SECONDARY METABOLITES BY CHEMICAL SCREENING

I. CALCIUM 3-HYDROXYQUINOLINE-2-CARBOXYLATE FROM A STREPTOMYCES

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Streptomyces griseoflavus subsp. (Gö 3592) was investigated by chemical screening methods. The mycelium contained gilvocarcin V (**1**). The culture filtrate contained the calcium salt of 3-hydroxyquinoline-2-carboxylic acid (**2**) confirmed by spectroscopic methods and by a 5-step partial synthesis of the free acid (**3**).

Chemical screening is an efficient method for detecting metabolites in crude extracts of microorganisms[1~3]. This method is particularly attractive for identifying secondary metabolites with very different types of biological activities.

A detailed understanding of the structure of microbial metabolites is important for several reasons. For example, metabolites could elicit selective biological responses, could belong to novel types of structural classes, could be useful as synthetic intermediates, and above all provide a detailed understanding of the metabolic pathways used by an organism.

From *Streptomyces griseoflavus* (Gö 3592) we isolated two substances which on silica gel TLC plates (Table 1) exhibited a strong green-yellow fluorescence in UV light (366 nm). The first was extracted from mycelium with acetone and identified as gilvocarcin V (**1**)[4~8], a remarkable antitumor antibiotic, whose *in vitro* activity is promoted by light[9]. The second was obtained from the culture filtrate by adsorption on Amberlite XAD-2 and elution with methanol. In this paper we describe the latter compound, the calcium salt (**2**) of 3-hydroxyquinoline-2-carboxylic acid (**3**).

Isolation and Characterization

The strain Gö 3592 was cultivated in a 120-liter fermentor, using soybean meal and mannitol as culture medium. After incubation for 93 hours at 28°C the mycelium was separated by filtration. From this gilvocarcin V (**1**) was isolated as given in the experimental section, yielding 20 mg/liter pure yellow antibiotic. The culture filtrate was adjusted to pH 6.5 and directly applied on Amberlite XAD-2. The fluorescent compound was eluted with methanol and further purified by chromatography on silica gel with ethyl acetate - methanol - water (6 : 2 : 1) and finally on Sephadex LH-20 with methanol.

Table 1. Rf values (TLC, silica gel) of gilvocarcin V (**1**) and the quinoline compounds **2** to **10**.

Solvent system	**1**	**2/3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
$CHCl_3$ - MeOH (9 : 1)	0.38	0.03	0.80	0.50	nt	nt	nt	nt	nt
$CHCl_3$ - acetone (3 : 1)	nt	nt	nt	nt	0.36	0.80	0.87	0.94	0
EtOAc - MeOH - H_2O (6 : 2 : 1)	nt	0.62	0.76	0.68	0.48	0.72	0.82	0.85	0.42
Toluene - acetone (9 : 1)	nt	0	0.52	0.18	0	0.18	0.49	0.55	0

nt: Not tested.

The colorless compound obtained (mp 170°C) is insoluble in diethyl ether, chloroform or acetone, sparingly soluble in methanol or water, soluble in dimethyl sulfoxide, aqueous bases and concentrated acids.

The elemental analysis of the optically inactive metabolite resulted in a non-combustible residue, which by flame photometric methods and atomic absorption was shown to be Ca^{2+} (half equivalent), while the presence of other metal ions (Na^+, K^+ and Mg^{2+}) was negligible. The calcium-free compound can be obtained by dissolving the salt in acidic methanol, followed by chromatography on Sephadex LH-20 with methanol or, more easily, by crystallization from methanol in the presence of a few drops

Fig. 1. IR spectra of calcium 3-hydroxyquinoline-2-carboxylate (**2**) and 3-hydroxyquinoline-2-carboxylic acid (**3**) in KBr.

