Synthesis and Biological Evaluation of a Siderophore-Virginiamycin Conjugate

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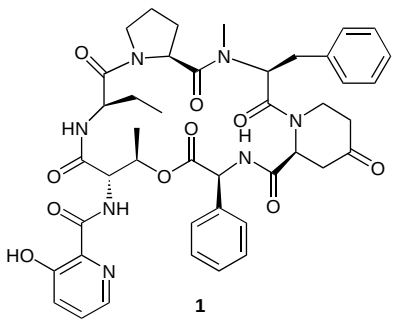
Abstract: Condensation of Virginiamycin S_1 (**1**) with aminooxyacetic acid afforded oxime **9**. EDC/HOAt-mediated coupling of **9** to the *N*-terminus of the tripeptide of N^5-hydroxy-N^5-acetyl-L-ornithine, the common iron chelator in most hydroxamate-derived siderophores, provided the desired siderophore-drug conjugate **5**. Preliminary biological evaluation of **5** against *E. coli* X580 under both iron sufficient and deficient conditions suggested uptake of siderophore-drug conjugate (**5**) by active iron transport systems.

Key words: oximes, drug delivery, iron

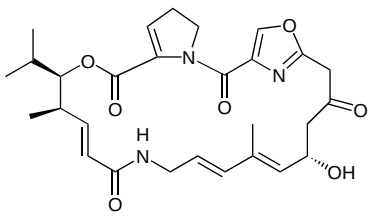
Virginiamycin S_1 (**1**, Figure 1) belongs to a family of Type B streptogramin antibiotics remarkable for their antibacterial activity and their unique mode of action. This cyclic macrolactone hexapeptide is usually coadministered with Type A streptogramins, such as Virginiamycin M (VM, **2**, Figure 1).[1] Streptogramins bind to the peptidyltransferase domain of bacterial ribosome and inhibit bacterial growth by disrupting the translation of mRNA into protein. VS_1 stimulates the dissociation of the peptidyl-tRNA complex and causes the premature release of polypeptides chains. The combination of Type A and Type B antibiotics greatly enhances their antibacterial potency. This synergistic effect results from an increased binding affinity of VS_1 for the ribosome due to a conformational change induced by VM.[2] Streptogramin antibiotics are much more effective towards Gram positive than Gram negative bacteria, presumably due to membrane permeability problems associated with Gram negative bacteria.[3]

The streptogramin antibiotics have been extensively employed as feed additives to elicit improved feed efficiency and increased rate of weight gain in swine and poultry[4] as well as in clinical treatment for humans. Synercid, a semisynthetic streptogramin, and pristinamycin have recently been used for clinical treatment of vancomycin-resistant *Enterococcus faecium* and methicillin-resistant *Staphylococcus aureus*.[5] Virginiamycin resistant strains of bacteria have been detected in *Enterococcus faecium* from pigs and chickens.[6] Streptogramin resistant bacterial strains have also been identified and isolated from hospitals where pristinamycin was extensively used.[7] In connection with interests in siderophore-mediated drug delivery systems in the Miller group and synthesis of VS_1 derivatives in the Helquist group, we decided to incorporate VS_1 into a siderophore component via a novel oxime linkage. This could be achieved by incorporating an aminooxyacetic acid spacer at the *N*-terminus of the tripeptide of N^5-hydroxy-N^5-acetyl-L-ornithine, the common iron chelator in most hydroxamate-derived siderophores.

Siderophores are secreted by microbes as microbial iron transport agents to sequester iron(III) under iron deficient conditions.[8] The siderophore–iron complex is recognized by outer membrane receptors regulated by the Fur protein, expressed under iron stressed conditions, and actively transported inside the cell.[9] Because iron is a limiting nutrient with the onset of infection, siderophores are essential for microbial pathogenicity.[10] Siderophore-drug conjugates (**3**, Figure 2) are composed of iron chelators, chemical linkers and biologically active components ("drug"). These siderophore-drug conjugates (**3**) could be recognized by microbes and transported inside the cell by the iron uptake system. Once inside the cell, the drug



1
Virginiamycin S_1



2
Virginiamycin M

Figure 1 Type A and Type B Streptogramin Antibiotics

could be released to induce an effective dosage to the microbes ("The Trojan Horse strategy").[11] Albomycins (**4**, Figure 2), antibiotics produced by *Streptomyces griseus* and *Streptomyces substropicus*, are naturally occurring examples of siderophore-drug conjugates.[12]

