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Journal of Pharmaceutical and Biomedical Analysis
32 (2003) 85–96
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Determination of degradation products of squalamine lactate using LC/MS

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Received 7 November 2002; received in revised form 2 January 2003; accepted 18 January 2003

Abstract

Heat, acid and base stress methods were applied to study the stability of squalamine lactate. Liquid chromatography coupled with mass spectrometry was used to analyze the degraded samples and tentative structural identifications were assigned based on their molecular weight measurements, reactivity and MS/MS fragmentation. Solid squalamine lactate generated a new amide, namely lactyl squalamide, when heated to 80 °C. Chemical structure for this new compound has been established by NMR and MS data interpretation and confirmed by direct comparison between the degradant and the synthesized compound. Squalamine lactate in pH 4 acetate buffer solution produced more degradants under stressed conditions. These degradants are formed due to the loss of the sulfate functionality. Squalamine lactate is stable in refrigerated conditions as well as in basic solution.

Keywords: Squalamine; Degradation; LC/MS

1. Introduction

Squalamine is a 7,24-dihydroxylated 24-sulfated cholestane steroid conjugated to spermidine at C-3, a natural product (Fig. 1) originally isolated from liver of the dogfish shark [1]. It has shown significant anti-angiogenic and antitumor, but not cytotoxic properties [2–5]. Squalamine lactate is currently in different phases of clinical trials as a new anticancer entity for the treatment of non-small cell lung cancer and ovarian cancer. Squalamine is used with other cytotoxic agents, such as paclitaxol or carboplatin, to achieve tandem anticancer effect. That is, squalamine is used to inhibit the growth the cancer tissue by limiting nutrition supply, while cyctotoxic agents are used to directly kill cancer cells. In order to assess the safety and potency of its products in clinical trials as well as the drug substance in manufacturing process, forced degradation testing was needed to quickly understand the degradant profile of squalamine

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0731-7085/03/$ - see front matter
doi:10.1016/S0731-7085(03)00047-5

lactate. Stability testing provides evidence for the quality of the bulk drug and its final drug product when they are exposed to influence of environmental factors such as pH, temperature and humidity. Forced degradation helps to determine the intrinsic stability of the molecule by establishing the degradation pathways.

Squalamine drug product (solid for injection) and the bulk substance (in a pH 4 acetate buffer, HCl and NaOH solutions) were stored under different temperature and relative humidity (RH) over different periods of time. HPLC/MS assays were used for analysis of samples at different testing time points to detect possible degradants and to provide structural information.

2. Experimental

2.1. Reagents and chemicals

HPLC grade water, acetonitrile, sodium hydroxide (electrophoresis grade), concentrated hydrochloric acid, glacial acetic acid, trifluoroacetic acid were purchased from J.T. Baker (Philipsburg, NJ). All chemicals were used without further purification.

2.2. Sample stress conditions

The samples of material were stressed as a dry solid, in a pH 4 acetate buffer (20 mM) solution (diluent), in acid and base solutions. Samples were stored in stability chambers or ovens under conditions of refrigerated 4 °C; 40 °C/60% RH, 60 and 80 °C for 1 day, 7 days, 2 weeks and 1 month and assayed in duplicates. At the time of assay, solution samples were transferred directly to LC vials for analysis, while the dry solid samples were dissolved to a concentration of 0.2 mg/ml in diluent for analysis. Sample solutions at 0.4 mg/ml in 0.1 N HCl and 0.1 N NaOH were stored in a 60 °C oven. After the storage of 4 h, 24 h and 3 days, duplicate samples were removed from the oven and neutralized by being mixed with the same volume of 0.1 N HCl or 0.1 N NaOH solution. Then the solution samples were transferred to LC vials for analysis.

2.3. Liquid chromatography

Chromatographic separation was performed on an Agilent 1100 LC system that includes a binary pump, a column heater oven and an autosampler. The system was programmed at 40 °C for the column oven, 10 μl for injection volume. A gradient elution was used to elute squalamine and all possible degradants from the column. The following table shows the gradient program.

Time (min)	%*B*
0.0	20.0
0.5	20.0
12.0	50.0
17.0	60.0
19.0	85.0
19.5	85.0

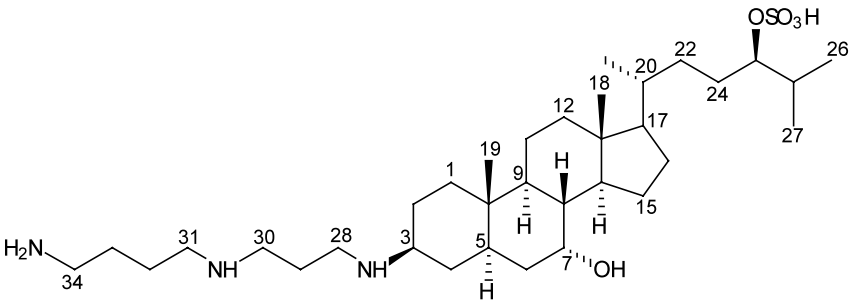


Fig. 1. Chemical structure of squalamine.

where:A = acetonitrile-water-TFA (10:90:0.025, v/v/v) and B = acetonitrile-water-TFA (90:10:0.025, v/v/v).

An Alltech Kromasil RP C_{18} LC column in the size of 4.6×250 mm was used with a guard column of NewGuard RP-18, 7 μm, 3.2×15 mm. The flow (1.0 ml/min) from the LC column was split to let only 0.3–0.4 ml/min to the mass spectrometer (MSD).

2.4. Evaporative light scattering detector conditions

A Sedere Model Sedex 75 evaporative light scattered detector (ELSD) was used. Detection unit was millivolt and the drift tube temperature was 40 °C.

2.5. Mass spectrometer conditions

An Agilent single quadrupole MSD was used for both negative and positive ion full scan modes to determine the molecular weights of the degradants. Drying gas temperature: 350 °C; Fragmentor setting can be adjusted depending upon the needs to collect fragment ion information or the degree of declustering solvents.

