INCLUSION COMPOUNDS OF LANKACIDIN-GROUP ANTIBIOTICS WITH CYCLODEXTRINS

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Lankacidin-group antibiotics formed inclusion compounds with β-cyclodextrin in molar ratios of about 1:1. These compounds showed remarkably improved water-solubility and stability in aqueous solutions. The structure of the inclusion compounds of lankacidin A is proposed based on the facility of their inclusion with β-cyclodextrin and the results of ^{1}H NMR spectral studies.

Lankacidin-group antibiotics[1~3] isolated from the culture filtrate of *Streptomyces rochei* var. *volubilis*[4] are 17-membered macrocyclic compounds.[1,5,6] They show strong protective effects in mice against Gram-positive bacteria including clinical isolates when orally or parenterally administered[7] and have low toxicities in mice and rats.[8] Studies on their structure-activity relationships, enzymatic reactions and metabolic fates have been reviewed.[9] Recently, one of these antibiotics, lankacidin A (**2**) was found to be active against *Treponema hyodysenteriae in vitro* and was strongly effective to experimental infections in mice and pigs.[10,11]

These antibiotics are scarcely soluble in water and the parts that dissolve are rapidly decomposed to compounds having no antimicrobial activity. To overcome this problem, we considered the use of cyclodextrins (CyDs) to improve water-solubility and stability in aqueous solutions by forming inclusion compounds with various kinds of organic compounds.[12] We prepared inclusion compounds of the antibiotics and examined their characteristics for use by injection. This report deals with the inclusion compounds of lankacidin-group antibiotics formed with CyDs.

Materials and Methods

Antibiotics and Reagents

Lankacidin-group and other antibiotics were prepared in our laboratories;[5,6,8] their structures appear in Table 1. The chemical nomenclature of **2** is: [1*S*,2*R*,7*S*,13*S*,15*R*,16*R*]-13-acetoxy-7-hydroxy-1,4,10,16-tetramethyl-17-oxo-2-pyruvamidocycloheptadeca-[3*E*,5*E*,9*E*,11*E*]-tetraene-1,15-carbolactone. This numbering system is based on the IUPAC nomenclature rules, and differs from that in Table 1. CyDs were purchased from Wako Pure Chemicals (Osaka, Japan).

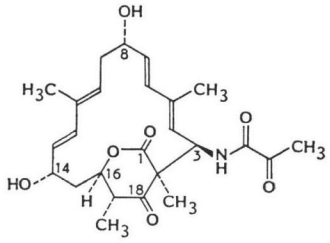
Estimation of Solubility

An excess amount of an antibiotic was added to a solution containing CyDs and the suspension was stirred at 7°C for 30 minutes to 2 hours. The insoluble part was removed with a filter paper (Toyoroshi, No. 5c) or a millipore filter (Millipore Corp, USA, 0.45 μm), and the amount of antibiotic in the filtrate was estimated by the UV absorbance at 226 to 228 nm.

Preparation of Inclusion Compounds

Method I: A small excess of the antibiotic calculated from the saturated solubility of the antibiotic

Table 1. Structures and solubilities of lankacidin-group antibiotics for β-CyD solutions.



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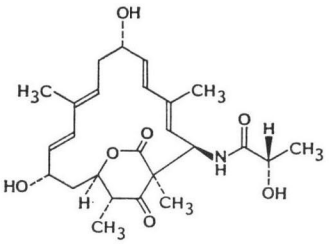

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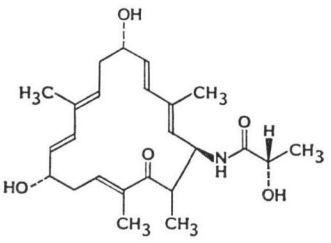

