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Studies on the reactions between daptomycin and glyceraldehyde

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Abstract

The objectives of this project were to determine the reaction pathways of daptomycin in the presence of glyceraldehyde in acidic solutions, and to quantitate the kinetics of the major pathways. In the presence of glyceraldehyde (pH range 1–7 at 25 to 60 °C), daptomycin formed two major products separable by RP-HPLC. The products were identified using UV spectroscopy, fluorimetry, mass spectrometry, and 2D-^{1}H NMR. The reaction scheme involved the reversible formation of imine and anilide derivatives. Carbinolamine was believed to be a common intermediate in formation pathways of both products. The carbinolamine intermediate underwent either acid catalyzed dehydration resulting in imine formation or intramolecular hydrogen bonding and bond cleavage giving rise to anilide formation. In mild acid conditions, both products reversed to daptomycin. The reaction between daptomycin and glyceraldehyde was first-order with respect to both reactants. In a pH range of 1–7, the imine formation rate was pH dependent with a maximum rate at approximate pH values of 3–4. The observed pH dependence was consistent with the pH dependence of typical amine–aldehyde reactions.

Keywords: NMR; Proteins; Kinetics; Aldehyde–amine reaction; Anilide formation; pH dependence

1. Introduction

Carbonyl amine reactions are pharmaceutically significant. The reaction of amine drugs with reducing sugar excipients to form Schiff's base and subsequent Maillard reaction products is a well-known degradation pathway (Blaug and Huang, 1972, 1973; Dubost et al., 1996). Additionally, carbonyl amine reactions have been used to synthesize useful prodrugs. Examples of Schiff's base prodrugs include hetacillin, pivampicillin, bacampicillin, and azomethine. (Klixbull and Bundgaard, 1985; Krause et al., 1996).

Aldehydes typically react with unprotonated amines at the N-terminal or ε-amino of side chain residues (i.e. histidine, lysine or ornithine) resulting

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in a carbinolamine intermediate that is dehydrated to form an imine (Schiff's base). Imine formation is reversible, e.g. glyceraldehyde-hemoglobin S adduct released free glyceraldehyde in phosphate-buffered saline at room temperature over a period of time (Acharya and Manning, 1980). The chemical structure of the reactive aldehyde affects imine formation rate. Klixbull and Bundgaard (1985) studied the reaction of ampicillin and various simple aldehydes and showed that imine formation rate decreased as the bulkiness of

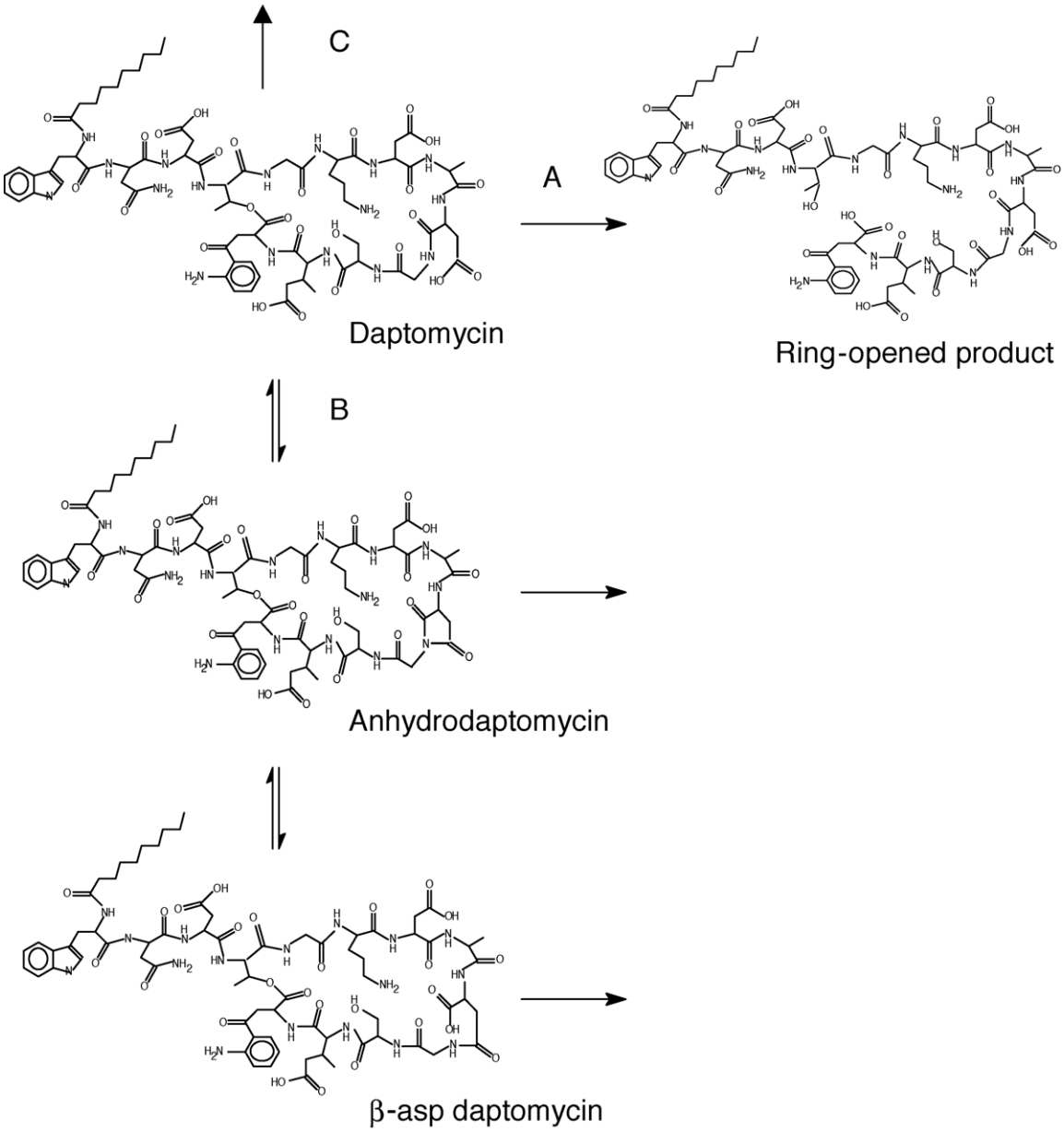


Fig. 1. The degradation pathways of daptomycin in acidic, neutral, and alkaline conditions. Pathway A (ester hydrolysis) occurs in alkaline condition. Pathway B (aspartyl transpeptidation) is the predominant pathway in the pH range 3–6. At low pH, unknown degradation (pathway C) occurs.

the attacking carbonyl increased. But the reversal rate was not significantly affected.

In excess of aldehyde, the reaction is first-order with respect to amine (Klixbull and Bundgaard, 1985). The pH rate profile is typically bell-shaped which has been interpreted as a change in rate-determining step from carbinolamine formation under low pH condition associated with amine protonation to rate-determining dehydration at higher pH values.

Daptomycin is a potent lipopeptide antibiotic against gram positive bacteria including vancomycin and/or methicillin resistant pathogens. Daptomycin is composed of 13 amino acid residues and a decanoyl side chain (Fig. 1) (Debono et al., 1988). In the pH range of 0–14, daptomycin contains six ionizable groups including four carboxylic acid side chains (three aspartic acids and one methyl-glutamic acid) and two primary amines (kynurenine and ornithine). One of the carboxylic acid groups has a pK_a of 3.0 while others have overlapping pK_a values at 5.3 (23 °C) (Kirsch et al., 1989). pK_a values of the aromatic amine, kynurenine, is 0.8 whereas the aliphatic amine pK_a, ornithine, is 10 at 25 °C (Muangsiri and Kirsch, 2001). Daptomycin contains two fluorophores, tryptophan and kynurenine.

Daptomycin is susceptible to hydrolytic degradation and concentration-dependent aggregation (Kirsch et al., 1989; Muangsiri and Kirsch, 2001). In the pH range of 3–8, daptomycin undergoes reversible aspartyl transpeptidation only at Asp-9 residue giving rise to the succinimide intermediate (anhydrodaptomycin) and the β-aspartyl peptides (β-asp daptomycin, see Fig. 1). The reaction also involves parallel (non-transpeptidation) pathways of loss. In alkaline solutions, daptomycin is quantitatively converted to a ring-opened variant by ester hydrolysis of the bond between the C-terminus and the side chain of Thr-4 residue. Ester hydrolysis was second-order overall: first-order with respect to hydroxide and substrate concentrations up to 1 mM (Muangsiri and Kirsch, 2001). At higher substrate concentrations, the reaction was less than first-order due to substrate aggregation.

