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Silver(I) complexes based on novel tripodal thioglycosides: synthesis, structure and antimicrobial activity

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Abstract—The reaction of tris(2-bromoethyl)amine hydrobromide with sugar thiols or thioacetates leads to the formation of novel carbohydrate substituted tripodal NS_3 ligands. Complexation with silver(I) ions gives stable complexes. NMR, X-ray, MS and EXAFS studies indicate their mononuclear C_3-symmetric structure. The highly water soluble complexes formed from the unprotected ligands show a wide spectrum of effective antimicrobial activities and their use lowers the cytotoxic and antiproliferative activities compared to the free silver salts.

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1. Introduction

Silver(I) complexes received attention because of their often displayed antimicrobial activity.[1–6] Furthermore, their anticancer[7–11] and antiviral[12] effects and the possible use of the radioisotope ^{111}Ag in radioimmunotherapy attracted attention.[13] Most of the antimicrobially active compounds displayed a slow release of silver ions, but stability and solubility in water are desired for their application. Carbohydrates are increasingly used to implement such properties to obtain bioactive metal complexes.[14–18] However, the chemistry of sugars together with silver(I) ions so far is limited to the detection of reducing sugars based on the reduction to silver (Tollen's reagent), the structural characterisation of a silver(I) complex of lactobionate,[19] the complexation of D-glucono-δ-lactone[20] and β-D-glucopyranosyl-thiol[21] and the study of the antiviral activity of silver(I) glycoporphyrin derivatives.[22]

Benzyl- and vinyl-substituted ethylene-bridged NS_3-open chain ligands have been reported recently to form stable Ag^I complexes in which the silver ions are not completely encapsulated and can be attacked by competing ligands to undergo transmetallation.[23] Although thioglycosides are very active intermediates—for instance in oligosaccharide synthesis where they are used as glycosyl donors especially in connection with metal salts as activators to transfer the sugar moiety to glycosyl acceptors[24]—we report herein the formation of stable silver(I) complexes derived from novel tripodal thioglycosidic ligands.

2. Results and discussion

The acetyl-protected compounds **5** and **6** were prepared by the reaction of tris(2-bromoethyl)amine hydrobromide **1**[25] with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-thiol **2** and 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-thiol **3**[26] in DMF and triethylamine. The corresponding mannose derivative **7** was synthesised in a one-pot reaction in DMF starting from 2,3,4,6-tetra-*O*-acetyl-α-D-thioacetyl-mannopyranose **4**,[27] in situ hydrolysis of the thioacetyl group by stirring with diethylamine at 0 °C and subsequent addition of triethylamine and the bromide **1**. Cleavage of the acetyl groups could not be carried out with conventional methods. Under basic conditions the removal of the formed salts from the extremely water soluble ligands **8–10** was not possible. Treatment with acids for the ester hydrolysis caused decomposition of the ligands. Only in a 1:1 mixture of water and ethanol using a hydroxyl group loaded DOWEX-resin the reaction proceeds smoothly (Scheme 1). After filtration over silica gel and evaporation the pure unprotected substances

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Keywords: Carbohydrates; Silver; *S*-Ligand; Biological activity.

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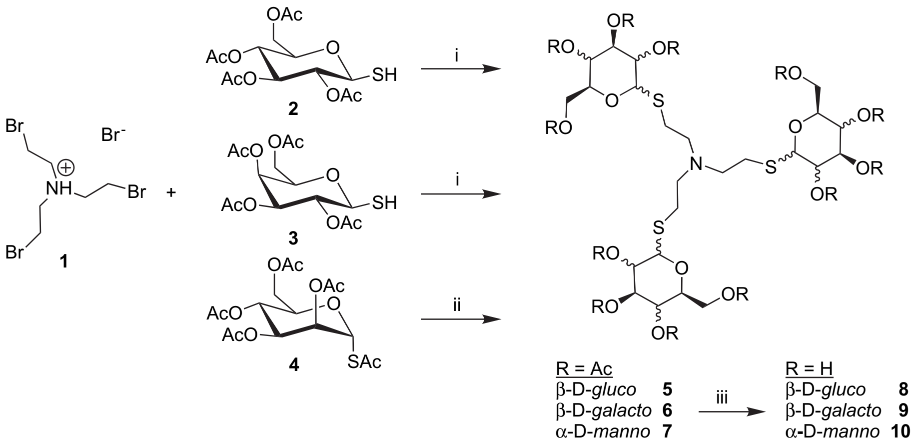


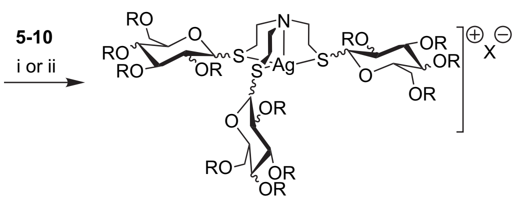
Scheme 1. Synthesis of the tripodal ligands: (i) NEt_3, DMF, 12 h, room temperature; (ii) $HNEt_2$, DMF, 0 °C, 15 min and then NEt_3, 12 h, room temperature; (iii) DOWEX-OH^-, H_2O/EtOH, 12 h, 60 °C.

8–10 could be obtained. All synthesised ligands show in solution C_3-symmetry. Only one set of signals of one 'arm' occurs in the ^1H and ^{13}C NMR spectra. Besides the signals typical for the respective sugar residue the ethylene protons show a multiplet from 2.7 to 2.8 ppm and two ^{13}C-signals around 55 ppm ($-CH_2-N-$) and 30 ppm ($-CH_2-S-$).

To determine the influence of the anions complexation of **5**–**7** was carried out in ethanol with silver(I) nitrate and silver(I) hexafluorophosphate by refluxing the mixtures for 15 min under light exclusion, **8**–**10** were stirred together with the appropriate silver salt in water overnight (Scheme 2). Degradation of the thioglycosides was not observed under these conditions and after filtration and evaporation the complexes were obtained as white powders. Only refluxing especially the water soluble complexes **17**–**22** for a longer time with an excess of silver salt resulted in the formation of dark precipitates and a mixture of side products. Also the NMR samples show the formation of a silver mirror when standing for several days. From the solid substances only the α-mannose derived compounds became yellow when stored over several weeks. ESI-MS of the compounds indicate the complete formation of the complexes. No signals for the free ligands were observed. The acetyl-protected substances **11**–**16** gave almost single peaks at *m*/*z* 1296 for the cations of the general formula $[AgL]^+$. All water soluble complexes **17**–**22** show besides the molpeak at *m*/*z* 792 the same characteristic degradation pattern under ESI conditions.

NMR measurements in acetone-d_6 or $CDCl_3$ (**11**–**16**) and D_2O (**17**–**22**) clearly indicate the C_3-symmetric structure of all complexes in solution. The ethylene protons around the silver ion are not equal anymore and show four separate signals at around 2.6 ppm, 2.9 ppm, 3.0 ppm and 3.2 ppm. The corresponding ^{13}C-signals were shifted to 35 ppm ($-CH_2-S-$) and 50 ppm ($-CH_2-N-$). The shifts and the separation of the peaks are only depending on the sugar residue and the used solvent. Almost no influence of the anion was observed so that an ionic structure of the complexes in solution can be assumed.

