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Synthesis, from nojirimycin, of N^1-alkyl-D-gluconamidines as potential glucosidase inhibitors

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Abstract

The labile reaction products from nojirimycin (5-amino-5-deoxy-D-glucopyranose, **1**) with butyl- and dodecyl-amine were dehydrogenated with iodine to the corresponding N^1-alkyl-D-gluconamidines. A major by-product from the direct dehydrogenation of **1** was D-glucono-1,5-lactam. Progress of the reaction and product isolation was monitored by measuring the inhibitory potency of reaction mixtures with mammalian lysosomal and cytosolic β-glucosidases. The latter was tentatively identified as a non-specific β-glucosidase/β-galactosidase in a commercial β-galactosidase preparation from bovine liver by its resistance to conduritol B epoxide and strong inhibition by taurodeoxycholate. Both enzymes were inhibited by the N^1-dodecyl derivative > 1000-fold better than by **1**. Unsubstituted D-gluconaminidine and its *N*-benzyl derivative could not be prepared by the method described, because ammonia was immediately transformed into insoluble NI_3 and benzylamine was rapidly dehydrogenated. The amidines were stable in aqueous solution at pH ≤ 6, but were hydrolyzed above pH 8 to D-gluconolactam and alkylamine (half-life 24 h at pH 8 and 25 °C). A detailed study with the C_{12}-derivative at pH 10 indicated that the hydrolysis proceeded with formation of the *N*-dodecylamide of 5-amino-5-deoxy-D-gluconic acid as intermediate which still had considerable inhibitory potency against the cytosolic β-glucosidase. A method is described for the quantification of cyclic amidines based on the reaction of amino groups with ninhydrin before and after hydrolysis with dilute alkali. © 1996 Elsevier Science Ltd.

Keywords: Glucosidases, inhibitors; Glucosidases, transition state mimics; D-Gluconamidines, N^1-alkyl derivatives; Nojirimycin, oxidative aminoalkylation

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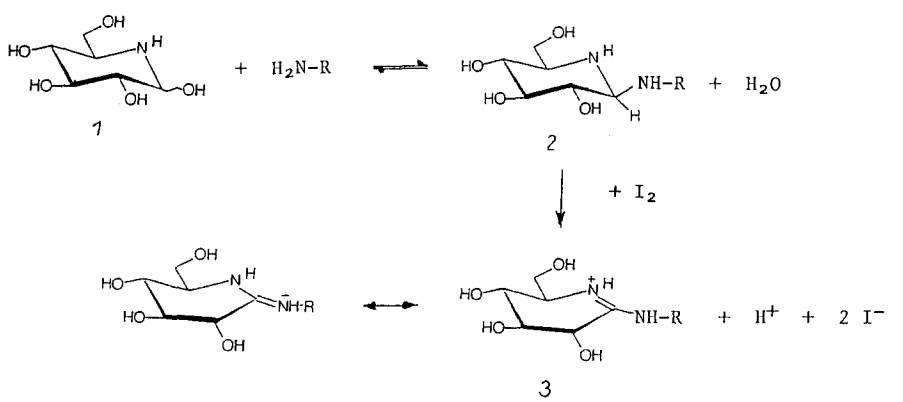
1. Introduction

Glycosidase inhibitors designed to mimic the transition state of the first bond breaking step should have, in addition to a hydroxylation pattern corresponding to that of the substrate, the following features [1]: (i) a flat (sp^2-like) geometry at C-1 or its equivalent; (ii) a basic center or positive charge at or adjacent to C-1; (iii) a substituent complementary to or at least able to interact with the aglycon binding site.

Whereas there are many glycon analogues fulfilling point (i) (glycono-lactones and -lactams [1] and the corresponding oximes [2–5]) and point (ii) (glycosylamines, nojirimycins, and 1-deoxynojirimycins [1]) there are only a few studies in which interactions with the aglycon site have been addressed (e.g. *N*-alkyl derivatives of glycosylamines and 1-deoxynojirimycins [6–10] and the *N*-phenyl carbamate esters of glyconolacton oximes [2]. A combination of point (i) and (ii) was first described by Ganem and co-workers [3–5] who synthesized D-gluconamidinium and -amidrazonium salts. Except for the trehalase inhibitor trehazoline [11] and the α-mannosidase inhibitor N^1-benzyl-D-mannonamidine [12], no glycon derivatives with all three features seem to have been described. A comparison of such compounds with structural analogues fulfilling only one or a combination of two of points, (i) to (iii), would provide information on the contributions of structural details to the binding energy and resemblance of such inhibitors with the transition state. We have, therefore, extended the structure of D-gluconamidine by alkyl substituents on the exocyclic nitrogen N^1, employing a novel route based on nojirimycin (5-amino-5-deoxy-D-glucopyranose, **1**) as starting material. Unsubstituted alkyl residues were chosen to simplify the preparative procedures and to permit a comparison with alkyl glucosides and glucosylamines (see next communication).

