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Development of Orthogonally Protected Hypusine for Solid-Phase Peptide Synthesis

Aimin Song,[†] Jeffrey Tom,[†] Zhiyong Yu,[‡] Victoria Pham,[§] Dajin Tan,[‡] Dengxiong Zhang,[‡] Guoyong Fang,[∥] Tao Yu,[∥] and Kurt Deshayes*[,†]

[†]Department of Early Discovery Biochemistry, Genentech, Inc., 1 DNA Way, South San Francisco, California 94080, United States

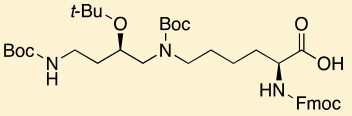
[‡]International Discovery Service Unit, WuXi AppTec (Shanghai) Co., Ltd., 288 Fute Zhong Road, Waigaoqiao Free Trade Zone, Shanghai 200131, China

[§]Department of Protein Chemistry, Genentech, Inc., 1 DNA Way, South San Francisco, California 94080, United States

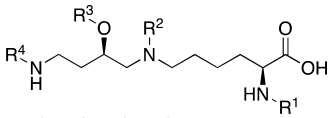
[∥]Center for Peptide Service, WuXi AppTec (Shanghai) Co., Ltd., 288 Fute Zhong Road, Waigaoqiao Free Trade Zone, Shanghai 200131, China

Supporting Information

ABSTRACT: An orthogonally protected hypusine reagent was developed for solid-phase synthesis of hypusinated peptides using the Fmoc/*t*-Bu protection strategy. The reagent was synthesized in an overall yield of 27% after seven steps from Cbz-Lys-OBzl and (*R*)-3-hydroxypyrrolidin-2-one. The side-chain protecting groups (Boc and *t*-Bu) are fully compatible with standard Fmoc chemistry and can be readily removed during the peptide cleavage step. The utility of the reagent was demonstrated by solid-phase synthesis of hypusinated peptides.



Unusual amino acids widely exist in organisms. Many of them are formed by posttranslational modifications (PTMs) of natural proteinogenic amino acids and play important roles in various biological processes.[1] (+)-Hypusine ((2*S*,9*R*)-2,11-diamino-9-hydroxy-7-azaundecanoic acid, **1**, Figure 1) is a PTM derivative of lysine that was first isolated from



1 $R^1 = R^2 = R^3 = R^4 = H$
2 R^1 = Fmoc, $R^2 = R^4$ = Cbz, R^3 = THP
3 R^1 = Fmoc, $R^2 = R^4$ = Boc, R^3 = *t*-Bu

Figure 1. Structures of hypusine derivatives.

homogenates of bovine brain in 1971.[2] Eukaryotic translation initiation factor 5A (eIF5A) is the only known cellular protein that contains hypusine. Research has shown that eIF5A plays a key role in the viability and proliferation of eukaryotic cells,[3] and is overexpressed in a number of human cancers.[4] The activity of eIF5A is modulated by the formation of hypusine that involves two enzymatic reactions. A specific lysine residue in eIF5A is first modified with the 4-aminobutyl moiety of spermidine by deoxyhypusine synthase (DHS), followed by the deoxyhypusine hydroxylase (DOHH)-catalyzed hydroxylation.[5]

Over the past decade, eIF5A and hypusination have attracted increasing attention as a potential diagnostic marker and a drug target.[4,6] Synthetic hypusinated peptides are of great interest in order to elucidate the physiological functions of eIF5A. Although several synthetic methods for free hypusine have been reported,[7] the orthogonal protection of the side-chain functionalities remains as a challenge for the incorporation of hypusine into peptide sequences. In 1997, Bergeron et al. developed a protected hypusine reagent (**2**, Figure 1) for peptide synthesis.[8] The α-amino group of hypusine was protected with Fmoc (9-fluorenylmethoxycarbonyl), and the side-chain amino groups and hydroxyl group were protected with Cbz (benzyloxycarbonyl) and THP (tetrahydropyranyl), respectively. The reagent was successfully used for the synthesis of hypusinated peptides.[8,9] However, the side-chain protecting groups cannot be removed by standard TFA (trifluoroacetic acid) cleavage, and the deprotection required the treatment with HBr in TFA. The extra deprotection step caused not only inconvenience but also decreased peptide purity due to the harsh reaction conditions.[10]

