**Design, Synthesis and Pharmacological Evaluation of
4,5-Diarylisoaxazols Bearing Amino Acid Residues within the 3-Amido
Motif as Potent Heat Shock Protein 90 (Hsp90) Inhibitors**

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Abstract

A structure-based medicinal chemistry optimization was conducted on the clinical Hsp90 inhibitor diarylisoxazole **3**. Several series of new compounds were designed and synthesized by incorporating diversified amino acid derivatives with various lengths to the 3-amido motif of the isoxazole scaffold. Compound **14j** was identified to have high Hsp90 binding potency (14 nM) and antiproliferative activity against H3122 human lung cancer and BT-474 breast cancer cells. Treatment of **14j** with H3122 cell led to degradation of client protein ALK, reduction of downstream phosphorylation of AKT and ERK, and up-regulation of Hsp70. Molecular docking suggested that the terminal valine moiety and the ethyleneglycol linker in compound **14j** formed additional apolar and polar interaction network with a number of amino acid residues.

Keywords: Diarylisoxazole; Resorcinol; Amino acid; Hsp90 inhibitor; Antiproliferative effect.

1. Introduction

Heat shock proteins (Hsps) are a family of molecular chaperones that consist of Hsp100, Hsp90, Hsp70, Hsp60 and many other smaller Hsps (15-30 kDa) [1,2]. Among this family, Hsp90 is highly conserved from bacteria to mammals and responsible for protein homeostasis with the capacity to facilitate protein folding, assembly, transportation and degradation [3,4]. Most oncogenic tyrosine kinases, transcription factors and cell-cycle proteins are client proteins of Hsp90 [5]. Under stress conditions such as in cancer cells, Hsp90 is generally overexpressed 2-10 fold higher than that in normal cells, and forms highly susceptible complexes with its clients under the assistance of cochaperones [6]. Inhibition of Hsp90 function has been shown to cause degradation of client proteins including some oncogenic proteins such as ALK, Her2, EGFR, Met and BCR-ABL, likely by disruption of cell growth and proliferation [7-12]. Therefore, Hsp90 has long been recognized as a viable molecular target for cancer treatment [13]. The majority of Hsp90 inhibitors currently in the clinic are Hsp90 N-terminal inhibitors that interact with the ATPase binding site in the N-terminal of Hsp90 structure [14-16]. The earlier Hsp90 inhibitors including the natural product geldanamycin and its analogues (e.g. 17-AAG, 17-DMAG) have showed acceptable objective responses in patients bearing different types of tumor, however, poor solubility, hepatotoxicity and many other adverse effects limited their further development [17,18].

The resorcinol substituted pyrazole **1** is a high-throughput screening hit of Hsp90 inhibitor from Vernalis Ltd., which has moderate inhibitory potency (IC_{50} , 280 nM) against Hsp90 enzyme and much poor antiproliferative effect in the HCT116 cells (5.8 μ M) (Figure 1) [19]. The co-crystal structure of **1** with the N-terminal domain of human Hsp90 showed that in addition to the key interaction network formed between

the highly conserved residue Asp93 in the ATP-binding pocket of Hsp90 and the resorcinol moiety through two molecules of water, the pyrazole 5-position is much closer to the Hsp90 and surrounded by many amino acid residues, especially Gly97. Based on this analysis, (*N*-ethyl amido) moiety was introduced to access the residue Gly97 and the resulting compound **2** not only showed 10-fold improved potency against Hsp90 (25 nM) and also showed >20-fold improved cellular potency in the HCT116 tumor cells with an IC₅₀ value of 260 nM. Further scaffold hopping and optimization of the solvent-interaction moiety at Vernalis resulted in diarylisoxazole **3** that showed significant antiproliferative effects against various human cancer cells with GI₅₀ values averaging 9 nM [20]. It also has good pharmacokinetic properties and showed significant tumor growth inhibition in a human colon cancer xenograft model. Compound **3** (AUY922) has been under a number of clinical trials for years either as monotherapy or in combination with other therapeutical agents [21-24].

[Insert Figure 1 here]

Encouraged by the improved biochemical and cellular potency of **3** and in view of the remaining untouched distal hydrophobic network around the 3-position of the isoxazole center in **3** formed by a number of amino acid residues (Ile96, Lys58, His154...., Figure 1), we speculate that there might be still potentials to develop potent Hsp90 inhibitors by introducing a peptide moiety with various lengths at this site to achieve more additional apolar and polar interactions with these amino acid residues. In fact, only limited variations on the amido moiety (Me, Et, Prⁱ) have been reported at the isoxazole-3-position of **3** by scientists at Veralis [20]. In this regard, we here report our recent SAR on the optimization of the 3-amido moiety of **3** by introduction of various peptides consisting of different D-amino acids. The preference of D-amino acids is based on the Hsp90 inhibitor **5** (San A-amide, Figure 1), a cyclic

depsipeptide derived from marine fungus of genus *Fusarium* [25-27]. **5** was proved to specifically bind to the *N*-middle domain of Hsp90 [28-30], which was believed to be related to the D-amino acid residues (Val, Valine; Leu, Leucine; Phe, Phenylalanine).

[Insert Scheme 1 here]

2. Results and Discussion

2.1. Chemistry

As shown in Scheme 1, the key intermediate **6** was synthesized following literature procedures [20]. Condensation of acid **6** with various amino acid derivatives (esters or amides) in the presence of HATU, HOAT and DIPEA gave amides **7a-j** and **9a-m**. Removal of benzyl protection groups by BCl_3 at 0 °C furnished final compounds **8a-j** in 15-63% two steps yields and **10a-m** in 15-57% two steps yields.

[Insert Scheme 2 here]

The synthesis of compounds **14a-d** was described in Scheme 2. Pyrimidine -5-carboxylic acid **11** was reacted with various methyl amino acid esters to form pyrimidine amides **12** in 44-53% yields, which was subsequently treated with TFA to give piperazine **13** in 50-91% yields. Condensation of isoxazole-3-carboxylic acid **6** with piperazine **13** in the presence of HATU, HOAT and DIPEA, followed by *O*-benzyl deprotection afforded the desired products **14a** and **14b** in 39% and 42% overall yields, respectively. Similarly, compounds **14c** and **14d** were obtained in 20% and 28% overall yields by condensation of isoxazole-3-carboxylic acid **6** with amine **16**.

[Insert Scheme 3 here]

As shown in Scheme 3, acids **17** with various linkers were treated with amino acid methyl esters to provide intermediates **18** in 69-92% yields. After *N*-deprotection of **18** by TFA, subsequent amide condensation with isoxazole-3-carboxylic acid **6**

followed by *O*-debenzylation by BCl₃ afforded compounds **14e-h** in 11-44% overall yields. Meanwhile, 3-azido-carboxylic acid **19** was condensed with amino acids to provide amides **20** in 83-92% yields. After reduction of azides **20** by Pd/C under hydrogen atmosphere, subsequent condensation with isoxazole-3-carboxylic acid **6** followed by *O*-debenzylation by BCl₃ afforded compounds **14i** and **14j** in 36% and 31% overall yields, respectively.

2.2 Structure-activity relationship Study (SAR)

The biochemical activity of the new compounds against Hsp90 α was determined by fluorescence polarization (FP) assay and the SRB assay was used to measure the antiproliferative effects in NCI-H3122 cancer cell lines. As summarized in Table 1, several natural and unnatural amino acid methyl esters were incorporated into the isoxazole-3-amido motif, and all the compounds (**8a-8g**) retained good potency against Hsp90 α with IC₅₀ values ranging between 0.07-0.84 μ M, although they were not as potent as compound **3** (AUY922). Compound **8a** bearing D-valine methyl ester and **8e** bearing methyl 2-amino-3-hydroxypropanoate were the most potent in this subseries with a nearly identical IC₅₀ value of ~0.07 μ M, however, **8a** was 8-fold more potent than **8e** in the NCI-H3122 cancer cells. Interestingly, compound **8b** bearing leucine methyl ester displayed moderate biochemical activity (0.151 μ M), but was much more potent in the cell with an IC₅₀ value of 0.019 μ M. Compound **8c** bearing a L-amino acid moiety was nearly inactive in the H3122 cell (> 4 μ M), though moderate potency was observed in the enzymatic assay. On the basis of these results, valine methyl ester was the best amino acid residue to be incorporated to the isoxazole-3-amido motif, and the corresponding compound **8a** was potent in both the enzymatic and cellular assays, with potency only 2- to 4-fold less potent than

reference compound **3**.