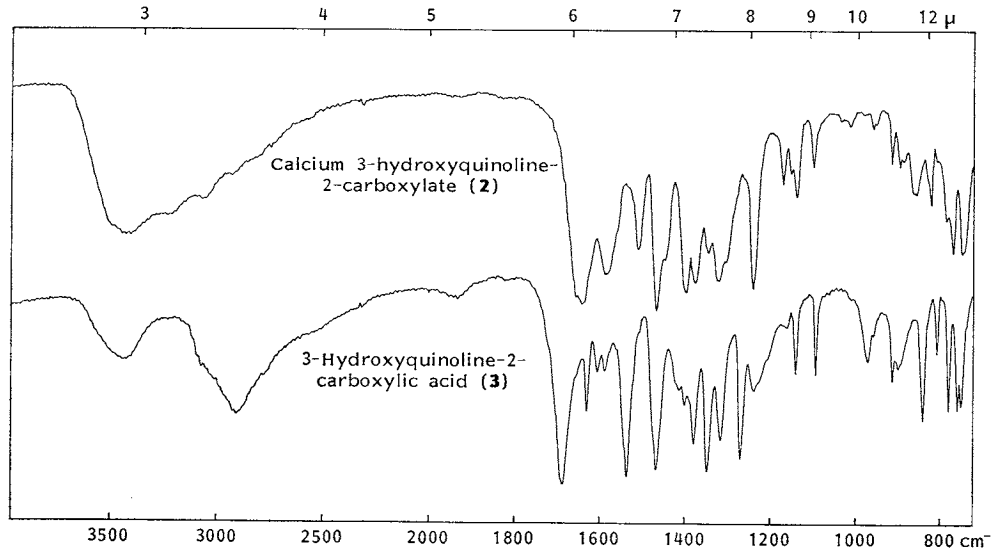
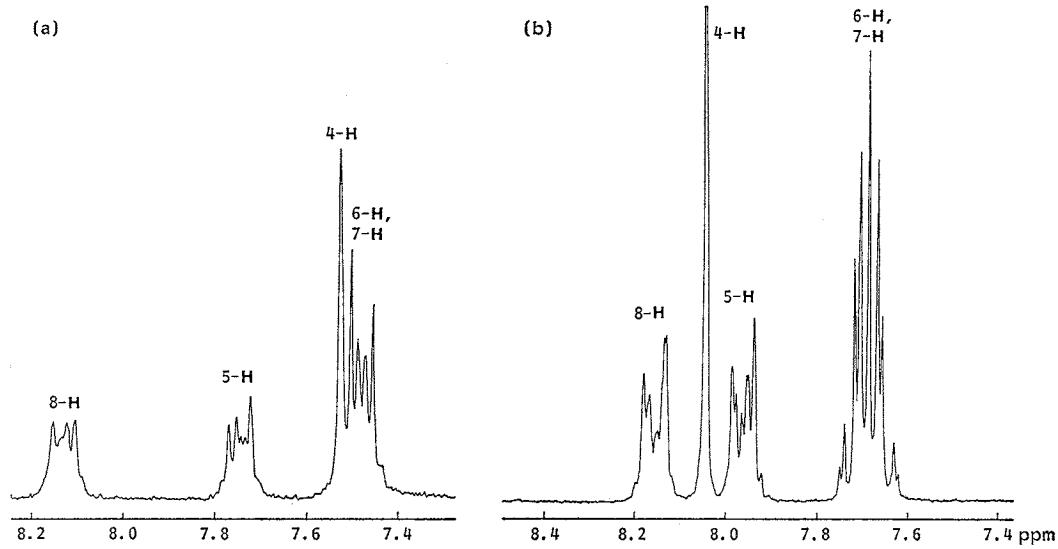
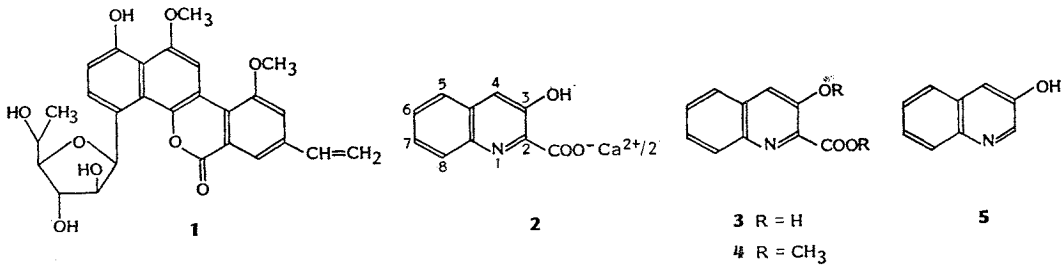


Fig. 2. ^1H NMR spectra of calcium 3-hydroxyquinoline-2-carboxylate (**2**) (a) and 3-hydroxyquinoline-2-carboxylic acid (**3**) (b) in DMSO-d_6 at 200 MHz.