Recrystallisation of **11** from ethanol resulted in the formation of crystals suitable for single crystal X-ray structural analysis. Figure 1 shows the ORTEP view of one of the three molecules found in the asymmetric unit. The Ag^I ion is trigonal-pyramidally coordinated by the ligand and deflected out of the plane defined by the three sulfur atoms away from the nitrogen atom at the top of the trigonal pyramid.



		β-D-*gluco*	β-D-*galacto*	α-D-*manno*
R = OAc	X = NO_3^-	**11**	**12**	**13**
	X = PF_6^-	**14**	**15**	**16**
R = H	X = NO_3^-	**17**	**18**	**19**
	X = PF_6^-	**20**	**21**	**22**

Scheme 2. Synthesis of the silver(I) complexes: (i) $AgNO_3$ and (ii) $AgPF_6$; for R=Ac: reflux in ethanol, 15 min; for R=H: stirring in water, room temperature, overnight.

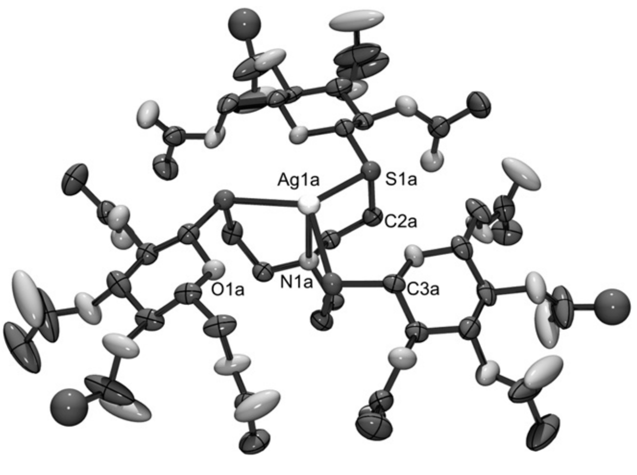


Figure 1. ORTEP view (50% probability) of one of the molecules in the structure of **11**. H-atoms and the coordinating nitrate anion are omitted for clarity. Selected distances [Å] and angles [°]: Ag1a-S1a 2.6806(13), Ag1a-N1a 2.607(7), N1a-Ag1a-S1a 77.48(3), S1a-Ag1a-S1a 115.43(2).

Additionally, in the solid state one nitrate anion (not shown in Fig. 1) is weakly coordinated to the silver ion (Ag1a-O1na 2.594(16)).

To verify the structure of the deprotected complexes EXAFS measurements have been carried out using complex **11** as a standard. The k^3-weighted EXAFS oscillations, $k^3\chi(k)$, resemble each other, suggesting a similar coordination structure of the Ag^I ions. The $k^3\chi(k)$ values were fitted with sum of two back-scattering contributions, namely, Ag–N ($k^3\chi(k)_N$) and Ag–S ($k^3\chi(k)_S$). The back-scattering amplitude and phase shift function, $F_i(k)$ and $\Phi_i(k)$, for single scattering pathways were estimated by means of ab initio self-consistent calculation using FEFF 8.2.[28] No empirical parameters are used in the analysis of EXAFS oscillation. Table 1 lists the best-fitted parameters, interatomic distances r, the coordination number N and the Debye–Waller factor σ. The parameters for **17**(BN) are close to those for **11**(BN). Therefore, **17** should have a similar coordination structure like **11**. The interatomic distances r_N and r_S for **11**(BN) are slightly shorter than that derived from X-ray crystallography. The amplitude of EXAFS oscillation of **17**(H_2O) is smaller than that for solid phase. However, the contribution ratio between Ag–N and Ag–S, i.e., N_N/N_S value, is 0.78 which is almost the same as for the solid state (0.83). The reduced EXAFS oscillation of the sample in aqueous solution may be caused by the difference in the amplitude reduction factor S_0^2. Consequently, the unprotected complex **17** has a similar coordination structure to that of the protected complex **11** and keeps this structure even in aqueous solution.

Table 1. Structural parameters derived from EXAFS analysis

Sample	Shell	N	r (Å)	dE	σ	R (%)
11(BN)[a]	Ag–N	2.9	2.684	−1.528	0.090	2.999
	Ag–S	3.9	2.600	−0.552	0.119	
17(BN)[a]	Ag–N	2.9	2.651	1.255	0.070	1.938
	Ag–S	3.5	2.560	1.886	0.105	
17(H_2O)[b]	Ag–N	1.4	2.508	1.721	0.177	0.981
	Ag–S	1.8	2.545	3.098	0.113	

[a] Boron nitrate pellets.
[b] Aqueous solution.

The antimicrobial activities of the obtained water soluble complexes **17–22** against bacteria, yeasts and fungi were examined qualitatively by agar diffusion tests (Table 2). Whereas the free ligands **8–10** show no activity the silver complexes possess a wide spectrum of effective antibacterial and antifungal activities. They inhibit the growth of Gram-positive and Gram-negative bacteria (*Bacillus subtilis*, sensitive and multiresistant (MRSA) *Staphylococcus aureus*, vancomycin resistant (VRE) *Enterococcus faecalis*, *Mycobacterium vaccae*, *Escherichia coli*, *Pseudomonas aeruginosa*), yeasts (*Candida albicans*, *Candida glabrata*) and fungi (*Penicillium notatum*, *Aspergillus fumigatus*, *Fusarium oxysporum*), but not of silver non-sensitive *Aspergillus terreus*.

Additionally compounds were evaluated by the determination of MIC values for selected organisms (Table 3). With MIC values of 15.6–62.5 μM the compounds exhibit effective activities against bacteria in the same range of those observed for water soluble silver complexes based on L-histidine and (*S*)-2-pyrrolidone-5-carboxylic acid,[6] but moderate compared to 0.05–0.8 μM for ciprofloxacin under the same conditions. For Gram-negative bacteria a two times higher activity of the complexes is observed compared to the silver salts.

The experiments show that the effects are independent from the used sugar residue or anion. Three mechanisms for the inhibition by aqueous silver(I) ions have been proposed, interference with electron transport, binding to DNA and interaction with the cell membrane.[29] Consistently with other studies the mechanism of the antimicrobial action has to be related to the replacement of the ligands in the silver(I)

Table 3. Antibacterial activities evaluated by minimal inhibition concentration of selected compounds[a]

	17	19	20	23	$AgNO_3$	$AgPF_6$
E. coli	15.6	15.6	15.6	15.6	31.3	46.9
P. aeruginosa	15.6	23.4	15.6	15.6	23.4	31.3
E. faecalis	62.5	62.5	62.5	62.5	62.5	62.5
M. vaccae	15.6	15.6	15.6	15.6	31.3	46.9

[a] MIC values in μmol/l.