[Insert Table 1 here]

Next, we optimized the valine residue in isoxazoline **8a** by incorporating various valine-derived esters and amides. As shown in Table 2, compared to methyl ester **8a**, the ethyl ester **10a** was 2-fold less potent, but the antiproliferative effect was 10-fold improved with an IC₅₀ value of 2 nM. Notably, the *tert*-butyl ester **10b** was also slightly less potent against Hsp90 (112 nM), but was highly potent in the H3122 cell (1 nM). Both esters **10a** and **10b** were more than 10-fold more potent than the reference compound **3** in the cell assay. Replacement of the methyl ester to simple methyl or ethyl amides yielded compounds **10c** and **10d**, both showed similar high potency in the enzymatic (~20 nM) as well as in the cellular (~30 nM) assays. Amides **10e-h** bearing cyclopropylamine, azetidine, piperazine, and morpholine component respectively also retained good potency in both assays, although they were not as potent as **10c** and **10d**. Incorporating a second amino acid (Phe, Val, Leu) residue to the valine moiety of **8a** led to dipeptide analogues **10i-k**. They retained moderate potency against Hsp90 (0.2 – 0.4 μM), but only the Val-Phe dipeptide analog **10i** showed good cellular potency with an IC₅₀ value of 79 nM and the other two were inactive (> 10 μM). Interestingly, tripeptide analog **10l** and tetrapeptide analog **10m** also retained moderate Hsp90 binding potency (0.26 μM vs 0.52 μM), but the tripeptide **10l** was nearly 20-fold more potent than the tetrapeptide **10m** with IC₅₀ values of 0.03 μM and 0.63 μM, respectively.

[Insert Table 2 here]

In the meantime, we also modified isoxazole-3-carboxylic acid methyl ester by keeping the phenylalanine methyl ester or valine methyl ester as the terminal moiety, but incorporating various cyclic or linear linkages. As shown in Table 3, compounds

14a and **14b**, though containing the same 2-(piperazin-4-yl)pyrimidin-5-carbonyl moiety as the linker, showed significantly different potency against Hsp90. Compound **14a** bearing phenylalanine methyl ester as the terminal moiety was inactive ($> 1 \mu\text{M}$), whereas compound **14b** with valine methyl ester as the terminus retained moderate potency ($0.47 \mu\text{M}$). Interestingly, both compounds showed nearly identical activity against the proliferation of H3122 cell ($0.16 \mu\text{M}$ vs $0.13 \mu\text{M}$). With 2-(aminomethylpiperidin-1-yl)-pyrimidine-5-carbonyl as the linker, compounds **14c** and **14d** retained moderate Hsp90 inhibitory potency ($0.75 \mu\text{M}$ vs $0.41 \mu\text{M}$), while **14d** bearing valine methyl ester as the terminus was 4-fold more potent in the cell ($1.1 \mu\text{M}$ vs $0.28 \mu\text{M}$). With the 5-aminopentanoyl moiety as the linker, compounds **14e** and **14f** showed good enzymatic and cellular potency, especially compound **14f** bearing valine methyl ester moiety showed IC_{50} values of 98 nM and 65 nM , respectively against Hsp90 enzyme and the cell growth. Inserting oxygen to the linker led to compounds **14g** and **14h**, of which only **14h** retained good potency both in the enzyme and in the cell assays (154 nM vs 90 nM). Further extending the linker afforded compounds **14i** and **14j**. Both compounds showed high antiproliferative effect with identical IC_{50} values of 40 nM , whereas the later compound bearing valine methyl ester as the terminus was more than 8-fold more potent against Hsp90 with an IC_{50} value of 14 nM . From the results above, it could be concluded that the water-soluble oxygen-containing linkers generally produced better cellular potency (**14h-j**) and compounds bearing valine methyl ester as the terminal moiety generally provided higher binding potency against Hsp90 than compounds bearing phenylalanine methyl ester as the terminus. In view of both high biochemical and cellular potencies (14 nM vs 42 nM) as well as the structural novelty, compound **14j** was selected for further evaluation.

[Insert Table 3 here]

2.3 Cancer cell lines panel test

Since Hsp90 is a ubiquitous molecular chaperone and is highly expressed in cancer cells, suppression of Hsp90 function would cause growth inhibition of a broad range of cancer cells [31]. In this regard, in addition to the H3122 lung cancer cell, the new potent Hsp90 inhibitor **14j** was subjected to a variety of additional cancer cells expressing different tumor types, including A549 and NCI-H460 (human lung cancer), HCT-116 (colorectal cancer), BT-474 and MDA-MB-231 (breast cancer), DU-145 (prostate cancer), and U87-MG (glioma cancer). As shown in Table 4, compound **14j** showed moderate to high growth inhibition (42-560 nM) against the tested cell lines. In addition to the lung cancer cell H3122, high potency was observed in the breast cancer BT-474 as well with an IC₅₀ value of 57 nM.

[Insert Table 4 here]

2.4 Inhibitory effects on client proteins

The fusion protein of tyrosine kinase anaplastic lymphoma kinase (ALK) with the echinoderm microtubule-associated protein-like 4 (EML4)—ALK) is an oncogenic protein and one of the highly sensitive clients of Hsp90 [7,8]. Since the H3122 cell is addicted to the fusion protein EML4-ALK, it was then incubated with compound **14j** in various concentrations. As shown in Figure 2, the new inhibitor **14j** induced significant degradation of ALK at the concentration of 100 nM. Meanwhile, Hsp70, the well-known feedback response and pharmacodynamic signature [32] to Hsp90 inhibition was up-regulated. Further, the downstream signaling, namely the phosphorylation of AKT and ERK (also clients of Hsp90), was also reduced after treatment with **14j**. Compared to compound **3** (AUY922), **14j** displayed similar inhibitory profile on client proteins.

[Insert Figure 2 here]

2.5 Molecular docking

To elucidate the interaction mode of the high potency Hsp90 inhibitor **14j**, a docking study of this compound with ATP binding site of Hsp90 *N*-terminal domain was performed (Figure 3). Similar to that of **3**, the two phenolic hydroxyls on the resorcinol motif together with the *N*- and *O*-atoms of the isoxazole center intensively interacted with the key residue Asp93 through two crystal water molecules [20]. Meanwhile, the amido moiety on the isoxazole-3-position formed hydrogen bonds with Gly97 and Lys58. Notably, the ethylene glycol moiety within the linker interacted with adjacent water molecule and formed additional hydrogen bonds. Unexpectedly, another two hydrogen bonds were observed between the valine methyl ester terminus (as hydrogen acceptor) and the residues of Gln23 and Lys112. In addition, the amido-NH of the valine moiety formed another hydrogen bond with Thr109. Moreover, a hydrophobic interaction was formed between the terminal isopropyl group of valine moiety and the isobutyl group of the residue I110. All these interactions formed a complex network and contributed to the high potency of compound **14j**.

[Insert Figure 3 here]

3. Conclusions

In summary, a structure-based medicinal chemistry optimization was conducted on the clinical Hsp90 inhibitor **3**, and several series of new compounds were designed and synthesized by incorporating diversified amino acid derivatives with various lengths to the 3-amido motif of the diarylisoxazole scaffold. Fluorescence polarization assay as well as cell antiproliferative experiments were conducted, which led to the identification of compound **14j** bearing a terminal valine methyl ester and an

ethyleneglycol-containing linear liker. This compound showed high binding potency of 14 nM against Hsp90 and inhibited cell growth of various cancer cells, especially H3122 human lung cancer cell and BT-474 breast cancer cell with IC₅₀ values of 42 and 57 nM, respectively. Treatment of **14j** with H3122 cell led to degradation of ALK, a highly sensitive client protein of Hsp90. Inhibition of downstream phosphorylation of AKT and ERK was also observed, together with up-regulation of Hsp70, the pharmacodynamic signature of Hsp90 inhibition, confirming the Hsp90 inhibition mechanism of this compound. Molecular docking suggested that in addition to the typical interaction pattern formed between Asp93 and the resorcinol motif and the central isoxazole center, compound **14j** formed additional apolar and polar interaction network through the terminal valine moiety and the ethyleneglycol linker with a number of amino acid residues, including Thr109, Gln23 and Lys112.

4. Experimental section

4.1. Chemistry

4.1.1 General methods

All reactions were performed in glassware containing a teflon-coated stir bar. All solvents and chemical reagents were obtained from commercial sources and used without further purifications unless otherwise stated. ¹H and ¹³C NMR spectra were recorded on a Varian Mercury 300, 400 or 500 NMR spectrometer and data were recorded using CDCl₃ as the solvent. Low-resolution mass spectrometry (MS) and high-resolution mass spectrometry (HRMS) analysis was recorded at anionizing voltage of 70 eV on a Finnigan/MAT95 spectrometer. Optical rotations were measured on Autopol VI, serial number 90079, manufactured by Rudolph Research Analytical, Hackettstown, N.J.. Flash column chromatography on silica gel (200-300

mesh) was used for the routine purification of reaction products. All reactions were monitored by TLC on silica gel (100-200 mesh) precoated on glass plates (15×50 mm), and spots were visualized by UV light at 254 nm.

