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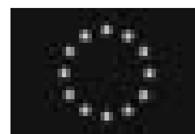

DOTTORATO DI RICERCA IN SCIENZE CHIMICHE

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Xenobiotics in the environment: abiotic transformations and toxicity

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1. Introduction

During recent years intensive agricultural methods and the large scale development of the agrochemical industry has drammatically increased the variety and levels of the agrochemicals in continental and marine natural waters.

The use of pesticides is an integral part of world food production as illustrated by the fact that more than 2.5 million tons of these antropogenic chemicals were applied to soil and foliage in 1996 (Brown L.R. *et al.*, 1996). When applied to a particular location, pesticides can enter ground and surface water in solution, in emulsion, or bound to soil colloids and may impair water for its designated uses. Recent findings cite the presence of these compounds in drinking water (Koplin D.W. *et al.*, 1996). Some types of pesticides are resistant to degradation and may persist and accumulate in aquatic ecosystems modifying their equilibrium by eliminating or reducing populations of organisms, including endangered species. In addition, they can destroy the food source of higher organisms, or reduce the amount of vegetation available for habitat and stabilization of soft sediments. A major source of contamination from pesticide use is a result of their normal application to the soil. Pollution of surface waters also depends on the quantity and nature of pesticides which can be used under different chemical formulations, and on the peculiarities of soils which may be easily permeable and near to the water systems. Other sources of pesticide contamination are atmospheric deposition, spray drift during the application process, misuse, and discharges that may be associated with pesticide storage, handling, and waste disposal.

Pesticides in drinking water and food (Cabras and Angioni, 2000) may have adverse effects for human health: carcinogenesis (Blair A. *et al.*, 1985), neurotoxicity (Tanner and Laangston *et al.*, 1990), effects on cell development (Gray L.E. *et al.*, 1994) are the possible chronic effects deriving from these compounds. The scientific community has shown great concern about these risks, which is supported by results from major monitoring studies performed over 20 years ago (Hörmann W.D. *et al.*, 1979), and confirmed by more recent investigations (Anon, 2000a; Anon, 2000b). With increasing global demand for vegetables, the situation does not look likely to improve. In fact, the current

situation might worsen with the appearance of new substances. In the European Union, the quality of water for human consumption is controlled by many regulations which establish the maximum admissible values of toxic pesticides and their degradation products at very low concentrations: a maximum permissible concentration for a particular pesticide and/or its derivative is 0.01 ppb and 0.5 ppb for the total load of all plaguicides (Prammer B., 1998; World Health Organization, 1993).Environmental concentrations of pesticides and their known metabolites are fixed in the maximum contaminant level parameter (CML) in USA, and in parameter of maximum allowable concentration (MAC) in Canada.The critical nature of this environmental problem has prompted the development of faster and more accurate methods for characterisation and quantization of the pesticides dispersed in the environment. These methods have generally been very successful, but until now, no completely efficient methods have been developed for remediation of contaminated waters (Bryant E.A. *et al*, 1992). Moreover, pesticides are only one component of a group of chemicals which are continually introduced in the environment: among these, pharmaceuticals must also be considered. Studies undertaken in USA, Europe and Canada have detected a wide range of drugs in groundwater, surface water and even drinking water systems (Zuccato E. *et al*., 2000; Jones O.A.H. *et al*., 2002). Levels of pharmaceuticals amount to thousands of tons per year which are similar to the amount of fertilizers and other chemicals used in agriculture (OECD 2001). After administration of drugs, only a limited quantity is assimilated and metabolized by the organisms, the remaining part is excreted and ultimately ends up in waste treatment plants. Most treatment plants are unable to remove drugs so they pass either into surface waters or groundwater. Runoff from farm animal operations contributes a significant amount to drugs into the environment, as do hospital discharges and the aquaculture industry. The widespread occurrence of pharmaceuticals in the aquatic environment explains the detection of their presence in drinking waters (Heberer T. *et al*., 1996; Ternes T.A., 2001). Figure 1 shows possible sources and pathways for the occurrence of pharmaceutically active products in the environment.

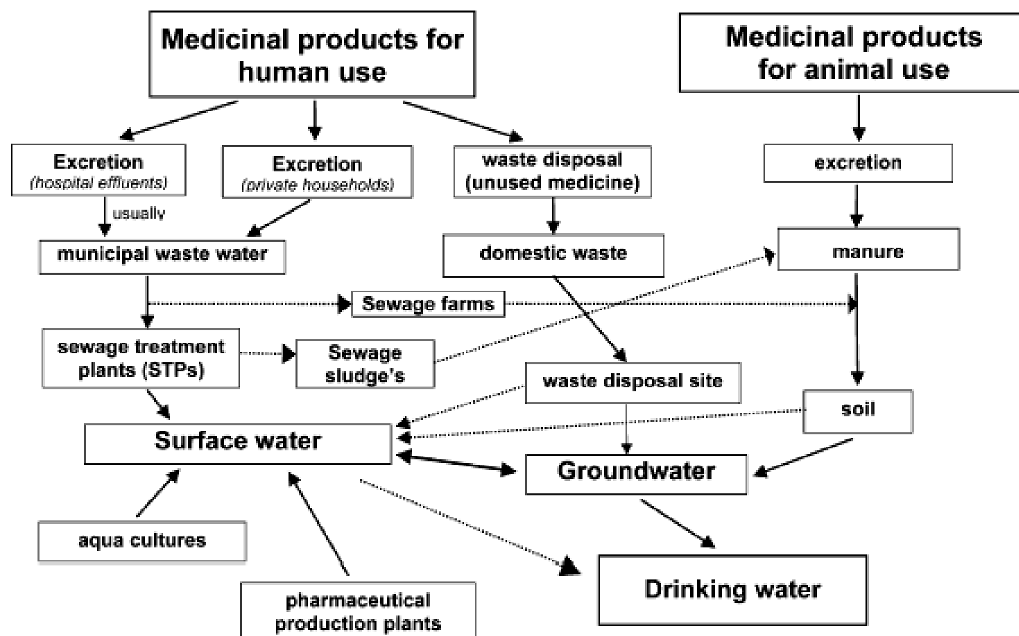


Figure 1. Possible sources and pathways for the occurrence of pharmaceutical residues in the aquatic environment.

The major issues associated with the origins and presence of these chemicals in surface, subsurface, and drinking waters have been featured in a number of reviews, books, and proceedings, among which some recent examples are Daughton (2001), Daughton and Jones-Lepp (2001), Daughton and Ternes (1999), Heberer (2002), Kümmerer (2001). Even if at low concentrations, pharmaceuticals cannot be considered harmless for living species in the environment and their toxicity should be examined (Colburn and Clement, 1992). In fact, it is worth noting that antibacterial drugs used in stock-breeding, have antialgal effects (Henschel K.P. *et al*., 1997) and previously, diazepam has been shown to have adverse effects on the diatoms *Hantzschia amphioxys* and *Surirella robusta* (Spurck and Pickett-Heaps,1994). Thus, the aquatic environment receives composite loads of contaminants including pesticides, pharmaceuticals, veterinary and human antibiotics, industrial compounds, hormones and sterols, in increasing amounts. A chemical-analytical approach to monitoring pollutants is not exhaustive, but it is necessary to know their toxicological effects by using target

aquatic organisms in order to obtain an overall measure of the environmental contamination risk.

Most toxicity assays are presented in the literature as acute toxicity tests. However, it is also necessary to perform chronic tests on target aquatic organisms to better identify whether these compounds have sublethal effects at concentrations which usually range from ng/l to µg/l. It is likely that these low concentrations do not represent an acute risk, but there are no significant data about their chronic toxicity, the active/ passive assimilation of xenobiotics by the organisms, or their accumulation in the tissues and diffusion through the food chain.

From 1998 the US Department of Health and Human Services has required all Federal agencies to assess the environmental impact of approving drugs when the expected concentration at the point of entry into the aquatic environment (EIC) is 1 µg/l or greater (US 1998). A note for the guidance of the European Agency for the evaluation of medicinal products EMEA (2003) states that an application for the marketing authorization for a medicinal product for human use must be accompanied by an environmental risk assessment when the predicted environmental concentration (PEC) is above 0.01 µg/l.

Little is known about the fate of pharmaceuticals and pesticides in the environment and limited researches have until now been conducted on their transformation products from both analytical and toxicological points of view. The disappearance of xenobiotic residues at a given location does not mean the end of the environmental problem, because they can be translocated, bioconcentrated or converted into more dangerous chemicals.

Given the potential human and wildlife health risks associated with toxic chemicals, it is important to have considerable information on their persistence in surface waters and/or in the soil by considering their reaction mechanisms under typical environmental conditions.

In fact, xenobiotics can be subjected to biotic (biotransformation by aquatic organisms such as algae, bacteria) and abiotic (hydrolysis, oxidation, photodegradation) processes in the environment giving derivatives that can be more persistent and more toxic than the parent compounds.

In this regard, the EMEA (European Agency for the evaluation of medicinal products) excludes an environmental risk assessment of metabolites formed at levels below 10%, while for transformation products exceed 10% (major metabolites) the risk assessment is performed using the defined PEC (predicted environmental concentration) and PNEC (predicted no-effect concentration) values. The opinion of the European Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE, 2001) differs from that of EMEA as CSTEE considers inappropriate to define major metabolites by their percentages. They should be those which may produce significant adverse effect on environmental species. In fact, a study on the toxicity of prednisolone, dexamethasone and their photochemical derivatives on aquatic organisms (DellaGreca *et al*., 2004) has evidenced that a photoderivative (5% yield) had a chronic toxicity one hundred times higher than prednisolone on *C. dubia*.

Levels of the pesticide malathion in water as low as 5 parts per million were shown to cause heart defects in certain types of fish, resulting in circulatory defects (Solomon and Weis, 1979). In addition, the metabolic breakdown products of parathion and malathion, paraoxon and malaoxon respectively, have been shown to decrease cell numbers, DNA synthesis and protein synthesis in cell cultures of chick pectoral muscle (Wilson and Stinnett, 1969). Thus, metabolites may be more detrimental than the parent compounds.

Many metabolites are included in the monitoring system of groundwater and surface water. For example, in Dutch underground waters analytical investigations are extended, in addition to the pesticide aldicarb widely used for treatment of soybeans and potatoes, also to its metabolites: aldicarb sulfoxide and aldicarb sulfone. (Bottoni P., 1994). The USEPA (US Environmental Protection Agency), in the context of the National Pesticide Survey, performed between 1985 and 1990, listed pesticides and their respective derivatives with high contaminant potential, which must be searched in underground waters (Table 1). (Funari E. *et al*., 2001)

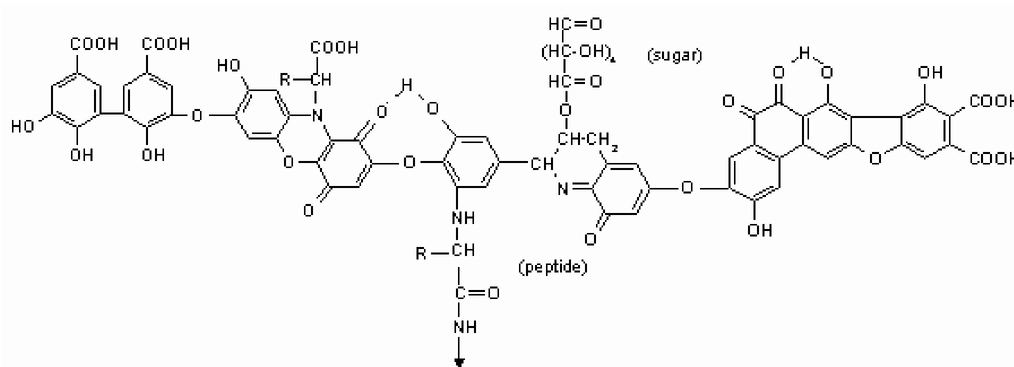
Table 1. Pesticides and their respective degradation products (USEPA) detected in the US underground water systems (National Pesticide Survey). Compounds with high contaminant potential are reported in bold

Acifluorfen	3,5-dichlorobenzoic acid	paraoxon methyl
Alachlor	1,2-dicloropropane	**Metolachlor**
Aldicarb	1,3-dichloropropene *cis*	**Metribuzin**
Aldicarb sulfone	1,3-dicloropropene *trans*	Metribuzin DA
aldicarb sulfoxide	Dichlorprop	metribuzin DADK
Baygon (propoxur)	Dichlorvos	Metribuzin DK
Bentazone	**Dinoseb**	MGK 264
Bromacil	**Diphenamid**	Molinate
Carbaryl	Disulfoton	Napropamide
Carbofuran	disulfoton sulfone	Neburon
3-OH-carbofuran	Disulfoton sulfoxide	4-nitrophenol
phenol carbofuran	**Diuron**	Norflurazon
Phenol-3-keto carbofuran	Endosulfan I	Pentachlorophenol
Carboxin	Endosulfan II	Permethrine *cis*
Chlorothalonil	Endosulfan sulphate	Permethrine *trans*
Chlorpropham	**Heptachlor**	**Picloram**
Cyanazine	**heptachlor epoxide**	Prometryn
Cycloate	EPTC	**Pronamide**
2,4-D	**hexachlorobenzene**	Pronamide metabolite
2,4-DB	hexachlorocyclohesane	**Propachlor**
Dalapon	Etoprop	Propanil
DCPA	**Ethylen bromide**	**Propham**
DCPA acid metabolites	**Fenamiphos**	**Simazine**
4,4-DDT	Fenamiphos sulfone	**2,4,5-T**
4,4-DDD	Fenamiphos sulfoxide	**2,4,5-TP**
4,4-DDE	Fenamirol	**Terbufos**
Diazinon	Linuron	Terbutryn
Dicamba	Methiocarb	Triadimefon
5-OH-dicamba	Methomyl	**Trifluralin**
3,5-dichlorobenzoic acid	**Metoxychlor**	Vernolate

It is obvious, in the light of these data, how the risk associated with the presence of both parent compounds and degradation products into the aquatic environment has become an important issue in environmental chemistry.

With regard to abiotic degradation, various researches (Zafiriou and True, 1979; Zepp *et al*., 1985; Scully and Hoigné, 1987) have revealed that in natural aquatic

environments abiotic transformations such as hydrolysis, and direct or indirect photodegrative processes can occur. Photochemical reactions are most important in the conversion and degradation of pollutants in aquatic systems (Mansour M., 1993a; Mansour M. *et al.*, 1993b; Durand G. *et al.*, 1990), while in the soil they are significant only at surface level (Scheunert I. *et al.*, 1993).
The phototransformation of a pollutant in surface water may result from light absorption by the pollutant itself (direct photolysis) or may be photoinduced by the dissolved natural organic matter or nitrate ions present in the water, as these chromophores are known to photoproduce reactive species (indirect photolysis). Several studies have been reported in the literature (Vialaton D. *et al.*, 1998; Welker and Steinberg, 2000; Krieger M.S. *et al.*, 2000) showing the relative importance of the dependence of the two pathways on the pollutant structure. As sunlight penetrates down into freshwater and marine waters, the great bulk of the radiation is absorbed by natural dissolved or particulate substances. A number of recent investigations has shown that the influence of natural sustances on photoreactions in freshwater and seawater is not limited to light attenuation (Zepp R.G. *et al.*, 1981a; Zepp R.G. *et al.*, 1981b; Wolff C.J.M. *et al.*, 1981). Sunlight-induced reactions involving free radicals may be initiated through photolysis of natural inorganic constituents such as nitrite (Zafiriou O.C., 1983) and hydrogen peroxide (Zika and Cooper, 1983; Draper and Crosby, 1983). Moreover, a significant portion of the solar radiation absorbed by freshwater humic substance results in formation of electronically excited molecules that are capable of participating in a variety of reactions with aquatic pollutants (Zepp R.G. *et al.*, 1981a; Zepp R.G. *et al.*, 1981b). These photosensitized reactions can greatly accelerate the light-induced transformation of trace chemicals in natural waters, in some cases resulting in the rapid photoreaction of compounds that are stable to sunlight in distilled water (Joussot-Dubien and Kadiri, 1970; Zepp R.G. *et al.*, 1981a; Zepp R.G. *et al.*, 1981b). Humic substances are aromatic structures (Figure 2) arising from the degradation processes of the lignins.

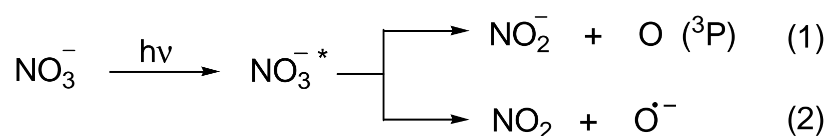


Model structure of humic acid

Figure 2

They may sensitize oxygen and other photoreactions of organic chemicals involving electronic energy transfer (Zepp R.G. *et al.*, 1981a). There is a dearth of information on factors that influence the rates of these processes in aquatic environments. Humic acids are able to absorb organic matter through different mechanisms such as ion exchange, hydrogen bond, Van der Waals strength, modifing solubility, biodegradability, photoreactivity and, hence, the persistence of pollutants in the environment.

The nitrate in aquatic environment has long been known to be involved as an electron acceptor in the biological oxidation of organic sustrates (Hutchinson G.E., 1975). Recent evidence indicates that nitrate ions also promote the photochemical oxidation of trace organic compounds in water (Zepp R. G. *et al.*, 1987). The irradiation of nitrate in its long-wavelength absorption band (maximum 302 nm) results in two primary photochemical processes (Scheme 1):



followed by

Scheme 1

$O^{\cdot -}$ is rapidly protonated to its conjugate acid, the hydroxyl radical (eq 3), a potent oxidant that reacts much more rapidly with most organic chemicals than does atomic oxygen O (^{3}P) (Huie and Herron, 1975). The major fate of the atomic oxygen produced in reaction 1 is likely to be a reaction with oxygen molecular to form ozone. The ozone is rapidly consumed by reaction with NO_2^- (Hoigné J. *et al.*, 1985), or by decomposition to $OH^{\cdot}$ (Hoigné and Bader, 1976; Staehelin and Hoigné, 1985).

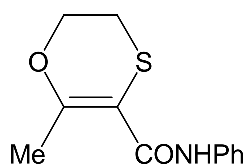
The aims of this thesis have been to study the photolitic and hydrolitic processes of certain xenobiotics and evaluate their toxicity as well as that of their degradation products, since as above reported, toxicological study is meaningful only if it includes both parent compounds and their derivatives.
Investigation has been devoted on some pesticides, in particular on carboxin and carbammates, and on different groups of pharmaceuticals, among these, steroidal anti-inflammatory drugs (prednisolone and dexamethasone) and non-steroidal antinflammatory drugs (naproxen sodium salt), diuretics (furosemide and hydrochlorothiazide), fibrate drugs (bezafibrate, fenofibrate and gemfibrozil), and proton pump inhibitors (lansoprazole and omeprazole). These chemicals have been selected on the basis of their sale and/or their presence into the aquatic environment.
Their abiotic degradation has been studied as close to natural conditions as possible. They have been dissolved (for analytical purposes) or dispersed (for preparative purposes) in aqueous media, using distilled water, distilled water with added nitrates or humic acids, in sewage treatment plant water, and irradiated by a solar simulator or with solar light. In certain cases, photolysis and hydrolysis have also been examined at the different pHs that are possible in polluted aquatic environments. Degradation products have been isolated by chromatographic techniques (silica gel chomatography, TLC, HPLC) and characterized by spectrometric means (one and two dimensional NMR, IR, EI-MS, UV).
The potential environmental impact of the selected xenobiotics and their derivatives has been evaluated, in collaboration with the "Seconda Università

degli Studi di Napoli", by performing acute and chronic toxicity tests on different organisms of the aquatic chain. For the acute toxicity, the bacterium *Vibrio fisheri*, the rotifer *Brachionus calyciflorus*, the anostracan crustacean *Thamnocephalus platyurus* and the cladoceran crustaceans *Daphnia magna* and *Ceriodaphnia dubia* have been used. The chronic toxicity has been evaluated on producers such as algae (*Selenastrum capricornutum*), and primary consumers (*Brachionus calyciflorus, Ceriodaphnia dubia*). Acute and chronic toxicity data are generally expressed as median effective concentrations (LC50 and EC50 in mg/l).

2. Pesticides

2.1.1 Fungicide: Carboxin

Carboxin (**1**) belongs to the class of carboxanilide fungicides used in agriculture for the seed treatment of wheat, barley, flax and cotton prior to planting (von Schmeling and Kulka, 1966) against diseases caused by *Basidiomycetes* (Snel M. *et al.*, 1970). In fact, the mycelium of these microrganisms penetrates deeply into the seed and thus cannot be controlled by superficial protectans.



Carboxin (**1**)

Carboxin reacts with receptors of mytocondrial membrane of fungi which are unable to oxidise succinate and the metabolism of the pesticide affords hydroxylation products which have been identified in both plants and animals. Since it is useful as a seed treatment for food crops, a clear understanding of its fate in the crops was necessary.

Degradation studies in soil (Balasubramanya and Patil, 1980) or in various plant species and animals (Chin W. T. *et al.*, 1970) have shown that carboxin degrades and its main metabolite is the sulfoxide that has a non-fungitoxic activity. Recently studies described the photochemical behaviour of the pesticide when irradiated with UV light (filter Pyrex) in organic solvent (Iesce M.R. *et al.*, 2002a) or in the presence of humic substances and soil (Hustert K. *et al.*, 1999) and with halogen lamp in the presence of sensitizers (Iesce and Cermola, 2002b). These irradiation conditions determine the photooxidative alteration of carboxin and give a variety of photoproducts deriving from the addition of singlet oxygen to the double bond or to sulfur. Sensitizers generally have an acceleration effect on the photolysis of carboxin and this effect was observed experimentally also by

exposing the pesticide to sunlight. These preliminary studies can be used as a starting point to investigate photolytic fate of this pesticide in the environment.

2.1.2 Results and Discussion

*Photolysis of carboxin (**1**)*

A dispersion of carboxin in pure water (20 mg/ 500 ml) was exposed to natural sunlight, under aerobic conditions. After 4 days, the dispersion was extracted with ethyl acetate and the organic and aqueous extracts were analyzed by ^{1}H NMR.

The organic extract was chromatographed by HPLC giving unreacted carboxin (50%) and five compounds (complessively 20%) which were identified as sulfoxide **2**, ketoamide **3**, acetate **4**, disulfide **5**, quinolinone **6** (Figure 3) by spectroscopic means (^{1}H NMR, ^{13}C NMR, MS, IR) and/or by comparison of spectroscopic data with those previously reported (Hustert K. *et al.*, 1999; Hahn H.G. *et al.*, 1995; Corbeil M.A. *et al.*, 1973).

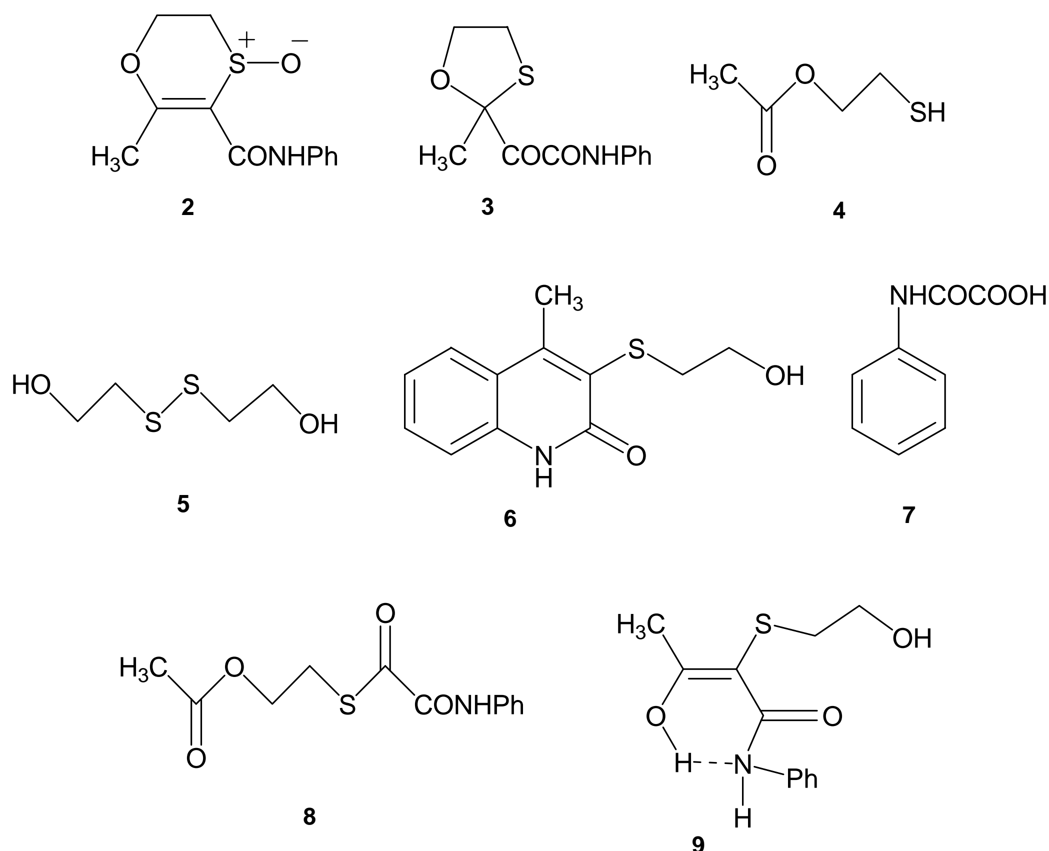


Figure 3

A similar procedure was carried out by sunlight exposing aqueous suspensions of carboxin at pH 2, pH 14, in the presence of humic acid (10 ppm) or in the presence of nitrate salts (5 ppm). The conversion percentage and the composition of each mixture were evaluated by ^{1}H NMR spectrum and HPLC of the organic extract and are reported in Table 2. In acidic conditions ester **8** and enol **9** were also identified. Under all the conditions used, evaporation of the aqueous layer furnished a compound which was spectroscopically identified as oxanilinic acid **7**

Table 2. Product distribution by sunlight irradiation of carboxin in water after 4 days.

Reaction condition[a]	Product distribution[b] (%)							
	1	**2**	**3**	**4**	**5**	**6**	**8**	**9**
pH 7	55	23	5	14	1	2	-	-
pH 10	76	20	3	-	-	1	-	-
pH 2	47	38	7	2	<1	-	5	2
Humic acid[c]	63	24	3	10	<1	<1	<1	-
KNO_3[d]	27	45	5	18	5	<1	<1	-

[a] Suspension of carboxin (20 mg) in 300 ml of water after saturating with oxygen. [b] The percentages have been deduced by ^{1}H NMR of the mixture extracted with ethyl acetate. [c] 10 ppm . [d] 5 ppm.

As shown in Table 2, carboxin is readily photodecomposed by natural sunlight giving mainly sulfoxide **2** and acetate **4**. Ketoamide **3** is also found while disulfide **5**, quinolinone **6** and ester **8** are obtained in very small amounts. Environmental effects such as pH variation or the presence of humate appear to have little influence on photodegradation rate, while a significant increase is observed in the presence of the nitrate, as expected on the basis of its inducing photo-oxidation ability (Zepp R.G. *et al*., 1987).

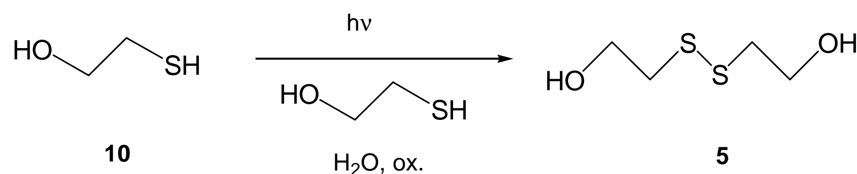
Carboxin was recovered unaltered when the above experiments were performed keeping its dispersions in the dark for four days.

Sulfoxide **2** was identified according to the molecular peak at *m/z* 251 in the EI-MS spectrum and to the pattern of signals in the 2.90 and 3.10 ppm range of the protons of the CH_2SO group correlated to the carbon at δ 43.5 in the HMQC experiment. IR spectrum showed strong bands at 1039 and 1079 cm^{-1} due to the stretching absorptions belonging to the S=O group.

Compound **3** in the EI-MS spectrum had its molecular ion peak at *m/z* 251, and strong peaks at *m/z* 103, due to the fragment $[M-COCONHC_6H_6]^+$, and at *m/z* 148, due to the $[COCONHC_6H_6]^+$ fragment. The multiplet at δ 2.95-3.15 of the CH_2S group in the ^1H NMR, the C-2 quaternary carbon at δ 92.1 in the ^{13}C NMR spectrum, the absence of the IR bands typical of the S=O bond in the range 1030-1100 cm^{-1} were in agreement with the proposed structure of compound **3**.

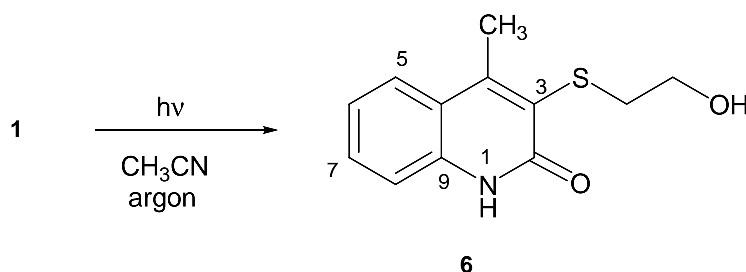
In the EI-MS spectrum of compound **4** peaks were present at *m/z* 87 and 43, belonging to fragments $[M-SH]^+$ and $[M-OCH_2CH_2SH]^+$ respectively.

Compound **5** was identified by comparison of spectral data with those of an authentic compound prepared by exposing aqueous solution of 1,2-mercaptoethanol **10** to sunlight (Scheme 2).



