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C-Lysine Conjugates: pH-Controlled Light-Activated Reagents for Efficient Double-Stranded DNA Cleavage with Implications for Cancer Therapy

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Abstract: Double-stranded DNA cleavage of light-activated lysine conjugates is strongly enhanced at the slightly acidic pH (<7) suitable for selective targeting of cancer cells. This enhancement stems from the presence of two amino groups of different basicities. The first amino group plays an auxiliary role by enhancing solubility and affinity to DNA, whereas the second amino group, which is positioned next to the light-activated DNA cleaver, undergoes protonation at the desired pH threshold. This protonation results in two synergetic effects which account for the increased DNA-cleaving ability at the lower pH. First, lysine conjugates show tighter binding to DNA at the lower pH, which is consistent with the anticipated higher degree of interaction between two positively charged ammonium groups with the negatively charged phosphate backbone of DNA. Second, the unproductive pathway which quenches the excited state of the photocleaver through intramolecular electron transfer is eliminated once the donor amino group next to the chromophore is protonated. Experiments in the presence of traps for diffusing radicals show that reactive oxygen species do not contribute significantly to the mechanism of DNA cleavage at the lower pH, which is indicative of tighter binding to DNA under these conditions. This feature is valuable not only because many solid tumors are hypoxic but also because cleavage which does not depend on diffusing species is more localized and efficient. Sequence-selectivity experiments suggest combination of PET and base alkylation as the chemical basis for the observed DNA damage. The utility of these molecules for phototherapy of cancer is confirmed by the drastic increase in toxicity of five conjugates against cancer cell lines upon photoactivation.

Introduction

Control of chemical reactions becomes especially challenging when chemical processes have to work in the complexity of biological environments. This is one of the reasons why the ability to design molecules with structure, reactivity, and biological activity "switchable" via an externally controlled factor continues to draw significant attention, from both the practical and fundamental points of view. Possible applications of such molecules include design of molecular machines and switches, logic gate mimics, optical sensors, drug delivery systems, etc. Since pH-dependent "switchable" molecules are of particular use for processes that occur in biochemical systems and in the environment, interesting pH-sensitive systems were developed to control such diverse phenomena as strand orientation and exchange in peptide assemblies,[1] charge densities in self-assembled monolayers,[2] encapsulation of guests in supramolecular polymers,[3] phase transitions in stimuli-sensitive polymers for drug delivery,[4] rotaxane switching,[5] properties of luminescent devices based on organic fluorophores[6] and metal complexes,[7] permeability of mesoporous materials,[8] growth of

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nanomaterials,[9] control of recognition-mediated reactions,[10] and the design of bioreactors.[11] Life itself is a pH-sensitive phenomenon, as many biochemical processes work only within a very narrow pH window.

In this study, we provide the first example of a "switchable" molecular system for pH-controlled double-stranded DNA cleavage designed for selective targeting of cancer cells. The more acidic extracellular environment of solid tumors, relative to that of the normal cells, leads to a pH gradient that has a dramatic effect on drug uptake in tumor cells[12] and can be explored in the design of tumor-specific DNA cleaving agents.[13] It is known that hyperglycemia (e.g., glucose infusion) and/or certain drugs, e.g., amiloride, nigericin, and hydralyzine, are also able to lower the intracellular pH of cancer cells. For example, administration of amiloride and nigericin at dosages that do not affect the normal cells drops the intracellular pH in a number of tumor cell types from 7.2 to 6.2−6.6.[14−17] Moreover, hyperglycemia as well as hypoxia leads to an even further acidification to pH as low as 5.5.[18,19]

Although research in this direction has been hampered by the scarcity of suitable pH-dependent cytotoxic agents, a number of approaches can be used for the rational design of such molecules.[20] An illustration of how these ideas can be applied to a highly potent class of natural enediyne antibiotics either through isomerization into more reactive functional groups or through unlocking of structural constraints is given in Figure 1.[21] In addition, there have been promising reports of acid-labile drugs that either hydrolyze at lower pH to give toxic products[22] or produce free radicals due to accelerated Co−R bond homolysis.[23] An interesting recent finding involves acid-promoted DNA cleavage by natural antibiotic varacin C.[24] The authors found a 2-fold increase in the extent of single-stranded (ss) DNA cleavage at pH 5.5 (47% at 5 μM antibiotic loading) compared with that at pH 7 (23%). Unfortunately, this increase only applies to the ss damage which is, unlike the double-stranded (ds) damage, usually repairable by the cell chemical machinery.

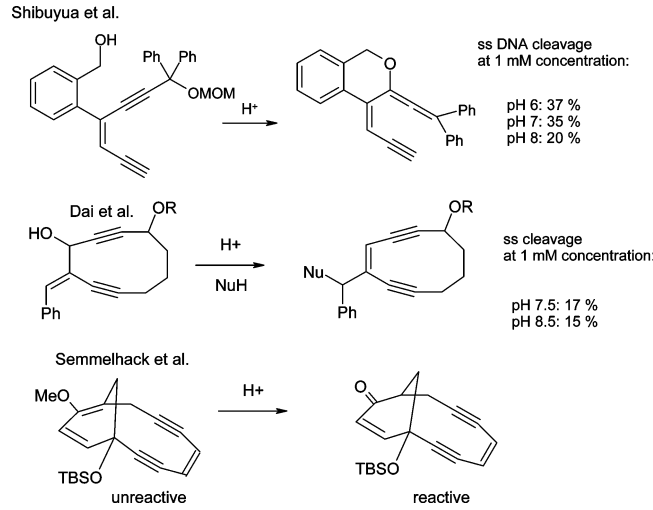

Figure 1. Approaches to pH-activated enediynes based on acid-catalyzed transformations of unreactive prodrugs.

Another approach to pH-regulated DNA-cleaving agents involves protonation of basic functional groups. Amine functionality is one of the obvious choices, and several elegant experimental designs based on the protonation of this functional group appeared in the literature (Figure 2). In particular, the groups of Kerwin, Chen, and Kraka and Cremer reported

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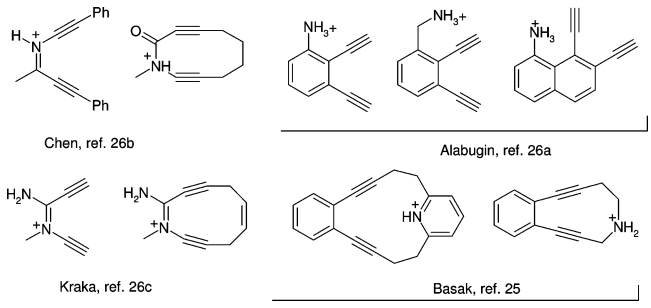

Figure 2. Literature examples of pH-controlled amino enediynes.

systems where protonation increased the efficiency of radical damage through simultaneous acceleration of the H-abstraction step and deceleration of *p*-benzyne diradical deactivation through the retro-Bergman ring-opening.[25] After detailed computational studies indicated that properly positioned cationic groups decrease the activation barrier for Bergman cyclization,[26] Basak and co-workers[27] pursued an alternative approach based on significant acceleration of cycloaromatization step imposed by a spatially close ammonium moiety.

Unfortunately, simple aliphatic amines are too basic for the change in the protonation state to occur at the pH window necessary for targeting hypoxic cancer cells. As a result, even when protonation has been shown to accelerate the Bergman cyclization of enediynes, the change in reactivity did not occur in the optimal pH range. Nevertheless, the basicity of nitrogen bases can be controlled in a number of ways, and thus the above obstacle is not insurmountable. For example, anilines are significantly less basic than aliphatic amines and can be fine-tuned through substitution[28] to accept the proton only at the desired pH range. Amides,[25b] suggested by Chen, and aldimines,[25c] designed by Kraka and Cremer, may offer an excellent solution for fine-tuning the nitrogen basicity.

The present work describes the development of the first pH-controlled system capable of inducing the much more therapeutically important *double-stranded* (ds) DNA cleavage. Several challenges presented themselves at the beginning of this study. First, the change in reactivity has to occur at a relatively narrow and predefined pH point (ideally $\sim$ pH 7). Second, an efficient DNA cleaver was needed which could operate within the physiological pH range and be attached to a pH-sensitive functionality without sacrificing its potency.

In order to solve the first problem, we utilized a simple but, to the best of our knowledge, new strategy for the design of pH-regulated molecules which is based on the presence of *two* amino moieties (or related functional groups) with different basicities. The first amino group should be sufficiently basic to be protonated at a wider range of physiological conditions. This auxiliary group plays three roles through (a) enhancing solubility of the conjugates in water, (b) increasing their affinity to the negatively charged backbone of DNA, and (c) modulating the basicity of the pH trigger. The pH trigger itself corresponds to the second amino group which should enable pH-switchable behavior at the desired pH range.

The bifunctional pH-regulated part in our design is derived from a diamino carboxylic acid (lysine) which is connected to the DNA-cleaving moiety through the carboxyl group of lysine (Figure 3). This mode of attachment is different from the most common way of lysine incorporation into conjugates or proteins through the formation of a peptide bond at the expense of the α-amino group.[29] Importantly, the use of the carboxyl group for the assembly preserves the two amino groups, both of which are essential for solubility, binding to DNA, and pH regulation of DNA cleavage.

