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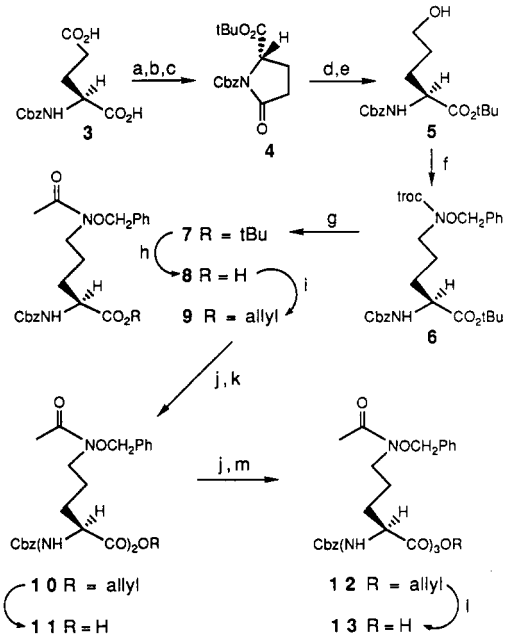
Communications to the Editor

N^5-Acetyl-N^5-hydroxy-L-ornithine-Derived Siderophore–Carbacephalosporin β-Lactam Conjugates: Iron Transport Mediated Drug Delivery

Sir:

In 1982 the structure of the iron-chelating siderophore and antibiotic albomycin δ_1 (X = S) (Figure 1) was firmly established by Benz and co-workers.[1] This microbial iron-transport agent was shown to contain 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 ferrichrome family of siderophores[2] and has been shown to utilize the ferrichrome mediated iron transport system.[3] In an elegant study by Benz,[4] the oxygen analogue of the deferri form of albomycin δ_1 (X = O) was synthesized, replacing the toxic thioribosyl moiety of the albomycins. It has been postulated that the toxic thioribosyl moiety is released by enzymatic processes.[5] This oxygen analogue, however, was reported to have no antimicrobial activity, suggesting that it is very important to have a specific or generally labile toxic moiety for successful enzymatic release. With this in mind, we envisioned that it may be possible to smuggle other antimicrobial agents such as β-lactam antibiotics into the microbial cells by this siderophore iron transport system.

Scheme I[a]



[a] (a) DCC, THF, 0 °C; (b) DCHA; (c) tBuOAc, catalytic $HClO_4$; (d) KOH, CH_3OH, H_2O, 0 °C; (e) Et_3N, ethyl chloroformate, THF followed by filtration and aqueous $NaBH_4$; (f) *N*-troc-OBHA, Ph_3P, DEAD, CH_3CN; (g) HOAc, Ac_2O, Zn; (h) TFA; (i) *O*-allyldiisopropylisourea, CH_3CN, 50 °C; (j) **9**, HBr/HOAc followed by neutralization; (k) **8**, EEDQ, CH_2Cl_2; (l) $(Ph_3P)_4Pd$, OBHA, CH_2Cl_2; (m) **11**, EEDQ, CH_2Cl_2.

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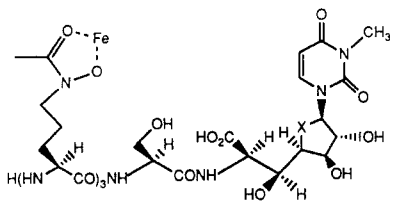
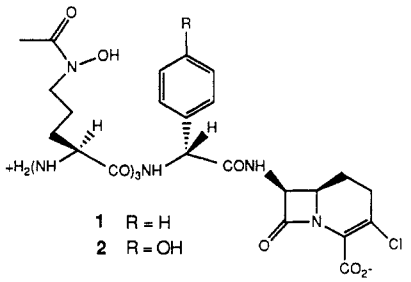


Albomycin δ_1 X = S
Oxygen Analog of Albomycin δ_1 X = O



1 R = H
2 R = OH

Figure 1.

Other investigators[6] have implicated the iron-transport system as a means of β-lactam antibiotic transport. Therefore, we now report preliminary results on the synthesis and antimicrobial activity of the N^5-acetyl-N^5-hydroxy-L-ornithine tripeptide siderophore–carbacephalosporin antibiotic conjugates **1** and **2**.

