# Design and Synthesis of N1,N8-Diacetylspermidine Analogues having a Linker with Desired Functional Groups

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| Complete List of Authors: | Ohta, Reiya; Osaka University, Graduate School of Pharmaceutical Sciences  
Oguro, Akihiro; Jikei University School of Medicine  
Nishimura, Kazuhiro; Graduate School of Pharmaceutical Sciences, Chiba University, Department of Clinical and Analytical Biochemistry  
Murai, Kenichi; Osaka University, Graduate School of Pharmaceutical Sciences  
Fujioka, Hiromichi; Osaka University, Graduate School of Pharmaceutical Sciences  
Arisawa, Mitsuhiro; Osaka University, Graduate School of Pharmaceutical Sciences |
Design and Synthesis of $N^1, N^8$-Diacetylspermidine Analogues Having A Linker with Desired Functional Groups

Reiya Ohta, Akihiro Oguro, Kazuhiro Nishimura, Kenichi Murai, Hiromichi Fujioka and Mitsuhiro Arisawa

A synthesis of new $N^1, N^8$-diacetylspermidine (DiAcSpd) analogues having a linker with desired functional groups in the methylene skeleton, which have been designed by theoretical calculation, is described. We have also achieved the preparation of DiAcSpd supported on solid-phase resins, which have the potential to be used for evolution of ligands by exponential enrichment (SELEX).

Introduction

Polyamines, such as putrescine, spermidine, or spermine, are low-molecular-weight and physiologically active compounds in which some of the amino groups are contained in an alkyl chain skeleton (Figure 1A). They play many important roles in cell proliferation and differentiation.\(^1\) It is known that cell proliferation is significantly affected when polyamines are deficient or in excess, therefore, their intracellular concentration is strictly controlled. It was furthermore estimated the polyamine distribution in cells, they interact mainly with RNAs of nucleic acids and have been shown to be greatly involved in protein synthesis.\(^2\) In particular, spermidine and its derivatives have been well studied because they are involved in human health and diseases.\(^3\) It is generally agreed upon that $N^1, N^8$-diamethylspermidine (DiAcSpd), which is a metabolite of spermidine via acetylationtransferase, has the potential to be a biomarker for the diagnosis of malignancies.\(^4\)

The amount of DiAcSpd is remarkably increased in the urine of cancer patients, whereas it is hardly elevated in the case of a healthy person, which means that the urinary level of DiAcSpd is a useful diagnostic and prognostic indicator for cancer. The development of a system for the easy and rapid detection of DiAcSpd in the urine is therefore required. For example, in 1997, Kawakita et al. reported the preparation of antibodies with high specificity to DiAcSpd.\(^5\)

In recent years, high-affinity oligonucleotides called ‘aptamers’ have drawn attention as biomolecules that highly recognize the three-dimensional structure of the target molecule.\(^6\) They can be obtained from randomized RNA libraries (ca. $\sim 10^{14}$ oligonucleotides) by in vitro selection, which is termed ‘Systematic Evolution of Ligands by EXponential enrichment (SELEX).’\(^7\) Aptamers also have properties that are not present in antibodies: (1) they are easy to chemically modify and (2) Their molecular weight are smaller and can be synthesized in large amounts. Aptamers are therefore suitable as diagnostic materials. In general, obtaining aptamers that target low-molecular-weight compounds is difficult and seldom reported. In our previous work, we succeeded in obtaining two RNA aptamers targeted for spermine by SELEX.\(^8\) We also looked at obtaining DiAcSpd-specific aptamers in the same way as we did with spermine, but unfortunately we were unsuccessful (Figure 1B).\(^9\) This is probably because the aptamers could not recognize the three-dimensional structure and functional groups of DiAcSpd due to DiAcSpd being immobilized on the solid-phase resin using one nitrogen atom of the substrate as a foothold. In SELEX, it is common to use analogues that target molecules immobilized on solid-phase resin as a support, and many aptamers are obtained by this technique. Based on the above study, we set out to design the analogues whose conformation and functional groups would be maintained as in the DiAcSpd.

We then hypothesized that DiAcSpd analogues having a linker in the methylene skeleton could keep the same or similar three-dimensional structure and functional groups as DiAcSpd (Figure 1C). To the best of our knowledge, this type of spermidine analogue has been overlooked and remain unreported, while many other spermidine analogues, including orthogonally protected or with side chains grown from the internal nitrogen atom of the substrate, have been reported.\(^10\) Not limited to polyamines, many bioactive compounds, such as histamine, serotonin, or adrenaline, use methylene skeletons to connect functional groups. It is important, therefore, to develop a synthesis of analogues having a linker with desired functional groups in the methylene skeleton in order to obtain the corresponding biomolecules.

