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Original article

Synthesis and QSAR of substituted 3-hydroxyanthranilic acid derivatives as inhibitors of 3-hydroxyanthranilic acid dioxygenase (3-HAO)

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Abstract – Novel 4,5-, 4,6-disubstituted and 4,5,6-trisubstituted 3-hydroxyanthranilic acid derivatives were synthesized and their ability to reduce the production of the excitotoxin quinolinic acid (QUIN) by inhibition of brain 3-hydroxyanthranilic acid dioxygenase (3-HAO) was subsequently investigated. The potency of the compounds to inhibit 3-HAO was assayed in rat brain homogenate, while chemical stability of certain compounds was studied by HPLC. The data were used to generate quantitative structure-activity relationship (QSAR) models for potency of 3-HAO inhibition and compound stability. Compounds with longer half-lives were obtained when the difference between the HOMO and LUMO was increased, while electron withdrawing groups in the 4- and 5-positions increased the potency of 3-HAO inhibition. Selected compounds that showed high potency in vitro were also found to be efficacious inhibitors in vivo after cerebral administration in rats.

3-HAO / QSAR / hydroxyanthranilic acid / enzyme inhibitors / neuroprotection

1. Introduction

The cytosolic, non-haem ferrous (Fe^{2+}) enzyme 3-hydroxyanthranilic acid dioxygenase (3-HAO; EC 1.13.11.6) plays an important role in the metabolic transformation of L-tryptophan to nicotinamide in the kynurenine pathway (*figure 1*). The enzyme oxidizes and ring opens 3-hydroxyanthranilic acid (3-HANA) to produce α-amino-β-carboxymuconic acid ω-semialdehyde, which subsequently, spontaneously cyclizes and forms quinolinic acid (QUIN) [1]. 3-HAO seems mainly to be localized to hepatic tissue, but it has also been demonstrated that the enzyme is expressed both in the brain [2, 3] and in inflammatory cells [2, 4] where the production of QUIN has been shown to be stimulated by certain cytokines [5–9]. Biochemical and immunological analysis in the rat suggest that the brain and liver 3-HAO are identical proteins [10]. Moreover, there appears to be a high degree of homology (94% similarity) between the rat and the human 3-HAO amino acid sequence [11].

QUIN is an NMDA receptor agonist and an excitotoxin [12] that has been reported to cause convulsions [13] or neurodegeneration [14] after intracerebral administration. It has been shown that intrastriatal injections of QUIN in rats induce biochemical and morphological alterations similar to those observed in Huntington's disease [15, 16]. Furthermore, increases in neuronal Huntingtin immunoreactivity [17] have recently been reported to occur after administration of QUIN into the striatum of mice, providing further support for a role of QUIN in the pathogenesis of Huntington's disease. It is also possible that QUIN is involved in epilepsy. For example, the levels of QUIN are higher in epilepsy-prone mice in a transgenic model of epilepsy than in the corresponding wild type mice [18]. Moreover, increased QUIN levels, or enhanced kynurenine pathway activity, have been implicated in inflammatory diseases of the central nervous system. For instance, QUIN has been

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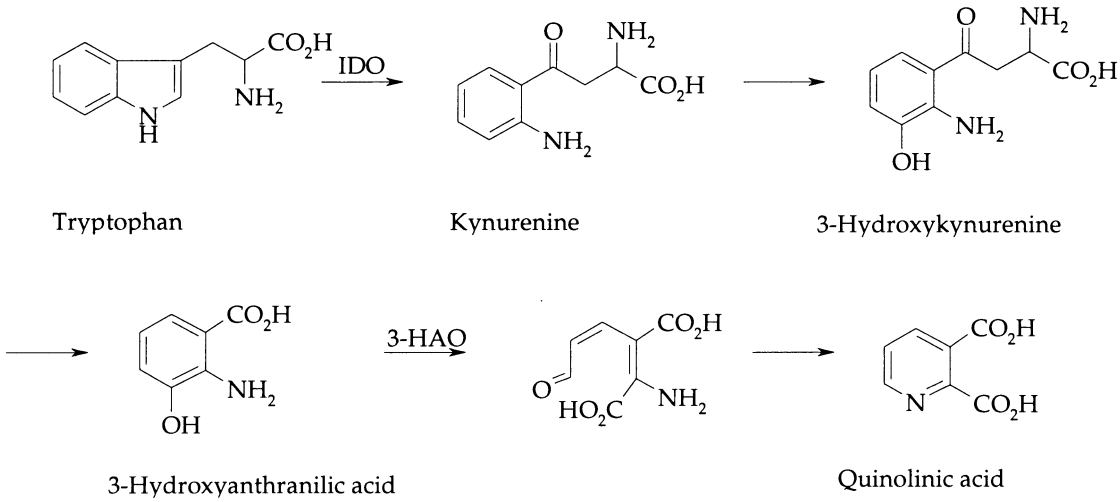


Figure 1. The biotransformation pathway for tryptophan yielding the excitotoxin QUIN.

associated with neurological dysfunction occurring following various viral or bacterial infections [19–23].

To elucidate the possible role of 3-HAO-mediated formation of QUIN in different neurological conditions, inhibitors of the enzyme are valuable tools, and may also find therapeutic utility. In this report, the synthesis of several analogues of 3-HANA and their structure-activity relationship as inhibitors of 3-HAO are presented. The main objective was to develop inhibitors with improved chemical and pharmacological properties compared to previously described 4-halogenated 3-HANA analogues [24]. A particular problem associated with 3-HANA analogues is the propensity of 3-HANA for auto-oxidation leading to its degradation and the formation of oxidized cinnabarinic acid [25, 26]. Hence, the stability of some of the synthesized compounds was studied by HPLC. The potency of the compounds to inhibit 3-HAO was assessed in rat brain tissue homogenates, while the ability of selected compounds to inhibit 3-HAO in vivo was also studied following intracerebral administration.

2. Chemistry

A series of 4,5-, 4,6-disubstituted and 4,5,6-trisubstituted 3-hydroxyanthranilic acids were prepared. The 4,5-dihalo substituted compounds were synthesized from the known 4-chloro- and 4-bromo-3-hydroxyanthranilic acids. The strong directing effect of the amino substituent gives access to derivatives **11** and **12** after reaction with bromine and chlorine, respectively. When the 5-position is substituted, the 4-substituted halogens can be obtained by a regio-selective reaction at the 4-position. Accordingly, compounds **8** and **10** were obtained by direct bromination of the 5-substituted-2-hydroxyanthranilic acids or esters (*figures 2* and *3*). To confirm the identity of the structures, 5-bromo-3-hydroxyanthranilic acid was chlorinated and shown to give an identical product as formed by bromination of 4-chloro-3-hydroxyanthranilic acid. The identity of the 4,5-dichloro and 4,5-dibromo derivatives was confirmed by comparison with the corresponding 4,6-disubstituted compounds and by synthesis either from the 4- or 5-halogenated 3-HANA leading to identical products (*figure 2*).

Compound **39** was synthesized by bromination of 5-hydroxy-2-methoxybenzoic acid, which after nitration and reduction of the nitro group gave the desired product

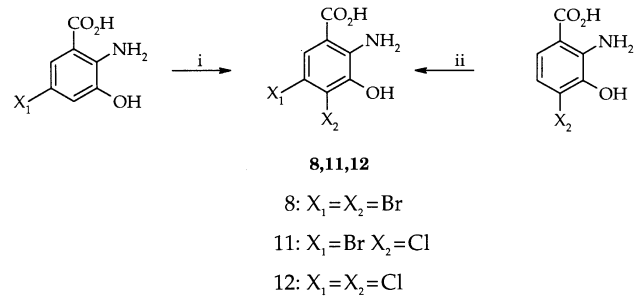
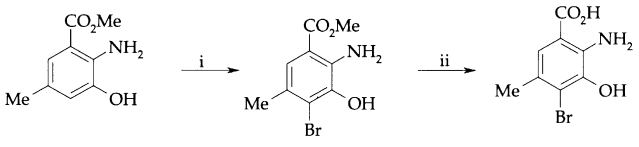


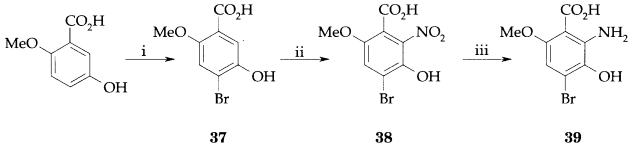
Figure 2. i) Br_2/HBr/HOAc, ii) Br_2/HBr/HOAc or Cl_2/HCl/HOAc.



9 **10**

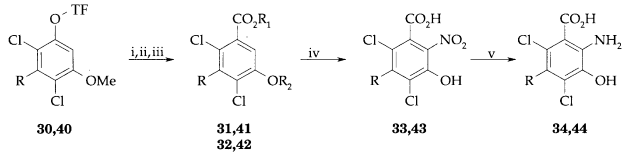
Figure 3. i) Br_2/HOAc, ii) KOH/EtOH.

(*figure 4*). Compounds **6**, **34** and **44** (*figures 5* and *6*) were prepared by nitration of suitably substituted mono or dihalogenated benzoic acid. The benzoic acids **32** and **42** were obtained by a palladium catalysed carbonylation of the corresponding triflates. The directing effect of the phenol gave the desired regio-selectivity for compounds **6** and **44**. The more easily oxidized compounds **17, 25** and **36** (*figure 7* and *8*) were obtained after halogenation of an intermediate without the strongly directing and activating amino-function. To make the tri-substituted compounds **21** and **29** and the 4,6-disubstituted **50** and **54** the carboxylic acid was introduced via ring closure of the corresponding isonitrosoacetanilid followed by oxidative ring-opening of the isatin-derivative (*figure 9*).



37 **38** **39**

Figure 4. i) Br_2/HOAc, ii) $NaNO_3$/$La(NO_3)_3$/HCl(aq.)/diethyl ether, iii) H_2/PtS_2/EtOH.



30,40 **31,41 32,42** **33,43** **34,44**

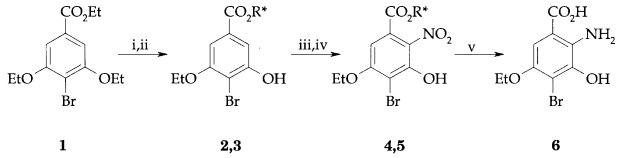
30: R=Me
40: R=H

31: R=R_1=R_2=Me
32: R=Me R_1=R_2=H
41: R=H R_1=R_2=Me
42: R=R_1=R_2=H

33: R=Me
43: R=H

34: R=Me
44: R=H

Figure 5. i) $Pd(OAc)_2$/CO/MeOH/dppp, ii) KOH/MeOH, iii) BBr_3 or HBr, iv) HNO_3/CH_3NO_2, v) Pd/H_2.

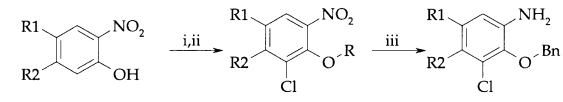


1 **2,3** **4,5** **6**

2: R* = H
3: R* = Me

4: R* = Me
5: R* = H

Figure 6. i) EtSNa/DMF, ii) HCl/MeOH, iii) HNO_3/CH_3NO_2/CH_2Cl_2, iv) KOH/EtOH, v) $SnCl_2xH_2O$/EtOH.



