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Mimicking the biological activity of diazobenzo[*b*]fluorene natural products with electronically tuned diazofluorene analogs

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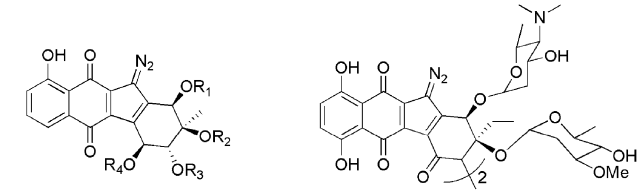
Abstract—Under appropriate electronic modulation, simple diazofluorene analogs recapitulate the DNA cleavage activity of kinamycin D under thiol-based reducing conditions. Achieving DNA cleavage under these reducing conditions is key to anticancer activity, as the most active compound, 1-methoxydiazofluorene, inhibits the proliferation of HeLa cells.

The kinamycins are a family of microbial metabolites that are characterized by an uncommon diazobenzo[*b*]fluorene skeleton.[1,2] In addition to their atypical architecture, they have been reported to possess both antitumor and antibiotic activities.[3,4] Interest in this class of natural products has been further enhanced by the recent disclosure of lomaiviticin A by researchers at Wyeth-Ayerst.[5] Lomaiviticin A is a glycosylated homodimeric diazobenzo[*b*]fluorene that was reported to possess potent anticancer activity against a wide range of cancer types (0.01–98 ng/mL), as well as activity against Gram-positive bacteria.[5] Lomaiviticin A was reported to cleave DNA under reducing conditions, and its cytotoxicity profile in the 24-cancer cell line panel was unique when compared to known DNA damaging agents (adriamycin and mitomycin C)[5] (Fig. 1).

We were attracted to this class of natural products because of its ability to mediate DNA cleavage under reducing conditions. Given this biological activity, and that reducing conditions are prevalent in intracellular space, there is potential to harness synthetic diazo compounds that cleave DNA under reducing conditions as cancer chemotherapeutics. There have been a number of reports that document the DNA cleavage activity of synthetic, diazo-based compounds;[6–10] however, none of these compounds have recapitulated the DNA cleaving ability of a diazobenzo[*b*]fluorene natural product under reducing conditions. All previously reported cases have relied upon photochemical[6,7,9,10] or oxidative[8] activation of the diazo group. Since the reactivity of a diazo compound is dictated by its electronic properties,[11] we posited that electronically tuned diazofluorene analogs would demonstrate similar cleavage activity as a diazobenzo[*b*]fluorene natural product, and that this activity would translate into antiproliferative activity. To test this hypothesis, we synthesized a range of diazofluorene analogs (Fig. 2) that were under differential electronic modulation, and compared their DNA cleaving activity to that of kinamycin D. Compounds that recapitulated the DNA cleavage activity of kinamycin D were then assayed for antiproliferative activity against HeLa cells.



kinamycin A: R_1 = H; R_2, R_3, R_4 = Ac
B: R_1, R_3, R_4 = H; R_2 = Ac
C: R_1, R_3, R_4 = Ac; R_2 = H
D: R_1, R_3 = Ac; R_2, R_4 = H

lomaiviticin A

Figure 1. Kinamycins A–D and lomaiviticin A.

The diazofluorene parent was synthesized as previously described.[12] Our synthesis of the 1-substituted and 4-substituted diazofluorenes is outlined in Scheme 1.