Scheme 1.



of aqueous HCl, yielding light yellow needles (mp 196~198°C).

The UV spectra of the calcium salt (**2**) as well as the free acid (**3**) exhibit typical absorption bands at 352, 292, 228 and 217 nm, under neutral or basic conditions and a bathochromic shift in acid to 369, 328 and 237 nm. Differences between both compounds are especially demonstrated in the OH and CO region of the IR spectra (Fig. 1). A carbonyl absorption at 1643 cm^{-1} is observed at 1688 cm^{-1} in the spectrum of the free acid, indicating an aromatic carboxylic acid. On the basis of high resolution mass spectroscopy of both compounds the free acid was assigned the molecular formula $C_{10}H_7NO_3$ (*m/z* 189) in accordance with the elemental analysis.

Table 2. Physico-chemical properties of the calcium salt (**2**) and the free acid (**3**).

		Calcium salt (2)	Free acid (3)
Molecular formula		$C_{20}H_{18}N_2O_6Ca$	$C_{10}H_7NO_3$
MW (*m/z*)		416	189
MP (°C)		170	196~198
IR (KBr) cm^{-1}		1643, 1582	1688
^1H NMR	4-H	7.53 s	8.05 s
	5-H	7.74 m	7.96 m
	6-H/7-H	7.48 m	7.69 m
	8-H	8.13 m	8.15 m
^{13}C NMR	C-2	142.2 s	138.0 s
(proton	C-3	155.3 s	152.4 s
decoupled	C-4	117.5 d	122.4 d
and APT)	C-4a	131.0 s	130.8 s
	C-5	125.6 d	126.6 d
	C-6	125.4 d	126.1 d
	C-7	127.3 d	128.8 d
	C-8	129.7 d	128.5 d
	C-8a	140.7 s	137.9 s
	C=O	170.2 s	166.8 s

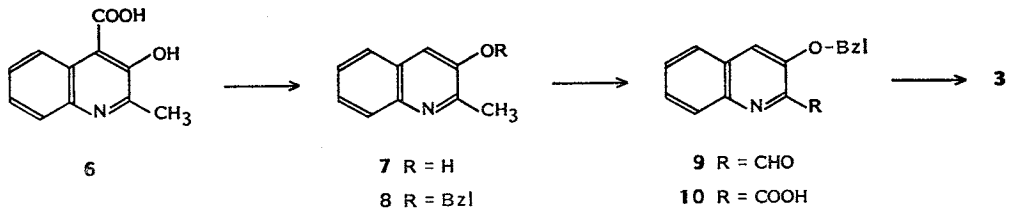
The ^1H NMR spectra of both compounds (Figs. 2a and 2b) reveal signals for five aromatic hydrogens, from which a singlet at δ 7.53 of the calcium salt is striking because of its shift to δ 8.05 in the free acid. The ^{13}C NMR spectrum of the former include the anticipated signals for ten carbon atoms (Table 2), exclusively in the sp^2 region (5 CH, 4 C, 1 CO). The observed data argued the presence of a quinoline or isoquinoline moiety in the novel metabolite, which is substituted by a hydroxy and a carboxy group.

This assumption was confirmed by the preparation of a permethylated derivative, which exhibited the expected data of a methyl ether methyl ester (**4**).

Decarboxylation

The structural nucleus as well as the type of substitution could not be deduced from spectroscopic data, because of the complicated coupling-systems in such heteroaromatic compounds and the small differences between isomers. However, the structure of the free acid was determined by thermal decarboxylation of the free acid in a xylol suspension at 180°C. The phenolic compound formed shows a molecular ion (electron impact mass spectrum (EI-MS)) at *m/z* 145, indicating the presence of the desired hydroxyquinoline or -isoquinoline. The UV spectrum with maxima at 331/324 nm respectively at 344 or 353 nm in acidic or basic MeOH, is also characteristic of 3-hydroxyquinoline (**5**) as are the

Scheme 2.