The first requirement was the development of an efficient synthesis of protected versions of N^5-acetyl-N^5-hydroxy-L-ornithine needed for successful peptide coupling to produce the necessary peptide fragments. Ideally, we desired to produce multigram quantities (15–20 g) of these intermediates in optically pure form so that sufficient quantities of the targets **1**, **2** and the peptide fragments would be available for biological evaluation. Therefore, *N*-carbobenzyloxy-L-glutamic acid (**3**) (Scheme I), which contains the appropriate carbon framework, was treated with dicyclohexylcarbodiimide in tetrahydrofuran at 0 °C, followed by filtration and treatment with dicyclohexylamine to produce the corresponding pyroglutamate. Acidification, extraction, and transesterification with *tert*-butyl acetate and catalytic perchloric acid provided the *tert*-butyl ester **4** in 30–40% yield following flash silica gel chromatography and crystallization. This material was optically pure as monitored by Pirkle chiral stationary phase HPLC chromatography[7] after the necessary derivatization. Hydrolysis of **4** with aqueous potassium hydroxide in tetrahydrofuran at 0 °C followed by conversion of the acid to the mixed anhydride with ethyl chloroformate and triethylamine, filtration, and reduction with an excess of sodium borohydride in water and tetrahydrofuran produced the protected norvaline derivative **5** in 80% yield. Production of **5** was routinely conducted on a 20–30-g scale to afford optically pure **5** as monitored by Pirkle chiral stationary phase HPLC chromatography. Mitsunobu reaction[8] of **5** in the presence of *N*-[(trichloroethoxy)carbonyl]-*O*-benzylhydroxylamine produced the fully protected hydroxamate **6**,[9] which upon treatment with acetic acid, acetic anhydride, and zinc dust gave the protected hydroxamic acid **7** in 50% overall yield from **5**. Deprotection of the *tert*-butyl ester proceeded smoothly with trifluoroacetic acid to give the acid **8**. Treatment of **8** with *O*-allyldiisopropylisourea in acetonitrile produced the allyl ester **9** in 78% yield after silica gel chromatography. Compound **9** also proved to be nearly homogeneous by Pirkle HPLC analysis.

Coupling of the amino acid fragments **8** and **9** in a stepwise fashion produced the protected siderophore **13**. Thus, treatment of the fully protected amino acid **9** with hydrobromic acid in acetic acid followed by neutralization and addition of the free acid **8** and EEDQ in dichloromethane produced the dipeptide **10** in 55% yield. Removal of the allyl ester from **10** with tetrakis(triphenylphosphine)palladium, in the presence of *O*-benzylhydroxylamine as an allyl cation scavenger, gave the acid **11** in 66% yield. The related tripeptide **12** was produced in a similar manner in 78% yield. Palladium-mediated removal of the allyl group from **12** produced the fully protected siderophore[10] **13** (74%) needed for coupling to the β-lactam derivatives.

In order to provide a physiologically effective side chain for the carbacephalosporin as in Loracarbef and a potential site for proteolytic enzyme release of the antibiotic once it has entered the cell, the D-*p*-hydroxyphenylglycyl and D-phenylglycyl residues were chosen for coupling to the protected carbacephalosporin **14**.[11] Treatment of **14** (Scheme II) with the *N*-tritylphenylglycines **15** and **16** in the presence of EEDQ and dichloromethane gave the modified antibiotics **17** (a protected version of Loracarbef) and **19** in 46 and 36% yields, respectively. Deprotection of the trityl protecting group with trifluoroacetic acid

