

Lithocholic acid analogues, new and potent α -2,3-sialyltransferase inhibitors†

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A new type of noncompetitive α -2,3-sialyltransferase inhibitor has been synthesized; we report the discovery, preparation and inhibitory activity of sixteen lithocholic acid analogues.

Sialic acids, nine-carbon amino sugars bearing a negative charge under physiological conditions, are cell-type specific markers that are widely distributed throughout the organism.¹ Sialyltransferases (ST), a class of glycosyltransferases, catalyze the transfer of sialic acids to the terminal positions of growing oligosaccharide chains of glycoconjugates.² Hypersialylation at the non-reducing end of glycoproteins or glycolipids is a vital biological process, which plays a role in various physiological and pathological events, such as cell-cell adhesion, immune defense, tumor cell metastasis, invasions, and inflammation.³ Therefore, the discovery of potent inhibitors of ST has become a promising strategy for chemotherapeutic treatment of cancer⁴ and can be an invaluable tool to study ST-dependent physiology in immune responses.⁵ Lately, ST inhibitors with a structural mimetic of transition-state analogues, bisubstrate analogues, donor analogues, and acceptor analogues have been developed based on cytidine monophosphate-*N*-acetylneuraminic acid (CMP-Neu5Ac) or disaccharides.⁶ Although these compounds have been demonstrated to effectively inhibit STs, the inhibitors display poor permeability across cell membranes and their clinical applications for studying the biology of glycoconjugates are relatively limited.⁷ To our knowledge, few ST inhibitors with a cell-permeable property have been reported so far.^{7–8} Here we describe the discovery and synthesis of lithocholic acid analogues, which employ a steroidal moiety to improve their uptake,⁹ and demonstrate noncompetitive inhibition of α -2,3-sialyltransferase (α -2,3-ST) in the presence of CMP-Neu5Ac.

Soyasaponin I (**1**),⁸ a rigid pentacyclic system with trisaccharide from soybean saponin, was a new type of ST inhibitor and showed a significant inhibition ($K_i = 2.1 \mu\text{M}$) toward α -2,3-ST *in vivo*. Compound **1**, where the pentacyclic ring is similar to the main skeleton of steroidal compounds (Fig. 1), exhibits important properties relating not only to cell permeability but also to invasive/metastatic behaviors of cancer cell lines.¹⁰ Attractive attention was stimulated by this report and we reasoned that some steroid-related compounds might have potential for inhibiting α -2,3-ST activity. As a result of our initial screening efforts, we have identified epiandrosterone succinyl ester (**2**), a potent *Schistosoma japonicum*

glutathione *S*-transferase inhibitor,¹¹ and lithocholic acid (**3**), a substrate of nuclear pregnane X receptor,¹² as potent inhibitors of α -2,3-ST with IC₅₀ values of 350 and 21 μM , respectively.

Synthesis of epiandrosterone derivatives **4–5** and **27–28** was achieved as depicted in Scheme 1. Epiandrosterone **23** was esterified with the required amino acids to yield the corresponding esters **24–26**, which were subsequently converted to the epiandrosterone-amino acid conjugates **4** and **27–28** by alkaline hydrolysis of the Fmoc and acidolytic cleavage of the remaining *t*Bu or Boc groups. Efficient formation of epiandrosterone-phosphate conjugate **5** was obtained from **23** via a three-step procedure (Scheme 1). The alcohol **23** was converted to the bis-(*p*-nitrophenyl)epiandrosteronyl phosphate intermediate **29** by carefully controlling the release of one equiv. *p*-nitrophenol with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), followed by alkaline-mediated replacement of the *p*-nitrophenyl with methyl. The final product **5** was accomplished by quantitative silyldemethylation of the *P,P*-dimethyl groups of **30** with

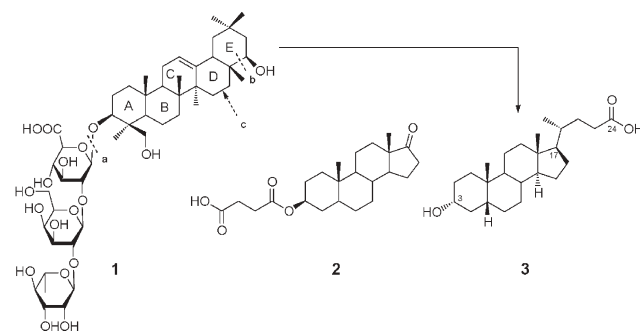
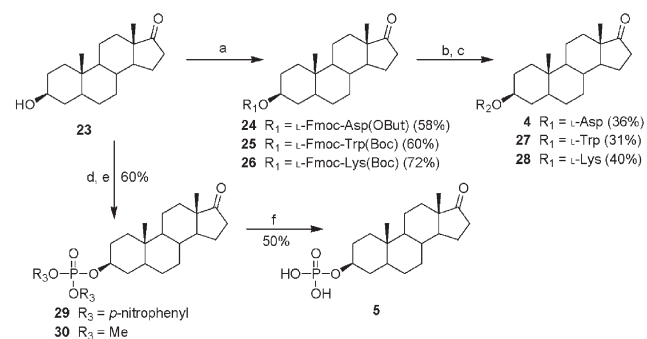


