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A Versatile Synthetic Route to Cycloheximide and Analogues that Potently Inhibit Translation Elongation

Yongho Park, Yumi Koga, Cindy Su, Amanda L. Waterbury, Christopher L. Johnny, and Brian B. Liau*[a]

Abstract: Cycloheximide (CHX) is an inhibitor of eukaryotic translation elongation that has played an essential role in the study of protein synthesis. Despite its ubiquity, few studies have been directed towards accessing synthetic CHX derivatives, even though such efforts may lead to protein synthesis inhibitors with improved or alternate properties. Here, we describe the total synthesis of CHX and analogues, and establish structure-activity relationships (SAR) responsible for translation inhibition. The SAR studies aided the design of more potent compounds, one of which irreversibly blocks ribosomal elongation, preserves polysome profiles, and may be a broadly useful tool for investigating protein synthesis.

Protein synthesis is a highly coordinated process that involves ribosomes, mRNA, aminoacyl-tRNAs, and various cofactors. Due to the essential role of protein synthesis for life, many organisms for self-defense have evolved to produce secondary metabolites targeting nearly every step of translation.[1] The biological activities of these natural products and their derivatives have been exploited to gain critical insights into tRNA decoding,[2] peptide bond formation,[3] resistance mechanisms to antibiotics,[4] and cellular protein synthesis.[5,6] In particular, cycloheximide (CHX, **1**) has been employed routinely for decades to rapidly and reversibly inhibit elongating ribosomes in order to study protein synthesis and measure protein half-lives (Figure 1).[7]

Recent biochemical[8] and crystallographic[9] studies demonstrated that **1** binds to the ribosome exit-site (E-site), competitively occupying a pocket where the 3' CCA sequences of deacylated tRNAs reside. By blocking translocation of tRNAs, **1** slows down all actively translating ribosomes on mRNA, leading to polysome stabilization. The stalled polysomes can then be visualized in vivo[10] or analyzed to probe post-transcriptional processes.[11] Moreover, polysome stabilization is essential to ribosome profiling,[12] which has revealed details regarding upstream open reading frames,[13] co-translational assembly of proteins,[14] and elongation rates of translating ribosomes.[15] One drawback of stabilizing polysomes with **1** is that binding of **1** to ribosomes is reversible and elongation continues to occur during the course of the experiment.[16] Even at high concentrations of **1**, it has been estimated that translation elongation still proceeds at 0.1 to 0.3 amino acids per second, resulting in dose- and time-dependent biases.[16a] Thus, irreversible stabilization of polysomes is highly desired to enable more robust downstream experiments to study translation.

Since protein synthesis can be inhibited by stabilizing or disrupting polysomes, several other natural products have been evolved to bind the same pocket as **1** with different effects on polysome stability.[9] For example, chlorolissoclimide (**2**) stabilizes polysomes in a similar manner to **1**,[17] lactimidomycin (LTM, **3**) only blocks translation at the initiating codon,[8] while phyllanthoside (PHY, **4**) induces dissociation of translating ribosomes from mRNA.[18] Even though high-resolution structures of these molecules are now available, how each inhibitor influences the overall stability of polysomes remains unclear and presents a challenge in designing analogues with additional biological function.[19] Motivated by these questions, herein we report a versatile synthetic route to **1** and potent analogues that enabled the development of an irreversible elongation inhibitor.

