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Design, Synthesis and *In vitro* Anti-tumor Evaluation of Novel Acrylohydrazide Thioglycosides

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Abstract

A facile, convenient and high yielding synthesis of novel Acrylohydrazide thioglycosides via one-pot reaction of the potassium thiolate salts of aglycon part - prepared from readily available starting materials - with 2,3,4,6-tetra-O-acetyl-*α*-D-gluco- and galactopyranosyl bromides . Pharmacological evaluation of compounds 8j, 8b, 8h, 8k, 8f and 5b *in vitro* against (MCF-7) cell line (Breast carcinoma cell line) showing high- moderate anti-tumor activities with IC50 values ranging from 3.69-14.93 (μM), moreover molecular modeling of these compounds revealed that they have high binding affinity through hydrophobic-hydrophobic interaction and moderate selectivity through the hydrogen bond interaction with the atypical nucleotide binding pocket in the amino terminus of Hsp90.

Keywords: Acrylohydrazide; Anti- tumor activity; Hsp90

Introduction

The incidence and mortality of cancer patients have become one of the important issues discussed worldwide [1]. Unfortunately, development of resistance to chemotherapeutic agents is a common obstacle in the treatment of different types of cancers [2]. Several important drugs including tamoxifen (TAM), 5- flurouracil (5FU), adriamycin (ADR) and vincristin (VCR) with different structures and mechanisms of anti-tumor activities fail to end these problems completely [3]. Due to the several side effects [4], drug resistance and failure of anti-tumor drugs to exert their effects in certain cases of cancers [5], looking for new chemotherapeutic agents with synthetic or natural origins is one of the hot topics in cancer research laboratories.

As a part of our program directed towards the development of new, simple and efficient procedures for the synthesis of antimetabolites [6-8], we described that pyridine thioglycosides exerted inhibitory effects on both DNA and RNA containing viruses [9-11]. It was reported that the dihydropyridine derivative exhibits strong P-glycoprotein (Pgp) antagonist effect and possesses activity against human colon carcinoma cells.

Based on these findings, it was of interest to prepare more effective agents. Heat shock protein 90 (Hsp90) represents an exciting target for the treatment of cancer, as inhibition of this chaperone can affect multiple proteins that are directly associated with all six hallmarks of cancer [12,13]. Hsp90 is a 90 kDa molecular chaperone and is intimately involved in the post-translational conformational maturation of nascent polypeptides [14] as well as the re-folding of denatured proteins and the re-solubilization of protein aggregates [15,16]. Hsp90 has emerged as a promising anti-cancer target, with more than 20 clinical trials currently in progress with small molecules that bind the N-terminal ATP binding site [17]. It was reported that Novobiocin, O-glycoside antibiotic shown to bind adjacent to the ATP-binding site of bacterial gyrase B and to interfere with nucleotide binding, was also able to interact with Hsp90 catalytic site C-Terminal, albeit with lower affinity than with gyrase B, and to disrupt the chaperone activity of Hsp90 in a manner similar to radicicol-Hsp90 inhibtor antitumor drug especially against MCF-7 SKBr-3 [18] with SAR showing that both the six membered sugar moeity at position 7 and the amide linker are essential for the highest activity in comparison with other analogues [19]. We have focused our attention on synthesis of new S-glycosides as anti-cancer agents which could be capable of inhibiting Hsp90 function by potential interaction towards the binding pocket in the amino terminus of Hsp90.

A library of acrylohydrazide thioglycosides was energy minimized using semi-empirical (PM3) calculations. The catalytic domain of HSP 90 was obtained from protein data bank (PDB) and was prepared for docking using Open Eye's Fred Receptor program Open Eye Omega application was used to generate different conformations of each ligand, in order to achieve flexible ligand docking, to be used in the docking simulations, this software package is able to perform consensus scoring which is essential filtering technique used to obtain more accurate predictions i.e. The lower consensus score, the better binding affinity of the ligands towards the receptor. It was done in Faculty of Science, South Dakota University, USA. This study revealed that some acrylohydrazide thioglycosides, such as 8b, 8c, 8e, 8k, 8j, 8l, 8g, 8d has hydrophobic – hydrophobic interaction towards ATP-binding site of Hsp 90 (2BZ5) and also for example 8b has hydrogen bonding between cyano group and Lys 58: A and 2 hydrogen bonds coming from oxygen of acetylated sugar moiety with ASN 51: A and Phe 138: A as shown at Figures 1-3. And other derivatives(8d, 8g, 8c) showed different binding mood but still 3 hydrogen bonds the cyano group binds to ti ALA 111:A, and the C=O of the acetylated sugar binds to LYS 112A Figure 4

Molecular modeling comparative consensus score of acrylohydrazide thioglycosides are listed in Table 1.

From these finding we found that some derivatives of acrylohydrazide thioglycosides has high binding affinity through hydrophobic-hydrophobic interaction and moderate selectivity through the hydrogen bond interaction with the atypical nucleotide

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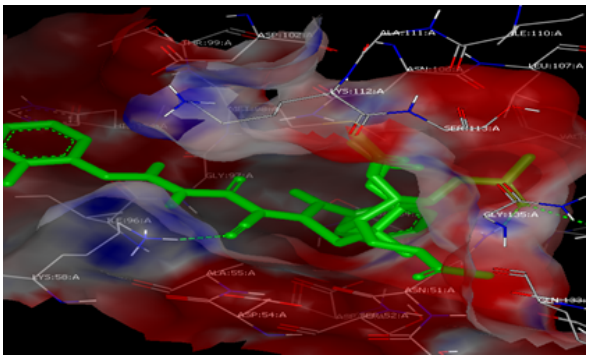


Figure 1: Visual representation for 8b showing the hydrophobic-hydrophobic interaction towards the ATP bindingsite of Hsp 90, the dashed lines showing the hydrogen bonding between the cyano group and Lys 58:A and 2 hydrogen bonds coming from oxygen of acetylated sugar moiety with ASN 51:A and Phe 138:A as shown by Vida.

