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Carbohydration of 1,4,8,11-tetraazacyclotetradecane (cyclam): synthesis and binding properties toward concanavalin A

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Dedicated to Dr. Hartmut Spies in memoriam

Abstract—Two novel glycocluster ligands with cyclam core bearing thiourea-linked D-glucose and 2-acetamido-2-deoxy-D-glucose at the periphery have been synthesized. The interaction with concanavalin A has been studied by isothermal titration microcalorimetry for characterizing protein-ligand interactions. The sugar-containing multivalent ligands showed higher association affinity compared to the sugar monomers, which is attributed to an entropy driven glycoside clustering effect.

Radiopharmaceuticals based on the metallic radionuclides $^{64/67}$Cu, ^{99m}Tc, $^{186/188}$Re and ^{90}Y are often used for diagnostic and therapeutic purposes.[1] Cyclam and its derivatives are one of the most important ligands for the radionuclides mentioned above.[2] Regarding to radiopharmaceutical applications radiometal complexes of cyclam derivatives with hydrophilic surface groups are attractive candidates. Recently, we could show that a star-like cyclam ligand appended with four PEG-arms rapidly forms stable copper(II) complexes.[3] Besides the pegylation the carbohydration of radio-labelled compounds is of considerable interest to improve the pharmacokinetics and tumor accumulation.[4] In this perspective, carbohydrate clusters are gaining in importance as interesting targets.[5] Thus, sugar-containing dendritic wedges show both unique cell uptake behavior and specific carbohydrate–protein interaction.[6,7] However, only a couple of examples of glycoclusters with metal complexing units are known. In connection with the work to be presented in this Letter, upper rim calyx[4]arene divalent glycoclusters have to be mentioned.[8] Glycosylated macrocyclic amphiphiles with cyclam as core element show unique self-aggregation properties and have the potential for use in enantioselective metal-catalyzed reactions.[9] Roy and Kim described dendritic bipyridyl-glycoclusters whereby particularly the corresponding Cu(II)-complexes show enhanced efficacy to inhibit the binding of asialoglycoprotein to the lectin VVA-HRP.[10] Recently, glycoconjugates possessing dendritic wedges with 12 glucose or galactose groups linked to a central gadolinium complex have been developed in view of MRI applications.[11]

Currently, we are focusing our attention on the development of dendritic ligands having both enhanced complex stability and improved bio-availability. In this perspective, the synthesis of two glycoclusters with a cyclam core modified with thiourea-linked sugar residues at the periphery of the molecules is presented. D-glucose and 2-acetamido-2-deoxy-D-glucose have been chosen as sugar moieties. The interaction of these sugar-containing ligands with concanavalin A has been studied using isothermal titration microcalorimetry to characterize protein-ligand interaction and possible cluster effects.

The glycocluster ligands **6** and **7** were prepared by glycosylation of the tetraamino-functionalized cyclam core predecessor **1**[3] using the *O*-acetyl-protected glucosyl isothiocyanates **2**[12] and **3**[13] (Scheme 1). The reaction course

