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Design, Synthesis, and Biological Evaluation of Ring-Constrained Novobiocin Analogues as Hsp90 C-Terminal Inhibitors

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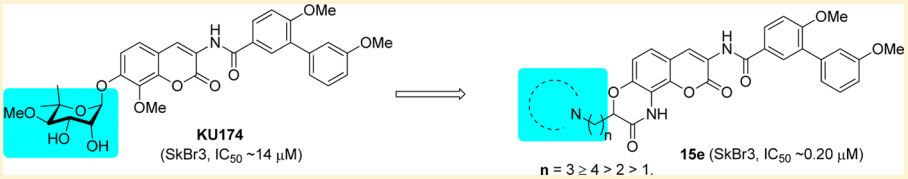
Supporting Information



ABSTRACT: Hsp90 C-terminal inhibitors represent a novel and alternative chemotherapeutic approach for the treatment of cancer. Novobiocin was the first natural product identified as an Hsp90 C-terminal inhibitor; however, it manifests poor antiproliferative activity. In contrast to N-terminal inhibitors, novobiocin does not induce the pro-survival heat shock response. Structural investigations on novobiocin have elucidated some structure−activity relationships and several promising compounds. On the basis of structure−activity relationships and computational studies, a library of ring-constrained novobiocin analogues was designed, synthesized, and evaluated in antiproliferative assays. Results obtained from these studies provide insights into the Hsp90 C-terminal binding site, and new analogues that were developed manifest low micromolar to mid-nanomolar antiproliferative activity resulting from Hsp90 inhibition.

KEYWORDS: *Heat shock protein 90, Hsp90 C-terminal inhibitors, ring-constrained novobiocin analogues, structure−activity relationship, breast cancer*

The 90 kDa heat shock protein 90 (Hsp90) is a molecular chaperone that plays a crucial role in homeostasis as well as an adaptive response to cell stress.[1,2] It is a key component of the cellular protein folding machinery and regulates the conformational maturation, activation, and integrity of a wide array of client proteins, many of which are associated with cell signaling, proliferation, and survival.[3] However, in cancer cells, many of these client substrates are mutated and/or overexpressed and contribute to malignant transformation and/or the maintenance of oncogenic phenotypes.[4] In fact, Hsp90 client proteins (e.g., Her2, Raf1, Akt, MET, Src, CDK4, etc.) are directly associated with all six hallmarks of cancer and are actively pursued as individual therapeutic targets for the treatment of cancer.[5,6] Consequently, Hsp90 inhibition provides an opportunity to simultaneously disrupt multiple oncogenic pathways and hence represents an attractive target for the development of cancer chemotherapeutics.

Hsp90 exists as a homodimer with each monomer consisting of three domains; (1) the N-terminal ATP-binding domain, (2) the middle domain, and (3) the C-terminal dimerization domain. The majority of research has focused on the development of Hsp90 N-terminal inhibitors. In fact, 17 small molecules that target this region have undergone clinical investigation for the treatment of cancer.[7] Although there has been great interest in the development of these Hsp90 inhibitors, challenges such as concomitant induction of the pro-survival heat shock response, hepatotoxicity, and multidrug resistance have dampened enthusiasm regarding their clinical promise.[8] In contrast, Hsp90 C-terminal inhibitors have emerged as an alternative strategy for Hsp90 modulation as these agents manifest similar inhibitory activity without induction of the pro-survival heat shock response and therefore could potentially circumvent some of the limitations associated with N-terminal inhibition.[9−11]

