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Synthesis and Evaluation of Pyrazine Derivatives with Herbicidal Activity

Martin Doležal[1] and Katarína Kráľová[2]
[1]*Faculty of Pharmacy in Hradec Králové, Charles University in Prague*
[2]*Faculty of Natural Sciences, Comenius University in Bratislava*
[1]*Czech Republic*
[2]*Slovak Republic*

1. Introduction

The pyrazine ring is a part of many polycyclic compounds of biological and/or industrial significance; examples are quinoxalines, phenazines, and bio-luminescent natural products pteridines, flavins and their derivatives. All these compounds are characterized by a low lying unoccupied π-molecular orbital and by the ability to act as bridging ligand. Due to these two properties 1,4-diazines, and especially their parent compound pyrazine, possess a characteristic reactivity. Pyrazine is a weak diacid base (pK_1 = 0.57; pK_2 = -5.51), weaker than pyridine, due to the induction effect of the second nitrogen (Bird, 1992). Its inherent bifunctionality and the low lying unoccupied molecular orbital permit pyrazine to form coordination polymers having unusual electrical and magnetic properties (Brown & Knaust, 2009). 1,4-Diazines may be employed to study inter- and intramolecular electron transfer in organic, inorganic and biochemical reactions. Autocondenzation of α-aminocarbonyle compounds to the dihydropyrazine derivative, which is followed by oxidation on the final substituted pyrazine, or the condenzation of α,β-dicarbonyle and α,β-diamino compounds forming during the fermentation of saccharides and peptides are the main routes of pyrazine ring building. Pyrazines are found mainly in processed food, where they are formed during dry heating processes *via* Maillard reactions (Maillard, 1912). They are also found naturally in many vegetables, insects, terrestrial vertebrates, and marine organisms, and they are produced by microorganisms during their primary or secondary metabolism (Adams et al., 2002; Beck et al., 2003; Wagner et al., 1999; Woolfson & Rothschild, 1990). The widespread occurrence of simple pyrazine molecules in nature, especially in the flavours of many food systems, their effectiveness at very low concentrations as well as the still increasing applications of synthetic pyrazines in the flavour and fragrance industry are responsible for the high interest in these compounds (Maga, 1992). Certain pyrazines, especially dihydropyrazines, are essential for all forms of life due their DNA strand-breakage activity and/or by their influencing of apoptosis (Yamaguchi, 2007). Synthetic pyrazine derivatives are also useful as drugs (antiviral, anticancer, antimycobacterial, etc.), fungicides, and herbicides (Doležal, 2006a). Furthermore, a simple pyrazine compound, 3-amino-6-chloro-pyrazine-6-carboxylic acid, has shown anti-auxin behaviour (Camper & McDonald, 1989). The importance of the pyrazine (1,4-diazine) ring for the biological

activity can be evaluated primarily according to the size of the studied molecules. In relatively small compounds, the pyrazine ring is necessary for biological action due to its resemblance (bioisosterism) to the naturally occurring compounds (e.g. nicotinamide, or pyrimidine nucleic bases). In bulky compounds the introduction of the pyrazine ring brings specific chemical and physicochemical properties for the molecule as a whole, such as basic and slightly aromatic character (Doležal, 2006a). A fully comprehensive study of the pyrazines including reactivity and synthesis is beyond the scope of this work but can be found in the literature (Brown, 2002; Joule & Mills, 2010).

Herbicides are generally considered as growth inhibitors, thus their different inhibitory responses have been studied in various culture systems. Plant tissue and cell cultures provide model systems for the study of various molecular, physiological, organism and genetic problems. These systems have been used in the study of herbicides and other xenobiotics (Linsmaier & Skoog, 1965).

2. Pyrazine herbicides

The most successful pyrazine derivative was diquat-dibromide (see Fig. 1, the structure I). This non-selective, contact herbicide has been used to control many submerged and floating aquatic macrophytes which interferes with the photosynthetic process, releasing strong oxidizers that rapidly disrupt and inactivate cells and cellular functions (at present banned in many EU countries). Severe oral diquat intoxication has been associated with cerebral haemorrhages and severe acute renal failure (Peiró et al., 2007). Also quinoxaline herbicides (containing the pyrazine fragment) are very useful herbicides. Among them propaquizafop (Fig. 1, II) and quizalofop-ethyl (Fig. 1, III) are the most important derivatives (Frater et al., 1987; Sakata et al., 1983).

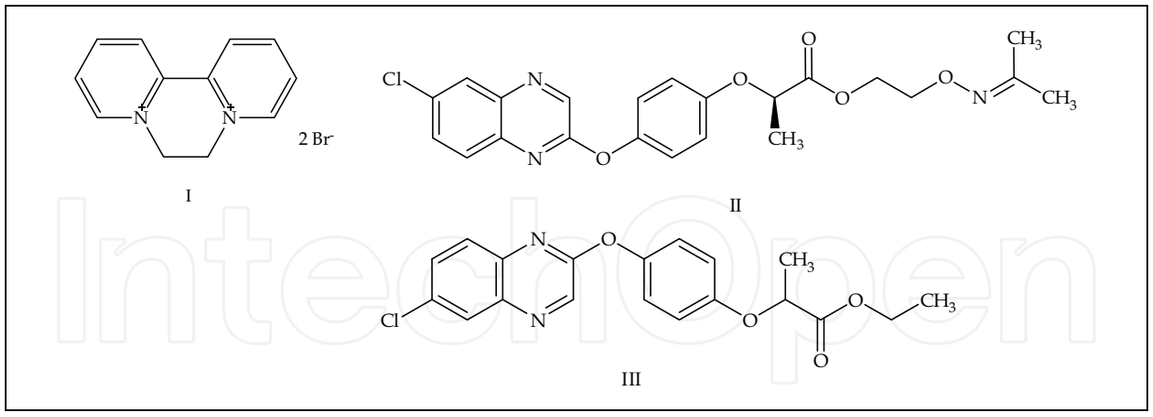


Fig. 1. Structures of diquat-dibromide (I), propaquizafop (II) and quizalofop-ethyl (III).

2.1 Diquat

Diquat-dibromide (6,7-dihydrodipyrido[1,2-a:2',1'-c]pyrazinediium-dibromide; for the structure see Fig. 1, I) is a quaternary ammonium salt used as a non-selective contact herbicide and desiccant, absorbed by the foliage with some translocation in the xylem. It is used for preharvest desiccation of many crops, as a defoliant on hops, for general weed control on non crop land etc. (Ritter et al., 2000; Ivany, 2005). It is applied as an aquatic

herbicide in many countries since the late 1950s for control of emergent and submerged aquatic weeds (Ritter et al., 2000). According to Massachusetts Department of Agricultural Resources (2010) following weeds are controlled by diquat: *i)* submersed aquatics: *Ultricularia*, *Ceratophyllum demersum*, *Elodea* spp., *Najas* spp., *Myriophyllum* spp., *Hydrilla verticillata*, *Potamogeton* spp.; *ii)* floating aquatics: *Salvinia* spp., *Eichhornia crassipes*, *Pistia Stratiotes*, *Lemna* spp., *Hydrocotyle* spp.; *iii)* marginal weeds: *Typha* spp. ; *iv)* algae: *Pithophora* spp. , *Spyrogyra* spp. (filamentous algae). Diquat is stable in neutral and acidic solutions but unstable in alkaline medium. It breaks down by the UV radiation and the degradation increases with pH > 9 (Diaz et al., 2002). It is also biodegraded in water by microorganisms that uses this herbicide as a source of carbon or nitrogen (Petit et al., 1995).

Trade names for diquat-dibromide formulations included Desiquat®, Midstream®, Reglone®, and Reglex®. Mixtures of diquat with another quaternary herbicide paraquat (1,1'-dimethyl-4,4'-bipyridinium-dichloride) were sold under trade names including Actor®, Dukatalon®, Opal®, Pathclear® (also includes simazine and aminotriazole), Preeglox®, Preglone®, Seccatutto®, Spray Seed®, and Weedol® (Lock & Wilks, 2001).

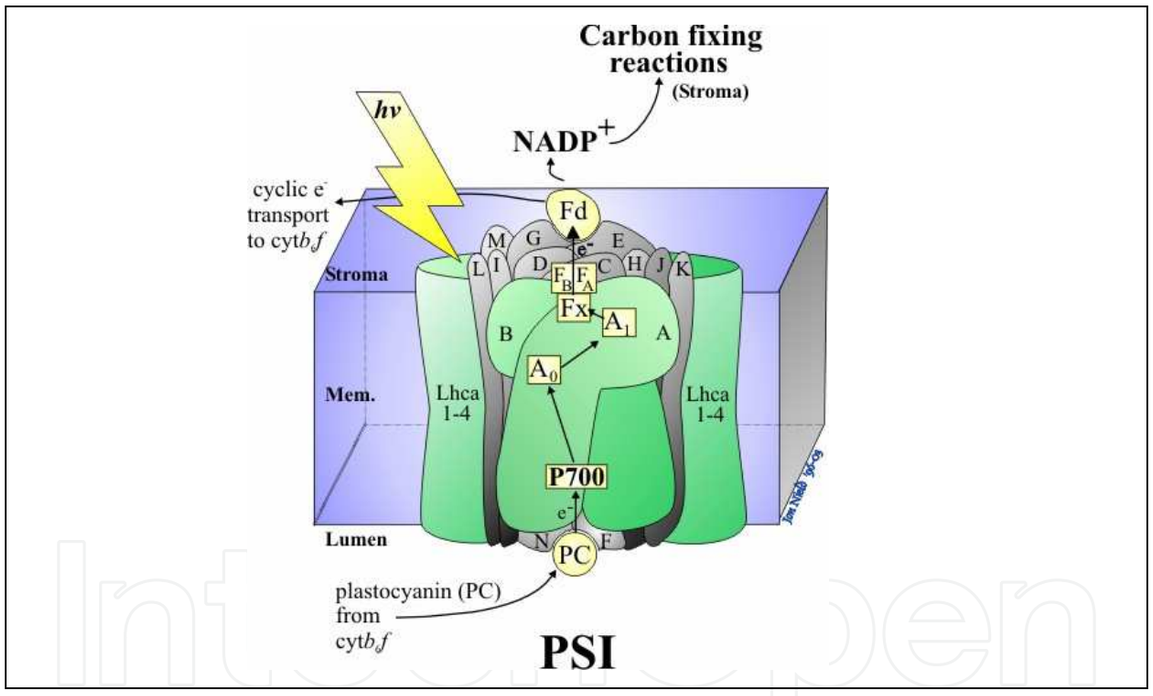


Fig. 2. Scheme of the photosynthetic electron transport in photosystem I (PS I). (Figure taken from http://www.bio.ic.ac.uk/research/barber/psIIimages/PSI.jpg with permission of Prof. Barber, Imperial College London).

The first paper dealing with the mode of action of diquat was published in 1960 by Mees who indicated that oxygen and light were essential for its herbicidal effect. Later Zweig et al. (1965) found that diquat caused a deviation of electron flow from photosystem (PS) I what resulted in an inhibition of $NADP^+$ reduction and the production of a reduced diquat radical. In Fig. 2 is shown scheme of the photosynthetic electron transport (PET) in PS I.

In plants, the PS I complex catalyzes the oxidation of plastocyanin and the reduction of ferredoxin (F_d). From the primary donor, P700, electrons are transferred to the primary

acceptor, A_0 and then to phylloquinone (A_1) operating as a single electron acceptor. From A_1 electrons are transferred to a 4Fe-4S cluster (F_X) and subsequently to two 4Fe-4S clusters, F_A and F_B, located on the stromal side of the reaction center close to F_X. PS I produces a strong reductant that transfers electrons to F_d. Ferredoxin, one of the strongest soluble reductants found in cells, operates in the stromal aqueous phase of the chloroplast, transferring electrons from PS I to ferredoxin-$NADP^+$ oxidoreductase. The final electron acceptor in the photosynthetic electron transport chain is $NADP^+$, which is fully reduced by two electrons (and one proton) to form NADPH, a strong reductant which serves as a mobile electron carrier in the stromal aqueous phase of the chloroplast (Whitmarsch, 1998).