Fig. 1 Main skeleton of lithocholic acid **3** is depicted as being formed from **1** by cleaving bonds a and b, followed by converting the D-ring into a 5-membered ring, and oxidizing the terminal alcohol into carboxylic acid.

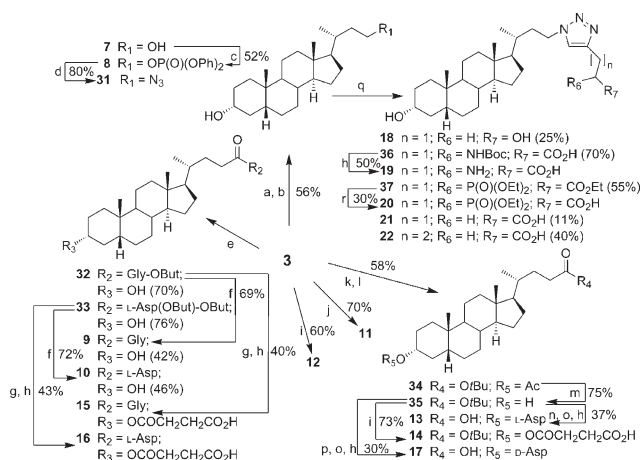


Scheme 1 (a) Protected amino acid, DCC, DMAP, DCM, r.t., 30 min; (b) DBU, DCM, r.t., 30 min; (c) trifluoroacetic acid (TFA), 2% H₂O or 4 N HCl, MeOH, r.t., 1 h; (d) P(O)(*p*-nitrophenyl)₃, DBU, DCM, r.t., 15 h; (e) DBU, MeOH, DCM, r.t., 15 h; (f) bromotrimethylsilane, DCM, r.t., 30 min.

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Scheme 2 (a) MeOH, Amberlite IR120, 80 °C, 12 h; (b) LAH, THF, 0 °C → r.t., 10 min; (c) DPPA, DBU, THF, r.t., 10 h; (d) NaN₃, TBAI, 15-crown-5, 110 °C, 15 h; (e) HBTU, DIPEA, H-Gly-OBu-HCl or L-Asp, *N,N*-dimethylformamide (DMF), r.t., 30 min; (f) TFA, DCM, r.t., 1 h; (g) succinic anhydride, DMAP, pyridine, 80 °C, 15 h; (h) TFA, 2% H₂O, r.t., 1 h; (i) succinic anhydride, DMAP, pyridine, 60 °C, 15 h; (j) CrO₃, acetic acid, 100 °C, 30 min; (k) acetic anhydride, pyridine, r.t., 5 h; (l) *t*-BuOH, DMAP, DCC, DCM, r.t., 30 min; (m) NaOCH₃, MeOH, r.t., 2 h; (n) Fmoc-L-Asp(OBu)-OH, DMAP, DCC, DCM, r.t., 30 min; (o) DBU, DCM, r.t., 1 h; (p) Fmoc-D-Asp(OBu)-OH, DMAP, DCC, DCM, r.t., 30 min; (q) alkyne, CuSO₄·5H₂O, sodium ascorbate, THF/H₂O (1 : 1), r.t., 8 h; (r) NaOH, EtOH/H₂O (1 : 1), r.t., 5 h.

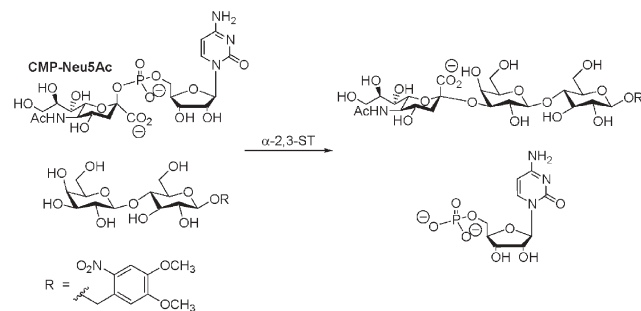
bromotrimethylsilane.¹³ Coupling of succinic anhydride to androsterone in the presence of 4-(dimethylamino)pyridine (DMAP) and pyridine produced the desired isomer **6**.

