Vaccine Synthesis

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Fully Synthetic Vaccines Consisting of Tumor-Associated MUC1 Glycopeptides and a Lipopeptide Ligand of the Toll-like Receptor 2**

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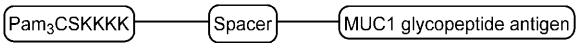
Dedicated to Professor Horst Kessler on the occasion of his 70th birthday

Mucin glycoprotein structures on epithelial tumor cells are characteristically different from the mucin structures on healthy cells.[1] However, the immunogenicity of these tumor-associated glycoproteins is too low to overwrite the endogenous tolerance of the immune system. Therefore, they can not be used directly as antitumor vaccines. Recently, it was demonstrated that glycopeptides from the tandem repeat region of tumor-associated mucin MUC1 conjugated to a T-cell epitope peptide from ovalbulmin furnish fully synthetic vaccines which elicit a strong, highly specific immune response in transgenic mice.[2] An even stronger and highly specific immune response was induced by immunization of wild-type balb/c mice with a vaccine containing the tumor-associated MUC1 glycopeptide bound to tetanus toxoid as the carrier protein.[3] This type of vaccines has the advantage of being applicable to humans. Of course, such MUC1 glycopeptide/tetanus toxoid vaccines also elicit immune reactions against tetanus toxoid. To suppress the generation of an anticarrier immune reaction, for example in booster immunizations, an alternative form of a synthetic vaccine must be developed in which the tumor-associated MUC1 glycopeptide is covalently bound to a general immunostimulating structure. Toll-like receptor ligands, for example tripalmitoyl-*S*-glycerylcysteine peptides like $Pam_3CysSer(Lys)_4$ described by Bessler, Jung et al.,[4] represent such immunostimulating structures. Recently, Boons et al.[5] reported vaccines consisting of a glycoundecapeptide of the tandem repeat unit of MUC1 containing the monosaccharide T_N-antigen side chain directly coupled to a T-cell epitope from polio virus[6] and the aforementioned TLR2 agonist. These constructs induced selective immune reactions in mice. During the synthesis of these vaccines the O-deacetylation of the *N*-acetylgalactosamine part was achieved by transesterification with hydrazine in methanol.[7] This procedure is not applicable to the synthesis of glycopeptides bearing neuraminic acid.

To benefit from the immunostimulating effects of Pam_3Cys Toll-like receptor ligands in synthetic MUC1 glycopeptide vaccines supplementing the tetanus toxoid conjugates, we developed a fragment condensation to attach the $Pam_3CSKKKK$ lipopeptide to tumor-associated MUC1 glycopeptides to give fully synthetic vaccines **A**.



A

To minimize the influence of the lipopeptide and its basic side chains on the conformation of the MUC1 glycopeptide antigen, an oligoethylene glycol spacer was placed between the TLR2 ligand and the B-cell epitope. Provided the activated carboxylic group of the lipopetide bears only acid-labile protecting groups and the saccharide part of the glycopeptide already is deprotected, the final acidolytic deprotection should not affect the palmitic esters and should afford a pure fully synthetic vaccine.

The N-terminally and side-chain-protected lipopeptide was synthesized on a resin functionalized with the 2-phenyl-2-trimethylsilylethylester (PMTSEL) anchor.[9] This anchor molecule is cleavable under neutral conditions by use of tetrabutylammonium fluoride trihydrate in dichloromethane. Fmoc-Lys(Boc)-OH was treated with 4-(2-hydroxy-1-trimethylsilylethyl)phenoxyacetic acid allyl ester[9,11] (**1**) according to the procedure reported by Steglich and Neises[10] to give the anchor ester molecule **2** (Scheme 1). The allyl ester **2** was cleaved selectively using catalytic amounts of tetrakis(triphenylphosphine)palladium(0) and *N*-methylaniline[12] as the allyl scavenger. The obtained anchor carboxylic acid **3** was coupled to amino-functionalized Tentagel[13] resin using TBTU/HOBt to yield the resin **4** preloaded with Fmoc-Lys(Boc) (Scheme 1). The lipopetide **5** was assembled on resin **4** following the Fmoc strategy. After cleavage of the PMTSEL anchor with fluoride,[9] the side-chain-protected TLR2 ligand hexapeptide **5** was isolated in 81% yield (Scheme 2).[15]

