Oxidation of the N-Terminal Gly-Residue of Peptides: Stress Study of Pexiganan Acetate in a Drug Formulation[1]

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Purpose. The purpose of this study was to identify four major degradation products, which were formed during a stress study of pexiganan (a 22-mer peptide) in a 1% formulation.

Methods. The degradation products were isolated and characterized by LC/MS, tryptic and aminopeptidase digests.

Results. One of the degradation products was shown to be des-gly$_1$-pexiganan. The other three are structural isomers of N-glyoxylyl-des-gly$_1$-pexiganan. These isomers undergo reversible inter-conversions, as well as decompose irreversibly to des-gly$_1$-pexiganan. Thus, all the impurities were formed from a single oxidation product of pexiganan, N-glyoxylyl-des-gly$_1$-pexiganan. The aldehyde group of the glyoxylyl residue and the NH-amide of the adjacent isoleucine residue form a piperazinedione derivative of des-gly$_1$-pexiganan. This heterocyclic compound rearranges to other tautomers or back to the N-glyoxylyl compound (see Fig. 3). Tryptic digests of the three degradation products showed that their N-terminal segment produced N-glyoxylyl-I-G-K whereas the N-terminal segment of pexiganan produced G-I-G-K. All the other tryptic-digest segments were identical to those formed in pexiganan. The LC/MS of the N-terminal segment and of synthetic N-glyoxylyl-I-G-K were identical. The enzymatic resistance of the three impurities to undergo aminopeptidase-M cleavage further supported the conclusion that their N-terminal amino residues are substituted.

Conclusions. After a year under stress conditions 1% pexiganan cream lost about 15% of the active component to oxidative-deamination,[4] where the N-terminal glycine residue was oxidized to N-glyoxylyl-des-gly$_1$-pexiganan. The other nine ε-amino lysine-residues of the peptide stayed intact. This oxidation product inter-converted and formed two additional impurities, tautomers of piperazinedionyl-des-gly$_1$-pexiganan, and decomposed to des-gly$_1$-pexiganan, the forth impurity.

KEY WORDS: N-glyoxylyl-des-gly$_1$-pexiganan; piperazinedionyl-des-gly$_1$-pexiganan; N-glyoxylyl-peptides; N-terminal oxidation of peptides in drug formulation.

INTRODUCTION

Pexiganan acetate was recently submitted for approval as a human therapeutic anti-microbial drug. It is a synthetic analog of the magainin peptides (23 amino-acid residues in length) originally isolated from the skin of the African clawed-frog, Xenopus laevis. These amphipathic, cationic, linear-peptides kill microbial targets by disrupting their membrane-permeability (1). They demonstrate selectivity for membranes which contain accessible acidic-phospholipids, a property characterizing the cytoplasmic membrane of many species of bacteria (2). Pexiganan is a potent, broad spectra antibiotic that demonstrated efficacy in topical treatment of infected diabetic foot-ulcers, exhibiting a clinical response in two Phase-III trials comparable to orally administered ofloxacin.

The structure of pexiganan is:

G–I–G–K–F–L–K–K–A–K–K–F–G–K–A–F–V–K–I–L–K–K–NH2 G-glycine, I-isoleucine, K-lysine, F-phenylalanine, L-leucine, A-alanine and V-valine

An essential part of drug development is the stability of the active ingredient in the formulation. More information is available on stability of proteins in pharmaceutical formulations than is available for peptides. The physical and chemical stability of proteins in drug formulations has been reviewed in the literature (3,4).

The aromatic amino acids (phenylalanine, histidine, tryptophan and tyrosine) and the sulfur-containing amino acids were found susceptible to photo-ionizing chemical-modification. These modifications occurred randomly along the protein chains. On the other hand, chemical-oxidation of proteins in drug-formulation occurs under milder conditions and was postulated to be a metal-catalyzed oxidation. These are often site-directed oxidations, where only one particular residue in a given protein is modified. Metal-catalyzed reactions are 'caged' processes in which an amino-acid residue at the metal-binding site is the specific target of the oxidation (5). For example, a methionine adjacent to a histidine residue is more prone to oxidation than otherwise positioned methionines at similarly accessible sites (6). Relevant to the present study is the oxidation of the lysine ε-amino-group to the appropriate aldehyde by H_2O_2 *via* a Fe^{2+}/Fe^{3+}-complex (7). Active-oxygen reagents are produced through the interaction of oxygen with a transition metal, as describe in the literature (4). Other peroxides may be introduced *via* the formulation excipients such as surfactants containing a polyethylene-glycol residue. It was reported that polysorbate 80 (which is also used in the 1%-pexiganan formulation) increased the amount of oxidation products in an interleukin-2 formulation (8). The addition of metal-chelating agents is one source that can introduce trace amounts of metals into the formulation, which then under certain circumstances could accelerate catalytic oxidations (3,4).

The biological conversion of amines to carbonyl compounds by transamination involving prototropic interconversion of Schiff bases was applied by Corey *et al*. to design reagents to oxidize primary amines (9). 3,5-Di-*t*-butyl-1,2-benzoquinone, mesitylglyoxal and others were used to convert in high yield under mild conditions primary amines to their corresponding carbonyl compounds. Kubo *et al* used the first reagent to convert 1-(alanylaminomethyl)-5,7,8-trimethoxy-6-methylisoquinoline to its 1-pyruvoylaminomethyl derivative (10). Conversion of a similar glycyl residue to the corresponding glyoxylyl derivative (although expected) is not described in the literature. In addition, selectively of these reagents, for example, to oxidize α-amino groups while leaving ε-amino groups of peptides intact is also not described in the literature. The anti-oxidant used in the pexiganan formulation was 2,6-di-*t*-butyl-4-methylphenol (BHT). When oxidized BHT might form compounds containing functional groups similar to those present in Corey's reagents. If

[1] After four years under ambient conditions, less than 5% of these impurities were formed.

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BHT can form such reagents, and if those formed can selectively oxidize the N-terminal amino group of pexiganan, one might consider an alternative route to the metal-catalyzed oxidation where formation of the particular degradation products occurred through oxidative transamination.

