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## Abstract

Tylosin is a widely used macrolide antibiotic for therapeutics and growth promotion in swine, beef cattle, and poultry production. Through various routes such as manure application, emission, inappropriate disposal, etc., tylosin enters the environment. The fate of tylosin in the environment is not yet fully understood. In this study, two photoreaction products of tylosin in water were identified as isotylosin A alcohol (E,Z) and isotylosin A aldol (E,Z). Tylosin A, B, C, D, isotylosin A alcohol, and isotylosin A aldol were purified, and immunological cross-reactivities of these tylosin-related compounds were tested with a specificity of 26% for tylosin B, 19% for tylosin C, 106% for tylosin D, 121% for isotylosin A alcohol, and 46% for isotylosin A aldol, compared to 100% for tylosin A. Competitive direct enzyme-linked immunosorbent assay (ELISA) for tylosin detection in water was compared with a high-performance liquid chromatography (HPLC) method by analyzing the same water samples from a study of tylosin dissipation in water. ELISA kits detect the other tylosin-related compounds besides tylosin A, which can result in differences in tylosin determination in water.

## Disciplines

Biochemistry | Entomology

## Comments

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# Identification of Tylosin Photoreaction Products and Comparison of ELISA and HPLC Methods for Their Detection in Water

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Tylosin is a widely used macrolide antibiotic for therapeutics and growth promotion in swine, beef cattle, and poultry production. Through various routes such as manure application, emission, inappropriate disposal, etc., tylosin enters the environment. The fate of tylosin in the environment is not yet fully understood. In this study, two photoreaction products of tylosin in water were identified as isotylosin A alcohol (E,Z) and isotylosin A aldol (E,Z). Tylosin A, B, C, D, isotylosin A alcohol, and isotylosin A aldol were purified, and immunological cross-reactivities of these tylosin-related compounds were tested with a specificity of 26% for tylosin B, 19% for tylosin C, 106% for tylosin D, 121% for isotylosin A alcohol, and 46% for isotylosin A aldol, compared to 100% for tylosin A. Competitive direct enzyme-linked immunosorbent assay (ELISA) for tylosin detection in water was compared with a high-performance liquid chromatography (HPLC) method by analyzing the same water samples from a study of tylosin dissipation in water. ELISA kits detect the other tylosin-related compounds besides tylosin A, which can result in differences in tylosin determination in water.

## Introduction

The macrolide veterinary antibiotic tylosin is naturally produced as a mixture of tylosin A, tylosin B, tylosin C, and tylosin D by the actinomycete *Streptomyces fradiae* and is active against most gram-positive bacteria, mycoplasma, and certain gram-negative bacteria. Tylosin exerts its antimicrobial activity by binding onto the bacterial 50S ribosomal subunit, where it prevents protein synthesis by interfering with peptide bond formation as well as by blocking the passage of the nascent peptide chain (1). Tylosin has been widely used for the treatment of disease and the promotion of growth in swine, cattle, and poultry. Tylosin was the most frequently used antibiotic at 31% of swine production facilities (2) and was detected in 14% of 139 streams in the United States (3).

Through application of manure with excreted tylosin residues from livestock on croplands, tylosin enters the water system as a pollutant in the environment. Because tylosin

and its related compounds have been shown to have biological activity (4), the fate and impact of tylosin in the environment have recently received more attention (4–9). Currently, the HPLC methods are the most widely used for detection and separation (10), and the ELISA kit is commercially available for rapid analysis of tylosin in various matrices. Use of ELISA for detection is convenient, but cross-reactivity information among tylosin-related compounds is not available for ELISA quantitative techniques.

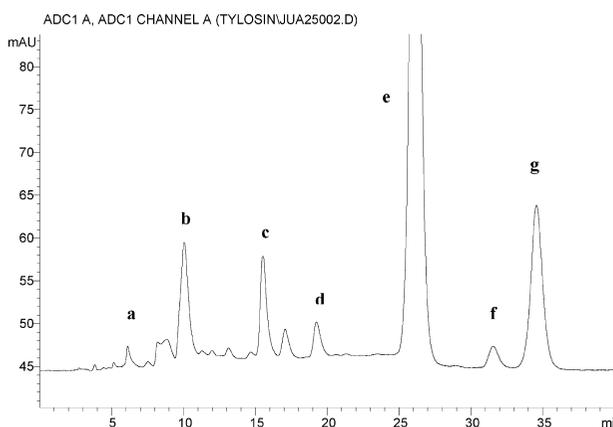
It has been shown that tylosin A is converted into different forms under acidic, neutral or alkaline conditions (11, 12). Tylosin A is converted into tylosin B in an acidic medium, and tylosin A aldol was found in neutral or alkaline medium, together with some unknown polar degradation products. A photoreaction product, isotylosin A, is reported by Paesen et al. (12). In this paper, the two primary objectives were to elucidate molecular structures of two additional photoreaction products and to compare the established HPLC and ELISA methods by analyzing the same tylosin water samples from a study of the dissipation of tylosin in water. In addition, cross-reactivity of tylosin-related compounds was tested, which accounts for the differences in detection of tylosin and related compounds in water samples analyzed by ELISA and HPLC.

