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Synthesis and Biological Evaluation of Antibody Conjugates of Phosphate Prodrugs of Cytotoxic DNA Alkylators for the Targeted Treatment of Cancer

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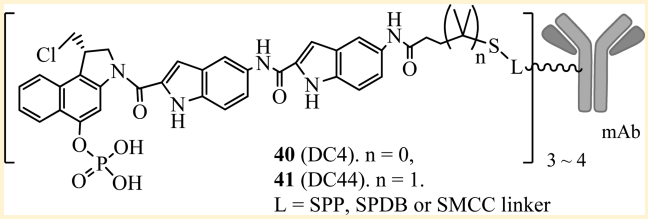
Supporting Information

ABSTRACT: The synthesis and biological evaluation of phosphate prodrugs of analogues of **1** (CC-1065) and their conjugates with antibodies are described. The phosphate group on the 1,2,9,9a-tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one (CBI) portion of the compounds confers enhanced solubility and stability in aqueous solutions. In the presence of phosphatases, these compounds convert into active DNA-alkylating agents. The synthesis of the prodrugs was achieved sequentially through coupling of CBI with a bis-indolyl moiety, followed by attachment of a thiol-containing linker, and conversion of the hydroxyl group of CBI into a phosphate prodrug. The linkers incorporated into the prodrugs enable conjugation to an antibody via either a stable disulfide or thioether bond, in aqueous buffer solutions containing as little as 5% organic cosolvent, resulting in exclusively monomeric and stable antibody-cytotoxic prodrug conjugates. Two disulfide-containing linkers differing in the degree of steric hindrance were used in antibody conjugates to test the effect of different rates of intracellular disulfide cleavage and effector release on biological activity. The prodrugs can be converted to the active cytotoxic compounds through the action of endogenous phosphatases. Antibody−prodrug conjugates displayed potent antigen-selective cytotoxic activity in vitro and antitumor activity in vivo.



40 (DC4). n = 0,
41 (DC44). n = 1.
L = SPP, SPDB or SMCC linker

INTRODUCTION

For cancer treatment, it is highly desirable to selectively target malignant cells and not healthy tissues. One approach that has been advanced through a series of preclinical and clinical studies is to use antibodies that recognize tumor-associated antigens expressed on the surface of tumor cells to selectively target cytotoxic agents to these cells.[1−3] In this approach, a cytotoxic agent is covalently linked to a tumor-targeted monoclonal antibody (mAb) forming an antibody−drug conjugate (ADC). Upon binding to cell surface antigens, a typical ADC is internalized via antigen-mediated endocytosis and then transported to lysosomes, where enzymes or reducing agents facilitate release of the cytotoxic effector molecule which then kills the tumor cells. A number of ADCs containing a highly potent cytotoxic compound are currently in various stages of preclinical and clinical development. The cytotoxic agents used include the antimicrotubule agents, the maytansinoids (DM1 and DM4)[1−4] and the auristatins,[5,6] the DNA damaging agents, calicheamicin,[7,8] and analogues of **1** and duocarmycin.[9−11]

The compound **1** bearing a cyclopropapyrroloindole pharmacophore (CPI) is a very potent antitumor antibiotic isolated from *Streptomyces* sp. in the late 1970s.[12] The synthetic and mechanistic aspects of **1** and its derivatives have been extensively studied by Boger and co-workers.[13,14] Compound **1** was not developed because it exhibited delayed toxicity in mice. The design of synthetic analogues of **1**, such as adozelesin, carzelesin, and bizelesin, overcame the delayed toxicity issue.[15,16] Thus, these analogues proceeded to clinical evaluation but were found to have limited therapeutic activity and their development was discontinued.[17] A simpler cyclopropabenzindole (CBI) subunit developed by Boger and co-workers[18] to replace the alkylating CPI subunit of **1** gave compounds that were chemically more stable (∼4-fold), biologically more potent (∼4-fold), and considerably more synthetically accessible.[19] A simplified analogue of **1** containing the CBI subunit, called DC1, has been explored in our laboratory as an effector moiety for conjugation with an antibody (Figure 1).[9] DC1 is about 1000-fold more cytotoxic than commonly used anticancer drugs, such as doxorubicin, methotrexate, and vincristine.[20] It binds to the minor groove of DNA, followed by alkylation of adenine residues by its CBI component.[13] A conjugate of DC1 with the humanized anti-CD19 antibody B4, huB4−DC1, was found to be highly cytotoxic to CD19-expressing cell lines, with IC_{50} values in the picomolar range. The conjugate was selective in its cytotoxicity being at least 1000-fold less cytotoxic toward antigen negative cells. In human tumor xenograft models in immunodeficient mice, huB4−DC1 was found to be superior in its antitumor

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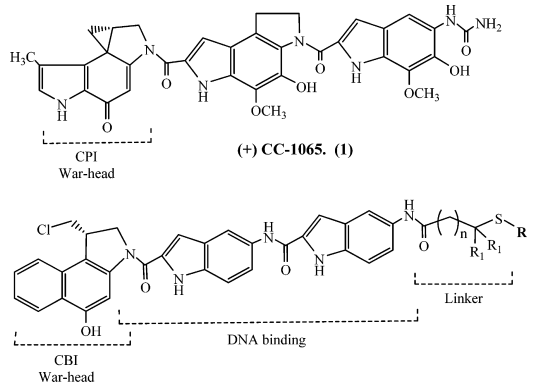
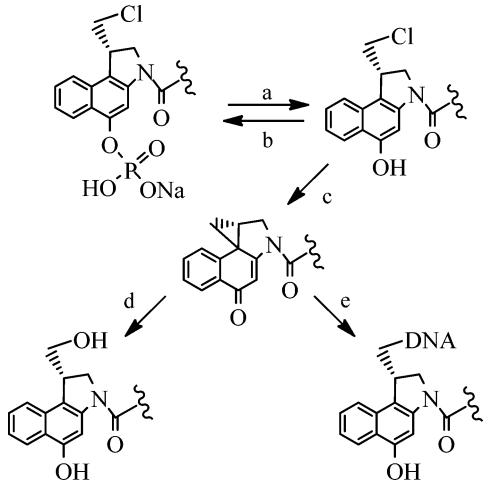


Figure 1. Structures of CC-1065 (**1**),[12] DC1 (**2**), DC41 (**3**), and huB4–DC1 conjugate.[9] DC1 (**2**): R = H, R_1 = H, n = 1; DC41 (**3**): R = H, R_1 = CH_3, n = 2; B4–DC1 conjugate: R = $SCH(CH_3)CON(CH_3)(CH_2)_3CON$-mAb, R_1 = H, n = 1.

activity to the clinically used anticancer drugs doxorubicin, vincristine, and cyclophosphamide.[9] Antibody–DC1 conjugates were not developed further for two main reasons, instability and poor solubility of the DC1 component in aqueous solutions. In physiological buffers, the *seco*-form of DC1 (*seco*-CBI) is spontaneously converted via Winstein cyclization[21] to the active cyclopropyl form, which then reacts with water, resulting in opening of the cyclopropyl ring yielding the inactive hydroxy compound (Scheme 1). The poor solubility of

Scheme 1. The Property of CBI[a]



[a]Conditions: (a) phophatases; (b) chemical phosphorylation; (c) aqueous buffer; (d) hydrolysis by H_2O; (e) DNA alkylation.

DC1 complicated the conjugation efforts, necessitating the use of 20% organic cosolvents which led to significant aggregation of the conjugates. Therefore, a prodrug of DC1 has been sought that would (i) remain stable in aqueous formulation and during circulation in vivo, (ii) convert into the active cyclopropyl form only upon reaching the target tumor cell (this might limit the extent of its inactivation in the absence of target DNA, and also might reduce toxic side effects), and (iii) have improved solubility in aqueous solutions.

Several prodrugs of various analogues of **1** have been reported in which the *seco* phenol was protected with a labile group, such as carbamoyl,[10,22,23] glycosyl,[24] *O*-(acylamino),[25] peptidyl,[26] and carbonyl.[23,27] Some of these prodrugs exhibited improved therapeutic efficacy in vivo compared to the parent compounds.[28,29] A few of these prodrugs were conjugated with antibodies, and the resulting conjugates displayed antigen-specific cytotoxicity in vitro.[26] However, most of these prodrugs, in particular the phenyl, piperazino, and piperidino carbamates, display low solubility in aqueous buffer solution in the pH range (6–8) typically used in antibody-conjugation reactions. Moreover, the activation of other piperazino or piperidino carbamate prodrugs, such as irinotecan, by human carboxylesterases is reported to be inefficient,[30] and interpatient variability in the level of enzyme activity has been reported.[31] Thus, the level of carbamate activation observed in preclinical models (e.g., mice or rats) may not translate to the human situation.

There are a few examples of anticancer drugs, unrelated to **1**, that have been converted into water-soluble prodrugs. The anticancer drug, etoposide phosphate, is an example of a water-soluble prodrug that has a phenolic phosphate protecting group and is rapidly converted into the active moiety during circulation in humans, presumably through hydrolysis by endogenous alkaline phosphatase.[32] Thus, a phosphate group appeared to be a good choice for protecting the phenolic hydroxyl of the CBI unit. Here we report prodrugs of DC1 in which seco-CBI is protected with a phosphate group. It has been previously reported that protected *seco*-CBI compounds generally do not alkylate DNA.[33] We found that these prodrugs were stable in aqueous solutions at physiological pH but were easily converted into active drugs in the presence of phosphatases. These prodrug conjugates were highly potent in killing cells in vitro, suggesting that the prodrugs were activated inside target cells, presumably by endogenous phosphatases.

RESULTS

DC1 comprises three different subunits: a DNA-alkylating unit CBI (A), binding unit bis-indole (B), and a linker unit for conjugation (C) to antibodies. CBI ((1,2,9,9a-tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one) is the key precursor required for the synthesis of DC1 and its derivatives.[34] The synthesis of CBI (Scheme 2) began with the commercially available 1,3-dihydroxynaphthlene **4**, which was treated with ammonia at high temperature to generate the unstable 3-aminonaphthelenol, which was converted without purification to the bis-BOC protected 1-hydroxy-3-naphthylamine (**5**) using excess BOC anhydride in the presence of a base in 30% yield. Regioselective electrophilic iodination of **5** with *N*-iodosuccinimide and a catalytic amount of TsOH at reduced temperature gave the iodide **6** as the sole identifiable product in 86% yield. Deprotonation of the carbamate **6**, using NaH in DMF, followed by *N*-alkylation of the resulting anion with commercially available (*E*:*Z*)-1,3-dichloropropene. gave a 93% yield of the (1:2) *E*:*Z* isomers of vinyl chloride **7**, the desired precursor for the key aryl radical cyclization. A deoxygenated solution of iodide **7** in dry benzene, refluxed for 3 h in the presence of one equivalent of tri-*N*-butyltin hydride and AIBN as the catalyst, gave the desired, fully protected alkylating subunit **8** in 94% yield as a mixture of optical isomers. The cyclized product **8** is formed in a highly chemoselective manner; the reaction is believed to proceed via an initial preferential homolysis of the weaker aryl C–I bond in **7** to generate an aryl radical which undergoes a preferred 5-exotrig intramolecular cyclization onto the tethered vinyl chloride acceptor to give **8**.[35] Resolution of the isomers of **8** was readily

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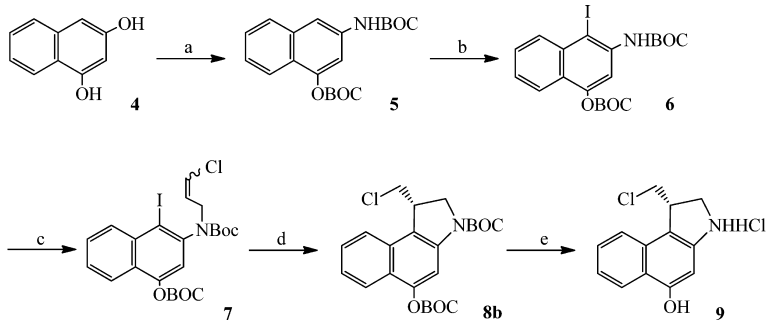
Scheme 2. Synthesis of CBI[a]



[a]Conditions: (a) (1) NH_3(l), 135 °C, 14 h, (2) BOC_2O, DIPEA, 30%; (b) NIS/TsOH, −40 °C to RT, 4 h, 86%; (c) NaH/DMF, ClCH═CH−CH_2Cl, 0 °C, 4 h, 93%; (d) (l) Bu_3SnH/AIBN, phH/80 °C, 3 h, 94%, (2) chiral OD column, 20% IPA/hexane; (e) HCl (conc), EtOAc, 95%.

accomplished by chiral chromatography on a preparative Chiralcel OD column providing multigram quantities of both enantiomers (+)-**8a** (t_R = 18.5 min) and (−)-(*S*)-**8b** (t_R = 35.8 min, >99% ee) in a single run. The slower eluting (−)-enantiomer of **8b** was assigned to be the desired natural (*S*)-configuration as it exhibited the more potent biological activity, and its DNA-alkylation selectivity was identical with that of the natural products. Acid-mediated (20% HCl in ethyl acetate) deprotection of (−)-(1*S*)-**8b** removed both BOC groups quantitatively to give the CBI salt **9**, which was directly used for the synthesis of DC1. The incorporation of these synthetic improvements provided CBI salt (**9**) in six steps and overall 21% conversion.

The linkers chosen to connect analogues of **1** to a monoclonal antibody were prepared using either commercially available 3-mercaptopropionic acid (**10**) or 4-mercapto-4,4-dimethyl-butanoic acid (**11**) prepared from isobutylene sulfide.[4] As depicted in Scheme 3, the reactive free thiols of these two linkers were protected as methylthio, acetyl, or pyridine-2-ylthio derivatives by reaction with an excess of $CH_3SSO_2CH_3$, Ac_2O, or aldrithiol-2 (PySSPy), respectively, in a neutral aqueous buffer.

Scheme 3. Synthesis of DCx Linkers[a]

10: n = 0;
11: n = 1.

12: R = SCH_3, n = 0;
13: R = SPy, n = 0;
14: R = Ac, n = 0;
15: R = SCH_3, n =1.

[a]Condition: MEOH/H_2O, pH 6−7.5, $CH_3SSO_2CH_3$, or PySSPy, or Ac_2O.

The synthesis of DC1 was accomplished through an initial amide bond coupling of the CBI subunit with the di-indole moiety followed by coupling with the linker (Scheme 4). Commercially available ethyl 5-nitroindole-2-carboxylate **16** was either hydrolyzed to the carboxylic acid **17** or reduced to ethyl 5-aminoindole-2-carboxylate **18** in excellent yields. Condensation of **17** and **18** in the presence of *O*-(benzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) in *N*,*N*-dimethyl acetamide (DMA) provided 80% yield of the bis-indolyl ester **19** after simple filtration and washing with water and methanol. Alkaline hydrolysis of the ester in **19** with a mixture of 1.0 M NaOH and DMA at 60 °C gave the bis-indole carboxylic acid **20** in 90% yield with no detected cleavage of any of the amide bonds. Coupling of the free acid of **20** with *seco*-CBI **9** using EDC in DMA provided the bis indolyl-*seco*-CBI compound **21** in 75% yield. Reduction of the nitro group in **21** with hydrogen over Pd/C under mildly acidic conditions provided the amino-bis-indolyl-*seco*-CBI compound **22** which was then coupled with the different linkers (**12**−**15**) to form DC1−SR2 (**23**−**26**, respectively). Initial efforts to reduce **21** with $TiCl_3$ in an acetone/water mixture led to poor yields of DC1−SR2 products due to incomplete reduction of **21** and the instability of both **22** and DC1−SR2 in aqueous solutions. Also the separation of DC1−SR2 from the mixtures of **21** and **22** was tedious and it had to be conducted in a mixed solvent system containing DMA or DMF through normal phase HPLC purification. The linkable compound **2** (DC1) can be generated from the reduction of **23** (DC1SMe) or **24** (DC1SPy) by either DTT or TCEP or from the hydrolysis of **25** (DC1Ac) by NH_2OH in DMA containing a small amount of a weak acid buffer. At high pH (>7.5), DC1−SR2, exemplified by compound **23**, readily underwent spirocyclic hydrolysis with displacement of the chloride of the CBI subunit to form the cyclopropylcycloheadienone compound **27** (DC10SMe), which would undergo N-3 alkylation with an adenine residue and thus form a DNA adduct.[33] Because of challenges encountered in the purification of DC1−SR2 from compound **22**, an alternative strategy involving a change of the sequence of amide bond coupling to prepare DC1−SR2 was explored. Thus, the linker was first coupled to the bis-indolyl moiety followed by coupling of the CBI unit.

