93579-62-5; **16**, 93579-63-6; **17**, 93579-59-0; **18**, 113111-32-3; **19**, 113111-33-4; **20**, 113111-34-5; **21**, 113111-35-6; **22**, 113111-36-7; **23**, 113111-37-8; **24**, 113111-38-9; **25**, 113111-39-0; H_2NCONH_2, 57-13-6; H_2C=CHCOOEt, 140-88-5; MeI, 74-88-4; EtI, 75-03-6; C_3H_7I, 107-08-4; C_4H_9I, 542-69-8; $H_3C(CH_2)_4I$, 628-17-1; *i*-C_3H_7I, 75-30-9; *i*-C_4H_9I, 513-38-2; $(CH_3)_3CCH_2I$, 15501-33-4; $C_6H_5CH_2Cl$, 100-44-7; $H_2NCH_2C(CH_3)_3$, 5813-64-9; H_2NNH_2, 302-01-2; H_2C=$CHCH_2Br$, 106-95-6; $HOCH_2CH_2NH_2$, 141-43-5; aromatase, 9039-48-9; CSCC enzymes, 37292-81-2; propargyl bromide, 106-96-7.

Coupling Products of Amino Acids to Penicillin V and Cephalothin: Synthesis and Susceptibility to Carboxypeptidases and Lysosomal Enzymes

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Amino acids have been coupled to the carboxyl group of penicillin V and cephalothin by methods that keep the β-lactam ring intact. Derivatives were successfully obtained with both neutral (Leu, Val, Ala, Ile, Trp, Tyr, Gly) and one acidic (Glu) amino acids. The new compounds were inactive in vitro against *Staphylococcus aureus* or *Micrococcus luteus*. Incubation in the presence of purified carboxypeptidases (A, B), soluble lysosomal fractions from liver, or cellular homogenates from liver, kidney, fibroblasts, and macrophages did not allow recovery of the antibacterial activity. Injection in mice also failed to cause liberation of microbiologically active compounds. HPLC studies confirmed that the amide linkage between the antibiotic and the amino acid was not hydrolyzed in the presence of soluble lysosomal fractions from liver. However, conversion of cephalothin and cephalothin–leucine to desacetyl derivatives was observed in the presence of soluble lysosomal fractions and extracts from liver and semipurified orange peel acetylesterase(s). It is concluded that amino acid derivatives of β-lactam antibiotics do not offer potential chemotherapeutic use as prodrugs.

β-Lactam antibiotics are of considerable value in the chemotherapy of bacterial infections because of their great potency, wide spectrum of activity, and low incidence of adverse reactions.[1] These drugs, however, do not accumulate in phagocytic cells[2-5] and, accordingly, are not efficacious against most intracellular bacteria.[6-8] Thus, bacteria that have been ingested by phagocytes but have not been killed by the natural host defense mechanisms appear largely protected from β-lactams. This may result in the development of chronic or recurrent infections.[9,10] β-lactam antibiotics display a free carboxyl group, and this acidic character is probably responsible for their lack of intracellular accumulation, as is observed for other weak organic acids.[11,12] Indeed, conversion of penicillin G into a basic derivative by amidation of its free carboxyl group with a (dimethylamino)propyl moiety results in a considerable enhancement of its accumulation by phagocytic cells and its partial localization in lysosomes.[13] This behavior is consistent with the models proposed to explain the unequal distribution of weak bases and weak acids across membranes separating compartments of different pH.[14] These observations allow the conception of various β-lactam prodrugs, hopefully with enhanced intracellular accumulation.[15] These could result from the coupling of the antibiotic with an amino acid or a small peptide bearing a suitable basic functional group in order to obtain a lysosomotropic drug that would enter cells by diffusion and would subsequently be segregated in lysosomes by proton trapping.[14,15] Coupling could also be made with peptides or proteins that enter cells by endocytosis. These groups would mask the carboxyl group of the β-lactams, while allowing its transport in cells by piggy-back endocytosis.[14-16] Obviously, the validity of these approaches rests upon the capacity for lysosomal enzymes to hydrolyze the amide linkage between the drug and the first amino acid since a free carboxyl group in position 3 of penicillins (or in position 4 of cephalosporins) is essential for antibacterial activity.[17] This paper describes the synthesis of deriva-

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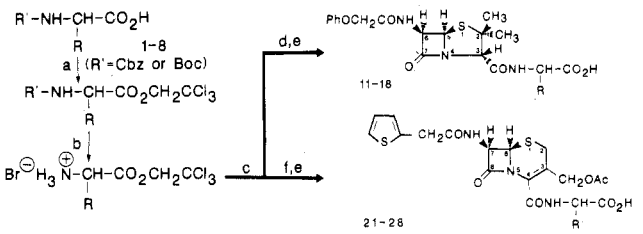
Scheme I. Coupling of Amino Acids to Penicillin V and Cephalothin[a]



[a] Reagents and conditions: (a) $HOCH_2$–CCl_3, *i*-C_3H_7N=C=N-*i*-C_3H_7 (DMAP), CH_2Cl_2, 0–20 °C; (b) HBr, HOAc, 20 °C; (c) K_2CO_3, H_2O (pH ~9), 0 °C or Et_3N, CH_2Cl_2, 0 °C; (d) Pen V-COOH, *i*-C_3H_7N=C=N-*i*-C_3H_7, CH_2Cl_2, 0 °C; (e) Zn, HOAc–DMF, 0 °C; (f) Ceph-COOH, $CH_3C{\equiv}CNEt_2$, CH_2Cl_2, −60 to 20 °C. R: $CH_2CH(CH_3)_2$ (1), $CH(CH_3)_2$ (2), CH_3 (3), H (4), $CH(CH_3)CH_2CH_3$ (5), $CH_2C_8NH_6$ (6), $CH_2CH_2CO_2CH_2CCl_3$ (7), $CH_2C_6H_4OCO_2CH_2CCl_3$ (8).

tives of penicillin V and cephalothin bearing one amino acid residue on the carboxyl group and a study of their behavior in the presence of carboxypeptidases from various sources.

Results

Chemistry. Penicillin V (Pen V) and cephalothin (Ceph) were selected as model antibiotics for our studies since they are stable in the pH range (4.5–7.0) over which they were expected to be exposed in vivo in the extracellular, cytoplasmic, and lysosomal milieus.[12,14] Actually, the half-lifes of Pen V and Ceph in acetate buffer at pH 4.5 were 15.6 and 29 h, respectively. Moreover, the chemical syntheses were expected to be simplified by the absence of reactive functional groups in the acylamino side chains. The syntheses of the modified antibiotics were modeled after procedures commonly used in peptide synthesis. No major effort was made to optimize the yields.

The benzyl, *p*-nitrobenzyl, *tert*-butyl, and trichloroethyl esters of L-leucine (1) were prepared by known methods.[18-20] Coupling of these protected amino acids to penicillin V with *N*,*N*-diisopropylcarbodiimide[21-24] (DMAP, CH_2Cl_2, 0 °C) gave the Pen V-Leu-OR″ (R″ = Bz, 46%; PNB, 85%; *t*-Bu, 52%; TCE, 67%). Deprotection of the benzyl ester by catalytic hydrogenation[25] (Pd 10% on C, EtOAc, or MeOH/HOAc) was a slow process leading to the destruction of the β-lactam. With the *p*-nitrobenzyl ester, the hydrogenolysis[26] was faster, but the purification of the resulting acid 11 was rather tedious. Treatment of the *tert*-butyl ester with trifluoroacetic acid[27] or trimethylsilyl iodide[28] was accompanied by a rapid disappearance of the β-lactam. The best results were obtained with the trichloroethyl ester, which was readily cleaved with zinc powder and acetic acid[29] in dimethylformamide solution to give a good yield of the pure acid 11. This procedure was successfully applied to the preparation of coupling products of penicillin V to various neutral and acidic amino acid derivatives (Scheme I, Table I).

Table I. Coupling Products of Penicillin V to Amino Acids

		$[\alpha]_D$ (concn, %)[a]		
compound	no.	a (TCE esters)	b (free acids)	yield,[b] %
Pen V-Leu	11	+141.6° (1.12)	+160.7° (0.50)	54
Pen V-Val	12	+168.8° (0.58)	+208.3° (0.44)	52
Pen V-Ala	13	+150.0° (1.10)	+207.8° (0.52)	56
Pen V-Gly	14	+169.6° (0.50)	+195.2° (0.50)	43
Pen V-Ile	15	+181.2° (0.50)	+263.7° (0.40)	57
Pen V-Trp	16	+117.8° (0.30)	+180.8° (0.50)	12
Pen V-Glu	17	+116.9° (0.52)	+162.3° (0.30)	12
Pen V-Tyr	18	+113.6° (0.47)	+112.3° (0.57)	29

[a] Measured in $CHCl_3$ solution. [b] Pure free acid from penicillin V (two steps).

