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Tyrenes: synthesis of new antiproliferative compounds with an extended conjugation

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Abstract—A series of substituted styryl-acrylonitriles was designed and synthesized. The new compounds, called tyrenes, were tested for the ability to inhibit acute lymphocytic leukemia (ALL) cancer cell growth, as well as on their toxicity to normal bone marrow (NBM) cells. The results showed that 3,4-dihydroxystyryl-acrylonitriles, in particular **CR-4**, revealed great potency as antitumor agents, and also exhibited low toxicity to normal cells. The effectiveness of these compounds with extended conjugation may be due to their possible functioning as reactive Michael acceptors.

1. Introduction

Protein kinases and phosphatases are involved in certain signaling pathways, which regulate various cell functions such as proliferation, differentiation, and apoptosis. Protein kinases (PK) catalyze the phosphorylation of the hydroxyl-containing amino acids, such as tyrosine, serine, and threonine.[1] PK activity appears to be the predominant way of passing proliferative signaling through the cell membrane and into cells. Therefore, an abnormal activity of PK may cause an extensive cell proliferation, which is the source of oncogenic diseases. In particular, phosphorylation of tyrosine catalyzed by protein tyrosine kinase (PTK) is known as a key process in unregulated cell growth. Therefore the synthesis of compounds, which inactivate PTK activity becomes an important tool for development of perspective anticancer drugs.

One of the existing approaches to block the PTK activity is the use of low molecular competitive inhibitors of PTK, which would mimic the parent tyrosine structure. One of the structurally similar naturally occurring potent PTK inhibitor was found to be erbstatin.[2] Subsequent studies have demonstrated that the inhibitory ability of erbstatin against PTK is connected to its ability to compete with tyrosine as a substrate to phosphorylation, because of its structural similarity to tyrosine,[3] that is because of the presence of two phenolic hydroxyls in erbstatin. It is possible that erbstatin resembles the specific conformation of tyrosine moieties within the peptide.[3] Some synthetic erbstatin analogs with different arrangement of hydroxyls in the benzene ring were synthesized. Among those, 2,3- and 3,4-dihydroxy analogs showed potent inhibitory activity comparable to erbstatin.[4]

In the past two decades, significant progress had been achieved in design and synthesis of various low molecular compounds based on the structures of erbstatin and related compounds. In particular, Knoevenagel condensation of various hydroxy-substituted benzaldehydes with malononitrile and its derivatives led to a number of compounds called tyrphostins that possessed a good inhibitory activity to various PTKs.[5,6] The common feature of erbstatin and synthetic tyrphostins is the presence of hydroxystyryl moiety, which mimics tyrosine and is apparently responsible for their biological activity. An enhanced acidity of the phenol or catechol groups caused by presence of the electrophilic acrylonitrile may promote binding of such molecules to the

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substrate subsite of the PTK domain, making them effective antiproliferative agents. Indeed blocking the phenol groups or saturating the double bond resulted in loss of antiproliferative activity.[5]

In the past few years, however, a number of tyrphostin-like compounds had been designed, where the phenolic hydroxyl group is absent[7] or blocked;[8] nevertheless those compounds retained PTK inhibiting activities. It should be noticed that all these compounds are all Michael acceptors in a broader sense, that is they have olefinic groups conjugated with electron-withdrawing substituents, having the structures Z′–CH=CH–Z.[9] Such functionalities are subject to attack by a suitable nucleophilic site on a corresponding protein or DNA, for example, by cysteinyl thiol groups on protein (for reviews on application of Michael acceptors for anticancer drug design see Refs. 10 and 11). In this study, we describe a new series of antiproliferative compounds, that we called 'tyrenes', which possess the two important functions: activated phenolic hydroxyls, combined with an extended conjugation that may serve as a reactive Michael acceptor.

2. Results and discussion

2.1. Design and synthesis

2-Benzylaminocarbonyl-3-arylacrylonitriles were previously reported as PTK blocking agents.[12] The activated styrylphenolic moiety in those compounds might play a crucial role as a competitive agent to tyrosine phosphorylation. We hypothesized that an extra-annular double bond added to the existing conjugated system, might serve as an effective acceptor in a conjugate Michael addition.

Based on this hypothesis, we have designed and synthesized a series of hydroxystyryl-acrylonitriles, shown on Figure 1. Previously, some compounds with similar structural features were obtained and used as components for nonlinear optical and photoreactive materials.[13]

The key step in obtaining these compounds is Knoevenagel condensation of α, β-unsaturated aryl substituted aldehydes with compounds having an activated methylene group. We have found that hydroxyl-substituted cinnamaldehydes showed themselves as very reactive agents, so the reaction proceeded smoothly in ethanol in the presence of equimolar amount of piperidine (1 equiv per hydroxyl) within 0.5 h at room temperature. However, applying these conditions to the condensation of protected, or nonphenolic cinnamaldehydes (such as unsubstituted cinnamaldehyde and its dimethoxy- or nitro-derivatives) led to the decomposition products only. To obtain the corresponding adducts, β-alanine was employed as a milder catalyst, and heating for several hours was required (Scheme 1).