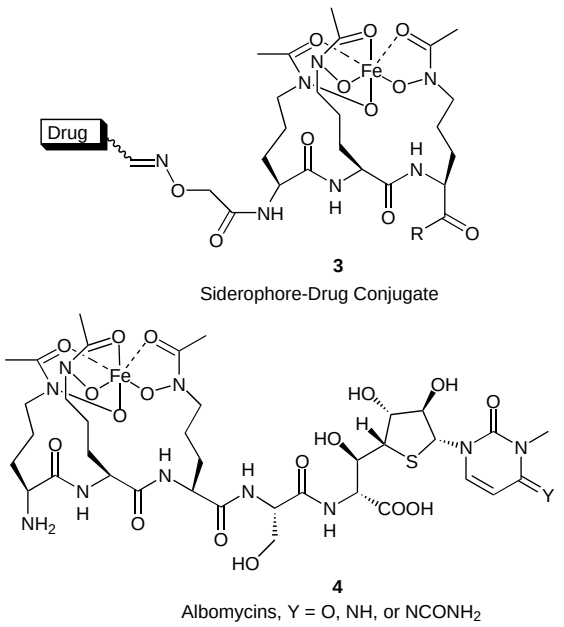


Figure 2 Siderophore-Drug Conjugate and Albomycins

Thus, VS_1 is an ideal drug component in testing the scope of this "Trojan Horse" active drug delivery strategy due to its structural features and unique mode of action. We therefore decided to use an oxime linkage which can take advantage of the oxo group found on the 4-oxopipecolic acid domain present in **1** for constructing the desired siderophore-drug conjugate (**5**, Figure 3). Once transported inside the cell, hydrolysis of the oxime linkage of the conjugate was anticipated to regenerate VS_1 and allow it to exert its lethal effect on the bacterial ribosome.

Herein, we report our synthesis of a novel virginiamycin oxime siderophore conjugate (**5**) and its preliminary biological evaluation against a strain of *E. coli*.

Our initial attempt in making an oxime linkage for a siderophore-drug conjugate started from siderophore component **6** (Scheme 1), readily available through the practical synthesis of the corresponding tripeptide trihydroxylamine by an indirect oxidation method.[13] Treatment of fully unprotected siderophore component **6** with the *N*-hydroxysuccinimide (NHS) active ester of Boc-protected aminooxyacetic acid afforded **7** in 72% yield. As a model study, the Boc group was cleaved and the resulting hydroxylamine was treated with an excess of acetone and pyridine to provide oxime **8** as its pyridinium salt in quantitative yield. Conceptually, this approach would provide a general synthetic route in condensing various oxo-containing drug components directly with the hydroxylamine moiety of the siderophore component to provide the desired oxime-linked conjugate at the last step of the synthesis. However, reaction of this hydroxylamine with a stoichiometric amount of VS_1 did not provide the desired conjugate, presumbly due to the instability of the hydroxylamine indicated by changes in its NMR spectra upon storage.

We then decided to preform an oxime by reaction of Virginiamycin S_1 (**1**) with aminooxyacetic acid followed by coupling to the *N*-terminus of a siderophore component (Scheme 2). Treatment of aminooxyacetic acid with **1** in the presence of pyridine provided the desired oxime **9** in 96% yield as a 1:1 mixture of *E, Z* isomers.[14]

The *C*-terminus of the siderophore component was protected by coupling **10** with the methyl ester of 6-aminocaproic acid to afford **11**. The 6-aminocaproic acid spacer not only served as a protecting group, but also provided the carboxy group for potential coupling to other drug components in future studies. Deprotection of the Cbz group followed by a EDC/HOAt-mediated coupling reaction with oxime **9** provided the desired conjugate **5** in good overall yield as an inseparable 1:1 mixture of *E* and *Z* isomers.

Biological evaluation of this conjugate mixture was then carried out at 10 μM and 20 μM using kinetic growth assays in Luria broth under both iron sufficient and deficient conditions against *E. coli* X580, a Gram-negative bacteria (Figure 4).