Table II. Coupling Products of Cephalothin to Amino Acids

		$[\alpha]_D$ (concn, %)[a]		
compound	no.	a (TCE esters)	b (free acids)	yield,[d] %
Ceph-Leu	21	−21.3° (0.58)	+53.3° (0.35)	55
Ceph-Val	22	−14.3° (0.52)	+68.4° (0.50)	51
Ceph-Ala	23	−10.8° (0.51)	+72.6° (0.26)[b]	59
Ceph-Gly	24	−6.8° (0.51)	+77.1° (0.66)	13
Ceph-Ile	25	−6.5° (0.29)	+38.8° (0.50)[c]	48
Ceph-Trp	26	−17.9° (0.50)	+48.3° (0.82)	24
Ceph-Glu	27	−2.9° (0.41)	+127.4° (0.50)[b]	22
Ceph-Tyr	28	−12.1° (0.30)	+54.6° (0.25)	41

[a] Measured in THF solution, unless otherwise mentioned. [b] Measured in $CHCl_3$ solution. [c] Measured in acetone solution. [d] Pure free acid from cephalothin (two steps).

The method did not apply as well to the coupling of cephalothin to amino acids since diisopropylurea could not be readily separated from the final products. We therefore used (diethylamino)propyne[30] as coupling reagent. This reagent is transformed into *N*-diethylpropionamide, which was easily separated from the coupling products by extraction with ether. This method allowed for the preparation of neutral and acidic amino acid derivatives of cephalothin, without migration of the cephem double bond[24] (Scheme I, Table II).

Protected basic amino acids (N^{ω}-Troc-Lys, N^{ω}-NO_2-Arg, N^{ω}-Tos-Arg) were also coupled to penicillin V and cephalothin according to Scheme I. However, the deprotection of the basic function was always accompanied by the destruction of the β-lactam ring.

All new derivatives 11–18 and 21–28 were fully characterized by their spectroscopic properties. Their purity and, in particular, the absence of contamination by free penicillin V or cephalothin was determined by analytical HPLC on Microstyragel columns. For biological evaluation, the free acids were transformed into their potassium salts.

Biological and Analytical Studies. Whereas penicillin V and cephalothin completely inhibited the growth of *Micrococcus luteus* or *Staphylococcus aureus* at concentrations above 0.02–0.1 mg/L (concentrations causing

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Table III. Conditions Used for Incubation of the Amino Acid Derivatives of Penicillin V and Cephalothin in the Presence of Enzymatic Preparations

preparation	protein concn, mg/mL	pH	time, h	compd no.
kidney homogenate	3	7	6	15–18
liver homogenate	3	7	6	15–18
fibroblasts homogenate	2.5	6	6	11–12
			48	11–12
macrophages homogenate	0.05	6	4	11–12
			48	11–12
lysosomal fraction	0.75	4.5	20	13–23
		5	48	12–11
		5.5	5	12–11
			18	15–18–22–21–27–25–28
			20	14
			45	21–22
		5.9	22	26
		6	48	11–12
		6.5	24	15–28
carboxy-peptidase A	3.2[a]	7.5	18	11–12
			48	11–12
carboxy-peptidase B	0.75[a]	7.5	4	21–22
			18	11–12–15–18–28–27–25
			20	14
			28	21–22
			48	11–12

[a] 113 units of enzyme activity.

50% of growth inhibition [ID_{50}]: 0.01–0.05 mg/L), none of the amino acid derivatives decreased the bacterial growth rate in the whole concentration range investigated (0–10 mg/L). After incubation of these derivatives with carboxypeptidases from various sources and under the conditions listed in Table III, no detectable amount of antibacterial activity was recovered. Samples were prepared at a concentration of 0.02 mM of the derivatives (which would correspond to ca. 7 mg/L of coupled penicillin or cephalothin). When penicillin V or cephalothin were incubated under the same conditions as those described in Table III, their antibacterial activity decreased up to 3- to 10-fold, but their ID_{50}'s remained in all cases lower than 0.1 mg/L. Thus, our results exclude hydrolysis of the derivatives yielding more than 2% of free, active penicillin V or cephalothin (or an equivalent amount of another microbiologically active substance). Yet, the soluble extract from lysosomes used in the present study was previously shown to hydrolyze a N-blocked dipeptide.[31] Furthermore lysosomes are known to contain several carboxypeptidases capable of releasing the C-terminal residue of peptides terminated by any of the amino acids used in this work.[32]

Injection of cephalothin–valine (22), cephalothin–tyrosine (28) or cephalothin–leucine (21) into mice also failed to reveal any significant conversion of these derivatives to microbiologically active substances. Thus, whereas the 48-h sample of urine of mice injected with cephalothin contained microbiologically active substance(s) equivalent to 40% of the injected dose, no detectable activity was

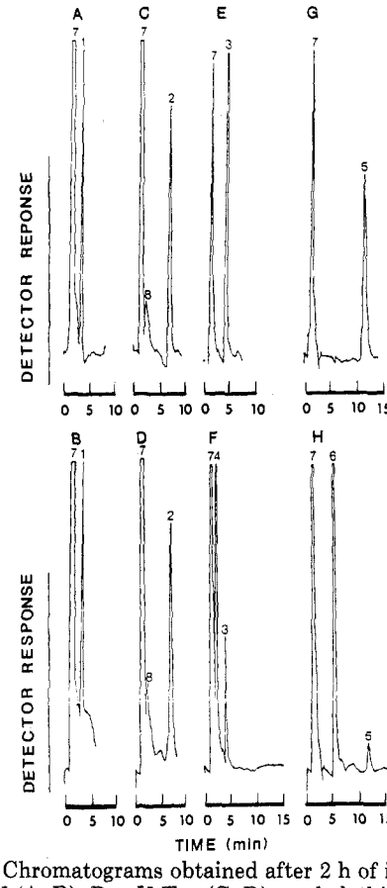

Figure 1. Chromatograms obtained after 2 h of incubation of penicillin V (A, B), Pen V-Tyr (C, D), cephalothin (E, F), and Ceph-Leu (G, H) in acetate buffer pH 4.5 (A, C, E, G) or in the presence of lysosomal enzymes in the same buffer (B, D, F, H). Peaks: 1 = penicillin V (t_R 3.32 min); 2 = Pen V-Tyr (t_R 7.1 min); 3 = cephalothin (t_R 4.04 min); 4 = desacetylcephalothin (t_R 2.3 min); 5 = Ceph-Leu (t_R 11.7 min); 6 = metabolite of Ceph-Leu (t_R 5.18); 7 = breakthrough peak (void volume); 8 = unknown.

recovered from the urine of animals injected with the amino acid derivatives (our technique would have detected an amount of antibacterial substance showing an activity equivalent to 0.5% of the injected cephalothin). Analysis of homogenates from liver, spleen, kidney, lung, heart, and intestine also failed to reveal any antibacterial activity related to the administration of the derivatives (the slight natural antibacterial activity of some organs from control animals was discounted).