The most pronounced difference between D-glucose and its nitrogen containing analogue **1** is the ease by which its anomeric hydroxyl group undergoes (formal) nucleophilic substitution reactions. This was first shown by Inouye et al. [13], who prepared the zwitterionic 1-deoxy-1-sulfonate of **1** by passing SO_2 into its aqueous solution. Regeneration of **1** occurs readily on treatment of a suspension of the sulfonate with Dowex 1 (OH^-) or with barium hydroxide. Similarly, cyanide reacts rapidly and



Scheme 1. Synthesis of N^1-alkyl-D-gluconamidines from nojirimycin by oxidative aminoalkylation.

practically irreversibly with **1** to give the 1-α-cyano-1-deoxy derivative [14]. These reactions occur at rates which are, in the case of HSO_3^-, of the same order of magnitude as with aliphatic aldehydes [15] and on the time scale of minutes in the case of CN^- [Legler, unpublished]. Based on these findings we thought aliphatic amines to react with similar ease. Dehydrogenation of the initial product, *N*-alkyl-(5-amino-5-deoxy-β-D-glucopyranosyl)amine (**2**), a labile analogue of an *N*-alkylglucosylamine, was expected to give the desired N^1-alkylgluconamidine, **3** (Scheme 1).

2. Results and discussion

The reaction between **1** and an aliphatic amine was expected to be readily reversible, thus precluding detection of **2** by TLC. As tests for amidines with Dragendorff's reagent [16] or iodoplatinate (IV) [17] were only moderately sensitive and non-specific, we employed the inhibition of bovine lysosomal or cytosolic β-glucosidases to monitor the reactions given in Scheme 1, using dodecylamine. Both enzymes are inhibited by **1** with K_I-values from 1 to 5 μM [9] and 45 μM, respectively [18], whereas *N*-dodecylglucosylamine inhibited with K_I-values near 1 nM [7,8]. Since the inhibitory potency of both, the primary product **2** (with $R = -C_{12}H_{25}$) and the resulting D-gluconamidine **3**, were expected to be similar or even larger than that of *N*-dodecylglucosylamine, we would be able to detect even a small degree of conversion to **3**, thus permitting an efficient optimization of the experimental conditions.

Inhibition tests, with mixtures containing up to 15 mM **1** and 35 mM of dodecylamine in 67% aqueous 2-propanol, showed a less than 1.5-fold higher inhibition than calculated for **1** after reaction times up to 1 h at 5 °C and addition of 3.5 mM dodecylammonium iodide as catalyst [19]. We ascribe our failure to detect significant amounts of **2** to an unfavorable equilibrium and/or a rapid hydrolysis of **2** during the dilution steps required for the assay. On addition of 1.1 M equiv of iodine, however, the inhibition had increased ~ 500-fold over that of **1** after 2 h and ~ 1000-fold after 20 h. Further improvements were seen when the reaction was carried out in 80% aq 2-propanol with 3 equiv of dodecylamine and 1.5 M equiv iodine.

Attempts to synthesize unsubstituted D-gluconamidine by this method were unsuccessful, because addition of iodine led to the immediate formation of a black precipitate, presumably NI_3. Our method also failed with benzylamine, because it was rapidly oxidized.

The products from the reaction with dodecylamine were separated by hydrophobic interaction chromatography on Sephadex LH-20 after removal of 2-propanol by repeated evaporations with water (Table 1). The column effluent was assayed for inhibition of cytosolic β-glucosidase and for iodide as calculated from A_{226}. The material in the first inhibitory peak (having low A_{226}) was identified as D-glucono-1,5-lactam by comparison with an authentic sample (TLC, melting point). Its yield, calculated from the inhibition data and its K_I-value (2.6 μM, Table 2, Experimental) ranged from 55 to 65%. The second peak contained the iodide salt of the gluconamidine (C_{12}-**3**), obtained in 30 to 40% yield as calculated from the iodide concentration. Its K_I-value was found to be 0.45 nM at pH 6.0. Dodecylammonium iodide eluted in the third peak (very low inhibition, high iodide).

