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PII: S0040-4039(14)01828-0
DOI: http://dx.doi.org/10.1016/j.tetlet.2014.10.122
Reference: TETL 45351

To appear in: *Tetrahedron Letters*

Received Date: 26 August 2014
Revised Date: 8 October 2014
Accepted Date: 22 October 2014

Please cite this article as: Kothayer, H., Morelli, M., Brahemi, G., Elshanawani, A.A., Abu Kull, M.E., El-Sabbagh, O.I., Shekhar, M.P.V., Westwell, A.D., Optimized synthesis of diamino-triazinylmethyl benzoates as inhibitors of Rad6B ubiquitin conjugating enzyme, *Tetrahedron Letters* (2014), doi: http://dx.doi.org/10.1016/j.tetlet.2014.10.122

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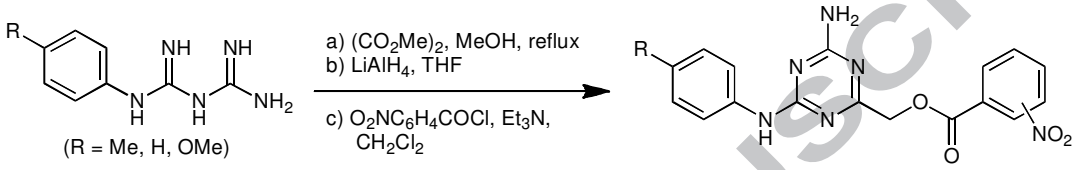

Tetrahedron Letters
journal homepage: www.elsevier.com

Optimized synthesis of diamino-triazinylmethyl benzoates as inhibitors of Rad6B ubiquitin conjugating enzyme

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ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
Triazines
Biguanides
Rad6B inhibitors
Antitumour agents

ABSTRACT

Recently, we have identified the first inhibitors of Rad6B, an E2 enzyme essential for post-replication DNA repair and a potential new drug target for treatment of breast cancer. We report two newly optimized synthetic routes to our [4-amino-6-(phenylamino)-1,3,5-triazin-2-yl]methyl 4-nitrobenzoate target compounds **TZ8** and **TZ9** with general applicability to further structure-activity relationship studies around this pharmacophore. The key step involved the condensation/cyclisation between a phenylbiguanide and either ethyl bromoacetate or dimethyloxalate in good yield.

The highly regulated ubiquitin-proteasome system responsible for degradation of >80% of cellular proteins, has proved to be a popular target for the development of new anticancer agents in recent years. Research in this field has culminated in the clinical approval of bortezomib (Velcade®) as the first-in-class small molecule proteasome inhibitor for treatment of relapsed multiple myeloma and mantle cell lymphoma.[1] A major function of the protein ubiquitination system serves to polyubiquitinate (tag with small proteins) cellular proteins destined for proteasomal degradation,[2] however the role of ubiquitination in other important cellular functions such as cell signalling and DNA repair is now becoming more widely appreciated.[3] Three successive classes of enzymes of increasing structural and mechanistic class diversity mediate protein ubiquitination. The process starts with the ubiquitin-activating enzyme (E1) that forms a thioester bond between its active site cysteine and the carboxyl terminus of the ubiquitin protein. Transfer of ubiquitin to the family of thioester-linked E2 ubiquitin conjugating enzymes results in either direct transfer of ubiquitin to the protein substrate, or interaction with the larger family of E3 ubiquitin-protein ligases resulting in substrate mono- or poly-ubiquitination.

