THE ANTIBIOTIC EDEINE. XII

ISOLATION AND STRUCTURE OF EDEINE F

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The peptide antibiotic edeine F produced by *Bacillus brevis* Vm_4, one of the components of edeine antibiotics complex, was isolated from a fermentation broth and was also obtained by amidination of edeine D.

Edeine F is composed of amino acids: (*S*)-β-phenyl-β-alanine, (*S*)-isoserine, (*S*)-2,3-diaminopropionic acid, (2*R*,6*S*)-diamino-(7*R*)-hydroxyazelaic acid, glycine and a polyamine guanidylspermidine. Enzymatic degradation of antibiotic with carboxypeptidase B, dinitrophenylation of edeine and of its enzymatic degradation products and synthesis of edeine F from edeine D of known structure permitted to postulate the chemical structure for edeine F.

Edeines $A^{1\sim4)}$, $B^{1\sim4)}$, and $D^{5,6)}$ were found in the complex of edeines produced by *Bacillus brevis* Vm_4 strain. They are composed of the amino acids: β-tyrosine (β-Tyr) in edeines A and B, β-phenyl-β-alanine (Iph) in edeine D, isoserine (Ise), 2,3-diaminopropionic acid (A_2pr), 2,6-diamino-7-hydroxyazelaic acid (A_2ha), glycine (Gly) in edeines A, B, D and the amines: spermidine (Spe) in edeines A and D, guanidylspermidine (Gsp) in edeine B.

Edeine B was also obtained by amidination of the primary spermidine amino group of edeine A[7].

It could be expected that besides edeine B, a close analog of edeine A, a similar analog of edeine D should be formed in the course of biosynthesis. The expected amidinated edeine D analog was found among the biologically active compounds formed by *B. brevis* Vm_4 strain. Such an analog was obtained simultaneously by semisynthesis. It appeared to be identical with an antibiotic isolated from fermentation broth and was named edeine $F^{6,8,9)}$.

Experimental

Paper chromatography (PC) was performed on Whatman 3 filter paper, thin-layer chromatography (TLC), on DC-Alufolien Kieselgel 60 (Merck), and high voltage electrophoresis (HVE) on Whatman 3 filter paper.

Determination of Biological Activity

The activity of edeine was determined by the standard cylinder plate method, using the *Bacillus subtilis* ATCC 6633 strain as test organism grown at 37°C during 18 hours. A suspension of spores in sterile water (85% light transmittance measured on a Specol colorimeter at 530 nm) was added to the medium poured on plates. Viomycin sulfate was used as reference, dissolved in a phosphate buffer at pH 6.0 whose initial concentration was 1 mg/ml. The initial concentration of edeine solutions in the phosphate buffer at pH 6.0 was also 1 mg/ml.

The activity of edeine was calculated as follows:

$$\log N = (\log 4) - a/b$$

$$a=(B_2+B_1)-(W_2+W_1);\ b=(B_2-B_1)+(W_2-W_1)$$

N: Ratio of the activity of the compound to the activity of the standard.

B_2: Sum of diameters of inhibition zones for the compound at high concentration.

B_1: Sum of diameters of inhibition zones for a compound with low concentration.

W_2,W_1: Corresponding values of the inhibition zones for a standard with high and low concentration.

The activity of a compound is determined in μg/ml equivalent to units/ml.

Isolation of the Edeine Complex

The crude antibiotic was isolated following the procedure of BOROWSKI and DZIEGIELEWSKI[10] with modifications. Fermentation broth[11] with the activity 250 units/ml and pH 7.0 was acidified with oxalic acid to pH 3.0~3.5, then stirred for 30 minutes. 30 g of Kieselguhr per 1 liter broth was added, filtered and the remainder was washed with water. The pH of the filtrate was brought to 7.0~7.5 with 20% of aqueous NaOH solution. The antibiotic was adsorbed from a solution of 220 units/ml activity on columns packed with Wofatite CP Na^+. The ion exchange bed was washed with water and the antibiotic was eluted with 0.5 M aqueous ammonia solution.

