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Synthesis and evaluation of novel 8-oxo-8*H*-cyclopenta[*a*]acenaphthylene-7-carbonitriles as long-wavelength fluorescent markers for hypoxic cells in solid tumor

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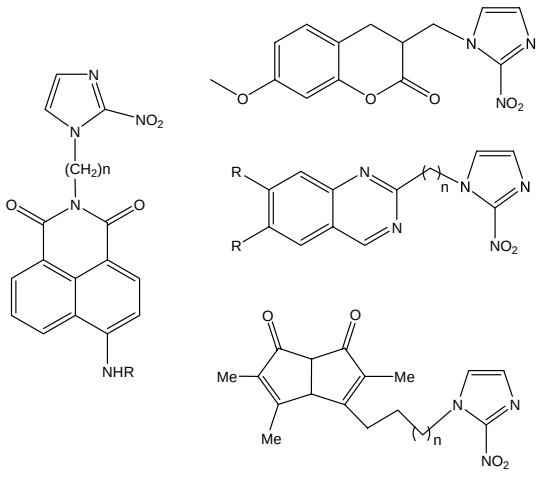
Abstract—Novel bioreductive and long-wavelength fluorescent markers for hypoxic cells in solid tumor, 9-isocyano-8*H*-acenaphtho[l,2-*b*]pyrrol-8-one with the side chain of 2-nitroimidazole, were designed, synthesized, and evaluated in V79 379A Chinese hamster cells in vitro. Compounds $\mathbf{A_2}$ and $\mathbf{A_4}$ showed good hypoxic–oxic fluorescence differential in vitro (V79 cells) by using fluorescence scan ascent.

Most of the solid tumor cells are hypoxic. There is now clear evidence that the oxygenation status of cells in tumors can influence the response of these tumors to therapy. Hypoxic cells are more resistant to radiation than cells under well-oxygenated conditions.[1] Tumor oxygenation status has also been shown to be an important factor in the response of tumors to certain chemotherapeutic agents, cytokines, hyperthermia, and photodynamic therapy. Therefore, if one could accurately measure the oxygenation status of individual tumors, one should be able to better predict treatment outcome and select appropriate therapies to improve it.[2]

Up to now, a number of methods have been proposed for measuring tumor oxygenation status, such as oxygen microelectrodes, histomorphometric analysis, DNA strand breaks, etc., however most of them invasive and not readily available to most investigators.[3] A simpler and easier especially non-invasive method for identifying hypoxic cells is suggested to be the use of fluorescent nitroaromatic compounds. The nitro group quenches the fluorescence of the aromatic ring system, but on bioreduction of the nitro group in hypoxic cells the compound becomes more fluorescent. Numerous nitroaromatic structures have been evaluated in model experiments in vitro (Scheme 1).[4] Although some promising



Scheme 1. Structures of some reported fluorescent markers for hypoxic cells.

