously for **7b**. Acetate deprotection of **11a** was achieved in THF−CH_3OH (4:3) and 2 N NaOH to give chimera **11b** (betulastatin 2).

Scheme 3 details the synthesis of C-30 betulin peptide chimera **16**. The diacetate was brominated using *N*-bromosuccinimide (NBS) as described in the literature[13] to give allylic bromide **12** and was then reacted with NaN_3 in dry dimethylformamide (DMF) to give azide **13** in high yield. The azide was next reduced to the deprotected amine **14** using a 1 M solution of $LiAH_4$ in THF followed by condensing with Boc-Dap **4** in the presence of DEPC and triethylamine (TEA) to give the Boc-protected peptide derivative **15a**. In order to proceed with removal of the Boc group from Dap with TFA and avoid acid-catalyzed rearrangement at C-20/C-29, **15a** was reacetylated in the presence of acetic anhydride and dimethylaminopyridine (DMAP) to give diacetate **15b**.

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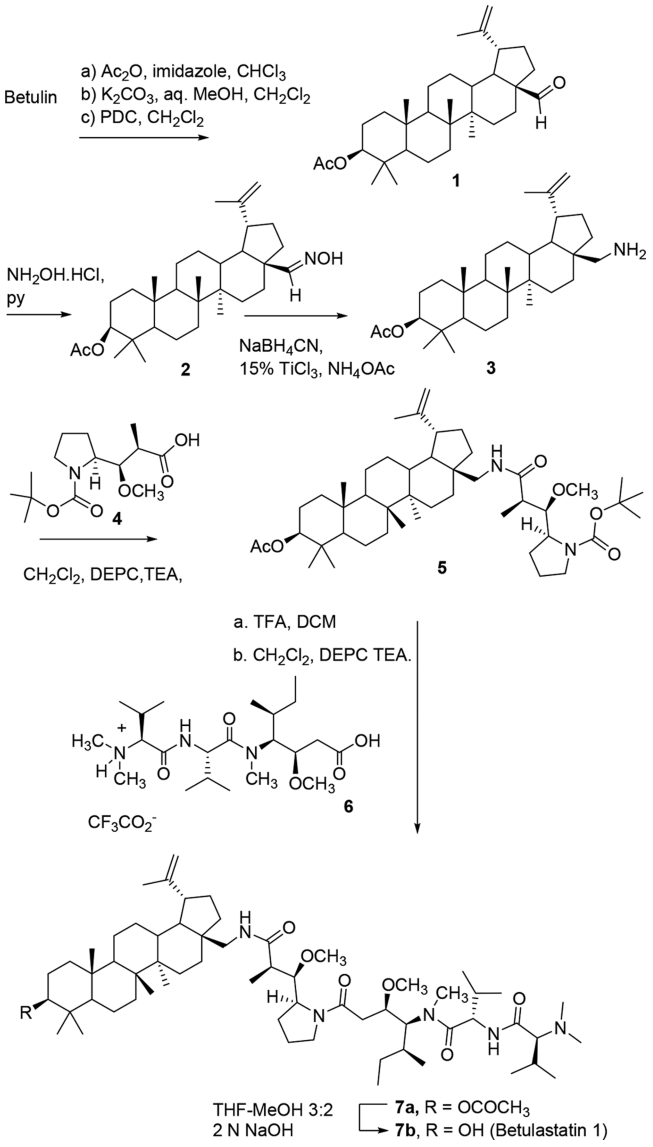
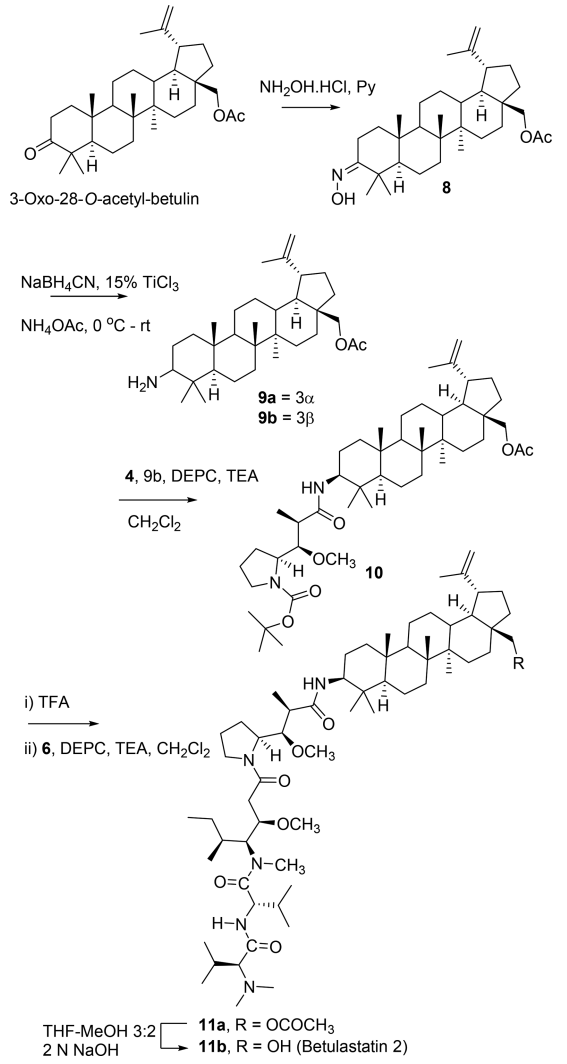
Scheme 1



Scheme 2



Coupling the tripeptide **6** with **15b** gave, as expected, **16a**, which was then deacetylated to peptide **16b** (betulastatin 3).

Scheme 4 describes extending the pentapeptide from the betulin molecule by a distance of two carbons by reacting 3-*O*-acetylbetulinic acid[8] and ethylenediamine in the presence of oxalyl chloride followed by the same coupling sequence with Boc-Dap **4** and peptide **6**, as outlined in the previous schemes. Interestingly, the reaction acid during the deprotection of the Boc-Dap amide caused a rearrangement to the lactam shown in Scheme 4 and supported by examination of the NMR data. The resonances for the methylene carbon (C-29) and the attached tertiary carbon (C-20), usually seen at 110 and 150 ppm, respectively, were not observed in the ^{13}C NMR spectrum for peptides **18a** and **18b** (betulastatin 4). The ^{1}H NMR spectrum provided further evidence. The resonances due to the geminal protons on the C-29 methylene group of betulin, usually observed as singlets near 4.7 and 4.6 ppm, were not detected. In the 2D HSQC spectrum of **18a**, the proton on C-19 was observed downfield at 3.75 ppm and correlated with C-19 at 86.8 ppm. Removal of the acetate protecting group at C-3 on peptide **18a** with 2 N NaOH, as described previously, gave peptide **18b** (betulastatin 4).

As can be seen from Table 1, chimera **7b**, amine **9b**, and chimeras **11b** and **18b** in general resulted in a 10-fold increase in cancer cell growth, while chimera **16b** provided a 100-fold increase, and this lead will be further developed.

EXPERIMENTAL SECTION

General Experimental Procedures. Both *N*-Boc-dolaproine[10a] and Dov-Val-Dil·TFA[11] were synthesized as described earlier. Other reagents including diethyl cyanophosphonate and anhydrous solvents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and were used as received. Small quantities of betulin (**1**) were purchased from Sigma-Aldrich, and larger quantities were isolated[5] from white birch bark collected in the state of Maine by Professor Robert Dunlap, Department of Chemistry, University of Maine, and Mr. B. Deane as well as Hardwood Products Company LLC, Guilford, Maine.