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No.	Antibiotic	Rf[a]	β-CyD (mol/liter)		
			(1) 0	(2) 10^{-2}	Inc.[b]
1	Lankacidin C	0.41	0.268	3.54	0.71
2	〃 A (=C 14-acetate)	0.64	0.181	2.69	0.50
3	〃 C 14-propionate	0.68	0.019	0.159	0.03
4	〃 C 8-acetate	0.56	0.074	2.77	0.53
5	〃 C 8-propionate	0.58	0.018	3.84	0.77
6	〃 C 8-*n*-butyrate	0.59	0.009	0.404	0.07
7	〃 C 8,14-diacetate	0.84	0.016	0.681	0.12
8	〃 C 8-propionyl-14-acetate	0.86	<0.001	0.025	<0.01
9	6,7,12,13-Tetrahydrolankacidin C	0.64	0.013	0.025	<0.01
10	Lankacidinol	0.19	0.655	3.78	0.67
11	〃 A (=14-acetate)	0.33	0.170	3.07	0.59
12	〃 8,14-diacetate	0.49	0.440	1.78	0.40
13	〃 2′,8,14-triacetate	0.76	0.037	0.332	0.05
14	〃 A 18-ol	0.10	0.52	1.07	<0.01
15	Lankacyclinol	0.15	0.824	3.61	0.67
16	〃 2′,8,14-triacetate	0.71	0.098	1.85	0.32
17	Leucomycin A_3	0.58	0.135	0.453	0.04
18	Folimycin	0.48	0.019	0.046	<0.01

[a] TLC, SiO_2 60 F-254 (Merck AG.), toluene - acetone=1: 1.

[b] Inclination: The increase of solubility of antibiotics/concentration of β-CyD.

in 0.01 M β-CyD solution was added to 0.01 M β-CyD solution, and the mixture was stirred at 7°C for 30 minutes. After filtration, the filtrate was concentrated and freeze-dried. The content of the antibiotic was calculated from the UV absorbance of the lyophilized powder.

Antibiotic (g)	β-CyD (g)/H_2O (liter)	Powder (g)
2 (5.0)	20.4/1.8	23.6
1 (1.1)	3.4/0.3	3.8
11 (1.0)	3.4/0.3	3.7
10 (1.25)	3.4/0.3	4.0

Method II: Amounts of the antibiotic and β-CyD, calculated from those at the turning point (T-point) shown in Fig. 1, were suspended in H_2O, and the mixture was stirred at 7°C for an hour. The mixture was treated as stated for Method I.

Antibiotic (g)	β-CyD (g)/H_2O (ml)	Powder (g)
2 (1.0)	4.64/172	3.7
1 (1.0)	2.98/40	3.2
10 (1.0)	2.80/20	3.0

Stability Constant

The calculation formula used was:

$$K=\frac{[ACyD]}{[A].[CyD]}$$

K; Stability constant, [A]; Concentration of free antibiotic: [Saturated concentration of the antibiotic for H_2O] (mol/liter), [CyD]; Concentration of free β-CyD: [Concentration of added β-CyD] − [Concentration of the inclusion compound] (mol/liter), [ACyD]; Concentration of the inclusion compound: [Saturated concentration of the antibiotic in the β-CyD solution] − [Concentration of free antibiotic] (mol/liter).

Molar Ratio

The molar ratio (M.R.) between the antibiotic and β-CyD in an inclusion compound was calculated with this formula:[13]

M.R.=[Concentration of the antibiotic at the T-point]−[Saturated concentration of the antibiotic for H_2O] (mol/liter): [Concentration of β-CyD at the T-point]−[Saturated concentration of β-CyD for H_2O] (mol/liter).

High-performance Liquid Chromatography (HPLC)

A Model 6000 A/660/440 instrument was used with a column of reverse-phase μBondapak C_{18} (Waters Assoc.). The flow rate of the mobile phase was 1 ml/minute with CH_3CN - 0.01 M phosphate buffer, pH 7.7, (4: 6). The retention time of **1** was 5.6 minutes under these conditions.

Results and Discussion

The solubilities for CyD solutions were first examined using lankacidin C (**1**) which is antimicrobially the most active among lankacidin-group antibiotics (Table 2). **1** showed remarkable solubility increase for 0.01 M β-CyD solution but no increase for α-CyD solutions. The solubility was increased by γ-CyD solutions but 10 times more γ-CyD than β-CyD was needed to obtain the same level of solubility. Lankacidin C 8-propionate (**5**) and 14-propionate (**3**) were subjected to the solubility test to find the effect of the substitution position on the inclusion ability of CyDs (Table 2). **5** showed a greater increase of solubility with β-CyD solution than γ-CyD solution. These data suggested that β-CyD is the best inclusion host for lankacidin-group antibiotics. However, the positional isomer, **3**, showed smaller increase of solubilities for CyD solutions than **5**.