Novobiocin (**1**), a DNA gyrase inhibitor, was the first natural product identified as a Hsp90 C-terminal inhibitor (Figure 1).[12] Unfortunately, it manifests poor antiproliferative activity (IC_{50} ≈ 700 μM aganist SKBr3 cells) and consequently has limited therapeutic application. Preliminary investigations on novobiocin revealed key structural elements important for Hsp90 activity, which ultimately transformed it from a DNA gyrase inhibitor into a selective Hsp90 inhibitor.[13,14] Subsequent studies elucidated additional structure−activity relationships (SARs) and led to the identification of several promising lead molecules (Figure 1) that manifest potent antiproliferative activity against multiple cancer cell lines.[15−28] Upon comparison of these lead compounds, the following SARs were observed: (1) the biaryl side chain appears optimal; (2) the amide linker provides hydrogen bonding interactions with the binding pocket; (3) incorporation of a hydrogen-bond acceptor at the 8-position improves inhibitory activity; and (4) modification of the phenolic ether is tolerated and allows for replacement of the

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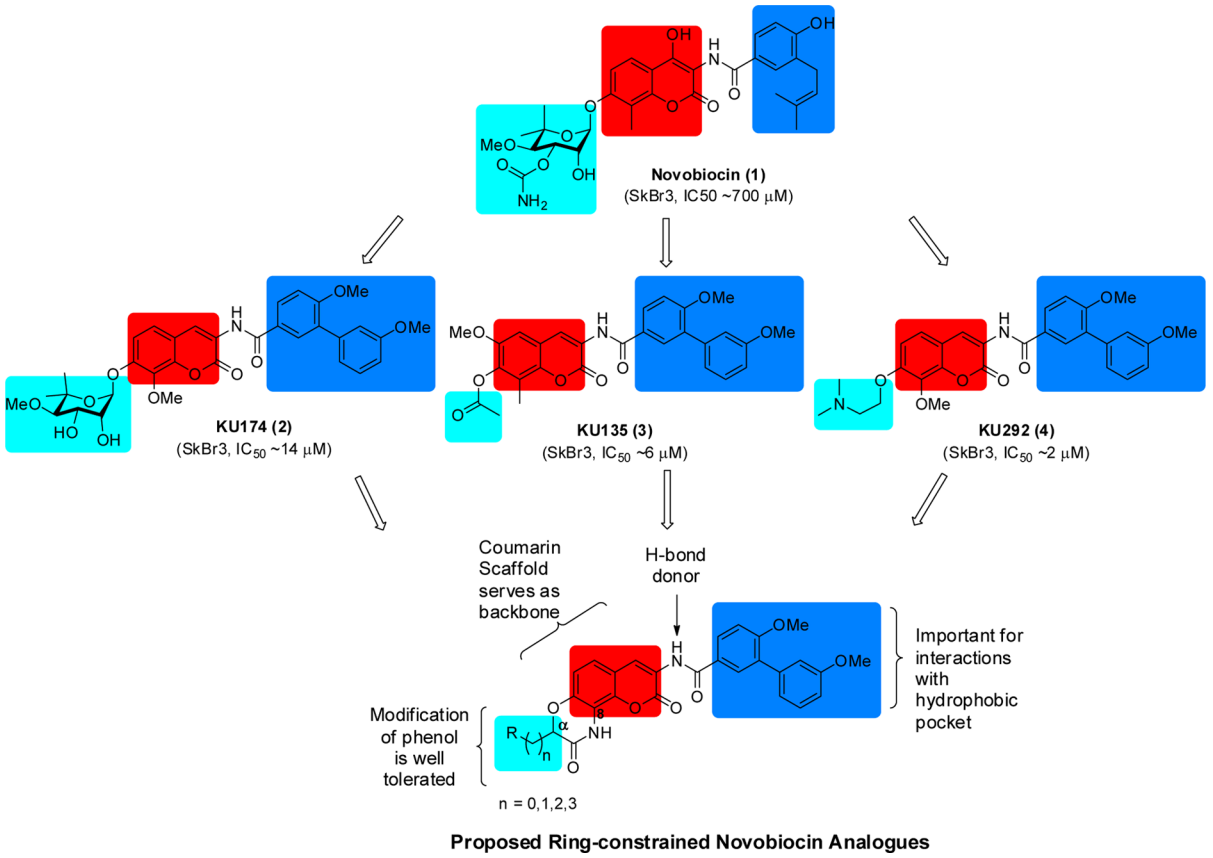

Figure 1. Rationale for ring-constrained novobiocin analogues.

