Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 1321–1328

BIOORGANIC & MEDICINAL CHEMISTRY

A Novel Approach Towards Studying Non-Genotoxic Enediynes as Potential Anticancer Therapeutics

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Received 5 September 2001; accepted 5 November 2001

Abstract—A novel uracil-containing enediyne **7** was synthesized by the fusion at N^1 and N^3 of uracil with an 11-membered cyclic enediyne. Compound **7** was found to be stable against cycloaromatization at 80 °C. Thus, it did not cause DNA-damage. Unlike other alkylated uracil derivatives **2**–**6**, highly strained uracil-containing enediyne **7** was reacted with methyl thioglycolate at 25 °C to produce uracil (**1**) and linear enediyne **8**. This reactivity toward a sulfhydryl group may play a significant role in the mechanism by which compound **7** directed its cytotoxicity toward tumor cell lines. Tumor cells were found to be more susceptible to enediyne **7** than normal human embryonic lung cells. A combination of **7** with adriamycin or 1-(β-D-arabinofuranosyl)cytosine resulted in synergistic anticancer activity against murine L1210 and P388 leukemias, Sarcoma 180, and human CCRF–CEM lymphoblastic leukemia. After treatment of Molt-4 cells with uracil-containing enediyne **7**, light microscope examination demonstrated the presence of cell shrinkage and nuclear segmentation. Treatment of cultured Molt-4 human leukemia cells with enediyne **7** resulted in a time-dependent depletion of glutathione (GSH) whereas the exposure of the cells to the GSH precursor *N*-acetylcysteine (NAC) resulted in a substantial suppression of this effect. As such, involvement of GSH depletion in the process of apoptosis may explain the mechanism of action of non-genotoxic enediyne **7** against malignant tumor cell lines.

Introduction

Anticancer agents are generally divided into two groups, genotoxic which cause damage to DNA, and non-genotoxic, which do not directly cause chemical damage to DNA. The formation of covalent adducts with non-genotoxic chemicals does not happen with DNA, rather with other cellular nucleophilic species such as protein, L-γ-glutamyl-L-cysteinylglycine (glutathione, GSH), and water. Adduct formation at these sites does not normally have profound genetic consequences as that on DNA, and is often a detoxification process.

GSH is the most prevalent intracellular thiol known to function in many important biological phenomena including suppression of apoptosis[1a] and the synthesis of proteins and deoxyribonucleotide precursors of DNA.[1b] In the presence of glutaredoxin, the substrates for ribonucleoside diphosphate reductase (RDPR) are GSH and each of the four ribonucleoside 5′-diphosphates.[2] Glutaredoxin contains about 89 amino acid residues including two half-cysteine residues that form a single disulfide bridge that is readily reduced to a dithiol by GSH and GSSG reductase. Such reduction does not occur with thioredoxin (TR) reductase.[1] Glutaredoxin and TR are structurally unrelated proteins, and apparently are separate gene products. Interestingly, loss of the TR system in a mutant of *Escherichia coli* does not lead to loss of ribonucleotide reduction.[3] Mutants deficient in GSSG reductase were also isolated;[4,5] they grew normally and contained normal amount of GSH, indicating that GSSG was reduced by another pathway, presumably involving TR. Thus, either GSH or TR system can mediate ribonucleotide reduction for DNA biosynthesis.[1] Consequently, the activity of ribonucleotide reductase are influenced by GSH and/or TR. Therefore, these coenzymes are crucial to metabolic pathways in cell division; their inhibitors may exhibit anticancer activity.

On the other hand, GSH transferase apparently increases the ionization of the thiol group of GSH, increasing its nucleophilicity toward electrophiles and conjugating with these potentially harmful electrophiles, thereby

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0968-0896/02/$ - see front matter
PII: S0968-0896(01)00393-5

protecting other vital nucleophilic centers in the cell such as nucleic acids and proteins.[6] GSH is also capable of reacting non-enzymically with electrophiles and excreted them in the bile and urine. As a result, significant connections between GSH and carcinogenesis have attracted attention.[7] Administration of certain carcinogens increases level of GSH for detoxication.[8,9] Thus, GSH can be considered as an inhibitor for cytotoxic drugs; yet inhibition of GSH is expected to enhance the activity of cytotoxic anticancer agents.

We designed and synthesized highly strained uracil-containing enediyne **7** possessing electrophilic centers at C-2 and C-9 positions to inhibit intracellular thiols such as GSH and/or TR. To verify the efficiency of this novel approach toward cancer chemotherapy, the reaction of enediyne **7** with model biological nucleophiles, the inhibitory property of **7** toward the enzymatic conversion of CDP to dCDP in the presence of TR, cell morphology in the presence of enediyne **7**, as well as its effect on the GSH level in Molt-4 human leukemia cells were studied.

