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Highly Efficient Chemoenzymatic Synthesis of α-Galactosyl Epitopes with a Recombinant α(1→3)-Galactosyltransferase

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Abstract: α-Galactosyl epitopes are carbohydrate structures bearing a Galα1-3Galβ terminus. The interaction of these epitopes on the surface of animal cells with anti-α-galactosyl antibodies in human serum is believed to be the main cause in antibody-mediated hyperacute rejection in xenotransplantation. This report describes an efficient chemoenzymatic approach based on the use of recombinant α(1→3)-galactosyltransferase (α1,3-GalT) for the synthesis of xenoactive α-galactosyl epitopes, which are highly desired in the research of xenotransplantation and immunotherapy. A truncated bovine α1,3-GalT (80−368) was cloned into the pET15b vector and subsequently transformed into *E. coli* BL21 strain. This expression system efficiently produced the soluble recombinant enzyme on a large scale with highly specific activity. A variety of α(1→3)-galactosylated epitopes were synthesized using such a recombinant enzyme. In a unique fashion, α-galactosyl pentasaccharide was synthesized via a one-pot, two-step enzymatic synthesis with in situ cofactor regeneration.

Introduction

Organ transplantation as one of the miracles of modern medicine is overshadowed by the severe worldwide shortage of human organ donors. One solution to this problem is the use of animal organs—xenotransplantation.[1] Pigs are considered the best organ donor candidates. However, human antibody mediated hyperacute rejection presents a formidable barrier. The major xenoactive antigens on porcine endothelial cells are carbohydrate structures bearing a Galα1-3Galβ terminus. Trisaccharides (Galα1-3Galβ1-4Glcβ-R and Galα1-3Galβ1-4GlcNAcβ-R) and pentasaccharide (Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-R) are considered as α-Gal epitopes binding specifically to human anti-Gal antibodies during the xenotransplantation.[2] Such epitopes are abundantly expressed on the cells of most mammals with the exception of humans, apes, and other Old World primates. The unique enzyme responsible for the formation of α-Gal epitopes is α(1→3)-galactosyltransferase (α1-3GalT) (EC 2.4.1.151), a protein which is absent in humans, apes, and other Old World primates due to mutational inactivation of the gene. Conversely, anti-Gal antibodies exist in humans, constituting 1−2% of total serum IgG and 3−8% of total serum IgM (Figure 1). The discovery of the interaction of anti-Gal and α-Gal epitopes has led to experimental attempts to overcome hyperacute rejection by either depleting the recipient's anti-Gal through α-Gal immobilized affinity columns or antagonizing anti-Gal by infusing soluble synthetic α-Gal oligosaccharides.[2b,3] However, such procedures would require access to a substantial amount of α-Gal oligosaccharides as well

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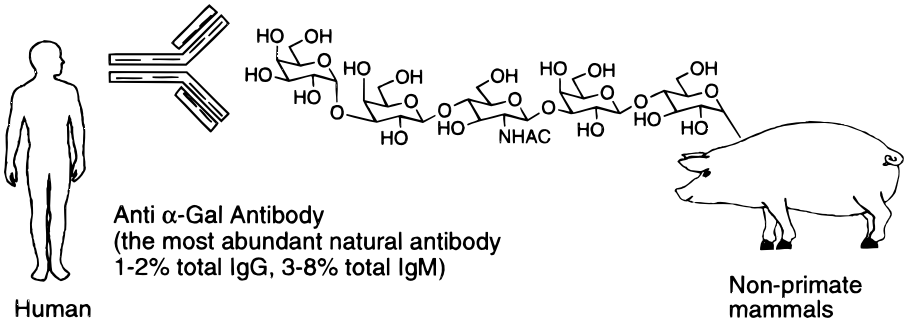

Figure 1. Interplay of α-galactosyl epitopes and anti-α-Gal antibody.

as synthetically derived α-Gal analogues and mimetics with high affinity to anti-Gal antibodies.

