SYNTHESIS OF 3-AMINOMETHYLGLAUCINE DERIVATIVES AND *IN VITRO* EVALUATION OF THEIR ANTI-TYROSINASE, ANTIVIRAL AND RADICAL SCAVENGING ACTIVITIES

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ABSTRACT

A series of amides was synthesized by coupling of 3-aminomethylglaucine with various amino acid analogues. The reaction was carried out under mild conditions by using various carbodiimides and in the presence of catalytic amount of N-hydroxybenzotriazole. Five of the compounds obtained were new, and their spectral data are presented and discussed here. Selected amides were tested for their ability to scavenge the stable free 1,1-diphenyl-2-picryl-hydrazyl (DPPH•) radical. The most active compounds were N-sinapoylphenylalanyl-3-aminomethylglaucine amide and N-feruloylphenylalanyl-3-aminomethylglaucine amide and their activity was due to the presence of hydroxycinnamoyl moiety in their molecules. The compounds were further tested in vitro for antiviral activity against viruses belonging to the Picornaviridae family.

Furthermore, anti-tyrosinase activity of the compounds was performed in vitro. The results obtained suggested that the most promising tyrosinase inhibitor was feruloylphenylalanyl-3-aminomethylglaucine.

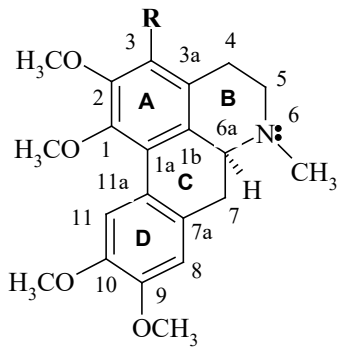
Keywords: glaucine, 3-aminomethylglaucine, N_{α}-protected amino acid amides of glaucine, antioxidant effect, tyrosinase activity, DPPH• test.

INTRODUCTION

An isoquinoline nucleus has been found in numerous native bioactive compounds. Due to the significance of this heterocyclic moiety in drugs and medicinal chemistry, the isolation and the functionalization of similar compounds continue to be a very attractive field of research.

Aporphines are well-known group of naturally occurring isoquinoline alkaloids. Their structure is based on the 4Hdibenzo [de,g]quinoline skeleton. Within this group of compounds, the ttention is focused on (+) glaucine [(*S*)-1,2,9,10-tetramethoxyaporphine], which is widely distributed extending from the Black Sea area to southern, western and north-western Europe [1].

(+) Glaucine (**1**, Fig.1) is the major alkaloid isolated by Ivanov & Ivanova [2] from the above-ground parts of *Glaucium flavum* Crantz (Papaveraceae). It is also present in various plant species such as *Glaucium oxylobum* [3], *Croton lechleri* [4] and *Corydalis yanhusuo* [5]. This alkaloid displays a broad range of biological effects, such as bronchodilating, anti-inflammatory, antinociceptive, PDE4 inhibitory and calcium channel blocking ones [6 - 12]. *Glaucine flavum* Cr. is used in traditional medicine as an antidiabetic agent [13 - 15]. Moreover, the effects of the aqueous extracts from different parts of *G. flavum* Cr. reveal that *G. flavurn* leaves have the most potent hypoglycemic *in vivo* activity [16, 17].



(**1**) glaucine, R=H;
(**2**) 3-aminoglaucine, R=NH_2;
(**3**) **AmGla**; 3-aminomethylglaucine, R=CH_2NH_2

Fig. 1. Chemical structures of glaucine and its derivatives.

There is literature data describing that glaucine is also used as a cough suppressant in Eastern Europe, including Bulgaria [18, 19].

Our previous results reveal the antiviral activity of some molecular hybrids of cinnamic acids and glaucine, especially against the replication of rhinovirus 14 (HRV-14) [20]. Data also exists referring to the antiviral effects of several aporphine alkaloids and their derivatives as isoboldine, oxoglaucine, 3-hydroxyglaucine and dehydroglaucine [21, 22].

Glaucine and its aporphinic analogue, e.g. boldine (1,10-dimethoxy-2,9-dihydroxyaporphine) are known to exhibit antioxidant and photoprotective properties that may be attributed to the above-mentioned activities [23, 24]. In contrast to diphenolic boldine, glaucine has no phenolic groups, therefore its antioxidant effect is mainly due to the presence of the biphenyl system. Indeed, in nature, glaucine is easily oxidized to 6a, 7-dehydroglaucine and oxoglaucine by photochemical and enzymatic processes. As mentioned in the literature, the benzylic C-6a-H bond is the initial point of the free radical attack. The resulting radicals are stabilized by extended delocalization beyond the biphenyl nucleus and the nitrogen electron pair [25].

Moreover, it is known that compounds of an antioxidant activity often reveal a whitening effect. The resemblance in the antioxidant properties of glaucine and its diphenolic analogue-boldine (and the determined melanin inhibition of the latter) provides to assume a potential tyrosinase inhibition effect of the glaucine derivatives [26].

The molecule of glaucine is modified in the course of the investigation reported continuing the previous synthetic work on natural compounds functionalization. The aim is to obtain its amide derivatives with various amino acid analogues and hybrids with hydroxycinnamic acids. Furthermore, the synthesized amides are screened for their potential radical scavenging as wel as their anti-viral and tyrosinase inhibitory activities.