Extensive efforts in recent years to target various classes of E3 ubiquitin ligases deregulated in cancer cells has led to the development of a number of potential cancer therapeutics. Amongst the most well developed examples are the Mdm2 (E3 ligase)–p53 protein-protein interaction inhibitors known as the Nutlins, currently being studied in clinical trials for cancer.[4] A further example is provided by the disulfiram-based inhibitors of the E3 ligase enzyme BCA2 (Breast Cancer Associated protein-2) discovered in our laboratories.[5]

Inhibitors of E2 ubiquitin conjugating enzymes are less well studied than their E3 counterparts, despite their potential as cancer drug targets in a number of cases. For example, the E2 enzyme Rad6 has been found to be essential for post-replication DNA repair, and Rad6B over-expression is reported in breast cancer cell lines and tumours. Constitutive Rad6B over-expression in non-transformed cells is associated with induction of cancer phenotypic changes including centrosome amplification, abnormal mitosis and aneuploidy.[6] The ability of Rad6B to ubiquitinate β-catenin leads to conjugates insensitive to proteasomal degradation. Consequent stabilization and activation of oncogenic β-catenin provides further evidence of the therapeutic potential of Rad6B as a drug target, particularly in breast cancer.[7]

An important therapeutic advance in this area was the recent report from our laboratories of the first selective inhibitors of Rad6 ubiquitin conjugating enzyme.[8] Virtual screening of a library of drug-like structures against a pharmacophore model generated from the conserved key residues stabilizing the E2-ubiquitin thioester intermediate, identified a substituted diamino-triazine core structure as a starting point for analogue synthesis. Triazine analogue synthesis coupled to *in vitro* anticancer evaluation in Rad6B-relevant models led to the identification of

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[4-amino-6-(phenylamino)-1,3,5-triazin-2-yl]methyl 4-nitrobenzoates **TZ8** and **TZ9** as novel and selective Rad6B-inhibitory anticancer lead compounds. **TZ8** and **TZ9** were found to inhibit proliferation, colony formation and migration, leading to G_2-M cell cycle arrest and apoptosis in MDA-MB-231 breast cancer cells.

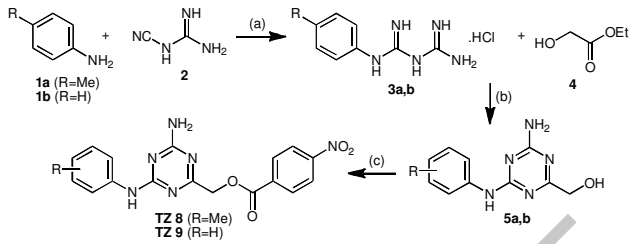
The trisubstituted 1,3,5-triazine scaffold plays an important role in medicinal chemistry, since a number of triazine-based compounds are reported to possess useful biological properties. Biologically relevant trisubstituted triazines include the antigastric ulcer agent irsogladine,[9] commonly used in Japan and also shown to possess anti-angiogenic/antimetastatic activity;[10] plus other agents with anticancer,[11] antimalarial,[12] antimicrobial[13] and anti-angiogenic[14] properties.

There are two major conceptual approaches used for the synthesis of trisubstituted 1,3,5-triazines. The first main method involves the successive base-promoted nucleophilic substitution of chlorine atoms starting from commercially available cyanuric chloride (2,4,6-trichloro-1,3,5-triazine). Although widely used, this method has significant drawbacks mainly relating to the harsh conditions and low yields associated with nucleophilic substitution of the third chlorine atom to obtain the final trisubstituted product. The method has recently been extended to the synthesis of substituted triamino-triazine derivatives using an efficient microwave-promoted aryl and heteroaryl amination to install the third triazine substituent.[15] In the case of our synthetic triazine targets **TZ8** and **TZ9**, this approach was discounted due to the difficulty of forming the required carbon-carbon bond to the methyl benzoate substituent. Alkyl group substitution into triazines is often accomplished through the use of highly reactive Grignard reagents, which was not appropriate for our target compounds.

The second major approach to the synthesis of trisubstituted triazines involves construction of the triazine ring through condensation/cyclisation chemistry. For example, a common approach involves the reaction of substituted biguanides with carboxylic acid derivatives such as esters,[16] nitriles,[17] acyl chlorides or acid anhydrides. This method has been used for example in the microwave-promoted synthesis of 2-(arylmethyl)amino-4-arylamino-6-alkyl-1,3,5-triazines via the reaction of phenylbiguanide derivatives and simple aliphatic esters.[16]

Further development of our trisubstituted triazine-based lead compounds was severely compromised by poor yielding and unreliable synthetic procedures. Following the discovery of the first selective E2 ubiquitin conjugating enzyme inhibitors of Rad6B, it was imperative to develop a general and reliable synthetic route to this target class of compounds. In this paper we report the discovery and development of two new synthetic approaches to Rad6B-inhibitory trisubstituted triazines, including new structural analogues.

