Pharmaceutical Research, Vol. 6, No. 5, 1989

Research Article

Kinetics of the Aspartyl Transpeptidation of Daptomycin, a Novel Lipopeptide Antibiotic

Lee E. Kirsch,[1,2] R. Michael Molloy,[1] Manual Debono,[1] Patrick Baker,[1] and Khadiga Z. Farid[1]

Received October 21, 1988; accepted January 2, 1989

Two degradation products of the lipopeptide antibiotic, daptomycin, were identified and the reaction pathway and kinetics were delineated in aqueous solution at 60°C, pH range 3 to 8 and ionic strength 0.01. The degradation products were 1) a succinimido intermediate (anhydro-daptomycin) formed by attack of side-chain carbonyl on the peptide-bond nitrogen in the asp–gly sequence and 2) a β-asp daptomycin isomer formed by rehydration of the anhydrodaptomycin succinimide. This aspartyl transpeptidation pathway was found to be reversible. Formation of the anhydrodaptomycin from either daptomycin or β-asp daptomycin was pH dependent but the pH–rate profiles for anhydrodaptomycin formation were not mechanistically interpretable. The pH–rate profiles for the formation of daptomycin or β-asp daptomycin from the anhydrodaptomycin were linear with slopes = 1, which is consistent with nucleophilic hydroxide ion attack of the succinimido intermediate at either the α-carbonyl, giving rise to the β-asp daptomycin, or the β-carbonyl, giving rise to daptomycin.

KEY WORDS: aspartyl transpeptidation; peptide degradation pathway; pH–rate profiles; daptomycin; lipopeptide antibiotic.

INTRODUCTION

Daptomycin is a lipopeptide currently under clinical investigation as a parenteral antibiotic for treatment of gram-positive infections. *In vitro* and *in vivo* animal testing have demonstrated a high activity versus methicillin-resistant staphylococci and bactericidal activity against *Streptococcus faecalis* (1). Its mechanism of action involves interruption of cell wall synthesis at sites different from those of vancomycin or β-lactam antibiotics (2).

Chemically, daptomycin is composed of a decanoyl side chain connected to the N-terminal tryptophan of a 13-amino acid peptide (Fig. 1) (1). The C-terminal amino acid, kynurenine, is connected via an ester bond to the hydroxyl side chain of threonine. Daptomycin contains two dissociable side-chain amines (primary amine at ornithine and an aromatic amine at kynurenine) and four side-chain carboxylic acids (three aspartic acids and one methyl-glutamic acid).

In the course of studies of the preparation of daptomycin, we observed that a pair of by-products was consistently formed and their concentration appeared to be temperature and pH dependent. It was of interest to determine the exact nature of these transformations, not only to define the nature of the formed products, but also to delineate conditions which would minimize their formation.

This paper reports on the kinetics and degradation products of the aspartyl transpeptidation of daptomycin in aqueous solutions.

MATERIALS AND METHODS

Isolation of Anhydro-Daptomycin. An aliquot (200 mg) of the chromatographically purified *t*-Boc-anhydrodaptomycin was dissolved in 10 ml of cold (5°C) trifluoroacetic acid containing 1 ml of anisole for 5 min. The solution was allowed to warm to room temperature (approximately 15 min) and concentrated to a syrup under reduced pressure. Trituration with ethyl ether (three times using 50 ml of solvent each time) gave a brown solid which was collected on a filter. After redissolving this solid in H_2O (10 ml) and adjusting to pH 6.2 (addition of neat pyridine), the solution was lyophilized to give 190 mg of a product which was of 80% purity by high-performance liquid chromatography (HPLC). This material was repurified using the same conditions described above to give a homogeneous product (retention time, 14.8 min, while daptomycin gave a retention time of 11.2 min using a reverse-phase C18 silica gel column, IBM4×150 mm).

Isolation of β-Asp Daptomycin. Preparations of daptomycin contained a minor impurity with retention time of 14.4 min when analyzed by reverse-phase HPLC using a Zorbax C8-150A column (4.6 × 250 mm), UV detection at 214 nm using a mobile phase consisting of 34% CH_3CN–66% aqueous 0.5% $NH_4H_2PO_4$ flowing at 1.5 ml/min. This component, isolated by purification of 500 g of an intermediate grade of daptomycin, was dissolved in 5 liters of a solvent mixture consisting of 65% (v/v) 0.5 *M* aqueous NaAc (pH adjusted to

[1] Lilly Research Laboratories, Eli Lilly & Co., Indianapolis, Indiana 46285.

[2] To whom correspondence should be addressed.

