ENZYMATIC PHOSPHORYLATION OF MACROLIDE ANTIBIOTICS

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Five macrolide antibiotics (erythromycin A, **1**; oleandomycin, **3a**; tylosin, **4a**; spiramycins, **5a**; leucomycin A_3, **6a**) have been phosphorylated enzymatically using cell-free extracts derived from *Streptomyces coelicolor* UC 5240. The necessary cofactors and the rates of the conversion have been determined.

Phosphorylation of antibiotics by microorganisms is a well-known phenomenon in such classes as the aminocyclitols, lincosaminides, and nucleosides[1]. Such phosphorylations are one of the ways in which microorganisms inactivate antibiotics *in vivo*[2]. Phosphorylating enzymes are widespread among *Streptomyces* and are found in other genera[1]. In a program extending microbial phosphorylation to macrolide antibiotics *Streptomyces coelicolor* was the organism chosen as it had been found effective in other cases[3]. Microbial phosphorylation of macrolides has not been reported prior to a preliminary report[4] of some of our results although chemical synthesis of erythromycin A phosphate has been reported[5]. The present communication reports further studies of macrolide phosphorylation involving erythromycin A (**1**), oleandomycin (**3a**), tylosin (**4a**), a mixture of spiramycins I, II, III (**5a**), and leucomycin A_3 (**6a**) by cell-free extracts of *S. coelicolor*. Some of the factors required in the conversion such as biochemical requirements, reaction rates and nucleotide specificity were studied.

Materials and Methods

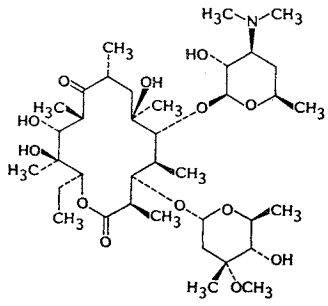
Microbiological

S. coelicolor UC 5240 (NRRL3532) was stored and maintained on sterile soils in the culture collection of The Upjohn Company. The organism was inoculated into a seed medium (GS-7) which contained Cerelose (C.P.C. International) and Pharmamedia (Procter and Gamble) each added at 25 g/liter of tap water. The medium was adjusted to pH 7.2 with NH_4OH and was autoclaved for 30 minutes. The inoculated 100 ml volumes of GS-7 were shaken in wide-mouth 500-ml fermentation flasks at 250 rpm for 48 hours at 28°C. The mature seed cultures were used as the source of inoculum (5% seed rate) for the fermentation medium. The latter was a modification of a medium reported by COATS and ARGOUDELIS[3] and contained glucose 20 g, NZ-amine B (Sheffield Chemicals) 5 g, yeast extract 2.5 g, $NaNO_3$ 1.5 g and $FeSO_4$ 10 mg per liter of deionized water. After formulation the medium was adjusted to pH 7.2 with NH_4OH and was sterilized by autoclaving. The medium was employed in the manner described for GS-7. After 24 hours of growth at 28°C the fermentation was centrifuged in the cold at $10^4 \times g$ for 15 minutes. The sedimented cellular mass was retained as the source of enzyme.

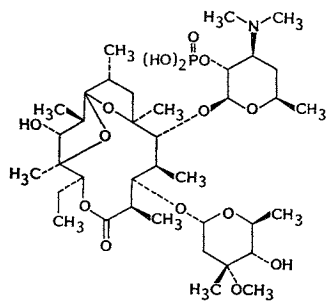
Biochemical

Sedimented cellular mass (100 g) was suspended in 100 ml of 100 mM potassium phosphate, pH 7, and was centrifuged as described previously. The washed cellular material was then resuspended in 100 ml of 10 mM potassium phosphate, pH 7.5, which contained EDTA at 500 mg/liter and was lysed by egg white lysozyme (Sigma, grade I) using the procedure of HEY and ELBEIN[6]. The resulting cell-free extract was used as the crude enzyme. Crude enzyme protein was quantitated by the Bio-Rad

Fig. 1. Structures of substrates and products.



