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Abstract

Ten new phenylalkyl-substituted iminosugars (**1**-**10**) and a cinnamic acid derived glucoside (**11**) were isolated from the roots of *Glyphaea brevis* (Malvaceae). Their structures were elucidated by 1D and 2D NMR analysis, as well as by HR-ESIMS. Compounds **1**-**10** retain an unprecedented structure composed of an iminosugar-like core identified as 1-deoxyfuconojirimycin in glyphaeaside A_1-A_4 (**1**, **2**, **5**, **6**), 1-deoxygalactonojirimycin in glyphaeaside B_1-B_5 (**3**, **4**, **7**- **9**) or 1-deoxynojirimycin in glyphaeaside C (**10**), substituted by a *β*-D-glucopyranose in compounds **2**, **4**, **6** and **9**. These compounds feature a di-, tri- or tetra-hydroxylated nine-carbon chain at the pseudo-anomeric position, substituted by a terminal phenyl group. All alkyl *C*-iminosugars displayed potent and selective inhibition towards *β*-glucosidase with IC_{50} values ranging from 0.15 to 68 µM. Compound **10** with an 1-deoxynojirimycin backbone was the most active and was found to act as a competitive inhibitor with K_i = 31 nM, therefore emerging as one of the most potent inhibitor of *β*-glucosidase reported to date. Inhibition of *β*-mannosidase was observed with compounds **1**, **3**, **7** and **10**, but only weak inhibition could be detected with the alkyl-*C*-iminosugars on the other tested glycosidases (*α*-glucosidase, *α*-fucosidase, *α*- and *β*-galactosidase).

1.Introduction

Naturally occurring iminosugars are monosaccharide analogues with the heterocyclic oxygen replaced by nitrogen. These structures display strong glycosidase inhibitory activity, generally attributed to their structural analogy with the oxocarbenium-like transition state which forms during the glycosidase-catalyzed reaction (Segraves and Crews, 2005). Glycosidases are involved in several important biological processes, such as digestion, biosynthesis of glycoproteins and lysosomal catabolism of glycoconjugates. As a consequence, glycosidase inhibitors are potential agents for the treatment of type 2 diabetes, viral infections and cancer (Kato et al., 2010, Mehta et al., 1998, Wordnigg et al., 2008). After the isolation of nojirimycin in the 1960s (Figure 1), a large series of 5-membered or 6-membered-ring iminosugars have been described from plant and microbial sources. Various configurations are fashioned by nature, affording a large panel of stereoisomers with specific biological properties (Asano et al., 2000, Watson et al., 2001). However, only few iminosugar derivatives with a long-side chain have been isolated. Broussonetines are polyhydroxy-pyrrolidines (5-membered iminosugars) featuring a 13-carbon substituent vicinal to nitrogen (Figure 1) and to date, about twenty compounds of the broussonetine family have been isolated from *Broussonetia kajinoki* (Moraceae), which differ in configuration and in the substitution pattern of the alkyl chain (Asano et al., 2004, Kato et al., 2010). Recently, new pyrrolidine derivatives with long side chain at the C-1 position were isolated from *Adenophylla triphylla* and *Suregada glomerulata* (Kato et al., 2010, Yan et al., 2013). Interestingly, six-membered iminosugars (piperidine derivatives) possessing additional alkyl substitution (n>4) are rare and are limited to the three batzellasides A-C isolated from a marine source and a few related analogues (Segraves and Crews, 2005) (Figure 1).

Glyphaea brevis (Spreng.) Monach. is a shrub or tree, widely distributed in Africa (Adjanohoun et al., 1984). In traditional African medicine, it is used for the treatment of various diseases and ailments. For example, its roots are used to treat sleeping sickness, paralysis, respiratory diseases, diarrhea, dysentery, convulsions and spasms (Adjanohoun et al., 1984, Bouquet and Debray, 1974, Oshomoh and Idu, 2012, Borokini and Omotayo).

In this paper, we describe the isolation and structural determination of ten new phenylalkyl-*C*-iminosugars (**1**-**10**) and a cinnamic acid derivative glucoside **(11**) from the roots of *Glyphaea brevis* (Figure 1). The ten new *C*-iminosugar derivatives contain a polyhydroxypiperidine core related to 1-deoxyfuconojirimycin (DFJ, in compounds **1**, **2**, **5**, and **6)**, 1-deoxygalactonojirimycin (DGJ, in compounds **3**, **4**, **7**, **8**, and **9**), or 1-deoxynojirimycin (DNJ, in compound **10**), functionalized with a C_9 side-chain at the C-1α position. In addition, their inhibitory activity toward glycosidases was evaluated.

2. Results and discussion

The 80% methanol extract of *Glyphaea brevis* roots was concentrated and partitioned successively with $CHCl_3$ and *n*-BuOH. The *n*-BuOH soluble fraction was subjected to normal-phase and RP-18 CC and repeated HPLC to yield eleven new compounds (**1**-**11**). The D-configuration for Glc in isolated compounds was verified by measurement of the optical rotation of purified glucose after acid hydrolysis of the *n*-BuOH soluble fraction.

Glyphaeaside A_1 (**1**) has a $C_{21}H_{35}NO_7$ molecular formula, deduced from the $[M+H]^+$ ion at *m/z* 414.2487 (calcd for $C_{21}H_{36}NO_7$, 414.2492) in the positive HRESIMS. The ^1H and ^{13}C NMR spectra of **1** showed signals corresponding to aromatic, glycosidic, and aliphatic chain protons and carbons. The ^1H NMR spectrum exhibited, for the aromatic part, signals characteristics of a

