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N-Butyl-L-Deoxynojirimycin (L-NBDNJ): Synthesis of an Allosteric Enhancer of α-Glucosidase Activity for the Treatment of Pompe Disease

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KEYWORDS: iminosugars; *de novo* synthesis; acid α-glucosidase; pharmacological chaperone; Pompe disease.

ABSTRACT: The highly stereocontrolled *de novo* synthesis of L-NBDNJ (the unnatural enantiomer of the iminosugar drug Miglustat) and a preliminary evaluation of its chaperoning potential are herein reported. L-NBDNJ is able to enhance lysosomal α-glucosidase levels in Pompe disease fibroblasts, either when administered singularly or when co-incubated with the recombinant human α-glucosidase. In addition, differently from its D-enantiomer, L-NBDNJ does not act as a glycosidase inhibitor.

INTRODUCTION

Acid α-glucosidase (GAA; EC 3.2.1.20) is a lysosomal enzyme enabling the degradation of glycogen to glucose (glycosyl hydrolase family: GH31).[1] A deficiency in GAA activity leads to intralysosomal glycogen storage, causing extensive and progressive damages to cardiac and skeletal muscles.[2] The genetic disorder due to mutations-induced functional defects of GAA is known as Pompe disease (PD; glycogen storage disease type II) and represents one of the most common lysosomal storage disorders (LSDs).[3,4] To date, the only clinically approved strategy for the treatment of the disease is the enzyme replacement therapy (ERT), based on intravenous administration of recombinant human GAA (rhGAA).[5] Even though ERT has shown to stabilize the disease course, use of rhGAA has some major limitations, regarding, *inter alia*, its adequate delivery to lysosomes and the stability to non-acidic conditions.[6] In the search for alternative approaches, the identification of small molecule chaperones able to restore functions and properties of the mutated enzyme – the core of the so-called pharmacological chaperone therapy (PCT) – is representing an emerging strategy and a formidable challenge of the modern biomedical research.[7,8] According to PCT, the use of suitable GAA ligands enables stabilization of protein conformation, inhibits premature misfolding and facilitates enzyme translocation into the lysosome, thereby preventing ER-associated degradation processes.[7] Among

pharmacological chaperones,[9-11] the iminosugar drug[12] *N*-butyl-D-deoxynojirimycin (**1**) (D-NBDNJ; Figure 1) currently represents one of the most promising candidates under development for the treatment of PD.[4] In early studies, **1** has been found to improve residual activity and lysosomal trafficking of mutated GAA in cultured fibroblasts from Pompe patients.[13] Even more remarkably, the chaperone has subsequently demonstrated to enhance the physical stability and therapeutic efficacy of rhGAA itself.[14] This finding has represented the first documented example of a synergistic effect deriving from the combined use of PCT and ERT, which has opened the way to the so-called "combination therapy" for the treatment of various LSDs.[7,8] Higher lysosomal GAA levels in blood as resulting from co-administration of rhGAA and **1** have been recently found in animal models and in PD patients.[15] Further trials aimed at addressing the clinical efficacy of the combination protocol are currently in progress.[16]

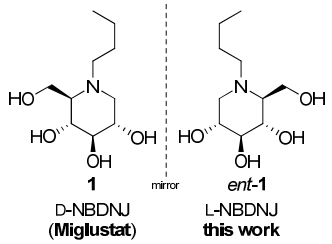


Figure 1. D- and L-iminosugars **1** and *ent*-**1**.

Despite these promising data, it must be noted that **1**, like most iminosugar chaperones, is an example of active-site specific chaperone (ASSC). Accordingly, in its protonated form **1** resembles the oxocarbenium ion produced during glucopyranoside hydrolysis and therefore acts as a competitive inhibitor of GAA as well as of a variety of other glucosidases.[17] Even though chaperoning activity typically occurs at sub-inhibitory concentrations,[13] GAA inhibition may clearly represent a limitation to clinical use, as it could hamper to reach the optimal drug dosage. Hence, the identification of new-generation chaperones, able to protect GAA from degradation

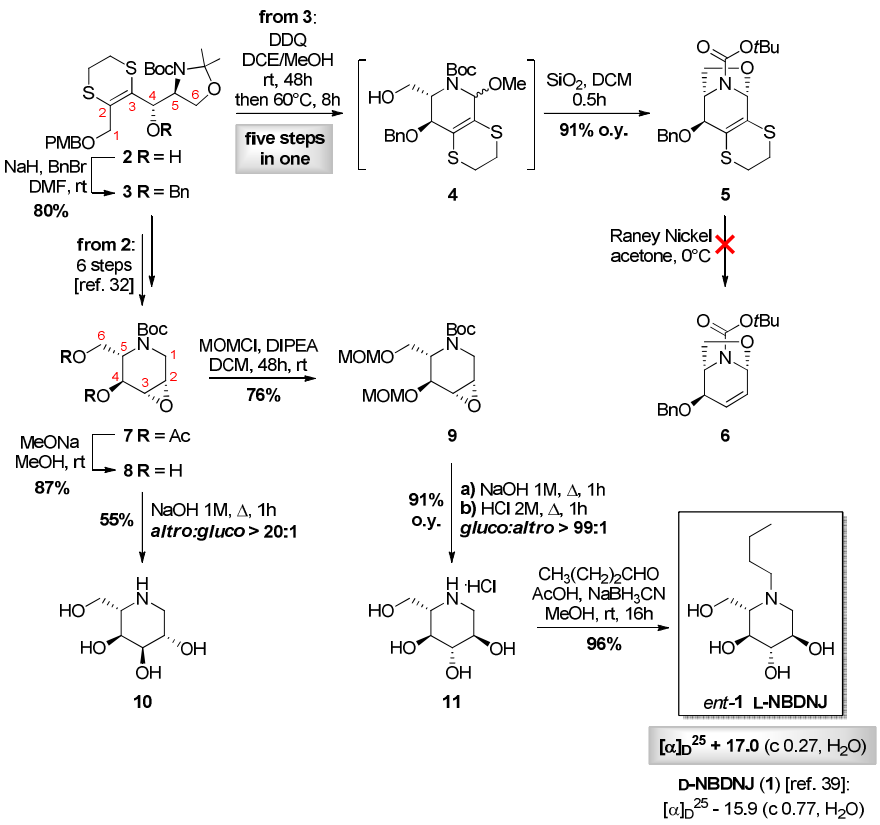
without interfering with its activity (allosteric chaperones), is likewise being pursued.[6] In this context, novel therapeutic opportunities are recently being offered by L-iminosugars.[18,19] Indeed, although typically acting as weaker[20] and non-competitive[18] glycosidase inhibitors, several L-iminosugars (either alone or in combination with their D-enantiomers)[21] have displayed noteworthy chaperoning activities toward the same enzymes in their mutated forms.[21,22] Based on these combined data, our research program aimed at exploring the stereoselectivity of biomolecular recognition processes[18,23-26] took us to study the role of iminosugar chirality in PCT through the analysis of the chaperoning potential of the unnatural enantiomer of **1**, i.e. *N*-butyl-L-deoxynojirimycin (*ent*-**1**) (L-NBDNJ; Figure 1). In this communication, the stereoselective synthesis of *ent*-**1**, the analysis of its glycosidase inhibition properties and a preliminary evaluation of its potential as enhancer of GAA activity both in its mutated and recombinant form are reported.

RESULTS AND DISCUSSION

Chemistry. To the best of our knowledge, the synthesis of *ent*-**1** has never been reported before.[27] More generally, the access to L-iminosugars is often problematic, due to the limited commercial availability of almost all L-hexoses, which hampers their use as starting materials. Alternatively, in an extension of a long studied[28] *de novo* methodology, already devised to obtain unnatural carbohydrates[28] and biomimetics[25,26,29-31] including iminosugars[32] and their precursors,[33] the synthesis of *ent*-**1** was explored from the synthetically available[32] alcohol **2** (Scheme 1). Particularly, having previously fixed the stereochemistry of C4 and C5 stereocentres,[34] we have herein addressed the synthetic problem of the stereoselective introduction of the two remaining stereocentres at C2 and C3 positions having a *trans*-

diequatorial orientation of the substituents. In early attempts, inspired by previous results on L-glucose synthesis,[35] the preparation and stereoselective functionalization of 1,6-anhydro-iminosugars was considered (Scheme 1). *Sec*-alcohol **2** was first benzylated under common conditions (NaH, BnBr). The resulting benzyl ether **3** was then treated with a DCE/MeOH solution of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) directly providing, after 48h at rt and then 8h at 60 °C, the bicyclic compound **4**. As already observed for similar substrates,[28] the reaction was the result of a multistep process, which was enabled by the excellent electron-donating properties of the *bis*-thioenol ether moiety.[31] Spontaneous cyclization of **4** into 1,6-anhydro-iminosugar precursor **5** was even observed during chromatographic purification procedures. In spite of the synthetic utility of the reaction, **5** could not be further functionalized as the subsequent double bond unmasking step (Ra-Ni) did not provide the expected olefin **6**; the corresponding over-reduced compound was instead obtained. Looking for alternative strategies, we then investigated the reactivity of oxirane **7**, obtained from alcohol **2** as previously described[32] (Scheme 1). Epoxide ring opening of **7** with refluxing NaOH directly produced the unprotected iminosugar with L-*altro* configuration **10**, additionally in a highly stereoselective fashion (*altro:gluco* > 20:1). Conversely, treatment of *bis*-MOM acetal **9** (from **7**: MeONa, then MOMCl/DIPEA) under the same conditions gave, after the subsequent HCl addition to the crude mixture, the desired **11** as the only detected stereoisomer (d.r. > 99:1).