Keywords: Fluorescent markers; Synthesis; Evaluation; Hypoxic cells; Solid tumor.
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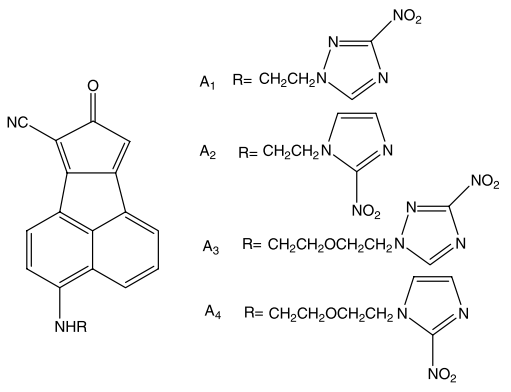
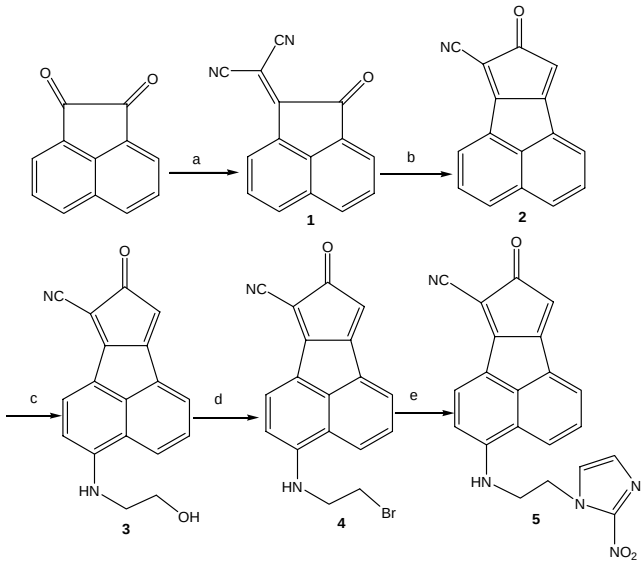
results have been obtained by using flow cytometry, which is expensive and not easily available to common researchers, the maximal absorption wavelengths of most compounds are less than 500 nm, the low sensitivity of the eye to the bluish fluorescence from these compounds making them less suitable for investigations involving microscopy.[5] In fact, the background emission (generally in short-wavelength range) from biological systems might be the interference in the accurate detection. Therefore, the compounds with long absorption or fluorescence wavelength are thought to be more suitable as fluorescent markers for hypoxic cells, and the relevant approach by using fluorescence scan ascent, which is cheaper and easily available, is also important and significant for measuring tumor oxygenation status.

8-Oxo-8*H*-cyclopenta[*a*]acenaphthylene-7-carbonitrile is a kind of novel fluorescent chromophore with long absorption and fluorescence wavelength, whose excitation and emission wavelengths can reach 530 nm and 590 nm, respectively.[6] Another characteristic is its lower DNA intercalating properties, which could avoid problems in vivo, as a compound with the high DNA affinity could usually make the probe become localized to blood vessels or the nuclear region of well-oxygenated cells in tumors and fail to reach those hypoxic cells. Therefore, in this study, 8-oxo-8*H*-cyclopenta[*a*]acenaphthylene-7-carbonitrile bound with a side chain of 2-nitroimidazole (**A_2** and **A_4**) or 3-nitro-l,2,4-triazole (**A_1** and **A_3**) as bioreductive moiety for hypoxic cells in solid tumor was designed, synthesized, and evaluated (Scheme 2).

These compounds were synthesized from acenaphthylene- 1,2-dione shown in Scheme 3 (with compound **A_2** as an example). Acenaphthylene-l,2-dione reacted with malononitrile at room temperature in CH_2C1_2 and silica gel to give red solid (**1**), which was refluxed for 1 h in CH_3CN in the presence of K_2CO_3 as a catalyst to afford umber solid of 8-oxo-8*H*-cyclopenta[*a*]acenaphthylene-7-carbonitrile (**2**). Then this solid reacted with $NH_2CH_2CH_2OH$ in CH_2C1_2 at room temperature for 1 h to give 3-(2-hydroxyethylamino)-8-oxo-8*H*-cyclopenta[*a*]acenaphthylene-7-carbonitrile (**3**). And (**3**) reacted with bromine in ethyl acetate with PCl_3 as a catalyst to provide 3-(2-bromoethylamino)-8-oxo-8*H*-cyclopenta[*a*]acenaphthylene-7-carbonitrile (**4**). Finally,



Scheme 2. Novel fluorescent markers for hypoxic cells.



Scheme 3. Syntheses of target compounds. Reagents and conditions: (a) malononitrile, CH_2Cl_2, silica gel, rt, 95% yield; (b) K_2CO_3, CH_3CN, reflux, 1 h, 90% yield; (c) $NH_2CH_2CH_2OH$, CH_2C1_2, rt, 1 h, 75% yield; (d) Br_2/PCl_3, ethyl acetate, reflux, 5 h, 85% yield; (e) 2-nitroimidazole or 3-nitro-1,2,4-triazole, CH_3ONa/DMF, reflux, 8 h, 35% yield.

a solution of (**4**) in DMF was added to the mixture of sodium methoxide and 2-nitroimidazole, and heated at 150 °C for 8 h to give target compounds (**A_1**–**A_4**). All the structures were confirmed by IR, ^{1}H NMR, and HR-MS.[7]

The structures of novel compounds are shown in Scheme 1. It was found from Table 1 that the maximal absorption and emission wavelengths of compounds **A_1**–**A_4** were about 510 and 592 nm, respectively, and their fluorescences were still strong, which mean that nitro group could not quench the fluorescence of the aromatic ring system completely.