EXPERIMENTAL

Materials

3-Aminomethylglaucine (AmGla) was synthesized from glaucine by following the procedure described in ref. [27]. With the only exception of Boc-Phe (3-F)-OH which was synthesized according to the Pozdnev's method [28], the other Boc-amino acids were purchased from Reanal, while the Fmoc-amino acids were provided by Novabiochem (Läufelfingen, Switzerland). HOBt, EDC (N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride) were purchased from Merck, while DIC (N,N′-Diisopropylcarbodiimide) was a Fluka product. The standard antioxidants used, as quercetin, tocopherol, isoeugenol, eugenol and DPPH• (1,1-Diphenyl-2-picrylhydrazyl radical), DOPA (3,4-Dihydroxy-L-phenylalanine Sigma-Aldrich were obtained from Sigma Chemical Co. US.

Preparative TLC was performed on a Merck Kiesegel 60 F_{254} plates. The spots were visualized by UV light and by spraying with the Dragendorff's reagent.

Analitical Methods

^1H and ^{13}C NMR spectra were recorded on a Bruker Avance II+ 600 and a Bruker Avance DRX-250 MHz spectrometers operating at 600 MHz and 250.13 MHz for ^1H. The NMR spectra of the synthesized amides were obtained in the course of measurements in $CDCl_3$ solutions. They were carried out at an ambient temperature (300 K) and tetramethylsilane (TMS) was used as an internal standard.

Agilent 8453 UV-Visible spectrophotometer was used to record the UV absorbance, for the measurement of the reduction of DPPH• (1,1-diphenyl-2-picrylhydrazyl radical) absorbance at 516 nm and for determination of absorbance of DOPAchrome (2-carboxy-2,3-dihydroindole-5,6-quinone) at 475 nm (ε 3600 M^{-1} cm^{-1}). ESI-MS spectra were obtained on Esquire 3000 plus.

Cells and viruses

The experiments referring to testing the antienteroviral activity of the newly synthesized compounds were carried out with poliovirus type 1 [PV-1 (LSc-2ab)] and human rhinovirus 14 (HRV-14) from the collection of The Stephan Angeloff Institute of Microbiology at the Bulgarian Academy of Sciences, Sofia, Bulgaria. The viruses were grown in FL and MRC-5 cells, respectively. The cells were grown in a humidified atmosphere at 37°C and 5 % CO_2 in a growth medium of Dulbecco modified Eagle's medium (DMEM) (Gibco®, Invitrogen) containing 5 % foetal bovine serum (Gibco®, Invitrogen) supplemented with antibiotics (penicillin 100 IU/mL, streptomycin 100 μg/mL and gentamycin 50 μg/mL) and 20 mM HEPES buffer (Gibco®, Invitrogen). The serum in the maintenance medium was reduced to 0.5 % for the antiviral assays. HRV-14 was grown at 33°C.

A general procedure for the synthesis of 3 – amino-methylglaucine with amino acid derivatives using EDC (DIC)/ HOBt method (4-14)

A mixture of the corresponding amino acid derivative (0.39 mM) and 0.093 g (0.39 mM) HOBt in 2 ml CH_2Cl_2 was stirred and 0.39 mM EDC (DIC) was added at 0 °C. After 5 min 0.150 g (0.39 mM) 3-aminomethylglaucine and 0.04 ml (0.39 mM) NMM were added to the reaction mixture. The stirring was continued for an hour at 0 °C and for further 21 h at a room temperature. The progress of the reaction was monitored by TLC (PE/$CHCl_3$/CH_3COCH_3/CH_3OH = 4:4:1:1 or 4:8:1:2). After the reaction completion the solvents were evaporated *in vacuo.* A mixture of CH_2Cl_2 and water was added and the organic phase extracted with 5 % $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness under reduced pressure. The residue was purified by preparative TLC on Kieselgel 60 F_{254} using a solvent system of PE/$CHCl_3$/CH_3COCH_3/CH_3OH = 4:8:1:2 or 4:4:1:1).

The identification of the compounds is listed as follows: ***Boc-AA-CONH-CH$_2$- glaucine*** **(4-9)**.

Fmoc-Glu(OBu^t)- CO NH-CH$_2$- glaucine **(10)**: ^{1}H NMR (250.13 MHz, $CDCl_3$, δ/ppm): 1.49 (9H, s, 3 × CH_3), 1.82 - 2.01(2H, m, CH_2), 2.30 (2H, m, CH_2), 2.67 (3H, s, NCH_3), 2.90-3.12 (6H, m, 3 x CH_2), 3.67 (3H, s, OCH_3, (C-1)), 3.89 (1H, s, H-6a), 3.94 (9H, s, 3 x OCH_3), 4.14-4.23 (3H, m, -CH- + -OCH_2), 4.33 (1H, m, CH), 4.42-4.50 (1H, m, CH), 4.51 (2H, m, -CH_2NH-), 5.28 (1H, d, -NH-), 6.77 (1H, s, Ar-H (H-8)), 7.29 (2H, t, Ar-H), 7.37 (2H, t, Ar-H), 7.61 (2H, d, Ar-H), 7.76 (2H, d, Ar-H), 7.92 (1H, s, Ar-H (H-11)), 8.21 (1H, d, -NH-); ESI-MS: 792.1 ($[M + H]^+$), 815.2 ($[M + Na]^+$).