0724-8741/89/0500-0387$06.00/0

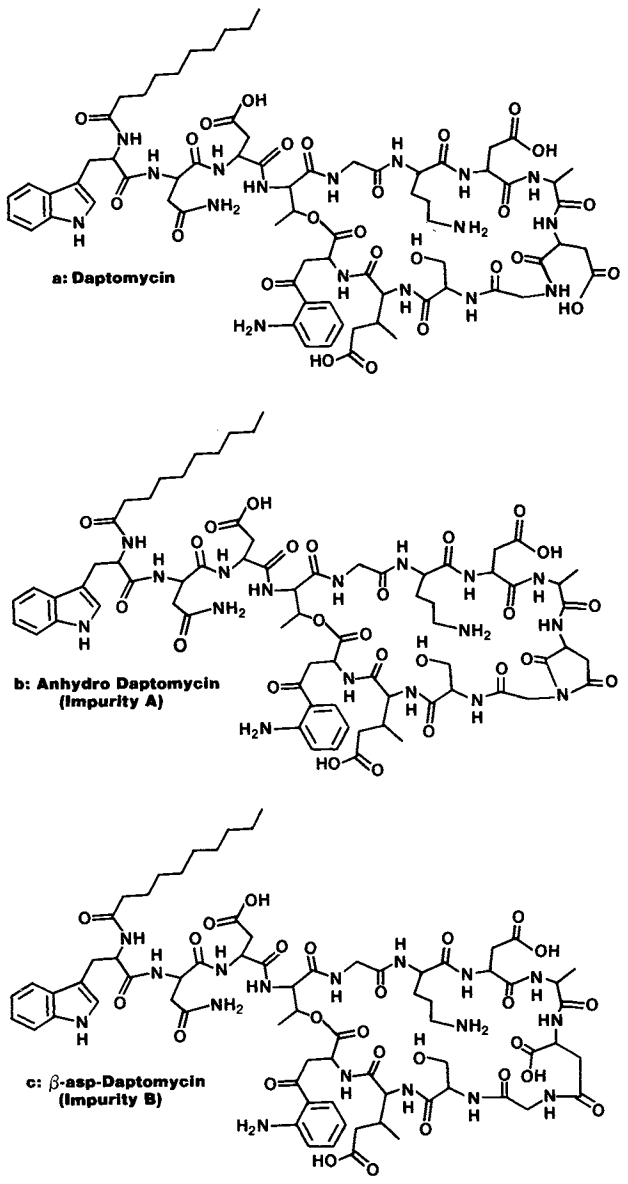


Fig. 1. Structures of daptomycin and transpeptidation products.

4.7 with NaOH), 25% CH_3CN, and 10% methanol, filtered through Super-Cel Hyflo and applied to a column containing 18 liters of HP-20SS resin (Diaion) which had been equilibrated with the latter solvent system. The column was eluted with 5 column vol (90 l) of this developing solvent that had been chilled to 7°C, and this effluent discarded. Thereafter fractions (4 liters) were collected and assayed by HPLC. Fractions 9, 10, and 11, which contained the desired material (retention time, 14.4 min), were pooled, diluted 1:1 with chilled water, and desalted on a 1-liter HP-20 resin (Diaion) column at a flow rate of 6 column vol/hr. The column was eluted with 3 column vol of chilled water and the effluent discarded. The desired compound was obtained by elution with 5 column vol of a chilled solution containing 60% aqueous CH_3CN. The fractions containing the desired material were concentrated and freeze-dried and gave 4.4 g of an enriched preparation of the desired product. This preparation was dissolved in 200 ml of a solvent mixture consisting of 27% CH_3CN, 28% methanol, with 1% acetic acid and adjusted to pH 4 with sodium hydroxide. This was applied to Chromatospac 100 column containing 4 liters of reverse-phase C_{18} silica gel packing and developed with the latter solvent system at a flow rate of 1 column vol/hr. Fractions of 500 ml were collected and each was analyzed by HPLC. The fractions containing the desired material were 14, 15, and 16. These fractions were combined, diluted 1:1 with chilled water, adjusted to pH 3.5 with sulfuric acid, and stirred with 100 ml of HP-20 resin in a chill room for 1 hr. The resin was collected on a filter and washed with 2 vol of chilled water. The resin was then slowly added to 10 vol of CH_3CN, packed in a column, and eluted with 90% CH_3CN and 10% water; five 50-ml fractions were collected. The column was eluted further with 85% aqueous CH_3CN, again collecting five 50-ml fractions. Analysis of these fractions by HPLC showed that the purest material was in fractions 4 and 5 (90% CH_3CN) and fractions 1 through 4 (85% CH_3CN). These fractions were combined, concentrated under reduced pressure, and freeze-dried to give 470 mg of β-asp daptomycin.

Analysis of Reaction Mixtures. Reverse-phase, isocratic HPLC with UV detection (214 nm) was used to assay daptomycin, anhydro-daptomycin, and β-asp daptomycin in mixtures. The mobile phase, prepared by combining 380 ml of acetonitrile and 620 ml of 0.5% $NH_4H_2PO_4$ (pH 3.5), was filtered and deaerated. Chromatography was performed on a Beckman Model 332 gradient liquid chromatograph equipped with a 250-mm Dupont column containing 150 A C-8 packing material. A Kratos Spectroflow UV detector (Model 757) was used and data were accumulated with a Shimadzu integrator (Model C-R3A). Analyses were performed at ambient conditions using 1.5 ml/min flow rate. The approximate retention times for daptomycin, anhydro-daptomycin, and the β-asp daptomycin were 25, 35, and 23 min, respectively.

