MICROBIAL TRANSFORMATION OF ANTIBIOTICS CLINDAMYCIN RIBONUCLEOTIDES*

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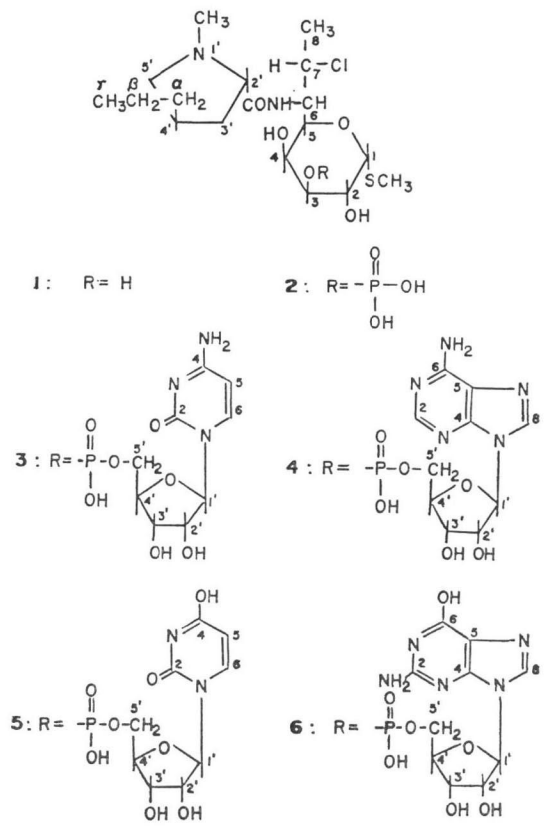
Addition of clindamycin to whole-cell cultures of *Streptomyces coelicolor* MÜLLER resulted in the loss of *in vitro* activity against organisms sensitive to clindamycin. Incubation of such culture filtrates with crude alkaline phosphatase generated a biologically active material identified as clindamycin. Fermentation broths containing inactivated clindamycin yielded clindamycin 3-ribonucleotides and clindamycin 3-phosphate the structure of which was established by physicochemical and enzymatic means. Attempts to transform clindamycin to clindamycin 3-ribonucleotides by lysates or partially purified enzyme preparations from *S. coelicolor* have failed.

In the past few years we have been involved in studies related to biomodification of antibiotics by actinomycetes. Previous papers have described the acylation of chloramphenicol by *Streptomyces coelicolor*[1], the phosphorylation of lincomycin by *Streptomyces rochei*[2], the transformation of clindamycin (**1**) to 1-demethylclindamycin and clindamycin sulfoxide by *Streptomyces punipalus* and *Streptomyces armentosus*[3] and the phosphorylation of clindamycin by whole cells and lysates of *Streptomyces coelicolor*[4]. Further studies have shown that whole cells (but not cell-free extracts) of *S. coelicolor* transform clindamycin to a mixture of clindamycin 3-phosphate and clindamycin 3-ribonucleotides. The present paper discusses the production, isolation, chemistry and biological properties of these clindamycin bioconversion products.

Fig. 1



Production and Isolation of Clindamycin 3-Ribonucleotides and Clindamycin 3-Phosphate

In our initial paper[4] describing the phosphorylation of clindamycin by *S. coelicolor* we

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reported that clindamycin was inactivated when added to 24-hour cultures of the organism grown in a complex medium. Clindamycin could be regenerated by treatment of the bioinactive fermentation broth with crude alkaline phosphatase. This observation led us to the isolation of clindamycin 3-phosphate. However, during the isolation studies we observed the presence of unidentified compounds that also yielded clindamycin when incubated with crude alkaline phosphatase. We decided therefore to study further the products of biotransformation of clindamycin by whole cell cultures of *S. coelicolor*.

The fermentation conditions used in the present study were identical with those reported earlier[4]. Clindamycin (50 mcg/ml) was added to whole cell cultures of *S. coelicolor* 24 hours after inoculation. Cultures were harvested after 48 hours of fermentation at which time clindamycin was no longer detectable. Clindamycin could be regenerated by the treatment of the bioinactive fermentation broth with either crude alkaline phosphatase or snake venom phosphodiesterase. Since clindamycin 3-phosphate was not converted to clindamycin by venom phosphodiesterase we concluded that *S. coelicolor* converted clindamycin not only to clindamycin 3-phosphate but, in addition, to compound(s) containing phosphodiester bonds.

Fermentation and isolation studies were followed by *in vitro* assays *vs. Sarcina lutea* before and after treatment of the sample under evaluation with crude alkaline phosphatase. This crude enzyme preparation exhibited both phosphomono- and phosphodiesterase activities. Fractions containing the clindamycin bioconversion products did not show any bioactivity before treatment with the enzyme. Incubation of these fractions with alkaline phosphatase afforded clindamycin which was easily identified by TLC.

The clindamycin bioconversion products, which for convenience would be designated as clindamycin 3-ribonucleotides and clindamycin 3-phosphate, were isolated from culture filtrates by adsorption on Amberlite XAD-2 followed by elution with aqueous methanol. Chromatography of the methanolic eluates on Dowex-1 (acetate) resulted in the separation of clindamycin 3-ribonucleotides (isolated from the spent) from most of clindamycin 3-phosphate (eluted from the column with 5% aqueous acetic acid). Amberlite XAD-2 chromatography of the crude clindamycin 3-ribonucleotides followed by counter double current distribution (1-butanol-water, 1:1 v/v) yielded a mixture of bioinactive, UV-absorbing compounds which afforded clindamycin by treatment with venom phosphodiesterase. Chromatography of the mixture on DEAE-Sephadex (acetate) using *tris*-acetate (pH 8.0, 0.1~0.2 M) buffer gave eight compounds designated A, B, C, D, E, F, G and H in order of elution from the column

