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# Liquid chromatography–thermospray mass spectrometric study of N-acylamino dilactones and 4-butyrolactones derived from antimycin A

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## ABSTRACT

Reversed-phase high-performance liquid chromatography–thermospray mass spectrometric (HPLC–MS) characteristics of four sets of lactonic complexes (one 4-butyrolactones and three dilactone complexes) derived from antimycin A were investigated. Three types of 8-hydroxy analogues were also included in the study. Pairs of a–b structures isomeric at the 8-acyloxy ester side-chains were best separated with a high-efficiency octadecylsilica column prior to analysis by HPLC–MS. Mass spectra of the a–b pairs each with identical molecular weights exhibited virtually indistinguishable fragmentation patterns, although their relative intensities were not superimposable. In some cases, HPLC–MS of the title compounds yielded mass chromatograms showing the minor components more easily recognizable than the HPLC–UV counter parts because of the apparent higher ionization efficiency of the minor isomers and increased resolution of subcomponents in the MS system. Under the mobile phase conditions employed, analyte ionization occurred with variable degrees of gas phase ammonolysis depending upon the ammonia concentration of the buffer. Potential applicability of the on-line HPLC–MS technique for the characterization of components in mixtures of antimycin analogues and isomers is demonstrated.

## INTRODUCTION

Antimycin A (an effective fish toxicant) is a mixture of closely related antibiotic substances produced by streptomyces fermentation [1]. This cluster of dilactonic compounds consists of at least ten components [2]. Of these, the five major homologues (a-isomers) and five minor homologues (b-isomers) are designated as a-subcomponents and b-subcomponents, respectively (Fig. 1). The presence of

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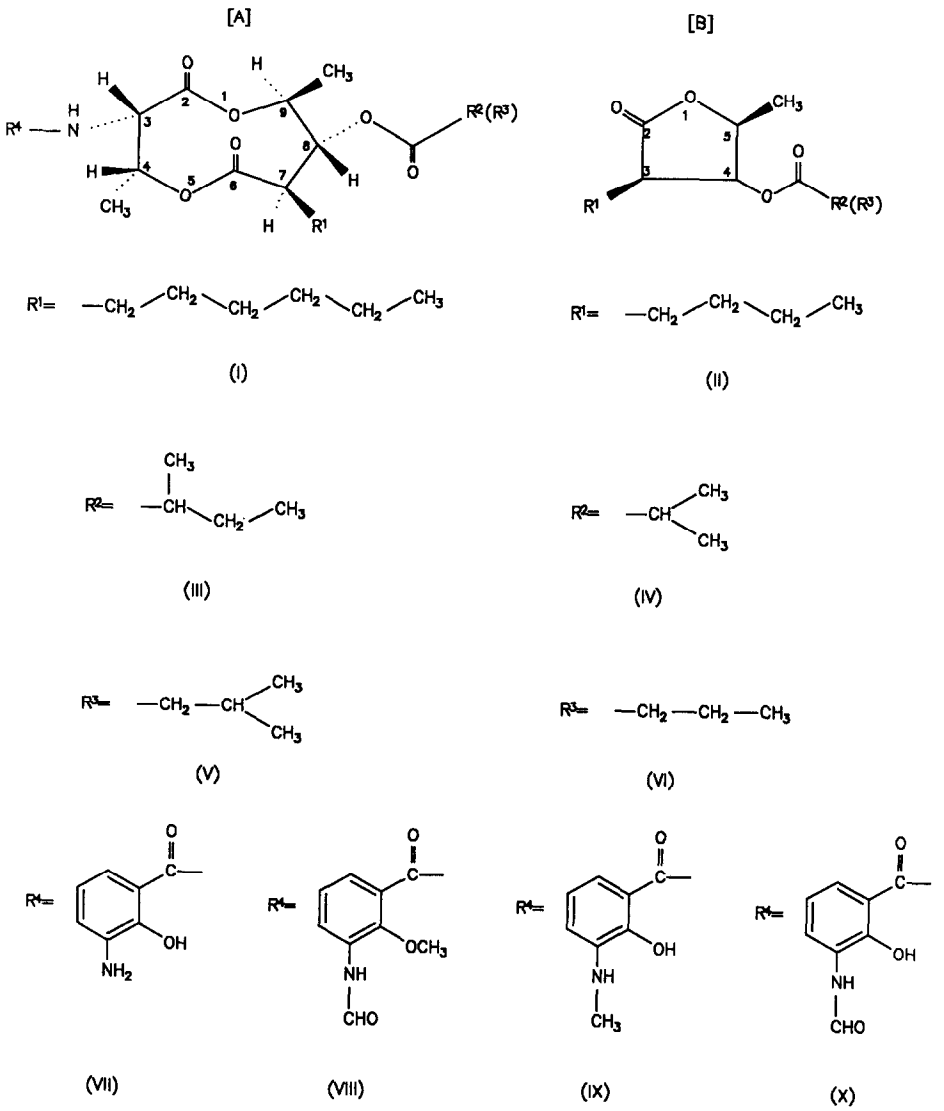



Fig. 1. Structures of (A) N-acylamino dilactones and (B) 4-butyrolactones (antimycin lactones). Subcomponents: 1a, $R^1 = I$, $R^2 = III$; 1b, $R^1 = I$, $R^3 = V$; 2a, $R^1 = I$, $R^2 = IV$; 2b, $R^1 = I$, $R^3 = VI$; 3a, $R^1 = II$, $R^2 = III$; 3b, $R^1 = II$, $R^3 = V$; 4a, $R^1 = II$, $R^2 = IV$; 4b, $R^1 = II$, $R^3 = VI$. Antimycins (AT): $R^4 = X$; methylated antimycins (AT-OMe): $R^4 = VIII$; deformylated antimycins (AT-DF): $R^4 = VII$; 3′-N-methylantimycins (AT-NMe): $R^4 = IX$; debenzoylated antimycins (AT-DB): $R^4 = H$.

structurally isomeric $R^2$ and $R^3$ alkyls of the acyloxy side chains in the structures is responsible for the existence of the a–b forms of antimycins. Most recent nuclear magnetic resonance (NMR) studies [3,4] revealed that structures of each of the corresponding a–b subcomponent pairs $A_{1a}$–$A_{1b}$, $A_{2a}$–$A_{2b}$, $A_{3a}$–$A_{3b}$, and $A_{4a}$–$A_{4b}$ are isomers of the same molecular weight (Fig. 1).