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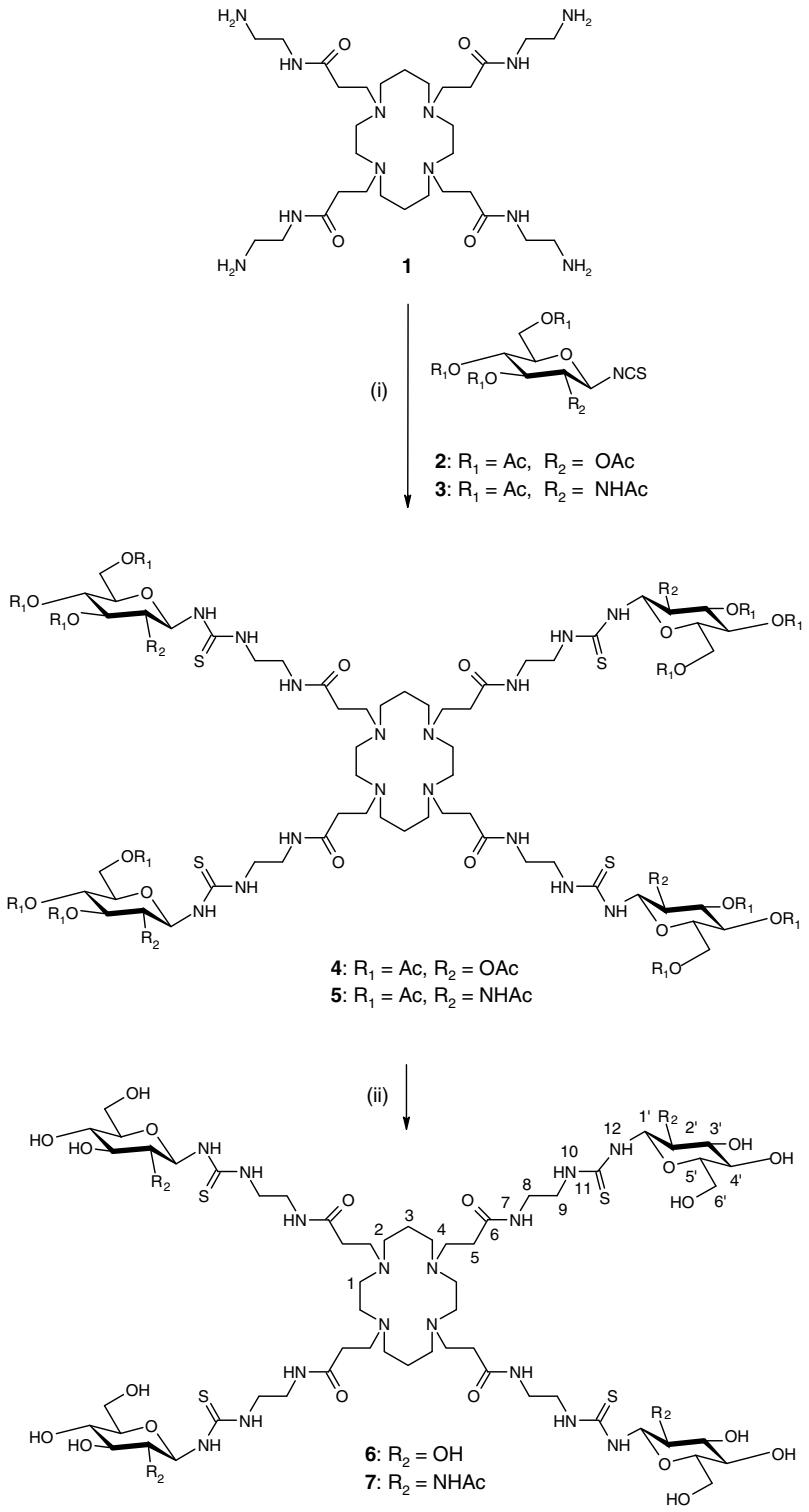

Scheme 1. Synthesis of **6** and **7**. Reagents and conditions: (i) excess of **2** and **3**, $CHCl_3$ (MeOH), rt, 6 days, Al_2O_3, $CHCl_3$/MeOH (12:1), (**4**: 85 %), 1 day (**5**: >50%); (ii) NaOMe/MeOH, pH 8–9, rt, 2 h, neutralization with DOWEX 50WX8, SEC (**6**: 69%; **7**: 37%).

was studied by thin layer chromatography [neutral alumina plates, methanol, $R_f = 0$ (**1**), $R_f = 0.92$ (**2**, **3**), $R_f = 0.33$ (**4**, **5**)]. The reaction was completed after 6 days for **4** and 1 day for **5**. The purification of the O-acetylated products was not trivial. Due to irreversible sorption of **4** and **5** on silica gel, flash chromatography failed. The replacement of acetyl- by benzoyl-protecting groups for the glucosyl isothiocyanates led to highly lipophilic cluster compounds allowing the purification by silica gel chromatography.

