A Simple Method of Preparation of Labelled Peptides Possessing Antibiotic Properties

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Preparation of derivatives of peptide antibiotics polymyxin B and colistin- substituted on free amino groups by aminocarboxylic acid is described. The first procedure is based on the condensation of the antibiotic with active ester of a suitably protected amino acid. The second one makes use of Woodward's reagent by condensation of the antibiotic with a protected amino acid. The following compounds were prepared: glycyll-14C-polymyxin B and glutaminyll-polymyxin B and glycyll-colistin, also under conditions suitable for a radioactive synthesis. All the preparations preserve a substantial part of the biological activity comparable with polymyxin B and colistin.

Note: As the free y-amino groups of Dab are practically equal, it may be assumed that the molecules of glycine are bound statistically uniformly to the molecule of polymyxin or colistin. Dab = L-γ-diaminobutyric acid, Leu = L-leucine, D-Leu = D-leucine, D-Phe = D-phenylalanine, Thr = L-threonine, Ipel = (+)-isopelargonic acid.

Iodination with iodine isotopes or acylation of free amino groups, for which both 14C and 3H may be used gave methods for the labelling of proteins. Holt and co-workers 1 acylated wool with p-nitrophenyl esters of 14C-labelled fatty acids and they observed that the acylation took place predominantly at ε-amino groups of lysine residues. Carpenter 2 described the synthesis of diaminoacyl- and triaminoacyl-insulins which still possessed 40 — 50% of the biological activity of insulin itself. Recently, Teuber 3 prepared N-acetyl-14C-derivatives of polymyxin B. The formed mixture of mono- to penta-acetyl-14C-derivatives could be resolved to unreacted polymyxin and mono-, di-, tri-, tetra-, and penta-acetyl derivatives using paper electrophoresis. Of these derivatives only monoacetyl derivative (the radioactivity of which made only 23.7% of the total radioactivity of the crude product) possessed suitable bactericidal properties against Salmonella typhimurium.

In this paper we demonstrate that in the case of basic polypeptides having antibiotic properties it is more convenient to carry the substitution of free hydrogen atoms of the amino groups by aminoacyl residues, as for example labelled glycine. The convenience of this method consists in the fact that the original basic character of the molecule remains intact, and hence, also the biological activity. Our reasoning was based on the assumption that the presence of free amino groups is indispensable for the preservation of the biological activity, while a small change in the length of the side chain might not be deleterious.

Thus, for example, Morozova and co-workers 4 announced the synthesis of a series of linear and cyclic analogues of polymyxin in which diaminoacylic acid was substituted by lysine in order to prepare antibiotics with a decreased toxicity.

For the preparation of glycyll-14C-polymyxin we made use of two alternative procedures:

1. For example, to the sulfate of polymyxin B (Aerosporin, B.P.-producer Burroughs Wellcome and Co., London) (36.3 mg; 25 μmol) suspended in 3.5 ml of acetonitrile in a centrifugal test tube 0.2 N NaOH (0.25 ml; 50 μmol) was added under stirring followed by a solution of 2,4,5-trichlorophenylglycine prepared by condensation of 2,4,5-trichlorophenylderivatives with 14C (prepared by condensation of 2,4,5-trichlorophenol in the presence of dicyclohexylcarbodiimide) (20.4 mg; 50 μmol) in dioxan (1.2 ml). The reaction mixture was shaken for 16 hours and then extracted 5-times with}

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2 D. LEVY and E. CARPENTER, Biochemistry 6, 3559 [1967].
4 E. A. MOROZova, M. A. ZEWAIL, E. S. OKSENOIT, and E. N.

Gorbacheva, Abstract of the paper read at the 7th International Symposium on the Chemistry of Natural Products, Riga 1970 — A 16, p. 35—36.

* Non-radioactive trichlorophenyl ester of o-nitrophenylsulfenylglycine was prepared for the first time by Dr. E. Kasarík of the Research Institute for Pharmacy and Biochemistry in Prague, to whom we express our gratitude for kindly donating a sample.
1.5 ml portions of light petroleum. The lower layer was concentrated to dryness in a rotatory evaporator and the residue was washed with ice-cold 0.01 N sulfuric acid (0.25 ml). After centrifugation the product was washed twice with icy water (0.25 ml and 0.15 ml). The washed intermediary product was dried in a vacuum desiccator, dissolved in 0.3 ml of glacial acetic acid, additioned with 0.15 ml of 1.46 N HCl in acetic acid, and allowed to stand at room temperature for 10 minutes. Dry ether was added to the mixture and the precipitated material was centrifuged and washed four times with dry ether. After drying in a vacuum desiccator the crude glycyl-2-14C-polymyxin was purified by preparative paper electrophoresis (Whatman M3 paper; 22 V/cm; buffer pH 5.7; 2 hours). After elution with 0.05 N acetic acid, evaporation in vacuo, and drying in a vacuum desiccator 19.9 mg (approx. 52%) of a product were obtained which was easily soluble in water and the specific activity of which was 0.15 mcg/mg, i.e. 0.177 mCi/mmol. The radiochemical yield was 2.99 mCi, i.e. 38%. On checking the purity by electrophoresis it was found that the product (Relative electrophoretic mobility: VPM = 0.9) does not contain either glycine or unsubstituted polymyxin.

