Cyclization kinetics and biological evaluation of an anticancer 1,2-dialkynylimidazole†

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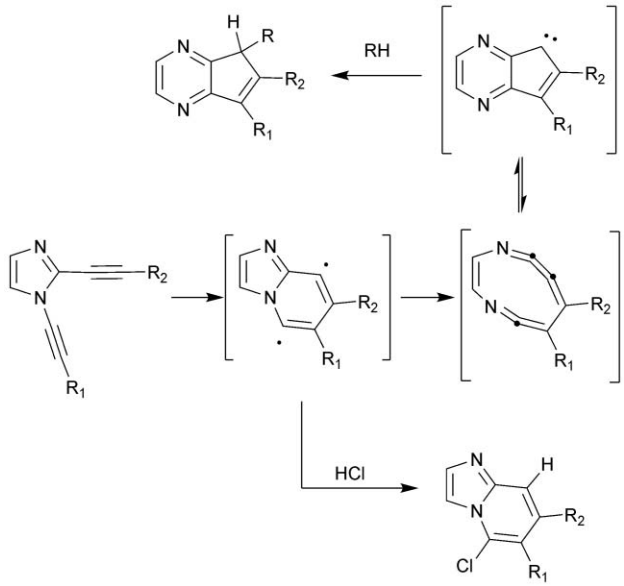
1,2-Dialkynylimidazoles have been reported to undergo thermal cyclization/rearrangement to diradical and carbene intermediates. Optimization of the synthesis of the 1,2-dialkynylimidazole 3 has provided sufficient material for kinetic and biological studies. The 1,2-dialkynylimidazole 3 is cytotoxic against a wide range of cancer cells and induces apoptosis in A549 cells. Experimentally-determined kinetics of the thermolysis of 3 (E_a = 30.0 kcal mol^{-1}) are in excellent agreement with DFT calculations of the cyclization/rearrangement to diradical and cyclopentapyrazine carbene intermediates (E_a = 29.7 kcal mol^{-1}). Commensurate with the relatively high barrier for cyclization of 3, no evidence for cleavage of supercoiled DNA under physiological conditions was found; however, under aqueous conditions at 70 °C 3 formed a covalent adduct with a model peptide. These studies indicate that if cyclization of 3 is involved in its anticancer activity, the cyclization must be facilitated, perhaps through initial protein binding, which could lead to covalent protein modification.

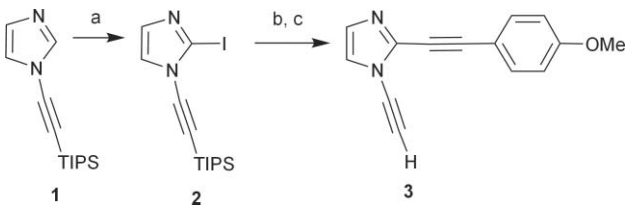
The enediyne structural moiety is present in several naturally occurring anticancer antibiotics.[1] These antitumor enediyne natural products exhibit their cytotoxicity as a result of their DNA cleavage ability based on the generation of 1,4-benzenoid diradical intermediates arising from the Bergman cyclization of the enediyne core.[2] Numerous efforts to design cytotoxic DNA-cleaving enediynes[3] or compounds that undergo alternative diradical-generating cyclizations[4] have been reported. Certain designed enediynes have been found to target proteins in addition to, or instead of, DNA.[5]

Efforts to design alternative diradical-generating cyclizations led to the preparation and study of 1,2-dialknylimidazoles (Scheme 1).[6,7] Initial reports of the synthesis of 1,2-dialkynylimidazoles as well as their thermal rearrangement to products derived from trapping of didehydroimidaza[1,2-*a*]pyridine diradical intermediates or cyclopentylpyrazine carbene intermediates have been reported (Scheme 1).[6]



Scheme 1 Thermolysis of 1,2-dialkynylimidazoles.