In addition, Scheme 2 illustrates the synthesis of lithocholic acid analogues **7–22**. Compound **3** was treated with Amberlite IR120 in anhydrous methanol, and reduction of the resulting methyl ester gave the alcohol **7**. The hydroxyl group of **7** was converted to a diphenylphosphate group of **8** in the presence of diphenyl phosphorylazide (DPPA) and DBU. Condensation of **3** and the corresponding protected amino acids, H-Gly-OBu and L-H-Asp(OBu)-OBu, using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/diisopropylethylamine (DIPEA) as coupling agent furnished the protected conjugates **32–33**. Compounds **9–10** were obtained after removal of the remaining *t*Bu groups. Analogues **15–16** were prepared by the esterification of **32–33** and succinic anhydride in a manner similar to **6**. Oxidation of **3** with CrO₃ in acidic conditions produced **11** in 70% yield. Compound **12** was synthesized in a manner similar to that described for **6**, **15** and **16** by esterification of **3** with succinic anhydride. In order to prepare analogues **13–14** and **17**, the hydroxyl group of **3** was transformed to an acetyl group, and subsequent esterification gave **34**. Intermediate **35**, derived from **34** by deacylation, was converted to the desired product **13** by dicyclohexylcarbodiimide (DCC)-promoted coupling followed by deprotection of the Fmoc, Boc and *t*Bu groups. Esterification of the secondary alcohol **35** gave compound **14**. The D-form amino acid–lithocholic acid conjugate **17** was synthesized in a manner similar to that described for **13**. The 1,2,3-triazoles **18–22** required first the synthesis of the terminal azide **31** (Scheme 2). The conversion of diphenylphosphate **8** to the corresponding azide **31** was achieved in the presence of excess sodium azide (NaN₃), a catalytic amount of tetrabutylammonium iodide (TBAI), and 15-crown-5.¹⁴ Reliable

preparation of various 1,4-disubstituted 1,2,3-triazoles **18**, **21–22** and **36–37** was successful using the copper(I)-catalyzed ligation of azide **31** and their respective alkynes according to the click chemistry concept approach.¹⁷ Subsequent Boc deprotection or saponification afforded the racemic derivatives **19** or **20**.

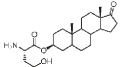
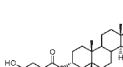
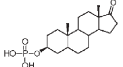
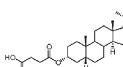
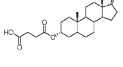
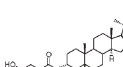
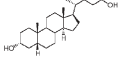
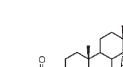
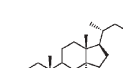
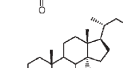
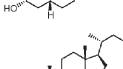
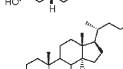
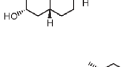
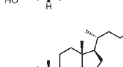
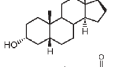
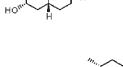
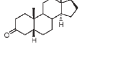
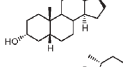
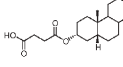
The inhibition assay of α -2,3-ST was done as described by Schmidt¹⁵ using CMP-Neu5Ac as a substrate and the modified disaccharide¹⁶ with a 4,5-dimethoxy-2-nitrobenzyl group as a UV-labelled acceptor (Scheme 3). To enhance the potency in compound **2**, we initially prepared four derivatives **4–5** and **27–28** with alternations of the succinyl ester unit. However, efforts to improve the IC₅₀ were unsuccessful, for example, replacement of the succinyl moiety of **2** with aspartyl, phosphate, Tyr, or Lys either reduced inhibitory activity (IC₅₀ > 350 μ M for **4–5**) or gave a product displaying no biological functions (**27–28**) toward α -2,3-ST (Table 1). In addition, a minor influence on the inhibitory property (IC₅₀ = 450 μ M for **6**) of the α isomer of **2** has been observed, suggesting negligible interaction in the recognition of epiandrosterone/androsterone analogues with succinyl-stereochemistry by the enzyme.