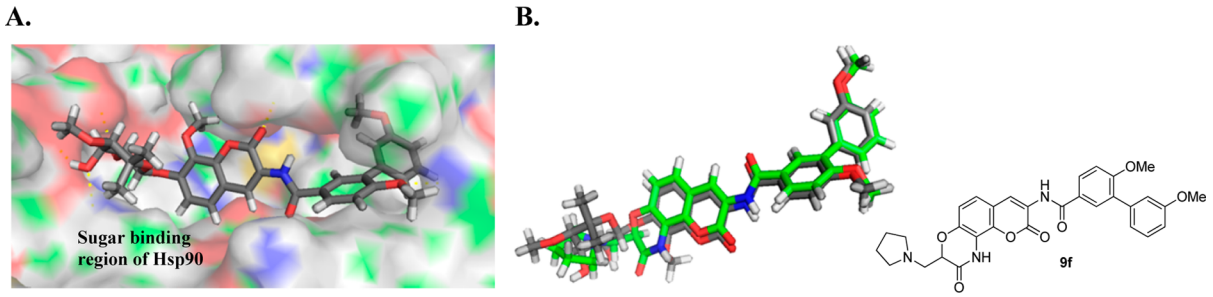

Figure 2. (A) Docked model of KU174 (gray) in the putative Hsp90 C-terminal binding site. (B) Molecular overlay of KU174 (gray) and investigational molecule, **9f** (green).

complex sugar with simple moieties without compromising inhibitory activity.

On the basis of these SARs as well as molecular docking studies,[28,29] it was hypothesized that lactams containing substituents at the α-position in lieu of the sugar moiety could provide ring-constrained analogues that possess these critical features (Figure 1). Furthermore, as a consequence of ring constraint, the analogues would exhibit less entropic penalty upon binding, compared to their freely rotating counterparts, and therefore could project the side chain into the region normally occupied by the novoise sugar of novobiocin. In addition, replacement of the hydrolytically sensitive ether linkage with a lactam would produce analogues with greater stability.

In support of this hypothesis, compound **9f**, which contains the physiologically ionizable pyrrolidine moiety appended to the α-position of the lactam, was designed and docked into the Hsp90 C-terminal model.[29] As shown in Figure 2B, compound **9f** aligned structurally with the lead compound, KU174.

Encouraged by these docking studies, molecules were designed to include a sugar surrogate at the α-position of the lactam and to project into the region typically occupied by the sugar moiety for interactions with the binding pocket. Since no cocrystal structure of Hsp90 bound to C-terminal inhibitors has been solved, there is limited information regarding the size and nature of this binding pocket. Therefore, lactams containing hydrophobic, rigid, flexible, ionizable, and nonionizable substituents were investigated to identify beneficial interactions with the binding site.

Retrosynthetically, we envisioned construction of these lactams from *O*-alkylation of phenol **6** with α-bromo ester **7**, followed by a tandem reduction/ring closing reaction that could be catalyzed upon Pd hydrogenolysis (Scheme 1). Phenol **6** was proposed to result from an Aldol condensation between aldehyde **4** and acid **3**, whereas aldehyde **4** could be obtained by the formylation of commercially available nitro resorcinol. Synthesis of acid **3** could be achieved via an amide coupling

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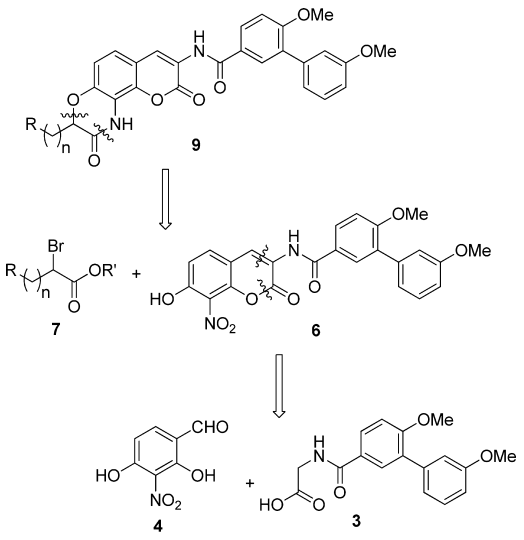
Scheme 1. Retrosynthetic Analysis of Ring-Constrained Novobiocin Analogues

