SYNTHESIS OF A CEPHALOSPORIN-DOXORUBICIN ANTITUMOR PRODRUG: A SUBSTRATE FOR AN ANTIBODY-TARGETED ENZYME#

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Abstract- Cephalosporin (**12**) substituted at the C-3' position with the potent oncolytic agent doxorubicin (**2**) was synthesized as a potential prodrug for the treatment of solid tumors. We envision the conversion of prodrug to free doxorubicin to be mediated by an immunoconjugate, consisting of a β-lactamase enzyme which is covalently attached to a monoclonal antibody, which has been prelocalized on the tumor cell surface. Doxorubicin was covalently attached to the cephalosporin nucleus via a carbamate linkage. This was accomplished by condensation of the amino sugar moiety of doxorubicin with cephem 3'-*p*-nitrophenyl carbonate (**10**).

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 non-targeted tissues. An alternative two-step approach utilizes a bifunctional antibody (a MoAb with affinity for both a tumor antigen and a hapten) and a hapten which is a radiopharmaceutical or cytotoxic agent.[2] We have reported [3] an alternative two-step approach [4] 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 in **Figure 1**. This approach, which we term Antibody-directed

Dedicated to Professor Edward C. Taylor on the occasion of his 70th birthday.

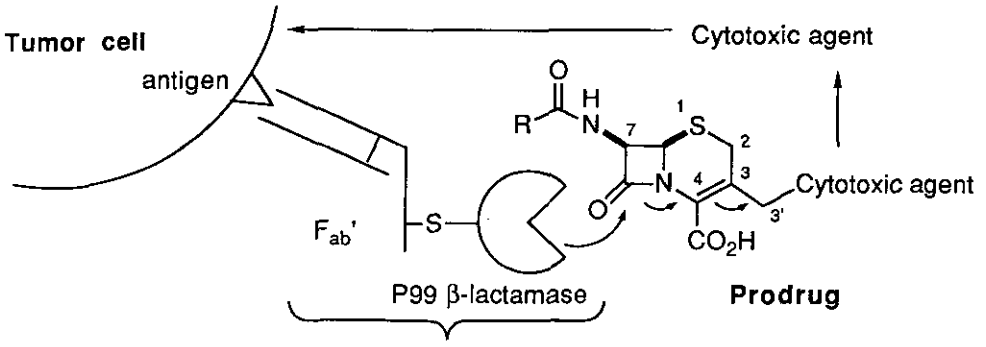


Figure 1. Antibody Directed Catalysis (ADC) Concept

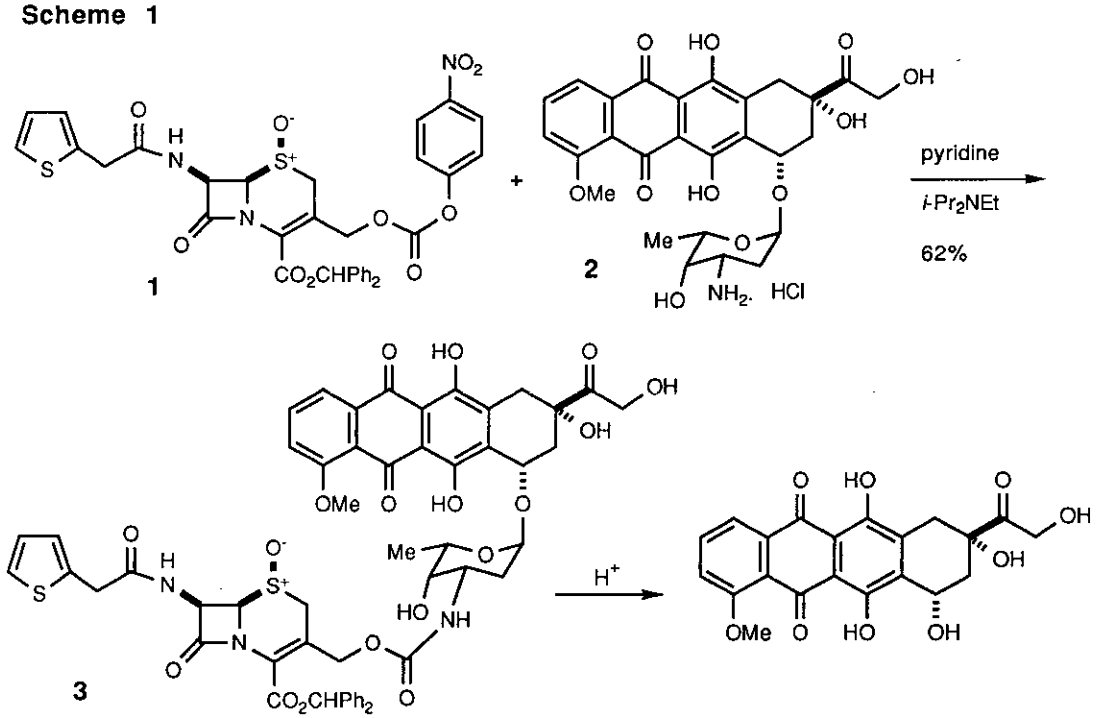
catalysis or ADC, has advantages over the other two systems. The catalytic nature of the immunoconjugate allows delivery of many drug molecules to a tumor cell utilizing a single MoAb-enzyme molecule. Thus lower conjugate doses may be used, and target antigens present in relatively low copy number may 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.

