PROTICIN, A NEW PHOSPHORUS-CONTAINING ANTIBIOTIC. II

CHARACTERIZATION AND CHEMICAL STUDIES

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Dedicated to Professor Dr. Heinrich Ruschig on his 65 th birthdy anniversary.

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This article concerns itself with the purification and characterization of the new antibiotic, proticin (I). Hydrogenation with 8 moles of hydrogen and subsequent esterification of proticin (I) yields derivative (II). On the basis of this derivative and of several degradation products the molecular formula of proticin was found to be $C_{31}H_{44}O_7PNa$. The functional groups of proticin include one OH capable of acetylation, one lactone group, and one monoester of phosphoric acid as enol ester. Proticin contains a conjugated triene. The presence of the triene and of a number of double bonds in the lactone ring as well as the 1,4 positions of alcohol and phosphate will be discussed.

The new phosphorus-containing antibiotic proticin (I) was obtained by fermentation of a strain of *Bacillus licheniformis* var. *mesentericus*. The strain characteristics, culturing, and isolation techniques were described in the previous article[1]. The present report deals with purification, physical and chemical properties, and the determination of the molecular formula of proticin. An inquiry has also been made into its structure by investigating its derivatives. The crude antibiotic[1] isolated by extraction, precipitation, and column chromatograpay on silica gel impregnated with phosphate buffer requires further purification. In our experiments it was separated by column chromatography on Sephadex LH-20 gel (Pharmacia Fine Chemicals; Uppsala, Sweden). The active fractions were examined and found to contain no impurities (thin-layer chromatography). The elementary analyses also revealed no significant differences, but the separated antibiotic was nevertheless inhomogeneous. The variations of the UV extinctions at wavelengths 235 and 272.5 nm reached up to 15 %, and with the rising number of fractions the specific rotation increased from the initial value of $-10°$ to over $-70°$. The antibiotic activity per weight unit also increased. Rechromatography of a highly active fraction showed essentially the same picture if no special precautions were taken. However, at 0°C and if the drying of the substance before loading the column was avoided, no variation of specific rotation occurred. Repeated tests indicated that the antibiotic should be assigned a specific rotation of $-78°$, and the UV maxima the extinction values given in Fig. 1.

The warming of proticin solutions to over 40°C or concentration to dryness *in*

vacuo led to gradual inactivation of the product. Since a highly purified antibiotic is not easily obtained, the majority of the experiments described below were carried out with preparations still containing transformation products.

The pure solvent-free antibiotic is a colorless amorphous substance. Proticin is easily soluble in chloroform, polar organic solvents, and water, and slightly soluble in petroleum ether, benzene, and acidic water.

Proticin can be colored with chlorosulfonic acid[2], $SbCl_3$[3], $KMnO_4$, and molybdate reagent[3], while ninhydrin, $FeCl_3$, and TTC[3] produce no reactions.

The elementary analysis[1] showed the presence of C, H, O, P (and Na if a sodium buffer was used for isolation). In the high

Fig. 1. Ultraviolet absorption spectrum of proticin.

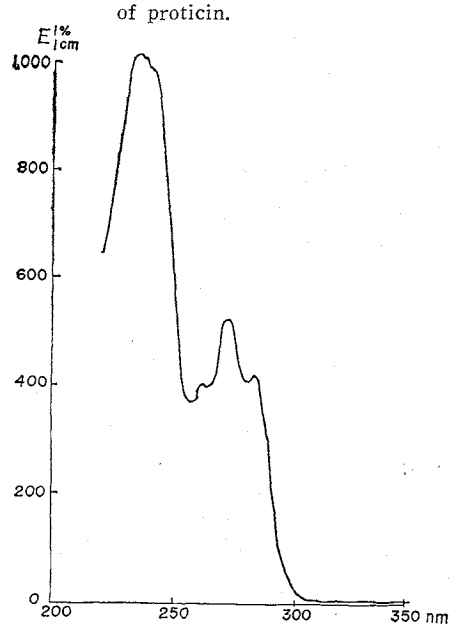
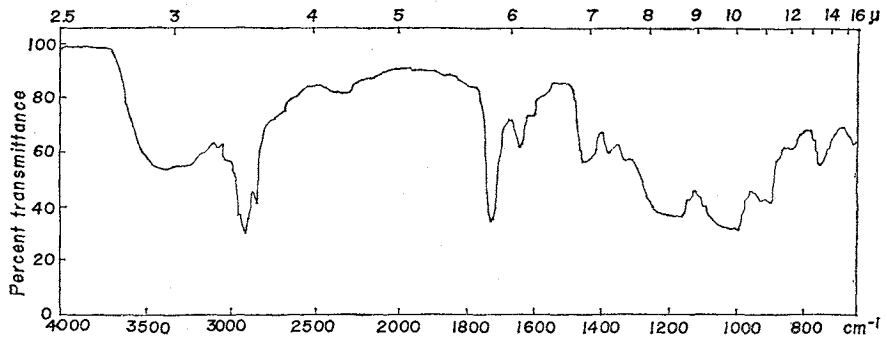


