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SYNTHESIS OF NEW PYRROLIDINE DERIVATIVES AS INHIBITORS OF α -MANNOSIDASE AND OF THE GROWTH OF HUMAN GLIOBLASTOMA CELLS

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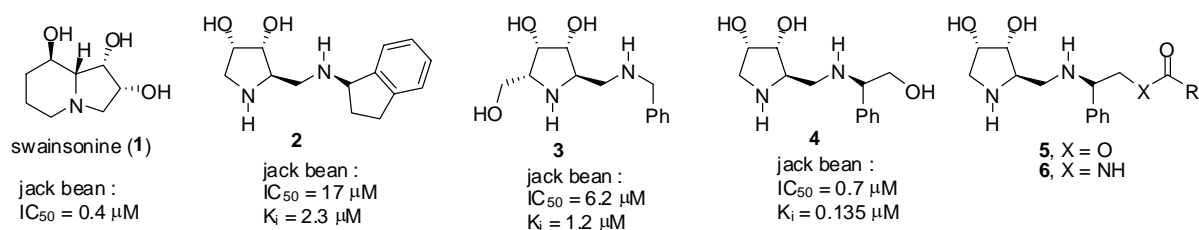
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Abstract – New 2-benzylamino-3,4-dihydropyrrolidines bearing aromatic and aliphatic amido side chains have been prepared. The influence of the amido substituents on the inhibitory activity of these diamines toward 24 commercially available glycosidases was determined. The most potent and selective α -mannosidase inhibitor (**6d**) (*N*-[(2*R*)-2-({[(2*R*,3*R*,4*S*)-3,4-dihydropyrrolidin-2-yl]methyl} amino)-2-phenylethyl]-3-bromobenzamide) of these series was also the most potent inhibitor of the growth of human glioblastoma cells.

INTRODUCTION

Very few therapeutic options exist for the treatment of human glioblastoma, in part due to their high proliferative and invasive potential and multiple resistance toward conventional chemotherapeutic agents. This cancer is also associated with poor prognosis.¹ Therefore new drugs have to be developed that are able to overcome resistance. One line of research is to target the glycosylation pathways of cancer cells. In tumor cells, the distribution of cell surface *N*-linked oligosaccharides is frequently altered and it correlates with disease progression and metastasis.² The Golgi α -mannosidase II (GMII), which is responsible for the specific trimming of 2 mannose residues from the branched GlcNAcMan₅GlcNAc₂ mannose intermediate with retention of the sugar anomeric configuration, is a key enzyme of the glycoprotein processing pathways. GMII has consequently been viewed as a potential target in the development of new anti-cancer chemotherapies.³ In clinical trials, swainsonine (**1**), a natural inhibitor of

GMII featuring a 4-amino-4-deoxy-mannofuranoside unit, has been shown to decrease the size of solid tumors and hematological dysfunctions.⁴ Nevertheless, the co-inhibition of lysosomal mannosidases prevents further development of this compound as a chemotherapeutic agent (scheme 1). Recently, we reported a new combinatorial methodology for the rapid discovery of selective and competitive inhibitors of α -mannosidase from jack bean. This led to the discovery of new selective inhibitors of α -mannosidase that contain the 3,4-dihydroxypyrrolidin-2-yl core.⁵ The inhibitory ability of the best congeners of this series (**2-4**) ranged from 2.3 to 0.135 μM (K_i measured for the inhibition of α -mannosidase from jack bean).⁶ However, these derivatives did not inhibit the growth of human tumor cells. This failure was postulated to be due to their hydrophilic character that prevents their internalization by cells. Esterification of **4** led to the formation of more lipophilic derivatives (**5**) that have demonstrated promising inhibitory activities on the growth of glioblastoma and melanoma cells.⁷ Cellular esterases probably release the parent inhibitor (**4**) within the tumor cells. Based on these results, we wondered whether aliphatic and aromatic amides (**6**) should present an improved uptake by human glioblastoma cells. Here we report the synthesis and characterization of new amido derivatives from **4** as well as their ability to inhibit commercially available glycosidases and the proliferation of human glioblastoma cells.



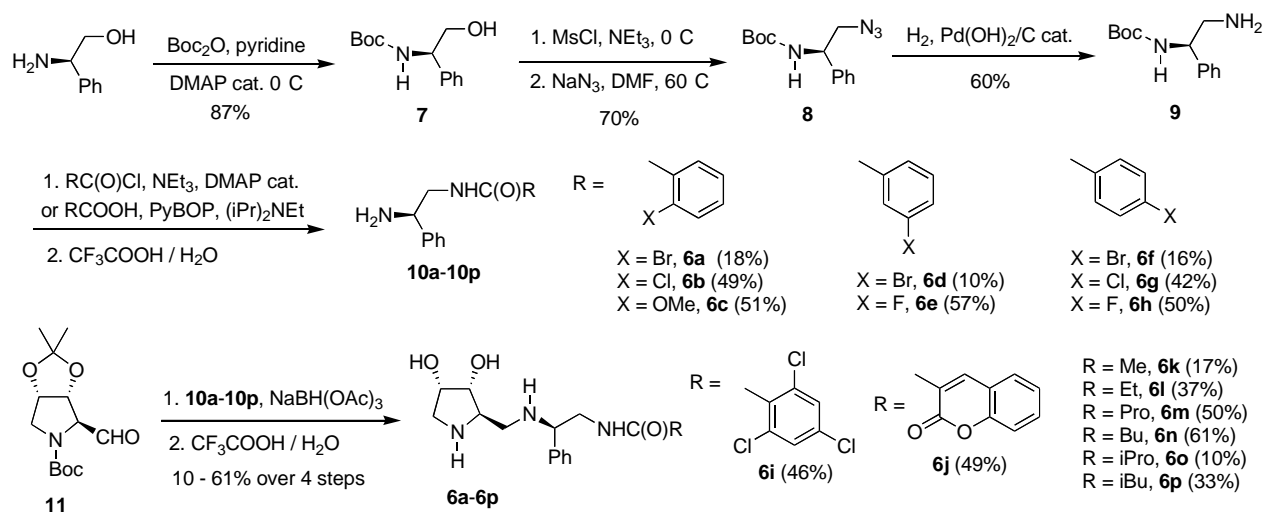
Scheme 1. Swainsonine and *cis*-3,4-dihydroxypyrrolidine analogues are inhibitors of α -mannosidases

RESULTS AND DISCUSSION

SYNTHESIS

D-(-)- α -Phenylglycinol was converted into the corresponding *tert*-butyl carbamate (87%). Esterification of the primary alcohol of **7** with methanesulfonyl chloride and Et_3N gave a mesylate that was displaced with sodium azide providing the azido derivative (**8**) in 70 % yield (two steps). Reduction of azide (**8**) by catalytic hydrogenation (H_2 , $\text{Pd}(\text{OH})_2/\text{C}$) afforded amine (**9**) that was then reacted with several aromatic (**a-j**) and aliphatic (**k-p**) acyl chlorides. The synthesis of the coumarin derivative utilized coumarin-3-carboxylic acid and PyBOP as coupling reagent (Scheme 2). The carbamate protecting group of the compounds so-obtained were removed under acidic conditions ($\text{CF}_3\text{COOH} / \text{H}_2\text{O}$) affording the corresponding aminoamides (**10a-10p**). These derivatives were engaged directly in a reductive amination procedure with carbaldehyde (**11**)⁸ and sodium triacetoxyborohydride. Subsequent acid-catalyzed

removal of acetone and Boc moieties provided the targeted pyrrolidines (**6a-6p**) in 10 to 61% yield over 4 steps (Scheme 2).



