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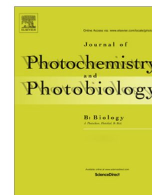

Screening of novel chemical compounds as possible inhibitors of carbonic anhydrase and photosynthetic activity of photosystem II

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ABSTRACT

Thirty novel chemical compounds were designed and synthesized expecting that they would be possible inhibitors. From this number eleven were organic bases, twenty-four were their organic derivatives and fourteen were metal complexes. Screening of these chemicals by their action on photosynthetic electron transfer (PET) and carbonic anhydrase (CA) activity (CAA) of photosystem II (PSII), α-CA, as well as β-CA was done. Several groups were revealed among them. Some of them are capable to suppress either one, two, three, or even all of the measured activities. As example, one of the Cu(II)-phenyl sulfonylhydrazone complexes (compound **25**) suppresses CAA of α-CA by 88%, CAA of β-CA by 100% inhibition; CAA of PSII by 100% and the PSII photosynthetic activity by 66.2%. The Schiff base compounds (**12, 15**) and Cu(II)-phenyl sulfonylhydrazone complexes (**25, 26**) inhibited the CAA and PET of PSII significantly. The obtained data indicate that the PSII donor side is a target of the inhibitory action of these agents. Some physico- or electrochemical properties such as diffusion coefficient, number of transferred electrons, peak potential and heterogeneous standard rate constants of the compounds were determined in nonaqueous media. p*K*a values were also determined in nonaqueous and aqueous media. Availability in the studied group of novel chemical agents possessing different inhibitory activity allow in future to isolate the "active part" in the structure of the inhibitors responsible for different inhibitory mechanisms, as well as to determine the influence of side substituters on its inhibitory efficiency.

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1. Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) is an enzyme that catalyzes a reversible hydration of carbon dioxide: $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$ [1]. Carbonic anhydrases (CA) are found in all forms of life: plants, animals, bacteria, and fungi [1–4]. According to the modern classification, CAs are divided into five main classes: α, β, γ, δ and ζ [1,5]. The CsoS3 protein was previously considered as the ancestor of a new ε-class of CAs [6]. However, according to its crystal structure analysis, it was finally attributed to a subclass of β-CAs [7]. At least three basic CA classes, α-CA, β-CA and γ-CA, were found in higher plants, such as spinach [8], parsley [9], pea [10], lettuce [11], tobacco [12], chickpea [13], potato [14], and wheat [15,16].

It has been demonstrated that this enzyme is involved in basic cell processes, such as photosynthesis, respiration, transport of inorganic carbon (Ci) and ions, calcification, and regulation of acid–base balance [1]. In algae and cyanobacteria, the CAs are primarily involved in the CO_2-concentrating mechanism (CCM), and they maintain an elevated concentration of CO_2 around Rubisco [17–20].

At the same time, information on the characteristics of the cyanobacterial or algal CAs is rather limited. CAs are essential participants in the regulation of the primary photosynthetic processes

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around PSII [21]. Several reports have indicated CA activity in isolated PSII particles. The first algal intracellular α-CA (Cah3) was identified in the green algae *Chlamydomonas reinhardtii* [22]. Immunogold analysis confirmed that Cah3 is located on the luminal side of thylakoid membranes [23]. Evidence for CA activity in the thylakoid fractions of other green algae has been well documented [24]. Moreover, the PS II associated CA activity has also been identified in higher plants [25]. Recent experiments by Dudoladova et al. [26] indicated that the β-CA of 60 kDa is expressed constitutively and associated with polypeptides of PSII in the cyanobacterium *Rhabdoderma lineare*. In addition, some reports suggested that CA activity with extremely unusual characteristics is associated with the extrinsic proteins from pea PSII particles and with isolated fractions enriched with PsbO [27]. Later, cah3 was shown to be a component of the core complex of PSII and required for stabilization and function of the water oxidizing complex (WOC) [28,29].

Four sources of carbonic anhydrase (CA) activity in submembrane preparations of PSII isolated from pea leaves were examined. Three of them belong to the hydrophilic proteins of the oxygen-evolving complex of PSII with molecular mass 33 kDa (protein PsbO), 24 kDa (protein PsbP), and 18 kDa (protein PsbQ). The fourth source of CA activity is associated with a pigment-protein complex of PSII after removing three hydrophilic proteins by salt treatment [30]. Since the found sources of CA activity demonstrated properties different from the ones of typical CA (need for Mn^{2+}, insensitivity or low sensitivity to acetazolamide or ethoxyzolamide) and such CA activity was found only among PSII proteins, we cannot exclude that they belong to the type of Mn-dependent CA associated with PSII [30].

The effects of suppression of the carbonic anhydrase (CA) activity by a CA-inhibitor, acetazolamide (AA), on the photosynthetic activities of PSII particles from higher plants were investigated. Along with CA-activity, AA also inhibits the PSII photosynthetic electron transfer and the AA-induced suppression is totally reversed by the addition of bicarbonate (3–5 mM). The data clearly indicate significance of CA-activity for the functioning of the PSII donor side in higher plants [31].

All CAs contain metals (Zn, Fe, or Co) in the active center and reveal different sensitivity to sulfanilamide inhibitors: α-CA, containing Zn cation in the active center, are most sensitive [32–34]. Both β-CA containing Zn cation and γ-CA, which can contain Zn, Fe, or Co in the active center, are inhibited weakly by sulfanilamide compounds [33,35,36].

The catalytically active zinc is bound to three histidine residues and one water molecule/hydroxide ion acting as a fourth ligand in a tetrahedral geometry. The hydroxide ion is bound to Zn(II) in the active form of the CA enzyme. The CO_2 molecule is bound to both zinc and OH ligand following a strong nucleophile attack. The formation of a bicarbonate ion proceeds with a penta-coordinated geometry. Then, the bicarbonate ion is displaced by a water molecule and goes into the solution. The resultant water-ligated enzyme is catalytically inactive. In order to revert to the active form, a proton is transferred from the active site of the enzyme to the medium. This proton transfer reaction is the rate limiting step in catalysis [37].

Inorganic anions and sulfonamides are two main classes of CA inhibitors [38–41]. To date, a few studies were reported on carbonic anhydrase activity in PSII and the effect of CA inhibitors (CAIs) on the kinetics of chlorophyll fluorescence in PSII [30,42–45]. Tavallali et al. showed that the net photosynthetic rate and the quantum yield of PSII were reduced under conditions of zinc deficiency and NaCl stress, and increasing salinity in soil under Zn-deficient conditions generally decreased CA activity, protein, chlorophyll *a* and *b* contents [46]. Khan et al. reported that the CA activity was increased at low $[Cd^{2+}]$ (10 mM) and inhibited at higher Cd^{2+} concentrations under low $[Zn^{2+}]$, and increasing $[Cd^{2+}]$ decreased the CA activity [47]. On the other hand, Moskvin et al. showed that ethoxyzolamide (lypophilic inhibitor) inhibited the CA activity of PSII membrane fragments (BBY particles), but acetazolamide (the hydrophilic, non-permeable inhibitors) had no significant effect [25]. Pronina reported that acetazolamide and imidazole inhibited the photoinduced yield of chlorophyll fluorescence [48]. Ignatova et al. showed that acetazolamide increased carbonic anhydrase activity of PSII membranes at concentrations lower than 10^{-6} M and suppressed this activity only at higher concentrations, but ethoxyzolamide effectively suppressed the CA activity of PSII membranes ($I_{50} = 10^{-9}$ M) [49]. They also reported that the proteins in the PSII membranes differed in their sensitivity to acetazolamide [50].