1



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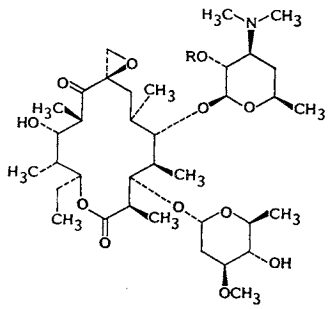
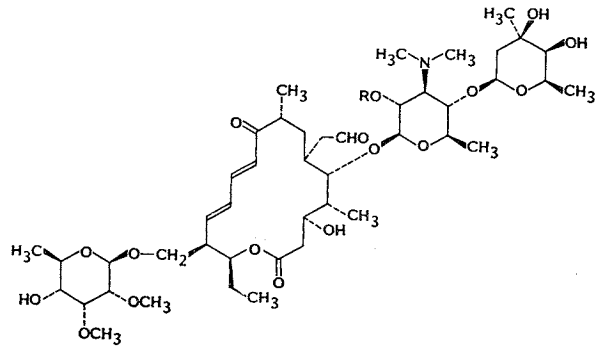


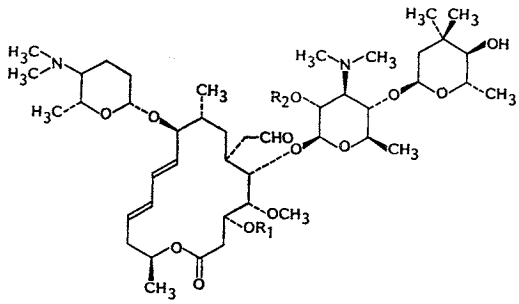
3a R = H

3b R = $\overset{O}{\overset{\|}{P}}(OH)_2$



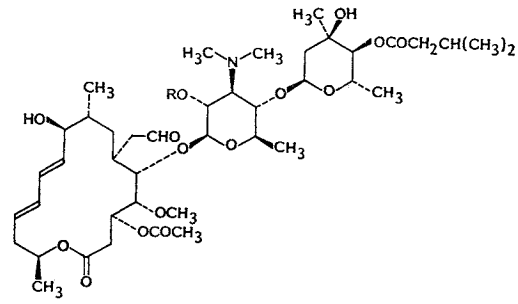
4a R = H

4b R = $\overset{O}{\overset{\|}{P}}(OH)_2$



5a R_1 = H, CH_3CO, CH_3CH_2CO
R_2 = H

5b R_1 = H, CH_3CO, CH_3CH_2CO
R_2 = $\overset{O}{\overset{\|}{P}}(OH)_2$



6a R = H

6b R = $\overset{O}{\overset{\|}{P}}(OH)_2$

method which is based on the procedure of BRADFORD[7]. The protein concentration of the enzyme preparation averaged 5 mg/ml.

Enzymatic phosphorylation of the macrolides was performed using reaction volumes ranging from 20 ml to 4 liters. Erlenmeyer flasks of sizes closely corresponding to the reaction volumes were employed as reaction vessels. The reaction mixtures contained nucleoside-5′-triphosphates (Sigma) 50 μmol, macrolide antibiotic 300 μg, enzyme protein 25 to 600 μg, $MgCl_2$ 40 μmol and potassium phosphate 5 μmol per ml of distilled water. These mixtures were adjusted to *ca.* pH 7 and the reac-

tions were stirred slowly using small magnetic stirring bars for the designated period of time at 25°C.

Analytical

Using the reaction conditions described the macrolide antibiotics were inactivated through conversion to their phosphates. Therefore, with the possible exception of the erythromycins, inactivation was considered to be equivalent to phosphorylation. A biological assay employing *Micrococcus luteus* UC 130 was used to quantitate the inactivations. One bio-unit of anti-*M. luteus* activity was defined as the amount of antibiotic that, when applied to a 12.7 mm paper disc (Schleicher and Schuell No. 740-E), produced a zone of growth inhibition of 20 mm on a seeded agar culture. Through comparison of the experimental bio-unit activities to those of standardized antibiotic solutions μg values were assessed.