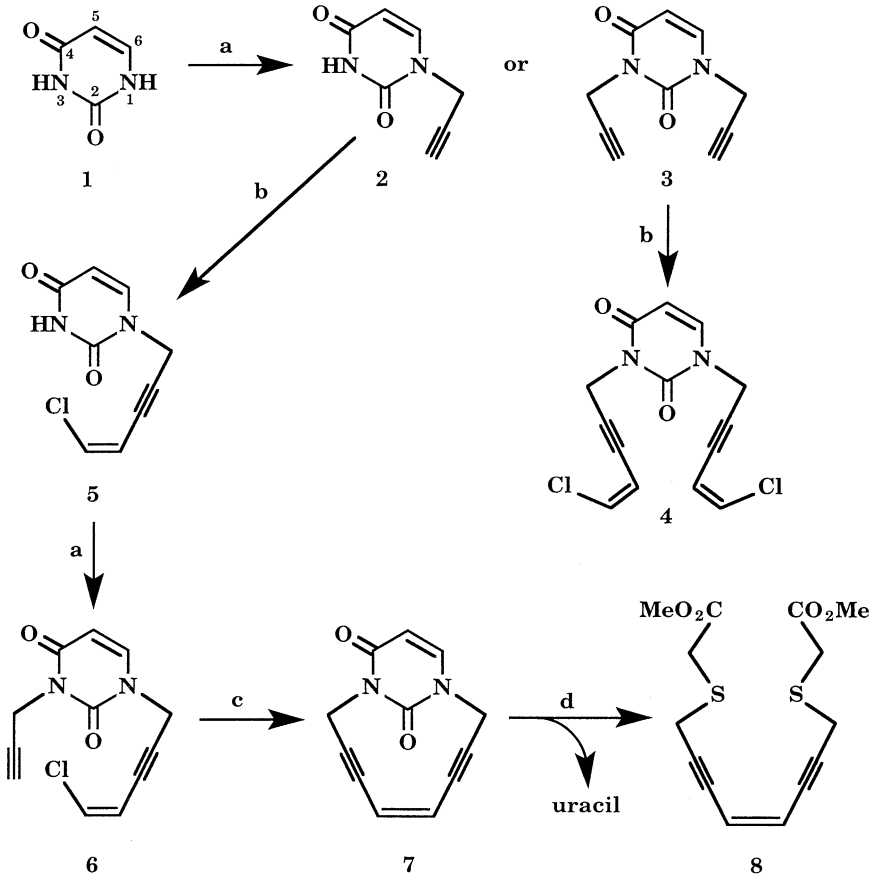
Compound **7** was found to be an anticancer agent, but did not exhibit toxicity toward normal human embryonic lung cells (HEL) up to 50 μM. It also exerted a large synergistic effect on anticancer properties of adriamycin and 1-(β-D-arabinofuranosyl)cytosine (ara-C) against murine L1210 and P388 leukemias, Sarcoma 180 (S-180), and human CCRF–CEM lymphoblastic leukemia.

Results

Synthesis of bicyclic uracil-containing enediyne 7 and linear enediyne 8 (Scheme 1)

We treated uracil **1** with propargyl bromide and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in CH_3CN at reflux to afford the corresponding N^1-alkylated product **2**[10] in 80% yield (Scheme 1). By use of two equivalents of propargyl bromide and DBU, N^1,N^3-dialkylated product **3** was produced in 90% yield. The terminal alkynes in **3** were then subjected to coupling with *cis*-1,2-dichloroethene under Nagata et al.[11] conditions to furnish the cyclic enediyne **7**; yet we obtained acyclic product **4** in 65% yield. The difficult cyclization is an indication of high steric strain of the target bicyclic system **7**.

Thus for the synthesis of **7**, we turned our attention to the conversion of alkyne **2** to chloroenyne **5** (Scheme 1). Treatment of **2** with *cis*-1,2-dichloroethene in the presence of $Pd(PPh_3)_4$/CuI[11] afforded **5** in 70% yield. Alkylation of N^3-position in **5** with propargyl bromide by use of DBU gave (N^1-chloroenyne-N^3-alkynyl)uracil **6** in 80% yield. Cyclization of **6** in the presence of $Pd(PPh_3)_4$/CuI and *n*-$BuNH_2$ produced bicyclic target molecule **7** in 66% yield. Uracil-containing enediyne **7** was found to be quite stable on silica gel or in solution. It did not give any cycloaromatized product on standing in solution for several days in the presence of



Scheme 1. Synthesis of bicyclic uracil-containing enediyne **7** and linear enediyne **8**. Reagents: **(a)** $BrCH_2C{\equiv}CH$, DBU, CH_3CN (80% for **2**, 90% for **3**, 80% for **6**); (b) *cis*-1,2-dichloroethene, *n*-$BuNH_2$, $(PPh_3)_4Pd$, CuI, C_6H_6 or THF (65% for **4**, 70% for **5**); (c) *n*-$BuNH_2$, $(PPh_3)_4Pd$, CuI, THF, 66%; (d) methyl thioglycolate, 1.0 M EtOH/AcOH, 53%.

1,4-cyclohexadiene as a hydrogen atom donor. Compound **7** was also stable in CH_3CN at reflux for 3 h.

Uracil-containing enediyne **7** was then reacted with methyl thioglycolate in 1.0 M EtOH/AcOH at 25 °C to produce uracil and 53% yield of the linear enediyne **8** after 48 h (Scheme 1). This result indicates that C-2 and C-9 positions in highly strained enediyne **7** are electrophilic and can conjugate with suitable nucleophiles.

On the other hand, treatment of compound **7** with purine and pyrimidine nucleotides did not result in adduct formation and enediyne **7** was recovered quantitatively.

Effect of uracil-containing enediyne 7 on intracellular GSH levels in Molt-4 human leukemia cells

Cultured Molt-4 cells were treated with 300 μM enediyne **7** for 12–60 h, and the total intracellular GSH content was determined (see Experimental). Treatment of the cells with enediyne **7** or a combination of **7** and *N*-acetylcysteine (NAC) resulted in a time-dependent decrease in the intracellular level of GSH to 15 or 52% of that of untreated cells at 60 h post-treatment (see Fig. 1).