6

7 R = H
8 R = Bzl

9 R = CHO
10 R = COOH

Bzl = Benzyl

short wavelength maxima at 277 and 232 nm[10]. The ^{1}H NMR spectrum of **5** is in accordance with the proposed structure, especially a doublet at δ 8.61 demonstrates a free 2-position of the quinoline nucleus, which must have been substituted in the parent acid. Thus the only possible structure for the free acid and consequently for its calcium salt is given back by formula **2** and **3**.

Partial Synthesis

The structure was confirmed by a short five step synthesis, which additionally allowed the preparation of larger amounts of **3**. The starting material was 3-hydroxy-2-methylquinoline-4-carboxylic acid (**6**)[11], which was decarboxylated[12] to yield 3-hydroxy-2-methylquinoline (**7**). The hydroxy group was protected by benzyl chloride to give **8**[13] and then oxidized in two steps first with SeO_2 yielding **9**[13] and then with $AgNO_3$ yielding **10**. Finally the protecting group was cleaved by treatment with conc HCl to give **3** in a 48% yield overall.

Results and Discussion

The calcium salt (**2**) of 3-hydroxyquinoline-2-carboxylic acid has not previously been isolated from microorganisms; however, the free acid (**3**) was published as a saponification-product from the cytostatic peptide antibiotic cinropeptin[14]. Obviously **3** possesses an affinity for calcium ions and, therefore, is obtained from the culture broth only as the calcium salt. A reasonable explanation would be that **2** is at least partially a metal complex. This assertion is supported by an UV-spectroscopic experiment: In alkaline solution addition of calcium ions causes a bathochromic shift of the long wavelength maximum 352 nm while the maximum at 287 nm disappears simultaneously. However the displacement of the maximum depends on the quantity of the calcium added and reaches a limit at 375 nm caused by 5 equiv of calcium.

The novel metabolite **2** is related to a number of biologically active molecules. The 1-, 6- and 7-substituted 4-hydroxyquinoline-3-carboxylic acids are known as antibacterial drugs[15] or inhibitors of dehydrogenase enzymes[16]. In addition, 3-hydroxy-6-methoxyquinoline-2-carboxylic acid as well as **3** itself are part of the cytostatic peptide antibiotics BBM-928[17] and cinropeptin[14]. **2** exhibit no cytostatic activity and no effect against bacteria and fungi in the agar diffusion test up to concentrations of 100 mg/ml. Against Herpes simplex viruses growth stimulation at a concentration of 0.1 mg/ml was observed. Further tests for pharmacological effects of **2** and **3** are under investigation.

Experimental

General

Melting points were determined on a Reichert hot stage microscope and are uncorrected. IR spectra in pressed KBr disks were recorded on a Perkin-Elmer Model 297 spectrometer, UV spectra

using a Zeiss DMR 21 spectrophotometer. The NMR spectra were determined with a Varian XL-100, XL-200 or FT 80 respectively. Chemical shifts are expressed in δ values (ppm) with TMS as an internal standard. The mass spectra were taken by a Varian MAT 311 A (EI).

Analyticals

Thin-layer chromatography (TLC) was carried out on Silica gel plates (Merck 60 F_{254}), preparative TLC on silica gel (Macherey-Nagel, P UV 254, plates 20×40 or 20×20 cm, layer 2 mm).

Hakomori-reagent[18,19]: 50 mg NaH in 1 ml of DMSO were heated 15 minutes to 90°C and used after cooling immediately.