Scheme 2

Quinolinone **6** presented a molecular peak at *m/z* 235 in the EI-MS spectrum. It is a photoisomerization product of carboxin as confirmed by control experiments. In fact, it was synthetized in 20% yields by irradiating carboxin solutions in CH_3CN under argon atmosphere with high pressure UV lamp (Scheme 3).



Scheme 3

In our irradiation conditions, quinolinone **6** was evidenced only in traces. In the EI-MS spectrum, along with the ion molecular peak at *m/z* 235 at low intensity, other peaks were present suggesting a sequence $–SCH_2CH_2OH$. These data were also confirmed by the presence, in the ^1H NMR spectrum, of two triplets at δ 3.04 and 3.70, reciprocally coupled in the H-H COSY experiment, and correlated to the signals at δ 38.8 and 60.2 in the HMQC experiment. Sequence t-d-t-d between δ 7.25 and 7.80 (with couplings of 7.5 Hz) in the ^1H NMR spectrum and four methine aromatic carbons between δ 116.0 and 132.0 in the DEPT experiment were consistent with a 1,2 disubstituted aromatic ring. In the HMBC experiment, the methyl singlet at δ 2.90 gave heterocorrelations with the C-3 olefinic quaternary carbon and the C-10 quaternary carbon. Methyl gave NOE contact with the doublet at δ 7.78 in a NOESY experiment in accordance with its position on the C-4 carbon.

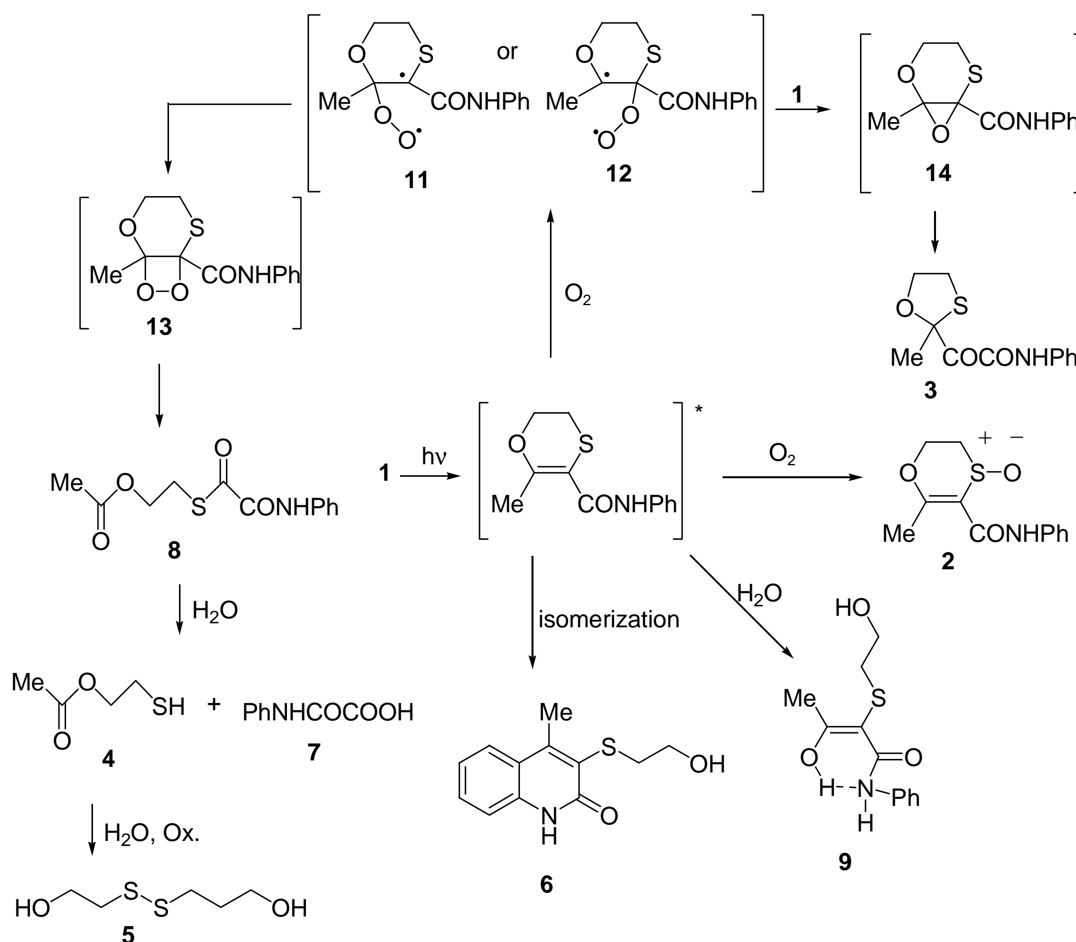
The structure of oxanilic acid **7** was proved by the ^1H NMR spectrum which showed only aromatic protons, and by mass spectrum which revealed peaks at *m/z* 168 and 148 due to the molecular ion peak and to the fragment $[M\text{-}OH]^+$ respectively.

Compound **8** showed the molecular ion peak at *m/z* 267 in the EI-MS. Its ^1H NMR spectrum showed a methyl singlet and two multiplet signals integrated for two protons in the aliphatic region and five protons in the aromatic region, while the ^{13}C NMR experiment revealed two carbons at δ 28.0 and 61.8 corresponding to CH_2S and CH_2O groups respectively, and three carbonyl carbons at δ 155.8 (CON), 170.6 (CO_2) and 191.6 (COS).

Compound **9** had molecular peak at *m/z* 253 in the MS spectrum and its enolic structure was identified by the presence in the ^1H NMR spectrum of the singlet at 15.5 ppm.

The formation of all the products can be explained, on the basis of photo-oxidative transformations, as the main light-induced pathways. According to previous reports (Iesce M.R. *et al*., 2002a), excited states of the pesticide, formed directly by the absorption of the solar radiation [carboxin exhibits an absorption band with a maximum at 292 nm (log ε 3.2)], can react with ground state which

adds to sulfur or to the double bond leading to sulfoxide **2** or to the radicals **11** or **12** (Scheme 4).

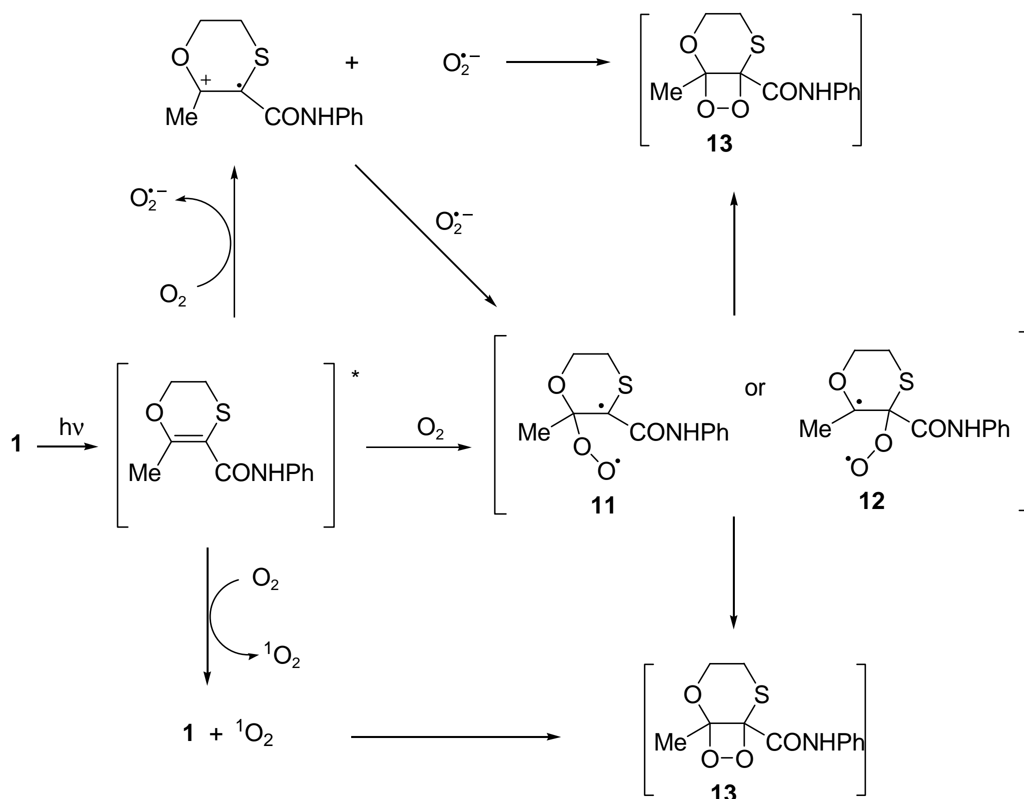


Scheme 4. Mechanism of the photodegradation of carboxin

The radicals **11** or **12** afford ester **8** via the unstable dioxetane **13** while intermolecular reactions should be involved in the formation of ketoamide **3** via the intermediate **14**. As proved by control experiments, hydrolysis of ester **8** leads to acetate **4** and acid **7**. Further decomposition of compound **4** gives disulfide **5**. Quinolinone **6** is a photoisomerization product, in fact it is formed also in the absence of oxygen (Iesce M.R. *et al.*, 2002a). Enol **9** is formed by both acid and light-induced addition of H_2O to carboxin. Indeed, this compound is not found under neutral and basic conditions and carboxin was quantitatively recovered under acid conditions in the dark after 4 days.

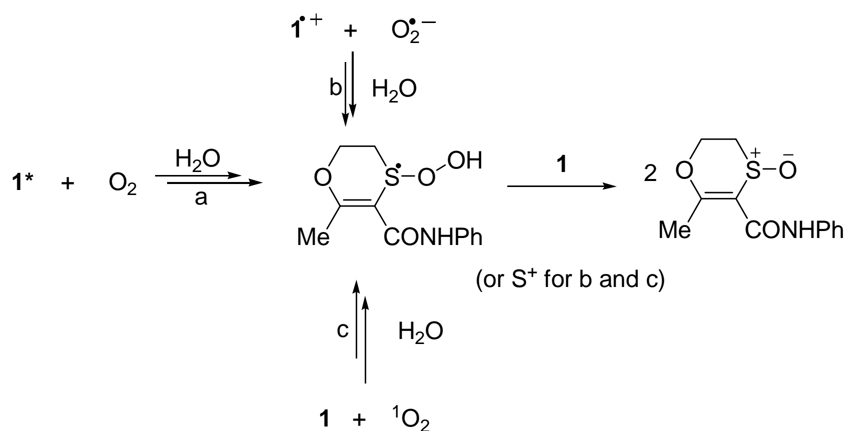
In order to obtain informations about photooxygenation mechanisms of carboxin, irradiation experiments were performed in water in presence of KO_2, which releases superoxide anion, since photooxidation processes might involve electron transfer mechanisms including the formation of the superoxide anion (Coyle, J.D., 1986), or in presence of D-Mannitol, an OH radical scavenger, to verify the involvement of radical reactions, or in presence of DABCO, a singlet oxygen quencher, to verify its role in the photooxidation mechanism.

Reaction mixtures were examined by ^{1}H NMR after exposure to sunlight for four days. No appreciable changes were observed when carboxin was irradited in the presence of KO_2 or D-Mannitol, while the most relevant results were obtained in presence of DABCO which remarkably slowed pesticide degradation. Thus, it is likely that exicited molecules of carboxin generate singlet oxygen which is able to add to double bond (Scheme 5) or to oxidize sulfur atom of carboxin (Scheme 6).



Scheme 5. Photooxigenation mechanisms of carboxin

On the other hand, oxygenated species might oxidize the sulfur atom giving sulfoxide **2** via S-hydroperoxy radical or reactive cation (Bonesi S.M. *et al*., 1998) (Scheme 6).



Scheme 6. Sulfur oxidation

Toxicity studies

Under all the conditions used, sulfoxide **2** is the main photoproduct (20-30%) and it also results highly photostable. Therefore we examined its toxicity on aquatic organisms

Table 3. Toxicity tests of carboxin and its sulfoxide **2** towards aquatic organisms

	L(E)C50 in mg/l for acute toxity tests		L(E)C50 in mg/l for chronic toxity tests		
Compound	*B. calyciflorus*	*T. platyurus*	*D.magna*	*C.dubia*	*P. subcapitata*
carboxin **1**	5.0 (2.60-7.32)	61.00 (55.25-67.35)	22.59 (19.09-26.80)	0.64 (0.52-0.73)	2.41 (2.09-2.77)
sulfoxide **2**	4.10 (2.72-6.19)	56.57 (40.94-78.17)	NE[b] (80 ppm)	0.66 (0.31-0.79)	NE[b] (30 ppm)

[a] 95% confidence limits in brackets. [b] NE = no effect at

Acute toxicity data, expressed as median effective concentrations (LC50 and EC50) of carboxin and its sulfoxide are reported in Table 3.

The photoproduct was found to be as toxic as the parent compound for two organisms tested, *B. calyciflorus* and *T. platyurus,* while no effect was found for *D. magna*.

Chronic tests showed higher toxicity than acute tests (Table 3). From these data it was possible to note that carboxin was bioactive at low concentrations mainly for the primary consumer *C. dubia* (0.64 mg/l) while it was one order of magnitude less inhibent towards algae (2.41 mg/l). No toxic potential for sulfoxide **2** was evidenced for algae at the maximum concentrations of 30 mg/l tested, while it showed a similar activity to that of carboxin towards the crustacean. No phototransformation of carboxin and sulfoxide was found at the end of the experiments with algae, after three days of test solution exposure at 10,000 lux.

2.1.3 Conclusion

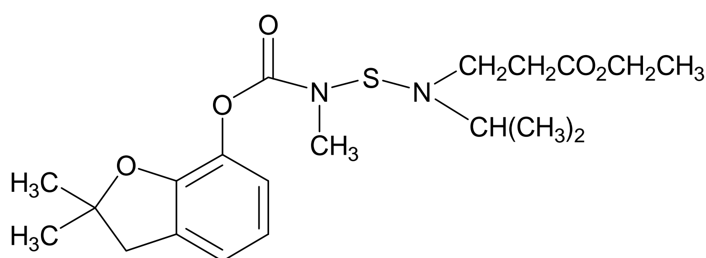
Carboxin is photodegraded by exposure to sunlight in water and, as found in organic solvents, is particularly sensitive to photo-oxidation conditions. Eight photoproducts have been isolated and characterized, confirming previously reported results (Hustert K. *et al*., 1999) and their formations have been rationalized. The main product, which results also the most photostable and the least hydrolizable, is sulfoxide **2**. It should be noted that carboxin is eventually oxidized to sulfoxide **2** in soil, or in various plant species and animals, too (Balasubramanya and Patil, 1980; Chin W.T. *et al*., 1970). The metabolite has a non-fungitoxic activity and, as results from our investigation, exhibits similar or even lower acute toxicity towards aquatic organisms.

2.2.1 Carbamic insecticides: benfuracarb, carbosulfan and carbofuran

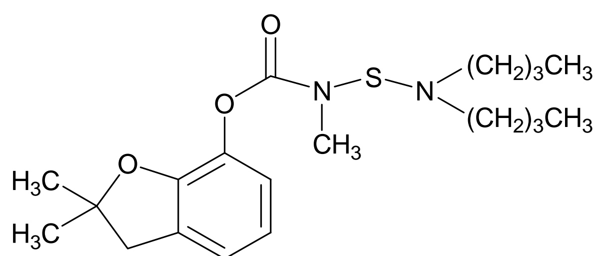
Objective in this study was to determine the main products of hydrolytic and photolytic cleavage of three carbamic insecticides: benfuracarb (**15**), carbosulfan (**16**) and carbofuran (**17**).

N-Methylcarbamate (NMC) insecticides are widely used for crop protection. The reason is that they proved to have a high insect toxicity but a generally low toxicity toward warm-blooded species. In addition, carbamates are much less persistent than organochlorine pesticides and produce fewer toxic degradation products (Bogialli S. *et al.*, 2004). Nevertheless, because carbamates are inhibitors of acetylcholinesterase, they are considered toxic for the environment and for human beings. In particular, carbofuran (**17**) is known to exhibit extreme mammalian toxicity (Fahmy M.A. *et al.*, 1970); thus, it has been classified as highly hazardous. This has compelled the introduction of EU regulations stating that the most toxic carbamates must not be present in fruits and vegetables at levels higher than 50 ng/g.

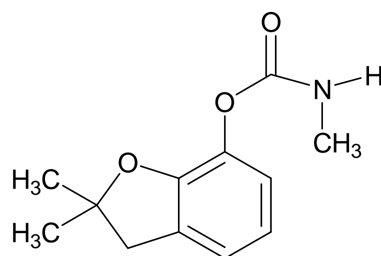
The intensive use of carbofuran could increase the possibility of environmental exposure to this pesticide and the potential major route of exposure to carbofuran is drinking water (Johnson and Lavy, 1995).



benfuracarb (**15**)



carbosulfan (**16**)



carbofuran (**17**)

Degradation has been observed for carbosulfan (**16**) in different buffer solutions (water/methanol ca 3:2 v/v) (Umetsu N. *et al*., 1980) as well as for benfuracarb (**15**) by photolysis in methanol (Dureja P. *et al*., 1990). Much more attention has been addressed to carbofuran which has proven scarcely sensitive to both hydrolysis and photolysis (Burrows H.D. *et al*., 2002). In particular, photodecomposition via C-O heterolysis of the carbamate group followed by ring opening has been observed in water using 254 nm UV light and leads to a substituted cathecol moiety with a tert-butyl alcohol substituent and its corresponding dehydration product (Bachman and Patterson, 1999). Moreover, several photoproducts, mainly deriving from oxidation, methylation, chlorination and rearrangement, have been detected by irradiation in variuos solvents under sunlight (Battacharya A. *et al*., 1994; Raha and Das, 1990).
In this study the behaviour of the three pesticides has been examined in MilliQ water solutions/dispersions using Pyrex tubes in the dark and under sunlight irradiation. All the three pesticides exhibit absorption spectra in the same region (λ_{max} 277-283 nm) with a tail extending to 350 nm. The effect of pH, humic acid and nitrate is also investigated.

2.2.2 Results and Discussion

Transformations of pesticides in water and/or sunlight

Benfuracarb (**15**) (205 mg/l) and carbosulfan (**16**) (190 mg/l) were dispersed in MilliQ water and exposed to sunlight, under aerobic conditions. Each experiment was performed in duplicate, with one set of dark controls. After 6 days, each

reaction mixture was evaporated in vacuum and the residues were analysed by ^{1}H-NMR and by HPLC. Control experiments showed that diluted solutions (4 ppm) of two pesticides afforded similar results.

Benfuracarb was unstable in water, decomposing to carbofuran (**17**) both in the dark and irradiating conditions (Table 4).

Table 4: Hydrolysis /photolysis of benfuracarb (**15**) and carbosulfan (**16**) in different conditions

Condition[a]	Starting pesticide (%)[b] Dark/Sunlight	Degradation products (%)[b] Dark/Sunlight	
	benfuracarb (**15**)	carbofuran (**17**)	Phenol (**18**)
H_2O	79/57	14/21	1/10
pH 5.1	63/55	26/31	-/2
pH 9.0	85/74	6/13	1/6
KNO_3[c]	81/70	13/18	-/3
Humic acid[d]	80/82	11/18	-/2
	carbosulfan (**16**)		
H_2O	93/87	4/5	-/3
pH 5.1	87/82	7/7	-/3
pH 9.0	85/80	11/6	-/4
KNO_3[c]	91/83	5/6	-/8
Humic acid[d]	58/55	27/26	2/7

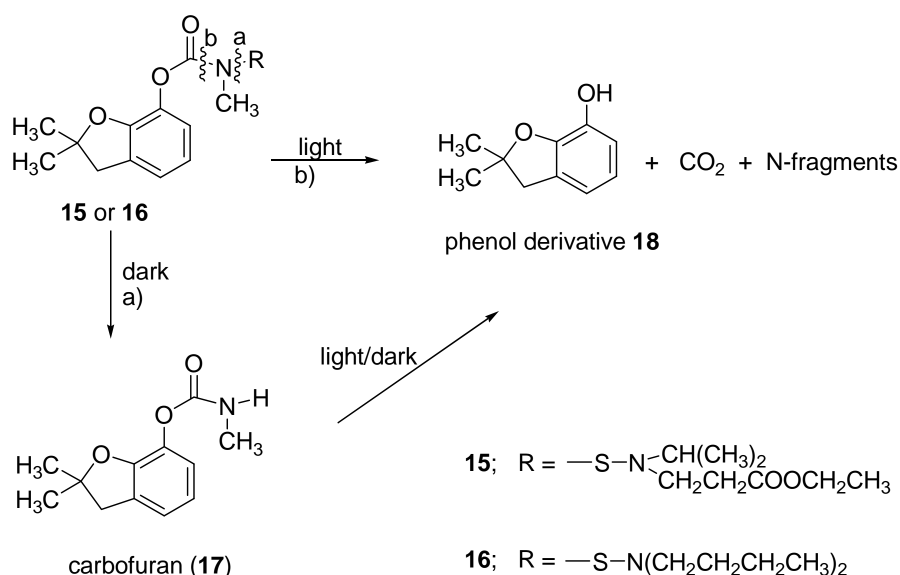
[a]Dispersion of the pesticide in MilliQ water (205 mg/l for **15**; 190 mg/l for **16**); r.t.; Pyrex tube. [b]Deduced by ^{1}H NMR and HPLC. [c]10 mg/l. [d]5 mg/l.

Under sunlight a small amount of phenol derivative **18** was found. It was identified by comparison of ^{1}H and ^{13}C-NMR data with those of an authentic sample obtained by treating carbofuran with methanolic KOH.

The pesticide was not sensitive to small pH variations or to the presence of nitrate or humic acid which give the same products in comparable amounts. Carbosulfan resulted more stable under all conditions examined except in the presence of humic acid, which led to carbofuran and/or phenol (about 40% degradation) either in the dark or in sunlight (Table 4).

These results showed that the cleavage of the weak S-N bond was the main process observed in the dark (Scheme 7). The finding of only carbofuran illustrates the greater lability of the carbamate nitrogen sulfur bond compared with the amino nitrogen-sulfur bond, which is in accordance with previous data reported in the literature (Umetsu N. *et al.*, 1980). Under sunlight, the

photocleavage of light-sensitive (Cameron and Frechet, 1990) carbamate bond also occurs, even to a small extent, leading to phenol derivative **18**. As reported for photolysis of carbamates in water (Givens R.S. *et al.*, 2004; Su and Zabik, 1972) the process should occur with the initial homolytic cleavage of the phenoxy bond to afford the fragments which liberate phenol derivative **18**, CO_2 and nitrogen-containing fragments (Scheme 7).



Scheme 7. Proposed degradation pathway of carbamic pesticides in water

Experiments using the same concentrations of pesticides were carried out at pH 5.0 and 9.0, in the presence of KNO_3 or with humic acid. After 6 days, each reaction mixture was evaporated in vacuum and analyzed by ^{1}H-NMR and HPLC. Both hydrolysis and photochemical processes do not appear to be affected by pH variations or by the presence of additives as humic acid or KNO_3. Only at pH 9 was a slight enhancement of phenol derivative **18** formation observed. The enhanced degradation of carbosulfan in the presence of humic acid might not be due to acidic or sensitizing effects but rather to adsorbtion phenomena which might make the pesticide more susceptible to hydrolysis. The role of suspended sediment or soil on the persistence of pesticides has been observed and appeared not to be strictly related to the chemical structure (Sharom M.S. *et al.*, 1980).

Carbofuran in MilliQ water was treated according to the standard procedure. The presence of only carbamic function made carbofuran more persistent and no appreciable degradation was observed after six days in the dark. Within the same time the degradation rate was barely enhanced by irradiation (Mansour M. *et al.*, 1997; Campbell S. *et al.*, 2004) and led to only about 7% phenol derivative production.

Effects of pH, humic acid and KNO_3 were evaluated by kinetics on dilute solutions in the dark and by UV irradiation and clearly evidenced the enhanced degradation induced by light (Table 5). The photolysis with this lamp is faster than that under sunlight, due to the higher UV lamp intensities compared to the natural light. The results at pH 9 are significant, in fact the basic medium, alone or with light, contributes to promoting the C-O bond cleavage. Experiments performed by flushing the solution with N_2 showed that phototrasformation of carbofuran to phenol does not require aerobic conditions (data not shown).

Table 5: Kinetics of carbofuran (**17**)

	UV[b]		Dark	
Condition [a]	K (h^{-1})	$t_{1/2}$ (h)	K (h^{-1})	$t_{1/2}$ (h)
pH 5.0	1.9×10^{-3}	365	1.0×10^{-4}	6931
pH 7.1	1.4×10^{-3}	495	9.0×10^{-5}	7701
pH 9.0	0.67	1.0	0.21	3.0
milliQ water	1.0×10^{-3}	693	1.0×10^{-4}	6931
humic acid[c]	8.0×10^{-4}	866	9.0×10^{-5}	7701
nitrate[d]	8.0×10^{-4}	866	9.5×10^{-5}	7296

[a]Solution of the pesticide (4 mg/l) in milliQ water; r.t.; Pyrex tube. [b]500W high-pressure mercury lamp (Pyrex filter). [c]5 mg/l. [d]10 mg/l.

Toxicity studies

Acute and chronic toxicity tests were performed on pesticides **15**, **16** and **17** and on their main degradation product, phenol derivative **18**. The otained data are reported in Tables 6 and 7, respectively.

Table 6. Acute toxicity tests L(E)C50 (in mg/l) with 95% confidence range

Compound	*B. calyciflorus*	*T. platyurus*	*Daphnia magna*
15	48% mortality at 200 mg/l	3.66 (2.27 - 5.29)	0.13 (0.11 - 0.15)
16	95.7 (85.5 - 103.4)	8.93 (6.02 – 13.26)	0.004 (0.003 - 0.006)
17	14.1 (13.3 - 14.9)	2.32 (1.53 - 3.51)	0.01 (0.01 - 0.02)
18	55.2 (41.6 - 73.2)	111 (102 - 122)	18.8 (10.4 – 38.0)

Table 7: Chronic toxicity tests EC50 (in mg/l) with 95% confidence range

Compound	*C. dubia*	*P. subcapitata*
15	1.0×10^{-6}	14.1 (10.4 – 26.0)
16	8.2×10^{-8} (5.4×10^{-8} – 1.8×10^{-7})	4.6 (3.4 - 6.2)
17	1.8×10^{-8} (1.2×10^{-8} - 2.6×10^{-8})	2.6 (2.2 - 3.2)
18	4.7×10^{-5} (3.5×10^{-5} - 7.0×10^{-5})	11.4 (10.9 – 18.7)

For all the compounds acute effects were found for concentrations ranging from 2.32 mg/l (carbofuran versus *T. platyurus*) to 48% mortality at 200 mg/l (benfuracarb versus *B. calyciflorus*) suggesting the limited acute ecotoxicity of these compounds. As an exception, *D. magna* was found to be more sensitive, particularly to parent molecules. Chronic results demonstrated that all the tested pesticides had a strong toxic potential for the crustacean *C. dubia* with EC50 values at least two orders of magnitude below the acute toxic level and five orders below the chronic level for the algae. Among the three investigated pesticides, carbosulfan and carbofuran were the most active and phenol derivative **18** was generally less toxic than the parent compounds.

By comparing data, it was found that the various species utilized were not of the same order of sensitivity, suggesting that the investigated pesticides and the phenolic product **18** showed a different toxic impact on non-target organisms.

2.2.3 Conclusion

Benfuracarb and carbosulfan, under natural conditions, decay to carbofuran and/or phenol derivative **18**, while carbofuran gives phenol derivative **18**. The S-N bond breakage occurs easily under all the conditions tested while carbamic bond cleavage is favoured by light and basic media. Accordingly, phenol is formed with difficulty by carbofuran and becomes appreciable at pH 9 and/or by irradiation. The high persistence of carbofuran accounts for the fact that many papers that have reported the detection of this pesticide in water, fruit and vegetables (Bogialli S. *et al*., 2004). Toxicological studies, reported in Tables 6-7, highlight the environmental risk of carbofuran that was found to be the most toxic towards all the exposed organisms.

The different results observed in our reaction conditions, compared with those reported in previous works, are probably due to the the absence of organic solvents, even in a small amounts, which might favour association processes, (Umetsu N. *et al*., 1980; Dureja P. *et al*., 1990; Battacharya A. *et al*., 1994) or the use of more diluted solutions (Raha and Das, 1990) or the use of natural sunlight (Bachman and Patterson J. *et al*., 1999).

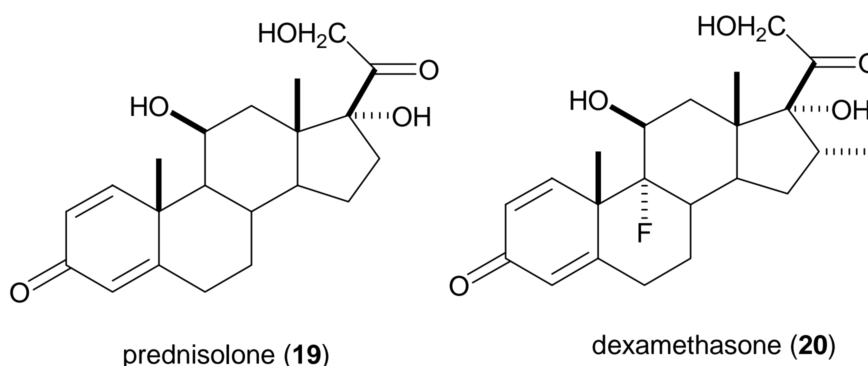
It is interesting to note that phenol derivative **18** has also been found as an enzymatic degradation product from soil microorganisms (Chaudhry and Ali, 1988). This finding may assume importance especially for chronic exposure of aquatic organisms to carbamic pesticides because of their effective concentrations, found to be active for *C. dubia*.