Melting points are uncorrected and were determined with a Fisher Scientific melting point apparatus. Optical rotations were measured by use of a Rudolph Research Autopol IV automatic polarimeter. The $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. The ^{1}H and ^{13}C NMR spectra were recorded on Varian Unity INOVA 400 and 500 and Bruker 400 NMR instruments with deuterated solvents. High-resolution mass spectra were obtained employing a JEOL LCMate instrument and a Bruker MicrOTOF-Q in the ESI positive mode

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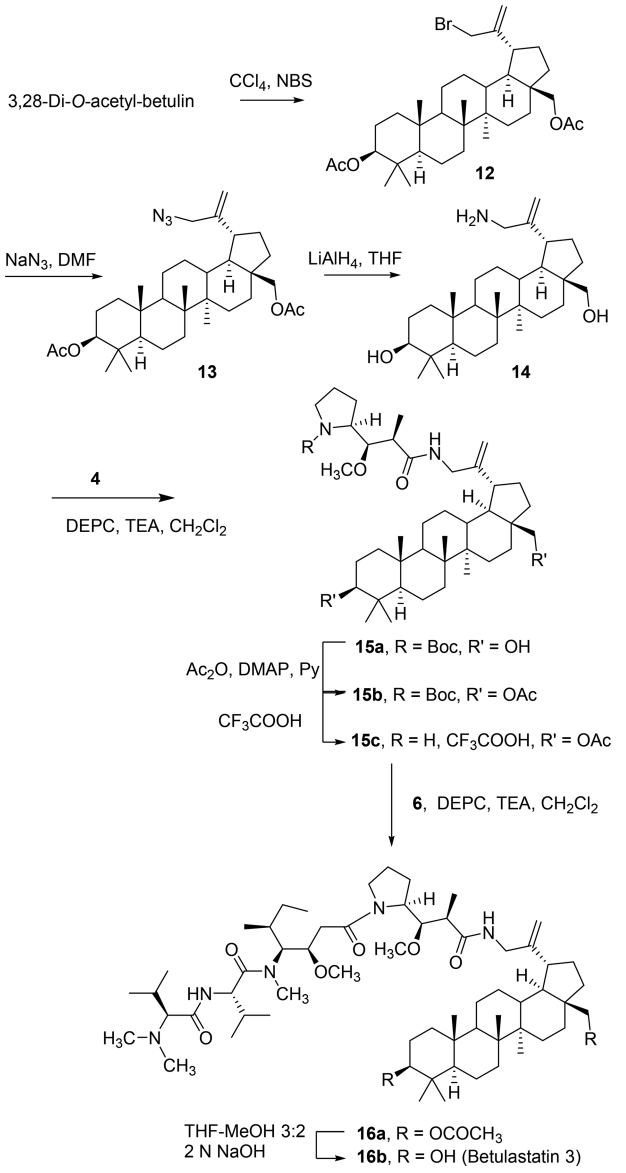
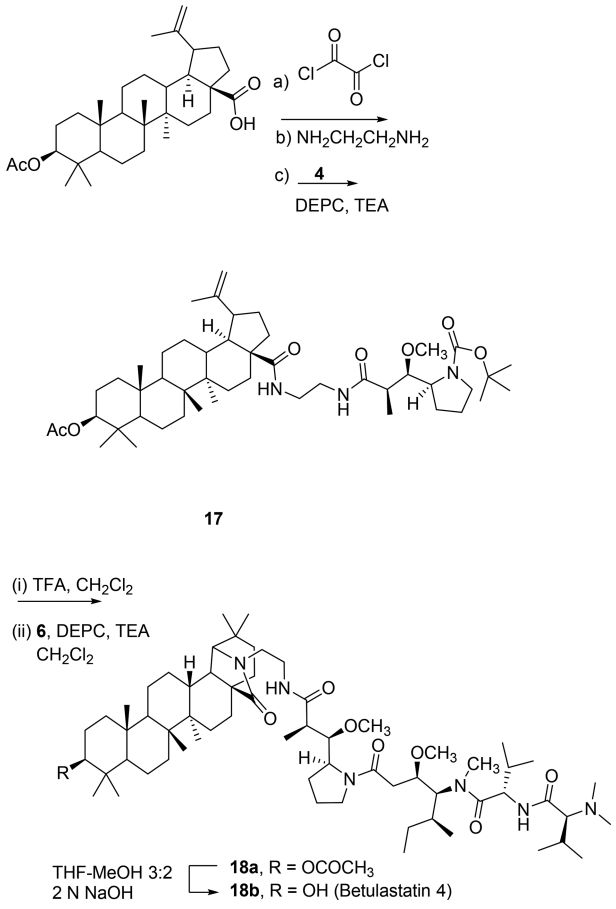
Scheme 3



Scheme 4



(direct infusion with internal calibration) at the Arizona State University CLAS High Resolution Mass Spectroscopy Laboratory. For thin-layer chromatography, Analtech silica gel GHLF Uniplates were used and visualized with short-wave UV irradiation and an iodine chamber. For column chromatography, silica gel (230−400 mesh ASTM) from E. Merck (Darmstadt, Germany) was employed.

3-*O*-Acetyl-28-betulinal (1). 3-*O*-Acetylbetulin was prepared from the diacetate as described previously[7a] and purified using column chromatography on silica gel eluting with hexanes−EtOAc (8:2). Recrystallization from CH_2Cl_2−MeOH yielded colorless plates: R_f = 0.7 (CH_2Cl_2−MeOH, 98:2); mp 260−261 °C; $[\alpha]^{20}_D$ +28.2 (*c* 0.7, $CHCl_3$), lit.[7a] mp 256−258 °C; lit.[7b] mp 258−260 °C; $[\alpha]^{20}_D$ +26 (*c* 0.9, $CHCl_3$); ^{1}H and ^{13}C NMR spectroscopic data in agreement with published values.[7a,c] 3-*O*-Acetylbetulinal (**1**) was prepared as described previously[7b] from 3-*O*-acetylbetulin to yield a colorless crystalline solid: mp 170−175 °C; $[\alpha]^{20}_D$ +22 (*c* 0.6, $CHCl_3$), lit.[7b] $[\alpha]^{20}_D$ +32 (*c* 0.7, $CHCl_3$); ^{1}H and ^{13} C NMR data in agreement with reported values.[7b]

3-*O*-Acetyl-28-oxime-betulin (2). 3-*O*-Acetylbetulinal (**1**) (0.25 g, 0.5 mmol) was dissolved in dry pyridine (5 mL), and NH_2OH·HCl (0.1 g, 1.5 mmol, 3 equiv) was added. The solution was heated at reflux for 2 h and monitored by TLC (hexanes−EtOAc, 8:2). The reaction mixture was cooled and added to iced water (100 mL), and the resulting white precipitate filtered and dried under vacuum to yield oxime **2** (0.22 g, 86% yield). Crystallization of **2** from hot MeOH gave colorless crystals: mp 255−257 °C; lit.[8] mp >260 °C; R_f = 0.5 (hexanes−EtOAc, 8:2); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.12 (1H, s, NOH), 7.52 (1H, s, H-28), 4.69 (1H, bs, H-29), 4.58 (1H, s, H-29), 4.45 (1H, dd, *J* = 10, 6 Hz, H-3), 2.49 (1H, dt, *J* = 12, 6 Hz, H-19), 2.02 (3H, s, $OCOCH_3$), 1.68 (3H, s), 0.97 (3H, s), 0.95 (3H,), 0.82 (6H, s), 0.81 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) 171.1, 155.4, 149.7, 110.1, 80.9, 55.3, 50.3, 49.7, 49.3, 47.9, 42.8, 40.9, 38.6, 38.3, 37.7, 37.04, 37.02, 34.2, 32.3, 29.7, 27.9, 27.8, 25.1, 23.7, 21.3, 20.8, 19.1, 18.1, 16.5, 16.1, 16.0, 14.7; (+)-HRAPCIMS *m*/*z* 498.3953 $[M + H]^+$ (calcd for $C_{32}H_{52}NO_3$, 498.3947).