Previous chemical syntheses of Galα1-3Galβ1-4Glcβ-R and Galα1-3Galβ1-4GlcNAcβ-R required lengthy protection and deprotection sequences.[4,5] Enzymatic synthesis of Galα-3Gal sequence was reported using glycosidase-catalyzed transglycosylation reactions.[6] Unfortunately, low yields and unpredictable regioselectivities for the formation of desired glycosidic linkages plague this approach. Alternatively, the "one enzyme−one linkage" concept[7] of glycosyltransferases makes them a viable strategic choice for the preparative synthesis of complicated oligosaccharides and glycoconjugates. Nevertheless, the amount of α1-3 GalT available from natural sources is very limited. Although several studies on cloning and characterization of α1-3GalT have been reported,[8] no practical production of the recombinant enzyme has been accomplished. We describe here a novel expression system which produces truncated, soluble α1-3GalT on a large scale. Using this enzyme, a variety of α-Gal epitopes and derivatives have been synthesized.

Results and Discussion

Full length bovine α1-3GalT is a type-II membrane protein with a short N-terminal cytosolic domain, a membrane spanning region, a stem, and a C-terminal catalytic region.[8a] It was found that a truncated catalytic domain of 80−368 was the minimal sequence for the enzymatic activity. Thus, the corresponding gene plus a six-histidine tag at the N-terminus was cloned into pET 15b vector (Novagen, Madison) which contained an ampicillin-resistant gene and a T7 promoter (Figure 2). The recombinant enzyme was expressed in *E. coli* BL21(DE3) (Novagen, Madison) transformed with pET15b-αGalT. In the cell lysate, the enzyme was expressed at a level of approximately 60 unit (U)/L. The tag of six histidines fused on the N-terminal simplified the purification of active soluble enzyme by Ni−NTA affinity chromatography.[9] The purified enzyme has an expected MW 36 000 with a specific activity of 10.6 U/mg. The specific activity is 100-fold higher than that reported from the bovine α1-3GalT expressed in Sf9 insect cells and 100 000-fold higher than that reported for the enzyme present in calf thymus, which is the tissue with the highest known specific activity.[8a] Thus, this expression system provides a viable access to a large quantity of α1-3GalT essential for the enzymatic synthesis of α-galactosyl epitopes.

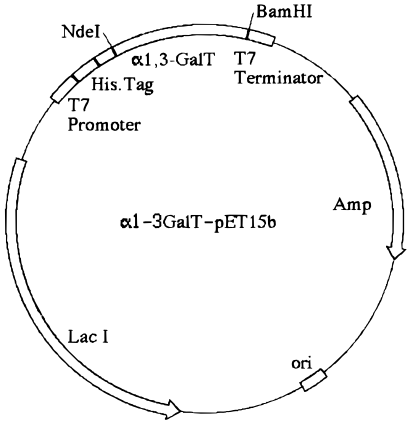

Figure 2. Genetic map of α(1→3)-galactosyltransferase expression plasmid.

Using the recombinant enzyme, α-galactosyl epitopes and a variety of derivatives were synthesized on preparative scales. The acceptors that were used in this study include lactose **1a**, β-lactosyl azide **1b**,[10] β-thiophenyl lactoside **1c**,[11] *N*-acetyllactosamine derivatives **1d,e**,[12] and lactosamine **1f**.[13] All of these disaccharides served as good acceptors and produced the corresponding α-Gal epitopes and derivatives **2a**−**f** in good yields (see the Experimental Section for details). It is worth noticing that **1f**, which has an unprotected amino group, still served as a good acceptor. The product **2f** bearing an amino group was easily isolated by cation-exchange resin chromatography.[13] Another advantage of this trisaccharide is that the amino group can be utilized for further synthetic manipulation.[14] Compounds **2b,c** can be directly used as trisaccharide building blocks for the convergent synthesis of larger α-galactosyl epitopes and glycopeptides.

