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Synthesis and Cytotoxicity of Substituted Ethyl 2-Phenacyl-3-phenylpyrrole-4-carboxylates

The substituted ethyl-2-phenacyl-3-phenylpyrrole-4-carboxylates were synthesized by a condensation of a beta-chloroenal and an alpha-aminoketone under neutral conditions. They proved to be potent cytotoxic agents against the growth of murine L1210 and P388 leukemias and human HL-60 promyelocytic leukemia, HuT-78 lymphoma, and HeLa-S^3 uterine carcinoma. Selective compounds were active against the growth of $Tmolt_3$ and $Tmolt_4$ leukemias and THP-1 acute monocytic leukemia, liver Hepe-2, ovary 1-A9, ileum HCT-8 adenocarcinoma, and osteosarcoma HSO. A mode of action study in HL-60 cells demonstrated that DNA and protein syntheses were inhibited after 60 min at 100 μM. DNA and RNA polymerases, PRPP-amido transferase, dihydrofolate reductase, thymidylate synthase, and TMP kinase activities were interfered with by the agent with reduction of d[NTP] pools. Nonspecific interaction with the bases of DNA and cross-linking of the DNA may play a role in the mode of action of these carboxylates.

Keywords: Antitumor; Pyrroles, Cytotoxicity

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Full Paper

Introduction

Previously it has been shown that alkyl-3,4-bis(4-methoxyphenyl) pyrrole-2-carboxylates [1] and 2,3,4-trisubstituted pyrroles [2] demonstrated potent cytotoxicity against the growth of murine and human tumors, i.e. leukemia, lymphomas, $Tmolt_4$ leukemia, THP-1 acute monocytic leukemia, and HeLa-S^3 uterine carcinoma. In addition, it also has been documented that 2,4-disubstituted and 2,3,4-trisubstituted brominated pyrroles [3] successfully demonstrated potent cytotoxicity against the growth of solid human tumors. These compounds afforded ED_{50} values of $\leq$ 4 μg/mL, which is required for significant activity. All of the compounds were active against the growth of human HL-60 cell leukemia suppressing the DNA and RNA syntheses but not protein synthesis in 60 min at 100 μM. Mode of action studies revealed the compounds suppressed DNA polymerase α, m-RNA polymerase, t-RNA polymerase, dihydrofolate reductase, and nucleoside kinase activities. In calf-thymus DNA studies, certain compounds caused alkylation of the bases of DNA. DNA fragmentation occurred after 24 h with certain compounds, while other compounds appeared to cause cross-linking of the DNA stands [3].

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Results

The substituted ethyl 2-phenacyl-3-phenylpyrrole-4-carboxylates demonstrated potent activity against the growth of murine L1210 lymphoid leukemia and P388 lymphocytic leukemias [Table 1]. ED_{50} values of less than

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Table 1. Cytotoxicity [ED_{50} values = µg/mL] of carboxylates in murine and human tumors.

Tumor cell line	**9 c**	**9 b**	**9 d**	**9 a**	**6-MP**	**Ara-C**	**VP-16 Etopo-side**	**Mitomy-cin C**	**5-FU**
L1210 mouse leukemia	3.09	3.09	3.59	2.64	2.43	3.07	1.83		1.41
P388 mouse lymphocytic leukemia	2.77	3.42	2.11	3.38	2.04	0.79	0.99		1.41
HL-60 human leukemia	2.22	1.44	3.79	1.79	3.35	4.00	4.43		5.28
$Tmolt_3$ T cell leukemia	4.70	6.28	3.22	4.17	1.62	2.67	1.00		2.14
T $molt_4$ T cell leukemia	5.88	5.72	3.40	3.20	2.67	2.36	1.92		2.75
HuT-78 lymphoma	3.22	2.13	2.75	2.96	1.68	2.50	1.33		5.81
THP-1 Acute monocytic leukemia	4.06	4.57	3.56	2.63	3.03	2.54	3.27		1.12
HeLa-S^3 uterine	3.98	2.48	2.59	2.64	2.12	2.13	1.69		2.47
KB nasopharynx	4.08	5.74	4.06	5.06	11.04	2.84	3.32	1.70	1.25
Lung A-549	5.15	6.22	6.04	4.00	4.71	5.62	4.74	0.14	3.58
Liver Hepe-2	3.14	3.24	4.23	3.26					
Ovary 1-A9	2.32	2.58	6.50	4.90	6.64	5.39	6.24		
Breast MCF-7	8.18	7.77	4.53	3.28	8.84	12.45	11.00	0.45	6.82
Glioma UM 86	5.63	8.69	6.87	2.47	4.46	1.88	2.44	1.60	1.28
Ileum HCT-8	3.23	3.28	1.41	4.42	1.15	2.54	1.13	0.15	1.30
Prostate PL	8.52	6.65	6.28	10.9			2.80	1.60	
Osteosarcoma HSO	2.35	4.21	2.14	9.57	9.13	0.86	3.57		8.73
Melanoma SK2	9.10	6.00	2.32	6.89	6.86	10.53	3.53		5.93
Normal RMPI 1788	7.84	6.12	7.34	8.73	–	–	–		

4 µg/mL were considered significant according to the NCI protocol [7]. This was the concentration of test compound required to cause 50 % tumor cell death after 3 days incubation. All four compounds were active against the growth of human HL-60 leukemia, HuT-8 lymphoma, and suspended HeLa-S^3 uterine growth. Compounds **9 a** and **9 d** were active against $Tmolt_4$ T cell leukemia, and THP-1 acute monocytic leukemia growth but only compound **9 d** was active against $Tmolt_3$ T cell leukemia growth. None of the compounds were active against the growth of the solid tumors: KB nasopharynx, lung A-549, prostate-PL, or human normal fibroblasts 1788. Yet, ileum adenocarcinoma HCT-8 growth was suppressed by compounds **9 b**, **9 c,** and **9 d,** osteosarcoma HSO by **9 c** and **9 d**, liver-Hepe-2 carcinoma by compounds **9 a**, **9 b**, and **9 c**, ovary A-1carcinoma by compounds **9 b** and **9 c**. Compound **9 d** reduced the growth SK-2 melanoma and compound **9 a** reduced the growth of breast MCK-7 and glioma UM-86.

A mode of action study in HL-60 leukemia cells with compound **9 a** showed that after 60 min at 100 µM, DNA synthesis was suppressed 37 % and protein synthesis 52 % but there was no effect on RNA synthesis [Table 2]. DNA polymerase α activity was reduced 51 %, m-RNA polymerase activity 47 %, r-RNA polymerase activity 39 % and t-RNA polymerase activity 50 % after 60 min at 100 µM. *De novo* purine synthesis was suppressed 23 % with the regulatory enzyme of the pathway, PRPP-amido transferase, being inhibited 88 %. Dihydrofolate reductase activity was reduced 93 %, thymidylate synthase activity 26 % and TMP kinase activity 32 %. The *de novo* pyrimidine pathway and its regulatory enzyme activities were not affected by compound **9 a** as was true for IMP dehydrogenase, thymidine kinase, and TDP kinase activities. The inhibition of the above enzyme activities was reflected in the deoxyribonucleotide pool levels in that dATP was reduced 24 %, dGTP, 19 %, dCTP 13 %, and TTP 17 % after 60 min at 100 µM. *ct*-DNA studies showed that compound **9 a** caused a slight hyperchomic shift in the UV absorption from 220–340 mm but there was no change in the T_M values for DNA denaturation or for DNA viscosity after 24 h incubation at 100 µM. HL-60 leukemia cells after 24 h incubation at 100 µM demonstrated evidence of cross-linking of the DNA molecules [Fig. 4]. Compound **9 a** did not inhibit DNA topoisomerase I or II activities or elevate caspase 1 or 3 cleavage in HL-60 cells at 100 µM.

