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Inhibition of Hsp90 with Resorcylic Acid Macrolactones: Synthesis and Binding Studies

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Abstract: A series of resorcylic acid macrolactones, analogues of the natural product radicicol has been prepared by chemical synthesis, and evaluated as inhibitors of heat shock protein 90 (Hsp90), an emerging attractive target for novel cancer therapeutic agents. The synthesis involves acylation of an *ortho*-toluic acid dianion, esterification, followed by a ring-closing metathesis to form the macrocycle. Subsequent manipulation of the protected hydroxymethyl side chain allows access to a range of new analogues following deprotection of the two phenolic groups. Co-crystallization of one of the new macrolactones with the *N*-terminal domain of yeast Hsp90 confirms that it binds in a similar way to the natural product radicicol and to our previous synthetic analogues, but that the introduction of the additional hydroxymethyl substituent appears to result in an unexpected change in conformation of the macrocyclic ring. As a result of this conformational change, the compounds bound less favorably to Hsp90.

Keywords: anticancer agents · enzyme inhibitors · macrocycles · macrocyclic compounds · metathesis · proteins

Introduction

One of the most promising cancer therapeutic strategies to emerge in recent years is the inhibition of heat shock protein 90 (Hsp90),[1–9] an abundant ATP-dependent chaperone that is central to the regulation and maintenance of a range of proteins, its so-called clients proteins.[5,10] Although many of these clients are essential to normal cellular processes, others are known oncogenic proteins such as CRAF, BRAF, ERBB2, AKT, telomerase and mutant p53. Hence inhibition of Hsp90 can cause client proteins to adopt abnormally folded conformations that are rapidly ubiquinated and subject to proteasomal degradation, and thereby disrupt multiple cancer causing pathways simultaneously. The approach has been successfully validated by the entry of at least 12 Hsp90 inhibitors into clinical trial.[11,12]

As with many other areas of medicinal chemistry, initial leads came from the natural world. Two natural products, geldanamycin (**1**) and radicicol (**2**) (see below) were discovered to be potent inhibitors of Hsp90, binding to the ATP-binding domain in the *N*-terminal region of the protein.[13–15] Although subsequent work has led to the discovery of further ansamycin geldanamycin analogues, three of which are now in clinical trial,[12] the early promise of radicicol, a member of the resorcylic acid lactone family,[16–19] against Hsp90 has yet to translate into a drug candidate molecule based on the molecular framework of the natural product. Whilst remaining one of the most potent Hsp90 inhibitors in vitro,[20] radicicol has little or no activity in vivo,[21,22] presumably as a result of its reactive epoxide and dienone moieties. Nevertheless, the compound continues to attract considerable attention, and a number of naturally derived and synthetic analogues, some of which do show in vivo activity, have been described.[23–28]

Our own work in this area has involved the synthesis and biological evaluation of a series of novel resorcylic acid macrolactones of varying ring size and conformation.[29] One of

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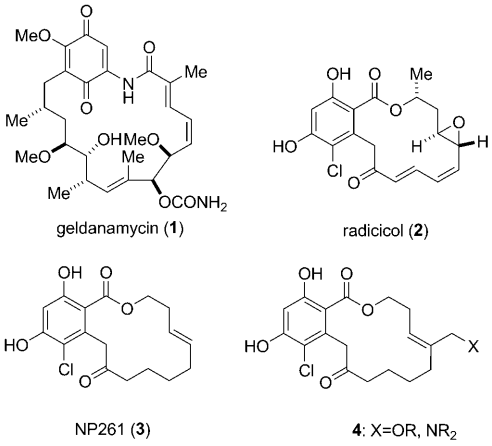
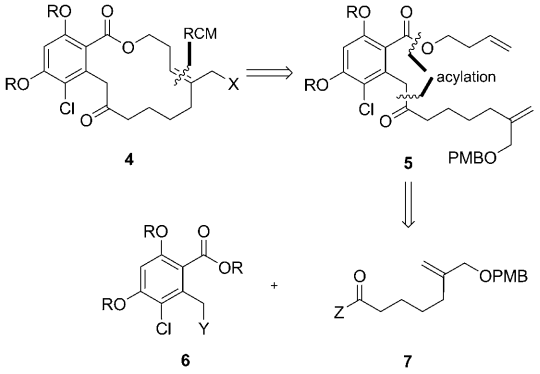




Scheme 1. Retrosynthetic analysis of the resorcylic acid macrolactones **4**.