Fmoc-His(Trt)- CO NH-CH$_2$- glaucine **(11)**: ^{1}H NMR (250.13 MHz, $CDCl_3$, δ/ppm): 2.70 (3H, s, NCH_3), 2.93-3.11 (8H, m, 4 x CH_2), 3.69 (3H, s, OCH_3, (C-1)), 3.78 (1H, s, H-6a), 3.91 (9H, s, 3 x OCH_3,), 4.03 (1H, m, CH), 4.42-4.48 (1H, m, 2x CH), 4.5-4.55 (4H, m, 2 x CH_2), 5. 9 (1H, br.s, NH), 6.78 (1H, s, Ar-H (H-8)), 7.1-7.8 (25H, m, Ar-H), 7. 72 (1H, br.s., NH), 7.93 (s, 1H, Ar-H (H-11)); ESI-MS: 986.1 ($[M]^+$), 1009.2 ($[M + Na]^+$).

Fmoc-Lys(Boc)-CO NH-CH$_2$- glaucine **(12)**: ^{1}H NMR (250.13 MHz, $CDCl_3$, δ/ppm): 1.17 (9H, s, 3 × CH_3), 1.41 - 1.62 (4H, m, 2x CH_2), 1.87 (2H, m, CH_2), 2.73 (3H, s, >NCH_3), 2.93-3.19 (8H, m, 4 x CH_2), 3.68 (3H, s, OCH_3, (C-1)), 3.75 (1H, t, CH), 3.87 (1H, s, H-6a), 3.91 (9H, s, 3 x OCH_3), 4.10-4.32 (3H, m, CH-CH_2), 4.53 (2H, m, -CH_2-NH-), 6.79 (1H, s, Ar-H (H-8)), 7.29-7.33 (2H, t, Ar-H), 7.37-7.44 (2H, t, Ar-H), 7.47 (1H, br.s., NH), 7.68 (1H, m, NH), 7.70-7.72 (2H, d, Ar-H), 7.86-7.87(2H, d, Ar-H), 7.91 (1H, s, Ar-H (H-11)), 8.67 (1H, t, NH). ESI-MS: 835.2 ($[M]^+$), 858.2 ($[M + Na]^+$).

Fmoc-Tyr(OBu^t)-CO NH-CH$_2$- glaucine **(13)**: ^{1}H-NMR (250.13 MHz, $CDCl_3$, δ/ppm): 1.49 (9H, s, 3 × CH_3), 2.76 (3H, s, NCH_3), 2.98-3.23 (10H, m, 5 x CH_2), 3.64 (3H, s, OCH_3, (C-1)), 3.89 (1H, s, H-6a), 3.94 (9H, s, 3 x OCH_3), 4.17-4.45 (3H, m, CH-CH_2-O), 4.57-5.0 (3H, m, CH_2-NH+CH), 6.08 (1H, d, -NH, J = 7.4 Hz), 6.74 (2H, d, Ar-H, J = 8.5 Hz), 6.78 (1H, s, Ar-H (H-8)), 6.98 (2H, d, Ar-H, J = 8.5 Hz), 7.04-7.12 (8H, m, Ar-H), 7.93 (1H, s, Ar-H (H-11)), 8.16 (1H, br.s., NH). ESI-MS: 826.1 ($[M]^+$).

Fmoc-Ser(OBu^t)-CO NH-CH$_2$- glaucine **(14)**: ^{1}H-NMR (250.13 MHz, $CDCl_3$, δ/ppm): 1.37 (9H, s, 3 × CH_3), 2.70 (3H, s, NCH_3), 2.91-3.16 (6H, m, 3 x CH_2), 3.31 (2H, d, NCH_2), 3.69 (3H, s, OCH_3, (C-1)), 3.87 (1H, s, H-6a), 3.93 (9H, s, 3 x OCH_3), 3.95-3.98 (2H, m, $CHCH_2$), 4.15 (1H, t, J=6.9 Hz, CH), 4.41 (2H, d, CH_2, J=7.0 Hz), 4.83 (1H, m, $CHCH_2$), 5.8 (1H, br.s, NH), 6.79 (1H, s, Ar-H (H-8)), 7.27-7.45 (4H, m, Ar-H), 7.6 (2H, d, Ar-H, J=6.9 Hz), 7.85 (2H, d, Ar-H, J=7.0 Hz), 7. 74 (1H, br.s., NH), 7.96 (1H, s, Ar-H (H-11)); ESI-MS: 750.1 ($[M + H]^+$).

The synthesis of the molecular hybrids was carried out in accordance with the mentioned coupling method by joining sinapic or ferulic acid via phenylalanine spacer with 3-aminomethylglaucine (**15, 16**).

