culture flasks were then shaken at 37 °C at 300 rpm. Aliquots were removed every 2 h for culture turbidity measurements at 600 nm.

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Registry No. 1, 18928-01-3; 2, 1155-62-0; 4, 32159-21-0; 5, 81470-51-1; 6, 5891-45-2; 7, 124620-51-5; 8, 90195-00-9; 9, 124620-52-6; 10, 92412-96-9; 11, 89969-29-9; 12, 124620-53-7; 13, 57732-63-5; 14, 130761-69-2; 15, 130761-70-5; 16, 130761-71-6; 17, 130761-72-7; 18, 124620-54-8; 19, 124620-55-9; 20, 130761-73-8; 21, 124650-79-9; 22, 124620-56-0; 23, 92412-99-2; 24, 130761-74-9; 25, 130761-75-0; 26, 130761-76-1; 27, 130761-77-2; 28, 130761-78-3; 29, 124620-57-1; 30, 27460-85-1; 31, 130761-79-4; 32, 130761-80-7; 33, 130761-81-8; 34, 130761-82-9; 35, 130761-83-0; 36, 130761-84-1; AcOBu-*t*, 540-88-5; 3,5-$(O_2N)_2C_6H_3COCl$, 99-33-2; *i*-PrNHC-$(OCH_2CH{=}CH_2){=}$NPr-*i*, 3116-52-7; H-Ser-OCH_2Ph, 1738-72-3; BOC-Ser-OH, 3262-72-4; H-D-Asp(OCH_2Ph)-OCH_2Ph, 4079-59-8; $PhCH_2Br$, 100-39-0; D-(-)-phenylglycine, 875-74-1.

Synthesis and Siderophore and Antibacterial Activity of N^5-Acetyl-N^5-hydroxy-L-ornithine-Derived Siderophore–β-Lactam Conjugates: Iron-Transport-Mediated Drug Delivery

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N^5-Acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithine, the functionally instrumental component of the albomycins and ferrichromes, has been incorporated as a "carrier" substructure into both carbacephalosporin and oxamazin type β-lactam antibiotics. The previously synthesized protected version of this tripeptide (14) was coupled with various β-lactam analogues 17, 19, 24, and 25 to give protected conjugates 21, 22, 26, and 27. Final deprotection by hydrogenolysis provided the deprotected siderophore–β-lactam antibiotic conjugates 1–4. The growth-promoting ability of each has been evaluated using either the siderophore-deficient mutant *Shigella flexneri* SA 100 or *S. flexneri* SA240 (SA 100 *iuc*D:Tn5). Measurement of the growth-promoting activity using two isogenic *Escherichia coli* strains differing only in the presence or absence of *fhu*A (hydroxamate ferrichrome receptor) suggests uptake by the hydroxamate iron-transport system. The antibacterial activity of these conjugates has been investigated, and the potential for use of the ferrichrome iron-transport system as a means of drug delivery is discussed.

The albomycins (Figure 1) are natural siderophores and antibiotics first isolated in 1947 from *Streptomyces griseus* and given the name grisein.[1] Several years later, another microbial iron-transport agent, named albomycin,[2] was isolated from *Streptomyces subtropicus* and subsequently determined to have the same structure as grisein.[3] Although early extensive structural studies seemed to indicate that albomycin was an iron-chelating siderophore based on the tripeptide N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithine, similar to the ferrichromes[4] (Figure 1), it was not until 1982 that Benz and co-workers[5] firmly established the structure of the albomycins. It has been demonstrated[6] that the linear tripeptide of N^5-acetyl-N^5-hydroxy-L-ornithine is the hexadenate, octahedral ligand for ferric ion responsible for intracellular transport of iron as determined by its growth-promoting ability in *S. flexneri* SA240 (SA 100 *iuc*D:Tn5). This tripeptide apparently utilizes the same ferrichrome iron-transport system[7] as implicated in the case of the albomycins.

The albomycins are not the only family of antibiotics and drugs based on iron-containing natural siderophores.[8] Siderophores and analogues have other potential uses, such as the treatment of iron metabolism disorders. The drug of choice[9] for treatment of the iron overload resulting from

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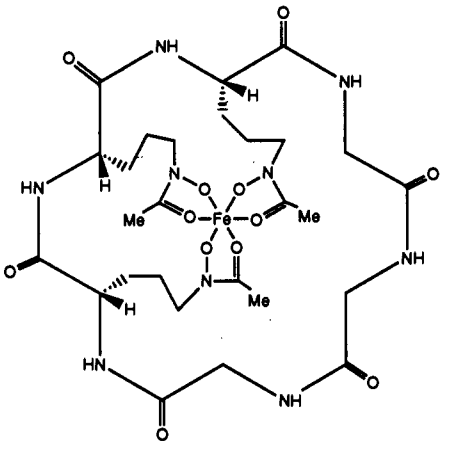
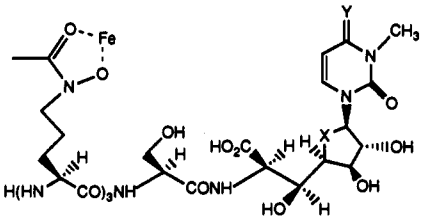


FERRICHROME



The Albomycins X = S, Y = $NCONH_2$ or O or NH

Oxygen Analog of Albomycin δ_1 X and Y = O

Figure 1.

the transfusional treatment of β-thalasemia, or Cooley's anemia, is the mesylate salt of the deferriform of the natural siderophore desferrioxamine. One problem associated with treatment of iron overload with siderophores, such as desferrioxamine mesylate, is that their use often leads to severe septicemia, especially in immunocompromised patients. It should be recalled that siderophores, such as desferrioxamine, are secreted and utilized under *natural* iron-depleted conditions by several types of microbes to promote iron transport, and therefore, their own growth. Thus, in order to avoid life-threatening septicemia, antibiotic therapy typically accompanies treatment of iron overload with desferrioxamine mesylate.

Amidst this background, one can rationalize several motives for attempting to imitate the rather wide antimicrobial activity of albomycins by attaching other potential antimicrobial agents to the iron-transporting moiety. First, development of an antibacterial agent which also chelated ferric ion effectively could facilitate iron-overload therapy while avoiding septicemia or other side effects. Secondly, a siderophore–antibiotic conjugate may be capable of selectively targeting microorganisms which are heavily dependent on the ferrichrome iron-transport system. Finally, the investigation of this type of drug-delivery system may allow extension to other types of compounds for drug delivery.[10]

Discussion

The earliest test of siderophore-mediated drug delivery was investigated by Zahner et al.[11] In this semisynthetic approach, various sulfonamides were covalently attached to several members of the ferrichrome family of siderophores. Of the four compounds synthesized, only two sulfanilamidonicotinic acid derivatives displayed weak antimicrobial activity which was limited to *Staphylococcus aureus*. Lately, catechol or hydroxamates have been exploited by attachment to β-lactam antibiotics in the hope that the corresponding iron complexes will be transported into microbial cells via the iron-transport system. Several of these derivatives have been shown to have improved activity against various microbes, including *Pseudomonas aeruginosa* strains which are less sensitive to β-lactam antibiotics.[12] Several new catechol-containing cephalosporins have been shown[13] to be dependent upon the *ton*B iron-transport system for their antimicrobial activity. In fact, a recently disclosed cephalosporin derivative, containing an iron-binding hydroxamate, displayed activity against both Gram-negative and Gram-positive bacteria.[14]

On the basis of the postulated mechanism of action of the albomycins[15] and what is known about the uptake and modification of ferrichrome,[16] the albomycin siderophore tripeptide N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithine was chosen for use as the iron-chelating ligand for our initial study. It has been suggested[15] that, after uptake of the albomycin–iron complex by a membrane receptor, two enzymes called peptidase N and peptidase A present in the cytoplasmic membrane hydrolytically release the toxic thioribosyl moiety which is subsequently transported into the intracellular cavity, where it exerts antimicrobial activity. This peptidase activity appears to be a convenient biochemical method by which other toxic moieties could by cleaved and smuggled into the intracellular fluid. However, in an elegant study, Benz and co-workers[17]

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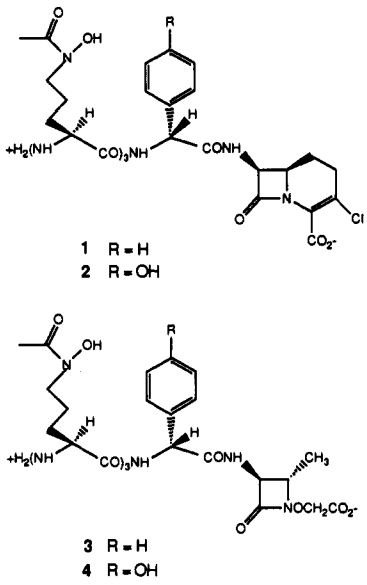
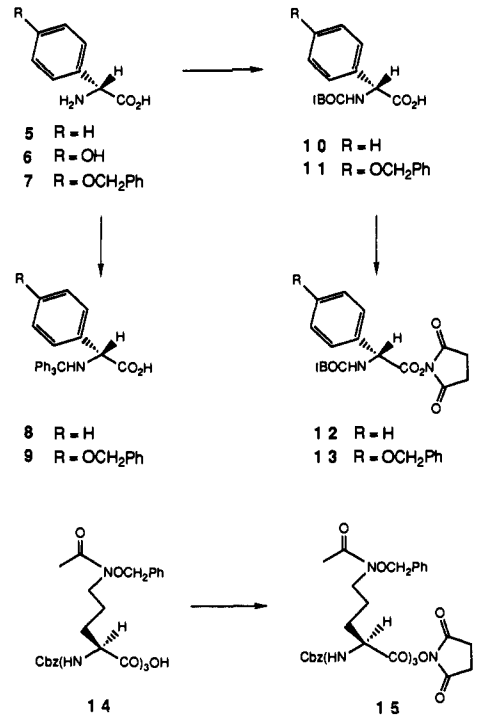


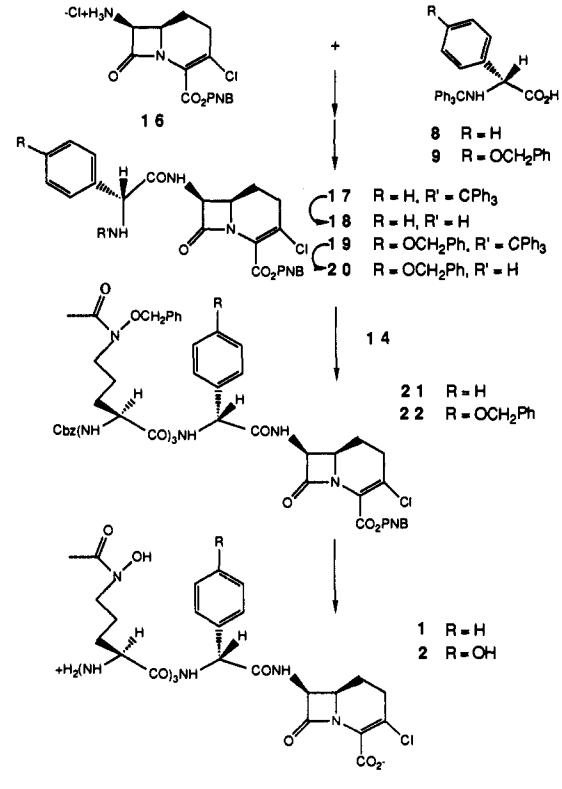
Figure 2.