More recently, a 1,2-dialkynylimidazole covalent inhibitor of p38α kinase was described, although the roles of either the diradical or carbene intermediates in this inhibition were not addressed.[8] Based on the potential of dialkynylimidazoles to behave like anticancer enediynes, computational and experimental studies of the structure, cyclization, DNA- and protein-interactions, and cancer cell cytotoxicity of a representative 1,2-dialkynylimidazole, 1-ethynyl-2-((4-methoxy-phenyl)ethynyl)-1*H*-imidazole (**3**) (Scheme 2) were undertaken.



Scheme 2 Synthetic route to 1,2-dialkynylimidazole **3**. (a) *n*-BuLi, THF, –78 °C 15 min., then I_2, –78 °C 30 min (83%); (b) $Pd(PPh_3)_4$, CuI, RCCH, Et_3N, 50 °C (100%); (c) TBAF, THF, –78 °C 30 min (90%).

The key step in 1,2-dialkynylimidazole synthesis involves the formation of the 1-alkynylimidazole moiety. In the previously reported synthesis of compound **3**, an inefficient coupling between imidazolate and a hypervalent iodine reagent was employed.[6c] Recently, the synthesis of 1-alkynylimidazoles by coupling reaction between imidazoles and bromoalkynes in the presence of

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catalytic copper salts and 2-acetylcyclohexanone (AcC) ligand[9] or PEG400[10] has been reported. The two-step conversion of 1-alkynylimidazoles to 1,2-dialkynylimidazoles *via* 2-position iodination followed by Sonogashira coupling has recently been described.[11] However, while this approach affords both superior yields and wider scope compared to previous routes, it suffers from the requirement for an excess of base and bromoalkyne partner in the ligand-mediated copper-catalyzed coupling reaction. Given the need to produce sufficient quantities of **3** to study both its chemistry and biology, the coupling reaction conditions were optimized with the objective of decreasing the quantities of bromoalkyne and base necessary for the preparation of the alkynylimidazole **1** (Scheme 2). Thus imidazole and 1-bromotriisopropylsilylacetylene were coupled under various conditions (see Electronic Supporting Information). These studies reveal that when employing 5 mole% of CuI and a 20 mole% of AcC ligand, the effect of decreasing the quantity of Cs_2CO_3 base from two to one equivalent was modestly unfavorable; whereas, decreasing the quantity of bromoalkyne had a much more pronounced deleterious effect on yield. However, the reaction exhibited better yields when lower quantities of copper/ligand were used. This observation corroborates the hypothesis of the formation of catalytically inactive aggregates of the copper ion and the imidazole.[9] Reducing the quantity of copper disfavors the presence of such polymorphic complexes. Thus, when employing just 0.5 mole% of CuI and 2 mole% of ligand, with 1.1 equiv. of both the bromoalkyne and Cs_2CO_3 the yield of **1** was 79%, which is comparable to the yield employing the originally described procedure, but only requires a slight excess of bromoalkyne and base.

The 1-alkynylimidazole **1** was converted into the dialkynylimidazole **3** *via* iodination to **2** followed by Sonogashira coupling and protodesilylation (Scheme 2). This sequence was carried out on 5 mmole of alkynylimidazole **1** to afford a 59% overall yield of **3**. Notably, the Sonogashira coupling (Scheme 2, step b) was realized in quantitative yield using only 1 mole% of $Pd(PPh_3)_4$ and 2 mole% of CuI. The structure of **3** was confirmed by X-ray crystallography (Fig. 1). The structure reveals a relatively short (N)Csp^1–C sp^1 bond length of 1.167 Å.[12] This, combined with exocyclic imidazole C2–N1–alkyne (126.0°) and imidazole N1–C2–alkynyl (120.8°) bond angles leads to a relatively short 4.129 Å c,d distance[13] between the two distal Csp^1 atoms C4 and C15.[14] In addition, the crystallographic packing, shown in Fig. 2, reveals intermolecular C–H···O interactions between the ether oxygen and the terminal Csp^1-H of adjacent molecules.

