Investigation of the Pigments of *Pycnoporus sanguineus* - Pycnosanguin and New Phenoxazin-3-ones

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Reinvestigation of the pigments of *Pycnoporus sanguineus* (Polyporaceae) yielded 4 new pigments of the 2-amino-phenoxazin-3-one type and the new phenoxazine ether pycnosanguin (**9**) besides cinnabarine (**1**). Unsubstituted phenoxazin-3-one was also shown among the pigments. Isolation and structure elucidation was performed after methylation of the crude acetone extract. The structures were established by spectral analysis and chemical degradations or interconversions.

Untersuchung der Pigmente von *Pycnoporus sanguineus* - Pycnosanguin und neue Phenoxazin-3-one

Erneute Untersuchung der Pigmente von *Pycnoporus sanguineus* (Polyporaceae) führte neben Cinnabarin (**1**) zur Isolierung von 4 neuen Pigmenten vom 2-Amino-phenoxazin-3-on-Typ und dem neuen Phenoxazinether Pycnosanguin (**9**). Unsubstituiertes Phenoxazin-3-on befand sich ebenfalls unter den isolierten Verbindungen. - Die Isolierung erfolgte nach Methylierung des rohen Aceton-Extraktes. Die Strukturen wurden durch Spektroskopie sowie chemische Umwandlungen und Abbau aufgeklärt.

The intensively coloured *Pycnoporus sanguineus* (Fr.) Murr (Polyporaceae) grows on dead wood in tropical and subtropical areas of Africa, America, and Asia.

Cinnabarine (**1**) is known to be its main colouring component[1,2]; but cinnabarinic acid (**2**) [3] and tramesanguin (**3**) [4] have also been shown to be among the minor constituents.

Obviously due to solubility and separation problems a more thorough-going analysis of the pigments of *Pycnoporus sanguineus* has not yet been performed [5].

Therefore, *P. sanguineus* (collected from dead palm trunks on the Seychelles) was defatted with petroleum ether and subsequently the pigments were extracted with acetone. To overcome separation problems, the total pigment mixture was methylated using CH_3I and then subjected to the chromatographic steps depicted in Fig. 1.

To discriminate the methyl groups originally present in the pigments from those introduced by the methylation procedure an aliquot part of the acetone extract was treated with CD_3I. The deuterated products were isolated and their molecular weights determined by mass spectrometry.

The individual (methylated) pigments **4** to **9** were isolated and their structures deduced mainly by spectroscopy.

The electron excitation spectra (λmax about 435 nm) [6] as well as a strong band at 1580 cm^{-1} in the IR-spectrum[7] indicate basic phenoxazin-3-one structures for all compounds **4** to **8**. Among these, only in **4** to **7** a bathochromic shift (10 to 15 nm) of the long wave maximum is caused by addition of acid, and this indicates an amino group at C-2 [6].

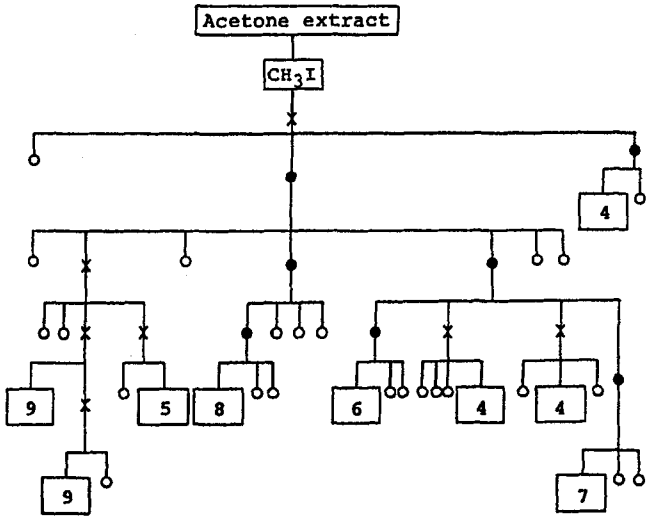
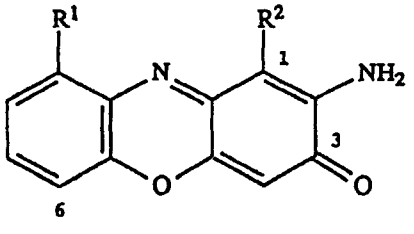


Fig. 1: Separation of the acetone extract from *P. sanguineus* by gel chromatography on columns (Either Fractogel PVA 500 or Sephadex LH 20 (–×–) were used. ○ indicates fractions not further examined.)