Scheme 2. Synthesis of functionalized pyrrolidines from phenylglycinol amides

INHIBITION OF PLANT GLYCOSIDASES

The inhibitory potential of compounds (**6a-6r**) toward 24 commercially available glycosidases⁹ was determined (Table 1).

| Compound | % inhibition | IC ₅₀ (μM) | Compound | % inhibition | IC ₅₀ (μM) |
|-----------|--------------|-----------------------|-----------|--------------|-----------------------|
| 6a | 89 | 145 | 6i | 88 | 130 |
| 6b | 85 | 149 | 6j | 69 | 333 |
| 6c | 95 | 95 | 6k | 88 | 91 |
| 6d | 95 | 89 | 6l | 85 | 133 |
| 6e | 94 | 92 | 6m | 83 | 178 |
| 6f | 90 | 230 | 6n | 83 | 164 |
| 6g | 94 | 108 | 6o | 84 | 291 |
| 6h | 90 | 145 | 6p | 82 | 91 |

Table 1. Inhibitory activities of pyrrolidine derivatives toward α -mannosidase from jack bean. Percent inhibition was determined at 1mM substrate and inhibitor concentrations and optimal pH of the enzyme.

These derivatives (at 1mM concentration) did not inhibit α -L-fucosidases (from bovine epididymis or human placenta), α - and β -galactosidases (from coffee beans, aspergillus niger, aspergillus orizae, escherichia coli or jack bean), α - and β -glucosidases (from yeast, rice, aspergillus niger, rhizopus mold, almond or caldocellum saccharol.), β -mannosidases (from helix pomatia), β -xylosidase (from aspergillus

niger), α -*N*-acetylgalactosaminidase and β -*N*-acetylglucosaminidase (from chicken liver) (results not shown). However, α -mannosidase from jack bean¹⁰ was inhibited by **6a-6p** with high selectivity and IC₅₀ ranging from 333 to 89 μ M. For the aromatic amides, the nature of halide substituents did not have significant influence on the inhibitory activity. However, the position of the halide group seems to be relevant since *meta*-substituted derivatives (**6d** and **6e**) displayed higher inhibitory activity than their *ortho*- and *para*-substituted analogues. The amide derivative bearing a coumarin moiety (**6j**) presented the lowest inhibitory activity toward α -mannosidase from jack bean. This is probably due to unfavourable rigidity and the bulk of the substituent which cannot adapt to the active site. In the case of aliphatic amides, the inhibitory activity decreased as the length of the chain increased (IC₅₀ = 91, 133, 178 μ M for **6k**, **6l** and **6m**, respectively). The large isopropyl group resulted in significant loss of inhibitory activity. Surprisingly, the isobutyl-substituted amide (**6p**) displayed similar inhibition of α -mannosidase from jack bean than its methyl analogue, despite its larger size. Nevertheless, as previously observed for the ester derivatives (**5**), compounds (**6a-6p**) were much less active than inhibitor (**4**), pointing out the importance of the primary alcohol moiety for high affinity binding to the α -mannosidase.

EVALUATION OF THE NEW PYRROLIDINE DERIVATIVES AS GROWTH INHIBITORS OF HUMAN GLIOBLASTOMA CELLS

The effects of derivatives (**6a-6p**) were first determined in human LN18 and LN 308 glioblastoma cells using the MTT ((3,4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium) assay, which determines the number of metabolically active cells and compared with the growth inhibitory activity of the inhibitor (**4**). Most of the aliphatic and aromatic amides did not display significant inhibition of both types of glioblastoma cell lines (data not shown). However, the 3-bromobenzamide (**6d**), which was the most potent α -mannosidase inhibitor of this series, presented a dose-dependent inhibition of glioblastoma cell growth, producing almost 80% inhibition within 24 hours at 300 μ M concentration (Table 2, Figure 1). At the same concentration, swainsonine (**1**), an α -mannosidase inhibitor with promising anti-tumoral properties,⁴ inhibited the growth of glioblastoma cell lines LN 18 and LN 308 by only 13 and 16%, respectively.

| Conc. (μ M) | Swainsonine (1) | | 4 | | 6d | |
|------------------|--------------------------|--------|----------|--------|-----------|--------|
| | LN 18 | LN 308 | LN 18 | LN 308 | LN 18 | LN 308 |
| 300 | 13 | 16 | 22 | 26 | 77 | 83 |
| 200 | 18 | 24 | 19 | 8 | 30 | 20 |
| 100 | 15 | 22 | 13 | 0 | 15 | 2 |

Table 2. *Growth inhibition of glioblastoma cells by functionalized pyrrolidines.* Cells were exposed for 24h to 0, 100, 200 or 300 μM of the various synthetic derivatives, then the MTT assay was performed for the 2 last hours of incubation. The % of residual mitochondrial activity was calculated as the ratio of treated to control cells.

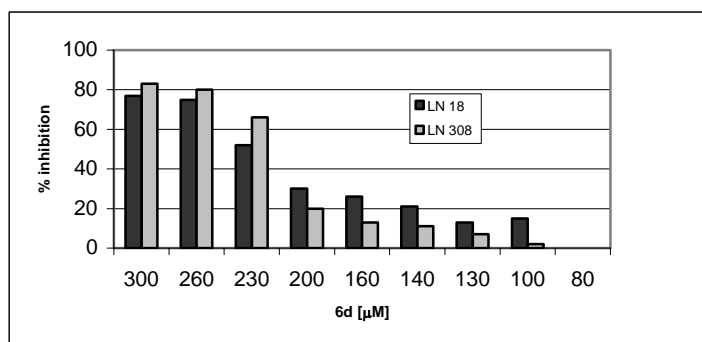


Figure 1. *Growth inhibition of human glioblastoma cells by 6d.* Cells were exposed for 24 h to increasing concentration of **6d**, then the MTT assay was performed for the 2 last hours and the percent of growth was calculated as the ratio of the MTT reduction of treated to untreated cells.

We then evaluated whether **6d** had the potential to diminish cell growth by inhibiting the synthesis of DNA, and/or of proteins. The degree of incorporation of [^3H]-thymidine and [^3H]-leucine following 6 hours exposure of the cells to **6d** was evaluated. It demonstrated that this compound inhibited thymidine incorporation, therefore DNA synthesis (Figure 2A), at slightly lower concentration and higher extent than leucine incorporation, therefore protein synthesis (Figure 2B). These results suggest that **6d** acts initially by inhibiting DNA synthesis (84% inhibition at 300 μM in LN18), then the rate of protein synthesis decreases (39% at 300 μM in LN18), resulting in diminished cell survival.