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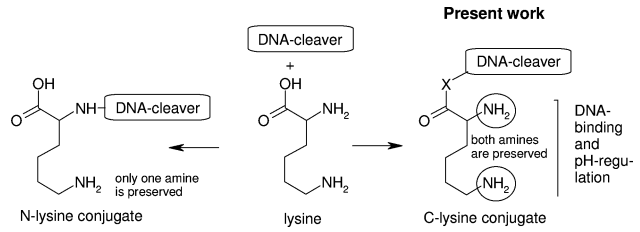

Figure 3. Two types of lysine conjugates.

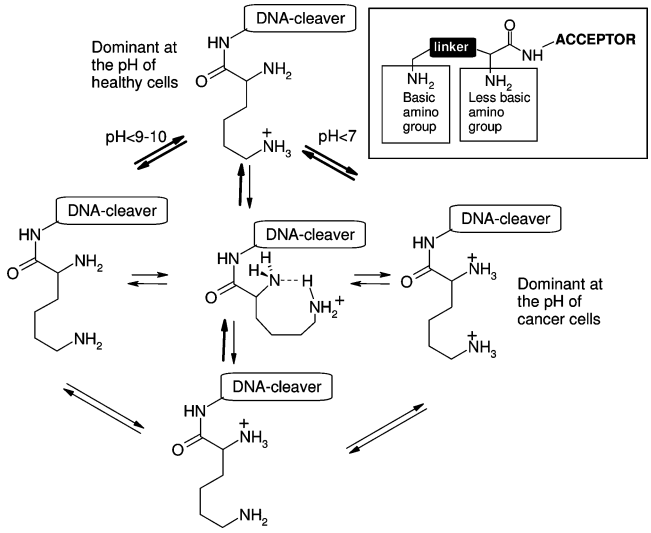

Figure 4. Design of pH-dependent DNA cleavers based on different stages of protonation of the lysine side chain. The dominant protonation pathway is indicated by the bold arrows.

The auxiliary amino group is at the remote terminus whereas the pH-switchable amine is next to the carbonyl group.[30] We were interested in determining whether the presence of a highly polarized acceptor moiety can differentiate the basicities of the two amino groups to the extent where distinct protonation states exist at the biologically relevant pH regions (Figure 4). If this approach is successful, sequential protonation of these groups at different pH can be used to control the photophysical properties of such molecules, their binding to DNA, and ultimately the efficiency of ds DNA cleavage.

The pH-activated part was combined with light-activated DNA cleavers in order to take advantage of the high degree of spatial and temporal controls over reactivity inherent to the photochemical activation.[31] We based the choice of the photochemically activated DNA part on our earlier discovery of the C1−C5 cyclization of enediynes which results in the net transfer of four hydrogen atoms to the enediyne moiety.[32,33] In particular, the tetrafluoropyridine (TFP)-substituted enediynes were found to be *both* photochemically and thermally stable, unless photoexcitation occurs in the proximity of a suitable electron donor, such as DNA. We have recently shown that these molecules are efficient reagents for the double-strand (ds) DNA photocleavage[34] and that they are amenable to two-photon activation.[35] We have also shown that the lysine conjugates of enediynes, as well as related fulvenes and acetylenes, perform guanosine-specific (oxidative) cleavage at the ends of AT-rich

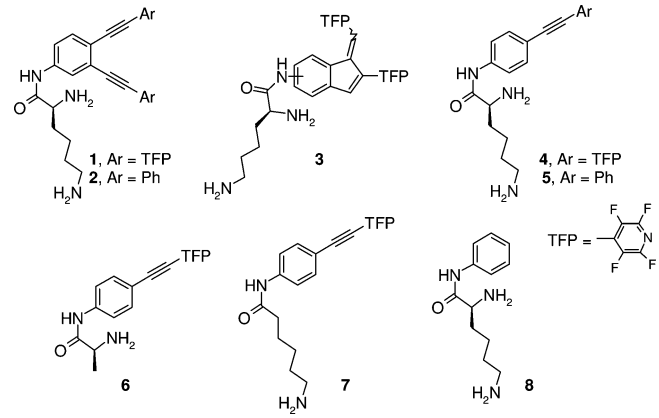

Figure 5. Structures of lysine conjugates **1**−**5** and related control compounds **6**−**8**.

sequences of DNA.[36] After a serendipitous discovery of the photochemical removal of the terminal radioactive label from ^{32}P-labeled oligonucleotides that suggested lysine conjugates are capable of recognition of terminal phosphate groups, we utilized this phenomenon for the design of reagents which can recognize the DNA-damage sites (nicked or gapped ss cleavage). These reagents allowed for the first time to achieve a controlled photochemical transformation of the easily repairable ss DNA cleavage to the much more therapeutically important ds cleavage.[37]

These considerations guided our design and prompted us to investigate DNA-cleavage activity and binding to DNA of five lysine conjugates **1**−**5** in the range of pH 5.5−8 which can be used to differentiate between cancer and healthy cells (Figure 5). After testing whether the pH-dependent DNA cleavage based on this molecular design is feasible, we will concentrate on the mechanistic basis of this phenomenon through a combination of photophysical techniques, NMR titrations, analysis of DNA binding trends, and effect of chemical additives at the cleavage efficiency.

Results and Discussion

pH-Dependent DNA Cleavage. The ability of lysine conjugates to cleave DNA upon irradiation was investigated using conversion of supercoiled plasmid DNA into the respective relaxed circular and linear forms (forms II and III). The relative amounts of the three DNA forms were determined by densitometric analysis[38] of the gel electrophoresis bands. Although enhancement of DNA-cleaving ability has been observed for all lysine conjugates (see Figure 8), we will focus our discussion below on compound **4**.

The most remarkable observation is the dramatic increase in efficiency of ds DNA cleavage caused by compound **4** when pH changes from neutral to slightly acidic (Figure 6). While control experiments clearly indicate that no additional DNA cleavage is caused at this pH by the conjugate alone in the dark

(34) Kovalenko, S. V.; Alabugin, I. V. *Chem. Commun.* **2005**, 1444.
(35) Kauffman, J. F.; Turner, J. M.; Alabugin, I. V.; Breiner, B.; Kovalenko, S. V.; Badaeva, E. A.; Masunov, A.; Tretiak, S. *J. Phys. Chem. A* **2006**, *110*, 241.
(36) Interestingly, this selectivity is observed not only for the TFP-substituted molecules but also for the Ph-substituted enediynes expected to undergo the photochemical Bergman cyclization. Breiner, B.; Schlatterer, J. C.; Kovalenko, S. V.; Greenbaum, N. L.; Alabugin, I. V. *Angew. Chem.* **2006**, *45*, 3666.
(37) Breiner, B.; Schlatterer, J. C.; Kovalenko, S. V.; Greenbaum, N. L.; Alabugin, I. V. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 13016.
(38) The cleavage was quantified by using SAFA software: Laederach, R. D. A.; Pearlman, S.; Herschlag, D.; Altman, R. *RNA* **2005**, *11*, 344.

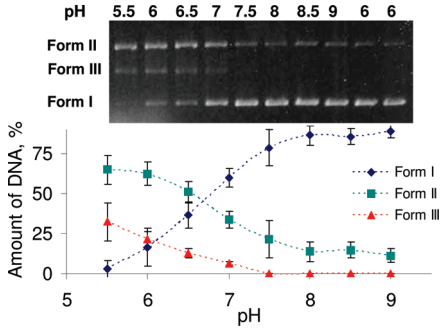

Figure 6. Plasmid relaxation assays with conjugate **4** (15 μM) and pBR 322 plasmid DNA (30 μM/bp) in 20 mM sodium phosphate buffer after 3 min of irradiation. Form I = intact supercoiled DNA, form II = relaxed form (ss cleavage), form III = linear form (ds cleavage). Lanes 1−8: from pH 5.5 to pH 9, lane 9: no compound + UV, lane 10: compound + no UV. Cleavage data were quantified through densitometry. Reported values represent the average of four experiments.

or by UV itself, the damage is amplified strongly when both light and compound **4** interact with DNA at the lower pH. At pH 6, only 16% of intact DNA (form I) remained after 3 min of irradiation in contrast to 60% and 86% of unreacted DNA at pH 7 and 8, respectively. Even more remarkable is that at the concentrations where hardly any ds cleavage is observed at pH 7, the amount of double-stranded cleavage at the lower pH reaches the 2:1 ss:ds ratio, which rivals DNA cleavage mediated by the natural antibiotics calicheamicin γ[39] and bleomycin![40] Higher concentrations of **4** lead to as much as 50% of ds cleavage which, to the best of our knowledge, is unprecedented for a small molecule-based DNA cleaver (natural or designed).

Interestingly, the dependences of all three forms of DNA cleavage from pH follow the classic titration curves, which afford the apparent pK_a values of 6.6, 6.8, and 6.2, respectively for forms I, II, and III (Figure 7).[41] All of these values agree that a significant change in the efficiency of DNA cleavage occurs at the pH 6−7 window ideal for targeting cancer cells.

