Cephalosporin Derivatives of Doxorubicin as Prodrugs for Activation by Monoclonal Antibody–β-Lactamase Conjugates

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The synthesis of a series of cephalosporin doxorubicin derivatives that differ with respect to the substituent at position 7 of the cephem nucleus is described. These compounds are designed as prodrugs of doxorubicin for activation by monoclonal antibody–β-lactamase conjugates. The key step in the synthesis of this series of compounds involves the use of the phenylacetamido group as an enzymatically removable protecting group for the 7-amino group on the cephem. In vitro cytotoxicity assays with H2981 lung adenocarcinoma cells revealed that cephalosporin doxorubicin derivatives were all less toxic than the released drug. Prodrugs containing negatively charged groups in the side chain, such as the δ-carboxybutanamido derivative **4** and the α-sulfophenylacetyl derivative **5**, displayed the least cytotoxic activity and were 46- and 26-fold less toxic than doxorubicin, respectively. The efficiency of activation of all the prodrugs was evaluated in cytotoxicity assays on H2981 cells with the β-lactamases from *Enterobacter cloacae* P99, *Escherichia coli* TEM-1, and *Bacillus cereus* (type II). In general, the *E. cloacae* enzyme was found to most rapidly activate the majority of these prodrugs. Phenylacetamido prodrug **2** and δ-carboxybutanamido prodrug **4** were both activated in an immunospecific manner by L6–*E. cloacae* β-lactamase, a monoclonal antibody conjugate that binds to receptors on H2981 lung adenocarcinoma cells.

Introduction

Recent research in several laboratories has involved the use of monoclonal antibody (mAb)–enzyme conjugates for the selective activation of antitumor prodrugs.[1] This is a two-step approach to cancer chemotherapy in which a mAb–enzyme conjugate is given systemically and binds in a selective manner to tumor-associated antigens. In the second step, an anticancer prodrug that undergoes activation by the targeted enzyme is administered. Several groups have demonstrated that this drug-targeting strategy can lead to significant levels of antitumor activity in vitro and in vivo.[1,2]

β-Lactamases offer considerable potential for prodrug activation. These microbial enzymes are involved in the hydrolysis of the β-lactam rings of penicillins and cephalosporins. A number of reports have demonstrated that β-lactam hydrolysis can initiate a fragmentation reaction leading to the release of cytotoxic drugs such as doxorubicin (**1**) from appropriately substituted cephalosporins (Scheme 1). Thus, it has been possible to use β-lactamase for the release of peptide antibiotics,[3] vinca alkaloids,[2d] nitrogen mustards,[4] and doxorubicin.[5]

Detailed structure–activity studies have demonstrated that the potencies, spectrum of activities, and β-lactamase reactivities of cephalosporins are influenced by the nature of the substituent at the 7-position.[6] It is quite likely that this position will also have an effect on the properties of cephalosporin-containing anticancer prodrugs. Indeed, this is supported by recent studies which show that the enzymatically catalyzed hydrolysis rates of 7-(phenylacetamido) and 7-(carboxybutanamido)cephalosporin nitrogen mustards were quite different.[4c] Kinetic differences may become significant in vivo as suggested by a mathematical model in which the amount of intratumorally generated drug is dependent on the K_m and V_{max} of the enzyme.[7] Thus, there is a need for a simple synthetic methodology that can be used to obtain a variety of 7-substituted cephalosporin drug derivatives.

In this paper, we describe the preparation of cephalosporin doxorubicin derivatives which differ at the 7-position of the bicyclic cephem ring. The key step in the synthesis involves the use of penicillin-G amidase for the hydrolysis of 7-(phenylacetamido)cephalosporin doxorubicin[5a] to form the corresponding 7-aminocephalosporin derivative. This amine is then used as a common intermediate to produce a wide range of other cephalosporin derivatives which differ in their substrate profiles and in vitro cytotoxic activities.

Results and Discussion

Synthesis. The known 7-(phenylacetamido)doxorubicin derivative **2**[5a] shown in Scheme 1 served as the starting material for the preparation of a variety of cephalosporin doxorubicin derivatives. It was possible to enzymatically hydrolyze the 7-phenylacetamido group of **2** in a selective manner using penicillin-G amidase as a catalyst. This resulted in the formation of **3** in yields of at least 70% after purification by reversed phase chromatography. This purification, however, was not required when **3** was used as an intermediate to generate other acylated derivatives, such as **4**–**7**.

