Elucidating the Pathways of Degradation of Denagliptin

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ABSTRACT: Stress testing or forced degradation studies of denagliptin (**1**) tosylate in solution and solid-state, its blends with excipients, and capsules were conducted in order to elucidate degradation pathways, aid formulation development, and generate data to support regulatory filings. In solution, denagliptin was stressed in acid, water, and base using organic cosolvents. In the solid-state, denagliptin was stressed under heat, humidity, and light. Blends of denagliptin with various excipients were stressed under heat and humidity in order to evaluate whether tablet was a viable dosage form. Capsules were stressed under heat, humidity, and light. It was found that denagliptin was stable in the solid-state, but degraded in solution, in blends with all excipients, and in capsules predominantly by cyclization to (3*S*,7*S*,8a*S*) amidine (**2**), which epimerized to (3*S*,7*S*,8a*R*) amidine (**3**). (3*S*,7*S*,8a*R*) amidine (**3**) subsequently hydrolyzed to the corresponding diketopiperazine (**4**). The purpose of this manuscript is to discuss the results of stress testing studies conducted during the development of denagliptin and the elucidation of its key degradation pathway. © 2010 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 99:3030–3040, 2010

Keywords: chemical stability; formulation; mass spectrometry; NMR spectroscopy; preformulation; solid state stability; stability

INTRODUCTION

Denagliptin (Scheme 1), (2*S*,4*S*)-4-Fluoro-1-[4-fluoro-β-(4-fluorophenyl)-L-phenylalanyl]-2-pyrrolidinecarbonitrile 4-methylbenzene sulfonic acid salt (1:1), was being developed for the treatment of type 2 diabetes due to its ability to inhibit dipeptidyl peptidase IV. Stress testing studies of denagliptin in solution, in the solid-state, in blends with various excipients, and bead-filled capsules were conducted to elucidate its degradation pathways, aid formulation development, and generate data for regulatory filings. The conditions used for stress testing and the threshold used for identification of degradation products were based on guidance provided in regulatory documents.[1,2]

Three key degradation products were observed arising primarily by intramolecular cyclization of amino and cyano groups in denagliptin (Scheme 1). The results of stress testing studies of denagliptin, preparation of the three predominant degradation products, and their structure elucidation are discussed in this manuscript. Intramolecular cyclization of amino and carboxylic acid, ester, or amide groups to diketopiperazines has been reported as a key degradation pathway for model dipeptides,[3,4] and dipeptide drugs like moexipril,[5–8] quinapril,[9–12] enalapril,[13–15] and lisinopril.[16] Detailed mechanisms of diketopiperazine formation by cyclization of dipeptide amides[17] and esters[18] has also been reported in the literature. However, to our knowledge, this is the first example of drug degradation by intramolecular cyclization to amidines.

EXPERIMENTAL

Materials and Methods

Denagliptin drug substance and capsules were manufactured in-house (Glaxo SmithKline, Research Triangle Park, NC). Monobasic sodium phosphate,

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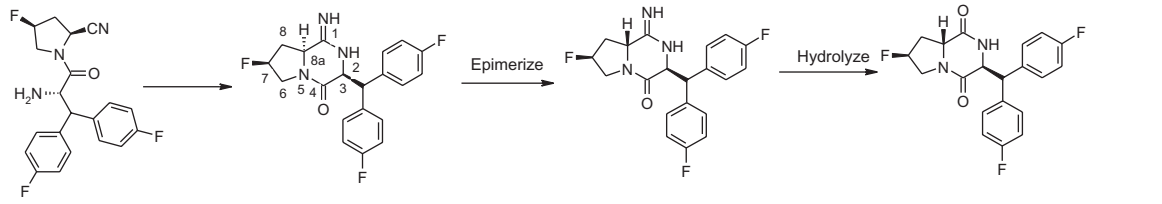

Scheme 1. Pathway of denagliptin degradation.

dibasic sodium phosphate, sodium hydroxide, hydrochloric acid, trifluoroacetic acid, dimethyl sulfoxide (DMSO), 1-methyl 2-pyrrolidinone (NMP), and 2-methoxy ethyl ether (Diglyme) were obtained from J.T. Baker (Springfield, NJ). All chemicals were of reagent grade and used as received. Solvents used for chromatography were HPLC grade. Deuterated DMSO was obtained from Cambridge Isotope Laboratories (Andover, MA). Mannitol was obtained from Roquette Pharma (Keokuk, IA), croscarmellose sodium from FMC Biopolymer (Philadelphia, PA), crospovidone from International Speciality Products Inc. (Wayne, NJ), magnesium stearate from Peter Greven (Bad Munstereifel, Germany), stearic acid from Mallinckrodt (Hazelwood, MO), sodium stearate from Witco Corporation (Greenwich, CT), alginic acid from Edward Mendell (Carmel, NY), and citric acid from Sigma–Aldrich (St. Louis, MO). Ovens were obtained from Baxter Scientific products and the light chamber was obtained from Forma Scientific Inc. (Model 3890). Amber flint glass vials (part number 223763) were obtained from Wheaton Scientific Products (Millville, NJ).

Stress Testing Studies of Denagliptin (1) Drug Substance

In solution, denagliptin was stressed at 80°C in 0.1 N HCl, in water under N_2, air, and O_2 headspaces, and in 0.01 N NaOH at a concentration of 2.0 mg/mL. Organic cosolvents were used to enhance the solubility of denagliptin in the aqueous reagents used for the study. Dimethyl sulfoxide was used as a cosolvent in 0.1 N HCl, NMP was used as a cosolvent in water, and diglyme in 0.01 N NaOH. Different cosolvents were used depending on the pH of the study in order to minimize degradation of solvents, and prevent interaction between cosolvent or its degradation products and denagliptin. The ratios of organic to aqueous reagents were 1:4 DMSO:0.1 N HCl, 1:4 NMP:water, and 1:1 diglyme:0.01 N NaOH.

In the solid-state, denagliptin was stressed for 2 weeks at 80°C (ambient RH), 2 weeks at 80°C/75% RH, and under UV (400 Wh/m^2) and fluorescent light (2.4 million lux hours). In order to prepare samples for stressing at 80°C and 80°C/75% RH, 60 mg of drug was weighed directly into two different 25-mL amber flint glass vials. One vial was sealed with rubber septum and aluminum flange collar whereas the other was left open. Both vials were placed in a microdessicator containing saturated sodium chloride solution, which was then placed in the 80°C oven. In order to prepare samples for stressing in light, 60 mg of drug was weighed directly into two clear polypropylene petri-plates, the drug was spread into a thin film using spatula, and the plates were placed uncovered in UV and fluorescent light chambers, respectively.

