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Synthesis of Tolypocladin and Isotolypocladin

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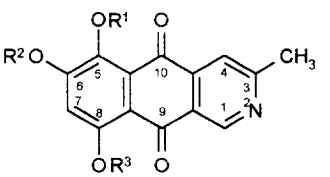
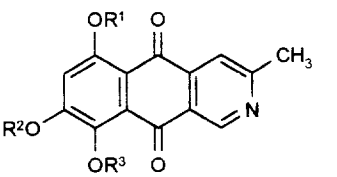
Abstract: Total synthesis of the microbial metabolite tolypocladin (**1**) and isomeric isotolypocladin (**2**) is described using Friedel-Crafts acylation for condensation of the 2-aza-anthraquinone-(9,10) ring.

INTRODUCTION

Tolypocladin (**1**) was isolated recently from *Tolypocladium inflatum* DSM 915 and identified as 3-methyl-5,6 (or 7),8-trihydroxy-2-aza-anthraquinone-(9,10)[1]. Apparently, its structure is related to bostrycoidin (**1a**) from *Fusarium solani* D_2 *purple* as the appropriate 6-methoxy derivative.[2]





1:	R^1, R^2, R^3 = H	Tolypocladin	**2**:	R^1, R^2, R^3 = H	Isotolypocladin
1a:	R^1, R^3, = H, R^2 = Me	Bostrycoidin	**2a**:	R^1, R^3 = H, R^2 = Me	Isobostrycoidin
1b:	R^1, R^2, R^3 = Ac	Triacetyl-tolypocladin	**2b**:	R^1, R^2, R^3 = Ac	Triacetyl-isotolypocladin

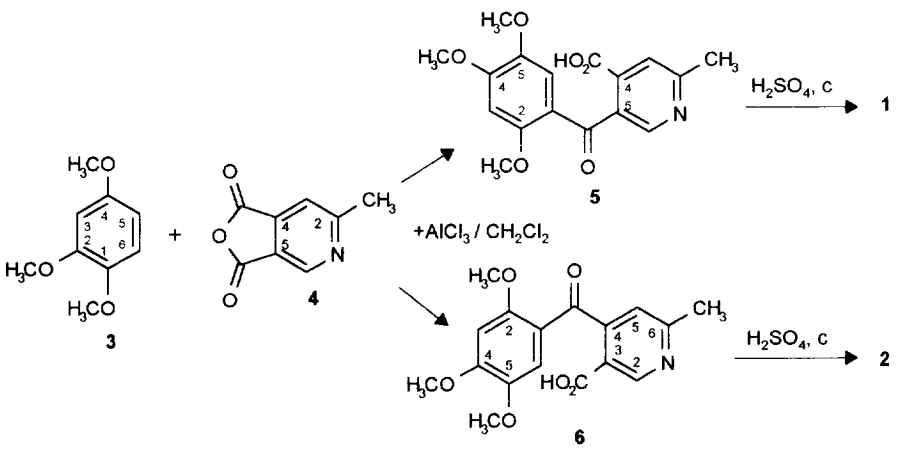
Though the NMR data settled most details of the chemical constitution of **1**, chemical total synthesis was necessary to determine the exact position of one of the hydroxy groups, located either at C-6 or C-7 atom of the aromatic skeleton.

Cameron[3,4,5] and Watanabe[6] synthesized **1a** *via* a series of rather difficult steps in low yields because the commonly used Friedel-Crafts synthesis failed to give reasonable results. Thus, Cameron et al. first prepared 2-methyl-6,8-dimethoxy-2-aza-anthraquinone-(9,10) by the addition of 1,1-dimethoxy ethene to 3-methyl-isoquinoline quinone (which is rather hardly accessible) as well as through radical acylation of 4-cyano-2-methyl-pyridine by 3,5-dimethoxy-benzaldehyde, followed by ring closure. The resulting key intermediate was photooxidized and demethylated partially to yield **1a**. Moreover, Watanabe et al. condensed a lithiated nicotinic acid amide with a trimethoxy-benzoic acid amide. There after, the pyridine ring was methylated in position 2, followed by reduction, ring closure, oxidation and partial demethylation.

