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ARTICLE *in* THE JOURNAL OF PHYSICAL CHEMISTRY A · JANUARY 2001

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Direct Characterization of Radical Species Generated on One-Electron Oxidation of 3,6-Diamino-10-methylacridan

A. Marcinek, J. Zielonka, J. Adamus, and J. Gębicki*

Institute of Applied Radiation Chemistry, Technical University, 90-924 Lodz, Poland

Matthew S. Platz

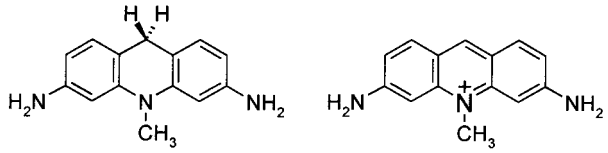
Newman and Wolfrom Laboratory of Chemistry, The Ohio State University, Columbus, Ohio 43210

Received: July 22, 2000; In Final Form: November 2, 2000

Sequential electron−proton−electron transfer processes in the oxidation of 3,6-diamino-10-methylacridan, an uncharged precursor of acriflavine, were studied. Transient products, i.e., radical cations and radicals, were spectroscopically characterized by pulse radiolysis and laser flash photolysis.

1. Introduction

A recent report from our laboratory has revealed that 3,6-diamino-10-methylacridan (**1H**) can be prepared in chemically pure form and oxidized spontaneously by molecular oxygen in water to acriflavine (**1**$^+$).[1] As the oxidation of **1H** to **1**$^+$ was found to be quantitative, therefore **1H** can be regarded as an uncharged precursor of **1**$^+$.



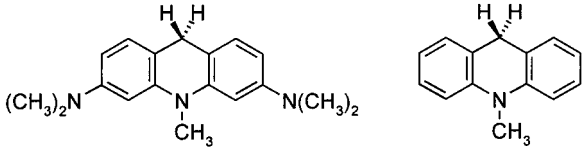
1H **1**$^+$

On the other hand, **1**$^+$ represents a group of cationic DNA intercalators that has outstanding antibacterial properties.[2] It is assumed that **1**$^+$ binds rapidly and saturates the surface of cellular membranes, which are negatively charged due to the presence of phosphate anions in the outer leaf.[3] Therefore, only a very small fraction of **1**$^+$ molecules can penetrate inside bacterial cells to interact with the genetic material. To overcome transportation barriers through the plasma membranes a "Trojan horse" strategy can be envisaged. This strategy may involve application of an uncharged, hydrophobic precursor, which can release **1**$^+$ inside bacterial cells. The above requirement for precursor **1**$^+$ seems to be fulfilled by **1H**. Indeed, the antimicrobial properties of **1H** were found to be superior to those of **1**$^+$**,** which seems to be associated with preferential diffusion of **1H** through the plasma membranes as well as with synergic antimicrobial effects of **1**$^+$ and reactive forms of oxygen.[4]

In view of the above discussion it seems important to establish the mechanism of **1H** oxidation and to characterize any reactive intermediates involved in this process. This paper describes our efforts aimed at direct characterization of the radical cation (**1H**$^{\bullet+}$) and the radical (**1**$^\bullet$). To support the proposed assignments, analogues of **1H**, namely 3,6-bis(dimethylamino)-10-methylacridan (**2H**) and 10-methylacridan (**3H**), were studied.

2. Results and Discussion

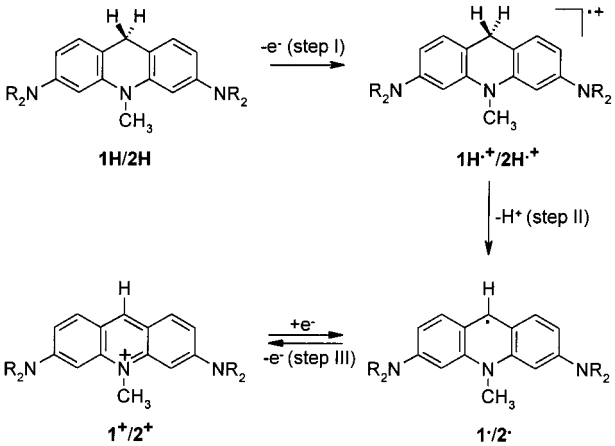
Based on literature concerning the oxidation of related systems by molecular oxygen, it is assumed that the oxidation



2H **3H**

of **1H** to **1**$^+$ follows the stepwise mechanism of electron−proton−electron transfer as shown in Scheme 1. In this mechanism two intermediates, radical cation and radical, play a key role and will be spectroscopically characterized.