Due to deviation of electron flow from F_d, an inhibition of $NADP^+$ reduction occurs and a reduced diquat radical is formed. Davenport (1963) found that in the presence of oxygen the reduced diquat free radical was reoxidized with the production of hydrogen peroxide. Thus, an one-electron reduction of diquat results in a cation free radical that reacts rapidly with molecular oxygen and generates reactive oxygen species such as the superoxide anion radical (Mason, 1990). Reactive oxygen species cause oxidative stress in the cell with consecutive damage of biological membranes. In herbicide classification diquat, similarly to paraquat, is classified as HRAC Group D herbicide causing PS I electron diversion (HRAC 2005). Injury to diquat–treated crop plants occurs in the form of spots of dead leaf tissue wherever spray droplets contact the leaves indicating that this herbicide belongs to membrane disruptors. The use of diquat for the control of aquatic weeds is widespread in the US (US Environmental Protection Agency, 1995) whereas it is forbidden in the EU (European Commission, 2001, 2002).

As mentioned above, diquat toxicity to both aquatic plants and animals originates from the formation of reactive oxygen species in both chloroplasts and mitochondria (Cedergreen et al., 2006; Sanchez et al., 2006). The field effects of diquat to natural strands of aquatic vegetation were studied by Peterson et al. (1997) and Campbell at al. (2000). The filamentous cyanobacteria were slightly less tolerant than the unicellular cyanobacteria and the most sensitive was genus *Anabena* (Peterson et al., 1997). Gorzerino et al. (2009) showed that diquat, used as the commercial preparation Reglone 2®, inhibited the growth of *Lemna minor* in indoor microcosms. According to findings of Campbell et al. (2000) diquat has a minimal ecological impact to benthic invertebrates and fish; on the other hand, aquatic plants in the vicinity of application to surface waters appear to be at risk (nevertheless this is expected, as diquat-dibromide kills aquatic plants). Howewer, Koschnick et al. (2006) observed that the accession of *Landoltia* from Lake County (Florida) had developed resistance to diquat and the resistance mechanism was independent of photosynthetic electron transport.

2.2 Patented pyrazine herbicides

The control of unwanted vegetation by means of chemical agents, *i.e.* herbicides, is an important aspect of modern agriculture and land management's. While many chemicals that are useful for the control of unwanted vegetation are known, new compounds that are more effective generally, are more effective for specific plant species, are less damaging to desirable vegetation, are safer to man or the environment, are less expensive to use or have other advantageous attributes, are desirable (Benko, 1997). Many structural variations of pyrazine compounds with herbicidal properties can be found in the patent literature.

Several thiazolopyrazines exhibited pre-emergent herbicidal activity when applied as aqueous drenches to soil planted with seeds of certain plants. For example, application of 4000 ppm of compound IV (Fig. 3) resulted in emergence inhibition of crabgrass (50% of the

control) and barnyard grass (***Echinochloa crus-galli*** (L.) P. Beauv.) (45% of the control). Due to the treatment with a dose of 2 lb per acre of compound V (Fig. 3), the emergence of cotton reached only 30% of the control (Tong, 1978).

Böhner & Meyer (1989a, 1989b, 1990) prepared a set of aminopyrazinones (Fig. 3, VI) and aminotriazinones and tested these compounds for their herbicidal action before emergence of the plants. It was found that application of 70.8 ppm of some compounds on the substrate vermiculite resulted in very potent inhibition of seed germination of *Nasturtium officinalis*, *Agrostis tenuis*, *Stellaria media* and *Digitaria sanguinalis*. Due to the treatment with compound where R^1 = CH_3, R^2 = OCH_3, R^3 = H, R^7 = H, R^8 = $COOCH_3$, X = O plants have not germinated and completely died. After spraying of 21 days old spring barley (*Hordeum vulgare*) and spring rye (Secale) plants shoots with an active substance VI (up to 100 g per hectare) new additional growth of plants reached only 60-90% of the control. For grasses *Lolium perenne*, *Poa pratensis*, *Festuca ovina*, *Dactylis glomerate* and *Cynodon dactylon* sprayed with the same dose of an active substance (Fig. 3, VII) reduction in new additional growth in comparison with the untreated control (10-30% of control) was observed, too (Böhner & Meyer, 1989a, 1989b, 1990).

Benko et al. (1997) patented a series of *N*-aryl[1,2,4]triazolo[1,5-a]pyrazine-2-sulfonamides as good pre- and post-emergence selective herbicides with good growth regulating properties. Excellent pre-emergence activity against pigweed and morning glory and very good post-emergence herbicidal activity against morning glory and velvet leaf (*Abutilon theophrasti*) have been exhibited by the title compounds.

Dietsche (1977) patented as herbicides a group of substituted 6,7-dichloro-3,4-dihydro-2*H*-pyrazino(2,3-*b*)(1,4)oxazines showing hundred-percent inhibitory effectiveness when applied as pre- as well as post-emergence herbicides (4000 ppm) for pigweeds.

Shuto et al. (2000) patented as useful active ingredients of herbicides a series of pyrazin-2-one derivatives (Fig. 3, VIII, IX) where R^1 is hydrogen or alkyl, R^2 is haloalkyl, R^3 is optionally substituted alkyl, alkenyl or alkynyl and Q is optionally substituted phenyl. Some compounds showed superb effectiveness against *Abtutilon theophrasti* and *Ipomoea hederacea* when applied as foliar or soil surface treatment on upland fields (2000 g/ha).

Griffin et al. (1990) patented alkylpyrazine compounds (Fig. 3, X) with plant growth regulating activity, where R^1 is C_1-C_4 alkyl optionally substituted with halogen or cyclopropyl, optionally substituted with C_1-C_4 alkyl; R^2 is C_1-C_8 alkyl, C_2-C_8 alkenyl, or C_2-C_8 alkynyl optionally substituted with halogen; C_3-C_6 cycloalkyl, C_3-C_6 cycloalkenyl. C_3-C_6 cycloalkylalkyl, C_3-C_6 cycloalkenylalkyl, phenylalkenyl or phenylalkynyl each optionally substituted on the ring group; R^3 is hydrogen or C_1-C_4 alkyl; R^4 is hydrogen, C_1-C_4 alkyl, halogen, alkylamino, cyano, or alkoxy; n is 0 or 1; and salts, ethers, acylates and metal complexes therof. The treatment of plants with these compounds can lead to the leaves developing a darker green colour. In dicotyledonous plants such as soybean and cotton, there may be promotion of side shooting. The compounds may be useful in rendering plants resistant to stress since they can delay the emergence of plants grown from seeds, shorten stem height and delay flowering. Engel et al. (1999) patented herbicidal pyrazine derivatives (Fig. 3, XI) which are suitable very effectively control weeds and grass weeds mainly in crops such as wheat, rice, corn, soybean and cotton, without significantly damaging the crops. It could be stressed that this effect occurs in particular at low application rates. In addition, these compounds can also be used in crops which have been made substantially resistant to the action of herbicides by breeding and/or by the use of genetic engineering methods.

N-pyrazinyl-haloacetamides (Fig. 3, XII) where R is hydrogen, hydrocarbonyl, halogen, epoxy, hydroxy, alkoxy, mercapto, alkylsulfanyl, nitro, cyano or amino, R´ is hydrogen or

hydrocarbonyl, X is halogen, m is integer from 1 to 4 and n is 0, 1 or 2 showed herbicidal activity. For example, spraying of the 2,2,2-trichloro-*N*-pyrazinyl acetamide on the soil resulted in 100% growth inhibition of wild oats (dosage 1.12 g m^{-2}) and yellow foxtail or cultured rice (dosage 1.12 g m^{-2}) (Fischer, 1988).

Novel pyrazine-sulfonylcarbamates and thiocarbamates (Fig. 3, XIII) (where Z is oxygen or sulfur and R is C_1-C_4 alkyl, phenyl or benzyl; whereas the pyrazine ring may be variously further substituted) have been found to be good selective herbicides and therefore they are suitable for use in crops of cultivated plants. Moreover, these compounds can damage problem weeds which till then have only been controlled with total herbicides (Böhner et al., 1987). By means of surface treatment it is possible to damage perennial weeds to their roots. Moreover, the compounds are effective when used in very low rates of application and they are able to potentiate the phytotoxic action of other herbicides against certain noxious plants and to reduce the toxicity of such herbicides to some cultivated plants. These compounds can be used also as plant growth regulators causing inhibition of vegetative plant growth what results in substantial increase of the yield of plants. Böhner et al. (1987) synthesized and patented also a set of novel pyrazinyl sulfonamides of the formula Q-SO_2-NH_2 where Q is substituted pyrazine group which could be useful in controlling weeds and are suitable for selectively influencing plant growth. The compounds can be used as pre- and post-emergence herbicides and as plant growth regulators for growth inhibition of cereals (e.g. *Hordeum vulgare* or summer rye (Secale)) and grasses (e.g. *Lolium perenne*, *Poa partensis*, *Festuca ovina*, *Cynodon dactylon*). Selective inhibition of the vegetative growth of many cultivated plants permits more plants to be grown per unit of crop area, resulting in significant increase in yield with the same fruit setting and in the same crop area.

Zondler et al. (1989) prepared a set of 2-arylmethyliminopyrazines (Fig. 3, XIV) and tested them for their pre-emergent and post-emergent herbicidal action, as well as for their plant growth regulating activity. Compounds with R^5 = 4-Cl, R^6 = 2-Cl, R^7 = H and R^1 = SCH_3H_7(n) or $SCH_2CH{=}CH_2$ showed excellent pre-emergent effect (dose 4 kg/ha) against *Echinochloa crus-galli* and *Monocharia vag*. The last compound was active already at application rate of 500 g/ha. The 2-arylmethylimino-pyrazines were found to be also effective post-emergence herbicides and can be used for growth inhibition of tropical leguminous cover crops (e.g. *Centrosema plumieri* and *Centrosema pubescens*), growth regulation in soybeans and growth inhibition of cereals, too.

Cyanatothiomethylthiopyrazines have been found to be active as pesticides and find particular usage as fungicides, bactericides, nematocides and herbicides (Mixan et al., 1978).

Arylsulfanylpyrazine-2,3-dicarbonitriles have high herbicidal activity (Takematsu et al., 1984; Portnoy, 1978). Takematsu et al. (1981) patented 2,3-dicyanopyrazines (Fig. 3, XV) as compounds with high herbicidal activity as well as useful active ingredients of herbicides. The compounds have ability to inhibit the germination of weeds and/or wither their stems and leaves, and therefore exhibit an outstanding herbicidal effect as an active ingredient of pre-emergence and/or post-emergence herbicides in submerged soil treatment, foliar treatment of weeds, upland soil treatment, etc.

Compounds where A represents a phenyl group which may have 1 or 2 substituents selected from the class consisting of halogen atoms and lower alkyl groups containing 1 to 3 carbon atoms and B represents an ethylamino, *n*-propylamino, *n*- or *iso*-butylamino, 1-carboxyethylamino, 1-carboxy-*n*-propylamino, 1-carboxy-*iso*-butylamino, 1-carboxy-*n*-pentylamino or allylamino group have the property of selectively blanching (causing

chlorosis, *i.e.* inhibiting the formation of chlorophyll and/or the acceleration of its decomposition) of weeds without chlorosis of useful crops. Hence, these compounds are most suitable as high selective herbicides of chlorosis type.