Lipopeptide **5** selectively deprotected at the terminal carboxylic group can now be used for fragment condensations. For coupling reactions, the fully deprotected spacer-

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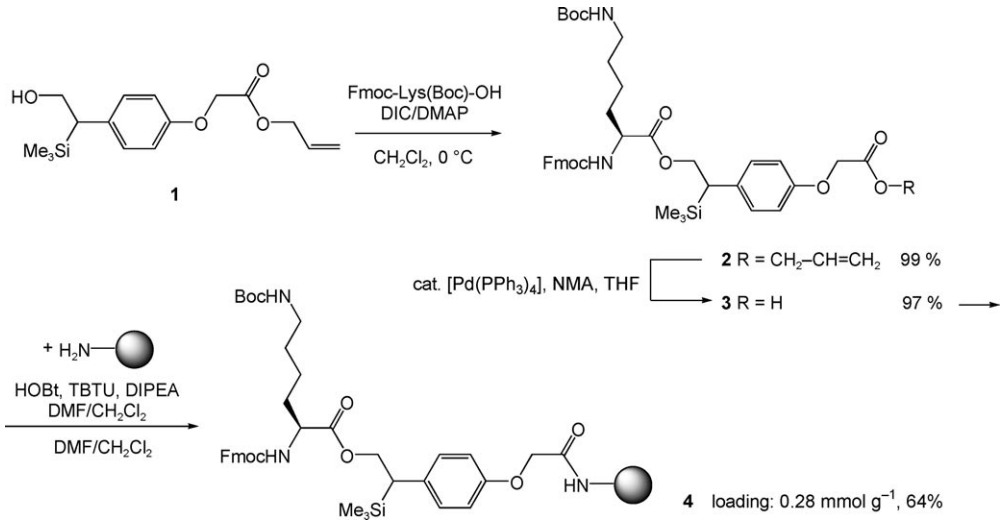
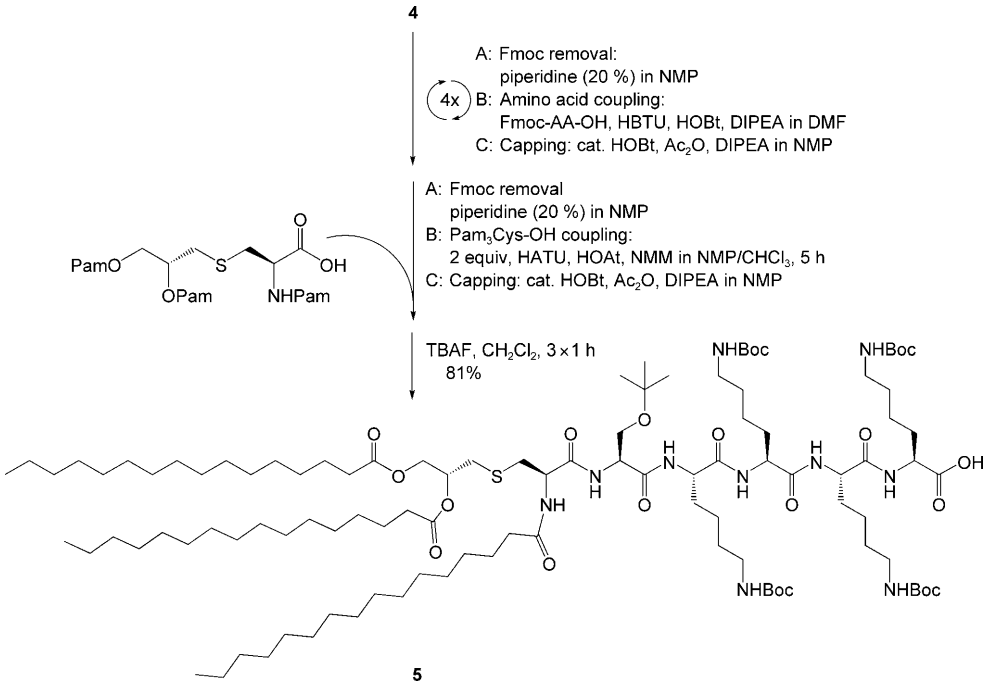


Scheme 1. Synthesis of the preloaded PMTSEL resin: Fmoc = fluorenyl-9-methoxycarbonyl, DIC = diisopropylcarbodiimide, DMAP = 4-dimethylaminopyridine, NMA = *N*-methylaniline, TBTU = *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate,[14] HOBt = 1-hydroxybenzotriazole, DIPEA = diisopropylethylamine (Hünig's base).