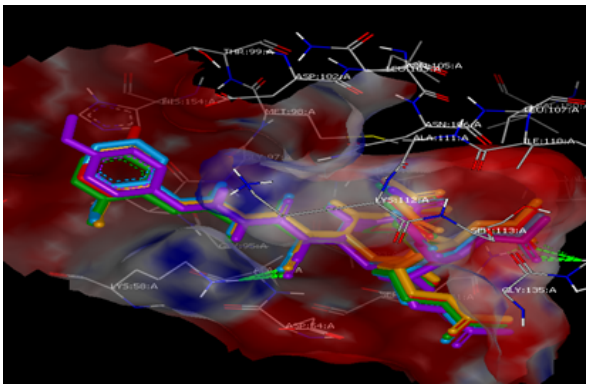


Figure 2: Overlay of 8b (green), 8e (light blue), 8k (golden yellow), 8j (red), 8l (purple) over 2BZ5.

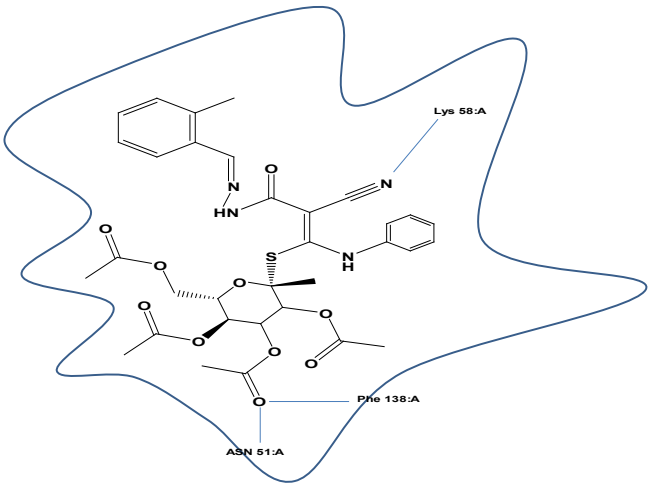


Figure 3: Graphical representation for 4b showing the hydrophobic-hydrophobic interaction towards the ATP binding site of Hsp 90, the dashed lines showing the hydrogen bonding between the cyano group and Lys 58:A and 2 hydrogen bonds coming from oxygen of acetylated sugar moiety with ASN 51:A and Phe 138:A representation.

binding pocket in the amino terminus of Hsp90 , In view of the above mentioned findings and our previous reports the purpose of the present work was to design, synthesize and investigate the anti-tumor activity

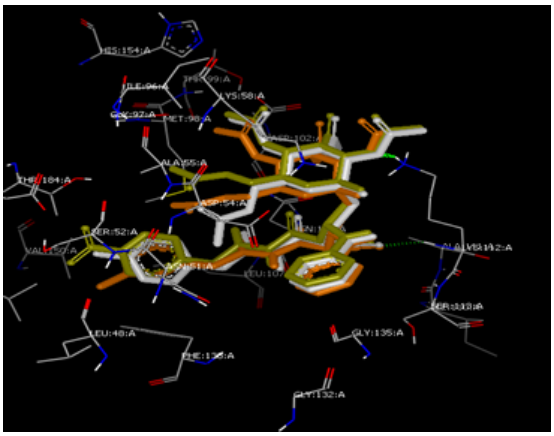


Figure 4: Visual representation for 8d: white, 8g: brownish green,,8c : orange Show different binding mode, sincethey didn't fill the whole hydrophobic binding site like 8b did, but they still have 3 hydogen bonds but binds to different amino acids, CN group is binding ti ALA 111:A, and the C=o of the acetylated sugar binds to LYS 112A.

Compound	Consensus Score
8b	151
8e	156
8k	169
8j	315
5b	384
8d	425
8g	463
8l	483
8c	502
Novobiocin	538

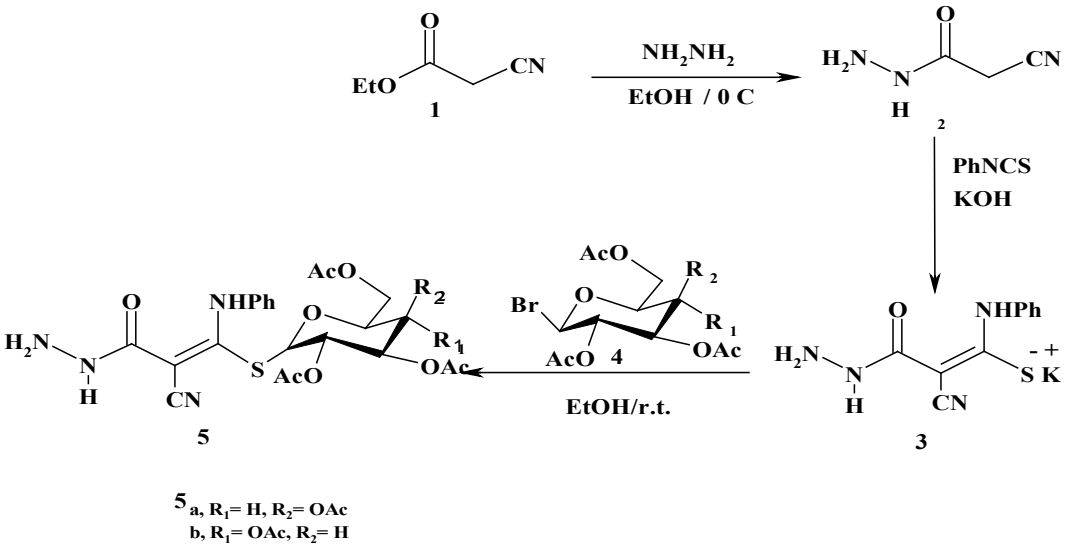
Table 1: Molecular modeling consensus scores of acrylohydrazide thioglycosides 8b, 8c, 8e, 8k, 8j, 8l, 8g, 8d, 5b and Novobiocin.

of some novel acrylohydrazide derivatives carrying carbohydrate residues through S-glycosidic bond formation.