monosubstituted aromatic ring at δ_H 7.26 (1H, tt, *J* = 7.3**,** 2.3 Hz, H-4′), 7.35 (2H, t, *J* = 7.3 Hz, H-3′, -5′) and 7.42 (2H, dd, *J* = 7.3, 2.3 Hz, H-2′, -6′), and their corresponding carbon resonances were assigned by HSQC at δ_C 128.2, 129.1, and 127.7, respectively (Table 1). The connectivity of the phenyl moiety to the C-1 of the aliphatic chain was defined unambiguously by the HMBC correlation H-2′ and H-6′/C-1 (δ_C 74.0) (figure 2). Analysis of the COSY spectrum showed correlations between H-1 (δ_H 4.91, d, *J*=3.5 Hz) and H-2 (δ_H 3.49, dd, *J*=6.8, 3.5 Hz), H-2 and H-3 (δ_H 3.57, ddd, *J*=9.1, 6.8, 3.5 Hz), and H-3 and H-4 (2H, δ_H 1.45, 1.73, m). On the other side of the aliphatic chain, the COSY spectrum showed correlations between H-9 (δ_H 3.76, ddm, *J*=7.4, 3.1 Hz) and H-8 (2H, δ_H 1.54, 1.63, m), and between H-8 and H-7 (2H, δ_H 1.43, 1.53, m). Their corresponding carbons C-1 (δ_C 74.0), C-2 (δ_C 79.6), C-3 (δ_C 72.6), C-4 (δ_C 33.9), C-7 (δ_C 26.5), C-8 (δ_C 26.5), and C-9 (δ_C 72.2) were attributed by analysis of HSQC spectrum. The HMBC cross-peaks observed between H-4 and C-5 (δ_C 26.6) and C-6 (δ_C 30.7) on one side (Figure 2), and between H-8 and C-7 (δ_C 26.5) and C-6, on the other side, indicated that a C_9 aliphatic chain was attached to C-1′ of the phenyl moiety. The chemical shift of carbons C-1, -2, -3, and -9 ranging from δ_C 72.2 to 79.6 suggested the presence of a 1,2,3,9-tetrahydroxy-1-phenyl-nonane moiety for compound **1**. This structure was verified by analysis using COSY, HSQC, and HMBC spectra (Figure 2).

The ^{1}H NMR spectrum of **1** also showed signals characteristic of an iminosugar unit. The pseudo-anomeric proton appeared at δ_H 3.76 (t, *J* = 3.1 Hz) and was correlated with its corresponding carbon at δ_C 71.9 in the HSQC spectrum. The presence of a methyl signal at δ_H 1.41 (δ_C 12.1) suggests a deoxyhexose-like unit. Complete assignments of ^{1}H and ^{13}C chemical shifts of the iminosugar moiety were accomplished by analysis of COSY, HSQC, HMBC, and ROESY experiments. The axial orientation of the alkyl chain of **1** was based on the relatively

small coupling constants $J_{1'',2''}$=3.1 Hz whereas the large coupling constants $J_{2'',3''}$=8.5 Hz indicated an equatorial orientation of hydroxyl groups at C-2" and C-3". In the same manner, the small coupling constants $J_{3'',4''}$ and $J_{4'',5''}$=3.1 Hz indicated axial orientation for hydroxyl group at C-4" and equatorial orientation for the methyl group at C-5". These data ascertained the relative position of all the substituents in the piperidine framework, allowing the identification of the iminosugar as DFJ (Saka et al., 2013) or its enantiomer D-DFJ. Since only the L-configured DFJ has been found in nature thus far, we suggest that Glyphaeaside A_1 (**1**) encompasses such a moiety rather than its enantiomer (structure shown in Figure 1). The HMBC correlations observed between H-1" (δ_H 3.76) and both carbons C-8 (δ_C 34.1) and C-9 (δ_C 72.7) confirm the $C_{1''}$-C_9 linkage. The assignment of the relative stereochemistry of the hydroxyl groups at C-1, -2, -3, and -9 of **1** was difficult to establish in acyclic system only by a combination of 3 bond H-H *J* values and analysis of the ROESY spectrum. The coupling constants $J_{1,2}$ and $J_{2,3}$ observed between the protons H-1, H-2 and H-3 are depending to the rotamer distribution and their interpretation is no easy to elucidated if these protons are in *erythro* or *threo*-configurations as formely reported (Bellucci et al., 1996, Kim et al., 2011, Matsumori et al., 1999). An unambiguous chemical synthesis of the aglycone will firmly establish the overall relative and absolute configurations. At this time, the structure of glyphaeaside A_1 was established as 9-*C*-(1-deoxyfuconojirimycin)-1,2,3,9-tetrahydroxy-1-phenyl-nonane.

The comparison of ^{1}H and ^{13}C NMR spectra of compounds **1**-**4** showed that the aglycone 1,2,3,9-tetrahydroxy-1-phenyl-nonane was common to these compounds. Full assignment of the proton and carbon resonances of the aglycone moiety of each of the isolated compounds (**2**-**4**) was achieved by analysis of 1D and 2D NMR spectra (Table 1).

Glyphaeaside A_2 (**2**) showed a pseudomolecular ion peak at *m/z* 576.3029 $[M + H]^+$ in the HRESIMS, corresponding to the molecular formula $C_{27}H_{46}NO_{12}$. The presence of signals for a second sugar unit in the ^1H and ^{13}C NMR spectra of **2** and the deshielding of C-3" (δ_C 82.0), suggest a disaccharide-like chain in compound **2**. The analysis of COSY, HSQC spectra and the measurement of coupling constants in the ^1H NMR spectrum (up to 7 Hz for protons H-1‴ to H-5‴) allowed assignment of all protons and carbons of a *β*-D-glucopyranose moiety (Glc) (Table 1). The HMBC correlation observed between H-1‴/C-3″ indicated that C-3″ was substituted by a *β*-D-glucopyranose unit whereas the correlation between the pseudo-anomeric proton H-1" and C-8 confirmed as in **1,** the position of the *C*-glycosylation. Thus, compound **2** was elucidated as 9-*C*-[*β*-D-glucopyranosyl-(1→3)-1-deoxyfuconojirimycin]-1,2,3,9-tetrahydroxy-1-phenyl-nonane.

Glyphaeasides B_1 (**3**) and B_2 (**4**) were found to have the molecular formula $C_{21}H_{35}NO_8$ and $C_{27}H_{46}NO_{13}$, respectively from the HRESIMS in positive ion mode ($[M + H]^+$ at *m/z* 430.2435 for **3** and *m/z* 592.2979 for **4**). Their 1D and 2D NMR spectra, compared to those of **1** and **2**, indicated that both compounds **3** and **4** differed by the absence of the methyl signals at δ_H 1.41 and δ_C 12.1 (as in **1**) and the presence of a supplementary hydroxymethylene group in **3** (δ_C 59.1 and δ_H 3.91 and 3.96) and **4** (δ_C 59.0 and δ_H 3.92 and 3.97) (Table 1), suggesting an imino-hexose instead of a desoxy imino-hexose. The analysis of 1D and 2D NMR data of **3** and **4** allowed identification of this imino-hexose as DGJ (Godin et al., 2004). This latter was characterized by the large coupling constants $J_{2'',3''}$ (8.6 Hz) and the small coupling constant $J_{3'',4''}$ (3.7 Hz) as summarized in Table 1. The axial orientation of the alkyl chain of **3** was based on the relatively small coupling constants $J_{1'',2''}$=4.1 Hz. In the ROESY spectrum, the rOe effects observed between H-1″ and H-2″, and between H-3″ and H-5″ support the orientation of

