J. Biochem. **136**, 659–664 (2004)
DOI: 10.1093/jb/mvh162

Role of the Carboxyl-Terminal Region in the Activity of *N*-Acetylglucosamine 6-*O*-Sulfotransferase-1

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Received July 27, 2004; accepted August 25, 2004

***N*-Acetylglucosamine 6-*O*-sulfotransferases (GlcNAc6STs) catalyze the transfer of sulfate from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to the C-6 position of non-reducing *N*-acetylglucosamine. *N*-acetylglucosamine 6-*O*-sulfotransferase-1 (GlcNAc-6ST-1) is the first cloned GlcNAc6ST and is involved in the synthesis of the L-selectin ligand. We noticed conserved C-terminal segments among GlcNAc6STs and produced mutant enzymes to reveal the functional significance. Mutant enzymes were transiently expressed as fusion proteins with protein A in COS-7 cells, and some of them were purified to homogeneity by IgG Sepharose column chromatography. Deletion of a C-terminal segment (amino acid numbers 479–483) resulted in a complete loss of the activity, when assayed using GlcNAcβ1-6ManOMe as a substrate. Upon site-directed mutagenesis of the C-terminal region, three mutants, L477A, L478A and L483A, exhibited reduced activity. The K_M values for GlcNAcβ1-6ManOMe of L477A and L478A were 4 times higher than the K_M of the wild-type enzyme, while that of L483A was unchanged. On the other hand the K_M for PAPS of L483A was 3 times higher than that of the wild-type enzyme, while the values of L477A and L478A were unchanged. Furthermore, the L477A mutant acted on a core 3 structure (GlcNAcβ1-3GalNAc-pNP), while the wild-type enzyme does not. These results demonstrate a role for leucine residues in the C-terminal region in the enzymatic activity.**

Key words: carbohydrate sulfation, *N*-acetylglucosamine 6-*O*-sulfotransferase, site-directed mutagenesis, substrate specificity, sulfotransferase.

Abbreviations: GlcNAc6ST, *N*-acetylglucosamine 6-*O*-sulfotransferase; ITS, insulin, transferrin and selenium; Me, methyl; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PCR, polymerase chain reaction; pNP, *p*-nitrophenyl; TST buffer, 50 mM Tris-HCl buffer, pH 7.6 containing 0.15 M NaCl and 0.05% Tween 20.

Sulfotransferases catalyze the transfer of a sulfate group from the ubiquitous donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to acceptor substrates. The sulfation of carbohydrate chains is an important step in the construction of most glycosaminoglycans and is also observed in glycoproteins and glycolipids (*1–4*). Sulfotransferases can be divided into two main classes: cytosolic and Golgi-resident enzymes (*4–7*). The structural-functional relationships of many cytosolic sulfotransferases have been clarified (*7–15*); notably a conserved motif is present for the binding of PAPS. Except for the conservation of the PAPS-binding site, only limited information is available concerning active sites of the Golgi-resident carbohydrate sulfotransferases (*16–20*).

N-Acetylglucosamine 6-*O*-sulfotransferases (GlcNAc-6STs) catalyze the transfer of sulfate to the 6-position of *N*-acetylglucosamine at the non-reducing end. To date 5 molecular species of GlcNAc6STs have been identified (*5*, *6*), GlcNAc6ST-1 (*21*, *22*), GlcNAc6ST-2 (HEC-GlcNAc-6ST, LSST) (*23*, *24*), GlcNAc6ST-3 (I-GlcNAc6ST) (*25*), GlcNAc6ST-4 (*26*, *27*) and GlcNAc6ST-5 (C-GlcNAc6ST) (*28*). GlcNAc6STs show homology to chondroitin 6-sulfotransferase and keratan sulfate Gal sulfotransferase, and together all these enzymes form the Gal/GalNAc/GlcNAc-6-*O*-sulfotransferase family (*4–6*). GlcNAc6STs are involved in the synthesis of both keratan sulfate and glycoproteins. The null mutation of GlcNAc6ST-5 in humans leads to the onset of macular corneal dystrophy (*28*). GlcNAc6ST-2 plays key roles in the synthesis of L-selectin ligands, and its deficiency in mice leads to a reduction in lymphocyte homing to peripheral lymph nodes and mesenteric lymph nodes, but not to Peyer's patches (*29*). GlcNAc6ST-1 is also involved in the synthesis of L-selectin ligands (*30*), and GlcNAc6ST-1 null mice exhibit reduced homing to peripheral lymph nodes, mesenteric lymph nodes and Peyer's patches (*31*). It is concluded that GlcNAc6ST-1 and -2 play complementary roles in the synthesis of L-selectin ligands. GlcNAc6ST-1 has broad specificity, and is widely distributed in organs of humans and mice (*21*, *22*, *32*). The enzyme is also expected to be a powerful tool for the modification of oligosaccharides in glycoproteins (*33*). The present study focuses on the role of the C-terminal segment of the enzyme.