## Materials and Methods

**Reagents.** Tylosin tartrate (95.0%, CAS NO. 74610–55–2) was purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile, methanol, and ammonium acetate were purchased from Fisher Scientific (Fair Lawn, NJ). ELISA kits were purchased from Immuno-Diagnostic Reagents (Vista, CA).

**Preparation of water samples and photoreaction products.** Tylosin at 50  $\mu\text{g}/\text{mL}$  in ultrapure water was maintained at a temperature of  $25 \pm 2$  °C under exposure to daylight in Ames, IA (42.01° N, 93.97° W) between April 15 and October 15 of 2005. Water samples were collected at days 0, 1, 3, 5, 7, 10, 14, 21 and 30, 60, and 180 post-treatment, and all samples were stored frozen at  $-70$  °C until analysis.

Solid-phase extraction (SPE) columns, Oasis Hydrophilic-Lipophilic Balance (HLB) cartridges (6  $\text{cm}^3$ ) (Waters, Milford, MA), were used to enrich tylosin from water. Tylosin A, B, C, D, and two new photoreaction products were purified from concentrated tylosin water samples by semipreparative chromatography with a Waters Atlantis dC18 column (10  $\times$  250 mm, 10- $\mu\text{m}$  particle size). The two photoreaction



**FIGURE 1.** HPLC–UV chromatogram showing separation of the mixture of tylosin and its related compounds: (a) tylosin B, (b) tylosin A ( $\text{NH}_4^+$ ), (c) tylosin C, (d) tylosin D, (e) tylosin A, (f) isotylosin 1, (g) isotylosin 2.

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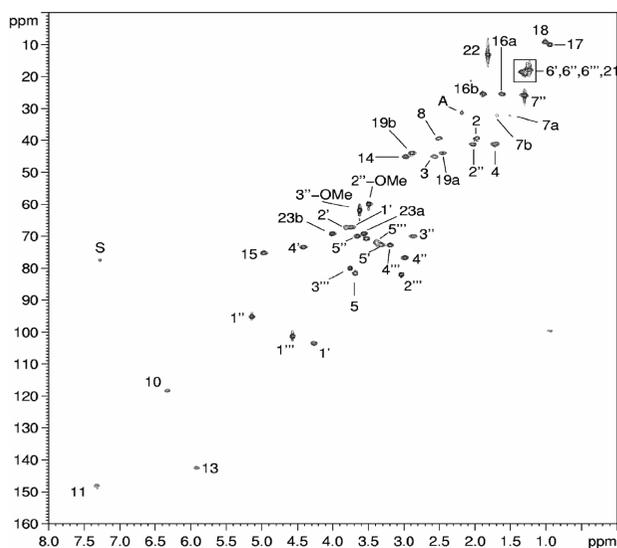
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**TABLE 1. Mean Recovery, Accuracy, and Limit of Detection (LOD) for Tylosin Determination in Water Using HPLC and ELISA ( $n = 4$ )**

matrix	spiked ( $\mu\text{g/mL}$ )	detected ( $\mu\text{g/mL}$ )	RSD <sup>a</sup>	recovery (%)	LOD (ng/mL)
HPLC	0.05	0.045	5.0	89.9	1
	0.02	0.017	10.6	83.3	
	0.005	0.0040	9.5	80.1	
ELISA	0.05	0.041	21	82	0.5
	0.02	0.016	18	81.6	
	0.005	0.0051	29	102.2	

<sup>a</sup> RSD: relative standard deviation.