In an effort to develop automated solid-phase synthesis of hypusinated peptides, we designed a new orthogonally protected hypusine derivative (**3**, Figure 1) using the standard Fmoc/*t*-Bu protection strategy. The α-amino group was protected with Fmoc for solid-phase peptide elongation. Boc (*tert*-butoxycarbonyl) and *t*-Bu (*tert*-butyl) were selected to protect the side-chain amino groups and hydroxyl group, respectively. The acid-labile side-chain protecting groups are fully compatible with standard Fmoc chemistry and can be readily removed during the peptide cleavage step. The hypusine molecule contains three amino groups, one hydroxyl group, and one carboxyl group. We envisioned that the selective protection would be difficult once the molecule was assembled. Therefore, the protection should start with the synthons.

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Scheme 1. Synthesis of Orthogonally Protected Hypusine

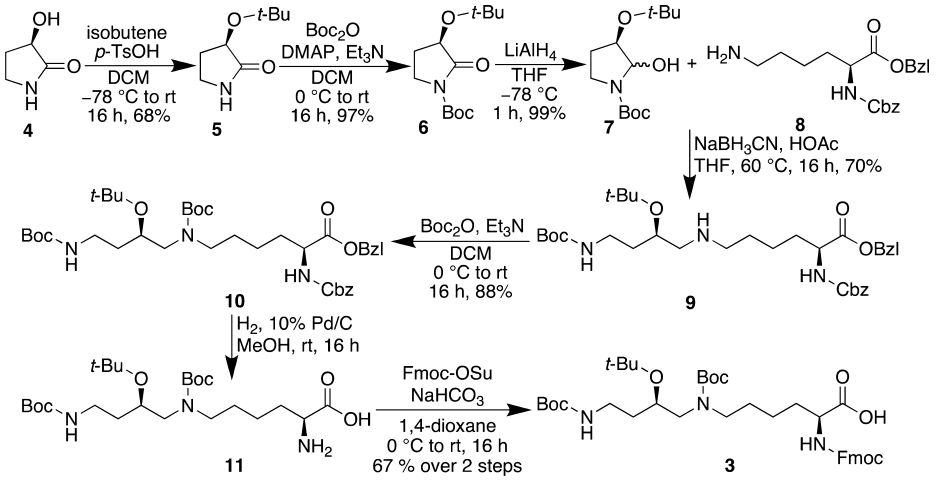
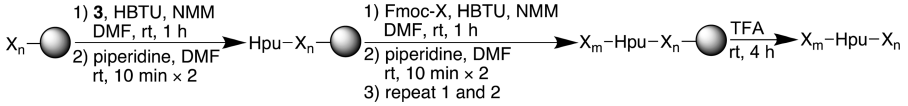


Table 1. Automated Solid-Phase Synthesis of Hypusinated Peptides[a]



				HRMS $[M + 2H]^{2+}$	
entry	peptide sequence	crude purity (%)	isolated yield[b] (%)	calcd	found
1	TG-Hpu-HGHAK	75	37	461.7645	461.7654
2	IVEMSTSKTG-Hpu-HGHAK	63	31	899.4856	899.4863
3	IVEMSTSK(Ac)TG-Hpu-HGHAK	46	24	920.4909	920.4906
4	Biotin-IVEMSTSK(Ac)TG-Hpu-HGHAK	49	21	1033.5297	1033.5295
5	YEDICPSTHNMDVPNI-Hpu-R	65	34	1110.0328	1110.0338
6	A-Hpu-AIIVEFAQQGLNAALFYENK	58	28	1263.1918	1263.1912

[a]Hpu represents hypusine, and X represents other amino acids. Standard single-letter codes are used for natural amino acids. [b]Yields were calculated based on the resin loading.

