AMINO AND CARBOXY-TERMINAL SEQUENCES OF NEOCARZINOSTATIN, AN ANTI-TUMOR POLYPEPTIDE ANTIBIOTIC

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1. Introduction

Neocarzinostatin (NCS) is an anti-tumor polypeptide antibiotic, isolated from a culture filtrate of *Streptomyces carzinostaticus* [1]. It is an acidic single-chain polypeptide with a molecular weight of about 9000 [2]. Biochemical studies have shown that NCS specifically inhibits deoxyribonucleic acid synthesis in susceptible microorganisms [3, 4] or in mammalian tumor cells [5]. Chemical investigations of the amino acid composition established the presence of two tryptophan residues [7] and the absence of histidine and methionine residues [2]. All optically active amino acids appear to be of the L configuration since no significant oxygen uptake was observed manometrically when an acid hydrolysate of NCS was treated with D-amino acid oxidase [2, 8]. NCS contains four half-cystine residues forming two disulfide bridges [2]. The occurrence of NH_2-terminal alanine and of COOH-terminal asparagine has been reported earlier [2, 6]. Investigations of amino-terminal and carboxy-terminal amino acid sequences are herein reported.

2. Materials and methods

Crude NCS was prepared by Kayaku Antibiotics Research Laboratory, Inc., Tokyo, and obtained through the courtesy of Dr. N.Ishida, Tohoku University, Sendai, Japan. It was purified five-fold by repeated chromatography on CM-cellulose, followed each time by Sephadex G-50 column chromatography as described previously [2]. For heat denaturation a 1% solution of NCS in 1% $NaHCO_3$ was heated in a water bath at 80° for 20 min. Reduction and *S*-benzylation in liquid ammonia gave single-chain tetra *S*-benzyl NCS [9]. Phenylisothiocyanate (PITC, Eastman Kodak, Rochester, N.Y., USA) was distilled *in vacuo*. Constant boiling HCl was twice distilled. All other chemicals were of reagent grade and were used without further purification. Leucine aminopeptidase (LAPC, 100 units/mg, EC 3.4.1.1) and diisopropylphosphofluoridate-treated carboxypeptidase A (COADFP, 45.8 units/mg, EC 3.4.2.1) were obtained from Worthington Biochemical Corp., Freehold, N.J., USA. The carboxypeptidase was further purified by washing four times with distilled water [10].

2.1. NH_2-*terminal analysis*

The dansylation reaction [11] and the identification after acid hydrolysis of the dansyl amino acids on polyamide thin-layer sheets † were done according to the procedure of Woods and Wang [12].

For the Edman degradation [13–15] native NCS (1.5 μmoles) in pyridine-water (1:1, v/v, 2–3 ml, ad-

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jsuted to pH 9.0–9.5 with 0.1 N NaOH) was treated with PITC (50 μl, approximately 200-fold excess) for 2 hr at 40° under nitrogen in a closed test tube. After the removal of excess PITC by five washings with thiophene-free benzene (Merck and Co., Inc., Rahway, N.J., USA) the solution was brought to dryness *in vacuo*. The phenylthiocarbamoyl-peptide was treated with trifluoroacetic acid for 15 min at 40° under nitrogen. After removal of trifluoroacetic acid *in vacuo* the residue was dissolved in 0.1 N acetic acid and the solution was washed four times with ethyl acetate to remove the phenylthiohydantoin derivative. An aliquot of this solution was taken for dansylation. The remaining solution was subjected to further Edman degradation.

2.2. COOH-*terminal analysis*

Heat-denatured NCS or tetra-*S*-benzyl NCS (5.6 mg, 0.6 μmole) was dissolved in 0.1 M $NaHCO_3$ (3 ml) and carboxypeptidase A (0.2 mg) was added. Digestions were done at 22°. Samples were taken at the intervals indicated in fig. 1 and reactions were stopped by addition of acetic acid (3–4 drops). The samples were thoroughly dried *in vacuo* and subjected to amino acid analysis [16, 17].

Asparagine and serine gave one single peak on the recorder of the amino acid analyzer. To identify asparagine and to determine the individual values of each of the two amino acids, asparagine was converted into aspartic acid which appeared in a different position on amino acid analysis. The serine values were obtained by subtraction of the aspartic acid values from the original peak. For these purposes a separate carboxypeptidase digest was prepared. The enzyme and residual NCS were removed from the reaction mixture by precipitation with trichloroacetic acid (7.5% in water) followed by centrifugation. Repeated washing of the supernatant with peroxide-free ether removed the trichloroacetic acid. The remaining amino acid mixture was then treated with 3.5 M HCl at 100° for 1.5 hr [18] and the liberated aspartic acid was determined by amino acid analysis.

3. Results and discussion

3.1. NH_2-*terminal sequence*

A combination of the dansyl aminoterminal determination and of the sequential Edman degradation methods was employed. Repeated experiments on native NCS gave unequivocal and reproducible results up to the fourth position, thus establishing the amino-terminal sequence as NH_2-Ala-Ala-Pro-Thr-. This sequence confirms the earlier report [2] that alanine is the NH_2-terminal amino acid of NCS. On treatment of native NCS with leucine aminopeptidase [19], only 10% of the expected amount of alanine was obtained, possibly due to the proline residue in position 3.

3.2. COOH-*terminal sequence*

Chronological studies [10] of the release of amino acids during carboxypeptidase A digestion were conducted. Because native NCS was digested too slowly to give useful results, both heat denatured and tetra-*S*-benzyl NCS were used. Both gave similar results; an example is shown in fig. 1. Asparagine and phenylalanine were released at almost identical rates and in almost the same quantities. During the first few minutes, however, the asparagine release was slightly higher. These observations confirmed the previous determination [6] of asparagine as the *C*-terminal amino acid of NCS by a tritiation-racemization procedure [20]. A total of six amino acids were released in the order: asparagine, phenylalanine, isoleucine, alanine, valine, and serine. After 6 hr incubation, the released amounts of each amino acid approached 90% of the theoretical yield. Neither larger amounts of any of these six amino acids nor any other amino acid were detected even after enzymatic treatment for 20 hr. The results (fig. 1) established the sequence -Ser-Val--Ala-Ile-Phe-Asn-OH as the COOH-terminal hexapeptide of NCS.

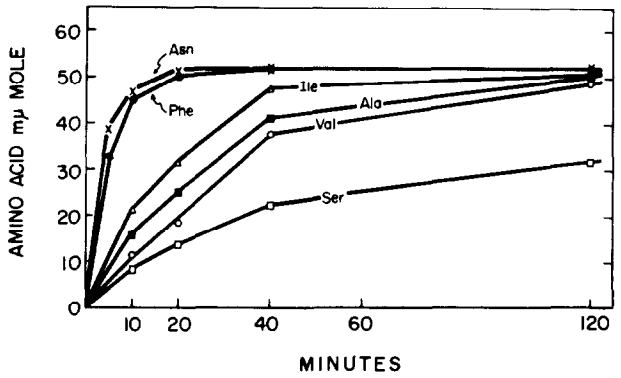

Fig. 1.

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