Since the biological experiments described above do not distinguish between catabolism of the amino acid derivatives of cephalothin or penicillin into inactive compounds, or mere resistance of the amide linkage between the amino acids and the penicillin V or the cephalothin, we examined by HPLC the metabolism of the derivatives upon incubation in vitro in the presence of a soluble fraction of lysosomes at pH 4.5. Penicillin–tyrosine (18) and cephalothin–leucine (21) were selected for this study because they were easily separated from penicillin V and cephalothin (retention times were 11.7 vs 4.0 min for cephalothin–leucine and cephalothin, and 7.05 vs 3.3 min for penicillin V–tyrosine and penicillin V, respectively). Penicillin V and penicillin V–tyrosine showed similar, prolonged half-lives in the presence or in the absence of lysosomal enzymes (Table IV), suggesting that their slow disappearance was solely due to chemical degradation in aqueous solution at pH 4.5 and 37 °C. In contrast, Table III shows that both cephalothin and cephalothin–leucine were quickly catabolized in the presence of the lysosomal extract. Each of these compounds was readily converted

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Table IV. Compared Half-Life of Penicillin V, Penicillin V-Tyrosine, Cephalothin, and Cephalothin-Leucine in Acetate Buffer (pH 4.5) or in the Presence of Lysosomal Enzymes in the Same Buffer

compound	half-life, h	
	acetate buffer	lysosomal enzymes
penicillin V	17	14
penicillin V-tyrosine	7	7
cephalothin	29	1.1
cephalothin-leucine	41	0.8

into a more polar metabolite (retention times 2.3 and 5.2 min, respectively; Figure 1). The metabolite arising from cephalothin was identified as desacetylcephalothin, on the basis of its behavior in HPLC and in thin-layer chromatography (in two systems; data not shown) when compared to a sample of pure desacetylcephalothin (kindly provided by Dr. H. Kirst, Lilly Research Laboratories, Indianapolis, IN) and to the product of the incubation of cephalothin with semipurified acetylesterase (EC 3.1.1.6) prepared from orange peel.[33] When cephalothin-leucine was exposed to orange peel acetylesterase, it formed the same polar compound as seen in the presence of liver lysosomal soluble extract, suggesting that the latter was desacetylcephalothin-leucine. Exposure of cephalothin and cephalothin-leucine to other subcellular fractions from rat liver yielded the same metabolite, in accordance with the known widespread distribution of acetylesterase in liver.[34,35]

Discussion

The present study is the first systematic approach to the design and synthesis of amino acid prodrugs of penicillins and cephalosporins in order to develop lysosomotropic forms of these antibiotics.[15] Derivatives have been successfully obtained with both neutral and acidic amino acids by a procedure that does not destroy the β-lactam ring of penicillin V or cause bond migration in cephalothin. Our attempt, however, has failed with respect to an essential requirement, which is the ability of the prodrug to regenerate free, active antibiotic through chemical or enzymatic hydrolysis of the amide linkage under conditions compatible with those prevailing in vivo. This is in sharp contrast with peptidic derivatives of drugs such as daunorubicin in which a free amino group is substituted by the amino acid and which can be quickly and almost completely reactivated by the action of aminopeptidase(s).[36] Thus, carboxypeptidase(s) appear unable to detach amino acids substituting the carboxyl function of penicillin V or cephalothin. We recently showed that lysosomal carboxypeptidase(s) do not easily hydrolyze *t*-Boc-D-prolyl-L-alanine, whereas *t*-Boc-L-prolyl-L-alanine is easily severed by these enzymes.[31] Thus the configuration of the carbon adjacent to the carbonyl appears critical for carboxypeptidase(s) action. This most likely explains the resistance of the amino acid derivatives of penicillin V, since the carbon adjacent to the carbonyl is also of the D configuration. Crystallographic data[37,38] indicate that the position of the carboxyl group in cephalosporins, penicillins, and D-proline is very similar with respect to the plane of the adjacent ring structure and largely different from that in L-proline. Moreover, the angles formed by the bonds N–C–CO are almost identical in cephalosporins, penicillins, and D-proline (113°, 114°, and 111°, respectively). This feature could therefore explain the common resistance of the amino acid derivatives of cephalothin, penicillin V, and D-proline to the hydrolysis by carboxypeptidase(s). Our data also suggest that the nature of the acidic or neutral amino acid attached to the β-lactam antibiotic is unimportant in this respect. Since no derivative of either cephalothin or penicillin V could be successfully obtained with a basic amino acid, we cannot, however, determine whether or not the carboxypeptidases that specifically act on peptides with a C-terminal basic amino acid would also show the same dependence on the absolute configuration at the carbon adjacent to the amide carbonyl.

Whatever the explanation and its degree of generalization, our results clearly show that substitution of the carboxyl group of β-lactam antibiotics by amino acids is not a promising approach for the development of prodrug derivatives directed against intracellular bacteria. Muto et al.[39] described ethyl and (di)methyl esters of cephalothin–tyrosine, cephalothin–aspartic acid, cephalothin–glutamic acid, and cephalothin–methionine. These compounds yielded small quantities of microbiologically active substances in vitro upon incubation at pH 7.4 with liver extracts in the presence of a NADPH-generating system. They also partly protected infected animals after oral administration. Since we could not detect reactivation of Ceph-Tyr (**28**) or Ceph-Glu (**27**) in the presence of lysosomal enzymes (Pen V-Tyr was also resistant to the same enzymes) or carboxypeptidases, it is likely that the reactivation of the compounds described by Muto et al. takes place in other cell compartments than lysosomes and that these compounds are therefore unsuitable for intralysosomal antibacterial therapy. Ester prodrugs such as phthalidyl or (pivaloyloxy)methyl esters of penicillins or cephalosporins, which are sensitive to unspecific esterases,[40] are not useful either for this purpose, since they tend to be quickly hydrolyzed extracellularly. Thus, other approaches will need to be followed for the successful design of prodrugs of β-lactam antibiotics capable of entering cells and susceptible to reactivation in lysosomes.

Experimental Section

Chemistry. IR spectra (CH_2Cl_2 unless otherwise mentioned, calibration with polystyrene) were recorded on Perkin-Elmer 257 and 297 spectrometers. ^1H NMR spectra ($CDCl_3$ unless otherwise mentioned, TMS as internal standard) were measured on a Varian XL200 spectrometer. Mass spectra were recorded on Varian MAT 111 or MAT 44 instruments (direct chemical ionization with isobutane or ammonia, DCI-IB or DCI-NH_3, 100 eV, gas pressure 200 μbar). Optical rotations were measured on a Perkin-Elmer 241 ML polarimeter. HPLC analyses were performed on a Waters instrument equipped with both UV (Model 440, 254 nm) and IR (Miran 1A, 1790 cm^{-1}) detectors, with a Microstyragel column eluted with $CHCl_3$ or THF (1 mL/min). Merck silica gel (70–230 mesh or 230–400 mesh) was used for column chromatography. TLC was performed on Merck silica gel 60 F_{254} plates. CH_2Cl_2 and DMF were dried over P_2O_5 (reflux) and then distilled. THF, ether, and petroleum ether were dried over Na and distilled. MeOH and EtOH were distilled over CaO.

Synthesis of the Trichloroethyl Esters of Amino Acids. All the reactions were conducted under dry atmosphere. The N-protected amino acid[17–19] (1 equiv) was dissolved in CH_2Cl_2 and

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treated at 0 °C with trichloroethanol (1 equiv) and then with *N,N*-diisopropylcarbodiimide (DIC, 1 equiv) and a catalytic amount of (dimethylamino)pyridine (5%). The mixture was then stirred at room temperature until disappearance of the absorption band at 2110 cm^{-1}. Filtration of the urea and removal of the solvent left a residue, which was purified by chromatography on silica gel (benzene or benzene–EtOAc, 90:10).

The N-protected amino ester (OTce) was then stirred for 1 h at room temperature with a saturated solution of HBr in dry HOAc. The hydrobromide of the H_2N amino ester was precipitated by addition of dry ether (100–200 mL) and recrystallized in CH_3OH–ether or EtOH–ether.

The free amines were obtained from the hydrobromides **1–8** by treatment of their aqueous solution, at 0 °C, with K_2CO_3 (5%) in order to reach pH 9. Extraction with CH_2Cl_2 (two times), drying ($MgSO_4$), and removal of the solvent quantitatively yielded the free amines, which were directly used in the coupling reactions with the antibiotics.

Coupling of the Amino Acids with the Antibiotics. All the reactions were conducted under a dry and inert atmosphere (argon).

Procedure A. The antibiotic (free acid) was dissolved in CH_2Cl_2 and treated at 0 °C with the amino acid (1 equiv, free amine in CH_2Cl_2 solution) and then with *N,N*-diisopropylcarbodiimide (DIC, 1 equiv). The mixture was allowed to slowly reach 20 °C and stirred until disappearance of DIC was complete as determined by IR spectroscopy. After filtration and removal of the solvent, the residue was treated with EtOAc (8–12 h, 0 °C) in order to precipitate the *N,N*-diisopropylurea, which was filtered off. The filtrate was concentrated, and the residue was chromatographed over silica gel, yielding pure coupling product (assayed by HPLC).

Procedure B. The antibiotic (free acid) was dissolved in CH_2Cl_2 and treated at 0 °C successively with the hydrobromide of the amino acid (1 equiv), Et_3N (1 equiv), and then DIC (1 equiv). The mixture was allowed to warm slowly to 20 °C and stirred until the reaction was complete (IR). Filtration, washing with water, drying ($MgSO_4$), and evaporation gave a crude coupling product, which was purified by chromatography on silica gel (assayed by HPLC).