5-Nitroindole-2-carboxylic acid **17** was converted to the *tert*-butyl ester **28** in 85% yield utilizing oxalyl chloride and potassium *tert*-butoxide in tetrahydrofuran (Scheme 5). Initial efforts on direct transesterification of the commercial available ethyl ester **16** to the *t*-butyl ester **28,** or from ethyl ester **19** to *t*-butyl ester **30** utilizing published *t*-BuOH and sulfated SiO_2 method[36] were unsuccessful. Pd/C catalyzed reduction of nitro group in **28** with hydrogen provided the amino ester **29** in quantitative yield. Amide bond coupling of **29** with 5-nitroindole-2-carboxylic acid **17** with TBTU in DMA provided the nitro-bis-indolyl ester **30** in 89% yields. Reduction of the nitro group in **30** by catalytic hydrogenation, followed by coupling of the resulting amino compound **31** with either linker **12** or **15**, provided linkable bis-indoles **32** and **33** in 80% and 82% yields, respectively. The *t*-butyl esters of **32** and **33** were hydrolyzed by 20% TFA in dichloromethane in the presence of a catalytic amount of Et_3SiH to give the carboxylic acids **34** and **35** in 85−92% yields after crystallization. Coupling of **34** or **35**

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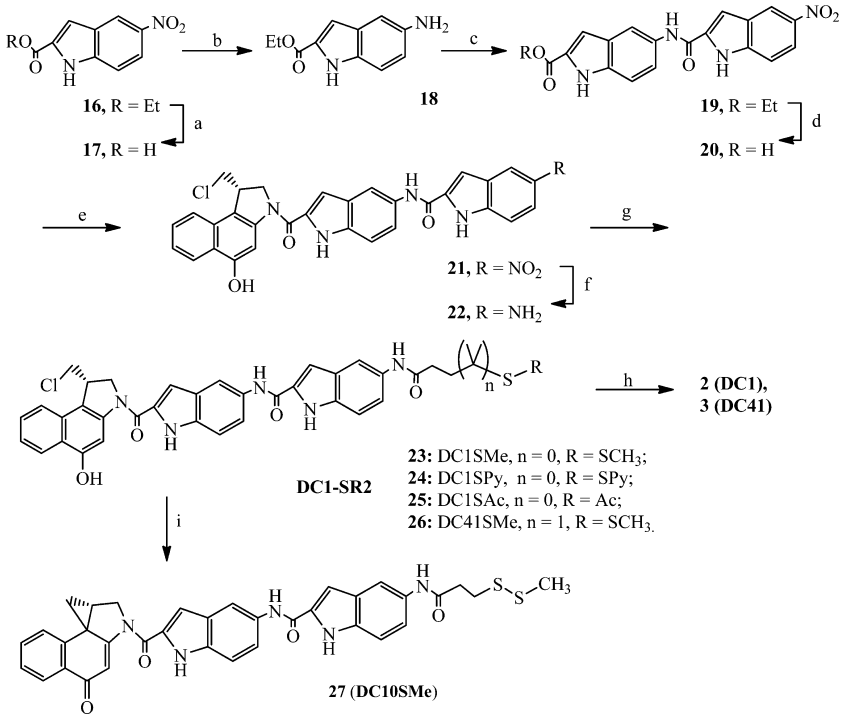
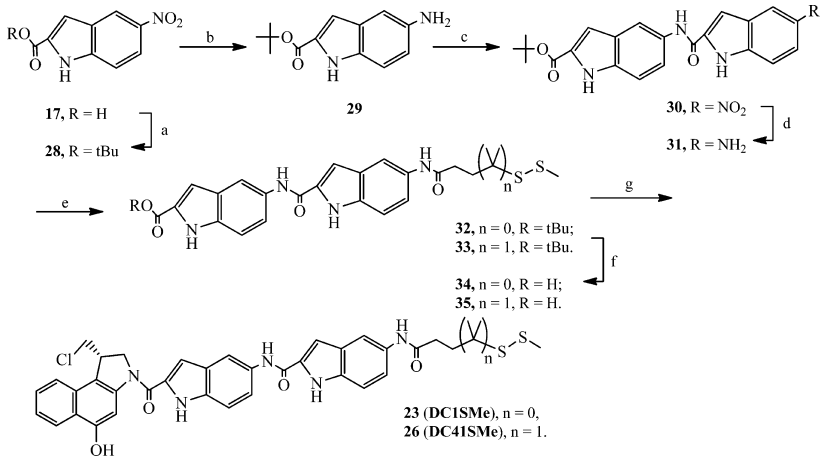
Scheme 4. Synthesis of DC1−SR2[a]



[a]Conditions: (a) NaOH/THF, 94%; (b) H_2/Pd/C, THF, 97%; (c) **17**, TBTU/DMA, 80%; (d) NaOH/DMA, 90%; (e) **9**, EDC/DMA, 75%; (f) H_2/Pd/C, DMA, 90%; (g) **12**, **13**, **14**, **15**, EDC/DMA; (h) DTT/DMA/TCEP, pH 6 for **23**, **24**, **26**, NH_2OH/DMA pH 6, for **25**; (i) from **23**, 5% $NaHCO_3$/THF, 80%.

Scheme 5. Alternative Strategy for Synthesis of DC1SMe and DC41SMe[a]



[a]Conditions: (a) (1) $(COCl)_2$, (2) *t*-BuOK, 85%; (b) H_2/Pd/C, THF, 97%; (c) **16**, TBTU/DMA, 89%; (d) H_2/Pd/C/DMA, 92%; (e) **12** or **15**, EDC/DMA, ∼80%; (f) 20% TFA/DCM/Et_3SiH (cat), ∼90%; (g) **9**, EDC/DMA, ∼75%.

with *seco*-CBI **9** in the presence of EDC in DMA provided **23** (DC1SMe) and **26** (DC41SMe) in 70−80% yields. Additionally compounds **23** and **26** were easily separated from compounds **34** and **35** using SiO_2 chromatography. Thus the method in Scheme 5 was more favorable than the one in Scheme 4 for the large scale production of DC1−SR2.

Compounds **23** and **26** were converted to the phosphate prodrugs **40** (DC4) and **41** (DC44), respectively, as shown in Scheme 6. Treatment of the phenols **23** or **26** with dibenzylphosphate, carbon tetrachloride, and base (DIPEA) in a mixture of THF and CH_3CN provided the DC1−dibenzylphosphates (**36**, **37**). Removal of the benzyl protecting groups and cleavage of the disulfide bonds of DC1−dibenzylphosphates (**36**, **37**) by methanesulfonic acid with the aid of DTT, provided **40** (DC4) and **41** (DC44). Alternatively reaction of **23** or **26** with phosphorus oxychloride in the presence of base (DIPEA) provided DC1−dichlorophosphates which hydrolyzed spontaneously at pH 4−6 to form **38** (DC4SMe) and **39** (DC44SMe), respectively. Reduction of **38** and **39** by TCEP or DTT in DMA containing pH 5−7 buffers provided **40** (DC4) and **41** (DC44) in high yields (>90%).

Solubility and Stability. To achieve conjugation of a cytotoxic agent to an antibody and provide a reasonable yield,

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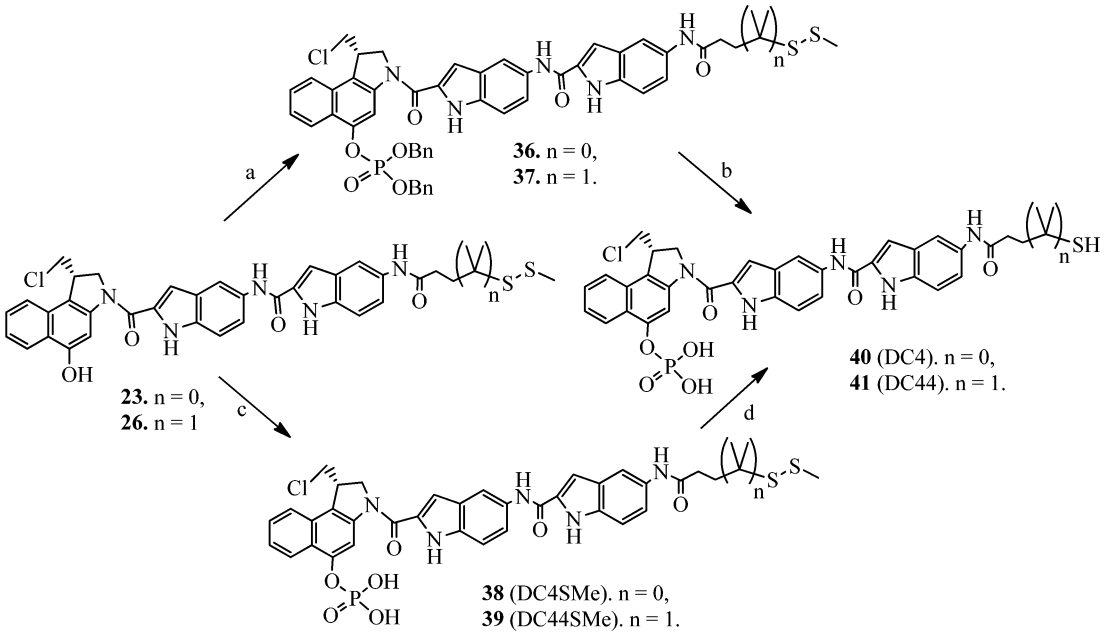
Scheme 6. Synthesis of DC4 and DC44[a]



[a]Conditions: (a) $HP(O)(OBn)_2$, CCl_4, THF/CH_3CN, DIPEA; (b) CH_3SO_3H/DTT; (c) (1) $POCl_3$/THF/CH_3CN/DIPEA, (2) NaH_2PO_4/H_2O; (d) TCEP or DTT, pH 5−7.

the conjugation reaction should be performed in a solvent in which they both are fully dissolved. Because antibodies can only be dissolved in either aqueous solutions or in a solution containing a small amount of a water-miscible organic solvent, the choice of an acceptable solvent is limited. An antibody of the IgG type has a molecular weight of approximately 150000, and in our experience its optimal concentration in the conjugation reaction should be around 3 mg/mL (~2×10^{-5} M). To achieve a cytotoxic agent per antibody ratio (CAR) of 3, at least 3 equiv of the cytotoxic agent should be added per mole of the antibody. Thus the minimum required solubility for the cytotoxic agent is at least 6×10^{-5} M. We found that the effectors **23**, **24**, **25**, **26**, **38**, **39**, **40**, and **41** were not soluble in water, toluene, ethyl acetate, hexane, chloroform, dichloromethane, ethanol, or methanol but were soluble in polar aprotonic solvents, such as DMSO, DMA, and DMF. DC1SMe (**23**) and DC41SMe (**26**) were also soluble in THF, dioxane, and acetone. The phosphate prodrugs (**38**, **39**, **40**, and **41**) are readily soluble in a mixture of water containing as little as 5% of a water miscible organic solvent, such as THF, acetone, dioxane, DMSO, DMA, or DMF. Because the thiol-bearing compounds **2** (DC1), **3** (DC41), **40** (DC4), and **41** (DC44) are prone to oxidation in aqueous solution, particularly in the presence of a trace of heavy metal salt, the methyl disulfide moieties **23** (DC1SMe), **26** (DC41SMe), **38** (DC4SMe), and **39** (DC44SMe) were used instead for the solubility study. While compounds **23** and **26** were not soluble in a buffer mixture containing up to 20% organic solvents (Table 1), the correspondent phosphate prodrugs **38** and **39** were soluble in buffers containing 5−20% DMA and were over 3000-fold more soluble than their respective precursors **23** and **26**.

Like the CBI-based agents of **1** detailed previously,[37] **23** and **26** are readily converted to the ring-closed cyclopropyl forms in aqueous solutions. In contrast, the phosphate prodrugs (**38**, **39**, **40**, and **41**) proved to be very stable such that no more than 3% of cyclopropyl ring formation was detected by HPLC even after storage for one year at room temperature in a phosphate buffer containing 5% DMA at pH ~ 7. Next, we tested if **38**, **39**, **40**, and **41** would be dephosphorylated by commercially available mammalian (human, bovine, rabbit, calf, and porcine) acid or alkaline phosphatases. All of the tested enzymes readily dephosphorylated **38**, **39**, **40**, and **41** at pH 7.4. In particular, **38** and **39** were completely converted to **23** and **26**, respectively, by alkaline phosphatase from bovine liver, bovine kidney, rabbit intestine, or porcine mucosa. Following dephosphorylation, the newly formed compound **23** readily converted into the active cyclopropyl form **27** (as detected by HPLC Figure 2).

Table 1. The Solubility of DCx Compounds

	solubility (μM) at pH 7.0			
compd	100% buffer	5% DMA, 95% buffer	10% DMA, 90% buffer	20% DMA, 80% buffer
23 (DC1SMe)	<0.1	<1	1.0	3.0
38 (DC4SMe)	19	3000	4700	>6000
26 (DC41SMe)	<0.1	<1	1.0	3.0
39 (DC44SMe)	19	2900	4600	>6000

Antibody Conjugates. DC4 (**40**) and DC44 (**41**) were conjugated to the humanized monoclonal antibody huB4[38] and to the humanized antibody huC242 (its parental murine C242 antibody was described previously).[39] These antibodies recognize the tumor-associated antigen CD19 and CanAg, respectively, which are expressed on many cancer cell lines. The preparation of ADCs through disulfide linkage using the antibody modifying agents *N*-succinimidyl-4-(2-pyridyldithio) butyrate (SPDB) and *N*-succinimidyl 4-(2-pyridyldithio)-pentanoate (SPP), or through a thioether linkage using *N*-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) was performed as previously described for maytansi-

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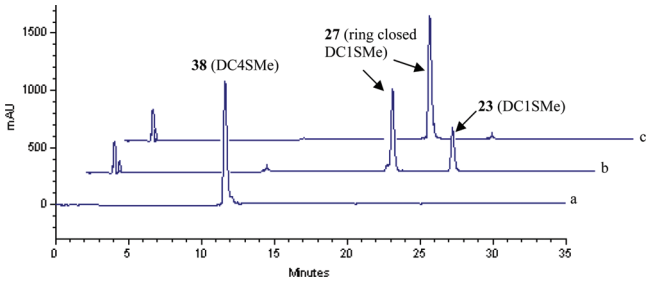

Figure 2. HPLC analysis of the hydrolysis of DC4SMe (0.2 μmol) by an alkaline phosphatase from bovine liver (2 units) in a phosphate-buffered saline, pH 7.5, containing 5% DMA at 37 °C. (a) Without a phosphatase, (b) after 2 h incubation with the phosphatase, (c) after 4 h incubation with the phosphatase.

noid conjugates.[4] In general, the antibodies were modified with ~6 equiv of either SPP, SPDB, or SMCC. Uncoupled linkers were removed by gel filtration over a small Sephadex-based desalting column. To remove any noncovalently bound free cytotoxins from the conjugates, in most cases, a second-step purification was applied using a Porapak column[9] that was found to selectively remove the noncovalently bound cytotoxic compound. However, the use of Porapak columns significantly reduced the yields of the antibody–DCx conjugates because the Porapak resin also bound the conjugates, although to a lesser degree than the free DCx compounds (data not shown). The molar ratios of linker to antibody incorporated using SPDB or SPP was between 3.5–5.0. Because compounds **40** (DC4) and **41** (DC44) are not soluble in 100% aqueous buffers, their conjugation to a linker-modified antibody, as well as subsequent purification by size exclusion chromatography, were performed in a phosphate-buffered saline, containing 5–20% DMA. DC4 and DC44 were used at 1.7 equiv of the conjugated linker. The amount of any residual free effector moiety remaining in the purified conjugate that was not covalently bound to the antibody was determined by first capping any free thiol with *N*-ethyl maleimide (NEM) then precipitating protein with addition of acetone and analyzing supernates by HPLC. The antibody–DC4 and antibody–DC44 conjugates were monomeric, soluble in the buffered aqueous solution, and stable for over one year upon storage at 4 °C.

In Vitro Cytotoxicity of DC1 Derivatives. We found that the compounds that contained free thiol were not very cytotoxic (data not shown), possibly due to the formation of mixed disulfides with a cystine in the cell culture medium resulting in charged compounds that would diffuse poorly across cellular membranes.[4] Therefore, we used the compounds whose sulfurhydryl groups were protected by either a methyldisulfide or a thioacetate to determine their in vitro cytotoxicity. In these cytotoxicity experiments, human cancer cell lines Ramos, Namalwa, HL60/s, and COLO 205 were exposed to these compounds for 72 h, and then the surviving fractions of cells was determined by their ability to form colonies (clonogenic assay). The disulfide-containing phosphate prodrugs **38** and **39** were evaluated both in the absence and in the presence of an acid phosphatase and compared with their parent compounds **23** and **26**. As shown in Table 2, DC1 derivatives **21**, **22**, **23**, **24**, **25**, and **26** were found to be highly potent with IC_{50} values in the 1 pM to 10 pM range on Ramos, Namalwa, and HL60/s cells, and in the 100 pM range when tested on COLO 205 cells. The IC_{50} value of **23** is consistent

Table 2. DC Drugs Cytotoxicity: IC_{50} (pM) against Ramos, Namalwa, HL60/s, and COLO 205 Cancer Cells

compd	Ramos cells	Namalwa cells	HL60/s cells	COLO 205
21 (DC0-NO_2)	nd	5 ± 3	7 ± 3	nd
22 (DC0-NH_2)	nd	5 ± 3	30 ± 10	nd
23 (DC1SMe)	22 ± 5	10 ± 2	32 ± 8	250 ± 85
24 (DC1Spy)	50 ± 20	90 ± 20	50 ± 25	nd
25 (DC1SAc)	47 ± 33	20 ± 14	45 ± 25	nd
26 (DC41SMe)	18 ± 5	21 ± 10	25 ± 5	220 ± 70
27 (DC10SMe)	15 ± 10	12 ± 5	12 ± 5	nd
38 (DC4SMe)	1900	2900	1800	>3000
39 (DC44SMe)	2000	2800	1900	>3000
38 (DC4SMe + phosphatase[a])	80	30	nd	nd
39 (DC44Me + phosphatase[a])	90	25	nd	nd

[a]Alkaline phosphatase from bovine liver.

with that previously reported.[9] Compound **24** (DC1SPy) was slightly less potent than **23** and **25**, possibly due to reaction of its pyridyl disulfide moiety with a thiol compound present either in the cells or in cell culture medium. The prodrugs **38** and **39** were 20- to 160-fold less cytotoxic (IC_{50} of 2 nM) than their parental compounds **23** and **26**, but following incubation with an acid phosphatase for 1.5 h, their cytotoxicity was fully restored. HPLC analysis confirmed the complete conversion of these prodrugs to the corresponding active cytotoxic moieties.