Fermentation and Isolation

Four liters of a sterile medium of soybean meal 2% and mannitol 2%, pH 7, in 24 Erlenmeyer flasks were inoculated with the strain Gö 3592 and shaken 48 hours at 28°C. This culture was used as inoculum for a 120-liter fermentation (93 hours, 100 rpm, aeration 150 liters/hour) at 28°C. The culture broth was adjusted to pH 6.5 and then filtered after addition of Celite. The mycelium was extracted twice with 40 liters of acetone and the yellow solution was evaporated under reduced pressure. The aqueous residue was extracted twice with 25 liters of chloroform and the combined organic layers were evaporated to dryness. The residue was suspended in 1.5 liters of MeOH and the sparingly soluble yellow substance filtered to yield 1.8 g of nearly pure gilvocarcin V (**1**)[4~8]. Additional **1** was precipitated by concentrating and cooling of the MeOH-solution. The culture filtrate was adsorbed at 11 kg Amberlite XAD-2, the fluorescent compound eluted with 40 liters of MeOH and the solution evaporated to dryness under reduced pressure. The residue was extracted twice with 750 ml of MeOH, the concentrated extract was chromatographed on silica gel (column, 11×20 cm) first with EtOAc - MeOH (7:3) and then EtOAc - MeOH - H_2O (6:2:1). **2** was eluted as a broad, green fluorescent area (6.9 g). The crude product was suspended in 200 ml of EtOAc, filtered and the residue (5 g) washed with 50 ml of cold MeOH. In this way 2.9 g of **2** were obtained of 90% purity and used for derivatization reactions. To yield pure **2** for elemental analysis and spectra, the enriched product was chromatographed on silica gel (preparative TLC, 20×40 cm) with EtOAc - MeOH - H_2O (6:2:1) and eluted with MeOH, followed by a final gel filtration on Sephadex LH-20 with MeOH. MP 170°C; Rf see Table 1; IR (KBr) cm^{-1} 3430, 1643, 1582; UV λ_{max}^{MeOH} nm (ε) 352 (2,100), 291 (1,500), 228 (16,100), 217 (16,300); $\lambda_{max}^{MeOH-NaOH}$ nm (ε) 353 (2,100), 286 (1,800), 228 (14,500); $\lambda_{max}^{MeOH-HCl}$ nm (ε) 369 (2,200), 328 (2,400), 237 (11,000); emission (DMF, λ_{Exc}=360 nm): λ_{max}=488 nm, $[\phi]_{Em}$=0.29; ^1H and ^{13}C NMR see Table 2; EI-MS (70 eV) *m/z* (abundant) 189 (61%, M^+, high resolution calcd for $C_{10}H_7NO_3$ and found: 189.04259), 171 (15%, M−H_2O), 145 (71%, M−CO_2), 143 (100%, M−H_2O−CO), 117 (32%), 115 (92%), 89 (37%).

Anal Calcd for $(C_{10}H_6NO_3)_2Ca$: C 57.69, H 2.90, N 6.72, Ca 9.62.
Found: C 57.69, H 2.90, N 6.74, Ca 8.39.

3-Hydroxyquinoline-2-carboxylic Acid (**3**)

2 was dissolved in MeOH with addition of some drops of 2 N HCl and chromatographed on Sephadex LH-20 with MeOH to yield **3** as a light yellow substance: MP 196~198°C (literature 195~197°C)[14]; Rf see Table 1; IR (KBr) cm^{-1} 3430, 2900, 1688; ^1H and ^{13}C NMR see Table 2; EI-MS indentical with **2**.

Anal Calcd for $C_{10}H_7NO_3$: C 63.48, H 3.72, N 7.40.
Found: C 63.55, H 3.92, N 7.33.

Methyl 3-Methoxyquinoline-2-carboxylate (**4**)

To a solution of 20 mg **2** in 4 ml DMSO at room temp 0.5 ml methyl iodide and 1 ml of Hakomori-reagent was added. After 30 minutes the reaction mixture was poured into 150 ml water, the aqueous solution extracted twice with chloroform, the organic layer washed with water and evaporated under reduced pressure. The residue was chromatographed on Sephadex LH-20 with MeOH to yield 20 mg (87%) **4**: MP 78~80°C; Rf see Table 1; IR (KBr) cm^{-1} 2940, 1738, 1602; UV λ_{max}^{MeOH} nm (ε) 333 (3,800), 285 (3,000), 236 (30,900); ^1H NMR (200 MHz, DMSO-d_6) δ 3.94 (s, OCH_3), 3.98 (s, OCH_3), 7.69 (m, 6-H and 7-H), 8.00 (m, 5-H and 8-H), 8.05 (4-H); EI-MS (70 eV) *m/z* (abundant) 217 (22%,

M^+, high resolution calcd for $C_{12}H_{11}NO_3$ and found: 217.07389), 202 (5%, M−CH_3), 186 (5%, M−OCH_3), 158 (100%, M−$COOCH_3$), 128 (85%).