Daptomycin is known to spontaneously form reversible conjugates with reactive carbonyls such as reducing sugars (Inman and Kirsch, 1990). We have investigated the use of daptomycin's reactivity toward aldehydes as a means of preparingpolysaccharide-based macromolecular conjugates, which may be useful in altering the biodistribution and pharmacokinetic properties of daptomycin. In order to understand the complexity of the reactions between two polymers, we studied the reactions of a model aldehyde (glyceraldehydes) and the peptide antibiotic.

The objective of studies reported herein was to determine the reaction products of daptomycin and glyceraldehydes, the reactive site of activated dextran, and to measure the rates of transformations. The chemical structure of reaction products were identified using UV spectroscopy, fluorescence spectroscopy, mass spectrometry, and two-dimensional proton nuclear magnetic resonance (2D-^{1}H NMR).

2. Materials and methods

2.1. Materials

Sodium phosphate monobasic monohydrate ($NaH_2PO_4{\cdot}H_2O$), sodium phosphate dibasic heptahydrate ($Na_2HPO_4{\cdot}7H_2O$), sodium chloride (NaCl), glacial acetic acid (CH_3COOH), sodium acetate (CH_3COONa), *o*-phosphoric acid (H_3PO_4), standardized sodium hydroxide and hydrochloric acid solutions (0.1N NaOH,1.0N NaOH, and 1.0N HCl) were obtained from Fisher Chemicals, Co. (Fair Lawn, NJ). All chemicals were analytical grade. HPLC grade acetonitrile was from Fisher Chemicals, Co. Standardized buffer solutions were from VWR® Scientific (West Chester, PA). DL-Glyceraldehyde was from Sigma Chemical Company (St. Louis, MO). Daptomycin (Lilly Research Laboratories, Indianapolis, IN) was used as received.

2.2. Methods

2.2.1. Daptomycin HPLC analysis

The reverse-phase high performance liquid chromatography (RP-HPLC) system consisted of a pump (Shimadzu LC-10ATVP), a system controller (Shimadzu SCL-10AVP), an UV detector (Shimadzu SPD-10AVP), an autoinjector (Shimadzu SIL-10ADVP), and a fraction collector (Shimadzu FRC 10A). The HPLC method was adapted from a previously reported method and employed a 250 mm × 4.6 mm i.d., 5 μm particle size, C-8 column (Zorbax 300SB-C8, Hewlett Packard®, Serial #HD2411) (Kirsch et al., 1989). The column was operated at room temperature using a sol-

vent flow rate of 1 ml min^{-1}. The mobile phase was acetonitrile:0.05 M phosphate buffer pH 5 (29:71). The sample injection volume was 10 μl, and the run time was 30 min. Daptomycin and related substances were detected at a wavelength of 214 nm, 0.64 AUFS. A typical chromatogram revealed peaks at retention times of 17, 16, and 24 min corresponding to daptomycin, β-asp daptomycin, and anhydrodaptomycin, respectively. Four-point calibration curves relating peak areas to concentration were linear ($R^2 > 0.99$) in the concentration range of 0.02–0.3 mM.

2.2.2. Generation of reaction products

Chromatograms obtained from samples of daptomycin degraded under identical conditions in the presence and absence of glyceraldehyde were compared. Two significant HPLC peaks with relative retention times of 0.77 and 0.82 to that of daptomycin were present in reactions that contained glyceraldehyde but not when this reagent was absent. Thus, these peaks appeared to result from the reaction of daptomycin and glyceraldehyde and were subjected to further studies. They are referred to herein as products **A** and **B** with corresponding relative retention time of 0.77 and 0.82, respectively.

Reactions were conducted for about 10 h at 40 or 60 °C in aqueous solution at pH 3.8. The solutions were analyzed by RP-HPLC using a Zorbax 300SB-C8 column and acetonitrile:0.50 M ammonium acetate pH 5.7 (29:71) as a mobile phase. The column was operated at room temperature using a flow rate of 1 ml min^{-1} and an analytical wavelength of 214 nm. Samples of products **A** and **B** were collected from 40 injections of 1 mM daptomycin and 20 mM glyceraldehyde reaction mixtures using a Shimadzu FRC 10A fraction collector. The fractions were pooled; the organic solvent was evaporated under nitrogen purge and the fractions were then lyophilized. The lyophilized residues were reconstituted in distilled water and re-injected into HPLC to check peak integrity. The lyophilized residues were kept at 4 °C in a refrigerator for further structural identification studies.

2.2.3. Structure identification of reaction products

Structure elucidations of the products **A** and **B** were done using UV spectroscopy, fluorimetry, mass spectrometry, and 2D-^{1}H NMR.

UV spectra of the reaction products and daptomycin were collected using a HP 8453A UV spectrophotometer with a 1 cm path length cuvette at room temperature. The lyophilized residues were accurately weighed and dissolved in distilled water. The absorbance spectra between 220 and 450 nm were taken.

Fluorescence spectrophotometry was employed to investigate the possibility of chemical modification at kynurenine residue of daptomycin using a Kontron SFM25 fluorospectrometer with a 1 cm path length cuvette. The degradation products were prepared by dissolving the lyophilized residues with distilled water. The emission spectra were obtained using the excitation wavelength of kynurenine at 364 nm. The relative fluorescence intensities of kynurenine at 465 nm in the reaction products were determined.

Products **A** and **B** and daptomycin were analyzed by Matrix Assisted Laser Desorption and Ionization (MALDI-TOF) mass spectrometer (Bruker Biflex III). In this study, the matrix solution was 10 mg ml^{-1} α-cyano-4-hydroxycinnamic acid in TFA:acetronitrile:water (0.1:50:50). The samples were dissolved in distilled water. Acetonitrile was added to the mixture. The sample solutions and the matrix solution were then mixed at 1:1 ratio directly on a welled plate with a final volume of 1 μl. White-colored protein/matrix crystals formed on the plate after a few minutes. The sample plate was then loaded on the instrument for time-of-flight (TOF) mass spectrometric analysis. MALDI-TOF operating conditions were set as follows: mode of operation was reflector; polarity was positive; an acceleration voltage was 20,000 V, and delayed extraction time was 200 μs. The analyte ions were separated based on mass-to-charge ratio (m/z). Time required for the analyte ions to reach the detector was measured and converted to mass-to-charge ratio of the analyte.

2D-^{1}H NMR including double quantum filtered correlation spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), and nuclear Overhauser enhancement spectroscopy (NOESY) were performed to obtain chemical shifts relating to the protons in the structure of daptomycin and product **B** using DMSO-d_6 as a solvent in order to minimize aggregation. The concentrations of daptomycin and product **B** in 99.9% DMSO-d_6 were 1 and 0.1 mM (based on tryptophan absorbance at 282 nm), respectively. One- and two-dimensional spectra were collected at 25 °C on

Table 1
^{1}H chemical shifts of daptomycin in DMSO

Residue number	Residue	Chemical shift (ppm)			
		NH	$C^{\alpha}H$	$C^{\beta}H$	Others
1	Trp	7.953	4.420	3.040, 2.882	$C^{\delta 1}H$ 7.130, $^{N\varepsilon 1}H$ 10.750
2	Asn	8.286	4.563	2.354, 2.440	
3	Asp	7.886	4.581	2.472	
4	Thr	8.008	4.480	5.254	$C^{\gamma}H_3$ 1.081
5	Gly	8.517	3.795		
6	Orn	8.421	4.185	1.670	$C^{\gamma}H_2$ 1.593, $C^{\delta}H_2$ 2.781
7	Asp	8.371	4.434	2.507, 2.627	
8	Ala	7.922	4.136	1.232	
9	Asp	7.853	4.597	2.383, 2.619	
10	Gly	8.226	3.586, 4.686		
11	Ser	8.110	4.273	3.642	
12	Met-Glu	8.064	4.295	0.867, 2.037, 2.415	
13	Kyn	7.988	4.461	2.389, 2.422	

a 500 MHz Varian Unity INOVA Spectrometer using standard procedures (Sawai et al., 2002; Welch et al., 2003). All spectra were referenced against a DMSO-d_6 chemical shift at 2.5 ppm. The spectral width was 6 kHz in each dimension for all spectra. DQF-COSY spectra had a resolution of 2048 complex points in f_2 and 512 complex points in f_1 with an average of 32 transients per f_1 increment. TOCSY and NOESY spectra were taken at 120 and 300 ms mixing times, respectively. In the NOESY and the TOCSY spectra the dataset were 1024 complex points in f_2 by 512 complex points in f_1 and 16 transients were averaged per f_1 increment.