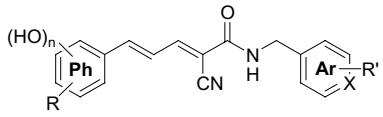


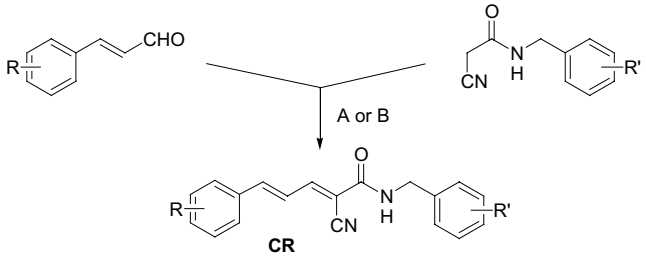
Figure 1. Basic structure of tyrenes.

In all cases, the single *E*,*E*-adducts were obtained, which was confirmed by HPLC (single peaks were detected) and ^{1}H NMR spectroscopy (mutual coupling constant was 14–15 Hz for all olefinic protons, and the proton at the α-position to CN group was at the vicinity of 8 ppm, which is in agreement with the spectra of another α, β, γ, δ-unsaturated nitriles of *trans*-configuration[14]).

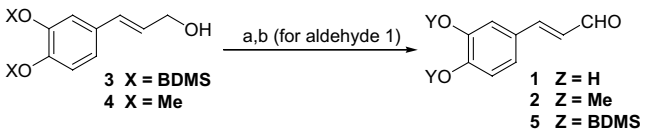
To obtain this series of compounds, various hydroxy-substituted cinnamaldehydes were required. Unlike homologous benzaldehydes, only few of them, such as sinapyl- and coniferyl aldehydes are commercially available. Therefore, a reliable synthetic pathway to such cinnamaldehydes, in particular, to 3,4-dihydroxy-cinnamaldehyde (caffeoyl aldehyde) had to be developed.

It was known, that Wittig condensation of hydroxy-substituted benzaldehydes with resonance-stabilized ylide formylmethylenetriphenylphosphorane is certainly not a reliable process for obtaining 3,4-substituted cinnamaldehydes, as a mixture of products with different degree of unsaturation was obtained.[15] Due to this, for obtaining caffeoyl aldehyde **1** and its 3,4-dimethoxy analog **2** we have chosen a straightforward approach, involving known transformations of commercially available substituted cinnamic acids. Following the described procedures,[16,17] the alcohols **3** and **4** were obtained and oxidized with activated manganese dioxide into corresponding aldehydes **5** and **2**. Deprotection of the aldehyde **5** led to caffeoyl aldehyde **1** (Scheme 2).

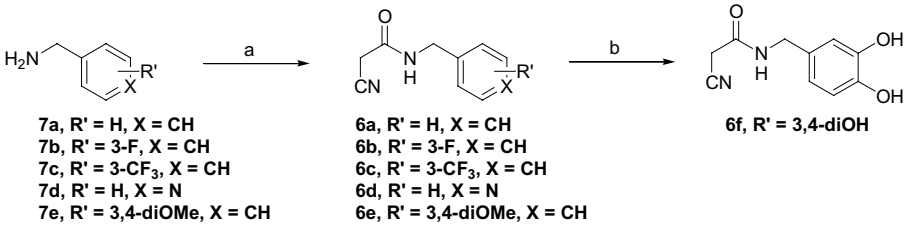
The synthons with activated α-methylene, *N*-benzyl-2-cyanoacetamides **6**, were obtained by a direct coupling of various benzylamines **7** with methyl cyanoacetate (Scheme 3). Cleavage of the *vic*-methoxy groups in the



Scheme 1. Reagents and conditions: (A) piperidine, EtOH, 0.5 h, 20 °C (when R = OH); (B) β-alanine, EtOH, 80 °C, 1–4 h (when R≠OH).



Scheme 2. Reagents and conditions: (a) activated MnO_2, CH_2Cl_2; (b) *n*-Bu_4NF, $CHCl_3$.



Scheme 3. Reagents and conditions: (a) $N{\equiv}CCH_2COOMe$ neat, 2 h, 20 °C; (b) **9e**, BBr_3, CH_2Cl_2, −78 °C → 20 °C.

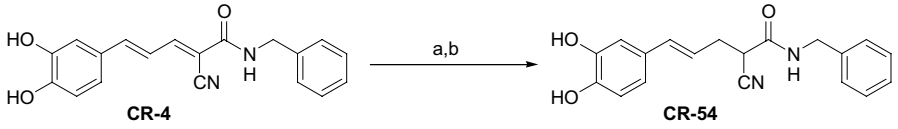
3,4-dimethoxybenzylamide yielding 3,4-dihydroxybenzylamide was achieved with BBr_3.

CR-54, the monosaturated analog of **CR-4** was obtained by treatment of **CR-4** with sodium borohydride in methanol as described[18] (Scheme 4). In our case, α, β-double bond in **CR-4** was selectively reduced, leading to 2-cyano-5-(3,4-dihydroxyphenyl)-4*E*-pentenoic acid benzylamide (**CR-54**).