Pexiganan has nine lysine residues that can undergo oxidative-degradation. Still, as will be seen later, the degradation products found in the drug stored under stressed conditions were formed through oxidation of the N-terminal glycine residue. In a similar study, van den Oetelaar, *et al*. attributed some of the degradation products of a peptide stored under stressed conditions to direct hydrolysis of N-terminal glycine groups, and others to oxidation (11). As will be seen later, oxidation of the N-terminal glycine group is a preceding step to the hydrolysis of the glycine residue.

Experimental

The chromatographic separations were done on a Model 1100 and the LC/MS on an Electrospray Model 5989 Hewlett Packard (San Fernando, CA). The columns used were Vydac 218TP54, 4.6 × 250 mm (Vydac, Hesperia, CA), Luna C18, 10 μm, 10 × 250 mm (Phenomenex, Torrance, CA) and PLRP-S, 100A, 101-15 μm, 4.6 × 250 mm (Polymer Laboratories, Amherst, MA). Using a HP auto-sampler, samples were withdrawn and sandwiched between two volumes of 1–5% TFA, mixed and injected. This injection-program allowed direct injection of reaction mixtures, instant quenching of the basic conditions, and accurately monitoring reactions carried out in small sample-vials (100–200 μl).

Extraction

The content of three 1%-pexiganan 15 ml-tubes (34.1 g), which were kept at 40°C and 60% RH for 12 months, was sonicated in 200 ml water/100 ml chloroform until uniform. The mixture was poured into a separatory funnel, and the two layers separated. The chloroform layer was shaken with an additional 100 ml of water and the layers separated. The combined aqueous layers were washed with chloroform (100 ml), and the aqueous layer was freeze-dried.

Materials

Immobilized aminopeptidase-M and immobilized trypsin were obtained from Pierce Chemical Company, Rockford, IL. Amberchrom-161c was obtained from Tosohaas, Montgomerville, PA.

Synthesis

Preparation of N-glyoxylyl-L-isoleucylbutanamide (14), for structures see Fig. 1. Di(isopropylthio)acetic acid was prepared according to Qasmi *et al*. (12). N-*t*-BOC-L-isoleucine (2 mmole) was converted to N-*t*-BOC-L-isoleucylbutanamide, the *t*-BOC removed and the product coupled with di(isopropylthio)-acetic acid to yield N-di(isopropylthio)acetyl-L-isoleucylbutanamide, 11. The reaction mixture (in chloroform) was washed with 5% tartaric acid and 5% sodium bicarbonate. The chloroform was removed under reduced pressure, the residue was suspended in 50% aq. acetonitrile and the precipitate filtered to yield 492 mg (1.3 mmole) of a white powder. The material

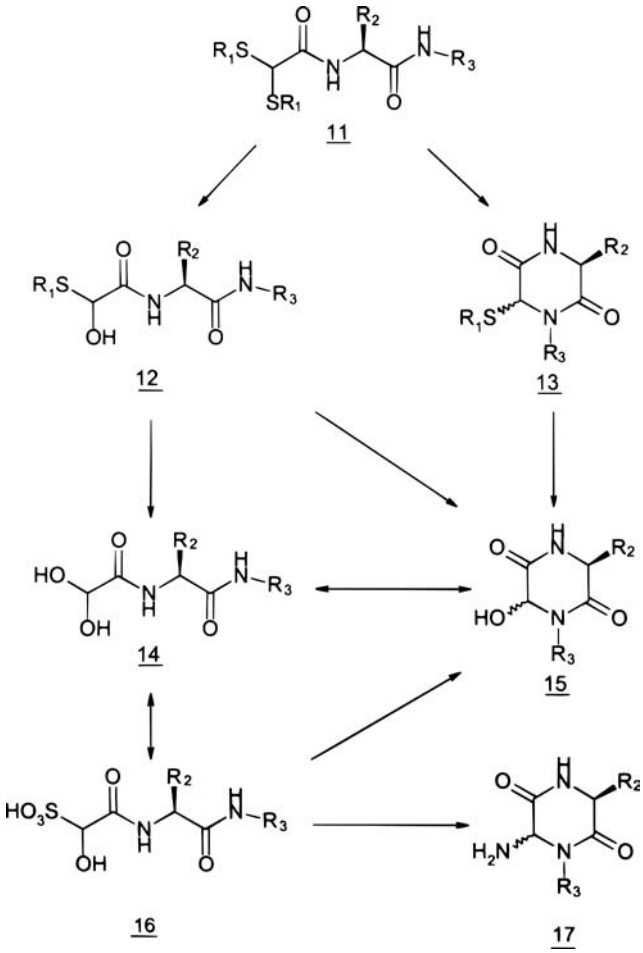

Fig. 1. Compounds 11–17; R_1 = −2-propyl, R_2 = −2S-butyl and R_3 = - butyl.

was 91% HPLC-pure, and 2.6% was the largest single impurity. ^{1}H NMR ($CDCl_3$) δ = 7.12 (d, J = 8.3 Hz, 1H), 6.02 (s, 1H), 4.34 (s, 1H), 4.17 (dd, J = 7.55 Hz, 1H), 3.24 (t, 2H), 3.12 (h, 2H), 2.1 − 1.05 (m, 19H) and 0.95 − 0.85 (m, 9H). For LC/MS see Table I. The thio protecting-groups were removed under conditions described by Corey *et al* (13). A solution of 189 mg (1.06 mmole) of N-bromosuccinimide (NBS) in 90% acetone (30 mL) was cooled to −5 °C, and a solution of 50 mg (0.13 mmole) of 11 in 10 mL 90% acetone (8:1 equivalents) was added with vigorous stirring. After 2 min the reaction was quenched with a mixture of 30 mL of 5% sodium thiosulfate and 30 mL chloroform. The phases were separated and the chloroform layer was washed with 5% tartaric acid. Samples of the chloroform and the tartaric acid layers were analyzed by HPLC. The chromatograms (which were similar) showed a major peak (Rt = 14.07 min). The LC/MS of this peak of the chloroform layer is shown in the Table I for 14.