2.6. Mass spectrometer (API-2000) conditions

Isolated or synthesized squalamine degradants were infused into the Sciex API-2000 MS/MS to acquire product ion information. Positive ion mode was used for both Q1 full scan and product ion scan. Ion Spray Voltage (IS): 5000 V; Nebulizer Gas (Gas 1): 10 psi; Turbo Gas (Gas 2): 30 psi; Turbo Gas Temperature (TEMP): 350 °C; Curtain Gas (CUR): 35 psi; Collision Gas (CAD): 2 psi. Focusing Potential (FP): 350 V; Entrance Potential (EP): −10 V; Declustering Potential (DP) and Collision Energy (CE) values were adjusted depending on the needs to collect product ion information or the degree of declustering solvents.

2.7. Isolated degradant A from thermal decomposition of solid squalamine lactate

One gram of squalamine lactate solid was heated for about 7 months at 80 °C. Preparative HPLC purification of the decomposed mixture yielded 550 mg degradant A. For NMR data, see Ref. [6].

2.8. Synthesis of lactyl squalamide

Lactic acid (3.38 g) in dimethyl acetamide was reacted with benzyl bromide (5.4 ml) in the presence of sodium hydride (3.3 g) to form O-benzyl lactic acid (1.29 g), which was then converted to the hydroxy succinimide active ester (1.3 g) by reacting with *N*-hydroxy succinimide (0.75 g) in the presence of *N*,*N*′-dicyclohexylcarbodiimide (1.34 g). Squalamine free base (974 mg) was coupled with this active ester to obtain O-benzyl lactyl squalamide (1.33 g), which, on hydrogenation, yielded N^3-lactyl squalamide (724 mg) after purification by reversed phase HPLC.

2.9. Sensitivity

This work was designed and performed more qualitatively than quantitatively, therefore no standard curve was used to calibrate the MSD. At this time, it is impractical to estimate the limit of detection for this class of compounds. However, the MSD seemed very sensitive in that it detected the 24S-squalamine, which had a concentration of less than 10 μg/ml (24S-squalamine had a content of less than 5% of starting material). Meanwhile, it should be noted that different compounds have different ionization efficiencies, and therefore have different responses. Amino sterols with a sulfate group are believed to form a strong inter- or intramolecular ion-pair, decreasing the sensitivity to MSDs [7], while the degraded amino sterols without a sulfate group (no ion-pair) should be much more sensitive.

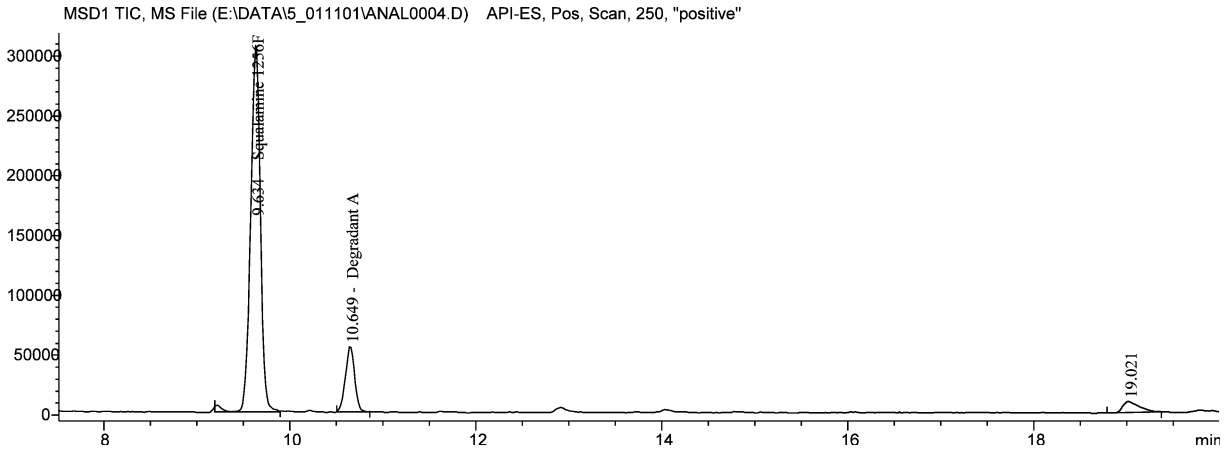


Fig. 2. Ion chromatogram of solid squalamine stored at 80 °C for 30 days.

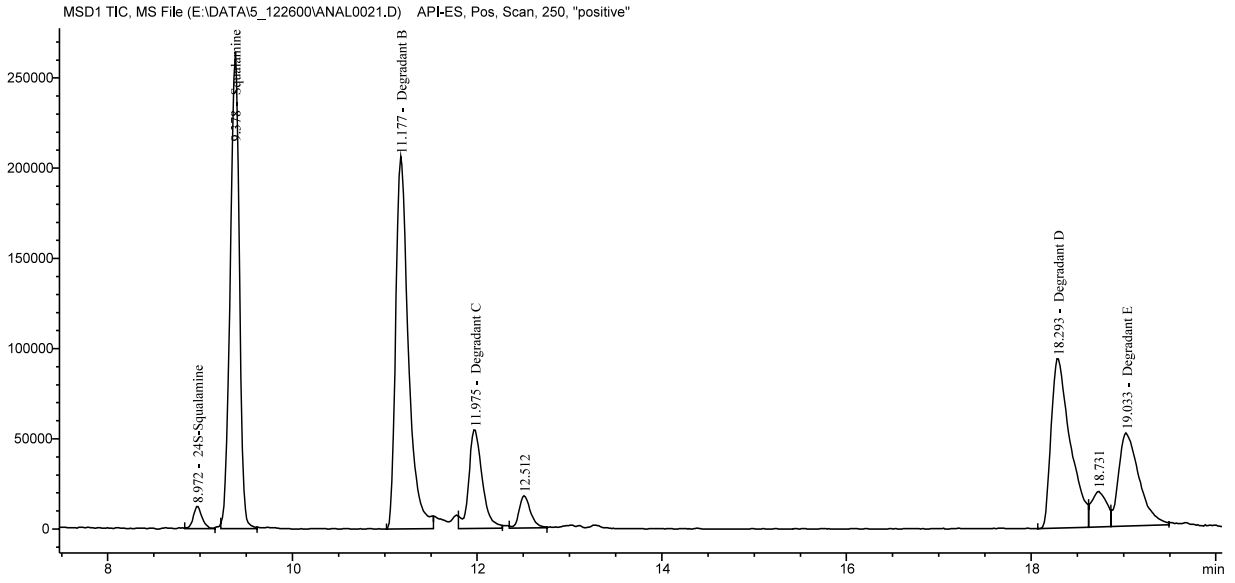


Fig. 3. Chromatogram of squalamine in pH 4 solution stored at 60 °C for 30 days.