SCHEME 1



Step I: Radical Cations. An excellent method of stabilization and characterization of radical cations is their generation by ionizing radiation in low-temperature matrices.[5−9] The inhibitory effect of the low-temperature matrices on recombination and fragmentation processes allows direct observation of radical cations.

It seems important to compare the spectra of radical cations generated from amino-substituted compounds with the spectrum generated from the unsubstituted molecule. It has been shown that photolysis of 10-methylacridan (**3H**) in acetonitrile results in the formation of radical cation. This radical cation has been characterized in the previous studies by its long-wavelength

10.1021/jp002606f CCC: $20.00 © 2001 American Chemical Society
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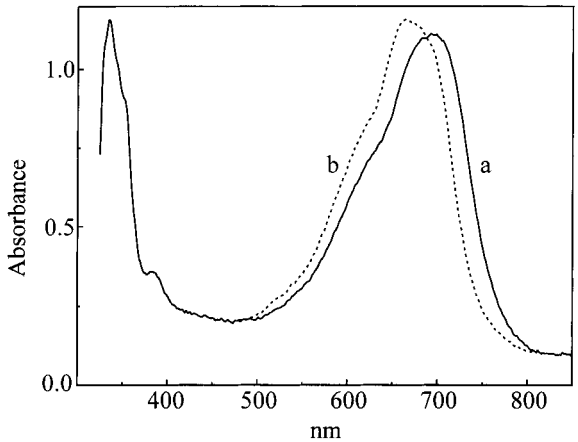
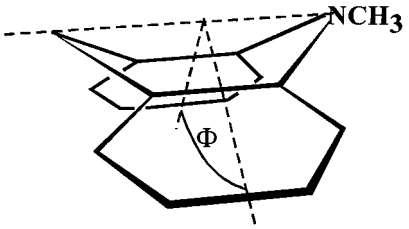


Figure 1. Absorption spectrum of irradiated 10-methylacridan (**3H**) (0.02 M) in a glassy MCH/n-BuCl matrix at 77 K (a). The spectrum (b) obtained after the sample was warmed to 85 K (10 min) and cooled back to 77 K. The sample was 2 mm thick and received a radiation dose of 7×10^3 Gy.

absorption with maximum at about 640 nm.[10−13] The spectrum of an irradiated matrix containing 10-methylacridan, presented in Figure 1, reveals some additional features. First, the maximum of long-wavelength absorption is red shifted compared to that observed in acetonitrile at ambient temperatures. Second, two additional absorption bands around 375 and 335 nm can be assigned to the radical cation $\mathbf{3H}^{\bullet+}$ on the basis of their behavior upon bleaching of the long-wavelength absorption and annealing of the sample. A similar sequence of three absorption bands was also observed for the radical cations of 10-methylacridine dimer, thioxanthene, and xanthene.[8−9]

Some changes have been monitored in this spectrum as a result of softening of the sample due to gentle warming. Especially interesting is a significant blue shift of a maximum of the long-wavelength band by over 50 nm, and this band was finally shifted to the position observed in solution.



10-Methylacridan and its derivatives are not planar molecules[14] and, according to calculations, may undergo a substantial geometry change after ionization. The B3LYP/6-31G* method predicts that the dihedral angle Φ between two aromatic side rings is 148.11° (compared to 142.7° from HF SCF calculations[14]) and 149.21° in neutral **3H** and **1H**, respectively.[15,16] The geometry changes on ionization and the resulting radical cations are more planar than their neutral parent compounds: $\Phi = 161.96°$ ($\mathbf{3H}^{\bullet+}$) and 165.29° ($\mathbf{1H}^{\bullet+}$). A complete flattening of the molecule on a route from the reduced to oxidized forms is reached only at the stage of radical formation.[17] It is very likely that the observed shift in the radical cation spectrum was due to the initial flattening of the molecule. In the rigid environment, the radical cation initially formed should retain the structure of the parent neutral precursor. Upon softening of the surrounding matrix, the radical cation may undergo relaxation to a thermodynamically more stable geometry. The calculated energy for the relaxation of $\mathbf{1H}^{\bullet+}$ and $\mathbf{3H}^{\bullet+}$ from the neutral geometry is only 5.8 and 3.4 kcal/mol, respectively.[15] This means that the potential energy surface for the relaxation process is flat and therefore the process can be significantly inhibited by a rigid matrix environment. Similar effects were observed for radical cations generated from thioxanthene and xanthene in glassy matrices.[9]