Although all lysine conjugates have similar pH-dependent trends (Figure 8), conjugates **1** and **4** possess the greatest DNA-cleaving ability. As a result, we focused our mechanistic discussion on acetylene **4** which is not only less toxic than enediyne **1** in the dark (vide infra) but also does not aggregate and precipitate at the higher pH values (~8). When necessary, we incorporated data for other conjugates as well.

In order to investigate the relative role of the two amino groups in these conjugates, we extended this study to include DNA cleavage activities of alanine and caproic acid conjugates **6** and **7**. These compounds have only one amino group (at the α- and ε-carbon, respectively) and, as expected, show different behavior (Figure 9). Even though the monoamines **6**, **7** are comparable in reactivity to diamine **4** at pH 8, the two

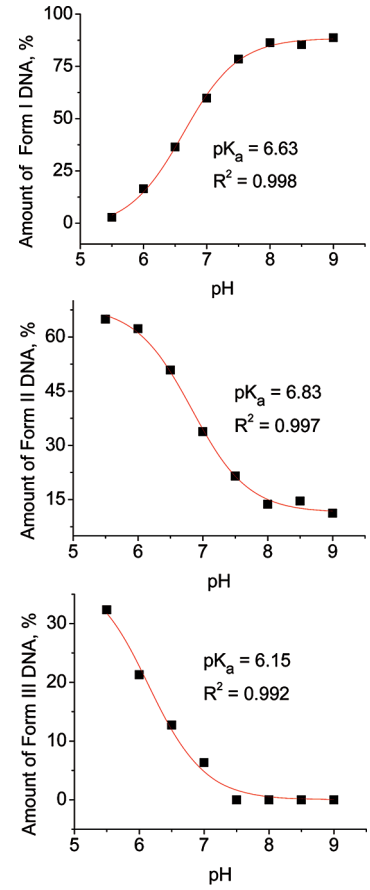

Figure 7. Percentage of unreacted DNA (form I), single-stranded cleavage (form II), and double-stranded cleavage (form III) as a function of pH, and the apparent pK_a values corresponding to these plots.

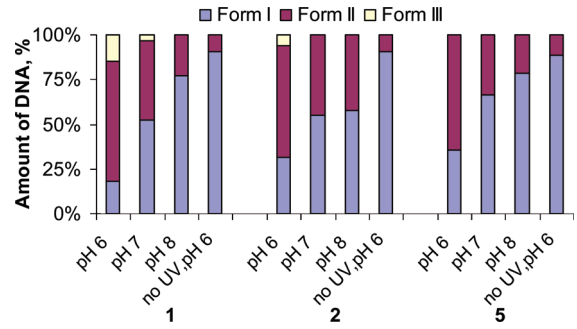

Figure 8. Quantified plasmid relaxation assays with 15 μM of compounds **1**, **2**, **5** and 30 μM/bp of pBR 322 plasmid DNA in 20 mM sodium phosphate buffer at pH 6, 7, and 8 after 3 min irradiation.

conjugates lacking one of the two amino groups rapidly fall far behind in their efficiency at the lower pH values[42] and do not produce any ds cleavage even at pH 6.

The fact that only the lysine conjugates produce clear ds cleavage under these conditions suggests that *both* amino groups are required for this process to occur. Several explanations are possible. For example, the remarkable ability of lysine to recognize a nick in DNA and facilitate a break of the complementary strand (the ss→ds cleavage conversion) discovered in our previous work[37] may play some role in the high

(39) The ds/ss ratio is 1:2 for plasmid DNA and 1:3 for cellular DNA: Elmroth, K.; Nygren, J.; Martensson, S.; Ismail, I. H.; Hammarsten, O. *DNA Repair* **2003**, *2*, 363.

(40) About 10% of the DNA strand breaks induced by bleomycin are double-strand breaks: Charles, K.; Povirk, L. F. *Chem. Res. Toxicol.* **1998**, *11*, 1580. Povirk, L. F.; Wubker, W.; Kohnlein, W.; Hutchinson, F. *Nucleic Acids Res.* **1977**, *5*, 3573. See also Lloyd, R. S.; Haidle, C. W.; Hewitt, R. R. *Cancer Res.* **1978**, *38*, 3191. Harsch, A.; Marzilli, L. A.; Bunt, R. C.; Stubbe, J.; Vouros, P. *Nucleic Acids Res.* **2000**, *28*, 1978. Stubbe, J.; Kozarich, J. W. *Chem. Rev.* **1987**, *87*, 1107. Burger, R. M. *Chem. Rev.* **1998**, *98*, 1153. Quada, J. C., Jr.; Zuber, G. F.; Hecht, S. M. *Pure Appl. Chem.* **1998**, *70*, 307. Hecht, S. M. *J. Nat. Prod.* **2000**, *63*, 158.

(41) Value for the ds cleavage (form III) is less accurate because of the greater experimental error bars.

(42) Interestingly, monoamines possess a certain (but lesser) degree of pH dependency and presence of an α-amine in **6** has a slightly larger effect than the presence of an ε-amine in **7**.

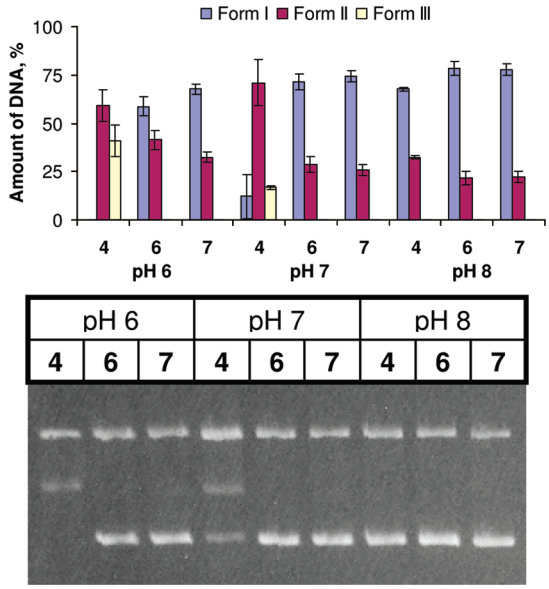

Figure 9. Plasmid relaxation assays for DNA photocleavage with 15 μM of compounds **4**, **6**, and **7** and pBR322 plasmid DNA (30 μM/bp) in 20 mM sodium phosphate buffer (pH 6, 7, 8) after 10 min of UV irradiation. Quantified cleavage data are presented on the top, and the original gels are on the bottom.

efficiency of the ds cleavage. However, the *enhancement* of this type of cleavage at the lower pH is an unprecedented phenomenon. This effect is likely to stem from the presence of a protonated α-amino group in compound **4** which enhances the efficiency of DNA cleavage through one of the three mechanisms outlined in Figure 11 (vide infra).

The difference in kinetics of DNA photocleavage by conjugate **4** at different pH values provides another illustration of the remarkable degree of pH control (Figure 10). DNA cleavage at pH 6 is remarkably fast: plasmid DNA is completely consumed within 5 min of irradiation, even though control experiments clearly show that there is very little of DNA damage by UV itself under these conditions. In a sharp contrast, DNA cleavage at pH 8 is slow: even after 1 h of irradiation, ~60% of DNA remains unreacted (note, however, that this reaction is still considerably faster than the damage by UV itself shown at the bottom part of Figure 10). These observations indicate that, depending on the pH, compound **4** can induce DNA damage in two kinetically different ways. The DNA-damaging activity at pH 7 can be described as a superposition of the two different cleavage patterns described above. These experiments also provide the control data to confirm that change in the pH per se does not induce acceleration of DNA photocleavage (lower part of Figure 10). Instead, the observed pH-dependence stems from the properties of lysine conjugates.

Mechanistic Alternatives for the Observed pH-Dependence of DNA Cleavage. Several possible mechanisms which could lead to the observed pH dependency for the interaction of lysine conjugates with DNA are outlined in Figure 11. Mechanism (a) involves the control of deactivating intramolecular electron transfer from the donor α-amino group to the excited chromophore through protonation. Electron transfer intercepts the excited state of the DNA cleaver, making it less active at higher pH. In contrast, at lower pH, both amino groups are protonated and the nitrogen lone pair is not available for deactivating the reactive excited state.

Another possibility is illustrated by a model for stronger binding of the diprotonated lysine moiety to DNA at lower pH,[43] as shown in Figure 11b. At neutral pH, only the more basic, remote amino group of the lysine moiety is protonated, leading to a relatively loose binding to DNA. At lower pH, the electrostatic interaction of positively charged ammonium groups and negatively charged phosphate groups of DNA brings the DNA-cleaving warhead close to the ribose backbone of DNA, providing a possible mechanism for the increase in the extent of DNA damage by this warhead.

Finally, we envisioned that a conformational change may occur upon the second protonation (Figure 11c). Although it was not clear a priori whether the coiled conformation may gain sufficient importance in the strongly hydrogen bonding aqueous environment, it is interesting to consider such possibility as a protonation-induced shape change may lead to a change in the DNA binding mode.