Table 2. Qualitative determination of antimicrobial activity by agar diffusion tests (diameter of inhibition zones in mm)

	8	**9**	**10**	**17**	**18**	**19**	**20**	**21**	**22**	$AgNO_3$	$AgPF_6$	Ciprofloxacin	Amphoter. B[a]	Nystatin
B. subtilis	0	0	0	14	13	14	14	13.5	13.5	12.5	11.5	28	—	—
S. aureus	0	0	0	14p	14p	14.5p	15p	14.5p	14.5	13p	12p	19	—	—
S. aureus MRSA	0	0	0	14.5P	14P	14.5P	14.5P	14.5P	14P	12.5P	12P	0	—	—
E. faecalis VRE	0	0	0	12	12	13	13	13	13	12	12	15	—	—
M. vaccae	0	0	0	14	13.5	14	14.5	14	14	12.5	12	21	—	—
E. coli	0	0	0	16	15	16	16.5	15	15	13	12.5	21/29p	—	—
P. aeruginosa	0	0	0	13.5p	14p	14p	14P	13p	14p	12p	11.5p	24.5	—	—
S. salmomicolor	0	0	0	17A	17	18P	17A	18A	16A	12P	12P	—	20P	—
C. albicans	0	0	0	17p	16.5p	15p	16p	16p	15	11	10	—	20.5	—
P. notatum	0	0	0	25	24	24	25	25	24	15	14.5	—	16/19p	—
A. fumigatus	0	0	0	19p	15P	19p	15p	18P	17P	12/14p	12A	—	—	22[b]
F. oxysporum	0	0	0	19P	15P	17P	15P	18P	16P	12p	12P	—	—	18[c]
A. terreus	0	0	0	0	0	0	0	0	0	11p	0	—	—	19[d]

p—Colonies in the inhibition zone; P—many colonies in the inhibition zone; A—indication of inhibition zone.
[a] 10 μg/ml.
[b] 50 μg/ml.
[c] 200 μg/ml.
[d] 100 μg/ml.

complexes by proteins of the microbes as sulfur donor ligands.[30,31] As a key factor the strength of binding of the Ag^I ions by N/S/O donors has been determined. Generally compounds with weaker bonds such as Ag–N and Ag–O bonds show a wider spectrum of antimicrobial activities. In our case the silver is bound only weakly to the thio-ether functions, which allow further replacement with biological ligands.

The antiproliferative and cytotoxic activities of the ligands **8–10** and the complexes **17–22** were determined using the cell lines K-562 (human chronic myeloid leukaemia) and L-929 (mouse fibroblast) for antiproliferative effects and HeLa (human cervix carcinom) for cytotoxic effects (Table 4). The results show that the carbohydrate based complexes exhibit only half of the cytotoxic and antiproliferative activities than the appropriate silver salt. No sugar specific action has been observed.

Table 4. Antiproliferative and cytotoxic activities[a]

Compound	Antiproliferative effect		Cytotoxicity
	L-929[b]	K-562[b]	HeLa[c]
17	5.7	2.2	43.1
18	5.7	3.0	45.4
19	5.7	2.6	44.2
20	7.7	3.6	54.3
21	10.0	2.9	79.5
22	8.6	3.0	68.0
$AgNO_3$	2.4	1.2	24.1
$AgPF_6$	5.9	2.4	30.5

[a] Ligands **8–10** show no effects up to 292 mmol/l.

[b] GI_{50} in μmol/l.

[c] CC_{50} in μmol/l.

3. Conclusion

In conclusion, we could develop highly practical synthetic methods to obtain new sugar substituted tripodal NS_3 ligands. Their complexation with silver(I) ions leads to stable C_3-symmetric complexes with defined mononuclear structure. The solubility of the Ag^I complexes is influenced by the sugar moieties and their residues. Independently from the sugar residue the water soluble silver compounds show a wide range of effective antimicrobial activities against bacteria, yeast and fungi. The use of the carbohydrate based substances enhances the antimicrobial activity and reduces the cytotoxicity and antiproliferative activities compared to the free silver(I) salts.

4. Experimental

4.1. General

All reagents and solvents were purchased from commercial sources and used as received. NMR spectra were measured on a JEOL JMTC-400/54/SS, ESI-MS were carried out on a JEOL JMS-T100LC, elemental analyses on a Perkin–Elmer PE2400 Series II CHNS/O Analyzer (Nara Institute of Science and Technology).

4.1.1. Tris[2-(2,3,4,6-tetra-*O*-acetyl-β-D-thio-glucopyranosyl)ethyl]amine (5). 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl-thiol (8.79 g, 24.1 mmol) was dissolved in 50 ml DMF and 4.7 ml triethylamine were added. At 0 °C under stirring 3.33 g (8.02 mmol) of tris(2-bromoethyl)amine hydrobromide were added and the solution was allowed to warm up to room temperature and stirred overnight. DMF was evaporated, the residue extracted with ethyl acetate and water. After drying the organic phase with sodium sulfate, ethyl acetate was removed and the raw product was recrystallised from ethanol to yield 8.70 g (91%) colourless needles. ^1H NMR (400 MHz, $CDCl_3$): 4.55 (d, 3H, H-1, $J_{1,2}$ 10.0 Hz), 5.24 (t, 3H, H-2, $J_{2,3}$ 9.5 Hz), 5.08 (t, 3H, H-3, $J_{3,4}$ 9.8 Hz), 5.03 (t, 3H, H-4, $J_{4,5}$ 9.8 Hz), 3.77 (ddd, 3H, H-5), 4.26 (dd, 3H, H-6, $J_{5,6}$ 4.6 Hz, $J_{6,6'}$ 12.4 Hz), 4.14 (dd, 3H, H-6′, $J_{5,6'}$ 2.2 Hz), 2.71–2.80 (m, 12H, 6×CH_2), 2.01, 2.03, 2.07, 2.09 (4s, 36H, CH_3-acetyl). ^{13}C NMR ($CDCl_3$): 170.3, 169.9, 169.2 (*C*O-acetyl), 83.07 (*C*1), 75.77 (*C*2), 73.71 (*C*3), 69.66 (*C*4), 68.20 (*C*5), 62.04 (*C*6), 53.70 (N–*C*H_2–), 27.58 (S–*C*H_2–), 20.7 (*C*H_3-acetyl). ESI-MS *m*/*z* (%): 1210.44 (100) $[M+Na]^+$. Anal. Calcd for $C_{48}H_{69}NO_{27}S_3$: C, 48.52; H, 5.85; N, 1.18. Found: C, 48.45; H, 5.86; N, 1.25%.

4.1.2. Tris[2-(2,3,4,6-tetra-*O*-acetyl-β-D-thio-galactopyranosyl)ethyl]amine (6). 2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl-thiol (3.432 g, 9.426 mmol) was reacted with 1.303 g (3.142 mmol) tris(2-bromoethyl)amine hydrobromide and 3.808 g triethylamine in DMF analogous to **5**. After extraction with ethyl acetate and water the organic phase was dried over sodium sulfate, filtered and ethyl acetate was evaporated. Chromatographic cleaning over silica gel with ethyl acetate/hexane 2:1 (R_f 0.5) yields 1.45 g (40%) of colourless solid. ^1H NMR (400 MHz, $CDCl_3$): 4.54 (d, 3H, H-1, $J_{1,2}$ 9.8 Hz), 5.21 (t, 3H, H-2, $J_{2,3}$ 10.0 Hz), 5.07 (dd, 3H, H-3, $J_{3,4}$ 3.0 Hz), 5.44 (d, 3H, H-4), 3.99 (t, 3H, H-5, $J_{5,6}$ 6.7 Hz), 4.13 (m, 6H, H-6, H-6′), 2.77 (m, 12H, 6×CH_2), 1.99, 2.05, 2.07, 2.16 (4s, 36H, CH_3-acetyl). ^{13}C NMR ($CDCl_3$): 170.1, 169.9, 169.8, 169.4 (*C*O-acetyl), 83.73 (*C*1), 74.35 (*C*2), 71.72 (*C*3), 67.23 (*C*4), 67.31 (*C*5), 61.35 (*C*6), 54.10 (N–*C*H_2–), 27.89 (S–*C*H_2–), 20.8, 20.7 (*C*H_3-acetyl). ESI-MS *m*/*z* (%): 1210.37 (100) $[M+Na]^+$. Anal. Calcd for $C_{48}H_{69}NO_{27}S_3$: C, 48.52; H, 5.85; N, 1.18. Found: C, 48.11; H, 5.87; N, 1.24%.