Preparative-scale separations and isolations of antimycin have been cumbersome, lengthy, and laborious [5]. A pure material of antimycin $A_1$, $A_2$ $A_3$, or $A_4$ containing an inherent pair of a–b subcomponents has recently been marketed in the form of individual antimycin homologues [6], but these individually isolated compounds are too expensive to purchase in quantity for practical largescale applications. None of the antimycin subcomponents $A_{1a}$, $A_{1b}$, $A_{2a}$, $A_{2b}$, $A_{3a}$, $A_{3b}$, $A_{4a}$ and $A_{4b}$ has been separated nor been isolated on a commercial scale. If available, the isomerically homogeneous materials would be costlier than the four antimycin homologues $A_1$, $A_2$, $A_3$ and $A_4$. In the premise of severe supply shortage of single-component antibiotics, relatively more affordable mixtures of antimycin A complex have frequently been used at our laboratories in a number of organic preparations and biological investigations. Furthermore in fishery management, field workers in practice have routinely employed the toxicant formulations solely prepared from mixtures of antimycin A complex. In consequence, for studying the biological and environmental fates of antimycins, it is necessary that analytical efforts should be directed toward mixture analysis in lieu of single-component analysis.

There are several reactive sites in the antimycin molecule (Fig. 1). Metal hydride reduction of antimycin A complex yields a host of heterogeneous mixtures in the products whose compositions vary depending on reaction parameters. Unless the acyloxy side chains have not been cleaved, the products are typical mixtures of homologous complexes each of which should have the same number of subcomponents as in antimycins (Fig. 1). In a few of our earlier studies [2,7] involving the precursor antimycin A complex, it was found that diagnostic and qualitative product analyses of both known and unknown mixtures of products were most conveniently and effectively carried out by using a combined high-performance liquid chromatography–thermospray mass spectrometry (HPLC–MS) technique [8]. Direct probe chemical ionization (CI) and electron impact (EI) MS techniques [9] proved to be useful for antimycin mixture analysis. However, the direct probe methods suffer from certain limitations that require the use of strictly pure compounds of known structures. Due to thermal instability, the title dilactonic compounds are not amenable to gas chromatographic (GC) analysis. Analysis of unusually complicated samples containing multiple sets of complexes must be accomplished by HPLC–MS. This paper reports the HPLC–MS results of N-acylamino dilactonic compounds and 4-butyrolactones derived from antimycin A.

## EXPERIMENTAL

### *Chemicals and reagents*

Crude antimycin A was a gift from Aquabiotics (Bainbridge Island, WA, U.S.A.). Pure analytical standards of antimycin A were obtained from Sigma (St. Louis, MO, U.S.A.) or prepared from the crude materials following a published method [6]. For preparation of methylated antimycin (AT-OMe) having the methoxy group at the 2′-position, a previously described procedure [2] was modified for improving yields and product purity. Thus, a solution of antimycins in diethyl ether was stirred with an equivalent amount of diazomethane at 35°C for 30 min. Another equal portion of diazomethane was added to the reaction mixture. The mixture was stirred at 35°C for additional 30 min and left standing at room temperature overnight.

Mixtures of deformylated antimycins, 3′-aminoantimycins (AT-DF), were prepared by treating antimycins with either hot hydrochloric acid [1] or diisobutylaluminum hydride. The 3′-N-methyl analogues (AT-NMe) were prepared from antimycins by reaction with diborane. Detailed procedures for the above hydride reductions are described in a separated paper [10]. The 4-butyrolactone complex (AT-LCT) was readily prepared by mild alkaline hydrolysis of antimycins at room temperature [11].

Adequately clean samples of individual dilactone mixtures were often desirable for use as reference standards. In such cases, the crude reaction products were purified by thin-layer chromatography (TLC). According to sample sizes, the TLC experiments were performed on analytical or preparative silica gel plates (Analtech, Newark, DE, U.S.A.) using solvent systems as described previously [2,12].

All organic reagents were obtained from Aldrich (Milwaukee, WI, U.S.A.). Chromatography-grade buffer salts and solvents were the products of J. T. Baker (Phillipsburg, NJ, U.S.A.).

*High-performance liquid chromatography–thermospray mass spectrometry*

A Waters liquid chromatograph equipped with a Model 600 multi-solvent delivery system and a Model 490-MS detector was used. For obtaining optimal separation of components within reasonable retention times, mobile phases were prepared from variable proportions of methanol and buffered water (0.1 *M* ammonium acetate). The buffer solutions were adjusted to pH 5 with acetic acid. Depending on the type of compounds analyzed, HPLC eluents were pumped isocratically at a flow-rate of 0.5–2 ml/min or with gradient elution [2]. All samples were frozen in dry state prior to analysis. Analytical samples were dissolved in methanol and aliquots (50–150 μg) were injected into a HPLC column via a Waters U6K injector. Analyte components were first separated with a Hibar Superspher RP-18e column (25 cm × 4 mm, 3 μm, EM Science) or an Altex Ultrasphere ODS column (25 cm × 4.6 mm, 5 μm) and then analyzed with an on-line HPLC–thermospray mass spectrometer.

The chromatograph was coupled via a Vestec Model 45 (for analyis of AT-OMe) or Model 701C [for analysis of AT-DF and 8-hydroxy-AT-DF (AT-DF-80H)] thermospray interface to a Finnigan Model 4600 triple-stage quadrupole mass spectrometer. The models 45 and 701C HPLC–MS systems were operated in the positive ion mode with the "filament off" and "filament on", respectively. For the analysis of compounds in the other series, a Vestec Model 201 thermospray mass spectrometer was used and all spectra were obtained in the positive ion discharge mode. The MS temperatures were adjusted to optimum conditions suitable for different compound types: temperature ranges used for the probe tip, the vaporizer, and the ion block were 178–236, 238–269, and 268–300°C, respectively. The mass spectrometers were scanned from $m/z$ 150-700 in 2 s.

## RESULTS AND DISCUSSION

The investigated N-acylamino dilactone- and 4-butyrolactone complexes (Fig. 1) include AT-OMe, AT-DF, AT-NMe, AT-LCT, 8-hydroxy-N-methyl antimycin (AT-NMe-8OH), AT-DF-8OH and 8-hydroxydebenzoyl antimycin (AT-DB-8OH). As mentioned earlier, mixtures of closely related compounds were invariably obtained whenever the antimycin A complex was used as the starting material in organic

preparations. Most of the nine-membered dilactone compounds under study were found to be fairly short-lived reaction intermediates, especially in solution. In our experience, a solution of a given sample in methanol gradually decomposed within a few days at room temperature. Isolation of individual components from each complex would be extremely tedious and particularly impractical in view of the lability of the nine-membered dilactone structures. The compounds isolated could be the decomposition products, which would lead to misinterpretation of results. Therefore, direct and timely analysis of the dilactonic compounds by on-line HPLC–MS provided the most effective means not only for accurate assessment of various reaction courses dealing with unstable compounds, but also for structural confirmation and identification of products. In consideration of the potential application of the analytical data in biochemical and fishery research, HPLC–MS approaches to mixture analysis of antimycin degradation products are of biochemical and environmental significance.