Excipient Compatibility Stress Testing Study

The excipient compatibility stress testing study of denagliptin was conducted using the same drug to excipient ratios as desired for the 7.5 mg tablet. Binary, ternary, and quaternary blends of denagliptin were prepared and stressed at 60°C and 60°C/75% RH for 2 days in order to evaluate the impact of single and a combination of excipients on the stability of denagliptin. Appropriate amounts of drug and excipient(s) were weighed directly into clear 25-mL glass vials, the contents were vortexed to mix, and stored sealed (60°C) or open (60°C/75% RH) in a dessicator containing saturated brine solution. The ratios of drug to various excipients used for preparation of the blends are summarized in Table 1. Amounts of sodium stearate and stearic acid were adjusted so that two equivalents were used relative to magnesium stearate. Alginic acid and anhydrous citric acid were evaluated as acid modifiers in quaternary blends using amounts to ensure that same number of moles of both were present in the blends. Excipient free denagliptin was used as control. Samples were prepared for HPLC analysis in duplicate by adding 20.0 mL of 1:1 water:acetonitrile directly to vial, vortexing to dissolve, filtering using 1 μm Gelman GF filter, discarding the first few milliliters transferring to a HPLC vial, and injecting 8 μL for analysis.

Stress Testing Studies of Denagliptin (1) Capsules

Capsules of 7.5 mg, 15.0 mg, and 30.0 mg strengths, prepared by coating drug on microcrystalline cellulose spheres, and placebo were stressed for 2 weeks at

Table 1. Denagliptin Excipient Ratios in Blends

Blend Type	Components	Blend Ratio
Binary	Denagliptin:mannitol	7.5:183.5
	Denagliptin:croscarmellose sodium	7.5:5.0
	Denagliptin:crospovidone	7.5:4.0
	Denagliptin:magnesium stearate	7.5:4.0
Ternary	Denagliptin:mannitol:croscarmellose sodium	7.5:183.5:5.0
	Denagliptin:mannitol:magnesium stearate	7.5:183.5:4.0
Quaternary	Denagliptin:mannitol:croscarmellose sodium:magnesium stearate	7.5:183.5:5.0:4.0
	Denagliptin:mannitol:croscarmellose sodium:stearic acid	7.5:183.5:5.0:3.8
	Denagliptin:mannitol:croscarmellose sodium:sodium stearate	7.5:183.5:5.0:4.1
	Denagliptin:mannitol:crospovidone:stearic acid	7.5:183.5:4.0:3.8
Acid modifiers	Denagliptin:mannitol:croscarmellose sodium:stearic acid:aliginic acid	7.5:183.5:5.0:3.8:20.0
	Denagliptin:mannitol:croscarmellose sodium:stearic acid:citric acid	7.5:183.5:5.0:3.8:2.6

80°C (ambient RH), 2 weeks at 80°C/75% RH, and under UV and fluorescent light. Five capsules each of drug or placebo were placed in 25-mL amber flint glass vials for stressing at 80°C and 80°C/75% RH. The vials were sealed for storage at 80°C and left open for storage at 80°C/75% RH. The vials were placed in a micro-dessicator containing saturated sodium chloride solution, which was then placed in an 80°C oven. The samples stressed under light were prepared by placing five capsules each in two clear polypropylene petri-plates, which were then placed uncovered in UV and fluorescent light chambers, respectively. Samples were prepared for HPLC analysis by opening the capsule shells, transferring the beads and empty capsule shells to an appropriate volumetric flask, adding 3:2 water:acetonitrile till the flask was two-third full, shaking for 40 min, and diluting to mark with 3:2 water:acetonitrile. The samples were filtered as described above in excipient compatibility section before HPLC analysis.

HPLC Separations

An Agilent 1100 analytical HPLC system was used for analyses of stressed drug substance, blends, and capsule samples. Stressed drug substance samples were analyzed using Zorbax bonus-RP C18 column (150 mm × 4.6 mm i.d., 3.5 μm particle size) with 40°C column compartment temperature and 215 nm detector wavelength. Mobile phase A consisted of 80/20 20 mM phosphate buffer/acetonitrile at pH 6.1 and mobile phase B contained acetonitrile. Gradient started at 0% B and increased to 50% B after 18.0 min with 1.5 mL/min flow rate. This method was modified to make it LC/MS compatible by using 80/20 10 mM ammonium acetate/acetonitrile at pH 6.1 instead of phosphate buffered mobile phase as mobile phase A. HPLC column, gradient, flow rate, and all other parameters were exactly the same for the two methods. Stressed blends and capsule samples were analyzed using Zorbax SB-phenyl column (150 mm × 4.6 mm i.d, 3.5 μm particle size) with 40°C column compartment temperature and 215 nm detector wavelength. Gradient started at 0% B and increased to 95% B after 15.0 min with 1.0 mL/min flow rate. Mobile phase A consisted of 0.05% (v/v) trifluoroacetic acid in 90/10 water/methanol and mobile phase B contained 0.05% (v/v) trifluoroacetic acid in 10/90 water/methanol. It was not possible to accurately quantitate the levels of (3*S*,7*S*,8a*S*) amidine **2** using either of the two methods because it epimerized to amidine **3**.

LC/MS Experiments

Mass spectral data were acquired with a Thermo Electron LTQ ITMS (San Jose, CA) and an Agilent Ion Trap SL (Santa Clara, CA) using positive electrospray ionization (ESI). Typical parameters for operation of the Finnigan LTQ were as follows: spray voltage, nominal 4.5 kV; heated capillary, 325°C; sheath gas, 60 psi; auxillary gas, 20 psi; sweep gas, 6 psi; scan range, 100–2000 Th (or other appropriate range for the expected mass of the compounds of interest); total "microscans," 1; maximum injection time, 10 ms and ion polarity, positive. The Agilent Ion Trap was operated with a spray voltage of 3.5 kV, a nebulization pressure of 70 psi, drying gas temperature of 350°C, and drying gas flow of 12 L/min. An accumulation time of 300 ms over a scan range of 100–1000 Th was used for the ion trap mass analyzer.