4.1.3. Tris[2-(2,3,4,6-tetra-*O*-acetyl-α-D-thio-mannopyranosyl)ethyl]amine (7). 2,3,4,6-Tetra-*O*-acetyl-α-D-thioacetyl-mannose (1 g, 2.46 mmol) was dissolved in 20 ml DMF and 180 mg (2.46 mmol) diethylamine were added drop wise at 0 °C. After stirring for 15 min an excess of triethylamine (1 ml) and 343 mg (0.82 mmol) tris(2-bromoethyl)amine hydrobromide were added and the solution was stirred overnight at room temperature. DMF was evaporated and the residue extracted with ethyl acetate and water. After drying the organic phase with sodium sulfate, ethyl acetate was evaporated and the raw product cleaned by column chromatography. ^1H NMR (400 MHz, $CDCl_3$): 5.33–5.28 (m, 9H, H-1, H-2, H-4), 5.26 (dd, 3H, H-3, $J_{3,4}$ 3.2 Hz, $J_{2,3}$ 10.0 Hz), 4.35–4.29 (m, 6H, H-5, H-6, $J_{5,6}$ 5.0 Hz, $J_{5,6'}$ 1.9 Hz), 4.11 (dd, 3H, H-6′, $J_{6,6'}$ 12.0 Hz), 2.80–2.62 (m, 12H, 6×CH_2), 1.99, 2.05, 2.10, 2.16 (4s, 36H, CH_3-acetyl). ^{13}C NMR ($CDCl_3$): 170.2, 169.6, 169.4 (*C*O-acetyl), 82.57 (*C*1), 69.30 (*C*2), 71.03 (*C*3), 69.12 (*C*4), 66.32 (*C*5), 62.40 (*C*6), 53.83 (N–*C*H_2–), 29.28 (S–*C*H_2–), 20.95, 20.85, 20.76, 20.68 (*C*H_3-acetyl). ESI-MS *m*/*z* (%): 1210.35 (100)

[M+Na]$^+$. Anal. Calcd for $C_{48}H_{69}NO_{27}S_3$: C, 48.52; H, 5.85; N, 1.18. Found: C, 48.33; H, 5.93; N, 1.25%.

4.1.4. Tris[2-(β-D-thio-glucopyranosyl)ethyl]amine (8). Tris[2-(2,3,4,6-tetra-*O*-acetyl-β-D-thio-glucopyranosyl)ethyl]amine **5** (870 mg, 0.73 mmol) and 4.9 g of OH$^-$-loaded DOWEX were dissolved in 45 ml of a mixture of water and ethanol 1:1. The reaction mixture was stirred at 60 °C until no starting material could be detected by TLC. Filtration and evaporation gave the raw product as colourless foam. It was cleaned by filtration over silica gel with methanol yielding 332 mg (60%). ^{1}H NMR (400 MHz, D_2O, DSS): 4.53 (d, 3H, H-1, $J_{1,2}$ 9.8 Hz), 3.31 (t, 3H, H-2, $J_{2,3}$ 8.8 Hz), 3.39 (t, 3H, H-3, $J_{3,4}$ 8.8 Hz), 3.44–3.50 (m, 6H, H-4, H-5), 3.89 (d, 3H, H-6, $J_{6,6'}$ 12.2 Hz), 3.69 (dd, 3H, H-6′, $J_{5,6'}$ 5.6 Hz), 2.86 (m, 12H, 6×CH_2). ^{13}C NMR (D_2O, DSS): 87.98 (*C*1), 82.44 (*C*2), 79.65 (*C*3), 74.77 (*C*4), 72.06 (*C*5), 63.45 (*C*6), 56.02 (N–*C*H$_2$–), 29.15 (S–*C*H$_2$–). ESI-MS *m*/*z* (%): 706.25 (100) [M+Na]$^+$. Anal. Calcd for $C_{24}H_{45}NO_{15}S_3\cdot 3H_2O$: C, 39.07; H, 6.97; N, 1.90. Found: C, 38.99; H, 7.00; N, 1.98%.

4.1.5. Tris[2-(β-D-thio-galactopyranosyl)ethyl]amine (9). Similar to **8**. ^{1}H NMR (400 MHz, D_2O, DSS): 4.47 (d, 3H, H-1, $J_{1,2}$ 9.5 Hz), 3.55 (t, 3H, H-2, $J_{2,3}$ 9.8 Hz), 3.64 (dd, 3H, H-3, $J_{3,4}$ 3.4 Hz), 3.96 (d, 3H, H-4), 3.68–3.78 (m, 9H, H-5, H-6, H-6′), 2.86 (m, 12H, 6×CH_2). ^{13}C NMR (D_2O, DSS): 88.51 (*C*1), 81.52 (*C*2), 76.39 (*C*3), 71.33 (*C*4), 72.12 (*C*5), 63.65 (*C*6), 55.98 (N–*C*H$_2$–), 29.18 (S–*C*H$_2$–). ESI-MS *m*/*z* (%): 706.25 (100) [M+Na]$^+$. Anal. Calcd for $C_{24}H_{45}NO_{15}S_3$: C, 42.15; H, 6.63; N, 2.05. Found: C, 39.85; H, 6.77; N, 2.11%.

4.1.6. Tris[2-(α-D-thio-mannopyranosyl)ethyl]amine (10). Similar to **8**. ^{1}H NMR (400 MHz, D_2O, DSS): 5.31 (s, 3H, H-1), 4.04 (d, 3H, H-2, $J_{2,3}$ 3.0 Hz), 3.77 (m, 6H, H-3, H-6′), 3.65 (t, 3H, H-4, $J_{3,4}$ 9.6 Hz, $J_{4,5}$ 9.6 Hz), 3.99 (m, 3H, H-5, $J_{5,6'}$ 6.1 Hz), 3.88 (d, 3H, H-6, $J_{6,6'}$ 12.2 Hz), 2.76–2.92 (m, 12H, 6×CH_2). ^{13}C NMR (D_2O, DSS): 87.51 (*C*1), 75.63 (*C*2), 74.19 (*C*3), 73.55 (*C*4), 69.61 (*C*5), 63.39 (*C*6), 55.25 (N–*C*H$_2$–), 30.16 (S–*C*H$_2$–). ESI-MS *m*/*z* (%): 706.25 (100) [M+Na]$^+$. Anal. Calcd for $C_{24}H_{45}NO_{15}S_3$: C, 42.15; H, 6.63; N, 2.05. Found: C, 40.94; H, 6.81; N, 2.12%.