The ^{1}H-NMR spectra of **4** to **7** exhibit a singlet (1H) around δ 6.5 ppm, a resonance value typical for H-4 of



1: R^1 = CH_2OH; R^2 = COOH

2: R^1 = COOH ; R^2 = COOH

3: R^1 = COOH ; R^2 = CHO

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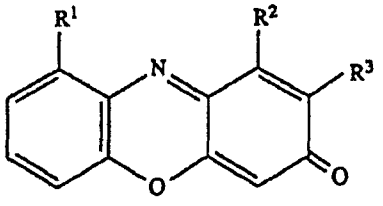
2-amino-phenoxazin-3-ones [8]; at lower field a system of 3 vicinal aromatic H is observed.

Further functional groups at the basic chromophoric skeleton **10** were deduced from IR- and NMR-spectra. The corresponding individual structures are summarized in Tab. 1:

The MS (and HR-MS) corroborate these structures, as do the results of chemical interconversions or degradations: **4** and **7** on treatment with alkali [9] yield 2-amino-3-hydroxybenzylalcohol, which demonstrates the hydroxymethyl group at C-9. **6** is produced by oxidation of **4** using pyridinium chlorochromate. Acetylation of **4** gives **5** as the major product.



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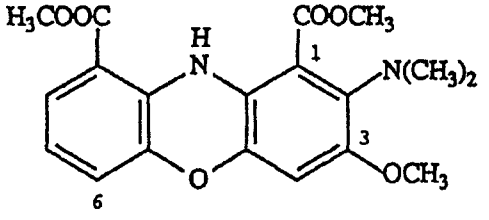

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Tab. 1: Substituents at basic skeleton **10** for the isolated methylated pigments **4** to **8** and the original - unmethylated - pigments in *P. sanguineus*.

	Isolated compound			Original pigment prior to methylation			
	R^1	R^2	R^3		R^1	R^2	R^3
4:	CH_2OH	$COOCH_3$	NH_2	**1:**	CH_2OH	CO_2H	NH_2
5:	CH_2OAc	$COOCH_3$	NH_2	**11:**	CH_2OAc	CO_2H	NH_2
6:	CHO	$COOCH_3$	NH_2	**12:**	CHO	CO_2H	NH_2
7:	CH_2OH	$COOCH_3$	$NHCH_3$	**13:**	CH_2OH	$COOCH_3$	$NHCH_3$
8:	H	H	H				

Tab. 2: Results of NOE-studies.

Irradiation at:		Irradiation at:	
δ [ppm]	Proton	δ [ppm]	Proton
7.38	H-8	6.63	H-7
		3.88	9-$COOCH_3$
6.78	H-6	6.63	H-7
6.63	H-7	7.38	H-8
		6.78	H-6
6.51	H-4	3.81	3-OCH_3
3.94	1-$COOCH_3$	3.88	9-$COOCH_3$
		2.68	$N(CH_3)_2$
3.88	9-$COOCH_3$	7.38	H-8
3.81	3-OCH_3	6.51	H-4

Tab. 3: Constituents of *P. sanguineus* and their approximate concentrations. [Content in % x 10^{-3} (dried *Pycnoporus* = 100 %)]

Compound class	Compound	Content
Sterols	Ergosterol	12.5
	5,6-Dihydroergosterol	11.2
	Ergosterolperoxide	6.2
Phenoxazine	Cinnabarine (1)	50.0
	O-Acetyl cinnabarine (11)	1.2
	2-Amino-9-formylphenoxazone-1-carbonic acid (12)	1.2
	9-Hydroxymethyl-2-methylamino-phenoxazone-1-carbonic acid methyl ester (7)	0.9
	Phenoxazone (8)	0.3
	Pycnosanguin (9)	1.3

8 contains only one N atom, and the low molecular weight as well as the electron excitation spectrum and MS fragmentation are in agreement with unsubstituted phenoxazin-3-one. This was corroborated by comparison with an authentic sample.

The least polar isolated pigment is yellow, and its electron excitation spectrum (λ max 399 nm) is significantly different from those discussed above. The ^{1}H- and ^{13}C-NMR-spectra indicate 4 aromatic protons, 3 OCH_3, 2 ester carbonyl, 1 NH, and 1 $N(CH_3)_2$, and NOE experiments demonstrate the positive effects summarized in Tab. 2:

From these observations structure **9** can be established.

9 respresents the first phenoxazine ether from a natural source; therefore, **9** was named pycnosanguin [10].

