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Bioorganic & Medicinal Chemistry Letters 18 (2008) 60–65
Bioorganic & Medicinal Chemistry Letters

Chemical modulators of heat shock protein 70 (Hsp70) by sequential, microwave-accelerated reactions on solid phase

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Received 25 September 2007; revised 31 October 2007; accepted 6 November 2007
Available online 17 November 2007

Abstract—Molecular chaperones, such as Hsp70 and Hsp90, are responsible for a variety of protective, anti-apoptotic functions. While inhibitors of Hsp90, such as geldanamycin and its derivative 17-AAG, are well known and important anti-cancer leads, Hsp70 has received less attention. Interesting lead candidates for Hsp70 share a dihydropyrimidine core; however, the preferred display of pendant functionality is still not clear. Here, we take advantage of the versatility of peptides to explore the requirements for activity. An exploratory compound collection was assembled by performing a Biginelli cyclocondensation at the terminus of a resin-bound β-peptide. Liberation from solid support yielded peptide-modified dihydropyrimidines and, within this series, we uncovered compounds that alter the ATPase activity of Hsp70 and its bacterial ortholog, DnaK. Moreover, we identified important contributions made by aromatic, hydrophobic groups. These chemical probes could be used to study the roles of this molecular chaperone in disease.

The heat shock proteins, including Hsp70 and Hsp90, are members of a family of molecular chaperones that regulate protein function, trafficking, and homeostasis.[1–7] Given their many roles within the cell, it is not surprising that molecular chaperones have been implicated in a variety of diseases, including cancer and neurodegenerative disease.[8,9] Notably, recent work has strongly linked Hsp70 and Hsp90 to cancer.[10,11] Hsp70 is expressed at high levels in numerous cancerous tissues, but its molecular targets and the mechanisms responsible for its anti-apoptotic function are not yet clear.[12–14] Conversely, more is known about Hsp90's roles; this chaperone is believed to promote aberrant survival via stabilizing interactions with oncogenic targets.[15–17]

In part, the important roles of Hsp90 have been elucidated through the use of the small molecules geldanamycin and radicicol.[18] These compounds permit rapid, reversible chemical suppression of Hsp90 function and illumination of the corresponding phenotypes. These experiments provide insight into how Hsp90 promotes survival of cancer cells. Moreover, derivatives of geldanamycin, such as 17-AAG, are being studied in more than a dozen clinical trials. Thus, these chemical tools have not only been useful as probes for understanding the roles of Hsp90 in the apoptotic cascade, but they have also given rise to important therapeutic drugs.

It is likely that insights into Hsp70's roles in disease would arise from the availability of potent and selective chemical inhibitors. Early work in this area yielded 15-deoxyspergualin (DSG; Fig. 1), which, at concentrations of approximately 10 μM, modifies the ATPase activity of Hsp70 by 20–40%.[19] Structurally related compounds, such as alkylated benzamides coupled to amino acids (e.g., *N*-tetradecanoyl-(4-aminomethylbenzoyl)-isoleucine; Fig. 1), were subsequently found to have related activity.[20,21] These principally linear hydrocarbons provide minimal opportunities for analog synthesis and this limitation prompted a focused search for more 'drug-like' scaffolds. These efforts led to the Hsp70-binding compound, NSC-630668-R/1 (R/1), which, like DSG, also modifies ATPase activity at ~300–600 μM.[22] Most recently, attention has shifted to compounds that resemble R/1, but are assembled on a dihydropyrimidine core.[23] These studies identified MAL3-101, which at approximately 300 μM inhibits

Keywords: Chaperone; Dihydropyrimidine; Biginelli; β-Peptide; Cancer; Neurodegenerative disease.
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doi:10.1016/j.bmcl.2007.11.027