Here we report on a new approach to **1, 1a** and **2, 2a** via Friedel-Crafts acylation which demonstrates this route[7] to be more encouraging than in the past.

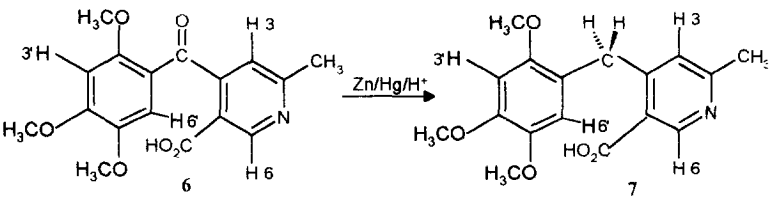
RESULTS AND DISCUSSION

The first step of our synthesis was the condensation of 1,2,4-trimethoxy-benzene (**3**) and 2-methyl-pyridine 4,5-dicarboxylic acid anhydride (**4**) under Friedel-Crafts conditions (Scheme 1). Although formation of not less than six structural isomers (3 protons in **3**, 2 acyl groups in **4**) appeared as possible, the acylation occurred regioselectively at position 5 of **3**, but the educt **4** reacted with each acyl group in different manners. The resulting key products **5** and **6** (Scheme 1) can be separated by recrystallization.



Scheme 1

The ^{1}H NMR spectra of **5** and **6** show two singlets of the remaining protons in positions 3' and 6' at the benzene ring. As neither ortho nor meta coupled protons have been observed, H-3' and H-6' are located in para position (Scheme 2). Conclusive evidence of hydroxyl position in **1** and **2** was readily inferred from the chemical shift data of the protons in **6** and **7** observed after Clemmensen reduction of the carbonyl group to the methylene compound **7** (Scheme 2).



Scheme 2

Table 1: ^{1}H NMR data, δ [ppm] of the aromatic hydrogen atoms in **6** and **7**, $CDCl_3$.

H Atoms	δ_6	δ_7	δ_6-δ_7
H-3'	6.67 s	6.68 s	-0.01
H-6'	7.10 s	6.76 s	+0.34
H-3	7.38 s	6.86 s	+0.52
H-6	8.92 s	8.77 s	+0.15

The greatest changes of the chemical shifts δ have been observed for H-6' and H-3 neighboured to the carbonyl and methylene groups (Table 1). Due to this position 7 is unequivocally proved for the hydroxyl group in **2** and vice versa position 6 for HO substituent in **1**. Consequently, cyclization and demethylation of the key products **5** and **6** by sulfuric acid yielded **1** and **2** (Scheme 1). Traces of bostrycoidin (**1a**) and/or isobostrycoidin (**2a**) were detected by EI/MS (M^+: m/z 285) as further products of the cyclocondensation due to the incomplete demethylation.

The purification of **1** and **2** by recrystallization or preparative chromatography is nearly impossible, because of intermolecular hydrogen bonds and insufficient solubility, but the easily accessible triacetyl derivatives **1b** and **2b** do not possess these disadvantages and they are suitable for recrystallization or column chromatography. You can get back pure **1** and **2** by saponification of **1b** and **2b**. Thin layer chromatography (TLC) and column chromatography are possible, if the silica gel sheets or adsorbents are prepared with a methanolic oxalic acid solution.

The isomers **1** and **2** are discernible by characteristic physico-chemical differences: The EI/MS fragmention pattern of **2** displays less fragments than that of **1**. Even the melting behaviour of **2** is characteristic in comparison to **1**: The microcrystalline shape of **2** changes at 230 °C to long red needles, which melt under decomposition while the microcrystalline **1** does not change its shape. The TLC spot of **2** fluoresces orange at λ 366 nm, but **1** fluoresces pale violet.- The ^{1}H NMR spectrum of **1** displays three distinguishable HO signals, while that of **2** indicates only one signal at 13.18 ppm attributable to two HO protons. However, high dilution of **2** in DMSO-d_6 yielded significant shift changes of signals towards deeper (or higher) field, probably due to the degradation of intermolecular associates, which are present in higher concentrated solutions.