V79 379A Chinese hamster cells were used for evaluation of the compounds, which were maintained as exponentially growing suspension cultures in Eagle's minimal essential medium with Earle's salts, modified for suspension cultures with 7.5% fetal calf serum. The compounds were added to cell suspensions to give the appropriate concentration at 10^{-4} M. Then the suspension was incubated in special gases (air + 5% CO_2, nitrogen + 5% CO_2) at 37 °C.[4] Samples from hypoxic and oxic cell suspensions incubated with the solution of compounds for different times were initially evaluated by fluorescence

Table 1. Spectra data of compounds **A_1**–**A_4**[a,b]

Compound	UV λ_{max} (lg ε)	FL λ_{max} (ϕ)
A_1	515 (4.48), 566 (4.93)	591 (0.16)
A_2	510 (4.54), 560 (4.91)	591 (0.13)
A_3	517 (4.49), 567 (4.92)	592 (0.19)
A_4	511 (4.47), 562 (4.92)	592 (0.15)

[a] In acetone.
[b] With quinine sulfate in sulfuric acid as quantum yield standard (ϕ = 0.55).

microscopy using 510 nm excitation wavelengths. Then a quantitative study of the time courses of accumulation of fluorescent metabolites in the same condition was carried out with fluorescence scan ascent. The results are shown in Figure 1.

From Figure 1, obvious differential of the fluorescence can be seen between the cells incubated under hypoxic and oxic conditions. It was found by using fluorescence scan ascent that the fluorescence of cells incubated under oxic condition had little change all the time, but the case was different with hypoxic cells. The compounds $\mathbf{A_2}$ and $\mathbf{A_4}$ (with 2-nitroimidazole as reductive moiety) in hypoxic cells were reduced by nitroreductase enzymes in two-electron steps in an oxygen-insensitive process, which resulted in significant fluorescence enhancement of cells incubated during three hours. After 3 h, the fluorescence in cells incubated reached the maximal value and then kept it. Of course, the hypoxic–oxic differential reached the largest at the same time.

The largest hypoxic–oxic fluorescence differential of $\mathbf{A_4}$ in cells (15 times) was higher than that of $\mathbf{A_2}$ (11 times). The difference might have been caused by two factors: (1) The introduction of an ether moiety as a hydrophilic group into the side chain of $\mathbf{A_4}$ promoted its water solubility, thus improving the transport of compound in vivo to tumor cells. (2) Weak fluorescence quenching caused by a slight intramolecular photoinduced electron transfer (PET) from the reduction products of the nitroimidazole moiety to the naphthalimide nucleus of $\mathbf{A_4}$.[8] It was reported that the PET efficiency was related to several aspects:[9,10] (a) It was directly related to the presence of the electron donors. That is, the electron-deficient moiety of 2-nitroimidazole would become electron-rich moiety of 2-aminoimidazole after bioreduction, which was easy to proceed with PET to quench fluorescence somewhat and balance the fluorescence enhancement. (b) Longer spacer can also weaken the quenching efficiency by preventing effective approach of electron from the donor to the receptor. In this paper, the side-chain length of $\mathbf{A_2}$ was shorter than that of $\mathbf{A_4}$, PET efficiency of the reduction product of $\mathbf{A_2}$ was higher than that of $\mathbf{A_4}$, and fluorescence quenching efficiency of $\mathbf{A_2}$'s reductive product should be also higher than that of $\mathbf{A_4}$, so that hypoxic–oxic fluorescence differential of $\mathbf{A_2}$ was lower than that of $\mathbf{A_4}$.