However, thiourea-bridging reaction of **1** with 2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl isothiocyanate proved to be rather slow and low yielding. Therefore,

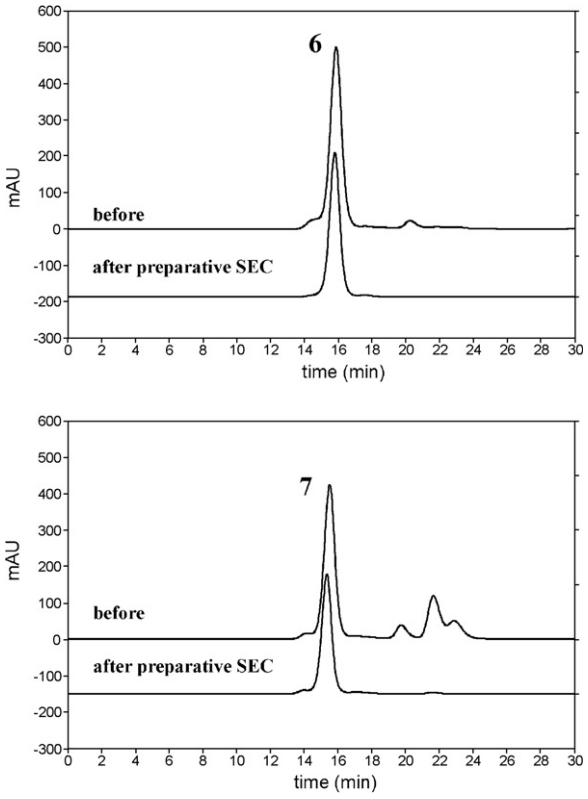

Figure 1. SEC traces of glycoclusters **6** and **7** (PSS HEMA 40, 8 × 300 mm, H_2O/acetic acid 1:0.025, 1 mL/min).

the crude O-acetylated products **4** and **5** were purified by column chromatography using neutral alumina gave the pure **4** in 85% yield. In the case of **5** the product contained persistently adhesive glucosyl isothiocyanate **3**. Deacetylation of **4** and **5** was performed according to Zemplén with sodium methanolate.[14] After neutralization with acidic ion exchange resin, the products were purified by size exclusion chromatography (see Fig. 1) giving compounds **6** and **7** in 69% and 37% yield. NMR and electrospray mass spectrometry confirm the structure of glycocluster compounds.[15,16] Precise accurate mass data were also obtained by high resolution mass spectrometry (**6**, calcd: 1541.6678, found: 1541.6683; **7**, calcd: 1705.7740, found: 1705.7777). Mass spectrometric analysis of fragmentation pattern of the glycocluster compounds and their transition metal complexes is described in an additional paper.[17]

Isothermal microcalorimetric titration (ITC) experiments were carried out at 298 K using a TAM microcalorimeter (Thermometric AB, Sweden) equipped with the high performance calorimetric module 2201 and a titration microreaction ampoule 2251. The sample cell was filled with 1.2–1.4 mL of a 0.23 mM (monomer) concanavalin A[18] solution in buffer (50 mM dimethyl glutaric acid, 250 mM sodium chloride, 1 mM calcium chloride, 1 mM manganese chloride, pH 5.2). The sugar (ca. 35 mM) and glycocluster (ca. 4 mM) solutions in the identical buffer were added in 8 μL increments from a 250 μL Hamilton syringe driven by a computer controlled syringe pump.

Titration experiments consisted of 30 injections with 30 min time intervals between the injections, allowing the reaction mixtures to equilibrate. The complete titration experiment and data acquisition were controlled by the instrument software (Digitam 4.1). The heat associated with each injection was calculated by integration of the peaks in the heat flow versus time curve using the Digitam software. The calibration of the calorimeter was performed by electrical heat pulses after each titration run. Blank experiments were performed under identical experimental conditions by titrating the ligand solution into pure buffer. The blank effects were subtracted to correct for dilution, mixing and injection effects. The experimental data were fitted to a theoretical titration curve based on a one-site reaction model[19] using a software written with the MATLAB (MathWorks Inc., USA) package, with the enthalpy change ΔH, stability constant K and the number of binding sites per concanavalin A monomer n as adjustable parameters.