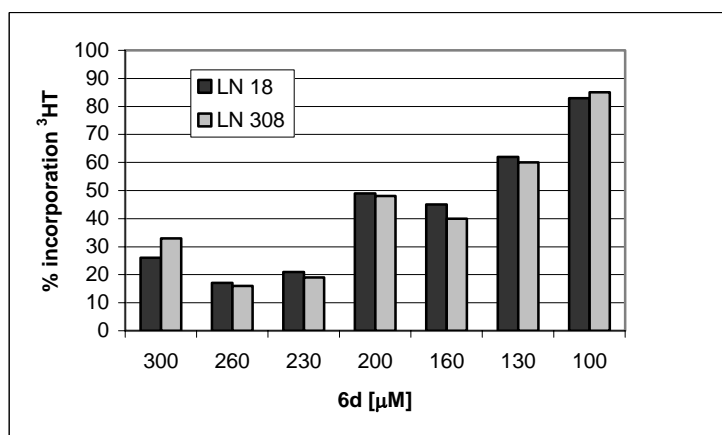


Figure 2A. *Inhibition of DNA synthesis by 6d in human glioblastoma cells*

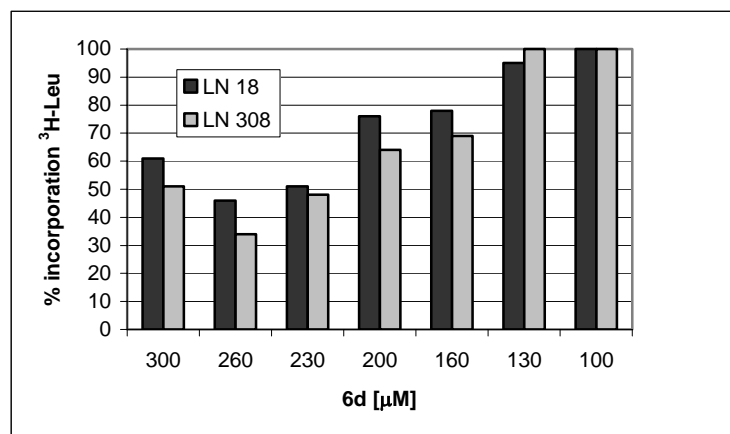


Figure 2B. Inhibition of protein synthesis by **6d** in human glioblastoma cells.

In summary, a series of new 2-benzylaminomethyl-*cis*-3,4-dihydropyrrolidines bearing aromatic and aliphatic amido side chains have been prepared. These compounds displayed moderate, but selective, inhibitory activities toward α -mannosidase from jack bean. The 3-bromobenzamide derivative (**6d**) is the most potent congener. It also inhibits the growth of human glioblastoma cells, more efficiently than swainsonine. Under the same conditions, our previously reported α -mannosidase inhibitor (**4**) did not induced glioblastoma cell death. The introduction of a lipophilic aromatic amide moiety probably increased the cellular uptake of the pyrrolidine derivative.

Relatively high concentrations (> 200 μ M) of inhibitor must be applied in order to achieve inhibition of glioblastoma cell growth. The bioavailability of this series of compounds should be optimized for the development of anticancer therapeutic agents. Studies are undergoing in our group to determine whether the introduction of peptide sequences would increase their internalization by cells.

EXPERIMENTAL

tert-Butyl ((1R)-2-amino-1-phenylethyl)carbamate (9). To a solution of **7**¹¹ (5.7 g, 24 mmol) in anhydrous CH₂Cl₂ (200 mL) were added, at 0°C, triethylamine (8.4 mL, 60 mmol) and methanesulfonyl chloride (2.3 mL, 28.8 mmol). The mixture was stirred for 3 h at 0°C and then poured into water (200 mL). The solution was extracted with CH₂Cl₂ (100 mL, 3 times). The combined organic extracts were dried (MgSO₄) and concentrated *in vacuo*. The residual oil was taken up in anhydrous THF (200 mL). Sodium azide (7.6 g, 116 mmol) was added and the mixture was stirred at 60°C for 14 h. The solution was poured into a sat. aq. solution of NaHCO₃ (60 mL) and extracted with EtOAc (40 mL, 3 times). The combined organic extracts were dried over MgSO₄ and concentrated *in vacuo*. Flash chromatography on silica gel (pentane / EtOAc 8:1) afforded **8** (4.4 g, 70%, 2 steps) as a white solid. A solution of **8** (2 g, 7.7 mmol) in anhydrous MeOH (50 mL) was stirred under 1 atmosphere of H₂, in the

presence of a catalytic amount of 10% Pd(OH)₂/C, for 7 h. The mixture was filtered over a pad of celite and concentrated *in vacuo*. Flash chromatography on silica gel (CH₂Cl₂ / MeOH 9:1) afforded **9** (1.1 g, 60%) as a white foam. $[\alpha]_{589}^{25} = -50$, $[\alpha]_{435}^{25} = -109$, $[\alpha]_{405}^{25} = -137$ ($c = 0.43$, CH₂Cl₂). UV (MeCN): 216 (3175). IR (film): 3580, 3430, 3055, 2980, 2530, 1705, 1495, 1455, 1390, 1365, 1265, 1170, 825 cm⁻¹. ¹H-NMR (400 MHz, CDCl₃): 7.34-7.23 (5d, 5H_{arom}) ; 4.67 (s, 1H) ; 2.98 (s, 2H) ; 2.22 (s, 2H) ; 1.41 (s, 9H). ¹³C-NMR (101 MHz, CDCl₃): 155.6, 140.5, 128.6, 127.3, 126.3, 79.5, 56.3, 46.8, 28.3. MALDI-TOF : 237 (M+H)⁺.

General procedure for the preparation of amide derivatives (6a-6p). To a solution of diamine (**9**) (0.45 to 0.65 mmol) in anhydrous CH₂Cl₂ (0.1 M) were added triethylamine (4 eq), an acyl chloride and a catalytic amount of DMAP (0.1 eq). The mixture was stirred at 25°C for 4 h and then poured into a sat. aq. solution of NaHCO₃ (10 mL). The aqueous layer was extracted with CH₂Cl₂ (10 mL, 3 times). The combined organic extracts were washed with a sat. aq. solution of NH₄Cl (10 mL), then brine (10 mL) and dried over MgSO₄ and concentrated *in vacuo*. The resulting crude amide was stirred for 1 h, at 25°C, in a 4:1 mixture of CF₃COOH and H₂O. The mixture was concentrated *in vacuo*. The residual oil was mixed with aldehyde (**11**)⁸(1eq) and NaBH(OAc)₃ (1.4 eq) in 1,2-dichloroethane. After stirring for 8 h at 25°C, the mixture was poured into a sat. aq. solution of NaHCO₃ (10 mL) and extracted with EtOAc (10 mL, 3 times). The combined organic extracts were dried over MgSO₄ and concentrated *in vacuo*. The resulting diamine was stirred for 7 h, at 25°C, in a 4:1 mixture of CF₃COOH and H₂O. The mixture was concentrated *in vacuo*. The residue was purified by flash chromatography on silica gel.