Fig. 2. DEAE-Sephadex chromatography of clindamycin ribonucleotides

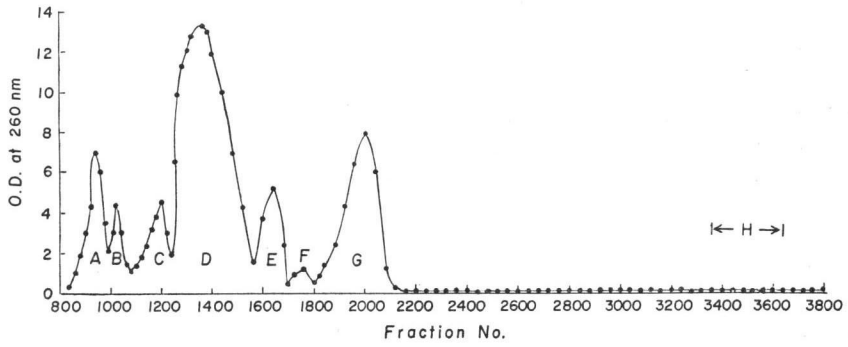


Table 1. Analytical data obtained on compounds A, D, E, G and H

Compound	Mol. formula	Calculated						Found					
		C	H	N	Cl	P	S	C	H	N	Cl	P	S
A	$C_{27}H_{45}N_5O_{12}ClPS$	44.48	6.17	9.60	4.87	4.25	4.39	45.62	6.99	9.80	4.04	3.44	4.61
D	$C_{28}H_{45}N_7O_{11}ClPS$	44.63	6.05	13.07	4.72	4.11	4.28	44.77	6.66	12.57	4.38	3.52	4.65
E	$C_{27}H_{44}N_4O_{13}ClPS$	44.27	6.33	7.68	4.86	4.24	4.39	44.62	6.19	7.79	4.32	4.22	4.04
G	$C_{28}H_{45}N_7O_{12}ClPS$	43.71	5.85	12.74	4.61	4.03	4.16	43.69	6.34	11.62	4.15	3.81	3.63
H	$C_{18}H_{34}N_2O_8ClPS$	42.85	6.79	5.55	7.02	6.15	6.35	42.91	7.04	6.34	6.74	5.29	5.56

Table 2. Characterization data

Compound	Mol. formula	Mol. weight		$[\alpha]_D^{25}$ [b]	$[M]_D$	UV $[\lambda_{max}$ ($\varepsilon \times 10^{-3}$)] [c]		
		Calcd	Found [a]			pH 2.0	pH 7.0	pH 11.0
A	$C_{27}H_{45}N_5O_{12}ClPS$	729	742	+61°	+445°	279 (9.60)	269 (6.80)	271 (6.60)
D	$C_{28}H_{45}N_7O_{11}ClPS$	753	726	+62.9°	+473°	257 (12.60)	261 (12.50)	261 (12.70)
E	$C_{27}H_{44}N_4O_{13}ClPS$	732	764	+79.5°	+578°	261 (8.20)	262 (8.40)	262 (6.50
G	$C_{28}H_{45}N_7O_{12}ClPS$	769	750	+69°	+530°	256 (11.10) 277 (7.50) (sh)	254 (12.50) 273 (8.00) (sh)	259 (10.70) 266 (10.60)
H	$C_{18}H_{34}N_2O_8ClPS$	504	530	+91.3°	+458°	No UV absorption		

[a] Molecular weights were determined by vapor pressure osmometry in methanol.

[b] Specific rotation was determined in water (*c* 1).

[c] Reported for: cytidine 5′-phosphate, pH 2.0, 280 (13,200); pH 7.0, 271 (9,100); pH 11.0, 271 (9,100). Adenosine 5′-phosphate, pH 2.0, 257 (15,000); pH 7.0, 259 (15,400); pH 11.0, 259 (15,400). Uridine 5′-phosphate, pH 2.0, 262 (10,000); pH 7.0, 262 (10,000); pH 11.0, 261 (7,800). Guanosine 5′-phosphate, pH 1.0, 256 (12,200); pH 7.0, 252 (13,700); pH 11.0, 258 (11,600).

(Fig. 2). All but compound H yielded clindamycin by treatment with venom phosphodiesterase and were followed during the chromatography by UV absorption at 260 nm. Compound H showed no UV absorption, was not effected by treatment with venom phosphodiesterase but yielded clindamycin by treatment with alkaline phosphatase. Removal of THAM-acetate buffer and isolation of pure compounds A, B, C, D, E, F, G and H was done by chromatographies over Amberlite XAD-2. Characterization of compounds A, D, E, G and H, the main components of the mixture, is discussed in the next section. Compounds B, C and F are not completely characterized. Possible structures for these compounds are proposed in the later part of this paper.

Characterization and Structure of Clindamycin 3-Ribonucleotides (A, D, E, G) and Clindamycin 3-Phosphate (H)

Clindamycin 3-ribonucleotides (compounds A, D, E and G) and clindamycin 3-phosphate (compound H) were isolated as amorphous colorless materials soluble in water and lower alcohols and practically insoluble in acetone, ethyl acetate and chlorinated and saturated hydrocarbon solvents. All five compounds are amphoteric and like clindamycin, contain a basic group, pKa′ *ca.* 7.5~7.7 (water). Analytical data obtained on compounds A, D, E, G and H (Table 1) combined with molecular weight determinations (vapor pressure osmometry in methanol; Table 2) indicated the molecular formulas shown in Tables 1 and 2. The IR spectra of compounds A, D, E, G and H, though different from each other, showed common features like broad absorption at 3300~3100 cm^{-1} (-OH; -NH) and strong amide carbonyl absorption (1685~1675 cm^{-1}, amide I; 1550~1530 cm^{-1}, amide II).

Compounds A, D, E and G showed UV absorptions (Table 2 and Fig. 3) identical to those reported for cytidine, adenosine, uridine and guanosine phosphate, respectively. Compound H showed no UV maxima.

Compound H was identified as clindamycin-3-phosphate (**2**, Fig. 1) by comparison ($[\alpha]_D$, IR and NMR spectra) to an authentic sample[4]. This material afforded clindamycin by treatment with alkaline phosphatase (Table 4) but remained unchanged after incubation with either snake venom or spleen phosphodiesterases.

The molecular formulae of compounds A, D, E and G (specifically the presence of one P atom per molecule), the UV spectra and the conversion of these compounds to clindamycin by treatment with crude alkaline phosphatase and venom phosphodiesterase suggested structures for these compounds in which clindamycin is linked to the phosphate group of cytidine phosphate (compound A), adenosine phosphate (compound D), uridine phosphate (compound E) and guanosine phosphate (compound G). Enzymatic and chemical hydrolytic studies, periodate oxidations, and PMR and CMR spectra (Table 7) established the structures of compound A as clindamycin 3-(5′-cytidylate) (**3**), compound D as clindamycin 3-(5′-adenylate) (**4**), compound E as clindamycin 3-(5′-uridylate) (**5**) and compound G as clindamycin 3-(5′-guanylate) (**6**) (Fig. 1).

The assignment of the nucleoside-5′-phosphate clindamycin linkage was established by enzymatic studies. Enzymatic and chemical hydrolyses were followed by TLC. The Rf values of the clindamycin 3-ribonucleotides (compounds A, D, E and G) and their degradation products are recorded in Table 3. Treatment of the four

Fig. 3. UV spectra of clindamycin 3-ribonucleotides.