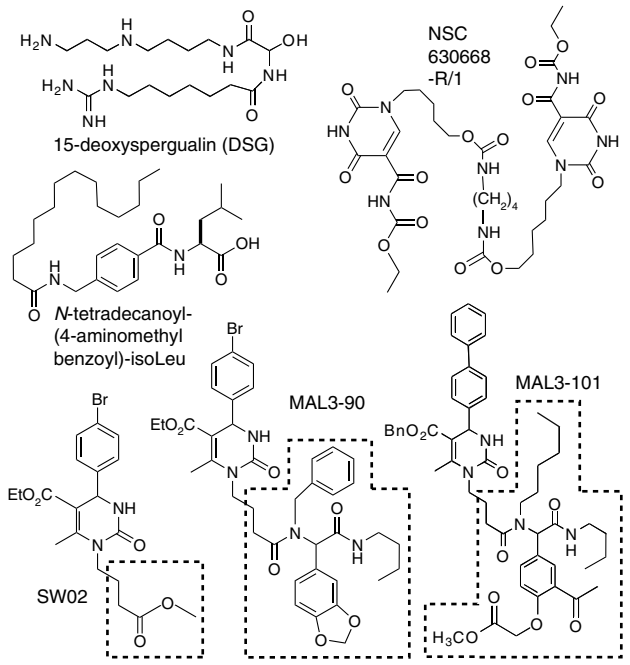


Figure 1. Chemical structures of known modulators of Hsp70 are shown, with the region under investigation highlighted.

co-chaperone stimulated ATP hydrolysis,[23] blocks endoplasmic reticulum trafficking of some targets[23], and, at 2 μM, is cytotoxic to carcinoma cells.[24] Interestingly, structurally related compounds, such as MAL3-90, enhance ATP turnover and another dihydropyrimidine, SW02, promotes the anti-aggregation functions of Hsp70 in vitro.[23,25] Thus, this family of compounds has members that either stimulate or inhibit known functions of Hsp70. These studies firmly establish dihydropyrimidines as modifiers of this chaperone; however, systematic structure–activity studies have not been reported.

The goal of this study is to generate a focused library of dihydropyrimidines and screen these for additional leads. Previous compounds, such as MAL3-101 and MAL3-90, were assembled from consecutive, multicomponent Biginelli and Ugi reactions, which produce highly functionalized dihydropyrimidines (Fig. 2). This core is shared amongst known Hsp70 partners, thus, it is believed to be important for activity. However, the role of the Ugi-centered functionality is still not clear because some interesting compounds, such as R/1 and SW02, have limited complexity in this region. Therefore, the specific goal of this study is to explore the structure–activity relationships in this portion of the molecule using protease-resistant β-peptides as a replacement for the Ugi product (Fig. 2), while keeping the identity of the dihydropyrimidine constant. Peptides provide a facile entry into rapid analysis of functional group effects and we reasoned that this approach would permit modular installation of common side chains. In the absence of structural information or knowledge of the binding site, we preferred an empirical approach in which polar and hydrophobic residues could be easily interchanged. Moreover, we envisioned that this approach would be amenable to routine Fmoc chemistry on solid support and that this method would provide the opportunity to assemble combinatorial libraries.

Our route begins with microwave-assisted assembly of β-peptides on Wang resin (Fig. 3).[26] A series of di- and tripeptides with a range of hydrophobic and polar side chains were used to explore the SAR in the target region and, based on work from the Gellman group, microwave irradiation was used to accelerate the historically poor yields and reaction times for these transformations.[27,28] Following installation of the final β-amino acid, one of two approaches was used to complete the products. For the dipeptides, a solution-phase Biginelli reaction afforded the desired dihydropyrimidine and then this pre-formed molecule was coupled to the terminus of the immobilized peptide. The identity of the dihydropyrimidine was chosen to resemble the core of the known, bioactive compounds, MAL3-90 and SW02. Coupling this unit via the pendant carboxylate proceeded smoothly to produce the dipeptide-modified dihydropyrimidine in adequate overall yields (between 5% and 32%; 6 steps, >90% purity). However, to investigate whether a more linear approach could be used, we also attempted the Biginelli reaction directly on the resin-coupled peptide. The Wipf group has reported that this reaction is amenable to solid-phase synthesis[29] and, further, the Kappe laboratory has established that it is accelerated by microwave irradiation.[30,31] Therefore, we envisioned that the microwave-assisted, solid-phase assembly of this scaffold might proceed smoothly;