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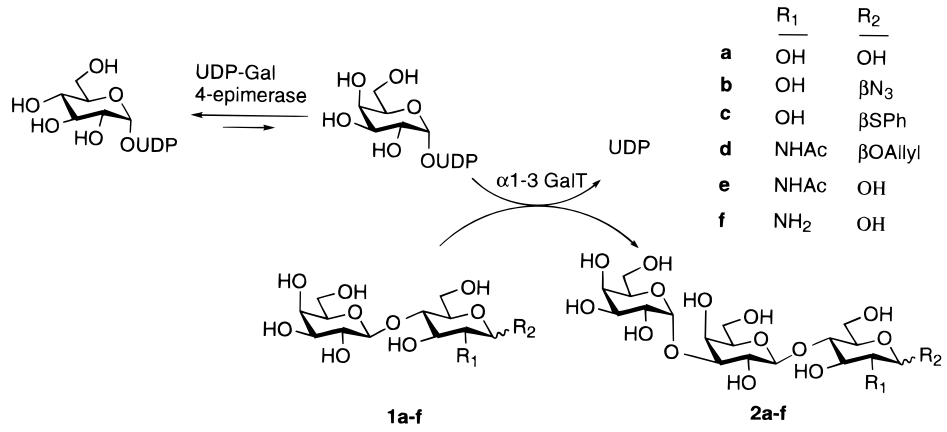
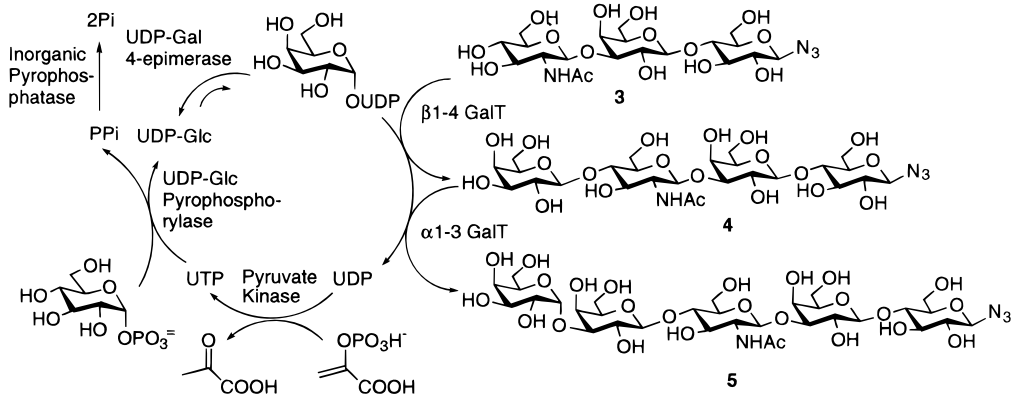
Scheme 1. Synthesis of α(1→3)-Galactosyl Trisaccharides Using α(1→3)-Galactosyltransferase and UDP-Galactose-4-epimerase



Scheme 2. Synthesis of Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc$\beta$$N_3$ Pentasaccharide with in Situ Cofactor Regeneration



To synthesize the Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-R pentasaccharide, we designed a one-pot enzymatic synthesis which included two sequential enzymatic glycosylations using α(1→3)-galactosyltransferase and β(1→4)-galactosyltransferase that share a common UDP-galactose donor.[15] In situ cofactor regeneration cycles of the donor[16] were applied to avoid stoichiometric use of the expensive nucleotide sugar donor and product inhibition of the transferases. The starting trisaccharide **3** was prepared through an established stereospecific glycosylation.[17] The azido group was purposefully introduced for its synthetic flexibility in the solid-phase synthesis of glycopeptides,[18] glycopolymers, or glycodendrimers.[19] It can also be transformed to other useful glycosylation functionalities such as glycosyl fluorides for orthogonal oligosaccharide synthesis.[20] It is noteworthy that almost all of the starting trisaccharide **3** was consumed in this high-yielding and efficient one-pot synthesis. The corresponding intermediate **4** and product **5** were obtained in 53% and 35% yields, respectively.

Conclusion

In summary, efficient chemoenzymatic syntheses of a variety of α-galactosyl epitopes with different functionalities were successfully achieved by the use of a novel recombinant α-(1→3)-galactosyltransferase. This approach provides an easy access to a wide spectrum of α-galactosyl epitopes and their derivatives to support the continuous studies on xenotransplantation as well as other pharmaceutical research.

