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Note

Fast and simple method for the separation of intermediates and cofactors involved in the biosynthesis of cephalosporin C using chemically bonded C_{12} reversed-phase thin-layer chromatography

M. FAUPEL, H. R. FELIX and E. VON ARX

Chemical and Biological Research Laboratories, Pharmaceutical Division, Ciba-Geigy Limited, CH-4002 Basle (Switzerland)

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Reversed-phase (RP) thin-layer chromatography (TLC) on a chemically bonded C_{12} stationary phase is a more effective method for the separation of β-lactam antibiotics and cofactors involved in their biosynthesis[1] than traditional analysis by paper chromatography[2] or cellulose TLC[3]. This RP method is also a pilot technique for high-performance liquid chromatography (HPLC)[4]. Cephalosporin C or penicillin N can be detected in small amounts (0.125 g/l) using bioautography as described earlier[5].

EXPERIMENTAL

TLC plates

Commercially available 20 × 20 cm thin-layer plates pre-coated with OPTI-UP C_{12} and containing a fluorescent indicator were obtained from Antec (Bennwil, Switzerland). They were used without pre-treatment.

Thin-layer chromatography conditions

A 20-μg amount of each substance (for bioautography 1 μg of penicillin N and cephalosporin C is used) was applied with a modified Hamilton syringe exactly as for adsorption chromatography. The chromatography was initiated on 20 × 20 cm OPTI-UP C_{12} plates, which were developed in a twin-trough[6] tank (Camag, Muttenz, Switzerland) without saturation of the tanks. The solvents move 15 cm in 90 min. The plates were dried at room temperature in a stream of cold air.

Detection

The detection techniques are indicated in Table I.

Chemicals

All chemicals were of analytical-reagent grade. Antibiotics were obtained from Ciba-Geigy Limited (Basle, Switzerland) and δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine was a gift from Dr. Shirafuji[8].

Preparation of bioautography plates

Three different strains of bacteria were used: *Alcaligenes faecalis* ATCC 8750 (AF), *Neisseria catarrhalis* ETH 4163 (NC) and *Sarcina lutea* ATCC 9341 (SL). The bacteria are preserved in lyo-ampoules or on agar slants and stored at 4 °C. For breeding, 500-ml erlenmeyer flasks with one baffle and 100 ml of BHI nutrient broth for NC and SL or 100 ml of Alc-nutrient broth for AF (3.0 g/l of L-asparagine, 0.125 g/l of L-cysteine, 0.1 g/l of peptone, 0.5 g/l of KH_2PO_4, 1.5 g/l of K_2HPO_4, 2.0 g/l of $Na_2SO_4 \cdot 10H_2O$, 0.05 g/l of $MgSO_4 \cdot 7H_2O$ and 5 g/l of sodium acetate, pH 7.0) are inoculated with 10, 2.5 or 0.3% of a 24-h culture of AF, NC or SL. The flasks are shaken for 24 h at 250 rpm and 25 °C.

The bioautography plates are prepared by pouring DST-agar (Oxoid) or Alc-agar (Alc-nutrient broth with 15 g/l of bacto-agar) into a rectangular plastic dish (22 × 15 cm). After the base layer has solidified, 100 ml of the agar inoculated

TABLE I

R_F VALUES FOR β-LACTAM ANTIBIOTICS, INTERMEDIATES AND COFACTORS OF THEIR BIOSYNTHESIS AND SOME OTHER COMPOUNDS

Solvents: I = water–dioxan (98:2); II = water; III = water + 10 g/l of Na_2SO_4.