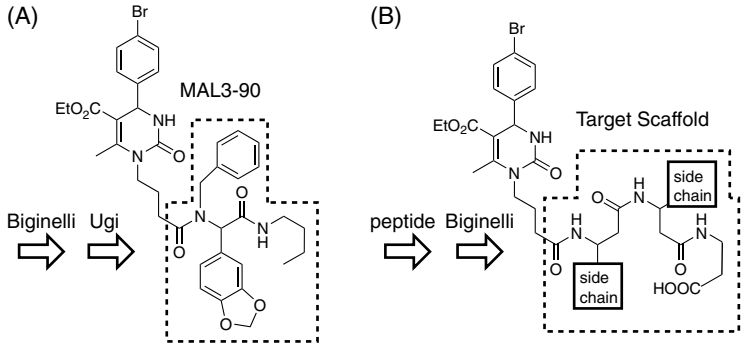


Figure 2. Design of dihydropyrimidines appended to β-peptides to mimic the functional group arrangement of the highly functionalized dihydropyrimidine, MAL3-90.

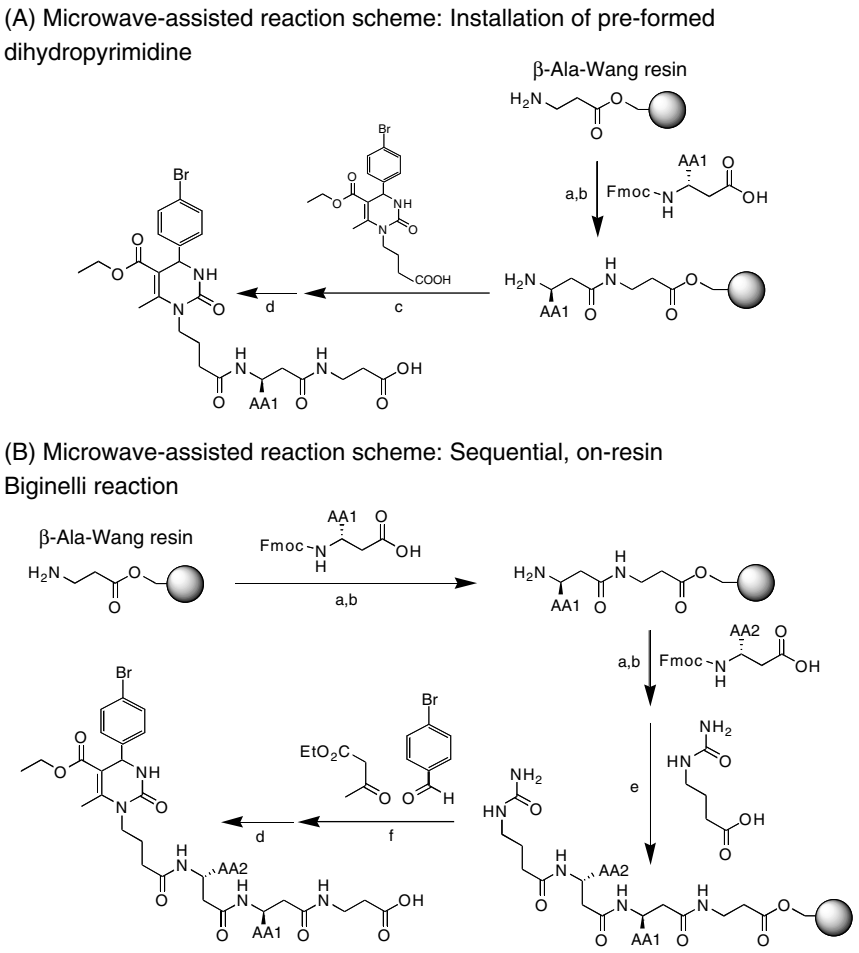
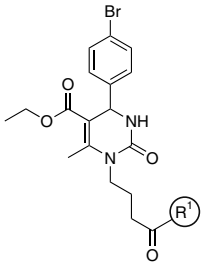


Figure 3. Two routes used to assemble potential modifiers of Hsp70. Compounds were either assembled by (A) coupling a pre-formed dihydropyrimidine to the terminus of the β-dipeptide or (B) the Biginelli cyclocondensation reaction was performed on solid phase by appending the urea to a β-tripeptide. (a) DIC, HOBt, 70 °C, μW, 20 min, DMF; (b) 20% piperidine/DMF; (c) DIC, HOBt, 60 °C, μW, 15 min; (d) 1:1 TFA:DCM, 30 min; (e) DIC, HOBt, 80 °C, μW, 20 min, DMF; (f) 4:1 DMF:HCl, μW, 120 °C, 40 min. Overall purified yields were between 1% and 32%. The amino acid side chains are signified by AA1 and AA2.