**FIGURE 2.**  $^1\text{H}$ – $^{13}\text{C}$  HSQC spectrum of tylosin A in  $\text{CDCl}_3$  at  $25\text{ }^\circ\text{C}$ . Labels S and A denote peaks from residual  $\text{CHCl}_3$  and acetone, respectively. Not shown in this chemical shift range is a peak at  $^1\text{H}$  chemical shift 9.66 ppm and  $^{13}\text{C}$  chemical shift 203.4 ppm due to the aldehydic group at position 20. Also not shown at this contour level is the weak peak at  $^1\text{H}$  chemical shift 2.06 ppm and  $^{13}\text{C}$  chemical shift 31.8 ppm due to the methyne group at position 6.

products were denoted as isotylosin 1 and isotylosin 2 in our previous study (9). The individual tylosin-related compounds with purity of more than 98% were used for the cross-reactivity test of ELISA kits, and the two photoreaction products were also studied through NMR analysis.

**NMR Spectroscopy.** A commercial standard of tylosin A, as well as isotylosin 1 and isotylosin 2, were each dissolved in chloroform- $D$  for NMR analysis. All NMR spectra were acquired at  $25\text{ }^\circ\text{C}$  on a Bruker Avance II 700 spectrometer equipped with a TCI ( $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ ) cryoprobe with Z-gradient. 1D  $^1\text{H}$ , 1D  $^{13}\text{C}$ , APT (13), 2D DQF-COSY (14), 2D  $^1\text{H}$ – $^{13}\text{C}$  HSQC (15), and 2D  $^1\text{H}$ – $^{13}\text{C}$  HMBC (16) spectra were acquired using standard experimental protocols for the tylosin A sample. For isotylosin 1 and isotylosin 2 there was insufficient material to acquire 1D  $^{13}\text{C}$ , APT, and HMBC high-quality spectra. Chemical shifts were internally referenced to tetramethylsilane (TMS). NMR spectra were processed and analyzed using Bruker Topspin 1.6 software.

**HPLC Assay.** HLB cartridges were used for cleanup of water samples before injection into HPLC for detection. An HLB cartridge was conditioned with 5 mL of acetonitrile, followed by 5 mL of wash solvent of acetonitrile/distilled water (85:15). The samples were loaded without modification onto the cartridge that was conditioned to retain tylosin. Flow rate was maintained at about 2 drops/s, and 5 mL

nanopure water was used to rinse the column. The column was dried under a vacuum for 2 min and the analytes were eluted with methanol.

Analysis of the samples was carried out on a Hewlett-Packard (HP) series 1100 HPLC system. Data were collected and analyzed using HP Chemstation system software (rev. B.02.01). The separation of tylosin-related compounds was accomplished using a Waters Atlantis dC18 column ( $4.6 \times 250\text{ mm}$ ,  $5\text{-}\mu\text{m}$  particle size) at room temperature, with a mobile phase consisting of acetonitrile/20 mM ammonium acetate (35:65, v/v, pH 6.0) and a flow rate of 1.0 mL/min. Detection was performed by measuring UV absorbance at a wavelength of 290 nm.

**ELISA Assay.** Immunoassay was conducted as described by the manufacturer's instruction. In brief,  $25\text{ }\mu\text{L}$  of standard solution or water sample were added to microtiter plate wells coated with anti-tylosin antibodies. Tylosin-alkaline phosphatase conjugate ( $100\text{ }\mu\text{L}$ ) was added to each well. After proper mixing, the microtiter plate was incubated for 40 min at room temperature. After incubation, the nonbound conjugate was removed by washing with PBS-Tween buffer, and then the enzyme-substrate, *p*-nitrophenyl phosphate, was added. The enzyme reaction was stopped after 20 min with  $50\text{ }\mu\text{L}$  of 3 N NaOH each well. Immunoassay was performed using a THERMOMax microplate reader with SOFTmax Pro V3.0 software (Molecular Devices; Sunnyvale, CA) at 405 nm. The measure range of the standard curve was from 1 to 50 ng/mL. All tylosin samples were diluted into this range for quantification.

**Immunological Cross-Reactivity.** Cross-reactivity of the ELISA to tylosin-related compounds was tested. Inhibition binding of 50% ( $\text{IC}_{50}$ ) was attained for tylosin A at a concentration of approximately  $10\text{ }\mu\text{g/L}$ . All tylosin-related compounds were prepared at a concentration of  $10\text{ }\mu\text{g/L}$ , and they were measured as described in the ELISA assay. Cross-reactivity was compared to tylosin A (set as 100%), as a function of the % B value ( $n = 3$ ); % B is defined as the absorbance of the sample calculated as a percentage of the negative control.