Fig. 2. Infrared absorption spectrum of proticin.



voltage electrophoresis the antibiotic migrated as an acid.

Fig. 2 shows the IR absorption spectrum of the antibiotic. The spectrum shows evidence of the acid character of the compound and reveals the presence of carbonyl. The UV spectrum indicates a highly unsaturated compound, in which three double bonds form a conjugated triene. Proticin causes a strong circular dichroism:

$$\Delta\varepsilon_{max} = -25.4 \text{ (at 227 nm)}, \ -20.6 \text{ (at 263 nm)}, \ -7.7 \text{ (at 278 nm)},$$
$$-6.9 \text{ (at 283 nm), and } +0.68 \text{ (at 300 nm)}^*.$$

The ^1H NMR spectrum is complex, a conspicuous feature being the large number of double bond protons, and a singlet at $\tau = 8.24$, which suggests the presence of methyl at one of the double bonds. The ^{31}P NMR spectrum contains a signal at -2.3 p.p.m., relative to phosphoric acid as standard.

* The measurements of circular dichroism were carried out in a few-day-old sample which might have already contained fransformation products.

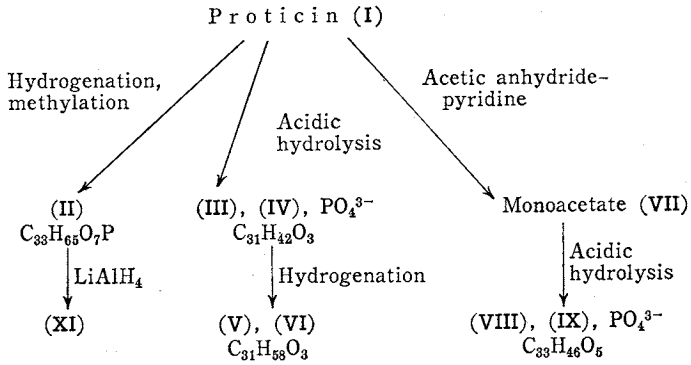
A number of phosphorus-containing antibacterial substances have already been described. They include, on the one hand, the antibiotics of the moenomycin group, *viz.* moenomycin[4], prasinomycin[5], 11837 R.P.[6], diumycin[7], and macarbomycin[8]; on the other hand, phosphonomycin[9]. Proticin, for example, differs from the antibiotics of the moenomycin group by the absence of nitrogen, and from phosphonomycin by its UV spectrum.

It follows from the previously mentioned results that proticin is a monosodium salt of an ester of phosphoric acid, and further, that its double bonds belong entirely or partially to a ring system, since otherwise it would be impossible to explain the strong effect of circular dichroism. The measured values correspond to an inherently asymmetrical chromophore as represented by nonplanar conjugated double bonds.

It is difficult to determine the molecular weight of a decomposable salt-like substance that is sensitive to acids. Therefore proticin was catalytically hydrogenated with 8 moles of hydrogen calculated on the basis of a molecular weight of 582 (*cf.* below), converted into the acid, and methylated with diazomethane. The resulting dimethyl phosphate (II) showed peaks in the mass spectrum at 604 and 605 mass units. The signal at 605 was more intensive than could be explained by an isotope peak. Treatment of II with acetic anhydride-pyridine yielded a monoacetate with its most intensive peak in the high mass area at 647, *i.e.* 42 mass units higher, owing to acetylation. Since the molecular weights of compounds consisting of C, H, O, and P must be even-numbered, 604 and 646 were taken to represent the respective molecular weights, and 605 and 647 the M+1 peaks.

