SYNTHESIS OF NOVEL BOLDINE AMIDES AND THEIR *IN VITRO* INHIBITORY EFFECTS ON A MUSHROOM TYROSINASE

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ABSTRACT

Boldine ((S)-2,9-dihydroxy-1,10-dimethoxy-aporphine) has been defined as the major alkaloid in Chilean boldo tree. Besides its diverse pharmacological activities, e.g. neuroprotective, cytoprotective, anti-inflammatory activities, boldine also exhibits tyrosinase-inhibiting effect.

Tyrosinase [EC 1.14.18.1] is well known as a bifunctional enzyme that is responsible for the melanin biosynthesis. Considering the diphenolic structural feature of the alkaloid, which is associated with its tyrosinase inhibitory properties, herein we first modified the boldine core and then linked it with the natural phenolic antioxidants such as: caffeic-, ferulic- and sinapic acids. Furthermore, the newly amides were tested in vitro on the mushroom tyrosinase. Our results indicated, that amongst the tested boldine derivatives, caffeoyl- and feruloylamides have shown the anti-tyrosinase activity closely correlated with a hydroquinone, used as a standard.

Keywords: boldine, hydroxycinnamoylamides, hydroquinone, antioxidant activity, anti-tyrosinase activity.

INTRODUCTION

Peumus boldus Mol., commonly known as boldo, is an evergreen Chilean tree, belonging to the family Monimiaceae. Boldo has been known in folk medicine for a long time and continue to be used as an herbal remedy, mentioned in various pharmacopoeias [1]. Diverse boldo preparations (infusions, tinctures, and extracts) have been widely applied in traditional medicine, usually for the treatment of digestive and hepatobiliary disorders [2].

Earlier studies have reported that the secondary metabolites of boldo leaves, and bark consist mainly of alkaloids, and other non-alkaloids compounds such as phenolic compounds, glycosides, and essential oil [3]. Amongst the main active alkaloid contents, boldine (*S*)-2,9-dihydroxy-1,10-dimethoxy-aporphine (1; Fig. 1) was the firstly isolated one by Bourgoin E. and Verne C. more than 120 years ago [4]. This alkaloid (1) is related to the aporphine alkaloid class, which is consisted of benzylisoquinoline nucleus.

Indeed, due to its diphenolic hydroxyl structure, boldine emerges as one of the most potent natural antioxidants, exhibiting several pharmacological activities, such as cytoprotective, anti-tumour promoting, anti-inflammatory, antipyretic and antiplatelet, which can be related to its potent free radical-scavenging ability [1, 5 - 7].

In correlation to its antioxidant properties, stands out also its tyrosinase inhibitory property [8]. Recently,

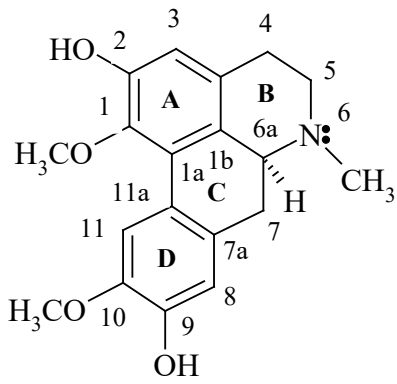


Fig. 1. Chemical structure of boldine (1).

extensive efforts have been spent for finding out of non - cytotoxic and potent tyrosinase inhibitors.

Tyrosinase (EC 1.14.18.1), being a polyphenoloxidase enzyme catalyzes the oxidation of phenols (substrates such *L*-tyrosine or 3,4-dihydroxyphenylalanine (*L*-DOPA)) under aerobic condition. It is generally accepted, that in mammals, tyrosinase plays a crucial role in the skin pigmentation process, in vegetables it is responsible for browning effects and be important for the formation of protective cuticles in insects [9 - 13]. Consequently, the potential applications of tyrosinase inhibitors extend to the treatment of a wide range of dermatological disorders associated with abnormal melanogenesis, as well as for aesthetic problems after sunburn injury. Additionally, mushroom tyrosinase has been clinically applied for the treatment of vitiligo as well [14]. From an agriculture point of view, the tyrosinase inhibitors might be also successfully used as bio-insecticides [8].