Table 1
Purification of C_{12}-**3** by column chromatography on Sephadex LH-20 (see Experimental for details)

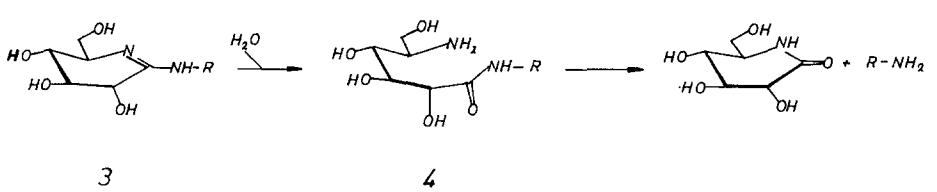
Fract. Nr.	V_e / V_o [a]		K_I(app) [b]
12–16	1.0	Blue dextran	–
28–37	2.9	D-Glucono-1,5-lactam	{·} [c]
46–51	4.0	C_{12}-**3**	0.2–0.7 nM [d]
52–60	4.7	Dodecylammonium iodide	700 nM

[a] Relative peak elution volume based on peak volume of blue dextran.
[b] Based on inhibitor concentration calculated from A_{226} with ε_{226} 14,000 M^{-1} cm^{-1} for iodide.
[c] A meaningful K_I(app) could not be calculated from A_{226} [(lactam) 246 M^{-1} cm^{-1}] because of unretarded UV-absorbing contaminants.
[d] K_I(app) increased from front to tail fractions because of partial overlap with dodecylammonium iodide.

As the C_{12}-**3** iodide salt isolated by lyophilization was hygroscopic and turned brown on storage, the LH-20 column effluent was converted into the chloride salt by ion exchange. The lyophilized salt was only slightly hygroscopic, readily soluble in water, methanol, acetone, and chloroform. Attempts to crystallize it by the addition of weak solvents like ether or toluene, were unsuccessful.

N^1-Butylgluconamidine (C_4-**3**) was prepared as described for C_{12}-**3**, using butylamine. Purification on Sephadex LH-20 or other hydrophobic chromatography supports was not possible, because of its much more hydrophilic character. Size exclusion chromatography, however, on the highly crosslinked polyacrylamide gel Biogel P-2 permitted sufficient separation of C_4-**3** from most of the butylammonium iodide, provided that ionic interactions with trace amounts of carboxylate groups in the gel were suppressed by the addition of 30 mM sodium chloride to the eluent.

Chemical characterization, quantification, and pK_a.—On TLC, the type **3** compounds gave a positive reaction with Dragendorff's reagent (dark orange) and iodoplatinate (IV) (purplish brown) but did not react with ninhydrin; permangante was decolorized slowly. The quantification of **3** in preparations containing alkylammonium salts or sodium chloride presented some problems, because amidines do not have a characteristic UV-maximum above 210 nm and no specific color reactions could be found in the literature. Therefore, we estimated the amount of **3** from the reaction with ninhydrin after hydrolysis with 0.03 M NaOH at 65 °C, using 2-amino-2-dehydropiperidine as reference. Contaminating amounts of alkylammonium salts and of the primary hydrolysis product **4** (Scheme 2) were corrected for by the ninhydrin reaction prior to the treatment with NaOH.



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Scheme 2. Two-step hydrolysis of N^1-alkyl-D-gluconamidines (modified from [20]).

The lability of unprotonated **3** (Scheme 2) precluded the determination of pK_a by potentiometric titration; on going from pH 11.5 to pH 4.0 after the titration (10 min) the solution had lost > 50% of its inhibitory potency and gave a positive reaction with ninhydrin. Therefore, pK_a was determined from the pH-value of a solution of **3**-hydrochloride to which 0.5 equiv of NaOH (corrected for dissolved CO_2) had been added. Protonated C_{12}-**3** was found to have pK_a 10.8 by this method. Unsubstituted D-gluconamidinium ion is reported to have pK_a 10.6 [4].