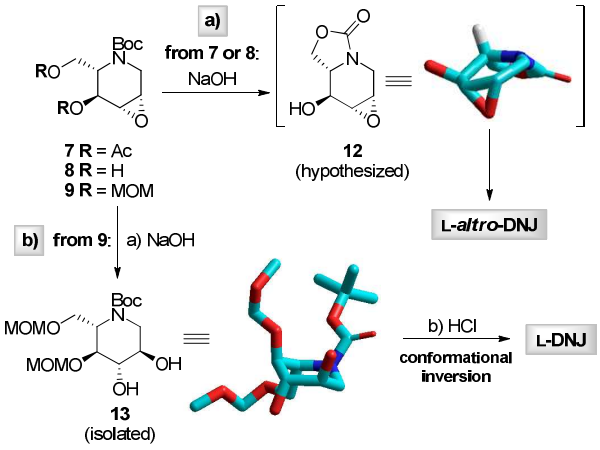
Scheme 1. Stereocontrolled *de novo* synthesis of *ent*-1.



The stereodivergent outcome of the reactions was assumed to rely on the conformational preferences of epoxides **7** and **9** (Scheme 2). We observed that diacetate **7** adopted a sugar conformation having both C4 and C6 substituents in pseudo-diaxial orientations.[32] In line with previous findings, this behavior was judged to be a consequence of the steric clash occurring between the *N*-Boc and the pseudo-equatorially oriented C6 acetate groups.[32] Accordingly, the formation of **10** from **7** was hypothesized to take place via the base-labile cyclic carbamate[36] intermediate **12** (Scheme 2a). In agreement with literature data,[37] an energy-minimized structure of **12** suggested that it was firmly locked in a quasi-planar conformation close to a ^{5}E form with a pseudo-equatorially oriented methyleneoxy moiety (Scheme 2a). The conformational inversion from epoxide **7** to **12** justified the formation of **10** after *trans*-diaxial epoxide ring opening of **12**. Conversely, when the Ac group was replaced by the base-stable MOM group, the oxirane ring

cleavage of *bis*-acetal **9** only provided the *gluco*-configured diol **13** (Scheme 2b). Even though the broadness of most NMR signals of epoxide **9** hampered the identification of its preferred conformation, NMR analysis of diol **13** indicated a conformation with all substituents in near axial orientations ($J_{1,2}$ 2.5 Hz; $J_{3,4}$ 1.8 Hz; $J_{4,5}$ 2.6 Hz). Close agreement between these *J* values and the theoretical values[38] obtained from an energy-minimized structure (Scheme 2b) suggested that a $^4C_1$ chair was adopted in this case. Eventually, the "all-equatorial" L-DNJ **11** was obtained by the concurrent removal of *N*-Boc and MOM groups, enabling as expected a conformational inversion from $^4C_1$ to $^1C_4$ chair (Scheme 2b). With the latter in hand, the access to the corresponding *ent*-**1** was achieved under standard *N*-alkylation conditions (Scheme 1).

Scheme 2. Reactivity and conformational preferences of iminosugar intermediates 7-9.



Biological evaluation. Glycosidase inhibition studies of *ent*-**1** were first carried out. The selected glycosidases were representative examples of various GH families (Table 1 and Supporting Information, Figures S1-S3). No inhibitory effect up to 1 mM was found for *ent*-**1** in most cases, except for the very weak inhibition of intestinal sucrase/isomaltase (38% inhibition, IC_{50} 2 mM, entry 2), lactase (30% inhibition, IC_{50} >5 mM, entry 4) mixed GBA1/GBA2 (18% inhibition, entry 7) and α-fucosidase (19%, entry 14). Instead, rhGAA and β-glucosidases from

P. furiosus and *S. solfataricus P2* even showed a 20-25% activation (entries 3, 5 and 7), even though no significant enhancement in the melting temperature (T_m) of rhGAA by *ent*-**1** was indicated by differential scanning fluorimetry experiments (Supporting Information, Figure S4). Conversely, **1** displayed inhibition of rice α-glucosidase (IC_{50} 275 μM, entry 1), sucrase/isomaltase (IC_{50} 1.5 μM, entry 2), GBA1/GBA2 (IC_{50} 180 μM, entry 7) and recombinant human β-glucocerebrosidase (rhGBA1 or Cerezyme™: IC_{50} 0.3 mM, entry 8). Inhibition of rhGAA was also observed (IC_{50} 158 μM, entry 2). In addition, **1** is reported to be a much more effective inhibitor of endogenous GAA (IC_{50} 5.3 μM).[39]

Table 1. Residual activity of various glycosidases in the presence of 1 and *ent*-1 (1 mM).

Entry	Enzyme	GH Family	Residual activity (%)	
			1	*ent*-**1**
1	Rice α-glucosidase	GH31	17	98
2	Intestinal sucrase/isomaltase[a]	GH31	3	62
3	rh-GAA	GH31	19	125
4	Intestinal lactase[a]	GH1	62	70
5	β-Glucosidase from *P. furiosus*	GH1	81	120
6	β-Glucosidase from *S. solfataricus* P2	GH1	94	124
7	Human GBA1/GBA2[b,c]	GH30/116	24	82
8	rh-GBA1	GH30	35	97
9	Human β-Hexosaminidase[b]	GH20	98	98
10	α-Galactosidase from *T. maritime*	GH36	82	100
11	rh-Gal A	GH27	100	104
12	β-Galactosidase from *A. acidocaldarius*	GH42	98	102

13	Human β-Galactosidase[b]	GH35	87	98
14	α-Fucosidase from *S. solfataricus*	GH29	83	81

Inhibition studies were conducted on purified enzymes unless otherwise specified. [a]Enzyme source: small intestine of WT BL6 mice. [b]Enzyme source: HL60 cells. [c]Measure of the overall β-glucosidase activity in HL60 cells.

The effect of *ent*-**1** in cell lines deriving from Pompe patients was then explored (Figures 2-3). In a first set of experiments, PD fibroblasts from a patient carrying the mutation p.L552P/p.L552P (omozygous mutation) were incubated in the absence or in the presence of 20 μM *ent*-**1** or its D-enantiomer[40] (Figure 2). The administration of *ent*-**1** effectively increased the residual activity of mutated GAA (Figure 2). The activating effect (1.5-fold increase) was slightly higher than that observed for **1** under the same experimental conditions (1.3-fold increase).

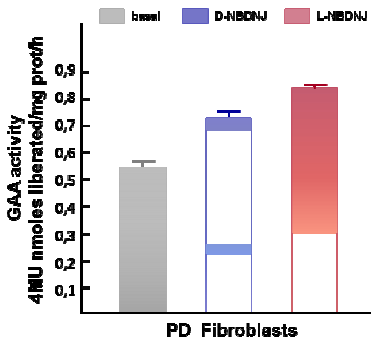


Figure 2. Effect of **1** and *ent*-**1** (20 μM) on residual activity of mutated GAA in fibroblasts from a PD patient carrying the p.L552P/p.L552P mutation.

The chaperoning activity of an L-piperidine iminosugar has been reported only seldom before;[21] in addition, to the best of our knowledge the higher enhancing effect of an L-piperidine iminosugar compared to that of the corresponding D-enantiomer is even unprecedented. The lack of inhibition of the deficient enzyme and of the other glycosidases further augments the activating potential of *ent*-**1**, especially if compared with its enantiomer. It must be noted that,

however, under these conditions the enhancement of GAA residual activity was observed only in cells with *ent*-**1** and it was not sufficiently high to be considered of therapeutic relevance.