## Results and Discussion

**HPLC Method.** Separation of tylosin-related compounds was accomplished by reversed-phase isocratic HPLC using a mobile phase of acetonitrile:ammonium acetate buffer. A typical HPLC chromatogram obtained from the separation of the tylosin-related compounds is shown in Figure 1. All tylosin-related compounds were confirmed by HPLC–ESI–MS because of the lack of standards except for tylosin A. Various methods of detecting tylosin have been discussed in a review article (10), and here we established a simple isocratic HPLC–UV method which is sensitive, selective and reproducible without using a corrosive acid. The method has been applied to detection of tylosin in soil and pond water samples with additional cleanup procedures in our laboratory (9).

No conversion between different tylosin forms was observed during sample preparation through comparison of directly injected samples and samples eluted from SPE cartridges. Background interference was evaluated by comparing the chromatograms of blank water samples with spiked water samples. Tylosin A, B, C, D, isotylosin 1, and isotylosin 2 were separated with good resolution in water samples. Extraction recovery and accuracy were evaluated by spiked samples at three concentrations in water. The limits of detection (LOD) of HPLC were calculated based on a signal-to-noise ratio of 3:1. The results of recovery, accuracy and LOD for both HPLC and ELISA methods are shown in Table 1. The ELISA method is less reproducible than HPLC, while it is able to detect lower levels of tylosin in water. The recovery with a range of 80–102% was acceptable for both methods.

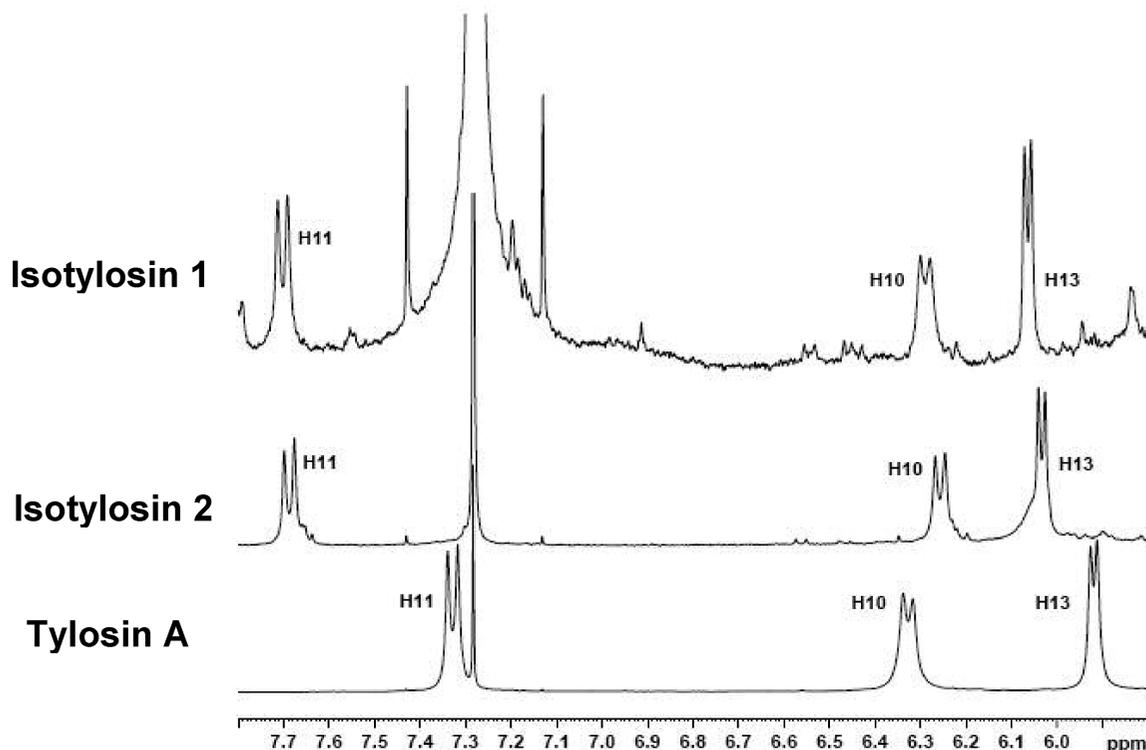


FIGURE 3. Conjugated double-bond region of proton NMR spectra in  $\text{CDCl}_3$  for the tylosin standard, isotylosin 1, and isotylosin 2.