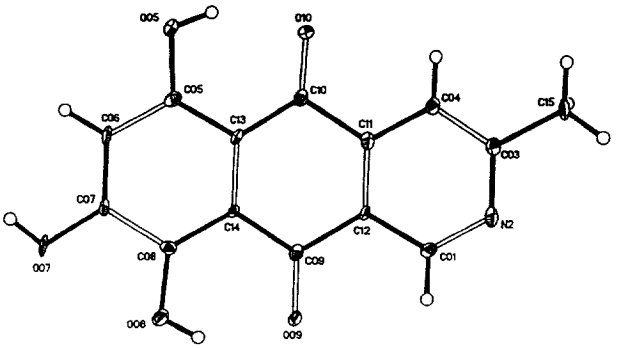


Figure 1. Molecular structure of **2** with the thermal ellipsoids (20%) and the atomic numbering.

The differences in the NMR spectra of **1** and **2** were the reason to measure the X-ray diffraction of **2** crystals (from chloroform/ethyl acetate), in order to examine if there are any differences in their structures too. It is shown in Figure 1 the molecular structure of **2**. The molecule is about planar. The dihedral angles of the two terminal benzene rings to the central para quinone ring are 2(2)° and 3(2)° respectively. Figures 2-4 display the projections of the crystal structure of **2** along the 0-x, 0-y, and the 0-z-axis. In Figures 2 and 3 it is shown, that two molecules are linked by two intermolecular N2...HO7 hydrogen bonds (2.735 Å). No other intermolecular hydrogen bonds are possible. There are, however, two intramolecular hydrogen bonds (O08...O09, O05...O010; 2.600 and 2.585 Å, respectively). Figure 4 displays the crystal structure with molecule chains, parallel to the crystallographic z-axis. The intramolecular distances show too, that the central ring, at least in the crystal, is para-quinoid.[8]

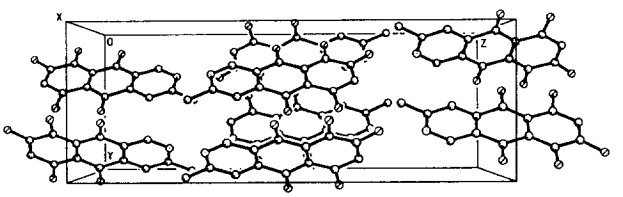


Figure 2. Projection of the crystal structure of **2** along the x-axis.

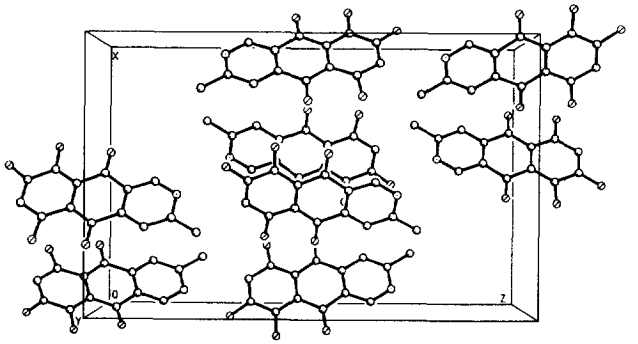


Figure 3. Projection of the crystal structure of **2** along the y-axis.

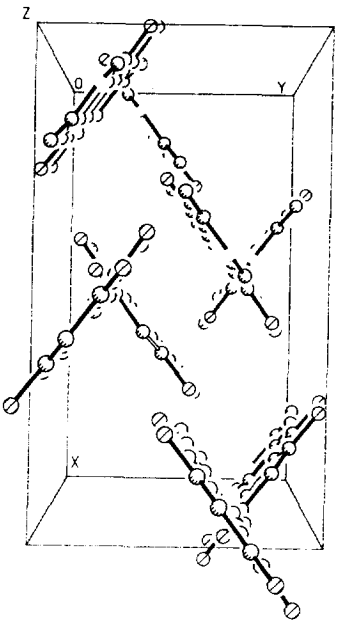


Figure 4. Projection of the crystal structure of 2 along the z-axis.

