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Research Letter

Synthesis of 5,6-dihydro-4*H*-benzo[*d*]isoxazol-7-one and 5,6-dihydro-4*H*-isoxazolo[5,4-*c*]pyridin-7-one Derivatives as Potential Hsp90 Inhibitors

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A novel class of 5,6-dihydro-4*H*-benzo[*d*]isoxazol-7-ones and 5,6-dihydro-4*H*-isoxazolo[5,4-*c*]pyridin-7-ones was designed, synthesized, and assayed to investigate the affinity toward Hsp90 protein. The synthetic route was based on a 1,3-dipolar cycloaddition of nitriloxides, generated *in situ* from suitable benzaldoximes, with 2-bromocyclohex-2-enones or 3-bromo-5,6-dihydro-1*H*-pyridin-2-ones. Whereas all the compounds bearing a benzamide group on the bicyclic scaffold were devoid of activity, the derivatives carrying a resorcinol-like fragment showed a remarkable inhibitory effect on Hsp90. Docking calculations were performed to investigate the orientation of the new compounds within the binding site of the enzyme.

Key words: anticancer, docking calculations, Hsp90 inhibitors, isoxazoles, synthesis

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Heat-shock protein 90 (Hsp90) is a molecular chaperone, which is essential for a wide range of protein assembly, trafficking, folding, and degradation processes (1). Multiple signal transduction pathways implicated in the regulation of cell proliferation and survival are dependent on Hsp90 (2). Several Hsp90 client proteins are involved in critical processes, including cell-cycle regulation and apoptosis. The heat-shock proteins are often overexpressed in tumor cells, and this supports their ability to survive under unfavorable stress conditions (e.g., hypoxia and acidosis), as well as to facilitate rapid somatic evolution (3).

The discovery and characterization of natural compounds inhibiting Hsp90, such as geldanamycin (GDA) and radicicol (Figure 1), has validated this molecular chaperone as a therapeutic target. GDA (4) inhibitory activity is mainly due to a competition with the ATP binding within the N-terminus of the protein. Radicicol (5), a natural macrocyclic antifungal antibiotic, inhibits Hsp90 by interacting within the same site of action of GDA. Due to its chemical instability, this compound could not be developed, but served as a template for the discovery of new Hsp90 inhibitors. In particular, the presence of a resorcinol-like fragment was found to be extremely important to drive its binding mode and to get a strong interaction with the enzyme. This mode of binding has also been verified with other synthetic series of compounds (6–8).

The investigation and clinical development of Hsp90 inhibitors continue to progress. Currently, a number of highly specific compounds are undergoing clinical trials (i.e. SNX5422, NVP-AUY922, and STA9090; Figure 1), and an impressive growth in scientific literature confirms the great interest toward this target (9). However, to date, there are still no FDA approved Hsp90-targeting agents. For all these reasons, the finding of novel chemotypes that fully satisfy requisites of safety and stability, moving forward the knowledge in this field, still remains an interesting and promising goal.

Previous reports supported the hypothesis that the presence of the isoxazole nucleus could exert a key role in the docking of compounds to the ATP binding site of the enzyme. In fact, synthetic compounds containing this heterocyclic moiety have shown potent and selective inhibition of Hsp90, see NVP-AUY922 and SST0116CL1 (6,10–12).

Recently, a structural investigation on the isoxazole scaffold led us to discover a new class of 4,5,6,7-tetrahydroisoxazolo-[4,5-*c*]pyridines containing an isoxazole nucleus fused with a tetrahydropyridine ring (13). Other structures described in recent papers, containing condensed bicyclic groups, have been very successful in targeting Hsp90 (7,8,14). Thus, we envisaged that the isoxazole scaffold could be fused to other rings to build novel series of potential Hsp90 inhibitors with a bicyclic core structure.