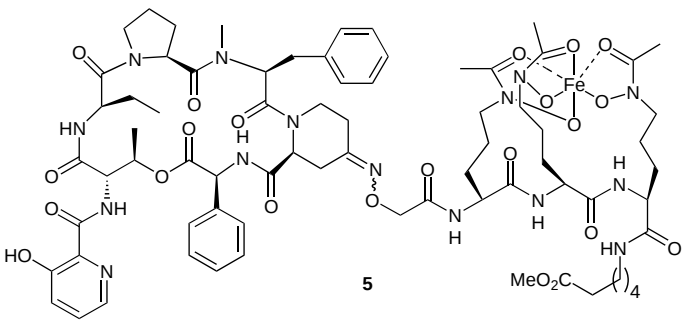
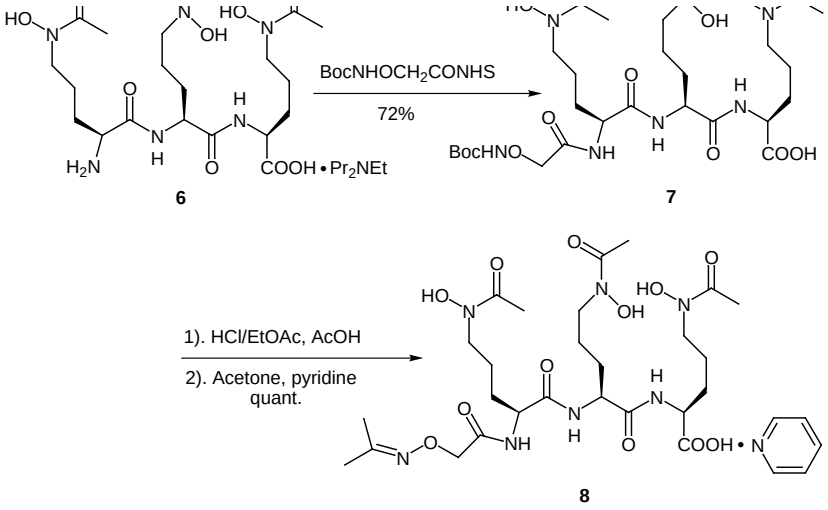


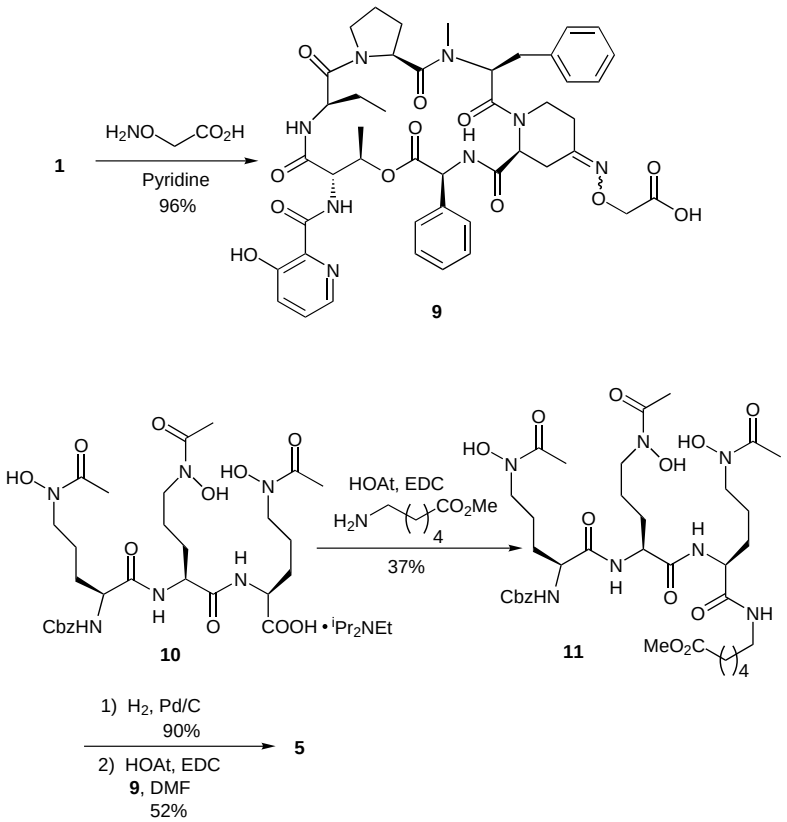
Figure 3 Virginiamycin-Siderophore Conjugate



