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CHEMICAL STUDIES ON LICHENS—XXXIII*

ROCCANIN, A NEW CYCLIC TETRAPEPTIDE FROM *ROCCELLA CANARIENSIS*

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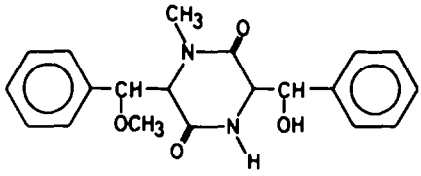
Abstract—A new cyclic peptide, cyclo-(*R*-β-phenyl-β-alanyl-L-prolyl-)$_2$ has been isolated from the lichen *Roccella canariensis* Darb. The structure has been established from chemical and spectral evidence and amino acid analyses.

NO OLIGO peptides have previously been isolated from lichens. Picroroccellin, a diketopiperazine derivative, was once (in 1877) isolated from the lichen *Roccella fuciformis*.[1] Forster and Saville, working with the original sample, proposed the structure I.[2] However it has not been possible to find this peptide again.



I

Substitution on the nitrogens may be reversed.

As earlier reported[3] a new cyclic tetrapeptide, roccanin, has been isolated from the lichen *Roccella canariensis* Darb. This peptide is identical with the unknown compound reported by Huneck *et al.* from *Roccella vicentina*[4] and *R. canariensis*.[5]

Acid hydrolysis of roccanin yielded two compounds, which were separated by preparative layer chromatography and identified as L-proline and *R*-β-phenyl-β-alanine by co-chromatography with authentic samples, mass spectrometry and polarimetry.

Contradictory conclusions about the absolute configuration of the optically active β-phenyl-β-alanine are reported in the literature. Lukeš *et al.*[6] suggested the *L*-configuration (corresponding to the *R*-configuration according to Cahn *et al.*) for the isolated enantiomer of β-phenyl-β-alanine ((+) in H_2O, (−) in HCl and in NaOH). The *R*-configuration of this isomer is further indicated by CD investigations, as the

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amino acid gives the same sign of the Cotton effect in the 220 region as the *R*(−)-3-phenylbutanoic, *R*(−)-3-phenylpentanoic and *S*(−)-4-methyl-3-phenylpentanoic acids.[7]

The identities of the amino acids were further confirmed by analytical ion exchange chromatography of the dipeptides obtained by reacting the acid hydrolysate with L-leucine-N-carboxyanhydride according to Manning and Moore.[8]

Quantitative amino acid analysis showed equimolar amounts of proline and β-phenyl-β-alanine, which accounted for the whole sample.

The mass spectrum of roccanin has a probable molecular peak at *m/e* 488 with the composition $C_{28}H_{32}N_4O_4$ (high resolution MS). This suggests the compound could be a cyclic tetrapeptide. In contrast the elemental analysis of the peptide, dried at 100°/0·1 mm Hg, gave the empirical formula $C_{28}H_{34}N_4O_5$ ($C_{28}H_{32}N_4O_4 + H_2O$), which would suggest a linear peptide. However, after sublimation at 340–360°/0·05–0·1 mm Hg the elemental analysis gave the formula $C_{28}H_{32}N_4O_4$. Furthermore the sublimation product and the unsublimed peptide have identical mass spectra and m.ps and virtually identical IR spectra.

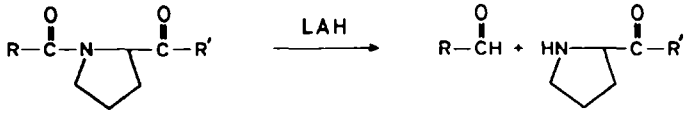
Since it is considered unlikely that cyclization occurs during the sublimation, roccanin must be cyclic, containing one mole of water (recrystallized from glacial acetic acid). The formation of strongly combined mono- or polyhydrates is common among cyclic peptides.[9,10]

Supporting evidence for the proposed cyclic structure is as follows: In the IR spectrum of roccanin the bands corresponding to $\geq NH^+$ and $—COO^-$ are absent. The insolubility in sodium hydroxide indicates the absence of any acidic group. The test for primary amino groups according to van Slyke was negative. No colour reaction was obtained with ninhydrin and no dinitrofluorobenzene derivative could be prepared by the method of Sanger indicating that Roccanin has no free amino groups. The peptide could not be acetylated with acetic anhydride and pyridine, which confirmed the absence of an amino group.

