THE ORIGIN OF METHYL GROUPS OF 4′-DEPROPYL-4′-ETHYLLINCOMYCIN (U-21699)

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(Received for publication December 11, 1972)

The bioorigin of the methyl groups of 4′-depropyl-4′-ethyllincomycin has been studied. Using radioactive techniques it was determined that in addition to the $-SCH_3$ and NCH_3 groups, the CCH_3 present in the amino acid moiety is derived from C_1 donors. These results are identical to the findings on the origin of the methyl groups of lincomycin, the main antibiotic produced by *Streptomyces lincolnensis*.

4′-Depropyl-4′-ethyllincomycin (I, Fig. 1) is an antibiotic produced[1] concomitantly with lincomycin (II, Fig. 1)[2,3] in fermentations of *Streptomyces lincolnensis* var. *lincolnensis*.

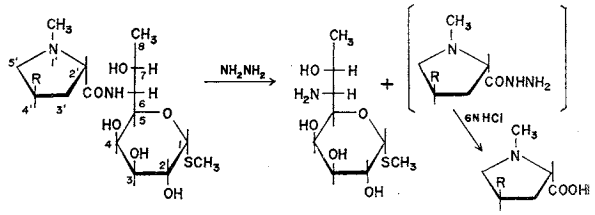
As part of our work on the biosynthesis of the antibiotics produced by *S. lincolnensis* we have examined the bioorigin of the methyl groups present in both antibiotics. The conclusions derived from our work with lincomycin were described in a previous communication[4]. The present paper discusses the bioorigin of the methyl groups present in 4′-depropyl-4′-ethyllincomycin.

Experimental

Counting Procedures: Radioactivity was determined with an automatic Packard Tri-Carb liquid scintillation spectrometer, Model 3000 (Packard Instrument Co., Inc.). The procedures used were identical to those described by ARGOUDELIS *et al.*[4]

Fermentation Procedures: Seed cultures of *S. lincolnensis* var. *lncolnensis* were prepared in a medium consisting of glucose monohydrate (Cerelose; 10 g/liter), N-Z-amine B (Sheffield Chemicals, Norwich, N. Y.; 5 g/liter), and Yeastolac (10 g/liter). The cultures were incubated at 28°C for 48 hours on a rotary shaker. A fermentation medium consisting of glucose (30 g/liter), sodium citrate (3 g/liter), $ZnSO_4 \cdot 7H_2O$ (0.001 g/liter) $FeSO_4 \cdot 7H_2O$ (0.001 g/liter, $MgSO_4$ (1 g/liter), K_2HPO_4 (2.5 g/liter), NaCl (0.5 g/liter), and NH_4NO_3 (2.0 g/liter) was inoculated at a rate of 5% (v/v) with the 48-hour medium. The fermentation beers were harvested after 144 hours. Antibiotic titers were measured by disk plate activity using *S. lutea* as assay organism.[5] The precursors were added to the broth after 24-hour fermentation at a concentration of 180 μC/liter. The broths were harvested after 120- or 144-hour fermentation.

Fig. 1.



I: R = CH_2CH_3
II: R = $CH_2CH_2CH_3$

III

IVa: R = CH_2CH_3
IVb: R = $CH_2CH_2CH_3$

Isolation of Antibiotics: Crude radioactive mixture of lincomycin and 4′-depropyl-4′-ethyllincomycin hydrochlorides was isolated by the procedure of HERR and BERGY[2] from 1 liter of fermentation broth. This material (*ca.* 1.2 g) was mixed with 500 mg of radioinactive 4′-depropyl-4′-ethyllincomycin and the resulting preparation was purified by countercurrrent distribution as described by ARGOUDELIS *et al.*[1] 4′-Depropyl-4′-ethyllincomycin was thus separated from lincomycin and isolated as the crystalline hydrochloride (420 mg). Crystalline lincomycin hydrochloride (600 mg) was also isolated by concentration of appropriate fractions of the countercurrent distribution.