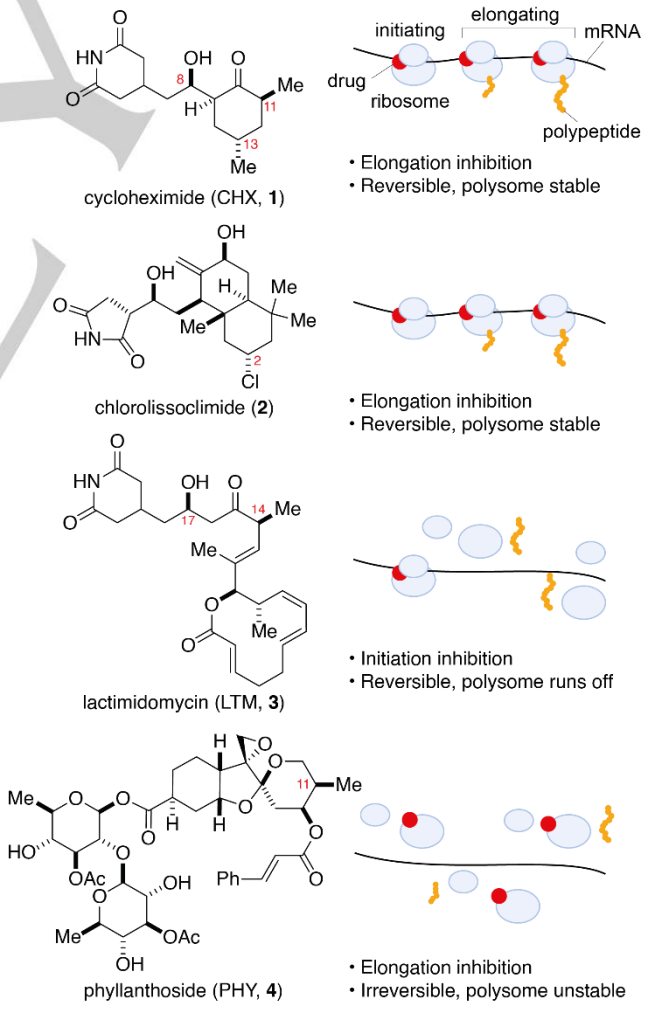

Figure 1. Natural products that bind the ribosome E-site and their effects on translation.

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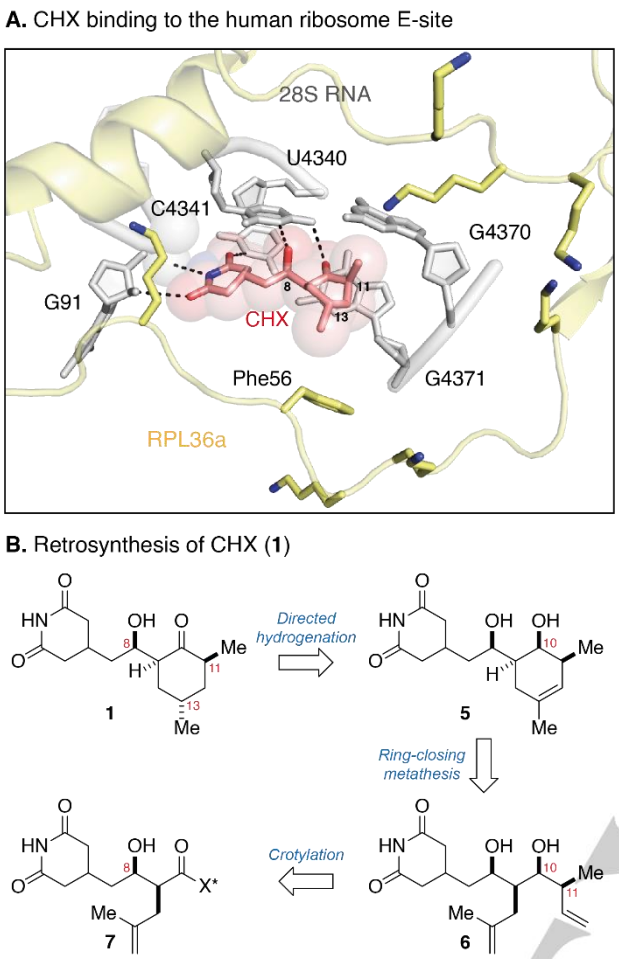

Figure 2. (A) Structural view of the human ribosome E-site bound by **1**. Critical bases in the 28S ribosomal RNA (gray) and RPL36a (yellow) are shown. PDB: 5lks. (B) Retrosynthetic analysis of **1**.

