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Isolation of decomposition products of tylosin using liquid chromatography

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Abstract

Decomposition products of tylosin A were isolated using open column chromatography and preparative liquid chromatography. Two decomposition products, formed in slightly alkaline medium, were identified as epimers of tylosin A aldol, one of which has been described previously. Another decomposition product was formed on exposure of a tylosin A solution to light. Isomerization of the double bond between C_{12}–C_{13} takes place, resulting in the formation of the hitherto unreported isotylosin A.

1. Introduction

Tylosin, a 16-membered ring macrolide antibiotic, produced by fermentation of Streptomyces strains, was first described by McGuire et al. [1]. It is a mixture of a number of structurally related antibiotics of which tylosin A is the main component. Commercial samples of tylosin have been shown to contain variable amounts of related substances in addition to tylosin A, such as tylosin B or desmycosin [2], tylosin C or macrocin [3], tylosin D or relomycin [4], demycinosyltylosin [5], 5-O-mycaminosyltylonolide (OMT) [6] and lactenocin [3]. Structures of these compounds are shown in Fig. 1. A large number of other 16-membered macrolide antibiotics related to tylosin have been isolated [7] or synthesized by chemical transformation [8].

During the analysis by liquid chromatography of tylosin containing solutions, a substance called tylosin A aldol (TAD) was separated [9]. Aldol condensation products, obtained from different tylosins in slightly alkaline medium, have been described [10]. By solid-state thermal degradation rosaramicin, another macrolide antibiotic, is also transformed into an aldol structure [11]. In all these reports no mention was made of the stereochemistry of the new asymmetric centre. The formation of aldol condensation was also observed during the study of the stability of tylosin A in aqueous alkaline solutions [12]. Fig. 2 shows the formation of TAD in function of time in the pH range 7.0–11.0. During the purification of TAD, described in this paper, two epimers were isolated, one of which corresponds to that described previously [10].

Recently, the formation of another decomposition product was observed in aqueous solutions

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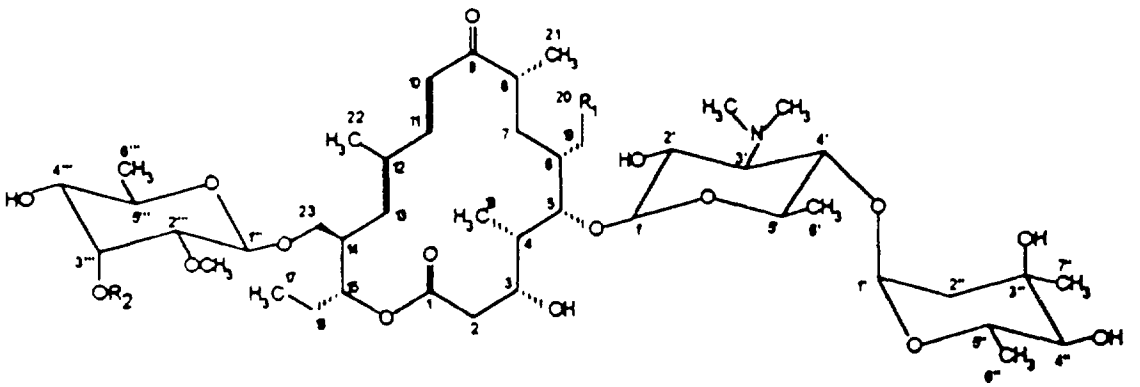

Mycinose Mycaminose Mycarose

	R1	R2	Mycarose	Mycinose
Tylosin A	CHO	CH_3	+	+
Tylosin B	CHO	CH_3	-	+
Tylosin C	CHO	H	+	+
Tylosin D	CH_2OH	CH_3	+	+
Lactenocin	CHO	H	-	+
OMT	CHO		-	-
DMT	CHO		+	-

+ = sugar present

- = sugar not present

Fig. 1. Structures of tylosin A and related substances.

