Inc. The disulfide form of tocinamide was supplied by Coast Scientific Products. The reduced dithiol forms of the peptides were produced in situ by reduction of the disulfides with D,L-1,4-dithiothreitol-d_{10} (DTT-d_{10}) (MSD Isotopes). Typically, reduction was complete within 10 min after addition of DTT-d_{10} to a pD 12–13.2 solution of disulfide. Because of their susceptibility to air oxidation, the dithiols were prepared and the pD of their solutions was adjusted for NMR measurement in the oxygen-free atmosphere of an air-tight glovebox. NMR samples were capped and sealed with Parafilm before removing them from the glovebox. L-cysteinylglycine and L-cysteine methyl ester were products of Research Plus Inc. and Fluka, respectively. D_2O was obtained from ICON Services Inc.; DCl and KOD were supplied by Sigma Chemical Co. All chemicals were used without further purification.

^1H NMR spectra were measured at 500 MHz. The residual HDO resonance was suppressed by presaturation with the decoupler channel. Probe temperature was 25 °C, and chemical shifts were measured relative to internal standard *tert*-butyl alcohol (1.2366 ppm). The thiolate protonation equilibria of cysteine methyl ester and cysteinylglycine were characterized by measuring their absorbance at 254 nm as a function of pD.

All protonation constants were determined in D_2O, at an ionic strength of 0.2–0.3 M (KCl) and 25 ± 2 °C. The peptide concentration was 1–3 mmol dm^{-3}. pD was adjusted by addition of DCl or KOD titrant solution; the titrant solution concentrations were between 0.1 and 0.3 mol dm^{-3}. pH electrodes were calibrated with Fisher pH = 4.000 and pH = 9.000 H_2O standard solutions. pH meter readings for D_2O solutions were converted to pD using the relationship pD = pH meter reading +0.40.[33]

Acknowledgment. This research was supported by National Institutes of Health Grant GM 37000. The NMR instrumentation was supported in part by BRSG 2 S07 RR07010-20 awarded by Biomedical Research Resources, National Institutes of Health.

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Synthesis of Acylhydrazido-Substituted Cephems. Design of Cephalosporin–Vinca Alkaloid Prodrugs: Substrates for an Antibody-Targeted Enzyme

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Cephalosporin **20** substituted at the C-3′ position with the potent oncolytic agent desacetylvinblastine hydrazide (**3**) was synthesized as a potential prodrug for the treatment of solid tumors. The design of this novel prodrug was based on the knowledge that hydrolysis of a cephalosporin's β-lactam bond can result in the expulsion of the C-3′ substituent. Proper selection of the linkage used to join the cephem to the vinca, e.g., **8** vs **20**, provided a releasable form of the drug as well as a chemically stable prodrug. We envisioned the conversion of prodrug to free vinca to be mediated by an immunoconjugate, consisting of a β-lactamase enzyme covalently attached to a monoclonal antibody, which has been prelocalized at the tumor. Treatment of candidate prodrugs with the P99 β-lactamase enzyme isolated from *Enterobacter cloacae* 265A efficiently catalyzed their conversion to the free drug form. A study of model compounds **11** and **18** indicated that cephem 1-β-sulfoxide **18** was a better substrate for the enzyme than its sulfide counterpart **11**. This finding prompted the synthesis of cephem sulfoxide **20** which was efficiently accomplished via condensation of desacetylvinblastine hydrazide with the cephalothin derived cephem 3′-*p*-nitrophenyl carbonate **15**.

Introduction

The targeting of oncolytic agents to tumor cells using monoclonal antibody (MoAb)-drug conjugates has received considerable attention in recent years.[1] Utilizing the ability of the MoAb to recognize and bind to specific tumor associated antigens, a cytotoxic agent covalently bound to the MoAb may exhibit both antitumor activity and decreased toxicity to nontargeted tissues. An alternative two-step approach has been reported in which radiopharmaceuticals, cytotoxic agents, or hapten-modified cytotoxic agents are localized at tumor targets by prelocalization of a bifunctional antibody (a MoAb with affinity for both a tumor antigen and a small molecule) followed by administration of the small molecule.[2] We have reported[3] another two-step approach[4] which was based upon the insight gained from work on bifunctional antibodies as well as covalent MoAb-cytotoxic agent constructs. Our system employs an enzyme covalently bound to a MoAb which localizes on the targeted tumor cell surface. Subsequent administration of a prodrug (which is a substrate of the enzyme) allows for the specific enzyme-catalyzed release of the cytotoxic agent at the tumor site, as depicted

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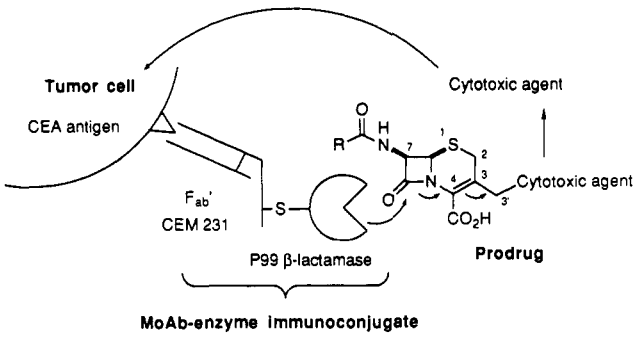
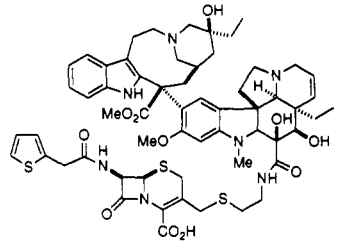


Figure 1. Antibody-Directed Catalysis (ADC) Concept.

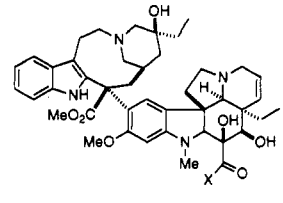
in Figure 1. This approach, which we term antibody-directed catalysis or ADC, has several possible advantages over the other two systems. Due to the catalytic nature of the immunoconjugate one can, in principle, deliver many drug molecules to a tumor cell utilizing a single MoAb-enzyme molecule. Thus, lower conjugate doses may be used, and target antigens present in relatively low copy number may be employed. If properly designed the prodrug may be less cytotoxic than the parent drug. Furthermore, several different drugs may be delivered by using multiple prodrugs and a single antibody–enzyme construct.

In defining a construct for ADC we considered the selection of the enzyme/substrate components to be most critical to the ultimate success of this approach. The main attributes considered for the enzyme were as follows: ability to catalyze a scission reaction; high specificity for only one substrate moiety; no endogenous interference from inhibitors, substrates, or related enzymes in a mammalian system; high catalytic activity without the need for cofactors; and low molecular weight for optimal pharmacological behavior. The P99 β-lactamase isolated from *Enterobacter cloacae* 265A possesses these attributes as well as being readily available, easily purified, and quite stable. The choice of a β-lactamase enzyme dictated the use of a β-lactam-derived prodrug. Cephalosporins were chosen as prodrug candidates because the controlled release of a cytotoxic agent covalently attached to the C-3′ position might be realized. Pratt[5] and others have shown that when cephalosporins are hydrolyzed by a β-lactamase enzyme the C-3′ substituent is expelled in accordance with its leaving-group propensity. Proper selection of the linkage, cephem to cytotoxic agent, should provide a stable prodrug which possesses the ability to release the drug upon contact with the β-lactamase. Judicious selection of the attachment site to the cytotoxic agent might also significantly reduce the cytotoxicity of the prodrug relative to the free drug. In addition, cephalosporins are known to exhibit minimal inherent mammalian toxicity.

