Synthesis of biotinylated retinoids for cross-linking and isolation of retinol binding proteins

Nasri Nesnas,a Robert R. Randob,∗ and Koji Nakanishi,a,∗

aDepartment of Chemistry, Columbia University, New York, NY 10027, USA
bDepartment of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA

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This article is dedicated to Professor Yoshito Kishi on his receipt of the Tetrahedron Prize

Abstract—The synthesis of (3R)-3-[Boc-Lys(biotinyl)-O]-11-cis-retinol bromoacetate and 3-[Boc-Lys(biotinyl)-O]-all trans-retinol chloroacetate is described. These biotinylated retinoids are instrumental in labeling the retinol binding proteins (RBPs) via a nucleophilic displacement of the haloacetate by a residue in the binding site of the protein. The covalently linked biotin will allow for a facile isolation and purification of the protein on a streptavidin column thus rendering the protein ready for a tryptic digest followed by mass spectrometric analysis. The 11-cis retinoid was synthesized via metal reduction of an alkyne intermediate generated from a Horner–Wadsworth–Emmons (HWE) reaction whereas the all-trans was synthesized via two consecutive HWE couplings. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

Retinoids are derivatives of vitamin A (all trans-retinol) which can generally be synthesized for studies directed to a better understanding of human and non-human vision. We hereby report the synthesis of (3R)-3-[Boc-Lys(biotinyl)-O]-11-cis-retinol bromoacetate and 3-[Boc-Lys(biotinyl)-O]-all trans-retinol chloroacetate (Fig. 1). These retinoids can act as ligands for proteins known as retinol binding proteins (RBPs) present in the retinal pigment epithelial (RPE) cells. These RBPs are believed to be responsible for the transport of retinols in RPE cells and some are involved in catalyzing esterification, hydrolysis, and isomerization of retinols at various stages in the visual cycle. A summary of the mammalian visual cycle is presented in Fig. 2. The visual protein, rhodopsin, is comprised of the protein opsin (ca. 348 amino acids in bovine) and 11-cis-retinal covalently linked via a Schiff base with Lys296 residue. Vision begins with the absorption of light by rhodopsin causing the isomerization of 11-cis-retinylidene to all trans-retinylidene. This occurs over several states of rhodopsin leading to a meta II state (unprotonated Schiff base) which is responsible for G-protein activation ultimately leading to neural signaling.∗

Keywords: biotinylated retinoids; biotin; retinol binding proteins; RBP.

* Corresponding author. Tel.: +1-212-854-2169; fax: +1-212-932-8273; e-mail: kn5@columbia.edu

Figure 1. The target retinoid biotinylated structures.
This isomerization is accompanied by various conformational changes of the protein which in turn trigger the binding of G-protein. These photo intermediates have been well studied through photoaffinity labeling experiments, protein digestion and mass spectrometric characterization.3 The meta II state is then protonated generating meta III (see Fig. 2) which undergoes hydrolysis regenerating the protein opsin and all trans-retinal. To complete the visual cycle, the all trans-retinal needs to be re-isomerized back to 11-cis-retinal. This is achieved through a cascade of several events involving proteins such as dehydrogenase which initially reduces all trans-retinal to all trans-retinol (vitamin A). The latter is then isomerized to 11-cis-retinol through the action of numerous RBPs which we are interested in further investigating.1 The actions of these proteins include the esterification of vitamin A with a palmitoyl group followed by an isomerization of the resulting palmitate through a possible hydrolytic mechanism. One such protein that has been already under investigation is known as lecithin retinol acyl transferase (LRAT).4,5 To regenerate the visual pigment, 11-cis-retinol undergoes oxidation via a dehydrogenase rendering 11-cis-retinal ready for incorporation into opsin forming the Schiff base rhodopsin.

One important advantage that is featured in the target structures needs to be emphasized. The biotin allows for the successful isolation of the protein due to its remarkable binding to streptavidin with association constants often exceeding 10^{15} M^{-1}.6,7 However, this often presents a challenge for the scientist who is interested in isolating the protein via breaking this association. The above structure features a labile ester of the primary alcohol at C-15 of the retinol hydroxyl which can be easily cleaved in basic media (pH 11).