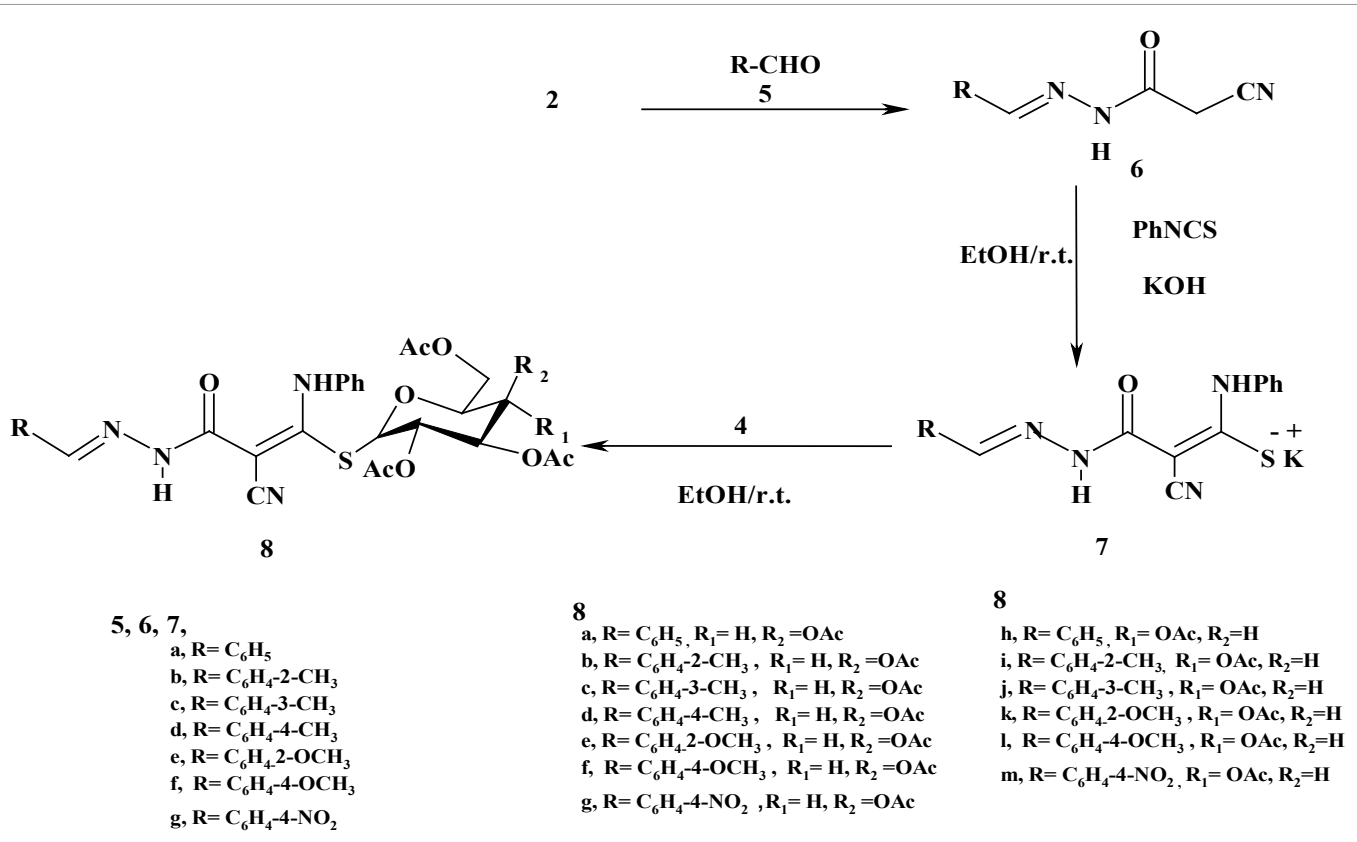
Chemistry

In order to explore the possibility of the coupling reaction between alkylidenethiolate salts and the halosugars to obtain the title thioglycosides 5 a,b and 8 a-m. Schemes 1-3 describe the synthesis of Acrylohydrazide thioglycosides 5a,b and 8a-m starting with cyano acetic acid hydrazide 2. Cyano acetic acid hydrazide **2** was prepared by the reaction of ethyl cyano acetate 1 with hydrazine in EtOH at 0°C [20], and then was reacted with phenyl isothiocyanate in KOH-EtOH at room temperature to give the corresponding stable potassium (*E*)-2-cyano-3-(phenylamino) acrylohydrazide -3-thiolates salts 3. The latter react with 2,3,4,6-tetra-O-acetyl-α-D-gluco- and galacto-pyranosyl bromides 4 in ethanol at room temperature to give the corresponding S-glucosides 5a or S-galactosides 5b , in high yield as shown in Scheme 1.

The structures of the reaction products 5a,b were established by their elemental analyses and spectral data (IR,^{1}H NMR). As an example, the analytical data for 5b revealed a molecular formula $C_{23}H_{28}N_4O_{10}S$. The ^{1}H NMR spectrum showed the anomeric proton as a doublet at δ5.25 ppm. The coupling constant $J_{1',2'}$=9.5 Hz indicated H-1′to be trans-diaxial to H-2′. The other six glucose protons resonated at 3.02-5.09 ppm and the four acetyl groups appeared as four singlets at δ 1.98-2.09 ppm. N'-Substituted cyano acetic acid hydrazide derivatives 6a–g were prepared by the reaction of cyano acetic acid hydrazide 2 with the corresponding aldehyde 5, and then the corresponding S-glucosides



Scheme 1: Synthethitic pathway for the synthesis of Cyano acid hydrazide and its derivatives of Acrylohydrazide thioglycosides.

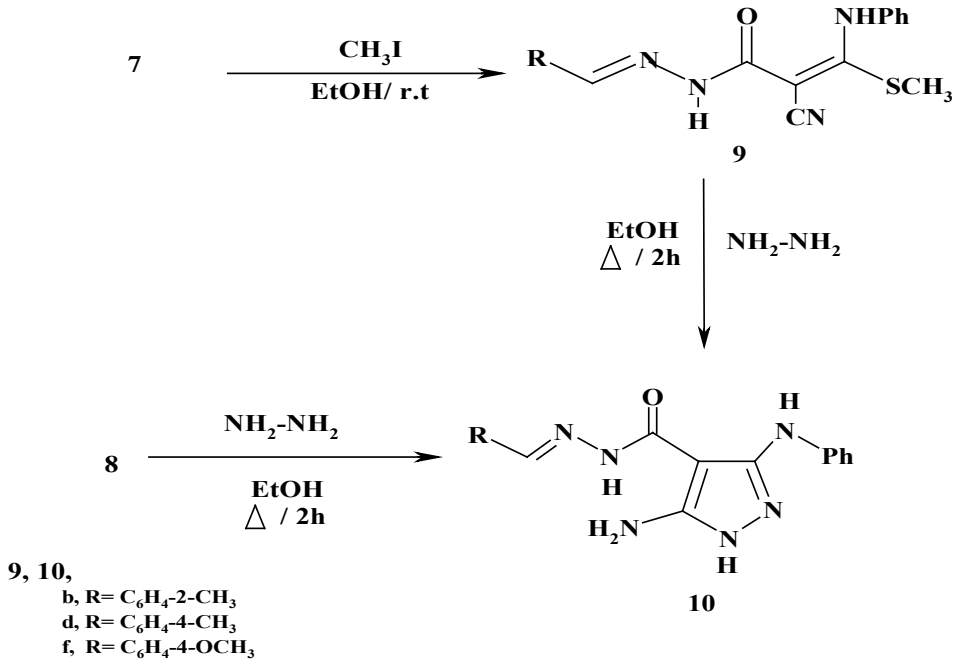


Scheme 2: Synthesis of N' Benzylidene derivatives of Cyano acid hydrazide and its thiogylcosides derivatives.