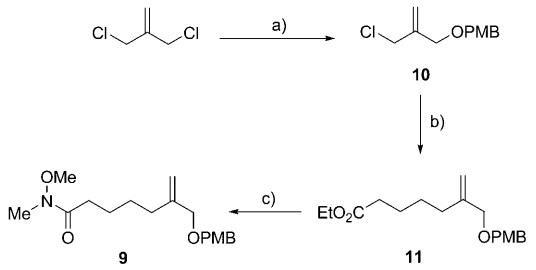
these analogues, NP261 (**3**), exhibited potent Hsp90 binding, with an $IC_{50} = \sim 40$ nM as measured by a fluorescence polarization (FP) binding assay, and in addition showed the established molecular signature of Hsp90 inhibitors, that is, depletion of client proteins with upregulation of Hsp70 in treated cancer cells. X-Ray crystallography studies revealed that **3** also was bound to the *N*-terminal ATP site of Hsp90 in a comparable way to the structurally more complex natural product.[29] The molecule adopts a similar folded conformation with the same key hydrogen-bonding interactions between the salicylate ester and phenolic groups and the protein (the carboxylate side-chain of Asp79, the main-chain amide group of Gly83, the hydroxyl side-chain of Thr171, the main-chain carbonyl of Leu34, and conserved water molecules; yeast Hsp90 numbering) (Figure 2C), although it obviously lacks the interaction with the ε-amino side-chain of Lys44, present through the epoxide oxygen in the natural product. In an attempt to restore this potentially useful H-bonding interaction, we now report the synthesis of a new series of resorcylic acid macrolactones incorporating heteroatoms, with the potential to bind to the side chain of Lys44, thereby increasing the potential potency of the compounds.[30]

Results and Discussion

It was clear from our earlier studies that an additional substituent on the macrolactone ring was required to form an H-bond to the side-chain of Lys44,[29,30] and examination of the Hsp90 bound structure of **3** suggested that compounds of the general structure **4** (X = OR, NR_2) might possess the desired characteristics, and therefore attention was focused on their synthesis. Many recent routes to resorcylic acid macrolactones, including our own,[29] rely on a ring-closing metathesis (RCM) reaction to form the macrocyclic ring, and given the reliability of this approach we saw no reason to change our strategy. Our approach to the required 14-membered macrolactone is outlined in Scheme 1, and incorporates an allylic alcohol protected with a *p*-methoxybenzyl (PMB) group to enable subsequent manipulation of this substituent. In common with our recent approach based on isocoumarins,[30] it also incorporates the chlorine atom from the start to obviate the need for late stage chlorination of every compound. Hence the problem reduces to the synthesis of the benzylic ketone **5**, and aside of the aforementioned approach based on isocoumarins, used by Lett et al. in the original synthesis of radicicol,[31,32] and Danishefsky's elegant use of the Diels–Alder reaction to construct the benzene ring,[33] most routes disconnect at the benzylic bond by some sort of acylation (or equivalent) reaction. Thus, benzylic chlorides **6** (Y = Cl) have been coupled with 1,3-dithiane anions, equivalent to an acyl anion derived from carbonyl compound **7** (Z = H),[29,34] or toluate anions derived from **6** (Y = H) have been acylated with, for example, Weinreb amides **7** (Z = NMeOMe). This latter approach, extensively employed by Winssinger and colleagues,[26,28,35–37] has proved successful for a range of resorcylic acid lactone derivatives, and therefore seemed ideally suited to our needs, requiring the known toluate ester **8** featuring ethoxymethyl protecting groups for the two phenols,[37] and Weinreb amide **9** as starting materials.

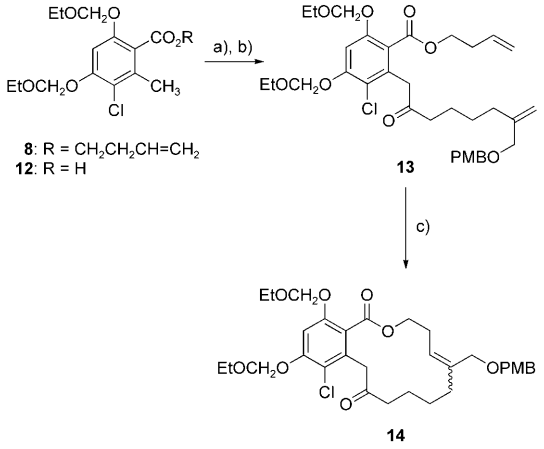
The Weinreb amide **9** could be prepared in three steps as shown in Scheme 2. 3-Chloro-2-(chloromethyl)prop-1-ene was reacted with the sodium salt of *p*-methoxybenzyl alcohol in THF to give the known 3-chloro-2-(4-methoxybenzyloxy)methylpropene (**10**).[38] Copper-catalyzed reaction[39] of the allyl chloride **10** and the organozinc derived from ethyl iodobutyrate[40] gave ester **11** in good yield. Direct conversion into the Weinreb amide **9** was achieved in excellent yield using the isopropylmagnesium chloride mediated reaction with *N*,*O*-dimethylhydroxylamine hydrochloride developed by Williams et al.[41]

The known *ortho*-toluate ester **8**[37] in THF was treated with LDA (2.5 equiv) at −78 °C, and the deep red colored solution was immediately treated with the Weinreb amide **9**. However, after work-up none of the desired acylated product was isolated. When the corresponding isopropyl ester was used, a mixture of decomposition products was observed. Previous studies with toluate anions have shown that



