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Novel 6-*O*-acylated vitamin C derivatives as hyaluronidase inhibitors with selectivity for bacterial lyases

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Abstract—Previously, we identified ascorbic acid 6-*O*-hexadecanoate as an up to 1500 times more potent inhibitor of bacterial and bovine hyaluronidases than the parent compound, vitamin C, and determined a crystal structure of hyaluronidase from *Streptococcus pneumoniae* in complex with the inhibitor. As the alkanoyl chain interacts with a hydrophobic patch of the enzyme we synthesized other 6-*O*-acylated vitamin C derivatives bearing various lipophilic residues and investigated the inhibition of *Streptococcus agalactiae* strain 4755 hyaluronidase (*Sag*Hyal$_{4755}$) and of bovine testicular hyaluronidases (BTH) in a turbidimetric assay. All compounds showed selectivity for the bacterial enzyme. Whereas vitamin C 6-*O*-hexanoate only weakly inhibited *Sag*Hyal$_{4755}$, the inhibition of both enzymes increased with the length of the aliphatic chain. In the case of the 6-*O*-octadecanoate, IC$_{50}$ values of 0.9 and 39 μM for *Sag*Hyal$_{4755}$ and BTH, respectively, were determined. Partial replacement of the aliphatic chain with a phenyl, p-phenylene or p-biphenylyl group resulted in inhibitors with activity in the lower micromolar range, too. The title compounds are among the most potent inhibitors of both enzymes known to date.

Hyaluronidases are enzymes degrading hyaluronic acid (hyaluronan), an important component of the extracellular matrix. Hyaluronan consists of 1 → 4-linked β-D-glucuronic acid-(1 → 3)-β-*N*-acetyl-D-glucosamine disaccharide units. According to their catalytic mechanism, the hyaluronidases are divided into three main families. In contrast to hyaluronidases from other sources, bacterial hyaluronan lyases (EC 4.2.2.1) degrade the substrate into unsaturated disaccharide units, cleaving the β-1,4-glycosidic bond between *N*-acetyl-β-D-glucosamine and D-glucuronic acid residues by an elimination reaction.[1] As the role of hyaluronidases is far from being understood, potent and selective inhibitors are required as pharmacological tools. Moreover, inhibitors of the bacterial and mammalian enzymes might be useful agents in the treatment of various diseases, for example, bacterial infections,[2] cancer,[3] or as contraceptives.[4]

Vitamin C, reported to inhibit the bovine testicular hyaluronidase (BTH),[5] was found to be a weak inhibitor of the hyaluronan lyase of *Streptococcus pneumoniae* (IC$_{50}$ value: 5.8 mM). X-ray analysis verifies that ascorbic acid binds to the active site[6] and suggests that additional interactions with a hydrophobic patch might increase the potency. Therefore, we investigated L-ascorbic acid-6-*O*-hexadecanoate (vitamin C palmitate, Vcpal, cpd. **13i** in Table 1), a highly effective antioxidant, for the inhibition of hyaluronan lyases from *S. pneumoniae* (*Spn*Hyal) and *Streptococcus agalactiae* strain 4755 (*Sag*Hyal$_{4755}$) and of BTH. Furthermore, *Spn*Hyal was crystallized in complex with Vcpal and the X-ray structure was determined.[7] Compared to vitamin C, Vcpal is an up to 1500 times more potent inhibitor of bacterial and bovine hyaluronidases.

As shown in Figure 1, Vcpal binds exactly in the same region of the catalytic cleft of *Spn*Hyal as a hexasaccharide substrate.[8] In addition to hydrogen bonds of the carboxylate group of the inhibitor (the ring-opened form of ascorbic acid was found in the crystal structure) with Tyr408 and Arg462, and of the hydroxy groups at C-4 and C-5 with Asn290, extensive interactions with the hydrophobic patch (Trp291, Trp292, Phe343) are

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Keywords: Ascorbic acid derivatives; Hyaluronidase inhibitors; Hyaluronan; Hyaluronan lyases; Bovine testicular hyaluronidase.

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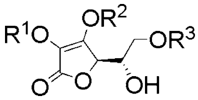
Table 1. Inhibitory effects of L-ascorbic acid derivatives on hyaluronan lyase from *S. agalactiae* strain 4755 (***Sag*****Hyal**$_{4755}$) and bovine testicular hyaluronidase (BTH; Neopermease®) at pH 5.0