8a-g or S-galactosides 8h-m were prepared as shown in the reaction sequence summarized in Scheme 2.

The structures of the reaction products 8a-m were established by their elemental analyses and spectral data (IR, ^{1}H NMR, ^{13}C NMR). As an example, the analytical data for 8g revealed a molecular formula $C_{31}H_{31}N_5O_{12}S$. The ^{1}H NMR spectrum showed the anomeric proton as a doublet at δ 5.30 ppm. The coupling constant $J_{1',2'}$=9.8 Hz indicated H-1′ to be trans-diaxial to H-2′. The other six glucose protons resonated at 3.35-4.09 ppm and the four acetyl groups appeared as four singlets at δ 1.93-2.03 ppm. The ^{13}C NMR spectrum of 8g contained a signal at δ 83.84 corresponding to the C-1' atom and five signals appearing at δ 61.81, 67.84, 70.24, 73.14 and 75.08 that were assigned to C-6', C-4', C-2', C-3' and C-5', respectively. Attempt removal of protecting groups in 8 by methanolic ammonia has not resulted in formation of the corresponding free glycosides.

It was suggested that structure 8a-m should be present in the *E*



Scheme 3: Synthesis of 5-amino pyrazole derivatives.

form and not in the *Z* form, this was shown by reacting compounds 8 with hydrazine in refluxing ethanol to give the corresponding 5-aminopyrazole derivatives 10 as shown in Scheme 3. The structures of 10 were established on the basis of spectral data (IR) as compound 10f showed the disappearance of CN group when compared with 8f. 10 could also be prepared by refluxing (*2E,15E*)-*N'*-benzylidene-2-cyano-3-(methylthio)-3-(phenylamino) acrylohydrazide 9 with hydrazine in ethanol. Compounds 9 were prepared by reaction of 7 with methyl iodide in ethanol [8] as shown in Scheme 3. In summary, we have achieved the synthesis of acrylohydrazide thioglycosides by the reaction of the potassium (*2E,15E*)-*N*-benzylidene-2-cyano -3-(phenylamino) acrylohydrazide-3-thiolate salts with α-glycosyl halides. These acyclic glycosides can be utilized as starting materials for the synthesis of other carbohydrate derivatives.

Pharmacology

Materials and methods

Potential cytotoxicity effect of the newly synthesized compounds in four concentrations, were evaluated in the National Institute of Cancer, Cairo, Egypt by SRB assay [21]. Cells were plated in 96-multiwell plate (104cells/well) for 24 h before treatment with the compounds to allow attachment of cell to the wall of plate. Different concentrations of each compound under test (0, 5, 12.5, 25 and 50 μg/ml) were added to the cell monolayer triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compound(s) for 48 h at 37°C and in atmosphere of 5% CO2. After 48 h, cells were fixed, washed and stained with sulforhodamine B strain. Excess stain was washed with acetic acid and attached stain was recovered with EDTA buffer. Color intensity was measured in an ELISA reader. Finally, the relation between surviving fraction and drug conc. is plotted to get the survival curve of each tumor cell line after the specified compound.

Anticancer screening studies

Seven of the newly synthesized compounds were screened for their anticancer activities against MCF-7 (Breast), IC50 was calculated with regard to saline control group and potency was calculated with regard to percentage of change of Novobiocin and tested compounds, as depicted, in Table 2.

Our SAR study shows that all the tested compounds have high or moderate anti-tumor activity towards Breast cell lines (MCF-7) with IC50 values ranging from 3.69-14.93 (μM). N- free Acrylohydrazide thioglycosides (5b) has lower activity than those linked with Arylidenyl moiety as they have lower ability to fill the hydrophobic space in ATP-binding site of Hsp 90 (2BZ5)[consensus score 384, IC_{50} 14.93 μM] , moreover Acrylohydrazide thioglycosides linked with Arylidenyl moieties substituted at ortho position (8b,8k) [consensus score 151, IC_{50} 4.50 μM and consensus score 169, IC_{50} 3.69 μM respectively] is more active than those substituted at meta position (8j) (consensus score 315, IC_{50} 6.00 μM respectively) then un-substituted moiety (8a,8h) [IC_{50} 8.73 μM and IC_{50} 6.89 μM] and para position (8f) [IC_{50} 12.83 μM] this because the ortho substituted moiety may improve the hydrophobic-hydrophobic interaction towards the ATP binding site of Hsp 90 and thus increase the ability of the ligands to fill the hydrophobic pocket of the target [22-24].

Conclusion

We have achieved the synthesis of Acrylohydrazide derivatives having cyclic carbohydrate residues through S-glycosidic bond formation in an efficient manner. Pharmacological evaluation of compounds 8a, 8d, 8j, 8h, 8f, 8k and 5b against cell lines MCF-7 (breast) revealed them to possess high or moderate anti-tumor activities. Hence, it could be a potential drug candidate for cancer treatment.

Experimental

All melting points were uncorrected on a Gallenkamp melting point apparatus. The IR spectra were recorded (KBr disk) on a Perkin Elmer 1650 FT-IR instrument. The NMR spectra were recorded on a Varian 500 MHz spectrometer in $(CD_3)_2SO$ using $Si(CH_3)_4$ as

Compound No.	IC50 (μM) Breast cancer cell line MCF-7
Novobiocin	481.30 Laura et al.,2010
8b	4.50
8k	3.69
8j	6.00
8f	12.83
8a	8.73
8h	6.89
5b	14.93

Table 2: Cytotoxcity of the synthesized candidates on breast (MCF-7) cancer cell lines.

an internal standard. Elemental analyses were obtained from the Microanalytical Data Center at Cairo University, Egypt. Progress of the reactions was monitored by TLC using aluminum sheets coated with silica gel F254 (Merck). Viewing under a short-wavelength UV lamp effected detection. All evaporations were carried out under reduced pressure at 40ºC. Compounds 2 and 6 were prepared following reported procedures [19].