In a previous attempt to synthesize 14, solutions of 1.6 mmole NBS and 0.27 mmole of 11 (6:1 equivalents) reacted under the same conditions, quenched with a mixture of 5% Na_2SO_3/methylene chloride and the two layers were separated. The aqueous layer was washed twice with chloroform. The chloroform extract of the aqueous layer contained mostly 13 and some of compounds 14 and 15 (see Fig. 1). The aqueous

Table I. Retention Time and Molecular Masses as Obtained by LCMS*

Comp.	Rt (min)		m/e
11	18.5[a]	377.3	(M + 1)
		301.1	(M − 75)
		753.6	(2M + 1)
		775.6	(2M + Na)
12	15.2[b]	319.1	(M + 1)
		341.1	(M + Na)
		659.4	(2M + Na)
13	15.75[b]	301.1	(M + 1)
		323.1	(M + Na)
		623.4	(2M + Na)
14	14.07[b]	243.1	(M − 17)[c]
		261.0	(M + 1)
		283.1	(M + Na)
		543.4	(2M + Na)
15	15.32[b]	243.1	(M + 1)
		265.1	(M + Na)
		507.3	(2M + Na)
16	11.47[b,d]	243.1	(M − 81)
	11.63	325.1	(M + 1)
		649.4	(2M + 1)
17	12.45[b,d]	242.1	(M + 1)
	12.77		

* Column/conditions: as in Fig. 4 except gradient.
[a] 30% B 1 min, 30% B to 100% B in 25 min.
[b] 5% B 1 min, 5% B to 70% B in 25 min.
[c] The aldehyde, −CH=O.
[d] Diastereoisomers.

layer contained only 16. The methylene chloride layer after washing with 5% sodium bicarbonate and 5% tartaric acid contained mostly 14 and 12. The retention time of compounds 12, 13, 14, 15 and 16, and the molecular masses as obtained by LC/MS are summarized in the Table I.

Preparation of N-glyoxylyl-L-isoleucyl-(N-methyl)butanamide (18).

Compound 18 was prepared in a similar synthetic route. The N-di(isopropylthio)acetyl-L-isoleucyl-(N-methyl)butanamide, (19), was purified on a semi-preparative RP-column. Its LC/MS showed the following m/e signals: 391.2 (M + 1), 413.1 (M + Na), 315.1 (M−75) and 803.5 (2M + Na). Compound 18 was prepared from 19 using 8 equivalents of NBS. The reaction mixture was quenched with 5% sodium thiosulfate/chloroform. The chloroform layer was washed with 5% tartaric acid to yield HPLC-pure 18 (Rt = 15.93 min under conditions b of Table I). Its LC/MS showed the following m/e signals: 257.2 (M - 17; M = the hydrate form, 275.1 (M + 1) and 297.1 (M + Na). Compound 18 was converted to its sulfite-adduct 20 with aq. 5% sodium sulfite. The aqueous layer was washed with methylene chloride, adsorbed on an Amberchrom-161c resin, the resin was washed with water to remove excess salts. Compound 20 was eluted with 60% acetonitrile/water and freeze dried. Compound 20 (two diasteromers) showed two peaks at 13.43 and 13.54 min under conditions b of Table I.

Preparation of N-glyoxylyl-L-isoleucylglycyl-L-lysine (22)

N-CBZ-L-isoleucylglycyl-L-(ε-BOC)-lysine was prepared by coupling N-CBZ-L-isolencylglycine with L-(εE-BOC)-lysine. The material was dissolved in ethanol and the CBZ group was removed in the presence of Pd/H_2. The product precipitated from the ethanol solution. The reaction mixture filtered, the precipitate washed with ethanol, and the product was recovered from the 10% Pd/C catalyst by extraction with 1:1:1 methanol:water:acetic acid. HPLC analysis [Rt = 14.40 min under conditions (b) of Table I] showed the product L-isoleucylglycyl-L-(ϵ-BOC)-lysine, in 85%-purity. The solvent was removed under reduced pressure, the residue was dissolved in dry DMF and converted with N-hydroxysuccinimido di(isopropylthio)acetate to N-di(isopropylthio)aceyl-L-isoleucylglycyl-L-(ϵ-BOC)-lysine, 23. Compound 23 was dissolved in chloroform, and the *t*-BOC protecting group was removed with 25% v/v trifluoroacetic acid. The volatiles were removed, the residue dissolved in chloroform and extracted twice with water. The aqueous layer contained the N-di(isopropylthio)acetyl-L-isoleucylglycyl-L-lysine (24) in 89% HPLC-purity [Rt = 18.11 min under conditions (b) of Table I]. Its LC/MS showed the positive m/e 507.2 (M + 1) and 1013.4 (2M + 1) signals. ^{1}H NMR δ = 0.89 (t, J = 7.6 Hz) and 0.96 (d, J = 6.0 Hz), 6H; 1.26 − 1.30 (m, 13H); 1.40 − 2.0 (m, 8H); 3.01 (t, J = 7.4 Hz, 2H); 3.12 (h, J = 6.9 Hz, 2H); 3.97 (s, 2H); 4.17 (d, J = 7.8 Hz, 1H); 4.38 (dd,, 1H) and 4.64 (s, 1H). Compound 24 was converted to 22 under the previous conditions using 8 eq. NBS and thiosulfate quenching. The aqueous layer was separated and extracted with chloroform. A portion of the aqueous layer was purified by HPLC to a single peak (Rt = 11.45 min, gradient 0% B to 15% B in 20 min), while the rest of the mixture was freeze-dried. The LC/MS of this peak had m/e of 373.1 (M + 1) (minor signal) and 391.0 (M + H_2O + 1), the major signal. Compound 22 had the same retention time as peak 2 under the conditions of Fig. 7. The portion that was freeze-dried showed later a different chromatographic pattern, where the single peak of 22 disappeared and two new peaks (Rt = 11.67 and 11.84 min) of its diasteromeric sulfite-adduct (25) appeared (the sulfite was probably formed from the excess thiosulfate used in quenching the NBS). Compound 25 was purified by HPLC. Its LC/MS showed m/e = 455.1 (M + 1) and 909.2 (2M + 1), and ^{1}H NMR δ = 0.89 (t, J = 7.6 Hz) and 0.96 (d, J = 6.0 Hz), 6H; 1.2–2.0 (5m, 9H); 3.00 (t, J = 6.6 Hz, 2H); 3.95 (s, 2H); 4.26 and 4.30 (dd, J = 7.1 and 6.6 Hz, 1H); 4.38 (m, 1H); and 5.05 (s, 1H).