Amide derivatives were prepared by reacting *in situ* generated **3** with glutaric anhydride, α-sulfophenylacetyl chloride,[8] and 2-thienylacetyl chloride to produce **4**–**6**, respectively (Scheme 1). Compound **5** proved to be a diastereomeric mixture, since racemic α-sulfophenylacetyl chloride was used in the synthesis. Bromoacetyl chloride also reacted with the amino group on **3**, forming an activated intermediate that was further elaborated through its reaction with thioglucose to give **7**. Thus, a wide variety of 7-substituted cephalosporin derivatives can be made through selective enzymatic

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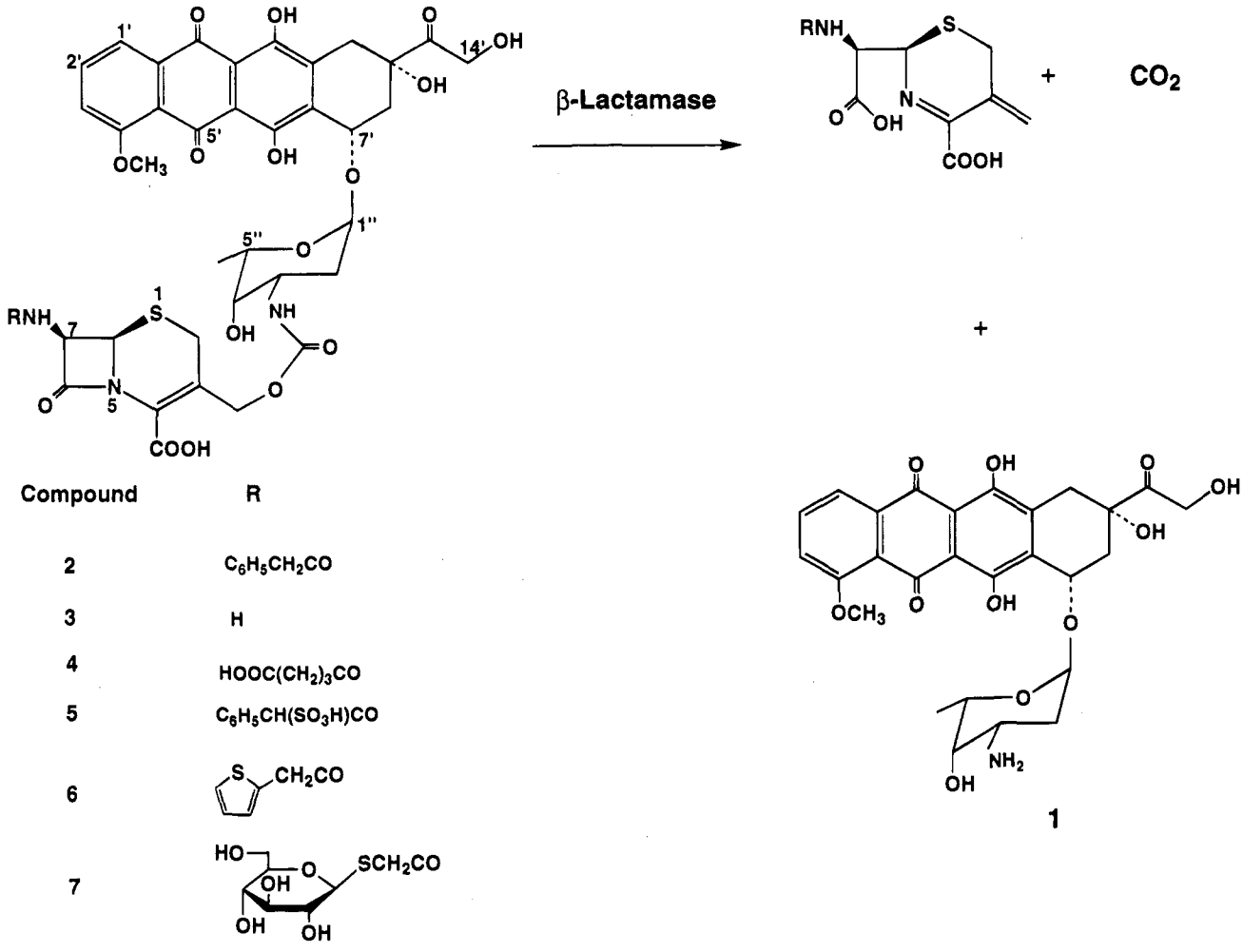
Scheme 1



Compound	R
2	$C_6H_5CH_2CO$
3	H
4	$HOOC(CH_2)_3CO$
5	$C_6H_5CH(SO_3H)CO$
6	
7	

hydrolysis followed by acylation of the cephem 7-amino group. These steps do not result in any perceptible modifications in the appended doxorubicin.

Cytotoxic Activities. Studies were undertaken to determine if **2**–**7** were cytotoxic to tumor cells and if β-lactamase affected their activities. The cytotoxic effects of **2**–**7** both in the presence and absence of *Enterobacter cloacae* P99 β-lactamase (EClbL) were tested on the human lung adenocarcinoma cell line H2981. This particular enzyme was used because of its high level of activity with a wide range of cephalosporin derivatives.[4,5] After exposing cells to the drugs alone or to drug/EClbL combinations, the cytotoxic effects were determined by measuring the inhibition of [^{3}H]-thymidine incorporation into DNA. Compounds **2**–**7** were all significantly less toxic to cells than doxorubicin (**1**) and could be activated in the presence of EClbL (Table 1). A slight variation in the IC_{50} value for **1** took place between different experiments. For that reason, the cytotoxic effects of each prodrug and **1** are shown in Table 1 for the same experiment. Independently run HPLC assays established that β-lactamase catalyzed hydrolyses of **2**–**7** and led to the formation of **1** (data not shown). Thus, **2**–**7** are prodrugs of doxorubicin that can be activated by β-lactamase.