4.1.2 General procedure for the amido condensation and O-benzyl deprotection

A mixture of acid **6** (0.097 mmol), an appropriate amino acid derivative (0.146 mmol), HATU (0.194 mmol), HOAT (0.194 mmol) and DIPEA (0.291 mmol) in DCM (5 mL) was stirred at room temperature until the completion of the reaction. The mixture was concentrated in vacuum and purified by chromatograph (petroleum/acetoacetate: 1/1) to give corresponding products **7** which were dissolved in dry DCM (5 mL) under nitrogen atmosphere. BCl₃ (0.388 mmol) was added to the solution at 0 °C and stirred for 2 h. The mixture was basified with sodium bicarbonate until pH > 7. After extraction with DCM, the combined organic phase was washed with brine, dried over sodium sulfate, filtered, and then evaporated. The crude product was purified by chromatograph (DCM/MeOH: 10/1) to afford the corresponding target compounds.

4.1.3 (R)-Methyl 2-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamido)-3-methylbutanoate (**8a**)

This compound was obtained as white solid in overall yield of 63% (two steps) from **6** and D-valine methyl ester hydrochloride. ¹H NMR (300 MHz, CDCl₃) δ 7.22 (s, 4H), 6.82 (s, 1H), 5.96 (s, 1H), 4.59 (m, 1H), 3.68 (s, 3H), 3.60 (s, 4H), 3.41 (s, 2H), 3.00 – 2.88 (m, 1H), 2.43 (s, 4H), 2.19 (m, 1H), 0.89 (m, 12H); ¹³C NMR (126 MHz, CDCl₃) δ 171.76, 167.61, 159.39, 156.78, 155.84, 153.44, 130.30, 129.81, 128.54, 127.74, 127.50, 115.50, 105.69, 104.04, 66.49, 63.11, 57.19, 53.46, 52.37, 31.35, 25.98, 22.35, 18.95, 17.79; ESI-MS: 552 [M + H]⁺; HRMS-ESI: m/z [M + H]⁺

calcd for C₃₀H₃₈N₃O₇: 552.2710, found: 552.2705; [α]_D²⁰ = -10.4 (*c* 0.067, CHCl₃).

4.1.4 (R)-Methyl 2-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamido)-4-methylpentanoate (8b)

This compound was obtained as white solid in 45% overall yield (two steps) from **6** and D-Leucine methyl ester hydrochloride. ¹H NMR (300 MHz, CDCl₃) δ 7.27 (s, 4H), 7.18 (d, *J* = 7.8 Hz, 1H), 6.88 (s, 1H), 6.11 (s, 1H), 4.74 (s, 1H), 3.73 (s, 3H), 3.68 (s, 4H), 3.50 (s, 2H), 3.07 – 2.90 (m, 1H), 2.51 (s, 4H), 1.69 (d, *J* = 7.2 Hz, 3H), 0.92 (d, *J* = 6.9 Hz, 12H); ¹³C NMR (126 MHz, CDCl₃) δ 172.26, 166.74, 158.41, 156.01, 155.39, 152.93, 129.82, 129.21, 127.12, 126.81, 114.97, 105.28, 103.61, 66.16, 62.61, 53.01, 51.98, 50.24, 40.88, 25.49, 24.42, 22.28, 21.81, 21.45; ESI-MS: 566 [M + H]⁺; HRMS-ESI: *m/z* [M + H]⁺ calcd for C₃₁H₄₀N₃O₇: 566.2866, found: 566.2850; [α]_D²⁰ = -4.0 (*c* 0.050, CHCl₃).

4.1.5 (S)-Methyl 6-(((benzyloxy)carbonyl)amino)-2-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamido)hexanoate (8c)

This compound was obtained as white solid in 29% overall yield (two steps) from **6** and L-Lysine (CBz) methyl ester hydrochloride. ¹H NMR (300 MHz, CDCl₃) δ 7.46 (d, *J* = 7.7 Hz, 1H), 7.31 (d, *J* = 8.2 Hz, 9H), 6.84 (s, 1H), 6.25 (s, 1H), 5.08 (s, 2H), 4.96 (d, *J* = 5.4 Hz, 1H), 4.69 (d, *J* = 5.3 Hz, 1H), 3.70 (d, *J* = 6.4 Hz, 7H), 3.51 (s, 2H), 3.18 (s, 2H), 3.00 (m, 1H), 2.50 (s, 4H), 1.93 (s, 1H), 1.80 (s, 1H), 1.52 (d, *J* = 6.2 Hz, 2H), 1.42 (d, *J* = 6.2 Hz, 2H), 0.89 (d, *J* = 5.5 Hz, 6H); ¹³C NMR (151 MHz, CDCl₃) δ 172.13, 167.72, 159.39, 157.16, 156.82, 155.67, 153.59, 136.34, 130.35, 129.77, 128.54, 128.18, 128.13, 127.76, 127.49, 115.53, 105.38, 104.01, 66.88, 66.42, 63.00, 53.37, 52.60, 52.22, 40.60, 31.75, 29.50, 25.91, 22.50, 22.37; ESI-MS: 715 [M + H]⁺; HRMS-ESI: *m/z* [M + H]⁺ calcd for C₃₉H₄₇N₄O₉: 715.3343, found: 715.3332; [α]_D²⁰ = +4.2 (*c* 0.048, CHCl₃).

4.1.6 (R)-Methyl 3-cyclohexyl-2-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamido)propanoate (**8d**)

This compound was obtained as white solid in 15% overall yield (two steps) from **6** and 3-cyclohexyl-D-alanine methyl ester hydrochloride. ¹H NMR (300 MHz, CDCl₃) δ 7.26 (s, 4H), 7.16 (d, *J* = 8.4 Hz, 1H), 6.89 (s, 1H), 5.95 (s, 1H), 4.81 – 4.69 (m, 1H), 3.73 (s, 3H), 3.66 (s, 4H), 3.45 (s, 2H), 2.98 (m, 1H), 2.47 (s, 4H), 1.80 – 1.57 (m, 8H), 1.36 (s, 2H), 1.17 (t, *J* = 10.2 Hz, 3H), 0.92 (d, *J* = 6.7 Hz, 6H); ¹³C NMR (151 MHz, CDCl₃) δ 172.99, 167.61, 159.33, 156.82, 155.77, 153.39, 130.27, 129.81, 128.55, 127.75, 127.55, 115.59, 105.65, 104.01, 66.49, 63.14, 53.48, 52.56, 50.22, 39.87, 34.13, 33.46, 32.43, 26.32, 26.13, 25.98, 25.94, 22.39, 22.37; ESI-MS: 604 [M - H]⁺; HRMS-ESI: *m/z* [M - H]⁺ calcd for C₃₄H₄₂N₃O₇: 604.3023, found: 604.3040; [α]_D²⁰ = -5.9 (*c* 0.051, CHCl₃).

4.1.7 (R)-Methyl 2-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamido)-3-hydroxypropanoate (**8e**)

This compound was obtained as white solid in 23% overall yield (two steps) from **6** and D-serine methyl ester hydrochloride. ¹H NMR (300 MHz, CDCl₃ + CD₃OD) δ 7.31 (s, 4H), 6.85 (s, 1H), 6.30 (s, 1H), 4.75 (s, 1H), 4.03 (dd, *J* = 11.5, 3.5 Hz, 1H), 3.92 (dd, *J* = 11.5, 3.1 Hz, 1H), 3.77 (d, *J* = 6.8 Hz, 7H), 3.62 (s, 2H), 3.04 (m, 1H), 2.61 (s, 4H), 0.94 (d, *J* = 6.7 Hz, 6H); ¹³C NMR (126 MHz, CDCl₃ + CD₃OD) δ 170.55, 167.99, 159.79, 157.51, 155.94, 153.83, 130.11, 129.49, 128.71, 127.85, 127.50, 115.61, 105.02, 103.06, 66.47, 62.99, 62.24, 54.60, 53.26, 52.69, 25.81, 22.34; ESI-MS: 540 [M + H]⁺; HRMS-ESI: *m/z* [M + H]⁺ calcd for C₂₈H₃₄N₃O₈: 540.2346, found: 540.2346; [α]_D²⁰ = -20.4 (*c* 0.054, CHCl₃).

4.1.8 (R)-Methyl 2-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamido)-3-phenylpropanoate (**8f**)

This compound was obtained as white solid in 37% overall yield (two steps) from **6** and D-phenylalanine methyl ester hydrochloride. ¹H NMR (300 MHz, CDCl₃) δ 7.26 (s, 7H), 7.12 (d, *J* = 6.8 Hz, 2H), 6.87 (s, 1H), 6.11 (s, 1H), 4.98 (d, *J* = 7.7 Hz, 1H), 3.71 (s, 3H), 3.67 (s, 4H), 3.48 (s, 2H), 3.21 – 3.14 (m, 2H), 3.03 – 2.94 (m, 1H), 2.49 (s, 4H), 0.91 (d, *J* = 6.7 Hz, 6H); ¹³C NMR (151 MHz, CDCl₃) δ 171.30, 167.68, 159.18, 156.94, 155.64, 153.47, 135.42, 130.25, 129.81, 129.27, 128.74, 127.70, 127.59, 127.30, 115.47, 105.55, 103.95, 66.35, 63.00, 53.35, 53.27, 52.55, 37.89, 25.96, 22.39; ESI-MS: 600 [M + H]⁺; HRMS-ESI: *m/z* [M + H]⁺ calcd for C₃₄H₃₈N₃O₇: 600.2710, found: 600.2705; [α]_D²⁰ = -42.2 (*c* 0.054, CHCl₃).