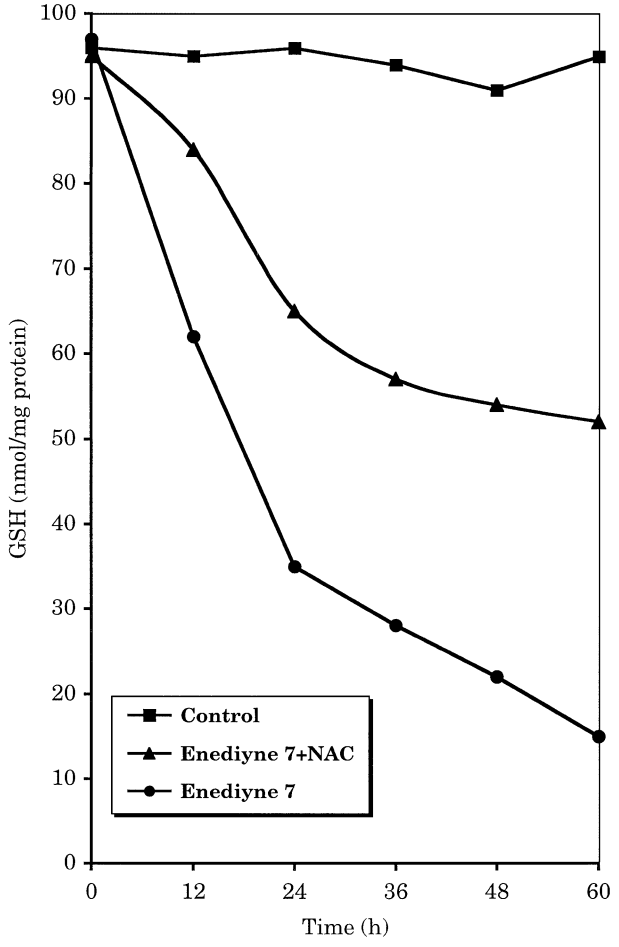


Figure 1. Effect of uracil-containing enediyne 7 as well as 7 + *N*-acetylcysteine on intracellular GSH levels in Molt-4 cells. Results are the mean of duplicate determinations.

Effect of uracil-containing enediyne 7 on the reduction of CDP to dCDP in the presence of *E. coli* RDPR and TR

Enediyne **7** was evaluated for a non-specific inhibitory property against *E. coli* RDPR. The method involved conversion of CDP to dCDP (see Experimental). With respect to a control experiment, we did not observe any significant change in the rate of reduction of CDP to dCDP in the presence of **7**.

DNA-binding and DNA-cleavage experiments

Enediyne **7** was evaluated for its binding property with a double-helical DNA. Moreover, a DNA-cleavage experiment was performed in the presence of enediyne **7**. With respect to a control experiment, it was found that compound **7** is not capable to bind or cleave DNA. Thus, for non-genotoxic enediyne **7**, the normal mode of action of genotoxic enediynes through DNA-damage was ruled out.

Apoptotic morphology of human leukemia Molt-4 cell

The ability of enediyne **7** to cause morphological changes in Molt-4 cell was evaluated by concentration of the compound required to cause microscopically visible change or disruption (cell shrinkage, condensation of the chromatin, and nucleus segmentation) in about 50% of the cell sheet (see Experimental).

Anticancer activity

The anticancer screening experiments for compounds **2**–**8**, a mixture of **7** and adriamycine (1:1 W/W), a mixture of **7** and ara-C (1:1 W/W), a mixture of **7** and NAC (1:1 W/W) as well as the reference compounds adriamycine and ara-C were carried out in vitro against murine L1210 and P388 leukemias, Sarcoma 180 (S-180), and human CCRF–CEM lymphoblastic leukemia. The activity is expressed as the concentration (μM) required to inhibit cell replication by 50% (IC_{50}) of each the given cell lines. Their cytotoxicity for normal human embryonic lung cells (HEL) were also determined. Among compounds **2**–**8**, bicyclic enediyne **7** exhibited interesting anticancer activity (Table 1). This compound, however, is about 3 times less active than the reference compounds. On the other hand, toxicity (IC_{50}) of **7** against HEL is > 50 μM; yet adriamycine and ara-C possess IC_{50} values of 7.56×10^{-4} and 1.98×10^{-3} μM against HEL, respectively.

Discussion

Genotoxic enediyne-class antibiotics have an exceptional antitumor potency, unusual chemical structure, and marked ability to cause DNA strand scission.[12] In the absence of DNA, the antitumor antibiotic neocarzinostatin reacts irreversibly with nucleophiles to form a covalent adduct which will bind but not cleave DNA.[13] On the other hand, the nonprotein component of neocarzinostatin undergoes rapid and irreversible reaction with thiols to produce a species which is capable of

cleaving DNA upon aerobic incubation.[14] The distance between the remote acetylenic carbons in enediyne systems provide a useful guide for determination the ease of their cycloaromatization at ambident temperature.[12] The distance between C-3 and C-8 acetylenic carbons in **7** is 3.74 Å (Fig. 2). Thus, it is quite stable toward cycloaromatization. Consequently, the mode of action of **7** against malignant tumor cell lines is not due to benzenoid diradical formation in biological systems. Furthermore, DNA-cleavage did not occur in the presence of enediyne **7**. In addition, enediyne **7** did not react with nucleophilic sites on purine and pyrimidine nucleoside-3′,5′-diphosphates that make up the structure of DNA. As such, its mode of action is not to crosslink DNA in the nucleus thereby preventing replication.