In Vitro Cytotoxicity of Antibody–Drug Conjugates. To test if an antibody conjugate of a phosphate prodrug would have cytotoxic activity, we conjugated DC4 (**40**) to the humanized C242 antibody via either a disulfide-containing linker (SPP or SPDB) or via a noncleavable SMCC linker. Neither of these modifications damaged the binding affinity of the antibody to CanAg-positive cells, as evaluated by an indirect flow cytometric assay (Supporting Information, Figure S3). As shown in Figure 3, all three conjugates displayed high cytotoxicity in vitro toward the antigen-expressing COLO 205 cells with IC_{50} values between 0.7 and 1.6 pM (antibody concentration) in the presence of a phosphatase and between 2.1 and 6.0 pM in the absence of a phosphatase, the latter values indicating that the prodrug moiety was dephosphorylated inside the cell. The conjugates were at least 30000-fold less cytotoxic toward the antigen-negative A375 cells (Figure 3). The cleavability of the linker in these three conjugates did not appear to affect the in vitro cytotoxicity of the conjugates. The conjugates were more potent (IC_{50} values in the picomolar range) than the free DC1SMe (**23**), which has an IC_{50} value of 250 pM for antigen-positive COLO 205 cells, suggesting that the antibody enhanced the delivery of the effectors. The modes of internalization of the free prodrugs and their conjugates differ. The free cytotoxic agent is expected to penetrate the cell via diffusion across the plasma membrane, and this diffusion is likely to slow down when the cytotoxic agent possesses a highly charged and hydrated phosphate group. Hydrolysis of the phosphate should enhance diffusion of the resulting non-charged hydrophobic cytotoxic molecules inside the cells, which is consistent with the 20- to 160-fold enhancement of their cytotoxicity upon acid phosphatase treatment. In contrast, antigen-mediated internalization of conjugates via endocytosis seems to be not dependent on the charge of the effector moiety, as reflected by only a modest (2- to 5-fold) enhancement of their cytotoxicity upon acid phosphatase

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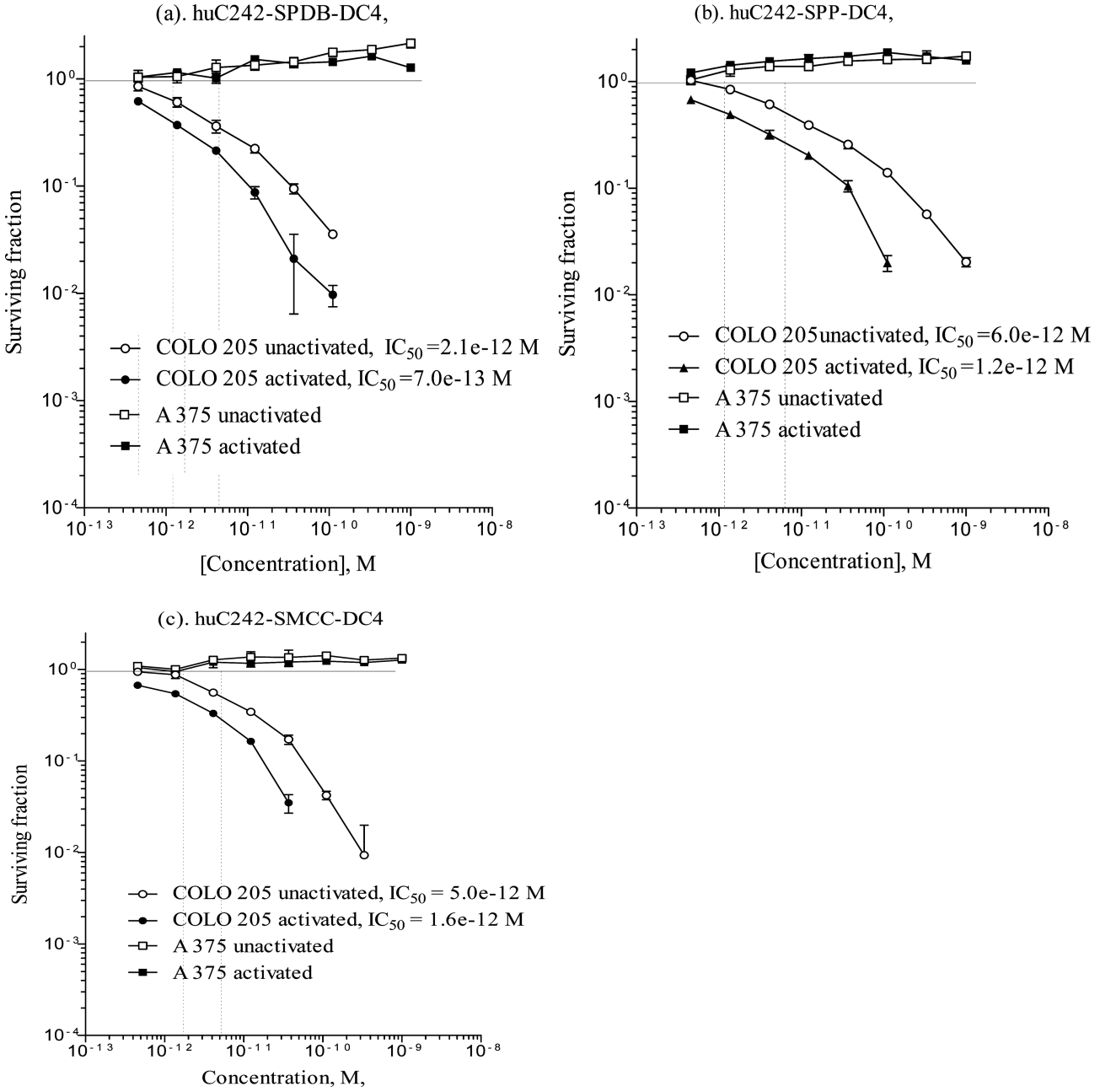

Figure 3. Cytotoxicity of conjugates of huC242−SPDB−DC4, huC242−SPP−DC4, and huC242−SMCC−DC4 against Colo 205 (Ag+) and A 375 (Ag−) cells in the presence and absence of an acid phosphatase from bovine prostate.

treatment. This small different in potency may simply reflect a difference in rate of cell killing between an ADC wherein the "payload" is already activated before it binds to cells versus an ADC that requires the payload to be activated by intracellular enzymes following binding and internalization.

To test whether antibody−DC4 conjugates targeting antigens other than CanAg would also be potent, we conjugated DC4 to the humanized B4 antibody (huB4). This antibody recognizes CD19, an antigen found on lymphomas and leukemias of B cell lineage.[40] Two huB4−DC4 conjugates were prepared, one conjugate with the disulfide-containing SPP linker, and the other conjugate with the noncleavable SMCC linker. In addition, we also prepared a conjugate bearing a hindered disulfide bond (huB4−SPP−DC44), **41**, and a disulfide-linked conjugate with the parent drug DC1 (**2**) (huB4−SPP−DC1). The latter served as a positive control, since in our previous studies we had established that DC1 conjugate was highly active and selective in killing CD19-positive cells.[9] HuB4−SPP−DC4 displayed high potency in vitro toward CD19-expressing Ramos cells both in the absence and in the presence of acid phosphatase, with IC_{50} values (in protein concentration) of 11 pM and 7.5 pM, respectively. This activity of the conjugate was antigen-selective because it was at least 200-fold less potent toward antigen-negative HL60/s cells, both with and without phosphatase treatment. This prodrug conjugate was only slightly less active than the corresponding nonprodrug conjugate, huB4−SPP−DC1 (IC_{50} = 1.4 pM toward Ramos cells) (Table 3), HuB4−SPP−DC44, which bears a more hindered disulfide bond than huB4−SPP−DC4, and the noncleavable B4−SMCC−DC4 displayed much poorer

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Table 3. Cytotoxicity of B4−SPP−DCx Conjugates against Ramos (Ag+) and HL60/s (Ag−) Cells

		IC_{50} (pM)	
compd	acid phosphatase treatment	Ramos cell (Ag +)	HL60/s cells (Ag−)
B4-SPP-DC4	no	11.0 ± 2.0	>3000
B4-SPP-DC4	yes	7.5 ± 2.0	>1500 ± 500
B4-SPP-DC44	yes	400	>1800
B4-SMCC-DC4	yes	1200	2600
B4-SPP-DC1	no	1.4	1300

potency, with IC_{50} values of 0.4 nM and 1.2 nM respectively, against CD19-expressing Ramos cells, and IC_{50} of 1.8 and 2.6 nM, respectively, against CD19-negative HL60/s cells. One explanation for the poorer potency of these two conjugates might be incomplete activation by intracellular phosphatases upon their internalization by the targeted tumor cells. To test this hypothesis, we examined the yield of dephosphorylated prodrugs in these conjugates by treatment with an acid phosphatase. HuB4−SPP−DC4 and huB4−SPP−DC44 (~5 μmol) were incubated with a large excess (~10 units) of a bovine acid phosphatase in phosphate-buffered saline, pH 6.5 at 37 °C for 24 h, denatured by acetone precipitation, and the linked DC4 and DC44 moieties were released by TCEP reduction. HPLC analysis showed that in both cases approximately 59% of the free DCx moieties were released from the conjugates by TCEP. The small molecule weight compounds released from huB4−SPP−DC4 consisted of 48% of DC1 and 52% of nonconverted DC4, while those released from huB4−SPP−DC44 consisted of 33% of DC1, the rest being nonconverted DC44. Thus a considerable amount of antibody-bound prodrug was not activated in these two conjugates even after extensive exposure to a phosphatase. The poor conversion rate coupled with a slower rate of cleavage of the more hindered linker in huB4−SPP−DC44 may account for the poorer potency of huB4−SPP−DC44 relative to the huB4−SPP−DC4 conjugate.

In Vivo Antitumor Activity. The antitumor activities of huB4−SPP−DC4 and huB4−SPP−DC44 conjugates were evaluated in SCID mice bearing CD19-positive xenograft tumors. Animals with established subcutaneous xenograft Ramos tumors were treated with either huB4−SPP−DC4 (DC4 dose of 75 μg/kg, qd ×5), or huB4−SPP−DC44 (DC44 dose of 75 μg/kg, qd ×5), unconjugated DC4 (75 μg/kg, qd ×5), unconjugated DC44 (75 μg/kg, qd ×5), or with phosphate-buffered saline vehicle (control), administered intravenously, and the tumor growth was monitored. As shown in Figure 4, the tumors in the control group of mice grew aggressively. The two prodrug conjugates, huB4−SPP−DC4 and huB4−SPP−DC44 were active and delayed tumor growth by 38 and 17 days, respectively. The corresponding unconjugated prodrugs DC4 and DC44 were inactive in this model, with the tumor growth rates tracking with that of the control mice.

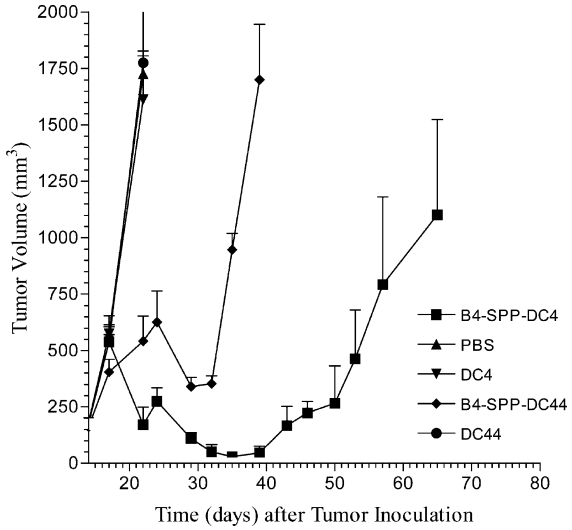

Figure 4. Comparisons of antitumor activities of free DC4, DC44, and conjugates of huB4−SPP−DC4 and huB4−SPP−DC44 on Ramos xenografts.

DISCUSSION

Previously we have known that DC1 conjugated via a disulfide bond to the huB4 antibody (huB4−DC1) was a highly efficacious immunoconjugate resulting in complete regression in the majority of animals in a Namalwa xenograft model. However, due to the lack of solubility and stability of DC1 in aqueous buffer, the conjugation was highly inefficient. To improve this conjugation, we were interested in developing a prodrug strategy that would increase both the aqueous solubility and stability of these analogues of **1** through the incorporation of a phosphate moiety. The ideal position for incorporation of this substituent is the phenol group of the CBI subunit as it would serve two purposes: (a) improve the hydrophilicity of the molecule and thus its water solubility and (b) improve the stability in physiological buffers by preventing the undesired premature cyclization reaction to form a cyclopropyl ring, which would then react with water causing loss of activity.

A key step in the synthesis of these phosphate prodrugs is the resolution of the enantiomers of *seco*(-)CBI during the CBI synthesis. The previously published procedure[18] used two different protecting groups for the *seco*-CBI, a *t*-BOC for the amino moiety, and a benzyl for the phenolic group. Resolution of the enantiomers on a chiral HPLC column was inefficient and not amenable to scale up. Through a slight modification of this procedure we found that using a di-*t*-BOC protected *seco*-CBI (**8**) resulted in a vastly improved separation of the enantiomers on a chiral HPLC column (Supporting Information, Figure S3) and allowed for the easier accumulation of larger amounts of material. In addition, using the same *t*-BOC protecting group for the amine and phenolic groups reduced the number of protection and deprotection steps, thus increasing the efficiency and overall yield of the process.

For the synthesis of linkable bis-indolyl CBI drugs, two different approaches were explored. In the first approach (Scheme 4), the bis-indole unit was first attached to *seco*-CBI, and the linker was connected in the last step. However, the insolubility of bis-indolyl compounds resulted in a difficult separation of the desired compound **23**, **24**, **25**, or **26** from the amino bis-indolyl *seco*-CBI precursor **22**. Thus, column chromatography required the use of polar aprotic cosolvents along with a large column size, resulting in product streaking and poor resolution of separation. Thus synthetic Scheme 5, wherein the linker was attached to the bis-indolyl unit in the first step, followed by incorporation of the *seco*-CBI moiety in the final step, was preferred. In this case, the difference in

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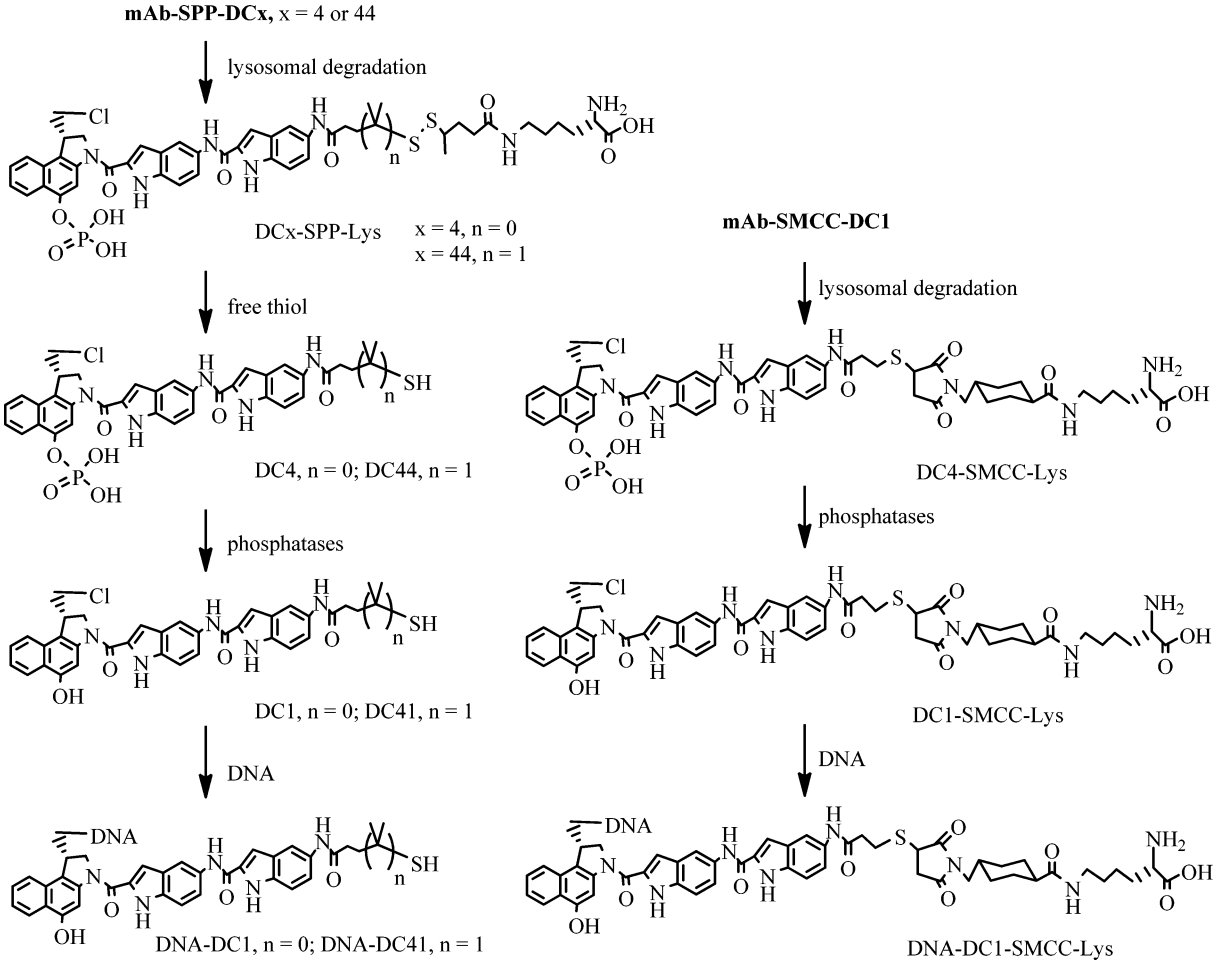
Scheme 7. The Possible Metabolism of mAb−SPP−DC4, mAb−SPP−DC44, and mAb−SMCC−DC4



polarity between the desired bis-indolyl-*seco*-CBI products and the corresponding bis-indole precursors allowed for a facile separation.