Procedure C. The antibiotic (free acid) was dissolved in CH_2Cl_2 and treated at −60 °C (dry ice–acetone bath) with the amino acid (1 equiv, free amine in CH_2Cl_2 solution) and then with (diethylamino)propyne (ynamine, 1 equiv). The mixture was allowed to slowly warm to 20 °C and stirred until complete reaction (IR). After removal of the solvent, the *N,N*-diethylpropionamide was extracted with ether–petroleum ether (1:1), the residue was chromatographed on silica gel, and the purity was assayed by HPLC.

The coupling products are isolated as gums (Pen V derivatives) or foams (Ceph derivatives).

Pen V-LeuOTce (11a). From 1.38 g (3.94 mmol) of Pen V in 50 mL of CH_2Cl_2, 1.035 g (3.94 mmol) of H_2N-LeuOTce (**1**) in 20 mL of CH_2Cl_2, and 0.64 mL (3.94 mmol) of DIC was prepared, according to procedure A, 1.012 g (67%) of **11a**: TLC benzene–EtOAc, 80:20, R_f 0.35; IR 1795, 1760, 1710–1680 cm^{-1}; mass (DCI–IB) 594 $(M + 1)^+$; ^1H NMR δ 0.97 (d, 3, J = 6.2 Hz), 0.99 (d, 3, J = 6.2 Hz), 1.53 (s, 3), 1.69 (m, 3), 1.75 (s, 3), 4.23 (s, 1), ~4.55 (AB q, 2), 4.65 (m, 1), 4.66–4.92 (AB q, 2, J = 11.9 Hz), 5.45 (d, 1, J = 4.6 Hz), 5.88 (dd, 1, J = 4.6 and 9.6 Hz), 6.78–7.37 (m, 7).

Pen V-ValOTce (12a). From 1.507 g (4.3 mmol) of Pen V in 50 mL of CH_2Cl_2, 1.07 g (4.3 mmol) of H_2N-ValOTce (**2**) in 10 mL of CH_2Cl_2, and 0.666 mL (4.3 mmol) of DIC was prepared, according to procedure A, 1.612 g (64%) of **12a**: TLC benzene–EtOAc, 80:20, R_f 0.42; IR 1795, 1745, 1710–1680 cm^{-1}; mass (DCI–IB) 580 $(M + 1)^+$; ^1H NMR δ 0.97 (d, 3, J = 6.9 Hz), 1.02 (d, 3, J = 6.9 Hz), 1.52 (s, 3), 1.75 (s, 3), 2.34 (d × sept, 1, J = 6.9 Hz), 4.26 (s, 1), ~4.55 (AB q, 2), 4.66–4.93 (AB q, 2, J = 11.8 Hz), 4.67 (dd, 1, J = 4.5 and 8.9 Hz), 5.46 (d, 1, J = 4.6 Hz), 5.89 (dd, 1, J = 4.6 and 9.5 Hz), 6.89–7.37 (m, 7).

Pen V-AlaOTce (13a). From 1.606 g (4.59 mmol) of Pen V in 60 mL of CH_2Cl_2, 1.012 g (4.59 mmol) of H_2N-AlaOTce (**3**) in 20 mL of CH_2Cl_2, and 0.717 mL (4.59 mmol) DIC was prepared, according to procedure A, 1.749 g (69%) of **13a**: TLC benzene–CH_2Cl_2–EtOAc, 40:40:20, R_f 0.31; IR 1795, 1760, 1680 cm^{-1}; mass (DCI–IB) 552 $(M + 1)^+$; ^1H NMR (CD_3COCD_3) δ 1.55 (d, 3, J = 7.3 Hz), 1.55 (s, 3), 1.75 (s, 3), 4.22 (s, 1), 4.63–4.69 (AB q, 2, J = 11.9 Hz), 4.70 (m, 1), 4.75 (s, 2), 5.46 (d, 1, J = 4.6 Hz), 5.87 (dd, 1, J = 4.6 and 9.5 Hz), 6.86–7.37 (m, 7).

Pen V-GlyOTce (14a). From 0.476 g (1.36 mmol) of Pen V in 35 mL of CH_2Cl_2, 0.391 g (1.36 mmol) of HBr·H_2N-GlyOTce (**4**), 0.137 g (1.36 mmol) of Et_3N, and 0.213 mL (1.36 mmol) of DIC was prepared, according to procedure B, 0.371 g (50%) of **14a**: TLC benzene–EtOAc, 70:30, R_f 0.22; IR 1790, 1760, 1695 cm^{-1}; Mass (DCI-IB) 538 $(M + 1)^+$; ^1H NMR δ 1.57 (s, 3), 1.77 (s, 3), ~4.22 (ABX m, 2, J = 18.5 Hz), 4.25 (s, 1), 4.55 (s, 2), 4.77–4.84 (AB q, 2, J = 11.9 Hz), 5.46 (d, 1, J = 4.6 Hz), 5.86 (dd, 1, J = 4.6 and 9.5 Hz), 6.91–7.38 (m, 7).

Pen V-IleOTce (15a). From 0.977 g (2.79 mmol) of Pen V in 40 mL of CH_2Cl_2, 0.772 g (2.79 mmol) of H_2N-IleOTce (**5**) in 10 mL of CH_2Cl_2, and 0.449 mL (2.79 mmol) of DIC was prepared, according to procedure A, 0.456 g (27%) of **15a**: TLC benzene–EtOAc, 80:20, R_f 0.37. An impure fraction (0.886 g) was chromatographed again (flash column,[41] CH_2Cl_2–CH_3CN, 95:5) to give 0.610 g (37%) of **15a**: IR 1795, 1760, 1710–1685 cm^{-1}; mass (DCI–IB) 594 $(M + 1)^+$; ^1H NMR (200 MHz, CD_3COCD_3) δ 0.92 (t, 3, J = 7.3 Hz), 1.01 (d, 3, J = 6.9 Hz), 1.35 (m, 2), 1.54 (m, 1), 1.56 (s, 3), 1.62 (s, 3), 4.40 (s, 1), 4.61 (dd, 1), 4.62 (s, 2), 4.84–5.00 (AB q, 2, J = 12.2 Hz), 5.62 (d, 1, J = 4.3 Hz), 5.72 (dd, 1, J = 4.3 and 8.2 Hz), 6.95–7.79 (m, 7).

Pen V-TrpOTce (16a). From 0.450 g (1.3 mmol) of Pen V in 25 mL of CH_2Cl_2, 0.438 g (1.3 mmol) of H_2N-TrpOTce (**6**) in 10 mL of CH_2Cl_2, and 0.190 mL (1.3 mmol) of DIC was prepared, according to procedure A, 0.219 g (25%) of impure **16a**: TLC CH_2Cl_2–CH_3CN, 85:15, R_f 0.73. This material was rechromatographed twice (benzene–EtOAc, 85:15, and cyclohexane–CH_2Cl_2–EtOAc, 45:35:20) to give 0.117 g (14%) of pure **16a**: IR 1795, 1760, 1680 cm^{-1}; mass (DCI–NH_3) 684 $(M + NH_4)^+$; ^1H NMR δ 1.44 (s, 3), 1.70 (s, 3), 3.39 (ABX m, 2), 4.16 (s, 1), 4.49 (s, 2), 4.62–4.78 (AB q, 2, J = 11.9 Hz), 5.01 (m, 1), 5.05 (d, 1, J = 4.2 Hz), 5.57 (dd, 1, J = 4.2 and 9.5 Hz), 6.88–7.39 (m, 11), 7.45 (br d, 1, J = 7.6 Hz), 8.45 (br s, 1).

Pen V-Glu(OTce)$_2$ (17a). From 0.350 g (1 mmol) of Pen V in 30 mL of CH_2Cl_2, 0.491 g (1 mmol) of HBr·H_2N-Glu(OTce)$_2$ (**7**), 0.101 g (1 mmol) of Et_3N and 0.156 mL (1 mmol) of DIC was prepared, according to procedure B, 0.192 g (26%) of **17a** (TLC benzene–EtOAc, 80:20, R_f 0.24; two flash chromatographies,[41] cyclohexane–EtOAc, 70:30): IR 1795, 1755, 1695–1680 cm^{-1}; ^1H NMR δ 1.54 (s, 3), 1.75 (s, 3), 2.30 (m, 2), 2.65 (m, 2), 4.21 (s, 1), 4.55 (AB q, 2), 4.66–4.97 (AB q, 2, J = 11.9 Hz), 4.72–4.79 (AB q, 2, J = 12 Hz), 4.75 (m, 1), 5.45 (d, 1, J = 4.6 Hz), 5.90 (dd, 1, J = 4.6 and 9.6 Hz), 6.91–7.37 (m, 7).