To verify the correctness of this assumption, proticin was subjected to degradation reactions. Already 0.2 N hydrochloric acid at room temperature led to complete hydrolysis. The hydrolysate was shown to contain inorganic phosphate and several nonpolar substances. A short meticulous hydrolysis yielded two primary products, III and IV, which were transformed into more polar substances under slight warming. The elementary analysis of the substances III and IV gave identical results, and the UV spectra displayed the same maxima as the UV spectrum of the initial substance, except for the maximum at 235 nm, the intensity of which was considerably lower. Both spectra showed an intensive peak at 462 mass units, but under certain recording

Fig. 3. Reaction scheme of proticin.



conditions ions of higher masses than 462 were also observed. The molecular weight as determined by osmometry was 482. Catalytic hydrogenation of the substances III and IV with 8 moles of hydrogen (based on the molecular weight 462) gave rise to two compounds, V and VI, the molecular peaks of which were both at 478. The occurrence of higher mass numbers than those corresponding to the molecular weight was observed in most of the highly unsaturated substances measured, and was attributed to polymerization.

In the cleavage products III and IV, the presence of two alcohol groups was expected. It was therefore surprising to find that neither III and IV nor the hydrogenation products, V and VI, contained such groups. In the IR spectrum, bands at more than 3100 cm^{-1} were missing; acetylation, dehydration of V and VI with thionyl chloride, and oxidation according to JONES[11], all gave negative results.

More careful degradation methods gave the same result. The silver salt of proticin could not be converted by the action of methyliodide into its methylester. Triesters of phosphoric acid could be hydrolyzed more easily than diesters or monoesters; in fact, in this case the cleavage occurred spontaneously. The degradation products were dimethyl phosphate as well as III and IV.

When treated with acetic anhydride in an excess of pyridine, proticin yielded a monoacetate VII. Acidic degradation of VII produced inorganic phosphate and the isomers VIII and IX, each of which carried one acetyl group. The UV spectra of VIII and IX were identical with the UV spectrum of the starting material.

Again, the mass spectrum contained an intensive peak at 462, but the molecular peak was at 522, which means that it was higher by the molecular weight of acetic acid. The high-resolution mass determination gave the value 522.331 ± 0.005, which corresponds to a compound $C_{33}H_{46}O_5$. Accordingly, the unstable, natural, nonacetylated alcohol (X) has the molecular weight 480 and the molecular formula $C_{31}H_{44}O_4$. On this basis the composition of proticin can be calculated by adding the group PO_3Na, the formula being $C_{31}H_{44}O_7PNa$, which compares favorably with the results of the elementary analysis. Similarly, the formula of derivative II, the dimethyl phosphate that has been hydrogenated with 8 moles of hydrogen, is $C_{33}H_{65}O_7P$, corresponding to a molecular weight of 604. These data were confirmed by the elementary analysis and the mass spectrum of II.

The presence of one carbonyl function in the molecular formula of the hydrogenated substance II suggests that the latter contains one ring, whereas the cleavage products III, IV, V, and VI are bicyclic systems. The second ring forms apparently spontaneously by dehydration from the two OH groups of the hypothetical alcohol (X) during the production of III and IV. The ring formation may be associated with the base peak of 81 in the mass spectra of III and IV, which is typical of alkyl furans[12]. On the one hand, spontaneous formation of a furan suggests a 1,4 position of phosphate and alcohol; on the other hand, it indicates the presence of enol phosphate, this being confirmed by an easy hydrolytic cleavage of the phosphoric ester bond of proticin. Hydrolytic cleavage of the hydrogenated antibiotic requires much more severe conditions.

The IR spectrum indicated that carbonyl function can be attributed to one lactone group. Preliminary trials to reduce the hydrogenated and methylated antibiotic (II) with lithium-aluminum hydride led to a highly polar compound (**XI**) under preservation of the $PO_4(CH_3)_2$ moiety. Such a polar compound can be expected if two alcohol groups are newly formed. No fragments other than **XI** could be detected in the reaction mixture by thin-layer or gas chromatography, as would have been the case if an ester had been present.

Experimental

The IR spectra were measured as chloroform films, the UV spectra in methanol, and the NMR spectra in deuteriochloroform ($CDCl_3$) with tetramethyl silane as internal standard. The mass spectra were recorded with an AEI MS-9 apparatus at 70 eV. As thin layer plates served DC alufoils of silica gel F254 (E. Merck, Darmstadt). Staining was done with a mixture of chlorosulfonic acid and glacial acetic acid by heating to 110°C.