1, 6-11, 13a-t

Compound	R^1	R^2	R^3	*Sag*Hyal$_{4755}$	BTH
				IC_{50} [µM] or % inhibition[a], pH 5.0	
1	H	H	H	6100 ± 100	Inactive[b]
6	Me	Me	H	Inactive[c]	Inactive[c]
7	Bn	Bn	H	355 ± 59	Inactive[c]
8	CH_2CH_2		H	24% (2000)	Inactive[c]
9	Me	Me	$CO(CH_2)_{14}CH_3$	5% (160)	Inactive[d]
10	Bn	Bn	$CO(CH_2)_{14}CH_3$	Inactive[d]	Inactive[d]
11	CH_2CH_2		$CO(CH_2)_{14}CH_3$	32% (190)	Inactive[d]
13a	H	H	$COC(CH_3)_3$	43% (1100)	Inactive[e]
13b	H	H	$CO(CH_2)_4CH_3$	475 ± 16	Inactive
13c	H	H	$CO(CH_2)_6CH_3$	772 ± 19	Inactive
13d	H	H	$CO(CH_2)_8CH_3$	102 ± 5	1380 ± 49
13e	H	H	$CO(CH_2)_9CH_3$	72 ± 2	580 ± 21
13f	H	H	$CO(CH_2)_{10}CH_3$	47 ± 2	208 ± 4
13g	H	H	$CO(CH_2)_{11}CH_3$	14.3 ± 0.3	96 ± 5
13h	H	H	$CO(CH_2)_{12}CH_3$	8.4 ± 0.2	71 ± 1
13i	H	H	$CO(CH_2)_{14}CH_3$	4.2 ± 0.1	57 ± 1
13j	H	H	$CO(CH_2)_{16}CH_3$	0.9 ± 0.1	39 ± 1
13k	H	H	COPh	132 ± 6	33% (1430)
13l	H	H	$COCH_2$-*p*-C_6H_4-Ph	358 ± 15	2006 ± 40
13m	H	H	$CO(CH_2)_5OPh$	717 ± 28	Inactive
13n	H	H	$CO(CH_2)_5OCH_2Ph$	437 ± 77	Inactive
13o	H	H	$CO(CH_2)_5O$-*p*-C_6H_4-Ph	61 ± 1	188 ± 3
13p	H	H	$CO(CH_2)_5OCH_2$-*p*-C_6H_4-Ph	102 ± 2	543 ± 15
13q	H	H	$CO(CH_2)_5O$-*p*-C_6H_4-C_2H_5	280 ± 9	Inactive
13r	H	H	$CO(CH_2)_5O$-*p*-C_6H_4-OCH_2Ph	76 ± 1	210 ± 3
13s	H	H	$CO(CH_2)_{10}O$-Ph	31 ± 0.4	105 ± 1
13t	H	H	$CO(CH_2)_{10}O$-*p*-C_6H_4-Ph	7.5 ± 0.2	37 ± 0.4

[a] Inhibition of enzyme expressed as IC_{50} (µM), mean values ± SEM ($N = 2$, experiments performed in duplicate), or as % inhibition at inhibitor concentration given in parentheses; substances were tested up to a concentration of 1.5 mM unless otherwise indicated.

[b] At concentrations $\leqslant$100 mM.

[c] At concentrations $\leqslant$ 13 mM.

[d] At concentrations $\leqslant$200 µM.

[e] At concentrations $\leqslant$ 1.1 mM.

evident. The lipophilic face of the vitamin C portion lies flat on the side chain of Trp292, and the palmitoyl group interacts with Trp291 and Phe343, also with His399 and Thr400. This prompted us to synthesize various ascorbic acid derivatives with increased lipophilicity as potential hyaluronidase inhibitors.

The ascorbic acid derivatives **9**–**11** were synthesized according to a modified four-step reaction sequence by analogy with the procedure described by Sanders[9] (Scheme 1). First the acetal **2** was formed, which was alkylated to obtain the 2,3-di-*O*-protected derivatives **3**–**5**. After cleavage of the isopropylidene-protecting group, acylation was carried out using palmitoyl chloride to obtain compounds **9**–**11**. The 6-*O*-acylated ascorbic acid derivatives **13a**, **b**, **i**–**k** were accessible by acylation of benzyl-protected ascorbic acid **7** followed by hydrogenolysis of the benzyl ether groups (Scheme 2, Method A). For the synthesis of the 6-*O*-acylated ascorbic acid derivatives **13c**–**h**, **l**–**t** an alternative procedure was used (Scheme 2, Method B): ascorbic acid was treated with the corresponding methyl esters in the presence of lipase from *Candida antarctica* immobilized on acrylic resin (Novozyme 435®) at 60 °C under reduced pressure (200 mbar).[10,11]

The synthesized compounds were investigated for inhibition of *Sag*Hyal$_{4755}$ and BTH at pH 5.0 in a modified turbidimetric assay[16] based on the method of Di Ferrante.[17] Under these conditions both enzymes show comparably high activity.[18] The results are summarized in Table 1 and exemplarily depicted in Figure 2.