MATERIALS AND METHODS

Materials—The following materials were obtained commercially from the sources indicated. [^{35}S]PAPS

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(51GBq/mmol; 1.38 Ci/mmol) from PerkinElmer Life Sciences (Boston, MA), GlcNAcβ1-6ManOMe from Sigma (St. Louis, MO), ITS (insulin, transferrin and selenium) premix for medium from BD Bioscience (Franklin Lakes, NJ) and GlcNAcβ1-6(Galβ1-3)GalNAc-pNP and GlcNAcβ1-3GalNAc-pNP from Toronto Research Chemicals (North York, Ontario, Canada).

Site-Directed Mutagenesis of Human GlcNAc6ST—A cDNA encoding human GlcNAc6ST-1 without a transmembrane domain (Ref. *22*, GenBank accession number AB01468, nucleotide number 495–1844) was fused with a sequence from pcDSA vector (*34*) encoding a protein A tag with an IgM signal peptide, and subcloned into pBluescript II SK- (Stratagene, La Jolla, CA) to construct pBluescript II SK-human GlcNAc6ST-1 as described previously (*33*). Site-directed mutagenesis was performed on this plasmid by PCR using specifically mutated primers. The sequences of primers for each mutant are as follows: the forward primer, 5′-GTGGACGACCGCGTGTGCAAG-3′; the reverse primer for K471A, 5′-TTAGAGACGGGGCTTCCGAAGCAGGGTCTTGCTGAGGTCTGCGAC-3′; that for D472A, 5′-TTAGAGACGGGGCTTCCGAAGCAGGGTCTTGCTGAGGGCTTTGAC-3′; that for L473A, TTAGAGACGGGGCTTCCGAAGCAGGGTCTTGCTGAGGTCTGCGAC-3′; that for S474A, 5′-TTAGAGACGGGGCTTCCGAAGCAGGGTCTTGGCGAGGTC-3′; that for K475A, 5′-TTAGAGACGGGGCTTCCGAAGCAGGGTCGCGCTGAG-3′; that for T476A, 5′-TTAGAGACGGGGCTTCCGAAGCAGGGCCTTGCT-3′; that for L477A, 5′-TTAGAGACGGGGCTTCCGAAGCGCGGTCTTGCTG-3′; that for L477T, 5′-TTAGAGACGGGGCTTCCGAAGCGTGGTCTTGCTG-3′; that for L478A, 5′-TTAGAGACGGGGCTTCCGAGCCAGGGTCTTG-3′; that for L478T, 5′-TTAGAGACGGGGCTTCCGAGTCAGGGTCTTG-3′; that for R479A, 5′-TTAGAGACGGGGCTTCGCAAGCAGGGTC-3′; that for K480A, 5′-TTAGAGACGGGGCGCCCGAAGCAGG-3′; that for P481A, 5′-TTAGAGACGGGCCTTCCGAAGCAG-3′; that for R482A, 5′-TTAGAGAGCGGGCTTCCGAAGCAG-3′; that for L483A, 5′-TTAGGCACGGGGCTTCCGAAGCA-3′; that for L483T, 5′-TTAGGTACGGGGCTTCCGAAGCA-3′; that for Δ479–483 , 5′-TTAAAGCAGGGTCTTGCTGAG-3′. PCR was carried out at 94°C for 2 min, with 35 cycles of 94°C for 0.5 min, 59°C for 0.5 min and 72°C for 1.5 min. The PCR products were subcloned into pGEM-T easy vector (Promega, Madison, WI); the orientation and nucleotide sequence of each mutant were confirmed by the dideoxy chain termination method (*35*) using an Applied Biosystems automated sequencer. Then, the correct plasmid was digested with *Nco*I and *Sal*I to release a 0.11-kbp fragment. This DNA fragment was used to replace the corresponding DNA sequence in the plasmid pBluescript II SK-human GlcNAc6ST-1 that was digested with the same restriction enzymes. Then the pBluescript II SK-human GlcNAc6ST-1 with mutations was digested with *Eco*RI, and the released 1.5-kbp fragments were subcloned into pcDSA vector digested with the same restriction enzyme to obtain the expression plasmid pcDSA-GlcNAc6ST-1 with mutations.

