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TARGET DIRECTED ENEDIYNE PRODRUGS: hER AND AhR DEGRADATION BY A SYNTHETIC OXO-ENEDIYNE

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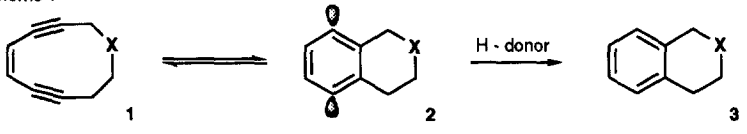
Abstract: An efficient route to oxo-enediynes is presented. A simple oxo-enediyne has been synthesized, which cyclizes to give an isochroman. The agent shows cytotoxicity for ER rich breast cancer cells and a model for its mode of action is proposed.

Interest in the preparation of cyclic enediynes has grown steadily over the past decade, fueled by reports of potent antitumoral activity for naturally occurring enediyne antibiotics, and many synthetic analogs.[1] The basic pharmacophore of the natural enediynes is often described by a cyclodec-3-ene-1,5 diyne unit (**1**, X=CH_2), and as part of our ongoing program in antitumor agent design,[2] we recently developed a synthetic approach to this core.[3] Biological or thermal activation results in Bergman cyclization of the core, yielding the reactive diyl radical species **2**, which, under appropriate conditions of hydrogen atom transfer, can form arene **3**.

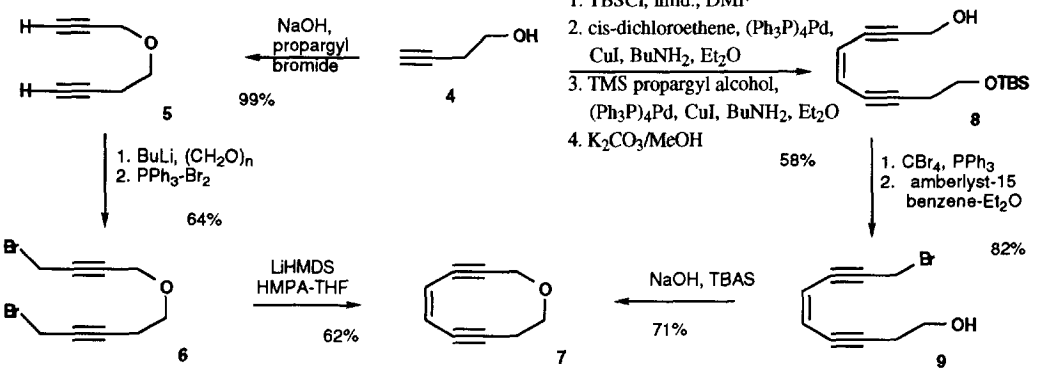
Scheme 1



In a cellular environment, this atom transfer event can result in formation of ribosyl radicals on DNA,[4] and has also been reported to result in the generation of peptide radicals,[5] in part accounting for the typically high biological activity of most cyclic enediynes. Interception of the diyl species **2** can also be encouraged using other radical donor sources,[6] as exemplified by Grissom in the synthesis of carbocyclic and oxygenated target molecules,[6b] and Semmelhack, in the preparation of 1,4 dihaloarenes.[6c] While most attention has been directed towards the preparation of carbocyclic enediynes, we wished to explore the utility of using heterocyclic enediynes for the synthesis of bioactive enediyne libraries, and became interested in oxo-enediynes (**1**, X=O) for a number of reasons: (a) a more economical route may be available; (b) the half-life of **1**, X=O at 37 °C is calculated to be slightly shorter than **1**, X=CH_2 (15 h vs 18 h)[7] making bioassay more convenient; and (c) more flexibility for subsequent synthetic transformation of the arene products of Bergman cyclization (**3**) would be offered, a consequence of the heteroatom.

The synthesis of oxo-enediyne **1**, X=O (**7**) was investigated using two independent routes, as shown in Scheme 2. While both routes commence from commercially available homopropargyl alcohol, they differ in the method of final ring closure, either involving a Williamson type closure via **9**, or the intramolecular carbenoid route from dibromide **6**, which was developed in this laboratory.[3] Overall, the carbenoid route *via* dibromide **6** proved far superior both in terms of efficiency and cost effectiveness, and could be conducted effortlessly on a multigram scale.