High Resolution Mass Spectrometry Experiments

High resolution mass spectrometry (HRMS) analyses were performed using an LTQ-Orbitrap Discovery XL hybrid mass spectrometer (ThermoFisher, San Jose, CA). HRMS experiments were performed by pneumatically assisted electrospray ionization (ESI) in positive ion detection mode with a nominal Orbitrap resolving power setting of 30,000 (at m/z 400). The instrument was operated at a spray voltage of 4.5 kV, a capillary temperature of 325°C, a capillary voltage

of 22 V and a tube lens voltage of 70 V. Gas flow rates, electronics settings and voltages for the ion optics were optimized and confirmed prior to performing HRMS experiments. Data acquisition for full scan experiments was performed in profile mode. Data acquisition for MS/MS fragmentation experiments using higher energy collisionally activated dissociation (HCD) were performed in centroid mode using collision energy settings optimized for each analyte.

Sample introduction was performed using an Agilent (Santa Clara, CA) 1200 HPLC/DAD system employing a linear, reverse-phase gradient chromatographic method with mobile phases comprised of water, acetonitrile and trifluoroacetic acid. The 1.0 mL/min flow of eluent from the HPLC system was split to achieve approximately 350 μL/min flow into the mass spectrometer with the remainder diverted to waste.

Single Crystal X-Ray Analysis of 3[1]

All measurements were made at 150(2) K using a Nonius KappaCCD diffractometer with graphite monochromated Mo Kα radiation ($\lambda = 0.71073$ Å) from a normal focus sealed tube source. The crystal was monoclinic, space group $P2_1$ (no. 4), with a unit cell of, $a = 13.3888(16)$ Å, $b = 6.3797(3)$ Å, $c = 15.047(3)$ Å, $\beta = 92.922(15)^\circ$, $V = 1283.6(3)$ Å^3, giving $Z = 2$, $D_{calc} = 1.412$ Mg m^{-3} and μ(Mo Kα) = 0.187 mm^{-1}. Data were corrected for Lorentz and polarization effects and a numerical absorption correction was applied (transmission = 0.950–0.983). The structure was solved by direct methods and refined (using data to 45° in 2θ) using full-matrix least-squares procedures: the function $\sum w(F_0^2 - F_c^2)^2$ was minimized in the Bruker-AXS SHELXTL software package (Ver. 6.10 [NT]). Co-ordinates and anisotropic atomic displacement parameters were refined for all non-hydrogen atoms, with some appropriate rigid bond and similarity atomic displacement restraints. Hydrogen atoms were included in calculated positions and were refined using a riding model. Isotropic atomic displacement parameters for the hydrogen atoms were used as multiples of U_{eq} for the attached atoms. The final refinement of 344 variables converged ($\Delta/\sigma_{max} = 0.000$) to give crystallographic residuals $R1$ [2210 reflections with $F_0 > 4\sigma(F_0)$] and $wR2$ (all 3129 data) of 0.0754 and 0.1059, respectively. The "goodness of fit" on F^2 was 1.093. A final difference Fourier synthesis showed residual electron density between −0.25 and 0.35 eÅ^{-3}. The absolute structure parameter refined to 0.02(16).

[1]CCDC 749088 contains the supplementary crystallographic data for this article. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: +44-122-336033.

Preparation of (3*S*, 7*S*, 8a*S*) Amidine (2)

(3*S*,7*S*,8a*S*) amidine (**2**) was prepared by heating a 2.5 mg/mL solution of denagliptin free base (25.98 mg) in $CHCl_3$ and $FeCl_3$ (12.49 mg; mole ratio 1:1.2) at 80°C for 2 h by modification of a procedure described in the literature[19] to prevent epimerization. A complex of amidine **2** with $FeCl_3$ precipitated out of solution and was 98.7% pure after filtration. Amidine **2** with purity of 97.5% was obtained by redissolving the complex in $CHCl_3$ followed by washing with 4 volumes of 0.01 N NaOH and rotary evaporation of the organic layer.

Preparation of (3*S*,7*S*,8a*R*) Amidine (3)

(3*S*,7*S*,8a*R*) amidine (**3**) was prepared by weighing 101.64 mg denagliptin free base and 50.6 mg K_2CO_3 (2 equivalents) directly into a 10-mL reactivial and adding 10 mL methanol. The vial was placed in a 40°C oven for 19 h after which it was removed, cooled to ambient temperature, and methanol was evaporated on a rotary evaporator. The residue was dissolved in 80 mL ethyl acetate and washed with 50 mL water. Ethyl acetate layer was dried over $MgSO_4$, filtered, and ethyl acetate was evaporated on a rotary evaporator. The residue (93.6 mg) was then dissolved in 2.5 mL CH_3CN and 95.0 mg toluene sulfonic acid monohydrate (2.0 equivalents) was added and the solution was stirred for 1 h to get a white precipitate of **3** with a purity of 98.6% by HPLC. This sample was used for NMR analysis. The sample used for single crystal X-ray diffraction analysis was prepared using exactly the same ratio of reagents and procedure as described above starting with 1.0 g denagliptin free base.

Preparation of Diketopiperazine (4)

Diketopiperazine formed with 99.0% purity when 98.1 mg denagliptin free base in a 25-mL amber flint glass vial was heated at 80°C for 2 weeks in the solid-state. This sample was used for NMR analysis.

NMR Experiments

All NMR spectra were recorded at 25°C on a VARIAN UNITY INOVA spectrometer operating at a ^{1}H frequency of 499.887 MHz and ^{13}C frequency of 125.709 MHz using a nalorac 3 mm indirect detection probe. Tetramethyl silane (TMS) was used as an internal chemical shift standard. The samples were prepared by dissolving 10–15 mg of compound in 250 μL $CDCl_3$ or DMSO-d_6, adding 5 μL TMS, and transferring to a 3 mm NMR tube.

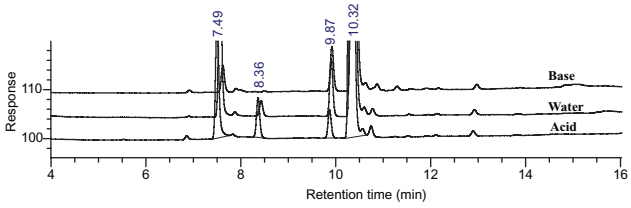

Figure 1. Overlaid HPLC chromatograms of denagliptin samples stressed in solution for 6 h at 80°C.

