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Main glucosidase conversion products of the gluco-alkaloids dolichantoside and palicoside

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Abstract

The enzymatic glucose cleavage of palicoside revealed the biosynthetic pathway to akagerine, whereas the conversion of dolichantoside led to a new quaternary heteroyohimbine alkaloid named N_b-methyl-21-β-hydroxy-mayumbine. The hypothetical models of reactions occurring after the conversion of both substrates are proposed. Dolichantoside and palicoside, as well as *Strychnos mellodora* stem bark crude ethanol extract, exhibit significant antimycotic activity against human pathogens in presence of specific glucosidase.

Keywords: *Strychnos mellodora*; *Loganiaceae*; Biosynthesis; Gluco-alkaloids; Dolichantoside; Palicoside; Akagerine; N_b-Methyl-21-β-hydroxy-mayumbine

1. Introduction

In our search for alkaloids in *Strychnos mellodora* S. Moore, an endemic species of East-Africa (Leeuwenberg, 1969) we isolated a wide range of gluco-alkaloids (Tits et al., 1996; Brandt et al., 1999) and particularly interesting amounts of the N_b-methylated alkaloids dolichantoside (**1**) and palicoside (**2**). Their structures are closely related to and present the same 3α configuration as strictosidine (**3**), the central intermediate in the biosynthesis of monoterpenoid indole and quinoline alkaloids (Rueffer et al., 1978; Bisset, 1980). The enzymatic cleavage of glucose in strictosidine (**3**) gives rise to a large variety of structure groups (Kisakürek et al., 1983). The intermediate isolated by Stevens (1994) after strictosidine conversion was 3α, 15α-$\Delta^{20(21)}$-didehydrocathenamine, a $\Delta^{20(21)}$-didehydro-heteroyohimbine. The amounts of dolichantoside (**1**) and palicoside (**2**) obtained from *Strychnos mellodora* gave us the opportunity to start phylogenetical studies in order to evaluate the potential biosynthetic pathway from those gluco-alkaloids. In our previous work, we mentioned the conversion of these two substrates by strictosidine glucosidase from *Catharanthus roseus* (SGD) and by a specific glucosidase isolated from *Strychnos mellodora* (SMGD) (Brandt et al., 2000). Here we present the isolation and identification of the main conversion products. An evaluation of the antimycotic activity before and after glucosidase conversion was carried out on the crude ethanol extract of *Strychnos mellodora* stem bark, as well as on dolichantoside (**1**) and palicoside (**2**).

2. Results and discussion

2.1. Identification and determination of conversion products

To check the invariability of the pattern of conversion products of dolichantoside (**1**) and palicoside (**2**), incubations were first compared in the presence of three different glucosidases: SGD, SMGD and a less specific one, the glucosidase from sweet almonds. Independent of the glucosidase used, the pattern of conversion products was the same. The kinetic parameters K_m and V_{max} were determined for the enzymes SGD and SMGD, in the presence of the substrates **1** and **2** (Brandt et al., 2000). This study clearly revealed that the conversion of substrates **1** and **2** was quicker and more complete with the enzyme SMGD (Table 1). The V_{max} or maximal