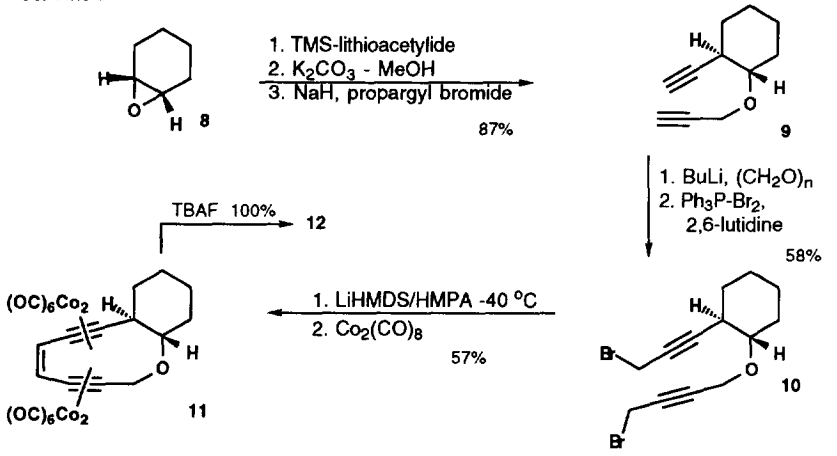
Scheme 2



With quantities of **7** in hand, Bergman cyclization was then investigated under controlled conditions; incubation of **7** at 37 °C in the presence of 1,4 cyclohexadiene (30 equiv) gave a nearly quantitative (>90%) yield of isochroman (**3**, X=O), presumably via the intermediacy of diyl **2**, X=O. Having established a high yielding and economical route to oxo-enediynes of type **1**, we sought to incorporate the method in the synthesis of more complex bicyclic systems. We envisoned that a readily available bicyclic oxo-enediyne **12** could function as a precursor to the tricyclic isochroman family, and thus embarked on its preparation.

The synthesis of **12** commenced from cyclohexene oxide which was ring opened with lithiotrimethylsilyl acetylide (Scheme 3). Acetylene desilylation proved more effective at this stage, and was then followed by

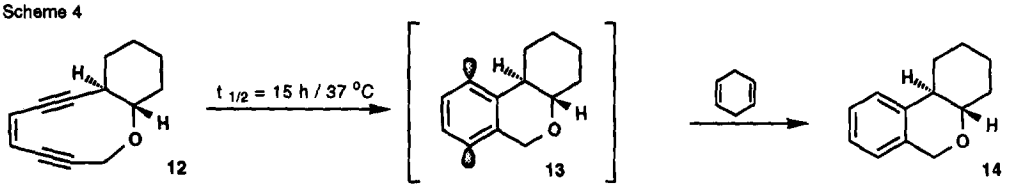
Scheme 3



propargylation to give bis alkyne **9**. Hydroxymethylation, followed by bromination under mild conditions, then provided enediyne precursor **10**. The intramolecular carbenoid addition-elimination reaction was again employed, which gave a high yield of the desired enediyne **12** directly. For practical purposes however, the cyclization was followed by immediate enediyne protection using dicobalt octacarbonyl to give **11** that was stable at 25 °C for extended periods.

Deprotection of **11** to liberate **12** was best achieved using TBAF. Thus the biological activity of the oxo-enediyne could be studied as required. The half-life of **12** was found to be approximately 15 h at physiological temperature, in good empirical agreement with its calculated intra-acetylenic ('c-d') distance of 3.23 A.[7] Incubation of **12** in the presence of 1,4 cyclohexadiene (30 equiv) led to conversion (>90%) to tricyclic product **14**, presumably via the intermediacy of diradical **13**. The process represents an efficient route to substituted isochromans (Scheme 4), and could also be extended by trapping with alternative atom donors.[6]

Scheme 4



With quantities of a potentially useful enediyne with a defined half-life, in hand (**12**), we sought means to investigate its biological activity. One of our main interests lies in the development of enediyne prodrugs of ligands that bind to identified biological receptors. Our rationale is that, if Bergman cyclization can be induced in a receptor-ligand complex, receptor cleavage may thus be possible, giving insight into ligand binding, mitigation of biological response, and cellular signalling events. A number of natural[8] and synthetic[5] enediynes are reported to possess proteolytic activity, and it is very likely that such events contribute to the observed *in vitro* and *in vivo* antitumoral activity of many of the bioactive enediynes examined to date.[9] Due to its importance in the regulation of breast cancer, the human estrogen receptor (hER) has become one of the most intensely studied of the nuclear receptor superfamily.[10] As a result, it has been firmly established that endogenous and exogenous estrogens contribute to the development of most breast cancers.[11] Clinical control of estrogen responsive breast cancers is often possible using triarylethylene partial antiestrogens such as tamoxifen, developed by ICI (Nolvadex).[12] Second generation 'pure' antiestrogens including the estradiol derived ICI 182, 780, and RU 58 668 are currently undergoing evaluation, but likely exert their antagonism via totally independent mechanisms.[13]