Significant differences in IC_{50} values were obtained for the prodrugs (Table 1). The two least toxic prodrugs were **4** and **5**, which were 46 and 26 times less cytotoxic than **1**, respectively. Both of these prodrugs contain negatively charged side chain residues which may play a role in reducing cellular uptake. The only prodrug that appeared to be more cytotoxic than the parent prodrug **2** was the glucose-containing derivative **7**. These results establish that the side chain has an influence on prodrug cytotoxicity.

Table 1. Cytotoxic Effects of Doxorubicin Derivatives on H2981 Human Lung Adenocarcinoma Cells[a]

	IC_{50}[b] (μM)		
compd	without EClbL	with EClbL (10 μg/mL)	doxorubicin[c]
2	7.4 ± 1.2	0.75 ± 0.17[d]	0.55 ± 0.25
3	8.2 ± 2.4	0.43 ± 0.06	0.46 ± 0.25
4	21.9 ± 11.6	0.47 ± 0.21	0.48 ± 0.25
5	14.8 ± 3.4	0.47 ± 0.05	0.56 ± 0.35
6	10.3 ± 2.2	0.55 ± 0.11	0.56 ± 0.31
7	2.5 ± 0.61	0.45 ± 0.13	0.46 ± 0.29

[a] Cells were exposed to the drugs ±EClbL for 1 h and washed, and the cytotoxic effects were compared to doxorubicin in the same experiment. [b] Drug concentration resulting in 50% incorporation of [^{3}H]thymidine compared to untreated control cells. The values represent the averages of at least three experiments except as noted. [c] Overall IC_{50} for **1** = 0.50 ± 0.24 μM. [d] This experiment was done twice.

Kinetics of Prodrug Activation. Experiments were performed to determine how much of a particular enzyme was required to obtain an IC_{50} value with H2981 cells in the presence of the prodrugs at 1 μM. This concentration was chosen since it is well below the IC_{50} value of the prodrugs but above that of **1** (Table 1). The enzymes used were EClbL, *Escherichia coli* TEM-1 β-lactamase (ECbL), and *Bacillus cereus* β-lac-

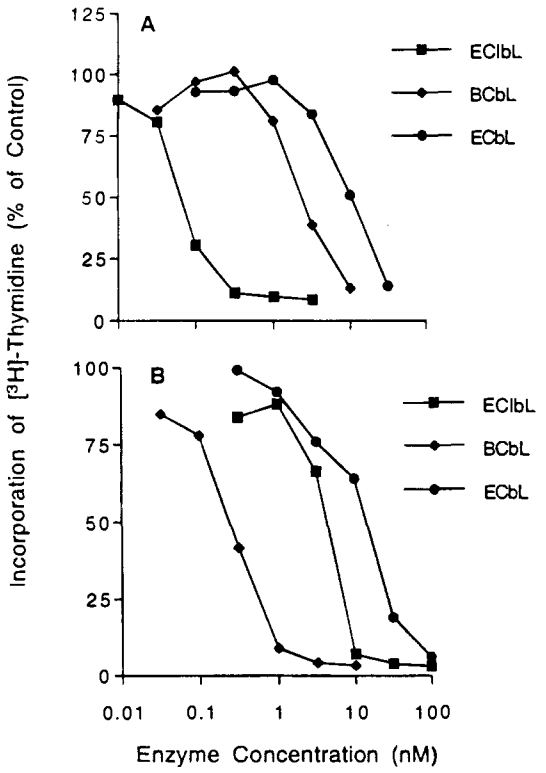


Figure 1. Effect of enzyme concentration on the cytotoxic activities of prodrugs 4 (A) and **5** (B) at 1 μM.

Table 2. Enzyme Concentrations Required To Achieve an IC_{50} Value on H2981 Lung Adenocarcinoma Cells[a]

	enzyme (nM)		
substrate	EClbL	BCbL	ECbL
2	0.012 ± 0.001	0.25 ± 0.13	0.68 ± 0.37
3	9.86 ± 2.1	24.9 ± 8.5	185 ± 29.9
4	0.039 ± 0.020	1.13 ± 0.48	6.5 ± 3.0
5	5.65 ± 1.53	0.39 ± 0.21	22.7 ± 11.9[b]
6	0.024 ± 0.01	0.21 ± 0.046	0.25 ± 0.098
7	0.13 ± 0.12	1.58 ± 0.61	1.13 ± 0.44

[a] Cells were exposed to the substrates at 1 μM, and various amounts of the enzymes were added. After 1 h, the cells were washed, and incubation was continued. IC_{50} values were determined from the cellular incorporation of [^{3}H]thymidine. The values represent the averages of at least three experiments. [b] This experiment was done twice.

tamase (type II) (BCbL). Figure 1 illustrates the results obtained using **4** and **5** as enzyme substrates. Under the conditions used, the concentration of EClbL required to achieve an IC_{50} with prodrug **4** as substrate (Figure 1A) was lower than that of both BCbL and ECbL. These results are in contrast with the observations made with **5** as the enzyme substrate (Figure 1B), in which the BCbL was apparently the most active of the enzymes tested.

