Bioorganic & Medicinal Chemistry Letters 24 (2014) 147–151

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and anticancer potential of benzothiazole linked phenylpyridopyrimidinones and their diones as mitochondrial apoptotic inducers

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ARTICLE INFO

Article history:
Received 28 August 2013
Revised 6 November 2013
Accepted 22 November 2013
Available online 1 December 2013

Keywords:
Benzothiazole linked-phenylpyridopyrimidinone
Cytotoxicity
Cell cycle arrest

ABSTRACT

A series of benzothiazole linked phenylpyridopyrimidinones (**8a–g**) and their diones (**9a–g**) have been designed, synthesized and evaluated for their anticancer activity. Among the series one of the conjugate **8b** showed significant cytotoxicity against human cervical cancer cell line ME-180 with IC_{50} value of 4.01 μM. This compound was tested on the cell cycle perturbations and DNA damage. Flow cytometry analysis revealed that the compound **8b** showed drastic cell cycle perturbations due to concentration dependent increase in the sub-G0 phase in ME-180 cell line. DNA fragmentation and Hoechst staining reveals that this compound induced cell death by apoptosis. Further caspase-3 and loss of mitochondrial membrane potential suggested that the compound induces cell death by apoptosis.

Cancer, the uncontrolled growth of cells, is a major cause of death throughout the world. Currently chemotherapy is one of the treatments for cancer and its ultimate goal is to produce a drug which can specifically destroy cancer cells without having any significant effect on the normal cells. Cancer chemotherapy has achieved significant success through the discovery of various new drugs. E7010 (**1**), a sulphonamide class of molecule, inhibits tubulin polymerization[1] and causes cell cycle arrest in M phase.[2,3] This compound exhibits good in vivo antitumor activity against several rodent as well as human tumours and is presently in phase II clinical trials.[4] Based on E7010 (**1**) we have previously designed and synthesized a series of 2-anilinonicotinyl linked aminobenzothiazoles (**2**) which showed potent cytotoxicity, cell cycle arrest and DNA fragmentation.[5] Similarly, 2-anilinonicotinyl sulfonylhydrazide conjugates (**3**) also showed promising anticancer activity,[6] however 2-anilinonicotinyl linked oxadiazoles (**4**) derivatives proved to be good inhibitors of tubulin polymerization.[7] Benzothiazoles are known to exhibit various biological properties including antimicrobial, anticancer, anti-amyloid, anti-rheumatic and anti-glutamate activities.[8–11] Various modified aryl benzothiazoles and substituted 2-aminobenzothiazoles are also known to possess significant anticancer activity both in vitro and in vivo models (Fig. 1).[12–16]

Our earlier efforts towards the synthesis of a variety of hybrid molecules led to the development of promising anticancer agents.[17–21] Based on these successes an attempt has been made to further focus on 2-anilino-pyridyl structural component of E7010. These newly designed benzothiazole linked phenylpyridopyrimidinones (**8a–g**) and their diones (**9a–g**) were synthesized by cyclization of 2-anilinonicotinyl linked aminobenzothiazoles by inserting of CH_2 group and carbonyl group, respectively. These newly designed compounds were evaluated for their cytotoxic potential on four different cancer cell lines with IC_{50} values lying in the range of 4.01–44.7 μM. Among the series one of the compound **8b** has shown promising cytotoxicity specifically against human cervical cancer cell line ME-180 with IC_{50} value of 4.01 μM. Flow cytometric analysis of this compound shows arrest of cell cycle in the G0/G1 phase. Hoechst 33258 staining and DNA fragmentation assay reveals that this compound induces cell death by apoptosis. Further, activation of caspase-3 and loss of mitochondrial membrane potential also suggested that this compound produces apoptotic cell death (Scheme 1).