Clinical drugs used as carbonic anhydrase inhibitors (CAIs) such as acetazolamide, dichlorophenamide, dorzolamide, and brinzolamide, are composed of a primary sulfonamide moiety [51]. It is well known that these inhibitors are typically organic, but Meggers et al. showed that high selectivity does not only depend on intermolecular interactions but also on a directed 3D arrangement of different functionalities. So the metal complexes offer ''natural-product-like'' structural complexities and strikingly high target specificities [52]. Thus, researchers have reported several organometallic complexes as CAIs [53–55]. For example, Salmon et.al determined the protein X-ray crystal structures of four organometallic inhibitors in complex with carbonic anhydrase II. They assumed that the barrel-shaped hydrophobic complex moieties provide an improved occupancy of the hydrophobic binding patch within the enzyme's active site [53].

So far, only a few studies were reported that simultaneously addressed the investigation of CA and photosynthetic activity of the same PSII preparations [30,45,48]. In this study, we investigated 16 organic compounds and 14 metal complexes as novel potent inhibitors against CA activity and photosynthetic electron transport of PSII, as well as bovine CA. In addition, the novel compounds have also been investigated from the electrochemical point of view in order to reveal a possible relationship between the inhibitors' effects and the electrode potentials of the compounds.

2. Materials and methods

2.1. Isolation of PS II particles

Photochemically active thylakoid membrane fragments enriched in PSII (PSII membrane complexes further termed as BBY (for Berthold–Babcock–Yocum)-particles) were prepared from leaves of pea plants (Pisum sativum grown for 2–3 weeks) by the method described earlier [56]. These PSII preparations contained 250 molecules of chlorophyll (Chl) per reaction center (RC) [57] and exhibited O_2 evolution rates of 400–500 μmol mg^{-1} of Chl h^{-1} under saturating light in the presence of 0.1 mM 2,5-dichloro-p-benzoquinone plus 1 mM $K_3Fe(CN)_6$ as electron acceptors. Samples were stored in liquid nitrogen until use.

2.2. Carbonic anhydrase activity measurements

Carbonic anhydrase activity was measured by the electrometric procedure of Wilbur and Anderson [58] as the rate of pH change in CO_2 hydration using Mettler Toledo InLab 413 pH electrode and cpX-2 pH/ion meter (Institute of Biological Instruments (IBI) of the Russian Academy of Sciences, Pushchino) interfaced with a computer. Measurements were carried out at 1.5–2 °C in a medium containing 25 mM veronal (pH 8.6), 50 mM KCl, and 15 mM $MgCl_2$. BBY particles (0.21 mL, 100 μg) were added to 1.29 mL of veronal buffer (the sample addition decreased pH by 0.1–0.15 units), and

then water (0.75 mL, saturated by bubbling with CO_2 at 0 °C for 1 h) was added to the reaction mixture (final Chl concentration was 44 μg mL^{-1}). The time required to decrease pH from 8.3 to 7.8 was calculated from the data of the pH change recorded by a special PC program "pX-meter". To express the value of CA-activity, Wilbur and Anderson units calculated per mg Chl were used. The calculations were performed according to the formula: (to − t)/(tm), where to and t are times for pH change from 8.3 to 7.8 in the control and in the sample, respectively; m is the amount of Chl in milligrams added to the reaction mixture. The measurements were carried out at least 3–4 times using three biological replications. To analyze the effect of inhibitors on CA-activity in the BBY particles an inhibitor (at final concentration of 100 μM) was added and the sample was incubated in the cell for 1 min before the measurements.

2.3. α-Carbonic anhydrase

In work α-carbonic anhydrase isozyme II from bovine erythrocytes (lyophilized powder, ⩾3000 W-A units/mg protein) (C2522, Sigma) was used.

2.4. -Carbonic anhydrase

β-Class carbonic anhydrase was obtained as described earlier [4]. The gene for β-class carbonic anhydrase (CA), which was designated as *cahB1*, was cloned from the genomic library of the alkaliphilic cyanobacterium *Microcoleus chthonoplastes*. The product of the *cahB1* gene was expressed in *Escherichia coli*. *E. coli* BL21(DE3)pLysS cells transformed with empty vector pET-32b(+) or recombinant pET32::*cahB1* were grown in presence of 1 mM IPTG for 4 h at 37 °C. Cells were harvested by centrifugation, resuspended in 30 mM HEPES–KOH buffer (pH 8.2), and supplied with equal volume of glass beads (G4649, Sigma). Cells were disrupted by vortexing twice for 30 s with a 30 s break (cells were kept on ice) at maximum speed (vortex Reax-top, Heidolf Instruments GmbH & Co., Schwabach, Germany). Glass beads were separated from cell extract by centrifugation at 700*g*, 4 °C, 1 min. Measurements were performed using the portions of total cellular extracts of *E. coli*, each contained 0.1 mg of protein. Samples were diluted into a final volume of 4 mL with 30 mM HEPES–KOH, pH 8.2. Electrometric assays revealed high CA activity, such as 53.47 ± 4.88 WAU mg protein^{-1}, in total cell extracts containing the recombinant protein. The rate of CO_2 conversion by *cahB1* was inhibited by the specific CA inhibitor, ethoxyzolamide, with a constant of inhibition (I_{50}) of $1.4 \pm 0.2 * 10^{-5}$ M.

2.5. Chlorophyll a fluorescence measurements

The initial minimum level of fluorescence (F_0), kinetics of photoinduced changes of Chl fluorescence yield (ΔF) at $\lambda > 660$ nm (related to photoreduction of Q_A) and the value of the maximum level of fluorescence (F_M) were measured in a 1-cm cuvette at 20 °C using a XE-PAM Fluorometer, "WALZ" (Germany). The measurements were carried out in a medium (non-depleted of the endogenous bicarbonate) containing 25 mM MES-NaOH (pH 6.5) and 10 mM NaCl. The Chl concentration was 10 μM mL^{-1}. Illumination conditions are given in the figure legends.

2.6. Chlorophyll concentration

Chlorophyll (Chl) concentrations and Chl *a* to Chl *b* ratios were assayed in 80% acetone according to the method of Arnon [59].

2.7. Preparation of novel inhibitor solutions

Novel chemical compounds were dissolved in dimethylsulfoxide (DMSO), for carbonic anhydrase and photosynthetic activity measurements.

2.8. Inhibition studies

Spontaneous reaction of CO_2 hydration was measured in the absence of other additions and the uncatalyzed rates were determined. Then this reaction was measured in the presence of novel compounds. It allowed clarifying if the uncatalyzed rates were changed by these chemicals. The rate of the reaction catalyzed an enzyme (α-carbonic anhydrase, β-carbonic anhydrase or BBY-particles) in the absence of other additions was determined. The data was used as control for the corresponding enzyme. The specific inhibition of CA was studied in the presence of novel compounds. They were supplied at final concentrations 0.1 mM and incubated with CA samples for determined time before the assays began. The measurements were carried out at least in 7–9 replicates. Acetazolamide (AAZ) was used as a standard inhibitor for all CAs investigated here.