In this study, we describe a synthesis to produce new DiAcSpd analogues 1 and 2 which have a linker with desired functional groups, such as hydroxide or amine groups, in the linker...
instead of the resin structure. It was observed that substitution at the α-position of the amino groups, such as the methylene skeleton that have been designed based on conformational studies using theoretical calculations. Furthermore, we synthesized DiAcSpd analogues supported on solid-phase resins that have the potential to be used for SELEX.

Results and discussion
First of all, we investigated the conformational study of DiAcSpd and its analogues having a linker in methylene skeleton in order to understand in which position the linker should be introduced. Our investigation revealed that DiAcSpd should adopt a linear conformation as its stable conformation (Figure 2A). We then performed conformational studies of DiAcSpd analogues having a linker in the methylene skeleton and in order to simplify the calculation, a butyl group was used as the model linker instead of the resin structure. It was observed that substitution at the α-position of the amino groups, such as the C5 and C8 positions, induced the DiAcSpd analogue into a bent conformation which greatly differed from the original (Figure 2B). These results suggest that it would be better to avoid introducing a linker at the α-position of each of the amino groups. We also examined the conformational effects of DiAcSpd analogues having a linker at the β-position of each of the amino groups, such as the C6 and C7 positions (Figure 2C). Substitution at the C7 position resulted to a non-linear conformation, which was caused by the hydrogen bond formation between acetoamide and internal amine. On the other hand, substitution at the C6 position led to a roughly linear conformation which was similar to the stable conformation of DiAcSpd. We therefore examined the synthesis of DiAcSpd analogues having a linker at the C6 position since we presumed that this design of the compound would keep a similar three-dimensional structure and functional groups as in DiAcSpd. We then developed two synthetic routes for synthesizing DiAcSpd analogues having a linker with desired functional groups at the C6 position (Figure 1C).
First, path a, was examined. Its strategy consisted of constructing the spermidine skeleton first and then extending the side chain moiety by metathesis reaction. We predicted that DiAcSpd analogue 7 which has a linker could be prepared from commercially available 4-amino-2-methylenebutan-1-ol (3) in just two steps (Scheme 1). Treatment of 3 with acetic anhydride in dichloromethane affords the acetamide 4, which was subjected to react with o-nitrobenzenesulfonyl (Ns) amide 5 that contained an acetyl group to construct the DiAcSpd skeleton with an exo-methylene substituent in the methylene skeleton (6). Next, we conducted the cross-metathesis of DiAcSpd analogue 6 with allylic alcohol derivatives using commercially available ruthenium catalysts, such as Grubbs 1st, Grubbs 2nd, Hoveyda-Grubbs 1st, and Hoveyda-Grubbs 2nd. However, the cross-metathesis product (7) was not observed at all. We attributed this result to the competition between the productive cross-metathesis and rapid self-cross-metathesis of allylic alcohol derivatives. We speculated that the steric bulkiness imposed by the Ns amide group at the β-position hindered the reaction of substrate (6) and disfavoured formation of the metallocyclobutane intermediate, resulting to the lack of productive cross-metathesis to afford 7. As is often the case with cross-metathesis, the reactivity of sterically demanding alkene is a lot poorer than terminal olefins. Of course, several syntheses of trisubstituted olefins by cross-
metathesis using sterically demanding alkenes such as 1,1-disubstituted alkenes as a substrate have been reported.\textsuperscript{11} However, this type of reaction remains challenging and is limited to simple substrates without functional groups. Therefore, we decided to carry out the cross-metathesis in the initial stage of synthesis. In addition, we decided to change the exo-methylene moiety of 4 to an allylic one (12) in order to improve the reactivity of cross-metathesis.