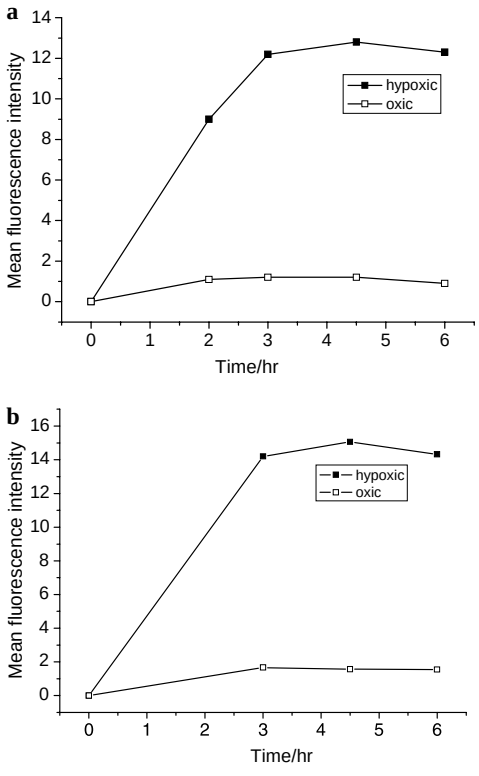


Figure 1. The time courses of accumulation of fluorescent metabolites in V79 379A Chinese hamster cells incubated with 10^{-4} M compounds at 37 °C. (a) $\mathbf{A_2}$; (b) $\mathbf{A_4}$.

The study of the time courses of accumulation of fluorescent metabolites in V79 cells incubated with $\mathbf{A_1}$ and $\mathbf{A_3}$, was also carried out by using fluorescence scan ascent, the hypoxic–oxic fluorescence differential incubated with $\mathbf{A_1}$ and $\mathbf{A_3}$ being 4 times and 6 times, respectively. The difference between $\mathbf{A_1}$ and $\mathbf{A_3}$ was caused by the same two factors as the case of $\mathbf{A_2}$ and $\mathbf{A_4}$.

The difference in fluorescence behavior between two kinds of compounds, $\mathbf{A_2}$, $\mathbf{A_4}$ and $\mathbf{A_1}$ $\mathbf{A_3}$, might be due to that of their bioreductive moieties in the side chains. 2-Nitroimidazole was a strong electron-deficient moiety by comparison with 3-nitro-l,2,4-triazole,[8] but the electron-donating ability for bioreductive product (corresponding to the PET efficiency for fluorescence quenching to balance the fluorescence enhancement during bioreduction) of the latter was higher than that of the former, which finally make $\mathbf{A_1}$ and $\mathbf{A_3}$ have small hypoxic–oxic fluorescence differential in cells. From the redox point of view, the oxidation potential of the electron donor species is one of the most important factors affecting the PET efficiency.[11] The strong electron-rich reductive product of 3-nitro-l,2,4-triazole was easier to lose electron for oxidation than the bioreductive product of 2-nitroimidazole did, which resulted in a higher PET efficiency of $\mathbf{A_1}$ and $\mathbf{A_3}$.

By fluorescence microscopy, we also got the fluorescence microphotographs of V79 cells incubated with 10^{-4} M of $\mathbf{A_2}$ and $\mathbf{A_4}$, respectively (Figs. 2 and 3).

It was found by using fluorescence microscopy that the cells incubated with compounds ($\mathbf{A_1}$–$\mathbf{A_4}$) showed red fluorescence under irradiation of light at 510 nm, although hypoxic–oxic fluorescence differential in the images was not obvious enough (Figs. 2 and 3). However, more importantly, obvious differential fluorescence between hypoxic and oxic cells was observed and recorded by using fluorescence scan ascent (Fig. 1). In fact, a number of nitroaromatic compounds have been evaluated as fluorescent markers for hypoxic cells in model experiments in vitro, emissions for most of the reduced products in hypoxic cells being in short wavelength, for example, blue, green, and yellow fluorescence.