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(10) Satisfactory IR, NMR (^{1}H and ^{13}C), mass spectra, and or combustion analysis were obtained for all new compounds. Characterization data for **(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 (13)**: white amorphous solid; mp 118–119 °C; IR (thin film) 3300, 3420–2700 (br), 1720, 1655 (br) cm^{-1}; $[\alpha]^{28}_D$ = +3.6° (*c* = 1.00, $CHCl_3$); ^{1}H NMR (CD_3OD, 300 MHz) δ 1.50–1.90 (m, 12 H, CH_2), 2.00 (s, 6 H, CH_3CON), 2.10 (s, 3 H, CH_3CON), 3.50–3.80 (m, 6 H, CH_2N), 4.10–4.25 (m, 1 H, NC*H*CO), 4.30–4.50 (m, 2 H, NC*H*CO), 4.75–4.96 (m, 6 H, benzylic H), 5.05 (AB q, 2 H, *J* = 15.6 Hz, benzylic H), 7.20–7.46 (m, 20 H, aromatic H), 7.88 (s, 1 H, N*H*), 8.00–8.20 (m, 2 H, N*H*); ^{13}C NMR (CD_3OD, 75 MHz) δ 20.50, 24.35, 29.77, 30.39, 44.5–45.0 (br m), 53.10, 53.76, 55.71, 67.57, 77.09, 128.68, 128.88, 129.39, 129.61, 129.88, 130.59, 135.92, 138.02, 158.23, 173.63, 174.19 (m), 174.33, 174.61; MS (positive ion FAB, glycerol–methanol matrix) *m*/*z* 940 (M + 1). Anal. Calcd for $C_{50}H_{62}O_{12}N_6$: C, 63.94; H, 6.66; N, 8.95. Found: C, 63.74; H, 6.50; N, 9.01.

(11) This generous gift of was provided by the Eli Lilly and Co., Indianapolis, IN.

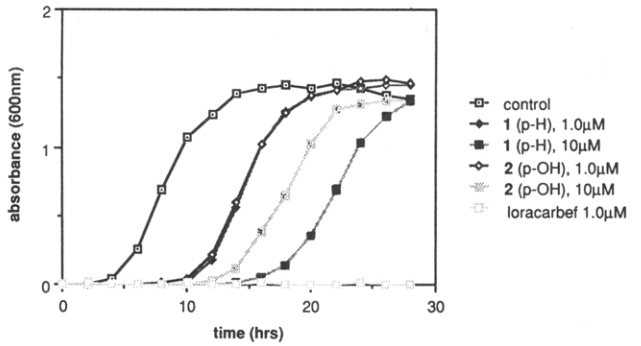
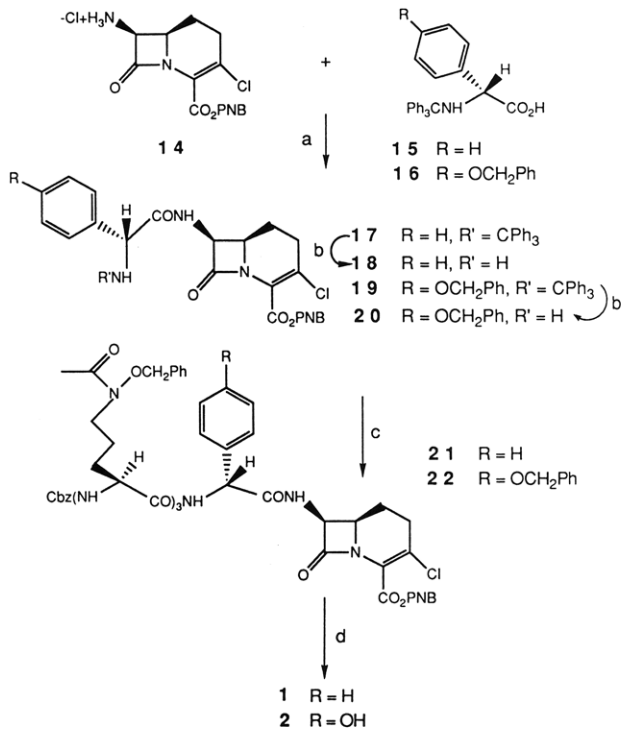


Figure 2. The effect of loracarbef (1.0 μM) and the preformed Fe(III) complexes of compounds **1** and **2** (1.0 and 10 μM) in Mueller-Hinton broth on the growth rate of *E. coli* X580.

followed by neutralization produced the unstable free amines **18** and **20**. Exposure of these amines to the protected siderophore **13** in the presence of EEDQ in dichloromethane gave the protected conjugates **21** (67%) and **22** (44%). Hydrogenation of these protected conjugates under the conditions of 5% aqueous dimethylformamide, 3.0 equiv of hydrogen chloride, 20% w/w of 10% palladium on carbon and hydrogen followed by filtration and lyophilization gave the desired target conjugates **1** and **2** as light amber semisolids[12] in near-quantitative yield.