RESULTS

Stress Testing Studies of Drug Substance

Denagliptin was stable on stressing in the solid-state, but unstable on stressing in solution. Three major impurities were observed in acid and water, and two major impurities were observed in base, as shown in Figure 1. LC/MS analysis of stressed samples using the modified ammonium acetate method indicated that peaks eluting with retention times 7.49 min (amidine **3**) and 8.36 min (amidine **2**) were isobaric to denagliptin with a molecular weight 373 Da, and the peak eluting with retention time of 9.87 min had a molecular weight of 374 Da (diketopiperazine **4**). Accurate mass HRMS measurements of amidine **2** demonstrated a measured *m/z* value of 374.1477 for the $[M+H]^+$ ion to be within 0.5 ppm of calculated (374.1475) at a resolving power of 34,700 and indicated a molecular formula of $C_{20}H_{18}F_3N_3O$. Accurate mass measurements of amidine **3** demonstrated a measured *m/z* value of 374.1475 for the $[M+H]^+$ ion to be within 0.1 ppm of calculated (374.1475) at a resolving power of 34,800 and indicated a molecular formula of $C_{20}H_{18}F_3N_3O$. Accurate mass measurements of diketopiperazine **4** demonstrated a measured *m/z* value of 375.1315 for the $[M+H]^+$ ion to be within 0.1 ppm of calculated (375.1315) at a resolving power of 34,600 and consistent with a molecular formula of $C_{20}H_{17}F_3N_2O_2$.

Amidine **3**, eluting with retention time of 7.49 min, was observed at levels between 0.88% and 5.9% area/area, amidine **2** eluting with 8.36 min retention time was present at <0.05% to 1.6% area/area, and diketopiperazine **4** eluting with 9.87 min retention time was observed at 1.1–2.7% area/area relative to denagliptin (Table 2).

Excipient Compatibility Stress Testing Study

Denagliptin was found to be unstable in the presence of all excipients, especially on exposure to 75% RH, and degraded to two predominant degradation products eluting with retention times of 12.22 and 13.93 min, respectively (Figure 2). Denagliptin eluted with retention time of 11.77 min. Different HPLC methods were used for analysis of stressed drug substance and blend samples, which led to different elution orders and retention times for the same degradation products. Different methods were used for analysis of stressed drug substance and drug product samples, including the excipient compatibility samples, because different methods were filed in the regulatory submissions of denagliptin for analysis of long-term and accelerated stability samples of drug substance and product. LC/MS analysis of stressed blend samples indicated that the molecular weight of 12.22 min peak (amidine **3**) was 373 Da and 13.93 min peak (diketopiperazine **4**) was 374 Da. Binary blends of denagliptin with mannitol were unstable and showed higher degradation than binary blends of denagliptin with croscarmellose sodium and magnesium stearate. All ternary and quaternary blends containing denagliptin and mannitol blended with other excipients showed higher degradation than binary blends prepared by mixing denagliptin and mannitol. Alginic and citric acid blends also showed higher degradation than denagliptin drug substance used as a control. The results, expressed as percent drop in denagliptin content, are summarized in Table 3.

Table 2. Stress Testing Data for Denagliptin (**1**) in Solution

Compound Name	0.1 N HCl:DMSO (4:1), Initial (%w/w)[a]	0.1 N HCl:DMSO (4:1), 80°C, 6 h (%w/w)[a]	Natural pH Degradation (4:1, Water:NMP), 80°C, 6 h (%w/w)[a]				0.01 N NaOH:Diglyme (1:1), Initial (%w/w)[a]	0.01 N NaOH:Diglyme, 80°C, 6 h (1:1) (%w/w)[a]
			Initial	N_2	Air	O_2		
Denagliptin	99.9	93.0	100.5	92.5	90.9	92.5	98.8	96.3
Amidine **3**	ND	4.1	<0.05	5.6	5.5	5.9	<0.05	0.88
Amidine **2**	<0.05	1.6	<0.05	0.67	0.65	0.66	<0.05	<0.05
Diketopiperazine **4**	ND	1.1	ND	2.4	2.3	2.7	ND	1.9
Other Impurities[b]	1.2 (9)	2.4 (13)	1.1 (10)	1.6 (13)	1.5 (9)	1.6 (10)	1.3 (10)	1.8 (11)
Total	101.1	102.1	101.6	102.8	100.9	103.4	100.1	100.9

ND, not detected.

[a]Data for initial and stressed samples are expressed in terms of %w/w relative to an external standard and impurities are in %area/area relative to denagliptin.

[b]Other impurites include sum of total of synthetic impurites and minor degradation products with numbers in brackets representing the total number of these impurities.

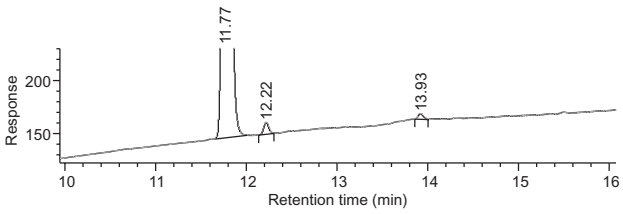

Figure 2. HPLC chromatogram of denagliptin–mannitol excipient compatibility sample after 2 days at 60°C/75% RH.

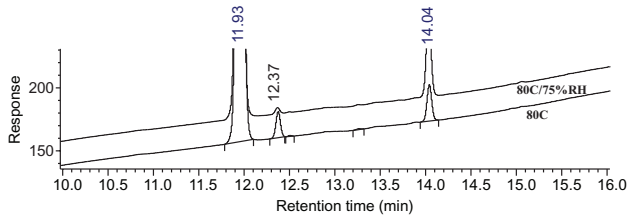

Figure 3. Overlaid HPLC chromatograms of 7.5 mg denagliptin capsule samples stressed for 2 weeks at 80°C and 80°C/75% RH.

Stress Testing Studies of Drug Product

Denagliptin capsules showed degradation to two major impurities, eluting with retention times of 12.37 and 14.04 min (Figure 3), after stressing for 2 weeks at 80°C and 80°C/75% RH. No degradation was observed on exposure to light. LC/MS analysis of stressed capsule samples indicated that the molecular weight of 12.37 min peak (amidine **3**) was 373 Da and 14.04 min peak (diketopiperazine **4**) was 374 Da. Amidine **3** was observed at 1.0–1.8% area/area relative to denagliptin in capsules stressed for 2 weeks at 80°C and at 0.24–0.42% area/area in capsules stressed for 2 weeks at 80°C/75% RH. Diketopiperazine **4** was observed at 1.6–3.2% area/area after stressing the capsules for 2 weeks at 80°C and at 3.5–7.9% area/area after stressing for 2 weeks at 80°C/75% RH. The levels of degradation products relative to denagliptin were higher in stressed 7.5 mg capsules compared to 15.0 mg and 30.0 mg capsules. Different HPLC systems were used for analysis of stressed blend and capsule samples resulting in different retention times for the two key impurities, but their relative retention time with respect to denagliptin were the same in both blend and capsule samples.