4.1.7. Tris[2-(2,3,4,6-tetra-*O*-acetyl-β-D-thio-glucopyranosyl)ethyl]-amine-silver(I)-nitrate (11). Compound **5** (300 mg, 0.252 mmol) in 10 ml ethanol was mixed with 42.6 mg (0.252 mmol) of silver(I) nitrate and refluxed for 15 min. After filtration the ethanol was evaporated and the residue recrystallised from ethanol yielding 280 mg (82%) of colourless crystals suitable for single crystal X-ray analysis. ^{1}H NMR (400 MHz, $CDCl_3$): 4.63 (d, 3H, H-1, $J_{1,2}$ 9.8 Hz), 5.24 (t, 3H, H-2, $J_{2,3}$ 9.3 Hz), 5.17 (t, 3H, H-3, $J_{3,4}$ 9.8 Hz), 5.09 (t, 3H, H-4, $J_{4,5}$ 9.8 Hz), 3.86 (d, 3H, H-5), 4.02 (d, 3H, H-6, $J_{6,6'}$ 12.0 Hz), 4.50 (dd, 3H, H-6′, $J_{5,6'}$ 2.2 Hz), 3.20 (d, 3H, C*H*, *J* 12.9 Hz), 2.90 (t, 3H, C*H*, *J* 12.3 Hz), 2.82 (t, 3H, C*H*, *J* 12.5 Hz), 2.44 (d, 3H, C*H*, *J* 12.9 Hz), 2.06, 2.05, 2.00, 1.98 (4s, 36H, CH_3-acetyl). ^{13}C NMR ($CDCl_3$): 169.8, 169.3 (*C*O-acetyl), 82.19 (*C*1), 77.10 (*C*2), 73.87 (*C*3), 67.74 (*C*4), 67.02 (*C*5), 60.67 (*C*6), 51.70 (N–*C*H$_2$–), 26.8 (S–*C*H$_2$–), 20.6, 20.5 (*C*H$_3$-acetyl). ESI-MS *m*/*z* (%): 1296.34 (100) [M]$^+$. Anal. Calcd for $C_{48}H_{69}AgN_2O_{30}S_3$: C, 42.45; H, 5.12; N, 2.06. Found: C, 42.18; H, 5.00; N, 2.19%.

4.1.8. Tris[2-(2,3,4,6-tetra-*O*-acetyl-β-D-thio-galactopyranosyl)ethyl]-amine-silver(I)-nitrate (12). Compound **6** (100 mg, 0.084 mmol) and 14.2 mg $AgNO_3$ were dissolved in 10 ml ethanol and the solution was heated up for 15 min. It was filtered and the solvent was evaporated to give a colourless powder. ^{1}H NMR (400 MHz, $CDCl_3$): 4.68 (d, 3H, H-1, $J_{1,2}$ 9.8 Hz), 5.22 (t, 3H, H-2, $J_{2,3}$ 10.0 Hz), 5.08 (dd, 3H, H-3, $J_{3,4}$ 3.3 Hz), 5.45 (d, 3H, H-4), 3.95 (m, 3H, H-5), 4.18–4.23 (m, 6H, H-6, H-6′), 2.14–3.12 (m, 12H, 6×CH_2), 1.98, 2.04, 2.11, 2.18 (4s, 36H, CH_3-acetyl). ^{13}C NMR ($CDCl_3$): 169.9, 169.4 (*C*O-acetyl), 82.24 (*C*1), 75.09 (*C*2), 71.44 (*C*3), 66.98 (*C*4), 66.49 (*C*5), 60.60 (*C*6), 51.00 (N–*C*H$_2$–), 27.40 (S–*C*H$_2$–), 20.7 (*C*H$_3$-acetyl). ESI-MS *m*/*z* (%): 1296.33 (100) [M]$^+$. Anal. Calcd for $C_{48}H_{69}AgN_2O_{30}S_3$: C, 42.45; H, 5.12; N, 2.06. Found: C, 40.82; H, 5.27; N, 2.08%.

4.1.9. Tris[2-(2,3,4,6-tetra-*O*-acetyl-α-D-thio-mannopyranosyl)ethyl]-amine-silver(I)-nitrate (13). Similar to **12**. ^{1}H NMR (400 MHz, $CDCl_3$): 5.46 (s, 3H, H-1), 5.29–5.36 (m, 9H, H-2, H-3, H-4), 4.20–4.35 (m, 6H, H-5, H-6), 4.11 (dd, 3H, H-6′, $J_{5,6'}$ 1.7 Hz, $J_{6,6'}$ 10.0 Hz), 3.06–2.74 (m, 12H, 6×CH_2), 2.19, 2.10, 2.06, 2.03 (4s, 36H, CH_3-acetyl). ^{13}C NMR ($CDCl_3$): 170.2, 170.0, 169.6, 169.4 (*C*O-acetyl), 81.34 (*C*1), 71.31 (*C*2), 70.40 (*C*3), 68.86 (*C*4), 65.91 (*C*5), 62.01 (*C*6), 51.05 (N–*C*H$_2$–), 29.39 (S–*C*H$_2$–), 20.99, 20.76 (*C*H$_3$-acetyl). ESI-MS *m*/*z* (%): 1296.23 (100) [M]$^+$. Anal. Calcd for $C_{48}H_{69}AgN_2O_{30}S_3$: C, 42.45; H, 5.12; N, 2.06. Found: C, 42.07; H, 5.18; N, 2.10%.

4.1.10. Tris[2-(2,3,4,6-tetra-*O*-acetyl-β-D-thio-glucopyranosyl)ethyl]-amine-silver(I)-hexafluorophosphate (14). Similar to **11**, with 100 mg (0.084 mmol) of **5** and 21.2 mg (0.084 mmol) of $AgPF_6$. Compound **14** directly precipitates from solution to yield 96 mg colourless solid. ^{1}H NMR (400 MHz, acetone-d_6): 4.63 (m, 6H, H-1, H-3), 5.31 (t, 3H, H-2, $J_{2,3}$ 9.3 Hz), 5.02 (t, 3H, H-4, $J_{4,5}$ 10.0 Hz), 4.08 (ddd, 3H, H-5, $J_{5,6}$ 5.4 Hz, $J_{5,6'}$ 2.2 Hz), 4.23 (dd, 3H, H-6, $J_{6,6'}$ 12.4 Hz), 4.14 (dd, 3H, H-6′), 3.37 (m, 3H, C*H*), 3.18 (m, 3H, C*H*), 2.99 (m, 3H, C*H*), 2.84 (m, 3H, C*H*), 2.13, 2.08, 2.03, 1.99 (4s, 36H, CH_3-acetyl). ^{13}C NMR (acetone-d_6): 170.4, 169.9 (*C*O-acetyl), 84.34 (*C*1), 77.10 (*C*2), 73.87 (*C*3), 70.72 (*C*4), 68.51 (*C*5), 62.77 (*C*6), 51.91 (N–*C*H$_2$–), 31.28 (S–*C*H$_2$–), 20.5 (*C*H$_3$-acetyl). ESI-MS *m*/*z* (%): 1296.19 (100) [M]$^+$. Anal. Calcd for $C_{48}H_{69}AgF_6NO_{27}PS_3$: C, 40.01; H, 4.83; N, 0.97. Found: C, 39.44; H, 5.01; N, 1.11%.

