3,6-Diamino-10-methylacridan: Uncharged Precursor of Acriflavine and Its Unique Antimicrobial Activity

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The vast majority of clinically important antibiotics and chemotherapeutic agents show some hydrophobicity, which allows them to diffuse across the lipid bilayer of the plasma membranes.[1] The plasma membranes are less permeable to compounds, which exist in charged forms. However, acriflavine (**Acr**$^+$) representing a group of cationic DNA intercalators plays an important role due to its outstanding antibacterial properties.[2] It is assumed that **Acr**$^+$ binds rapidly to saturate the surface of the membrane, which is negatively charged due to the presence of phosphate anions in the outer leaf.[3] Therefore only very small fractions of acriflavine molecules can penetrate inside bacterial cells to interact with the genetic material.

To overcome transportation barriers through the plasma membranes a strategy of "Trojan horse" can be envisaged. This strategy may involve application of an uncharged, hydrophobic **Acr**$^+$ precursor, which can release **Acr**$^+$ inside bacterial cells.

This communication presents our successful approach to the preparation of a stable acriflavine precursor of unique chemical properties, namely 3,6-diamino-10-methylacridan (**AcrH**). It is shown that this precursor, which can be regarded as a reduced form of **Acr**$^+$, can be quantitatively reoxidized by oxygen in polar solvents and it can also serve as a thermal source of the superoxide radical anion ($O_2^{\bullet-}$). Thus, the parallel formation of both **Acr**$^+$ and $O_2^{\bullet-}$ (being a precursor of other reactive forms of oxygen) from **AcrH** may be of practical importance due to the fact that both **Acr**$^+$ and reactive forms of oxygen possess antimicrobial properties and in principle, at least in part, these species can be formed inside the cell attacking directly cell organelles.

The reduced (leuco) form (**AcrH**) of **Acr**$^+$ can be generated by light under reductive conditions;[4,5] however the leuco product has not been recovered from the solution due to easy reoxidation by the dissolved oxygen. Also the leuco forms are considered as intermediates in the synthesis of acridine dyes.[6] Our efforts to prepare **AcrH** in the chemically pure form turned out to be successful, and to our surprise **AcrH** is stable if handled properly.

3,6-Diamino-10-methylacridan (**AcrH**) was prepared by $NaBH_4$ reduction of 3,6-diamino-10-methylacridinium chloride which was isolated from Acriflavine neutral (Aldrich) by the method of Gilliot.[7] **AcrH** was purified by standard methods to give a light-orange crystalline solid which melts and decomposes at about 127 °C. Structure of the **AcrH** molecule was confirmed by ^{1}H NMR (Bruker 250 MHz, $CDCl_3$): δ ppm 3.26 (s, 3H, NCH_3), 3.56 (bs, 4H, NH_2), 3.69 (s, 2H, CH_2), 6.20 (s, 2H), 6.26 (d, 2H, $J = 7.5$ Hz), 6.90 (d, 2H, 7.4 Hz). Anal. ($C_{14}H_{15}N_3$) C: calcd, 74.64; found, 73.59; H, N. The absorption spectrum of **AcrH** in methanol shows a maximum at 305 nm (ϵ_{max} 10 000).

AcrH while stored under argon at 5 °C is practically stable. It decomposed to a degree of about 2% over 9 months. In the presence of air at room temperature a graduate oxidation of **AcrH** was noticed (with the rate about 1−2% per week). At the same conditions negligible decomposition of **AcrH** was observed when stored as a salt with inorganic or organic acids.

It is shown that upon dissolving of **AcrH** in aerated water or other polar solvent spontaneous reoxidation takes place and **Acr**$^+$ is reformed quantitatively (Figure 1). This process is understandable in terms of relatively low oxidation potential of **AcrH** which was determined by cyclic voltammetry to be 0.52 V vs SCE in acetonitrile. Reoxidation takes place during several hours in H_2O, and it can be much longer in less polar solvents or in solvents of poor solubility of oxygen. 3,6-Diamino-10-methylacridan-9-one (**AcrO**, λ_{max} 364 nm, ϵ_{max} 27 000; prepared according to the known procedure[8]) could be also expected as oxidation product; however only traces of this compound could be detected by a more sensitive, in this case, fluorescence technique. Carefully deaerated solutions of **AcrH** are quite stable, however a prolonged storage in solution leads to a graduate decomposition of **AcrH**. The rate of the reoxidation process can be substantially increased by light, both by exciting the reduced form in the UV range (300 nm) or the oxidized form in the visible (450 nm).

