# ISOLATION AND IDENTIFICATION OF 7,8-DIDEMETHYL-8-HYDROXY-5-DEAZARIBOFLAVIN, AN UNUSUAL COSYNTHETIC FACTOR IN STREPTOMYCETES, FROM *STREPTOMYCES LINCOLNENSIS*

MING-S. T. KUO, DAVID A. YUREK, JOHN H. COATS and GRACE P. LI

Chemical and Biological Screening, The Upjohn Company, Kalamazoo, Michigan 49001, U.S.A.

(Received for publication September 21, 1988)

A study of complementing lincomycin non-producing mutants of *Streptomyces lincolnensis* resulted in the discovery of a low molecular weight compound that functions as a catalytic agent, lincomycin cosynthetic factor (LCF), in the biosynthesis of propylproline.[1] This note reports the isolation and identification of LCF and its detection in fermentation broths of many actinomycetes.

For the purification of LCF, *S. lincolnensis* mutant NTG-5 fermentation broth was adjusted to pH 3.0, filtered, passed over an Diaion HP-20 chromatography column and eluted with a $H_2O$ - MeOH gradient. Active fractions, identified by bioassay,[1] were pooled, adjusted to pH 9.0, absorbed to Amberlite IRA 458 ($OH^-$) resin, and eluted with $H_2O$ - 1 M AcOH. The Diaion HP-20 column chromatography and later steps were also followed by TLC (silica plates developed with methyl ethyl ketone - acetone - water, 71 : 19 : 10, Rf 0.41) with long wavelength UV light as the detection method. The eluate from the Amberlite IRA column was rechromatographed on an Diaion HP-20 column to remove acetate, absorbed on a silica column and eluted with methyl ethyl ketone - acetone - $H_2O$ (71 : 19 : 10). After removing solvents, active fractions were absorbed on a second silica column and eluted with a $CH_2Cl_2$ - MeOH - $H_2O$ - TFA gradient (from 90 : 9 : 1 : 0.1 to 65 : 31.5 : 3.5 : 0.1). An amorphous-yellow solid was obtained by Sephadex G-10 chromatography and lyophilization.

LCF is soluble in basic water and DMSO, slightly soluble in lower alcohols and insoluble in ethyl acetate, ether, chlorinated and saturated hydrocarbons. The UV-vis spectrum of LCF showed $\lambda_{max}$ at 424, 299, 267 (sh), and 249 nm in neutral and basic MeOH. The absorption maxima shifted to 378, 272, 250, and 230 nm in acidic solution, suggesting the existence of a phenolic or an enolic functional group. This was also suggested by the weakly acidic nature of LCF exhibited during isolation. The IR absorption of LCF (not shown) was also sensitive to pH changes. Fast atom bombardment (FAB)-MS showed molecular ion peaks at *m/z* 364 $(M+H)^+$ and 386 $(M+Na)^+$. High-resolution measurement of *m/z* 364 peak indicated 364.1140 as the exact molecular weight suggesting a molecular formula of $C_{16}H_{17}N_3O_7$ (calcd 364.1145).

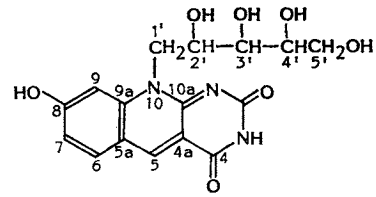
The 500 MHz $^1H$ NMR spectrum of LCF displayed eleven nonexchangeable protons (Table 1) which were comprised of seven carbinol protons and four aromatic protons. The chem-

Table 1. NMR data of LCF.

| Carbon atom | $^{13}C$ Chemical shift[a] | $^1H$ Chemical shift[b] |
|---|---|---|
| 2 | 162.5 (s) | |
| 8 | 158.5 (s) | |
| 4 | 158.1 (s) | |
| 10a | 156.7 (s) | |
| 9a | 144.3 (s) | |
| 5 | 140.1 (d) | 8.56 |
| 6 | 133.4 (d) | 7.65 (d, $J$=8) |
| 7 | 117.6 (d) | 6.95 (d, $J$=3) |
| 4a | 114.5 (s) | |
| 5a | 108.0 (s) | |
| 9 | 102.1 (d) | 6.74 (dd, $J$=8, 3) |
| 3′ | 73.8 (d) | 3.75 (dd, $J$=7, 5) |
| 4′ | 72.6 (d) | 3.86 |
| 2′ | 69.8 (d) | 4.35 (ddd, $J$=9, 7, 4) |
| 5′ | 63.2 (t) | 3.83, 3.66 |
| 1′ | 47.7 (t) | 5.04, 4.67 |

[a] Reference to MeOH-$d_4$ (39.5 ppm); multiplicity (in parentheses) determined by DEPT experiment.