The covalent attachment of the P99 β-lactamase to the Fab′ fragment of antibody CEM231, which recognizes carcinoembryonic antigen (CEA), has been described previously.[3] We also showed that this β-lactamase-Fab′ conjugate retained the same level of immunoreactivity and enzymatic activity as the parent proteins. The complete evaluation of this bioconjugate will be discussed in a more appropriate setting.[6] The viability of the ADC concept has been demonstrated in vitro utilizing cephem–vincathiol prodrug 1 and the MoAb-enzyme conjugate described above.[3] Herein, we report on the design and synthesis of a new prodrug derived from cephalothin and the potent cytotoxic agent desacetylvinblastine hydrazide[7] (**3**) (DAVLBH).



1 (LY191026)



2 X = $HS(CH_2)_2NH$-
3 X = NH_2NH- (DAVLBH)

Results and Discussion

When considering possible cytotoxic agents for use in the ADC system we felt compounds with potent antitumor activity, but dose-limiting toxicity, were excellent candidates for conversion to prodrugs. The vinca alkaloids are attractive targets as there is considerable clinical experience with this class of agents. The initial proof of the ADC concept was demonstrated employing a prodrug of the vincathiol analogue **2**.[3] Another attractive drug candidate is DAVLBH (**3**). This compound is a potent cytotoxic agent, and in-house experience suggested substitution on the hydrazide moiety could reduce the potency of this agent.[8] Furthermore, a covalently attached MoAb–DAVLBH immunoconjugate has undergone phase I clinical evaluation.[1] Thus, preparation of an appropriate prodrug would allow us to evaluate the potential of the ADC system relative to the covalent immunoconjugate and nontargeted DAVLBH.

We initially selected the directly attached C-3′ acylhydrazido cephem **8** as our target prodrug (Scheme I). To our knowledge C-3′-substituted cephalosporins of this type (–NHNHCOR) are unprecedented in the literature. Key to the preparation of this compound was the successful conversion of allyl cephalothin **5** to the C-3′ iodocephem **6**. Thus, treatment of **5** with 2 equiv of iodotrimethylsilane[9] in methylene chloride chemoselectively cleaved the acetoxy moiety, in the presence of the allyl ester, giving rise to the desired C-3′ iodocephem **6** in 79% yield. The iodide was displaced with DAVLBH in DMF containing sodium bicarbonate to give the requisite N-alkylated product **7**. Palladium(0)-catalyzed removal of the allyl ester[10] completed the synthesis of prodrug candidate **8**.

For our purposes a successful prodrug must be a good substrate for the P99 enzyme and release the free drug upon enzymatic hydrolysis of the β-lactam bond. The β-lactam moiety in prodrug **8** was rapidly hydrolyzed by the P99 enzyme; however, release of the free DAVLBH was determined to be too slow to be of practical use in the ADC delivery system.[11] This finding necessitated the design of a better leaving group which would ultimately allow for the attachment and more efficient release of free DAVLBH from the cephem nucleus.

(5) Faraci, W. S.; Pratt, R. F. *Biochem.* **1986**, *25*, 2934. Pratt, R. F.; Faraci, W. S. *J. Am. Chem. Soc.* **1986**, *108*, 5328. Mobashery, S.; Johnston, M. J. *J. Biol. Chem.* **1986**, *261*, 7879 and references cited therein.

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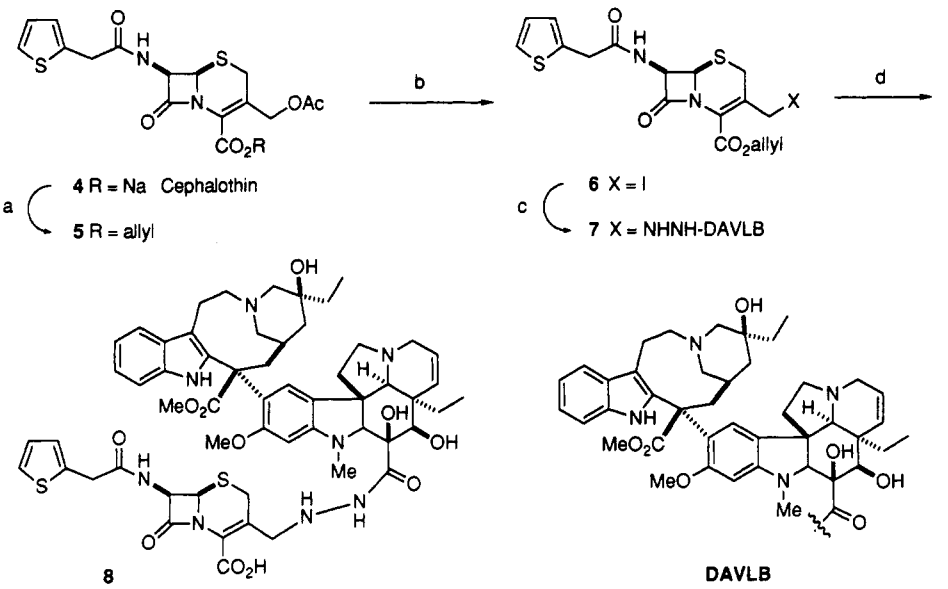
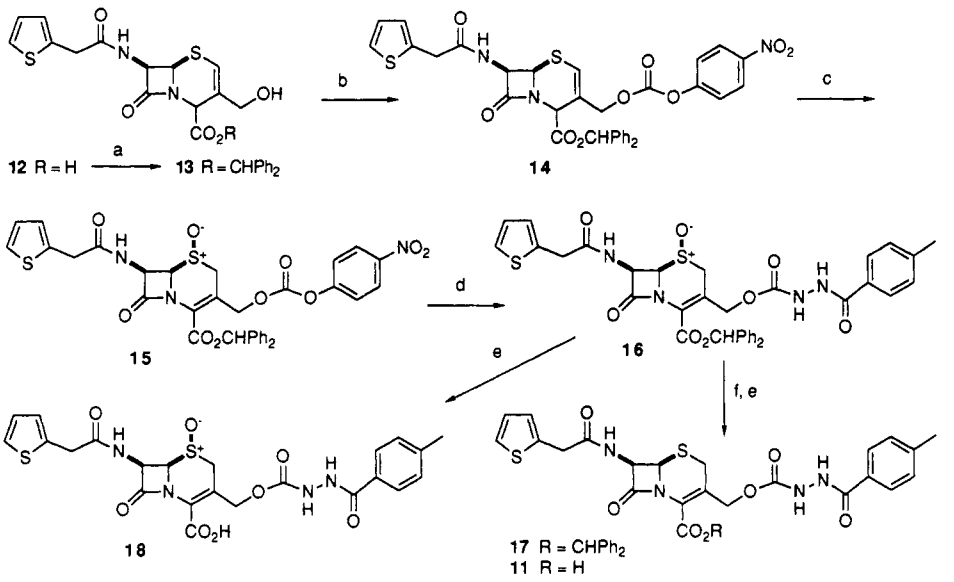
Scheme I[a]