The synthesis of the orthogonally protected hypusine derivative is illustrated in Scheme 1. The key step of the synthesis involved the reductive alkylation of a commercially available lysine derivative, Cbz-Lys-OBzl (**8**), with an aldehyde equivalent (**7**) that was derivatized from (*R*)-3-hydroxypyrrolidin-2-one (**4**). The hydroxyl group of **4** was first protected with *t*-Bu in 68% yield by the treatment with isobutene in the presence of *p*-TsOH (*p*-toluenesulfonic acid).[11] Subsequent reaction of the lactam **5** with Boc_2O (di-*tert*-butyl dicarbonate) afforded intermediate **6** in 97% yield.[12] The product was analyzed with chiral HPLC, and no racemization was observed.

Initial attempts to reduce intermediate **6** with DIBAL-H failed to give the desired product.[13] Reduction of **6** with $NaBH_4$ at −40 °C afforded intermediate **7** in 28% yield, but the yield decreased at higher temperature due to the formation of the overreduction product. Finally, the aldehyde equivalent **7** was obtained by reduction with $LiAlH_4$ at −78 °C in quantitative yield.

Various combinations of reagents, solvents, and conditions were tested for the reductive alkylation of Cbz-Lys-OBzl (**8**) with intermediate **7**. The best result was achieved with $NaBH_3CN$ in THF at 60 °C in the presence of HOAc. Under these conditions, the key intermediate **9** was obtained in 70% yield. SFC (supercritical fluid chromatography) and NMR analysis indicated that compound **9** was the sole product of the reductive alkylation. There was no detectable epimerization probably because the reaction proceeded via a cyclic *N*-acyliminium ion intermediate instead of a ring-opened imine.[14]

Intermediate **9** was then reacted with Boc_2O to complete the protection of the side-chain functionalities in hypusine. The fully protected hypusine derivative **10** was obtained in 88% yield. Hydrogenation of **10** catalyzed by Pd/C resulted in intermediate **11**, which was subsequently protected with Fmoc-OSu without purification.[15] The final product **3** was obtained in 67% yield from **10** over two steps. A small amount of **3** was treated with 10% piperidine in ACN (acetonitrile) and subsequently 4 M HCl in 1,4-dioxane to afford hypusine **1** in 72% yield as its dihydrochloride salt ($[\alpha]_D^{23}$ +7.4 (*c* 0.5, 6 M HCl); lit. $[\alpha]_D^{23}$ +7.6 (*c* 0.5, 6 M HCl),[7d] $[\alpha]_D^{25}$ +7.3 (*c* 0.52, 6 M HCl)[7e]). The analytical data for this substance matched those reported in the literature.[7]

The orthogonally protected hypusine reagent **3** was readily incorporated into peptide sequences using standard solid-phase Fmoc chemistry (Table 1).[16] The coupling of **3** was complete in 1 h at room temperature using HBTU (*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate)/NMM (4-methylmorpholine) as activating reagents. LC-MS analysis of the crude hypusinated peptides showed that the side-chain protecting groups were completely removed during the TFA cleavage step. To date, 47 hypusinated peptides were synthesized using reagent **3** in our group with various modifications, including acetylation, biotinylation, PEGylation, and stable isotope labeling. The hypusinated peptides were

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obtained in 42−81% crude purity and 12−37% isolated yield after HPLC purification, which was comparable to that of control peptides without hypusination.