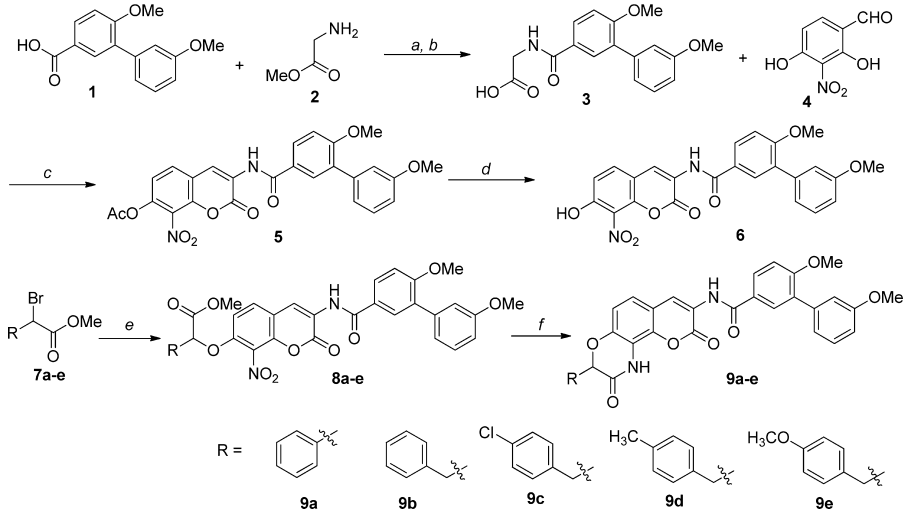


reaction between the glycine ester and the previously reported biaryl acid **1**,[1] followed by base-catalyzed hydrolysis.

As shown in Scheme 2, the synthesis of aromatic analogues began via an amide coupling reaction between biaryl acid **1** and amine **2** to afford amide **3**, which underwent an Aldol condensation with benzaldehyde **4** to give acetylated-coumarin **5**. Subsequent deacetylation of **5** gave the coumarin intermediate **6**, in high yield. Phenol **6** was *O*-alkylated with the α-bromo esters, **7a−e**, to yield the coumarin ethers, **8a−e**. The resulting nitro aromatics (**8a−e**) were then reduced with Pd/C under a hydrogen atmosphere to furnish the corresponding anilines, which underwent an acid-catalyzed cyclization to afford lactams **9a−e**. In parallel, a series of amino lactams was synthesized as shown in Scheme 3.

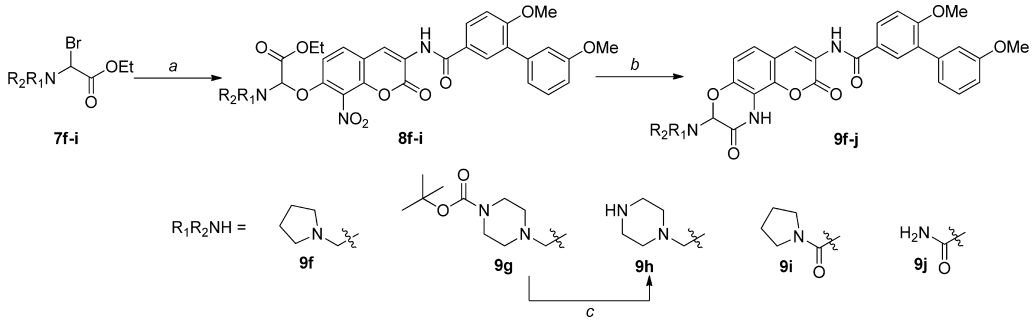
Upon construction, the lactams were evaluated for antiproliferative activity against SKBr3 (estrogen receptor negative, Her2 overexpressing breast cancer cells) and MCF-7 (estrogen receptor positive breast cancer cells) cell lines (Table 1). In general, the aromatic-containing lactams were less effective than their flexible counterparts. Direct attachment of the aromatic moiety to the lactam was detrimental, as compound **9a** exhibited an IC_{50} value of ∼50 μM. Although, the benzyl analogue (**9b**) manifested improved activity, compounds **9c** and **9d** were inactive. Interestingly, the inclusion of a polar group onto the aromatic ring resulted in improved activity, as analogue **9e** exhibited 3−4-fold greater antiproliferative activity than **9b**. These results suggest that a polar moiety attached to a linear tether may be beneficial. Consistent with this hypothesis, compound **9f** manifested comparable activity to KU174.