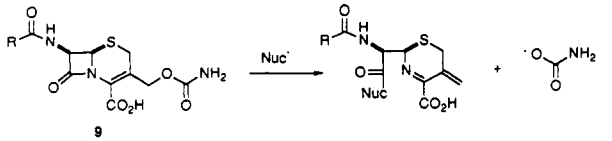
[a] Key: (a) allyl bromide, DMF, H_2O, 48%; (b) Me_3SiI (2 equiv), CH_2Cl_2, 79%; (c) DAVLBH, DMF, $NaHCO_3$, 21%; (d) $(Ph_3P)_2PdCl_2$, Et_3SiH, HOAc, CH_2Cl_2, then flash chromatography, 24%.

Scheme II[a]

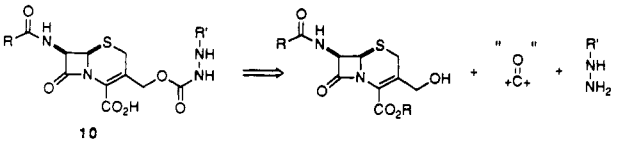


[a] Key: (a) Ph_2CN_2, CH_3CN, Me_2CO, 60%; (b) p-$O_2NC_6H_4OC(O)Cl$, THF, DMAP, 2,6-lutidine, 74%; (c) m-CPBA, CH_2Cl_2, 86%; (d) toluohydrazide, pyridine, i-Pr_2NEt (cat.), 65%; (e) TFA, Et_3SiH, CH_2Cl_2, 0 °C, 68% for 18, 52% for 11; (f) $SnCl_2$, AcCl, DMF, 0 °C, 76%.

A carbamoyloxy group on the C-3′ position of a cephalosporin, e.g., **9**, is known to be a good leaving group.[12] We



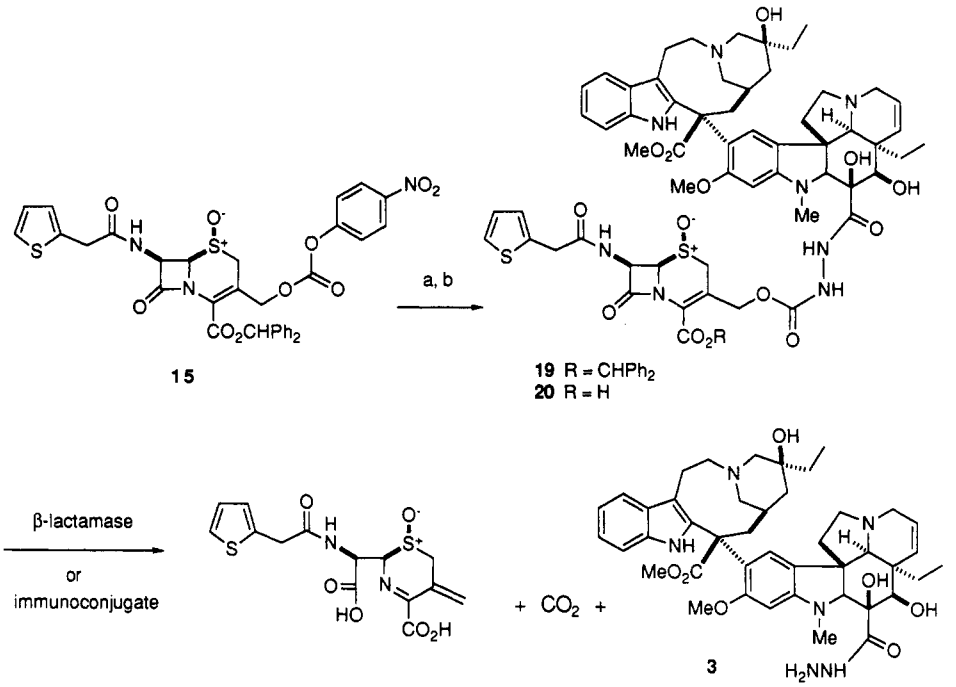
proposed that an aza carbamate derived from DAVLBH's hydrazide moiety might also be a good leaving group upon lactam hydrolysis and that rapid decarboxylation would follow, under physiological conditions, to release free DAVLBH. Thus, our attention focused on the synthesis of azacarbamate **10**. Cephalosporins of this general type



(C-3′, $-O_2CNHNHCOR$) are also, to the best of our knowledge, unknown in the literature. Retrosynthetic analysis suggested the target should be obtained by combination of a C-3′ hydroxy-substituted cephalosporin, DAVLBH, and a phosgene equivalent. Considering the cost and limited supply of DAVLBH we chose to first focus on the synthesis of a model compound **11**, which substituted p-toluohydrazide in place of DAVLBH (Scheme II).

(12) Boyd, D. B. in *Chemistry and Biology of β-Lactam Antibiotics*; Morin, R. B., Gorman, M., Eds.; Academic Press: New York, 1982; Vol 1, pp 437–545.

Scheme III[a]

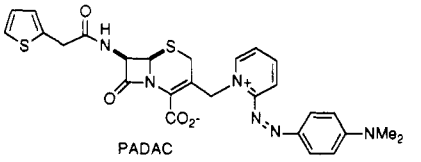


[a] Key: (a) DAVLBH, pyridine, *i*-Pr_2NEt (cat), 56%; (b) TFA, Et_3SiH, CH_2Cl_2, 0 °C.

After considerable experimentation the following route to the target cephem azacarbamates was devised[13] (Scheme II). (Hydroxymethyl)cephem **12** is readily available from cephalothin.[14] Esterification of **12** with diphenyldiazomethane gave benzhydryl ester **13**. Acylation of the C-3′ hydroxyl moiety with *p*-nitrophenyl chloroformate provided carbonate **14** in good yield. Double-bond isomerization was affected in the usual fashion[15] by oxidation to the cephem sulfoxide **15** with *m*-CPBA. Under these conditions only the β-sulfoxide isomer was obtained.[16] The pivotal displacement of the *p*-nitrophenyl moiety was cleanly affected with *p*-toluohydrazide and a catalytic amount of Hunig's base in pyridine to give carbamate **16** in 65% yield. Displacement by the more basic amine nitrogen was supported by the presence of three distinct amide NH resonances in the NMR of **16**. The sulfoxide was efficiently reduced with stannous chloride and acetyl chloride to give the desired Δ-3 cephem **17** in 76% yield. Acid-catalyzed hydrolysis of the benzhydryl ester in the presence of triethylsilane as a carbonium ion trap provided model compound **11**. In order to determine whether or not a cephem sulfoxide could serve as a substrate for β-lactamase, **16** was deesterified (TFA, Et_3SiH) to give the sulfoxide analogue **18**.