3. Drugs

3.1.1 Steroidal anti-inflammatory drugs: prednisolone and dexamethasone

The aim of this study was to asses the behaviour of prednisolone (**19**) and dexamethasone (**20**) under sunlight irradiation and evaluates their toxicity.

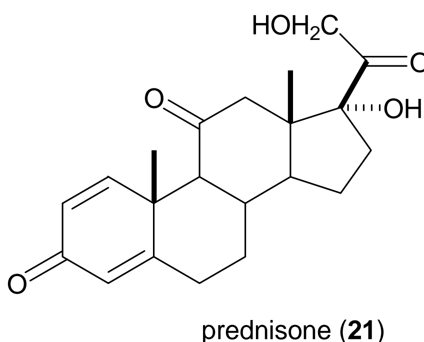
The wide range of pharmacological properties puts corticosteroids among the most widely used drugs in the world. Prednisolone (**19**) and dexamethasone (**20**) are used for their potent anti-inflammatory effects and the former is a metabolite of prednisone (**21**) in man (Maayan R. *et al.*, 1988). Furthermore, both drugs are reported to be sensitive to light (Takacs M. *et al.*, 1991).



prednisolone (**19**) dexamethasone (**20**)

Preliminary studies on photochemical behaviour of corticosteroids were performed by Williams (1979) who focused attention on prednisone acetate. It was sensitive to light in pure solvents such as methanol or dioxane producing several products by ultraviolet radiations.

A recent study on prednisone (**21**), a corticosteroid, (DellaGreca *et al.*, 2003) has shown that this drug undergoes transformation by sunlight giving seven photoproducts.



prednisone (**21**)

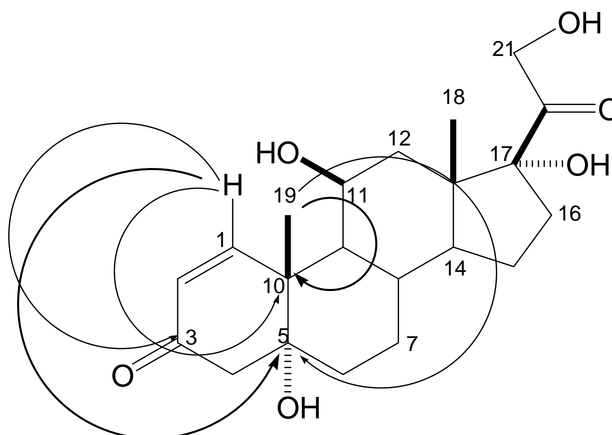
3.1.2 Results and discussion

*Phototransformations of prednisolone (**19**)*

Irradiation of an aqueous suspension of prednisolone (**19**) by a solar simulator for 4 h gave a complex mixture, which was resolved into its components by several chromatographies.

Along with unreacted prednisolone, the photoproducts **22 – 28**, identified by their spectroscopic features, were isolated.

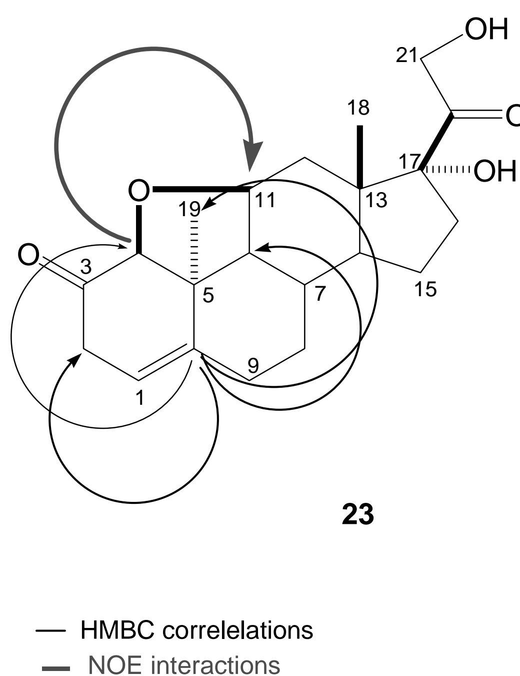
The first compound was identified as the 5α-hydroxyderivative **22**, by comparison of its spectral data with those of the analogous photoproduct of prednisone (DellaGreca *et al.*, 2003). According to the structure, the MS showed a molecular peak at *m/z* 378 for the molecular formula $C_{21}H_{30}O_6$. Furthermore in the HMBC experiment the H-1 proton was correlated to the C-3, C-5 and C-10 carbons and the H-19 protons gave heterocorrelations with the C-5 and C-10 carbons.



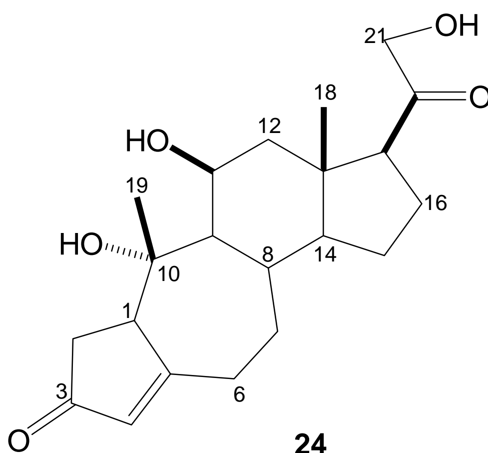
22

Structure **23** was attributed to the second photoproduct. It had molecular formula $C_{21}H_{28}O_5$ according to the molecular peak at *m/z* 360 in its MS spectrum. The ^{1}H and ^{13}C NMR resonances were assigned by combination of COSY, TOCSY, DEPT, HMQC and HMBC experiments. The HMBC spectrum showed the correlations of the C-10 olefinic carbon with the H-2, H-4, H-6, H-7 and H-19 protons, as well as that of the C-3 carbonyl carbon with H-4 protons and that of the C-5 carbon with the H-4, H-6, H-7, H-9 and H-19 protons. The correlation in a

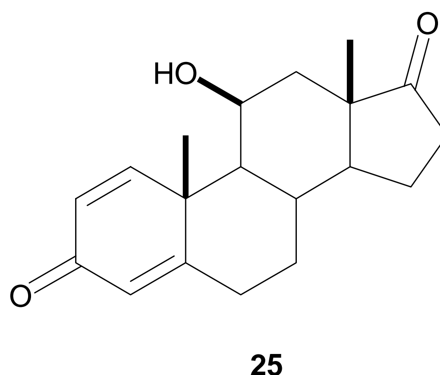
TOCSY experiment between the H-4 and H-11 protons supported the presence of an ethereal bridge between the C-4 and C-11 carbons. The stereostructure of **23** derived from a ROESY experiment. The correlations with the H-18 methyl and the H-21 methylene protons allowed the assignment of the β-orientation to the H-12 proton at δ 2.20 and, consequently, the α-one to the H-12 proton at δ 2.53. These NOE's pointed out a chair conformation of the C ring and the small couplings of the H-11 proton with the H-12 protons agreed with its α equatorial orientation. The correlation of the H-4 proton with the H-11 revealed its α-orientation and, consequently the β-one of the ethereal bridge. Finally the α axial orientation of H-19 methyl was supported by the nOe interaction of its protons with the H-4α proton.



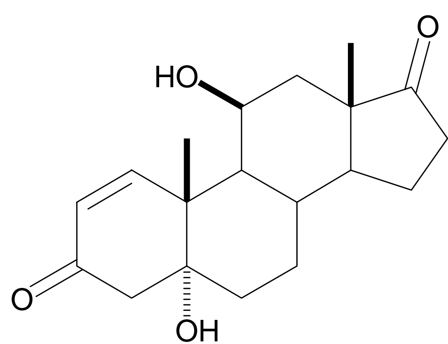
The third compound was identified as **24**. It had the molecular formula $C_{21}H_{30}O_6$ according to the molecular peak at *m/z* 360 in the EIMS spectrum. The ^{1}H-NMR spectrum showed the H-4 olefinic proton at δ 5.90, and the H-19 and H-20 methyls. The ^{13}C-NMR spectrum identified the C-3 and C-5 carbonyl carbons, the C-4 and C-5 olefinic carbons. These data, compared with those of the corresponding photoderivative of prednisone, justified the structural assignment.

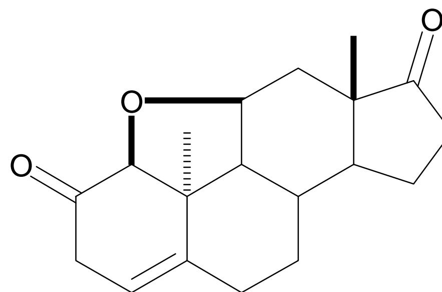


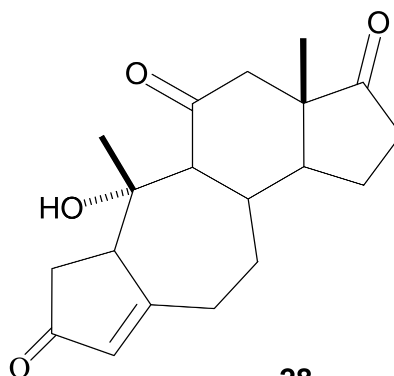
As already verified on prednisone (DellaGreca *et al.*, 2003) light also caused the degradation of the side chain at C-17. In fact the fourth photoproduct was identified as 11β-hydroxy-androsta-1,4-diene-3,17-dione (**25**) by comparison with an authentic sample obtained by MnO_2 oxidation of prednisolone.



Structures **26 – 28** were attributed to the remaining compounds owing to the strong analogies of their physical features with those of **22** – **24** and by comparison of their spectral data with those of authentic compounds obtained from irradiation of compound **25** in same conditions of prednisolone.

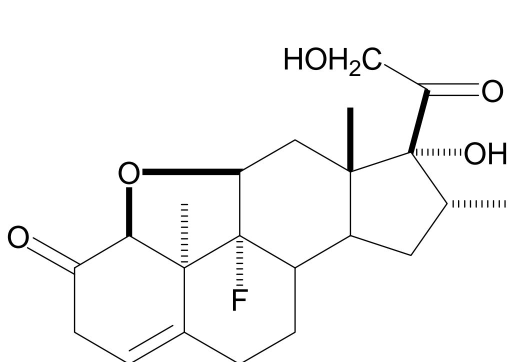


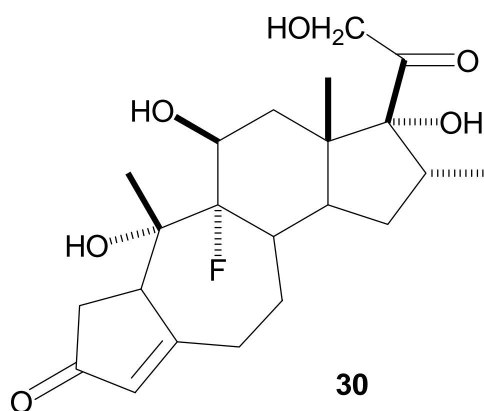




*Phototransformation of dexamethasone (**20**)*

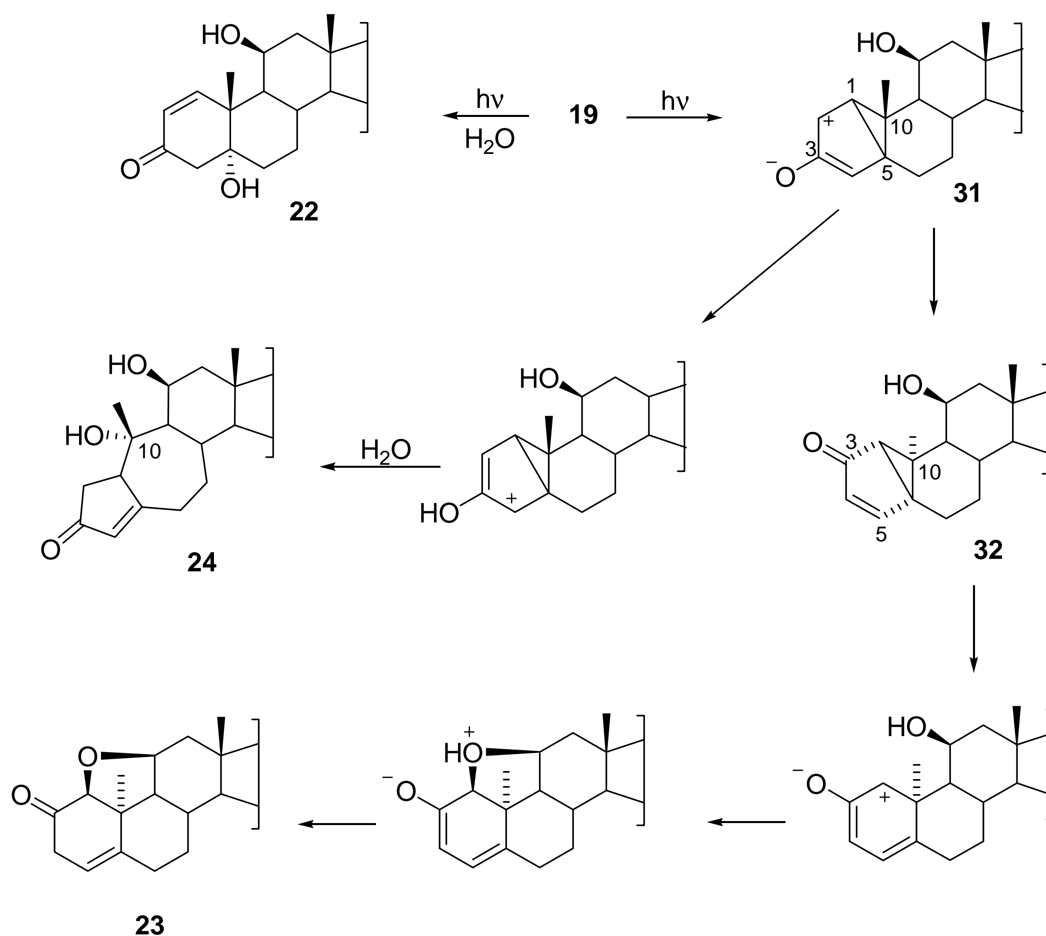
The photochemical behaviuor of dexamethasone (**20**) only partly matched that of prednisone and prednisolone (**19**). In fact, the irradiation with the solar simulator for 8 h of its aqueous suspension converted dexamethasone (**20**) only in 15% amount and photoderivatives **29** and **30** were isolated, without trace of the products obtained by degradation of the side chain at C-17. The data of compounds **29** and **30** compared with those of **23** and **24**, respectively, justified the structure assignments.





The formation of 5α-hydroxyderivative **22** may be easily justified by a photoinduced hydration reaction on the less hindered α-face of the Δ4 double bond (Scheme 8). The formation of prednisolone photoderivatives **23** and **24** finds its explanation in the generally accepted mechanism for photoisomerization of cross-conjugated steroidal dienones (Williams J.R. *et al*., 1979). The light induced formation of cyclopropyl derivative **31**, protonation and subsequent attack of H_2O on the α-face generates **24**.

Isomerization of **31** into lumiprednisolone **32** followed by the attack of the hydroxyl group at C-11 on the C-4 position affords the ether **23**. The phototransformation of **19** into **25** by side chain degradation, and the same steps reported in Scheme 8 for prednisolone, justify the formation of **26 – 28**.



Scheme 8. Mechanism of phototransformation of prednisolone **19**

Toxicity studies

Acute toxicity data are reported in Table 8 for prednisolone and its photoproducts, and in Table 9 for dexamethasone and the respective derivatives. Despite of the high concentrations tested, prednisolone **19** did not demonstrate a measurable value of LC50/EC50 except for rotifers. All the other compounds showed values by the orders of units or dozens of mg/l except compound **27** which is slightly more toxic for the all organisms tested.

Dexamethasone **20** and its derivatives **20** and **30** demonstrated similar activity as prednisolone photoproducts but also for these compounds the concentrations ranged from 10.88 to 60.11 mg/l. These orders of concentration should not present a problem because drug quantities found in the surface waters are usually below parts per billion (Aherne and Briggs, 1989; Raloff J., 1998; Ternes and Wilken, 1999).

Table 8. Acute median effective concentrations concentrations in mg/l (95% confidence limits in brackets) of prednisolone and its phototrasformation products.

Compound	*D. magna*	*T. platyurus*	*B. calyciflorus*
19	NE[a] 85	23% mortal[b] 140	22.29 (20.82-24.56)
22	5.09 (3.98-6.54)	26.53 (18.44-38.13)	15.39 (12.58-18.84)
23	3.80 (2.70-5.33)	22.92 (17.16-30.61)	24.54 (20.82-28.92)
24	17.88 (14.06-22.74)	40.77 (25.18-66.01)	35.46 (30.46-41.29)
25	9.05 (7.20-11.37)	10.79 (8.52-13.67)	9.19 (5.52-15.23)
26	5.74 (4.81-6.85)	10.57 (8.21-13.59)	10.36(7.47-14.37)
27	1.79 (1.38-2.32)	0.71 (0.5-1.0)	1.43(1.14-1.81)
28	11.89 (9.78-14.46)	10.0 (7.58-13.18)	9.96 (8.46-11.74)

[a]NE= no effect at [b]Mortal= mortality at

Table 9. Acute median effective concentrations in mg/l (95% confidence limits in brackets) of dexamethasone and its phototrasformation products

Compound	*D. magna*	*T. platyurus*	*B. calyciflorus*
20	48.30 (39.91-58.45)	60.11 (44.21-81.73)	48.22 41.37-56.20)
29	10.88 (7.28-16.26)	20.9 16.49-26.50)	13.20 11.43-15.23)
30	17.82 (13.84-22.94)	30.52 25.54-46.66)	44.66 38.91-51.25)

The long-term effects are shown in Tables 10 and 11. The detected drugs demonstrated a different toxic potential depending on the organism tested. Daphnies were found to be significantly more sensitive than algae. This result was particularly evident for the parental drugs where a median effective concentration of 0.23 mg/l was found for prednisolone on *C. dubia* against no effect at 160 mg/l for *P. subcapitata*.

Also dexamethasone, while it inhibited *C. dubia* 50% population growth at 0.05 mg/l, showed no effect on the algal growth at 100 mg/l. In this study algae showed median effective concentrations of the same order of magnitude as LC50 and EC50 found for acute toxicity tests. Compound **27** evidenced acute values for the all biota tested less than the chronic ones found for the algae. Both the photoderivatives of prednisolone and dexamethasone showed effects on C. dubia that lead to long term action, except compound **26** that was one hundred and ten times more active than prednisolone and compounds **27** and **28** respectively. Other significant differences were not expressed. These chronic data differ from those of prednisone and its photoderivatives (DellaGreca *et al*., 2003) where no toxicity was found at concentrations harmful for the aquatic environment.

Table 10. Chronic median effective concentrations in mg/l (95% confidence limits in brackets) of prednisolone and its phototrasformation products.

Compound	*C. dubia*	*P. subcapitata*
19	0.23 (0.16-0.28)	NE[a] 160
22	0.22 (0.16-0.30)	27.46 (25.07-30.07)
23	0.12 (0.07-0.19)	30.42 (28.10-32.94)
24	0.22 (0.14-0.35)	24.65 (21.32-28.50)
25	0.51 (0.31-1.16)	14.14 (8.68-23.03)
26	0.007 (0.00026-0.026)	19.84 (17.98-21.88)
27	0.04 (0.018-0.06)	23.78 (11.75-48.13)
28	0.025 (0.014-0.038)	25.62 (20.32-28.90)

[a]NE= no effect at

Table 11. Chronic median effective concentrations in mg/l (95% confidence limits in brackets) of dexamethasone and its phototrasformation products.

Compound	*C. dubia*	*P. subcapitata*
20	0.05 (0.042-0.076)	NE[a] 100
29	0.13 (0.11-0.15)	12.15 (8.96-16.49)
30	0.06 (0.04-0.08)	40.75 (36.35-45.69)

[a]NE= no effect at

3.1.3 Conclusion

Prednisolone and dexamethasone are transformed by sunlight giving seven and two photoproducts, respectively.

Chronic exposure to this class of pharmaceuticals causes inhibition of growth population on the freshwater crustacean *C. dubia* while the alga *P. subcapitata* seems to be less affected by the presence of these products. The low values of acute toxicity found for *B. calyciflorus*, *D. magna* and *T. platyurus* do not determine an acute environmental risk. Photoderivatives showed higher toxicity than parental compounds but the order of magnitude of effective concentrations was lower than the drug quantities generally found in surface waters.

3.2.1 Non-steroidal antinflammatory drug: naproxen sodium salt

Naproxen toghether with benoxaprofen, carprofen, ketoprofen, tiaprofenic acid and suprofen, is a 2-arylpropionic acid derivative (Figure 4), and belongs to nonsteroidal anti-inflammatory agents. Their photophysical and photochemical properties were reviewed *in vivo* experimental studies (Ophaswongse and Maibach, 1993) in order to understand their photobiological properties and to explain (or, in the case of new drugs, to predict) the appearance of photosensitizing side effects.

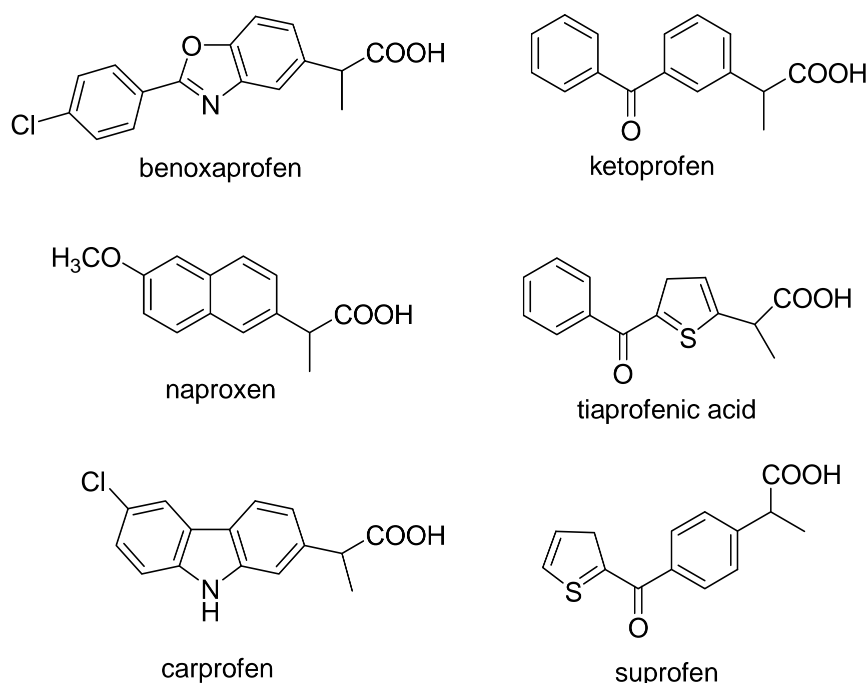
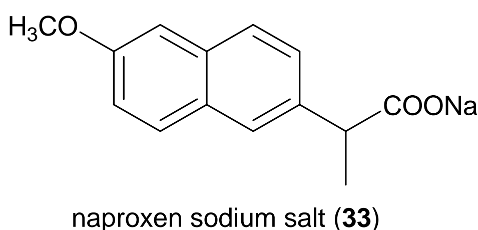


Figure 4. Nonsteroidal anti-inflammatory drugs (NSAID) with photosensitizing side effects

Naproxen and its water-soluble sodium salt (**33**) are used for oral administration as tablets or suspensions.



naproxen sodium salt (**33**)

According to IMS Health Canada Ltd (2002), Canadian physicians wrote almost 2.5 million naproxen prescriptions in 2001 corresponding to more than 26 tons of the drug. Pharmacokinetic studies on naproxen have shown that approximately 95% of the dose is excreted in the urine (Boost G., 1975), so that naproxen may be considered a major organic pollutant. As a result, studies run in Germany, Italy, and other countries have reported the presence of the drug in rivers at median concentrations higher than 2.5 mg/l (Ternes T.A., 1998). Further, Boscá *et al*. (1990) have studied the photodegradation of the drug under different conditions, on the basis of previous investigations showing that naproxen and other non-steroidal anti-inflammatory drugs are phototoxic in vivo (Diffey B.L. *et al*., 1983; Ljunggren and Ludberg, 1985). The authors (Boscá F. *et al*., 1990) reported data on irradiation of naproxen as either free acid or carboxylate ion (**33**) in aqueous oxygenated solutions affording the ethyl derivative **34**, the carbinol **35**, the ketone **38**, and the olefin **39** (Figure 5).

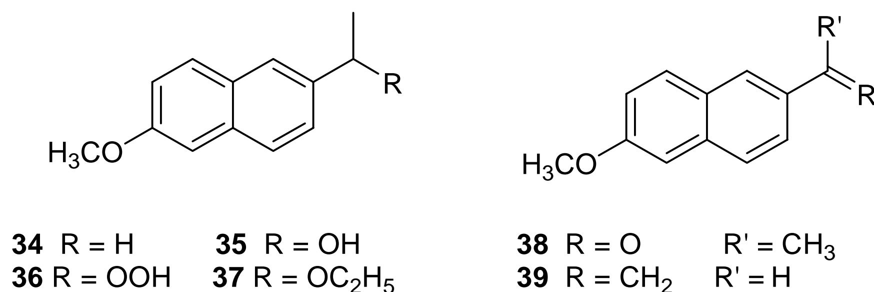


Figure 5. Photoproducts of naproxen Na in distilled water

3.2.2 Results and discussion

*Phototransformation of naproxen Na (**33**)*

Irradiation of naproxen sodium salt was conducted in distilled water, and then in drinking water by a solar simulator. Water was evaporated in vacuum and residues purified on preparative TLC.

First, in distilled water, irradiation of a solution of naproxen sodium salt (**33**) with a solar simulator for 72 h quantitatively transformed the drug into photoproducts

34, **35**, **38**, and **39** (Figure 5). As reported in the literature (Boscá F. *et al*., 1990), the formation of **35** and **38** was explained as the result of the oxygen trapping by a benzyl radical intermediate and subsequent breaking down of the unstable hydroperoxide **36**.

The products, isolated by chromatographic methods, were identified on the basis of their spectroscopic data (Boscá F. *et al*., 1990). To isolate hydroperoxide **36**, the reaction was stopped after 4 h and only ketone **38** (4%) and the hydroperoxide **36** (6%) were isolated. The latter compound, positive to KI and identified on the basis of its spectral data, was quantitatively transformed into alcohol **35** and ketone **38** by its irradiation under the previously described reaction conditions.

Second, in drinking water, naproxen sodium salt (**33**) was transformed, after 72 h in 84% amount, into photoproducts **35** (15 %), **37** (5 %), **38** (17 %), **39** (4 %), **40** (9 %), **41** (2 x 9%).

Compound **37** showed the molecular ion at *m/z* 230 in the electronic impact mass spectrum and fifteen carbon signals in the ^{13}C NMR spectrum according to the molecular formula $C_{15}H_{18}O_2$. The presence of the ethoxyl group at C-11 was justified by the presence of the methylene quartet at δ 3.39, coupled with the methyl triplet at δ 1.20 in the ^{1}H NMR spectrum and by the presence of the methylene carbon at δ 63.9 and the methyl carbon at δ 15.4 in the ^{13}C NMR spectrum.

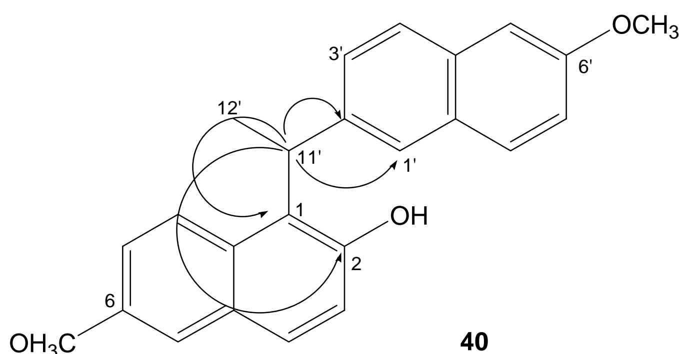
The MALDI-TOF mass spectrum of compound **40** showed the molecular peak at *m/z* 358 in agreement with the molecular formula $C_{24}H_{22}O_3$. In the ^{1}H NMR spectrum eleven aromatic protons were present which, on the basis of their multiplicities and a COSY experiment, were attributed to three ABX and one AB system (Table 12). Besides, two methoxy methyl groups, a methyl doublet and a methine quartet were also present in the spectrum.

Table 12. NMR data of compound **40**

C	DEPT	δ_C	δ_H	HMBC
1	C	123.9		
2	C	150.3		
3	CH	120.0	6.98 d (9.0)	C-1 C-10
4	CH	127.9	7.59 d (9.0)	C-2 C-5 C-9
5	CH	107.2	7.16 d (2.5)	C-4 C-6
6	C	155.6		
7	CH	119.1	7.16 dd (2.5, 9.0)	C-6 C-9
8	CH	124.1	8.12 d (9.0)	C-1 C-6 C-10
9	C	129.0		
10	C	128.1		
OCH_3	CH_3	55.5	3.91 s	
1'	CH	124.1	7.88 d (2.0)	
2'	C	138.9		
3'	CH	127.1	7.28 dd (2.0, 9.0)	C-1' C-10' C-11'
4'	CH	127.4	7.64 d (9.0)	C-2' C-5' C-9'
5'	CH	105.8	7.10 d (2.5)	C-4' C-6' C-9'
6'	C	157.8		
7'	CH	118.9	7.16 dd (2.5, 9.0)	C-6' C-9'
8'	CH	129.3	7.74 d (9.0)	C-1' C-6' C-10'
9'	C	128.1		
10'	C	133.4		
11'	CH	35.3	5.22 q (7.0)	C-1 C-1' C-2 C-2' C-12'
12'	CH_3	17.3	1.87 d (7.0)	C-1 C-2' C-11'
OCH_3	CH_3	55.5	3.92 s	

[a] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by multiplicity and the coupling constants (J in Hz)

The ^{13}C NMR spectrum showed only twenty-one carbon signals, being the signals at δ 128.1, 124.1 and 55.5 integrated for two carbons in an inverse-gated experiment. On the basis of an HMQC experiment the protons were correlated to the corresponding carbons. According to the structure, in the HMBC spectrum, the H-11’ proton was correlated to both aromatic moieties (C-1’, C-2’, and C-1, C-2).