2.9. Synthesis of the novel compounds

Compounds used in this study were prepared as described in our previous study [60–65]. They were characterized by melting points, elemental analysis, MS, IR and NMR spectroscopy and the data were found to be in agreement with those of the assigned molecular structures.

2.10. Electrochemical studies

Voltammetric measurements were performed using an Ivium Stat potentiostat. A glassy carbon working electrode (BAS; ∅: 3 mm, diameter), a commercial Ag/Ag^+ (BAS Co., Ltd.) reference electrode and a platinum wire counter electrode in a standard one-compartment three-electrode cell of 10 mL capacity were used in all experiments. Before each measurement the glassy carbon electrode was polished manually with aqueous slurry of alumina powder (∅: 0.01 μm) on a damp smooth polishing cloth (BAS velvet polishing pad). All measurements were realized at room temperature. 0.1 M tetrabutylammonium tetraflouroborate (TBATFB) was used as supporting electrolyte. 1 mM of the compounds was prepared in the DMSO. The ultramicro electrode (UME) studies were carried out using 10 μm diameter Pt ultramicro disc electrode and carbon ultramicro electrode in DMSO and in aqueous media, respectively. The electrochemical properties were determined in the range of pH 4.1–4.7 in aqueous media. The UME responses and chronoamperometric Cottrell slopes of the ligands at 1 mM concentration on Pt disc electrode were used to determine the diffusion coefficients and the number of electrons transferred.

Some physico- or electrochemical properties like the diffusion coefficient, transferred electron number, peak potential and heterogeneous standard rate constants of the compounds were determined in nonaqueous media according the literature [66,67]. p*K*a values were also determined in nonaqueous and aqueous media according to the literature [68].

3. Results and discussion

Taking into account the available information of known inhibitors of CA, thirty novel chemical compounds were designed and synthesized expecting that they would be possible inhibitors. From this number eleven were organic bases, twenty-four were their

organic derivatives and fourteen were metal complexes. Chemical structures of organic-based and metal-based compounds are shown in Figs. 1 and 2. All of them were checked by their effects on CAA of α-CA, β-CA as well as CA- and photochemical activity of PSII.

3.1. Effects of novel compound on CAA of α-CA

Fig. 3 shows the kinetics of pH changes characteristic for carbonic anhydrase activity (reaction of CO_2 hydration) of α-CA. Final concentration of protein was 0.0005 mg mL^{-1}. Initial carbonic anhydrase activity of protein (without additions) was equal to 2500 Wilbur–Anderson units per 1 mg of protein (curve 2). The value of the reaction rate was used as control for all novel compounds. In the presence of compound **19** (Ni(II)-naphthyl sulfonylhydrazone complex) the reaction was suppressed by 38% (curve 3). Kinetics of spontaneous reaction of CO_2 hydration occurring in the measuring medium in the absence of enzyme is shown by curves 1 and 4, without any additions (curve 1) and in the presence of compound **19** (curve 4). The fact that curve 4 coincides with curve 1 testifies that the novel compound does not affect the spontaneous reaction of CO_2 hydration. The buffer concentration in the measuring medium was 25 mM, whereas the final concentration of any added novel compound was only 0.1 mM. All compounds were tested in the same manner. The obtained data are presented in Table 1. As shown in Table 1 not all of these compounds are capable to inhibit CAA of α-CA. Only eight compounds have inhibiting effects. In addition to compound **19**, they are: compound **5**–55% inhibition; compound **12**–65% inhibition; compound **18**–46% inhibition; compound **25**–88% inhibition; compound **26**–33% inhibition; compound **30**–65% inhibition. It is interesting that similar Cu(II)-phenyl sulfonylhydrazone complexes (compounds **25** and **26**) as well as Ni(II)-naphthyl sulfonylhydrazone complexes (compounds **18** and **19**) revealed different inhibitory efficiency. Probably it is due to the nature side substitutes. Furthermore, it is evident that the nature of metal is also crucial to the inhibitory efficiency. Efficiency of the Zn-containing complex (compound **30**–65%) is higher than that of the Cu-containing complex (compound **26**–33%). Surprisingly, organic bases not containing metal ion also revealed high inhibitory activity (compounds

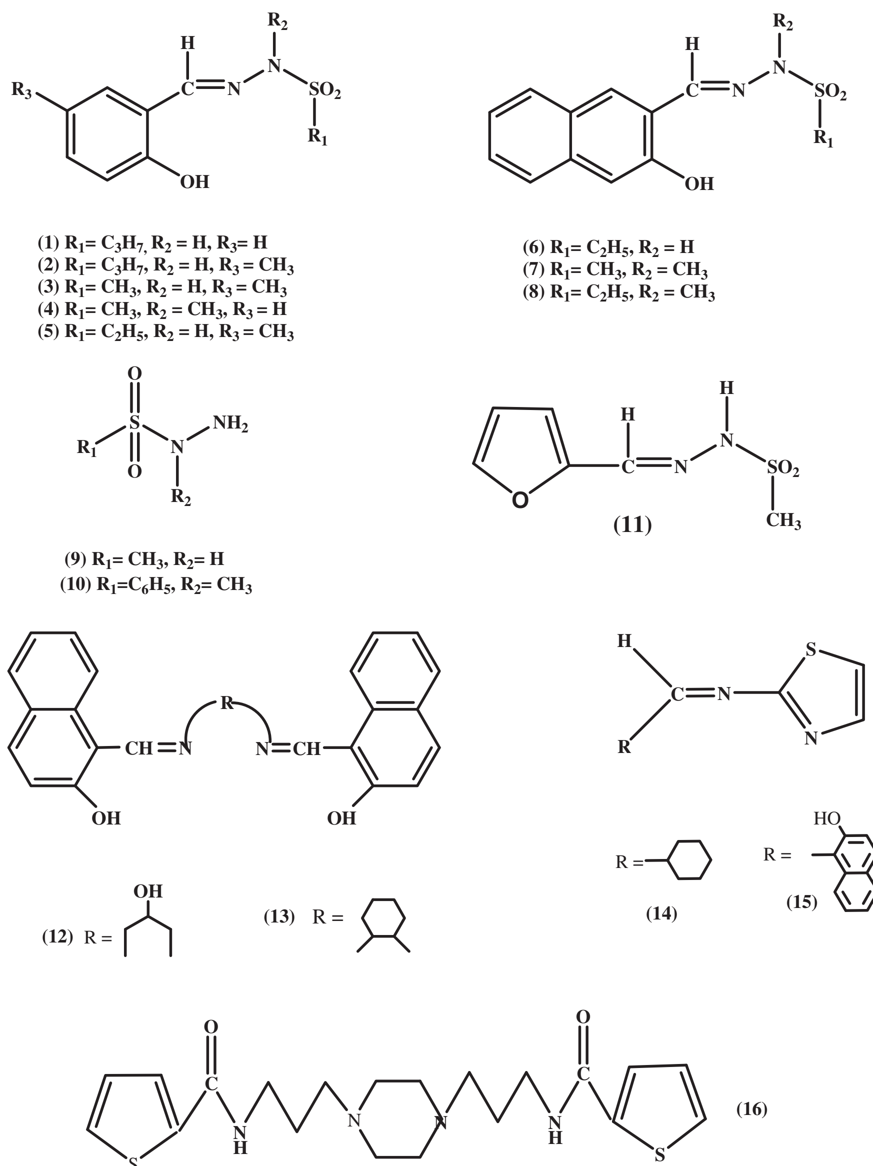

Fig. 1. Structure of the ligands.

