# SYNTHESIS OF N-ACETYLDEACETOXY-CEPHALOSPORIN C BY A MUTANT OF *CEPHALOSPORIUM ACREMONIUM*

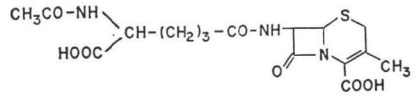
P. TRAXLER, H.J. TREICHLER and J. NÜESCH

Research Laboratories of the Pharmaceuticals Division of Ciba-Geigy Ltd., Basel, Switzerland

(Received for publication May 26, 1975)

Mutants of *Cephalosporium acremonium* C.M.I. 49137, M8650, blocked in the synthesis of cephalosporin C were investigated for accumulation of unknown $\beta$-lactam compounds. The noncephalosporin C producers were isolated after subjecting the superior cephalosporin C (CPC) producing parental strain M8650/C462 to mutagenic treatment. Mutants were screened for the inability to synthesize CPC by the method described in a previous publication[1]. All mutants blocked for CPC synthesis were grown at 23°C in shaken (250 r.p.m.) Erlenmeyer flasks (200ml) containing 50 ml of complete fermentation medium. After 7 days the cells were removed by filtration and the broths were immediately assayed for penicillin N, deacetoxycephalosporin C (DXPC), deacetylcephalosporin C (DCPC) and CPC. These antibiotics were separated by paper chromatography using a *n*-butanol - acetic acid - water (11 : 3 : 7) mixture. The cephalosporins were detected by placing the dried chromatograms on plates seeded with *Neisseria catarrhalis* and penicillinase. Antibiotics were identified by Rf and concentrations were measured by comparing zone sizes with those of standards. Among 40 isolates selected for their inability to synthesize CPC, a number of mutants which accumulated penicillin N alone, either DXPC or DCPC or both, and penicillin N, but only traces of CPC, have been found as described in the preceeding publication[1]. Beside the four classes, which were discernible based on the accumulation of penicillin N, DXPC and DCPC[1,2], a new class has been found by examining a number of DXPC producers. Mutant CP 71 accumulated DXPC (0.5 mg/ml), penicillin N (1.5 mg/ml), traces of CPC, and a new cephem compound, which had not previously been detected in any of the cultures of *C. acremonium* mutants. The new compound was identified as N-acetyldeacetoxycephalosporin C by ultra-violet and bioactivity spectrum, co-migration with authentic standard in several chromatography systems, masspectrum, nuclear magnetic resonance and infrared spectrum. In contrast, the parent of CP 71 produced high levels of CPC as well as DCPC (2 mg/ml) and penicillin N (1.5 mg/ml), but only traces of DXPC and no N-acetyldeacetoxycephalosporin C. The appearance of N-acetyldeacetoxycephalosporin C was observed in variable amounts (0.05 $\sim$ 0.2 mg/ml) in three independently obtained DXPC-producers, which were blocked in the synthesis of CPC. More isolates are required to furnish proof of a concomitance of DXPC- and N-acetyl-DXPC-production in the respective mutants.

Fig. 1. Structure of N-acetyldeacetoxycephalosporin C




Isolation and identification of N-acetyldeacetoxycephalosporin C:

A sample of the whole broth at the end of the fermentation was centrifuged to remove cells and particulates. The resulting broth was adjusted to pH 2.0 and extracted with *n*-butanol to remove lipophilic impurities and cephalosporin P. The aqueous phase was concentrated *in vacuo* and chromatographed on a column of Amberlite XAD-2 resin. After washing with water the activity was eluted with isopropanol - water (12:88). The active fractions were combined and concentrated *in vacuo*. The concentrate was applied to a column of DEAE-Sephadex A-25 and the column was developed with 0.1M $NH_4Br$+0.01M $CH_3COOH$. With this chromatography N-acetyldeacetoxycephalosporin C could be separated from DXPC and traces of CPC. The fractions containing N-acetyldeacetoxycephalosporin C were combined and desalted through a column of Amberlite XAD-2. Crude N-acetyldeactoxycephalosporin C was about 50 % pure. This material was further purified by preparative thin-layer chromatography on silicagel (*n*-butanol - acetic acid - water, 11:3:7) to obtain

N-acetyldeacetoxycephalosporin C of about 80~90% purity. Chromatographical analyses on thin-layer cellulose plates with 4 systems (Table 1) and the retention times in high pressure liquid chromatography (Table 2) afforded good evidence for the identity of this material with authentic N-acetyldeacetoxycephalosporin C prepared from DXPC by N-acetylation. Further, some fractions of the DEAE-Sephadex A-25 chromatography showed in liquid chromatography a peak with the same retention time as authentic N-acetylcephalosporin C.