RESULTS AND DISCUSSION

Pexiganan acetate was used in the preparation of topical cream. The peptide used for the preparation of the cream had 98.7% purity, with no single impurity larger than 0.2%. None of the impurities present in the original material could be identified with the degradation products formed in the stressed drug. Tubes containing 15 mL of cream were stored for 12 months at 40°C, and in 60% relative humidity. The peptide-content of three 15mL-tubes was extracted (see experimental) and analyzed on a C18-column. The chromatogram obtained is shown in Fig. 2a. Compound 2 is pexiganan accompanied by ~5% each of impurities 1 (peak 1), 3 (peak 3) and the mixture 4–5 (peaks 4 and 5). The peptide-extract was fractionated on a semi-prep RP-column to fractions A to J, and compounds 1, 3 and mixture 4–5 were isolated in reasonably good purities, see Fig. 2b, 2d and 2e, respectively.

LC/MS for compounds 1, 2, 3, 4 and 5 gave positive masses, calculated for m/e of 2420.5, 2477.6, 2476.6, 2494.7

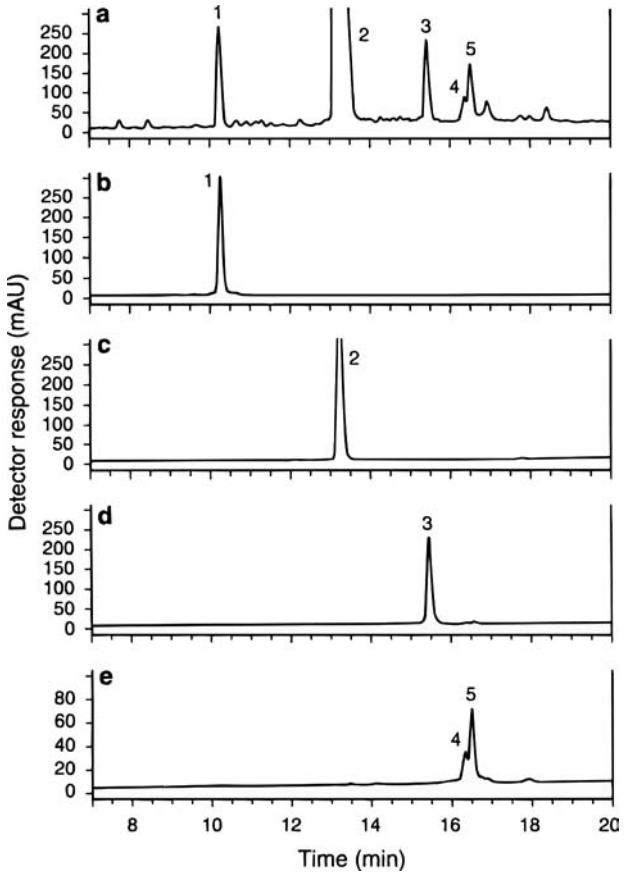

Fig. 2. Chromatograms of (a) the aq. extract of the stressed pexiganan-cream, (b) fraction A of the semi-prep purification, the des-gly$_1$-pexiganan, (c) fraction E of the semi-prep purification, pexiganan, (d) fraction H of the semi-prep purification, compound 3, and (e) fraction J of the semi-prep purification, compounds 4 and 5. Column: Vydac 218TP54, 4.6 × 250 mm; flow rate: 1 ml/min; col. temperature: 40 °C; detector: UV 215 nm. Buffers: A—0.1% TFA in 99/1 H_2O/AcN; B—0.1% TFA in 10/90 H_2O/AcN; gradient: 25% B 1min, 25% to 45% B in 20 min.

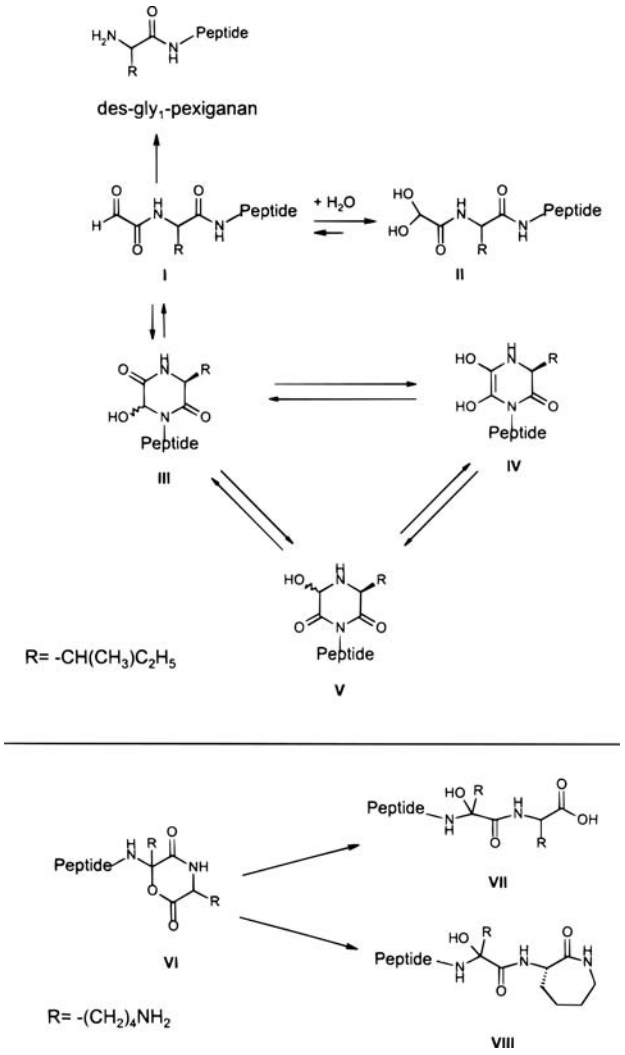

Fig. 3. Alternative degradation-products initiated by oxidation reaction (i) and (ii).

and 2476.7, respectively. Compound 1 was identified as des-gly$_1$-pexiganan, as it had the molecular weight of des-gly$_1$-pexiganan and co-eluted with an authentic sample of the synthetic compound. Compound 3 and 5 were 1 amu lighter, while 4 was 17 amu heavier than pexiganan (or 18 amu heavier than 3 or 5). A survey of possible oxidation products with molecular weights 1 amu less than pexiganan, and 17 amu heavier than pexiganan is shown in Fig. 3. These *hypothetical* compounds (marked for this reason with roman numerals) could be formed through alternative site-selective oxidations involving the N-terminal glycine-residue (reaction i), the C-terminal segment of pexiganan (reaction ii), or oxidation of one of the nine lysine-residues (reaction iii).