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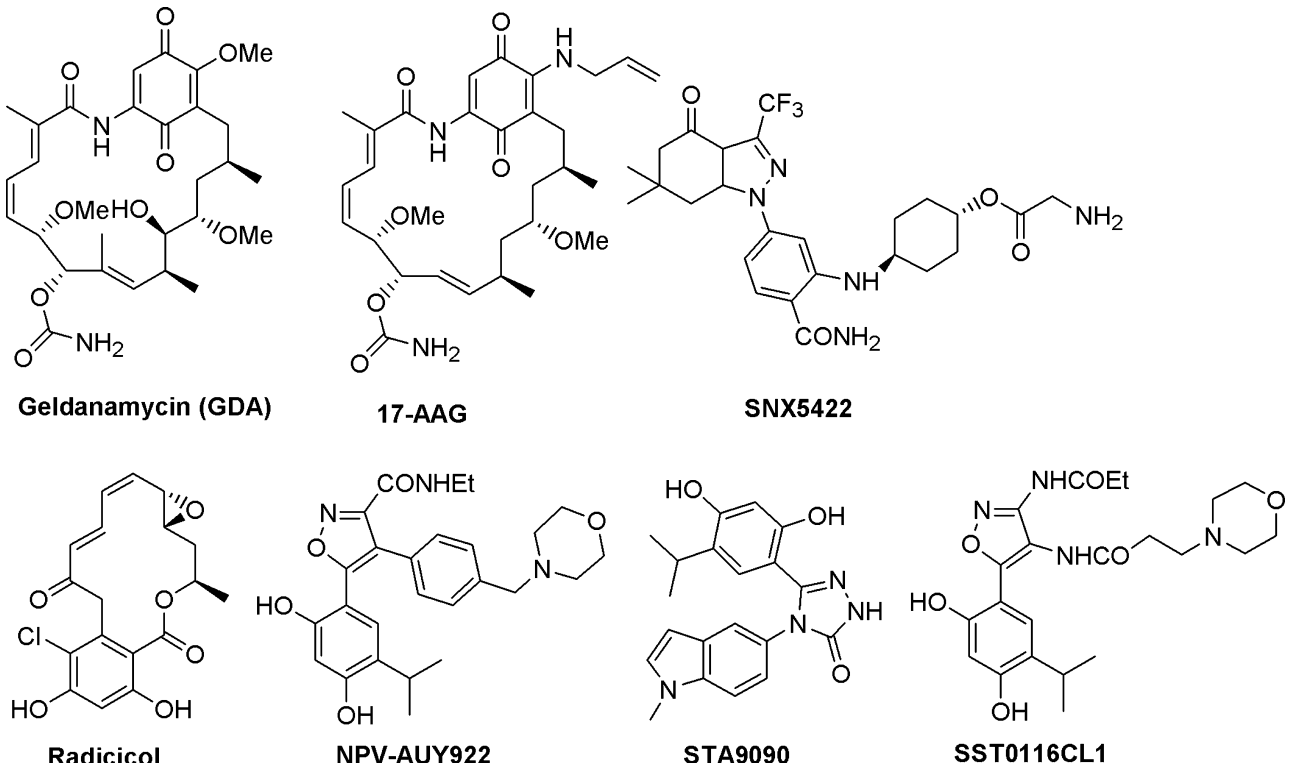
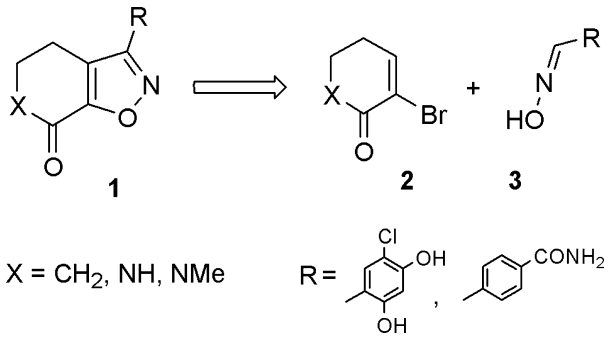


Figure 1: Hsp90 inhibitors.

Based on preliminary computational studies on isoxazole-based molecules using Hsp90 X-ray structure, we selected compounds with a 5,6-dihydro-4*H*-benzo[*d*]isoxazol-7-one (**1,** X = CH_2, Scheme 1) or a 5,6-dihydro-4*H*-isoxazolo [5,4-*c*]pyridin-7-one (**1,** X = NH, Scheme 1) scaffold as starting points for further investigation. Our exploration was focused on the expansion of the core structure within the ATP binding site, by adding groups aimed at improving the fitting to the pocket. In particular, we planned to link the bicyclic system either to a resorcinol-like group or to a primary benzamide moiety, being both these fragments able to confer tight binding into the ATP binding pocket (15).