Preparation of (3*S*,7*S*,8a*S*) Amidine (2)

The preparation of (3*S*,7*S*,8a*S*) amidine (**2**) was challenging because its epimerization to (3*S*,7*S*,8a*R*) amidine (**3**) was facile and occurred at room temperature in the presence of $FeCl_3$ and ethanol, which were used for its synthesis. In addition, three impurities arising out of reaction between ethanol and the cyano group of denagliptin were observed at retention times of 12.84, 13.64, and 13.76 min. Amidines **2** and **3** eluted with retention times 12.44 and 12.33 min, respectively whereas denagliptin eluted with retention time of 11.95 min. In order to minimize the side reaction with the solvent and epimerization of amidine 2 to 3, three aprotic solvents, tetrahydrofuran (THF), dichloromethane (CH_2Cl_2), and chloroform ($CHCl_3$) were evaluated at temperatures of 60 and 80°C. Reactions carried out in THF and CH_2Cl_2 were slow and incomplete, but amidine **2** with purity of 97.5% was obtained when $CHCl_3$ was used for the reaction.

NMR Experiments

The ^1H NMR spectrum of amidine **2**, acquired immediately after preparation of the sample in $CDCl_3$, indicated the presence of 10% (by mole) of amidine **3** as an impurity (Figure 4). During the course of acquiring ^{13}C, HMQC (one bond H–C correlation), and HMBC (two to three bond H–C correlation) data almost complete epimerization of 2 to 3 was observed, which was confirmed by both NMR and LC/MS analyses (Figure 4). After 48 h in the NMR spectrometer, resonances attributable to **3** grew

Table 3. Stress Testing Data for Denagliptin Excipient Compatibility Studies

Drug	Diluent/Filler	Disintergrant	Lubricant	Acidifier	%Degradation, 60°C for 2 Days	%Degradation, 60°C/75% RH for 2 Days
Denagliptin	—	—	—	—	0.23	0.33
Denagliptin	Mannitol				0.32	1.6
Denagliptin	—	Croscarmellose sodium	—	—	0.17	0.5
Denagliptin	—	—	Magnesium stearate	—	0.22	0.63
Denagliptin	Mannitol	Croscarmellose sodium	—	—	0.26	1.7
Denagliptin	Mannitol	—	Magnesium stearate		0.63	5.9
Denagliptin	Mannitol	Croscarmellose sodium	Magnesium stearate	—	0.55	6.4
Denagliptin	Mannitol	Croscarmellose sodium	Stearic acid	—	2.6	16.6
Denagliptin	Mannitol	Croscarmellose sodium	Sodium stearate	—	1.7	12.0
Denagliptin	Mannitol	Croscarmellose sodium	Stearic acid	Alginic acid	1.7	2.7
Denagliptin	Mannitol	Croscarmellose sodium	Stearic acid	Citric acid	3.5	8.9
Denagliptin	Mannitol	Crospovidone	Stearic acid	—	2.6	16.5

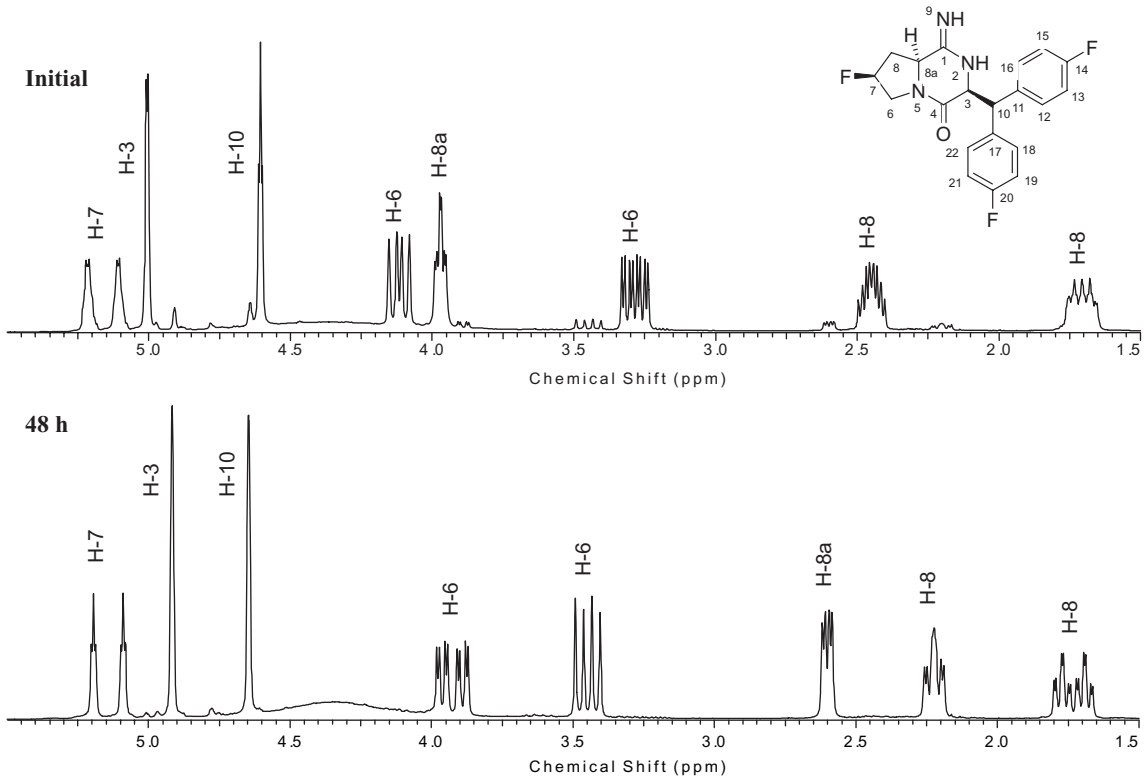

Figure 4. Expansions of aliphatic region of ^{1}H NMR spectra of amidine **2**—initial and 48 h.