Based on the structure of **1** bound to the human ribosome,[9b] we hypothesized that installation of electrophilic functionality extending from the cyclohexanone of **1** may allow covalent interception of nearby lysine residues of RPL36a – a ribosomal protein that comprises the E-site – and subsequent irreversible inhibition of translation (Figure 2A). Such a strategy would require alteration of cyclohexanone of **1** without disrupting **1**'s activity, but the relative contribution of the cyclohexanone's substituents to **1**'s activity remains elusive. The lack of information could potentially be attributed to the strategy employed in **1**'s lone synthesis,[20] wherein the cyclohexanone moiety was prepared in the first stage. Since the importance of glutarimide and C(8)-OH of **1** has already been established,[21] we devised an alternative route to **1** which would allow for stereodivergent assembly of C11- and C13-substituents late stage. We envisioned cyclohexene **5** as a key intermediate for incorporating different functionality and stereochemistry across the alkene. To access **1** from **5**, the desired C13-streeocenter could be installed by directed hydrogenation. The C11-methyl stereocenter in **6** could be secured via a diastereoselective crotylation, while the C8- and C9-stereocenters in **7** could be generated by an Evans' aldol reaction.

The synthesis commenced with an Evans' aldol reaction with *N*-acyl oxazolidinone **8** and aldehyde **9**, each of which was obtained in three steps from commercial materials (Scheme 1).[22] The aldol product was then protected to provide TBS ether **10** in 98% yield over two steps. Chemoselective conversion of **10** to a thioester was achieved using a lithium thiolate,[23] and subsequent Fukuyama reduction[24] delivered aldehyde **11** in 55% yield over two steps. Diastereoselective crotylation of **11** was achieved using (*E*)-crotylboronic acid pinacol ester to yield homoallylic alcohol **12** with the correct C10- and C11-stereochemistry. Ring-closing metathesis of **12** to prepare cyclohexene **13** followed by directed hydrogenation yielded cyclohexanol **14** as a single diastereomer. The C10-carbinol was then oxidized to furnish the corresponding cyclohexanone, and global deprotection of this intermediate using ceric ammonium nitrate completed the total synthesis of **1** in 10% and 12 steps from commercial 2-(2,6-dioxopiperidin-4-yl)acetic acid.

Following the established route, we synthesized several analogues of **1** with altered C11- and C13-substituents, and evaluated them in a cell-based assay using *O*-propargyl puromycin (OPP) incorporation (Supporting Information, Figure S1).[5] Overall, the C11- and C13-methyl substituents were vital to **1**'s activity. Inversion of the C11-stereocenter (**15**), addition of a second methyl group (**16**), and removal of the methyl group (**17**) all abolished inhibitory activity (Figure 3A). Inversion of the C13-stereocenter (**18**) and elongation of C16-methyl to *n*-butyl (**19**) also resulted in highly diminished activity. The activity could be recovered when the *n*-butyl chain was positioned pseudoequatorially (**20**) instead. These data indicate that CHX analogues likely need to interact with G4370, G4371, and RPL36a Phe56 for successful inhibition. It is noteworthy that other E-site binders (**2**-**4**) maintain similar contacts with those residues. For instance, the corresponding methyl groups in LTM (**3**) are similarly positioned (Supporting Information, Figure S2).[9a] Moreover, C(2)–Cl of chlorolissoclimide (**2**)[17a] and C11-methyl of PHY (**4**)[9a] are also oriented in a similar fashion to the C11-methyl group of **1**.

Based on these observations, we considered if incorporation of an additional substituent at the C13-position of **1** could lead to a more potent analogue with a functional group handle. Toward this goal, we first targeted streptovitacin A (**23a**) (Scheme 1). Mukaiyama hydration[25] of **13** afforded the C13-diastereomeric tertiary alcohols **21a** and **21b** in 48% and 52% yield, respectively. Subsequent oxidation to the cyclohexanone and global deprotection then yielded streptovitacin A (**23a**, 11% yield over two steps) and C13-*epi*-streptovitacin A (**23b**, 18% yield over two steps). In the OPP assay, **23a** exhibited robust translation inhibition, thus suggesting the feasibility of derivatizing the C13-position to generate potent analogues. We then further leveraged the synthetic versatility of **13** to install a quaternary center through a radical-mediated olefin cross-coupling reaction (Scheme 1). Hydrogen atom transfer to **13** and subsequent reaction with benzyl acrylate installed the side-chain in 2:1 diastereoselectivity.[26] The C13-diastereomers were separated after Dess–Martin oxidation to give cyclohexanones **25a** and **25b** in 21% and 46% yield, respectively, over two steps. Global deprotection then afforded benzyl esters **26a** and **26b**.

