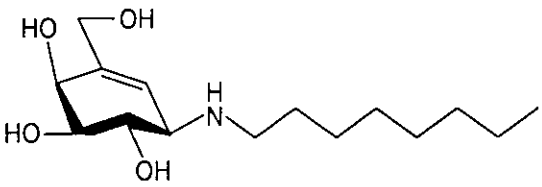
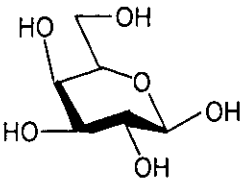


N-オクチル-4-エピ-β-バリエナミン
(NOEV)



ガラクトース

1. 分子量 287.40
2. 室温で安定
3. 水 (3 mM),メタノール,DMSO に溶解
4. 試験管内：酵素活性を阻害 (競合的-高濃度：IC_{50}=0.2 μM)
5. 細胞内：酵素活性を復元 (低濃度)

図3 NOEV の構造と物性

コア構造はガラクトースに似ているが，ガラクトースのC1側鎖基部とC1～5結合部はガラクトースと異なる．

(NOEV) がわれわれの目的にもっとも有効であることを確認した．

この化合物には炭化水素側鎖がついているため，水に対する親和性は高くない．しかしこのフリー体のままでも3～5 mM 濃度であれば容易に水に溶解する．室温で安定であり，試験管内での50%酵素阻害濃度は 0.2 μM である．この化合物をヒト β-ガラクトシダーゼ欠損症患者由来の線維芽細胞培養液に添加すると，特定の変異遺伝子に対して活性化効果のあることがわかった．

V. β-ガラクトシダーゼ欠損症に対するケミカルシャペロン療法の試み

上記の予備的な結果をもとに，ヒト線維芽細胞，マウス線維芽細胞，われわれが開発したモデルマウス個体に対してこの薬剤投与実験を開始した．その成果は最近のわれわれの報告[11]にまとめた．

1．ヒト線維芽細胞

ヒト患者由来線維芽細胞の培養液に 0.2～2 mM の濃度に NOEV を添加し，4日間培養したところ，酵素活性が著しく上昇する細胞株があった．とくに若年型あるいは成人型症例にみられる R 201 C，R 201 H 変異ではその反応が大きかった．全体として若年型症例にもっとも有効であり，乳児型，成人型症例にも3倍以上の活性上昇を示す症例があった．すべての症例をあわせて 30%の細胞が陽性反応を示した．モルキオ B 病にははっきりした反応がみられなかった．

2．マウス線維芽細胞

すでに確立し，報告した β-ガラクトシダーゼ欠損ノックアウトマウス[12)13)]の線維芽細胞を培養し，それにヒト β-ガラクトシダーゼ欠損患者の病型特異的な変異をもつ cDNA を導入して，この変異を発現する細胞株を確立した[10]．これらの細胞の培養液に NOEV を投与したところ，ヒト細胞とは多少の違いがあるが，ファブリー病で確認した 1-デオキシガラクトノジリマイシンの 1,000～2,000 倍の活性化効果を認めた．そしてやはり R 201 C 変異がもっとも有効であった[11]．

この培養系にガングリオシドを負荷したところ，正常遺伝子を発現する細胞には基質の蓄積がみられなかったのに対し，R 201 C 発現細胞ではガングリオシド G_{M1} とそのアシアロ体 G_{A1}

表 G_{M1}-ガングリオシドーシスモデルマウスへのNOEV投与効果

NOEV濃度	大脳	小脳	心	肺	肝	脾	腎	精巣 卵巣	筋肉
0 mM	5.3	8.7	12.0	8.1	23.6	8.6	35.4	26.6	3.6
0.1 mM	9.6	13.5	79.3	50.6	165.8	48.9	119.8	194.3	14.0
1 mM	24.4	28.6	225.8	114.9	165.2	142.4	183.4	195.1	61.6

マウス：R 201 C変異酵素を発現するノックアウト・トランスジェニックマウス
NOEV：水溶液としてアドリブに1週間投与
数字はβ-ガラクトシダーゼ活性：nモル/mg蛋白質/30分
脳を含むすべての組織でNOEV濃度依存的に著しい酵素活性上昇を認めた．

の著しい蓄積を示した．そして培養液にNOEVを添加したところ，これらの基質が著しく減少した[11]．

これらの結果を踏まえ，以後の実験はR 201 C変異を対象とした実験を行った．

3．モデルマウスの作成と個体へのNOEV投与

動物個体実験のために，まず上記のノックアウトマウスにR 201 Cその他のヒトβ-ガラクトシダーゼ欠損症の病型特異的な変異遺伝子をトランスジーンとして導入したモデルマウスを作成した．

このなかで，正常なβ-ガラクトシダーゼを発現する遺伝子GP 8を導入したマウスは病気を発現せず，まったく正常の個体として生存した．つまり，この遺伝子過剰発現系の動物個体については遺伝子治療ができたということになる．全身病に対する遺伝子治療は困難であるが，このアプローチであれば操作は容易である．もちろん，この結果をすぐにヒト個体に適用するわけにはいかないが，理論的には操作が可能であるということになる．ただしヒトのように生存期間が長い個体では，トランスジーンによる長期の酵素活性過剰発現の影響を考慮しなければならない．

次にR 201 CマウスにNOEVの1 mM水溶液を1週間経口投与した．脳を含むすべての組織の酵素活性が著しく上昇し（表），病理学的，免疫組織化学的にも，脳組織のガングリオシド蓄積が消失した．これは発症前のマウス実験であり，臨床効果を観察することはできなかったが，今後は発症前から長期の薬剤投与を行い，発症予防効果があるかどうか検討する必要がある．

VI. NOEVについてわかったこと，検討すべきこと

以上の細胞，動物個体の実験から，次のようなことがわかった．

① 低分子化合物NOEVが腸管内で吸収され，血液に入り，血液脳関門を通って脳組織に入ること：これは現在試みているNOEVの血液内，組織内濃度の定量的測定によっても確かめられた．つまり，これまでに試みられたことのない，脳の遺伝病に経口薬を投与するという新しい治療的アプローチが理論的に可能となったということである．

② 培養細胞実験で確認されたように，NOEVが脳細胞のなかで変異蛋白質を安定化，活性化し，実際に異常に蓄積した基質（脂質）を分解すること：ガングリオシドG_{M1}もそのアシアロ体もともに分解された．今回は1 mMという一定の濃度での実験データであるが，今後は投与量や投与方法による効果の違いが出るか

どうかを検討する.

③ NOEV の臨床効果：形態的，化学的分析により確かめた治療効果が，実際に臨床症状を修飾し，発症を予防し，症状を軽快させることの確認が必要である．現在検討中である.

④ NOEV が安定な化合物であること：これまでの実験では長期間，室温でまったく変化しないことがわかった．これは薬剤としての必須の条件であり，さらに安定性についての吟味を続ける.

⑤ 毒性の検討：薬剤として開発するためには，効果だけでなく長期投与に伴う毒性の検討が必須である．これまでのところ，5週間までは，体重，飲水量，血液生化学などにみるべき変化はなかった．さらに長期の投与による影響を確認する.

⑥ より有効な化合物の検索：NOEV がわれわれの目的にもっとも優れた化合物であるという保証はない．現在，いくつかの化合物をテスト中である．なかには同程度の活性をもち，かつ薬剤としての物性が優れている化合物もある.

⑦ NOEV の変異遺伝子特異的な効果：この化合物がすべての変異蛋白質に有効であるわけではない．変異遺伝子が分子として活性をもつ変異蛋白質を発現する，という条件が必須である．つまり同じ名前の病気のすべての患者に一様に適用できる手法ではない．しかし，現在まったく治療法のない病気の一部でもこの方法で症状の軽減，予防が可能になれば，きわめて大きな学問的，社会的な意味をもつ.

Ⅶ. 他のライソゾーム病・他の遺伝病への応用

すでに述べたように，この研究はファブリー病から始まった．現在の主要な研究対象は脳病変を発現する β-ガラクトシダーゼ欠損症候群，すなわち G_{M1}-ガングリオシドーシスである．モルキオ B 病については効果が確認できていない．しかし原理的にこのアプローチはすべてのライソゾーム病に適用できるはずである．実際，β-グルコシダーゼ欠損症候群（ゴーシェ病）でも，グルコース類似の化合物が，少なくとも培養細胞で有効であることが確認できている[14]．また α-ガラクトシダーゼに有効な NOEV 類縁体も検索中である.

このように，現在のところ，ガラクトースとグルコースの α および β 結合を認識する酵素についての分析が進行中であるが，今後はほかのライソゾーム酵素欠損症について，順次対象を広げる予定である.

たまたまわれわれはライソゾーム病という細胞内分子病態がかなり明らかにされた疾患群を対象としてきたが，ほかのカテゴリーの遺伝病でも，もしその分子病態，つまり変異遺伝子の発現，変異蛋白質の分子修飾，活性発現部位への細胞内移動，活性発現機構などが明らかにされれば，同じ原理の治療的アプローチが可能になるはずである.

その意味で，この分子治療法，ケミカルシャペロン療法が，今後多くの病気について検討され，実用化されることを期待している.

本稿の β-ガラクトシダーゼ欠損症の研究は国際医療福祉大学をはじめ，以下の多くの施設の研究者との共同研究により進行中である．黒澤美枝子(国際医療福祉大学基礎医学研究センター)，岩崎博之，渡辺浩史（国際医療福祉大学臨床医学研究センター)，松田潤一郎(国立感染症研究所獣医科学部)，難波栄二(鳥取大学生命機能研究支援センター)，大野耕策（鳥取大学医学部)，飯田真己(生化学工業中央研究所)，小川誠一郎（慶應義塾大学理工学部)．以上の代表者とともに，各施設の多くの共同研究者のご協力をいただいた．また α-ガラクトシダーゼ欠損症の研究には石井達（東京都臨床医学総合研究所，現・帯広畜産大学）と樊建強（東京都臨床医学総合研究所，現・マウントサイナイ医科大学）両氏の貢献が大きい．ここに

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N-Octyl-β-valienamine up-regulates activity of F213I mutant β-glucosidase in cultured cells: a potential chemical chaperone therapy for Gaucher disease

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Abstract

Gaucher disease (GD) is the most common form of sphingolipidosis and is caused by a defect of β-glucosidase (β-Glu). A carbohydrate mimic *N*-octyl-β-valienamine (NOV) is an inhibitor of β-Glu. When applied to cultured GD fibroblasts with F213I β-Glu mutation, NOV increased the protein level of the mutant enzyme and up-regulated cellular enzyme activity. The maximum effect of NOV was observed in F213I homozygous cells in which NOV treatment at 30 μM for 4 days caused a ~ 6-fold increase in the enzyme activity, up to ~ 80% of the activity in control cells. NOV was not effective in cells with other β-Glu mutations, N370S, L444P, 84CG and RecNciI. Immunofluorescence and cell fractionation showed localization of the F213I mutant enzyme in the lysosomes of NOV-treated cells. Consistent with this, NOV restored clearance of ^{14}C-labeled glucosylceramide in F213I homozygous cells. F213I mutant β-Glu rapidly lost its activity at neutral pH in vitro and this pH-dependent loss of activity was attenuated by NOV. These results suggest that NOV works as a chemical chaperone to accelerate transport and maturation of F213I mutant β-Glu and may suggest a therapeutic value of this compound for GD.

Keywords: Gaucher disease; β-glucosidase; Valienamine; Glucosylceramide; Chaperone

1. Introduction

Gaucher disease (GD) is an inherited lipid storage disorder, characterized by lysosomal accumulation of glucocerebroside (glucosylceramide; GlcCer) in monocyte-macrophage cells [1]. It is caused by a defect of acid β-glucosidase (β-Glu; glucocerebrosidase EC 3.2.1.45). Patients with GD exhibit hepatosplenomegaly, anemia, bone lesions and respiratory failure, with or without progressive neurological symptoms. Patients without neurological symptoms are classified as type 1, whereas those with neurological symptoms are classified into type 2 (acute infantile form) and type 3 (juvenile form).