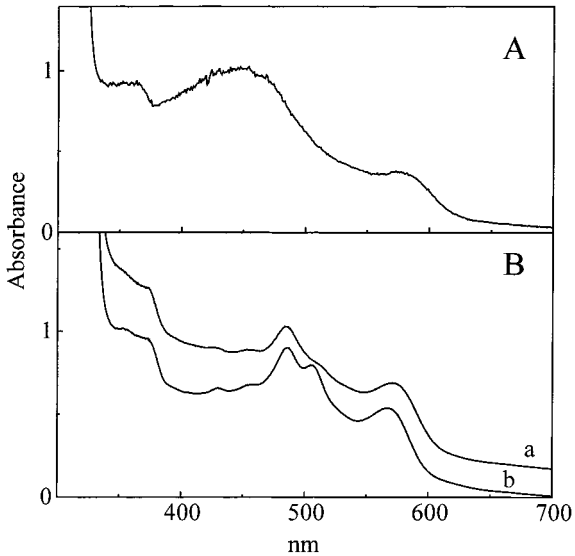


Figure 2. Electronic absorption spectra obtained after irradiation of (A) **1H** (0.02 M) in 2-chlorobutane at 77 K and (B) **2H** (saturated solution) in a glassy matrix MCH/n-BuCl at 77 K. The samples were 2 mm thick and received radiation doses of 8×10^3 Gy and 7.5×10^3 Gy, respectively. The spectrum (B−b) obtained after the sample was warmed to 85 K (10 min) and cooled back to 77 K.

An electronic absorption spectrum recorded as a result of irradiation of **1H** in a 2-chlorobutane matrix is presented in Figure 2A. The most distinct features are the absorption bands observed at 365 and 580 nm, which are accompanied by a broad absorption around 450 nm. The 580 nm band underwent a slight blue shift (finally to 570 nm) and narrowing after thermal annealing of the matrix to 85 K.

3,6-Bis(dimethylamino)-10-methylacridan (**2H**), contrary to **1H**, is sufficiently soluble in methylcyclohexane/1-chlorobutane (1 M) mixture (MCH/n-BuCl) to allow studies in this matrix. On freezing this sample forms glasses of better quality than does 2-chlorobutane. The electronic absorption spectrum of irradiated **2H** in MCH/n-BuCl matrix at 77 K is presented in Figure 2B (spectrum a). This spectrum consists of a few distinct features: a 575 nm absorption band, a group of bands at 420−500 nm, and a broad band at 375 nm. Upon slight warming of the matrix the 575 nm band is shifted to shorter wavelength and is narrowed. The observed shift, finally to 565 nm was irreversible, and cooling the sample back to 77 K stabilized the species represented by the blue shifted absorption.

Similar absorption spectra were obtained on laser flash photolysis (LFP) of **1H** and **2H** in acetonitrile solution at room temperature (Figure 3).

On the basis of the bleaching and matrix annealing experiments, the long-wavelength bands in the spectra (around 570 nm) can be assigned to the radical cations of $\mathbf{1H}^{\bullet+}$ and $\mathbf{2H}^{\bullet+}$. Moreover, the parallel behavior of the bands located around 370 and 570 nm in matrix experiments and the same lifetime (1.75 μs in acetonitrile) observed for both transitions in LFP experiments support the assignment of the absorption bands around 370 nm also to the radical cations.