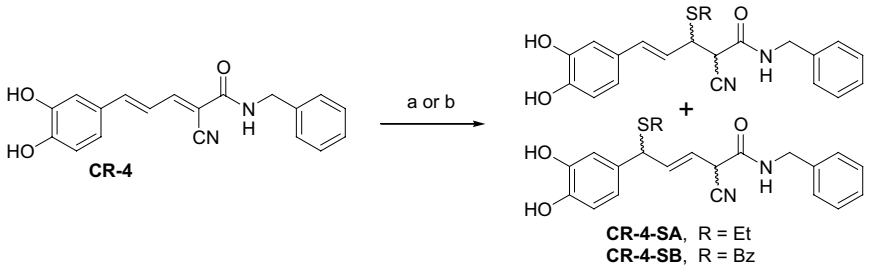
To prove the hypothesis that tyrenes are Michael acceptors, **CR-4** was treated with simple nucleophiles, mimicking cysteine residues, such as mercaptans (Scheme 5). Indeed, **CR-4** smoothly and quantitatively reacted with ethanethiol in DMSO in the presence of piperidine giving a less polar product with a remarkably different UV spectrum (λ_{max} 205 and 280 nm). Benzyl mercaptan appeared to be more reactive in this process, as no catalyst was required. The products were unstable in solutions, and easily gave back the parent **CR-4** when they were treated with a base. The NMR spectrum of the mixture of the ethanethiol derivatives (**CR-4-SA**) showed the absence of the signal at 8 ppm indicating that the system of the two double bonds was partially saturated. The appearance of the complex multiplet at 6.6–6.8 ppm suggested that the obtained adduct is in fact a diastereomeric mixture of the products of 1,2- and 1,4-addition of ethanethiol. Partial separation of the four possible regio/stereoisomers of **CR-4-SA** and **CR-4-SB** was achieved on an Agilent SB-CN column in the reverse-phase mode (see Section 5). The full scan Q1 mass spectra of all the peaks recorded in LC–MS mode showed the molecular ions at m/z 383 (for **CR-4-SA**) and at 445 (for **CR-4-SB**), as well as the prominent daughter ion m/z 321 ($[M - Et]^+$) and ($[M - Bz]^+$) for the two derivatives. To prove that the ion with m/z 321 is a daughter ion and not the molecular ion of **CR-4** itself, the molecular ions of **CR-4** (m/z 321) and the derivatives **CR-4-SA** and **CR-4-SB** (m/z 383 and 445) were used for the daughter ion scan. Both molecular ions of the compounds **CR-4-SA** and **CR-4-SB** gave a number of daughter ions, whereas the parent molecular ion of **CR-4** gave almost no fragmentation in the same ionization conditions.

3. Biological activity

The synthesized compounds were tested for their ability to inhibit abnormal cell proliferation. At the same time, the compounds were tested for their toxicity to normally growing cells and the most promising ones showed no deleterious effect on normal cell growth. In those experiments, acute lymphoblastic leukemia (ALL) cells, and normal human bone marrow (NBM) cells were chosen as models. In a typical experiment, leukemic and normal bone marrow cells were exposed to various



Scheme 4. Reagents and conditions: (a) $NaBH_4$, MeOH, 1 h, 20 °C; (b) HPLC, Nova-Pak C18 19.0×300 mm, gradient MeCN–H_2O, 20:80 → MeCN–H_2O, 80:20.



Scheme 5. Reagents and conditions: (a) EtSH, DMSO, piperidine, 0.5 h, 20 °C; (b) BzSH, DMSO, 0.5 h, 20 °C.

styryl-acrylonitriles and the number of colonies counted after 9 and 14 days, respectively. The most effective compounds were not only able to block ALL cells growth, but also permitted the unaffected growth of NBM cells. The IC_{50} values for the compounds against ALL and NBM cells are listed in Table 1.

The compounds containing free phenol or catechol groups were effective against ALL cells; some of them eliminated ALL cell growth at sub-micromolar doses. The activity of the compounds where the phenolic hydroxyls were absent or blocked, was significantly reduced, however, this fact might also be related to their high level of hydrophobilicy. The most active compounds toward ALL cells were shown to be the ones having the catechol group. In particular, the compounds with the 3,4-dihydroxystyryl moiety showed an exceptional activity. One of them, **CR-4**, significantly inhibited Philadelphia positive ALL cells growth at the level as low as 0.12 μM (Table 1; the detailed report on **CR-4** inhibitory ability toward various cancer cell lines see Ref. 19). When one of the double bonds in **CR-4** was selectively saturated, the inhibitory activity of such compound (**CR-54**) was significantly diminished, however, not blocked completely (compare to Ref. 5). The IC_{50} to NBM growth, exhibited by **CR-4**, was as high as 9 μM, which makes the compound a possible therapeutical candidate.

Other compounds of this sub-series with different substitutions at Ar-ring (**CR-21**, **CR-43**, **CR-66**, **CR-72**, **CR-74**) showed similar, yet lower level of activity toward ALL cells. Noteworthy, the presence of hydrophilic amide moiety in **CR-21** led to decreasing of ALL inhibitory activity, suggesting that the hydrophobic amide group in **CR-4** might be important to this function. An attempt to prepare more hydrophobic halogen-substituted compounds (**CR-66**, **CR-72**) was not successful, as the compounds demonstrated lower therapeutic differential (TD) to inhibiting cell growth; in particular, a significant toxicity to NBM cells was observed (Table 1).