(*E*)-2-cyano-3-(2`,3`,4`,6`-tetra-*O*-acetyl–β-D-gluco-and/orgalactopyranosylthio)-3 (phenylamino)acrylohydrazide. (5a,b)

General procedure: A mixture of cyano acetic acid hydrazide 2 (0.01 mol) and phenyl isothiocyanate (0.01 mol) was stirred for 10 to 20 min in ethanol (25 ml) containing potassium hydroxide (0.01 mol). After cooling, A solution of 2, 3, 4, 6-tetra-*O*-acetyl –α-D-gluco- and/or galactopyranosyl bromides (0.01 mol) in 10 ml acetone was stirred at room temperature for 16 hour. The solution was evaporated and the formed residue was washed with distilled water to remove the formed potassium bromide salt. The resulting product was recrystallized from ethanol.

(*E*)-2-cyano-3-(2`,3`,4`,6`-tetra-*O*-acetyl – *β* -D-glucopyranosylthio)-3-(phenylamino)acrylohydrazide. (5a)

Yellow solid; yield 52%; m.p. 111 ºC; IR (KBr cm^{-1}) 3472.2 (NH2), 2227.38 (CN), 1750.08 (CO); ^{1}H NMR(500MHz δ ppm $CDCl_3$) 1.88-2.08 (4s, 12H, 4 x CH_3CO), 3.13 (s, 2H, 6`-$H_2$), 3.26-3.44 (m, 2H, *J*=9.34, 4`-H, 5-H), 4.02-4.22 (m, 1H, 3`-H), 4.86-5.09 (m, 1H, 2`-H), 5.23-5.27 (d, 1H, *J*=9.55 1`-H), 6.98-7.55 (m, 5H,C_6H_5), 10.68 (s, 2H, NH_2); Anal. Calcd For. $C_{23}H_{28}N_4O_{10}S$ (552.55): C, 49.99; H, 5.10; N, 10.14. Found: C, 49.73; H, 5.35; N, 10.22. .

(*E*)-2-cyano-3-(2`,3`,4`,6`-tetra-*O*-acetyl – *β* -D-galactopyranosylthio)-3-(phenylamino)acrylohydrazide. (5b)

Yellow solid; yield 65%; m.p. 175 ºC ; IR (KBr cm^{-1}) 3463.53 (NH2), 2360.44 (CN), 1750.08 (CO); ^{1}H NMR(500MHz δ ppm $CDCl_3$) 1.98-2.09 (4s, 12H, 4 x CH_3CO), 3.02 (s, 2H, 6`-$H_2$), 3.77-3.78 (m, 2H, *J*=9.54 4`-H, 5`-H), 4.22-4.32 (m, 1H, 3`-H), 4.63-5.09 (m, 1H, 2`-H), 5.24-5.25 (d, 1H, *J*=9.33 1`-H), 7.24-7.35 (m, 5H,C_6H_5), 9.68 (s, 2H, NH_2); Anal. Calcd For. $C_{23}H_{28}N_4O_{10}S$ (552.55): C, 49.99; H, 5.10; N, 10.14 . Found: C, 49.65; H, 5.27; N, 10.42.

(*2E*)-*N*'-benzylidene-2-cyano-3-(2`,3`,4`,6`-tetra-*O*-acetyl –*β*-D-gluco- and/or galactopyranosylthio)-3-(phenylamino) acrylohydrazide. (8 a-n)

General procedure: A mixture of N-substituted Arylidenyl acrylohydrazide derivatives 6a–d (0.01 mol) and phenyl isothiocyanate (0.01 mol) was stirred for 10 to 20 min in ethanol (25 mL) containing potassium hydroxide (0.01 mol). After cooling,A solution of 2,3,4,6-tetra-*O*-acetyl –α-D-gluco- and/or galactopyranosyl bromides (0.01mol) in 10 ml acetone was stirred at room temperature for 16 hour. The solution was evaporated and the formed residue was washed with distilled water to remove the formed potassium bromide salt. The resulting product was recrystallized from ethanol.

(*2E*)-*N*'-benzylidene-2-cyano-3-(2`,3`,4`,6`-tetra-*O*-acetyl–*β*-D-glucopyranosylthio)-3-(phenylamino) acrylohydrazide. (8a)

Yellow solid; yield 87%; m.p. 179 ºC; IR (KBr cm^{-1}) 3330.46 (NH), 2198.45 (CN), 1745 (CO) ; ^{1}H NMR(500MHz δ ppm $CDCl_3$) 1.93-2.02 (4s, 12H, 4 x CH_3CO), 2.50 (s, 1H, CH), 3.35 (s, 2H, 6`-$H_2$), 3.97-4.02 (m, 2H, *J*=9.55 4`-H, 5`-H), 4.83-4.90 (m, 1H, *J*=9.3 3`-H), 5.17-5.24 (m, 1H, *J*=9.5 2`-H), 5.27-5.30 (d, 1H, *J*=9.45 1`-H), 7.24-7.70 (m, 10H, NH-C_6H_5, CH-C_6H_5), 11.37 (s,1H, CONH), 12.03 (s, 1H, NH-C_6H_5) ; ^{13}C NMR (DMSO, d_6 δppm) 61.81 (CH_2, C-6`), 67.85 (C-4`), 70.24 (C-2`), 73.15 (C-3`), 75.06 (C-5`), 83.84 (C-1`),117.85 (CN), 126.41–130.37 (2C_6H_5), 134.73 (C-2), 160.18 (C-3), 163.70 (C-1), 169.00-170.36 (4 x CO)Anal.Calcd For. $C_{31}H_{32}N_4O_{10}S$ (652.62): C, 57.05; H, 4.94; N,8.58. Found: C, 57.11; H, 4.99; N, 8.72.