The summary of the results obtained using all of the substrates in combination with the three enzymes is shown in Table 2. The results reveal large differences between the enzyme/prodrug combinations. For example, 470 times as much EClbL is required to achieve an IC_{50} value for **5** compared to **2**. This would indicate that in settings where enzyme is limiting much more doxorubicin would be generated from **2** compared to **5** with EClbL as catalyst. Under these conditions it would also be expected that the rate of hydrolysis of **2** would be approximately 21 times higher with EClbL compared to BCbL. In general, it appears that the EClbL enzyme

Table 3. Kinetic Constants for β-Lactamases[a]

	substrate			
	2		**4**	
enzyme	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})
EClbL	46 ± 11	280 ± 67	83 ± 26	210 ± 39
BCbL	38 ± 7.8	30 ± 3.0	56 ± 12	6.6 ± 0.55
ECbL	25 ± 4.4	8.4 ± 0.54	73 ± 11	2.8 ± 0.31

[a] The values represent the average of at least three separate determinations.

is more effective than the other two enzymes at prodrug conversion (with the only exception being with **5** as substrate) and that prodrugs **2**, **4**, and **6** represent the best overall enzyme substrates.

Studies were undertaken to determine if the amount of enzyme needed to achieve a prodrug IC_{50} value correlated with the K_m and k_{cat} values. This correlation was made with all three enzymes with two representative prodrugs, **2** and **4** (Table 3). The highest k_{cat}/K_m ratios and absolute turnover numbers for both substrates were obtained using EClbL. This is in agreement with the data shown in Table 2, in that less EClbL is required than the other enzymes for the activation of both **2** and **4**. It is worth noting that the same trend was observed for the nitrogen mustard analogues of **2** and **4**.[4c]

Immunologically Specific Prodrug Activation. In vitro cytotoxicity assays using mAb–EClbL conjugates were performed in order to demonstrate the utility of the prodrugs **2** and **4**, the two prodrugs that appeared to have the most favorable kinetic and cytotoxic properties. The conjugates were prepared by attaching approximately two F(ab') fragments of the L6 and P1.17 mAbs to each EClbL molecule as previously described.[9] As before, the enzyme activity and binding properties of the conjugates were preserved.

H2981 cells (L6 antigen positive, P1.17 antigen negative) were exposed to **1**, **2**, or **4** for 1 h, washed, and incubated for another 18 h before being pulsed with [^{3}H]-thymidine. The cytotoxic effects were compared to cells that were treated with the mAb–EClbL conjugates (10 nM) prior to exposure to **2** and **4**. The cytotoxic effects of both **2** (Figure 2A) and **4** (Figure 2B) were greatly enhanced on cells that were pretreated with L6–EClbL. The activation was shown to be immunologically specific, since P1.17–EClbL did not enhance prodrug activity and unconjugated L6 at saturating amounts was able to abrogate the effect of L6–EClbL.

Conclusion

One of the goals of this project was to develop a convenient method for synthesizing cephalosporin derivatives of doxorubicin that could serve as prodrugs for activation by various β-lactamases. In principle, each prodrug could be prepared starting with a cephalosporin containing the desired side chain already in place. This was deemed as being too cumbersome, since separate synthetic pathways would be required for each compound produced. Having a common intermediate that is late in the synthetic pathway was considered to be a simpler strategy. The finding that penicillin-G amidase could convert **2** to **3** without affecting the doxorubicin moiety was a key step in the development of the prodrugs described here. The results described complement other studies with penicillin-G amidase for the

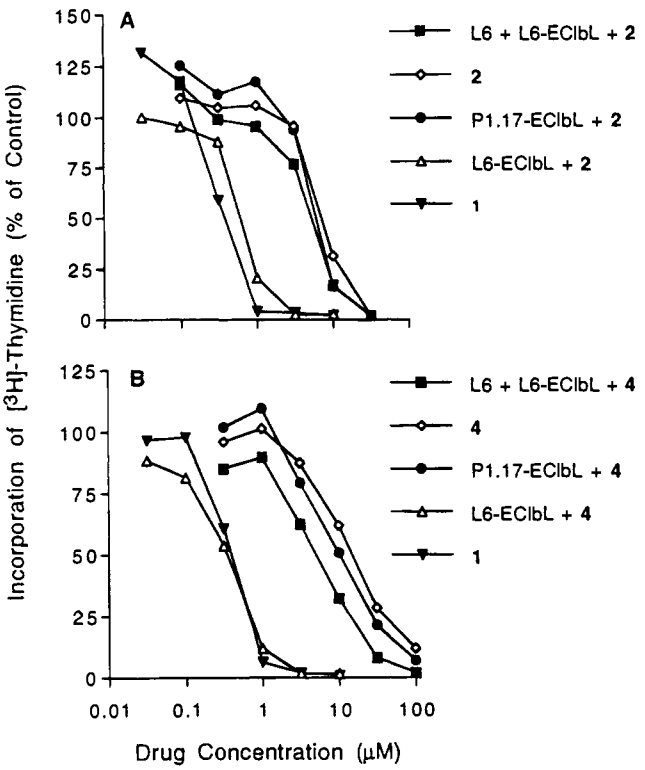


Figure 2. Immunologically specific activation of **2** (A) and **4** (B). H2981 cells (L6 antigen positive, P1.17 antigen negative) were treated with the mAb–EClbL conjugates and washed prior to treatment with prodrugs.