(i) H_2NCH_2CO–peptide $\xrightarrow{[O]}$ O=CHCO–peptide (I)

(ii) peptide–$CONH_2$ $\xrightarrow[\text{(peracid)}]{[O]}$ peptide–$COO_2H \rightarrow$ VI

(iii) [peptide]–$(CH_2)_4NH_2$ $\xrightarrow{[O]}$ [peptide]–$(CH_2)_3CHO$ (IX)

Site-selective oxidation usually involves a transition-metal complex with the site adjacent to the oxidation (see reviews 3 and 4). In pexiganan the N-terminal glycine and the two adjacent amide groups can furnish the only complexing-site for a metal ion (see review 14). The presence of des-gly$_1$-pexiganan indicates that oxidation of the N-terminal residue occurred and suggests the probable oxidation-pathway that produced the degradation products. Still, the identity of compounds 3, 4 or 5 with any of the compounds produced through the alternative pathways should not be dismissed.

Hypothetical compounds I, III, IV, V, VI, VIII (see Fig. 3) and IX (formula iii) have MW = 2476.6 (1 amu less than pexiganan), and compounds II and VII have MW = 2494.7 (17 amu heavier than pexiganan). If for example compound 3 had the structure VI, strong basic conditions should open the lactone ring to produce VII and possibly lactam VIII. Compound 3 dissolved in 5% sodium carbonate (pH = 11.5) was rapidly converted to 5 (40% conversion in 8 min and almost full conversion in 2 hrs). The amount of 5 peaked after about 2 hrs then gradually decreased. The amount of compounds 4 and 1, on the other hand, gradually increased. After 7 hrs, 1, 4 and 5

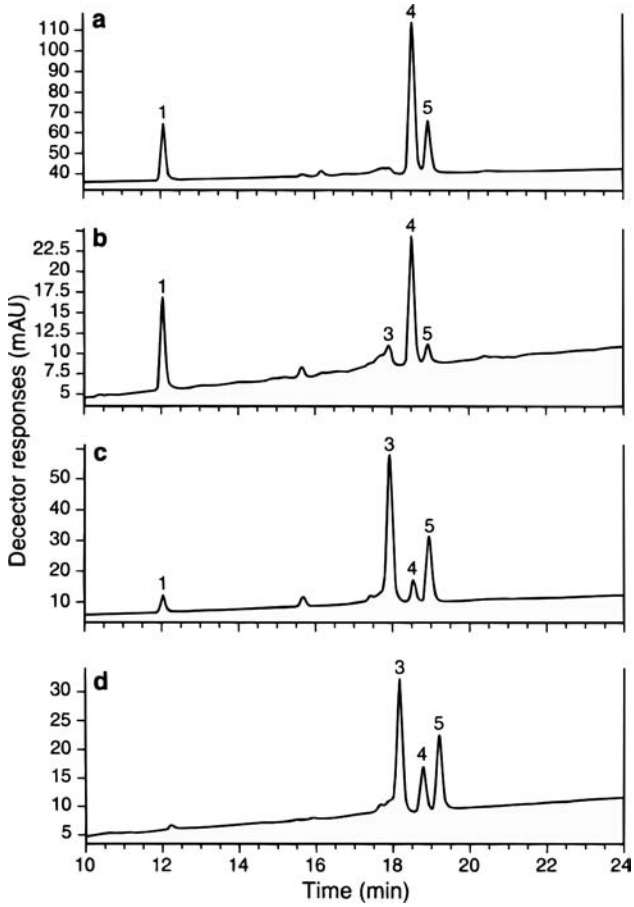

Fig. 4. Stability of compound 3 (fraction H) under different pH-conditions: (a) pH = 11.5 (5% Na_2CO_3), RT, t = 21.5 hrs, (b) pH = 8.0 (50mM NH_4HCO_3), T = 35 °C, t = 19 hrs, (c) pH = 7.0 (200 mM Na/HPO_4), T = 35 °C, t = 19 hrs and (d) pH = 4.4 (20 mM NaOAc adjusted with acetic acid), T = 35 °C, t = 21 hrs. Column/conditions: as in Fig. 2 except col. temp. 15 °C and gradient 25% B 1 min, 25% to 48% B in 25.5 min.

were approximately in the ratios of 1:3:3, and after 21.5 hrs they were approximately at 1:3:1. In a separate experiment, the reaction was monitored and quenched with acetic acid when peaks 3, 4 and 5 were still reasonably intense. LC/MS of 3, 4 and 5 formed in this mixture gave the same molecular weights found originally in the stressed drug. Formation of 1, 4 and 5 from 3, and 1 and 4 from 5 strongly suggested that all the major degradation products were produced from a single compound, namely from I.

In order to select conditions for enzymatic cleavage where compounds 3, 4 or 5 do not (chemically) decompose to 1, solutions of compound 3 were monitored under different conditions. After 21 hrs at pH = 4.4 and 35 °C, 3 formed a mixture of compounds 3, 4, 5 and traces of 1 (see Fig. 4d)**. After 19 hrs at pH = 7.0 and 35 °C, still mostly 3, 4, and 5 were present along with some small amounts of 1, the des-gly_1-pexiganan (see Fig. 4c). After 19 hrs at pH = 8.0 and 35 °C, mostly 1 and 4 were present (see Fig. 4b). It was interestingly to note that when a solution of compounds 4 and 5, and a solution of compound 3, respectively, were exposed to the same conditions, the resulting mixtures of 3, 4 and 5 had the same composition. This indicated that both mixtures reached equilibrium under these conditions (compare Fig. 5b with Fig. 4c).