Figures 2 and 3 show exemplarily the titration curves (top) of D-glucose and **6** when bound to conconavalin A and the resulting one-site fit of the differential molar enthalpies (bottom), respectively. All fits yielded n values between 0.98 and 1.02 indicative for monovalent binding of conconavalin A. This is as expected because the glycoclusters **6** and **7** are too small to bind to two

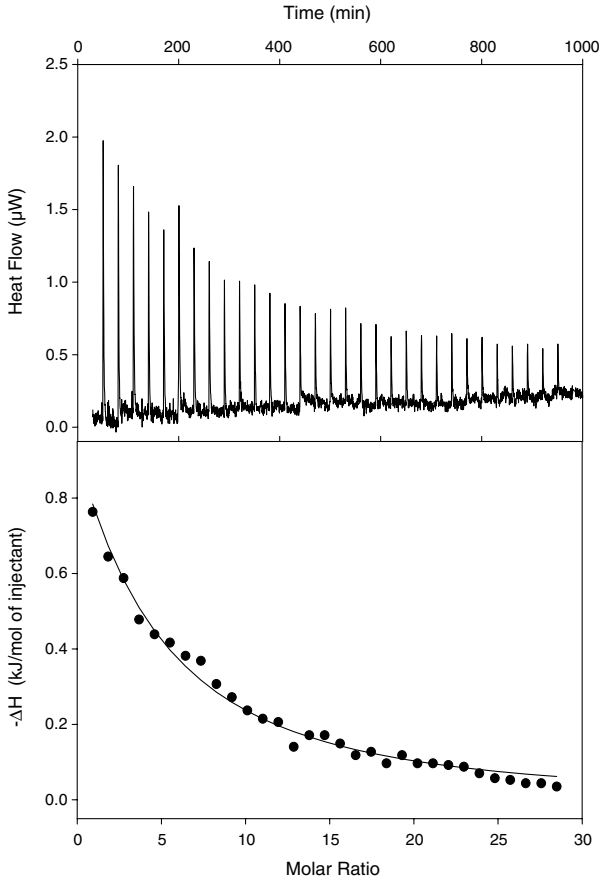

Figure 2. Calorimetric data for the titration of concanavalin A (0.2 mM) with D-glucose (28.5 mM). Top: Raw data. Bottom: Fit of the integrated data (points) using a one-site model (line).

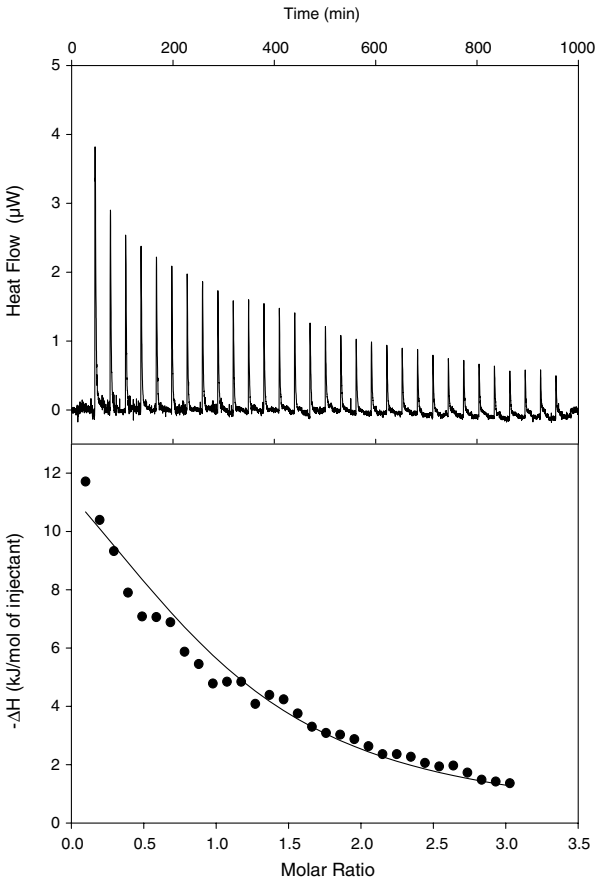

Figure 3. Calorimetric data for the titration of concanavalin A (0.23 mM) with **6** (4.13 mM). Top: Raw data. Bottom: Fit of the integrated data (points) using a one-site model (line).