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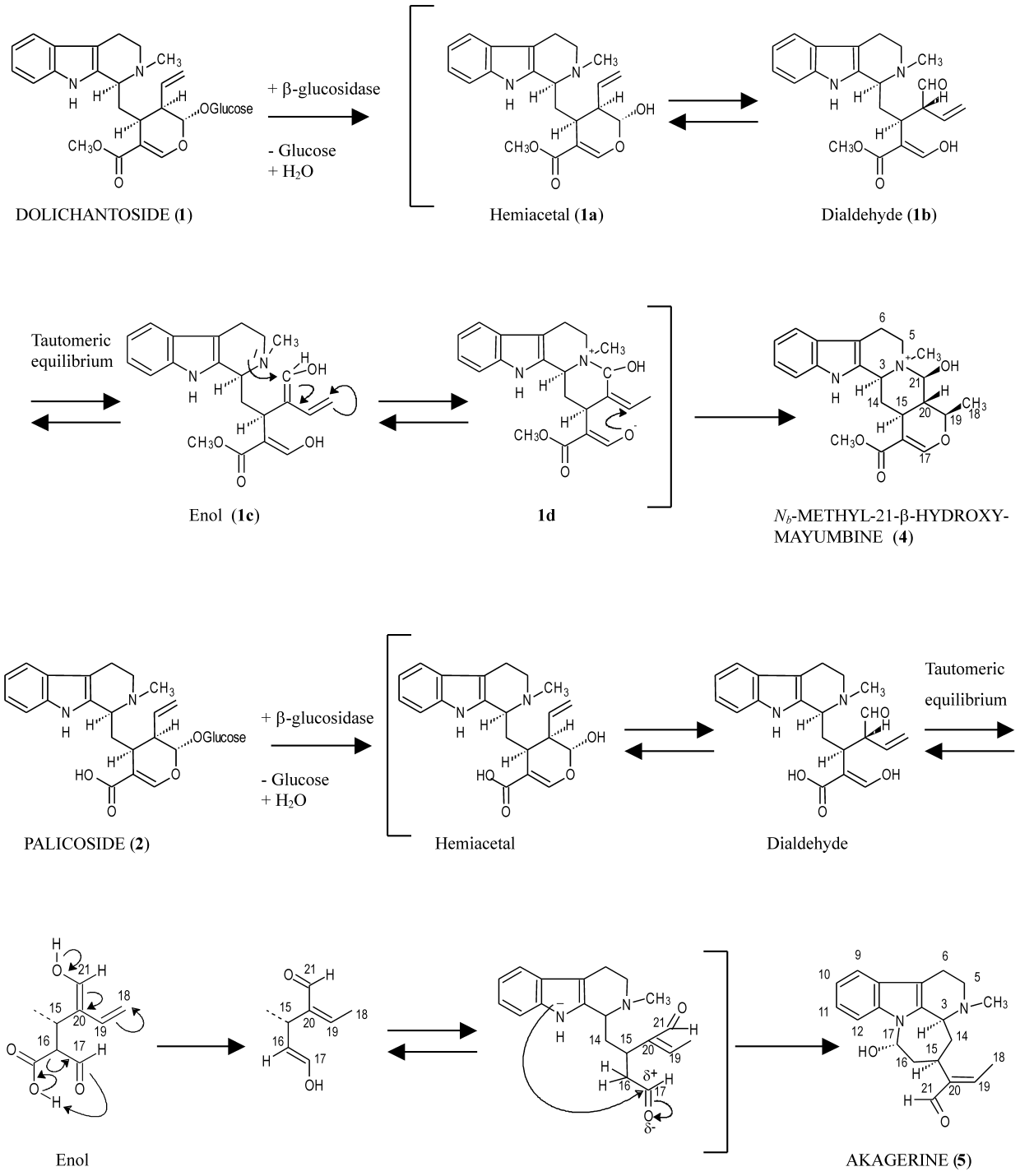

Scheme 2. Hypothetical model for the conversion of palicoside into akagerine.

conversion velocities were higher in the presence of SMGD and reached with lower substrate concentrations, as K_m represents half of the substrate concentration necessary to reach the maximal conversion velocity.

We thus incubated a larger amount of dolichantoside (**1**) in the presence of SMGD. After 72 h of incubation, dolichantoside was nearly completely converted into a main product (**4**) that was more polar. This product was compared in TLC and HPLC and stated to be identical to the one obtained after the 15 min incubation of a 1 mM dolichantoside solution. It was separated (see Experimental) from the incubation mixture and its structure determined by spectroscopic methods as N_b-methyl-21-β-hydroxy-mayumbine (**4**), a new heteroyohimbine quaternary alkaloid. Its UV spectrum revealed maxima of tetrahydro-β-carboline alkaloids. The high resolution mass spectrum exhibited a molecular peak corresponding to the molecular formula $C_{22}H_{27}N_2O_4^+$.

Table 1
Kinetic parameters of SGD and SMGD towards dolichantoside (**1**) and palicoside (**2**). Conditions used to determine these parameters are found in literature (Brandt et al., 2000)

Substrate	SGD		SMGD	
	K_m (μM)	V_{max} (nkat mg $^{-1}$ protein)	K_m (μM)	V_{max} (nkat mg $^{-1}$ protein)
(**1**)	1130	21.2	564	224
(**2**)	7015	11.1	335	39