Table I shows HPLC–MS data for the ten methylated antimycin components, AT-OMe ([A] in Fig. 1). After on-line HPLC separation, mass spectra of individual AT-OMe components in all cases exhibited protonated molecules along with fragment ions characteristic of the homologous series. Structures of some of the observed ions are given in Fig. 2 which generally depicts the four major fragment ions commonly

TABLE I

HPLC–MS DATA FOR METHYLATED ANTIMYCIN A COMPLEX (AT-OMe)

| Component[a] | *m/z* Value of observed ion and its relative abundance in parenthesis[b] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | [E] | [F]$-R^{2(3)}CO$ | [D]$-CO_2$ | [C] | [D] | [F]$-NH_3$ | [F] | M−CO | M+1 | M+$NH_4$ |
| $(AT\text{-}OMe)_{1a}$ | 195 | 218 | 235 | 261 | 279 | 285 | 302 | 535 | 563 | 580 |
| | (2.0) | (8.0) | (22) | (100) | (60) | (14) | (14) | (6.5) | (54) | (16) |
| $(AT\text{-}OMe)_{1b}$ | 195 | 218 | 235 | 261 | 279 | 285 | 302 | 535 | 563 | 580 |
| | (8.7) | (10) | (24) | (100) | (71) | (19) | (22) | (15) | (75) | (20) |
| $(AT\text{-}OMe)_{2a}$ | 195 | 218 | 235 | 261 | 279 | 271 | 288 | 521 | 549 | 566 |
| | (2.0) | (7.0) | (22) | (100) | (47) | (9.9) | (12) | (5.2) | (40) | (12) |
| $(AT\text{-}OMe)_{2b}$ | 195 | 218 | 235 | 261 | 279 | 271 | 288 | 521 | 549 | 566 |
| | (6.5) | (6.2) | (20) | (100) | (60) | (13) | (19) | (11) | (59) | (14) |
| $(AT\text{-}OMe)_{3a}$ | 195 | 190 | 235 | 261 | 279 | 257 | 274 | 507 | 535 | 552 |
| | (2.3) | (9.9) | (29) | (100) | (32) | (14) | (17) | (2.0) | (41) | (14) |
| $(AT\text{-}OMe)_{3b}$ | 195 | 190 | 235 | 261 | 279 | 257 | 274 | 507 | 535 | 552 |
| | (11) | (3.7) | (26) | (100) | (84) | (16) | (26) | (4.7) | (92) | (28) |
| $(AT\text{-}OMe)_{4a}$ | 195 | 190 | 235 | 261 | 279 | 243 | 260 | 493 | 521 | 538 |
| | (1.6) | (8.4) | (20) | (100) | (53) | (13) | (15) | (16) | (80) | (34) |
| $(AT\text{-}OMe)_{4b}$ | 195 | 190 | 235 | 261 | 279 | 243 | 260 | 493 | 521 | 538 |
| | (5.1) | (8.0) | (19) | (100) | (59) | (13) | (23) | (19) | (71) | (28) |
| $(AT\text{-}OMe)_{5a}$ | 195 | 190 | 235 | 261 | 279 | 229 | 246 | 479 | 507 | 524 |
| | (1.6) | (5.8) | (34) | (100) | (59) | (13) | (30) | (13) | (57) | (25) |
| $(AT\text{-}OMe)_{5b}$ | 195 | 190 | 235 | 261 | 279 | 229 | 246 | 479 | 507 | 524 |
| | (0.9) | (2.1) | (10) | (39) | (51) | (8.6) | (13) | (25) | (100) | (40) |

[a] For compound abbreviations, see Results and Discussion.

[b] For ion identification, see Figs. 1 and 2.

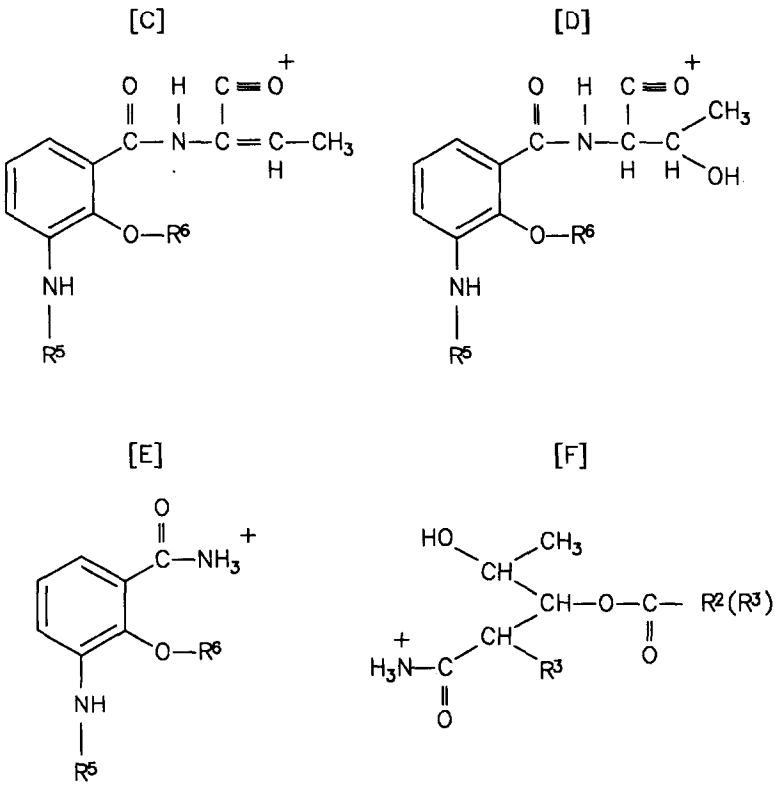



Fig. 2. Significant fragments observed in HPLC–MS of the N-acylamino dilactones: (A) methylated antimycins (AT-OMe), (B) deformylated antimycins (AT-DF), and (C) 3′-N-methylantimycins (AT-NMe).