Data obtained from the COSY, TOCSY, and NOESY spectra along with the known primary sequence were analyzed to assign proton chemical shifts of daptomycin. Assignment of spin systems was performed using COSY and TOCSY spectra and sequential assignment technique was employed to map the extracted spin system to the peptide primary sequence. Finally, chemical shifts of the daptomycin were assigned (Table 1).

2.2.4. Kinetic studies of daptomycin and glyceraldehyde

Aqueous daptomycin (0.3 to 6.0 mM) was reacted with 0 to 454.0 mM glyceraldehyde at 25, 40 and 60 °C in buffers pH range of 1.0–7.0 using HCl, phosphate, or acetate buffers (μ = 0.154 M) or in aqueous solutions adjusting the pH value using standardized hydrochloric acid or sodium hydroxide (Table 2). The reaction was allowed to proceed for at least two to three half-lives. Aliquots were removed from reaction mixtures at appropriate times and immediately analyzed using HPLC. The aliquots were diluted with distilled water prior to assay if necessary.

2.2.5. Degradation of products **A** and **B**

In a series of four aqueous degradation studies product **A** or **B** was degraded at 60 °C at pH 2.91, 2.94 and 4.05 (adjusting the pH value using standardized hydrochloric acid). The reaction was allowed to proceed for at least two to three half-lives. Aliquots were removed from reaction mixtures at appropriate times and immediately analyzed using HPLC.

3. Results

Daptomycin (1 mM) was reacted with 20 mM glyceraldehyde at 25 °C in aqueous solution in the pH range of 1–7 (reactions #1–5, Table 2). Chromatographic separation of reaction mixture aliquots from reaction #2 which contained glyceraldehyde gave several degradation peaks that were different than those observed in the absence of glyceraldehyde (e.g. reaction #5). Two degradation products with relative retention times of 0.77 (product **A**) and 0.82 (product **B**) were of primary interest because they were present only in reactions conducted with glyceraldehyde, and they formed to a significant extent. Preliminary UV characteristics of the two products and daptomycin peaks were obtained as they eluted by stopping the mobile phase flow and

Table 2
Experimental conditions for the kinetic studies of the daptomycin and glyceraldehyde reaction

Reaction #	Solvent	Initial concentration of daptomycin (mM)	Initial concentration of glyceraldehydes (mM)	pH	Temperature (°C)	Initial rate of daptomycin loss (% peak area per hour)	$t^*_{1/2}$ (h)	Initial rate of product **A** formation (% peak area per hour)	Initial rate of product **B** formation (% peak area per hour)
1	HCl	1.01	18.1	2.56	25	2.86	24.3	0.315	0.258
2	HCl	1.00	18.1	3.70	25	2.52	27.5	0.555	0.244
3	NaOH	0.956	18.1	5.10	25	0.862	80.4	0.098	0
4	NaOH	0.987	18.1	6.14	25	0.306	226	0	0
5	20.0 mM glycerin	0.997	0	3.02	25	0.097	715	0	0
6	0.17 M phosphate	0.996	0	3.08	40	0.158	439	0	0
7	0.17 M phosphate	0.998	0.101	3.13	40	0.292	237	0.041	0
8	0.17 M phosphate	1.01	0.502	3.11	40	0.393	173	0.080	0.00313
9	0.17 M phosphate	0.993	1.00	3.10	40	0.478	145	0.096	0.0125
10	0.17 M phosphate	0.984	2.00	3.06	40	0.691	100	0.246	0.0386
11	0.17 M phosphate	0.998	7.58	3.05	40	2.07	33.5	0.496	0.104
12	0.17 M phosphate	0.981	10.0	3.08	40	2.71	25.6	0.461	0.145
13	0.17 M phosphate	1.01	19.9	3.10	40	4.01	17.3	1.31	0.182
14	0.17 M phosphate	1.00	49.9	3.05	40	5.32	13.0	2.66	0.433
15	0.17 M phosphate	0.310	454	3.03	40	46.1	1.50	36.1	–
16	0.17 M phosphate	0.501	454	3.03	40	73.3	0.95	12.8	–
17	0.17 M phosphate	1.00	454	3.08	40	64.6	1.07	19.0	–
18	0.17 M phosphate	1.97	454	3.08	40	61.1	1.14	15.1	–
19	0.17 M phosphate	3.03	454	3.09	40	78.7	0.880	51.8	–
20	0.17 M phosphate	5.94	454	3.13	40	67.4	1.03	21.2	–
21	0.1N HCl/NaCl	1.01	20.0	1.19	40	6.07	11.4	0.000366	0.00135
22		1.02	0	1.25	40	0.192	361	0.000891	0
23	0.5 M phosphate	0.988	20.2	2.15	40	2.07	33.5	0.216	0.000653
24		1.00	0	2.19	40	0.141	492	0.000523	0
25	0.17 M phosphate	1.01	19.9	3.10	40	3.66	19.0	1.35	0.00200
26		1.00	0	3.08	40	0.252	275	0.000404	0
27	0.50 M acetate	1.00	20.3	4.05	40	5.24	13.2	1.30	0.00500
28		0.994	0	4.03	40	0.465	149	0.000256	0
29	0.20 M acetate	1.00	20.9	5.01	40	2.63	26.3	0.554	0.00800
30		0.966	0	4.98	40	0.526	132	0.000047	0
31	0.06 M phosphate	0.994	20.3	6.96	40	3.36	20.6	0.748	0.00100
32		0.990	0	6.95	40	0.108	639	0	0
33	HCl	0.998	18.6	3.25	60	14.2	4.90	2.64	0.971
34	HCl	1.01	0	2.85	60	3.37	20.5	0.418	0
35	HCl	0.996	20.4	3.96	60	11.8	5.90	2.70	0.924
36	HCl	0.984	0	4.05	60	2.42	28.7	0.137	0

$t^*_{1/2}$ estimated based on initial rates of daptomycin loss.

scanning the peaks. Product **A** possessed an absorption maximum at 375 nm corresponding to the kynurenine maximum which appeared to have undergone a bathochromic shift by 10 nm relative to the kynurenine residue in daptomycin. Contrariwise, product **B** lost the characteristic of kynurenine maximum but showed an absorption maximum at 325 nm. Fractions of the two products were collected, pooled and lyophilized prior to further studies.

The integrity of lyophilized products **A** and **B** was evaluated by HPLC analysis. Lyophilized product **A** was re-dissolved in distilled water and re-injected into the HPLC to check the integrity of the compound after the separation and lyophilization. The chromatogram suggested that product **A** degraded to additional product with a relative retention time of about 0.69 and perhaps reversibly formed daptomycin during the isolation and lyophilization processes. Chromatograms of reconstituted product **B** showed the presence of product **B**, trace amounts of daptomycin and product **A**. It was speculated that daptomycin was reversibly formed from product **B** while product **A** was present as a contaminant during fraction collection. Peak areas obtained from the HPLC chromatograms implied that 70 and 80% of products **A** and **B** remained intact after separation and lyophilization.

3.1. Structure identification of reaction products

3.1.1. Spectral properties of isolated products **A** *and* **B**

The UV spectrum of product **A** showed absorption maxima at 375 nm which was identical to the preliminary UV analysis described above. The fluorescence intensity of product **A** solution was measured employing the excitation and emission wavelengths of kynurenine at 364 and 465 nm, respectively. The apparent quantum yield for product **A** was 1.7 times greater than that of daptomycin.

Product **B** lost the UV characteristic of kynurenine but showed an absorption maximum at 325 nm. The quantum yield was about one-half of that of daptomycin using the excitation and emission wavelengths of kynurenine.

Alteration in the UV absorption maxima and quantum yield of the kynurenine residue of both products suggested that the kynurenine primary amine was involved in the formation of both products.

Concentrations of products **A** and **B** were estimated based on UV absorbance of the tryptophan residue (282 nm using Beer's law curve based on daptomycin standards at the same analytical wavelength). The assumption made was that tryptophan was not the site of glyceraldehyde reaction. Thus, the molar absorptivities of tryptophan in products **A** and **B** were assumed to be equivalent to that of daptomycin.

3.1.2. Determination of mass

The mass spectrum of lyophilized product **A** showed two different masses of 1642.57 and 1714.64 *m/z* corresponding to masses of 1619.59 and 1691.66 ionized by one sodium atom. 1619.59 Da was identical to the mass of daptomycin. The mass of product **A** was estimated to be 1691.66 which was equivalent to the mass of glyceraldehyde and daptomycin less one water molecule. The presence of daptomycin in lyophilized product **A** was consistent with the chromatographic result previously reported that daptomycin was present as a contaminant in the isolated product **A**.