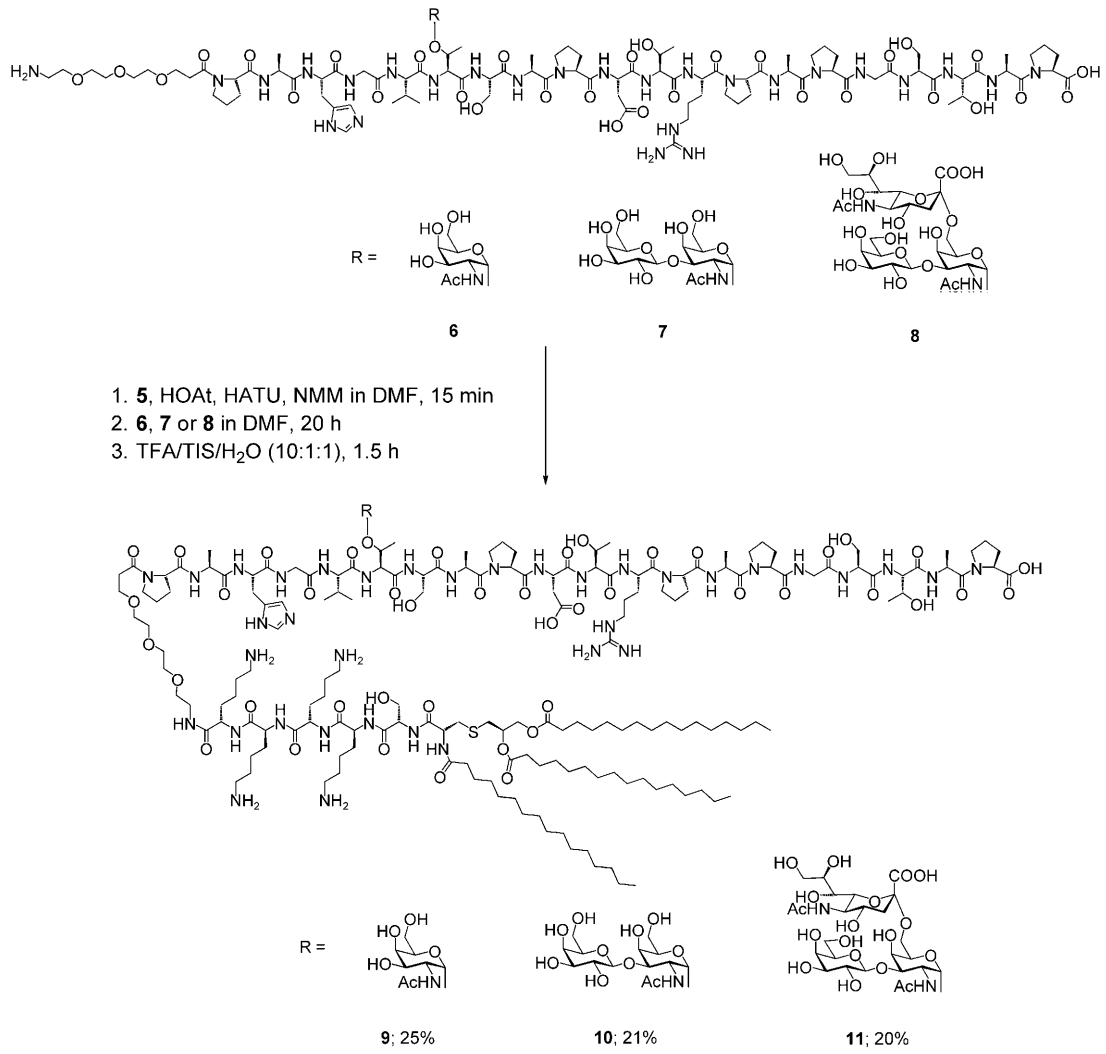


Scheme 2. Synthesis of the Pam_3Cys lipopeptide **5**. HBTU = *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOAt = 7-aza-1-hydroxybenzotriazole,[16] NMM = *N*-methylmorpholine, NMP = *N*-methylpyrrolidone.