When only one hydroxyl in the Ph-ring was exposed, the activity on the cancer cells was lowered, so the IC_{50} values were in low-micromolar range. One of the compounds of this sub-series, namely **CR-11**, showed 10-fold decrease in activity in comparison with **CR-4** (IC_{50} 1.4 μM). When the only hydroxyl in the Ph-ring was less sterically hindered (**CR-56**, **CR-57**), the compound became significantly more active (IC_{50} 0.32–0.34 μmol). On the other hand, **CR-11** showed proportionally less toxicity to normal cells, with TD of 20.

Some compounds of another sub-series, where the Ph ring remained unsubstituted, and Ar ring was a catechol (**CR-19**, **CR-28**), also revealed a significant activity to ALL cells inhibition. However, those compounds were particularly toxic to the NBM cells, with TD of 12–16. Those results suggested that the presence of activated hydroxyls in the Ph-ring is highly desirable, yet is not a crucial factor in the anti-cancer ability. We have concluded that the extended conjugation adjacent to electron-withdrawing substituents in the side chain played an independent role in making the compounds remarkably strong cancer cell growth inhibitors. It is known that substituents at the olefinic function generally decrease reactivity; therefore tyrenes with totally exposed γ,δ-double bond appear to be very reactive Michael acceptors. It is therefore possible that these compounds might be more effective than compounds having only one olefinic block partially substituted with the cyano groups. In support of this hypothesis, AG-490 demonstrated inhibitory activity to ALL at 5–25 μM level[20] while **CR-4** was effective in nM concentration.[19]

4. Conclusion

In this report, we have synthesized a new series of tyrenes—compounds, containing an extended hydroxy-styryl-acrylonitrile conjugation system. The obtained compounds were evaluated on their ability to inhibit ALL cells growth, as well as their effect on the development of normal cells. 3,4-Dihydroxy-tyrenes showed a remarkable activity on ALL cells ($IC_{50} = 0.1–0.2$ μM). *O*-Methyl derivatives were significantly less active ($IC_{50} = 0.5–1.0$ μM), however, they revealed much lower

Table 1. Biological activity of tyrenes

Code	R	R′	IC_{50}, ALL (μM)	IC_{50}, NBM (μM)
CR-1	H	H	10 ± 0.2	7 ± 2
CR-2	3,4-$(OMe)_2$	H	2.28 ± 0.06	29 ± 1
CR-4	3,4-$(OH)_2$	H	0.12 ± 0.04	9 ± 2
CR-11	4-OH, 3,5-$(OMe)_2$	3,4-$(OH)_2$	1.4 ± 0.2	28 ± 2
CR-19	H	3,4-$(OH)_2$	0.09 ± 0.03	2.5 ± 0.3
CR-21	3,4-$(OH)_2$	3,4-$(OH)_2$	1.5 ± 0.36	18.6 ± 1
CR-28	4-NO_2	3,4-$(OH)_2$	0.29 ± 0.07	43.8 ± 0.3
CR-43	3,4-$(OH)_2$	3,4-$(OMe)_2$	0.21 ± 0.05	6.2 ± 0.8
CR-54	Partially saturated **CR-4**		0.6 ± 0.2	29.4 ± 0.6
CR-56	4-OH, 3-OMe	H	0.34 ± 0.06	9.8 ± 3
CR-57	4-OH, 3-OMe	3,4-$(OH)_2$	0.32 ± 0.09	19.2 ± 1.7
CR-66	3,4-$(OH)_2$	3-CF_3	0.33 ± 0.06	2.8 ± 0.2
CR-72	3,4-$(OH)_2$	3-F	0.16 ± 0.02	3.3 ± 0.3
CR-74	3,4-$(OH)_2$	Py	0.2 ± 0.02	16 ± 1.1

toxicity toward NBM cells ($IC_{50} > 10$–15 μm), and also promoted normal cells growth. These data suggest that the extended conjugation, acting as an active Michael acceptor, promote PTK inhibitory activity of this group of compounds. Among synthesized compounds, **CR-4** was the most promising candidate for development of antitumor agents. We now continue further chemical modifications and pharmacological evaluations of these compounds.

5. Experimental

5.1. General procedures

^{1}H NMR spectra were obtained on a Varian Unity Plus spectrometer at 500 MHz in acetone-d_6 with Me_4Si as an internal standard ($\delta = 0$). Electrospray mass spectra of the compounds **CR-4-SA** and **CR-4-SB** were acquired on an API 4000 LC/MS/MS mass spectrometer (PE Sciex, Thornhill, Canada). UV–vis spectra were recorded in MeCN containing 0.1% H_3PO_4 on a Beckman DU 640 spectrophotometer. High-resolution mass spectroscopic analyses (HRMS) and elemental analyses were performed at the Chemistry Department of the University of Toronto, Canada. Elemental analyses were within ±0.4% of the theoretical value unless otherwise noted. Melting points were recorded on a Barnstead Mel-Temp capillary melting point apparatus and are uncorrected. The final compounds were re-crystallized from PEG400-H_2O. Thin layer chromatography was performed with UV-254 aluminum-backed TLC sheets of 0.25 mm thickness (Kieselgel 60 F_{254}, Merck). HPLC chromatograms were obtained on a model 600 liquid chromatograph (Waters, USA) with a model 996 PDA detector. The column was 5 μm Zorbax SB-CN 4.6×250 mm (Agilent Technologies, USA). Gradient separations of the final compounds were conducted using the following buffers: (A) 0.1% H_3PO_4 in H_2O; (B) MeCN–H_2O, 4:1 + 0.1% H_3PO_4. The following gradient system was employed: buffer A for 10 min, followed by linearly increasing the percentage of the buffer B from 20% to 90% during 20 min, flow rate 1.0 mL/min. Separations of the derivatives **CR-4-SA** and **CR-4-SB** were achieved using the system MeCN–H_2O, 50:50, isocratic mode, flow rate 1.0 mL/min. Vacuum distillations were done using Kugelrohr apparatus (Aldrich) at stated temperatures of an oven.

