Synthesis of Fluorescent Jasplakinolide Analogues for Live-Cell STED Microscopy of Actin

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ABSTRACT: The nanometer thickness of filaments and the dynamic behavior of actin—a protein playing a crucial role in cellular function and motility—make it attractive for observation with super-resolution optical microscopy. We developed the solution-phase synthesis of des-bromo-des-methyl-jasplakinolide-lysine, used as the “recognition unit” (ligand) for F-actin in living cells. The first amino acid—Fmoc-O-TIPS-β-tyrosine—was prepared in 78% yield (two steps in one pot). The new solution-phase synthesis involves 2-phenylisopropyl protection of the carboxyl group and does not require excesses of commercially unavailable amino acids. The overall yield of the target intermediate obtained in nine steps is about 8%. The 2-phenylisopropyl group can be cleaved from carboxyl with 2−3% (v/v) of TFA in acetonitrile (0−10 °C), without affecting TIPS protection of the phenolic hydroxyl in β-tyrosine and N-Boc protection in lysine. Des-bromo-des-methyl-jasplakinolide-lysine was coupled with red-emitting fluorescent dyes 580CP and 610CP (via 6-aminohexanoate linker). Actin in living cells was labeled with 580CP and 610CP probes, and the optical resolution measured as full width at half-maximum of line profiles across actin fibers was found to be 300−400 nm and 100 nm under confocal and STED conditions, respectively. The solution-phase synthesis of des-bromo-des-methyl-jasplakinolide-lysine opens a way to better fluorescent probe perspective for actin imaging.

INTRODUCTION

Actin protein plays a crucial role in cellular function and motility.1 It can be present either as a monomer (G-actin; globular) or, upon polymerization, it may form filaments (F-actin): flexible fibers with a diameter of 4−7 nm and length of up to several micrometers. In living cells, both forms of actin are present in equilibrium; they are essential for the proper mobility and contraction of cells during cell division, cell motility, cytokinesis, vesicle and organelle movement, cell signaling, as well as the establishment and maintenance of cell junctions and cell shapes. The nanometer thickness and dynamic behavior of actin filaments make them an attractive object for observation with super-resolution optical microscopy.

The fluorescent probes for super-resolution and live imaging of actin2−5 incorporate the so-called des-bromo-des-methyl-jasplakinolide-lysine (Figure 1),6 as the ligand or “recognition unit” for F-actin in living cells. This macrocyclic depsipeptide has a reactive amino group, and its salts can be readily generated from N-tert-butoxycarbonyl derivative (7-H in Scheme 3) which represents the key intermediate and stable precursor of the conjugates with organic dyes. Compound 7-H is commercially unavailable, and the solid-phase synthesis of 7-H has been outlined only briefly.2,6 The aim of the present work was to develop the new and productive route to macrocyclic depsipeptide 7-H, compare the syntheses on a solid phase and in solution, prepare the conjugates of compound 7-H with fluorescent dyes, and apply them as fluorescent probes for the super-resolution microscopy of actin filaments in living cells. As cell-permeate fluorescent dyes, we have chosen carbopyronines 580CP and 610CP which demonstrated high imaging performance as conjugates with various ligands.20,21 The absorption and emission spectra of these dyes are given in Figure 1 and the photophysical properties in Table 1.

RESULTS AND DISCUSSION

Both synthesis routes—on the solid phase and in solution—involves Fmoc-O-TIPS-β-tyrosine as the first amino acid (AA1;
Schemes 1 and 2). This compound was initially obtained via a multistep procedure including the Michael addition of a chiral dibenzyl amine to p-coumaric acid ester, separation of the diastereomers, N-debenzylation, and manipulation with O Protecting groups. We found a shorter route to Fmoc-O-TIPS-β-tyrosine, which starts from commercially available Fmoc-β-tyrosine (Scheme 2). The two-step procedure includes silylation with triisopropyl silyl chloride on both oxygen centers, followed by the hydrolytic cleavage of the more labile (triisopropylsilyl)ester group under mild basic conditions and affords the required amino acid AA1 (Scheme 2). The solid-phase synthesis (Scheme 1) provides triamide 1-H-TIPS as the key intermediate. Compound 1-H-TIPS was isolated with an overall yield of about 22% (52%, when calculated on the loading degree of the first amino acid AA1). However, these yields are based on the use of large excess of amino acids AA1, AA2, and AA3. The first two are not commercially available and have to be prepared separately. Therefore, the use of large excess of AA1 and AA2 is not cost- and time-efficient. Another important detail of the solid-phase synthesis is that the cleavage from the resin is performed in the presence of weakly acidic hexafluoroisopropanol. The latter (b. p. 58 °C) concentrates in the reaction mixture in the course of solvent evaporation (DCM) and causes (partial) removal of the triisopropyl silyl group. We added a higher boiling solvent (ethyl acetate) into the solution in order to prevent this undesirable effect and suppress the formation of deprotected phenol 1-H-H.