In the OPP assay, **26a** was approximately an order of magnitude more potent than **1** (Figure 3B). The cytotoxicity of **26a** was similar to **1**, with slightly enhanced selectivity toward certain cancer cell lines (Supporting Information, Figure S3). Based on the available structural data,[8a] we speculate that the increased potency of **26a** may be driven by stabilizing interactions involving the benzyl ester with the E-site in a manner similar to the

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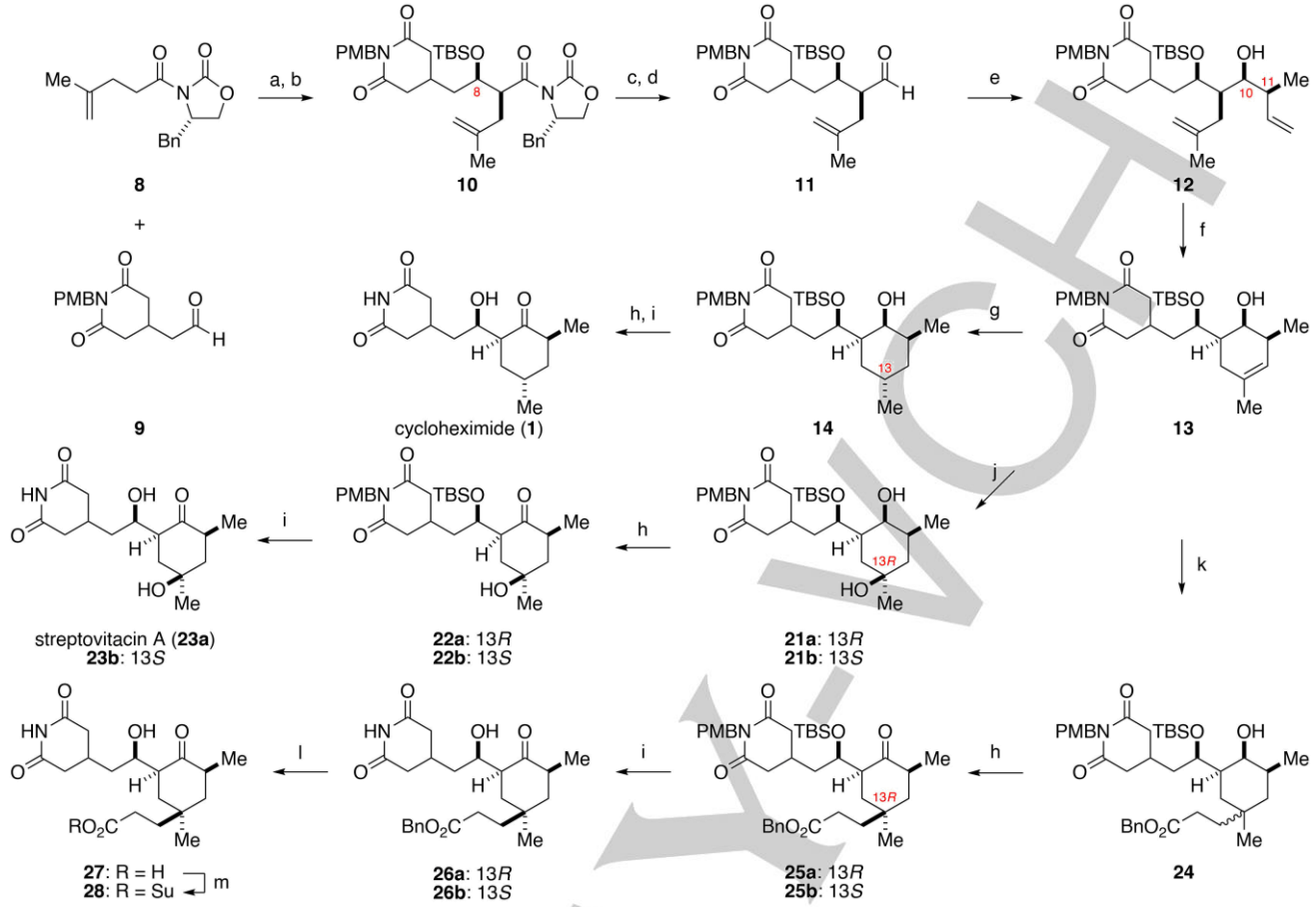