Chemical

General Procedure

In all cases the initial isolation and purification was the same and is illustrated by the following procedure for leucomycin A_3.

A two-liter enzymatic conversion in which 400 mg of **6a** had been used as the substrate was passed over 85 ml of XAD-7. The column was washed with 200 ml of H_2O and eluted with 60% CH_3OH collecting 53×5 ml. On the basis of a weight analysis fractions $11 \sim 39$ were combined and evaporated *in vacuo* to a white solid, weight 320 mg. This material showed a single UV and I_2 visible spot, Rf 0.74, on TLC [SiO_2; $CH_3COOC_2H_5$ - 95% C_2H_5OH - 15% NH_4OAc (6 : 7 : 8)]. It was dissolved in 25 ml of H_2O, and the pH was adjusted to 4.40 with 0.1 N HCl. The solution was evaporated to dryness *in vacuo* and extracted with 15 ml of $CHCl_3$ filtering through a fine sinter glass funnel. Evaporation *in vacuo* gave 297 mg. The residue was chromatographed on 29 g of silica gel eluting with CH_3OH - H_2O (95 : 5) and collecting 110×4 ml. On the basis of a weight analysis and TLC in the above system fractions $11 \sim 24$ were combined and evaporated *in vacuo* to give 213 mg.

In all cases elemental analyses were relatively poor. On the basis of our analyses it was concluded that inorganic materials were present which were not removed by extensive efforts at purification including solvent extraction, resin chromatography and countercurrent distribution.

Anhydroerythromycin 2'-Phosphate (**2**)

A four-liter enzymatic conversion in which 1.2 g of **1** had been used as the substrate gave 400 mg of **2**. Two hundred mg was subjected to countercurrent distribution in a 335-ml Ito coil using the two phases of a $CHCl_3$ - CH_3OH - H_2O (5 : 2 : 5) system with the lower phase being the mobile phase and collecting 50×21 ml. On the basis of a weight analysis fractions $26 \sim 40$ were combined and evaporated *in vacuo*, weight 94 mg. TLC [SiO_2; $CH_3COOC_2H_5$ - 95% C_2H_5OH - 15% NH_4OAc (9 : 7 : 8)] showed a single spot, Rf 0.43, visualized with H_2SO_4 and I_2. The ^1H NMR and ^{13}C NMR (CD_3OD) were consistent with the proposed structure.

Fast atom bombardment (FAB)-MS *m/z* $(M-H)^-$ 794.4077.
Calcd for $C_{37}H_{65}NO_{15}P$, 794.4071.
Anal Calcd for $C_{37}H_{66}NO_{15}P$: C 55.83, H 8.31, N 1.76, P 3.89.
Found: C 54.52, H 8.51, N 1.60, P 4.03.

Oleandomycin 2'-Phosphate (**3b**)

A four-liter enzymatic conversion containing 1.2 g of oleandomycin was subjected to the general procedure for isolation and purification to give 338 mg of **3b**. TLC [SiO_2; CH_3OH - H_2O (9 : 1)] showed a single spot, Rf 0.33, detected with H_2SO_4 and I_2. The ^1H NMR and ^{13}C NMR (CD_3OD) were consistent with the proposed structure.

FAB-MS *m/z* $(M-H)^-$ 766.3770.
Calcd for $C_{35}H_{61}NO_{15}P$, 766.3778.
Anal Calcd for $C_{35}H_{62}NO_{15}P$: C 54.74, H 8.12, N 1.82, P 4.04.
Found: C 52.25, H 7.86, N 1.74, P 4.29.