however, it was unclear whether the steric environment of highly peptide-modified resin would be suitable for this transformation. To explore this idea, bulky tripeptides were assembled by Fmoc chemistry on Wang resin as illustrated in Figure 3, followed by installation of 1-ureidobutyric acid to the N-terminus, and subsequent Biginelli cyclocondensation (Fig. 3B).[32,33] While the Biginelli reaction is known to be relatively functional group tolerant,[31] we were pleased to find that this transformation proceeded readily, even in the presence of polar and sterically challenging amino acid side chains. Overall purified yields were modest (1–10%; 9 steps) but, using this sequential, microwave-accelerated approach, the prospective Hsp70 ligands could be built entirely on solid support in excellent purity (>90%). Using these routes, we generated an exploratory collection of 17 compounds (Table 1).

The many functions of Hsp70 are derived from its ATPase activity.[2,34] Thus, using a recently developed assay,[35] we screened **1**–**17** for their ability to influence ATP turnover.[36] Specifically, we examined two related members of the Hsp70 family: DnaK from *Escherichia coli* and bovine Hsc70. Although Hsp70 proteins are highly conserved,[37] we were interested in understanding whether selective inhibitors of individual chaperones could be uncovered. For both proteins, the inclusion of the stimulatory co-chaperone, DnaJ, improved signal intensity and provided robust screening conditions.[35] When compounds **1**–**17** were screened at 100 μM in this assay, we found that some of them stimulated ATP turnover, while others inhibited this activity. This finding is consistent with previous reports and this dual capacity seems to be a feature of dihydropyrimidine-based ligands.[23,25] The mechanistic origin of this plurality is currently unknown, but it likely involves an unidentified, allosteric pathway. Importantly, both types of Hsp70 regulators (activators and inhibitors) might be interesting as chemical probes for exploring the roles of the chaperone in disease; thus, we pursued both classes and defined compounds that either reduced or improved signal by at least 20% as 'hits'. The EC_{50} values for these hits were then determined from dose-dependence experiments. We found that, under these conditions, MAL3-101 has an effect on the ATPase activity of Hsc70 but had no significant activity against DnaK (Fig. 4). Similarly, com-

Table 1. Structures of dihydropyrimidine-based compounds



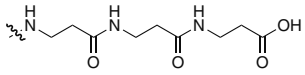
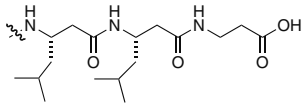
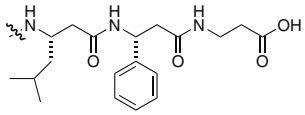
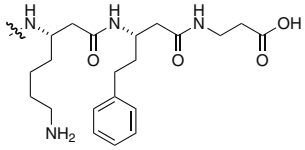
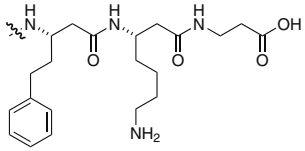
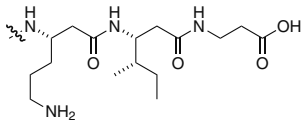
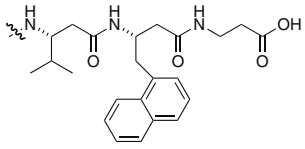
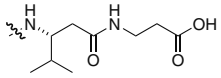
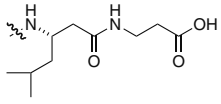
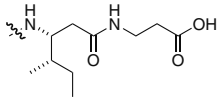
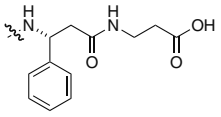
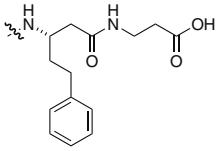
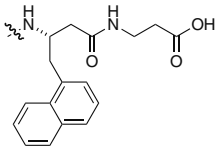
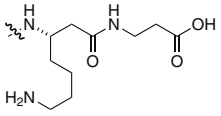
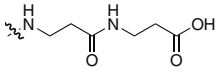
Compound	R′	Mass spec[a]
1	OH	c 424.00 o 425.01
2	OCH_3	c 439.30 o 438.90
3		c 638.17 o 638.20
4		c 750.30 o 750.72
5		c 770.27 o 770.31
6		c 812.31 o 812.30
7		c 812.31 o 812.30
8		c 693.27 o 693.30
9		c 819.28 o 819.30
10		c 609.51 o 609.20
11		c 623.54 o 623.21
12		c 623.54 o 623.22