2-Chloronicotinic acid was converted into ethyl 2-chloronicotinate (**2**) by refluxing with ethanol and H_2SO_4 at 80 °C for 2 h. The substituted ethyl 2-anilinonicotinate is prepared by using standard method[5] from ethyl 2-chloronicotinate by reaction with various substituted anilines in ethylene glycol at 160 °C for 8 h. Further, hydrolysis of ester (**4a–d**) by using solution of 2N NaOH in ethanol for 2 h under reflux afforded corresponding substituted 2-anilinonicotinic acid (**5a–d**). Synthesis of **7a–g** was carried out by the reaction of respective 2-anilinonicotinic acids with 6-substituted 2-amino benzothiazoles (**6a–d**) by using EDCI/HOBt in dry DMF as solvent. The target substituted benzothiazole linked

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http://dx.doi.org/10.1016/j.bmcl.2013.11.057

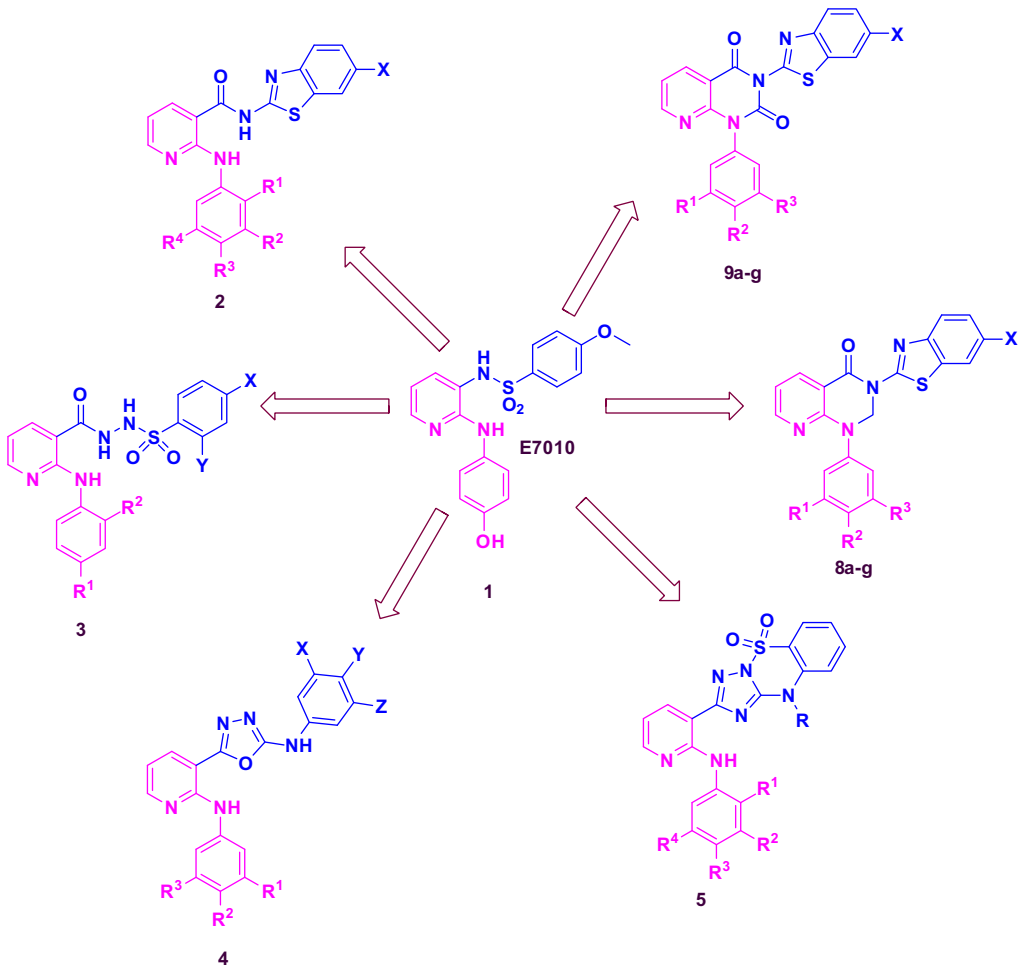


Figure 1. Flow chart of various series derived from E7010 in our laboratory.