The covalent attachment of the P99 β-lactamase to the Fab' fragment of antibody CEM231, which recognizes carcinoembryonic antigen (CEA), has been described previously.[3a,5] This β-lactamase-Fab' conjugate retains the same level of immunoreactivity and enzymatic activity as the parent proteins.[5] The viability of the ADC concept was demonstrated *in vitro* and *in vivo* utilizing a cephem-vinca alkaloid prodrug and the MoAb-β-lactamase conjugate.[3] The exciting *in vivo* efficacy exhibited by the desacetylvinblastine hydrazide based prodrug LY266070 [3b] in mouse xenograft models against solid tumors have prompted us to explore the feasibility of synthesizing cephem based prodrugs of other efficacious cytotoxic agents. Herein, we report on the synthesis of a new prodrug derived from cephalothin and the potent cytotoxic agent doxorubicin (**2**).

Our earlier results with vinca alkaloid based prodrugs prompted us to select cephem sulfoxide (**12**) (Scheme 3) as our target doxorubicin prodrug. It is known that a carbamate moiety at the C-3' position of the cephem nucleus serves as a good leaving group upon enzyme-catalyzed hydrolysis of the β-lactam. Furthermore, the literature suggested that acylation of the amino sugar moiety on doxorubicin usually provided analogues with reduced cytotoxicity relative to the parent drug.[6]

We envisioned a straightforward synthesis analogous to that employed for our desacetylvinblastine hydrazide prodrug LY266070.[3b] Thus, condensation of *p*-nitrophenyl carbonate (**1**) with doxorubicin hydrochloride (**2**) in pyridine provided the desired carbamate (**3**) in good yield (Scheme 1). Unfortunately *N*-acylation of the amino sugar renders the glycosidic linkage particularly acid labile. We tried a variety of acidic reaction conditions and were not able to remove the benzhydryl ester without concomitant hydrolysis of the glycosidic linkage.[7]