3-Hydroxyquinoline (**5**)

A suspension of 30 mg calcium salt **2** in 10 ml *o*-xylol was heated 3 hours to 180°C in a closed sovirel flask. The solvent was then evaporated under reduced pressure and the brown colored residue chromatographed on silica gel (preparative TLC, 20×20 cm) with $CHCl_3$ - MeOH (9:1). The blue fluorescent (366 nm, UV light) zone was eluted with $CHCl_3$ - MeOH (4:1) and the solvent evaporated to give 14 mg (61%) **5** as a white solid: MP>150°C (dec); Rf see Table 1; IR (KBr) cm^{-1} 3420, 1600; UV λ_{max}^{MeOH} nm (ε) 331 (2,700), 324 (2,700), 277 (1,600), 232 (16,700); $\lambda_{max}^{MeOH-HCl}$ nm (ε) 344 (4,000), 314 (2,500), 238 (13,000), 225 (14,200); $\lambda_{max}^{MeOH-NaOH}$ nm (ε) 352 (3,700), 243 (23,200); ^1H NMR (80 MHz, DMSO-d_6) δ 7.53 (m, 4-H, 6-H and 7-H), 7.82 (m, 5-H), 7.92 (m, 8-H), 8.61 (d, 2-H); EI-MS (70 eV) *m/z* (abundant) 145 (100%, M^+, high resolution calcd for C_9H_7NO and found: 145.05276), 117 (5%, M−CO), 116 (6%, M−CHO), 90 (13%), 89 (12%).

3-Hydroxy-2-methylquinoline (**7**)[13]

A suspension of 1 g (4.9 mmol) 3-hydroxy-2-methylquinoline-4-carboxylic acid in 25 ml diethyl succinate was heated for 15 minutes to 230°C. After cooling to room temp **7** crystallized from the dark brown, blue fluorescent solution. The crystallization was finished in an ice bath, the brown colored precipitate filtered and washed with cold *n*-pentane to yield 711 mg (91%) **7**. A small amount was chromatographed on silica gel (preparative TLC, 20×20 cm) with $CHCl_3$ - MeOH (9:1) to give a white solid used for chemical and spectroscopic characterization: MP>200°C (dec); Rf see Table 1; IR (KBr) cm^{-1} 3420 (weak), 1600; UV λ_{max}^{MeOH} nm (ε) 328 (5,300), 318 (sh, 4,900), 269 (2,300), 233 (23,300); $\lambda_{max}^{MeOH-NaOH}$ nm (ε) 350 (6,500), 272 (4,600), 243 (30,300); $\lambda_{max}^{MeOH-HCl}$ nm (ε) 339 (7,700), 246 (19,100); ^1H NMR (100 MHz, DMSO-d_6) δ 2.56 (s, CH_3), 7.42 (m, 4-H, 6-H and 7-H), 7.75 (m, 5-H and 8-H); EI-MS (70 eV) *m/z* (abundant) 159 (100%, M^+, high resolution calcd for $C_{10}H_9NO$ and found: 159.06841), 130 (64%, M−CHO), 103 (12%, M−CHO−HCN).

