116. Structures and Mutagenic Properties of Products Obtained by *C*-Nitrosation of Opipramol

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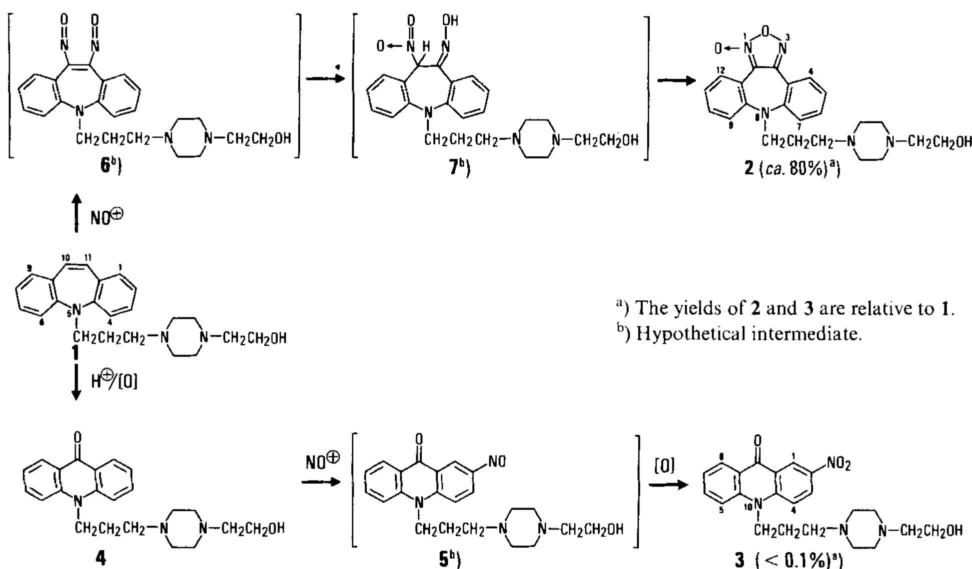
Reaction of the tricyclic psychotropic drug opipramol (**1**) with an excess of HNO_2 affords a product mixture mutagenic for *Salmonella typhimurium*. The main product is a tetracyclic furoxan **2** (yield *ca.* 80%), resulting from nitrosation at C(10) and C(11) of **1**. Compound **2** is not mutagenic. The essential mutagen is a nitroarene **3** formed *via* contraction of the central ring of **1**, and nitrosation at C(2). Its yield is extremely low (< 0.1%). Nitroarenes have previously not been encountered as mutagenic products of the interaction of drugs with nitrite.

Introduction. – Opipramol hydrochloride (**1**·HCl; *Insidon*®) is a well established psychotropic drug [1] [2]. Chemically, it is a tricyclic basic compound possessing the structure of 4-[3-(5*H*-dibenz[*b,f*]azepin-5-yl)propyl]piperazine-1-ethanol dihydrochloride *(cf. Scheme)*. Earlier studies have shown that opipramol and other tricyclic

Scheme. *Proposed Pathways for the Formation of* **2** *and* **3** *by Reaction of Opipramol* (**1**) *with an Excess of Nitrous Acid* in vitro



a) The yields of **2** and **3** are relative to **1**.
b) Hypothetical intermediate.

drugs react with HNO_2 *in vitro* and that the crude reaction mixtures display mutagenic or DNA-damaging activities in several instances [3–10]. The authors imply that these activities be attributable to *N*-nitroso compounds present in the mixtures. However, the structures and properties of individual reaction products have not been explored. Hypothetically, nitrosation can also occur in the human stomach during therapeutic use of a drug. The gastric juice is in fact known to contain nitrite, although at low concentrations only [11–13].

In our laboratories, we recently found that opipramol undergoes *C*-nitrosation with HNO_2 *in vitro*, one of the products being strongly mutagenic [14]. The present paper deals with the structure analysis of nitrosation products of **1** by spectroscopic and chemical methods. It includes an assessment of the quantitative aspects of nitrosation *in vivo*.