Degradation of 4′-Depropyl-4′-Ethyllincomycin and Lincomycin. Isolation of Methyl α-Thiolincosaminide (III) and *trans*-4-Ethyl-L-Hygric (IVa) and *trans*-4-*n*-Propyl-L-Hygric (IVb) acids: The procedure described by SCHROEDER *et al.*[6] was used for the hydrazinolysis of 4′-depropyl-4′-ethyllincomycin to methyl α-thiolincosaminide and *trans*-4-ethyl-L-hygric acid hydrazide. Lincomycin treated under the same conditions yielded methyl α-thiolincosaminide and *trans*-4-*n*-propyl-L-hygric acid hydrazide. The acid hydrazides were converted to the corresponding 4-ethyl-L-hygric acid and 4-*n*-propyl-L-hygric acid hydrochlorides by hydrolysis with 6 N aqueous hydrochloric acid.[6]

Degradation of Methyl α-Thiolincosaminide. Isolation of 2,4-Dinitrophenyl Methyl Sulfide: Methyl α-thiolincosaminide obtained from degradation of either lincomycin or 4′-depropyl-4′-ethyllincomycin was treated with 5 N aqueous sulfuric acid as described by ARGOUDELIS *et al.*[4] Crystalline 2,4-dinitrophenyl methyl sulfide was recrystallized from 95% ethanol.

Decarboxylation of *trans*-4-Ethyl-L-Hygric Acid (IVa) by Lead Tetraacetate: *Trans*-4-Ethyl-L-hygric acid hydrochloride was treated with lead tetraacetate according to the procedure used for the decarboxylation of *trans*-4-*n*-propyl-L-hygric acid hydrochloride.[4] The evolved carbon dioxide was isolated as barium carbonate.

Permanganate Oxidation of *trans*-4-Ethyl-Hygric Acid (IVa) to Ethyl Succinic Acid (V): The procedure described by MAGERLIEN *et al.*[7] was followed. The obtained material was purified by chromatography on silica gel plates using benzene - methanol - glacial acetic acid (100 : 2 : 1, v/v) or benzene - methanol - glacial acetic acid (100 : 20 : 10, v/v) as the solvent systems. For identification of the spots the plates were heated at 50°C for 30 minutes and then sprayed with 2% solution of bromophenol blue.

KUHN-ROTH Oxidations: KUHN-ROTH oxidation of methyl α-thiolincosaminide and *trans*-4-ethyl-L-hygric acid were run by Huffman Laboratories, Inc. The sodium acetate obtained was transformed to crystalline S-benzyl isothiouronium acetate.

Decarboxylation of Sodium Acetate: The procedure described by ARGOUDELIS *et al.*[4] was followed. Carbon dioxide evolved was isolated as barium carbonate.

Discussion and Results

Methyl groups attached to oxygen, nitrogen and sulfur as well as many of those attached to carbon originate from C_1 donor systems like methionine through transmethylation[8]. When L-[methyl-^{14}C] methionine, [2-^{14}C] glycine, or L-[U-^{14}C] serine was added to fermentations of *S. lincolnensis*, a high incorporation of radioactivity into lincomycin was observed[4]. As showin in Table 1 the mixture of lincomycin and 4′-depropyl-4′-ethyllincomycin obtained from cultures of *S. lincolnensis* grown in the presence of L-[methyl-^{14}C]-methionine contained 18% of the radioactivity added in the fermentation media. Separation of the two antibiotics by countercurrent distribution afforded 4′-depropyl-4′-ethyllincomycin containing 1% of the radioactivity added in the fermentation (Table 1) or 5.5% of the radioactivity present in the mixture of the two antibiotics. The isolated lincomycin contained 17% of the

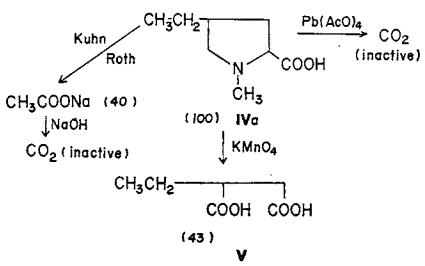
Table I. Incorporation of radioactivity*

	% Incorpration**	% Antibiotic present in mixture	Ratio (% Incorporation/% antibiotic present)	Ratio of sp. act. (α-Methyl thiolincosaminide/aminoacids)
Antibiotic mixture	18	—	—	—
Lincomycin	17	94.5	0.18	0.53
4′-Depropyl-4′-ethyl-lincomycin	1	5.5	0.20	0.52

* L-[Methyl-^{14}C]-methonine was used as the radioactive precursor.
** % Incorporation indicates the percentage of radioactivity added in the fermentation media which was found in the indicated compounds.

radioactivity present in the antibiotic mixture. Vapor phase chromatography indicated[4] that the composition of the mixture of antibiotics produced by *S. lincolnensis* is *ca.* 95% lincomycin and 5% 4′-depropyl-4′-ethyllincomycin. We therefore concluded that the absolute rate of incorporation (ratio of % of incorporation to % of antibiotic present) of radioactivity in both antibiotics is almost identical.