Planning the solution-phase synthesis of compound 1-H-TIPS (5-H-H in Schemes 2 and 3), we realized that its success is determined by the correct choice of carboxyl protection in AA1 (Scheme 2). We used the 2-phenylisopropyl protecting group because 2-chlorotrityl esters partially cleaved in the course of work-up and isolation procedures (chromatography), when the synthesis was carried out according to Scheme 2. The synthesis was carried out according to Scheme 2, using water-soluble carbodiimide (free base) in the presence of H2A and 2,4,6-collidine in DCM. Under these conditions, no racemization was observed. 9-Fluorenylethoxycarbamate groups were cleaved using diethylamine (the excess of which was removed by several evaporations with toluene); intermediate compounds with free amino groups were not isolated but used directly in the following amidation reactions. In this approach, the excess of N-protected amino acids is not required (which is an advantage over the solid-phase methodology). The final step—removal of 2-phenylpropyl protecting group—was achieved using 2–3% solution of TFA in acetonitrile at 0...+5 °C. Under these conditions, N-tert-butoxycarbonyl protection of amines is stable. Moreover, TIPS protection of the phenolic hydroxyl group turned out to be stable as well. However, we detected and isolated compound 5-H-C(CH3)2C6H4 (17%), which was formed when 2-phenylpropyl residue was transferred to another nucleophilic center—nitrogen atom of tryptophan. The synthesis in solution is attractive not only because of the relatively high overall yield (35%; Scheme 2) but also because it is not necessary to apply (unrecoverable) excess of exotic and expensive amino and (S)-2,4-dimethylpent-4-enoic acids. The final steps of the assembly of macrocyclic depsipeptide 7-H are common for the solid- and solution-phase syntheses and are given in Scheme 3. The first reaction—formation of ester 6 from carboxylic acid 5-H-H (1-H-TIPS in Scheme 1) and (S)-S-hexen-2-ol in the presence of carbodiimide (EDC*HCl)—requires 4-(N,N-dimethylamino)pyridine (DMAP) as a catalyst. We found that the use of more than 10 mol % of Scheme 1. Solid-Phase Synthesis Affords the Key Intermediate 1-H-TIPS

![Scheme 1](https://dx.doi.org/10.1021/acs.joc.0c00653)
DMAP is counterproductive, as it converts the intermediate O-acylurea (active acylating reagent) into the stable N-acylurea which is inert and does not react any further. The undesired O-to N-migration of the acetyl residue consumes carboxylic acid, and the required esterification does not take place. The metathesis of dialkene 6 was performed as reported. Along with macrocyclic alkene 7-TIPS, we isolated isomer 8-TIPS which, after removal of the TIPS group, gave compound 8-H (mixture of 2 epimers; the structure established by NMR spectroscopy). Formally, compound 8-TIPS is formed, if the
Table 1. Spectral Properties of Carbopyronines 580CP, 610CP, and SiR (Reference Dye) in Aqueous PBS Buffer (pH 7.4) at Room Temperature

| dye     | absorption λmax nm (ε, M−1 cm−1) | fluores. dmax nm (Φfl) | brightness rel. to SiR² | Dλs² | fluorescence lifetime τ, ns |
|---------|----------------------------------|------------------------|-------------------------|------|---------------------------|
| 580CP   | 582 (90,000)                     | 607 (0.69)             | 1.63                    | 34.6 | 3.6                       |
| 610CP   | 609 (100,000)                    | 634 (0.59)             | 1.55                    | 36.4 | 3.1                       |
| SiR     | 645 (93,000)                     | 661 (0.41)             | 1 (ref.)                | 64.5 | 2.7                       |

"Fluorescence quantum yield. °Expressed as (ε × Φfl)_{SiR}/(ε × Φfl)_{sample}.