Table 1. Rf values of cephalosporin C, deacetoxycephalosporin C and their N-acetyl-derivatives on cellulose thin-layer in different systems

| Compound | Rf value | | | |
|---|---|---|---|---|
| | A | B | C | D |
| N-Acetyldeacetoxycephalosporin C (from fermentation) | 0.79 | 0.59 | 0.72 | 0.77 |
| Deacetoxycephalosporin C | 0.32 | 0.38 | 0.45 | 0.51 |
| Cephalosporin C | 0.25 | 0.42 | 0.60 | 0.49 |
| N-Acetylcephalosporin C | 0.76 | 0.63 | 0.77 | 0.71 |
| N-Acetyldeacetoxycephalosporin C (authentic sample) | 0.79 | 0.60 | 0.72 | 0.76 |

Systems:
A. Isopropanol-formic acid-water (77:4:19)
B. *n*-Butanol-acetic acid-pyridin-water (37.5:7.5:25:30)
C. 66% Aqueous acetonitrile
D. *n*-Butanol-acetic acid-water (11:3:7)

Table 2. Retention times of cephalosporin C, deacetoxycephalosporin C and their N-acetyl-derivatives in liquid chromatography

| Compound | Retention time in minute |
|---|---|
| N-Acetyldeacetoxycephalosporin C (from fermentation) | 35 |
| Deacetoxycephalosporin C | 17.5 |
| Cephalosporin C | 22 |
| N-Acetylcephalosporin C | 48 |
| N-Acetyldeacetoxycephalosporin C (authentic sample) | 35 |

The chromatography was performed on a Varian liquid chromatograph 4000 by using a Zipax Sax column (1.8×150 cm; mobile phase 0.5~0.25 M borate buffer at pH 9.6; flow rate 0.45~0.7 ml per minute)

Further, some fractions of the DEAE-Sephadex A-25 chromatography showed in liquid chromatography a peak with the same retention time as authentic N-acetylcephalosporin C.

To prove the structure the material of the DEAE-Sephadex A-25 chromatography was treated with diazomethane in methanol/diethylether and the resulting dimethylester was chromatographed on a preparative silicagel thin-layer plate ($CHCl_3$-$CH_3OH$, 95:5) to obtain pure N-acetyldeacetoxycephalosporin C-dimethylester. It showed a UV-maximum at 264 nm. The masspectrum (by high resolution) gave a molecular ion *m/e* 427 ($C_{18}H_{25}N_3SO_7$) and two fragmentations at *m/e* 256 ($C_{11}H_{16}N_2O_5$) and *m/e* 172 ($C_6H_{10}NSO_2$) which are typical for the masspectroscopical cleavage of the β-lactam ring in cephalosporins. In the NMR-spectra of the dimethylester the singuletts at 1.82 and 2.0 ppm belong to the acetate methyl group respectively the methyl group in position 3 of the thiazolidine ring. Natural N-acetyldeacetoxycephalosporin C-dimethylester was in all physical-chemical properties identical with the authentic samples.

Acknowledgements

The authors are grateful to Dr. J. GRUNER for his contributions and the excellent technical assistance of Miss ROSTETTER and Mr. LACH is acknowledged with many thanks.

## References

1) NÜESCH, J.; A. HINNEN, M. LIERSCH & H. J. TREICHLER: A biochemical and genetical approach to the biosynthesis of cephalosporin C. *In*: K.D. MACDONALD (ed.). Genetics of Industrial Microorganisms: Proceedings of the second international symposium. Academic Press, London, New York, San Francisco (in press).
2) QUEENER, S.W.; J.J. CAPONE, A.B. RADUE & R. NAGARAJAN: Synthesis of deacetoxycephalosporin C by a mutant of *Cephalosporium acremonium*. Antimicr. Agents & Chemoth. 6: 334~337, 1974