4.1.9 (R)-Methyl 2-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamido)-3-(1H-indol-3-yl)propanoate (8g)

This compound was obtained as light yellow solid in 50% overall yield (two steps) from **6** and D-tryptophan methyl ester hydrochloride. ¹H NMR (300 MHz, CDCl₃) δ 8.33 (s, 1H), 7.50 (d, *J* = 7.8 Hz, 1H), 7.30 (d, *J* = 7.6 Hz, 2H), 7.20 (d, *J* = 8.9 Hz, 4H), 7.16 – 7.05 (m, 2H), 6.95 (s, 1H), 6.83 (s, 1H), 6.07 (s, 1H), 5.01 (d, *J* = 7.7 Hz, 1H), 3.64 (s, 7H), 3.46 (s, 2H), 3.36 (d, *J* = 5.2 Hz, 2H), 3.02 – 2.93 (m, 1H), 2.47 (s, 4H), 0.89 (d, *J* = 6.8 Hz, 6H); ¹³C NMR (151 MHz, CDCl₃) δ 171.88, 167.94, 159.69, 157.11, 155.75, 153.56, 136.17, 130.16, 130.03, 127.87, 127.72, 127.20, 123.40, 122.10, 119.52, 118.33, 115.54, 111.51, 108.96, 105.42, 103.94, 65.91, 62.66, 53.04, 52.86, 52.67, 27.49, 25.93, 22.43; ESI-MS: 639 [M + H]⁺; HRMS-ESI: *m/z* [M + H]⁺ calcd for C₃₆H₃₉N₄O₇: 639.2819, found: 639.2813; [α]_D²⁰ = -44.3 (*c* 0.061, CHCl₃).

4.1.10 (R)-Ethyl 2-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamido)-3-methylbutanoate (10a)

This compound was obtained as white solid in 55% overall yield (two steps) from **6** and D-valine ethyl ester hydrochloride. ¹H NMR (300 MHz, CDCl₃) δ 7.27 (s, 4H),

6.88 (s, 1H), 6.01 (d, $J = 2.4$ Hz, 1H), 4.63 (m, 1H), 4.25 – 4.12 (m, 2H), 3.66 (s, 4H), 3.48 (s, 2H), 3.06 – 2.95 (m, 1H), 2.50 (s, 4H), 2.25 (d, $J = 6.1$ Hz, 1H), 1.27 (t, $J = 7.1$ Hz, 3H), 0.95 (m, 12H); ^{13}C NMR (126 MHz, CDCl_3) δ 171.31, 167.92, 159.61, 157.06, 155.77, 153.50, 130.33, 129.97, 129.28, 127.70, 115.53, 105.56, 103.93, 66.03, 62.79, 61.59, 57.27, 53.12, 31.36, 25.99, 22.40, 18.93, 17.77, 14.20; ESI-MS: 566 $[\text{M} + \text{H}]^+$; HRMS-ESI: m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{31}\text{H}_{40}\text{N}_3\text{O}_7$: 566.2866, found: 566.2859; $[\alpha]_{\text{D}}^{20} = -8.3$ (c 0.060, CHCl_3).

4.1.11 *(R)-tert-Butyl 2-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamido)-3-methylbutanoate (10b)*

This compound was obtained as white solid in 57% overall yield (two steps) from **6** and D-valine tert-butyl ester hydrochloride. ^1H NMR (300 MHz, CDCl_3) δ 7.32 (d, $J = 9.4$ Hz, 1H), 7.27 (s, 4H), 6.87 (s, 1H), 5.93 (s, 1H), 4.55 (m, 1H), 3.64 (s, 4H), 3.44 (s, 2H), 3.01 (m, 1H), 2.47 (s, 4H), 2.23 (m, 1H), 1.47 (s, 9H), 0.99 – 0.89 (m, 12H); ^{13}C NMR (151 MHz, CDCl_3) δ 170.44, 167.68, 159.51, 156.89, 156.00, 153.45, 136.19, 130.29, 129.86, 128.83, 127.72, 127.59, 115.48, 105.70, 104.03, 82.54, 66.43, 63.14, 57.63, 53.46, 31.55, 28.05, 25.98, 22.40, 22.36, 18.88, 17.72; ESI-MS: 592 $[\text{M} - \text{H}]^+$; HRMS-ESI: m/z $[\text{M} - \text{H}]^+$ calcd for $\text{C}_{33}\text{H}_{42}\text{N}_3\text{O}_7$: 592.3023, found: 592.3034; $[\alpha]_{\text{D}}^{20} = -10.9$ (c 0.064, CHCl_3).

4.1.12 *(R)-5-(2,4-Dihydroxy-5-isopropylphenyl)-N-(3-methyl-1-(methylamino)-1-oxobutan-2-yl)-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamide (10c)*

This compound was obtained as white solid in 30% overall yield (two steps). ^1H NMR (300 MHz, CDCl_3) δ 7.29 (d, $J = 5.0$ Hz, 4H), 6.78 (s, 1H), 6.32 (s, 1H), 4.24 (d, $J = 7.4$ Hz, 1H), 3.70 (s, 4H), 3.49 (s, 2H), 3.05 – 2.97 (m, 1H), 2.76 (s, 3H), 2.47 (s, 4H), 2.15 (m, 1H), 0.94 (d, $J = 6.7$ Hz, 6H), 0.88 (d, $J = 6.8$ Hz, 6H); ^{13}C NMR (126 MHz, CDCl_3) δ 171.75, 168.03, 160.13, 157.33, 155.82, 153.70, 130.24, 129.88,

128.90, 115.34, 105.25, 103.96, 66.28, 62.96, 59.14, 53.32, 30.89, 26.32, 25.90, 22.42, 19.23, 18.36; ESI-MS: 551 $[M + H]^+$; HRMS-ESI: m/z $[M + H]^+$ calcd for $C_{30}H_{39}N_4O_6$: 551.2870, found: 551.2863; $[\alpha]_D^{20} = +25.4$ (c 0.071, $CHCl_3$).

4.1.13 *(R)-5-(2,4-Dihydroxy-5-isopropylphenyl)-N-(1-(ethylamino)-3-methyl-1-oxobutan-2-yl)-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamide (10d)*

This compound was obtained as light yellow solid in 47% overall yield (two steps). 1H NMR (300 MHz, $CDCl_3$) δ 7.37 (d, $J = 8.2$ Hz, 1H), 7.25 (d, $J = 9.5$ Hz, 4H), 6.81 (s, 1H), 6.45 (s, 1H), 6.19 (s, 1H), 4.27 (t, $J = 7.9$ Hz, 1H), 3.68 (s, 4H), 3.54 (s, 2H), 3.36 – 3.16 (m, 2H), 3.08 – 2.94 (m, 1H), 2.54 (s, 4H), 2.14 (d, $J = 6.4$ Hz, 1H), 1.09 (t, $J = 7.1$ Hz, 3H), 0.92 (m, 12H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 170.89, 168.01, 160.13, 157.37, 155.86, 153.71, 130.24, 129.94, 129.08, 127.79, 127.72, 115.34, 105.23, 103.90, 66.16, 62.83, 59.24, 53.20, 34.66, 30.94, 25.92, 22.44, 19.23, 18.40, 14.51; ESI-MS: 563 $[M - H]^+$; HRMS-ESI: m/z $[M - H]^+$ calcd for $C_{31}H_{39}N_4O_6$: 563.2870, found: 563.2878; $[\alpha]_D^{20} = +17.8$ (c 0.050, $CHCl_3$).

4.1.14 *(R)-5-(2,4-Dihydroxy-5-isopropylphenyl)-N-(1-((1-(hydroxymethyl)cyclopropyl)amino)-3-methyl-1-oxobutan-2-yl)-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamide (10e)*

This compound was obtained as light yellow solid in 17% overall yield (two steps). 1H NMR (300 MHz, $CDCl_3 + CD_3OD$) δ 7.32 (s, 1H), 7.28 (s, 4H), 6.77 (s, 1H), 6.29 (s, 1H), 4.20 (d, $J = 6.8$ Hz, 1H), 3.71 (s, 4H), 3.59 (d, $J = 11.8$ Hz, 1H), 3.51 (t, $J = 5.5$ Hz, 3H), 3.01 (m, 1H), 2.48 (s, 4H), 2.12 (m, 1H), 0.96 – 0.87 (m, 12H), 0.85 – 0.75 (m, 4H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.16, 167.96, 160.31, 157.47, 155.86, 153.78, 130.25, 129.53, 128.62, 127.68, 127.55, 115.30, 104.93, 103.35, 67.88, 66.57, 63.02, 58.82, 53.35, 34.98, 30.81, 25.80, 22.35, 22.31, 19.13, 18.09, 12.00, 11.93; ESI-MS: 605 $[M - H]^+$; HRMS-ESI: m/z $[M - H]^+$ calcd for

C₃₃H₄₁N₄O₇: 605.2975, found: 605.2960; [α]_D²⁰ = +14.2 (*c* 0.064, CHCl₃).