In the development of ADCs, the aqueous solubility and stability of the cytotoxic agent used are two key factors in determining the efficiency of conjugation and the usefulness of the conjugate, respectively. The extreme insolubility of the bis-indolyl-*seco*-CBI compounds, such as DC1 (**2**) in water hampered the efficient production of ADCs. In our earlier attempts, conjugation of DC1 with an antibody could only be performed in a buffer containing 20% organic cosolvents, which led to a significant amount of antibody aggregation. In addition, because of the propensity of the DC1 component to undergo hydrolytic inactivation in water, the conjugate had to be stored in a frozen or lyophilized state. Thus, we sought a prodrug approach that would address the issue of aqueous solubility and stability while also ensuring conversion to the active drug under physiological conditions. Our data shows that the choice of a phosphate prodrug fulfilled all these requirements. Thus, incorporation of a charged phosphate group into the cytotoxic agent dramatically improved the solubility of the compound under conjugation conditions. For example, in the presence of as little as 5% of a water-miscible organic cosolvent, such as DMA or DMF, the solubility of the prodrugs was improved over 3000 times. The resulted ADCs with these phosphate prodrugs were soluble in physiological buffers even after the organic cosolvents were completely removed by dialysis and stable for an extended period of time when stored at 4 °C.

Our in vitro studies with the unconjugated prodrugs indicate that these unnatural phenolic phosphate esters are not active against all tested tumor cell lines but can be fully activated by mammalian phosphatases (Table 2). In vitro studies with two different ADCs of these prodrugs demonstrated potent and good antigen-selective killing of target cells without the addition of exogenous phosphatases, suggesting that tumor cells possess phosphatases that are capable of activating the ADC once delivered into the cell. On the basis of our previous studies on the mechanism of intracellular activation of ADCs comprising of maytansinoid drugs,[41] we propose that phosphatases do not act on the intact ADC of the prodrug but on a cellular metabolite. Thus, upon internalization into the cell, the disulfide-linked antibody−SPP−DC4 conjugate would undergo lysosomal degradation of the antibody component to give a lysine−SPP−DC4 metabolite (Scheme 7), which is then enzymatically dephosphorylated to produce the lysine−SPP−DC1 metabolite. This metabolite can directly alkylate DNA or can first undergo disulfide reduction to give DC1 followed by alkylation of DNA. In the case of the noncleavable thioether-linked conjugate, antibody−SMCC−DC4, the sole metabolite would be lysine−SMCC−DC4, which would be dephosphorylated to lysine−SMCC−DC1. The huC242−SMCC−DC4 conjugate is quite potent in vitro, suggesting that the proposed metabolite lysine−SMCC−DC1 is capable of binding to, and

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alkylating a DNA sequence. We did find that another ADC, huB4−SMCC−DC4, was not very potent in vitro. One explanation for the differential potency of the huC242−SMCC−DC4 and huB4−SMCC−DC4 conjugate toward their respective target cells could lie in the relative level of antigen expression: the CanAg antigen that binds to huC242 is expressed at a high level (>10^6 antigens/cell) on the surface of the target COLO 205 cells, while the antigen for huB4 on Ramos cells is expressed at a much lower level (<10^5 antigens/cell). Thus, a threshold level of active metabolite in the cytosol sufficient to cause cell killing is probably not achieved with the huB4−SMCC−DC4 conjugate. In vivo, both the huB4−SPP−DC4 and the huB4−SPP−DC44 conjugates displayed antitumor activity by slowing tumor growth. The respective free prodrugs were inactive in this model demonstrating the benefits of antibody-mediated delivery of the charged molecules. For the two conjugates, the relative disulfide bond strength appears to play a role in the level of antitumor activity. The huB4−SPP−DC44, which has a more sterically hindered disulfide bond than the corresponding DC4 conjugate, was less active, suggesting that the more labile disulfide bond of the DC4 conjugate makes it more susceptible to release of DC4 at the tumor. The phosphate-containing DC4 prodrug can undergo activation to DC1 which then, in addition to killing the targeted cell, can also diffuse into neighboring cell causing bystander killing resulting in an enhanced antitumor effect.[42] Our study suggests that a phosphate prodrug may be a suitable choice for ADCs of effector molecules that do not possess sufficient water solubility or stability.

EXPERIMENTAL SECTION

Melting points were measured using an Electrothermal apparatus and are uncorrected. NMR spectra were recorded on a Bruker AVANCE400 (400 MHz) spectrometer. Chemical shifts are reported in ppm relative to TMS as an internal standard. Low-resolution mass spectra were obtained using a Bruker Esquire 3000 system. Ultraviolet spectra were recorded on a Hitachi U1200 spectrophotometer. Analytical HPLC was performed using a Beckman Coulter system GOLD 168 variable wavelength detector. The analytical HPLC columns were Alltech's Altima C18 column, Vydac analytical C-18 column (both are 4.6 mm × 150 mm 10 μm) and a Chiralcel's OD 4.6 mm × 250 mm chiral column. Preparative HPLC was performed on R & S Technology Zonator system equipped with a Hitachi UV detector, using a self-packed Chiralcel OD 7.5 mm × 50 cm column. Thin layer chromatography was performed on analytical GF silica gel TLC plates. Silica gel for flash column chromatography was from Baker. All solvents used were reagent grade or HPLC grade. All the tested compounds (**21**, **22**, **23**, **24**, **25**, **26**, **27**, **38**, **39**, **40**, and **41**) were over 95% pure as determined by HPLC.

Synthesis of CBI. *3-N-(tert-Butyloxycarbonyl)amino-1-O-(tert-butyloxycarbonyl)-1-naphthol (**5**).* Naphthoresorcinol (**4**) (50.0 g, 0.312 mol) was dissolved in liquid ammonia (200 mL) at −78 °C. This solution was sealed in a 1 L steel bomb containing a glass linear. The reaction mixture was kept at 135 ± 10 °C and 1300 psi for 14 h with vigorous stirring. The vessel was allowed to cool to 60 °C, and the NH_3 was released slowly. The remaining traces of NH_3 were removed by coevaporation of THF (2 × 150 mL) under a stream of argon at 60 °C. Di-*tert*-butyl dicarbonate (175 g, 0.801 mol) in dry THF (300 mL) and *N*,*N*-diisopropylethylamine (140 mL, 0.803 mol) were successively added to the bomb. The bomb was resealed, and the contents were warmed at 100 °C with stirring for 4 h. The bomb was cooled to room temperature and opened and the residue partitioned between saturated aqueous NaCl (800 mL) and EtOAc (500 mL). The aqueous phase was extracted with EtOAc (200 mL × 2). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated under reduced pressure. Chromatography on SiO_2 (1:8 to 1:4 EtOAc/hexane) and crystallization with EtOAc/ethanol/hexane provided 33.66 g (30%) of the title compound (**5**). ^{1}H NMR ($CDCl_3$, 400 MHz) 8.14 (d, 1H, *J* = 8.1 Hz), 7.66 (d, 1H, *J* = 8.1 Hz), 7.43 (dd, 1H, *J* = 6.8, 8.2 Hz), 7.35 (dd, 1H, *J* = 6.8, 8.2 Hz), 7.22 (d, 1H, *J* = 1.8 Hz), 7.15 (br, 1H, NH), 6.69 (s, 1H), 1.59 (s, 9H), 1.37 (s, 9H). ^{13}C NMR ($CDCl_3$) 153.71, 152.9, 136.11, 135.20, 128.12, 128.01, 126.81, 126.03, 123.61, 107.94, 102.95, 82.98, 82.10, 28.93, 27.69. ESI MS *m*/*z* 382.52 $(M + Na)^+$. HRMS ($C_{20}H_{25}NO_5$ + Na), *m*/*z*+ 382.1630, calcd 382.1641 $(M + Na)^+$.

*2-N-(tert-Butyloxycarbonyl)amino-4-O-(tert-butyloxycarbonyl)-oxy-1-iodo-naphthalene (**6**).* 3-*N*-(*tert*-Butyloxycarbonyl)amino-1-*O*-(*tert*-butyloxycarbonyl)-1-naphthol (**5**) (24.50 g, 68.24 mmol) and *N*-iodosuccinimide (17.70 g, 74.73 mmol) were dissolved in THF/CH_3OH (250 mL, 1:1). After the solution was stirred at −40 °C under Ar in the dark for 5 min, TsOH (0.86 g 4.52 mmol) was added. The reaction mixture was stirred under Ar in the dark at −40 °C for 2 h and then at room temperature for 2 h. The mixture was diluted with Et_2O (800 mL), washed successively with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl, dried over $MgSO_4$, filtered, and concentrated in vacuo. Flash chromatography on SiO_2 (EtOAc/hexane 1:10) and crystallization with ethanol/ethyl acetate/hexane afforded 28.46 g (86%) of the title compound (**6**). R_f = 0.48 (10% EtOAc/hexane). ^{1}H NMR ($CDCl_3$, 400 MHz) 8.27 (d, 1H, *J* = 8.0 Hz), 7.98 (dd, 1H, *J* = 1.5, 8.1 Hz), 7.83 (s, 1H), 7.55 (m, 2H), 7.18 (br, 0.8H, NH), 1.62 (m, 18H). MS *m*/*z* 508.36 $(M + Na)^+$. HRMS *m*/*z*+ 508.0575, calcd 508.0597

*2-[N-(tert-Butyloxycarbonyl)-N-(E,Z-3-chloro-2′-propen-1′-yl)-amino]-4-O-(tert-butyloxycarbonyl)oxy-1-iodo-naphthalene (**7**).* To 2-*N*-(*tert*-butyloxycarbonyl)amino-4-*O*-(*tert*-butyloxycarbonyl)oxy-1-iodo-naphthalene (**6**) (940 mg,1.86 mmol) in 20 mL of dry DMF was added NaH (150 mg of 60% in mineral oil, 3.75 mmol) under Ar. After the mixture was stirred at 0 °C for 30 min, *E*,*Z*-1,3-dichloropropene (1.50 mL, 14.57 mmol) was added. The reaction mixture was stirred at 0 °C under Ar for 2 h, then neutralized with 1.0 M NaH_2PO_4, extracted with EtOAc, dried with dried over $MgSO_4$, filtered, and concentrated in vacuo. Flash silica gel chromatography (ethyl acetate/hexane 1:9) afforded 1.01 g (93%) of the vinyl chloride compound (**7**). R_{fZ} = 0.37, R_{fE} = 0.32 (1:8 EtOAc/hexane) (*E*:*Z* vinyl chloride rotamers). ^{1}H NMR ($CDCl_3$, 400 MHz) 8.26 (d, 2H, *J* = 7.7 Hz), 7.96 (m, 2H), 7.59 (br, 4H), 7.20 (s, 1H), 7.16 (s, 1H), 6.17−6.07 (m, 4H), 4.64 (dd, 1H, *J* = 6.2, 15.2 Hz), 4.53 (dd, 1H, *J* = 6.2, 14.7 Hz), 4.31 (dd, 1H, *J* = 6.0, 15.0 Hz), 3.84 (dd, 1H, *J* = 7.5, 15.0 Hz), 1.58 (S, 9H), 1.33 (s, 9H). ^{13}C NMR ($CDCl_3$) 153.78, 151.08, 150.98, 133.31, 133.29, 128.66, 128.61, 127.50, 127.41, 126.41, 121.68, 119.03, 84.22, 84.11, 80.99, 77.20, 28.20, 27.66. MS *m*/*z*+ 582.8 $(M + Na)^+$, 598.03 $(M + K)^+$. HRMS *m*/*z*+ 582.0491 $(M + Na)^+$, calcd 582.0520

*5-(O-tert-Butyloxycarbonyl)oxy-3-[N-(tert-butyloxycarbonyl)-amino-1-(chloromethyl)-1,2-dihydro-3H-benz(e)indole (**8**).* To a solution of the aryl iodide (**7**) (1.36 g, 2.43 mmol) in dry benzene (100 mL) were added tri-*N*-butyltin hydride (0.70 mL, 2.52 mmol) and catalytic ammount of 2,2′-azobis(isobutyronitrile) (AIBN) (30 mg, 0.18 mmol). The mixture was stirred under Ar at room temperature for 30 min and then refluxed at 80 °C for 2 h. The reaction mixture was cooled, and the solvent was removed in vacuo. Trituration of the crude oil with hexane provided a solid which was filtered and washed with hexane to give the title compound **8** (~93% pure) as an off-white solid. Direct purification, after removal of the solvent, by silica gel flash chromatography (ethyl acetate/hexane 1:9) afforded 1.01 g (94%) of the indoline compound (**8**). R_f = 0.34 (1:9 EtOAc/hexane). ^{1}H NMR ($CDCl_3$) 8.12 (br, 1H), 7.91 (d, 1H, *J* = 8.4 Hz), 7.69 (d, 1H, *J* = 8.4 Hz), 7.50 (dt, 1H, *J* = 1.0, 6.9, 7.0 Hz), 7.37 (dt, 1H, *J* = 0.9, 6.9, 6.9 Hz), 4.27 (br, 1H), 4.12 (t, 1H, *J* = 9.0 + 10.0 Hz), 3.99 (m, 1H), 3.90 (dd, 1H, *J* = 2.4, 11.0 Hz), 3.45 (t, 1H, *J* = 10.8 +10.8 Hz), 1.58 (S, 18H). ^{13}C NMR ($CDCl_3$) 152.27, 151.84, 147.99, 130.17, 127.62, 124.33, 122.46, 122.22, 108.95, 83.78, 52.80, 46.13, 28.36, 27.79. MS *m*/*z* 456.9 $(M + Na)^+$. HRMS *m*/*z* 456.1550, calcd 456.1554 $(M + Na)^+$.

*Resolution of (**8**).* The mixture of enantiomers of (**8**) (1.5 g in 20 mL of EtOAc) were resolved on a HPLC preparative column (7.5 cm

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× 50 cm, packed with Diacel Chiralcel OD gel in our laboratory) eluted with 15% *i*-PrOH-hexane at 180 mL/min with an R & S Technology Zonator system. The enantiomers eluted with retention times of 18.5 min [**8a** (+) enantiomer] and 35.8 min [**8b** (−) natural (1*S*) enantiomer]. ent **8b** (−)-(1S): $[\alpha]^{25} = -49.6°$ (c = 5.25 $CHCl_3$). The resolution is $R_s = 2(t_{RB} - t_{RA})/(w_B + w_A)$, where t_{RB} = retention time of solute B, t_{RA} = retention time of solute A, w_B = Gaussian curve width of solute B, w_A = Gaussian curve width of solute A. (see the Supporting Information data).