Keywords: Kinamycins; Lomaiviticins; DNA cleavage; Diazofluorenes; Antiproliferative activity.
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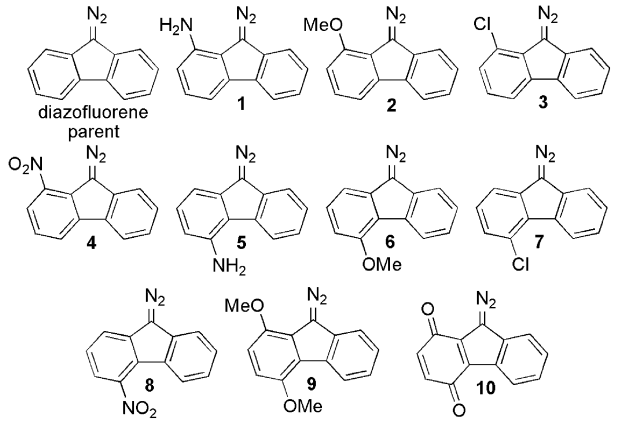
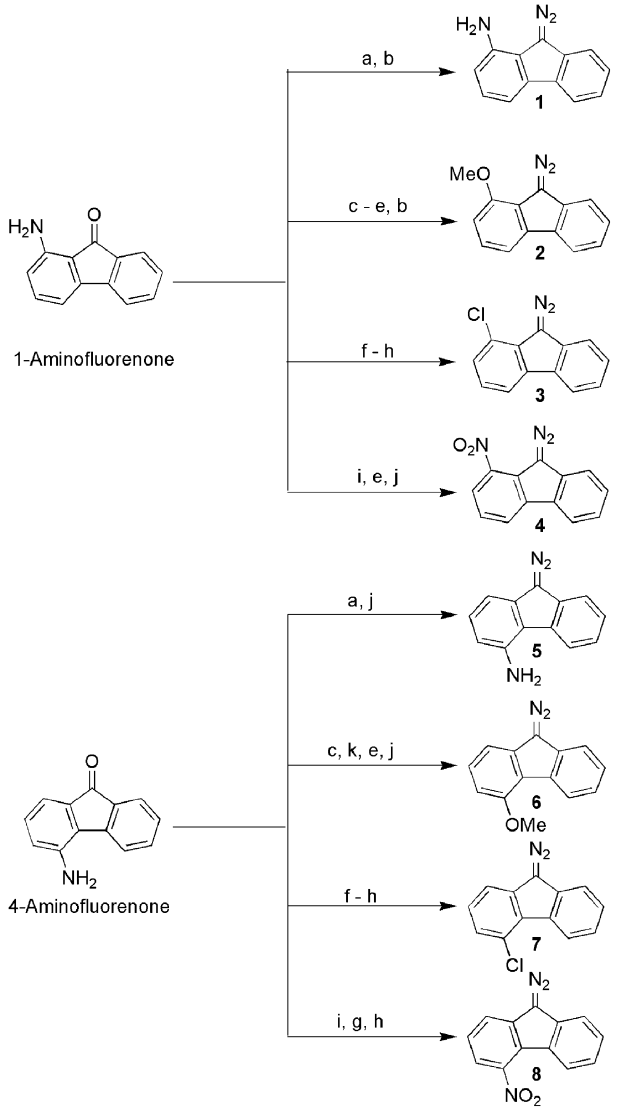


Figure 2. Diazofluorene probes.

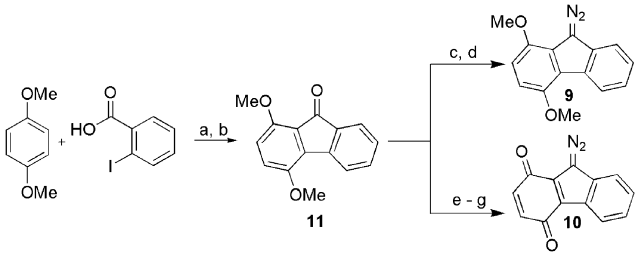


Scheme 1. Reactions and conditions: (a) NH_2NHTs, EtOH, HCl, reflux; (b) NaOMe, MeOH, reflux; (c) $NaNO_2$, H_2SO_4, H_2O; (d) NaH, DMF then MeI; (e) NH_2NHTs, THF, HCl, reflux; (f) $NaNO_2$, HCl, CuCl, H_2O; (g) N_2H_4, *n*-butanol, reflux; (h) HgO, toluene, reflux; (i) Tf_2O, H_2O_2, CH_2Cl_2; (j) 50% NaOH, dioxane, 50 °C; (k) NaOH, Me_2SO_4, EtOH, reflux.