Scheme 2. Synthesis of Aromatic-Containing Lactams[a]



[a]Reagents and conditions: (a) EDCI, DMAP, Et_3N, THF, rt, 12 h, 80%; (b) LiOH·H_2O, MeOH/THF/H_2O, rt, 12 h, 95%; (c) NaOAc, Ac_2O, 110 °C, 3 h, 60%; (d) K_2CO_3, MeOH, 2 h, 85%; (e) **6**, NaH/K_2CO_3, DMF, 12 h, 20−70%; (f) 10% Pd/C, H_2, AcOH, MeOH/EtOAc, rt, 2−3 d, 20−60%.

Scheme 3. Synthesis of Amine-Containing Lactams[a]



[a]Reagents and conditions: (a) **6**, NaH/K_2CO_3, DMF, 12 h, 20−30%; (b) 10% Pd/C, H_2, AcOH, MeOH/EtOAc, rt, 2−3 d, 20−40%; (c) 40% TFA/DCM, DCM, rt, 4 h, 60%.

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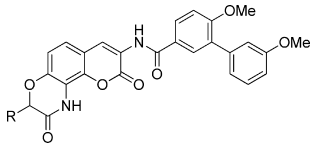
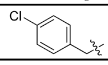
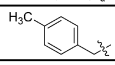
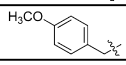
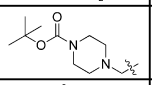
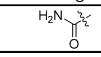
Table 1. Antiproliferative Activity of Aromatic- and Amine-Containing Lactams



Entry	R	SKBr3 (IC_{50}, μM)	MCF-7 (IC_{50}, μM)
KU174	-	13.9 ± 0.15	9.0 ± 0.33
9a		48.18 ± 2.28[a]	>50
9b		21.49 ± 0.70	22.11 ± 0.64
9c		>50	>50
9d		>50	>50
9e		8.37 ± 1.41	6.12 ± 0.26
9f		14.97 ± 4.3	22.90 ± 0.96
9g		>50	>50
9h		5.78 ± 0.91	5.27 ± 0.29
9i		>50	>50
9j		>50	33.35 ± 0.97

[a]Values represent mean ± standard deviation for at least two separate experiments performed in triplicate.

Compound **9h**, which contains a piperazine moiety, manifested improved antiproliferative activity over compound **9f**, while compound **9g** was inactive. Interestingly, replacement of the amine with an amide, resulted in inactive analogues (**9f** vs **9i** and **9j**), suggesting the physiologically ionizable amine may form key interactions with the protein that are important for antiproliferative activity.