Abbreviations: α-Gal A, α-galactosidase A; α-Glu, α-glucosidase; β-Gal, β-galactosidase; β-Glu, β-glucosidase; β-Hex, β-hexosaminidase; NOEV, *N*-octyl-β-epi-valienamine; NOV, *N*-octyl-β-valienamine; DGJ, 1-deoxy-galactonojirimycin; ER, endoplasmic reticulum; GD, Gaucher disease; GlcCer, glucosylceramide; HPTLC, high performance thin layer chromatography; NN-DGJ, *N*-(*n*-nonyl)-deoxy-nojirimaycin

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Current therapeutic strategies for GD include enzyme replacement and substrate depletion. Enzyme replacement has been achieved by intravenous administration of macrophage-targeted recombinant β-Glu [2] and it has been proven to be quite effective for visceral, hematologic and skeletal abnormalities [3,4]. Unfortunately, the efficacy to neurological manifestations of this therapy is, if any, limited [5–7]. A high cost as well as necessity to continue the infusion every 2 weeks is not negligible, when indication of enzyme replacement is considered in practice [4]. Substrate depletion has been achieved by oral administration of *N*-butyl-deoxynojirimycin, which inhibits glucosyltransferase and decreases substrate biosynthesis. This therapy has been reported to be beneficial for non-neuropathic GD [8,9].

We have proposed a novel therapeutic strategy for glycolipid storage disorders to accelerate transport and maturation of mutant enzymes by using enzyme inhibitors as a chemical chaperone. This strategy was first applied to Fabry disease (α-galactosidase A [α-Gal A] deficiency) and we found that 1-deoxy-galactonojirimycin (DGJ), an inhibitor of α-Gal A, markedly enhanced activity of mutant enzymes in lymphoblasts from Fabry patients [10]. Although up-regulation of enzyme activity by an inhibitor appeared paradoxical, evidence was presented that DGJ prevented pH-dependent degradation of mutant α-Gal A at the site of its synthesis [i.e., the endoplasmic reticulum (ER)]. With the aid of DGJ, mutant α-Gal A escaped the ER quality control system and was transported to the lysosome where it is stabilized because of the acidic condition and restored cellular enzyme activity. This strategy was then tested in GM1-galactosidosis [β-galactosidase (β-Gal) deficiency] and we found that DGJ as well as another derivative *N*-(*n*-butyl)-deoxy-galactonojirimycin could up-regulate activity of mutant human β-Gal expressed in fibroblasts from β-Gal knockout mice [11]. In pursuit of the same therapeutic strategy, Sawkar et al. [12] reported that an inhibitor of β-Glu, *N*-(*n*-nonyl)-deoxynojirimaycin (NN-DNJ) up-regulated activity of N370S mutant β-Glu in GD fibroblasts. Although it is yet to be tested whether NN-DNJ can correct GlcCer accumulation in N370S GD cells, their findings suggested that this strategy might be extended to GD.

Valienamine is a synthetic carbohydrate mimic and we have prepared various *N*-alkyl and *N*,*N*-dialkyl-β-valienamines in continuation of a chemical modification program [13]. Among these substances, *N*-octyl-β-epivalienamine (NOEV) is an inhibitor of β-Gal and we have provided evidence that NOEV worked as a chemical chaperone to up-regulate mutant β-Gal activity both in cultured cells and in mice [14]. *N*-octyl-β-valienamine (NOV) is an isomer of NOEV and exerted the strongest inhibition of β-Glu activity in the mouse liver [15]. The purpose of the current studies was to test a possibility that NOV could up-regulate mutant β-Glu activity in cultured human cells. Preliminary findings of the current studies have been reported [16].

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum (FCS) and dialyzed serum were obtained from GibcoBRL. NOV was synthesized in our laboratory (Central Research Laboratories, Seikagaku). Stock solution of NOV was prepared in H_2O at 3 mM and stored at −20 °C. A mouse monoclonal antibody against human β-Glu (clone 8E4, Ref. [17]) was a kind gift from Dr. Barranger JA. Rabbit polyclonal anti-hexosaminidase A (HexA) has been described [18]. Rabbit polyclonal anti-calnexin was from Calbiochem. [1-^{14}C]Serine (1.85 GBq/mmol) was from American Radiolabeled Chemicals (St. Louis, MO).

2.2. Cell culture

Human skin fibroblasts were cultured in DMEM/10% FCS at 37 °C in 5% CO_2. We used two lines of control cells (H11, H34) and five lines of GD cells with β-Glu mutations of 754A(F213I)/754A(F213I), 754A(F213I)/1448C(L444P), 1126G(N370S)/84GG, 1448C(L444P)/RecNciI, and 1448C(L444P)/1448C(L444P) [19]. 84GG causes premature termination of the encoded protein and RecNciI causes amino acid substitutions L444P and A456P [1]. Culture medium was replaced every 2 days with fresh media supplemented with or without NOV at the concentrations indicated.

2.3. Enzyme assays

Lysosomal enzyme activities in cell lysates were determined as described [14,18,20,21]. Briefly, cells were scraped into ice-cold H_2O (10^6/ml) and lysed by sonication. Insoluble materials were removed by centrifugation at 12,000 × *g* for 10 min at 4 °C and protein concentrations were determined with a BCA microprotein assay kit (Piearce). Ten microliters of the lysates was incubated at 37 °C with 20 μl of the substrate solution in 0.1 M citrate buffer, pH 4.5. The substrates were 4-methylumbelliferone-conjugated β-D-galactopyranoside (for β-Gal, Ref. [14]), β-D-glucopyranoside (for β-Glu, Ref. [20]), α-D-glucoside (for α-Glu, Ref. [21]) and *N*-acetyl-β-D-glucosaminide (for β-Hex, Ref. [18]). The reaction was terminated by adding 1.0 ml of 0.2 M glycine sodium hydroxide buffer (pH 10.7). One unit of enzyme activity was defined as nanomoles of 4-methyl-umbelliferone released per hour.

2.4. Western blotting

Cell lysates (20 μg protein) were electrophoresed on a 10% SDS-PAGE and transferred to a PVDF membrane.

The blots were probed with antibodies against β-Glu (1:500) or HexA (1:1000) and developed with an ECL kit (Amersham Pharmacia). Densitometry was performed by using an NIH image software.

2.5. Immunofluorescence

We used staining procedures described previously [22]. Briefly, cells grown on cover glasses were fixed with 4% paraformaldehyde and permiabilized with 1% Triton X-100. Cells were incubated with anti-β-Glu (1:100), followed by Alexa488-conjugated anti-mouse IgG. Fluorescent images were collected by using a Bio-Rad MRC1024 confocal laser microscope. For localization of lysosomes, cells were exposed to Lysotracker Red (5 μg/ml; Molecular Probe) for 1 h prior to fixation.

2.6. Subcellular fractionation

Cell homogenates were fractionated by using Opti-prep (Axis-Shield plc., Dundee, UK) as described [23]. Briefly, cells were homogenized with a potter homogenizer in ice-cold buffer [HEPES 10 mM pH 7.0, 1 mM EDTA, 1 mM EGTA supplemented with a protease inhibitor cocktail (Boehringer)]. Homogenates were overlaid on Opti-prep and centrifuged at 100,000 × *g* for 16 h at 4 °C. Twelve fractions were recovered from the top and numbered accordingly.

2.7. Metabolic labeling of GlcCer

Cellular glycolipids were labeled with [^{14}C]serine as described [24]. Briefly, cells were cultured for 1 week in dialyzed serum supplemented with essential amino acids except for serine, and incubated with [^{14}C]serine (1 μCi/ml) for 3 days. The labeled cells were cultured in fresh DMEM/10% FCS for 5 days with or without NOV. We analyzed labeled lipids by high-performance thin layer chromatography (HPTLC) as described [25]. In brief, cells were harvested at the time indicated, and lipids were extracted with chloroform/methanol (2:1 v/v) and purified by an alkaline treatment. Neutral glycolipids were purified by C18 affinity chromatography. HPTLC was performed with chloroform/methanol/water (55:25:4) as a developing solvent. Labeled lipids were visualized by autoradiography (Fuji-BAS 2500: Fuji, Tokyo, Japan) and densitometry was performed using an NIH image software.

2.8. pH-dependent stability of β-Glu in vitro

Cell lysates were incubated in 0.1 M citrate-phosphate buffer at pH 5, 6 or 7 at 37 °C for the time indicated. Incubation was terminated by the addition of 3 volumes of 0.2 M citrate-phosphate buffer (pH 4.5), immediately followed by chilling on ice. The enzyme assay was done at pH 4.5 as described above.

3. Results

3.1. Inhibition of human β-Glu by NOV in vitro

Chemical structures of NOV and its isomer NOEV are shown in Fig. 1a. NOV inhibited β-Glu activity in mouse liver extracts with an IC_{50} value of 0.03 μM [15]. To test whether it also works on the human enzyme, we determined β-Glu activity in lysates from control human fibroblasts in the absence or presence of NOV. NOV caused dose-dependent inhibition of β-Glu activity with an IC_{50} value of 3 μM (Fig. 1b), indicating that it also works on human β-Glu. NOV contains a C8 fatty acid acyl moiety chain (Fig. 1a). A related compound with a C6

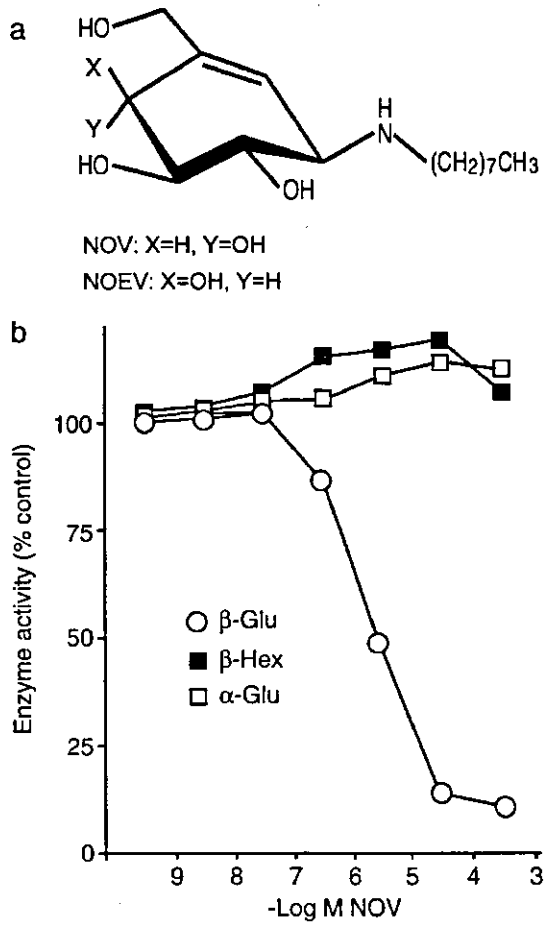

Fig. 1. Effects of NOV on lysosomal enzyme activities in lysates from control human fibroblasts. (a) Chemical structures of NOV and its isomer NOEV. (b) Dose dependence. Enzyme activity in H11 cell lysates was determined in the absence or presence of increasing concentrations of NOV. Each point represents means of triplicate determinations obtained in a single experiment. Values were expressed as relative to activity in the absence of NOV (100%). Values in the absence of NOV in this experiment were β-Glu 142, α-Glu 94 and β-Hex 5089 (units/mg protein). Similar results were obtained in two other experiments.

fatty acid acyl moiety chain inhibited human β-Glu with an IC_{50} value of 30 μM (data not shown), suggesting that the inhibitory activity can be regulated by the length of this chain. NOV caused no inhibition of other lysosomal enzymes α-Glu and β-Hex in the same cell lysates, suggesting a specificity of NOV as an inhibitor of β-Glu (Fig. 1b).