Potential synergistic effects deriving from co-administration of *ent*-**1** and rhGAA in PD fibroblasts were also considered. PD fibroblasts from three patients carrying various mutations (Figure 3) were incubated with rhGAA and *ent*-**1** or its D-enantiomer (20 μM). In all cases, co-incubation of *ent*-**1** and rhGAA provided a higher increase in GAA levels than those provided by rhGAA when incubated singularly. Especially when PD fibroblasts carrying the mutation p.L552P/p.L552P were evaluated, a 2-fold increase of GAA activity was found; this value was the same observed by treatment of the cells with **1**/rhGAA. In the remaining cases, the enhancing effect was less marked (and lower than that found for **1**/rhGAA) (Figure 3).

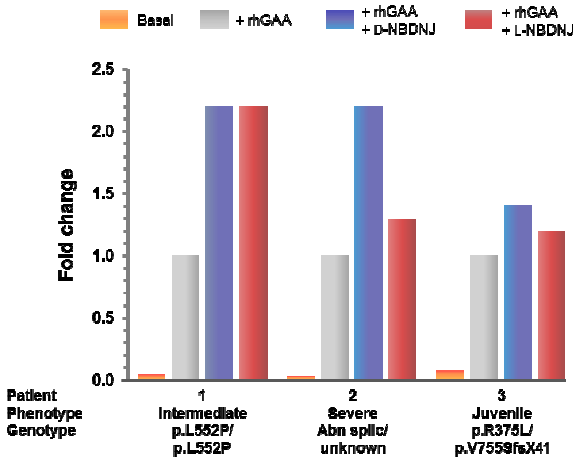


Figure 3. Synergy between rhGAA and **1** or *ent*-**1** in PD fibroblasts.

Western blot analysis was eventually performed in *ent*-**1**-treated and untreated fibroblasts carrying the mutation p.L552P/p.L552P (Figure 4), to provide first clues on the mechanisms enabling the observed enhancement of GAA activity. In this case, density scans of rhGAA bands indicated that the mature 76 KDa isoform was more represented in the presence of both

enantiomers, even though the increase with rhGAA/*ent*-**1** was less marked with respect to that observed in cells treated with rhGAA/**1**.

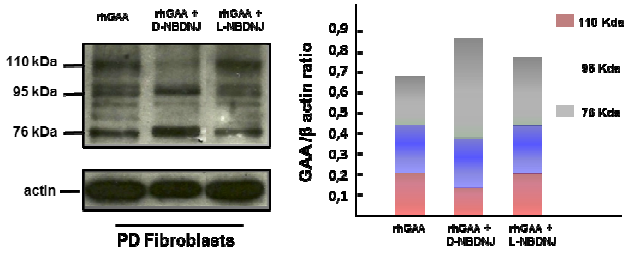


Figure 4. Western blot analysis of rhGAA processing in Pompe fibroblasts carrying the p.L552P/p.L552P mutation incubated with rhGAA alone, rhGAA + **1**, or rhGAA + *ent*-**1**.

CONCLUSION

The synthesis and a preliminary evaluation of the potential of *ent*-**1** as enhancer of GAA activity have been herein reported. The access to *ent*-**1** has been performed by a highly stereocontrolled *de novo* strategy, exploiting the different reactivity and conformational preferences of epoxide intermediates **7-9**. Differently from its D-enantiomer, *ent*-**1** did not act as a glycosidase inhibitor; however, it was found to enhance the activity of lysosomal α-glucosidase in PD fibroblasts. Especially when co-incubated with rhGAA, we found a synergistic enhancing effect, which was comparable to that observed for rhGAA/**1**. From these early studies, *ent*-**1** seems not to affect the stability of the enzyme, while enhancing the activity of the latter in cells. Thus, although the molecule cannot be considered a pharmacological chaperone of GAA, it represents a promising new candidate for the combination therapy of Pompe disease. Further studies aimed at elucidating the nature of the enhancing effect of *ent*-**1** are currently ongoing and will be published in due course.

EXPERIMENTAL SECTION

1) Chemical synthesis

General information. All chemicals and solvents were purchased with the highest degree of purity (Sigma-Aldrich, Alfa Aesar, VWR) and used without further purification. All moisture-sensitive reactions were performed under nitrogen atmosphere using oven-dried glassware. The reactions were monitored by TLC (precoated silica gel plate F254, Merck) and the products were detected by exposure to ultraviolet radiation, iodine vapor and chromic mixture. Column chromatography: Merck Kieselgel 60 (70-230 mesh); flash chromatography: Merck Kieselgel 60 (230-400 mesh). The purity of the synthetic intermediates and the final compounds was determined either by HPLC, NMR, CHNS analysis or by optical rotation and was ≥ 95% in all cases. HPLC analysis was performed using an Agilent 6420 Triple Quadrupole LC-MS/MS system with a HPLC 1100 series binary pump. HRMS analysis was performed on a Thermo LTQ Orbitrap XL mass spectrometer coupled to a Thermo U3000 HPLC system. Mono- and bidimensional NMR spectra were recorded on NMR spectrometers operating at 200 MHz (Varian), 400 MHz (Bruker DRX, Bruker AVANCE) or 500 MHz (Varian Inova), using $CDCl_3$ solutions unless otherwise specified. Combustion analyses were performed using a CHNS analyzer. Optical rotations were measured at 25 ± 2 °C in the stated solvent.

Benzyl ether 3. NaH (31.5 mg, 1.31 mmol) was added to a solution of **2** (0.50 g, 1.01 mmol) in anhydrous DMF (10 mL) at 0 °C under nitrogen atmosphere. After 10 min, BnBr (0.14 mL, 1.21 mmol) was added in one portion. The reaction mixture was warmed to room temperature, stirred for 2h, then carefully quenched with 10% aq NH_4Cl. The mixture was extracted with EtOAc, the combined organic phases washed with brine, dried (Na_2SO_4) and evaporated under reduced pressure. Chromatography of the crude residue over silica gel (hexane/Et_2O = 7:3)

afforded the pure **3** (0.48 g, 80% yield). Oily; ^{1}H NMR (400 MHz): δ 1.42 (s, 9H), 1,44 (s, 3H), 1,51 (s, 3H), 3.09-3.28 (m, 4H), 3.79 (s, 3H), 3.80-3.92 (m, 2H), 4.18-4.40 (m, 4H), 4.42-4.47 (m, 4H), 6.85 (d, 2H, *J* = 8.2 Hz), 7.28-7.40 (m, 7H). ^{13}C NMR (100 MHz): ppm 27.1, 28.3, 29.1, 30.1, 55.2, 59.6, 63.1, 64.1, 69.8, 70.7, 71.6, 80.0, 94.5, 113.7, 126.5, 127.9, 128.4, 128.9, 129.4, 129.6, 133.2, 137.5, 159.3, 162.4. Anal. calcd for $C_{31}H_{41}NO_6S_2$: C, 63.34; H, 7.03; N, 2.38 S, 10.91. Found: C, 63,22; H, 7.05; N, 2,39; S, 10,94.

Tricyclic compound 5. To a stirring solution of alcohol **3** (0.58 g, 0.99 mmol) in a DCE/MeOH solution (3/1 v/v, 20 mL), DDQ (0.56 g, 2.50 mmol) was added in one portion at rt. The resulting mixture was stirred at the same temperature in the dark. After 48h, the reaction was heated to 60 °C and stirred at the same temperature for 8 h. The mixture was then cooled to rt, transferred into a separatory funnel, washed with brine and extracted repeatedly with DCM. The collected organic layers were dried (Na_2SO_4) and the solvent evaporated under reduced pressure. The crude residue was dissolved in DCM (10 mL) and treated with silica gel (2 g) at rt for 30 min. The mixture was then filtered, and the filtrate was concentrated under reduced pressure. Chromatography of the crude residue over silica gel (hexane:acetone = 8:2) gave the pure **5** (0.37 g, 91% overall yield). Oily; ^{1}H NMR (200 MHz): δ 1.43 (s, 9H), 3.08-3.32 (m, 4H), 3.45 (dd, 1H, *J* = 8.0, 1.2 Hz), 3.69 (d, 1H, *J* = 2.1 Hz), 3.85 (dd, 1H, *J* = 8.0, 6.6 Hz), 4.62 (d, 1H, *J* = 11.6 Hz), 4.76 (d, 1H, *J* = 11.6 Hz), 4.89 (bd, 1H, *J* = 6.2 Hz), 5.46 (s, 1H), 7.22-7.45 (m, 5H). ^{13}C NMR (50 MHz): ppm 27.6, 28.0, 28.2, 64.2, 70.5, 79.0, 80.1, 81.6, 85.6, 120.9, 126.2, 127.8, 128.1, 128.4, 137.8, 153.4. Anal. calcd for $C_{20}H_{25}NO_4S_2$: C, 58.94; H, 6.18; N, 3.44; S, 15.74. Found: C, 59.08; H, 6,16; N, 3.43; S, 15.69.