Lithocholic acid (**3**), which potentially mimics a pentacyclic ring of **1** (Fig. 1), showed a promising inhibition constant (IC₅₀ = 21 μ M), indicating an acceptable pharmacophore. Among the 16 synthetic analogues (Table 1), the terminal alcohol **7** and its derivative **8** displayed a 5–17-fold decrease over **3**, suggesting that a carboxylic acid group is important for promoting affinity. This was further confirmed by utilizing the method of peptide coupling to extend the terminal carboxylic acid; the inhibitory properties of compounds **9** and **10** can be restored completely compared to those of **7–8**. To determine the importance of the 3-hydroxyl position of **3**, the inhibition of α -2,3-ST activity by compound **11**, which has a ketone moiety in place of a hydroxyl group, was evaluated. Compound **11** was found to have an IC₅₀ of 139 μ M, representing 7-fold lower potency than **3**. Furthermore, compounds **12** and **13** exhibited a 2-fold potency increase over **3**, indicating that construction of the 3-hydroxyl portion of **3** is tunable to the interaction of binding affinity. When the terminal carboxylic acid of **12** was varied, the analogues **14–16** appeared to reach a low micromolar affinity plateau (Table 1). Surprisingly, replacement of the D-Asp with L-Asp, as in **17**, gave a 2-fold improvement in potency over **13**. In addition, compound **3** was further optimized by replacing the carboxylic acid with a 1,4-disubstituted 1,2,3-triazole ligand using the click chemistry approach. Compounds **20** and **21** showed an 8–12-fold potency increase over **19**, suggesting that adding the positively charged amino group could abolish affinity. The primary alcohol **18** was at least 10-fold less potent than **21** and 20-fold less potent than **22**.



Scheme 3 α -2,3-ST catalyzed sialylation of acceptor.

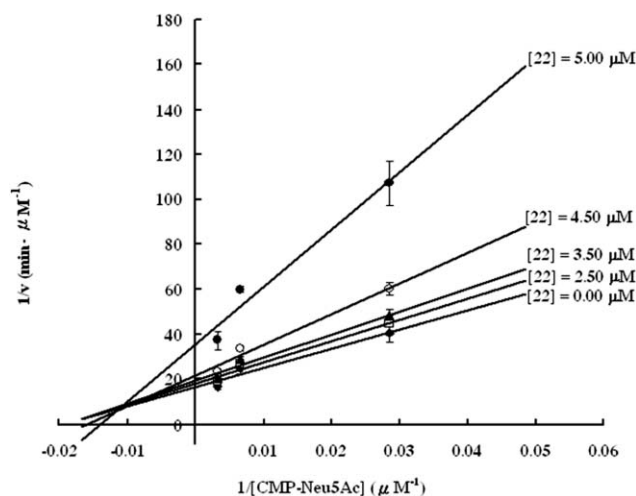
Table 1 α -2,3-Sialyltransferase^a inhibitors

Compound	#	IC ₅₀ (μM) ^b	Compound	#	IC ₅₀ (μM) ^b
	4	480		14	13
	5	400		15	22
	6	450		16	18
	7	351		17	6
	8	> 100		18	> 100
	9	25		19	83
	10	16		20	7
	11	139		21	10
	12	12		22	5
	13	12			

^a Note that rat liver α -2,3-ST was obtained from CalBiochem at a concentration of 1 mU/μL. ^b The inhibition assay was carried out as described in ref. 15, except for the use of the modified disaccharide¹⁶ as the acceptor.

These results are consistent with previous observations concerning the value of the terminal carboxylic acid in binding. Analogue **22** was the most active compound with an IC₅₀ of 5 μM. The Lineweaver–Burk plot (Fig. 2) shows that compound **22** exhibits a noncompetitive inhibition ($K_i = 2.2$ μM) toward cytidine monophosphate *N*-acetylneuraminic acid (CMP-Neu5Ac). However, we failed to identify the mode of inhibition between **22** and acceptor due to the solubility problem derived from the modified disaccharide with a 4,5-dimethoxy-2-nitrobenzyl moiety. It is possible that compound **22** binds to α -2,3-ST and alters the structure of the active site, resulting in the decrease of enzyme activity. Due to the potential of compounds **11**, **13**, **15–16**, and **19–22** as anticancer drugs, our collaborators recently evaluated their cytotoxicity using *in vitro* assays against several tumor cell lines. These results will be published elsewhere.

Because of the physiological and pathological significance of ST, there is strong demand for an efficient approach to searching for cell-permeable inhibitors. This is the first report of a strategy for designing effective α -2,3-ST inhibitors based on the structure of an authentic inhibitor. Analogues **17**, **20**, and **22** display more potent

**Fig. 2** Lineweaver–Burk plot of the results of rat α -2,3-ST inhibition assay for the synthetic inhibitor **22**.

inhibitory activities among these synthetic derivatives. The results demonstrated here should pave the way for the design of other ST inhibitors.

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