Photophysical properties of lysine conjugates with acceptor substituents will help us to determine whether the choice of photochemical DNA cleavers activated through photoinduced electron transfer (PET) introduces a new component in the pH-controlled behavior (Figure 11a). NMR analysis of different protonated species will elucidate the possibility of conformation change in Figure 11c whereas pH dependence in DNA binding will help to determine whether tighter binding is achieved for dications as illustrated in Figure 11b.

However, before comparing the relative importance in the three possible pathways for the observed pH dependence of DNA cleavage for the lysine conjugates, it is important to obtain reliable answers regarding the basicities of the two amino groups in the lysine conjugates. Is the presence of an acceptor moiety next to the α-amino group and another already protonated amino group sufficient to decrease the basicity of the α-amino group to the extent where the change in its protonation will occur in the vicinity of pH 6−7? If yes, how are these changes in the protonation state transferred in the changes in the efficiency of DNA binding? In order to address these questions, we proceeded to determine pK_as for the two amino groups of the lysine moiety as well as binding affinities of the different protonated states to DNA.

Determination of pK_a Values. Direct potentiometric pH determination of both pK_a values is inaccurate for most of the conjugates because, at a pH where both ammonium groups are deprotonated and these molecules lose all their charge, the solubility in water becomes very low (<1 mM). The decrease in solubility leads to the apparent aggregation and distortion of potentiometric curves at the higher pH. This distortion prevents accurate curve-fitting (see Figure S1, Supporting Information). Thus, we had to resort to alternative spectral methods. Fortunately, there is a selection of convenient procedures which provide complementary information which can be used for cross-checking the accuracy of the alternative approaches.

NMR Titrations. The advantage of NMR titrations[44] is that they provide direct structural information, allowing differentiation of the two protonation events through observations of

(43) Standke, K. H. C.; Brunnert, H. *Nucleic Acids Res.* **1975**, *2*, 1839. Ahmad, J.; Ashok, B. T.; Ali, R. *Immunol. Lett.* **1998**, *62*, 87. Tyler, J. K.; Allen, K. E.; Everett, R. D. *Nucleic Acids Res.* **1994**, *22*, 270.

(44) Gao, G.; DeRose, E. F.; Kirby, T. W.; London, R. E. *Biochemistry* **2006**, *45*, 1785. Kakehashi, R.; Shizuma, M.; Yamamura, S.; Maeda, H. *J. Colloid Interface Sci.* **2005**, *289*, 498. Rush, J. R.; Sandstrom, S. L.; Yang, J.; Davis, R.; Prakash, O.; Baures, P. W. *Org. Lett.* **2005**, *7*, 135.

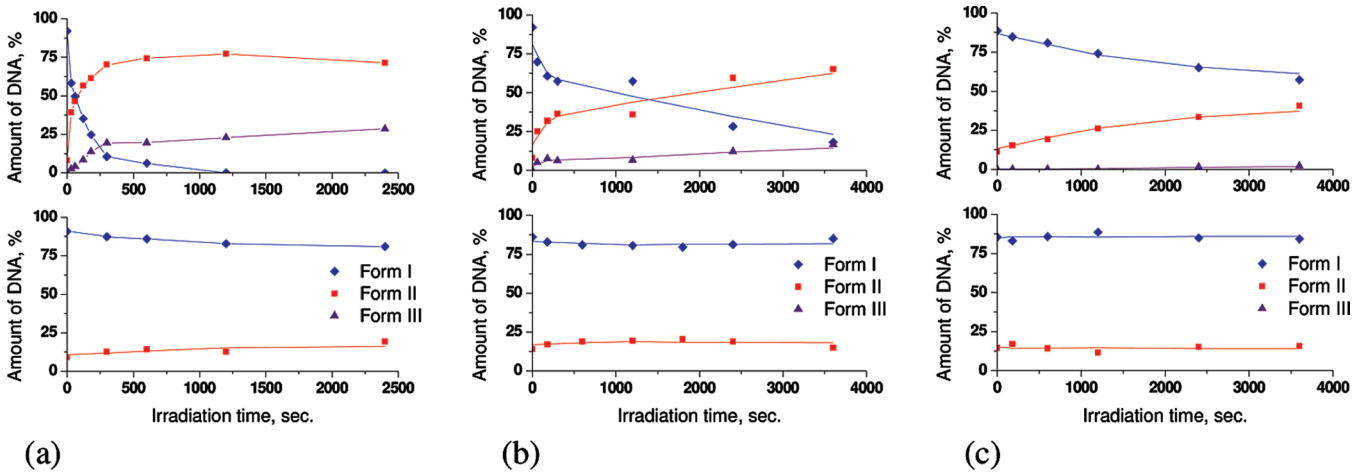

Figure 10. Quantified plots of pBR 322 plasmid (30 μM/bp) relaxation assays for 10 μM of compound **4** in 20 mM sodium phosphate buffer at pH 6 (a), 7 (b), and 8 (c) as a function of the irradiation time. Plots on the bottom show the effect of UV on the DNA without the conjugate at different pH. Plots on the top illustrate reactivity in the presence of compound **4**.

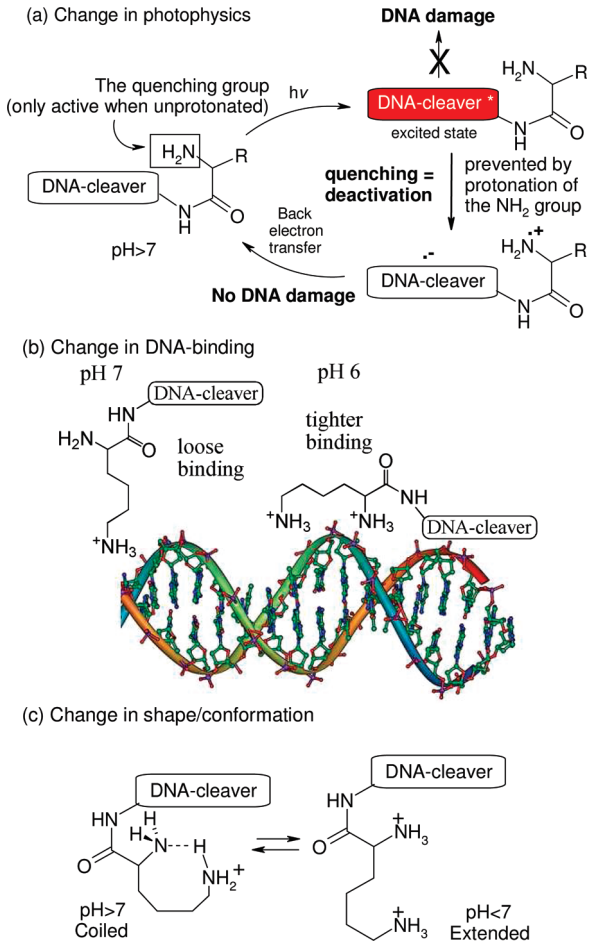

Figure 11. Three possible mechanisms for pH-regulated DNA modification by lysine conjugates.

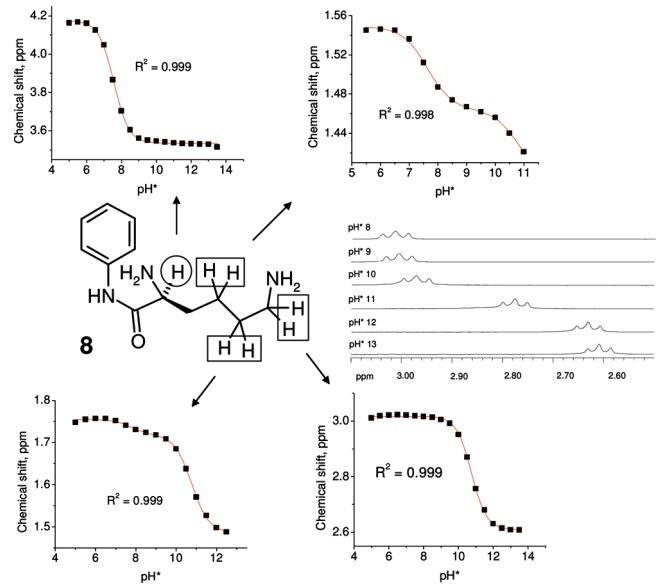

Figure 12. ^{1}H NMR spectra of α- and ε-hydrogens in compound **8** (5 mM) in D_2O at pD 5, 6, 7, 8, 9, 10, 11, 12, 13, and chemical shift−pD titration data for α- and ε-hydrogens in compound **8**. These pK_a values change to 7.44 and 10.40 when correction for going from pK_a^{H*} to pK_a is applied.

chemical shifts for hydrogens spatially close to one of the two amino groups.[45] Such differentiation can confirm unambiguously whether the presence of a carbonyl group next to the amino group leads to decrease in the pK_a of the respective ammonium ion.