Unsubstituted cinnamaldehyde and its 4-hydroxy-3-methoxy-, 3,5-dimethoxy-4-hydroxy-, and 4-nitro-derivatives, as well as all other reagents were purchased from Aldrich and were used as received. Solvents were purchased from Caledon (Canada).

The key Knoevenagel condensation was carried out in ethanol using piperidine (method A) or β-alanine (method B) as catalysts.

5.2. Synthesis of key intermediates

5.2.1. Substituted cinnamaldehydes. ^{1}H NMR spectra of cyanoacetamides are listed in Table 2.

5.2.2. 3,4-Dihydroxycinnamaldehyde (caffeoyl aldehyde) (1). 3,4-Bis-(*t*-butyldimethylsilyloxy)cinnamyl alcohol (**3**) (7.88 g, 20 mmol) was dissolved in 1000 mL CH_2Cl_2, 17.2 g activated MnO_2 (200 mmol) were added and the mixture was thoroughly stirred for 24 h at 20 °C. MnO_2 was filtered off and the filtrate was taken to dryness. To the obtained 3,4-diBDMS caffeoyl aldehyde (**5**) 250 mL of $CHCl_3$ was added followed by addition of *n*-Bu_4NF monohydrate (11.6 g, 40 mmol). The mixture was stirred at 20 °C for 30 min and worked up with 300 mL of 5% HCl. Chloroform layer was separated, washed with H_2O, dried with Na_2SO_4, and taken to dryness. The residue was purified on a silica gel column, eluent MeOH–$CHCl_3$, 1:4 + 1% AcOH. The solvents were evaporated and the crystalline residue was washed with $CHCl_3$ to give caffeoyl aldehyde (**1**) (1.77 g, 54%). Melting point 208–210 °C.

5.2.3. 3,4-Dimethoxycinnamaldehyde (2). Obtained in a similar manner by oxidizing of 970 mg (5 mmol) 3,4-dimethoxycinnamalcohol (**4**) with 1720 mg (20 mmol) of activated MnO_2 in 400 mL CH_2Cl_2. The product was re-crystallized from ethyl acetate–heptane to give 865 mg (90%) of the aldehyde (**2**). Melting point 91 °C.

5.2.4. Substituted cyanoacetamides. ^{1}H NMR spectra of cyanoacetamides are listed in Table 3.

5.2.5. General method of synthesis of compounds 6a–e. In a typical experiment, equimolar amounts of methyl cyanoacetate and corresponding amine were mixed and kept at room temperature for 12 h. The formed white solid was re-crystallized from ethanol or ethanol–water. The average yield of cyanoacetamides was 60–80%. Compound **6a**: mp 128 °C; **6b**: mp 106 °C; **6c**: mp 118 °C; **6d**: mp 130 °C; **6e**: mp 142 °C.

Table 2. ^{1}H NMR Spectra of substituted cinnamaldehydes (δ, ppm; J, Hz)

No	Hα	Hβ	CHO	H^2	H^3	H^4	H^5	H^6	Other
1	6.54 (dd, $J = 7.7, 15.8$)	7.52 (d, $J = 15.8$)	9.62 (d, $J = 7.7$)	7.21 (br s)	—	—	6.91 (d, $J = 8.2$)	7.12 (br d, $J = 8.2$)	—
2	6.69 (dd, $J = 16.0$)	7.60 (d, $J = 16.0$)	9.66 (d, $J = 7.6$)	7.37 (d, $J = 1.5$)	—	—	7.05 (d, $J = 8.3$)	7.28 (dd, $J = 1.5, 8.3$)	3.90
5	6.60 (dd, $J = 7.7, 15.9$)	7.59 (d, $J = 15.9$)	9.64 (d, $J = 7.7$)	7.27 (m)	—	—	7.27 (m)	7.00 (dd, $J = 1.5, 8.2$)	0.25, 1.00

Table 3. ^{1}H NMR Spectra of substituted cyanoacetamides (δ, ppm; J, Hz)