3. Results and discussion

3.1. LC/MS analysis

Squalamine lactate is stable under refrigerated conditions (2–4 °C) in both solid and solution forms. No new peak was detected from samples stored under this condition for 30 days. However, at harsher conditions up to 80 °C and acid, squalamine lactate produced degradants due to the reactivity of either the sulfate group or the polyamine group.

Two additional peaks (Fig. 2) were found from squalamine lactate solid stored at 60 or 80 °C. The degradant eluted at 10.65 min is named as degradant A. The degradant eluted at 19.02 min was slowly formed as it was not found from samples of the first three time points.

Four degradants, namely degradants B, C, D and E, were first detected from acetate buffer

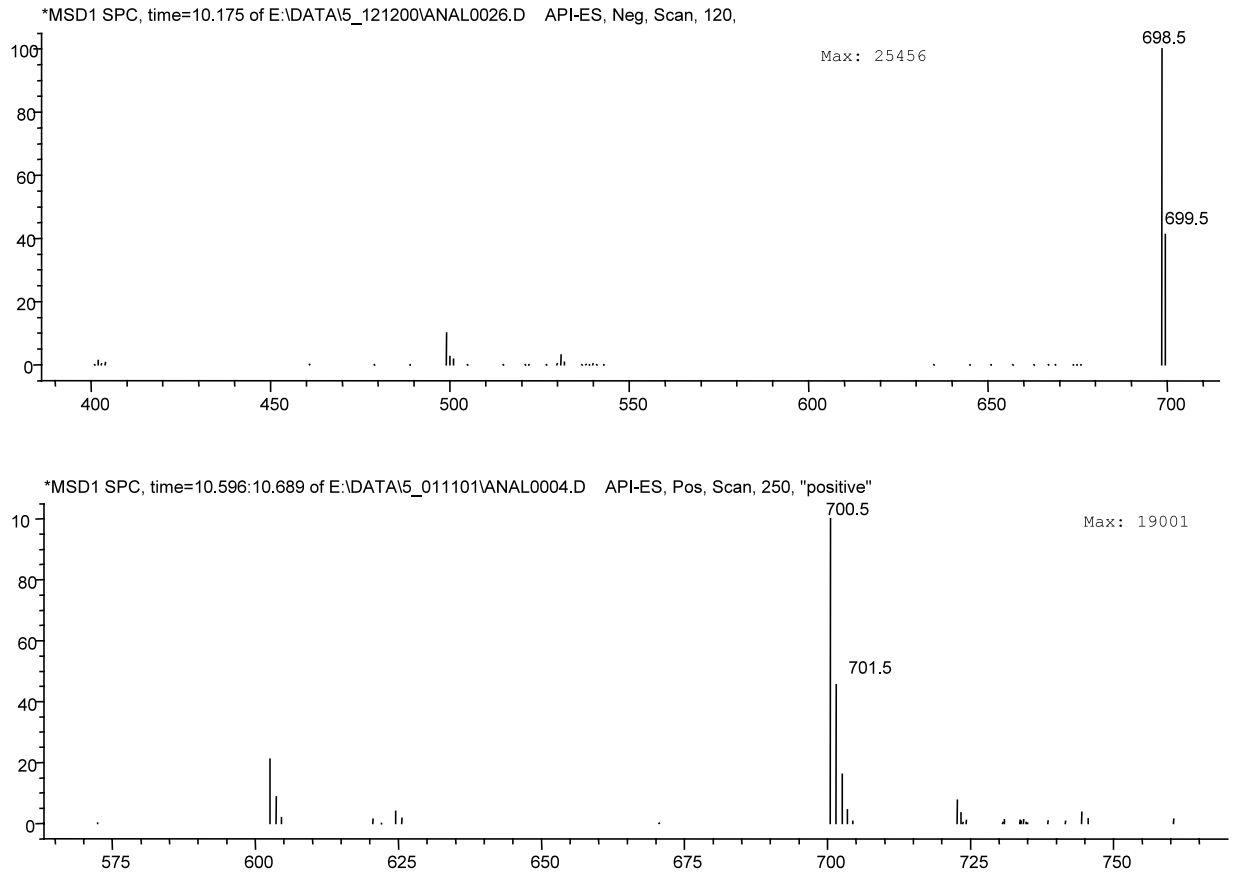


Fig. 4. MSD mass spectra of degradant A in negative (upper panel) and positive (lower panel) modes.

solutions stored for 24 h in Thermolyne oven set at 80 °C. Their retention times are 11.18, 11.98, 18.29 and 19.03 min, respectively. Their concentrations increased as the storage time increased. These same degradants (based on retention times) were detected in the solutions stored at 60 and 45 °C/60% humidity from Day-7, Day-14 and Day-30 tests. Two additional degradants were detected from squalamine solution stored at 60 °C for 30 days (Fig. 3) eluted at 12.51 (m/z 548.5) and 18.73 (m/z 530.5) min. Since they were not detected from samples of the first three time points, they are believed to be either secondary degradants or products due to slow reaction. Their mass spectra indicate they are also desulfate products.

Squalamine lactate in 0.1 N NaOH was stable, since no additional peak was detected. One more peak at the retention time of 10.5 min was detected from solutions of squalamine lactate in 0.1 N HCl stored at 60 °C.