The biological role of metabolites as **1**, either as metal-chelating agents or of constituents of a particular respiratory chain, has been subject to discussion.[9] The biological importance of pigment production for the producing microbe itself is not yet understood, but it seems to be possible that metal-chelating agents as **1** can act as scavenger of trace elements or as detoxifying ligand for high concentrations of heavy metals. Gräfe et al.[1] investigated the electron spectral properties and the complex formation with di- or trivalent metal ions of naturally occurring **1** and found characteristic bathochromic shifts of the electron spectral pattern.

Our interest was to study the ultraviolet/visible and fluorescence spectra of synthetic **1** and **2** and to compare the spectra of their complexes with Al^{3+} ions in different ratios, because, as mentioned above, the TLC spots of the isomers **1** and **2** showed characteristic fluorescence under ultraviolet light. Furtheron it was useful to detect and to identify the **1**- and **2**-Al^{3+} ion complexes in methanol by UV during the isolation, separation and purification processes of natural **1** and synthetic **1** and **2**.

The ultraviolet/visible spectrum of **1** displays a broad band absorption in the orange-red region (480 to 600 nm) resulting in a violet-blue colour of its methanolic solution. The orange-red colour of **2** is caused by its narrower absorption band in the blue yellow region (490 to 515 nm) (Figure 5). The fluorescence emission spectra of **1** and **2** are clearly discernible: The maximum of **1** ranges near 550 nm (weak emission at 405 nm), that of **2** near 405 nm (weak emission at 550 nm), when excitated with 305 nm light (Figure 6).

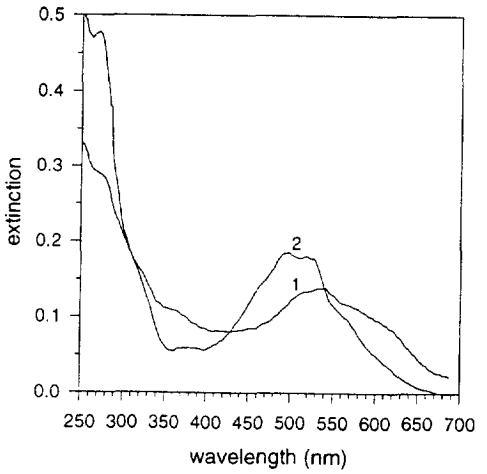


Figure 5. UV/vis-absorption spectra of **1** and **2**.

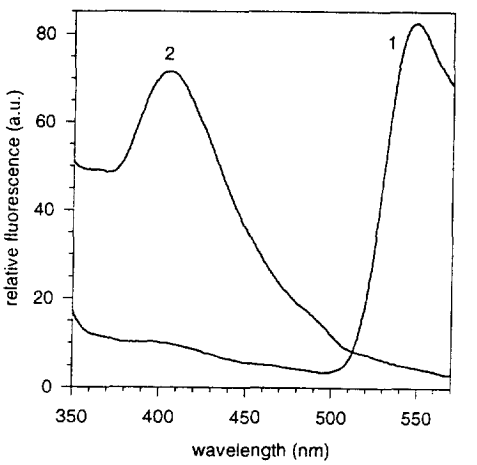


Figure 6. Fluorescence emission spectra of **1** and **2**. excitation 305 nm.

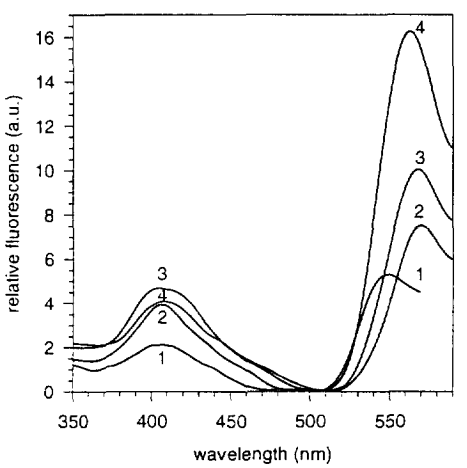


Figure 7. Fluorescence spectra of **1**-Al^{3+}complexesformed by addition of Al^{3+} ions in molar ratios 1:0 (1); 1:0.5 (2); 1:1 (3); 1:2 (4); excitation 305 nm.