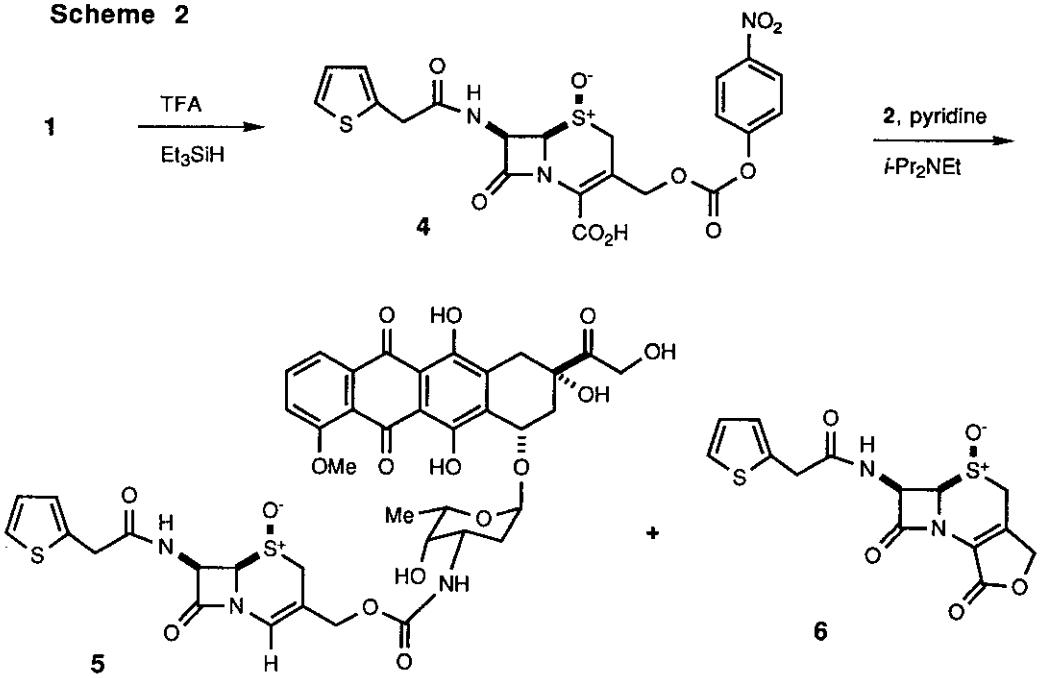
Scheme 1



Alternatively, we decided to perform the ester hydrolysis first and then attempt coupling the free acid (**4**) to doxorubicin. We assumed the success of this approach would depend on the relative rates of two competing reactions, nucleophilic displacement of the *p*-nitrophenyl carbonate vs. lactone formation (Scheme 2). In practice, coupling of free acid (**4**) with doxorubicin, under the conditions described above, gave a mixture of products. Lactone (**6**) was obtained in variable amounts along with only one product which contained the elements of the cephem nucleus and doxorubicin, as observed by nmr. Mass spectral characterization of this

product revealed a parent ion which was fourty four mass units less than expected for the desired product (**12**). Proton and carbon nmr ultimately revealed the presence of a cephem C-4 methine proton and an extra methine carbon.[8] These data are consistent with the descarboxycephem analogue (**5**). There is literature precedent for the decarboxylation of cephem sulfoxides under similar reaction conditions, involving an apparent equilibration of Δ-2 and Δ-3 olefin isomers.[9]