3-*O*-Acetyl-28-aminobetulin (3). To a stirred solution of 3-*O*-acetyl-28-oxime-betulin (**2**) (0.35 g, 0.7 mmol) in MeOH (50 mL) and cooled to 0 °C (ice-bath) were added NH_4OAc (0.75 g, 9.7 mmol, 14 equiv) and $NaBH_4CN$ (0.6 g, 0.97 mmol). $TiCl_3$ (10 wt % in 20−30 wt % HCl) (3 mL) was then added dropwise to the cooled mixture. The reaction flask was removed from the cold bath after 2 h, and the mixture stirred at rt for 1 h. Next, 2 N NaOH (20 mL) was added dropwise to the mixture until pH 10. MeOH was removed under vacuum, and the aqueous suspension was extracted with CH_2Cl_2 (200 mL). The organic fraction was separated and washed with water until it was at neutral pH (3 × 30 mL), dried (Na_2SO_4), and concentrated to a solid (0.25 g, 71% yield): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.66 (1H, bs, H-29), 4.56 (1H, bs, H-29), 4.45 (1H, dd, *J* = 10, 6 Hz, H-3), 2.85 (1H, d, *J* = 13 Hz, H-28a), 2.35 (2H, m), 2.15 (1H, m), 2.02 (3H, s, $OCOCH_3$), 1.66, 1.00, 0.95, 0.83, 0.82, 0.81 (3H, s, 6 × CH_3), 2.0−0.7 (CH, CH_2, NH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) 171.0, 150.6, 109.6, 80.9, 55.3, 50.3, 48.9, 47.4, 47.3, 42.6, 40.9, 39.2, 38.3, 37.8, 37.0, 36.9, 34.1, 34.0, 29.7, 29.3, 27.9, 27.0, 25.1, 23.7, 21.3, 20.9, 19.1, 18.2, 16.5, 16.1, 15.9, 14.7; (+)-HRAPCIMS *m*/*z* 484.4147 $[M + H]^+$ (calcd for $C_{32}H_{54}NO_2$, 484.4155). The product was used immediately without further purification in the next reaction.

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Table 1. Human Cancer Cell Line (GI_{50} μg/mL in DMSO) Growth Inhibition Results from Comparison Experiments

	cell line[a]					
compound	BXPC-3	MCF-7	SF-268	NCI-H460	KM20L2	DU-145
betulin[b,S]	9.3	>10	>10	7.4	>10	>10
1	>10	>10	>10	>10	>10	>10
2	15.5	4.5	13.2	4.1	5.1	>10
3	1.9	1.8	1.8	1.9	1.9	1.8
5	>10	>10	>10	>10	>10	>10
7a	4.1	3.6	2.6	4.2	2.4	3.7
7b	0.60	0.40	0.30	>1	0.31	0.40
8	10.4	5.9	>10	>10	14.1	>10
9a	>10	2.1	1.2	6.0	3.0	1.3
9b	1.1	0.20	0.21	0.21	0.35	0.13
10	>10	>10	>10	>10	>10	>10
11a	10.0	4.1	3.8	>10	7.0	2.5
11b	2.0	0.70	0.40	2.1	0.85	0.40
12	>10	>10	>10	>10	>10	>10
13	>10	>10	>10	>10	>10	>10
14	3.0	3.1	4.2	4.0	2.9	2.1
15a	>10	9.0	>10	>10	>10	>10
15b	>10	>10	5.9	>10	7.0	>10
16a	8.0	6.3	4.0	>10	4.0	3.0
16b	0.40	0.08	0.04	0.50	0.090	0.040
18a	3.8	3.6	1.3	5.1	3.1	0.39
18b	0.31	0.21	0.09	0.60	0.22	0.06

[a]Cancer cell lines in order: pancreas (BXPC-3); breast (MCF-7); CNS (SF-268); lung (NCI-H460); colon (KM20L2); prostate (DU-145). [b]Source: Sigma-Aldrich.

3-*O*-Acetyl-28-amino-*N*-(Boc-Dap)-betulin (5). To a stirred solution of amine **3** (0.16 g, 0.33 mmol) in anhydrous CH_2Cl_2 (10 mL) at 0 °C was added Boc-Dap (**4**)[10] (0.08 g, 0.27 mmol). The solution was cooled in an ice bath, and TEA (0.16 mL, 3.5 equiv) followed by DEPC (0.1 mL, 0.62 mmol, 2 equiv) were added to the reaction mixture. Stirring with warming to room temperature (rt) was continued for 24 h. The mixture was then concentrated and purified using silica gel chromatography with gradient elution (100% hexane → hexanes−EtOAc, 7:3) to give **5** (0.1 g, 46%) as a waxy solid. Crystallization from hexanes with cooling gave a colorless solid: mp 116−119 °C; $[\alpha]^{20}_D$ −11 (*c* 0.65, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) 6.39 (1H, bs), 5.57 (1H, bs), 4.68 (1H, s), 4.58 (1H, s), 4.46 (1H, dd, *J* = 9.6, 6.4 Hz), 3.86−3.76 (2H, m) 3.55 (1H, m), 3.44 (3H, s, OCH_3), 3.39 (1H, m), 3.26 (1H, m) 3.13−3.02 (1H, m), 2.47 (2H, m, H-19, H-28), 2.03 (3H, s, $OCOCH_3$), 1.68, 1.05, 0.95 (3H, s, CH_3), 0.84 (2 × CH_3) 0.83 (3H, s), 0.7−1.95 (CH, CH_2 hydrogens); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 174.6, 170.9 150.4, 109.4, 80.9, 60.8, 55.4, 50.3, 48.9, 47.3, 46.8, 44.5, 42.6, 40.9, 38.3, 37.8, 37.1, 37.0, 36.6, 34.9, 34.0, 30.3, 28.5, 27.9, 27.1, 25.9, 24.7, 23.7, 23.4, 21.3, 20.8, 19.2, 18.1, 16.5, 16.1, 16.09, 14.7; (+)-HRAPCIMS *m/z* 753.5776 $[M + H]^+$ (calcd for $C_{46}H_{77}N_2O_6$, 753.5782).

