17. V. F. Pozdnev, USSR Author's Certificate No. 585,165. Otkr. Izobr. Tov. Znaki, No. 47, 81 (1977).
18. V. F. Pozdnev, Zh. Obshch. Khim., 48, 476 (1977).
19. K. Inouye and K. Otsuka, J. Org. Chem., 27, 4236 (1962).
20. B. Handford, T. Hylton, Hung Tsung-Wang, and B. Weinstein, J. Org. Chem., 33, 4251 (1968).
21. J. L. Roosemount, Anal. Biochem., 88, 314 (1978).
22. IUPAC Inform. Bull., App. No. 23, 3 (1972).
23. J. H. Jones and W. I. Ramage, J. Chem. Soc., Chem. Commun., 472 (1978).
24. H. Matthews and H. Rapaport, J. Am. Chem. Soc., 95, 2297 (1973).
25. H. Rink and B. Riniker, Helv. Chim. Acta, 57, 831 (1974).
26. V. F. Pozdnev, E. A. Smirnova, N. N. Podgornova, N. K. Zentsova, and U. O. Kalei, Zh. Org. Khim., 15, 106 (1979).

CHEMICAL CHARACTERIZATION OF THE CRYSTALLINE PROTEINS OF *Bac. thuringiensis*. CLEAVAGE WITH CYANOGEN BROMIDE

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The crystalline proteins from *Bac. thuringiensis* of the third and fourth serotypes with a molecular weight of 130,000 have been isolated and carboxymethylated, and their amino acid compositions has been determined. The CM-proteins have been found to be labile in acid media (80% CF_3COOH and 65% HCOOH). Conditions have been found for the cleavage of the CM-protein of the third serotype with cyanogen bromide. A BrCN fragment with a molecular weight of 16,000 has been isolated, the intensity of which in 5% polyacrylamide gel on scanning amounts to about half the intensity of the sum of all the fragments. The amino-acid composition of this fragment has been determined.

The crystalline protein formed by *Bac. thuringiensis* during sporulation is pathogenic for a number of insects and is therefore of great interest for chemical and biochemical investigations. In the majority of cases (this form of bacterium consists of 13 serotypes) the crystalline inclusions consist of a single protein with a molecular weight of 130-140 thousand daltons [1]. According to available literature information [2-4], these proteins differ little from one another in amino acid composition, but, nevertheless, the toxicities of the crystals of the different serotypes have not a broad, but a restricted action spectrum. The interrelationship between the features of the chemical structure and the biological activity of these proteins is the most interesting aspect of the study of the entomopathogenic crystalline inclusions of *Bac. thuringiensis*.

We have investigated the third and fourth serotypes, the crystals of which consists of a single protein with a molecular weight of 135 thousand daltons. After three days' growth at 28°C, the culture liquid was centrifuged, and the precipitate was treated with 0.1 N Na_2HPO_4 containing 8 M urea and 10^{-2} M mercaptoethanol for 7 min in the boiling water bath. The insoluble fraction was separated off by centrifuging. The protein present in the solution was carboxymethylated with the aid of iodoacetic acid. As can be seen from the results of electrophoresis in 5% polyacrylamide gel (Fig. 1a, b), the CM-protein isolated from the biomass contained as impurity a very small amount of a protein with a molecular weight of 23 thousand daltons (the accurate molecular weight and percentage of this impurity were not determined) and only in this way did it differ from the CM-protein obtained from the pure crystals.

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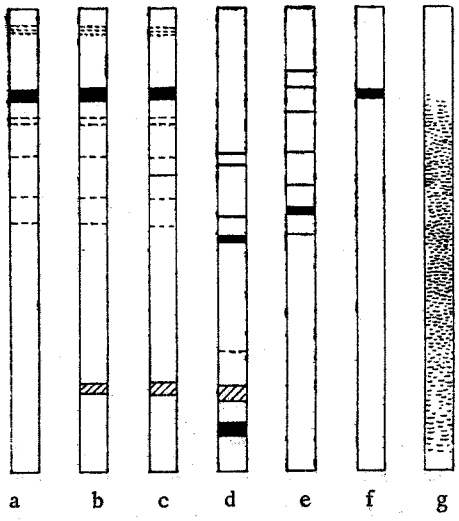

Fig. 1. Disk electrophoresis in polyacrylamide gel in 8 M urea and 1% SDS (a, b, c, d, f, and g — in 5% gel, and e in 10% gel): a) CM-protein of the third serotype obtained from the pure crystals; b) obtained from the biomass; c) CM-protein of the third serotype + 60% HCOOH, T_b, 24 h; d, e) CM-protein of the third serotype + BrCN in 60% HCOOH, T_b, 24 h; f) CM-protein of the fourth serotype; g) CM-protein of the fourth serotype + 80% CH_3COOH, 28°C, 24 h.