TABLE 2.  $^{13}\text{C}$  and  $^1\text{H}$  Chemical Shift Assignments for the Aglycone Rings of Tylosin A, Isotylosin 1, and Isotylosin 2<sup>a</sup>

carbon	tylosin A standard		isotylosin 1		isotylosin 2	
	$^{13}\text{C}$	$^1\text{H}$	$^{13}\text{C}$	$^1\text{H}$	$^{13}\text{C}$	$^1\text{H}$
1	174.1	none	na	none	na	none
2	39.3	1.97	na	2.24	40.1	2.227
3	45.0	2.56	46.5	3.08	na	na
4	41.1	1.71	na	1.34	40.9	1.76
5	81.4	3.68	na	3.95	78.1	3.59
6	31.8	2.06	na	na	na	na
7	32.0	1.68,1.50	na	1.72	35.5	1.73, 1.31
8	39.3	2.50	na	2.77	64.8	none
9	177.1	none	na	none	na	none
10	118.3	6.32	126.1	6.29	126.4	6.24
11	148.1	7.32	140.5	7.69	140.3	7.67
12	135.2	none	na	none	133.0	none
13	142.4	5.91	140.8	6.01	140.6	6.02
14	45.1	2.97	42.1	3.04	42.2	3.04
15	75.2	4.97	75.9	4.93	76.0	4.93
16	25.3	1.88, 1.61	24.9	1.86,1.64	24.5	1.85, 1.64
17	9.9	0.93	9.60	0.93	9.7	0.92
18	9.0	1.00	na	na	8.3	0.95
19	43.8	2.88,2.44	na	na	40.0	2.48,2.27
20	203.6	9.64	na	9.80, na	na	9.81,9.67
21	17.8	1.22	na	1.14	18.0	1.27
22	13.0	1.81	na	na	20.5	1.91
23	69.1	4.00, 3.55	69.0	3.93,3.61	69.1	3.93,3.61

<sup>a</sup> None, unprotonated carbons; NA: unassigned atoms.

**Structural Elucidation.** Photoreaction products, isotylosin 1 and isotylosin 2 were initially detected in our previous study of tylosin dissipation in ultrapure water and in pond water (9), through comparison of dissipation profiles in light and in dark. They were accumulating over the 6-month study period, though their stability is not yet known as they individually exist in the environment. Tylosin A and two photoreaction products presented a similar fragment pattern on HPLC–ESI–MS, indicating they were structurally similar (9). The relative antibacterial activity of some tylosin-related

compounds reportedly is 31–83% of tylosin A (4). Slight changes in the chemical structure could cause significant loss of biological activity, which could greatly reduce the selective pressure for bacterial drug-resistance.

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR frequencies for spectra of the tylosin A standard were assigned in Figure 2 by analysis of 1D  $^1\text{H}$ , 1D  $^{13}\text{C}$ , APT, DQF-COSY, and  $^1\text{H}$ – $^{13}\text{C}$  HSQC spectra. The assigned  $^{13}\text{C}$  shifts corresponded very closely with those reported by Paesen et al. for tylosin A (12). A notable exception was the  $^{13}\text{C}$  shift for carbonyl carbon 9. We observed this resonance at 174.3 ppm whereas Paesen et al. reported 202.7 ppm. Spectra were reacquired on a freshly prepared sample of tylosin A giving identical results. Finally, an HMBC spectrum showed a cross peak at proton shift 2.49 ppm (H8) and carbon shift 174.1 ppm, indicating that the resonance at 174.1 ppm is indeed C9. The  $^1\text{H}$  and  $^{13}\text{C}$  peaks for positions 6 and 7 were very weak, probably because of conformational motions of the aglycone ring in the intermediate exchange regime for these atoms.

The presence of a single set of peaks in the conjugated double bond region of the  $^1\text{H}$  and  $^{13}\text{C}$  spectra indicated that a single geometric isomer was present for carbons C8 to C17 of the aglycone ring. On the basis of the close similarities to shifts reported by Paesen et al. for tylosin A, we assign the conjugated system between C8 and C14 to be in the (E,E) configuration. That is, H10 is trans to H11 and H13 is trans to the methyl group on C12. The magnitude of the scalar coupling between H10 and H11 ( $J_{\text{H10,H11}} = 16.3$  Hz) is consistent with these atoms being trans.