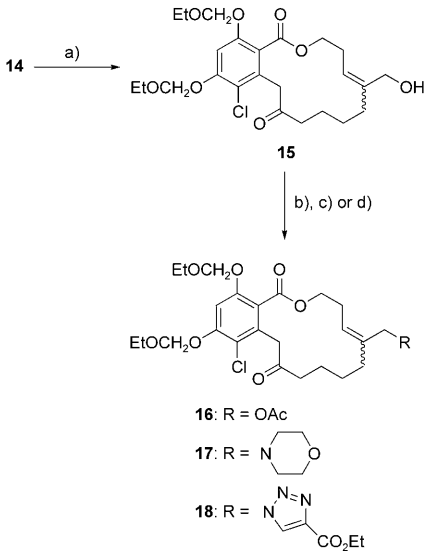
Scheme 2. a) NaH, 4-$MeOC_6H_4CH_2OH$, THF, 0°C to RT (56%); b) $EtO_2CCH_2CH_2CH_2ZnI$ (from the iodide, Zn in DMA), DMA/THF, CuCN, 70°C (68%); c) i) MeONHMe.HCl, *i*PrMgCl, THF, −20 to −5°C; ii) saturated aq. NH_4Cl, −5°C to RT (92%).

by-products such as those resulting from self condensation are common in such reactions.[42] In order to circumvent the formation of decomposition products, it was reasoned that use of the dianion from an *ortho*-toluic acid instead of the ester-derived monoanion might be preferable, since such dianions have been successfully used in synthesis previously.[43–46] Thus the corresponding toluic acid **12**[47] was treated with 2.2 equivalents of *sec*-butyllithium. Quenching the resulting dilithiated toluate with the Weinreb amide **9** resulted in a mixture of starting acid **12** and acylated material after work-up. The crude mixture was reacted with 3-butenol under Mitsunobu conditions [diisopropyl azodicarboxylate (DIAD), Ph_3P and THF], and product could be easily separated to give the required ester **13** in 41% yield over two steps. Ring-closing metathesis using Grubbs' second-generation catalyst {benzylidene[1,3-bis(2,4,6,-trimethylphenyl)-2-imidazolidinylidene]dichloro(tricyclohexylphosphine)ruthenium} proceeded in excellent yield to give the desired macrocycle **14**, unfortunately as an inseparable mixture (48:52) of double bond isomers (Scheme 3). The reaction was most conveniently carried out on about 50 mg scale since increasing the scale of the reaction (>200 mg) resulted in much longer reaction times and reduced yields.



Scheme 3. For compound **12**, a) i) *s*BuLi, THF, −78°C; ii) **9**, THF, −78°C; b) DIAD, PPh_3, THF, $HOCH_2CH_2CHCH_2$, 0°C to RT (41% over two steps); c) Grubbs' II catalyst, CH_2Cl_2, 45°C (92%).

The PMB-protecting group could be removed oxidatively from macrocycle **14** using cerium(IV) ammonium nitrate (CAN) to give allyl alcohol **15** in reasonable yield (Scheme 4), ready for conversion into other substituents.

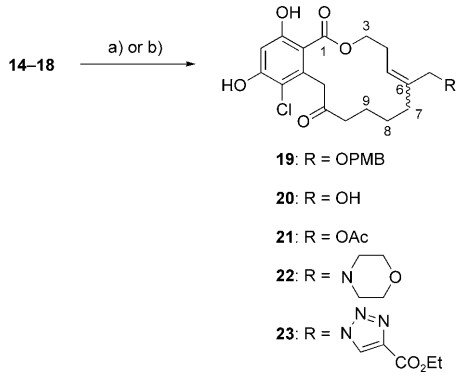


Scheme 4. a) CAN, MeCN/H_2O, 0°C (54%); b) Ac_2O, NEt_3, CH_2Cl_2 (75%); c) i) MsCl, NEt_3, CH_2Cl_2, 0°C; ii) LiBr, THF/CH_2Cl_2, 0°C; iii) morpholine, NaI, *i*Pr_2NEt, DMF (75% over three steps); d) i) MsCl, NEt_3, CH_2Cl_2, 0°C; ii) NaN_3, DMF; iii) $EtO_2CC{\equiv}CH$, $CuSO_4$, Na-ascorbate, *t*BuOH (20% over three steps).

Thus, allyl alcohol **15** could be acetylated using acetic anhydride to give acetate **16**, or mesylated and then reacted with lithium bromide in a mixture of tetrahydrofuran and dichloromethane. The resulting allyl bromide was reacted with morpholine in the presence of sodium iodide and Hunig's base to give tertiary amine **17** in good yield over three steps. In addition, the mesylate was reacted with sodium azide and the product subjected to the copper-catalyzed Huisgen cycloaddition[48] with ethyl propiolate to give triazole **18** in 20% over three steps.

Removal of the EOM-protecting groups from compounds **14**–**18** using TFA in dichloromethane or HCl in dioxane was readily achieved in moderate to excellent yield (26–98%) to give a range of novel radicicol analogues **19**–**23** (Scheme 5). After deprotection of PMB-ether **14**, both *Z* and *E* isomers of compound **19** could be separated by chromatography, the double-bond assignment being based on NOE spectroscopy. Thus, in the *Z* isomer an NOE enhancement was seen between the alkene proton at C5 and the methylene at C7. Also, the *E* isomer of allyl alcohol **20** was successfully recrystallized (from the *E*/*Z* mixture) and its structure confirmed by X-ray crystallography (Figure 1).