functionalized MUC1 glycopeptides with T_N- (**6**), T- (**7**), and 2,6-sialyl-T-antigen side chains (**8**) were synthesized on a resin equipped with a trityl anchor according to an already described procedure[17] (Scheme 3). To accomplish the fragment condensation, lipopeptide **5** was converted to its active ester by reaction with HATU/HOAt.[16] Subsequently, a solution of the amino-functionalized MUC1 glycopeptide **6, 7**, or **8** (0.5 equiv) was added (see the Supporting Information). After completion of the reaction, acidolytic deprotection, and workup, the lipoglycopeptide vaccines **9, 10**, and **11** were isolated in yields of 20–25 %. They were characterized by MALDI-TOF and high-resolution ESI mass spectrometry and by two-dimensional ^1H NMR spectroscopy.[18] The synthesis of the Pam_3Cys MUC1 glycopeptide vaccine **11** gives evidence that the described fragment condensation between the activated protected lipopetide and the completely deprotected glycopeptide antigen is useful for the conversion of important tumor-associated glycopeptide antigens containing neuraminic acid into TLR2-ligand-based vaccines.

To evaluate the immunogenicity of the TLR2 ligand glycopeptide vaccines, balb/c-J mice were immunized with Pam_3Cys-icosaglycopeptide conjugate **10** in combination with complete Freund's adjuvant (CFA). After every 20 days, booster immunizations with **10** and incomplete Freund's adjuvant (IFA) were performed. Five days after the second boost, the induced antibodies were determined regarding their binding to the immobilized conjugate **12** of the MUC1 antigen glycopeptide with bovine serum albumin[2a,3] (Scheme 4) by means of an ELISA based on double-antibody technique (Figure 1).

The results of the ELISA analysis indicate that a specific humoral immune response had been elicited in all three mice. The antiserum titers were not as high as those for the corresponding MUC1 tetanus toxoid vaccine,[3] but the effect is reproducible and shows that the general mechanism of the immunological activation by TLR2 agonists can be applied to antitumor vaccines based on MUC1 glycopeptides.



Scheme 3. Synthesis of MUC1 glycopeptide TLR2 agonist vaccines **9**–**11** by fragment condensation. TIS = triisopropylsilane.