3.2. Up-regulation of F213I mutant β-Glu activity in GD cells treated with NOV

To explore an effect of NOV on mutant β-Glu activity, GD cells with five different genotypes were cultured for 4 days with increasing concentrations of NOV, and β-Glu activity in cell lysates was determined (Fig. 2a, right). NOV

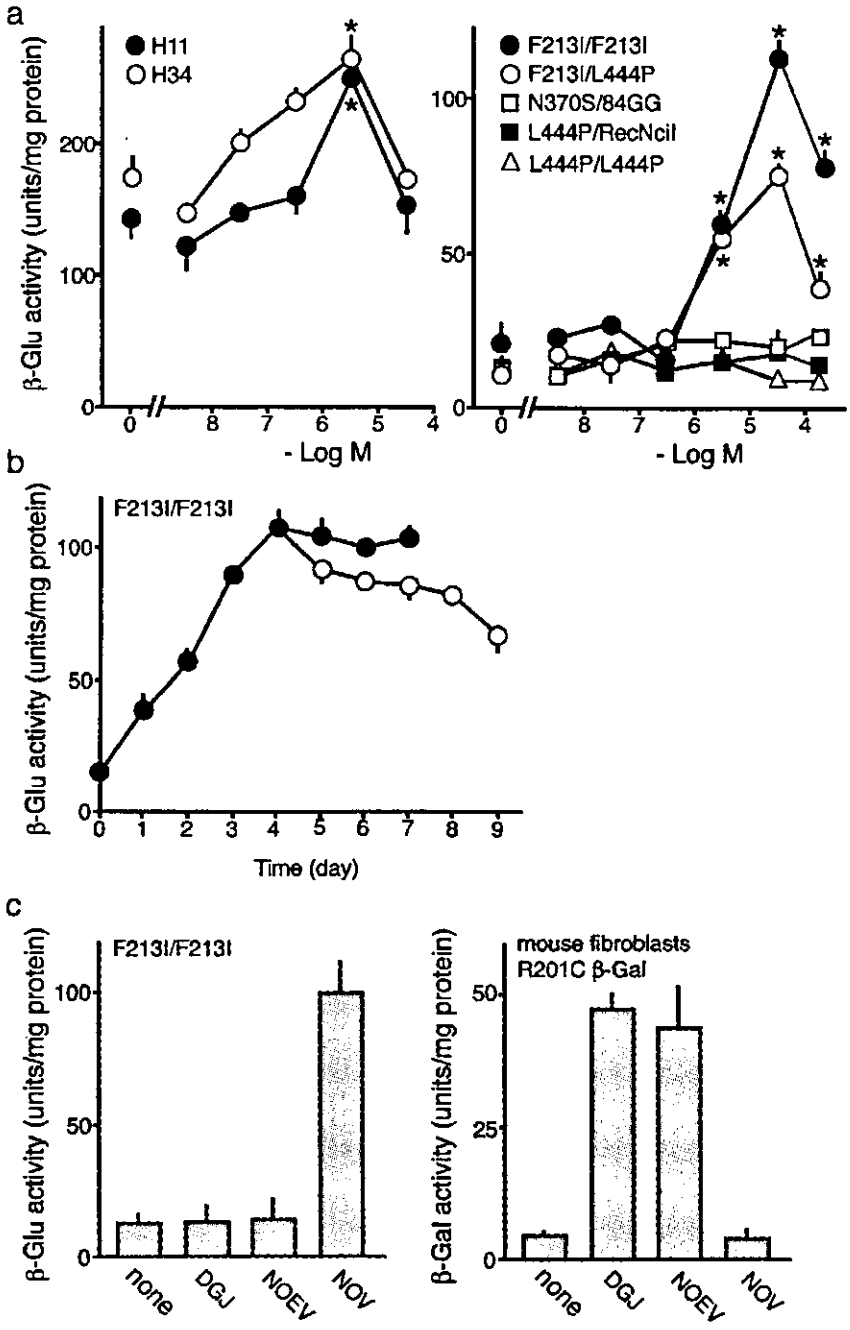

Fig. 2. Effects of NOV on cellular β-Glu activity. (a) Dose dependence. Two lines of control cells (left) and five lines of GD cells (right) were cultured for 4 days in the absence or presence of increasing concentrations of NOV and β-Glu activity in cell lysates was determined. (b) Time course. F213I/F213I cells were cultured in the presence of 30 μM NOV up to 7 days (●). A subset of cells was cultured with NOV for 4 days, washed and further cultured without the drug for 5 days (○). At the time indicated, cells were harvested and β-Glu activity in cell lysates was determined. (c) Specificity of the effects of NOV. F213I/F213I cells were cultured in the presence of indicated drugs (all at 20 μM) for 4 days and β-Glu activity in cell lysates was determined (left). In separate experiments, the same drugs were applied to mouse fibroblasts that express human R201C β-Gal and β-Gal activity in cell lysates was determined (right). Each point or bar represents mean ± S.E. of three determinations each done in triplicate. $^*P < 0.05$, statistically different from the values in the absence of the drug (*t* test).

caused dose-dependent increases in β-Glu activity in two lines of GD cells, F213I/F213I and F213I/L444P. The maximum effect of NOV was observed in F213I homozygous cells in which treatment at 30 μM caused a ~ 6-fold increase in the enzyme activity, up to ~ 80% of the basal activity in control cells. NOV at the same concentration caused a ~ 3-fold increase in F213I/L444P cells. There appeared to be an optimal concentration of NOV, because it was less potent at a higher concentration (100 μM). NOV caused no substantial increase in the enzyme activity of GD cells with other mutations, indicating a specificity of this up-regulation for F213I mutant β-Glu. This effect of NOV, however, was also observed in wild-type β-Glu with a different dose dependence profile; NOV at 3 μM caused a ~ 1.5-fold increase in the two control cell lines but was not effective at 30 μM (Fig. 2a, left).

Time-course analysis using F213I/F213I cells showed that in the presence of NOV (30 μM), β-Glu activity increased in a time-dependent manner and reached a plateau on day 4. When cells were deprived of NOV on

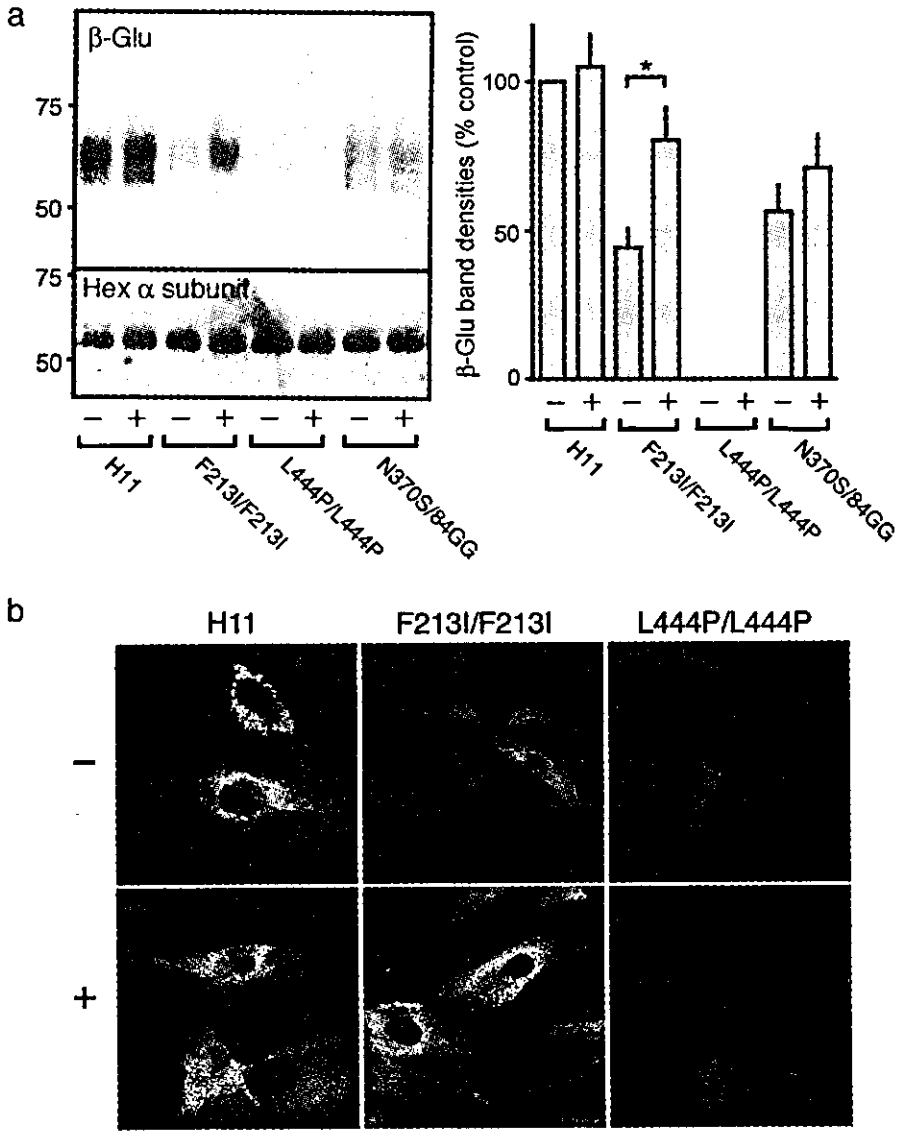

Fig. 3. Effects of NOV on the protein level and intracellular localization of mutant β-Glu. Cells were cultured with (+) or without (−) NOV at 30 μM for 4 days. (a) Western blotting. Cell lysates were analyzed by Western blotting with antibodies against β-Glu or Hex α subunit (left). Twenty micrograms of protein was loaded in each lane. Molecular weights are given on the left (kDa). Densities of β-Glu bands were quantified by densitometry (right). Each bar represents mean ± S.E. of 3 determinations. $^{*}P<0.05$, statistically different from each other (*t* test). (b) Anti-β-Glu immunofluorescence. (c) Double labeling of F213I/F213I cells with anti-β-Glu and Lysotracker Red. In b and c, shown are the representative images obtained with a confocal microscope. All the images were obtained at the same laser intensity and window level. (d) Cell fractionation. Cells were cultured with (●) or without (○) NOV at 30 μM for 4 days. Cell homogenates were subjected to Opti-prep fractionation and each fraction was assessed for activity of β-Glu (upper) or β-Hex (lower). Fractions from F213I/F213I cells were also subjected to anti-calnexin Western blotting. Each point represents mean values of triplicate determinations obtained in a single experiment. Similar results were obtained in two other experiments.