Tylosin 2'-Phosphate (**4b**)

A four-liter enzymatic conversion, in which 1.2 g of **4a** as its tartrate was the substrate was sub-

Table 1. FAB-MS data on the phosphorus-containing fragments.

Compound	Fragment composition	Found	Calcd
Erythromycin	$C_8H_{19}NO_6P$	256.0956	256.0950
Oleandomycin	$C_8H_{17}NO_5P$	238.0854	238.0844
Tylosin	$C_8H_{17}NO_6P$	254.0793	254.0793
Spiramycins	$C_8H_{17}NO_6P$	254.0770	254.0793
Leucomycin A_3	$C_8H_{17}NO_6P$	254.0795	254.0793

jected to the general procedure for isolation and purification, gave 488 mg of **4b**. This was subjected to countercurrent distribution in a 335-ml Ito coil using the two phases of $CH_3COOC_2H_5$ - $CHCl_3$ - CH_3OH - H_2O (1 : 5 : 3 : 5) with the lower phase being used as the mobile phase and collecting 21 × 20 ml. The stationary phase was removed and evaporated *in vacuo*, weight 244 mg. TLC [SiO_2; $CH_3COOC_2H_5$ - 95% C_2H_5OH - 15% NH_4OAc (6 : 7 : 8)] showed a single UV visible spot, Rf 0.64. The ^1H NMR and ^{13}C NMR (CD_3OD) were consistent with the proposed structure.

FAB-MS *m/z* $(M-H)^-$ 994.4787.
Calcd for $C_{46}H_{77}NO_{20}P$, 994.4776.
Anal Calcd for $C_{46}H_{78}NO_{20}P$: C 55.47, H 7.89, N 1.41, P 3.11.
Found: C 54.46, H 7.75, N 1.25, P 3.53.

Spiramycins I, II, III 2′-Phosphate (**5b**)

A two-liter enzymatic conversion in which 600 mg of a mixture of spiramycins I, II, III had been used as the substrate was subjected to the general procedure for isolation and purification to give 288 mg of **5b**. TLC [SiO_2; $CH_3COOC_2H_5$ - 95% C_2H_5OH - 15% NH_4OAc (6 : 7 : 8)] showed a single UV visible spot, Rf 0.49.

FAB-MS *m/z* 922.4770, 978.4914.
Calcd for $C_{43}H_{75}N_2O_{17}P$, 922.4803.
Calcd for $C_{46}H_{79}N_2O_{18}P$, 978.5065.
Anal Calcd for $C_{45}H_{77}N_2O_{18}P$: P 3.21.
Found: P 2.73.

Leucomycin A_3 2′-Phosphate (**6b**)

The material from the general procedure was dissolved in 10 ml of H_2O, and the solution was adjusted to pH 4.40 with 0.1 N HCl. The solution was evaporated to dryness *in vacuo*. The residue was mixed with 15 ml of $CHCl_3$, and the mixture was filtered through a fine sinter glass funnel. The filtrate was evaporated to dryness *in vacuo*, weight 210 mg. This material was chromatographed on 21 g of silica gel eluting with CH_3OH - H_2O (95 : 5) and collecting 43 × 5 ml. On the basis of a TLC analysis [SiO_2; $CH_3COOC_2H_5$ - 95% C_2H_5OH - 15% NH_4OAc (6 : 7 : 8)] fractions 19～23 were combined and evaporated to dryness *in vacuo*, weight 54 mg. TLC in the above system showed a single UV visible spot, Rf 0.48. ^1H NMR and ^{13}C NMR (CD_3OD) were consistent with the proposed structure.

FAB-MS *m/z* $(M-H)^-$ 906.4263.
Calcd for $C_{42}H_{69}NO_{18}P$, 906.4252.
Anal Calcd for $C_{42}H_{70}NO_{18}P$: C 55.56, H 7.77, N 1.54, P 3.52.
Found: C 52.99, H 7.34, N 1.52, P 2.94, Ash 8.95.