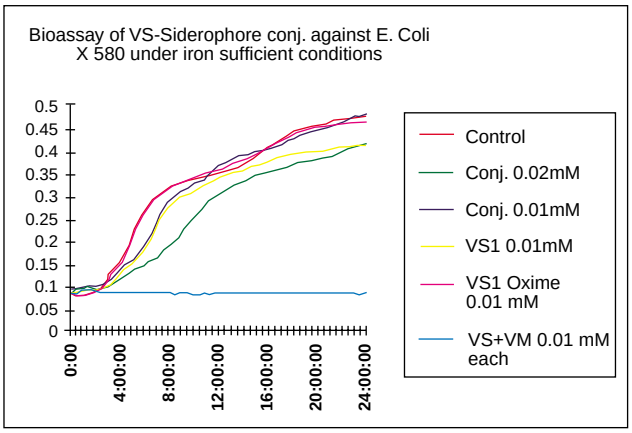
Scheme 1

Scheme 1

Under iron sufficient conditions, VS_1 siderophore conjugate (**5**), VS_1 itself (**1**), and VS_1 oxime (**9**) displayed no significant antibacterial activity while combination of VS_1 and VM displayed potent synergistic activity, as expected. However, under iron deficient conditions, significant antibacterial activity was observed with siderophore drug conjugate (**5**) at 10 μM and 20 μM. These results suggested receptor-mediated active transport of the drug conjugate since outer membrane receptors are expressed under iron stressed conditions. Interestingly, VS_1 also showed significant antibacterial activity under iron deficient conditions. It is noteworthy that oxime **9** did not display any



Scheme 2



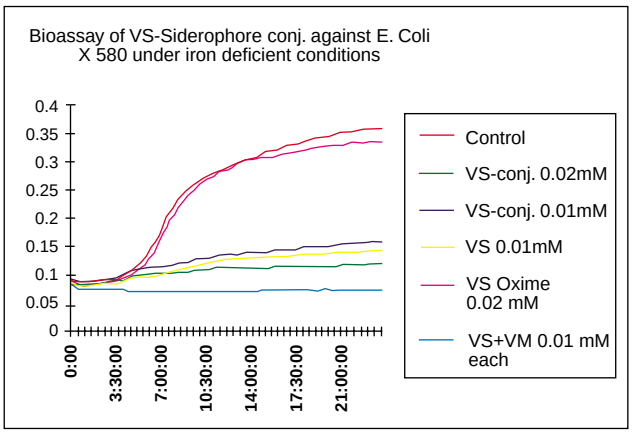


Figure 4 Growth Curves of *E. Coli* X580 in Luria Broth Culture

activity under iron deficient conditions. This suggested that the siderophore-drug conjugate was transported inside the cell and either the intact conjugate was active or intracellular cleavage of the oxime linkage release of the drug occurred by hydrolysis of the oxime.

In summary, we have described the synthesis and antibacterial activity of a novel siderophore drug conjugate through an oxime linkage. The biological testing results suggested that the conjugate was taken up by *E. coli* through the receptor-mediated active transport pathway. It would be interesting to test if a VM-siderophore conjugate will display a synergistic effect with the VS_1-conjugate. The synthesis of a virginiamycin M siderophore conjugate and its biological study are currently underway. This modification of VS_1 also shows that Type B streptogamin antibiotics can be derivatized to target Gram negative bacteria. A broad spectrum assay of VS_1 conjugate (**5**) is also underway.

General. All reactions were carried out under N_2 unless otherwise stated. Instruments and general methods used have been described earlier.[15] Biological evaluation procedures have been described before.[16]

Protected Siderophore Component 7

To a solution of Boc-aminooxyacetic acid (46 mg, 0.24 mmol), NHS (30 mg, 0.26 mmol), in THF (2.0 mL) at 0 °C, was added DCC (52 mg, 0.25 mmol). The mixture was then stirred at r.t. for 15 h. The mixture was used as the active ester solution in the following reaction.

To a solution of **6** (100 mg, 0.15 mmol), and $NaHCO_3$ (150 mg, 1.78 mmol) in THF/H_2O (5 mL/5 mL) at r.t., was added the above active ester solution. The mixture was stirred at r.t. for 20 h. The volatiles were removed and the residue was partitioned between H_2O (20 mL) and EtOAc (20 mL). The pH of the mixture was adjusted to 2 by adding 0.2 M HCl. The layers were separated and the aqueous layer was extracted with EtOAc (2 x 10 mL). The combined extracts were dried (Na_2SO_4), filtered, concentrated and purified by reverse phase column chromatography (C_{18}, 4:1 H_2O/CH_3OH to 2:1 H_2O/CH_3OH) to give 42 mg (72%) of **7** as a white foam. Unreacted **6** (26 mg) was recovered as its free acid.