5-Hydroxy-3-amino-1-[S]-(chloromethyl)-1,2-dihydro-3H-benz-(e)indole (9). To a solution of 5-(*O-tert*-butyloxycarbonyl)oxy-3-[*N*-(*tert*-butyloxycarbonyl)amino-1-[*S*]-(chloromethyl)-1,2-dihydro-3*H*-benz(*e*)indole (**8b**) (400 mg, 0.923 mmol) in 10 mL of 1:5 HCl (conc)/ethyl acetate was added 0.05 mL of triethylsilane. After stirring for 3 h under Ar, the mixture was diluted with 10 mL of 1:1 CH_2Cl_2/toluene and evaporated to dryness 252 mg (93%). The dry solid was again coevaporated three times with CH_2Cl_2/toluene and used directly for coupling to di-indole compounds without further purification (∼92% pure). ^{1}H NMR (DMSO-d_6, 400 MHz) 10.93 (s, 0.8 H), 8.18 (d, 1H, J = 8.6 Hz), 7.92 (d, 1H, J = 8.3 Hz), 7.61 (t, 1H, J = 7.9 + 7.2 Hz), 7.48 (t, 1H, J = 7.6 + 7.4 Hz), 6.86 (s, 1H), 4.27 (br, 1H), 4.04 (dd, 1H, J = 3.1, 11.0 Hz), 3.93−3.79 (m, 3H). MS m/z 234.78 (M + H)$^+$. $C_{13}H_{13}ClNO$ HRMS ($C_{13}H_{13}ClNO$ + H), m/z+ 234.0686, calcd 234.0657 (M + H)$^+$.

The Synthesis of DC1 Linkers. *General Procedure for Synthesis of Linkers 12, 13, 14, and 15.* To the solution of the mercapto acids (**10**) or (**11**) (20 mmol) in a mixture of 100 mL of 100 mM NaH_2PO_4, pH 7.5, and 30 mL of THF at 0 °C was added the agents [either methylmethanethiosulfonate (50 mmol) or Aldrithiol-2 (80 mmol) or acetic anhydride (40.0 mmol)] in THF (60 mL). After addition, the reaction mixture was stirred at 0 °C for 1 h and room temperature for 1−2 h and concentrated to ∼100 mL in vacuo. The mixture then was washed twice with CH_2Cl_2 (2 × 50 mL), and the aqueous solution was acidified to pH = 3.0 with 2 M HCl and then extracted with EtOAc (4 × 100 mL). The EtOAc layers were combined, dried over $MgSO_4$, filtered, concentrated, and purified on silica gel chromatography (ethyl acetate/hexane/acetic acid 3:1:0.05%) to afford the linkers **12**, **13**, **14**, and **15**.

3-(Methyldithio)propionic Acid (12). Yield 2.61 g (86%); R_f = 0.31 (1:100:300 HOAc/EtOAc/hexane). ^{1}H NMR ($CDCl_3$), 11.23 (b, 1H), 2.94 (m, 2H), 2.82 (m, 2H), 2.41 (s, 3H). ^{13}C NMR, 178.34, 33.99, 31.95, 23.17. MS m/z 153.38 (M + H)$^+$. HRMS ($C_4H_8O_2S_2$ + Na), m/z+ 174.9845, calcd 174.9863 (M + Na)$^+$.

3-(Pyridin-2-yldisulfanyl)propanoic Acid (13). 3.42 g (80%); ^{1}H NMR (CD_3COCD_3), 8.45 (ddd, 1H, J = 1.3, 2.7, 4.8 Hz), 7.80 (m, 2H), 7.21 (m, 1H), 3.09 (t, 2H, J = 6.9 Hz), 2.55 (t, 2H, J = 6.9 Hz), ^{13}C NMR, 172.66, 160.12, 150.46, 138.23, 121.81, 120.10, 34.57, 33.87; MS m/z 238.4 (M + Na)$^+$; HRMS ($C_8H_9O_2S_2$ + Na), m/z+ 237.9972, calcd 237.9950 (M + Na)$^+$.

3-(Acetylthio)propanoic Acid (14). Yield 2.60 g (88%). ^{1}H NMR ($CDCl_3$), 11.90 (b, 1H), 3.04 (t, 2H, J = 7.0 Hz), 2.68 (t, 2H, J = 6.9 Hz), 2.31 (s, 3H). ^{13}C NMR, 190.10, 173.34, 32.19, 31.25, 30.09. MS m/z 171.8 (M + Na)$^+$. HRMS ($C_5H_8O_3S$ + Na), m/z+ 171.0092, calcd 171.0068 (M + Na)$^+$.

4-Methyl-4-(methyldisulfanyl)pentanoic Acid (15). Yield 3.22 g (83%); R_f = 0.3 (1:200:400 HOAc/EtOAc/hexane). ^{1}H NMR ($CDCl_3$), 2.48 (m, 2H), 2.41 (s, 3H), 1.95 (m, 2H), 1.31 (s, 6H). ^{13}C NMR, 179.15, 50.52, 35.73, 29.93, 25.21, 18.47. MS m/z− 193.1 (M − H). HRMS ($C_7H_{14}O_2S_2$ + Na), m/z+ 217.0333, calcd 217.03358 (M + Na)$^+$.

The Synthesis of Bis-indole Component of DC1. *5-Nitroindole-2-carboxylic Acid (17).* To a stirred solution of ethyl-5-nitroindole-2-carboxylate (**16**) (25.0 g, 106.8 mmol) in 500 mL of THF-methanol (1:1, v/v) at room temperature was added a solution of NaOH (40 g, 1.0 mmol) in 300 mL of water. The resulting deep-red−brown solution was stirred for 3 h and then quenched by acidification to pH 1 with dilute HCl. The precipitated product was collected by vacuum filtration, and the remaining dissolved product was extracted with THF/ethyl acetate (1:2, v/v, 2 × 400 mL). The precipitate was dissolved in THF, and its solution was combined with organic layers from the extractions. Drying over $MgSO_4$, filtration, concentration in vacuo, and crystallization with THF/ethyl acetate/hexane afforded 20.5 g (94%) of 5-nitroindole-2-carboxylic acid. ^{1}H NMR (DMSO), 11.50 (s, 1H), 7.20 (d, 1H, J = 8.4 Hz), 6.85 (s, 1H), 6.70 (m, 2H); MS m/z−205.2 (M − H). HRMS ($C_9H_6N_2O_4$ − H), m/z− 205.0262, calcd 205.0249 (M − H)$^-$.

Ethyl 5-Aminoindole-2-carboxylate (18). A 500 mL Parr hydrogenation bottle was charged with ethyl 5-nitroindole-2-carboxylate (**16**) (5.0 g, 21.36 mmol), Pd/C (0.3 g, 10% of Pd, 50% wet), CH_3OH/THF (150 mL, 1:4 v/v), and purged with H_2. The reaction mixture was shaken with 40 psi H_2 overnight. The catalyst was removed by filtration, and the solvent was evaporated to give 4.2 g (97%) of the title compound as brown solid. ^{1}H NMR ($CDCl_3$), 8.77 (s, 1H), 7.26 (s, 1H), 7.23 (t, 1H, J = 0.8 Hz), 7.21 (d, 1H, J = 0.7 Hz), 7.03 (dd, 1H, J = 0.7, 1.5 Hz), 6.93 (dd, 1H, J = 0.7, 1.6 Hz), 6.80 (dd, 1H, J = 2.2, 8.6 Hz), 4.38 (dd, 2H, J = 7.2, 14.3 Hz), 1.40 (t, 3H, J = 7.2 Hz). ^{13}C NMR ($CDCl_3$) 162.02, 140.30, 138.14, 131.87, 128.45, 127.77, 117.12, 112.50, 107.36, 105.86, 60.87, 14.41. MS m/z− 205.20 (M + H). HRMS m/z+ 227.0791 (M + Na)$^+$, calcd 227.0797. This product is unstable and it was directly used for next step.

Ethyl 5-(5′-Nitroindol-2′-yl-carbonyl amino)indole-2-carboxylate (19). To a mixture of 5-nitroindole-2-carboxylic acid (**17**) (1.02 g, 5.00 mmol) and ethyl 5-aminoindole-2-carboxylate (**18**) (1.02 g, 4.95 mmol) in DMA (30 mL) were added TBTU (4.00 g, 12.40 mmol) and DIPEA (0.3 mL, 1.72 mmol) under Ar. The reaction mixture was stirred overnight, concentrated, and the mixture was diluted with 30 mL of ethyl acetate and 150 mL of $NaHCO_3$ (satd), and the solid was suspended between the two layers. The solid compound was filtered, washed with water, and then resuspended with 1 M NaH_2PO_4, pH 3.0, filtered, washed again with water and 10% methanol in water, dried under oil pump vacuum to afford 1.55 g (80%) of the title compound (**19**). R_f = 0.31 (1:2 THF/hexane). ^{1}H NMR (DMSO), 12.45 (s, 1H), 11.90 (s, 1H), 10.43 (s, 1H), 8.77 (d, 1H, J = 1.9 Hz), 8.15 (s, 1H), 8.13 (dd, 1H, J = 2.2, 9.1 Hz), 7.70 (s, 1H), 7.61 (m, 2H), 7.46 (d, 1H, J = 8.9 Hz), 7.18 (s, 1H), 4.35 (dd, 2H, J = 7.1, 14.1 Hz), 1.35 (t, 3H, J = 7.1 Hz). ^{13}C NMR (DMSO), 161.22, 158.68, 141.32, 139.50, 135.37, 134.60, 131.47, 128.01, 126.56, 126.38, 119.92, 119.27, 118.59, 113.27, 112.87, 112.60, 107.77, 105.69, 60.43, 14.31. MS m/z 443.85 (M + Na)$^+$. HRMS m/z + 415.1012 (M + Na)$^+$, calcd 415.1019.

5-(5′-Nitroindol-2′-yl-carbonyl amino)indole-2-carboxylic Acid (20). To ethyl 5-(5′-nitroindol-2′-yl-carbonyl amino)indole-2-carboxylate (**19**) (1.26 g, 3.20 mmol) in 35 mL of DMA was added 1.0 g of NaOH in 10 mL of H_2O. After stirring for 1 h, the mixture was concentrated and coevaporated three times with 10 mL of H_2O at 40 °C. The solution was diluted with cold CH_2OH and H_2O, adjusted pH to 3 with HCl (conc), and a solid was precipitated. The solid was filtered, washed with 10% methanol in water, and dried under oil-pump vacuum to afford 1.06 g (90%) of the title compound (**20**). ^{1}H NMR (DMSO), 12.48 (s, 1H), 11.75 (s, 1H), 10.44 (s, 1H), 8.77 (s, 1H), 8.15 (s, 1H), 8.10 (d, 1H, J = 9.3 Hz), 7.69 (s, 1H), 7.60 (m, 2H), 7.44 (d, 1H, J = 8.9 Hz), 7.10 (s, 1H). ^{13}C NMR (DMSO), 161.91, 158.66, 141.32, 139.52, 135.45, 134.44, 131.26, 128.01, 126.72, 126.39, 119.47, 119.25, 118.02, 113.24, 112.88, 112.48, 107.23, 105.71. ESI MS m/z 386.66 387.85 (M + Na)$^+$. HRMS m/z+ 387.0697 (M + Na)$^+$, calcd 387.0706.

1-[S]-(Chloromethyl)-5-hydroxy-3-{{5-[5′-nitroindol-2′-yl-carbonyl amino]indole-2-carbonyl}-1,2-dihydro-3H-benz[e]indole (21). To a solution of 5-hydroxy-3-amino-1-[*S*]-(chloromethyl)-1,2-dihydro-3*H*-benz(*e*)indole, hydrochloride salt (**9**) (200 mg, 0.74 mmol), [fresh prepared from 5-(*O-tert*-butyloxycarbonyl)oxy-3-[*N*-(*tert*-butyloxycarbonyl)amino-1-[*S*]-(chloromethyl)-1,2-dihydro-3*H*-benz-(*e*)indole, (**7**)] and 5-[5′-nitroindol-2′-yl-carbonyl amino]indole-2-carboxylic acid (**20**) (250 mg, 0.69 mmol) in 30.0 mL of DMA was added EDC (400 mg, 0.20 mmol) under Ar. After stirring overnight, two drops of 50% acetic acid were added to the mixture and the mixture was evaporated to dryness, purified on silica gel chromatographic column (40% THF in toluene) to afford 301 mg (75%) of $DC0NO_2$ (**21**). ^{1}H NMR (DMF-d_7) 12.54 (s, 1H), 11.73 (s, 1H), 10.60 (s, 1H), 10.58 (s, 1H), 8.80 (d, 1H, J = 2.3 Hz), 8.42 (d, 1H, J = 1.9 Hz), 8.25 (d, 1H, J = 8.5 Hz), 8.19 (dd, 1H, J = 2.1, 9.1 Hz), 8.09

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(br, 1H), 7.95 (d, 1H, J = 8.3 Hz), 7.82 (d, 1H, J = 1.5 Hz), 7.79 (d, 1H, J = 9.1 Hz), 7.74 (dd, 1H, J = 2.0, 8.9 Hz), 7.62 (d, 1H, J = 8.8 Hz), 7.58 (dt, 1H, J = 1.7, 7.0 + 7.0 Hz), 7.42 (dt, 1H, J = 1.2, 7.0 + 7.0 Hz), 7.33 (d, 1H, J = 1.7 Hz), 4.91 (t, 1H, J = 11.0 Hz), 4.77 (dd, 1H, J = 2.1, 11.1 Hz), 4.33 (m, 1H), 4.13 (dd, 1H, J = 3.1, 11.1 Hz), 3.97 (dd, 1H, J = 7.9, 11.1 Hz). ^{13}C NMR 163.35, 161.48, 160.05, 155.79, 142.98, 137.18, 135.03, 133.22, 133.16, 131.50, 128.85, 128.45, 128.11, 124.62, 124.02, 123.76, 120.33, 119.36, 118.70, 116.45, 114.00, 113.08, 106.97, 105.02, 101.53. MS m/z 602.96 (M + Na)$^+$, 604.78, 603.81, 618.64 (M + K)$^+$, 620.48. HRMS m/z+ 602.1215 (M + Na)$^+$, calcd 602.1207.

*General Procedure for DC1−SR2 (**23**, **24**, **25**, **26**,).* A flask was charged with 1-[*S*]-(chloromethyl)-5-hydroxy-3-{{5-[5′-nitroindol-2′-yl-carbonyl amino]indole-2-yl}carbonyl}-1,2-dihydro-3*H*-benz[e]-indole (**21**) (∼58 mg, 0.1 mmol), Pd/C (30 mg, 10% Pd, 50% wet), HCl (conc) (15 μL), and DMA (25 mL). After the air was removed by vacuum suction, H_2 was conducted through hydrogen balloon overnight. The mixture was filtered through Celite and the solution was evaporated to give ∼50 mg (∼90%) of a brown solid (**22**), which was used directly without further purification. To this solid amine compound in 10 mL of DMA was added 0.12 mmol of the linker (either **12** or **13** or **14** or **15**) and 0.35 mmol of EDC under Ar. After stirred overnight, two drops of 50% acetic acid was added, the mixture was evaporated to dryness, and purified by preparative silica gel TLC (10% DMA, 30% THF, 60% toluene) to afford DC_1−SR_2.

*1-[S]-(Chloromethyl)-5-hydroxy-3-{{5-[5′-(3″-methyldithiopropionyl)indol-2′-yl-carbonyl amino]indole-2-yl}-carbonyl}-1,2-dihydro-3H-benz[e]indole (**23**) (DC1SMe).* Yield 48.1 mg (63%); R_f = 0.40 (3:7 acetone/toluene). ^{1}H NMR (CD_3COCD_3) 10.91 (s, 1H), 10.88 (s, 1H), 9.64 (s, 1H), 9.56 (s, 1H), 9.27 (s, 1H), 8.35 (d, 1H, J = 1.9 Hz), 8.25 (d, 1H, J = 8.0 Hz), 8.17 (d, 1H, J = 1.9 Hz), 8.07 (s, 1H), 7.88 (d, 1H, J = 8.3 Hz), 7.64 (dd, 1H, J = 2.0, 8.1 Hz), 7.58−7.50 (m, 3H), 7.38−7.35 (m, 2H), 7.31 (d, 1H, J = 1.7 Hz), 7.26 (d, 1H, J = 1.7 Hz), 4.86 (dd, 1H, J = 8.7, 11.0 Hz), 4.80 (dd, 1H, J = 2.3, 10.9 Hz), 4.30 (m, 1H), 4.07 (dd, 1H, J = 3.1, 11.0 Hz), 3.83 (dd, 1H, J = 8.4, 11.2 Hz), 3.09 (t, 2H, J = 7.1 Hz), 2.83 (t, 2H, J = 7.1 Hz), 2.45 (s, 3H). ^{13}C NMR 169.56, 161.10, 160.43, 155.13, 143.50, 134.78, 134.46, 133.55, 133.34, 133.03, 132.57, 131.21, 128.80, 128.69, 128.21, 124.22, 124.02, 123.53, 123.44, 120.16, 118.79, 116.45, 113.91, 113.02, 112.95, 112.73, 106.78, 103.72, 101.63, 56.01, 47.73, 43.10, 37.25, 34.01, 23.00. MS m/z 706.66 (M + Na)$^+$. HRMS $C_{35}H_{30}ClN_5O_4S_2$ 706.1306 (M + Na), calcd 706.1325

*1-[S]-(Chloromethyl)-5-hydroxy-3-{{5-[5′-(3″-pyridi-2′-yl-dithiopropionyl)indol-2′-yl-carbonyl amino]indole-2-yl}carbonyl}-1,2-dihydro-3H-benz[e]indole (**24**) (DC1SPy).* Yield 50.6 mg (61%), R_f = 0.35 (1:2 THF/toluene). ^{1}H NMR (DMF-d_7) 11.87 (br, 1H), 11.68 (s, 2H), 10.58 (s, 1H), 10.25 (s, 1H), 10.04 (s, 1H), 8.51 (m, 1H), 8.40 (d, 1H, J = 1.8 Hz), 8.25 (d, 1H, J = 7.9 Hz), 8.20 (d, 1H, J = 1.9 Hz), 8.10 (s, 1H), 7.94 (d, 1H, J = 8.3 Hz), 7.86 (m, 2H), 7.71 (dd, 1H, J = 2.0, 8.1 Hz), 7.60 − 7.52 (m, 3H), 7.48 (d, 1H, J = 1.5 Hz), 7.42 (m, 2H), 7.30−7.26 (m, 2H), 4.90 (dd, 1H, J = 8.7, 11.0 Hz), 4.75 (dd, 1H, J = 2.3, 10.9 Hz), 4.32 (m, 1H), 4.13 (dd, 1H, J = 3.2, 11.0 Hz), 3.96 (dd, 1H, J = 8.4, 11.2 Hz), 3.24 (t, 2H, J = 7.1 Hz), 2.80 (t, 2H, J = 7.1 Hz). ^{13}C NMR 169.70, 161.50, 160.95, 160.85, 155.76, 150.78, 143.91, 138.78, 135.14, 134.86, 134.14, 133.72, 133.02, 129.30, 128.84, 128.78, 128.43, 124.41, 124.18, 123.88, 123.74, 122.20, 120.32, 116.43, 113.92, 113.38, 113.29, 112.63, 106.92, 104.26, 101.75, 56.37, 48.53, 43.04, 36.97, 35.57. MS m/z 748.6 (M + H)$^+$. HRMS $C_{39}H_{31}ClN_6O_4S_2$ 747.1611 (M + H)$^+$, calcd 747.1615.