1.1. Retrosynthetic analysis of 1 and 2

Scheme 1 represents the two approaches for the installation of a biotin on one end of the retinoid and a haloester on the other end. Structure 7 represents the general formula for retinoids 1 and 2. Structure 3 represents the retinoid backbone with some orthogonal protection on the hydroxy groups at C-3 and C-15. Route A will entail installing the Boc-Lys(biotinyl)-OH (B-OH) first directly after deprotection of the 3-hydroxy and then attachment of the haloester, and finally deprotection of the 3-hydroxy
followed by esterification with B-OH. The first route is favored as the labile haloester which is attached last will not need to endure the steps presented in Route B. However, both routes were used as will become apparent in due course owing to the nature of the retinoid precursor 3 for each of the two target molecules, the cis and the trans.

The retrosynthesis of 4a is outlined in Scheme 2. The 11-cis double bond can be generated via a zinc metal reduction of the alkyne intermediate 8. The latter can be constructed via a Horner–Wadsworth–Emmons (HWE) coupling of the 3-hydroxy-β-ionone (R)-9 and phosphonate 10 followed by a Sonogashira coupling with vinyl iodide 11, which can be obtained from a reduction of 12 followed by iodination with I2.

The 3-hydroxy-β-ionone can also be used in the synthesis of 4b as shown in the retrosynthetic analysis in Scheme 3. Two consecutive HWE couplings: the first between (±)-9 and 14 followed by DIBAH reduction to generate an aldehyde ready for the second coupling with phosphonate 15 thus generating 13. Reduction of the latter with LiAlH4 will afford the desired 3-siloxylated-all trans-retinol 4b. Retrosynthetic Schemes 2 and 3 indicate the key role of the compound 3-hydroxy-β-ionone 9. The stereochemistry at the 3-hydroxy position does not need to be defined for the biological assays since the biotin linker is not directly involved in the binding of the protein. We have in fact generated 3-hydroxy-β-ionone 9 from two independent routes and have arbitrarily proceeded with (R)-9 for the synthesis of 1 and the racemic 9 for the synthesis of 2. The stereoselective route for the generation of (R)-9 was needed for another purpose that will be published elsewhere. Scheme 4 presents that two routes for the generation of 9. The non-stereoselective route will involve the introduction of a double at the C-3, followed by hydroboration treatment to afford (±)-9. The stereoselective route will involve the stereoselective reduction of 18 followed by a second reduction of the unhindered carbonyl resulting in 17. The latter can be transformed into (R)-9 via triflation followed by a Heck reaction.

2. Results and discussion

The synthesis of the racemic 3-hydroxy-β-ionone (±)-9 from β-ionone 16 was carried out according to Scheme 5. Treatment of the latter with N-bromosuccinimide (NBS) under basic and refluxing conditions yielded a 4-bromo derivative which eliminated when heated in pyridine for 2 h affording 3,4-dehydro-β-ionone 19 in a 50% overall yield. Protection of the ketone occurred smoothly with ethylene glycol in benzene catalyzed by p-toluenesulfonic acid in the presence of triethylorthoformate. The latter acted as a water trap and its presence resulted in enhancement of the yield from 50% (with a Dean–Stark distillation) to 80%.
Hydroboration of 20 with 9-BBN in THF followed by oxidative manethanolic work up afforded a mixture of hydroxylated compound enriched in the desired 21 (racemic) with a yield of 55%.13 Deprotection of the latter in 5% HCl in acetone for 5 min afforded (±)-9 in quantitative yield. Silylation of 3-hydroxy-β-ionone 9 was carried out, as necessary for further transformation, using triethylsilyl as a protecting group with a yield up to 97% resulting in 22. The overall yield of transformation from 16 to 22 was 21%.

The stereoselective route for the synthesis of (3R)-3-hydroxy-β-ionone (R)-9 shown in Scheme 6 involved an enzymatic reduction of 2,6,6-trimethyl-2-cyclohexen-1,4-dione (4-oxoisophorone) 18 with baker’s yeast to generate 23 after which a Raney Nickel reduction resulted in 17.15 A generous amount of 17 was provided to us by Hoffmann-LaRoche from which the rest of the synthesis was actually carried out. Silylation of the latter under standard conditions of DMAP and triethylamine resulted in a quantitative yield of 24.16 Triflation was carried out on 24 with 1.1 equiv. of LDA and Tf$_2$NPh to afford 25 in a modest 68% yield.16 This was followed by a Heck reaction with methyl vinyl ketone (MVK) catalyzed by Pd(0), which forms in situ from Pd(II), to give 26 in 67% yield.17 The overall yield of transformations from compound 17 to the desired (3R)-3-silyloylated-β-ionone 26 was 44%.