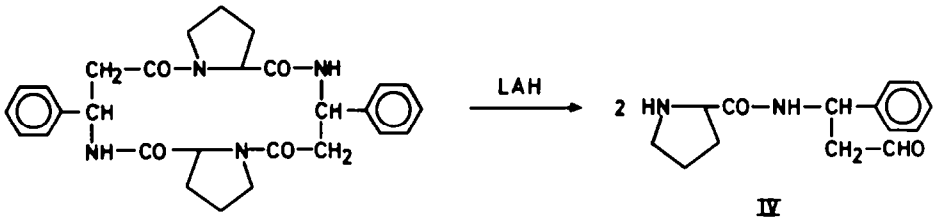
Consequently roccanin ought to have structure II or III.

Ruttenberg *et al.* found that the reactivity of amides when treated with LAH was in the order tertiary amide ≫ primary amide > secondary amide.[11]

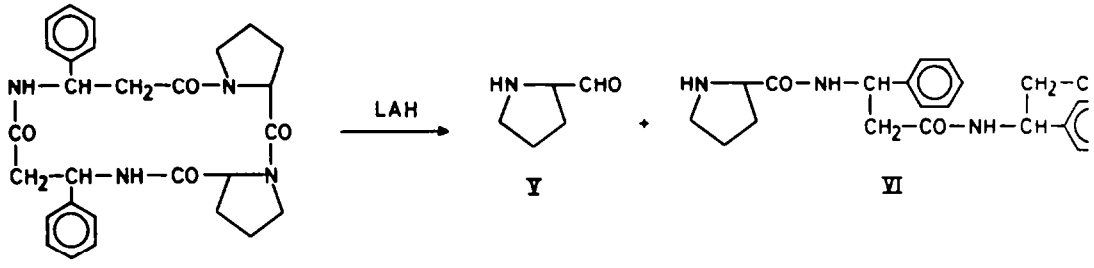
Therefore in order to determine the amino acid sequence roccanin was treated with LAH to get a specific reductive cleavage at the acyl proline linkage yielding an aldehyde and an amino-terminal proline.



II would be cleaved into two equal parts, IV, which upon acid hydrolysis would give proline and an amino aldehyde. III would be cleaved in two unequal parts, V and VI, which upon acid hydrolysis would give, apart from amino aldehydes, equimolar amounts of proline and β-phenyl-β-alanine. Quantitative amino acid analysis of the cleavage product of roccanin gave proline and β-phenyl-β-alanine in the molar ratio 1·00 : 0·06.



II IV



III V VI

The results above show that II must be the correct structure of roccanin.

In recent years mass spectrometry has become a helpful tool for determining the amino acid sequence of oligo peptides and in some instances even of cyclic peptides.[12]

Theoretically the fragmentation of both II and III could give the fragment at *m/e* 244 originating from the dipeptide prolyl-β-phenyl-β-alanine. It will be clear from Fig 1 that this makes a prediction of the amino acid sequence of roccanin by mass spectrometry very uncertain. In Fig 1 the fragmentation of the peptide is shown and probable explanations of the fragments are given. The origin of the fragment at *m/e* 103 from the fragment at *m/e* 131 is confirmed by a "metastable peak" at *m/e* 81·0. The elemental composition of the fragments are confirmed by high resolution mass measurements.

β-Phenyl-β-alanine has three times been reported as a component of naturally occurring peptides. Marumo isolated the toxic metabolite islanditoxin, cyclo-(L-seryl-L-seryl-L-dichloroprolyl-*R*-β-phenyl-β-alanyl-L-α-aminobutyryl-), from *Penicillium islandicum* Sopp.[9] Tatsuno *et al.*, independently of Marumo, reported cyclochlorotine, a toxic cyclic pentapeptide, cyclo-(L-dichloroprolyl-L-α-aminobutyryl-L-seryl-L-seryl-L-dichloroprolyl-*R*-β-phenyl-β-alanyl-L-α-aminobutyryl-), from *Penicil*- Koyama *et al.* isolated γ-L-glutamyl-*R*-β-phenyl-β-alanine from *Phaseolus angularis* W. F. Wight (Azuki bean).[14]

Because most peptide antibiotics are cyclic in character and many of them contain proline, it was of interest to test the antibiotic and toxic properties of roccanin. This test was difficult to perform, owing to the low solubility of the peptide in aqueous solution. A suspension of roccanin in agar–agar and water was tested on rats (400 mg/kg "oral" and 200 mg/kg "intraperitoneal". The peptide showed no toxic properties and caused no other obvious acute symptoms.

