# STRUCTURE OF A NEW ANTIBIOTIC CURROMYCIN A PRODUCED BY A GENETICALLY MODIFIED STRAIN OF *STREPTOMYCES HYGROSCOPICUS*, A POLYETHER ANTIBIOTIC PRODUCING ORGANISM

Sir:

Antibiotic production, formation of aerial mycelia (Amy), and nutrient auxotrophy of some streptomycetes have been reported to be related to the loss of plasmids,[1~3] the movement of a transposable element[4,5], or the appearance of reiterated DNA sequences in the bacterial chromosome[6~8]. We have been engaged in an investigation to analyze in detail these relationships in streptomycetes which produce polyether antibiotics. During course of these studies, we found that when *Streptomyces hygroscopicus* 358AV$_2$, a carriomycin producing strain, was subjected to protoplast regeneration[9], the regeneration rate in this strain was as high as 90%, and it lost the ability to produce the antibiotic at high frequency (about 90%) with the formation of aerial mycelium being unaffected.

Protoplast regeneration has been reported to result in the loss of plasmids of streptomycetes[10,11] and alteration of several phenotypes[12]. For example, the pSV1 plasmid, which codes for synthesis of and resistance to methylenomycin A, was eliminated by protoplast regeneration[10]. Therefore, this result suggests that carriomycin non-producing mutants are formed by the loss of plasmid(s) or the deletion of DNA during protoplast regeneration.

Treatment of the parent strain with ethidium bromide gave Amy$^-$ mutant strains retaining the productivity of carriomycin at high frequency (about 70%), but no carriomycin non-producing mutants. The productivity of carriomycin in these mutant strains was inferior to that of the parent strain. Except for the formation of aerial mycelium, most of these mutants had the same phenotypic properties as the parent strain.

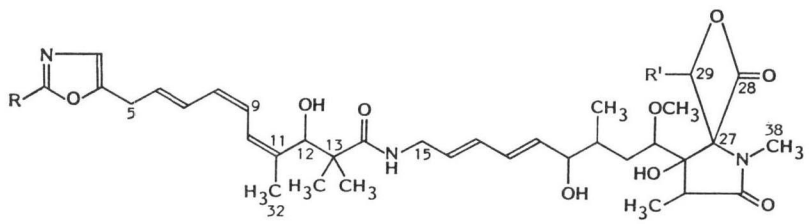
Table 1. Assignments of the chemical shifts of protons and carbons of curromycin A.

| | | | | |
|---|---|---|---|---|
| $-CH_3$ | 10.2 | 17.1 | 21.6 | 26.0 |
| | (1.15) | (0.94) | (1.03) | (1.29) |
| $-NCH_3$ | 26.5 | | | |
| | (2.87) | | | |
| $-OCH_3$ | 56.8 | 59.5 | | |
| | (3.30) | (3.39) | | |
| $=\!<_{CH_3}$ | 13.9 | 19.6 | | |
| | (2.36) | (1.74) | | |
| $-CH_2-$ | 32.8 | | | |
| | (1.30, 1.98) | | | |
| $-OCH_2-$ | 70.7 | | | |
| | (4.01, 4.14) | | | |
| $-NCH_2-$ | 41.1 | | | |
| | (3.85) | | | |
| $=\!<_{CH_2-}$ | 29.1 | | | |
| | (3.40) | | | |
| $-CH<$ | 37.2 | 43.3 | | |
| | (1.70) | (2.42) | | |
| $-OCH<$ | 75.2 | 76.7 | 78.5 | 82.5 |
| | (4.58) | (3.92) | (4.75) | (3.49) |
| $=CH-$ | 122.4 | 123.8 | 124.8 | 128.0 |
| | (6.55) | (6.14) | (6.36) | (5.89) |
| | 128.0 | 128.9 | 129.5 | 130.0 |
| | (6.60) | (5.71) | (5.63) | (6.14) |
| | 131.5 | 134.2 | | |
| | (6.14) | (5.63) | | |
| $>C<$ | 44.9 | 81.6 | 86.2 | 138.5 |
| | 150.2 | 160.7 | | |
| $>C=O$ | 169.0 | 175.1 | 177.9 | |

These data were established by 2D C-H correlation spectrum. The chemical shift of protons are designated in parentheses.