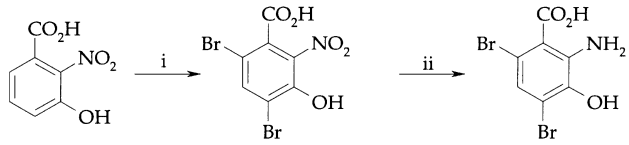
14,22 **15,23 16,24** **17,25**

14: R_1 = R_2 = -$(CH_2)_4$-
22: R_1 = R_2 = Me

15: R_1 = R_2 = -$(CH_2)_4$- R = H
16: R_1 = R_2 = -$(CH_2)_4$- R = Bn
23: R_1 = R_2 = Me R = H
24: R_1 = R_2 = Me R = Bn

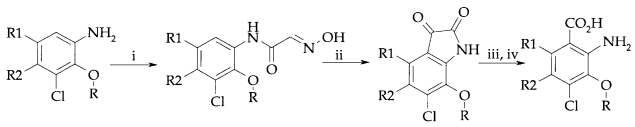
17: R_1 = R_2 = -$(CH_2)_4$-
25: R_1 = R_2 = Me

Figure 7. i) Cl_2/$CHCl_3$, ii) BnBr/K_2CO_3, iii) Cu(I)Cl/KBH_4.



35 **36**

Figure 8. i) Br_2/HOAc/NaOAc, ii) H_2/PtS_2.



18,26,47,51 **19,27,48,52** **20,28,49,53 21,29,50,54**

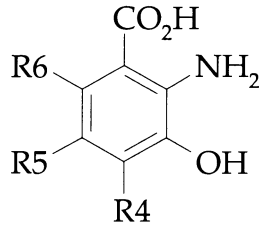
18: R_1 = R_2 = -$(CH_2)_4$- R = Bn
26: R_1 = R_2 = Me R = Bn
47: R_1 = Ph R_2 = H R = Me
51: R_1 = Me R_2 = H R = Me

19: R_1 = R_2 = -$(CH_2)_4$- R = Bn
27: R_1 = R_2 = Me R = Bn
48: R_1 = Ph R_2 = H R = Me
52: R_1 = Me R_2 = H R = Me

20: R_1 = R_2 = -$(CH_2)_4$- R = Bn
21: R_1 = R_2 = -$(CH_2)_4$- R = H
28: R_1 = R_2 = Me R = Bn
29: R_1 = R_2 = Me R = H
49: R_1 = Ph R_2 = H R = Me
50: R_1 = Ph R_2 = H R = H
53: R_1 = Me R_2 = H R = Me
54: R_1 = Me R_2 = H R = H

Figure 9. i) $CCl_3CH(OH)_2$/NH_2OH/DMF/H_2O, ii) H_2SO_4/Dioxane or polyphosphoric acid, iii) NaOH/H_2O_2, iv) H_2/Pd or BBr_3.

Table I. Inhibition of 3-HAO and chemical stability of 3-HANA derivatives.



Compound	R_4	R_5	R_6	IC_{50}[a] nM	Stability[b]
55 [24, 32]	Cl	H	H	6	38
56 [24]	Br	H	H	2	
57 [24]	F	H	H	24	
6	Br	OEt	H	56	
8	Br	Br	H	0.3	21
10	Br	Me	H	2.3	
11	Cl	Br	H	0.26	
12	Cl	Cl	H	0.3	
36	Br	H	Br	5.8	90
39	Br	H	MeO	120	0
44	Cl	H	Cl	10.1	89
50	Cl	H	Ph	11 000	79
21	Cl	$(CH_2)_2$	$(CH_2)_2$	200	
29	Cl	Me	Me	8.2	0[c]
34	Cl	Me	Cl	4.4	62
54	Cl	H	Me	7.8	53

[a]In vitro inhibition of 3-HAO in rat cortex. [b]Percent remaining after 24 h in a PBS buffer at pH 7.5 and 37 °C. [c]Percent remaining after 24 h in a PBS buffer at pH 7.5 and 50 °C.

3. QSAR

A quantitative structure-activity relationship was developed for the 16 compounds presented in *table I*. The σ of the R_1, R_2 and R_3 substituents, the π and MR for the R_4, R_5 and R_6 substituents were obtained from the literature [27]. The pKa values for the CO_2H, NH_2 and OH substituents and logP for the molecules were calculated using the Pallas software [28]. Semi-empirical calculations using AM1 (Spartan) [29] provided values for HOMO and LUMO energies, e-neg, hardness, heat of formation, polarizability, surface area, molecular volume and ovality [30] which were subsequently used as descriptors in the QSAR calculations.

4. Pharmacology

The compounds were tested for their ability to inhibit 3-HAO in homogenates of rat brain tissue according to the method of Foster et al. [18]. The production of radioactive QUIN was measured after addition of [1-^{14}C]3-HANA to determine inhibition of the enzyme. Test compound concentrations resulting in a 50% inhibition of the enzyme (IC_{50}) are reported.

Also, the ability of selected compounds to inhibit cerebral 3-HAO in vivo was studied following intracerebroventricular (i.c.v.) administration of the compounds in rats. The de novo formation of QUIN in hippocampal tissue after concomitant i.c.v. administration of the test compounds and QUIN was measured by GC/MS.

5. Results and discussion

A PLS analysis[#] (Codex, AP Scientific Service) of potency resulted in a four component model, accounting for 97% of the variance in pIC_{50} (cross-validated Q^2 = 0.85) (*figure 10*). All the components in the model were statistically significant according to cross-validation. The two most important components accounted for 86% of the variance. A plot of the PLS-weights for these components indicated that the most important factors influ-

[#] Descriptors used in the PLS analysis are available on the Internet on request

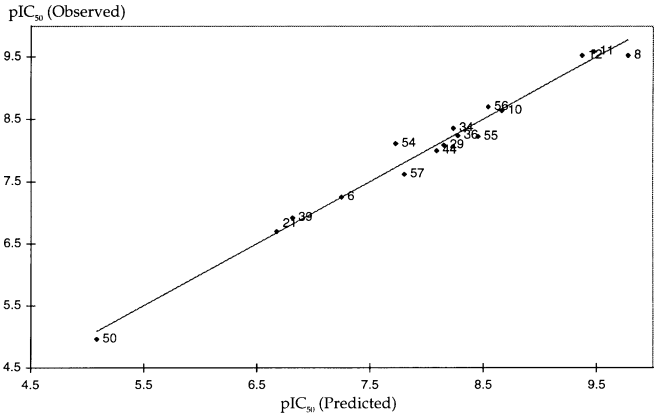


Figure 10. Predicted vs. observed IC_{50} values for 16 3-HAO inhibitors.

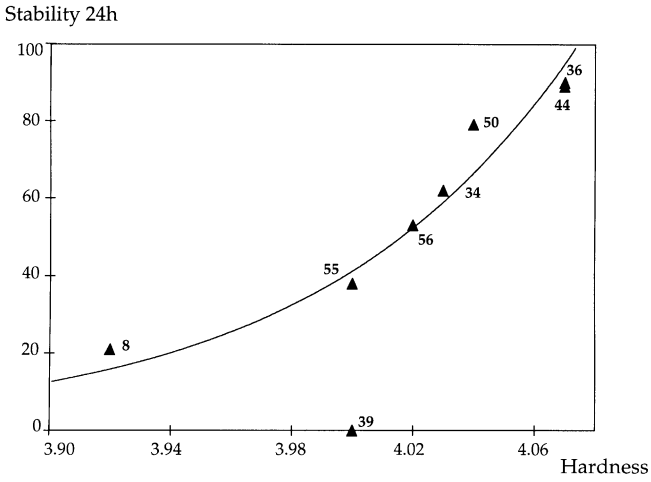


Figure 11. Plot of stability versus hardness.

encing the IC_{50} values are the size of the R_6 substituent, the lipophilicity of the R_5 substituent and the pKa of the phenol. This implies that small, electron-withdrawing groups at R_6 and lipophilic, electron-withdrawing groups at R_5 would increase the potency of the compounds.

Nine of the compounds were used to develop a QSAR model for chemical stability. The PLS analysis resulted in a two-component model, accounting for 94% of the variance in stability. The single most important factor for the model was the difference between the HOMO and LUMO energies (hardness) of the molecules (*figure 11*). The very labile compound **39**, having the strongly electron-donating 6-methoxy substituent could not be accounted for in the model. This may indicate a different mechanism for the degradation of this compound. The increased instability observed for compounds with a small difference in HOMO and LUMO-orbitals is in agreement with the observation that dimerization has been reported to be involved in the degradation of 4-bromo-3-hydroxyanthranilic acid [25, 26].

Several compounds that were found to be potent in vitro inhibitors of 3-HAO were also efficacious in vivo after intracerebral administration. There was a general agreement between the in vitro and in vivo ability of the 3-HANA analogues to inhibit 3-HAO (*tables I* and *II*). Hence, compounds which showed low nM IC_{50} values were able to almost totally inhibit de novo production of QUIN in vivo when given at doses that were equivalent to or higher than those of 3-HANA, while **21**, which showed a high nM IC_{50} value in vitro, displayed no in vivo activity. On the other hand, certain differences in potency observed in vitro were not reflected in vivo. This may, at least partially, depend on the inherent differences in chemical stability of the compounds.

For example, the low stability of the very potent compound **8** may have reduced its in vivo actions, but distribution and local metabolism in vivo could also contribute to the discrepancies seen.

Importantly, the finding that potency and stability depend on separate structural features present possibilities to further improve the characteristics of 3-HANA analogues as useful inhibitors of 3-HAO.

Table II. In vivo effects of 3-HAO inhibitors on 3-HANA-induced de novo production of QUIN after i.c.v. administration in rat.

Compound	Ratio dose[a] 3-HANA:compound	% Inhibition
55	1:1	96.9 ± 0.1
8	1:1	87.4 ± 2.0
36	1:0.3	67.2 ± 6.6
	1:1	81.9 ± 1.4
	1:30	95.2 ± 1.4
44	1:30	91.9 ± 1.9
21	1:30	0 ± 13
29	1:30	95.1 ± 1.2
34	1:0.3	69.6 ± 7.9
	1:30	93.9 ± 0.6

[a]3-HANA was given in a dose of 10 nmol i.c.v., while the different compounds were given in doses of either 3, 10 or 300 nmol i.c.v. at the same time. The cerebral levels of QUIN at 2 h after administration were determined in hippocampal tissue by GC/MS. Data are the mean ± SEM of 5–7 determinations.

6. Experimental protocols

6.1. Chemical methods

Melting points were determined on a Büchi SMP-20 apparatus. ^{1}H and ^{13}C NMR spectra were recorded at ambient temperature on a Varian Unity 400 or Varian Gemini 300 instrument. Chemical shifts are given in ppm from internal standards. For ^{1}H NMR and ^{13}C NMR spectra the internal references were tetramethylsilane (0.0 ppm), $CDCl_3$ (δ 7.26 or δ 77.0 ppm), CD_3OD (δ 3.38 or δ 49.3 ppm) or DMSO-d_6 (δ 2.49 or δ 39.5 ppm), respectively. Coupling constants are given in Hertz, and the splitting patterns are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet and br, broad. Mass spectra were obtained on an LKB 2091 (ELI, 70 eV or CI/CH_4) or on a Finnigan-MAT TSQ 70 (thermospray) spectrometer. Elemental analyses were performed by MIKRO KEMI AB, Uppsala, Sweden. The values were within ± 0.4% of theoretical if not otherwise indicated.