Results. – Opipramol (**1**) reacted readily with $NaNO_2$ *in vitro* when treated with a 10-fold molar excess of the reagent at pH 3.5 and 37°. After 4 h, unchanged **1** was no longer detectable by TLC. The organic products were completely extractable from the mixture at pH 8 with Et_2O. Both the crude mixture and the extract showed direct mutagenicity in the *Ames* test [14]. The extract was composed of one major and numerous minor compounds, according to TLC. The major component **2** was obtained by crystallization of its dimaleate salt in a yield of 81% with respect to **1**. By spectroscopic methods, **2** was identified as *8-{3-[4-(2-hydroxyethyl)piperazinyl]propyl}-8*H*-dibenz-*[b,f]*[1]oxa[2,5]diazolo[3,4-*d*]azepine 1-oxide* (for details see below). However, pure **2** proved virtually inactive in the *Ames* test [14].

The main mutagen **3** was isolated by prep. liquid chromatography, starting with *ca.* 20 g of extractable nitrosation products of **1**. The procedure comprised five successive separation stages. At each stage, there was only one fraction with high specific mutagenicity which always contained $\geqslant 77\%$ of the total mutagenicity recovered in all fractions of that stage [14]. Only these highly active fractions were further processed, *i.e.* *Fractions 4, 4.1, 4.1.6, 4.1.6.5*, and *4.1.6.5.2*. The yield of enriched **3** in the last fraction was 2.2 mg. It was identified as *10-{3-[4-(2-hydroxyethyl)piperazinyl]propyl}-2-nitro-9(10*H*)-acri-*

Table. *Spectroscopic Data of Opipramol* (**1**), *and of Compounds* **2** *and* **3** *Formed by Reaction of* **1** *with Nitrous Acid* in vitro

No.	Relevant spectroscopic data
1	^{1}H-NMR (CD_3OD): 7.15 (H–C(3), H–C(7)); 7.05 (H–C(1), H–C(4), H–C(6), H–C(9)); 6.97 (H–C(2), H–C(8)); 6.69 (H–C(10), H–C(11)); 3.76 (CH_2–N(5)); 3.63 (CH_2O); 2.3–2.6, 1.72 (remaining CH_2)
2	EI-MS: 405 ($M^{+\cdot} - O$), 404 ($M^{+\cdot} - OH$), 390 ($M^{+\cdot} - CH_2OH$), 374 ($M^{+\cdot} - O - CH_2OH$), 264 ($M^{+\cdot} - CH_2CH_2$–$(C_4H_8N_2)$–CH_2CH_2OH), 248 (264 – O), 143 (CH_2–$(C_4H_8N_2)$–CH_2CH_2OH) FD-MS: 421 ($M^{+\cdot}$) ^{1}H-NMR (CD_3OD): 8.00, 7.80, 7.2–7.6 (aryl H); 3.89 (*m*, CH_2–N(8)); 3.62 (CH_2O); 2.3–2.6, 1.67 (remaining CH_2)
3	EI-MS: 410 ($M^{+\cdot}$, $C_{22}H_{26}N_4O_4$), 392 ($M^{+\cdot} - H_2O$), 379 ($M^{+\cdot} - CH_2OH$), 362 (392 – NO), 253 ($M^{+\cdot} - CH_2CH_2$–$(C_4H_8N_2)$–CH_2CH_2OH), 207 (253 – NO_2), 157 (CH_2CH_2–$(C_4H_8N_2)$–CH_2CH_2OH), 143 (CH_2–$(C_4H_8N_2)$–CH_2CH_2OH), 139 (157 – H_2O) FD-MS: 410 ($M^{+\cdot}$) ^{1}H-NMR ($CDCl_3$): 9.35 (*d*, *J* = 2, H–C(1)); 8.53 (*dd*, *J* = 7, 1.5, H–C(8)); 8.45 (*dd*, *J* = 9, 2, H–C(3)); 7.81 (*d*, *J* = 9, H–C(4)); 7.80 (H–C(6)); 7.72 (H–C(5)); 7.39 (*td*, *J* = 7, 1.5, H–C(7)); 4.56 (CH_2–N(10)); 3.68 (CH_2O); 2.0–2.8 (remaining CH_2)

dinone. When tested with *Salmonella typhimurium* TA98 and TA100, *Fraction 4.1.6.5.2* had a specific mutagenicity of 17 500 and 10 400 mutants per µg, respectively. The specific activity of pure synthetic **3** was 34 000 and 25 000 mutants per µg [14].