For the preparation of **6j**, the first step of the procedure was carried out as follow. A solution of coumarin 3-carboxylic acid (88 mg, 0.46 mmol) and PyBOP (1.2 eq, 287 mg, 0.55 mmol) in CH₂Cl₂ (2 mL) was added, at 0°C, to a solution of **9** (108 mg, 0.46 mmol) in CH₂Cl₂ (3 mL). Was added ⁱPr₂NEt and the temperature was raised to 25°C for 4 h. The mixture was poured into a sat. aq. solution of NaHCO₃ (10 mL) and extracted with CH₂Cl₂ (10 mL, 3 times). The combined organic extracts were washed with sat. NH₄Cl (10 mL), brine (10 mL) and then dried over MgSO₄ and concentrated *in vacuo*.

N-[(2R)-2-({[(2R,3R,4S)-3,4-dihydroxypyrrolidin-2-yl]methyl}amino)-2-phenylethyl]-2-bromobenzamide (6a) Yield 18%, 4 steps, pale yellow solid, mp 97-99 °C. $[\alpha]_{589}^{25} = +8$, $[\alpha]_{577}^{25} = +10$, $[\alpha]_{435}^{25} = +21$, $[\alpha]_{405}^{25} = +24$ ($c = 0.54$, MeOH). IR (film) : 3410, 3055, 2985, 2305, 1680, 1455, 1265, 1210, 1140, 1030, 800, 740, 705 cm⁻¹. ¹H-NMR (400 MHz, MeOD): 7.65 (d, 1H_{arom}, ³J = 9.0), 7.47-7.27 (m, 8H_{arom}), 4.26 (ddd, 1H, ³J = 2.3, 4.0, 4.0), 4.00-3.96 (m, 2H), 3.67 (dd, 1H, ³J = 6.6, ²J_{gem} = 13.6), 3.61 (dd, 1H, ³J = 6.7, ²J_{gem} = 13.6), 3.47-3.42 (m, 2H), 3.25 (dd, 1H, ³J = 2.3, ²J_{gem} = 12.4), 2.94 (dd, 1H, ³J = 4.2, ²J_{gem}

= 13.1), 2.75 (dd, 1H, $^3J = 9.1$, $^2J_{gem} = 13.1$). $^{13}\text{C-NMR}$ (101 MHz, MeOD): 163.3, 142.4, 139.7, 134.2, 132.3, 129.8, 129.7, 128.9, 128.6, 128.6, 119.5, 74.8, 71.2, 63.0, 62.8, 50.6, 47.5, 46.5. MALDI-TOF : 436 (M+2)⁺, 434 (M)⁺. MALDI-HRMS (M+H, C₂₀H₂₅N₃O₃Br): calcd. 434.1080, found 434.1079.

***N*-[(2*R*)-2-([(2*R*,3*R*,4*S*)-3,4-dihydroxypyrrolidin-2-yl]methyl)amino]-2-phenylethyl]-2-chloro-**

benzamide (6b) Yield 49%, 4 steps, pale yellow oil. $[\alpha]_{589}^{25} = +11$, $[\alpha]_{577}^{25} = +13$ ($c = 0.45$, MeOH). IR (film) : 3070, 2910, 2775, 2330, 1790, 1665, 1435, 1310, 1195, 1140, 840, 800, 750, 725 cm⁻¹. $^1\text{H-NMR}$ (400 MHz, MeOD): 7.48-7.32 (m, 9H_{arom}), 4.26 (m, 1H), 4.03-3.98 (m, 2H), 3.70 (dd, 1H, $^3J = 6.6$, $^2J_{gem} = 13.7$), 3.64 (dd, 1H, $^3J = 6.9$, $^2J_{gem} = 13.7$), 3.51-3.44 (m, 2H), 3.27 (dd, 1H, $^3J = 2.0$, $^2J_{gem} = 12.5$), 2.98 (dd, 1H, $^3J = 4.1$, $^2J_{gem} = 13.1$), 2.79 (dd, 1H, $^3J = 9.3$, $^2J_{gem} = 13.1$). $^{13}\text{C-NMR}$ (101 MHz, MeOD): 170.5, 139.7, 137.1, 132.3, 131.9, 131.0, 130.0, 129.9, 129.6, 129.0, 128.1, 74.9, 70.8, 63.9, 61.3, 50.9, 47.2, 45.4. MALDI-TOF : 413 (M+Na)⁺, 390 (M)⁺. MALDI-HRMS (M+H, C₂₀H₂₅N₃O₃Cl): calcd. 390.1585, found 390.1586.

***N*-[(2*R*)-2-([(2*R*,3*R*,4*S*)-3,4-dihydroxypyrrolidin-2-yl]methyl)amino]-2-phenylethyl]-2-methoxy-**

benzamide (6c) Yield 51%, 4 steps, colorless oil. $[\alpha]_{589}^{25} = +10$, $[\alpha]_{577}^{25} = +12$, $[\alpha]_{435}^{25} = +26$, $[\alpha]_{405}^{25} = +33$ ($c = 0.58$, MeOH). IR (film) : 3055, 2900, 2360, 1680, 1555, 1470, 1435, 1265, 1200, 1130, 840, 800, 740, 705 cm⁻¹. $^1\text{H-NMR}$ (400 MHz, MeOD): 7.94 (d, 1H_{arom}, $^3J = 7.8$), 7.54-7.42 (m, 6H_{arom}), 7.13-7.06 (m, 2H_{arom}), 4.24 (ddd, 1H, $^3J = 2.1$, 3.9, 3.9), 4.02-3.98 (m, 2H), 3.81 (s, 3H), 3.79 (dd, 1H, $^3J = 6.1$, $^2J_{gem} = 13.5$), 3.71 (dd, 1H, $^3J = 6.6$, $^2J_{gem} = 13.5$), 3.49 (ddd, 1H, $^3J = 3.9$, 9.0, 9.0), 3.46 (dd, 1H, $^3J = 3.9$, $^2J_{gem} = 12.4$), 3.27 (dd, 1H, $^3J = 2.1$, $^2J_{gem} = 12.4$), 3.01 (dd, 1H, $^3J = 3.9$, $^2J_{gem} = 13.2$), 2.82 (dd, 1H, $^3J = 9.0$, $^2J_{gem} = 13.2$). $^{13}\text{C-NMR}$ (101 MHz, MeOD): 168.6, 159.1, 142.2, 134.4, 132.1, 129.9, 129.0, 128.6, 122.5, 122.0, 112.9, 74.8, 71.1, 64.1, 62.5, 56.4, 50.6, 47.4, 46.1. MALDI-TOF : 424 (M+K)⁺, 408 (M+Na)⁺, 386 (M+H)⁺. MALDI-HRMS (M+H, C₂₁H₂₈N₃O₄), calcd. 386.2081, found 386.2079.