The LC-MS analysis result of the synthetic stable isotope labeled eIF5A(48-55) peptide (Thr-Gly(U-$^{13}C_2$,^{15}N)-hypusine-His-Gly(U-$^{13}C_2$,^{15}N)-His-Ala(U-$^{13}C_3$,^{15}N)-Lys, $[M + 2H]^{2+}$ Calcd 466.7718) is shown in Figure 2, in comparison with

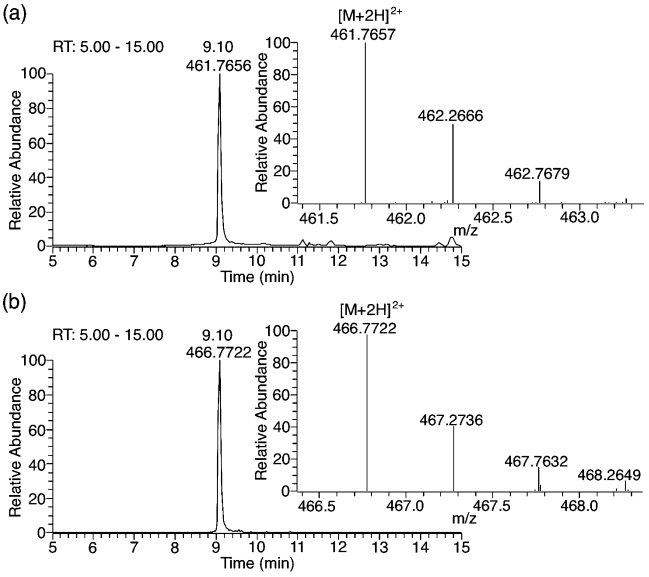

Figure 2. LC-MS analysis results of endogenous (a) and synthetic heavy isotope labeled (b) eIF5A(48-55) peptides.

that of the endogenous peptide ($[M + 2H]^{2+}$ Calcd 461.7645). The stable isotope-labeled hypusinated peptide eluted at the same time as the endogenous eIF5A(48-55) with the matching mass accuracy, isotopic envelope, and MS/MS fragmentation pattern (see the Supporting Information), confirming the authenticity of the synthetic hypusinated peptide.

In summary, we have developed an orthogonally protected hypusine reagent for solid-phase synthesis of hypusinated peptides. The reagent was synthesized in an overall yield of 27% after seven steps and was successfully used for the synthesis of hypusinated peptides. The side-chain protecting groups are fully compatible with standard Fmoc chemistry and can be readily removed during the peptide cleavage step. The reagent is ideally suited for the automated synthesis of hypusinated peptides.

EXPERIMENTAL SECTION

General Experimental Methods. All solvents and reagents were used without further purification. The purity of compounds was determined by LC-MS analysis (C18 column, 2 × 25 mm, 1.6 μm, 1.5 mL/min, 1.5 min gradient from 5% to 95% ACN/water containing 0.1% TFA) or HPLC analysis (C18 column, 2.1 × 30 mm, 3 μm, 1.2 mL/min, 8 min gradient from 10% to 80% ACN/water containing 0.1% TFA, 220 nm). The optical purity of compounds was determined by SFC analysis (chiral column, 4.6 × 150 mm, 3 μm, 2.5 mL/min, 10 min gradient from 5% to 40% methanol or ethanol (0.05% DEA) in CO_2, 220 nm). Peptides were purified by preparative HPLC (C18 column, 30 × 250 mm, 15 μm, 70 mL/min, 50 min gradient from 10% to 90% ACN/water containing 0.1% TFA, 220 nm).