Two compounds with different Rfs (petroleum ether/ acetone 9:1) were isolated from irradiation mixture. Spectral data of these compounds are reported in Table 13.

In the MALDI-TOF spectrum both of them showed the molecular peak at m/z 386 and also in the EI-MS the peak at m/z 185, due to the cleavage of the C–O bond. The compounds exhibited in the ^{1}H NMR spectra (Table 13) six aromatic protons for two ABX systems, a methine proton as a quartet, three methyl protons as doublet and the methoxyl methyl.

Table 13. Nuclear magnetic resonance data of compounds **41**

C	δ_C (Rf = 0,77)	δ_H (Rf = 0,77)	δ_C (Rf = 0,69)	δ_H (Rf = 0,69)	HMBC
1	124.8 (t)[a]	7.65 d (2.0)	124.9 (t)[a]	7.58 d (2.0)	C-8 C-10 C-11
2	139.4 (q)		139.2 (q)		
3	125.2 (t)	7.38 dd (2.0 9.0)	125.1 (t)	7.46 dd (2.0 9.0)	C-1 C-10
4	128.7 (t)	7.66 d (9.0)	127.2 (t)	7.77 d (9.0)	C-2 C-5 C-9
5	105.7 (t)	7.10 d (2.5)	105.8 (t)	7.17 d (2.5)	C-4 C-6 C-7
6	157.5 (q)		157.6 (q)		
7	118.6 (t)	7.14 dd (2.5, 9.0)	118.8 (t)	7.17 dd (2.5, 9.0)	C-5 C-9
8	129.3 (t)	7.66 d (9.0)	129.3 (t)	7.72 d (9.0)	C-1 C-6 C-10
9	128.7 (q)		128.7 (q)		
10	133.9 (q)		134.1(q)		
11	74.5 (t)	4.69 q (7.0)	74.6 (t)	4.41 q (7.0)	C-1 C-2 C-13
13	22.8 (p)	1.56 d (7.0)	24.6 (p)	1.46 d (7.0)	C-2 C-11
OCH_3	55.3 (p)	3.91 s	55.3 (p)	3.95 s	

[a] Letters p, s, t and q, in parentheses indicate, respectively, the primary, secondary, tertiary and Quaternary carbons, assigned by DEPT. [b]^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by multiplicity and the coupling constants (J in Hz)

The ^{13}C NMR spectra showed 13 carbon signals defined in a DEPT as seven methines, two methyls and two quaternary carbons. HMQC and HMBC experiments defined the structures. Spectral data of two compounds fit structure **41** exactly and, in particular due to the small differences in the ^{1}H and ^{13}C chemical shifts, with the two diastereomeric forms (d,l and meso forms) as expected, owing to the presence of the two stereogenic centers (Figure 6).

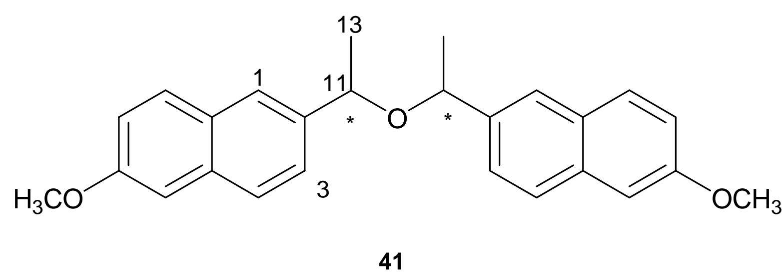


Figure 6

The main difference between irradiation in drinking and distilled water was the formation of **40**, and dimers **41**. In the light of the above data, it should be deduced that the formation of these compounds is due to the action of inorganic salts present in the drinking water. Dimer **40** could be formed from **36** through a photochemical process, resembling the formation of phenol from cumene hydroperoxide (Osamu F., *et al*., 2001). This step, which introduces the hydroxyl function on the naphtalene ring, could be followed by a second step consisting of the reaction of the above intermediate with the benzyl radical. The same **36** could be taken into account in the formation of naphtene [1,1'-oxydiethylidene]bis **41**. The cleavage of the O-O bond of the hydroperoxide (Alcantara R. *et al*., 2000) followed by the coupling with the benzyl radical produces the dimers.

Toxicity studies

The photoproducts obtained in more than 5% yield have been investigated for their potential environmental risk. Acute toxicity data of naproxen sodium salt and their photoderivatives to the different aquatic organisms are reported in Table 14. LC50 and EC50 values for all compounds ranged between two orders of magnitudes (1–100 mg/l) for all species tested. Photoproducts were significantly more toxic than the parent compound. The highest activity was registered for compound **40** towards rotifers (4.51 mg/l). Dimers **41**, tested on the crustacean *D. magna*, showed different toxicity, thus evidencing the role of stereostructure-activity relationship.

Table 14: Acute toxicity tests L(E)C50 (in mg/l) with 95% confidence range

Compound	*V. fischeri*	*B.calyciflorus*	*T. platyurus*	*C. dubia*	*D. magna*
33	42.95 (38.01-53.11)	54.64 (35.9-83.1)	43.54 (35.35-53.62)	43.64 (34.64-54.96)	59.44 (44.14-80.04)
35	20.61 (19.49-21.81)	14.46 (11.5-18.19)	14.01 (11.7-16.77)	16.49 (10.20-26.44)	12.61 (6.87-23.15)
37	NE 50	11.37 (9.43-13.71)	5.30 (4.60-6.12)	10.09 (8.63-11.81)	10.51 (8.21-13.45)
38	16.17 (14.68-17.82)	9.45 (8.06-11.07)	8.23 (7.21-9.39)	16.70 (13.55-20.58)	13.65 (10.08-18.63)
40	30.41 (21.08-35.45)	4.51 (3.78-5.39)	11.63 (10.02-13.51)	6.30 (1.55-25.59)	6.43 (4.00-10.33)
41 (Rf=0,77)	ND	ND	ND	ND	NE 60
41 (Rf=0,69)	ND	ND	ND	ND	50.00 (44.60-57.80)

NE= no effect at; ND = not determined

As expected, chronic tests showed higher toxicity than acute tests. From the chronic data, reported in Table 15, it was possible to note that the class of compounds tested was bioactive at low concentrations mainly for the primary consumers *B. calyciflorus* and *C. dubia*. Algae showed toxicity values two orders of magnitude lower than rotifers and crustaceans. Even if the alga *P. subcapitata*

appeared not to be very sensitive to naproxen sodium salt, photoderivatives showed a significant difference in toxic activity in comparison with parent compound. The photoderivatives revealed the greatest effects on *C. dubia* with compounds **37** and **40** showing the lowest EC50s, respectively 0.026 mg/l and 0.062 mg/l.

Table 15: Chronic toxicity tests EC50 (in mg/l) with 95% confidence range

Compound	*P. subcapitata*	*B. calyciflorus*	*C. dubia*
33	39.31 (33.16-46.61)	0.79 (0.64-0.89)	0.68 (0.39-1.32)
35	6.86 (5.05-9.31)	0.25 (0.14-0.35)	1.06 (0.46-2.65)
37	1.9 (1.14-3.16)	0.45 (0.10-0.86)	0.026 (0.015-0.064)
38	3.86 (2.93-5.08)	0.46 (0.10-0.95)	0.10 (0.07-0.16)
40	ND	0.67 (0.55-0.87)	0.062 (0.01-0.09)

ND = not determined

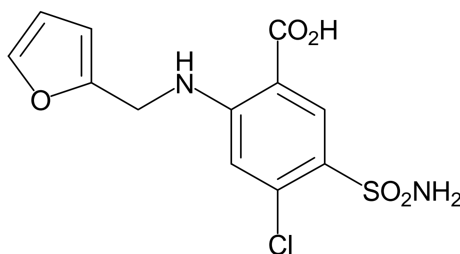
3.2.3 Conclusion

Naproxen sodium salt was found to be light sensitive in biomimetic conditions affording several photoproducts. Toxicity data indicate that exposure of aquatic organisms to the parental drug and its photoderivatives caused effects at mg/l concentrations. These concentrations do not represent the amounts expected in aquatic environment, but they might be found in sewage treatment plants where the daily load of naproxen is in the order of grams (Ternes T.A., 1998).

3.3 Diuretics: furosemide and hydrochlorothiazide

3.3.1.1 Furosemide (42)

Furosemide is a potent diuretic used to treat high blood pressure and some pathologis including heart or liver diseases.



furosemide (**42**)

Furosemide is a white to off-white odourless crystalline powder slightly soluble in water. It is among the most worldwide prescribed pharmaceuticals. About 90% of the intake drug is excreted as parent compound and its presence in the Northern Italy rivers Po and Lambro has been recently reported (Calamari D. *et al.*, 2003). In this work a mass balance was made in both rivers to compare predicted environmental concentration (PEC) and measured environmental concentration (MEC) of several pharmaceuticals. The predicted concentrations were obtained by dividing the theoretical loads (annual sales loads corrected for the metabolic rates) by the average flow rate of the rivers at each sampling site. The MEC/PEC ratio for furosemide was about 0.3 in both rivers. As stated in the article when the MEC/PEC ratio is in the range 0.01-0.3, the ratio is possibly affected by the behaviour of the drug and the extent of its degradation in the environment.
Photochemical studies on the drug, which exhibits absorption spectrum in the sunlight region above 280 nm (λ_{max} 330 nm), have been performed under a variety of irradiation conditions and have evidenced its high photodegradability. Reduction, dechlorination, hydrolysis, decarboxylation, oxygenation (Moore and

Sithipikas, 1983; Bundgaard H. *et al.*, 1988; Zanocco A. *et al.*, 1998) have been found to occur, depending on the reaction conditions and, in many cases, the related photoproducts have been isolated and characterized.

3.3.1.2 Results and Discussion

*Phototransformations of furosemide (**42**)*

A solution of furosemide (**42**) (24 μM) in distilled water was irradiated for 36 hr by a solar simulator.

Reverse phase C-18 HPLC analysis of the reaction mixture after 36 hr showed the presence of a transformation product (Figure 7) which, by repeating the reaction starting from a 0.6 mM solution, was isolated by silica gel flash column chromatography, purified by HPLC and identified as dimer **43** on the basis of its spectroscopic data.

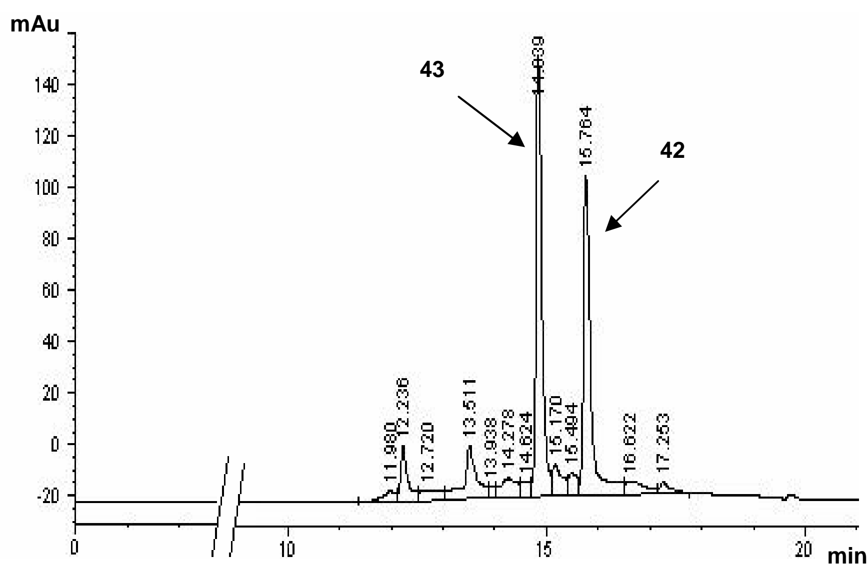
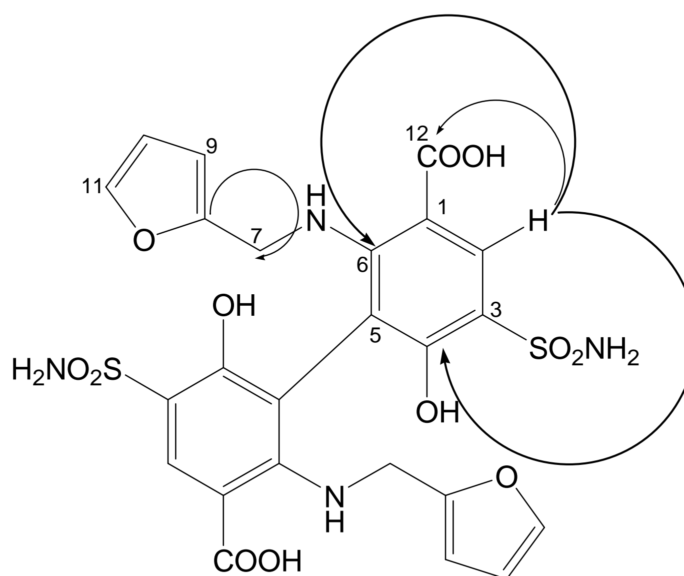


Figure 7

The ^{1}H NMR spectrum showed the H-2 benzene singlet proton at δ 8.01 besides the H-7 methylene singlet at δ 4.24 and the H-9, H-10 and H-11 protons at δ 6.30, 6.24 and 7.35 of the furyl moiety.

The ^{13}C NMR spectrum revealed the presence of a carbonyl carbon at δ 173.2, a methylene carbon at δ 37.3 and ten aromatic carbons, only four protonated. On the basis of HMQC and HMBC experiments, the protonated carbons at δ 108.3, 104.8 and 140.2 were attributed to the furane C-9, C-10 and C-11 respectively. The carbon at δ130.7 was assigned to the C-2 according to the long range correlations of the H-2 proton with the carboxyl carbon and the C-4 and C-6 carbons at δ 164.3 and 152.6 respectively.



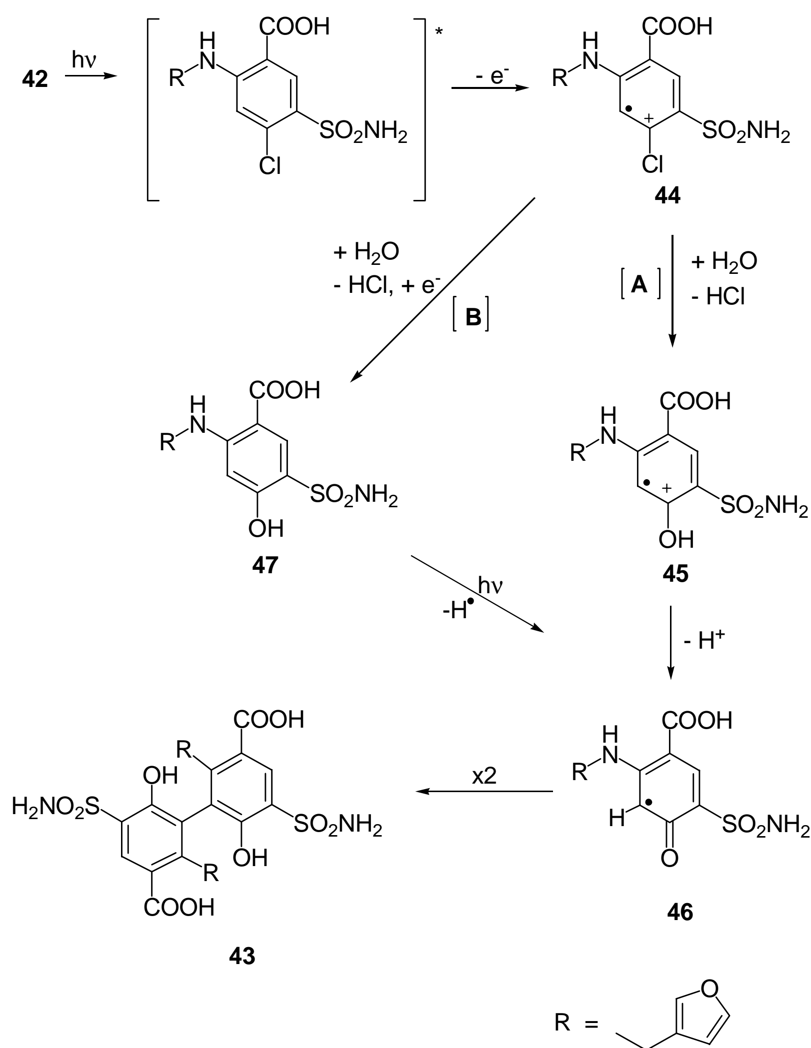
43

The substituted aromatic carbon at δ150.2 was assigned to the furane C-8 owing to its correlation with the H-7, H-9, H-10 and H-11 protons, while the carbons at δ 104.4, 113.4, 164.3 and 152.6 were assigned to the benzene C-1, C-3, C-4 and C-6, respectively. The chemical shift values of C-6 as well as of C-1, C-3 and C-5 were consistent with the presence of a hydroxyl group at C-4.

The MALDI MS spectrum showed peaks at m/z 560 due to the fragment [M-CO_2- H_2O]$^+$, at 543 corresponding to the loss of fragment SO_2NH [M- SO_2NH]$^+$ and at 526 due to fragment [543 – OH]$^+$. All these data were in agreement with the molecular formula $C_{24}H_{22}N_4O_{12}S_2$.

The photochemical behaviour of furosemide has been widely explored, but the formation of dimer **43** has never been described. To investigate its formation the reaction was run in the dark and in the light under argon atmosphere. In the first case, no trace of the dimer was detected, while under argon compound **43** was still obtained in 45 % amount. Thus, the formation of **43** cannot be ascribed to the attack of $^3O_2$ on the excited state of **42**, but to a photoinduced nucleophilic substitution.

On the basis of these data and those previously reported (Moore and Sithipikas, 1983; Bundgaard H. *et al.*, 1988), formation of **43** can be easily rationalized by assuming photoionization as the primary photochemical process leading to cation radical **44** (Scheme 9).



Scheme 9

The presence of a positive charge on **44** makes this intermediate more susceptible to nucleophilic attack by the solvent (water). The loss of HCl leads to cation radical **45** (pathway A) and expulsion of the proton affords radical **46**. Dimerization of **46** should be the final event leading to **43**. It cannot be excluded that, after the substitution process of the chlorine by the hydroxyl group in **44**, a back electron-transfer could lead to compound **47** (pathway B), which affords radical **46** by O-H homolytic cleavage. However compound **47** was not evidenced in our experimental conditions.

Photochemical aromatic substitution ($S_{RN}1Ar^*$) is a well known process (Karapire and Icli, 2004), and, in particular, photohydrolysis of halobenzenes (Stegeman M.H. *et al.*, 1993) as well as the dimerization of phenol derivatives (Horspool W.M., 2003) under radical conditions are reported.

The dehalogenation of furosemide has been previously observed by irradiating in acid methanol or acid aqueous methanol and leads to a mixture of substitution and reduction products (Moore and Sithipikas, 1983). It is likely that neutral aqueous medium favours the substitution rather than reduction and the OH function is determinant for the dimerization.

The phototransformation of furosemide was also investigated under different conditions. The experiments were run in distilled water with nitrate added (5 mg/l), in distilled water in the presence of humic acids (10 mg/l), in drinking water and in STP water. In all cases the only photoproduct was dimer **43** and the yields of photoproduct after 36 hr, calculated by HPLC, were comparable.

The drug dissolved in water at the same concentration was also exposed in open tube to the direct solar light and, after 3 days, the only identified product was still dimer **43**, formed at about 46 % yield.

Toxicity studies

Acute and chronic toxicity tests are reported in Tables 16 and 17.
Acute results showed that furosemide was more bioactive than compound **43** on *D.magna* and *T. platyurus*, while the rotifers *B. calyciflorus* and the bacteria *V. fischeri* did not undergo any effects up to 200 mg/l of tested compound.

Table 16: Acute toxicity tests EC50 (in mg/l) with 95% confidence range

Compound	*V. fischeri*	*B.calyciflorus*	*T. platyurus*	*C. dubia*	*D. magna*
42	NE (200 ppm)	NE (101ppm)	70.57	84.09 (70.11-91.01)	60.62 (30.86-119.08)
43	NE (120 ppm)	NE (120 ppm)	81.02 (75.98-86.40)	75.79 (64.31-79.12)	NE (100 ppm)

NE= no effect at

Chronic values were one hundred times lower than the acute ones. In fact the bioactive concentrations ranged from 0.50 to 2.50 mg/l and compound **43** was more toxic than the parent compound for all tested organisms.

Table 17: Chronic toxicity tests EC50 (in mg/l) with 95% confidence range

Compound	*C. dubia*	*P. subcapitata*	*D. magna*
42	2.35 (1.38-6.49)	NE 70 ppm	2.49 (2.0-3.10)
43	0.56 (0.27-3.01)	ND	1.03 (0.76-1.38)

NE= no effect at

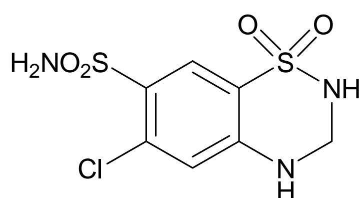
3.3.1.3 Conclusion

Furosemide is not stable in the aquatic environment and undergoes photolysis under solar irradiation conditions. The drug is largely transformed in dimer **43** under aerobic or anaerobic conditions. Such behaviour could justify the low

MEC/PEC ratio found by Calamari *et al*. (2003) in the rivers Po and Lambro and should be considered in the analytical measurement on the presence of the drug in surface waters. The possible presence of the transformation product in surface waters should be taken into account also when eco-toxicological evaluations are made since, in chronic results, it was more toxic than the parent compound.

3.3.2.1 Hydrochlorothiazide (48)

6-chloro-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7 sulfonamide 1,1-dioxide, hydrochlorothiazide (**48**), as furosemide, is a sulfonamide diuretic and antihypertensive and is supplied as tablets for oral use.



hydrochlorothiazide (**48**)

It is a white, or practically white, crystalline powder, which is slightly soluble in water. It is well-known that hydrochlorothiazide is not metabolized and at least 61 percent of the oral dose is eliminated by the kidney unchanged within 24 hours (O'Grady P. *et al.*, 1999). In a recent study a survey was made in the Po and Lambro rivers in Italy to check presence of therapeutic drugs in the aquatic environment and HCTZ was detected in concentrations ranging from 10 to 250 ng/L (Calamari P. *et al.*, 2003).

Previous studies on the photostability of hydrochlorothiazide are present in literature. Tamat and Moore (1983) studied its photocatalytic decomposition. In their work the drug is reported to decompose upon irradiation with near UV-light ($\lambda > 310$ nm) in methanol and aqueous solutions.

In the aqueous (5% methanol) solution, the primary photoprocesses were photodehalogenation and photohydrolysis obtaining only small amounts of **52** and the hydrolyzed and dechlorinated **53** (Figure 8). Revelle L.K. *et al.* (1997) re-investigated the photolytic decomposition in methanol by UV-A fluorescent lamp with wavelengths ranging from 300 to 400 nm. Photodehalogenation was reported to be the primary degradation process in which the chlorine of HCTZ is replaced by hydrogen (**49**) or by a methoxyl group (**50**) from the methanol solvent (Figure

8). They also observed the formation of **54** and a HCTZ photodehydrogenation process that led to derivatives **52**, **56** and chlorothiazide (**55**) (Figure 8). **49** and **52** were the main products obtained by Ulvi and Tammilehto (1989) when HCTZ was irradiated in ethanolic solution with a high-pressure mercury lamp.

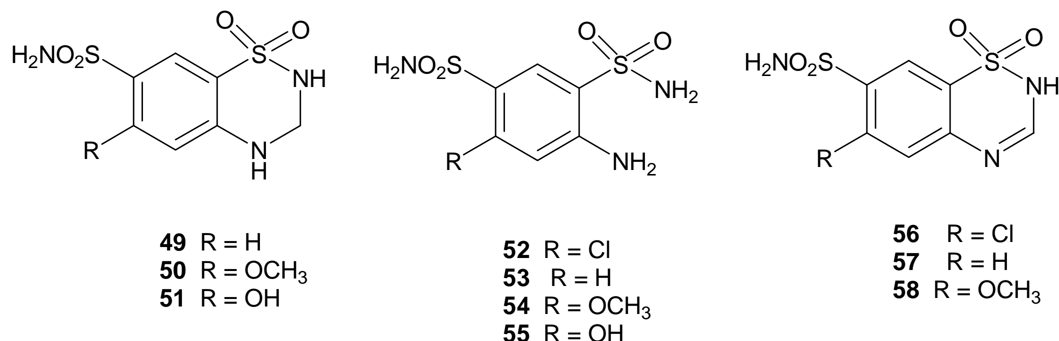


Figure 8

3.3.2.2 Results and Discussion

*Phototransformations of hydrochlorothiazide (**48**)*

In order to understand the fate of hydrochlorothiazide when exposed to sunlight in surface and sewage treatment plant waters, the photochemical behaviour of hydrochlorothiazide (**48**) was investigated in water. It was suspended in pure water (100 μM) and irradiated at different times with a solar simulator. Transformation of HTCZ was followed by injecting an aliquot of the water sample withdrawn at various times in a HPLC–UV system. After 5 h, a new peak was observed in the HPLC chromatogram (Figure 9), this peak grew with the irradiation time and became constant after 30 h of irradiation. Only after 30 h was the appearance of two other peaks observed in minor amounts and after 200 h it was evident that three main photoproducts (**51**, **52**, **55**) were present. To isolate and characterise the three photoproducts obtained, irradiation of HTCZ was performed in a preparative scale.

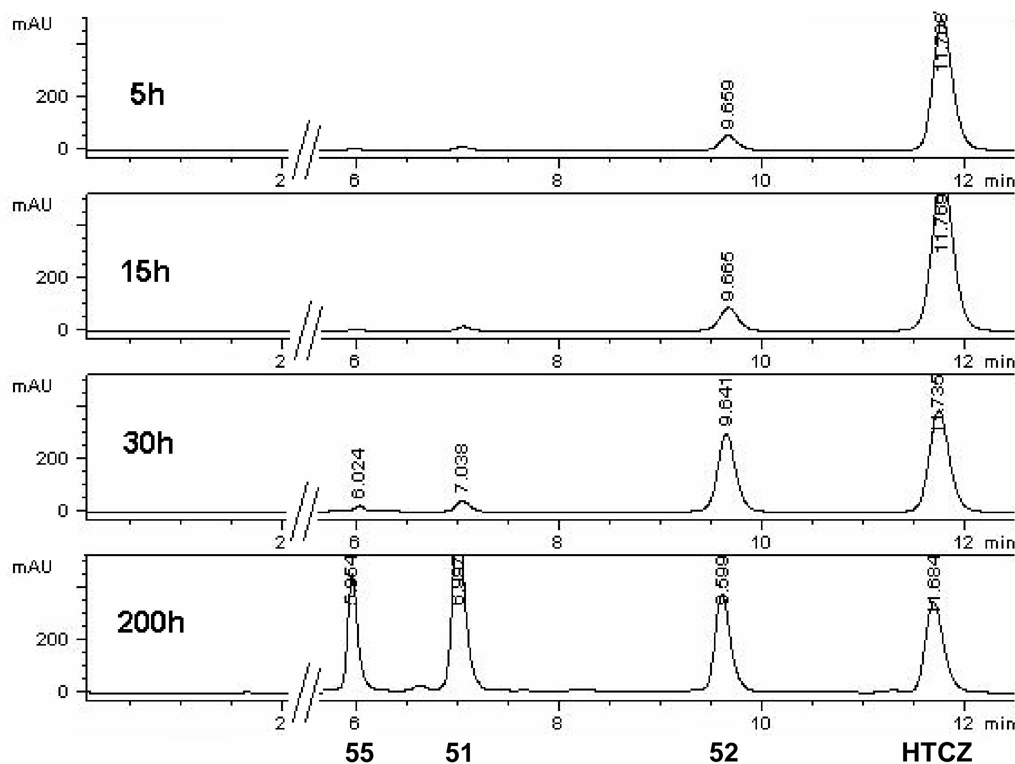
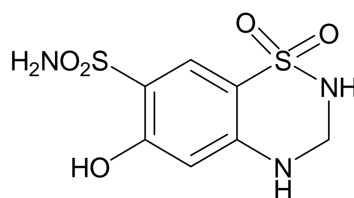


Figure 9. HPLC chromatograms at different times of irradiation

A dispersion of hydrochlorothiazide (0.7 mM) in distilled water was irradiated for 200 h by a solar simulator. After evaporation of the water, the mixture was submitted to flash chromathography yielding three compounds along with hydrochlorothiazide.

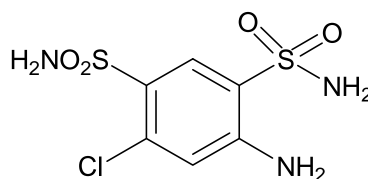
The three photoproducts were further purified by TLC and HPLC.

Compound **51**, formed in 15% after 200 h irradiation, was identified as 6-hydroxy-3,4-dihydro-2H-1,2,4-benzothiadazine-7-sulfonamido-1,1-dioxide. This compound was suggested as intermediate in the mechanism of photodegradation by Tamat and Moore (1983), but it had never isolated and described before now.