Dielectrical constant of dioxane (ε) = 2.38; that of PBS buffer (pH 7.4) at Room Temperature = 80. The values were calculated from the refractive index and density of the respective solutions. The refractive index values were counted from Handbook (7). The density values were calculated from the molar mass and Pycnometer measurements.

We labeled actin in living human osteosarcoma cells (U-2 OS) (Figure 2A,B) and in kidney cells derived from the African green monkey (COS-7) (Figure 2C,D) using 580CP- and 610CP-jasplakinolide probes, respectively. Both probes (for structures, see Scheme 4) performed well in confocal and STED (stimulated emission depletion) microscopy. The optical resolution (full width at half maximum of a line profile) in the STEED mode improved: the apparent diameters of actin fiber bundles under confocal and STEED conditions were 300–400 nm and ca. 100 nm, respectively (see Figure 2). Both dyes (for spectral properties, see Table 1) have some residual emission at 775 nm (wavelength of the STEED laser; see Figure 1) but virtually no absorption at this wavelength. These valuable spectral features provide an efficient STEED effect and, as a result, optical resolution improvement without undesirable reexcitation with the STEED beam. Importantly, the conjugates of carbopyronine dye 580CP enable two-color STEED microscopy in living cells with standard optical settings (e.g., in combination with SiR dye; see Table 1).

CONCLUSIONS

In vitro labeling of actin filaments (Figure 2) with 580CP- and 610CP-jasplakinolide conjugates exhibits different patterns in different cell lines and at different concentrations: the best imaging results were achieved when 580CP probe was applied at 5 μM for 30 min and 610CP-jasplakinolide—at 1 μM for 60 min. Compared to 610CP-, 580CP-jasplakinolide enables an enhanced labeling of intricate actin structures. The overall performance can be affected by the specific dye residue coupled to the (same) jasplakinolide ligand, as the dye was shown to influence the core characteristics of the whole fluorescent probe, such as binding parameters (kinetics, affinity, equilibrium between F- and G-actin), cytotoxicity, and, most importantly, cell entry and/or retention. Other (less toxic, more specific, brighter) fluorescent probes for actin in cells and tissues may help further to understand the role of this protein in cell functions and motility(16). The proposed methodology enables the scalable synthesis of compounds 7–H, 9, their analogs (e.g., by varying the structure of unsaturated alcohol in Scheme 3), and their conjugates with fluorescent dyes, in order to reveal new important aspects of actin behavior in the living matter.

EXPERIMENTAL SECTION

General Remarks. The reactions (in solution phase synthesis) were performed with magnetic stirring under an argon atmosphere. Evaporations in vacuum were performed in a rotary evaporator with bath temperature not exceeding 45 °C. Automated flash column chromatography was carried out using cartridges with regular silica gel.
Figure 2. In vitro labeling of actin filaments in U-2 OS and COS-7 cells with S80CP-jasplakinolide and 610CP-jasplakinolide probes (see Scheme 4 and ref s). (A,B) Human osteosarcoma (U-2 OS) cells and (C,D) kidney cells derived from the African green monkey (COS-7) were incubated with (A,C) S80CP-jasplakinolide (5 μM for 30 min) or (B,D) 610CP-jasplakinolide (1 μM for 60 min) probes, respectively (followed by a washing step of additional 30 min). Live-cell STED images were acquired using a quad scanning STED microscope (Åbberior Instruments, Göttingen, Germany) equipped with a UPlanSApo 100×/1.40 Oil objective (Olympus, Tokyo, Japan). (1–4) Line profiles (with a line width of 60 nm (three pixels) for averaging) were taken at locations indicated by arrows in the enlarged sections (right). The averaged data were fitted with a Lorentzian function and plotted. Except for contrast stretching, no further image processing was applied. Scale bars: 5 μm (overviews), 500 nm (enlarged sections).