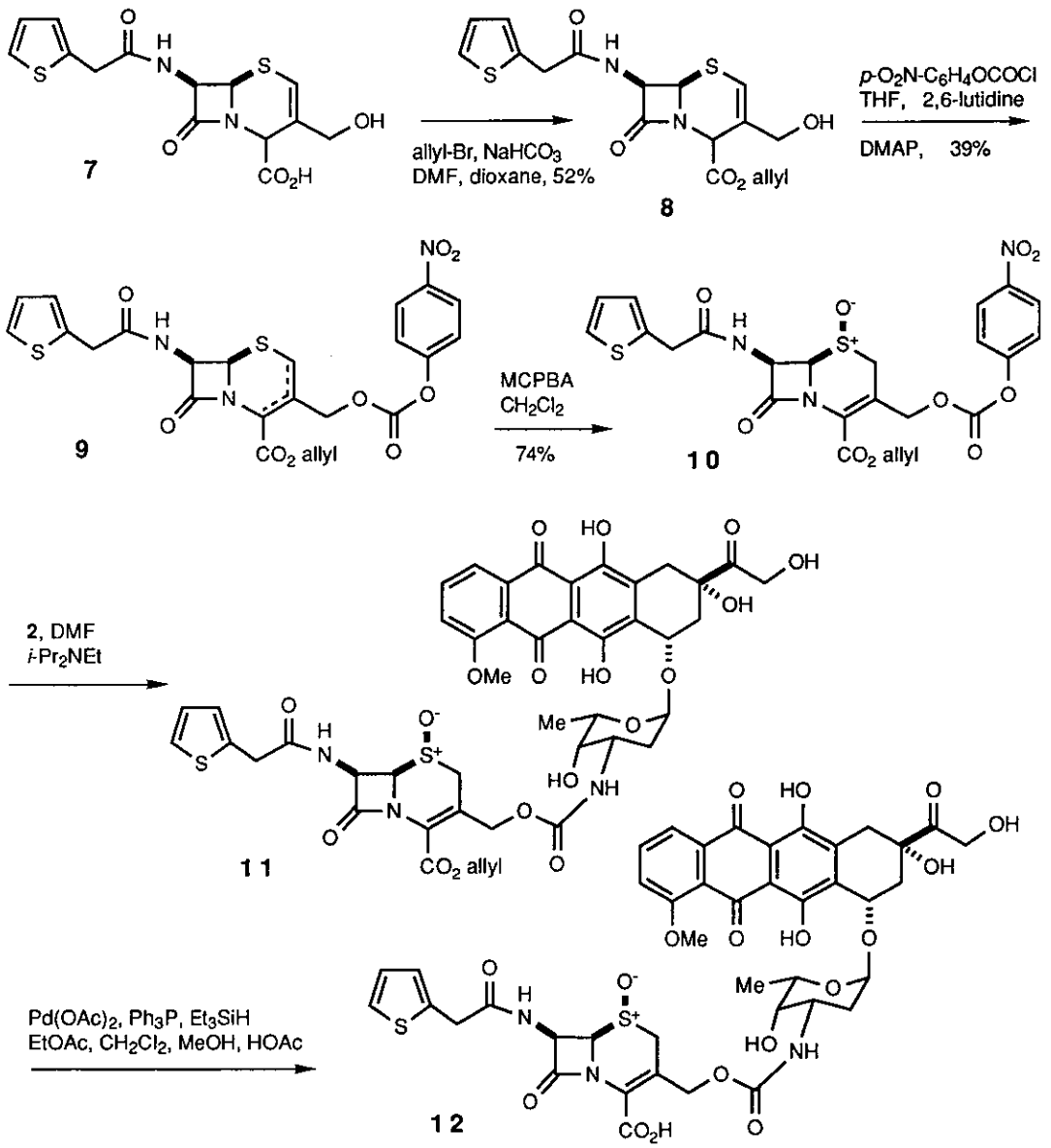
Scheme 2



It was apparent that we required a carboxylic acid protecting group that could be readily removed under neutral conditions. Prior experience suggested the use of allyl esters in this situation, thus, an efficient synthesis of key intermediate (**10**) was required. Esterification of Δ-2 cephem (**7**) with allyl bromide provided the desired ester (**8**) in good yield (Scheme 3). Acylation of the C-3' hydroxyl group with *p*-nitrophenyl chloroformate provided the cephem carbonate (**9**) as a mixture of olefin isomers. Oxidation with MCPBA provided the key intermediate Δ-3-cephem-1-sulfoxide (**10**). Condensation of carbonate (**10**) with doxorubicin hydrochloride

(2) provided the desired carbamate (11) in good yield. Palladium (0) catalyzed hydrolysis[10] of the allyl ester followed by reverse phase hplc purification provided the long-sought-after prodrug (12).

Scheme 3



Prodrug (**12**) is a good substrate for β-lactamase and release of free doxorubicin is observed upon incubation with the enzyme[11]. A MoAb-enzyme immunoconjugate bound to human tumor cells catalyzes the release of free doxorubicin *in vitro*, thus establishing (**12**) as a viable candidate for use in ADC[12]. Details of the preclinical evaluation of prodrug (**12**) will be published in due course.[13]

EXPERIMENTAL SECTION

General Procedure.

All reactions were run under a positive pressure of dry nitrogen. Fast atom bombardment mass spectra (FABms) were obtained on a VG ZAB-3 instrument. Nmr spectra were obtained on a GE QE300 instrument. Flash chromatography was carried out on E. Merck Kieselgel 60 (230-400 mesh). Doxorubicin was obtained from Meiji Seika Kaisha LTD., Tokyo, Japan.

Allyl 7-β-[2-(Thien-2-yl)acetamido]-3-hydroxymethyl-2-cephem-4-carboxylate (8).