Transient Expression of Protein-A–Tagged GlcNAc6ST-1 and Its Mutants by COS-7 Cells—COS-7 cells (3×10^6 cells in a 10-cm dish) were transfected with 4 μg of a relevant plasmid using Lipofectamine PLUS (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. After 24 h of culture in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, the medium was replaced with Dulbecco's modified Eagle's medium containing 1% IgG-free fetal calf serum and ITS. After culturing for another 48 h, the culture medium was collected and centrifuged at 3,000 × *g*, at 4°C for 20 min to remove the cellular debris. An aliquot of the supernatant was subjected to Western blot analysis, enzyme-linked immunoassay and sulfotransferase assay after binding to 10 μl of IgG Sepharose. The culture supernatant was applied to an IgG Sepharose column, and GlcNAc6ST-1 and the mutant proteins were purified as described before (*33*).

Assay of Sulfotransferase Activity—The reaction mixture contained 0.05 M Tris-HCl, pH 7.2, 0.01 M $MnCl_2$, 2 mM AMP, 0.1 M NaF, 0.05% Triton X-100, 1 mM GlcNAcβ1-6ManOMe or other acceptors, 6 μM [^{35}S]PAPS (8.3×10^{-3} mCi), and recombinant GlcNAc6ST-1s in a final volume 50 μl. For the determination of K_M values, the concentration of the acceptors or PAPS was changed. After 1 h at 37°C, the reaction was stopped by immersing the reaction tube in a boiling water bath for 1 min. ^{35}S-labeled products were separated by Superdex 30 column chromatography. The radioactivity in the oligosaccharide fraction was determined with a liquid scintillation counter.

Other Methods—SDS-PAGE was performed in a 10% running gel under reducing conditions. For Western blotting, the membrane was first incubated with rabbit IgG and then with horseradish-peroxidase–labeled goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) followed by detection with an ECL detection kit (Amersham Biosciences, Piscataway, NJ). Enzyme-linked immunosorbent assays were performed also with rabbit IgG and horseradish-peroxidase-labeled goat anti-rabbit IgG and the color was developed with *o*-phenylenediamine dihydrochloride. The amount of protein was determined by a Micro BCA Kit (Pierce, Rockford) using bovine serum albumin as a standard.

RESULTS

Alteration of GlcNAc6ST-1 Activity by Mutation in the C-Terminal Region—A comparison of the amino acid sequences of human GlcNAc6STs revealed leucine 477 of GlcNAc6ST-1 to be conserved in all GlcNAc6STs, except that a gap should be introduced to observe conservation in GlcNAc6ST-3 (Fig. 1). This amino acid is also conserved in the related enzymes chondroitin 6-sulfotransferase (*36*) and keratan sulfate galactose-6-sulfotransferase (*37*), but not in other sulfotransferases such as chondroitin 4 sulfotransferase-1 and -2 (*38*, *39*), HNK-sulfotransferase-1 (*40*), heparan sulfate 2-sulfotransferase (*41*) heparan sulfate 6-sulfotransferases (*42*) or cerebroside sulfotransferase (*43*) (Fig. 1). In addition to this leucine residue, other conserved features are present in the C-terminal region of GlcNAc6STs. Lysine 471 in GlcNAc6ST-1 is homologous to the arginine residue conserved in other GlcNAc6STs. Leucine 473 is conserved in other GlcNAc6STs except for GlcNAc6ST-4.