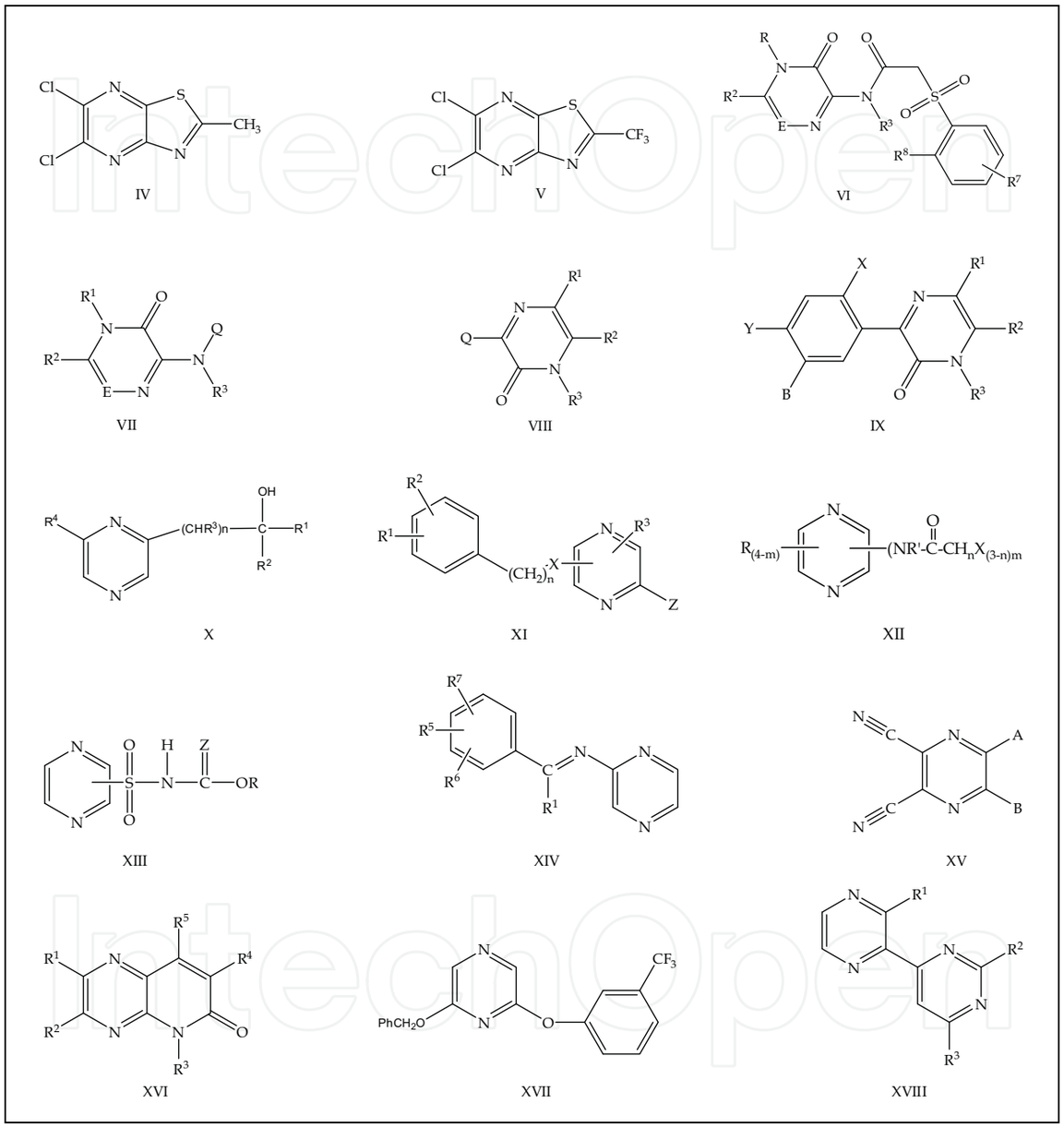


Fig. 3. Structures of patented thiazolopyrazines (IV,V), aminopyrazinones (VI,VII), substituted pyrazin-2-ones (VIII,IX), arylalkylpyrazines (X, XI), *N*-pyrazinyl-haloacetamides (XII), pyrazine-sulfonylcarbamates and thiocarbamates (XIII), 2-arylmethyliminopyrazines (XIV), substituted 2,3-dicyanopyrazines (XV), pyridopyrazines (XVI), aryloxopyrazines (XVII) and pyrimidinopyrazines (XVIII).

Takematsu et al. (1984) also patented a set of 2,3-dicyano-6-phenylpyrazine herbicides with outstanding herbicidal activities on paddy weeds in submerged soil treatment. Because they

are not phytotoxic to rice, they can effectively control weeds in paddies. The compounds exhibited herbicidal activity against important upland weeds such are *Digitaria adscendens*, *Polygonum persicaria*, *Galinsoga ciliata*, *Amaranthus viridis*, *Chenopodium album*, *Chenopodium ficifolium*, *Echinochloa crus-galli* (without damaging upland crops) as well as against a very broad range of other upland weeds including *Galium aparin*, *Rumex japonicus*, *Erigeron philadelphicus*, *Erigeron annuus*, and *Capsella bursapastoria*.

Cordingley et al. (2008) prepared herbicidal effective pyridopyrazines (Fig. 3, XVI) with R^1,R^2 independently = H, alkyl, halo, CN, aryl, etc.; R^3 = H, (halo)alkyl, alkenyl, etc.; R^4 = (un)substituted heteroaryl; and R^5 = OH or group metabolizable to OH) or a salt or *N*-oxide thereof. XVI applied post-emergence at 1000 g/ha completely controlled *Solanum nigrum* and *Amaranthus retroflexus*. Also substituted aryloxopyrazines (Fig. 3, XVII) possess interesting herbicidal effect (Niederman & Munro, 1994). For example, in tests against 8 plants, title compound XVII at 5 kg/ha (foliar spray) gave complete kill of *Echinochloa crus-galli* with no damage to rice. Test data include foliar, pre-emergence, and soil drench applications against the 8 plants for most compounds. Sato et al. (1993) patented pyrimidinopyrazines (Fig. 3, XVIII) (R^1 = H, halo, alkoxy, alkylamino, alkyl, haloalkyl; R^2 = Ph, substituted Ph, benzyl, pyridyl, thienyl, furyl; R^3 = SR^4, OR^5, NR^6R^7; R^4,R^5,R^6,R^7 = H, alkyl, alkenyl, alkynyl; NR^6R^7 may form 3-7 membered ring), useful as herbicides, were prepared and showed herbicidal activity against *Stellaria neglecta* at 0.63 kg/ha.

2.2.1 Structure-activity relationships in series of herbicidal 2,3-dicyanopyrazines

Nakamura et al. (1983) synthesized sixty six 2,3-dicyano-5-substituted pyrazines and measured their herbicidal activities against barnyard grass in pot tests to clarify the relationship between chemical structure and activity. The activity of 59 derivatives showed parabolic dependence on the hydrophobic substituent parameter at the 5-position of the pyrazine ring, indicating that the compounds should pass through a number of lipoidal-aqueous interfaces to reach a critical site for biological activity. It was found that the moiety of 2,3-dicyanopyrazine is essential for herbicidal activity, and the 5-substituent on the pyrazine ring plays an important role in determining the potency of this activity and that *para*-substituted phenyl derivatives show undesirable effects on the potency of the activity at the ultimate site of herbicidal action.

Nakamura et al. (1983a) also synthesized sixty eight 6-substituted 5-ethylamino and 5-propylamino-2,3-dicyanopyrazines and tested their herbicidal activities against barnyard grass using pot tests. In general, these compounds induced chlorosis against young shoots of barnyard grass and inhibited their growth. The most active compound was 2,3-dicyano-5-propylamino-6-(*m*-chlorophenyl)-pyrazine. The results indicated that the structure of the 5-ethylamino and 5-propylamino-2,3-dicyanopyrazine moieties is an important function for the herbicidal activity and that the potency of activity of these two series of compounds is determined by the hydrophobic and steric parameters of substituents at the 6-position of the pyrazine ring.

3. Design, synthesis and evaluation of the pyrazinecarboxamides with herbicidal activity

The structural diversity of organic herbicides continues to increase; therefore classification of herbicides should be based on their chemical structure. The chlorinated aryloxy acids dominated for long period, later were replaced by chemicals of many distinct chemical

classes, including triazines, amides (haloacetanilides), benzonitriles, carbamates, thiocarbamates, dinitroanilines, ureas, phenoxy acids, diphenyl ethers, pyridazinones, bipyridinium compounds, ureas and uracils, sulfonylureas, imidazolinones, halogenated carboxylic acids, and many other compounds. Carboxamide or anilide moieties are present in many used herbicides, *i.e.* alachlor, acetochlor, benoxacor, butachlor, diflufenican, dimethenamid, diphenamid, isoxaben, karsil, napropamide, pretilachlor, propyzamide, dicryl, diflufenican, flufenacet, mefenacet, mefluidide, metolachlor, naphtalan, picolinafen, propachlor, propanil, propham, solan (The Merck Index, 2006). Carboxamide or anilide herbicides are nonionic and moderately retained by soils. The sorption of several carboxamide herbicides has been investigated (Weber & Peter, 1982). The *N*-substituted phenyl heterocyclic carboxamides are an important class of herbicides as protoporphyrinogen-IX oxidase inhibitors with advantages such as high resistance to soil leaching, low toxicity to birds, fish, and mammals, and slow development of weed resistance (Hirai, 1999).

We have designed and prepared a series of 113 carboxamide herbicides derived from pyrazinecarboxylic acid and various substituted anilines. The final compounds XIX were prepared by the anilinolysis of substituted pyrazinoylchlorides (Doležal, 1999, 2000, 2002, 2006b, 2007, 2008a, 2008b). Their chemical structure, hydrophobic parameters (log *P* calculated by ACD/logP ver. 1.0, 1996), and photosynthesis-inhibiting activity, structure-activity relationship (SAR) were studied. We synthesized in preference: *i)* the compounds with the lipophilic and/or electron-withdrawing substituents on the benzene moiety (R^3), *ii)* the compounds with the hydrophilic and/or electron-donating groups on the benzene part of molecule (R^3), and finally *iii)* the compounds with the lipophilic alkyl (R^2), *i.e.* methyl (-CH_3) or *tert*-butyl (-$C(CH_3)_3$) and/or halogen (chlorine) substitution (R^1) on the pyrazine nucleus, for their synthesis and structure see Fig. 4 and Table 1.

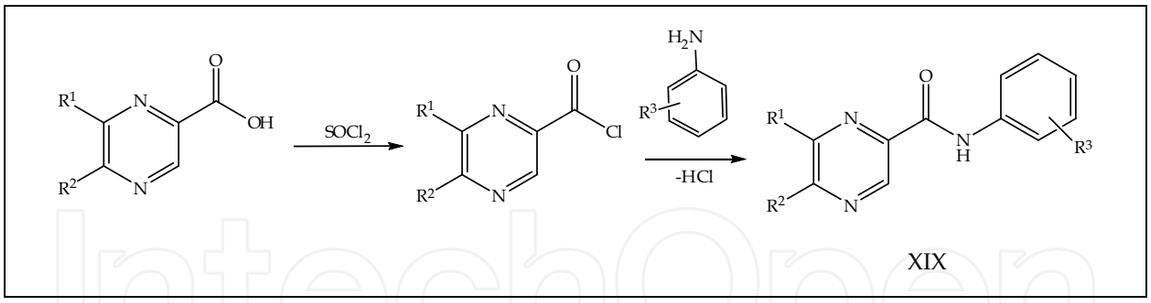


Fig. 4. Synthesis and structure of substituted *N*-phenylpyrazine-2-carboxamides (XIX).