The $^1H$ and $^{13}C$ NMR spectra were taken in $CDCl_3$ with TMS as internal reference.

Fig. 1. The total structures of curromycin A and curromycin B.



Curromycin A R=$CH_3$, R′=$CH_2OCH_3$
Curromycin B R=$CH_3$, R′=$CH_3$
Oxazolomycin R=H, R′=H

Surprisingly, however, half of these mutants produced an unknown antibacterial substance in addition to carriomycin. In this paper we wish to report isolation and structural elucidation of this antibiotic, which we have named curromycin A.

One of the mutant strains, *S. hygroscopicus* EtBr No. 32, was cultivated at 27°C for 4 days in two 50-liter jar fermentors containing 30 liters of a medium consisting of 2.5% soluble starch, 1.5% soybean meal, 0.2% dry yeast and 0.4% calcium carbonate. The broth filtrate (pH 7.7, 45 liters) was extracted with ethyl acetate (15 liters×2) and, after the extract was evaporated to a small volume *in vacuo*, the concentrate (2 liters) was washed succesively with 0.1 N HCl, 0.1 N NaOH and water, and then evaporated to dryness. The oily residue thus obtained was dissolved in a mixed solvent ($CHCl_3$-MeOH, 24: 1) and chromatographed on a silica gel column developing with the same solvent to give two fractions active against *Bacillus subtilis*. Carriomycin was obtained from the first fraction. The second one containing curromycin A was concentrated and subjected to rechromatography on a silica gel column ($CHCl_3$ - MeOH, 24: 1). Further purification by column chromatography on a Toyopearl HW 45 column (MeOH) gave a pure sample of the new antibiotic curromycin A in an overall yield of 90 mg.

The physico-chemical properties of curromycin A are as follows: Yellowish amorphous powder; mp 103~105°C; $[\alpha]_D^{22}$ +39.0° (*c* 0.115, MeOH); *Anal* [calcd for $C_{38}H_{55}O_{10}N_3$: C 63.96, H 7.71, O 22.44, N 5.89%; found: C 63.65, H 7.41, O 23.05, N 5.25%]; FAB-MS *m/z* 714 $(M+H)^+$, 752 $(M+K)^+$; IR $\nu_{max}^{KBr}$ $cm^{-1}$ 3350, 1825, 1690 and 1640; UV $\lambda_{max}^{MeOH}$ nm ($\varepsilon$) 228 (19,800), 267 (sh 16,300), 275 (19,800) and 285 (sh 15,100). The IR absorption bands at 1825, 1690 and 1640 $cm^{-1}$ suggested $\beta$-lactone and amide structures. The UV spectrum of curromycin A indicated the presence of a triene and a diene system. Such characteristics suggested that the antibiotic curromycin A is very similar to the antitumor antibiotic oxazolomycin[13].

In accord with the molecular formula $C_{38}H_{55}O_{10}N_3$, established from the FAB mass spectrum and the elemental analysis, the $^{13}C$ NMR spectrum of curromycin A taken in $CDCl_3$ revealed 38 carbons as summarized in Table 1. The assignments of functional groups were established by the aid of the INEPT and 2D C-H correlation spectra of curromycin A. The analysis of the $^{13}C$ NMR spectrum accounted for 51 protons directly attached to carbons. The $^1H$ NMR spectrum of curromycin A taken at 45°C revealed the four remaining exchangeable protons at $\delta_H$ 6.75 (t, 1H, $J$=5.2 Hz), 4.81 (br d, 1H, $J$=4.8 Hz), 4.23 (br s, 1H) and 2.98 (br s, 1H).

Analysis of the 2D-COSY spectrum of curromycin A revealed straightforwardly three partial structures **1**, **2** and **3** as shown in Fig. 2. The C-32 methyl carbon in the partial structure **1** is connected to an $sp^2$ carbon C-11, because long range coupling was observed between H-10

Fig. 2. The partial structures **1**, **2** and **3** proved by the 2D COSY spectrum, LSPD and NOE experiments.