hydroxyl and hydroxymethylene groups of the DGJ. The detailed analysis of 1D and 2D NMR spectra of compound **4** led to the identification of a C-3" (δ_C 81.8) monosubstituted DGJ. As in compound **2** the iminosugar was substituted by a terminal β-D-glucopyranose (Table 1). The HMBC correlation between H-1″ and C-9 (δ_C 72.8) of compound **3** defined the site of *C*-glycosylation. On the basis of these data, the structure of glyphaeaside B_1 (**3**) was established as 9-*C*-(1-deoxygalactonojirimycin)-1,2,3,9-tetrahydroxy-1-phenyl-nonane. The structure of compound **4** was therefore elucidated as 9-*C*-[β-D-glucopyranosyl-(1→3)-(1-deoxygalactonojirimycin]-1,2,3,9-tetrahydroxy-1-phenyl-nonane, confirmed by the HMBC cross peaks between H-1‴ and C-3″ (δ_C 81.8) and between H-1″ and C-9 (δ_C 73.9).

As previously observed for compounds **1**-**4**, the comparison of ^{1}H and ^{13}C NMR resonances of compounds **5**-**9** showed that they possess the same phenyl alkyl part. The ^{1}H and ^{13}C NMR spectra of **5**-**9** were similar to those of compounds **1**-**4**, except for the absence of signal associated with a carbon bearing a hydroxyl group at C-3 in the alkyl chain (Tables 1 and 2). The identification of 1,2,9-trihydroxy-1-phenyl-nonane moiety was achieved by analysis of the COSY, HSQC and HMBC spectra, of each isolates compounds (**5**-**9**). Glyphaeasides A_3 (**5**) and A_4 (**6**) showed pseudomolecular ion peaks at *m/z* 398.2541 $[M + H]^+$ and *m/z* 560.3070 $[M + H]^+$ in the HRESIMS, corresponding to molecular formula $C_{21}H_{36}NO_6$, and $C_{27}H_{45}NO_{11}$, respectively. Further analysis of COSY, ROESY, HSQC, and HMBC data allowed full assignment of all ^{1}H and ^{13}C NMR resonances of a DFJ unit in **5**, as in **1**, and a disaccharide β-D-glc-(1→3)-1-DFJ in **6**, as in **2** (Tables 1 and 2). HMBC correlations were observed between H-1‴/C-3" and H-1"/C-9 confirming the interglycosidic linkage and the site of the *C*-glycosylation. Therefore, the structure of glyphaeasides A_3 (**5**) was elucidated as 9-*C*-(1-

deoxyfuconojirimycin)-1,2,9-trihydroxy-1-phenyl-nonane, and of glyphaeaside A_4 (**6**) as 9-*C*-[*β*-D-glucopyranosyl-(1→3)-1-deoxyfuconojirimycin]-1,2,9-trihydroxy-1-phenyl-nonane.

Glyphaeasides B_3 (**7**) and B_4 (**8**) had the same molecular formula $C_{21}H_{35}NO_7$ (HRESIMS, *m/z* 414.2485 $[M + H]^+$), whereas glyphaeaside B_5 (**9**) was assigned the molecular formula $C_{27}H_{45}NO_{12}$ (HRESIMS, *m/z* 576.3018 $[M + H]^+$). Comparison of their 1D and 2D NMR spectra, with those of **3** and **4**, indicated that compounds **7-8** share the same DGJ unit linked at C-9 position, as in **3**, and compound **9** possess the disaccharide *β*-D-glc-(1→3)-1-DGJ, as in **4** (Tables 1 and 2). The signals of the DGJ and the aglycone parts of **8**, assigned from 1D and 2D NMR spectra, were almost superimposable to those of **7**. In the ^{1}H NMR spectrum, the slight differences in the signals of H-1 (d, J = 5.5 Hz, $\Delta\delta_H$ = + 0.11) and H-2 (ddd, J = 8.2, 5.5, 2.3 Hz, $\Delta\delta_H$ = + 0.08) (Table 2) suggested that **8** was a stereoisomer of **7** in C-2 position. The coupling constants $J_{1,2}$=5.5 Hz and $J_{2,3}$=8.2, 2.3 Hz were different from those obtained in **7** for these protons suggesting a 1,2-*erythro* relative configuration in **8** instead of the 1,2-*threo* orientation in compound **7,** or inversely**.** Based on the above-mentioned evidence, the structures of glyphaeaside B_3 and glyphaeasides B_4 were established as 9-*C*-(1-deoxygalactonojirimycin)-1,2,9-trihydroxy-1-phenyl-nonane, and glyphaeaside B_5 as 9-*C*-[*β*-D-glucopyranosyl-(1→3)-1-deoxygalactonojirimycin]-1,2,9-trihydroxy-1-phenyl-nonane.

Glyphaeaside C (**10**) gave an pseudomolecular ion peak at *m/z* 414.2495 $[M + H]^+$ in the positive HRESIMS, from which the same molecular formula as that of **7** and **8** ($C_{21}H_{35}NO_7$) was deduced. Comparison of the ^{1}H and ^{13}C NMR spectroscopic data of **10** with those of **7** revealed a similar type of complexity for the iminosugar core aglycone (Table 2). The ^{1}H NMR spectrum exhibited, for the aromatic part, signals characteristic of a *para*-disubstituted aromatic ring at δ_H 7.09 and δ_H 6.72 (each 2H, dd, J=6.4, 2.1 Hz) whose carbon resonances were assigned by HSQC