The long-wavelength absorption bands of radical cations of both substituted acridans are significantly blue shifted compared

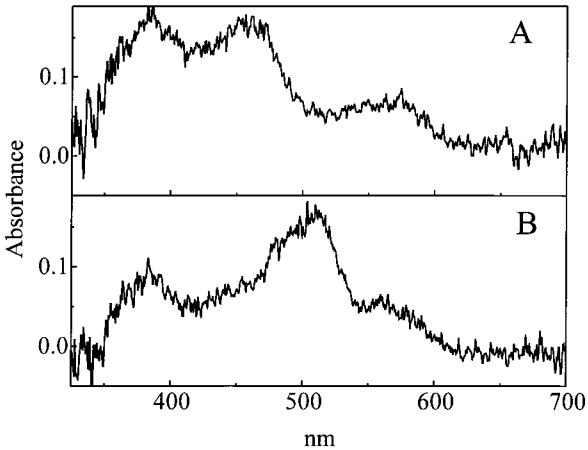
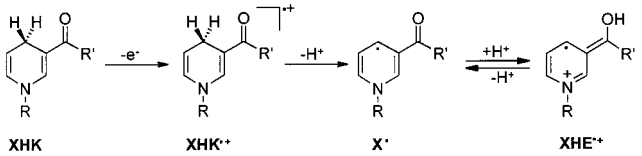


Figure 3. Transient absorption spectra measured 200 ns after 308 nm laser excitation of (A) **1H** and (B) **2H** in aerated acetonitrile at room temperature.

SCHEME 2



to the absorption of the radical cation of parent 10-methylacridan. This may indicate that both amino groups strongly participate in the delocalization of the "positive hole" in these radical cations. Localization of the positive charge completely on one amino group can be excluded because the radical cations of aniline and N,N-dimethylaniline absorb at 400−450 nm.

It is worth mentioning that only the method of direct ionization of the stable reduced compounds **1H** and **2H** unambiguously enables spectroscopic characterization of the primary radical cations $\mathbf{1H}^{\bullet+}$ and $\mathbf{2H}^{\bullet+}$. Radical cations can also be formed upon reduction of the appropriate cations in an acidic environment or by direct reaction of hydrogen atoms with the closed shell cations. In the case of $\mathbf{1}^+$ and $\mathbf{2}^+$**,** this leads to the proton/hydrogen atom attachment to the most electronegative site in the radical/cation, i.e., to the one of two amino groups and therefore to a different type of radical cation.

By analogy, with our previous studies of NADH analogues, we have found that the reduction of NAD^+ model compounds in acidic media leads to the radical cations in the enol form ($\mathbf{XHE}^{\bullet+}$, see Scheme 2), instead of keto radical cations $\mathbf{XHK}^{\bullet+}$, that are formed on direct ionization of NADH compounds.[18]

Step II: Radicals. Contrary to the absorption bands at 370 and 570 nm, the group of bands seen in Figure 2 in the region 400−500 nm cannot be assigned to radical cations. The observation of their further growth upon matrix relaxation while the radical cation absorption was decreasing indicates that they may belong to the products of secondary reactions involving radical cations. Although the radical cations are stable in glassy matrices at 77 K, the presence of the products of their decomposition at this temperature can be explained as a result of the excess energy released on ionization.[7]

In the spectrum collected after irradiation of 10-methylacridan at 77 K, the presence of a small amount of the radical $\mathbf{3}^\bullet$ could be distinguished directly by the absorption band of this radical at 355 nm.[8] The initial concentration of radical was estimated as less than 5×10^{-5} M, i.e., less than 5% of radical cation concentration. The radical absorption initially grew upon

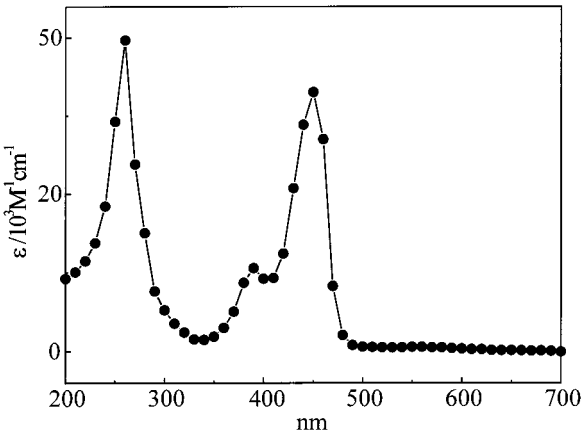


Figure 4. Electronic absorption spectrum of $\mathbf{1}^\bullet$ obtained on reduction of $\mathbf{1}^+$ (10^{-4} M) by pulse radiolysis in N_2O saturated aqueous solution (pH = 7) containing 2-propanol (1 M). The spectrum was measured 20 μs after the electron pulse and corrected for bleaching of $\mathbf{1}^+$.

annealing of the matrix accompanied, in part, by its subsequent fast decay to the final product of 10-methylacridinium cation $\mathbf{3}^+$.