The mechanism of **AcrH** reoxidation to **Acr**$^+$ involves a sequential electron−proton−electron transfer (Scheme 1).[9,10] We have been able to characterize directly the adequate radical cation (**AcrH**$^{\bullet+}$) and radical (**Acr**$^\bullet$) by laser flash photolysis and pulse radiolysis.[11]

Thus, the observations mentioned above can be summarized in Scheme 2. A quantitative formation of **Acr**$^+$ and $O_2^{\bullet-}$ upon thermal or photochemical reoxidation of **AcrH** seems to be of interest and maybe of practical use if one compares the system presented here with other thermal sources of superoxide radical anions invented recently.[12]

To test the antimicrobial properties of **AcrH** vs **Acr**$^+$ the comparative studies were undertaken for representative species of bacteria, fungi, and viruses.[13] Due to limited stability of **AcrH** in the presence of Mueller−Hinton, bouillon and Sabouroud media the studied species of bacteria and fungi were exposed to **AcrH** in physiologic solution of sodium chloride. Microbial strains were purchased from National Institute of Hygiene. As expected it was found in the time-dependent studies (0.5−24 h) that **Acr**$^+$ and $O_2^{\bullet-}$ generated from **AcrH** inactivate Gram-negative bacteria *(Escherichia coli, Proteus vulgaris,* and *Pseudomonas aeruginosa*) about 5 times faster and Gram-positive (*Staphylococcus au-*

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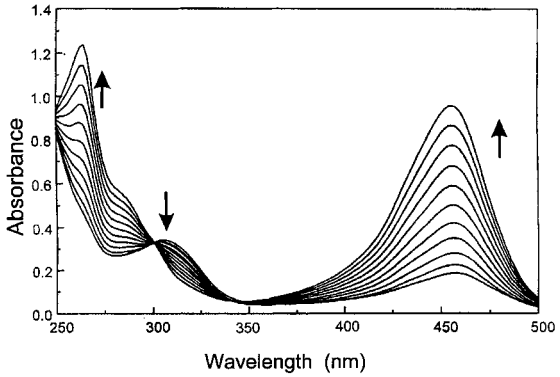
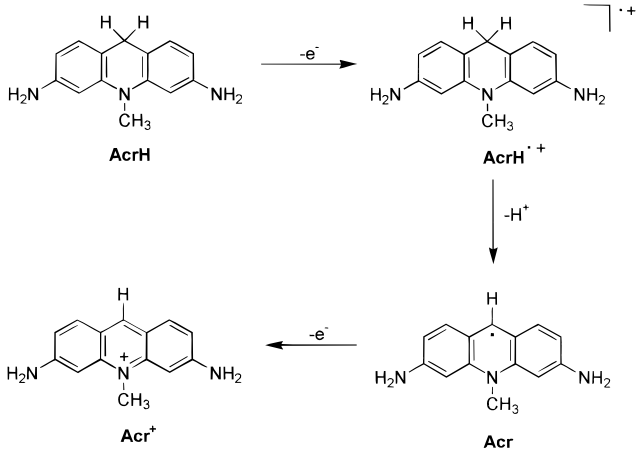
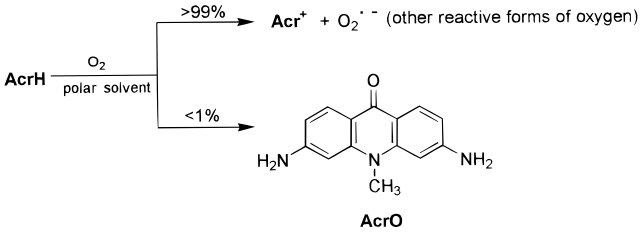


Figure 1. Changes in absorption of **AcrH** (λ_{max} = 302 nm) and **Acr**$^+$ (λ_{max} = 263 and 454 nm) in air-saturated water−acetonitrile mixture (9:1, v/v) detected every hour from dissolving of **AcrH** (concentration of **AcrH**, 3.4×10^{-5} M).