experiment at δ_C 131.4 (C-2′, -6′) and 116.1 (C-3′, -5′), respectively (Table 2). HMBC cross-peaks were observed between H-2′, -6′ and the hydroxylated carbon C-4′ (δ_C 156.6) suggesting a *p*-hydroxy-phenyl moiety in **10**. In the COSY spectrum, for the alkyl chain, H-2 (δ_H 3.59, ddd, *J*=8.5, 5.5, 3.4) coupled with protons H-1 (δ_H 2.64, dd, *J*=13.8, 8.0 and 2.80, dd, *J*= 13.8, 5.5) and H-3 (δ_H 3.41, m). Thus, two oxygen-bearing methines were assigned in C-2 (δ_C 76.6) and C-3 (δ_C 74.0) of the alkyl chain. Connectivity of the *p*-hydroxy-phenyl moiety to the C-1 of the aliphatic chain was defined unambiguously by the HMBC correlations between H-2′, -6′ and C-1 (δ_C 39.7) and between H-3 and C-1′ (δ_C 131.4). In the same manner as in compounds **1**-**9**, a C_9 aliphatic chain was mapped by analysis of 1D and 2D NMR spectra suggesting that **10** was a 2,3-dihydroxy-(*p*-hydroxy-phenyl)-nonane derivative. The iminosugar part was identified as 1-*α*-deoxynojirymicin ($\delta_{H\text{-}1''}$ 3.88 and $\delta_{C\text{-}1''}$ 70.1) by analysis of 1D and 2D NMR spectra (Asano et al., 1998). The DNJ was characterized by large coupling constants ($J_{2'',3''}$, $J_{3'',4''}$, $J_{4'',5''} \geq 6.7$ Hz) as summarized in Table 2. The *α*-pseudo-anomeric configuration of the deoxynojirymicin unit was determined by the relatively small $J_{1''eq\,2''ax}$ coupling constant (2.5 Hz). In the ROESY spectrum, correlations observed between H-2″/H-4″ and between H-3″/H-5″ support the *α-gluco* pattern of this imino-hexose. The HMBC correlation between H-1″ and C-9 (δ_C 34.6) and the COSY correlation between H-1″ and H-9 (δ_H 1.64) established the $C_{1''}$-C_9 linkage. On the basis of the foregoing evidence, glyphaeaside C was concluded to be 9-*C*-(1-deoxynojirymicin)-1-2,3-dihydroxy-(*p*-hydroxy-phenyl)-nonane.

Compound **11** had the molecular formula $C_{19}H_{27}NO_9$ according to the pseudomolecular ion peak at *m/z* 414.1761 $[M + H]^+$ in the HRESIMS. The ^1H NMR spectrum exhibited signals of two olefinic protons at δ_H 6.54 and 7.94 (each 1H, d, *J*=15.5 Hz), indicating their *trans*-configuration, and two *meta*-coupled aromatic protons at δ_H 6.85 and 6.93 (each 1H, d, *J*=2.8 Hz). The ^{13}C

NMR spectrum exhibited signals at δ_C 105.4, 107.3, 120.9, 129.8, 140.5, 144.2, 152.9, 157.7 and 172.3 in accordance with the presence of a *E*-cinnamoyl derivative moiety substituted by three hydroxyl groups. Its ^{1}H and ^{13}C resonances were assigned using 2D NMR experiments (COSY, HSQC, and HMBC). HMBC correlations between H-7/C-1, -2, -6, H-4/C-2, -3, -5, and H-6/C-5 indicated a 2,3,5-trihydroxy-cinnamic acid derivative for compound **11**. In addition, signals of four methyl groups were observed in the ^{1}H and ^{13}C NMR spectra at δ_H 2.19 (6H, s), δ_H 3.76 (3H, s), and 3.79 (3H, s), and δ_C 30.7 (2C), 56.2 and 62.5, respectively. The methyl groups at δ_H 2.19 were correlated in the HMBC spectra with the carbonyl at δ_C 172.3, and were assigned to methyls linked to nitrogen, suggesting an amide group, in agreement with the molecular formula. The other methyls were included into methoxy groups and were assigned at C-2 and C-5, according to the HMBC connectivities between the carbons at δ_C 144.2 (C-2) and 157.7 (C-5) and the protons at δ_H 3.76 and 3.79, respectively. The aglycone of compound **11** was thus identified as 3-hydroxy-2,5-dimethoxy-*N*,*N*-dimethyl-cinnamide. All of the remaining signals in the ^{1}H and ^{13}C NMR spectra were assigned to a β-D-glucopyranosyl moiety with its anomeric signals at δ_H 4.47 (d, J=7.5 Hz) and δ_C 102.6. This glucose unit was linked to the hydroxyl in position C-3 of the aglycone, as deduced by the HMBC cross peak observed between H-1' and C-3 and reversely H-3 and C-1'. Therefore, the structure of compound **11** was established as 3-*O*-β-D-glucopyranosyl-3-hydroxy-2,5-dimethoxy-*N*,*N*-dimethyl-cinnamide.

A variety of plant-derived iminosugars are known that are structurally related to **1-10**, yet none exhibit the same theme of alkyl substitution. Thus, structures **1-10** provide a nice extension of the molecular framework for simple nojirimycins. Their glycosidase inhibitory activity was assayed with respect to α-glucosidase (rice), β-glucosidase (almond), β-galactosidase (*Aspergillus orizae*), α-galactosidase (green coffee beans), α-fucosidase (bovine kidney) and β-

mannosidase (*Helix pomatia*). In all these experiments, a concentration of 2 mM was used for the appropriate *p*-nitrophenyl-glycoside. In a first set of experiments, percent inhibition was evaluated relative to control at 1 mM or 100 µM concentration of alkyl-*C*-iminosugar (Table 3). In cases where the highest concentration tested (100 μM of drug for fucosidase, 1 mM for all the other enzymes) resulted in greater than 90% inhibition, the IC_{50} value was calculated (Table 3).

From the data collected for the compounds **1**-**10** (Table 3), the following conclusion can be drawn: only weak inhibition (less than 50% inhibition at the maximum tested concentration) was observed with all the alkyl *C*-iminosugars toward α-glucosidase and α-fucosidase. This results is contrasting with former studies from the literature, which emphasize some inhibition of both enzymes with 1-*C*-alkyl-DNJ or 1-*C*-alkyl-DFJ derivatives respectively (Godin et al., 2004; Saka et al., 2013). Interestingly, these inhibitors carried a simple alkyl chain lacking any hydroxyl substituent. Thus, the presence of a polyhydroxylated nonyl chain in the glyphaesides described herein, seems to affect drastically the binding of the piperidine ring in the catalytic site of glycosidases. The lack of affinity of glyphaesides toward α-glucosidase and α-fucosidase, which process α-D-glucose and α-L-fucose respectively, also raises the issue of the absolute configuration of the iminosugars. Indeed, unnatural L-DNJ, L-DGJ or D-DFJ show generally weaker inhibition potencies than their enantiomers. Though these compounds have not been isolated up to now, their inclusion in the structures of glyphaesides cannot be entirely ruled out at this stage. Furthermore, compound **10** showed some ability to inhibit β-galactosidase (82% at 1 mM), whereas analogues **3**, **7** and **8** where slightly active against α-galactosidase with 74%, 76% and 59% inhibition respectively. All other alkyl *C*-iminosugars were inactive towards the galactosidases. Variable inhibition features were obtained with β-mannosidase : compounds **1**, **3** and **7** displayed similar affinities (IC_{50} = 75 µM, 43 µM and 57 µM respectively), despite the