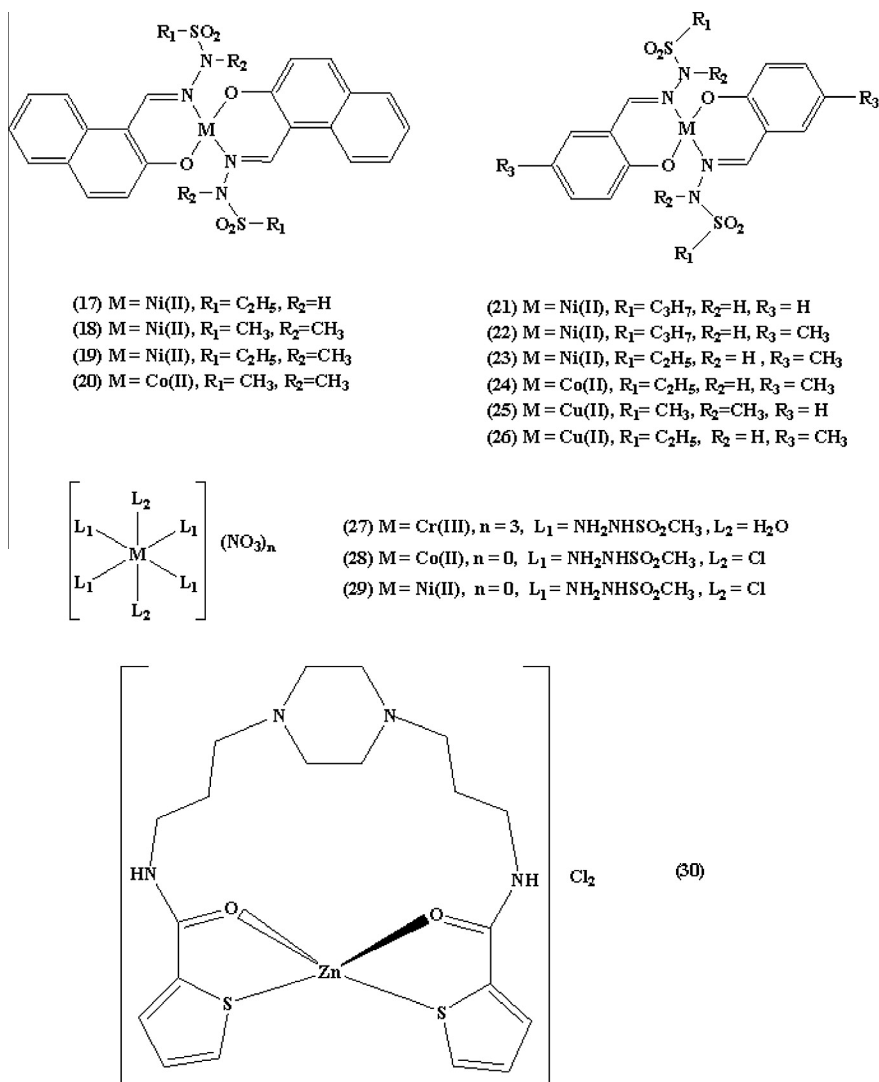

Fig. 2. Structures of metal-based complexes.

5–55% and **12**–65%). Inhibitory constant of α-CA for well-known inhibitors, acetazolamide (AA) and ethoxzolamide (EZ) are in nanomolar range [17,31].

3.2. Effects of novel compound on CAA of -CA

It is known that some of β-CA-s are less sensitive to inhibition by AA and EZ than α-CA-s. It was interesting to study the effects of known CA inhibitors as well as novel compounds on recently revealed recombinant β-CA from the alkaliphilic cyanobacterium *M. chthonoplastes*. The rate of CO_2 conversion by the β-CA was inhibited by the specific CA inhibitor, ethoxyzolamide, with a constant of inhibition (I_{50}) of $1.4 \pm 0.2 * 10^{-5}$ M [4]. Effects of novel compound of CA of the β-CA was studied in the manner similar to α-CA (see above). The obtained data are presented in Table 2. The rate of the reaction measured in the absence of inhibitors was used as control for all novel compounds (0% of inhibition). As in the case of α-CA not all of these compounds inhibit CAA of β-CA with high efficiency. Only fourteen compounds have inhibitory effects with an incubation time for formation of the E–C (enzyme–compound) complex of 30 min. They are in order of decreasing efficiency: compound **25**–100% inhibition; **27**–86.6%; **17**–76.7%; **30**–67.7%; **21**–47.2%; **12**–44.9%; **18**–43.4%; **24**–37%; **22**–31.8%; **5**–28.6%; **4**–26.8%; **14**–24.1%; **15**–17%. However if the incubation time of the E-C complex was only 5 min the inhibitory efficiency was drastically decreased practically for all indicated compounds except for novel agents 21 and 30. These chemicals suppressed CAA of β-CA with the same efficiency after 5 and 30 min of incubation. It is probably due to higher penetration of these compounds to the reaction center of the enzyme. The Cu(II)-phenyl sulfonylhydrazone complexes (compounds **25**–100% inhibition) was two times more effective than Ni(II)-phenyl sulfonylhydrazone (compounds **21**–47.2% inhibition). This also indicates the crucial significance of metal ion in providing maximal efficiency. The observed efficiency of Ni containing complexes with different organic bases (compounds **21**, **17**) have shown that naphthyl sulfonylhydrazone (**17**) was more favorable for inhibition than phenyl sulfonylhydrazones (**21**). However, Ni-complexes with similar organic bases but with different side substituters have different efficiency (**17** and **18**).

3.3. Effects of novel compound on CA- and photochemical activity of PSII

Novel compound were also studied for their action of CA of PSII. The obtained data are presented in Table 3. As shown in Table 3 only three compounds were effective inhibitors of the PSII CAA:

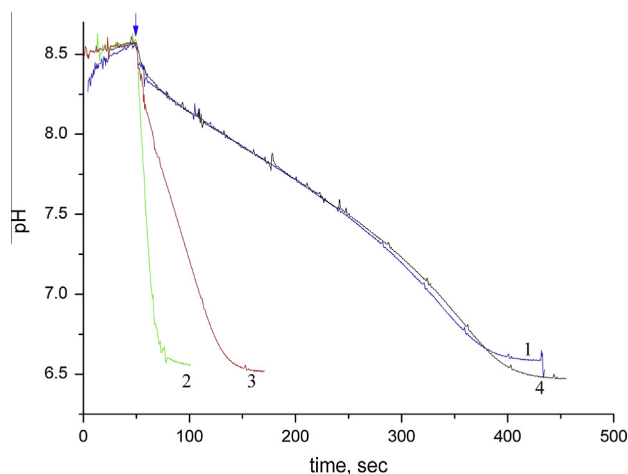

Fig. 3. Kinetics of pH changes characterizing carbonic anhydrase activity (reaction of CO_2 hydration) of α-CA (0.001125 mg, final concentration of protein 0.0005 mg mL^{-1}) in the absence of other additions (curve 2) and in the presence of 0.1 mM compound **19** (NAFESMH-Ni) (curve 3). Kinetics of spontaneous reaction of CO_2 hydration occurring in the measuring medium in the absence of enzyme (curves 1 and 4) without any additions (curve 1) and in the presence of compound **19** (curve 4). Downward arrow indicates the moment of addition of CO_2-satured water. Chlorophyll concentration was 44 μg mL^{-1}. Each trace is an average of at least seven separate measurements. 100% carbonic anhydrase activity of protein (without additions) was equal to 2500 Wilbur–Anderson units per 1 mg of protein.