at the expense of corresponding **2** resonances so all correlations observed in HMQC and HMBC experiments were attributable to **3**. Key correlations shown by H-3 (δ 4.917), H-8a (δ 2.602), and H-8 (δ 1.732) to C-1 (δ 157.10) and by H-3 (δ 4.917), H-10 (δ 4.646), and H-6 (δ 3.927) to C-4 (δ 168.28) confirmed the presence of 6-member cyclic amidine ring. The chemical shift of H-8a in **2** was significantly downfield (δ 3.971) compared to **3** (δ 2.602), which suggested that C-8a was the likely site of epimerization. The relative stereochemistry at C-3, C-7, and C-8a positions in **3** was obtained by single crystal X-ray diffraction analysis (Figure 5), which confirmed that it was the 3*S*,7*S*,8a*R* isomer based on the known configuration at C-7. Spectral data of diketopiperazine (**4**) were similar to the data for amidine **3**. Chemical shift assignments were made by analysis of its ^{1}H, ^{13}C, DQCOSY, HMQC, and HMBC data. The presence of 6 member diketopiperazine ring was confirmed by HMBC correlations shown by H-3 (δ 4.624–4.573), H-8a (δ 4.201), and H-8 (δ 2.063) to C-1 (δ 168.89 ppm) and by H-3 (δ 4.624–4.573) and H-6 (δ 3.728) to C-4

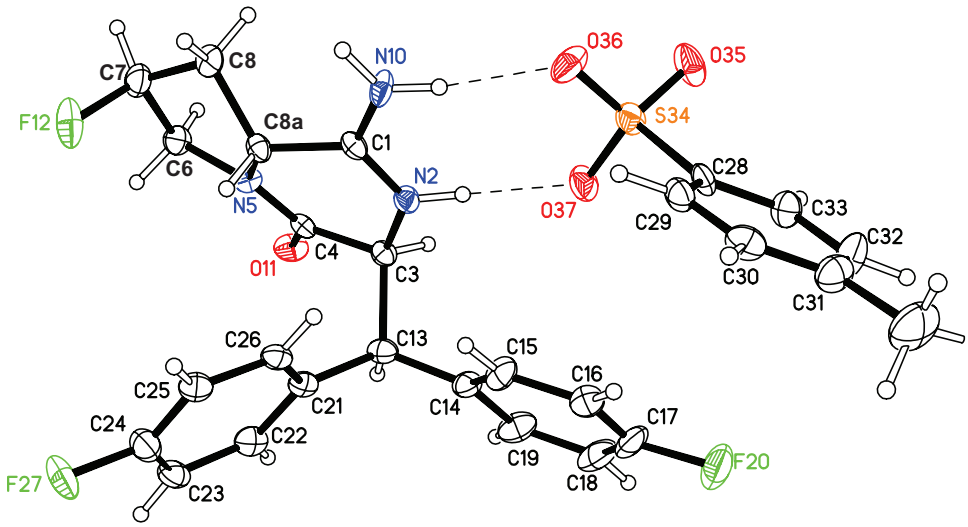

Figure 5. View of cation and anion from crystal structure of amidine **3**.

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Table 4. ^{1}H NMR Data of **2**, **3**, and **4** at 25°C

Position	δ_H (ppm)[a,b] (**2**)	δ_H (ppm)[a,b] (**3**)	δ_H (ppm)[a,c] (**4**)
2	Exchanged with solvent	Exchanged with solvent	8.471
18,22	7.442	7.509	7.489
12,16	7.184	7.095	7.432
19,21	6.974	6.980	7.147
13,15	6.900	6.926	7.096
7	5.162	5.143	5.279
3	5.007	4.917	4.624–4.573
10	4.606	4.646	4.624–4.573
8a	3.971	2.602	4.201
6	4.116	3.927	3.728
6	3.285	3.448	3.406–3.324
8	2.449	2.224	2.350
8	1.705	1.732	2.063

[a]Referenced to TMS = 0.000 ppm.
[b]Acquired in $CDCl_3$.
[c]Acquired in DMSO-d_6.

(δ 164.48). As the resonances for H-3 and H-10 overlapped, the relative stereochemistry of **4** could not be established by NMR spectroscopy. The stereochemistry of **4** was determined to be the same as **3** by an additional single crystal X-ray diffraction analysis. ^{1}H NMR chemical shift assignment data for **2**, **3**, and **4** are included in Table 4.

DISCUSSION

Regulatory Requirements for Conducting Stress Testing Studies

Stress testing studies of denagliptin drug substance and capsules were performed according to recommendations in the regulatory guidance for stress testing of new drug substances and products.[1] The guidance indicates that the susceptibility of drug to hydrolysis must be examined across a wide range of pH values in solution or suspension. In the solid-state and in drug products, the stress testing study should use temperatures higher than used for accelerated testing (40°C), humidities of 75% RH or greater, and photostability testing on at least one batch of drug substance and product. The guidance also indicates that degradation products that are not formed under accelerated or long-term conditions need not be evaluated. Data from the study are typically also used to evaluate mass balance and stereochemical stability. The thresholds for identification and qualification of impurities in new drug substance and products indicated in regulatory guidance[2] required the identification of all impurities greater than 0.2% in stressed samples. As amidine **2**, amidine **3**, and diketopiperazine **4** were the only three impurities exceeding identification thresholds on accelerated stability testing, they were prepared and characterized by NMR and single crystal X-ray diffraction analysis. As one key purpose of conducting a stress testing study is to develop a HPLC method capable of monitoring performance of drug substance and product under accelerated or long-term conditions, the extent of degradation in stress testing studies was limited to 5–10% of the active.

Stress Testing Study Results for Denagliptin Drug Substance

Denagliptin was stressed in acid, water, and base in solution and it degraded to three key cyclization products under all conditions (Table 2). Hydrolysis of the cyano group and epimerization of the cyano stereocenter (C-8a) was observed at low levels in samples stressed in solution, but not in solid-state stress testing studies of drug substance or blends and in capsules stored under stress testing, accelerated or long term conditions. Hydrolysis of the amide linkage, epimerization at two other stereocenters, sequence inversion, and oxidation was not observed for denagliptin. The preference of denagliptin to undergo cyclization can be attributed to the basicity of the amino group ($pK_a = 6.12$; calculated from its pH solubility curve) and the steric bulk of the N-terminal residue as reported in the literature[3] for cyclization of C-terminal proline containing peptides. The cyclization rate for C-terminal proline peptides was shown to depend on the pK_a of the amino group, steric bulk of the N-terminal residue, ability of the peptide bond to undergo *cis–trans* isomerization, and conformational stability of resulting diketopiperazines.[3] The rate of cyclization of denagliptin would be expected to be significantly different relative to the rates reported for dipeptide analogs. The presence of a cyano group in denagliptin instead of a carboxylic acid, ester, or amide groups present in dipeptide analogs could significantly impact the rates of *cis–trans* isomerization of the peptide bond. In addition, the mechanism

of cyclization of denagliptin would also be different compared to dipeptide analogs because the initially formed intermediate in aminolysis of cyano group is not tetrahedral. Therefore the comparison between denagliptin and dipeptide cyclization is restricted to the pathway, for example, cyclization by aminolysis, and not mechanism of degradation.