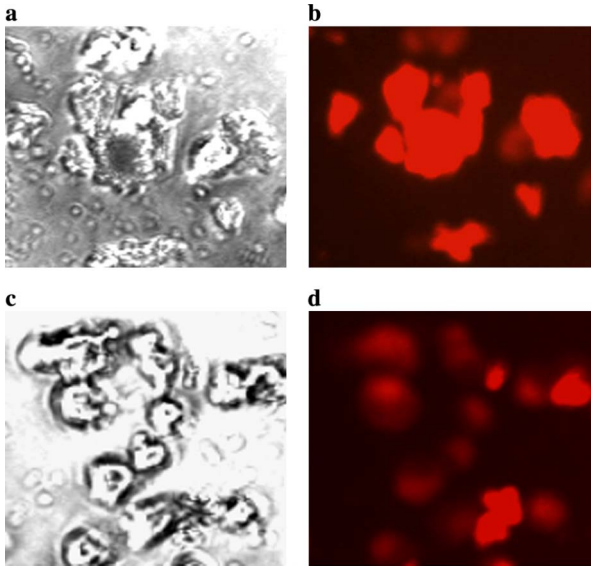


Figure 2. Fluorescence microphotographs of V79 cells incubated with 10^{-4} M of $\mathbf{A_2}$ at 37 °C. After 3.5 h incubation, scanning was taken. Magnification was 1000×. (a) Scanning was taken on brightfield, cells under hypoxic condition (incubated in nitrogen and 5% CO^2); (b) excited at 510 nm, cells under hypoxic condition; (c) scanning was taken on brightfield, cells under oxic condition (incubated in air and 5% CO^2); (d) excited at 510 nm, cells under oxic condition.

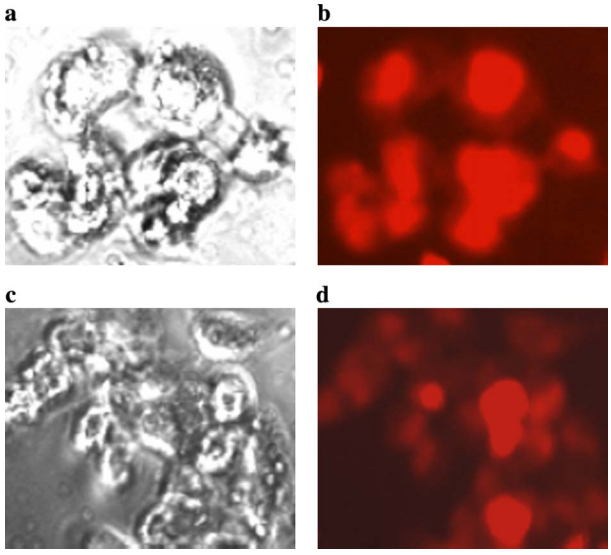


Figure 3. Fluorescence microphotographs of V79 cells incubated with 10^{-4} M of $\mathbf{A_4}$ at 37 °C After 3.5 h incubation, scanning was taken. Magnification was 1000×. (a) Scanning was taken on brightfield, cells under hypoxic condition (incubated in nitrogen and 5% CO_2); (b) excited at 510 nm, cells under hypoxic condition; (c) scanning was taken on brightfield, cells under oxic condition (incubated in air and 5% CO_2); (d) excited at 510 nm, cells under oxic condition.

In summary, the present work demonstrated novel long-wavelength fluorescent markers for hypoxic cells in solid tumor. Some of the compounds ($\mathbf{A_2}$ and $\mathbf{A_4}$) showed obvious fluorescence differential between hypoxic and oxic cells (V79 cells) in vitro by using fluorescence scan ascent. When time was approaching 3 h, the hypoxic–oxic fluorescence differential incubated with $\mathbf{A_2}$ and $\mathbf{A_4}$ in V79 cells could reach 11 times and 15 times, respectively, which revealed they might be promising candidate markers for hypoxic cells.