observed in the mass spectra of the dilactonic compounds related to antimycin. With the exception of $(AT\text{-}OMe)_{5b}$, each homologue gave rise to a base peak at *m/z* 261 attributable to fragment [C] presumably produced by a concerted rearrangement leading to cleavage at the 1–2 and 5–6 bonds (Fig. 1) with hydrogen transfer to the esterified oxygen to yield initially ion [D], followed by the loss of water [9]. Other less abundant ions at *m/z* 279, *m/z* 235 ($[D]-CO_2$), and *m/z* 195 [E] were universally present in the spectra. It should be noted that these fragments devoid of $R^1$ and $R^{2(3)}$ groups have no bearing on the distinction of isomers of interest. In the spectra of the homologous series, there were six additional weaker, but diagnostically significant, peaks. These were attributed to $(M+NH_4)^+$, $(M+1)^+$, $(M-CO)^+$, $[F]^+$,

$([F]-NH_3)^+$, and $([F]-R^{2(3)}CO)^+$. All but the last of these ions contain the $R^1$ and $R^{2(3)}$ groups contributing to the observation of homologous mass ions. Analysis of the five sets of fragmentation data (Table I) confirmed the homologous relationships among the AT-OMe components. Because ammonium acetate was used in the HPLC mobile phase system, occurrence of gas phase ammonolysis [9] during positive ion reversed-phase HPLC–MS of methylated antimycins was evident. Fig. 2 shows possible structures of some adduct ions.

Comparisons of the HPLC–MS results (Table I) between a-components and b-components of AT-OMe indicated that mass spectra of these structural isomers with identical molecular weights exhibited virtually indistinguishable fragmentation patterns except for notable differences in their relative intensities of the corresponding ions. In general, HPLC–MS of the minor $(AT\text{-}OMe)_b$ compounds tended to give more abundant ions (including the protonated molecules) than the corresponding major a-isomers, $(AT\text{-}OMe)_a$ (Table I). Fig. 3 presents a typical reconstructed total ion current chromatogram and a mass chromatogram showing separation of a pair of a–b subcomponents.

The HPLC–MS results for the deformylated antimycin A complex, AT-DF([A] in Fig. 1), are summarized in Table II. The most abundant base peak was located at *m/z* 237 ([D], Fig. 2) in every spectrum of the ten components in the series. Besides the protonated molecule $(M+1)^+$ and the $([F]-NH_3)^+$ fragment, the two non-isomer-specific peaks at *m/z* 219 and 193 correspond respectively to [C] and $[D]-CO_2$ (Fig. 2). Also, several ammonia adducts [F], $[F]-R^{2(3)}CO$, $[F]-R^{2(3)}CO-H_2O$, and [E] were present in the spectra. The detection of ammonia adducts of moderate intensity is indicative of some degree of gas phase ammonolysis of the ions. Examples of reconstructed mass chromatograms of the a–b subcomponents $(AT\text{-}DF)_{1a}$ and $(AT\text{-}DF)_{1b}$ are shown in Fig. 4. Although the a–b subcomponents in the series showed partial resolution in HPLC–UV (not shown here), superior separations of the two subcomponents of identical mass were clearly demonstrated in analysis by HPLC–MS and reconstruction of appropriate mass chromatograms.

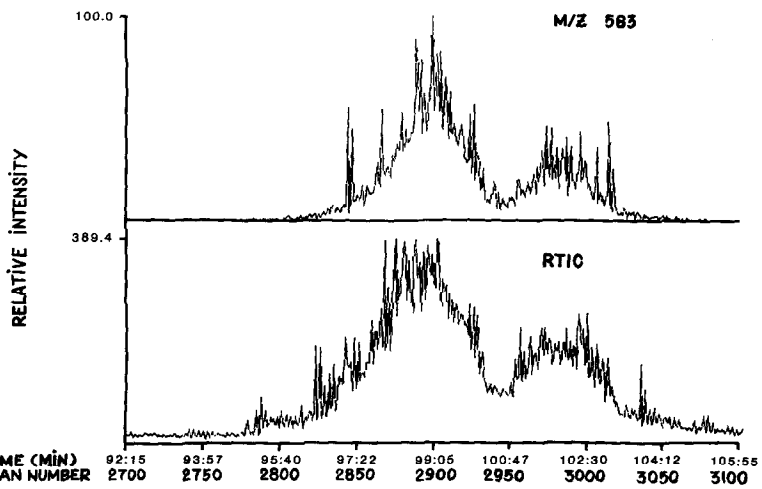



Fig. 3. HPLC–MS chromatograms of a selected pair of methylated antimycin subcomponents: $(AT\text{-}OMe)_{1a}$, left; $(AT\text{-}OMe)_{1b}$, right. RTIC = Reconstructed total ion current chromatogram. Sample injected: 10 μg.

TABLE II

HPLC–MS DATA FOR THE DEFORMYLATED ANTIMYCIN A COMPLEX (AT-DF)

| Component[a] | *m/z* Value of observed ion and its relative abundance in parenthesis[b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | [E] | [D]$-CO_2$ | [F]$-R^{2(3)}CO-H_2O$ | [F]$-R^{2(3)}CO+1$ | [C] | [D] | [F]$-NH_3$ | [F] | M+1 |
| $(AT\text{-}DF)_{1a}$ | 153 | 193 | 200 | 218 | 219 | 237 | 285 | 302 | 521 |
| | (34) | (26) | (9.8) | (12) | (72) | (100) | (21) | (55) | (49) |
| $(AT\text{-}DF)_{1b}$ | 153 | 193 | 200 | 218 | 219 | 237 | 285 | 302 | 521 |
| | (47) | (36) | (12) | (10) | (94) | (100) | (24) | (64) | (50) |
| $(AT\text{-}DF)_{2a}$ | 153 | 193 | 200 | 218 | 219 | 237 | 271 | 288 | 507 |
| | (23) | (20) | (6.6) | (9.7) | (59) | (100) | (19) | (41) | (45) |
| $(AT\text{-}DF)_{2b}$ | 153 | 193 | 200 | 218 | 219 | 237 | 271 | 288 | 507 |
| | (35) | (29) | (8.9) | (14) | (63) | (100) | (20) | (50) | |
| $(AT\text{-}DF)_{3a}$ | 153 | 193 | 172 | 190 | 219 | 237 | 257 | 274 | 493 |
| | (39) | (28) | (13) | (16) | (75) | (100) | (23) | (58) | (52) |
| $(AT\text{-}DF)_{3b}$ | 153 | 193 | 172 | 190 | 219 | 237 | 257 | 274 | 493 |
| | (41) | (26) | (18) | (15) | (76) | (100) | (29) | (60) | (68) |
| $(AT\text{-}DF)_{4a}$ | 153 | 193 | 172 | 190 | 219 | 237 | 243 | 260 | 479 |
| | (19) | (25) | (10) | (11) | (60) | (100) | (20) | (50) | (45) |
| $(AT\text{-}DF)_{4b}$ | 153 | 193 | 172 | 190 | 219 | 237 | 243 | 260 | 479 |
| | (26) | (26) | (13) | (12) | (67) | (100) | (21) | (55) | (50) |
| $(AT\text{-}DF)_{5a}$ | 153 | 193 | 172 | 190 | 219 | 237 | 229 | 246 | 465 |
| | (20) | (29) | (8.3) | (9.3) | (65) | (100) | (23) | (48) | (49) |
| $(AT\text{-}DF)_{5b}$ | 153 | 193 | 172 | 190 | 219 | 237 | 229 | 246 | 465 |
| | (35) | (25) | (8.9) | (10) | (71) | (100) | (26) | (53) | (55) |

[a] For compound abbreviations, see Results and Discussion.