Incubation of both model compounds **11** and **18** with the MoAb-enzyme immunoconjugate provided the results shown in Table I. Both compounds proved to be excellent substrates for the β-lactamase enzyme and rapidly released a molar equivalent of *p*-toluohydrazide upon cleavage of the β-lactam bond. It is interesting to note that the sulfoxide **18** proved to be a better substrate than the parent cephalosporin **11**.[3b] At relatively low prodrug (substrate) concentration, expected in vivo, efficient hydrolysis of the prodrug and concomitant release of free drug will be determined by the k_{cat}/K_M of the enzyme-substrate interaction. Prodrugs which are rapidly hydrolyzed by β-lactamase, and which exhibit high affinity for the enzyme (high k_{cat}/K_M) are expected to offer a therapeutic advantage in an ADC delivery system. Qualitatively the sulfoxide exhibited superior solution stability as well. Furthermore, the presence of the sulfoxide moiety precludes double-bond migration to the undesired Δ-2 cephem olefin isomer, which caused considerable problems in the synthesis of our original vincathiol prodrug **1**. These exciting findings prompted us to select cephalosporin sulfoxide analogue **20** (LY266070) as our target prodrug.

Table I. Hydrolysis of Cephem Prodrugs by the MoAb-Enzyme Immunoconjugate



compd	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M
4	55	5	10
PADAC	135	55	2.5
11	16	1.8	9
18	1000	38	26
20	1700	160	11

Synthesis of the cephem-DAVLBH prodrug **20** was accomplished in straightforward fashion as outlined in Scheme III. Condensation of DAVLBH and *p*-nitrophenyl carbonate **15** in pyridine containing a catalytic amount of Hunig's base provided the desired cephem

(13) After this work was completed several reports on similar approaches to cephem 3′-*N*-alkylcarbamates appeared. From cephem 3′-chloroformates: Albrecht, H. A.; Keith, D. D.; Konzelmann, F. M.; Rossman, P. L.; Wei, C. C.; Weigele, M.; Yang, R. Eur. Pat. Appl. EP 335,297; *Chem. Abstr.* **1990**, *112*, 235053q. From cephem 3′-(pentafluorophenyl)carbonates: Eaton, M. A. W.; Pratt, A. J.; Alexander, R. P. Eur. Pat. Appl. EP 392,745. From tetrachlorocarbonate intermediates: Demuth, T. P., Jr.; White, R. E.; Tietjen, R. A.; Ebetino, F. H.; Kraft, W. G.; Andersen, J. A.; McOsker, C. C.; Walling, M. A.; Davis, B. W.; Rourke, F. J. 200th National Meeting of the American Chemical Society; Washington, DC, Aug 1990, Med. Chem. Abstr. 154.

(14) Kukolja, S. *J. Med. Chem.* **1970**, *13*, 1114.

(15) Kaiser, G. V.; Cooper, R. D. G.; Koehler, R. E.; Murphy, C. F.; Webber, J. A.; Wright, I. G.; Van Heyningen, E. M. *J. Org. Chem.* **1970**, *35*, 2430.

(16) Cooper, R. D. G.; DeMarco, P. V.; Cheng, J. C.; Jones, N. D. *J. Am. Chem. Soc.* **1969**, *91*, 1408.

carbamate **19** in 56% yield. TFA, in the presence of Et_3SiH, removed the benzhydryl group to give the target prodrug **20**.

Prodrug **20** was found to be an excellent substrate for the MoAb-enzyme immunoconjugate (Table I), and concomitant release of free DAVLBH was observed by HPLC. Differential in vitro cytotoxicity of prodrug **20** versus DAVLBH was measured using the LS174T colon tumor cell line. A reproducible 5-fold (molar basis) differential in cytotoxicity was observed between DAVLBH and the less toxic prodrug **20** at short incubation times. Preexposure of antigen positive tumor cells to the antibody–enzyme immunoconjugate prior to administration of the prodrug reversed this differential, indicating the immunoconjugate catalyzes release of free DAVLBH in vitro. Mouse xenograft studies employing the antibody–enzyme immunoconjugate with prodrug **20** caused regression of established tumors, and this combination was significantly more active than either the prodrug **20** or DAVLBH administered alone.[17] These data demonstrate the ability of the ADC system to mediate antigen dependent cytotoxicity both in vitro and in vivo. Details of these studies will be published in due course.

Experimental Section

General Procedure. All reactions were run under a postive pressure of dry nitrogen. Fast atom bombardment mass spectra (FABMS) were obtained on a VG ZAB-3 instrument. ^1H NMR spectra were obtained on a GE QE300 instrument. J values are given in Hz. Flash chromatography was carried out on E. Merck Kieselgel 60 (230–400 mesh). Melting points were determined in open capillaries and are uncorrected.

Preparation of Allyl 7β-(2-(Thien-2-yl)acetamido)-3-(acetoxymethyl)-3-cephem-4-carboxylate (5). To a solution of cephalothin sodium salt **4** (11 g, 26.3 mmol, available from Sigma) in 1:1 DMF/H_2O (100 mL) was added allyl bromide (3.6 g, 29.7 mmol). After being stirred at room temperature for 48 h the mixture was diluted with EtOAc and washed with 0.1 N HCl and the organic phase dried ($MgSO_4$) and concentrated in vacuo to give an orange oil. Flash chromatography (EtOAc) followed by recrystallization from 1:1 $CHCl_3/Et_2O$ gave 5.5 g, 48%, of the title compound **5** as a white powder: mp 143–145 °C; NMR ($CDCl_3$) δ 7.25 (d, 1, J = 7), 7.0 (m, 2), 6.27 (br d, 1, J = 9), 6.0–5.8 (m, 1), 5.83 (dd, 1, J = 5, 9), 5.4–5.2 (m, 2), 5.06 ($^1/_2$ of ABq, 1, J = 14), 4.96 (d, 1, J = 5), 4.81 ($^1/_2$ of ABq, 1, J = 14), 4.74 (br d, 2, J = 6), 3.85 (s, 2), 3.55 ($^1/_2$ of ABq, 1, J = 18), 3.33 ($^1/_2$ of ABq, 1, J = 18); 2.06 (s, 3); IR (KBr) 1776, 1745, 1707, 1658 cm^{-1}. Anal. Calcd for $C_{19}H_{20}N_2O_6S_2$: C, 52.29; H, 4.59; N, 6.42. Found: C, 52.34; H, 4.71; N, 6.39.