demonstrated that the synthetic oxygen analogue of the deferriform of δ_1-albomycin (Figure 1) had no antimicrobial activity. Therefore, it appears that an appropriate type of toxic moiety must be attached to the siderophore iron-binding portion in order for antimicrobial activity to be manifested. We have demonstrated[6] that the corresponding tetrapeptides derived from D-phenylglycine and *p*-hydroxy-D-phenylglycine and the tripeptide N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithine are also siderophores as determined by their growth-promoting ability for *S. flexneri* SA 240 (SA 100 *iuc*D:Tn5). Although peptides containing D-amino acids are often poor substrates for both peptide permeases and peptidases,[18] these were chosen for our initial studies[6b] since the β-lactam antibiotic loracarbef (LY163892/KT3777),[19] a new carbacephalosporin, contains a D-phenylglycyl moiety. The carbacephalosporin and an oxamazin were chosen as the antibiotic components since they do not contain sulfur and were compatible with planned hydrogenolytic removal of all protecting groups at the end of the synthesis. This approach was anticipated to avoid extensive purification of the final products of unknown stability. Thus, the synthesis of the siderophore–β-lactam antibiotic conjugates 1–4 were considered. This paper reports the successful synthesis and biological activity of all four of these conjugates (Figure 2).

Scheme I



Scheme II



Results

Synthesis of Siderophore–Carbacephalosporin Conjugates (1 and 2). The synthetic sequences used for the preparation of the siderophore–carbacephalosporin conjugates 1 and 2 are shown in Schemes I and II. The amino group of D-phenylglycine 5 was protected as the triphenylmethyl (trityl) derivative to give 8. The trityl group[20] was chosen since it can be easily deprotected and

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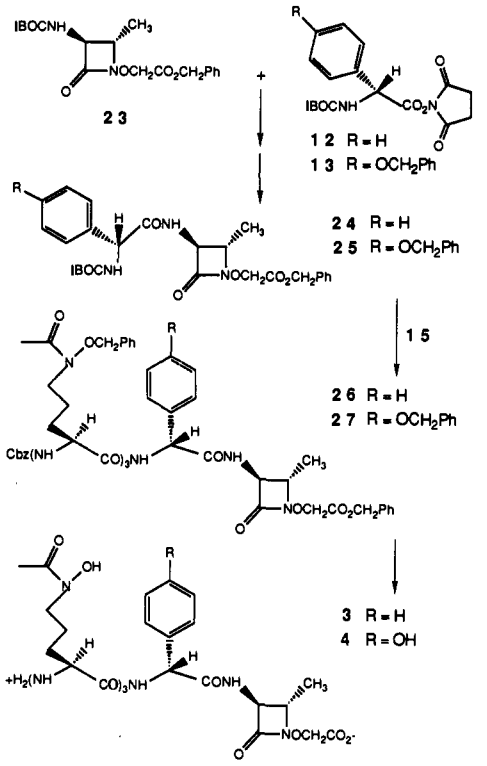
was anticipated to prevent racemization of the sensitive stereogenic center.[21] A control reaction which supported the retention of optical integrity during this protecting group introduction is described in the Experimental Section. However, based on the precedent that trityl protected amino acid esters are often very resistant to hydrolysis,[20b] the ability of this compound to couple effectively with an amino group of the carbacephalosporin and the oxamazin was a concern. Neutralization of the hydrochloride salt of carbacephalosporin 16[19] and extractive isolation provided the crude free amine which was treated with 8 and 2-ethoxy-*N*-(ethoxycarbonyl)-1,2-dihydroquinoline[22] (EEDQ) to give a 46% isolated yield of 17. Variation in experimental conditions failed to increase the yield, perhaps because of the slight instability of the free amine of 16, although it could be isolated by chromatography and characterized by infrared and ^{1}H NMR analysis before decomposition.

Coupling of modified carbacephalosporin 17 and tripeptide acid 14 was relatively straightforward. Brief treatment of 17 with excess trifluoroacetic acid (TFA) in THF provided free amine 18. In order to verify that deprotection had occurred, pure amine 18 was initially isolated by chromatography and spectral data collected. However, it eventually decomposed. Free amines of phenylglycyl derivatives of carbacephalosporins are prone to rearrangement to the corresponding diketopiperazine.[23] Treatment of crude amine 18 with the tripeptide acid 14 and EEDQ produced the fully protected conjugate 21 in 67% isolated yield.

Deprotection of fully protected conjugate 21, however, proved problematic due to the presence of the double bond in the carbacephem nucleus. As described in the Experimental Section, initial deprotection attempts were not effective. Successful deprotection, with retention of the double bond and without reductive elimination of the chlorine, was eventually achieved[24] by treatment of the fully protected conjugate with 5% aqueous DMF, concentrated HCl, 10% Pd–C, and hydrogen. Filtration and lyophilization provided 1 as a light tan solid in near quantitative yield. The allylic carbon signal at 31.44 ppm in the ^{13}C NMR spectrum indicated that the double bond had remained intact. The presence of the double bond was also verified biologically. A sample in which it was known that the double bond was reduced was shown to be devoid of antimicrobial activity when compared to 1 in selective studies.

Synthesis of the *p*-hydroxyphenylglycine analogue 2 followed a similar course (Schemes I and II). Protection

Scheme III



of the hydroxyl group of *p*-hydroxy-D-phenylglycine as the benzyl ether was accomplished by the method of Kamiya and co-workers[25] to provide 7 in 55% yield. The amino group of 7 was protected as the trityl derivative to give 9 in 61% yield. Coupling of the carbacephalosporin hydrochloride salt 16 with 9 was accomplished by treatment with triethylamine and EEDQ to provide 19 in 36% purified yield. As previously described, deprotection provided 20. Subsequent treatment with EEDQ and tripeptide acid 14 afforded fully protected conjugate 22 in 44% yield. Hydrogenolysis of 22, as previously described, gave 2 as a light tan solid in near quantitative yield. Again, the ^{13}C NMR spectrum indicated the presence of the allylic carbon signal at 31.33 ppm, confirming that the double bond was intact.

Synthesis of Siderophore–Oxamazin Conjugates (3 and 4). Syntheses of siderophore–oxamazin conjugates 3 and 4 are shown in Schemes I and III. The starting oxamazin,[26] 23, was synthesized by our modified method.[27] As in the case of the siderophore–carbacephalosporin conjugates 1 and 2, the *N*-triphenylmethyl-protected phenylglycines 8 and 9 failed to couple to oxamazin 23 with EEDQ as a carbonyl-activating agent. The use of the *N*-hydroxysuccinimide ester of *N*-trityl-protected 8 produced a 10% yield of desired product 24. It was later discovered that the free amine derived from 23 is extremely unstable and *immediately* decomposes upon attempted isolation. This instability appeared to be responsible, in part, for the unacceptable yield.

In order to determine whether the bulky trityl group also contributed to this rather low yield, *N*-Boc-*N*-hydroxy-

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succinimide active ester analogue **12** was synthesized (Scheme I). D-Phenylglycine was treated with di-*tert*-butyl dicarbonate to provide **10**. Subsequent treatment of **10** with DCC in the presence of *N*-hydroxysuccinimide gave crystalline active ester **12**. The Boc group of **23** was removed with TFA. Addition of **12** and 110 mol % of potassium bicarbonate in a two-phase system of ethyl acetate and water produced an inseparable mixture of active ester **12** and the desired product **24** as determined by ^{1}H NMR. The remaining starting active ester **12** was easily destroyed by addition of excess potassium bicarbonate to give **24** as a white solid in 25% yield. Modification of the reaction conditions failed either to increase the yield or resulted in formation of epimers as determined by NMR.

Removal of the Boc group from **24** and treatment of the product with tripeptide acid **14**, triethylamine and EEDQ gave protected conjugate **26** in 16% purified yield. The instability of the free amine derived from **24**, in conjunction with the use of EEDQ carbonyl-activating agent, was thought to be responsible for the low yield. Therefore, **14** was first converted to *N*-hydroxysuccinimide active ester **15** (Scheme I). Reaction with the free amine of **24** in a two-phase system of ethyl acetate and water with potassium bicarbonate as a base gave **26** in 48% yield. This product was identical with that isolated from the EEDQ-mediated reaction. Deprotection of the fully protected conjugate **26** by hydrogenolysis provided **3** in 95% yield as a light tan solid.

Synthesis of the *p*-hydroxyphenylglycine analogue **4** is shown in Scheme I and III. Protection of the amino group of **7** as Boc derivative **11** and conversion to the crystalline active ester **13** was accomplished as previously described. Removal of the Boc group and coupling with **13** afforded an inseparable mixture of starting active ester **13** and desired product **25** as determined by ^{1}H NMR analysis. The remaining active ester was destroyed as described in the Experimental Section. Aqueous workup and radial silica gel chromatography provided a 26% yield of **25**. Removal of the Boc group of **25** and coupling with active ester **15** produced a 38% yield of **27** after purification. Hydrogenolysis of **27** gave **4** in quantitative yield.

Biological Results

Siderophore Activity and Antimicrobial Activity. Synthetic conjugates **1–4** were anticipated to be transported by the ferrichrome hydroxamate iron transport system in microbes, thereby bypassing the normal β-lactam-transport mechanisms.[29] We have previously shown[6] that the tetrapeptides N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithyl-D-phenylglycine and N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithinyl-D-*p*-hydroxyphenylglycine are active siderophores, thereby promoting bacterial growth. A separate study using *E. coli* X580[30] revealed that these two peptides themselves are devoid of antimicrobial activity. Several types of assays and microbes were utilized to determine whether conjugates **1–4** were either siderophores (iron-transporting agents) or effective antimicrobial agents. Details of these assays are given at the end of the Experimental Section. The microbes used include *S. flexneri* SA100 and *S. flexneri* SA240 (SA100 *iuc*D:Tn5), a mutant of SA100 which lacks the ability to synthesize siderophores. Two isogenic *E. coli* strains that differ only in *fhu*A were also examined. The RW193 strain is an *E. coli* K12 entA, fhuA positive organism and the AN193 strain is an entA, fhuA negative mutant which is deficient in the hydroxamate ferrichrome receptor. Finally, *E. coli* X580,[30] a β-lactam hypersensitive organism commonly used to screen for antibiotic activity, was used for further testing.