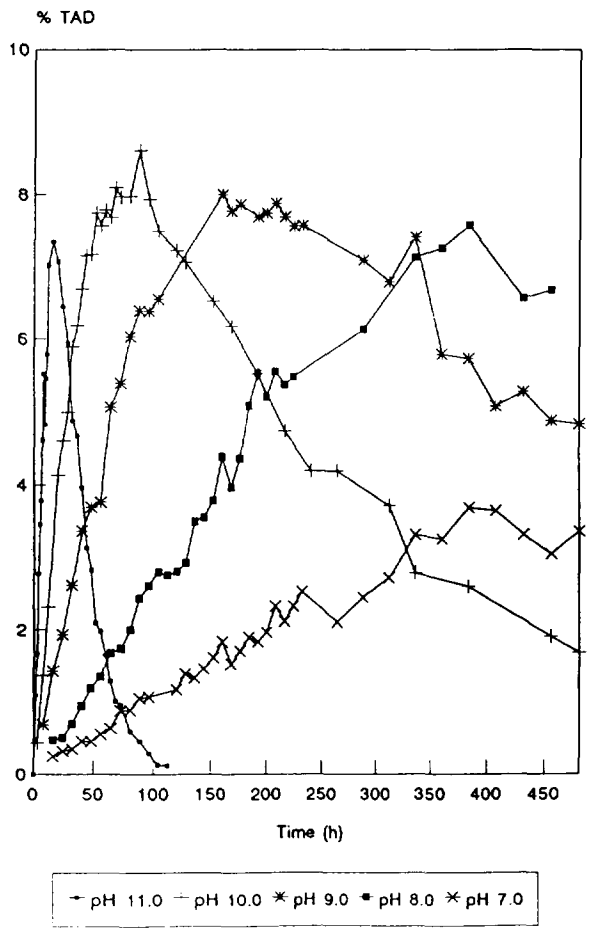

Fig. 2. Formation of tylosin aldol in function of time at different pH values (pH 7–11). Solutions containing 1.0 mg tylosin A/ml in 0.05 *M* potassium phosphate buffers pH 7.0–11.0 were used.

of tylosin, when these solutions were exposed to daylight. Here the preparation, purification and identification is described of this photochemical reaction product, which is called isotylosin A (isoTA).

2. Experimental

2.1. Sample, solvents and reagents

Commercial-grade tylosin base was obtained from Eli Lilly (Indianapolis, IN, USA). 2-Methoxy-2-methylpropane or *tert.*-butyl methyl ether (BME), diethylamine, triethylamine and dichloromethane were purchased from Janssen Chimica (Beerse, Belgium). LC-grade tetrahydrofuran was obtained from Rathburn (Walkerburn, UK). Water was distilled twice. All other chemicals were of analytical-reagent grade from Janssen Chimica.

2.2. Preparative and analytical chromatographic methods

Open column chromatography was performed using silica gel 60 H, 15 μm (Merck, Darmstadt, Germany) (80 g) packed in a glass column of 25 cm × 3.5 cm. Fines were removed from the silica gel by flotation in water. Thin-layer chromatography (TLC) using precoated silica gel plates (Kieselgel 60 F_{254}, Merck) and a mixture of BME and diethylamine (96:4 or 97:3) was used for purity control of the fractions obtained by open column chromatography. Spots were detected by spraying with a mixture of 4-methoxybenzaldehyde–ethanol–sulphuric acid (1:9:1) and heating at 105–110°C for 5 min.

The preparative liquid chromatographic (LC) system used for the purification of TAD consisted of a 25 cm × 1.25 cm O.D. stainless-steel column packed with silica gel 60 H, 15 μm (Merck) from which the fines were removed as mentioned above, a Milton Roy minipump (Laboratory Data Control, Riviera Beach, FL, USA), a Model CV-6-UHPa-N60 manual injector (Valco, Houston, TX, USA) equipped with a fixed loop of 0.5 ml, a Model 150 UV detector set at 280 nm (Altex, Berkeley, CA, USA) and a BD 40 recorder (Kipp en Zonen, Delft, Netherlands). The mobile phase consisted of a mixture BME–diethylamine (97:3) and was delivered at a flow-rate of 5 ml/min. For the purification of isoTA, a similar preparative LC system was used, with a column of 25 cm × 2.5 cm O.D., a mobile phase BME–diethylamine (98:2) at a flow-rate of 7 ml/min and detection at 254 nm.