3.1 Inhibition of photosynthetic electron transport by substituted *N*-phenylpyrazine-2-carboxamides

3.1.1 Photosynthetic electron transport in photosystem II

Photosystem II uses light energy to drive two chemical reactions: the oxidation of water and the reduction of plastoquinone. Five of redox components of PS II are known to be involved in transferring electrons from H_2O to the plastoquinone pool: the water oxidizing manganese cluster $(Mn)_4$, the amino acid tyrosine (Y_z), the reaction center chlorophyll (P680), pheophytin, and two plastoquinone molecules, Q_A and Q_B (Fig. 5). Tyrosine, P680, pheophytin (Pheo), Q_A, and Q_B are bound to two key polypeptides (D_1 and D_2) that form the reaction center core of PS II and also provide ligands for the $(Mn)_4$ cluster (Whitmarsh,

1998). After primary charge separation between P680 (chlorophyll *a*) and pheophytin (Pheo), $P680^+/Pheo^-$ is formed. Then electron is subsequently transferred from pheophytin to a plastoquinone molecule Q_A (permanently bound to PS II) acting as a one-electron acceptor.

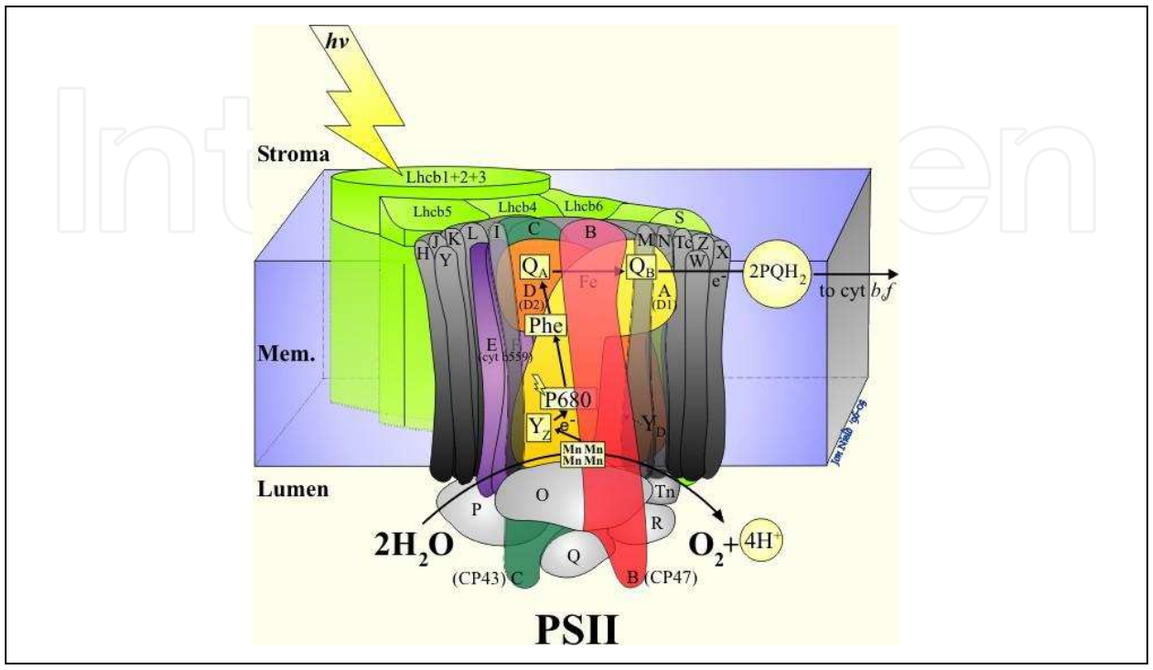


Fig. 5. Scheme of the photosynthetic electron transport in photosystem II (PS II). (Taken from Photosystem II in http://www.bio.ic.ac.uk/research/barber/psIIimages/PSII.jpg with permission of Prof. Barber, Imperial College London).

From Q_A^- the electron is transferred to another plastoquinone molecule Q_B (acting as a two-electron acceptor); two photochemical turnovers of the reaction centre are necessary for the full reduction and protonation of Q_B. Because Q_B is loosely bound at the Q_B-site, reduced plastoquinone then unbinds from the reaction centre and diffuses in the hydrophobic core of the membrane and Q_B-binding site will be occupied by an oxidized plastoquinone molecule (Whitmarsh, 1998). Several commercial herbicides inhibit Photosynthetic elektron transport (PET) by binding at or near the Q_B-site, preventing access to plastoquinone (*e.g.* Oettmeier, 1992). Photosystem II is the only known protein complex that can oxidize water, which results in the release of O_2 into the atmosphere. Oxidation of water is driven by the oxidized primary electron donor, $P680^+$ which oxidizes a tyrosine on the D_1 protein (Yz) and four Mn ions present in the water oxidizing complex undergo light-induced oxidation, too. Water oxidation requires two molecules of water and involves four sequential turnovers of the reaction centre whereby each photochemical reaction creates an oxidant that removes one electron. The net reaction results in the release of one O_2 molecule, the deposition of four protons into the inner water phase, and the transfer of four electrons to the Q_B-site (producing two reduced plastoquinone molecules) (Whitmarsh & Govindjee, 1999).

PET in chloroplasts can be estimated by electrochemical measurements of oxygen concentration using Clark electrode (PET through the whole photosynthetic apparatus is registered) or by spectrophotometric methods enabling the monitoring of PET through individual parts of photosynthetic apparatus. The site of action of PET inhibitors can be

more closely specified by the use of chlorophyll fluorescence (*e.g.* Joshi & Mohanty, 2004) or by electron paramagnetic resonance (EPR) (*e.g.* Doležal et al., 2001a).

3.1.2 Hill reaction activity of *N*-phenylpyrazine-2-carboxamides

The Hill reaction is formerly defined as the photoreduction of an electron acceptor by the hydrogens of water, with the evolution of oxygen. *In vivo*, or in the organism, the final electron acceptor is $NADP^+$, in isolated chloroplasts an artificial electron acceptor that changes colour as it is reduced, is applied. We tested a large series of pyrazinecarboxamides (XIX) for their activity related to oxygen evolution rate (OER) using spinach chloroplasts and 2,6-dichlorophenol-indophenol (DCPIP) as an electron acceptor what intercepts the electrons before they transfer to cytochrome *bf* complex. Because the site of DCPIP action is plastoquinone pool (PQ) on the acceptor side of PS II (Izawa, 1980) this method is suitable for PET monitoring through PS II. The PET-inhibiting activities of the studied compounds XIX (expressed as IC_{50} values) are summarized in Table 1.

No.	R^1	R^2	R^3	IC_{50}	Ref.	No.	R^1	R^2	R^3	IC_{50}	Ref.
1	Cl	H	2-Br	334	a	**58**	Cl	tBu	2-Cl,5-OH	652	i
2	H	tBu	2-Br	171	a	**59**	H	CH_3	3-Br	648	b
3	Cl	tBu	2-Br	315	a	**60**	H	CH_3	3-C≡CH	668	b
4	Cl	H	3,5-Br-4-OH	995	a	**61**	Cl	tBu	3-C≡CH	385	b
5	H	tBu	3,5-Br-4-OH	404	a	**62**	Cl	tBu	3-C≡N	375	b
6	Cl	tBu	3,5-Br-4-OH	590	a	**63**	H	CH_3	3-Cl	174	b
7	Cl	H	3-OCH_3	500	a	**64**	H	CH_3	3-NO_2	402	b
8	H	tBu	3-OCH_3	800	a	**65**	H	CH_3	2-C≡N-4-NO_2	550	b
9	Cl	tBu	3-OCH_3	644	a	**66**	H	CH_3	3-I-4-CH_3	317	b
10	Cl	H	3,5-OCH_3	533	a	**67**	H	CH_3	2-COOH	75	b
11	H	tBu	3,5-OCH_3	317	a	**68**	Cl	tBu	3-F	262	c
12	Cl	tBu	3,5-OCH_3	435	a	**69**	H	tBu	3-OH-4-Cl	105	c
13	Cl	H	5-Br-2-OH	146	a	**70**	Cl	tBu	3-OH-4-Cl	44	c
14	H	tBu	5-Br-2-OH	80	a	**71**	Cl	tBu	2-Cl	43	c
15	Cl	tBu	5-Br-2-OH	42	a	**72**	H	tBu	2-Cl	371	c
16	Cl	H	3,4-Cl	105	a	**73**	H	H	2-Cl	47	c
17	H	tBu	3,4-Cl	1525	a	**74**	Cl	H	2-CH_3	1072	e
18	Cl	tBu	3,4-Cl	130	a	**75**	H	tBu	2-CH_3	440	e
19	Cl	H	3-F	565	d	**76**	Cl	tBu	2-CH_3	244	e
20	Cl	H	2,4-F	539	d	**77**	Cl	H	3-CH_3	486	e
21	Cl	H	4-Cl	486	d	**78**	H	tBu	3-CH_3	148	e
22	Cl	H	4-$CH(CH_3)_2$	118	d	**79**	Cl	tBu	3-CH_3	118	e
23	H	tBu	3-F	313	d	**80**	H	tBu	2-OCH_3	286	e
24	H	tBu	2,4-F	371	d	**81**	Cl	tBu	2-OCH_3	97	e
25	H	tBu	4-Cl	1502	d	**82**	Cl	H	3-Br	313	e
26	H	tBu	4-$CH(CH_3)_2$	110	d	**83**	H	tBu	3-Br	81	e
27	Cl	tBu	3-F	129	d	**84**	Cl	tBu	3-Br	107	e
28	Cl	tBu	2,4-F	106	d	**85**	Cl	H	3,5-CF_3	26	e

No.	R^1	R^2	R^3	IC_{50}	Ref.	No.	R^1	R^2	R^3	IC_{50}	Ref.
29	Cl	tBu	4-Cl	43	d	**86**	H	tBu	3,5-CF_3	114	e
30	Cl	tBu	4-$CH(CH_3)_2$	52	d	**87**	Cl	tBu	3,5-CF_3	241	e
31	Cl	H	2-OH	66	f	**88**	Cl	H	2,6-CH_3	649	e
32	Cl	H	3-OH	2288	f	**89**	H	tBu	2,6-CH_3	229	e
33	Cl	H	4-OH	3322	f	**90**	Cl	tBu	2,6-CH_3	242	e
34	Cl	H	2-OH-5-Cl	8	f	**91**	H	H	2-Cl-5-OH	722	g
35	H	tBu	2-OH	205	f	**92**	H	H	4-F	480	g
36	H	tBu	3-OH	431	f	**93**	H	H	2-CF_3	376	g
37	H	tBu	4-OH	314	f	**94**	H	H	3-CF_3	130	g
38	H	tBu	2-OH-5-Cl	465	f	**95**	H	H	4-CH_3	1475	g
39	Cl	tBu	2-OH	435	f	**96**	Cl	H	2-Cl-5-OH	624	g
40	Cl	tBu	3-OH	262	f	**97**	Cl	H	4-F	384	g
41	Cl	tBu	4-OH	43	f	**98**	Cl	H	2-CF_3	557	g
42	Cl	tBu	2-OH-5-Cl	105	f	**99**	Cl	H	3-CF_3	229	g
43	Cl	H	4-Cl-3-CH_3	595	h	**100**	Cl	H	4-CH_3	1524	g
44	Cl	H	3-I-4-CH_3	51	h	**101**	H	tBu	4-F	524	g
45	H	tBu	4-Cl-3-CH_3	190	h	**102**	H	tBu	2-CF_3	55	g
46	Cl	tBu	2-F	69	h	**103**	H	tBu	3-CF_3	283	g
47	Cl	tBu	4-CF_3	184	h	**104**	H	tBu	4-CH_3	164	g
48	H	H	4-F	480	i	**105**	Cl	tBu	2-Cl-5-OH	625	g
49	Cl	H	4-F	384	i	**106**	Cl	tBu	4-F	103	g
50	H	tBu	4-F	524	i	**107**	Cl	tBu	2-CF_3	205	g
51	Cl	tBu	4-F	103	i	**108**	Cl	tBu	3-CF_3	173	g
52	H	H	3-Cl	290	i	**109**	Cl	tBu	4-CH_3	73	g
53	Cl	H	3-Cl	262	i	**110**	Cl	H	2,4,6-CH_3	495	j
54	H	tBu	3-Cl	47	i	**111**	H	tBu	2,4,6-CH_3	434	j
55	H	tBu	3-Cl	103	i	**112**	Cl	tBu	2,4,6-CH_3	195	j
56	H	H	2-Cl-5-OH	722	i	**113**	H	tBu	4-$COCH_3$	664	j
57	Cl	H	2-Cl-5-OH	624	i	**-**	-	-	-	-	-

Table 1. IC_{50} values (in μmol dm^{-3}) related to PET inhibition in spinach chloroplasts by substituted pyrazinecarboxamides XIX (Ref. Doležal et al., 2006b[(a)], 2008a[(b)], 2001b[(c)], 2000[(d)], 2002[(e)], 1999[(f)], 2008b[(g)], 2007[(h)], 2004[(i)], 2001a[(j)]).