The molecular peak also present in the ES–MS–MS spectrum at m/z 383 $(M)^+$ exactly corresponded to the loss of a glucose moiety from dolichantoside (**1**); an ion at m/z 365 suggested the easy loss of water and thus the presence of a hydroxyl function. In the ^{1}H-^{1}H NMR auto-correlation spectra, the most characteristic point for the formation of a heteroyohimbine structure was the presence of a methyl group at 1.55 ppm (d, $J_{18–19}$ = 6.7 Hz) (CH_3-18) correlated to H-19 at 3.75 ppm (q, $J_{19\text{-}20}$ = 8.6 Hz). Chemical shifts of those protons, in particular shielding of H-19, as well as high coupling constants, by comparison with the literature data concerning the eight possible diastereoisomers of heteroyohimbine type (Phillipson and Supavita, 1983), suggested α-configuration of H-19 and consequently β-configuration of the methyl group C-18 (19 *R* series). This configuration is related to 19-epiajmalicine or mayumbine (3α, 20β, *normal* diastereoisomeric type) (Uusvuori and Lounasmaa, 1981). The formation of a *normal* type alkaloid is predictable considering the initial H_3-α, H_{20}-α configuration of dolichantoside. On the other hand, the configuration α of H_{19}, characteristic of 19-epiajmalicine, is less evident but plausible if one considers the biomimetic scheme named "La Ronde" established by Lounasmaa and Hanhinen (1998). This scheme shows interconversions existing between the four $\Delta^{20(21)}$-didehydroheteroyohimbines (cathenamines), which, when combined with reduction steps, allow access to the eight basic heteroyohimbines. The option of H-19 α or β configuration takes place during the *Z–E* epimerisation of the ethylidene side chain (Lounasmaa et al., 1992; Uskoković et al., 1979). Closing of *E* ring is then achieved by a Michael addition of the enolate stereochemically exclusively possible on the β-face on the ethylidene chain (Brown, 1980). This ethylidene chain is moreover directly connected to H-20 (3.15 ppm), itself coupling with H-21 appearing in the form of a deshielded doublet at 4.45 ppm ($J_{20–21}$ = 7.5 Hz), compatible with the presence of an hydroxyl function on C-21 and the presence of an adjacent quaternary nitrogen. The coupling constant $J_{20–21}$ suggested the antiperiplanar position of H-20 and H-21, and thus the α position of H-21. H-20 is also correlated to H-15 at 2.85 ppm. The proton chemical shift of the *N*-methyl group is in accord with a C/D *cis* ring junction. Indeed, this stereochemistry is supported by the relatively low field position (^{1}H: 3.27; ^{13}C: 47.0 ppm) of the quaternary N_b-methyl, suggesting a *cis*-relationship between the N_b-methyl and the H-3 (Arata et al., 1975; Sugiura et al., 1977). The resonances of H-14a, H-14b and H-3 appear respectively at 2.45, 1.90 and 5.0 ppm. This latter deshielded value is likewise consistent with the presence of the adjacent quaternary nitrogen. All aromatic protons, including H-17 at 7.49 ppm, are detected. Carbons chemical shifts were determined on the base of HMQC spectra.

The second substrate, palicoside (**2**), was incubated with SMGD for 72 h. Its unfinished conversion gave rise to two main products. The first one, **5**, was apparently less polar than palicoside on TLC, in contrast to the second one, **6**, more polar. After a rapid chloroform extraction of **5** from the incubation mixture, its UV and MS spectra were measured. The UV spectrum exhibited maxima of an indole chromophore. The mass spectrum showed a molecular peak at m/z 325, corresponding to the molecular formula $C_{20}H_{24}N_2O_2$. We confronted this product **5** with available reference substances of identical molecular mass, and in particular to akagerine. They showed to possess the same behaviour when compared by HPLC and TLC in different systems. Moreover, the revelation of TLC plates by iodoplatinate reagent (Svendsen and Verpoorte, 1983) produced in each case a pink colour turning into red after a few minutes. We completed this identification by comparison of their 1D ^{1}H NMR spectra. The recorded resonances were identical, which confirmed that akagerine was the non-polar conversion product of palicoside (**2**). Akagerine (**5**) is a perhydroazepine alkaloid that accumulates in numerous species of *Strychnos* as *Strychnos usambarensis* (Angenot et al., 1975). It has showed convulsivant (Rolfsen et al., 1980) and antiprotozoal (Wright et al., 1991) properties on mice. Its enantioselective synthesis was proposed by Danieli et al. (1995). We completed the spectral data of this reference alkaloid by further ^{1}H and ^{13}C NMR contribution. The polar conversion product **6** could not be separated in sufficient amount to be identified and is, thus, still under studies.