Stability in aqueous solutions.—The stability of **3** in aqueous buffers was evaluated by measuring the inhibitory potency of the C_{12}-derivative against the bovine cytosolic β-glucosidase as expressed by $1/K_I$(app). Compound C_{12}-**3** was stable (decrease of $1/K_I$(app) < 5%) for at least four weeks at 4 °C and pH 2 to pH 6 and for at least 48 h at 25 °C and 36 °C at pH 3 to pH 6. After 24 h at 25 °C and pH 8 and pH 9, however, $1/K_I$(app) had decreased by 50 and 77%, respectively. The decomposition rate of C_{12}-**3** at the higher pH-values differed from that of its non-alkylated counterpart which is reported to be decomposed in water at pH 8 to D-gluconolactam with a half-life of 1 h [5].

A more detailed study at pH 10 and above revealed that the loss of inhibitory potency did not occur with direct formation of the expected hydrolysis products D-gluconolactam (K_I 5.1 μM) and dodecylamine (K_I 0.71 μM) in a single first order process (Fig. 1). Instead, a more slowly decomposing intermediate appeared to accumulate which still had about 10% of the inhibitory potency of C_{12}-**3**, i.e. about 300-fold more than calculated for the inhibition by the hydrolysis products. A tentative explanation is that the hydrolysis of C_{12}-**3** follows a two-step mechanism as described by Perrin and Arrhenius [20] for the unsubstituted parent amidine, 2-amino-1,2-dehydropiperidine. If this mechanism also holds for C_{12}-**3** the *N*-dodecylamide of 5-amino-5-deoxy-D-gluconic acid (**4**) would be formed as intermediate (Scheme 2). As **4** can be regarded as alkyl derivative of non-cyclic and oxidized **1** it might well be responsible for the inhibition remaining after the initial decrease. That non-cyclic analogues of **1** are similar or even better inhibitors than the cyclic parent compound has been demonstrated by Fowler et al. [21] with α-glucosidase from yeast.

Support for the formation of an intermediate with a free NH_2-group came from TLC results with solutions of **3** kept at pH ≥ 10. They revealed a ninhydrin positive compound slightly less polar than **3**. Its proportion increased with time and then decreased. After 24 h, this compound could no longer be detected, leaving alkylamine and D-gluconolactam as the only products. Some preparations of C_4-**3** contained the new compound even before the pH 10 treatment, presumably because of an insufficient removal of water before the oxidation step. Additional evidence for the putative intermediate **4** was obtained by reacting pH 10 treated solutions with 1-fluoro-2,4-dinitrobenzene. On TLC, a yellow spot was seen; its polarity was intermediate between that of the DNP-derivatives of glucosamine and alkylamine.

A comparison of the rates for the formation of the putative intermediate **4** (k_1) and its decomposition (k_2) calculated from Fig. 1 with those reported by Perrin and Arrhenius for the unsubstituted reference amidine (k_1 0.12 min^{-1}; k_2 0.0012 min^{-1} [20]) shows them to be very similar. However, if we consider that the reference amidine was studied at about pH 11.9 (a 0.5 M aqueous solution of the free base) the type **3** amidines are