No	N≡CCH$_2$	CH$_2$Ar	H^2	H^3	H^4	H^5	H^6	Other
6a	3.62 (s)	4.49 (s)	7.26 (m)	7.32 (m)	7.32 (m)	7.32 (m)	7.26 (m)	—
6b	3.14 (s)	4.44 (s)	7.11 (m)	—	7.02 (m)	7.37 (m)	7.16 (m)	—
6c	3.14 (s)	4.53 (s)	7.56–7.68 (m)	—	7.56–7.68 (m)	7.56–7.68 (m)	7.56–7.68 (m)	—
6d	3.18 (s)	4.49 (s)	7.32 (m)	8.50 (m)	—	8.50 (m)	7.32 (m)	—
6e	3.82 (s)	4.38 (s)	6.97 (d, $J = 2.1$)	—	—	6.92 (d, $J = 8.1$)	6.87 (dd, $J = 2.1, 8.1$)	2.87
6f	3.60 (s)	4.25 (s)	6.79 (d, $J = 2.0$)	—	—	6.75 (d, $J = 8.1$)	6.63 (dd, $J = 2.0, 8.1$)	—

5.2.6. 2-Cyano-*N*-(3,4-dihydroxybenzyl)acetamide 6f. To 2-cyano-*N*-(3,4-dimethoxybenzyl)acetamide **6e** (2.68 g, 11.45 mmol) in 200 mL of CH_2Cl_2, boron tribromide was added dropwise at −10 °C (8.75 g, 35 mmol).

After 0.5 h the reaction was brought to room temperature and stirred for an additional 1 h. The reaction was cooled to 0 °C, 200 mL of water was carefully added, and the organic layer was separated. The aqueous phase was saturated with NaCl and extracted with 3 × 100 mL of ethyl acetate. The combined organic phase was dried with Na_2SO_4, and taken to dryness. The solidified residue was dissolved in methanol and passed through a column with silica gel, eluent 5% MeOH in EtOAc. The solvents were evaporated, and the residue was dissolved in water and lyophilized to give an off-white powder. Yield 1.24 g (53%). Melting point 153–154 °C.

5.2.7. Synthesis and transformations of tyrenes. ^{1}H NMR Spectra of tyrenes are listed in Table 4. Physical data of tyrenes are listed in Table 5.

5.2.8. General method A. To a solution of 0.1 mmol of hydroxyl-substituted cinnamaldehyde **1** and 0.1 mmol of amide **6** in 3–4 mL of ethanol, an equimolar amount of piperidine was added. The deep red solution was stirred at 20 °C for 0.5–1.0 h until the starting material disappeared. 1 N HCl (0.2 mL) was added followed by addition of 10 mL H_2O, and the mixture was kept at 0 °C for 2 h. The precipitated powder was washed with H_2O, re-crystallized from MeCN–H_2O, and dried in a desiccator over NaOH. The average yield of the CR compounds was 50–70%.

5.2.9. General method B. To a solution of 0.1 mmol of cinnamaldehyde, or its 3,5-dimethoxy-4-hydroxy derivative, or its 4-nitro derivative, or cinnamaldehyde **2** and 0.1 mmol of amide **6** in 3–4 mL of ethanol, a few crystals of β-alanine were added. The mixture was stirred at 80 °C for 2.0–4.0 h until the starting material disappeared. H_2O (10 mL) was added, and the mixture was kept at 0 °C for 2 h. The precipitated powder was washed with H_2O, re-crystallized from MeCN–H_2O,

Table 4. ^{1}H NMR Spectra of tyrenes (δ, ppm; J, Hz)