*1-[S]-(Chloromethyl)-5-hydroxy-3-{{5-[5′-(3″-acetylthiopropionyl)indol-2′-yl-carbonyl amino]indole-2-yl}-carbonyl}-1,2-dihydro-3H-benz[e]indole (**25**) (DC1SAc).* Yield 47.2 mg (62%); R_f = 0.38 (1:2 THF/toluene). ^{1}H NMR (DMF-d_7) 11.68 (s, 1H), 11.67 (s, 1H), 10.58 (s, 1H), 10.25 (s, 1H), 9.88 (s, 1H), 8.37 (d, 1H, J = 1.9 Hz), 8.25 (m, 1H), 8.22 (d, 1H, J = 1.9 Hz), 8.10 (s, 1H), 7.94 (d, 1H, J = 8.3 Hz), 7.73 (dd, 1H, J = 2.0, 8.1 Hz), 7.60−7.50 (m, 3H), 7.45−7.39 (m, 3H), 7.30 (d, 1H, J = 1.7 Hz), 4.90 (dd, 1H, J = 8.7, 11.0 Hz), 4.77 (dd, 1H, J = 2.1, 10.7 Hz), 4.32 (m, 1H), 4.14 (dd, 1H, J = 3.1, 11.0 Hz), 3.96 (dd, 1H, J = 8.4, 11.2 Hz), 3.07 (t, 2H, J = 7.1 Hz), 2.68 (t, 2H, J = 7.1 Hz), 2.35 (s, 3H). ^{13}C NMR 196.07, 170.01, 162.85, 161.51, 160.86, 155.77, 143.91, 135.09, 134.86, 133.93, 133.55, 133.02, 131.48, 128.84, 128.77, 128.42, 124.41, 123.89, 123.75, 120.26, 116.45, 113.92, 113.32, 113.28, 112.54, 106.92, 104.72, 101.63, 56.38, 48.53, 43.09, 38.65, 29.60. MS m/z 702.66 (M + Na)$^+$. HRMS $C_{36}H_{30}ClN_5O_5S$ 702.1542 (M + Na), calcd 702.1554.

*1-[S]-(Chloromethyl)-5-hydroxy-3-{{5-[5′-(4″-methyldithio-3″,3″-dimethyl butyryl)indol-2′-yl-carbonyl amino]indole-2-yl}carbonyl}-1,2-dihydro-3H-benz[e]indole (**26**) (DC41SMe).* Yield 52.5 mg (65%); R_f = 0.40 (2:3 THF/toluene). ^{1}H NMR (DMF-d_7) 11.68 (dd, 2H, J = 1.6, 6.7 Hz), 10.58 (s, 1H), 10.25 (s, 1H), 9.92 (s, 1H), 8.41 (d, 1H, J = 1.9 Hz), 8.25 (d, 1H, J = 8.0 Hz), 8.22 (d, 1H, J = 1.8 Hz), 8.10 (s, 1H), 7.94 (d, 1H, J = 8.3 Hz), 7.72 (dd, 1H, J = 2.0, 8.8 Hz), 7.60−7.52 (m, 3H), 7.48−7.39 (m, 3H), 7.29 (d, 1H, J = 1.7 Hz), 4.89 (dd, 1H, J = 8.7, 11.0 Hz), 4.75 (dd, 1H, J = 2.0, 10.9 Hz), 4.31 (m, 1H), 4.13 (dd, 1H, J = 3.3, 11.0 Hz), 3.96 (dd, 1H, J = 7.8, 11.1 Hz), 2.53 (m, 2H), 2.48 (s, 3H), 2.03 (m, 2H), 1.34 (s, 6H). ^{13}C NMR 171.72, 163.30, 161.45, 160.83, 155.72, 143.85, 135.04, 134.81, 134.04, 133.50, 132.96, 131.42, 128.80, 128.75, 128.38, 124.36, 124.13, 123.83, 123.70, 120.21, 118.77, 116.38, 113.88, 113.30, 113.25, 112.47, 108.52, 106.87, 104.20, 101.71, 67.74, 56.33, 48.48, 42.99, 37.67, 33.51, 24.78 21.93. MS m/z+ 764.0 (M + K)$^+$, 748.0 (M + Na)$^+$, 728.1 (M + K − Cl)$^+$, 712.1(M + Na − Cl)$^+$; m/z− 724.1 (M − H), 688.2 (M − Cl − H). HRMS $C_{38}H_{36}ClN_5O_4S_2$ 748.1784 (M + Na), calcd 748.1795.

*5-(3-(Methyldisulfanyl)propanamido)-N-(2-((9aS)-4-oxo-2,4,9,9a-tetrahydro-1H-benzo[e]cyclopropa[c]indole-2-carbonyl)-1H-indol-5-yl)-1H-indole-2-carboxamide (**27**) (DC10SMe).* 1-[*S*]-(Chloromethyl)-5-hydroxy-3-{{5-[5′-(3″-methyldithiopropionyl)indol-2′-yl-carbonyl amino]indole-2-yl}carbonyl}-1,2-dihydro-3*H*-benz[*e*]indole (**23**) (15 mg, 21.9 mmol) in THF (8 mL) was 5% $NaHCO_3$ (2 mL). After stirred at room temperature for 1 h, the mixture was diluted with EtOAc, and the aqueous phase separated and extracted twice with EtOAc/THF (2:1). The organic layers were combined, dried (Na_2SO_4), filtered, concentrated, and purified on short SiO_2 column eluted with THF/CH_2Cl_2 (1:2 to 1:1) to afford 11.5 mg (83% yield) of the title compound. ^{1}H NMR (DMF-d_7) 11.69 (s, 1H), 11.67 (s, 1H), 10.58 (s, 1H), 10.24 (s, 1H), 9.92 (s, 1H), 8.40 (d, 1H, J = 1.9 Hz), 8.25 (d, 1H, J = 8.3 Hz), 8.22 (d, 1H, J = 1.8 Hz), 8.10 (s, 1H), 7.95 (d, 1H, J = 8.3 Hz), 7.73 (dd, 1H, J = 2.0, 8.1 Hz), 7.61−7.52 (m, 3H), 7.47 (d, 1H, J = 1.8 Hz), 7.45−7.39 (m, 2H), 7.20 (s, 1H), 4.75 (dd, 1H, J = 2.1, 10.7 Hz), 3.98−3.95 (m, 1H), 3.83−3.78 (m, 1H), 2.94 (t, 2H, J = 7.1 Hz), 2.75 (m, 1H), 2.58 (t, 2H, J = 7.1 Hz), 2.46 (s, 3H), 1.55 (m, 1H), 1.27 (m, 1H). ^{13}C NMR 201.37, 171.03, 162.80, 161.82, 160.90, 156.77, 144.96, 135.11, 134.88, 133.96, 133.77, 133.62, 131.49, 128.89, 128.80, 128.52, 124.42, 123.91, 123.77, 120.27, 117.05, 113.93, 113.37, 113.29, 112.55, 106.93, 104.73, 101.65, 58.30, 43.09, 38.69, 37.55, 29.60, 27.90, 21.02. MS m/z 670.40 (M + Na)$^+$. HRMS $C_{35}H_{29}N_5NaO_4S_2$ 670.1672 (M + Na), calcd 670.1559.

*t-Butyl 5-Nitroindole-2-carboxylate(**28**).* To a stirred solution of 5-nitroindole-2-carboxylic acid (**17**) (12.8 g, 61.2 mmol) in 200 mL of dry THF under Ar was added oxalyl chloride (12.0 mL, 137.5 mmol), followed by 0.1 mL of DMF, which caused a vigorous evolution of gas. After stirring for 40 min, the reaction mixture was evaporated to dryness. The resulting solid was redissolved in 150 mL of dry THF and cooled at ∼30 °C under Ar. A solution of potassium *t*-butoxide (1.0 M in THF, 140 mL, 140 mmol) was then added dropwise over 45 min, and stirring was continued for an additional 45 min. The reaction was quenched with 600 mL of water, neutralized with few drops of H_3PO_4, and extracted with ethyl acetate (3 × 400 mL). The organic extracts were washed with saturated aqueous $NaHCO_3$ and water, and then dried over $MgSO_4$, filtered, concentrated, and crystallized with ethanol/hexane to afford 9.62 g (85%) of the title compound (**28**). R_f = 0.35 (1:5 ethyl acetate/hexane). ^{1}H NMR ($CDCl_3$), 11.63 (s, 1H), 8.66 (dd, 1H, J = 0.5, 1.3 Hz), 8.20 (dd, 1H, J = 0.5, 9.0 Hz), 7.48 (dd, 1H, J = 0.5, 9.1 Hz), 7.28 (dd, 1H, J = 0.9, 11.1 Hz), 1.63 (s, 9H). ^{13}C NMR 160.39, 142.12, 138.11, 132.10, 126.78, 120.22, 119.83, 111.98, 109.82, 82.91, 28.26. ESI MS m/z 285.43 (M + Na)$^+$. HRMS $C_{13}H_{14}N_2O_4$ 285.0859 (M + Na), calcd 285.0851.

*t-Butyl 5-Aminoindole-2-carboxylate (**29**).* A 500 mL of Parr hydrogenation bottle was charged with *t*-butyl 5-nitroindole-2-carboxylate (**28**) (5.80 g, 22.14 mmol), Pd/C (0.6 g, 10% Pd, 50%

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wet), THF (150 mL), and purged with H_2. The reaction mixture was shaken with 50 psi H_2 overnight. The catalyst was removed by filtration, and the solvent was evaporated to give 4.98 g (97%) of the title compound (**29**) as brown solid. ^{1}H NMR (DMSO), 11.42 (s, 1H), 7.18 (d, 1H, J = 8.3 Hz), 6.83 (s, 1H), 6.71 (s, 1H), 6.67 (d, 1H, J = 8.4 Hz), 1.62 (s, 9H). ESI MS m/z 255.40 $(M + Na)^+$. This product is unstable and it was directly used for next step.

t-Butyl 5-(5′-Nitroindol-2′-yl-carbonyl amino)indole-2-carboxylate (30). To the solution of 5-nitroindole-2-carboxylic acid (**17**) (4.50 g, 21.81 mmol) and *t*-butyl 5-aminosindole-2-carboxylate (**29**) (4.98 g, 21.45 mmol) in 200 mL of DMA were added TBTU (10.5 g, 32.70) and DIPEA (2.0 mL, 45.83 mmol) of under Ar. The reaction mixture was stirred overnight, concentrated, diluted with ethyl acetate and $NaHCO_3$ (satd) and a suspended solid was formed. The solid compound was filtered, washed with water, and then resuspended with 1 M NaH_2PO_4, pH 3.0, filtered, washed with water and 10% methanol in water again, and dried over oil vacuum pump to afford 8.40 g (89%) of the title compound (**30**). R_f = 0.31 (1:2 THF/hexane). ^{1}H NMR (DMSO), 12.43 (s, 1H), 11.69 (s, 1H), 10.41 (s, 1H), 8.77 (d, 1H, J = 2.2 Hz), 8.13 (dd, 2H, J = 2.3, 9.0 Hz), 7.64 (t, 2H, J = 9.2 Hz), 7.47 (d, 1H, J = 8.9 Hz), 7.08 (s, 1H), 1.59 (s, 9H). ^{13}C NMR (DMSO), 161.48, 159.53, 142.19, 140.38, 136.30, 135.27, 132.28, 130.30, 127.43, 127.25, 120.57, 120.12, 114.08, 113.74, 108.22, 106.64, 81.74, 28.84. ESI MS m/z 443.85 $(M + Na)^+$. HRMS $C_{22}H_{20}N_4O_5$ 443.1314 (M + Na), calcd 443.1331.

t-Butyl 5-(5′-Aminoindole-2′-yl-carbonyl amino)indole-2-carboxylate (31). A 250 mL of Parr hydrogenation bottle was charged with *t*-butyl 5-(5′-nitroindol-2′-yl-carbonyl amino)indole-2-carboxylate (**30**) (2.40 g, 5.71 mmol), Pd/C (0.3 g, 10% Pd, 50% wet), DMA (50 mL), and purged with hydrogen. The reaction mixture was shaken with 40 psi H_2 overnight, filtrated through Celite, and evaporated over an oil pump to give 2.05 g (92%) of the title compound (**31**) as brown solid. ^{1}H NMR (DMSO), 11.75, (s, 1H), 11.67 (s, 1H), 10.17 (s, 1H), 8.10 (d, 1H, J = 1.2 Hz), 7.59 (t, 2H, J = 8.8 Hz), 7.45 (m, 1H), 7.35 (m, 1H), 7.17 (dd, 1H, J = 0.8, 8.0 Hz), 7.06 (d, 1H, J = 2.0 Hz), 1.57 (s, 9H). ESI MS m/z 413.40 $(M + Na)^+$. HRMS for $C_{22}H_{22}N_4O_3$, 413.1598 (M + Na), calcd 413.1590. This product is unstable and it was directly used for next step.

t-Butyl 5-[5′-(3″-Methyldithiopropionyl)indol-2′-yl-carbonyl amino]indole-2-carboxylate (32). To a solution of *t*-butyl 5-(5′-aminoindol-2′-yl-carbonyl amino)indole-2-carboxylate (**31**) (2.00 g, 5.12 mmol) in 30 mL of DMA was added of 3-(methyldithio)-propionic acid (**12**) (0.90 g, 5.92 mmol) and EDC (3.0 g, 15.33 mmol). After stirring under Ar overnight, the mixture was diluted with 70 mL of 1.0 M NaH_2PO_4, pH 6.0, and extracted with THF/ethyl acetate (1:1, 6 × 100 mL). The organic layers were combined, dried over $MgSO_4$, filtered, evaporated, purified with silica gel chromatography (1:3 acetone/toluene) and crystallized with THF/hexane to yield 2.15 g (80%) of the title compound (**32**). mp = 279−283 (dec), R_f = 0.31 (1:3 THF/toluene). ^{1}H NMR (CD_3COCD_3), 10.75 (d, 2H, J = 3.07 Hz), 9.50 (s, 1H), 9.14 (s, 1H), 8.20 (d, 1H, J = 2.0 Hz), 8.14 (d, 1H, J = 1.8 Hz), 7.62 (dd, 1H, J = 2.0, 8.9 Hz), 7.46 (dd, 2H, J = 0.7, 8.1 Hz), 7.34 (dd, 1H, J = 2.0, 10.8 Hz), 7.26 (d, 1H, J = 1.5 Hz), 7.07 (dd, 1H, J = 0.9, 2.1 Hz), 3.05 (t, 2H, J = 7.1 Hz), 2.76 (t, 2H, J = 7.0 Hz), 2.42 (s, 3H), 1.57 (s, 9H). ^{13}C NMR 169.42, 161.58, 160.32, 135.31, 134.76, 133.56, 133.40, 133.12, 130.86, 128.72, 128.27, 120.27, 118.75, 113.69, 113.09, 113.02, 112.69, 108.27, 103.58, 81.66, 37.28, 34.00, 28.41. MS m/z 547.88 $(M + Na)^+$. HRMS for $C_{26}H_{28}N_4O_4S_2$, 547.1457 (M + Na), calcd 547.1450.

t-Butyl 5-[5′-(4″-Methyldithio-3″,3″-dimethyl butyryl)indol-2′-yl-carbonyl amino]indole-2-carboxylate (33). To a solution of *t*-butyl 5-(5′-aminoindol-2′-yl-carbonyl amino)indole-2-carboxylate (**31**) (301 mg, 0.78 mmol) in 30 mL of DMA was added 4-methyldithio-3,3-dimethyl butyric acid (**15**) (155 mg, 0.79 mmol) and EDC (206 mg, 1.07 mmol). After stirring under Ar overnight, the mixture was diluted with 70 mL of 1.0 M NaH_2PO_4, pH 6.0, and extracted with THF/EtOAc (1:1, 4 × 70 mL). The organic layers were combined, dried over $MgSO_4$, filtered, evaporated, purified with silica gel chromatography (1:3 THF/toluene), and crystallized with THF/hexane to yield 344 mg (78%) of the title compound (**33**). R_f = 0.30 (1:3 THF/toluene). ^{1}H NMR (DMF-d_7), 11.71 (s, 1H), 11.64 (s, 1H), 10.21 (s, 1H), 9.91 (s, 1H), 8.29 (d, 1H, J = 2.0 Hz), 8.20 (d, 1H, J = 1.8 Hz), 7.72 (dd, 1H, J = 2.0, 8.9 Hz), 7.51 (dd, 2H, J = 0.76, 9.0 Hz), 7.51 (dd, 1H, J = 0.76, 9.0 Hz), 7.44 (m, 2H), 7.12 (dd, 1H, J = 0.8, 2.0 Hz), 2.53 (m, 2H), 2.48 (s, 3H), 2.02 (m, 2H), 1.60 (s, 9H), 1.33 (s, 6H). ^{13}C NMR 171.32, 162.91, 161.54,, 160.43, 135.32, 134.65, 133.62, 133.57, 133.22, 130.63, 128.36, 127.87, 120.16, 118.38, 113.34, 113.05, 112.91, 112.08, 108.03, 103.82, 81.56, 51.51, 37.30, 33.12, 28.31, 27.74, 25.10. MS m/z+ 589.1 $(M + Na)^+$, 605.1 $(M + K)^+$. m/z− 565.3 $(M - H)^-$. HRMS for $C_{29}H_{34}N_4O_4S_2$, 589.1932 (M + Na), calcd 589.1919.