To a solution of 7-β-[2-(thien-2-yl)acetamido]-3-hydroxymethyl-2-cephem-4-carboxylic acid (**7**)[14] (17.5 g, 49.5 mmol) in DMF (250 ml) and dioxane (150 ml) was added sodium bicarbonate (4.5 g, 53.6 mmol) followed by allyl bromide (5.9 ml, 68.3 mmol). The resulting solution was refluxed for 1 h, cooled to room temperature, then partitioned between EtOAc (500 ml) and brine (500 ml). The organic phase was washed several times with water and brine, then dried (Na_2SO_4), and concentrated in vacuo to give 10.2 g (52%) of the title compound (**8**) as a brown oil which was used in the next step without further purification. An analytical sample was obtained by crystallization of the crude product from a mixture of EtOAc/CH_2Cl_2/hexanes to give a white solid. Nmr ($CDCl_3$) δ 7.27 (m, 1H); 7.00 (m, 2H); 6.38 (d, 1H, J=9); 6.28 (s, 1H); 5.90 (m, 1H); 5.65 (dd, 1H, J=4,9); 5.30 (m, 3H); 5.08 (s, 1H); 4.66 (d, 2H, J=6); 4.20 (ABq, 2H, J=13); 3.85 (s, 2H). Ir ($CHCl_3$) 1779, 1750, 1684, 1510 cm^{-1}. Uv (EtOH) λ_{max} 234 (ε=14,384), 201 (ε=15,766). FABms Calcd for $C_{17}H_{18}N_2O_5S_2Li$: 401.0817. Found: 401.0844. Anal. Calcd for $C_{17}H_{18}N_2O_5S_2$: C, 51.76; H, 4.50; N, 7.10. Found: C, 51.50; H, 4.56; N, 6.93.

Allyl 7-β-[2-(Thien-2-yl)acetamido]-3-[[[(4-nitrophenoxy)carbonyl]oxy]methyl]-2-cephem-4-carboxylate (9).

To a 0°C solution of hydroxymethylcephem (**8**) (10.2 g, 25.8 mmol) in dry THF (50 ml) was added 2,6-lutidine (4.8 ml, 41.3 mmol), *p*-nitrophenyl chloroformate (8.50 g, 41.3 mmol) followed by DMAP (100 mg,

0.8 mmol). The resulting solution was allowed to warm to room temperature then stirred an additional 30 min. 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 5.66 g (39%) of carbonate (**9**) as a yellow gum. This material was a mixture of olefin isomers as observed by nmr. An analytical sample was obtained by performing a second flash chromatography (5% EtOAc in CH_2Cl_2), 0.28 g of the mixture gave 0.08 g of pure Δ-2 isomer as a colorless oil. Nmr ($CDCl_3$) δ 8.28 (d, 2H, J=9); 7.38 (d, 2H, J=9); 7.25 (m, 1H); 7.01 (m, 2H); 6.56 (s, 1H); 6.32 (m, 1H); 5.91 (m,1H); 5.68 (dd, 1H, J=4,9); 5.33 (m, 3H); 5.09 (s, 1H); 4.92 (d, 1H, J=12); 4.77 (d, 1H, J=12); 4.68 (d, 2H, J=7); 3.86 (s, 2H). Ir ($CHCl_3$) 1779, 1730, 1685, 1510 cm^{-1}. Uv (EtOH) λ_{max} 239(ε=17,365), 201(ε=24,226). FABms Calcd for $C_{24}H_{21}N_3O_9S_2Li$: 566.0879. Found: 566.0870. Anal. Calcd for $C_{24}H_{21}N_3O_9S_2$: C, 51.51; H, 3.78; N, 7.51; S, 11.46. Found: C, 51.72; H, 3.88; N, 7.27; S, 11.22.

Allyl 7-β-[2-(Thien-2-yl)acetamido]-3-[[[(4-nitrophenoxy)carbonyl]oxy]methyl]-2-cephem-4-carboxylate 1β-Sulfoxide (10).