9 also was synthesized by oxidation of 2-amino-3-hydroxybenzoic acid with MnO_2 and subsequent treatment with $Na_2S_2O_4$ and $(CH_3O)_2SO_2$ according to a known synthetic route in the phenoxazine series [11].

Besides the pigments mentioned, ergosterol, 5,6-dihydroergosterol and ergosterol-5,8-peroxide were isolated from the petrol ether extract as major components.

Tab. 3 summarizes the isolated compounds and their approximate concentrations in *P. sanguineus*.

Thanks are due do Prof. Dr. Leif Ryvarden, University of Oslo, Norway, for the classification of the Pycnoporus. We also thank the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie for financial support.

Experimental Part

TLC on Nano-plates Sil-20 UV (Macherey-Nagel); solvent system toluene-EtOAc (6:4), detection by UV. - IR in KBr, if not stated otherwise. - UV/VIS in MeOH, if not stated otherwise. - MS: Finnigan 4500 at 70 eV. - NMR: Jeol GX 400, ^{1}H at 400 MHz, ^{13}C at 100 MHz; solvent: $CDCl_3$, if not stated otherwise. TMS as internal standard. NOE effects were measured by the difference method without quantitative results for enhancement.

Origin of fungi

P. sanguineus (Fr.) Murr was collected in May 1986 from dead palm trunks on La Digue Island (Seychelles) and identified by Prof. Dr. *Leif Ryvarden*, University of Oslo, Norway. A voucher specimen is deposited in the institute's collection.

Extraction, preparation, and isolation

Successive extraction of the dried and ground fungi (150 g) with petroleum ether and then acetone at room temp. gave 1.1 g and 2.1 g of crude acetone extracts, respectively. 1.6 g of the acetone extract were methylated with CH_3I/K_2CO_3 in acetone to yield 1.72 g residue (extract A); 0.5 g of the acetone extract were methylated with CD_3I/K_2CO_3 in the same way to yield 0.55 g residue (extract B).

Extract A was subjected to column chromatography (= CC) on Sephadex LH 20 and then Fractogel PVA using acetone and acetone-dioxane mixtures according to Fig. 1. Extract B was worked-up by prep. TLC.

Cinnabarine methyl ester (4)

Orange crystals (81 mg from extract A); m.p. 252 °C (decomp.); TLC R_f = 0.35. - IR: 1660; 1580 cm^{-1}. - UV/VIS: λ max (log ε) = 234 (4.48), 255 (sh) (4.18), 265 (sh) (4.12), 416 (sh) (4.25), 436 nm (4.37); (MeOH/HCl) λ max (log ε) = 231 (4.38), 268 (sh) (4.02), 279 (sh) (3.92), 422 (sh) (4.26), 443 (4.22), 485 (sh) nm (3.91). - ^{1}H-NMR: δ (ppm) = 4.00 (s, 3H, CO_2CH_3), 5.04 (s, 2H), 5.65 (br, OH), 6.51 (s, 1H, H-4), 7.10 (br, NH), 7.22 (dd, 1H, J_1 = 7.5, J_2 = 1.5 Hz, H-6), 7.31 (dd, 1H, J_1 = 8.0, J_2 = 1.5 Hz, H-8), 7.40 (dd, 1H, J_1 = 8.0, J_2 = 7.5 Hz, H-7), 8.82 (br, NH). - ^{13}C NMR (DMSO-d_6)*): δ (ppm) = 178.1 (C-3), 167.2 ($\underline{C}O_2CH_3$), 149.2, 148.5, 143.9, 141.1, 140.5, 130.1, 129.3 (C-7), 122.9 (C-8), 113.9 (C-6), 103.7 (C-4), 98.4, 58.9 (CH_2OH), 51.6 ($CO_2\underline{C}H_3$). - MS m/z (rel.Int.) = 300.07468 (M^+, 100: $C_{15}H_{12}N_2O_5$), 299 (39), 271 (14), 268 (34), 267 (37), 240 (74), 239 (43), 238 (13), 222 (13), 212 (51), 211 (22), 156 (9), 77 (14), 76 (17).

*) assignments by heteronuclear COSY.