Another phenolic-backbone structures that have emerged as active tyrosinase inhibitors in cosmetics are attributed to natural/ synthetically derived cinnamic acids and their derivatives [15 - 18]. These derivatives are widely recognized for their antioxidant activities and have been evaluated as versatile pharmacologically active compounds [19].

As part of our ongoing research program directed toward the phenol - derived compounds, we investigated several hydroxycinnamic acids amides that showed promising anti-tyrosinase activities [20, 21].

In this sense, in continuation of our efforts in searching for potent, non-toxic tyrosinase inhibitors, herein, we conjugated natural bioactive compounds - boldine and versatile hydroxycinnamic (ferulic, sinapic and caffeic) acids, both of which possess anti-tyrosinase properties.

EXPERIMENTAL

General information

(*E*)-4-Hydroxy-3-methoxycinnamic-(ferulic) acid, (*E*)-3,4-dihydroxycinnamic-(caffeic) acid, (*E*)-3,5-dimethoxy-4-hydroxycinnamic (sinapic) acid, tyrosinase [EC 1.14.18.1], *L*-DOPA, *L*-tyrosine, *N*-hydroxymethylacetamide, *N*-methylmorpholine (NMM) were purchased from Sigma Aldrich, whereas standard tyrosinase inhibitor, hydroquinone was obtained from the company Ferak (Germany). Boldine (2,9-dihydroxy-1,10-dimethoxy aporphine), HOBt, *N*-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC) were purchased from Merck. All solvents were reagent grade and were used without further purification. The water used for tyrosinase inhibition assay was deionized. Thin layer chromatography (TLC) spots were visualized as orange or brown spots after spraying with Dragendorff's reagent.

The structures and purity of all the amides were confirmed by spectral methods –^{1}H and ^{13}C NMR spectroscopy and ESI-MS. The ^{1}H and ^{13}C NMR spectra were acquired by a Bruker Avance III 400 spectrometer, operating at 400.15 MHz for protons and 100.62 MHz for carbons. ESI-MS analyses were performed on a Bruker Compact QTOF-MS (Bruker Daltonics, Bremen, Germany.

Synthetic procedures of novel boldine analogues

3-(Acetamidomethyl) boldine (2)

To a solution of 2.5 g (7.6 mmol) boldine, 0.8 g (8.7 mmol) *N*-hydroxymethylacetamide in 7.5 mL of glacial CH_3COOH, and concentrated H_2SO_4 (2.5 mL) was added dropwise at 0°C. The mixture was stirred for 22 hours at room temperature and reaction monitoring was provided using chromatographic system (PE: $CHCl_3$: CH_3COCH_3 : $HOCH_3$ = 1 : 1 : 1 : 2). After completion of the reaction, the mixture was diluted 1:1 with H_2O, alkalized with 20 % NaOH (at 0°C), and extracted with CH_2Cl_2. The combined organic extracts were dried over anhydrous Na_2SO_4, and then the solvent was evaporated under vaccum. The crude residue was purified by

column chromatography over neutral alumina (Merck, act. II-III), using the PE:EtOAc system with increasing polarity.

3-(Acetamidomethyl) boldine (2): Yield 61 %; ^{1}H NMR (400 MHz, chem. shifts in ppm, solvent DMSO-d_6): d 1.85 (s, 3H, $OCCH_3$), 2.25 (m, 2H, $ArCH_2a$ + NCH_2a), 2.40 (s, 3H, NCH_3), 2.68 (m, 1H, $CH_{2a}CH_2N$), 2.75 (m, 1H, $CHNCH_3$), 2.84 (m, 1H, $CH_{2b}CH_2N$), 2.94 (m, 1H, $ArCH_{2b}$), 2.98 (m, 1H, NCH_{2b}), 3.54 (s, 3H, OCH_3), 3.78 (s, 3H, OCH_3), 4.21 (2H, $(CO)HNCH_2$), 6.73 (s, 1H, Ar-CH-COH), 7.83 (s, 1H, Ar-CH-$COCH_3$), 8.36 (1H, NH), 9.14 (s,1H, OH), 9.40 (s, 1H, OH). ^{13}C NMR (100.62 MHz, in ppm, solvent DMSO-d_6): d 21.5 (CH_3CO), 25.8 (CH_2CH_2N), 33.6 (CH_2CH), 34.6 $(C(O)NHCH_2)$, 43.8 (NCH_3), 52.7 (NCH_2), 56.0 (OCH_3), 59.4 (OCH_3), 62.8 (NCH), 111.8 (ArCH), 115.2 (ArCH), 122.0 (Cq), 122.6 (Cq), 125.6 (Cq), 128.6 (Cq), 129.6 (Cq), 129.6 (Cq), 143.1 ($CqOCH_3$), 145.9 (CqOH), 146.1($CqOCH_3$), 148.1 (CqOH), 170.7 (CONH).