Results and Discussion

The macrolides **1**, **3a**, **4a**, **5a** and **6a** were subjected to the action of *S. coelicolor* cell-free extract, and the products of the conversion were isolated and purified. The products were identified by high resolution mass spectrometry and elemental analysis. In all cases it was found that phosphorylation occurred on the hydroxyl group of the amino sugar as shown in **2**, **3b**, **4b**, **5b** and **6b**. FAB-MS peak matching of the fragments arising from the sugars identified those fragments containing the phosphorous

Table 2. Enzyme and cofactor requirements for conversion of tylosin tartrate.

Reaction conditions	μg Tylosin tartrate/ml				
	0 hour	17 hours	24 hours	41 hours	46 hours
Complete	250	125	54	0	0
Boiled enzyme	250	250	221	221	203
Minus enzyme	250	221	221	203	203
Minus Mg^{++}	250	221	221	203	203
Minus ATP	250	221	221	221	203

Reaction volumes were 20 ml and reaction conditions were those reported in Biochemical Methods. The components of the reaction mixtures were as listed in Biochemical Methods with crude enzyme protein being present at 400 μg/ml.

Fig. 2. Reaction pH optimum using tylosin tartrate as a substrate.

Reaction volumes were 20 ml and reaction rates were determined by assays performed at regular intervals throughout the first 24 hours of the reaction. The components of the reaction mixtures and the conditions of the reactions were as listed in Biochemical Methods with crude enzyme protein being present at 100 μg/ml.

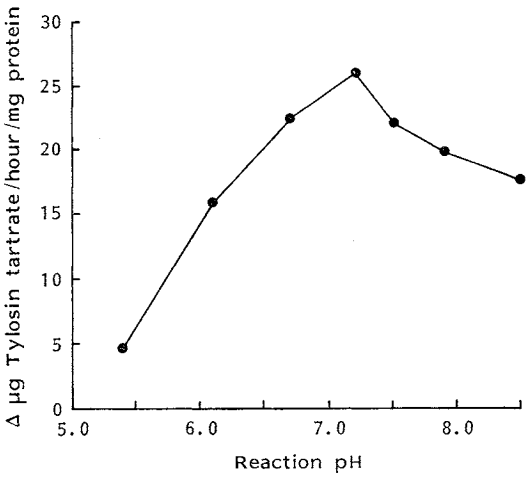


Fig. 3. Crude enzyme specific activity.

Reaction volumes employed were 20 ml and reaction rates were determined by assays performed at regular intervals throughout the first 24 hours of the reaction. The components of the reaction mixtures and conditions of the reactions were as listed in Biochemical Methods with tylosin tartrate being employed as the substrate.

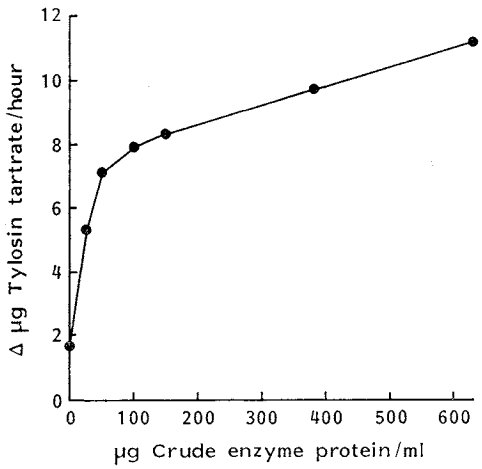


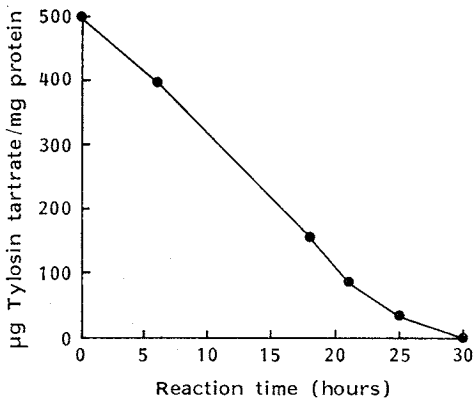
Table 3. Enzymic conversion of macrolide antibiotics.