selective deprotection of amines.[10] This synthetic methodology is in keeping with recently reported synthetic trends in which multistep organic reactions are carried out in aqueous solvents using enzymes as catalyst.[11]

This work was initiated with the notion that the cephalosporin side chain might influence prodrug cytotoxicity. Indeed, this appears to be the case, since **4** was 46 times less cytotoxic than **1**, while **7** was only 5 times less cytotoxic. It is quite possible that other side chain modifications will further reduce prodrug cytotoxicity. Studies along these lines are currently underway. Another important consideration concerns whether the kinetics of prodrug conversion influence the amount of drug that can be generated intratumorally, as suggested by a mathematical model.[7] The prodrugs described here may function as probes to test the validity of this model, since they possess widely different kinetic parameters. Finally, in vivo therapy studies are probably warranted, since **2** and **4** are activated in an immunologically specific manner and are significantly less toxic than doxorubicin.

Experimental Section

General. Penicillin-G amidase (PGA) was obtained from Boehringer-Mannheim. Crude β-lactamases EClbL and BCbL were obtained from Sigma Chemical Co. and purified according to published procedures.[4a,c] ECbL was purified from the periplasmic space of DH5-α cells transformed with the pUC 18 vector. Osmotic shock was used to release protein from the periplasm,[12] and ECbL was then purified by boronic acid affinity chromatography as previously described.[4a] EClbL was conjugated to the L6[13] and P1.17 mAbs as previously described.[9] The human lung adenocarcinoma cell line H2981 (L6 antigen positive, P1.17 antigen negative) has previously been described.[13] CF_3COOH was distilled prior to use. CH_2Cl_2 was dried over type 3 Å molecular sieves. Fast atom bombardment (FAB) mass spectra were acquired on a Kratos MS 25 mass spectrometer, and high-resolution FAB accurate mass measurements were obtained on a Kratos MS 50 mass spectrometer. Both are equipped with a saddle field FAB gun (Ion Tech) with xenon as the primary particle source and *m*-nitrobenzyl alcohol as the matrix. NMR spectra (300 MHz) were run on Bruker AC-300 or AM-300 instruments. Chemical shifts are reported in ppm downfield from $(CH_3)_4Si$. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. TLC was performed on Whatmann silica gel plates. HPLC analyses were performed on a Hewlett Packard 1090 liquid chromatograph equipped with a UV/vis detector set at 495 nm and a spheri 5 RP18 reversed phase column (220 × 2.1 mm; Applied Biosystems). All runs were performed at a flow rate of 0.2 mL/min. A linear gradient of 30–80% CH_3CN in 50 mM triethylammonium formate buffer at pH 2.8 was employed as the eluting solvent.

3-[[(*N*-Doxorubicinylcarbonyl)oxy]methyl]-7-(phenylacetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (2). This compound was prepared according to a reported procedure[5a] with some minor modifications involving the deprotection of the diphenylmethyl protecting group. A stirred solution of the diphenylmethyl ester-protected cephalosporin derivative of doxorubicin (1.74 g, 1.12 mmol) in CH_2Cl_2 (17 mL) was cooled to 0 °C and treated with $(C_2H_5)_3SiH$ (0.766 mL). Anhydrous CF_3COOH (4.35 mL) was added in one lot to the red solution, and after 70 s, the reaction mixture was poured quickly into an ice cold vigorously stirring mixture of ethyl acetate (250 mL), ethanol (25 mL), and 0.25 M $NaHCO_3$ (260 mL). After 10 min, the layers were separated and the organic layer was washed with water (100 mL). The aqueous extracts (pH 7.8) were combined, concentrated to approximately 300 mL in vacuo, and applied to a bed of C-18 silica gel (4 cm × 5.5 cm) equilibrated with H_2O. The column was eluted with H_2O (400 mL) and then CH_3CN:H_2O (1:4, 400 mL). Fractions containing **2** and a more polar impurity were combined, evaporated to a 50 mL volume, and applied to another column of C-18 silica gel (2 × 24 cm). The column was eluted with H_2O (100 mL) and then CH_3CN:H_2O (1:4, 400 mL). Fractions containing **2** were combined and lyophilized to give 350 mg (33% yield) of a fine red solid. The compound thus obtained was pure by HPLC (t_R = 14.1 min) and displayed the reported spectral characteristics.[5a] Anal. ($C_{44}H_{43}N_3O_{17}$-SNa·6H_2O) C, H, N, S.