The macrocycle **20** was co-crystallized (as its *Z* isomer from the *E*/*Z* mixture) with the *N*-terminal domain of yeast Hsp90, and the structure of the resulting complex solved by molecular replacement. Comparison with the previously determined structure of Hsp90-bound NP261 **3**[29] showed that



Scheme 5. a) TFA, CH_2Cl_2; b) HCl, dioxane.

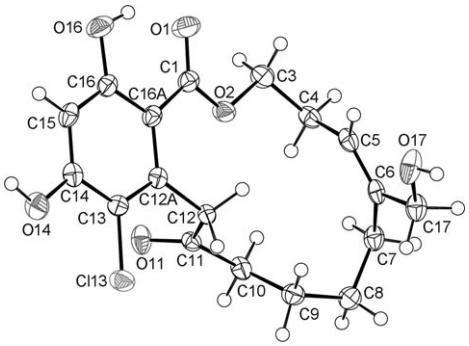
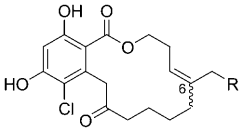


Figure 1. X-ray crystal structure of the *E* isomer of resorcylic acid macrolactone **20**.

the compounds bind with overall similarity, except for the macrolactone ring between carbons atoms C6 to C9 (same numbering as Figure 1), where a distinctly different conformation is adopted (Figure 2B). Unfortunately, the electron density for macrocycle **20** in this region of the macrolactone ring was weak relative to that for the structure of **3**, and therefore the H-bonding interactions with the protein are less easy to define with certainty. Figure 2C shows all the possible bonding interactions for the most populated conformation of **20**, and illustrates that our planned H-bond between the side chain of Lys44 and the hydroxymethyl substituent at C6 is indeed present, notwithstanding the fact that the macrocyclic ring now adopts an unexpected conformation. The glycerol molecule, which was found in complex with **20**, probably originates from the cryoprotection of the crystals and forms a number of hydrogen bounds with compound **20**. It is likely that these bonds were also previously present, but mediated via a water molecule, that may have then been displaced by the incoming glycerol molecule.

The macrocyclic lactones **19–23** were evaluated for binding to the ATP site of the *N*-terminal domain of human Hsp90β in two Hsp90 assays: the fluorescence polarization (FP) assay,[49,50] and the TR-FRET assay.[51] Their growth inhibitory activity against a human colon cancer cell line (HCT116) was also measured by the SRB assay. As can be seen in Table 1, introduction of groups into the 6-position of

Table 1. Biological activity of the macrocyclic lactones **19–23** compared to radicicol **2** and NP261 **3**.



Entry	Compound	R	TR-Fret IC_{50} [μM]	FP IC_{50} [μM]	HCT116 SRB GI_{50} [μM]
1	**2**	–	0.011	0.0043	0.00061
2	**3**	–	0.35	0.038	7.6
3	(*E*)-**19**	OPMB	18% @ 10 μM	0.85	9.4
4	(*Z*)-**19**	OPMB	7.70	1.65	8.2
5	**20**	OH	0.90	0.65	45
6	**21**	OAc	4.20	1.90	10
7	**22**	morpholinyl	32.5% @ 10 μM	4.80	34
8	**23**	triazolyl	9.60	3.85	43

the macrocyclic ring results in a significant reduction in binding to Hsp90 compared to the quite potent (IC_{50} ~40 nM in FP assay) NP261 **3**. However, compounds **19–23** are only marginally less potent than **3** in the SRB growth inhibition assay. The loss of potency in inhibition of Hsp90 is presumably a result of the change in conformation of the macrolactone ring upon introduction of the substituent at C6, something that was not foreseen in our original analysis.

Conclusion

In summary, Hsp90 is an important drug target for molecular cancer therapeutics, and we have previously synthesized and evaluated a simple analogue of the natural product radicicol known as NP261 **3**. In an attempt to increase potency by introducing additional H-bonding substituents, we decided to incorporate heteroatom groups at the C6-position of the NP261 macrocycle. Unfortunately, as demonstrated by X-ray crystallography, this induced a significant and unexpected change in conformation of the macrocyclic ring, resulting in compounds that bound less favorably than the unsubstituted ring. The unexpected change in ring conformation suggests an alternative approach to the design of analogues of macrolactone **3** for Hsp90 inhibition.

Experimental Section

Full experimental procedures and spectroscopic characterization data are given in the Supporting Information. Data for the compounds that were evaluated in biological assays are given below.