3.2. Degradant structure characterization by LC/MS and MS/MS

Degradant A generated in squalamine lactate solid gave molecular ions at m/z 700.5 $[M+1]^+$ and 698.5 $[M-1]^-$ under positive and negative scan modes, respectively (Fig. 4), indicating it has a molecular weight of 699.5 Da. The compound also generated a fragment ion under positive product ion scan mode (MS/MS) at m/z 602.5, which can be assigned as the product ion due to a loss of a molecule of sulfuric acid from the precursor ion. This finding confirms the compound has retained the sulfate moiety intact in

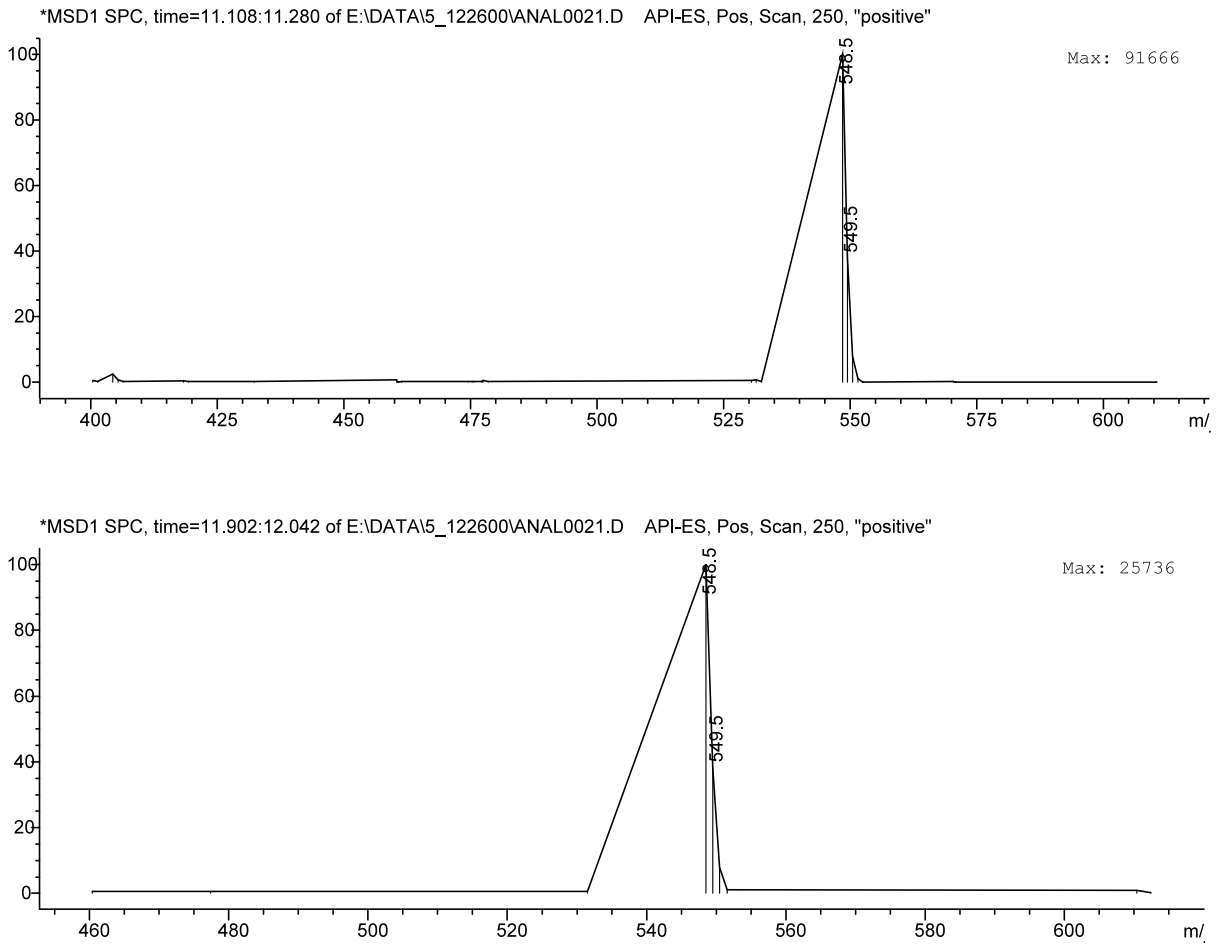


Fig. 5. Mass spectra of degradants B (upper panel) and C (lower panel) appeared in Fig. 3 chromatogram.

this degradant product. Since the molecular weight difference of 72 amu between the degradant A and squalamine corresponds to a lactyl moiety, this compound must be produced by the reaction between lactic acid and squalamine. The exact location of the reaction could be on the C-3 polyamine, forming an amide, or could be on the C-7 hydroxyl group, forming an ester. To characterize the chemical structure, degradant A was isolated with reversed phase HPLC for further NMR and MS/MS study. The triplet proton signal at δ 7.75 ($J = 6.0$ Hz) showed long range correlation respectively to the carbonyl carton at δ 174.46 and a methylene carbon at δ 37.31. The chemical shift and coupling constant indicate this proton has to be a primary amide proton, and therefore the lactyl group is assigned to the primary amine at C-34. The new primary amide, lactyl squalamide, was synthesized and it showed the same LC retention times, molecular weights, mass and NMR spectra of degradant A.

Degradants B and C (eluted at 11.18, 11.98 min) produced in acetate buffer solution showed almost the same mass spectra. Both compounds gave molecular ions at m/z 548.5 $[M+1]^+$ under positive scan mode, 80 amu less than the molecule of squalamine (Fig. 5). This molecular weight difference indicates the loss of sulfur trioxide from squalamine. Therefore these two degradants are proposed as desulfate squalamines. This would be expected since the sulfate group is labile to aqueous hydrolysis. Molecular ions for the two