Accordingly, the cytotoxicity of **12** was assessed using a breast cancer cell line known to contain a functional hER (T-47D), and also against a cell line that is deficient of the hER (T-47D-Y). Using a conventional thymidine uptake assay, whilst the T-47D-Y cells grew at rates unaffected by enediyne concentrations 10^{-8} through 10^{-4} M, *the T-47D cells were sensitive, with agent 12 proving cytotoxic at concentrations as low as 10^{-6} M, and causing 83% growth inhibition at 10^{-4} M* (Figure 1).[14] As expected, when stimulated with estradiol (E_2, 10^{-9}M) only the T-47D cell line showed enhanced proliferation, suggesting that the observed antiproliferative effects of **12** may be due to interaction with the transcriptional machinery. Since enediyne **12** and its terminal product **14** are presumably incapable of acting as traditional antagonists, it is tempting to conclude that interaction of **13** with hER is responsible for the observed antiproliferative effects. Encouraged by these results, we conducted experiments to monitor hER *expression* in MCF-7 breast cancer cells, over a range of concentrations of **12**.[15] The results showed a nearly 60% reduction in protein at 10^{-5} M, but no clear correlation between enediyne and protein at lower concentrations (Figure 2). An alternative explanation for the observed *in vitro* antitumor activity of **12** could, however, be interaction with the aryl hydrocarbon receptor (AhR), which is

present in a wide variety of cells, including MCF-7 breast cancer cells.[16] Safe has reported that ligands with affinity for the Ah receptor cause a decrease in nuclear ER levels, and as such, can behave as antiestrogens.[16] Two agents with high affinity for AhR are dibenzodioxin and α-naphthoflavone,[17] both of which have residual structural similarity to the aromatised product **14**. Monitoring for AhR protein in MCF-7 cells showed a clear correlation between enediyne concentration and depletion of cellular protein (Figure 3).[15]

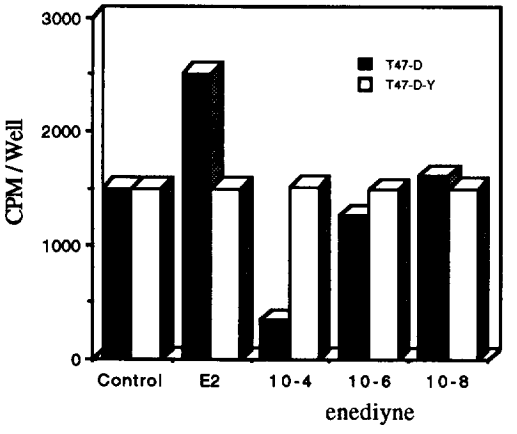
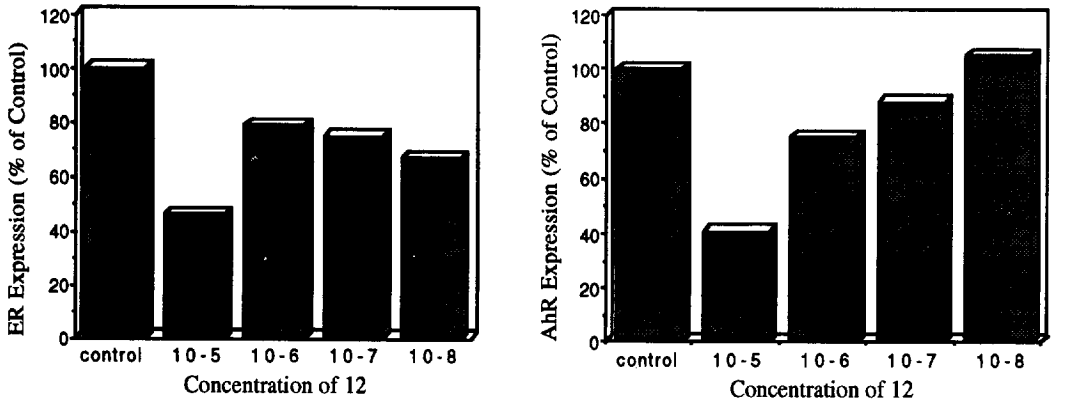


Figure 1. Antiproliferative effects of **12** versus T47-D and T47-D-Y breast cancer cells.[14] Cells received estradiol (E2) at 1x 10^{-9}M, or enediyne **12** at indicated concentrations (M). All cells received ^{3}H thymidine (1 x 10^{-6}M); after 6 h nucleii were recovered and incorporated ^{3}H thymidine measured.