(*2E*)-*N*'-benzylidene-2-cyano-3-(2`,3`,4`,6`-tetra-*O*-acetyl–*β*-D-galactopyranosylthio)-3-(phenylamino) acrylohydrazide. (8b)

Yellow solid; yield 74%; m.p. 170 ºC ; ^{1}H NMR(500MHz δ ppm $CDCl_3$) 1.93-2.13 (4s, 12H, 4 x CH_3CO), 2.49 (s, 1H, CH), 3.38 (s, 2H, 6`-$H_2$), 4.02-4.22 (m, 2H, *J*=9.3 4`-H, 5`-H), 4.87-5.01 (m, 1H, *J*=9.5 3`-H), 5.15-5.18 (m, 1H, *J*=9.5 2`-H), 5.29-5.35 (d, 1H, *J*=9.2 1`-H), 7.24-7.86 (m, 10H, NH-C_6H_5, CH-C_6H_5) Anal.Calcd For. $C_{32}H_{34}N_4O_{10}S$ (666.65): C, 57.65; H, 5.14; N, 8.40. Found: C, 57.90; H, 5.37; N, 8.75.

(*2E*)-*N*'-(3-methyl benzylidene)-2-cyano-3-(2`,3`,4`,6`-tetra-*O*-acetyl –*β*-D-glucopyranosylthio)-3-(phenylamino) acrylohydrazide. (8c)

Yellow solid; yield 76%; m.p. 177ºC; IR (KBr cm^{-1}) 3308.29 (NH), 2202.31 (CN), 1746.2 (CO) ; ^{1}H NMR(500MHz δ ppm $CDCl_3$) 1.92-2.02 (4s, 12H, 4 x CH_3CO), 2.49 (s, 3H, CH_3), 2.50-2.52 (s, 1H, CH), 3.35 (s, 2H, 6`-$H_2$), 3.62-3.71 (m, 2H, *J*=9.4 4`-H, 5`-H), 3.97-4.02 (m, 1H, *J*=9.55 3`-H), 4.81-4.89 (m, 1H, *J*=9.34 2`-H), 5.17-5.29 (m, 1H, *J*=9.2 1`-H), 7.23-7.52 (m, 9H, NH-C_6H_5, CH-C_6H_4), 11.36 (s,1H, CONH), 12.01 (s, 1H, NH-C6H5) ^{13}C NMR (DMSO, d_6 δppm) 41.01(CH_3), 61.81 (CH_2, C-6`), 67.85 (C-4`), 69 (C-2`), 73 (C-3`), 75 (C-5`), 83 (C-1`),117 (CN), 124.95–129.33 (2C_6H_5), 134.68 (C-2), 138.22 (C-3), 150 (C-1), 169.57-170.36 (4 x CO) Anal. Calcd For. $C_{32}H_{34}N_4O_{10}S$ (666.65): C, 57.65; H, 5.14; N, 8.40. Found: C, 57.40; H, 5.03; N, 8.09.

(*2E*)-*N*'-(4-methyl-benzylidene)-2-cyano-3-(2`,3`,4`,6`-tetra-*O*-acetyl–*β*-D-glucopyranosylthio)-3-(phenylamino) acrylohydrazide. (8d)

Yellow solid; yield 85%; m.p. 196 ºC; IR (KBr cm^{-1}) 3329.5 (NH), 2197.49 (CN), 1745.2 (CO) ; ^{1}H NMR(500MHz δ ppm $CDCl_3$) 1.92-2.02 (4s, 12H, 4 x CH_3CO), 2.49 (s, 3H, CH_3), 2.50-2.52 (s, 1H, CH) , 3.34 (s, 2H, 6`-$H_2$), 3.61-3.71 (m, 2H, *J*=9.2 4`-H, 5`-H), 3.96-4.02 (m, 1H, *J*=9.5 3`-H), 4.81-4.89 (m, 1H, *J*=9.55 2`-H), 5.17-5.29 (m, 1H, *J*=9.55 1`-H), 7.24-8.41 (m, 9H, NH-C_6H_5, CH-C_6H_4), 11.30 (s,1H, CONH), 12.02 (s, 1H, NH-C_6H_5) ^{13}C NMR (DMSO, d_6 δppm) 51.05 (CH_3), 61.31 (CH_2, C-6`), 67.85 (C-4`), 69 (C-2`), 70 (C-3`), 75.01 (C-5`), 79.5 (C-1`),110 (CN), 127.55–132.02 (2C_6H_5), 134.68 (C-2), 138.22 (C-3), 148.98 (C-1), 169.56-170.35 (4 x CO) Anal.Calcd For. $C_{32}H_{34}N_4O_{10}S$ (666.65): C, 57.65; H, 5.14; N, 8.40. Found: C,

(*2E*)-*N'*-(2-methoxy-benzylidene)-2-cyano-3-(2`,3`,4`,6`-tetra-*O*-acetyl-*β*-D-glucopyranosylthio)-3-(phenylamino) acrylohydrazide. (8e)

Yellow solid; yield 80%; m.p. 172 °C; IR (KBr cm^{-1}) 3198.36 (NH), 1956.43 (CN), 1704.76 (CO); ^{1}H NMR(500MHz δ ppm $CDCl_3$) 1.92-2.02 (4s, 12H, 4 × CH_3CO), 2.49 (s, 1H, CH), 3.34 (s, 2H, 6`-$H_2$), 3.60-3.70 (m, 2H, *J*=9.55 4`-H, 5`-H), 3.85 (s, 3H, $OCH_3$), 3.96-4.02 (m, 1H, *J*=9.3 3`-H), 4.81-4.89 (m, 1H, *J*=9.55 2`-H), 5.18-5.29 (m, 1H, *J*=9.5 1`-H), 6.97-7.44 (m, 9H, NH-C_6H_5, CH-C_6H_4)^{13}C NMR (DMSO, d_6 δppm) 56.16 (OCH_3), 61.31 (CH_2, C-6`), 69 (C-2`), 73.15 (C-3`), 79.5 (C-1`),117.85 (CN), 129.31 (2C_6H_5), 134.68(C-2), 138.00(C-3), 142.98(C-1), 170.35(4xCO), Anal.Calcd For. $C_{32}H_{34}N_4O_{10}S$ (682.65): C, 56.30; H, 5.02; N, 8.20. Found: C, 56.62; H, 5.24; N, 8.53.