2.2. Hypothetical models for the conversion reactions

For both conversions, we present hypothetical models of reactions occurring after enzymatic hydrolysis and removal of the glucose moiety. It should be noted that none of the intermediates depicted in Schemes 1 and 2 has ever been isolated as a product from any incubation mixture, or has ever been demonstrated in any other direct way. Their occurrence is described by mere theoretical organic chemistry. It has been reported that the deglucosylation of strictosidine leads also to reactive

and unstable intermediates, which may readily and quickly undergo different intra-molecular rearrangements (Hemscheidt and Zenk, 1980; Stöckigt, 1980; Stevens, 1994). The same situation is observed in our hands using systems with dolichantoside or palicoside as analogous substrates.

Reactions leading from dolichantoside (**1**) to N_b-methyl-21-β-hydroxy-mayumbine (**4**) are similar to those proposed in the conversion model of strictosidine (Stevens, 1994). They can be schematised as follows (scheme 1): removal of the glucose moiety of dolichantoside leads to an unstable hemiacetal (**1a**), which is rapidly converted into a high reactive aldehyde by opening of E ring. This aldehyde (**1b**) is in equilibrium with its enol tautomer (**1c**). This intermediate is then stabilised by a nucleophilic attack of the nitrogen N_b on C-21 to close D ring, with concomitant transformation of the vinyl chain into a *Z*-ethylidene chain (**1d**). Protonation of C-18 is ensured by the C-17 hydroxyl function, acting as proton donor. Closing of *E* ring is then achieved by a Michael addition of the enolate on the β face of the ethylidene side chain to form the quaternary alkaloid N_b-methyl-21-β-hydroxy-mayumbine (**4**).

Besides the cleavage of the glucose moiety, palicoside (**2**), during its conversion, further loses a group of 44 mass units equivalent to the carbonic gas CO_2. The conversion is thought to start by the two first steps occurring with dolichantoside (scheme 2). The loss of CO_2, happening in totally soft conditions (30°C, neutral pH), can take place at the stage of the tautomeric equilibrium between aldehyde and enol functions. This step is just characterised by the presence of a carboxylic function in β position from an aldehydic function where the start of CO_2 is favoured by a cyclic elimination process at six centres, identical to what occurs in the decarboxylation of malonic acid or β-cetonic acids (Carey and Sundberg, 1997). The double bond shift and the appearance of an ethylidene chain take place at the same time. Closing of perhydroazepine ring is then realised by the nucleophilic attack of the indole nitrogen N_a on C-17, to form a carbinolamine, akagerine (**5**). The loss of CO_2 would not be allowed in the case of dolichantoside, because of the presence of the methyl group on the carboxylic function, which prevents the six centres elimination process to take place.

2.3. Antimycotic activity of the substrates in presence of specific glucosidase

The antimycotic activity of dolichantoside (**1**) and palicoside (**2**), as well as of the crude ethanol extract of *Strychnos mellodora* stem bark (EtOH extract), was tested before and after enzymatic hydrolysis by SMGD against three standard strains of human pathogens, the yeasts *Candida albicans*, *Candida glabrata* and a filamentous fungus, *Aspergillus niger*. The substrates applied alone showed no activity at the higher tested concentrations (1.9 mg/ml of **1**; 1.8 mg/ml of **2**; 3.8 mg/ml of EtOH extract). This is in contrast with the significant antimycotic activity found against those same three pathogens when the substrates were tested in the presence of SMGD. The minimum inhibitory concentration (MIC) measured for each substrate in presence of SMGD against each strain is presented in Table 2. These products are, of course, far from competing with other antifungal agents such as miconazole that inhibits the growth of yeast strains *C. albicans* (1.6 μg/l), *C. glabrata* (0.2 μg/l) and *Aspergillus niger* (0.8 μg/l) at much lower concentrations in the same conditions (Piel et al., 1999).