It was completely arbitrary and a result of synthetic timing that resulted in the usage of 26 in the synthesis of 11-cis retinoid 1 and the usage of the racemic precursor 22 in the synthesis of the all trans retinoid 2. Scheme 7 presents the synthesis of the 11-cis precursor monoprotected retinoid 4a.

The silyloylated β-ionone 26 was treated with phosphonoate$^{18}$ 10 and BuLi to afford the protected alkyne 27 in a quantitative yield with 4:1 of the desired E/Z ratio. The isomeric mixture inseparable at that stage was subjected to a global deprotection of both silyl groups with TBAF resulting in the terminal alkyne 28 ready for a Sonogashira coupling with vinyl iodide 11 generating 8 in quantitative yield. The 9E and 9Z isomers were then easily separated at that stage due to the presence of the bulky silyl group via column chromatography. Vinyl iodide 11 was prepared via reduction of 2-butyn-1-ol 12 with titanocene dichloride$^{9}$ followed by quenching with I$_2$ and protection of the primary alcohol with tert-butyldiphenylsilyl chloride (TBDPSCI). The key step of generating the 11-cis from alkyn 8 utilized a copper/silver pretreated zinc reduction$^8$ of the latter in a 1:1 MeOH/H$_2$O mixture over a period of ca. 24 h resulting in a nearly quantitative yield of exclusively the 11-cis isomer 4a.

Synthesis of the all trans retinoid 13 involved a series of two HWE couplings outlined in Scheme 8.$^{10,11}$ The silyloylated β-ionone 22 was treated with the NaH generated anion of phosphonate 14 to afford 29 in a mixture of 3:5:1 of 9E/9Z which were separated after DIBAH reduction resulting in the desired 9E 30 in an overall yield of 61%. The second HWE of the latter with 15 pretreated with BuLi resulted in a 4:1 of 13E/13Z mixture separable by column chromatography to afford 13.

Due to the nature of the structures of the retinoid precursors 4a and 13, both routes A and B (see Scheme 1) were utilized in installing the biotin group. For instance, in the case of 13 the only means of introducing the primary alcohol at C-15 is through an LAH reduction. Therefore the Boc-Lys-biotin (B) had to be introduced last (according to route B) as it would not have survived the latter reduction. Thus 13 must be first reduced with LAH to the primary alcohol, esterified with the appropriate α-haloacid, then desilylated without disturbing the labile haloester, and finally bioinylated with Boc-Lys(biotinyl)-OH. The 11-cis was fortunately carried through as the protected primary alcohol and a free C-3 hydroxyl allowing for the transformations depicted by route A (the more desirable of the two routes).

Scheme 9 describes the transformations required for the generation of the target biotinylated retinoid 1 according to route A. The monoprotected 3-hydroxy retinoid 4a was subjected to EDC coupling conditions with Boc-Lys(biotinyl)-OH affording 5a in 78% yield. This was followed by TBAF deprotection of the primary alcohol resulting in 6a with a yield of 79%. The unstable primary alcohol was immediately treated with bromoacetic acid under EDC
The synthesis of biotinylated retinoids 1 and 2 was accomplished using two different routes. Installing the biotin first, route A, was preferred due to the labile nature of α-haloesters (in particular chloroesters) which were preferably attached last. Route A was used in the synthesis of 1, however, to avoid multiple protection and deprotection procedures, route B (attaching the chloroester first) was employed in the synthesis of 2. The overall yield for the synthesis of 1 and 2 were 20% (from 17) and 0.7%, respectively. The biotinylated targets are currently in use for labeling RBP s in the retinal pigment epithelial (RPE) cells allowing for facile isolation via avidin-affinity chromatography, hydrolysis off the column, and final characterization by mass spectrometry.

3. Conclusion

The transformations that brought about the final stitches in the construction of 2 in Scheme 10 were certainly a lot more cumbersome due to the undesirable route B that was pursued. Needless to say, several attempts at completing this route proved unsuccessful due to the labile nature of the chloroester (which indeed is much more unstable than its bromo analog). The latter information regarding the stability of the bromoester versus the chloroester was not available to us until after the completion of this work and hence the reason for the synthesis of the chloroester analog 2. Moreover, preliminary unpublished biological assay results, which were not available during the time of the synthesis of the chloroester, have shown no preference toward having one halogen over the other, and therefore future targets will employ the exclusive use of α-bromoesters.