Scheme 1. Total synthesis of cycloheximide (**1**), streptovitacin A (**23a**) and irreversible inhibitor (**28**). Conditions: (a) *n*-Bu_2BOTf, Et_2N*i*-Pr, CH_2Cl_2, 0 °C; then **9**; (b) TBSOTf, 2,6-lutidine, CH_2Cl_2, 98% (2 steps); (c) *n*-BuLi, $C_{12}H_{25}SLi$, 0 °C; then **10**; (d) Pd/C (10 wt%), Et_3SiH, acetone, 55% (2 steps); (e) (*E*)-crotylboronic acid pinacol ester, PhMe, 90%; (f) Hoveyda–Grubbs' catalyst, 2nd generation (2 mol%), 1,2-dichloroethane, 60 °C, 81%; (g) Crabtree's catalyst (17 mol%), H_2 (1 atm), CH_2Cl_2, 84%; (h) Dess–Martin periodinane, CH_2Cl_2, 53% for **22a**, 64% for **22b**, 21% for **25a** (2 steps), 46% for **25b** (2 steps); (i) CAN, MeCN/H_2O, 42% for **1** (2 steps), 20% for **23a**, 28% for **23b**, 73% for **26a**, 66% for **26b**; (j) $Co(acac)_2$ (20 mol%), $PhSiH_3$, O_2 (1 atm), THF, 48% for **21a**, 52% for **21b**; (k) $Fe(acac)_3$ (5 mol%), $PhSiH_3$, benzyl acrylate, EtOH, 40 °C; (l) Pd/C (20 wt%), H_2 (1 atm), EtOH; (m) TSTU, Et_3N, CH_2Cl_2, 41% (2 steps).

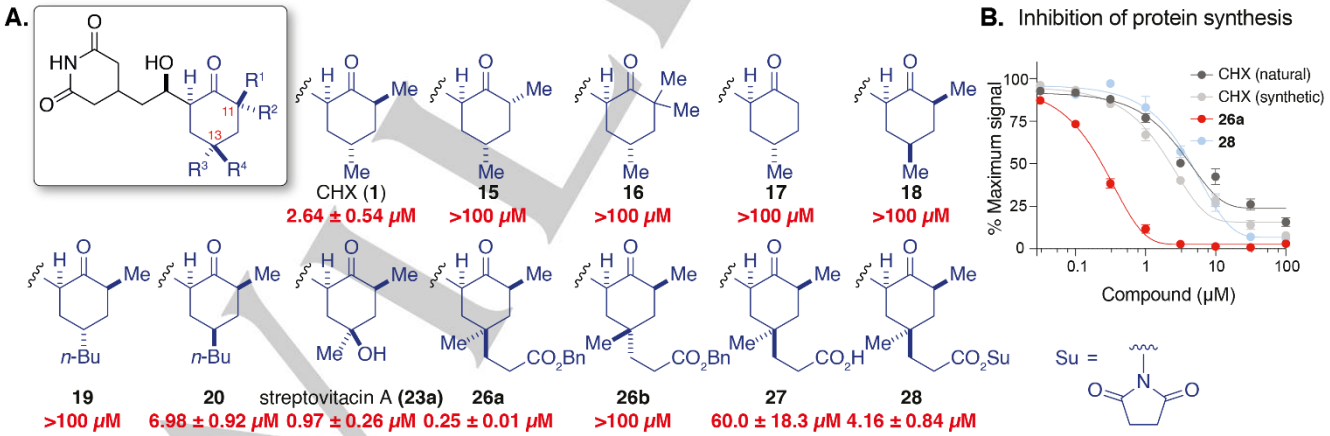

Figure 3. (A) Structures of CHX derivatives synthesized in this study. For each compound, IC_{50} values for translation inhibition are shown in red. Error represent standard deviation (SD) for n =3. (B) Dose-response curves show relative protein synthesis levels (%max, y axis) after treatment with natural **1**, synthetic **1**, **26a**, or **28** versus vehicle control (0.1% DMSO v/v). Error bars represent standard error (SE) for n = 3.