Table 2
Minimal inhibitory concentration (MIC) of dolichantoside (**1**), palicoside (**2**) and the crude EtOH extract of *Strychnos mellodora* stem bark in presence of the glucosidase from *Strychnos mellodora* (SMGD) against three ATCC reference strains

	Minimum inhibitory concentration (mg/ml)		
	Candida albicans 10231	*Candida glabrata* 90030	*Aspergillus niger* 16404
Dolichantoside (**1**)/SMGD	0.38	0.76	0.38
Palicoside (**2**)/SMGD	0.14	1.48	0.74
EtOH extract /SMGD	2.28	0.38	0.76

3. Conclusion

The hypothesis formulated by Massiot and Delaude (1988) putting forward that N_b-methylated precursors lead to the formation of akagerine-type bases, monoterpenoid alkaloids with N_a–C_{17} bond, has been verified by the isolation and identification of a stable alkaloid, akagerine (**5**), after the conversion of palicoside (**2**). We have shown, moreover, that even in the presence of a methyl group, addition on N_b is possible and produces polar alkaloid, as in the case of the conversion of dolichantoside (**1**) into a new quaternary alkaloid, N_b-methyl-21-β-hydroxy-mayumbine (**4**).

We detected no presence of those conversion products in *Strychnos mellodora* organs, which may indicate that at the plant level substrates and glucosidase are contained in different cell compartments. Although the results of antimycotic activity do not concern vegetal pathogens, they may nevertheless suggest a constitutive "trigger mechanism" in the plant, in which an infection, by damage of the plant cell, results in substrate deglucosidation, thus allowing the active aglycone(s) to act against the intruder.

4. Experimental

4.1. Materials and methods

Dolichantoside (**1**) and palicoside (**2**) were isolated from *Strychnos mellodora* S. Moore stem bark according to Tits et al. (1996). Strictosidine β-glucosidase (SGD) was obtained by purification from suspension cultured cells of *Catharanthus roseus* L. G. Don according to Geerlings (1999). Strictosidine β-glucosidase from *Strychnos mellodora* (SMGD) was obtained by purification from dry leaves of *Strychnos mellodora* (Brandt et al., 2000). The β-glucosidase from sweet almonds was purchased from Merck (Germany). The reference akagerine has been isolated from *Strychnos usambarensis* and is kept in our laboratory.

The comparison of conversion products pattern was based on a HPLC method described by Pennings et al. (1989), but with a 4 × 250 mm Lichrospher 60 RP-Select B column (particle size 5 μm, cat. No. 00961; Merck). With this system the retention time of the substrates and conversion products were: **1** (18.5 min), **2** (5.4 min), **4** (24.2 min), **5** (4.3 min).

Protein concentrations were determined using the method described by Bradford (1976). UV spectra were recorded on a spectrophotometer Kontron Uvikon 922 with Hellma type 100-QS 10.00 mm thickness precision cuvettes.

^{1}H NMR and ^{13}C NMR 1D and 2D spectra of **4** were determined at 400 and 100 MHz respectively on a Bruker 400 MHz spectrometer. ^{1}H NMR and ^{1}H–^{1}H autocorrelation spectra of **5** and reference akagerine were determined at 600 MHz; ^{13}C NMR and HMQC spectra of reference akagerine were recorded at 150 MHz on a Bruker DMX 600 MHz spectrometer. Samples were run in deuterated solvents with TMS as internal standard.

HRMS was performed on a Micromass Ultima spectrometer (5.0 mDa resolution) in electrospray (MS–MS), positive ion; ES–MS–MS spectra was taken on a Micromass VG-Quattro II in electrospray, positive ion, ionisation energy 20 eV.

4.2. Conversion of dolichantoside (*1*) into N_b-methyl-21-β-hydroxy-mayumbine (*4*)

Half a milliliter of Na-Pi buffer pH 6.3 containing 50 mg dolichantoside (**1**) and 1 ml of enzymatic preparation (36.2 μg protein ml^{-1}) obtained from the leaves of *Strychnos mellodora* were mixed, homogenised and incubated in a bain-marie at 30°C until complete conversion of dolichantoside, after 72 h. The conversion was monitored by TLC analysis on silica gel with the mobile phase EtOAc–*iso*–prOH–NH_4OH 4.25% (60:25:15). In this system, dolichantoside (**1**) appears at R_f 0.43 and N_b-methyl-21-β-hydroxy-mayumbine (**4**) at R_f 0.13.