Table 1 (*continued*)

Compound	R′	Mass spec[a]
13		c 643.53 o 643.20
14		c 671.58 o 671.20
15		c 707.61 o 707.20
16		c 638.55 o 638.12
17		c 567.43 o 567.05

[a] Mass: [*m*/*z* + H]; c = calculated; o = observed.

pound **2**, which we had previously shown to modify Hsp70's anti-aggregation functions,[25] had weak stimulatory activity against Hsc70 and was inactive against DnaK. From the exploratory screen, we identified compounds **6**–**9**, **14**, and **15** for further analysis. Compound **6** partially blocked function of both Hsc70 and DnaK with EC_{50} of ~110 μM. Conversely, **14** and **15** were relatively good activators and compound **14** was selective for Hsc70, while **15** stimulated both chaperones. The potency of these compounds is modest (EC_{50} ~ 100–200 μM), but it is important to note that, despite their similarly small impact on ATP turnover, DSG, SW02, and MAL3-101 have dramatic effects in biological settings.[23–25] Thus, in vitro changes in ATPase activity may under-represent potency in more complex environments. From these studies, we have identified additional modulators of Hsp70 and, given the low number of comparable reagents, we expect that these compounds will find use in exploring the function of this chaperone.

In these assays, two evolutionary distant members of the Hsp70 family were used, DnaK from *E. coli* and mammalian Hsc70. We chose to study these chaperones because we were interested in understanding whether selective modulators of distinct Hsp70 isoforms could be uncovered. In these experiments, we found that, even though the extent of homology between the two isoforms is high (~46% identical, 64% similar),[38] some compounds, such as **14**, were highly selective for one type over the other. However, other structurally related compounds, such as **15**, were equally effective against both chaperones. Although the chemical and structural basis for this selectivity is not yet clear, these results sug-

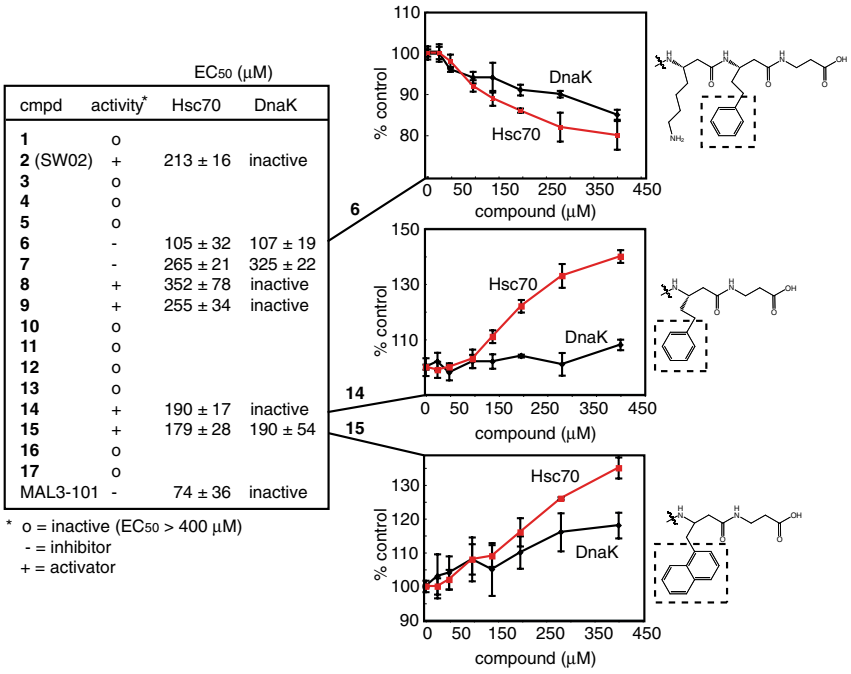


Figure 4. Activity of compounds **1**–**17** against the ATPase activity of mammalian Hsp70 and bacterial DnaK. The dose dependence of promising compounds and the structure of their peptide regions are shown on the right. Aromatic groups in either the first or second amino acid position were important for activity.

gest, for the first time, that compounds in this class may serve as important reagents for selective modulation of chaperone function in environments containing contributions from multiple Hsp70s.