Favorable solubility of *N*-phenyl amide **8** in D_2O rendered this molecule a particularly convenient substrate for the ^{1}H NMR studies. These studies followed changes in the chemical shifts of the lysine chain protons upon the protonation/deprotonation of the two amino groups during the pH (pD) titration. The titrations curves clearly showed that α-amino group is less basic (pK_a = 7.56) than ε-amino group (pK_a = 10.75). To allow comparison of these pK_as with pK_as obtained by other methods, one has to take into account the difference between pK_a^{H*}, which is obtained by a direct reading of the H_2O-calibrated pH meter in a D_2O solution, and pK_a (pK_a = 0.929pK_a^{H*} + 0.42).[46]

Interestingly, monitoring of protons on the *β*-, *γ*-, and *δ*-carbons produced two protonation curves with their relative sensitivity being directly proportional to the proximity to the

(45) As the control experiment, we tested two pK_a values of ethylene diamine with NMR titration. After correcting measured pK_a values with the equation (ref 42), the values of 7.03 and 9.99 are obtained experimentally (R^2 = 0.999). These data are in good agreement with the corresponding potentiometric literature values of 7.19 and 9.98. Avdeef, A.; Kearney, D. L.; Brown, J. A.; Chemotti, A. R. *Anal. Chem.* **1982**, *54*, 2322.

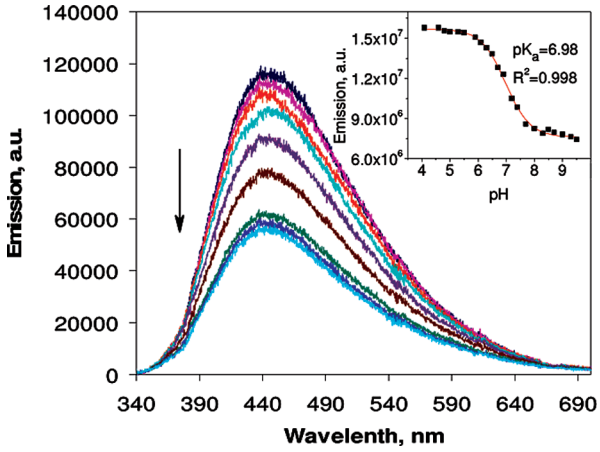

Figure 13. Fluorescence spectra of compound **4** (10 μM) at pH 4.1, 5.0, 6.1, 6.5, 6.9, 7.2, 8.0, 9.0, and 9.5. Insert: Quantified changes in fluorescence intensity as a function of pH and their fit to the Henderson−Hasselbalch equation for an acid−base equilibrium involving one amino group.

respective amino groups. The observation that α-hydrogens are not sensitive to the change in protonation of the ε-amino group suggests a lack of communication between the two amino groups, thus eliminating the cyclic H-bonded structure in the monoprotonated species where the ammonium group is hydrogen bonded to the other NH_2 group of the same molecule (the central structure in Figure 4 which corresponds to mechanism (c) of pH regulation in Figure 11).[47]

Fluorometric Determination of pK_a of the α-Amino Group. Highly sensitive fluorescence methods are especially convenient for compounds of limited solubility. We determined the basicity of the α-amino group using intramolecular quenching of fluorescence through photoinduced electron transfer (PET) from the nitrogen lone pair to the excited chromophore. At the lower pH, amino groups are protonated and the lone pair of electrons at nitrogen is unavailable for inter- or intramolecular electron transfer. However, as the ammonium group adjacent to the chromophore loses the proton at the higher pH, electron transfer becomes possible and quenches the fluorescence (Figure 13). Although the validity of this approach is based on the assumption that changes in intermolecular PET and other fluorescence quenching pathways with the pH are insignificant relative to intramolecular PET at the 10^{-5} M concentration, the advantage of fluorescence is that it is more sensitive to changes in the immediate vicinity of the chromophore and, thus, directly reports protonation of the α-amino group.

Lifetimes. To gain further insight into the effect of protonation on the excited state properties, we measured the singlet excited state lifetimes for compounds **4** and **6** as a function of pH. Within the error of measurement, all TFP-substituted compounds show a clear decrease in the lifetime of the singlet excited state at the higher pH. An illustration of the pH dependence for the singlet excited state lifetimes of compound **4** and **6** is provided in Figure 14.

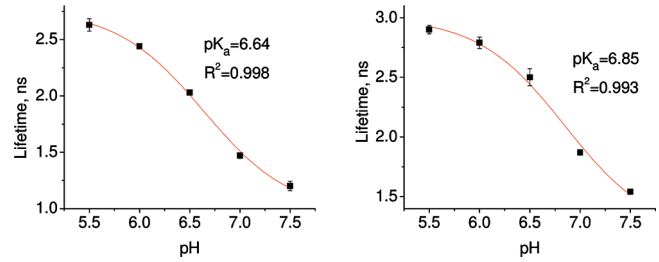

Figure 14. The pH-dependent lifetimes of the singlet excited state for conjugates **4** and **6** (10 μM), measured in 20 mM sodium phosphate buffer.

Table 1. The pK_a Values of Amino Groups in Lysine Conjugates **4**, **8**, and Related Monoamines **6**, **7** According to Emission/Absorption and NMR Spectra

compound	4	6	7	8	lysine
method	emission/ absorption	emission/ absorption	emission/ absorption	^{1}H NMR	−
pK_a1	6.98/6.9	7.6/7.4	10.8/9.4	7.56 (7.44)[a]	8.95[b]
pK_a2	−/9.5	−	−	10.75 (10.40)[a]	10.53[b]

[a] Data in parentheses include a correction factor from ref 46. [b] Data from ref 49.

The observed pH dependency in the lifetimes means that the S_1 state is less available for triggering DNA cleavage at the higher pH, a factor which should contribute to the observed decrease in activity under these conditions. At the lower pH, both amino groups are protonated, and hence electron transfer from the nitrogen lone pair does not deactivate the excited state, exactly as it is described in Figure 11a.

UV-Absorption Titrations. Similar pK_a values can be obtained using pH-dependent changes in the UV−vis absorption spectra of the conjugates. This method is less sensitive, as the changes observed are much smaller (about 10% increase in absorption upon transition from pH 11 to 5.5) than the analogous changes in the fluorescence spectra discussed in the previous section. In contrast to fluorescence, there are two titration curves for compound **4** which correspond to absorption changes of almost equal magnitude (Figure S4, Supporting Information). The pK_a for the first of these curves corresponds exactly to the pK_a obtained from the fluorescence titrations. Interestingly, pK_a for the ε-ammonium group of compound **7** is >1 pK_a units lower than that derived from the fluorescence changes. Although the reasons for this interesting behavior of the terminal amino group extend beyond the scope of this paper,[48] this subtle difference is irrelevant at the biologically relevant pH region where we observe the enhancement of ds-DNA cleavage. It is clear that, independent of the method, the pK_a of the auxiliary ammonium group is sufficiently high to allow more than 99% of the respective amine to be protonated even at neutral pH. The above

(46) pK_a values measured with pH* are assumed to be similar to the corresponding values in H_2O: Primrose, W. U. *NMR of Macromolecules. A Practical Approach*; Poberts, G. C. K., Ed.; Oxford University Press: Oxford, 1993; pp 22−23. Scheller, K. H.; Scheller-Krattiger, V.; Martin, R. B. *J. Am. Chem. Soc.* **1981**, *103*, 6833. Alternatively, one can use a formula for correlating pK_a values determined in D_2O and H_2O: Krezel, A.; Bal, Wojciech, A. *J. Inorg. Biochem.* **2004**, *98*, 161.

(47) Another interesting observation is that chemical shifts of aromatic hydrogens show small but measurable response to the changes in pH in the vicinity of the pH 10 region (see the Supporting Information), suggesting that there may some contribution from cation−π interaction. Gallivan, J. P.; Dougherty, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 9459. Ma, J. C.; Dougherty, D. A. *Chem. Rev.* **1997**, *97*, 1303. Mercozzi, S.; West, A. P., Jr.; Dougherty, D. A. *J. Am. Chem. Soc.* **1996**, *118*, 2307. Dougherty, D. A. *Science* **1996**, *271*, 163.

(48) A possible explanation is that fluorescence indicates properties of an excited molecule, whereas absorption indicates a ground-state counterpart. Acidities of ground and excited states are often different: Tolbert, L. M.; Solntsev, K. M. *Acc. Chem. Res.* **2002**, *35*, 19. The validity of this explanation depends on the relative rates of protonation/deprotonation and the key photophysical events. This complicated question is outside the scope of this paper.

Table 2. NBO Analysis of Electronic Properties of α-and ε-Nitrogen Lone Pairs of Diamines **4** and **8** at the B3LYP/6-31G(d,p) Level of Theory with the Polarizable Continuum Model (PCM)[52] for Solvation in H_2O

	neutral			protonated at the ε-nitrogen atom		
compound **4**	occupancy, e	energy, a.u.	hybrid character, n in sp^n orbital	occupancy, e	energy, a.u.	hybrid character, n in sp^n orbital
α	1.92791	−0.30618	4.42	1.92765	−0.30724	4.49
ε	1.96564	−0.29939	3.50	–	–	–
compound **8**	occupancy, e	energy, a.u.	hybrid character, n in sp^n orbital	occupancy, e	energy, a.u.	hybrid character, n in sp^n orbital
α	1.92941	−0.30457	4.39	1.92906	−0.30557	4.46
ε	1.96555	−0.29893	3.52	–	–	–

experiments do confirm unequivocally that the two amino groups of lysine conjugates possess different basicities and that the less basic α-group has the right properties to serve as a pH trigger for selective targeting of cancer cells.