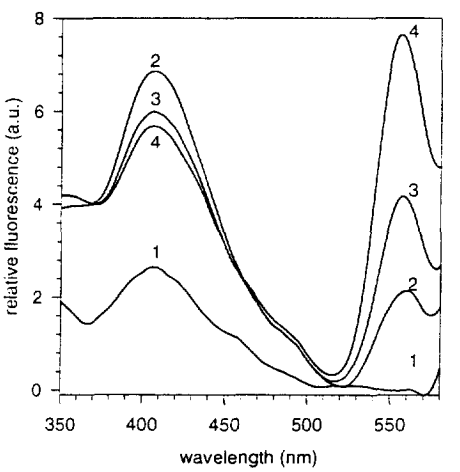


Figure 8. Fluorescence spectra of **2**-Al^{3+}complexes formed by addition of Al^{3+} ions in molar ratios 1:0 (1); 1:0.5 (2); 1:1 (3); 1:2 (4); excitation 305 nm.

The fluorescence intensities of methanolic solutions of **1** at 550 nm increases strongly with growing amounts of Al^{3+} ions, while the smaller maximum at 410 nm increases less applying the ratios 1:0.5 (2) and 1:1 (3), but decreases continuing the Al^{3+} ions addition to 1:2 (4) (Figure 7). In contrary to **1** the isomer **2** fails to show a fluorescence emission at 530-570 nm, but a very strong fluorescence light is developed while adding adequately increasing quantities of Al^{3+} ions (Figure 8, curves 1-4). The maximum near 405 nm increases strongly after the first addition of Al^{3+} ions (curve 2), but then slightly decreases as to be seen in Figure 8 (curves 3 and 4).

EXPERIMENTAL

M. p.: Boetius M (corr.). TLC. Aluminium foils (sheets), silica gel 60, F_{254} (Merck) TLC of **1** and **2**. Silica gel sheets (Merck) soaked with 5% oxalic acid in MeOH, dried at room temperature. TLC solv. 1: (v/v). $CHCl_3/Me_2CO/AcOH/H_2O$ = 8/2/1/0.6. Solv. 2:(v/v). $Me_2CO/CHCl_3$=2/1. IR spectra: Specord 75 IR, Carl Zeiss Jena, KBr. Electron impact mass spectra (EIMS). Jeol JMS-D100. UV spectra: Specord 500 UV-VIS (Carl Zeiss Jena), MeOH,c: 0.02 mM. Fluorescence spectra: Spectrofluometer SFM 25 (Kontron Instruments Inc). The ^1H NMR (200 MHz) and ^{13}C NMR (200 MHz) spectra were recorded on a Bruker AC 200-E spectrometer, $DMSO_{D6}$ solution unless otherwise stated. δ[ppm], s: singlet.

2-Methyl-pyridine-4,5-dicarboxylic anhydride (**4**).- 18.1 g (100 mmol) 2-Methyl-pyridine-4,5-dicarboxylic acid (prepared according to ref.[10], m.p. 245-250 °C, dec.; m.p. found 240-280 °C, dec.) are refluxed 30 min in 150 mL acetic anhydride, cooled, the small residue is removed and the filtrate evaporated. 15.5 g (95%) bright crystals, m. p. 102-103 °C ($CHCl_3$). Anal. found: C, 58.71; H, 3.25; N, 8.79. Calc. for $C_8H_5NO_3$ (163.1): C, 58.90; H, 3.09; N, 8.59. IR: 1860 and 1786 cm^{-1} (anhydride). ^1H NMR: δ = 9.13 (s, H-6), 7.70 (s, H-3), 2.75 (s, CH_3). ^{13}C NMR 167.5, 161.3, 161.0, 146.5, 138.9, 122.3, 117.7, 25.2 (CH_3).