An analytical LC method employing a polymeric stationary phase, described in a companion paper [13], was used to determine the composition of the different fractions obtained after preparative LC. NMR spectra were re-

corded in C^2HCl_3 solution using a Jeol FX90Q spectrometer (Tokyo, Japan) with tetramethylsilane as internal standard.

2.3. Preparation of crude TAD

A 5.0-g amount of tylosin base was dissolved in a mixture of ethanol–water–triethylamine (250:250:2) and stored, protected from light, at a temperature of 50°C for 12 days. The reaction mixture was evaporated under reduced pressure.

2.4. Preparation of crude isoTA

A 5.0-g amount of tylosin base was dissolved in 500 ml of BME and stored in a glass erlenmeyer flask in daylight for 4 weeks. The solution was filtered and evaporated under reduced pressure.

3. Results and discussion

3.1. Purification of TAD

The TAD content in the crude mixture was 28%, as determined by LC. Open column chromatography of the crude product was performed using an aliquot of 2.5 g, dissolved in 10 ml of dichloromethane and BME–diethylamine (96:4) as the mobile phase. Fractions containing TAD, with $R_F = 0.10$ in TLC, were combined and evaporated under reduced pressure. The residue was dissolved in BME (25 mg/ml) and 0.5-ml aliquots were purified on the preparative LC system. The fractions which contained mainly TAD were analysed separately and combined according to their TAD content, resulting in: fraction H1 (>90% TAD), fraction H2 (80–90% TAD) and fraction H3 (<80% TAD). Fraction H3 was further purified using the same LC system to contain 87.8% TAD and 7.1% TA. Further attempts of purification did not result in an increase of the TAD content. In an analogous way but using preparative LC with BME–diethylamine (99.5:0.5) as the mobile phase, a minor substance (TAD 2) was also isolated. TAD 2 was eluted in the preparative LC system before the major compound, now called TAD 1. Fig. 3 shows an analytical chromatogram of TAD 1. The position of TAD 2 is also indicated.

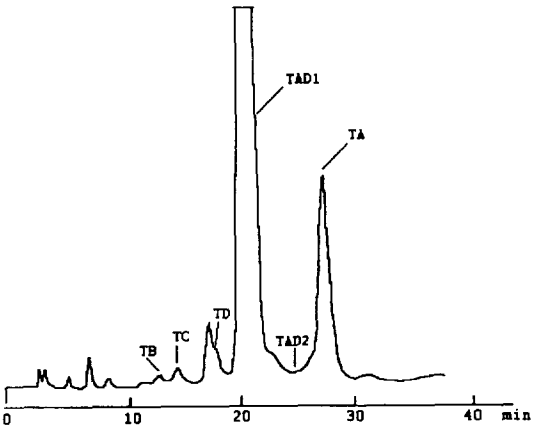

Fig. 3. Analytical LC chromatogram of the purified tylosin A aldol. TAD 1 = tylosin A aldol 20-R epimer, TAD 2 = tylosin A aldol 20-S epimer, TA, TB, TC, TD = tylosin A, B, C, D.

3.2. Characterization of TAD

The structure of the purified aldol condensation products TAD 1 and TAD 2 was elucidated by ^{13}C NMR. Table 1 reports the chemical shifts in C^2HCl_3 solution for TA, TAD 1 and TAD 2. Chemical shifts for TA and TAD 1 correspond very well with those reported previously [10].