(*Z*) and (*E*)-13-Chloro-14,16-dihydroxy-6-(4-methoxybenzyloxy)methyl-3,4,7,8,9,10-hexahydro-1*H*-benzo[*c*][1]oxacyclotetradecine-1,11(12*H*)-dione (19)

Z Isomer: colorless solid; m.p. 142–144 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 11.72 (s, 1H, OH), 7.20 (d, 2H, J = 8.4 Hz, ArH), 6.86 (d, 2H, J = 8.4 Hz, ArH), 6.57 (s, 1H, ArH), 6.20 (s, 1H, OH), 5.41 (t, 1H, J = 6.9 Hz, =CH), 4.41 (t, 2H, J = 5.6 Hz, CH_2), 4.37 (s, 2H, CH_2), 4.22 (s,

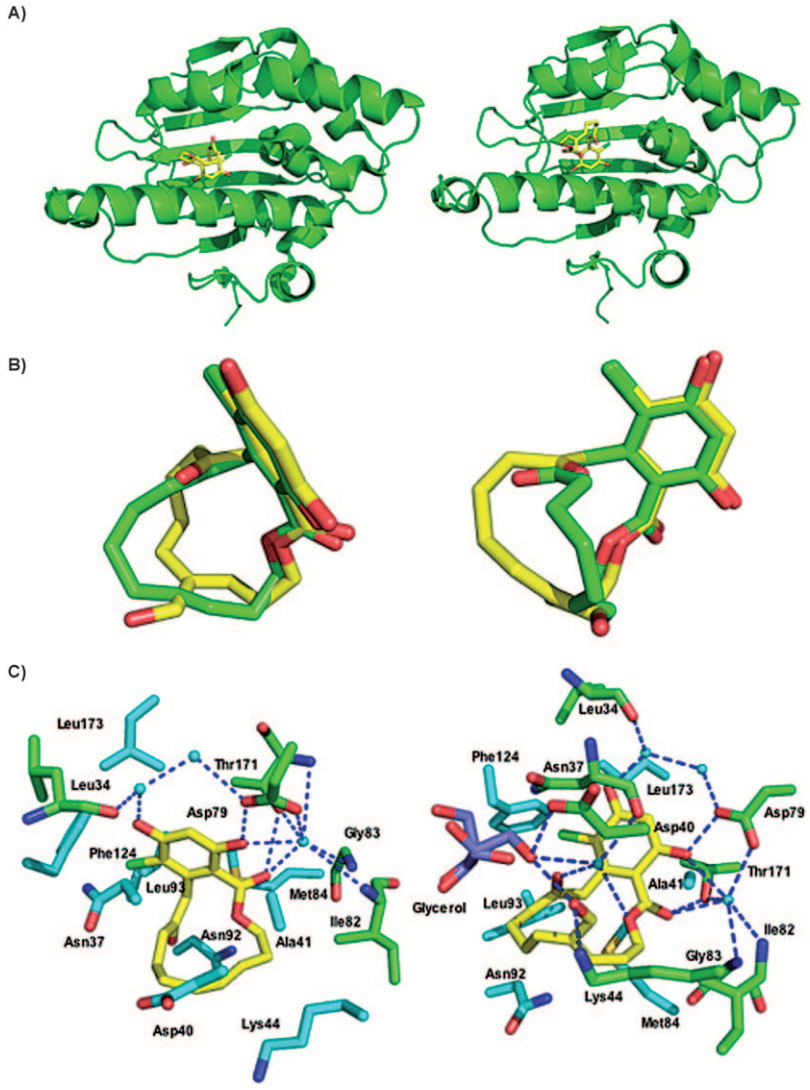


Figure 2. A) Cartoon showing NP261 **3** (left) and macrocycle **20** (right) bound in the *N*-terminal domain of yeast Hsp90. B) Two orthogonal views of the superimposition of NP261 **3** (green) and **20** (yellow), showing that both compounds bind with overall similarity, except for the macrolactone ring of compound **20** between carbons atoms C6 to C9 (same numbering as Figure 1). The structures for both compounds were obtained at 2.2 Å resolution. For atomic coordinates and structure factors, see PDB codes 2CGF and 2XD6, respectively. C) PyMOL diagram showing binding interactions of NP261 **3** (left) and **20** (right). The electron density for macrocycle **20** from carbon 6 to 9 of the macrolactone ring was very weak relative to that for NP261 structure. However, we have shown all the possible bonding interactions for the most populated conformation of **20**. It should be noted that bond interactions to the disordered section of **20** are therefore weak. Dotted blue lines, hydrogen bonds; green, amino acid residues involved in H-bonding; cyan-colored spheres, water molecules; cyan residues, residues solely in van der Waals contact; magenta, glycerol molecule.