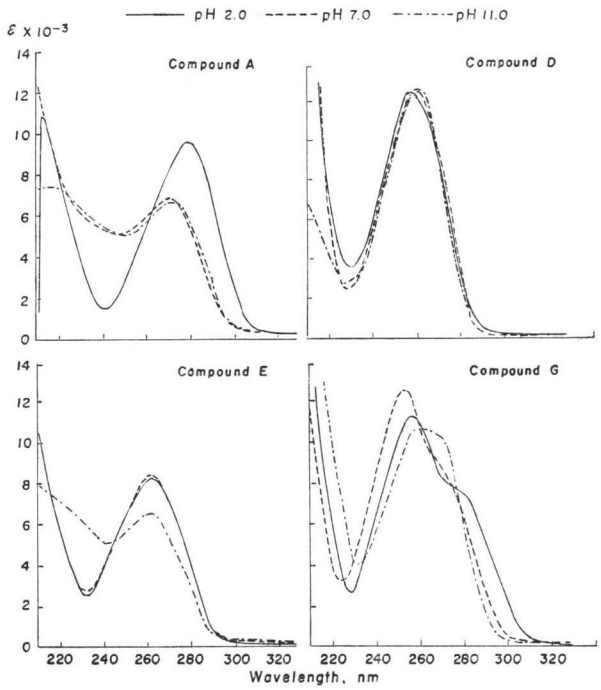


Table 3 Thin-layer chromatography of clindamycin 3-ribonucleotides and their degradation products

Compound	Rf[a]		
	A[b]	B[c]	C[d]
Clindamycin 3-(5′-cytidylate) (A)	0.18	0.77	—
Cytosine	0.66	0.72	0.22
Cytidine	0.81	0.69	0.14
Cytidine 5′-phosphate	0.88	0.42	0.05
Clindamycin 3-(5′-adenylate) (D)	0.12	0.77	0.50
Adenine	0.54	0.84	0.35
Adenosine	0.73	0.79	0.24
Adenosine 5′-phosphate	0.86	0.62	0.05
Adenosine 3′-phosphate	0.86	0.70	0.06
Clindamycin 3-(5′-uridylate) (E)	0.25	0.75	—
Uracil	0.88	0.72	0.46
Uridine	0.93	0.66	0.29
Uridine 5′-phosphate	0.96	0.41	0.09
Clindamycin 3-(5′-guanylate) (G)	0.21	0.77	—
Guanine	—	0.73	0.16
Guanosine	0.90	0.70	0.13
Guanosine 5′-phosphate	0.93	0.38	0.05

[a] Spots were detected by a short wave length UV lamp.
[b] A: Silica gel GF plates (Analtech, Inc.); water as the solvent system.
[c] B: Silica gel GF plates (Analtech, Inc.); *n*-propyl alcohol-conc. ammonium hydroxide - water (55: 10: 35).
[d] C: MN-Polygram Cellulose 300 (Brinkman Instruments, Inc.); solvent: *n*-butyl alcohol - water - formic acid (77: 13: 10).

Table 4. Compounds[a] produced by degradation of clindamycin-ribonucleotides

Compound	Enzymatic hydrolysis			Chemical hydrolysis		
	Crude alkaline phosphatase[b]	Snake venom diesterase[c]	Spleen diesterase[c]	1 N HCl[d]	6 N HCl or 72% ClO_4H[e]	0.1 N NaOH[f]
A	Clindamycin; cytidine	Clindamycin; cytidine-5′-P	No reaction	Cytidine	Cytidine Cytosine	Cytidine
D	Clindamycin; adenosine	Clindamycin; adenosine-5′-P	No reaction	Adenine ribose	Adenine	Adenosine
E	Clindamycin; uridine	Clindamycin; uridine-5′-P	No reaction	Uridine	Uridine Uracil	Uridine
G	Clindamycin; guanosine	Clindamycin; guanosine-5′-P	No reaction	Guanine ribose	Guanine	Guanosine
H	Clindamycin	No reaction	No reaction	—	—	—

[a] Identified by TLC (Silica Gel GF) in three solvent systems.
[b] Adenine and guanine were isolated as the crystalline hydrochlorides.
[c] Crude alkaline phosphatase (Worthington Biochemicals) exhibited both mono- and diesterase activities.
[c] Worthington Biochemicals Corp., Freehold, New Jersey.
[d] 100°C, 1 hour.
[e] 100°C, 1 hour.
[f] 100°C, 2.5 hours.

clindamycin 3-ribonucleotides A, D, E and G with crude alkaline phosphatase (Table 4) yielded, as expected, clindamycin and cytidine, adenosine, uridine and guanosine. Venom phosphodiesterase* afforded clindamycin and the corresponding nucleoside 5′-phosphates; cytidine 5′-phosphate (compound A), adenosine 5′-phosphate (compound D), uridine 5′-phosphate (compound E) and guanosine 5′-phosphate (compound G) (Table 4). Furthermore, spleen phosphodiesterase** failed to degrade

Table 5. Periodate consumption[a,b]

Compound	Time (hour)							
	0.16	0.3	0.5	1	2	3	4	5
Clindamycin	1.98	2.34	2.50	2.52	2.60	2.60	2.74	3.01
Clindamycin-3-(5′-cytidylate) (A)	1.34	1.54	1.93	2.06	2.11	2.11	2.15	2.25
Cytidine	1.08	1.04	1.07	1.11	1.09	1.17	1.12	—
Cytidine 5′-phosphate	0.83	0.83	—	0.90	0.83	0.90	0.90	—
Cytidine 3′-phosphate	0.02	0.14	0.23	0.11	0.02	0.00	0.00	—
Clindamycin-3-(5′-adenylate) (D)	1.28	1.56	1.65	1.56	—	1.80	2.03	2.20
Adenosine	0.98	1.13	1.13	1.05	—	1.23	—	1.03
Adenosine 5′-phosphate	0.95	1.12	1.03	0.95	—	1.18	1.02	—
Clindamycin-3-(5′-uridylate) (E)	1.37	1.63	1.93	2.15	2.15	2.22	2.18	2.29
Uridine	0.98	0.97	0.98	1.05	1.10	1.13	1.16	—
Uridine 5′-phosphate	0.82	0.79	0.90	1.02	0.90	1.06	0.90	—
Clindamycin-3-(5′-guanylate) (G)	1.40	1.59	1.59	1.62	1.68	—	1.97	—
Guanosine 5′-phosphate	0.73	0.75	0.77	0.77	0.78	—	0.80	0.84
Guanosine 3′-phosphate	0.05	0.05	0.00	0.00	0.01	—	0.00	0.00

[a] Moles of periodate per mole of the compounds listed at the designated time intervals.
[b] For details *see* Experimental.