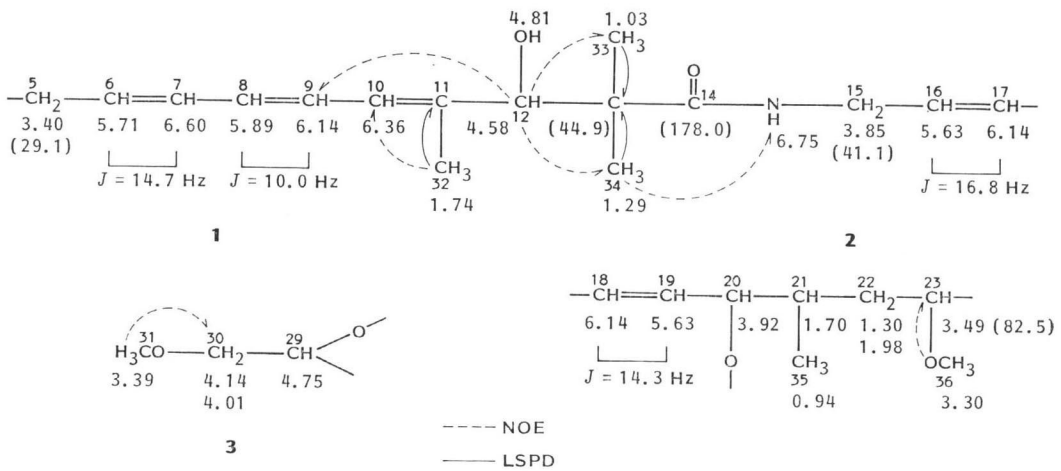
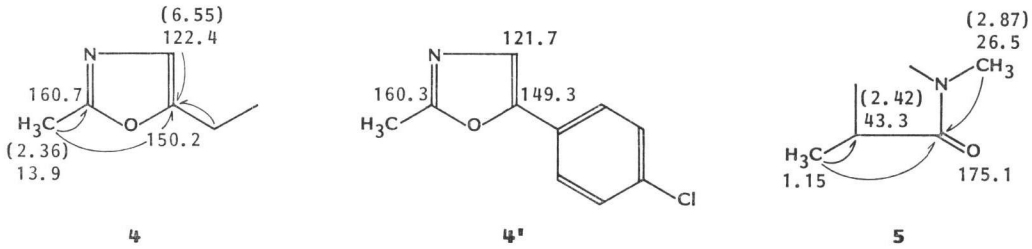


Fig. 3. The partial structures **4** and **5** established by the LSPD experiments.
Compound **4'** is 2-methyl-4-*p*-chlorophenyl-oxazole.




**4** **4'** **5**

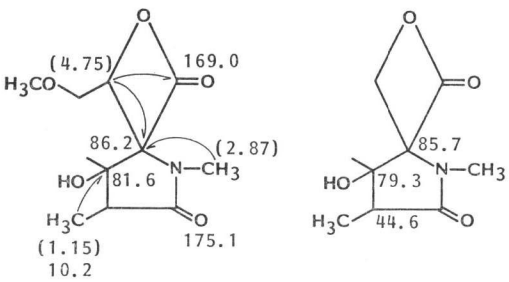
methine proton and H-32 methyl protons in the relayed COSY spectrum of curromycin A. Furthermore, a long range selective proton decoupling experiment (LSPD)[14] irradiating the C-32 methyl protons collapsed the C-11 carbon resonance. NOE enhancements observed between the olefinic proton H-9 at $\delta_H$ 6.14 and the oxymethine proton H-12 at $\delta_H$ 4.58 connected the C-12 hydroxymethine carbon to C-11.

In the partial structure **2**, an exchangeable proton at $\delta_H$ 6.75, which is ascribed to an amide function from its chemical shift, was coupled to the methylene protons H-15 at $\delta_H$ 3.85. The quaternary carbon C-13, observed at $\delta_C$ 44.9, which was connected to a *gem*-dimethyl group by LSPD experiments, was linked to the amide carbonyl carbon by observing an NOE enhancement between the amide and *gem*-dimethyl protons as shown in Fig. 2. Likewise the NOE enhancement observed between these *gem*-dimethyl protons and the hydroxymethine proton in the partial structure **1** proved the linkage of the partial structures **1** and **2**.