Diol 8. Zemplén deacetylation was accomplished by treatment of a solution of **7** (0.24 g, 0.76 mmol) in MeOH (5 mL) with MeONa (41 mg, 0.76 mmol) for 4h at room temperature. The

mixture was neutralized with few drops of acetic acid, then the volatiles were removed under reduced pressure. The crude residue was dissolved in $CHCl_3$ and filtered through a short pad of silica gel. The resulting filtrate was eventually concentrated to dryness, giving **8**, which was used in the following step without further purification. Oily; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.39 (s, 9H), 3.14 (bs, 1H), 3.35 (bs, 1H), 3.41-3.57 (m, 1H), 3.87 (bt, 1H, J = 7.2 Hz), 3.91-3.98 (m, 1H), 4.02-4.13 (m, 1H), 4.14-4.23 (m, 1H), 4.62 (bt, 1H, J = 4.9 Hz). ^{1}H NMR (400 MHz, 55 °C, DMSO-d_6): δ 1.40 (*s*, 9H), 3.11-3.20 (m, 2H), 3.32-3.41 (m, 2H), 3.46-3.55 (m, 1H), 3.84-3.96 (m, 2H), 4.13 (bs, 1H), 4.47 (t, 1H, J = 5.7 Hz), 5.19 (d, 1H, J = 5.2 Hz). ^{13}C NMR (100 MHz): ppm 28.4, 43.1, 50.2, 53.6, 60.0, 61.1, 63.1, 81.2, 156.9. Anal. calcd for $C_{11}H_{19}NO_5$: C, 53.87; H, 7.81; N, 5.71. Found: C, 53.94; H, 7.79; N, 5.73. HRMS: *m/z* $[M + Na]^+$, calcd: 268.1155; found: 268.1144.

***Bis*-acetal 9**. To a stirring solution of the crude diol **8** (26 mg, 0.1 mmol) in DCM (2 mL), DIPEA (35 μL, 0.2 mmol) and MOMCl (15 μL, 0.2 mmol) were sequentially added at room temperature. The resulting mixture was stirred at the same temperature for 48 h; then the reaction was quenched by the addition of H_2O (1 mL). The mixture was diluted with DCM and washed with brine (pH 7). The organic phases were dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Chromatography of the crude residue gave the *bis*-acetal **9** (25 mg, 76% yield) as a colourless oil. ^{1}H NMR (400 MHz): δ 1.45 (s, 9H), 3.31 (bs, 1H), 3.45 (s, 3H), 3.37-3.46 (m, 4H), 3.56-3.70 (m, 4H), 4.18 (bs, 1H), 4,30 (bd, 1H, J = 13.3 Hz), 4.54-4.68 (m, 2H), 4,71 (d, 1H, J = 6.7 Hz), 4.79 (d, 1H, J = 6.7 Hz). ^{1}H NMR (400 MHz, 45°C): δ 1.53 (s, 9H), 3.31-3.35 (m, 2H), 3.38 (t, 3H), 3.45 (t, 3H), 3.47-3.53 (m, 1H), 3.60-3.64 (m, 1H), 3.68 (t, 2H, J = 7.9 Hz), 4.20-4.45 (m, 2H), 4.61 (d, 1H, J = 6.6 Hz), 4.63 (d, 1H, J = 6.7 Hz), 4.73 (d, 1H, J = 6.6 Hz), 4.82 (d, 1H, J = 6.8 Hz). ^{13}C NMR (100 MHz): ppm 28.3, 50.4, 51.1, 53.8, 55.2, 55.7, 65.1,

69.7, 72.8, 80.2, 96.0, 155.5. Anal. calcd for $C_{15}H_{27}NO_7$: C, 54.04; H, 8.16; N, 4.20. Found: C, C, 54.20; H, 8.14; N, 4.19.

L-*Altro*-deoxynojirimycin (**10**). Acetate **7** (33 mg, 0.1 mmol) was suspended in a 1M NaOH solution (3 mL). The resulting suspension was warmed to reflux and stirred at the same temperature for 1 h. Temperature was then cooled to rt; the mixture was eluted through a short pad containing a Dowex 50WX8 (H^+ form) resin. The resulting filtrate was concentrated; chromatography of the crude residue provided **10** (9.0 mg, 55% yield) as an oil. NMR spectra of **10** were fully in line with those reported elsewhere.[32] HRMS: *m/z* $[M + H]^+$, calcd: 164.085; found: 164.0902.

L-Deoxynojirimycin hydrochloride (**11**). Oxirane **9** (25 mg, 75 μmol) was suspended in a NaOH 1M solution (2 mL). The reaction mixture was warmed to reflux for 1 h; then temperature was cooled to rt. The mixture was eluted through a short pad containing a Dowex 50WX8 (H^+ form) resin. The resulting filtrate was concentrated, acidified with HCl 2M (3 mL) and refluxed for 1 h. Removal of the volatiles under reduced pressure yielded **11** (15 mg, 91% over two steps) as a white solid. ^{1}H NMR (400 MHz, D_2O): δ 3.14 (dd, 1H, *J* = 12.6, 11.6 Hz), 3.37 (ddd, 1H, *J* = 3.2, 5.2, 10.5 Hz), 3.67 (dd, 1H, *J* = 12.6, 5.4 Hz), 3.76 (dd, 1H, *J* = 10.5, 9.3 Hz), 3.87-3.98 (m, 2H), 4.03 (dd, 1H, *J* = 5.2, 12.8 Hz), 4.11 (dd, 1H, *J* = 3.2, 12.8 Hz). ^{13}C NMR (100 MHz, D_2O): ppm 41.5, 53.6, 55.8, 62.8, 63.7, 72.2. Anal. calcd for $C_6H_{14}ClNO_4$: C, 36.10; H, 7.07; N, 7.02. Found: C, 36.22; H, 7.05; N, 7.00. HRMS: *m/z* $[M + H]^+$, calcd: 164.0917; found: 164.0910.

Diol 13. A small amount of the crude residue deriving from oxirane ring cleavage of **9** was subjected to column chromatography (hexane:ethyl acetate, from 100:0 to 70:30), to provide the pure **13** as a pale yellow oil. ^{1}H NMR (400 MHz): δ 1.47 (s, 9H), 3.36 (s, 3H), 3.39 (bd, 1H, *J* =

14.3 Hz), 3.43 (s, 3H), 3.71 (bd, 1H, *J* = 2.7 Hz), 3.77 (dd, 1H, *J* = 4.8, 10.3 Hz), 3.85 (dd, 1H, *J* = 5.5, 10.3 Hz), 3.88 (bt, 1H, *J* = 2.0 Hz), 3.91 (bt, 1H, *J* = 3.3 Hz), 4.07 (dd, 1H, *J* = 3.0, 14.3 Hz), 4.47 (bt, 1H, *J* = 5.1 Hz), 4.62 (d, 1H, *J* = 6.6 Hz), 4.65 (d, 1H, *J* = 6.6 Hz), 4.71 (d, 1H, *J* = 7.0 Hz), 4.74 (d, 1H, J = 7.0 Hz). ^{13}C NMR (100 MHz, acetone-d_6): ppm 27.8, 51.6, 51.8, 54.3, 55.0, 64.8, 69.3, 69.6, 74.3, 78.7, 95.4. 95.9, 155.3. Anal. calcd for $C_{15}H_{29}NO_8$: C, 51.27; H, 8.32; N, 3.99. Found: C, 51,40; H, 8.30; N, 3.98. HRMS: *m/z* $[M + Na]^+$, calcd: 374.1785; found: 374.1769.

***N*-Butyl-L-deoxynojirimycin** (*ent*-**1**). Butanal (16.4 μL, 0.18 mmol) was added to a stirring solution of L-DNJ (**11**, 28 mg, 0.14 mmol) in an acetic acid/methanol (2 mL, 1:200 v/v) solution. After a few minutes, $NaBH_3CN$ (8.8 mg, 0.14 mmol) was added. The resulting mixture was stirred at the same temperature for 16 h; then the volatiles were removed under reduced pressure. Flash chromatography of the crude residue (DCM:MeOH:NH_4OH = 70:28:2) yielded the pure *ent*-**1** (30 mg, 96% yield) as a colorless oil. $[\alpha]_D$ +17.0, *c* 0.27 (**1**: -15.9, *c* 0.77).[39] ^{1}H NMR (400 MHz, D_2O): δ 0.92 (t, 3H, *J* = 7.3 Hz), 1.31 (sext, 2H, *J* = 7.3 Hz), 1.47-1.59 (m, 2H), 2.40-2.55 (m, 2H), 2.68-2.80 (m, 1H), 2.82-2.95 (m, 1H), 3.10-3.21 (m, 1H), 3.32 (t, 1H, *J* = 9.3 Hz), 3.45 (t, 1H, *J* = 9.5 Hz), 3.61 (ddd, 1H, *J* = 10.7, 9.3, 4.9 Hz), 3.89 (dd, 1H, *J* = 12.9, 2.5 Hz), 3.94 (dd, 1H, *J* = 12.9, 2.5 Hz). ^{13}C NMR (100 MHz, CD_3OD): ppm 14.2, 21.4, 27.0, 53.7, 56.5, 57.7, 67.4, 69.6, 70.8, 79.6. Anal. calcd for $C_{10}H_{21}NO_4$: C, 54.77; H, 9.65; N, 6.39. Found: C, 54.88; H, 9.62; N, 6.37. HRMS: *m/z* $[M + H]^+$, calcd: 220.1471; found: 220.1533.