Hydrolysis of 3-(Acetamidomethyl) boldine to 3-(Aminomethyl) boldine (3):

1.5 g (3.8 mmol) of 3-(Acetamidomethyl) boldine was heated at 100ºC in 16 mL of concentrated HCl and 32 mL H_2O. After 4 h, the reaction mixture was cooled in an ice-bath to 0ºC, then it was alkalized with NH_4OH and extracted 5 times with CH_2Cl_2. The combined organic phases were dried over Na_2SO_4 and evaporated *in vacuo*.

3-(Aminomethyl) boldine (3): Yield 73 %; ^{1}H NMR (400 MHz, chem. shifts in ppm, solvent DMSO-d_6): d 2.22 (m, 2H, $ArCH_2a$ + NCH_2a), 2.39 (s, 3H, NCH_3), 2.54 (m, 1H, $CH_{2a}CH_2N$), 2.74 (m, 2H, $CH_{2b}CH_2N$ + $CHNCH_3$), 2.95 (m, 2H, $CH_2CH_{2b}N$ + $ArCH_{2b}$), 3.44 (2H, (H_2NCH_2), 3.60 (s, 3H, OCH_3), 3.78 (s, 3H, OCH_3), 3.9 (br s, 4H, NH_2 + 2 x OH), 6.72 (s, 1H, Ar-CH-COH), 7.83 (s, 1H, Ar-CH-$COCH_3$). ^{13}C NMR (100.62 MHz MHz, in ppm, solvent DMSO-d_6): d 26.3 (CH_2CH_2N), 33.9 ($\mathbf{C}H_2CH$), 39.0 (H_2NCH_2), 39.8 (CH), 43.9 (NCH_3), 53.0 ($N\mathbf{C}H_2$), 56.0 (OCH_3), 56.2 ($\mathbf{C}H_2$), 59.2 (OCH_3), 62.9 (NCH), 112.0 (ArCH), 115.0 (ArCH), 121.4 (Cq), 123.0 (Cq), 124.6 (Cq), 126.4 (Cq), 129.5 (Cq), 142.8 ($CqOCH_3$), 145.6 (CqOH), 146.1 (CqOH), 150.8 (Cq).

General procedure for the amidation of hydroxycinnamic acids with 3 - (Aminomethyl) boldine using EDC/ HOBt method (4 - 6)

To the stirring CH_2Cl_2 solution (10 mL) of the corresponding hydroxycinnamic acid (1.12 mmol), 0.16 g (1.12 mmol) HOBt was added 0.22 g (1.12 mmol) EDC in an ice-water bath. Then, equimolar quantities of 3-aminomethyl boldine (0.4 g (1.12 mmol)) and 0.12 mL (1.12 mmol) NMM were added. Stirring was continued for an hour at 0°C and for further 20 hours at room temperature. After completion of reaction the solvent was evaporated *in vacuo.* Then, 30 mL ethylacetate was added, and the organic phase extracted with 5 % $NaHCO_3$ (3x 20 mL) and brine, dried over anhydrous Na_2SO_4, then filtered and evaporated to dryness under reduced pressure. The residue was purified by preparative TLC on Kieselgel 60 F_{254} using solvent system PE/ $CHCl_3$/ CH_3OH = 1:5:3; 1:5:2 or 1:2:2). Recrystallization of products were done by H_3COH/ Et_2O. Yields were between 38 and 59 %.