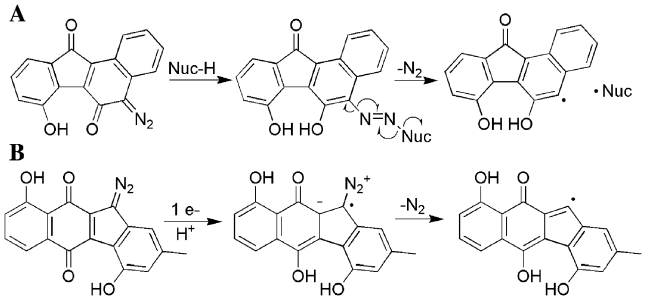
To access either the 1- or 4-nitrofluorenone, we oxidized 1- or 4-aminofluorenone with Tf_2O/H_2O_2.[13] The chlorofluorenones were synthesized from the respective aminofluorenone under Sandmeyer conditions ($NaNO_2$/HCl/CuCl/H_2O),[14] while the methoxyfluorenone derivatives were synthesized by converting the aminofluorenones to hydroxyfluorenones ($NaNO_2$, H_2SO_4, H_2O)[15] followed by methylation (either NaH/MeI or NaOH/Me_2SO_4). Finally, the diazo group was installed by either: (1) condensing the fluorenone with hydrazine followed by oxidation with mercuric oxide, or (2) condensing the fluorenone with tosyl hydrazine followed by elimination of the tosyl group under basic conditions.

The 1,4-substituted compounds were synthesized as outlined in Scheme 2. Briefly, 1,4-dimethoxybenzene and 2-iodobenzoic acid were coupled under the action of triflic anhydride.[16] Pd(II)-mediated coupling then delivered 1,4-dimethoxyfluorenone (**11**) for further elaboration.[16] To access the 1,4-dimethoxydiazofluorene **9**, **11** was condensed with tosyl hydrazine and subjected to base-mediated elimination with sodium methoxide. To access the 1,4-diazoquinone derivative **10**, **11** was quantitatively demethylated with BBr_3[17] and condensed with hydrazine. The resulting crude hydrazone was treated with Fetizon's reagent (Ag_2CO_3/celite),[17] which affected the tandem oxidation of the hydrazone and 1,4-hydroquinone to the target quinone **10**.

First, we established the conditions under which kinamycin D-mediated DNA cleavage. There have been a limited number of reports that address the mechanism of action (MOA) of the diazobenzo[*b*]fluorenes. Lomaiviticin A was reported to cleave DNA under reducing conditions;[5] however, no experimental details were documented. Using the diazobenzo[*a*]fluorene isoprekinamycin as a model substrate, Laufer and Dmitrienko[18] postulated that the electrophilicity of the diazo group would mediate formation of a nucleophilic adduct that would subsequently decompose to form a diradical and induce DNA damage (Scheme 3A). Using prekinamycin as their model substrate and guided by the established biological chemistry of other quinone natural products,[19] Feldman and Eastman[20] have postulated that bioreduction of the quinone to a hydroquinone would destabilize the diazo moiety, resulting in decomposition to a carbon-based radical with concomitant release of N_2 (Scheme 3B).



Scheme 2. Reactions and conditions: (a) Tf_2O, TFA, reflux; (b) $Pd(OAc)_2$, NaOAc, DMA, 125 °C; (c) NH_2NHTs, THF, HCl, reflux; (d) NaOMe, MeOH, reflux; (e) BBr_3, CH_2Cl_2; (f) N_2H_4, EtOH, reflux; (g) Fetizon's reagent (Ag_2CO_3 on celite), TEA, CH_2Cl_2.



Scheme 3. Diazo decomposition. (A) Nucleophile-mediated; (B) reduction-mediated.