The compounds **1**-**18** inhibited PET in spinach chloroplasts; however the inhibitory activity of the majority of these compounds was relatively low. The IC_{50} values varied in the range from 42 to 1589 μmol dm^{-3}, the most efficient inhibitors was 5-*tert*-butyl-6–chloro-*N*-(5-bromo-2-hydroxyphenyl)-pyrazine-2-carboxamide (**15**, Table 1). The dependence of PET-inhibiting activity of compounds **1**-**18** on the lipophilicity of the compounds (log *P*) is shown in Fig. 6, A. Markedly lowered solubility of **4**-**6** as well as **17** due to insertion of two halogen atoms (Br or Cl) in R^3 substituent resulted in decreased inhibitory activity of these compounds. Based on the dependence of PET-inhibiting activity on log *P* of the rest compounds, these can be divided into two groups. In both groups increase of compound activity with increasing lipophilicity can be observed. Thus, with the exception of compounds **14** and **15** (R^2 = 5-Br-2-OH) it can be assumed, that the introduction of lipophilic

R^1 (Cl) and R^2 (*tert*-butyl, tBu) substituents, respectively, can result in partial decrease of the aqueous solubility and so in reduced inhibitory activity.

In other set of studied compounds **19-30**, compound **25** exhibited very low activity due to its low aqueous solubility (Table 1). As shown (Fig. 6, B), the PET-inhibiting activity of other compounds from the set expressed as log ($1/IC_{50}$) increased linearly with increasing compound lipophilicity (log *P*). The most active compounds from the set were 5-*tert*-butyl-6–chloro-*N*-(4-chlorophenyl)-pyrazine-2-carboxamide (**29**, IC_{50} = 43 μmol dm^{-3}) and 5-*tert*-butyl-6–chloro-*N*-(4-isopropylphenyl)-pyrazine-2-carboxamide (**30**, IC_{50} = 52 μmol dm^{-3}).

The inhibitory activity of the compounds **31-42** (Table 1) was affected not only by the lipophilicity of the compounds but also by the value of Hammett's constants of R^3 substituents. Very low activity of compounds **32** and **33** was connected with their low aqueous solubility. The most active compounds from this set were 6–chloro-*N*-(5-chloro-2-hydroxyphenyl)-pyrazine-2-carboxamide (**34**, IC_{50} = 8 μmol dm^{-3}) and 5-*tert*-butyl-6–chloro-*N*-(4-hydroxyphenyl)-pyrazine-2-carboxamide (**41**, IC_{50} = 43 μmol dm^{-3}), the activity of rest compounds from the set varied between 66 (**31**) and 465 μmol dm^{-3} (**38**).

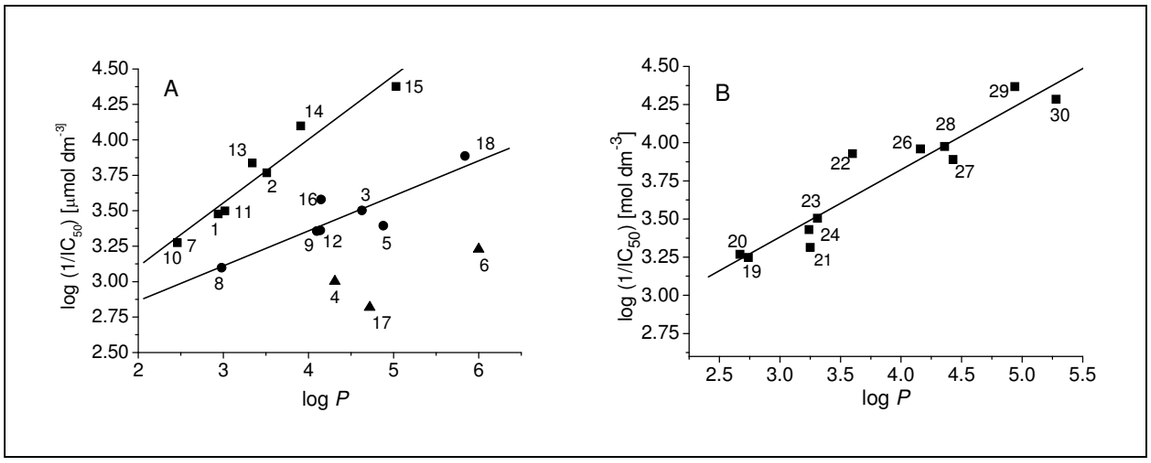


Fig. 6. The dependence of PET-inhibiting activity of compounds **1-18** (A) and compounds **19-30** (B) on the lipophilicity of the compounds (log *P*).

It was found that from the aspect of inhibitory activity it is much more favourable when on the phenyl ring (R^3 substituent) halogen atom occurs in *meta* and methyl moiety in *para* position (**44**, IC_{50} = 51 μmol dm^{-3}) in comparison with compound **43** where R^3 =4-Cl-3-CH_3 (IC_{50} = 595 μmol dm^{-3}). However, the inhibitory activity of the above mentioned compound **43** can be increased by introduction of *tert*-butyl substituent instead of H in R^2 (**45**, IC_{50} =190 μmol dm^{-3}).

The IC_{50} values related to PET-inhibiting activity of compounds **48-58** varied in the range from 47.0 (**54**) to 722 μmol dm^{-3} (**56**). The inhibitory activity of majority of these compounds was relatively low, the most efficient inhibitors were 5-*tert*-butyl-6–chloro-*N*-(4-fluorophenyl)-pyrazine-2-carboxamide (**51**), *N*-(2-chloro-5-hydroxyphenyl)-pyrazine-2-carboxamide (**55**, both IC_{50} = 103.0 μmol dm^{-3}), and especially 5-*tert*-butyl-6–chloro-*N*-(3-chlorophenyl)-pyrazine-2-carboxamide (**54**, IC_{50} = 47.0 μmol dm^{-3}). Their log *P* values calculated ranged between 3.28 and 4.18.

In the set of compounds **59-67** the PET-inhibiting activity of compounds **61**, **62**, **63**, **66** and **67** (Fig. 7, A) expressed as log ($1/IC_{50}$) showed a linear decrease with increasing values of lipophilicity parameter (log *P*). On the other hand, the biological activity of compounds **59**,

60, **64** and **65** was significantly lower and linear decrease of PET-inhibiting activity with increasing log *P* values was less sharp indicating that the biological activity of compounds **59-67** depended both on the compound lipophilicity as well as on Hammett's constants σ of the substituent R^2. The most active PET inhibitor from this set was found to be 2-(5-methyl-pyrazine-2-carboxamido)-benzoic acid (**67**, IC_{50} = 75.0 μmol dm^{-3}) (Doležal et al., 2008a).
From the set of compounds **68-73** the most active inhibitors with comparable inhibitory activity were compounds 5-*tert*-butyl-6–chloro-*N*-(3-chloro-4-hydroxyphenyl)-pyrazine-2-carboxamide (**70**, IC_{50} = 44 μmol dm^{-3}), 5-*tert*-butyl-6–chloro-*N*-(2-chlorophenyl)-pyrazine-2-carboxamide (**71**, IC_{50} = 43 μmol dm^{-3}) and *N*-(2-chlorophenyl)-pyrazine-2-carboxamide (**73**, IC_{50} = 47 μmol dm^{-3}).

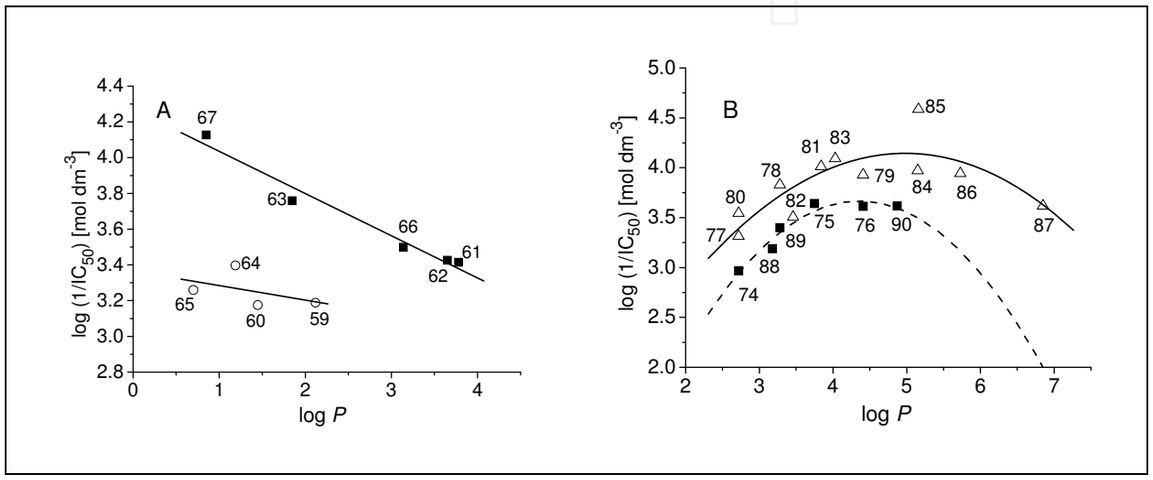


Fig. 7. The dependence of PET-inhibiting activity of compounds **59-67** (A) and compounds **74-90** (B) on the lipophilicity of the compounds (log *P*).

In the set of compounds **74-90** the IC_{50} values related to PET inhibition varied in the range from 26 (**85**) to 1072 μmol dm^{-3} (**74**), see Table 1. In general, the inhibitory activity of these compounds depended on their lipophilicity showing a quasi-parabolic trend (Fig. 7, B). However, the studied compounds could be divided into two groups. The compounds with 2-CH_3 substituents on the phenyl ring (**74**, **75**, **76**, **88**, **89** and **90**, squares in Fig. 7, B) had lower biological activity than the other investigated compounds with comparable log *P* values. Consequently, it can be assumed that the methyl substituent in *ortho* position of the benzene ring is disadvantageous from the viewpoint of interactions with the photosynthetic apparatus. On the other hand, compound **85** (6–chloro-*N*-(3,5-trifluoro-methylphenyl)-pyrazine-2-carboxamide) exhibited higher inhibitory activity than expected.
The majority of compounds **91-109** inhibited PET in spinach chloroplasts; however their inhibitory activity was rather low. From the obtained results it can be concluded that the activity depended on the lipophilicity and also on the electron accepting or withdrawing power of R^3 substituent(s). The most effective inhibitor was compound **102** (5-*tert*-butyl-*N*-(2-trifluoromethylphenyl)-pyrazine-2-carboxamide, IC_{50} = 55 μmol dm^{-3}). Among the three most active compounds **102**, **109** and **106** the optimal values of lipophilicity ranges from log *P* = 4.02-4.41. On the other hand, for the group of compounds **105**, **108** and **107** with the highest lipophilicity, the PET-inhibiting activity showed a decrease with increasing compound lipophilicity. The most effective inhibitor from the compounds with R^3= 2,4,6-

CH_3 was 5-*tert*-butyl-6–chloro-*N*-(2,4,6-methylphenyl)-pyrazine-2-carboxamide (**112**, IC_{50} = 195 μmol dm^{-3}) (Doležal et al., 2001a).