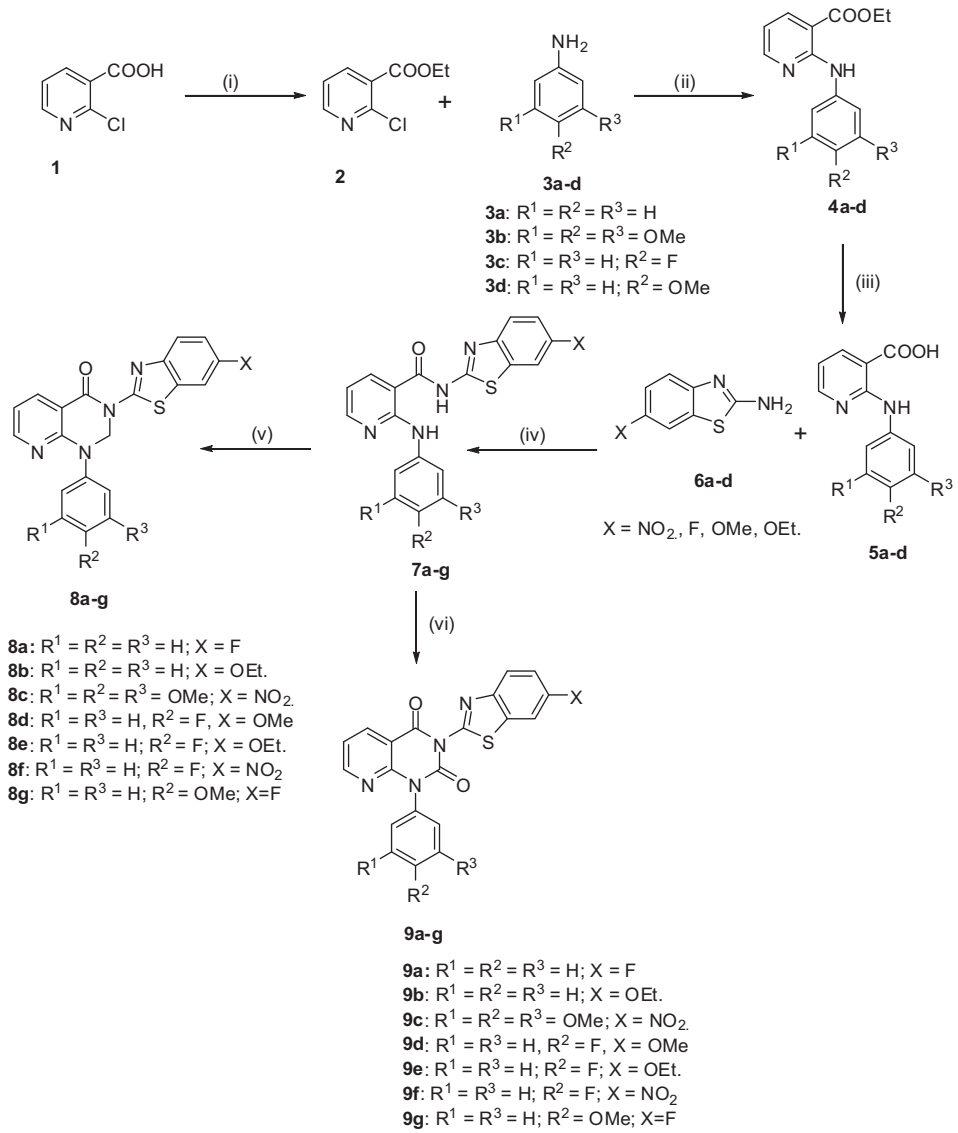
phenylpyridopyrimidinones (**8a**–**g**) and diones (**9a**–**g**) are prepared by the using standard methodology from **7a**–**g** in good yields. Compounds **8a**–**g** obtained by the reaction of **7a**–**g** with formaldehyde in water and acetic acid (9:1) for 1 h and **9a**–**g** were prepared by the reaction of **7a**–**g** in THF with triphosgene and triethylamine was added as a base and the reaction mixture was refluxed at 60 °C for 2 h. The chemical structures of target substituted conjugates **8a**–**g** and **9a**–**g** were confirmed by IR, NMR and MS spectral analysis and the molecular formula of all the compounds were determined by elemental analysis.

The synthesized compounds have been tested for their cytotoxicity against a different set of human cancer cell lines MCF-7 (breast), B-16 (melanoma), ME-180 (cervical), DU-145 (prostate cancer) using MTT assay[22] and the values obtained were compared to the standard drug like 5-flurouracil as shown in Table 1. The screening results suggest that **8b** exhibits significant activity in ME-180 cell line with IC_{50} value 4.01 μM that is comparable to the standard 5-flurouracil as shown in Table 1. To study the cytotoxic effect on normal cell lines, compound **8b** was screened on HEK-293 and the result suggested that this conjugate (**8b**) was 10 fold less toxic to normal cell exhibiting an IC_{50} value of 42.1 μM. Therefore, it was considered of interest to examine the effect of **8b** on cell cycle progression by FACS analysis.