Table 1
Inhibition (%) of CAA of α-CA from bovine erythrocytes by novel chemical compounds. CAA without additions of any inhibitors was used as control (0% of inhibition). Compound and enzyme solutions were preincubated together for 1 min at temperature of 2 °C and stirring prior to assay, in order to allow for the formation of the E–C complex (for more information see materials and methods).

Compound (0.1 mM)	Inhibition (%) of CAA of α-CA
No additions	100% Activity 0% inhibition
1	[a]
2	[a]
3	[a]
4	[a]
5	55
6	[a]
7	[a]
8	[a]
10	[a]
11	[a]
12	65
13	[a]
14	[a]
15	[a]
16	[a]
17	[a]
18	46
19	38
20	6
21	[a]
22	[a]
23	[a]
24	[a]
25	88
26	33
27	[a]
28	[a]
29	[a]
30	65

[a] No effects.

12, **25**–100%; **30**–95.6%. Less effective were: **15**–31%; **19**–25%; **26**–40%. Remained compounds had no effect on the PSII CAA. It is worth to note that organic bases of these effective compounds include several aromatic rings. Organic base not containing metal ion (**12**) had the same efficiency as complexes including Cu(II) and Zn(II) (**25**, **30**).

Table 2
Inhibition (%) of CAA of β-CA by some of novel chemical compounds. CAA without additions of any inhibitors was used as control (0% of inhibition). Compound and enzyme solutions were preincubated together for 5 or 30 min at room temperature prior to assay, in order to allow for the formation of the E–C complex (for more information see materials and methods).

Compound (0.1 mM)	Incubation time (min)	
	5	30
No additions	100% Activity 0% Inhibition	100% Activity 0% Inhibition
4	[b]	26.8
5	[b]	28.6
8	[a]	[a]
12	18.6	44.9
14	[a]	24.1
15	[b]	17
17	[b]	76.7
18	[b]	43.4
19	[a]	[a]
21	48.7	47.2
22	[a]	31.8
23	[b]	[a]
24	[a]	37
25	[b]	100
26	[a]	[a]
27	68.1	86.6
30	68.7	67.7

[a] No effects.
[b] Not measured.

It was shown that AA inhibits both CA and photosynthetic activity of PSII. This indicates that the PSII photosynthetic activity partially depends on its CAA [31]. We checked all novel compounds also for their effect on PSII photosynthetic activity. The photoinduced changes of PSII Chl *a* fluorescence (F) yield (variable $F - F_V$ or ΔF) was used as a measure of PSII photosynthetic activity.

Inhibition (%) of PSII photosynthetic activity was calculated according to equation $[(F_{Vcont.} - F_{Vexp.})/F_{Vcont.}] * 100\%$, where $F_{Vcont.}$ is F_V of control (without inhibitor); $F_{Vexp.}$ is F_V measured in the presence of inhibitor. Effects of novel compounds of PSII photosynthetic activity measured in PSII subchloroplast membrane particles are shown in Fig. 4. In the absence of other additions typical kinetic of the photoinduced ΔF (related to photoreduction of the PSII primary electron acceptor, plastoquinone Q_A) was observed (curve 1). The value of F_V obtained from curve 1 was used as control (100% activity, 0% inhibition).

Novel compounds (**17**–41% inhibition and **15**–24% inhibition) reduced only F_M level (which is equal to $F_0 + \Delta F$) by suppressing the photoinduced changes in fluorescence yield ΔF, without affecting F_0 level and the rate of ΔF relaxation in the dark (which reflects Q_A re-oxidation) (Fig. 4, curves 2 and 3). Subsequent additions of artificial electron donor of PSII, sodium ascorbate (1 mM), to the sample restored level of photochemical activity (data not shown). Furthermore, these inhibited ΔF are reversible after removing of inhibitors by washing. Similar results are obtained for all novel compounds, which suppressed the photoinduced ΔF (Table 3).

It has been shown earlier [57] that inhibition of the PSII donor side due to a complete removal of Mn from PSII membrane fragments results in similar effect – a 15–20-fold decrease of photoinduced ΔF which can be restored by Mn^{2+} added at a catalytic (0.1–0.2 μM) concentration. Thus, inhibition of the photoinduced ΔF without an increase in the level of F_0 and subsequent reactivation of the ΔF with electron donors is a characteristic of a reversible blocking of electron transfer on the donor side of PS-II [57]. The donor side inhibition sharply distinguishes from “Diuron-type

Table 3
Inhibition (%) of CA- and photosynthetic activity of PSII by novel chemical compounds. CAA of PSII without additions of any inhibitors was used as control (0% of inhibition). Inhibition (%) of photosynthetic activity of PSII was calculated as $[(F_{Vcont.} - F_{Vexp.})/F_{Vcont.}] \times 100\%$. Compound and enzyme solutions were preincubated together for 1 min at temperature of 2 °C and stirring prior to assay, in order to allow for the formation of the E–C complex (for more information see materials and methods).

Compound (0.1 mM)	Inhibition of CAA of PSII (%)	Inhibition of the PSII photosynthetic activity (%)
No additions	100% Activity 0% Inhibition	100% Activity 0% Inhibition
1	[a]	4.1
2	[a]	[a]
3	[a]	1.0
4	[a]	10.9
5	[a]	13.2
6	[a]	4.7
7	[a]	7.0
8	[a]	6.6
10	[a]	4.6
11	[a]	0.8
12	100	17.6
13	[a]	4.3
14	[a]	9.9
15	31	24.4
16	[a]	1.1
17	[a]	41.0
18	[a]	13.4
19	25	4.1
20	[a]	3.2
21	[a]	17.6
22	[a]	9.2
23	[a]	18.7
24	[a]	6.6
25	100	66.2
26	40	24.8
27	[a]	9.0
28	[a]	5.1
29	[a]	2.3
30	95.6	1.9
AA (0.1 mM)	20–30[b]	23[c]
EZ (0.1 mM)	100[b]	10[c]

[a] No effects.
[b] [44,49].
[c] [31].

effect". It is known that addition of Diuron (known inhibitor of electron transfer in PSII) to PSII preparations resulted in the characteristic "Diuron-type effect", i.e., an increase in the F_0 level to a new level F_0' and in a slower dark relaxation of ΔF (data not shown), the effects repeatedly reported in the literature [69].

It should be noted that the restoration is not total, but only to about 85–90% from initial. The fact that restoration of photochemical activity is not total indicate that there is an additional unspecific site for CA of novel compounds. This is similar to the inhibitory effects previously observed for AA on CA and photosynthetic activity of the PSII samples [31].