6-Methyl-4-(2,4,5-trimethoxy-benzoyl)-nicotinic acid (**6**).- In a three necked bottle are placed 6.4 g (48 mmol) anhydrous aluminium chloride and suspended with 40 mL water-free methylene chloride. The mixture is stirred and cooled at about 5 °C and two separate solutions of 2.6. g (16 mmol) **4** (in 25 mL methylene chloride) and 8.1 g (48 mmol) commercially available 1,2,4-trimethoxy-benzene (in 25 mL methylene chloride) are added during 15 min by each two dropping funnels. Let the temperature rise to 15 °C during 15 min and stir 2 h at room temperature. The red-brown resinous mass is then decomposed by ice and conc. HCl (2:1) under stirring and ice water cooling from outside. From the resulting yellow solution a microcrystalline precipitate separates, which is centrifuged. The water phase is decanted and the solids are sucked off. A further crop can be isolated from the aqueous filtrate. The yield amounts to 4.5 g (75%) 6-hydrochloride-hemihydrate, m. p. 233-236 °C (diluted HCl). Anal. found. C, 54.28; H, 5.35; Cl, 10.01; N, 3.70. Calcd. for $C_{17}H_{17}NO_6$ HCl 0.5 H_2O (376.8): C, 54.19; H, 5.08; Cl, 9.41; N, 3.72. Water, found 1.76. Calcd. 2.4. MS: m/e = 331.3 (M^+). Calcd. for $C_{17}H_{17}NO_6$ (331.3). IR: 1634 and 1724 cm^{-1}. ^1H NMR: (Numbering see scheme 2) δ = 8.99 (s, H-6), 7.39 (s, H-3), 7.31 (s, H-6'), 6.68 (s, H-3'), 3.87 and 3.78 (s, CH_3O-2', s, CH_3O-4'), 3.40 (s, CH_3O-5'), 2.60 (s, CH_3-2). The 6-hydrochloride-hemihydrate is dissolved in water, adjusted with a sodium acetate solution to pH 6 and colourles crystals are precipitated: M. p. 250-251 °C (water). Rf = 0.53 (solv. 1). Anal. found: C, 61.53; H, 5.21; N, 4.16. Calcd. for $C_{17}H_{17}NO_6$ (331.3): C, 61.63; H, 5.17; N, 4.23. MS: m/e = 331.2 (M^+). Calcd. m = 331.3. IR: 1638 and 1707 cm^{-1} (COOH, CO). ^1H NMR: δ = 8.92 (s, H-6), 7.38 (s, H-3), 7.10 (s, H-6'), 6.67 (s, H-3'), 3.87 and 3.78 (s, CH_3O-2', s, CH_3O-4'), 3.38 (s, CH_3O-5'), 2.53 (s, CH_3-2).

6-Methyl-4-(2,4,5-trimethoxy-benzyl)-nicotinic acid (**7**): 2.08 g $HgCl_2$ (7.6 mmol) dissolved in 30 mL water are given to 20.8 g (317 mmol) Zn dust. 1 mL conc. HCl is added under shaking, and the mixture is decanted after 5 min. 1.66 g (5 mmol) of the keto acid **6** is solved in diluted hydrochloric acid (275 mL water/ 4.14 mL conc. HCl), and the mixture is put by stirring to the Zn amalgam. The reduction starts at once, and after 30 min the process is finished (TLC). After 2.5 h the filtrate is evaporated, the residue, suspended in 15 mL water, is solved in ammonia and H_2S gas introduced untill all ZnS is precipitated. The mixture is heated for 1 h to facilitate the following filtration. The filtrate is evaporated, the residue dissolved in 30 mL hot water and acidified with acetic acid (20%) to the isoelectric point, where 1.5 g 7 (94%) crystallize, colourless prisms, m. p. 158-159 °C, Rf – 0.33 (solv.

1). Anal. found: C, 64.31; H, 6.12; N, 4.47. Calcd. for $C_{17}H_{19}NO_5$ (317.3): C, 64.34; H, 6.03; N, 4.41. MS: m/e found 317.2, calcd. 317.1247. IR: 3430, 2990, 2830, 1618, 1508,1395. ^{1}H NMR: (Numbering see scheme 2) δ = 8.77 (s, H-6), 6.86 (s, H-3), 6.76 (s, H-6'), 6.68 (s, H-3'), 4.18 (s, CH_2), 3.77, 3.69, 3.63 (3 s, OCH_3), 2.39 (s, CH_3-2).