51

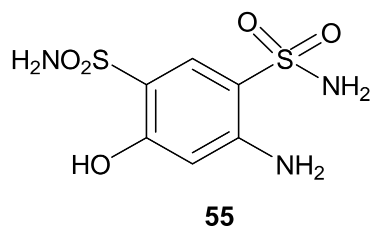
In the ESI-MS spectrum the molecular peak at 278 was present in agreement with the molecular formula $C_7H_9N_3O_5S_2$. The ^{1}H NMR spectrum showed two aromatic protons, as singlets at δ 6.24 and 7.92, and two protons of a methylene as a singlet at δ 4.70. In the ^{13}C NMR spectrum seven carbon signals were identified. Three protonated carbons at δ 127.0, 101.9 and 56.0 and four aromatic quaternary carbons at δ 160.0, 150.0, 120.2, and 114.6 were detectable. All these data were consistent with the structure proposed. This compound was formed by photosubstitution from hydrochlorothiazide in a process where the chlorine is replaced by OH from the solvent. To verify that oxygen was not involved in this process, a hydrochlorothiazide suspension was irradiated under argon atmosphere. The most abundant compound was determined to be 4-amino-6-chloro-1,3-benzenedisulfonamide (**52**).



52

Two singlet aromatic protons at δ 8.34 and 6.97 were present in the ^{1}H NMR spectrum, and six aromatic carbon signals were identified in the ^{13}C NMR spectrum. These NMR data were consistent with literatura data (Revelle L.K. *et al*., 1997). Further structural information was obtained by ESI-MS analysis which showed the peak at m/z 278 corresponding to the molecular formula $[C_6H_8O_4N_3ClS_2 - 1]^+$. Thus, the photolysis of hydrochlorothiazide in pure water leads to fragmentation of the thiadazine ring as dominant product. After 200 h, the formation of compound **51** was observed in 50% yield.

The structure 4-amino-6-hydroxy-1,3-benzenedisulfonamide was attributed to compound **55** (5%).



The molecular peak at 266, along with the elemental analysis defined the molecular formula $C_6H_9N_3O_5S_2$. The ^{1}H NMR spectrum exhibited two aromatic protons, as singlets at δ 6.18 and 8.07. In the ^{13}C NMR spectrum six carbon signals were present: two protonated carbons at δ 131.9, 116.5 and four aromatic quaternary carbons at δ 152.6, 145.6, 119.5 and 116.2.

All compounds were used as standards to evaluate the phototransformation yields by HPLC analysis. The yields were also confirmed by ^{1}H NMR integration analysis of the mixture after irradiation. The yields of photoproduct **51**, **52** and **55** after 200 h were 15%, 35% and 5%, respectively.

To verify the phototransformation in a simulated aquatic environment an irradiation experiment was also performed suspending hydrochlorothiazide in sewage treatment plant (STP) water. The photoproducts **51**, **52** and **55** were obtained after 200 h. The only difference observed in this case was the slight yield of compound **55** (1%).

Finally, an experiment irradiating the hydrochlorothiazide suspension was performed in pure water under sunlight for 5 days in January. HPLC and NMR analysis of the irradation mixture showed that hydrochlorothiazide was almost completely transformed and the main product was compound **51** (75% yield).

The occurrence of the same three photoproducts in all irradiation conditions tested suggests a unique photolysis pathway for hydrochlorothiazide in water, where the main processes are photoinduced fragmentation of the thiadazine ring and the photosubstitution of the chlorine with the hydroxyl group.

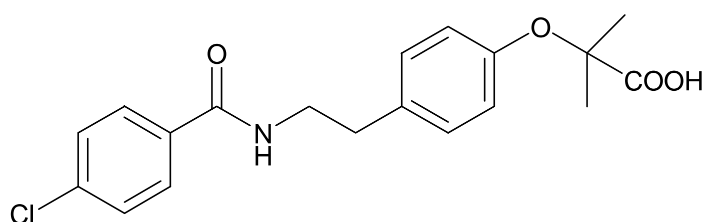
3.3.2.3 Conclusion

Hydrochlorothiazide was irradiated under biomimetic conditions for 200 hours leading to three photoproducts that were isolated and characterized. Two of them were isolated in percentages significantly higher than 10 %.

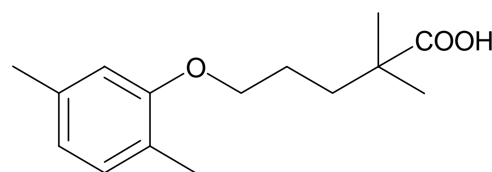
We are currently investigating on eco-toxicity of hydrochlorothiazide and its phototransformation products, to assess the environmental risk.

3.4.1 Fibrates: bezafibrate, gemfibrozil, fenofibrate

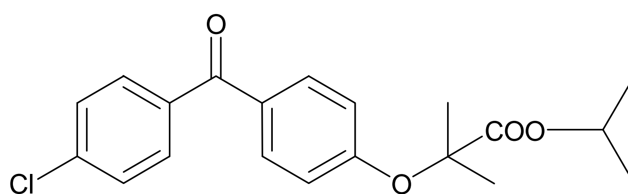
During the past 20 years, fibric acid derivatives (ethyl-2-[4-chlorophenoxy]-2-methyl propionate) have been the major drugs used in the treatment of hyperlipidaemia (Baker R. *et al.*, 1982; Harvengt C. *et al.*, 1982) when raised cholesterol levels are associated with raised levels of triglycerides. Clofibrate and gemfibrozil were widely prescribed in the United States (The Helsinki Heart Study, 1996). After about 10 years, a new generation of fibric acid derivatives was developed in Europe and the prescription of such agents as bezafibrate (**59**), is common (Drouin P. *et al.*, 1980; Michell H. *et al.*, 1979), due to their greater potency and more satisfactory reduction at low density lipoprotein-cholesterol levels.



bezafibrate (**59**)



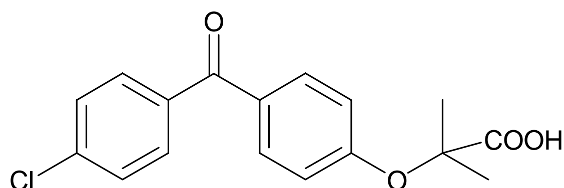
gemfibrozil (**60**)



fenofibrate (**61**)

Bezafibrate (**59**), gemfibrozil (**60**) and fenofibrate (**61**) have been selected for our study because they are the most prescribed fibrate and they are included in the 2002 list of the most used drugs in the world. As fenofibrate is rapidly

metabolized to fenofibric acid (**62**) after administration, this metabolite has also been investigated.



fenofibric acid (**62**)

Bezafibrate has been frequently identified in the environment (Andreozzi R. *et al.*, 2003). In his investigations on effluents of German sewage treatment plants (STP) Ternes T.A. (1998) reported concentrations up to 4.6 μg/l of this drug. Calamari D. *et al.* (2003) in a recent investigation on Naples STP water have found concentrations of 116 ng/l.

The second fibrate under investigation is gemfibrozil. The drug has been found in surface waters (Ternes T.A., 1998) and in STP effluents in Canada (Metcalfe C. *et al.*, 2000). Andreozzi R. *et al.* (2003) have found 4.76 μg/l concentrations in Naples STP effluent.

No trace of fenofibrate has been found in the aquatic environment. These data agree with the almost quantitative conversion of fenofibrate to its metabolite fenofibric acid (**62**) after its administration (Elsom L.F. *et al.*, 1976). Concentrations up to 1.2 μg/l of this metabolite have been found in German STP effluents and rivers (Ternes T.A., 1998).

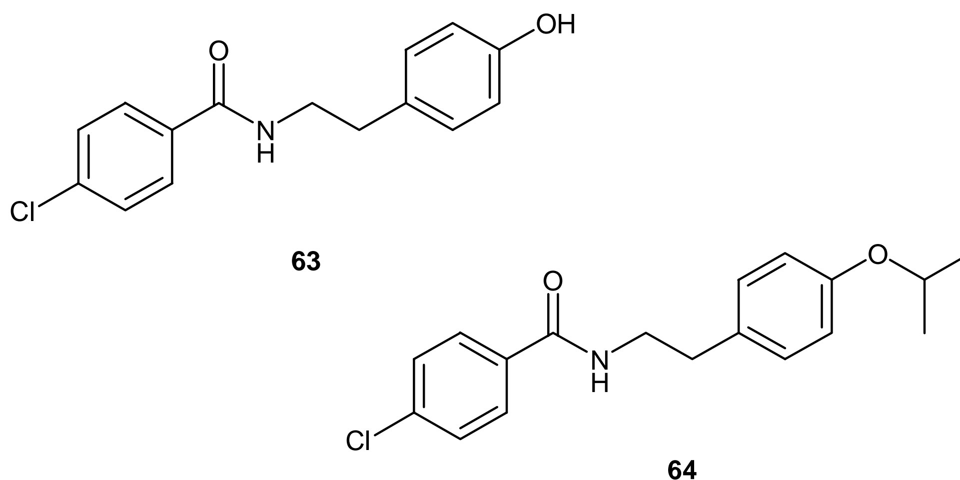
In recent years, light-mediated cutaneous reactions, such as pruritus, dry skin, maculopapular rashes (Blane G.F., 1987), erythema multiforme (Arif and Vahrman, 1975) and photosensitivity following the taking of these pharmaceuticals have been described. In connection with these facts, studies on photodegradation (by UV-B 290-329 nm) and phototoxicity *in vitro* (photohemolysis) of bezafibrate, gemfibrozil and fenofibrate were performed (Vargas F. et al., 1993). Their photoxicity can be explained by the involvement of

free radicals, singlet oxygen and stable photoproducts (Miranda M.A. *et al.*, 1994a).

3.4.2 Results and Discussion

*Phototransformations of bezafibrate (**59**)*

2-[4-2-[4-chlorobenzamido]ethylphenoxy]-2-methylpropanoic acid, bezafibrate (**59**) was irradiated in distilled water and its transformation monitored by thin layer chromatography at 50, 100 and 200 hr. Appreciable amounts of transformation products were obtained only after 200 hr. After removal of the water in vacuo, the residue was chromatographed on silica gel to give, along with unreacted bezafibrate (88 %), phenol **63** (2%) and ether **64** (3 %).



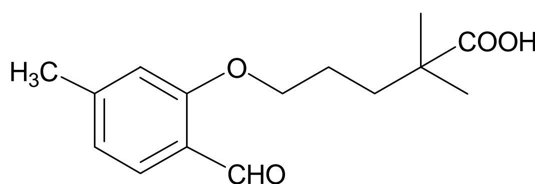
Compound **63** was identified by comparing its spectral data with those reported in literature. In fact, it was isolated in a previous study (Canudas N. *et al.*, 1996) on phototoxicity of bezafibrate (**59**) which was irradiated with a 125 W medium pressure Mercury lamp in biological conditions. Structure **64** was attributed on the basis of spectroscopic data. The ^{1}H NMR spectrum, in addition to four aromatic doublets, showed the signals of the geminal methyls as a doublet at δ 1.32 and the methine proton as a quartet at δ 4.53 according to the presence of an isopropyloxy group. The corresponding carbons in the ^{13}C NMR spectrum were at δ 22.0 and 69.9, respectively.

The same photoproducts **63** and **64** were also obtained when bezafibrate was irradiated for 200 hr in pure water in the presence of nitrates or humic acids. The yields were comparable, so that the presence of environmental photosensitizers seems not to influence the phototransformation of bezafibrate. Instead, when using STP water after 200 hr irradiation no significant photodegradation was observed. Irradiation of bezafibrate in distilled water saturated with argon gave only compound **64** (2 mg).

The experiments were also performed by sunlight irradiation, and after 200 hr similar results were obtained.

*Phototransformations of gemfibrozil (**60**)*

5-(2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid, gemfibrozil (**60**), dispersed in distilled water, was irradiated by the solar simulator. After 200 hr, water was evaporated in vacuo and the reaction mixture was purified by silica gel flash chromatography and preparative thin layer chromatography giving gemfibrozil and a crude photoproduct in about 9% yield. The compound isolated was identified as aldehyde **65**. The EI-MS spectrum showed a M^+ at *m/z* 264 with a base peak at *m/z* 136. ^{1}H-NMR spectrum showed that aromatic protons were shifted at δ values higher than the ones of gemfibrozil, in agreement with the oxidation of a methyl group. In fact, a singlet at δ 10.38 in the ^{1}H-NMR spectrum and a carbonyl carbon at δ 189.7 in the ^{13}C NMR spectrum attested to the presence of the formyl group. The ortho position of the formyl group was established on the basis of a NOESY spectrum where the methyl gave NOE with the doublet at δ 6.82 and with the singlet at δ 6.73.

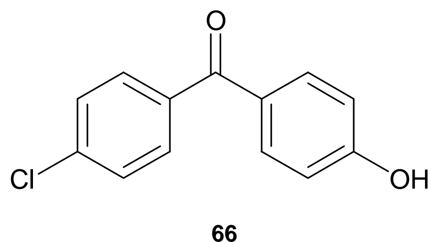


65

Irradiations in distilled water in the presence of nitrates or humic acids as well as in STP water, or in distilled water in argon atmosphere left gemfibrozil unaltered even after 300 hr. The same results were obtained by sunlight irradiation.

*Phototransformations of fenofibrate (**61**)*

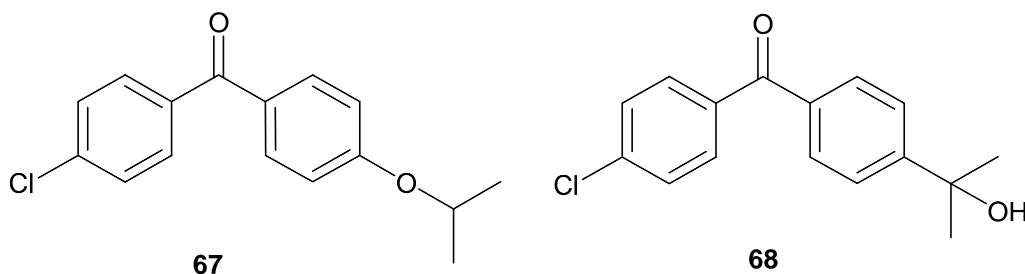
2-[4-(4Chlorobenzoyl phenoxy)]-2-methyl propionic acid-isopropyl ester, fenofibrate (**61**) was dispersed in distilled water and was irradiated for 200 hr by the solar simulator. Water was evaporated and the residue was chromatographed to give unreacted fenofibrate (88%), compound **62** and compound **66** with an overall yield of about 8%. These compounds were identified by comparison with authentic samples (from Aldrich).



Its irradiation in distilled water in the presence of nitrate ions, humic acids or in STP water gave products **62** and **66** in similar yields to those in pure distilled water. Fenofibrate (**61**) by irradiation in argon gave only fenofibric acid **62** in traces.

*Phototransformations of fenofibric acid (**62**)*

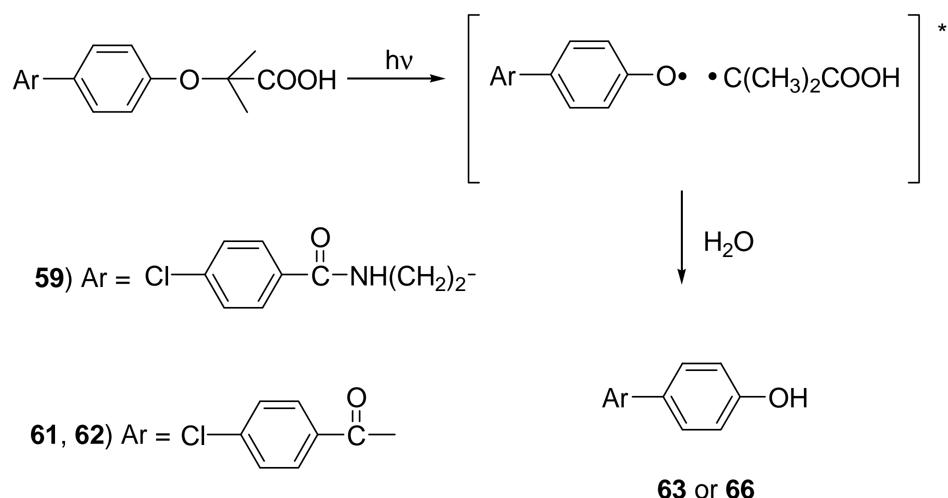
Irradiation of a distilled water solution of fenofibric acid (**62**), performed as for the other fibrate, gave phenol **66** (13%), ether **67** (70%) and alcohol **68** (18%).



Fenofibric acid (**62**) was the most photolabile among tested drugs and after 60 hr it was quantitatively converted. Similar behaviour was observed in STP water as well as by changing the lamplight with the direct sunlight. Instead, irradiation in argon gave only compounds **67** (70%) and **68** (15%). These latter were previously isolated by Miranda et al. in a study on photosensitization by fenofibrate **62** (1994a).

On the basis of the experimental and literature data, mechanistic pathways may be drawn for the formation of the photoproducts. All involve the aryloxy moiety as the key reactive site and well-stabilized radicals (or radical ions) as intermediates.

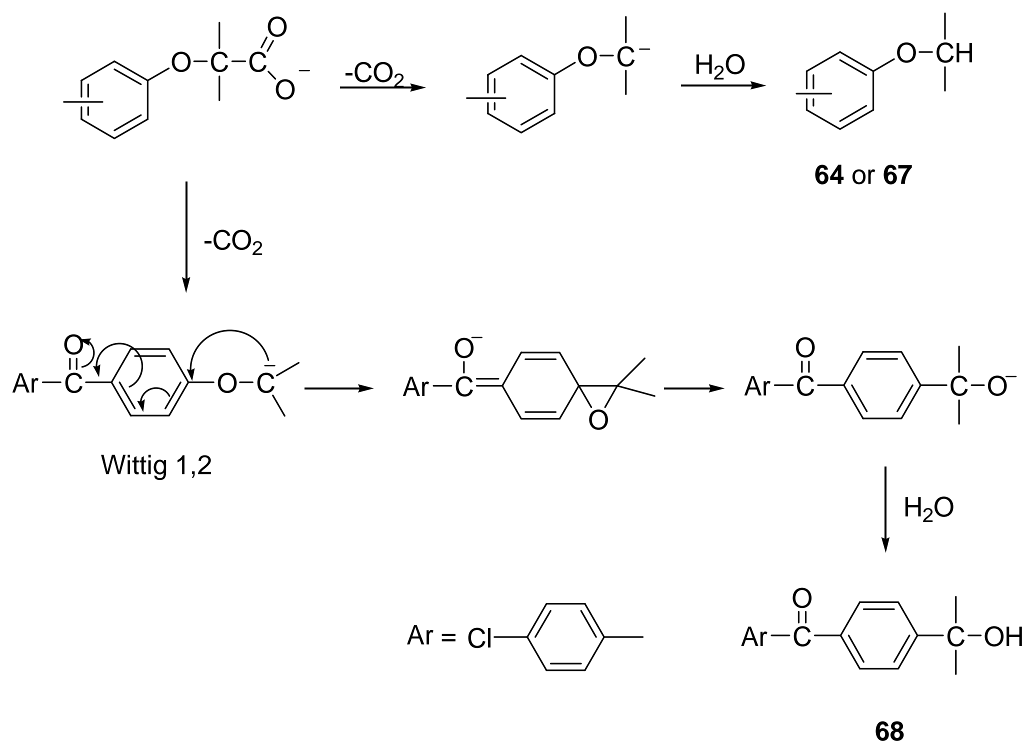
Formation of phenols **63** and **66** occurs by a homolytic cleavage of the aryloxy bond followed by hydrogen abstraction from the solvent (Scheme 10), as already proposed by Canudas N. *et al*. (1996) for bezafibrate, and requires aerobic conditions, as confirmed by the control experiment performed in the absence of oxygen.



Scheme 10

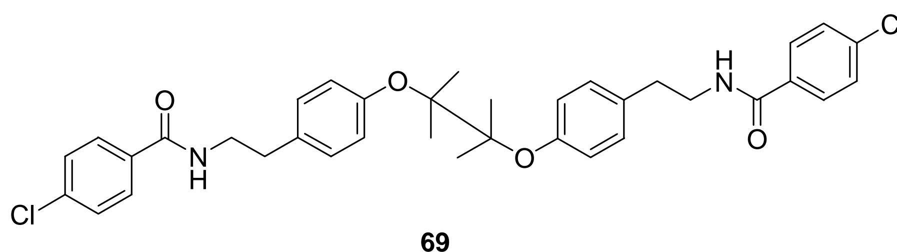
Formation of ethers **64** and **67** can be explained by an ionic photodecarboxylation process of the dissociated acids to aryloxy-substituted carbanions which are protonated by water (Scheme 11). Photodecarboxylation is also the first event

followed by a [1,2]-Wittig rearrangement in the formation of **68** from **62** (Scheme 11) and has been already justified by Miranda M.A. *et al.* (1994b). These phototransformations do not require aerobic conditions.



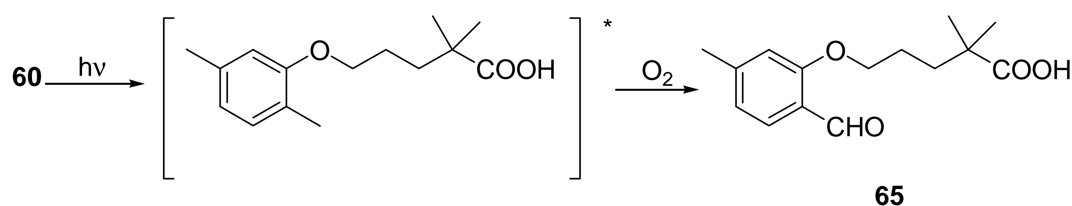
Scheme 11

The high photodegradation of **62** may be justified by the presence of the easily photoexciting benzophenone group. It is to be noted that in a previous work by Canudas N . *et al.* (1996) only dimer **69** together with phenol **63** was found.



This different result may be due to different irradiation conditions (UV lamp vs solar lamp) and also to the more diluted sample (10^{-3} M vs 10^{-4} M). Indeed, as reported by Monti S. *et al*. (1997), a pathway for decarboxylation may occur which is scarcely efficient especially at low light levels. This pathway would involve a radical species formed via a photoionization process upon electron release followed by loss of CO_2, and dimer **69** would form from a radical-radical recombination.

Formation of aldehyde **65** may be rationalized by a photooxidation promoted by the ortho aryloxy function (Scheme 12), in agreement with the easy oxidation of alkyl-substituted phenols (Horspool W.M., 2003). We are currenly investigating the detailed mechanism.



Scheme 12

Toxicity studies

Results of acute toxicity for fibrates and their photoproducts are reported in Table 18. Data showed that the drugs had a limited acute toxicity on the tested organisms. Parental compounds showed no effects or low acute effects towards all the organisms ranging from 39.69 mg/l (bezafibrate vs *T. platyurus*) to 161.05 (gemfibrozil vs *T. platyurus*). Concentrations found to exercise a toxic potential were far from environmental concentrations. No effect of environmental concern was found for photoderivatives of bezafibrate and gemfibrozil, while the fenofibrate derivative showed the highest acute effects for all the organisms and

resulted the most active among all tested compounds. Preliminary acute tests performed on *T. platyurus* and *B. calyciflorus* for fenofibric acid and its derivatives showed compounds **67** and **68** were more toxic than the parent compound.

Table 18. Acute toxicity tests with confidence limits (95% probability)

Compound	*V.fischeri*	*C.dubia*	*T.platyurus*	*B.calyciflorus*	*D.magna*
59	NE (110 ppm)	75.70 (60.13-81.01)	39.69 (24.93-63.17)	60.91 (54.03-68.66)	100.08 (80.02-120.54)
63	NE (130 ppm)	77.11 (65.41-84.09)	NE (70 ppm)	NE (70 ppm)	NE (120 ppm)
64	37.24 (29.74-46.61)	90.57 (81.31-99.65)	45.96 (44.41-47.57)	109.32 (85.91-139.10)	NE (80 ppm)
60	85.74 (77.22-91.74)	NE (200 ppm)	161.05 (136.98-189.34)	77.30 (59.12-101.08)	74.30 (66.15-88.45)
65	NE (100 ppm)	NE (100 ppm)	NE (190 ppm)	64.97 (57.12-72.36)	50.12 (44.78-58.55)
61	NE (100 ppm)	NE (100 ppm)	NE (190 ppm)	64.97 (57.12-72.36)	50.12 (44.78-58.55)
66	22.16 (17.15-25.62)	42.24 (35.47-49.66)	27.16 (23.35-34.40)	0.35 (0.27-0.41)	17.68 (10.32-22.15)
62	ND	ND	82.03 (74.40-91.23)	74.30 (60.95-82.11)	ND
67	ND	ND	28.06 (21.07-37.37)	30.06 (23.31-40.38)	ND
68	ND	ND	31.27 (24.64-39.70)	55.49 (40.49-76.06)	ND

NE = no effects at
ND = not determined

Chronic data on the inibition of reproduction for *B. calyciflorus* and *C. dubia* are reported in Table 19. Results confirmed the trend of acute data for bezafibrate and its derivatives even if the bioactive concentrations ranged from 0.13 to 7.36 mg/l while no significant difference was found between fenofibrate, gemfibrozil and their respective derivatives that, however, showed EC50 values less than 1 mg/l. Chronic data also demonstrated that bezafibrate is the most toxic.

Table 19. Chronic toxicity tests with confidence limits (95% probability)

Compound	*C.dubia*	*B.calyciflorus*
59	0.133 (0.038 - 0.260)	0.44 (0.25 – 0.51)
63	1.49 (0.74-2.65)	1.44 (1.08 -1.91)
64	7.35 (5.30 - 9.62)	7.36 (5.52-9.78)
60	ND	0.44 (0.17 – 0.69)
65	0.43 (0,35-0,51)	0.36 (0,15 – 0,54)
61	0.76 (0.66 - 0.88)	ND
66	0.92 (0.80 - 0.98)	ND

ND = not determined

3.4.3 Conclusion

From an environmental point of view it is noteworthy that bezafibrate and gemfibrozil are stable in STP effluent, probably due to the filter action of this medium which has a large absorption band at λ_{max} 214 nm with a code up to 300 nm. Consequently, only fenofibrate **61** and its mainly biological metabolite fenofibric acid **62** call for deeper attention. Their transformations in STP water allow us to expect similar behaviours in surface waters; furthermore **62** undergoes fast and complete degradation by sunlight. According to U.S. and Europe Scientific Committees, analytical and ecotoxicological investigations should also be addressed toward their environmental metabolites **67** and **68** to assess the environmental risk.

However, preliminary acute and chronic data, here reported, indicate that high, environmentally uunrealistic concentrations of fibrates and their photoproducts are needed to cause toxicity.

3.5.1 Proton Pump Inhibitors: lansoprazole and omeprazole

Lansoprazole (**70a**) and omeprazole (**70b**), two substituted pyridylmethylsulfinyl benzimidazole derivatives, are proton pump inhibitors.

lansoprazole (**70a**)   omeprazole (**70b**)

Proton pump inhibitors (PPIs) are widely used for the treatment of acid-related disorders including gastroesophageal reflux disease and for peptic-ulcer disease caused by stress, nonsteroidal antiinflammatory drugs, and *Helicobacter pylori* infection (Langtry and Wilde, 1998; Zimmermann and Katona, 1997). These compounds inhibit acid secretion by irreversibly interacting with H^+ -K^+-ATPase, the terminal proton pump of the parietal cell (Sachs G., 1997). In the acid space of the secreting parietal cell or in the vicinity of the enzyme, these drugs are converted to thiophilic sulfenamide or sulfenic acid, which reacts with Cys-813 residue in the catalytic subunit of the H^+ -K^+ -ATPase, which is critical for enzyme inactivation (Wolfe and Sachs, 2000).

Omeprazole has been extensively used to control these disorders (Langtry and Wilde, 1998), lansoprazole, with benzimidazole containing a trifluoroethoxy group, has also been used more recently (Zimmermann and Katona, 1997).

Omeprazole has been found in surface waters of Lambro river (Calamari D. *et al.*, 2003). Stability studies especially on omeprazole have revealed their poor stability in acid medium or to light and heat (Wallmark B. *et al.*, 1987), and this has been particularly noticed in the settlement of methods for their determination (Karljikovic-Rajic K. *et al.*, 2003; El-Kousy and Bebawy, 1999; Castro D. *et al.*, 1999).

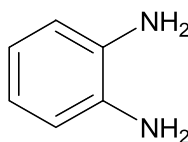
The objective of this study was to determine the main products of hydrolytic and photolytic cleavage of lansoprazole (**70a**) and omeprazole (**70b**) under environmental conditions, in particular, in water, in water with added of humic acid or nitrates, and at different pHs.

3.5.2 Results and discussion

*Transformations of lansoprazole (**70a**)*

Dispersions of lansoprazole **70a** in pure water were irradiated by the solar simulator. After 72 hr, water was evaporated and irradiation mixture was purified on preparative TLC. Unreacted lansoprazole, compounds **71a-75a** and an intractable red material were obtained (Table 20).

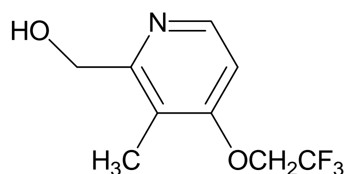
Compound **71a** was identified as dianiline by comparison of its spectral data with those of the commercially available compound.



71a

The ^{1}H NMR spectra of compound **72a** showed two doublets at δ 8.33 and 7.06 of the pyridine moiety; in the aliphatic region, in addition to the methylene quartet of the CH_2CF_3 group and the methyl singlet at δ 2.13, a singlet at δ 4.62, integrated for two protons.

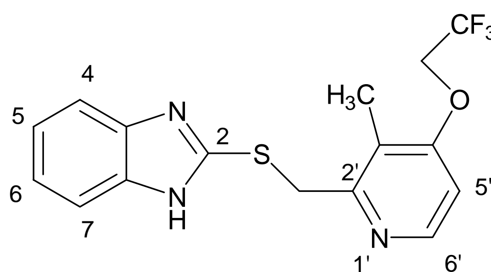
The ^{13}C NMR resonances were assigned on the basis of HMQC and HMBC experiments. This latter showed the correlations of the signal at δ 4.62 with the C-2' and C-3' carbons. In the EI-MS spectra the molecular peak at m/z 221, the peaks at 206 and 190 due to the fragments $[M-CH_3]^+$ and $[M-CH_2OH]^+$, respectively, were present. All these data were in agreement with the structure of compound **72a**.