from Biotage (10, 25 or 50 g SiO2) on a Biotage Isolera One device. For analytical TLC, Merck Millipore ready-to-use plates with silica gel 60 (F254) were used. The spots were visualized by illumination with a UV lamp (λ = 254 and 365 nm), staining with phosphomolybdic acid or ninhydrin solutions.1H and 13C{1H}NMR spectra were recorded at 25 °C on Agilent 400-MR (400 MHz 1H and 100.5 MHz 13C), Bruker AVANCE NEO 600 MHz (TBO probe) and Bruker AVANCE III HD 500 MHz (BBO Prodigy probe) instruments. Chemical shifts are given in parts per million (ppm) using the residual solvent peak (s) as references. Multiplicities of the signals are described as follows: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet or overlap. J values are given in Hz. Mass-spectra with electro-spray ionization (ESI-MS) were recorded on a Varian 500-MS spectrometer (Agilent). ESI-HRMS were measured on a MICROTOF spectrometer (Bruker) equipped with an Apollo ion source and a direct injector with an LC-autosampler. LC-MS analyses were performed with an Interchim puriFlash 4250 device with a 250 × 21.2 mm column PF5C18AQ; flow rate 20 mL/min, gradient of acetonitrile in 50 mM aqueous of Et3N × 2H2O buffer (pH = 7.5).

Fmoc-O-TIPS-β-tyrosine (AA1 in Schemes 1 and 2)2 was prepared from Fmoc-β-tyrosine (Fmoc-(3-[(dimethylamino)propyl]-carbodiimide (EDC; free base) was from Sigma-Aldrich. HOAt (1-hydroxy-7-azabenzotriazole) was bought from Gen Script. (S)-2,4-Dimethylpent-4-en-2-ol were purchased from Enamine (Ukraine) and Carbosynth (UK), respectively. Grubbs Catalyst M2a (C848) was from Sigma-Aldrich. All other starting materials and reagents were purchased from commercial suppliers (Acros, Alfa Aesar, Merck, Novabiochem) and used without
purification. Anhydrous CH2Cl2 (DCM), N-methylpyrrolidone-2 (NMP), N,N-dimethylformamide (DMF), and hexafluoro-2-propanol-2 (HFIP) were stored over molecular sieves (4 Å). The temperature 0 °C corresponds to the cooling of the stirred reaction mixture with an ice bath.

**Solid-Phase Peptide Synthesis.** Loading. 2-Chlorotritol chloride resin (200–400 mesh, 1.2–1.4 m mol g⁻¹ CI loading; 1% cross-linked polystyrene) was purchased from Sigma-Aldrich. Anhydrous DCM and DMF were used in the following protocol. Under an argon atmosphere, a fritted 20 mL syringe was charged with dried resin (1.29 mmol, 937 mg), 9.4 mL of DCM was added, and the resin washed with DCM (9.4 mL), DCM (3 × 9.4 mL), and DCM (3 × 9.4 mL). For better coupling efficiency, incubation with AA1 and washing steps were repeated with half of the amount of AA1.

**Fmoc Cleavage.** The solvent was removed and the resin dried for 12 h at 0.2 mbar. Two portions of the resin (5.5 mg each) were used to determine the loading degree of AA1 (0.44 mmol g⁻¹) using a mixture of DBU/piperidine/NMP (2:2:96 v/v) for cleaving the Fmoc group and measuring the optical density (at 304 nm) against the blank sample. The main part of resin (1.25 g, 0.55 mmol) was washed with NMP (9.4 mL) and subjected to deprotection by re-suspending with a mixture of DCM/hexane/EtOAc (2:2:96 v/v) and shaking (300 rpm) for 10 min at 23 °C. This operation was repeated (with 30 min exposure at 23 °C). Completion of the cleavage was controlled by TLC (hexane/EtOAc 75:25): the application of the second cleavage cocktail revealed no appreciable UV active spot(s) of the Fmoc derivatives.