The active fraction was concentrated under reduced pressure to small volume, cooled to 15°C, then neutralized with 20% aqueous H_2SO_4 solution to pH 4.8~5.3. To this solution activated H-extra charcoal (1.5% m/v) was added and stirred for 1 hour. The adsorbent was filtered off, washed with water and the filtrate concentrated: a solution obtained from 50 liters of broth was concentrated to 200 ml. Seventy seven per cent of the activity was recovered. The solution was added dropwise to 12 volumes of methanol with stirring and then left for 30 minutes. The formed edeine sulfate was filtered, washed with methanol - water (12: 1, v/v) mixture, methanol and acetone and the residue dried under reduced pressure. The activity of the product was 220 units/mg, (with less than 5% moisture). Upon the precipitation 90% of activity was recovered.

Separation of the Edeine Complex into Components

2 g of the edeine sulfate was dissolved in 10 ml water and applied on a 2.6×100 cm column packed to 90% with DE-32 OH^- cellulose. Antibiotics were eluted with water or 0.5 M aqueous ammonia with hydrostatic pressure of 0.8~1.0 m/m of bed ion exchanger in the following sequence: edeine F, D, B and A. Elution was monitored by TLC in the systems 5* (Rf value: A 0.42, B 0.29, D 0.42, F 0.12) and 3* (Rf value of edeines: A 0.21, B 0.10, D 0.32, F 0.06). Fractions containing individual edeines were combined and neutralized to pH 5.0~6.0 with 0.1 M *p*-toluenesulfonic acid. The strongly alkaline fractions were neutralized with a 2 M solution of the same acid. The solutions were evaporated under reduced pressure to dryness.

The edeines *p*-toluenesulfonates were purified on columns packed with Sephadex LH-20, with 50% aqueous methanol as eluent. Fractions containing edeine F were concentrated 20-fold under reduced pressure, stirred with 20 volumes of methanol and edeine F sulfate precipitated by the treatment with a 50% aqueous triethylamine sulfate solution. The precipitate was filtered, washed three times with methanol, with acetone and then dried under reduced pressure. Recovery: 80%. Activity of the compound 341 units/mg.

Anal. Calcd. for $C_{34}H_{64}N_{12}O_{17}S_2$: C 41.79, H 6.60, N 17.20, S 6.56.
for $C_{34}H_{64}N_{12}O_{17}S_2\cdot 3H_2O$: C 39.60, H 6.84, N 16.30, S 6.21.
Found: C 39.01, H 6.58, N 15.73, S 6.31.

Edeine F Obtained by Amidination of Edeine D

56 mg of edeine D sulfate (0.045 mmol) and 117 mg of *O*-methylisourea hydrochloride (1.063 mmol) were dissolved in 4.3 ml of solution composed of water - ethanol - triethylamine, 4.4: 3.5: 1.5, while the pH of the solution was brought to 11.0.

The reaction was conducted at 25°C for 24 hours, whereupon the pH of the solution was brought to 2~3 with 1 M aqueous HCl solution. The solution was concentrated to a small volume and applied to a column packed with Sephadex LH-20 (1×94 cm). Elution was conducted with water at a flow rate of 1.2 ml/hour. Edeine F was found in fractions collected at 27~39 ml of the effluent. Samples from the reaction and isolation were tested in the solvent system 3* on Whatman 3 filter paper. The chroma-

* see Table 1.

tograms were developed with ninhydrin and the SAKAGUCHI reagent. Bioautography was carried out using a *Serratia marcescens* strain. The solution containing edeine F sulfate was concentrated to a small volume and added dropwise to methanol. The precipitate was centrifuged, washed with acetone and ethyl ether and dried under reduced pressure over NaOH 45 mg of edeine F sulfate were obtained[8] activity 350 units/mg. The results of elemental analysis were practically the same as found for edeine F obtained by biosynthesis.