Table I. Liquid Growth Assay for Siderophore Activity for the Carbacephalosporin Conjugate **2**[a]

microorganism	strain	$t = 0$	$t = 2$ h	$t = 8$ h
S. Flexneri	SA240			
SA240 only		0.001	0.002	0.278
SA240 plus **2**		0.001	0.107	0.974
E. coli	RW193			
RW193 only		0.001	0.003	0.647
RW193 plus **2**		0.001	0.347	1.590
E. coli	AN193			
AN193 only		0.001	0.003	0.592
AN193 plus **2**		0.001	0.019	0.691

[a] The liquid growth assay was completed as follows: overnight cultures of each strain were diluted 1:500 into Luria broth with 10 μg/mL of EDDA with or without 100 μg/mL of the test compound **2**. Growth was monitored by measuring turbidity by absorbance at 650 nm.

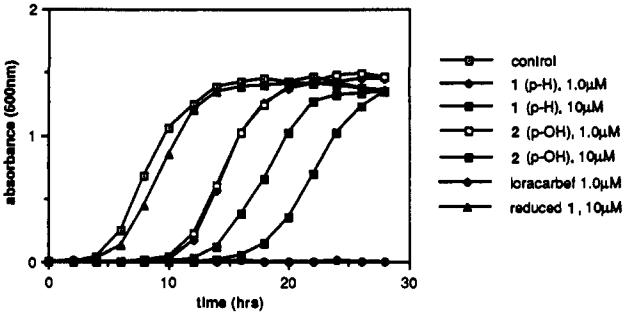

Figure 3. Effects of the preformed Fe(III) complexes of conjugates 1 and 2 and reduced 1 on the growth rate of *E. coli* X580 in Mueller–Hinton broth.

As shown in Table I, the liquid growth assay using an iron-deficient media containing the iron chelator ethylenediaminebis(*o*-hydroxyphenylacetic acid) revealed that carbacephalosporin conjugate **2**, in fact, promotes growth compared to the control. Although it could be argued that a β-lactamase is responsible for this activity, this was unlikely since *S. flexneri* SA240 (SA100 *iuc*D:Tn5) is sensitive to ampicillin. Use of *E. coli* RW193 also revealed some growth-promoting activity, apparently produced by the ferrichrome hydroxamate transport system. This was supported by the assay using *E. coli* AN193, which is deficient in the ability to produce the ferrichrome transport receptor system. In this assay, microbial growth in the presence of **2** was marginal at best. The fact that these organisms could still proliferate by apparent siderophore-promoted growth in the presence of a β-lactam antibiotic was at first puzzling. In separate plate bioassays with the above organisms, neither conjugates **1** nor **2** displayed any antimicrobial activity since no zones of inhibition were observed. Since these conjugates revealed growth-promoting activity, the absence of net antibiotic activity in the plate method of screening may have several causes. It is not unreasonable to expect that some organisms may not have a peptidase needed for release of

(29) (a) Nikaido, H.; Nakae, T. *Adv. Microbiol. Physiol.* **1979**, *19*, 163. (b) Osborn, M. J.; Wu, H. C. P. *Annu. Rev. Microbiol.* **1980**, *34*, 369. (c) Jaffe, A.; Chabbert, Y. A.; Semonin, O. *Antimicrob. Agents Chemother.* **1982**, *22*, 942. (d) Nikaido, H.; Rosengerg, E. Y.; Foulds, J. *J. Bacteriol.* **1983**, *153*, 232. (e) Sawai, T.; Hiruma, R.; Kawana, N.; Kaneko, M.; Taniyasu, F.; Inami, A. *Antimicrob. Agents Chemother.* **1982**, *22*, 585. (f) Harder, K. J.; Nikaido, H.; Matsuhashi, M. *Antimicrob. Agents Chemother.* **1981**, *20*, 549. (g) Nikaido, H.; Rosenberg, E. Y. *J. Bacteriol.* **1983**, *153*, 241.

(30) We thank Eli Lilly and Co. for the generous gift of this microorganism.

Table II. Selected Minimum Inhibitory Concentrations[a]

Organism	strain	MIC, μg/mL loracarbef	cefaclor	1	2
Salmonella	X514	0.25	0.25	0.125	0.06
Salmonella	1135	0.50	1.0	>128	>64
Pseudomonas	X528	>128	>128	>128	>128
Staphylococcus epidermidis	222	2	2	>128	>128
Streptococcus pneumonia	PARK	1	0.5	>64	>64
E. coli	EC14	0.5	1.0	>64	>32

[a] Performed at Eli Lilly and Co. by using the standard agar dilution method.[28]

the antibiotic, or it is possible that mutant organisms have been selected which lack the necessary iron-transport components needed for absorption. Results from incubation of siderophore–antibiotic conjugates 1 and 2 with β-lactam hypersensitive *E. coli* X580 are shown in Figure 3. The conjugates 1 and 2 displayed *significant* antimicrobial activity as shown by the delayed observed microbial growth. The bacteria that did eventually grow were isolated and separately incubated again in the presence of each of the carbacephalosporin derivatives 1 and 2. In all cases, *no delay* of bacterial growth was observed. These results suggest that, in the first incubation, the delayed microbial growth may be due to either selection of a mutant of the parent strain or repression of the biosynthesis of the ferrichrome transport proteins. This observation, however, is not surprising since it is known that bacterial resistance to the albomycins often is acquired quickly.[31] A comprehensive description of these observations will be reported elsewhere.[32] Bacteria resistant to compounds entering via iron-transport pathways may not be expected to be virulent pathogens, relative to the parent strain, due to their inability to assimilate iron from the environment. *In fact, use of iron-deficient Luria broth (EDDA) with E. coli X580 in the presence of 1 resulted in greater than a 5 log kill.* It is interesting to note that a derivative of conjugate 1, in which it is known that the double bond of the cephem nucleus is reduced, had no antimicrobial activity as compared to the control (Figure 3).

Selective antimicrobial activity of carbacephalosporin conjugates 1 and 2 is shown in Table II. The minimum inhibitory concentrations (MIC) values indicate that conjugates 1 and 2 are very active against *Salmonella* X514 yet are poorly active against *Salmonella* 1135 and other Gram-positive and Gram-negative organisms.

The siderophore growth-promoting ability of the oxamazin–siderophore conjugates 3 and 4 are shown in Table III. With *S. flexneri* SA100, it is clear that growth is promoted by these compounds in the iron-deficient media used. In separate assays using β-lactam hypersensitive *E. coli* X580, no antimicrobial activity was observed for either 3 or 4. The absence of a release mechanism may be responsible. However, the lack of activity was not surprising since the antimicrobial activity of oxamazins are known[26] to be very sensitive to the type of side chain present in the molecule.

Conclusions

In this article, we have described the syntheses and biological activity of several siderophore–β-lactam conjugates. We have presented nonradiolabel-derived biological evidence which suggests that these compounds are transported into the microbe by the ferrichrome iron-transport system and that some of the conjugates (1 and 2) display significant antimicrobial activity against selected organisms. Our synthetic approach allows flexibility which will enable extension of this approach to other antibiotics and drugs in the hope that iron-transport-mediated drug transport will become viable.

Table III. Plate Bioassay for Siderophore Activity for the Oxamazin Conjugates

concn, mM	zone of stimulation,[a] mm 3	4
10	28	31
1	12	16
0.1	0	0

[a] *S. flexneri* SA 100 overnight culture, 100 mL of 10^{-3} dilution into 100 mL of Luria–EDDA (250 mg/mL) agar. Plates were splotted with 10-mL samples of siderophores at dilutions indicated. Plates were incubated at 37 °C for 18 h and examined for zones of stimulation around compounds.

Experimental Section

General Methods. Melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Infrared spectra (IR) were recorded on a Perkin-Elmer 1420 spectrophotometer. TF refers to thin-film and KBr refers to potassium bromide disks for infrared spectra. Proton spectra were obtained on a Magnachem A-200 or a General Electric GN-300 spectrometer. Carbon-13 NMR were obtained on a General Electric GN-300 spectrometer. Chemical shifts are reported in ppm relative to tetramethylsilane (deuterochloroform), the upfield methyl signal of deuterodimethylformamide (2.74 ppm), or 1,4-dioxane (3.55 ppm in deuterium oxide) for proton NMR. Carbon NMR references were the center peak of deuterochloroform (77.0 ppm), the center peak of the upfield methyl signal of deuterodimethylformamide (30.1 ppm), or, for deuterium oxide, the signal for 1,4-dioxane (66.5 ppm). Electron impact mass spectra, chemical ionization mass spectra and fast atom bombardment (FAB) mass spectra were recorded on an AEI Scientific Apparatus MS 902, Du Pont DP 102, Finnigan MAT Model 8430, or ZAB-SE spectrometer. Exact mass FAB spectra were kindly performed by Dr. Richard Milberg, Department of Chemistry, University of Illinois. Optical rotations were obtained with a Rudolf Research Autopol III polarimeter. Analytical TLC was carried out with commercially available aluminum-backed 0.2-mm silica gel 60 F-254 plates. HPLC analyses were performed with an ISCO Model 2350 system fitted with a Model 2360 gradient programmer and an Alltech C8 10-μm reversed-phase column (250 × 4.6 mm) with UV detection at 254 nm. Gradients were formed as specified with two solvents (A = 100 mM potassium phosphate and 1 mM EDTA at pH = 5.8, B = acetonitrile). Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ. Radial preparative silica gel chromatography was performed with a Harrison Research Chromatotron Model 7924 and flash silica gel column chromatography was conducted with Merck silica gel 60. Solvents used were dried and purified by standard methods.[33] The term "dried" refers to the drying of a organic solution over anhydrous magnesium sulfate.

N^5-Acetyl-N^5-(benzyloxy)-N^5-(benzyloxycarbonyl)-L-ornithyl-N^5-acetyl-N^5-(benzyloxy)-L-ornithyl-N^5-acetyl-N^5-(benzyloxy)-L-ornithine (14) was synthesized by the procedure of Miller et al.[6]

N^5-Acetyl-N^5-(benzyloxy)-N^2-(benzyloxycarbonyl)-L-ornithyl-N^5-acetyl-N^5-(benzyloxy)-L-ornithyl-N^5-acetyl-N^5-(benzyloxy)-L-ornithine Succinimido Ester (15). To a solution of tripeptide acid 14 (0.205 g, 0.219 mmol) and *N*-hydroxysuccinimide (0.025 g, 0.219 mmol) in 1.0 mL of anhydrous THF at 0 °C under nitrogen was added dropwise a solution of

(31) Garson, W.; Waksman, S. A. *Proc. Natl. Acad. U.S.A.* 1948, *34*, 232.

(32) A complete report on the siderophore and antimicrobial activity of all the peptide fragments and conjugates will be reported elsewhere.

(33) Gordon, A. J.; Ford, R. A. The Chemist's Companion—a Handbook of Practical Data, Techniques, and References; John Wiley & Sons: New York, 1971; pp 408–455.