***N- Feruloylphenylalanine amide of 3-aminomethyl-glaucine* (15)**: ^{1}H NMR (600 MHz, $CDCl_3$, δ/ppm): 2.45 (3H, s, NCH_3), 2.67 (2H, m, $>CH_2$), 2.90-3.10 (6H, m, 3 x CH_2), 3.57 (3H, s, OCH_3, (C-1)), 3.75 (3H, s, OCH_3,), 3.81 (3H, s, $OCH_{3\,(FA)}$), 3.86 (6H, s, 2x OCH_3), 4.24-4.39 (2H, m, >N-CH_2), 4.66 (1H, m, CH), 5.9 (1H, d, NH) 6.2 (1H, d, CH=, J = 15.6 Hz,), 6.38 (1H, d, NH), 6.70 (1H, d, Ar-H (m), J = 8.2 Hz,), 6.8 (d, 1H, Ar-H (o), J = 1.8 Hz,), 6.90 (1H, s, Ar-H (H-8)), 6.95 (1H, dd, Ar-H (o)), 7.08-7.14 (5H, m, Ar-H), 7.4 (1H, d, >CH=, J = 15.6 Hz,), 7.84 (s, 1H, Ar-H (H-11)); ^{13}C NMR (600 MHz, $CDCl_3$, δ / ppm):): 34.1, 35.1, 38.8 (3 CH_2), 44.0 (NCH_3), 53.0, 53.1, 54.7, 55.8, 55.9 (5 OCH_3), 56.2, 60.1 (2 CH_2), 61.1, 63.2 (2 CH), 109.4, 110.7, 111.6, 111.6, 114.8, 117.6, 122.5, 122.5, 124.0, 126.9, 126.9, 127.1, 127.2, 127.3, 127.6, 128.6, 128.6, 129.3, 136.5, 136.6 (20 ArC and ArCH), 141.7, 146.8, 147.5, 147.6, 148.3, 151.4, (6 ArCOR), 165.8, 170.3 (C=O); ESI-MS: 708.4 ($[M + H]^+$), 730.2 ($[M + Na]^+$); UV (EtOH) (λ max/ nm)): 218, 284, 316.

***N- sinapoylphenylalanine amide of 3-aminomethyl-glaucine* (16)**: ^{1}H NMR (600 MHz, $CDCl_3$, δ/ppm): 2.46 (s, 3H, NCH_3), 2.65 (m, 2H, $>CH_2$), 2.90-3.11 (m, 6H, 3 x CH_2), 3.58 (s, 3H, OCH_3, (C-1)), 3.76 (s, 3H, OCH_3,), 3.82 (s, 6H, 2 x $OCH_{3\,(SA)}$), 3.86 (s, 6H, 2x OCH_3), 4.24-4.40 (m, 2H, >N-CH_2), 4.65 (m, 1H, CH), 5.88 (d, 1H, NH) 6.20 (d, 1H, CH=, J = 15.6 Hz), 6.37 (d, 1H, NH), 6.65 (s, 2H, Ar-H), 6.70 (s, 1H, Ar-H (H-8)), 7.09-7.14 (m, 5H, Ar-H), 7.42 (d, 1H, CH=, J = 15.6 Hz), 7.84 (s, 1H, Ar-H (H-11)); ^{13}C NMR (600 MHz, $CDCl_3$, δ / ppm):): 35.1, 38.9, 39.0 (3 CH_2), 44.1 (NCH_3), 53.0, 53.1, 53.1, 54.7, 55.8, 56.2 (6 OCH_3), 56.3, 60.1 (2CH_2), 61.1, 63.2 (2CH), 104.8, 110.7, 111.3, 111.6, 111.6, 118.1, 124.0, 126.1, 126.9, 126.9, 127.2, 127.3, 127.6, 128.6, 128.6, 129.3, 136.5, 136.6, 136.7 (19 ArC), 140.8, 141.8, 147.2, 147.5, 148.2, 148.3, 151.4 (7 Ar COR), 165.6, 170.2 (2 C=O); ESI-MS: 738 ($[M + H]^+$), 760 ($[M + Na]^+$), 761.1 ($[M+Na+H]^+$); UV (EtOH) (λ max/ nm)): 221, 284, 315.

***A general procedure for Boc removal of Boc-protected amides* (4-9)**

2.3 M HCl/ EtOAc (2 ml) was added at a room temperature to the stirred solution of 0.23 mmol Boc-AA-CONH-CH_2-glaucine (**4 - 9)** in EtOAc (3 ml). After 30 min, the solvent was evaporated *in vacuo* and H_2O was added to the residue. This was followed by continuous extraction with PE and EtOAc. After its completion, NH_4OH was added to the aqueous layer to make it alkaline. Then extraction with EtOAc was carried out. The ethyl acetate layers were combined, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness under reduced pressure. The crude product was purified by preparative TLC on Kieselgel 60 F_{254} using a solvent system of PE/$CHCl_3$/CH_3COCH_3/CH_3OH = 4:8:1:2 or 4:8:1:1).

***H-Gly-CONH-CH$_2$- glaucine* (4a)**: ^{1}H NMR (250.13 MHz, $CDCl_3$, δ/ppm): 2.58 (3H, s, NCH_3), 2.89-3.28 (6H, m, 3 x CH_2), 3.64 (3H, s, OCH_3, (C-1)), 3.89 (9H, s, 3 x OCH_3), 4.47 (2H, m, -CH_2-NH-), 6.77 (1H, s, Ar-H (H-8)), 7.54 (1H, t, NH), 7.93 (1H, s, Ar-H (H-11)); ESI-MS: 441.9 ($[M + H]^+$); UV (EtOH) (λ max/ nm)): 222, 283, 305.

***H-Ala-CONH-CH$_2$- glaucine* (5a)**: ^{1}H NMR (250.13 MHz, $CDCl_3$, δ/ppm): 1.37 (3H, d, CH-CH_3, J = 6.9 Hz), 2.61 (3H, s, NCH_3), 2.95-3.26 (6H, m, 3 x CH_2), 3.64 (3H, s, OCH_3, (C-1)), 3.88 (9H, s, 3 x OCH_3), 4.47-4.52 (3H, m, -CH_2-NH + CH), 6.77 (1H, s, Ar-H (H-8)), 7.54 (1H, t, NH), 7.93 (1H, s, Ar-H (H-11)); ESI-MS: 455.9 ($[M]^+$); UV (EtOH) (λ max/ nm)): 223, 284, 307.