72a

The third compound isolated from the irradiation mixture was identified as sulfide **73a** according to ^{1}H NMR showing seven patterns of signals with almost identical chemical shifts in comparison with those of the lansoprazole, except the exchange of chemical shifts of two methylene groups. The ^{13}C NMR signals were assigned by combination of the HMQC and HMBC experiments. The HMBC spectrum showed the correlations of the CH_2CF_3 methylene protons with the CF_3 and C-4' carbons, as well as that of the CH_2S methylene protons with the C-2', C-3' and C-6' carbons, and that of the H-6' proton with the C-4', C-2' and C-5' carbons. The shielded methylene carbon at δ 36.1 attached to the pyridine 2-position. The absence of IR band at 1050 cm^{-1} typical of the streching of SO group and the molecular peak at *m/z* 353 in the EI-MS spectra were in agreement with sulfur compound **73a**.

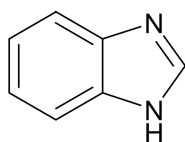
Structure **73a** was confirmed chemically. Indeed, an experiment was performed by adding *m*-chloroperbenzoic acid (0.8 mM) to a solution of **73a** in anhydrous dichloromethane (1 mM). After two hours, TLC showed the presence of a compound which was identified as lansoprazole by comparison of its Rf value and spectral data with those of the corresponding standard.

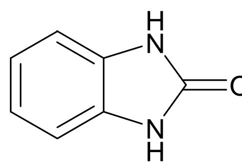


73a

Benzimidazole **74a** and benzimidazolone **75a** were identified by comparing of their spectral data with those of commercially available compounds. ^{1}H NMR of benzimidazole **74a** showed three signals: a singlet proton at δ 8.16, and two multiplet protons at δ 7.61 and 7.28 of the benzimidazole moiety.

The signal at δ 6.85 was present in the ^{1}H NMR spectrum of compound **75a**. The HMBC experiment showed the correlations of this signal with the aromatic carbons at δ 120.2 and 109.9.





74a **75a**

Attempts to characterize the red material failed due to its complexity and changeable nature.

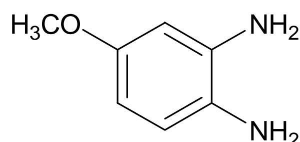
The dispersion of lansoprazole in water milliQ, kept in the dark for 72 h gave, after evaporation of the water, the red-coloured residue which was cromathographed on preparative TLC affording sulfide **73a**, lansoprazole, the intractable red fraction and benzimidazolone **75a** at decreasing Rfs (Table 20).

*Transformations of omeprazole (**70b**)*

Irradiation for 42 hours of omeprazole **70b** in milliQ water by the solar simulator gave, after evaporation of water and purification of the reaction mixture on preparative TLC, five photoproducts and an intractable red fraction (Table 20).

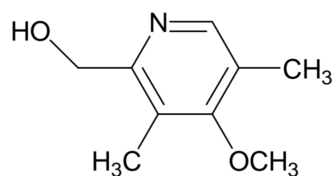
The first isolated photoproduct was compound **71b**. The ^{1}H NMR spectrum showed in the aromatic region a doublet integrated for one proton and a multiplet integrated for two protons, while in the aliphatic region the methoxyl singlet at δ 3.79 was present. In the ^{13}C NMR spectra there were seven signals, four protonated carbons and three quaternary carbons. All the resonances were

assigned on the basis of the HMQC and HMBC experiments. The EI-MS spectra showed the molecular peaks at m/z 138 in agreement with the structure of the dianiline **71b**.



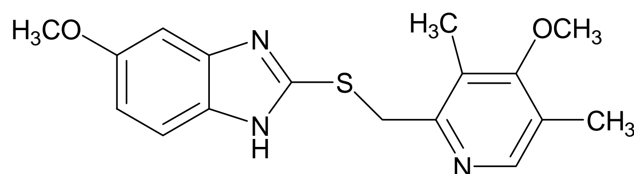
71b

Compounds **72b** and **74b** were identified by ^{1}H NMR and LC-MS due to their low amounts. ^{1}H NMR of compound **72b** revealed the presence of the only pyridine proton at δ 8.14 while LC-MS showed the molecular peak at m/z 167.



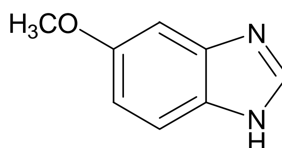
72b

All the considerations concerning spectral data and control experiments we reported for compound **73a** were found also for compound **73b**.



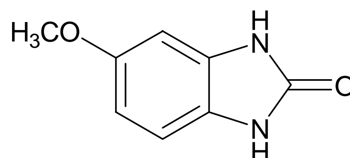
73b

The singlet at δ 8.04 of the H-1 proton together with the signals typical of the benzimidazole moiety and the molecular peak at m/z 148 in the LC-MS confirmed the structure of compound **74b**.



74b

Compound **75b** was identified as benzimidazolone by the presence of the signals at δ 6.92, 6.66 and 6.63 in the ^{1}H NMR spectrum and by the presence of the C-2 carbonyl carbon at δ 158.4 in the ^{13}C NMR spectrum. IR spectrum showed the band at 1720 cm^{-1} due to the carbonyl group. Definitively, the EI-MS spectra revealed the presence of the molecular peak at *m/z* 164.



75b

As for lansoprazole, the red material was intractable by chromatographic means and all attempts to characterize it failed.

When the omeprazole was dispersed in milliQ water and kept at dark for 43 h, after water evaporation, it led to an intense red-coloured residue which was separated on preparative TLC giving **71b,** omeprazole, the intractable red residue and **75b** at decreasing Rfs.

Photochemistry and hydrolysis of two drugs were also investigated at pHs 4.0 or 9.0 and after neutralization of the dispersions and evaporation of water, the mixtures were analyzed by ^{1}H NMR and purified on preparative TLC showing that both lansoprazole and omeprazole degradation was accelerated in acid

conditions, also in accordance with previous results reported by Lagerström and Persson (1984).

The same products in the same yields were obtained when the drugs were irradiated in milliQ water in the presence of humic acids (5 ppm) or KNO_3 (10 ppm).

As shown in Table 20, degradation is accelerated by light (**70a** and **70b** exhibit absorption bands at λ_{max} 292 and 300 nm, respectively). After 72 h in milliQ water, lansoprazole was present only for 24% while after 43 h omeprazole was completely degraded. The effect of light on the degradation is particularly evidenced by comparing the results at buffered pH 7.0 with those at the same conditions in the dark where the drugs are instead stable.

Table 20

Drug[c]	Condition	**70a**	**71a**	**72a**	**red material**	**73a**	**74°**	**75a**
70°	light[e]	24	19	5	15	10	5	5
70°	dark	57	-	-	17	10	-	3
70°	pH 7.0/light[e]	22	19	3	8	5	5	8
70°	pH 7.0/dark	100	-	-	-	-	-	-
70°	pH 4.0/dark	50	-	-	15	20	-	5
70°	pH 9.0/dark	100	-	-	-	-	-	-
		70b	**71b**	**72b**	**red material**	**73b**	**74b**	**75b**
70b	light[e]	-	10	<1	20	16	<1	20
70b	dark	20	-	-	15	25	-	10
70b	pH 7.0/light[e]	5	16	<1	15	12	<1	20
70b	pH 7.0/dark	100	-	-	-	-	-	-
70b	pH 4.0/dark	-	-	-	14	50	-	28
70b	pH 9.0/dark	100	-	-		-	-	-

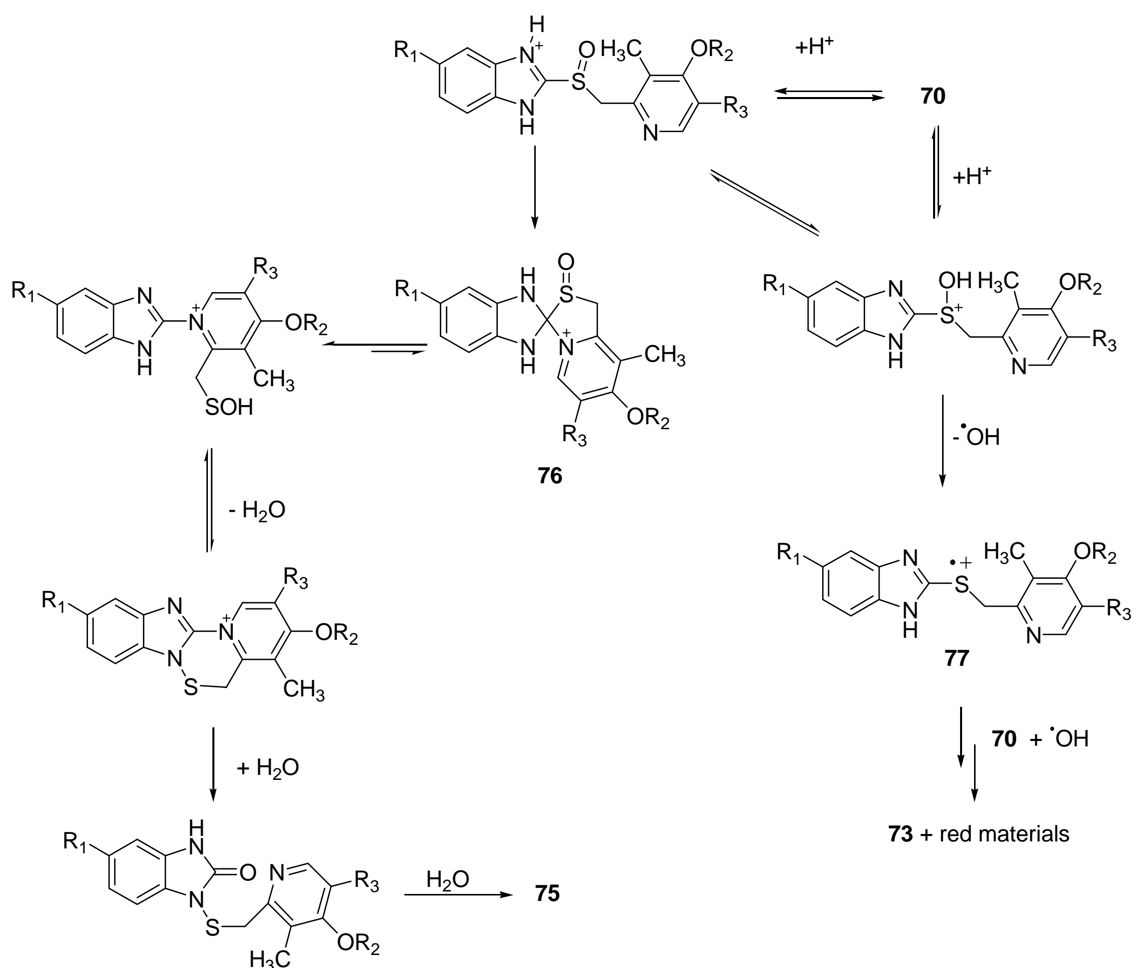
[a]Reaction time 72 h. [b]Reaction time 43 h. [c]40 mg in 500 mL of milliQ water. [d]By TLC. [e]By a solar simulator.

Control experiments showed that product distribution both by hydrolysis and irradiation were not to be affected under argon-saturated conditions. Moreover, it was verified that sulfides **73** were stable to hydrolysis at the dark while by

irradiation they led to dianilines **71** and to benzimidazoles **74**. These conversions were almost quantitative, because of the absorption bands at λ_{max} 292 and 300 nm which are similar to those of the respective parent compounds. In contrast, benzimidazoles **74** and benzimidazolones **75** resulted stable both to hydrolysis and photolysis.

As shown in Table 20, the dark degradation of two drugs was significant leading, among the others, to sulfides **73**.

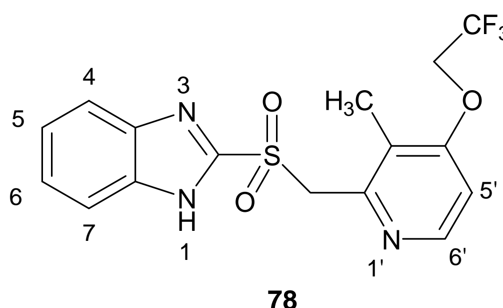
An interpretation is reported in Scheme 13 and is based on literature data.



Scheme 13. Isolated degradation products from drugs **70** in aqueous sispension

Sulfides **73** have been evidenced mainly under physiological pH in the presence of thiols, in a model, for studying the mechanism of (H^+- K^+)-ATPase inhibition by the sulfoxides (Im W.B., *et al.* 1985; Sturm E. *et al.*, 1987; Brandstrom A. *et al.*, 1989). Their formation was explained by assuming that the sulfoxides rearrange in acidic media to a spirointermediate as **76** which, through subsequent steps, one involving reaction with thiols, leads to the sulfides, which contain the original molecular backbone (Lindberg P. *et al.*, 1986). In our case the formation of sulfides cannot be easily justified in this way.

Another pathway is possible for formation of sulfides **73**. It is reported that aromatic sulfoxides are fragmented and/or reduced via cations or radical cations as **77** and OH radical in acid solution, and the convertion, which may be slow if the acid is weak, occurs more easily with heterocyclic compounds (Shine H.J., 1967). So, the alteration of drugs **70** even in MilliQ water might be due to the mild acid medium (initially measured ca. pH 5.0) as expected on the basis of the pKa at 3.83 for **70a** and 4.06 for **70b** (Shin J.M. *et al.*, 2004). Therefore it is likely that protonation involves cation (Im W.B. *et al.* 1985) or radical cation and/or OH radical formation which might trigger diverse oxidation reactions, presumably on the aromatic groups of the drugs, leading to the sulfides **73** and the red material. This hypothesis agrees with recent studies which have evidenced the antioxidant role of lansoprazole and omeprazole as OH-radical scavengers during ulceration in addition to acting as proton pump inhibitors (Biswas K. *et al.*, 2003). The authors identified only sulfones among four oxidation products formed by incubation of the drug with Cu^{2+}-ascorbate system. In our case sulfone was not formed. In fact control experiments showed that the sulfone **78** (spectral data are reported in appendix) purposely prepared and treated as the parent **70a** was found to be stable under dark conditions.



78

Benzimidazolones **75** could be formed by oxidation at benzimidazole group or, better, via the pathway suggested above, by hydrolysis of the spirointermediate **76** and the easy N-S bond breakage (Umetsu N. *et al.*, 1987).

The faster degradation of omeprazole might be due to the presence of activating groups such as 5-OMe or Me on the pyridinium moiety which should favour oxidation.

When irradiated, the degradation of both drugs is accelerated. The only identified products are dianilines **71**, pyridines **72**, benzimidazoles **74** in addition to sulfides **73**, benzimidazolones **75** and the red material. By irradiation the excited drug undergoes a series of fragmentations which are difficult to rationalize due to the low concentrations of other unidentified products and low mass balance. It has been ascertained that photodegradation does not involve oxygen. So, compounds **72** might form via decomposition of a photoisomerization product as an unstable sulfenate intermediate (Still I.W.J., 1988; Hogg D.R., 1990) while compounds **71** and **74**, which are also found by irradiation of sulfides **73**, might form via simple homolytic benzimidazole-sulfoxide and/or -sulfide bond cleavage (Still I.W.J, 1988) or photoinduced water addition to the benzimidazole moiety.

3.5.3 Conclusion

Lanzoprazole and omeprazole result stable enough at pH 7.0 or higher, while mild acid medium or solar light induce significant degradation, so justifying the difficulty of their determination (Karljikovic-Rajic K. *et al.*, 2003). Redox reactions and fragmentations are mainly involved and do not require oxygen. This aspect is of particular interest and fits in with recent observations that these drugs act as both proton pump inhibitors (Horn J., 2000) and antioxidant and antiapoptotic agents (Biswas K. *et al.*, 2003).

4. Summary

This PhD thesis has examined abiotic transformations of chemicals selected on the basis of their sale and/or their presence into the aquatic environment. In particular, reaction conditions as close as possible to natural ones in the aquatic systems have been used (aqueous solutions, sunlight irradiation, aerobic conditions). The effects of pH or of natural photosensitizers such as humic acids or nitrates have also been considered. The degradation products have been isolated and fully characterised; in many cases their mechanicistic pathways have been discussed.

In particular it has been observed that:

- Carboxin pesticide is easily photodegradated leading to eight products. Among them sulfoxide is the most photostable and least hydrolyzable. The toxicity tests have revealed that this metabolite exhibits similar or even lower activity than the parent compound.
- Benfuracarb and carbosulfan hydrolyze selectively to carbofuran and, under irradiation, to a phenol derivative, too. Degradation of carbofuran leads exclusively to the phenol derivative and occurs slowly even under sunlight irradiation. These results are in contrast with literature data which report many photoproducts likely due to the different reaction conditions used. Toxicity tests have revealed that carbosulfan and carbofuran are the most active and the phenol derivative is generally less toxic than the parent compounds.
- Irradiation of corticosteroids, prednisolone and dexamethasone, leads to seven products, among which four compounds are unprecedented and derive from cleavage, type *Norrish I*, of the side chain at C-17.
- Naproxen sodium salt is light-sensitive under biomimetic conditions and leads to nine products. It is to be noted that dimeric forms, previously unreported, have been isolated by irradiation in drinking water, probably due to the action of dissolved inorganic salts.
- For the first time a dimeric compound has been isolated from furosemide under sunlight irradiation. The formation of dimer has been rationalized by the formation of a radical cation intermediate. Dehalogenation-hydroxylation

($S_{RN}1Ar^*$) followed by deprotonation and dimerization are the events leading to the dimer. The degradation rate of the drug is fast and could justify the low MEC/PEC ratio found.

- Irradiation of hydrochlorothiazide in water affords three photoproducts. Among these, the dehalogenation-hydroxylation product has been suggested as intermediate in the photodegradation by UV-light of the parent compound but, until this study, it had not been isolated and described.
- Degradation of fibrates under environmental conditions is very slow and lead to photoproducts previously isolated from irradiation of these drugs in organic solvents. As regards gemfibrozil, the first investigation on its photochemical behaviour has been performed which has evidenced the formation of "peculiar" oxidation product.
- Photochemical and hydrolytic behaviour of lansoprazole and omeprazole has been investigated for the first time. It has been found that both drugs degrade in water leading to sulfides, benzimidazolones and a red complex material. Benzimidazoles, dianilines and pyridines have also been identified. Degradation is accelerated in acid medium or by light. Redox reactions and fragmentations are mainly involved and do not require oxygen. This study is of particular interest because it agrees with the recent observations that these drugs act as both proton pump inhibitors and antioxidant and antiapoptotic agents and with the difficulty of their determination (pKas of lansoprazole and omeprazole 3.83 and 4.06, respectively).

All the examined drugs and respective derivatives have been found to be bioactive towards aquatic organisms only at concentrations of mg/l orders, more high than the environmental ones. However, even if they are usually detected into aquatic environment at very low concentrations (ng/l, μg/l), drugs have structural properties to be bioaccumulated in tissues of aquatic organisms bringing on long-term effects, which must be considered.

Special remarks can be drawn by this PhD thesis and are listed as follows:

1. Attention has been focused on the isolation and spectral characterization of metabolites. Their nature is very important, in fact, also in agreement with toxicity data obtained in this thesis, many derivatives are more

persistent and exhibit toxicity higher than the parent compounds, in particular in chronic results. Thus, the possible presence of transformation products in surface waters should be taken into account by including metabolites in monitoring systems of groundwater and surface waters. These analytical investigations are still limited because of the dearth of data on the environmental fate of xenobiotics and the lack of standard metabolites.

2. According to the preceding remark, a study on eco-toxicity not only of the parent compounds but also of their metabolites has been performed and evaluated towards aquatic organisms. This combination of studies is not frequent in the literature but during recent years has increasingly become of interest to the environmental chemistry community. Indeed, the validity of toxicological studies is meaningful only if they include both the parent compounds and their derivatives.
3. Finally, besides confirming known photochemical processes, this study has evidenced new photoinduced routes which can represent the starting point for further studies in the field of photochemical reactions (e.g., the photoisomerization of carboxin **1** to quinolinone **6** or the photooxidation of gemfibrozil **60** to aldehyde **65**).

5. Experimental Section

Equipment and methods.

Nuclear magnetic resonance (NMR) spectra were recorded at 500 MHz for [^{1}H] and 125 MHz for [^{13}C] on a Fourier Transform NMR Varian 500 Unity Inova spectrometer. Carbon multiplicity was evidenced by DEPT experiments. The proton couplings were evidenced by ^{1}H-^{1}H COSY experiments. The heteronuclear chemical shift correlations were determined by HMQC and HMBC pulse sequences.

Electronic impact mass spectra (EIMS) were obtained with a HP 6890 spectrometer equipped with a MS 5973N detector (SIS Instruments). Matrix assisted laser desorption ionization (MALDI) mass spectra were recorded using a Voyager-DE MALDI-TOF spectrometer. Electrospray ionization (ESI) spectra were recorded using a Finnigan LCQ operating in negative ion mode. The scan range was 80-2000 *m/z*.

Infrared spectra (IR) were determined on a Fourier Transform Infrared Perkin-Elmer 1740 spectrometer in $CHCl_3$ solutions. Ultraviolet spectra (UV) were recorded on a Perkin-Elmer LAMBDA 7 spectrophotometer.

Irradiation experiments were performed with a 150-W solar simulator equipped with a Xenon lamp. The lamp had a spectral output 200 to 2.400 nm and an irradiance at 0.5 m higher than 10 mW m^{-2} nm^{-1}; a filter was used to simulate irradiation at the earth surface (Oriel Instruments) or by 500 W high-pressure mercury lamp (Helios Italquartz).

The HPLC apparatus consisted of an Agilent 1100 HPLC system equipped with UV or refractive index detector or on a Varian Vista 5500 apparatus equipped with a refractometric detector

Analytical TLC was made on Kieselgel 60 F_{254} or RP-18 F_{254} plates with 0.2 mm layer thickness (Merck). Preparative TLC was performed on Kieselgel 60 F_{254} plates with 0.5 or 1 mm film thickness (Merck). Flash column chromatography (FCC) was conducted on Kieselgel 60, 230-400 mesh (Merck), at medium pressure.

Sewage treatment plant (STP) water was obtained from Mercato S. Severino treatment plant (Salerno, Italy). KNO_3 and humic acids were obtained from Aldrich.

5.1 Fungicides: carboxin (1)

Chemicals Carboxin, analytical standard grade (99%), was supplied by Labservice Analytika S.r.l.

Photolysis of carboxin by natural solar light

Suspension of carboxin (20 mg) in 300 mL of deionized water was exposed to natural sunlight in Pyrex flask, under aerobic conditions, at Naples in October 2003. Similar experiments were carried out adjusting pH of suspension at 2 by HCl 1 mM and at 10 by KOH 0.1 mM, in the presence of KNO_3 (10 mg/l), with humic acid (5 mg/l). Each experiment was performed in duplicate, with one set of dark controls. After 4 days sunlight exposure, each reaction mixture was extracted with ethyl acetate. The organic layer and the aqueous extract were analyzed by ^{1}H-NMR. The organic extract was chromatographed by reverse phase C-18 HPLC [Agilent 1100 HPLC system equipped with refractive index detector. The column was a Phenomenex HYDRO RP-18, 4 μm, 250 x 4.5 mm, eluent H_2O-CH_3OH-CH_3CN (5:3:2)] to give unreacted carboxin (30-55%) and the photoproducts (complessively 20-35%). The aqueous extract was acidified with HCl 2M and extracted with ethyl acetate. The organic layer gave pure oxanilic acid **7**. All the products were fully characterized by spectral means.

Spectral data

Compound **2**: IR ($CHCl_3$) ν 1721, 1673, 1079 cm^{-1}, 1039; ^{1}H NMR ($CDCl_3$) δ 2.38 (s,3H, Me), 2.90 e 3.10 (2m, 2H, CH_2S), 4.40-4.70 (m, 2H, CH_2O), 7.10-7.60 (m, 5H, ArH), 8.35 (brs, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 20.7 (Me), 43.5 (CH_2S), 56.9 (CH_2O), 110.7 (C-3), 120.5 (C-2'), 124.6 (C-4'), 129.2 (C-3'), 137.8 (C-1'), 163.6 (CON), 166.6 (C-2); EIMS: *m/z* 251 $[M]^+$, 234, 159, 131.

Compound **3**: IR ($CHCl_3$) ν 3377, 1718, 1694 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.11 (s, 3H, Me), 2.90 - 3.26 (m, 2H, CH_2S), 4.01 e 4.42 (2m, 2H, CH_2O), 7.10-7.60 (m, 5H, ArH), 8.83 (brs, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 25.3 (Me), 33.8 (CH_2S), 71.8

(CH_2O), 92.1 (C-2), 119.8 (C-2'), 125.5 (C-4'), 129.2 (C-3'), 136.1 (C-1'), 156.2 (CON), 189.7 (CO). EIMS: *m/z* 251 $[M]^+$, 148, 103.

Compound **4**: IR ($CHCl_3$) ν 2852, 1717 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.08 (s, 3 H, Me), 2.93 (t, J = 6.3 Hz, 2H, CH_2S), 4.33 (t, J = 6.3 Hz, 2H, CH_2O). ^{13}C NMR ($CDCl_3$) δ 23.7 (Me), 38.7 (CH_2S), 68.1 (CH_2O), 172.6 (CO_2). EIMS: *m/z* 87 $[M-SH]^+$, 60, 43.

Compound **5**: ^1H NMR ($CDCl_3$) δ 2.88 (t, J = 6.3 Hz, 4H, $2CH_2S$), 2.98 (brs, 2H, 2OH), 3.89 (t, J = 6.3 Hz, 4H, $2CH_2O$); ^{13}C NMR ($CDCl_3$) δ 41.2 (CH_2S), 60.3 (CH_2O); EIMS *m/z* 154 $[M]^+$, 92, 79, 64, 45.

Compound **6**: IR ($CHCl_3$) ν 3389, 1641 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.92 (s, 3H, Me), 3.04 (t, J = 5.2 Hz, 2H, CH_2S), 3.70 (t, J = 5.2 Hz, 2H, CH_2O), 4.35 (brs, 1H, OH), 7.30 (t, J = 7.5 Hz, 1H, H-6), 7.45 (d, J = 7.5 Hz, 1H, H-8), 7.58 (t, J = 7.5 Hz, 1H, H-7), 7.78 (d, J = 7.5 Hz, 1H, H-5), 11.75 (brs, 1H, NH). ^{13}C NMR ($CDCl_3$) δ 18.2 (Me), 38.8 (CH_2S), 60.2 (CH_2O), 116.7 (C-8), 120.6 (C-10), 123.2 (C-6), 124.4 (C-3), 125.5 (C-5), 131.5 (C-7), 137.2 (C-9), 155.7 (C-4), 161.1 (C-2). EIMS: *m/z* 235 $[M]^+$, 204, 143, 77, 43.

Compound **7**: IR (KBr, wafer) ν 3473, 1697 cm^{-1}; ^1H NMR (DMSO) δ 7.03 (t, J = 7.3 Hz, 1H, H-4'), 7.27 (t, J = 7.3 Hz, 2H, H-3') and 7.75 (d, J = 7.3 Hz, 2H, H-2'), 10.18 (brs, 1H, NH). ^{13}C NMR (DMSO) δ 119.8 (C-2'), 125.5 (C-4'), 129.2 (C-3'), 136.1 (C-1'), 163.3 (CON), 165.5 (COOH); EIMS: *m/z* 167 $[M]^+$, 148 $[M-OH]^+$.

Compound **8**: IR ($CDCl_3$) ν 3377, 1741, 1703, 1677 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.07 (3H, s, Me), 3.26 (m, J = 6.3, 2H, CH_2S), 4.27 (m, J = 6.3, 2H, CH_2O), 7.10-7.70 (m, 5H, ArH), 8.49 (brs, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 20.1 (Me), 28.0 (CH_2S), 61.8 (CH_2O), 119.9 (C-2'), 125.6 (C-4'), 129.2 (C-3'), 135.7 (C-1'), 155.8 (CON), 170.6 (CO_2), 191.6 (COS); EIMS *m/z* 267 $[M]^+$, 224, 92.

Compound **9**: IR ($CDCl_3$) ν 3680, 3620, 1674 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.33 (s, 3H, Me), 2.71 (t, J = 6.3, 2H, CH_2S), 3.76 (t, J = 6.3, 2H, CH_2O), 7.02-7.70 (m, 5H, ArH), 9.35 (brs, 1H, NH), 15.42 (s, 1H, OH); ^{13}C NMR ($CDCl_3$) δ 21.3 (Me), 39.1 (CH_2S), 59.7 (CH_2O), 93.1 (C-3),

120.3 (C-2’), 124.5 (C-4’), 129.0 (C-3’), 137.3 (C-1’), 171.1 (C-2), 183.1 (C-7); EIMS: *m/z* 253 $[M]^+$, 193, 135.

5.2 Carbamic insecticides: benfuracarb (15), carbosulfan (16) and carbofuran (17)

Chemicals

Benfuracarb, carbosulfan and carbofuran were commercially available by Aldrich-Fluka and used without further purification.

Transformations of pesticides

Irradiations were performed by exposure of the compounds to sunlight or to UV lamp. In a standard procedure suspensions of benfuracarb (205 ppm) and carbosulfan (190 ppm) in MilliQ water were exposed to sunlight in Pyrex flasks, under aerobic conditions. Each experiment was performed in duplicate, with one set of dark controls. After 6 days, each reaction mixture was evaporated in vacuum and residues were analysed by ^{1}H-NMR and by HPLC [Agilent 1100 system equipped with UV detector. The column was a Spherex 10μm OH (DIOL), eluent hexane-ethyl acetate (4:1), λ = 280 nm]. Control experiments showed that diluted solutions (4 ppm) of benfuracarb and carbosulfan afforded similar results.