Overall, preliminary structure−activity relationships suggest an amine and a linear tether are important for antiproliferative activity. To gain further insight into this region of the binding site, amino analogues **12a** and **15a**, which contain a pyrrolidine moiety tethered to the lactam core, were docked into the Hsp90 C-terminal model (Figure 1S, see Supporting Information). The docking studies suggested the pyrrolidine amine may provide hydrogen bonding interactions with Glu537 within the binding site. Interestingly, this pyrrolidine ring projects into a subpocket that allows for the inclusion of additional substituents. However, homologation of the linker length may diminish hydrogen-bonding interactions and therefore produce less efficacious analogues. Consequently, it was hypothesized that the incorporation of a two- or three-carbon amino tether could project deeper into the hydrophobic subpocket, while maintaining interactions between the ammonium ion and amino acid side chains. The amines were selected based on their potential to provide hydrogen bonding interactions and include various ring sizes and steric bulk.

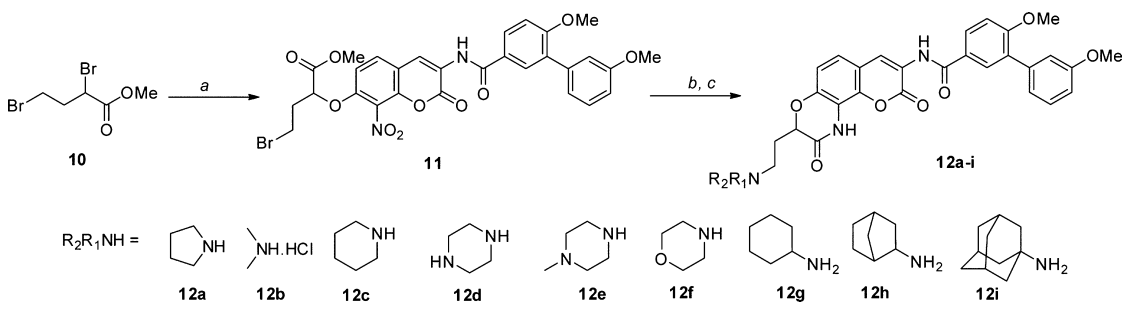
As shown in Scheme 4, the syntheses of ethylene-containing lactams was initiated by *O*-alkylation of phenol **6** with 2,4-dibromo ether **10** to give coumarin ether **11** in high yield. The resulting coumarin ether (**11**) was then treated with primary or secondary amines to give the corresponding products, which were then subjected to tandem reduction/intramolecular ring closing, enlisting a Pd/C catalyst under hydrogen atmosphere to furnish compounds **12a−i**.

Upon their preparation, these two-carbon tethered lactams were evaluated for antiproliferative activity against SKBr3 and MCF-7 cell lines (Table 2). Consistent with the docking studies, ethylene-containing lactams exhibited improved antiproliferative activity compared to their methylene-containing counterparts (**9f** vs **12a**). Introduction of an acyclic amine (**12b**) resulted in decreased potency, emphasizing the need for a cyclic ring system. Increasing the ring size to six atoms (**12c**) maintained activity and suggested that various ring structures could be accommodated. It was found that the inclusion of a second hydrogen bond donor/acceptor (**12d**, **12e**, and **12f**) decreased activity, indicating the subpocket may be hydrophobic in nature. Interestingly, the secondary amines manifested better activity than the tertiary amines (**12g**, **12h**, and **12i**).

For comparison with 1- and 2-carbon linkers, a series of three-carbon tethers was synthesized as shown in Scheme 5. Confirmation of the optimal linker length was investigated through construction of a 4-carbon tethered lactam **18** (see Supporting Information).

Upon completion, the lactams were evaluated for antiproliferative activity against both cancer cell lines. As shown in Table 3, the analogue containing a three-carbon linker exhibited improved antiproliferative activity over the corresponding 2-carbon tethered lactams (**15a** vs **12a**). In addition, compound **18** showed comparable activity to **15a**, suggesting further homologation of the linker length does not provide better interactions with the binding pocket. Similar to the trend observed with ethylene-containing analogues, the piperidine

Scheme 4. Synthesis of Two-Carbon Tethered Lactams[a]



[a]Reagents and conditions: (a) **6**, K_2CO_3, DMF, rt, 12 h, 78%; (b) $\mathbf{R_1R_2NH}$, DMF, rt, 12 h; (c) 10% Pd/C, H_2, CH_3COOH, MeOH/EtOAc, rt, 2−3 d, 28−55%.