Experimental Section

General Method. ^{1}H and ^{13}C spectra were recorded on 400-MHz Varian VXR400 NMR and 500-MHz Varian Unity spectrometers. Mass spectra (FAB or ESI) were run at the mass spectrometry facility at the University of California, Riverside. Thin-layer chromatography was conducted on Baker Si_{250F} silica gel TLC plates with a fluorescent indicator.

T7 promoter and T7 terminator primers and pET15b vector were purchased from Novagen. Ni^{2+}−NTA resin and DNA miniprep spin kit were from Qiagen. Taq DNA polymerase, DNA ligase, and Magic PCR Preps DNA purification systems were obtained from Promega Corp. DNA ligase, BamHI, and Nde I were from New England Biolabs. UDP-galactose-[galactose-6-^{3}H] was purchased from Amersham. UDP-galactose and DOWEX 1×8 resin were obtained from Sigma.

The plasmid containing the gene sequence of bovine α1-3GalT in pSV-SPORT vector was obtained from Dr. L. Inverardi (Diabetes Research Institute, Cell Transplant Center, University of Miami School of Medicine, Miami, FL). Both *E. coli* cell lines DH5α (Gibco-BRL) and BL21(DE3) (Novagen) are commercially available.

Amplification of the α(1→3)-Galactosyltransferase Gene. PCR amplifications were performed in a 50-μL reaction mixture containing 5 μL of template DNA, 1 μM primers BE80GT-N (5′CGAATATCATATGGAAAGCAAGCTTAAGCTATCG3′) and BGT-C (5′CGCGGATCCCAAAGTCAGACATTATTTCTAACCAC3′), 2.5 mM $MgCl_2$, 5 μL of 10× buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3), 1 mM of dNTPs, and 2.5 U of Taq DNA polymerase. The reaction mixture

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was overlayed with 50 μL of mineral oil and subjected to 30 cycles of amplifications with an annealing temperature of 55 °C.

Construction of the α(1→3)-Galactosyltransferase Expression Plasmid. The DNA obtained from the first PCR amplification (Extension PCR) was used as the template for the second amplification after purification by agarose gel electrophoresis. The DNA product of the second PCR was directly used for TA cloning. Positive colonies were picked up and screened by restriction mapping with NdeI and BamHI. The appropriate insert was selected and was cloned into the pET15b vector previously cleaved by the same restriction enzymes after purification by a agarose gel electrophoresis. The resulting α1-3GalT-pET15b plasmid was transformed into *E. coli* DH5α competent cells, and a selected clone was grown up for minipreps and characterization by restriction mapping and DNA sequencing. The plasmids from the same culture were transformed into *E. coli* strain BL21(DE3) for overexpression of the enzyme.

Preparation of α(1→3)-GalT from Transformed *E. coli* Strain. Cells were cultured in LB medium containing 100 μg/mL ampicillin with rapid shaking (250 rpm) at 37 °C in a C25 incubator shaker. The cultures were monitored by absorbance at 600 nm using a Beckman DU-600 spectrometer. When the $A_{600\,nm}$ of the culture reached 0.8−1.0, IPTG (isopropyl-1-thio-β-D-galactopyranoside) was added to a concentration of 400 μM to induce the expression of α1-3GalT. After shaking at 37 °C (250 rpm), the cells were harvested by centrifugation at 4000 rpm for 20 min and washed with washing buffer (pH 8.5, 20 mM Tris-HCl, 20% sucrose). Lysis buffer (pH 8.5, 20 mM Tris-HCl, 1 mM EDTA, 1% Triton ×100, 200 μg/mL lysozyme) was added, and the mixture was stirred vigorously for 10 min at room temperature. DNaseI (2 μg/mL) was added. The mixture was shaken at 37 °C in a water bath for 40 min, and the lysate was collected by centrifugation at 11 000 rpm for 20 min.

Purification of Recombinant α(1→3)-Galactosyltransferase. The enzyme was purified using a Ni−NTA affinity column (Qiagen) which binds to the six-histidine-containing sequence. Purification was performed at 4 °C. The Ni^{2+} column was equilibrated with 3 volumes of 1× binding buffer (5 mM imidazole, 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl) before loading the cell lysate. The column was then washed exclusively with 6 volumes of 1× binding buffer, followed by 6 volumes of 1× washing buffer (60 mM imidazole, 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl)) and 6 volumes of 1× elution buffer (200 mM imidazole, 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl). Elution buffer containing the purified enzyme was dialyzed at 4 °C against dialysis buffer (10% glycerol, 20 mM Tris-HCl, pH 7.9) before using in synthesis.

