Preparation of biologically active mono-*N*-acetyl(^{14}C)-derivatives of the membrane-specific polypeptide antibiotic polymyxin B

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(Z. Naturforsch. **25 b**, 117 [1970]; eingegangen am 22. Dezember 1969)

Working on the mechanism of action of polymyxin B [1, 2], the availability of radioactive labelled polymyxin became necessary in order to determine the exact amount of the antibiotic which has to be bound per cell to produce a lethal event. I want to report a simple and convenient method for the preparation, identification and isolation of mono-*N*-acetyl(^{14}C)-polymyxin B having a well defined, high specific activity together with the original bactericidal properties. Polymyxin B sulfate (sterile powder) was generously supplied by Pfizer GmbH, Karlsruhe, Germany. Acetic anhydride-1-^{14}C (spec. activity: 62 mc/mmole) was purchased from the Radiochemical Center, Amersham, England. Acetylation of polymyxin B: 2 mg ($\sim$ 1.6 μmoles) of the active base dissolved in 0.4 ml of water were mixed with 0.1 ml of saturated sodium bicarbonate solution (final pH = 8.0) and added to 100 μc of free acetic anhydride-1-^{14}C (1.62 μmoles). After a reaction time of 30 min at 12 °C, samples were withdrawn and the reaction products were separated by high-voltage paper electrophoresis at pH 2 (formic acid : acetic acid : H_2O = 50 : 150 : 800). On a preparative scale, separations were performed on cellulose coated aluminium foils (Merck, Darmstadt, Germany) using n-butanol : pyridine : water (6 : 4 : 3) as a solvent. The partially *N*-acetylated compounds were quantitatively eluted from the cellulose with 1% sodium chloride solution. The bactericidal activity was determined against *Salmonella typhimurium* G 30 [3].

Fig. 1 demonstrates that the reaction mixture contained 5 radioactive products (B – F) corresponding to ninhydrin positive spots of a nonradioactive reference preparation. The polymyxin B molecule has 5 free γ-aminogoups of the L-α,γ-diaminobutyric acid residues [4]. Substitution of these aminogroups by *N*-acetylation should lead to a loss of one electrical charge per substituted group. Based on a positive net charge of 5 for free polymyxin B (spotA, Fig. 1) at pH 2, net charges of 4.4, 3.26, 2.24, 1.12 and 0 were calculated from their rate of migration for compounds B, C, D, E and F, respectively. Since the electrophoretic mobility of a substance at a given molecular weight is proportional to its electrical net charge, compounds B to E could identified unequivocally as mono-, di-, tri-, tetra- and penta-*N*-acetyl-polymyxins. That this assignment was true, was substantiated by the observed behaviour of the *N*-acetyl-polymyxins on thin-layer chromatography. According to their lipophilic character, their R_f-values increased with the number of *N*-acetylated amino groups (polymyxin B: 0.26; B: 0.38; C: 0.52; D: 0.68; E: 0.90; F: 0.97). No *O*-acetylation of the two threonine hydroxylgroups occurred as indicated by the absence of radioactivity from spot A (Fig. 1). Final prove for the correct identification of the *N*-acetylated polymyxins was obtained by visual estimation of their reactivity with ninhydrin after chromatographic separation:

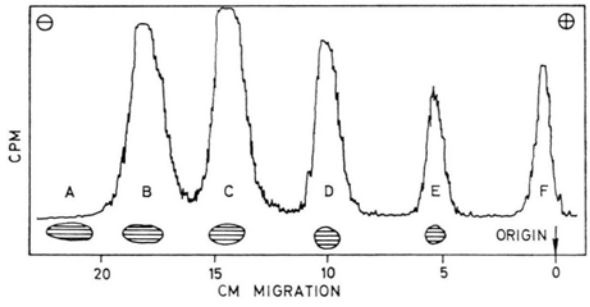

Fig. 1. Paper electrophoretic separation of *N*-acetyl(^{14}C)-substituted polymyxin B derivatives. A: polymyxin B (PX); B: mono-*N*-acetyl-PX; C: di-*N*-acetyl-PX; D: tri-*N*-acetyl-PX; E: tetra-*N*-acetyl-PX; F: penta-*N*-acetyl-PX. Shaded spots are ninhydrin positive areas.

Compound F did not stain at all, whereas compound B was the most reactive one. Under these conditions, the yields of the single compounds could be calculated from the spec. activity of the used acetic anhydride (Table 1).

Compound	Yield [μc]	Yield [μmoles]	Biol. Act
polymyxin B (PX)	0	–	+
nono-*N*-acetyl-PX	8.2	0.26	+
di-*N*-acetyl-PX	7.2	0.12	+
tri-*N*-acetyl-PX	6.4	0.07	(+)
tetra-*N*-acetyl-PX	5.9	0.05	0
penta-*N*-acetyl-PX	6.9	0.05	0

Table 1. Yield and biological activities of *N*-acetyl(^{14}C)-substituted polymyxin B derivatives.

The biological activity as estimated by the agar diffusion test is shown in the last column of table 1. But only the mono-*N*-acetyl-polymyxin B was equally active as the parent polymyxin B when determined quantitatively by a dilutions series in liquid culture. The minimal concentration necessary to halt growth of *Salmonella typhimurium* (7.5×10^8 cells/ml) completely was 4 μg/ml both for polymyxin B and its mono-*N*-acetyl(^{14}C)-derivatives. At bactericidal levels, mono-*N*-acetyl(^{14}C)-polymyxin B was not deacetylated by *S. typhimurium*. Despite the fact that mono-*N*-acetyl(^{14}C)-polymyxin B is naturally a mixture of 5 isomers, it should be an useful tool to do meaningful binding studies [5].

This investigation was supported by a grant from the Deutsche Forschungsgemeinschaft.

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