Solubility changes of **2**, **1** and lankacidinol (**10**), which displayed typically distinctive solubilities for water and the Rf values on TLC (Table 1), were found for the β-CyD solution. Three antibiotics increased their solubilities for β-CyD solutions as the β-CyD concentration increased (Table 3). The stability constants in 0.01 M β-CyD solutions were calculated to be 2,800 for **2**, 4,270 for **1** and 1,480

Table 2. Solubilities of **1**, **5** and **3** for β-CyD solutions (mg/ml).

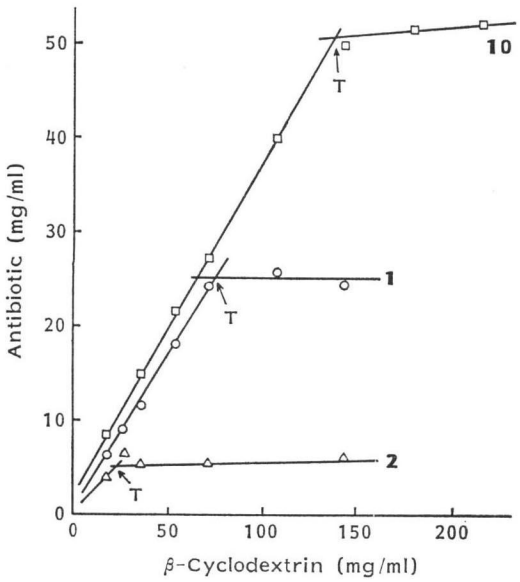
Compound	CyD	Concentration (mol/liter)				
		0	10^{-4}	10^{-3}	10^{-2}	10^{-1}
1	α-	0.260	0.250	0.240	0.270	
	β-		0.280	0.650	3.73	
	γ-		0.260	0.460	2.39	3.79
5	β-	0.018	0.046	0.328	3.84	
	γ-		0.019	0.054	0.249	1.61
3	β-	0.019	0.021	0.067	0.159	
	γ-		0.019	0.035	0.114	0.273

Table 3. Solubilities of **2**, **1** and **10** for β-CyD solutions (mg/ml).

Compound	Concentration (mol/liter)							
	0	10^{-4}	2×10^{-4}	5×10^{-4}	10^{-3}	2×10^{-3}	5×10^{-3}	10^{-2} [a]
2	0.181	0.195	0.201	0.265	0.400	0.632	1.37	2.69
1	0.237	0.262	0.304	0.409	0.566	0.934	1.78	3.35
	0.268	0.330			0.715			3.54
10	0.655	0.731			0.725			3.78

[a] The maximum solubility of β-CyD for H_2O was 11.3 mg/ml at 7°C.

Fig. 1. Phase-stability diagrams of **1**, **2** and **10**.



for **10**, on the basis of the postulated 1:1 stoichiometric ratio for the interactions. These values suggest that the antibiotics form fairly stable inclusion compounds with β-CyD.

Molar ratios between antibiotics and β-CyD in the inclusion compounds were established. Excess antibiotics were added to the β-CyD suspension containing excess more than the saturated concentration of β-CyD for water (18 mg/ml at 25°C). The suspension was stirred and filtered. The amounts of antibiotics in the filtrate increased linearly to the T-points and reached the saturated states as shown in Fig. 1, giving a type A phase-solubility diagram.[13] Concentrations of **2**, **1** and **10** at the T-points were 6.05, 24.4 and 50 mg/ml, respectively, and their stability constants were calculated to be 2,440, 5,840 and 3,890, respectively. Molar ratios between antibiotics and β-CyD were estimated to be 1:1.26, 1:1.03 and 1:1.09, respectively. From these data, we concluded that lankacidin-group antibiotics and β-CyD form inclusion compounds at the molar ratio of approximately 1:1.

When the β-CyD inclusion compounds of these antibiotics prepared by Method I were dissolved

Table 4. Solubilities of freeze-dried powders for H_2O.

Method	(Compound)	Initial samples		Recovered samples
		Contents of antibiotics (μg/mg)	Solubilities for H_2O [a] (mg/ml)	Contents of antibiotics (μg/mg)
I	(**2**)	162	62.0	252
	(**1**)	238	78.4	252
	(**11**)	171	45.5	281
	(**10**)	238	50.5	258
II	(**2**)	178	60.7	245
	(**1**)	253	80.9	252
	(**10**)	275	74.2	270

[a] Antibiotic concentrations in the dissolved solutions.