Kinetics in Aqueous Solutions. Reactions were initiated with either daptomycin, anhydro-daptomycin, or β-asp daptomycin in order to determine the reaction scheme and to obtain accurate rate constant estimates for each pathway involved in the aspartyl transpeptidation scheme. Twenty-five-milligram samples of purified daptomycin or degradation product were added to 50 ml buffered reaction medium (described in Table I) which was previously temperature equilibrated at 60°C. Reaction pH was measured at 60°C before and after each reaction run and remained constant. The initial reactant concentration was approximately 0.001 *M*.

Reaction mixture aliquots were removed periodically, quenched in an ice bath, and refrigerated until assayed. Samples of reactions at pH 6.60 and 7.77 initiated with anhydro-daptomycin were pH quenched using HCl solutions which were prepared such that, when combined with a equivolume reaction aliquot, the resultant pH was 4.

HPLC peaks resulting from sample analyses were normalized in terms of the total peak area of the first reaction aliquot. Thus concentrations were determined as fractions of the total initial concentration.

Determination of Dissociation Constants. Daptomycin and β-asp daptomycin solutions (10 mg/ml) were prepared in distilled-deionized water. Purities were determined by HPLC. Fifty milliliters of daptomycin or 25 ml of β-asp dap-

Table I. Experimental Conditions and Estimated Rate Constants for the Hydrolysis of Daptomycin and Related Substances at 60°C and $\mu = 0.10$[a]

Buffer Composition				Estimated rate constant (day^{-1})						
	Conc. (*M*)									
Identity	Acid	Base	pH	k_1	k_{10}	k_2	k_4	k_{20}	k_3	k_{30}
Citrate	0.006	0.010	2.97	0.22	0.47	0.03	0.06	0.28	1.50	0.50
Acetate	0.030	0.006	4.02	0.73	0.10	0.10	0.24	0.22	1.49	0.06
Acetate	0.025	0.045	4.80	0.64	0.16	0.39	0.98	0.00	0.95	0.15
Acetate	0.005	0.009	4.80	0.64	0.16	0.33	0.94	0.00	0.94	0.16
Acetate	0.006	0.030	5.33	0.53	0.08	0.52	1.62	0.27	0.76	0.05
Phosphate	0.006	0.006	6.59	0.38	0.09	6.39	19.8	5.57	0.49	0.08
Tris	0.012	0.012	7.77	0.37	1.33	46.8	153.	32.4	0.17	1.51

[a] Rate constants are defined in Scheme 1 and were estimated using SIMUSOLV as described in the text.

tomycin solution was equilibrated at 23°C in a water-jacketed reaction vessel, pH-adjusted to 9, and then titrated manually in the pH range 9.0 to 2.0 with 0.050- or 0.025-ml aliquots of 1.00 *N* HCl (Fischer Scientific Certified Solution) using a Metrohm Dosemat 665 Titrator. The pH was measured after acid addition with a Metrohm pH meter (Model 632).

The moles of bound protons after each titrant addition was calculated from the difference between the expected pH change in the absence of peptide and the observed pH change. The moles of proton dissociated per mole of peptide (ν) was plotted versus pH and the data were fit using the SAS NLIN procedure to

$$\nu = \sum_{i=1}^{4} \frac{n_i K_i a_H^{-1}}{(1 + K_i a_H^{-1})}$$

where i is the ith carboxylic acid, n_i is the number of i protons dissociating with dissociation constant K_i, and a_H is the hydronium ion activity.

RESULTS

Identification of Transpeptidation Intermediate and Product. Examination of solutions of daptomycin at various conditions of pH and temperature by HPLC on reverse-phase silica gel revealed the formation of a substance at a longer retention time, anhydro-daptomycin, and one at a slightly shorter retention time from daptomycin, β-asp daptomycin. The FAB-mass spectrum of anhydro-daptomycin showed a molecular weight of 1602 (P + 1) and an empirical formula of C_{72} H_{99} N_{17} O_{25}, corresponding to the loss of a mole of water from daptomycin.

The Fourier Transform Infrared spectrum on anhydro-daptomycin had a band at 1790 cm^{-1}, which is a characteristic absorption band for a succinimide. Degradative studies on anhydro-daptomycin using hydrazine selectively cleaved the imide group at the aspartyl residue (9). The cleavage site was confirmed by taking advantage of the newly formed amino terminus in the cleaved peptide. Edman degradation of this material gave the sequential release of glycine (10), serine (11), and 3-methyl-glutamyl (12), thereby establishing imide location at amino acids 9 and 10. The structure of anhydro-daptomycin is given in Fig. 1b.

The UV spectrum and the amino acid analysis of β-asp daptomycin were the same as those of daptomycin. FAB-mass spectrometry showed a molecular ion (P + 1) at 1620 which was the same as that of daptomycin and corresponded to the addition of a molecule of water to anhydro-daptomycin. These data are consistent with the view that β-asp daptomycin is the β-asp isomer of daptomycin (Fig. 1c).