The tumor suppressor gene p53 is a multifunctional protein responsible for maintaining genomic integrity and its mutation is known to cause tumors in humans. In response to DNA damage, aberrant growth signals, or chemotherapeutic drugs, p53 repair, induces apoptosis and/or cell cycle arrest.[23] Data from MTT assay showed that conjugate **8b** induced significant inhibition of cervical cancer cells. It was of interest to understand whether this inhibition of cell growth was on account of cell cycle arrest. Hence, we studied the cell cycle distribution of this conjugate by treating the cells with propidium iodide-labelling and followed by fluorescence-activated cell sorting analysis. Results (Fig. 2 and Table 2) indicated that **8b** showed cell cycle arrest at G0/G1 phase in 48 h as compared to the untreated control that activates signalling for DNA repair initially and in failure of DNA as shown in Figure 3.

Apoptosis was also assessed by electrophoresis of extracted genomic DNA from cells. Endonuclease mediated cleavage of nuclear DNA results in the formation of oligonucleosomal DNA fragment (180–200 base pairs long) a biochemical hallmark of apoptosis in a number of cell types.[24,25] DNA laddering assay was performed with ME-180 cells by treatment of **8b** at a concentration of 4 μM for 24 h, then the genomic DNA was isolated and fallowed by electrophoresis in 1.8% agarose gel. The conjugate **8b** induces DNA fragmentation which is a characteristic ladder pattern in ME-180 cells at 4 μM concentration whereas such laddering was observed in the control as shown in Figure 4.

Further confirmation of the ability of the most potent compound **8b** to induce apoptosis was obtained by analysis of drug-treated ME-180 cell populations (4 μM, 24 h) by fluorescence microscopy.[26] Following drug treatment morphological changes of ME-180 cells was observed. The untreated cells possess large sized nuclei, whereas the incubation for 24 h at 4 μM concentration of



Scheme 1. Reagents and conditions: (i) ethanol, H_2SO_4, 80 °C, 2 h; (ii) ethylene glycol, 160 °C, 8 h; (iii) 2N NaOH, ethanol, 80 °C 2 h; (iv) EDCI, HOBT, DMF, 8 h; (v) formaldehyde, acetic acid, H_2O (1:9), 1 h; (vi) triphosgene, THF, 60 °C, 2 h.

Table 1
Cytotoxic effects of compounds **8a–g** and **9a–g** on various cancer cell lines. The bold values signifies the Potent cytotoxic effect against the corresponding cell lines.

Compound	IC_{50} (μM)			
	ME-180[a]	MCF-7[b]	B-16[c]	DU-145[d]
8a	8.30	>20	**6.1**	8.1
8b[e]	**4.01**	**7.8**	**12.1**	**15.2**
8c	>20	>20	>20	**6.4**
8d	9.9	>20	>20	**5.1**
8e	**6.10**	>20	**5.1**	12.1
8f	13.23	14.2	12.1	>20
8g	16.2	>20	13.8	**5.2**
9a	>20	>20	15.2	12.5
9b	15.8	>20	12.5	19.9
9c	>20	>20	19.9	>20
9d	>20	12.3	17.5	9.3
9e	13.9	11.6	>20	17.5
9f	19.0	13.7	7.4	8.3
9g	18.9	19.9	14.6	>20
5-Fluorouracil	4.9	3.5	4.2	—

[a] Cervical cancer.
[b] Breast cancer.
[c] Mouse macrophages cell line.
[d] Prostrate cancer.
[e] Normal cell line HEK-293 (IC_{50} of **8b** is 42.1 μM).

8b revealed fragmentation of nuclei due to apoptosis in ME-180 cells (Fig. 5).