**Activation and Coupling.** AA2 (AA3, A4) (1.65 mmol, 3 equiv) was added into an oven-dried round bottom flask filled with argon and dissolved in NMP (7.5 mL). Then, a solution of HOBt (4.0 equiv, 2.2 mmol, 343 mg) in NMP (750 μL) was used for rinsing the Fmoc resin and then addeted CA2 (AA3, A4) (1.65 mmol, 3 equiv) in anhydrous DCM (3 mL per 1 g resin); diethylamine (2 mL) was added, and the reaction mixture was left at room temperature for 8–10 h. All volatile materials were removed in vacuum, and the residue was co-evaporated in vacuum with toluene (3 × 10 mL). After keeping in high vacuum (0.1 mbar) for 2 h, the residue was dissolved in anhydrous DCM (ca. 3 mL per 1 g resin) and filtered from the polymeric materials (if there were any) obtained in the course of Fmoc removal. The filtrate was evaporated in vacuum, and the residue was re-dissolved in DCM and used in the next (coupling) step. Coupling step. Coupling step. HOAt (1.1 equiv) and 2,4,6-collidine (TMF; 1.1 equiv) were added to a cooled (ice bath) solution of the corresponding Fmoc-protected amino acid (1.1 equiv) in anhydrous DCM (3.0 mL per 1 g resin). Then, EDC (free base, 1.1 equiv) was added dropwise with stirring at 0 °C. After 10–15 min, the solution of the appropriate N-deprotected peptide (1.0 equiv) and TMP (2.0 equiv) in DCM (ca. 2 mL per 1.0 mmol) was added at 0 °C. The ice bath was removed, the reaction mixture was stirred overnight at room temperature, diluted with ethyl acetate (EtOAc) (30 mL), washed with 1 M aq. KHSO4, 0.5% aq. NaHCO3, 2 mL (2 × 5 mL), water (2 × 5 mL), 5% Na2CO3, 400 mesh, 1.29 mmol; 9.4 mL of DCM was added, and the resin was washed with DCM (9.4 mL), CH2Cl2 (3 × 9.4 mL), and subjected to deprotection. The product is isolated (0.72 g, GP1) by 90% EtOAc in the mixture of EtOAc/H2O (90:10). 1H NMR (CD3CN, 500 MHz, major isomer): δ 7.69 (d, 1H, J = 7.9), 7.58 (d, 2H, J = 8.0), 7.53–6.80 (m, 15H, H), 6.57 (m, 1H, CHN), 4.35 (m, 1H, CHMe2), 1.08 (d, 18H, J = 7.4, 3 × CHMe3); 13C NMR (CD3CN, 101 MHz, major isomer): δ 169.7, 156.3, 155.7, 144.7, 144.8, 141.8 (2×), 135.9 (all 7 signals—Cq), 128.9 (2×), 128.8, 128.5, 128.0, 126.0, 126.1 (2×), 121.0, 120.2 (2×; all 9 signals—CH), 82.3 (Cq – O), 66.3 (CH2O), 52.7 (CHN), 47.8 (2CH), 42.9 (2CH), 29.1 (2× Me), 18.5 (6× Me), 13.3 (3× CH3S); HRMS (ESI) calc for C26H28NO6Si [M + Na]⁺, 700.4349; found, 700.4319.

**General Procedure (GP1) for Peptide Coupling in Solution.** Synthesis of Compounds 3, 4, and 5-C(CH3)2-C6H5=CH2-H. Deprotection step. Compound 2 or Fmoc-protected peptides (3, 4, see Scheme 2 and text below) were dissolved in MeCN (3 mL per 1 mmol); diethylamine (2 mL) was added, and the reaction mixture was left at room temperature for 8–15 h. All volatile materials were removed in vacuum, and the residue was co-evaporated in vacuum with toluene (3 × 10 mL). After keeping in high vacuum (0.1 mbar) for 2 h, the residue was dissolved in anhydrous DCM (ca. 3 mL per 1 mmol) and filtered from the polymeric materials (if there were any) obtained in the course of Fmoc removal. The filtrate was evaporated in vacuum, and the residue was re-dissolved in DCM and used in the next (coupling) step. Coupling step. Coupling step. HOAt (1.1 equiv) and 2,4,6-collidine (TMF; 1.1 equiv) were added to a cooled (ice bath) solution of the corresponding Fmoc-protected amino acid (1.1 equiv) in anhydrous DCM (3.0 mL per 1.0 mmol). Then, EDC (free base, 1.1 equiv) was added dropwise with stirring at 0 °C. After 10–15 min, the solution of the appropriate N-deprotected peptide (1.0 equiv) and TMP (2.0 equiv) in DCM (ca. 2 mL per 1.0 mmol) was added at 0 °C. The ice bath was removed, the reaction mixture was stirred overnight at room temperature, diluted with ethyl acetate (EtOAc) (30 mL), washed with 1 M aq. KHSO4, 0.5% aq. NaHCO3, 3.0 mL, NMP (1.5 mL), and subjected to dehydration by re-suspending with a mixture of DCM/PFIP (4:1 v/v) and stirring (300 rpm) for 10 min at 23 °C. This operation was repeated (with 30 min exposure at 23 °C). Completion of the cleavage was controlled by TLC (hexane/EtOAc 75:25): the application of the second cleavage cocktail revealed no appreciable UV active spot(s) of the Fmoc derivatives.