The shifts for TAD 2 are very similar to those of TAD 1, except for C-5, C-6, C-7, C-8, C-9, C-19, C-20 and C-21. The substantial downfield shift for C-9 for TAD 2 points to the presence of a hydrogen bridge between the C-9 carbonyl and the C-20 OH, which is, as can be seen on a model, only likely when the hydroxy group is in the up-position. TAD 2 is therefore assigned the 20-*S*-configuration. In consequence, TAD 1 must be the 20-*R* epimer, in which the hydroxy group is in the down-position. An additional argument in favour of TAD 2 being the 20-*S* epimer, is the downfield shift for C-21 and C-5, which in the *S* epimer are in a less hindered *trans* relationship to the 20-OH group. It can thus be concluded that the major compound TAD 1 was the 20-*R*

Table 1
^{13}C NMR chemical shifts for tylosin A, two epimers of tylosin A aldol and isotylosin A

Carbon No.	Tylosin A[a]	TAD 1[b]	TAD 2[c]	Isotylosin A[d]
1	173.6 S	174.2 S	174.5 S	172.6/171.4
2	39.2 T	39.1 T	39.1 T	na
3	71.5 D	71.7 D	71.9 D	na
4	44.7 D	42.3 D	42.3 D	na
5	81.3 D	79.9 D	81.1 D	na
6	32.0 D	37.2 D	36.4 D	na
7	32.7 T	34.7 T	33.6 T	na
8	40.1 D	57.9 S	54.2 S	na
9	202.7 S	205.2 S	209.6 S	204.5/204.3
10	118.6 D	122.3 D	121.9 D	126.5
11	147.8 D	144.7 D	145.8 D	140.1/139.0
12	134.6 S	134.7 S	134.9 S	132.6
13	142.0 D	139.1 D	140.5 D	139.6
14	44.4 D	44.8 D	44.8 D	na
15	75.0 D	75.4 D	75.5 D	na
16	25.2	25.4 T	25.6 T	na
17	8.8 Q	9.3 Q	9.5 Q	na
18	9.4 Q	9.3 Q	9.5 Q	na
19	43.6 T	36.9 T	35.8 T	na
20	202.6 D	73.0 D	79.8 D	202.7
21	17.1 Q	17.2 Q	19.9 Q	na
22	12.7 Q	12.6 Q	12.7 Q	na
23	67.9 T	69.3 T	69.4 T	na
Mycaminose				
1′	103.6 D	103.9	103.6	103.5
2′	68.8 D	68.8	68.9	69.1
3′	68.6 D	67.4	67.6	68.8
4′	75.0 D	75.3	75.5	75.5
5′	73.0 D	73.1	73.0	72.8
6′	18.8 Q	18.9	19.1	19.0
3′-NMe_2	41.7 Q	41.8	41.8	41.8
Mycarose				
1″	96.3 D	96.4	96.6	96.1
2″	40.8 T	41.0	41.0	40.7
3″	69.2 S	69.3	69.4	69.2
4″	76.3 D	76.4	76.4	76.2
5″	65.8 D	65.9	66.0	65.8
6″	18.0 Q	18.0	18.2	18.1
7″	25.2 Q	25.2	25.4	25.2
Mycinose				
1‴	100.8 D	100.9	101.0	100.9
2‴	81.8 D	81.9	82.0	81.6
3‴	79.6 D	79.7	79.8	79.7
4‴	72.5 D	72.7	72.7	72.5
5‴	70.4 D	70.4	70.6	70.3
6‴	17.6 Q	17.6	17.7	17.5
2‴-OMe	59.4 Q	59.2	59.4	59.3
3‴-OMe	61.4 Q	61.3	61.5	61.5

S = Singlet; D = doublet; T = triplet; Q = quadruplet; multiplicities observed in the "single-frequency off-resonance (SFOR)"-decoupled spectrum; na = not assigned.
[a] Chemical shifts for tylosin A and TAD 1 correspond very well with those reported previously by Kennedy [10].
[b] Isolated using BME–diethylamine (96:4) as the mobile phase.
[c] Isolated using BME–diethylamine (99.5:0.5) as the mobile phase.
[d] Multiple peaks are observed for the aglycone part of isotylosin. Assignments are therefore not given.