DCC (0.045 g, 0.219 mmol) in 1.0 mL of anhydrous THF. The mixture was stirred overnight at room temperature and then evaporated. The residue was suspended in anhydrous benzene and filtered to remove solid dicyclohexylurea. The solvent was removed by evaporation to provide 0.230 g of crude **15** (100%) as a foam. This unstable material was not purified but used as such for further coupling reactions after spectral identification: IR (KBr) 3300, 1820, 1790, 1745, 1650 (br) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 1.50–1.95 (m, 12 H, CH_2), 2.03 (s, 3 H, CH_3CON), 2.04 (s, 3 H, CH_3CON), 2.10 (s, 3 H, CH_3CON), 2.68 (s, 4 H, CH_2CH_2), 3.40–4.10 (m, 6 H, CH_2N), 4.30–4.50 (m, 1 H, NC*H*CO), 4.60–4.70 (m, 2 H, NC*H*CO), 4.75–4.90 (m, 6 H, benzylic H), 5.05 (s, 2 H, benzylic H), 5.90 (d, 1 H, J = 9 Hz, N*H*), 7.20–7.45 (m, 20 H, aromatic H), 7.75 (d, 1 H, J = 9 Hz, N*H*); MS (positive ion FAB, glycerol) m/z 1036 (M^+), 940 ($M - C_4H_4NO_2 + 1$).

***N*-(Triphenylmethyl)-D-phenylglycine (8)** was prepared by the method of Miller et al.[6] In order to determine if any racemization had occurred under the introduction conditions for the trityl group, a control reaction was conducted in which D-phenylglycine was regenerated from its *N*-trityl derivative by deprotection. The protected product **8** was treated with a mixture of 1.45 M HCl and THF, followed by careful evaporation of the THF and extraction of the aqueous phase with ethyl acetate, which removed the byproduct triphenylmethanol (tritanol). Comparison of the optical rotation of both the starting D-phenylglycine and the deprotected material present in the aqueous phase revealed values of $[\alpha]_D$ = −151° and −149°, respectively, at the same concentration, temperature, and in the same solvent system. Therefore, we were confident that no extensive racemization had occurred under the basic (triethylamine) conditions used for introduction of this group.

4-Nitrobenzyl 7β-[[*N*-(Triphenylmethyl)-D-phenylglycyl]amino]-1-carba-3-chloro-3-cephem-4-carboxylate (17). A solution of 93.5 wt % (0.019 g, 0.050 mmol) of **16** in saturated sodium carbonate was repeatedly extracted with ethyl acetate. The organic phases were washed with brine, then dried, filtered, and evaporated. Immediately, EEDQ (0.017 g, 0.066 mmol) and **8** (0.021 g, 0.053 mmol) in 1.0 mL of anhydrous dichloromethane were added followed by stirring under nitrogen for 19 h. The solvents were evaporated, ethyl acetate was added, and the solution was washed with saturated sodium bicarbonate, 1.0 M HCl, water, and brine. The organic phase was dried, then filtered, and evaporated to give 0.036 g of a foam. This foam was purified by radial silica gel chromatography eluting with ethyl acetate–hexanes (1:2) to provide 0.018 g (46%) of **17** as a foam. Different reaction conditions including excess EEDQ, excess **8**, in situ generation of the free amine of **16** with triethylamine, use of absolute ethanol as a solvent, or a modified workup involving a wash with 1 M copper sulfate in an attempt to remove quinoline and to minimize loss of the triphenylmethyl group (in contrast to washing with aqueous acid) failed to improve the yield above 46%. The mixed anhydride method of carbonyl activation with ethyl chloroformate and triethylamine resulted in production of a mixture of diastereomers as determined by NMR spectroscopy. Characterization data for diastereometrically pure **17**: TLC (ethyl acetate-hexanes 1:2) R_f = 0.51; IR (TF) 3320, 1780, 1745, 1670 cm^{-1}; $[\alpha]^{21}_D$ = −60.0° (c = 1.30, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz) δ 0.86–1.10 (m, 1 H, C-1 H CH_2), 1.35–1.47 (m, 1 H, C-1 H CH_2), 2.33–2.50 (m, 2 H, C-2 allylic H CH_2), 3.50 (d, 1 H, J = 9 Hz, N*H*), 3.57–3.67 (m, 1 H, C-8 H), 4.33 (t, J = 6 Hz, 1 H, C-7 H), 4.71 (d, 1 H, J = 6 Hz, NC*H*CO benzylic H), 5.35 (AB quartet, 2 H, J = 12 Hz, benzylic H), 6.61 (d, 1 H, J = 6 Hz, N*H*), 7.00–7.45 (m, 20 H, aromatic H), 7.57 (d, 2 H, J = 9 Hz, aromatic H), 8.20 (d, 2 H, J = 9 Hz, aromatic H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 21.18, *31.54* (allylic), 52.17, 58.59, 61.93, 66.06, 72.28, 119.98, 120.03, 122.91, 123.64, 125.49, 126.76, 126.85, 127.66, 127.85, 128.64, 128.97, 129.76, 130.99, 140.87, 142.10, 145.51, 147.72, 159.90, 164.33, 173.67; MS (positive ion FAB, dithioerythritol–dithiothreitol) m/z 691 (M − Cl). Examples of reductive eliminations under FAB mass spectral conditions are known.[34]

4-Nitrobenzyl 7β-(D-Phenylglycylamino)-1-carba-3-chloro-3-cephem-4-carboxylate (18). To a solution of **17** (0.014 g, 0.019 mmol) in 1 mL of anhydrous THF under nitrogen was added 80 μL (1.00 mmol) of anhydrous TFA. TLC monitoring revealed that starting material had been consumed and the solvent was evaporated to give a yellow semisolid. This material was dissolved in THF and water (which led to disappearance of the yellow color) and the resulting mixture was stirred for 0.25 h. The THF was evaporated and the resulting aqueous solution was diluted with saturated sodium carbonate and extracted repeatedly with ethyl acetate. The organic phases were washed with brine, then dried, filtered, and evaporated to give 0.016 g of a solid. This solid was purified by radial silica gel chromatography eluting with methanol–ethyl acetate (1:5) to provide 6.6 mg (69%) of **18** as an oil. The oil slowly decomposed to give the diketopiperazine as monitored by silica gel thin-layer chromatography since **18** disappeared, resulting in the formation of a faster moving compound. Compound **18** was isolated and purified only to verify deprotection, since in all further reactions the amine was used as a crude product: TLC (methanol–ethyl acetate 1:5) R_f = 0.55; IR (TF) 3320, 1775, 1735, 1675 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 1.38–1.62 (m, 1 H, C-1 H CH_2), 1.66–2.00 (br s overlapping a m, 3 H, C-1 H CH_2 and NH_2), 2.50–2.64 (m, 2 H, C-2 allylic CH_2), 3.77–3.89 (m, 1 H, C-8 NC*H*), 4.47 (s, 1 H, C-7 NC*H*CO), 5.15–5.38 (t overlapping a AB q, 3 H, benzylic H and NC*H*CO benzylic H), 7.17–7.32 (m, 5 H, aromatic H), 7.50 (d, 2 H, J = 9 Hz, aromatic H), 7.60 (d, 1 H, J = 6 Hz, N*H*), 8.13 (d, 2 H, J = 9 Hz, aromatic H).

4-Nitrobenzyl 7β-[[N^5-Acetyl-N^5-(benzyloxy)-N^2-(benzyloxycarbonyl)-L-ornithyl-N^5-acetyl-N^5-(benzyloxy)-L-ornithyl-N^5-acetyl-N^5-(benzyloxy)-L-ornithyl-D-phenylglycyl]amino]-1-carba-3-chloro-3-cephem-4-carboxylate (21). To a solution of (0.130 g, 0.170 mmol) of **17** in 5 mL of anhydrous THF was added 656 μL (8.51 mmol) of anhydrous TFA under nitrogen. The reaction was monitored by TLC which revealed a disappearance of starting material. The mixture was concentrated, diluted with 2 mL of THF and 2 mL of water and stirred for 0.25 h. The THF was evaporated and the residue was diluted with saturated sodium carbonate and ethyl acetate. The organic layer was washed with brine, then dried, filtered, and concentrated to give an oil which was not purified but used immediately. To this oil was added 2 mL of anhydrous dichloromethane followed by solid tripeptide **14** (0.182 g, 0.170 mmol) and EEDQ (0.055 g, 0.221 mmol). The mixture was stirred under nitrogen for 18 h. The reaction mixture was evaporated to an oil and purified by radial silica gel chromatography eluting with methanol–ethyl acetate (3:97) to give 0.178 g (67%) of **21** as a foam: TLC (methanol–ethyl acetate 1:10) R_f = 0.33; IR (KBr) 3295, 1775, 1725, 1655, 1635 cm^{-1}; $[\alpha]^{21}_D$ = −13.7° (c = 1.70, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.15–1.40 (m, 2 H, C-1 CH_2), 1.45–1.85 (m, 12 H, CH_2), 1.90 (s, 3 H, CH_3CON), 2.00–2.15 (m, 6 H, CH_3CON), 2.45–2.60 (m, 2 H, C-2 allylic CH_2), 3.40–4.05 (m, 7 H, C-8 NC*H* and CH_2N), 4.15–4.65 (m, 4 H, NC*H*CO), 4.70–4.85 (m, 6 H, benzylic H), 4.95–5.10 (m, 2 H, benzylic H), 5.25–5.35 (m, 2 H, benzylic H), 5.50 (d, 1 H, J = 7 Hz, benzylic NC*H*CO), 5.97 (d, 1 H, J = 7 Hz, N*H*), 7.20–7.40 (m, 25 H, aromatic H), 7.52 (d, 2 H, J = 8 Hz, aromatic H), 761 (d, 1 H, J = 4 Hz, N*H*), 8.13 (d, 2 H, J = 8 Hz, aromatic H), 8.30 (d, 1 H, J = 7 Hz, N*H*); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 15.25, 20.08, 20.35, 21.22, 23.05, 23.22, 23.41, 27.85, 29.14, 29.86, *31.39* (allylic), 43.51, 44.63, 51.99, 52.74, 53.07, 54.49, 57.87, 59.15, 63.62, 66.21, 66.66, 76.17, 103.94, 123.21, 123.56, 127.63, 127.71, 127.82, 127.89, 128.28, 128.34, 128.60, 128.68, 128.82, 128.88, 128.94, 129.09, 129.70, 134.06, 134.11, 134.27, 136.28, 136.48, 142.14, 147.63, 156.44, 160.35, 164.67, 170.94, 171.52, 172.52; MS (positive ion FAB, dithioerythritol–dithiothreitol) m/z 1406 (M + 1), 1428 (M + Na).