Compound (1 µl of a 2% solution in water)	*Solvent*			*Detection*
	I	*II*	*III*	
Cephalosporin C	0.42	0.32	0.23	UV_{254}, ninhydrin
Deacetylcephalosporin C	0.76	0.73	0.60	UV_{254}, ninhydrin
Deacetoxycephalosporin C	0.56	0.51	0.40	UV_{254}, ninhydrin
Penicillin N	0.56	0.47	0.39	Ninhydrin, TDM**
α-Aminoadipylcysteinylvaline (dimer)	0.24	0.06	0.07	Ninhydrin
α-Aminoadipylcysteinylvaline (monomer)	0.45	0.32	0.32	Ninhydrin
α-Aminoadipic acid*	0.88	0.88	0.83	Ninhydrin
Valine	0.69	0.69	0.66	Ninhydrin
Cysteine	0.84	0.84	0.82	Ninhydrin
Cystathionine*	0.91	0.92	0.93	Ninhydrin
Adenosine 5'-triphosphate	0.43	0.35	0.33	UV_{254}, TDM
Acetate (sodium salt)	0.80	0.81	0.71	Bromocresol green
α-Ketoglutaric acid	0.86	0.91	0.82	I_2, UV_{254}
Acetylcoenzyme A	0.06	0.02	0.03	UV_{254}, TDM
Coenzyme A	0.11	0.03	0.04	UV_{254}, TDM
Alanine	0.86	0.86	0.85	Ninhydrin
Fusidic acid (= cephalosporin P)	0.00	0.00	0.00	I_2
Glutamic acid	0.92	0.91	0.89	Ninhydrin
Glutamine	0.85	0.83	0.82	Ninhydrin
Glutaric acid	0.82	0.85	0.71	Bromocresol green
Glutathione (reduced)	0.83	0.83	0.71	Ninhydrin
Glutathione (oxidized)	0.90	0.89	0.75	Ninhydrin
Glycine	0.90	0.91	0.91	Ninhydrin
Homocysteine	0.76	0.76	0.73	Ninhydrin
α-Ketoadipic acid	0.83	0.89	0.77	I_2, UV_{254}
α-Ketoisovaleric acid	0.67	0.68	0.57	I_2, UV_{254}
Lysine	0.86	0.85	0.90	Ninhydrin
Methionine	0.60	0.59	0.58	Ninhydrin
Serine	0.93	0.95	0.93	Ninhydrin

* One drop of HCl was added to 100 µl of solution.

** TDM = 4,4'-tetramethyldiaminodiphenylmethane[7].

with 1.5, 2.5 or 0.3% of a shaken flask culture of AF, NC or SL are poured on to it. When this seed layer has solidified the bioautography plate is dried at 37 °C for about 45 min. No residual water droplets should be visible on the surface of the agar.

The thin-layer plate is placed very carefully on top of the seed agar layer. No air bubbles should occur between the agar surface and the thin-layer plate. The bioautography plate is kept at 4 °C for 30 min in order to allow diffusion into the inoculated agar to take place. The thin-layer plate is then removed and the bioautography plate is incubated at 37 °C for 18–24 h.

Antibiotically active substances are visible on the plate as clear zones, without growth of the bacteria.

RESULTS AND DISCUSSION

The R_F values are given in Table I. Each value is the average of five to seven independent migrations. Cephalosporin C and penicillin N can be distinguished clearly. With this separation technique, enzyme reactions such as the acetylation of deacetylcephalosporin C[9] can be measured.

Using reversed-phase thin-layer plates in bioautography assays (Fig. 1), cephalosporin C and penicillin N can be detected with *Neisseria catarrhalis*, cephalosporin C with *Alcaligenes faecalis* and penicillin N with *Sarcina lutea*. Even a quantitative determination of the antibiotics is possible using a standard calibration graph constructed with 2 μl of a 0.25–1.5 g/l solution of penicillin N.

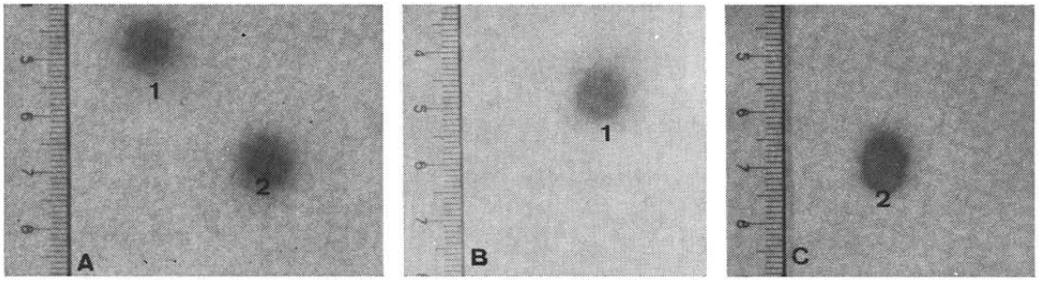

Fig. 1. Bioautography with cephalosporin C (1) and penicillin N (2). A = *Neisseria catarrhalis*: B = *Alcaligenes faecalis*: C = *Sarcina lutea*. Contrast photography was used; therefore the clear zones in the original appear as dark regions here.

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