Fig. 2.



* Numbers in parentheses indicate percentage of radioactivity in the indicated compounds.

The radioactive 4′-depropyl-4′-ethyllincomycin (I) and lincomycin (II) were degraded (Fig. 1) to methyl α-thiolincosaminide (III) and the corresponding *trans*-4-ethyl-L-hygric (**IVa**) and *trans*-4-*n*-propyl-L-hygric (**IVb**) acids and the specific activity of these compounds was determined.

As indicated in Table 1 the ratio of the specific activity of methyl α-thiolincosaminide (obtained from 4′-depropyl-4′-ethyllincomycin) to that of *trans*-4-ethyl-L-hygric acid is *ca.* 0.52. Similarly the ratio of the specific activity of methyl α-thiolincosaminide, obtained from lincomycin, to that of *trans*-4-*n*-propyl-L-hygric acid is *ca.* 0.53. These results indicate that the amino acids are twice as radioactive as methyl α-thiolincosaminide, and furthermore suggest identical bioorigin of the methyl groups in both antibiotics.

It has been shown[4] that of the two methyl groups present in the amino sugar III of lincomycin only the $-SCH_3$ originates from methionine. Acid hydrolysis of methyl α-thiolincosaminide obtained from 4′-depropyl-4′-ethyllincomycin afforded methanethiol isolated as the crystalline 2,4-dinitrophenyl methyl sulfide. This compound contained 96% of the radioactivity present in methyl α-thiolincosaminide indicating that of the two methyl groups present in the aminosugar moiety only the SCH_3 originates from methionine.

Oxidative decarboxylation of *trans*-4-ethyl-L-hygric acid (Fig. 2) using lead tetraacetate gave radioinactive carbon dioxide.

On the other hand, permanganate oxidation of **IVa** gave ethylsuccinic acid (**V**) containing 43% of the radioactivity present in 4-ethyl-L-hygric acid. This indicates that the $-NCH_3$ group contains the remaining 57% and thus also originates from methionine.

KUHN-ROTH oxidation of *trans*-4-ethyl-L-hygric acid (**IVa**) yielded sodium acetate containing 40% of the radioactivity present in **IVa**. Sodium hydroxide fusion gave radioinactive carbon dioxide indicating that the methyl group of the ethyl side chain of **IVa** is derived from the methyl group of methionine.

The results obtained show that the bioorigin of the methyl groups of 4′-depropyl-4′-ethyllincomycin is identical to that of the methyl groups of lincomycin[4]. It is therefore reasonable to assume that on the basis of biosynthetic relationships the methyl groups in both antibiotics originate from C_1 fragments (C-2 of glycine, C-3 of serine, or $-CH_3$ of methionine) and that these fragments are incorporated as methyl groups rather than as oxidized forms.*

Lincomycin and 4′-depropyl-4′-ethyllincomycin differ only in the aminoacid part of their molecules. The fact that the $N-CH_3$ and the $C-CH_3$ groups of the respective aminoacids are derived from methionine indicates that *trans*-4-ethyl-L-hygric acid (**IVa**) is not a precursor of *trans*-4-*n*-propyl-L-hygric acid (**IVb**), but that both aminoacids are derived in the same manner from precursors differing by one carbon. WITZ and his coworkers[9] have shown recently that L-tyrosine is the precursor of *trans*-4-*n*-propyl-L-hygric acid. They have also proposed a pathway involving L-dopaquinone as a key intermediate which by cyclization, further oxidation and methylation on carbon** and nitrogen leads to both *trans*-4-*n*-propyl-L-hygric and *trans*-4-ethyl-L-hygric acids.

Acknowledgements

The technical assistance of Mr. K. J. GEIPEL is highly appreciated.

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* For discussion on the biosynthesis of $-SCH_3$ by transmethylation or "trans thiomethylation" (transfer of $-SCH_3$ of methionine) *see* Ref. 4.

** For discussion of methylation on carbon *see* Ref. 4.