The dolichantoside (**1**) incubation mixture was then frozen and lyophilised. The dry residue was suspended in 2 ml MeOH, stirred and centrifuged 10 min at 3500 *g*. The supernatant was separated and evaporated to give a residue which was taken in 2 ml H_2O. This aq. soln was then extracted twice by *n*-BuOH and evaporated under reduced pressure. The residue dissolved in a few drops of MeOH gave by addition of Et_2O a voluminous white ppt. This latter was separated by centrifugation 10 min at 2500 *g*, dried and washed with Et_2O to obtain N_b-methyl-21-β-hydroxy-mayumbine (**4**) (27 mg) as an amorphous white powder; UV (MeOH) λ_{max} nm (log ε): 222 (4.19), 267 (3.54), 278 (3.55), 287 (3.43); ^{1}H NMR spectral data (400 MHz, CD_3OD): δ 1.55 (3H, *d*, $J=6.7$ Hz, H-18), δ 1.90 (1H, *m*, H-14b), δ 2.45 (1H, *m*, H-14a), δ 2.85 (1H, *m*, H-15), δ 3.15 (1H, *m*, $J=8.6$, 7.5 Hz, H-20), δ 3.20 (1H, H-6b), δ 3.27 (3H, *s*, N^+CH_3), δ 3.50 (1H, *d*, H-5a), δ 3.71 (3H, *s*, OCH_3), δ 3.75 (1H, *q*, $J=6.7$, 8.6 Hz, H-19), δ 4.30 (1H, H-5b), δ 4.45 (1H, *d*, $J=7.5$ Hz, H-21), δ 5.00 (1H, *d*, H-3), δ 7.07 (1H, *t*, $J=7.8$ Hz, H-0), δ 7.17 (1H, *t*, $J=8.2$ Hz, H-11), δ 7.37 (1H, *d*, $J=8.2$ Hz, H-12), δ 7.49 (1H, *s*, H-17), δ 7.50 (1H, *d*, $J=7.8$ Hz, H-9); ^{13}C NMR spectral data (100 MHz, CD_3OD): δ 10.8 (C-18), δ 17.5 (C-6), δ 25.6 (C-14), δ 32.7 (C-15), δ 47.0 (N^+CH_3), δ 51.3 (OCH_3), δ 59.5 (C-5), δ 61.7 (C-19), δ 69.0 (C-3), δ 75.0 (C-20), δ 97.0 (C-21), δ 114.4 (C-12), δ 118.2 (C-9), δ 119.6 (C-10), δ 122.4 (C-11), δ 144.0 (C-17); HRMS-MS–(EI) *m*/*z* (rel. int.): 383.1978 $[C_{22}H_{27}N_2O_4]^+$ (100), 365.2310 $[M-H_2O]^+$ (16), 352.2046 (20), 340.1962 (69), 322.1725 (41), 308.1646 (27), 144.0979 (53); Positive ES-MS-MS *m*/*z* (rel. int.): 383 $[M]^+$ (100), 365 $[M–H_2O]^+$ (13), 340 (28), 222 (30), 194 (64), 170 (50), 144 (97), 130 (42);

4.3. Conversion of palicoside (*2*) into akagerine (*5*)

Twenty milligrams of palicoside (**2**) dissolved in 200 μl of Na–Pi buffer pH 6.3 and 0.5 ml of enzymatic preparation (36.2 μg proteinml^{-1}) obtained from the leaves of *Strychnos mellodora* were mixed, homogenised and incubated in a bain-marie at 30°C. Lower specificity of the glucosidase in the presence of palicoside prevented to obtain a complete conversion of this substrate. Incubation was stopped after 72 h. In the TLC monitoring system described above palicoside (**2**) reaches a R_f of 0.09. Its conversion products, **5** and **6**, are characterised by R_f of 0.05 and 0.78 respectively. The palicoside (**2**) incubation mixture was then frozen and lyophilised. The residue was taken in 2 ml H_2O and then extracted by 2 ml CH_2Cl_2 until complete disappearance of **5** from the aq. layer. Each organic layer was separated by centrifugation 10 min at 2500 *g*. The joined organic layers were then conc. under reduced pressure and submitted to a prep. TLC in the system composed of silica gel and the mobile phase EtOAc–*iso*–prop-NH_4OH 4.25% (60:25:15). The elution was performed by CH_2Cl_2 and allowed