Table 2. Effects of compound **9 a** on Hl-60 leukemia cell metabolism after 60 min incubation.

Assay ($N = 6$)	Percent of control ($\bar{X}$ ± S.D.). Control	25 µM	50 µM	100 µM
DNA synthesis	100 ± 6[a]	79 ± 5*	72 ± 4*	63 ± 4*
RNA synthesis	100 ± 5[b]	106 ± 6	105 ± 5	100 ± 4
Protein synthesis	100 ± 6[c]	52 ± 6*	49 ± 5*	48 ± 4*
DNA polymerase α	100 ± 5[d]	43 ± 4*	41 ± 4*	39 ± 3*
mRNA polymerase	100 ± 4[e]	87 ± 5	58 ± 4*	53 ± 5*
rRNA polymerase	100 ± 6[f]	78 ± 4*	72 ± 4*	61 ± 3*
tRNA polymerase	100 ± 6[g]	89 ± 5	87 ± 6	50 ± 4*
Ribonucleotide reductase	100 ± 6[h]	101 ± 4	98 ± 5	95 ± 4
De novo purine synthesis	100 ± 6[i]	100 ± 5	86 ± 3	77 ± 3*
PRPP amido transferase	100 ± 6[j]	60 ± 4*	18 ± 3*	12 ± 2*
IMP dehydrogenase	100 ± 7[k]	105 ± 5	96 ± 3	91 ± 4
De novo pyrimidine synthesis	100 ± 6[l]	93 ± 4	91 ± 3	88 ± 3
Carbamyl phosphate synthetase	100 ± 6[m]	97 ± 5	93 ± 4	90 ± 4
Aspartate transcarbamylase	100 ± 7[n]	128 ± 6*	103 ± 7	86 ± 4
Thymidylate synthase	100 ± 6[o]	100 ± 5	89 ± 5	74 ± 4*
Thymidine kinase	100 ± 5[p]	109 ± 6	133 ± 7	132 ± 5
TMP kinase	100 ± 4[q]	141 ± 6	95 ± 4	68 ± 4*
TDP kinase	100 ± 4[r]	113 ± 6	98 ± 5	95 ± 5
Dihydrofolate reductase	100 ± 6[s]	29 ± 6*	13 ± 4*	7 ± 3*
d(ATP)	100 ± 4[t]			76 + 5*
d(GTP)	100 ± 6[u]			81 ± 4*
d(CTP)	100 ± 6[v]			87 ± 5
d(TTP)	100 ± 6[w]			83 ± 6*

* $P \leq 0.001$;

[a] 45011 dpm [b] 4226 dpm [c] 5343 dpm [d] 7125 dpm [e] 5693 dpm [f] 8394 dpm [g] 5151 dpm [h] 63565 dpm [i] 17646 dpm [j] 0.164 OD units [k] 4658 dpm [l] 7316 dpm [m] 1.242 µmoles citrulline [n] 1.030 mol *N*-carbamyl aspartate [o] 13890 dpm [p] 1511 dpm [q] 320 dpm [r] 286 dpm [s] 0.092 OD units [t] 9.02 pmoles [u] 11.21 pmoles [v] 13.65 pmoles [w] 16.73 pmoles

Discussion

The substituted ethyl 2-phenacyl-3-phenylpyrrole-4-carboxylates demonstrated good cytotoxic activity against murine and human cancers similar to previously reported related compounds in that they were more active against suspended tumor cells and more selective towards inhibiting cell growth of cells derived form solid tumors. It should be noted that no marked structure activity relationship existed for the cytotoxicity of the pyrrole derivatives tested. The suppression of DNA synthesis in HL-60 leukemia cells by the pyrrole derivative was due to multiple enzymes being targeted by the drug. DNA template utilization was affected since all of the polymerase activities were reduced in a concentration manner. The agent also seemed to be directed towards *de novo* purine synthesis at its regulatory enzyme, PRPP-amido transferase, but the *de novo* pyrimidine pathway appeared not to be affected after 60 min. The marked reduction in dihydrofolate reductase activity afforded by the compound would also reduce one-carbon transfer for *de novo* purine synthesis. Nucleoside kinase activity was marginally reduced as was the d[NTP] pools after 60 min. These pool levels are the net of two metabolic events in that the d[NTP] pools should rise due to the inhibition of DNA polymerase activity which would not allow the incorporation of d[NTP] into the new strand of DNA whereas inhibition of purine synthesis would reduce the available d[NTP]s for the incorporation. The observation that RNA synthesis was not affected significantly by the agent in 60 min is not unusual in that RNA

to DNA ratios in mammalian cells is 10:1 and the blockage of purine synthesis would affect DNA synthesis earlier than RNA synthesis. The DNA molecule itself can not be eliminated as a target of the agent in that a slight hyperchromic shift occurred. This binding of the drug to DNA is probably not by alkylation of the nucleoside bases by the pyrrole ring but rather it probably is a nonspecific interaction between the drug and the chromatin materail. The drug did not cause intercalation between nucleoside base pairs and no DNA fragmentation occurred in 24 h. This is consistent with the agent having no effect on DNA topoisomerase I and II activities or the repair DNA β polymerase activity. The agent did not appear to function in a manner so that it caused tumor cell death due to activation of apoptosis since the caspase proteolytic enzymes were not activated. HL-60 cells did demonstrate cross-linking of the DNA-strands after 24 h incubation suggesting the possibility of a nonspecific interaction with chromatin structure.

Compared to the previous pyrrole derivatives, the current pyrrole demonstrated more potent inhibition of protein synthesis and dihydrofolate reductase activity but was similar in its reduction of PRPP-amido transferase and TMP kinase activities and cross-linking of the DNA strands [1–3]. However, the current pyrrole did not cause inhibition of IMP dehydrogenase or ribonucleotide reductase activities as observed with other pyrroles [1–3].

Chemistry

The 2,3,4-trisubstituted pyrroles (**9 a**–**9 d**) were prepared using methods previously reported [1–5] by heating an α-aminoketone (**7** or **8**) and a β-chloroenal (**4 a–4 d**) in DMF under neutral conditions. In this [3+2]-cycloaddition reaction, the nitrogen and C-1 are from the aminoketone and the remaining three carbons are provided from the β-chloroenal. The chloroenal was prepared from an acetophenone in three steps. A Claisen-type condensation [6] of the acetophenone (**1 a–1 d**) and diethyl carbonate is carried out in toluene and sodium

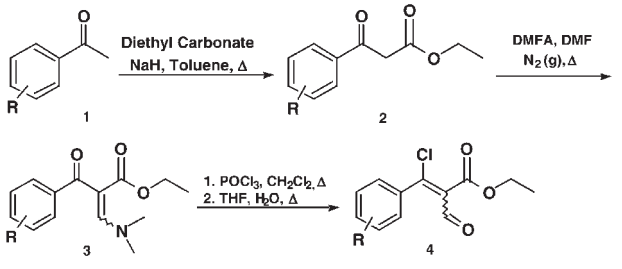

Figure 1. Preparation of the β-chloroenal from an acetophenone. R = 3-Cl (a), 4-Cl (b), 4-Br (c), 4-CH_3 (d).

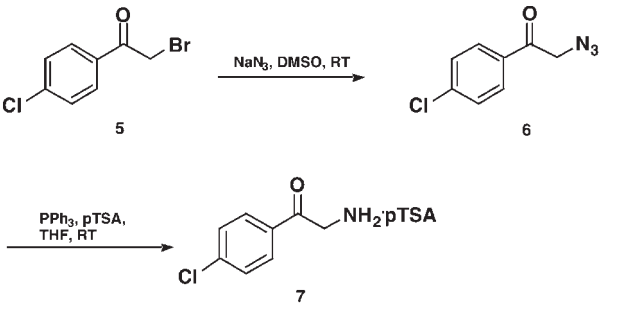

Figure 2. Preparation of the salt of the α-aminoketone from a 2-bromoacetophenone.