[b] Reference to TMS ($J$=coupling constant in Hz).




I

Table 2. Concentrations of LCF in randomly selected microbial fermentations (assayed by HPLC).

| Organism | UC No.[a] | LCF ($\mu$g/ml) |
|---|---|---|
| *Actinomadura kijaniata* | 8349 | 183 |
| *Actinoplanes azureus* | 8164 | 0.0 |
| *A. missouriensis* | 5015 | 22 |
| *A. nipponensis* | 8166 | 0.0 |
| *Actinopycnidium caerulium* | 8198 | 1.0 |
| *Actinosporangium violaceum* | 8044 | 14 |
| *Actinosynnema pactiosum* | 8412 | 3.4 |
| *Amycolatopsis orientalis* | 8326 | 4.7 |
| *Chainia antibiotica* | 2833 | 28 |
| *C. kunmingensis* | 8709 | 84 |
| *Kitasatoa kauaiensis* | 5543 | 65 |
| *Micromonospora melanosporeus* | 2318 | 0.5 |
| *Micromonospora* sp. (*coerulea*?) | 2488 | 0.8 |
| *Microtetraspora caesia* | 8461 | 1.3 |
| *M. glauca* | 5956 | 0.2 |
| *M. niveoalba* | 5959 | 1.1 |
| *Nocardia lactamdurans* | 5683 | 85 |
| *Nocardia* sp. | 2811 | 0.2 |
| *N. uniformis* subsp. *tsuyamanensis* | 5838 | 1.5 |
| *N. vaccinii* | 2536 | 0.1 |
| *Rhodoccocus* sp. | 5444 | 0.8 |
| *Saccharopolyspora erythraea* | 2427 | 18 |
| *Streptomyces achromogenes* subsp. *rubradiris* | 2630 | 137 |
| *S. achromogenes* subsp. *streptozoticus* | 2522 | 200 |
| *S. albus* | 2631 | 19 |
| *S. aureofaciens* | 2589 | 25 |
| *S. avermitilis* | 8346 | 80 |
| *S. coelicolor* | 8389 | 15 |
| *S. flocculus* | 2632 | 5.0 |
| *S. griseus* subsp. *desideus* | 2528 | 41 |
| *S. hygroscopicus* subsp. *odoratus* | 2548 | 15 |
| *S. lincolnensis* mutant NTG-3 | 8292 | 0.0 |
| *S. lincolnensis* mutant NTG-5 | 8293 | 23 |
| *S. lincolnensis* mutant #1404 | | 77 |
| *S. lividans* | 8718 | Contains LCF, concentration not determined |
| *S. rochei* | 2540 | 28 |
| *Streptomyces* sp. CMRI A16 | 2597 | 111 |
| *Streptomyces* sp. Ill. 155-2 | 2539 | 10 |
| *Streptomyces* sp. NRRL S-1182 | 2542 | 31 |
| *Streptosporangium roseum* | 2549 | 0.1 |
| *Streptoverticillium album* | 2641 | 0.7 |
| *S. ladakanum* | 2654 | 3.0 |
| *Streptoverticillium* sp. A-23 | 2663 | 31 |
| *Streptoverticillium* sp. B-8 | 2680 | 30 |
| *Streptoverticillium* sp. #179 | 2619 | 9.0 |

[a] Upjohn Culture collection No.

ical shifts and coupling constants of 6-H, 7-H, and 9-H suggested that LCF contained a 1,2,4-trisubstituted benzene ring. A two-dimensional COSY[2] experiment (spectrum not shown) confirmed this hypothesis. Furthermore, COSY results also suggested that 5-H, an isolated vinyl proton, was long-range coupled to 7-H and that the seven carbinol protons were part of a ribitol moiety.