In the latter two cases, extrapolation of the reported reaction conditions to a cellular environment remains ambiguous. Dmitrienko utilized β-naphthol as a nucleophile in THF,[18] while Feldman employed AIBN/Bu_3SnH in refluxing benzene as the source of a one-electron reductant.[20] Neither mechanistic study addressed DNA cleavage.

Based upon these two hypotheses, we explored kinamycin D cleavage of the supercoiled plasmid pBR322 under a variety of nucleophilic and reductive conditions. Kinamycin D was first incubated with pBR322 (Fig. 3, lane 2), and no DNA cleavage was noted, even after 3 days. Kinamycin D was then incubated with pBR322 and either dithiothreitol (DTT), nicotinamide adenine dinucleotide phosphate (NADPH), or sodium cyanide (NaCN). Only in the presence of DTT (Fig. 3, lane 4) did we observe any appreciable DNA damage. We also conducted a time course of kinamycin D-mediated DNA cleavage in the presence of DTT (data not shown) and noted maximal cleavage after 2 days.

Under these optimized conditions (DNA, DTT, 2 days), we assayed each of our diazofluorene derivatives for their ability to cleave DNA. Despite our best efforts, 1-aminodiazofluorene was unstable and decomposed (even storing at −80 °C). We observed that the 1-methoxydiazofluorene and the 4-aminodiazofluorene derivatives cleaved DNA with similar efficiency as kinamycin D (Fig. 4, lanes 1 and 3, compounds **9** and **10** are included for reference). All other diazo compounds, including the diazofluorene parent, did not induce extensive damage to the supercoiled plasmid.

Next, we assayed for the ability of 1-methoxydiazofluorene **2** and 4-aminodiazofluorene **5** to cleave DNA in the absence of DTT. Under these conditions, 4-aminodiazofluorene **5** retained DNA cleavage activity, while 1-methoxydiazofluorene **2** displayed minimal activity. 1-Methoxydiazofluorene **2** did not cleave DNA in the presence of either NaCN or NADPH (Fig. 5). We also confirmed (data not shown) that the diazo group was essential for DNA cleavage activity by assaying for the ability of 1-methoxyfluorenenone and 4-aminofluorenone to induce DNA damage. Neither compounds possessed DNA cleavage activity.

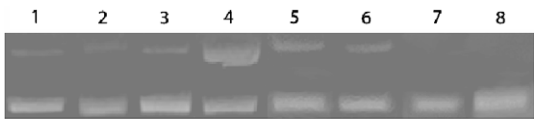


Figure 3. Plasmid cleavage assay, top band is type II nicked DNA, the bottom band is type I supercoiled DNA. Conditions: all lanes contain 714 ng of plasmid DNA. Lanes 2, 4, 6, and 8, final [kinamycin D] = 1.0 mM. Lane 1, DNA; lane 2, kinamycin D + DNA; lane 3, DNA/DTT (1.0 M); lane 4, kinamycin D + DNA/DTT (1.0 M); lane 5, DNA/NADPH (0.5 M); lane 6, kinamycin D + DNA/NADPH (0.5 M); lane 7, DNA/NaCN (1.0 M); lane 8, kinamycin D + DNA/NADPH (0.5 M).

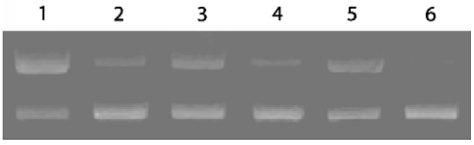


Figure 4. Plasmid cleavage assay, top band is type II nicked DNA, the bottom band is type I supercoiled DNA. Conditions: all lanes contain 714 ng of plasmid DNA, final [DTT] = 1.0 M, final [compound] = 1.0 mM. Lane 1, **2**; lane 2, **9**; lane 3, **5**; lane 4, **10**; lane 5, kinamycin D; lane 6, DNA only.