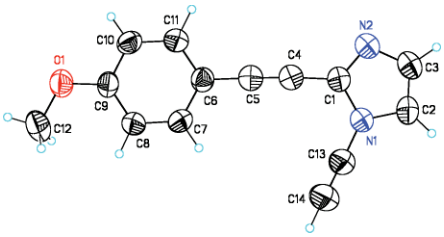


Fig. 1 View of **3** showing the atom labelling scheme. Displacement ellipsoids are scaled to the 50% probability level.

The cytotoxicity[15] of **3** has been evaluated by the National Cancer Institute against 60 human cancer cell lines. The dialkynylimidazole **3** displayed activity against a range of cell lines with GI_{50} values from 10^{-8} to 10^{-6} M and a mean GI_{50} of 3 μM. A COMPARE analysis[16] of the spectrum of activity of **3** against that of standard anticancer drugs revealed no similarities (correlation co-efficient < 0.5); however, within the set of compounds selected by the NCI for *in vivo* testing, the activity of **3** correlated most strongly (P = 0.727 to 0.602) to a series of quinoxaline 1,4-dioxides. These specific quinoxaline 1,4-dioxides have been shown to generate hydroxyl radicals and cleave DNA under aerobic conditions through redox cycling involving the NADPH/cytochrome P450 reductase system.[17] Under anaerobic conditions, the one-electron reduction of quinoxaline 1,4-dioxides is accompanied by radical fragmentation to afford stoichiometric hydroxyl radicals.[18]

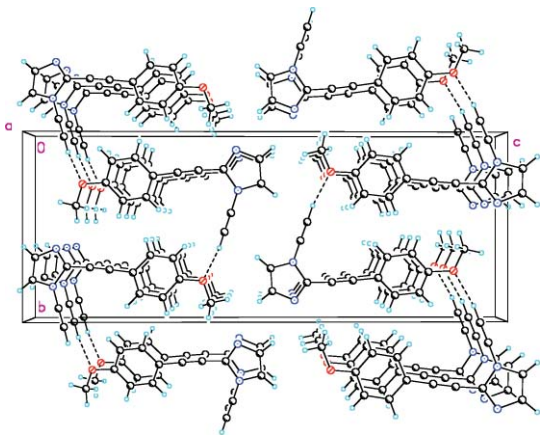


Fig. 2 Unit cell packing diagram for **3**. The view is approximately down the a axis. Dashed lines illustrate close C–H···O contacts between adjacent molecules. The geometry of this C14-H14···O1 interaction is: C-O 3.231(3) Å, H···O 2.25(2) Å, C–H···O 171(2)°.

In order to gain more insight into the mechanism of action of the dialkynylimidazole **3**, studies were conducted in the human non-small cell lung cancer cell line A549,[19] which is a well-characterized standard human alveolar epithelial cell line. A549 cells were incubated with various concentrations of **3** and the extent of apoptosis was determined by flow cytometry (Fig. 3).

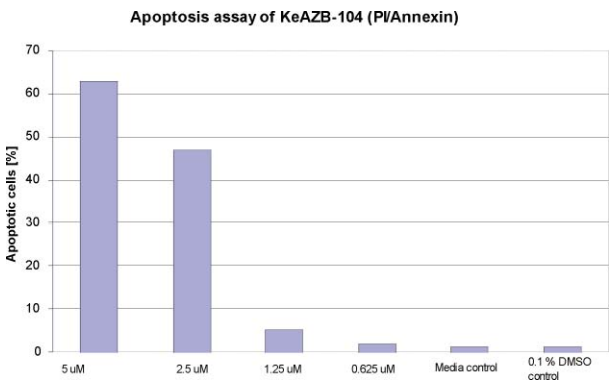


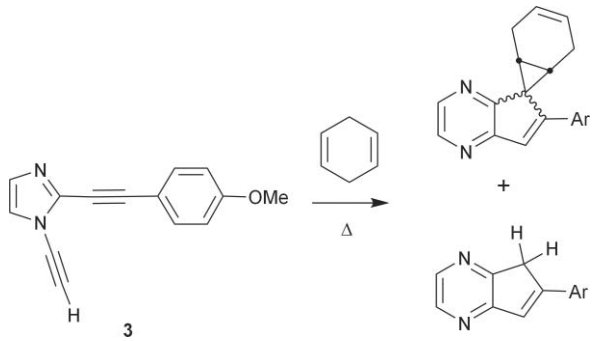
Fig. 3 Apoptosis of A549 cancer cells in the presence of **3**.