6.1.1. Ethyl 4-bromo-3,5-diethoxybenzoate **1**

4-Bromo-3,5-dihydroxybenzoic acid [31] (4.68 g, 20 mmol), potassium carbonate (8.28 g) and diethyl sulfate (7.84 mL) in dry acetone (50 mL) under nitrogen atmosphere were stirred at room temperature for 20 min and then refluxed for 8 h. The salts were filtered off and the solvent was evaporated, and the remainder was dissolved in diethyl ether. The organic layer was washed with water, sodium hydroxide (aq., 5%), ammonium hydroxide (conc.), hydrochloric acid, water and finally dried (Na_2SO_4). The organic phase was then evaporated to give the title compound (2.63 g, 51%). ^{1}H NMR ($CDCl_3$) δ 7.22 (s, 2H), 4.39 (q, *J* = 7.1 Hz, 2H) 4.18 (q, *J* = 7.0 Hz, 4H), 1.5 (t, *J* = 7.0 Hz, 6H), 1.41 (t, *J* = 7.1 Hz, 3H), ^{13}C NMR ($CDCl_3$) δ 166.8, 157.0, 130.8, 107.9, 106.8, 65.4, 61.6, 14.7, 14.4.

6.1.2. 4-Bromo-5-ethoxy-3-hydroxybenzoic acid **2**

A solution of **1** (1.02 g, 3.2 mmol) in *N*,*N*-dimethylformamide (5 mL) was added to sodium ethanethiolate (4.53 g, 51.2 mmol) in *N*,*N*-dimethylformamide (25 mL) under nitrogen atmosphere. The mixture was stirred at 100 °C for 8 h. After cooling, the mixture was poured into water. The aqueous phase was acidified by the addition of hydrochloric acid (1 M) and extracted with diethyl ether. The organic phase was dried (Na_2SO_4) and evaporated The remainder was purified by flash chromatography (SiO_2, toluene-acetic acid 5:1) to give the title compound (115 mg, 14%). ^{1}H NMR (DMSO-d_6) δ 7.13 (d, *J* = 1.8 Hz, 1H), 6.95 (d, *J* = 1.8 Hz, 1H) 4.08 (q, *J* = 7.0 Hz, 2H), 3.4 (br, 2H), 1.30 (t, *J* = 7.0Hz, 3H), ^{13}C NMR (DMSO-d_6) δ 167.5, 156.5, 155.8, 131.3, 109.7, 104.5, 104.3, 64.6, 14.6, MS (TSP) m/z: 263/261 (M + 1).

6.1.3. Methyl 4-bromo-5-ethoxy-3-hydroxybenzoate **3**

A solution of **2** (115 mg, 0.44 mmol) in dry methanol (10 mL) was saturated with hydrogen chloride at 0 °C and the mixture was stirred overnight at room temperature. The solution was evaporated and the remainder was dissolved in chloroform. The organic phase was washed with sodium bicarbonate (sat.), water, brine and then dried (Na_2SO_4) and evaporated to give the title compound (94 mg, 79%). ^{1}H NMR ($CDCl_3$) δ 7.35 (d, *J* = 1.8 Hz, 1H), 7.14 (d, *J* = 1.8 Hz, 1H) 5.85 (br, 1H), 4.17 (q, *J* = 7.0 Hz, 2H), 3.91 (s, 3H), 1.49 (t, *J* = 7.0 Hz, 3H), ^{13}C NMR ($CDCl_3$) δ 167.2, 156.5, 154.4, 130.8, 110.1, 106.0, 105.5, 65.4, 52.7, 14.7, MS (TSP) m/z: 277/275 (M + 1).

6.1.4. Methyl 4-bromo-5-ethoxy-3-hydroxy-2-nitrobenzoate **4**

A solution of **3** (89.7 mg, 0.326 mmol) in nitromethane (1 mL) and dichloromethane (1 mL) was stirred with nitric acid (90%, 15 μL) at 40 °C for 10 min. Ice was added, and the layers were separated. The aqueous layer was extracted with chloroform, and the combined organic layer was washed with water and evaporated. Purification of the remainder by flash chromatography (SiO_2, toluene-ethyl acetate 10:1) gave the title compound (37 mg, 35%). ^{1}H NMR ($CDCl_3$) δ 6.59 (s, 1H), 4.21 (q, *J* = 7.0 Hz, 2H) 3.90 (s, 3H), 1.47 (q, *J* = 7.0 Hz, 3H), ^{13}C NMR ($CDCl_3$) δ 167.1, 162.2, 154.4, 132.2, 126.9, 105.4, 103.3, 66.6, 53.9, 14.6, MS (TSP) m/z: 339/337 (M + NH_4).

6.1.5. 4-Bromo-5-ethoxy-3-hydroxy-2-nitrobenzoic acid **5**

Methyl 4-bromo-5-ethoxy-3-hydroxy-2-nitrobenzoate **4** (30.2 mg, 0.094 mmol) was dissolved in ethanol (1.2 mL) and potassium hydroxide (62 mg) in water (0.54 mL) was added. The mixture was stirred at 40 °C for 16 h and then cooled and acidified to pH 2 by the addition of hydrochloric acid. The remainder was triturated with water, filtered and dried to give the title compound (24.6 mg, 86%). ^{1}H NMR (CD_3OD) δ 6.82 (s, 1H), 4.74 (br, 2H) 4.00 (q, *J* = 7.0 Hz, 2H), 1.26 (t, *J* = 7.0 Hz, 3H), ^{13}C NMR (CD_3OD) δ 167.3, 159.7, 151.0, 135.3, 128.2, 106.9, 106.3, 67.4, 15.4, MS (EI, 70 eV) m/z: 307/305 (M +, 99/100).

6.1.6. 4-Bromo-5-ethoxy-3-hydroxyanthranilic acid **6**

A mixture of **5** (19.8 mg, 0.065 mmol) and $SnCl_2xH_2O$ (73 mg) in ethanol (2.0 mL) was heated at 70 °C for 5.5 h under nitrogen atmosphere. After cooling, water (2.0 mL) was added and the pH was adjusted to 7 by adding

sodium bicarbonate (aq., 5%). The mixture was extracted with ethyl acetate, and the organic phase was washed with brine and dried (Na_2SO_4). The organic phase was evaporated to give the title compound (8.4 mg, 47%). ^{1}H NMR (CD_3OD) δ 6.93 (s, 1H), 4.80 (br, 4H) 3.89 (q, J = 7.0 Hz, 2H), 1.30 (t, J = 7.0 Hz, 3H), ^{13}C NMR (CD_3OD) δ 171.7, 147.7, 145.1, 137.9, 109.5, 108.7, 107.2, 67.0, 15.7, MS (TSP) m/z: 278/276 (M + 1).

6.1.7. 5-Bromo-3-hydroxyanthranilic acid **7**

To 3-hydroxyanthranilic acid (5 g, 32.6 mmol) in acetic acid (450 mL) was bromine (10.4 g, 65.2 mmol) in acetic acid (50 mL) added drop-wise. The thick slurry was stirred for 1 h at room temperature and was then evaporated. The remainder was dissolved in methanol (60 mL) and water was added. The precipitate was filtered and dried to give the title compound (7.38 g, 98%), m.p.: 210–212 °C. ^{1}H NMR (DMSO-d_6) δ 10.20 (br, 1H), 7.30 (d, J = 2.4 Hz, 1H) 6.88 (d, J = 2.4 Hz, 1H), ^{13}C NMR (DMSO-d_6) δ 169.2, 146.4, 141.2, 123.2, 118.9, 110.9, 104.2, MS (EI, 70 eV) m/z: 233/231 (M +, 14/13).

6.1.8. 4,5-Dibromo-3-hydroxyanthranilic acid **8**

5-Bromo-3-hydroxyanthranilic acid **7** (7.38 g, 31.8 mmol) was dissolved in methanol (120 mL) and dichloromethane (250 mL) saturated with hydrogen bromide was added. More dichloromethane (300 mL) was added and the mixture was stirred for 48 h whereafter the solvent was evaporated. The residue (8.92 g) was dissolved in acetic acid (400 mL) and hydrobromic acid (48%, 100 mL) and bromine (5.5 g, 34.4 mmol) in acetic acid (50 mL) were added drop-wise for 20 min. The mixture was heated to 70 °C and additional bromine (5.50 g, 34.4 mmol) in acetic acid (50 mL) was added. After 14 h at 90 °C the solvent and excess reagent were evaporated. The remainder was crystallized from ethanol-water to give the title compound (8.45 g, 85%), m.p.: 223–223.5 °C. ^{1}H NMR (DMSO- d_6) δ 9.3 (br, 1H), 7.56 (s, 1H), 6.0 (br, 1H), 5.3 (br, 1H). ^{13}C NMR (DMSO-d_6) δ 168.9, 143.5, 142.5, 125.9, 117.9, 110.3, 107.2. MS (EI, 70 eV) m/z: 313/311/309 (M +, 51/100/50). Anal. for the hydrochloride $C_7H_5Br_2NO_3$xHCl (C, H, N).

6.1.9. Methyl 4-bromo-3-hydroxy-5-methylanthranilate **9**

To a solution of methyl 3-hydroxy-5-methylanthranilate hydrochloride (50 mg, 0.23 mmol) dissolved in acetic acid (2.5 mL) under argon atmosphere was bromine (36 mg, 0.23 mmol) in acetic acid (2.5 mL) added drop-wise. After 3 h at room temperature, additional bromine (18 mg, 0.11 mmol) in acetic acid (1 mL) was added. The solvent and excess reagent were evaporated, and the remainder was purified by repeated preparative thin layer chromatography (SiO_2, chloroform-methanol-ammonium hydroxide 300:10:1, chloroform-methanol 1 000:1) to give the title compound (9 mg, 15%). ^{1}H NMR (DMSO- d_6) δ 7.22 (s, 1H), 6.25 (br, 3H), 3.74 (s, 3H), 2.17 (s, 3H), MS (EI, 70 eV) m/z: 261/259 (M +, 85/86).

6.1.10. 4-Bromo-3-hydroxy-5-methylanthranilic acid **10**

Methyl 4-bromo-3-hydroxy-5-methylanthranilate **9** (14 mg, 0.05 mmol) was dissolved in ethanol (0.7 mL) and flushed with argon. Potassium hydroxide (aq, 0.35 mL, 0.05 mmol) was added and the reaction was stirred at 40 °C for 6 h. The mixture was acidified to pH 2 by the addition of hydrochloric acid (2 M). The solvent was removed and the remainder was triturated with ice-water. The crude product was purified by preparative HPLC (Lichrosorb C18) using methanol-ammonium hydroxide (0.05 M) (40:60) as the eluent to give the title compound (2 mg, 16%). ^{1}H NMR (DMSO-d_6) δ 7.23 (s, 1H), 3.35 (br, 2H), 2.13 (s, 2H), 2.05 (s, 3H), MS (EI, 70 eV) m/z: 247/245 (M +, 82/86). High resolution MS $C_8H_8BrNO_3$.

6.1.11. 5-Bromo-4-chloro-3-hydroxyanthranilic acid **11**

To 4-chloro-3-hydroxyanthranilic acid [23] (50 mg, 0.27 mmol) dissolved in acetic acid (2.0 mL) were hydrobromic acid (47%, 0.02 mL, 0.54 mmol) and then bromine (86 mg, 0.54 mmol) in acetic acid (3 mL) added, and the mixture was stirred at room temperature and under nitrogen atmosphere for 2 d. The solvent was evaporated and the remainder was crystallized from ethanol (40%), to give the title compound (37 mg, 51%), m.p.: 227–228 °C. ^{1}H NMR (CD_3OD) δ 7.72 (s, 1H), 5.0 (br, 4H), ^{13}C NMR (CD_3OD) δ 170.3, 143.5, 143.0, 127.2, 125.4, 111.0, 106.7. MS (TSP) m/z: 270/268/266 (M +, 28/100/80). Anal. $C_7H_5BrClNO_3$ (C, H, N, O).