2-Methyl-5-(2,4,5-trimethoxy-benzoyl)-isonicotinic acid (5): The isomer **5** is enriched in all acid filtrates resulting from the isolation and purification of the described isomer **6**. By fractionated precipitation with sodium acetate solution colorless crystals are separated. The yield of **5** amounts to 0.9 g (17%) ; m. p. 286-287 °C (dec.) (water); Rf = 0.4 (solv. 1). Anal. found: C, 60.98; H, 5.16; N, 4.32. Calcd. for $C_{17}H_{17}NO_6$ (331.3): C, 61.63; H, 5.17; N, 4.23. MS: m/e found 331.2, calcd. 331.1057. ^{1}H NMR: (Numbering see scheme 1), pyridine ring: δ – 8.36 (s, H-6), 7.60 (s, H-3), 2.58 (s, CH_3-2); benzene ring: δ = 7.32 (s, H-6), 6.67 (s, H-3), 3.87 and 3.76 (s, CH_3O-2, s, CH_3O-4), 3.43 (s, CH_3O-5).

3-Methyl-5,7,8-trihydroxy-2-aza-anthraquinone-(9,10) (2): 331 mg (1 mmol) **6** is heated in 5 mL conc. sulfuric acid to 120 °C during 2 h. After cooling the solution is put on ice. The most of the acid is neutralized with sodium hydroxide solution, the rest finally adjusted with sodium acetate to the pH value 6. Then the product is extracted fully with chloroform, the orange red extract dried with sodium sulfate, and the filtrate is evaporated. The yield amounts ca. 150 mg (55%) microcrystalline red powder, which crystallizes sometimes in dark red solids from chloroform. During heating from 230 to 260 °C long red crystals are generated, m. p. 317 °C (dec.), sublimation 230-260 °C, vermillion powder, Rf = 0.6 (solv. 2). Anal. found: C, 61.69; H, 3.42; N, 5.13. Calcd. for $C_{14}H_9NO_5$ (271.2): C, 62.00; H, 3.35; N, 5.16. MS: m/e found 271.0451, calc.271.0481. IR: 3425, 3000, 2600, 1623, 1586, 1445 cm^{-1}. ^{1}H NMR: δ = 13.18 (s, 2 OH), 9.19 (s, H-1), 7.82 (s, H-4), 6.59 (s, H-7), 2.68 (s, CH_3). ^{13}C NMR : 24.68 (CH_3), 105.75, 109.36, 112.14, 117.53, 123.63, 139.18, 148.00, 149.50, 158.20, 161.09, 165.57, 181.96 (CO), 186.09 (CO).

X ray diffraction: $C_{14}H_{29}NO_5$, M_r = 271.2, orthorhombic, Pbca, a = 13.428 (3), b = 7.696 (2), c = 21.5.22 (4), Å, V = 2224.1 (9) Å^3, Z = 8, D_X = 1.620 Mg m^{-3}, λ (Mo Kα) = 0.71073 Å, μ = 0.125 mm^{-1}, F(000) = 1120, T = 193 K, R = 0.076 ($I > 2\sigma(I)$, wR = 0.199 for all 2674 unique diffractometer data.- Crystallization succeeded from $CHCl_3$. One crystal of the dimensions 0.48 * 0.28 * 0.01 mm^3 was sealed in a Lindemann-glas capillary. 25 reflections with $\vartheta > 5°$ were used to determine the cell parameters on a four circle computer controlled diffractometer (R3m/V Siemens). The intensities were measured on the same apparatus: MoKα radiation, $\vartheta_{max} < 28°$, 3239 reflections ($-2 \le h \le 17$, $-10 \le k \le 10$, $-28 \le l \le 8$), of which 2474 were unique, which were used for the structure analysis. Direct methods for solving the phase problem (Sheldrick, 1990), refinement of the structure parameters by Least-Squares Methods (full-matrix minimization of $(|F_o|^2 - |F_o|^2)^2$ weighting scheme: $w = 1/\sigma^2$ (F) according to the counting statistics, 185 parameters, coordinates of the H atoms were got by geometrical considerations, S = 0.85, R = 0.076 ($I > 2\sigma(I)$), R_W = 0.199 (all data), 10 largest peaks in the difference map. All calculations were made by a microVAX II with the SHELXTL-PLUS, SHELXS and SHELXL programs (Sheldrick, 1990,1993).[8] The results are given in the figures and the tables, which are available as Supplementary Material.[8]