Determination of Carboxylic Acid Dissociation Constants. Analysis of daptomycin and β-asp daptomycin aqueous titration data (Fig. 2) revealed the dissociation of the four side-chain carboxylic acid groups in the pH range 3 to 6.

Both compounds had one carboxylic acid group dissociate at pH 3.0 and three acid side chains with overlapping pK_a's of 5.3 (daptomycin) and 5.0 (β-asp daptomycin). The observation of a downshifted titration curve for the β-asp isomer was probably indicative of the greater acidity of the free α-asp side chain.

Identification of Degradation Scheme. At pH 5.3 the hydrolysis of daptomycin gave rise to anhydro-daptomycin and β-asp daptomycin (Fig. 3a). Under identical conditions the reaction initiated with anhydro-daptomycin gave rise to daptomycin and β-asp daptomycin (Fig. 3b). Therefore the daptomycin to anhydro-daptomycin pathway was shown to be reversible. Furthermore, hydrolysis of β-asp daptomycin

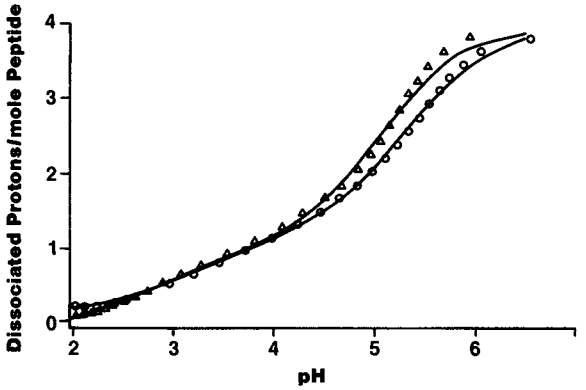


Fig. 2. Titration of daptomycin (○) and β-asp daptomycin (△) with hydrochloric acid. Curves were generated using estimated dissociation constants (see text).

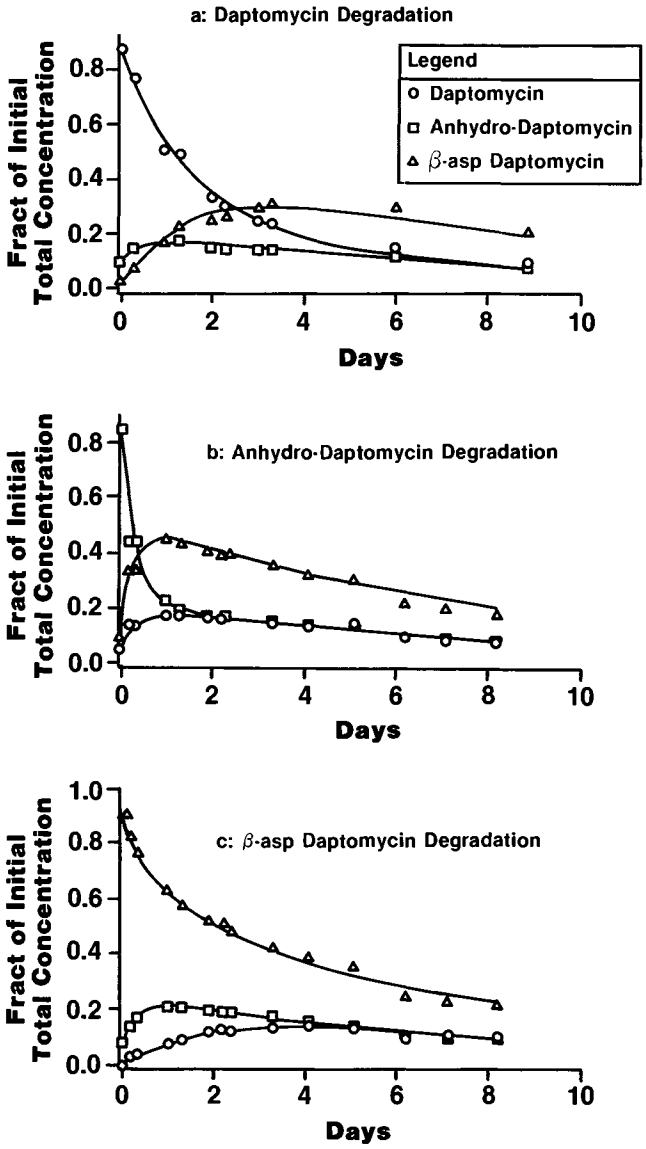
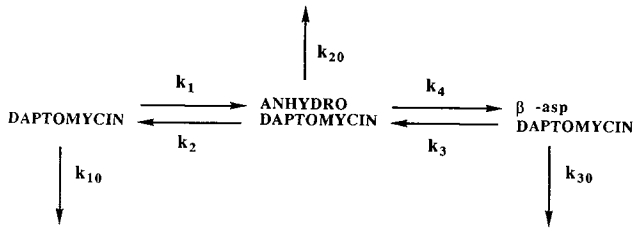


Fig. 3. Hydrolysis of daptomycin and related substances at 60°C, μ = 0.10, and pH 5.3. Reactions were initiated with daptomycin (a), anhydro-daptomycin (b), or β-asp daptomycin (c) under identical experimental conditions. Curves were generated using estimated rate constants (Table I).

under these conditions gave rise to anhydro-daptomycin and daptomycin (Fig. 3c). Therefore the anhydro-daptomycin to β-asp daptomycin was also shown to be reversible.