4.1.11. Tris[2-(2,3,4,6-tetra-*O*-acetyl-β-D-thio-galactopyranosyl)ethyl]-amine-silver(I)-hexafluorophosphate (15). Similar to **11**. ^{1}H NMR (400 MHz, $CDCl_3$): 4.75 (d, 3H, H-1, $J_{1,2}$ 9.8 Hz), 5.14 (t, 3H, H-2, $J_{2,3}$ 10.0 Hz), 5.07 (dd, 3H, H-3, $J_{3,4}$ 2.9 Hz), 5.44 (d, 3H, H-4), 3.90 (m, 3H, H-5), 4.15–4.20 (m, 6H, H-6, H-6′), 3.20 (t, 3H, C*H*, *J* 13.0 Hz), 3.05 (d, 3H, C*H*, *J* 14.4 Hz), 2.90 (t, 3H, C*H*, *J* 12.3 Hz), 2.69 (d, 3H, C*H*, *J* 12.9 Hz), 1.99, 2.05, 2.08, 2.28 (4s, 36H, CH_3-acetyl). ^{13}C NMR ($CDCl_3$): 170.2, 169.6, 169.8, 169.4 (*C*O-acetyl), 86.48 (*C*1), 75.18 (*C*2), 71.25 (*C*3), 67.06 (*C*4, *C*5), 61.73 (*C*6), 50.57 (N–*C*H$_2$–), 34.48 (S–*C*H$_2$–), 20.7 (*C*H$_3$-acetyl). ESI-MS *m*/*z* (%): 1296.22 (100) [M]$^+$. Anal. Calcd for $C_{48}H_{69}AgF_6NO_{27}PS_3$: C, 40.01; H, 4.83; N, 0.97. Found: C, 40.69; H, 4.88; N, 0.98%.

4.1.12. Tris[2-(2,3,4,6-tetra-*O*-acetyl-α-D-thio-mannopyranosyl)ethyl]-amine-silver(I)-hexafluorophosphate (16). Ligand **7** (395 mg, 0.333 mmol) and 84.1 mg (0.333 mmol) of $AgPF_6$ were dissolved in 30 ml ethanol by refluxing the mixture for 15 min. The solution was allowed to cool to room temperature. Overnight crystals were formed. They were collected and recrystallised from ethanol. ^{1}H NMR (400 MHz, $CDCl_3$): 5.40–5.36 (m, 9H, H-1, H-2, H-4), 5.29 (dd, 3H, H-3, $J_{3,4}$ 2.9 Hz, $J_{2,3}$ 10.0 Hz), 4.31–4.23 (m, 6H, H-5, H-6), 4.11 (dd, 3H, H-6′, $J_{6,6'}$ 10.0 Hz), 3.12–2.99 (m, 6H, 3×CH_2), 2.83 (s, 6H, 3×CH_2), 2.21, 2.11, 2.06 (3s, 36H, CH_3-acetyl). ^{13}C NMR ($CDCl_3$): 170.3, 169.2 (*C*O-acetyl), 81.34 (*C*1), 70.85 (*C*2), 71.28 (*C*3), 69.20 (*C*4), 65.49 (*C*5), 61.89 (*C*6), 49.27 (N–*C*H_2–), 30.50 (S–*C*H_2–), 20.91, 20.83, 20.73 (*C*H_3-acetyl). ESI-MS *m*/*z* (%): 1296.23 (100) $[M]^+$. Anal. Calcd for $C_{48}H_{69}AgF_6NO_{27}PS_3$: C, 40.01; H, 4.83; N, 0.97. Found: C, 39.49; H, 4.89; N, 1.07%.

4.1.13. Tris[2-(β-D-thio-glucopyranosyl)ethyl]-amine-silver(I)-nitrate (17). Compound **8** (277 mg, 0.405 mmol) and 68.5 mg (0.405 mmol) $AgNO_3$ were dissolved in water (5 ml) and the solution was stirred overnight. After filtration the solvent was evaporated. By diffusion of acetone into a aqueous solution of the raw product 310 mg (89%) of colourless needles were obtained. ^{1}H NMR (400 MHz, D_2O, DSS): 4.71 (d, 3H, H-1, $J_{1,2}$ 9.8 Hz), 3.31 (t, 3H, H-2, $J_{2,3}$ 9.3 Hz), 3.40 (t, 3H, H-3, $J_{3,4}$ 9.5 Hz), 3.47–3.52 (m, 6H, H-4, H-5), 3.96 (d, 3H, H-6, $J_{6,6'}$ 12.2 Hz), 3.72 (dd, 3H, H-6′, $J_{5,6'}$ 6.1 Hz), 3.25 (d, 3H, C*H*, *J* 12.2 Hz), 3.06 (d, 3H, C*H*, *J* 15.1 Hz), 2.84–2.99 (m, 3H, C*H*), 2.61 (d, 3H, C*H*, *J* 13.7 Hz). ^{13}C NMR (D_2O, DSS): 88.88 (*C*1), 82.63 (*C*2), 79.51 (*C*3), 75.38 (*C*4), 71.85 (*C*5), 63.53 (*C*6), 53.30 (N–*C*H_2–), 34.57 (S–*C*H_2–). ESI-MS *m*/*z* (%): 792.16 (100) $[M]^+$, 630.09 (60) $[M-C_6H_{10}O_5]^+$, 468.03 (70) $[M-2(C_6H_{10}O_5)]^+$, 305.97 (95) $[M-3(C_6H_{10}O_5)]^+$. Anal. Calcd for $C_{24}H_{45}AgN_2O_{18}S_3$: C, 33.77; H, 5.31; N, 3.28. Found: C, 32.90; H, 5.68; N, 3.07%.

4.1.14. Tris[2-(β-D-thio-galactopyranosyl)ethyl]-amine-silver(I)-nitrate (18). Similar to **17**. ^{1}H NMR (400 MHz, D_2O, DSS): 4.66 (d, 3H, H-1, $J_{1,2}$ 10.0 Hz), 3.54 (t, 3H, H-2, $J_{2,3}$ 9.5 Hz), 3.80–3.66 (m, 12H, H-3, H-5, 2×H-6), 3.99 (s, 3H, H-4), 3.22 (m, 3H, CH_2), 3.06 (d, 3H, CH_2), 2.90 (m, 9H, 3×CH_2). ^{13}C NMR (D_2O, DSS): 89.70 (*C*1), 81.59 (*C*2), 76.09 (*C*3), 71.30 (*C*4), 72.43 (*C*5), 63.86 (*C*6), 55.98 (N–*C*H_2–), 29.18 (S–*C*H_2–). ESI-MS *m*/*z* (%): 792.19 (100) $[M]^+$, 630.12 (40) $[M-C_6H_{10}O_5]^+$, 468.05 (40) $[M-2(C_6H_{10}O_5)]^+$, 305.98 (50) $[M-3(C_6H_{10}O_5)]^+$. Anal. Calcd for $C_{24}H_{45}AgN_2O_{18}S_3$: C, 33.77; H, 5.31; N, 3.28. Found: C, 33.33; H, 5.74; N, 3.07%.