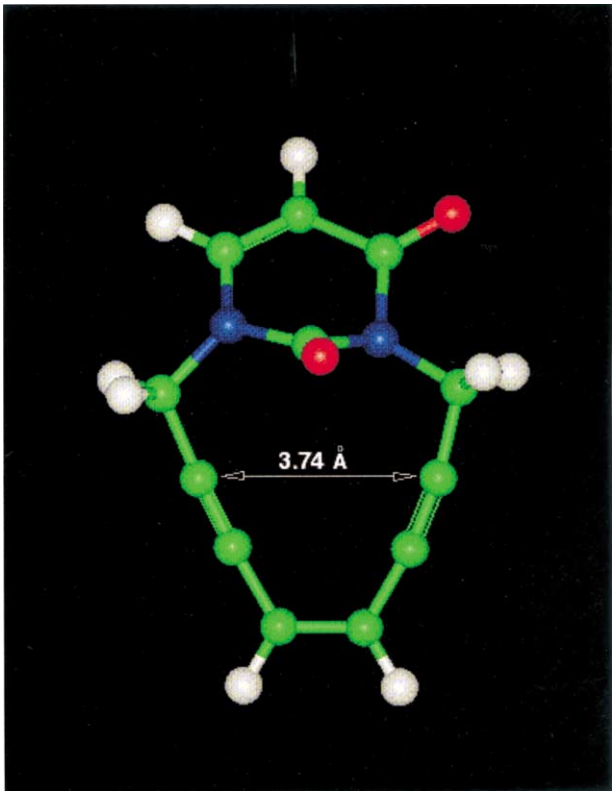


Figure 2. The structure of uracil-containing enediyne **7** represented by ball-and-stick with the lowest energy obtained by the ESFF calculations.

It is well known that deoxyribonucleotides are formed by direct reduction of ribonucleotides by ribonucleotide reductase in the presence of TR and TR reductase.[1,15] A mutant of *E. coli* was also found that lacked TR, however, it is active in NADPH-dependent ribonucleotide reduction.[3,7–9] In this mutant, GSH and glutaredoxin function in the reduction for production of DNA precursors.[2,3,16,17] As an example, chemically reduced glutaredoxin is enzymically active in conversion of CDP to dCDP.[1]

Newly synthesized uracil-containing enediyne **7** having electrophilic centers at C-2 and C-9 positions exhibited notable anticancer activity (Table 1). Unlike intermediates **2**–**6**, strained macrocycle **7** was found to be reactive toward a model biological nucleophile, methyl thioglycolate. Similarly, the inhibition of biologically important sulfhydryl groups, involved in DNA biosynthesis, cell division, or cell protection by compound **7** is expected to play a significant role in the mechanisms by which this novel enediyne exerts its anticancer activity. While the physiological reducing agent, TR, was not inhibited by compound **7**, the GSH level of the enediyne **7**-treated Molt-4 cells dropped to 28 and 15% of that of untreated cells at 36 and 60 h post-treatment, respectively. On the other hand, the exposure of cells to the GSH precursor, NAC, resulted in reduction of GSH depletion by enediyne **7** to 57 and 52% of that of untreated cells after 36 and 60 h, respectively (see Fig. 1). Thus the activity of non-genotoxic enediyne **7**, in the presence of NAC, against malignant tumor cell lines was reduced. Consequently, GSH depletion with enediyne **7** may lead to stimulation of apoptosis which was reversed by NAC. In fact, cytomorphology observation of human leukemia Molt-4 cells in the presence of **7** further stressed this viewpoint that such modification in intracellular GSH content may have played an important role in apoptosis.

GSH can also reduce the efficiency of cytotoxic drugs through the process of detoxication.[8,9] Thus, inhibition of GSH is expected to increase the activity of cytotoxic anticancer agents. As such, a combination of enediyne **7** with cytotoxic drugs (i.e., adriamycine or Ara-C) exhibited superior activity in comparison with individual drug applied alone. Moreover, enediyne **7** did not render normal cells more sensitive to chemotherapeutic

Table 1. Inhibitory effects of compound **7**, a mixture of **7** and adriamycine (1:1 W/W), a mixture of **7** and ara-C (1:1 W/W), a mixture of **7** and NAC (1:2 W/W) as well as adriamycine and ara-C on the growth of malignant tumor cell lines and normal human embryonic lung cells (HEL) in vitro by 50% (IC_{50})

Compound	IC_{50}[a] (μM)				
	L1210	P388	S-180	CCRF-CEM	HEL
7	0.23±0.01	0.46±0.03	1.55±0.40	0.38±0.05	>50
Adriamycine+**7**	0.009±0.001	0.07±0.01	0.03±0.00	0.03±0.01	4.39 ×&10^{-2}
ara-C+**7**	0.02±0.00	0.01±0.00	0.06±0.01	0.007±0.002	8.23 ×&10^{-1}
NAC+**7**	1.57±0.43	2.39±0.87	8.74±1.25	3.03±0.76	>50
Adriamycine	0.06±0.01	0.23±0.02	0.67±0.11	0.21±0.01	7.56 ×&10^{-4}
ara-C	0.18±0.02	0.12±0.01	0.39±0.07	0.04±0.00	1.98 ×&10^{-3}

[a]50% Inhibitory concentration, or compound concentration required to inhibit cell proliferation by 50%. For 1:1 (W/W) mixtures, IC_{50} in μM refers to known anticancer drugs; in the case of **7**+NAC (1:2 W/W) refers to enediyne **7**.

anticancer agents (see Table 1). Consequently, malignant tumor cells are evidently more susceptible to the effect of GSH depletion than are the normal cells.

Conclusions

A new uracil-containing enediyne **7** was synthesized by chemical methods and its structure–activity relationship was explored. Compound **7** was found to be stable against cycloaromatization. As such, it did not cleave DNA. On the other hand, it was reactive toward a model biological nucleophile and capable to deplete GSH content of Molt-4 cells. Such modification in intracellular GSH content may have played an important role in apoptosis, as evidenced by morphology of human leukemia Molt-4 cells in the presence of enediyne **7**. Bicyclic enediyne **7** exhibited interesting activity against murine L1210 and P388 leukemias, Sarcoma 180 (S-180), and human CCRF–CEM lymphoblastic leukemia. This compound was not toxic up to a level as high as 50 μM on the noncancerous cells (HEL). Non-genotoxic enediyne **7** may be an important adjuvant in a wide range of chemotherapy. Such an approach requires more knowledge of the GSH status of the host cells and of the tumor cells.