After treating A549 cells with 5 μM of compound **3** for 24 h, the proportion of apoptotic cells increased to 63%, 25-times higher than the proportion of apoptotic cells in the absence of **3**. Even at concentrations as low as 1.25 μM, compound **3** caused a significant increase in the proportion of apoptotic cells.

The spectrum of anticancer activity of **3** analyzed by COMPARE correlates with the known DNA cleaving quinoxaline 1,4-dioxides. Agents of a wide variety of mechanistic actions, including DNA-damaging agents, are able to induce apoptosis in A549 cells.[20] In order to probe more deeply the possible role of DNA damaging intermediates arising from cyclization of **3** in the anticancer activity of this compound, kinetic studies were undertaken. While the cyclization of **3** is reported to produce diradical and carbene intermediates (Fig. 1),[6] the kinetics of this process have not been previously investigated, either computationally or experimentally.

Although the Bergman and related diradical-generating cyclizations present significant challenges to computational methods, seminal work by Kraka and Cremer has shown that broken spin-symmetry unrestricted density-functional theory (BS-UDFT) methods employing hybrid functionals can perform as well as much more expensive methods on these systems.[21] This BS-UDFT method was applied to the computer design of new enediyne warheads,[22] although no experimental data for these are available. BS-UDFT calculations were carried out on the cyclization of **3** using the sum-corrected M1 method of Sherer and co-workers.[23] Briefly, these B3LYP/6-31G(d,p)//B3LYP/6-31G(d,p) calculations were performed using the broken-spin-symmetry, unrestricted method for the diradical and the transition states to and from this species, and the energies of the resulting singlets were corrected for triplet contamination using the sum method employing triplet energies of the BS-UB3LYP singlet geometries. These calculations indicate that the singlet diradical **5** resulting from aza-Bergman cyclization of **3** (Fig. 4) should rapidly rearrange to the cyclic cumulene **7**; the transition state **6** for this process lies just 2.4 kcal mol^{-1} above the singlet diradical **5**, and the S-T gap for **5** is 9.3 kcal mol^{-1}. There is a relatively low barrier for rearrangement of **7** to the cyclopentacarbene **9**. Despite an unfavorable equilibrium between the carbene **9** and the cumulene **7**, the reactivity of the former[24] leads to its efficient trapping. Thus, the rate-limiting step for formation of carbene-trapping products is the initial aza-Bergman cyclization.

Thermolysis of 1,2-dialkynylimidazole **3** in neat 1,4-cyclohexadiene yielded a mixture of cyclopentapyrazine trapping products (Scheme 3).[6d] Solutions of **3** and internal standard 5,6-benzoquinoline in 1,4-cyclohexadiene were heated at 80, 90, 100, 120 or 150 °C, and the rate of disappearance of **3** was monitored by LC-MS (Fig. 5).



Scheme 3 Products obtained from the thermolysis of 1,2-dialkynylimidazole **3** in neat 1,4-cyclohexadiene.

The kinetic experiments showed first-order disappearance of starting material with half-lives ranging from 13.5 h to 7 min. An Arrhenius plot of the first-order rate constants provides activation parameters E_a = 30.0 kcal mol^{-1}; $A = 6 \times 10^{12}$ (Fig. 6). The calculated activation energy ($E_a = \Delta H^{\ddagger} - RT$) at 115 °C for the aza-Bergman cyclization of **3** to the diradical **5** from the DFT calculations is 29.7 kcal mol^{-1}, in excellent agreement with the experimental value.