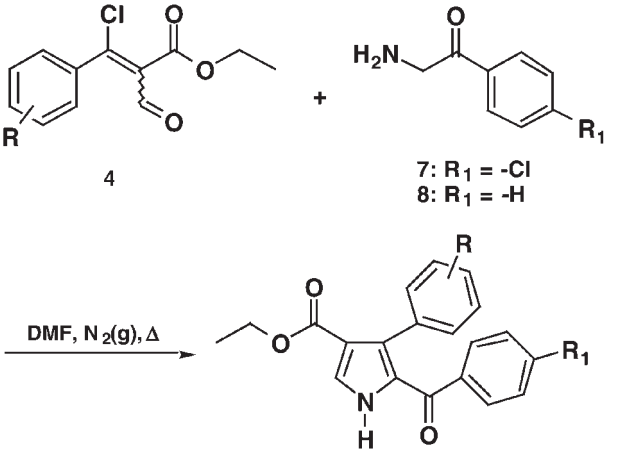

Figure 3. Synthesis of the 2,3,4-trisubstituted pyrroles.

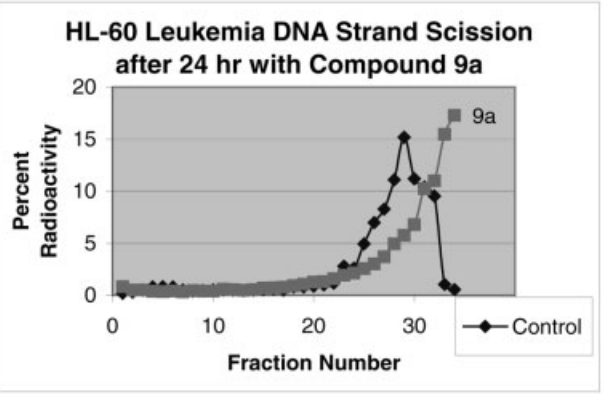

Figure 4. HL-60 DNA strand scission after 24 h at 100 μM.

hydride, which produces a β-ketoester (**2 a–2 d**) in good yield. The ketoester is treated with DMF-acetal quantitatively yielding a vinylogous amide (**3 a–3 d**), which is then refluxed in $POCl_3$, and CH_2Cl_2 for 2–3 h followed by hydrolysis in THF/H_2O affording the β-chloroenal (4 a–4 d) yields of 14–34 % (Figure 1). The pTSA salt of

an α-amino ketone (**7**) is conveniently prepared in two steps [7] by first forming an α-azidoketone (**6**) from the 2-bromoacetophenone (**5**) and NaN_3 in DMSO followed by a reduction carried out by Ph_3P and *p*TSA (Figure 2). Finally, heating the β-chloroenal and the salt of the α-aminoketone in dry DMF forms the 2,3,4-trisubstituted pyrrole (**9 a–9 d**, Figure 3).

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Experimental

Materials and methods

All chemicals and reagents were obtained from Aldrich Chemical Company (Milwaukee, WI) and used as received except for dry solvents, which were dried and distilled using standard procedures [8]. TLC was performed using silica gel 60F 254 plates (silica gel on plastic, Aldrich Chemical Company). Melting points were obtained on a Thomas-Hoover Uni-melt apparatus (capillary method), and were uncorrected. IR spectra were obtained on a Perkin-Elmer 1600 FTIR spectrometer on sodium chloride plates or in a potassium chloride liquid cell in $CHCl_3$ or $CDCl_3$. NMR spectra were obtained on a 300 MHz Bruker Avance FT-NMR spectrometer using tetramethylsilane as an external standard for ^1H and ^{13}C spectra (δ = 0 ppm). Elemental analyses were performed by Quantitative Technologies, Inc. (Whitehouse, NJ).

Preparation of ethyl 3-(3′-chlorophenyl)-3-oxopropionate (**2 a**)

Sodium hydride (6.5 g, 0.16 mol, 2.5 Eq) as a 60 % dispersion in mineral oil was placed in a 1L 3-neck flask under nitrogen atmosphere. The mineral oil was removed by washing 2× with 50 mL hexanes. To the NaH was added 150 mL toluene and diethyl carbonate (38.21 g, 0.323 mol, 5 Eq) with stirring. 3′-Chloroacetophenone (10.0 g, 0.0647 mol) was added dropwise to the flask containing the NaH and diethyl carbonate over 7 min. The mixture was stirred at reflux under N_2 (*g*) for 12 h. After cooling to RT, 25 mL of glacial acetic acid was added to the stirring mixture. The mixture was then poured over ice (40 mL) and conc. HCl (10 mL). The aqueous layer was washed 3× with 50 mL ethyl acetate. The combined organic layers were washed 2× with 50 mL sat. $NaHCO_3$ (*aq*) and 2 × 50 mL brine. The solution was dried over anhydrous $MgSO_4$, filtered, and the solvent was removed under reduced pressure. The crude oil was purified by Kugelrohr distillation, bp = 114–116 °C at 0.5 mm Hg, affording 6.96 g (0.031 mol) of a clear oil in a 47 % yield. TLC showed a single spot with an R_f = 0.43 in 9:1 hexanes:ethyl acetate. IR (neat on NaCl): ν = 1744 cm^{-1} (C=O), 1693 cm^{-1} (C=O), 1628 cm^{-1} (C=O), 1594 cm^{-1} (aromatic C-C). ^1H NMR of keto tautomer ($CDCl_3$): δ = 7.83 (s, 1 H, 7.02–7.75 (m, 3 H), 4.11 (q, 2 H, *J* = 7.2 Hz), 3.85 (s, 2 H), 1.22 (t, 3 H, *J* = 7.2 Hz) ppm. ^{13}C NMR ($CDCl_3$): δ = 192, 167, 138, 136, 134, 131, 129, 127, 62, 46, 14 ppm. ($C_{11}H_{11}ClO_3$).

Preparation of ethyl 3-(4′-chlorophenyl)-3-oxopropionate (**2 b**)

Sodium hydride (6.78 g, 0.169 mol, 2.2 Eq) as a 60 % dispersion in mineral oil was placed in a 1L 3-neck flask under nitrogen atmosphere. The mineral oil was removed by washing with 2× 50 mL hexanes. To the NaH was added 400 mL toluene and diethyl carbonate (47 mL, 45.8 g, 0.389 mol, 5.1 Eq) with stirring. A solution of 4′-chloroacetophenone (10 mL, 11.9 g, 0.077 mol) in 10 mL toluene was added dropwise to the flask containing the NaH and diethyl carbonate. The mixture was stirred at reflux under N_2 (*g*) for 18 h. After cooling to RT, 25 mL of glacial acetic acid was added to the stirring mixture. The mixture was then poured over ice (500 mL) and conc. HCl (10 mL). The aqueous layer was washed with 3× 50 mL ethyl acetate. The combined organic layers were washed with 3× 50 mL cold H_2O, 3× 50 mL sat. $NaHCO_3$ (*aq*) and 3× 50 mL brine. The solution was dried over anhydrous $MgSO_4$, filtered and the solvent was removed under reduced pressure. The crude oil was purified by Kugelrohr distillation, bp = 121–123 °C at 0.5 mm Hg, affording 9.49 g (.0419 mol) of a pale yellow oil in a 54 % yield. TLC showed a single spot with an R_f = 0.53 in 7:3 hexanes:ethyl acetate. IR (neat on NaCl): ν = 1742 cm^{-1} (C=O), 1684 cm^{-1} (C=O), 1607 cm^{-1} (aromatic C-C). ^1H NMR of keto tautomer ($CDCl_3$): δ = 7.81 (d, 2 H, *J* = 8.2 Hz), 7.37 (d, 2 H, *J* = 8.2 Hz), 4.13 (q, 2 H, *J* = 7.2 Hz), 3.88 (s, 2 H), 1.18 (t, 3 H, *J* = 7.2 Hz) ppm. ^{13}C NMR ($CDCl_3$): δ = 192, 168, 141, 132, 130.3, 129.5, 62, 46, 15 ppm. ($C_{11}H_{11}ClO_3$).