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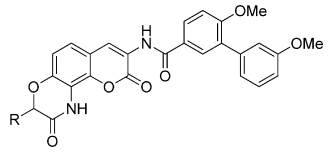
Table 2. Antiproliferative Activity of Two-Carbon Tethered Lactams



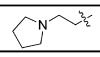
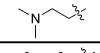
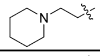
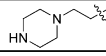
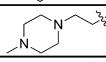
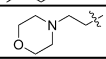
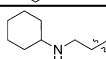
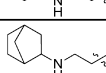
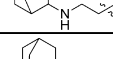
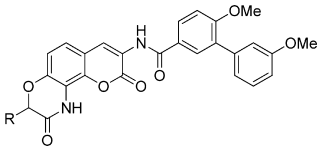
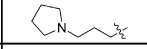
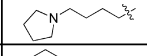
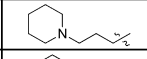
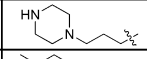
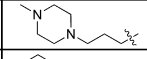
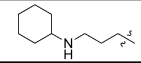
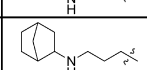
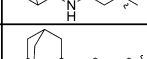
Entry	R	SKBr3 (IC_{50}, μM)	MCF-7 (IC_{50}, μM)
12a		3.49 ± 0.20	5.2 ± 0.38
12b		8.06 ± 0.82	11.40 ± 0.16
12c		1.03 ± 0.07	1.04 ± 0.01
12d		5.44 ± 0.12	7.63 ± 0.10
12e		4.1 ± 0.07	5.68 ± 0.37
12f		6.66 ± 0.47	7.44 ± 0.86
12g		0.78 ± 0.07	1.16 ± 0.04
12h		0.68 ± 0.04	1.22 ± 0.14
12i		0.88 ± 0.02	1.09 ± 0.17

Table 3. Antiproliferative Activity Manifested by Three-Carbon Tethered Lactams

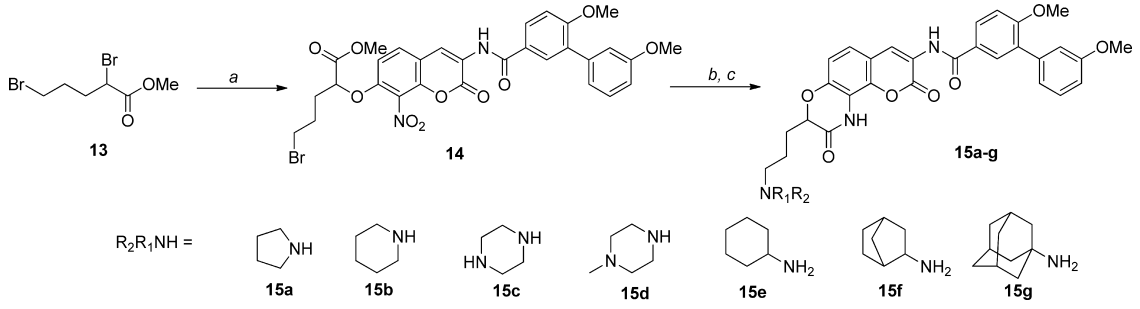


Entry	R	SKBr3 (IC_{50}, μM)	MCF-7 (IC_{50}, μM)
15a		1.02 ± 0.19	1.49 ± 0.02
18		2.50 ± 0.53	1.53 ± 0.61
15b		0.78 ± 0.14	0.85 ± 0.05
15c		9.08 ± 0.02	12.85 ± 0.13
15d		1.76 ± 0.39	2.43 ± 0.45
15e		0.20 ± 0.08	0.35 ± 0.01
15f		1.26 ± 0.01	1.34 ± 0.11
15g		0.62 ± 0.01	0.72 ± 0.01

(**15b**) analogue manifested improved antiproliferative activity, while the piperazine (**15c**) and *N*-methylpiperazine (**15d**) resulted in decreased inhibitory activity. Interestingly, cyclohexylamino analogue **15e** manifested better antiproliferative activity compared to the bicycloalkyl and tricyclic amino analogues (**15f** and **15g**), indicating limitations within the hydrophobic subpocket.