2) Computational studies

The optimal conformation of compounds **12-13** was simulated using the semiempirical method PM3 (software package Hyperchem 8.0).

3) Biochemical studies

Glycosidase inhibition experiments. Glycosyl hydrolase activities were assayed by spectroscopic techniques using artificial sugar-substrates containing 2-nitrophenyl (2Np), 4-nitrophenyl (4Np) or 4-methylumbelliferyl (4-MU) moieties.

The inhibition assays of rice α-glucosidase (8 μg) and recombinant human α-glucosidase (rhGAA; Myozyme, 5 μg) were performed on 4Np-α-D-glucopyranoside (4Np-α-Glc, 25 and 20 mM, respectively), in 100 mM sodium acetate buffer, pH 4.0, at 37 °C. The inhibition assays of the β-glucosidases from *Pyrococcus furiosus* (0.15 μg) and *Sulfolobus solfataricus P2* (0.1 μg), and the β-galactosidase from *Alicyclobacillus acidocaldarius* (0.05 μg) were performed on 2.5 mM 4Np-β-D-glucopyranoside (4Np-β-Glc), 6 and 20 mM, respectively, 2Np-β-D-galactopyranoside (2Np-β-Gal), in 50 mM citrate/phosphate buffer, pH 5.5, at 65 °C. The inhibition assays of the recombinant human α-galactosidase A (Fabrazyme, 0.4 μg) were performed on 30 mM 4Np-α-D-galactopyranoside (4Np-α-Gal) in 50 mM citrate/phosphate pH 4.5, at 37°C. The inhibition assays of the α-galactosidase from *Thermotoga maritima* (0.1 μg) were performed on 2.5 mM 4Np-α-Gal, in 50 mM sodium acetate pH 5.0, at 65 °C. The inhibition assays of the α-fucosidase from *Sulfolobus solfataricus* (2 μg) were performed on 1 mM 4Np-α-L-fucopyranoside (4Np-α-Fuc), in 50 mM sodium phosphate buffer, pH 6.5, at 65 °C. For all the above enzymes, the % of inhibition was measured in the presence of **1** and *ent*-**1** (1 mM). The reactions were stopped in ice by adding 0.8 mL of 1 M sodium carbonate pH 10.2. The absorbance values were measured by a Varian Cary 50 Bio UV-visible spectrophotometer at 420 nm at room temperature and the molar extinction coefficients were 17.2 $mM^{-1} \times cm^{-1}$ (4Np) and 4.83 $M^{-1} \times cm^{-1}$ (2Np). The activities of the enzymes without inhibitors were reported

as 100% (Figure S2). The IC_{50} values of **1** were measured for rice α-glucosidase and rhGAA at their standard conditions, in the range 25-500 μM and 1-1000 μM inhibitor, respectively (Figure S3). All kinetic data were calculated as the average of the two experiments and were plotted and refined with the programme GraphPad Prism.

The remaining assays were performed as follows. β-Glucosidase, β-galactosidase and β-hexosaminidase activity: the enzyme sources were HL60 cell pellets (human promyelocytic leukemia cells) homogenised in PBS with 0.1% TritonX-100. HL-60 cells were cultured in RPMI-1640 supplemented with 20% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine. Cultures were maintained at 37 °C with 5% CO_2. For the enzyme assays, 30 x 10^6 cells were collected, centrifuged, washed with PBS, centrifuged again and stored as dry pellets at -20°C. For the determination of β-galactosidase activity, 1 mM 4-MU β-D-galactoside (4-MU-Gal), in assay buffer (200 mM acetate, pH 4.3, 100 mM NaCl, 0.1% TritonX-100) was used. The substrate for β-glucosidase and Cerezyme™ activity was 4.5 mM 4-MU β-D-glucoside (4-MU-Glc) in assay buffer (200 mM sodium citrate, pH 5.5, 0.1% TritonX-100). For the determination of β-hexosaminidase activity, 3 mM 4-MU *N*-acetyl-β-D-glucosaminide (4-MU-GlcNAc) in assay buffer (200 mM sodium citrate, pH 4.5, 0.1% TritonX-100) was used. Assays (in triplicate) containing homogenate, different concentrations of iminosugar inhibitors and 4-MU substrates were incubated at 37 °C for 30 min. The reaction was stopped by adding cold 0.5 M Na_2CO_3 pH 10.7, and released 4-MU was measured with a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany) with excitation at 360 nm and emission at 460 nm. Sucrase/isomaltase and lactase activity: supernatant from homogenised small intestines of C57BL/6 control mice[41] (5 weeks old) was used as the enzyme source. Small intestines of C57BL/6 control mice were dissected (2-3cm), carefully washed in PBS and centrifuged in PBS for 10 min at 3.000 rpm. The

supernatant was discarded and clean intestines were set up at 100 mg wet weight/ml 100 mM sodium citrate / 100 mM citric acid buffer, pH 6. The intestines were homogenised by using an Ultraturrax T25 probe homogeniser (Janke&Kunkel, IKA-Labortechnik, Germany). The homogenate underwent three repeated cycles of freezing and thawing before second centrifugation for 10 min at 3.000 rpm. Finally, the pellet was discarded and the supernatant was kept at -20 °C for enzyme assays. Sucrase/isomaltase and lactase activities were assayed by measuring glucose released from sucrose or lactose substrate using a Glucose Assay Kit (Sigma-Aldrich). Assays (in triplicate) contained a range of concentrations of iminosugar inhibitors and were incubated at 37 °C for 30 min. Absorbance at 340 nm was measured with a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany).

Differential scanning fluorimetry (DSF) of rhGAA. Thermal stability scans of rhGAA were performed as already described.[6] Briefly, 2.5 μg of enzyme were incubated in the absence and in the presence of **1** and *ent*-**1** (0.1 mM and 1 mM in both cases), SYPRO Orange dye, 25 mM sodium phosphate buffer and 150 mM NaCl, pH 7.4. Thermal stability scans were performed at 1 °C/min in the range 25–95 °C in a Real-Time Light Cycler (Biorad, Milan, Italy). SYPRO Orange fluorescence was normalized to maximum fluorescence value within each scan to obtain relative fluorescence.

4) Biological studies

Cell lines. Fibroblasts from the PD patients carrying different mutations (pt. 1: phenotype, intermediate; genotype, p.L552P/p.L552P; pt. 2: phenotype, severe; genotype, aberrant splicing/unknown; pt. 3: phenotype, juvenile; genotype, p.R375L/p.V755SfsX41) were available

in the laboratory of Department of Translational Medical Sciences, section of Pediatrics, Federico II, University of Naples, Italy. All cell lines were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY) and 20% fetal bovine serum (Sigma-Aldrich, St Louis, MO), supplemented with 100U/ml penicillin and 100 mg/ml streptomycin.

Reagents. rhGAA (Myozyme) was purchased from Genzyme Co. (Naarden, Netherlands). Compound **1** was purchased from (Sigma-Aldrich, St Louis, MO). The primary antibodies used for western blot analysis were anti-human GAA, purchased from (PRIMM s.r.l., Milan, Italy); anti-β-actin, mouse monoclonal antibody, purchased from (Sigma-Aldrich, St Louis, MO). The secondary antibodies were goat anti-rabbit IgG (H + L)-HRP conjugated and goat anti-mouse IgG (H + L)-HRP conjugated purchased from Biorad (Hercules, CA).

Treatment of fibroblasts with iminosugars. Fibroblasts were incubated for 4 days with **1** or *ent*-**1** (20 μmol/L). The same cell lines were cultured in parallel in the absence of iminosugars for comparison. After each experiment, the cells were harvested and the cell extracts were used for the assay of GAA activity.