Scheme II[a]



[a] (a) Et_3N, EEDQ, CH_2Cl_2; (b) TFA followed by neutralization; (c) EEDQ, CH_2Cl_2, **13**; (d) aqueous DMF, HCl, 10% palladium on carbon, hydrogen, 24 h.

Results from incubation[13] of the preformed iron complexes of the siderophore–antibiotic conjugates **1** and **2** with the β-lactam hypersensitive bacterial microorganism *Escherichia coli* X580 are shown in Figure 2. The conjugates **1** and **2** displayed significant antimicrobial activity at both 1.0 and 10.0 μM concentrations as shown by the delayed 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 was due to selection of a mutant of the parent *Escherichia coli* strain that was deficient in some form of iron transport since its growth was unaffected by the presence of the siderophore–antibiotic conjugates. While verification of this postulate is in progress, it should be pointed out that such iron transport deficient mutants might not be expected to be virulent pathogens due to their inability to assimilate iron from the environment.[6d] In fact, under the conditions of iron deficient-media (Luria broth, EDDA), more potent antimicrobial activity was observed to the point that greater than a 5 log kill occurred.[14] The fact that conju-

(12) Characterization data for **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-phenylglycylamino]-1-carba-3-chloro-3-cephem-4-carboxylic acid (1)**: light amber semisolid; $FeCl_3$ positive (red-purple); IR (KBr) 3700–2400 (br), 1765, 1655 (br) cm^{-1}; ^1H NMR (d_7-$(CD_3)_2NCDO$, 300 MHz) 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 dimethylformamide), 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), 4.90 (s, 1 H, benzylic H), 5.40–5.55 (m, 1 H, NH), 5.65–5.75 (m, 1 H, NH), 7.20–7.50 (m, 5 H, aromatic H), 7.55–10.40 (br m, 8 H, NH, NH_3^+, and NOH); ^{13}C NMR (d_7-$(CD_3)_2NCDO$, 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, 34.96 (allylic methylene), 46.88, 47.14, 52.87, 53.60, 53.65, 53.75, 54.19, 57.67, 58.86, 73.66, 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; mass spectrum (positive ion FAB, glycerol) m/z 866 (M^+). **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-(4-hydroxy)phenylglycyl)amino]-3-chloro-1-carba-3-cephem-4-carboxylic acid (2)**: light amber semisolid; $FeCl_3$ positive (red-purple); IR (KBr) 3700–2400 (br), 1760, 1660 (br) cm^{-1}; ^1H NMR (d_7-$(CD_3)_2NCDO$, 300 MHz) 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 dimethylformamide), 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, NCHCO), 4.89 (s, 1 H, benzylic H), 5.35–5.60 (m, 2 H, NH), 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, 8 H, NH, NH_3^+, and NOH); ^{13}C NMR (d_7-$(CD_3)_2NCDO$, 75 MHz, all signals observed at 20 °C reported) δ 20.72, 20.80, 22.39, 22.83, 24.05, 28.20, 28.85, 34.86 (allylic methylene), 46.87, 47.16, 53.00, 53.41, 53.83, 53.91, 54.17, 57.40, 59.03, 73.66, 78.19, 79.29, 79.70, 80.16, 115.91, 128.00–130.00 (br m with major signals at 128.87, 129.34, 129.26), 158.37, 162.97, 163.54, 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); mass spectrum (positive ion FAB, glycerol) m/z 882 (M + 1).

(13) Growth curve procedure: The preformed iron complex of the respective siderophore conjugates **1** and **2** was added to sterile Luria broth (with and without ethylenediaminebis((*o*-hydroxyphenyl)acetic acid) (EDDA)) or to Mueller-Hinton broth by filtration through an Acro-Disc 0.2-μm filter assembly to give solutions 10 μM in each of the conjugates. Immediately, 20 μL of a 25-h-old trypticase soy 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, and the culture turbidity was measured at 600 nm.