^{1}H NMR (300 MHz, CD_3OD): δ = 1.47 (s, 9H) 1.69–1.88 (m, 12H), 2.10 (s, 9H), 3.61–3.69 (m, 6H), 4.29 (AB, *J* = 15.9 Hz, 2H), 4.36–4.47 (m, 3H).

^{13}C NMR (75 MHz, CD_3OD): δ = 20.7, 20.8, 24.5, 24.6, 24.8, 29.0, 30.3, 30.58, 30.6, 48.9, 54.0, 54.4, 54.6, 76.9, 83.6, 160.3, 172.2, 174.2, 174.3, 174.5, 175.6.

IR (neat): ν = 1543, 1636, 1641, 1650, 1724, 3244 cm^{-1}.

FABMS: calcd. for $C_{28}H_{49}O_{14}N_7Na$ $(M+Na)^+$:730.3235, found:730.3209.

Oxime 8

To a solution of **7** (23 mg, 0.032 mmol) in HOAc (6 mL) at r.t., was added HCl/EtOAc (2 mL, saturated at 0 °C). The mixture was stirred at r.t. for 1 min and the volatiles were removed in vacuo to give 21 mg (quant.) of hydroxylamine as a slightly pink oil.

To a sample of the above hydroxylamine (3 mg, 0.0047 mmol) at r.t., was added acetone (2 mL) and H_2O (0.5 mL). Pyridine (0.5 mL, 6.19 mmol) was then added and the solution was stirred at r.t. for 6 h. The volatiles were removed to afford 4 mg (quant.) of **8** as a white foam. ^{1}H NMR (600 MHz,CD_3OD): δ = 1.68–1.82 (m, 12H), 1.86 (s, 3H), 1.95 (s, 3H), 2.10 (s, 9H), 3.55–3.72 (m, 6H), 4.37–4.50 (m, 5H), 8.11 (m, 2H), 8.66 (t, *J* = 7.8 Hz, 1H), 8.88 (d, *J* = 5.4 Hz, 2H).

^{13}C NMR (150 MHz, CDOD): δ = 16.3, 20.8, 22.1, 24.4, 24.6, 24.8, 30.2, 30.6, 31.1, 48.6, 48.7, 48.8, 53.9, 54.1, 54.6, 73.4, 129.2, 143.7, 148.5, 159.8, 173.2, 174.3, 174.5, 175.4.

IR (neat) ν = 1540, 1637, 1729, 3250, 3400 cm^{-1}.

FABMS: calcd. for $C_{26}H_{46}O_{12}N_7$ $(M+H)^+$:648.3204, found:648.3215.

Siderophore Component (11)

To a mixture of **10** (88 mg, 0.11 mmol), methyl 6-aminocaproate hydrochloride (30 mg, 0.16 mmol), HOAt (18 mg, 0.13 mmol), DMAP (27 mg, 0.22 mmol), Et_3N (0.05 mL, 0.39 mmol) in DMF (2 mL) at 0 °C, was added EDC (25 mg, 0.13 mmol). The yellow mixture was stirred to r.t. for 38 h. H_2O (10 mL) was added and the aqueous solution was washed with CH_2Cl_2 (2 x 10 mL), concentrated and purified by reverse phase column chromatography (C_{18}, 2:1 H_2O/CH_3OH to 1:1 H_2O/CH_3OH) to give 28 mg (37%) of **11** as a white foam. Unreacted **10** (10 mg) was recovered as a free acid.

^{1}H NMR (500 MHz, CD_3OD): δ = 1.33 (m, 2H), 1.52 (m, 2H), 1.62–1.67 (m, 14H), 2.08 (s, 3H), 2.09 (s, 3H), 2.10 (s, 3H), 2.31 (t, *J* = 7.5 Hz, 2H), 3.12 (m, 2H), 3.64 (s, 3H), 3.58–3.68 (m, 6H), 4.12

(m, 1H), 4.27 (m, 1H), 4.33 (m, 1H), 5.09 (AB, *J* = 12.5 Hz, 2H), 7.33 (m, 5H).