Pen V-OTroc-TyrOTce (18a). From 0.760 g (2.17 mmol) of Pen V in 40 mL of CH_2Cl_2, 1.061 g (2.17 mmol) of H_2N-OTroc-TyrOTce (**8**) in 10 mL of CH_2Cl_2, and 0.34 mL (2.17 mmol) of DIC was prepared, according to procedure A, 0.626 g (36%) of **18a** (TLC benzene–EtOAc, 80:20, R_f 0.21; flash chromatography,[41] cyclohexane–EtOAc, 70:30): IR 1795, 1775–1760, 1690 cm^{-1}; mass (DCI–NH_3) 835 $(M + NH_4)^+$; ^1H NMR δ 1.47 (s, 3), 1.73 (s, 3), 3.20 (d, 2, J = 5.7 Hz), 4.18 (s, 1), 4.54 (s, 2), 4.68–4.81 (AB q, 2, J = 11.9 Hz), 4.88 (s, 2), 5.33 (d, 1, J = 4.7 Hz), 5.83 (dd, 1, J = 4.7 and 9.5 Hz), 6.80–7.37 (m, 11).

Ceph-LeuOTce (21a). From 1.508 g (3.81 mmol) of cephalothin in 50 mL of CH_2Cl_2 (suspension), 0.794 g (3.81 mmol) of H_2N-LeuOTce (**1**) in 20 mL of CH_2Cl_2, and 0.542 mL (3.81 mmol) of ynamine was prepared, according to procedure C, 1.894 g (77%) of **21a**: TLC benzene–EtOAc, 60:40, R_f 0.17; IR 1790, 1765–1740, 1710–1680 cm^{-1}; mass (DCI–IB) 640 $(M + 1)^+$; ^1H NMR δ 0.96 (d, 6, J = 5.8 Hz), 1.75 (m, 3), 2.08 (s, 3), 3.35–3.49 (AB q, 2, J = 18.2 Hz), 3.85 (s, 2), 4.65–4.91 (AB q, 2, J = 11.9 Hz), 4.76 (m, 1), 4.88–4.96 (AB q, 2, J = 13.4 Hz), 4.96 (d, 1, J = 4.8 Hz), 5.8 (dd, 1, J = 4.8 and 9 Hz), 6.62 (br d, 1, J = 9 Hz), 7.19–7.41 (m, 4).

Ceph-ValOTce (22a). From 1.619 g (4.08 mmol) of cephalothin in 50 mL of CH_2Cl_2, 1.016 g (4.08 mmol) of H_2N-ValOTce (**2**) in 20 mL of CH_2Cl_2, and 0.582 mL (4.08 mmol) of ynamine was prepared, according to procedure C, 1.901 g (74%) of **22a**: TLC benzene–EtOAc, 70:30, R_f 0.25; IR 1790, 1745, 1710–1681

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cm^{-1}; mass (DCI–IB) 626 $(M + 1)^+$; ^1H NMR δ 0.99 (d, 3, J = 6.9 Hz), 1.05 (d, 3, J = 6.9 Hz), 2.07 (s, 3), 2.36 (d × sept, 1), 3.29–3.56 (AB q, 2, J = 18.1 Hz), 3.85 (s, 2), 4.65–4.93 (AB q, 2, J = 11.9 Hz), 4.77 (dd, 1, J = 4.2 and 8.4 Hz), ~4.93 (AB q, 2), 4.98 (d, 1, J = 4.9 Hz), 5.83 (dd, 1, J = 4.9 and 8.7 Hz), 6.48 (br d, 1, J = 8.7 Hz), 6.98–7.37 (m, 3), 7.30 (br d, 1, J = 8.4 Hz).

Ceph-AlaOTce (23a). From 1.813 g (4.58 mmol) of cephalothin in 60 mL of CH_2Cl_2, 1.010 g (4.58 mmol) of H_2N-AlaOTce (**3**) in 20 mL of CH_2Cl_2, and 0.653 mL (4.58 mmol) of ynamine was prepared, according to procedure C, 2.137 g (78%) of **23a**: TLC benzene–EtOAc, 50:50, R_f 0.38; IR 1795, 1760–1740, 1680 cm^{-1}; mass (DCI–NH_3) 615 $(M + NH_4)^+$; ^1H NMR (CD_3COCD_3) δ 1.48 (d, 3, J = 7.3 Hz), 2.07 (s, 3), 3.46–3.63 (AB q, 2, J = 17.9 Hz), ~3.88 (AB q, 2), ~4.71 (m, 1), 4.82–4.99 (AB q, 2, J = 12.2 Hz), 4.88–4.97 (AB q, 2, J = 12.9 Hz), 5.07 (d, 1, J = 4.7 Hz), 5.75 (dd, 1, J = 4.7 and 9.1 Hz), 6.93–7.35 (m, 3), 8.15 (m, 2).

Ceph-GlyOTce (24a). From 1.36 g (3.43 mmol) of cephalothin in 60 mL of CH_2Cl_2, 0.99 g (3.43 mmol) of HBr·H_2N-GlyOTce (**4**), 0.347 g (3.43 mmol) of Et_3N, and 0.538 mL (3.43 mmol) of DIC was obtained, according to procedure B, 0.561 g (28%) of **24a** (TLC CH_2Cl_2–CH_3CN, 85:15, R_f 0.18, flash chromatography,[41] CH_2Cl_2–EtOAc, 70:30): IR ($CHCl_3$) 1795, 1765–1740, 1680 cm^{-1}; mass (DCI–NH_3) 601 $(M + NH_4)^+$; ^1H NMR (CD_3COCD_3) δ 2.07 (s, 3), 3.45–3.62 (AB q, 2, J = 18 Hz), ~3.88 (AB q, 2), ~4.22 (ABX m, 2), 4.89–4.92 (AB q, 2, J = 12.2 Hz), 4.98 (s, 2), 5.10 (d, 1, J = 4.8 Hz), 5.76 (dd, 1, J = 4.8 and 8.8 Hz), 6.92–8.33 (m, 5).

Ceph-IleOTce (25a). From 0.948 g (2.39 mmol) of cephalothin in 40 mL of CH_2Cl_2, 0.662 g (2.39 mmol) of H_2N-Ile-OTce (**5**) in 10 mL of CH_2Cl_2, and 0.341 mL (2.39 mmol) of ynamine was prepared, according to procedure C, 1.377 g (87%) of **25a**: TLC benzene–EtOAc, 60:40, R_f 0.27; IR 1795, 1745, 1680 cm^{-1}; mass (DCI–NH_3) 657 $(M + NH_4)^+$; ^1H NMR (CD_3CN) δ 0.96 (t, 3, J = 7.2 Hz), 1.05 (d, 3, J = 6.9 Hz), ~1.60 (m, 2), ~1.90 (m, 1), 2.07 (s, 3), 3.46–3.62 (AB q, 2, J = 18.1 Hz), 3.83 (s, 2), 4.64 (dd, 1, J = 5.5 and 8.1 Hz), 4.83–4.91 (AB q, 2, J = 13 Hz), 4.83–4.97 (AB q, 2, J = 12.2 Hz), 5.08 (d, 1, J = 4.9 Hz), 5.76 (dd, 1, J = 4.9 and 8.1 Hz), 7.01–7.43 (m, 5).

Ceph-TrpOTce (26a). From 0.762 g (1.92 mmol) of cephalothin in 30 mL of CH_2Cl_2, 0.646 g (1.92 mmol) of H_2N-TrpOTce (**6**) in 10 mL of CH_2Cl_2, and 0.275 mL (1.92 mmol) of ynamine was prepared, according to procedure C, 0.426 g (31%) of **26a**: TLC CH_2Cl_2–CH_3CN, 80:20, R_f 0.45; IR ($CHCl_3$) 1790, 1760–1750, 1680 cm^{-1}; mass (DCI–NH_3) 730 $(M + NH_4)^+$; ^1H NMR (CD_3COCD_3) δ 2.07 (s, 3), ~3.37 (ABX m, 2), 3.42–3.57 (AB q, 2, J = 18 Hz), 3.89 (s, 2), 4.83 (s, 2), 4.84–4.88 (AB q, 2, J = 13 Hz), ~4.89 (m, 1), 5.02 (d, 1, J = 4.8 Hz), 5.75 (dd, 1, J = 4.8 and 8.7 Hz), 6.94–7.61 (m, 11).