Table 5. Stabilities of a freeze-dried inclusion compound of **1** in 0.01 M β-CyD solution at 25°C.

Time (hour)	Residual rate (%)[a]					
	1/H_2O			Inclusion compound of **1**/β-CyD soln		
	0.1 mg/ml			0.1 mg/ml	1.0 mg/ml	10 mg/ml
	1	D_1[b]	D_2[b]	**1**	**1**	**1**
0	100	1.9	0	100	100	100
0.5	99.6	2.0	0.8	99.3	100.1	—
1.0	100.3	2.9	0.9	99.0	100.6	99.9
2.0	96.0	5.2	2.4	99.3	100.4	99.8
4.0	92.1	8.6	3.8	99.2	101.7	99.9

[a] HPLC detection.
[b] D_1 and D_2 are the decomposition products.

in water, their solubilities (Table 4) were about 330 times higher for **2** and about 340 times for **1** than the solubilities of the antibiotics for water (Table 1). Powders of **1** and **10** recovered by freeze-drying the solutions showed almost the same level of antibiotic content as compared with the initial samples. In the case of **2**, the contents in the recovered powder increased slightly to reach the same level as **1** and **10**. This suggests that free β-CyD used in the preparation of the inclusion compound of **2** was excluded by re-dissolution and therefore, the molar ratios between the antibiotics and β-CyD are consistent among the inclusion compounds. The freeze-dried powders prepared by Method II showed similar antibiotic contents and solubilities for water to those prepared by Method I.

The freeze-dried inclusion compound of **1** was remarkably stable in the β-CyD solution at 25°C in comparison with **1** in water as shown in Table 5. These findings indicate that the powder prepared by the freeze-drying method may keep some stable form(s) of β-CyD inclusion compounds, which im-

Table 6. ^1H NMR spectral data of **2** and its β-CyD inclusion compound (400 MHz in D_2O, δ_{ppm} J(Hz), Jeol GX-400).