Figures 2-3. Protein (hER/AhR) expression as a function of exposure to enediyne **12**, in MCF-7 cells. Cells were incubated with either DMSO (control) or **12** at indicated concentrations (M), for 16 h. Cells were then harvested, lysed, and subjected to Western blot analysis.

Since the enediyne **12** has a half-life of 15 h at physiological temperature, additional assays were performed at 10^{-5} M enediyne, with increased incubation times before analysis for protein. The results showed (Figure 4) that depletion levels off by 16 h of incubation, and AhR levels do not recover. It is thus entirely possible that antagonsim of AhR may result in the observed lowering of hER levels, and account for the previously observed antiproliferative activity.

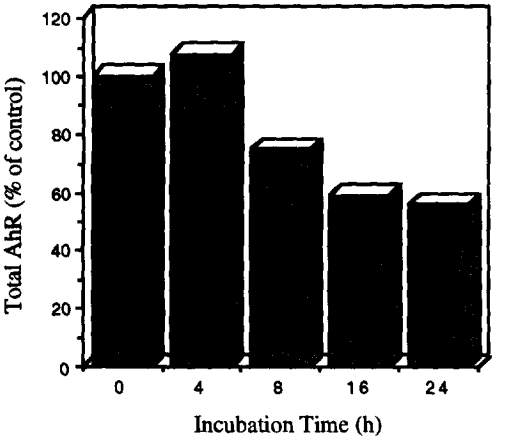


Figure 4. Effect of incubation time in the degradation of AhR by enediyne **12**. Triplicate plates of MCF-7 cells were treated with 2 x 10^{-5} M **12** for 4, 8, 16, or 24 h. Cells were then harvested, resolved by SDS-PAGE, blotted and stained for AhR, hER, and actin.

A tempting explanation for these observations is receptor proteolysis, mediated by diyl **13**. Ongoing investigations are designed to reveal the exact nature of the enediyne-receptor interactions, and could have ramifications in the design of enediyne based devices for the modulation of cellular processes.[18] Development of enediyne prodrugs with higher relative binding affinity for both hER and AhR, together with in depth biological data will thus be reported in due course.[19]

Acknowledgments:
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7. 'c-d' distance for **1**, X=CH_2 = 3.293 A (PM3); **1**, X=O = 3.212 A (PM3); **12** = 3.230 A (PM3). Preminimization was performed using the SYBYL field and followed by semi empirical (PM3) minimization (SPARTAN).
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14. Full details of all bioassay protocols will be published elsewhere: Briefly, following treatment with charcoal stripped serum, control cells were treated with essential media to achieve growth levels indicated. Cells received $1x10^{-9}$ M estradiol or enediyne **12** at concentrations 10^{-4} through 10^{-8} M; All cells received ^3H thymidine (1 x 10^{-6} M); after 6h nucleii were recovered and incorporated ^3H thymidine measured by scintillation counter.
15. Briefly: MCF-7 cells were incubated in stripped FBS for 30 h, then with enediyne 2 x 10^{-5} through 2 x 10^{-8} M for 16 h. Cells were then harvested, lysed, and subjected to Western blot analysis for hER, AhR, and actin. Following densitometry, AhR and hER levels were normalized by actin levels.
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18. At this stage it is impossible to rule out interaction of diyl **13** with other aspects of the transcriptional machienery. DNA damage induced by diyl **13** is however only significant (ΦX174 RFI DNA) at enediyne concentrations of 10^{-3} M and higher, suggesting DNA is not the ultimate target of **12**. Control assays using both isochromans and monocyclic enediynes support the involvement of diyl **13** in the observed receptor degradation and antiproliferative effects, since no significant effects were observed at the concentrations indicated (data not shown). Full details, including competition experiments using partial and pure antiestrogens will be reported in due course.
19. For an example of a potentially useful enediyne-steroid hybrid see: Wang, J.; DeClercq, P. J. *Angew. Chem. Intl. Ed.* **1995**, *34*, 1749.

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