***N*-[(2*R*)-2-([(2*R*,3*R*,4*S*)-3,4-dihydroxypyrrolidin-2-yl]methyl)amino]-2-phenylethyl]-3-bromo-**

benzamide (6d) Yield 10%, 4 steps, white solid, mp 162-164 °C. $[\alpha]_{589}^{25} = +5$, $[\alpha]_{435}^{25} = +15$, $[\alpha]_{405}^{25} = +16$ ($c = 0.31$, MeOH). IR (film): 3615, 3580, 3425, 3055, 2360, 1675, 1465, 1265, 1190, 1140, 845, 815, 730 cm⁻¹. $^1\text{H-NMR}$ (400 MHz, MeOD): 7.93 (s, 1H_{arom}), 7.72 (dd, 2H_{arom}, $^3J = 8.0$, $^3J = 15.2$) ; 7.41-7.31 (m, 6H_{arom}) ; 4.25 (m, 1H), 3.99 (m, 2H), 3.67 (dd, 1H, $^3J = 7.1$, $^2J_{gem} = 13.5$), 3.59 (dd, 1H, $^3J = 7.1$, $^2J_{gem} = 13.5$), 3.51-3.43 (m, 2H), 3.28 (dd, 1H, $^3J = 2.1$, $^2J_{gem} = 12.5$), 2.96 (dd, 1H, $^3J = 4.1$, $^2J_{gem} = 13.2$), 2.73 (dd, 1H, $^3J = 9.1$, $^2J_{gem} = 13.2$). $^{13}\text{C-NMR}$ (101 MHz, MeOD): 169.0, 142.4, 137.7, 135.6, 131.4, 129.8, 128.9, 128.4, 127.1, 123.5, 74.7, 71.1, 64.1, 62.7, 50.6, 47.4, 46.8. MALDI-TOF: 436 (M+2)⁺, 434 (M)⁺. MALDI-HRMS (M+H, C₂₀H₂₅N₃O₃Br): calcd. 434.1080, found 434.1078.

***N*-[(2*R*)-2-({[(2*R*,3*R*,4*S*)-3,4-dihydroxypyrrolidin-2-yl]methyl}amino)-2-phenylethyl]-3-fluoro-**

benzamide (6e) Yield 57%, 4 steps, white solid, mp 85-87 °C. $[\alpha]_{577}^{25} = +10$, $[\alpha]_{435}^{25} = +23$, $[\alpha]_{405}^{25} = +32$ ($c = 0.35$, MeOH). IR (film) : 3580, 3350, 3060, 2360, 2340, 1700, 1555, 1455, 1205, 1140, 1020, 845, 800, 725 cm^{-1} . $^1\text{H-NMR}$ (400 MHz, MeOD): 7.61-7.59 (m, 1 H_{arom}), 7.54-7.28 (m, 8 H_{arom}), 4.25 (ddd, 1H, $^3J = 1.8, 3.9, 3.9$), 4.18 (m, 1H), 4.00 (dd, 1H, $^3J = 3.9, 8.4$), 3.77 (dd, 1H, $^3J = 6.7, ^2J_{\text{gem}} = 14.2$), 3.72 (dd, 1H, $^3J = 6.1, ^2J_{\text{gem}} = 14.2$), 3.56 (ddd, 1H, $^3J = 3.8, 8.4, 8.4$), 3.49 (dd, 1H, $^3J = 3.9, ^2J_{\text{gem}} = 12.5$), 3.30 (dd, 1H, $^3J = 1.8, ^2J_{\text{gem}} = 12.5$), 3.09 (dd, 1H, $^3J = 3.8, ^2J_{\text{gem}} = 12.8$), 3.01 (dd, 1H, $^3J = 8.4, ^2J_{\text{gem}} = 12.8$). $^{13}\text{C-NMR}$ (101 MHz, MeOD): 165.3, 147.8, 140.3, 137.6, 131.6, 130.1, 129.5, 128.7, 124.2, 119.7, 115.4, 75.0, 70.8, 64.2, 61.5, 50.9, 47.8, 45.9. MALDI-TOF : 374 (M+H) $^+$. MALDI-HRMS (M+H, $\text{C}_{20}\text{H}_{25}\text{N}_3\text{O}_3\text{F}$): calcd. 374.1881, found 374.1883.

***N*-[(2*R*)-2-({[(2*R*,3*R*,4*S*)-3,4-dihydroxypyrrolidin-2-yl]methyl}amino)-2-phenylethyl]-4-bromo-**

benzamide (6f) Yield 16%, 4 steps, colorless oil. $[\alpha]_{589}^{25} = +5$, $[\alpha]_{577}^{25} = +6$ ($c = 0.61$, MeOH). IR (film) : 3585, 3055, 2985, 2355, 1650, 1435, 1265, 1205, 1140, 1075, 1010, 800, 740, 705 cm^{-1} . $^1\text{H-NMR}$ (400 MHz, MeOD): 7.69-7.63 (m, 4 H_{arom}), 7.41-7.32 (m, 5 H_{arom}), 4.24 (ddd, 1H, $^3J = 2.0, 4.1, 4.1$), 4.00-3.95 (m, 2H), 3.69 (dd, 1H, $^3J = 6.6, ^2J_{\text{gem}} = 13.5$), 3.58 (dd, 1H, $^3J = 6.4, ^2J_{\text{gem}} = 13.5$), 3.48-3.41 (m, 2H), 3.26 (dd, 1H, $^3J = 2.0, ^2J_{\text{gem}} = 12.5$), 2.96 (dd, 1H, $^3J = 4.1, ^2J_{\text{gem}} = 13.2$), 2.72 (dd, 1H, $^3J = 9.2, ^2J_{\text{gem}} = 13.2$). $^{13}\text{C-NMR}$ (101 MHz, MeOD): 169.5, 142.5, 134.6, 132.8, 130.1, 129.8, 128.8, 128.4, 127.1, 74.7, 71.1, 64.1, 62.7, 50.6, 47.4, 47.0. MALDI-TOF : 459 (M+2+Na) $^+$, 457 (M+Na) $^+$, 436 (M+2) $^+$, 434 (M) $^+$. MALDI-HRMS (M+H, $\text{C}_{20}\text{H}_{25}\text{N}_3\text{O}_3\text{Br}$): calcd. 434.1080, found 434.1079.