3.1.3 Determination of the site of inhibitory action of *N*-phenylpyrazine-2-carboxamides in the photosynthetic electron transport chain by electron paramagnetic resonance spectroscopy and chlorophyll a fluorescence measurements

The site of inhibitory action of some *N*-phenylpyrazine-2-carboxamides XIX in the photosynthetic electron transport chain was investigated using spinach (*Spinacia oleracea* L.) chloroplasts. For this purpose electron paramagnetic resonance spectroscopy (EPR) and measurement of chlorophyll *a* fluorescence were used.

Intact chloroplasts of algae and vascular plants exhibit EPR signals in the region of free radicals (g = 2.00), which are stable during several hours (Hoff, 1979) and could be registered at laboratory temperature by conventional continual wave EPR apparatus. These signals were denoted as signal I (g = 2.0026, ΔB_{pp} = 0.8 mT) and signal II (g = 2.0046, ΔB_{pp} = 2 mT) indicating their connection with photosystem (PS) I and PS II, respectively (Weaver, 1968). Signal II consists from two components, namely signal II_{slow} which is observable in the dark and signal $II_{very\ fast}$ which occurs at irradiation of chloroplasts by visible light and represents intensity increase of signal II at irradiation of chloroplasts by the visible light. It was found that signal II_{slow} belongs to the intermediate $D^{\bullet}$ and signal $II_{very\ fast}$ belongs to the intermediate $Z^{\bullet}$. Intermediates $Z^{\bullet}$ and $D^{\bullet}$ are tyrosine radicals which are situated at 161st position in D_1 and D_2 proteins which are located on the donor side of PS II (Svensson et al., 1991). The EPR signal I is associated with cation radical of chlorophyll *a* dimmer situated in the core of PS I (Hoff, 1979).

Using EPR spectroscopy it has been found that the studied compounds XIX affect predominantly the intensity of EPR signal II, mainly the intensity of its constituent signal II_{slow}. As mentioned above, the signal II_{slow} is well observable in the dark (see Fig. 8, full line) and it belongs to the $D^{\bullet}$ intermediate, *i.e.* tyrosine (Tyr_D or Y_D) radical which is located on the donor side of PS II in the 161st position in D_2 protein (Svensson et al., 1991; see Fig 5). From Fig. 8 it is evident that the intensity of signal II_{slow} has been decreased by the studied compounds (see Fig. 8, B and C, full lines). That means that in the suspension of spinach chloroplasts the 5-*tert*-butyl-6–chloro-*N*-(3-fluorophenyl)-pyrazine-2-carboxamide (**68**) and 5-*tert*-butyl-*N*-(3-hydroxy-4-chlorophenyl)-pyrazine-2-carboxamide (**69**) interact with the $D^{\bullet}$ intermediate. Due to this interaction of the studied anilides with this part of PS II, the photosynthetic electron transport from the oxygen evolving complex to the reaction centre of PS II is impaired. Consequently, the electron transport between PS II and PS I is inhibited as well and a pronounced increase of signal I intensity in the light can be observed (see Fig. 8, B and C, dashed lines). The signal I (g = 2.0026, ΔB_{pp} = 0.8 mT) belongs to the cation radical of chlorophyll *a* dimmer in the reaction centre of PS I (Hoff, 1979).

Similar site of action in the photosynthetic apparatus of spinach chloroplasts was confirmed for 2-alkylsulfanylpyridine-4-carbothioamides (Kráľová et al., 1997) and substituted benzanilides and thiobenzanilides (Kráľová et al., 1999). From Fig. 8 it is evident that the decrease of signal II_{slow} is greater in the presence of compound **69** (Fig. 8, B) than in presence of compound **68** (Fig. 8, C). These results are in agreement with those obtained for OER inhibition in spinach chloroplasts (Table 1, IC_{50} = 105 μmol dm^{-3} for **69** and 262 μmol dm^{-3} for **68**).

1,5-Diphenylcarbazide (DPC) is an artificial electron donor acting in $Z^{\bullet}/D^{\bullet}$ intermediate (Jegerschöld & Styring, 1991). By addition of DPC to chloroplasts inhibited by PET inhibitors the supply of electrons to P680 is secured. However, the complete restoration of the electron transport to PS I occurs only in the case that photosynthetic electron transport chain between $Z^{\bullet}/D^{\bullet}$ and plastoquinone is not damaged. After addition of DPC to chloroplasts inhibited by the studied anilides up to 70-80%, the OER in the suspension of spinach chloroplasts was not completely restored. It was restored only up to 55-75% of the untreated control sample what indicated that also some member of the photosynthetic electron transport chain between $Z^{\bullet}/D^{\bullet}$ intermediate and plastoquninone is partially damaged by the studied compounds in the light (dashed lines).

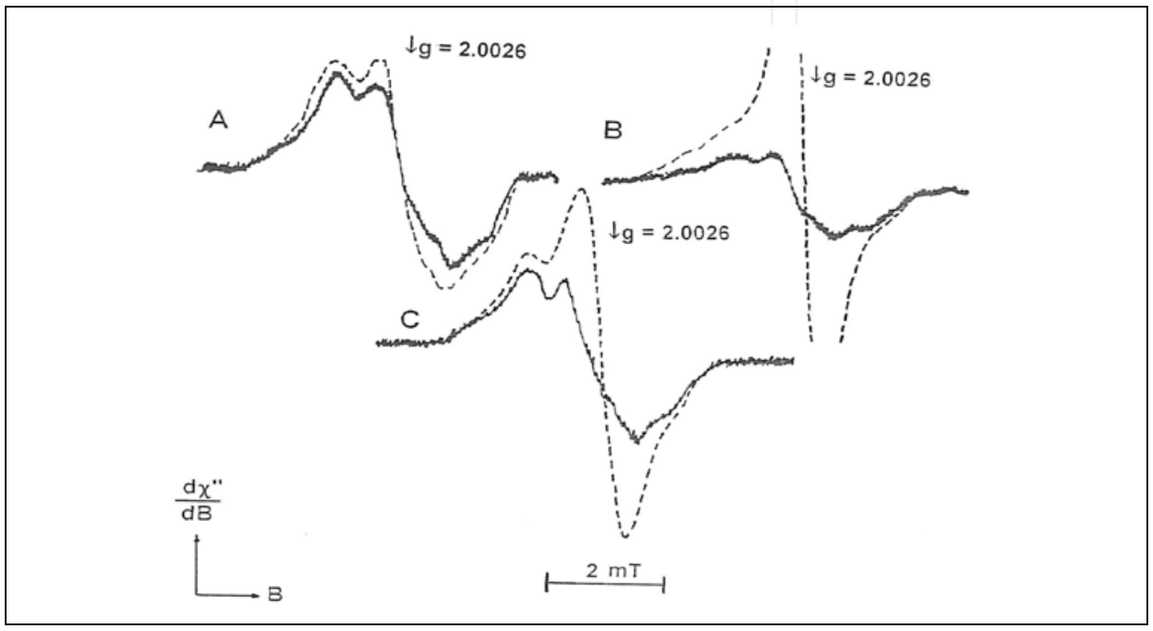


Fig. 8. EPR spectra of the untreated spinach chloroplasts (A) and in the presence of 0.05 mol dm^{-3} of 5-*tert*-butyl-*N*-(3-hydroxy-4-chlorophenyl)-pyrazine-2-carboxamide (**69**, B) and 5-*tert*-butyl-6–chloro-*N*-(3-fluorophenyl)-pyrazine-2-carboxamide (**68**, C) registered in the dark (full lines) and in the light (dashed lines). (Ref. Doležal et al., 2001a; reprinted with permission of editor).

The effects of *N*-phenylpyrazine-2-carboxamides XIX on the photosynthetic centres of spinach chloroplasts were investigated by studying chlorophyll *a* fluorescence. Fluorescence emission spectra of spinach chloroplasts were recorded on fluorescence spectrophotometer F-2000 (Hitachi, Japan) using excitation wavelength λ_{ex} = 436 nm for monitoring fluorescence of chlorophyll *a* and the samples were kept in the dark 10 min before measuring (Doležal et al., 2001a). When chloroplasts were irradiated with the light of λ_{ex} = 436 nm, an emission band with the maximum at λ = 686 nm was observed. This band belongs to the pigment-protein complexes present mainly in photosystem II (Govindjee, 1995). It was found that chloroplasts treated with the studied compounds exhibited quenching of the emission of Chl *a* molecules. Fig. 9 presents the dependence of F/F_{contr} in the suspension of spinach chloroplasts (F_{contr} – fluorescence intensity at λ = 686 nm in the control, F – fluorescence intensity at λ = 686 nm in the presence of the studied compound)

on the concentration of 5-*tert*-butyl-*N*-(3-hydroxy-4-chlorophenyl)-pyrazine-2-carboxamide (**69**), 5-*tert*-butyl-6–chloro-*N*-(3-hydroxy-4-chlorophenyl)-pyrazine-2-carboxamide (**70**), 5-*tert*-butyl-6–chloro-*N*-(2-chlorophenyl)-pyrazine-2-carboxamide (**71**), and 5-*tert*-butyl-(2-chlorophenyl)-pyrazine-2-carboxamide (**72**). The greater is the fluorescence quenching, the more efficient is the interaction of the inhibitor with pigment-protein complexes in photosystem II. For the investigated compounds the intensity of this interaction showed a decrease in the following order: **70** > **69** > **72** > **71** (Fig. 9).

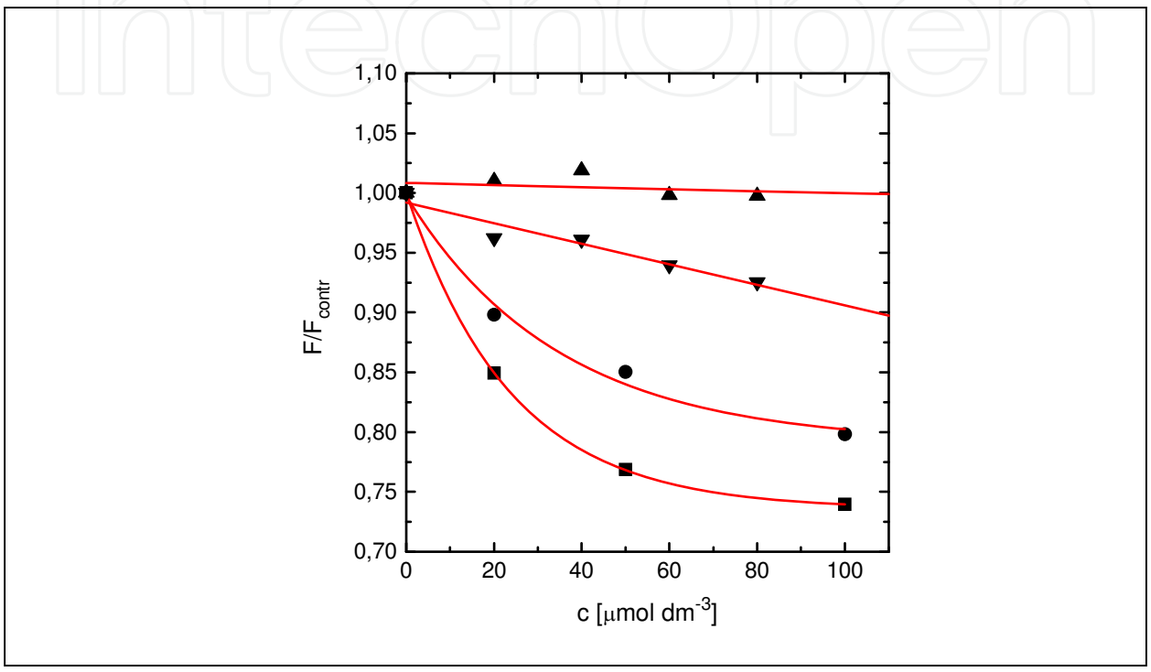


Fig. 9. Dependence of the fluorescence quenching on the concentration of 5-*tert*-butyl-6–chloro-*N*-(3-hydroxy-4-chlorophenyl)-pyrazine-2-carboxamide (**70**, squares), 5-*tert*-butyl-*N*-(3-hydroxy-4-chlorophenyl)-pyrazine-2-carboxamide (**69**, circles), 5-*tert*-butyl-(2-chlorophenyl)-pyrazine-2-carboxamide (**72**, down triangles) and 5-*tert*-butyl-6–chloro-*N*-(2-chlorophenyl)-pyrazine-2-carboxamide (**71**, up triangles) ($F_{contr.}$ = fluorescence of the untreated suspension of spinach chloroplasts; F = fluorescence of anilide treated suspension of spinach chloroplasts; λ = 686 nm). (Ref. Doležal et al., 2001a; reprinted with permission of editor).