To elucidate the functional significance of the C-terminal region in GlcNAc6ST-1, we produced a deletion

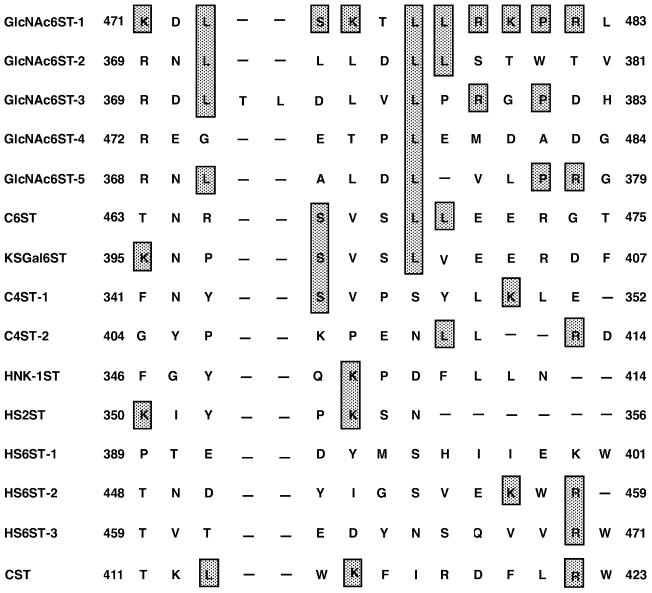

Fig. 1. **Comparison of the C-terminal regions of human Golgi-located sulfotransferases.** Conserved residues are boxed. The sequences are from the references cited below, and some of the abbreviations were also explained; GlcNAc6ST-1, (*22*, *44*); GlcNAc6ST-2 (*45*); GlcNAc6ST-3 (*25*); GlcNAc6ST-4 (*26*); GlcNAc6ST-5 (*28*); C6ST, chondroitin 6-sulfotransferase (*46*); KSGal6ST, keratan sulfate Gal-6-sulfotransferase (*37*); C4ST-1, chondroitin 4-sulfotransferse-1 (*38*); C4ST-2, chondroitin 4-sulfotransferase-2 (*39*); HNK-1, HNK-1 sulfotransferase (*47*); HS2ST, heparan-sulfate 2-*O*-sulfotransferase (*48*); HS6ST-1, heparan-sulfate 6-*O*-sulfotransferase-1 (*42*); HS6ST-2, heparan-sulfate 6-*O*-sulfotransferase-2 (*49*); HS6ST-3, heparan-sulfate 6-*O*-sulfotransferase-3 (*50*); CST, cerebroside sulfotransferase (*43*).

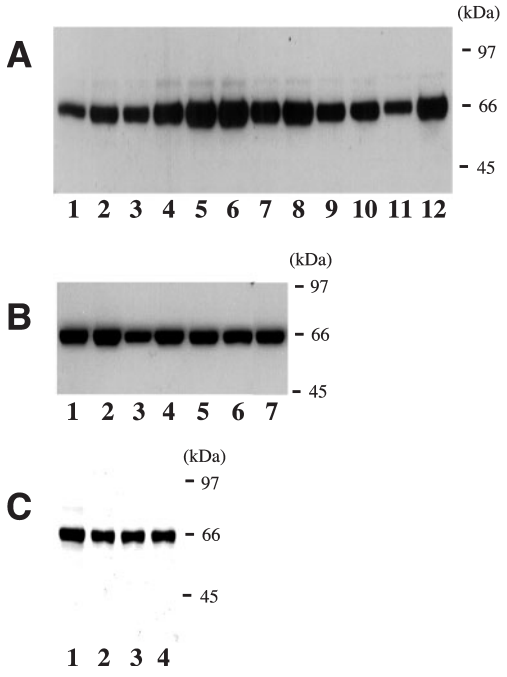

Fig. 2. **SDS-PAGE of GlcNAc6ST-1 mutants.** The culture supernatant (A, B) or purified enzymes (C) were subjected to SDS-PAGE in 10% gels, and theprotein bands were revealed by Western blotting (A, B) or silver staining (C). A; lane1, L477A; lane 2, L477T; lane 3, L478A; lane 4, L478T; lane 5, R479A; lane 6, K480A; lane 7, P481A; lane 8, R482A; lane 9, L483A; lane 10, L483T; lane 11, Δ479–483; lane 12, wild-type enzyme. B: lane 1, K471A; lane 2, D472A; lane 3, L473A; lane 4, S474A; lane 5, K475A; lane 6, T476A; lane 7, wild-type enzyme. C: lane 1, wild-type enzyme; lane 2, L477A; lane 3, L478A; lane 4, L483A.