***N*-[(2*R*)-2-({[(2*R*,3*R*,4*S*)-3,4-dihydroxypyrrolidin-2-yl]methyl}amino)-2-phenylethyl]-4-chloro-**

benzamide (6g) Yield 42%, 4 steps, white solid, mp 87-89 °C. $[\alpha]_{589}^{25} = +10$, $[\alpha]_{577}^{25} = +15$, $[\alpha]_{435}^{25} = +31$, $[\alpha]_{405}^{25} = +48$ ($c = 0.54$, MeOH). IR (film) : 3585, 3200, 3055, 2900, 2360, 1650, 1470, 1440, 1265, 1200, 1150, 840, 800, 740 cm^{-1} . $^1\text{H-NMR}$ (400 MHz, MeOD): 8.60 (d, 2 H_{arom} , $^3J = 6.7$), 8.24 (d, 2 H_{arom} , $^3J = 6.8$), 8.18-8.07 (m, 5 H_{arom}), 4.40 (ddd, 1H, $^3J = 2.0, 4.0, 4.0$), 4.17 (dd, 1H, $^3J = 6.9, 6.2$), 4.10 (dd, 1H, $^3J = 4.0, 8.5$), 3.74 (dd, 1H, $^3J = 6.9, ^2J_{\text{gem}} = 13.6$), 3.69 (dd, 1H, $^3J = 6.2, ^2J_{\text{gem}} = 13.6$), 3.54 (ddd, 1H, $^3J = 8.5, 9.0, 4.0$), 3.49 (dd, 1H, $^3J = 4.0, ^2J_{\text{gem}} = 12.5$), 3.27 (dd, 1H, $^3J = 2.0, ^2J_{\text{gem}} = 12.5$), 2.93 (dd, 1H, $^3J = 4.0, ^2J_{\text{gem}} = 13.1$), 2.70 (dd, 1H, $^3J = 9.0, ^2J_{\text{gem}} = 13.1$). $^{13}\text{C-NMR}$ (101 MHz, MeOD): 169.5, 141.9, 138.8, 134.1, 130.1, 129.9, 129.7, 129.0, 128.5, 74.8, 71.0, 64.2, 62.3, 50.7, 47.4, 46.7. MALDI-TOF : 412 (M+Na) $^+$, 390 (M+H) $^+$. MALDI-HRMS (M+H, $\text{C}_{20}\text{H}_{25}\text{N}_3\text{O}_3\text{Cl}$): calcd. 390.1585, found 390.1583.

***N*-[*(2R)*-2-({[*(2R,3R,4S)*-3,4-dihydroxypyrrolidin-2-yl]methyl)amino]-2-phenylethyl]-4-fluoro-**

benzamide (6h) Yield 50%, 4 steps, pale yellow solid, mp 93-95 °C. $[\alpha]_{589}^{25} = +8$, $[\alpha]_{577}^{25} = +10$, $[\alpha]_{435}^{25} = +17$, $[\alpha]_{405}^{25} = +20$ ($c = 0.72$, MeOH). IR (film) : 3585, 3350, 3055, 2985, 2360, 1675, 1505, 1455, 1265, 1205, 1140, 850, 800, 740 cm^{-1} . $^1\text{H-NMR}$ (400 MHz, MeOD): 7.88-7.85 (m, 2 H_{arom}), 7.55-7.47 (m, 5 H_{arom}), 7.21-7.16 (m, 2 H_{arom}), 4.50 (m, 1H), 4.26 (m, 1H), 4.05 (dd, 1H, $^3J = 3.9, 9.0$), 3.95 (dd, 1H, $^3J = 5.8, ^2J_{\text{gem}} = 14.4$), 3.90 (dd, 1H, $^3J = 6.4, ^2J_{\text{gem}} = 14.4$), 3.74 (ddd, 1H, $^3J = 3.7, 9.0, 9.0$), 3.56 (dd, 1H, $^3J = 3.9, ^2J_{\text{gem}} = 12.7$), 3.47 (m, 1H), 3.36 (dd, 1H, $^3J = 1.3, ^2J_{\text{gem}} = 12.7$), 2.82 (dd, 1H, $^3J = 3.5, ^2J_{\text{gem}} = 13.5$). $^{13}\text{C-NMR}$ (101 MHz, MeOD): 170.2, 167.6, 165.2, 136.7, 131.1, 130.4, 129.1, 116.5, 116.3, 75.3, 70.3, 64.5, 59.4, 51.4, 47.1, 44.4. MALDI-TOF : 374 (M+H) $^+$. MALDI-HRMS (M+H, $\text{C}_{20}\text{H}_{25}\text{N}_3\text{O}_3\text{F}$): calcd. 374.1881, found 374.1879.

***N*-[*(2R)*-2-({[*(2R,3R,4S)*-3,4-dihydroxypyrrolidin-2-yl]methyl)amino]-2-phenylethyl]-2,4,6-trichloro-**

benzamide (6i) Yield 46%, 4 steps, white solid, mp 68-71 °C. $[\alpha]_{589}^{25} = +17$, $[\alpha]_{577}^{25} = +19$, $[\alpha]_{435}^{25} = +37$ ($c = 0.61$, MeOH). IR (film) : 3585, 3055, 2360, 1670, 1650, 1580, 1435, 1265, 1200, 1135, 840, 800, 740, 705 cm^{-1} . $^1\text{H-NMR}$ (400 MHz, MeOD): 7.53-7.33 (m, 7 H_{arom}), 4.27 (ddd, 1H, $^3J = 2.2, 4.0, 4.0$), 4.01 (m, 1H), 4.00 (dd, 1H, $^3J = 4.0, 8.0$), 3.75 (dd, 1H, $^3J = 5.8, ^2J_{\text{gem}} = 13.5$), 3.69 (dd, 1H, $^3J = 7.2, ^2J_{\text{gem}} = 13.5$), 3.50 (m, 1H), 3.48 (dd, 1H, $^3J = 4.0, ^2J_{\text{gem}} = 12.5$), 3.28 (dd, 1H, $^3J = 2.2, ^2J_{\text{gem}} = 12.5$), 2.97 (dd, 1H, $^3J = 4.2, ^2J_{\text{gem}} = 13.1$), 2.90 (dd, 1H, $^3J = 9.1, ^2J_{\text{gem}} = 13.1$). $^{13}\text{C-NMR}$ (101 MHz, MeOD): 166.8, 141.6, 136.9, 136.0, 133.9, 129.9, 129.2, 129.1, 128.8, 74.7, 71.0, 63.9, 62.4, 50.7, 47.2, 46.1. MALDI-TOF : 481 (M+Na) $^+$, 458 (M) $^+$. MALDI-HRMS (M+H, $\text{C}_{20}\text{H}_{23}\text{N}_3\text{O}_3\text{Cl}_3$): calcd. 458.0806, found 458.0803.

***N*-[*(2R)*-2-({[*(2R,3R,4S)*-3,4-dihydroxypyrrolidin-2-yl]methyl)amino]-2-phenylethyl]-2-oxo-2*H*-chro-**

mene-3-carboxamide (6j) Yield 49%, 4 steps, white solid, mp 105-107 °C. $[\alpha]_{577}^{25} = +2$, $[\alpha]_{435}^{25} = +10$, $[\alpha]_{405}^{25} = +16$ ($c = 0.52$, MeOH). IR (film) : 3585, 3055, 2915, 2350, 1680, 1650, 1440, 1265, 1210, 1180, 1145, 845, 800, 740 cm^{-1} . $^1\text{H-NMR}$ (400 MHz, MeOD): 8.93 (s, 1H), 7.90 (m, 1 H_{arom}), 7.80 (m, 1 H_{arom}), 7.51-7.34 (m, 7 H_{arom}), 4.29 (m, 1H), 4.03 (dd, 1H, $^3J = 3.9, 8.1$), 3.91 (dd, 1H, $^3J = 5.0, 8.7$), 3.83 (dd, 1H, $^3J = 5.0, ^2J_{\text{gem}} = 13.6$), 3.61-4.49 (m, 3H), 3.35 (m, 1H), 3.04 (dd, 1H, $^3J = 3.7, ^2J_{\text{gem}} = 13.2$), 2.65 (dd, 1H, $^3J = 9.4, ^2J_{\text{gem}} = 13.2$). $^{13}\text{C-NMR}$ (101 MHz, MeOD): 163.8, 162.6, 155.9, 149.5, 141.8, 135.7, 131.3, 130.0, 129.9, 129.0, 128.1, 126.6, 120.0, 117.5, 74.5, 71.2, 64.2, 62.7, 50.8, 47.1, 46.5. MALDI-TOF : 446 (M+Na) $^+$, 424 (M+1) $^+$. MALDI-HRMS (M+H, $\text{C}_{23}\text{H}_{26}\text{N}_3\text{O}_5$): calcd. 424.1873, found 424.1870.