Experimental

General methods

For anhydrous reactions, glassware was dried overnight in an oven at 120 °C and cooled in a desiccator over anhydrous $CaSO_4$ or silica gel. Reagents purchased from Sigma (St. Louis, USA), ICN (1090 VB, Amsterdam, The Netherlands), or Fluka (Switzerland). Solvents, including dry ether and tetrahydrofuran (THF), were obtained by distillation from the sodium ketyl of benzophenone under nitrogen. Other solvents, including chloroform, dichloromethane, ethyl acetate, and hexanes were distilled over CaH_2 under nitrogen. Absolute methanol and ethanol were purchased from Merck (Germany) and used as received. Deoxyribonucleic acid, from calf thymus, was obtained from ICN (USA). Hematoxylin, cresyl violet, and Hoechst 33342 stain were obtained from Sigma.

Melting points were obtained with a Büchi 510 melting point apparatus. Infrared (IR) spectra were recorded on a Perkin-Elmer spectrophotometer. The wavenumbers reported are referenced to the 1601 cm^{-1} absorption of polystyrene. Proton NMR spectra were obtained on a Varian XL-300 (300 MHz) Spectrometer. Chloroform-d was used as solvent; Me_4Si (δ 0.00 ppm) was used as an internal standard. All NMR chemical shifts are reported as δ values in parts per million (ppm) and coupling constants (J) are given in hertz (Hz). The splitting pattern abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet; br, broad; m, unresolved multiplet due to the field strength of the instrument; and dd, doublet of doublets. UV–vis spectroscopy was carried out using an HP8452A diode array spectrophotometer. Mass spectra were carried out on a VG 70-250 S mass spectrometer. Microanalyses were performed on a Perkin–Elmer 240-B microanalyzer. Aristo Mic-O-Lite microscope (New York, USA) was used to study cell morphology.

Molt-4 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum at 37 °C in a humidified environment with 5% CO_2. Cells were seeded and grown to near 3×10^5 /mL before use.

Purification on silica gel refers to gravity column chromatography on Merck Silica Gel 60 (particle size 230–400 mesh). Analytical TLC was performed on precoated plates purchased from Merck (Silica Gel 60 F_{254}). Compounds were visualized by use of UV light, I_2 vapor, or 2.5% phosphomolybdic acid in ethanol with heating.

Measurement of GSH level in cells

Intracellular GSH content was determined by an established procedure.[18,19] Briefly, cells (2×10^7) were suspended in 400 μL of 10% 5-sulfosalicylic acid. The precipitated proteins were pelleted by centrifugation at 4 °C for 15 min at 2000g. Aliquots of the acid-soluble supernatant were mixed with sodium phosphate (125 mM, pH = 7.5), EDTA (6.3 mM), NADPH (0.21 mM), and 5,5-dithio-*bis*-(2-nitrobenzoic acid) (0.60 mM). Upon addition of GSH reductase, the increase in absorption at 412 nm was monitored and used to determine the amount of GSH in the sample by comparison to a reference curve generated with known amounts of GSH standard. Results from the treatment of cells with enediyne **7** (300 μM) or **7** (300 μM) + NAC (600 μM) are illustrated in Figure 1. Protein content was estimated by the Bradford dye binding assay[20] using bovine serum albumin as standard.[21]

Evaluation of the effectiveness of enediyne 7 toward inhibition of RDPR

E. coli RDPR was isolated as described[22] and used by the method of Baker et al.[23] Briefly, incubation of HEPES (50.0 mM, pH = 7.60), $MgSO_4$ (15.0 mM), EDTA (1.00 mM), ATP (1.60 mM), NADPH (5.00 mM), TR (12.0 μM), TRR (0.790 μM), R1 subunit (12.8 μM), and R2 subunit (12.8 μM) with 0.40 mM of **7** at 37 °C did not result in the reduction of enzyme activity. The reaction mixture was assayed for activity as described by Steeper and Steuart.[24] This method involves conversion of CDP to dCDP. Control experiment was performed in the absence of enediyne **7**.

Determination of binding or cleaving ability of enediyne 7 with DNA

By use of an established procedure,[25] the interaction of enediyne **7** with a highly polymerized DNA from calf thymus was examined. The reference solution contained the same DNA to which had been added 200 μL of water. No binding between compound **7** and DNA was detected. As such, the cleavage of the examined DNA by enediyne **7** did not occur.[14]

Cytomorphology observation by light microscope

Molt-4 cells were treated with various concentrations of enediyne **7** for different time intervals. Afterward, the treated cells, as well as control cells, were fixed with paraformaldehyde and extracted with cytoskeleton extraction buffer as described.[26] Cells were then stained with hematoxylin or cresyl violet (2% stain in 0.1% acetic acid) for microscopic observation. To better visualize the nucleus, cells were also grown on coverslips. After treatment with enediyne **7**, coverslips were air-dried, fixed with 4% paraformaldehyde in phosphate-buffered saline for 30 min, permeabilized for 2 min on ice with 0.1% Triton X-100 in 0.1% sodium citrate, and blocked for 30 min with 20% fetal bovine serum and 3% bovine serum albumin.[27] Nuclei were then labeled with Hoechst 33342 stain (1.0 μg/mL) for 15 min. Concentration of enediyne **7** required to cause microscopically visible change on about 50% of Molt-4 cells was measured ($CC_{50} = 3.58$ μM) by use of the linear regression method.[28]