4.1.15 (R)-N-(1-(3,3-Difluoroazetidin-1-yl)-3-methyl-1-oxobutan-2-yl)-5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamide (**10f**)

This compound was obtained as white solid in 22% overall yield (two steps). ¹H NMR (300 MHz, CDCl₃) δ 7.33 (d, *J* = 8.4 Hz, 1H), 7.27 (s, 2H), 7.22 (d, *J* = 7.7 Hz, 2H), 6.82 (s, 1H), 6.08 (s, 1H), 4.82 – 4.68 (m, 1H), 4.38 (m, 4H), 3.67 (s, 4H), 3.50 (s, 2H), 3.04 – 2.96 (m, 1H), 2.50 (s, 4H), 2.08 (m, 1H), 0.97 (t, *J* = 6.6 Hz, 6H), 0.90 (d, *J* = 6.6 Hz, 6H); ¹³C NMR (151 MHz, CDCl₃) δ 171.50, 167.76, 159.75, 157.07, 155.65, 153.49, 130.26, 129.87, 128.70, 127.85, 127.62, 115.38, 114.75(t, *J* = 273 Hz), 105.43, 103.95, 66.42, 63.07, 62.36 (t, *J* = 30.2 Hz), 60.22 (t, *J* = 28.7 Hz), 54.53, 53.42, 31.01, 25.95, 22.38, 19.14, 18.13; ESI-MS: 611 [M - H]⁺; HRMS-ESI: *m/z* [M - H]⁺ calcd for C₃₂H₃₇N₄O₆F₂: 611.2681, found: 611.2698; [α]_D²⁰ = +1.9 (*c* 0.052, CHCl₃).

4.1.16 (R)-5-(2,4-Dihydroxy-5-isopropylphenyl)-N-(3-methyl-1-(4-methylpiperazin-1-yl)-1-oxobutan-2-yl)-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamide (**10g**)

This compound was obtained as white solid in 34% overall yield (two steps). ¹H NMR (300 MHz, CDCl₃) δ 7.51 (d, *J* = 8.4 Hz, 1H), 7.26 (q, *J* = 7.9 Hz, 4H), 6.79 (s, 1H), 6.29 (s, 1H), 4.91 (s, 1H), 3.66 (d, *J* = 15.6 Hz, 8H), 3.53 (s, 2H), 3.09 – 2.95 (m, 1H), 2.51 (s, 8H), 2.32 (s, 3H), 2.05 (d, *J* = 6.0 Hz, 1H), 0.92 (m, 12H); ¹³C NMR (151 MHz, CDCl₃) δ 169.68, 167.88, 159.79, 157.32, 156.06, 153.87, 130.26, 129.75, 128.92, 127.77, 127.58, 115.31, 105.31, 103.88, 66.44, 62.99, 54.81, 54.40, 53.87, 53.35, 45.61, 31.52, 25.89, 22.44, 19.71, 17.42; ESI-MS: 618 [M - H]⁺; HRMS-ESI: *m/z* [M - H]⁺ calcd for C₃₄H₄₄N₅O₆: 618.3292, found: 618.3301; [α]_D²⁰ = -20.8 (*c* 0.048,

CHCl₃).

4.1.17 (R)-5-(2,4-Dihydroxy-5-isopropylphenyl)-N-(3-methyl-1-((2-morpholinoethyl)amino)-1-oxobutan-2-yl)-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamide (10h)

This compound was obtained as light yellow solid in 29% overall yield (two steps). ¹H NMR (300 MHz, CDCl₃) δ 7.42 (d, *J* = 7.6 Hz, 1H), 7.29 – 7.22 (m, 4H), 7.01 (s, 1H), 6.76 (s, 1H), 6.26 (s, 1H), 4.31 (t, *J* = 7.4 Hz, 1H), 3.64 (s, 8H), 3.50 (s, 2H), 3.37 (s, 2H), 3.05 – 2.95 (m, 1H), 2.49 (s, 10H), 2.13 (d, *J* = 6.9 Hz, 1H), 0.96 – 0.83 (m, 12H); ¹³C NMR (151 MHz, CDCl₃) δ 171.18, 168.12, 160.15, 157.39, 155.79, 153.88, 130.27, 129.79, 128.83, 127.90, 127.64, 115.39, 105.21, 103.79, 66.39, 66.32, 62.93, 59.20, 56.71, 53.34, 53.12, 35.74, 30.83, 25.89, 22.43, 19.21, 18.34; ESI-MS: 648 [M - H]⁺; HRMS-ESI: *m/z* [M - H]⁺ calcd for C₃₅H₄₆N₅O₇: 648.3397, found: 648.3408; [α]_D²⁰ = -4.0 (*c* 0.050, CHCl₃).

4.1.18 Methyl 2-((R)-2-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamido)-3-methylbutanamido)-3-phenylpropanoate (10i)

This compound was obtained as white solid in 43% overall yield (two steps). ¹H NMR (300 MHz, CDCl₃) δ 7.46 (d, *J* = 8.6 Hz, 1H), 7.25 (d, *J* = 6.3 Hz, 4H), 7.16 (d, *J* = 5.5 Hz, 3H), 7.06 (d, *J* = 7.2 Hz, 2H), 6.80 (s, 2H), 6.17 (s, 1H), 4.90 – 4.81 (m, 1H), 4.33 (m, 1H), 3.66 (d, *J* = 8.2 Hz, 7H), 3.48 (s, 2H), 3.09 – 2.98 (m, 3H), 2.48 (s, 4H), 2.14 (m, 1H), 0.90 (t, *J* = 6.2 Hz, 12H); ¹³C NMR (151 MHz, CDCl₃) δ 171.72, 170.64, 167.81, 159.90, 157.14, 155.75, 153.61, 135.44, 130.31, 129.82, 129.24, 128.65, 127.81, 127.67, 127.20, 115.44, 105.46, 104.07, 66.39, 63.07, 58.89, 53.42, 53.34, 52.51, 37.74, 30.85, 25.95, 22.40, 19.12, 18.16; ESI-MS: 697 [M - H]⁺; HRMS-ESI: *m/z* [M - H]⁺ calcd for C₃₉H₄₅N₄O₈: 697.3237, found: 697.3256; [α]_D²⁰ = -30.4 (*c* 0.056, CHCl₃).

4.1.19 Methyl 2-((*R*)-2-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholino methyl)phenyl)isoxazole-3-carboxamido)-3-methylbutanamido)-3-methylbutanoate (**10j**)

This compound was obtained as orange solid in 43% overall yield (two steps). ¹H NMR (300 MHz, CDCl₃) δ 7.50 (d, *J* = 7.9 Hz, 1H), 7.24 (m, 4H), 6.80 (s, 2H), 6.23 (s, 1H), 4.53 – 4.45 (m, 1H), 4.40 (t, *J* = 7.6 Hz, 1H), 3.72 (s, 7H), 3.57 (s, 2H), 3.06 – 2.95 (m, 1H), 2.57 (s, 4H), 2.17 (m, 2H), 0.95 (d, *J* = 5.6 Hz, 6H), 0.88 (t, *J* = 7.7 Hz, 12H); ¹³C NMR (151 MHz, CDCl₃) δ 172.18, 171.02, 167.84, 159.98, 157.16, 155.80, 153.57, 130.30, 129.92, 127.74, 127.66, 115.37, 105.39, 104.00, 66.16, 62.89, 59.09, 57.51, 53.24, 52.32, 31.01, 30.92, 25.96, 22.41, 19.17, 18.92, 18.30, 17.86; ESI-MS: 649 [M - H]⁺; HRMS-ESI: *m/z* [M - H]⁺ calcd for C₃₅H₄₅N₄O₈: 649.3237, found: 649.3253; [α]_D²⁰ = +13.0 (*c* 0.046, CHCl₃).

4.1.20 Methyl 2-((*R*)-2-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholino methyl)phenyl)isoxazole-3-carboxamido)-3-methylbutanamido)-4-methylpentanoate (**10k**)

This compound was obtained as orange solid in 56% overall yield (two steps). ¹H NMR (300 MHz, CDCl₃) δ 7.53 (d, *J* = 8.2 Hz, 1H), 7.33 – 7.18 (m, 4H), 6.79 (s, 2H), 6.14 (s, 1H), 4.59 (s, 1H), 4.39 (t, *J* = 7.7 Hz, 1H), 3.70 (s, 7H), 3.54 (s, 2H), 3.08 – 2.96 (m, 1H), 2.54 (s, 4H), 2.28 – 2.15 (m, 1H), 1.59 (d, *J* = 6.5 Hz, 2H), 0.97 (t, *J* = 6.7 Hz, 6H), 0.86 (m, 12H); ¹³C NMR (151 MHz, CDCl₃) δ 173.30, 170.90, 167.77, 160.04, 157.17, 155.77, 153.51, 130.37, 129.90, 127.87, 127.64, 115.35, 105.36, 104.04, 66.27, 63.03, 58.94, 53.37, 52.46, 51.01, 40.90, 31.02, 25.92, 24.75, 22.72, 22.40, 21.72, 19.13, 18.18; ESI-MS: 663 [M - H]⁺; HRMS-ESI: *m/z* [M - H]⁺ calcd for C₃₆H₄₇N₄O₈: 663.3394, found: 663.3396; [α]_D²⁰ = +18.6 (*c* 0.059, CHCl₃).