* Snake venom phosphodiesterase (phosphodiesterase I) catalyzes the hydrolysis of oligonucleotides (ribo- or deoxyribo-) with free 3′-hydroxyl end group to yield mononucleoside 5′-phosphates[5].

** Spleen phosphodiesterase (phosphodiesterase II) acts as an exonuclease and yields nucleoside-3′-phosphates. A free 5′-hydroxyl group is necessary[5].

Table 6. Proton magnetic resonance spectra of clindamycin 3-ribonucleotides. Chemical Shift (δ)[a,b]

Compounds[c]				Assignment[c]
Clindamycin 3-(5′-cytidylate) (A)	Clindamycin 3-(5′-adenylate) (D)	Clindamycin 3-(5′-uridylate) (E)	Clindamycin 3-(5′-guanylate) (G)	
0.92, t, 3H	0.91, t, 3H	0.91, t, 3H	0.90, t, 3H	$^{7}CH_3CH_2$-
1.48, d, 3H	1.41, d, 3H	1.48, d, 3H	1.41, d, 3H	$^{8}CH_3CHCl$-
2.20, s, 3H	2.00, s, 3H	2.18, s, 3H	2.05, s, 3H	-SCH_3
2.91, s, 3H	2.90, s, 3H	2.90, s, 3H	2.90, s, 3H	-NCH_3
4.0～4.9, complex	3.9～4.9, complex	4.0～4.9, complex	2.9～4.9, complex	-CHOH (clindamycin and ribose)
5.42, d, 1H	5.32, d, 1H	5.42, d, 1H	5.35, d, 1H	Anomeric H (clindamycin)
6.03, d, 1H	6.11, d, 1H	5.91, d, 1H	5.90, d, 1H	Anomeric H (ribose)
6.18, d, 1H	8.19, d, 1H	6.04, d, 1H	8.10, s, 1H	Purine and
7.93, d, 1H	8.48, d, 1H	7.95, d, 1H		pyrimidine protons

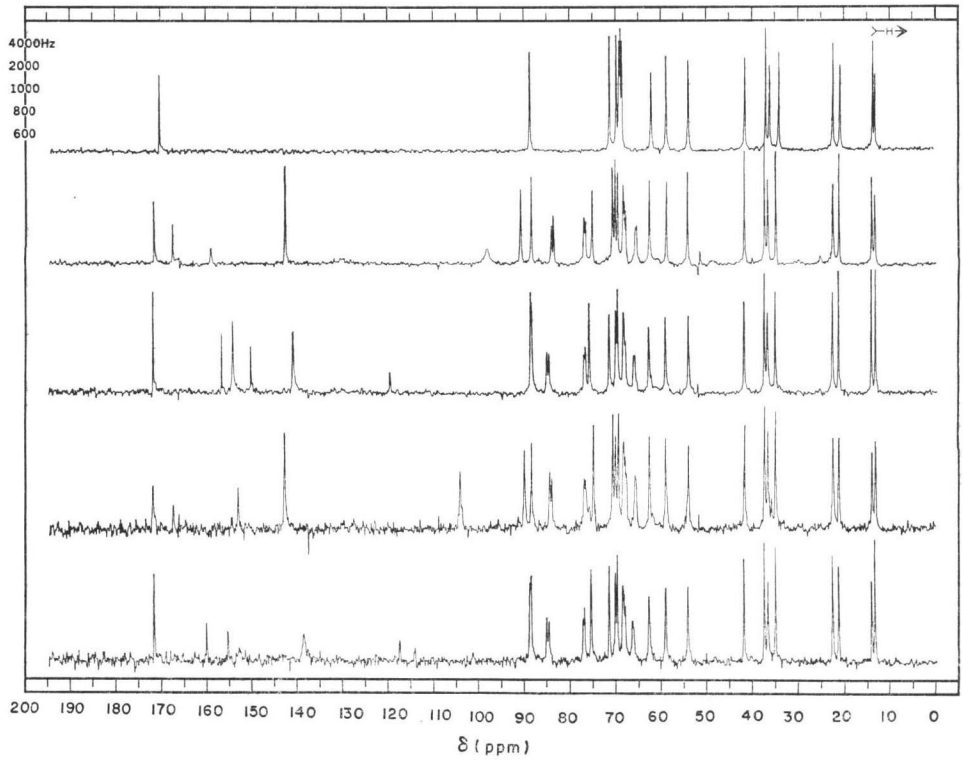
[a] D_2O was used as solvent.
[b] Multiplicity of absorption signals: s=singlet; d=doublet; t=triplet.
[c] See structures in Fig. 1.

Table 7. ^{13}C-NMR Spectra of clindamycin and clindamycin-3-ribonucleotides. Chemical shift (δ)[a]

Clindamycin (1)	Compounds[b]				Assignment[c]	
	A (3)	D (4)	E (5)	G (6)		
88.7	88.1	88.2	88.2	88.2	C-1	Clindamycin[e] part
68.5	68.0[d]	67.9[d]	68.0[d]	67.9[d]	C-2	
71.2	76.3	76.3	76.4	76.3	C-3	
69.1	67.5[d]	67.4[d]	67.5[d]	67.5[d]	C-4	
68.9	69.2	69.2	69.2	69.1	C-5	
54.0	53.8	53.6	53.7	53.6	C-6	
58.9	58.5	58.7	58.8	58.5	C-7	
22.8	22.5	22.6	22.5	22.5	C-8	
13.6	13.3	13.2	13.3	13.2	-SCH_3	
69.7	69.7	69.5	69.7	69.5	C-2′	
36.5	36.6	36.6	36.6	36.4	C-3′	
37.3	37.3	37.3	37.3	37.2	C-4′	
62.1	62.2	62.3	62.3	62.1	C-5′	
34.5	34.9	35.0	35.0	34.8	α-CH_2	
21.3	21.2	21.3	21.2	21.1	β-CH_2	
14.1	14.0	14.1	14.0	13.9	γ-CH_3	
41.8	41.6	41.6	41.6	41.6	-N-CH_3	
169.9	171.0	171.3	171.3	170.8	-Amide carbonyl	
—	90.4	88.0	89.7	88.0	C-1′	Ribose[f] part
—	70.3	70.9	70.3	70.8	C-2′	
—	74.7	75.3	74.5	74.8	C-3′	
—	83.4	84.4	83.9	84.2	C-4′	
—	65.2	65.4	65.3	65.6	C-5′	
—	158.6	153.7	152.6	154.6	C-2	Purine or pyrimidine part[f]
—	166.9	149.6	166.9	152.1	C-4	
—	97.8	119.3	103.9	117.0	C-5	
—	142.1	156.2	142.4	159.3	C-6	
—	—	140.4	—	137.9	C-8	

[a] Relative to tetramethylsilane using dioxane as an internal standard; D_2O was used as solvent.
[b] Compounds: A, clindamycin-3-(5′-cytidylate), (3); D, clindamycin 3-(5′-adenylate) (4); E, clindamycin 3-(5′-uridylate) (5); G, clindamycin 3-(5′-guanylate).
[c] See structures in Fig. 1.
[d] Assignments could be reversed.
[e] For discussion of ^{13}C-NMR spectra of clindamycin, see Tetrahedron Letters 1977; 721～724, 1977
[f] For ^{13}C-NMR spectra of purine and pyrimidine nucleosides and nucleotides, see Ref. 7 and 8.