Most of the proteolytic cleavages during apoptosis result from the activation of caspases, a family of cysteine-dependent proteases.[27] Caspases can initiate apoptotic signals and execute the apoptotic programs. Hence, the involvement of various caspases and their role in the process of apoptosis has been investigated. Compounds that showed apoptosis induction in flow cytometric assay was also evaluated for their ability to induce caspase activity in ME-180 cells. Conjugate **8b** upon treatment at 2 and 4 μM concentrations for 48 h in ME-180 cells activated caspase-3 by 4–6 folds when compared to control as shown in the Figure 6. The activation of caspase-3 by **8b** at both concentrations indicates that it induces apoptosis through both the intrinsic as well as extrinsic apoptotic pathway in ME-180 cells.

The loss of mitochondrial membrane potential is a major characteristic of apoptosis.[28] Our results have shown that **8b** disrupts mitochondrial membrane potential. The result of JC-1 staining indicates loss of $\Delta\Psi m$ which displays strong evidence of apoptosis in this conjugate **8b**. Thus, the data generated has shown high percentage of chromatin condensation, nucleus fragmentation, and loss of $\Delta\Psi m$ in this conjugate.

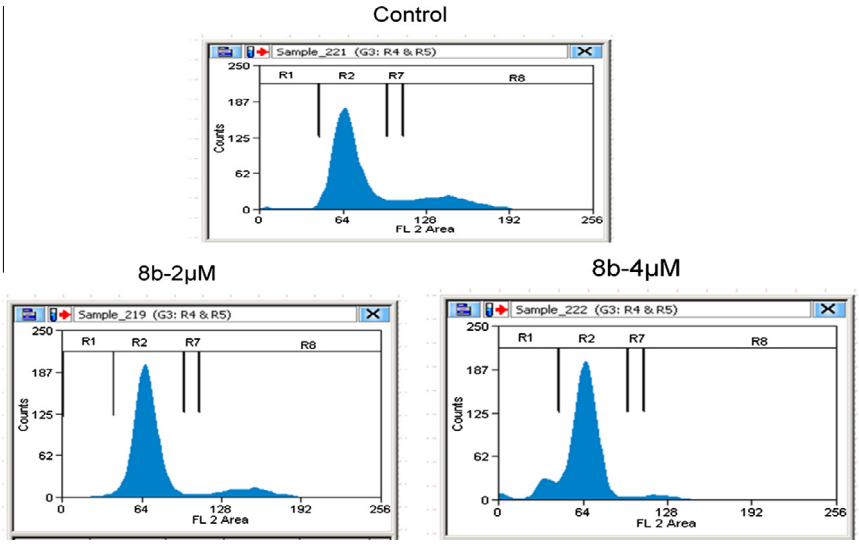


Figure 2. Flow-activating cell sorting (FACS) analysis of compounds **8b** at 2 and 4 μM. Effect of compound **8b** on cell cycle distribution of ME-180 cell line was examined by staining cells with propidium iodide.

Table 2

Compound	G0	G1	S	G2/M
Control	2.97	69.80	3.40	23.82
8b (2 μM)	2.38	79.78	1.57	16.41
8b (4 μM)	13.18	79.54	1.49	5.79

% of DNA content in G0, G1, S and G2/M phase in ME-180 cells.

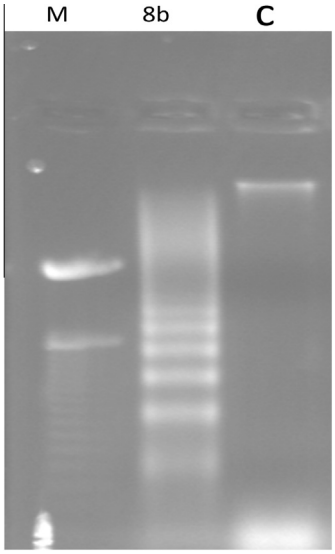


Figure 3. Effect of compound **8b** on DNA fragmentation on ME-180 cells. Cells were incubated for 24 h with 4 μM concentration.

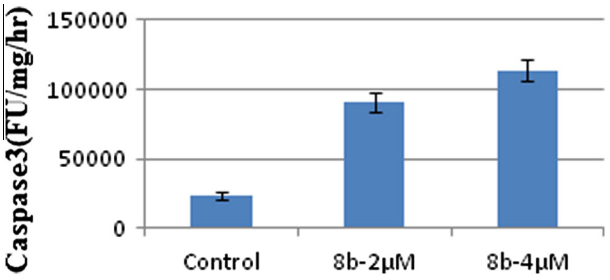


Figure 5. Effect of compound **8b** on caspase-3, activities in ME-180 cells determined by fluorimetry at 2 and 4 μM after 48 h incubation. Error bars represents data from two independent experiments with each performed in duplicate. Data are mean ± SD from three independent experiments.

