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# Synthesis and biological activity of simplified denoviose-coumarins related to novobiocin as potent inhibitors of heat-shock protein 90 (hsp90)

Christine Radanyi,[b] Gaëlle Le Bras,[a] Samir Messaoudi,[a] Céline Bouclier,[b] Jean-François Peyrat,[a] Jean-Daniel Brion,[a] Véronique Marsaud,[b] Jack-Michel Renoir[b] and Mouâd Alami[a,*]

[a]*Univ Paris-Sud, CNRS, BioCIS-UMR 8076, Laboratoire de Chimie Thérapeutique, Faculté de Pharmacie, IFR 141, 5 rue J.-B. Clément, Châtenay-Malabry F-92296, France*
[b]*Univ Paris-Sud, CNRS, UMR 8612, Laboratoire de Pharmacologie Cellulaire et Moléculaire des Anticancéreux, Faculté de Pharmacie, IFR 141, 5 rue J.-B. Clément, Châtenay-Malabry F-92296, France*

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**Abstract**—A new series of coumarin inhibitors of hsp90 lacking the noviose moiety as well as substituents on C-7 and C-8 positions of the aromatic ring was synthesised and their hsp90 inhibitory activity has been delineated: for example, their capacity to induce the degradation of client proteins and to inhibit estradiol-induced transcription in human breast cancer cells. In cell proliferation assay, the most active compound **5g** was ~8 times more potent than the parent novobiocin natural compound.


The aminocoumarins novobiocin and clorobiocin are well-established antibiotics agents that act as inhibitors of the bacterial ATP binding gyrase B, a type II DNA topoisomerase.[1] The natural product novobiocin (Nvb) has attracted renewed attention because of its antitumour activity.[2] The mechanism of action of novobiocin was found to inhibit the heat-shock proteins 90 (hsp90) via interaction with a previously unrecognized C-terminal ATP binding site.[3] This ATP-dependent molecular chaperone is well known to be involved in the folding of many client proteins including, among others, kinases (Akt, Raf-1, Her-2, cdk-4), transcription factors like steroid receptors and p53, that are directly associated with all six hallmarks of cancer.[4] Inhibition of hsp90 by Nvb induced the degradation of the hsp90 client proteins via the ubiquitin–proteasome pathway.[2] Consequently, hsp90 has evolved as an exciting new target in cancer drug discovery.[5]

Unfortunately, the ability of Nvb to induce degradation of hsp90 client protein (e.g., ErbB2 in SkBr3 breast cancer)[2] is relatively weak (700 μM) and requires further investigation to provide more potent compounds with improved pharmaceutical properties. Among the most active analogues, Blagg et al.[6] highlighted the crucial role of the noviose moiety at the 7-position of the coumarin ring for the biological activity, whereas the 4-hydroxy and 8-methyl groups in Nvb are not indispensable. In more recent studies,[7] 3′-descarbamoyl-4-deshydroxynovobiocin DHN2 proved to be a more effective and selective hsp90 inhibitor (Fig. 1).

As part of our research devoted to nonsugar coumarin analogues that target hsp90, we recently showed that the removal of the noviose moiety together with the introduction of a tosyl substituent at C-4 coumarins provides 4-tosylcyclonovobiocic acid (4TCNA) as a lead compound having a C-7 free-phenolic function and a C-8 methyl group.[8] Biological evaluation of 4TCNA revealed higher potency than Nvb itself to induce client-protein degradation. In our continuing structure–activity relationship (SAR) study, our aim was to determine what modifications are necessary to selectively provide more potent nonsugar coumarin com-

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* Corresponding author. Tel.: +33 146 83 58 87; fax: +33 146 83 58 28; e-mail: mouad.alami@u-psud.fr