Anticancer test procedures in vitro

Murine L1210 leukemia, P388 leukemia, S-180 cells, human CCRF–CEM lymphoblastic leukemia cells, and human embryonic cells (HEL) were maintained as suspension cultures in Fisher's medium. The medium was supplemented with 10% horse serum and all cells maintained at 37 °C in humidified atmosphere of 5% CO_2/95% air. Under this condition, the generation time for L1210, P388, S-180, CCRF–CEM, and HEL cells was about 12, 12, 18, 20 and 30 h, respectively. Compounds **2**–**8**, a mixture of **7** and adriamycine (1:1 W/W), a mixture of **7** and ara-C (1:1 W/W), a mixture of **7** and NAC (1:2 W/W) as well as the reference compounds adriamycine and ara-C, at various concentrations, were added to L1210, P388, S-180, CCRF-CEM and HEL cells (2×10^4 cells/mL) in their exponential phase of growth. The cell number of the drug-free cultures (control), as well as that of the cultures supplemented with the test compounds, were determined after 34, 49, and 72 h of growth. The IC_{50} values were estimated from dose–response curves compiled from two independent experiments and represent the drug concentration (μM) required to inhibit replication of the respective L1210, P388, S-180, CCRF–CEM, and HEL cell lines by 50% after 72 h incubation (Table 1).

***N*¹-(Prop-2-yne-1-yl)uracil (2).** To a solution of **1** (2.010 g, 17.84 mmol) in CH_3CN (60 mL) and DBU (2.985 g, 19.63 mmol) was added propargyl bromide (2.120 g, 17.84 mmol) dropwise within 1.5 h. The reaction mixture was stirred at reflux for 1 h. The solution was concentrated under reduced pressure and EtOAc (160 mL) was added. The EtOAc solution was washed with NH_4Cl aqueous solution (5%, 3 × 100 mL). Then, it was dried over $MgSO_4$ (s) and filtered. Evaporation under reduced pressure and purification of the residue by use of column chromatography (hexanes/EtOAc = 7:3) afforded **2** as a white solid. Crystallization (hexanes/EtOAc = 1:1) gave **2** (2.140 g, 14.27 mmol) in 80% yield: mp 169–170 °C; R_f (hexanes/EtOAc = 1:1) 0.23; IR (CH_2Cl_2) ν 3550-3640 (NH), 3264 (≡CH), 2934 (C≡C), 1690 (C=O),1635 (C=O), 1286, 674 cm^{-1}; UV (EtOH) λ_{max} 262 (ε 6 100); ^1H NMR ($CDCl_3$) δ3.37 (t, $J = 3.0$ Hz, 1 H, ≡CH), 4.49 (d, $J = 3.0$ Hz, 2 H, CH_2), 5.61 (d, $J = 6.0$ Hz, 1 H, C_5H), 7.68 (d, $J = 6.0$ Hz, 1 H, C_6H), 11.35 (br s, 1 H, NH); ^{13}C NMR ($CDCl_3$) δ 36.62 (CH_2), 75.82 (≡CH), 78.44 (≡C),101.69 (C_5), 144.48 (C_6), 150.38 (C_2=O), 163.55 (C_4=O); MS *m*/*z* 150 (M^+). Anal. calcd for $C_7H_6N_2O_2$: C, 56.00; H, 4.03; N, 18.66. Found: C, 56.04; H, 4.08; N, 18.55.

***N*¹,*N*³-Bis(prop-2-yne-1-yl)uracil (3).** To a solution of **1** (2.010 g, 17.84 mmol) in CH_3CN (60 mL) and DBU (5.970 g, 39.25 mmol) was added propargyl bromide (4.240 g, 35.68 mmol). The reaction mixture was stirred at reflux for 16 h. The solution was concentrated under reduced pressure and EtOAc (160 mL) was added. The EtOAc solution was washed with NH_4Cl aqueous solution (5%, 3 × 100 mL). Then, it was dried over $MgSO_4$ (s) and filtered. Evaporation under reduced pressure and purification of the residue by use of column chromatography (hexanes/EtOAc = 7:3) afforded **3** as a white solid. Crystallization (hexanes/EtOAc = 1:1) gave **3** (3.030 g, 16.06 mmol) in 90% yield: mp 96–98 °C; R_f (hexanes/EtOAc = 1:1) 0.52; IR (CH_2Cl_2) ν 3303 (≡CH), 2306 (C≡C), 1717 (C=O),1676 (C=O), 1275, 636 cm^{-1}; UV (EtOH) λ_{max} 261 (ε 7 250); ^1H NMR ($CDCl_3$) δ 2.14 (t, $J = 3.0$ Hz, 1 H, ≡CH), 2.48 (t, $J = 3.0$ Hz, 1 H, ≡CH), 4.57 (d, $J = 3.0$ Hz, 2 H, CH_2), 4.68 (d, $J = 3.0$ Hz, 2 H, CH_2), 5.80 (d, $J = 7.5$ Hz, 1 H, C_5H), 7.44 (d, $J = 7.5$ Hz, 1 H, C_6H); ^{13}C NMR ($CDCl_3$) δ 36.62 (CH_2), 38.71 (CH_2), 75.82 (≡CH), 76.79 (≡CH), 78.44 (≡C),78.97 (≡C),99.75 (C_5), 142.97 (C_6), 152.19 (C_2=O), 162.99 (C_4=O); MS *m*/*z* 188 (M^+). Anal. calcd for $C_{10}H_8N_2O_2$: C, 63.83; H, 4.28; N, 14.89. Found: C, 63.80; H,4.29; N, 14.91.