To confirm the antiproliferative activity exhibited by these lactams resulted from Hsp90 inhibition, Western blot analysis was performed on lysates treated with **15b**, **15e**, and **15g**. Actin, an Hsp90-independent protein, was used as a negative control.

Scheme 5. Synthesis of Three-Carbon Tethered Lactams[a]



[a]Reagents and conditions: (a) **6**, K_2CO_3, DMF, rt, 12 h, 75%; (b) $\mathbf{R_1R_2NH}$, DMF, rt, 12 h; (c) 10% Pd/C, H_2, CH_3COOH, MeOH/EtOAc, rt, 2−3 d, 20−40%.

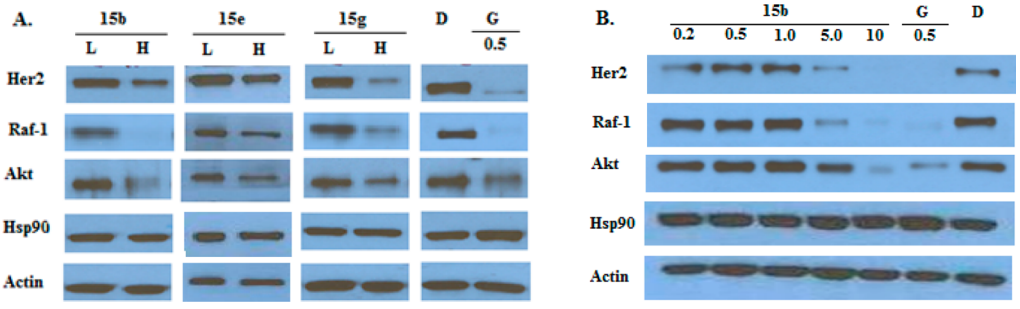


Figure 3. Western blot analyses of lactams after incubation with MCF-7 breast cancer cells for 24 h. (A) Western blot analyses of the Hsp90 client protein degradation after treatment with lactams **15b**, **15e**, and **15g**. (B) Western blot analyses of the Hsp90 client protein degradation after treatment with lactam **15b**. Concentrations (in μM) were indicated above each lane. L represents a concentration 1/2 of the antiproliferative activity. H represents a concentration equal to 5-fold of the antiproliferative activity. Geldanamycin (G, 0.5 μM) and dimethyl sulfoxide (D, 100%) serve as positive and negative controls, respectively.

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As shown in Figure 3A, incubation with these compounds induced the degradation of Hsp90-dependent client proteins Her2, Raf-1, and Akt, confirming these analogues manifest their cellular activity through Hsp90 inhibition. In addition, treatment of MCF-7 cells with **15b** induced the degradation of the Hsp90-dependent client proteins Her2, Raf-1, and AKt in a concentration-dependent manner (Figure 3B). Actin levels remained unaffected, indicating selective degradation of Hsp90-dependent client proteins. Additionally, Hsp90 levels were unaltered, which is a hallmark exhibited by Hsp90 C-terminal inhibitors.

In summary, a series of ring-constrained novobiocin analogues was designed, synthesized, and evaluated against MCF-7 and SKBr3 breast cancer cell lines. Incorporation of ring-constrained analogues led to the development of molecules that manifest submicromolar to mid-nanomolar inhibitory activity. SAR studies revealed that amines attached to a three-carbon linker produced the most efficacious analogues. Furthermore, these studies suggest the presence of a hydrophobic subpocket that can be exploited for the development of more efficacious Hsp90 inhibitors, which will be reported in due course.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures for the synthesis and characterization of new compounds (^{1}H and ^{13}C NMR, HRMS). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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