3-*O*-Acetyl-28-amino-*N*-(Dap-Dil-Val-Dov)-betulin (7a). 3-*O*-Acetyl-28-*N*-(Boc-Dap)-betulin (**5**) (0.07 g, 0.086 mmol) was dissolved in anhydrous CH_2Cl_2 (4 mL) and cooled in an ice bath. TFA (1.5 mL) was added, and the solution stirred for 1 h. The product mixture was then concentrated to remove the solvent, and TFA was removed under reduced pressure to yield 3-*O*-acetyl-28-amino-*N*-(Dap)-betulin TFA salt as a foamy solid. The salt was then stirred together with Dov-Val-Dil-TFA (**6**)[11] (0.045 g, 0.083 mmol) in anhydrous CH_2Cl_2 (5 mL) under N_2 at 0 °C. TEA (0.06 mL, 0.42 mmol) and DEPC (0.02 mL, 0.15 mL, 1.8 equiv) were added in succession, and the reaction mixture was stirred for 24 h with warming to rt, concentrated, and purified on silica gel eluting with CH_2Cl_2−MeOH (95:5) to give **7a** as an off-white solid (0.05 g, 45% yield). Attempts at crystallizing **7a** from hexanes−acetone gave a fine powder: mp 129−131 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.07 (1H, bs), 6.41 (1H, bs), 4.80−4.64 (2H, m), 4.53 (1H, s), 4.42 (1H, m), 4.12−4.02 (2H, m), 3.89 (1H, m), 3.42−2.99 (8H, m), 3.10−3.04 (1H, m), 2.98 (3H, s), 2.60−2.20 (11H, m), 2.08−1.92 (8H, m), 1.86−1.42 (16 H, m), 1.40−1.14 (13H, m), 1.04−0.73 (40 H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 174.7, 173.5, 171.5, 171.2, 170.5, 150.5, 109.8, 82.2, 81.1, 78.3, 77.5, 61.5, 60.7, 59.7, 58.2, 55.5, 54.1, 50.5, 49.2, 48.8, 47.5, 47.2, 44.6, 42.8, 42.7, 41.1, 38.5, 37.9, 37.7, 37.4, 37.2, 35.2, 34.2, 33.4, 31.0, 30.5, 29.9, 28.1, 27.9, 27.3, 25.9, 25.3, 25.1, 25.08, 23.8, 21.5, 20.9, 20.2, 19.7, 19.5, 18.3, 18.1, 16.7, 16.3, 16.2, 16.0, 14.9, 14.87, 10.7; (+)-HRFABMS *m/z* 1064.8355 (calcd for $C_{63}H_{110}N_5O_8$, 1064.8354).

Betulin-28-amino-*N*-(Dap-Dil-Val-Dov) (7b). To a solution of acetate **7a** (0.024 g, 0.022 mmol) in a mixture of THF−MeOH, 3:2 (0.7 mL), was added aqueous 2 N NaOH (0.15 mL). The reaction mixture was stirred at rt for 24 h. Water (1 mL) was added, the mixture was extracted into EtOAc (2 × 1 mL), and the combined organic layers were concentrated and separated by flash silica gel column chromatography (CH_2Cl_2−MeOH, 96:4) to yield a colorless solid, 12 mg (50% yield): mp 150 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.88 (1H, d, *J* = 8.8 Hz), 6.45 (1H, bs), 4.78 (1H, dd, *J* = 8.4, 6.8 Hz), 4.68 (1H, s), 4.57 (1H, s), 4.12 (2H, m), 3.91(1H, d, *J* = 8 Hz), 4.38 (2H, m), 3.40, 3.32 (OCH_3, s), 3.41−3.26 (10 H, m), 3.16 (2H, m), 3.10 (1 H, s), 3.01 (3H, s), 2.48−2.29 (5H, m), 2.24 (6H, s), 2.11−1.95 (6H, m), 1.88-l.58 (12H, m), 1.46−1.32 (6H, m), 1.30−1.18 (8H, m), 1.13 (1H, d, *J* = 8.6 Hz), 1.09−0.88 (27H, m), 0.81 (6H, t, *J* = 7.5 Hz), 0.75 (3H, s), 0.67 (1H, m); (+)-HRAPCIMS *m/z* 1022.8246 $[M + H]^+$ (calcd for $C_{61}H_{108}N_5O_7$, 1022.8250).

3-Oxime-28-*O*-acetylbetulin (8). A solution of 3-oxo-28-*O*-acetylbetulin[12] (0.1 g, 0.21 mmol) and $NH_2OH{\cdot}HCl$ (0.06 g, 4 equiv) in anhydrous pyridine (5 mL) was heated at 80 °C for 2 h. The reaction was cooled to rt, diluted with CH_2Cl_2 (15 mL), washed with 20% HCl (3 × 15 mL) and brine (3 × 15 mL), dried over Na_2SO_4, and filtered, and the filtrate was concentrated under reduced pressure. The crude product was separated by chromatography on silica gel and by eluting with 5:1 hexanes−EtOAc gave a colorless foamy solid (90 mg, 87%), which was crystallized from $CHCl_3$−MeOH: mp 205 °C; lit.[12] mp 203−204 °C; R_f 0.4 (9:1 hexanes−EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.17 (1H, s, OH), 4.66, 4.56 (1H each, s, H-29), 4.22, 3.83 (1H each, d, *J* = 11 Hz, H-28), 2.96 (1H, dt, *J* = 14, 4 Hz, H-2 equiv), 2.42 (1H, m, H-19), 2.20 (1H, m), 2.04 (3H, s, $OCOCH_3$), l.66, 1.11, l.03, 1.02, 0.93, 0.90 (3H each, s, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.6, 167.0, 150.1, 109.9, 62.8, 55.5, 49.9, 48.7, 47.7, 46.3, 42.7, 40.9, 40.3, 38.8, 37.5, 37.2, 34.5, 33.8, 29.7, 29.5, 27.2, 27.0, 25.2, 22.9, 21.0, 19.1, 19.0, 17.1, 16.0, 15.8, 14.6; (+)-HRAPCIMS *m/z* 498.3949 $[M + H]^+$ (calcd for $C_{32}H_{51}NO_3$, 498.3947).

3-α/β-Amino-28-*O*-acetylbetulin (9a/9b). Oxime **8** (0.38 g, 0.76 mmol) was suspended in MeOH (50 mL) at rt, and NH_4OAc (0.88g, 11.4 mmol, 15 equiv) and $NaBH_3CN$ (0.96 g, 15.2 mmol, 20 equiv) were added. Next $TiCl_3$ (15 wt % in 20−30 wt % HCl, 2.3 mL) was added in 0.1 mL aliquots over 45 min. The mixture was stirred at rt for 18 h; then 2 N NaOH was added dropwise until pH 10. The mixture was concentrated under reduced pressure to an aqueous residue, which was extracted with CH_2Cl_2 (2 × 100 mL). The combined organic extract was washed with H_2O until neutral pH, dried (Na_2SO_4), and concentrated under reduced pressure to a colorless solid (0.3 g, 80% yield). After separation by column chromatography eluting with CH_2Cl_2−MeOH−NH_4OH, 95:4:l, 3α amine (**9a**) was obtained as a colorless glassy solid (0.065 g, 18% yield): R_f 0.2 (CH_2Cl_2−MeOH−NH_4OH, 95:4:l); mp 80−85 °C; $[\alpha]^{23}_D$ +0.31 (*c* 1.9, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.66 (1H, s), 4.56 (1H, s), 4.22 (1H, d, *J* = 12 Hz), 3.83 (1H, d, *J* = 12 Hz), 2.56 (l H, nm, H-3β), 2.42 (1H, td, *J* = 11, 6 Hz), 2.05 (3H, s, $OCOCH_3$), 2.0−1.88 (2H, m), 1.82 (1H, ddd, *J* = 10, 2, 4 Hz), 1.74 (1H, dd, *J* = 12, 8 Hz), 1.20−0.80 (22 H, m, CH_2, CH ring protons), l.66, 1.00, 0.97, 0.85, 0.83, 0.81 (18H, s, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.9, 150.4, 110.0, 63.1, 56.7, 50.6, 49.0, 48.98, 47.9, 46.5, 43.0, 41.3, 37.7, 37.7, 34.8, 34.3, 33.6, 30.0, 29.8, 29.0, 27.2, 25.9, 25.4, 23.6, 21.3, 20.9, 19.3, 18.5, 16.3, 16.2, 15.1; (+)-HRAPCIMS *m/z* 484.4156 $[M + H]^+$ (calcd for $C_{32}H_{54}NO_2$, 484.4155). The 3β amine (**9b**) was also obtained as a colorless crystalline solid (0.24 g, 65%):