The most effective compounds (**70** and **71**) contained two Cl substituents in their molecules. The results of fluorescence study obtained for compounds **70**, **69** and **72** are in agreement with those obtained for OER evolution in spinach chloroplasts (Table 1; IC_{50} = 44 (**70**), 105 (**69**) and 371 μmol dm^{-3} (**72**)). However, the fluorescence of the chloroplast suspension was not affected by 5-*tert*-butyl-6–chloro-*N*-(2-chlorophenyl)-pyrazine-2-carboxamide (**71**) which can be considered as relatively effective inhibitor of OER (IC_{50} = 43 μmol dm^{-3}). This can be explained with the decreased aqueous solubility of this compound. Whereas in the OER experiments the investigated compounds were dissolved in dimethyl sulfoxide, in fluorescence experiments ethanolic solutions were used and after evaporation of the solvent the compound was dissolved directly in the aqueous chloroplast suspension. Consequently, it can be assumed that the fluorescence was not affected due to insolubility of compound **71**

in this suspension. The quenching of the fluorescence intensity at λ = 686 nm produced by the studied compounds suggested PS II as the site of action of the studied compounds.

3.1.4 Inhibition of oxygen evolution rate in suspensions of *Chlorella vulgaris* by *N*-phenylpyrazine-2-carboxamides

The inhibition of oxygen evolution rate (OER) in the suspension of *Chlorella vulgaris* was investigated with two model inhibitors (compounds **69** and **72**). The dependences of OER (expressed as the percentage of the untreated control sample) on the concentrations of compounds 5-*tert*-butyl-*N*-(3-hydroxy-4-chlorophenyl)-pyrazine-2-carboxamide (**69**) and 5-*tert*-butyl-*N*-(2-chlorophenyl)-pyrazine-2-carboxamide (**72**) are shown in Fig. 10. It is evident that both investigated compounds inhibited OER in the suspension of *Chlorella vulgaris* algae. Compound **69** was more effective inhibitor than compound **72** what is reflected in the corresponding IC_{50} values (99 μmol dm^{-3} for **69** and 329 μmol dm^{-3} for **72**). These results are in good agreement with those obtained for inhibition of OER in spinach chloroplasts (Table 1). The introduction of hydroxyl moiety in compound **69** enhanced its photosynthesis-inhibiting activity with respect to that of compound **72** approximately threefold.

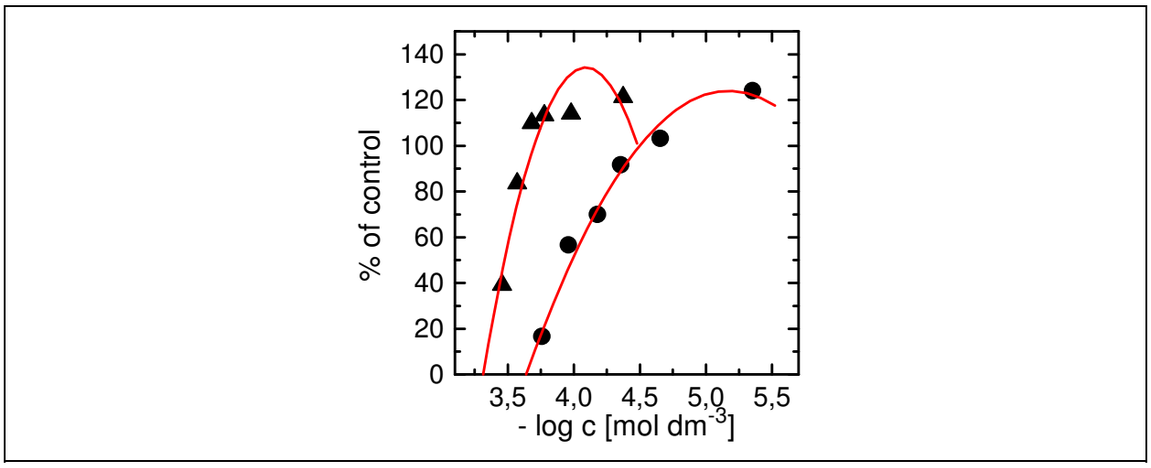


Fig. 10. Dependence of OER in the suspension of *Chlorella vulgaris* (expressed as the percentage of the control) on the concentration of 5-*tert*-butyl-*N*-(2-chlorophenyl)-pyrazine-2-carboxamide (**72**, triangles) and 5-*tert*-butyl-*N*-(3-hydroxy-4-chlorophenyl)-pyrazine-2-carboxamide (**69**, circles). (Ref. Doležal et al., 2001a; reprinted with permission of editor).

3.1.5 Reduction of chlorophyll content in *Chlorella vulgaris* by *N*-phenylpyrazine-2-carboxamides

Toxic effects of environmental pollutants on algae which are essential components of aquatic ecosystems can directly affect the structure and function of ecosystem (Campanella et al., 2000). Herbicides can alter species composition of an algal community what could result in modified structure and function of aquatic communities. Ma et al. (2000) examined the effects of 40 herbicides (belonging to 18 different chemical classes with nine different modes of action) on the green alga *Raphidocelis subcapitata* (formerly named *Selenastrum capricornutum*) and found that the highest acute toxicity exhibited herbicides acting as photosynthesis inhibitors. Photosynthetic pigments have often been used as biomarkers of exposure to different classes of herbicides in autotrophic plants including algae (Blaise, 1993;

Sandmann, 1993). The inhibitory effectiveness of some substituted pyrazinecarboxamides related to reduction of chlorophyll content in *Chlorella vulgaris* expressed by IC_{50} values is summarized in Table 2. The dependence of log ($1/IC_{50}$) on the compound lipophilicity (log *P*) showed a quasi-parabolic course (Fig. 11).

No.	R^1	R^2	R^3	IC_{50}	Ref.
43	Cl	H	4-Cl-3-CH_3	80	h
44	Cl	H	3-I-4-CH_3	44	h
45	H	tBu	4-Cl-3-CH_3	89	h
79	Cl	tBu	3-CH_3	63	e
84	Cl	tBu	3-Br	67	e
85	Cl	H	3,5-CF_3	125	e
86	H	tBu	3,5-CF_3	208	e
87	Cl	tBu	3,5-CF_3	356	e
88	Cl	H	2,6-CH_3	79	e
95	H	H	4-CH_3	71	b
97	Cl	H	4-F	32	b
100	Cl	H	4-CH_3	37	b
102	H	tBu	2-CF_3	33	b

Table 2. IC_{50} values (in μmol dm^{-3}) related to reduction of chlorophyll content in *Chlorella vulgaris* of some substituted pyrazinecarboxamides XIX. Cultivation conditions: 7 days; photoperiod 16 h light/8 h dark; photosynthetic active radiation 80 μmol m^{-2} s^{-1}; pH = 7.2; Chl content in the suspensions at the beginning of the cultivation was 0.01 mg dm^{-3}. (Ref. Doležal et al., 2007[h], 2002[e], 2008a[b]).

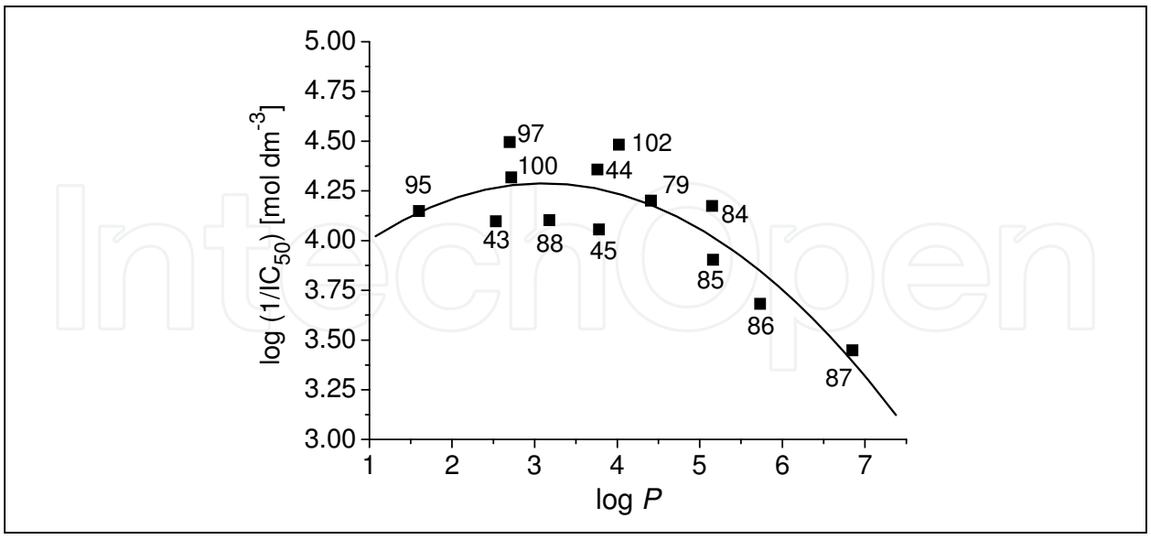


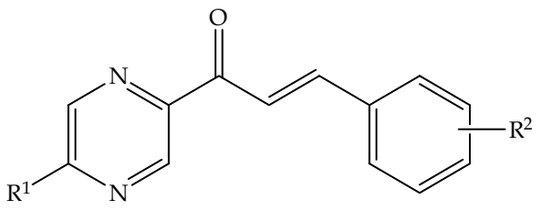
Fig. 11. The dependence of antialgal activity expressed as log ($1/IC_{50}$) on the lipophilicity (log *P*) of some substituted pyrazinecarboxamides XIX.

However, differences in IC_{50} values of compounds with comparable lipophilicity indicate that the biological activity is affected beside of lipophilicity also by the electronic properties

of R^3 substituent(s). Because of too low aqueous solubility of many compounds from the tested set of pyrazinecarboxamides XIX the compounds fall out during experiment (7 days) and the corresponding IC_{50} values could be determined only for limited number of compounds.

4. Photosynthesis-inhibiting pyrazine analogues of chalcones

Chalcones and related compounds "chalconoids" are aromatic ketones containing two aromatic rings linked with three carbon chain. The presence of an unsaturated double bound is typical for chalcones. Hence, chalcones are 1,3-diarylprop-2-ones. They show antibacterial, antifungal, antitumor and anti-inflammatory properties (Dimmock et al., 1999). The aim of our project was the isosteric replacement of a phenyl moiety in chalcones with the pyrazine ring to form some pyrazine analogues of chalcones ("diazachalcones"). Several series (thirty two compounds) of ring substituted (*E*)-3-phenyl-1-(pyrazin-2-yl)-prop-2-en-1-ones XX (Fig. 12) were prepared in our laboratories by means of modified Claisen–Schmidt condensation of acetylpyrazines with aromatic aldehydes (Opletalová et al., 2002, Opletalová et al., 2006, Chlupáčová et al., 2005).



XX

Fig. 12. Pyrazine analogues of chalcones XX (R^1 = H, alkyl; R^2 = OH, NO_2, Cl).