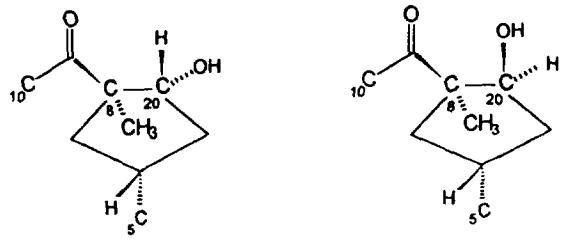

Fig. 4. Partial structures for the 20-*S* epimer and the 20-*R* epimer of tylosin A aldol.

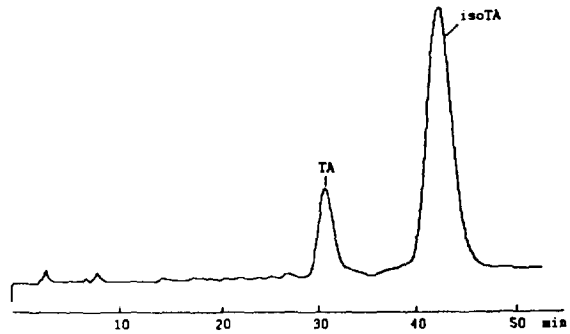

Fig. 5. Analytical LC chromatogram of the purified isotylosin A.

epimer. Fig. 4 shows partial structures of TAD 1 and TAD 2.

3.3. Purification of isoTA

The content of isoTA in the crude product was 14% as determined by analytical LC. The crude material was dissolved in dichloromethane (5 g/20 ml) and open column chromatography was performed using BME–diethylamine (97:3) as the mobile phase. Fractions containing the new compound with $R_F = 0.17$ in TLC (R_F TA = 0.20), were combined and evaporated under reduced pressure. The residue was redissolved in methanol and evaporated, yielding 1 g of material with following composition: 52% TA, 32% isoTA and 16% of other substances. This product was further purified on the preparative LC system (40 mg per injection). The eluate was collected in two fractions: the first contained mainly TA, the second (0.2 g) contained about 50% of isoTA. Three more preparative LC purifications were performed to obtain 80 mg of a product with 81% of isoTA and 17% of TA. As the content of isoTA did not change between the third and the fourth purification step, no further purification was performed. There apparently exists an equilibrium between TA and isoTA in solution. Fig. 5 shows a LC chromatogram of the isoTA fraction finally obtained.

3.4. Characterization of isoTA

For the conjugated double bond structure (C-10 to C-13) of the aglycone, theoretically four isomers can be written, as is shown in Fig. 6: tylosin A, the parent compound having a (*E*,*E*) configuration, and three possible isomers, isoTA (*E*,*Z*), isoTA′ (*Z*,*Z*) and isoTA″ (*Z*,*E*). A ^{13}C NMR spectrum of the purified isoTA was recorded in C^2HCl_3. Although the interpretation