6.1.12. 4,5-Dichloro-3-hydroxyanthranilic acid **12**

To a solution of 4-chloro-3-hydroxyanthranilic acid [32] (150 mg, 0.80 mmol) in acetic acid (12 mL) and under argon, were hydrochloric acid (12 M, 336 μL, 4.8 mmol) and then chlorine (1.76 mmol) in acetic acid (1.78 mL) added drop-wise. The reaction was stirred for 20 h and then the precipitated material was filtered. The crude material was recrystallized from ethanol (50%) giving the title compound (14 mg, 8%). ^{1}H NMR (CD_3OD) δ 7.57 (s, 1H), 4.98 (br, 4H), ^{13}C NMR (CD_3OD) δ 170.7, 143.6, 143.4, 124.0, 118.7, 110.6, 109.5. MS (TSP) m/z: 224/222 (M +, 53/100). Anal. $C_7H_5Cl_2NO_3$ (C, H, N).

6.1.13. 6-Methoxy-7-nitrotetralin **13**

To a solution of 6-methoxytetralin (6.80 g, 41.9 mmol) in dichloromethane (135 mL) under argon atmosphere and at 0 °C was nitric acid (90%, 3.92 mL, 83.8 mmol) added drop-wise. The mixture was stirred for 25 h when iodomethane (7.85 mL, 126 mmol), tetra-*n*-butylammonium hydrogensulfate (28.4 g, 83.8 mmol) and sodium hydroxide (2 M, 8 mL, 16 mmol) were added and the mixture was heated at reflux for 4 h. The organic layer was collected, washed with brine, sodium hydroxide (2 M), brine, dried ($MgSO_4$) and evaporated. The remainder was purified by flash chromatography (SiO_2, chloroform-hexane 2:1) to give the title compound (2.62 g, 30%). ^{1}H NMR ($CDCl_3$) δ 7.60 (s, 1H), 6.74 (s, 1H), 3.90 (s, 3H), 2.79 (m, 2H) 2.71 (m, 2H) 1.81–1.77 (m, 4H), ^{13}C NMR ($CDCl_3$) δ 151.5, 145.5, 137.6, 130.0, 126.8, 114.2, 57.0, 30.5, 28.8, 23.3 23.1, MS (EI, 70 eV) m/z: 207 (M +, 59).

6.1.14. 6-Hydroxy-7-nitrotetralin **14**

To **13** (2.92 g, 14.1 mmol) dissolved in dichloromethane (450 mL) at –65 °C was boron tribromide (1 M, 28.2 mmol) in dichloromethane (28.2 mL) added for 15 min under an atmosphere of argon. The solution was stirred for 2 h when the temperature was gradually increased to 0 °C. The organic phase was collected, washed with sodium bicarbonate (sat., 200 mL), brine, dried ($MgSO_4$) and evaporated. The remainder was purified on a short column (SiO_2, chloroform-hexane 2:1) to give the title compound (2.10 g, 77%). ^{1}H NMR (DMSO-d_6) δ 10.47 (br, 1H), 7.62 (s, 1H), 6.80 (s, 1H), 2.7–2.6 (m, 4H), 1.65–1.71 (m, 4H), ^{13}C NMR (DMSO-d_6) δ 150.0, 146.1, 134.0, 128.3, 124.7, 118.5, 28.9, 27.5, 22.3, 22.0, MS (EI, 70 eV) m/z: 193 (M +, 100).

6.1.15. 5-Chloro-6-hydroxy-7-nitrotetralin **15**

To a solution of **14** (2.44 g, 12.6 mmol) in chloroform (290 mL) under argon atmosphere was chlorine (0.99 M, 25.6 mmol) in chloroform (25.6 mL) added. The mixture was stirred at room temperature for 6 h when the solvent was evaporated. The remainder was purified by flash chromatography (SiO_2, chloroform-hexane 1:1) to give the title compound (2.29 g, 80%). ^{1}H NMR (DMSO-d_6) δ 10.62 (br, 1H), 7.71 (s, 1H), 2.75–2.69 (d, 4H), 1.77–1.64 (d, 4H), ^{13}C NMR (DMSO-d_6) δ 146.3, 143.6, 134.6, 129.7, 123.4, 122.8, 28.3, 27.9, 21.7, 21.6, MS (EI, 70 eV) m/z: 229/227 (M +, 32/100).

6.1.16. 6-Benzyloxy-5-chloro-7-nitrotetralin **16**

To a solution of **15** (2.28 g, 10.0 mmol) in dry *N*, *N*-dimethylformamide (40 mL) under argon atmosphere, were benzyl chloride (11.5 mL, 100 mmol), tetra-*n*-butylammonium hydrogensulfate (95 mg, 0.25 mmol) and potassium carbonate (41.5 g, 30.0 mmol) added. The mixture was stirred at room temperature for 24 h, the salts were then filtered and the solvent was evaporated. The remainder was dissolved in ethyl acetate, and the organic phase was washed with brine, dried ($MgSO_4$) and evaporated. The crude material was purified by flash chromatography (SiO_2, chloroform-hexane 1:1) to give the title compound (2.20 g, 69%), m.p.:80–82 °C. ^{1}H NMR (DMSO-d_6) δ 7.73 (s, 1H), 7.48–7.37 (m, 5H), 5.06 (s, 2H), 2.79–2.75 (m, 4H), 1.79–1.69 (m, 4H). ^{13}C NMR (DMSO-d_6) δ 144.8, 142.1, 135.8, 135.6, 129.6, 128.4, 123.4, 106.2, 105.5, 28.6, 27.6, 21.6, 21.4. MS (TSP) m/z: 337/335 (M + NH_4, 30/100). Anal. $C_{17}H_{16}ClNO_3$ (C, H, N).

6.1.17. 7-Amino-6-benzyloxy-5-chlorotetralin **17**

To a suspension of **16** (2.56 g, 8.33 mmol) in methanol at 1 °C was copper(I) chloride (4.95 g, 25.0 mmol) and potassium borohydride (3.25 g, 60.1 mmol) added portion-wise for 8 h. The mixture was filtered and evaporated. The remainder was dissolved in ethyl acetate and the organic phase was washed with water, brine, dried (Na_2SO_4) and evaporated. The crude material was purified by flash chromatography (SiO_2, chloroform) to give the title compound (1.82 g, 76%). ^{1}H NMR (DMSO-d_6) δ 7.55 (dd, J = 1.6 Hz, J = 8.1 Hz, 2H), 7.41–7.34 (m, 3H), 6.42 (s, 1H), 4.84 (s, 2H), 4.79 (s, 2H), 2.58–2.52 (m, 4H), 1.71–1.60 (m, 4H), ^{13}C NMR (DMSO-d_6) δ 140.1, 139.3, 137.3, 134.0, 128.3, 128.2, 128.1, 127.9, 127.0, 121.8, 114.0, 72.8, 29.0, 26.2, 22.7, 22.3, MS (TSP) m/z: 290/288 (M + 1, 27/100). Anal. $C_{17}H_{18}ClNO$ (C, H, N).

6.1.18. 6-Benzyloxy-5-chloro-7-isonitrosoacetaminotetralin **18**

To a solution of **17** (1.84 g, 6.41 mmol) in *N*, *N*-dimethylformamide (80 mL) and water (8 mL) under argon atmosphere, were hydrochloric acid (12 M, 0.53 mL) and chloral hydrate (1.17 g, 7.05 mmol) added. The mixture was heated to 110 °C when hydroxylamine hydrochloride (1.78 g, 25.6 mmol) in water (8 mL) was added. The mixture was heated to 100 °C for 1 h and the solvent was evaporated. The remainder was dissolved in ethyl acetate, and the organic phase was washed with water, brine, dried (Na_2SO_4) and evaporated. The crude material was purified by flash chromatography (SiO_2, ethyl acetate-chloroform 1:5) to give the title compound (1.35 g, 59%). ^{1}H NMR (DMSO-d_6) δ 12.33 and 9.72 (E/Z) (2s, 1H), 9.20 and 8.28 (E/Z) (2s, 1H), 7.84 and 7.72 (E/Z) (2s, 1H), 7.60 (s, 1H), 7.57–7.37 (m, 5H), 4.90 and 4.88 (E/Z) (2s, 2H), 2.71–2.65 (m, 4H), 1.75–1.68 (m, 4H), ^{13}C NMR (DMSO-d_6) δ 160.3, 160.0, 143.4, 143.0, 136.6, 136.1, 134.4, 134.3, 131.2, 130.6, 129.8, 129.5, 128.8, 128.3, 128.2, 128.1, 127.0, 120.7, 120.3,

74.8, 74.3, 29.1, 26.7, 22.2, 22.0, MS (TSP) m/z: 361/359 (M + 1, 28/100).

6.1.19. 9-Benzyloxy-8-chloro-1H-4,5,6,7-tetrahydro[e] benzindole-2,3-dione **19**

To sulfuric acid (conc., 5 mL) at 60 °C, **18** (500 mg, 1.39 mmol) was added portion-wise for 1 h. The mixture was poured on ice (50 mL) and extracted with ethyl acetate (200 mL). The organic phase was dried (Na_2SO_4) and evaporated. The remainder was dissolved in *N*, *N*-dimethylformamide (3 mL) under argon atmosphere, and benzyl bromide (0.16 mL, 1.39 mmol) and potassium carbonate (192 mg, 1.39 mmol) were added. The mixture was stirred at room temperature for 18 h. The salt was filtered off and the solvent was evaporated. The remainder was purified by repeated flash chromatography (SiO_2, ethyl acetate-methanol 20:1, chloroform-methanol 50:1) to give the title compound (56 mg, 12%). ^{1}H NMR (DMSO-d_6) δ 11.40 (s, 1H), 7.58 (d, *J* = 7.3 Hz, 2H), 7.42–7.36 (m, 3H), 4.90 (s, 2H), 2.92 (t, *J* = 7.3 Hz, 2H), 2.63 (t, *J* = 6.0 Hz, 2H), 1.74–1.66 (m, 4H), ^{13}C NMR (DMSO-d_6) δ 183.8, 159.4, 142.3, 137.3, 137.1, 136.2, 136.2, 129.9, 128.8, 128.2, 128.1, 114.7, 74.6, 26.7, 25.4, 21.7, 20.8, MS (EI, 70 eV) m/z: 343/341 (M +, 5/15).

6.1.20. 6-Amino-7-benzyloxy-5-carboxy-8-chlorotetralin **20**

To a suspension of **19** (51 mg, 0.15 mmol) in sodium hydroxide (0.68 M, 0.60 mmol) and water (0.46 mL) at 10 °C, was hydrogen peroxide (30%, 86 μL, 0.85 mmol) in sodium hydroxide (0.68 M, 0.90 mmol) added. The mixture was stirred for 3 h and then filtered. Water and acetic acid were added and the mixture was extracted with ethyl acetate (40 mL). The organic phase was washed with brine, dried (Na_2SO_4) and evaporated to give the title compound (35 mg, 70%). ^{1}H NMR (DMSO-d_6) δ 7.56 (d, *J* = 7.0 Hz, 2H), 7.43–7.36 (m, 3H), 4.84 (s, 2H), 3.3 (br, 3H), 2.73 (m, 2H), 2.59 (m, 2H) 1.70–1.61 (m, 4H), ^{13}C NMR (DMSO-d_6) δ 169.3, 139.9, 139.5, 136.8, 133.1, 129.5, 128.3, 128.1, 122.3, 116.5, 73.1, 28.1, 26.8, 22.2, 22.0, MS (EI, 70 eV) m/z: 333/331 (M +, 7/18).