^{13}C NMR (125 MHz, CD_3OD): δ = 20.8, 24.7, 24.8, 26.1, 27.8, 30.2, 30.4, 30.4, 30.6, 30.7, 35.2, 40.7, 48.7, 48.8, 52.5, 55.0, 55.2, 56.8, 68.3, 129.3, 129.6, 130.0, 138.6, 159.2, 174.2, 174.28, 174.3, 174.5, 175.7, 176.3.

IR (neat) ν = 1536, 1640, 1713, 3285 cm^{-1}.

FABMS: calcd. for $C_{36}H_{58}N_7O_{13}(MH)^+$:796.4093, found:796.4059.

VS_1-siderophore Conjugate (5)

A solution of **11** (16 mg, 0.02 mmol) in CH_3OH (3 mL) was stirred with Pd/C (10%, 3 mg) under H_2 (1 atm) at r.t. for 1 h. The catalyst was filtered off and rinsed with CH_3OH. The filtrate was concentrated to give 12 mg (90%) of unprotected siderophore component as a colorless oil.

A solution of oxime **9** (25 mg, 0.028 mmol), unprotected siderophore component (16 mg, 0.024 mmol), EDC (7 mg, 0.037 mmol), HOAt (5 mg, 0.037 mmol), and DMAP (6 mg, 0.049 mmol) in DMF (0.5 mL) was stirred at r.t. for 17 h. H_2O (10 mL) and $CHCl_3$ (20 mL) were added and the layers were separated. The aqueous layer was extracted with $CHCl_3$ (2 x 10 mL). The combined organic layers were washed with brine (2 x 10 mL), dried (Na_2SO_4), filtered, concentrated and purified by reverse phase column chromatography (C_{18}, 1:1 H_2O/CH_3OH to 1:2 H_2O/CH_3OH) to give 19.5 mg (52%) of **5** as a white foam.

^{1}H NMR (600 MHz, CD_3OD): δ = 0.86–0.90 (m, 3H), 1.04–1.08 (m, 1H), 1.25–1.96 (m, 26H), 2.03–2.08 (m, 11H), 2.24–2.32 (m, 2H), 2.41–2.60 (m, 2H), 3.07–3.37 (m, 8H), 3.49–3.63 (m, 10H), 4.22–4.70 (m, 8H), 4.79–4.81 (m, 1H), 4.96–4.98 (m, 1H), 5.16–5.38 (m, 2H), 5.55–5.63 (m, 1H), 5.78 (m, 1H), 7.12–7.58 (m, 12H), 7.86–7.90 (m, 1H).

^{13}C NMR (150 MHz, CD_3OD): δ = 10.8, 16.9, 17.2, 17.3, 17.6, 20.79, 20.8, 24.5, 24.7, 24.8, 25.1, 25.2, 25.7, 26.06, 26.1, 26.3, 26.4, 27.4, 27.7, 27.8, 29.2, 29.9, 30.1, 30.4, 30.6, 30.7, 30.8, 31.6, 32.0, 32.3, 32.7, 35.2, 37.6, 37.8, 38.4, 38.6, 39.9, 40.0, 40.4, 40.7, 48.7, 48.8, 52.5, 53.5, 54.7, 54.9, 55.0, 55.2, 55.6, 57.4, 57.5, 57.6, 57.9, 58.2, 59.0, 59.6, 71.4, 71.5, 73.6, 73.8, 73.9, 108.4, 128.4, 129.4, 129.5, 129.7, 129.8, 129.9, 130.1, 130.49, 130.5, 130.79, 130.8, 131.0, 131.1, 131.2, 131.3, 131.5, 132.5, 132.7, 137.9, 138.1, 138.2, 147.4, 156.7, 157.2, 158.1, 170.2, 170.8, 170.9, 171.1, 171.5, 172.19, 172.2, 172.3, 172.8, 173.2, 173.6, 173.9, 174.0, 174.1, 174.2, 174.3, 174.4, 174.68, 174.7, 176.3.

IR (neat): ν = 1247, 1448, 1523, 1628, 1645, 1653, 1678, 1737, 3275, 3367 cm^{-1}.

FABMS: calcd: for $C_{73}H_{101}N_{15}O_{22}(M^+)$:1540, found:1540.

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