Preparation of ethyl 3-(4′-bromophenyl)-3-oxopropionate (**2 c**)

Sodium hydride (6.94 g, 0.174 mol, 2.6 Eq) as a 60 % dispersion in mineral oil was placed in a 1L 3-neck flask under nitrogen atmosphere. The mineral oil was removed by washing 2× with 50 mL hexanes. To the NaH was added 300 mL toluene and diethyl carbonate (48 mL, 46.8 g, 0.396 mol, 5.9 Eq) with stirring. A solution of 4′-bromoacetophenone (13.29 g, 0.0668 mol) in 100 mL toluene was added dropwise to the flask containing the NaH and diethyl carbonate. The mixture was stirred at reflux under N_2 (*g*) for 18 h. After cooling to RT, 30 mL of glacial acetic acid was added to the stirring mixture. The mixture was then poured over ice (500 mL) and conc. HCl (20 mL). The aqueous layer was washed 2× with 50 mL ethyl acetate. The combined organic layers were washed with 3× 50 mL cold H_2O, 3× 50 mL sat. $NaHCO_3$ (*aq*), and 3× 50 mL brine. The solution was dried over anhydrous $MgSO_4$, filtered, and the solvent was removed under reduced pressure. The crude oil was purified by Kugelrohr distillation, bp = 129–131 °C at 0.6 mm Hg, affording 14.85 g (0.05477 mol) of a dark brown oil in a 82 % yield. TLC showed a single spot with an R_f = 0.59 in 7:3 hexanes:ethyl acetate. IR (neat on NaCl): ν = 1688 cm^{-1} (C=O), 1620 cm^{-1} (C=O), 1586 cm^{-1} (aromatic C-C). ^1H NMR of keto tautomer ($CDCl_3$): δ = 7.76 (d, 2 H, *J* = 8.5 Hz), 7.76 (d, 2 H, *J* = 8.5 Hz), 4.08 (q, 2 H, *J* = 7.2 Hz), 3.82 (s, 2 H), 1.13 (t, 3 H, *J* = 7.2 Hz) ppm. ^{13}C NMR ($CDCl_3$): δ = 191, 167, 140, 135, 131, 129, 62, 46, 14 ppm. ($C_{11}H_{11}BrO_3$).

Preparation of ethyl 3-(4′-methylphenyl)-3-oxopropionate (**2 d**)

Sodium hydride (11.2 g, 0.28 mol, 2.5 Eq) as a 60 % dispersion in mineral oil was placed in a 1L 3-neck flask under nitrogen atmosphere. The mineral oil was removed by washing 2× with 50 mL hexanes. To the NaH was added 400 mL toluene and diethyl carbonate (27 mL, 0.22 mol, 2 Eq) with stirring. A solution of 4′-methylacetophenone (15.0 mL, 15.1 g, 0.112 mol) in 100 mL toluene was added dropwise to the flask containing the NaH and diethyl carbonate. The mixture was stirred at reflux under N_2 (*g*) for 10 h. After cooling to RT, 60 mL of glacial acetic acid was added to the stirring mixture. The mixture was then poured over ice (500 mL) and conc. HCl (30 mL). The aqueous layer was washed 3× with 50 mL $CHCl_3$. The combined organic layers were washed 3× with 50 mL cold H_2O, 3× with 50 mL sat. $NaHCO_3$ (*aq*) and 3× with 50 mL brine. The solution was dried over anhydrous $MgSO_4$, filtered and the

solvent was removed under reduced pressure. The crude oil was purified by Kugelrohr distillation, bp = 139 °C at 0.2 mm Hg, affording 13.06 g of a clear oil in a 58 % yield. TLC showed a single spot with an R_f = 0.67 in 7:3 hexanes:ethyl acetate. IR (neat on NaCl): ν = 1742 cm^{-1} (C=O), 1684 cm^{-1} (C=O), 1607 cm^{-1} (aromatic C-C). ^1H NMR of keto tautomer ($CDCl_3$): δ = 7.81 (d, 2 H, *J* = 8.1 Hz), 7.23 (d, 2 H, *J* = 8.1 Hz), 4.18 (q, 2 H, *J* = 7.2 Hz), 3.91 (s, 2 H), 2.38 (s, 3 H), 1.23 (t, 3 H, *J* = 7.2 Hz) ppm. ^{13}C NMR ($CDCl_3$): δ = 192, 167, 144, 134, 130, 129, 62, 46, 22, 14 ppm. ($C_{12}H_{14}O_3$).

Preparation of ethyl 1-(3′-chlorophenyl)-3-(dimethylamino)-prop-2-enone-2-carboxylate (**3 a**)

To a solution of 21.2 g (0.178 mol, 4 Eq) dimethylformamide dimethyl acetal (DMFA) in 175 mL of dry DMF was added 10.0 g (0.044 mol) of the β-keto ester (**2 a**). The solution was stirred at reflux under N_2 (*g*) for 18 h. The solvents were removed under reduced pressure and by Kugelrohr distillation. The dark oily product was purified by Kugelrohr distillation affording 7.37 g (29.9 mmol) of a dark yellow oil, bp = 167 °C at 1.00 mm Hg, in 68 % yield. TLC showed a single spot with an R_f = 0.66 in 7:3 hexanes:ethyl acetate. IR (neat on NaCl): ν = 1698 cm^{-1} (C=O), 1651 cm^{-1} (C=O). ^1H NMR of *Z* isomer ($CDCl_3$): δ = 7.92 (s, 1 H), 7.30–7.82 (m, 4 H), 4.21 (q, 2 H, *J* = 7.2 Hz), 3.03 (br.s., 6 H), 1.25 (t, 3 H, *J* = 7.2 Hz) ppm. ^{13}C NMR of *Z* isomer ($CDCl_3$): δ = 191.70, 167.48, 163.37, 157.09, 130.49, 129.72, 129.64, 129.61, 128.95, 127.76, 127.02, 62.04, 14.45 ppm. ($C_{14}H_{16}ClNO_3$).

Preparation of ethyl 1-(4′-chlorophenyl)-3-(dimethylamino)-prop-2-enone-2-carboxylate (**3 b**)

To a solution of 19.10 g (0.160 mol, 4.1 Eq) dimethylformamide dimethyl acetal (DMFA) in 100 mL of dry DMF was added 8.77 g (0.039 mol) of the β-keto ester (**2 b**). The solution was stirred at reflux under N_2 (*g*) for 18 h. The solvents were removed under reduced pressure and by Kugelrohr distillation. The dark oily product was purified by Kugelrohr distillation affording 10.10 g (0.0358 mol) of a dark yellow oil, bp = 181 °C at 0.77 mm Hg, in 93 % yield. TLC showed a single spot with an R_f = 0.08 in 7:3 hexanes:ethyl acetate. IR (neat on NaCl): ν = 1727 cm^{-1} (C=O), 1683 cm^{-1} (C=O), 1631 cm^{-1} (C=O), 1587 cm^{-1} (aromatic C-C). ^1H NMR of *Z* isomer ($CDCl_3$): δ = 7.53–7.76 (m, 3 H), 7.29 (d, 2 H, *J* = 8.7 Hz), 3.90 (q, 2 H, *J* = 7.2 Hz), 2.92 (br.s., 6 H), 0.88 (t, 3 H, *J* = 7.2 Hz) ppm. ^{13}C NMR of *Z* isomer ($CDCl_3$): δ = 192.91, 168.79, 156.61, 130.61, 130.46, 129.92, 128.58, 128.17, 60.19, 14.34 ppm. ($C_{14}H_{16}ClNO_3$).