The most widely used method of fragmenting proteins is that of BrCN cleavage, which is carried out in a strongly acid medium. The solvents used are, as a rule, trifluoroacetic and formic acids. However, we have observed that the proteins of the crystals are extremely unstable under these conditions, and on incubation for a day at 20-30°C give a complex mixture of cleavage products. The CN-protein of the crystals of *Bac. thuringiensis* of the fourth serotype proved to be particularly sensitive to acids (80% CF_3COOH and 65% HCOOH). As can be seen from the results of disk electrophoresis (Fig. 1g), the product of the incubation of this protein in 80% CF_3COOH consists of a difficultly separable mixture of proteins with molecular weights ranging from 10 thousand to 120 thousand.

The CM-protein of the crystals of *Bac. thuringiensis* of the third serotype was less sensitive to acids, but in 80% CF_3COOH it also underwent considerable decomposition. The reason for the increased acid lability of the protein remains unclear at this point. It may be assumed that cleavage of certain peptide bonds sensitive to acids (Asp-Asp, Pro-Asp) [5, 6] takes place, which, as is well known, causes various complications on working with proteins under acid conditions. In order to account for this phenomenon, simultaneously with the presence of such bonds labile under acid conditions in the protein one must also assume their frequent repetition in the protein molecule. Other explanations of the increased sensitivity of the protons of the crystal to acids are apparently possible.

We have found that 60% HCOOH causes only a slight decomposition of the protein of the crystals, and therefore it was used as the solvent for the BrCN treatment of the protein of the crystals of *Bac. thuringiensis* of the third serotype (Alesti variant). A further lowering of the concentration of HCOOH led to incomplete cleavage at the methionine residues. Under the optimum conditions of performing the reaction, according to the results of electrophoresis in 5% and 10% polyacrylamide gel, several fragments were formed (Fig. 1d).

The scanning of the gel showed that only four fragments were present in amounts of 10% and more each. The intensity of a fragment with a molecular weight of 16 thousand amounted to about half the intensity of all the fragments of the BrCN hydrolysate, which leads to the idea of the existence of a repetition of this fragment in the initial protein. A sample of this fragment isolated with the aid of preparative electrophoresis in 5% polyacrylamide gel was homogeneous on electrophoresis in 5, 10, and 15% gels.

Below we give the amino acid compositions of the proteins of the crystals of *Bac. thuringiensis* of the third and fourth serotypes, and of the cyanogen bromide fragment with a molecular weight of 16,000 (number of residues/100 residues) (see Table 1). The amino acid composition of the fragment with a molecular weight of 16,000 differs only slightly from that of the initial protein. The investigation of the BrCN hydrolysate is continuing.

EXPERIMENTAL

Carboxymethylation of the Protein in the Crystals. A precipitate of the crystals or the biomass (from 10-12 flasks) was dissolved in 150 ml of a hot (97-100°C) 8 M urea, 0.01 M EDTA,

TABLE 1

Amino acid	Serotype 4	Serotype 3	Fragment with mol. wt. 16,000
Aspartic acid	10.9	10.5	10.9
Threonine	6.6	6.9	7.0
Serine	7.5	6.6	7.2
Glutamic acid	14.2	11.7	11.5
Proline	5.3	4.7	5.9
Glycine	7.4	8.1	7.7
Alanine	5.9	8.3	7.2
Valine	7.1	11.8*	10.6*
Methionine	0.7	1.6	—
Isoleucine	4.9	5.0	4.7
Leucine	7.8	7.1	7.1
Tyrosine	4.6	2.5	2.3
Phenylalanine	4.1	6.2	5.7
Lysine	2.3	1.9	1.3
Histidine	2.1	1.7	2.6
Arginine	7.1	4.7	6.7

*The peak of an unknown substance is superposed on the valine peak in the chromatogram.

0.01 M mercaptoethanol, and 0.1 N Na_2HPO_4 (pH 8.9-9.1) solution and the mixture was heated in the boiling water bath for 7 min. The undissolved part was separated off by centrifuging, 1.5 ml of mercaptoethanol was added to the solution, argon was passed through it for 5 min, and it was left overnight at 20°C. The excess of mercaptoethanol was eliminated by passing the protein solution through a column (5.0 × 46 cm) of Sephadex G-25 equilibrated with a 0.1 N solution of Na_2HPO_4 containing 8 M urea. Argon was passed through the solution of the protein fraction and then 400 mg of iodoacetic acid in 5 ml of 0.1 N Na_2HPO_4 solution containing 8 M urea was added and the mixture was incubated at 20°C for 30-40 min. Then the protein solution was desalted on a column (5.0 × 46 cm) of Sephadex G-25 equilibrated with distilled water, and the CM-protein was freeze-dried.

Cleavage of the CM-Protein with Cyanogen Bromide. A solution of 110 mg of the CM-protein of the crystals in 28 ml of 60% HCOOH was treated with 200 mg of BrCN and was left in the dark at 20°C for a day. Then it was diluted with water and freeze-dried.

The complete hydrolysis of the protein was carried out in evacuated tubes with 5.7 N hydrochloric acid at 105°C for 24 h, and the hydrolysate was analyzed on a Durrum automatic amino acid analyzer (USA).