At 24 hr the sums of the concentrations of daptomycin, anhydro-daptomycin, and β-asp daptomycin were 78, 82, 90, 86, and 24% of the total initial concentrations at pH 3.0, 4.0, 5.3, 6.6, and 7.8, respectively. Therefore, significant, nontranspeptidation, parallel pathways of loss were present under all conditions.

Initial rate estimates were used to determine which of the three compounds were subject to significant nontranspeptidation hydrolysis. The generalized reaction scheme which includes parallel (nontranspeptidation) pathways of loss is depicted in Scheme 1. The differential equations which define this scheme are given by



Scheme 1

$$\frac{d[\text{daptomycin}]}{dt} = k_2[\text{anhydro-daptomycin}] - (k_1 + k_{10})[\text{daptomycin}]$$

$$\frac{d[\text{anhydro-daptomycin}]}{dt} = k_1[\text{daptomycin}) + k_3(\beta\text{-asp daptomycin}) - (k_2 + k_4 + k_{20})[\text{anhydro-daptomycin}]$$

$$\frac{d[\beta - \text{asp-daptomycin}]}{dt} = k_4[\text{anhydro-daptomycin}] - (k_3 + k_{30})[\beta - \text{asp daptomycin}]$$

The difference between the initial rate of daptomycin loss and the initial rate of anhydro-daptomycin appearance when the reaction was initiated with daptomycin indicated the approximate contribution of nontranspeptidation pathway to daptomycin hydrolysis. For example, at pH 4.02, the rate constant for daptomycin loss estimated from the initial rate was 0.86 day^{-1}, whereas the estimated rate constant for anhydro-daptomycin appearance was 0.60 day^{-1}. Furthermore, at pH 4.80 and 5.33 the initial rate constants for daptomycin loss versus anhydro-daptomycin appearance were 0.41 versus 0.24 and 0.82 versus 0.58 day^{-1}, respectively. Since the rate constants for daptomycin loss were greater than those for anhydro-daptomycin appearance, the simplest reaction scheme for daptomycin loss included a pathway for nontranspeptidation hydrolysis (k_{10} in Scheme 1). For reactions begun with β-asp daptomycin, comparisons of the rate constants estimated from initial rate data for β-asp daptomycin loss versus anhydrodaptomycin appearance at pH 4.02, 4.80, and 5.33 were 1.61 versus 1.26 day^{-1}, 0.80 versus 0.71 day^{-1}, and 0.52 versus 0.39 day^{-1}, respectively, suggesting that nontranspeptidation hydrolysis of β-asp daptomycin was also significant (k_{30} in Scheme 1). Additionally, for reactions begun with anhydro-daptomycin, the comparisons of the rate constants estimated from initial rate data for the loss of anhydro-daptomycin versus the sum of the rate constants for appearance of daptomycin and β-asp daptomycin at pH 4.02, 4.80, and 5.33 were 0.32 versus 0.18, 0.93 versus 0.95, and 2.90 versus 2.07 day^{-1}, respectively. Therefore under some of the conditions studied all of the parallel pathways of loss depicted as Scheme 1 were needed to describe the concentration–time profiles.

Determination of Kinetic Constants. The rate constants depicted in Scheme 1 were estimated by nonlinear regression at each pH condition. Rate constant estimates obtained from initial rate data were refined by simultaneously "fitting" the

nine concentration time profiles at each pH condition to the above equations using SIMUSOLV (Table I). Goodness of fit was judged visually by comparing concentration–time profiles generated using rate constant estimates to experimental data (Figs. 3–5).

DISCUSSION

Degradation Pathway. Transpeptidation of aspartyl residues in amphomycin (3) and ACTH hexapeptide (4) has been previously reported to occur via succinimido-intermediate formation. Intermediate hydrolysis was the result of the rehydration of the imide bond. Since this rehydration can take place on either side of the imide nitrogen group, two products are possible: opening on the side of the imide closest to the aspartyl amino group results in the formation of α-aspartyl peptide, while hydration of the other side results in the formation of a β-aspartyl linkage. The kinetic data reported herein were very comparable to the work on an ACTH hexapeptide which also contained the asp–gly sequence found in daptomycin (Table II).

Transpeptidation is related by virtue of a common succinimido intermediate to (i) aspartyl deamidation (Scheme 2), which is a frequent protein and peptide instability problem (5,6), and (ii) aspartyl ester hydrolysis, which can lead to impurities in protein synthesis (7). In the case of the aspartyl transpeptidation of daptomycin, both anhydro-daptomycin and β-asp daptomycin are microbiologically inactive. The effect of pH on transpeptidation has not been reported.