***N*¹,*N*³-Bis(5-chloropent-4-ene-2-yne-1-yl)uracil (4).** To a solution of **3** (0.190 g, 1.03 mmol) in benzene (15 mL) and n-butylamine (0.370 g, 5.05 mmol), under argon atmosphere, were added *cis*-1,2-dichloroethene (0.130 g, 1.34 mmol) and $Pd(PPh_3)_4$ (60.0 mg, 0.0520 mmol). After stirring at 25 °C for 30 min, CuI (20.0 mg, 0.100 mmol) was added and the mixture was stirred at 25 °C for 12 h. The brown solution was quenched with NH_4Cl aqueous solution (5%, 20 mL) and extracted with EtOAc (3 × 25 mL). The EtOAc solution was dried over $MgSO_4$ (s) and filtered. Evaporation under reduced pressure and purification of the residue by use of column chromatography (hexanes/EtOAc = 7:3) afforded **4** (0.21 g, 0.65 mmol) in 65% yield as a foam: R_f (hexanes/EtOAc = 1:1) 0.60; IR (CH_2Cl_2) ν 3055 (CH=CH), 2986 (≡CH), 2305 (C≡C), 1715 (C=O), 1672 (C=O), 1262, 990, 895 cm^{-1}; UV (EtOH) λ_{max} 232 (ε 16 487), 262 (ε 10 250); ^1H NMR ($CDCl_3$) δ 4.78 (s, 2 H, CH_2), 4.88 (s, 2 H, CH_2), 5.79 (d, $J = 6.0$ Hz, 1 H, CH), 5.83 (d, $J = 8.0$ Hz, 1 H, C_5H), 5.89 (d, $J = 6.0$ Hz, 1 H, CH), 6.34 (d, $J = 6.0$ Hz, 1 H, CHCl), 6.48 (d, $J = 6.0$ Hz, 1 H, CHCl), 7.54 (d, $J = 8.0$ Hz, 1 H, C_6H); ^{13}C NMR ($CDCl_3$) δ 31.28 (CH_2), 38.69 (CH_2), 77.51, 81.45, 88.87, 91.55 (2 C≡C), 102.16 (C_5), 110.80, 111.56 (2=CH), 129.05, 130.74 (2=CCl), 140.67 (C_6), 150.36 (C_2=O), 161.65 (C_4=O); MS *m*/*z* 308 (M^+, Cl-cluster). Anal.

calcd for $C_{14}H_{10}Cl_2N_2O_2$: C, 54.39; H, 3.26; Cl, 22.94; N, 9.06. Found: C, 54.37; H, 3.14; Cl, 22.87; N, 9.11.

N^1-(5-Chloropent-4-ene-2-yne-1-yl)uracil (5). To a solution of **2** (0.600 g, 3.99 mmol) in THF (150 mL) and n-butylamine (0.590 g, 8.04 mmol), under argon atmosphere, were added *cis*-1,2-dichloroethene (0.580 g, 6.03 mmol) and $Pd(PPh_3)_4$ (0.23 g, 0.20 mmol). After stirring at 25 °C for 30 min, CuI (76.0 mg, 0.380 mmol) was added and the mixture was stirred at 25 °C for 10 h. The reaction was quenched with NH_4Cl aqueous solution (5%, 30 mL) and extracted with EtOAc (3 × 30 mL). The EtOAc solution was dried over $MgSO_4$ (s) and filtered. Evaporation under reduced pressure and purification of the residue by use of column chromatography (hexanes/EtOAc = 6:4) afforded **5** (0.599 g, 2.79 mmol) in 70% yield as a foam: R_f (hexanes/EtOAc = 1:1) 0.38; IR (CH_2Cl_2) ν 3381 (NH), 3050 (CH=CH), 2986 (≡CH), 2359 (C≡C), 1700 (C=O), 1694 (C=O), 1275, 1131, 864 cm^{-1}; UV (EtOH) λ_{max} 235 (ε 17,300), 260 (ε 13,250); ^1H NMR ($CDCl_3$) δ 4.74 (s, 2 H, CH_2), 5.78 (d, J = 8.0 Hz, 1 H, C_5H), 5.88 (d, J = 6.0 Hz, 1 H, CH), 6.47 (d, J = 6.0 Hz, 1 H, CHCl), 7.52 (d, J = 8.0 Hz, 1 H, C_6H), 9.21 (br s, 1 H, NH); ^{13}C NMR ($CDCl_3$) δ 37.79 (CH_2), 81.32, 88.91 (C≡C), 102.86 (C_5), 110.81 (=CH), 130.77 (=CCl), 142.47 (C_6), 150.34 (C_2=O), 163.34 (C_4=O); MS m/z 210 (M^+, Cl-cluster). Anal. calcd for $C_9H_7ClN_2O_2$: C, 51.32; H, 3.35; Cl, 16.83; N, 13.30. Found: C, 51.23; H, 3.30; Cl, 16.95; N, 13.27.