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mp 185 °C; $[\alpha]^{23}_{D}$ +3.4 (*c* 2.8, CH_2Cl_2); R_f 0.13 (CH_2Cl_2–MeOH–NH_4OH, 95:4:1); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.66 (1H, s), 4.56 (1H, s), 4.22 (1H, d, J = 12 Hz), 3.83 (1H, d, J = 12 Hz), 2.42 (1H, td, J = 11, 6 Hz), 2.26 (1H, m, H-3α), 2.04 (3H, s, $OCOCH_3$), 2.00–1.86 (2H, m), 1.81 (1H, ddd, J = 10, 4, 2 Hz) 1.74 (1H, dd, J = 12, 8 Hz), 1.70–0.60 (22H, m, CH_2, CH ring protons), 1.65, 1.00, 0.95, 0.89, 0.77, 0.67 (18H, s, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.8, 150.3, 110.0, 63.0, 59.8, 56.1, 50.6, 49.0, 47.9, 46.5, 42.9, 41.0, 39.6, 37.8, 37.5, 34.7, 34.4, 30.0, 29.8, 28.5, 27.2, 25.4, 21.3, 20.9, 19.3, 18.9, 16.2, 15.6, 15.0; (+)-HRAPCIMS m/z 484.4150 (calcd for $C_{32}H_{54}NO_2$, 484.4155).

3-β-Amino-*N*-(Boc-Dap)-28-*O*-acetylbetulin (10). Boc-Dap[10] (0.03 g, 0.10 mmol) was dissolved in dry CH_2Cl_2 (l mL) and added to a solution of **9b** (0.05 g, 0.01 mmol) in dry CH_2Cl_2 (3 mL), the solution was cooled to 0 °C (ice bath), and TEA (80 μL, 3.5 equiv) followed by DEPC (0.025 mL, 0.16 mmol, 1.6 equiv) were then added. The solution was stirred for 18 h with warming to rt over time and concentrated to an amber oil. The crude product was separated by column chromatography on silica gel eluting with hexanes–EtOAc (3:7) to yield a colorless oil, which crystallized from $CHCl_3$–hexanes (0.042 g, 56% yield): mp 105 °C; $[\alpha]^{22}_{D}$ −7.1 (*c* 1.3, $CHCl_3$); R_f 0.3 (hexanes–EtOAc, 2:8); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.83 (0.5H, bs, NH), 5.51 (0.5H, bs, NH), 4.65(1H, s), 4.56 (1H, s), 4.22 (1H, d, J = 11 Hz), 3.98–3.74 (3H, m), 3.62–3.48 (2H, m), 3.41 (3H, s, OCH_3), 3.20 (1H, m), 2.41 (1H, m), 2.27 (1H, m), 2.04 (2H, s, $OCOCH_3$), 1.99–0.70 (34 H, m), 1.65, 0.99, 0.94, 0.83, 0.78, 0.72 (21 H, 6s, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) (two conformers observed)[10c] δ 173.7, 173.2, 171.8, 154.7, 154.5, 150.2, 110.0, 84.1, 82.3, 79.9, 79.3, 62.9, 60.9, 60.5, 59.0, 56.5, 56.2, 56.1, 50.4, 48.9, 47.8, 47.1, 46.6, 46.4, 44.5, 43.9, 42.8, 40.9, 39.2, 37.9, 37.7, 37.1, 34.7, 34.2, 29.8, 29.7, 28.8, 27.2, 26.3, 25.7, 25.5, 25.3, 24.9, 24.7, 24.3, 21.2, 20.8, 19.2, 18.7, 16.6, 16.1, 14.8, 14.6, 14.3; (+)-HRAPCIMS m/z 753.5768 $[M + H]^+$ (calcd for $C_{46}H_{77}N_2O_6$, 753.5782).

3β-Amino-*N*-(Dap-Dil-Val-Dov)-28-*O*-acetylbetulin (11a). Amide **10** (0.05 g, 0.067 mmol) was dissolved in anhydrous CH_2Cl_2 (1 mL) and cooled in an ice bath. TFA (0.1 mL, 0.149 g. 1.3 mmol, 19 equiv) was added, and the solution stirred for 3 h. Excess TFA was removed under reduced pressure for 2 h to yield a foamy solid. The TFA salt (0.43 g, 0.07 mmol) was stirred together with Dov-Val-Dil-TFA[11] (0.038 g, 0.07 mmol) in anhydrous CH_2Cl_2 (2 mL) under N_2 at 0 °C. TEA (0.05 mL, 0.36 mmol, 15 equiv) and DEPC (0.011 mL, 0.7l mmol, 10 equiv) were then added. The reaction mixture was stirred for 18 h at rt, then concentrated and separated using column chromatography eluting with CH_2Cl_2–MeOH (97:3) to give **11a** as a colorless frothy solid (0.052 g, 74% yield). Further purification on Sephadex LH-20 eluting with methanol gave a colorless crystalline solid: mp 135 °C; R_f 0.3 (CH_2Cl_2–MeOH, 95:5); ^{1}H NMR ($CDCl_3$, 500 MHz) (two conformers were present and some signals were doubled) δ 6.90 (1H, d, J = 9.0 Hz), 5.92 (1H, dd, J = 9.5, 4.5 Hz), 4.87 (1H, m), 4.79 (1H, dd, J = 9.2, 6.6 Hz), 4.68 (1H, m), 4.59 (1H, m), 4.27–4.09 (3H, m), 4.02 (1H, d, J = 6.8 Hz), 3.96 (1H, m), 3.88–3.74 (2H, m), 3.71–3.50 (2H, m), 3.49–3.36 (7H, m), 3.41 (s, OCH_3), 3.33 (4H, m), 3.32 (s, OCH_3), 3.15 (1H, s), 3.02 (3H, s), 2.51–2.37 (4H, m), 2.25 (9H, s), 2.07 (3H, s, $OCOCH_3$), 2.15–0.72 (CH, CH_2, and CH_3 protons, betulin ring, and peptide protons); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.6, 173.56, 171.9, 171.8, 170.2, 150.32, 110.0, 88.11, 86.3, 82.3, 78.2, 76.6, 62.9, 61.8, 60.4, 59.3, 58.1, 56.6, 56.2, 53.9, 51.1, 50.4, 48.9, 47.9, 47.8, 46.9, 46.8, 46.4, 45.5, 45.0, 43.8, 43.0, 42.9, 42.8, 41.6, 41.2, 41.0, 40.9, 40.7, 39.3, 38.0, 37.9, 37.7, 37.3, 37.2, 36.8, 36.4, 33.8, 31.9, 31.1, 29.9, 29.7, 28.9, 28.6, 27.8, 27.2, 26.6, 26.5, 26.0, 25.9, 25.5, 25.3, 25.1, 24.7, 23.7, 21.2, 20.9, 20.3, 19.9, 19.8, 19.2, 18.7, 17.9, 16.7, 16.6, 16.2, 15.9, 14.9, 14.1, 13.7, 11.0; (+)-HRAPCIMS m/z 1064.8360 $[M + H]^+$ (calcd for $C_{63}H_{110}N_5O_8$, 1064.8350).