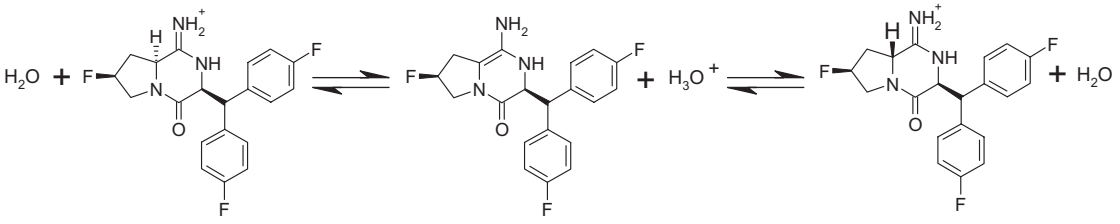
Denagliptin was stable on stressing in the solid-state due to the rigidity of its crystal lattice attributable to hydrogen bonds between denagliptin cation and tosylate anion in the unit cell, which kept the amino group protonated and trans to the cyano group. The solid-state stress testing study was stopped after 2 weeks of storage at 80°C and 80°C/75% RH because the kinetic equivalent of 6 months at 40°C/75% RH exposure recommended in the regulatory guidance[1] was achieved. The extrapolation of storage time from 80°C/75% RH to 40°C/75% RH was based on doubling of reaction rate for every 10°C rise in temperature using a conservative estimate of 12.2 kcal/mol for energy of activation, E_a.[20]

Excipient Compatibility Stress Testing Study

Isothermal excipient compatibility stress study of denagliptin in combination with various excipients was performed early in development to evaluate the feasibility of preparing a stable tablet formulation. Mannitol was evaluated as a diluent in order to avoid the use of hygroscopic excipients like microcrystalline cellulose, which accelerated cyclization of quinapril and moexipril.[7,10] However, just after 2 days of stressing at 60°C/75% RH the denagliptin–mannitol binary blend showed higher degradation compared to unblended drug used as a control. Denagliptin–mannitol binary blend was stable after 2 days of stressing at 60°C under ambient RH, but ternary and quaternary blends showed higher degradation than the unblended denagliptin. As mannitol is polar and protic, it would not be expected to enhance *trans–cis* isomerization of denagliptin amide bond[21,22] to cause higher degradation in the binary blends. The rate of amide bond isomerization in denagliptin and its blends could not be determined by NMR spectroscopy[23,24] due to low aqueous solubility of denagliptin (2.6 mg/mL) and close chemical shifts of *cis* and *trans* isomer resonances. The natural pH of a saturated aqueous solution (i.e., 2.6 mg/mL) of denagliptin tosylate is ca. 4.3, which is below the apparent pH_{max} of 5.3. pH_{max} is defined as the pH at which both the salt and free base species of a drug are simultaneously saturated. Moreover, the surface acidity of mannitol expressed as pH_{eq} is reported to be 3.23.[25] These factors preclude the formation of solid free base by disproportionation in blends with mannitol.[26] It is more likely that the degradation of denagliptin–mannitol binary blend at 60°C/75% RH occurred in microsolutions formed by adsorption of water, which was exacerbated by higher surface interaction between drug and mannitol due to low drug:excipient ratio (1:25). This hypothesis is consistent with greater degradation on exposure to 60°C/75% RH compared to 60°C, and the observation that degradation in blends followed the order sodium stearate > magnesium stearate > croscarmellose sodium. This order is consistent with the basicity of these excipients and the base catalyzed nature of cyclization of dipeptides.[3,17] Higher surface interaction due to low drug excipient ratio has also been reported as a cause for cyclization in enalapril.[14] Acidic excipients like stearic acid, alginic acid, and citric acid did not improve the stability of the blends significantly, which could be due to their ability to enhance the rate of epimerization of amidine **2** (Scheme 2) and/or hydrolysis of amidine **3** to diketopiperazine **4** (Scheme 1). Higher degradation for all stearic acid blends could be attributed to the melting of stearic acid (mp ≥ 54°C),[27] which could dissolve the drug and enhance mobility relative to a solid. Denagliptin exists as anhydrous form 1 and no evidence for any other form was obtained in any of the stressed samples by XRD. Since denagliptin was found to be chemically unstable in the presence of all excipients, including mannitol, the development of tablet was discontinued and bead filled capsules were progressed for further development.

Stress Testing Results for Denagliptin Capsules

Amidine **3** and diketopiperazine **4** were key degradation products observed in stressed capsule samples, confirming that the key degradation pathway in capsules was the same as in solution and excipient blends. However, the rate of degradation was much



Scheme 2. Proposed mechanism for epimerization of amidine 2 in acid.

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slower compared to excipient blends, justifying the progression of bead filled capsules as the formulated product.

Epimerization of (3*S*,7*S*,8a*S*) Amidine (2)

Results of solution phase stress testing experiments suggest that epimerization of amidine **2** occurred in all the samples. The absence of epimerized denagliptin in stressed samples, including samples stressed for 4 h at 80°C and 22 h at 60°C (data not reported) where the extent of denagliptin degradation was <5%, suggests epimerization occurred predominantly after cyclization to amidine **2**. This observation is consistent with results reported in the literature for epimerization of diketopiperazines.[28] The synthesized sample of amidine **2** also epimerized rapidly in $CDCl_3$ in the NMR tube, which supports epimerization after cyclization. The rapid epimerization of amidine **2** can be explained by the possibility of existence of tautomeric forms in equilibrium (Scheme 2) and steric factors. Cyclic dipeptides containing an aromatic amino acid residue have been known to adopt folded F conformation in solution as well as in the solid-state.[29] In this conformation, the aromatic side-chain ring stacks over the diketopiperazine ring and is favored by 13 kJ/mol over other possibilities in polar solutions. If amidine **2** were to adopt this conformation, the steric interaction between methylene group on 5-member ring and phenyl rings would be higher due to *cis* orientation compared to amidine **3**, which has hydrogen *cis* to phenyl ring. The preference for *cis–trans* epimerization at ring junction stereocenter over the ring stereocenter was also reported for diketopiperazine derivatives of enalapril and lisinopril.[30] Single crystal X-ray diffraction data for amidine **3** and diketopiperazine **4** are consistent with aromatic ring stacked over the amidine and diketopiperazine rings, respectively in the solid-state. It may be possible for them to adopt a similar conformation in $CDCl_3$ solution.[31]

Summary

Denagliptin degraded by cyclization on stressing in solution, excipient blends, and bead filled capsules. The structures of three key degradation products were elucidated by analysis of NMR and single crystal X-ray diffraction data. Amidine **2** was unstable and epimerized to amidine **3**, which hydrolyzed further to the corresponding diketopiperazine **4**. All the blends of denagliptin with excipients, including acidic and basic excipients, showed higher degradation compared to unblended denagliptin. Bead filled capsules of denagliptin were developed in order to minimize the interaction of denagliptin with excipients and moisture. The capsules showed adequate stability and may be a good alternative for formulating low dose products of moisture sensitive drugs that require high excipient to drug ratio.