The silylated ester 13 was reduced with LAH resulting in 4b which was subsequently used without purification in an EDC coupling reaction with chloroacetic acid affording 5b which was purified rapidly by flash chromatography to avoid major decomposition. The latter was mildly desilylated by treatment with 5% HF/pyridine in THF for 5 min affording 6b in a modest overall yield of 27% from the starting ester 13. An EDC coupling of 6b with Boc-Lys(biotiny1)-OH resulted in the formation of an apparent quantitative yield of 2 by TLC; however a maximum of 40% recovery was achieved after workup and purification of the desired compound 2.

4. Experimental

4.1. General

All chemicals were obtained from Aldrich except for Boc-Lys(biotiny1)-OH which was purchased from Bachem. The solvents THF, Et2O, and CH2Cl2 were obtained dry from the solvent purification system via passage through an activated alumina cartridge. Column chromatography was performed using ICN silica gel (32–63 mesh). 1H NMR spectra were obtained on Bruker DXP 400 or 500 MHz spectrometers and are reported in parts per million (ppm) relative to TMS (δ), with coupling constants (J) in Hertz (Hz). The residual protic solvents (in CDCl3 or C6D6) were used as an internal reference. Low and high resolution FAB mass spectra were measured on a JEOL JMS-DX303 HF mass spectrometer using a glycerol matrix and Xe ionizing gas. ESI and APCI spectra were measured on a JEOL LC mate mass spectrometer. The all trans retinoids were handled under dim light conditions especially when containing more than 3 conjugated double bonds. The 11-cis compounds were handled strictly in the darkroom under dim red lights. Retinoids are best stored in benzene solutions frozen at −78°C, unless they are not soluble.

4.2. Synthesis of biotinylated retinoid 1

4.2.1. (R)-5-(tert-Butyldimethylsiloxy)-1,3,3-trimethyl-2-[3-methyl-6-(trimethylsilyl)-hexa-1,3-dien-5-ynyl]-cyclohexene (27). Dimethyl (3-trimethylsilyl-2-propynyl)phosphonate 10 (0.550 g, 2.5 mmol) in 10 mL of THF at 0°C was treated with nBuLi (2.5 mmol) to give a red solution. The reaction mixture was then stirred at room temperature for 15 min, after which time 26 (0.400 g, 1.24 mmol) in 1 mL of THF was added. The mixture was stirred for an additional 2 h, and was then quenched with aqueous NH4Cl, extracted with Et2O, washed with brine, and dried over Na2SO4. The product was purified by column chromatography using 2% Et2O in hexanes as an eluent to afford 27 as an oil (0.511 g, 99% yield; 4:1 9E/9Z). 1H NMR (500 MHz, CDCl3): δ=6.90 (d, 1H of 9Z, J=16.4 Hz), 6.31 (d, 1H of 9Z, J=16.4 Hz), 6.21 (d, 1H of 9E, J=16.4 Hz), 6.07 (d, 1H of 9E, J=16.4 Hz), 5.44 (s, 1H of 9E), 5.57 (s, 1H of 9Z), 3.93 (m, 1H), 2.22 (m, 1H), 2.07 (s, 3H of 9E), 2.1–2.0 (m, 1H), 1.92 (s, 3H of 9Z), 1.78 (s, 3H of 9Z), 1.68 (s, 3H of 9E),
1.49 (t, 1H, J=12.2 Hz), 1.11 and 1.08 (2s, 2H), 0.91 (s, 6H), 0.20 (s, 9H). [C25H44O2Si2+H+]**: calculated 417.30; found 417.2 (APCI+).