Complete assignment and structural elucidation of isotylosin 1 and isotylosin 2 was not possible because of lack of sufficient pure materials to obtain high quality NMR spectra comparable to those obtained for tylosin A. However, some structural conclusions can be made. First, isotylosin 1 and isotylosin 2 have very similar carbon connectivities and multiplicities for the aglycone ring as for tylosin A, as can be elucidated from DQF-COSY spectra. However, the C9 to C13 conjugated system exists in a distinct configuration in isotylosin 1 and isotylosin 2 compared to standard tylosin A as shown by  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts in Figure 3 and Table 2.

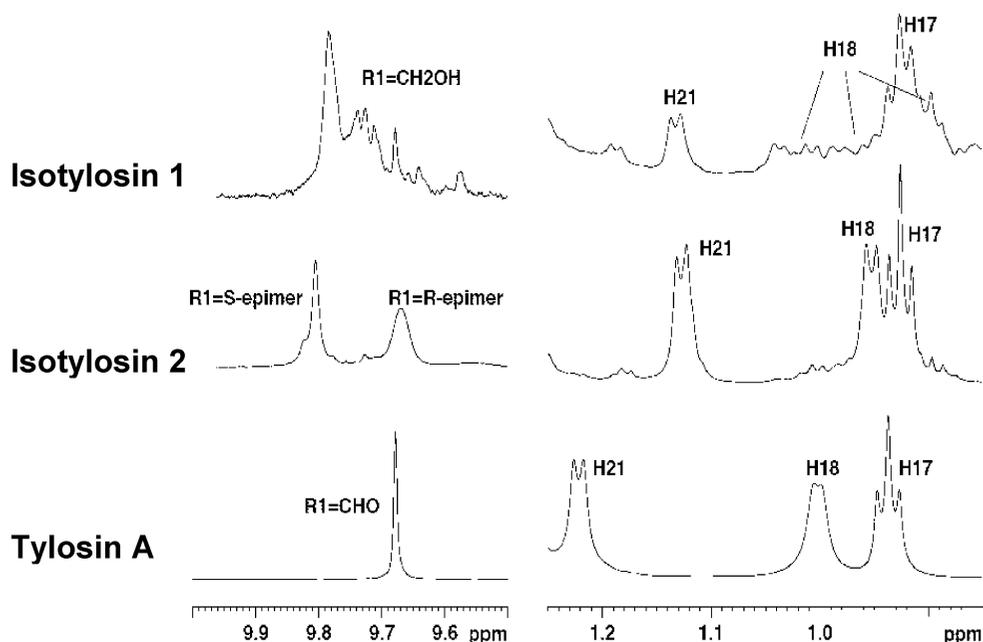


FIGURE 4. Isotylosin 1 and isotylosin 2 are aldol and alcohol derivatives, respectively, of tylosin A.

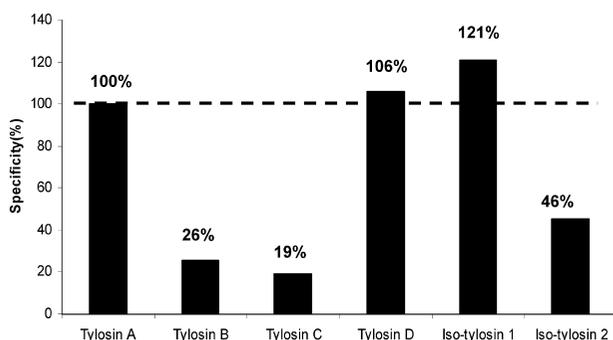


FIGURE 5. Cross-reactivity of the ELISA to tylosin-related compounds.