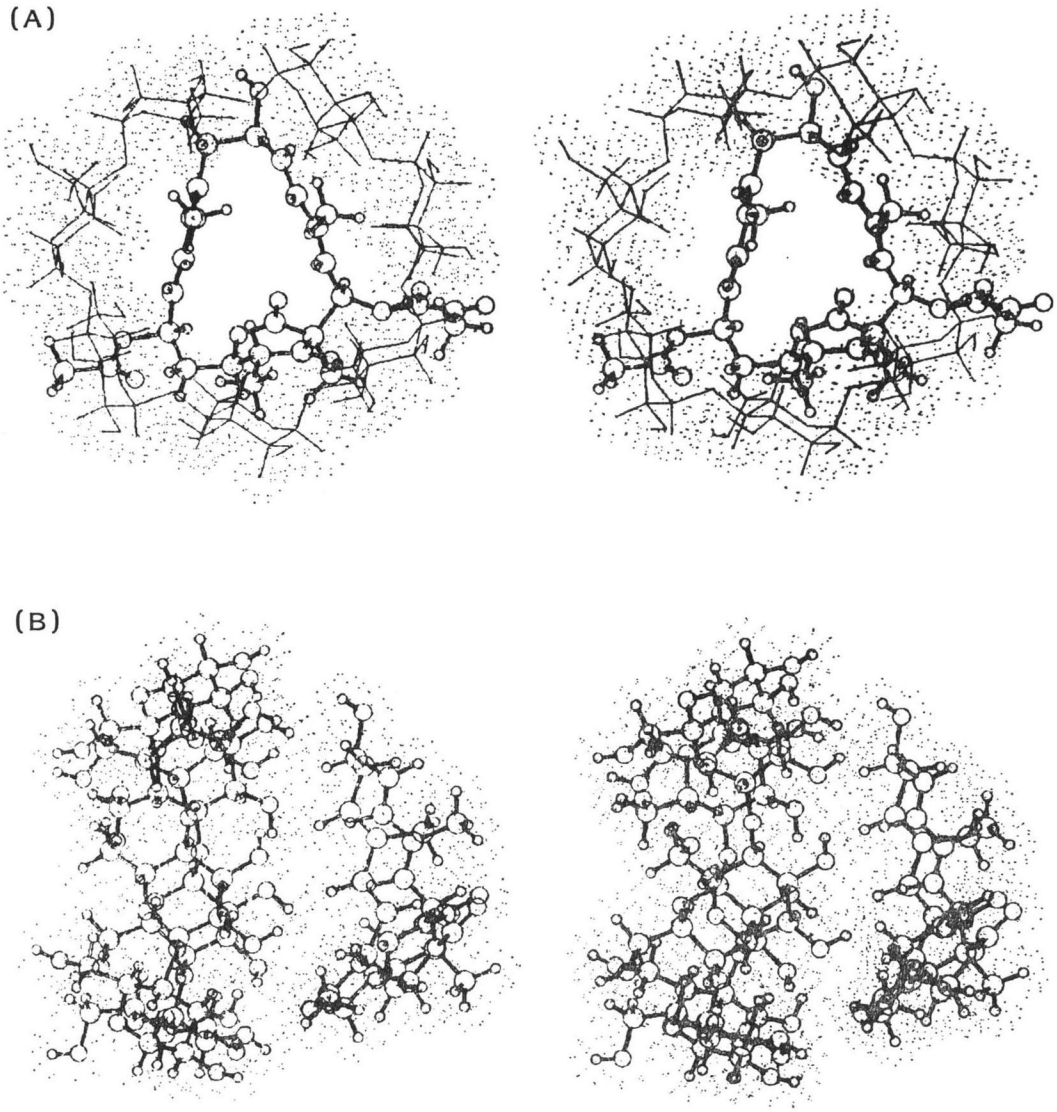
Proton	**2**[a]	**2**/β-CyD[a]	Δ
H-12	6.478 (d, J=15)	6.368 (d)	−0.11
H-13	5.678 (dd, J=10, 15)	5.728, 5.718 (dd, J=10, 15)	
H-6	5.765, 5.760 (d, J=16)	5.552, 5.576 (d, J=15.5)	−0.21
H-7	5.470 (m)	5.389 (m)	
H-3	5.364, 5.312 (d, J=11)	5.330, 5.320 (d, J=11)	
H-14	5.57 (m)	5.45 (m)	−0.12
H-10	5.54 (bt, J=8)	5.419 (t, J=8)	−0.12
H-8	4.16, 4.12 (q like)	3.90 (q)	+0.26
H-17	2.66 (m)	2.47, 2.36 (m)	−0.19
3′-CH_3	2.45 (s)	2.33 (s)	−0.12
9-CH_2	2.40 (m)	2.34 (m)	
15-CH_2	2.38 (m)	2.22 (m)	−0.16
14-CH_3	2.067, 2.062 (s)	1.948, 1.968 (s)	
5-CH_3	1.841, 1.94 (d)	1.892, 1.863 (d)	
11-CH_3	1.531, 1.54 (d)	1.504, 1.47 (d)	
2-CH_3	1.43 (s)	1.34 (s)	
17-CH_3	1.28 (d, J=6.5)	1.245, 1.234 (d, J=6.5)	

[a] Although two corresponding signals were observed in each spectrum, the samples tested were ascertained not to decompose in D_2O by the recovery experiment. The presence of conformational isomers may be ascribable.

Fig. 2. Proposed structure of the β-CyD inclusion compound of **2** which expressed by the stereoscopic views (dots: van der Waals radii).

(A): Lankacidin A; lower side, β-CyD; upper side (for the paper surface).

(B): Lankacidin A; right side, β-CyD; left side.



proves the stabilities of the antibiotics in aqueous solutions.

The inclination in the phase-solubility diagram (Fig. 1 and Table 1) is considered to indicate how easily inclusion occurs in CyD solution. Although the fat-solubilities of the compounds assumed from the Rf values on TLC had no relation to the facility of inclusion, interesting findings were obtained with respect to the structure and the facility of inclusion; 1) the lengths of the side chains at positions, 3, 8 and 14, have important limitation according to the data for **2** and **3**, **5** and 8-*n*-butyrate (**6**), and lankacidinol 8,14-diacetate (**12**) and 2′,8,14-triacetate (**13**), 2) the lactone moiety does not affect to the inclusion according to the data of lankacyclinol (**15**) and its triacetate (**16**), 3) two di-substituted double bonds are essential for the inclusion due to conformational changes from the data of 6,7,12,13-tetra-

hydrolankacidin C (**9**) and 4) the 18-carbonyl group is also essential according to the data for lankacidinol A (**11**) and its 18-ol compound (**14**).