Macrolide	μg Macrolide/ml			
	0 hour	18 hours	46 hours	72 hours
Erythromycin A	250	32	1	0
Erythromycin B	256	64	1	0
Oleandomycin	250	96	0	0
Leucomycin	251	117	7	0
Tylosin tartrate	250	96	39	0
Spiramycin	252	144	24	0

Reaction volumes were 20 ml and reaction conditions were those reported in Biochemical Methods. The components of the reaction mixtures were as listed in Biochemical Methods with crude enzyme protein being present at 400 μg/ml.

Fig. 4. Rate of enzymatic phosphorylation of tylosin tartrate.

Reaction volumes employed were 20 ml. The components of the reaction mixtures and the conditions of the reactions were as listed in Biochemical Methods with crude enzyme protein being present at 100 μg/ml.



atom and showed that they also were derived from the amino sugars (Table 1). In the earlier report[3], it was suggested that phosphorylation occurred on the cladinose moiety, but it has been found that this is not the case. In that report it was mistakenly published in the abstract that the macrolide converted was erythromycin B.

The high resolution mass spectrum of erythromycin A suggested that during either the enzymatic conversion or the workup, conversion to the anhydro compound (**2**) occurred. The ^{13}C NMR spectrum was consistent with such an interpretation as no peak for a ketonic carbonyl was observed.

The substrate for the spiramycin conversion was a mixture of spiramycins I, II and III, and no effort was made to separate the mixture of products which was formed. However, in all three of the spiramycins a high resolution mass spectrum of the phosphorylated fragment would be the same if phosphorylation occurred on the mycaminose moiety. It was found that only one fragment showed the expected $C_8H_{17}NO_6P$ composition.

Table 2 shows the biochemical requirements for the enzymatic phosphorylation of tylosin tartrate as catalyzed by a crude enzyme preparation of *S. coelicolor*. Data derived from the complete reac-

Table 4. Nucleoside-5′-triphosphate requirements for enzymic conversion of macrolide antibiotics.

Cofactor	Reaction period (hours)	Erythromycin A (μg/ml)	Erythromycin B (μg/ml)
ATP	0	144	144
	20	1	11
	48	1	1
UTP	0	144	144
	20	29	71
	48	2	1
ITP	0	144	144
	20	116	116
	48	116	116
CTP	0	144	144
	20	126	116
	48	126	116
GTP	0	144	144
	20	144	116
	48	126	116
None	0	144	144
	20	126	116
	48	126	116

Reaction volumes were 20 ml and reaction conditions were those reported in Biochemical Methods. The components of the reaction mixtures were as listed in Biochemical Methods with crude enzyme protein being present at 400 μg/ml.

tion mixture demonstrate that total inactivation occurred in 41 hours. However, in the presence of boiled enzyme or in the absence of ATP, Mg^{++} or native enzyme only minimum inactivation occurred. These data show the reaction to be enzyme catalyzed and to require ATP and Mg^{++}. Fig. 2 shows the pH optimum of this reaction using tylosin tartrate as a substrate.

The time course of inactivation with several macrolide substrates is shown in Table 3. The components are listed roughly in the order of their abilities to serve as substrates in this reaction. Fig. 3 shows crude enzyme specific activity using tylosin tartrate as a substrate. Fig. 4 shows the rate of enzymatic phosphorylation of tylosin tartrate.

Table 4 shows the specificity of crude enzyme for nucleoside-5′-triphosphates. These data indicate that only ATP and UTP function as cofactors in these reactions.

These results suggest that phosphorylation of other macrolide antibiotics containing the same type of amino sugar could be brought about by *S. coelicolor*.

Acknowledgment

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