5-[5′-(3″-Methyldithiopropionyl)indol-2′-yl-carbonyl amino]-indole-2-carboxylic Acid (34). A mixture of *t*-butyl 5-[5′-(3″-methyldithiopropionyl)indol-2′-yl-carbonyl amino]indole-2-carboxylate (**32**) (300 mg, 0.57 mol) and Et_3SiH (20 μL, 0.12 mmol) in 30 mL of CH_2Cl_2 was added 7.0 mL of TFA and the mixture to become clear solution. After stirring for 0.5 h, the reaction mixture was diluted with 25 mL of toluene. The mixture was evaporated to dryness and crystallized with THF/toluene/hexane to yield of 245 mg (92%) of the title compound (**34**). ^{1}H NMR (DMSO), 11.71 (s, 1H), 11.61 (s, 1H), 10.10 (s, 1H), 9.92 (s, 1H), 8.11 (d, 1H, J = 1.9 Hz), 8.02 (d, J = 1.7 Hz), 7.55 (dd, 1H, 2.0, 11.0 Hz), 7.42 (d, 1H, J = 8.8 Hz), 7.39 (d, 1H, J = 8.8 Hz), 7.34 (d, 1H, J = 2.0 Hz), 7.31 (dd, 1H, J = 2.0, 8.8 Hz), 7.08 (d, 1H, J = 1.3 Hz), 3.06 (t, 2H, J = 7.0 Hz), 2.75 (t, 2H, J = 7.0 Hz), 2.45 (s, 3H). ^{13}C NMR (DMSO), 168.70, 162.79, 159.47, 134.37, 133.56, 132.44, 131.98, 131.64, 126.96, 126.75, 119.62, 117.74, 113.04, 112.46, 112.35, 111.44, 107.36, 103.37, 36.03, 33.01. MS 490.81 $(M + Na)^+$. HRMS for $C_{22}H_{20}N_4O_4S_2$ 491.0810, cal: 491.0824.

5-[5′-(4″-Methyldithio-3″,3″-dimethyl butyryl)indol-2′-yl-carbonyl amino]indole-2-carboxylic Acid (35). A mixture of t-butyl 5-[5′-(4″-Methyldithio-3″,3″-dimethyl butyryl)indol-2′-yl-carbonyl amino]-indole-2-carboxylate (**33**) (150 mg, 0.26 mmol) and Et_3SiH (5 μL 0.031 mmol) in CH_2Cl_2 (21 mL) was added 5.0 mL of TFA and the mixture became clear. After stirring for 1 h, the reaction mixture was diluted with 25 mL of toluene. The mixture was evaporated to dryness and crystallized with THF/toluene/hexane to yield of 125 mg (90%) of the title compound (**35**). ^{1}H NMR (DMF-d_7), 13.17 (br, 0.75H, COOH), 11.70 (s, 1H), 11.64 (s, 1H), 10.21 (s, 1H), 9.91 (s, 1H), 8.31 (d, 1H, J = 1.6 Hz), 8.20 (d, J = 1.0 Hz), 7.71 (dd, 1H, J = 2.0, 8.9 Hz), 7.52 (dd, 1H, J = 8.0, 9.7 Hz), 7.46 (d, 1H, J = 1.5 Hz), 7.42 (dd, 1H, J = 1.9, 8.8 Hz), 7.13 (dd, 1H, J = 1.3 Hz), 3.06 (t, 2H, J = 7.0 Hz), 2.54 (m, 2H), 2.50 (s, 3H), 2.03 (m, 2H), 1.33 (s, 6H); ^{13}C NMR (DMSO), 171.32, 163.50, 162.91, 160.42, 135.46, 134.65, 133.65, 133.57, 133.14, 130.23, 128.36, 128.03, 120.05, 118.37, 113.43, 113.07, 112.91, 112.08, 108.10, 103.81, 67.93, 51.51, 37.30, 33.12, 27.74, 25.11; MS m/z- 509.1 $(M-H)^-$, m/z+ 550.6 $(M + K + H)^+$; HRMS for $C_{25}H_{26}N_4O_4S_2$ 511.1466 $(M + H)^+$, cal: 511.1474.

1-[S]-(Chloromethyl)-5-hydroxy-3-{{5-[5′-(3″-methyldithiopropionyl)indol-2′-yl-carbonyl amino]indole-2-yl}-carbonyl}-1,2-dihydro-3H-benz[e]indole (23) (DC1SMe). To a solution of 5-hydroxy-3-amino-1-[*S*]-(chloromethyl)-1,2-dihydro-3*H*-benz(*e*)indole, hydrochloride salt (**9**) (55 mg, 0.20 mmol) and 5-[5′-(3″-methyldithiopropionyl)indol-2′-yl-carbonyl amino]indole-2-carboxylic acid (**34**) (100 mg, 0.21 mmol) in 7.0 mL of DMA was added EDC (120 mg, 0.62 mmol) under Ar. After stirring overnight, the mixture was evaporated to dryness, purified by silica chromatography (25% to 70% THF in toluene) and crystallized with THF/toluene/hexane to afford 116 mg (85%) of DC1SMe (**23**). R_f = 0.40 (3:7 acetone/toluene); (the NMR data and HRMS are the same described above).

1-[S]-(Chloromethyl)-5-hydroxy-3-{{5-[5′-(4″-methyldithio-3″,3″-dimethyl butyryl)indol-2′-yl-carbonyl amino]indole-2-yl}carbonyl}-1,2-dihydro-3H-benz[e]indole (26) (DC41SMe). To the dry salt of 5-hydroxy-3-amino-1-[*S*]-(chloromethyl)-1,2-dihydro-3*H*-benz(*e*)indole, hydrochloride salt (**9**) (67.2 mg, 0.25 mmol) was added 5-[5′-(4″-methyldithio-3″,3″-dimethyl butyryl)indol-2′-yl-carbonyl amino]indole-2-carboxylic acid (**35**) (122 mg, 0.239 mmol), 10 mL of DMA and EDC (220 mg, 1.14 mmol) under Ar. After stirred overnight, the mixture was evaporated to dryness, purified by silica gel chromatography (25% to 70% THF in toluene) and crystallized with THF/

toluene/hexane to afford 117 mg (83%) of DC41SMe (**26**). R_f = 0.40 (2:3 THF/Toluene); (The NMR data and HRMS are the same described above).

*1-[S]-(Chloromethyl)-5-dibenzylphosphonoxy-3-{{5-[5-(3-methyldithiopropionyl)indol-2-yl-carbonyl amino]indole-2-yl}-carbonyl}-1,2-dihydro-3H-benz[e]indole (DC4—dibenzylphosphate, **36**).* To a solution of 1-[*S*]-(chloromethyl)-5-hydroxy-3-{{5-[5′-(3″-methyldithiopropionyl)indol-2′-yl-carbonyl amino]indole-2-yl}-carbonyl}-1,2-dihydro-3*H*-benz[*e*]indole (**23**) (DC1SMe) (50 mg, 0,073 mmol) in 10 mL of THF/CH_3CN (1:1) was added CCl_4 (100 μL, 1.036 mmol), DIPEA (55.0 μL, 0.316 mmol), dibenzylphosphite (0.100 mL, 0.452 mmol), and DMAP (0.2 mg, 0.0016 mmol) under Ar. After stirring under Ar overnight, the mixture was diluted with 5 mL of 1.0 M NaH_2PO_4, pH 4.0, and 10 mL of ethyl acetate. The organic layer was separated, and the aqueous solution was extracted with THF/EtOAc (1:1, 4 × 25 mL). The organic layers were combined, dried over $MgSO_4$, filtered, evaporated, and purified with silica gel chromatography (3:7 acetone/toluene) to afford 62 mg (89%) of the title compound (**36**). (R_f = 0.37 acetone/toluene 1:2). ^{1}H NMR (DMF-d_7) 11.84 (s, 1H), 11.74 (s, 1H), 10.31 (s, 1H), 10.04 (s, 1H), 8.77 (s, 1H), 8.44 (s, 1H), 8.22 (s, 1H), 8.10 (t, 2H, *J* = 7.3 Hz), 7.74 (dd, 1H, *J* = 1.7, 8.8 Hz), 7.66−7.61 (m, 2H), 7.55−7.29 (m, 15H), 5.37 (t, 4H, *J* = 7.5 Hz), 5.01 (m, 2H), 4.84 (dd, 1H, *J* = 1.9, 10.9 Hz), 4.51 (m, 1H), 4.20 (dd, 1H, *J* = 3.2, 10.9 Hz), 4.11 (dd, 1H, *J* = 6.9, 11.1 Hz), 3.13 (t, 2H, *J* = 7.2 Hz), 2.87 (t, 2H, *J* = 7.1 Hz), 2.50 (s, 3H). ^{13}C NMR 169.52, 161.18, 160.41, 147.88, 147.20, 136.74, 136.67, 134.68, 134.56, 133.70, 133.36, 133.20, 132.11, 130.83, 129.25, 129.23, 129.21, 129.19, 128.84, 128.78, 128.62, 128.42, 128.32, 127.90, 124.28, 124.22, 123.96, 123.33, 122.87, 120.03, 118.32, 113.51, 112.94, 112.12, 108.42, 106.87, 70.75, 70.69, 67.91, 55.90, 47.96, 42.59, 37.02, 34.04, 23.08. ^{31}P NMR −4.49; MS *m*/*z* 966.17 (M + Na)$^+$, 968.14 (M + 2 + Na), 967.17. HMMS *m*/*z* for $C_{49}H_{43}ClN_5O_7PS_2$ 966.1957, (M + Na)$^+$, calcd 966.1928.

*1-[S]-(Chloromethyl)-5-phosphonoxy-3-{{5-[5-(3-methyldithiopropionyl)indol-2-yl-carbonyl amino]indole-2-yl}-carbonyl}-1,2-dihydro-3H-benz[e]indole (**38**, DC4SMe).* 1-[*S*]-(Chloromethyl)-5-hydroxy-3-{{5-[5′-(3″-methyldithiopropionyl)indol-2′-yl-carbonyl amino]indole-2-yl}carbonyl}-1,2-dihydro-3*H*-benz[*e*]-indole (**23**) (DC1SMe) (50 mg, 0.073 mmol) was dissolved in a mixture of THF (5 mL), CH_3CN (4 mL), and DMA (0.5 mL) under Ar. To the mixture was added $POCl_3$ (80 μL, 0.858 mmol), DIPEA (150 μL, 0.862 mmol), and DMAP (5 mg, 0.040 mmol). After stirring for 2 h, both TLC and HPLC indicated that DC1SMe was completed consumed. Then 5 mL of 1.0 M NaH_2PO_4, pH 4, was added, and the mixture was stirred overnight. The mixture was concentrated, purified with C-18 chromatography column eluted with water/THF, and crystallized with THF/H_2O/CH_3OH to afford 47 mg (84%) of the title compound (DC4SMe, **38**). ^{1}H NMR (DMF-d_7) 11.77 (s, 1H), 11.70 (s, 1H), 10.26 (s, 1H), 10.02 (s, 1H), 8.74 (s, 1H), 8.42 (s, 1H), 8.30 (d, 1H, *J* = 7.6 Hz), 8.22 (s, 1H), 7.72 (d, 1H, *J* = 8.3 Hz), 7.59 (m, 2H), 7.55−7.43 (m, 4H), 7.33 (s, 1H), 4.96 (t, 1H, *J* = 9.8 Hz), 4.81 (d, 1H, *J* = 10.2 Hz), 4.42 (m, 1H), 4.18 (m, 1H), 4.05 (dd, 1H, *J* = 7.8, 11.0 Hz), 3.11 (t, 2H, *J* = 7.0 Hz), 2.87 (t, 2H, *J* = 7.1 Hz), 2.49 (s, 3H). ^{13}C NMR 169.96, 161.45, 160.85, 143.10, 135.12, 134.93, 134.15, 133.81, 133.58, 132.80, 131.60, 128.86, 128.78, 128.02, 124.50, 124.05, 120.22, 118.61, 113.97, 113.40, 113.32, 112.57, 108.02, 104.27, 56.18, 48.60, 43.21, 37.47, 34.49, 23.53. ^{31}P NMR −3.37; ESI MS *m*/*z*$^-$ 762.20 (M − H). HMMS *m*/*z* for $C_{35}H_{27}ClN_5O_7PS_2$ 762.1021 (M − H)$^-$, calcd 762.1013.