2H, CH_2), 3.87 (s, 2H, CH_2), 3.81 (s, 3H, CH_3), 2.54–2.49 (m, 4H, 2×CH_2), 2.20–2.17 (m, 2H, CH_2), 1.68 (quin, 2H, J = 6.2 Hz, CH_2), 1.59–1.57 ppm (m, 2H, CH_2); ^{13}C NMR (125 MHz, $CDCl_3$): δ=206.3 (C), 171.1 (C), 163.8 (C), 159.3 (C), 156.2 (C), 138.7 (C), 136.0 (C), 130.3 (C), 129.4 (CH), 125.5 (CH), 114.6 (C), 113.8 (CH), 107.3 (C), 103.6 (CH), 73.9 (CH_2), 72.0 (CH_2), 67.0 (CH_2), 55.3 (CH_3), 46.2 (CH_2), 41.6 (CH_2), 26.9 (CH_2), 26.3 (CH_2), 25.0 (CH_2), 21.8 ppm (CH_2); IR (dichloromethane): ν_{max}=3514, 1721, 1660, 1611, 1590, 1513 cm^{-1}; MS (ESI): *m/z* (%): 513/511 (35/100) $[M+Na]^+$, 508/506 (7/21) $[M+H_4N]^+$; *m/z*: calcd for $C_{26}H_{29}{}^{35}ClNaO_7$: 511.1494; found: 511.1474 $[M+Na]^+$.

***E* Isomer**: colorless solid; m.p. 164–166 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ=7.26 (d, 2H, J = 8.6 Hz, ArH), 6.88 (d, 2H, J = 8.6 Hz, ArH), 6.58 (s, 1H, ArH), 6.16 (s, 1H, OH), 5.51 (t, 1H, J = 6.8 Hz, =CH), 4.44 (s, 2H,CH_2), 4.42 (t, 2H, J = 5.6 Hz, CH_2), 4.30 (s, 2H, CH_2), 3.94 (s, 2H, CH_2), 3.80 (s, 3H, CH_3), 2.63–2.55 (m, 4H, 2×CH_2), 2.21 (t, 2H, J = 6.4 Hz, CH_2), 1.73 (quin, 2H, J = 6.6 Hz, CH_2), 1.58–1.56 ppm (m, 2H, CH_2); phenolic OH not observed; ^{13}C NMR (125 MHz, $CDCl_3$): δ=206.5 (C), 170.5 (C), 163.3 (C), 159.2 (C), 156.3 (C), 138.9 (C), 136.1 (C), 130.2 (CH), 129.3 (CH), 125.9 (CH), 114.7 (C), 113.8 (CH), 107.3 (C), 103.5 (CH), 72.3 (CH_2), 67.3 (CH_2), 66.2 (CH_2), 55.3 (CH_3), 46.8 (CH_2), 40.8 (CH_2), 35.0 (CH_2), 27.0 (CH_2), 23.2 (CH_2), 22.0 ppm (CH_2); IR (dichloromethane): ν_{max}=3684, 3512, 1720, 1657 cm^{-1}; MS (ESI): *m/z* (%): 513/511 (37/100) $[M+Na]^+$, 508/506 (4/2); *m/z*: calcd for $C_{26}H_{29}{}^{35}ClNaO_7$: 511.1494; found: 511.1482 $[M+Na]^+$.

13-Chloro-14,16-dihydroxy-6-hydroxymethyl-3,4,7,8,9,10-hexahydro-1*H*-benzo[*c*][1]oxacyclotetradecine-1,11-(12*H*)-dione (20)

Mixture of double bond isomers 49:51; colorless solid; m.p. 203–206 °C; ^{1}H NMR (400 MHz, [D$_6$]acetone): mixture of isomers δ=9.72 (brs, 1H, OH), 6.53 (s, 1H, ArH), 6.52 (s, 1H, ArH), 5.54 (t, 1H, J = 6.9 Hz, =CH), 5.35 (t, 1H, J = 6.9 Hz, =CH), 4.44 (t, 4H, J = 5.6 Hz, CH_2), 4.40 (s, 2H, CH_2), 4.33 (s, 2H, CH_2), 4.09 (s, 2H, CH_2), 4.05 (s, 2H, CH_2), 2.66–2.56 (m, 8H, 3×CH_2), 2.24–2.19 (m, 4H, 2×CH_2), 1.73 (quin, 2H, J = 6.6 Hz, CH_2), 1.67–1.55 ppm (m, 6H, 3×CH_2); ^{13}C NMR (125 MHz, [D$_6$]acetone): mixture of isomers δ=205.0 (C), 171.4 (C), 170.8 (C), 163.1 (C), 162.8 (C), 158.0 (C), 157.99 (C), 141.6 (C), 141.5 (C), 137.5 (C), 137.3 (C), 123.6 (CH), 122.4 (CH), 115.0 (C), 107.0 (CH), 106.6 (CH), 102.8 (CH), 102.7 (CH), 67.2 (CH_2), 66.4 (CH_2), 65.7 (CH_2), 59.3 (CH_3), 46.1 (CH_2), 45.7 (CH_2), 40.9 (CH_2), 40.1 (CH_2), 33.5 (CH_2), 26.6 (CH_2), 26.4 (CH_2), 25.8 (CH_2), 25.0 (CH_2), 23.0 (CH_2), 22.0 (CH_2), 21.5 ppm (CH_2); IR (dichloromethane): ν_{max}=3552, 3491, 1692, 1645 cm^{-1}; MS (ESI): *m/z* (%): 393/391 (34/100) $[M+Na]^+$; *m/z*: calcd for $C_{18}H_{21}{}^{35}ClNaO_6$: 391.0919; found: 391.0923 $[M+Na]^+$.