Synthesis of (*R*)-3-*tert*-Butoxypyrrolidin-2-one (5). Isobutene (27.7 g, 494 mmol) was added to a solution of (*R*)-3-hydroxypyrrolidine-2-one (**4**, 5.0 g, 49.5 mmol) in DCM (dichloromethane) (35 mL) in a pressure vessel at −78 °C. The vessel was sealed, and the reaction mixture was stirred at room temperature for 16 h. The reaction vessel was chilled in a dry ice/isopropanol bath, and the inner pressure was carefully released. TLC analysis (EtOAc, R_f = 0.4) indicated complete reaction. Saturated $NaHCO_3$ solution was added into the mixture until pH = 7, followed by the addition of DCM (100 mL). The organic phase was separated, and the aqueous phase was extracted with DCM (100 mL × 2). The combined organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give compound **5** (5.3 g, 68%) as a white solid. The product was used for the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.13 (t, J = 8.0 Hz, 1H), 3.36−3.31 (m, 1H), 3.24−3.21 (m, 1H), 2.34−2.30 (m, 1H), 1.99−1.94 (m, 1H), 1.20 (s, 9H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 175.7, 74.0, 69.2, 37.6, 31.0, 28.2; $[\alpha]_D^{20}$ +46.3 (c 1.0, CH_3OH).

Synthesis of (*R*)-*tert*-Butyl 3-*tert*-Butoxy-2-oxopyrrolidine-1-carboxylate (6). Boc_2O (14.7 g, 67.4 mmol) was added to a stirred solution of compound **5** (5.3 g, 33.7 mmol), triethylamine (6.8 g, 67.4 mmol), and DMAP (4.1 g, 33.7 mmol) in DCM (60 mL) at 0 °C. The resulting mixture was stirred at room temperature for 16 h. TLC analysis (20% EtOAc in petroleum, R_f = 0.6) indicated complete reaction. The reaction volatiles were removed under reduced pressure, and the residue was purified by flash column chromatography (5% EtOAc in petroleum) to give compound **6** (8.5 g, 97%) as a white solid. Chiral HPLC analysis showed no racemization. ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.36(dd, J = 10.0, 8.5 Hz, 1H), 3.62−3.58 (m, 1H), 3.43−3.40 (m, 1H), 2.22−2.16 (m, 1H), 1.74−1.64 (m, 1H), 1.44 (s, 9H), 1.16 (s, 9H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.8, 149.9, 81.8, 74.6, 70.4, 41.6, 28.0, 27.7; $[\alpha]_D^{20}$ +46.0 (c 1.0, CH_3OH).

Synthesis of (2*RS*,3*R*)-*tert*-Butyl 3-*tert*-Butoxy-2-hydroxypyrrolidine-1-carboxylate (7). A solution of compound **6** (7.0 g, 27.2 mmol) in THF (30 mL) was added to a stirred solution of $LiAlH_4$ (1.55 g, 40.8 mmol) in THF (50 mL) at −78 °C. The resulting mixture was stirred at −78 °C for 1 h. TLC analysis (20% EtOAc in petroleum, R_f = 0.5) indicated complete reaction. The reaction was quenched by adding ice water (1.3 mL), 10% NaOH (1.3 mL), and water (2.6 mL) sequentially at −78 °C. The resulting mixture was warmed up to 27 °C and then filtered. The filter cake was washed with THF (10 mL × 2). The filtrate was concentrated under reduced pressure to give compound **7** (7.0 g, 26.9 mmol, 99.2%) as a colorless oil. The product was used for the next step without further purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.74 (d, J = 3.6 Hz, 0.5H, OH), 5.54 (d, J = 3.6 Hz, 0.5H, OH), 4.88 (dd, J = 20.0, 3.6 Hz, 1H), 3.79 (dd, J = 20.0, 3.6 Hz, 1H), 3.25−3.21 (m, 2H), 2.07−2.04 (m, 1H), 1.56 (brs, 1H), 1.38 (s, 9H), 1.13 (s, 9H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 153.6, 85.7, 78.4, 76.3, 75.5, 73.5, 44.0, 43.7, 30.2, 29.4, 28.1, 28.0; HRMS (ESI-TOF) m/z: $[M + Na]^+$ Calcd for $C_{13}H_{26}NO_3Na$ 282.1681; Found: 282.1695.