Uptake of recombinant enzyme. To study rhGAA uptake and correction of GAA activity, PD and control fibroblasts were incubated with 50 μmol/L rhGAA for 24 hours in the absence and in the presence of **1** or *ent*-**1** (20 μmol/L). The cells were then harvested and cell pellets were washed twice with phosphate-buffered saline, resuspended in water and disrupted by five cycles of freeze-thawing. GAA activity was assayed as described above. Protein concentrations were measured according to Lowry *et al.*[42]

GAA enzyme assay. Fibroblasts were harvested by trypsinization and disrupted by freezing and thawing (3x). Cell homogenates (10-20 μg of proteins) were incubated at 37°C for 60 min

with 2 mM 4 methylumbelliferyl α-D-glucopyranoside as substrate in a 0.2 acetate buffer pH 4 in an incubation mixture of 20 µl. Reactions were stopped with 1 ml glycine carbonate buffer pH 10.7 and fluorescence was read on a Turner biosystem fluorometer Modulus 9200 (360 nm excitation, 450 nm emission). Protein concentration in cell homogenates was measuring according to Lowry *et al*.[42]

Western blot analysis. To study GAA processing fibroblast extracts were subjected to western blot analysis. The cells were harvested, washed in phosphate-buffered saline, resuspended in water, and disrupted by five cycles of freeze-thawing. Equal amounts (20 µg protein) of fibroblast extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (7.5% acrylamide) and proteins were transferred to PVD membrane (GE Healthcare). An anti-human antiserum GAA was used as primary antibody to detect GAA polypeptides; to detect β-actin, a monoclonal mouse antibody was used. Immunoreactive proteins were detected by chemiluminescence (ECL; GE Healthcare). Quantitative analysis of band intensity was performed using ImageJ.

ASSOCIATED CONTENT

Copies of NMR spectra, LC-MS data, glycosidase inhibition data and DSF experiments. Molecular formula strings of compounds **1** and *ent*-**1** (CSV). This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

ASSC, active-site specific chaperone; Bn, benzyl; DCE, dichloroethane; DCM, dichloromethane; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; ECL, enhanced chemiluminescence; ERT, enzyme replacement therapy; Fuc, fucopyranoside; GAA, acid α-glucosidase; Gal, galactopyranoside; GBA, glucocerebrosidase; GH, glycosyl hydrolase; Glc, glucopyranoside; HRP, horseradish peroxidase; IgG, immunoglobulin G; LSD, lysosomal storage disorder; MOM, methoxymethyl; MU, methylumbelliferyl; NBDNJ, *N*-butyldeoxynojirimycin; NMR, nuclear magnetic resonance; NP, nitrophenyl; PBS, phosphate-buffered saline; PCT, pharmacological chaperone therapy; PD, Pompe disease; PVD, polyvinylidene.

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Intramolecular Rearrangement Reactions as Advanced Synthetic Methods in Glycoscience, 1st Edition; Witczak, Z. J., Bielski, R., Eds.; John Wiley & Sons Ltd.: Hoboken, 2016, pp. 97-113.

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(34) As already reported (see ref. 32), a carbohydrate-like numbering was applied for *ent*-**1** and its precursors.

(35) D'Alonzo, D.; Guaragna, A.; Napolitano, C.; Palumbo, G. Rapid access to 1,6-anhydro-β-L-hexopyranose derivatives via domino reaction: synthesis of L-allose and L-glucose. *J. Org. Chem.* **2008**, *73*, 5636-5639.

(36) The formation of **12** allows explaining the NaOH-mediated cleavage of the *N*-Boc group, despite the recognized stability of the latter to alkaline conditions, see: Agami, C.; Couty, F. The reactivity of the *N*-Boc protecting group: an underrated feature. *Tetrahedron* **2002**, *58*, 2701-2724.

(37) Singh, A.; Kim, B.; Lee, W. K.; Ha, H.-J. Asymmetric synthesis of 1-deoxyazasugars from chiral aziridines. *Org. Biomol. Chem.* **2011**, *9*, 1372-1380.

(38) Theoretical *J* values were obtained by applying the Haasnoot–de Leeuw–Altona equation: Navarro-Vázquez, A.; Cobas, J. C.; Sardina, F. J.; Casanueva, J.; Díez, E. A graphical tool for the prediction of vicinal proton-proton $^{3}J_{HH}$ coupling constants. *J. Chem. Inf. Comput. Sci.* **2004**, *44*, 1680-1685.

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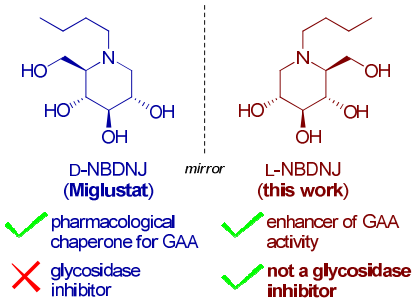
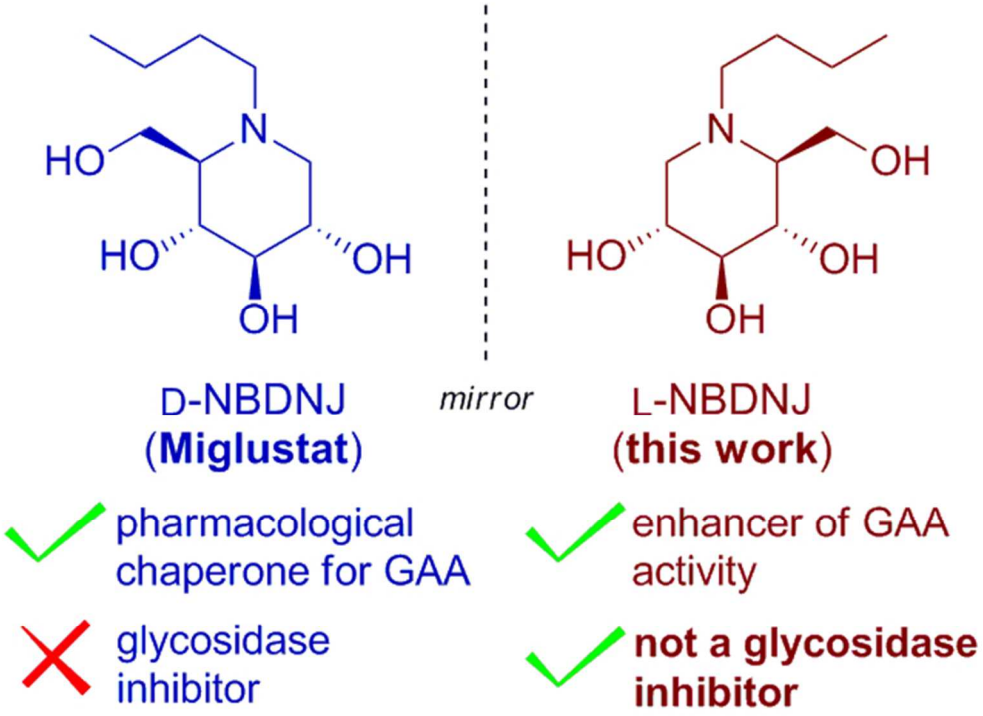
(40) For a comparative analysis of the biological activity of the two enantiomers, experiments using 20 μM solutions of *ent*-**1** – the optimal concentration value found for **1** (ref. 13) – were conducted in this study.

(41) All animal studies were approved by the UK Home Office for the conduct of regulated procedures under licence (Animal Scientific Procedures Act, 1986).

(42) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *1*, 265-275.

TABLE OF CONTENTS GRAPHIC





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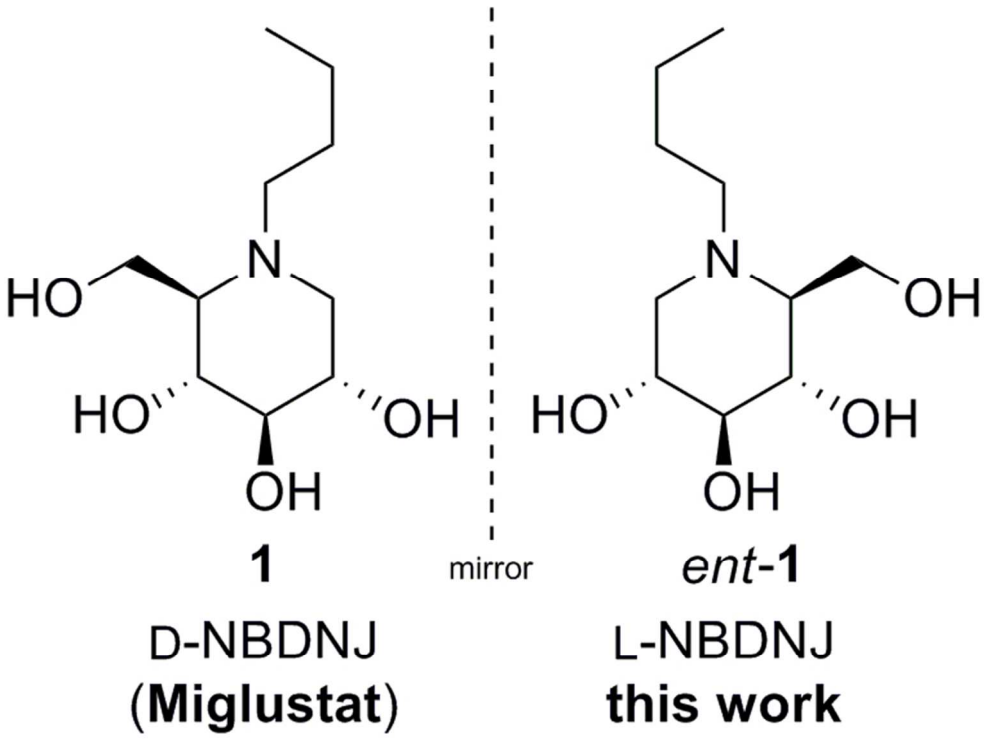