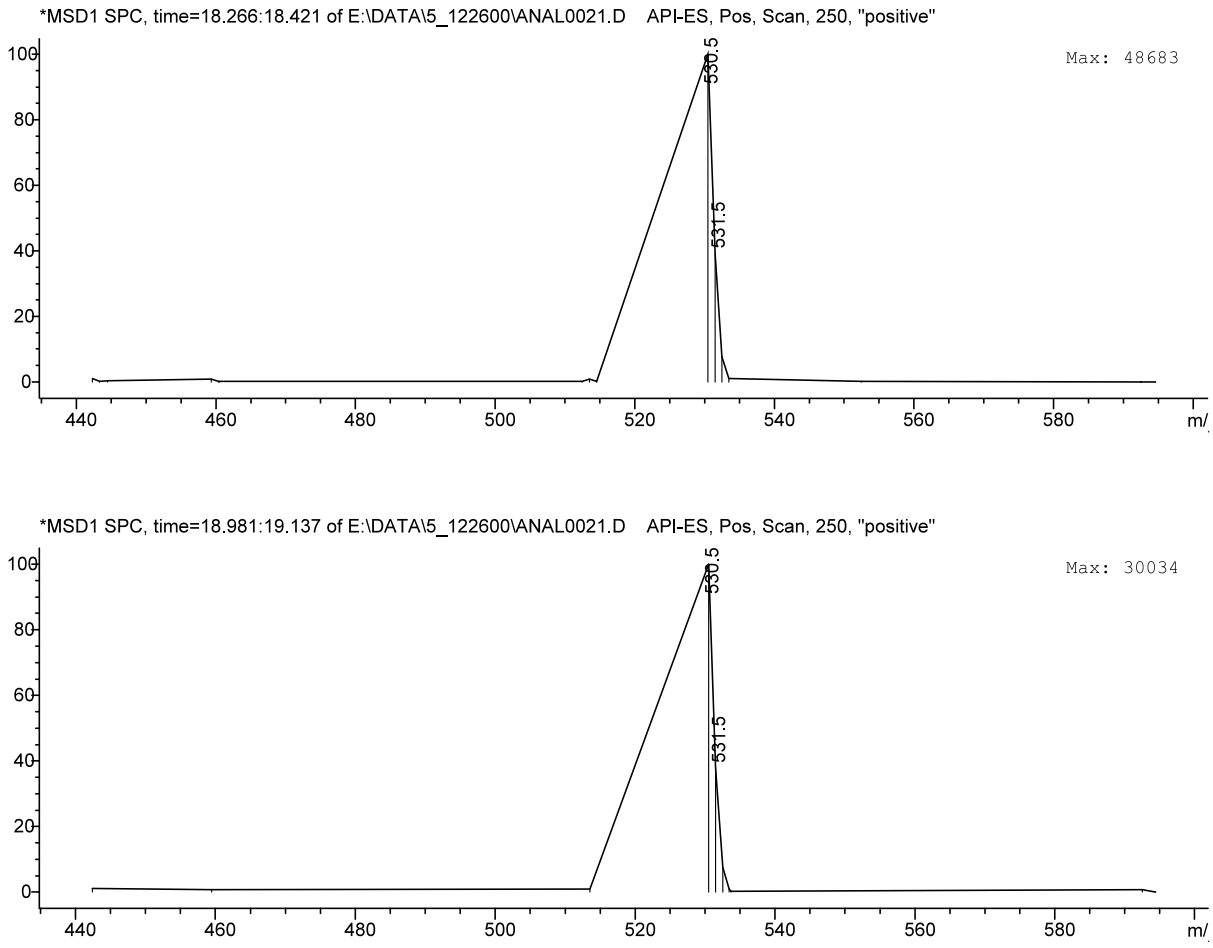


Fig. 6. Mass spectra of degradants D (upper panel) and E (lower panel) appeared in Fig. 3 chromatogram.

compounds cannot be unambiguously identified under negative scan mode. This again indicates that the compounds are desulfate products, losing the ability of carrying a negative charge.

Degradants D and E of retention times at 18.29 and 19.03 min generated in solutions also showed the same mass spectra, which showed molecular ions at m/z 530.5 for the two compounds (Fig. 6). The molecular weight difference between the two compounds and squalamine, 98 amu, suggests they are produced by elimination of sulfuric acid from squalamine. Their retention times also indicate the compounds are much less polar than squalamine.

The degradant eluted at 19.02 min from degraded solid squalamine lactate showed molecular ion at m/z 530.5. Another degradant eluted at 18.73 min from degraded squalamine solution also showed the same molecular ion. They are proposed as the products of losing a molecule of SO_3 and a molecule of water from squalamine. The third minor degradant eluted at 12.51 min from degraded squalamine solution showed its molecular ion at m/z 548.5, indicating it is the desulfated squalamine.

Peak purity was checked using the CHEMSTATION software. All the degradant peaks were purely formed by single ions in the unit resolution range. For example, the degradant B peak appeared in Fig. 3 was due to the response of ions of m/z 548–549 amu.

Degradant from squalamine lactate in hydrochloric acid solutions was not analyzed by LC/