O-Acetyl cinnabarine methyl ester (**5**)

Orange crystals (2 mg from extract A); m.p. 234 °C (decomp.); TLC R_f = 0.49. - IR ($CHCl_3$): 1735; 1672; 1646; 1580 cm^{-1}. - UV/VIS: λ max (log ε) = 234 (4.36), 260 (sh) (4.06), 415 (sh) (4.22), 431 nm (4.26); (MeOH/HCl) λ max (log ε) = 229 (4.25), 267 (sh) (3.88), 278 (sh) (3.83), 420 (sh) (3.95), 445 (4.05), 485 (sh) nm (3.86). - ^{1}H-NMR: δ (ppm) = 2.15 (s, 3H, $OCOCH_3$), 3.99 (s, 3H, CO_2CH_3), 5.67 (s, 2H, CH_2OAc), 6.48 (s, 1H, H-4), 7.35 (m, 1H) and 7.44-7.49 (m, 2H) (H-6, H-7, H-8). - MS m/z (rel.Int.) = 342.08539 (M^+, 80: $C_{17}H_{14}N_2O_6$), 300 (34), 299 (63), 268 (36), 267 (100), 251 (12), 240 (26), 239 (14), 224 (12), 223 (24), 222 (39), 212 (11), 140 (13), 77 (9), 76 (9), 43 (33).

2-Amino-9-formyl-1-methoxycarbonylphenoxazone (**6**)

Orange crystals (2 mg from extract A); m.p. 228 °C (decomp.); TLC R_f = 0.51. - IR ($CHCl_3$): 1690; 1650; 1580 cm^{-1}. - UV/VIS: λ max (log ε) = 234 (4.18), 260 (sh) (3.87), 420 (sh) (4.04), 436 nm (4.05); (MeOH/HCl) λ max (log ε) = 232 (4.11), 277 (sh) (3.72), 307 (sh) (3.47), 426 (sh) (3.82), 450 (3.90), 486 (sh) nm (3.75). - ^{1}H-NMR: δ (ppm) = 3.99 (s, 3H, CO_2CH_3), 6.52 (s, 1H, H-4), 7.50 (dd, J_1 = 8.0, J_2 = 7.5 Hz, 1H, H-7), 7.63 (dd, J_1 = 8.0, J_2 = 1.5 Hz, 1H, H-6), 7.94 (dd, J_1 = 7.5, J_2 = 1.5 Hz, 1H, H-8), 7.1 (br, NH), 8.7 (br, NH), 11.17 (s, 1H, CHO). - MS m/z (rel.Int.) = 298.05896 (M^+, 9: $C_{15}H_{10}N_2O_5$), 271 (11), 270 (80), 239 (15), 238 (100), 210 (14), 127 (7), 77 (6), 76 (6).

9-Hydroxymethyl-1-methoxycarbonyl-2-(methylamino)phenoxazone (**7**)

Red crystals (3 mg from extract A); m.p. 182 °C (decomp.); TLC R_f = 0.31. - IR: 1710; 1628; 1582; 1515 cm^{-1}. - UV/VIS: λ max (log ε) = 240 (4.65), 264 (sh) (4.41), 417 (sh) (4.56), 439 nm (4.60); (MeOH/HCl) λ max (log ε) = 236 (4.65), 270 (sh) (4.29), 416 (sh) (4.41), 442 (4.50), 494 (sh) nm (4.26). - ^{1}H-NMR: δ (ppm) = 3.07 (d, J = 5.5 Hz, 3H, $NHCH_3$), 4.00 (s, 3H, CO_2CH_3), 5.01 (d, J = 5.5 Hz, 2H, CH_2OH), 6.42 (s, 1H, H-4), 6.79 (br, 1H, $NHCH_3$), 7.24 (dm, J = 7.5 Hz, 1H, H-6), 7.31 (dd, J_1 = 8.0, J_2 = 1.5 Hz, 1H, H-8), 7.38 (dd, J_1 = 8.0, J_2 = 7.5 Hz, 1H, H-7). - MS m/z (rel.Int.) = 314.09014 (M^+, 37: $C_{16}H_{14}N_2O_5$), 265 (13), 264 (100).

Phenoxazone (**8**)

Orange-brown crystals (0.5 mg from extract A); m.p. 198 °C (decomp.); TLC R_f = 0.45. - IR ($CHCl_3$) 1645; 1616; 1570; 1517 cm^{-1}. - UV/VIS: λ max (log ε) = 244 (3.92), 252 (sh) (3.90), 262 (sh) (3.68), 345 (3.78), 446 nm (3.70). - ^{1}H-NMR: δ (ppm) = 6.34 (d, J = 2 Hz, 1H, H-4), 6.87 (dd, J_1 = 10, J_2 = 2 Hz, 1H, H-2), 7.34 (dd, J_1 = 8, J_2 = 1.5 Hz, 1H, H-6), 7.38 (ddd, J_1 = 8, J_2 = 7.5, J_3 = 1.5 Hz, 1H, H-8), 7.45 (d, J = 10 Hz, 1H, H-1), 7.55 (ddd, J_1 = 8, J_2 = 7.5, J_3 = 1.5 Hz, 1H, H-7), 7.81 (dd, J_1 = 8, J_2 = 1.5 Hz, 1H, H-9). - MS m/z (rel.Int.) = 197.04758 (M^+, 100: $C_{12}H_7NO_2$), 169 (89), 140 (12), 114 (8), 63 (8).