***H-Val-CONH-CH$_2$- glaucine* (6a)**:^{1}H NMR (250.13 MHz, $CDCl_3$, δ/ppm): 0.83 (3H, d, CH_3, J = 6.9 Hz), 0.98 (3H, d, CH_3, J = 6.9 Hz), 2.29 (1H, m, >CH-CH-), 2.54 (3H, s, NCH_3), 2.99-3.09 (6H, m, 3 x CH_2), 3.70 (3H, s, OCH_3, (C-1)), 3.83 (1H, s, H-6a), 3.93 (9H, s, 3 x OCH_3), 4.46-4.56 (3H, m, -CH_2-NH + CH), 6.77 (1H, s, Ar-H (H-8)), 7.32 (1H, t, NH), 7.93 (1H, s, Ar-H (H-11)); ESI-MS: 484.1 ($[M + H]^+$), 506.1 ($[M + Na]^+$), 967.3 ($[2M + H]^+$); UV (EtOH) (λ max/ nm)): 223, 284, 307 nm; UV (EtOH) (λ max/ nm)): 223, 284, 307.

***H-Phe-CONH-CH$_2$- glaucine* (7a)**: ^{1}H NMR (250.13 MHz, $CDCl_3$, δ / ppm): 2.72 (3H, s, NCH_3), 2.93-3.11 (6H, m, 3 x CH_2), 3.29 (2H, d, CH_2), 3.35-3.37 (2H, d, -CH_2-C_6H_5), 3.68 (3H, s, OCH_3, (C-1)), 3.87 (1H, s, H-6a), 3.94 (9H, s, 3 x OCH_3), 4.42-4.50 (1H, m, CH), 4.53 (2H, m, -CH_2-NH-), 6.78 (1H, s, Ar-H (H-8)), 7.20 -7.32 (5H, m, Ar-H), 7.47 (1H, br.s., NH), 7.93 (1H, s, Ar-H (H-11)); ESI-MS: 531.9 ($[M + H]^+$); UV (EtOH) (λ max/ nm)): 221, 284, 307.

***H-Phe(3-F)-CONH-CH$_2$- glaucine* (8a)**: ^{1}H NMR (250.13 MHz, $CDCl_3$, δ/ppm): 2.60 (3H, s, NCH_3), 2.64 (2H, s, NH), 2.99-3.26 (6H, m, 3 x CH_2), 3.29 (2H, d, CH_2), 3.25-3.29 (2H, d, -CH_2-C_6H_5), 3.68 (3H, s, OCH_3, (C-1)), 3.85 (1H, s, H-6a), 3.93 (9H, s, 3 x OCH_3), 4.40-

4.56 (1H, m, CH), 6.77 (1H, s, Ar-H (H-8)), 6.85-7.00 (3H, m, Ar-H), 7.23 (1H, m, Ar-H), 7.39 (1H, t, NH), 7.91 (1H, s, Ar-H (H-11)); ESI-MS: 549.9 ($[M]^+$); UV (EtOH) (λ max/ nm)): 220, 284, 306.

H-His-CONH-CH$_2$- glaucine (**9a**): ^{1}H NMR (250.13 MHz, $CDCl_3$, δ/ppm): 2.69 (3H, s, NCH_3), 3.06-3.17 (6H, m, 3 x CH_2), 3.68-3.70 (2H, d, -CH_2-), 3.94 (12H, s, 4 x OCH_3), 4.42-4.54 (1H, m, CH), 6.75 (1H, s, Ar-H (H-8)), 6.87 (1H, d, (Ar-H)^{imid}), 7.57 (1H, br.s., -NH), 7.89 (1H, s, Ar-H (H-11)); ESI-MS: 522.0 ($[M + H]^+$); UV (EtOH) (λ max/ nm)): 222, 283, 309.

Evaluation of DPPH free radical scavenging activity *(RSA)*

Each compound of the concentration tested (3.6 mM) was mixed with 0.1 mM ethanolic solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH•) stable free radical. The absorption at 516 nm was measured at the start, at the 10-th min and at the 20-th min. The tests were performed in triplicate at 27ºC. The results are expressed as percent radical scavenging activity: % RSA = [$Abs_{516\ nm\ (t = 0}$) -$Abs_{516\ nm\ (t = t0)}$ x 100/$Abs_{516\ nm\ (t = 0)}$], as proposed by *Nenadis N.et al.* [29].

Antiviral tests

The antiviral activity of the newly synthesized compounds was tested by the multicycle viral cytopathic effect (CPE) inhibition assay. Monolayer cells in 96-well plates were inoculated with 0.1 mL of the corresponding virus suspension containing 100 $CCID_{50}$. The mock infected cells served as toxicity and cell controls. After an hour for virus adsorption at 37ºC for PV-1 and 33^{0}C for HRV-14, virus inocula were discarded and the cells treated so far were further inoculated with 0.2 mL of maintenance medium containing 0.5 lg of the compounds tested. The cells were further incubated in a humidified atmosphere at 37ºC for PV-1 and 33ºC for HRV-14 and 5 % CO_2. When the virus cytopathic effect (CPE) reached its maximum (usually on the 48-th hour p.i. for PV-1 and 72-96h p.i. for HRV-14), the viable cells were stained according to MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] procedure with slight modifications. The optical density (OD) of each well was read with a spectrophotometer at 540 nm with 690 nm as a reference read-out [30, 31]. The percentage of CPE inhibition was calculated according to the following formula: % CPE = [$OD_{test\ sample}$ – $OD_{virus\ control}$] / [$OD_{toxicity\ control}$ – $OD_{virus\ control}$] x 100 [30, 31].