to obtain 7.38 mg of **5** as an amorphous white powder. UV (MeCN) λ_{max} nm (log ε): 229 (4.51), 276 (3.78), 283 (3.80), 293 (3.68); ^{1}H NMR spectral data (600 MHz, $CDCl_3$): δ 1.99 (1H, *d*, $J=12.8$ Hz, H-14b), δ 2.07 (3H, *d*, $J=7.2$ Hz, H-18), δ 2.09 (1H, *m*, H-16b), δ 2.19 (1H, *q*, $J=10.9$, 12.8 Hz, H-14a), δ 2.33 (1H, *t*, H-16a), δ 2.53 (3H, *s*, NCH_3), δ 2.72–2.78 (3H, *m*, H-5b, H-6a, H-6b), δ 3.07 (1H, *m*, H-5a), δ 3.66 (1H, *t*, $J=11.7$, 1.4 Hz, H-15), δ 3.82 (1H, *d*, $J=10.9$ Hz, H-3), δ6.24 (1H, *d*, $J=3.7$ Hz, H-17), δ 6.55 (1H, *q*, $J=7.2$ Hz, H-19), δ 7.11 (1H, *t*, $J=7.7$ Hz, H-10), δ7.18 (1H, *t*, $J=8.2$ Hz, H-11), δ 7.29 (1H, *d*, $J=8.2$ Hz, H-12), δ 7.48 (1H, *d*, $J=7.7$ Hz, H-9), δ 9.31 (1H, *d*, $J=1.4$ Hz, H-21); Positive ES–MS–MS *m*/*z* (rel. int.): 325 $[M+H]^+$ (93), 307 $[M+H\text{-}H_2O]^+$ (5), 282 (100), 264 (73), 144 (5), 130 (3); **5** has the same behaviour as reference akagerine in the TLC system described above ($R_f=0.78$), and with the mobile phases $CHCl_3$–CH_3OH (80:20) ($R_f=0.5$) and EtOAc–*iso*–prOH–NH_4OH 4.25% (45:35:5) ($R_f=0.73$); iodoplatinate reagent produces a pink color turning into red in a few minutes.

4.4. Reference akagerine

^{1}H NMR spectral data (600 MHz, $CDCl_3$) exactly match with **5** ones; ^{13}C NMR spectral data (150 MHz, $CDCl_3$): δ 15.2 (C-8), δ 19.8 (C-6), δ 29.1 (C-15), δ 36.3 (C-14), δ 37.4 (C-16), δ 42.5 (NCH_3), δ 50.4 (C-5), δ 60.8 (C-3), δ 75.7 (C-17), δ 108.3 (C-12), δ 108.8 (C-7), δ 118.3 (C-9), δ 119.4 (C-10), δ 121.3 (C-11), δ 126.7 (C-8), δ 136.0 (C-13), δ 137.0 (C-2), δ 147.9 (C-20), δ 150.5 (C-19), δ 194.9 (C-21).

4.5. Measure of antimycotic activity

The minimal inhibitory concentration (MIC) of **1**, **2** and the crude *Strychnos mellodora* EtOH extract against three ATCC® (American Type Culture Collection) reference strains were determined by using a broth microdilution method, based upon the National Committee for Clinical Laboratory Standards (NCCLS-USA) guidelines for broth microdilution technique (M-27A and M-38P) (NCCLS, 1997 and 1998). The three references strains, *Candida albicans* (ATCC 10231), *Candida glabrata* (ATCC 90030) and *Aspergillus niger* (ATCC 16404), were tested in duplicate and in parallel with the substrates alone and in presence of SMGD. Preparation of tested antifungal stock solns was not possible because of the low availability of the substrates. **1**, **2** and EtOH extract were dissolved in H_2O as acetates (by addition of the HOAc molar equivalent), filtered, frozen and then lyophilised. The residues were then weighed and dissolved in RPMI-1640 standard medium to give 7.6 mg/ml EtOH extract, 3.8 mg/ml **1** and 3.6 mg/ml **2**. These solns were incubated for 24 h in the presence of SMGD, then filtered to give solns of 6.08 mg/ml hydrolysed EtOH extract, 3.04 mg/ml hydrolysed **1** and 2.88 mg/ml hydrolysed **2**. Strains were pre-cultivated following the NCCLS guidelines. Cultures were realised in 96 wells microplates with lid. Wells 2–11 contained twofold decreasing dilutions of the different substrates. Well 1 served as sterility control with 0.1 ml RPMI 1640 medium and 0.1 ml sterile physiologic liquid while well 12 served as growing control with 0.1 ml strain suspension and 0.1 ml sterile physiologic liquid. Each well was inoculated with 0.1 ml of one of the three inoculum suspensions. The plates were then incubated at 35°C and examined on a spectrophotometer at 625 nm every 24 h until positive control reaches a optical density of 0.2. CMI is determined as the substrate concentration that allows a reduction of the strain growth to 20% of the positive control.

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