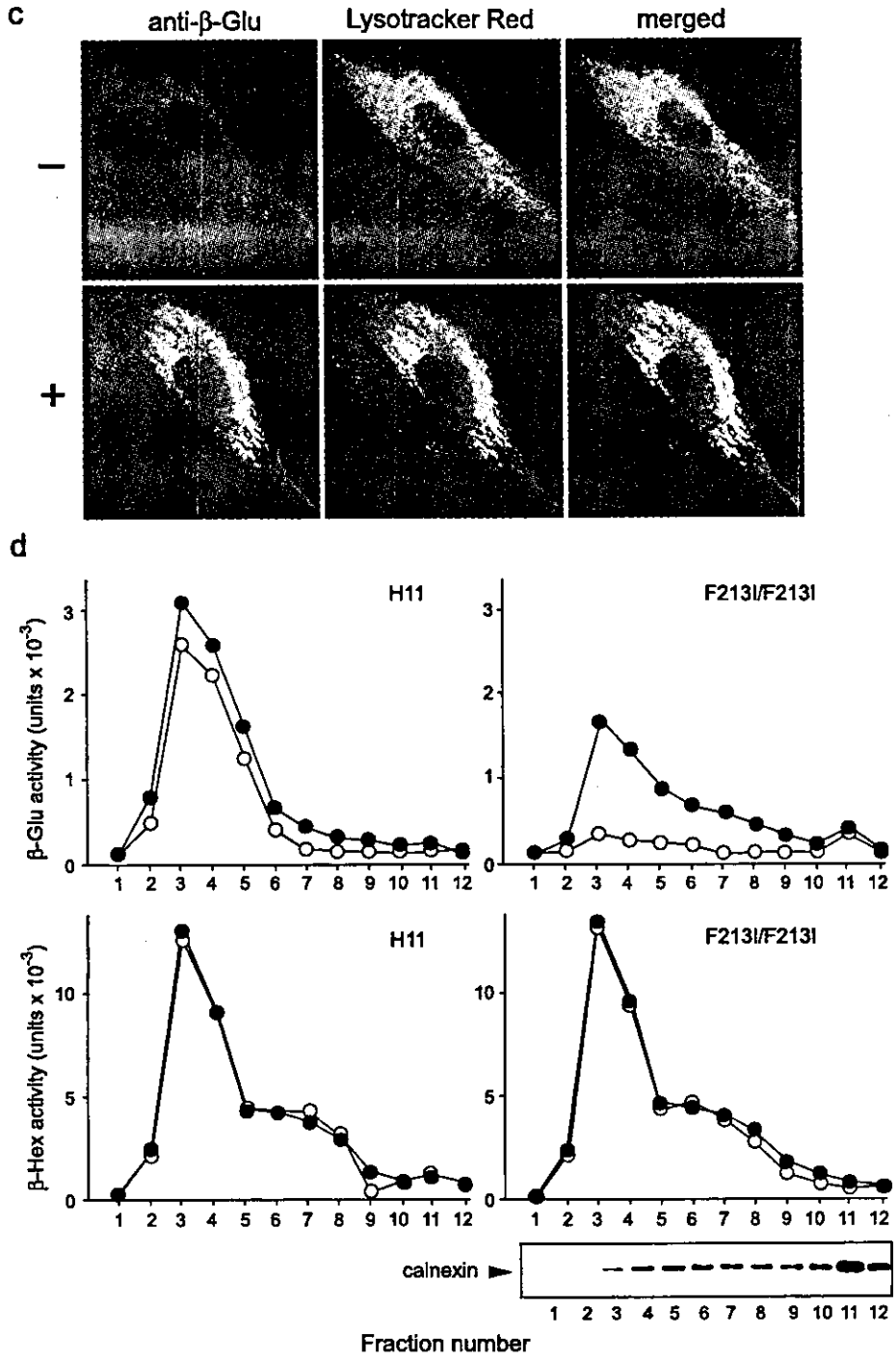

Fig. 3 (*continued*).

day 4, the activity gradually decreased thereafter but was still more than three times higher than the basal level on day 9 (Fig. 2b).

We have shown that both DGJ and NOEV could up-regulate the activity of R201C mutant β-Gal that was expressed in fibroblasts derived from β-Gal knockout mice [11,14]. To test specificity of NOV, first, we examined whether DGJ or NOEV could up-regulate F213I β-Glu activity, and found that neither of these substances caused any changes in the activity (Fig. 2c, left). Second, we examined whether NOV could up-regulate the activity of R201C mutant β-Gal, and found that unlike DGJ and NOEV, NOV had no effect on this mutant enzyme (Fig. 2c, right).

3.3. NOV increased the protein level of F213I mutant β-Glu and restored its localization in the lysosome

To examine the effect of NOV on the protein level, cell lysates were subjected to Western blotting with 8E4 monoclonal anti-β-Glu antibody and the protein levels were estimated by densitometry. This analysis showed that NOV treatment (30 μM for 4 days) of F213I/F213I cells caused a significant increase in the protein level of F213I mutant β-Glu. The same treatment did not increase the protein levels in N370S/84GG and control cells. As reported previously [26], the antibody did not recognize L444P mutant β-Glu. As a control, NOV treatment caused no changes in the protein levels of Hex α subunit (Fig. 3a).

Next, we examined intracellular localization of β-Glu by immunofluorescence and cell fractionation. Anti-β-Glu staining of control cells showed localization of β-Glu immunoreactivity in perinuclear punctate structures and this localization was not affected by NOV treatment. β-Glu immunoreactivity in F213I/F213I cells was lower than in control cells and there was no clear localization in perinuclear punctate structures. When these cells were treated with NOV, however, the immunoreactivity was clearly seen in these structures (Fig. 3b). Localization of F213I mutant β-Glu in the lysosome of NOV-treated cells was evidenced by co-localization of the immunoreactivity and a lysosome marker Lysotracker Red (Fig. 3c). β-Glu immunoreactivity was not detectable in L444P/L444P cells, indicating the specificity of this antibody staining (Fig. 3b).

When control cells were subjected to subcellular fractionation on Opti-prep, β-Glu activity was recovered in fractions #3–5. The same analysis of F213I/F213I cell fractions showed broad distribution of mutant β-Glu activity with peaks at #3 and #11. Both peaks were small but were consistently observed in three independent determinations. Anti-calnexin Western blotting showed that #11 contained a high amount of this ER marker protein [27]. NOV treatment of control cells caused marginal increases (~ 1.1-fold) in β-Glu activity recovered in #3–5. The same treatment of F213I/F213I cells caused ~ 4-fold increases in #3–5. As a control, we measured β-Hex activity in each fraction. Both in control and F213I/F213I cells, β-Hex activity was recovered in #3–4 and to a lesser degree, in #5–8. β-Hex activity in each fraction was not affected by NOV treatment in either cell line (Fig. 3d).

3.4. NOV restored clearance of ^{14}C-labeled GlcCer in F213I/F213I cells

NOV-induced increase of mutant β-Glu activity in the lysosome of F213I/F213I cells prompted us to examine whether NOV could correct abnormal catabolism of GlcCer in this cell line. By using conventional HPTLC analysis of cellular lipid extracts, accumulation of GlcCer was barely detectable in GD skin fibroblasts, most likely because of the low level of this lipid in these cells (data not shown). Therefore, we employed metabolic labeling of cellular glycolipids with [^{14}C]serine and assessed clearance of ^{14}C-labeled GlcCer. When control cells were chased for 5 days after the metabolic labeling, the content of ^{14}C-GlcCer decreased by ~ 50%. This clearance of ^{14}C-GlcCer was retarded in F213I/F213I cells in which there was only a ~ 10% decrease. Inclusion of NOV (30 μM) in the chase medium had no effect in control cells but accelerated the clearance in F213I/F213I cells. In the presence of NOV, the content of ^{14}C-GlcCer in F213I/F213I cells decreased by ~ 50% and reached a level that was comparable to that in control cells (Fig. 4b). The HPTLC analyses showed that besides ^{14}C-GlcCer, clearance of ^{14}C-LacCer was retarded in F213I/F213I cell and again it was accelerated by NOV (Fig. 4a). In addition, NOV caused decreases in the levels of ^{14}C-labeled lipids that corresponded to the positions of CTH, SM and

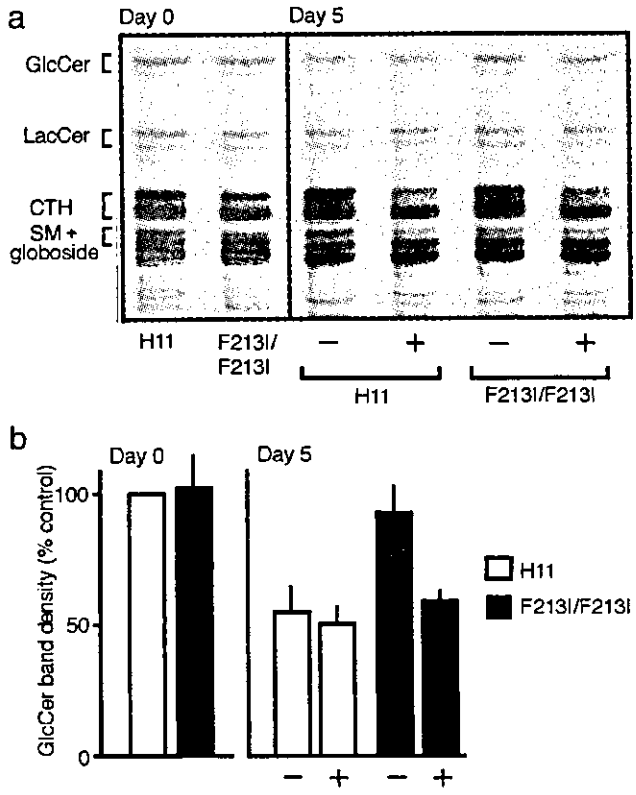

Fig. 4. Effects of NOV on cellular clearance of ^{14}C-labeled GlcCer. (a) HPTLC separation of ^{14}C-labeled cellular lipids. Cells were labeled with ^{14}C-serine for 3 days and then chased up to 5 days in the presence or absence of NOV (30 μM). At the beginning (day 0) and the end (day 5) of the chase period, cells were harvested and neutral glycolipid fraction was analyzed by HPTLC. Positions of standard lipids are given on the left (LacCer, lactosylceramide; CTH, ceramide trihexoside; SM, sphingomyelin). (b) Densitometry. Densities of ^{14}C-GlcCer bands on autoradiographs were quantified using an NIH image software. Values were expressed as relative to the band density of H11 cell extracts at the beginning of the chase period (day 0) as 100%. Each bar represents mean ± S.E. of three determinations.

globoside. The decreases, however, were observed both in control and F213I/F213I cells and the identities of these lipids were left unresolved.

3.5. NOV attenuated pH-dependent loss of F213I mutant β-Glu activity in vitro

Some mutations of lysosomal enzymes affect pH-dependent protein stability [1] and we reported that an α-Gal A inhibitor DGJ prevented in vitro degradation of mutant α-Gal A at neutral pH [10]. To examine whether a similar mechanism underlined NOV effects on F213I mutant β-Glu, we compared pH-dependent stability of wild-type and F213I mutant β-Glu and tested an effect of NOV. In these experiments, we used cell lysates prepared from untreated control cells and from F213I/F213I cells that had been treated with NOV at 30 μM for 4 days and further cultured without the drug for 1 day. When F213I/F213I cell lysates were incubated at pH 7, mutant β-Glu activity was rapidly lost and there remained less than 5% activity at 1 h. Mutant β-Glu activity also decreased in acidic conditions at pH 5 or 6, but ~ 60% activity retained at 1 h under these conditions. In contrast, there were only marginal decreases of wild-type β-Glu activity in control cell lysates and more than 80% activity retained after 1-h incubation at every pH (Fig. 5a). The decrease of F213I mutant β-Glu activity at neutral pH was attenuated by NOV in a dose-dependent manner (Fig. 5b).

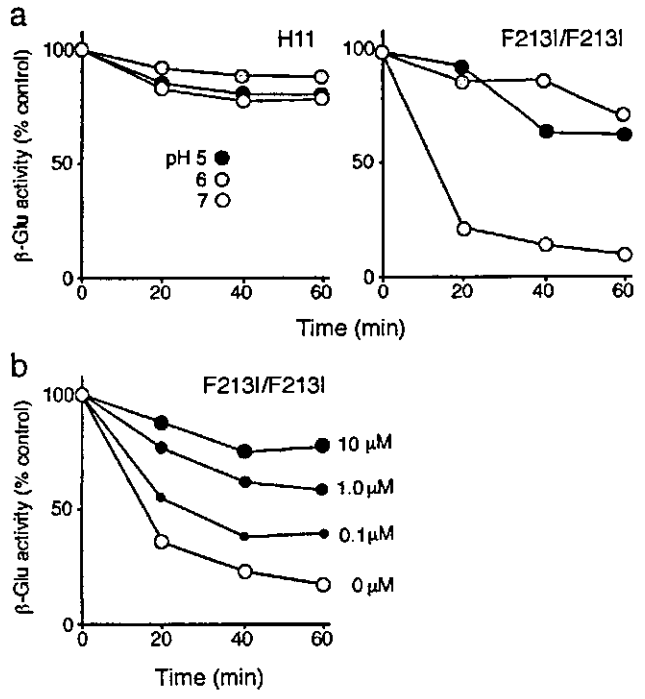

Fig. 5. Effects of NOV on pH-dependent loss of F213I mutant β-Glu activity in vitro. (a) Time course. Cell lysates were incubated at 37 °C in 0.1 M citrate-phosphate buffer at the indicated pH and the enzyme activity was determined at pH 4.5. (b) Effects of NOV. F213I/F213I cell lysates were incubated at pH 7 in the absence or presence of NOV at the concentrations indicated. Each point represents mean values of triplicate determinations obtained in a single experiment. Values were expressed as relative to the activity before the incubation (100%). Similar results were obtained in two other experiments.