marked difference in the nature of the iminosugar framework which belongs either to the DFJ or to the DGJ series. The *gluco* analogue **10**, which features a DNJ-like backbone was the most potent inhibitor of *β*-mannosidase, outperforming its relatives by at least one order of magnitude (IC_{50} = 4.5 µM). Analogues **2, 4-6, 8, 9** retained also some activity towards *β*-mannosidase, though to a lesser extent. Again, these results show that the inhibition potency is mainly modulated by the polyhydroxy side chain and arises independently of the nature of the piperidine ring. Most remarkably, all the new alkyl *C*-iminosugars displayed potent inhibition of *β*-glucosidase (IC_{50} = 0.15-68 µM), regardless of the configuration of the iminosugar. Together, these results suggest that the nature of the piperidine core might not be essential for the binding of glyphaesides to glycosidases, but most of the biological activity would be potentiated by the polyhydrohylated side chain. Further chemical synthesis and biological evaluation of the sole side chain could help confirm or reject this hypothesis. At this stage, it is difficult to establish structure-activity relationships, regarding the substitution pattern of the alkyl chain. For instance, *C*-iminosugars **1** and **3**, which bear structurally equivalent chains, display similar inhibition potencies towards *β*-glucosidase, whereas compounds **5** and **7** show a difference in activity of about one order of magnitude. Moreover, the presence of an additional glucose residue in the structure seems detrimental for binding: monomers **1, 3, 7** are better inhibitors of *β*-glucosidase than the corresponding imino-*C*-disaccharides **2, 4, 9**. Finally, glyphaeaside C (**10)**, which features the less substituted alkyl chain, offered an advantage of at least one order of magnitude in inhibitory power. Lineweaver-Burk plots (Figure 3) revealed that glyphaeaside C acted as a competitive inhibitor of *β*-glucosidase with IC_{50}=0.15µM and K_i = 31 nM. Since DNJ is a moderate inhibitor of almond *β*-glucosidase by itself (IC_{50}=81µM, Godin et al., 2004), the alkyl chain must play a critical role in the binding of inhibitor **10** into the catalytic site of the enzyme

improving the affinity by ca 3 orders of magnitude. The lack of hydroxyl groups at C-9 and C-1 in the alkyl chain when compared to the other glyphaesides, could explain, at least in part, the potent activity of the less sterically constrained glyphaeaside C (**10**) on *β*-mannosidase and *β*-galactosidase.

3. Conclusions

Ten new phenylalkyl-substituted iminosugars, glyphaeaside A_1-A_4 (**1**, **2**, **5**, **6**), glyphaeaside B_1-B_5 (**3**, **4**, **7**-**9**) and glyphaeaside C (**10**), were isolated from the 80% hydromethanolic extract of the *Glyphaea brevis* roots with a cinnamic acid derived glucoside (**11**). Their structures were elucidated by 1D and 2D NMR analysis, as well as by HR-ESIMS. Compounds **1**-**10** retain an unprecedented structure composed of an iminosugar-like core substituted by a di-, tri- or tetra-hydroxylated nine-carbon chain at the pseudo-anomeric position, substituted by a terminal phenyl group. All alkyl *C*-iminosugars displayed potent and selective inhibition towards *β*-glucosidase with IC_{50} values ranging from 0.15 to 68 µM. Compound **10** with a DNJ backbone was the most active and was found to act as a competitive inhibitor with K_i = 31 nM, therefore emerging as one of the most potent inhibitor of *β*-glucosidase reported to date. Inhibition of *β*-mannosidase was observed with compounds **1**, **3**, **7** and **10**, but only weak inhibition could be detected with the alkyl-*C*-iminosugars on the other tested glycosidases (*α*-glucosidase, *α*-fucosidase, *α*- and *β*-galactosidase). Unlike unsubstituted nojirimycins, the inhibition potencies of glyphaesides might be modulated by the side-chain, which induces most of the favorable or disfavorable contributions to binding.

4. Experimental

4.1. General experimental procedures

Optical rotations were determined in MeOH with a Perkin-Elmer 341 polarimeter. ^{1}H and ^{13}C NMR spectra were recorded in CD_3OD on a Bruker Avance III 500 spectrometer, equipped with a BBFO+5 mm sonde (^{1}H at 500 MHz and ^{13}C at 125 MHz). 1D and 2D NMR experiments were performed using standard Bruker microprograms. Chemical shift referencing was carried out using the internal solvent resonances at δ_H 3.33 (CHD_2OD) and δ_C 49 (CD_3OD) calibrated to TMS at 0.00 ppm. HRESIMS experiments were recorded on a ESI-Q-TOF micro instrument (Manchester, UK). TLC was performed on precoated silica gel 60 F_{254} plates (Merck) or Alugram UV_{254} (Macherey-Nagel) and detection was carried out by spraying with 50% H_2SO_4 followed by heating at 100 °C. Column chromatography (CC) was carried out on Kieselgel 60 (63-200 mesh) Merck or LiChroprep RP-18 (40-63 μm) Merck. High performance flash chromatography (HPFC) was carried out using a Grace Reveleris Flash System with prepacked silica gel or RP-18 columns. HPLC was performed on a Dionex apparatus equipped with an ASI-100 autosampler, a P580 pump, a UVD 340S diode array detector, and a Chromeleon® software. RP-18 column (Phenomenex 250x10 mm, Luna 5 μm, 100 A) was used for semipreparative HPLC with a binary gradient eluent (Solvent A: H_2O, pH 2.4, with 0.0025% TFA; Solvent B: MeCN or MeOH) and a flow rate of 3.5 mL min^{-1}. The chromatogram was monitored at 205, 210, 250, and 300 nm.

4.2. Plant material

The roots of *Glyphaea brevis* (Spreng.) Monach. were collected in the area of the lagoons, Abidjan, Ivory Coast, in October 2009. A voucher specimen (No Aké Assi S.N-01) has been

deposited in the herbarium of the National Center of Floristic of FHB University of Cocody (Ivory Coast).