mutant and point mutants of the enzyme. The mutants fused with protein A were expressed in COS-7 cells. The purity of the mutant proteins was confirmed by SDS-PAGE and Western blotting; all mutant proteins exhibited single bands of 66 kDa (Fig. 2, A and B). The activities of mutants were determined after adsorption to IgG Sepharose, and the activities are expressed relative to the amount of Protein A as determined by enzyme-linked immunoassay. The deletion of five C-terminal amino acids (Δ479–483) resulted in a complete loss of enzymatic activity, indicating the importance of the C-terminal segment to GlcNAc6ST activity (Fig. 3). Next we assayed point mutants; every amino acid residue in the C-terminal region starting from lysine 471 was individually mutated to alanine. Among the 13 mutants, L477A, L478A and L483A exhibited activity less than 50% of the wild-type level (Fig. 3). For these amino acids, we also produced mutants in which leucine was changed to threonine. All these mutants showed reduced levels of activity, although the reduction in the activity of L478T was less than that of L478A (Fig. 3).

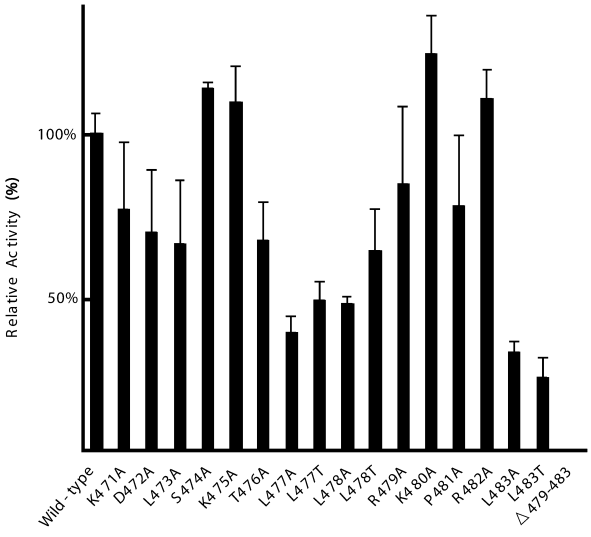

Fig. 3. **Activities of GlcNAc6ST-1 mutants.** Activities were normalized to the amount of Protein A, and the results are expressed relative to the wild-type enzyme.

Enzymatic properties of L477A, L478A and L483A Mutants—The three mutant proteins were purified by affinity chromatography on IgG columns; they exhibited single bands upon SDS-PAGE and silver staining (Fig. 2C). A kinetics analysis confirmed that the V_{max} values were decreased in all mutants (Table 1). We found that the three mutants could be classified into two groups (Table 1). For L477A and L478A, the K_M values for PAPS were not significantly different from that of the wild-type enzyme (Table 1); however, their K_M values for GlcNAc β1-6ManOMe were about 4 times higher than that of the

Table 1. **Comparison of K_M values of the wild-type enzyme and mutants.**

	GlcNAcβ1–6ManOMe (mM)	PAPS (μM)
Wild-type enzyme	0.26	4.6
Mutants		
L477A	1.06	5.0
L478A	0.98	4.4
L483A	0.33	13.3

The V_{max} values under saturated accepter concentration were 1.55, 0.24, 0.27 and 0.31 nmol/min/mg protein for the wild-type enzyme, L477A, L478A and L483A, respectively

wild-type enzyme. On the other hand, for L483A, the K_M for PAPS was about 3 times that of the wild-type enzyme, while the K_M for GlcNAcβ1-6ManOMe was unchanged. We also compared the substrate specificity of the L477A mutant with that of the wild-type enzyme. We found that the mutant enzyme acted on core 3 structure (Table 2), although the wild-type enzyme is unable to do so (*32*).