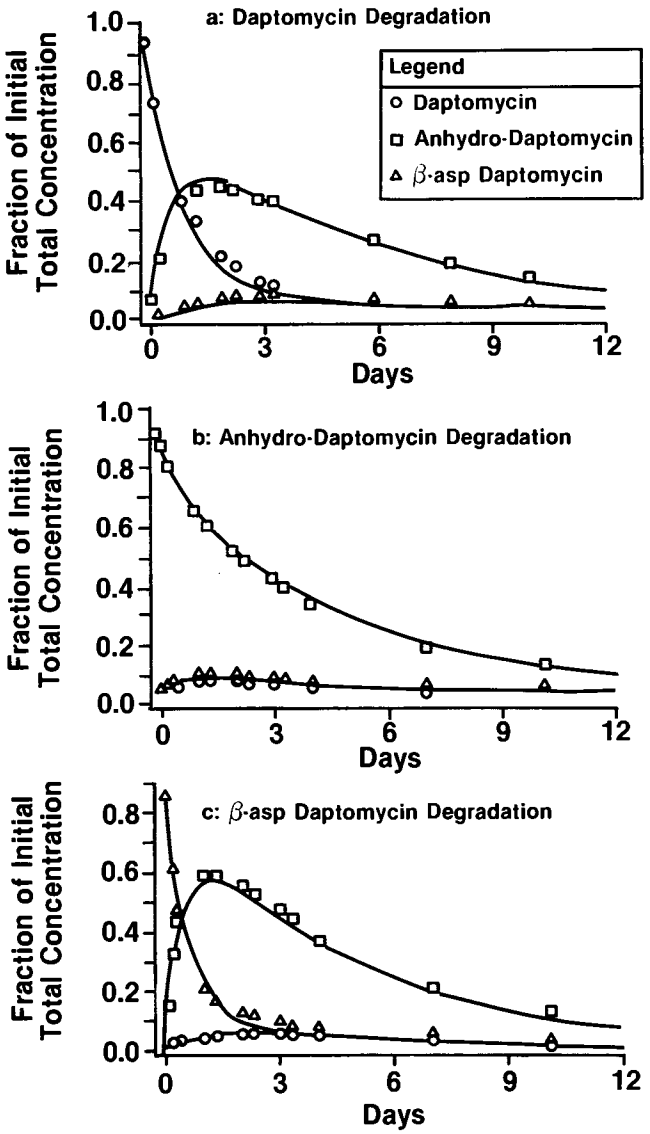


Fig. 4. Hydrolysis of daptomycin and related substances at 60°C, μ = 0.10, and pH 4.0. Reactions were initiated with daptomycin (a), anhydro-daptomycin (b), or β-asp daptomycin (c) under identical experimental conditions. Curves were generated using estimated rate constants (Table I).

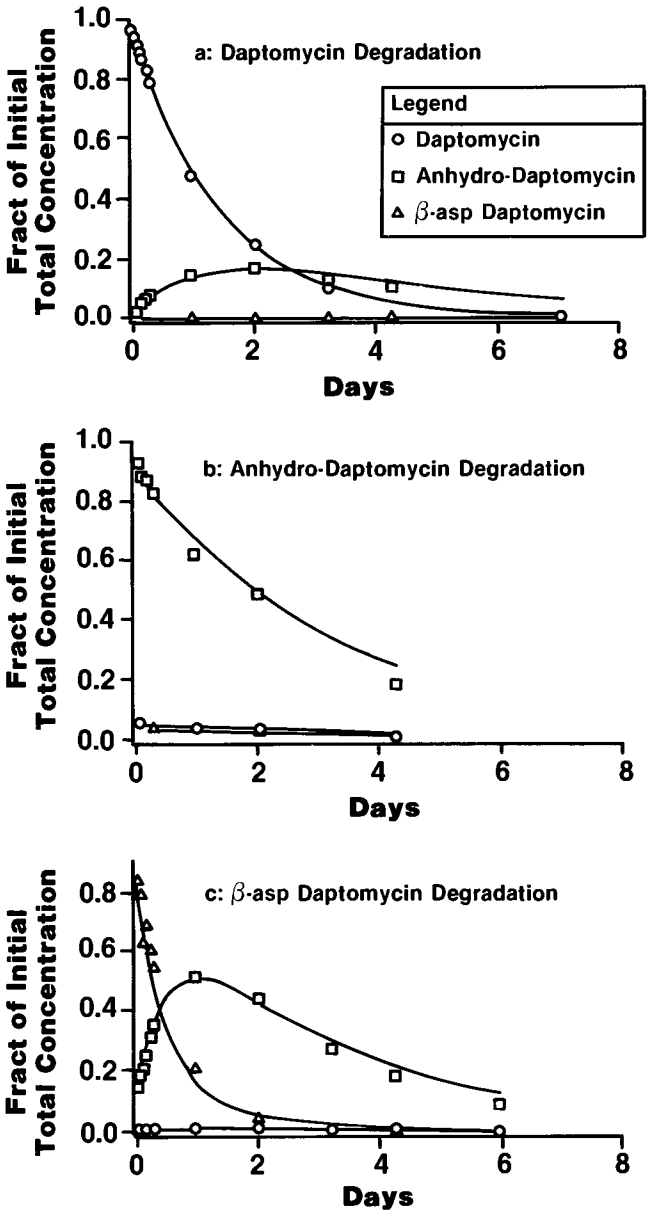


Fig. 5. Hydrolysis of daptomycin and related substances at 60°C, μ = 0.10, and pH 3.0. Reactions were initiated with daptomycin (a), anhydro-daptomycin (b), or β-asp daptomycin (c) under identical experimental conditions. Curves were generated using estimated rate constants (Table I).