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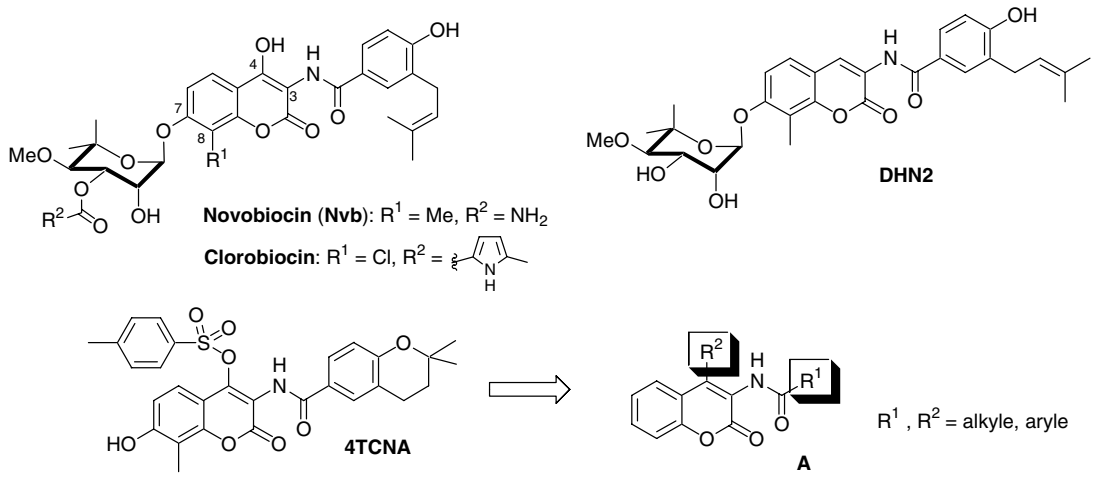
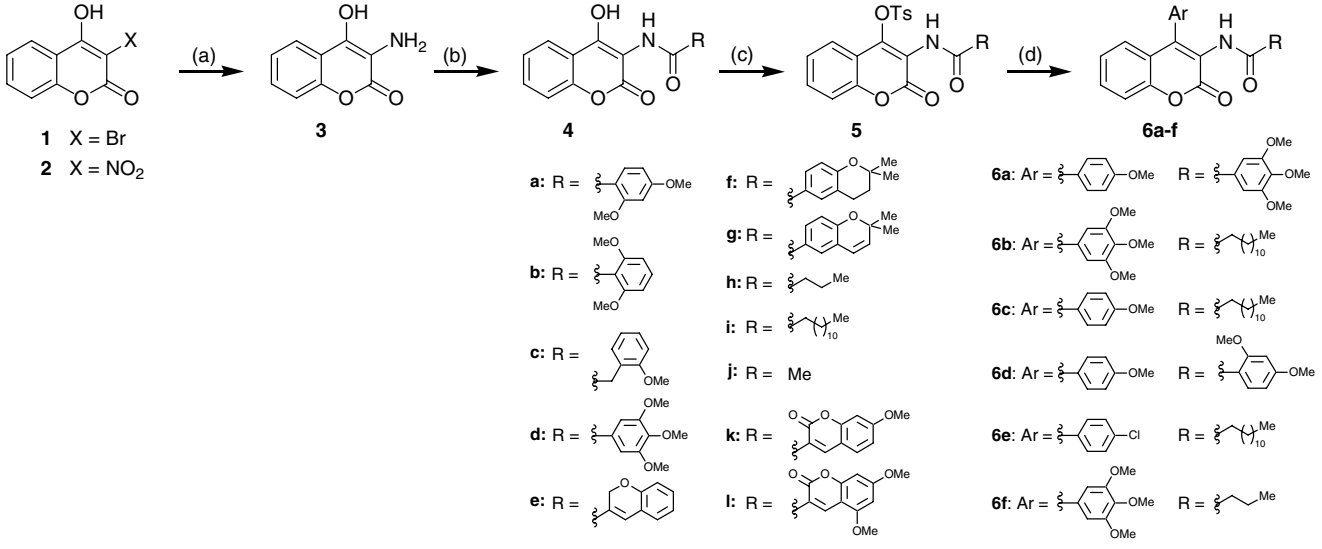



**Figure 1.** Hsp90 inhibitors and general structure **A** of the synthesised compounds.

pounds that target hsp90. Herein we report the synthesis of a simplified scaffold 3-(N-substituted) aminocoumarin of type **A** (Fig. 1), which includes two centres for introduction of diversity into coumarin molecule and disclose the impact of the absence of the 7-hydroxy moiety and the 8-methyl groups on the hsp90 inhibitory activity. The potencies of newly synthesised coumarins were evaluated for their capacity to inhibit estradiol ($E_2$)-induced transcription in human breast cancer cells, to induce the proteasome-mediated degradation of several known hsp90 client proteins, including Raf-1, the estrogen receptor α (ERα) and the progesterone receptors A and B (PRA, PRB).

Initially, the synthesis of 3-aminocoumarin derivatives **4** was examined by the palladium-catalysed C–N bond coupling reaction of known 3-bromo-4-hydroxy-coumarin **1** with a series of amide nucleophiles as we previously described.[9] All our attempts to react **1** with 2,4-dimethoxybenzamide to provide directly **4a** using various combinations of Pd/L/base mixtures (e.g., $Pd(OAc)_2$, $Pd_2(dba)_3$/Xantphos, BINAP, Xphos, dppf/$Cs_2CO_3$, $K_3PO_4$, $K_2CO_3$, etc.) resulted unfortunately in unsuccessful results, presumably for steric considerations. Therefore, the requisite aminocoumarins of general structure **A** were prepared by the route shown in Scheme 1. The synthesis was initiated with the reduction of the nitro function of commercially available 3-nitro-4-hydroxycoumarin **2** under Pd/C-catalysed hydrogenation to afford the corresponding 3-amino-4-hydroxycoumarin **3** in 84% yield. Further coupling with a series of aryl and alkyl carboxylic acids in the presence of PyBOP and DIEA in DMF at room temperature gave 3-acylaminocoumarin derivatives **4a**–**l** in moderate to good yields (44–91%). Because of the strong insolubility derivatives **4k** and **4l** in the usual organic solvents, their biological activity could not be delineated.