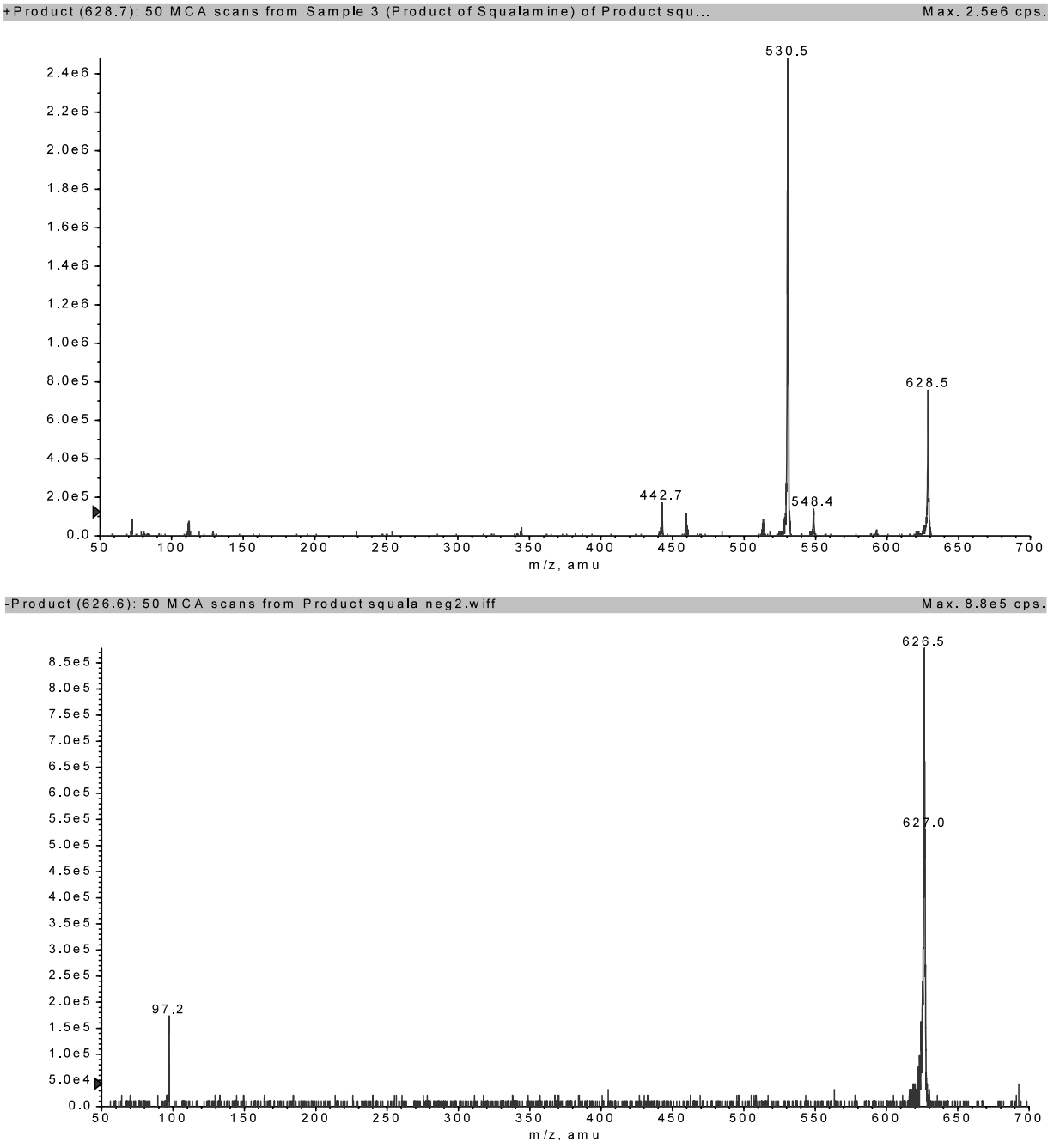
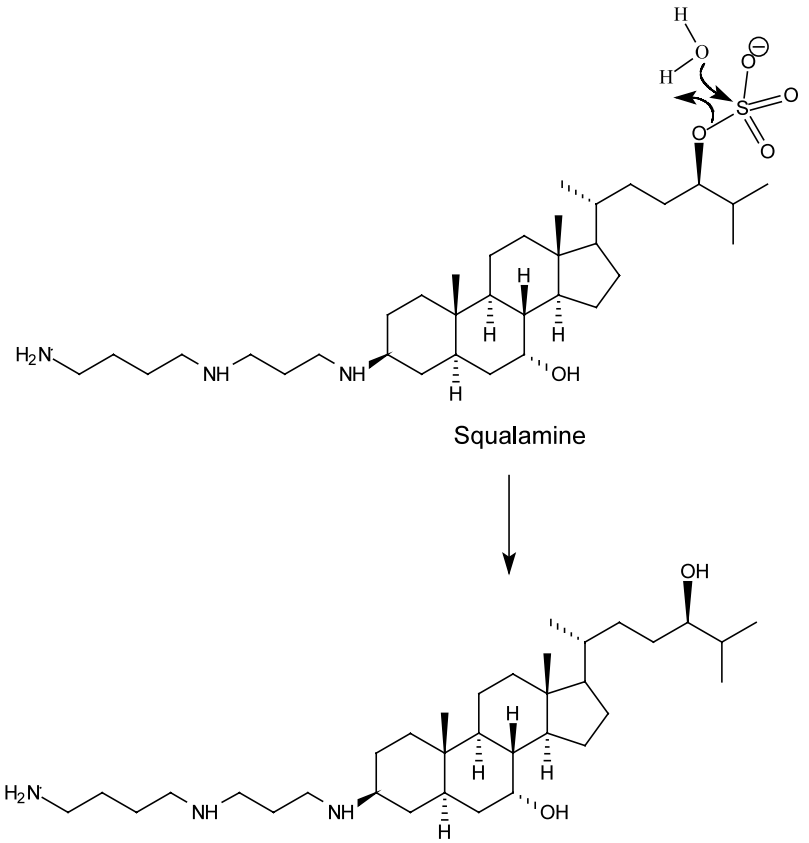


Fig. 7. Product ion scan of squalamine lactate in positive and negative modes.

MSD method. But based on the retention time detected with ELSD, 10.5 min, it is highly proposed as desulfate squalamine, possibly identical to degradant B.

From the generation of degradants B, C, D and E, it appears that the sulfate group on C-24 of squalamine is the most active part, when squalamine is dissolved in acidic solution. To confirm