4.1.21 (*R*)-Methyl 2-((*R*)-2-((*S*)-2-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamido)-3-methylbutanamido)-3-methyl

butanamido)-3-methylbutanoate (10l)

This compound was obtained as orange solid in 41% overall yield (two steps). ¹H NMR (300 MHz, CDCl₃) δ 7.79 (s, 1H), 7.24 (s, 4H), 6.77 (s, 1H), 6.39 (s, 1H), 4.49 (s, 2H), 4.36 (s, 1H), 3.72 (s, 7H), 3.59 (s, 2H), 3.00 (s, 1H), 2.58 (s, 4H), 2.16 (s, 2H), 2.06 (s, 1H), 0.88 (s, 24H); ¹³C NMR (151 MHz, CDCl₃) δ 172.22, 171.47, 171.27, 167.79, 160.21, 157.24, 155.99, 153.68, 130.26, 129.86, 127.71, 127.53, 115.36, 105.25, 103.96, 66.10, 62.80, 59.18, 57.34, 57.30, 53.16, 52.24, 30.96, 30.79, 30.72, 25.91, 22.42, 19.23, 19.11, 18.89, 18.48, 18.34, 17.86; ESI-MS: 748 [M - H]⁺; HRMS-ESI: *m/z* [M - H]⁺ calcd for C₄₀H₅₄N₅O₉: 748.3922, found: 748.3942; [α]_D²⁰ = +31.8 (c 0.054, CHCl₃).

4.1.22 (3R,6R,9R,12R)-Methyl 1-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholinomethyl)phenyl)isoxazol-3-yl)-3,6,9,12-tetraisopropyl-1,4,7,10-tetraoxo-2,5,8,11-tetraazatridecan-13-oate (10m)

This compound was obtained as white solid in 15% overall yield (two steps). ¹H NMR (300 MHz, CDCl₃ + CD₃OD) δ 7.84 (d, *J* = 7.5 Hz, 1H), 7.30 (d, *J* = 0.9 Hz, 1H), 7.24 (s, 3H), 6.77 (s, 1H), 6.31 (s, 1H), 4.53 (d, *J* = 4.4 Hz, 2H), 4.44 (d, *J* = 7.7 Hz, 1H), 4.28 (d, *J* = 6.5 Hz, 1H), 3.73 (s, 3H), 3.69 (s, 4H), 3.48 (s, 2H), 3.06 – 2.97 (m, 1H), 2.47 (s, 4H), 2.18 – 2.06 (m, 2H), 2.03 – 1.93 (m, 2H), 0.87 (t, *J* = 6.5 Hz, 30H); ¹³C NMR (126 MHz, CDCl₃ + CD₃OD) δ 172.54, 171.82, 171.29, 170.99, 167.41, 160.03, 157.34, 156.33, 153.73, 130.08, 129.47, 128.74, 127.60, 127.53, 115.23, 105.18, 103.52, 66.56, 63.03, 58.67, 58.47, 57.17, 57.08, 53.34, 52.21, 31.29, 31.11, 30.95, 30.60, 25.81, 22.37, 22.34, 19.01, 18.97, 18.84, 18.46, 18.42, 18.31, 17.83; ESI-MS: 849 [M + H]⁺; HRMS-ESI: *m/z* [M + H]⁺ calcd for C₄₅H₆₅N₆O₁₀: 849.4762, found: 849.4763; [α]_D²⁰ = +31.5 (c 0.064, CHCl₃).

4.1.23 (R)-Methyl 2-(2-(4-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholino

methylphenyl)isoxazole-3-carbonyl)piperazin-1-yl)pyrimidine-5-carboxamido)-3-phenylpropanoate (14a)

This compound was obtained as white solid in 42% overall yield (two steps) from **6**. ¹H NMR (300 MHz, CDCl₃) δ 8.52 (s, 2H), 7.18 (m, 7H), 7.07 – 6.99 (m, 3H), 6.59 (d, *J* = 7.6 Hz, 1H), 6.10 (s, 1H), 4.96 (m, 1H), 3.69 (s, 7H), 3.44 (s, 4H), 3.34 (s, 2H), 3.26 (s, 3H), 3.22 – 2.96 (m, 4H), 2.30 (s, 4H), 0.94 (d, *J* = 6.8 Hz, 6H); ¹³C NMR (126 MHz, CDCl₃) δ 171.91, 165.27, 163.45, 160.94, 160.42, 157.19, 157.00, 153.03, 135.24, 129.73, 128.69, 128.45, 128.22, 127.39, 126.80, 115.61, 113.48, 104.79, 103.09, 65.63, 62.28, 53.00, 52.70, 52.12, 45.98, 43.02, 42.67, 41.31, 37.25, 25.59, 22.00; ESI-MS: 790 [M + H]⁺; HRMS-ESI: *m/z* [M + H]⁺ calcd for C₄₃H₄₈N₇O₈: 790.3564, found: 790.3558; [α]_D²⁰ = -37.9 (*c* 0.048, CHCl₃).

4.1.24 (R)-Methyl 2-(2-(4-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholino methyl)phenyl)isoxazole-3-carbonyl)piperazin-1-yl)pyrimidine-5-carboxamido)-3-methylbutanoate (14b)

This compound was obtained as white solid in 39% overall yield (two steps) from **6**. ¹H NMR (300 MHz, CDCl₃) δ 8.62 (s, 2H), 7.19 (d, *J* = 6.9 Hz, 4H), 6.97 (s, 1H), 6.71 (d, *J* = 7.9 Hz, 1H), 6.11 (s, 1H), 4.64 (d, *J* = 4.9 Hz, 1H), 3.68 (s, 7H), 3.45 (s, 4H), 3.31 (d, *J* = 24.1 Hz, 6H), 3.00 (d, *J* = 6.6 Hz, 1H), 2.30 (s, 4H), 2.17 (d, *J* = 6.2 Hz, 1H), 0.91 (d, *J* = 5.1 Hz, 12H); ¹³C NMR (151 MHz, CDCl₃) δ 172.79, 165.70, 164.34, 161.50, 160.89, 157.73, 157.56, 157.41, 153.51, 130.20, 128.94, 127.85, 116.35, 113.93, 105.30, 103.60, 66.20, 62.81, 57.42, 53.23, 52.41, 46.48, 43.50, 43.17, 41.79, 31.40, 26.07, 22.47, 19.01, 18.09; ESI-MS: 740 [M - H]⁺; HRMS-ESI: *m/z* [M - H]⁺ calcd for C₃₉H₄₆N₇O₈: 740.3408, found: 740.3426; [α]_D²⁰ = -18.7 (*c* 0.054, CHCl₃).

4.1.25 (R)-Methyl 2-(2-(4-((5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholino

methyl)phenyl)isoxazole-3-carboxamido)methyl)piperidin-1-yl)pyrimidine-5-carboxamido)-3-phenylpropanoate (**14c**)

This compound was obtained as white solid in 20% overall yield (two steps) from **6**. ¹H NMR (300 MHz, CDCl₃) δ 8.59 (s, 2H), 7.32 – 7.24 (m, 7H), 7.13 (d, *J* = 6.8 Hz, 2H), 6.85 (s, 1H), 6.63 (d, *J* = 7.9 Hz, 1H), 6.32 (s, 1H), 5.02 (d, *J* = 6.8 Hz, 1H), 4.79 (d, *J* = 13.0 Hz, 2H), 3.73 (s, 3H), 3.69 (s, 4H), 3.58 (s, 2H), 3.22 (m, 4H), 3.05 – 2.97 (m, 1H), 2.85 (t, *J* = 12.3 Hz, 2H), 2.57 (s, 4H), 1.89 (s, 1H), 1.78 (d, *J* = 12.6 Hz, 2H), 1.16 (s, 2H), 0.91 (d, *J* = 6.6 Hz, 6H); ¹³C NMR (126 MHz, CDCl₃) δ 172.42, 167.87, 164.38, 161.72, 159.97, 157.48, 157.23, 156.14, 153.70, 135.82, 130.35, 129.85, 129.22, 128.71, 127.73, 127.63, 127.27, 115.54, 114.81, 105.43, 103.94, 66.04, 62.72, 53.39, 53.12, 52.54, 44.75, 43.88, 42.07, 37.81, 36.40, 25.98, 22.42; ESI-MS: 818 [M + H]⁺; HRMS-ESI: *m/z* [M + H]⁺ calcd for C₄₅H₅₂N₇O₈: 818.3877, found: 818.3878; [α]_D²⁰ = -32.1 (*c* 0.053, CHCl₃).

