Short Communications

Studies on Coumarins.

Pyrrolocoumarin

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Naturally occurring furocoumarins of the psoralen and angelicin type were isolated by Baerheim Svendsen *et al.*¹ from the roots of different *Heracleum* species of the family *Umbelliferae* which also occur in Scandinavia. They are all structurally related to umbelliferone (7-hydroxycoumarin). Martin *et al.*² have investigated the fungicidal effect of these same furocoumarins on some fungi, primarily *Gloeosporium limetticola* and *Alternaria tenuis*.

Other naturally occurring coumarins with biocidal and physiological properties have been reviewed by Seshadri³ and Soine.⁴ On the other hand, the nitrogen analogues of furocoumarins, *viz.* pyrrolocoumarins, seem not to have been found in Nature and even not been prepared. Only a few papers on synthesis (by Skraup's method) of pyranquinolines have been published.^{5–7}

In order to investigate the biocidal properties of pyrrolocoumarins, one such substance was prepared starting from 6-amino-4,7-dimethylcoumarin. This was obtained by reduction of the corresponding nitrocoumarin with tin(II) chloride in conc. hydrochloric acid rather than iron and acetic acid,⁸ since the tin method gave a better yield. The aminocoumarin was then treated with benzoin following Bischler's method, analogously to the preparation of 4- and 6-hydroxy-2,3-diphenylindole.^{9–11}

The cyclization to a pyrrolocoumarin took place in two steps with *p*-toluenesulfonic acid or hydrochloric acid as a catalyst. The intermediate product, an α -anilidoketone, was obtained on refluxing the reactants in ethanol or benzene for 18–24 h. This intermediate was then treated in the presence of acid catalyst without any solvent at 150–180 °C for some hours. The overall yield was rather poor. In the reaction scheme the synthetic pathway is shown for the novel pyrrolocoumarin III.

Scheme 1. Synthesis of the pyrrolocoumarin. R' = CH₃, R'' = C₆H₅.

Experimental. All melting points are uncorrected. The ¹H NMR spectra were recorded on a Varian A 60 spectrometer and the mass spectra on a LKB 9000 mass spectrometer (temperature of the ion source 270 °C, electron energy 70 eV).

6-Amino-4,7-dimethylcoumarin (I, R' = CH₃). The nitrocoumarin (11.0 g, 50 mmol) and tin(II) chloride dihydrate (35.0 g, 155 mmol) were suspended in 60 ml of conc. hydrochloric acid at 20 °C. The mixture was heated with stirring on a boiling water bath for 20 h, cooled, and the undissolved amine hydrochloride removed by filtration with suction. The crude product was dissolved in hot water, filtered with suction, and the filtrate neutralized with sodium hydrogen carbonate. The precipitate, containing stannic salts, was filtered and dried. After Soxhlet extraction with dichloromethane the aminocoumarin was recrystallized once from dioxane (10 ml/g). Yield 5.1 g = 56.6 %. M.p. 210–211 °C (Lit.⁸ 203–205 °C). The ¹H NMR spectrum in DMSO-*d*₆ exhibits the following δ -values (assignment, multiplicity, *J* Hz): 2.20 (7-CH₃, 1), 2.37 (4-CH₃, 2, 1.0), 5.0 (6-NH₂, 1), 6.30 (3-H, 2, 1.0), 7.0 and 7.12 (5- and 8-H or *vice versa*, 2, 0.5).

6-(1',2'-Diphenyl-2'-ketoethyl)amino-4,7-dimethylcoumarin. (II). Compound I (3.8 g), benzoin (4.3 g) and *p*-toluenesulfonic acid (*p*-TS, 0.1 g) were dissolved in 75 ml of absolute ethanol and refluxed for 24 h. The coumarin II, which had already precipitated from the hot solution, was collected after cooling and re-

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crystallized once from ethanol. Yield 3.8 g = 49.7 %. M.p. 192–194 °C. (Found: C 77.5; H 5.6; N 3.8. Calc. for $C_{25}H_{21}NO$: C 78.3; H 5.5; N 3.7). Mass spectrum (rel. intensity): M^+ 383, 279 (20.5), 278 (100), 105 (20.0), 91 (24.7), 77 (24.7).

4,5-Diphenyl-2,3-b-pyrrolo-4,7-dimethylcoumarin (III). Coumarin I (1.9 g) and benzoin (2.2 g), or alternatively coumarin II (3.8 g), and *p*-TS (0.02 g) were mixed thoroughly and heated in an oil bath at 180 °C for 15 h. The crude product was treated with sodium hydrogen carbonate in water, dried and recrystallized twice from dimethylformamide (10 ml/g). Yield 0.7 g = 19.2 %. M.p. 323–325 °C. 0.2 g was chromatographed twice on preparative thin layer alumina with carbon tetrachloride-ethanol 25:1 for analysis. M.p. 330–331 °C. (Found C 82.3; 81.9; H 5.3; 5.0; N 3.7, 4.0. Calc. for $C_{25}H_{21}NO$: C 82.2; H 5.2; N 3.8). Mass spectrum M^+ 365 (100), 337 (42.6), 323 (23.0).

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Pseudomonas Cytochrome *c* Peroxidase. X. The Effect of *Pseudomonas* Neutral Proteinase on the Enzyme Molecule

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Pseudomonas cytochrome *c* peroxidase (PsCCP, EC 1.11.1.5) has been isolated in a homogeneous form as revealed by disc electrophoresis and analytical ultracentrifugation.^{1,2} It consists of a single polypeptide chain with an apparent molecular weight of $40\,000 \pm 1\,500$ according to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS).³ In some preparations, however, two smaller components were observed in SDS-gel electrophoresis. *Pseudomonas aeruginosa* was found to produce several proteolytic enzymes,⁴ and therefore the smaller components were suspected to derive from PsCCP by proteolysis by a proteinase still present in the preparation.

In this study the effect of a partly purified *Pseudomonas* neutral proteinase on PsCCP was investigated.

Experimental. PsCCP was prepared as described previously.^{1,2} The absorbance ratio A_{467}/A_{280} of the preparations was 4.50–4.66. The purity of the preparations was checked by disc electrophoresis according to Maurer⁵ (system No. 1a, pH 8.9, 7 % gel).

Pseudomonas neutral proteinase (elastase, "semialkaline proteinase") was prepared according to Morihara *et al.*⁶ from a semisynthetic growth medium. After elution from DEAE-cellulose, the preparation was concentrated by ultrafiltration in a Diaflo apparatus (Amicon N.V., UM-10 membrane) and used without further treatment.

Incubation of PsCCP with the partly purified proteinase was carried out in 0.01 M Na-phosphate buffer pH 7.0 at 40 °C at an enzyme-substrate ratio appr. 1:100 (w/w). The reaction was stopped by inactivating proteinase by heating at 100 °C for 5 min.

Analytical SDS-polyacrylamide gel electrophoresis was performed according to Weber and Osborn⁷ in 10 % gels. Standard proteins used for molecular weight estimation were: bovine serum albumin (Fraction V, Armour), ovalbumin (Grade V, Sigma), pepsin (crystallized, Sigma), and horse heart cytochrome *c* (Type III, Sigma). Analytical SDS-polyacrylamide gel electrophoresis in 12.5 % gels in the presence of 8 M urea according to Swank and Munkres⁸ was used to separate the peptides in the 1 500 to 10 000 molecular weight range. 10–30 μ g of protein were applied per gel. After electrophoresis the gels were cut in the middle of the marker dye band, stained with