Fig. 4. ^{13}C-NMR spectra of clindamycin and clindamycin-3-ribonucleotides
The order of the spectra from top to bottom is: Clindamycin and compounds A, D, E and G.



the clindamycin 3-ribonucleotides A, D, E and G.

The assignment of the phosphate diester linkage at the C-3 position of the aminosugar moiety of clindamycin is based on periodate oxidation studies.* In this oxidation (Table 5), it was found that cytidine, adenosine, guanosine and their respective 5′-phosphates consumed one mole of periodate in less than 15 minutes with no overoxidation. It was also found that clindamycin rapidly consumed two moles of periodate by cleavage of the glycol groupings at C-2, C-3 and C-4 and slowly an additional mole by oxidation of the sulfur. Compounds A, D, E and G consumed two moles of periodate (one rapidly and one slowly) which indicates phosphodiester attachment at C-3 since alternative attachments (C-2 or C-4) would require consumption of three moles of periodate.

The results obtained by chemical hydrolyses (Table 4) support the structures postulated above. As expected[6], the purine ribonucleotides (compounds D and G) afforded purines** and ribose*** by treatment with 1 N HCl while the pyrimidine nucleotides A and E gave the corresponding nucleosides, cytidine and uridine respectively. Hydrolysis of all four nucleotides with 6 N HCl or 72% perchloric acid resulted in liberation of purine** or pyrimidine bases, while treatment with 0.1 N NaOH gave in all cases the corresponding nucleosides.

The PMR spectra of compounds A, D, E and G (Table 6) are in agreement with the assigned struc-

* See also arguments based on CMR spectra.
** Adenine and guanine were isolated crystalline and compared to authentic materials.
*** Ribose was identified by TLC [silica gel G, methyl ethyl ketone - acetone - water (186: 52: 20, v/v)].

Table 8. CMR-chemical shifts reported[a] for purine and pyrimidine ribonucleosides-5′-phosphates.

Compounds[c]				Assignment	Chemical Shift (δ)[b]
Cytidine-5′-phosphate	Adenosine-5′-phosphate	Uridine-5′-phosphate	Guanosine-5′-phosphate		
90.0	87.9	89.4	87.9	C-1′	Ribose part
70.6	71.4	71.0	71.5	C-2′	
75.2	75.4	74.9	75.2	C-3′	
84.2	85.1	84.9	85.0	C-4′	
64.2	64.4	64.4	64.8	C-5′[d]	
158.4	153.3	152.7	154.5	C-2	
167.1	149.3	167.1	151.9	C-4	
97.4	118.9	103.5	116.6	C-5	
142.7	155.9	143.0	159.2	C-6	
—	140.8	—	138.2	C-8	

[a] See Ref. 7 and 8.
[b] Relative to tetramethylsilane.
[c] See structures in Fig. 1.
[d] The chemical shift of C-5′ of cytidine, adenosine, uridine and guanosine appears at δ 61.9, 62.7, 61.9 and 62.2 respectively.

tures **3**, **4**, **5** and **6** respectively. All compounds contain the $CH_3CH_2CH_2$-, CH_3CHCl, -SCH_3 and -NCH_3 groups present in clindamycin. Furthermore, the spectra of all four ribonucleotides have absorptions due to the anomeric protons at C-1 of clindamycin and C-1′ of ribose. In addition, absorptions due to the protons of the respective pyrimidine or purine nucleus were also observed.

CMR studies show that a simple comparison of the spectra of clindamycin and of the ribonucleotides A, D, E and G (Table 7 and Fig. 4) is sufficient to establish the structure of these compounds. As shown in Table 7 there is an excellent agreement between the chemical shifts of 17 of the carbons of clindamycin and the corresponding carbons of the clindamycin 3-ribonucleotides. The chemical shift of the remaining carbon (C-3) of clindamycin appears at δ 71.2 while C-3 of compounds A, D, E and G have chemical shifts of δ 76.3~76.4 (see Table 7). This is due to the deshielding effect of the phosphate group attached at C-3 of these compounds[7]. Furthermore, the chemical shifts of the carbons of ribose and the purine or pyrimidine moieties of compounds A, D, E and G are almost identical to the chemical shifts (see Table 8) of the corresponding nucleoside 5′-phosphates, *i.e.* cytidine 5′-phosphate, adenosine 5′-phosphate, uridine 5′-phosphate and guanosine 5′-phosphate respectively. It should be noted that the chemical shifts of C-5′ of the nucleoside 5′-phosphates (δ 64.2~64.8, Table 8) and of the clindamycin 3-ribonucleotides (δ 65.2~65.6, Table 7) are higher than the chemical shifts of the corresponding nucleosides (δ 61.9~62.7). This is also due to the deshielding effect of the phosphate group at C-5′ of ribose. We conclude, therefore, that the CMR studies indicate a phospho diester* bond between C-3 of clindamycin and C-5′ of the ribose moiety and specifically that compounds A, D, E and G have structures **3**, **4**, **5** and **6** respectively.

* The ^{31}P-^{13}C coupling constants (Hz) observed were as follows:
$J_{P\text{-}C\text{-}5'}$ (ribose): 3.9 (comp. A); 4.2 (comp. D); 3.7 (comp. E) and 5.2 (comp. G). $J_{P\text{-}C\text{-}4'}$ (ribose): 8.3 (comp. A); 8.4 (comp. D); 7.9 (comp. E) and 9.6 (comp. G). $J_{P\text{-}C\text{-}3}$ (clindamycin): 5.7 (comp. A); 5.5 (comp. D); 5.7 (comp. E) and 5.6 (comp. G). $J_{P\text{-}C\text{-}2}$ or $_{C\text{-}4}$ (clindamycin): 5.8 (comp. A); 4.7 (comp. E) and 5.8 (comp. G).