Table 1. ITC results for binding of D-glucose, 2-acetamido-2-deoxy-D-glucose, glycoclusters 6 and 7 to concanavalin A at 25°

Compound	K [M^{-1}]	ΔG [kJ/mol]	ΔH [kJ/mol]	ΔS [J/K mol]
D-Glucose	368 ± 4	−14.6	-16.8 ± 0.04	−7.4
2-Acetamido-2-deoxy-D-glucose	278 ± 6	−13.9	-14.0 ± 0.2	−0.3
6	7930 ± 260	−22.2	-16.3 ± 0.2	19.8
7	2800 ± 92	−19.7	-15.9 ± 0.4	12.7

binding sites of a conconavalin dimer.[20] In principle, there is a possibility of cross-linked agglutination, however, no indications like aggregation or precipitation have been found in the ITC experiments. Thermodynamic binding data derived from ITC results for the tetravalent glycoclusters **6** and **7** and the monovalent sugars are summarized in Table 1. A strong enhancement of the binding affinity of the glycoclusters relative to the appropriate sugar monomers was observed, which is typical for a distinct glycoside clustering (or more exactly proximity/statistical) effect.[20]

Furthermore, the binding energy remained approximately constant suggesting that the affinity enhancement arises completely from an entropy advantage of glycocluster binding. Favorable entropy effects have also been reported for multivalent glycodendrimer binding by Toone et al.[21] Comparing the binding behavior of both the different sugars and glycoclusters shows a higher binding affinity and energy for the D-glucose moiety. The modification at the C_2 position of glucose with the bulky acetamido substituent leads apparently to a reduced shape complementary for the lectin binding site.

In summary, we have synthesized two novel cyclam derivatives with four sugar moieties on the periphery by thiourea-bridging of a tetraamino-functionalized predecessor and the O-acetyl-protected glucosyl isothiocyanates. Isothermal titration calorimetry was used to characterize the protein-glycocluster interactions. Thermodynamic data derived from ITC show a distinct glycoside clustering effect, which can be understood by an entropy advantage of glycocluster binding. Preliminary experiments show that the sugar-containing cyclam ligands are able to form stable complexes with the diagnostically relevant radioisotopes ^{99m}Tc and ^{64}Cu.[22] Kinetics of copper(II) complex formation are rather slow probably due to the binding of Cu(II) by the thiourea unit in an initial complexation step. Synthetic work to replace the thiourea linker by an amido group are underway.