macrolactone of LTM (**3**) (Supporting Information, Figure S2 and S4). Given the increased potency of **26a**, installation of electrophilic functionality was explored to potentially transform **26a** into an irreversible inhibitor. Thus, benzyl ester **26a** was converted to *N*-hydroxysuccinimidyl (NHS)-ester **28** in two steps. Comparable activities were obtained for **28** and **1** in the OPP assay (Figure 3).

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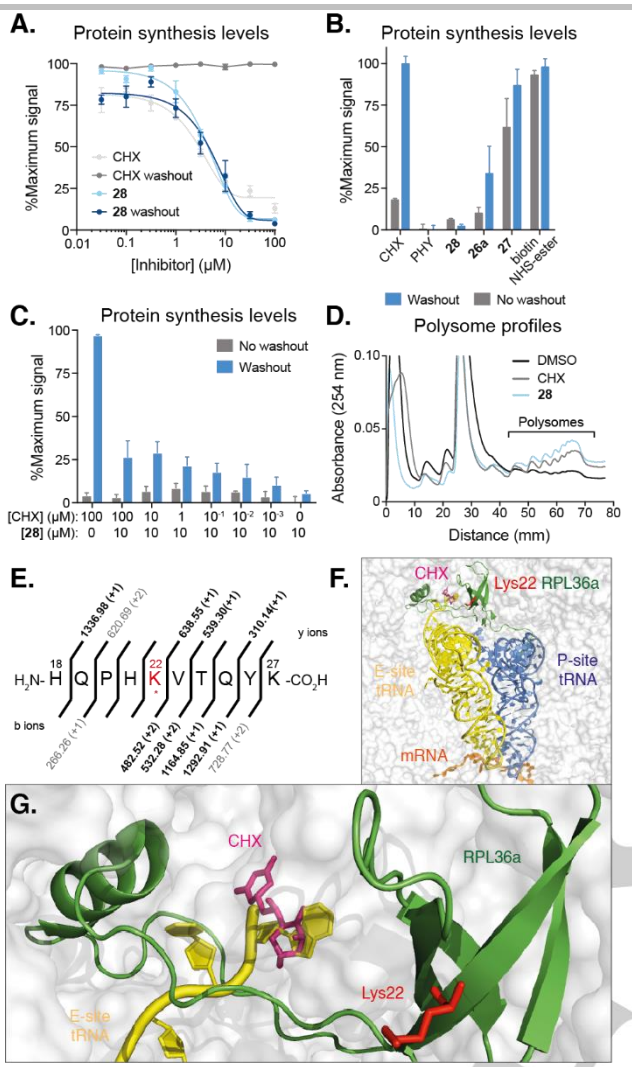

Figure 4. (A) Dose-response curves show relative protein synthesis levels for CHX and **28** treatments after being retained or washed out from the media for 30 min. Error bars represent SE for n = 3. (B) Relative protein synthesis levels (%max, y axis) as measured by OPP incorporation after compounds (100 μM) are retained or washed out for 30 min. Error bars represent SE for n = 3. (C) Relative protein synthesis levels after co-treatment with CHX and **28** at various doses. Cells were preincubated with CHX for 5 min before **28** was added. Error bars represent SE for n = 3. (D) Polysome profiles obtained from 293T cells treated with vehicle (0.1% DMSO v/v), CHX (100 μM), or **28** (100 μM) for 90 min. (E) HPLC-MS/MS analyses of the lysine 22-modified peptide resulting from trypsin digestion (* indicates modification by **28** (100 μM, 90 min), while # indicates alkylation of cysteine resulting from iodoacetamide treatment). Observed mass-to-charge (m/z) values of y and b ions are shown, with charge states in brackets. Ions in bold: |error| < 500 ppm; ions in gray: 500 ppm < |error| < 1000 ppm. (F) Structural view of the human ribosome. E-site tRNA (yellow), P-site tRNA (blue), mRNA (orange), Lys22 (red) of RPL36a (green), CHX (pink) are shown. PDB: 5lks aligned to 5lzt. (G) Zoomed-in view of the E-site.