The structures of the nitrosation products **2** and **3** were deduced from the spectroscopic data in the *Table*.

The molecular weight of **2** is established by FD-MS. In the EI-MS, no molecular ion, but only the $M^{+\cdot} - O$ ion is detectable. This observation is often made with *N*-oxides. The basic fragments result from cleavages α to the N-atoms, which is in agreement with the proposed structure. The structure of **2** is corroborated by its ^{1}H-NMR spectrum using **1** as reference compound. The aromatic protons of **2** are shifted to lower field by the introduction of the furoxan (= 1-oxa-2,5-diazol) ring into the molecule. The degeneracy of the aromatic rings is removed, since the mirror plane of the original molecule is no longer present. For the same reason, the CH_2–N(8) protons show two different chemical shifts. The remaining signals of **2** are very similar to those of **1**.

The structure of **3** is established on the basis of its ^{1}H-NMR spectrum using 10-{3-[4-(2-hydroxyethyl)-piperazinyl]propyl}-9(10*H*)-acridinone (**4**) as reference compound. The aliphatic part of the two spectra is almost identical. The aromatic part of the spectrum of **3** indicates that one benzo ring is unchanged, and the other substituted by a strongly electron-withdrawing group (the NO_2 group) in 2-position. The position of the substituent is based on the low-field shift of H–C(1) and H–C(8) in **4** (8.48 ppm, CD_3OD) and in 9(10*H*)-acridinone (8.27 ppm, (D_6)DMSO). Compound **3** gives an intense $M^{+\cdot}$ peak in its EI-MS which is further confirmed by FD-MS. The empirical formula ($C_{22}H_{26}N_4O_4$) is obtained from accurate mass measurement with the peak-matching technique. The fragmentation which generally characterizes an aromatic nitro compound (loss of NO followed by expulsion of CO) is not very pronounced. The main fragments are obtained from α-cleavages at the N-atoms. In its spectroscopic properties, **3** isolated as nitrosation product does not differ from the independently synthesized reference compound **3**.

Discussion. – Reaction of opipramol (**1**) with HNO_2 *in vitro* leads to a non-mutagenic main product **2** and a mutagenic trace component **3**, among other, non-identified products. It can be assumed that the mutagenicity of the original nitrosation mixture is largely attributable to **3**. In bacterial test systems, **3** shows a high mutagenic potency comparable to that of some other nitroarenes [14]. Both, **2** and **3** are formed by *C*-nitrosation rather than *N*-nitrosation.

Compound **2** carries a furoxan (= 1-oxa-2,5-diazol) ring condensed to the tricyclic skeleton of **1**. This ring results from a two-fold attack of HNO_2 at C(10) and C(11). It is likely that the reaction proceeds *via* the dinitrosoalkene **6** which can be converted to the nitrooxime **7** by addition of H_2O and rearrangement *(Scheme)*. Dinitrosoalkenes have independently been proposed as short-lived precursors of furoxans [15] [16], and nitrooximes are established intermediates of the above sequence [17]. The reaction of alkenes with HNO_2 is known since long, although the structure of the resulting furoxans has only been identified with the advent of modern NMR spectroscopy [18–21].

Compound **3** is a nitroacridinone derivative. Its formation begins with a contraction of the 7-membered central ring of **1** yielding the acridinone **4** *(Scheme)*. This reaction has independently been found to occur, when **1** is kept in an oxidative acid medium [22]. In the present study, **4** had in fact been recognized by TLC as a strongly fluorescent by-product at the 3rd separation stage. Nitrosation of **4** yields **5** which is then oxidized to **3**. Nitrosation takes place at C(2), *i.e. para* to the tertiary amino group of the ring system, as it is also known for other tertiary aromatic amines. For example, imipramine forms 2-nitroso-imipramine, when treated with HNO_2 [23]; *N,N*-dialkylanilines form *p*-nitroso *derivatives* [24] [25]. Such nitroso compounds are readily oxidized *in situ* if an excess of nitrite is used [26]. In this reaction sequence, introduction of the NO group is the rate-limiting step.