[b] For ion identification, see Figs. 1 and 2.

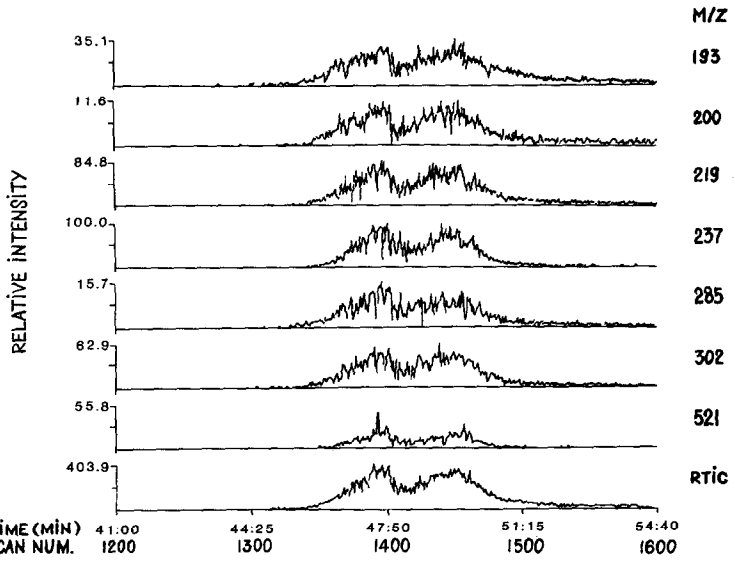



Fig. 4. HPLC–MS chromatograms of a selected pair of deformylated antimycin subcomponents: $(AT\text{-}DF)_{1a}$ (left) and $(AT\text{-}DF)_{1b}$ (right). Sample injected: 10 $\mu$g.

TABLE III

HPLC–MS DATA FOR THE N-METHYLATED ANTIMYCIN A COMPLEX (AT-NMe)

| Component[a] | *m/z* Value of observed ion and its relative abundance in parenthesis[b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | [E]−$CH_3$ | [E] | [D]−$CO_2$ | [C] | [D] | [F]−$NH_3$ | [F] | M+1 |
| $(AT\text{-}NMe)_{1a}$ | 153 | 167 | 207 | 233 | 251 | 285 | 302 | 535 |
| | (16) | (81) | (18) | (75) | (95) | (69) | (97) | (100) |
| $(AT\text{-}NMe)_{1b}$ | 153 | 167 | 207 | 233 | 251 | 285 | 302 | 535 |
| | (18) | (90) | (19) | (77) | (97) | (74) | (95) | (100) |
| $(AT\text{-}NMe)_{2a}$ | 153 | 167 | 207 | 233 | 251 | 271 | 288 | 521 |
| | (21) | (98) | (13) | (63) | (93) | (76) | (68) | (100) |
| $(AT\text{-}NMe)_{2b}$ | 153 | 167 | 207 | 233 | 251 | 271 | 288 | 521 |
| | (25) | (98) | (15) | (66) | (93) | (80) | (73) | (100) |
| $(AT\text{-}NMe)_{3a}$ | 153 | 167 | 207 | 233 | 251 | 257 | 274 | 507 |
| | (12) | (58) | (15) | (60) | (97) | (78) | (45) | (100) |
| $(AT\text{-}NMe)_{3b}$ | 153 | 167 | 207 | 233 | 251 | 257 | 274 | 507 |
| | (15) | (62) | (16) | (69) | (96) | (79) | (53) | (100) |
| $(AT\text{-}NMe)_{4a}$ | 153 | 167 | 207 | 233 | 251 | 243 | 260 | 493 |
| | (36) | (94) | (15) | (80) | (93) | (35) | (20) | (100) |
| $(AT\text{-}NMe)_{4b}$ | 153 | 167 | 207 | 233 | 251 | 243 | 260 | 493 |
| | (23) | (89) | (19) | (77) | (95) | (58) | (53) | (100) |
| $(AT\text{-}NMe)_{5a}$ | 153 | 167 | 207 | 233 | 251 | 229 | 246 | 479 |
| | (23) | (58) | (12) | (58) | (83) | (98) | (14) | (100) |
| $(AT\text{-}NMe)_{5b}$ | 153 | 167 | 207 | 233 | 251 | 229 | 246 | 479 |
| | (10) | (83) | (13) | (71) | (90) | (87) | (33) | (100) |

[a] For compound abbreviations, see Results and Discussion.
[b] For ion identification, see Figs. 1 and 2.

TABLE IV

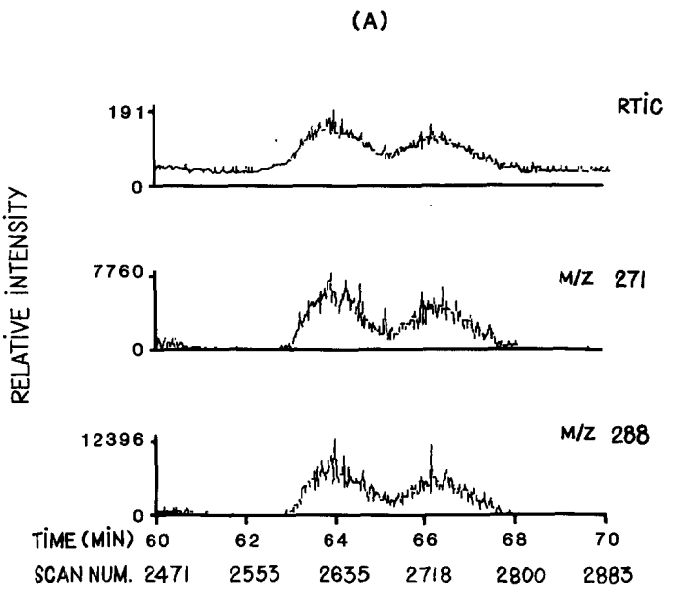
HPLC–MS DATA FOR THE ANTIMYCIN LACTONE COMPLEX [4-ACYLOXY-3-ALKYL-5-METHYL-γ-BUTYROLACTONE (AT-LCT)]