4.1.26 (R)-Methyl 2-(2-(4-((5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholino methyl)phenyl)isoxazole-3-carboxamido)methyl)piperidin-1-yl)pyrimidine-5-carboxamido)-3-methylbutanoate (14d**)**

This compound was obtained as white solid in 28% overall yield (two steps) from **6**. ¹H NMR (300 MHz, CDCl₃) δ 8.68 (s, 2H), 7.28 (s, 4H), 6.82 (s, 1H), 6.76 (s, 1H), 6.33 (s, 1H), 4.78 (d, *J* = 10.7 Hz, 2H), 4.72 – 4.65 (m, 1H), 3.74 (s, 3H), 3.68 (s, 4H), 3.57 (s, 2H), 3.27 (s, 2H), 3.06 – 2.97 (m, 1H), 2.83 (d, *J* = 12.0 Hz, 2H), 2.56 (s, 4H), 2.23 (d, *J* = 6.1 Hz, 1H), 1.88 (s, 1H), 1.77 (d, *J* = 10.5 Hz, 2H), 1.19 (s, 2H), 1.01 – 0.94 (m, 6H), 0.90 (d, *J* = 6.6 Hz, 6H); ¹³C NMR (151 MHz, CDCl₃) δ 173.00, 167.96, 164.87, 161.67, 160.08, 157.54, 157.30, 156.11, 153.77, 130.30, 129.82, 127.82, 127.58, 115.59, 114.91, 105.36, 103.85, 66.06, 62.74, 57.43, 53.10, 52.36, 43.90, 42.10, 36.36, 31.33, 25.93, 22.42, 19.05, 18.15; ESI-MS: 768 [M - H]⁺; HRMS-ESI:

m/z $[M - H]^+$ calcd for $C_{41}H_{50}N_7O_8$: 768.3721, found: 768.3733; $[\alpha]_D^{20} = -46.8$ (c 0.056, $CHCl_3$).

4.1.27 (R)-Methyl 2-(6-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamido)hexanamido)-3-phenylpropanoate (14e**)**

This compound was obtained as white solid in 37% overall yield (two steps) from **6**. 1H NMR (300 MHz, $CDCl_3$) δ 7.37 – 7.30 (m, 5H), 7.25 – 7.20 (m, 2H), 7.08 (d, $J = 6.3$ Hz, 2H), 6.83 (s, 1H), 6.29 (s, 1H), 6.21 (d, $J = 7.8$ Hz, 1H), 4.88 (m, 1H), 3.71 (s, 7H), 3.54 (s, 2H), 3.35 (s, 2H), 3.19 – 3.00 (m, 3H), 2.52 (s, 4H), 2.20 (t, $J = 7.1$ Hz, 2H), 1.61 (d, $J = 7.1$ Hz, 4H), 1.32 (d, $J = 7.1$ Hz, 2H), 0.87 (d, $J = 6.8$ Hz, 6H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 173.29, 172.17, 167.57, 159.45, 157.46, 156.21, 153.57, 135.68, 130.41, 129.73, 129.19, 128.65, 127.90, 127.34, 127.21, 115.39, 105.22, 104.01, 66.39, 62.99, 53.35, 53.14, 52.48, 39.28, 37.80, 36.03, 28.89, 26.15, 25.84, 25.08, 22.35; ESI-MS: 711 $[M - H]^+$; HRMS-ESI: m/z $[M - H]^+$ calcd for $C_{40}H_{47}N_4O_8$: 711.3394, found: 711.3420; $[\alpha]_D^{20} = -40.4$ (c 0.047, $CHCl_3$).

4.1.28 (R)-Methyl 2-(6-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamido)hexanamido)-3-methylbutanoate (14f**)**

This compound was obtained as white solid in 11% overall yield (two steps) from **6**. 1H NMR (300 MHz, $CDCl_3$) δ 7.32 (s, 4H), 6.83 (s, 1H), 6.32 (s, 1H), 6.25 (d, $J = 9.2$ Hz, 1H), 4.56 (s, 1H), 3.73 (s, 7H), 3.55 (s, 2H), 3.38 (s, 2H), 3.02 (s, 1H), 2.54 (s, 4H), 2.28 (s, 2H), 2.19 – 2.10 (m, 1H), 1.65 (d, $J = 13.8$ Hz, 4H), 1.43 (s, 2H), 0.90 (m, 12H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 173.64, 172.65, 167.52, 159.35, 157.46, 156.23, 153.57, 130.46, 129.71, 127.93, 127.25, 115.38, 105.22, 104.04, 66.52, 63.05, 57.17, 53.41, 52.32, 39.31, 36.25, 31.23, 28.96, 26.28, 25.84, 25.30, 22.35, 18.96, 17.88; ESI-MS: 663 $[M - H]^+$; HRMS-ESI: m/z $[M - H]^+$ calcd for $C_{36}H_{47}N_4O_8$: 663.3394, found: 663.3412; $[\alpha]_D^{20} = -1.8$ (c 0.054, $CHCl_3$).

4.1.29 (R)-Methyl 12-benzyl-1-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholinomethyl)phenyl)isoxazol-3-yl)-1,10-dioxo-5,8-dioxo-2,11-diazatridecan-13-oate (**14g**)

This compound was obtained as white solid in 44% overall yield (two steps) from **6**. ¹H NMR (300 MHz, CDCl₃) δ 7.50 (s, 1H), 7.28 (s, 5H), 7.23 (s, 2H), 7.11 (d, *J* = 6.7 Hz, 2H), 6.84 (s, 1H), 6.18 (s, 1H), 4.95 – 4.87 (m, 1H), 3.99 (s, 2H), 3.68 (s, 7H), 3.56 (s, 8H), 3.52 (s, 2H), 3.15 – 3.09 (m, 2H), 3.05 – 2.97 (m, 1H), 2.51 (s, 4H), 0.90 (d, *J* = 6.7 Hz, 6H); ¹³C NMR (151 MHz, CDCl₃) δ 172.05, 170.13, 167.63, 159.95, 157.16, 156.13, 153.63, 135.73, 130.29, 129.71, 129.26, 128.60, 127.68, 127.17, 115.60, 105.49, 103.92, 70.92, 70.25, 70.04, 69.65, 66.24, 62.93, 53.27, 52.75, 52.55, 39.25, 38.00, 25.94, 22.42; ESI-MS: 745 [M + H]⁺; HRMS-ESI: *m/z* [M + H]⁺ calcd for C₄₀H₄₉N₄O₁₀: 745.3449, found: 745.3440; [α]_D²⁰ = -13.0 (*c* 0.062, CHCl₃).

4.1.30 (R)-Methyl 1-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholinomethyl)phenyl)isoxazol-3-yl)-12-isopropyl-1,10-dioxo-5,8-dioxo-2,11-diazatridecan-13-oate (**14h**)

This compound was obtained as white solid in 38% overall yield (two steps) from **6**. ¹H NMR (300 MHz, CDCl₃) δ 7.56 (s, 1H), 7.30 (s, 4H), 6.84 (s, 1H), 6.27 (s, 1H), 4.57 (m, 1H), 4.06 (m, 2H), 3.71 (s, 9H), 3.66 (s, 6H), 3.59 (s, 2H), 3.03 (m, 1H), 2.58 (s, 4H), 2.17 (m, 1H), 0.92 (t, *J* = 5.8 Hz, 12H); ¹³C NMR (126 MHz, CDCl₃) δ 172.48, 170.30, 167.68, 160.03, 157.25, 156.15, 153.67, 130.31, 129.80, 129.53, 127.68, 127.60, 115.52, 105.42, 103.90, 71.02, 70.29, 70.09, 69.74, 66.00, 62.71, 56.71, 53.08, 52.36, 39.26, 31.35, 25.95, 22.43, 18.94, 17.92; ESI-MS: 697 [M + H]⁺; HRMS-ESI: *m/z* [M + H]⁺ calcd for C₃₆H₄₉N₄O₁₀: 697.3449, found: 697.3447; [α]_D²⁰ = -4.0 (*c* 0.050, CHCl₃).