Aminopeptidase-M selectively cleaves N-terminal amino-acid residues. It cleaves them one residue at a time, as long as the terminal amino group is free. It should be added that the enzyme cleaves more slowly at non-hydrophobic residues as is the gly_1-residue of pexiganan. The previous experiments strongly suggested that compounds 3, 4 and/or 5 are within the group of compounds I–V and should not undergo aminopeptidase-M cleavage, while pexiganan should undergo the cleavage at a slow rate. The conditions selected for the cleavage (pH = 7.0 and 35 °C) were adjusted for minimum formation of 1 through 'chemical' decomposition and optimum Aminopeptidase-M activity. Under these conditions, fraction J (4 and 5) and pexiganan were exposed to immobilized aminopeptidase-M. Only pexiganan underwent the cleavage and produced des-gly_1-pexiganan, 1. Compounds 4 and 5 just inter-converted to reach equilibrium with 3 without formation of 1 (see Fig. 5), indicating again that none of peptides 3, 4 and/or 5 contains

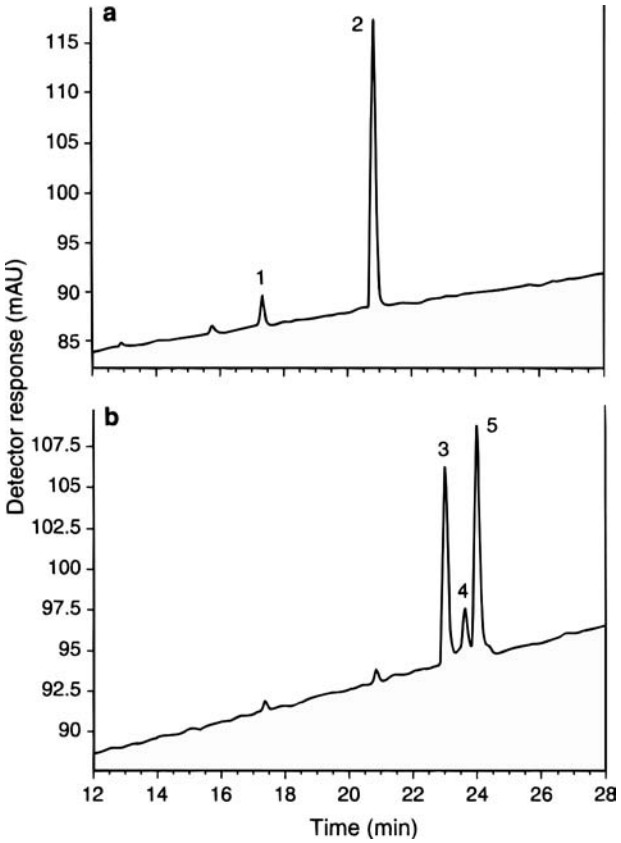

Fig. 5. Immobilized aminopeptidase-M (40 μl/ml) in pH = 7.0 (200 mM Na/HPO_4), T = 35 °C, t = 26 hrs; with (a) pexiganan (0.5 mg/ml), and with (b) fraction J (0.5 mg/ml), mixture of compounds 4 and 5. Column/conditions: as in Fig. 2 except col. temp. 15 °C and gradient: 20% B 1 min, 20% to 48% B in 30 min.

** Interestingly, the composition of 3, 4 and 5 formed from 3 under these conditions is similar to that found in the original stressed drug-extract. The pH-range specified for the cream is 4.5–5.5.

the necessary free N-terminal amino group to undergo the cleavage. The traces of 1 present in the mixture were most likely formed chemically.

Immobilized trypsin selectively cleaves peptide bonds in which the carboxyl group is contributed by a lysine or an arginine residue. The bonds susceptible to cleavage in pexiganan are marked by the letter t. Pexiganan, compound 3 and the mixture of compounds 4–5 were exposed to the enzyme for 5 hrs, at 35 °C, pH = 8.0. (trypsin is not active at a lower pH-range and shortening the exposure time was a compromise necessary to reduce the loss of the N-glyoxylyl residue). After centrifugation (to remove the immobilized enzyme) the solutions were acidified and analyzed, see Fig. 6. The LC/MS of the products formed gave M + 1 = 374.0, 373.0, 372.9, 350.9, 464.1, 373.1 and 407.1 for peaks 1, 2, 3, 4, 5 and 6, respectively, which corresponded to: 1- GIGK; 2- glyoxylyl-IGK; 3- FGK; 4- FLK; 5- ILK and 6- AFVK (7 is an impurity which showed-up also in a blank run, probably a residual surfactant). Peak 2 was present only in the tryptic digests of compounds 3 and of mixture 4–5, peak 1 only in the tryptic digest of pexiganan, while the other peaks showed-up in the three digests. Synthetic glyoxylyl-IGK (22) had the same retention time and mass spectra as peak 2. These experiments emphasized again the position where oxidation of pexiganan occurred.