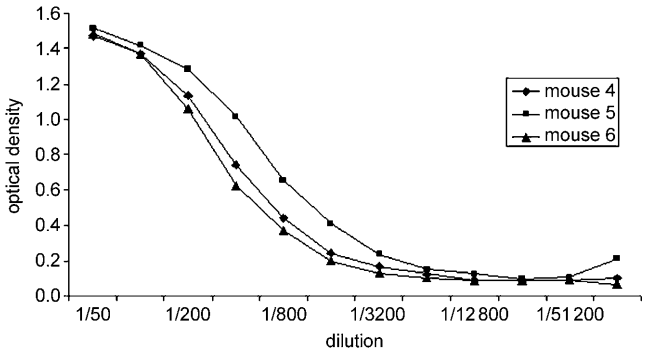
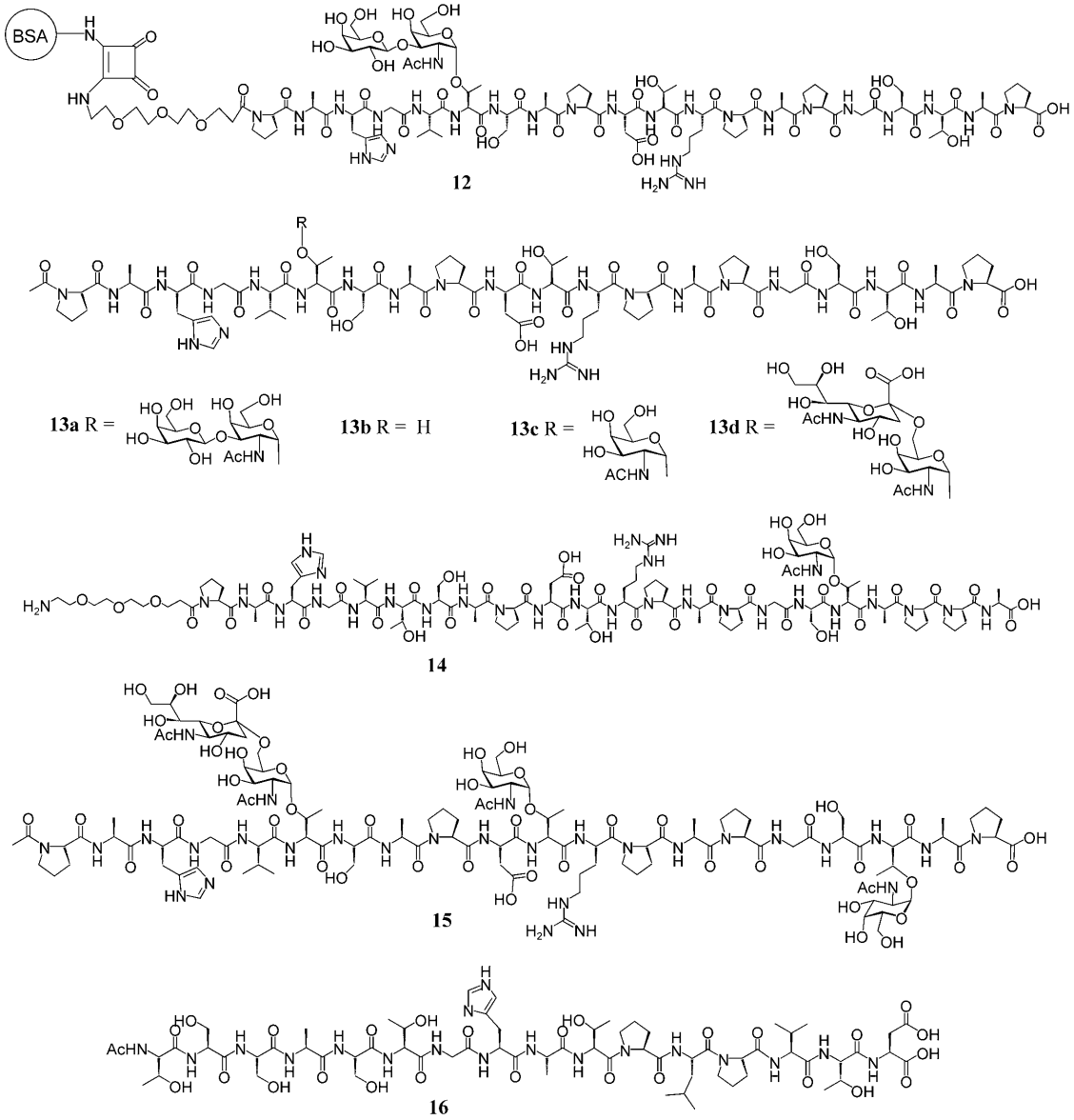


Figure 1. Determination of the induced immune reaction against **10** in balb/c-J mice.

To determine the specificity of the induced immune reaction, the antibodies were subjected to incubation with the already described MUC1 glycopeptides **13**–**15**[2a] and a MUC4 peptide[19] **16** (Scheme 4). These neutralization experiments with the antisera induced by vaccine **10** showed that the T-antigen MUC1 glycopeptide **13a** as well as the unglycosylated MUC1 peptide **13b** and MUC1 glycopeptides that are glycosylated in the same position but with different tumor-associated carbohydrate antigens such as T_N-antigen (**13c**) or sialyl-T_N-antigen (**13d**) do bind to the induced antibodies. However, the antibodies do not recognize MUC1 glycopeptides of the same sequence that are glycosylated in a different position, as for example **14** and **15**,[2a] and also not the peptide sequence **16** from mucin MUC4[19] (Figure 2).