***N*-[(2*R*)-2-([(2*R*,3*R*,4*S*)-3,4-dihydroxypyrrolidin-2-yl]methyl)amino]-2-phenylethyl] acetamide (6k)**

Yield 17%, 4 steps, colorless oil. $[\alpha]_{589}^{25} = +7$ ($c = 0.50$, MeOH). IR (film) : 3580, 3270, 3055, 2985, 2355, 1650, 1420, 1265, 1115, 735, 705 cm^{-1} . $^1\text{H-NMR}$ (400 MHz, MeOD): 7.42-7.30 (m, 5 H_{arom}), 4.22 (m, 1H), 3.91 (dd, 1H, $^3J = 4.2$, 7.8), 3.81 (t, 1H, $^3J = 6.6$), 3.41 (ddd, 1H, $^3J = 6.9$, 13.5, 13.5), 3.39-3.37 (m, 3H), 3.19 (dd, 1H, $^3J = 2.1$, $^2J_{\text{gem}} = 12.4$), 2.87 (dd, 1H, $^3J = 4.3$, $^2J_{\text{gem}} = 12.9$), 2.66 (dd, 1H, $^3J = 8.9$, $^2J_{\text{gem}} = 12.9$), 1.94 (s, 3H). $^{13}\text{C-NMR}$ (101 MHz, MeOD): 179.5, 142.6, 129.8, 128.7, 128.3, 74.9, 71.2, 64.0, 62.7, 50.6, 47.6, 46.7, 22.6. MALDI-TOF : 316 ($\text{M}+\text{Na}$)⁺, 294 ($\text{M}+\text{H}$)⁺. MALDI-HRMS ($\text{M}+\text{H}$, $\text{C}_{15}\text{H}_{24}\text{N}_3\text{O}_3$): calcd. 294.1818, found 294.1817.

***N*-[(2*R*)-2-([(2*R*,3*R*,4*S*)-3,4-dihydroxypyrrolidin-2-yl]methyl)amino]-2-phenylethyl] propanamide (6l)**

Yield 37%, 4 steps, colorless oil. $[\alpha]_{589}^{25} = +11$, $[\alpha]_{577}^{25} = +13$, $[\alpha]_{435}^{25} = +25$, $[\alpha]_{405}^{25} = +31$ ($c = 0.42$, MeOH). IR (film) : 3305, 3055, 2985, 2355, 1650, 1555, 1455, 1265, 1205, 1140, 800, 740, 705 cm^{-1} . $^1\text{H-NMR}$ (400 MHz, MeOD): 7.42-7.30 (m, 5 H_{arom}), 4.25 (m, 1H), 3.95 (dd, 1H, $^3J = 4.0$, 7.8), 3.83 (t, 1H, $^3J = 6.5$), 3.45 (ddd, 1H, $^3J = 6.8$, 13.4, 13.4), 3.44-3.40 (m, 3H), 3.25 (dd, 1H, $^3J = 2.1$, $^2J_{\text{gem}} = 12.5$), 2.90 (dd, 1H, $^3J = 4.2$, $^2J_{\text{gem}} = 13.1$), 2.69 (dd, 1H, $^3J = 9.2$, $^2J_{\text{gem}} = 13.1$), 2.19 (q, 2H, $^3J = 7.6$), 1.10 (t, 3H, $^3J = 7.6$). $^{13}\text{C-NMR}$ (101 MHz, MeOD): 177.4, 142.5, 129.7, 128.7, 128.4, 74.7, 71.1, 64.0, 62.8, 50.6, 47.4, 46.3, 30.1, 10.5. MALDI-TOF : 330 ($\text{M}+\text{Na}$)⁺, 308 ($\text{M}+\text{H}$)⁺. MALDI-HRMS ($\text{M}+\text{H}$, $\text{C}_{16}\text{H}_{26}\text{N}_3\text{O}_3$): calcd. 308.1975, found 308.1976.

***N*-[(2*R*)-2-([(2*R*,3*R*,4*S*)-3,4-dihydroxypyrrolidin-2-yl]methyl)amino]-2-phenylethyl] butanamide (6m)**

Yield 50%, 4 steps, pale pink oil. $[\alpha]_{589}^{25} = +18$, $[\alpha]_{577}^{25} = +20$, $[\alpha]_{435}^{25} = +47$, $[\alpha]_{405}^{25} = +49$ ($c = 0.42$, MeOH). IR (film) : 3585, 3055, 2985, 2360, 1650, 1555, 1455, 1265, 1205, 1145, 840, 800, 725, 705 cm^{-1} . $^1\text{H-NMR}$ (400 MHz, MeOD): 7.42-7.33 (m, 5 H_{arom}), 4.26 (m, 1H), 3.96 (dd, 1H, $^3J = 3.9$, 7.9), 3.85 (t, 1H, $^3J = 6.3$), 3.51 (ddd, 1H, $^3J = 6.2$, 13.5, 13.5), 3.47-3.39 (m, 3H), 3.27 (dd, 1H, $^3J = 2.0$, $^2J_{\text{gem}} = 12.5$), 2.94 (dd, 1H, $^3J = 4.1$, $^2J_{\text{gem}} = 13.2$), 2.73 (dd, 1H, $^3J = 9.5$, $^2J_{\text{gem}} = 13.2$), 2.14 (t, 2H, $^3J = 7.4$), 1.60 (qt, 2H, $^3J = 7.4$, 7.4), 0.92 (t, 3H, $^3J = 7.4$). $^{13}\text{C-NMR}$ (101 MHz, MeOD): 176.7, 141.8, 129.8, 129.0, 128.5, 74.8, 71.0, 64.1, 62.4, 50.6, 47.3, 45.9, 38.9, 20.3, 14.0. MALDI-TOF : 344 ($\text{M}+\text{Na}$)⁺, 322 ($\text{M}+\text{H}$)⁺. MALDI-HRMS ($\text{M}+\text{H}$, $\text{C}_{17}\text{H}_{28}\text{N}_3\text{O}_3$): calcd. 322.2131, found 322.2132.