Lichen mass spectrum[15] of *Roccella canariensis* shows a small peak at *m/e* 502, which follows roccanin during isolation and purification and probably originates

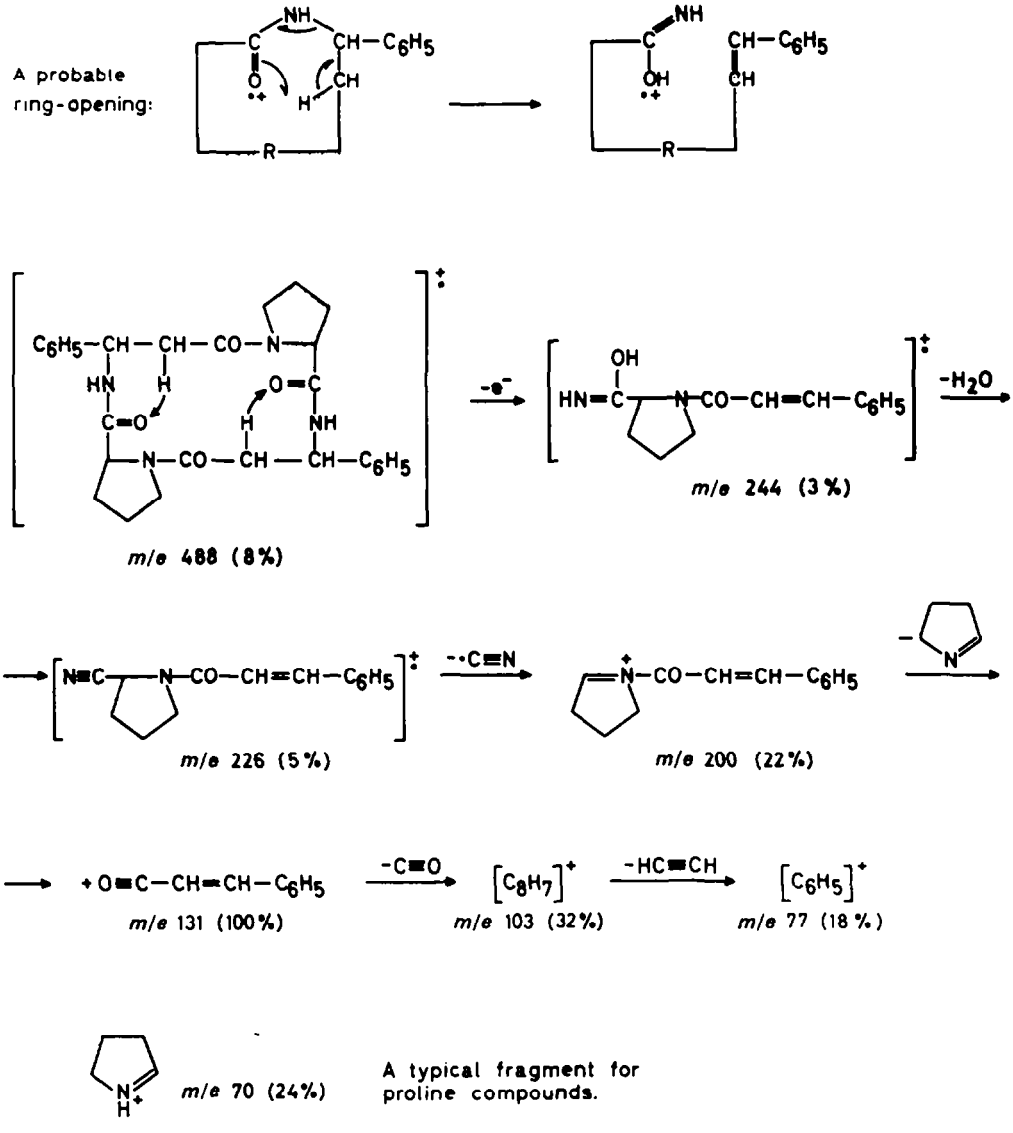


FIG 1. The fragmentation in mass spectrometry (70 eV) of roccanin. The relative intensity of the fragment is given within parentheses.

from a corresponding N-methylated peptide. This peptide occurs in too small an amount to be isolated.