Table II. Comparison of the Asp–Gly Transpeptidation Rates of Daptomycin and ACTH Hexapeptide at 60°C and pH 7.4[a]

Reaction Pathway	Half-life (hr)	
	Daptomycin	ACTH Hexapeptide
Anhydro-daptomycin formation	43.7 and 66.5	86.6
α-Asp peptide formation	0.7	0.6
β-Asp peptide formation	0.2	0.2

[a] Daptomycin rate constants were interpolated from pH–rate profiles. ACTH hexapeptide data were extrapolated from Arrhenius plots (3).

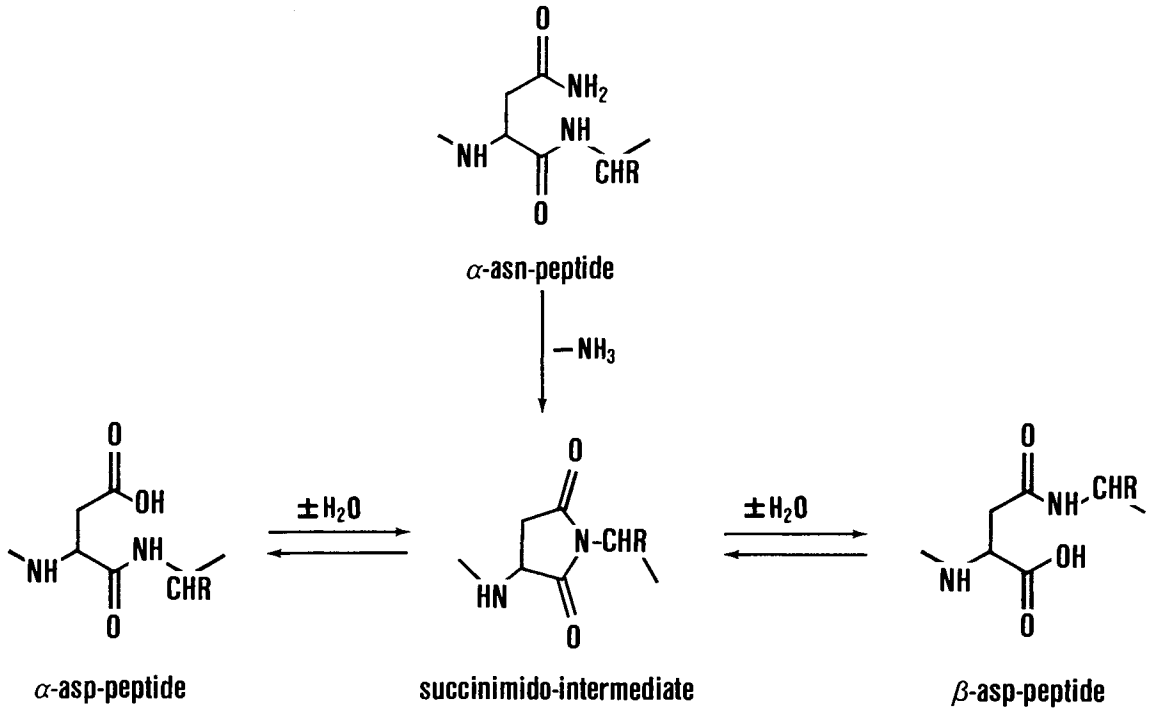
Effect of Buffer Concentration on Transpeptidation Kinetics. Two series of kinetic studies were conducted at pH 4.80 using 0.014 and 0.070 *M* acetate buffers. The estimated rate constants for all the pathways defined in Scheme 1 were nearly identical (Table I). These results demonstrate that buffer catalysis does not occur to any appreciable extent under the conditions studied and that the rate constant estimation procedures and experimental methods were reproducible.

pH Effect on Anhydro-Daptomycin Formation. The pH–rate profiles for the formation of anhydro-daptomycin from daptomycin and β-asp daptomycin are shown in Fig. 6. In general the rates of anhydro-daptomycin formation were similar regardless of the starting material except at pH 3.0, where β-asp daptomycin conversion to anhydro-daptomycin was threefold greater than daptomycin conversion. This difference was reflected in the greater instability of the β-asp daptomycin versus daptomycin at pH 3 (Fig. 5). The reason for this difference is unknown but may reflect confirmational differences between daptomycin and β-asp daptomycin when all side-chain carboxylic acids are nonionized.

In the pH region 4 to 8 the anhydro-daptomycin formation rate increased as hydronium ions increased but the magnitude of the effect was not indicative of a first-order hydronium ion dependence. Interpretation of pH effects was complicated by the fact that the four carboxylic acid side chains which dissociate in this pH region gave rise to a multitude of ionic daptomycin species, all of which may or may not have different propensities to transpeptidate.