**Scheme 1.** Reagents and conditions: (a) $H_2$, Pd/C, MeOH, HCl (1%), 3 h, rt, 84%; (b) $RCO_2H$ (1.2 equiv), PyBOP (1 equiv), DIEA (3 equiv), DMF, 18 h, rt; (c) TsCl (2 equiv), pyridine, 16 h, rt; (d) $ArB(OH)_2$ (1.3 equiv), $K_3PO_4$ (3 equiv), $Bu_4NBr$ (10 mol %), $PdCl_2dppf$ (5 mol %), MeCN, 80 °C, overnight.

In order to evaluate the importance of the free 4-hydroxyl group in derivatives **4**, we planned to introduce an aryl substituent by metal-catalysed cross coupling reaction. For this purpose, the use of aryl tosylates as electrophiles for the Suzuki-type coupling is a very attractive procedure.[10] The synthesis of the required 4-tosylcoumarins **5** was achieved from coumarins **4** using tosyl chloride in the presence of pyridine as a solvent at room temperature. Compounds **5** were then submitted to cross-coupling with various arylboronic acids under our previously best conditions ($K_3PO_4$, $PdCl_2$(dppf), $Bu_4NBr$, MeCN, 80 °C).[8] As expected, the corresponding coupling products **6** were obtained in good yields (52–96%).

We had previously established that 4TCNA inhibits estradiol (E2)-induced transcription in MELN cells, and targets several hsp90-client proteins including ERα to proteasome-mediated degradation. In addition, 4TCNA promotes apoptosis through activation of caspases 7 and 8 in ER-positive MCF-7 human breast cancer cells. With Nvb and 4TCNA in hands as reference compounds, in a large screening experiment, we found that among all the novobiocin analogues from the three series **4**–**6**, compounds **4b**, **4c**, **4j**, **4k**, **5e**, **5g**, **5i**, **5h** and **6c** were able to decrease the levels of ERα and Raf-1, whereas compounds **4f** and **5d** were able to induce the loss of only ERα without affecting Raf-1 (Fig. 2A). The activity of all these compounds was inhibited by the proteasome-inhibitor MG-132, except that of **6c** (not shown). Other derivatives proved to be inactive. These data suggest that different analogues may affect selectively various hsp-90/client protein complexes leading to depletion of different key signalling proteins. As shown in Figure 2A, compound **6c** induced the degradation of other proteins reflecting a strong toxicity likely unrelated to hsp90 inhibition and was not further evaluated. In dose–response analysis (Fig. 2B) compound **5g** revealed to be the strongest inhibitor since processing of ERα, Raf-1 and both progesterone receptors (PRB and PRA) occurred over a range of 40–100 μM .

We further assessed the potency of these novobiocin analogues to affect transcription in MELN cells. As shown in Figure 3, compound **4g** enhances both the basal and the $E_2$-induced transcription, whereas **4h**, **5d**, **5c**, **5i**, **5e**, **5h** and **5g** inhibit the $E_2$-induced Luciferase (LUC) expression. Surprisingly, almost all compounds

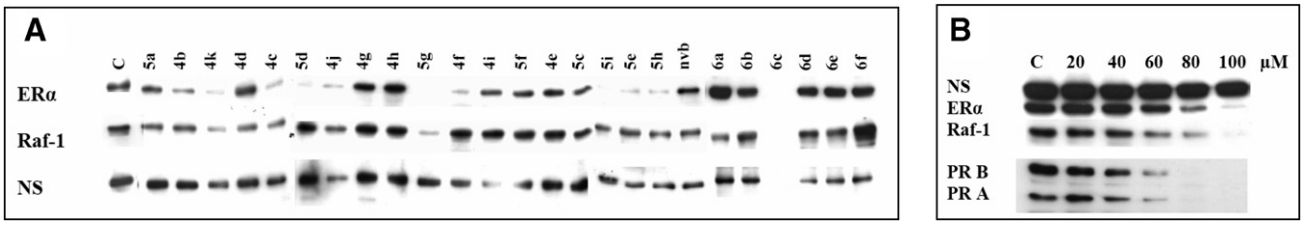



**Figure 2.** Stability of hsp90 client proteins in the presence of various novobiocin analogues. (A) MCF-7 breast cancer cells were exposed to 100 μM Nvb or analogues for 16 h and cell lysates were analyzed by Western blotting with regard to the levels of ERα and Raf-1. (B) In addition to detection of these two hsp90 client proteins, PRA and PRB fate was analyzed in cell lysates following exposure of MCF-7 cells to increasing amounts of **5g**. Primary antibodies were: ERα (HC20), Raf-1 (C12) from Santa Cruz; PR (Ab-8) from Labvision Corp., all used at 1 μg/mL. Complexes were detected with peroxidase-labelled secondary antibody. C, control cell lysate from cell exposed to DMSO. The visualization of a nonspecific (NS) band served as constant protein loading.