To a 0°C solution of cephem (**9**) (5.66 g, 10.1 mmol, mixture of olefin isomers) in CH_2Cl_2 (225 ml) was added dropwise over 15 min a solution of 55% m-CPBA (3.23 g, 10.3 mmol eq.) in CH_2Cl_2 (50 ml). After an additional 10 min at 0° the solvent was removed *in vacuo* and the residue triturated with MeOH to give 4.24 g, (74%) of the 1-β-sulfoxide (**10**) as a tan solid. Nmr (DMSO- d_6) δ 8.48 (d, 1H, J=8); 8.29 (d, 2H, J=9); 7.52 (d, 2H, J=9)); 7.33 (m, 1H); 6.92 (m, 2H); 5.90 (m, 2H); 5.41-5.20 (m, 3H); 4.91 (d, 1H, J=4); 4.87 (d, 1H, J=13); 4.74 (d, 2H, J=5); 4.04 (d, 1H, J=18); 3.82 (ABq, 2H, J=15); 3.66 (d, 1H, J=18). Ir ($CHCl_3$) 1808, 1772, 1733, 1689 cm^{-1}. Uv (EtOH) λ_{max} 400(ε=688), 332(ε=2,394), 325(ε=2,353), 270(ε=17,094), 239(ε=14,438), 201(ε=28,842). Anal. Calcd for $C_{24}H_{21}N_3O_{10}S_2$: C, 50.08; H, 3.68 N, 7.30. Found: C, 50.02; H, 3.63 N, 7.04.

Reaction of Cephem-3'-carbonate (10) with Doxorubicin. Preparation of Carbamate (11).

To a slurry of doxorubicin hydrochloride (0.87 g, 1.5 mmol) in Aldrich sure seal DMF (5 ml) was added cephem (**10**) (0.87 g, 1.51 mmol) followed by *i*-Pr_2NEt (0.3 ml, 1.7 mmol). This mixture was stirred while being protected from light for 2.5 h. Ether (200 ml) was added to the mixture and the red solid (1.29 g) which precipitated was typically utilized in the next step without further purification. Hplc analysis indicated that approximately 90% of this material was the desired carbamate (**11**) (Waters C18 μbondapak, 8x100mm, 35%

MeOH/35% MeCN/ 0.5% NH_4OAc in water, flow rate 2 ml/min, retention time 3.6 min). An analytical sample was obtained from chromatography on a Chromatotron (1 micron plate), 130 mg of the crude red solid was eluted with 6% MeOH in CH_2Cl_2 to give 29 mg of the desired carbamate as a red solid. Nmr (DMSO-d_6) δ 13.90 (s, 1H); 13.20 (s,1H); 8.35 (d, 1H, J=8); 7.84 (m, 2H); 7.56 (m, 1H); 7.31 (d, 1H, J=4); 6.90 (m, 3H); 5.90-5.75 (m, 1H) with overlapping 5.76 (dd, 1H, J=5,9); 5.32 (m, 1H); 5.16 (m, 2H); 5.02 (d, 1H, J=13); 4.80 (m, 2H); 4.65 (m, 2H); 4.53 (br s, 2H); 4.44 (d, 1H, J=13); 4.10 (d, 1H, J=5); 3.92 (s, 3H); 3.88-3.70 (m, 4H); 3.62 (m, 1H); 3.50-3.10 (m, 2H) with overlapping 3.28 (s, 2H); 2.83 (ABq, 2H, J=18); 2.20-2.00 (m, 2H); 1.76 (m, 1H); 1.40 (m, 1H); 1.08 (d, 3H, J=6). Ir ($CHCl_3$) 1806, 1724, 1700, 1610 cm^{-1}. Uv (EtOH) λ_{max} 532(ε=4,274), 496(ε=7,857), 480(ε=7,839), 349(ε=1,467), 251(ε=21,091), 234(ε=30,361). FABms Calcd for $C_{45}H_{45}N_3O_{18}S_2Li$: 986.2300. Found: 986.2308. Anal. Calcd for $C_{45}H_{45}N_3O_{18}S_2$: C, 55.15; H, 4.63 N, 4.29. Found: C, 55.07; H, 4.51 N, 4.52.