4.1.15. Tris[2-(α-D-thio-mannopyranosyl)ethyl]-amine-silver(I)-nitrate (19). Similar to **17**. ^{1}H NMR (400 MHz, D_2O, DSS): 5.35 (s, 3H, H-1), 4.14 (s, 3H, H-2), 3.85 (dd, 3H, H-3, $J_{2,3}$ 3.2 Hz, $J_{3,4}$ 9.5 Hz), 3.72 (t, 3H, H-4, $J_{3,4}$ 9.6 Hz, $J_{4,5}$ 9.6 Hz), 3.97 (t, 3H, H-5, $J_{5,6'}$ 7.3 Hz), 3.90 (d, 3H, H-6, $J_{6,6'}$ 12.4 Hz), 3.78 (dd, 3H, H-6′), 2.76–3.08 (m, 12H, 6×CH_2). ^{13}C NMR (D_2O, DSS): 85.15 (*C*1), 76.77 (*C*2), 73.72 (*C*3), 73.37 (*C*4), 69.33 (*C*5), 63.21 (*C*6), 50.80 (N–*C*H_2–), 30.96 (S–*C*H_2–). ESI-MS *m*/*z* (%): 792.20 (70) $[M]^+$, 630.13 (30) $[M-C_6H_{10}O_5]^+$, 468.06 (50) $[M-2(C_6H_{10}O_5)]^+$, 305.98 (100) $[M-3(C_6H_{10}O_5)]^+$. Anal. Calcd for $C_{24}H_{45}AgN_2O_{18}S_3$: C, 33.77; H, 5.31; N, 3.28. Found: C, 31.46; H, 5.55; N, 3.31%.

4.1.16. Tris[2-(β-D-thio-glucopyranosyl)ethyl]-amine-silver(I)-hexafluorophosphate (20). Compound **8** (313 mg, 0.458 mmol) together with 115.7 mg (0.458 mmol) $AgPF_6$ was stirred in 20 ml water at room temperature overnight. The solution was filtered and solvent evaporated to give 385 mg (90%) white solid. ^{1}H NMR (400 MHz, D_2O, DSS): 4.71 (d, 3H, H-1, $J_{1,2}$ 9.8 Hz), 3.31 (t, 3H, H-2, $J_{2,3}$ 9.3 Hz), 3.40 (t, 3H, H-3, $J_{3,4}$ 9.5 Hz), 3.47–3.55 (m, 6H, H-4, H-5), 3.96 (d, 3H, H-6, $J_{6,6'}$ 12.0 Hz), 3.72 (dd, 3H, H-6′, $J_{5,6'}$ 6.1 Hz), 3.25 (d, 3H, C*H*, *J* 12.2 Hz), 3.06 (d, 3H, C*H*, *J* 15.1 Hz), 2.88–2.99 (m, 3H, C*H*), 2.60 (d, 3H, C*H*, *J* 13.2 Hz). ^{13}C NMR (D_2O, DSS): 88.98 (*C*1), 82.65 (*C*2), 79.53 (*C*3), 75.47 (*C*4), 71.87 (*C*5), 63.55 (*C*6), 53.28 (N–*C*H_2–), 34.66 (S–*C*H_2–). ESI-MS *m*/*z* (%): 792.17 (100) $[M]^+$, 630.10 (60) $[M-C_6H_{10}O_5]^+$, 468.04 (70) $[M-2(C_6H_{10}O_5)]^+$, 305.97 (95) $[M-3(C_6H_{10}O_5)]^+$. Anal. Calcd for $C_{24}H_{45}AgF_6NO_{15}PS_3$: C, 30.78; H, 4.84; N, 1.50. Found: C, 29.02; H, 5.13; N, 1.42%.

4.1.17. Tris[2-(β-D-thio-galactopyranosyl)ethyl]-amine-silver(I)-hexafluorophosphate (21). Similar to **20**. ^{1}H NMR (400 MHz, D_2O, DSS): 4.68 (d, 3H, H-1, $J_{1,2}$ 9.8 Hz), 3.53 (t, 3H, H-2, $J_{2,3}$ 9.5 Hz), 3.84–3.74 (m, 9H, H-5, 2×H-6), 3.67 (dd, 3H, H-3, $J_{3,4}$ 2.3 Hz), 3.99 (d, 3H, H-4), 3.26 (t, 3H, C*H*, *J* 12.5 Hz), 3.06 (d, 3H, C*H*, *J* 14.9 Hz), 2.93 (t, 6H, C*H*, *J* 16.0 Hz), 2.61 (d, 3H, C*H*, *J* 13.2 Hz). ^{13}C NMR (D_2O, DSS): 89.87 (*C*1), 81.61 (*C*2), 76.06 (*C*3), 72.47 (*C*4), 71.31 (*C*5), 63.92 (*C*6), 53.36 (N–*C*H_2–), 35.02 (S–*C*H_2–). ESI-MS *m*/*z* (%): 792.20 (90) $[M]^+$, 630.13 (70) $[M-C_6H_{10}O_5]^+$, 468.06 (80) $[M-2(C_6H_{10}O_5)]^+$, 305.98 (100) $[M-3(C_6H_{10}O_5)]^+$. Anal. Calcd for $C_{24}H_{45}AgF_6NO_{15}PS_3$: C, 30.78; H, 4.84; N, 1.50. Found: C, 29.28; H, 4.97; N, 1.38%.

4.1.18. Tris[2-(α-D-thio-mannopyranosyl)ethyl]-amine-silver(I)-hexafluorophosphate (22). Similar to **20**. ^{1}H NMR (400 MHz, D_2O, DSS): 5.35 (s, 3H, H-1), 4.14 (s, 3H, H-2), 3.85 (dd, 3H, H-3, $J_{2,3}$ 3.2 Hz, $J_{3,4}$ 9.5 Hz), 3.72 (t, 3H, H-4, $J_{3,4}$ 9.6 Hz, $J_{4,5}$ 9.6 Hz), 3.97 (t, 3H, H-5, $J_{5,6'}$ 6.6 Hz), 3.90 (d, 3H, H-6, $J_{6,6'}$ 12.0 Hz), 3.78 (dd, 3H, H-6′), 2.71–3.08 (m, 12H, 6×CH_2). ^{13}C NMR (D_2O, DSS): 85.12 (*C*1), 76.77 (*C*2), 73.73 (*C*3), 73.38 (*C*4), 69.33 (*C*5), 63.21 (*C*6), 50.73 (N–*C*H_2–), 30.93 (S–*C*H_2–). ESI-MS *m*/*z* (%): 792.20 (80) $[M]^+$, 630.13 (20) $[M-C_6H_{10}O_5]^+$, 468.06 (40) $[M-2(C_6H_{10}O_5)]^+$, 305.98 (100) $[M-3(C_6H_{10}O_5)]^+$. Anal. Calcd for $C_{24}H_{45}AgF_6NO_{15}PS_3$: C, 30.78; H, 4.84; N, 1.50. Found: C, 28.76; H, 4.99; N, 1.39%.