6.1.21. 6-Amino-5-carboxy-8-chloro-7-hydroxytetralin **21**

6-Amino-7-benzyloxy-5-carboxy-8-chlorotetralin **20** (33 mg, 0.10 mmol) in ethanol (3 mL) was hydrogenated at room temperature and at atmospheric pressure for 2 h with Pd/C (5%, 4 mg) as the catalyst. The mixture was filtered, evaporated and dried to give the title compound (21 mg, 87%), m.p.: 147 °C (dec.). ^{1}H NMR (DMSO-d_6) δ 7.9 (br, 4H), 2.66 (t, *J* = 7 Hz, 2H), 2.54 (t, *J* = 7 Hz, 2H), 1.70–1.64 (m, 2H), 1.63–1.57 (m, 2H), ^{13}C NMR (DMSO-d_6) δ 169.7, 138.1, 136.4, 128.1, 123.3, 121.4, 115.2, 27.9, 26.9, 22.4, 22.2, MS (EI, 70 eV) m/z: 333/331 (M +, 21/65).

6.1.22. 4,5-Dimethyl-2-nitrophenol **22**

To a solution of 3,4-dimethylphenol (20.0 g, 164 mmol) in dichloromethane (400 mL) under argon atmosphere and at 2 °C was nitric acid (90%, 7.7 mL, 165 mmol) added drop-wise. The mixture was stirred for 90 min while the temperature was slowly increased to ambient temperature. To the mixture was dichloromethane (500 mL) added and the organic phase was washed with brine, water, dried ($MgSO_4$) and evaporated. The remainder was purified by flash chromatography (SiO_2, ethyl acetate-hexane 1:1) to give the title compound (10 g, 36%), m.p.: 82–83 °C. ^{1}H NMR (DMSO-d_6) δ 11.5 (br, 1H), 7.71 (s, 1H), 6.92 (s, 1H), 2.22 (s, 3H), 2.17 (s, 3H), ^{13}C NMR (DMSO-d_6) δ 150.8, 146.3, 133.4, 128.0, 124.9, 119.7, 19.7, 18.1, MS (EI, 70 eV) m/z: 167 (M +, 100).

6.1.23. 2-Chloro-3,4-dimethyl-6-nitrophenol **23**

To a solution of **22** (7.0 g, 41.9 mmol) dissolved in chloroform (300 mL) under argon atmosphere was chlorine (0.99 M, 83.7 mmol) in chloroform (84.8 mL) added. The mixture was stirred for 26 h and then the solvent and excess reagents were evaporated. The remainder was dissolved in dichloromethane (400 mL) and the organic phase was washed with brine, dried ($MgSO_4$) and evaporated. The crude material was purified by flash chromatography (SiO_2, chloroform-hexane 1:1) to give the title compound (6.47 g, 77%), m.p.: 62–63 °C. ^{1}H NMR (DMSO-d_6) δ 11.5 (br, 1H), 7.80 (s, 1H), 2.34 (s, 3H), 2.27 (s, 3H). ^{13}C NMR (DMSO-d_6) δ 146.9, 143.8, 134.1, 128.8, 123.7, 122.9, 19.4, 17.4. MS (EI, 70 eV) m/z: 203/201 (M +, 37/100).

6.1.24. 2-Benzyloxy-3-chloro-4,5-dimethylnitrobenzene **24**

To a solution of **23** (6.44 g, 31.9 mmol) in dry *N*, *N*-dimethylformamide (105 mL) under argon atmosphere, were benzyl bromide (4.17 mL, 35.1 mmol) and potassium carbonate (13.2 g, 95.7 mmol) added. The mixture was stirred at room temperature for 8 h and was then filtered. The solvent was evaporated, and the remainder was purified by flash chromatography (SiO_2, chloroform-hexane 1:1) affording the title compound (8.44 g, 91%), m.p.: 74–75 °C. ^{1}H NMR (DMSO-d_6) δ 7.80 (s, 1H), 7.48–7.37 (m, 5H), 5.06 (s, 2H), 2.37 (s, 3H), 2.34 (s, 3H), ^{13}C NMR (DMSO-d_6) δ 145.2, 142.2, 142.1, 135.7, 134.7, 129.6, 128.4, 128.4, 123.6, 75.7, 19.7, 17.1. MS (EI, 70 eV) m/z: 293/291 (M +, 8/37).

6.1.25. 2-Benzyloxy-3-chloro-4,5-dimethylaniline **25**

To a solution of **24** (3.00 g, 10.3 mmol) in methanol (420 mL) at 2 °C, were copper(I) chloride (6.11 g, 30.8 mmol) and potassium borohydride (4.4 g, 81.6 mmol) added in portions. The mixture was filtered and evaporated. The remainder was dissolved in ethyl acetate, and the organic phase was washed with water, dried ($MgSO_4$) and evaporated. The crude material was purified by flash chromatography (SiO_2, chloroform) to give the title compound (2.14 g, 79%), m.p.: 57–58 °C. ^{1}H NMR (DMSO-d_6) δ 7.54 (d, J = 6.5 Hz, 2H), 7.42–7.34 (m, 3H), 6.52 (s, 1H), 4.83 (s, 2H), 4.79 (s, 2H), 2.12 (s, 6H), ^{13}C NMR (DMSO-d_6) δ 140.0, 138.9, 137.3, 133.0, 128.3, 128.2, 127.9, 127.3, 121.2, 115.4, 72.7, 20.2, 15.4, MS (EI, 70 eV) m/z: 286/284 (M + 23, 42/100).

6.1.26. 2-Benzyloxy-3-chloro-4,5-dimethylisonitroso-acetanilide **26**

To a solution of **25** (2.14 g, 8.19 mmol) in *N,N*-dimethylformamide (60 mL) and water (2 mL) were hydrochloric acid (conc., 0.68 mL, 8.19 mmol) and chloral hydrate (1.49 g, 9.00 mmol) added. The mixture was heated to 105 °C and hydroxylamine hydrochloride (2.28 g, 32.8 mmol) dissolved in water (4 mL) was added. The mixture was stirred for 1 h and the solvent was evaporated. The remainder was dissolved in ethyl acetate, and the organic layer was washed with water, dried ($MgSO_4$) and evaporated. The crude material was purified by flash chromatography (SiO_2, chloroform-ethyl acetate 10:1) affording the title compound (1.28 g, 47%). ^{1}H NMR (DMSO-d_6) δ 12.33 and 9.71 (E/Z) (2s, 1H), 9.20 and 8.27 (E/Z) (2s, 1H), 7.91 and 7.83 (2s, 1H), 7.60 (s, 1H), 7.65–7.36 (m, 5H), 4.90 (s, 1H), 4.87 (s, 1H), 2.26–2.23 (m, 6H), ^{13}C NMR (DMSO-d_6) δ 160.2, 160.0, 143.4, 143.1, 136.6, 136.1, 133.4, 133.3, 131.0, 130.4, 129.7, 129.4, 128.7, 128.4, 128.4, 128.3, 128.2, 128.1, 128.0, 127.4, 127.2, 121.5, 121.2, 74.7, 74.2, 20.3, 16.1, MS (EI, 70 eV) m/z: 334/332 (M +, 4/12).

6.1.27. 7-Benzyloxy-6-chloro-4,5-dimethylisatin **27**

To sulfuric acid (conc., 6 mL) at 80 °C was **26** (700 mg, 2.10 mmol) added. The mixture was stirred at 80 °C for 10 min and then poured into ice-water (200 mL). The mixture was extracted with ethyl acetate (200 mL) and the organic phase was dried ($MgSO_4$) and evaporated. The remainder was dissolved in *N,N*-dimethylformamide and benzyl bromide (0.28 mL, 2.30 mmol) and potassium carbonate (318 mg, 2.30 mmol) were added. The mixture was stirred at ambient temperature for 30 h and filtered. Acetic acid (1.5 mL) was added, and the solvents were evaporated. The remainder was purified by repeated flash chromatography (SiO_2, dichloromethane-methanol 50:1) to give the title compound (13 mg, 2%). ^{1}H NMR (DMSO-d_6) δ 11.42 (s, 1H), 7.57 (d, J = 6.2 Hz, 2H), 7.42–7.35 (m, 3H), 4.92 (s, 2H), 2.44 (s, 3H), 2.22 (s, 3H), ^{13}C NMR (DMSO-d_6) δ 184.3, 159.3, 141.9, 137.0, 137.0, 136.3, 135.4, 130.2, 128.8, 128.3, 128.2, 115.6, 74.6, 15.3, 14.2, MS (EI, 70 eV) m/z: 317/315 (M +, 3/7).

6.1.28. 3-Benzyloxy-4-chloro-5,6-dimethylanthranilic acid **28**

To a suspension of **27** (13 mg, 0.04 mmol) in dioxane (0.5 mL) and sodium hydroxide (0.68 M, 0.14 mmol) at 10 °C, was hydrogen peroxide (30%, 7 μL, 0.22 mmol) dissolved in sodium hydroxide (0.41 mL) added in portions. The mixture was concentrated in a stream of nitrogen, when acetic acid (38 μL) was added. The mixture was dissolved in ethyl acetate (3 mL) and the organic phase was washed with brine, dried ($MgSO_4$) and evaporated to give the title compound (11 mg, 90%). ^{1}H NMR (DMSO-d_6) δ 7.55 (d, J = 7.0 Hz, 2H), 7.43–7.35 (m, 3H), 4.82 (s, 2H), 2.21 (s, 3H), 2.19 (s, 3H), MS (EI, 70 eV) m/z: 307/305 (M +, 7/19).

6.1.29. 4-Chloro-5,6-dimethyl-3-hydroxyanthranilic acid **29**

3-Benzyloxy-4-chloro-5,6-dimethylanthranilic acid **28** (10 mg, 0.03 mmol) in ethanol (1.5 mL) was hydrogenated at ambient temperature and atmospheric pressure for 5 h with Pd/C (10%, 2 mg) as the catalyst. The mixture was filtered and the solvent was evaporated. The remainder was purified by preparative HPLC (Lichrosorb-C18, methanol-phosphate buffer, pH 3, 1:1). The fractions containing the product were pooled and evaporated. To the residue was ethyl acetate added and the organic phase was washed with sodium carbonate (sat), dried and evaporated to give the title compound (3 mg, 46%). ^{1}H NMR (DMSO-d_6) δ 3.35 (br, 1H), 3.16 (s, 2H), 2.16 (s, 3H), 2.15 (s, 3H), ^{13}C NMR (DMSO-d_6) δ 169.9, 137.9, 135.2, 126.4, 123.5, 121.4, 117.3, 17.7, 16.0, MS (EI, 70 eV): m/z 217/215 (M +, 21/63).