Acknowledgments

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7. Compound $\mathbf{A_1}$: mp > 300 °C. ^{1}H NMR (SF: 500 MHz, $CDCl_3$-d_1), δ (ppm), 8.86 (d, 1H, J = 8.64 Hz), 8.75 (d, 1H, J = 7.29 Hz), 8.72 (d, 1H, J = 8.01 Hz), 8.42 (d, 1H, J = 7.99 Hz), 7.99 (t, 1H, J_1 = 7.43 Hz, J_2 = 7.55 Hz,), 7.05 (s, 1H, triazole-5′H), 4.47 (t, 2H, J_1 = 5.41 Hz, J_2 = 5.39 Hz, –CH_2–triazole), 4.01 (t, 2H, J_1 = 5.46 Hz, J_2 = 5.33 Hz, –NHCH_2–). HRMS (ESI): $C_{19}H_{11}N_7O_3$ calculated: 385.0923; found: 385.0925; IR (KBr), cm^{-1}: 3310, 1672, 1595, 1570. Compound $\mathbf{A_2}$: mp > 300 °C. ^{1}H NMR (SF: 500 MHz, $CDCl_3$-d_1), δ (ppm), 9.35 (d, 1H, J = 2.00 Hz), 9.16 (d, 1H, J = 2.05 Hz), 8.80 (d, 1H, J = 7.29 Hz), 8.45 (d, 1H, J = 7.25 Hz), 7.97 (t, 1H, J_1 = 7.79 Hz, J_2 = 7.75 Hz), 7.92 (d, 1H, J = 7.48, imidazole- 4′H), 6.78 (d, H, J = 8.43, imidazole-5′H), 4.50 (t, 2H, J_1 = 4.48 Hz, J_2 = 5.38 Hz, –CH_2–imidazole), 4.01 (t, 2H, J_1 = 5.42 Hz, J_2 = 5.42 Hz, –NHCH_2–). HRMS (ESI): $C_{20}H_{12}N_6O_3$ calculated: 384.0971; found: 384.0975; IR (KBr), cm^{-1}: 3455, 2212, 1625, 1585, 1570. Compound $\mathbf{A_3}$: mp > 300 °C ^{1}H NMR (SF: 500 MHz, MeOD-d_4), δ (ppm), 8.42 (m, 2H,), 8.25 (d, 1H, J = 8.56 Hz), 7.54 (d, 1H, J_1 = 7.60 Hz, J_2 = 8.19 Hz), 7.04 (s, 1H, triazole-5′H), 6.74 (d, 1H, J = 8.56Hz), 4.31

(m, 4H, –NHCH_2CH_2O–), 3.78 (t, 2H, OCH_2CH_2–, J_1 = 5.71 Hz, J_2 = 5.8 Hz), 3.49 (t, 2H, J_1 = 5.76 Hz, J_2 = 5.72 Hz, -OCH_2CH_2–). HRMS (ESI): $C_{21}H_{15}N_7O_4$ calculated: 429.1185; found: 429.1188; IR (KBr), cm^{-1}: 3320, 2215, 1638, 1580, 1492. Compound **A_4**: mp > 300 °C. ^{1}H NMR (SF: 500 MHz, acetone-d_6), δ (ppm), 8.12 (d, 1H, J = 8.10 Hz), 8.06 (d, 1H, J = 7.10 Hz), 7.82 (t, 1H, J_1 = 7.53 Hz, J_2 = 7.55 Hz), 7.64 (d, 2H, J = 7.45 Hz, imidazole-5′H), 7.55 (d, 1H, J_1 = 7.30 Hz, J_2 = 7.17 Hz), 7.25 (d, 1H, J = 7.32 Hz, imidazole-4′H), 4.11 (m, 6H, –NH$CH_2CH_2OCH_2CH_2$–), 3.59 (d, 2H, J = 4.7 Hz, –OCH_2CH_2–). HRMS (ESI): $C_{22}H_{16}N_6O_4$ calculated: 428.1233; found: 428.1236; IR (KBr), cm^{-1}: 3320, 1632, 1570, 1490.

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