Ceph-Glu(OTce)$_2$ (27a). From 1.025 g (2.59 mmol) of cephalothin in 40 mL of CH_2Cl_2, 1.064 g (2.59 mmol) of H_2N-Glu-$(OTce)_2$ (**7**) in 10 mL of CH_2Cl_2, and 0.369 mL (2.59 mmol) of ynamine was prepared, according to procedure C, 0.868 g (42%) of **27a**: TLC CH_2Cl_2–CH_3CN, 75:25, R_f 0.37; IR (CH_3CN) 1790, 1770–1740, 1680 cm^{-1}; mass (DCI–NH_3) 803 $(M + NH_4)^+$; ^1H NMR (CD_3CN) δ 2.07 (s, 3), ~2.40 (m, 2), 2.75 (t, 2), 3.47–3.63 (AB q, 2, J = 18.3 Hz), 3.83 (s, 2), ~4.80 (m, 1), 4.84–4.99 (AB q, 2, J = 12.1 Hz), 4.86–4.94 (AB q, 2, J = 13 Hz), 4.87 (s, 2), 5.10 (d, 1, J = 4.9 Hz), 5.76 (dd, 1, J = 4.9 and 8.6 Hz), 7.00–7.40 (m, 4), 7.50 (br d, 1, J = 7.6 Hz).

Ceph-OTroc-TyrOTce (28a). From 0.621 g (1.57 mmol) of cephalothin in 25 mL of CH_2Cl_2, 0.77 g (1.57 mmol) of H_2N-OTroc-TyrOTce (**8**) in 5 mL of CH_2Cl_2, and 0.224 mL (1.57 mmol) of ynamine was prepared, according to procedure C, 0.743 g (55%) of **28a**: TLC benzene–EtOAc, 70:30, R_f 0.21; IR 1795, 1785–1750, 1690–1680 cm^{-1}; mass (DCI–IB) 864 $(M + 1)^+$, ^1H NMR (CD_3CN) δ 2.07 (s, 3), ~3.26 (ABX m, 2), 3.42–3.57 (AB q, 2, J = 18 Hz), 3.83 (s, 2), 4.81 (s, 2), 4.87 (s, 2), ~4.88 (m, 1), 4.98 (s, 2), 5.06 (d, 1, J = 4.9 Hz), 5.73 (dd, 1, J = 4.9 and 8.5 Hz), 7.01–7.65 (m, 9).

Deprotection of the Coupling Products. The trichloroethyl ester of the coupling product was dissolved in dimethylformamide and HOAc and stirred in the presence of zinc dust (~9 equiv) for 3 h at 0 °C. After filtration and addition of cold water (10 times the volume of DMF), the solution was extracted with EtOAc (3 times), and the organic layers were washed with HCl (5%) and brine. Drying ($MgSO_4$) and evaporation yielded the free acid derivative, which was precipitated from CH_2Cl_2–petroleum ether or ether–petroleum ether (purity assessed by HPLC).

Pen V-LeuOH (11b). Compound **11a** (0.851 g, 1.43 mmol) was treated with 3.57 mL of DMF, 1.027 mL of HOAc, and 0.829 g (12.72 mmol) of Zn to give 0.539 g (81%) of **11b**: IR 1795, 1720, 1710–1680 cm^{-1}; mass (DCI–IB) 464 $(M + 1)^+$; ^1H NMR δ 0.95 (d, 6, J = 5.7 Hz), 1.53 (s, 3), 1.65 (m, 3), 1.73 (s, 3), 4.27 (s, 1), ~4.56 (AB q, 2), ~5.05 (m, 1), 5.50 (d, 1, J = 4.6 Hz), ~5.65 (m, 2), 5.90 (dd, 1, J = 4.6 and 9.6 Hz), 6.92–7.41 (m, 5).

Pen V-ValOH (12b). Compound **12a** (1.407 g, 2.4 mmol) was treated with 6 mL of DMF, 1.8 mL of HOAc, and 1.392 g (21.36 mmol) of Zn to give 0.901 g (82%) of **12b**: IR 1795, 1725, 1700–1680 cm^{-1}; mass (DCI–NH_3) 467 $(M + NH_4)^+$; ^1H NMR δ 0.97 (d, 3, J = 7 Hz), 0.99 (d, 3, J = 7 Hz), 1.52 (s, 3), 1.74 (s, 3), 2.21 (d × sept, 1), 4.30 (s, 1), ~4.56 (AB q, 2), 4.56 (dd, 1), 5.50 (d, 1, J = 4.6 Hz), 5.95 (dd, 1, J = 4.6 and 9.7 Hz), 6.91–7.47 (m, 8).

Pen V-AlaOH (13b). Compound **13a** (0.644 g, 1.16 mmol) was treated with 3 mL of DMF, 0.874 mL of HOAc, and 0.676 g (10.32 mmol) of Zn to give 0.403 g (82%) of **13b**: IR (CH_3CN) 1790, 1740, 1700–1680 cm^{-1}; mass (DCI–NH_3) 422 $(M + 1)^+$; ^1H NMR (CD_3COCD_3) δ 1.40 (d, 3, J = 7.2 Hz), 1.56 (s, 3), 1.64 (s, 3), 4.26 (s, 1), ~4.47 (m, 1), 4.62 (s, 2), 5.60 (d, 1, J = 4.4 Hz), 5.71 (dd, 1, J = 4.4 and 8.9 Hz), 6.95–7.36 (m, 7).

Pen V-GlyOH (14b). Compound **14a** (0.311 g, 0.57 mmol) was treated with 1.425 mL of DMF, 0.427 mL of HOAc, and 0.330 g (5.07 mmol) of Zn to give 0.206 g (87%) of **14b**: IR 1790, 1710, 1690 cm^{-1}; mass (DCI–NH_3) 425 $(M + NH_4)^+$; ^1H NMR (CD_3CN) δ 1.55 (s, 3), 1.68 (s, 3), 3.95 (d, 2, J = 5.7 Hz), 4.26 (s, 1), 4.62 (s, 2), 5.57 (d, 1, J = 4.4 Hz), 5.73 (dd, 1, J = 4.4 and 9.1 Hz), 7.00–7.51 (m, 8).

Pen V-IleOH (15b). Compound **15a** (0.450 g, 0.74 mmol) was treated with 1.85 mL of DMF, 0.555 mL of HOAc, and 0.429 g (6.586 mmol) of Zn, to give 0.284 g (89%) of **15b**: IR ($CHCl_3$) 1795, 1715, 1690–1670 cm^{-1}; mass (DCI–NH_3) 437 $(M - CO_2 + NH_4)^+$; ^1H NMR δ 0.89 (t, 3, J = 6.5 Hz), 0.97 (d, 3, J = 6.8 Hz), ~1.23 (m, 2), ~1.50 (m, 1), 1.54 (s, 3), 1.75 (s, 3), 4.31 (s, 1), 4.57 (s, 2), 4.62 (dd, 1), 5.52 (d, 1, J = 4.6 Hz), 5.98 (dd, 1, J = 4.6 and 9.5 Hz), 6.93–7.44 (m, 7).

Pen V-TrpOH (16b). Compound **16a** (0.274 g, 0.41 mmol) was treated with 0.93 mL of DMF, 0.308 mL of HOAc, and 0.196 g (3.65 mmol) of Zn to give 0.196 g (89%) of **16b**: IR (CH_3CN) 1795, 1710, 1690–1670 cm^{-1}; mass (DCI–NH_3) 510 $(M - CO_2 + NH_4)^+$; ^1H NMR (CD_3COCD_3) δ 1.37 (s, 3), 1.58 (s, 3), ~3.34 (ABX m, 2), 4.19 (s, 1), 4.62 (s, 2), ~4.78 (m, 1), 5.47 (d, 1, J = 4.4 Hz), 5.67 (dd, 1, J = 4.4 and 9.1 Hz), 6.95–7.72 (m, 13).