4. Discussion

We found in the current study that, when applied to GD cells with F213I mutations, NOV up-regulated cellular β-Glu activity (Fig. 2) and accelerated cellular clearance of GlcCer (Fig. 4). NOV caused a modest but significant increase in the protein level of the mutant enzyme and increased its activity in the lysosome (Fig. 3). We also found pH-dependent loss of F213I mutant β-Glu activity in vitro and its prevention by NOV (Fig. 5). These findings are most likely explained by an activity of NOV as a chemical chaperone to accelerate transport and maturation of F213I mutant β-Glu. Although details are yet to be proven, we suppose that F213I mutant β-Glu is degraded in the ER because of its instability at neutral pH. With the aid of NOV, this mutant β-Glu is protected from degradation and is transported to the lysosome where it is stabilized because of the acidic condition and cellular enzyme activity is restored. Since effects of NOV on ER enzymes responsible for β-Glu degradation have not been examined, an alternative possibility remains to be excluded that the observed effects of NOV were secondary to inhibition of such enzyme(s). Although we have shown negative effects of NOV on some other lysosomal enzymes in vitro (Fig. 1b), potential effects of this compound on ER enzymes must be the subject of future studies.

Because NOV is an inhibitor of β-Glu (IC_{50} = 3 μM, Fig. 1b), it should inhibit β-Glu activity at the lysosome if it reaches to an appropriate concentration in this compartment. NOV was most effective in inducing F213I mutant β-Glu activity at 30 μM in the medium (Fig. 2a), which was 10 times higher than its IC_{50} value. NOV at this concentration, however, failed to inhibit ^{14}C-GlcCer clearance both in control and F213I/F213I cells (Fig. 4). One possible explanation for this apparent lack of inhibition is that at 30 μM in the medium, the concentration of NOV in the lysosome did not rise high enough to inhibit β-Glu activity (whereas the concentration in the ER did rise high enough to prevent mutant β-Glu degradation). If this is the case, the action of NOV as a β-Glu inhibitor may emerge at inappropriately high concentrations. Indeed, NOV was less effective at 100 μM in increasing F213I mutant β-Glu activity (Fig. 2a), although it is yet to be proven that it was due to this action of NOV. We noticed similar dose dependence for DGJ to increase mutant α-Gal A activity in Fabry lymphocytes [10]. Thus, it is apparent that there is an appropriate concentration range for an enzyme inhibitor to up-regulate cellular enzyme activities.

The effect of NOV on cellular enzyme activity was specifically observed in GD cells with F213I mutations

but not in cells with other point mutations N370S, L444P and RecNciI (L444P and A456P) (Fig. 2a). Although a precise molecular basis is left unknown, this selectivity might depend on the differences in the stability of individual enzymes, or alternatively, on the differences of NOV-binding capacities. In this context, the lack of NOV effects on N370S/84GG cells is in contrast to the reported effects of NN-DNJ that caused a twofold increase of β-Glu activity in N370S homozygous cells [12]. It should be clarified in future studies whether this difference in the effects of NOV and NN-DNJ is due simply to the cell lines employed, or to differential binding capacities of N370S mutant enzyme to these substances.

N370S is the most common mutation in GD patients and is associated only with type 1 non-neuronopathic GD [1]. F213I is one of the two prevalent mutations in Japanese GD patients, found in 15% of alleles [19]. Clinically, patients with F213I mutations have either non-neuronopathic or neuronopathic GD. NOV may be of particular therapeutic value in the latter group, since there is no established therapy against GD brain lesions. We have shown that NOEV, an isomer of NOV, could penetrate the blood–brain barrier in mice [14]. It must be determined in a future study whether NOV penetrates the blood–brain barrier and exerts its activity on mutant β-Glu in the brain.

Finally, besides F213I, other β-Glu mutant forms are a potential target of NOV or related compounds. In this context, it should be noted that in a report of a neuronopathic GD patient with G202R mutations, ultrastructural immunohistochemistry revealed the absence of the mutant protein in the lysosome, despite its abundant presence in the ER [28]. So far, nojirimycin derivatives have been an only known class of carbohydrate mimics with chemical chaperone activity for lysosomal enzymes [10–12]. Together with NOEV [14], NOV represents a novel class of carbohydrate mimics with a potential chemical chaperone activity. It is a subject of future studies to test whether NOV and related compounds work as a chemical chaperone for other β-Glu mutant forms, and further for other mutant lysosomal enzymes.

Acknowledgements

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Convenient synthesis and evaluation of glycosidase inhibitory activity of α- and β-galactose-type valienamines, and some *N*-alkyl derivatives

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Abstract—Valienamine analogues having α- and β-galactose-type structures were synthesized by racemic modification from (1*SR*,2*RS*,3*SR*)-6-methylenecyclohex-4-ene-1,2,3-triol. Four *N*-alkyl derivatives of the β-anomer were readily prepared selectively by treatment of key intermediate 2,6-di-*O*-acetyl-3,4-*O*-isopropylidene-5a-carba-α- and β-L-*arabino*-hex-5(5a)-enopyranosyl bromides with alkyl amines. All compounds were assayed for inhibitory activity against six glycosidases, and the *N*-dodecyl derivative was shown to be a very strong inhibitor of β-galactosidase (IC_{50} 0.01 μM, bovine liver).

1. Introduction

Mutant forms of enzyme proteins have been shown to be labile and rapidly degraded in somatic cells from patients with lysosomal storage diseases.[1] However, they can be stabilized and transported to the lysosomes by competitive inhibitors of low molecular weight (chemical chaperones) for therapeutic purposes. This phenomenon was confirmed for the mutant enzyme causing Fabry disease (α-galactosidase deficiency),[2] and very recently this strategy has been extended to two other diseases involving β-galactosidase deficiency in the central nervous system: GM1-gangliosidosis and Morquio B disease. Some unsaturated carbaglycosylamine derivatives have recently been found with remarkable effects: galactose-type[3] [*N*-octyl-β-D-5aCGal(5,5a)enamine,† GalX **1**] and glucose-type *N*-octyl-β-valienamine[4,5] [*N*-octyl-β-D-5aCGlc(5,5a)enamine, GlcX **2**] for β-galactosidase and β-glucosidase, respectively (Fig. 1). Compound **1** was actually demonstrated[3] to be a very potent inhibitor of human β-galactosidase (IC_{50} = 0.3 μM), and has been extensively studied as a candidate novel therapeutic agent for treatment of several human genetic diseases. Thus, such unsaturated 5a-carba-sugars are now regarded as important lead compounds.

2. Results and discussion

We report here a sequence worked out by modification of the route[6] for 5a-carba-α-fucopyranosylamines[7] with nucleophilic substitution of the primary bromo group of 2,3,4-tri-*O*-acetyl-6-bromo-6-deoxy-5a-carba-α- and β-DL-*arabino*-hex-5(5a)-enopyranosyl bromides‡ (**5**α,β), derived from the alkadiene[8] **4** (Scheme 1). Selective preparation of **4** was also here achieved in a 72% yield

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† For convenience, we herewith propose abbreviations for naming the carba sugar and unsaturated carba sugars as follows: 5a-Carba-α-D-glucopyranose: α-D-5aCGlc; 2-Acetamido-2-deoxy-5a-carba-α-D-glucopyranose: α-D-5aCGlcNAc; 5a-Carba-α-D-glucopyranosylamine (validamine): α-D-5aCGlcamine; 5a-Carba-α-D-*xylo*-hex-(5,5a)-enopyranosylamine (valienamine): α-D-5aCGlc(5,5a)enamine not β-L-5aCIdo(5,5a)enamine. As exemplified above, the (5,5a)-unsaturated 5a-carba-sugar is a named derivative of the parent D-hexopyranose.

‡ In the text use of carba-sugar nomenclature following the IUPAC-IUBMB Nomenclature of Carbohydrates (Recommendation 1996: *Carbohydr. Res.*, **1997**, *297*, 1-92) is discussed. However, in the experimental section, IUPAC nomenclature for bi- and tri-cyclic compounds was used throughout for the sake of general understanding.

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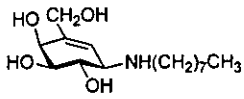

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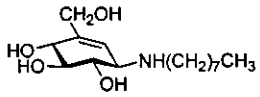

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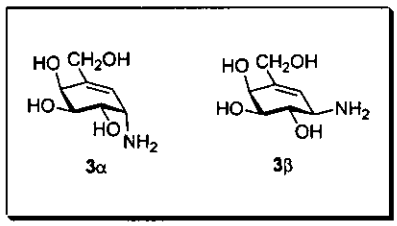
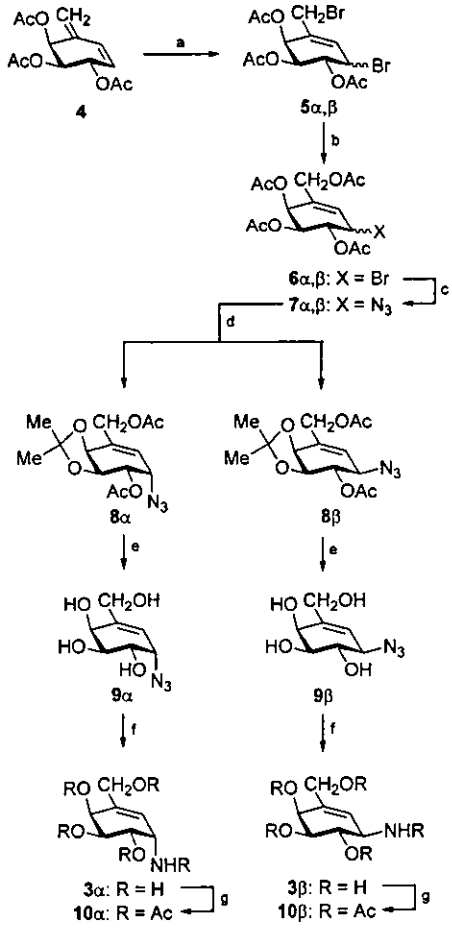


Figure 1.



Scheme 1. Reagents and conditions: (a) Br_2 (molar equiv), CCl_4, 1 h, rt; (b) NaOAc (molar equiv), DMF, 20 h, rt; (c) NaN_3, DMF, r.t.; (d) 1M NaOMe-MeOH, 1 h, rt; $(MeO)_2CMe_2$;, DMF, TsOH hydrate, 1 day, rt; Ac_2O, pyridine; (e) 4 M HCl:THF (1:1), 1 h, reflux; f) Ph_3P (3 molar equiv), 50% aq THF, 1 day, rt; Dowex 50 W×2 (H^+) resin, 1% aq NH_3; g) Ac_2O, pyridine.

by treatment of (1*SR*,2*SR*,3*RS*,4*SR*,6*RS*)-1,2,3-triacetoxy-4-bromo-6-(bromomethyl)cyclohexane[8] with sodium acetate in HMPA at 120 °C.