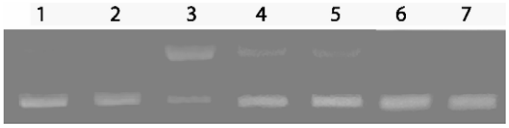


Figure 5. Plasmid cleavage assay, top band is type II nicked DNA, the bottom band is type I supercoiled DNA. Conditions: all lanes contain 714 ng of plasmid DNA. Final [compound] = 1.0 mM. Lane 1, DNA only; lane 2, **2**/DNA; lane 3, **5**/DNA; lane 4, 0.5 M NADPH/DNA; lane 5, **2**, 0.5 M NADPH/DNA; lane 6, 1.0 M NaCN/DNA; lane 7, **2**, 1.0 M NaCN/DNA.

We quantitated the extent of conversion from type I supercoiled DNA to type II nicked DNA for each compound. This is summarized in Table 1. In the presence of DTT, 1-methoxydiazofluorene **2** exhibited 150% of the cleavage potential of kinamycin D, while 4-aminodiazofluorene **5** exhibited 64% activity in comparison to kinamycin D. Interestingly, 4-aminodiazofluorene **5** exhibited increased cleavage activity in the absence of DTT. We are conducting extensive mechanistic studies to delineate the differential reactivity between 1-methoxydiazofluorene **2** and 4-aminodiazofluorene **5**.

Table 1. Quantitation of DNA cleavage (%) by kinamycin D and diazofluorene analogs

Compound	No promoter	DTT
Kinamycin D	<5	28 ± 7
Diazo parent	<5	<5
1-Aminodiazofluorene (**1**)	nd	nd
1-Methoxydiazofluorene (**2**)	11 ± 9	41 ± 6
1-Chlorodiazofluorene (**3**)	<5	<5
1-Nitrodiazofluorene (**4**)	<5	<5
4-Aminodiazofluorene (**5**)	44 ± 7	18 ± 8
4-Methoxydiazofluorene (**6**)	<5	13 ± 6
4-Chlorodiazofluorene (**7**)	<5	<5
4-Nitrodiazofluorene (**8**)	<5	<5
1,4-Dimethoxydiazofluorene (**9**)	<5	9 ± 8
Diazoquinofluorene (**10**)	<5	10 ± 9

nd, not determined.

Finally, we have assayed 1-methoxydiazofluorene **2**, 4-aminodiazofluorene **5**, and the diazo parent for antiproliferative activity against HeLa cells. We chose this compound set to provide validation that achieving DNA cleavage under reducing conditions was the key to achieving antiproliferative activity. Previous studies have shown that the diazofluorene parent possesses DNA cleavage activity; however, it is only active under oxidizing conditions (Cu^{2+}).[8] Therefore, this set allows us to assay compounds that achieve maximal DNA cleavage under disparate reaction conditions: thiol-mediated reducing conditions (diazofluorene **2**), no promoter (diazofluorene **5**), and oxidizing conditions (diazofluorene parent). All antiproliferative studies were conducted at 100 nM, the solubility limit of the synthetic diazo compounds in cell culture media. Although IC_{50} values could not be established due to this limited solubility, we did observe that 1-methoxydiazofluorene **2** had the highest activity of the three diazo compounds. 1-Methoxydiazofluorene **2** demonstrated a time-dependent inhibition of HeLa cell proliferation and inhibited cell growth 35–40% at 12 h. This activity is similar to the antiproliferative activity of the most active kinamycins.[3]

In conclusion, we have successfully identified 1-methoxydiazofluorene **2** as a simplified diazo compound that recapitulates the DNA cleaving activity of kinamycin D under thiol-mediated conditions. In addition, we have demonstrated that achieving DNA cleavage activity under thiol-mediated conditions translates into antiproliferative activity, and we have identified simple diazo compounds that have similar anticancer activity as the structurally complex kinamycins. We are currently working toward improving solubility and incorporating these structural elements into simplified lomaiviticin A analogs, and will report on their antiproliferative activity shortly.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.07.024.

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