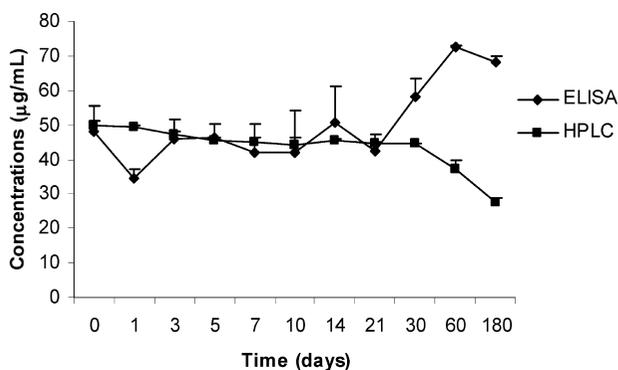


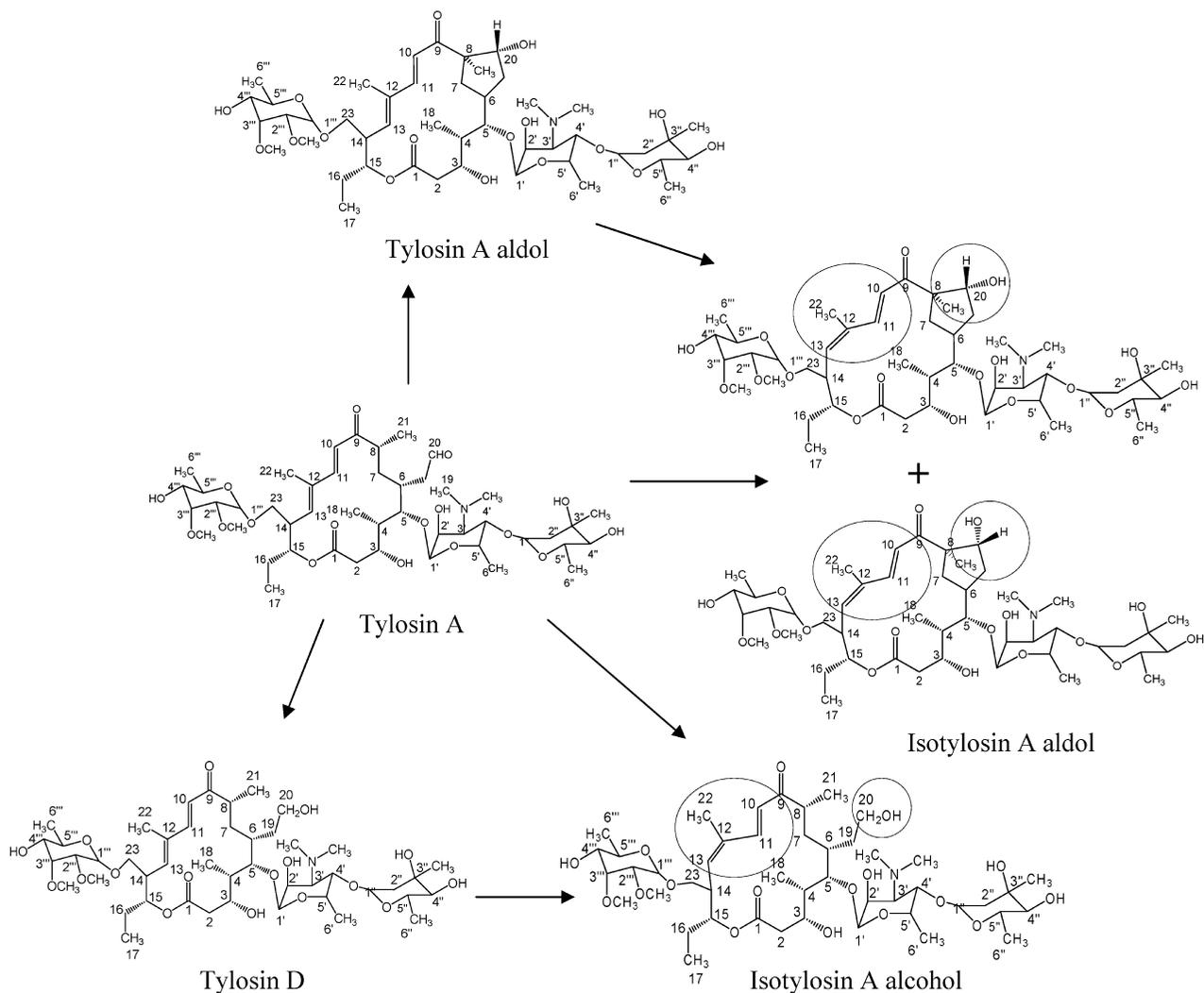
FIGURE 6. Tylosin dissipation in water measured by HPLC and ELISA ( $n = 4$ ).

Scalar coupling constants between H10 and H11 measured by DQF-COSY are 16.5 and 18.5 Hz for isotylosin 1 and isotylosin 2, respectively, which indicates that the trans configuration for H10 and H11 is maintained in both photoreaction products. The only configuration for isotylosin 1 and isotylosin 2 consistent with the scalar coupling and chemical shift data is (E, Z) in which H13 and the methyl on C12 is cis. This indicates that the light exposure causes the transformation of the (E, E) into the (E, Z) configuration at the double-bond conjugated region of isotylosin 1 and isotylosin 2, because the structure was found only in water samples exposed to the light.