| Component[a] | *m/z* Value of observed ion and its relative abundance in parenthesis[b] | | | | |
|---|---|---|---|---|---|
| | $M-R^{2(3)}CO+1$ | $M+1$ | $M+NH_4-1$ | $M+NH_4$ | $M+NH_4+1$ |
| $(AT\text{-}LCT)_{1a}$ | 200 (20) | 285 (38) | 301 (23) | 302 (100) | 303 (15) |
| $(AT\text{-}LCT)_{1b}$ | 200 (22) | 285 (49) | 301 (21) | 302 (100) | 303 (17) |
| $(AT\text{-}LCT)_{2a}$ | 200 (16) | 271 (32) | 287 (25) | 288 (100) | 289 (16) |
| $(AT\text{-}LCT)_{2b}$ | 200 (20) | 271 (33) | 287 (27) | 288 (100) | 289 (16) |
| $(AT\text{-}LCT)_{3a}$ | 172 (25) | 257 (61) | 273 (35) | 274 (100) | 275 (22) |
| $(AT\text{-}LCT)_{3b}$ | 172 (26) | 257 (69) | 273 (40) | 274 (100) | 275 (20) |
| $(AT\text{-}LCT)_{4a}$ | 172 (13) | 243 (40) | 259 (29) | 260 (100) | 261 (17) |
| $(AT\text{-}LCT)_{4b}$ | 172 (14) | 243 (45) | 259 (30) | 260 (100) | 261 (19) |

[a] For compound abbreviations, see Results and Discussion.
[b] For ion identification, see Figs. 1 and 2.

Table III shows the HPLC–MS data for the 3′-N-methyl-analogues of antimycin A complex, AT-NMe ([A] in Fig. 1). In the spectra of the homologous series, the most intense ion was the protonated molecule $(M+1)^+$ under the HPLC–MS conditions employed. It was noteworthy that no detectible ions were produced by deacylation. This mode of fragmentation was significant in the case of the deformylated compounds (described in the preceding paragraph) differing merely at the 3′-N-substituents from the compounds in the present case. By careful examination of fragmentation patterns and protonated molecules, we were able to determine for the first time structures of the hitherto unknown AT-NMe compounds present in the crude reaction products. The structures were later confirmed by $^1H$ and $^{13}C$ NMR spectrometry [10].

4-Butyrolactone complex ([B] in Fig. 1), known as antimycin lactone (AT-LCT), is a mixture of the primary degradation products of antimycin A in mildly alkaline media. Since the acyloxy side-chains of the lactone products have been established to be identical with those of parent antimycins [10], it was deemed logical to include these environmentally important lactones in this study. The HPLC–MS results for the compounds in this series are presented in Table IV. Interestingly, the base peaks were the ammonia adducts of the intact molecules, $(M+NH_4)^+$, which is illustrative of the molecular stability of the five-membered ring systems in 4-butyrolactones under the ionization conditions. In contrast, the base peaks in the spectra of the dilactones were ascribed to various ions other than the ammonia adducts of the intact molecules. As



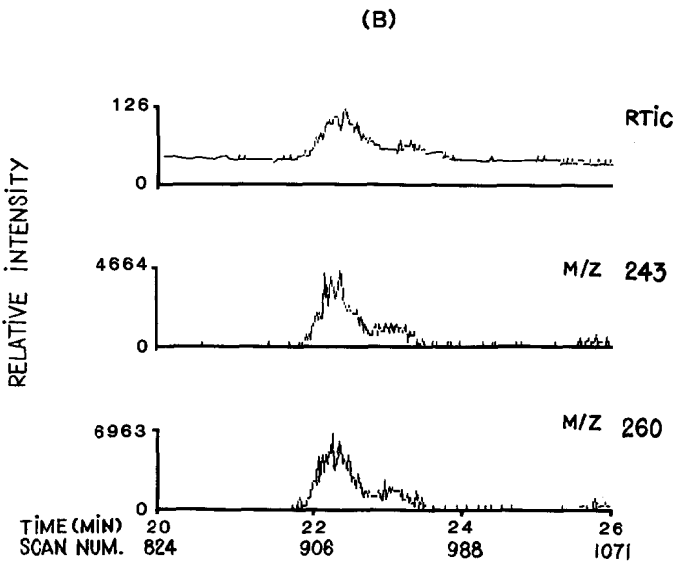




Fig. 5. HPLC–MS chromatograms of minor antimycin lactone homologues (AT-LCT)$_2$ and (AT-LCT)$_4$ with subcomponents: (A) (AT-LCT)$_{2a}$ (left) and (AT-LCT)$_{2b}$ (right), sample injected: 10 $\mu$g, and (B) (AT-LCT)$_{4a}$ (left) and (AT-LCT)$_{4b}$ (right). These subcomponents correspond to the 2a, 2b, 4a, and 4b peaks in Fig. 6. Each sample injected: 10 $\mu$g.