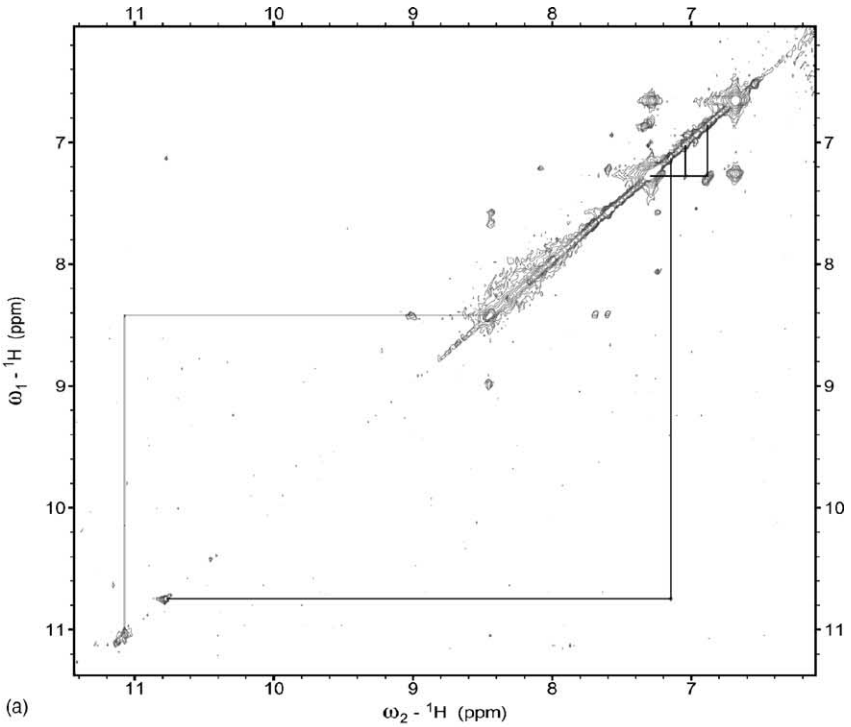
Mass spectrum of product **B** showed the mass of 1670.71 *m/z* corresponding to 1647.73 Da ionized by one sodium atom which was equivalent to an addition of either an aldehyde or ethyl group to daptomycin.

3.1.3. NMR spectra of product **B**

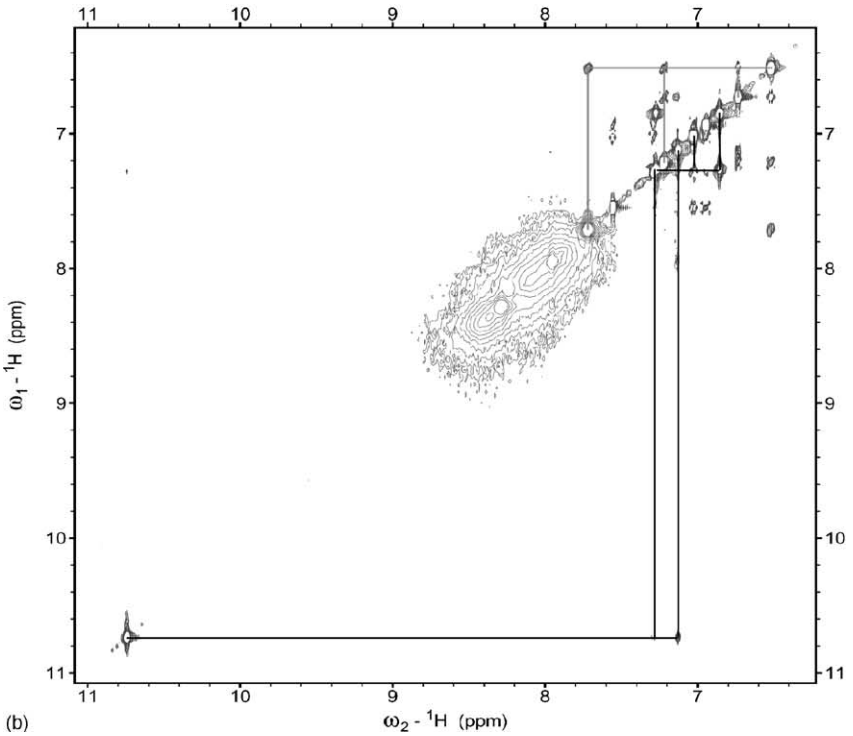
NOESY spectrum of product **B** in DMSO-d_6 taken over 80 h was compared to NOESY spectrum of daptomycin (Fig. 2). The NOESY spectrum of product **B** showed additional peaks (at 8.449 and 11.09 ppm) and cross peaks, which did not belong to daptomycin. Moreover, some peaks and cross peaks of product **B** in a range of 6.5–9.0 ppm were distinctively different from peaks and cross peaks of daptomycin.

3.2. Kinetics of reaction between daptomycin and glyceraldehyde

Reactions of 1 mM daptomycin and 20.0 mM glyceraldehyde were conducted in the pH range of 1–5 at 25 °C (reactions #1–5, Table 2). Reactions between 1 mM daptomycin and 20 mM glycerin were used as controls (reaction #5, Table 2). Concentration–time profiles showed 40 to 80% loss of daptomycin at pH 2.6, 3.7, and 5.1 in 100 to 200 h in the presence of glyceraldehyde. Formation and loss of products **A** and **B** were described as percent of initial total peak area.



(a)



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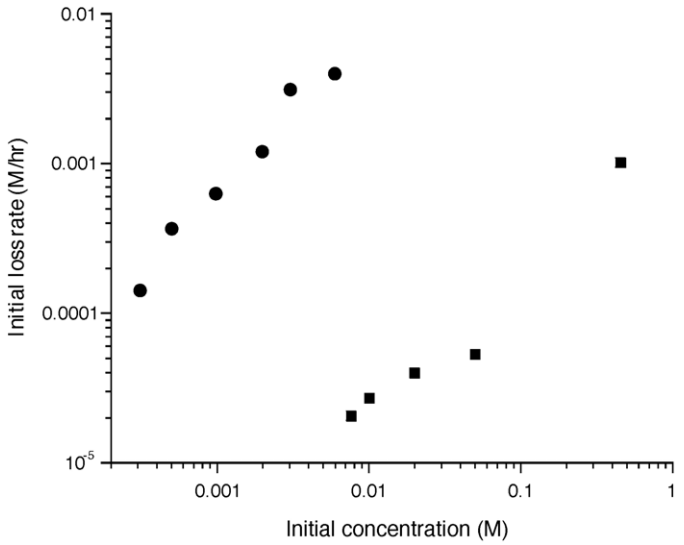

Fig. 3. Plot of initial loss rate of daptomycin vs. initial concentration of daptomycin on the logarithmic scales. Initial daptomycin loss rates were employed to determine order with respect to daptomycin concentration (●) and order with respect to glyceraldehyde concentration (■). The reactions were performed in 0.17 M phosphate buffer pH 3 (μ = 0.154 M) at 40 °C.

Product **A** was observed to accumulate from 3 to 25% in reactions conducted at pH values varying from 2.6 to 5.1. Product **B** was only observed in reactions at pH 2.6 and 3.7 wherein it accumulated to 5 to 7% of the initial substrate concentration. The initial rate of daptomycin loss and initial rates of products **A** and **B** formation are reported in Table 2.

Mass balance was determined by plotting the sum of all peak areas as a percent of the initial total peak area versus time. This data treatment assumes that the molar absorptivities of daptomycin and the two products were the same at the analytical wavelength (214 nm). Molar absorptivity of a peptide in the 190–230 nm range depends on the number of chromophores (i.e. tryptophan, tyrosine, and peptide bonds) present in its structure. Thus, peptides which have the same number of peptide bonds have equivalent molar absorptivities. Both products had masses within 106% to that of daptomycin which suggests that they possess nearly the same number of peptide bonds. Therefore, daptomycin reaction products were assumed to have the same molar absorptivities as that of daptomycin at 214 nm analytical wavelength.

Time-dependent mass loss was observed in the reaction of 1 mM daptomycin with 20 mM glyceraldehyde at pH 3.7, 25 °C but not in the absence of glyceraldehyde. The observation of 50% mass loss in the presence of glyceraldehyde indicated that other reaction products were formed.

3.3. *Degradation of products* **A** *and* **B**

Aliquots of products **A** and **B** were subjected to degradation at 60 °C, pH 3 and 4 using 0.1N standardized hydrochloric acid. The reactions were monitored using HPLC. Degradation of product **A** gave rise to two major products corresponding to daptomycin and an unknown. Daptomycin was a major degradation product of product **B**. In both reactions, mass balance was not maintained.