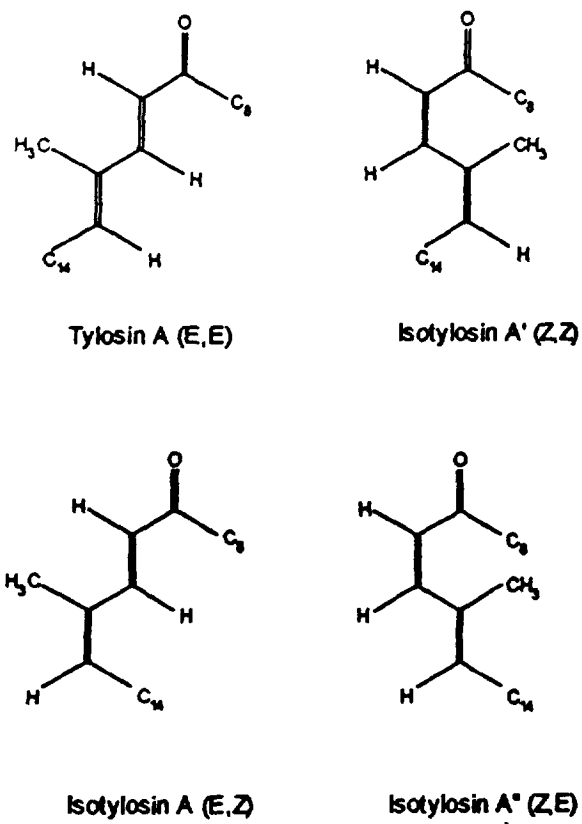

Fig. 6. Four possible isomers of the conjugated system in tylosin.

of the spectrum was not straightforward because some of the resonances were double (apparently due to the presence of a second isomer), all the sugar peaks could be clearly assigned (see Table 1), leaving the aglycone peaks at positions which did not differ much from those of TA. This observation, together with the shifts for the sp^2 carbons, pointed towards an isomerization of the double bond structure, with the macrocyclic lactone ring having almost the same conformation as in TA. Since, as can be seen in a model, the (*Z*,*E*) isomer, isoTA″, would have a clearly different conformation of the macrocyclic lactone ring, with expected concomitant large shifts of the carbon resonances, it is most improbable that the compound was the (*Z*,*E*) isomer. Based on the intensity of the two clearly separated carbonyl peaks (δ = 204.5 and 204.3 ppm), two lactone peaks (δ = 172.6 and 171.4 ppm) and two C-11 peaks (δ = 140.1 and 139.0 ppm), it was concluded that the two isomers, present in a 1:1 ratio, were (*E*,*Z*) and (*Z*,*Z*). When the isoTA fraction, isolated from BME, was dissolved in water and evaporated again, the ratio of the two isomers changed drastically to 95:5. Apparently the formation of only one isomer (aldehyde = 204.3, lactone = 171.4, C-11 = 139.0 ppm) is favoured in water. This explains why only one peak corresponding to isotylosin was observed in the aqueous conditions of the analytical LC chromatogram (Fig. 5). Analysis of the UV spectrum of this peak, using a diode array detector, indicated that the peak corresponded to a single product.

From the ^{13}C NMR spectrum it is not possible to determine which of these four isomers corresponds to the purified compound. However, from the ^{1}H NMR spectrum a coupling constant (J) between H-10 and H-11 of 15.4 Hz was found, which is identical to J_{10-11} in TA. This definitely points to a *trans* relationship of the hydrogens of this double bond. It can be concluded that the isomer predominantly present in water, is the (*E*,*Z*) isomer isoTA.

The UV spectrum of isoTA showed a maximum at 298 nm, while the spectrum of TA shows a maximum at 289 nm. This batochromic shift observed for isoTA may be explained by small conformational rearrangements, which affect the coplanarity of the conjugated system. The infrared (IR) spectrum of isoTA was recorded using KBr discs. Compared to the IR spectrum of TA, the main differences are changes in the relative intensity for the lactone carbonyl (1720 cm^{-1}) and the conjugated double bonds. The bands for the conjugated carbonyl and the double bonds are shifted from 1665 to 1680 cm^{-1} and from 1620 to 1625 cm^{-1}, respectively.

4. Conclusions

TA, upon standing in slightly alkaline medium is partly converted into the 8,20 aldol derivative TAD. Careful analysis showed that the two possible C-20 epimers are present. The two epimers TAD 1 and TAD 2 were isolated and purified here and were tentatively assigned the 20-*R* and 20-*S* configuration, respectively. TA in solution is slowly converted under the influence of light into isoTA through an isomerization of the conjugated double bonds, if the solution is stored in light. In aqueous solution the isomerisation favours a single isomer (*E*,*Z*), while in BME two isomers (*E*,*Z*) and (*Z*,*Z*) seem to be present in equal amounts.

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