4.2. Crystal structure of 11

The intensity data for the compound were collected on a Nonius KappaCCD diffractometer, using graphite-monochromated Mo Kα radiation. Data were corrected for Lorentz and polarisation effects, but not for absorption effects.[32,33] The structures were solved by direct methods (SHELXS)[34] and refined by full-matrix least squares techniques against Fo^2 (SHELXL-97).[35] The hydrogen atoms were included at calculated positions with fixed thermal parameters. All non-hydrogen atoms of the cations were refined anisotropically.[35] ORTEP and POV-ray were used for structural representation.

$[C_{48}H_{69}AgNO_{27}S_3]^+$ NO_3^-, Mr=1358.10 g mol^{-1}, colourless prism, size 0.04×0.04×0.03 mm^3, trigonal, space group *P*3, *a*=19.0275(3), *b*=19.0275(3), *c*=14.4848(3) Å, *V*=4541.6(1) Å^3, *T*=−90 °C, *Z*=3, ρ_{calcd}=1.490 g cm^{-3}, μ (Mo Kα)=5.26 cm^{-1}, *F*(000)=2118, 32,600 reflections in *h*(−23/24), *k*(−24/24), *l*(−18/16), measured in the range 1.87°≤Θ≤27.47°, completeness Θ_{max}=99.7%, 13,470 independent reflections, R_{int}=0.039, 11,036 reflections with $F_o>4\sigma(F_o)$, 736 parameters, 1 restraints, $R1_{obs}$=0.054, wR^2_{obs}=0.137, $R1_{all}$=0.071, wR^2_{all}=0.148, GOOF=1.024, Flack-parameter 0.01(2), largest difference peak and hole: 0.865/−0.652 eÅ^{-3}. CCDC 291388 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336 033; or deposit@ccdc.cam.ac.uk).

4.3. Extended X-ray absorption fine structure (EXAFS)

Measurements were performed at beam line 10B of the Photon Factory of the High Energy Acceleration Research Organization (KEK-PF), Tsukuba, Japan. A channel-cut Si(311) monochromator was used. The ring current was 300–450 mA, and the storage ring was operated with electron energy of 2.5 GeV. The experiments at the Ag K edge (25,516.5 eV) were carried out at room temperature in the transmission mode. The samples are prepared as BN pellets of **11** and **17** for solid state, and aqueous solution of **17** using a cell having pathlength of 2 cm.

4.4. Antimicrobial activity

Antimicrobial activity was determined qualitatively by agar diffusion tests according to literature.[36] A suspension (100 µl) of the test organism with a density of McFarland standard 0.5 was inoculated into 32 ml of sterile melted agar medium and poured into Petri dishes. Holes of 9 mm in diameter were cut in the agar and filled with 50 µl of a 0.2 mM solution of the compound. Inhibition zones were read after overnight incubation.

Antimicrobial activity of the selected compounds was studied quantitatively by the determination of minimal inhibitory concentrations (MICs) according to the NCCLS guidelines[37] using the broth microdilution method.[38] The bacteria were grown overnight at 37 °C in Mueller–Hinton broth (MHB) (Difco). A 50 µl compound solution of 2 mM were serially diluted by a factor of two with MHB. Then the wells were inoculated with 50 µl of test organisms to give a final concentration of 5×10^5 CFU/ml. After microtiter plates were incubated at 37 °C for 24 h, the MIC values were read with a Nepheloscan Ascent 1.4 automatic plate reader (Labsystems, Vantaa, Finland) as the lowest dilution of antibiotic allowing no visible growth. *C. albicans* and *C. glabrata* were grown in yeast nitrogen base (Difco) supplemented with 1% glucose and incubated at 30 °C.

4.5. Antiproliferative and cytotoxic activities

Antiproliferative and cytotoxic activities of the compounds were determined as described using the cell lines K-562 (human chronic myeloid leukaemia) and L-929 (mouse fibroblast) for antiproliferative effects and HeLa (human cervix carcinom) for cytotoxic effects.[39]

Cells of established suspended cell lines K-562 (DSM ACC 10) and adherent L-929 (DSM ACC 2) were cultured in RPMI 1640 medium (GIBCO BRL 42402-016), supplemented with 25 µg/ml gentamicin sulfate (BioWhittaker 17-528Z), 10% heat inactivated foetal bovine serum (GIBCO BRL 10500-064), and L-glutamine (GIBCO BRL 25030-024) at 37 °C in high density polyethylene flasks (NUNC 156340).

HeLa (DSM ACC 57) cells were grown in RPMI 1640 culture medium (GIBCO BRL 21875-034) supplemented with 25 µg/ml gentamicin sulfate (BioWhittaker 17-528Z), and 10% heat inactivated foetal bovine serum (GIBCO BRL 10500-064) at 37 °C in high density polyethylene flasks (NUNC 156340).

The compounds were assayed against cell lines K-562 (human chronic myeloid leukaemia) and L-929 (mouse fibroblast) for their antiproliferative effects. The adherent cells of L-929 were harvested at the logarithmic growth phase after soft trypsinization, using 0.25% trypsin in PBS containing 0.02% EDTA (Biochrom KG L2163).

For each experiment with K-562, L-929, and HeLa approximately 10,000 cells were seeded with 0.1 ml RPMI 1640 (GIBCO BRL 21875-034), containing 25 µg/ml gentamicin sulfate (BioWhittaker 17-528Z), but without HEPES, per well of the 96-well microplates (K-562: NUNC 163320, L-929, HeLa: NUNC 167008).

For the cytotoxic assay HeLa cells were preincubated for 48 h without the test substances. The dilutions of the compounds were carried out carefully on the monolayers of HeLa cells after the preincubation time.

Cells of L-929, K-562, and HeLa were incubated for 72 h at 37 °C in a humidified atmosphere and 5% CO_2.

Suspension cultures of K-562 in microplates were analysed by an electronic cell analyser system CASY 1 (SCHÄRFE, Reutlingen, Germany). The software for data evaluation CASYSTAT (SCHÄRFE) offers a fast graphical evaluation of the measurement parameters, for example, as diagrams of cell diameter distributions, overlays of different curves, and cell volume distributions. The principles of measurement and evaluation of data were described. The 0.2 ml content of each well in the microplate was diluted 1:50 with CASYTON (NaCl: 7.93 g/l; Na_2EDTA: 0.38 g/l; KCl: 0.4 g/l; $NaH_2PO_4\cdot1H_2O$: 0.22 g/l; $NaH_2PO_4\cdot2H_2O$: 2.45 g/l; NaF: 0.3 g/l; SCHÄRFE). Every count/ml was automatically calculated from the arithmetic mean of three successive counts of 0.4 ml each. From the dose–response curves the GI_{50} values (concentration which inhibited cell growth by 50%) were calculated with CASYSTAT. The GI_{50} value was defined as being where the concentration–response curve intersected the 50% line, determined by means of the cell counts/ml, compared to control. The essential parameters for the estimation of growth inhibition and for changes in diameter distribution curves are expressed as diagrams.

The adherent L-929 and HeLa cells were fixed by glutaraldehyde and stained with a 0.05% solution of methylene blue for 15 min. After gentle wash the stain was eluted with 0.2 ml of 0.33 N HCl in the wells. The optical densities were measured at 630 nm in SUNRISE microplate reader (TECAN). Comparisons of the different values were performed with software Magellan (TECAN).

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