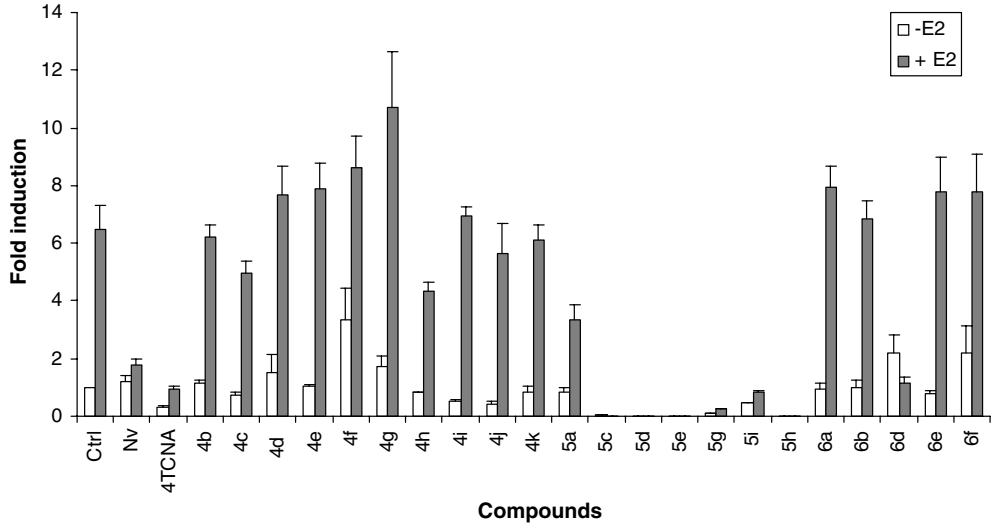



**Figure 3.** Effects of novobiocin analogues on ER-mediated transcription in MELN breast cancer cells. Cells were grown and treated for 18 h as described before[8] with 200 μM Nvb and analogues in the presence (+) or not (−) of 0.1 nM $E_2$. LUC activity was measured and expressed as fold induction relative to untreated control cells.

**Table 1.** Anti-proliferative activity of the most active inhibitors

| Compound | $IC_{50}$[a] (μM) |
|---|---|
| **5d** | 40 (±6) |
| **5g** | 35 (±3) |
| **5i** | 50 (±5) |
| **5e** | 70 (±4) |
| **5h** | 75 (±4) |
| 4TCNA | 45 (±8) |
| Nvb | 260 (±15) |

[a] Values are means of three experiments, carried out in sextuplate and standard deviation is given in parentheses.

of series **5** having a tosyl substituent on the C-4 position of the coumarin nucleus, behave as stronger inhibitors of ER-mediated transcription, except compound **5a**, as compared to Nvb.

The in vitro activity of these derivatives was further evaluated by their growth-inhibitory potency in MCF-7 cells. The quantification of cell survival in MCF-7 cells (initially 5000 cells/well of a 96-well plate, exposed after 24 h to serial dilution of the different analogues for 72 h) was established by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT, Sigma). The experiment was conducted as described before[8] and the $IC_{50}$ values were measured as the drug concentration that inhibits the cell growth by 50% compared with growth of vehicle-treated cells. As shown in Table 1, the $IC_{50}$ of the most active analogues was in the range of 35–75 μM not strongly different from that of our lead compound 4TCNA. Furthermore, all the analogues exhibited improved growth inhibition potentials as compared to the parent compound Nvb. Consistent with the degradation of hsp90 client proteins, the most active inhibitors are those with the lowest $IC_{50}$.

In summary, results from these studies demonstrate that the newly synthesised novobiocin analogues, based on a simplified 3-aminocoumarin scaffold exhibited increased inhibitory activity against the hsp90 protein folding process. In this SARs study, we highlighted in denoviose analogues **5**, bearing a tosyl group on the 4-position, that removal of C7/C8 substituents is not detrimental for hsp90 inhibitory activity and strongly enhances the capacity of the analogues to inhibit hsp90. Among these analogues, compound **5g** was identified to be the most potent representative of the new family of simplified coumarins. Our results suggest that **5g** and 4TCNA which exerted similar biological profile may be considered interesting compounds for the development of more potent novobiocin analogues.

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