Summary of Experimental pK_a Trends. The results for conjugates **4**, **6**, **7**, and **8** are summarized in Table 1. The α-pK_a for the lysine conjugates **4** is ∼0.5 units lower than the pK_a for the analogous alanine conjugate **6**. This difference indicates that, as expected, the presence of a second cationic moiety does play some role in the decrease of α-amino moiety basicity. However, the relatively close pK_a values for the α-ammonium groups in compounds **4** and **6** together with their large deviation from the pK_a values for the ε-ammonium groups in compounds **4** and **7** illustrate that the lowered basicity of the α-amino group in lysine is mostly due to its proximity to the amide moiety. It is also interesting that the presence of the acceptor tetrafluoropyridinyl moiety in **4** leads to a noticeable (∼0.5−0.6 pK_a units) decrease in the basicity of the α-amino group relative to that in a simple phenyl conjugate **8**.

Computational Analysis of pK_a Trends. In order to provide a theoretical rationale to the observed differences in basicity, we investigated electronic properties of the two amino groups in diamines **4** and **8**. Natural Bond Orbital (NBO)[50] analysis of electronic structure of these compounds at the B3LYP/6-31G(d,p) level clearly shows that the α-nitrogen lone pair is lower in energy and has a significantly lower population than the ε-nitrogen lone pair (see Table 2 and the Supporting Information). Both of these factors render the α-nitrogen a less efficient donor[28,51] and a weaker base than the ε-nitrogen.

Proton affinities calculated at the B3LYP/6-31G(d,p) level in water (Figure 15) support the above experimental findings very well. For both compounds **4** and **8**, proton affinity of the ε-nitrogen is significantly higher than that of the α-nitrogen. In agreement with the experimental pK_a values for monoamines **6** and **7** relative to the two pK_a values of the diamines **4** and **8**, the first protonation has some effect on the basicity of the second amino group, but the effect is not dramatic (1.2−1.5 kcal/mol difference in the proton affinity values). In addition, and again in a full agreement with the experiment, the α-amino group in compound **8** is noticeably more basic than the α-amino group in compound **4** (1.2 kcal/mol difference).

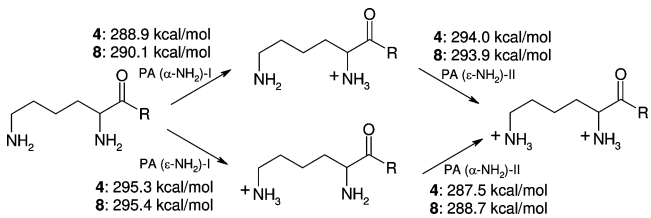

Figure 15. Proton affinities (in kcal/mol) of α- and ε-nitrogen lone pairs of diamines **4** and **8** at the B3LYP/6-31G(d,p) level of theory with PCM solvation in H_2O.

pH-Dependent DNA Binding. From a practical perspective, the pK_a value of 7.0 for the TFP-acetylene **4** suggests that this compound exists mostly as the monocation at the slightly basic pH, and it should be converted into >90% of dication at the same pH region (pH < 6) where we observe the amplification of the ds DNA cleavage. The following section concentrates on interaction (binding) of DNA and lysine conjugates in the different protonation states. We approached this question through a combination of several methods outlined below. Note that qualitative treatments of binding affinities in these systems are difficult due to the presence of several species with different spectroscopic properties (the three protonation states of diamines) and inherent complexity of calf thymus (CT) DNA which offers numerous types of binding environments to the DNA cleaver. As the result, we will limit ourselves in providing sufficient information for illustrating the *differences* in binding at the semiquantitative basis.

Absorbance and Fluorescence Titration. Titrations of 10 μM solutions of compounds **1**−**5** with calf thymus (CT) DNA

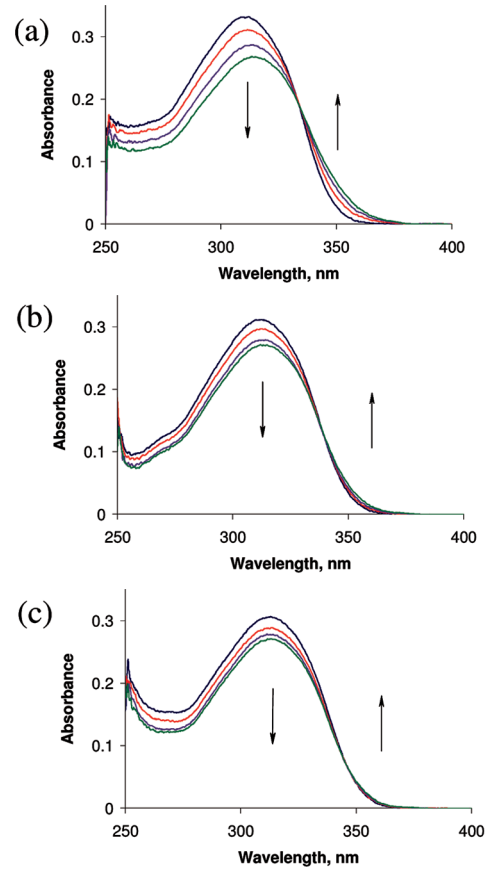

Figure 16. UV−vis absorbance titrations of **4** (10 μM) in 20 mM sodium phosphate buffer with 0, 1, 2, 3 equiv of CT DNA (bp) at pH 5.5 (a), pH 7 (b), and pH 8 (c).

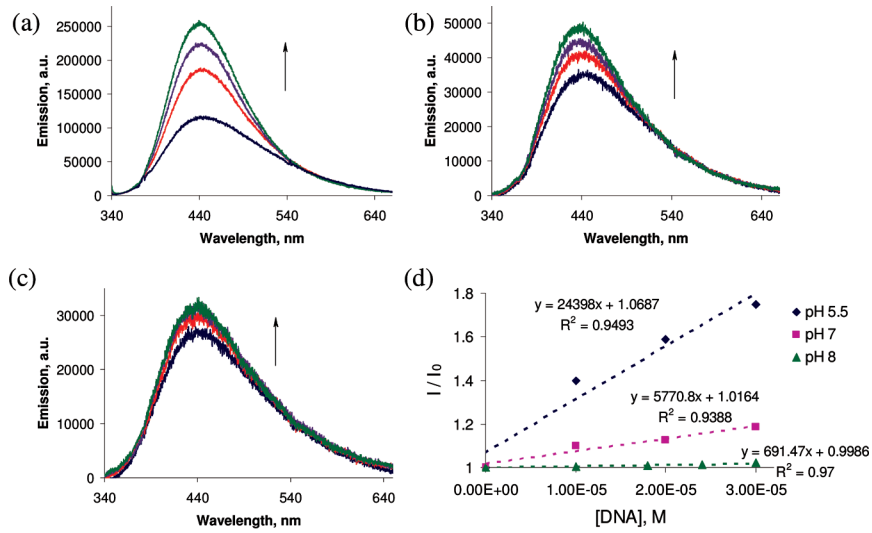

Figure 17. Emission titrations of **4** (10 μM) in 20 mM sodium phosphate buffer with 0, 10, 20, 30 μM/bp of CT DNA at pH 5.5 (a), pH 7 (b), and pH 8 (c). The initial slopes of plots illustrating changes in fluorescence of **4** as a function of DNA concentration (d).

revealed a significant difference in absorbance and emission changes at different pH values. Results for compound **4** are discussed in more detail in this section. While little or no shift of λ_{max} and small hypochromicity (14%, 12%, respectively) are observed for the titrations of conjugate chromophore with DNA at pH 7 and 8, the λ_{max} changes from 310 to 315 nm during the titrations with the same amounts of DNA at pH 5.5 and 20% hypochromicity is observed. The greater change of absorbance at pH 5.5 indicates the larger interaction between the chromophore and DNA.

Changes in fluorescence upon the DNA titrations followed a similar pattern where greater changes were observed at the lower pH, indicating a stronger binding affinity to DNA. Interestingly, while emission of TFP−enediyne−lysine conjugate **1** was quenched with DNA (Figure S6, Supporting Infromation), most likely through an electron transfer pathway,[53] we observed an increase in fluorescence for compound **4**. This finding is likely to indicate that the DNA-binding modes for these two compounds are different. In particular, the fluorescence increase displayed by compound **4** suggests that it may be a DNA groove binder.[54] Although fluorescence increase is often observed for intercalated chromophores as well,[55] intercalation is unlikely in this system because close proximity to the nucleobases should quench fluorescence of **4** through electron transfer.