7β-[[N^5-Acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithyl-N^5-acetyl-N^5-hydroxy-L-ornithyl-D-phenylglycyl]amino]-1-carba-3-chloro-3-cephem-4-carboxylic Acid (1). Initial deprotection attempts under hydrogenolysis conditions using a two-phase system of deionized distilled water and ethyl acetate in the presence of a catalytic amount of 10% palladium on carbon at atmospheric hydrogen pressure for short periods consistently resulted in reduction of the double bond. This overreduction was determined by the disappearance of the allylic proton signals located between 2.4 and 2.8 ppm in the ^1H NMR spectrum. Despite monitoring of the reaction by ^1H NMR, the rate of double bond reduction was faster than removal of the

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benzyl groups. Stoichiometric in situ generation of hydrogen by catalytic transfer hydrogenation[35] or use of trimethylsilyliodide,[36] or Lindlar's catalyst (5% palladium on calcium carbonate and lead oxide) were also unsuccessful. However, the following conditions led to the desired product. To a solution of **21** (0.094 g, 0.060 mmol) in 2.0 mL of 5% aqueous DMF (prepared from deionized distilled water and HPLC grade DMF) was added 16 μL (0.180 mmol, 3.0 equivalents) of concentrated HCl and 0.020 g of 10% Pd–C. This mixture was exposed to hydrogen for 24 h at atmospheric pressure followed by filtration of the catalyst. The DMF–water was removed by evaporation under high vacuum. The residue was redissolved in absolute ethanol in an attempt to remove water azeotropically and remove traces of DMF under high vacuum. This process was repeated three times to give 0.063 g (100%) of **1** as a light amber semisolid. Even though a ^{1}H NMR spectrum could be obtained for this compound in deuterium oxide, traces of DMF from the deprotection remained despite all attempts to remove it. Compounds of this nature have a relatively high affinity for DMF.[19f] Therefore, due to the solubility problem and the fact that traces of DMF were present, deuterated DMF was used as an NMR solvent: $FeCl_3$ positive (red-purple); HPLC (RP-C8) t_R = 44.8 min (eluent = 100% solvent A for 10 min, then a linear gradient to 1:1 of solvent A and B for 25 min, followed by this same ratio for an additional 10 min before reequilibrating the column); IR (KBr) 3700–2400 (br), 1765, 1655 (br) cm^{-1}; ^{1}H NMR (300 MHz, DMF-d_7) δ 1.20–1.35 (m, 2 H, C-1 CH_2), 1.45–1.90 (m, 12 H, CH_2), 2.00–2.15 (m, 9 H, CH_3CON), 2.50–3.00 (C-2 allylic H obscured by NMR solvent peak and residual DMF), 3.40–3.80 (m, 7 H, CH_2N and C-8 CHN), 3.85–4.00 (m, 1 H, NCHCO), 4.20–4.40 (m, 1 H, NCHCO), 4.45–4.70 (m, 2 H, NCHCO), 5.47 (dd, 1 H, J = 8 Hz, NCHCO benzylic H), 5.70 (d, 1 H, J = 8 Hz, NH), 7.20–7.50 (m, 5 H, aromatic H), 7.55–10.40 (br m, 9 H, NH, NH_3^+, and NOH); ^{13}C NMR (DMF-d_7, 75 MHz, all signals observed at 20 °C reported) δ 20.73, 20.80, 22.44, 22.78, 22.86, 22.99, 24.03, 28.24, 28.85, *31.44* (allylic), 46.88, 47.14, 52.87, 53.60, 53.65, 53.75, 54.19, 57.67, 58.86, 125.67, 126.42, 128.02, 128.40, 129.07, 130.48, 139.21, 165.69, 169.75, 171.01, 171.20–172.00 (br m with major signals at 171.36, 171.57, 171.81), 172.23, 172.51; MS (positive ion FAB, glycerol) m/z 866 (M$^+$); exact mass FAB calcd for (M + H) 866.3451, found 866.3437.

D-4-(Benzyloxy)phenylglycine (7). This was prepared by a known literature method[25] in 55% yield: mp 218–219 °C (lit.[25] mp 225–228 °C); IR (KBr) 3700–2700 (br), 2910, 1600, 1515, 1395, 1250 cm^{-1}; $[\alpha]^{22}_D$ = −58.0° (c = 0.3, 0.1 M NaOH) [lit.[25] $[\alpha]_D$ = −55° (c = 0.3, 0.1 M NaOH)].

D-*N*-(Triphenylmethyl)-4-(benzyloxy)phenylglycine (9). To a slurry of **7** (1.29 g, 5.00 mmol) in 10 mL of anhydrous DMF was added 0.67 mL (5.25 mmol) of trimethylsilyl chloride to give a clear solution. The solution was stirred for 10 min followed by addition of triphenylmethyl chloride (1.46 g, 5.25 mmol) and 1.5 mL (10.50 mmol) of distilled Et_3N. The mixture was stirred for 2 h and diluted with water and ether followed by acidification to pH 3 with 1.0 M HCl. The organic layer was washed with water and brine, then dried, filtered, and evaporated to give 2.32 g of a tan foam. This foam was purified by flash silica gel chromatography eluting first with ethyl acetate–hexanes (1:2) then methanol–ethyl acetate (1:5) to afford 1.63 g (61%) of **9** as a light tan foam: TLC (ethyl acetate–hexanes 1:2) R_f = 0.31; IR (KBr) 3700–2300 (br), 1710, 1610, 1510 cm^{-1}; $[\alpha]^{22}_D$ = −150° (c = 3.54, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.29 (s, 1 H, NCHCO benzylic H), 5.08 (s, 2 H, benzylic H), 6.90–7.50 (m, 24 H, aromatic H); MS (positive ion FAB, glycerol) m/z 500 (M + 1). Anal. ($C_{34}H_{29}NO_3$) C, H, N.

4-Nitrobenzyl 7β-[[*N*-(Triphenylmethyl)-D-4-(benzyloxy)phenylglycyl]amino]-1-carba-3-chloro-3-cephem-4-carboxylate (19). To a suspension of **16** (0.042 g, 0.10 mmol) in 0.5 mL of anhydrous dichloromethane was added 14 μL (0.10 mmol) of distilled Et_3N to give a clear light amber solution. Immediately, **9** (0.134 g, 0.25 mmol) and EEDQ (0.062 g, 0.25 mmol) were added. The mixture was stirred for 20 h, evaporated, dissolved in ethyl acetate, and washed with several portions of 0.5 M copper sulfate solution. The solution was washed with brine, then dried, filtered, and evaporated to give 0.204 g of a tan foam. This foam was purified by radial silica gel chromatography eluting with ethyl acetate–hexanes (1:4) to give 0.031 g (36%) of **19** as a white solid: TLC (ethyl acetate–hexanes 2:1) R_f = 0.29; IR (KBr) 3320, 1780, 1735, 1650, 1610 cm^{-1}; $[\alpha]^{23}_D$ = −79.0 (c = 0.83, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.79–1.50 (m, 2 H, C-1, CH_2), 2.32–2.50 (m, 2 H, C-2 allylic CH_2), 3.42 (br s, 1 H, NH), 3.50–3.74 (m, 1 H, C-8 NCH), 4.21 (s, 1 H, C-7 NCHCO), 4.79 (t, 1 H, J = 6 Hz, NCHCO benzylic H), 5.06 (s, 2 H, benzylic H), 5.37 (AB q, 2 H, J = 12 Hz, benzylic H), 6.47 (d, 1 H, J = 6 Hz, NH), 6.92 (d, 2 H, J = 8 Hz, aromatic H), 7.00–7.50 (m, 17 H, aromatic H), 7.61 (d, 2 H, J = 9 Hz, aromatic H), 8.24 (d, 2 H, J = 9 Hz, aromatic H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 21.33, *31.58* (allylic), 52.23, 58.65, 61.48, 66.11, 69.98, 31.58, 52.23, 58.65, 61.48, 66.11, 69.98, 72.37, 115.10, 123.04, 123.69, 126.81, 127.36, 127.91, 127.96, 128.05, 128.55, 128.71, 129.05, 130.94, 133.33, 136.82, 142.16, 145.55, 147.81, 158.31, 159.96, 164.45, 173.95; MS (positive ion FAB, dithioerythritol–dithiothreitol); no molecular ion was observed. However, considerable reductive elimination[36] appeared to occur: m/z 755 (parent ion), 831 (755 + C_6H_5).

4-Nitrobenzyl 7β-[[D-4-(Benzyloxy)phenylglycyl]amino]-1-carba-3-chloro-3-cephem-4-carboxylate (20). To a solution of **19** (0.026 g, 0.03 mmol) in 1.0 mL of anhydrous THF was added 115 μL (1.50 mmol) of anhydrous TFA to give a bright yellow solution. Monitoring by TLC revealed starting material to be absent. The mixture was evaporated to give a yellow semisolid, which was dissolved in THF and water and stirred for 0.25 h. The THF was removed by evaporation and diluted with a mixture of ethyl acetate and saturated aqueous sodium carbonate. The aqueous phase was extracted with ethyl acetate. The organic phases were washed with brine, then dried, filtered, and evaporated to give 0.020 g of a tan solid. This solid was purified by radial silica gel chromatography eluting with methanol–ethyl acetate (1:5) to give 0.011 g (59%) of **20** as an oil. As monitored by TLC, this compound slowly decomposed to the diketopiperazine. Compound **20** was isolated and purified only to verify deprotection (this compound was utilized as crude material in all further reactions): TLC (methanol–ethyl acetate 1:5) R_f = 0.41; IR (TF) 3320, 1770, 1735, 1680 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.48–1.65 (m, 2 H, CH_2), 2.60–2.70 (m, 2 H, C-2 CH_2), 3.87–3.96 (m, 1 H, C-8 CH_2), 4.53 (s, 1 H, C-7 NCHCO), 5.06 (s, 2 H, benzylic H), 5.25–5.47 (t overlapping AB q, 3 H, benzylic H and NCHCO benzylic H), 6.95 (d, 2 H, J = 9 Hz, aromatic H), 7.00–7.25 (m, 7 H, aromatic H), 7.61 (d, 2 H, J = 9 Hz, aromatic H), 7.65 (d, 1 H, J = 7 Hz, NH), 8.23 (d, 2 H, J = 9 Hz, aromatic H).