Next, we examined the alternative synthesis route (Figure 1C, path b). In this pathway, we extended the side chain moiety by metathesis reaction first and then the spermidine skeleton was constructed. We supposed that this pathway could solve the low reactivity of the metathesis reaction which was the weakness of path a. As shown in Scheme 2, DiAcSpd analogues 1 and 2, which satisfied the required molecular design based on prior conformational studies, were synthesized from commercially available methyl 4-((tert-butoxy)carbonyl)amino)butanoate (9). We started the synthesis by reacting 9 with allylbromide to give adduct 10. Reduction of 10 via mixed acid anhydride then gave alcohol 11 in 98% yield. The tert-butoxycarbonyl (Boc) group in 11 was deprotected with HCl and subjected to acetylation to afford 12 in 81% yield over two steps. Cross-metathesis of 12 with (allyloxy)(tert-butyldimethylsilyl)dimethylsilane proceeded smoothly with Grubbs 1\textsuperscript{st} as the catalyst to give the metathesis product.
was accomplished by treatment with 2-mercaptoethanol and 1,8-diaza-15-crown-5-dodec-7-ene (DBU) to afford the resin-bound DiAcSpd analogue 15A. The product was then confirmed based on disappearance of the absorption peak of the Ns group (ca. 1550 cm⁻¹), and the Kaiser test using ninhydrin to check the loading of amines. Next, a sepharose resin, NHS-activated sepharose (GE Healthcare Life Sciences), was used as support similar to polystyrene resin (Scheme 3B). In SELEX, the use of different types of resin is effective for subtracting the pseudo-positive aptamers that bind to solid-phase resins. DiAcSpd analogue having amine linker 2 was immobilized on NHS-activated sepharose by amine coupling through its primary amino group. Subsequently, deprotection of the Ns group was accomplished by treatment with 2-mercaptoethanol and Cs₂CO₃ to afford resin-bound DiAcSpd analogue 15B. These reactions were also monitored by ATR-IR, and the products were confirmed based on the appearance or disappearance of the absorption peak of the Ns group (Figure 4). We are currently examining SELEX in detail in order to obtain DiAcSpd-specific aptamers using DiAcSpd analogues 15A and 15B. We are also investigating their biochemical and physicochemical properties which will be reported in due course.

**Conclusion**

In conclusion, we have developed a synthesis to produce new DiAcSpd analogues that have a linker with desired functional groups, such as hydroxide or amine in the methylene skeleton. Furthermore, it was possible to immobilize these analogues on solid-phase resins for potential use in SELEX.

**Experimental**

**General experimental details**

All reagents were purchased from commercial sources and used without further purification, unless otherwise noted. Reactions were performed under a nitrogen atmosphere using purchased anhydrous solvent. All reactions were monitored by thin-layer chromatography using Merck SiO₂ gel 60 F254. The products were purified by column chromatography over SiO₂ 60N (40-50 μm, spherical neutral) purchased from Kanto Chemical. ¹H NMR and ¹³C NMR spectra were recorded at 25 °C on a JEOL JNM-AL300 (at 300 MHz and 75 MHz, respectively), a JEOL JNM-ECS 400 (at 400 MHz and 100 MHz, respectively) or a JEOL JNM-LA 500 (at 500 MHz and 125 MHz, respectively), and the chemical shifts are reported relative to internal TMS (δ = 0.00 ppm), CDCl₃ (δ = 7.26 ppm), CD₂OD (δ = 3.31 ppm, ¹³C, δ = 49.0 ppm). Data for ¹H NMR spectra are reported as follows: chemical shift (δ ppm) (integration, multiplicity, coupling constant (Hz)). Multiplicity and qualifier abbreviations are as follows: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad. IR spectra were recorded with Shimadzu IR Affinity-1 spectrometers equipped with Specac Quest Single Reflection Attenuated Total Reflection (ATR) Accessory (P/N GS 10800). High-resolution mass spectra (MALDI-TOF) were performed by the Elemental Analysis Section of Graduate School of Pharmaceutical Science in Osaka University. S and 9 (commercially available) are known compounds. DiAcSpd was synthesized based on the method of the literature.

**Experimental procedures**

10: To a solution of 9 (835.0 mg, 3.85 mmol) in THF (15.4 mL) was added dropwise potassium bis(trimethylsilyl)amide (ca. 0.5 M in toluene, 20 mL, 10.0 mmol, 2.6 equiv.) at −78 °C. After 0.5 h, the reaction mixture was added dropwise allyl bromide (1.6 mL, 19.2 mmol, 5.0 equiv.), and the mixture was stirred at the same temperature for 6 h. Then, sat. NH₄Cl aq. was added, and the mixture was warmed to room temperature, the organic layer was separated subtracting n-hexane/EtOAc = 9/1 to 5/1) to give 10 (404.1 mg, 1.57 mmol, 40%) as a colorless oil.

**HRMS** (MALDI-TOF) m/z [M + Na]⁺ calcd for C₁₇H₁₇NO₃Na 280.1519, found 280.1511.
(Step 1): To a solution of 10 (1.04 g, 4.04 mmol, 1.0 equiv.) in THF (13.5 mL) was added 1 M LiOH aq. (6.06 mL, 6.06 mmol, 1.5 equiv.) at room temperature, and the mixture was stirred for 24 h. Then, 1 M HCl aq. and EtOAc was added. The organic layer was separated and the aqueous layer was extracted with EtOAc. The organic layers were combined, and the solution was dried over anhydrous Na2SO4 and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (CHCl3/MeOH = 30/1 to 20/1) to give 11 (152.4 mg, 0.889 mmol, 81% in 2 steps) as a yellow oil.