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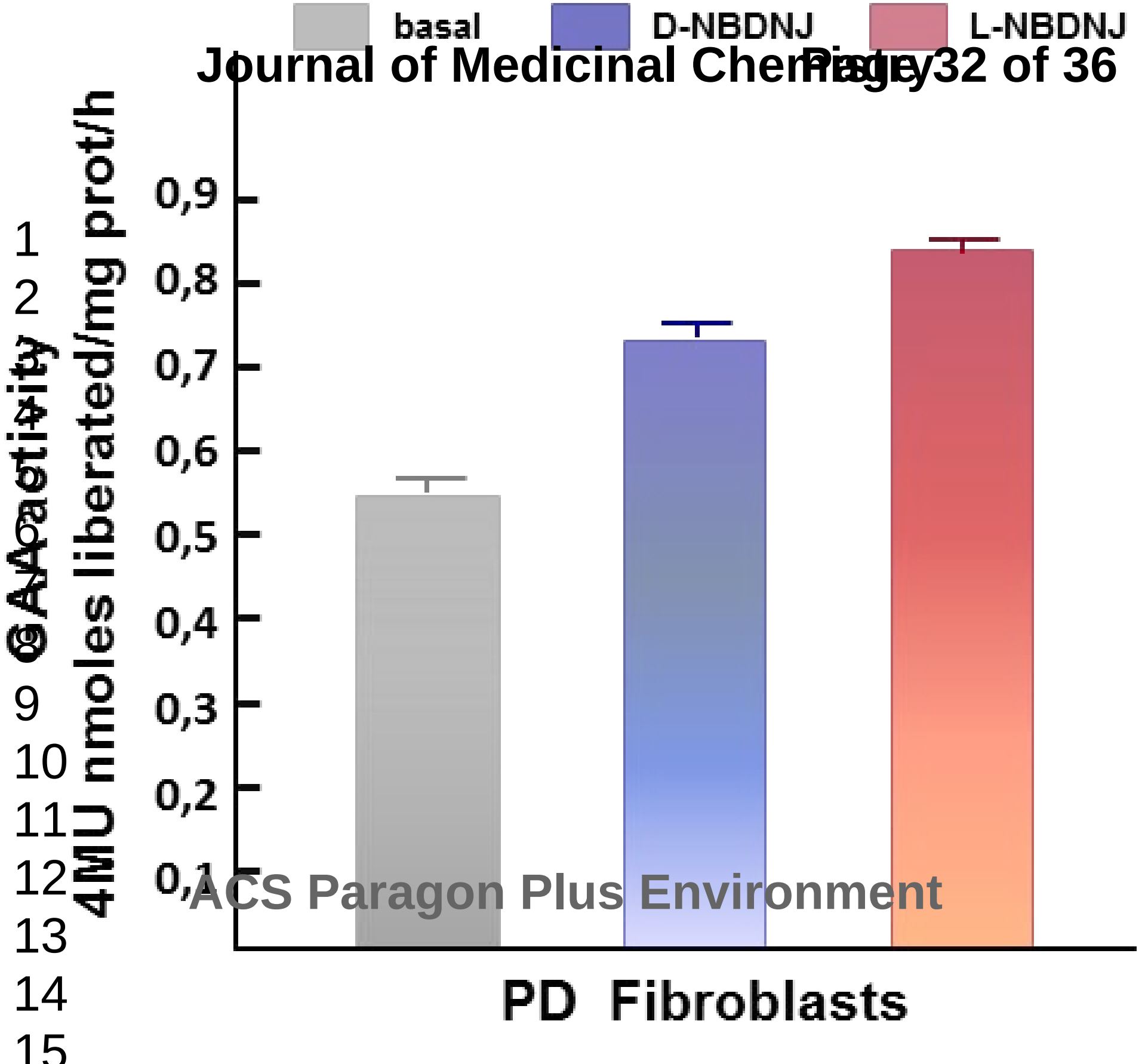

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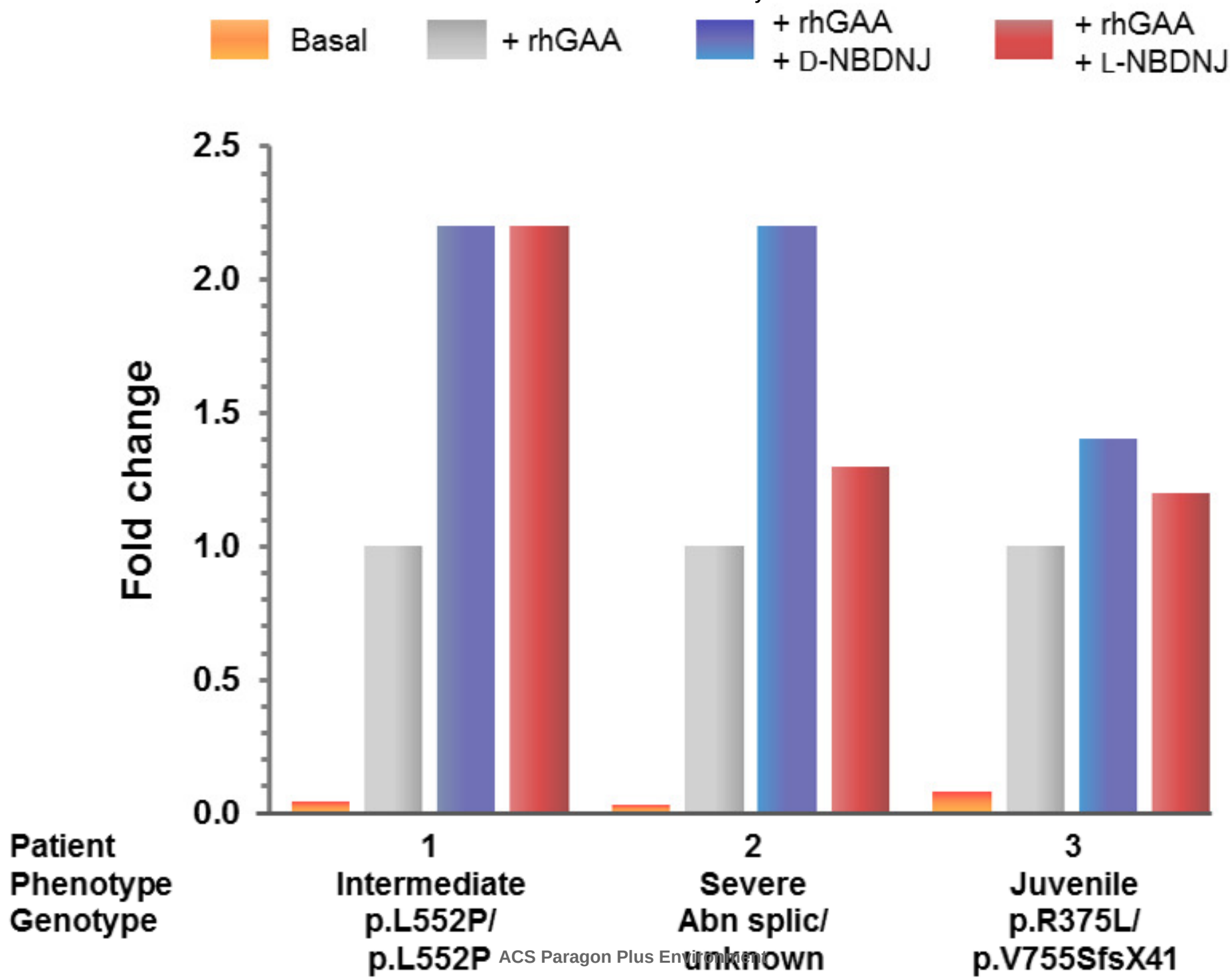