3-[[(*N*-Doxorubicinylcarbonyl)oxy]methyl]-7-amino-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (3). A solution of **2** (45 mg, 0.048 mmol) in 45 mL of phosphate-buffered saline at pH 7.2 (consisting of 0.14 M NaCl, 2.7 mM KCl, 1.4 mM NaH_2PO_4, and 8 mM $Na_2HPO)_4$) was treated with PGA (0.5 mL, 1.08 mg/mL). After 1 h, TLC ($CHCl_3$:CH_3OH:HCOOH = 15:4:1) revealed completion of the reaction with the formation of a single more polar compound. The reaction mixture was applied to a C-18 column (2 cm × 15 cm) equilibrated with H_2O. After washing the column with 100 mL of H_2O, the product was eluted with CH_3CN:H_2O (1:4). Fractions 4–8 (15 mL each) were combined and lyophilized to give 27.5 mg (70% yield) of amine **3** as a red powder which was pure by HPLC (t_R = 7.5 min): MS (M + Na) = 844.1611 (calcd), 844.1631 (found); ^1H NMR (CD_3OD) δ 7.96 (d, 1 H, H-1′, $J_{1',2'}$ = 7.9 Hz), 7.72 (t, 1 H, H-2′, $J_{2',3'}$ = 7.9 Hz), 7.47 (d, 1 H, H-3′), 5.41 (d, 1 H, H-7, $J_{6,7}$ = 4.5 Hz), 5.11 (br s, 1 H, H-1″), 4.74 (m, 3 H, H-7′ and H-14′), 4.60 (d, 1 H, H-6), 4.25 (q, 1 H, H-5″, $J_{5'',6''}$ = 6.5 Hz), 4.02 (s, 3 H, $ArOCH_3$), 3.70–2.90 (m, 4 H, H-2 and H-10′), 2.40–1.65 (m three sets), 4 H, H-8′ and H-2″), 1.26 (d, 3 H, H-6″); UV_{max} (nm) (PBS) 495, 253, 232 (log ε = 3.94, 4.39, 4.51).

3-[[(*N*-Doxorubicinylcarbonyl)oxy]methyl]-7-(∂-carboxybutyramido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (4). A solution of **2** (390 mg, 0.42 mmol) in 0.1 M $NaHCO_3$ (390 mL, pH 9) was treated with 2.5 mL of PGA (1.08 mg/mL). After 45 min at 23 °C, formation of **3** was complete as determined by SiO_2 TLC ($CHCl_3$:CH_3OH:HCOOH = 8:1:1). The red solution was cooled in an ice–water bath for 10 min and then treated with 1 M glutaric anhydride in acetone (7.9 mL, 20 equiv). TLC (as above) after 15 min indicated complete conversion to a compound which was less

polar than the amine **3** and more polar than **2**. The reaction mixture was applied to a C-18 silica gel column (2.1 × 30 cm) which was equilibrated with H_2O and eluted with H_2O (200 mL) and CH_3CN:H_2O (1:9, 200 mL). The fraction obtained by elution with water was discarded. When the red-colored solution started eluting from the column, fractions of 10 mL each were collected. After analyzing by HPLC, fractions 4–10 were combined and lyophilized to give **4** (190 mg) as a red powder. Fractions 1–3 were combined and evaporated, and the residue in 20 mL water was reapplied to a C-18 column (same dimensions as above) which was equilibrated with water. The column was eluted with CH_3CN:H_2O (1:19, 200 mL) and CH_3CN:H_2O (1:9, 200 mL). Fractions were analyzed by HPLC as mentioned above, and those containing **4** were combined to give an additional 67 mg (combined yield = 64%) of 4: HPLC t_R = 9.2 min; MS (M + 2Na − 2H) = 956; ^{1}H NMR (CD_3OD) ∂ 7.90 (d, 1 H, H-1′, $J_{1',2'}$ = 7.9 Hz), 7.80 (t, 1 H, H-2′, $J_{2',3'}$ = 7.9 Hz), 7.52 (d, 1 H, H-3′), 5.55 (d, 1 H, H-7, $J_{6,7}$ = 4.5 Hz), 5.40 (d, 1 H, H-6), 5.01 (br s, 1 H, H-1″), 4.13 (q, 1 H, H-5″, $J_{5'',6''}$ = 6.5 Hz), 3.88 (s, 3 H,, $ArOCH_3$), 2.24–2.00 (m, 6 H, $COCH_2CH_2CH_2CO$ and H-8′), 2.00–1.74 (m, 4 H, $COCH_2$-CH_2CH_2CO and H-2″), 1.17 (d, 3 H, H-6″); UV_{max} (nm) (PBS) 495, 253, 232 (log ϵ = 3.98, 4.43, 4.53). Anal. ($C_{41}H_{41}N_3O_{19}$-SNa_2·$6H_2O$) C, H, N, S.