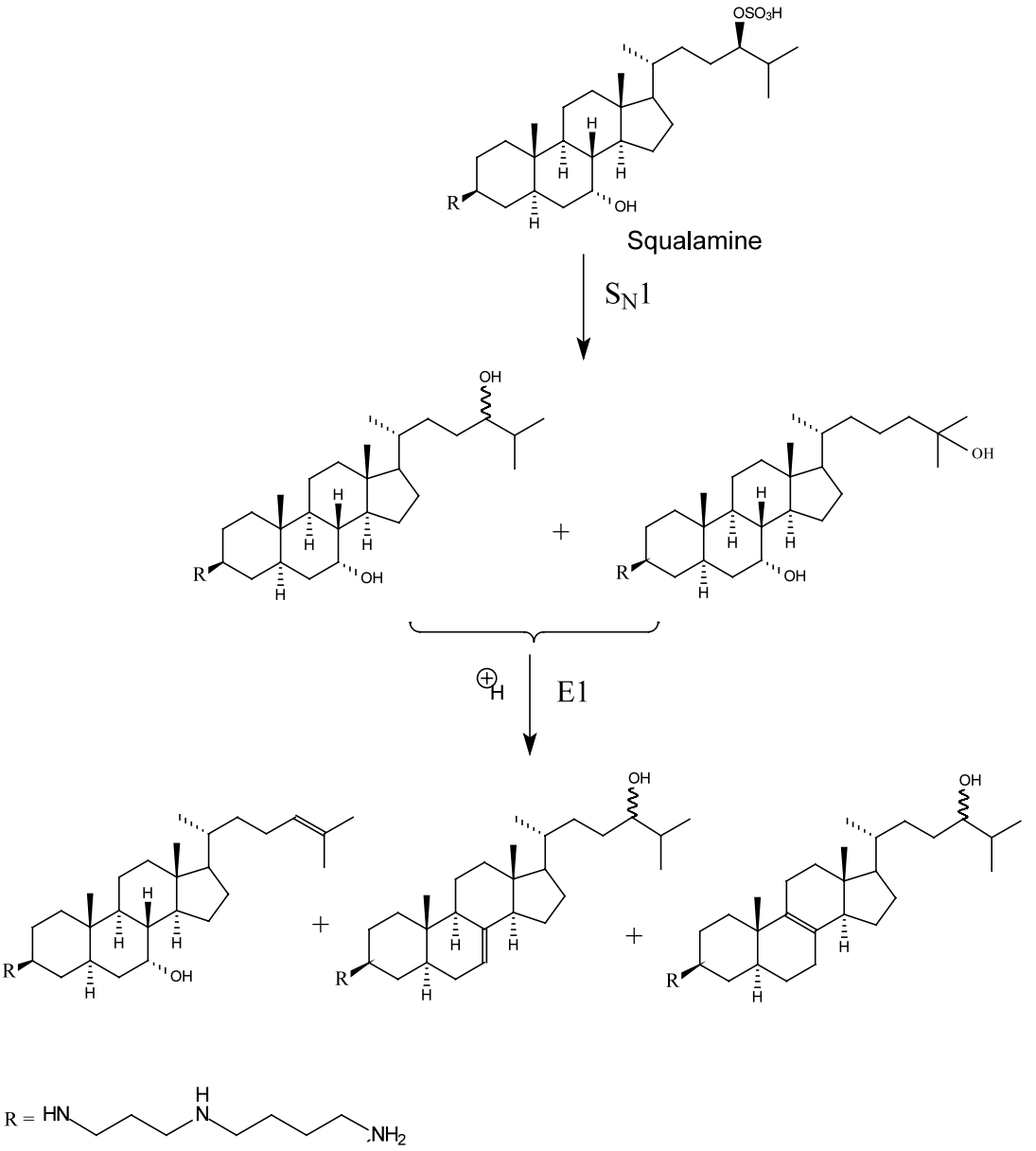


Scheme 1. Desulfate process for squalamine in acidic solution with heating.

this, a product ion scan test was performed using the Sciex API-2000 MS/MS instrument. In the test, squalamine ions in both positive and negative gas phase were selected (in Q1) to collide with the collision gas (N_2) (in Q2), and the dominant reaction was to lose a molecule of sulfuric acid (Fig. 7).

3.3. Degradation mechanism

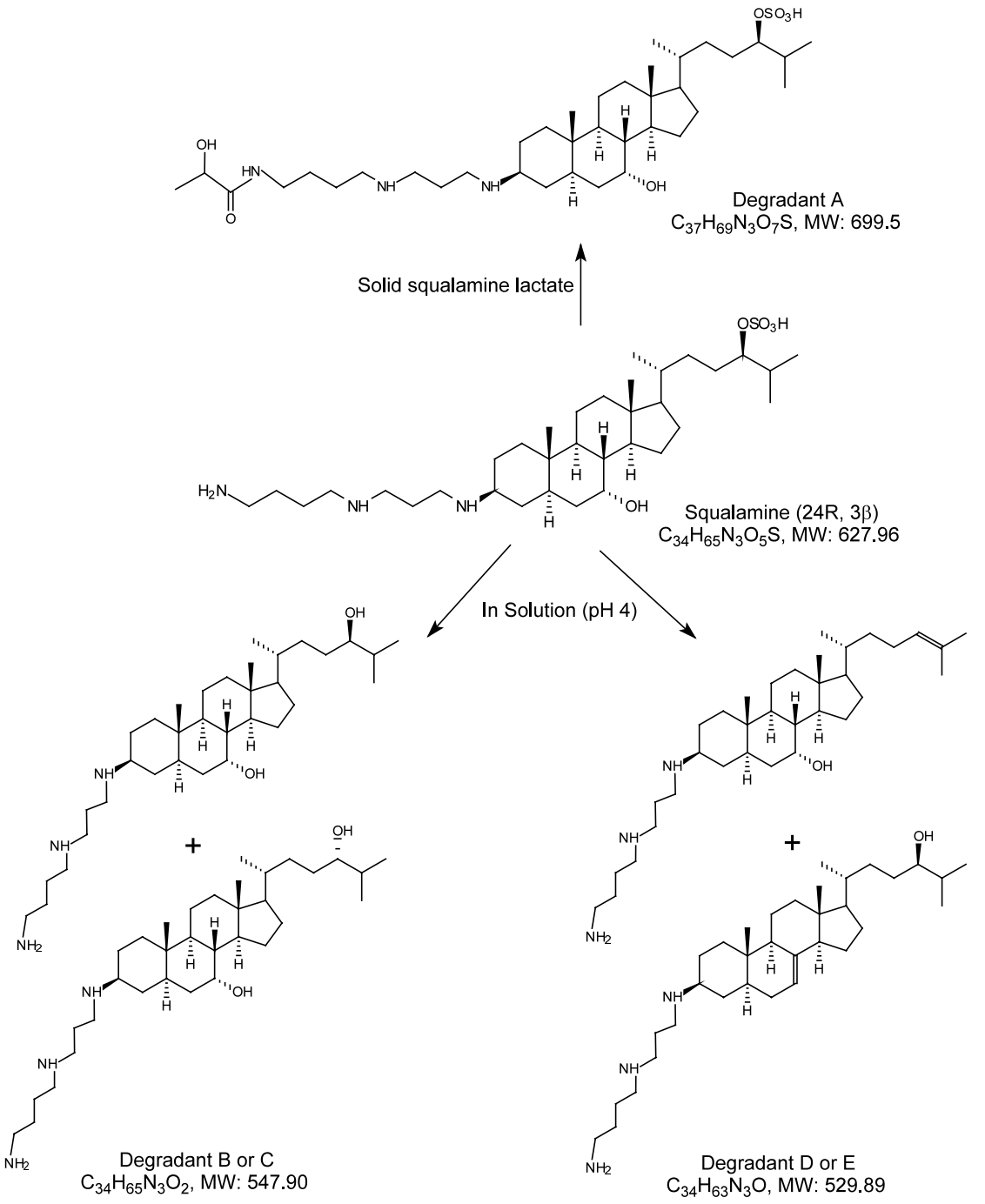
It is easily understandable that solid squalamine reacts with lactic acid when heated, generating a primary amide and water. However, squalamine in acidic solution may undergo complex mechanisms as indicated in Fig. 3. At least 3 desulfate composition isomers were formed (having different retention times but the same molecular ion). This phenomenon could be explained with Schemes 1 and 2. In Scheme 1, a water molecule, regarded as a nucleophile, approaches the sulfur atom and cause to lose a molecule of sulfur trioxide. In this reaction, the configuration of C-24 is retained. In Scheme 2, squalamine could undergo a typical S_N1 substitution first, generating a carbocation (C-24) intermediate. This intermediate is then attacked by water molecule and both 24R-OH and 24S-OH products could be formed. The carbocation could further migrate to C-25, resulting in a product with a 25-OH. Both squalamine and desulfated products could undergo a typical E1 elimination reaction, forming a double bond at C-24, C-7 or C-8. Apparently, the possible $\Delta^{8,9}$ and $\Delta^{8,14}$ products are due to the carbocation rearrangement. Scheme 3 summarizes proposed structures for all 5 major degradants.