4.1.31 (R)-Methyl 15-benzyl-1-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-

(morpholinomethyl)phenyl)isoxazol-3-yl)-1,13-dioxo-5,8,11-trioxa-2,14-diazahexadecan-16-oate (14i)

This compound was obtained as white solid in 36% overall yield (two steps) from **6**. ¹H NMR (300 MHz, CDCl₃) δ 7.44 (s, 1H), 7.29 (s, 4H), 7.25 (d, *J* = 9.7 Hz, 3H), 7.12 (d, *J* = 6.7 Hz, 2H), 6.84 (s, 1H), 6.20 (s, 1H), 4.86 (m, 1H), 3.99 (s, 2H), 3.67 (s, 7H), 3.61 (s, 10H), 3.49 (d, *J* = 12.0 Hz, 4H), 3.06 (m, 3H), 2.50 (s, 4H), 0.90 (d, *J* = 6.8 Hz, 6H); ¹³C NMR (151 MHz, CDCl₃) δ 171.95, 170.39, 167.64, 159.88, 157.10, 156.13, 153.64, 135.87, 130.32, 129.64, 129.22, 128.60, 127.72, 127.65, 127.14, 115.66, 105.58, 103.98, 70.92, 70.41, 70.32, 70.23, 70.22, 69.41, 66.36, 63.01, 53.34, 52.82, 52.44, 39.29, 37.83, 25.94, 22.43; ESI-MS: 787 [M - H]⁺; HRMS-ESI: *m/z* [M - H]⁺ calcd for C₄₂H₅₁N₄O₁₁: 787.3554, found: 787.3577; [α]_D²⁰ = -16.7 (*c* 0.048, CHCl₃).

4.1.32 (R)-Methyl 1-(5-(2,4-dihydroxy-5-isopropylphenyl)-4-(4-(morpholinomethyl)phenyl)isoxazol-3-yl)-15-isopropyl-1,13-dioxo-5,8,11-trioxa-2,14-diazahexadecan-16-oate (14j)

This compound was obtained as light yellow solid in 31% overall yield (two steps) from **6**. ¹H NMR (300 MHz, CDCl₃) δ 7.41 (s, 1H), 7.22 (s, 4H), 6.76 (s, 1H), 6.14 (s, 1H), 4.46 (m, 1H), 4.00 (d, *J* = 2.8 Hz, 2H), 3.66 – 3.54 (m, 19H), 3.45 (s, 2H), 3.01 – 2.89 (m, 1H), 2.44 (s, 4H), 2.10 (m, 1H), 0.84 (m, 12H); ¹³C NMR (151 MHz, CDCl₃) δ 172.32, 170.62, 167.67, 159.90, 157.13, 156.12, 153.65, 130.32, 129.64, 127.71, 127.64, 115.64, 105.55, 103.98, 71.08, 70.45, 70.38, 70.29, 70.28, 69.43, 66.37, 63.02, 56.81, 53.34, 52.27, 39.30, 31.10, 25.93, 22.42, 18.99, 17.94; ESI-MS: 739 [M - H]⁺; HRMS-ESI: *m/z* [M - H]⁺ calcd for C₃₈H₅₁N₄O₁₁: 739.3554, found: 739.3566; [α]_D²⁰ = -9.4 (*c* 0.053, CHCl₃).

4.2 Fluorescence polarization (FP) enzymatic assay

In vitro inhibitory activity of compounds on Hsp90 was carried out in 384-well plates by fluorescence polarization assay using Hsp90 α and Geldanamycin-FITC (Abcam# ab141589, Cambridge, USA) according to the manufacturer's instructions. The assay buffer contained 20 μ M HEPES pH 7.3, 50 μ M KCl, 5 μ M MgCl₂, 20 μ M Na₂MoO₄, 0.01% Triton X- 100. Before each use, 100 μ g/mL BSA and 2 μ M DTT were freshly added. In brief, 10 μ L of 5 nM FITC-labeled geldanamycin and 10 μ L appropriate serials of diluted compounds was added to the plate. The reactions were initiated by adding 20 μ L of Hsp90 α recombinant enzyme (22.2 nM), after 1 hours incubation in 4 °C, the Fluorescence was measured with excitation wavelength at 485 nm and emission wavelength at 530 nm on EnVision Multilabel Reader (Perkin Elmer, Inc.). The data were analyzed using Graphpad Prism5 (Graphpad Software, Inc).

4.3 Cell proliferative assay

Proper amount of Cells were seeded into 96-well plates and grown for 24 h. Cells were then treated with increasing concentrations of compounds and grown for further 72 h. At the end of exposure time, cell proliferation was determined using sulforhodamine B methods, measured by multiwall spectrophotometer (SpectraMax, Molecular Devices, U.S.A). The inhibition rate was calculated as $(1 - A_{515} \text{ treated} / A_{515} \text{ control}) \times 100\%$. The cytotoxicity of compounds was expressed as an IC₅₀, determined by the Logit method.

4.4 Western blot analysis

Tumor cells were cultured under regular growth conditions to exponential growth phase. Then the cells were treated with the indicated concentration of compounds for 24 h at 37 °C and lysed in 1 $\times$ SDS sample buffer. Those cell lysates were subsequently resolved on 10% SDS-PAGE, and transferred to nitrocellulose membranes. The blots were incubated with specific primary antibodies, then

subsequently with anti-rabbit or anti-mouse IgG horseradish peroxidase. Immunoreactive proteins were detected using an enhanced chemiluminescence detection reagent.

4.5 Molecular docking

Docking study was performed using Maestro 9.3. X-ray cocrystal structure of Hsp90 N-terminal (2VCI) was downloaded from RCSB Protein Data Bank and prepared by Protein Preparation Wizard Workflow in the Schrödinger program suite. Compound **14j** was prepared by Ligand Preparation and docked into the defined binding site without constraint. Based on the Glide-score, top ranking compounds were submitted and generated by Pymol.

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Graphic Abstract

**Design, Synthesis and Pharmacological Evaluation of
4,5-Diarylisoxazols Bearing Amino Acid Residues within the 3-Amido
Motif as Potent Heat Shock Protein 90 (Hsp90) Inhibitors**

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Figure 1. Reported and our new designed Hsp90 inhibitors. A, co-crystal structure of AUY922 with ATP binding site of hHsp90; B, amplification of the interaction domain of the 5-amido moiety.

Figure 2. Effects of **14j** on degradation of client onco-proteins of NCI-H3122 cell lines. NCI-H3122 cells were treated with **14j** or **3** (AUY922) at 11.1, 33.3 and 100 nM for 24 h. Cell lysates were prepared and analyzed with immunoblotting.

Figure 3. Binding mode of compound **14j** with Hsp90 N-terminal ATP pocket.

Table 1. Hsp90 binding activity and cell antiproliferative effects of compounds **8a-g**.^{*}

<div> <p>8a-g</p> </div>			
Compound	R	IC ₅₀ (μM)	
		Hsp90α	NCI-H3122 cells
8a		0.079	0.028
8b		0.151	0.019
8c		0.514	4.471
8d		0.836	>10
8e		0.072	0.227
8f		0.260	0.077
8g		0.368	0.149
3 (AUY922)	/	0.022	0.014

^{*} The IC₅₀ values are shown as the mean (μM) from two separate experiments.

Table 2. Hsp90 binding activity and cell antiproliferative effects of compounds **10a-m**.^{*}

<div> <p>10a-m</p> </div>			
Compound	R	IC ₅₀ (μM)	
		Hsp90α	NCI-H3122 cell
10a		0.189	0.002
10b		0.112	<0.001
10c		0.021	0.039
10d		0.028	0.034
10e		0.106	0.081
10f		0.039	0.037
10g		0.429	0.149
10h		0.206	0.139
10i		0.397	0.079

10j		0.206	>10
10k		0.285	>10
10l		0.259	0.034
10m		0.528	0.638
3	/	0.022	0.014

* The IC₅₀ values are shown as the mean (μM) from two separate experiments.

Table 3. Hsp90 binding activity and cell antiproliferative effects of compounds **14a-j**.^{*}

14a-j				
Compound	X	R	IC ₅₀ (μM)	
			Hsp90α	NCI-H3122 cell
14a			>1	0.164
14b			0.473	0.135
14c			0.749	1.128
14d			0.407	0.278
14e			0.307	0.090
14f			0.098	0.065
14g			>1	1.534

14h			0.154	0.090
14i			0.122	0.040
14j			0.014	0.042
3	/	/	0.022	0.014

* The IC₅₀ values are shown as the mean (μM) from two separate experiments.

Table 4. Antiproliferative effects of compound **14j** in various cancer cell lines.*

Cancer cell	IC ₅₀ (μM)
NCI-H3122 (NSCLC)	0.042
A549 (lung cancer)	0.351
NCI-H460 (NSCLC)	0.247
HCT-116 (colorectal cancer)	0.116
BT-474 (breast cancer)	0.057
MDA-MB-231 (breast cancer)	0.130
DU-145 (prostate cancer)	0.239
NCI-N87 (gastric cancer)	0.564
U87-MG (glioma cancer)	0.109

* Various cancer cells were treated with increasing concentrations of compound **14j** and grown for further 72 h. The cytotoxicity of compounds was expressed as an IC₅₀, determined by the Logit method. The IC₅₀ values are shown as the mean (μM) from two separate experiments.

Figure 1. Reported and our new designed Hsp90 inhibitors. A, co-crystal structure of AUY922 with ATP binding site of hHsp90; B, amplification of the interaction domain of the 5-amido moiety.

Figure 2. Effects of **14j** on degradation of client onco-proteins of NCI-H3122 cell lines. NCI-H3122 cells were treated with **14j** or **3** (AUY922) at 11.1, 33.3 and 100 nM for 24 h. Cell lysates were prepared and analyzed with immunoblotting.