Nature of Compounds B, C and F

The isolation of compounds B, C and F was mentioned earlier in this paper (see Fig. 2). Compound B had UV spectra identical to those of clindamycin 3-(5′-adenylate) (**4**). Treatment with alkaline phosphatase resulted in production of clindamycin sulfoxide and adenosine. Spleen phosphodiesterase did not affect compound B, while venom phosphodiesterase yielded clindamycin sulfoxide and adenosine 5′-phosphate. This enzymatic behavior suggests the clindamycin sulfoxide-(5′-adenylate) structure for compound B. The point of the phosphodiester attachment at the aminosugar moiety of clindamycin is not known. The formation of clindamycin sulfoxide-(5′-adenylate) is not surprising since clindamycin can be transformed to clindamycin sulfoxide by streptomycetes[9].

Compound C had UV, IR and NMR spectra identical to those of clindamycin 3-(5′-adenylate) (**4**). Compound C yielded clindamycin and adenosine or adenosine 5′-phosphate by treatment with alkaline phosphatase or venom phosphodiesterase, respectively, and was not affected by spleen phosphodiesterase. These data indicate a clindamycin-(5′-adenylate). The clindamycin site of the phosphodiester linkage is not known. A clindamycin 4-(5′-adenylate) structure is possible and would be produced by rearrangement of the nucleotide moiety from C-3 to C-4 of clindamycin under the isolation conditions.

Compound F had UV, IR and enzymatic behavior identical to that of clindamycin 3-(5′-guanylate) (**6**). We consider the structure of clindamycin 4-(5′-adenylate) for compound F although the point of attachment of the phosphodiester bond at C-4 of clindamycin has not been established.

Biological Properties of Clindamycin 3-Ribonucleotides

As mentioned earlier, the isolated clindamycin 3-ribonucleotides and clindamycin 3-phosphate were inactive *in vitro* against several organisms including *Staphylococcus aureus*. However, these compounds were found to protect *S. aureus* infected mice when they were administered subcutaneously. The observed CD_{50}'s[9] were as follows: clindamycin 3-(5′-adenylate), 30 mg/kg; clindamycin 3-(5′-uridylate), 37 mg/kg; clindamycin 3-(5′-guanylate), 26 mg/kg and clindamycin 3-phosphate, 9.8 mg/kg. The *in vivo* activity of the clindamycin 3-ribonucleotides and clindamycin 3-phosphate is presumably due to clindamycin and/or its bioactive metabolites produced by biotransformation of these compounds.

Attempts to Obtain Clindamycin 3-Ribonucleotides by Using Cell-Free Extracts of *S. coelicolor*

In the preliminary report of the present work we noted that the formation of clindamycin 3-ribonucleotides resembles the adenylation of streptomycin and other aminocyclitol antibiotics by enzymes isolated from R-factor carrying *Escherichia coli* strains and from strains of *Pseudomonas aeruginosa* and *S. aureus*[10].

Although adenylating enzymes are easily isolated from *E. coli* we have been unable to obtain enzyme preparations from *S. coelicolor* capable of forming the nucleotides of clindamycin in a cell-free system. Crude lysates and purified enzyme preparations were obtained from cultures of *S. coelicolor* by the procedures described by COATS and ARGOUDELIS[4]. Clindamycin was incubated under a variety of conditions with crude lysates or enzyme preparations of different degree of purity. Adenosine, cytidine, uridine and guanosine 5′-triphosphate alone or in mixture were used. In all cases clindamycin was rapidly inactivated. However, clindamycin 3′-phosphate was the only product of

inactivation.

Enzymatic reactions involving ribonucleotides and leading "active intermediates" (aminoacid activation, coenzyme A biosynthesis) are well known. However, the described biotransformation of clindamycin is the first reported example of modification of an antibiotic involving either adenosine-5′-phosphate (adenylation) or one of the remaining 5-ribonucleotides, guanosine-5′-phosphate, cytidine-5′-phosphate and uridine-5′-phosphate.

Experimental

Spectroscopic Methods

Proton magnetic resonance spectra were recorded on a Varian XL-100-15 spectrometer operating at 100 MHz. Spectra were run in D_2O using sodium 2,2-dimethyl-2-silapentane-5-sulfonate (SDSS) as internal reference.

Carbon magnetic resonance spectra were recorded on a Varian CFT-20 spectrometer. D_2O was used as the solvent and dioxane as an internal reference.

PMR and CMR chemical shifts are reported as ppm relative to tetramethylsilane.

Infrared spectra were obtained in mineral oil suspension on a Digilab Model 14D FOURIER Transform Spectrometer.

Assay of Clindamycin and Clindamycin Bioconversion Products

Since clindamycin 3-ribonucleotides and clindamycin 3-phosphate lack *in vitro* antibacterial activity, their formation from clindamycin can be followed easily by measuring the loss of such antibiotic activity. To determine the amounts of clindamycin in culture filtrates or reaction mixtures a standard assay with *Sarcina lutea* ATCC 9341 as described by COATS and ARGOUDELIS[4] was employed. To assay for the presence of clindamycin 3-ribonucleotides and clindamycin 3-phosphate in fermentation beers, extracts and purified materials, the phosphodiester or phosphate ester bond was first hydrolyzed with crude alkaline phosphatase by the procedures described below. Clindamycin in the hydrolysate was determined by standard assay.

Enzymatic Hydrolyses

Alkaline Phosphatase: Stock solutions (0.5 mg/ml) of calf intestine alkaline phosphatase, EC 3.1.3.1 (Calbiochem) were prepared in *tris* (hydroxymethyl) aminomethane (*Tris*)-hydrochloride buffer, 0.5 M pH 8.0. Samples to be treated were diluted 1 : 2 with the enzyme buffer mixture and were incubated at 28°C for 18 hours.

Snake Venom Phosphodiesterase: Stock solutions (1 mg/ml) of purified snake venom phosphodiesterase (Worthington Biochemicals, Freehold, N.J.) were prepared in distilled water. Incubation mixtures contained 0.3 ml of a solution (1 mg/ml) of the sample to be treated in water, 0.2 ml of 0.1 M *Tris*-hydrochloride buffer, pH 8.6 and 0.1 ml of the enzyme stock solution. Incubation was carried out at 28°C for 18~24 hours.

Spleen Phosphodiesterase: Stock solutions of spleen phosphodiesterase (Worthington Biochemicals) were prepared in distilled water so that the activity (Worthington) was *ca* 2~3 units/ml. Incubation mixtures contained 0.3 ml of a solution (1 mg/ml) of the sample to be treated in water, 0.1 ml of 0.25 M sodium succinate-hydrochloride buffer, pH 6.5 and 0.2 ml of the enzyme stock solution. Incubation was carried out at 28°C for 18~24 hours.