Scheme 2. Squalamine degradation mechanism in acidic solution with heating.

4. Conclusions

Squalamine lactate appears to be a stable compound. It is stable under refrigerated conditions (2–4 °C) in both solid and solution forms. Squalamine lactate in 0.1 N NaOH solution is stable also. However, under accelerated conditions, solid squalamine lactate form a new amide called lactyl squalamide. Squalamine in acidic solution quickly (24 h) produced degradants due to the loss of either sulfur trioxide or sulfuric acid. In other words, the sulfate functionality is the most labile part in the molecule. This has been confirmed by the gas phase ion fragmentation test



* The exact positions for double bond remain unknown.

Scheme 3. Proposed degradants of squalamine.

using tandem mass spectrometry technique. This preliminary study will make it possible to assess the level of degradation taking place in any future stability studies by checking for the appearance of any degradants listed above.

References

[1] K.S. Moore, S. Wehrli, H. Roder, M. Rogers, J.N. Forest, Jr., D. McCrimmon, M. Zasloff, Proc. Natl. Acad. Sci. USA 90 (1993) 1354–1358.

[2] A.K. Sills, Jr., J.I. Williams, B.M. Tyler, D.S. Epstein, E.P. Sipos, J.D. Davis, M.P. McLane, S. Pitchford, K. Cheshire, F.H. Gannon, W.A. Kinney, T.L. Chao, M. Donowitz, J. Laterra, M. Zasloff, H. Brem, Cancer Res. 58 (1998) 2784–2792.

[3] B.A. Teicher, J.I. Williams, H. Takeuchi, G. Ara, R.S. Herbst, D. Buxton, Cancer Res. 58 (1998) 2567–2792.

[4] J.A. Schiller, G. Bittner, Clin. Cancer Res. 5 (1999) 4287–4294.

[5] D. Li, J.I. Williams, R.J. Pietras, Oncogene 21 (2002) 2805–2814.

[6] NMR data of degradant A (AMX-400, DMSO-d_6): **^{1}H-NMR (400 MHz)** resolved signals: δ 8.51 (3H, br), 7.75 (1H, t, $J = 6.0$ Hz, N^3-H), 3.94 (1H, q, $J = 6.77$ Hz, H-α of lactyl group), 3.82 (1H, dd, $J = 10.33$ and 4.86 Hz, H-24), 3.61 (1H, s, H-7), 3.09 (1H, dd, $J = 13.3$ and 6.7 Hz, H-34), 1.20 (3H, d, $J = 6.77$ Hz, CH_3 of lactyl group), 0.87 (3H, d, $J = 6.3$ Hz, H-21), 0.82 (3H, d, $J = 7.8$ Hz, H-26 or 27), 0.80 (3H, d, J-7.1 Hz, H-26 or 27), 0.75 (3H, s, H-19), 0.61 (3H, s, H-18); **^{13}H-NMR (100 MHz)(DEPT):** δ 35.91 (CH_2, C-1), 24.18 (CH_2, C-2), 56.09 (CH, C-3), 30.14 (CH_2, C-4), 36.42 (CH, C-5), 36.57 (CH_2, C-6), 65.33 (CH, C-7), 38.87 (CH, C-8), 44.69 (CH, C-9), 35.19 (C, C-10), 20.46 (CH_2, C-11), 40.85 (CH_2, C-12), 41.86 (C, C-13), 49.94 (CH, C-14), 23.01 (CH_2, C-15), 27.67 (CH_2, C-16), 55.66 (CH, C-17), 11.71 (CH_3, C-18), 10.68 (CH_3, C-19), 35.12 (CH, C-20), 18.51 (CH_3, C-21), 30.79 (CH_2, C-22), 26.30 (CH_2, C-23), 80.98 (CH, C-24), 29.97 (CH, C-25), 17.90 (CH_3, C-26), 17.66 (CH_3, C-27), 37.31 (CH_2, C-28), 22.54 (CH_2, C-29), 43.95 (CH_2, C-30), 46.53 (CH_2, C-31), 22.85 (CH_2, C-32), 26.23 (CH_2, C-33), 37.31 (CH_2, C-34), 174.46 (C, lactyl carbonyl), 67.20 (CH, lactyl C-α), 21.11 (CH_3, lactyl C-β). This ^{13}C signal assignment is based partly on the HMBC correlations, and partly on the literature report for squalamine [8]. **Key HMBC correlations:** From ^{1}H signal at δ 7.75 to ^{13}C signals at δ 37.31 and 174.46, respectively; From ^{1}H signal at δ 3.09 to ^{13}C signals at δ 22.85, 26.23 and 174.46, respectively; **Key COSY45 correlations:** From signal at δ 7.75 to signal at δ 3.09; From signal at δ 3.94 to signal at δ 1.20.

[7] C.-J. Li, et al., J. Pharm. Biomed. Anal., submitted for publication.

[8] S.L. Wehrli, K.S. Moore, H. Roder, S. Durell, M. Zasloff, Steroids 58 (1993) 370–378.