Preparation of ethyl 1-(4′-bromophenyl)-3-(dimethylamino)-prop-2-enone-2-carboxylate (**3 c**)

To a solution of 14.7 mL (13.2 g, 0.111 mol, 4.5 Eq) dimethylformamide dimethyl acetal (DMFA) in 100 mL of dry DMF was added 6.71 g (0.0247 mol) of the β-keto ester (**2 c**). The solution was stirred at reflux under N_2 (*g*) for 18 h. The solvents were removed under reduced pressure and by Kugelrohr distillation. The dark oily product was purified by Kugelrohr distillation affording 5.35 g (16.4 mmol) of a dark yellow oil, bp = 181 °C at 1.10 mm Hg, in 66 % yield. TLC showed a single spot with an R_f = 0.09 in 7:3 hexanes:ethyl acetate. IR (neat on NaCl): ν = 1728 cm^{-1} (C=O), 1682 cm^{-1} (C=O), 1633 cm^{-1} (C=O), 1588 cm^{-1} (aromatic C-C). ^1H NMR of *Z* isomer ($CDCl_3$): δ = 7.66 (s, 1 H), 7.56 (d, 2 H, *J* = 8.4 Hz), 7.45 (d, 2 H, *J* = 8.4 Hz), 3.90 (q, 2 H, *J* = 7.2 Hz), 2.88 (br.s., 6 H), 0.87 (t, 3 H, *J* = 7.2 Hz) ppm. ^{13}C NMR of *Z* isomer ($CDCl_3$): δ = 191.65, 167.42, 161.49, 155.31, 131.10, 130.68, 130.14, 129.79, 128.81, 58.80, 12.92 ppm. ($C_{14}H_{16}BrNO_3$).

Preparation of ethyl 3-(dimethylamino)-1-(4′-methylphenyl)-prop-2-enone-2-carboxylate (**3 d**)

To a solution of 19.07 g (160 mmol, 4 Eq) dimethylformamide dimethyl acetal (DMFA) in 150 mL of dry DMF was added 8.2 g (40 mmol) of the β-keto ester **2 d**). The solution was stirred at reflux under N_2 (*g*) for 48 h. The solvents were removed under reduced pressure and by Kugelrohr distillation. The dark oily product was purified by Kugelrohr distillation affording 8.88 g (34.0 mmol) of a dark yellow oil, bp = 138–143 °C at 3 mm Hg, in 85 % yield of *E* and *Z* isomers. TLC showed a single spot with an R_f = 0.66 in 7:3 hexanes:ethyl acetate. IR (neat on NaCl): ν = 1742 cm^{-1} (C=O), 1678 cm^{-1} (C=O), 1634 cm^{-1} (C=O), 1607 cm^{-1} (aromatic C-C). ^1H NMR of *Z* isomer ($CDCl_3$): δ = 7.93, (s, 1 H), 7.58 (d, 2 H, *J* = 8.1 Hz), 7.10 (d, 2 H, *J* = 8.1 Hz), 3.90 (q, 2 H, *J* = 7.1 Hz), 2.86 (br.s., 6 H), 2.30 (s, 3 H), 0.87 (t, 3 H, *J* = 7.1 Hz) ppm. ^{13}C NMR of *Z* isomer ($CDCl_3$): δ = 194, 169, 155, 142, 139, 129.3, 129.0, 126, 60, 44, 22, 14 ppm. ($C_{15}H_{19}NO_3$).

Preparation of ethyl 3-(3′-chlorophenyl)-3-chloropropenal-2-carboxylate (**4 a**)

To a solution of 5.47 g (22.2 mmol) of the vinylogous amide (**3 a**) in 100 mL CH_2Cl_2 was added 4.5 mL (7.4 g, 48 mmol, 2.2 Eq) of phosphorus oxychloride. The solution was stirred at reflux under N_2 (*g*) for 3 h. The solvent was removed under reduced pressure. To the resulting dark oil was added 100 mL of 1:1 THF:H_2O. The solution was stirred at reflux overnight. The volatiles were removed under reduced pressure and the aqueous layer was washed 2× 50 mL with $CHCl_3$. The combined $CHCl_3$ layers were washed 4× 50 mL with water. The $CHCl_3$ layer was dried over anhydrous $MgSO_4$, filtered, and the solvent removed under reduced pressure. The crude dark oil was purified by column chromatography on silica gel using 8:2 hexanes:ethyl acetate, R_f = 0.61. A pale yellow oil, 2.04 g (7.45 mmol), bp = 134 °C at 0.3 mm Hg, was afforded in 34 % yield as a mixture of *E* and *Z* isomers. IR (neat on NaCl): ν = 1734 cm^{-1} (C=O), 1685 cm^{-1} (C=O), 1628 cm^{-1} (C=O), 1594 cm^{-1} (aromatic C-C). ^1H NMR of *Z* isomer ($CDCl_3$): δ = 10.07 (s, 1 H), 7.84 (s, 1 H), 7.74 (d, 1 H, *J* = 7.7 Hz), 7.25–7.41 (m, 2 H), 4.14 (q, 2 H, *J* = 7.2 Hz), 1.18 (t, 3 H, *J* = 7.2 Hz) ppm. ^{13}C NMR of *Z* isomer ($CDCl_3$): δ = 187.62, 167.50, 137.90, 135.56, 134.06, 131.51, 130.50, 128.95, 128.39, 127.02, 62.04, 14.44 ppm. ($C_{12}H_{10}Cl_2O_3$).

Preparation of ethyl 3-(4′-chlorophenyl)-3-chloropropenal-2-carboxylate (**4 b**)

To a solution of 10.10 g (0.0358 mol) of the vinylogous amide (**3 b**) in 100 mL CH_2Cl_2 was added 3.50 mL (5.76 g, 0.0375 mol, 1.05 Eq) of phosphorus oxychloride. The solution was stirred at reflux under N_2 (*g*) for 2 h. The solvent was removed under reduced pressure. To the resulting dark oil was added 100 mL of 1:1 THF:H_2O. The solution was stirred at reflux for 18 h. The volatiles were removed under reduced pressure and the aqueous layer was washed 3× 50 mL with ethyl acetate. The combined ethyl acetate layers were washed 2× 50 mL with water. The ethyl acetate layer was dried over anhydrous $MgSO_4$, filtered and the solvent removed under reduced pressure. The crude dark oil was purified by column chromatography on silica gel using 7:3 hexanes:ethyl acetate, R_f = 0.73. A pale yellow oil, 4.95 g (0.0181 mol), bp = 132–134 °C at 0.35 mm Hg, was afforded in 50.6 % yield of *E* and *Z* isomers. IR (neat on NaCl): ν = 1732 cm^{-1} (C=O), 1682 cm^{-1} (C=O), 1590 cm^{-1} (aromatic C-C). ^1H NMR of *Z* isomer ($CDCl_3$): δ = 9.28 (s, 1 H), 7.42 (d, 2 H, *J* = 8.8 Hz), 7.32 (d, 2 H, *J* = 8.8 Hz), 4.31 (q, 2 H, *J* = 7.2 Hz), 1.29 (t, 3 H, *J* = 7.2 Hz) ppm. ^{13}C NMR of *Z* isomer ($CDCl_3$): δ = 186.07, 164.00, 153.88, 138.73, 136.34, 132.54, 131.54, 131.53, 129.61, 62.77, 14.45 ppm. ($C_{12}H_{10}Cl_2O_3$).