Feruloyıol amide of 3-aminomethylboldine (4; FA-Boldine): ^{1}H NMR (400 MHz, chem. shifts in ppm, solvent DMSO-d_6): d 2.25 (m, 2H, $ArCH_2a$ + NCH_2a), 2.40 (s, 3H, NCH_3), 2.74 (m, 2H, $C\mathbf{H}_{2a}CH_2N$ + $C\mathbf{H}NCH_3$), 2.94 (m, 3H, $C\mathbf{H}_{2b}CH_2N$, $ArCH_{2b}$, NCH_{2b}), 3.54 (s, 3H, OCH_3), 3.77 (s, 6H, 2 x OCH_3), 4.34 (2H, $(CO)HNC\mathbf{H}_2$), 6.50 (d, J = 15.3 Hz, 1H, =CH), 6.72 (s, 1H, Ar-CH-COH), 6.77 (d, J = 9.2 Hz, 1H, Ar(m)), 6.97 (dd, J = 9.2, 1.4 Hz, 1H, Ar (o)), 7.10 (d, J = 1.4 Hz, 1H, Ar(o)), 7.37 (d, J = 15.3 Hz, 1H, =CH), 7.80 (s, 1H, Ar-C**H**-$COCH_3$), 8.43 (br t, J = 6.2 Hz, 1H, NH), 9.13 (br s,1H, OH), 9.50 (br s, 1H, OH). ^{13}C NMR (100.62 MHz, in ppm, solvent DMSO-d_6): d 25.8 ($\mathbf{C}H_2CH_2N$), 33.5 ($\mathbf{C}H_2CH$), 34.4 $(C(O)NH\mathbf{C}H_2)$, 43.7 (NCH_3), 52.7 ($N\mathbf{C}H_2$), 55.5 (OCH_3), 59.3 (OCH_3), 62.7 (NCH), 110.7 (Ar-o-CH), 111.9 (ArCH), 115.1 (ArCH), 115.5 (Ar-m-CH), 117.9 (=CH), 121.5 (Ar-o-CH), 122.0 (Cq), 122.6 (Cq), 125.6 (Cq), 128.6 (Cq), 129.6 (Cq), 129.6 (Cq), 139.7 (=CH), 143.1 ($CqOCH_3$), 145.8 (CqOH), 148.1 (CqOH), 164.1 (CONH). ESI-MS: 533.2 ($[M+H]^+$), 1065 ($[2M+H]^+$).

N - Sinapoylamide of 3-aminomethylboldine (5; SA-Boldine): ^{1}H NMR (400 MHz, chem. shifts in ppm, solvent DMSO-d_6): 2.46 (s, 3H, NCH_3), 2.88-3.11 (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 $OCH_{3\ (SA)}$), 4.24-4.40 (m, 2H, >N-CH_2), 5.88 (t, 1H, NH), 6.23 (d, 1H, =CH, J = 15.5 Hz), 6.75 (s, 2H, Ar-H(o)), 6.70 (s, 1H, Ar-H (H-8)), 7.42 (d, 1H, =CH, J = 15.5 Hz), 7.80 (s, 1H, Ar-H), 7.84 (s, 1H, Ar-CH-$COCH_3$), 8.43 (br t, J = 6.2 Hz, 1H, NH), 9.13 (br s, 1H, OH), 9.50 (br s, 1H, OH). ^{13}C NMR (100.62 MHz, in ppm, solvent DMSO-d_6): 24.1, 35.1 (2 x CH_2), 35.4 (C(O)N**C**H_2), 43.5 (NCH_3), 52.8 (NCH_2), 53.0 (OCH_3), 53.1 (OCH_3), 54.7 (OCH_3), 55.8 (OCH_3), 63.2 (NCH), 109.4 (Ar-o-CH), 110.7 (ArCH), 115.6 (ArCH), 118.9 (=CH), 122.5 (Ar-o-CH), 123.0 (Cq), 122.6 (Cq), 125.6 (Cq), 128.6 (Cq), 129.6 (Cq), 129.6 (Cq), 141.7 (=CH), 145.1 (2 x CqOCH_3), 147.8 (CqOH), 149.1 (CqOH), 162.3 (C-OH), 167.1 (CONH). ESI-MS: 563.1 ($[M+H]^+$), 585.2 ($[M+Na]^+$), 1125.1 ($[2M+H]^+$).