In the present study, two new series of benzothiazole linked phenylpyridopyrimidinones **8a–g** and their dione conjugates **9a–g** have been designed, synthesized and evaluated for their cytotoxic activity against four human cancer cell lines (ME-180, DU-145, MCF-7, B-16). Some of these compounds exhibit promising cytotoxicity at micromolar (μM) concentration. Among the series one compound **8b** exhibited significant anticancer activity (IC_{50}, 4.1 μM) against ME-180 cancer cell line and flow cytometric analysis of this compound shows arrest of cell cycle in the G0/G1 phase. Hoechst 33258 staining and DNA fragmentation assay reveals that this conjugate induces cell death by apoptosis. Further, activation of caspase-3 loss of mitochondrial membrane potential also suggested that this compound produces apoptotic cell death. Therefore this is an interesting lead to take up future structural modifications on this scaffold.

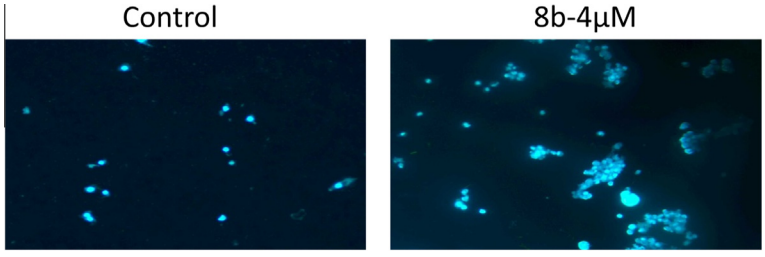


Figure 4. Fluorescent microscopy pictures showing morphological changes in ME-180 cells treated with 4 μM, compound **8b** for 24 h.

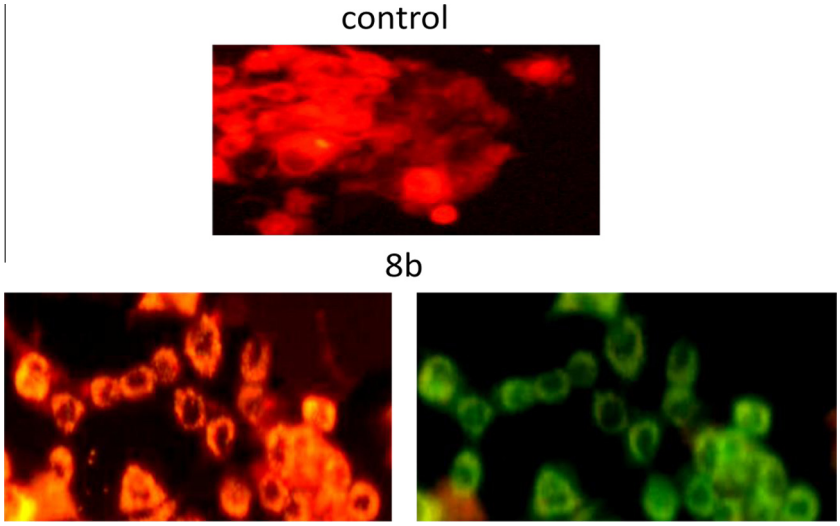


Figure 6. The loss of mitochondrial membrane potential was monitored by JC-1 stain. High ΔΨm in control mitochondria causes association with the dye in an aggregated state leading to red-orange fluorescence emission. Compound **8b** lead to dissipation of ΔΨm, and the dye is now partitioned into cytosol, resulting in green fluorescence.

Acknowledgments

M.A. and S.F. acknowledge CSIR-UGC, New Delhi and for the award of senior research fellowship. We also acknowledge CSIR for financial support under the 12th Five Year plan project 'Affordable Cancer Therapeutics (ACT)' (CSC0301).

Supplementary data

Supplementary data (experimental procedures and spectroscopic characterization of the compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.11.057.

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