In our nitrosation experiments, we used one of the established *in vitro* tests [6] [27]. It provides high, non-physiological concentrations of both drug and nitrite, *i.e.* 40 and 400 mmol/l, respectively. Thus, the reaction is strongly promoted which makes it easier to identify the products. However, the maximum concentrations to be expected in the human stomach are only 0.22 and 0.64 mmol/l, respectively. These figures are based on a physiological model [7] [28] which assumes that the recommended single dose (50 mg of **1**·HCl) is given after a meal containing the highest amount of nitrite reported (0.32 mmol). The total volume of the stomach contents is taken as 500 ml.

C-Nitrosation is brought about by the nitrosonium ion NO^+ [29] which arises from nitrite in acid solution. At constant pH, the rate is proportional to the concentrations of the substrate S and HNO_2 as shown by *Eqn. 1*. In the formation of the nitroarene **3**, the intermediate **4** serves as the substrate *(Scheme)*.

$$\text{Rate} = k \cdot [\text{S}] \cdot [\text{HNO}_2] \cdot [\text{H}^+] \qquad (1)$$

Assuming nitrosation to be rate limiting, *Eqn. 1* and the above concentration data allow one to estimate that the formation rate of **3** *in vivo* would be about 10^5 times lower than that *in vitro* under the present conditions.

In conclusion, when opipramol (**1**) is reacted with HNO_2, trace amounts of a mutagenic nitroarene **3** are formed via *C*-nitrosation. This is a hitherto unnoted pathway in the interaction of drugs with nitrite.

Experimental Part

General. Opipramol·HCl (**1**·HCl; pure active substance), 10-{3-[4-(2-hydroxyethyl)piperazinyl]propyl}-9(10*H*)-acridinone (**4**), and 4-(3-chloropropyl)piperazine-1-ethanol dihydrochloride were provided by *Ciba-Geigy Ltd.* (Basle, Switzerland). Chemicals and solvents were of anal.-reagent grade (from *E. Merck*, Darmstadt, FRG, or from *Ciba-Geigy*). The 2-nitro-9(10*H*)-acridinone was prepared as described in [30]. TLC: silica gel *(Merck Si 60, F 254)* was used to characterize samples or fractions containing nitrosation products of **1**, with 1,2-dichloroethane/MeOH/conc. aq. NH_3 80:20:1 (*v/v*) and toluene/EtOH/conc. aq. NH_3/H_2O 60:40:1:1. LC: silica gel (*Merck Si 60;* 0.063–0.200 mm; 160 g) in a glass column was used to purify **2**, with toluene/MeOH 99.75:0.25 and then toluene/MeOH 92.5:7.5. This silica gel also served to separate the crude mixture of nitrosation products on a prep. scale (1st separation stage), on a 67 × 7 cm (i.d.) column with hexane/EtOH/conc. aq. NH_3 60:40:1. High-resolution LC: *LiChrosorb Si 60* (*Merck*, 10 μm) was employed at the higher stages of prep. separation; glass columns (55 × 2.5, 30 × 2.5 and 30 × 1.3 cm i.d.; *Comptronix*, Wohlen, Switzerland), packed according to [31]; with 1,2-dichlorethane/MeOH/conc. aq. NH_3 80:20:1 and 1,2-dichloroethane/cyclohexane/2-propanol/conc. aq. NH_3 in ratios varying from 60:20:20:1 to 40:40:20:1; and flow rates of 2–4 ml/min; metering pump (*Milton Roy LDC 711, Comptronix;* or *Altex 110A, Kontron*, Zürich, Switzerland). UV detector (*CE 212, Cecil*, Cambridge, UK) set at 340 or 280 nm, recorder (*WW 1100, Kontron*), and fraction collector (*Labortec FS 2402, Schmidiger*, Münchenstein, Switzerland, or *Ultrorac 7000, LKB*, Bromma, Sweden). ^{1}H-NMR spectra: *Bruker WM 250* NMR spectrometer. MS: *Varian MAT CH5-DF* mass spectrometer equipped with a combined EI/FI/FD-ion source (EI: 70 eV ionizing energy, 180° source temp., direct sample insertion; FD: 50° source temp., emitters activated with benzonitrile, 18 mA emitter heating current); the empirical formula of the molecular ion of **3** was determined with the peak-matching technique by means of a *Finnigan MAT 212* mass spectrometer (70 eV ionizing energy, 190° ion source temp., resolution = 20000).