Purification of proticin:

A solution of crude proticin obtained by adsorption chromatography was concentrated *in vacuo* at bath temperature <20°C to a syrupy liquid and dissolved in chloroform-methanol (volume ratio 1:1). Three liters of this solvent mixture and 500 g of Sephadex LH 20 were allowed to swell overnight and were poured into a 6×100 cm glass column. Subsequently 1.2 g of crude proticin dissolved in 20 ml of the solvent were transferred and eluted. After the elution of the front, fractions of 20 ml were collected and examined by diffusion test for antibiotic activity. Beginning with fraction 98 a slight antibiotic activity against *Proteus vulgaris* was exhibited. At fraction 126 proticin concentration increased sharply and dropped again abruptly at fraction 148. Fractions 135~147 were collected and separated again as described, this time in a 200 cm column with an inside diameter of 2.4 cm. The volume of the fractions was 6 ml. Maximum activity was observed at fractions 85~96. Tubes No. 91~96 contained 40 mg of proticin with the following reproducible data: The specific rotation $[\alpha]_D^{22}$ was −78° (*c* 0.35 in ethanol).

Calculated ($C_{31}H_{44}O_7PNa$): C 63.9, H 7.6, O 19.2, P 5.4, Na 3.9 %.

Observed: C 63.8, H 7.8, O 19.8, P 4.4, Na 3.2 %.

λ_{max}: 284, 272.5, 264, and 235 nm ($E_{1cm}^{1\%}$ 415, 510, 395, and 1010)

Absorption bands in the IR spectrum were at 3400, 3080, 2920, 2820, 1725, 1640, 1445, 1370, 1160, 985, 890, and 750 cm^{-1}. The Rf value (with chloroform-methanol (3:2) as solvent) was 0.38.

To obtain the acetate (**VII**) 300 mg of proticin were dissolved in 6 ml of pyridine, treated with 1 ml of acetic anhydride, allowed to stand overnight, and after the conventional treatment with chloroform-methanol (4:1) chromatographed on silica gel impregnated with phosphate buffer. This procedure yielded 120 mg of a homogeneous substance (thin-layer chromatography), characterized by the following data:

The Rf value (with chloroform-methanol (7:3) as solvent) was 0.69.

λ_{max}: 284, 272.5, 264, and 235 nm ($E_{1cm}^{1\%}$ 400, 495, 380, and 1020)

Acetyl: 7.5 % CH_3-CO- (calculated 6.7 %).

Perhydroproticin methyl ester (II):

A suspension of 120 mg of PtO_2 in 10 ml of ethanol was prehydrogenated. Subsequently 306 mg of proticin in 20 ml of ethanol were added and hydrogenated under a pressure slightly above atmospheric. The consumption at 23°C and 770 mmHg was 100 ml H_2, which corresponds to 7.8 moles of hydrogen (based on the molecular weight 582). The hydrogenation product was an amorphous substance, which showed no maximum in the UV wavelengths above 220 nm. The specific rotation $[\alpha]_D^{23}$ was found to be −7° (*c* 0.5, methanol). The hydrogenated proticin was dissolved in 30 ml of water, treated with 3 ml

of 2 N hydrochloric acid, and shaken with *n*-butanol. The two clear phases were separated, the organic phase was washed with water, concentrated *in vacuo*, and after addition of tetrahydrofuran methylated with diazomethane. The substances so obtained were the main product (II) and two nonpolar side products. They were separated on silica gel with benzene and increasing quantities of isopropanol. The presence of the products was demonstrated by thin-layer chromatography, using benzene - isopropanol (95 : 5). The Rf value of the main substance (II) was 0.14, and of the side products 0.16 and 0.19, respectively. The specific rotation $[\alpha]_D^{22}$ of II was $-21°$ (*c* 0.39, ethanol).

Calculated: C 65.5, H 10.8, O 18.5, P 5.1 %.
Observed: C 65.5, H 10.1, O 18.3, P 3.3 %.

Strong peaks in the mass spectrum were at 41, 43, 55, 69, 81, 95, 127 (base peak), 361, 391, 460, 505, 602, 604, and 605. Typical of the NMR spectrum was a sharp doublet at $\tau=6.27$. The coupling constant was 11 Hz in both the 60 MHz and 100 MHz spectrum, corresponding to a ^{31}P-O-C-^{1}H coupling[18]. Bands in the infrared spectrum were at 3460, 2920, 1735, 1460, 1380, 1260, 1190, 1042, 1010, and 848 cm^{-1}.