In vitro determination of tyrosinase mushroom activity by the modified dopachrome method using L-DOPA as a substrate **[32]**

Each cuvette contained the reaction mixture (3ml): a phosphate buffer (1.0 ml, 0.1 M, pH 6.8); *L*-DOPA (1.0 ml, 4 mM) sonochemically dissolved in deionized water, a tested inhibitor (0.1 ml, 0.2 mM) dissolved in DMSO and deionized water (0.8 ml). After adding a mushroom tyrosinase aqueous solution (0.1 ml, 192 U/ml), the reaction mixture was incubated at 37˚C for 10 min. Then the further enzyme reaction was interrupted by cooling the mixture for 5 min.

The reference solution was prepared in the same manner, but 0.1 ml of DMSO was added instead of an inhibitor.

The UV absorbance of the obtained DOPA chrome was measured at 475 nm. The percentage of mushroom tyrosinase activity was calculated using the following equation:

Mushroom tyrosinase activity % = B/A x 100,

where A stands for the absorbance of the reference solution, while B is the absorbance of test sample solution.

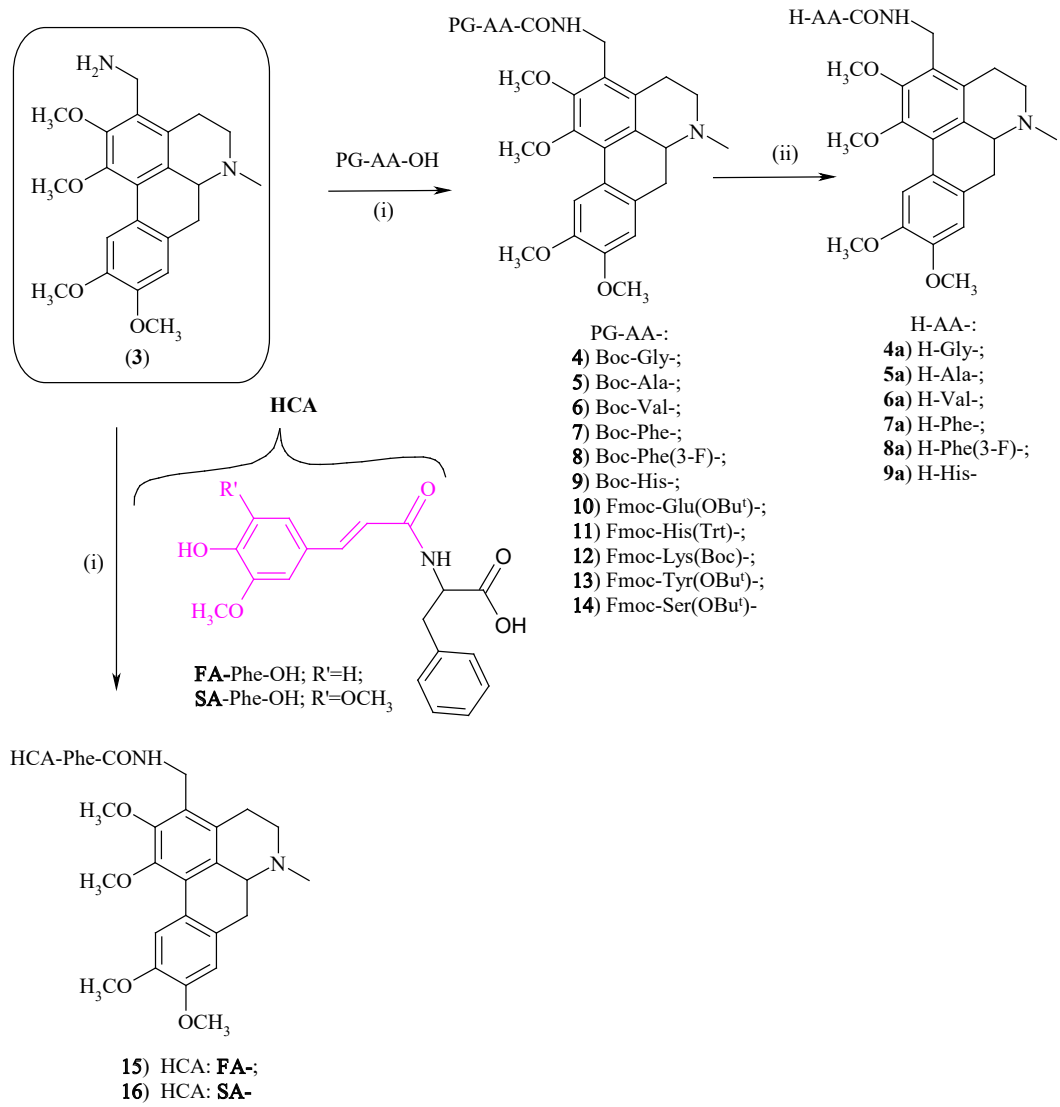
Each experiment was performed in triplicate and the data was averaged. Hydroquinone was used as a positive control.