The $^{13}C$ NMR spectrum of LCF displayed 16

signals. DEPT experiments[3] identified two methylene, seven methine, and seven quaternary carbons (Table 1). A two-dimensional $^{1}H$-$^{13}C$ chemical shift correlation experiment[4] established the correlations between protonated carbons. Three interesting observations can be made in reviewing the NMR data. First, the relatively high chemical shift value of C-8 suggests that the C-8 substituent is a hydroxyl group.[5] Second, the 15 ppm chemical shift difference between C-7 and C-9 suggests that there is another heteroatom (oxygen or nitrogen) *ortho* to the C-9 proton. Third, the $^{13}C$ NMR chemical shift value of C-1′ suggests that C-1′ is probably attached to a nitrogen atom. The anomalously high proton chemical shift values of C-1′ H's suggested that the nitrogen atom was part of a large aromatic system. Similarly, the relatively large C-5 proton chemical shift value can also be explained by the diamagnetic ring current effect caused by the aromatic system. A nuclear Overhauser enhancement (NOE) experiment provided further support to the preceding arguments. Irradiation of the 9-H enhanced the intensities of the C-1′ protons, suggesting their close proximity. On the other hand, irradiation of the C-5 proton enhanced the intensity of the C-6 proton, indicating that C-5 was *ortho* to the 6-H. These data suggested a partial structure reminiscent of a deazariboflavin. The unassigned $^{13}C$ NMR signals and the molecular formula support this assumption. The structure of LCF was, therefore, identified as **I**.

After the structure of LCF was assigned, a literature search revealed that **I** has been reported previously as a cosynthetic factor in tetracycline biosynthesis.[6] Comparison of spectroscopic data (IR, $^{13}C$ NMR, $^{1}H$ NMR, UV-vis) convinced us that LCF was identical to cosynthetic factor 1. After this work was completed, a report by DANIELS *et al.*[7] describing the presence of a 5-deazaflavin cofactor in nine species of *Streptomyces* and one *Nocardia* species was called to our attention. However, it was not clear from their report that the cofactor was identical to cosynthetic factor 1.

The fact that both *S. lincolnensis* and *Streptomyces aureofaciens* (tetracycline producer) produced cosynthetic factor 1 prompted us to examine other microbial fermentations for the presence of this compound. An analytical procedure utilizing HPLC (5 $\mu$m C-18 reverse-phase column eluted with a gradient of 0.1 M ammonium acetate and MeOH; 10% to 85% within 20 minutes at 1 ml/minute flow rate) equipped with fluorescence detection (excitation and emission at 420 and 480 nm, respectively) was developed to facilitate the process. The results are listed in Table 2. The majority of the organisms listed were grown for 4 days in medium F-13.[1]

The results in Table 2 from an examination of 16 genera of actinomycetes clearly indicate that **I** was found in almost every fermentation screened. Since no attempt was made to optimize the fermentation conditions, the results may be biased on the low end. The presence of LCF in a variety of antibiotic producing actinomycete genera, together with the fact that it plays an indispensable role in lincomycin and chlorotetracycline biosynthesis,[8] suggest that it may have a similar role in the biosynthesis of other secondary metabolites. Further evidence suggesting that the cofactor is specific for secondary metabolism in actinomycetes was found by examining fermentations of *Streptomyces achromogenes* var. *rubradiris* where a glucose effect was observed for both antibiotic production (rubradirin) and synthesis of cosynthetic factor 1.

## References


1) COATS, J. H.; G. P. LI, M.-S. T. KUO & D. A. YUREK: Discovery, production, and biological assay of an unusual flavenoid cofactor involved in lincomycin biosynthesis. J. Antibiotics 42: 472~474, 1989
2) BAX, A. & R. FREEMAN: Investigation of complex networks of spin-spin coupling by two-dimensional NMR. J. Magn. Reson. 44: 542~561, 1981
3) PEGG, D. T.; D. M. DODDRELL & M. R. BENDELL: Proton-polarization transfer enhancement of a heteronuclear spin multiplet with preservation of phase coherency and relative component intensities. J. Chem. Phys. 77: 2745~2752, 1982
4) BAX, A.: Two Dimensional Nuclear Magnetic Resonance in Liquids. Riedel, Boston, 1982
5) LEVY, G. C.; R. L. LICHTER & G. L. NELSON: Carbon-13 Nuclear Magnetic Resonance Spectroscopy. 2nd Ed. p. 11, John Wiley & Sons, New York, 1980
6) MCCORMICK, J. R. D. & G. D. MORTON: Identity of cosynthetic factor 1 of *Streptomyces aureofaciens* and fragment FO from coenzyme

F420 of *Methanobacterium* species. J. Am. Chem. Soc. 104: 4014~4015, 1982

7) DANIELS, L.; N. BAKHIET & K. HARMON: Widespread distribution of a 5-deazaflavin cofactor in actinomyces and related bacteria. Syst. Appl. Microbiol. 6: 12~17, 1985

8) MILLER, P. A.; N. O. SJOLANDER, S. NALESNYK, N. ARNOLD, S. JOHNSON, A. P. DOERSCHUK & J. P. D. MCCORMICK: Cosynthetic factor I, a factor involved in hydrogen-transfer in *Streptomyces aureofaciens*. J. Am. Chem. Soc. 82: 5002~5003, 1960