Isolation of BrCN Fragment with mol. wt. 16,000. The BrCN hydrolysate of the CM-protein of the crystals was dissolved in a mixture containing 0.1 N Na_2HPO_4, 8 M urea, 0.1% SDS, and 0.01 M mercaptoethanol and the solution was deposited on tubes containing 5% polyacrylamide gel and was subjected to electrophoresis by the Weber method. A control tube was stained and on it the sections of the gel containing the protein fragment with a molecular weight of 16,000 were marked. The gel was comminuted, the protein was extracted with distilled water and the extract was dialyzed against distilled water and was freeze-dried.

SUMMARY

1. The lability of the proteins of the crystals produced by *Bac. thuringiensis* (third and fourth serotypes) in an acid medium has been shown.

2. Conditions have been selected for the cleavage of the carboxymethylated protein of the crystals of the third serotype with cyanogen bromide. A cyanogen bromide fragment with a molecular weight of 16,000 has been isolated and its amino acid composition has been determined.

LITERATURE CITED

1. I. A. Zalunin, G. G. Chestukhina, and V. M. Stepanov, Biokhimiya, 44, No. 4, 693 (1979).
2. G. G. Chestukhina, L. I. Kostina, I. A. Zalunin, T. S. Kotova, S. P. Katrukha, Yu. S. Kuznetsov, and V. M. Stepanov, Biokhimiya, 42, No. 9, 1660 (1977).
3. M.-F. Glatron, M.-M. Lecadet, and R. Dedonder, Eur. J. Biochem., 30, 330 (1972).

4. B. H. Herbert, H. J. Gould, and E. B. Cham, Eur. J. Biochem., 24, 366 (1971).
5. D. Piszkiewiz, M. Landon, and E. L. Smith, Biochim. Biophys. Res. Commun., 40, 1173 (1970).
6. Ya. I. Lapuk, Yu. S. Kuznetsov, and V. M. Stepanov, Khim. Prir. Soedin., 540 (1964).

INSECT PHEROMONES AND THEIR ANALOGS.

IV. THE SYNTHESIS OF THE SEX ATTRACTANT OF THE HONEY BEE *Apis mellifera*

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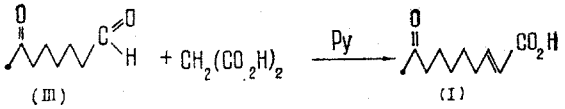
A new and convenient method for the synthesis of 9-oxodec-2E-enoic acid (I) from the readily accessible octa-2E,7-dienyl acetate is proposed. From the latter compound by a series of transformations (oxidation of the terminal double bond to a ketone, saponification, hydrogenation, and oxidation with Corey's reagent) 7-oxooctanal is obtained, and this is condensed by the Knoevenagel reaction with malonic acid to give compound (I) with a yield of 69%.

The synthesis of the pheromone of the honey bee, 9-oxodec-2E-enoic acid (I), has been considered in a number of papers [1-5]. In the majority of cases, as the initial compounds for obtaining the acid (I) difficultly accessible compounds are used or the syntheses are characterized by a large number of stages.

We have proposed a new approach to the synthesis of the acid (I) which is based on the use as the key compound the readily accessible octa-2E,7-dienyl acetate (II). The latter was obtained by the telomerization of butadiene with acetic acid under the action of low-valence phosphine complexes of palladium [6].

In particular, the synthesis of the acid (I) is effected by the Knoevenagel condensation of malonic acid with 7-oxooctanal (III) in pyridine [7]. Under these conditions, the yield of compound (I) is not less than 90%.



(III) (I)

To obtain the aldehyde (III), the acetate (II) was first converted into 7-oxooct-2E-enyl acetate (IV) by a literature method [8], and the hydrolysis of the latter with 5% NaOH in methanol led to the alcohol (V) in quantitative yield. Hydrogenation of the unsaturated compound (V) over 5% Pd/C in ethyl acetate gave the oxo alcohol (VI) with a yield of ∿100%. Compound (VI) was readily oxidized by the Corey reagent at room temperature into compound (III) with a yield of 80% [9].

It must be mentioned that in the reduction of the acetate (IV) with 5% Pd/C hydrogenolysis of the acetate group takes place, leading with high yield to 2-oxooct-6E-ene (VII). Thus, we have proposed a simple and convenient route for the synthesis of the attractant of the honey bee from octa-2,7-dienyl acetate and malonic acid (see Scheme 1).

EXPERIMENTAL

We used octa-2E,7-dienyl acetate with a purity of 98%. The compounds obtained were analyzed on a Khrom-41 chromatograph with a 1.2-m column containing 15% of SE-30. PMR spectra were recorded on a Tesla 480 BS instrument in CCl_4 solution (with HMDS as internal standard). IR spectra were taken on a UR-20 instrument (film) and mass spectra on a MKh-1306 instrument with an energy of the ionizing electrons of 70 eV at a chamber temperature of 200°C.

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