4.2.2. (R)-5-Hydroxy-1,3,3-trimethyl-2-[3-methyl-6-hexa-1,3-dien-5-ynyl]-cyclohexene (28). Acetylene 27 (0.511, 1.23 mmol) in 5 mL THF at 0°C was treated with TBAF (5 mL, 1 M in THF, 5 mmol) and the mixture was stirred at room temperature for 2 h. The mixture was then quenched with aq. NH4Cl, extracted with Et2O, washed with brine, and dried over Na2SO4. The product was purified by column chromatography using 30% EtOAc in hexanes as an eluent to afford 28 as an oil (0.28 g, 99% yield). 1H NMR (500 MHz, CDCl3): δ=7.81 (m, 4H), 7.71 (m, 6H), 6.86 (d, 1H, J=12.0 Hz), 6.36 (t, 1H, J=11.8 Hz), 6.31 (d, 1H, J=16.1 Hz), 6.17 (d, 1H, J=16.1 Hz), 5.99 (t, 1H, J=6.2 Hz), 5.88 (d, 1H, J=11.6 Hz), 4.37 (d, 2H, J=6.3 Hz), 3.79 (m, 1H), 2.21 (dd, 1H, J=16.8, 5.3 Hz), 1.95 (dd, 1H, J=16.8, 9.4 Hz), 1.82 (s, 3H), 1.68 (s, 3H), 1.47 (d, 1H, J=16.8 Hz), 1.42 (t, 1H, J=12.0 Hz), 1.17 (s, 9H), 1.05 and 1.04 (2s, 2x3H). [C30H48O2Si+H+]**: calculated 541.35; found 541.3 (APCI+).

4.2.3. (R)-3-Hydroxy-retinyl-11-ene-O-tetrt-butylidiphenylsilyle (8). Vinyl iodide 11 (872 mg, 1.6 mmol) was dissolved in iPrNH2 (4 mL) and tetrakis(triphenylphosphine)palladium (13.8 mg, 0.0124 mmol) was then added. The solution was stirred at room temperature for 5 min prior to the addition of CuI (2.4 mg, 0.0124 mmol). After 5 min of stirring, acetylene 28 (282 mg, 1.23 mmol) was introduced, and the mixture was stirred at room temperature for 2 h. The reaction was then quenched by the removal of solvent followed by the addition of Et2O and then NH4Cl. The resultant mixture was extracted with Et2O, washed with brine, and dried over Na2SO4. The product was purified by column chromatography using 12–20% EtOAc in hexanes as an eluent to afford 8 as an oil (432 mg of 9E, and 660 mg total weight, 99% yield). 1H NMR (500 MHz, CDCl3): δ=7.69 (m, 4H), 7.42 (m, 6H), 6.19 (d, 1H, J=16.0 Hz), 6.10 (d, 1H, J=16.0 Hz), 6.02 (t, 1H, J=5.1 Hz), 5.54 (s, 1H), 4.29 (d, 2H, J=6.28 Hz), 4.00 (m, 1H), 2.38 (dd, 1H, J=16.9, 5.2 Hz), 2.1–2.0 (m, 1H) ovlp with 2.06 (s, 3H), 1.77 (ddd, 1H, J=12.1, 3.3, 2.1 Hz), 1.72 (s, 3H), 1.67 (s, 3H), 1.47 (t, 1H, J=11.9 Hz), 1.35 (d, 1H, J=4.9 Hz), 1.06 and 1.05 (2s, 2x3H) ovlp with 1.05 (s, 9H). [C26H45O2Si+H+]**: calculated 539.33; found 539.3 (APCI+).

4.2.4. (R)-3-Hydroxy-11-cis-retin-O-tert-butylidiphenylsilyle (4a). Argon was bubbled through a suspension of Zn dust (10 g) in distilled H2O (60 mL) for 15 min after which Cu(OAc)2 (1 g) was added and the flask sealed immediately. The mixture was stirred for 30 min. The resultant activated Zn was then filtered and washed successively with H2O, MeOH, acetone, and Et2O. The moist activated Zn was then transferred to a flask to which 20 mL of H2O and 20 mL of MeOH were added. Acetylene 8 (194 mg, 0.36 mmol) dissolved in 2 mL MeOH was then added to the activated Zn and the reaction mixture was stirred at room temperature in the dark for 24 h. The Zn dust was then filtered through Celite, washed with Et2O and H2O. The aqueous layer was extracted further with EtOAc and the combined organic layers were washed with brine and dried over Na2SO4. The solvent was removed and the residue was suspended in 50% EtOAc/hexanes and filtered through a silica plug to afford purified 4a as an oil with an exclusive 11-cis geometry (194 mg, 99% yield). 1H NMR (500 MHz, CDCl3): δ=6.50 (d, 1H, J=12.0 Hz), 6.39
The reaction mixture was stirred for 2 h at room temperature. The mixture was then quenched with cold H₂O and extracted with CH₂Cl₂. The combined organic extracts were washed with Et₂O. The combined extracts were dried and purified by column chromatography using 20% Et₂O in hexanes to afford 2 as an oil. The NMR spectra of 2 show the expected chemical shifts and coupling constants. The compound was characterized by 1H and 13C NMR and high-resolution mass spectrometry. The elemental analysis of 2 indicates a purity of 86%.