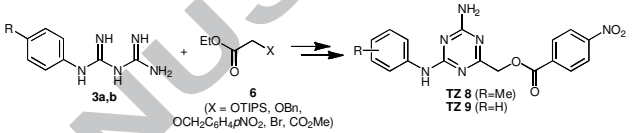
Our initial synthesis of Rad6-inhibitory triazines **TZ8** and **TZ9** was adapted from the method of Saczewski et al.,[11] and was accomplished in three chemical steps from commercially available starting materials as outlined in Scheme 1.[8a] Reaction of toluidine (**1a**) or aniline (**1b**) with dicyandiamide (**2**) with heating under acidic conditions (3 M HCl) gave the required phenylbiguanide hydrochlorides **3a,b** in high yields (84% and 73%, respectively).[18] Formation of trisubstituted triazines by reaction of biguanides **3a,b** with ethyl glycolate (**4**) to give the {4-amino-6-(phenylamino)-[1,3,5]triazin-2-yl}methanol intermediates **5a,b** proved to be a low yielding and unreliable synthetic step, severely compromising the quantity of pure triazine intermediates **5a,b** that could be obtained for conversion into final compounds for anticancer testing. Following extensive chromatographic purification, reaction of intermediates **5a,b** with 4-nitrobenzoyl chloride under basic conditions provided the required target triazines **TZ8** and **TZ9**.



[a]Reagents and conditions: (a) 3 M HCl (aq), 90 °C, 5h; (b) NaOEt, EtOH, reflux, 3 h; (c) 4-nitrobenzoyl chloride, Et_3N, CH_2Cl_2, reflux, 12 h.

Scheme 1. Initial synthetic approach to substituted diamino-s-triazinylmethyl benzoates **TZ8** and **TZ9**.

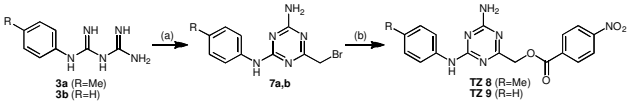
The reagent, ethyl glycolate, was considered to be the main reason for the complex mixture of products arising from the second step of Scheme 1, since we postulated that the unprotected nucleophilic alcohol function would be capable of participating in side reactions (for example self-condensation) under the strongly basic conditions of the reaction. We therefore tested a number of related functionalized esters in the triazine ring-forming step. These alternative synthetic approaches are outlined in Scheme 2.



Scheme 2. Alternative synthetic approaches to diamino-s-triazinylmethyl benzoates, avoiding the use of ethyl glycolate.

Initial studies were focused on protection of ethyl glycolate using an alcohol protecting group (**6**; X = OTIPS, OBn, $OCH_2C_6H_4p$-NO_2) that could later be readily removed to reveal the {4-amino-6-(phenylamino)-[1,3,5]triazin-2-yl}methanol intermediates **5a,b** for conversion into the final products. The use of the triisopropylsilyl (TIPS) or benzyl (Bn) protecting groups was found not to be successful, with both these protecting groups being unstable under the basic conditions of the triazine cyclisation reaction (sodium ethoxide in ethanol under reflux), and giving rise to complex product mixtures. The *p*-nitrobenzyl derivative of ethyl glycolate was found to be unstable under the strongly basic conditions of triazine formation, meaning that potential direct access to final triazine products **TZ8** and **TZ9** could not be realised.