To determine if **28** irreversibly inhibits protein synthesis, OPP-translation assays were conducted after inhibitor washout (Figure 4). Consistent with the literature, reversible inhibition was observed with **1**[7b] and irreversible with **4**.[18] Treatment with **28** resulted in irreversible inhibition, which was not due to hydrolysis of **28** or non-specific effects of an NHS-ester (Figure 4A and 4B). By contrast, inhibition induced by benzyl ester **26a** could be rescued by inhibitor washout, albeit to a reduced extent; this partial effect may reflect the increased potency of **26a** relative to **1**. Additionally, competition experiments between **28** and **1** strongly suggest that **1**, in a dose-dependent manner, prevents **28** from binding the same site as **1** (Figure 4C). Moreover, one minute of incubation time was sufficient to induce irreversible inhibition by **28** at 100 μM (Supporting Information, Figure S5). Altogether, our data support the notion that NHS-ester **28** effectively irreversibly inhibits protein synthesis and targets the same ribosome E-site as **1**.

Mechanistically, translation inhibition could be driven by either stabilization or dissociation of polysomes (e.g. PHY, **4**). To distinguish between the two possibilities, polysome profiles were obtained after treatment of 293T cells with vehicle, **1**, or **28**. Similar levels of polysomes were observed in samples treated with **1** and **28**, demonstrating **28**'s ability to globally inhibit translation elongation unlike LTM (**3**) or PHY (**4**). It is noteworthy that a single treatment with **28** at 100 μM prior to lysis was sufficient to preserve the integrity of polysome. Currently, typical polysome profiling experiments require a high concentration of **1** in all buffers after cell lysis due to the reversibility of **1**.[27]

To understand how irreversible inhibition by **28** might be mediated, we conducted immunoprecipitation (IP) mass spectrometry (MS) experiments on RPL36a. Mass shifts corresponding to acylation of Lys22 by **28** were observed in both trypsin and chymotrypsin digests (Figure 4E and Supporting Information, Figure S6); no other adducts of **28** were detected. Since Lys22 is located outside of the putative binding pocket of **28**, it remains to be established how the modification of Lys22 is mechanistically relevant to **28**'s binding to the E-site and its effect on protein synthesis.[28]

In conclusion, NHS-ester **28** acts as an effectively irreversible inhibitor of protein synthesis by maintaining polysome stability. We anticipate that **28** may serve as an important tool compound to study translation, especially in applications of ribosome profiling where **1** is suspected of displaying bias due to reversibility.[16] Future studies using these CHX analogues to investigate structure-function relationships in the ribosome E-site and interrogate protein synthesis will be reported in due course.

Experimental Section

Experimental details are reported in the Supporting Information.

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Keywords: cycloheximide • proteins • inhibitors • irreversible • polysome

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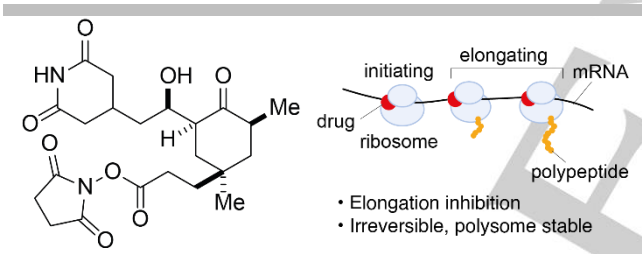

An irreversible inhibitor of protein synthesis based on the cytotoxic natural product cycloheximide is presented. The inhibitor preserves polysome profiles even after the compound is washed out.

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