Preparation of Allyl 7β-(2-(Thien-2-yl)acetamido)-3-(iodomethyl)-3-cephem-4-carboxylate (6). To a solution of allyl cephalothin **5** (5.01 g, 11.5 mmol) in CH_2Cl_2 (55 mL) was added dropwise iodotrimethylsilane (3.27 mL, 23 mmol), and the resulting solution was stirred at room temperature for 70 min. The reaction mixture was diluted with EtOAc and washed with ice-cold 10% sodium thiosulfate solution, saturated $NaHCO_3$, and brine. The organic phase was dried (Na_2SO_4) and concentrated in vacuo to give 4.6 g, 79%, of the title compound **6** as a yellow-orange tinted foam. This material was used immediately without further purification: NMR ($CDCl_3$) δ 7.25 (d, 1, J = 7), 7.0 (m, 2), 6.42 (d, 1, J = 9), 6.0–5.85 (m, 1), 5.78 (dd, 1, J = 5, 9), 5.4–5.2 (m, 2), 4.95 (d, 1, J = 5), 4.74 (d, 2, J = 6), 4.38 (ABq, 2, J = 10), 3.85 (s, 2), 3.72 ($^1/_2$ of ABq, 1, J = 18), 3.44 ($^1/_2$ of ABq, 1, J = 18).

N-Alkylation of Desacetylvinblastine Hydrazide with Allyl 7β-(2-(Thien-2-yl)acetamido)-3-(iodomethyl)-3-cephem-4-carboxylate (6). Preparation of 3′-Hydrazido Cephem 7. To a solution of allyl cephalothin **5** (872 mg, 2.0 mmol) in CH_2Cl_2 (20 mL) was added dropwise iodotrimethylsilane (0.57 mL, 4 mmol), and the resulting solution was stirred at room temperature for 1 h. The reaction mixture was diluted with EtOAc and washed with ice-cold 10% sodium thiosulfate solution, saturated $NaHCO_3$, and brine. The organic phase was dried (Na_2SO_4) and concentrated in vacuo. The residue was dissolved in DMF (4 mL), and solid $NaHCO_3$ (0.51 g, 6 mmol) was added followed by desacetylvinblastine hydrazide[7] (1.74 g, 2.0 mmol) in DMF (8 mL). The resulting solution was stirred at room temperature for 5 h. Water (100 mL) was added, and a yellow precipitate formed which was collected by filtration. This material was dissolved in $CHCl_3$, dried (Na_2SO_4), and concentrated in vacuo to give 1.9 g of a foam. Flash chromatography (10% i-PrOH in $CHCl_3$) gave 470 mg, 21% from **5**, of the desired 3′-hydrazido cephem **7** as a white powder: NMR ($CDCl_3$) δ 9.6 (br s, 1), 8.28 (d, 1, J = 6), 8.04 (s, 1), 7.55 (d, 1, J = 7), 7.3–7.0 (m, 6), 6.60 (s, 1), 6.35 (d, 1, J = 9), 6.1 (s, 1), 5.95 (m, 1), 5.84 (dd, 1, J = 5, 9), 5.75 (d, 1, J = 9), 5.30 (m, 2), 5.01 (d, 1, J = 5), 5.0–4.65 (m, 3), 4.20 (m, 1), 3.87 (s, 2), 3.80 (s, 3), 3.62 (s, 3), 3.60 (ABq, 2, J = 17), 2.75 (s, 3), 0.95 (2 overlapping t, 6, J = 7); the remaining protons appear as an envelope from 4.0–1.0 which closely resembles the spectrum of desacetylvinblastine hydrazide; IR ($CHCl_3$) 1783, 1724, 1674, 1504 cm^{-1}; UV (EtOH) λ_{max} 265 (ε 23 500); FABMS calcd for $C_{60}H_{73}N_8O_{11}S_2$ 1145.4840, found 1145.4825 (M + 1).

Hydrolysis of Ester 7. Preparation of the 3′-Hydrazido Cephem-4-carboxylic Acid 8. To a solution of the allyl ester **7** (340 mg, 0.3 mmol) in CH_2Cl_2 (6 mL) was added glacial HOAc (0.16 mL, 2.8 mmol), bis(triphenylphosphine)palladium(II) chloride (21 mg, 0.03 mmol), and triethylsilane (0.16 mL, 1 mmol). The reaction mixture was stirred at room temperature for 22 h and then loaded directly onto a flash chromatography column. Elution (20% i-PrOH in $CHCl_3$) gave 80 mg, 24%, of the desired 3′-hydrazido cephem-4-carboxcylic acid **8** as a white powder: NMR (DMSO-d_6) δ 9.9 (s, 1), 9.4 (br s, 1), 9.15 (d, 1, J = 9), 8.75 (br s, 1), 7.4 (m, 1), 7.36 (dd, 1, J = 1, 5), 7.27 (d, 1, J = 9), 7.05–6.9 (m, 4), 6.43 (s, 1), 6.20 (s, 1), 5.75 (dd, 1, J = 5, 9), 5.70 (dd, 1, J = 4, 10), 5.58 (d, 1, J = 10), 5.10 (d, 1, J = 5), 4.30 (d, 1, J = 7), 4.12 (ABq, 2, J = 17), 3.76 (s, 2), 3.70 (s, 3), 3.53 (s, 3), 2.83 (s, 3), 0.80 (t, 3, J = 7), 0.72 (t, 3, J = 7); the remaining protons appear as an envelope from 3.8–1.1 which closely resembles the spectrum of desacetylvinblastine hydrazide; IR (KBr) 1786, 1720, 1684 cm^{-1}; UV (EtOH) λ_{max} 264 (ε 23 200); FABMS calcd for $C_{57}H_{69}N_8O_{11}S_2$ 1105.4527, found 1105.4538. Anal. Calcd for $C_{57}H_{68}N_8O_{11}S_2$: C, 61.94; H, 6.20; N, 10.14. Found: C, 61.68; H, 6.09; N, 10.16.

Preparation of Benzhydryl 7β-(2-(Thien-2-yl)acetamido)-3-(hydroxymethyl)-2-cephem-4-carboxylate (13). To a solution of 7β-(2-(thien-2-yl)acetamido)-3-(hydroxymethyl)-2-cephem-4-carboxylic acid (**12**)[14] (5.0 g, 14.1 mmol) in 50 mL of 1:1 acetone/acetonitrile was added dropwise a solution of diphenyldiazomethane (2.7 g, 13.9 mmol) in CH_3CN (20 mL). The resulting solution was stirred at room temperature for 1 h and then concentrated in vacuo. The residue was triturated with CH_3CN and the insoluble material collected by filtration and dried in vacuo to give 4.4 g, 60%, of the title compound **13** as a beige solid: mp 144–146 °C; NMR ($CDCl_3$) δ 7.40–7.23 (m, 11), 7.20–6.95 (m, 2), 6.88 (s, 1), 6.45 (d, 1, J = 7.6), 6.25 (s, 1), 5.61 (dd, 1, J = 5, 8), 5.20 (d, 1, J = 5), 5.15 (s, 1), 4.10 (q, 2, J = 8), 3.82 (s, 2); IR ($CHCl_3$) 1778, 1743, 1683 cm^{-1}. Anal. Calcd for $C_{27}H_{24}N_2O_5S_2$: C, 62.29; H, 4.64; N, 5.38. Found: C, 62.07; H, 4.73; N, 5.51.