The synthetic route used for the preparation of compounds **1** was based on a 1,3-dipolar cycloaddition of nitriloxides, generated *in situ* from suitable benzaldoximes to 2-bromocyclohex-2-enones or 3-bromo-5,6-dihydro-1*H*-pyridin-2-ones. (Scheme 1).

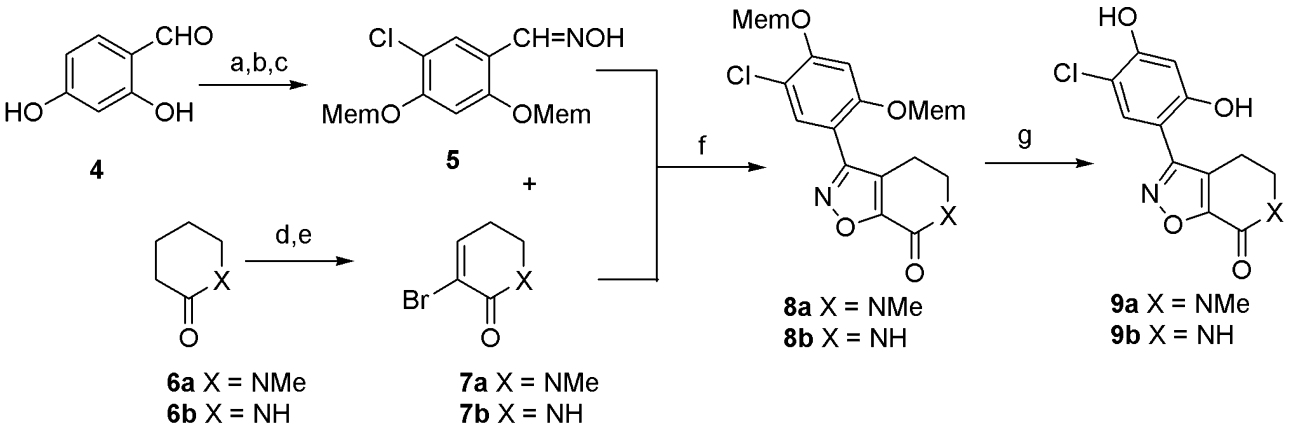


Scheme 1: Retrosynthetic approach to compound **1**.