The behavior of the antibodies induced by the T-antigen MUC1 TLR2 agonist vaccine **10** suggests that the recognition process is more dominated by the peptide sequence of the antigen and its conformation than was previously observed for vaccines containing neuraminic acid and a T-cell epitope[2] or the tetanus toxoid.[3] The incomplete neutralization of the antibodies by the T-antigen MUC1 structure **13a** contained in the vaccine **10** could indicate an influence of the Pam_3Cys lipopeptide in **10** on the B-cell epitope.

In conclusion, the combination of tumor-associated mucin glycopeptide antigens with lipopeptide Toll-like receptor 2 ligands furnished efficient fully synthetic vaccines, which can be generally synthesized through fragment condensation with unprotected amino-functionalized glycopeptides. These vaccines can be combined advantageously with vaccines[3] based



Scheme 4. The MUC1 glycopeptide BSA conjugate **12** and MUC1 and MUC4 peptides and glycopeptides **13–16** used for the neutralization of the antibodies induced by vaccine **10** (Figure 2). BSA = bovine serum albumine.

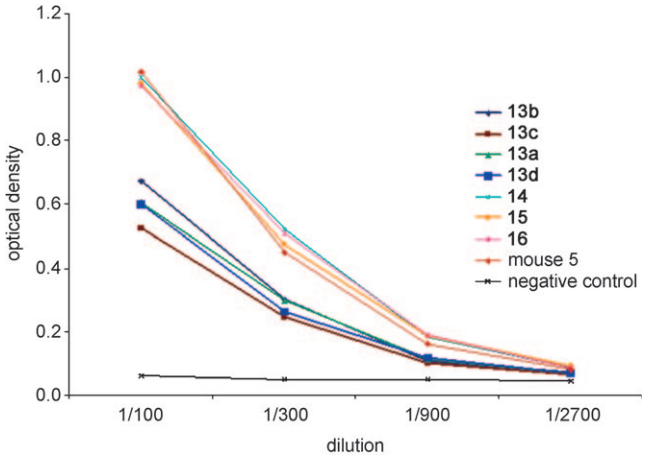


Figure 2. Neutralization of the antibodies induced by vaccine **10** using MUC1 and MUC4 peptide and glycopeptides **13–16** (Scheme 4).

on tetanus toxoid and principally are applicable to humans. They also offer the possibility to prevent the application of the complete Freund's adjuvant.

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[15] **5**: $[\alpha]_D^{24} = -5.6\ \mathrm{deg\,cm^3\,g^{-1}\,dm^{-1}}$ ($c = 1\ \mathrm{g\,cm^{-3}}$, $CHCl_3$); HR-ESI-MS (positive): *m*/*z*: 1554.19 ($[M+H-4\times Boc]^+$, calcd 1553.62). ^1H NMR data are shown in the Supporting Information.
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[17] See Schemes 1–3 in Ref. [3] and Scheme 2 in Ref. [2a].
[18] Details concerning the fragment condensation, deprotection, and characterization are given in the Supporting Information. **9**: 8 mg (from 20 mg of **6**); $[\alpha]_D^{23} = -66.5\ \mathrm{deg\,cm^3\,g^{-1}\,dm^{-1}}$ ($c = 0.37\ \mathrm{g\,cm^{-3}}$, H_2O); MALDI-TOF (dhb, positive): *m*/*z*: 3786.36 $[M+H]^+$, calcd 3785.29. **10**: 6.7 mg (from 20 mg of **7**); $[\alpha]_D^{22} = -48.4\ \mathrm{deg\,cm^3\,g^{-1}\,dm^{-1}}$ ($c = 0.3\ \mathrm{g\,cm^{-3}}$, H_2O); $t_R = 39.1$ min; MALDI-TOF (dhb, positive): *m*/*z*: 3947.64 $[M+H]^+$, calcd 3964.32. **11**: 6.1 mg (from 20 mg of **8**); $[\alpha]_D^{22} = -37.4\ \mathrm{deg\,cm^3\,g^{-1}\,dm^{-1}}$ ($c = 0.5\ \mathrm{g\,cm^{-3}}$, H_2O); HR-ESI-MS (positive): *m*/*z*: 1413.14 $[M+3H]^{3+}$; calcd 1413.14.
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