4.3.2. 5-(4-Hydroxy-2,6,6-trimethyl-cyclohex-1-enyl)-3-methyl-penta-2,4-dienitrile (29). In a 25 mL round-bottom flask, NaH (124 mg, 5.3 mmol) in 5 mL THF was added to the reaction mixture at 0°C. The reaction mixture was stirred for 1 h at room temperature. The reaction mixture was then quenched with cold H₂O, extracted with Et₂O, and dried over Na₂SO₄. The product was purified by column chromatography using 20% Et₂O in hexanes to afford 29 as an oil. The NMR spectra of 29 show the expected chemical shifts and coupling constants. The compound was characterized by 1H and 13C NMR and high-resolution mass spectrometry. The elemental analysis of 29 indicates a purity of 89%.

4.3.3. 3-Methyl-penta-2,4-dienal (30). A solution of 29 (447 mg, 1.3 mmol) in CH₂Cl₂ (5 mL) at −60°C was added DIBAH (2 mL of 1 M, 2.0 mmol) and the reaction stirred for 1 h prior to quenching with cold wet H₂O. The residue was then suspended in 10% EtO/phexanes and loaded on a column for purification to afford 30 as an oil (263 mg, 94% yield). The NMR spectra of 30 show the expected chemical shifts and coupling constants. The compound was characterized by 1H and 13C NMR and high-resolution mass spectrometry. The elemental analysis of 30 indicates a purity of 92%.
4.3.7. 3-Hydroxy-all trans-retinol chloroacetate (6b). To a 2 mL solution of 5b (the entire sample from the above procedure) in THF at 0°C, and two beads of activated molecular sieves 4 Å, was added a mixture of Boc-Lys(biotinyl)-OH and 835.3402 (HRMS) due to Cl isotope effect.

4.3.8. 3-Boc-Lys(biotinyl)-O- all trans-retinol chloroacetate (2). To a 2 mL solution of 6b (8 mg, 0.02 mmol) in CH2Cl2 at -10°C, and five beads of activated molecular sieves 4 Å, was added a mixture of Boc-Lys(biotinyl)-OH (20 mg, 0.04 mmol) in 200 μL DMSO and EDC (12 mg, 0.063 mmol) in 1 mL of CH2Cl2. After 5 min of stirring, DMAP (1 mg, 0.008 mmol) was added and the reaction mixture was stirred for 4 h which was sufficient for a quantitative amount of product by TLC. The reaction mixture was washed with NaHCO3, NH4Cl, extracted with CH2Cl2, dried over Na2SO4 and chromatographed rapidly with 5% MeOH/CH2Cl2 affording 2 as a film coating the inside of a vial (6.8 mg, 40% yield). 1H NMR (500 MHz, CDCl3): δ=6.66 (dd, 1H, J=15.1, 11.3 Hz), 6.23 (d, 1H, J=15.1 Hz), 6.11 (d, 1H, J=11.3 Hz), 5.17 (br, 2H), 5.09 (m, 1H), 4.85 (d, 2H, J=7.3 Hz), 4.53 (dd, 1H, J=7.2, 4.9 Hz), 4.34 (dd, 1H, J=7.7, 4.8 Hz), 4.23 (br, 1H), 4.07 (s, 2H), 3.24 (br, 2H), 3.17 (dd, 1H, J=11.9, 7.3 Hz), 2.93 (dd, 1H, J=12.8, 4.9 Hz), 2.75 (d, 1H, J=12.8 Hz), 2.43 (dt, 1H, J=16.4, 5.1 Hz), 2.20 (br t, 2H), 2.12 (dd, 1H, J=17.2, 9.6 Hz), 1.96 (s, 3H), 1.91 (s, 3H), 1.9–1.7 (m, 8H), overlapped with 1.71 (s, 3H), 1.7–1.2 (m, 6H), overlapped with 1.45 (s, 9H), 1.10 and 1.07 (2s, 2×3H). [C43H65ClN4O8S+]$: calcd 379.2040; found 379.2038 (HRMS).

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