Preparation of ethyl 3-(4′-bromophenyl)-3-chloropropenal-2-carboxylate (**4c**)

To a solution of 6.44 g (19.7 mmol) of the vinylogous amide (**3c**) in 100 mL CH_2Cl_2 was added 1.84 mL (3.03 g, 19.7 mmol, 1 Eq) of phosphorus oxychloride. The solution was stirred at reflux under N_2 (*g*) for 4 h. The solvent was removed under reduced pressure. To the resulting dark oil was added 100 mL of 1:1 THF:H_2O. The solution was stirred at RT for 3 days, then at reflux for 5 h. The volatiles were removed under reduced pressure and the aqueous layer was washed 3× 50 mL with ethyl acetate. The combined ethyl acetate layers were washed 2× with 50 mL water. The ethyl acetate layer was dried over anhydrous $MgSO_4$, filtered, and the solvent removed under reduced pressure. The crude dark oil was purified by column chromatography on silica gel using 8:2 hexanes:ethyl acetate, R_f = 0.53. A pale yellow oil, 1.37 g (4.31 mmol), bp = 138–140 °C at 0.35 mm Hg, was afforded in 22 % yield of *E* and *Z* isomers. IR (neat on NaCl): ν = 1736 cm^{-1} (C=O), 1676 cm^{-1} (C=O), 1584 cm^{-1} (aromatic C-C). ^{1}H NMR of *Z* isomer ($CDCl_3$): δ = 9.28 (s, 1 H), 7.54 (d, 2 H, *J* = 8.7 Hz), 7.30 (d, 2 H, *J* = 8.7 Hz), 4.31 (q, 2 H, *J* = 7.2 Hz), 1.28 (t, 3 H, *J* = 7.2 Hz) ppm. ^{13}C NMR of *Z* isomer ($CDCl_3$): δ = 187.21, 165.14, 155.09, 137.49, 134.18, 133.76, 132.83, 128.26, 63.92, 15.63 ppm. ($C_{12}H_{10}BrClO_3$).

Preparation of ethyl 3-chloro-3-(4′-methylphenyl)propenal-2-carboxylate (**4d**)

To a solution of 8.88 g (34.0 mmol) of the vinylogous amide (**3d**) in 100 mL CH_2Cl_2 was added 3.65 mL (6.00 g, 39.2 mmol, 1.2 Eq) of phosphorus oxychloride. The solution was stirred at reflux under N_2 (*g*) for 3 h. The solvent was removed under reduced pressure. To the resulting dark oil was added 100 mL of 1:1 THF:H_2O. The solution was stirred at RT for 3 days, then at reflux for 5 h. The volatiles were removed under reduced pressure and the aqueous layer was washed 3× with 50 mL ethyl acetate. The combined ethyl acetate layers were washed 2× with 50 mL water. The ethyl acetate layer was dried over anhydrous $MgSO_4$, filtered, and the solvent removed under reduced pressure. The crude dark oil was purified by column chromatography on silica gel using 8:2 hexanes:ethyl acetate, R_f = 0.65. A pale yellow oil, 1.20 g (4.75 mmol), bp = 122–123 °C at 0.3 mm Hg, was afforded in 14 % yield as a mixture of *E* and *Z* isomers. IR (neat on NaCl): ν = 1735 cm^{-1} (C=O), 1602 cm^{-1} (aromatic C-C). ^{1}H NMR of *Z* isomer ($CDCl_3$): δ = 9.40 (s, 1 H), 7.43 (d, 2 H, *J* = 8.1 Hz), 7.29 (d, 2 H, *J* = 8.1 Hz), 4.41 (q, 2 H, *J* = 7.2 Hz), 2.44 (s, 3 H), 1.40 (t, 3 H, *J* = 7.2 Hz) ppm. ^{13}C NMR of *Z* isomer ($CDCl_3$): δ = 186.48, 164.24, 155.46, 142.94, 135.83, 131.55, 130.29, 129.80, 62.40, 21.70, 14.37 ppm. ($C_{13}H_{13}ClO_3$).

Preparation of 2-azido-4′-choroacetophenone (**6**)

To a stirring suspension of 2-bromo-4′-chloroacetophenone (11.67 g, mmol) in 60 mL of DMSO at 10 °C was added 4.0 g (61 mmol, 1.2 Eq) of sodium azide. The mixture was allowed to come to RT and the resulting solution stirred at RT for 20 min. The solution was added to 200 mL of H_2O and the aqueous mixture was washed 3× with 50 mL ethyl acetate. The combined ethyl acetate layers were washed 3x with 50 mL water. The organic layer was dried over anhydrous sodium sulfate, filtered, and the solution was concentrated under reduced pressure. The product was crystallized from the concentrated ethyl acetate solution. The crystals were collected by suction filtration, washed with hexanes, and allowed to air dry. Yellow needles, 7.06 g (36.1 mmol) were afforded in 72 % yield. mp = 68–69 °C. TLC showed a single spot with an R_f = 0.64 in 7:3 hexanes:ethyl acetate. IR ($CDCl_3$ cell): ν = 2106 cm^{-1} (N_3), 1700 cm^{-1} (C=O), 1591 cm^{-1} (aromatic C-C). ^{1}H NMR ($CDCl_3$): δ = 7.83 (d, 2 H, *J* = 8.3 Hz), 7.46 (d, 2 H, *J* = 8.3 Hz), 4.41 (s, 2 H) ppm. ^{13}C NMR ($CDCl_3$): δ = 192.10, 140.80, 133.06, 129.46, 129.43, 54.97 ppm. ($C_8H_6ClN_3O$).

Preparation of 2-ammonium-4′-chloroacetophenone 4-toluenesulfonate (**7**)

2-Azido-4′-choroacetophenone (**6**), 4.46 g (22.8 mmol), triphenylphosphine, 5.98 g (22.8 mmol, 1 Eq), and *p*-toluenesulfonic acid, 13.02 g, (68.46 mmol, 3 Eq) were added to 150 mL of THF and the mixture was stirred under N_2 (*g*) at RT overnight. The resulting white solid was filtered and washed with THF affording 6.31 g (18.5 mmol) of pure salt, mp = 232–233 °C, in 81 % yield. TLC showed a single spot with an R_f = 0.41 in 7:3 hexanes:ethyl acetate. IR (Nujol): ν = 3176, 3085, 3057 cm^{-1} (NH_3), 1695 cm^{-1} (C=O), 1611 cm^{-1} (aromatic C-C). ^{1}H NMR (DMSO): δ = 8.20 (br. s., 3 H), 7.99 (d, 2 H, *J* = 8.7 Hz), 7.64 (d, 2 H, *J* = 8.7 Hz) 7.50 (d, 2 H, *J* = 8.1 Hz), 7.11 (d, 2 H, *J* = 8.1 Hz), 4.53 (s, 2 H), 2.28 (s, 3 H) ppm. ^{13}C NMR ($CDCl_3$): δ = 192.22, 145.40, 139.90, 138.42, 132.70, 130.33, 129.49, 128.45, 125.85, 45.30, 21.02 ppm. ($C_{15}H_{16}ClNO_4S$).