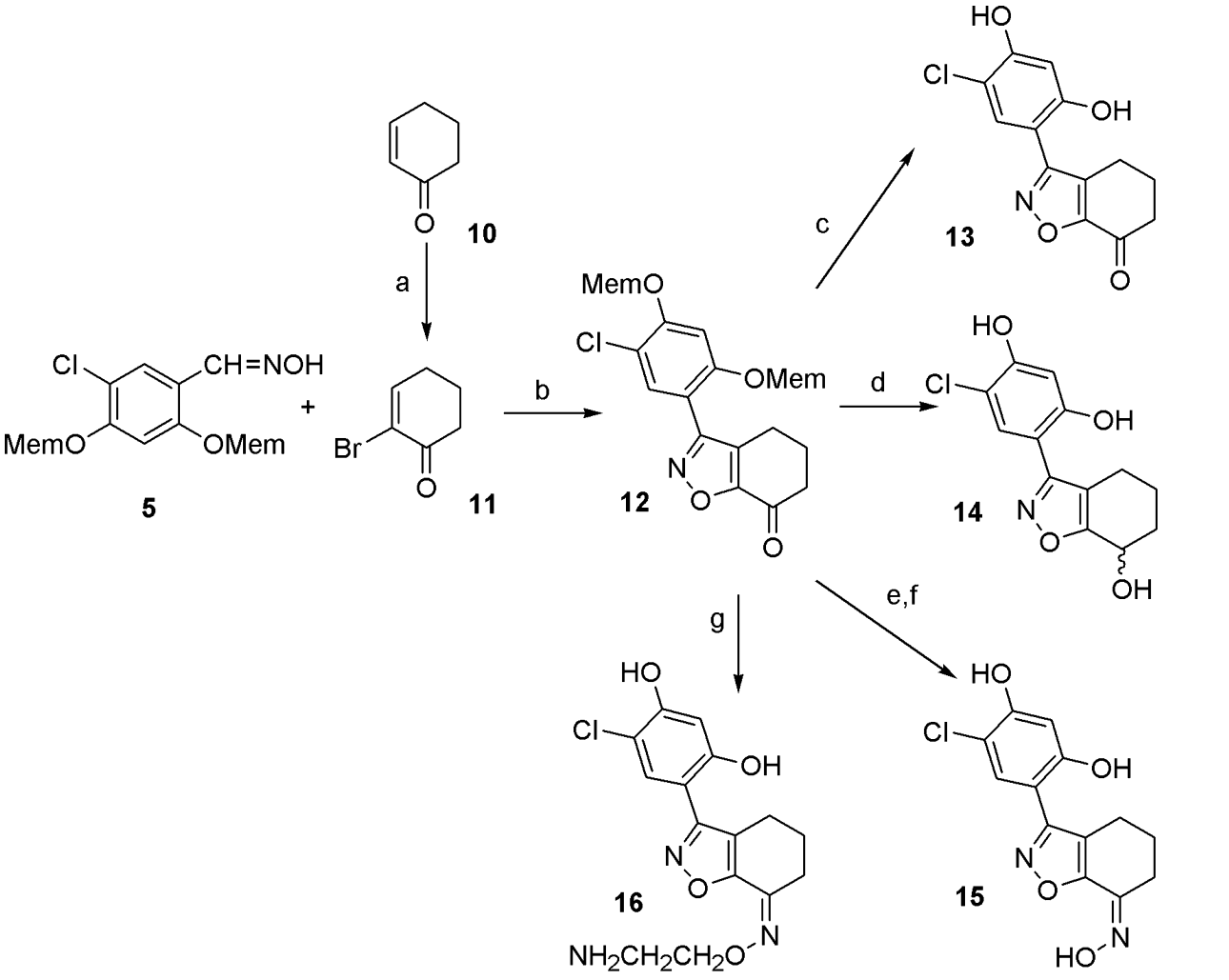
Literature reports (16) show that cycloaddition of arylnitriloxides to cyclohexenones affords 4-acylisoxazolines. Similarly, cycloaddition to α,β-unsaturated lactams affords mainly 4-carboxamidoisoxazolines with high regioselectivity (17). Thus, to reverse the regiochemistry of the reaction, we planned to use lactams and ketones with a bromine atom in alpha position with respect to the carbonyl group. Following this strategy, the isoxazole could be obtained in one step, due to the spontaneous isoxazoline dehydrobromination.

The key fragment **5** was obtained in three steps, starting from commercially available 2,4-dihydroxybenzaldehyde **4** (Scheme 2). Chlorination of **4** with NCS, protection of the phenol groups with 2-methoxyethoxymethyl chloride, followed by reaction with hydroxylamine hydrochloride in ethanol, in the presence of pyridine, gave the oxime **5**.

Compound **7a** was obtained starting from N-methylpiperidone, which was brominated to obtain 1-methyl-3,3-dibromo-2-piperidone (18). Dehydrobromination with $CaCO_3$ in DMF at 80 °C gave **7a** in good yield (19). Similarly, **7b** was obtained from δ-valerolactone (20). Cycloaddition of **5** to **7a** and **7b** was performed in dichloromethane at room temperature by generating the nitriloxide *in situ* with NCS



Scheme 2: Synthesis of compounds **9a**,**b**. Reagents and conditions: (a) NCS, $CHCl_3$, 6h, reflux, 89%; (b) 2-methoxyethoxymethyl chloride, iPr_2EtN, THF, 24 h, r.t.; (c) $NH_2OH{\cdot}HCl$, py, EtOH, 4h, reflux, 71%; (d) PCl_5, $ZnCl_2$, Br_2, $CHCl_3$, 0 °C, r.t., 54% from **6a** and 60% from **6b**; (e) $CaCO_3$, DMF, 80 °C, 88% for **7a** and 69% for **7b**; (f) NCS, Al_2O_3, CH_2Cl_2, r.t., 67% for **8a** and 57% for **8b**; (g) HCl (10%), CH_3OH, reflux, 43% for **9a** and 76% for **9b.**