Ascorbic acid (**1**) is only a very weak inhibitor of the bacterial enzyme. Structural modifications of the enediol system as in **6** and **8** are not tolerated, whereas the 2-*O*-, 3-*O*-dibenzylated analog **7** is more active than **1**. This is in agreement with the hypothesis that the potency of hyaluronidase inhibitors derived from vitamin C may be increased by additional hydrophobic interactions

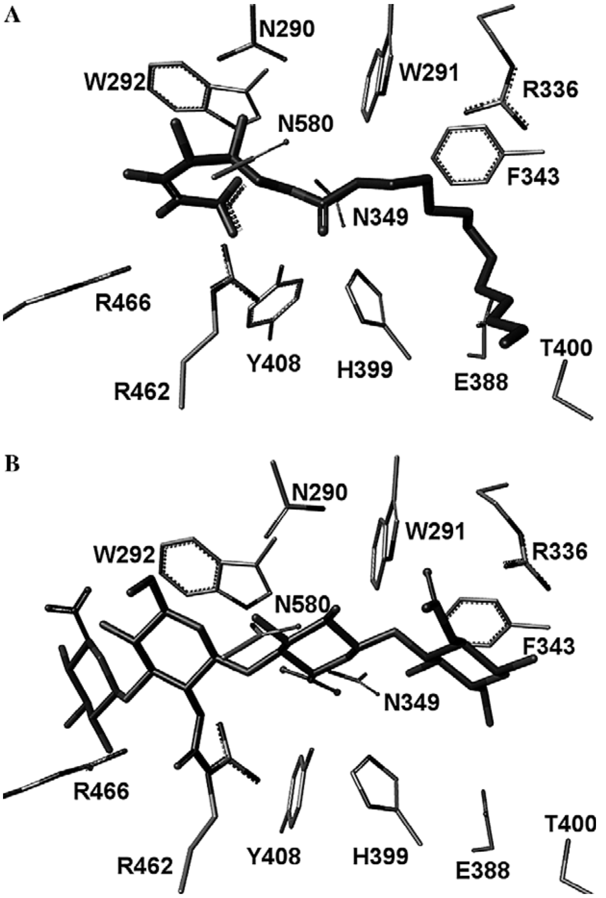
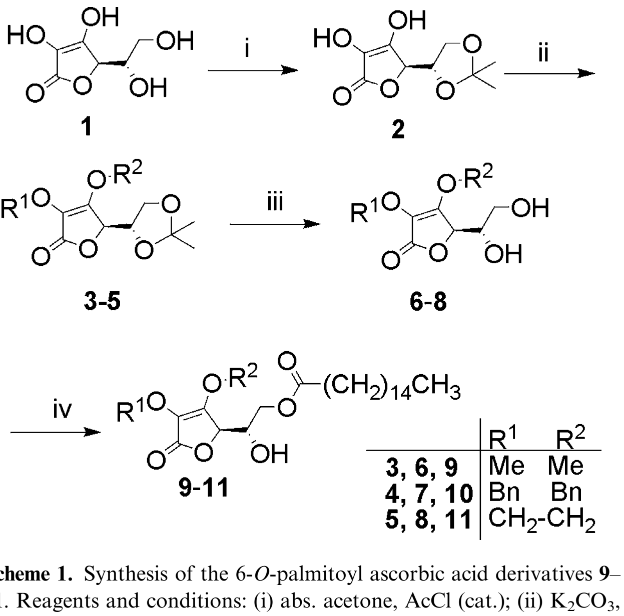
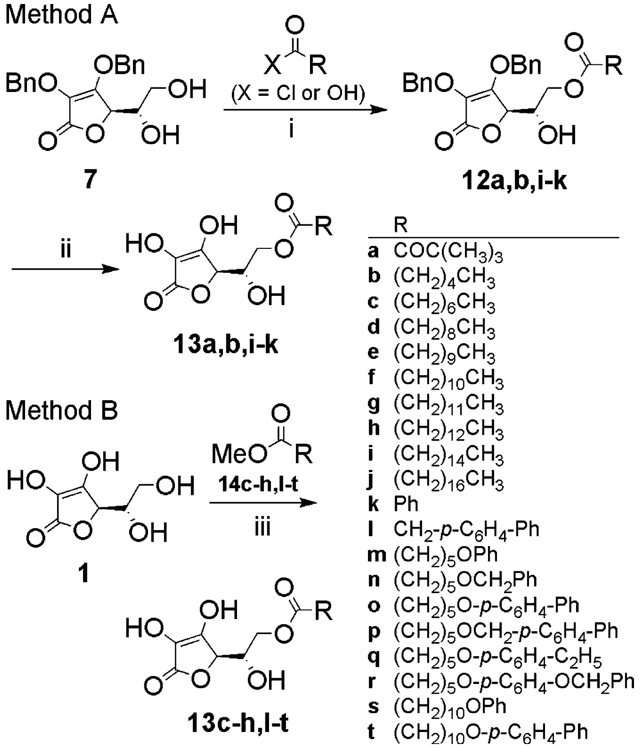


Figure 1. Binding modes of Vcpal and a hyaluronan hexasaccharide. (A) Binding mode of Vcpal (**13i**) in the active site of *Spn*Hyal (PDB code 1w3y). The inhibitor was found to bind in a ring-opened form. Missing electron density did not permit modeling of the last three carbon atoms of the palmitoyl chain. (B) *Spn*Hyal in complex with a hyaluronic acid hexasaccharide (PDB code 1loh, for clearity only the tetrasaccharide is shown).