3.4. *Substrate concentrations effects on the daptomycin–glyceraldehyde reaction*

Loss of daptomycin at various concentrations of daptomycin (0.3 to 6.0 mM) and glyceraldehyde (0

Fig. 2. NOESY spectrum of product **B** in DMSO (a) and daptomycin (b). Dark lines correlate peaks and cross peaks of tryptophan aromatic protons. Gray lines in (a) correlate peaks and cross peaks of an aldehydic proton to an unknown amide proton. Gray lines in (b) correlate peaks and cross peaks of kynurenine aromatic protons.

to 454 mM) were studied in phosphate buffer pH 3 (μ = 0.154 M) at 40 °C (reactions #6 and #11–20, Table 2).

Initial rates of daptomycin loss were determined from slopes of daptomycin concentration–time profiles during the initial time interval wherein the loss of daptomycin was less than 10%. Initial rates of daptomycin loss increased as daptomycin and glyceraldehyde concentrations increased at constant glyceraldehyde and daptomycin concentrations, respectively (Fig. 3).

3.5. pH dependence

The effect of pH on the reaction between daptomycin and glyceraldehyde was investigated at 40 °C in hydrochloric acid, phosphate, and acetate buffers with ionic strength of 0.154 M (reactions #21–32, Table 2). The degradation of daptomycin in the absence of glyceraldehyde under the same experimental conditions was used as control.

In a pH range of 1–7, initial loss rates of daptomycin in the presence of glyceraldehyde were faster than the initial loss rates of daptomycin in an absence of glyceraldehyde. The observed rates of daptomycin loss appeared to be pH dependent (Table 2). The rate was 6.07% per hour at pH 1 and then decreased at pH 2. The observed rate of daptomycin loss reached another maximum at pH 4 with the rate of 5.24% per hour and again decreased at pH 5.

The formation of products **A** and **B** were observed in the presence of glyceraldehyde. The peak concentration of products **A** and **B** represent 16 and 5% of the initial daptomycin, respectively.

3.6. Effect of glyceraldehyde on formation and loss of anhydrodaptomycin

Loss of anhydrodaptomycin in the absence and in the presence of glyceraldehyde and formation of anhydrodaptomycin from daptomycin as a function of glyceraldehyde concentrations were investigated in 0.5 M acetate buffer pH 3 (μ = 0.154 M) at 40 °C.

For anhydrodaptomycin degradation (0.3 mM in the absence and in the presence of 6 mM glyceraldehydes) substrate disappearance was first-order and the initial loss rate were 3.60×10^{-3} mM h^{-1} and 0.60×10^{-3} mM h^{-1} in the presence and absence of glyceraldehydes, respectively. Mass balance was maintained in both reactions.

In the absence of glyceraldehyde, the major degradation product of anhydrodaptomycin was daptomycin which was consistent with the previous results (Kirsch et al., 1989). In the presence of glyceraldehyde, anhydrodaptomycin gave rise to daptomycin and two other major products: product **A** and an unknown product with a relative retention time of 1.10 to that of daptomycin.

The effect of glyceraldehyde concentration on anhydrodaptomycin formation was studied from the reactions of 1.0 mM daptomycin with 0 to 20.0 mM glyceraldehyde in 0.5 M acetate buffer pH 3 (μ = 0.154 M) at 40 °C (reactions #6–13, Table 2). Under these conditions, initial formation rate of anhydrodaptomycin decreased from 0.17 M h^{-1} to 5.3×10^{-4} M h^{-1} as glyceraldehyde concentration increased from 0 to 20.0 mM.

4. Discussion

The reaction of daptomycin and glyceraldehyde was studied in aqueous acid solutions at 25, 40 and 60 °C. Under these conditions, daptomycin is known to hydrolyze to a variety of products but mainly to anhydrodaptomycin and β-aspartyl daptomycin (Kirsch et al., 1989). In the presence of glyceraldehyde, daptomycin degradation was much faster and the hydrolytic products were almost absent. Thus, the objectives of the studies presented here on daptomycin–glyceraldehyde reactions were to identify major reaction products, define a reaction scheme, propose a reasonable mechanism for intermediate formation, and explain apparent anomalous hydrolytic degradation behavior, namely the absence of the major daptomycin hydrolysis product (anhydrodaptomycin).

In contrast to daptomycin hydrolysis, in the presence of glyceraldehyde a loss of up to 50% of initial starting material was unrecovered by HPLC during the complete loss of daptomycin. Moreover, two transient products representing about 25 and 5% of the starting material were formed in the presence of glyceraldehyde which were not observed in its absence. These two transient products were isolated, identified and degraded to develop a reaction scheme. The rate constants associated with the reaction scheme were used to explain

the chemistry of intermediate formation and apparent anomalous hydrolytic behavior.

4.1. Proton chemical shift assignment of daptomycin

Proton chemical shifts of daptomycin were assigned from COSY, TOCSY and NOESY spectra. Most of protons belonging to the cyclic peptide of daptomycin were assigned in this study. Orn δ-CH_2, Ser β-CH_2, and Trp α-CH were assigned to be 2.78, 3.64, and 4.42 ppm, respectively, which were consistent with the chemical shifts previously reported. Debono et al. (1988) reported three chemical shifts of daptomycin nucleus, the cyclic peptide without the decanoyl side chain. In his study, proton chemical shifts of daptomycin nucleus in particular the Orn δ-CH_2, Ser β-CH_2, and Trp α-CH were reported to be 2.78, 3.68, and 3.87 ppm, respectively.

4.2. Identification of degradation products

In the pH range 1–7, the aromatic amine of daptomycin is unprotonated (pK_a = 0.8) and therefore reactive, whereas the aliphatic amine (pK_a = 10.7) is protonated and unlikely to react. The reaction of daptomycin and glyceraldehyde gave rise to two previously unobserved intermediates, products **A** and **B**.

Product **A** showed a UV bathochromic shift of kynurenine absorption maximum (365 nm) by 10 nm. Fluorescence intensity of kynurenine in product **A** was 1.7 times greater than that of daptomycin. Molecular mass of product **A** equalled the molecular mass of daptomycin and glyceraldehyde less one water molecule. Degradation of product **A** gave rise to daptomycin. Thus, product **A** was identified as a Schiff's base (imine) product formed from the reaction of glyceraldehyde and the kynurenine residue of daptomycin.

In product **B**, the UV absorption maximum at 365 nm was absent but a maximum was present at 325 nm. Fluorescence of product **B** was twofold less than daptomycin. The spectral properties of product **B** suggested that kynurenine was involved in product formation. The molecular mass of product **B** was higher than that of daptomycin by 28 Da equivalent to the either addition of an aldehyde or ethyl group. 2D-^{1}H NMR was employed to elucidate chemical structure of product **B**.

The NOESY spectrum of product **B** was compared to that of daptomycin. Difference of cross-peaks in the NOESY spectrum of product **B** was due to chemical modification on daptomycin structure. In particular, the chemical shifts at 11.09 and 8.449 ppm were unique to product **B**. Generally, chemical shifts in ranges of 9–11.5 and 8–9 correspond to aldehydic and amide protons, respectively (Abraham et al., 2003). In this study, cross peaks of an aldehydic proton (11.09 ppm) to an amide proton at 8.449 ppm implied that product **B** contained an aldehyde functional group which was in close proximity to an unknown amide proton (Fig. 2a).

In addition, chemical shifts of the aromatic protons in product **B** were different from those in daptomycin. Daptomycin possesses two aromatic spin systems; i.e. tryptophan and kynurenine. Both aromatic spin systems overlapped in the region of 6.5 to 7.5 ppm (Fig. 2b). One of these corresponded to the aromatic protons in tryptophan since COSY and TOCSY spectra showed cross peaks between aromatic protons to the aromatic amine proton at 10.75 ppm (Evans, 1995). Thus, the other aromatic spin system was mutually assigned to kynurenine.

The NOESY spectrum of product **B** also contained two aromatic spin systems. The chemical shifts of tryptophan aromatic protons were unchanged while the chemical shifts of kynurenine aromatic protons were drastically shifted downfield to the region of 7.2 to 9.0 ppm which suggested nearby structural changes (Fig. 2a).

The molecular mass of product **B** was higher than that of daptomycin by 28 Da. Kynurenine UV absorption maximum at 365 nm was absent and the fluorescence at 465 nm was significantly reduced. NMR evidence showed cross peaks between an aldehydic proton (11.09 ppm) and an aromatic nitrogen proton of kynurenine (8.449 ppm). These results suggested the presence of an amide-like benzyl side chain. Moreover, degradation of product **B** regenerated daptomycin which is consistent with formanilide hydrolysis (Bergstrand, 1983). Taken together, product **B** was hypothesized to be an anilide derivative of daptomycin (Fig. 4).