RESULTS AND DISCUSSION

Chemistry

The previous work [20] demonstrates that the initial attempts to obtain amides of glaucine analogue (3-aminoglaucine (**2,** Fig. 1**)**) with hydroxycinnamic acids fail. This is the reason to conduct the same reaction but using the more reactive 3-aminomethylglaucine (AmGla; **3**). As outlined in Scheme 1, compound **3** obtained by the known procedure [27] is further subjected to *N*-acylation with different *N*-Boc- or Fmoc-protected amino acids (PG-AA-OH) or *N*-hydroxycinnamoylphenylalanines (FA-Phe-OH or SA-Phe-OH) using a carbodiimide coupling reaction [33 - 35].

The synthesized amides (**4-16**) are purified by preparative TLC. Their yields are satisfactory. Then the Boc-amino acid amides (**4-9**) obtained follow the deprotection step with 2.3 M HCl/EtOAc and afforded compounds **4a-9a**. The UV, NMR and ESI-MS data is consistent with the structures assigned.



Reagents and conditions: (i) EDC(DIC), HOBt, NMM, CH_2Cl_2; (ii) 2.3 M HCl/EtOAc, then NH_4OH

Scheme 1. A synthetic route of 3-aminomethylglaucine amides.

Biological Activity

Effect of DPPH free radical scavenging activity

An insight into radical scavenging activities of the synthesized amides of 3-aminomethylglaucine is gained by studying their abilities to quench the DPPH• radical [29]. The results are compared with those obtained for the starting compounds, glaucine and 3-methylaminogalucine and those referring to standard antioxidants - sinapic acid, ferulic acid, quercetin, α-tocopherol, eugenol and isoeugenol (Table 1). As seen in Table 1, the tested amino acid amides scavenge the DPPH radical to a lesser extent.

However, it is observed that 3-aminomethylglaucine (**3**) is 2-fold more active DPPH radical scavenger than glaucine (1). Additionally, the phenylalanine amide of 3-aminomethylglaucine tested as a free base (**7a**) also shows 2-fold higher activity than its corresponding Boc-protected amide (**7**). On the other hand, the radical scavenging activity (%RSA) of *N*-Boc-protected phenylalanine analogues (**7, 9**) increases about 1.5 fold in respect to *3*-fluoro substituted phenylalanine amide (**9**).

As expected, the incorporation of an additional antioxidant hydroxycinnamoyl (feruloyl- or sinapoyl-) fragment in compound (**7a**) provides hybrid molecules (**15, 16),** which increase the antiradical activity. Compound (**16**) containing sinapoyl moiety strongly

scavenges DPPH radical, even better when compared to the references tested (with the exception of sinapic acid and quercetin). Therefore compound (**16)** can be considered a promising antioxidant.

% RSA for typical antioxidants are given for a comparison. All tests are performed in triplicate. The values presented in this table are the mean value of three measurements ± confidence interval, calculated at a level of significance of 0.05

It is interesting to note that the presence of a phenylalanyl linker between the hydroxycinnamoyl fragment and 3-aminomethylglaucine (hybrids **15**, **16**) reveals better % RSA than the antiradical results (%RSA ~30-35%) reported in the previous study [20] of directed linking of 3-aminomethylglaucine to ferulic or sinapic acid obtained under identical conditions. It can be

Table 1. DPPH• scavenging activities of glaucine amides and tested antioxidants.

	Compds	**(%) RSA** 3.6 mM Reaction period (20 min)
	Ferulic acid (FA)	**44.6±1.1**
	Sinapic acid (SA)	**69.7±1.6**
	Quercetin	**78.1±0.1**
	D,L α-Tocopherol	**58.1±1.0**
	Eugenol	**37.6±0.1**
	Isoeugenol	**25.3±0.1**
1	Glaucine	1.6±0.1
3	3-Aminomethyl-glaucine	3.8±0.1
4	Boc-Gly-$NHCH_2$-glaucine	0.2±0.1
5	Boc-Ala-$NHCH_2$-glaucine	0.5±0.1
6	Boc-Val-$NHCH_2$-glaucine	0.9±0.1
7	Boc-Phe-$NHCH_2$-glaucine	3.1±0.1
7a	H-Phe-$NHCH_2$-glaucine	6.0±0.1
8	Boc-Phe(3-F)-$NHCH_2$-glaucine	4.9±0.1
9	Boc-His-$NHCH_2$-glaucine	5.7±0.1
14	Fmoc-Ser(OBut)-$NHCH_2$-glaucine	1.1±0.1
15	N-(Feruloyl)-Phe-$NHCH_2$-glaucine	40.2±0.1
16	N-(Sinapoyl)-Phe-$NHCH_2$-glaucine	62.5±0.1

suggested that the differences in the antiradical activity between the two types of hydroxycinnamoyl hybrids could be due to the presence of a phenylalanyl unit.

Antienteroviral effects

Based on the likelihood that the presence of structural fragments of antioxidant and antiviral effects could positively affect the antienteroviral activity, the most potent radical scavengers (**15, 16**) are tested for their activity against the *in vitro* replication of PV-1(LSc-2ab) and HRV-14. The observed antiviral effect, although present, is weaker (the data is not shown) especially when compared with those of the previously synthesized cinnamoyl- and hydroxycinnamoyl amides of 3-aminomethylglaucine [20].

An effect of the newly synthesized amides of 3-aminomethylglaucine on tyrosinase activity

Considering the structural similarity of glaucine and its aporphine analogue, boldine [26], the anti-tyrosinase effect of the newly synthesized glaucine derivatives is evaluated.