**Dipeptide 3.** The product is isolated (0.69 g, GP1) by flash chromatography on regular SiO2. Glass-like foamy solids are obtained after drying in high vacuum (<0.1 mbar).
spherical SiO₂ and elution with 10–90% EtOAc in the mixture of hexane/DCM (3:1) over 11 column volumes. ¹H NMR (CDCl₃, 400 MHz): δ 9.62 (br s, 1H, NH indole), 7.74 (d, 2H, J = 7.5), 7.58 (d, 1H, J = 7.7), 7.51 (2H, J = 5 and 5.2), 7.36 (3H, m), 7.30–7.02 (m, 11H, H²), 6.94 (s, 1H), 6.78 (m, 2H), 5.65 (dd, 1H, J = 12.2, 4.6; CHN-Tyr), 5.53 (d, 1H, J = 6.6, NH), 5.37 (q, 1H, J = 7.4; CHN-Lys), 4.75 (d, 2H, J = 10.5, 6.9), 4.14 (m, 1H), 3.39 (dd, 1H, J = 16.2, 4.6, CH² = H²), 3.24 (dd, 1H, J = 16.1, 12.3; CH² = H²), 2.91 (m, 1H, CH² = H²-Tyr), 2.81 (m, 1H, CH² = H²-Tyr), 2.71 (s, 3H, NMe²), 1.64/1.62 (2x 2H, s, 6CH₃), 1.52 (s, 9H, Boc CH₃), 1.39 (m, 1H, CH₃), 1.07 (d, 18H, J = 7.3), 1.06 (m, 1H, 0.85 (m, 2H), ¹³C NMR (CDCl₃, 101 MHz): δ 173.2, 169.3, 169.1, 156.5, 156.0, 155.2, 145.2, 143.8, 143.6, 141.3, 136.4, 133.1 (12 signals C), 128.2, 127.7, 127.5, 127.1, 126.8, 125.0, 124.5, 121.8, 121.7, 119.93, 118.90, 118.5, 118.4, 111.4 (14 signals CH = CH), 110.4 (C), 92.0, 79.8 (2x CH₂), 67.0 (CH₂O), 56.6, 51.1, 49.2, 47.1 (4 signals CH), 41.5 (CH₂), 40.5 (CH₂), 31.3 (CH₂), 30.5 (NMe₂), 30.0 (CH₂), 28.7 (CH₂E), 28.5 (fbu), 28.0, 23.0 (CH₃), 21.3 (CH₂), 17.9 (6x Me), 16.3 (3x CH₃), HRMS (ESI) calcd for C₉₇H₁₳N₅O₉Si [M + Na]⁺, 1128.5852; found, 1128.5845.

Compound 5-C(CH₃)₂-C₆H₅-H.

The product is isolated (0.60 g, 43%) as colorless, voluminous, and very viscous material in a 30% yield (pH = 7.5) in 30 min (for other conditions, see in General Remarks). Lioprophilization of appropriate fractions gave compound 5-H-H (42 mg, 71%) and 5-H-C(CH₃)₂C₆H₅ (11 mg, 17%) as colorless, colorless, and very light powders. 5-H-H: ¹H NMR (600 MHz, DCM-D₂O): δ 9.47 (s, 1H, indole NH), 7.59 (d, 1H, J = 7.9, Trp 4), 7.48 (s, 1H, βTyr NH), 7.33 (d, 1H, J = 8.1, Trp 7), 7.12–7.19 (2m, 3H, Trp 6/Trp 2/6, J = 6.8, 7.08 (t, 1H, J = 7.5, Trp 7), 6.96 (s, 1H, Trp 2), 6.79 (2H, J = 8.6, Trp 3/5), 6.61 (d, 1H, J = 7.2, Lys NH), 5.65 (dd, 1H, J = 11.2, 3.3, Trp α), 5.35 (q, 1H, J = 6.8, βTyr β), 4.73 (s, 1H, S), 4.65–4.71 (2m, 2H, S/ Lys δ), 4.62 (q, 1H, J = 6.5, Lys α), 3.38 (dd, 1H, J = 16.0, 4.4, Trp β), 3.31 (dd, 1H, J = 16.0, 12.1, Trp β), 2.88–3.02 (5m, 2H, Trp NMe/Lys e), 2.72–2.80 (2m, 2H, βTyr a), 2.41–2.48 (1m, 1H, 2), 2.33 (dd, 1H, J = 14.3, 6.3, 2.01 (dd, 1H, J = 14.3, 7.9, 3.1), 1.66 (3s, 1H, 7), 1.50 (s, 9H, Boc CH₃), 1.16–1.34 (6m, 6H, TIPS CH/Lys β), 1.02–1.14 (11m, 2H, TIPS CH₂/6/Lys β), 0.80–0.95 (2m, 2H, Lys γ).