3β-Amino-*N*-(Dap-Dil-Val-Dov)-betulin (11b). A solution of **11a** (0.042 g, 0.04 mmol) in THF–MeOH (0.8 mL:0.6 mL) was treated with 2 N NaOH (0.3 mL) as described in the procedure for the synthesis of **7b**. Following separation of the reaction mixture by chromatography on a silica gel column (gradient elution CH_2Cl_2–MeOH, 97:3 → 94:4) and drying, **11b** was obtained as an amorphous powder (0.008 g, 19% yield): R_f 0.23 (CH_2Cl_2–MeOH, 96%:4%); ^{1}H NMR ($CDCl_3$, 500 MHz) 6.88 (1H, d, J = 8.6 Hz), 5.91 (1H, d, J = 9.6 Hz), 4.86–4.72 (2H, m), 4.68 (1H, s), 4.58 (1H, s), 4.23–4.10 (2H, m), 4.02 (1H, m), 3.80 (1H, d, J = 10.8 Hz), 3.67–3.50 (2H, m), 3.50–3.30 (9H, m), 3.40, 3.32 (s, OCH_3), 3.14 (1H, s), 3.01(s, 2H), 2.51–2.31 (5H, m), 2.28–2.22 (6H, nm), 2.25 (s, $N(CH_3)_2$, 2.13–0.74 (CH, CH_2, and CH_3 betulin ring and peptide protons, m); (+)-HRAPCIMS m/z 1022.8243 $[M + H]^+$ (calcd for $C_{61}H_{108}N_5O_7$, 1022 0.8250).

3,28-Di-*O*-acetyl-30-bromobetulin (12). The bromination of diacetoxy betulin was carried out according to the literature.[13] Diacetoxybetulin (0.5 g, 0.8 mmol) was dissolved in dry CCl_4 (10 mL), NBS (0.3 g, 1.7 mmol) was added, and the reaction mixture was heated at reflux for 1.5 h. The precipitated solid was collected and crystallized from ethanol to give a colorless solid: 0.35 g (61%); mp 189–190 °C; R_f 0.43 (hexanes–EtOAc, 8:2); ^{1}H and ^{13}C NMR spectroscopic data in accord with published data.

3,28-Di-*O*-acetyl-30-azidobetulin (13). To a solution of bromobetulin **12** (1.25 g, 2.0 mmol) in anhydrous DMF (28 mL) was added NaN_3 (0.60 g, 9.2 mmol, 4.5 equiv), the reaction mixture was heated to 90 °C for 30 min and cooled, and EtOAc (40 mL) was added followed by water (40 mL). The organic layer was extracted and washed with brine (2 × 40 mL), dried ($MgSO_4$), and concentrated to an off-white residue, and crystallization from EtOAc gave azide **13** as a colorless solid (0.82 g, 70%): mp 185 °C [lit.[14] 192–193 °C]; R_f 0.5 (EtOAc–hexanes, 2:8); ^{1}H and ^{13}C NMR spectroscopic data consistent with published data;[14] (+)-HRAPCIMS m/z 540.4052 $[M + H - N_2]^+$ (calcd for $C_{34}H_{54}NO_4$, 540.4053).

30-Aminobetulin (14). To a cooled (ice bath) solution of azide **13** (0.l g, 0.18 mmol) in dry THF (2.5 mL) was added $LiAlH_4$ (l M in THF, 0.75 mL, 0.75 mmol, 4 equiv). The reaction mixture was stirred with warming to rt for 2 h and cooled, and water (0.03 mL), 15% NaOH (0.03 mL), and water (0.09 mL) were added successively. The resulting mixture was stirred for 20 min before extraction with ether (25 mL). Anhydrous sodium sulfate was added, and the mixture was allowed to stand overnight, filtered, and concentrated to yield **14** as an off-white solid (68 mg, 85% yield), which was recrystallized from $CHCl_3$–CH_3OH: mp 210–215 °C; $[\alpha]^{22}_{D}$ −2.1 (*c* 0.52, EtOH); R_f 0.1 (CH_2Cl_2–CH_3OH, 9:1); ^{1}H NMR ($CDCl_3$, 400 MHz, sparingly soluble) δ 4.83 (2H, s), 3.77 (1H, d, J = 11 Hz), 3.69 (1H, m), 3.30 (1H, d, J = 11 Hz), 3.25 (1H, d, J = 7.4 Hz), 3.21–3.14 (1H, m), 2.32–2.21 (1H, m), 2.13–2.00 (2H, m), 1.95–1.81 (4H, m), 1.73–1.01 (21 H, CH_2, CH ring protons), 1.00, 0.96, 0.95, 0.80, 0.74 (15H, 5s, CH_3), 0.66 (1H, d, J = 9 Hz); ^{13}C NMR (CD_3OD, 100 MHz) δ 156.7, 106.6, 79.6, 62.8, 60.1, 56.7, 51.5, 50.6, 48.9, 43.8, 42.1, 39.9, 38.6, 38.3, 35.5, 35.0, 30.9, 30.4, 30.1, 28.6, 28.2, 28.0, 27.8, 22.1, 19.4, 16.7, 16.6, 16.2, 15.2; (+)-HRAPCIMS m/z 458.3998 $[M + H]^+$ (calcd for $C_{30}H_{52}NO_2$, 458.3998).

30-*N*-(Boc-Dap)-betulin (15a). To a cooled (ice bath) solution of amine **14** (0.15 g, 0.33 mmol) in dry CH_2Cl_2 (10 mL) was added a solution of Boc-Dap[10] (0.09 g, 0.30 mmol) in CH_2Cl_2 (3 mL). TEA (0.24 mL, 1.71 mmol) and DEPC (0.033 mL, 2.13 mmol) were added, and the reaction mixture was stirred for 20 h before terminating with removal of the solvent under low pressure overnight. Separation by flash silica gel chromatograpy (eluent: CH_2Cl_2–CH_3OH, 98:2) gave amide **15a** as a frothy solid (85 mg, 50% yield): mp 129–131 °C; R_f 0.2 (CH_2Cl_2–CH_3OH, 96:4); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.56 (0.5H, bs), 5.84 (0.5H, bs), 4.81 (1H, s), 4.72 (1H, s), 3.93–3.64 (5H, m), 3.59–3.31 (1H, m), 3.40 (3H, s, OCH_3), 3.29–3.08 (3H, m), 2.45–2.19 (2H, m), 2.12–1.97 (1H, m), 1.96–0.67 (CH, CH_2 ring protons and Boc-Dap protons), 0.97, 0.93, 0.78, 0.73 (15H, 4s, CH_3), 0.65 (1H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (two conformers present) 174.2, 173.7, 154.9, 154.6, 151.7, 107.9, 84.2, 82.4, 80.9, 79.5, 79.0, 60.9, 60.3, 59.4, 58.8, 55.4, 50.5, 49.5, 47.9, 47.2, 46.7, 44.7, 44.2, 42.8, 41.1, 39.0, 38.9, 37.4, 37.3, 34.4, 34.0, 29.4, 28.7 (m), 28.2, 27.5, 27.2, 26.2, 25.6, 24.8, 24.4, 21.1, 18.4, 16.3, 16.1, 15.6, 14.9; (+)-HRAPCIMS m/z 727.5631 $[M + H]^+$ (calcd for $C_{44}H_{75}N_2O_6$, 727.5625).