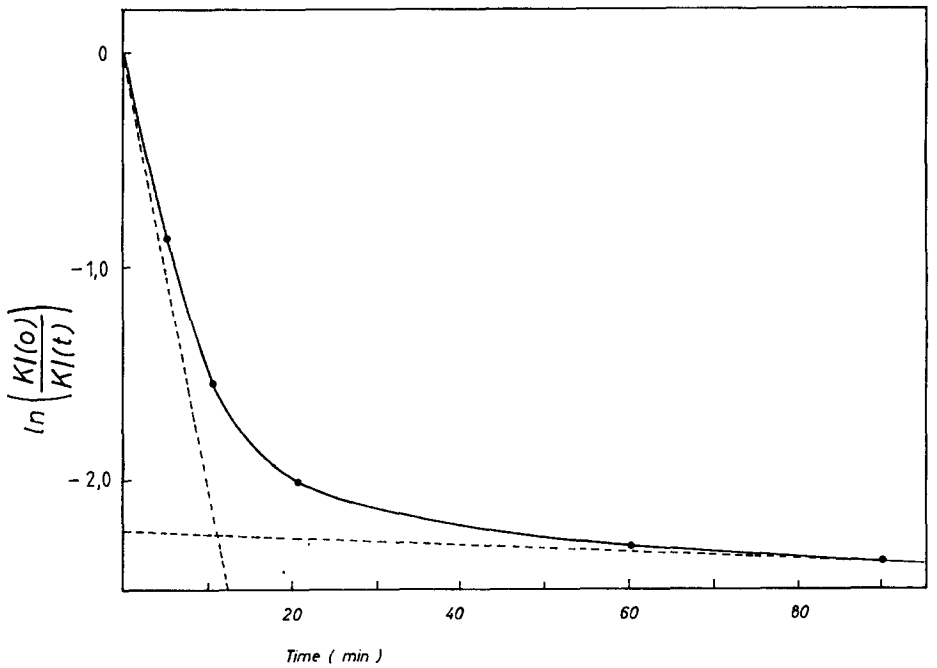

Fig. 1. Time course of the inhibition constants K_I(app) of N^1-dodecyl-D-gluconamidine with bovine cytosolic β-glucosidase plotted as $\ln[K_I(0)/K_I(t)]$. Stock solutions of inhibitor were kept at pH(app) 10.8 in 50% aq ethanol at 25°. Rate constants calculated from the initial and final slopes were 0.16 min^{-1} and 0.0018 min^{-1}, respectively.

seen to decompose about 10-fold more rapidly. The reasons for the different rates of lactam formation from D-gluconamidine ($t_{1/2}$ 1 h at pH 8 [5]) and for the decay of inhibitory potency of C_{12}-**3** ($t_{1/2}$ 24 h under the same conditions) are not clear at present.

3. Experimental

General methods.—TLC was done on aluminum sheets precoated with silica gel (E. Merck 5554) in the solvent systems given with the respective experiments. UV and vis spectral measurements were made on a Zeiss PM2K spectrophotometer, fluorescence measurements on a Zeiss PMQ 2 spectrophotometer with ZFM 4 fluorescence adaptor. NMR Spectra were run on a Bruker AM 300.

Syntheses.—Nojirimycin (**1**).—The hydrogen sulfite adduct of **1** was prepared from D-glucurono-6,3-lactone (Fluka) according to Stasik et al. [22]. The free base was released from the adduct (250 mg) by stirring with Dowex 1 (OH^--form, freshly prepared from the Cl^--form, 3 g) in water (15 mL) for 2 h at 0–5 °C. The yield, determined enzymatically (see below) after filtration and concentration to 7.5 mL was from 75 to 85%.

N^1-*Dodecyl-D-gluconamidine* (C_{12}-**3**).—An aqueous solution of **1** (0.3 mmol, 3 mL)

was diluted with *i*PrOH (6 mL), concentrated to 3 mL and supplied with dodecylamine (0.6 mmol) in *i*PrOH (6 mL), and kept at ≤ 5 °C for 20 min. A solution of iodine (0.33 equiv) in *i*PrOH (6 mL) was then added in small portions in the course of 5 h, maintaining a light brown color. After the addition of another 0.3 mmol of dodecylamine and iodine (0.15 equiv) in *i*PrOH the mixture was kept over night at ≤ 5 °C. Unreacted iodine and dodecylamine were extracted in the cold with toluene after water (10 mL) had been added to provide phase separation. The aqueous phase was adjusted to pH 5 to 6 with dilute acetic acid and repeatedly evaporated and diluted with water to remove *i*PrOH and concentrated to a final volume of 3 mL. An antifoam agent had to be added at the last stage of evaporation. Inhibition tests with bovine lysosomal (pH 5) or cytosolic β-glucosidase (pH 7, see below) gave apparent K_I-values of 2.5 nM and 0.5 ± 0.2 nM, respectively. K_I-Values were calculated with apparent inhibitor concentrations based on yields of 100%.

The products were purified by hydrophobic interaction chromatography on Sephadex LH-20 (Pharmacia) with water as eluant (column size 1.5×105 cm, flow rate 4.6 mL/h). Blue dextran (Pharmacia) was added to mark the volume of unretarded material, v_o. The effluent was monitored for iodide content by A_{226} (ε_{226} 14,000 M^{-1} mL^{-1}) and β-glucosidase inhibition (Table 1). Fractions (4.6 mL) were pooled according to their inhibitory potency and iodide content. Fractions with overlapping C_{12}-**3** and dodecylammonium iodide (~ 25% of the C_{12}-**3** peak) were pooled separately and added to another run of C_{12}-**3** purification.

D-Glucono-1,5-lactam was isolated from fractions 35 to 45 which yielded 178 μmol (59%) as calculated from the inhibition of cytosolic β-glucosidase with K_I 5.2 μM. Identification was done by taking the fractions to dryness, crystallization from water–EtOH 1–9 and comparison with an authentic sample by TLC (R_f 0.24 in 3:1:0.1:0.1 $CHCl_3$–MeOH–AcOH–H_2O); mp and mixed mp 202–204 °C.