Phenyl sulfonylhydrazones **(1–4)**, naphthyl sulfonylhydrazones **(6–8)**, sulfonamides **(9**, **10)** and furan sulfonylhydrazone **(11)** exhibited inefficient inhibitory effects on photosynthetic activity and α-CAA, but one of the phenyl sulfonylhydrazone **(5)** displayed weak inhibitory effect on photosynthetic activity and good α-CAA inhibition. Additionally, the symmetric bis-2-hydroxynaphthyl Schiff base compounds **(12–13)** exhibited different activity depending on the bridge moiety. For example, compound **(13)** with a 1,2-dimethylcyclohexyl bridge displayed very weak photosynthetic inhibitory effect, whereas compound **(12)** with a 3-hydroxypentyl bridge inhibited photosynthetic activity weakly, α-CA activity moderately, and CA activity of PSII significantly. It was also shown that, the phenyl-bearing thiazole Schiff base compound **(14)** was inefficient; however, the 2-hydroxynapthyl-bearing thiazole Schiff base compound (**15**) exhibited moderate photosynthetic inhibitory effect and moderate CA inhibitory activity in PSII.

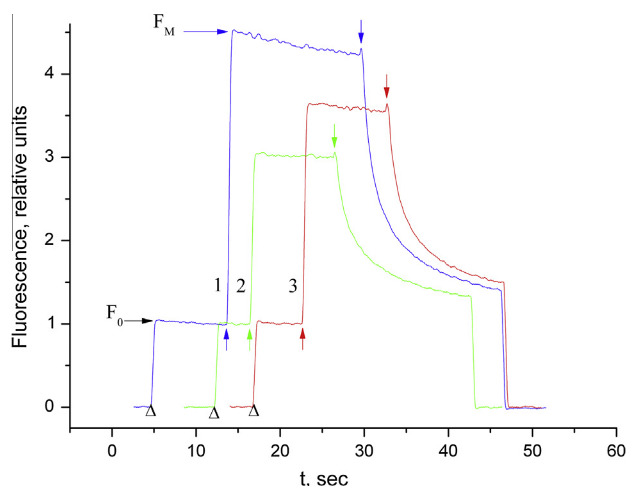

Fig. 4. Kinetics of the photoinduced changes of chlorophyll fluorescence yield (ΔF) with excitation by $\lambda > 660$ nm (related to photoreduction of the PSII primary electron acceptor, plastoquinone Q_A) measured in PSII subchloroplast membrane particles in the absence of other additions (curves 1) and in the presence of: 0.1 mM compound **17** (curve 2); 0.1 mM compound **15** (curve 3). Symbol Δ shows the moment of switching on the measuring light ($\lambda = 490$ nm, 0.01 μmol photons $m^{-2} s^{-1}$) which excites PSII chlorophyll fluorescence, F_0 ($\lambda \geqslant 650$ nm). The upward and downward arrows indicate the moment of switching on and off, respectively, of the actinic light ($\lambda > 600$ nm, 2 mmol photons $m^{-2} s^{-1}$). Chlorophyll concentration was 10 μg mL^{-1}. Each trace is at least an average of seven separate measurements.

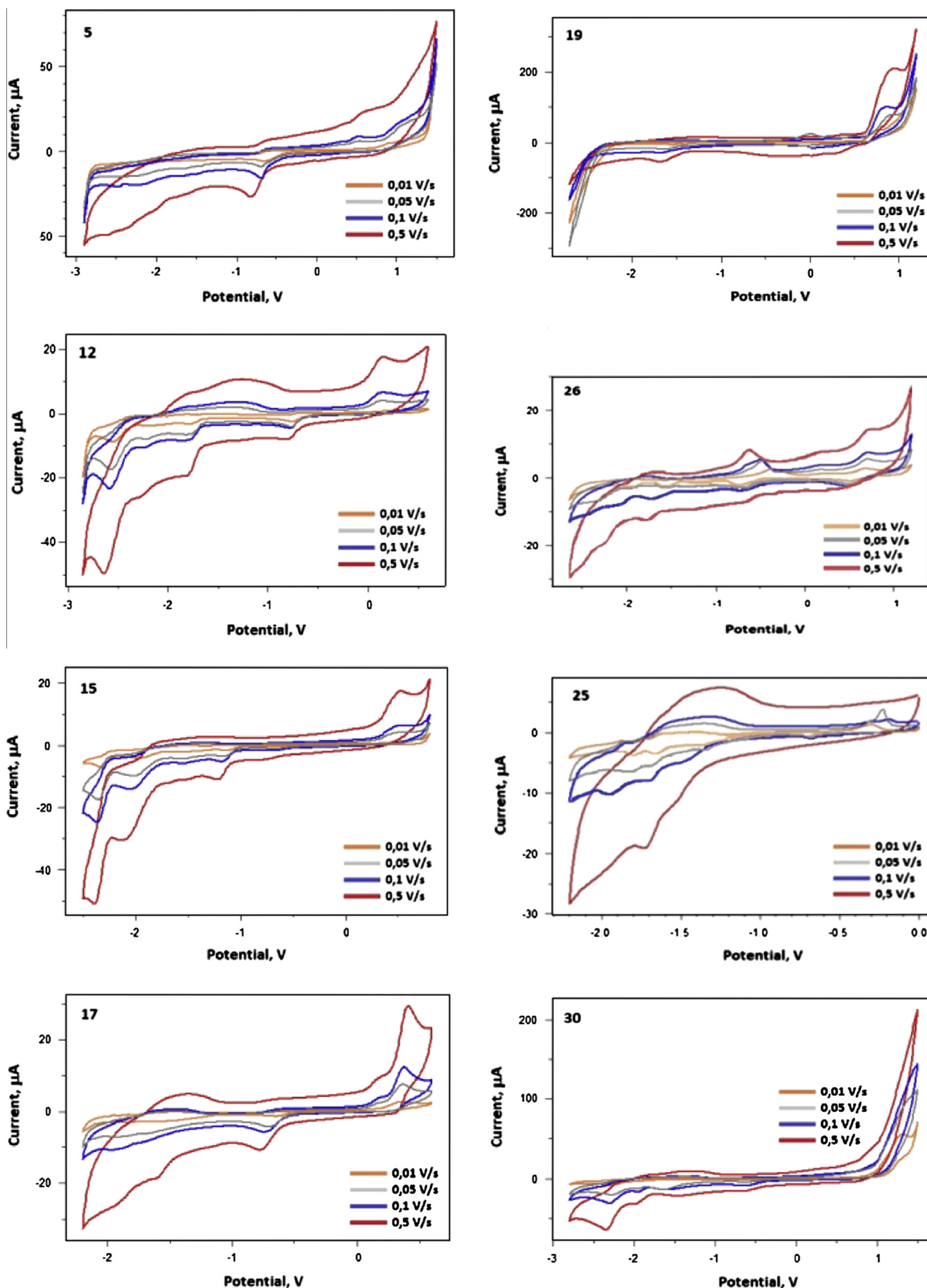

Fig. 5. Cyclic voltammograms of compounds, which show an inhibition effect ($1{\cdot}10^{-3}$ M), measured in DMSO at different scan rates (0.01; 0.05; 0.1; 0.5 V/s) at the glassy carbon electrodes, vs. Ag/Ag^{+} electrode.

Moreover, 2-thiophenecarboxamide bridging by 1,4-piperazine **(16)** showed no inhibitory activity.