When incubated with supercoiled DNA at 37 °C for 24 h, dialkynylimidazole **3** showed no evidence of DNA cleavage ability. This is to be expected, since the extrapolated half-life for cyclization of **3** at 37 °C is longer than 5 years. Thus, it does not

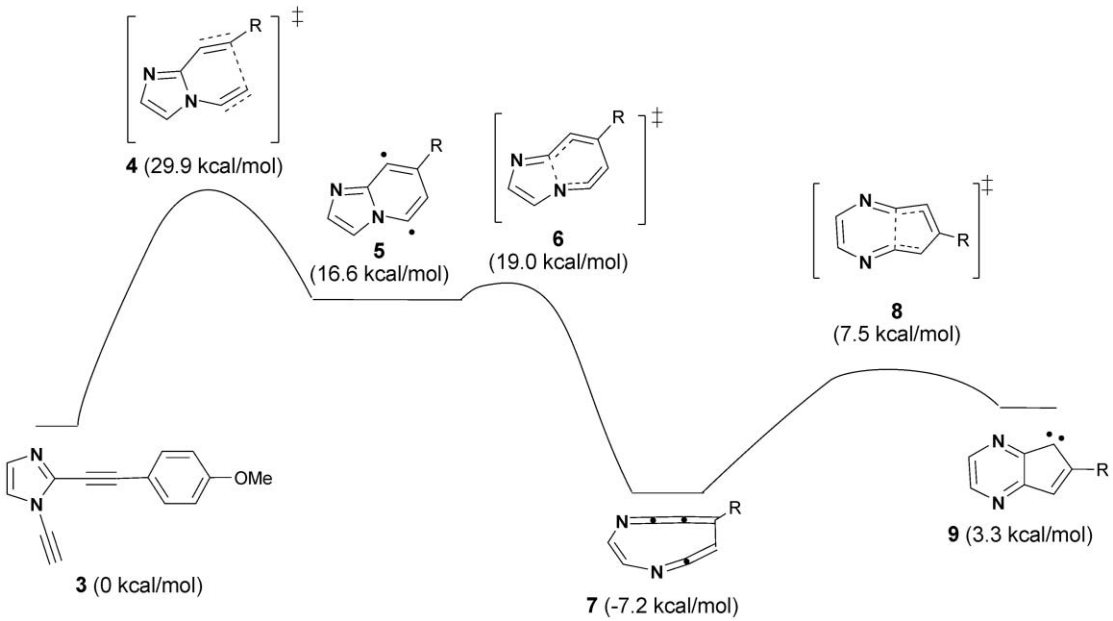


Fig. 4 Relative electronic energies + ZPE calculated at the BS-(U)B3LYP/6-31G(d,p) level for the thermal rearrangement of **3**.

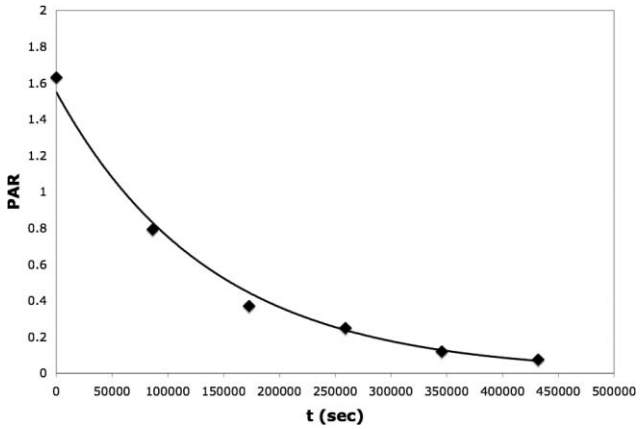


Fig. 5 Data obtained from the thermolysis of **3** at 90 °C.