1H NMR (400 MHz, CDCl3) δ 5.98 (1H, brs), 5.77 (1H, ddt, J = 17.2, 10.3, 6.9 Hz), 5.07-5.02 (2H, m), 3.65 (1H, dd, J = 10.9, 4.6 Hz), 3.65 (1H, dd, J = 10.9, 6.3 Hz), 3.39-3.34 (2H, m), 2.41 (1H, brs), 2.14-2.02 (2H, m), 1.98 (3H, s), 1.71-1.63 (1H, m), 1.59-1.54 (1H, m); 13C NMR (100 MHz, CDCl3) δ 170.5, 136.4, 116.7, 65.3, 38.0, 37.7, 36.0, 31.0, 23.2; HRMS (MALDI-TOF) m/z [M+] calcd for C19H19N2O2 323.1332, found 323.1332.

(Step 2): To a solution of 11 (252.0 mg, 1.10 mmol, 1.0 equiv.) in Et3O (1.8 mL) were added HCl (4 M in 1,4-dioxane, 1.1 mL, 4.4 mmol, 4.0 equiv.) at room temperature, and the mixture was stirred for 24 h. Then, the solution was concentrated under reduced pressure to give the crude amine 12 (184.0 mg, 1.0 mmol).

(Step 3): To a solution of 12 (184.0 mg, 1.10 mmol) in CH2Cl2 (5.5 mL) was added Ac2O (0.31 mL, 3.30 mmol, 3.0 equiv.) and Et3N (0.47 mL, 3.41 mmol, 3.1 equiv.) in an ice bath, and the mixture was stirred for 24 h. The reaction mixture was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (CHCl3/MeOH = 30/1 to 20/1) to give 12 (152.4 mg, 0.889 mmol, 81% in 2 steps) as a yellow oil.

1H NMR (400 MHz, CDCl3) δ 7.77 (2H, d, J = 8.3 Hz), 7.35 (2H, d, J = 8.3 Hz), 5.59 (1H, brs), 4.00-3.86 (2H, m), 3.53 (2H, t, J = 6.4 Hz), 3.26-
To a solution of 13 (109.7 mg, 0.233 mmol, 1.0 eq.) and 5 (77.1 mg, 0.256 mmol, 1.1 eq.) in DMF (1.0 mL) was added Cs₂CO₃ (113.7 mg, 0.349 mmol, 1.5 eq.) at room temperature, and the mixture was stirred at 80 °C for 5 h. After cooling to room temperature, water and CHCl₃ were added. The organic layer was separated and the aqueous layer was extracted with CHCl₃. The organic layers were combined, and the solution was washed with brine, dried over anhydrous Na₂SO₄, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (CHCl₃/MeOH = 30/1 to 20/1) to give 14 (116.2 mg, 0.193 mmol, 83%) as a colorless oil.

To a solution of 14 (109.7 mg, 0.233 mmol, 1.0 eq.) in THF (1.2 mL) was added n-BuNF (1.0 M in THF, 0.35 mL, 0.35 mmol, 3.0 equiv.) in an ice bath, and the mixture was stirred at room temperature for 6 h. Then, water and CHCl₃ were added. The organic layer was separated and the aqueous layer was extracted with CHCl₃. The organic layers were combined, and the solution was washed with brine, dried over anhydrous Na₂SO₄, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (CHCl₃/MeOH = 20/1) to give 15A (51.9 mg, 0.107 mmol, 92%) as a colorless oil.

1H NMR (500 MHz, CDCl₃) δ 7.95-7.93 (1H, m), 7.72-7.60 (3H, m), 6.46 (1H, brs), 6.01 (1H, brs), 3.58-3.55 (2H, m), 3.43-3.30 (2H, m), 3.26-3.12 (6H, m), 1.97 (3H, s), 1.97 (3H, s), 1.81-1.66 (3H, m), 1.52-1.41 (4H, m), 1.33-1.21 (4H, m), 0.87 (9H, s), 0.03 (6H, s); 13C NMR (125 MHz, CDCl₃) δ 170.6, 170.4, 148.1, 133.6, 132.7, 131.7, 130.5, 124.1, 62.8, 52.8, 46.3, 36.9, 36.8, 34.0, 32.9, 31.1, 30.9, 28.0, 26.0, 23.3, 23.2, 22.5, 18.3, -5.3; HRMS (MALDI-TOF) m/z [M + Na]+ calcd for C₂₁H₂₃N₂O₅Na₂S 623.2905, found 623.2904.