The mixture was kept in an ice bath. HPLC control: column = 5 C65H83N5O9Si [M + Na]⁺, 1128.5852; found, 1128.5845.

Compound 5+H-(C²H₅)₂H.

The mixture of two amide rotamers in the mixture was kept in an ice bath. HPLC control: column = 5 C65H83N5O9Si [M + Na]⁺, 1128.5852; found, 1128.5845.
Compound 7-TIPS and 8-TIPS. Compound 6 (62 mg, 65 μmol) was dissolved in dry DCM (60 mL) in a screw-cap tube. The tube was closed with septum, and a gentle argon stream was blown through the reaction mixture was stirred at 40 °C for 10 min with stirring and gradual heating up to 40 °C. Then, the solution of the Grubs’ catalyst (2nd generation, 5.0 mg, 9% mol) in degassed anhydrous toluene (0.6 mL) was added under an argon atmosphere, the septum was replaced with a screw-cap, and the reaction mixture was stirred at 40 °C for 90 min. HPLC control: purified Flash column C18AQ (Interchrom), 5 μm, 250 × 4.6 mm, flow rate 1.2 mL/min, A/B = 30/70 → 0/100 (20 min), then 100% B. Compound 6: tR = 24.7 min (3% HPLC area), compound 7-TIPS: tR = 23.8 min (~87% HPLC area), and compound 8-TIPS: tR = 23.5 min (~10% HPLC area); the toluene peak at 12.5 min was not integrated. The reaction mixture was evaporated in vacuo, and the residue subjected to flash chromatography on regular silica (Biotage cartridge with 25 g spherical silica gel). Elution with hexane-ethyl acetate (80:20–30:70) afforded a mixture of compounds 7-TIPS and 8-TIPS (51 mg, 85%) as a glass-like foam (compound 6 has higher Rr value). Compound 7-TIPS (compound 11 from ref 2). 1H NMR (CDCl3, 400 MHz): δ 8.99 (s, 1H, NH indole), 7.60 (dd, 1H, J = 7.5 and 1.3), 7.34 (d, 1H, J = 9.0), 7.17–7.04 (m, 5H), 6.93 (br s, 1H, NH), 6.81 (d, 2H, J = 8.6), 6.58 (d, 1H, J = 6.4, NH), 5.67 (dd, 1H, J = 12.1 and 4.7, CH=), 5.22 (dt, 1H, J = 7.8 and 4.1), 4.98 (t, J = 6.9, 1H), 4.66–4.65 (m, 3H), 3.36 (dd, 1H, J = 16.3 and 12.1), 3.26 (dd, 1H, J = 16.9 and 4.2), 3.03 (br s, 1H), 2.92 (d, 1H, J = 14.5 Hz), 2.88 (s, 3H, NMe), 2.76 (dd, 1H, J = 15.5 and 4.2), 2.55 (dd, 1H, J = 15.5, 4.1), 2.43 (m, 1H), 1.86–1.74 (m, 3H), 1.51 (s, 9H, Bu), 1.44 (d, 1H, J = 1.3, 3H, CH3), 1.32–1.15 (m, 6H, 3CH2Pr + CH2Chloro), 1.11 (d, 3H, J = 6, CH3CH), 1.08 (m, 20H, TIPS + CH3), 1.00 (m, 1H, CH3), 0.66 (m, 1H, CH2Cl). 13C NMR (CDCl3, 101 MHz): δ 174.5, 173.9, 170.4, 163.9, 156.6, 155.4, 136.5, 133.9, 132.9, 127.2 (all—C), 127.1 (2CH2), 124.4 (CH2), 121.7 (CH2), 119.5 (2CHX), 119.0 (CH), 118.3 (CH2), 111.4 (CH), 79.8 (C-O), 69.4 (CHO), 56.0 (CH2O), 49.7 (CHO), 44.2 (CH2), 40.5 (CH2), 38.8 (CH2), 35.2 (CH2), 35.2 (CH2), 31.4 (CH2), 31.0 (NMe2), 30.2 (CH2), 28.5 (Bu), 33.3 (CH2), 22.5 (CH2), 21.8 (CH2), 18.0 (60 Me), 17.3 (Me), 12.9 (3CH3). HRMS (ESI) calc for C24H33N3O5Si [M + Na]+, 796.4526; found, 796.4525.