One goal of this study was to begin to understand the functional groups that are important for the activity of dihydropyrimidine-based ligands for Hsp70. Strikingly, we found that aromatic, hydrophobic functional groups (benzyl and naphthyl rings) attached via short, flexible, methylene spacers were important for both activator and inhibitor functions. Specifically, the most potent compounds **6**, **14**, and **15** all possess these functional groups, whereas inactive examples, such as **3**, **4**, and **17**, lack aromatic substitutions. Although the binding site for these molecules is not yet known, these results suggest that there is a key hydrophobic contact within this region. Consistent with this idea, polar and charged functional groups, such as are present in **6**–**8** and **16**, had less impact on activity. Interestingly, the position of the hydrophobic group (i.e., whether it was appended to the second or third amino acid position) seemed less important, which might indicate a flexible or complex binding site. Together, these results suggest that, although other portions of the molecule remain to be explored, the peptide region makes an important contribution to potency.

The diverse cellular roles of Hsp70 likely contribute to its involvement in a number of diseases, including cancer and neurodegeneration.[8,9] However, there are few small molecules that are known to interact with Hsp70 and this limitation has slowed progress in the field. The most widely used of the known inhibitors, MAL3-101, inhibits the ATPase activity of yeast Ssa1 at concentrations of approximately 300–600 μM under some conditions.[23] The best of the new molecules described here have similar potency (~100 μM) and some of these, such as **6** and **15**, were unique in that they had activity against multiple Hsp70 isoforms. Thus, although extensive work remains, these findings may assist the development of new chemical probes that will help reveal the roles of this chaperone in disease.

Acknowledgments

The authors thank P. Wipf and J. Brodsky (University of Pittsburgh) for supplying MAL3-101 and helpful advice. C.G.E. is supported by the NIH Cellular Biotechnology Training Program. This work was additionally supported by Pilot Grants from the U. Michigan's Alzheimer's Disease Research Center, the Biomedical Research Council, and the McKnight Foundation Fund for Neuroscience.