3-Benzyloxy-2-methylquinoline (**8**)

To a solution of 640 mg (4 mmol) **7** in 6 ml 0.4 M KOH in ethanol 0.8 ml of benzyl chloride were added and the mixture was refluxed. After 2 hours 5 ml of ethanolic KOH were added and heated for one more hour. Then the cooled solution was filtered, the residue washed with ethanol and the solution evaporated under reduced pressure. The oily residue congeals after some hours to a brown solid. Yielding 905 mg (90%) **8**. A small amount of the crude **8** was chromatographed on silica gel (preparative TLC, 20×20 cm) with $CHCl_3$ - MeOH (95:5) to give a product, which crystallized as white long needles: MP 65°C; Rf see Table 1; IR (KBr) cm^{-1} 1610; UV λ_{max} (MeOH and MeOH - NaOH) nm (ε) 325 (5,000), 314 (4,500), 237 (32,000), 235 (32,200); $\lambda_{max}^{MeOH-HCl}$ nm (ε) 335 (7,600), 247 (26,000); ^1H NMR (100 MHz, $CDCl_3$) δ 2.74 (s, CH_3), 5.20 (s, OCH_2), 7.50 (m, 9-H), 8.00 (m, 8-H); EI-MS (70 eV) *m/z* (abundant) 249 (16%, M^+, high resolution calcd for $C_{17}H_{15}NO$ and found: 249.11536), 91 (100%).

3-Benzyloxy-2-formylquinoline (**9**)

To a stirred solution of 0.33 g (3 mmol) SeO_2 in 4 ml dioxane and 0.25 ml of H_2O a solution of 0.75 g (3 mmol) **8** in dioxane was added dropwise at room temp. The mixture was refluxed for 4 hours, during which time the white precipitate formed at the beginning of the reaction dissolved and the solution turned red. The reaction mixture was evaporated and the dark red residue chromatographed on silica gel with *n*-pentane - ether (1:1) to yield 0.64 g (81%) **9** as a light yellow solid: MP 121~123°C; Rf see Table 1; IR (KBr) cm^{-1} 1712, 1597; UV λ_{max} (MeOH and MeOH - NaOH) nm (ε) 328 (4,400), 318 (4,300), 233 (36,400); $\lambda_{max}^{MeOH-HCl}$ nm (ε) 341 (6,800), 246 (25,200), 226 (25,700); ^1H NMR (80 MHz, $CDCl_3$) δ 5.28 (s, CH_2O), 7.48 (m, 9-H), 8.13 (m, 8-H), 10.48 (s, CHO); EI-MS (70 eV) *m/z* (abundant) 263 (4%, M^+, high resolution calcd for $C_{17}H_{13}NO_2$ and found: 263.09462), 235 (4%, M−CO), 234 (3%), 91 (100%).

3-Benzyloxyquinoline-2-carboxylic Acid (**10**)

A solution of 1.82 g (10 mmol) $AgNO_3$ in 50 ml H_2O was poured into 50 ml of a 0.04 M NaOH.

0.9 g (3.4 mmol) **9**, dissolved in 18 ml THF was added dropwise to that suspension. The mixture was stirred 2 hours at 50°C, filtered, washed with hot water and the solvent THF evapoarted under reduced pressure. The remaining aqueous solution was adjusted to pH 4~5 with 2 M HCl and extracted with ether, yielding 0.785 g (82%) **10** as a white solid: MP 144°C; Rf see Table 1; IR (KBr) cm^{-1} 1728, 1603; UV λ_{max} (MeOH and MeOH - HCl) nm (ε) 328 (4,400), 237 (34,000); $\lambda_{max}^{MeOH-NaOH}$ nm (ε) 331 (3,200), 286 (2,400), 235 (32,600); ^1H NMR (100 MHz, D_2O - NaOD) δ 7.31 (m, 9-H), 7.75 (d, 8-H), 4.91 (OCH_2, interferes with H_2O-signal); EI-MS (70 eV) *m/z* (abundant) 279 (10%, M^+, high resolution calcd for $C_{17}H_{13}NO_3$ and found: 279.08954), 91 (100%).

3-Hydroxyquinoline-2-carboxylic Acid (3)

50 mg **10** were stirred in 10 ml of conc HCl 24 hours at room temp. The mixture was diluted with H_2O to 40 ml and the free acid purified by ion exchange chromatography (Dowex 1-X2/OH^-, 6 M acetic acid as eluant). After evaporation of acetic acid 30 mg (89%) of a light yellow solid were obtained, which was shown to be identical with the above isolated **3** in all physico-chemical properties.

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