In the case of amino acridans, it is not immediately obvious, however, whether the absorption in the region between 400 and 500 nm belongs to the radicals or to closed-shell cations. The cations absorb very strongly in this spectral region, and their presence might mask that of any other products formed. Generation of the radicals upon reduction of the appropriate salts in frozen alcoholic matrices indicated that their absorption might be expected in the same spectral region as that of the corresponding cations. Strong overlap of these bands, however, disabled their characterization under these conditions, because quite high concentration of salts had to be used. This can be avoided in experiments utilizing the reduction of salts dissolved in water either by direct reduction on reaction with electrons, or with isopropyl ketyl radicals $(CH_3)_2C^\bullet OH$ (see Experimental Section). The spectrum observed 20 μs after electron pulse radiolysis of $\mathbf{1}^+$ is characterized by a strong absorption at 380−390 nm and bleaching of the cation absorption in the region between 400 and 500 nm. The spectrum corrected for the bleaching presented in Figure 4 reveals three absorption bands at 260, 390, and 440 nm that can be assigned to the radical. Similar spectra were obtained in the case of irradiated $\mathbf{2}^+$ (see Table 1). The lifetimes of these radicals (pseudo first-order kinetics of a decay) in aqueous solutions depend on the initial concentration of the parent cation. However, the dimer radical cations initially formed must further disproportionate, based on 50% recovery of the cations reduced in pulse radiolysis experiments. These observations are in agreement with assignment obtained for semireduced acridine orange.[19−23]

These spectral features of the radicals indicate that the absorption bands observed between 400 and 500 nm in the initial spectrum detected under the matrix conditions (Figure 2) can be assigned to the radicals of interest formed upon deprotonation of the radical cations. Longer maintenance of the matrix at higher temperatures led to a decay of the radical cations bands accompanied by slight initial growth of the radicals bands. Under these conditions further reactions of radicals could also be seen.

Step III: Oxidation of Radicals. Prolonged thermal relaxation of the matrix led to the decay of the radical cations and radicals, and the only products observed after extensive thermal relaxation above 95 K were the closed-shell cations. In the case of MCH/n-BuCl matrix containing irradiated **2H**, the formation of the closed-shell cation $\mathbf{2}^+$, with absorption maximum at 505

TABLE 1: Spectroscopic Characterization of the Transient Species Detected

	low-temp matrices (77 K)			solution	
transient species	**1H** λ_{max} [nm]	**2H** λ_{max} [nm]	**3H** λ_{max} [nm]	**1H** λ_{max} [nm]	**2H** λ_{max} [nm]
radical cations	365, 570[a]	375, 565[b]	335, 375, 640[b]	380, 570[c]	385, 565[c]
radicals	~450[a]	430, 455, 485[b]	355, 480, 520[b,c]	260, 390, 440[d]	270, 410, 480[d]

[a] 2-Chlorobutane glass. [b] MCH/n-BuCl glass. [c] LFP in acetonitrile. [d] Pulse radiolysis in aqueous solution.

nm in this matrix, is already observed after initial thermal relaxation of the matrix (see spectrum b in Figure 2B). The solubility of $\mathbf{2}^+$ in this solution is rather poor, and most of the ionic products precipitated from the solution. Thus, the absorption growth at 505 nm on prolonged annealing was limited. However, formation of the monomer $\mathbf{2}^+$ was clearly followed by its dimerization, which was identified on the basis of a characteristic difference in the spectra of dimer dication (two close bands at 490 and 505 nm) and monomer cation.[24,25]

Superposition of the absorptions due to the radical and closed-shell cation are also responsible for the strong, broad band between 400 and 500 nm seen in the spectrum shown in Figure 2A and in the spectra obtained in the laser flash photolysis experiments (see Figure 3).

3. Conclusions

Transient radical cations and radicals observed during sequential oxidation of **1H** and **2H**, embedded in cryogenic matrices, have been stabilized and spectroscopically characterized. Spectroscopic features of the identified species are summarized in Table 1.