0.256 mmol, 1.1 equiv.) in an ice bath, and the mixture was stirred at room temperature for 6 h. Then, water and CHCl₃ were added. The organic layer was separated and the aqueous layer was extracted with CHCl₃. The organic layers were combined, and the solution was concentrated under reduced pressure. The residue was passed through a short silica gel column chromatography (CHCl₃/MeOH = 15/1) to give the crude tosylation product 56 (200.2 mg, 0.312 mmol).

(Step 2): To a solution of 56 (200.2 mg, 0.312 mmol, 1.0 equiv.) in DMF (3 mL) was added NaN₃ (101.5 mg, 1.560 mmol, 5.0 equiv.) in an ice bath, and the mixture was stirred at room temperature for 6 h. Then, water and CHCl₃ were added. The organic layer was separated and the aqueous layer was extracted with CHCl₃. The organic layers were combined, and the solution was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (CHCl₃/MeOH = 15/1) to give 15A (145.4 mg, 0.284 mmol).

(Step 3): To a solution of 57 (145.4 mg, 0.284 mmol, 1.0 equiv.) in THF (3 mL) and water (0.3 mL) was added PPh₃ (223.5 mg, 0.852 mmol, 3.0 equiv.) at room temperature, and the mixture was stirred for 12 h. Then, water and CHCl₃ were added. The organic layer was separated and the aqueous layer was extracted with CHCl₃. The organic layers were combined, and the solution was concentrated under reduced pressure. The residue was purified by C18-reversed phase silica gel column chromatography (CH₃CN/H₂O = 20/1) to give 2 (119.6 mg, 0.246 mmol, 64% for 3 steps) as a colorless oil.

1H NMR (400 MHz, CDCl₃) δ 8.05-8.02 (1H, m), 7.82-7.74 (3H, m), 3.27-3.09 (6H, m), 2.64-2.61 (2H, m), 1.92 (6H, s), 1.76-1.67 (3H, m), 1.52-1.29 (8H, m); 13C NMR (100 MHz, CDCl₃) δ 173.3, 173.1, 149.6, 135.3, 133.7, 131.1, 131.6, 125.4, 53.1, 46.7, 42.2, 37.92, 37.89, 34.9, 33.8, 31.8, 31.7, 29.2, 24.2, 22.6 (2C); HRMS (MALDI-TOF) m/z [M + Na]+ calcd for C₂₁H₂₃N₂O₅NaS 508.2205, found 508.2200.

Please do not adjust margins
EDC·HCl (107.4 mg, 0.560 mmol, 2.6 eq.) and DMAP (40.1 mg, 0.328 mmol, 1.5 eq.) at r.t. After shaking at 1500 rpm by fast shaker (EYELA #CM-1000) for 24 h at r.t., the resin was then filtered, alternating washed 5 times with CH<sub>3</sub>Cl<sub>2</sub>, MeOH, Et<sub>2</sub>O, H<sub>2</sub>O, followed by CH<sub>3</sub>Cl<sub>2</sub> and then dried in vacuo at 60 °C for 8 h to give the S<sub>8</sub> (199.8 mg). IR (ATR, cm<sup>-1</sup>): 3288, 3069, 3027, 2924, 2853, 1707, 1654, 1648, 1638, 1543, 1492, 1450, 1369, 1344, 1274, 1161, 1107, 1019, 752, 698.

(Step 2): We referred to the literature method. To a suspension of S<sub>8</sub> (~0.219 mmol/g, 100.0 mg, ~0.219 mmol) in DMF (1 mL) were added DBU (0.98 mL, 6.57 mmol, 30 eq.) at r.t. After shaking at 1500 rpm for 24 h by fast shaker for 12 h at r.t., the resin was then filtered, alternating washed 5 times with DMF, CH<sub>3</sub>Cl<sub>2</sub>, MeOH, Et<sub>2</sub>O, H<sub>2</sub>O, followed by CH<sub>3</sub>Cl<sub>2</sub> and then dried in vacuo at 60 °C for 6 h to give the 15A (71.9 mg, 0.0728 mmol, 37% loading). Kaiser test was positive. IR (ATR, cm<sup>-1</sup>): 3286, 3064, 3024, 2924, 2852, 1707, 1641, 1544, 1535, 1492, 1450, 1369, 1273, 1180, 1107, 1028, 752, 698.

Notes and references
9 We have also examined capillary electrophoresis (CE)-SELEX. However, we could not obtain DiAcSpd-specific aptamers because of their cationic properties.