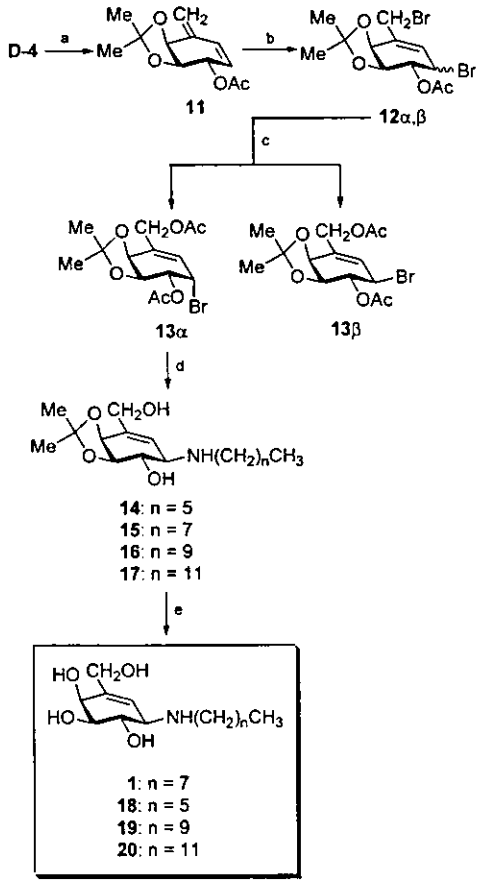
Treatment of **4** with a slight excess of bromine in carbon tetrachloride gave a 90% yield of approximately 1:1 mixture of the 1,4-addition products. The mixture was separable by silica gel chromatography to afford the dibromides[8] **5α** and **5β**. Compound **5α** was treated with sodium acetate to give a mixture of 2,3,4,6-tetra-*O*-acetyl-5a-carba-DL-*arabino*-hex-5(5a)-enopyranosyl bromides (**6α,β**). These results suggest that, although the allylic secondary bromo group remains unchanged, epimerization at C-1 occurs through nucleophilic attack by bromide ions generated in situ. Therefore, the mixture of **5α,β** was directly converted into the bromides **6α,β**, which was without purification treated with sodium azide in DMF at room temperature to give an inseparable 1:1 mixture of the azides[8] **7α,β** in 85% yield. In this case, neighboring group participation by the 2-acetoxyl group at C-1 was first anticipated to give rise to the β-azide **7β** selectively through formation of an intermediate 1,2-acetoxonium ion. However, the allylic carbon atom seems sufficiently active to suffer rear-side attack by an azide anion. Therefore, the mixture of **6α,β** was treated with sodium azide for conversion into a mixture of **7α,β** (85%), which was subsequently subjected to reduction with triphenylphosphine to generate free bases. The free bases were then converted into the *N*-acetyl and penta-*N*,*O*-acetyl derivatives in the usual manner. However, none of the above anomeric pairs could be separated by conventional silica gel chromatography.

O-Deacetylation of **7α,β** under Zemplén conditions gave the tetrols **9α,β**, which were treated with 2,2-dimethoxypropane-TsOH in DMF to afford, after acetylation, a mixture of the 2,3-*O*-isopropylidene derivatives **8α,β** selectively. These compounds were found to be separable on a silica gel column with 1:6 EtOAc/hexane as an eluent, giving **8α** (40%) and **8β** (42%), the ^1H NMR spectra of which showed a doublet of doublets (δ 4.25, $J = 3.8$ and 4.1 Hz) and a broad doublet (δ 3.97, $J = 8.9$ Hz) due to the pseudo-equatorial and axial protons on carbon atoms attached to the azido functions, respectively, supporting the proposed structures. Removal of the acetyl and isopropylidene groups of **8α** and **8β** was effected by treatment with 4 M hydrochloric acid at reflux temperature to give the respective azides **9α** (80%) and **9β** (74%). Reduction of the azido group of **9α** and **9β** with triphenylphosphine in 70% aqueous THF afforded, after purification over a column of Dowex 50 W×2 (H^+) resin with 5% aqueous NH_3 as eluent, the free amines[9] **3α** and **3β** in 86 and 86% yields, respectively. Their structures were further verified with ^{13}C and ^1H NMR spectra of the respective penta-*N*,*O*-acetyl derivatives[10] **10α** and **10β** obtained by conventional acetylation.

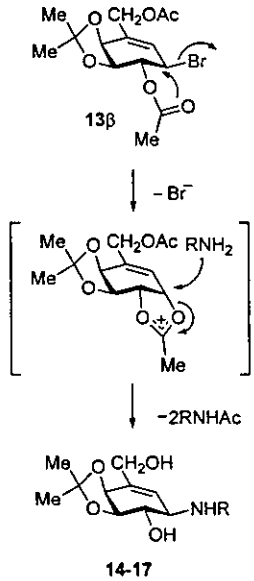
Incorporation of an alkylamino function at C-1 of the allyl bromides **6α,β** was attempted by treatment with an excess of *n*-octylamine. However, a complex mixture of products was formed. Then, the two *cis*-hydroxyl

groups of 6α,β were protected, with the aim of restricting conformational flexibility and avoiding a possible participation of the 4-hydroxyl (Scheme 2). Thus, the optically pure diene[8,11] **D-4** was first converted into the 3,4-*O*-isopropylidene derivative **11**, which was subsequently treated with bromine to afford the 1,4-addition products, ca. 1.7:1 mixture (76%) of **12α** and **12β**. Treatment of **12α,β** with sodium acetate gave selectively a mixture of the bromides **13α,β**, which was separable by silica gel chromatography, affording **13α** (48%) and **13β** (23%). The ^{1}H NMR signals due to C-1 appeared as a doublet of doublets (δ 4.83, J = 3.9 and 7.7 Hz) and a broad doublet (δ 4.50, J 8.3 Hz), respectively. Reaction of the α-bromide **13α** with 4 molar equiv of octylamine proceeded smoothly in an S_N2 fashion to give the protected *N*-octyl derivative as a single β-amine **15** (68%), which was then treated with aqueous acetic acid and subsequently purified on a column of Dowex 50W×2 (H^+) resin with aqueous ammonia, affording **1**[3] (56%). Similar treatment with hexyl-, decyl- and dodecyl-amines produced the corresponding *N*-alkyl derivatives[12] **14**, **16**, and **17**, which were deprotected to provide the β-amines **18**, **19**, and **20** in 30–65% total yields.

It is interesting to note that, on treatment with alkyl amines, the β-bromide **13β** also gave the β-amines as sole products, conceivably through neighboring group participation with the 2-acetoxyl or hydroxyl group (Scheme 3). Therefore, synthesis of **1** and its analogues would be much improved by use of an intact mixture of **13α,β** as the starting material.



Scheme 2. Reagents and conditions: (a) 1 M NaOMe-MeOH, 1 h, rt; $(MeO)_2Me_2$, DMF, TsOH hydrate, 1 day, rt; Ac_2O, pyridine; (b) Br_2 (molar equiv), CCl_4; rt; (c) NaOAc (1.4 molar equiv), 2 days, rt; (d) for example; decylamine (6 molar equiv), 2-propanol, 3 days, rt; 80% aq AcOH, 8 h, 80 °C, 8 h; Dowex 50 W×2 (H^+) resin, 1% NH_3-MeOH.



Scheme 3.

3. Biological assay

Results of biological assays[13] for inhibitory activity toward several glycohydrolases are listed in Table 1. None of the compounds showed any inhibitory activity against α-fucosidase (bovine kidney), α-glucosidase (Baker's yeast), or α-mannosidase (Jack beans). Although, for synthetic reasons,[3] only the *N*-octyl derivative **1** of **3β** has so far received attention as a β-galactosidase inhibitor, the present work provides the first description of inhibitory activity against glycohydrolases obtained by a series of N-substituted derivatives of **3β**. As expected,[4,6] *N*-alkylation dramatically improved the inhibitory activity against α- and β-galactosidases. It is worthy of note that the β-galactose-type valienamines **1** and **18–20** have both been shown to be very strong inhibitors of β-galactosidase and β-glucosidase, with no specificity regarding the 4-epimeric structures of the substrates. This characteristic is in good accordance with the cases of isofagomine[14] and calystegins.[15] Very recently, compound **1** has extensively been studied[16] as an important candidate for generation of novel therapeutic agents for treatment of GM_1-gangliosidosis and β-galactosidosis. Development of such enzyme-inhibitors might advantageously be accelerated by provision of various N-substituted derivatives, including **19** and **20**, readily prepared by use of the versatile precursors **13α,β**.

The present work describes a convenient synthetic route for the β-galactose-type valienamine **3β** and some *N*-alkyl derivatives thereof, demonstrating that enzyme-inhibitory activity can be significantly increased by a suitable N-substitution.

Table 1. Inhibitory activity (IC_{50}, μM) of compounds **1**, **3α,β**, and **18–20** against four glycosidases[a]

Compd	IC_{50} (μM)			
	α-Galactosidase (Green coffee beans)	β-Galactosidase (Bovine liver)	β-Glucosidase (Almonds)	α-Mannosidase (Jack beans)
1	3.1	0.87	3.1	NI
3α	56	NI	NI	370
3β	12	NI	NI	190
18	2.7	2.3	1.2	NI
19	1.9	0.13	2.5	NI
20	4.4	0.01	0.87	NI
DMJ	NT	NT	NT	150

[a] Compounds **3α** and **3β** are racemic; DMJ: deoxymannonojirimycin; NI: $IC_{50} > 0.1$ mg/mL; NT: Not tested.

4. Experimental

4.1. General methods

Optical rotations were measured with a JASCO DIP-370 polarimeter, and $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. ^1H NMR spectra were recorded for solutions in deuteriochloroform and deuteriomethanol with internal tetramethylsilane (TMS) as a reference with a JEOL JNM LAMDA-300 (300 MHz) instrument. ^{13}C NMR spectra were recorded with the same instrument (75 MHz). IR spectra were recorded with a JASCO IR-810 or HITACHI Bio-Rad Digital Lab FTS-65 spectrometer. Mass spectra were determined with HITACHI M-8000 ion trap mass spectrometer using electrospray ionization (ESI). TLC was performed on silica gel 60 F-254 (E. Merck, Darmstadt). The silica gel used for a column chromatography was Wakogel C-300 (Wako Junyaku Kogyo Co., Osaka, 200–300 mesh) or silica gel 60 KO (Katayama Kagaku Kogyo Co., Osaka, 70–230 mesh). Organic solutions were dried over anhydrous Na_2SO_4 and concentrated at >45 °C under diminished pressure.

4.1.1. (1*SR*,2*RS*,3*SR*)-1,2,3-Triacetoxy-4-methylenecyclohex-5-ene (4). A mixture of (1*RS*,2*RS*,3*RS*,4*RS*,6*RS*)-1,2,3-triacetoxy-4-bromo-6-(bromomethyl)cyclohexane[8] (1.64 g, 3.81 mmol) and anhydrous sodium acetate (1.25 g, 15.2 mmol) in HMPA (65 mL) was stirred for 2 h at 120 °C. After cooling, the mixture was diluted with ethyl acetate (210 mL), and the solution was washed thoroughly with water, dried, and evaporated. The residue was chromatographed on a silica gel column (90 g, 1:8 acetone/hexane) to give the conjugated diene **4** (0.73 g, 72%) as a syrup, TLC: R_f 0.45 (1:3 acetone/hexane); ^1H NMR (300 MHz, $CDCl_3$) δ 6.25 (br d, 1H, J = 10.0 Hz, H-6), 5.79 (d, 1H, J = 2.8 Hz, H-3), 5.70 (br d, 1H, J = 10.0 Hz, H-5), 5.64 (br d, 1H, J = 7.6 Hz, H-1), 5.33 and 5.28 (2 s, each 1H, CH_2), 5.17 (dd, 1H, J = 2.8 and 7.6 Hz, H-2), 2.10 and 2.10 (3 s, each 3H, 3 Ac). This compound was identified with an authentic sample[8] on comparison with spectral data.