Code	H^2	H^3	H^4	H^5	H^6	PhCH	PhC=CH	CHCN	Other
CR-1	7.24–7.51 (m)	7.24–7.51 (m)	7.24–7.51 (m)	7.24–7.51 (m)	7.24–7.51 (m)	7.24–7.51 (m)	7.24–7.51 (m)	8.05 (d, $J = 11.7$)	4.55; 7.24–7.51
CR-2	7.39 (d, $J = 2.0$)	—	—	7.07 (d, $J = 8.3$)	7.30 (dd, $J = 2.0, 8.3$)	7.37 (d, $J = 15.3$)	7.20 (dd, $J = 11.6, 15.3$)	8.05 (d, $J = 11.6$)	3.90; 3.91; 4.57; 7.26–7.41
CR-4	7.19 (d, $J = 2.1$)	—	—	6.87 (d, $J = 8.2$)	7.05 (dd, $J = 2.1, 8.2$)	7.34 (d, $J = 15.2$)	7.02 (dd, $J = 11.2, 15.2$)	7.99 (d, $J = 11.2$)	4.54; 7.24; 7.28–7.36
CR-11	7.07 (br s, 1H)	—	—	—	7.07 (br s)	7.37 (d, $J = 15.1$)	7.16 (dd, $J = 11.7, 15.1$)	7.98 (d, $J = 11.7$)	2.81; 3.89; 4.39; 6.68; 6.76; 6.86
CR-19	7.72 (m)	7.46 (m)	7.46 (m)	7.46 (m)	7.72 (m)	7.48 (d, $J = 15.7$)	7.29 (dd, $J = 11.7, 15.7$)	8.04 (d, $J = 11.7$)	2.82; 4.40; 6.70 6.76; 6.86
CR-21	7.22 (d, $J = 2.1$)	—	—	6.90 (d, $J = 8.6$)	7.09 (dd, $J = 2.1, 8.6$)	7.34 (d, $J = 15.3$)	7.05 (dd, $J = 11.8, 15.3$)	8.00 (d, $J = 11.8$)	4.38; 6.68; 6.76; 6.85
CR-28	8.03 (d, $J = 8.8$)	8.32 (d, $J = 8.8$)	—	8.32 (d, $J = 8.8$)	8.03 (d, $J = 8.8$)	7.64 (d, $J = 15.8$)	7.46 (dd, $J = 11.7, 15.8$)	8.10 (d, $J = 11.7$)	4.37; 6.73; 6.81; 6.89
CR-43	7.21 (d, $J = 2.1$)	—	—	6.89 (d, $J = 8.3$)	7.07 (dd, $J = 2.1, 8.3$)	7.34 (d, $J = 15.2$)	7.05 (dd, $J = 11.7, 15.2$)	8.00 (d, $J = 11.7$)	4.46; 3.79; 3.80; 6.90; 7.01
CR-54	6.93 (d, $J = 2.1$)	—	—	6.77 (d, $J = 8.1$)	6.71 (dd, $J = 2.1, 8.1$)	6.44 (d, $J = 15.7$)	6.01 (dt, $J = 7.3, 15.7$)	2.79 (m)	3.15; 4.44; 7.22–7.30
CR-56	7.37 (d, $J = 2.1$)	—	—	6.91 (d, $J = 8.2$)	7.20 (dd, $J = 2.1, 8.2$)	7.35 (d, $J = 15.1$)	7.14 (dd, $J = 11.6, 15.1$)	8.02 (d, $J = 11.6$)	3.92; 4.55; 7.25; 7.31–7.42
CR-57	7.37 (d, $J = 2.1$)	—	—	6.91 (d, $J = 8.2$)	7.20 (dd, $J = 2.1, 8.2$)	7.39 (d, $J = 15.3$)	7.13 (dd, $J = 11.6, 15.3$)	8.00 (d, $J = 11.6$)	3.92; 4.38; 6.68; 6.76; 6.86
CR-66	7.22 (d, $J = 2.2$)	—	—	6.90 (d, $J = 8.2$)	7.08 (dd, $J = 2.2, 8.2$)	7.36 (d, $J = 15.1$)	7.05 (dd, $J = 11.6, 15.1$)	8.02 (d, $J = 11.6$)	4.65; 7.60; 7.70
CR-72	7.22 (d, $J = 2.2$)	—	—	6.90 (d, $J = 8.2$)	7.07 (dd, $J = 2.2, 8.2$)	7.35 (d, $J = 15.3$)	7.05 (dd, $J = 11.7, 15.3$)	8.01 (d, $J = 11.7$)	4.56; 7.02; 7.14; 7.20; 7.36
CR-74	7.23 (d, $J = 2.2$)	—	—	6.91 (d, $J = 8.2$)	7.09 (dd, $J = 2.2, 8.2$)	7.38 (d, $J = 15.3$)	7.07 (dd, $J = 11.7, 15.3$)	8.04 (d, $J = 11.7$)	4.59; 7.38; 8.51

Table 5. Physical data of tyrenes

Code	Mp (°C)	λ_{max} (nm) (ε_{max})	HRMS, m/z, found (calcd)	Microanalysis (C,H,N)
CR-1	195–196	334 (31,694)	288.1271 (288.1263)	$C_{19}H_{16}N_2O$
CR-2	179–180	381 (35,570)	348.1467 (348.1474)	$C_{21}H_{20}N_2O_3$
CR-4	245–246	380 (32,328)	320.1169 (320.1161)	$C_{19}H_{16}N_2O_3$
CR-11	247–248	387 (29,702)	396.1315 (396.1321)	$C_{21}H_{20}N_2O_6$
CR-19	198–199	335 (28,149)	320.1144 (320.1161)	$C_{19}H_{16}N_2O_3$
CR-21	261–263	378 (28,137)	352.1051 (352.1059)	$C_{19}H_{16}N_2O_5$
CR-28	244–245 (dec)	346 (31,851)	365.1023 (365.1011)	$C_{19}H_{15}N_3O_5 \cdot 1/3H_2O$
CR-43	212–213	378 (37,288)	380.1363 (380.1372)	$C_{21}H_{20}N_2O_5 \cdot H_2O$
CR-54	161–163	263 (9851)	322.1316 (322.1317)	$C_{19}H_{18}N_2O_3$
CR-56	171–172	379 (33,680)	334.1308 (334.1317)	$C_{20}H_{18}N_2O_3$
CR-57	225–226	378 (31,274)	366.1215 (366.1216)	$C_{20}H_{18}N_2O_5 \cdot H_2O$
CR-66	216–218	378 (32,584)	388.1047 (388.1035)	$C_{20}H_{15}F_3N_2O_3$
CR-72	229–230	380 (34,654)	338.1071 (338.1067)	$C_{19}H_{15}FN_2O_3$
CR-74	250–252	384 (29,859)	321.1116 (321.1113)	$C_{18}H_{15}N_3O_3 \cdot 1/4H_2O$

and dried in a desiccator over NaOH. The average yield of the CR compounds was 70–75%.