Edeine Hydrolysis

a) Acid Hydrolysis: To each sample of natural edeines B, F and edeine F obtained in a result of amidination of edeine D, 0.4 ml of 6 M aqueous HCl solution was added. Hydrolysis was conducted in sealed ampoules for 20 hours at 100°C. Hydrolysate was evaporated under reduced pressure over P_2O_5 and NaOH and were analyzed by HVE at pH 3.5 (1 hour, 45 V/cm, 12°C) and TLC with standards.

b) Enzymatic Hydrolysis: Parallel reactions of edeines B, D and F with carboxypeptidase B were carried out as described earlier[4,12]. The composition of the reaction mixtures was determined by HVE at pH 3.5 (90 minutes, 40 V/cm, 13°C).

Peptides obtained by enzymatic degradation were isolated preparatively on filter paper with the aid of HVE at pH 3.5 and subjected to acid hydrolysis as in a).

Dinitrophenylation

Edeines F-natural, F-semisynthetic (1 mg of each) and products of enzymatic degradation obtained as above were dissolved in 0.2 ml of 1% water solution of triethylamine and treated with 0.2 ml of 2% 2,4-dinitrofluorobenzene in absolute ethanol. The method of SANGER and THOMPSON was used[13].

DNP-derivatives were hydrolyzed with concentrated HCl for 3 days at 36°C and with 6 M HCl for 6 hours at 100°C. The hydrolysates were evaporated *in vacuo* over KOH pellets. The residues were dissolved in water and examined in the solvent systems 6* and 7* by TLC with glycine and isoserine as standards[14]. To the aqueous solution acetone was added and solution of DNP-derivatives was examined by TLC[7,14], and by HVE at pH 1.9[4,14] (DNP-derivatives of A_2pr were separated).

Results and Discussion

Edeine F was isolated from the edeine mixture by ion exchange chromatography on DE-32 OH^- cellulose. This compound was also obtained by amidination of edeine D with *O*-methylisourea in a buffer of pH 11.0 at 25°C. The reaction conditions ensured selectivity in the amidination[15]. No other products were formed. The semisynthetic edeine F exhibited the same Rf values as those for edeine F obtained by biosynthesis (Table 1).

The natural and semisynthetic antibiotics gave positive ninhydrin, SAKAGUCHI and sodium nitroprusside tests pointing to the presence of free amino, guanidyl and secondary amino groups in their molecules.

Table 1. TLC and paper chromatography of edeine F.

	Rf values of edeine F natural and semisynthetic				
Solvent systems	1	2	3	4	5
TLC	0.12	0.25	0.05	0.05	0.30
Paper chromatography	0.16	0.38	0.57	0.22	0.67

1. Butanol - pyridine - acetic acid - water, 6: 2: 3: 3.
2. Butanol - pyridine - acetic acid - water, 6: 2: 3: 5.
3. Propanol - NH_4OH - $CHCl_3$, 12: 8: 1.
4. Propanol - NH_4OH - water, 60: 35: 5.
5. Propanol - NH_4OH - water, 6: 4: 3.
6. Phenol - water, 3: 1.
7. Propanol - water, 64: 37.

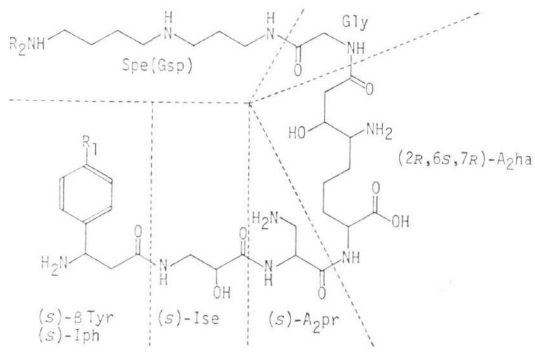
The two antibiotic preparations were subjected to acid hydrolysis and their composition was studied by means of HVE in the presence of standards. The following components Gly −2.5 cm, Ise −3.2 cm, A_2ha −5.9 cm, A_2da −5.0 cm, A_2pr −13.4 cm, Gsp −21.6 cm were identified. 2,6-Diamino-7-dehydroazelaic acid (A_2da) was formed from A_2ha in β-elimination of water molecule during acid hydrolysis[16]. With the aid of TLC in 1* and 6* solvent systems the presence of Iph was demonstrated (Rf value: 0.79 (1*), 0.53 (6*)), but not β-Tyr which is a *N*-terminal amino acid in the edeines A and B.