Purified C_{12}-**3** iodide (75 μmol) was converted to the chloride salt by passing over Dowex 1, Cl^--form (10 g), concentrated and lyophilized. Yield 27.5 mg, K_I 0.17 nM with cytosolic β-glucosidase at pH 7. The material was pure by TLC (R_f 0.58, solvent system as above) except for a trace of dodecylamine (R_f 0.82). It gave a positive reaction with $KMnO_4$ (0.5% in 5% aq Na_2CO_3), Dragendorff's reagent [16], and tetraiodoplatinate [17], but did not react with ninhydrin.

^{1}H NMR data (2:1 $CDCl_3$–CD_3OD, 300 MHz): δ 0.65 (t, *J* 6.68 Hz, 3 H, CH_3); 1.03–1.13 (m, 18.5 H, CH_2); 1.40–1.48 (m, 2 H, β-CH_2); 3.09–3.15 (m, α-CH_2 and ^{1}H in CD_3OD); 3.22–3.28 (m, 1 H, H-5); 3.43 (1 H, pseudo-t, $J_{3,4} = J_{4,5} = 9.45$ Hz, H-4); 3.52–3.58 (m, 2 H, H-3/H-6); 3.68 (dd, 1 H, $J_{4,5}$ 10.9, $J_{5,6}$ 4.0 Hz, H-6′); 3.98 (d, 1 H, $J_{2,3}$ 9.45 Hz, H-2). ^{13}C NMR ($CDCl_3$, 75.47 MHz): −13.47 (CH_3); 22.22–42.06 (11 signals, CH_2 of C_{12}-chain); 59.85 (C-6); 60.34, 67.61, 68.22, 72.31 (C-2 to C-5); 163.76 (C-1). The ^{1}H NMR spectrum taken in $CDCl_3$ gave sharp signals at 0.9 (t) and 1.25 (m) ppm and broad, unresolved peaks at 1.8, 3.1, 4.0, and 5.1 ppm, indicating the association of the hydrocarbon moiety of C_{12}-**3** with formation of inverse micelles. In CD_3OD, the carbohydrate signals were well resolved and the alkyl signals somewhat broader than in $CDCl_3$–CH_3OH.

N′-*Butyl*-D-*gluconamidine* (C_4-**3**).—Compound C_4-**3** was prepared as described for C_{12}-**3**, using butylamine. Inhibition of cytosolic β-glucosidase before work-up gave

K_I(app) 0.5 ± 0.15 μM at pH 7.0. Product purification was done by size exclusion chromatography on Biogel P-2 (Bio-Rad) with 30 mM NH_4-acetate as eluent. The other conditions were as described for C_{12}-**3**. Fractions containing C_4-**3** (fr. 26 to 40 with peak at $v/v_0 = 1.85$) were localized by A_{226} and glucosidase inhibition. K_I-Values calculated with inhibitor concentration from A_{226} were much too low, however, because iodide had been exchanged for acetate to a large extent. Overlap with butylammonium iodide (peak at $v/v_0 = 2.8$) was more pronounced than with C_{12}-**3** and dodecylamine. For complete conversion to the acetate and for characterization, the pooled fractions were passed over Dowex 1, AcO^- (10 g), concentrated and lyophilized. For complete removal of NH_4-acetate, lyophilization was repeated after dissolution in D_2O (5 mL). Yield 75 mg from 600 μmol **1**. Corrected for 35.6% Bu-NH_3^+ AcO^- (see below), this amounts to 48 mg (27%) C_4-**3**; K_I 0.11 μM. Examination by TLC (1.5:1:0.3 $CHCl_3$–MeOH–AcOH) showed the presence of a compound with the same staining properties as C_{12}-**3** at R_f 0.62 and, in addition, a ninhydrin positive spot at R_f 0.67 which co-migrated with butylammonium acetate (butylammonium iodide migrated wit R_f 0.72).