Tyrosinase (polyphenol oxidase, EC 1.14.18.1) is known as the rate-limiting enzyme controlling melanin biosynthesis. It catalizes two distinct reactions: the hydroxylation of monophenol (*L*-tyrosine) and the further conversion of *o*-diphenol (*L*-DOPA; dihydroxyphenylalanine) to the corresponding *o*-quinone (dopaquinone) [36]. Being readily available, the mushroom tyrosinase is widely used in pharmaceutical and food bioprocessing technologies [36].

The newly synthesized amides are subjected in this study to an evaluation of a mushroom tyrosinase activity. Following the modified dopachrome method [32], the diphenolase activity of the enzyme is determined spectrophotometrically by using *L*-DOPA as a substrate. For comparison, hydroquinone, a tyrosinase inhibitor, serves as a positive control. The results are shown in Fig. 2.

Compared to the positive control, all tested amides exhibit a stronger inhibitory activity in respect to mushroom tyrosinase, with the exception of compounds **4**, **7a** and **8**.

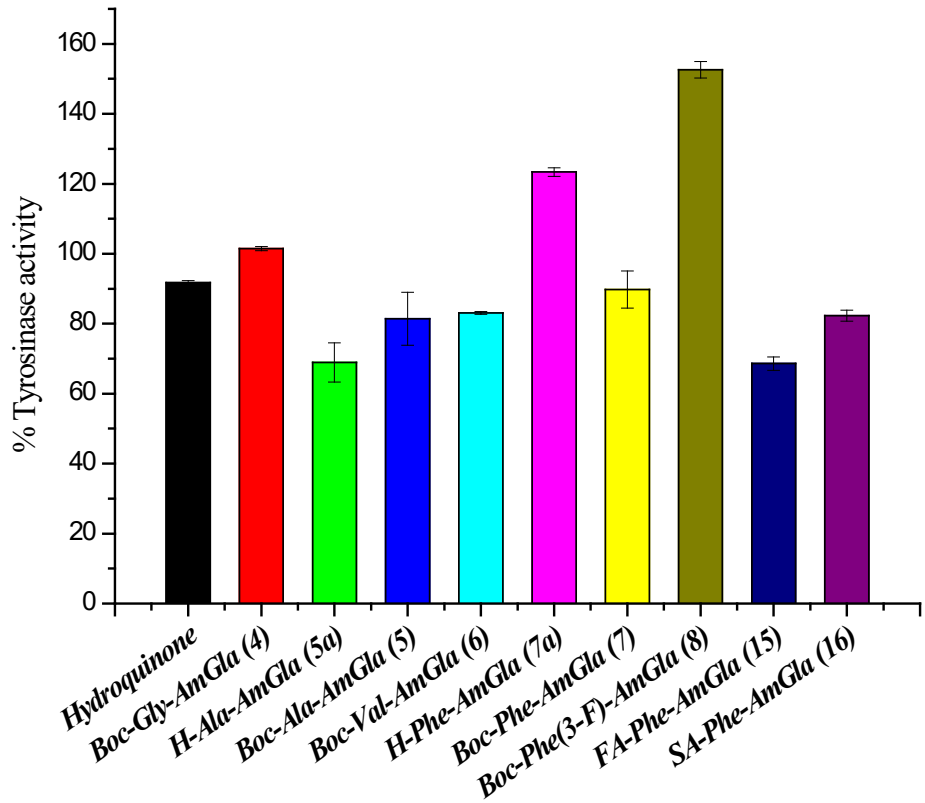


Fig. 2. Mushroom tyrosinase activity of tested compounds. Each experiment was performed in triplicate and averaged. The values are given as the mean ± confidence interval, calculated at level of significance 0.05.

Interestingly, amide **8** with an electron-withdrawing *F*-substituent at *m*-position of the phenylalanyl moiety, decreases dramatically the tyrosinase inhibitory activity unlike that caused by phenylalanine-containing compounds **7** and **7a**. Considering the Boc-protected amide (**7**) and its deprotected analogue (**7a**), it is worth noting that amide (**7**) has a diphenolase inhibitory activity nearly twice that of amide (**7a**). The tyrosinase inhibitory potency shown by phenylalanine amides **7** suggests that the hydrophobicity of that compound, due to its tert. butyloxycarbonyl protecting group (Boc-group) might affect the hydrophobic tyrosinase pocket. This assumption is supported by other studies [37-40], reporting that the addition of a hydrophobic moiety augments the tyrosinase inhibitory potency.

On the other hand, in contrast to compound **7a**, the introduction of an additional feruloyl- or sinapoyl fragment to hybride structures **15** or **16**, respectively, reveals a more potent inhibitory effect against mushroom tyrosinase, even than that of the tyrosinase inhibitor hydroquinone.

CONCLUSIONS

A series of different protected amino acid amides of 3-aminomethylglaucine and two hybride molecules (**15, 16**) obtained by functionalization of phenylalanyl-3-aminomethylglaucine with hydroxycinnmic acids (ferulic or sinapic acid, respectivly) are synthesized. The compounds are evaluated for their DPPH scavenging, antiviral and anti-tyrosinase activies.

The preliminary studies of DPPH test demonstrate that hydroxycinnamoyl compounds **15** and **16** show the most potent free DPPH scavenging activity amongst the tested amides. It is comparable with those of the corresponding free hydroxycinnamic acids.

Meanwhile, all compounds, with the exception of compounds **4**, **7a** and **8**, inhibit *in vitro* mushroom tyrosinase. The best tyrosinase inhibitory activity is shown by compound **15** containing a feruloyl moiety.

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