Ring substituted (*E*)-3-phenyl-1-(pyrazin-2-yl)-prop-2-en-1-ones XX were tested for their activity related to OER inhibition in spinach chloroplasts and *Chlorella vulgaris* as well as reduction of chlorophyll content in statically cultured suspensions of freshwater alga *Chlorella vulgaris*. The corresponding IC_{50} values are summarized in Tables 3 and 4.

No.	R^1	R^2	OER inhibition/IC_{50}	
			S. oleracea	*C. vulgaris*
114	tBu	2-OH	167	78
115	isoBu	2-OH	144	63
116	nBu	2-OH	184	147
117	nPro	2-OH	187	100
118	tBu	4-OH	315	279
119	isoBu	4-OH	235	232
120	nBu	4-OH	306	265
121	nPro	4-OH	399	514

Table 3. IC_{50} values (in μmol dm^{-3}) related to OER inhibition in spinach chloroplasts and *Chlorella vulgaris* by diazachalcones XX. (Ref. Opletalová et al., 2002).

The inhibition of OER in spinach chloroplasts by substituted diazachalcones XX (**114-121**) (Fig. 12) has been investigated spectrophotometrically, using DCPIP as an electron acceptor (Kráľová et al., 1992). For the study of OER inhibition in the algal suspensions a Clark type electrode has been used. The IC_{50} values of compounds **114-121** related to OER inhibition varied in the range of 144-399 μmol dm^{-3} for spinach chloroplasts) and 63-514 μmol dm^{-3} for algal suspension of *Chlorella vulgaris* (Table 3). 2-Hydroxy substituted derivatives were found to be more effective inhibitors of photosynthesis than the 4-hydroxy substituted ones. The inhibitory activity of 2-hydroxy substituted derivatives was affected also by the branching of R^1 substituent: OER inhibition in photosynthesizing organisms by the isomers with branched alkyl chain (*tert*-butyl, *iso*butyl) was more pronounced than by the isomer with unbranched alkyl substituent (*n*-butyl) (Opletalová et al., 2002).

No.	R^1	R^2	IC_{50} PET inhibition *S. oleracea*	IC_{50} Chl. content reduction *C. vulgaris*	Ref.
122	H	2-NO_2	ND	70.6	k
123	tBu	2-NO_2	325.0	ND	k
124	isoBu	2-NO_2	ND	118.0	k
125	nBu	2-NO_2	393.0	585.0	k
126	nPro	2-NO_2	ND	123.0	k
127	H	3-NO_2	658.0	19.6	k
128	tBu	3-NO_2	461.0	ND	k
129	isoBu	3-NO_2	340.0	62.8	k
130	nBu	3-NO_2	236.0	ND	k
131	nPro	3-NO_2	ND	18.6	k
132	H	4-NO_2	ND	44.9	k
133	tBu	4-NO_2	ND	ND	k
134	isoBu	4-NO_2	ND	ND	k
135	nBu	4-NO_2	706.0	ND	k
136	nPro	4-NO_2	ND	238.3	k
137	H	3-OH	877.0	32.5	l
138	tBu	3-OH	105.0	238.3	l
139	isoBu	3-OH	256.0	65.5	l
140	nBu	3-OH	ND	95.9	l
141	nPro	3-OH	ND	69.9	l
142	H	4-Cl	ND	24.5	l
143	tBu	4-Cl	181.0	ND	l
144	isoBu	4-Cl	246.0	ND	l
145	nBu	4-Cl	374.0	ND	l

Table 4. IC_{50} values (in μmol dm^{-3}) related to PET inhibition in spinach chloroplasts and IC_{50} values (in μmol dm^{-3}) related to reduction of chlorophyll content in statically cultivated *Chlorella vulgaris* determined for diazachalcones XX. (Ref. Opletalová et al., 2006[k], Chlupáčová et al., 2005[l]), ND – not determined.

The effects of substituted diazachalcones XX (**114-121**) on the photosynthetic centres of chloroplasts were investigated by studying chlorophyll *a* fluorescence. The decreased intensity of the emission band at 686 nm, belonging to the pigment-protein complexes in photosystem (PS) II, suggested PS II as the site of action of the studied compounds (Kráľová et al., 1998).

Using EPR spectroscopy it has been found that in spinach chloroplasts the intensity of EPR signal II, mainly the intensity of its constituent signal II_{slow}, showed a decrease by the studied compounds **114-121**. Consequently it can be concluded that the studied compounds, similarly to *N*-phenylpyrazine-2-carboxamides (Doležal et al., 2001a), interact with $D^{\bullet}$ intermediate, *i.e.* with the tyrosine radical in 161st position (Tyr_D; Y_D) which is located in D_2 protein on the donor side of PS II (Fig. 5). Due to interaction of the studied compounds with $D^{\bullet}$ intermediate PET from the oxygen evolving complex to the core of PS II is impaired. A pronounced increase of EPR signal I intensity in the light belonging to the cation-radical of chlorophyll *a* dimmer in the core of PS I indicated that the electron transport between PS II and PS I is inhibited as well. However, addition of DPC to chloroplasts inhibited by the studied compounds completely restored the reduction of DCPIP indicating that the core of PS II (P680) and a part of the electron transport chain - at least up to plastoquinone - remained intact. These results are in accordance with those obtained with 2-alkylsulfanylpyridine-4-carbothioamides (Kráľová et al., 1997). Similar study with anilides of 2-alkylpyridine-4-carboxylic acids has shown that also the core of PS II was partially impaired by these inhibitors of photosynthetic electron transport (Kráľová et al., 1998a). On the other hand, after addition of DPC to chloroplasts inhibited by the studied *N*-phenylpyrazine-2-carboxamides **68** and **69** up to 70-80%, the OER in the suspension of spinach chloroplasts was restored only up to 55-75% of the untreated control sample indicating that also some member of the photosynthetic electron transport chain between $Z^{\bullet}/D^{\bullet}$ and plastoquininone was partially damaged by the these compounds (Doležal et al., 2001a).

In general, in the series of diazachalcones **122-145** the most effective reduction of chlorophyll content in the suspensions of *C. vulgaris* showed compounds with R^1 = H (Table 4): **127** (R^2 = 3-NO_2; IC_{50} = 19.6 μmol dm^{-3}), **142** (R^2 = 4-Cl; IC_{50} = 24.5 μmol dm^{-3}), **137** (R^2 = 3-OH; IC_{50} = 32.5 μmol dm^{-3}), **132** (R^2 = 4-NO_2; IC_{50} = 44.9 μmol dm^{-3}) and **122** (R^2 = 2-NO_2; IC_{50} = 70.6 μmol dm^{-3}). However, the highest anti-algal activity from this series showed compound **131** (R^1 = $CH_3CH_2CH_2$, R^2 = 3-NO_2; IC_{50} = 18.6 μmol dm^{-3}). On the other hand, the most effective inhibitors of PET in spinach chloroplasts were found to be two compounds with R^1 = $C(CH_3)_3$, namely **138** (R^2 = 3-OH; IC_{50} = 105 μmol dm^{-3}) and **143** (R^2 = 4-Cl; IC_{50} = 181 μmol dm^{-3}) whereby IC_{50} values for several compounds could not be determined due to too low solubility of these compounds.

5. Conclusion

Pyrazines are a class of compounds that occur almost ubiquitously in nature. The worldwide distribution of pyrazines in plants, insects, terrestrial vertebrates, marine organisms, fungi and bacteria, their specific properties, including their using as drugs, fungicides and herbicides invite reasonable attention. Our review brings the basic information about some commercially produced pyrazine herbicides including their mechanism of action as well as survey of patented herbicidal pyrazine derivatives. Special attention was paid to the original compounds from series of 113 substituted *N*-

phenylpyrazine-2-carboxamides XIX and 32 diazachalcones XX prepared and evaluated in our laboratories. In first series, pyrazinecarboxamides XIX connected via –CONH– bridge with substituted anilines can form centrosymmetric dimer pairs with the peptidic carboxamido group of some peptides, needed for binding to the receptor site, possibly by formation of hydrogen bonds. All compounds were tested as potential inhibitors of the photosynthetic electron transport in spinach chloroplasts. Based on the obtained results it could be assumed that the biological activity of the studied substituted pyrazinecarboxamides did not depend exclusively on the compound lipophilicity but it was also affected by electron accepting or withdrawing power of the substituents on the aromatic benzene ring. The site of action of some substituted *N*-phenylpyrazine-2-carboxamides XIX in the photosynthetic apparatus of spinach chloroplasts was studied using fluorescence and EPR spectroscopy. It was found that the studied compounds cause quenching of the chlorophyll *a* fluorescence at 685 nm belonging mainly to the pigment—protein complexes in photosystem (PS) II. The extent of the fluorescence quenching correlated with the effectiveness of the compounds concerning inhibition of oxygen evolution rate (OER) in spinach chloroplasts. Using EPR spectroscopy it was confirmed that the title compounds interact with the intermediate $D^{\bullet}$ (Tyr_D), *i.e.* with the tyrosine radical, which is situated on the donor side of PS II at the 161th position of D_2 protein. It was found that the studied compounds inhibit OER not only in the suspension of spinach chloroplasts but also in the suspensions of *Chlorella vulgaris*. Introducing of Cl substituents into aromatic ring as well as pyrazine moiety of the studied molecules enhanced the effectiveness of OER—inhibiting activity. Some *N*-phenylpyrazine-2-carboxamides XIX reduced chlorophyll content in *Chlorella vulgaris* whereby their biological activity was affected beside of lipophilicity also by the electronic properties of R^3 substituent(s). The most effective inhibitor from the series XIX was 6–chloro-*N*-(5-chloro-2-hydroxyphenyl)-pyrazine-2-carboxamide (**34**, IC_{50} = 8 μmol dm^{-3}; Doležal, 1999).

The studied pyrazine analogues of chalcones, diazachalcones XX also reduced the rate of oxygen evolution in spinach chloroplasts and *C. vulgaris*, whereby the inhibitory activity of *ortho*-hydroxyl substituted derivatives XX was greater than that of *para*-hydroxyl substituted ones. The lowest IC_{50} values were found with compounds having a branched alkyl group on the pyrazine ring. The photosynthesis-inhibiting activity of nitro derivatives was lower than that of the corresponding hydroxylated analogs. In general, in the series of diazachalcones with R^2 = 2-NO_2; 3-NO_2; 4-NO_2; 3-OH and 4-Cl, the most effective reduction of chlorophyll content in the suspensions of *C. vulgaris* showed compounds with R^1 = H. It was confirmed that studied diazachalcones interact with $D^{\bullet}$ intermediate, *i.e.* with the tyrosine radical in 161st position (Tyr_D) which is located in D_2 protein on the donor side of PS II and that they do not damage the core of PS II (P680) and a part of the electron transport chain - at least up to plastoquinone.

6. Acknowledgments

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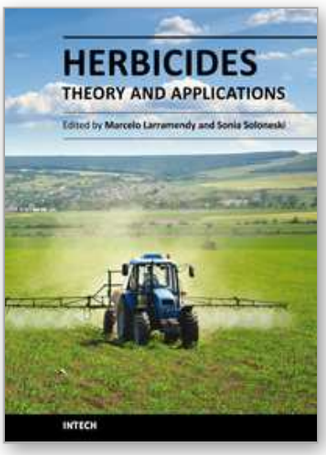

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The content selected in Herbicides, Theory and Applications is intended to provide researchers, producers and consumers of herbicides an overview of the latest scientific achievements. Although we are dealing with many diverse and different topics, we have tried to compile this "raw material" into three major sections in search of clarity and order - Weed Control and Crop Management, Analytical Techniques of Herbicide Detection and Herbicide Toxicity and Further Applications. The editors hope that this book will continue to meet the expectations and needs of all interested in the methodology of use of herbicides, weed control as well as problems related to its use, abuse and misuse.

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