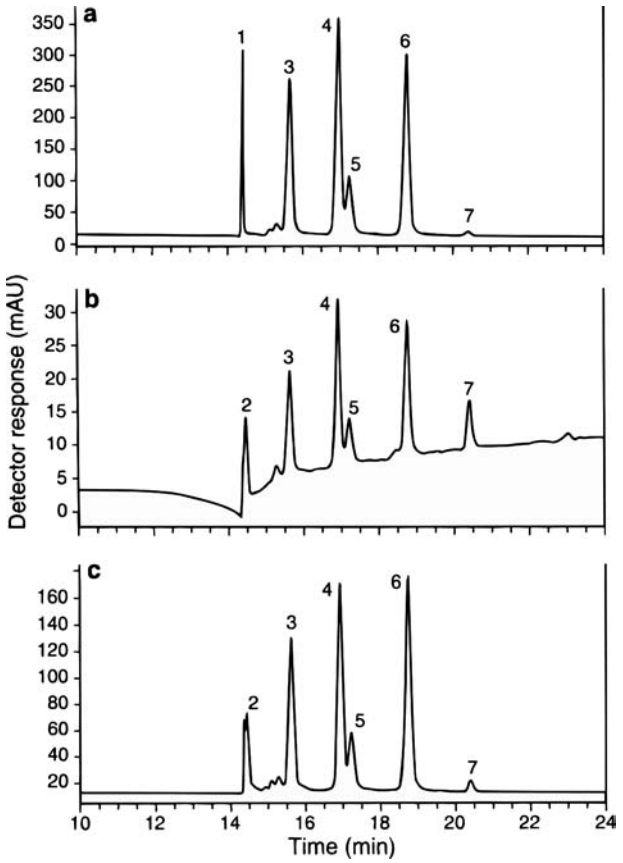

Fig. 6. Immobilized trypsin (200 μl/mL) in 100 mM NH_4CO_3 (pH = 8.0), T = 37 °C, t = 5 hrs with (a) 1.0 mg/mL pexiganan, (b) 0.3 mg/mL compound 3 and (c) 0.3 mg/mL mixture 4–5. Column PLRP-S, 100A, 15 μm, 4.6 × 250 mm; flow rate: 1.0 mL/min; col. temperature: 40 °C; detector UV 215 nm. Buffers: A—10mM NH_4HCO_3 in 99/1 H_2O/AcN, B—10/90 H_2O/AcN. Gradient: 0% B 5 min, 0% B to 70% B in 20 min. These buffer conditions gave improved LC/MS s/n-levels than those obtained under regular-RP 0.05% TFA buffer-conditions.

G-I-G-K-F-L-K-K-A-K-K-F-G-K-A-F-V-K-I-L-K-K-NH2
(t: K-F, K-K, K-A, K-K, K-F, G-K, K-A, F-V, K-I, L-K, K-K)

Behavior of Synthetic Glyoxylyl-Analogs

Chastrette *et al*. studied the ^{13}C NMR of aqueous solutions of glyoxylic acid and reported that 69 to 88% of the compound is present in the hydrate form, while the rest is present as dimeric hemiacetals (15). In the present study pexiganan had one impurity, 4, with MW = 2494.7 (the proposed hydrated II), and two impurities, 3 and 5, with M = 2476.6 (the proposed heterocyclics III, IV and/or V). As was shown, compound 3, which was isolated and was stable under 0.1% TFA-conditions, rapidly formed 5 and consequently hydrate 4 under basic conditions. These conversions are been driven by the relative stability of the anions involved, and might suggest compound 5 as being IV (the enolate) and 3 as being III. Unfortunately, more supportive data to assign 3 and/or 5 within a specific structure of III–V was not available, and the framework and the scope of this study did not permit additional efforts.

To study the tendency of glyoxylyl-des-gly_1-pexiganan to form the heterocyclic impurities, two smaller analogs were prepared; N-glyoxylyl-L-isoleucylbutanamide, 14 and N-glyoxylyl-L-isoleucyl-(N-methyl)butanamide, 18. The first had a primary amide (-NH-) in position 6 to the aldehyde and could form the cyclic compounds, while the second had an secondary amide [-N(CH_3)-] at this position and can not form them. The LC/MS of 14 showed M + 19 (for the hydrated aldehyde) and M + 1 (for the free aldehyde) at, respectively, 100% and 30% signal intensities. This compound also showed the mass for 2(M + 18) + Na, the aggregate of the hydrate, but not the 2M + 1 or 2M + Na for the aggregate of the free aldehyde. On the other hand, compound 15 (the cyclic analog which was formed from 14, see Fig. 1) showed M + 1 as the most intense signal, no M + 19, and the 2M + Na signal for the aggregate, see Table I.

Compound 16 is a stable sulfite-adduct of 14. It was easily purified, and was expected to regenerate the free aldehyde under mild conditions. Unfortunately, 16 was found to be fairly stable under the acidic conditions (5% aq. TFA, 14 days at ambient temperature). When 16 was dissolved in 5% ammonium carbonate, adjusted to pH = 10.0 with sodium hydroxide, it formed quantitatively a cyclic compound, 3-amino-4-butyl-6S-(2S-butyl)-2,5-piperazinedione, 17 (see Fig. 7b). On the other hand, when 16 was dissolved at the same pH = 10.0 in 5% sodium bicarbonate/4% sodium carbonate, it decomposed to 14, 15 and isoleucylbutanamide (see Fig. 7d). This mixture of the aldehyde, the cyclic analog(s) and the des-glyoxyl compound, was similar to the mixture formed from 3 under the basic conditions (see Fig. 4a).

Compound 20 is the N-methyl derivative of 16. The second amide is 'blocked' from forming the hetrocylic ring. Compound 20 was less stable, and when dissolved in water formed substantial amounts the hydrate of the free aldehyde, 18. Exposing 20 to pH = 10.0/RT/2 hrs in a sodium bicarbonate/sodium carbonate solution produced the hydrate 18 almost quantitatively, while in ammonium carbonate/sodium hydroxide solution 20 produced a mixture of 18 and 21. Compound 21 is the amino analog of