Scheme 1



Scheme 2



reus) over 15 times faster than **Acr**$^+$ in reference solution of similar concentration. For model species of fungi similar comparisons indicated that **Acr**$^+$ and $O_2^{\bullet-}$ generated from **AcrH** inactivate *Candida albicans* about 60 and *Trychophyton mentagrophytes* over 300 times faster than **Acr**$^+$ in reference solution.

AcrH carries also some potential as an antiviral agent where performance of **Acr**$^+$ against enveloped viruses is poor. For example, **AcrH** at concentrations of 0.01% and 0.1% reduces a titer of Herpes simplex type-1 virus after 2 h of incubation by 1.6 and 3.7 in log $TCID_{50}$ units, respectively. In addition it was found that **AcrH** at 0.1% concentration can degrade the surface antigen (HBsAg) of Hepatitis B virus in dilute serum after 2 h of incubation.

There is no doubt based on these preliminary studies that the antimicrobial properties of **AcrH** are superior to those of **Acr**$^+$. In contrast to **Acr**$^+$, **AcrH** is not planar (AM1 calculated[14] dihedral angle between the peripheral rings is 156.7°) and it seems that **AcrH** cannot be considered as an efficient DNA intercalator. So, we assume that **AcrH** itself has no pronounced antimicrobial activity, and the differences observed may result from preferential diffusion of **AcrH** through the plasma membranes as well as from synergic antimicrobial effects of **Acr**$^+$ and reactive forms of oxygen.

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Supporting Information Available: Details on the preparation of **AcrH** and its salt, decomposition of **AcrH** during the storage, electronic absorption spectra of **AcrH** and **Acr**$^+$, characterization of **AcrH**$^{\bullet+}$ and **Acr**$^{\bullet}$, and details on antibacterial and antifungal tests (9 pages). Ordering information is given on any current masthead page.

References

(1) Nikaido, H. Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* **1994**, *264*, 382−388.
(2) Dean, A. C. R. The Antibacterial Action of Acridines. In *Acridines*, 2nd ed.; Acheson, R. M., Ed.; Interscience Publishers: New York, 1973; pp 789−813.
(3) Nakamura, H. Biological action of acridine compounds. VII. What is action point of acriflavine. *Mem. Konan Univ. Sci. Ser.* **1995**, *42*, 93−104.
(4) Oster, G. Fluorescence quenching by nucleic acids. *Trans. Faraday Soc.* **1951**, *47*, 660−666.
(5) Oster, G.; Millich, F. Photoreduction of acridine dyes. *J. Am. Chem. Soc.* **1959**, *81*, 1357−1363.
(6) Tilak, B. D., Ayyangar, N. R. Acridine Dyes. In *Acridines*, 2nd ed.; Acheson, R. M., Ed.; Interscience Publishers: New York, 1973; pp 579−613.
(7) Gilliot, P. Composition and solubility of derivatives of 3,6-diaminoacridine used in therapy. *Bull. Soc. Chim. Fr.* **1934**, *1*, 796−806.
(8) Ehrlich, P.; Benda, L. Über die Einvirkung von Cyankalium auf Pyronin- und Acridinium-Farbstoffe. *Ber.* **1913**, *46*, 1931−1951.
(9) Marcinek, A.; Rogowski, J.; Adamus, J.; Gębicki, J.; Platz, M. S. Sequential electron-proton-electron transfer in the radiolytic and photochemical oxidation of thioxanthene and xanthene. *J. Phys. Chem.* **1996**, *100*, 13539−13543.
(10) Gębicki, J.; Marcinek, A.; Adamus, J.; Paneth, P.; Rogowski, J. Structural aspects and rearrangements of radical cations generated from NADH analogues. *J. Am. Chem. Soc.* **1996**, *118*, 691−692.
(11) Adamus, J.; Kiszka, M.; Marcinek, A.; Gębicki, J. Unpublished results.
(12) Ingold, K. U.; Paul, T.; Young, M. J.; Doiron, L. Invention of the first azo compound to serve as a superoxide thermal source under physiological conditions: concept, synthesis and chemical properties. *J. Am. Chem. Soc.* **1997**, *119*, 12364−12365.
(13) Ciebiada, I.; Korczak, E.; Denys, A. Unpublished results.
(14) HyperChem 5.01 for Windows.

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