Pycnosanguin (**9**)

Yellow needles (total amount: 3 mg from extract A); m.p. 246-249 °C; TLC R_f = 0.67. - IR ($CHCl_3$): 3310; 1690 cm^{-1}. - UV/VIS: λ max (log ε) = 213; 399 nm (3.94); (MeOH/HCl) λ max (log ε) = ~207; 238 (sh) (4.40), 424 nm (4.05). - ^{1}H-NMR (acetone-d_6): δ (ppm) = 2.68 (s, 6H, $N(CH_3)_2$), 3.81 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 6.51 (s, 1H, H-4), 6.63 (dd, J_1 = 8, J_2 = 8 Hz, 1H, H-7), 6.78 (dd, J_1 = 8, J_2 = 1.5 Hz, 1H, H-6), 7.38 (dd, J_1 = 8, J_2 = 1.5 Hz, 1H, H-8), 9.22 (s, br, 1H, NH). - ^{13}C-NMR (acetone-d_6)*): δ (ppm) = 168.3 (9-$\underline{C}O_2CH_3$), 167.3 (1-$\underline{C}O_2CH_3$), 153.7, 144.5 (C-5a), 141.1, 136.5, 136.2, 136.0, 125.9 (C-8), 122.2, 120.3 (C-7), 119.6 (C-6), 111.8 (C-9), 104.3 (C-4), 56.4 (OCH_3), 52.6 ($CO_2\underline{C}H_3$), 52.3 ($CO_2\underline{C}H_3$), 43.5 ($N(CH_3)_2$). - MS m/z (rel.Int.) = 372.13199 (M^+, 100: $C_{19}H_{20}N_2O_6$), 357 (13), 341 (14), 340 (35), 325 (26), 312 (10), 297 (8), 293 (33), 267 (11), 170 (17), 154 (12).

Alkaline degradation of **4** *and* **7**

4 (5 mg) (or **7**) in 5N NaOH (2 ml) were refluxed for 6 min. After cooling to room temp. and addition of water (2 ml), the solution was neutralized with 5N HCl and evaporated. The residue was extracted with MeOH and the methanolic solution again evaporated. Prep. TLC yielded 2-amino-3-hydroxybenzyl alcohol.

Acetylation of **4**

4 (5 mg) in 2 ml Ac_2O/pyridine (room temp., 12 h) gave 3.5 mg of a product identical with **5**.

Oxidation of **4**

To **4** (10 mg) suspended in 2 ml CH_2Cl_2 pyridinium chlorochromate (10 mg) was added and the mixture was stirred at room temp. for 12 h. Diethyl ether (5 ml) was added and the insoluble residue was filtered off. Evaporation and prep. TLC of the residue yielded 4 mg of orange crystalls identical with the isolated pigment **6**.

Synthesis of **9**

a) Synthesis of cinnabarinic acid (**2**) *(Lit.* [11]*)*

To 3-hydroxyanthranilic acid (500 mg) dissolved in 20 ml dimethylformamide/water (3:1) NaH_2PO_4 (2.4 g) and Na_2HPO_4 (0.7 g) were added. After 10 min. "active" MnO_2 (1.5 g) was added and the mixture was stirred at room temp. for 90 min and then poured into a solution of $FeSO_4$ (15 g) in 100 ml 2N HCl. The precipitate consisted of crude **2** (485 mg), which was filtered off and washed with water.

b) Reductive methylation of **2** *(Lit.* [6]*)*

2 (485 mg) was suspended in 200 ml acetone/water (2:1) under N_2. A solution of K_2CO_3 (3.9 g) in 20 ml water was added dropwise during 30 min followed by 6.5 ml $(CH_3O)_2SO_2$ during 45 min; this mixture was further stirred for 1 h at room temp. Adding a solution of $Na_2S_2O_4$ (2.6 g) in 30 ml water changed the intense red colour to light-yellow. After 1 h of stirring the acetone was removed by evaporation and the remaining aqueous solution was extracted with $CHCl_3$. Purification of the residue of the $CHCl_3$-extract by CC on SiO_2 yielded **9** (30 mg, light-yellow needles) identical with the compound isolated from *P. sanguineus*.

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*) cf. compound **4**.