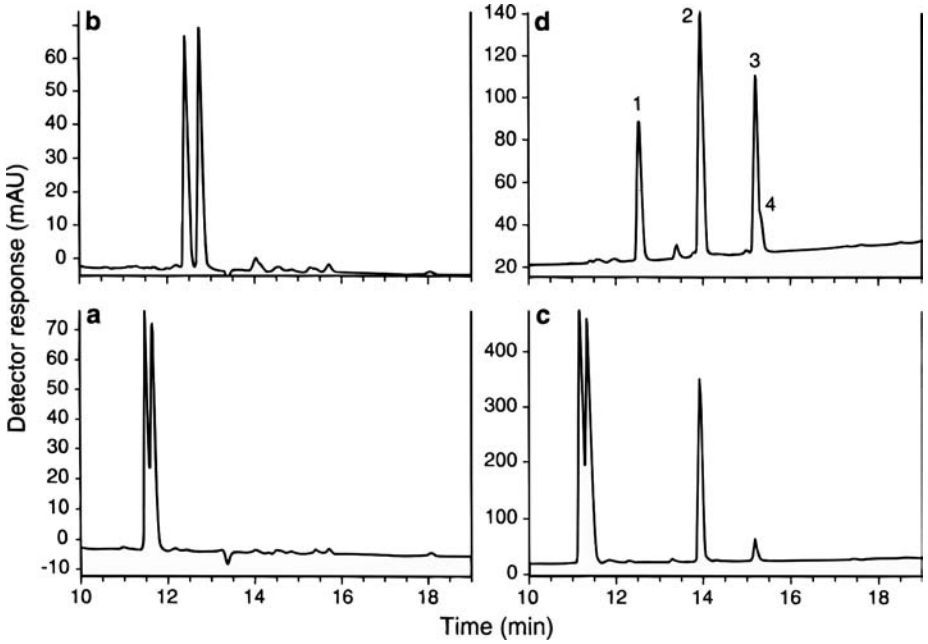

Fig. 7. Chromatograms of (a) compound 16, (b) compound 17, formed from 16 in $(NH_4)_2CO_3$/NaOH, pH = 10.0, RT, 2 hrs, (c) 16 after HPLC-purification and freeze-drying, and (d) reaction mixture of 16 in $NaHCO_3/Na_2CO_3$, pH = 10.0, RT, t = 72 hrs; peak 1- L-isoleucylbutanamide (as shown by LC/MS, M + 1 = 187.1); 2- compound 14 and 3- compound 15; column/conditions: as in Fig. 4 except gradient: 5% B 1 min, 5% B to 70% B in 25 min, and for (c) and (d) temp. 35 °C.

20, $HO_3SCH(NH_2)$CO-isoleucyl-(N-methyl)butanamide; its LC/MS signals showed m/e: 256.1 (M − 82) and 338.1 (M + 1). It can be postulated that formation of 17 from 16 involved a similar amino intermediate.

CONCLUSIONS

Four impurities were found in relatively large quantities in the 1% pexiganan cream kept under stressed conditions at 40°C and 60% relative humidity for 12 months. These impurities were introduced through a specific single-site oxidation of the N-terminal glycine residue. First pexiganan was oxidized to N-glyoxylyl-des-gly_1-pexiganan. The glyoxylyl residue then reacted with the adjacent amide group of the peptide to form tautomers of the piperazinedione derivative of des-gly_1-pexiganan and partially decomposed to des-gly_1-pexiganan. Oxidative-deamination of N-terminal residues of peptides should not be limited to peptides containing a N-terminal glycine-residue. Other N-terminal amino-acid residues should undergo similar reactions under similar conditions.

The study showed that under mild acidic-conditions (pH 4.5–5.5) the N-glyoxylyl and the piperazinedionyl derivatives of des-gly_1-pexiganan have good stabilities, and decompose to des-gly_1-pexiganan at extremely slow rates. The tendency of N-glyoxylyl-des-gly_1-pexiganan to form the heterocyclic compounds was demonstrated by the tendency of N-glyoxylyl-L-isoleucylbutanamide (an analog of N-glyoxylyl-des-gly_1-pexiganan) to form similar piperazinedione derivatives.

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REFERENCES

1. M. Zasloff. Magainins, a class of antimicrobial peptides from Xenopus skin; isolation, characterization of two active forms, and partial cDNA of a precursor. *Proc. Natl. Acad. Sci. USA* **84**:5449–5453 (1987).
2. L. Jacob and M. Zasloff. Potential therapeutic applications of magainins and other antimicrobial agents of animal origins. In *Antimicrobial Peptides, Ciba Foundation Symposium* **186**:197–216, John Wiley & Sons, New York, 1994.
3. T. M. Nguyen. Oxidation degradation of protein pharmaceuticals. In *Formulation and Delivery of Proteins and Peptides, ACS Symposium Series* **567**:59–71 (1994).
4. S. Li, C. Schoneich, and R. T. Borchardt. Chemical instability of protein pharmaceuticals; mechanisms of oxidation and strategies for stabilization. *Biotech. Bioeng.* **48**:490–500 (1995).
5. E. R. Stadtman. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annu. Rev. Biochem.* **62**:797–821 (1993).
6. S. Li, T. H. Nguyen, C. Schoneich, and R. T. Borchardt. Aggregation and precipitation of human relaxin induced by metal-catalyzed oxidation. *Biochemistry* **34**:5762–5772 (1995).
7. E. R. Stadtman. Metal ion-catalyzed oxidation of proteins; biochemical mechanism and biological consequences. *Free Radical Biol. Med.* **9**:315–325 (1990).
8. M. S. Hora, R. K. Rana, C. L. Wilcox, N. V. Katre, P. Hirtzer, S. N. Wolfe, and J. W. Thompson. Development of lyophilized formulation of interleukin-2. In *International Symposium on Biological product freeze-drying and Formulation, Dev. Biol. Std.,* **74**:295–306 (1991).
9. E. J. Corey and K. Achiwa. A new method for the oxidation primary amines to ketones. *J. Am. Chem. Soc.* **91**:1429–1432 (1969).
10. A. Kubo, Y. Kitahara, S. Nakahara, R. Iwata, and R. Numata. Synthesis of mimocin, an isoquinolinequinone antibiotic from

Streptomyces lavendulae, and its congeners. *Chem. Pharm. Bull.* **36**:4355–4363 (1988).
11. P. J. M. van den Oetelaar, P. S. L. Jansen, P. A. T. A. Melgers, G. N. Wagenaars and P. B. W. ten Kortenaar. Stability assessment of peptide and protein drugs. *J. Contr. Rel*. **21**:11–22 (1992).
12. D. Qasmi, E. de Rosny, L. Ren'e, B. Badet, I. Vergely, N. Boggetto, and M. Rebound-Ravaux. Synthesis of N-glyoxylyl peptides and their in vitro evaluation as HIV-1 protease inhibitors. *Bioorg. Med. Chem.* **5**:707–714 (1997).
13. E. J. Corey and B. W. Erickson. Oxidative hydrolysis of 1,3-dithiane derivatives to carbonyl compounds using N-halosuccinimide reagents. *J. Org. Chem.* **36**:3553–3560 (1971).
14. H. Sigel and R. B. Martin. Coordinating properties of the amide bond. Stability and structure of metal ion complexes of peptides and related ligands. *Chem. Rev.* **82**:385–426 (1982).
15. F. Chastrette, C. Bracoud, M. Chastrette, G. Mattioda and Y. Christidis. Etude de la composition de solutions aqueuses d'acide glyoxylique en RMN de ^{13}C. *Bull. Soc. Chim. Fr.* **1985** II:66–74.