4-Nitrobenzyl 7β-[[N^5-Acetyl-N^5-(benzyloxy)-N^2-(benzyloxycarbonyl)-L-ornithyl-N^5-acetyl-N^5-(benzyloxy)-L-ornithyl-N^5-acetyl-N^5-(benzyloxy)-L-ornithyl-D-4-(benzyloxy)phenylglycyl]amino]-1-carba-3-chloro-3-cephem-4-carboxylate (22). To a solution of **19** (0.083 g, 0.10 mmol) in 2.5 mL of anhydrous THF under nitrogen was added 374 μL (4.85 mmol) of anhydrous TFA. The reaction was monitored by TLC which showed that starting **19** had been consumed. The solution was evaporated, diluted with THF and water, and stirred for 0.25 h. The THF was removed by evaporation and the residue was diluted with ethyl acetate and saturated aqueous sodium carbonate. The aqueous phase was extracted with ethyl acetate, and the organic phases were washed with brine, then dried, filtered, and evaporated to give an oil. This unstable crude amine (**20**) was not purified but used immediately. To this residue was added 4 mL of anhydrous dichloromethane, solid tripeptide **14** (0.104 g, 0.10 mmol), and EEDQ (0.032 g, 0.13 mmol). This mixture was stirred under nitrogen for 20 h. The reaction mixture was evaporated and purified by radial silica gel chromatography eluting first with methanol–ethyl acetate (3:97) and then with methanol–ethyl acetate (1:5). This gave 0.082 g (44%) of **22** as a clear oil: TLC (methanol–ethyl acetate 1:50) R_f = 0.39; IR (KBr) 3295, 1775, 1735, 1655, 1630 cm^{-1}; $[\alpha]^{21}_D$ = −23.9° (c = 0.82, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.17–1.35 (m, 2 H, C-1 CH_2), 1.40–1.80 (m, 12 H, CH_2), 1.88 (s, 3 H, CH_3CON), 2.00–2.15 (m, 6 H, CH_3CON), 2.25–2.60 (m, 2 H, C-2 allylic CH_2), 3.40–3.90 (m, 7 H, C-6 NCH and CH_2N), 3.95–4.65 (m, 4 H, NCHCO), 4.68–4.85 (m, 6 H, benzylic H), 4.95–5.10 (m, 4 H, benzylic H), 5.18 (d, 1 H, J = 6 Hz, NCHCO benzylic H), 5.25–5.35 (m, 2 H, benzylic

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H), 5.36–5.45 (m, 2 H, NH), 5.94 (d, 1 H, J = 8 Hz, NH), 6.91 (d, 2 H, J = 9 Hz, aromatic H), 7.20–7.45 (m, 27 H, aromatic H), 7.52 (d, 2 H, J = 9 Hz, aromatic H), 8.14 (d, 2 H, J = 9 Hz, aromatic H), 8.21 (d, 1 H, J = 8 Hz, NH); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 17.97, 20.15, 20.42, 21.30, 23.07, 23.19, 23.52, 27.78, 29.14, 29.92, *31.44* (allylic), 43.6–44.8 (m), 51.95, 52.09, 52.86, 53.28, 54.63, 57.52, 59.34, 63.69, 66.29, 66.74, 69.95, 76.27, 115.16, 123.36, 123.62, 127.33, 127.68, 127.93, 128.40, 128.49, 128.67, 128.73, 128.88, 128.95, 129.01, 129.13, 129.51, 134.11, 134.20, 134.36, 136.34, 136.77, 142.19, 147.72, 156.49, 158.77, 160.45, 164.45, 164.72, 171.22, 171.22, 171.54, 172.73 (m); MS (positive ion FAB, dithiothreitol–dithioerythriol) m/z 1511 (M + 1).

7β-[(N⁵-Acetyl-N⁵-hydroxy-L-ornithyl-N⁵-acetyl-N⁵-hydroxy-L-ornithyl-N⁵-acetyl-N⁵-hydroxy-L-ornithyl-D-4-hydroxyphenylglycyl)amino]-3-chloro-1-carba-3-cephem-4-carboxylic Acid (2). To a solution of **22** (0.059 g, 0.031 mmol) in 2.0 mL of 5% aqueous DMF (prepared from deionized distilled water and HPLC-grade DMF) was added 8.0 μL (0.092 mmol, 3.0 equiv) of concentrated HCl and 0.012 g of 10% Pd–C. This mixture was exposed to hydrogen for 24 h at atmospheric pressure followed by filtration of the catalyst. The DMF–water was removed by evaporation under high vacuum. The residue was dissolved in deionized distilled water (5 mL) and ethyl acetate (5 mL). The organic layer was discarded and the aqueous phase was lyophilized to give 0.038 g (100%) of **2** as a light amber semisolid: $FeCl_3$ positive (red-purple); HPLC (RP-C8) t_R = 44.0 min (eluent = 100% solvent A for 10 min, then a linear gradient to 1:1 of solvent A and B for 25 min, followed by this same ratio for an additional 10 min before reequilibrating the column); IR (KBr) 3700–2400 (br), 1760, 1660 (br) cm^{-1}; ^{1}H NMR (300 MHz, DMF-d_7) δ 1.20–1.35 (m, 2 H, C-1 CH_2), 1.45–1.90 (m, 12 H, CH_2), 1.95–2.15 (m, 9 H, CH_3CON), 2.50–3.00 (C-2 allylic CH_2 obscured by NMR solvent peak and residual DMF), 3.30–3.80 (m, 7 H, CH_2N and C-8 NCH), 3.85–3.95 (m, 1 H, NCHCO), 4.20–4.40 (m, 1 H, NCHCO), 4.45–4.60 (m, 2 H, NCHCO), 5.35–5.60 (m, 2 H, NH and NCHCO benzylic H), 5.90–6.30 (br s, 1 H, phenol OH), 6.85 (d, 2 H, J = 7 Hz, aromatic H), 7.34 (d, 2 H, J = 7 Hz, aromatic H), 7.60–10.30 (br m, 9 H, NH, NH_3^+, and NOH); ^{13}C NMR (75 MHz, DMF-d_7, all signals observed at 20 °C reported) δ 20.72, 20.80, 22.39, 22.83, 24.05, 28.20, 28.85, *31.33* (allylic), 46.87, 47.16, 53.00, 53.41, 53.83, 53.91, 54.17, 57.40, 59.03, 115.91, 128.00–130.00 (br m with major signals at 128.87, 129.34, 129.26), 158.37, 163.78, 165.61, 169.75, 171.00–173.00 (br m with major signals at 171.51, 171.54, 171.79, 172.16, 172.54); MS (positive ion FAB, glycerol) m/z 882 (M + 1); exact mass FAB calcd 882.3400 (M + H), found 882.3403.

Benzyl [[4(S)-Methyl-3(S)-(tert-butoxyformamido)-2-oxo-1-azetidinyl]oxy]acetate (23) was prepared by the method of Miller et al.:[27] TLC (ethyl acetate–hexanes 1:1) R_f = 0.61; IR (TF) 3340, 3040, 2980, 1780, 1710 cm^{-1}; $[\alpha]^{21}_D$ = −42.6° (c = 5.10, $CHCl_3$), ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.40–1.50 (m, 12 H, CH_3 and $C(CH_3)_3$), 3.84 (dq, 1 H, J = 2 and 6 Hz, $NCH(CH_3)$), 4.09 (m, 1 H, NCHCO), 4.61 (AB q, 2 H, J = 17 Hz, OCH_2CO), 5.21 (AB q superimposed over a br s, 3 H, NH and benzylic H), 7.36 (s, 5 H, aromatic H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 15.82, 27.93, 60.12, 63.71, 66.70, 72.38, 79.83, 128.19, 128.28, 134.68, 154.73, 162.59, 167.89. Anal. ($C_{18}H_{24}N_2O_6$) C, H, N.

N-(tert-Butoxycarbonyl)-D-phenylglycine (10). To a solution of *D*-(−)-phenylglycine (**5**) (1.00 g, 6.61 mmol) and potassium bicarbonate (0.682 g, 6.81 mmol) in 20 mL of water was added di-*tert*-butyl dicarbonate (1.44 g, 6.61 mmol) in 20 mL of THF. This mixture was stirred for 18 h, evaporated, and diluted with ethyl acetate, and the ethyl acetate phase was discarded. The aqueous phase was acidified with 10% citric acid and repeatedly extracted with ethyl acetate. The pooled organic phases were washed with brine, then dried, filtered, and evaporated to give 1.65 g (99%) of **10** as a clear oil: IR (TF) 3700–2200 (br), 3310, 2980, 1740, 1660 cm^{-1}; $[\alpha]^{21}_D$ = −127° (c = 16.47, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.20 (s, 9 H, $C(CH_3)_3$), 5.13 (d, 1 H, J = 5 Hz, NCHCO benzylic H), 7.25–7.50 (m, 5 H, aromatic H), 8.18 (d, 1 H, J = 5 Hz, NH); exact mass (EI) calcd for $C_9H_8NO_4$ (M − $C(CH_3)_3$) 195.0532, found 195.0532.

N-(tert-Butoxycarbonyl)-D-phenylglycine N-Succinimido Ester (12). To a solution of **10** (0.840 g, 3.34 mmol) and *N*-hydroxysuccinimide (0.432 g, 3.68 mmol) in 5 mL of anhydrous THF at 0 °C under nitrogen was added dropwise DCC (0.759 g, 3.68 mmol) in 10 mL of anhydrous THF. The mixture was stirred for 43 h, evaporated, diluted with benzene, and filtered through Celite to remove dicyclohexylurea. Evaporation of the solvents gave a crude white solid which was recrystallized from ethyl acetate–hexanes to give 1.14 g (98%) of **10** as a white solid: mp 170–172 °C; IR (KBr) 3360, 2940, 1830, 1785, 1740, 1710 cm^{-1}; $[\alpha]^{21}_D$ = −48.1° (c = 1.14, $CHCl_3$); ^{1}H NMR (300 MHz, $CHCl_3$) δ 1.45 (s, 9 H, $C(CH_3)_3$), 2.78 (s, 4 H, CH_2CH_2), 5.35 (s, 1 H, NCHCO benzylic H), 5.75 (br s, 1 H, NH), 7.30–7.60 (m, 5 H, aromatic H); MS (CI, ammonia) m/z 366 (M + NH_4^+). Anal. ($C_{17}H_{20}N_2O_6$) C, H, N.

Benzyl [[4(S)-Methyl-3(S)-(N-Boc-D-phenylglycyl)-2-oxo-1-azetidinyl]oxy]acetate (24). To a solution of **23** (0.364 g, 1.00 mmol) in 2.0 mL of anhydrous dichloromethane at 0 °C under nitrogen was added anhydrous TFA (386 μL, 5.00 mmol). The reaction was monitored by TLC and, after 3 h, no starting **23** remained. The solution was evaporated, diluted with anhydrous benzene, and evaporated again. Hexanes were added, then evaporated, to afford a pale yellow semisolid. Immediately, 1.0 mL of water, 1.0 mL of ethyl acetate together with active ester **12** (0.166 g, 0.33 mmol), and potassium bicarbonate (0.330 g, 3.30 mmol) were added. The mixture was stirred vigorously overnight. The reaction mixture was diluted with ethyl acetate and water and the aqueous phase was extracted repeatedly with ethyl acetate. The organic phases were washed with brine, then dried, filtered, and evaporated to give an oil. This oil was purified by repeated radial silica gel chromatography eluting with ethyl acetate–hexanes (1:1) to give 0.042 g (25%) of **24** as an oil. This oil was crystallized from ether–hexanes to give a white solid: mp 117–119 °C; IR (TF) 3320, 1785, 1770, 1715, 1680 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.36 (s, 9 H, $C(CH_3)_3$), 1.40 (d, 3 H, J = 6 Hz, CH_3), 3.73 (dq, 1 H J = 2 and 6 Hz, $NCH(CH_3)$), 4.18–4.25 (m, 1 H, NCHCO), 4.50 (AB q, 2 H, J = 12 Hz, OCH_2CO), 5.05–5.20 (m, 3 H, benzylic H and NCHCO benzylic H), 5.55–5.70 (m, 1 H, NH), 6.98 (d, 1 H, J = 6 Hz, NH), 7.22–7.38 (m, 10 H, aromatic H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.30, 28.25, 58.51, 59.53, 63.75, 67.18, 72.66, 80.35, 127.18, 127.30, 128.46, 128.57, 128.66, 129.03, 134.80, 137.58, 155.00, 162.26, 167.99, 170.98; MS (CI, ammonia) m/z 498 (M + 1), 515 (M + NH_4^+). Anal. ($C_{26}H_{31}N_3O_7$) C, H, N.