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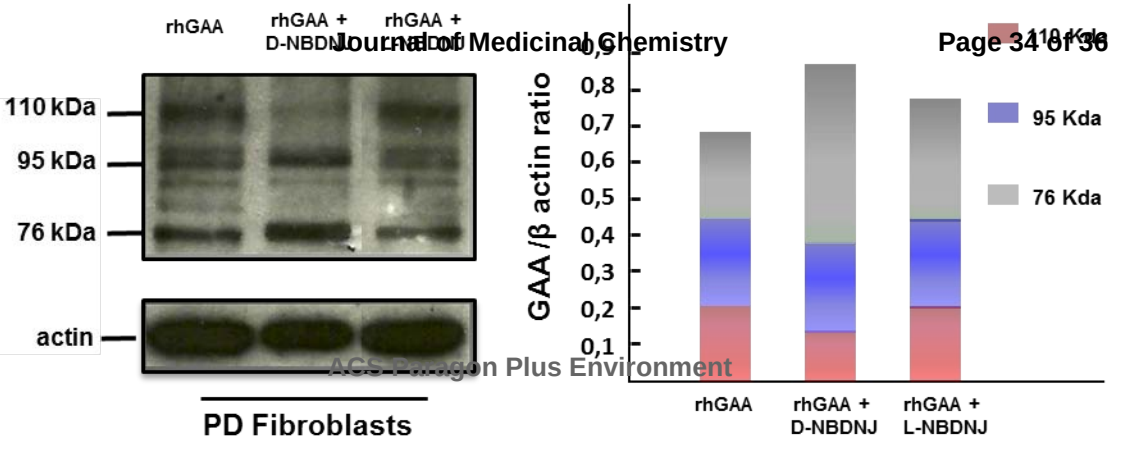

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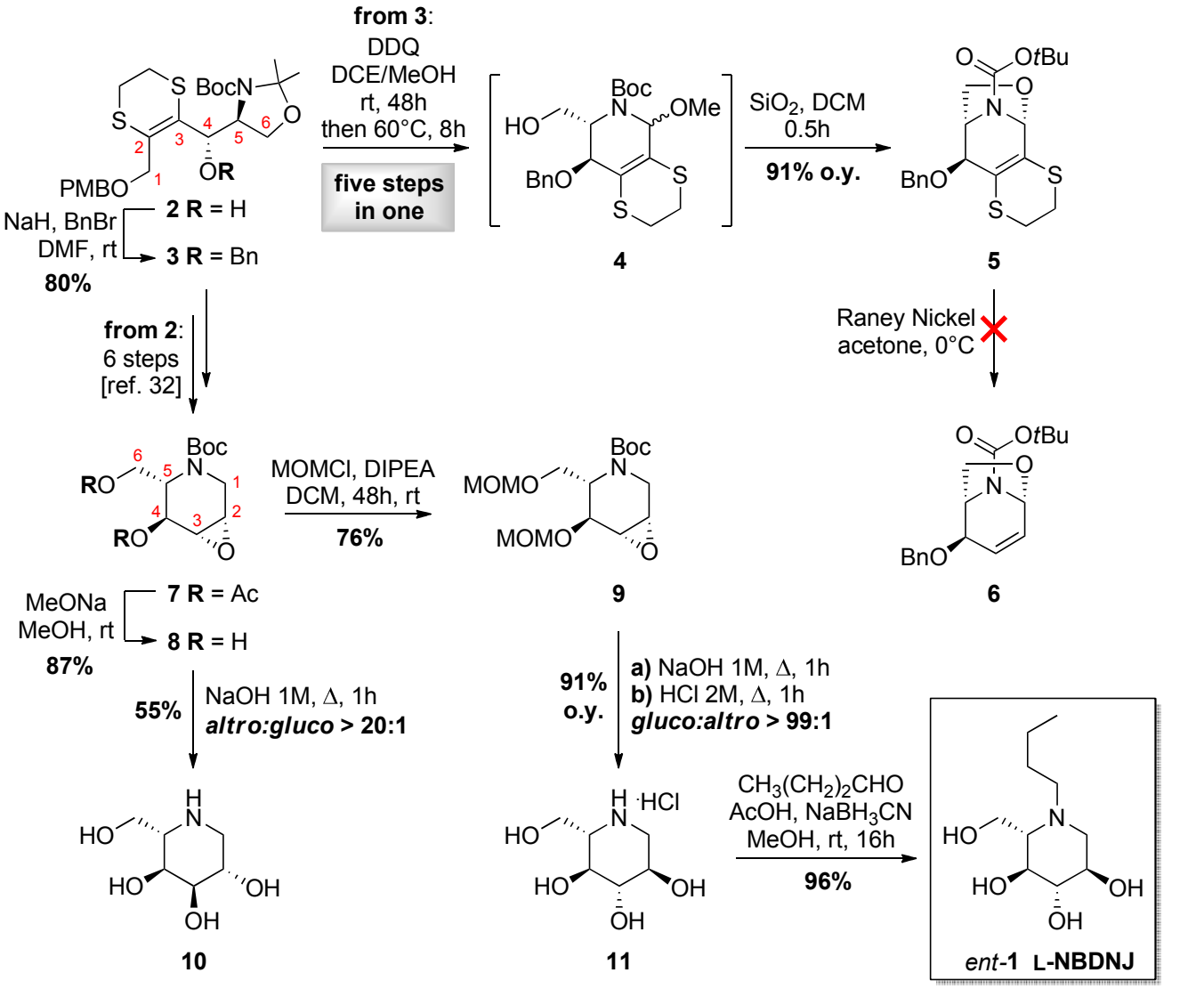
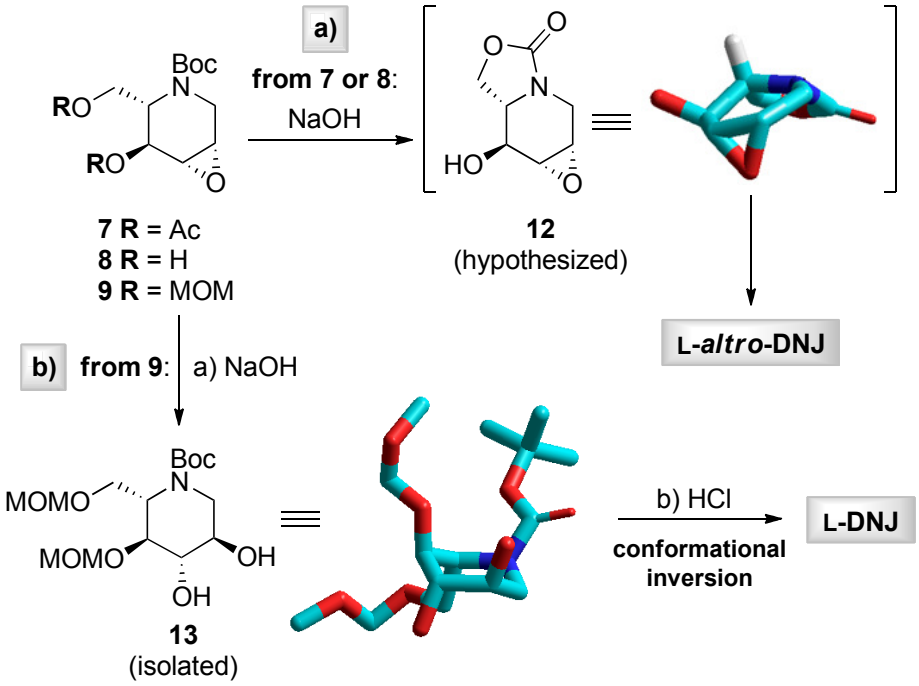

from 3:
DDQ
DCE/MeOH
rt, 48h
then 60°C, 8h
five steps in one
SiO2, DCM
0.5h
91% o.y.
NaH, BnBr
DMF, rt
80%
2 R = H
3 R = Bn
4
5
from 2:
6 steps
[ref. 32]
Raney Nickel
acetone, 0°C
MOMCl, DIPEA
DCM, 48h, rt
76%
MeONa
MeOH, rt
87%
7 R = Ac
8 R = H
6
9
55%
NaOH 1M, Δ, 1h
altro:gluco > 20:1
91% o.y.
a) NaOH 1M, Δ, 1h
b) HCl 2M, Δ, 1h
gluco:altro > 99:1
CH3(CH2)2CHO
AcOH, NaBH3CN
MeOH, rt, 16h
96%
10
11
ent-1 L-NBDNJ
[α]D25 + 17.0 (c 0.27, H2O)
D-NBDNJ (1) [ref. 39]:
[α]D25 - 15.9 (c 0.77, H2O)


a)
from 7 or 8:
NaOH
Boc
N
RO
RO
O
7 R = Ac
8 R = H
9 R = MOM
O
O
N
HO
O
12
(hypothesized)
L-altro-DNJ
b) from 9: a) NaOH
Boc
N
MOMO
MOMO
OH
OH
13
(isolated)
b) HCl
conformational
inversion
L-DNJ