Cleavage products III and IV:

Four hundred mg of proticin were dissolved in 20 ml of water and a 10 % silver nitrate solution was added under stirring till the precipitate turned flocculent. After centrifugation the silver salt was dissolved in 10 ml of chloroform, dried, concentrated *in vacuo*, treated with 4 ml of CH_3I, and stirred under exclusion of light for 6 hours at 4°C. The cleavage products (III) and (IV) were then separated in silica columns with benzene-diisopropyl ether. The Rf values determined by thin-layer chromatography with benzene-diisopropyl ether (5 : 1) were 0.63 for (III) and 0.65 for (IV).

(III) The specific rotation $[\alpha]_D^{23}$ was $-150°$ (*c* 0.35, chloroform)
Calculated: C 80.5, H 9.1 %.
Observed: C 79.9, H 9.1 %.
λ_{max}: 284, 272.5, 264, and 234 nm. ($E_{1cm}^{1\%}$ 680, 820, 700, and 825).

(IV) The specific rotation $[\alpha]_D^{23}$ was $+40°$ (*c* 0.3, chloroform)
Calculated: C 80.5, H 9.1 %.
Observed: C 79.9, H 9.0 %.
λ_{max}: 284, 272.5, 264, and 235 nm. ($E_{1cm}^{1\%}$ 710, 860, 725, and 730).

Owing to the sensitiveness of substances (III) and (IV) the data regarding specific rotation and UV extinctions are inaccurate. Typical IR bands of both these compounds are at 1728, 1643, 1590, and 890 cm^{-1}. Both mass spectra are characterized by the following intensive ion masses: 41, 55, 67, 79, 81 (base peak), 91, 95, 97, 105, 107, 125, 231, 338, 365 (only in IV), 418, 444, 462, 476, 508, 522, and 567. In the last four the intensity differs according to the recording technique used.

The cleavage products III and IV can also be obtained by acidic hydrolysis of proticin, following the same principles as the degradation of acetate (VII) described below.

Cleavage products VIII and IX:

Two hundred mg of proticin acetate (VII) were dissolved in a mixture of 30 ml of acetonitrile and 10 ml of water and covered with an 80 ml layer of cyclohexane. Subsequently 40 ml of 0.3 N hydrochloric acid were added under stirring and the mixture was stirred for $^1/_2$ hour. In addition to (III) and (IV), the cyclohexane phase contained 35 mg of the cleavage products (VIII) and (IX). It was separated, washed until neutral, and dried.

Separation by column chromatography on silica gel with benzene-diisopropyl ether mixtures yielded pure VIII and IX. The Rf values determined by thin-layer chromatography were 0.47 for VIII and 0.35 for IX in benzene - diisopropyl ether (5 : 1) as solvent. The λ_{max} were 284, 272.5, 264, and 235, their relationships in intensity corresponding approximately to those found in the initial product. The ^1H NMR spectrum revealed an acetyl group at $\tau=7.93$ in VIII and $\tau=8.02$ in IX.

In the reaction solution of the acidic degradation products the phosphate ions were

demonstrated chromatographically with the solvent system *n*-propanol – 25 % ammonia – water (6 : 2 : 1) by the method of HANES and ISHERWOOD[14], whereas degradation products obtained by the same method via dimethyl ester revealed the presence of dimethyl phosphate.

Conversion of III and IV into V and VI by hydrogenation :

A mixture of 173 mg of **III** and **IV** was hydrogenated in ethanol with a reduced PtO_2 catalyst. The consumption was 71 ml of H_2, corresponding to 7.9 mole of H_2 per mole of the substance. The resulting substances, **V** and **VI**, were separated on silica gel in the same manner as **VIII** and **IX**. The Rf values of the substances were 0.61 for **V** and 0.54 for **VI** in the solvent system indicated for **III** and **IV**. The specific rotation $[\alpha]_D^{23}$ was $-25°$ (*c* 0.56, chloroform) for **V** and $-10°$ (*c* 0.33, chloroform) for **VI**.

The UV spectra included no maximum above 220 nm. The IR spectrum was 1730 cm^{-1}; the MS spectra were 41, 43, 55 (base peak), 56, 69, 81, 83, 95, 109, 127, 165, 183, 297, 361 (strong in **VI**), and 478. The ion mass for $C_{31}H_{55}O_3$ as determined by high resolution was 478.438±0.003 (by calculation 478.4386).

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