Preparation of ethyl 3-(3′-chlorophenyl)-2-phenacylpyrrole-4-carboxylate (**9a**)

1.48 (3.84 mmol) of the β-chloroenal (**4a**) and 0.73 g (4.23 mmol, 1.1 Eq) of α-aminoacetophenone hydrochloride (**8**) were dissolved in 25 mL of dry DMF and stirred with heating under N_2 (*g*) for 3 days. The solvents were concentrated under reduced pressure, then removed by Kugelrohr distillation. The crude dark solid was purified by column chromatography on silica gel using 7:3 hexanes:ethyl acetate, R_f = 0.35, followed by recrystallization in ethyl acetate/hexanes. Pale yellow crystals, 75 mg (0.21 mmol), mp = 135–137 °C, was afforded in 5.5 % yield. IR ($CDCl_3$ cell): ν = 3428 cm^{-1} (N-H), 2249 cm^{-1} (N-C), 1717 cm^{-1} (C=O), 1622 cm^{-1} (C=O), 1600 cm^{-1} (aromatic C-C). ^{1}H NMR ($CDCl_3$): δ = 9.95 (br. s., 1 H), 7.73 (d, 1 H, *J* = 3.6 Hz), 7.32 (d, 2 H, *J* = 8.1 Hz), 7.23 (t, 2 H, *J* = 7.5 Hz), 6.99–7.11 (m, 4 H), 6.88–6.98 (m, 2 H), 4.15 (q, 2 H, *J* = 7.2 Hz), 1.16 (t, 3 H, *J* = 7.2 Hz) ppm. ^{13}C NMR ($CDCl_3$): δ = 187.98, 163.90, 137.60, 135.47, 133.27, 131.67, 130.28, 129.66, 129.01, 128.93, 128.86, 128.45, 127.97, 127.89, 127.42, 117.62, 60.32, 14.35 ppm. Elemental analysis ($C_{20}H_{16}ClNO_3$): C: 67.90 %, H: 4.56 %, 3.96 % (theoretical), C: 67.82 %, H: 4.46 %, N: 3.94 % (found).

Preparation of ethyl 3-(4′-chlorophenyl)-2-(4′-chlorophenacyl)pyrrole-4-carboxylate (**9b**)

0.828 g (3.03 mmol) of the β-chloroenal (**4b**) and 1.03 g (3.01 mmol, 1 Eq) of the α-aminoketone *p*TSA salt (**7**) was dissolved in 20 mL of dry DMF and stirred with heating under N_2 (*g*) for 3 days. The solvents were concentrated under reduced pressure, then removed by Kugelrohr distillation. The crude dark solid was purified by column chromatography on silica gel using 8:2 hexanes:ethyl acetate, R_f = 0.37, followed by recrystallization in ethyl acetate/hexanes. Tan crystals, 46.5 mg (0.120 mmol), mp = 164–167 °C, was afforded in 4.0 % yield. IR ($CDCl_3$ cell): ν = 3247 cm^{-1} (N-H), 2360 cm^{-1} (N-C), 1726 cm^{-1} (C=O), 1609 cm^{-1} (C=O), 1588 cm^{-1} (aromatic C-C). ^{1}H NMR ($CDCl_3$): δ = 9.69 (br. s., 1 H, 7.73 (d, 1 H, *J* = 3.4 Hz), 7.24 (d, 2 H, *J* = 6.7 Hz), 7.04 (d, 2 H, *J* = 6.1 Hz), 7.00 (d, 2 H, *J* = 6.7 Hz), 6.98 (d, 2 H, *J* = 6.1 Hz), 4.17 (q, 2 H, *J* = 7.2 Hz), 1.18 (t, 3 H, *J* = 7.2 Hz) ppm. ^{13}C NMR ($CDCl_3$): δ = 186.48, 163.74, 138.37, 135.83, 134.09, 132.73, 132.08, 131.88, 130.44, 130.03, 128.92, 128.21, 127.66, 117.64, 60.36, 14.43 ppm. Elemental analysis ($C_{20}H_{15}Cl_2NO_3$): C: 61.87 %, H: 3.89 %, N: 3.61 % (theoretical),): C: 61.94 %, H: 3.68 %, N: 3.47 % (found).

Preparation of ethyl 3-(4′-bromophenyl)-2-phenacylpyrrole-4-carboxylate (**9 c**)

0.615 g (1.94 mmol) of the β-chloroenal (**4c**) and 0.367 g (2.14 mmol, 1.1 Eq) of α-aminoacetophenone hydrochloride (**8**) was dissolved in 25 mL of dry DMF and stirred with heating under N_2 (*g*) for 3 days. The solvents were concentrated under reduced pressure, then removed by Kugelrohr distillation. The crude dark solid was purified by column chromatography on silica gel using 7:3 hexanes:ethyl acetate, R_f = 0.46, followed by recrystallization in ethyl acetate/hexanes. Tan crystals, 132 mg (0.332 mmol), mp = 152–154 °C, was afforded in 17 % yield. IR ($CDCl_3$ cell): ν = 3234 cm^{-1} (N-H), 2358 cm^{-1} (N-C), 1724 cm^{-1} (C=O), 1609 cm^{-1} (C=O). ^1H NMR ($CDCl_3$): δ = 9.72 (br. s., 1 H), 7.69 (d, 1 H, *J* = 3.4 Hz), 7.24 (d, 2 H, *J* = 7.4 Hz), 7.21 (d, 2 H, *J* = 7.4 Hz), 7.07 (m, 2 H, *J* = 8.5 Hz), 6.99 (t, 2 H, *J* = 7.4 Hz), 6.86 (d, 2 H, *J* = 8.5 Hz), 4.11 (q, 2 H, *J* = 7.2 Hz), 1.14 (t, 3 H, *J* = 7.2 Hz) ppm. ^{13}C NMR ($CDCl_3$): δ = 187.98, 163.97, 137.34, 133.07, 132.42, 132.15, 131.86, 130.48, 130.12, 129.13, 128.91, 128.00, 121.86, 117.23, 60.45, 14.56 ppm. Elemental analysis ($C_{20}H_{16}BrNO_3$): C: 60.32 %, H: 4.05 %, N 3.52 % (theoretical), C: 60.03 %, H: 3.82 %, N 3.55 % (found).

Preparation of ethyl 3-(4′-methylphenyl)-2-(4′-chlorophenacyl)pyrrole-4-carboxylate (**9 d**)

1.00 g (3.96 mmol) of the β-chloroenal (**4 d**) and 1.54 g (3.96 mmol, 1 Eq) of the α-aminoketone *p*TSA salt (**7**) was dissolved in 20 mL of dry DMF and stirred with heating under N_2 (*g*) for 66 h. The solvents were concentrated under reduced pressure, then removed by Kugelrohr distillation. The crude dark solid was purified by column chromatography on silica gel using 7:3 hexanes:ethyl acetate, R_f = 0.22, followed by recrystallization in ethyl acetate/hexanes. White crystals, 221 mg (0.601 mmol), mp = 155–157 °C, was afforded in 15 % yield. IR ($CDCl_3$ cell): ν = 3431 cm^{-1} (N-H), 2253 cm^{-1} (N-C), 1718 cm^{-1} (C=O), 1617 cm^{-1} (C=O). ^1H NMR ($CDCl_3$): δ = 9.66 (br. s., 1 H), 7.64 (d, 1 H, *J* = 3.6 Hz), 7.14 (d, 2 H, *J* = 8.7 Hz), 6.88 (d, 2 H, *J* = 8.7 Hz), 6.84 (d, 2 H, *J* = 8.0 Hz), 6.77 (d, 2 H, *J* = 8.0 Hz), 4.09 (q, 2 H, *J* = 7.2 Hz), 2.18 (s, 3 H), 1.11 (t, 3 H, *J* = 7.2 Hz) ppm. ^{13}C NMR ($CDCl_3$): δ = 186.83, 163.94, 137.74, 137.57, 136.05, 133.88, 131.38, 130.45, 130.25, 130.02, 128.90, 128.13, 127.93, 117.60, 60.19, 21.30, 14.44 ppm. Elemental analysis ($C_{21}H_{18}ClNO_3$): C: 68.57 %, H: 4.93 %, N: 3.81 % (theoretical), C: 68.25 %, H: 4.81 %, N: 3.77 % (found).