Considering the metal complexes, Ni(II)-naphthyl sulfonylhydrazone complexes **(17–19)** showed higher inhibitory activity than corresponding ligands **(6–8)**, whereas Ni(II)-phenyl sulfonylhydrazones complexes **(21–23)** showed no significant effect in similarity with the parent ligands **(1–3)**. The inhibitory action mechanism of Ni(II)-complexes (**17** and **19**) and the absence of any inhibitory action of their ligands **6** and **8** are still unexplained. Probably, higher efficiency of inhibitory action may be due to bigger molecular size of the metal complexes. In addition, Co(II) sulfonyl hydrazone complexes **(20**, **24)** did not show strong inhibitory properties, similarly

to corresponding ligands **(3**, **7).** Moreover, sulfonamide complexes containing Cr(III), Co(II) and Ni(II) metals **(27–29)** possessed no inhibitory activity. On the other hand, Cu(II)-phenyl sulfonylhydrazone complexes **(25**, **26)** exhibited stronger inhibitor activity than parent ligands **(4**, **5)**. In addition, the Zn(II)-thiophenecarboxamide complex **(30)** showed a good CA inhibitory effect in contrast to its ligand **(16)**.

In brief, our results demonstrate that **12, 25,** and **26** inhibited both CA activity and photosynthetic activity of PSII significantly. Cu(II) complex **(25)** at 0.1 mM concentration inhibited the PSII CA-activity to 100% and 33.8% of the photosynthetic activity of PSII. Moreover, Cu(II) complex **(26)** at the same concentration inhibited 60% of the CA activity of PSII and 75.2% of the photosynthetic activity of PSII. On the other hand, Schiff base compound (**12**) at the same concentration inhibited 100% of the CA activity of PSII and 82.4% of the photosynthetic activity of PSII.

3.4. Electrochemical properties of the compounds

Some electrochemical properties (diffusion coefficient, transferred electron number, standard heterogeneous rate constants, peak potentials E_{pc}) of the compounds were determined by cyclic voltammetry, square wave voltammetry, chronoamperometry, and bulk electrolysis (Fig. 5, Table 4). Only cyclic voltammograms of compounds which showed inhibitory effects are given in Fig. 5. These properties were obtained from the first reduction peak in the cyclic voltammograms. p*K*a values of the compounds were determined in both, nonaqueous and aqueous media by cyclic voltammetry (Table 5).

The slopes of $\log i_{pc}$ vs. $\log v$, which are equal to approximately 0.5 for all compounds, indicate that the reductions of the compounds are diffusion-controlled at the glassy carbon electrode surface in DMSO.

For the compounds with inhibitory action, the transferred electron numbers were 2 and the reduction peaks appeared at E_{pc} values of about −0.6 V. (Table 4), p*K*a values were between 5.86 and 8.71 (Table 5).

For some of the compounds, several peaks could not be observed with increasing scan rate. This is due to the fact that the electron transfer rate was slower than the scanning speeds, which means that the reduction does not take place. In some compounds, some of the peaks were not observed at both low and high scanning speeds; as a reason for that, the size of the rate of electron transfer is considered. Some of the lowest and the highest peaks of the few voltammograms scan speeds were observed. Here, the rate of electron transfer with a scan speed of discrepancies between low and high speeds is to be considered. The negative shift of the E_{pc} values indicates that the high scanning speeds are an indication of the irreversibility of the reduction process (Table 5).

As shown from Table 1, compound **25** and **26** showed the best values for photosynthetic inhibition and good inhibition parameters for bovine CA and PSII CA activity. These compounds had also larger diffusion coefficients and heterogeneous rate constants among all compounds tested (Table 4). Compounds of **17** and **15** exhibited good photosynthetic inhibition and also larger diffusion coefficients and heterogeneous rate constants (Tables 1–3 and 4). Although compound **30** showed good CA inhibition, it had bigger diffusion coefficient and heterogeneous rate constant (Tables 1–3 and 4). Compounds of **15**, **17**, **25** and **26** inhibited the photosynthetic activity of PSII significantly.

As shown from Table 3, the solvent DMSO ($pK_{DMSO} = 37$) is differentiating for acidic compounds in large range. Most of the compounds had acidic properties in this solvent (p*K*a; 6–10). Sulfonamides (**9**, **10**) showed basic properties in the aqueous solutions, but they behaved as weak acid in the DMSO. Sulfonamides had basic properties in aqueous solutions however their Cr(III),

Table 4
Transferred electron numbers (n), diffusion coefficients and heterogeneous rate constants of the compounds in nonaqueous media.

Compound	E_{pc}. V (0.01 V/s)	Cottrell slope (S)	Diffusion coefficient (D, cm^2/s) $\times 10^7$	UME Limit current (i, A) $\times 10^{10}$	Transferred electron number (n)	Heterogeneous rate constant ks (cm/s) $\times 10^6$
1	−0.56	3.00 ± 0.12	2.10 ± 0.05	4.06 ± 0.03	1.92 ± 0.01	2.23 ± 0.06
2	−0.56	2.56 ± 0.02	2.05 ± 0.03	3.42 ± 0.02	1.96 ± 0.02	2.17 ± 0.04
3	−0.65	4.19 ± 0.02	3.68 ± 0.05	7.50 ± 0.04	2.02 ± 0.03	3.31 ± 0.03
4	−0.60	7.59 ± 0.03	20.7 ± 0.20	32.20 ± 0.58	1.75 ± 0.02	20.5 ± 0.13
5	−0.65	7.23 ± 0.02	10.6 ± 0.37	22.00 ± 0.43	2.05 ± 0.02	9.69 ± 0.22
6	−0.57	4.50 ± 0.01	4.91 ± 0.08	9.30 ± 0.02	1.88 ± 0.01	6.30 ± 0.05
7	−0.58	9.95 ± 0.02	34.7 ± 0.21	54.70 ± 0.31	1.56 ± 0.01	38.2 ± 0.25
8	−1.53	2.50 ± 0.03	1.43 ± 0.02	2.79 ± 0.03	1.94 ± 0.02	1.63 ± 0.04
9	−0.63	6.14 ± 0.08	0.069 ± 0.004	1.50 ± 0.01	2.17 ± 0.05	0.060 ± 0.005
10	−2.03	4.60 ± 0.03	6.93 ± 0.02	11.30 ± 0.13	1.82 ± 0.01	2.03 ± 0.02
11	−0.55	4.75 ± 0.02	8.34 ± 0.02	12.80 ± 0.21	1.82 ± 0.01	2.49 ± 0.05
12	−0.75	2.28 ± 0.03	0.80 ± 0.001	1.90 ± 0.03	2.36 ± 0.01	0.62 ± 0.003
13	−0.71	1.60 ± 0.04	0.74 ± 0.002	1.28 ± 0.04	1.73 ± 0.02	0.61 ± 0.004
14	−0.58	2.00 ± 0.01	1.24 ± 0.03	2.08 ± 0.01	1.66 ± 0.01	1.36 ± 0.04
15	−0.55	4.50 ± 0.03	22.4 ± 0.25	10.20 ± 0.43	1.72 ± 0.02	12.0 ± 0.35
16	−0.60	2.50 ± 0.03	1.72 ± 0.01	3.06 ± 0.01	1.76 ± 0.01	0.48 ± 0.02
17	−0.64	1.99 ± 0.01	26.8 ± 0.59	1.73 ± 0.09	1.98 ± 0.02	24.9 ± 0.35
18	−0.87(0.05v/s)	8.12 ± 0.03	18.1 ± 0.21	32.20 ± 0.13	1.77 ± 0.01	12.4 ± 0.25
19	−1.53	4.46 ± 0.03	4.27 ± 0.02	8.60 ± 0.02	2.00 ± 0.01	1.66 ± 0.03
20	−0.63	9.94 ± 0.03	29.2 ± 0.16	50.10 ± 0.74	1.70 ± 0.02	27.9 ± 0.33
21	−0.69	3.00 ± 0.08	2.32 ± 0.02	4.26 ± 0.01	1.83 ± 0.01	2.03 ± 0.02
22	−0.57	3.26 ± 0.02	2.77 ± 0.04	5.06 ± 0.03	1.81 ± 0.02	2.89 ± 0.06
23	−0.61	8.98 ± 0.03	1.45 ± 0.01	40.40 ± 0.25	1.72 ± 0.02	2.27 ± 0.02
24	−0.68	3.28 ± 0.02	2.56 ± 0.08	4.90 ± 0.02	1.90 ± 0.02	2.24 ± 0.06
25	−0.60	2.98 ± 0.02	22.8 ± 0.41	42.68 ± 0.25	1.89 ± 0.03	17.0 ± 0.37
26	−0.61	8.65 ± 0.02	26.7 ± 0.34	41.70 ± 0.31	1.85 ± 0.02	26.5 ± 0.26
27	−0.53	2.87 ± 0.02	1.99 ± 0.04	3.78 ± 0.02	1.88 ± 0.04	1.69 ± 0.03
28	−0.57	4.19 ± 0.03	4.36 ± 0.04	8.16 ± 0.02	1.86 ± 0.04	4.54 ± 0.03
29	−0.58	2.72 ± 0.02	2.11 ± 0.02	3.69 ± 0.03	1.73 ± 0.02	2.16 ± 0.03
30	−0.58	8.41 ± 0.02	19.0 ± 0.13	34.20 ± 0.16	1.79 ± 0.01	39.6 ± 0.28