Preparation of Benzhydryl 7β-(2-(Thien-2-yl)acetamido)-3-[[[(4-nitrophenoxy)carbonyl]oxy]methyl]-2-cephem-4-carboxylate (14). To a 0 °C solution of (hydroxymethyl)cephem **13** (3.0 g, 5.77 mmol) in dry THF (5 mL) was added DMAP (2 mg) followed by p-nitrophenyl chloroformate (1.74 g, 8.65 mmol). Dry 2,6-lutidine (1.0 mL, 8.65 mmol) was added dropwise, and the resulting solution was allowed to warm to room temperature overnight. Some insoluble material was removed by filtration, and the filtrate was concentrated in vacuo. The residue was purified by flash chromatography (5% EtOAc in CH_2Cl_2) to give 2.9 g, 74%, of carbonate **14** as a white foam: mp 69–71 °C; NMR ($CDCl_3$) δ 8.28 (d, 2, J = 9.1), 7.42–7.28 (m, 13), 7.05–7.0 (m, 2), 6.92 (s, 1), 6.55 (s, 1), 6.35 (d, 1, J = 8.7), 5.65 (dd, 1, J = 4, 8.7), 5.24 (d, 1, J = 4), 5.21 (s, 1), 4.82 (d, 1, J = 12), 4.72 (d, 1, J = 12), 3.88 (s, 2); ^{13}C NMR ($CDCl_3$) δ 169.99, 165.90, 164.17, 155.26, 152.04, 145.46, 138.76, 138.69, 134.59, 128.84,

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128.77, 128.60, 128.47, 127.90, 127.55, 127.14, 126.79, 126.07, 125.30, 124.64, 121.72, 117.93, 79.49, 69.90, 60.45, 53.52, 50.14, 37.07; IR ($CHCl_3$) 1777, 1749, 1686, 1528 cm^{-1}; UV (EtOH) λ_{max} 244 (ϵ 16800); FABMS calcd for $C_{34}H_{28}N_3O_9S_2$ 686.1267, found 686.1245 (M + 1). Anal. Calcd for $C_{34}H_{27}N_3O_9S_2$: C, 59.55; H, 3.97; N, 6.13. Found: C, 59.34; H, 4.05; N, 5.93.

Oxidation of Cephem 14 to Benzhydryl 7β-(2-(Thien-2-yl)acetamido)-3-[[[(4-nitrophenoxy)carbonyl]oxy]methyl]-3-cephem-4-carboxylate 1β-Sulfoxide (15). To a 0 °C solution of cephem 14 (2.5 g, 3.65 mmol) in CH_2Cl_2 (40 mL) was added a solution of 55% *m*-CPBA (1.1 g, 3.65 mmol equiv) in CH_2Cl_2 (10 mL). After 1 h at 0 ° TLC (20% EtOAc in CH_2Cl_2) indicated the starting material had been consumed. The solvent was removed in vacuo and the residue subjected to flash chromatography (20% EtOAc in CH_2Cl_2) to give 2.2 g, 86%, of the 1β-sulfoxide 15 as a white solid: mp 143 °C dec; NMR (DMSO-d_6) δ 8.49 (d, 1, *J* = 8), 8.27 (d, 2, *J* = 9), 7.5–7.2 (m, 13), 6.92 (m, 3), 5.94 (dd, 1, *J* = 4.5, 8), 5.25 (d, 1, *J* = 13), 4.93 (d, 1, *J* = 4.5), 4.85 (d, 1, *J* = 13), 4.04 (d, 1, *J* = 18.7), 3.88 (d, 1, *J* = 15.3), 3.78 (d, 1, *J* = 15.3), 3.66 (d, 1, *J* = 18.7); IR (KBr) 1765, 1720, 1658 cm^{-1}; UV (EtOH) λ_{max} 265 (ϵ 3570). Anal. Calcd for $C_{34}H_{27}N_3O_{10}S_2$: C, 58.20; H, 3.88; N, 5.99. Found: C, 58.48; H, 4.08; N, 5.90.

Reaction of 15 with *p*-Toluohydrazide. Preparation of Benzhydryl 7β-(2-(Thien-2-yl)acetamido)-3-[[[[(4-methylbenzoyl)hydrazino]carbonyl]oxy]methyl]-3-cephem-4-carboxylate 1β-Sulfoxide (16). Cephem sulfoxide 15 (0.5 g, 0.71 mmol) and *p*-toluohydrazide (0.14 g, 0.93 mmol) were placed in a dry flask under N_2 and then dissolved in pyridine (4 mL). Two drops of *i*-Pr_2NEt were added, and the resulting solution was stirred at room temperature for 16 h. The solvent was removed in vacuo and the residue triturated with 5% MeOH in CH_2Cl_2. The insoluble material was collected by filtration and dried in vacuo to give 0.33 g, 65%, of the desired carbamate 16 as a white solid: NMR (DMSO-d_6) δ 10.21 (br s, 1, NH), 9.34 (br s, 1, NH), 8.44 (d, 1, *J* = 8, NH of cephem), 7.70 (d, 2, *J* = 7.6), 7.48 (d, 2, *J* = 7.6), 7.4–7.2 (m, 11), 6.94–6.87 (m, 3), 5.90 (dd, 1, *J* = 4, 8), 5.06 (d, 1, *J* = 13.5), 4.93 (d, 1, *J* = 4), 4.66 (d, 1, *J* = 13.5), 3.8 (m, 3), 3.58 (d, 1, *J* = 18), 2.31 (s, 3); IR (KBr) 1791, 1717, 1655 cm^{-1}; FABMS calcd for $C_{36}H_{33}N_4O_8S_2$ 713.1740, found 713.1751 (M + 1). Anal. Calcd for $C_{36}H_{32}N_4O_8S_2$: C, 60.66; H, 4.52; N, 7.86. Found: C, 60.94; H, 4.62; N, 8.00.

Hydrolysis of Ester 16. Preparation of 7β-(2-(Thien-2-yl)acetamido)-3-[[[[(4-methylbenzoyl)hydrazino]carbonyl]oxy]methyl]-3-cephem-4-carboxylic Acid 1β-Sulfoxide (18). To a 0 °C solution of benzhydryl ester 16 (93 mg, 0.13 mmol) in CH_2Cl_2 (5 mL) was added triethylsilane (0.5 mL) and TFA (5 mL). The resulting solution was stirred at 0 °C for 45 min and then diluted with cold CH_3CN (10 mL). The solvent was removed in vacuo, and additional CH_3CN was added and then removed in vacuo. The residue was triturated with cold CH_3CN, and the insolubles were collected by filtration then dried in vacuo to give 48 mg, 68%, of the desired acid 18 as a white solid: mp 170–172 °C; NMR (DMSO-d_6) δ 10.21 (s, 1, NH), 9.31 (s, 1, NH), 8.40 (d, 1, *J* = 8, NH of cephem), 7.70 (d, 2, *J* = 7.8), 7.33 (m, 1), 7.24 (d, 2, *J* = 7.8), 6.90 (s, 2), 5.80 (dd, 1, *J* = 4.5, 8), 5.18 (d, 1, *J* = 13), 4.87 (d, 1, *J* = 4.5), 4.62 (d, 1, *J* = 13), 3.90–3.40 (m, 4), 2.31 (s, 3); IR (KBr) 1791, 1736, 1721, 1660, 1652 cm^{-1}; UV (EtOH) λ_{max} 237 (ϵ 22400); FABMS calcd for $C_{23}H_{23}N_4O_8S_2$ 547.0957, found 547.0955 (M + 1).