Generally, amide bond formation in aqueous solution is unfavourable (Aman and Brown, 1999). However, intramolecular catalysis may facilitate the formation of an amide. The proximity of potential hydrogen bonding in the carbinolamine intermediate at the kynurenine side chain was investigated employing

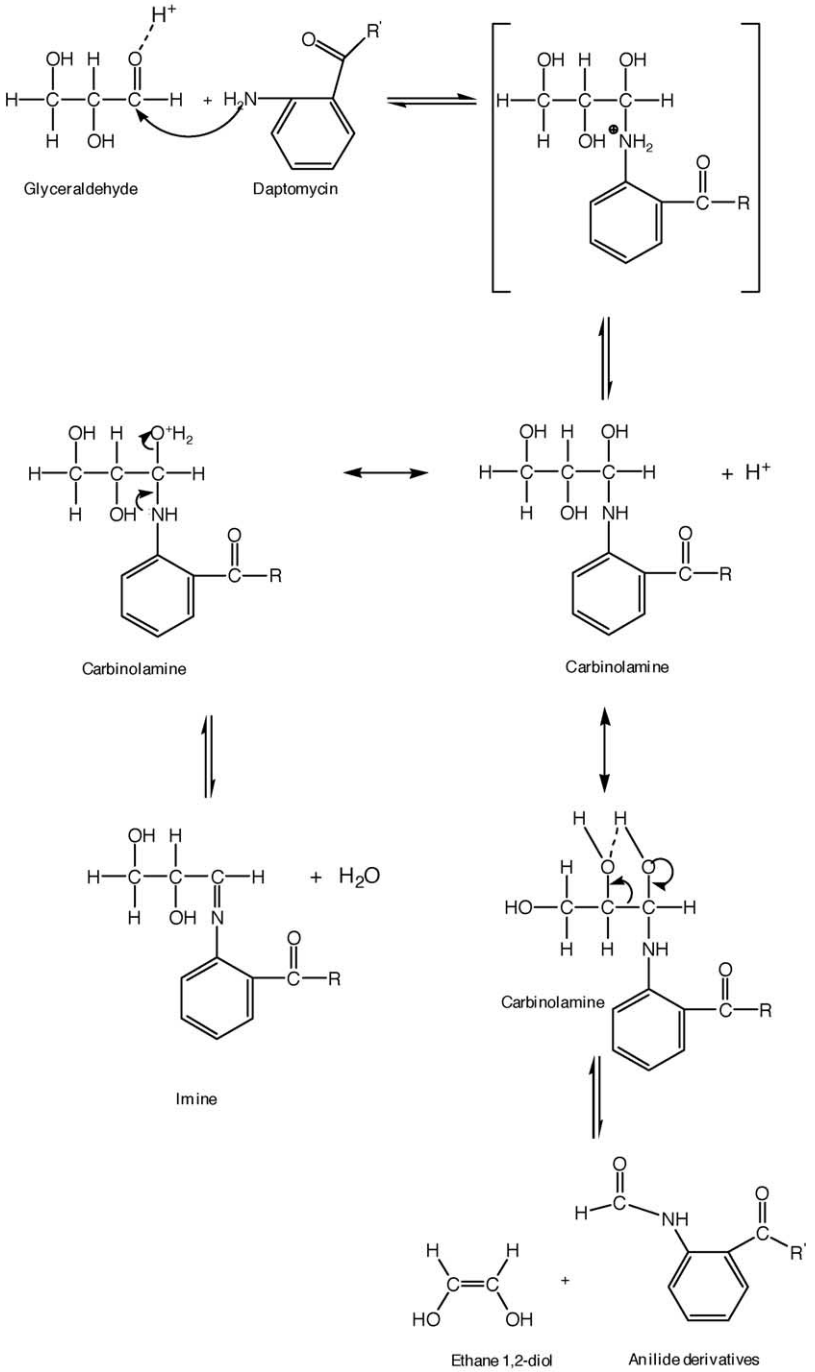

Fig. 4. Proposed formation scheme of products **A** and **B** from the reaction of daptomycin and glyceraldehyde.

MM2 energy minimization function available in CS Chem3D Ultra® by CambridgeSoft Corporation (Cambridge, MA). The distances between hydroxyl proton and the unshared pair of electrons in the other neighboring hydroxyl groups of the carbinolamine glycerol side chain were estimated to be about 1.78 Å and the distances between hydroxyl group oxygens were 2.63 Å. Generally, hydrogen bond could form where distance between hydroxyl proton and unshared pair electrons of the neighboring hydroxyl group is less than 1.8 to 1.9 Å (Loudon, 2002). Therefore, the intramolecular hydrogen bonding in the carbinolamine intermediate is plausible mechanism for anilide formation (Fig. 4).

4.3. Determination of degradation scheme

The reaction of glyceraldehyde and daptomycin in acidic solutions apparently gave rise to an imine and anilide. Both products likely share the same intermediate, a carbinolamine, formed from the attack of kynurenine on the carbonyl carbon of glyceraldehyde (Fig. 5). Upon further hydrolysis, the intermediate underwent acid dehydration to form an imine (product **A**) or intramolecular hydrogen bonding and resulting in a formation of an anilide (product **B**) and ethane-1,2-diol.

The degradation of the imine or anilide at 60 °C at pH 3 and 4 in the absence of glyceraldehyde regenerated daptomycin. Area under the curve (AUC) analysis was employed to demonstrate whether parallel loss of product **A** occurred. The rational and derivation of AUC analysis was described elsewhere (Joshi and Kirsch, 2002). The AUC for the daptomycin concentration–time profile from product **A** ($AUC_{dap/A}$) is given by

$$AUC_{dap/A} = \frac{k_{AD}[A]_0}{k_A k_{D0}} \quad (1)$$

where k_A is the sum of all routes of product **A** loss ($k_{AD} + k_{A0}$), k_{AD} is rate constant for daptomycin formation from product **A**, k_{A0} is a overall rate constant for parallel loss of product **A**, k_{D0} is rate constant of daptomycin hydrolysis, and $[A]_0$ is the initial concentration of product **A**. For a reaction initiated with daptomycin, $[D]_0$, the AUC for daptomycin loss is given by

$$AUC_{dap\,loss} = \frac{[D]_0}{k_{D0}} \quad (2)$$

Therefore, if $[D]_0$ is equal to $[A]_0$ the ratio of Eqs. (1) and (2) is equal to the ratio of the rate constants for conversion of product **A** to daptomycin (k_{AD}) to the sum of all rate constant for product **A** loss (k_A).

$$f_{dap/A} = \frac{k_{AD}}{k_A} \quad (3)$$

This ratio ($f_{dap/A}$) represents the fraction product **A** that is converted to daptomycin. If $f_{dap/A}$ is 1 then product **A** is quantitatively converted to daptomycin; i.e. there is no parallel loss of product **A**.

The area under the curve for the formation and loss of daptomycin from product **A** ($AUC_{dap/A}$) at pH 3 was estimated using the trapezoidal rule and area under the curve from $t_{end\to\infty}$ after correcting the observed AUC for the portion due to daptomycin contaminant present. In other words,

$$AUC_{dap/A} = AUC_{dap\ using\ trapezoidal\ rule} + \frac{[D]_{tend}}{k_{D0}} - \frac{[D]'_0}{k_{D0}}$$

where $[D]'_0$ was initial concentration of daptomycin contaminant present.

$AUC_{dap\,loss}$ was estimated by dividing the initial concentration of product **A** by k_{D0} and the fraction of product **A** that was converted to daptomycin ($f_{dap/A}$) was estimated to be 0.75 using Eq. (3). In other words, about 75% of product **A** converted to daptomycin while 25% of product **A** degraded to unidentified products.

In the same manner, fractions of product **A** converted to daptomycin at pH 4 was estimated to be 0.24 and fractions of product **B** converted to daptomycin

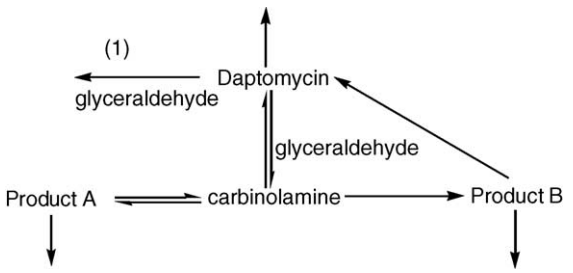

Fig. 5. Degradation scheme of daptomycin loss in the presence of glyceraldehyde. Additional loss of daptomycin due to reaction with glyceraldehyde was shown as pathway (1).