[N^1-(5-Chloropent-4-ene-2-yne-1-yl)-N^3-(prop-2-yne-1-yl)]uracil (6). To a solution of **5** (0.19 g, 0.93 mmol) in CH_3CN (40 mL) and DBU (0.190 g, 1.22 mmol) was added propargyl bromide (0.140 g, 1.22 mmol). The reaction mixture was stirred at 45 °C for 12 h. The solution was concentrated under reduced pressure and EtOAc (120 mL) was added. The EtOAc solution was washed with NH_4Cl aqueous solution (5%, 3 × 90 mL). Then, it was dried over $MgSO_4$ (s) and filtered. Evaporation under reduced pressure and purification of the residue by use of column chromatography (hexanes/EtOAc = 7:3) afforded **6** (0.185 g, 0.744 mmol) in 80% yield as a foam: R_f (hexanes/EtOAc = 1:1) 0.53; IR (CH_2Cl_2) ν 3304 (≡CH), 3089 (CH=CH), 3058 (C≡C), 1715 (C=O),1678 (C=O), 1450, 671 cm^{-1}; UV (EtOH) λ_{max} 235 (ε 15,100), 260 (ε 10,941); ^1H NMR ($CDCl_3$) δ 2.15 (t, J = 4.5 Hz, 1 H, ≡CH), 4.68 (d, J = 4.5 Hz, 2 H, CH_2), 4.77 (s, 2 H, CH_2), 5.82 (d, J = 8.0 Hz, 1 H, C_5H), 5.87 (d, J = 6.0 Hz, 1 H, CH), 6.47 (d, J = 6.0 Hz, 1 H, CHCl), 7.52 (d, J = 8.0 Hz, 1 H, C_6H); ^{13}C NMR ($CDCl_3$) δ 30.39 (CH_2), 38.64 (CH_2), 70.75 (≡CH), 77.81 (≡C),81.49, 88.83 (C≡C),102.18 (C_5), 110.78 (=CH), 130.76 (=CCl), 140.64 (C_6), 150.39 (C_2=O), 161.59 (C_4=O); MS m/z 248 (M^+, Cl-cluster). Anal. calcd for $C_{12}H_9ClN_2O_2$: C, 57.96; H, 3.65; Cl, 14.26; N, 11.27. Found: C, 57.90; H, 3.61; Cl, 14.29; N, 11.21.

1,10 - Diazabicyclo[8.3.1]trideca - 5,12 - diene - 3,7 - diyne - 11,14 - dione (7). To a solution of **6** (72.0 mg, 0.280 mmol) in THF (80 mL) and *n*-butylamine (1.0 mg, 0.014 mmol), under argon atmosphere, was added $Pd(PPh_3)_4$ (17.0 mg, 0.0147 mmol). After stirring at 25 °C for 30 min, CuI (5.0 mg, 0.026 mmol) was added and the mixture was stirred at 50 °C for 24 h. The reaction was quenched with NH_4Cl aqueous solution (20%, 20 mL) and extracted with EtOAc (3 × 20 mL). The EtOAc solution was dried over $MgSO_4$ (s) and filtered. Evaporation under reduced pressure and purification of the residue by use of column chromatography (hexanes/EtOAc = 7:3) afforded **7** (0.0393 g, 0.185 mmol) in 66% yield as a white solid: mp 114–116 °C; R_f (hexanes/EtOAc = 1:1) 0.33; IR (CH_2Cl_2) ν 3054 (CH=CH), 2360, 2306 (2 C≡C), 1715 (C=O),1676 (C=O), 1450, 1277, 888 cm^{-1}; UV (EtOH) λ_{max} 230 (ε 4 017), 262 (ε 3 288); ^1H NMR ($CDCl_3$) δ4.70 (s, 2 H, CH_2), 4.74 (s, 2 H, CH_2), 5.79 (d, J = 8.1 Hz, 1 H, C_5H), 5.99 (d, J = 5.13 Hz, 1 H, CH), 6.25 (d, J = 5.13 Hz, 1 H, CH), 7.50 (d, J = 8.1 Hz, 1 H, C_6H); ^{13}C NMR ($CDCl_3$) δ 30.88 (CH_2), 38.65 (CH_2), 66.85, 72.51, 81.35, 88.89 (2 C≡C), 102.01 (C_5), 112.82 (=CH), 115.07 (=CH), 140.81 (C_6), 150.17 (C_2=O), 161.39 (C_4=O); MS m/z 212 (M^+). Anal. calcd for $C_{12}H_8N_2O_2$: C, 67.92; H, 3.80; N, 13.20. Found: C, 67.81; H, 3.75; N, 13.20.

***cis*-1,2-(Dimethoxycarbonylmethylthiopropyn-1-yl)ethene (8).** To a solution of **7** (94.2 mg, 0.444 mmol) in EtOH (20 mL) and AcOH (1.26 mL), under argon atmosphere, was added methyl thioglycolate (106.4 mg, 1.004 mmol). After stirring at 25 °C for 48 h, the reaction was quenched with H_2O (30 mL) and extracted with EtOAc (3 × 20 mL). The aqueous solution contained uracil. The EtOAc solution was dried over $MgSO_4$ (s) and filtered. Evaporation under reduced pressure and purification of the residue by use of column chromatography (hexanes/EtOAc = 9:1) afforded **8** (73.0 mg, 0.234 mmol) in 53% yield as an oil: R_f (hexanes/EtOAc = 8:2) 0.64; IR (CH_2Cl_2) ν 3062 (CH=CH), 2298 (2 C≡C), 1736 (2 C=O), 1480, 1200, 878 cm^{-1}; UV (EtOH) λ_{max} 225 (ε 1 973); ^1H NMR ($CDCl_3$) δ 3.27 (s, 4 H, 2 CH_2), 3.80 (s, 6H, 2 CH_3), 3.95 (br s, 4 H, 2 ≡CCH_2), 5.89 (s, 2 H, CH=CH); MS m/z 312 (M^+, S-cluster). Anal. calcd for $C_{14}H_{16}O_4S_2$: C, 53.83; H, 5.16; S, 20.52. Found: C, 53.70; H, 5.29; S, 20.67.

Acknowledgements

For financial support, we thank Academia Sinica and the National Science Council of Republic of China.

References and Notes

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