(*2E*)-*N'*-(4-methoxy-benzylidene)-2-cyano-3-(2`,3`,4`,6`-tetra-*O*-acetyl-*β*-D-glucopyranosylthio)-3-(phenylamino) acrylohydrazide. (8f)

Yellow solid; yield 67%; m.p.200°C; IR (KBr cm^{-1}) 3208 (NH), 2189.43 (CN), 1755.76 (CO); ^{1}H NMR(500MHz δ ppm $CDCl_3$) 1.92-2.02 (4s, 12H, 4 × CH_3CO), 2.49 (s, 1H, CH), 3.34 (s, 2H, 6`-$H_2$), 3.61-3.71 (m, 2H, *J*=9.5 4`-H, 5`-H), 3.80 (s, 3H, $OCH_3$), 3.96-4.02 (m, 1H, *J*=9.4 3`-H), 4.80-4.89 (m, 1H, *J*=9.3 2`-H), 5.15-5.29 (m, 1H, *J*=9.1 1`-H), 6.99-8.39 (m, 9H, NH-C_6H_5, CH-C_6H_4), 11.30 (s,1H, CONH), 12.04 (s, 1H, NH-C_6H_5)^{13}CNMR (DMSO, d_6 δppm) 55.76 (OCH_3), 61.31 (CH_2, C-6`), 67.85 (C-4`), 69 (C-2`), 70 (C-3`), 75.01 (C-5`), 79.5 (C-1`),117.85 (CN), 129.18 -129.32 (2C_6H_5), 130.68 (C-2), 138.00 (C-3),169.57-170.36(4xCO) Anal.Calcd For. $C_{32}H_{34}N_4O_{10}S$ (682.65): C, 56.30; H, 5.02; N, 8.20. Found: C, 56.23; H, 5.00; N, 8.10.

(*2E*)-*N'*-(4-nitro-benzylidene)-2-cyano-3-(2`,3`,4`,6`-tetra-*O*-acetyl-*β*-D-glucopyranosylthio)-3-(phenylamino) acrylohydrazide. (8g)

Yellow solid; yield 65%; m.p.199 °C; IR (KBr cm^{-1}) 3341.07 (NH), 2197.49 (CN), 1752.98 (CO); ^{1}H NMR(500 MHz δ ppm $CDCl_3$) 1.93-2.03 (4s, 12H, 4 × CH_3CO), 2.50 (s, 1H, CH), 3.35 (s, 2H, 6`-$H_2$), 3.63-3.72 (m, 2H, *J*=9.55 4`-H, 5`-H), 3.98-4.03 (m, 1H, *J*=9.1 3`-H), 4.84-4.90 (m, 1H, 2`-H), 5.19-5.30 (m, 1H, 1`-H), 7.24-8.37 (m, 9H, NH-C_6H_5, CH-C_6H_4), 11.07-12.01 (s,2H, CONH, NH-C_6H_5), ^{13}C NMR (DMSO, d_6 δppm 61.81 (CH_2, C-6`), 67.84 (C-4`), 70.24 (C-2`), 73.14 (C-3`), 75.08 (C-5`), 83.84 (C-1`),117.85 (CN), 124.51–129.35 (2C_6H_5), 141.05 (C-3), 148.25 (C-1), 169.55-170.36 (4 × CO), Anal.Calcd For. $C_{31}H_{31}N_5O_{12}S$ (697.6): C, 53.37; H, 4.47; N, 10.03. Found: C, 53.58; H, 4.53; N,10.22.

(*2E*)-*N'*-benzylidene-2-cyano-3-(2`,3`,4`,6`-tetra-*O*-acetyl-*β*-D-galactopyranosylthio)-3-(phenylamino) acrylohydrazide. (8h)

Yellow solid; yield 71%; m.p. 110°C; IR (KBr cm^{-1}3439.42 (NH), 2200.38 (CN), 1749.12 (CO) ; ^{1}H NMR(500MHz δ ppm $CDCl_3$) 1.93-2.10 (4s, 12H, 4 × CH_3CO), 2.19 (s, 1H, CH), 3.35 (s, 2H, 6`-$H_2$), 4.07-4.18 (m, 2H, *J*=9.55 4`-H, 5`-H), 4.80-4.90 (m, 1H, *J*=9.5 3`-H), 4.99-5.17 (m, 1H, *J*=9.3 2`-H), 5.29-5.48 (d, 1H, *J*=9.55 1`-H), 7.21-7.89 (m, 10H, NH-C_6H_5, CH-C_6H_5), 11.5 (s,1H, CONH), 12.88 (s, 1H, NH-C_6H_5) ; Anal. Calcd For. $C_{31}H_{32}N_4O_{10}S$ (652.62): C, 57.05; H, 4.94; N, 8.58. Found: C, 57.34; H, 4.64; N, 8.32.

(*2E*)-*N'*-(2-methyl-benzylidene)-2-cyano-3-(2`,3`,4`,6`-tetra-*O*-acetyl-*β*-D-galactopyranosylthio)-3-(phenylamino) acrylohydrazide. (8i)

Yellow solid; yield 87%; m.p. 125°C ; IR (KBr cm^{-1}) 3254 (NH), 2201.35 (CN), 1750.08 (CO); ^{1}H NMR(500MHz δ ppm $CDCl_3$) 1.92-2.03 (4s, 12H, 4 × CH_3CO), 2.48 (s, 3H, CH_3), 3.66 (s, 1H, CH), 3.88 (s, 2H, 6`-$H_2$), 3.93-3.94 (m, 2H, *J*=9.55 4`-H, 5`-H), 4.70-4.72 (m, 1H, *J*=9.2 3`-H), 4.91 (m, 1H, *J*=9.3 2`-H), 5.15-5.32 (d, 1H, *J*=9.55 1`-H), 7.25-8.32 (m, 9H, NH-C_6H_5, CH-C_6H_4), 9.4 (s,1H, CONH), 12.6 (s, 1H, NH-C_6H_5); Anal. Calcd For. $C_{32}H_{34}N_4O_{10}S$ (666.65): C, 57.65; H, 5.14; N, 8.40. Found: C, 57.12; H, 5.04; N, 8.15.