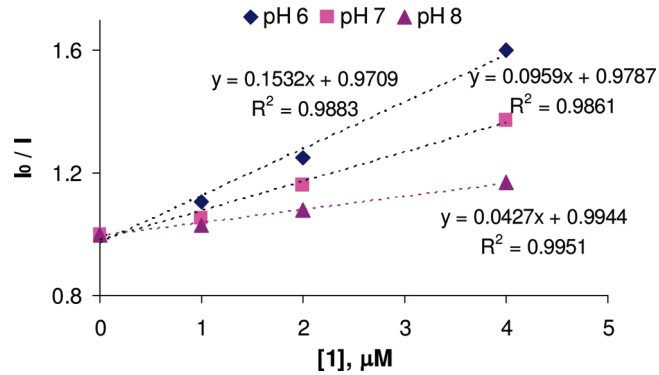

Figure 18. Changes in fluorescence of ethidium bromide (10 μM, the excitation wavelength = 535 nm) in CT DNA (10 μM/bp) as a function of displacement by compound **1**.

Further evidence for a significant difference in binding modes of enediyne **1** and acetylene **4** is provided by the ethidium bromide displacement assays (Figure 18). Only compound **1** displaces intercalated ethidium bromide from its complex with DNA, whereas addition of compound **4** does not lead to the displacement. This observation indicates again that compound **4** is not an intercalator. Nevertheless, despite the above differences in the DNA-binding mode, both lysine conjugates display similar pH dependence in their DNA cleavage.

Overall, both compounds show a much greater spectral response at the lower pH. As pH increases, the changes become

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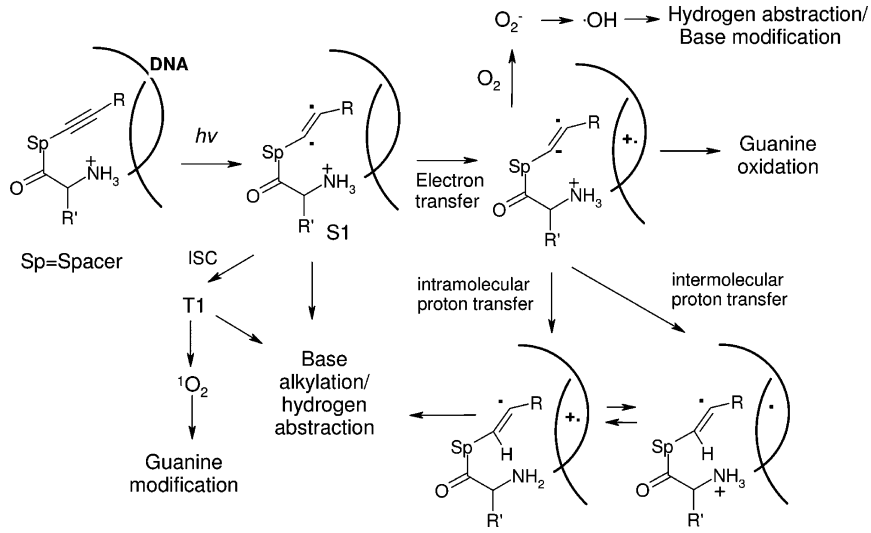

Figure 19. Summary of possible mechanistic alternatives for the observed DNA cleavage by amino acid/acetylene conjugates.

less pronounced, indicating a lower degree of interaction between the compounds and DNA at the higher pH. This trend was also shown in measurement of binding affinity of compound **4** to CT DNA by isothermal titration calorimetry (ITC; see the Supporting Information for details). These observations are consistent with the protonation behavior of these diamines discussed in the previous sections. At the lower pH, both amino groups are protonated to provide a higher degree of attraction between the two positive charges of the ammonium groups and the negative charges of the DNA phosphate backbone.

Mechanistic Considerations and pH Dependence of the Involvement of Diffusing Radicals in the DNA Cleavage. The potential complexity of chemical mechanisms which can be involved in DNA photocleavage by lysine acetylene conjugates is illustrated in Figure 19. A priori, a number of mechanisms may be considered as an explanation for the observed photochemical DNA cleavage by compound **4**.[56] In the first step, photochemical excitation leads to the formation of an acetylene excited singlet state. After this necessary step, the list of diverging possibilities includes a number of choices such as generation of reactive oxygen species like singlet oxygen, superoxide, and OH radical;[57] direct hydrogen abstraction from the DNA sugar backbone by photogenerated radicals, or singlet and triplet excited states;[58] oxidative damage due to electron transfer from DNA nucleobases;[59] and photoinduced DNA alkylation.[60]

We have shown previously that cleavage by the enediyne and acetylene derivatives discussed in the work proceeds with selectivity for guanine-rich parts of DNA which flank AT tracts because of a compromise between AT selectivity for binding and G selectivity for the formation of reactive species through PET.[36] Both the selectivity and the fact that the lesions in DNA oligomers required piperidine treatment clearly indicated that PET is involved in the damage. However, purely oxidative damage could not be the only mechanism because several compounds including **4** caused noticeable damage at a single G inside of a 12-bp AT tract. This observation suggested that these photocleavers may damage DNA through base alkylation as well. Subsequent work confirmed that compound **4** is capable of localized DNA cleavage, and the damage does not dissipate through hole-hopping when the same single G site is chosen as a target using phosphate−lysine recognition.[37] Furthermore, we also observed frank cleavage in 5′-(AATT)$_n$GG(AT)$_m$G-G(AAATTT)$_l$-3′ oligomers. The cleavage is amplified by heat which is indicative of either base pair alkylation or abstraction of a C1′-hydrogen by reactive radical species.[40] We have confirmed that triplet excited states of TFP−acetylenes behave as strongly electrophilic radicals which can alkylate π-systems.[61] Taken together, these observations suggest base pair alkylation is likely to complement direct PET damage as an important mechanism for the DNA damage, especially at the lower pH and tighter binding.

Further mechanistic details are unclear at the moment, and a number of possible scenarios can be suggested. In particular, intersystem crossing (ISC) may convert this excited state into a triplet state of the TFP−acetylene moiety, shown to behave as a highly electrophilic diradical.[61] In addition, the triplet state of the photocleaver may sensitize the formation of singlet oxygen. Alternatively, PET from DNA can lead to the formation of acetylene radical-anion and electron hole at one of the DNA bases. Electron transfer from acetylene radical-anion to dissolved oxygen would form superoxide anion which can be transformed

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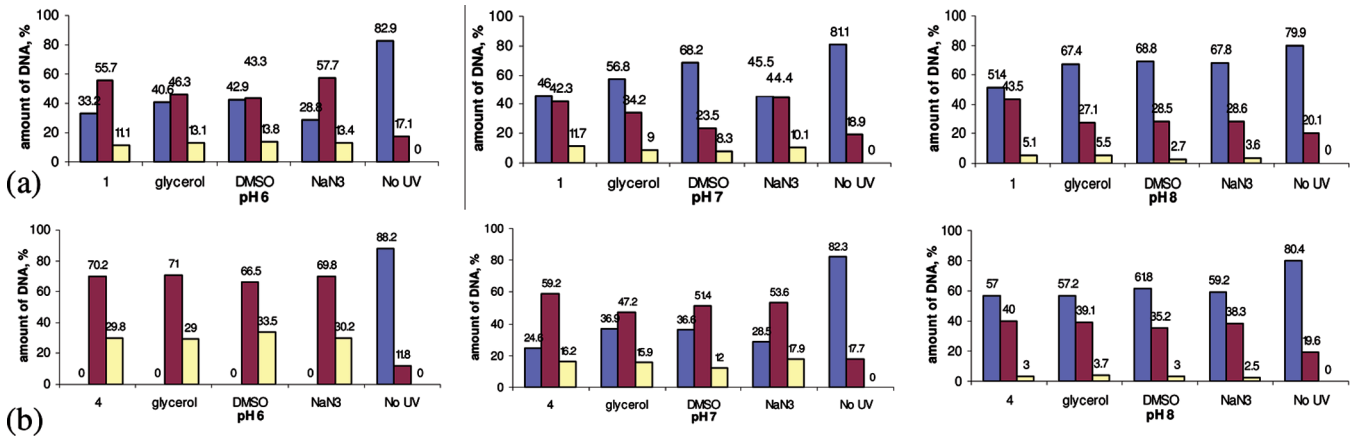

Figure 20. Effect of hydroxyl radical/singlet oxygen scavengers (10 mM) upon the efficiency of DNA cleavage at pH 6, 7, and 8 by conjugates **1** (a) and **4** (b) after 5 min irradiation. Color coding: light blue, form I; dark purple, form II; light yellow, form III.

into other reactive oxygen species. Depending on the relative rates of hole-hopping and hole-trapping, the DNA electron hole can either be trapped at a suitable location to produce an oxidized DNA base (likely, a guanine) or transfer a proton to the DNA-cleaver radical-anion. This proton transfer (which can be classified as proton-assisted electron transfer)[62] would transform the charge separated ion-pair into two new radicals, one at DNA and another at the partially reduced triple bond. The latter vinyl radical can be also formed when the intermediate radical anion of the DNA cleaver receives a proton (possibly through a solvent-mediated mechanism) from the adjacent α-ammonium group (Figure 19). Independent of the formation pathway, this vinyl radical can either abstract hydrogen from the deoxyribose backbone or alkylate an adjacent base.