For isotylosin 2, no peak was observed in the  $^{13}\text{C}$  spectrum at 200 ppm, suggesting that C20 is no longer a carbonyl. Yet low-field proton peaks were observed at 9.67 and 9.81 ppm. Further, we do not observe a correlation connecting methyl group C21 to the aglycone ring and cannot assign H8, suggesting that C8 is tertiary in isotylosin 2. We suggest that isotylosin 2 is a cyclic aldol derivative of tylosin A obtained by reducing aldehydic carbon C20 to a hydroxyl, with C19, C6, C7, C8, and C20 forming a five-membered ring. The two signals at 9.67 and 9.81 are due to the two possible epimers of the C20 hydroxyl. The C20 hydroxyl is apparently engaged in an intramolecular hydrogen bond with carbonyl C9, which accounts for the downfield shifts (17).

In the case of isotylosin 1, only partial assignments could be made because of the limited amount of material and the weak NMR spectra. Furthermore, the aglycone ring exhibited a slow exchange among multiple conformations, resulting in several peaks being divided into weak multiplets, as shown in Figure 4. However, in contrast to isotylosin 2, a COSY connection between C21 and C8 could be identified. As well, a major peak was observed at 9.78 ppm plus numerous minor peaks. We suggest that isotylosin 1 has a primary alcohol at the C20 position on its structure. Such a structure would have increased conformational flexibility compared to the cyclic aldol of isotylosin 2 and may be able to form intramolecular hydrogen bonds from C20 to both C9 and C1. This would be consistent with the multiple conformational forms observed. On the basis of this structure, we renamed isotylosin 1 to isotylosin A alcohol.

**Cross-Reactivity and Comparison of ELISA and HPLC Methods.** Tylosin-related compounds were individually purified by semipreparative HPLC. The purity of each was more than 98.0% after three times through the purification process. The results of the cross-reactivity test are illustrated in Figure 5. All tylosin-related compounds tested had cross-reactivity with the main component tylosin A to some extent, and tylosin D and isotylosin A alcohol have a specificity of more than 100%. The cross-reactivity increase in tylosin D and isotylosin 1 indicated that the OH group at the C20 position is critical for antibody recognition. The (E, Z) configuration of the conjugated double-bond region, mycinose and mycarose groups are other important determinants for cross-reactivity. Based on the results, the ELISA



**FIGURE 7.** Possible transformation pathways of tylosin in water. Circles indicate the location of modification sites on the structures. Isotylosin A aldol and isotylosin A alcohol have an (E, Z) configuration of the double-bond conjugated site, whereas the others have an (E, E) configuration.

antibody recognized multiple epitopes on the tylosin molecules; however, the extent to which each position has an effect on the cross-reactivity remains to be discussed.

For quantitative analysis of tylosin in water, results obtained by both HPLC and ELISA methods are shown in Figure 6. Agreement between both methods was good except for the samples on the 24th hour. Differences between HPLC and ELISA detection of tylosin were found for those samples after 1 month, which was coincident with appearance of isotylosin A alcohol in water (9). The concentrations determined by the HPLC method were expected to be lower because it only detects tylosin A. In cross-reactivity testing, isotylosin A alcohol showed a specificity of 121% compared to 100% for tylosin A, which indicates that the divergence might be caused by formation of isotylosin A alcohol.

Two photoreaction products both have an (E, Z) configuration at the double-bond conjugated site, and isotylosin 1 is isotylosin A alcohol (E, Z), and isotylosin 2 is isotylosin A aldol (E, Z) with two epimers. The possible transformation pathway in water is illustrated in Figure 7, which complements the degradation pathway in the environment proposed in a previous study (9). Isotylosin A aldol (E, Z) can be formed from tylosin A or from an intermediate tylosin A aldol, and isotylosin A alcohol (E, Z) can be formed from tylosin A or from tylosin D, which is usually present with tylosin A and possibly can be biologically formed from tylosin A. In our

study, no direct evidence was found to support formation of tylosin A aldol and tylosin D from tylosin A (9).

The ELISA detects not only tylosin A but also other tylosin-related compounds, including at least tylosin B, C, D, isotylosin A aldol, and isotylosin A alcohol. The broad spectrum of detection and the high sensitivity makes the method a particularly valuable screening tool, although it only provides semiquantitative results. Potentially the ELISA method, in combination with conventional methods such as HPLC/MS, can be useful for sensitive and specific detection of tylosin-related residues in environmental matrices.

### Acknowledgments

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