Benzyl [[4(S)-Methyl-3(S)-[[N⁵-Acetyl-N⁵-(benzyloxy)-N²-(benzyloxycarbonyl)-L-ornithyl-N⁵-acetyl-N⁵-(benzyloxy)-L-ornithyl-N⁵-acetyl-N⁵-(benzyloxy)-L-ornithyl-D-phenylglycyl]amino]-2-oxo-1-azetidinyl]oxy]-acetate (26). To a solution of **24** (0.062 g, 0.125 mmol) in 0.25 mL of anhydrous dichloromethane at 0 °C under nitrogen was added anhydrous TFA (48 μL, 0.622 mmol). The reaction was monitored by TLC which indicated that all starting **24** had been consumed after 2.5 h: The mixture was evaporated, diluted with benzene, and evaporated again. This procedure was repeated three times to give a semisolid. To this residue was added 1.0 mL of water, 1.0 mL of ethyl acetate, crude active tripeptide ester **15** (0.145 g, 0.125 mmol), and finally potassium bicarbonate (0.015 g, 0.150 mmol). The resulting mixture was stirred overnight, then diluted with ethyl acetate and saturated sodium bicarbonate solution. The aqueous phase was extracted repeatedly with ethyl acetate. The combined organic phases were washed with brine, then dried, filtered, and evaporated to give an oil. This oil was purified by radial silica gel chromatography eluting with methanol–ethyl acetate (3:97) to give 0.079 g (48%) of **26** as a foam: TLC (methanol–ethyl acetate 1:15) R_f = 0.41; IR (KBr) 3300, 1785, 1720, 1655 (br); $[\alpha]^{21}_D$ = −24.3° (c = 0.74, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.41 (d, 3 H, J = 6 Hz, CH_3), 1.50–1.80 (m, 12 H, CH_2), 1.88 (s, 3 H, CH_3CON), 2.00–2.15 (m, 6 H, CH_3CON), 3.40–3.90 (m, 7 H, CH_2N and $NCH(CH_3)$), 4.00–4.40 (m, 4 H, NCHCO), 4.52 (AB q, 2 H, J = 15 Hz, OCH_2CO), 4.70–4.85 (m, 6 H, benzylic H), 5.00–5.25 (m, 4 H, benzylic H), 5.49 (d, 1 H, J = 8 Hz, NCHCO benzylic H), 5.97 (br d, 1 H, J = 8 Hz, NH), 7.25–7.45 (m, 30 H, aromatic H), 7.50 (br d, 1 H, J = 8 Hz, NH), 7.55 (br d, 1 H, J = 8 Hz, NH), 8.20 (br d, 1 H, J = 8 Hz, NH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.79, 20.13, 20.40, 23.16, 23.29, 27.79, 29.21, 29.60, 29.86, 43.5–44.6 (br m), 51.86, 53.18, 54.57, 57.73, 59.33, 64.02, 66.73, 67.11, 72.91, 76.21, 127.80, 127.92, 128.18, 128.38, 128.56, 128.66, 128.83, 128.94, 129.11, 134.16, 134.38, 134.81, 136.34, 136.62, 156.48, 163.08, 167.99, 170.92, 171.50, 172.0–173.5 (br m with major signals at 172.77, 713.13; MS (positive ion FAB, dithioerythritol–dithiothreitol) m/z 1318 (M + 1), 1340 (M + Na);

exact mass (FAB) calcd for (M + H) 1318.6036, found 1318.6032.

[[4(*S*)-Methyl-3(*S*)-[(*N*^{5}-Acetyl-*N*^{5}-hydroxy-L-ornithyl-*N*^{5}-acetyl-*N*^{5}-hydroxy-L-ornithyl-*N*^{5}-acetyl-*N*^{5}-hydroxy-L-ornithyl-D-phenylglycyl)amino]-2-oxo-1-azetidinyl]oxy]-acetic Acid (3). A mixture of **26** (0.031 g, 0.021 mmol), 10% Pd–C (0.062 g, 200% w/w), 2.0 mL of ethyl acetate, and 2.0 mL of deionized distilled water were exposed to hydrogen for 1 h at atmospheric pressure. The catalyst was removed by filtration and the aqueous phase was separated from the organic phase. The aqueous phase was lyophilized to give 0.017 g (95%) of **3** as a light tan solid: IR (KBr) 3700–2400 (br), 1772, 1630 (br) cm^{-1}; ^{1}H NMR (300 MHz, D_2O) δ 1.46 (d, 3 H, J = 6 Hz, CH_3); 1.50–1.88 (m, 12 H, CH_2), 1.90–2.10 (m, 9 H, CH_3CON), 3.50–3.80 (m, 7 H, CH_2N and NCH(CH$_3$)), 3.95–4.12 (m, 2 H, NCHCO), 4.25–4.50 (m, 4 H, OCH_2CO and NCHCO), 5.42 (s, 1 H, NCHCO benzylic H), 7.25–7.60 (m, 5 H, aromatic H); MS (positive ion FAB, glycerol) 747 (M – C_6H_5 + H), 826 (M + 1), 842 (M + H_2O), 864 (M + H_2O + Na), (negative ion FAB, glycerol) 824 (M – 1), 841 (M + H_2O – H), (field desorption) 747 (M – C_6H_5 + H), 864 (M + H_2O + Na); exact mass (FAB) calcd for (M + H_2O + H) 842.3896, found 842.3876.

***N*-Boc-4-(benzyloxy)-D-phenylglycine (11).** To a suspension of **7** (1.00 g, 3.89 mmol) and potassium bicarbonate (0.402 g, 4.01 mmol) in 20 mL of water was added dropwise di-*tert*-butyl dicarbonate (0.850 g, 3.89 mmol) in 20 mL of THF. This mixture was stirred for 35 h, then diluted with ethyl acetate and water and filtered. The organic phase was discarded and the aqueous phase acidified to pH 3–4 with 10% citric acid. The resulting aqueous solution was repeatedly extracted with ethyl acetate. The combined organic phases were washed with brine, then dried, filtered, and evaporated to give 0.635 g (46%) of **11** as a tan foam: IR (KBr) 3700–2200 (br), 1720 (br), 1660 cm^{-1}; $[\alpha]^{21}_D$ = –118° (c = 0.500, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.32 (s, 9 H, C(CH_3)$_3$), 5.04 (s, 2 H, benzylic H), 5.30 (br s, 1 H, NCHCO benzylic H), 5.54 (br s, 1 H, NH), 6.95 (d, 2 H, J = 7 Hz, aromatic H), 7.24–7.50 (m, 7 H, aromatic H), 7.92 (br s, 1 H, COOH); MS (CI, ammonia) m/z 375 (M + NH_4^+), 319 (M + NH_4^+ – C(CH$_3$)$_3$).

***N*-Boc-4-(benzyloxy)-D-phenylglycine *N*-Succinimido Ester (13).** To a solution of **11** (1.50 g, 4.21 mmol) and *N*-hydroxysuccinimide (0.533 g, 4.63 mmol) in 20 mL of anhydrous THF at 0 °C under nitrogen was added dropwise DCC (0.956 g, 4.63 mmol) in 20 mL of anhydrous THF. The mixture was stirred overnight, evaporated, diluted with anhydrous benzene, and filtered to remove dicyclohexylurea. The solvents were evaporated to provide an oil/foam which was crystallized from ethyl acetate–hexanes to give 1.45 g (76%) of **13** as a white solid: mp 147–149 °C; IR (KBr) 3340, 1820, 1790, 1745, 1720 cm^{-1}; $[\alpha]^{21}_D$ = –55.4° (c = 0.500, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.46 (s, 9 H, C(CH_3)$_3$), 2.81 (s, 4 H, CH_2CH_2), 5.06 (s, 2 H, benzylic H), 5.41 (br s, 1 H, NCHCO benzylic H), 5.71 (br d, 1 H, NH), 7.00 (d, 2 H, J = 7 Hz, aromatic H), 7.28–7.63 (m, 7 H, aromatic H); MS (CI, ammonia) m/z 472 (M + NH_4^+). Anal. ($C_{24}H_{26}N_2O_7$) C, H, N.

Benzyl [[4(*S*)-Methyl-3(*S*)-(*N*-Boc-4-(benzyloxy)-D-phenylglycyl)-2-oxo-1-azetidinyl]oxy]acetate (25). To a solution of **23** (0.500 g, 1.37 mmol) in 2.0 mL of anhydrous dichloromethane at 0 °C under nitrogen was added anhydrous TFA (529 μL, 6.86 mmol). The reaction was monitored by TLC and, after 4 h, no starting **23** remained. The mixture was evaporated, diluted with benzene, evaporated again, then diluted with hexanes and then evaporated to provide an off-white foam. Immediately, 2.0 mL of ethyl acetate, 2.0 mL of water, active ester **13** (0.623 g, 1.37 mmol), and potassium bicarbonate (0.412 g, 4.12 mmol) were added. The mixture was stirred overnight, then diluted with ethyl acetate and saturated potassium bicarbonate. The organic layer was washed with brine, then dried, filtered, and evaporated to give an oil. Although analysis by TLC revealed only one spot with the *same* R_f value as starting active ester **13** [R_f = 0.63 in ethyl acetate–hexanes 1:1), analysis by ^{1}H NMR showed this material to be a mixture of desired product **25** and active ester **13**. Attempts to destroy the remaining active ester selectively with saturated potassium bicarbonate in ethyl acetate failed. Therefore, this oil was dissolved in 5 mL of ethyl acetate and 5 mL of water, and ammonium acetate (0.160 g) was added. The solution was stirred overnight, then diluted with ethyl acetate. The organic phase was washed with brine, then dried, filtered, and evaporated to give an oil. This oil was purified by radial silica gel chromatography eluting with ethyl acetate–hexanes (1:1) to provide 0.218 g (26%) of **25** as an oil. This oil was crystallized from ether–hexanes to give a fine, cottonlike, white solid: mp 139–140 °C; IR (KBr) 3410, 3340, 1780, 1750, 1705, 1675 cm^{-1}; $[\alpha]^{21}_D$ = –87.6° (c = 0.500, $CDCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.36–1.44 (m, 12 H, CH_3 and C(CH_3)$_3$), 3.79 (dq, 1 H, J = 2 and 6 Hz, NCH(CH$_3$)), 4.25 (d, 1 H, J = 6 Hz, NCHCO), 4.52 (AB q, 2 H, J = 17 Hz, OCH_2CO), 4.98 (s, 2 H, benzylic H), 5.09–5.20 (m, 3 H, NCHCO benzylic H and benzylic H), 5.77 (d, 1 H, J = 7 Hz, NH), 6.90 (d, 2 H, J = 9 Hz, aromatic H), 7.23–7.41 (m, 12 H, aromatic H), 7.45–7.52 (br m, 1 H, NH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.26, 28.31, 58.24, 59.77, 63.51, 67.12, 70.25, 72.71, 80.22, 115.24, 127.35, 127.90, 128.45, 128.51, 128.63, 130.17, 135.05, 136.96, 155.05, 159.05, 162.33, 167.86, 171.32; exact mass (EI) calcd for $C_{33}H_{37}N_3O_8$ 603.2581, found 603.2570. Anal. ($C_{33}H_{37}N_3O_8$) C, H, N.