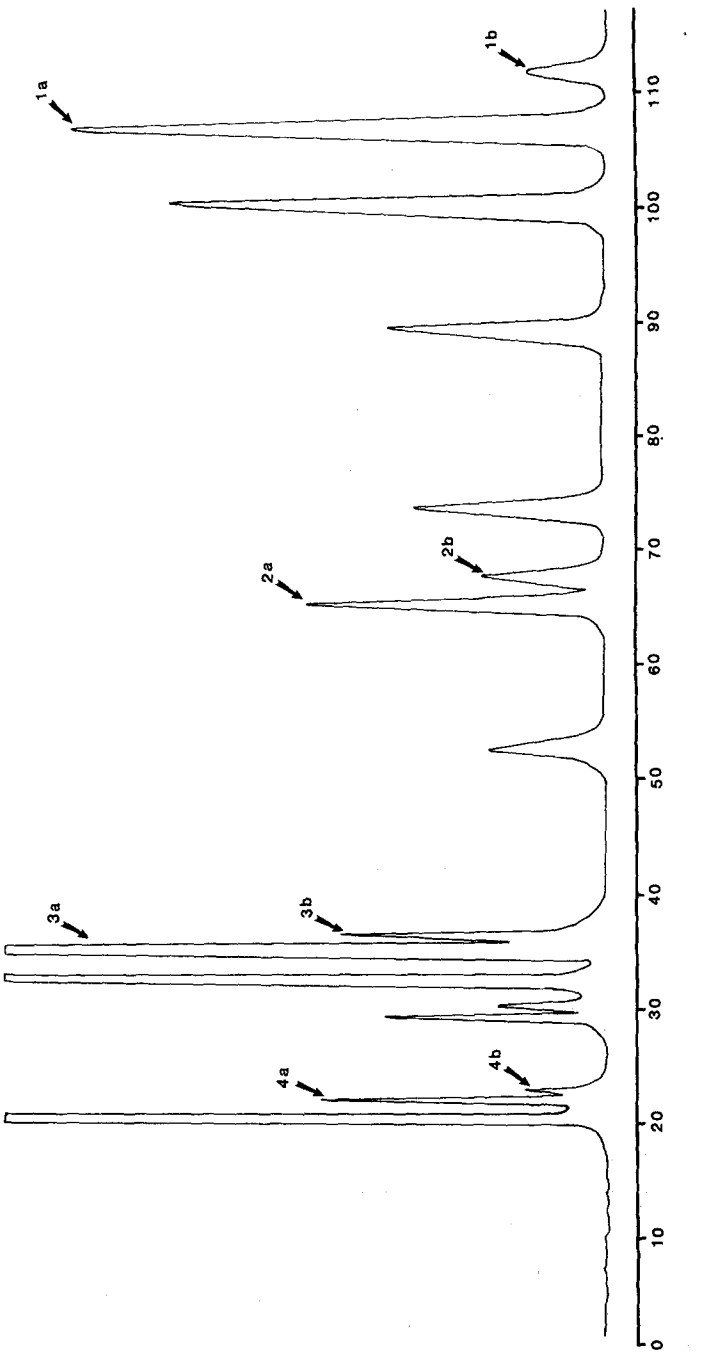



Fig. 6. HPLC–UV chromatogram of a crude sample of antimycin lactones. Sample injected: 80 μg. The chromatogram was obtained under HPLC conditions identical with those in Fig. 5.

compared with the spectral data for the dilactones described in the foregoing paragraphs fewer ions were generated by HPLC–MS of homologues in the 4-butyrolactone series. Nonetheless, the specific spectral features for the latter lactones were informative and sufficed for structure identification. In the absence of other detectible ions, the major fragmentation pathway for AT-LCT compounds seemed to proceed via a deacylation process leading to the $(M-R^{2(3)}CO+1)^+$ ion. However, under the experimental conditions employed, there were no fragments formed by alkyl scissions normally observed for some simple lactones [13–16]. Nearly identical spectra were obtained with thermospray MS in the "filament on" mode or in the "discharge" mode as the mechanisms of ion production are identical. Fig. 5 presents HPLC–MS chromatography tracings showing separations of a–b subcomponents in minor homologues $(AT\text{-}LCT)_2$ and $(AT\text{-}LCT)_4$. Without mass spectral data, it was very difficult, if not impossible, to identify these components in the crude degradation products by HPLC–UV (Fig. 6). The latter technique lacks the auxiliary spectral information as provided by HPLC–MS. It is postulated that the vacuum system from the mass spectrometer causes a pressure drop at the end of the LC column resulting in increased resolution. This phenomenon is very observable in GC–MS where the end of the capillary GC column is at the entrance of the ion source, and a larger pressure drop occurs than when the end of the GC column is at ambient pressure. This phenomenon could also occur in HPLC–MS. This may explain why the a–b subcomponents were somewhat better resolved in HPLC–MS than in HPLC–UV. In addition, it was apparent that the minor b-isomers in some cases showed more intense chromatographic peaks in HPLC–MS than HPLC–UV because of the apparent high ionization efficiency of the minor isomers in the former system.

The HPLC–MS data for three different types of 8-hydroxy homologues are shown in Table V. The mixture in each type consists of two homologues that differ in molecular weight by 28 u due to the difference in *n*-butyl and *n*-hexyl of the 7-$R^1$ alkyl group. Fragmentation of $(AT\text{-}NMe\text{-}8OH)_{1,2}$ under the HPLC–MS conditions employed, yielded a base peak at *m/z* 302 $[(M-ArCO+1)^+$, Fig. 1], whereas under identical conditions the most intense peak for the 7-butyl analogue, $(AT\text{-}NMe\text{-}8OH)_{3,4}$, was found at *m/z* 172 $([F]-R^{2(3)}CO-H_2O+1$, Fig. 2). While spectral characteristics of homologues in other series were alike and the observed most abundant ions corresponded to the same fragment or $(M+1)^+$ ions in the homologous series, it is unclear why HPLC–MS of the two homologues of AT-NMe-8OH led to base peaks of different fragments shown above. The HPLC–MS spectra of deformyldeacylantimycins, AT-DF-8OH, exhibited fragmentation patterns similar to those of the corresponding N-methyl analogues, except that the dearylcarbonylation process was apparently unimportant in the ionization of the AT-DF-8OH compounds. The $(M+1)^+$ ions of both $(AT\text{-}DF\text{-}8OH)_{1,2}$ and $(AT\text{-}DF\text{-}8OH)_{3,4}$ were responsible for the base peaks observed. Fig. 7 shows a reconstructed total ion current chromatogram and mass chromatograms from the analysis of $(AT\text{-}DF\text{-}8OH)_{1,2}$ which was one of the many ill-resolved, early eluting (first 5 min) components in the crude samples. For unsubstituted aminodilactones (Fig. 1), $(AT\text{-}DB\text{-}8OH)_{1,2}$ and $(AT\text{-}DB\text{-}8OH)_{3,4}$, thermospray ionization of these 3-amino-dilactones afforded the base peaks at $(M+1)^+$ attributable to the protonated molecules. As seen in Table V, relatively simple spectral data for the debenzoylated compounds (AT-DB-8OH) were obtained under the conditions used, though these compounds are the least stable compounds of all types investigated.