Assembly of Hypusine Intermediate 9 via Reductive Alkylation. $NaBH_3CN$ (2.14 g, 34.0 mmol) and HOAc (1.36 g, 22.7 mmol) were added to a stirred solution of compound **8** (6.0 g, 11.3 mmol) and compound **7** (4.41 g, 17.0 mmol) in THF (90 mL) at room temperature. The resulting mixture was stirred at 60 °C for 16 h. TLC analysis (EtOAc, R_f = 0.3) indicated complete reaction. The reaction mixture was diluted with saturated $NaHCO_3$ solution (30 mL) and extracted with EtOAc (100 mL × 2). The combined organic phase was washed with brine (60 mL × 2), dried over anhydrous Na_2SO_4, and concentrated to dryness under reduced pressure. The residue was purified by flash column chromatography (50% EtOAc in petroleum) to give compound **9** (4.9 g, 70%) as a colorless oil. SFC analysis showed no racemization. ^{1}H NMR (400 MHz, CD_3OD) δ 7.34−7.30 (m, 10H), 5.20−5.08 (m, 4H), 4.25−4.22 (m, 2H), 3.92 (brs, 2H), 3.07−2.93 (m, 6H), 1.88−1.72 (m, 1H), 1.71−1.66 (m, 5H), 1.42 (s, 9H), 1.23 (s, 9H); ^{13}C NMR (100 MHz, CD_3OD) δ 173.8, 158.9, 138.3, 137.4, 129.8, 129.6, 129.5, 129.0, 76.6, 68.1, 67.8, 66.6, 55.3, 52.7, 35.6, 32.1, 28.9, 28.8, 26.2, 24.0; HRMS (ESI-TOF) m/z: $[M + Na]^+$ Calcd for $C_{34}H_{51}N_3O_7Na$ 636.3625; Found: 636.3677.

Synthesis of Fully Protected Hypusine 10. Boc_2O (7.47 g, 34.2 mmol) and triethylamine (4.62 g, 45.6 mmol) were added to a stirred solution of compound **9** (14.0 g, 22.8 mmol) in DCM (200 mL) at 0

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°C. The resulting mixture was stirred at room temperature for 16 h. TLC analysis (20% EtOAc in petroleum ether, R_f = 0.4) indicated complete reaction. The reaction mixture was concentrated under reduced pressure, and the residue was purified by flash column chromatography (30% EtOAc in petroleum) to give fully protected hypusine **10** (14.4 g, 20.1 mmol, 88.4%) as a colorless oil. ^{1}H NMR (400 MHz, CD_3OD) δ 7.32−7.27 (m, 10H), 5.18−5.03 (m, 4H), 4.20 (brs, 1H), 3.83 (brs, 1H), 3.33−3.31 (m, 1H), 3.23−2.93 (m, 6H), 1.86−1.79 (m, 1H), 1.67−1.65 (m, 1H), 1.59−1.56 (m, 2H), 1.48−1.40 (m, 20H), 1.30 (brs, 2H), 1.14 (s, 9H); ^{13}C NMR (100 MHz, CD_3OD) δ 172.1, 156.8, 156.4, 155.6, 155.5, 136.3, 135.4, 127.7, 127.6, 127.4, 127.1, 126.9, 79.3, 79.0, 78.0, 73.4, 68.0, 67.3, 65.9, 65.8, 53.6, 50.8, 36.1, 33.9, 30.4, 26.9, 26.3, 22.3; HRMS (ESI-TOF) m/z: $[M + Na]^+$ Calcd for $C_{39}H_{59}N_3O_9Na$ 736.4149; Found: 736.4157.