Experiments using the same concentrations of pesticides were carried out at pH 5.0 and 9.0 using NaH_2PO_4/Na_2HPO_4 adjusting the pH by HCl 0.2 M and KOH 0.2 M. After 6 days, each reaction mixture was neutralized and analyzed by ^{1}H-NMR and HPLC. Experiments in presence of KNO_3 (10 mg/l) and of humic acid (5 mg/l) were also performed. After 6 days, each reaction mixture was evaporated in vacuum and analyzed by ^{1}H-NMR and HPLC.

Carbofuran (110 ppm) in MilliQ water was treated according to the standard procedure. After six days it was recovered unchanged at the dark while by irradiation it decomposed for about 7 % leading only to phenol derivative **18** (^{1}H NMR and HPLC).

Kinetic experiments of carbofuran (4 ppm) in MilliQ water were performed in Pyrex tubes and this compound was irradiated with UV lamp. At selected time intervals, samples were collected and analyzed directly using HPLC [Agilent

1100 system equipped with UV detector. The column was a Synergy 4 μm MAX-RP80A, eluent water-methanol-acetonitrile (21:14:15), λ = 254 nm].

Carbofuran-phenol (**18**) was isolated from irradiation experiments by repeated TLC [hexane-ethyl acetate (7:3)], and identified by comparison of ^1H and ^{13}C-NMR data with those of an authentic sample which was obtained by treating carbofuran (0.09 M) with methanolic KOH (5%): IR ($CHCl_3$) ν 3568 cm^{-1}; ^1H-NMR ($CDCl_3$) δ 6.72 (m, 3H), 3.04 (s, 2H), 1.50 (s, 6H). ^{13}C-NMR ($CDCl_3$): δ 145.8 (C-2), 140.3 (C-1), 127.8 (C-3), 120.7 (C-4, C-5), 117.0 (C-6), 88.0 (C-7), 43.5 (C-8), 28.2 (C-9, C-10).

5.3 Steroidal anti-inflammatory drugs: prednisolone (19) and dexamethasone (20)

Chemicals

Prednisolone and dexamethasone were purchased from Sigma–Aldrich and used without further purification.

*Irradiation of prednisolone (**19**)*

A suspension of prednisolone (100 mg) in water (500 ml) was irradiated by the solar simulator for 4 hr under slow magnetic stirring. The reaction mixture was extracted with ethyl acetate and the residue was subjected to silica gel flash chromatography. Elution with $CHCl_3$-acetone (19:1) gave a mixture of products **25** - **28** (5 mg), while elution with $CHCl_3$-CH_3OH (19:1) gave unreacted prednisolone (**19**) (55 %), pure **22** (13%) and crude **23** and **24**. TLC chromatography on silica gel ($CHCl_3$-CH_3OH 19:1) gave pure **23** (10%). Reverse-phase C-18 HPLC [Varian Vista 5500 HPLC system equipped with a refractometric detector and Lichrosorb RP-8 columns, eluent H_2O-CH_3OH-CH_3CN (6: 2: 2)] gave pure **24** (11%).

*Synthesis of 1,4-androstadien-11β-olo-3,17-dione (**25**)*

To a solution of prednisolone (**19**) (200 mg) in ethyl acetate (10 ml) MnO_2 (4 g) was added. After 1 hr at room temperature the reaction mixture was filtered on celite eluting with ethyl acetate and methanol. Chromatography on silica gel ($CHCl_3$-acetone 19:1) of the filtrate gave 1,4-androstadien-11β-olo-3,17-dione (**5d**) (95%).

*Irradiation of 1,4-androstadien-11β-olo-3,17-dione (**25**)*

A suspension of 1,4-androstadien-11β-olo-3,17-dione (**25**) (180 mg) in water (1 l) was irradiated by the solar simulator for 4 hr under magnetic stirring. The reaction mixture was separated by silica gel flash chromatography ($CHCl_3$-acetone 19:1) into its components **25** (78%), **26** (8%), **27** (4%) and **28** (11%).

Irration of dexamethasone (**20**)

Dexamethasone (**20**) (100 mg) suspended in water (500 ml) was irradiated by the solar simulator for 8 hr. The organic material was extracted with ethyl acetate (2×150 ml) and chromatographed by flash chromatography on silica gel. Elution with $CHCl_3$-acetone (7:3) gave three fractions A-C. Fraction A (84%) consisted of unreacted **20**. TLC chromatography on silica gel of fraction B [$CHCl_3$-CH_3OH (93:7)] gave **30** (4%) while TLC chromatography [organic phase of the mixture hexane-CH_2Cl_2-CH_3OH-H_2O (10:40:17:8)] of fraction C gave **29** (1%).

Specral data

Infrared spectra (IR) were determined in $CHCl_3$ solutions (0.025 M), while ultraviolet spectra (UV) were recorded in ethanol (10^{-4} M).

Compound **22**: $[\alpha]_D$ +18.0° (c 0,5); IR ($CHCl_3$): ν_{max} 3677, 3409, 1710 cm^{-1}; UV λ_{max} 231 nm; ^{1}H-NMR (C_5D_5N) δ 7.78 (d, *J* = 5,6 Hz, 1H, H-1), 6.69 (d, *J* = 5,6 Hz, 1H, H-2), 5.26 (d, *J* = 19.2 Hz, 1H, H-21), 4.82 (d, *J* = 19.2 Hz, 1H, H-21), 4.72 (brs, 1H, H-11), 3.20 (d, *J* = 19.2 Hz, 1H, H-4), 3.12 (m, 1H, H-16), 2.05 (d, *J* = 19.2 Hz, 1H, H-4), 1.25 (s, 3H, H-18), 1.20 (s, 3H, H-19); ^{13}C-NMR (C_5D_5N) δ 168.2 (C-1), 134.2 (C-2), 209.1 (C-3), 46.4 (C-4), 74.7 (C-5), 34.3 (C-6), 28.2 (C-7), 31.8 (C-8), 50.8 (C-9), 54.5 (C-10), 69.2 (C-11), 39.5 (C-12), 47.7 (C-13), 51.7 (C-14), 24.0 (C-15), 34.6 (C-16), 89.6 (C-17), 17.9 (C-18), 23.4 (C-19), 213.4 (C-20), 67.6 (C-21); EIMS *m/z* 378 $[M]^+$, 360 $[M-H_2O]^+$, 319 $[M-C_2H_3O_2]^+$.

Compound **23**: $[\alpha]_D$ -159,0° (c 0,7); IR ($CHCl_3$): ν_{max} 3685, 3505, 1727, 1710 cm^{-1}; UV λ_{max} 256 nm; ^{1}H-NMR (C_5D_5N) δ 5.34 (dt, *J* = 2.1 and 7.2 Hz, 1H, H-1), 5.27 (d, *J* = 19.2 Hz, 1H, H-21), 4.82 (d, *J* = 19.2 Hz, 1H, H-21), 3.11 (ddd, *J* = 2.1, 4.1 and 15.2 Hz, 1H, H-2α), 3.02 (ddd, *J* = 3.1, 11.6 and 14.7 Hz, 1H, H-16β), 2.62 (dd, *J* = 7.2 and 15.2 Hz, 1H, H-2β), 2.53 (dd, *J* = 4.5 and 14.5 Hz, 1H, H-12α), 2.20 (dd, *J* = 1.7 and 14.5 Hz, 1H, H-12β); ^{13}C-NMR (C_5D_5N) δ 116.6 (C-1), 38.4 (C-2), 204.3 (C-3), 86.6 (C-4), 54.0 (C-5), 26.2 (C-6), 26.9 (C-7), 31.2

(C-8), 54.3 (C-9), 145.6 (C-10), 77.9 (C-11), 32.8 (C-12), 48.2 (C-13), 49.1 (C-14), 22.9 (C-15), 34.2 (C-16), 89.3 (C-17), 17.5 (C-18), 25.0 (C-19), 213.0 (C-20), 67.5 (C-21); EIMS *m/z* 360 $[M]^+$, 342 $[M\text{-}H_2O]^+$, 301 $[M\text{-}C_2H_3O_2]^+$.

Compound **24**: ^{1}H-NMR ($CDCl_3$) δ 5.90 (d, *J* = 1.3 Hz, 1H, H-4), 4.69 (brs, 1H, H-11), 4.64 (d, *J* 19.2 Hz, 1H, H-21), 4.27 (d, *J* = 19.2 Hz, 1H, H-21), 0.90 (s, 3H, H-19), 1.15 (s, 3H, H-18); ^{13}C-NMR (CD_3OD) δ 58.3 (C-1), 37.3 (C-2), 212.8 (C-3), 131.5 (C-4), 188.3 (C-5), 32.1 (C-6), 30.9 (C-7), 40.4 (C-8), 62.7 (C-9), 76.8 (C-10), 70.3 (C-11), 40.3 (C-12), 40.3 (C-13), 53.7 (C-14), 26.0 (C-15), 34.7 (C-16), 90.8 (C-17), 18.7 (C-18), 20.4 (C-19), 213.8 (C-20), 68.1 (C-21); EIMS *m/z* 360 $[M]^+$.

Compound **25**: ^{1}H-NMR (CD_3OD) δ 7.45 (d, *J* = 5.9 Hz, 1H, H-1), 6.24 (d, *J* = 5.9 Hz, 1H, H-2), 6.01 (brs, 1H, H-4), 4.39 (brs, 1H, H-11), 1.18 (s, 3H, H-18), 1.52 (s, 3H, H-19); ^{13}C-NMR (CD_3OD) δ 161.0 (C-1), 128.0 (C-2), 189.5 (C-3), 123.0 (C-4), 174.9 (C-5), 35.0 (C-6), 33.1 (C-7), 32.5 (C-8), 57.5 (C-9), 46.8 (C-10), 71.1 (C-11), 42.0 (C-12), 46.8 (C-13), 53.2 (C-14), 23.7 (C-15), 37.0 (C-16), 214.4 (C-17), 17.5 (C-18), 22.5 (C-19).

Compound **26**: ^{1}H-NMR ($CDCl_3$) δ 7.70 (d, *J* = 5.9 Hz, 1H, H-1), 6.18 (d, *J* = 5.9 Hz, 1H, H-2), 4.46 (brs, 1H, H-11), 2.84 (d, *J* = 19.5 Hz, 1H, H-4), 1.92 (d, *J* = 19.5 Hz, 1H, H-4), 1.15 (s, 3H, H-19), 1.09 (s, 3H, H-18); ^{13}C-NMR ($CDCl_3$) δ 167.1 (C-1), 134.8 (C-2), 209.9 (C-3), 45.9 (C-4), 74.8 (C-5), 33.9 (C-6), 26.8 (C-7), 31.0 (C-8), 51.8 (C-9), 54.3 (C-10), 69.6 (C-11), 40.2 (C-12), 47.0 (C-13), 51.5 (C-14), 23.2 (C-15), 35.5 (C-16), 219.3 (C-17), 16.1 (C-18), 21.7 (C-19).

Compound **27**: ^{1}H-NMR ($CDCl_3$) δ 5.44 (dt, *J* = 2.1 and 7.2 Hz, 1H, H-1), 4.45 (brs, 1H, H-11), 4.18 (s, 1H, H-4), 1.08 (s, 3H, H-18), 1.40 (s, 3H, H-19); ^{13}C-NMR ($CDCl_3$) δ 116.4 (C-1), 37.9 (C-2), 204.1 (C-3), 86.4 (C-4), 53.9 (C-5), 25.7 (C-6), 25.2 (C-7), 30.5 (C-8), 54.7 (C-9), 144.8 (C-10), 76.7 (C-11), 33.0 (C-12), 47.4 (C-13), 48.9 (C-14), 25.2 (C-15), 35.3 (C-16), 219.7 (C-17), 15.6 (C-18), 21.0 (C-19).

Compound **28**: ^{1}H-NMR (CD_3OD) δ 5.94 (s, 1H, H-4), 4.68 (m, 1H, H-11), 1.17 (s, 6H, H-18, H-19); ^{13}C-NMR (CD_3OD) δ 58.2 (C-1), 36.7 (C-2), 212.6 (C-3), 131.7 (C-4), 187.9 (C-5), 32.0 (C-6), 29.4 (C-7), 40.4 (C-8), 63.4 (C-9), 76.8 (C-

10), 69.7 (C-11), 41.1 (C-12), 41.1 (C-13), 53.9 (C-14), 23.9 (C-15), 36.7 (C-16), 215.0 (C-17), 17.1 (C-18), 20.5 (C-19).

Compound **29**: ^{1}H-NMR (CD_3OD) δ 5.45 (dt, *J* = 2.0 and 7.0 Hz, 1H, H-1), 4.63 (d, *J* = 19,5 Hz, 1H, H-21), 4.27 (d, *J* = 19.5 Hz, 1H, H-21), 4.21 (s, 1H, H-4), 4.19 (m, 1H, H-11), 3.18 (m, 1H, H-6), 3.02 (m, 1H, H-16β), 3.00 (m, 1H, H-2), 2.75 (dd, *J* = 7.0 and 15.6 Hz, 1H, H-2), 2.38 (brs, 1H, H-6), 2.25 (m, 3H, H-7, H-12 and H-14), 1.80 (m, 1H, H-12), 1.65 (m, 1H, H-15), 1.53 (m, 1H, H-7), 1.37 (s, 3H, H-19), 1.17 (m, 1H, H-15), 0.92 (4H; m, 1H, H-8; d, *J* = 5.6 Hz, 3H, H-22), 0.90 (s, 3H, H-18); ^{13}C-NMR (CD_3OD) δ 116.8 (C-1), 38.0 (C-2), 203.5 (C-3), 85.7 (C-4), 53.0 (C-5), 25.1 (C-6), 30.0 (C-7), 36.2 (C-8), 99.7 (C-9), 143.8 (C-10), 98.1 (C-11), 31.2 (C-12), 49.6 (C-13), 42.5 (C-14), 21.2 (C-15), 34.3 (C-16), 90.1 (C-17), 17.0 (C-18), 20.5 (C-19), 212.1 (C-20), 67.8 (C-21), 14.9 (C-22).

Compound **30**: ^{1}H-NMR (CD_3OD) δ 5.98 (s, 1H, H-4), 4.62 (d, *J* = 19.1 Hz, 1H, H-21), 4.52 (m, 1H, H-11), 4.29 (d, *J* = 19.1 Hz, 1H, H-21), 3.09 (m, 1H, H-16β), 3.01 (m, 1H, H-2), 2.65 (m, 1H, H-2), 2.51 (m, 2H, H-6 e H-6’), 2.51 (m, 1H, H-7), 2.38 (m, 1H, H-12), 2.21 (m, 1H, H-14), 1.77 (m, 2H, H-1, H-7), 1.68 (m, 1H, H-15), 1.,46 (m, 1H, H-12), 1.3 (m, 1H, H-15), 1.25 (m, 1H, H-8), 1.22 (s, 3H, H-19), 1.00 (s, 3H, H-18), 0.89 (d, *J* = 17.2 Hz, 3H, H-22); ^{13}C-NMR (CD_3OD) δ 50.3 (C-1), 37.2 (C-2), 212.5 (C-3), 131.8 (C-4), 187.9 (C-5), 32.7 (C-6), 35.2 (C-7), 41.6 (C-8), 103.8 (C-9), 79.1 (C-10), 72.4 (C-11), 40.4 (C-12), 49.9 (C-13), 45.8 (C-14), 25.3 (C-15), 37.2 (C-16), 92.6 (C-17), 18.3 (C-18), 20.6 (C-19), 213.2 (C-20), 68.5 (C-21), 15.9 (C-22).

5.4 Non-steroidal antinflammatory drug: naproxen sodium salt (33)

Chemicals

Naproxen Na was purchased from Sigma–Aldrich and used without further purification.

Irradiation of naproxen Na in distilled water

A 7.8 x 10^{-4} M solution of naproxen sodium salt (**33**) in distilled water was irradiated at 20°C for 72 h by the solar simulator. The water was evaporated in vacuo and the residue, dissolved in acetone, was filtered on HV13 Millex filter, Millipore Co. The residue was filtered on Sep-Pak C-18 cartridges, Water Co, to

give fractions A–C. Fraction A eluting 20 ml H_2O-CH_3CN 7:3 contained alcohol **35** (21%). Fraction B eluting 20 m H_2O-CH_3CN 1:1 contained ketone **38** (48%) and olefin **39** (9%). Fraction C, eluting 20 ml H_2O-CH_3CN 3:7, contained **34** (8%) and olefin **39** (11%). Each compound was purified by reverse phase C-18 HPLC [Agilent 1100 system equipped with a ultraviolet detector and a Synergy Hydro column, eluent H_2O-CH_3CN (3:7), λ = 254 nm].

Irradiation of naproxen sodium salt in drinking water

A 7.8 x 10^{-4} M solution of naproxen sodium salt (**33**) in drinking water was irradiated at 20°C for 72 h by the solar simulator. The water was evaporated in vacuo and the residue, dissolved in acetone, was filtered on HV13 Millex filter. The residue has been filtered on Sep-Pak C-18 to give fractions A–B. Fraction A eluting 20 ml H_2O-CH_3CN (7:3) contained naproxen Na (16%), alcohol **35** (16%) and dimers **41** (2 x 5%). Fraction B eluting 20 ml H_2O-CH_3CN (1:1) contained ketone **38** (15%), ether **37** (4%), olefin **39** (3%) and dimer **40** (6%). Each compound has been purified by reverse phase C-18 HPLC.

Spectral data

Compound **34** was identified by comparison of spectral data with those previously reported by Boscá F. *et al*. (1990).

Compound **35**: ^{1}H-NMR ($CDCl_3$) δ 7.77 (m, 3H, H-1, H-4 e H-8), 7.52 (dd, *J* = 1.8 and 8.7 Hz, 1H, H-3), 7.20 (dd, *J* = 2.0 and 9.0 Hz, 1H, H-7), 7.18 (s, 1H, H-5), 5,04 (q, *J* = 7.0 Hz, 1H, H-11), 3.97 (s, 3H, OCH_3), 1,62 (d, *J* = 7.0 Hz, 3H, H-13); ^{13}C-NMR ($CDCl_3$) δ 157.6 (C-6), 140.9 (C-2), 134.0 (C-10), 129.4 (C-1), 128.7 (C-9), 127.1 (C-8), 124.4 (C-3), 123.7 (C-4), 118.9 (C-7), 105.7 (C-5), 70.5 (C-11), 55.3 (OCH_3), 25.0 (C-13).

Compound **36**: ^{1}H NMR ($CDCl_3$) δ 7.75 (3H, H-1, H-4 and H-8), 7.47 (dd, *J* = 1.8 and 8.7 Hz, 1H, H-3), 7.16 (dd, *J* = 2.5 and 9.0 Hz, 1H, , H-7), 7.14 (d, *J* = 2.5 Hz, 1H, H-5), 5.12 (q, *J* = 7.0 Hz, 1H, H-11), 3.93 (3H, s, OCH_3), 1.55 (d, *J* = 7.0 Hz, 3H, H-13).

Compound **37**: ^{1}H-NMR ($CDCl_3$) δ 7.74 (d, *J* = 8,0 Hz, 1H, H-4), 7.72 (d, *J* = 8.5 Hz, 1H, H-8), 7.66 (d, *J* = 1.3 Hz, 1H, H-1), 7.45 (dd, *J* = 1.3 and 8.0 Hz, 1H, H-3), 7.15 (dd, *J* = 2.0 and 8.5 Hz, 1H, H-7), 7.14 (d, *J* = 2.0 Hz, 1H, H-5), 4.55 (q, *J* = 7.1 Hz, 1H, H-11), 3.92 (s, 3H, OCH_3), 3.39 (q, *J* = 7.0 Hz, 2H, OCH_2), 1.52 (d,

J = 7.1 Hz, 3H, H-13), 1.20 (t, J = 7.0 Hz, 3H, CH_3); ^{13}C-NMR ($CDCl_3$) δ 157.6 (C-6), 139.3 (C-2), 134.1 (C-10), 129.3 (C-8), 128.7 (C-9), 127.1 (C-4), 124.8 (C-1), 124.7 (C-3), 118.7 (C-7), 105.7 (C-5), 77.8 (C-11), 63.9 (OCH_2), 55,3 (OCH_3), 24,1 (C-13), 15,4 (CH_3).

Compound **38**: ^{1}H-NMR ($CDCl_3$) δ 8.40 (d, J = 1.5 Hz, 1H, H-1), 8.01 (dd, J = 1.5 and 8.5 Hz, 1H, H-3), 7.86 (d, J = 9.0 Hz, 1H, H-8), 7.77 (d, J = 8.5 Hz, 1H, H-4), 7.21 (dd, J = 2.5 and 9.0 Hz, 1H, H-7), 7.16 (d, J = 2.5 Hz, 1H, H-5), 3.96 (s, 3H, OCH_3), 2.70 (s, 3H, H-13); ^{13}C-NMR ($CDCl_3$) δ 197.8 (CO), 159.7 (C-6), 137.2 (C-2), 132.5 (C-10), 131.1 (C-1), 131.1 (C-8), 130.0 (C-9), 127.0 (C-4), 124.6 (C-3), 119.6 (C-7), 105.7 (C-5), 55.4 (OCH_3), 26.5 (C-13).

Compound **39**: ^{1}H-NMR ($CDCl_3$) δ 7.70 (3H; d, J = 8.0 Hz, 1H, H-4; d, J = 8 Hz, 1H, H-8; s, 1H, H-1), 7.61 (dd, J = 2.0 and 8.7 Hz, 1H, H-3), 7.13 (2H; 1H, dd, H-7; 1H, s, H-5), 6.82 (dd, J = 10.5 and 16.5 Hz, 1H, H-11), 5.82 (dd, J = 1.0 and 16.5 Hz, 1H, H-13), 5.28 (dd, J = 1.0 and 10.5 Hz, 1H, H-12), 3.93 (s, 3H, OCH_3); EIMS: m/z 184 $[M]^+$, 152 $[M\text{-}OCH_3]^+$.

Compound **40**: ^{1}H-NMR ($CDCl_3$) δ 8.12 (d, J = 9.0 Hz, 1H, H-8), 7.88 (d, J = 2.0 Hz; 1H, H-1'), 7.74 (d, J = 9.0 Hz, 1H, H-8'), 7.64 (d, J = 9.0 Hz, 1H, H-4'), 7.59 (d, J = 9.0 Hz, 1H, H-4), 7.28 (dd, J = 2.0 and 9.0 Hz, 1H, H-3'), 7,16 (3H; d, J = 2.5, 1H, H-5; dd, J = 2.5 and 9.0 Hz, 1H, H-7; dd, J = 2.5 and 9.0 Hz, 1H, H-7'), 7,10 (d, J = 2.5 Hz, 1H, H-5'), 6.98 (d, J = 9.0 Hz, 1H, H-3), 5.22 (q, J = 7.0 Hz, 1H, H-11'), 3.92 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 1.87 (d, J = 7.0 Hz, 3H, H-13'); ^{13}C-NMR ($CDCl_3$) δ 157.8 (C-6'), 155.6 (C-6), 150.3 (C-2), 138.9 (C-2'), 133.4 (C-10'), 129.3 (C-8'), 129.0 (C-9), 128.1 (C-9'), 128.1 (C-10), 127.9 (C-4), 127.4 (C-4'), 127.1 (C-3'), 124.1 (C-1'), 124.1 (C-8), 123.9 (C-1), 120.0 (C-3), 119.1 (C-7), 118.9 (C-7'), 107.2 (C-5), 105.8 (C-5'), 55.5 (2 x OMe), 35.1 (C-11'), 17.3 (C-13'); MALDI-TOF m/z 358 $[M]^+$, EIMS m/z 185 $[M\text{-}C_{11}H_9O_2]^+$, 156 $[C_{11}H_9O_2 - OH]^+$.

Compound **41** (Rf = 0,77): ^{1}H-NMR ($CDCl_3$) δ 7.66 (2H; d, J = 9.0 Hz, 1H, H-4; d, J = 9.0 Hz, 1H, H-8), 7.65 (d, J = 2.0 Hz, 1H, H-1), 7.38 (dd, J = 2.0 and 9.0 Hz, 1H, H-3), 7.14 (dd, J = 2.5 and 9.0, 1H, H-7), 7.10 (d, J = 2.5 Hz, 1H, H-5), 4.69 (q, J = 7.0, 1H; H-11), 3.91 (s, 3H, OCH_3), 1.56 (d, J = 7.0, 1H; 3H, H-13); ^{13}C-NMR ($CDCl_3$) δ 124.8 (C-1), 157.5 (C-6), 139,4 (C-2), 133.9 (C-10), 129.3

(C-8), 128.7 (C-9), 128.7 (C-4), 125,2 (C-3), 118.6 (C-7), 105.7 (C-5), 74.5 (C-11), 55.3 (OCH_3), 22.8 (C-13); MALDI-TOF *m/z* 386 $[M]^+$, EIMS *m/z* 185 $[M-C_{13}H_9O_2]^+$.

Compound **41** (Rf = 0,69): ^{1}H-NMR ($CDCl_3$) δ 7.77 (d, *J* = 9.0 Hz, 1H, H-4), 7.72 (d, *J* = 9.0 Hz, 1H, H-8), 7.58 (d. *J* = 2.0, 1H, H-1), 7.46 (dd, *J* = 2.0 and 7.0 Hz, 1H, H-3), 7.17 (2H; d, *J* = 2.5 Hz, 1H, H-5; dd, *J* = 2.5 e 9.0 Hz, 1H, H-7), 4.41 (q, *J* = 7.0 Hz, 1H; H-11), 3.95 (s, 3H, OCH_3), 1.46 (d, *J* = 7.0 Hz, 3H, H-13); ^{13}C-NMR ($CDCl_3$) δ 157.6 (C-6), 139.2 (C-2), 134.1 (C-10), 129.3 (C-8), 128.7 (C-9), 127.2 (C-4), 125.1 (C-3), 124.9 (C-1), 118.8 (C-7), 105.8 (C-5), 74.6 (C-11), 55.3 (OCH_3), 24.6 (C-13); MALDI-TOF *m/z* 386 $[M]^+$, EIMS *m/z* 185 $[M-C_{13}H_9O_2]^+$.

5.5 Diuretics: furosemide (42) and hydrochlorothiazide (48)

Chemicals

Furosemide and hydrochlorothiazide were purchased from Sigma–Aldrich and used without further purification.

*Irradiation of furosemide (**42**) in water (24μM)*

A solution of furosemide (24 μM) in distilled water was irradiated at room temperature for 36 hr by the solar simulator. The water was concentrated and the residue was filtered on HV13 Millex filter (Millipore Co) and injected in a HPLC equipped with a ultraviolet detector. Experiments under the same irradiation conditions were also run in distilled water under argon atmosphere, in distilled water added of nitrate ions (5 mg/l), in distilled water in the presence of humic acids (10 mg/l), in drinking water and in effluent of a sewage treatment plant (STP). In all cases the only photoproduct was dimer **8a** and the yields of the photoproduct after 36 h, calculated by HPLC, were 45, 46, 47, and 45% respectively.

*Phototrasformation of furosemide (**42**) in distilled water (preparative scale)*

A solution of furosemide (0.6 mM) in distilled water was irradiated at room temperature for 36 hr by the solar simulator. The water was concentrated and the residue was filtered on HV13 Millex filter (Millipore Co). The mixture (100 mg) separated by silica gel flash column chromatography eluting with $CHCl_3$-acetone-

CH_3OH (3:1:1), yielded two fractions: A (56 mg) and B (38 mg). Fraction A contained mainly furosemide. Fraction B was further purified by reverse phase C-18 HPLC performed on an Agilent 1100 apparatus equipped with a UV-detector and a Synergy Hydro column which was equilibrated with a mixture of A (H_2O containing 1% acetic acid) - B (MeOH containing 1% acetic acid) 10:0 and the run was with the following program: an increase of B up to 5% in 5 min and then an increase of B up to 100% in 10 min, finally isocratic run for 5 min. The detector was set at 325 nm. From HPLC compound **8a** (40%) was obtained.

Spectral data

Compound **43**: ^{1}H NMR (D_2O) δ 8.01 (s, 1H, H-2), 7.35 (d, *J* = 3.5 Hz, 1H, H-11), 6.30 (m, 1H, H-10), 6.24 (d, *J* = 5.5 Hz, 1H, H-9), 4.24 (s, 2H, H-7).

^{13}C NMR (D_2O) δ 173.2 (C-12), 164.3 (C-4), 152.6 (C-6), 150.2 (C-8), 140.2 (C-11), 130.7 (C-2), 113.4 (C-3), 108.3 (C-9), 108.3 (C-5), 104.8 (C-10), 104.4 (C-1), 37.3 (C-7); MALDI-TOF *m/z* 560 $[M – CO_2 – H_2O]^+$, 543 $[M – SO_2NH]^+$ and 526 $[543 – OH]^+$.

*Irradiation of HCTZ (**48**) in water (100 μM)*

In a typical procedure a sospension of HCTZ (100 μM) in distilled water was irradiated at room temperature for 200 hr by the solar simulator. To follow the irradiation experiment, an aliquot was withdrawn at various times, concentrated and the residue dissolved in methanol, and injected in a HPLC-UV system (Agilent 1100 system). The column used was a RP-18 column (Phenomenex HYDRO RP-18, 4 μm, 250 x 4.5 mm) and eluted with a mixture of A (H_2O containing 1% acetic acid) - B (MeOH containing 1% acetic acid) 90:10, detection was at 260 nm and the flow rate was 0.7 ml/min. Experiments in the same irradiation conditions were run also in distilled water under argon atmosphere, and in water of a sewage treatment plant (STP).