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15. *Experimental details and data for* **6**: A solution of **1** (91 mg, 0.138 mmol) in $CHCl_3$ (6 ml) was dropped into a solution of **2** (440 mg, 1.13 mmol) in $CHCl_3$ (10 ml) and MeOH (200 μl). The mixture was stirred for 6 days until clearing off. The solvent was removed under reduced pressure and the residue was purified by alumina column chromatography using $CHCl_3$ (elution of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate) and $CHCl_3$/MeOH (12:1) as eluents. Product **4** was isolated in 85% yield as a white solid.
^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.65$ (s, 4H, H-3), 1.99–2.06 (q, 48H, Oac), 2.01–2.08 (m, 8H, H-5), 2.25–2.40 (m, 8H, H-4), 2.40–2.60 (m, 16H, H-1, -2), 2.60–2.85 (m, 8H, H-9), 2.28 (m, 8H, H-8), 3.45–3.80 (m, 4H, H-5′), 3.80–3.98 (m, 4H, H-4′), 4.00–4.20 (m, 4H, H-3′), 4.20–4.28 (m, 4 H, H-1′), 4.95–5.18 (m, 8 H, H-6′), 5.25–5.38 (m, 4 H, H-2′). ESI-MS: *m/z* calculated for $C_{90}H_{140}N_{16}O_{40}S_4$ ($[M+H]^+$): 2213.8; found: 2213.9.
NaOMe (1 M solution in MeOH) was added to the solution of **4** (260 mg, 0.117 mmol) in MeOH (40 ml) to adjust pH 8–9. The mixture was stirred for 2 h and afterwards diluted with water (30 ml) and neutralized with Dowex 50WX8. The solution was filtered and the solvent was removed under reduced pressure. The residue was purified by size exclusion chromatography (PSS HEMA 40, 10 μm/Polymer Standards Service Inc., 20 × 300 mm, H_2O/acetic acid 1:0.025, 1.5 mL/min) and lyophilized. Product **6** was isolated as tri-acetate in 69% yield as a white solid.
^{1}H NMR (400 MHz, DMSO-d_6): $\delta = 1.52$ (m, 4H, H-3), 1.87 (s, 9H, CH_3COOH), 2.21 (t, 8H, H-5), 2.43 (m, 16H, H-1, -2), 2.62 (t, 8H, H-4), 3.08 (m, 8H, H-9), 3.17 (m, 8H, H-8), 3.35–3.55 (m, 24H, H-1′, -3′, -4′, -5′, -6′), 3.56–3.65 (m, 4H, H-2′), 7.65–8.21 (m, 12H, H-7, -10, -12). ^{13}C NMR (100 MHz, D_2O): $\delta = 21.59$ (C-3), 30.33 (C-5), 39.09 (C-9), 43.78 (C-8), 47.90 (C-1), 48.23 (C-2), 49.55 (C-4), 60.77 (C-6′), 69.40 (C-4′), 72.12 (C-2′), 76.66 (C-3′), 77.37 (C-5′), 83.19 (C-1′), 173.64 (C=O, C-6), 182.90 (C=S C-11). ESI-MS: *m/z* calculated for $C_{58}H_{108}N_{16}O_{24}S_4$ ($[M+H]^+$): 1541.6; found: 1541.6. Anal. Calcd for $C_{58}H_{108}N_{16}O_{24}S_4 \cdot (CH_3COOH)_3 \cdot (H_2O)_{10}$ (1902.14): C, 40.41; H,7.42; N, 11.78; S, 6.74. Found: C, 40.28; H, 7.21; N, 11.47; S, 6.76.
16. *Experimental details and data for* **7**: A solution of **1** (91 mg, 0.138 mmol) in $CHCl_3$ (6 ml) was dropped into a solution of **3** (440 mg,1,13 mmol) in $CHCl_3$ (10 ml) and MeOH (200 μl). The mixture was stirred for 24 h until clearing off. The solvent was removed under reduced pressure and the residue was purified by alumina column chromatography using $CHCl_3$ (elution of 2-acetamido-2-deoxy-3,4,6-tri-acetyl-β-D-glucopyranosyl isothiocyanate) and $CHCl_3$/MeOH (12:1) as eluents. 450 mg of a crude product **5** was isolated and treated without further purification. NaOMe (1 M solution in MeOH) was added to 450 mg of **5** dissolved in MeOH (40 ml) to adjust pH 8–9. The mixture was stirred for 2 h and afterwards diluted with water (30 ml) and neutralized with Dowex 50WX8. The solution was filtered and the solvent was removed under reduced pressure. The residue was purified by size exclusion chromatography (PSS HEMA 40, 10 μm/Polymer Standards Service Inc., 20 × 300 mm, H_2O/acetic acid 1:0.025, 1.5 mL/min) and lyophilized. Product **7** was isolated as tri-acetate in 37% yield as a white solid.
^{1}H NMR (400 MHz, DMSO-d_6, 60 °C): $\delta = 1.52$ (m, 4H, H-3), 1.84 (s, 12H, NH*ac*), 1.86 (s, 9H, CH_3COOH), 2.20 (t, 8H, H-5), 2.46 (m, 16H, H-1, -2), 2.63 (t, 8H, H-4), 3.18 (m, 16H, H-8, -9), 3.30–3.65 (m, 28H, H-1′, -2′, -3′, -4′, -5′, -6′), 7.45 (d, 4H, NHac), 7.78–7.95 (m, 12H, H-7, -10, -12). ^{13}C NMR (100 MHz, D_2O): $\delta = 21.46$ (C-3), 22.18 (ac-CH_3), 30.27 (C-5), 38.93 (C-9), 43.62 (C-8), 47.71 (C-1), 48.40 (C-2), 49.54 (C-4), 54.60 (C-2′), 60.78 (C-6′), 69.78 (C-4′), 74.09 (C-3′), 77.39 (C-5′), 82.88 (C-1′), 173.50 (C=O ac), 175.14 (C=O C-6), 182.90 (C=S C-11). ESI-MS: *m/z* calculated for $C_{66}H_{120}N_{20}O_{24}S_4$ ($[M+H]^+$): 1705.8; found: 1705.9. Anal. Calcd for $C_{66}H_{120}N_{20}O_{24}S_4 \cdot (CH_3COOH)_3 \cdot (H_2O)_8$ (2030.32): C, 42.59; H, 7.35; N, 13.80; S, 6.32. Found: C, 42.40; H, 7.29; N, 13.77; S, 6.26.
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