(14) Complete manuscripts for the synthesis, nuclear magnetic resonance behavior, growth promoting ability, and antimicrobial activity of each peptide fragment including **1** and **2** are in preparation.

gates 1 and 2 display different growth curves compared to Loracarbef seems to rule out the possibility that siderophore hydrolysis is occurring and releasing Loracarbef prior to cellular absorption. Use of two isogenic *Escherichia coli* strains (RW193, *fhuA* positive; AN193, *fhuA* negative) differing only in the presence or absence of the hydroxamate ferrichrome receptor system suggests uptake by the iron-transport system.[14] In any event, this greater killing effect, use of isogenic strains, and other evidence[14] suggest that it may be entirely possible to smuggle other toxic moieties into microbes via the ferrichrome iron transport system. The possibility of expanding this mode of drug delivery is currently being investigated.

Acknowledgment. We gratefully acknowledge the National Institutes of Health for support of this research and Eli Lilly and Company for the sample of carbacephalosporin 14 and suggested deprotection conditions. We also sincerely appreciate the studies with the isogenic strains of *E. coli* which were performed by Professor Shelley M. Payne (University of Texas, Austin).[14]

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Articles

Design and Synthesis of 4*H*-3,1-Benzoxazin-4-ones as Potent Alternate Substrate Inhibitors of Human Leukocyte Elastase[1]

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Syntex Research (Canada), 2100 Syntex Court, Mississauga, Ontario L5N 3X4, Canada. *Received March 28, 1989*

4*H*-3,1-Benzoxazin-4-ones are alternate substrate inhibitors of the serine proteinase human leukocyte elastase (HL elastase) and form acyl enzyme intermediates during enzyme catalysis. We have synthesized a large variety of benzoxazinones using specific methods that have been adapted to achieve the pattern of ring substitution dictated by theoretical considerations. The results of the inhibition of HL elastase by 175 benzoxazinones are reported herein with reference to hydrophobicity constants D, alkaline hydrolysis rates k_{OH^-}, inhibition constants K_i, and their component acylation and deacylation rate constants, k_{on} and k_{off}, respectively. The ranges for the compounds are considerable; alkaline hydrolysis rates and k_{on} span 6, k_{off} covers 5, and K_i spans 8 orders of magnitude. Multiple regression on this large data set has been used to isolate the contributions of electronic and steric effects, as well as other factors specific to compound stability and elastase inhibition. Essentially, a simple electronic parameter is sufficient to account for almost all the variance in the alkaline hydrolysis data, indicating that electronic factors are the major determinants of this type of benzoxazinone reactivity. Factors that significantly enhance the potency of benzoxazinones I are R_5 alkyl groups and electron withdrawal by R_2. Bulk in R_7 and R_8 and compound hydrophobicity are not significant, but substitution in R_6 is highly unfavorable as are substituents linked via carbon to C_2. The physicochemical factors that underlie these trends in K_i are further analyzed in terms of equations that describe k_{on} and k_{off}. A conclusion that emerges is that chemically stable, potent benzoxazinone inhibitors of HL elastase with inhibition constants in the nanomolar range can be designed with (1) R_5 alkyl groups to inhibit enzyme-catalyzed deacylation, (2) small alkyl substituents linked via heteroatoms to C_2 to enhance acylation and limit deacylation rates, and (3) strongly electron-donating groups at C_7 to stabilize the oxazinone ring to nucleophilic attack. Thus, 2-(isopropylamino)-5-*n*-propyl-7-(dimethylamino)benzoxazinone **95** has $k_{OH^-} = 0.01\ M^{-1}\ s^{-1}$, which extrapolates to a half-life at pH 7.4 of over 8.5 years, and 2-ethoxy-5-ethylbenzoxazinone **38** has $K_i = 42$ pM.

Serine proteinases are attractive targets for medicinal chemists[2] engaged in the design of enzyme inhibitors since the catalytic mechanisms of this class of enzymes have been extensively investigated over the past few decades.[3] A rather compelling picture of enzyme catalyzed hydrolysis of amides and esters has emerged featuring formation and breakdown of tetrahedral and acyl enzyme intermediates.[3] This mechanistic framework, centered on carbonyl chemistry, has provided the basis for the design of a growing number of active site directed reagents conceived as affinity labels,[4] transition-state analogues,[5] and suicide inhibitors.[6] In addition to the broad range of compounds that can serve as substrates for serine proteinases and form acyl enzyme ester intermediates, a great deal is known from physical organic chemistry about ester reactivity and the means of controlling it,[7] which makes the acyl enzyme a natural focal point for rational drug design.

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(1) Contribution no. 309 from the Institute of Bioorganic Chemistry.
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