3-[[(*N*-Doxorubicinylcarbonyl)oxy]methyl]-7-(α-sulfophenylacetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (5). A solution of **2** (55 mg, 0.0586 mmol) in 0.25 M $NaHCO_3$ (55 mL) was converted to the amine **3** as in the preparation of **4** with quantities adjusted to the reaction scale. The crude amine was treated *in situ* with 1.2 equiv of an acetone solution of α-sulfophenylacetyl chloride etherate.[8] After purification by C-18 chromatography with water and CH_3CN:H_2O (1:9) as eluants, fractions containing **5** were combined and lyophilized to yield 27 mg of a red powder: HPLC t_R = 11.4 min (the diastereomers were inseparable); MS (M − Na + H) = 997; ^{1}H NMR (CD_3OD) for the major diastereomer ∂ 7.75–7.15 (m, 8 H, ArH), 5.63 (d, 1 H, H-7, $J_{6,7}$ = 4.7 Hz), 5.37 (d, 1 H, H-6), 5.07 (br s, 1 H, H-1″), 4.64 (m, 3 H, H-7′ and H-14′), 4.13 (q, 1 H, H-5″, $J_{5'',6''}$ = 6.4 Hz) 3.89 (s, 3 H, $ArOCH_3$), 1.25 (d, 3 H, H-6″, $J_{5'',6''}$ = 6.4 Hz); for the minor diastereomer (distinctly separable proton absorptions) ∂ 5.68 (d, 1H, H-7, $J_{6,7}$ = 4.8 Hz), 3.90 (s, 3H, $ArOCH_3$), 1.26 (d, 3H, H-6″, $J_{5'',6''}$ = 6.6 Hz); integration of the H-7 proton revealed that the two diastereomers were formed in a 2:7 ratio; UV_{max} (nm) (PBS) 495, 253, 232 (log ϵ = 3.90, 4.35, 4.49). Anal. ($C_{44}H_{41}N_3O_{20}S_2Na_2$·$7H_2O$) C, H, N, S.

3-[[(*N*-Doxorubicinylcarbonyl)oxy]methyl]-7-(2-thiopheneylacetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (6). PGA (0.51 mL, 1.38 mg/mL in PBS)-mediated hydrolysis of **2** (46 mg, 0.049 mmol) in 0.25 M $NaHCO_3$ followed by reaction of the product at 0 °C with 2-thiopheneacetyl chloride (0.0062 mL, 0.05 mmol) and chromatography on C-18 silica gel with H_2O, CH_3CN:H_2O (3:17), and CH_3CN:H_2O (1:4) gave **6** (33 mg, 71% yield): HPLC t_R = 13.7 min; MS (M + H) = 946.1775 (calcd), 946.1810 (found); ^{1}H NMR (DMSO-d_6) ∂ 8.98 (d, 1 H, NH, $J_{NH,7}$ = 8.5 Hz), 7.87 (m, 2 H, H-1′ and H-3′), 7.62 (m, 1 H, H-2′), 7.33 (m, 1 H, -SCH=C), 6.92 (m, 4 H, thiopheneyl =CHCH= and NH or OH), 5.43 (dd, 2 H, H-7 and OH, $J_{6,7}$ = 4.5 Hz), 5.20 (br s, 1 H, H-1″), 5.00–4.80 (m, 5 H, CH_2CONH, H-7′, H-3″, and H-4″), 4.56 (s, 2 H, H-14′), 4.13 (q, 1 H, H-5″, $J_{5'',6''}$ = 6.6 Hz), 3.96 (s, 3H, $ArOCH_3$), 3.72 (s, 2H, thiopheneylacetyl CH_2), 2.93 (m, 2 H, H-10′), 2.13 (m, 2 H, H-8′), 1.81 (m, 1 H, H-2″A), 1.45 (m, 1 H, H-2″B), 1.10 (d, 3 H, H-6″); UV_{max} (nm) (PBS) 495, 254, 232, 204 (log ϵ = 3.90, 4.37, 4.55, 4.42). Anal. ($C_{42}H_{40}N_3O_{17}S_2$-Na·$6.5H_2O$) C, S; H: calcd, 4.99; found, 4.54. N: calcd, 3.96; found, 4.39.