5.2.10. (*E*,*E*)-2-(Benzylaminocarbonyl)-3-(3,4-dihydroxystyryl)acrylonitrile (CR-4) (method A). To a solution of 16 mg of 3,4-dihydroxycinnamaldehyde **1** and 17 mg of amide **6a** in 3 mL of ethanol, an equimolar amount of piperidine was added. The solution was stirred at 20 °C for 1.0 h. 1 N HCl (0.2 mL) was added followed by addition of 10 mL H_2O, and the mixture was kept at 0 °C for 2 h. The precipitated powder was washed with H_2O, re-crystallized from MeCN–H_2O, and dried in a desiccator over NaOH. Yield 68%.

5.2.11. (*E*,*E*)-2-(3,4-Dihydroxybenzylaminocarbonyl)-3-(3,5-dimethoxy-4-hydroxystyryl)acrylonitrile (CR-11) (method B). To a solution of 21 mg of 3,5-dimethoxy-4-hydroxycinnamaldehyde and 20 mg of amide **6f** in 4 mL of ethanol, a few crystals of β-alanine were added. The mixture was stirred at 80 °C for 4.0 h until the starting material disappeared. H_2O (10 mL) was added, and the mixture was kept at 0 °C for 2 h. The precipitated powder was washed with H_2O, re-crystallized from MeCN–H_2O, and dried in a desiccator over NaOH. Yield 75%.

5.2.12. 2-Cyano-5-(3,4-dihydroxyphenyl)-4*E*-pentenoic acid benzylamide (CR-54). To a solution of **CR-4** (32 mg) in 8 mL MeOH 200 mg of $NaBH_4$ was added, and the mixture was stirred at room temperature for 1 h. The mixture was acidified with 1 N HCl, MeOH was evaporated and the residue was extracted with ethyl acetate. The solvent was evaporated and the residue was purified on a reverse-phase HPLC. Yield 25 mg (78%).

5.2.13. Modeling experiment. Condensation of CR-4 with mercaptans. To a solution of **CR-4** (16 mg, 0.05 mmol) in 0.3 mL DMSO, 0.5 mmol of mercaptan RSH (R = Et, Bz) was added. In case of ethanethiol, 20 μL of piperidine was added, and the mixture was kept for 0.5 h at room temperature. The mixture was acidified with 1 N HCl, and the product was extracted with ethyl acetate. The solvent was evaporated and the residue was purified on a reverse-phase HPLC. The products **CR-4-SA** and **CR-4-SB** were obtained in a quantitative yield. **CR-4-SA**: ^{1}H NMR (δ, ppm): 1.20, 1.26 (2×t, 2×3H, $J = 7.3$ Hz, SCH_2CH_3), 2.30–2.40 (m, SCH_2CH_3), 4.38 (m, $NHCH_2Ph'$), 6.60–6.82 (m, olefinic H + Ph), 7.22–7.36 (m, Ph′). MS (ESI, full scan Q1, m/z, rel intensity, %): 383.4 ($[M+1]^+$, 20), 321.2 ($[M-SEt]^+$, 100), 237.3 (17), 209.3 (7), 188.4 (32), 175.3 (12). HPLC (retention times, min): 6.5, 8.2, 8.4. **CR-4-SB**: MS (ESI, full scan Q1, m/z, rel intensity, %): 445.4 ($[M+1]^+$, 59), 321.5 ($[M-SBz]^+$, 100). HPLC (retention times, min): 9.2, 16.8 (a wide peak), 17.4.

5.3. Biological assays

5.3.1. Leukemia colony assay. To permit the assessment of individual colony formation, ALL cell lines and primary cells were cultured in semi-solid media containing 0.8% (v/v) methylcellulose at 5×10^3 cells/mL supplemented with 20% FCS. Primary ALL patient cells were prepared as previously described.[21] Briefly, nonadherent cells and T cell were depleted from bone marrow or peripheral blood samples and plated in Iscoves modified Dulbecco medium supplemented with 10% FCS and PHA-T cell conditioned medium (PHA-TCM). Cell lines were grown in standard medium with FCS. All assays were performed in duplicate dishes.

5.3.2. CFU-GEMM assay. The CFU-GEMM assay was performed according to Fauser and Messner[22,23] with some minor variations. In brief, heparinized bone marrow cells were layered over Percoll and centrifuged at 400*g* at 4 °C for 10 min to remove neutrophils and RBCs. The fractionated BM cells at 2×10^5 cells/mL were cultured in IMDM containing 0.9% (v/v) methylcellulose supplemented with 20% FCS or normal human plasma, a cocktail of cytokines containing G-CSF (10 ng/mL), IL-3 (40 U/mL), MGF (50 ng/mL), erythropoietin (2 U/mL), and 5×10^{-5} M β-2-mercaptoethanol. All cultures were evaluated at 14 days for the number of BFU-E colonies (defined as aggregates of more than 500

hemaglobinized cells or, three or more erythroid subcolonies), CFU-GM colonies (defined as granulocyte or monocyte-macrophage cells or both), and CFU-GEMM colonies (a mixed population comprising of all elements). To prepare haematopoietic stem cells, normal bone marrow cells were isolated as above and $CD34^+$ cells positively selected with anti-CD34 magnetic beads, utilized the MACS magnetic cell sorting system (Miltenyi Biotec Inc, CA).

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