Chemical Hydrolyses

Hydrolysis Using 1 N Aqueous Hydrochloric Acid: The procedure of HOTCHKISS[11] was used. The sample to be hydrolyzed (*ca* 30~50 mg) was dissolved in 5 ml of 1 N aqueous hydrochloric acid. The solution was kept at 100°C for 1 hour. The hydrolysate was then concentrated to dryness. The residue was dissolved in 2 ml of water and the solution was analyzed by UV determination and thin-layer chromatography.

Hydrolysis Using 6 N Aqueous Hydrochloric Acid: The sample to be hydrolyzed (*ca* 30~50 mg) was dissolved in 5 ml of 6 N aqueous hydrochloric acid. The solution was kept at reflux for 2 hours;

it was then cooled and analyzed by UV determination and thin-layer chromatography.

Hydrolysis Using Perchloric Acid: The procedure of MARSHAK and VOGEL[12] was used. The sample to be hydrolyzed (30~50 mg) was dissolved in 5 ml of 72% perchloric acid and the solution was kept at 100°C for 1 hour. The hydrolysate was analyzed by determination of its UV spectra at different pH's, and thin-layer chromatography.

Hydrolysis Using 0.1 N Sodium Hydroxide: The procedure of LORING and his co-workers[13] was used. The sample to be hydrolyzed (30~50 mg) was dissolved in 5 ml of 0.1 N aqueous sodium hydroxide, and kept at 100°C for 2.5 hours. The hydrolysate was then analyzed by UV determination and thin-layer chromatography.

Thin-Layer Chromatographic Analysis of Preparations and Chemical or Enzymatic Hydrolysates

The production and purification of clindamycin 3-ribonucleotides and clindamycin 3-phosphate was followed by assay against *S. lutea* (see above) and by TLC using silica gel G and methyl ethyl ketone-acetone - water (186: 52: 20, v/v) or ethyl acetate - acetone - water (8: 5: 1) as the solvent systems. Clindamycin and other bioactive metabolites of clindamycin were detected by bioautography on agar seeded with *S. lutea*.

The products of enzymatic or chemical hydrolysis of clindamycin 3-nucleotides and clindamycin 3-phosphate were separated by the following TLC systems:

A: Silica gel GF plates (Analtech Inc.); water as the solvent system.

B: Silica gel GF plates; *n*-propyl alcohol-conc. ammonium hydroxide - water (55: 10: 35, v/v).

C: NM-Polygram Cellulose 300 (Brinkman Instruments Inc.); 1-butanol - water - formic acid (77: 13: 10, v/v).

UV absorbing materials were detected by a short wavelength UV lamp. Bioinactive, UV-nonabsorbing materials were detected by a permanganate-periodate spray reagent. Ribose was detected by spraying with aniline acid phthalate reagent (1.66 g of phthalic acid and 0.93 g of aniline in 100 ml of water-saturated 1-butanol) and by permanganate-periodate. Bioactive materials (*i.e.* clindamycin) were detected by bioautography on agar seeded with *S. lutea*.

Periodate Oxidation

Material to be oxidized (*ca* 0.2 mmoles) was dissolved in 10 ml of water and mixed with 10 ml of sodium metaperiodate solution (4 g of $NaIO_4$ in 100 ml of water; 1.88 mmoles of periodate per 10 ml of solution). The consumption of periodate was determined at different time intervals using the FLEURY-LANGE procedure[14].

Fermentation Procedures

Seed cultures of *S. coelicolor* MÜLLER UC-5240 (NRRL 3532) were prepared in a medium consisting of glucose monohydrate (Cerelose), 25 g/liter and Pharmamedia (Traders Oil Mill Co., Fort Worth, Texas) 25 g/liter. The cultures were incubated at 28°C for 72 hours on a rotary shaker. A fermentation medium consisting of glucose monohydrate, 20 g/liter; yeast extract, 2.5 g/liter; NZ-amine B (Sheffield Chemical, Norwich, N.Y.), 5 g/liter; sodium nitrate, 3.0 g/liter; dipotassium phosphate, 1.0 g/liter; magnesium sulfate, 0.5 g/liter; potassium chloride, 0.5 g/liter and ferrous sulfate, 0.1 g/liter was inoculated with the 72-hour seed culture at a ratio of 5% (v/v). The pH of the medium was adjusted to 7.2. Cultures were incubated at 28°C on a rotary shaker (250 rpm, 6-cm stroke). Clindamycin hydrochloride (50 mg/liter) was added 24 hours after inoculation. Culture filtrates were harvested after 48 hours of incubation at which time the added clindamycin was found to be completely inactivated.

Isolation and Purification Procedures

Isolation of Clindamycin 3-Ribonucleotides and Clindamycin 3-Phosphate from Fermentation Broth. Adsorption on Amberlite XAD-2: Fermentation broth (*ca* 490 liters) containing inactivated clindamycin was filtered at harvest pH (6.5) by using filter aid. The mycelial cake was washed with 50 liters of water and discarded. The clear filtrate and the wash were combined and passed over a column prepared from 22 kg of Amberlite XAD-2 (Rohm and Haas Co., Philadelphia, Pa.), at a flow rate of 1 liter/minute. The column was washed with 100 liters of water and then eluted with 120 liters of methanol - water (60: 40, v/v). Fractions containing inactivated clindamycin were combined and

this solution was purified further by ion-exchange chromatography as described below.

Dowex-1 Chromatography: The column was prepared from 22 kg Dowex-1 (X-4) in the acetate form. The solution containing inactivated clindamycin, obtained as described above, was passed through the column. The spent, found to contain mainly clindamycin 3-ribonucleotides and small amounts of clindamycin 3-phosphate was concentrated to dryness to give 877 g of crude preparation (Prep A) which was purified as discussed below. The column was washed with 100 liters of water then eluted with 70 liters of 5% aqueous acetic acid. The acetic acid eluates yielded 89.4 g of material which contained clindamycin 3-phosphate and small amounts of clindamycin 3-ribonucleotides.

Amberlite XAD-2 Chromatography: Prep A, 776 g, (isolated as described above) was dissolved in 1.5 liters of water. The pH of the solution was adjusted to 7.5 with concentrated ammonium hydroxide and the solution was passed over a column containing 4 liters of Amberlite XAD-2. The spent was collected as one fraction and was discarded. The column was washed with 8 liters of water which was also discarded. The column was then eluted with methanol - water (90: 10). Selected fractions (20-ml each) were tested for activity against *S. lutea* before and after treatment with alkaline phosphatase. Fractions yielding bioactive materials after enzymatic treatment were combined, concentrated to an aqueous solution which was then freeze-dried to yield 64.7 g of a preparation containing clindamycin 3-ribonucleotides and clindamycin 3-phosphate. This material was purified by counter double current distribution described below.