2. The second, alternative, procedure makes use of the activation of the carboxyl group of a suitably protected amino acid (for example o-nitrophenyl-sulphenyl-glycine) by means of Woodward’s reagent, i.e. 2-ethyl-5-phenylisoxazolium-3'-sulphonate: To a solution of o-nitrophenyl-sulphenyl-glycine (set free from its dicyclohexylammonium salt) (11.4 mg; 50 µmol) in 1 ml acetonitrile 0.20 ml of a solution of N-ethylpiperidine (containing 5.66 mg, i.e. 50 µmol) in acetonitrile and 2-ethyl-5-phenyl-isoxazolium-3'-sulphonate (12.7 mg; 50 µmol) were added and the mixture was shaken until the reagent was dissolved (30 min). The obtained solution was added to a suspension of polymyxin B sulfate (36.3 µg; 25 µmol) in 3.5 ml of acetonitrile containing 0.5 N NaOH (0.1 ml), and the mixture was shaken for 22 hours. The reaction mixture was worked up as in the 1st procedure, and the result was practically the same. However, as the second procedure, utilizing Woodward’s reagent, is one synthetic step shorter, it seems more suitable.

K. Poduška, private communication.
Biosynthesis and biological properties of aminocylated derivatives of basic antibiotics

a) The determination of the antibiotic activity of polymyxin and colistin derivatives was carried out using the plate diffusion method.

Average activity of the derivatives of basic antibiotics, compared with a respective non-substituted standard (in aqueous solutions in the 100—10000 μg/ml concentration range).

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Escherichia coli [%]</th>
<th>Bacillus subtilis [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycyl-polymyxin</td>
<td>84</td>
<td>83</td>
</tr>
<tr>
<td>Glutaminyl-polymyxin</td>
<td>42</td>
<td>70</td>
</tr>
<tr>
<td>Glycyl-colistin</td>
<td>39 (108 *)</td>
<td>126 **</td>
</tr>
</tbody>
</table>

* When the activity of glycyl-colistin was compared with that of colistin-methanesulfonate as standard. ** At concentrations up to 500 μg/ml the activity is lower than in the case of un-substituted colistin, while at higher concentrations the activity is also increased. This is probably caused by the change in the diffusion properties of substituted colistin.

b) It was shown that the prepared derivatives distinctly put a stop to the postgerminal development of spores and the vegetative growth of Bacillus cereus.

Chemical investigation, as well as biological testing of the prepared derivatives is continued.

It can be assumed that the easy method of preparation described here will also be useful in the case of other basic peptides possessing antibiotic activity.

For the kind performance of microbiological tests our thanks are due to Dr. V. Musilek and Dr. M. Blumauerova of the Institute of Microbiology of the Czechoslovak Academy of Sciences, Prague. We are also grateful to Mr. Z. Zbrozek of the Institute of Organic Chemistry and Biochemistry of the Czechoslovak Academy of Sciences for the analyses of amino acids.

Dielectric Studies of Aggregation Phenomena with Separated α and β Chains of Human Hemoglobin

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The concentration dependence of the dielectric dispersion between 100 kHz and 15 MHz has been investigated for separated α and β chains of human hemoglobin. It is shown that such dielectric measurements can give very detailed information on aggregation phenomena. Specific dielectric increments and dipole moments of monomeric and aggregated chains could be obtained.

In a previous paper we have discussed the mechanism of the dielectric dispersion at 1 MHz for aqueous hemoglobin and myoglobin solutions in some detail and have found that the only possible explanation for this dispersion is an orientational relaxation mechanism. For this mechanism the theory of Onsager gives a connection between the macroscopic dielectric parameters and the microscopic quantities of the individual molecules. A further proof of this mechanism and also an interesting application of this knowledge provides the study of an aggregating system. Such a system was found in the separated α and β chains of human hemoglobin. From the dielectric study of such an