Reduction of 1β-Sulfoxide 16 to Benzhydryl 7β-(2-(Thien-2-yl)acetamido)-3-[[[[(4-methylbenzoyl)hydrazino]carbonyl]oxy]methyl]-3-cephem-4-carboxylate (17). To a 0 °C solution of 1β-sulfoxide 16 (0.30 g, 0.42 mmol) in DMF (4 mL) was added stannous chloride (0.24 g, 1.05 mmol) followed by dropwise addition of acetyl chloride (0.75 mL, 10.5 mmol). The resulting solution was stirred at 0 °C for 15 min, the cooling bath was removed, and stirring was continued for 45 min. The reaction mixture was partitioned between EtOAc and ice–water. The organic phase was washed with water, dried ($MgSO_4$), and concentrated in vacuo. The residue was purified via flash chromatography (5% MeOH in CH_2Cl_2) to give 0.22 g, 76%, of the desired sulfide 17 as an off-white solid: NMR (DMSO-d_6) δ 10.22 (s, 1, NH), 9.31 (s, 1, NH), 9.12 (d, 1, *J* = 8, NH of cephem), 7.71 (d, 2, *J* = 7.5), 7.46 (d, 2, *J* = 7.5), 7.37–7.2 (m, 11), 6.92–6.87 (m, 3), 5.74 (dd, 1, *J* = 4.6, 8), 5.13 (d, 1, *J* = 4.6), 4.88 (d, 1, *J* = 13), 4.71 (d, 1, *J* = 13), 3.72 (s, 2), 3.63 (d, 1, *J* = 18), 3.51 (d, 1, *J* = 18), 2.31 (s, 3); IR ($CHCl_3$) 1789, 1727, 1673 cm^{-1}; FABMS calcd for $C_{36}H_{33}N_4O_7S_2$ 697.1791, found 697.1820 (M + 1). Anal. Calcd for $C_{36}H_{32}N_4O_7S_2$: C, 62.06; H, 4.63; N, 8.04. Found: C, 61.97; H, 4.61; N, 7.75.

Hydrolysis of Ester 17. Preparation of 7β-(2-(Thien-2-yl)acetamido)-3-[[[[(4-methylbenzoyl)hydrazino]carbonyl]oxy]methyl]-3-cephem-4-carboxylic Acid (11). To a 0 °C slurry of benzhydryl ester 17 (0.50 g, 0.72 mmol) in CH_2Cl_2 (3 mL) was added triethylsilane (1.0 mL) and TFA (1.0 mL). The resulting solution was stirred at 0 °C for 45 min at which time TLC (10% MeOH in CH_2Cl_2) indicated the ester had been consumed. The mixture was diluted with cold CH_3CN (25 mL). The solvent was removed in vacuo, and additional CH_3CN was added and then removed in vacuo to give 400 mg of the desired acid as an off-white solid. A 200-mg portion of this material was purified by reversed phase HPLC (Rainin C18 column, 20% CH_3CN, 1% NH_4OAc in H_2O, then 25% CH_3CN, 1% NH_4OAc in H_2O) to give 100 mg, 52%, of the desired acid 11 as a white powder: mp 122–125 °C; NMR (DMSO-d_6) δ 10.21 (br s, 1, NH), 9.23 (br s, 1, NH), 9.04 (d, 1, *J* = 8, NH of cephem), 7.68 (m, 2), 7.40–7.20 (m, 3), 6.93 (m, 2), 5.65 (dd, 1, *J* = 4, 8), 5.12 (d, 1, *J* = 4), 5.03 (d, 1, *J* = 14), 4.75 (d, 1, *J* = 14), 3.77 (s, 2), 3.50 (d, 1, *J* = 17), 3.30 (m, 1), 2.34 (s, 3); IR ($CHCl_3$) 1780, 1658, 1593 cm^{-1}. UV (EtOH) λ_{max} 237 (ϵ 25200); FABMS calcd for $C_{23}H_{23}N_4O_7S_2$ 531.1008, found 531.1024 (M + 1). Anal. Calcd for $C_{23}H_{22}N_4O_7S_2$: C, 52.07; H, 4.18; N, 10.56. Found: C, 52.06; H, 4.31; N, 10.77.

Reaction of Cephem-3′-carbonate 15 with Desacetylvinblastine Hydrazide. Preparation of Carbamate 19. Desacetylvinblastine hydrazide sulfate[7] (0.5 g, 0.57 mmol) was dissolved in saturated aqueous $NaHCO_3$ solution (10 mL) and extracted with CH_2Cl_2. The organic phase was dried (Na_2SO_4) and concentrated in vacuo to give the free base. The free base was dissolved in dry pyridine (10 mL) and added to a flask containing cephem-3′-carbonate 15 (0.32 g, 0.45 mmol). Two drops of *i*-Pr_2NEt were added, the resulting solution was stirred at room temperature overnight, and the reaction mixture was then diluted with EtOAc and concentrated in vacuo to give a solid residue. This residue was purified by flash chromatography (10% MeOH in CH_2Cl_2) to give 0.34 g, 56%, of the desired carbamate 19 as an off-white solid: NMR ($CDCl_3$) δ 9.91 (s, 1), 8.66 (br s, 1), 8.01 (s, 1), 7.53–6.86 (m, 23), 6.55 (s, 1), 6.08 (s, 1), 6.05 (dd, 1, *J* = 4.5, 10), 5.8–5.6 (m, 2), 5.3–5.2 (m, 1), 4.85 (br s, 1), 4.47 (d, 1, *J* = 4.5), 3.83 (s, 2), 3.74 (s, 3), 3.57 (s, 3), 0.87 (t, 6, *J* = 7); the remaining protons appear as an envelope from 4.10 to 1.10 which closely resembles the spectrum of desacetylvinblastine hydrazide: IR ($CHCl_3$) 1803, 1730, 1690 cm^{-1}; UV (EtOH) λ_{max} 268 (ϵ 25200); FABMS calcd for $C_{71}H_{79}N_8O_{14}S_2$ 1331.5157, found 1331.5122 (M + 1).