6.1.30. 2,4-Dichloro-5-methoxy-3-methylphenyl trifluoro-methanesulfonate **30**

To a solution of 2,4-dichloro-5-methoxy-3-methyl-phenol [33] (7.73 g, 37.3 mmol) in dichloromethane (180 mL) were triethylamine (10.4 mL, 74.7 mmol) and *N,N*-dimethylaminopyridine (10 mg, 0.08 mmol) added. The solution was cooled to –78 °C and trifluoromethane-sulfonic anhydride (9.4 mL, 74.7 mmol) was added dropwise. The mixture was then stirred for 20 min while the temperature slowly reached 0 °C. To the solution was added dichloromethane (200 mL) and the organic phase

was washed with water (150 mL), brine, dried ($MgSO_4$) and evaporated. The remainder was purified by flash chromatography (SiO_2, ethyl acetate-hexane 1:3) affording the title compound (12.3 g, 97%), m.p.: 58.5–59.0 °C. ^{1}H NMR (DMSO-d_6) δ 7.30 (s, 1H), 3.92 (s, 3H), 2.49 (s, 3H), ^{13}C NMR (DMSO-d_6) δ 154.2, 143.9, 137.0, 122.8, 118.1, 118.0 (q, J = 321Hz), 105.3, 57.2, 18.0, MS (EI, 70 eV) m/z: 340/338 (M +, 47/64).

6.1.31. Methyl 2,4-dichloro-5-methoxy-3-methylbenzoate **31**

To a solution of **30** (7.60 g, 22.4 mmol) in dioxane (75 mL) were 1,3-bis(diphenylphosphino)propane (0.37 g, 0.90 mmol) and palladium acetate (0.20 g, 0.90 mmol) added. The mixture was flushed with carbon monoxide, then triethylamine (6.90 mL, 49.4 mmol) and methanol (23 mL) were added. The mixture was stirred at 70 °C and atmospheric pressure for 25 h and was then filtered and evaporated. The remainder was dissolved in diethyl ether and the organic phase was washed with ammonium hydroxide (2 M, 150 mL), brine (150 mL), dried ($MgSO_4$) and evaporated. The crude material was purified by flash chromatography (SiO_2, ethyl acetate-hexane 1:3) to give the title compound (4.18 g, 75%), m.p.: 73.5–74 °C. ^{1}H NMR (DMSO-d_6) δ 7.33 (s, 1H), 3.88 (s, 3H), 3.86 (s, 3H), 2.45 (s, 3H), ^{13}C NMR (DMSO-d_6) δ 165.7, 153.3, 136.1, 130.5, 125.2, 123.0, 110.8, 56.6, 52.7, 17.9, MS (EI, 70 eV) m/z: 250/248 (M +, 53/80).

6.1.32. 2,4-Dichloro-5-hydroxy-3-methylbenzoic acid **32**

To a solution of **31** (0.24 g, 0.96 mmol) in methanol (30 mL) under argon atmosphere, was potassium hydroxide (0.31 g, 4.8 mmol) added and the mixture was stirred at 50 °C for 19 h. The solvent was evaporated, and hydrobromic acid (48%, 30 mL) was added and the mixture was stirred at 110 °C for 3 d. The acid was evaporated, and the remainder was dissolved in ethyl acetate (40 mL). The organic phase was extracted with ammonium hydroxide (dil., 11 mL) and the pH of the aqueous phase was lowered to 1. The aqueous phase was extracted with ethyl acetate (40 mL), the organic phase was washed with brine, dried ($MgSO_4$) and evaporated to give, after crystallization from diethyl ether, the title compound (0.20 g, 94%), m.p.: 203.5–204.5 °C. ^{1}H NMR (DMSO-d_6) δ 10.68 (br, 1H), 7.15 (s, 1H), 2.42 (s, 3H), ^{13}C NMR (DMSO-d_6) δ 166.7, 151.8, 135.9, 131.2, 123.7, 121.2, 114.4, 17.9, MS (EI, 70 eV) m/z: 222/220 (M +, 56/100).

6.1.33. 4,6-Dichloro-3-hydroxy-5-methyl-2-nitrobenzoic acid **33**

To a solution of **32** (90 mg, 0.41 mmol) in nitromethane (9 mL) at 40 °C, was nitric acid (90%, 20 mL, 0.43 mmol) added. The mixture was stirred for 4 h, when the solvent was removed by evaporation. The remainder was purified by flash chromatography (SiO_2, ethyl acetate-acetic acid 30:1) to give the title compound (79 mg, 72%), m.p.: 197–199 °C. ^{1}H NMR (DMSO-d_6) δ 2.45 (s, 3H). ^{13}C NMR (DMSO-d_6) δ 164.3, 147.1, 139.6, 136.3, 128.4, 126.5, 118.9, 18.6. MS (EI, 70 eV) m/z: 267/265 (M +, 66/100).

6.1.34. 4,6-Dichloro-3-hydroxy-5-methylanthranilic acid **34**

4,6-Dichloro-3-hydroxy-5-methyl-2-nitrobenzoic acid **33** (69 mg, 0.26 mmol) in acetic acid (10 mL) and hydrochloric acid (conc., 33 mL) was hydrogenated at atmospheric pressure and room temperature for 2 h with Pd/C (10%, 10 mg) as the catalyst. Methanol was added and the mixture was filtered. The solvent was evaporated and the remainder was purified by flash chromatography (SiO_2, ethyl acetate-acetic acid 45:1) affording the title compound (55 mg, 90%), m.p.: 192 °C (dec.). ^{1}H NMR (DMSO-d_6) δ 2.27 (s, 3H), ^{13}C NMR (DMSO-d_6) δ 167.4, 139.2, 136.0, 122.9, 121.3, 120.4, 116.9, 17.0, MS (EI, 70 eV) m/z: 237/235 (M +, 45/79).

6.1.35. 4,6-Dibromo-3-hydroxy-2-nitrobenzoic acid **35**

To a cooled solution of 3-hydroxy-2-nitrobenzoic acid (10.5 g, 0.057 mol) and sodium acetate (9.85 g, 0.57 mol) in acetic acid (100 mL) was bromine (6.15 mL, 0.12 mol) added drop-wise. The mixture was stirred at 60 °C for 68 h and then cooled and filtered. The solution was evaporated, and the remainder was dissolved in ethyl acetate. The organic phase was washed with dilute hydrochloric acid, dried ($MgSO_4$) and evaporated. The crude product was purified by flash chromatography (SiO_2, toluene-acetic acid 5:1). The pure compound was crystallized from methanol to give the title compound (15.4 g, 79%), m.p.: 201–202 °C (dec.). ^{1}H NMR (DMSO-d_6) δ 8.20 (s, 1H), ^{13}C NMR (DMSO-d_6) δ 164.4, 146.9, 139.1, 130.5, 116.2, 108.1, MS (EI, 70 eV) m/z: 343/341/339 (M +, 46/98/49). Anal. $C_7H_3Br_2NO_5$ (C, H, N).

6.1.36. 4,6-Dibromo-3-hydroxyanthranilic acid **36**

4,6-Dibromo-3-hydroxy-2-nitrobenzoic acid **35** (4.09 g, 12 mmol) in ethanol (150 mL) was hydrogenated at atmospheric pressure and room temperature for 45 h, with PtS_2 (0.16 g, 0.62 mmol) as the catalyst. The mixture was filtered and the solvent was removed by evaporation. The remainder was purified by flash chromatog-

raphy (SiO_2, toluene-acetic acid 5:1) affording, after crystallization from methanol-water, the title compound (2.51 g, 63%), m.p.: 162–164.5 °C. ^{1}H NMR (DMSO-d_6) δ 6.96 (s, 1H), ^{13}C NMR (DMSO-d_6) δ 167.7, 140.6, 140.0, 121.8, 117.2, 112.5, 110.4. MS (EI, 70 eV) m/z: 313/311/309 (M +, 36/72/34). Anal. $C_7H_5Br_2NO_3$ (C, H, N).

6.1.37. 4-Bromo-5-hydroxy-2-methoxybenzoic acid **37**

To a solution of 5-hydroxy-2-methoxybenzoic acid [34] (1.24 g, 73.7 mmol) in acetic acid (100 mL) was bromine (0.38 mL, 7.4 mmol) added drop-wise. The mixture was stirred for 3 h and the solvent was then evaporated. The remainder was purified by flash chromatography (SiO_2, toluene-acetic acid 10:1) to give the title compound (1.5 g, 83%). ^{1}H NMR (CD_3OD) δ 7.38 (s, 1H), 7.26 (s, 1H), 3.84 (s, 3H), ^{13}C NMR (CD_3OD) δ 169.0, 154.1, 149.6, 121.3, 119.6, 119.0, 116.6, 57.7.

6.1.38. 4-Bromo-3-hydroxy-6-methoxy-2-nitrobenzoic acid **38**

To a solution of sodium nitrate (361 mg, 4.25 mmol), lanthanum nitrate hexahydrate (18 mg, 0.042 mmol) and hydrochloric acid (12 M, 4 mL) in water (4 mL) at 0 °C, was **37** (1.05 g, 4.25 mmol) in diethyl ether (20 mL) added in portions. The mixture was stirred for 7 h, while the solution reached ambient temperature. Dichloromethane (90 mL) and water (20 mL) were added, and the organic phase was collected, dried ($MgSO_4$), filtered and evaporated. The remainder was purified by flash chromatography (SiO_2, toluene-ethyl acetate-acetic acid 8:2:1) affording the title compound (600 mg, 48%). ^{1}H NMR (CD_3OD) δ 7.57 (s, 1H), 3.85 (s, 3H), MS (EI, 70 eV) m/z 293/291 (M +, 21/19).

6.1.39. 4-Bromo-3-hydroxy-6-methoxyanthranilic acid **39**

4-Bromo-3-hydroxy-6-methoxy-2-nitrobenzoic acid **38** (52 mg, 0.18 mmol) in ethanol (7 mL) was hydrogenated at atmospheric pressure and room temperature for 18 h with PtS_2 as the catalyst. The solution was filtered and the solvent was evaporated. The remainder was purified by flash chromatography (SiO_2, toluene-ethyl acetate-acetic acid 8:2:1) to give the title compound (30 mg, 64%). ^{1}H NMR (CD_3OD) δ 6.40 (s, 1H), 3.88 (s, 3H), ^{13}C NMR (CD_3OD) δ 170.8, 154.8, 145.6, 137.8, 115.5, 102.1, 101.6, 57.6, MS (EI, 70 eV): m/z 263/261 (M +, 79/80).

6.1.40. 2,4-Dichloro-5-methoxyphenyl trifluoromethanesulfonate **40**

To a solution of 2,4-dichloro-5-methoxyphenol [35] (4.43 g, 22.9 mmol), triethylamine (6.40 mL, 45.9 mmol) and *N*,*N*-dimethylaminopyridine (5 mg, 0.04 mmol) in dichloromethane (10 mL) at –70 °C, was trifluromethanesulfonic anhydride (5.79 mL, 34.4 mmol) added. The mixture was stirred for 20 min while the temperature was increased to ambient temperature. Dichloromethane (150 mL) was added and the organic phase was washed with water (100 mL), brine (100 mL), dried ($MgSO_4$) and evaporated. The remainder was purified by flash chromatography (SiO_2, dichloromethane-hexane 1:2) affording the title compound (6.37 g, 86%). ^{1}H NMR (DMSO-d_6) δ 7.99 (s, 1H), 7.42 (s, 1H), 3.92 (s, 3H), ^{13}C NMR (DMSO-d_6) δ 154.6, 144.0, 130.9, 122.3, 118.0 (q, J = 321 Hz), 117.1, 108.2, 57.3, MS (EI, 70 eV) m/z: 328/326/324 (M +, 6/37/55).