Synthesis of Orthogonally Protected Hypusine 3. A mixture of compound **10** (14.4 g, 20.2 mmol), 10% Pd/C (3.0 g), and MeOH (methanol) (50 mL) was agitated at room temperature under H_2 (40 psi) for 16 h. TLC analysis (30% EtOAc in petroleum) indicated complete reaction. The reaction mixture was filtered. The filtrate was concentrated under reduced pressure to afford a white solid, which was dissolved in a mixture of 1,4-dioxane (200 mL) and 5% $NaHCO_3$ in H_2O (200 mL). Fmoc-OSu (8.2 g, 24.2 mmol) was added with stirring at 0 °C. The resulting mixture was stirred at room temperature for 16 h. TLC analysis (60% EtOAc in petroleum, R_f = 0.3) indicated complete reaction. The reaction mixture was carefully acidified with 1 M HCl to pH 5 and was then extracted with EtOAc (200 mL × 2). The combined organic phase was washed with water (200 mL × 3), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to dryness. The residue was purified by flash column chromatography (5% MeOH in DCM) to give orthogonally protected hypusine **3** (9.6 g, 67% over two steps) as a white solid. SFC analysis showed no racemization. ^{1}H NMR (400 MHz, CD_3OD) δ 7.82−7.81 (m, 2 H), 7.70−7.68 (m, 2 H), 7.43−7.39 (m, 2 H), 7.35−7.31 (m, 2 H), 4.38−4.35 (m, 2 H), 4.26−4.18 (m, 2 H), 3.88 (brs, 1 H), 3.41 (brs, 1 H), 3.27−3.01 (m, 6 H), 1.91−1.86 (m, 1 H), 1.74 (brs, 1 H), 1.62−1.58 (m, 4 H), 1.47−1.44 (m, 20 H), 1.18 (s, 9 H); ^{13}C NMR (100 MHz, CD_3OD) δ 174.5, 157.3, 156.9, 143.9. 143.8, 141.1, 127.4, 126.7, 124.8, 119.5, 79.8, 79.5, 78.4, 73.9, 68.4, 67.8, 66.5, 53.8, 53.7, 51.2, 36.5, 34.3, 31.0, 27.4, 22.9; HRMS (ESI-TOF) m/z: $[M + Na]^+$ Calcd for $C_{39}H_{57}N_3O_9Na$ 734.3993; Found: 734.3954; $[\alpha]_D^{20}$ −4.4 (c 1.0, CH_3OH).

Hypusine 1 Dihydrochloride. Piperidine (200 μL) was added to a solution of compound **3** (200 mg, 0.28 mmol) in ACN (2 mL) at 0 °C. The resulting mixture was stirred at room temperature for 30 min. LC-MS analysis indicated complete reaction. The reaction mixture was concentrated under reduced pressure, and the residue was purified by preparative HPLC. The obtained intermediate **11** (104 mg, 0.21 mmol) was dissolved in 4 M HCl in 1,4-dioxane (2 mL). The resulting solution was agitated at room temperature for 2 h and was then diluted with anhydrous ether (45 mL). The precipitate was separated by centrifugation, washed with anhydrous ether (45 mL × 3), and dried over NaOH in vacuo. The crude product was dissolved in MeOH (2 mL) and precipitated again with anhydrous ether (45 mL) to give hypusine **1** dihydrochloride as a white hygroscopic solid (62 mg, 72% over two step). ^{1}H NMR (500 MHz, D_2O) δ 3.92 (tt, 1H J = 3.1, 9.7 Hz), 3.85 (t, 1H, J = 6.2 Hz), 3.08−2.90 (m, 6H), 1.89−1.76 (m, 3H), 1.72−1.61 (m, 3H), 1.45−1.31 (m, 2H); ^{13}C NMR (125 MHz, D_2O) δ 172.6, 64.6, 53.1, 52.0, 47.1, 36.3, 31.3, 29.3, 24.9, 21.4; HRMS (ESI-FTMS) m/z: $[M + H]^+$ Calcd for $C_{10}H_{24}N_3O_3$ 234.1817; Found: 234.1810; $[\alpha]_D^{23}$ +7.4 (c 0.5, 6 M HCl).