(*2E*)-*N'*-(3-methyl-benzylidene)-2-cyano-3-(2`,3`,4`,6`-tetra-*O*-acetyl-*β*-D-galactopyranosylthio)-3-(phenylamino) acrylohydrazide (8j)

Yellow solid; yield 86%; m.p. 134°C; IR (KBr cm^{-1}) 3374.82 (NH), 2255.34 (CN), 1674.87 (CO) ^{1}H NMR(500 MHz δ ppm $CDCl_3$) 1.98-2.20 (4s, 12H, 4 x CH_3CO), 2.48 (s, 1H, CH), 2.88 (s, 3H, OCH_3), 3.89 (s, 2H, 6`-$H_2$), 4.03-4.13 (m, 2H, *J*=9.5 4`-H, 5`-H), 4.28-4.53 (m, 1H, *J*=9.55 3`-H), 5.07-5.14 (m, 1H, *J*=9.4 2`-H), 5.24-5.28 (m, 1H, *J*=9.2 1`-H), 7.12-7.51 (m, 9H, NH-C_6H_5, CH-C_6H_4), 10.27 (s,1H, CONH) ; Anal.Calcd For. $C_{32}H_{34}N_4O_{10}S$ (666.65): C, 57.65; H, 5.14; N, 8.40. Found: C, 57.66; H, 5.23; N, 8.76.

(*2E*)-*N'*-(2- methoxy-benzylidene)-2-cyano-3-(2`,3`,4`,6`-tetra-*O*-acetyl -*β*-D-galactopyranosylthio)-3-(phenylamino) acrylohydrazide (8k)

White solid; yield 88%; m.p. 120°C; IR (KBr cm^{-1}) 3238.86 (NH), 2200.38 (CN), 1752..01 (CO) ; ^{1}H NMR(500MHz δ ppm $CDCl_3$) 1.94-1.96 (4s, 12H, 4 x CH_3CO), 2.46 (s, 1H, CH), 3.36 (s, 2H, 6`-$H_2$), 3.51 (s, 3H, $OCH_3$) 3.98-4.13 (m, 2H, *J*=9.5 4`-H, 5`-H), 4.74-4.85 (m, 1H, *J*=9.3 3`-H), 4.88-4.95 (m, 1H, *J*=9.5 2`-H), 5.22 (m, 1H, *J*=9.21`-H), 7.29-7.44 (m, 9H, NH-C_6H_5, CH-C_6H_4) , Anal.Calcd For. $C_{32}H_{34}N_4O_{10}S$ (682.65): C, 56.30; H, 5.02; N, 8.20. Found: C, 56.55; H, 5.34; N, 8.39.

(*2E*)-*N'*-(4-methoxy-benzylidene)-2-cyano-3-(2`,3`,4`,6`-tetra-*O*-acetyl-*β*-D-galactopyranosylthio)-3-(phenylamino) acrylohydrazide (8l)

Yellow solid; yield 85%; m.p. 195°C; IR (KBr cm^{-1}) 3198.36 (NH), 1956.43 (CN), 1704.76 (CO); ^{1}H NMR(500MHz δ ppm $CDCl_3$) 1.92-2.02 (4s, 12H, 4×CH_3CO), 2.49 (s, 1H, CH), 3.34 (s, 2H, *J*=9.55 6`-$H_2$), 3.61-3.71 (m, 2H, *J*=9.3 4`-H, 5`-H), 3.80 (s, 3H, $OCH_3$), 3.96-4.02 (m, 1H, *J*=9.5 3`-H), 4.80-4.89 (m, 1H, *J*=9.55 2`-H), 5.15-5.29 (m, 1H, *J*=9.2 1`-H), 6.99-8.39 (m, 9H, NH-C_6H_5, CH-C_6H_4), 11.30 (s,1H, CONH), 12.04 (s, 1H, NH-C_6H_5);Anal.Calcd For. $C_{32}H_{34}N_4O_{10}S$ (682.65): C, 56.30; H, 5.02; N, 8.20. Found: C, 56.67; H, 5.40; N, 8.48.

(*2E*)-*N'*-(4-nitro-benzylidene)-2-cyano-3-(2`,3`,4`,6`-tetra-*O*-acetyl-*β*-D-galactopyranosylthio)-3-(phenylamino) acrylohydrazide (8m)

Yellow solid; yield 86%; m.p.188°C; IR (KBr cm^{-1} 3483.78 (NH), 2193.63 (CN), 1753.94 (CO); ^{1}H NMR(500MHz δ ppm $CDCl_3$) 1.90-2.07 (4s, 12H, 4 × CH_3CO), 2.52 (s, 1H, CH), 3.35 (s, 2H, *J*=9.55 6`-$H_2$), 3.86 (m, 2H, *J*=9.3 4`-H, 5`-H), 3.88 (m, 1H, *J*=9.5 3`-H), 4.11 (s, 1H, 2`-H), 5.22-5.23 (m, 1H, *J*=9.55 1`-H), 7.20-8.30 (m, 9H, NH-C_6H_5, CH-C_6H_4), 11.65 (s,2H, CONH, NH-C_6H_5) ,^{13}C NMR (DMSO, d_6 δppm 61.39 (CH_2, C-6`), 67.29 (C-4`), 67.62 (C-2`), 71.20 (C-3`), 75.08 (C-5`),114.05 (CN), 124.48-29.37 (2C_6H_5), 141.11 (C-3), 148.22 (C-1),169.78-170.29 (4 x CO) Anal.Calcd For. $C_{31}H_{31}N_5O_{12}S$ (697.6): C, 53.37; H, 4.47; N, 10.03. Found: C, 53.23; H, 4.80; N,10.33

(*2E*)-*N'*-benzylidene-2-cyano-3-(methylthio)-3-(phenylamino) acrylohydrazide (9)

General procedure: A mixture of N-substituted Arylidenyl acrylohydrazide derivatives 1a–d (0.01 mol) and phenyl isothiocyanate (0.01 mol) was stirred for 10 to 20 min in ethanol (25 ml) containing

potassium hydroxide (0.01 mol). After cooling, then methyl iodide (0.01 mol) added at room temperature and stirred for 16 hour. The solution was evaporated and the formed residue was washed with distilled water to remove the formed potassium bromide salt. The resulting product was recrystallized from ethanol [8].

(*E*)-5-amino-*N*'-benzylidene-3-(phenylamino)-*1H*-pyrazole-4-carbohydrazide (10)

General procedures: A mixture of compounds 8 or 9 (0.01 mol) and hydrazine hydrate 99% (0.01 mol) in ethanol (30 ml) was refluxed for two hours. The resulting solid product was collected by filtration and recrystallized from ethanol to give the title compound 10 in 74%-78% or 50%-55% yield respectively [8].

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