6.1.41. Methyl 2,4-dichloro-5-methoxybenzoate **41**

To a solution of **40** (6.35 g, 19.5 mmol) in *N*, *N*-dimethylformamide (65 mL) flushed with carbon monoxide were 1,3-bis(diphenylphosphino)propane (314 mg, 0.76 mmol), palladium acetate (171 mg, 0.76 mmol), triethylamine (6.0 mL) and methanol (14.5 mL) added. The reaction was stirred at 70 °C and atmospheric pressure for 5 h, the solvent was then evaporated. The remainder was dissolved in diethyl ether (600 mL) and the organic phase was washed with ammonium hydroxide (2 M, 300 mL), brine (200 mL), dried ($MgSO_4$) and evaporated. The crude product was purified by flash chromatography (SiO_2, dichloromethane-hexane 2:3) affording the title compound (1.65 g, 36%), m.p.: 88–89 °C. ^{1}H NMR (DMSO-d_6) δ 7.73 (s, 1H), 7.49 (s, 1H), 3.90 (s, 3H), 3.86 (s, 3H), ^{13}C NMR (DMSO-d_6) δ 164.8, 153.4, 131.3, 129.6, 125.3, 123.2, 114.2, 56.7, 52.7, MS (EI, 70 eV) m/z: 238/236/234 (M +, 8/55/83).

6.1.42. 2,4-Dichloro-5-hydroxybenzoic acid **42**

To a solution of **41** (600 mg, 2.55 mmol) in dichloromethane (10 mL) at –70 °C and under argon atmosphere was boron tribromide (0.72 mL, 7.66 mmol) added. The mixture was stirred for 4 h while the temperature slowly reached ambient temperature. Dichloromethane (20 mL) and sodium hydroxide (2 M, 15 mL) were added and stirring was continued for 30 min. The aqueous phase was washed with dichloromethane (10 mL) and was then acidified to pH 1 by the addition of hydrochloric acid (12 M). The aqueous phase was extracted with ethyl acetate (40 mL), and the organic phase washed with brine, dried ($MgSO_4$) and evaporated to give the title compound (350 mg, 66%), m.p.: 200.5–201.5 °C. ^{1}H NMR (DMSO-d_6) δ 10.8 (br, 1H) 7.56 (s, 1H), 7.38 (s, 1H), ^{13}C NMR (DMSO-d_6) δ 165.8, 152.0, 131.3, 130.3, 123.6, 121.6, 118.1, MS (EI, 70 eV) m/z: 210/208/206 (M +, 11/64/100).

6.1.43. 4,6-Dichloro-3-hydroxy-2-nitrobenzoic acid **43**

To a solution of **42** (280 mg, 1.35 mmol) in nitromethane (35 mL) at 45 °C was nitric acid (90%, 63 μL, 1.35 mmol) added. The mixture was stirred for 4 h and then the solvent was evaporated. The remainder was dissolved in ethyl acetate (100 mL), and the organic phase was washed with water (5 mL), brine (5 mL), dried ($MgSO_4$) and evaporated. The crude product was purified by flash chromatography (SiO_2, ethyl acetate-acetic acid 50:1) affording the title compound (323 mg, 95%), m.p.: 186 °C (dec.). ^{1}H NMR (DMSO-d_6) δ 7.88 (s, 1H), ^{13}C NMR (DMSO-d_6) δ 163.8, 147.2, 139.0, 133.1, 128.1, 126.3, 118.2, MS (EI, 70 eV) m/z: 253/251 (M +, 34/49).

6.1.44. 4,6-Dichloro-3-hydroxyanthranilic acid hydrochloride **44**

4,6-Dichloro-3-hydroxy-2-nitrobenzoic acid **43** (223 mg, 0.88 mmol) dissolved in ethanol (50 mL) was hydrogenated at atmospheric pressure and room temperature for 1 h with Pd/C (10%, 30 mg) as the catalyst. The mixture was filtered and the solvent was evaporated. The remainder was purified by flash chromatography (SiO_2, ethyl acetate-acetic acid 50:1) affording the free base. The base was dissolved in tetrahydrofuran (0.6 mL) and ethereal hydrogen chloride (3 M, 1 mL) was added to give the title compound (32 mg, 14%), m.p.: 231 °C (dec.). ^{1}H NMR (DMSO-d_6) δ 6.68 (s, 1H), ^{13}C NMR (DMSO-d_6) δ 167.2, 140.3, 139.1, 122.5, 121.9, 116.2, 114.0, MS (EI, 70 eV) m/z: 223/221 (M +, 42/65). Anal. $C_7H_5Cl_2NO_3$xHCl (C, H, N).

6.1.45. 3-Chloro-2-methoxy-5-phenylnitrobenzene **45**

A solution of 2-chloro-6-nitro-4-phenylphenol [36] (21.2 g, 0.08 mol), potassium carbonate (17.6 g, 0.13 mol) and iodomethane (13.5 mL, 0.22 mol) in dry *N*,*N*-dimethylformamide (150 mL) was stirred at room temperature under nitrogen atmosphere for 19 h, when the solvent was evaporated. The remainder was purified by flash chromatography (SiO_2, hexane → ethyl acetate-hexane 10:1) affording, after crystallization from ethyl acetate-hexane, the title compound (22.2 g, 99%), m.p.: 76–77.5 °C. ^{1}H NMR ($CDCl_3$) δ 7.88 (d, *J* = 2.3 Hz, 1H), 7.81 (d, *J* = 2.3 Hz, 1H), 7.53–7.40 (m, 5H), 4.05 (s, 3H), ^{13}C NMR ($CDCl_3$) δ 148.8, 145.5, 138.1, 137.1, 132.8, 130.8, 129.4, 129.2, 128.7, 126.8, 121.8, 62.6, MS (EI, 70 eV) m/z: 265/263 (M +, 30/98). Anal. $C_{13}H_{10}ClNO_3$ (C, H, N).

6.1.46. 3-Chloro-2-methoxy-5-phenylaniline **46**

3-Chloro-2-methoxy-5-phenylnitrobenzene **45** (9.1 g, 34.5 mmol) was hydrogenated in tetrahydrofuran (100 mL), methanol (100 mL) and hydrochloric acid (2 M, 34.5 mL) at atmospheric pressure and room temperature for 3 h with Pd/C (10%, 180 mg) as the catalyst. The mixture was filtered and the solvent was evaporated. The remainder was dissolved in ethyl acetate and the organic phase was washed with ammonium hydroxide (2 M), dried ($MgSO_4$) and evaporated. The crude material was crystallized from ethyl acetate-hexane affording the title compound (5.05 g, 62%), m.p.: 65–66.5 °C. ^{1}H NMR ($CDCl_3$) δ 7.49–7.31 (m, 5H), 6.96 (d, *J* = 2.1 Hz, 1H), 6.82 (d, *J* = 2.1 Hz, 1H), 3.97 (s, 2H), 3.86 (s, 3H), ^{13}C NMR ($CDCl_3$) δ 142.5, 141.4, 139.9, 138.4, 128.7, 127.9, 127.4, 126.8, 118.2, 112.9, 59.8, MS (EI, 70 eV) m/z: 235/233 (M +, 24/48). Anal. $C_{13}H_{12}ClNO$ (C, H, N).

6.1.47. 3-Chloro-2-methoxy-5-phenylisonitrosoacetanilide **47**

To a stirred solution of chloralhydrate (1.08 g, 6.53 mmol) and sodium sulfate (5.0 g, 35.2 mmol) in water (20 mL) was **46** (1.01 g, 4.33 mmol) dissolved in a mixture of water (5 mL), *N*,*N*-dimethylformamide (10 mL) and hydrochloric acid (conc., 0.43 mL) added. The mixture was stirred at 95 °C for 90 min, when hydroxylamine hydrochloride (1.36 g, 19.5 mmol) was added, and the stirring was continued for another 22 h. The mixture was cooled and evaporated and then ethyl acetate was added. The organic phase was washed with water, dried ($MgSO_4$) and evaporated. The remainder was purified by flash chromatography (SiO_2, dichloromethane → ethyl acetate) to give the title compound (1.04 g, 79%). ^{1}H NMR (DMSO-d_6) δ 9.51 (s, 1H), 8.39 (s, 1H), 7.80 (s, 1H), 7.60 (d, 2H), 7.51–7.34 (m, 5H), 3.83 (s, 3H), ^{13}C NMR (DMSO-d_6) δ 160.4, 145.3, 143.6, 138.4, 137.3, 132.8, 129.0, 128.8, 127.9, 127.0, 126.6, 126.4, 123.3, 118.6, 60.8, MS (EI, 70 eV) m/z: 306/304 (M +, 6/20). Anal. $C_{15}H_{13}ClN_2O_3$ (C, H, N).

6.1.48. 6-Chloro-7-methoxy-4-phenylisatin **48**

3-Chloro-2-methoxy-5-phenylisonitrosoacetanilide **47** (6.5 g, 21.3 mmol) and polyphosphoric acid (66 g) were stirred at 80 °C for 3 h when ice, water and ethyl acetate were added. The organic phase was washed with water, dried (Na_2SO_4) and evaporated. The remainder was purified by flash chromatography (SiO_2, dichloromethane → ethyl acetate-hexane 1:1) to give the title compound (5.06 g, 83%). ^{1}H NMR (DMSO-d_6) δ 11.54 (br s, 1H), 7.54–7.42 (m, 5H), 7.10 (s, 1H), 3.82 (s, 3H), ^{13}C NMR (DMSO-d_6) δ 181.8, 159.3, 145.4, 139.8, 138.0, 135.6, 134.9, 128.9, 128.8, 128.1, 124.4, 114.5, 61.2, MS (EI, 70 eV) m/z: 289/287 (M +, 27/100). Anal. $C_{15}H_{10}ClNO_3$, (C, H, N).

6.1.49. 4-Chloro-3-methoxy-6-phenylanthranilic acid **49**

To a solution of **48** (2.15 g, 7.47 mmol) in dioxane (20 mL) and sodium hydroxide (0.68 M, 60 mL) at 0 °C

and under nitrogen atmosphere, was hydrogen peroxide (30%, 4.0 mL, 39.2 mmol) in sodium hydroxide (0.68 M, 50 mL) added drop-wise. The solution was stirred at ambient temperature for 6 h when acetic acid was added to pH 5. The mixture was extracted with ethyl acetate, the organic phase was dried (Na_2SO_4) and evaporated. The remainder was purified by flash chromatography (SiO_2, ethyl acetate-hexane 1:2 → toluene-ethyl acetate-acetic acid 4:1:1) to give the title compound (1.37 g, 66%). ^{1}H NMR (DMSO-d_6) δ 7.38–7.25 (m, 5H), 6.52 (s, 1H), 3.75 (s, 3H), ^{13}C NMR (DMSO-d_6) δ 169.3, 142.9, 141.6, 141.1, 139.0, 128.0, 127.9, 127.1, 117.5, 114.6, 59.4, MS (EI, 70 eV) m/z: 280/278 (M +, 15/47). Anal. $C_{14}H_{12}ClNO_3$ (C, H, N).