Benzyl [[4(*S*)-Methyl-3(*S*)-[[*N*^{5}-acetyl-*N*^{5}-(benzyloxy)-*N*^{2}-(benzyloxycarbonyl)-L-ornithyl-*N*^{5}-acetyl-*N*^{5}-(benzyloxy)-L-ornithyl-*N*^{5}-acetyl-*N*^{5}-(benzyloxy)-L-ornithyl-4-(benzyloxy)-D-phenylglycyl]amino]-2-oxo-1-azetidinyl]oxy]acetate (27). To a solution of **25** (0.052 g, 0.086 mmol) in 0.25 mL of anhydrous dichloromethane at 0 °C under nitrogen was added anhydrous TFA (33 μL, 0.430 mmol). This reaction was monitored by TLC and, after 2 h, no starting **25** remained. The solvent was evaporated and the residue was dissolved in anhydrous benzene, evaporated, diluted with hexanes, and evaporated again to give an oil. Immediately, 1.0 mL of water and 1.0 mL of ethyl acetate were added followed quickly by the crude tripeptide active ester **15** (0.100 g, 0.859 mmol) and potassium bicarbonate (0.0103 g, 0.103 mmol). The mixture was stirred overnight, then diluted with ethyl acetate and saturated sodium bicarbonate solution. The organic phase was washed with brine, then dried, filtered, and evaporated to give an oil. This oil was purified by radial silica gel chromatography eluting with methanol–ethyl acetate (3:97) to give 0.050 g (38%) of **27** as an oil: TLC (methanol–ethyl acetate, 1:15) R_f = 0.78; IR (KBr) 3390, 1772, 1760, 1720, 1650, 1630 cm^{-1}; $[\alpha]^{21}_D$ = –32.4° (c = 0.460, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.40 (d, 3 H, J = 6 Hz, CH_3), 1.50–1.85 (m, 12 H, CH_2), 1.89 (s, 3 H, CH_3CON), 2.00–2.15 (m, 6 H, CH_3CON), 3.40–3.90 (m, 7 H, CH_2N and NCH(CH$_3$)), 3.95–4.40 (m, 4, NCHCO), 4.52 (AB q, 2 H, J = 16 Hz, OCH_2CO), 4.70–4.85 (m, 6 H, benzylic H), 4.90–5.25 (m, 6 H, benzylic H), 5.42 (d, 1 H, J = 8 Hz, NCHCO benzylic H), 5.95 (br d, 1 H, J = 8 Hz, NH), 6.90 (d, 2 H, J = 9 Hz, aromatic H), 7.25–7.45 (m, 35 H, aromatic H and NH), 8.13 (br d, 1 H, J = 8 Hz, NH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.88, 20.19, 20.45, 23.21, 23.34, 27.74, 29.21, 29.64, 29.88, 43.5–45.0 (br m), 51.85, 53.26, 54.66, 57.28, 59.39, 64.11, 66.78, 67.15, 69.93, 72.97, 76.26, 115.12, 127.37, 127.81, 127.90, 127.95, 128.41, 128.52, 128.60, 128.70, 128.87, 129.00, 129.16, 134.17, 134.41, 134.84, 136.35, 136.81, 156.52, 158.68, 163.19, 168.01, 171.19, 171.48, 172.0–174.0 (br m with the major signals at 172.60 and 172.82); MS (positive ion FAB, dithiothreitol–dithioerythriol) m/z 1511 (M + 1), (negative ion FAB, dithiothreitol–dithioerythriol) 1509 (M – 1), (field desorption) 1534 (M + Na^+).

[[4(*S*)-Methyl-3(*S*)-[[*N*^{5}-acetyl-*N*^{5}-hydroxy-L-ornithyl-*N*^{5}-acetyl-*N*^{5}-hydroxy-L-ornithyl-*N*^{5}-acetyl-*N*^{5}-hydroxy-L-ornithyl-4-hydroxy-D-phenylglycyl]amino]-2-oxo-1-azetidinyl]oxy]acetic Acid (4). To a solution of **27** (0.024 g, 0.015 mmol) in 1.0 mL of deionized distilled water and 1.0 mL of ethyl acetate was added 10% Pd–C (0.047 g, 200% w/w). This mixture was exposed to hydrogen for 1 h at atmospheric pressure. The catalyst was removed by filtration and the aqueous phase separated. Lyophilization gave 0.0128 g (100%) of **4** as a light tan solid: IR (KBr) 3700–2300 (br), 1773, 1655 (br), 1620 (br); ^{1}H NMR (300 MHz, D_2O) δ 1.50 (d, 3 H, J = 6 Hz, CH_3), 1.55–1.85 (m, 12 H, CH_2), 1.90–2.20 (m, 9 H, CH_3CON), 3.50–3.80 (m, 7 H, CH_2N and NCH(CH$_3$)), 3.93–4.15 (m, 2 H, NCHCO), 4.30–4.53 (m, 4 H, OCH_2CO and NCHCO), 5.38 (s, 1 H, NCHCO benzylic H), 6.94 (d, 2 H, J = 8 Hz, aromatic H), 7.29 (d, 2 H, J = 8 Hz, aromatic H); MS (positive ion FAB, glycerol) 840 (M + 1), (negative ion FAB, glycerol) 838 (M – 1); exact mass (FAB) calcd for (M + H) 840.3739, found 840.3740.

Bioassay Testing Procedures. Plate Bioassay To Measure Siderophore Activity by Zones of Growth Stimulation. *S. flexneri* SA100 was grown overnight in Luria broth. Luria agar

containing the iron chelator ethylenediaminebis(o-hydroxyphenylacetic acid) (EDDA; 250 μg/mL) was seeded with the bacterial strain at a concentration of 10^3/mL, poured into plates, and allowed to solidify.

Compounds were prepared as 10 mM stock solutions in water and were diluted in water to give appropriate test concentrations. Sterile Sensi-disks (BBL) containing 10 μL of the compounds were placed on the surface of the seeded agar and the plates were incubated at 37 °C for 18 h. Diameters of zones of stimulation were measured.

Liquid Growth Bioassay for Siderophore Activity. This assay was completed with *S. flexneri* SA240 (SA100 *iuc*D:Tn5), a siderophore biosynthesis deficient mutant and *E. coli* strains RW193, an *E. coli* K12 *ent*A, *fhu*A, and AN193, a *ent*A, *fhu*A negative mutant (deficient in ability to give the ferrichrome receptor). Overnight cultures of each strain were diluted 1:500 into Luria broth with 10 μg/mL of EDDA with or without 100 μg/mL of the test compound **2**. Growth was monitored by measuring turbidity by absorbance at 650 nm.

Liquid Growth Bioassay for Antimicrobial Activity. The preformed iron complex of each respective siderophore peptide or conjugate was added by filtration through an Acro-Disc 0.2-μm filter assembly to sterile Luria broth containing EDDA (either 0.1 or 1.0 mg/mL) to give solutions of 10 or 50 μM final concentration in each case. Immediately, 20 μL of a 26-h-old Luria broth culture of *E. coli* X580 was added. The culture flasks were then shaken at 37 °C at 300 rpm. Aliquots were removed every 2 h for culture turbidity measurements at 600 nm.

Minimum inhibitory concentration values (MIC) were determined by Eli Lilly and Co. using their standard cephalosporin broad screen assay.

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Registry No. 1, 124650-78-8; **2**, 124620-50-4; **3**, 131080-76-7; **4**, 131080-77-8; **5**, 875-74-1; **7**, 69489-40-3; **8**, 124620-57-1; **9**, 124620-58-2; **10**, 33125-05-2; 11, 127526-64-1; **12**, 39249-27-9; **13**, 131080-78-9; **14**, 124620-56-0; **15**, 131080-79-0; **16**, 123932-46-7; **17**, 124620-59-3; **18**, 124620-60-6; **19**, 124620-61-7; **20**, 124620-62-8; **21**, 124620-63-9; **22**, 124620-64-0; **23**, 90849-37-9; **24**, 131080-80-3; **25**, 131080-81-4; **26**, 131080-82-5; **27**, 131080-83-6; Ph_3CCl, 76-83-5; *N*-hydroxysuccinimide, 6066-82-6.

Quinazoline Antifolates Inhibiting Thymidylate Synthase: 4-Thio-Substituted Analogues

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We report the synthesis of four new 4-thio-5,8-dideazafolic acid analogues and a 4-(methylthio) analogue structurally related to the thymidylate synthase (TS) inhibitor N^{10}-propargyl-5,8-dideazafolic acid. Three N^{10}-propargyl-4-thio-5,8-dideazafolic acid analogues had C^2 amino, hydrogen, and methyl substituents. A 4-thio and a 4-(methylthio) compound each with hydrogen at C^2 and ethyl at N^{10} were also synthesized. In general, the synthetic route involved thionation of the appropriate 4-oxoquinazoline; the sulfur thus introduced was then protected by methylation. Further protection with a pivaloyl group was required for the quinazoline bearing a 2-amino substituent. The protected quinazolines were treated with *N*-bromosuccinimide and the resulting 6-(bromomethyl) compounds were then coupled to the appropriate N-monoalkylated diethyl *N*-(4-aminobenzoyl)-L-glutamate in *N,N*-dimethylacetamide with calcium carbonate as base. The 4-thio-5,8-dideazafolic acids were obtained by removal of the methylthio group with sodium hydrosulfide, followed by deprotection of the carboxyl groups with cold dilute alkali. For the compound containing a pivaloyl protecting group, hot dilute alkali was used. To obtain the 5,8-dideazafolic acid containing a 4-(methylthio) substituent, the corresponding diester was treated with lithium hydroxide which selectively deprotected the carboxyl groups. The five compounds were tested as inhibitors of L1210 TS. It was found that replacement of the 4-oxygen of the quinazoline moiety by sulfur did not alter the TS inhibition. However, the introduction of a methylthio substituent at position 4 severely impaired TS inhibition. All 4-thio compounds were less cytotoxic to L1210 cells in culture than their 4-oxo counterparts.

It is well established that N^{10}-propargyl-5,8-dideazafolic acid[1] is a potent inhibitor of thymidylate synthase (TS)[2-4] and that its in vivo antitumor activity stems from this property alone.[5,6] In clinical trials it gave responses in patients with refractory ovarian, breast, liver, and lung cancer which indicated the potential of an antimetabolite acting cleanly upon TS.[7-11] However, the compound was nephrotoxic and this was thought to result from its poor aqueous solubility. Removal of the 2-amino group from N^{10}-propargyl-5,8-dideazafolic acid gave a much more soluble compound which was 8-fold worse a TS inhibitor yet 8.5-fold more cytotoxic against L1210 cells.[12] 2-

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