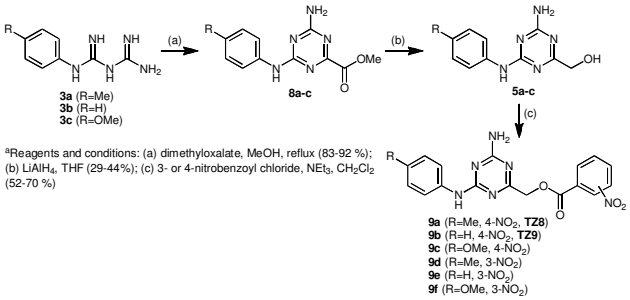
A more successful approach to the target trisubstituted triazines involved the use of ethyl bromoacetate (**6**; X=Br) as the biguanide cyclisation partner. For these reactions, we found that it was necessary to use the free base of phenylbiguanide, obtained by treatment of the hydrochloride salt with 25% sodium methoxide in methanol, to give the best results (see Supplementary Data). Reaction of the phenylbiguanide (**3a,b**) free bases with ethyl bromoacetate in methanol gave the required 6-(bromomethyl)-*N*2-aryl-1,3,5-triazine-2,4-diamines **7a,b** in moderate yields (39-43%) following chromatographic purification (Scheme 3). Nucleophilic substitution of the bromo group using 4-nitrobenzoic acid promoted by tetrabutylammonium fluoride (TBAF) in refluxing THF gave rise to the target triazines **TZ8** and **TZ9** in high yields (79-86%) following column chromatography. Although this alternative synthetic route using ethyl bromoacetate as a key reagent was successful, the moderate yields for the triazine formation step encouraged us to search for alternative methods to deliver the target compounds in high yields and quantities sufficienty for full biological characterization.



[a]Reagents and conditions: (a) $BrCH_2COOEt$, MeOH (39-43 %); (b) 4-$NO_2C_6H_4COOH$, TBAF, THF, reflux (79-86 %)

Scheme 3. Synthetic route to diamino-s-triazinylmethyl benzoates using ethyl bromoacetate.

Further success in finding alternative synthetic routes to our target triazines was achieved using dimethyl oxalate as the biguanide reaction partner in place of the originally used ethyl glycolate, as outlined in Scheme 4. Heating a mixture of arylbiguanide free base **3a-c** with excess dimethyl oxalate gave the intermediate methyl 4-amino-6-(arylamino)-1,3,5-triazine-2-carboxylates **8a-c** in high yields (83-92%) following recrystallisation from methanol. Reduction of the intermediate triazine carboxylates **8a-c** using lithium aluminium hydride in anhydrous THF then gave the key {4-amino-6-(phenylamino)-[1,3,5]triazin-2-yl}methanol intermediates **5a-c** in moderate yields following purification. Finally, esterification of the hydroxymethyl intermediate using 3- or 4-nitrobenzoyl chlorides gave the target triazines **9a-f**, including **TZ8** and **TZ9**, in good yields following recrystallisation from methanol or acetonitrile.



Scheme 4. Synthetic route to diamino-*s*-triazinylmethyl benzoates **9a-e** using dimethyloxalate.

In conclusion, we have successfully developed two general and reliable synthetic routes to trisubstituted diamino-*s*-triazines bearing carbon-based substituents (methyl benzoates). This development work facilitates the availability of lead compounds **TZ8** and **TZ9** in sufficient quantities and purity for further development, and provides a general route of access to new compounds bearing this pharmacophore. The generality of the new synthetic route is further illustrated by the preparation of new analogues **9c-f** for structure-activity relationship studies. Further work on related trisubstituted *s*-triazines is continuing to further elaborate the potential therapeutic activity of this novel class of compounds.

Acknowledgments

The authors are grateful to the Higher Education Ministry of the Arab Republic of Egypt for the award of a Channel Scholarship (to H.K.). We acknowledge the support of the Algerian Consulate (PhD studentship to G.B.). We thank Miss Frances Harwood (MPharm student, Cardiff University) for preliminary chemistry studies, and the EPSRC National Mass Spectrometry Facility (Swansea, U.K.) for provision of accurate mass spectrometric analysis.

Supplementary data

Supplementary data (experimental procedures and characterization data, including NMR and mass spectrometry data for all isolated compounds) associated with this article can be found in the online version.

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