α(1→3)-Galactosyltransferase Assay. Enzyme activity for different acceptors was assayed at 37 °C for 12 min in a final volume of 100 μL containing 10 mM Tris-HCl (pH 7.0), 10 mM $MnCl_2$, 0.1% BSA, 0.3 mM UDP-[6-^{3}H]Gal (final specific activity of 500 cpm/nmol), 0.04 mg/mL enzyme, and 50 mM acceptor. Acceptor was omitted for the blank. The reaction was stopped by adding 100 μL of ice-cold 0.1 M EDTA, and the mixture was passed through 2 mL of Dowex 1×8-200 chloride anion exchange column and washed with 0.5 mL and 1 mL of H_2O consecutively. The flow-through was collected in a 20-mL plastic vial. ScintiVerse BD (5 mL, Fisher) was added, and the vial was vortexed completely. The radioactivity of the enzyme was counted in a liquid scintillation counter (Beckmann LS counter). One unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the transfer of 1 μmol of galactose from UDP-Gal to lactose per minute at 37 °C.

General Procedure for Galactosylation of Lactose and LacNAc Acceptors (1a−f) Using the Two-Enzyme System. Trisaccharides 2a−f. To a mixture of the acceptor (480 μmol, 40 mM), UDP-glucose (576 μmol, 48 mM), $MnCl_2$ (10 mM), and bovine serum albumin (BSA) (0.1%) in Tris-HCl buffer (100 mM, pH = 7.0, 12 mL) were added the enzymes UDP-galactose-4-epimerase (10 U) and α(1→3)-galactosyltransferase (7 U). The reaction was shaken under an argon atmosphere at room temperature (ca. 25 °C) for 3 days. The mixture was passed through Dowex-Cl anion-exchange resin and purified with gel permeation chromatography (Bio-Gel P2). The following α-galactosyl trisaccharides were prepared according to this method:

2a (126 mg, 52%). Selected anomeric ^{1}H NMR (D_2O): δ 4.33 (d, J = 7.5 Hz, 1H), 4.48 (d, J = 8.0 Hz), 4.96 (d, J = 4.0 Hz, 1H), 5.04 (d, J = 3.5 Hz). Selected anomeric ^{13}C NMR (D_2O): δ 91.69, 95.28, 95.64, 102.70. HRFABMS calcd for $C_{18}H_{32}O_{16}$ (M + Na): 527.1588. Found: 527.1582.

2b (155 mg, 61%). ^{1}H NMR (D_2O): δ 3.13 (t, J = 9.0 Hz, 1H), 3.46−3.84 (m, 15H), 4.00 (d, J = 2.5 Hz, 1H), 4.01 (t, J = 6.5 Hz, 1H), 4.34 (d, J = 8.0 Hz, 1H), 4.59 (d, J = 8.5 Hz, 1H), 4.96 (d, J = 3.5 Hz, 1H). ^{13}C NMR (D_2O): δ 59.78, 60.80, 60.90, 64.69, 68.08, 69.00, 69.16, 69.45, 70.71, 72, 38, 74.30, 74.94, 76.55, 77.03, 77.77, 89.84, 95.30, 102.67. HRFABMS calcd for $C_{18}H_{31}N_3O_{15}$ (M + Na): 552.1652. Found: 552.1639.

2c (132 mg, 46%). ^{1}H NMR (D_2O): δ 3.25 (t, J = 9.5 Hz, 1H), 3.47−3.86 (m, 15H), 4.02 (d, J = 3.0 Hz, 1H), 4.03 (t, J = 6.5 Hz, 1H), 4.36 (d, J = 8.0 Hz, 1H), 4.67 (d, J = 10.0 Hz, 1H, H-1), 4.98 (d, J = 4.0 Hz, 1H). ^{13}C NMR (D_2O): δ 60.06, 60.91, 62.36, 64.71, 68.09, 69.01, 69.16, 69.46, 70.71, 71.30, 74.93, 75.74, 77.05, 78.02, 78.57, 87.04, 95.30, 102.66, 128.04, 129.22, 131.53. HRFABMS calcd for $C_{24}H_{36}O_{15}S$ (M + Na): 619.1672. Found: 619.1665.