EXPERIMENTAL

All m.ps are uncorrected. IR spectra were recorded on a Perkin-Elmer 157 spectrophotometer (KBr discs). Optical rotations were measured with a Perkin-Elmer 141 polarimeter. Mass spectra were recorded with an LKB 9000 mass spectrometer.

The high resolution mass spectrometry was carried out at the Laboratory of Mass Spectrometry, Karolinska Institutet, Stockholm 60. The elemental microanalyses were performed at the Microanalytic

Laboratory, Ultuna, Uppsala. The test for primary amino groups according to van Slyke was carried out at Alfred Bernhardt's Laboratory, Germany. The automatic amino acid analyses were carried out at the Central Amino Acid Analysis Laboratory, Institute of Biochemistry, Uppsala.

The lichen *Roccella canariensis*, collected on Teneriffa in the Canary Islands, was dried and extracted with acetone (14 days). Roccanin, which precipitated from the acetone extract together with erythritol and some lecanoric acid, was treated with acetone, ether and water and recrystallized from glacial AcOH (yield 0·2%), m.p. 320° (sinters at 285°), $[\alpha]_D^{25} = -92° \pm 1°$ ($c = 1$, DMSO), IR: 3465, 3330, 1675–1625, 1520 and 1440 cm^{-1}. (Found: C, 66·3; H, 6·7; N, 10·9%. Calc. for $C_{28}H_{32}N_4O_5$: C, 66·38; H, 6·77; N, 11·06%. After sublimation: Found: C, 68·7; H, 6·5; N, 11·1%. Calc. for $C_{28}H_{32}N_4O_4$: C, 68·83; H, 6·60; N, 11·47%).

The tetrapeptide was hydrolyzed with 6 N HCl at 110° for 24 hr in a sealed tube and the hydrolysate was evaporated to dryness. The hydrolysate was co-chromatographed on thin layer plates of silica gel (chloroform–methanol–ammonium hydroxide (17%), 2:2:1 v/v/v) with proline and β-phenyl-β-alanine.

β-Phenyl-β-alanine was prepared by the method of Posner[16] with some modifications and additions.[17] The *R*-antipode was obtained by resolution of the *R,S*-formyl derivative via the chinidine salt according to Fischer.[18] The formyl derivative was synthesized by a modified Fischer method: Ac_2O (70 ml) was added dropwise, at 5°, to β-phenyl-β-alanine (0·1 mol) dissolved in formic acid (98%; 210 ml). After stirring for 1 hr at room temp ice–water (80 ml) was added and the soln evaporated to dryness. Recrystallization from water gave colourless crystals (81%).

The automatic amino acid analyser was a Beckman Spinco instrument, equipped with a 0·9 × 60 cm column of Bio-Rad A4 resin at 57°. Operation conditions refer to an 0·35M sodium citrate buffer, pH 5·28, pumped at a flow rate of 50 ml/hr, with the ninhydrin soln being pumped at 25 ml/hr.

β-Phenyl-β-alanine could not be eluted in a reasonable time with the 0·185M sodium citrate buffer, pH 4·20. Another property of β-phenyl-β-alanine is its very low colour constant with ninhydrin.

Quantitative amino acid analysis of the hydrolysate showed β-phenyl-β-alanine and proline in the molar ratio 0·96:1·00.

The L-leucyl-dipeptides (for the determination of the configuration of the amino acids) were prepared as described by Manning and Moore.[8]

Not a trace of the dipeptides L-leucyl-D-proline or L-leucyl-*S*-β-phenyl-β-alanine could be detected from the derivatized hydrolysate of roccanin. The *R*-antipode from the resolution of *R,S*-β-phenyl-β-alanine was controlled in the same way and was found to be optically pure within the limits of detection of this method (< 0·2%).

The LAH reduction was carried out by adding a soln of roccanin in dry dioxane dropwise to a tenfold excess LAH in dry dioxane. After refluxing for 1 hr the mixture was filtered and the filtrate was evaporated to dryness. After treatment with acetone and recrystallization from dioxane colourless crystals were obtained, m.p. 245°, darkens (sinters at 200°).

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