Compound 8-H. 1H NMR (CDCl3, 500 MHz): δ 9.88 (s, 1H, indole NH), 7.59 (d, 1H, J = 7.8, Trp 4), 7.42 (d, 1H, J = 8.1, βTyr NH), 7.34 (d, 1H, J = 8.2, Trp 7), 7.05–7.16 (m, 4H, 3Tyr 5/6/βTyr 2/6), 6.93 (d, 1H, J = 2.2, Trp 2), 6.72–6.76 (m, 3H, βTyr 3/5/lys Nh), 5.68 (dd, 2H, J = 11.7, 5.1, Trp α), 5.18 (td, 1H, J = 8.0, 4.1, βTyr β), 4.97 (t, 1H, J = 7.1, 5), 4.83–4.89 (m, 1H, Lys α), 4.78 (ddq, 1H, J = 9.6, 3.7, 8), 4.69–4.75 (m, 1H, NH lys side chain), 3.33 (dd, 1H, J = 16.2, 12.1, Trp β), 3.28 (dd, 1H, J = 16.2, 5.3, Trp β), 2.97–3.08 (m, 1H, Lys ε), 2.89 (s, 3H, Trp NMe), 2.77–2.85 (m, 1H, Lys ε), 2.73 (dd, 1H, J = 15.5, 4.1, βTyr α), 2.55 (dd, 1H, J = 15.5, 8.0, βTyr α), 2.44–2.49 (m, 1H, 2), 2.36–2.43 (m, 1H, 3), 1.86 (d, 1H, J = 13.8, 3), 1.73–1.82 (m, 2H, 6), 1.46–1.56 (m, 1H, Boc CHβ, 7, lys β), 1.40 (s, 3H, 11), 1.28–1.36 (m, 1H, 7), 1.15–1.27 (2H, Lys δ), 1.12 (d, 3H, J = 6.6, 10), 1.08 (d, 3H, J = 6.3, 9), 0.89–0.93 (m, 2H, Lys β/γ), 0.56–0.67 (m, 1H, lys γ); 13C NMR (125 MHz, CDCl3): δ 175.3 (1), 173.7 (lys CO), 170.6 (βTyr CO), 169.4 (Trp CO), 156.6 (Boc CO), 155.2 (βTyr 4), 136.5 (Trp 7a), 133.5 (4), 132.5 (βTyr 1), 127.3 (βTyr 2/6), 127.1 (Trp 3a), 124.9 (5), 121.6 (Trp 2), 119.0 (Trp 5), 118.3 (Trp 4), 115.5 (βTyr 3/5), 111.4 (Trp 7), 109.9 (Trp 4), 79.9 (Boc C6), 69.6 (8), 56.2 (Trp α), 49.8 (lys α), 49.2 (βTyr β), 43.3 (βTyr δ), 40.7 (lys ε), 39.6 (2), 39.6 (βTyr α), 35.4 (7), 30.8 (lys β), 30.2 (Trp NMe), 30.0 (lys δ), 29.0 (Boc CH3), 23.1 (6), 22.4 (βTyr β), 20.9 (lys γ), 20.4 (9), 20.2 (10), 19.5 (11). HRMS (ESI) calc for C24H33N3O5Si [M + Na]+, 796.4526; found, 796.4525.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c00653.

Experimental details of cell culture, in vitro labeling, and STED microscopy; LC–MS analysis of the degradation reaction of 5-C(CH3)2C6H4=N–H; HPLC trace and ESI-MS for amine 9 (Scheme 4); HPLC data for 580CP- and 610CP-lysasparaginol conjugates; full 1H- and 13C-NMR assignments for isomers 5-C(CH3)2C6H4=N–H and 5-H-C(CH3)2C6H4=N, as well as 7-H and 8-H; and copies of NMR spectra (PDF)
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**ADDITIONAL NOTE**

“STED: stimulated emission depletion.”

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