N - Caffeoylamide of 3-aminomethylboldine (6; CafA-Boldine)*:* ^{1}H NMR (DMSO-d_6, d ppm): 2.36-2.11 (m, 2H,CH_2), 2.40 (s, 3H, NCH_3), 3.10-2.60 (5H, NCH+ NCH_2+CH_2), 3.53 (s, 3H, OCH_3), 3.76 (s, 3H, OCH_3),4.31 (brs, 2H, CH_2NH), 6.15(d, 1H, J = 15.8 Hz, =CH), 6.41(d, 1H, J = 15.6 Hz, =CH), 6.73 (s, 1H, H-Ar), 6.74 (d, 1H, J = 8.4 Hz, m-H), 6.81(dd, 1H, J = 8.4, 2.1 Hz, o-H), 6.94 (d, 1H, J = 2.1 Hz, o-H), 7.28 (d, 1H, J = 15.6 Hz, =CH), 7.79 (s, 1H, H-Ar), 8.60 (t, 1H, J = 5.1 Hz, NH), 9.23 (br s, 1H, OH), 9.59 (s, 1H, OH).^{13}C NMR (DMSO-d_6, d ppm): 26.1 (CH_2), 33.7 (CH_2), 35.0 (C(O)N**C**H_2), 44.0 (NCH_3), 53.1 (NCH_2), 56.1 (OCH_3), 59.8 (OCH_3), 63.1 (NCH), 112.5 (CH-Ar), 114.4(o-C), 115.9 (2C,m-C+ CH-Ar), 117.8 (=CH), 120.9 (1C, o-CH), 140.3 (=CH), 170.1 (CONH). ESI-MS: 519.2 ($[M+H]^+$), 1037.1 ($[2M+H]^+$).

Estimation of mushroom tyrosinase activity *in vitro*

The spectrophotometric assay was conducted *in vitro* using mushroom tyrosinase [21, 22]. The experiments were performed in 3 mL quarz cuvettets, containig a phosphate buffer (1.0 mL, 0.1 M, pH 6.8); *L*-DOPA (1.0 mL, 4 mM) dissolved in deionized H_2O under sonication, and putative inhibitor (0.1 mL, 0.2 mM) was dissolved in DMSO and deionized H_2O (0.8 mL).

After the addition of 0.1 mL tyrosinase solution (192 U mL^{-1}), the reaction mixture was incubated for 10 min at 37°C and then the enzyme reaction was terminated by cooling for 5 min.

Simillary, the reference solution contains the whole reaction mixture, but instead of an inhibitor, 0.1 mL DMSO was added.

Thus, during oxidation the absorbance of formed DOPAchrome was measured at 475 nm. Tyrosinase activity was calculated by the following equation:

Mushroom tyrosinase activity % = B/A x 100, where A represents for the absorbance of the reference solution, while B is the absorbance of test sample solution.

All experiments were performed in triplicate and the data were averaged. Hydroquinone was utilized as a potent tyrosinase inhibitor.

RESULTS AND DISCUSSION

Amidation strategy

Since the tyrosinase inhibitors have been supposed to have wide applications in different industries as medicine, agriculture, and cosmetic, undoubtedly there is a significant and unremitting interest in obtaining of such kind inhibitors. However, the existing tyrosinase inhibitors suffer from undesirable side effects such as carcinogenicity and limited clinical efficacy [23]. Accordingly, a huge number of anti-tyrosinase agents has been developed from synthetical and natural origin. In a previous paper of Chochkova et al. the synthesis of *N*-hydroxycinnamoyl amino acid amides, containing another aporphine alkaloid - glaucine (dimethoxy analogue of boldine) was reported [21]. In addition, it was demostrated that the addition of hydroxycinnamoyl moiety to the analogues is responsible for better tyrosinase inhibition on mushroom tyrosinase, even more potent than the used standard – hydroquinone.