Hydrolysis of Ester 19. Preparation of Cephem Acid 20 (LY266070). To a 0 °C solution of benzhydryl ester 19 (105 mg, 0.079 mmol) in CH_2Cl_2 (3 mL) was added triethylsilane (0.5 mL) and TFA (0.5 mL). The resulting solution was stirred at 0 °C for 15 min and then diluted with cold acetonitrile (10 mL). The solvent was removed in vacuo, and additional acetonitrile was added and then removed in vacuo. The residue was triturated with Et_2O to give 100 mg of the desired acid as a granular solid (presumed TFA salt): NMR (DMSO-d_6) δ 9.76 (s, 1), 9.46 (s, 1), 9.40 (s, 1), 8.43 (d, 1, *J* = 8), 7.47 (d, 1, *J* = 8), 7.4–6.9 (m, 16), 6.68 (s, 1), 6.28 (s, 1), 5.80 (dd, 1, *J* = 6, 8), 5.75 (s, 1), 5.16 (d, 1, *J* = 13), 4.85 (m, 1), 4.64 (d, 1, *J* = 13), 3.88 (s, 2), 3.78 (s, 2), 3.71 (s, 3), 3.53 (s, 3), 2.78 (br s, 3), 0.80 (t, 3, *J* = 7), 0.70 (t, 3, *J* = 7); the remaining protons appear as an envelope from 4.30 to 1.00 which closely resembles the spectrum of desacetylvinblastine hydrazide: IR (KBr) 1792, 1723, 1680, 1617, 1505 cm^{-1}; UV (EtOH) λ_{max} 266 (ϵ 19300); FABMS calcd for $C_{58}H_{69}N_8O_{14}S_2$ 1165.4374, found 1165.4329 (M + 1). This material was converted into the HCl salt by dissolving 25 mg of the above prepared acid in 0.1 N HCl (5 mL), filtering off a small amount of insoluble material, and freeze-drying the filtrate. The residue was dissolved 0.1 N HCl (5 mL) and then freeze dried. Finally, the resulting residue was dissolved in water (5 mL) and freeze-dried to give 22 mg of a white fluffy powder, mp >240 °C. Reversed phase HPLC analysis (40% CH_3CN, 0.3% H_3PO_4, water; Waters C-18 μ-bondapak) indicated this material was at least 95% pure, retention time 3.03 min. The free vinca hydrazide has a retention

time of 1.71 min under these conditions. Anal. Calcd for $C_{58}H_{68}N_8O_{14}S_2 \cdot 6HCl$: C, 50.33; H, 5.39; N, 8.10. Found: C, 50.62; H, 5.30; N, 8.06. In the absence of a Cl analysis these data are also consistent with $C_{58}H_{68}N_8O_{14}S_2 \cdot 3HCl \cdot CF_3CO_2H \cdot 2H_2O$: C, 50.58; H, 5.37; N, 7.86. A second lot of material prepared by this procedure and stored in the freezer for approximately 1 year was analyzed: calcd for $C_{58}H_{68}N_8O_{14}S_2 \cdot 2HCl \cdot CF_3CO_2H \cdot H_2O$: C, 52.59; H, 5.36; N, 8.17; Cl, 5.17. Found: C, 52.89; H, 5.38; N, 8.11; Cl, 5.45.

Enzymatic Hydrolysis of Cephalosporins. β-Lactamase activity of the MoAb-enzyme immunoconjugate was measured by monitoring the change in absorbance of the chromogenic substrate PADAC (Calbiochem) at 570 nm in a stirred cuvette at 37 °C in PBS. Absorbance was measured every 5 s for 120 s using a Hewlett-Packard 8451A spectrophotometer, and linear portions of the rate plots were used to obtain reaction velocities. K_M and k_{cat} were determined from the slope and intercept of Lineweaver-Burk plots of the velocity data. PADAC ϵ_{570} was taken to be 4.8×10^4 (product label).

Due to PADAC's low solubility and high extinction coefficient, the assay was run under conditions in which the rate is dependent on the substrate concentration. Consequently, the runs comparing the activity between different preparations were all performed with a starting A_{570} of approximately 0.5.

Kinetic parameters for cephalothin (4), 11, and 18 were measured as for PADAC, except that the change in absorbance was monitored at around 260 nm. Compounds 11 and 18 have a residual absorbance after hydrolysis so $\Delta\epsilon$s were obtained by completely hydrolyzing a known concentration of the substrate and calculating $\Delta\epsilon$ from the change in absorbance: $\Delta\epsilon_{258}$ for 11 = 6.6×10^3 (cm M)$^{-1}$; and $\Delta\epsilon_{260}$ for 18 = 8.4×10^3 (cm M)$^{-1}$.

The spectral change upon β-lactamase catalyzed hydrolysis of prodrug 20 was too small to be useful for quantitative determination of the rates. Consequently, HPLC methods were developed to monitor this reaction. Vials containing 1.5-mL solutions of varying concentrations of substrate at 37 °C in PBS, pH 7.4, were treated with 0.11 nM of the MoAb-enzyme immunoconjugate. Samples were quenched after 90 s by adding 0.5 mL of the reaction solution to 0.5 mL of 34% CH_3CN in 200 mM potassium phosphate, pH 3.0. Control experiments were performed to determine appropriate concentrations so that less than 10% of the substrate would be consumed during the 90-s reaction to assure a linear reaction rate. Duplicate samples of the quenched reaction mixtures were injected onto a 0.46- × 15-cm C18 reversed-phase HPLC column eluted (34% CH_3CN in 200 mM potassium phosphate) at 1 mL/min. The prodrug and product concentrations were monitored by absorbance at 266 nm.

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Use of a Thiol Tether for the Site-Specific Attachment of Reporter Groups to DNA

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The sequence-specific incorporation of a thiol tether into oligodeoxynucleotides provides for the rapid and facile attachment of a wide variety of reporter groups employing thiol-specific alkylating functionalities. The thiol residue is introduced into the DNA by oxidation of an internucleotide H-phosphonate at a unique site within the sequence in the presence of cystamine. After synthesis and purification of the cystamine-containing oligodeoxynucleotide, the sulfhydryl residue is unmasked by a short treatment with dithiothreitol. The tethered sulfhydryl residue is amenable to modification by a variety of thiol-specific reporter groups. Nuclease digests of the modified and unmodified sequences confirm that labeling occurs at the site of the tether. Duplex sequences containing a variety of fluorophores covalently bound through this thiol tether exhibit thermal stabilities that are very similar to that of the unlabeled sequence.

Introduction

The site-specific attachment of reporter groups to DNA would facilitate the detailed study of the structure and dynamics of unusual nucleic acid forms as well as ligand-DNA or protein-DNA complexes. In many previous studies (for a recent review see Goodchild[1]), the introduction of reporter groups has relied upon either the chemical synthesis of a modified nucleoside residue carrying the reporter group attached to the base residue[2] or a variety of related procedures which exploit the reactivity of functional groups attached to the 5′ or 3′ terminus of the DNA fragment.[3]

Reporter groups tethered to the base residue can be introduced site-specifically (depending upon the sequence location of the base). These procedures typically employ a linker arm attached to the C5-position of thymine or the N4-position on cytosine, the latter being a functional group normally involved in Watson-Crick hydrogen bonding. This approach can lead to destabilization of the helix

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