Measurements of the Cotton effect and $[\alpha]_D$ for amino acids isolated from an acid hydrolysate of edeine F showed the same values as those determined for the amino acids isolated from the hydrolysate of an edeine complex or from an acid hydrolysate of individual edeines. The configurations are *S* for Iph, Ise, A_2pr and 2*R*, 6*S*, 7*R* for A_2ha[16,17].

The natural and semisynthetic edeine F and the edeines B and D were cleaved with carboxypeptidase B which hydrolyzes the amide bond in edeines between A_2pr and A_2ha. In the course of this reaction a dipeptide amides and tripeptides were formed[4,12]. The reaction mixtures were studied by HVE. The *N*-terminal tripeptides from the edeines D and F had identical migration distances (−13.0 cm) in contrast to the tripeptide from edeine B (−11.8 cm). Dipeptide amides obtained from edeines B and F had identical migration distances (−22.8 cm). This suggests that the tripeptides generated from edeine F preparations in enzymatic reaction have approximately similar or identical structure as the tripeptide from edeine D: Iph-Ise-A_2pr and the dipeptide amide corresponds to the edeine B dipeptide amide: A_2ha-(Gly-Gsp). The hydrolysis products of tripeptides from both edeines F samples showed the presence of Iph, Ise and A_2pr, whereas the hydrolysis products of amides contained A_2ha, Gly and Gsp[7,17]. Hydrolysis of dinitrophenylated edeines D and F and of their degradation products yielded the same components except for the DNP-derivatives of their respective bases: DNP-Gsp for hydrolysate of edeine F and Di-DNP- Spe for edeine D. The following free amino acids were found in the hydrolysates: of edeines F, D-Gly and Ise; of tripeptides only Ise and of dipeptide amides-only Gly. In edeines A, B and D, it was found, that besides the active compound also an inactive isomer was always present, in which the dipeptide Iph (or β-Tyr)-Ise is linked with the β-amino group of A_2pr[4,14,18].

Migration of the substituent from the amino group ($\alpha \rightleftarrows \beta$ A_2pr) takes place in solutions and is characteristic for A_2pr derivatives[19,20]. The isomeric compounds have so similar properties that they are difficult to separate[4]. The two isomers of edeine F, the active and the inactive ones, can partially be separated, similarly to edeine D isomers, by ion exchange chromatography on DE-32 OH^- cellulose, with water as eluent. The activity of the fractions were determined on agar plates with *S. marcescens* as a test organism[14]. The active isomer containing the free β-amino group of A_2pr slightly contaminated with the inactive isomer was the first to leave the column. The ratio of the two isomers was determined by comparison of amounts of α and β DNP derivatives of A_2pr present in hydrolyzates of DNP derivatives of active and inactive isomers[4,14].

Fig. 1. Chemical structures of the edeine antibiotics.
Edeine A: R_1=OH, R_2=H
Edeine B: R_1=OH, R_2=CNH_2(=NH)
Edeine D: R_1=H, R_2=H
Edeine F: R_1=H, R_2=CNH_2(=NH)



The natural and semisynthetic edeine F showed the same properties and their degradation led to the formation of compounds with identical properties and composed of the same constituents. Edeine F was also obtained, as a result of selective amidination of the primary amino group of spermidine in edeine D that has known chemical structure, confirmed by synthesis[21]. This permits to postulate the following structure of edeine F:

(*S*)-Iph-(*S*)-Ise-(*S*)-A_2pr-(2*R*, 6*S*, 7*R*)-A_2ha-(Gly-Gsp)

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