***N*-[(2*R*)-2-([(2*R*,3*R*,4*S*)-3,4-dihydroxypyrrolidin-2-yl]methyl)amino]-2-phenylethyl] pentanamide (6n)**

Yield 61%, 4 steps, colorless oil. $[\alpha]_{589}^{25} = +13$, $[\alpha]_{577}^{25} = +14$, $[\alpha]_{435}^{25} = +25$, $[\alpha]_{405}^{25} = +35$ ($c = 0.62$, MeOH). IR (film) : 3580, 3055, 2915, 2770, 2300, 1650, 1560, 1435, 1265, 1200, 1130, 845, 740, 705 cm^{-1} . $^1\text{H-NMR}$ (400 MHz, MeOD): 7.42-7.30 (m, 5 H_{arom}), 4.26 (m, 1H), 3.96 (dd, 1H, $^3J = 3.9$, 7.8), 3.81

(t, 1H, $^3J = 6.5$), 3.49 (ddd, 1H, $^3J = 6.4$, 13.5, 13.5), 3.47-3.39 (m, 3H), 3.27 (dd, 1H, $^3J = 1.9$, $^2J_{gem} = 12.5$), 2.92 (dd, 1H, $^3J = 4.1$, $^2J_{gem} = 13.1$), 2.70 (dd, 1H, $^3J = 9.4$, $^2J_{gem} = 13.1$), 2.16 (t, 2H, $^3J = 7.4$), 1.55 (tt, 2H, $^3J = 7.4$, 7.4), 1.31 (qt, 2H, $^3J = 7.4$, 7.4), 0.93 (t, 3H, $^3J = 7.4$). $^{13}\text{C-NMR}$ (101 MHz, MeOD): 176.8, 142.1, 129.8, 128.9, 128.5, 74.7, 71.1, 64.0, 62.5, 50.6, 47.3, 46.1, 36.8, 29.1, 23.3, 14.1. MALDI-TOF : 358 (M+Na) $^+$, 336 (M+H) $^+$. MALDI-HRMS (M+H, C₁₈H₃₀N₃O₃): calcd. 336.2288, found 336.2290.

***N*-[(2*R*)-2-([(2*R*,3*R*,4*S*)-3,4-dihydroxypyrrolidin-2-yl]methyl)amino]-2-phenylethyl]-2-methylpropanamide (6o)** Yield 10%, 4 steps, colorless oil. $[\alpha]_{589}^{25} = +16$ ($c = 0.50$, MeOH). IR (film) : 3580, 3260, 3050, 2985, 2350, 1650, 1550, 1410, 1265, 1185, 1145, 895, 740 cm⁻¹. $^1\text{H-NMR}$ (400 MHz, MeOD): 7.40-7.29 (m, 5H_{arom}), 4.27 (m, 1H), 3.98 (dd, 1H, $^3J = 4.0$, 7.9), 3.88 (t, 1H, $^3J = 6.6$), 3.52-3.44 (m, 4H), 3.27 (dd, 1H, $^3J = 2.0$, $^2J_{gem} = 12.5$), 2.94 (dd, 1H, $^3J = 4.0$, $^2J_{gem} = 13.1$), 2.75 (dd, 1H, $^3J = 9.4$, $^2J_{gem} = 13.1$), 2.44 (m, 1H), 1.08 (d, 3H, $^3J = 6.8$), 1.07 (d, 3H, $^3J = 6.8$). $^{13}\text{C-NMR}$ (101 MHz, MeOD): 180.6, 142.4, 129.7, 128.8, 128.5, 74.7, 71.1, 64.0, 62.8, 50.6, 47.4, 46.0, 36.2, 19.9, 19.8. MALDI-TOF : 345 (M+Na) $^+$, 322 (M+H) $^+$. MALDI-HRMS (M+H, C₁₇H₂₈N₃O₃): calcd. 322.2131, found 322.2129.

***N*-[(2*R*)-2-([(2*R*,3*R*,4*S*)-3,4-dihydroxypyrrolidin-2-yl]methyl)amino]-2-phenylethyl]-3-methylbutanamide (6p)** Yield 33%, 4 steps, colorless oil. $[\alpha]_{577}^{25} = +12$, $[\alpha]_{435}^{25} = +26$, $[\alpha]_{405}^{25} = +31$ ($c = 0.46$, MeOH). IR (film) : 3200, 3055, 2900, 2360, 1650, 1555, 1470, 1265, 1180, 895, 840, 800, 740, 705 cm⁻¹. $^1\text{H-NMR}$ (400 MHz, MeOD): 7.42-7.30 (m, 5H_{arom}), 4.26 (m, 1H), 3.96 (dd, 1H, $^3J = 4.0$, 7.7), 3.82 (t, 1H, $^3J = 6.5$), 3.52 (ddd, 1H, $^3J = 6.1$, 13.5, 13.5), 3.46-3.40 (m, 3H), 3.28 (dd, 1H, $^3J = 2.0$, $^2J_{gem} = 12.5$), 2.91 (dd, 1H, $^3J = 4.1$, $^2J_{gem} = 13.1$), 2.69 (dd, 1H, $^3J = 9.3$, $^2J_{gem} = 13.1$), 2.02 (m, 3H), 0.92 (d, 3H, $^3J = 6.1$), 0.90 (d, 3H, $^3J = 6.1$). $^{13}\text{C-NMR}$ (101 MHz, MeOD): 176.1, 141.9, 129.8, 128.9, 128.5, 74.7, 71.0, 64.1, 62.4, 50.6, 47.3, 46.2, 46.0, 27.3, 22.7. MALDI-TOF : 336 (M+H) $^+$. MALDI-HRMS (M+H, C₁₈H₃₀N₃O₃): calcd. 336.2288, found 336.2286.

Inhibition of commercially available plant glycosidases The experiments were performed essentially as previously described.⁹ Briefly, 0.01 to 0.5 units/mL of enzyme (1 unit = 1 μmol of glycoside hydrolyzed/min) were preincubated for 5 min at 20°C with the inhibitor, and increasing concentration of aqueous solution of the appropriate *p*-nitrophenyl glycoside substrates buffered to the optimum pH of the enzymes were incubated for 20 min at 37°C (45°C for the amyloglucosidases). The reaction was stopped by addition of 2.5 volume of 0.2 M sodium borate buffer pH 9.8. The *p*-nitrophenolate formed was quantified at 410 nm and IC₅₀ were calculated or double-reciprocal (Lineweaver and Burk) plots were used to determine the inhibition characteristics.

Evaluation of cell proliferation by the MTT assay MTT ((3,4,5-dimethylthiazol-yl)-2,5-diphenyl tetrazolium, Sigma, Buchs, Switzerland) was used to quantify the number of metabolically active cells. Following treatment with pyrrolidine derivatives, the cells were exposed to 0.2 mg/mL MTT in DMEM medium for 2 h, the supernatant was aspirated, and the precipitated formazan was dissolved in 0.1 N HCl in 2-propanol and quantified at 540 nm in a multiwell plate reader (iEMS Reader MF, Labsystems, Bioconcepts, Switzerland).

Evaluation of the inhibition of DNA and protein synthesis Thymidine and leucine incorporation were used to assess DNA and protein synthesis, respectively. [³H]-Thymidine (Amersham-Pharmacia, Dübendorf, Switzerland, 400 nCi/well) or [³H]-Leucine (American Radiolabeled Chemicals, St-Louis MO, USA, 400 nCi/well) were added to treated or control cells for the last 2 h of incubation with the pyrrolidine derivatives. The cell layer was washed, precipitated with 10% trichloroacetic acid and the precipitate was dissolved in 1% SDS in 0.1 N NaOH, then 5 ml of scintillation cocktail (Optiphase, Wallac, Regensdorf, Switzerland) was added, and the radioactivity was counted in a β -counter (WinSpectral, Wallac).

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