4.1.2. (1*RS*,2*RS*,3*SR*,6*SR*)- and (1*RS*,2*RS*,3*SR*,6*RS*)-1,2,3-Triacetoxy-6-bromo-4-(bromomethyl)cyclohex-4-ene [2,3,4-tri-*O*-acetyl-6-bromo-6-deoxy-5a-carba-α and β-DL-*arabino*-hex-5(5a)-enopyranosyl bromide] (5α and 5β). To a solution of the diene **4** (1.96 g, 7.34 mmol) in carbon tetrachloride (40 mL) was added dropwise bromine (1.2 g, 7.6 mmol) for 1 h at room temperature. The mixture was then diluted with chloroform (400 mL) and the solution was washed thoroughly with saturated aqueous sodium thiosulfate, aqueous sodium hydrogen carbonate, and water, dried, and evaporated. The residue was chromatographed on a silica gel column (280 g, 6:1 ethyl acetate/hexane) to give about 1:1 mixture of the dibromides **5α** and **5β** (2.82 g, 90%) as colorless crystals.

The mixture of the products obtained from **4** (320 mg) was carefully fractionated by chromatography on silica gel (10:1 EtOAc/hexane) to give pure **5α** (120 mg) and **5β** (43 mg), together with **5α,β** (220 mg): ^1H NMR (300 MHz, $CDCl_3$) **5α**: δ 6.22 (d, 1H, J = 4.6 Hz, H-5), 5.94 (d, 1H, J = 4.1 Hz, H-3), 5.48 (dd, 1H, J = 4.1 and 10.3 Hz, H-2), 5.10 (dd, 1H, J = 3.7 and 10.3 Hz, H-1), 5.08 (dd, 1H, J = 3.7 and 4.6 Hz, H-6), 3.93 (s, 2H, CH_2Br); **5β**: δ 6.18 (d, 1H, J = 2.7 Hz, H-5), 5.88 (d, 1H, J = 3.6 Hz, H-3), 5.69 (dd, 1H, J = 7.6 and 10.5 Hz, H-1), 5.10 (dd, 1H, J = 3.6 and 10.5 Hz, H-2), 4.61 (dd, 1H, J = 2.7 and 7.6 Hz, H-6), 3.91 (s, 2H, CH_2Br).

These compounds were identified with authentic samples[8] on comparison with spectral data.

4.1.3. (1*RS*,2*RS*,3*SR*,6*SR*)- and (1*RS*,2*RS*,3*SR*,6*RS*)-1,2,3-Triacetoxy-4-(acetoxymethyl)-6-bromocyclohex-4-ene [2,3,4,6-tetra-*O*-acetyl-5a-carba-α and β-DL-*arabino*-hex-5(5a)-enopyranosyl bromide] (6α and 6β). A ca. 1:1 mixture (912 mg, 2.13 mmol) of the dibromides **5α** and **5β**, anhydrous sodium acetate (175 mg, 2.13 mmol) in DMF (14 mL) was stirred for 20 h at room temperature. The mixture was then diluted with ethyl acetate (180 mL), and the solution was washed with saline (3×60 mL), dried, and evaporated. The residue was chromatographed on a silica gel column (80 g, 1:3 ethyl acetate/hexane) to give a 1:1 inseparable mixture (743 mg, 86%) of the bromides **6α** and **6β** as colorless crystals, TLC: R_f 0.35 (1:2 EtOAc/hexane). This mixture of the compounds was identified with an authentic sample[8] on comparison with spectral data.

Compound **5α** (45 mg) was similarly treated with sodium acetate (8.6 mg) in DMF (1 mL) to give products, which were shown to be a mixture of **6α,β** by ^1H NMR spectrum.

4.1.4. (1*RS*,2*RS*,3*SR*,6*SR*)- and (1*RS*,2*RS*,3*SR*,6*RS*)-1,2,3-Triacetoxy-4-(acetoxymethyl)-6-azidocyclohex-4-ene [2,3,4,6-tetra-*O*-acetyl-5a-carba-α and β-DL-*arabino*-hex-5(5a)-enopyranosyl azide] (7α and 7β). A ca. 1:1 mixture (676 mg, 1.58 mmol) of the bromides **5α** and **5β**, anhydrous sodium acetate (130 mg, 1.58 mmol) in DMF (10 mL) was stirred for 15 h at room temperature. When TLC demonstrated almost disappearance of **5α** and **5β**, sodium azide (205 mg, 3.15 mmol) was added to the mixture and it was stirred for further 24 h at room temperature. The mixture was diluted with ethyl acetate (120 mL), and a solution was washed with saline (3×40 mL), dried, and evaporated. The residue was chromatographed on a silica gel column (50 g, 1:4 ethyl acetate/hexane) to give about 1:1 inseparable mixture (456 mg, 78%) of the azides **7α** and **7β** as a colorless syrup, TLC: R_f 0.51 (1:1 EtOAc/hexane); ^{1}H NMR (300 MHz, $CDCl_3$) (inter alia) δ 5.94 (d, 0.5H, J=4.9 Hz, H-5 of 7α), 5.88 (d, 0.5H, J=1.2 Hz, H-5 of 7β), 5.73 (br d, 0.5H, J=2.1 Hz, H-3 of 7α), 5.70 (d, 0.5H, J=3.5 Hz, H-3 of 7β), 5.48 (dd, 0.5H, J=8.1 and 11.0 Hz, H-1 of 7β). This mixture was identified with an authentic sample[8] on comparison with spectral data.

4.1.5. (1*SR*,4*SR*,5*SR*,6*SR*)- and (1*SR*,4*RS*,5*SR*,6*SR*)-5-Acetoxy-2-(acetoxymethyl)-4-azido-8,8-dimethyl-7,9-dioxabicyclo[4.3.0]non-2-ene [2,6-di-*O*-acetyl-3,4-*O*-isopropylidene-5a-carba-α and β-DL-*arabino*-hex-5(5a)-enopyranosyl azide] (8α and 8β). A solution of the azides **7α,β** (113 mg, 0.305 mmol) in methanol (1.1 mL) was treated with 1 M methanolic sodium methoxide (0.57 mL) for 1 h at room temperature. After neutralization by treatment with Amberlite IR-120 (H^+) resin, the solution was evaporated and the residue was dissolved in dry DMF (0.87 mL), to which 2,2-dimethoxypropane (0.132 mL, 1.07 mmol) and a catalytic amount of *p*-toluenesulfonic acid monohydrate were added. The mixture was stirred for 24 h at room temperature, neutralized with triethyl amine, and then co-evaporated with *n*-butanol and toluene to dryness. The residue was treated with acetic anhydride (0.5 mL) and pyridine (1.0 mL) overnight at room temperature. After quenched by addition of methanol (1 mL), the mixture was evaporated to dryness. The residue was chromatographed on a silica gel column (8.3 g, 1:8 ethyl acetate/hexane) to give **8α** (44 mg, 44%) and **8β** (42 mg, 42%) as a colorless syrup.

For **8α**: TLC: R_f 0.53 (1:2 EtOAc/hexane); ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.88 (d, 1H, J=4.1 Hz, H-3), 5.21 (dd, 1H, J=3.8 and 7.4 Hz, H-2), 4.75 and 4.67 (2 d, each 1H, J=14.9 Hz, CH_2OAc), 4.63 (d, 1H, J=6.2 Hz, H-1), 4.46 (dd, 1H, J=6.2 and 7.4 Hz, H-6), 4.25 (dd, 1H, J=3.8 and 4.1 Hz, H-4), 2.13 and 2.17 (2 s, each 3H, 2 Ac), 1.38 and 1.42 (2 s, each 3H, CMe_2); ITMS-ESI (positive mode): m/z 349 $[M+Na]^+$, 365 $[M+K]^+$.

For **8β**: TLC: R_f 0.40 (1:2 EtOAc/hexane); ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.77 (d, 1H, J=1.5 Hz, H-3), 5.15 (dd, 1H, J=8.9 and 8.9 Hz, H-5), 4.66 and 4.74 (2 d, each 1H, J=13.7 Hz, CH_2OAc), 4.59 (d, 1H, J=6.0 Hz, H-1), 4.22 (dd, 1H, J=6.0 and 8.9 Hz, H-6), 3.97 (br d, 1H, J=8.9 Hz, H-4), 2.12 and 2.17 (2 s, each 3H, 2 Ac), 1.38 and 1.51 (2 s, each 3H, CMe_2); ITMS-ESI (positive mode): m/z 349 $[M+Na]^+$, 364 $[M+K]^+$.

4.1.6. (1*SR*,2*SR*,3*SR*,6*SR*)-6-Azido-4-(hydroxymethyl)-cyclohex-4-ene-1,2,3-triol [5a-carba-α-DL-*arabino*-hex-5(5a)-enopyranosyl azide] (9α). A solution of **8α** (37 mg, 0.113 mmol) in a mixture of 4 M hydrochloric acid (0.5 mL) and THF (0.5 mL) for 1 h at reflux temperature. The mixture was evaporated and the residue was chromatographed on silica gel (1.1 g, 1:10 MeOH/$CHCl_3$) to give **9α** (18 mg, 80%) as a colorless syrup; TLC: R_f 0.40 (1:4 MeOH/$CHCl_3$); ^{1}H NMR (300 MHz, $CHCl_3$): δ 5.70 (d, 1H, J=4.2 Hz, H-5), 4.17 (dd, 1H, J=4.2 and 4.5 Hz, H-6), 4.10 (d, 1H, J=3.9 Hz, H-3), 4.01 (s, 2H, CH_2OH), 3.93 (dd, 1H, J=4.5 and 10.0 Hz, H-1), 3.68 (dd, 1H, J=3.9 and 10.0 Hz, H-2).

4.1.7. (1*SR*,2*RS*,3*SR*,6*RS*)-6-Azido-4-(hydroxymethyl)-cyclohex-4-ene-1,2,3-triol [5a-carba-β-DL-*arabino*-hex-5(5a)-enopyranosyl azide] (9β). Compound **8β** (31 mg, 0.094 mmol) was hydrolyzed as described in the preparation of **9α** to give **9β** (14 mg, 74%) as a colorless syrup; TLC: R_f 0.40 (1:4 MeOH/$CHCl_3$); ^{1}H NMR (300 MHz, $CHCl_3$): δ 5.57 (d, 1H, J=1.3 Hz, H-5), 4.07 (d, 1H, J=4.2 Hz, H-3), 4.00 (s, 2H, CH_2OH), 3.83 (dd, 1H, J=1.3 and 8.3 Hz, H-6), 3.62 (dd, 1H, J=8.3 and 10.7 Hz, H-1), 3.47 (dd, 1H, J=4.2 and 10.7 Hz, H-2).

4.1.8. (1*SR*,2*RS*,3*SR*,6*SR*)-6-Amino-4-(hydroxymethyl)-cyclohex-4-ene-1,2,3-triol [5a-carba-α-DL-*arabino*-hex-5(5a)-enopyranosylamine] (3α). A solution of **9α** (19 mg, 92 μmol) in 50% aqueous THF (2.5 mL) containing triphenylphosphine (73 mg, 0.28 mmol) was stirred for 24 h at room temperature. The mixture was then passed through a column of Dowex 50W×2 (H^+) resin (1 mL) with 1% aqueous ammonia as eluent to give **3α** (14 mg, 86%) as a white powder; TLC: R_f 0.35 (1:1:2H_2O/AcOH/*n*-BuOH); ^{1}H NMR (300 MHz, D_2O): δ 5.61 (d, 1H, J=3.9 Hz, H-5), 4.11 (d, 1H, J=3.9 Hz, H-3), 4.00 and 3.95 (ABq, J=15.1 Hz, CH_2OH), 3.81 (dd, 1H, J=9.5 and 9.5 Hz, H-1), 3.72 (dd, 1H, J=3.9 and 9.5 Hz, H-2), 3.47 (br d, 1H, H-6); ^{13}C NMR (75 MHz, D_2O): δ 139.16 (C-4), 126.96 (C-5), 69.79 (C-3 or 1), 69.44 (C-1 or 3), 67.40 (C-2), 63.15 (C-7), 49.48 (C-6); ITMS-ESI (positive mode): m/z 177 $[M+H]^+$, 198 $[M+Na]^+$.