*Irradiation of HCTZ (**48**) in distilled water (preparative scale)*

A solution of HCTZ (0.7 mM) in distilled water was irradiated at room temperature for 200 hr by the solar simulator. The water was evaporated to dryness. The mixture (100 mg) separated by silica gel flash column chromatography eluting with hexane-ethylic ether-acetone (3:3:4), yielded two

fractions: A (70 mg) and B (26 mg). Fraction A contained HCTZ and compound **52**. Compound **52** (30%) was separated from HCTZ on a preparative TLC (0,5 mm) eluting with hexane-ethylic ether-acetone (3:3:4). Fraction B was purified from reverse phase-HPLC (for experimental conditions see above) and compounds **51** (21%) and **55** (2%) were obtained.

Spectral data

Compound **51**: ^{1}H NMR (CD_3OD) δ 7.92 (s, 1H, H-8), 6.24 (s, 1H, H-5), 4.70 (s, 2H, H-3); ^{13}C NMR (CD_3OD) δ 160.0 (C-6), 150.0 (C-4'), 127.0 (C-8), 120.2 (C-7), 114.6 (C-8'), 101.9 (C-5), 56.0 (C-3); ESI-MS: *m/z* 278 $[M–1]^+$.

Compound **52**: ^{1}H NMR (CD_3OD) δ 8.34 (s,1H, H-2), 6.97 (s, 1H, H-5); ^{13}C NMR (CD_3OD) δ 150.5 (C-4), 137.0 (C-1), 132.1 (C-2), 128.2 (C-6), 123.0 (C-3), 119.2 (C-5); ESI-MS: *m/z* 278 $[M–1]^+$.

Compound **55**: ^{1}H NMR (CD_3OD) δ 8.07 (1H, s, H-2), 6.18 (1H, s, H-5); ^{13}C NMR (CD_3OD) δ 152.6 (C-6), 145.6 (C-4), 131.9 (C-2), 119.5 (C-1), 116.5 (C-5), 116.2 (C-3); ESI-MS: *m/z* 266 $[M–1]^+$.

5.5 Fibrates: bezafibrate (59), gemfibrozil (60), fenofibrate (61)

Chemicals

Compounds **59** - **61** were purchased from Aldrich. Fenofibric acid (**62**) was obtained by dissolving fenofibrate (**61**) (1g) in 5 % methanolic KOH (40 ml) and keeping the reaction mixture at 25 °C for 24 hours. After neutralization of the solution by Amberlite IR-120, methanol was evaporated in vacuum and **62** was obtained quantitatively.

General procedure

A distilled water suspension (solution) of the selected drug (24 μM) in a beaker, equipped with a jacket thermostated at 25° C, was irradiated from the top by the solar simulator. The transformation course was monitored by thin layer chromatography after 50, 100 and/or 200 hr. For preparative purposes, 0.5-0.6 mM concentrations were used. Further experiments were run in distilled water added of nitrate ions (5 mg/l), in distilled water in the presence of humic acids (10 mg/l), in an effluent of the sewage treatment plant (STP), in distilled water after saturating with argon for 30 min.

*Irradiation of bezafibrate (**59**)*

Bezafibrate (108 mg) in distilled water (500 ml) was irradiated for 200 hr by the solar simulator. Water was evaporated in vacuo, the residue was chromatographed by silica gel flash cromathography [$CHCl_3$-acetone-CH_3OH (7:2:1)] to give unreacted bezafibrate (73%) and a fraction A (20 mg). Fraction A was subject to preparative TLC [hexane-acetone (7:3)] to give bezafibrate (11%), pure products **63** (2%) and **64** (3%).

From the irradiation of bezafibrate in distilled water (108 mg/ 500 ml) in the presence of humic acid or KNO_3 and in the irradiation with STP water, products **63** (2%) and **64** (2%) were isolated. Irradiation of bezafibrate in distilled water saturated with argon gave only compound **64** (2%).

Compound **63**: ^{1}H NMR ($CDCl_3$) δ 7.74 (d, *J* = 9.0 Hz, 2H, H-2 and H-6), 7.45 (d, *J* = 9.0 Hz, 2H, H-3 and H-5), 7.06 (d, *J* = 8.5 Hz, 2H, H-2' and H-6'), 6.71 (d, *J* = 8.5 Hz, 2H, H-3' and H-5'), 3.58 (m, *J* = 5.5 Hz, 2H, H-8'), 2.80 (t, *J* = 6.9 Hz, 2 H, H-7'); ^{13}C NMR ($CDCl_3$) δ 169.4 (C-7), 157.4 (C-4'), 139.0 (C-1), 134.9 (C-1'), 131.7 (C-4), 131.2 (C-2' and C-6'), 130.3 (C-3 and C-5), 130.1 (C-2 and C-6), 116.7 (C-3' and C-5'), 43.4 (C-8'), 36.1 (C-7').

Compound **64**: ^{1}H NMR ($CDCl_3$) δ 7.62 (d, *J* = 8.5 Hz, 2H, H-2 and H-6), 7.38 (d, *J* = 8.5 Hz, 2H, H-3 and H-5), 7.12 (d, *J* = 8.9 Hz, 2H, H-2' and H-6'), 6.85 (d, *J* = 8.9 Hz, 2H, H-3' and H-5'), 6.02 (brs, 1H, NH), 4.53 (q, *J* = 6.1 Hz, 1H, H-9'), 3.68 (m, *J* = 5.5 Hz, 2H, H-8'), 2.86 (t, *J* = 6.9 Hz, 2H, H-7'), 1.32 (d, *J* = 6.1 Hz, 6H, H-10' and H-11'); ^{13}C NMR ($CDCl_3$) δ 164.6 (C-7), 156.9 (C-4'), 137.8 (C-1), 133.0 (C-1'), 130.5 (C-4), 129.7 (C-2' and C-6'), 128.8 (C-3 and C-5), 128.2 (C-2 and C-6), 116.2 (C-3' and C-5'), 69.9 (C-9'), 41.3 (C-8'), 34.7 (C-7'), 22.0 (C-10' and C-11').

*Irradiation of gemfibrozil (**60**)*

Gemfibrozil (100 mg) was dispersed in distilled water (700 ml) and irradiated by the solar simulator. After 200 hr, water was evaporated in vacuo and the reaction mixture was chromatographed by silica gel flash cromatography [hexane-ethyl acetate (4:1)] giving gemfibrozil (85%) and crude photoproduct **65** (9%) that was purified by preparative TLC eluting with hexane-ethyl acetate (7:3).

Irradiating in distilled water in the presence of nitrate ions or humic acids, in STP water or in distilled water in Argon atmosphere no trasformation products were obtained, even after 300 hr.

Compound **65**: IR ($CHCl_3$) ν 3300-2500, 2952, 2867, 1670, 1604 cm^{-1}; ^1H NMR ($CDCl_3$) δ 10.38 (s, 1H, H-14), 7.71 (d, *J* = 7.8 Hz, 1 H, H-3), 6.81 (d, *J* = 7.5 Hz, 1H, H-4), 6.70 (brs, 1H, H-6), 4.03 (t, *J* = 6.1 Hz, 2H, H-7), 2.38 (s, 3H, H-15), 1.80 (m, 2H, H-8), 1.78 (m, 2H, H-9), 1.26 (s, 6H, H-12 and H-13); ^{13}C NMR ($CDCl_3$) δ 189.5 (C-14), 182.1 (C-11), 161.4 (C-1), 147.4 (C-5), 128.3 (C-3), 122.6 (C-2), 121.6 (C-4), 112.9 (C-6), 68.4 (C-7), 41.8 (C-8), 36.7 (C-9), 25.0 (C-12 and C-13), 24.8 (C-10), 22.3 (C-15)]; EIMS: *m/z* 264 $[C_{15}H_{20}O_4]^+$, 136 $[C_8H_8O_2]^+$.

*Irradiation of fenofibrate (**61**)*

The suspension of fenofibrate (108 mg) in distilled water (500 ml) was irradiated for 200 hr by the solar simulator. Water was evaporated and the residue was chromatographed by silica gel flash cromathography [hexane-ethyl acetate (9:1)] to give unreacted fenofibrate (88%) and a fraction A that was cromatographed by preparative TLC [hexane-ethyl acetate (4:1)] to give fenofibric acid (**62**) (4%) and compound **66** (4%).

Irradiation in distilled water in the presence of nitrate ions, humic acids, or in STP water gave products **62** and **66** in similar yields as in pure distilled water. Fenofibrate (**61**) by irradiation in argon gave only fenofibric acid **13** in traces

Compound **66**: ^1H NMR ($CDCl_3$) δ 7.76 (d, *J* = 8.5 Hz, 2H, H-2 and H-6), 7.71 (d, *J* = 9.0 Hz, 2H, H-2' and H-6'), 7.46 (d, *J* = 8.5 Hz, 2H, H-3 and H-5), 6.91 (d, *J* = 8.5 Hz, 2H, H-3' and H-5'); ^{13}C NMR ($CDCl_3$) δ 196.3 (C-7), 165.3 (C-4'), 139.1 (C-1), 138.3 (C-1'), 134.0 (C-2 and C-6), 132.2 (C-3 and C-5), 129.5 (C-2' and C-6'), 128.8 (C-4), 116.7 (C-3' and C-5').

*Irradiation of fenofibric acid (**62**)*

Fenofibric acid (100 mg) was dispersed in distilled water (500 mL) and irradiated by the solar simulator. After 100 hr TLC showed the disappearance of **62**. Water was evaporated under vacuum and the residue was chromatographed on silica gel eluting with hexane-acetone (4:1) to give compound **66** (3%), compound **67** (70%), compound **68** (14%).

Very similar results were obtained by irradiations in the presence of nitrate ions and of humic acids or from the experiment in STP water. Differently, irradiation in argon gave only compounds **67** (82%) and **68** (16%).

Compound **67**: ^{1}H NMR ($CDCl_3$) δ 7.77 (d, *J* = 8.9 Hz, 2H, H-2 and H-6), 7.70 (d, *J* = 8.7 Hz, 2H, H-2' and H-6'), 7.44 (d, *J* = 8.7 Hz, 2H, H-3 and H-5), 6.92 (d, *J* = 8.7 Hz, 2H, H-3' and H-5'), 4.59 (m, *J* = 6.1 Hz, 1H, H-7'), 1.30 (d, *J* = 6.1 Hz, 6H, H-8' and H-9'); ^{13}C NMR ($CDCl_3$) δ 194.0 (C-7), 161.6 (C-4'), 137.9 (C-1), 136.3 (C-4), 132.2 (C-2 and C-6), 130.8 (C-3 and C-5), 129.0 (C-1'), 128.2 (C-2' and C-6'), 114.7 (C-3' and C-5'), 69.8 (C-7'), 21.6 (C-8' and C-9').

Compound **68**: ^{1}H NMR ($CDCl_3$) δ 7.76 (d, *J* = 8.2 Hz, 4H, H-2 and H-6; H-2' and H-6'), 7.61 (d, *J* = 8.2 Hz, 2H, H-3 and H-5), 7.46 (d, *J* = 8.7 Hz, 2H, H-3' and H-5'), 1.98 (brs, 1H, OH); ^{13}C NMR ($CDCl_3$) δ 195.2 (C-7), 154.0 (C-4'), 138.8 (C-1), 136.0 (C-4), 135.6 (C-1'), 131.4 (C-2 and C-6), 130.1 (C-3 and C-5), 128.6 (C-2' and C-6'), 124.5 (C-3' and C-5'), 72.6 (C-7'), 31.7 (C-8' and C-9'); EIMS: *m/z* 274 $[M]^+$, 259 $[M-CH_3]^+$.

5.6 Proton Pump Inhibitors: lansoprazole (70a) and omeprazole (70b)

Chemicals

Lanzoprazole and omeprazole were obtained from Sigma and used as received. All the other chemicals have been purchased from Aldrich.

General procedure

Dispersions were prepared by suspending the drug (40 mg) in milliQ water (500 ml). Experiments at pH 7.1 were carried out using the same concentration in pure water, buffered with NaH_2PO_4/Na_2HPO_4, and at pH 4.0 or 9.0 by adjusting the pH values using HCl 2M or KOH 2M, respectively. In a typical procedure each dispersion of the drug was kept in the dark or irradiated at room temperature (in the latter case the sample was irradiated from the top and maintained in a thermostated pyrex beaker). The water was then evaporated, and the residue was first analyzed by ^{1}H NMR and then chromatographed by TLC. The dispersions investigated at pH 4.0 or 9.0 were neutralized before water evaporation. Experiments in the presence of humic acid (5 ppm) and KNO_3 (10 ppm) were

carried out using the same concentration of the drug and then analyzing the mixture in the dark or by irradiation as above.

Experiments were carried out using the same concentration of the drug in closed pyrex tube after saturating with oxygen or argon and then analyzing the mixture in the dark or by irradiation, as above.

*Hydrolysis of lansoprazole (**70a**)*

The dispersion of lansoprazole (40 mg) in water milliQ (500 ml), kept in the dark for 72 h, led, after evaporation of water, to a red-coloured residue (30 mg). The latter was chromatographed on preparative TLC [CH_2Cl_2-$(CH_3)_2CO$ (9:1)] affording sulfide **73a** (10%), lansoprazole (**70a**) (57%), a red fraction (17%) and benzimidazolone **75a** (3%) at decreasing Rfs.

*Photolysis of lansoprazole (**70a**)*

The dispersion of lansoprazole (40 mg) in milliQ water (500 ml), irradiated by solar simulator for 72 h, after water evaporation, gave a residue (38 mg) which was purified on preparative TLC [$CHCl_3$/CH_3OH (95:5)] giving sulfide **73a** (10%), dianiline **71a** (8%), fraction A (16 mg), a red fraction (15%), benzimidazole **74a** (5%) and benzimidazolone **75a** (5%) at decreasing R_fs. Fraction A (16 mg) was purified on preparative TLC [CH_2Cl_2/CH_3OH (97:3)] giving dianiline **71a** (11%), pyridine **72a** (5%), lansoprazole (24%) at decreasing R_fs.

*Hydrolysis of omeprazole (**70b**)*

The dispersion of omeprazole (**70b**), kept in the dark for 43 h, led, after water evaporation, to an intense red-coloured residue which was separated on preparative TLC [CH_2Cl_2-CH_3OH (95:5)], giving compound **73b** (25%), omeprazole (20%), an intractable red fraction (15%) and compound **75b** (10%), at decreasing R_fs. The red fractions deriving from both drugs consisted of diverse products (TLC and ^1H NMR, data not shown). Attempts to separate and/or characterize the red materials failed due to their alteration over time or during chromatographic processes.

*Photolysis of omeprazole (**70b**)*

A suspension of omeprazole (80 ppm) in water milliQ was exposed to the solar simulator for 43 h. After evaporation of water, the residue (25 mg) was

chromatographed on TLC [CH_2Cl_2-CH_3OH (95:5)] leading, at decreasing Rfs, to dianiline **71b** (10%), sulfide **73b** (16%), benzimidazolone **75b** (20%), pyridine **72b** (traces, <1%) benzimidazole **74b** (traces, <1%) and a red fraction (20%).

*Photostability of derivatives sulfides (**73**), benzimidazoles (**75**), benzimidazolones (**75**).*

Suspensions of benzimidazoles **74a**, **75a**, derivatives of lansoprazole, and **74b**, **75b** derivatives of omeprazole, (10 ppm) in MilliQ water were exposed to the solar simulator for 72 or 43 h, respectively. Each experiment was performed in duplicate, with one set of dark controls. Each reaction mixture was evaporated in vacuum and each residue was analysed by ^{1}H-NMR and TLC showing only the starting material.

When sulfides **73a** and **73b** were treated in the same conditions, analysis of dark samples showed only starting materials, while by irradiation they led to a mixture of products. The mixture from **73a** (8 mg) was subjected to preparative TLC [CH_2Cl_2-CH_3OH (93:7)] affording dianiline **71a** (30%) and benzimidazole **74a** (38%). The mixture (7mg) deriving from irradiation of **73b** was subjected to preparative TLC [CH_2Cl_2/CH_3OH (93:7)] giving dianiline **71b** (43%) and benzimidazole **74b** (28%).

*Synthesis of 2-((4-(2,2,2-trifluoroethoxy)-3-methylpyridin-2-yl)methylsulfonyl)-1H benzo[d]imidazole (**78**)*

To a solution of compound **70a** (18 mg) in anhydrous dichloromethane (0.02 M), *m*-chloroperbenzoic acid (1 equiv.) was added and the resulting mixture kept at room temperature under magnetic stirring. After two hours, TLC showed that compound **70a** disappeared. Then, the mixture was washed with water and anhydrified with Na_2SO_4. After filtration and evaporation of dichloromethane, the sulfone **78** (64%) was purified by TLC [eluent CH_2Cl_2-methanol (96:4)]. When the sulfone was dispersed in milliQ water and kept in the dark, analysis by TLC and ^{1}H NMR after 72 h showed the sulfone unchanged.

Spectral data

Infrared spectra (IR) were determined in $CHCl_3$ solutions (0,025 M), while ultraviolet spectra (UV) were recorded in ethanol (10^{-4} M). Compounds **72b** and **74b** were identified by ^{1}H NMR and LC-MS due to their low amounts.

Compound **71a**: IR ($CHCl_3$) ν_{max} 3448, 2929 cm^{-1}; ^{1}H-NMR (CD_3OD) δ 7.20 (m, 4H, Ar-H); ^{13}C-NMR (CD_3OD) δ 133.8 (C-1 and C-6), 124.0 (C-3 and C-4), 110.9 (C-2 and C-5).

Compound **72a**: IR ($CHCl_3$) ν_{max} 3357, 1592, 1170 cm^{-1}; ^{1}H-NMR (CD_3OD) δ 8.33 (d, *J* = 5.5 Hz, 1H, H-6'), 7.06 (d, *J* = 5.5 Hz, 1H, H-5'), 4.82 (q, *J* = 8.4 Hz, 2H, CH_2CF_3), 4.62 (s, 2H, CH_2OH), 2.13 (s, 3H, CH_3); ^{13}C-NMR (CD_3OD) δ 164.2 (C-4'), 160.3 (C-2'), 148.7 (C-6'), 122.6 (C-3'), 120.0 (CF_3), 108.3 (C-5'), 67.2 (OCH_2), 64.5 (CH_2OH), 10.3 (CH_3); EIMS *m/z* 221 $[M]^+$.

Compound **73a**: IR ($CHCl_3$) ν_{max} 3100, 1581, 1168 cm^{-1}; UV λ_{max} 208, 292 nm; ^{1}H-NMR (CD_3OD) δ 8.27 (d, *J* = 5.6 Hz,1H, H-6'), 7.50 (brs, 2H, H-4, H-7), 7.22 (m, 2H, H-5, H-6), 7.02 (d, *J* = 5.6 Hz, 1H, H-5'), 4.70 (q, *J* = 8.4 Hz, 2H, CH_2CF_3), 4.59 (s, 2H, CH_2S), 2.29 (s, 3H, CH_3); ^{13}C-NMR (CD_3OD) δ 163.0 (C-4'), 155.5 (C-2'), 149.4 (C-6'), 147.5 (C-2), 139.8 (C-8, C-9), 122.1 (C-5, C-6), 123.0 (C-3'), 122.0 (CF_3), 113.0 (C-4, C-7), 106.4 (C-5'), 64.8 (CH_2CF_3), 36.1 (CH_2S), 9.3 (CH_3); EIMS *m/z* 353 $[M]^+$.

Compound **74a**: ^{1}H-NMR (CD_3OD) δ 8.15 (s, 1H, H-2), 7.60 (m, 2H, H-4 and H-7), 7.26 (m, 2H, H-5 and H-6); ^{13}C-NMR (CD_3OD) δ 142.5 (C-2), 123.8 (C-5 and C-6), 122.2 (C-8 and C-9), 110.2 (C-4 and C-7); EIMS *m/z* 118 $[M]^+$.

Compound **75a**: ^{1}H-NMR (DMSO) δ 7.01 (m, 4H, ArH); ^{13}C-NMR (CD_3OD) δ 156.1 (CO), 128.0 (C-8 and C-9), 120.9 (C-5 and C-6), 109.5 (C-4 and C-7).

Compound **71b**: IR ($CHCl_3$) ν_{max} 3290, 3195, 1626 cm^{-1}; ^{1}H-NMR (CD_3OD) δ 7.08 (d, *J* = 9.4, 1H, H-6), 6.78 (m, 2H, H-2, H-5), 3.79 (s, 3H, OCH_3). ^{13}C-NMR (CD_3OD) δ 157.1 (C-1), 133.4 (C-3), 126.7 (C-4), 110.5 (C-6), 110.2 (C-5), 94.7 (C-2), 55.1 (OCH_3); EIMS *m/z* 138 $[M]^+$.

Compound **72b**: ^{1}H-NMR (CD_3OD) δ 8.14 (s, 1H, H-6), 4.66 (s, 2H, CH_2O), 3.80 (s, 3H, OCH_3), 2.29 (s, 3H, CH_3), 2.26 (s, 3H, CH_3); LC-MS *m/z* 167 $[M]^+$.

Compound **73b**: IR ($CHCl_3$) ν_{max} 3100, 1591 cm^{-1}; UV λ_{max} 214, 300 nm; ^{1}H-NMR (CD_3OD) δ 8.13 (s, 1H, H-6'), 7.38 (d, *J* = 8.8 Hz, 1H, H-7), 7.00 (d, *J* = 2.4 Hz, 1H, H-4), 6.81 (dd, *J* = 8.8 and 2.4 Hz, 1H, H-6), 4.54 (s, 2H, CH_2S), 3.79 (s, 3H, OCH_3), 3.74 (s, 3H, OCH_3), 2.24 (s, 3H, CH_3), 2.22 (s, 3H, CH_3); ^{13}C-NMR (CD_3OD) δ 166.4 (C-4'), 158.1 (C-5), 155.6 (C-2'), 149.8 (C-6'), 150.3 (C-2), 140.8 (C-9), 135.0 (C-8), 127.7 (C-3'), 127.3 (C-5'), 116.1 (C-4), 113.1 (C-6),

97.8 (C-7), 60.6 (OCH_3), 56.2 (OCH_3), 37.8 (CH_2S), 13.4 (CH_3), 11.3 (CH_3); EIMS *m/z* 329 $[M]^+$.

Compound **74b**: ^{1}H-NMR (CD_3OD) δ 8.04 (s, 1H, H-2), 7.47 (d, *J* = 9.0 Hz, 1H, H-7), 7.09 (brs, 1H, H-4), 6.90 (dd, *J* = 9.0 and 2.5 Hz, 1H, H-6), 3.83 (s, 3H, OCH_3); LC-MS *m/z* 148 $[M]^+$.

Compound **75b**: IR ($CHCl_3$) ν_{max} 3140, 1720 cm^{-1}; ^{1}H-NMR (CD_3OD) δ 6.92 (d, *J* = 8.0 Hz, 1H, H-7), 6.66 (d, *J* = 2.5 Hz, 1H, H-4), 6.63 (dd, *J* = 8.5 and 2.5 Hz,1H, H-6), 3.77 (s, 3H, OCH_3). ^{13}C-NMR (CD_3OD) δ 158.4 (C-2), 157.0 (C-5), 131.6 (C-9), 124.7 (C-8), 110.7 (C-7), 108.6 (C-6), 96.9 (C-4), 56.2 (OCH_3); EIMS *m/z* 164 $[M]^+$.

Compound **78**: IR ($CHCl_3$): ν_{max} 3198, 1581, 1341, 1172, 1144 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 8.27 (d, *J* = 5.3 Hz, 1H, H-6'), 7.65 (brs, 2H, H-4, H-7), 7.37 (m, 2H, H-5, H-6), 6.68 (d, *J* = 5.3 Hz, 1H, H-5'), 5.10 (s, 2H, SO_2CH_2), 4.37 (q, *J* = 8.4, 2H, CH_2CF_3), 2.35 (s, 3H, CH_3). ^{13}C-NMR ($CDCl_3$) δ 164.0 (C-4'), 162.5 (C-2'), 148.1 (C-6'), 147.5 (C-2), 147.3 (C-8, C-9), 128.1 (C-5, C-6), 125.4 (C-3'), 125.3 (CF_3), 115.0 (C-4, C-7), 106.5 (C-5'), 65.3 (CH_2CF_3), 60.2 (CH_2SO_2), 11.4 (CH_3); EIMS *m/z* 385 $[M]^+$.

6. General Procedure Toxicity Tests

Acute toxicity testing

Acute toxicity was determined on primary consumers typical of the aquatic chain: the rotifer *B. Calyciflorus* and the crustaceans *D. magna* and *T. platyurus*. All the organisms were provided in cryptobioticstages by MicroBioTests Inc., Nazareth, Belgium. The test on *D. magna* was performed according to the ISO (International Organization for Standardization) 6341, the test on *B. calyciflorus* following the ASTM (American Society for Testing and Materials, 1991) E1440-91, while the test on *T. platyurus* following the manufacturer procedure.

Parental compounds and their photoderivatives were dissolved in dimethylsulphoxide (DMSO) and further diluted in double-deionized water to

make the final stock solutions. The DMSO concentration in the exposure solutions, including controls, was 0.01% (v/v) that is a non-effect dose as estimated in preliminary tests. All bioassays were conducted under static conditions, with no renewal of the test solution, measuring dissolved oxygen and pH in each sample both at the start and at the end of testing. At the same time as toxicity testing, reference tests were performed with potassium dichromate (Aldrich) for all the organisms. Juveniles (age, 0–2 h) of the rotifer *B. Calyciflorus* were hatched from cysts after 16–18 h of incubation under a light source of 3000–4000 lux at 25 °C in synthetic reconstituted medium (moderately hard medium EPA-600/4-85-013) and then exposed to the test sample.

Hardness was 80–100 mg/l CaCO3 and the dissolved oxygen content was at least 90% saturation at the beginning of the test. Tests were run in 36-well plates, five rotifers per well (0.3 ml of test solution, slightly different from the ASTM procedure), six replicates for each of the five concentrations. Test duration was 24 h, temperature 25°C, in the dark. The test parameter considered was mortality and the concentration found to kill 50% rotifers in 24 h was indicated as LC50.

The bioassay on the anostracan crustacean *T. platyurus* was conducted using second- and third-instar fairy shrimp larvae hatched from cysts after 20–22 h of incubation at 25°C in synthetic reconstituted freshwater (the same moderately hard EPA medium as rotifers) under continuous illumination (light source 3000–4000 lux). Tests were performed in 24-well plates, ten crustaceans per well (1.0 ml of test solution), three replicates for each of the .ve concentrations. Test duration was 24 h, temperature 25°C, in the dark. The test parameter considered was mortality and the concentration found to kill 50% crustaceans in 24 h was indicated as LC50.

The test on *D. magna* Straus was performed using juveniles (age< 24 h), hatched from ephippia after 3–4 days of incubation at 20°C under continuous illumination (light source 10 000 lux). The synthetic reconstituted freshwater, aerated before use, was the ISO hard medium (hardness 250 mg/l expressed as $CaCO_3$). Tests were performed with neonates< 24 h, five daphnids per vessel (10 ml of test solution), four replicates for each of the five concentrations. Daphnies were exposed to the samples at temperature of 20°C in the dark. After 24 h the number

of immobile daphnies was recorded to determine the sample concentration able to achieve 50% immobilization and it was indicated as EC50. *D. magna* was considered to be immobilized if it was not able to swim after gentle agitation of the liquid in 15 s of observation even if it can still move its antennae (ISO, 1996).

Chronic toxicity testing

The effects of the investigated drugs and their derivatives on the population growth inhibition were assessed using standard methods for chronic toxicity tests on the alga *P. subcapitata* (already known as *Selenastrum capricornutum*) and the crustacean *C. dubia.* The algal growth inhibition test was run in 72 h according to the ISO procedure 8692 (ISO, 1987). The *P. subcapitata* inoculum (1 · 104 cells/ml) was taken from an exponentially growing pre-culture (strain CCAP 278/4) and poured in 25 ml of test solution in five concentrations and three replicates. Flasks were placed in a growth chamber at 25°C under continuous illumination (8000 lux). The cell density was measured at 0 time and every 24 h for 3 days by an electronic particle dual threshold counter (Coulter Counter Z2, 100 lm capillary, Instrumentation Laboratory, Miami, FL, USA).

The test on *C. dubia* was run in 7 days and performed on young daphnids (<24 h old at the start of the exposure), obtained by acyclical parthenogenesis of individual adult females for at least three generations. The first females were born from the hatching of ephippia provided by MicroBioTests. Organisms were exposed individually to seven concentrations in beakers with 20 ml of synthetic reconstituted aerated hard ISO medium (total hardness 250 mg/l as CaCO3) and the desired concentration of single compounds. Each treatment consisted of ten replicates per concentration incubated at 25°C with a 16:8-h light: dark cycle (500 lux). Daphnies were fed daily with 100 ll of a suspension of the alga *P. subcapitata* (4 · 108 cells/ml), food fish (5 g/l) and yeast (5 g/l). Also test solutions were renewed daily as well as survival and offspring production assessed. From comparison between the number of o.spring born from live or dead mothers at the end of the test in the sample batch and the control it was possible to calculate the concentration which gave rise to 50% population growth inhibition (ISO, 2001).

Data analysis

Raw data for all bioassays, except algal test, were analyzed using the ToxcalcTM (1996). For acute toxicity tests, the LC50s and EC50s were calculated by concentration/response regression using probit or trimmed Spearman–Karber method, as appropriate (Peltier and Weber, 1985). For the test with *C. dubia*, the value of the concentration that gave 50% population growth inhibition was calculated using Maximum Likelihood-Logit method. Raw test data from algae were analyzed by a Microsoft Excel 5.0 program (Phoenix, AZ, USA) tailored for this test. Algal growth inhibition (%) was calculated by integrating the mean values of cell density from t_0 to t_{72} h. Inhibition (%) values were tabulated against log-transformed data of concentrations to evaluate the test concentration corresponding to 50% algal growth inhibition.

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