4.3. Extraction and isolation

The dried roots of *Glyphaea brevis* (500 g) were macerated with 20% aqueous MeOH (10 l) for 24h. After lixiviation, the hydromethanolic extract was concentrated *under vacuum* to a volume of 2 l. The aqueous residue was then partitioned successively with $CHCl_3$ and *n*-BuOH (each 3x1 l). The *n*-BuOH extract (6.2 g) was subjected to a silica gel vacuum-liquid chromatography (9 x 9 cm) eluted successively with $CHCl_3$-MeOH-H_2O (9:1:0, 8:2:0, 7:3:0, 7:3:0.5, and 5:5:0), each 2 l to give five fractions (A: 0.38 g, B: 2.2 g, C: 2 g, D: 0.61 g, and E: 0.8 g, respectively). Fraction C was subjected to RP-18 CC (80 g) using a gradient of MeOH-H_2O (2:8 to 10:0)) to give 44 fractions of 50 ml. Fractions [5-8] (384 mg), eluted with MeOH-H_2O (2:8), were purified by silica gel CC with a gradient of $CHCl_3$-MeOH (8:2 to 5:5) and fractions [33-45], eluted with $CHCl_3$-MeOH (7:3), were purified by semi-preparative HPLC with the elution program: 30-35% of MeOH for 35 min to give compounds **3** (R_t 8 min, 5 mg) and **10** (R_t 14 min, 11 mg). Fractions [9-11] (400 mg), eluted with MeOH-H_2O (2:8), were subjected to silica gel CC (19 g) using a gradient of $CHCl_3$-MeOH (8:2 to 5:5), and fractions of 30 ml were collected. Fractions [9-21], eluted with $CHCl_3$-MeOH (8:2), were purified by semi-preparative HPLC using an elution program: 15-18% of MeCN for 30 min to afford compounds **8** (R_t 13.8 min, 4 mg) and **7** (R_t 17.5 min, 5 mg). Fractions [39-52], eluted with $CHCl_3$-MeOH (7:3), were purified by semi-preparative HPLC using the binary gradient from 30% to 35% of MeOH, for 30 min yielding compounds **10** (R_t 13 min, 5 mg) and **6** (R_t 19.5 min, 17 mg). Fractions [16-20] (44 mg), eluted with MeOH-H_2O (25:75), were purified by semi-preparative HPLC, with the elution program: 35-37% of MeOH for 30 min to give compounds **11** (R_t 12 min, 3.5 mg) and **5** (R_t 16 min, 3 mg).

Fractions D and E were combined and separated by CC over RP-18 (65 g) eluting with a gradient of MeOH-H_2O (3:7 to 7:3) to give 32 fractions of 40 ml. Fraction 3, eluted with MeOH-H_2O (3:7), was purified by RP-18 high performance flash chromatography using a gradient of MeCN-H_2O from 5-55% of MeCN for 40 min, and a flow rate of 18 ml/min. Fractions [4-5] were then purified by semi-preparative HPLC using the binary gradient from 8% to 15% of MeCN for 30 min affording compounds **4** (R_t 12.5 min, 5 mg), **2** (R_t 14 min, 8 mg), **3** (R_t 15.8 min, 9 mg) and **1** (R_t 17.5 min, 6 mg). The mixture of fractions [13-21], eluted with MeOH-H_2O (35:65 to 5:5) were subjected to silica gel CC (7.5 g) with a gradient of $CHCl_3$-MeOH (3:7 to 5:5), and fractions of 10 ml were collected. Fractions [10-41] were further separated by semi-preparative HPLC with the elution program: 15-18% of MeCN for 30 min to afford compounds **10** (R_t 9.25 min, 3 mg), **9** (R_t 11.3 min, 15 mg) and **6** (R_t 12.5 min, 10 mg).

4.4. Acid hydrolysis

A part of the *n*-BuOH soluble fraction (200 mg) was refluxed (90 °C) with 5 ml of 2M TFA for 3 h, and then 10 ml of H_2O was added. After extraction with ethyl acetate (3 x 10 ml), the aqueous layer was evaporated to furnish the monosaccharide residue (55 mg). Glucose was identified by comparison with authentic sample on TLC in MeCOEt-iso-PrOH-Me_2CO-H_2O (20:10:7:6). The monosaccharide residue was subjected to a preparative TLC using the same solvent. The optical rotation of the purified sugar was measured to afford glucose {10 mg; R_f 0.48; $[\alpha]^{20}_D$ + 29.8, (*c* 0.5, H_2O)}.

4.5. Glyphaeaside A_1 (1)

Brown lacquer; $[\alpha]^{20}_D$ +27.1 (*c* 0.52, MeOH); ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 414.2487 $[M + H]^+$ (calcd for $C_{21}H_{36}NO_7$, 414.2492).

4.6. Glyphaeaside A_2 (2)

Brown lacquer; $[\alpha]^{20}_D$ +5.1 (*c* 0.59, MeOH); ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 576.3029 $[M + H]^+$ (calcd for $C_{27}H_{46}NO_{12}$, 576.3020).

4.7. Glyphaeaside B_1 (3)

Brown lacquer; $[\alpha]^{20}_D$ +8.3 (*c* 0.73, MeOH); ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 430.2435 $[M + H]^+$ (calcd for $C_{21}H_{36}NO_8$, 430.2441).

4.8. Glyphaeaside B_2 (4)

Brown lacquer; $[\alpha]^{20}_D$ -8.1 (*c* 0.43, MeOH); ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 592.2979 $[M + H]^+$ (calcd for $C_{27}H_{46}NO_{13}$, 592.2969).

4.9. Glyphaeaside A_3 (5)

Brown lacquer; $[\alpha]^{20}_D$ + 7 (*c* 0.1, MeOH); ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 398.2541 $[M + H]^+$ (calcd for $C_{21}H_{36}NO_6$, 398.2543).

4.10. Glyphaeaside A_4 (6)

Brown lacquer; $[\alpha]^{20}_D$ +3 (*c* 0.94, MeOH); ^1H and ^{13}C NMR data, see Table 2; HR-ESIMS *m/z* 560.3070 $[M + H]^+$ (calcd for $C_{27}H_{46}NO_{11}$, 560.3071).

4.11. Glyphaeaside B_3 (7)

Brown lacquer; $[\alpha]^{20}_D$ +18 (*c* 0.52, MeOH); ^1H and ^{13}C NMR data, see Table 2; HRESIMS *m/z* 414.2502 $[M + H]^+$ (calcd for $C_{21}H_{36}NO_7$, 414.2492).

4.12. Glyphaeaside B_4 (8)

Brown lacquer; $[\alpha]^{20}_D$ 0 (*c* 0.25, MeOH); ^1H and ^{13}C NMR data, see Table 2; HRESIMS *m/z* 414.2485 $[M + H]^+$ (calcd for $C_{21}H_{36}NO_7$, 414.2492).