The absolute configuration of **2** has been determined by X-ray crystallographic analysis of its 2′-*p*-bromophenyl hydrazone.[14] According to the data, the space atomic distances from C-8 to C-17 and from C-3 to C-15 are 6.32 Å and 6.43 Å, respectively, which are almost the same as the internal cavity size of β-CyD, 6.0~6.4 Å.[12] Therefore, the skeleton of the antibiotic can not enter the β-CyD cavity.

^{1}H NMR spectral studies were carried out to elucidate the structure of the β-CyD inclusion compound of **2**. When the added spectrum of **2** (60 μg/0.3 ml at 5°C) and β-CyD alone (5 mg/0.3 ml) in deuterium oxide was compared to the spectrum of the β-CyD inclusion compound of **2** (5 mg/0.3 ml as the antibiotic) (Table 6), the proton signal at position 3 showed an upfield shift (−0.088) from 3.844 ppm to 3.756 while other proton signals originating from β-CyD did not shift. This indicates that the host inclusion position is the large cavity site of β-CyD. Shifts (more than 0.1 ppm) in the proton signals originating from the antibiotic were observed at the methyl signals, 3′-CH_3 and 2-CH_3, the vinyl proton signals, H-12, H-6 and H-10, and the methine proton signals, H-8, H-14 and H-17. Most of these protons are located at the 18-carbonyl site in the absolute structure of **2**. Thus, the included position of the antibiotic is the 18-carbonyl site.

The binding between the antibiotics and β-CyD is assumed to be a loose one, like hydrogen bonding or van der Waals forces, because the antibiotics can be easily recovered by ethyl acetate extraction from an aqueous solution of the β-CyD inclusion compounds and the enzymatic reactions at position 14 proceed in β-CyD solution without any resistance.

Table 7. Protective effects of the β-CyD inclusion compounds of **2**, **1**, **11** and **10** on experimental *Treponema hyodysenteriae* (*T. hyo.*) infection in Ta: CF#1 mice.[a]

Expt	Compound	Dosage[b] (mg/kg)	No. of positive/examined	
			Gross cecal lesions	Colonization of *T. hyo.* in cecum
I	Infected control	0	15/15	15/15
	2/β-CyD	2.5	1/10	10/10
		10	0/13	4/13
	1/β-CyD	2.5	0/5	4/5
		10	0/5	1/5
	10/β-CyD	2.5	3/5	5/5
		10	0/5	5/5
	2/suspension[c]	2.5	5/10	10/10
		10	0/10	0/10
II	Infected control	0	5/5	5/5
	2/β-CyD	10	0/5	1/5
		20	0/5	0/5
	11/β-CyD	10	4/5	5/5
		20	1/5	5/5
	10/β-CyD	10	1/5	5/5
		20	0/5	3/5
	2/suspension[c]	10	0/5	2/5
		20	0/5	0/5

[a] A single oral inoculation of *T. hyodysenteriae* DJ70P3 (10^7 cfu/mouse).
[b] Administered subcutaneously for consecutive 2 days.
[c] Suspension with 5% gum arabic.

The structure of the β-CyD inclusion compound of **2** was deduced to be that shown in Fig. 2. The figure is depicted by 3-dimensional computer graphic techniques with van der Waals radii.

The solubilities of leucomycin A_3[15] (=josamycin), a 16-membered macrolide, and folimycin[16] (=concanamycin A),[17] an 18-membered macrocyclic lactone compound, were tested by the same method. They did not show the improved solubilities for the β-CyD solution, although they have similar structures and Rf values on TLC (Table 1). These findings suggested that this type of inclusion occurs specifically in compounds having the skeleton of the lankacidin-group antibiotics.

Table 7 shows the protective effects of the β-CyD inclusion compounds of **2**, **1**, **11** and **10**, which are important antimicrobially active metabolites,[18] on experimental *Treponema hyodysenteriae* infection in mice. **2** and **1** had effects similar to those of **2** given by suspension, but **11** and **10** were inferior to **2** and **1**. The β-CyD inclusion compound of **2** was effective against swine dysentery in the field at similar doses to those in mice by intramuscular administration. Trials for application is now in progress.

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