Scheme 3: Synthesis of compounds **13-16**. Reagents and conditions: (a) Br_2, TEA, CH_2Cl_2, 0 °C then rt, 86%; (b) NCS, Al_2O_3, CH_2Cl_2, r.t., 49%; (c) HCl (10%), CH_3OH, reflux, 61%; (d) $CeCl_3{\cdot}7H_2O$, $NaBH_4$, CH_3OH, HCl (10%), 55%; (e) $NH_2OH{\cdot}HCl$, py, EtOH, reflux; (f) TFA, CH_2Cl_2, 75%; (g) $NH_2OCH_2CH_2NH_2$ HCl, py, EtOH, reflux, 44%.

and Al_2O_3, to obtain **8a** and **8b**, respectively (21). Finally, deprotection of the phenol groups gave compounds **9a-b**.

Cycloaddition of **5** with **11**, on its turn obtained from cyclohexen-2-one (22), afforded **12**, which was deprotected by 10% HCl to give **13** (Scheme 3). To investigate the role of the carbonyl group of **13**, a series of analogs were prepared starting from the intermediate **12**. Treatment with $NaBH_4$ and $CeCl_3$, followed by HCl, gave the reduced compound **14**. Reaction of **12** with hydroxylamine hydrochloride or O-(2-aminoethylhydroxylamine) gave the oximes **15** and **16**, respectively.

A series of analogs with the benzamide moiety were prepared following the same synthetic strategy (Scheme 4). The 4-(hydroxyiminomethyl)benzonitrile **18** was prepared from 4-cyanobenzaldehyde **17** by reaction with hydroxylamine hydrochloride in ethanol, in the presence of pyridine (23). The cycloaddition reaction, performed following the conditions previously described, afforded **19** in 30% yield. The yield was increased to 38% when the 4-cyanobenzaldehye chlorooxime obtained reacting **19** with N-chlorosuccinimide in DMF was reacted with the dipolarophile **11** in the presence of $(Bu_3Sn)_2O$ at room temperature (24). Amide **20** was obtained by reaction of **19** with H_2O_2 and NaOH. Similarly to compound **12**, compound **20** was converted to oximes **21a-c** by reaction with suitable hydroxylamines, whereas compound **22** was obtained by reduction with $NaBH_4$, followed by treatment with H_2O_2 and NaOH.