Effect of pH of Formation of Daptomycin and β-Asp Daptomycin from Anhydro-Daptomycin. The pH–rate profiles for anhydro-daptomycin hydrolysis to daptomycin and β-asp daptomycin (Fig. 6) revealed an approximately first-order hydroxide ion dependence. An inflection point at approximately pH 5 probably reflects the ionization of side-chain carboxylic acids and accounts for the deviations in linearity seen in both pH–rate profiles. Furthermore, the two profiles were essentially parallel; the rate constant for anhydro-daptomycin hydrolysis to β-asp daptomycin was between two- and fourfold greater than the corresponding rate constant for daptomycin formation under all conditions. This is in agreement with Geiger and Clarke's observation of a 3:1 ratio of β-asp to α-asp peptide formation in the asparaginyl deamidation of ACTH hexapeptide (4).

The kinetic data were consistent with nucleophilic hydroxide ion attack on anhydro-daptomycin at the succinim-



Scheme 2. Typical pathways for asparaginyl deamidation and aspartyl transpeptidation.

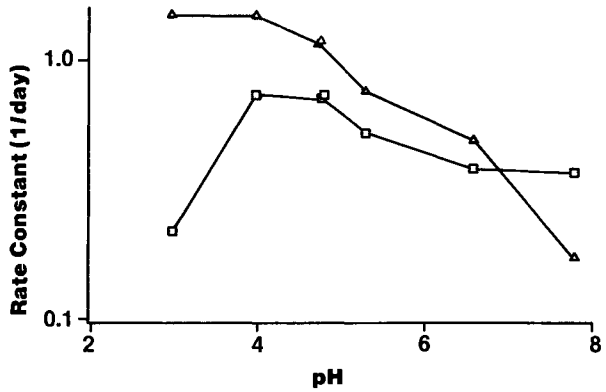


Fig. 6. pH–rate profiles for the formation of Anhydrodaptomycin from daptomycin (□); k_1) and β-asp daptomycin (△; k_3 at 60°C and μ = 0.10.

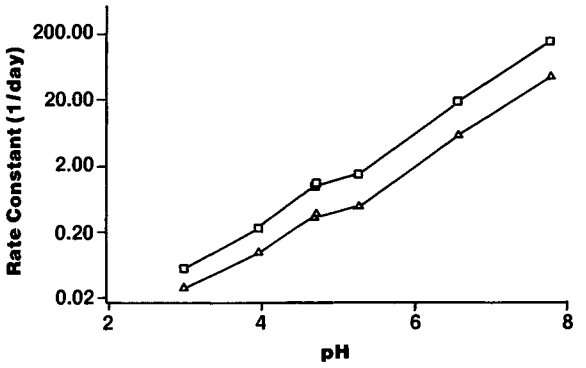


Fig. 7. pH–rate profiles for the hydrolysis of anhydro-daptomycin to daptomycin (△; k_2) and β-asp daptomycin (□; k_4) at 60°C and μ = 0.10.

ide at either the α-carbonyl (giving rise to the β-asp peptide) or the β-carbonyl (giving rise to daptomycin). The preference for β-asp daptomycin formation was probably because of the greater electrophilicity of the α-carbonyl.

Nontranspeptidation Hydrolysis Pathways. At present, we are continuing to investigate the parallel pathways of daptomycin, anhydro-daptomycin, and β-asp daptomycin hydrolysis. The estimated rates constants for nontranspeptidation hydrolyses are likely composite constants which may reflect asparaginyl deamidation (residue 2), ester hydrolysis, racemization, peptide backbone hydrolysis, etc. Interpretation of the pH–rate profiles associated with parallel loss pathways awaits conclusive identification of those pathways.

ACKNOWLEDGMENTS

The authors wish to thank Ms. Toni Cummings, Debra Gearhart, and Joan Ragozzino for their technical assistance in performing the kinetic studies and HPLC assays.

REFERENCES

1. M. Debono, B. J. Abbott, R. M. Molloy, D. S. Fukuda, A. H. Hunt, D. P. Daupert, F. G. Counter, J. L. Ott, C. B. Carrell, L. C. Howard, and R. L. Hamil. *J. Antibiot.* 41:10993–1105 (1988).
2. N. E. Allen, J. N. Hobbs, Jr., and W. E. Alborn, Jr. *Anti. Agents Chem.* 31:1093–1099 (1987).
3. M. Bodanszky, G. F. Sigler, and A. Bodanszky. *JACS* 95:2352–2357 (1973).
4. T. Geiger and S. Clarke. *J. Biol. Chem.* 262:785–794 (1987).
5. A. B. Robinson, J. H. McKerrow, and P. Cary. *Proc. Natl. Acad. Sci.* 66:753–757 (1970).
6. C. M. Harris, H. Kopecka, and T. M. Harris. *JACS* 105:6915–6922 (1983).
7. M. Bodanszky and J. Z. Kwei. *Int. J. Peptide Protein Res.* 12:69–74 (1978).