*1-[S]-(Chloromethyl)-5-phosphonoxy-3-{{5-[5-(4″-methyldithio-3″,3″-dimethyl butyryl)indol-2-yl-carbonyl amino]indole-2-yl}-carbonyl}-1,2-dihydro-3H-benz[e]indole (**39**, DC44SMe).* 1-[*S*]-(Chloromethyl)-5-hydroxy-3-{{5-[5′-(4″-methyldithio-3″,3″-dimethyl butyryl)indol-2′-yl-carbonyl amino]indole-2-yl}carbonyl}-1,2-dihydro-3*H*-benz[*e*]indole (**26**) (85 mg, 0.117 mmol) in a mixture of 3.5 mL of THF, 4 mL of CH_3CN at 0 °C was added DIPEA (100 μL, 0.575 mmol) and $POCl_3$ (90 μL, 0.96 mmol) under argon. After stirring for 2.5 h, both TLC and HPLC showed that $DC_{41}SMe$ was completely consumed. Five mL of 1.0 M NaH_2PO_4, pH 7.0 was added, and the mixture was stirred at 0 °C overnight. The mixture was concentrated, purified on a C-18 chromatography column, eluted with water/THF, and crystallized with THF/H_2O/CH_3OH to afford 81.5 mg (84%) of the title compound (DC44SMe, **39**). ^{1}H NMR (DMF-d_7) 11.75 (s, 1H), 11.68 (s, 1H), 10.25 (s, 1H), 9.92 (s, 1H), 8.73 (s, 1H), 8.41 (s, 1H), 8.31 (d, 1H, *J* = 7.6 Hz), 8.20 (s, 1H), 7.71 (d, 1H, *J* = 8.3 Hz), 7.59 (m, 2H), 7.53−7.43 (m, 4H), 7.31 (s, 1H), 4.94 (m, 1H), 4.80 (d, 1H, *J* = 10.2 Hz), 4.41 (m, 1H), 4.15 (m, 1H), 4.00 (dd, 1H, *J* = 7.8, 11.0 Hz), 2.53 (m, 2H), 2.48 (s, 3H), 2.03 (m, 2H), 1.34 (s, 6H). ^{13}C NMR 171.33, 170.31, 161.00, 160.42, 142.93, 134.65, 134.49, 133.68, 133.56, 133.15, 132.38, 130.71, 128.43, 128.35, 128.09, 124.86, 124.43, 123.55, 120.70, 118.36, 112.92, 112.88, 112.07, 107.56, 106.96, 106.64, 103.89, 69.02, 61.30, 50.30, 42.97, 37.29, 33.13, 25.10, 23.35. MS *m*/*z* 804.30 (M − H)$^-$, 806.30. HMMS *m*/*z* for $C_{38}H_{38}ClN_5O_7PS_2$ 804.1461 (M − H)$^-$, calcd 804.1482

*1-[S]-(Chloromethyl)-5-phosphonoxy-3-{{5-[5-(3-mercaptopropionyl)indol-2-yl-carbonyl amino]indole-2-yl}-carbonyl}-1,2-dihydro-3H-benz[e]indole (**40**, DC4).* A solution of TCEP (30 mg, 0.104 mmol) in 2 mL of H_2O was adjusted to pH 6.5−7.0 with addition of $NaHCO_3$ powder. To the solution was added 1-[*S*]-(chloromethyl)-5-phosphonoxy-3-{{5-[5-(3-methyldithiopropionyl)indol-2-yl-carbonyl amino]indole-2-yl}-carbonyl}-1,2-dihydro-3*H*-benz[*e*]indole (**38**, DC4SMe) (26 mg, 0.034 mmol) in 3 mL of DMA/H_2O (1:1). After stirring for 2 h under Ar, a few drops of 10% H_3PO_4 were added to adjust pH to ∼4.0. The mixture was evaporated and purified by preparative HPLC (*c*18, 20 mm × 250 mm, *v* = 8.0 mL/min., mobile phase: A, 0.01% acetic acid in H_2O; B, 50% DMA in CH_3CN (metal free); time table, 0−10′, 5% of B; to 20′, 20% of B; to 50′, 50% of B. The DC4 (**40**) was eluted out at 30−35 min. The fractions were pooled, concentrated, and dried over the oil vacuum pump to yield 22 mg (89%) of the title compound. ^{1}H NMR (DMF-d_7) 11.76 (s, 1H), 11.69 (s, 1H), 10.26 (s, 1H), 10.02 (s, 1H), 8.78 (s, 1H), 8.41 (s, 1H), 8.29 (d, 1H, *J* = 8.3 Hz), 8.21 (s, 1H), 7.79 (d, 1H, *J* = 5.1 Hz), 7.60−7.43 (m, 6H), 7.27 (s, 1H), 4.96 (t, 1H, *J* = 9.2 Hz), 4.80 (d, 1H, *J* = 10.6 Hz), 4.42 (m, 1H), 4.23 (dd, 1H, *J* = 2.2, 9.4 Hz), 4.06 (dd, 1H, *J* = 7.4, 10.6 Hz), 3.12 (t, 2H, *J* = 7.1 Hz), 2.88 (t, 2H, *J* = 7.1 Hz). HMMS *m*/*z* for $C_{34}H_{28}ClN_5O_7PS$ 716.1130 (M − H)$^-$, calcd 716.1136.

Alternative Procedure. A solution of 1-[*S*]-(chloromethyl)-5-dibenzylphosphonoxy-3-{{5-[5-(3-methyldithiopropionyl)indol-2-yl-carbonyl amino]indole-2-yl}carbonyl}-1,2-dihydro-3*H*-benz[*e*]indole (**36**) (20 mg, 0.021 mmol), 5 mL of methylsulfonic acid, thiolphenol (100 μL, 0.94 mmol) and DTT (15 mg, 0.097 mmol) was stirred 4 h under Ar. After the reaction was indicated completion by HPLC, the mixture was diluted with toluene, evaporated, and purified by the preparative HPLC as above-described to yield 7 mg (45%) of the DC4 (**40**).

*1-[S]-(Chloromethyl)-5-phosphonoxy-3-{{5-[5-(4″-mercapto-3″,3″-dimethyl butyryl)indol-2-yl-carbonyl amino]indole-2-yl}-carbonyl}-1,2-dihydro-3H-benz[e]indole (**41**, DC44).* A solution of TCEP (106 mg, 0.367 mmol) in 5 mL of H_2O was adjusted to pH 6.5−7.0 with addition of $NaHCO_3$ powder. To the solution was added 1-[*S*]-(chloromethyl)-5-phosphonoxy-3-{{5-[5-(4″-methyldithio-3″,3″-dimethyl butyryl)indol-2-yl-carbonyl amino]indole-2-yl}carbonyl}-1,2-dihydro-3*H*-benz[*e*]indole (**39**, DC44SMe) (65 mg, 0.076 mmol) in 10 mL of DMA/H_2O (1:1) followed added 50 mg of DTT. After stirring for 12 h under Ar, a few drops of 10% H_3PO_4 were added to pH 4.0. The mixture was evaporated, coevaporated 3 times with DMA/toluene (2:1, 30 mL), and purified with preparative HPLC (*c*18, 20 mm × 250 mm, *v* = 8.0 mL/min, mobile phase: A, 0.01% HOAc in H_2O; B, 50% DMA in CH_3CN; time table, 0−10′, 5% B; to 20′, 20% B; to 50′, 50% B. The DC44 was eluted out at 30′−35′. The DC44 fractions were pooled, concentrated, and dried over the oil vacuum pump to yield 56 mg (91%) of the title compound. ^{1}H NMR (DMF-d_7) 11.75 (s, 1H), 11.68 (s, 1H), 10.25 (s, 1H), 9.92 (s, 1H), 8.73 (s, 1H), 8.41 (s, 1H), 8.31 (d, 1H, *J* = 7.6 Hz), 8.20 (s, 1H), 7.71 (d, 1H, *J* = 8.3 Hz), 7.59 (m, 2H), 7.53−7.43 (m, 4H), 7.31 (s, 1H), 4.94 (m, 1H), 4.80 (d, 1H, *J* = 10.2 Hz), 4.41 (m, 1H), 4.15 (m, 1H), 4.00 (dd, 1H, *J* = 7.8, 11.0 Hz), 2.53 (m, 2H), 2.03 (m, 2H), 1.34 (s, 6H). ^{13}C NMR 171.33, 170.31, 161.00, 160.42, 142.93, 134.65, 134.49, 133.68, 133.56, 133.15, 132.38, 130.71, 128.43, 128.35, 128.09, 124.86, 124.43,

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123.55, 120.70, 118.36, 112.92, 112.88, 112.07, 107.56, 106.96, 106.68, 103.89, 69.32, 61.30, 50.30, 42.97, 37.29, 33.13, 25.10. MS m/z 804.1 $(M - H)^-$, 805.2, 806.2. HMMS m/z for $C_{37}H_{35}ClN_5O_7PS_2$ 758.1642 $(M - H)^-$, calcd 758.1605.

Solubility Test. The DC compound (~1.5 mg) in the appropriate solvent was sonicated for 45 min, then centrifuged at 1400g for 45 min. The concentration of the compound in the supernatants was measured by UV/vis spectrometry at 340 nm. At pH 7.0, DC_1-SR_2 (**23**, **24**, **25**, and **26**) ε = 41500 M^{-1} cm^{-1}; the phosphate prodrugs (**38**, **39**, **40**, and **41**) ε = 34500 cm^{-1} M^{-1}. For in the buffers containing 5% or less DMA, stock solutions (50 mM) of the subject compounds (**23**, **26**, **38**, **39**) in DMA was diluted 20−100 times with the buffers, then the same sonication, centrifuged, and UV measurement procedure were followed.

HPLC Analysis. HPLC analysis of the hydrolysis of DC4SMe (0.2 μmol) by an alkaline phosphatase from bovine liver (2 units) in a pH 7.5 phosphate buffer at 37 °C was performed using a Vydac analytical C-18 column (length, 150 mm; i.d., 4.6 mm; particle size, 10 μm) operating at 25 °C and at a flow rate of 1 mL/min, eluting with gradients of water (containing 0.5% acetic acid) (A) and acetonitrile/DMA (1:1) (B): 0 min, 20% B; 5 min, 20% B; 5−30 min, 20−65% B; 30−35 min, 65−95%. Under these conditions, compounds **38** (DC4SMe), **27**, and **23** eluted with retention time of 12, 23, and 27 min, respectively.

HPLC monitoring the hydrolysis of DC4 by a phosphatase was performed using a Vydac analytical C-18 column (length, 150 mm; i.d., 4.6 mm; particle size, 10 μm) at a flow rate of 1 mL/min, eluting with a gradient of 1% acetic acid in water (A) and acetonitrile/DMA (1:1) (B): 0 min, 5% B; 5 min, 5% B; 5−30 min, 5−75% B.

HPLC analysis of the target compounds (**21**, **22**, **23**, **24**, **25**, **26**, **27**, **38**, **39**, **40**, **41**) was performed using an Alltech's Altima C18 column (length, 150 mm; i.d., 4.6 mm; particle size, 10 μm) at a flow rate of 1 mL/min, eluting with a gradient of 1% acetic acid in water (A) and acetonitrile/DMA (1:1) (B): 0 min, 15% B; 5 min, 15% B; 5−30 min, 15−80% B; 30−35 min, 80−95% B.

Preparation of Antibody Conjugates with DC4 (40) and DC44 (41). DC4 and DC44 conjugations were conjugated to humanized C242 (huC242) and humanized B4 (huB4) antibodies in two steps. First, the antibody was modified with either *N*-succinimidyl-4-(2-pyridyldithio) butyrate (SPDB) or 4-(2-pyridyldithio)pentanoic acid-*N*-hydroxysuccinimide ester (SPP) to incorporate a linker bearing a pyridyl disulfide moiety, or antibody was modified with *N*-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) to introduce a linker bearing a maleimido group, following procedures described in the preparation of maytansinoid conjugates.[3,4] In general, the antibodies were modified with ~6 equiv of either SPP or SPDB or SMCC in phosphate buffer A (0.1 M potassium phosphate buffer, pH 7.5) with either 5% DMA or 5% DMSO for 90 min at room temperature. Noncoupled linkers were removed by gel filtration over a Nap 25 column equilibrated in 0.1 M potassium phosphate buffer, pH 7. The ratios of the linker versus antibody resulting from reaction of the antibody with SPDB and SPP were determined by measure UV absorbance at both 325 and 280 nm (the linker chromophore absorbance versus mAb absorbance). On average, 3.5−5.0 linkers were incorporated per antibody molecule. The SMCC linker has no unique chromophore by which its level of incorporation concentration can be determined by UV once coupled to an antibody. Therefore, an assumption was made that the reaction yielded four equiv of SMCC incorporated per antibody, similar to the level of incorporation for the other linkers. In the second step, 1.7 equiv of DC4 or DC44 over incorporated linker was added to the purified linker-modified antibody solutions in sodium phosphate buffer, pH 6.5, containing 5−20% DMA (v/v), and the mixture was incubated at room temperature for 3 h. Then ~5 equiv of 1 mM of *N*-ethylmaleimide was added to quench any unreacted free thiol group on DC4 or DC44. The mixtures were purified by size exclusion on Sephadex G-25 columns, equilibrated with 50−100 mM sodium phosphate buffer, pH 6.5, containing 5−20% DMA. In most cases, the second step purification of using a Porapak column (Waters Corp, part no. 35683) to remove noncovalent bound free DCx compounds from the conjugates was the same as previous reported.[9] The final concentration of the conjugates was determined spectrophotometrically using the known extinction coefficients for the antibody (ε_{280nm} = 217560 M^{-1} cm^{-1}) and for the DCx compounds (ε_{325nm} = 33500 M^{-1} cm^{-1} and ε_{280nm} = 22500 M^{-1} cm^{-1}) at pH 7.0.

To analyze any residual free DCx compounds in the conjugates, two aliquots of the conjugates, were either added a solution of *N*-ethyl maleimide (NEM) (20 equiv) or a buffer, respectively, and diluted 2−3-fold with cold acetone. The resulting solutions were vortexed briefly and placed on dry ice for at least 1 h then centrifuged at 14000g for 45 min. The supernatants were concentrated and analyzed by HPLC. A internal standard, which either used NEM-adducted DC44 (DC44NEM) when analyzing DC4, or used DC4NEM when analyzing DC44, or used DC4-acetamide, was added to the samples for HPLC quantification.

Activation of Prodrugs and the Conjugates by Phosphatases. The phosphatases used to test the activation of the prodrugs were acid phosphatase from prostatic acid phosphatase from bovine prostate (P6409), prostatic acid phosphatase from bovine semen (P3147), prostatic acid phosphatase from human semen (P1649), wheat germ (Sigma P3627), alkaline phosphatase from bovine (P8361), alkaline phosphatase from bovine intestinal mucosa (P7640), alkaline phosphatase from bovine kidney (P4653), alkaline phosphatase from bovine liver (P7034), alkaline phosphatase from calf intestine (P7923), alkaline phosphatase from canine intestine (P8639), alkaline phosphatase from porcine intestine mucosa (P4002), alkaline phosphatase from porcine kidney (P4439), alkaline phosphatase from rabbit intestine (P2256), alkaline phosphatase from human placenta (P3895), alkaline phosphatase from calf intestine mucosa (P79390). In general, 0.2−5.0 unit of above individual phosphatase was added to 0.5 mg of free DC4 or DC44 drug in phosphate-buffered saline, pH 6.5 or 7.5, with 10% DMA. The mixture was incubated at room temperature or 37 °C for 0−24 h and analyzed by HPLC. Each antibody−DCx drug conjugate (500 μL) was activated with 1−1.7 units of acid phosphatase in PBS, pH 6.5 containing 10% DMA. The mixtures were incubated overnight at 37 °C. A portion (100 μL) of each sample was removed, mixed with acetone (200 μL), and the DCx moiety was released by addition of TCEP (4 equiv) and incubation for 1−2 h, and then analyzed by HPLC.

In Vitro Cytotoxicity Assays. The cell lines used in cytotoxicity assays were Namalwa (human Burkitt's lymphoma, ATCC CRL-1432), Ramos (human Burkitt's lymphoma), A subclone of the human acute promyelocytic leukemia HL60 cell line, HL-60/s,[43] COLO 205 (human colon adenocarcinoma, ATCC CCL-222), and A375 (human malignant melanoma, ATCC CRL-1619). Cell cultures were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified incubator at 37 °C, 6% CO_2. The cytotoxicity clonogenic assay was described previously.[44] Briefly, the test cell lines were plated into 6-well culture dishes at a constant number of 1000−5000 cells per well and incubated with varying concentrations (0−3 nM) of a test-agent for 72 h. The medium was then aspirated from the plates and replaced with fresh medium, and the cultures were allowed to grow and form colonies for a total of 7−10 days after plating. Cells were then fixed and stained with 0.2% crystal violet in 10% formalin/PBS, and the colonies were counted. Plating efficiency of nontreated cells (medium alone) was determined by dividing the number of colonies counted by the number of cells plated. The surviving fraction of cells exposed to a toxic agent was determined by dividing the number of colonies in wells that were exposed to the agent by the number of colonies in the control wells.

Antitumor Activity In Vivo. The in vivo efficacy of conjugates of DC4 (**40**) and DC44 (**41**) and their conjugates with the huB4 antibody was evaluated in a human Burkitt's lymphoma tumor xenograft model established with Ramos cells. Five-week-old female CB 17 SCID mice (36 animals, supplied by Taconic Farms, Inc.) were inoculated subcutaneously in the area under the right shoulder with Ramos human lymphoma carcinoma cells (5×10^6 cells/mouse) in 0.1 mL of serum-free medium. The tumors were grown for 7 days to an average size of 100 mm^3. The animals were then randomly divided into five groups (six animals per group). The first group of mice served as

the control group and was treated with the phosphate-buffered saline vehicle. The remaining four groups were treated with either huB4–SPP–DC4 (DC4 dose of 75 μg/kg, qd ×5), or huB4–SPP–DC44 (DC44 dose of 75 μg/kg, qd ×5), or DC4 (75 μg/kg, qd ×5) or DC44 (75 μg/kg, qd ×5), administered intravenously. Three dimensions of the tumor were measured twice weekly using the LabCat system (Innovative Programming Associates, Inc. Princeton, NJ), and the tumor volumes were calculated using the formula tumor volume = $^1/_2$(length × width × height). The weight of the animals was also measured twice per week. A mouse was sacrificed when any one of the following criteria was met: (1) loss of body weight of more than 20% from pretreatment weight, (2) tumor volume larger than 1500 mm^3, (3) too sick to reach food and water, or (4) skin necrosis. A mouse was considered to be tumor-free if no tumor was palpable.

■ ASSOCIATED CONTENT

Supporting Information

HPLC monitoring activation of phosphate prodrugs with a phosphatase, chiral HPLC separation of CBI and binding assays of the antibody-DC4 drug conjugates, and analytical data (HPLC purity data) for target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

CBI, 5-hydroxy-3-amino-1-[*S*]-(chloromethyl)-1,2-dihydro-3*H*-benz(*e*)indole; CAR, cytotoxic agent per antibody ratio; DTT, dithiothreitol; DMA, dimethylacetamide; DCx, DC4 and/or DC44; mAb, monoclonal antibody; NEM, *N*-ethyl maleimide; PBS, phosphate-buffered saline; SMCC, succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; SPP, *N*-succinimidyl 4-(2-pyridyldithio)pentanoate; SPDB, *N*-succinimidyl 4-(2-pyridyldithio)butanoate; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TCEP, Tris-(2-carboxyethyl)phosphine

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