^{1}H NMR (D_2O, 300 MHz, integration normalized to the signal at 4.39 ppm): δ 0.98 (t, 7.02 H, *J* 7.3 Hz, CH_3); 1.40 (m, 4.61 H, $-CH_2$); 1.66 (m, 4.60 H, β-CH_2); 1.96 (s, 6.85 H, CH_3 of AcO^-); 3.01 (t, 2.62 H, *J* 7.3 Hz, α-CH_2 of Bu-NH_3^+); 3.40 (t, 2.10 H, *J* 7.3 Hz, α-CH_2 C_4-**3**); 3.60 (dd, 1.03 H, *J* 9.2, 6.0 Hz, H-4); 3.76–3.90 (m, 4.23 H, H-3, H-5, H-6.6′); 4.39 (d, 1.00 H, $J_{2,3}$ 10.0 Hz, H-2). The signal at 3.01 ppm and the excess of alkyl protons over those calculated for C_4-**3** at 0.98, 1.40, and 1.66 ppm point to the presence of 57 mol% (35.6 wt%) of butylammonium acetate in this preparation of C_4-**3**. This was confirmed by the quantitative determination of amino groups with ninhydrin (see below).

Analytical procedures.—Determination of **1**.—(a) With glucose oxidase/peroxidase (Merckotest Glucose, E. Merck 14365): the procedure recommended by the supplier for D-glucose was adhered to except that the reaction time was extended to 40 min. (b) With hexokinase/glucose-6-phosphate dehydrogenase: the phosphorylation of **1** by ATP and hexokinase proceeded at about the same rate as with D-glucose whereas the reaction of nojirimycin-6-P with Glc-6-P dehydrogenase was considerably slower [23]. We have, therefore, modified the conditions recommended for D-glucose [24] by using a five-fold higher concentration of Glc-6-P dehydrogenase.

Both methods gave identical results with a freshly prepared solution of **1**, whereas with aged solutions (20 d at 4 °C and pH 7) the hexokinase method indicated 30% decomposition while no decrease of **1** could be seen with glucose oxidase. Apparently, decomposition products of **1** are still reactive with glucose oxidase.

Determination of alkylamines and type **3** *compounds.*—Butyl- and dodecyl-amine were determined with ninhydrin as described [25]. Concentrations were read from standard curves of A_{570} prepared with known amounts of the respective amines. Under these conditions the reference amidine, 2-amino-1,2-dehydropiperidine (Fluka), gave a molar color equivalent of 3 ± 1%; with the best preparation of C_{12}-**3** 12 ± 2% were found. Whether this difference resulted from the trace amount of dodecylamine detected by TLC and/or a greater lability under the assay conditions was not investigated.

The low color yield of the amidines in the ninhydrin reaction and their facile hydrolysis at pH > 12 [20] formed the basis of their determination by the following

procedure: samples (100 μL) and 0.03 M NaOH (400 μL) were heated to 65 °C for 20 min in tightly closed screw-cap vessel, cooled in ice water for 10 min with occasional shaking (to redissolve volatilized amines in the aqueous phase), adjusted to pH 5 by the addition of 0.04 M AcOH (500 μL), and reacted with ninhydrin as described above. The molar color yield from A_{570} of the reference amidine was 99 ± 3%. To correct for the presence of alkylamines in samples of C_4- and C_{12}-**3** their concentration was calculated from the differences of A_{570} measured with and without NaOH-treatment. Samples containing appreciable amounts of buffer should be adjusted to pH 12. As doubling the time of reaction with NaOH gave < 3% higher values of A_{570}, we conclude that D-gluconolactam (Scheme 2) is stable under the assay conditions.

Aqueous samples which had been treated at pH 10 (75 μL containing 2 to 5 μmol C_4- or C_{12}-**3**), 2-propanol (75 μL), $NaHCO_3$ sat. in water (20 μL), and 1-fluoro-2,4-dinitrobenzene (10% v/v in EtOH, 10 μL) were kept for 2 h at room temp and the reaction stopped by addition of acetic acid (20 μL). Reference solutions with butyl- or dodecylamine and 2-amino-2-deoxy-glucose HCl were treated under the same conditions. Samples with C_4-**3** subjected to TLC in 5:1 $CHCl_3$—MeOH showed yellow spots with R_f 0.98 (DNP-butylamine) and 0.69 (DNP-C_4-**4**). DNP-Glucosamine had R_f 0.37, unreacted C_4-**3** (detected with $KMnO_4$) had R_f 0.13. Samples with C_{12}-**3** were chromatographed in 20:1 $CHCl_3$–MeOH, showing yellow spots at R_f 0.97 (DNP-dodecylamine) and R_f 0.43 (DNP-C_{12}-**4**). DNP-Glucosamine remained at the origin; unreacted FDNB showed up as dark spot under UV(254 nm) with R_f slightly less than the DNP-alkylamines. The spots assigned to the DNP-derivatives of **4** were no longer seen in samples kept at pH 10 for 24 h.