Pen V-Glu(OH)$_2$ (17b). Compound **17a** (0.192 g, 0.258 mmol) was treated with 1.29 mL of DMF, 0.387 mL of HOAc, and 0.299 g (4.50 mmol) of Zn to give 0.057 g (46%) of **17b**: IR 1795, 1740, 1700–1680 cm^{-1}; mass (DCI–NH_3) 453 $(M - CO_2 + NH_4)^+$; ^1H NMR (CD_3COCD_3) δ 1.57 (s, 3), 1.64 (s, 3), 2.08 (m, 2), 2.47 (t, 2), 4.27 (s, 1), ~4.61 (m, 1), 4.63 (s, 2), 5.60 (d, 1, J = 4.7 Hz), 5.71 (dd, 1, J = 4.7 and 8.9 Hz), 6.96–7.36 (m, 5), 7.74 (br d, 1), 7.87 (br d, 1).

Pen V-TyrOH (18b). Compound **18a** (0.335 g, 0.409 mmol) was treated with 2 mL of DMF, 0.615 mL of HOAc, and 0.480 g (7.28 mmol) of Zn to give 0.171 g (81%) of **18b**: IR (CH_3CN) 1790, 1710, 1660 cm^{-1}; mass (DCI–NH_3) 532 $(M + NH_4)^+$; ^1H NMR (CD_3COCD_3) δ 1.53 (s, 3), 1.77 (s, 3), ~3.21 (ABX m, 2), 4.34 (s, 1), 4.78 (s, 2), ~4.81 (m, 1), 5.71 (d, 1, J = 4.4 Hz), 5.88 (dd, 1, J = 4.4 and 9.2 Hz), 6.92–7.66 (m, 13).

Ceph-LeuOH (21b). Compound **21a** (0.270 g, 0.42 mmol) was treated with 1 mL of DMF, 0.315 mL of HOAc, and 0.243 g (3.74 mmol) of Zn to give 0.156 g (72%) of **21b**: IR (CH_3CN) 1790, 1745, 1700–1680 cm^{-1}; mass (DCI–IB) 492 $(M + 1 - H_2O)^+$; ^1H NMR (CD_3COCD_3) δ 0.91 (d, 3, J = 6.1 Hz), 0.93 (d, 3, J = 6.1 Hz), ~1.64 (m, 2), ~2.00 (m, 1), 2.09 (s, 3), 3.44–3.62 (AB q, 2, J = 18 Hz), 3.89 (s, 2), ~4.60 (m, 1), 4.89–4.98 (AB q, 2, J = 13.1 Hz), 5.05 (d, 1, J = 4.8 Hz), 5.77 (dd, 1, J = 4.8 and 8.9 Hz), 6.93–8.22 (m, 5).

Ceph-ValOH (22b). Compound **22a** (0.590 g, 0.94 mmol) was treated with 2.3 mL of DMF, 0.705 mL of HOAc, and 0.545 g (8.37 mmol) of Zn to give 0.326 g (69%) of **22b**: IR (KBr) 1785, 1740, 1700, 1650 cm^{-1}; mass (DCI–NH_3) 513 $(M + NH_4)^+$; ^1H NMR (CD_3COCD_3) δ 0.69 (d, 3, J = 6.9 Hz), 1.02 (d, 3, J = 6.9 Hz), 2.06 (s, 3), ~2.17 (d × sept, 1), 3.46–3.63 (AB q, 2, J = 18 Hz), 3.86 (s, 2), 4.53 (dd, 1, J = 5 and 8.5 Hz), 4.86–4.98 (AB q, 2, J = 13.1

Hz), 5.06 (d, 1, J = 4.9 Hz), 5.82 (dd, 1, J = 4.9 and 8.9 Hz), 6.93–8.18 (m, 5).

Cep-AlaOH (23b). Compound **23a** (1.197 g, 2 mmol) was treated with 5 mL of DMF, 1.5 mL of HOAc, and 1.16 g (17.8 mmol) of Zn to give 0.709 g (76%) of **23b**: IR (KBr) 1785, 1740, 1710, 1670–1650 cm^{-1}; mass (DCI–NH_3) 485 $(M + NH_4)^+$; ^1H NMR (CD_3COCD_3) δ 1.41 (d, 3, J = 7.2 Hz), ~2.05 (s, 3), 3.45–3.62 (AB q, 2, J = 18 Hz), 3.88 (s, 2), 4.57 (m, 1), 4.87–4.96 (AB q, 2, J = 13 Hz), 5.07 (d, 1, J = 4.8 Hz), 5.77 (dd, 1, J = 4.8 and 8.6 Hz), 6.25–7.34 (m, 3), 7.86 (br d, 1), 8.16 (br d, 1).

Ceph-GlyOH (24b). Compound **24a** (0.250 g, 0.427 mmol) was treated with 1 mL of DMF, 0.32 mL of HOAc, and 0.247 g (3.80 mmol) of Zn to give 0.090 g (46%) of **24b**: IR (KBr) 1785, 1740, 1700, 1670–1650 cm^{-1}; mass (DCI–NH_3) 471 $(M + NH_4)^+$; ^1H NMR (CD_3COCD_3) δ 2.05 (s, 3), 3.45–3.62 (AB q, 2, J = 18 Hz), 3.88 (AB q, 2), ~4.10 (ABX m, 2), 5.11 (d, 1, J = 4.9 Hz), 5.77 (dd, 1, J = 4.9 and 8.5 Hz), 6.24–7.32 (m, 3), 7.98 (br d, 1), 8.07 (br d, 1).

Ceph-IleOH (25b). Compound **25a** (0.654 g, 1 mmol) was treated with 2.5 mL of DMF, 0.75 mL of HOAc, and 0.580 g (8.9 mmol) of Zn to give 0.264 g (50%) of **25b**: IR (KBr) 1785, 1740, 1710, 1660–1650 cm^{-1}; mass (DCI–NH_3) 257 $(M + NH_4)^+$; ^1H NMR (CD_3COCD_3) δ 0.90 (t, 3, J = 7.3 Hz), 0.99 (d, 3, J = 6.9 Hz), ~1.31 (m, 2), ~1.53 (m, 1), 2.07 (s, 3), 3.46–3.63 (AB q, 2, J = 18 Hz), 3.89 (s, 2), 4.58 (dd, 1, J = 5.1 and 8.4 Hz), 4.85–4.97 (AB q, 2, J = 13 Hz), 5.06 (d, 1, J = 4.9 Hz), 5.82 (dd, 1, J = 4.9 and 8.8 Hz), 6.93–7.32 (m, 3), 7.62 (br d, 1), 8.12 (br d, 1).

Ceph-TrpOH (26b). Compound **26a** (0.250 g, 0.35 mmol) was treated with 0.875 mL of DMF, 0.262 mL of HOAc, and 0.203 g (3.115 mmol) of Zn to give 0.160 g (78%) of **26b**: IR (KBr) 1785, 1740, 1710, 1680–1665 cm^{-1}; ^1H NMR (CD_3COCD_3) δ 2.07 (s, 3), 3.32 (d, 2), 3.40–3.56 (AB q, 2, J = 18.2 Hz), 3.89 (s, 2), 4.83 (AB q, 2), 4.87 (m, 1), 5.01 (d, 1, J = 4.9 Hz), 5.77 (dd, 1, J = 4.9 and 8.7 Hz), 6.93–8.17 (m, 11).

Ceph-Glu(OH)$_2$ (27b). Compound **27a** (0.868 g, 1.1 mmol) was treated with 5.5 mL of DMF, 1.65 mL of HOAc, and 1.276 g (19.58 mmol) of Zn to give 0.311 (53%) of **27b**: IR (CH_3CN) 1790, 1745, 1720–1700, 1690–1680 cm^{-1}; ^1H NMR (CD_3CN) δ 2.07 (s, 3), ~2.40 (m, 2), 2.56 (t, 2), 3.50–3.68 (AB q, 2, J = 18.1 Hz), 3.89 (s, 2), ~4.58 (m, 1), 4.87–5.00 (AB q, 2, J = 13 Hz), 5.14 (d, 1, J = 4.9 Hz), 5.81 (dd, 1, J = 4.9 and 8.6 Hz), 7.06–7.49 (m, 5).

Ceph-TyrOH (28b). Compound **28a** (0.808 g, 0.933 mmol) was treated with 4.66 mL of DMF, 1.39 mL of HOAc, and 1.08 g (16.60 mmol) of Zn to give 0.384 g (74%) of **28b**: IR (KBr) 1790, 1740, 1700, 1660 cm^{-1}; mass (DCI–IB) 560 $(M + 1)^+$; ^1H NMR (CD_3COCD_3) δ 2.07 (s, 3), 3.05 (d, 2, J = 6.5 Hz), 3.42–3.58 (AB q, 2, J = 18.1 Hz), 3.89 (s, 2), 4.75 (m, 1), 4.80–4.87 (AB q, 2, J = 12.8 Hz), 5.06 (d, 1, J = 4.8 Hz), 5.78 (dd, 1, J = 4.8 and 8.9 Hz), 6.16–8.11 (m, 11).