4.13. Glyphaeaside B_5 (9)

Brown lacquer; $[\alpha]^{20}_D$ 0 (*c* 0.12, MeOH); ^1H and ^{13}C NMR (CD_3OD) see Table 2; HRESIMS *m/z* 576.3018 $[M + H]^+$ (calcd for $C_{27}H_{46}NO_{12}$, 576.3020).

4.14. Glyphaeaside C

Brown lacquer; $[\alpha]^{20}_D$ – 0.8 (*c* 1.18, MeOH); ^1H and ^{13}C NMR data, see Table 2; HRESIMS *m/z* 414.2495 $[M + H]^+$ (calcd for $C_{21}H_{36}NO_7$, 414.2492).

3.14. 3-O-β-D-glucopyranosyl-3-hydroxy-2,5-dimethoxy-N,N-dimethyl-cinnamide (11)

brown lacquer; $[\alpha]^{20}_D$ -18.5 (*c* 0.18, MeOH); ^1H NMR (CD_3OD, 500 MHz) δ 2.19 (6H, s, 2 × N-CH_3), 3.41 (1H, t, 8.6 Hz, H-4′), 3.47 (1H, ddd, 8.6, 5.8, 2.2 Hz, H-5′), 3.49 (1H, t, 8.5 Hz, H-3′), 3.53 (1H, dd, 8.5, 7.5 Hz, H-2′), 3.71 (2H, dm, 12.1 Hz, H-6′), 3.76 (3H, s, 2-OCH_3), 3.79 (3H, s, 5-OCH_3), 4.47 (1H, d, 7.5 Hz, H-1′), 654 (1H, d, 15.5 Hz, H-8), 685 (1H, d, 2.8 Hz, H-6), 693 (1H, d, 2.8 Hz, H-4), 7.94 (1H, d, 15.5 Hz, H-7); ^{13}C NMR (CD_3OD, 125 MHz) δ 30.7 (2 × N-CH_3), 56.2 (2-OCH_3), 62.5 (5-OCH_3), 62.52 (C-6′), 71.4 (C-4′), 75.0 (C-2′), 78.2 (C-3′),78.4 (C-5′), 102.6 (C-1′), 105.4 (C-6), 107.3 (C-4), 120.5 (C-8), 129.8 (C-1), 140.5 (C-7), 144.2 (C-2), 152.9 (C-3), 157.7 (C-5), 172.3 (C-9); HRESIMS *m/z* 414.1761 $[M + H]^+$ (calcd for $C_{19}H_{28}NO_9$, 414.1764).

4.15. Glycosidase inhibitory activities assay

α-Glucosidase (from rice), *β*-glucosidase (from almond), *β*-galactosidase (from *Aspergillus oryzae*), *α*-fucosidase (from bovine kidney), *α*-galactosidase (from green coffee beans), *β*-mannosidase (from *Helix pomatia*) and *p*-nitrophenyl glycosides used for the bioassay were purchased from Sigma Chemical Co. The Nitrophenol experimental procedure was used for the measurement of glycosidase activity with some modifications (Hottin et al., 2013). In a typical experiment, the glycosidase (0.013 U/ml) was pre-incubated at 33°C for 5 min in the presence of the inhibitor in 50 mM acetate buffer (pH 5.6). The reaction was started by addition of the appropriate *p*-nitrophenyl glycoside substrate (2 mM) to a final volume of 250 µl. The reaction was stopped after 15 min by addition of 400 µl of 0.4 M Na_2CO_3. The released *p*-nitrophenol was quantified spectrometrically at 410 nm. The inhibitory effects of the tested compounds were expressed as the concentration that inhibits 50% of the enzyme activity (IC_{50}). Ten compounds (**1**-**10**) were tested at 1 mM against *α*-glucosidase, *β*-glucosidase, *β*-galactosidase, *α*-galactosidase and *β*-mannosidase and at 100 μM against *α*-fucosidase. In cases where the highest concentration tested resulted in greater than 90% inhibition, the IC_{50} value was determined after assaying decreasing concentrations of inhibitor and exploiting the results by regression analysis of 1/v against [I] (Dixon plot).

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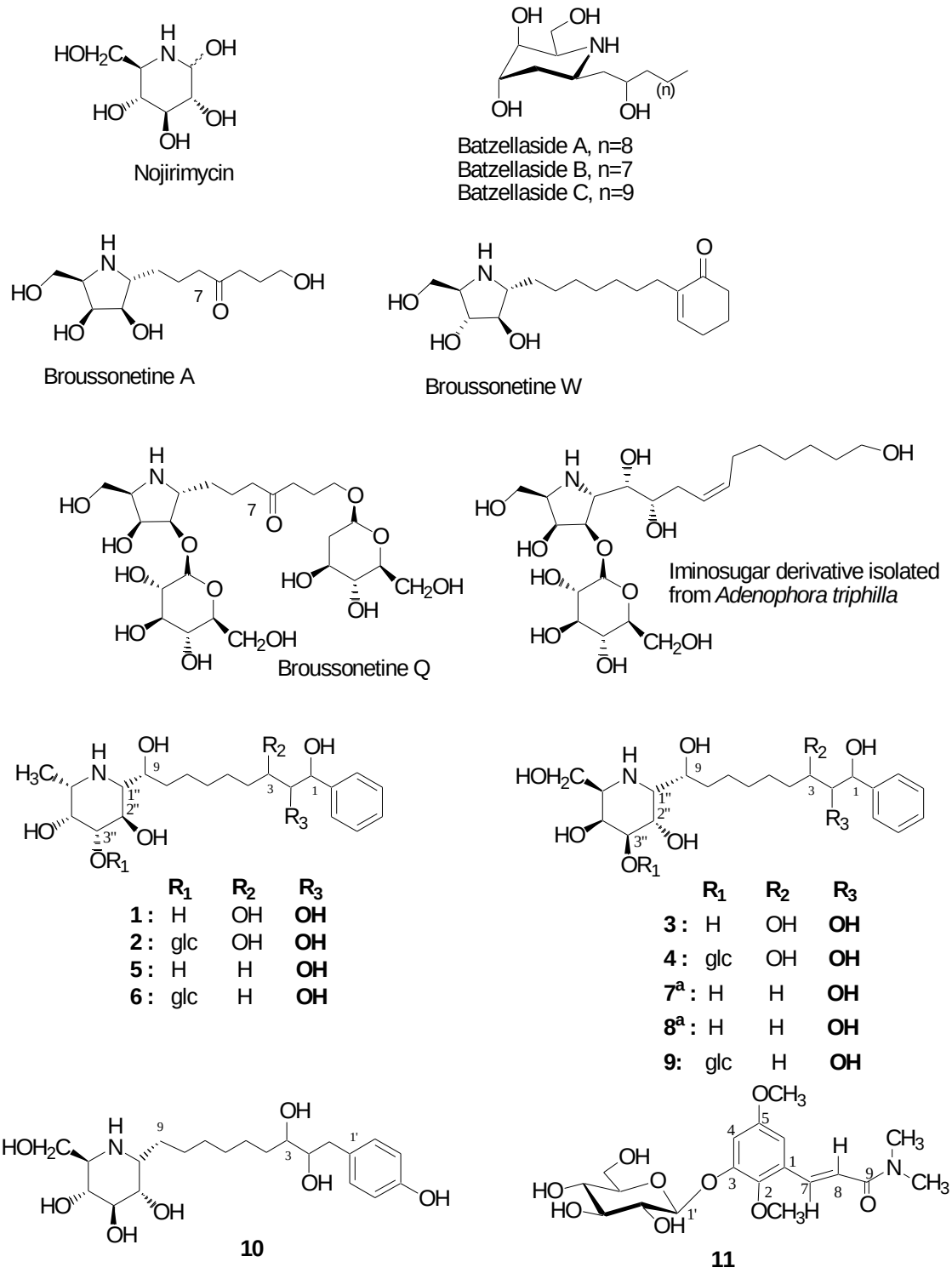