Solid-Phase Peptide Synthesis. Automated solid-phase synthesis of hypusinated peptides was carried out on a multiplex peptide synthesizer using standard Fmoc chemistry. For the coupling step, a solution of the Fmoc-protected amino acid (0.2 M) in DMF (*N*,*N*-dimethylformamide) (1.5 mL) was added to the resin-bound amine (0.1 mmol), followed by the addition of a solution of HBTU (0.2 M) and NMM (0.4 M) in DMF (1.5 mL) and DCM (1 mL). The resulting mixture was purged with nitrogen at room temperature for 1 h. The supernatant was drained, and the resin was washed with DMF (4 mL × 3). For Fmoc deprotection, 20% piperidine/DMF (4 mL) was added to the resin. The resulting mixture was purged with nitrogen at room temperature for 10 min. The supernatant was drained, and the process was repeated once. The resin was then washed thoroughly with DMF (4 mL × 6). The coupling−deprotection cycle was repeated until the peptide assembly was complete. For peptide cleavage, the resin was washed thoroughly with MeOH (4 mL × 3) and DCM (4 mL × 3), and then dried in vacuo. A mixture of TFA/phenol/water/thioanisole/3,6-dioxa-1,8-octanedithiol/triisopropylsilane (v/w/v/v/v 82.5:3.75:5:3.75:2.5:2.5, 4 mL) was added to the resin at 0 °C. The resulting mixture was slowly warmed to room temperature and allowed to mix for 4 h. The supernatant was collected by filtration, and the resin was washed with the cleavage mixture (1 mL × 3). The combined filtrate was concentrated under reduced pressure to a small volume, which was diluted with anhydrous ether (45 mL). The resulting mixture was allowed to stand at 0 °C for 30 min and then centrifuged. The precipitate was washed with anhydrous ether (45 mL × 3) and dried in vacuo. The crude peptide was analyzed with LC-MS and purified by HPLC.

Sample Preparation of eIF5A(48-55) Peptides for LC-MS/MS Analysis. Protein samples were reduced with 10 mM DTT (dithiothreitol) in 1× SDS sample buffer at 95 °C for 5 min and loaded on 4−12% Bis-Tris gel. The protein band corresponding to eIF5A was excised and further destained in 50 mM NH_4HCO_3 in 30% ACN/water, and then dehydrated in 100% ACN. Gel pieces were rehydrated with 10 ng/μL trypsin in 25 mM NH_4HCO_3 and chilled on ice for 1 h. Excess trypsin solution was removed, and digestion was performed in 25 mM NH_4HCO_3 at 37 °C overnight. Peptides were extracted with 50% ACN/water containing 0.1% TFA, followed by 100% ACN. Heavy isotope labeled hypusinated peptides were spiked in at 250 fmol. The peptide mixture was dried to completion and resuspended in 2% ACN/water containg 0.1% FA (formic acid).

LC-MS/MS Analysis of eIF5A(48-55) Peptides. Peptide digest containing 250 fmol of heavy isotopically labeled peptides was analyzed with LC-MS/MS (C18 column, 1.7 mm, 0.1 × 100 mm) with a flow rate of 1 μL/min and a gradient of 2−25% Solvent B (where Solvent A is 0.1% FA/2% ACN/water and Solvent B is 0.1% FA/2% water/ACN) applied over 35 min with a total analysis time of 60 min. Precursor ions were analyzed in the FTMS at 60 000 resolution; MS/MS was performed in data dependent mode, whereby the top 15 most abundant ions were subjected for fragmentation. Extracted ion chromatograms of the endogenous (light) and heavy labeled peptides were generated, and area under the curves (AUC) was integrated. Abundance measurements were calculated for each analyte peptide as the ratio of light to heavy peptides.

■ ASSOCIATED CONTENT

Supporting Information

Copies of spectra and chromatograms. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: deshayes.kurt@gene.com (K.D.).

Notes

The authors declare no competing financial interest.

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