Results are average of three measurements and standard deviation.

Table 5
p*K*a values of the compounds in nonaqueous and aqueous media.

Compound	p*K*a (nonaqueous)	p*K*a (aqueous)	Compound	p*K*a (nonaqueous)	p*K*a (aqueous)	Compound	p*K*a (nonaqueous)	p*K*a (aqueous)
1	6.42	4.48	**11**	7.81	6.40	**21**	6.96	5.27
2	6.48	5.01	**12**	6.05	4.69	**22**	6.95	4.84
3	6.25	4.75	**13**	4.95	3.88	**23**	8.66	6.74
4	8.58	6.72	**14**	8.45	6.12	**24**	7.85	5.84
5	8.51	6.48	**15**	5.86	4.01	**25**	6.63	4.36
6	6.96	5.04	**16**	6.70	4.86	**26**	8.71	6.84
7	6.61	4.88	**17**	7.14	5.45	**27**	8.19	6.04
8	7.67	5.47	**18**	6.88	4.90	**28**	8.48	6.28
9	8.61	7.54	**19**	7.75	5.66	**29**	8.29	6.32
10	10.2	7.55	**20**	6.72	5.33	**30**	6.71	4.71

Co(II) and Ni(II) coordination compounds (**27**–**29**) were weakly acidic. It is possible to say that coordination can cause acidity. This conclusion can be seen in the ligand; **16** and its Zn(II) coordination compound **30**. However, some sulfonylhydrazones **(1**, **3**, **5**–**8)** and their Ni(II) and Cu(II) coordination compounds have adverse properties. In these compounds acidity have been slowly decreased after coordination.

4. Conclusions

Our compounds showed completely different inhibition profiles against both CA activities (α-CA and PSII CA). For example, **12**, **19**, **25**, **26** and **30** suppressed both CA activities, but **5** and **18** inhibited only α-CA activity. In contrast, **15** acted only as CA inhibitor of PSII complexes. These results show that homology between these two CAs is insufficient to display similar activity.

Shitov et al. found that the efficiency of the inhibition of CA and photosynthetic activity is similar, and these authors claimed that the inhibition of photosynthetic activity and suppression of CA-activity may be based on the same mechanism of action [31]. However, our inhibitors such as **12, 25** and **30** affected CA and photosynthetic activities differently. For that reason, we can propose that these two types of inhibition may be based on different mechanisms.

Again, the 2-hydroxynaphthyl Schiff base compound **12** bearing 1,2-dimethylcyclohexyl bridge ($\log P = 5.21$ ($\log P$ is logarithm of the distribution coefficient in the system octanol/water)) inhibited the CA activity of PSII completely, and suppressed the photosynthetic activity of PS II moderately. However, compound **13** bearing 1,2-dimethylcyclohexyl bridge ($\log P = 7.14$) had no considerable inhibitory activity probably due to increasing lipophilic properties of **13**. This suggests that there are strong interactions between the more lipophilic compound **13** and the protein molecules. On the other hand, the p*K*a value of compound **12** (p*K*a = 6.05) ensures that the compound will be completely anionic under pH neutral or basic conditions (pH $\leqslant$ 7). Thus, the anionic nature of the compound is helpful for the interaction with the active site of CA (Table 5).

Our inhibitor analysis results using PSII exogenous electron donors demonstrate that along with suppression of CA activity of PSII, compound **12** and **25** also inhibit photosynthetic activity of the PSII donor side. This result indicates the significance of CA activity for the functioning of the PSII donor side in higher plants and also supports the previous finding [31].

To make a prediction, Mulliken net atomic charges on Cu, HOMO and LUMO energy levels and electrophilicity (ω) of compounds **25** and **26** were calculated using Gaussian 03 software at the B3LYP/6–31G(d,p) level. Mulliken net atomic charges on Cu of **25** and **26** were calculated as +0.821 and +0.844, respectively. Thus, the higher inhibitor efficiency of **26** may result from the relatively high positive atomic charge on Cu. In addition, LUMO energy levels and electrophilicity of the compounds can provide useful information regarding the donor–acceptor interactions. For example, the LUMO energy levels (−2.08 eV and −2.09 eV) and electrophilicities (2.48 and 2.54) of **25** and **26**, showed that the compound with low LUMO energy and high electrophilicity exhibits better activity. In other words, electron transfer from HOMO in the receptor to LUMO in compound **26** is relatively easier than in the case of **25**.

Compounds of **25**, **26**, **17**, **15** exhibit good photosynthetic inhibition effects and larger diffusion coefficients as well as heterogeneous rate constants. Higher diffusion coefficients and larger rate constants mean that compound transport to the electrode and electron transfer are fast, and therefore the reduction process is rapid. However, compounds **7** and **20** have also higher diffusion coefficients and larger rate constants, but these compounds are inefficient in terms of photosynthetic inhibition. This relationship will be investigated in the following researches.

Thus, novel chemical agents were revealed. Screening of these compounds for their effects on CAA of α-CA, β-CA, CA- and photosynthetic activity of PSII was made. Some of them are capable to suppress either one, two, three, or all of the checked activities. Availability in the studied group of novel chemical agents compounds possessing different inhibitory activity allow to isolate the "active part" in the structure of the inhibitor responsible for different inhibitory mechanisms, as well as to determine the influence of side substituters on its inhibitory efficiency. The obtained data allow hoping that in perspective some of the novel chemical compounds may be used as plant growth inhibitors as well as precursors of drugs in veterinary and human medicine.

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