Formation of the Potassium Salts. The free acids **11b–18b** and **21b–28b** were dissolved in CH_2Cl_2 and water at 0 °C and treated with stirring with $NaHCO_3$ (1 or 2 equiv for Glu derivatives). After 30 min, the aqueous phase was separated and lyophilized.

Biological and Analytical Studies. Antibacterial Properties of the Derivatives. The antibacterial activity of the amino acid derivatives of penicillin V and cephalothin was determined, after dissolution in 3 mM phosphate buffer pH 7.2, toward *S. aureus* (ATCC 25923) or *M. luteus* (*Sarcina lutea*; ATCC 9341) by using a turbidimetric method,[42] which evaluates the inhibitory effect of the antibiotic on bacteria growing in suspension. Results are expressed as ID_{50}, i.e., the antibiotic concentration reducing bacterial growth to 50% of that observed in a control culture without antibiotic.

In Vitro Hydrolysis of the Amino Acid Derivatives. The potential hydrolysis of the amino acid derivatives into free penicillin V or cephalothin or other microbiologically active products was examined by exposure to the following enzyme preparations.

(a) Homogenates of Rat Liver or Kidney. Tissue was homogenized with a Potter-Elvejhem tissue grinder in 3 mM phosphate buffer, pH 7.4, in 0.9% NaCl at a final protein concentration of 3 mg/mL. Incubation was carried out in the presence of 2 mM cysteine for 6 h. After incubation, the samples were diluted, centrifuged at 50 000 rpm for 30 min, and filtered on Millipore filters (0.22 μm) before microbiological assay.

(b) Homogenates of Cultured Rat Fibroblasts or Mouse Peritoneal Macrophages. Cells were grown as previously described,[43,44] collected, homogenized in 3 mM phosphate buffer–0.9% NaCl, and used at a protein concentration of 2.5 and 0.05 mg/mL, respectively. The incubation was carried out in 5 mM acetate buffer pH 6.0–2 mM cysteine–2 mM EDTA, and the samples were thereafter treated as above.

(c) Purified Carboxypeptidase A (EC 3.4.17.1) and Carboxypeptidase B (3.4.17.2) from Pancreas. These enzymes were obtained from Boehringer-Mannheim, GmbH, West Germany. The incubation was carried out for 4–48 h in 0.05 M Tris·HCl buffer pH 7.5–0.45 M KCl under sterile conditions, and the samples were directly used for microbiological assay.

(d) Soluble Fraction of Lysosomes. This fraction was purified from rat liver according to Trouet[45] and used at protein concentration of 0.75 mg/mL. Incubation was carried out for up to 48 h in 10 mM acetate buffer (pH ranging from 4.5 to 6.5)–2 mM EDTA and 2 mM cysteine under sterile conditions, and the samples were used directly for microbiological assays.

The concentration of the derivatives in the presence of the enzyme preparations was 0.9–1.6 mM. Before microbiological assay, the samples were diluted at least 100-fold. Controls included penicillin V and cephalothin treated in a similar fashion with the enzyme preparations, and enzyme preparations without antibiotic added. The latter had no or only negligable antimicrobial activity under our conditions of assay. The product of the exposure of the amino acid derivatives to lysosomal enzymes at pH 4.5 was further examined by HPLC. Aliquots were taken after various times of incubation at 37 °C; the protein was precipitated with 50% (vol/vol) acetonitrile. After centrifugation, the supernatant was diluted with the HPLC eluent. A Hewlett-Packard 1084B HPLC instrument was used with Altex reversed-phase silica gel column (Ultrasphere ODS, 250 × 4.6 mm), eluted at a flow rate of 1.5 mL/min with 20% or 25% acetonitrile in 50 mM phosphate buffer, pH 6.3. Detection was made by UV spectroscopy at 254 nm with a PYE-Unicam (Model LC/UV) UV detector.

In Vivo Experiments. Three groups of two mice (NMRI) were injected intraperitoneally with cephalothin, cephalothin–leucine (**21**), cephalothin–valine (**22**), or cephalothin–tyrosine (**28**). The drug was administered as a single dose of 20 mg in physiological saline solution. Animals were placed in metabolic cages, and urine was continuously collected over the next 48 h. Blood samples were obtained 1, 3, and 24 h after injection, and plasma was separated by centrifugation of the heparinized blood. Organs were removed immediately after decapitation. Tissue specimens were weighted and homogenized in 3 mM phosphate buffer pH 6.4–0.09% NaCl with a Potter-Elvehjem homogenizer (liver, spleen, kidney) or an Ultra-Turrax tissue disperser (stomach, small intestine, lung, heart). After centrifugation at 2000 rpm for 10 min, the supernatants were diluted in phosphate-buffered saline and microbiological assay was performed with a disk–plate diffusion method with *S. aureus* as test organism.[42]

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Quinolone Antibacterial Agents. Synthesis and Structure-Activity Relationships of 8-Substituted Quinoline-3-carboxylic Acids and 1,8-Naphthyridine-3-carboxylic Acids

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A series of 7,8-disubstituted 1-cyclopropyl-6-fluoroquinoline-3-carboxylic acids, 7-substituted 1-cyclopropyl-6-fluoro-1,8-naphthyridine-3-carboxylic acids, and 10-substituted 9-fluoropyridobenzoxazine-6-carboxylic acids has been prepared and evaluated for antibacterial activity. The side chains examined at the 7-position (benzoxazine 10-position) included piperazinyl (**g**), 3-aminopyrrolidinyl (**a**), 3-(aminomethyl)pyrrolidinyl (**b**), and alkylated 3-(aminomethyl)pyrrolidinyl (**c-f**). Variations at C-8 of the quinolone ring system included hydrogen, nitro, amino, fluorine, and chlorine. The relative enhancement of in vitro activities by the side chains on the 8-hydrogen quinolone and 1,8-naphthyridine against Gram-negative organisms was **a** > **b** > **g** > **c-f**. The activity imparted to the substituted quinolone nucleus by the 8-substituent was in the order F > Cl > naphthyridine > H > benzoxazine > NH_2 > NO_2. These trends were retained in vivo.

Since the discovery of nalidixic acid by Lesher in 1962,[1] the quinolone antibacterials have emerged as a significant class of chemotherapeutic agents. The initial compounds possessed oral activity against Gram-negative bacteria but suffered as a class in their inability to affect Gram-positive strains.[2] The initial structure-activity relationship (SAR) correlation by Meltzer and Kaminsky[3] resulted in compounds with a slightly broader spectrum of activity. Oxolinic acid, which possessed some Gram-positive in vitro activity as well as an enhanced antipseudomonal effect, was synthesized during this study. The next series of quinolones emerged with the synthesis of flumequine (**9**) (Figure 1).

The major structural change in these types was the introduction of a fluorine at C-6, which gave rise to compounds that were more potent than nalidixic acid and were comparable to oxolinic acid in vitro.[4] This modification continues to be a structural feature of all current synthetic analogues and is one of the major factors for the greatly increased activity of all of the current quinolones.

As research in this area became more extensive, positional SAR studies established the desirability of having a basic group at position 7.[5,6] This research resulted in the nearly simultaneous discovery of norfloxacin (**10**) and enoxacin (**11**) (Figure 1), which are characterized by having a piperazine moiety at C-7. These two compounds, which possess both broad-spectrum activity and oral efficacy, were the next members of this class of compounds. These discoveries produced a resurgence of interest in the quinolone area, resulting in the synthesis of many new and highly active agents such as ofloxacin (**12**), pefloxacin (**13**), difloxacin (**14**), and one of the most active quinolones to date, ciprofloxacin (**1g**).

These examples indicate the desirability of the piperazine substituent at C-7. The greatly increased Gram-negative activity as well as the appearance of some Gram-positive activity, in vitro, is a characteristic of compounds having a piperazine moiety at C-7.[6] It also appears that the piperazine substituent allows better in vivo activity without a proportional increase in side effects.[2d] For these reasons, the compounds currently in clinical trials feature the 7-piperazinyl moiety.

Our initial SAR studies investigated deviations from the set substitutional pattern by the use of a piperazine mimic,

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