Pharmacology and biochemical studies

Cytotoxicity

Compounds **9 a–9 d** (Table 1) were tested for cytotoxic activity by homogenizing the drugs and preparing a 1 mg/mL solution in 0.05 % Tween 80/H_2O. These solutions were sterilized by passing them through an acrodisc (0.45 μm). The following cell lines were maintained by literature techniques [9]: murine L_{1210} lymphoid leukemia, and P388 lymphocytic leukemia, human $Tmolt_3$ and $Tmolt_4$ acute lymphoblastic T cell leukemia, HL-60 leukemia, Hut-78 cutaneous lymphoma, THP-1 acute monocytic leukemia, HCT-8 ileocecal adenocarcinoma, liver Hepe-2, A-549 lung carcinoma, HSO osteosarcoma, KB epidermoid nasopharynx, HeLa-S^3 suspended cervical carcinoma, ovary 1-A9, SK-MEL-2 malignant melanoma, breast effusion MCF-7 and U-87-MG glioma. Normal human fibroblast 1788 were also used to test cytotoxicity of the agents. The NCI protocol was used to assess the cytotoxicity of the test compounds and standards in each cell line. Values for cytotoxicity were expressed as ED_{50} = μg/mL, i.e. the concentration of the compound inhibiting 50 % of cell growth. ED_{50} values were determined by the trypan blue exclusion technique [9]. A value of less than 4 μg/mL was required for significant activity of growth inhibition. Solid tumor cytotoxicity was determined utilizing crystal violet/MeOH and read at 580 nm (Molecular Devices) [10].

Incorporation studies

Incorporation of labeled precursors into ^3H-DNA, ^3H-RNA, and ^3H-protein for 10^6 HL-60 leukemia cells was obtained [11] using a concentration range of 25, 50, and 100 μM of the test agent **9 a** over a 60 min incubation. The incorporation of ^{14}C-glycine (53.0 mCi/mmol) into purines [12] and the incorporation of ^{14}C-formate (53.0 mCi/mmol) into pyrimidines [13] was determined in a similar manner.

Enzyme assays

Inhibition of various enzyme activities was performed by first preparing the appropriate HL-60 leukemia cell homogenates or subcellular fraction, then adding the drug to be tested during the enzyme assay. For the concentration response studies, inhibition of enzyme activity was determined at 25, 50, and 100 μM of compound **9 a,** after 60 min incubations. DNA polymerase α activity was determined in cytoplasmic isolated extracts [14] . Nuclear DNA polymerase β was determined by isolating nuclei [15]. The polymerase activity for both α and β was determined with ^3H-TTP [16]. Messenger-, ribosomal-, and transfer-RNA polymerase enzymes were isolated with different concentrations of ammonium sulfate; individual RNA polymerase activities were determined using ^3H-UTP [17, 18]. Ribonucleotide reductase activity was measured using ^{14}C-CDP with dithioerythritol [19]. The deoxyribonucleotides ^{14}C-dCDP were separated from the ribonucleotides by TLC on PEI plates. Thymidine, TMP and TDP kinase activities were determined using ^3H-thymidine (58.3 mCi/mmol) [20]. Carbamyl phosphate synthetase activity was determined [21] with citrulline quantitated colorimetrically [22]. Aspartate transcarbamylase activity was measured [21] and carbamyl aspartate was quantitated colorimetrically [23]. Thymidylate synthase activity was analyzed by the ^3H_2O released which was proportional to the amount of TMP formed from ^3H-dUMP [24]. Dihydrofolate reductase activity was determined by a spectrophotometric method [25]. PRPP amidotransferase activity was determined by the method of Spassova et al. [26]. IMP dehydrogenase activity was analyzed with 8-^{14}C-IMP (54 mCi/mmol) (Amersham, Arlington Heights, IL) after separating XMP on PEI plates (Fisher Scientific) by TLC [27]. Protein content was determined for the enzymatic assays by the Lowry et al. technique [28].

ct-DNA studies

After deoxyribonucleoside triphosphates were extracted [29], levels were determined by the method of Hunting and Henderson [30] with calf thymus DNA, *E. coli* DNA polymerase I, nonlimiting amounts of the three deoxyribonucleoside triphosphates not being assayed, and either 0.4 mCi of (^3H-methyl)-dTTP or (5-^3H)-dCTP. The effects of compound **9 a** on DNA strand scission was determined by the methods of Suzuki et al. [31], Pera et al. [32], and Woynarowski et al. [33]. HL-60 leukemia cells were incubated with 10 μCi thymidine [methyl-^3H, 84.0 Ci/mmol] for 24 h at 37 °C. HL-60 cells (10^7) were harvested and then centrifuged at 600 *g* × 10 min in PBS. They were later washed and suspended in 1 mL of PBS. Lysis buffer (0.5 mL; 0.5 M NaOH, 0.02 M EDTA, 0.01 % Triton X-100 and 2.5 % sucrose) was layered onto a 5–20 % alkaline-sucrose gradient (5 mL; 0.3 M NaOH, 0.7 M KCl, and 0.01 M EDTA); this was followed by 0.2 mL of the cell preparation. After the gradient was incubated for 2.5 h at room temperature, it was centrifuged at 12,000 RPM at 20 °C for 60 min (Beckman rotor SW60). Fractions (0.2 mL) were collected from the bottom of

the gradient, neutralized with 0.2 mL of 0.3 N HCl, and measured for radioactivity. Thermal calf thymus DNA denaturation studies, ct-DNA UV absorption studies and DNA viscosity studies were conducted after incubation of compound **9a** at 100 µM at 37 °C for 24 h [34].

Human DNA topoisomerase inhibition

Sample drugs were prepared in DMSO so that the stock final concentration was 5 mM [*w/v*]. The enzyme assay consisted of test drugs at 50–200 µM, 1 unit of human DNA topoisomerase II (p170 isoform) [TopoGen, Inc., Columbus, OH], ca. 0.5 µg of supercoiled PBR322 DNA in 50 mM Tris buffer, pH 7.5, 15 mM β-mercaptoethanol, 30 mg/mL bovine serum albumin, 1 mM ATP, 10 mM $MgCl_2$, and 150 mM KCl [35]. After 30 min incubation at 37 °C the reaction was terminated with 1 % SDS and 1 mg/mL proteinase K (*v/v*). After an additional hour of incubation, aliquots were applied to a 0.8 % agarose TBE gel (*v/v*) containing 0.5 mg/mL ethidium bromide and 1 % SDS (*w/v*). Following overnight electrophoresis at 30 V (constant), the gel was destained and photographed using a UV-transilluminator and Polaroid film. DNA topoisomerase I activity inhibition was assayed by a similar method. The enzyme reaction consisted of test drugs, 0.5 units of human topoisomerase I [TopoGen, Inc., Columbus, OH], 0.5 µg of supercoiled PBR322 DNA in 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 30 µg/mL nuclease-free BSA.

Flourogenic caspase assays

HL-60 cells (10^6) were incubated with test compound **9a** (100 µM), VP-16 (50 µM), or 0.05 % tween 80 overnight. Cells were collected at 600 × *g* for 3 min, washed with PBS (4 °C) and re-suspended in 200 µL of lysis buffer (10 mM Hepes, pH 7.4, 5 mM CHAPS, 5 mM DTT). Cells were allowed to swell on ice for 10 min. then subjected to 3 cycles of freeze/thaw. Cellular debris was removed by microfuging for 10 min. Aliquots (10 µg) were added to 200 µL of reaction buffer (5 mM HEPES, pH 7.4, 0.1 % CHAPS, 5 mM DTT, 2 µM caspase 3 or caspase 8 substrate (Ac-DEVD-AMC or Ac-YVAD-AMC, Alexis Biochemical). Additional reactions were performed in the same manner but in the presence of inhibitor (Ac-DEVD-CHO or Ac-YVAD-CHO, Alexis Biochemical.)

Statistic analysis

Data is displayed in Tables and Figures as the means ± standard deviations of the mean expressed as a percentage of the control value. *N* is the number of samples per group. The Student's "t"-test was used to determine the probable level of significance (*p*) between test samples and control samples.

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