Deblocking of Allyl Ester (11). Preparation of Cephalosporin-doxorubicin Prodrug (12). Palladium acetate (0.04 g, 0.18 mmol) and triphenylphosphine (0.2 g, 0.76 mmol) were slurried in EtOAc (1 ml) and sonicated for 1 min to give a white solid suspended in EtOAc. EtOAc (50 ml), MeOH (25 ml), and HOAc (0.75 ml) were added to the catalyst mixture. Crude cephem carbamate (**11**) (1.36 g, 1.39 mmol) was dissolved in 125 ml of 10% MeOH/ CH_2Cl_2 . This solution was added to the above prepared suspension along with Et_3SiH (0.35 ml, 2.2 mmol). The resulting mixture was stirred for 4 h, at which time the reaction was only partially complete. Additional palladium acetate, triphenylphosphine, and Et_3SiH were added in the same portions as before and stirring was continued overnight. The reaction mixture was concentrated in vacuo to give 1.4 g of crude (**12**) as a red solid. This material was purifed in aliquots by reverse phase hplc. Two 25x100 mm columns (Waters C18 μbondapak) were connected in series and eluted with 30% MeCN/0.1% TFA/ water. Flow rate was 8 ml/min and the eluant was monitored at 480 nm. After 25 min the amount of MeCN was increased to 40%. Under these conditions the product eluted after about 55 min. In a typical run, 76 mg of crude material was chromatographed and fractions of >95% analytical purity (Waters C18 μbondapak, 8x100mm, 35% MeOH/35% MeCN/ 0.5% NH_4OAc in water, flow rate 2 ml/min, retention time 6.2 min, under these conditions doxorubicin elutes in 3.3 min) were combined and freeze dried to give 24 mg of the desired prodrug (**12**) as a red solid. Nmr (DMSO-d_6) δ 14.00 (s, 1H); 13.20 (s,1H); 8.33 (d, 1H, J=8); 7.91 (d, 2H, J=4); 7.64 (t, 1H, J=5); 7.35 (m, 1H); 6.91 (m, 3H); 5.71 (dd, 1H, J=5,8); 5.43 (s, 1H); 5.19 (m, 1H); 5.05 (m, 1H);

4.92 (m, 1H); 4.82 (m, 2H); 4.70 (d, 1H, J=5); 4.55 (d, 2H, J=6); 4.46 (m, 1H); 4.12 (d, 1H, J=9); 3.97 (s, 3H); 3.80 (ABq, 2H, J=15); HOD obstructs the spectrum from 3.40-3.00; 2.96 (m, 2H); 2.12 (m, 2H); 1.82 (m, 1H); 1.43 (m, 1H); 1.09 (d, 3H, J=6). Ir ($CHCl_3$) 1780 1724, cm^{-1}. Uv (EtOH) λ_{max} 532(ε=4,274), 496(ε=7,857), 480(ε=7,839), 349(ε=1,467), 251(ε=21,091), 234(ε=30,361). FABms Calcd for $C_{42}H_{41}N_3O_{18}S_2Li$: 946.1987. Found: 946.1955.

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7. The successful acid catalyzed removal of the benzhydryl moiety from the analogous non-oxidized cephalosporin, in 15% yield, has recently been reported by the group from Bristol-Myers Squibb: European Patent Application 0 484 870 A2.
8. In $CDCl_3$ the nmr spectra of (**5**) and (**12**) are quite similar, in DMSO-d_6 the C-4 methine proton of compound (**5**) is found at δ 7.08 (s, 1H) and an NOE was observed between this proton and the C-3' methylene protons. FABms for (**5**): Calcd for $C_{41}H_{41}N_3O_{16}S_2Li$: 902.2088. Found: 902.2120. Analytical hplc: Waters C18 µbondapak, 8x100mm, 40% MeCN/ 0.5% NH_4OAc in water, flow rate 2 ml/min, retention time 6.4 min, under these conditions doxorubicin elutes in 2.9 min.

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11. D. Meyer and S. Mikolajczyk (Hybritech Incorporated, San Diego) unpublished observations.

12. J. Starling and K. Law (Lilly Research Laboratories) unpublished observations.

13. We wish to acknowledge the efforts of S. Mikolajczyk (Hybritech Incorporated, San Diego) for his assistance in working out successful conditions for the purification of compound (**12**) and the physical chemistry department at Lilly, especially J. Paschal and T. Elsey for spectral characterization and structural assignments for compounds (**5**) and (**12**).

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