2d (188 mg, 67%). ^{1}H NMR (D_2O): δ 1.84 (s, 3H), 3.38−4.02 (m, 19H), 4.15 (dd, J = 5.0, 13.0 Hz, 1H), 4.35 (d, J = 8.0 Hz, 1H), 4.39 (d, J = 8.5 Hz, 1H), 4.95 (d, J = 3.5 Hz, 1H), 5.06−5.14 (m, 2H), 5.67−5.75 (m, 1H). ^{13}C NMR (D_2O): δ 22.01, 54.88, 59.98, 60.78, 60.88, 64.66, 68.07, 68.98, 69.15, 69.47, 70.36, 70.70, 72.43, 74.62, 74.92, 77.00, 78.49, 95.27, 99.85, 102.65, 118.07, 133.13, 174.46. HRFABMS calcd for $C_{23}H_{39}NO_{16}$ (M + Na): 608.2167. Found: 608.2169.

2e (147 mg, 56%). Selected anomeric ^{1}H NMR (D_2O): δ 4.39 (d, J = 8.0 Hz, 1H), 4.57 (d, J = 7.5 Hz), 4.99 (d, J = 4.0 Hz, 1H), 5.05 (d, J = 2.0 Hz). Selected anomeric ^{13}C NMR (D_2O): δ 90.44, 94.80, 95.34, 102.75.

2f (120 mg, 50%). Selected anomaric 1H NMR (D_2O): δ 4.35 (d, J = 7.5 Hz, 1H), 4.79 (d, J = 8.1 Hz), 4.96 (d, J = 3.6 Hz, 1H), 5.27 (d, J = 3.6 Hz). Selected anomeric ^{13}C NMR (D_2O): δ 88.68, 92.35, 95.21, 102.70. HRFABMS calcd for $C_{18}H_{34}NO_{15}$ (M^+): 504.1928. Found: 504.1955.

One-Pot Enzymatic Synthesis of Pentasaccharide Galα1-3Galβ1-4Gluβ1-3Galβ1-4GlcNAcβN$_3$ (5). To trisaccharide **3** (60 mg, 0.11 mmol), glucose 1-phosphate (67 mg, 0.22 mmol), KCl (28 mg, 0.37 mmol), PEP (48 mg, 0.23 mmol), and UTP (11 mg, 0.02 mmol) in Tris-buffer (100 mM, pH 7.0, 10 mL) were added BSA (0.1%), $MgCl_2$ (10 mg, 0.1 mmol), and $MnCl_2$ (16 mg, 0.1 mmol). The solution was degassed with argon followed by addition of the enzymes (UDP-glucose-pyrophosphorylase (5 U), inorganic pyrophosphatase (10 U), UDP-galactose-4-epimerase (5 U), α(1→3)-galactosyltransferase (10 U), β(1→4)-galactosyltransferase (5 U), and pyruvate kinase (20 U)). The reaction was conducted at room temperature for 4 days. The mixture was passed through Dowex-Cl anion-exchange resin and purified with gel permeation chromatography (Bio-Gel P2) to afford pentasaccharide **5** (33 mg, 35%). Selected anomeric ^{1}H NMR (D_2O): δ 4.24 (d, J = 8.0 Hz, 1H), 4.36 (d, J = 8.0 Hz, 1H), 4.51 (d, J = 8.5 Hz, 1H), 4.58 (d, J = 9.0 Hz, 1H), 4.95 (d, J = 3.5 Hz, 1H). Selected anomeric ^{13}C NMR (D_2O): δ 89.83, 95.31, 102.66, 102.66, 102.78. HRFABMS calcd for $C_{32}H_{54}N_4O_{25}$ (M + Na): 917.2975. Found: 917.3008.

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Supporting Information Available: Spectral data for compounds **2a−f** and **3−5** (22 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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