(13-Chloro-14,16-dihydroxy-1,11-dioxo-3,4,7,8,9,10,11,12-octahydro-1*H*-benzo[*c*][1]oxacyclotetradecin-6-yl)methyl acetate (21)

Colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$): mixture of isomers δ=6.61 (s, 1H, ArH), 6.60 (s, 1H, ArH), 6.16 (m, 2H, OH), 5.52–5.45 (m, 2H, =CH), 4.55 (s, 4H, CH_2), 4.46 (q, 4H, J = 5.5 Hz, 2×CH_2), 4.31 (s, 2H, CH_2), 4.26 (s, 2H, CH_2), 2.63–2.58 (m, 6H, 3×CH_2), 2.52 (t, 2H, J = 6.4 Hz, CH_2), 2.22–2.16 (m, 4H, 2×CH_2), 2.10 (s, 3H, CH_3), 2.02 (s, 3H, CH_3), 1.82 (m, 2H,CH_2), 1.74 (m, 2H,CH_2), 1.66–1.61 ppm (m, 2H,CH_2);

^{13}C NMR (125 MHz, $CDCl_3$): mixture of isomers δ = 206.5 (C), 206.2 (C), 171.1 (C), 171.0 (C), 170.9 (C), 170.4 (C), 163.8 (C), 163.7 (C), 156.37 (C), 156.36 (C), 136.5 (C), 136.4 (C), 136.0 (C), 135.9 (C), 127.2 (CH), 126.8 (CH), 114.66 (C), 114.63 (C), 107.18 (C), 107.14 (C), 103.6 (CH), 68.0 (CH_2), 66.7 (CH_2), 66.0 (CH_2), 62.2 (CH_2), 46.8 (CH_2), 46.2 (CH_2), 41.6 (CH_2), 40.5 (CH_2), 34.2 (CH_2), 27.0 (CH_2), 26.9 (CH_2), 26.5 (CH_2), 25.0 (CH_2), 22.9 (CH_2), 22.0 (CH_2), 21.7 (CH_2), 21.0 (CH_2), 20.9 ppm (CH_2); IR (dichloromethane): ν_{max} = 3686, 3512, 1732, 1659, 1606 cm^{-1}; MS (ESI): *m*/*z* (%): 435/433 (35/100) $[M+Na]^+$, 430/428 (7/21) $[M+H_4N]^+$; *m*/*z*: calcd for $C_{20}H_{23}{}^{35}ClNaO_7$: 433.1025; found: 433.1024 $[M+Na]^+$.

13-Chloro-14,16-dihydroxy-6-(morpholinomethyl)-3,4,7,8,9,10-hexahydro-1*H*-benzo[*c*][1]oxacyclotetradecine-1,11(12*H*)-dione (22)

Off white foam; m.p. 85–89 °C; ^{1}H NMR (400 MHz, $CDCl_3$): mixture of isomers δ = 11.72 (s, 1H, OH), 11.67 (s, 1H, OH), 6.62 (s, 1H, ArH), 6.60 (s, 1H, ArH), 5.49–5.37 (m, 2H, =CH), 4.42 (t, 4H, *J* = 4.9 Hz, CH_2), 4.31 (s, 2H, CH_2), 4.27 (s, 2H, CH_2), 3.67 (brs, 8H, 4×CH_2), 2.90 (brs, 2H, CH_2), 2.84 (brs, 2H, CH_2), 2.58 (m, 6H, 3×CH_2), 2.48 (t, 2H, *J* = 6.4 Hz, CH_2), 2.36 (brs, 8H, 4×CH_2), 2.20 (m, 4H, 2×CH_2), 1.76–1.59 ppm (m, 16H, 8×CH_2); ^{13}C NMR (125 MHz, $CDCl_3$): mixture of isomers δ = 206.94 (C), 206.86 (C), 171.1 (C), 170.6 (C), 163.7 (C), 163.5 (C), 157.0 (C), 138.1 (C), 137.6 (C), 136.0 (C), 136.0 (C), 126.8 (CH), 126.0 (CH), 115.1 (C), 115.0 (C), 106.9 (C), 106.7 (C), 103.62 (CH), 103.59 (CH), 66.9 (CH_2), 66.7 (CH_2), 66.2 (CH_2), 65.4 (CH_2), 57.4 (CH_2), 53.7 (CH_2), 53.6 (CH_3), 46.7 (CH_2), 46.2 (CH_2), 41.1 (CH_2), 40.7 (CH_2), 35.0 (CH_2), 27.1 (CH_2), 26.93 (CH_2), 26.91 (CH_2), 25.1 (CH_2), 23.1 (CH_2), 22.0 (CH_2), 21.6 ppm (CH_2); IR (dichloromethane): ν_{max} = 3627, 3512, 1720, 1659, 1592 cm^{-1}; MS (ESI): *m*/*z* (%): 440/438 (35/100) $[M+H]^+$; *m*/*z*: calcd for $C_{22}H_{29}{}^{35}ClNO_6$: 438.1683; found: 438.1704 $[M+H]^+$.