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26. *Peptide synthesis*: To 1 equiv Wang resin, 2 equiv of the Fmoc-β-amino acid, 3 equiv DIC, and 3 equiv HOBt were added. The reaction was performed in DMF (5 mL DMF per 0.25 mmol Wang resin), and the mixture was irradiated with microwaves at 70 °C for 20 min. The resin was washed with 4× 10 mL DMF, followed by incubation with 5 mL of 20% acetic anhydride for 20 min in order to block uncoupled sites before Fmoc deprotection. The resin was washed (4× 10 mL DMF) followed by deprotection of the N-terminus with 20% piperidine in DMF. The coupling efficiency was estimated by transferring approximately 1–2 mg resin into 3 mL of 20% piperidine in DMF in two quartz cuvettes. After 2–3 min of stirring, the absorbance at 290 nm was determined, using 20% piperidine in DMF as a reference. The average coupling efficiency was 80%.
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32. *Synthesis of 1-ureido-butyric acid*: Reaction at 60 °C: 25 mmol δ-amino butyric acid and 16.7 g KOCN were added to 55 mL H_2O. After 4 h at 60 °C, the mixture was cooled on ice and the solution acidified by adding concentrated HCl dropwise. The white precipitate was filtered, washed with ice-cold water, and dried in air (yield: ~30%).
33. *Biginelli reaction*: Five equivalents of 1-ureido-butyric acid was coupled to the tripeptide in the presence of 3 equiv DIC and 3 equiv HOBt in 10 mL DMF per 0.25 g resin. The conditions for the subsequent Biginelli reactions were as follows: to 0.250 mmol of resin loaded with tripeptide, 4 equiv *p*-bromobenzaldehyde, 4 equiv ethylacetoacetate, and 250 μL 4:1 DMF:HCl (conc.) were added. Microwave conditions: 80 °C for 20 min followed by four washing steps: 3× 10 mL DMF, 3× 10 mL hexanes, 3× 10 mL MeOH, 3× 10 mL CH_2Cl_2. The product was cleaved with 5 mL 1:1 CH_2Cl_2:TFA for 30 min, followed by washing the resin with CH_2Cl_2. The solvent was evaporated under reduced pressure and the product was purified to over 90% using reverse phase HPLC (C18; acetonitrile:water; 20–80%, 1% per min). Products were isolated as white solids. The identities were verified by mass spectrometry (see Table 1) and ^{1}H NMR: compound **6**: [3-((3*S*)-3-((3*S*)-7-amino-3-(4-(4-(4-bromophenyl)-5-(ethoxycarbonyl)-6-methyl-2-oxo-3,4-dihydropyrimidin-1(2H)-yl)butanamido)heptanamido)-5-phenylpentanamido)propanoic acid] ^{1}H NMR: δ 1.09–1.12 (t, 3H), δ 1.25–1.26 (m, 1H), δ 1.33–1.37 (m, 2H), δ 1.44–1.48 (m, 2H), δ 1.50 (s, 2H), δ 1.69 (s, 2H), δ 2.04 (s, 2H), δ 2.21 (s, 4H), δ 2.10–2.29 (m, 2H), δ 2.34 (t, 2H), δ 2.47 (s, 3H), δ 2.54–2.57 (d, 1H), δ 2.75 (s, 2H), δ 3.21–3.22 (d, 2H), δ 3.77 (br s, 1H), δ 4.01–4.04 (t, 3H), δ 5.13 (d, 1H), δ 7.16 (s, 4H), δ 7.21–7.25 (t, 2H), δ 7.52 (d, 2H), δ 7.67 (s, 3H), δ 7.78 (d, 1H), δ 7.95–7.97 (d, 2H), δ 12.22 (br s, 1H). Compound **14**: [3-(3-(4-(4-(4-Bromophenyl)-5-(ethoxycarbonyl)-6-methyl-2-oxo-3,4-dihydropyrimidin-1(2H)-yl)butanamido)-5-phenylpentanamido)propanoic acid] ^{1}H NMR: δ 1.07–1.13 (t, 3H), δ 1.57–1.68 (m, 2H), δ 1.68–1.78 (m, 2H), δ 2.06 (t, 2H), δ 2.17–2.28 (m, 2H), δ 2.35 (t, 2H), δ 2.47 (s, 3H), δ 3.22 (d, 2H), δ 3.76–3.85 (m, 1H), δ 4.00–4.04 (q, 3H), δ 5.13 (d, 1H), δ 7.16–7.20 (m, 5H), δ 7.24–7.30 (m, 2H), δ 7.50 (d, 1H), δ 7.73 (d, 1H), δ 7.94 (t, 1H),δ 7.99 (d, 1H), δ 12.40 (s, 1H). Compound **15**: [3-(3-(4-(4-(4-Bromophenyl)-5-(ethoxycarbonyl)-6-methyl-2-oxo-3,4-dihydropyrimidin-1(2H)-yl) buta-namido)-4-(naphthalen-1-yl)butanamido)propanoic acid] ^{1}H NMR: δ 1.09–1.13 (q, 3H), δ 1.43–1.47 (m, 1H), δ 1.57 (m, 1H), δ 1.87–1.97 (m, 2H), δ 2.24 (s, 2H), δ 2.31 (s, 1H), δ 2.33 (d, 2H), δ 2.38–2.40 (t, 2H), δ 3.03–3.08 (m, 1H), δ 3.19–3.32 (m, 3H), δ 3.32–3.34 (t, 2H), δ 3.62 (m, 1H), δ 3.99–4.05 (t, 2H), δ 3.39–4.42 (t, 2H), δ 5.11 (s, 1H), δ 7.14 (d, 1H), δ 7.18 (d, 1H), δ 7.31–7.39 (m, 2H), δ 7.47–7.54 (m, 2H), δ 7.55–7.57 (m, 2H), δ 7.82–7.85 (t, 1H), δ 7.96 (s, 1H), δ 8.00 (s, 1H), δ 8.31 (d, 1H).
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36. The malachite green-based ATPase assay was performed as described in Ref. 35, except that the reactions were performed without GrpE and the incubation time was 4 h.
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