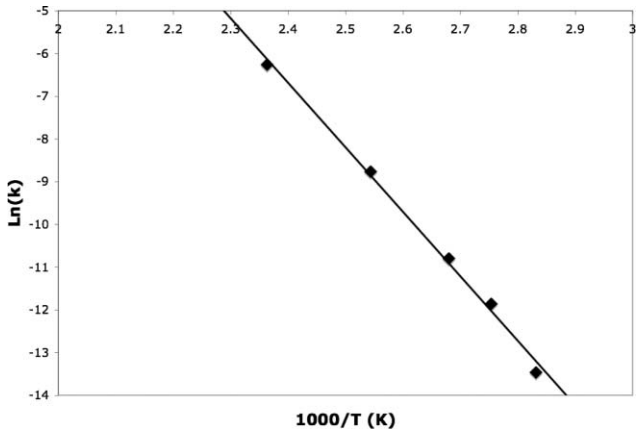


Fig. 6 Arrhenius plot from thermolysis of **3**.

appear likely that the cytotoxicity or the apoptosis of cancer cells due to **3** is a result of DNA strand scission. Perhaps the similarity in the cytotoxicity profile of **3** with that of quinoxaline 1,4-dioxides is due to the ability of both compounds to produce other types of DNA damage, or to interact with key proteins involved in cell proliferation and apoptosis.[25]

In light of the recent report of a 1,2-dialkynylimidazole covalent p38α inhibitor,[8] the ability of reactive intermediates derived from **3** to covalently interact with a model peptide under aqueous conditions was explored. Incubation of **3** (1 mM) with the peptide bradykinin [RPPGFSPFR] (1 mM) at 70 °C in 10 mM Tris buffer (pH 7.0) containing 2.5% DMSO (to solubilize **3**) for 24 h followed by MALDI-MS analysis demonstrates the formation of a small amount of an adduct corresponding to addition of **3** (or a reactive intermediate derived from it, see Scheme 3) to the peptide (Fig. 7).

Thus, in water, at temperatures where its rate of cyclization is significant, **3** is capable of covalently attacking peptides.

In summary, an improved procedure for the synthesis of 1-alkynylimidazole derivatives has been employed to prepare sufficient quantities of **3** for biological evaluation. The 1,2-dialkynylimidazole **3** is cytotoxic against a wide range of cancer cells and induces apoptosis in A549 cells. DFT calculations are in excellent agreement with experimentally determined kinetic analysis of the thermolysis of **3**; however, the rate of conversion of **3** to the diradical **5** or cyclopentapyrazine carbene **9** at physiological temperature is insignificant, ruling out any role for unassisted

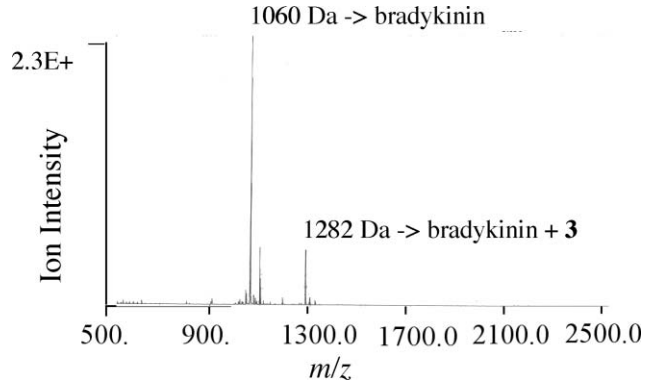


Fig. 7 MALDI-TOF analysis of bradykinin incubated at 70 °C for 24 hours with **3**.

cyclization in the cytotoxicity of this compound. While **3** does not cleave DNA at physiological conditions, at higher temperatures **3** can covalently adduct the peptide bradykinin. Thus, one intriguing possibility for the origin of the anticancer activity of **3** involves facilitated cyclization of **3** by protein binding, leading to the formation of protein-interactive intermediates such as diradical **5** or carbene **9** under physiological conditions. Further work to identify the specific molecular target(s) of **3** is underway.

Acknowledgements

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