Table 3
Degradation rate constants of products **A** and **B** at 60 °C pH 3 and 4

Reaction pathway	Reaction pH 3	Reaction pH 4
Rate constant (h^{-1})		
Loss of product A ($k_{A\,loss}$)	0.075	0.127
Loss of product B ($k_{B\,loss}$)	0.125	0.064
Loss of daptomycin ($k_{D\,loss}$)	0.192	0.209
Formation of daptomycin from product A (k_{DA})	0.056	0.030
Parallel pathway of product A loss (k_{A0})	0.019	0.096
Formation of daptomycin from product B (k_{DB})	0.125	0.036
Parallel pathways of B loss (k_{B0})	0.000	0.028
Daptomycin hydrolysis (k_{D0})	0.029	0.027
Formation of product A from daptomycin (k_{AD})	0.045	0.037
Formation of product B from daptomycin (k_{BD})	0.010	0.008
Unidentified parallel pathways of daptomycin loss due to glyceraldehydes reactions ($k_{D0/GA}$)	0.101	0.135

($f_{dap/B}$) at pH 3 and 4, 60 °C were estimated to be 1.14 and 0.56, respectively.

Thus, the imine product underwent reversible formation during the reaction of daptomycin and glyceraldehydes whereas hydrolysis of the anilide regenerated daptomycin without reformation of the carbinolamine intermediate since the molecular mass of the anilide was less than the molecular mass of the carbinolamine (Fig. 5).

4.4. Kinetic analysis of degradation scheme

The kinetic analyses were employed to test and refine the degradation scheme by quantitative analysis of concentration–time profiles. Rate constants associated with the scheme were estimated using data obtained from degradation of products **A** and **B** and degradation of daptomycin in the presence and absence of glyceraldehyde at 60 °C, pH 3 and 4 (reactions #6–14 and #33–36, Table 2).

The degradation of products **A** and **B** were used to estimated $k_{A\,loss}$ and $k_{B\,loss}$, overall degradation rate constants of products **A** and **B**, respectively. AUC analysis was used to estimate k_{AD}, k_{A0}, k_{BD}, and k_{B0}. Rate constants for products **A** and **B** formation from daptomycin in the presence of glyceraldehyde (k_{DA} and k_{DB}) were estimated from the initial appearance rate (reactions #33 and 35, Table 2). The hydrolysis rate constants for daptomycin (k_{D0}) were determined in the absence of glyceraldehyde (reactions #34 and 36, Table 2). A summary of the estimated rate constants are presented in Table 3.

Kinetic simulation software, Stella® V.7.0.3 by Isee systems, Inc. (Lebanon, NH), was employed to predict concentration–time profiles for daptomycin, the imine and anilide according to the proposed reaction scheme and the estimated rate constants.

The predicted profiles from a reaction scheme without an additional pathway due to the presence of glyceraldehyde underestimated loss of daptomycin (Fig. 6a). Thus, the additional pathway of daptomycin loss due to the glyceraldehyde reaction was proposed (pathway 1 in Fig. 5). The rate constant for the additional pathway ($k_{D0/GA}$) at pH 3 was estimated to be 0.101 h^{-1} using the difference between the rate constant for the overall loss of daptomycin in the presence of glyceraldehyde ($k_{D\,loss}$) estimated from initial rate and the rate constant estimates for individual pathways of daptomycin loss (Table 3), i.e.

$$k_{D0/GA} = k_{D\,loss} - (k_{D0} + k_{DA} + k_{DB})$$

The predicted profile according to the reaction scheme in Fig. 5 described the experimental data with reasonable accuracy (Fig. 6b). The presence of the additional loss due to the glyceraldehyde reaction was consistent with the previous observation that up to 50% of initial daptomycin concentration was unrecovered by HPLC. The additional pathways may involve parallel loss and glyceraldehydes catalysis.

4.5. Reaction order

Reaction order with respect to daptomycin and glyceraldehyde concentration was determined at 40 °C in 0.17 M phosphate buffer, pH 3 (μ = 0.154 M) (reactions #6–20, Table 2) using the initial rate method by varying concentrations of daptomycin and glyceraldehyde. In this method, rate of daptomycin loss is directly proportional to its initial concentrations raised to the empirical order

$$\frac{d[Dap]}{dt} = k[Dap]^n[GA]^m$$

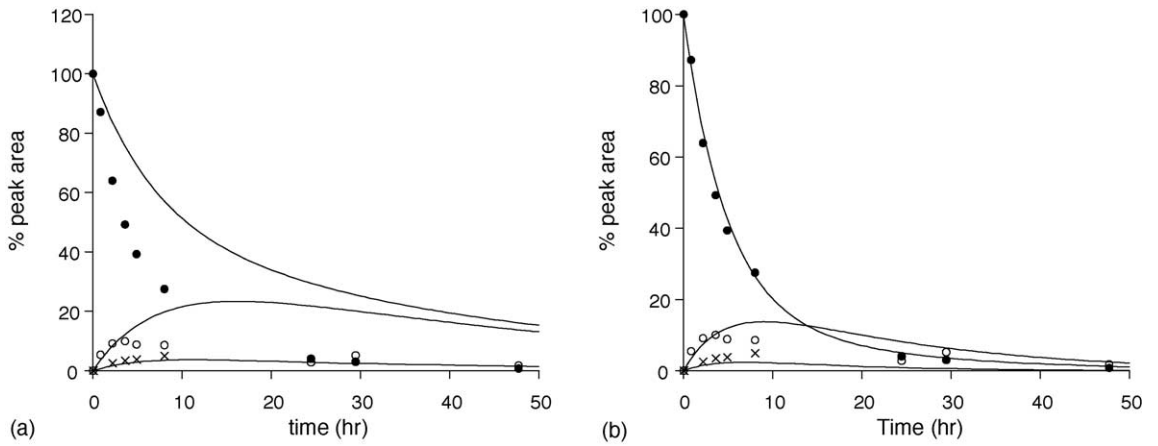

Fig. 6. Plots of loss of daptomycin (●) and formation and loss of products **A** (◯) and **B** (×) in the presence of glyceraldehyde at 60 °C, pH 3. Lines represent simulated data of daptomycin, formation and loss of products **A** and **B** obtained from Stella® using a model without (a) and with the additional loss (b) of daptomycin and glyceraldehyde reaction.

where k is the observed degradation rate constant, n and m are the order with respect to daptomycin and glyceraldehyde concentration, respectively. In the initial time interval, where the loss of daptomycin is less than 10%, the daptomycin concentration is approximately equal to its initial concentration, i.e. $[\text{Dap}]_t \cong [\text{Dap}]_0 \cong$ constant and the glyceraldehyde concentration is approximately $[\text{GA}]_0$. Then,

$$\frac{\text{d}[\text{Dap}]}{\text{d}t} = k'[\text{Dap}]^n \tag{4}$$

where $k' = k[\text{GA}]^m$

Thus, logarithmic transformation of Eq. (4) yields

$$\ln\left(\frac{\text{d}[\text{Dap}]}{\text{d}t}\right) = \ln\, k' + n\, \ln[\text{Dap}]_0 \tag{5}$$

and the slope of a plot from this equation equals the order of the reaction with respect to daptomycin concentration.

The initial rates of daptomycin loss were estimated from the reactions of 0.3 to 6.0 mM daptomycin with 0.454 M glyceraldehyde (reactions #15–20, Table 2). The initial rates were plotted according to Eq. (5) (Fig. 3). The slope was approximately unity and the reaction was first-order with respect to daptomycin concentration.

The reaction order with respect to glyceraldehyde concentration was determined in the same manner. The initial rate of daptomycin loss increased with the initial glyceraldehyde concentration with a slope of 0.93. The reaction was a first-order with respect to glyceraldehyde concentration and is second-order overall.

Based on kinetic analysis, other products resulted from the reaction of daptomycin and glyceraldehyde in addition to the formation of products **A** and **B**. The net loss of HPLC peak area over the reaction course demonstrates that these additional products were largely undetectable with the current chromatographic system. A number of possible side chain peptide reactions that could have occurred, the reaction of glyceraldehyde with the hydroxyl side chain of serine to form a hemiacetal or hemiketal (Chen et al., 2002; Louie et al., 2001) and glyceraldehyde reaction with the side chain amide of asparagines (Lakner and Negrete, 2002) have been reported. However, it is not likely that these minor side chain modifications would result in undetected products using the current chromatographic system. Contrariwise dimerization would likely result in uneluted products. Dimerization might be expected to result if a reaction order with respect to daptomycin was greater than first-order. However, this result was not obtained.