In order to get further insight into the mechanism of the DNA cleavage by the conjugates, we used the plasmid relaxation assays for the cleavage with enediyne conjugate **1** and TFP−acetylene conjugate **4** in the presence of hydroxyl radical (glycerol, DMSO) and singlet oxygen (NaN_3) scavengers. The results are summarized in Figure 20.

For compound **1**, a small protecting effect of the hydroxyl radical scavengers is observed at all pH values. The protecting effect seems to be slightly lower at pH 6 than at the higher pH values. It is noteworthy, however, that the ds cleavage at pH 6 is not affected by any of the additives. Singlet oxygen scavengers show no effect upon the efficiency of DNA damage by compound **1** at pH 6 and 7.

Remarkably, neither of the additives is able to protect DNA from damage by acetylene−lysine conjugate **4** at pH 6. Some protecting effect is observed only at the higher pH. These results clearly indicate that reactive oxygen species are not involved in the mechanism of DNA cleavage at the lower pH, perhaps due to the tighter binding under these conditions. This observation provides additional support for a direct mechanism of DNA damage such as base pair alkylation. From a practical perspective, this is valuable not only because many solid tumors are hypoxic but also because cleavage which does not depend on diffusing species is more localized and efficient.

The final insight on the nature of lysine conjugates/DNA interaction came from the effect of ionic strength on the efficiency of DNA cleavage (Figure 21). Increasing concentration of phosphate buffer from 10 mM to 50 mM decreases the

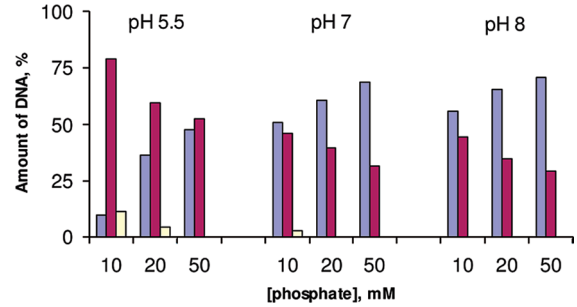

Figure 21. Quantified cleavage data of pBR 322 plasmid (30 μM/bp) relaxation assays of compound **4** (10 μM) at different concentrations of sodium phosphate buffer at pH 5.5, 7, and 8. Color coding: light blue, form I; dark purple, form II; light yellow, form III.

total DNA cleavage activity of compound **4** by 48%, 40%, 34% at pH 5.5, 7, and 8, respectively.[63] This result suggests that the electrostatic interaction between the positive charges of ammonium group of compound **4** and the negative charges of DNA backbone is an important factor for the cleavage. Again, this observation indicates relatively close binding between the DNA cleaver and its target which should be particularly important for the DNA-cleavage mechanisms based on alkylation and hydrogen abstraction. In contrast, the cleavage at pH 8 has greater contributions from processes not affected strongly by the ionic strength, such as the generation of diffusing reactive oxygen species.

Cancer Cell Proliferation Assays. The ability of compounds **1**−**5** to inhibit cell proliferation in three human clear cell renal cell carcinoma (RCC) cell lines was tested in the dark and under photoactivation (Figure 22). No difference was detected in the control experiments between cells not exposed to UV versus those that were exposed to UV at 350 nm for 10 min in any of the three RCC cell lines tested (Figure 22a−c; first two columns). On the other hand, all five compounds inhibited growth in each of the three cell lines but to varying degrees both in the dark and after UV radiation. Although UV treatment always enhanced the ability of conjugates **1**− **3** to inhibit cell proliferation, these compounds had strong growth inhibitory activity even without photoactivation in all three cell lines

(62) Reece, S. Y.; Hodgkiss, J. M.; Stubbe, J.; Nocera, D. G. *Phil. Trans. R. Soc. B* **2006**, *361*, 1351.

(63) The total percentage of cleavage for each pH was calculated as (2 × [form III] + [form II])/([form III] + [form II] + [form I]).

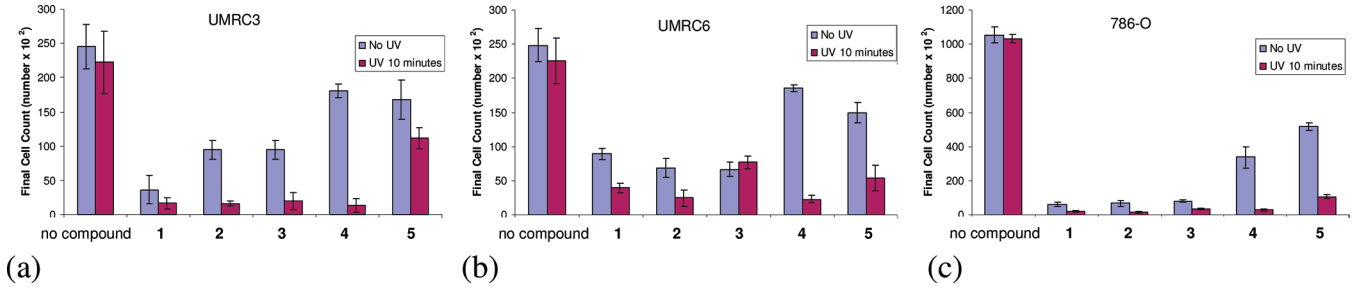

Figure 22. Cell proliferation assays using three different RCC cell lines: (a) UMRC3, (b) UMRC6, and (c) 786-O, and compounds **1**−**5**. RCC cells were resuspended in media and treated with UV radiation (350 nm) for 10 min with and without indicated compounds. Control cells were exposed to the indicated compound **1**−**5** at final concentration of 10 μM. Cell numbers determined after 72 h as described in the Supporting Information.

(Figure 22a−c) with a range of 62−94% inhibition.[64] On the other hand, compounds **4** and **5** were considerably less cytotoxic in the dark. Compound **4** seems to be an especially promising lead for the future development of anticancer agents because the decrease of cell numbers in control UV-irradiated cells as compared to control without UV irradiation in UMRC3 and UMRC6 cells was not statistically significant (Figure 22a,b), but when exposed to UV radiation and the conjugate, these cells displayed mortality of 94% in UMRC3 and 90% in UMRC6 cells.

Encouraged by these results, we studied the concentration effects of compound **4** at the proliferation of in LNCaP human prostate adenocarcinoma cells in the dark and under photoactivation conditions in more detail (Figure 23). Remarkably, even at the low 10 nM concentration, this system destroys more than 90% of LNCaP cells in one treatment whereas almost no toxicity is observed without light.

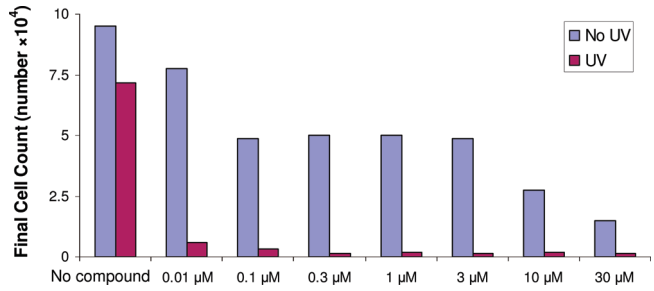

Figure 23. LNCaP cell proliferation assays with varying concentrations of compound **4**. The cells were exposed to UV for 10 min. Cells were counted 48 h after the exposure. Full experimental details are described in the Supporting Information.

Conclusion

Hybrid molecules which combine pH-regulated lysine moiety with a powerful DNA photocleaver display dramatically increased DNA-cleaving reactivity at the lower pH. Synergistic effects associated with the protonation of the α-amino group, such as pH-dependent changes in photophysical properties as well as in pH-dependent DNA binding (Figure 11), account for the increase in reactivity. Deactivation of the internal quenching pathway (Figure 11a) increases the lifetime of reactive singlet state while transformation of the conjugates into their dicationic form (Figure 11b) changes their binding mode with the polyanionic backbone of DNA.

Remarkably, the observed switch from negligible to record-breaking efficiency of the most therapeutically useful form of DNA cleavage (the ds DNA cleavage) occurs upon a relatively small change in the pH which can be used to differentiate healthy cells from hypoxic cancer tissues. The amplification of DNA-cleaving activity seems to be quite general and manifests itself for DNA-cleaving agents which bind DNA in different way and attack DNA through different chemical mechanisms. Cleavage at the lower pH for the conjugate **4** is not affected by the reactive oxygen species scavengers, suggesting possible value of this DNA cleaver for targeting hypoxic cancer tissues. Cancer cell proliferation assays further confirm remarkable potential of these molecules for the development of light-activated anticancer agents.

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Supporting Information Available: Details for experimental procedures, synthesis of DNA photocleavers and photophysical experiments, extended survey of DNA binding studies, Cartesian coordinates and energies of different protonation states of lysine conjugates, and ^{1}H and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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(64) We currently have no clear explanation for activity of the conjugates in the dark, but for compounds and **2**, it is unlikely to be associated with thermal Bergman reaction which is too slow under these conditions.