4.1.9. (1*SR*,2*RS*,3*SR*,6*RS*)-6-Amino-4-(hydroxymethyl)-cyclohex-4-ene-1,2,3-triol [5a-carba-β-DL-*arabino*-hex-5(5a)-enopyranosylamine] (3β). Compound **9β** (20 mg, 0.10 mmol) was treated with triphenylphosphine (53 mg, 0.20 mmol) in 50% aqueous THF (3.5 mL) and a crude product was purified, as in the preparation of **3α**, to give **3β** (15 mg, 86%) as a white powder; TLC: R_f 0.36 (1:1:2H_2O/AcOH/*n*-BuOH); ^{1}H NMR (300 MHz, D_2O): δ 5.54 (d, 1H, J=1.7 Hz, H-5), 4.10 (d, 1H, J=3.9 Hz, H-3), 4.01 (s, 2H, CH_2OH), 3.57 (dd, 1H, J=3.9 and 10.3 Hz, H-2), 3.53 (dd, 1H, J=8.3 and 10.3 Hz, H-1); ^{13}C NMR (75 MHz, D_2O): δ 139.46 (C-4), 127.49 (C-5), 73.34 (C-3 or 1), 72.34 (C-1 or 3), 67.40 (C-2), 62.84 (C-7), 54.41 (C-6); ITMS-ESI (positive mode): m/z 176.43 $[M+H]^+$, 198 $[M+Na]^+$.

4.1.10. (1*SR*,2*RS*,3*SR*,6*SR*)-6-Acetamido-1,2,3-triacetoxy-4-(acetoxymethyl)cyclohex-4-ene (10α). Compound **3α** was treated with acetic anhydride and pyridine in the usual manner to give the penta-*N*,*O*-acetyl derivative **10α**, as a syrup, quantitatively; TLC: R_f 0.42 (1:1 acetone/toluene); ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.86 (d, 1H, J = 4.4 Hz, H-5), 5.70 (d, 1H, J = 2.9 Hz, H-3), 5.57 (d, 1H, J = 11.3 Hz, NH), 5.35 (dd, 1H, J = 4.2 and 9.6 Hz, H-1), 5.32 (dd, J = 2.9 and 9.6 Hz, 1H, H-2), 5.10 (ddd, 1H, J = 4.2, 4.4, and 11.3 Hz, H-6), 4.59 and 4.46 (ABq, each 1H, J = 13.4 Hz, CH_2OAc), 2.09, 2.08, 2.06, 2.05 and 2.02 (5 s, each 3H, 5 Ac); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.38, 170.25, 170.09, 169.84 (2) (5 *C*H_3*C*O), 133.00 (C-4), 127.77 (C-5), 67.21 (C-3 or 1), 66.65 (C-1 or 3), 65.36 (C-2), 63.42 (C-7), 45.29 (C-6), 23.26, 21.59, 20.82, 20.69, 20.65 (5 CH$_3$*C*O); ITMS-ESI (positive mode): m/z 387 $[M+H]^+$, 408 $[M+Na]^+$.

4.1.11. (1*SR*,2*RS*,3*SR*,6*RS*)-6-Acetamido-1,2,3-triacetoxy-4-(acetoxymethyl)cyclohex-4-ene (10β). Compound **3β** was acetylated in the usual manner to give the penta-*N*,*O*-acetyl derivative **10β**, as a syrup, quantitatively; TLC: R_f 0.42 (1:1 acetone/toluene); ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.84 (d, 1H, $J_{1,5a}$ = 1.4 Hz, H-5), 5.79 (d, 1H, J = 3.9 Hz, H-3), 5.73 (d, 1H, J = 8.5 Hz, NH), 5.29 (dd, 1H, J = 8.5 and 10.0 Hz, H-1), 5.19 (dd, 1H, J = 3.9 and 10.0 Hz, H-2), 4.80 (ddd, 1H, J = 1.4, 8.5 and 8.5 Hz, H-6), 4.55 and 4.46 (ABq, each 1H, J = 13.4 Hz, CH_2OAc), 2.11, 2.05 and 1.98 (3 s, each 3H, 3 Ac), 2.08 (br s, 6H, 2 Ac); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.46, 170.37, 170.06, 169.97, 169.61 (5 *C*H_3*C*O), 131.08 (C-4), 130.49 (C-5), 69.39 (C-3 or 1), 68.92 (C-1 or 3), 65.76 (C-2), 63.64 (C-7), 51.07 (C-6), 23.23, 20.83, 20.77, 20.74, 20.54 (5 CH$_3$*C*O); ITMS-ESI (positive mode): m/z 387 $[M+H]^+$, 408 $[M+Na]^+$.

4.1.12. (1*S*,2*S*,6*S*)-2-Acetoxy-8,8-dimethyl-5-methylene-7,9-dioxabicyclo[4.3.0]non-3-ene (11). A solution of the diene[8,11] **D–4** (1.36 g, 5.06 mmol) in methanol (21 mL) was treated with sodium methoxide (55 mg, 1.0 mmol) for 2 h at room temperature. After neutralization with Amberlite IR-120 (H^+) resin, the mixture was evaporated. The residue was treated with 2,2-dimethoxypropane (2.0 mL, 1.6 mmol) and *p*-toluenesulfonic acid monohydrate (0.21 g, 1.1 mmol) for 23 h at room temperature. After neutralization with triethylamine, the mixture was evaporated to dryness and the residual product was acetylated with acetic anhydride (5.5 mL) and pyridine (11 mL) overnight at room temperature in the usual manner. The product was chromatographed on a silica gel column (30 g, 1:11 EtOAc/hexane) as eluent to give **11** (807 mg, 71%) as a colorless syrup; TLC: R_f 0.52 (1:1 EtOAc/hexane), $[\alpha]_D^{23}$ +149° (*c* 0.89, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.30 (br d, 1H, J = 10.3 Hz, H-4), 5.67 (br d, 1H, H-3), 5.43 (d, 1H, J = 5.4 Hz, H-2), 5.42 and 5.44 (2 s, each 1H, CH_2), 4.71 (d, 1H, J = 5.5 Hz, H-6), 4.21 (dd, 1H, J = 5.4 and 5.5 Hz, H-1), 2.11 (s, 3H, Ac), 1.44 and 1.49 (2 s, each 3H, CMe_2); ITMS-ESI (positive mode): m/z 247 $[M+Na]^+$, 263 $[M+K]^+$.

4.1.13. (1*S*,4*S*,5*R*,6*S*)- and (1*S*,4*R*,5*R*,6*S*)-5-acetoxy-4-bromo-2-(bromomethyl)-8,8-dimethyl-7,9-dioxabicyclo-[4.3.0]non-2-ene [2-*O*-acetyl-6-bromo-6-deoxy-3,4-*O*-isopropylidene-5a-carba-α and β-L-*arabino*-hex-5(5a)-enopyranosyl bromide] (12α and 12β). To a solution of **11** (0.657 g, 2.93 mmol) in carbon tetrachloride (6.6 mL) was added dropwise bromine (0.15 mL, ca. 3 mmol) for 7 min at room temperature. After treatment with saturated aqueous sodium thiosulfate, the mixture was diluted with chloroform (300 mL), and the solution was thoroughly washed with saturated sodium hydrogen carbonate and water, dried, and evaporated. The residue was chromatographed on silica gel (20 g, 1:50 EtOAc/toluene) to give about 1.7:1 inseparable mixture (824 mg, 76%) of **12α** and **12β** as a colorless syrup.

For **12α**: ^{1}H NMR (300 MHz, $CDCl_3$) (inter alia): δ 6.17 (br d, 1H, J = 6.4 Hz, H-3), 4.99 (br d, 1H, J = 8.3 Hz, H-5), 4.81 (dd, 1H, J = 3.7 and 6.4 Hz, H-4), 4.76 (dd, 1H, J = 6.8 and 8.3 Hz, H-6), 4.02 and 4.20 (2 d, each 1H, J = 10.5 Hz, CH_2Br), 2.19 (s, 3H, Ac), 1.42 and 1.46 (2 s, each 3H, CMe_2).

For **12β**: ^{1}H NMR (300 MHz, $CDCl_3$) (inter alia): δ 6.17 (br s, 1H, H-3), 5.37 (dd, 1H, J = 8.3 and 8.3 Hz, H-5), 4.87 (d, 1H, J = 5.5 Hz, H-1), 4.49 (br d, 1H, H-4), 4.20 (s, 2H, CH_2Br), 2.15 (s, 3H, Ac), 1.41 and 1.51 (2 s, each 3H, CMe_2).

4.1.14. (1*S*,4*S*,5*R*,6*S*)- and (1*S*,4*R*,5*R*,6*S*)-5-Acetoxy-2-(acetoxymethyl)-4-bromo-8,8-dimethyl-7,9-dioxabicyclo-[4.3.0]non-2-ene [2,6-di-*O*-acetyl-3,4-*O*-isopropylidene-5a-carba-α and β-L-*arabino*-hex-5(5a)-enopyranosyl bromide] (13α and 13β). A mixture (27 mg, 70 μmol) of **12α,β** and sodium acetate (6.3 mg, 0.10 mmol) in DMF (1 mL) was stirred for 2 days at room temperature. The mixture was then diluted with ethyl acetate (12 mL), the solution was thoroughly washed with saline and water, dried, and evaporated. The residual product (27 mg) was chromatographed on silica gel (4 g, 1:8 EtOAc/hexane) to give **13α** (12 mg, 48%) and **13β** (6 mg, 23%) as a colorless syrup.

For **13α:** TLC: R_f 0.36 (1:2 ethyl acetate/hexane); $[\alpha]_D^{20}$ +277° (*c* 1.13, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.09 (d, 1H, J = 7.7 Hz, H-3), 4.83 (dd, 1H, J = 3.9 and 7.7 Hz, H-4), 4.79 (dd, 1H, J = 3.9 and 8.5 Hz, H-5), 4.71 (d, 1H, J = 6.5 Hz, H-1), 4.67 and 4.74 (ABq, each 1H, J = 14.3 Hz, CH_2OAc), 4.55 (dd, 1H, J = 6.5 and 8.5 Hz, H-6), 2.13 and 2.19 (2 s, each 3H, 2 Ac), 1.40 and 1.46 (2 s, each 3H, CMe_2); ITMS-ESI (positive mode): m/z 283 $[M-^{79}Br]^+$, 305 $[M+Na-^{79}Br]^+$, 385 $[M+Na]^+$.

For **13β**: TLC: R_f 0.31 (1:2 EtOAc/hexane); $[\alpha]_D^{21}$ +33° (*c* 0.78, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.04 (br s, 1H, H-3), 5.39 (dd, 1H, J = 8.3 and 8.3 Hz, H-5), 4.67 and 4.75 (ABq, each 1H, J = 13.6 Hz, CH_2OAc), 4.58 (d, 1H, J = 5.9 Hz, H-1), 4.50 (br d, 1H, J = 8.3 Hz, H-4), 4.17 (dd, 1H, J = 5.9 and 8.3 Hz, H-6), 2.12 and 2.15 (2 s, each 3H, 2 Ac), 1.38 and 1.52 (2 s, each 3H, CMe_2); ITMS-ESI (negative mode): m/z 361 $[M-H]^-$.

4.1.15. (1*S*,4*R*,5*R*,6*S*)-4-Hexylamino-5-hydroxy-2-(hydroxymethyl)-8,8-dimethyl-7,9-dioxabicyclo[4.3.0]non-2-ene [*N*-hexyl-3,4-*O*-isopropylidene-5a-carba-α-L-*arabino*-hex-