4.6. pH dependence

The initial rate of daptomycin loss and initial rate of products **A** and **B** formation were estimated in the pH range of 1–7 at 40 °C (reactions #21–32, Fig. 7;

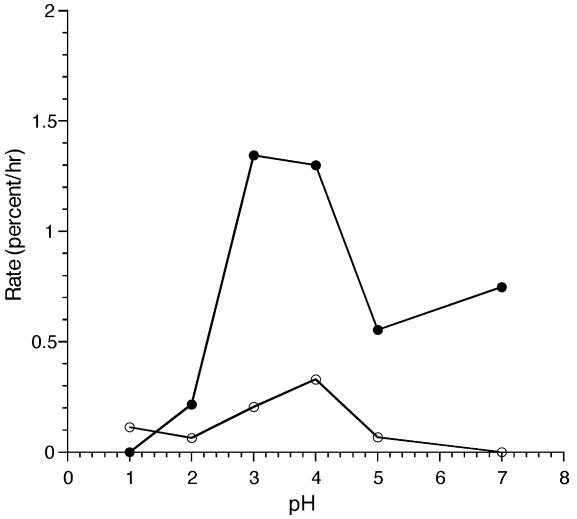

Fig. 7. Initial formation rate of products **A** (●) and B (○) from the reactions of 1 mM daptomycin with 20 mM glyceraldehyde at 40 °C in a pH range of 1–7.

Table 2). The reaction rate estimates appeared to show a pH dependence in which the reaction rates reached a maximum in a pH range 3–4.

A bell-shaped pH-rate profile is typical for amine aldehyde reactions (Jencks, 1969). The observed bell-shaped profile is due to change in rate-limiting steps from the attack of unprotonated amines on the carbonyl carbon at low pH to acid catalyzed dehydration of the carbinolamine intermediate at higher reaction pH (Jencks, 1969). In reactions between a simple aldehyde and amine, the change in rate-limiting step occurs near the amine pK_a (Jencks, 1969; Klixbull and Bundgaard, 1985). However, for the reactions between weakly basic amines and the aldehyde, the change in rate-determining step occurs at the same or at slightly higher pH compared with the reactions of the more basic amines (Jencks, 1969). An increase in optimum pH by 1–2 pH units was also observed when the substrates were changed from a dipeptide (Gly–Ser) to a tripeptide (Gly–Ser–Ala) (Mori et al., 1989). The anomalous changes in rate-determining step were proposed to be due to the presence of secondary structure of the tripeptide but not in the dipeptide. In this study, the reaction rate was maximum at pH 3–4, about 2 pH units higher than the expectation (at pH 1).

The rate constants for product **A** conversion to daptomycin (k_{AD}) increased from 0.03 to 0.06 h^{-1} as the reaction pH decreased from 4 to 3. This observation is consistent with acid hydrolysis of an imine which is specific acid catalyzed wherein protonation of the imine is the rate-determining step (El-Taher, 1996; Jencks, 1969).

Increases in the rate constant of daptomycin formation from product **B** (k_{BD}) were from 0.036 to 0.125 h^{-1} as the reaction pH decreased from 4 to 3. This result suggests that hydrolysis of product **B** was acid catalyzed which is consistent with anilide hydrolysis (Ahmed et al., 1988). Moreover, amide formation proceeds by formation of carbinolamine intermediate. This mechanism is consistent with the proposed formation scheme of product **B** in which the carbinolamine is the intermediate of anilide product formation (Fig. 4).

4.7. Anomalous hydrolytic degradation

The extent of anhydrodaptomycin formation from reactions of 1 mM daptomycin in the presence and absence of glyceraldehyde (20 mM) was determined in the pH range of 1–7 at ionic strength of 0.145 M (reactions #22–33, Table 2). In the presence of glyceraldehyde, anhydrodaptomycin formation was much less than in the absence of this reagent. This result was more pronounced in a pH range of 3–4 where the conversion rate of anhydrodaptomycin from daptomycin is the fastest (Kirsch et al., 1989).

To investigate the possible facilitation of anhydrodaptomycin degradation by glyceraldehyde, 0.3 mM anhydrodaptomycin was reacted with 6 mM glyceraldehyde in 0.5 M acetate buffer pH 3 (μ = 0.154 M) at 40 °C. In the absence of glyceraldehyde, anhydrodaptomycin degraded to daptomycin. In the presence of glyceraldehyde, anhydrodaptomycin gave rise to three products; i.e. daptomycin, product **A** and an unknown product with a relative retention time of 1.10 to that of daptomycin. The initial rates of anhydrodaptomycin loss in the absence and in the presence of glyceraldehyde were determined to be 0.60×10^{-3} mM h^{-1} and 3.60×10^{-3} mM h^{-1}, respectively. Therefore, glyceraldehyde facilitated anhydrodaptomycin degradation.

Three hypotheses were proposed to explain the observed formation of product **A** in this reaction. Anhydrodaptomycin formed product **A**, anhydrodaptomycin formed unknown product which co-eluted with product **A**, or anhydrodaptomycin converted to daptomycin resulting in product **A** formation.

Anhydrodaptomycin was likely to react with glyceraldehyde and form products in the same fashion as in the daptomycin and glyceraldehyde reaction. However, the Schiff's base product of anhydrodaptomcin was unlikely to co-elute with product **A**, the imine product of daptomycin. Anhydrodaptomycin is more lipophilic than daptomycin due to the presence of a succinimide group at the Asp-10 residue. The higher lipophilicity of anhydrodaptomycin is seen in the HPLC chromatogram wherein anhydrodaptomycin has a longer retention time than that of daptomycin. Therefore, products of anhydrodaptomycin and glyceraldehyde should possess a higher lipophilicity than the Schiff's base product of daptomycin, product **A**. Thus, the observed formation of product **A** occurred by conversion of anhydrodaptomycin to daptomycin which subsequently reacted with glyceraldehyde.

To determine whether the presence of glyceraldehyde retarded anhydrodaptomycin formation or facilitated parallel loss of daptomycin, daptomycin was reacted with glyceraldehyde at various concentrations (0 to 20.0 mM) in buffer pH 3 ($\mu = 0.154$ M), at 40 °C (reactions #7–14, Table 2). The initial formation rate of anhydrodaptomycin was determined from slopes of the anhydrodaptomycin formation time profiles and plotted against initial glyceraldehyde concentration on logarithmic scales. In a glyceraldehyde concentration range of 0–7.0 mM, the initial formation rate of anhydrodaptomycin was about 0.1 mM h^{-1}. At glyceraldehyde concentration above 7 mM, the observed initial formation rate of anhydrodaptomycin decreased to 0.5 mM h^{-1} at 20.0 mM glyceraldehyde. This observation implied that, in the presence of high glyceraldehyde concentration, parallel pathway competed with anhydrodaptomycin formation resulting in facilitation of daptomycin loss. Thus, glyceraldehyde facilitated loss of anhydrodaptomycin and facilitated parallel loss of daptomycin thereby the extent of anhydrodaptomycin formation was reduced.

5. Conclusion

Reaction of daptomycin and glyceraldehyde gave rise to two significant products, products **A** and **B**, which were separable by HPLC. Structural identification of reaction products were carried out using UV-spectroscopy, fluorescence, mass spectrometry, and 2D-^{1}H NMR. Product **A** was a reversible imine product of glyceraldehyde and daptomycin at the kynurenine primary amine. Product **B** was appeared to be anilide derivative of daptomycin. The mechanism of product **B** formation was proposed in which intramolecular hydrogen bonding of the carbinolamine intermediate resulting in electron transfer and sequentially bond cleavage. The proposed mechanism was chemically plausible (Fig. 4).

A reaction scheme was proposed that kynurenine primary amine of daptomycin reacted with glyceraldehyde and formed carbinolamine intermediate. Products **A** and **B** were formed through two separate pathways but shared one common intermediate, carbinolamine. The reaction was estimated to be first-order with respect to daptomycin and glyceraldehyde concentrations. In a pH range of 1–7, the initial formation rate of product **A** was pH dependent in which the formation rate increased as the pH increased. The formation rate reached a maximum at pH about 3 and then the formation rate of product **A** decreased as the pH increased.

In another study, the presence of glyceraldehyde was shown to reduce the formation of anhydrodaptomycin from daptomycin. This was believed that in the presence of glyceraldehyde, formation of the carbinolamine intermediate was relatively faster than the conversion of daptomycin to anhydrodaptomycin. Therefore, the overall reaction direction was moved towards formation of carbinolamine in the presence of glyceraldehyde.

In conclusion, the unprotonated primary amine of kynurenine reacted with the aldehyde group of glyceraldehyde to form a carbinolamine intermediate. The intermediate went through two separate pathways to form Schiff's base and anilide products. Degradation of each product gave rise to daptomycin formation. It was shown that the formation rate of imine product was pH dependent.

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