Furthermore, LSPD experiments established the two partial structures **4** and **5** as shown in Fig. 3. The presence of an oxazole ring was confirmed by good agreement of the carbon chemical shifts of the partial structure **4** with those of 2-methyl-4-*p*-chlorophenyloxazole[15]. This oxazole ring was connected to C-5 in the partial structure **1** due to the C-H long range coupling observed as shown in Fig. 3. In the partial structure **5**, the last nitrogen in curromycin A was assigned to an amide moiety since the *N*-methyl proton was long range coupled to the amide carbonyl carbon at $\delta_C$ 175.0. Therefore two remaining exchangeable protons were due to hydroxyl groups.

At this point, there remained for assignment two quaternary carbons ($\delta_C$ 81.6 and 86.2), one ester carbonyl carbon assigned to a $\beta$-lactone

Fig. 4. The spiro ring system established by the modified selective INEPT experiments: On the right is oxazolomycin's and, left, curromycin's.




($\delta_C$ 169.0), and two hydroxyl groups. Taking account of the degree of unsaturation, another ring structure must be present in curromycin A in addition to the oxazole ring and the $\beta$-lactone ring.

Although the conventional LSPD technique is powerful as visualized above in connecting proton spin systems separated by quaternary carbons or hetero-atoms when the number of protons in the systems under investigation is limited, it is not of use when the carbons of interest are coupled to too many protons. Therefore we used modified selective INEPT experiments in order to detect small changes in complicated splitting patterns of carbon multiplet resonances.

As the results show, modified selective INEPT experiments[16] revealed the combination of two ring structures (Fig. 4). Irradiation of a methine proton at $\delta_H$ 4.75, which is H-27 in the partial structure **3**, revealed the C-H long range couplings with a carbonyl carbon at $\delta_C$ 169.0 and a quaternary carbon at $\delta_C$ 86.2. Similarly, the *N*-methyl protons (H-38) at $\delta_H$ 2.87 were long range coupled with a carbonyl carbon at $\delta_C$ 175.1 and a quaternary carbon at $\delta_C$ 86.2.

Since the presence of a $\beta$-lactone was indicated

by the IR absorption at 1825 $cm^{-1}$ as described above, C-29 and C-28 must be connected through an oxygen. Thus, the $\beta$-lactone has the partial structure **3** on the $\beta$-position and partial structure **5** on the $\alpha$-position. Furthermore a C-H long range coupling was observed between the methyl protons at $\delta_H$ 1.15 and the quaternary carbon at $\delta_C$ 81.6. Because of the degree of unsaturation, still another ring structure must be present in curromycin A in addition to the oxazole ring and the $\beta$-lactone ring.

In order to accomodate an additional cyclic moiety the quaternary carbon at $\delta_C$ 81.6 must be connected to the $\alpha$-position of the $\beta$-lactone, resulting in the formation of a $\gamma$-lactam ring with a hydroxyl group on this quaternary carbon. The $\beta$-position (C-24) of this $\gamma$-lactam residue was connected to the already established main structure comprising partial structures **1**, **2** (Fig. 2) and **4** (Fig. 3). The chemical shifts of this spiro ring system were in good agreement with those of the analogous part in oxazolomycin as shown in Fig. 4.

Furthermore the last hydroxyl group was located to the C-20 oxymethine carbon in the partial structure **2**.

The above detailed NMR spectral analysis resulted in the establishment of the total structure of curromycin A as shown in Fig. 1. The stereochemistries of the triene and diene systems were determined by the spin coupling constants and the observed NOE enhancements as shown in Fig. 2.

Curromycin A is different from oxazolomycin in that the $\beta$-position of the $\beta$-lactone ring and C-2 position of the oxazole ring are substituted with $CH_3OCH_2$ and $CH_3$, respectively.

Curromycin A is active against some kinds of Gram-positive bacteria such as *Bacillus subtilis* IAM 1026, and showed cytotoxic activities against mouse B16 melanoma, P388 leukemia and Friend leukemia cells. Curromycin A therefore has a narrower antibacterial spectrum than oxazolomycin. Details about this will be reported elsewhere.

## Note Added in Proof

During the course of an attempt to improve the production yield of curromycin A, we found that modification of the production medium resulted in the accumulation of a new structurally related antibiotic, which we have named curromycin B. It differs from curromycin A in possessing a methyl, instead of a methoxymethylene residue (Fig. 1). Details will be published elsewhere[17].

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