Determination of pK_a *of* C_{12}-**3**.—Potentiometric titration of C_{12}-**3** iodide gave pK_a(app) 10.0. The solution became turbid at pH > 9.5, but the turbitity redissolved on readjustment to pH 4.5 from 11.5 after the titration. The molar color equivalent in the ninhydrin reaction without NaOH-treatment increased from 18% to 77%. To reduce the complications caused by the low solubility and hydrolysis of unprotonated C_{12}-**3** half the calculated equivalent of 0.02 M NaOH was added to 10 μmol C_{12}-**3** chloride in 2 mL water. The initial pH 10.4 fell to pH 9.8 within 8 min; the solution had become turbid after 90 s after the addition of NaOH. To correct for dissolved CO_2, the volume of NaOH required in a blank titration to reach pH 10.4 was used to calculate the degree of deprotonation α of C_{12}-**3** at this pH. Insertion of α into the Henderson–Hasselbalch equation gave the corrected pK_a 10.8.

Measurement of β-glucosidase inhibition.—Enzymes: (a) bovine lysosomal β-glucosidase was isolated and purified to electrophoretic homogeneity as described [26]. Enzyme activity was measured with 4-nonylumbelliferyl-β-D-glucopyranoside [27] in 50 mM Na-citrate/-phosphate pH 5.0 containing 0.6% Na-taurocholate; (b) bovine cytosolic β-glucosidase was a commercial preparation of β-galactosidase from bovine liver (Sigma G 1875) which was treated with 0.1 mM conduritol B-epoxide (CBE). As first noted by Ermert et al. [28], this enzyme is active against both β-galactosides and β-glucosides with an about two-fold higher activity against the latter. Our suspicion that it might in fact be a crude preparation of the cytosolic (non-specific) β-glucosidase [29] was confirmed by the following data: (i) Only 25% of the activity measured with 4-methylumbelliferyl-β-D-glucopyranoside was lost on incubation with 0.1 mM CBE for

Table 2
Inhibition constants K_I in μM for starting materials and by-products in crude preparations of C_4-**3** and C_{12}-**3** with bovine lysosomal and cytosolic β-glucosidases

β-Glucosidases	C_4-NH_2	C_{12}-NH_2	**1**	D-Glucono-1,5-lactam
Lysosomal, pH 5.0	n.d.	22	0.8	120
Cytosolic, pH 7.0	280	0.71	42	2.6

Abbreviations: n.d. not determined; C_4-NH_2 butylamine; C_{12}-NH_2 dodecylamine.

1 h. The other 75% and the activity with the corresponding β-galactoside were reduced < 15% after 24 h. The mammalian cytosolic β-glucosidase is one of the few enzymes which are practically resistant to inactivation by the covalent inhibitor CBE [30], whereas the lysosomal enzyme is exceptionally reactive. (ii) The β-glucosidase activity remaining after CBE treatment was strongly inhibited by taurodeoxycholate (K_I(app) 90 μM), a characteristic property of the cytosolic enzyme; (iii) K_M of 4-MU-β-glucoside (40 μM) and K_I of *N*-dodecyl-D-glucosylamine (0.1 nM) were very similar to the values published for the purified enzyme [29]. Significant differences (up to four-fold) were seen with inhibition constants of taurodeoxycholate and N-dodecyl D-glucosylamine measured with 4-MU-β-galactoside; we ascribe this to the presence of an additional, presumably lysosomal, β-galactosidase.

Inhibition constants K_I were calculated from rates measured in the presence of inhibitor (v_i) and in its absence (v_o), assuming competitive inhibition where I and S are the concentrations of inhibitor and substrate respectively:

$$K_I = \frac{I}{(v_o/v_i - 1)(1 + S/K_M)}$$

In order to estimate possible errors in the inhibition constants calculated for crude and partially purified preparations of C_4-**3** and C_{12}-**3**, we determined the inhibition constants of known or suspected contaminants (Table 2). The data show that the K_i-values of **1** and the alkylamines are > 400-fold larger than those of C_4-**3** (0.13 μM) and C_{12}-**3** (0.16 nM). Their presence, even in large excess, will thus not affect the accuracy of K_i-values of the amidines determined in crude preparations. The error caused by a three-fold molar excess of D-glucono-1,5-lactam in preparations of C_4-**3** would amount to about 17%.

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