TABLE V

HPLC–MS DATA DESACYLATED ANTIMYCIN COMPOUNDS AT-NMe-8OH, AT-DF-8OH AND AT-DB-8OH

| Component[a] | *m/z* Value of observed ion and its relative abundance in parenthesis[b] | | | | |
|---|---|---|---|---|---|
| | [E] | [D]−ArCO | [F]−$R^{2(3)}CO-H_2O+1$ | [D]−$CO_2$ | [F]−$R^{2(3)}CO+1-NH_3$ |
| $(AT\text{-}NMe\text{-}8OH)_{1,2}$ | 167 (66) | | 200 (72) | 207 (72) | |
| $(AT\text{-}NMe\text{-}8OH)_{3,4}$ | 167 (82) | | 172 (100) | 207 (48) | |
| $(AT\text{-}DF\text{-}8OH)_{1,2}$ | 153 (16) | | 200 (21) | 193 (16) | |
| $(AT\text{-}DF\text{-}8OH)_{3,4}$ | 153 (25) | | 172 (45) | 193 (10) | |
| $(AT\text{-}DB\text{-}8OH)_{1,2}$ | | 102 (19) | | | 201 (8.6) |
| $(AT\text{-}DB\text{-}8OH)_{3,4}$ | | 102 (18) | | | 173 (11) |

[a] For compound abbreviations, see Results and Discussion.
[b] For ion identification, see Figs. 1 and 2. Ar = aryl group.

In general, reversed-phase HPLC–MS results of the title dilactones can be interpreted partly based on gas phase ammonolysis [9] as corroborated by the observation of ammonia-containing fragments [E] and [F] in all cases (Fig. 2), and the adduct $[M+NH_4]^+$ in some cases. The occurrence of equimass subcomponents in

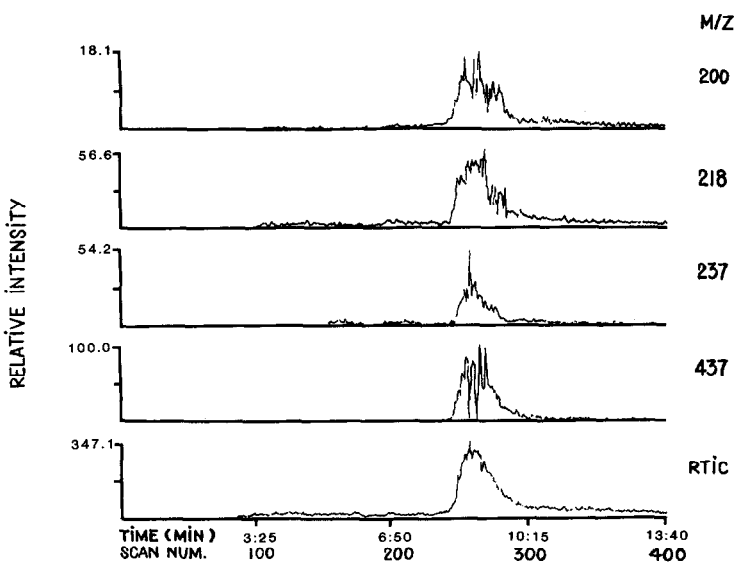



Fig. 7. HPLC–MS chromatograms of deformyldeacylantimycin $(AT\text{-}DF\text{-}8OH)_{1,2}$. Sample injected: 10 μg.

| [F]$-R^{2(3)}CO+1$ | [C] | [D] | $M-ArCO-NH_3+1$ | $M-ArCO+1$ | $M+1$ |
|---|---|---|---|---|---|
| 218 | 233 | 251 | 285 | 302 | 451 |
| (43) | 15) | (12) | (79) | (100) | (13) |
| 190 | 233 | 251 | 257 | 274 | 423 |
| (74) | (33) | (25) | (19) | (34) | (27) |
| 218 | 219 | 237 | | | 437 |
| (77) | (33) | (28) | | | (100) |
| 190 | 219 | 237 | | | 409 |
| (80) | (43) | (33) | | | (100) |
| 218 | | | | | 302 |
| (13) | | | | | (100) |
| 190 | | | | | 274 |
| (16) | | | | | (100) |

lactones having 8-acyloxy side-chains has been unequivocally established and the results were consistent with those reported by NMR assignments [3,4]. The potential analytical utility of the HPLC–MS technique for qualitative analysis of the complex lactone mixtures is demonstrated notwithstanding the noise spikes observed in many cases (Figs. 3, 4, 5 and 7) presumably caused by the unavoidable fluctuation in HPLC pumping systems [17]. This technique is not appropriate for trace analysis due to low sensitivity. The method may be applicable to the quantification of mixtures of antimycin conjugates at sufficient concentrations in biochemical systems. The on-line HPLC–MS technique provides a unique means to direct mixture analysis of enriched samples from tissues and environmental waters treated with antimycin A complex. Logarithmic peak pattern recognition of HPLC chromatograms combined with the mass spectral information facilitate analysis of antimycin residues and identification of complex degradation products. The method reported here has proven to be the sole instrumental technique for the characterization of new dilactonic products obtained from reaction of antimycin A complex with various reagents. Further, diagnostic product analysis of mixtures of unknown derivatives of antimycin A complex can be carried most effectively by HPLC–MS.

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REFERENCES

1 E. E. Van Tamelen, J. P. Dickie, M. E. Loumans, R. S. Dewey and F. M. Strong, *J. Am. Chem. Soc.*, 83 (1961) 1639; and references cited therein.
2 S. L. Abidi, *J. Chromatogr.*, 447 (1988) 595.
3 S. L. Abidi and B. R. Adams, *Magnet. Reson. Chem.*, 25 (1988) 1078.

4 S. L. Abidi, T. K. Ha and C. L. Wilkins, *Anal. Chem.*, 61 (1989) 404.
5 S. L. Abidi and G. Mack, presented at the *39th Pittsburgh Conference and Analytical Chemistry and Applied Spectroscopy, New Orleans, LA, February 22–27, 1987.*
6 S. L. Abidi, *J. Chromatogr.*, 234 (1982) 187.
7 S. L. Abidi and S. Callister, unpublished results.
8 M. L. Vestal, *Science (Washington, D.C.)*, 226 (1984) 275.
9 K. D. Haegele and D. M. Desiderio, Jr., *J. Org. Chem.*, 39 (1974) 1078; and references cited therein.
10 S. L. Abidi and R. T. Rosen, *J. Org. Chem.*, submitted for publication.
11 G. M. Tener, F. M. Bumpus, B. R. Dunshee and F. M. Strong, *J. Am. Chem. Soc.*, 75 (1953) 1100.
12 S. L. Abidi, *J. Chromatogr.*, 464 (1989) 453.
13 L. Friedman and F. L. Long, *J. Am. Chem. Soc.*, 75 (1953) 2832.
14 W. H. McFadden, E. A. Day and M. J. Diamond, *Anal. Chem.*, 37 (1965) 89.
15 E. Honkanen, T. Moisio and P. Karvonen, *Acta Chem. Scand.*, 19 (1965) 370.
16 B. J. Millard, *Org. Mass. Spectrom.*, 1 (1968) 279.
17 M. L. Vestal, Vestec Corp., Houston, TX, personal communication.