Scheme 1. Synthesis of the 6-*O*-palmitoyl ascorbic acid derivatives **9**–**11**. Reagents and conditions: (i) abs. acetone, AcCl (cat.); (ii) K_2CO_3, MeI or BnBr or 1,2-dibromoethane, DMF, 40–60 °C; (iii) 50% HOAc, MeOH, 80 °C; (iv) $CH_3(CH_2)_{14}COCl$, pyridine, dichloromethane (DCM), 4-dimethylaminopyridine (DMAP) (catalytic amount).



Scheme 2. Synthesis of the 6-*O*-acylated ascorbic acid derivatives **13a**–**t**, Reagents and conditions: (i) X = Cl: pyridine/DCM, DMAP (cat.); X = OH: 1,1′-carbonyl-diimidazole (CDI) or dicyclohexyl carbodiimide (DCC), DCM, DMAP (cat.); (ii) 10% Pd/C (cat.), H_2, 4 bar, EtOH. (iii) Lipase acrylic resin from *Candida antarctica*, *tert*-amyl alcohol, 60 °C, 200 mbar, 16–24 h. The alkanoic acids and acid chlorides used for the preparation of **12a**, **b**, **i**–**k** are commercially available. The building blocks **14c**–**h**, **l**–**t** were prepared according to standard procedures starting from the corresponding commercially available alkanoic acids, ω-bromo-alkanoic acids, methyl ω-bromoalkanoates or from ε-caprolacton.[12–15]

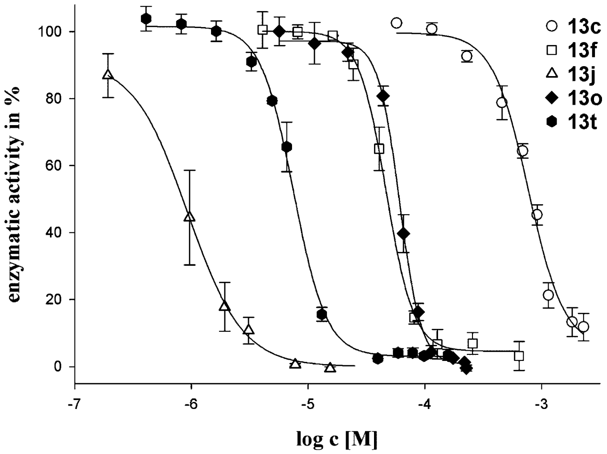


Figure 2. Enzymatic activity of hyaluronan lyase from *S. agalactiae* strain 4755 in the presence of compounds **13c**, **f**, **j**, **o**, **t** at pH 5.0.

with amino acids in the active site. Regarding compounds **13b**–**j**, the inhibition of both enzymes increases with the length of the 6-*O*-acyl residue, hence the octadecanoate **13j** inhibits *Sag*Hyal$_{4755}$ and BTH with the lowest IC_{50} values of 0.9 and 39 μM, respectively. Compound **13k**, bearing only a relatively small benzoyl residue, is more active than the alkanoyl analogs **13b** and **13c** on the bacterial enzyme. Structural modifications at the hydroxy groups of the enediol system of the strong hyaluronidase inhibitor **13i** (see compounds **9**–**11**) led to significantly lower potency on the bacterial enzyme and to inactivity on the BTH, respectively. Comparing compounds **13m** with **13o** and **13s** with **13t**, a remarkably increased inhibitory potency on both enzymes results when the phenyl groups are replaced with *p*-biphenylyl residues. The synthetic approach using an ether linkage to introduce aromatic residues makes the desired compounds easily accessible. Moreover, the ether group is capable of forming H-bonds in the active site by analogy with the substrate hyaluronan. The more rigid phenyl and biphenylyl residues are suitable alternatives to the flexible alkyl chains, as the inhibitory activities are in the same range as those of the corresponding aliphatic compounds.

In summary, the presented 6-*O*-acyl derivatives of L-ascorbic acid are among the most potent inhibitors of hyaluronidases known so far. The title compounds are rather selective for the bacterial enzyme and represent promising lead structures for rational optimization in ongoing work.

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