3-[[(*N*-Doxorubicinylcarbonyl)oxy]methyl]-7-(1-thio-β-D-glucosylacetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid (7). To a solution of **2** (120 mg, 0.128 mmol) in 0.1 M $NaHCO_3$ (120 mL) at 23 °C was added PGA (0.04 mL of 13.4 mg/mL ammonium sulfate suspension). After 1 h when TLC revealed complete reaction, the red solution was cooled on ice and $BrCH_2COCl$ (0.22 mL, 2.7 mmol) was added in three equal portions. The reaction was monitored by TLC ($CHCl_3$:CH_3OH:HCOOH = 8:1:1) 10 min after each addition. When all the amine **3** was converted to a less polar product, the reaction mixture was applied to a column of C-18 silica gel (2.1 × 30 cm) and eluted with H_2O (200 mL) followed by CH_3CN:H_2O. The entire red band was collected as a fraction which was concentrated to approximately 50 mL before adding $NaHCO_3$ (500 mg) and β-D-thioglucose sodium salt (95 mg, 0.37 mmol). The red solution was stored at ambient temperature for 1 h and then applied to a column of C-18 silica gel (2.1 × 30 cm) equilibrated with water. The fraction with H_2O (200 mL) as eluant was rejected. Subsequent elution with CH_3CN:H_2O (1:4, 200 mL) gave the desired product which was concentrated to 20 mL and lyophilized to give the sodium salt of the thioglucose derivative **7** (88 mg, 65% yield from **2**). It was subsequently found that **7** could be prepared on the same scale without the use of reversed phase chromatography after the bromoacetylation: HPLC t_R = 7.3 min; MS $(M)^+$ = 1057; ^{1}H NMR (DMSO-d_6) ∂ 8.82 (d, 1 H, NH, $J_{NH,7}$ = 9.0 Hz), 7.91 (d, 2H, Ar-H, $J_{1',2'}$ = 6.0 Hz), 7.66 (t, 1H, Ar-H, $J_{2',3'}$ = 6.0 Hz), 6.93 (d, 1H, H-6, $J_{6,7}$ = 6.0 Hz), 5.51 (s, 1 H, OH), 5.45 (dd, 1 H, H-7), 5.23 (s, 1 H, OH), 5.21 (br s, 1 H, H-1″), 5.10 (d, 1 H, OH, J = 3.4 Hz), 4.80–5.00 (m, 6H, H-3″, H-4″, H-7′, 3-CH_2OCONH, and OH or NH), 4.73 (br s, 2 H, -SCH_2-), 4.58 (d, 2 H, H-14′, $J_{14',OH}$ = 6.0 Hz), 4.53 (br s, 1 H, OH), 4.39 (d, 1 H, gluco H-1, J = 9.5 Hz), 4.16 (q, 1 H, H-5″, $J_{5'',6''}$ = 6.4 Hz), 4.00 (s, 3 H, $ArOCH_3$), 3.68 (d, 1 H, OH, J = 12 Hz), 3.18, 3.09 (2 × d, 2 H, H-2, J_{AB} = 18.0 Hz), 2.92 (m, 2 H, gluco CH_2), 2.18 (m, 1 H, H-8′), 1.84 (m, 1H, H-2″A) 1.46 (m, 1 H, H-2″B), 1.03 (d, 3H, H-6″); UV_{max} (nm) (PBS) 495, 254, 232 (log ϵ = 3.85, 4.31, 4.55, 4.45). Anal. ($C_{44}H_{48}N_3O_{22}S_2Na$·$5H_2O$) C, N, S; H: calcd, 5.50; found, 4.75.

Enzyme Kinetics. The kinetics of β-lactam hydrolysis of **2** and **4** were determined spectrophotometrically using ECbL, EClbL, and BCbL as catalysts for the reactions. Various concentrations of **2** (10.9–84.3 μM) and **4** (11.3–76.4 μM) were incubated with each of the three enzymes (0.1 μg/mL for EClbL and 1.0 μg/mL for BCbL and ECbL) at 23 °C in PBS containing 12.5 μg/mL bovine serum albumin. The enzyme concentrations were chosen to ensure linearity of the initial velocity for the duration of 1 min. The initial velocities were determined from the linear portions obtained from plots of absorbance (230 nm for **2**, $\Delta\epsilon_{230}$ = 9.3 × 10^3 M^{-1} cm^{-1}; 260 nm for **4**, $\Delta\epsilon_{260}$ = 6.7 × 10^3 M^{-1} cm^{-1}) as a function of time. The kinetic parameters calculated using direct linear plots[14] for each enzyme/substrate combination represent the mean of at least three independent experiments.

In Vitro Cytotoxicity Assays. H2981 cells in 0.1 mL of IMDM (Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum (v/v), 0.1 mg/mL streptomycin, and 60 μg/mL penicillin-G) were plated out at 4000 or 8000 cells/well in 96-well microtiter plates and allowed to adhere for 18 h at 37 °C. The cells were then washed with RPMI (Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum (v/v)), various concentrations of drugs or prodrugs were added, and incubation was continued for 1 h at 37 °C. The cells were washed with IMDM, and incubation was continued for 18 h at 37 °C. This was followed by either a 6 h pulse (if 8000 cells/well were plated out) or an 18 h pulse (if 4000 cells/well were plated out) with [^{3}H]thymidine at 1 μCi/well. The cells were washed with PBS, detached with a solution of trypsin/EDTA, harvested onto filter mats with a TOMTEC Harvester 96, and counted on an LKB WALLAC 1205 liquid scintillation counter. The incorporation of [^{3}H]-thymidine was expressed as a percentage of untreated control cells. For experiments utilizing L6–EClbL and P1.17–EClbL, cells were treated with the conjugates (10 nM mAb component) in 200 μL of RPMI media for 30–45 min at 4 °C, washed three times with 200 μL of RPMI media, and then treated with prodrug as described above.

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