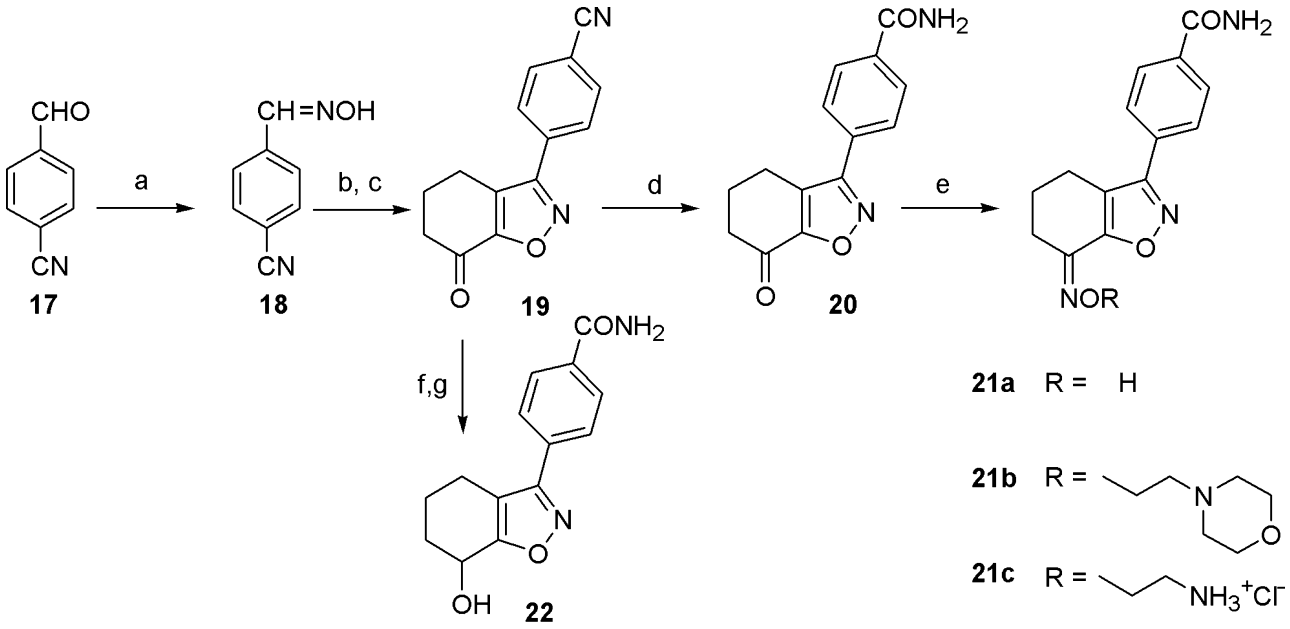
The binding affinity of these compounds to Hsp90 was determined by a fluorescence polarization (FP) assay, according to a protocol described previously (25). The results are summarized in Table 1.

The most disappointing result was the lack of activity showed by compounds carrying the benzamide moiety (**20**, **21a-c**, **22**). The compounds with a resorcinol-like moiety appeared more promising. In fact, almost all the tested molecules showed inhibitory activity with $IC_{50} < 10$ μM. Compound **15**, with an oxime group, showed a notable binding ability ($IC_{50} = 0.8$ μM). The reduction of the carbonyl group (as in **14**) caused a decrease of activity ($IC_{50} = 26$ μM), similarly to the introduction of a methyl group on the dihydropyridone moiety (**9b** versus **9a**).

According to the previous data, the compounds with the resorcinol fragment (**9a-b**, **13-16**) showed the most interesting profile, and two of them (namely, **9b** and **15**) had ability to bind Hsp90 comparable to or slightly better than that of the reference compound 17-AAG (1.6 and 0.8 μM versus 1.1 μM, respectively).

To gain a more precise picture of the interaction mode of these compounds with Hsp90, a computational protocol consisting in molecular docking calculations and energy minimization of the resulting complexes was set up. For this purpose, the structure of Hsp90 was taken from the crystallographic co-ordinates of its complex with NVP-AUY933 (PDB entry 2VCI) (6) and used as a template. Docking simulations and energy minimization were performed as previously described for other Hsp90 triazole inhibitors (26).

Analysis of the complexes resulting from docking calculations showed that the orientation of the new compounds



Scheme 4: Synthesis of compounds **21a-c** and **22**. Reagents and conditions: (a) $NH_2OH{\cdot}HCl$, py, EtOH, reflux, 71%; (b) NCS, CH_2Cl_2, r.t.; (c) 11, $(Bu_3Sn)_2O$, CH_2Cl_2, rt, 38%; (d) H_2O_2, 6N NaOH, EtOH, 57%; (e) $NH_2OR{\cdot}HCl$, py, EtOH, reflux, 58%; **21a:** 58%, **21b**: 41%, **21c**: 50%; (f) $CeCl_3{\cdot}7H_2O$, $NaBH_4$, CH_3OH, HCl (10%), 55%; (g) H_2O_2, 6N NaOH, EtOH, 94%.