Reaction of Opipramol Hydrochloride (**1**·HCl) *with Nitrite.* To a soln. of **1**·HCl (40 mmol, 17.46 g) in H_2O (800 ml), $NaNO_2$ (27.60 g) was added under stirring (molar ratio 1:10 [6]). The soln. was then adjusted to pH 3.5 with 1N HCl (240 ml) and stirred for 4 h at 37°. After cooling to 5°, sat. aq. $NaHCO_3$ soln. was added until a pH of

8 was reached. The mixture was extracted with Et_2O (3 times), the extract dried (Na_2SO_4), and the solvent evaporated.

In a second batch, 58 mmol of **1**·HCl were reacted correspondingly.

Isolation of **2**. The extractable products obtained by nitrosation of 40 mmol of **1**·HCl were dissolved in CH_2Cl_2 (180 ml) and mixed with a soln. of maleic acid (11.3 g) in EtOH (50 ml). The main component **2** of the mixture crystallized as the dimaleate salt in a yield of 81% (with respect to **1**·HCl). The product was chromatographed in free-base form (8 g) as described above and reconverted to the dimaleate salt. The latter was recrystallized in EtOH/H_2O 95:5. The pure **2**·dimaleate, m.p. 155–157°, was used for structure analysis and mutagenicity testing.

Isolation of **3**. The larger nitrosation batch (58 mmol of **1**·HCl) yielded 20.254 g of extractable products. They were submitted to prep. LC (see *General*). At the 1st separation stage, 5 fractions were collected arbitrarily and their mutagenicities determined. The most active *Fraction 4* (3.925 g) was again separated by LC (UV detection), and individual peaks or peak clusters were collected. Mutagenic activity at the 2nd stage was almost exclusively located in *Fraction 4.1* (1.336 g). This process was repeated to further remove inactive by-products. At the 3rd, 4th and 5th stage, mutagenicity was mainly found in *Fractions 4.1.6* (47 mg), *4.1.6.5* (8.7 mg), and *4.1.6.5.2* (2.2 mg), respectively. The weights are corrected for the amounts taken off for mutagenicity testing. For total recoveries of mutagenicity, see [14]. The last fraction contained **3** in enriched form, pure enough for structure analysis.

Mutagenesis Assay. Samples or fractions of nitrosation products were submitted to the *Ames* test [32] with *S. typhimurium* TA98 and TA100 as tester strains. The standard method was modified by including a pre-incubation of the sample with the bacteria. For details of the assay and the evaluation of data, see [14].

*10-{3-[4-(2-Hydroxyethyl)piperazinyl]propyl}-2-nitro-9(10*H*)-acridinone* (**3**). A suspension of 2-nitro-9(10*H*)-acridinone (1.2 g, 5 mmol) in *N,N*-dimethylformamide (50 ml) under N_2 was mixed with powdered K_2CO_3 (2.8 g, 20 mmol) and 4-(3-chloropropyl)piperazine-1-ethanol dihydrochloride (1.7 g, 6 mmol). Heating to 95° for 20 h led to a dark-reddish mixture which was cooled, poured on ice-water (200 ml), acidified with methanesulfonic acid and filtered. After adding an excess of aq. K_2CO_3 soln., the filtrate was extracted with CH_2Cl_2 (3 times). The extract was dried ($MgSO_4$) and evaporated, yielding 0.9 g of crude **3**. Part of the product (43 mg) was purified by LC on a *LiChrosorb* column (30 × 2.5 cm i.d.) with 1,2-dichloroethane/cyclohexane/2-propanol/conc. aq. NH_3 40:40:20:1 and crystallized in EtOH: 18 mg of **3**. M.p. 165° (dec.). Its structure was confirmed by spectroscopic analysis.

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