6.1.50. 4-Chloro-3-hydroxy-6-phenylanthranilic acid **50**

To **49** (110 mg, 0.40 mmol) dissolved in diethyl ether was ethereal hydrogen chloride added. The solvent was evaporated and dichloromethane (3 mL) was added. The solution was cooled to –70 °C under nitrogen atmosphere and boron tribromide (0.08 mL, 0.84 mmol) was added. The solution was stirred for 40 min while the temperature reached ambient temperature. The mixture was left over night, and then sodium bicarbonate (sat, 15 mL) was added and the aqueous phase was stirred with dichloromethane for 1 h. Then the pH was lowered to 4 by the addition of hydrochloric acid (2 M), and the aqueous phase was extracted with dichloromethane, the organic phase was dried ($MgSO_4$) and evaporated. The crude material was purified by flash chromatography (SiO_2, toluene-ethyl acetate-acetic acid 4:1:1) affording the title compound (23 mg, 22%). ^{1}H NMR (DMSO-d_6) δ 7.33–7.22 (m, 5H), 6.52 (s, 1H), ^{13}C NMR (DMSO-d_6) δ 172.5, 143.6, 141.2, 141.1, 137.2, 129.6, 129.2, 128.0, 123.0, 119.8, 115.2, MS (EI, 70 eV) m/z: 265/263 (M +, 23/79).

6.1.51. 3-Chloro-2-methoxy-5-methylisonitrosoacetanilide **51**

To a stirred solution of chloral hydrate (0.99 g, 6.0 mmol) and sodium sulfate (3.4 g, 24 mmol) in water (15 mL) was 3-chloro-2-methoxy-5-methylaniline [37] (0.51 g, 3.0 mmol) in *N*,*N*-dimethylformamide (7 mL), water (9 mL) and hydrochloric acid (conc., 0.37 mL) added. The mixture was stirred at 90 °C for 25 min, when hydroxylamine hydrochloride (1.24 g, 18 mmol) was added and stirring was continued for another 18 h. The mixture was cooled and ethyl acetate (100 mL) was added. The organic phase was washed with water (100 mL), dried (Na_2SO_4) and evaporated. The remainder was purified by flash chromatography (SiO_2, ethyl acetate-hexane 1:1) affording the title compound (0.62 g, 85%). ^{1}H NMR (DMSO-d_6) δ 9.36 (s, 1H), 7.90 (d, J = 1.6 Hz, 1H), 7.74 (s, 1H), 7.07 (d, J = 2.1 Hz, 1H), 3.75 (s, 3H), 2.25 (s, 3H), ^{13}C NMR (DMSO-d_6) δ 160.2, 143.6, 134.8, 132.1, 125.9, 125.5, 120.8, 60.7, 20.5, MS (TSP) m/z: 262/260 (M + 18, 30/100). Anal. $C_{10}H_{11}ClN_2O_3$ (C, H, N).

6.1.52. 6-Chloro-7-methoxy-4-methylisatin **52**

3-Chloro-2-methoxy-5-methylisonitrosoacetanilide **51** (0.49 g, 2.0 mmol) and polyphosphoric acid (6.3 g) were stirred at 80 °C for 3 h when ice was added. Ethyl acetate (150 mL) was added and the organic phase was washed with water (50 mL), dried (Na_2SO_4) and evaporated. The remainder was purified by flash chromatography (SiO_2, ethyl acetate-hexane 1:2 → ethyl acetate) to give the title compound (0.37 g, 82%). ^{1}H NMR (DMSO-d_6) δ 11.41 (br, 1H), 7.00 (s, 1H), 3.74 (s, 3H), 2.38 (s, 3H), ^{13}C NMR (DMSO-d_6) δ 183.8, 159.4, 144.5, 138.6, 136.0, 135.8, 125.1, 116.1, 61.1, 16.6, MS (TSP) m/z: 245/243 (M + 18, 37/100). Anal. $C_{10}H_8ClNO_3$ (C, H, N).

6.1.53. 4-Chloro-3-methoxy-6-methylanthranilic acid **53**

To a solution of **52** (320 mg, 1.4 mmol) in dioxane (5 mL) at 5 °C and under nitrogen atmosphere was hydrogen peroxide (30%, 0.73 mL, 7.2 mmol) in water (10 mL) and sodium hydroxide (0.67 M, 26 mL) added drop-wise. The mixture was stirred for 3 h while the temperature slowly reached ambient temperature. Water (25 mL) was added and the pH was adjusted to 5 by the addition of acetic acid. The mixture was extracted with ethyl acetate (120 mL) and the organic phase was dried (Na_2SO_4) and evaporated. The remainder was purified by flash chromatography (SiO_2, ethyl acetate → ethyl acetate-acetic acid 1 000:1) affording the title compound (210 mg, 70%). ^{1}H NMR (CD_3OD) δ 6.45 (s, 1H), 3.76 (s, 3H), 2.37 (s, 3H), ^{13}C NMR (CD_3OD) δ 171.9, 146.4, 142.9, 138.3, 131.0, 120.3, 114.5, 60.2, 23.2, MS (TSP) m/z: 218/216 (M + 1, 34/100). Anal. $C_9H_{10}ClNO_3$ (C, H, N).

6.1.54. 4-Chloro-3-hydroxy-6-methylanthranilic acid **54**

To **53** (49 mg, 0.23 mmol) in diethyl ether was ethereal hydrogen chloride (3 M) added. The solvent was evaporated and dichloromethane (2 mL) was added. The solution was cooled to –65 °C under nitrogen atmosphere when boron tribromide (0.1 mL, 1.12 mmol) was added. The reaction was stirred for 43 h while the temperature reached ambient temperature. Sodium bicarbonate (sat., 15 mL) was added, and the pH of the aqueous phase was adjusted to 2 by the addition of hydrochloric acid (2 M). The aqueous phase was extracted with ethyl acetate, and the organic phase was dried (Na_2SO_4) and evaporated. The remainder was purified by flash chromatography

(SiO_2, toluene-acetic acid 10:1 → 5:1) affording the title compound (22 mg, 47%). ^{1}H NMR (CD_3OD) δ 6.43 (s, 1H), 2.35 (s, 3H), ^{13}C NMR (CD_3OD) δ 172.2, 142.9, 139.9, 133.4, 123.8, 119.8, 113.8, 22.9, MS (TSP) m/z: 204/202 (M + 1, 28/100). Anal. $C_8H_8ClNO_3$ (C, H, N).

6.2. Stability assay

The compounds were dissolved in PBS buffer at pH 7.5 and the solution was left in the dark for 24 h at 37 °C. Aliquots were analysed by HPLC (Bondapak C18, 150 × 3.9, phosphate buffer pH 3-acetonitrile-acetic acid 75:25:1) with UV detection (254 nm).

6.3. Pharmacological methods

6.3.1. In vitro measurement of 3-HAO inhibition

Male Sprague-Dawley rats (150–200 g) were anaesthetized and perfused before decapitation. The cortex was rapidly dissected out on ice and stored at –70 °C. To prepare a homogenate, the cortex was thawed, minced and sonicated in 9 volumes (w/v) of ice-cold distilled water by 3 times 5 s bursts of sonication using a cell disrupter (Branson sonifier). Aliquots of the crude homogenate were stored in vials (400 μL/vial) at –70 °C prior to assay. To prepare cell-free homogenate, the vials were thawed in an ice-bath, and to each vial, 600 μL 150 mM (*N*-morpholino)-2-ethanesulfonic acid (MES)/NaOH buffer (pH 6.5), was added. The vials were then vortexed and centrifuged at 15 000 *g* for 10 min at 4 °C. 100 μL of the supernatant was incubated at 37 °C under gently shaking in a water bath for 1 h in a solution containing $FeSO_4$ (0.3 mM), ascorbic acid (aq, 0.1%) and [^{14}C]3-HANA (specific activity of 5.5 mCi/mmol, 5 μM, Astra Arcus AB, final volume 200 μL). Test compounds were added in a volume of 10 μL prior to the substrate. The incubation was terminated by addition of $HClO_4$ (aq., 6%, 50 μL), the tubes cooled on ice and the precipitate removed by centrifugation at 10 000 *g* for 4 min at 4 °C. An aliquot (230 μL) of the supernatant was applied to a Dowex 50W (200–400 mesh) cation-exchange column (0.5 × 2 cm), which was thereafter washed with distilled water (1 mL) to elute the [^{14}C]-QUIN. Scintillation fluid (10 mL, Beckman, Ultima Gold) was added to the eluate, and the radioactivity was determined by liquid scintillation spectrophotometry.

6.3.2. In vivo measurement of 3-HAO inhibition

Male Sprague-Dawley rats (150–200 g) were acclimatized to the animal quarters for at least 7 d before initiation of the experiments. The rats were housed five animals per cage under controlled conditions of temperature (21 °C), relative humidity (55–65%) and light-dark cycle (12:12 h, lights on 6 a.m.). Food and tap water were available ad libitum. The rats were anaesthetized with enflurane (Efrane; Abbott) in a flow mixture of O_2 and N_2O. They were thereafter placed in a stereotaxic frame. The anaesthesia was maintained by free breathing into a mask fitted over the nose of the rat of 3.5–5.0% of enflurane maintained at a flow of 3 L/min of O_2 and 7 L/min of N_2O. Body temperature was kept at 37 °C using a heating pad controlled via a rectal thermometer (CMA/12). The skull was oriented with the horizontal plane passing through bregma and lambda and through the intraural line. The skin over the skull was opened and a unilateral hole on the right side of the skull bone was made by drilling with a 1 mm burr. A Hamilton microlitre syringe (gauge 22S, 25 μL) was lowered 3.9 mm vertically from the surface of the brain at AP 0 mm (bregma) and L 1.0 mm. An intracerebroventricular (i.c.v.) injection of 3, 10, or 300 nmol of test compound together with 10 nmol 3-HANA dissolved in 10 μl 10 mM phosphate buffer, containing 105 mM NaCl, 2.5 mM KCl, 1.18 mM $MgCl_2$, 1.26 mM $CaCl_2$ and 0.1% ascorbic acid was performed for 1 min. The syringe was then removed and the skin was closed by wound clips. At various timepoints after the i.c.v. injection, the rats were decapitated, and their brains rapidly removed and placed on an ice-chilled petri-dish. The right hippocampus was dissected out and stored at –70 °C until QUIN determinations.

For QUIN determination, the samples were homogenized in 10 volumes (w/w) 0.3 M formic acid with [^{18}O]$_4$-quinolinic acid added as internal standard. After filtration, the samples were applied to a pretreated anion-exchanger column (0.5 mL Bio-Rad AG 1 × 8, 200–400 mesh, formate form). The column was washed with water (4 mL) before use. QUIN and internal standard were eluted with 3 mL 6.0 M formic acid. After evaporation, the samples were esterified at 70 °C with hexafluoroisopropanol and trifluoroacetylimidazole for 1 h. The esters were partitioned between water (0.4 mL) and *n*-heptane (3 mL) by whirlimixing for 1 min followed by centrifugation at 1 000 *g* for 5 min. The organic phase was transferred to a new tube and washed with 0.4 mL of water by whirlimixing. After a second centrifugation, the water was discarded and the samples evaporated in a stream of nitrogen at room temperature to about 5 mL. Two μL of the final solution was assayed using gas chromatography coupled to a mass spectrometer (GC/MS). The GC (Carlo Erba MFC 500) was operated in splitless injection mode, and a capillary column (NB-225, 25 m, i.d. 0.32 mm) was used. Helium was employed as carrier gas. The oven was run from 70–135 °C, with a ramp of 20 °C/min. The MS (VG,

TS-250) was adjusted to record m/e 467 (QUIN) and m/e 471 (internal standard) by negative chemical ionization. Methane was used as reagent gas. The peak height ratio (QUIN/internal standard) from standards with known amounts of QUIN was calculated. The calibration curve was plotted as peak height ratio against concentration of QUIN in the standards. QUIN amount in the samples was then calculated from the standard curve.

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