Figure 1. Structures of some known alkyl *C*-iminosugars and of new compounds **1-11** (relative configurations are specified, when known) isolated from the roots of *Glyphaea brevis*. [a] stereoisomers in C-2 position.

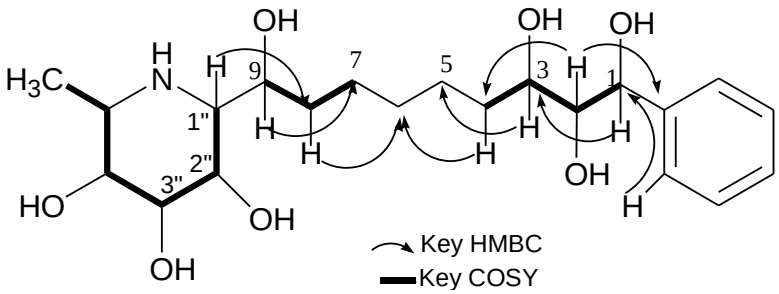

Figure 2. Selected COSY and HMBC correlations of compound **1**.

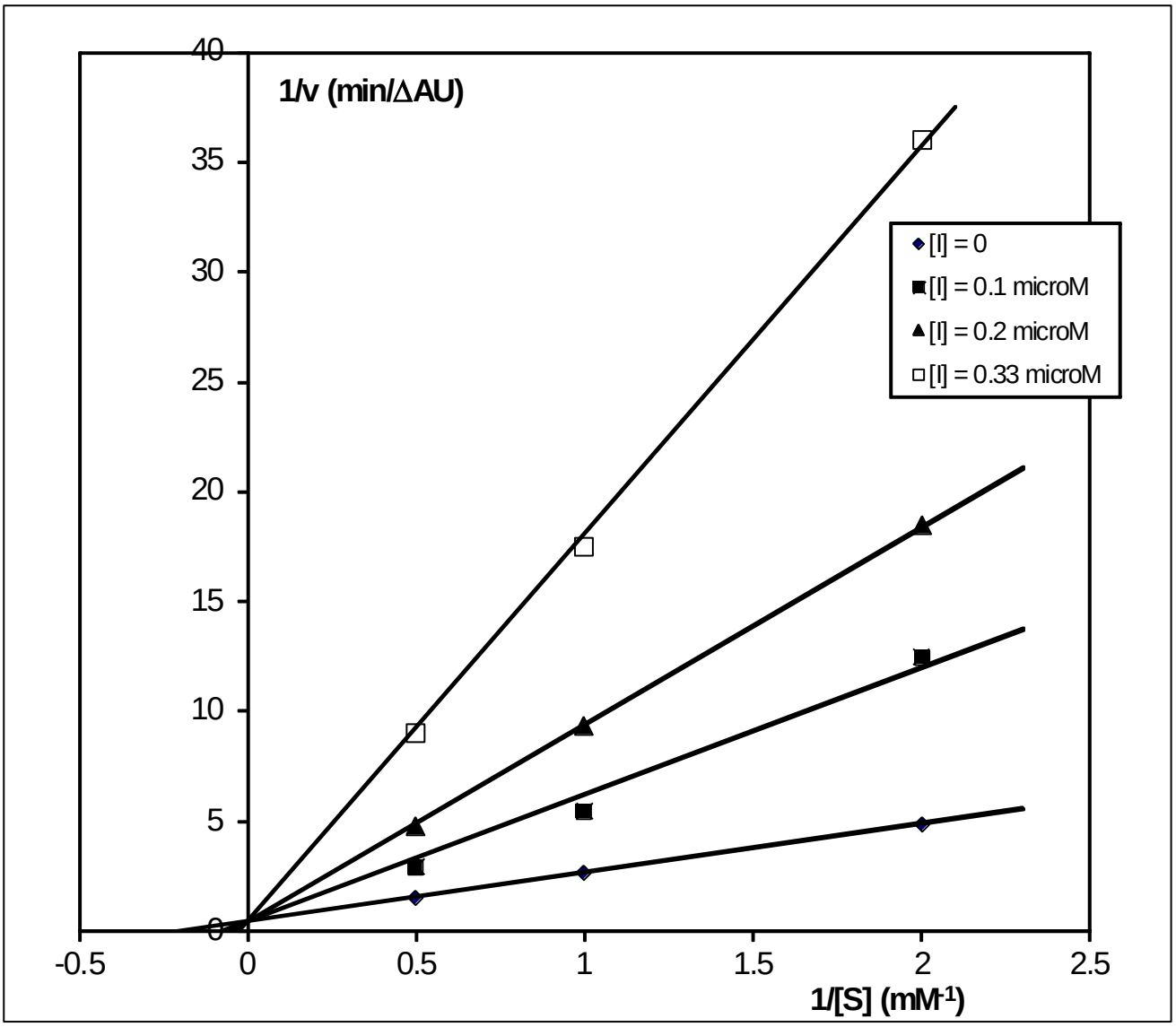

Figure 3. Lineweaver-Burk plots revealing competitive inhibition of *β*-glucosidase by compound **10** (*K*i=31 nM).