DISCUSSION

Three dimensional structures have been elucidated for certain sulfotransferases including steroid sulfotransferases (*7*, *14*, *15*), heparan sulfate *N*-deacetylase/sulfotransferase-1 (*17*) and heparan sulfate 3-*O*-sulfotransferase (*18*). These enzymes exhibit conserved structural motifs, especially at the PAPS-binding site. Site-directed mutagenesis of GlcNAc6ST-1 in the conserved motif revealed three amino acids critical for PAPS binding: R174, R332 and R341 (*19*). R174 is equivalent to a lysine residue, which is required for PAPS binding and is conserved in phenol sulfotransferase, estrogen sulfotransferase, monoamine sulfotransferase and heparan sulfate *N*-deacetylase/sulfotransferase-1. Very recently, it has been shown that the stem region of GlcNAc6ST-1, which is located on the N-terminal side, influences the substrate specificity of the enzyme (*20*).

The present study was conducted because we found an additional conserved domain in the C-terminal region of GlcNAc6STs and other members of the Gal/GalNAc/GlcNAc-6-*O*-sulfotransferase family. First, we demonstrated the importance of the C-terminal region using a deletion mutant lacking the 5 C-terminal amino acids (amino acids 479–483); the mutant completely lost enzyme activity. Then, we mutated each single amino acid in the 13 amino acid stretch of the C-terminal region. Among them, a marked reduction in activity occurred with the mutations of L477, L478 and L483. By kinetic analysis, L477A and L478A were shown to have reduced substrate-binding activity, and L483A to have reduced PAPS-binding activity. The importance of L477 is reasonable, since L477 is the conserved amino acid in the C-terminal region of GlcNAc6STs. That the mutation of leucine residues resulted in a significant loss of activity suggests that the C-terminal region interacts with another portion of the enzyme, and that the interaction is important for enzymatic activity. The complete loss of activity in the mutant lacking the 5 C-terminal amino acids might be due to the loss of the conformation necessary for this interaction.

X-ray crystallographic analyses of steroid sulfotransferases (*7*, *12*, *14*, *15*) heparan sulfate *N*-deacetylase/sulfotransferase (*17*) and heparan sulfate 3-*O*-sulfotransferase (*18*) revealed them to be single α/β globular proteins with a characteristic five-strand parallel β-sheet. The C-terminal segment is outside the globular domain. The C-terminal segment takes the form of an α-helix in the case of heparan sulfate *N*-deacetylase/sulfotransferase, but not in the case of other sulfotransferases. The results of the present investigation confirm the necessity of elucidating the three-dimensional structure of GlcNAc6ST-1. Most importantly, it should be clarified whether the C-terminal segment is in the globular domain in the case of GlcNAc6ST-1 or is away from the globular domain but plays a decisive role in the activity of the enzyme.

The change in the substrate specificity of the L477A mutant is of interest. GlcNAc6ST-1 does not act on the core 3 structure, while GlcNAc6ST-2 does (*32*). That the L477A mutant acts on the core 3 structure relatively weakly might indicate that the substrate-binding site of the mutant is altered to somewhat resemble that of GlcNAc6ST-2. This can happen when the C-terminal segment is near the substrate-binding site. However, the possibility can not be excluded that L477 interacts with another portion of the enzyme, and that the interaction affects the conformation of the substrate-binding site.

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and from the Mizutani Foundation for Glycoscience.

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Table 2. **Comparison of the substrate specificity of wild-type and mutant GlcNAc6ST-1.**[a]

Substrates	GlcNAc6ST-1		GlcNAc6ST-2
	wild-type[b]	L477A	wild-type[b]
GlcNAcβ1-6ManOMe	100	100	100
GlcNAcβ1-6[Galβ1-3]GalNAc- pNP (core 2)	191	165	145
GlcNAcβ1-3GalNAc-pNP (core 3)	ND[c]	20	184

[a]Results are the activities relative to that of GlcNAcβ1-6ManOMe. [b]Taken from Ref. *32*. [c]Not detected (less than 0.5).

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