Ethyl 1-((13-chloro-14,16-dihydroxy-1,11-dioxo-3,4,7,8,9,10,11,12-octahydro-1*H*-benzo[*c*][1]oxacyclotetradecin-6-yl)methyl)-1*H*-1,2,3-triazole-4-carboxylate (23)

Mixture of double bond isomers 47:53; colorless solid; m.p. 212–214 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): mixture of isomers δ = 10.59 (brs, 1H, OH), 10.33 (brs, 1H, OH), 8.74 (s, 2H, 2×triazole), 6.53 (s, 1H, ArH), 6.50 (s, 1H, ArH), 5.53 (t, 1H, *J* = 6.8 Hz, =CH), 5.45 (t, 1H, *J* = 6.9 Hz, =CH), 5.02 (s, 2H, CH_2), 5.00 (s, 2H, CH_2), 4.33–4.27 (m, 8H, 4×CH_2), 4.03 (s, 2H, CH_2), 3.84 (s, 2H, CH_2), 2.67–2.62 (m, 2H, CH_2), 2.38 (t, 2H, *J* = 6.0 Hz, CH_2), 1.95 (t, 2H, *J* = 6.8 Hz, CH_2), 1.87 (m, 2H, CH_2), 1.55 (quin, 2H, *J* = 6.5 Hz, CH_2), 1.45 (brs, 4H, 2×CH_2), 1.38 (quin, 2H, *J* = 6.5 Hz, CH_2), 1.29 ppm (t, 6H, *J* = 7.0 Hz, 2×CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): mixture of isomers δ = 206.1 (C), 206.0 (C), 169.0 (C), 168.3 (C), 160.7 (C), 158.0 (C), 156.7 (C), 156.4 (C), 156.0 (C), 139.3 (C), 139.2 (C), 135.7 (C), 134.6 (C), 133.6 (C), 129.8 (CH), 129.2 (CH), 128.63 (CH), 113.7 (C), 113.6 (C), 113.0 (C), 111.5 (C), 102.9 (CH), 102.8 (CH), 65.9 (CH_2), 65.5 (CH_2), 61.0 (CH_2), 55.6 (CH_2), 48.8 (CH_2), 45.6 (CH_2), 45.3 (CH_2), 32.9 (CH_2), 27.8 (CH_2), 26.8 (CH_2), 26.2 (CH_2), 25.2 (CH_2), 23.2 (CH_2), 22.4 (CH_2), 21.7 (CH_2), 14.6 ppm (CH_3); IR (dichloromethane): ν_{max} = 3687, 1722, 1660, 1557 cm^{-1}; MS (ESI): *m*/*z* (%): 516/514 (35/100) $[M+Na]^+$, 494/492 (6/17) $[M+H]^+$; *m*/*z*: calcd for $C_{23}H_{26}{}^{35}ClN_3NaO_7$: 514.1357; found: 514.1343 $[M+Na]^+$.

Protein crystallography: The expression, purification and crystallization of the *N*-terminal domain of yeast Hsp90 has been previously described.[52] Co-crystallizations were conducted by dissolving the inhibitor in 100% DMSO at 50 mM and adding 5 μL to 1 mL of Hsp90 *N*-terminal domain at 4 mg mL^{-1} in 20 mM Tris pH 7.5 and 1 mM EDTA. The complex was then concentrated to 200 μL (20 mg mL^{-1}) and crystallized as previously described.[52] Single crystals appeared overnight of approximate dimensions 0.3×0.2×0.2 mm. These were flash frozen after stepwise addition of glycerol to 30% and data collected on station ID23.1 and ID23.2 at the ESRF. The data were integrated using MOSFLM and scaled and merged using SCALA in CCP4.

The complex was initially solved by isomorphous replacement using a previously determined *N*-terminal structure (PDB ID 1AH6) in the usual space group, P4$_3$22. The model was refined in REFMAC5 in CCP4 and rebuilt using COOT. The FreeR did not refine below 30% and so other space groups were investigated. Refinement proceeded satisfactorily in C2, with four molecules in the asymmetric unit. The inhibitor library was built using SKETCHER. The inhibitor molecule and the waters were added in the final stages.

Biology: Compounds were assayed for their ability to bind to Hsp90 using two assays.

TR-Fret assay: A highly robust time-resolved fluorescence energy transfer (TR-FRET) assay was used to measure the binding of biotinylated geldanamycin (600 nM) to the full length human Hsp90 His-tagged protein (40 nM), as described previously.[51] Briefly, the europium labelled anti-His-tagged protein antibody (Perkin–Elmer Prod. No. AD0110) was added at 1 nM and the streptavidin Sureligh APC (Perkin–Elmer Prod. No. AD0201) at 90 nM. Compounds were tested across a ten point concentration range up to 10 μM and the IC_{50} determined.

FP assay: This is a measurement of binding competition with a fluorescent probe as described previously.[49,50]

Growth inhibition assay: The colorimetric sulforhodamine B assay (SRB) was used to measure growth inhibition studies as described previously.[53] The IC_{50} was calculated as the drug concentration that inhibits cell growth by 50% compared with control growth.

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