Counter Double Current Distribution: Twenty-one g of the material obtained as described above was dissolved in 100 ml of each phase of the solvent system consisting of equal volumes of 1-butanol - water (1: 1). The solutions were added in the center tubes of an all glass counter double current distribution apparatus (100 tubes, 25 ml/phase). The distribution was analyzed after 100 transfers by determination of bioactivity of selected fractions before and after treatment with crude alkaline phosphatase. Fractions yielding bioactive materials after treatment with alkaline phosphatase were combined, concentrated to an aqueous solution and freeze-dried to give 6.9 g of purified mixture of clindamycin 3-ribonucleotides containing small amounts of clindamycin 3-phosphate.

Two additional counter double current distribution runs using conditions identical to those described above yielded an additional 14.0 g of material.

DEAE-Sephadex Chromatography: Five hundred g of DEAE-Sephadex (A-25) was stirred for 1 hour with water and for 2 hours with 0.5 N aqueous sodium hydroxide. The ionic exchanger was washed with water till the pH was *ca* 7.5. The material was then stirred for 2 hours with 0.5 N aqueous acetic acid washed with water to a neutral pH, and poured into a glass column. The column was washed with 4 liters of water and 4 liters of 0.1% aqueous solution of *tris*-(hydroxymethyl)-aminomethane (THAM).

Thirteen g of the material obtained by counter double current distribution was dissolved in 100 ml of water. This solution was adjusted to pH 9.0 with concentrated ammonium hydroxide and added on the top of the column. The column was then eluted as follows. Fractions (20 ml each) were collected.

15 liters of 0.05 M THAM acetate, pH 8.0 (Fractions 1~722)
40 liters of 0.1 M THAM acetate, pH 8.0 (Fractions 723~2920)
20 liters of 0.2 M THAM acetate, pH 8.0 (Fractions 2921~3985)
20 liters of 0.3 M THAM acetate, pH 8.0 (Fractions 3986~5000).

Selected fractions were tested for bioactivity against *S. lutea* before and after treatment with alkaline phosphatase and by UV determination. Results are presented in Fig. 2. The following fractions were combined.

Pool	Fraction No.	Volume (ml)	UV_{max} (at pH 7.0) (nm)
A	850~965	2,300	270
B	990~1090	1,000	260
C	1110~1225	2,300	260
D	1240~1535	5,200	261
E	1550~1680	2,600	262
F	1705~1750	1,300	254; 278 sh
G	1771~2125	7,000	254; 278 sh
H	3401~3590	3,600	no maximum

Clindamycin 3-ribonucleotides and clindamycin 3-phosphate were obtained free of THAM acetate buffer by chromatographies over Amberlite XAD-2 as described below:

Isolation of Pure Clindamycin 3-Ribonucleotides and Clindamycin 3-Phosphate. Removal of THAM Acetate Buffer: The solutions (A~H) obtained by combination of appropriate fractions from the chromatography described above were passed over columns containing Amberlite XAD-2. The spents were discarded. The columns were washed with water and the eluted with methanol - water (80: 20, v/v). Fractions were analyzed by UV and by testing for bioactivity before and after treatment with crude alkaline phosphatase. Appropriate fractions were combined and concentrated to an aqueous solution which was freeze-dried. Details on the amount of Amberlite XAD-2 used for each pool, the amount of water wash and the amount of material obtained are listed in the following table.

Pool	Amberlite XAD-2 Used (ml)	Water Wash (ml)	Isolated Material (mg)
A	150	1,000	750
B	100	1,000	380
C	100	1,000	500
D	400	4,000	3,400
E	150	1,000	740
F	100	1,000	60
G	200	3,000	1,400
H	150	1,000	600

The materials obtained were designated as compounds A, B, C, D, E, F, G and H. Characterization of these materials has been discussed earlier in this paper.

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References

1) ARGOUDELIS, A. D. & J. H. COATS: Microbial transformation of antibiotics. VI. Acylation of chloramphenicol by *Streptomyces coelicolor*. J. Antibiotics 24: 206~208, 1971
2) ARGOUDELIS, A. D. & J. H. COATS: Microbial transformation of antibiotics. II. Phosphorylation of lincomycin by *Streptomyces* species. J. Antibiotics 22: 341~343, 1969
3) ARGOUDELIS, A. D.; J. H. COATS, D. J. MASON & O. K. SEBEK: Microbial transformation of antibiotics. III. Conversion of clindamycin to 1'-demethylclindamycin and clindamycin sulfoxide by *Streptomyces* species. J. Antibiotics 22: 309~314, 1969
4) COATS, J. H. & A. D. ARGOUDELIS: Microbial transformation of antibiotics. Phosphorylation of clindamycin by *Streptomyces coelicolor* Müller. J. Bact. 108: 459~464, 1971
5) RAZZELL, W. E.: Phosphodiesterases. Methods in Enzymology, Volume VI (S. P. COLOWICK & N.O. KAPLAN ed.). Academic Press, New York, p. 236, 1963
6) CHARGRAFF, E. & J. N. DADIDSON: The nucleic acids. Volume I, Chapter 5. Academic Press Inc., New York, N.Y., 1955
7) STOTHERS, J. B.: Carbon-13 NMR Spectroscopy. Academic Press, New York, N.Y., p. 471, 1972
8) STOTHERS, J. B.: Carbon-13 NMR Spectroscopy. Academic Press, New York, N.Y., p. 469, 1972
9) FINNEY, D. J.: Statistical Methods in Biological Assay. Hafner Publishing Co., New York, N. Y., 2nd Edition, pp. 524~530
10) DAVIES, J.; M. BREZEZINSKA & R. BEVENISTE: R Factors: Biochemical mechanisms of resistance to aminoglycoside antibiotics. Ann. N. Y. Acad. Science 182: 226~233, 1971
11) HOTCHKISS, R. D.: The quantitative separation of purines, pyrimidines and nucleosides by paper chromatography. J. Biol. Chem. 175: 315~332, 1948

12) MARSHAK, A. & H. J. VOGEL: Microdetermination of purines and pyrimidines in biological materials. J. Biol. Chem. 189: 597~605, 1951
13) LORING, H. S.; H. W. BORTNER, L. W. LEVY & M. L. HAMMEL: Preparation and properties of the isomeric cytidylic acids derived from yeast ribonucleic acid. J. Biol. Chem. 196: 807~819, 1952
14) DYER, J. R.: "Methods of Biochemical Analysis," Volume 3, p. 111. Interscience Publishers, Inc., New York, N.Y., 1956