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REFERENCES

1. Q1A (R2) Stability testing of new drug substances and products, November 2003. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm073369.pdf.
2. Q3B (R) Impurities in new drug products (revision 2), August 2006. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm073389.pdf.
3. Goolcharan C, Borchardt RT. 1998. Kinetics of diketopiperazine formation using model peptides. J Pharm Sci 87:283–287.
4. Leung SS, Grant DJW. 1997. Solid state stability studies of model dipeptides: Aspartame and aspartylphenylalanine. J Pharm Sci 86:64–71.
5. Gu L, Strickley RG. 1987. Diketopiperazine formation, hydrolysis, and epimerization of the new dipeptide angiotensin-converting enzyme inhibitor RS-10085. Pharm Res 4:392–397.
6. Strickley RG, Visor GC, Lin L, Gu L. 1989. An unexpected pH effect on the stability of moexipril lyophilized powder. Pharm Res 6:971–975.
7. Gu L, Strickley RG, Chi L, Chowan ZT. 1990. Drug-excipient incompatibility studies of the dipeptide angiotensin-converting enzyme inhibitor, moexipril hydrochloride: Dry powder vs wet granulation. Pharm Res 7:379–383.
8. Gu L, Strickley RG. 1990. A profound solvent effect on the diketopiperazine formation of the new dipeptide angiotensin-converting enzyme inhibitor, moexipril. Int J Pharm 60:99–107.
9. Guo Y, Byrn SR, Zografi G. 2000. Physical characteristics and chemical degradation of amorphous quinapril hydrochloride. J Pharm Sci 89:128–143.
10. Guo Y, Byrn SR, Zografi G. 2000. Effects of lyophilization on the physical characteristics and chemical stability of amorphous quinapril hydrochloride. Pharm Res 17:930–935.
11. Li J, Guo Y, Zografi G. 2002. The solid-state stability of amorphous quinapril in the presence of β-cyclodextrins. J Pharm Sci 91:229–243.
12. Li J, Guo Y, Zografi G. 2002. Effects of a citrate buffer system on the solid-state chemical stability of lyophilized quinapril preparations. Pharm Res 19:20–26.
13. Wang S, Lin S, Chen T. 2001. Reaction kinetics of solid-state cyclization of enalapril maleate investigated by isothermal FT-IR microscopic system. Chem Pharm Bull 49:402–406.
14. Cotton ML, Wu DW, Vadas EB. 1987. Drug-excipient interaction study of enalapril maleate using thermal analysis and scanning electron microscopy. Int J Pharm 40:129–142.
15. Wang S, Lin S, Chen T, Cheng W. 2004. Eudragit E accelerated the diketopiperazine formation of enalapril maleate determined by thermal FTIR microspectroscopic technique. Pharm Res 21:2127–2132.

16. Wang S, Lin S, Chen T. 2000. Thermal dependant dehydration process and intramolecular cyclization of lisinopril dihydrate in the solid state. Chem Pharm Bull 48:1890–1893.
17. Capasso S, Vergara A, Mazzarella L. 1998. Mechanism of 2,5-dioxopiperazine formation. J Am Chem Soc 120:1990–1995.
18. Purdie JE, Benoiton NL. 1973. Piperazinedione formation from esters of dipeptides containing glycine, alanine, and sarcosine: The kinetics in aqueous solution. J Chem Soc II 1845–1852.
19. Fuks R. 1973. N-Alkylation of nitriles: I. Tetrahedron 29:2147–2151.
20. Connors KA, Amidon GL, Stella VJ. 1986. Chemical stability of pharmaceuticals. 2nd edition. New York: John Wiley & Sons. p. 24.
21. Eberhardt ES, Loh SN, Hinck AP, Raines RT. 1992. Solvent effects on the energetics of prolyl peptide bond isomerization. J Am Chem Soc 114:5437–5439.
22. Fischer G. 2000. Chemical aspects of peptide bond isomerisation. Chem Soc Rev 29:119–127.
23. Grathwohl C, Wuthrich K. 1981. NMR studies of the rates of proline cis-trans isomerization in oligopeptides. Biopolymers 20:2623–2633.
24. Sui Q, Borchardt D, Rabenstein DL. 2007. Kinetics and equilibria of cis/trans isomerization of backbone amide bonds in peptoids. J Am Chem Soc 129:12042–12048.
25. Scheef CA, Oelkrug D, Schmidt PC. 1995. Surface acidity of solid pharmaceutical excipients. III. Excipients for solid dosage forms. Eur J Pharm Biopharm 46:209–213.
26. Guerrieri P, Taylor LS. 2009. Role of salt and excipient properties on disproportionation in the solid-state. Pharm Res 26:2015–2026.
27. Wade A, Weller PJ. 1994. Handbook of pharmaceutical excipients. 2nd edition. Washington DC: American Pharmaceutical Association and. Royal Pharmaceutical Society of Great Britain. pp. 494–497.
28. Gaines SM, Bada JL. 1988. Aspartame decomposition and epimerization in the diketopiperazine and dipeptide products as a function of pH and temperature. J Org Chem 53:2757–2764.
29. Liwo R, Ciarkowski J. 1985. Origin of the ring-ring interaction in cyclic dipeptides incorporating an aromatic amino acid. Tett Lett 26:1873–1876.
30. Demeter A, Fodor T, Fischer J. 1998. Stereochemical investigations on the diketopiperazine derivatives of enalapril and lisinopril by NMR spectroscopy. J Mol Struct 471:161–174.
31. Suaifan GA, Mahon MF, Arafat T, Threadgill MD. 2006. Effects of steric bulk and stereochemistry on the rates of diketopiperazine formation from N-aminoacyl-2,2-dimethylthiazolidine-4-carboxamides (Dmt dipeptide amides)—A model for a new prodrug linker system. Tetrahedron 62:11245–11266.