3-Methyl-5,7,8-triacetoxy-2-aza-anthraquinone-(9,10) (2b): 270 mg (1 mmol) of **2** in a solution of 30 mL acetic anhydride and 3 mL pyridine are heated 30 min to 100 °C . The dark red mixture clears to a yellow brown solution. After cooling yellow crystals separate, and they are collected. Together with a further crop from the filtrate the yield amounts 330 mg of **2b** (83%), m.p. 210-211 °C (ethyl acetate), Rf: = 0.9 (solv. 1). Anal. found: C, 60.29; H, 3.82; N, 3.69. Calcd. for $C_{20}H_{15}NO_8$ (397.3): C, 60.46; H, 3.80; N, 3.53. MS: m/e found 397.2, calcd. 397.07870 (M^+). ^{1}H NMR ($CDCl_3$): 9.31 (s, H-1), 7.80 (s, H-4), 7.41 (s, H-6), 2.76 (s, CH_3-3), 2.49, 2.47, 2.36 (3 s, CH_3COO).

3-Methyl-5,6,8-trihydroxy-2-aza-anthraquinone-(9,10) (1): The procedure is the same as described above for **2** . The crystals look reddish brown after sublimation in vacuo (230-260 °C, 67 Pa), or dark violet if recrystallized from $CHCl_3$ / EtOAc. The yield amounts 48% of the theory; m. p. >300 °C (dec.) [ref.[1] m. p. >320 °C (dec.)]. Rf = 0.6 (solv. 2). MS: m/e found 271.0472 (M^+), calcd. 271.0481, . Anal. found: C, 61.80; H, 3.65; N, 5.20. Calcd. for $C_{14}H_9NO_7$ (271.2): C, 62.00; H, 3.35; N, 5.16.- IR: 3600, 2905 (broad), 1623 (CO), 1585 (CO), 1456, 1412, 1309 (max.), 1118, 928, 763. ^{1}H NMR: 2.72 (s, CH_3), 6.74 (s, H-7), 7.79 (s, H-4), 9.29 (s, H-1), 11.80 (broad), 12.80 (broad), 13.33 (s, H-6). ^{13}C NMR: 24.7 (CH_3), 105.0, 110.3, 113.0, 117.6, 124.2, 138.3, 148.0, 149.7, 157.3, 160.5, 164.9, 183.0 (CO), 186.0 (CO).

3-Methyl-5,6,8-triacetoxy-2-aza-anthraquinone-(9,10) (1b): 1 mmol **1** is acylated in the same manner as described for **2**. The yield amounts 0.35 g (88%), purification by column chromatography (v/v: $CHCl_3$ / Me_2CO = 9/1) or recrystallization, yellow crystals, m. p. 217-219 °C (ethyl acetate), Rf: 0.9 (solv. 1). Anal. found: C, 60.31; H, 3.90; N 3.80. Calcd. for $C_{20}H_{15}NO_8$ (397.3): C, 60.46; H, 3.80; N, 3.53. MS: m/e found 397.2, calcd. 397.07889 (M^+). ^{1}H NMR ($CDCl_3$): 9.32 (s, H-1), 7.76 (s, H-4), 7.42 (s, H-6), 2.74 (s, CH_3-3), 2.48, 2.47, 2.35 (3 s, CH_3COO). ^{13}C NMR ($CDCl_3$): 181.05 (CO), 179.99 (CO), 168.92, 167.96, 166.98, 165.56, 149.38, 148.69, 148.53, 140.41, 138.35, 127.05, 125.65, 123.99, 123.09, 117.79, 77.63, 76.99, 76.35, 25.16 (CH_3).

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Crystallographic Data Centre, University Chemical Laboratory, Lensfield Road, Cambridge CB2 IEW. Any request should be accompanied by the full literature citation for this communication.

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