Table 1: Binding affinity to Hsp90 of synthesized compounds

Compound	Hsp90 (FP) (IC_{50}, μM)
17-AAG	1.09 ± 0.05
9a	10.0 ± 0.8
9b	1.60 ± 0.04
13	4.8 ± 0.1
14	26 ± 1
15	0.8 ± 0.1
16	6.3 ± 0.1
20	>100
21a	>100
21b	>100
21c	>100
22	>100

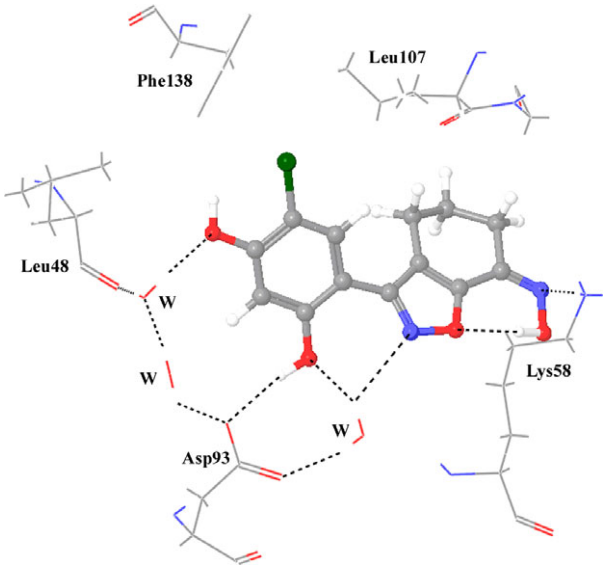


Figure 2: Graphical representation of the complex between Hsp90 and **15** (ball and stick notation) as derived from molecular docking calculations and energy minimization. The resorcinol hydroxy groups and the isoxazole nitrogen atom make hydrogen bond interactions mediated by water molecules (W). The carboxy terminus of Asp93 interacts directly with the *o*-hydroxy group of the ligand. The oxime OH group makes an intramolecular hydrogen bond with the heterocyclic oxygen, while the oxime nitrogen atom interacts by hydrogen bond with the terminal ammonium group of Lys58 side chain. Hydrophobic contacts between the chlorine substituent and the side chains of Phe138 and Leu107 are also found. For the sake of clarity, only few amino acid residues are displayed and labeled, while hydrogen bonds are depicted as dashed black lines.

9a-b, **13-16** within the binding site is very similar to that previously found for different Hsp90 inhibitors (26), with the resorcinol moiety deeply located within the cavity, while the remaining part of the molecule pointed toward the solvent (Figure 2). In further detail, the *o*-hydroxy group is involved in a direct and in a water-bridged (HOH2233, one of the four structural water molecules accommodated within the Hsp90 binding site) hydrogen bond with the terminal carboxyl group of Asp93. On the other hand, the *p*-hydroxy group makes a water-mediated (HOH2232, another structural water molecule present in the binding site) hydrogen bond with the carbonyl group of Leu48. The chlorine atom is accommodated in a large hydrophobic cavity delimited by Phe138 and Leu107 side chains. Moreover, also the isoxazole nitrogen atom interacts with the carboxyl terminus of Asp93 through a water-mediated (HOH2233) hydrogen bond. The oxime nitrogen of the most active compound (**15**) interacts with the ammonium group of the Lys58 side chain, whereas the endocyclic oxygen is involved in an intramolecular hydrogen bond with the terminal OH group of the oxime moiety. The saturated portion of the six-membered condensed ring does not show any significant hydrophobic interaction with the protein.

In summary, we have designed and synthesized a novel class of 5,6-dihydro-4*H*-benzo[*d*]isoxazol-7-ones and 5,6-dihydro-4*H*-isoxazolo[5,4-*c*]pyridin-7-ones to investigate their affinity toward Hsp90 protein. Whereas all the compounds having a benzamide group on the bicyclic scaffold were devoid of activity, all the derivatives carrying a resorcinol-like fragment showed inhibitory effect on the enzyme. In particular, **15** possessed a remarkable binding ability (IC_{50} = 0.8 μM), slightly better than that of the reference compound (17-AAG). On this basis, it could be considered as a useful starting point for medicinal chemists involved in designing new scaffolds in the field of Hsp90 inhibitors.

Work is in progress to investigate structural changes of the resorcinol portion, as well as of the bicyclic moiety, to enhance the fitting into the Hsp90 ATP binding pocket, and to improve the activity of the compounds.

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Conflict of Interest

The authors declare that there are no conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Experimental details for synthesis and evaluation of new compounds. NMR spectral data of new compounds.