30-*N*-(Boc-Dap)-3,28-di-*O*-acetoxybetulin (15b). Alcohol **15a** (0.08 g, 0.11 mmol) was taken up in dry pyridine (2 mL). Acetic

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anhydride (0.05 mL, 0.5 mmol) and DMAP (1 mg, 0.008 mmol) were added, and the reaction mixture was stirred at rt under N_2 for 24 h. Then, CH_2Cl_2 (10 mL) was added, and the organic fraction was washed with cold 3 N H_2SO_4 (4 mL), saturated $NaHCO_3$ (5 mL), and brine (7 mL), dried ($MgSO_4$), and concentrated. Column chromatography (CH_2Cl_2–MeOH, 98:2 →86:4) on silica gel provided **15b** as an off-white frothy solid (78 mg, 87% yield): mp 98–100 °C; R_f 0.5 (CH_2Cl_2–CH_3OH, 96:4); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.28 (0.5H, bs), 5.81 (0.5H, bs), 4.82 (1H, m), 4.73 (1H, m), 4.42 (1H, m), 4.20 (1H, d, J = 12 Hz), 3.92–3.70 (5H, m), 3.56–3.27 (4H, m), 3.39 (s, OCH_3), 3.19 (1H, m), 2.48–2.25 (2H, m), 2.01 (3H, s, $OCOCH_3$), 1.99 (3H, s, $OCOCH_3$), 2.00 (1H, m), 1.93–1.50 (12H, m), 1.50–1.30 (16H, m), 1.43, 1.49 (s, Boc-CH_3), 1.28–1.00 (8H, m), 1.00–0.75 (16H, m), 0.98, 0.91, 0.79 (× 2), 0.78 (s, CH_3), 0.73 (1H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) (doubling of signals observed for the peptide portion of the molecule) δ 174.1, 173.6, 171.65, 171.12, 155.0, 154.6, 151.3, 108.5, 84.4, 82.6, 81.0, 79.4, 62.6, 60.8, 59.3, 58.8, 55.5, 50.4, 49.6, 47.1, 46.7, 46.5, 45.1, 44.2, 42.8, 42.3, 41.1, 38.5, 37.9, 34.6, 34.3, 31.2, 29.9, 28.7, 28.1, 27.2, 26.6, 26.2, 25.6, 24.8, 24.4, 23.8, 21.4, 21.1, 21.0, 16.6, 16.3, 16.2, 14.5 × 2; (+)-HRESIMS m/z 811.5813 $[M + H]^+$ (calcd for $C_{48}H_{78}N_2O_8$, 811.5831).

30-*N*-(Dap)-3,28-Di-*O*-acetoxybetulin Trifluoroacetate (15c). TFA (0.15 mL, 0.22 g, 1.96 mmol) was added to a cooled solution of **15b** (0.78 g, 0.096 mmol) in dry CH_2Cl_2 (1.5 mL), and stirring was continued with warming to rt under N_2 for 4 h. The product mixture was concentrated in the presence of toluene to aid in TFA removal followed by further drying overnight under low pressure to give crude **15c** as a frothy orange residue: R_f 0.2 (CH_2Cl_2–MeOH, 92:8). The salt was used in the next step without further purification.

30-*N*-(Dap-Dil-Val-Dov)-3,28-Di-*O*-acetoxybetulin (16a). Dov-Val Dil-TFA salt (0.055 g, 0.10 mmol) was added to a solution of amide **15c** (0.096 mmol) in dry CH_2Cl_2 (2 mL) at rt. The reaction mixture was cooled in an ice bath, and TEA (0.2 mL) and DEPC (0.17 mL, 0.18 g, 1.1 mmol) were added. Stirring was continued for 24 h with warming to rt over time. The solvent was removed under vacuum to yield a brown residue. Separation by column chromatography on flash silica gel (eluent: CH_2Cl_2–MeOH, 96:4) gave **16a** as an off-white solid (50 mg). Further purification on a Sephadex LH-20 column gave a frothy off-white solid (38 mg, 35% yield): R_f 0.5 (CH_2Cl_2–MeOH, 96:4); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.90 (1H, d, J = 9 Hz), 6.37 (1H, m), 4.90–4.74 (3H, m), 4.47 (1H, dd, J = 11, 5.4 Hz), 4.25 (1H, d, J = 10.8 Hz), 4.20–4.09 (2H, m), 3.97 (1H, dd, J = 7.6, 2.3 Hz), 3.83 (2H, m), 3.80 (1H, d, J = 10.8), 3.54–3.40 (2H, m), 3.42 (3H, s, OCH_3), 3.32 (3H, s, OCH_3), 3.03 (3H, s, NCH_3), 2.53–2.29 (5H, m), 2.25 (6H, s), 2.13–1.96 (11 H, m), 1.88–1.74 (4H, m), 1.73–1.56 (7H, m), 1.54–1.32 (9H, m), 1.32–1.23 (5H, m), 1.20–1.05 (3H, m), 1.04–0.91 (24H, m), 0.88–0.74 (13H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 174.0, 173.6, 171.9, 171.7, 171.2, 170.5, 151.4, 108.2, 86.2, 82.4, 81.0, 78.5, 77.4, 76.7, 62.7, 60.6, 59.4, 58.1, 55.5, 53.9, 50.4, 49.5, 47.9, 46.5, 44.9, 44.3, 43.1, 42.8, 41.1, 38.6, 38.0, 37.8, 37.7, 37.2, 34.6, 34.3, 33.3, 31.2, 31.1, 29.9, 28.1, 27.8, 27.2, 26.6, 25.9, 25.2, 25.1, 23.9, 23.7, 21.5, 21.2, 21.1, 20.9, 20.3, 20.0, 19.7, 18.3, 17.9, 16.7, 16.3, 16.2, 16.0, 15.0, 14.5, 10.9; (+)-HRESIMS m/z 1122.8411 $[M + H]^+$ (calcd for $C_{65}H_{112}N_5O_{10}$, 1122.8404).

30-*N*-(Dap-Dil-Val-Dov)-Betulin (16b). To a solution of 30-*N*-(Dap-Dil-Val-Dov)-3,28-di-*O*-acetoxybetulin (**16a**) (0.02 g, 0.018 mmol) in a mixture of THF–MeOH (1.4 mL, 0.8:0.6) was added 2 N NaOH (0.3 mL). The solution was stirred at rt for 24 h. The solvent was evaporated, and the residue separated on a silica gel column eluting with CH_2Cl_2–CH_3OH (95:5) to give the product as a colorless solid, 0.013 g (70% yield): mp 145 °C; R_f 0.3 (CH_2Cl_2–MeOH, 92:8); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.98–6.85 (1H, m), 6.42 (1H, m), 4.93–4.72 (3H, m), 4.15 (2H, m), 3.96 (1H, m), 3.90–3.65 (3H, m), 3.51–3.29 (7H, m), 3.42 (3H, s, OCH_3), 3.33 (3H, s, OCH_3), 3.19 (1H, m), 3.10, 3.03 (3H, s, NCH_3), 2.59–2.39 (3H, m), 2.33 (1H, m), 2.39–2.20 (7H, m), 2.25 (3H, s, $N(CH_3)_2$), 2.15–0.74 (CH, CH_2, and CH_3 protons), 0.68 (1H, d, J = 10.1 Hz); (+)-HRESIMS m/z 1038.8202 $[M + H]^+$ (calcd for $C_{61}H_{108}N_5O_8$, 1038.8192).