Conformational relaxation of the radical cations in rigid matrices, induced by thermal softening of the matrix, led to flattening of the intermediate radical cations. This was associated with a blue shift of their long-wavelength electronic transitions. It is interesting to note that flattening of the acridans during oxidation begins immediately upon ionization. The removal of one electron produces the radical cations which further deprotonate to give the radicals. Oxidation of the radicals, in turn, results in formation of the adequate closed shell cations.

For all of the compounds studied a quantitative oxidation can be achieved upon photolysis in aerated polar solvents. Laser flash photolysis experiments revealed participation of the radical cations in these processes.

4. Experimental Section

Compounds. 3,6-Diamino-10-methylacridan (**1H**) was prepared by sodium borohydride reduction of 3,6-diamino-10-methylacridinium chloride, which was isolated from acriflavine neutral (Aldrich) following a published procedure.[26] The reduction product was purified by crystallization from $CHCl_3$ to give light-yellow needles (30%, mp. 127 °C, dec).

3,6-Bis(dimethylamino)-10-methylacridan (**2H**) was prepared by sodium borohydride reduction of 3,6-bis(dimethylamino)-10-methylacridinium methyl sulfate, which was in turn obtained by reaction of acridine orange (Aldrich) with dimethyl sulfate (Aldrich) in toluene solution. The reduction product was crystallized from $CHCl_3$ to give orange needles (45%, mp. 103 °C, dec).

10-Methylacridan (**3H**) was prepared by sodium borohydride reduction of 10-methylacridinium chloride according to a known procedure and recrystallized three times from 96% EtOH. 10-Methylacridinium chloride was prepared from acridine (Fluka, purum) via 10-methylacridinium methyl sulfate.[11]

Low-Temperature Radiolysis. The steady-state measurements of 2-chlorobutane glassy samples containing **1H** (saturated solution) were performed in a temperature controlled nitrogen-cooled cryostat (Oxford Instruments). The optical spectra were measured with a Cary 5 (Varian) spectrophotometer. Irradiation of the samples (a few pulses of 600 Gy each) was made with the ELU-6 linear electron accelerator. A detailed description of the pulse radiolysis system is given elsewhere.[27,28]

Pulse Radiolysis. Reduction of $\mathbf{1}^+$ was achieved by reaction with 2-propanol ketyl radicals generated in aqueous solutions of 2-propanol (1 M) saturated with N_2O. This method enables a quantitative conversion of the major products of water radiolysis (i.e., e_{aq} (2.6), $^{\bullet}OH$ (2.7), $H^{\bullet}$ (0.6) − numbers in parentheses are the *G* values, i.e., yields of radicals per 100 eV of energy absorbed)[29] to a reducing agent, namely 2-propanol ketyl radical, over a wide pH range.

$$e_{aq} + N_2O \rightarrow {}^{\bullet}OH + OH^- + N_2 \quad (1)$$

$$^{\bullet}OH + (CH_3)_2CHOH \rightarrow (CH_3)_2C^{\bullet}OH + H_2O \quad (2)$$

$$H^{\bullet} + (CH_3)_2CHOH \rightarrow (CH_3)_2C^{\bullet}OH + H_2 \quad (3)$$

α-Hydroxy alkyl radicals formed in reactions 2 and 3 with the 85% yield (remaining 15% are unreactive *β*-hydroxy radicals) are strongly reducing species that react with many compounds via one-electron transfer. The pulse radiolysis measurements in solution were made on system equipped with the ELU-6 linear accelerator (17ns pulses delivering a dose of 10−50 Gy).[27]

Laser Flash Photolysis. The laser flash photolysis system employed a Lambda Physik EMG 101 excimer laser utilizing XeCl for 308 nm emitting line with a pulse energy of 150 mJ and a pulse width of ca. 20 ns. The concentrations of the 1-cm thick samples were kept to have absorbance of 0.3−0.5 at 308 nm. An EG&G Princeton Applied Research model 1460 optical multichannel analyzer (OMA) recorded transient absorption spectra. The laser flash photolysis apparatus is described in more detail elsewhere.[30]

Acknowledgment. This work was supported by the State Committee for Scientific Research (grant no. 3/*T*09A/037/17), the M. Skłodowska-Curie Joint Fund (grant no. MEN/NSF-97-303), and the Lodz City Research Council. J.G. thanks Foundation on behalf of Polish Science for support from the program "Subsidies for Scholars". We thank also Professor Thomas Bally and Dr. Paweł Bednarek for calculation of the dihedral angles.

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