3-*O*-Acetyl-28-amidoallobetulin-28-*N*-1′-ethyldiamine-*N*-2′-Dap-Dil-Val-Dov (18a). Betulinic acid and 3-*O*-acetylbetulinic acid were prepared as previously described.[8] 3-*O*-Acetylbetulinic acid (0.113 g, 0.2 mmol) and oxalyl chloride (2 M in CH_2Cl_2) (1 mL, 2 mmol) were stirred together for 10 min, then concentrated under vacuum to remove excess reagent. The residue was dissolved in CH_2Cl_2 (2 mL), and diethylamine (0.15 mL) was added. The mixture was stirred at rt under N_2 for 5 h, the solution was concentrated, and water was added. The resulting white precipitate was collected, washed with water, dissolved in ethanol, and filtered. The ethanol solution was then concentrated to a light yellow colored residue, which was dried under reduced pressure. The residue (0.08 g, 0.15 mmol) was dissolved in anhydrous CH_2Cl_2 (2 mL), and a solution of Boc-Dap (0.043 g, 0.15 mmol, 1 equiv) in CH_2Cl_2 (2 mL) was added. The solution was cooled in an ice bath, and TEA (60 μL, 3.5 equiv) followed by DEPC (0.036 mL, 1.5 equiv) were added. The reaction mixture was stirred for 24 h under N_2 and allowed to warm to rt over time, before concentrating and separating on silica gel by eluting with hexanes–ethyl acetate (8:2) followed by hexane–acetone (7:3) and yielded the Boc-protected amide **17** as a colorless solid: 78 mg; mp 135 °C; R_f 0.14 (hexane–CH_3COCH_3, 4:1); (+)-HRAPCIMS m/z 810.5978 $[M + H]^+$ (calcd for $C_{48}H_{80}N_3O_7$, 810.5996). The Boc-protected amide **17** was used directly in the next step. This compound **17** (0.077 g, 0.096 mmol) was dissolved in anhydrous CH_2Cl_2 (4 mL), and the solution was cooled (ice bath). TFA (1.5 mL) was added, and the reaction was stirred for 1 h, then concentrated under reduced pressure overnight. The TFA salt was dissolved in CH_2Cl_2 (4 mL), and Dov-Val-Dil-TFA (0.05 g) was added. The reaction mixture was cooled (ice bath), and TEA (0.06 mL) and DEPC (0.02 mL) were added, then stirred under nitrogen for 24 h with warming to rt. Concentration to a yellow oil followed by separation on silica gel (gradient elution: hexanes–CH_3COCH_3, 7:3→ 6.5 → 4:5) gave **18a** as a colorless solid (32 mg, 18% yield from Boc-Dap): mp 114–115 °C; R_f 0.4 (hexanes–CH_3COCH_3, 4:6); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.85 (1H, d, J = 9.0 Hz), 6.51 (1H, m), 4.88, 4.69 (0.5 H, 1H, m), 4.42 (1H, m), 4.19–4.06 (2H, m), 3.91 (1H, m), 3.82–3.72 (2H, m), 3.52–3.22 (13H, m), 3.37, 3.29 (3H, s, OCH_3), 2.99, 3.10 (3H, s, NCH_3), 2.48–2.34 (3H, m), 2.22 (6H, s, $N(CH_3)_2$), 1.83–0.73 (CH, CH_2, and CH_3 protons); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.9, 171.9, 171.8, 171.2, 170.3, 170.2, 82.5, 81.0, 76.8, 76.7, 61.9, 61.8, 60.4, 59.2, 59.0, 58.3 58.2, 55.8, 54.0, 53.9, 51.3, 47.8, 47.1, 47.05, 46.8, 45.9, 45.7, 45.3, 45.2, 43.8, 43.1, 43.0, 40.7, 40.4, 40.3, 38.8, 38.0, 37.9, 37.4, 35.9, 35.8, 34.5, 34.3, 34.2, 33.9, 33.4, 32.7, 31.2, 31.1, 29.5, 29.1, 28.1, 27.9, 27.4, 26.5, 26.3, 25.9, 25.3, 25.0, 24.3, 24.2, 23.8, 21.5, 21.1, 20.3, 18.3, 18.0, 17.9, 16.7, 16.6, 15.7, 15.69, 15.6, 14.1, 13.9, 11.0; (+)-HRAPCIMS m/z 1121.8560 $[M + H]^+$ (calcd for $C_{65}H_{113}N_6O_9$, 1121.857).

28-Amidoallobetulin-28-*N*-1′-ethyldiamine-*N*-2′-Dap-Dil-Val-Dov (18b). To a solution of peptide **18a** (0.012 g, 0.011 mmol) in a mixture of THF–MeOH (1.4 mL, 0.8:0.6) was added 2 N NaOH (0.3 mL). The solution was stirred at rt for 24 h. The solvent was evaporated, and the residue separated on a silica gel column eluting with CH_2Cl_2–CH_3OH (95:5) to give the C-3-deprotected product as a colorless solid: 2 mg (17% yield); R_f 0.13 (CH_2Cl_2–MeOH, 92:8); ^{1}H NMR ($CDCl_3$, 500 MHz) (conformational isomers observed) δ 6.94, 6.89 (1H, d, J = 9.0 Hz), 6.51, 6.14 (1H, m), 4.95–4.70 (2H, m), 4.25–4.10 (2H, m), 3.96 (1H, m), 3.82 (1H, m), 3.57–3.28 (13H, m), 3.42, 3.41, 3.34, 3.33 (12H, s, OCH_3), 3.21 (1H, dd, J = 12, 4 Hz), 3.14, 3.03 (3H, s, NCH_3), 2.63–2.38 (4H, m), 2.36–2.22 (7H, m), 2.27, 2.26 [s, $N(CH_3)_2$], 2.19–1.96 (5H, m), 1.87–0.80 (CH, CH_2, and CH_3 protons), 0.78 (3H, s, CH_3), 0.70 (1H, m); (+)-HRESIMS m/z 1079.8472 (calcd for $C_{63}H_{111}N_6O_8$, 1079.8458).

Cancer Cell Line Testing Procedures. Inhibition of human cancer cell growth was assessed using the standard sulforhodamine B assay of the U.S. National Cancer Institute, as previously described.[15] To begin, cells in a 5% fetal bovine serum/RPMI1640 medium were inoculated in 96-well plates and incubated for 24 h. Next, serial dilutions of the compounds were added. After 48 h, the plates were fixed with trichloroacetic acid, stained with sulforhodamine B, and read with an automated microplate reader. Each plate contains its own

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positive and negative controls as well as a series of plates set up with the only purpose of establishing a baseline at the time of the addition of the compounds under evaluation. Within an experiment, a compound is evaluated in five serial dilutions and optical density data are measured in duplicates for each end point. A growth inhibition of 50% (GI_{50}, or the drug concentration causing a 50% reduction in the net protein increase) was calculated from optical density data with Immunosoft software.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00536.

^{1}H and ^{13}C NMR spectral data for compounds **3**, **5**, **7a**, **9a**, **9b**, **10**, **11a**, **14**, **15a**, **15b**, **16a**, and **18a**; ^{1}H NMR and HRMS data for betulastatins 1 (**7b**), 2 (**11b**), 3 (**16b**), and 4 (**18b**) (PDF)

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Notes

The authors declare no competing financial interest.

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