Therefore, in the current study we hypothesized that the hybridization of two natural structural fragments - hydroxycinnamic acids and diphenolic boldine molecule with recorded tyrosinase inhibitory activities will lead to enhance inhibitory effects.

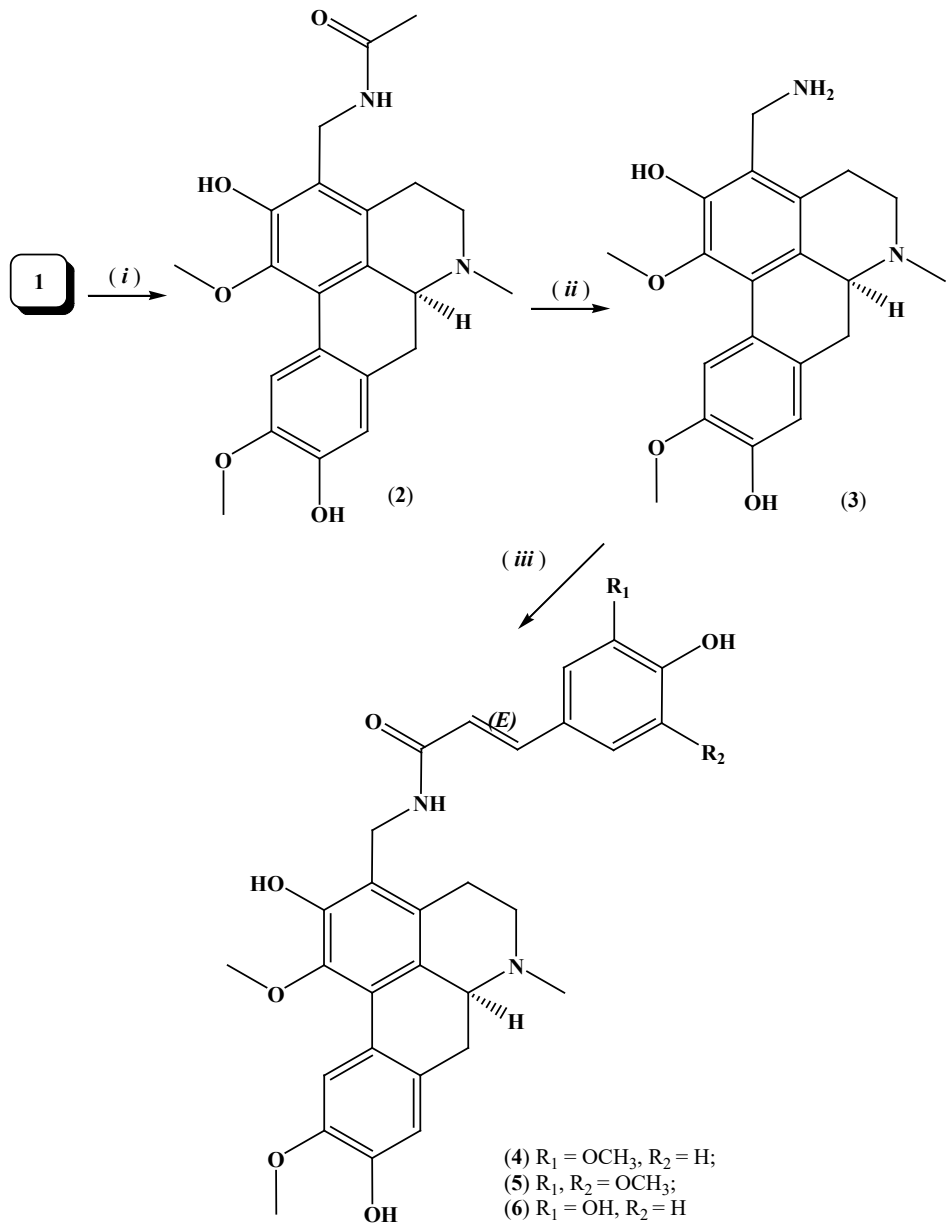
Thus, for the linking of above-mentioned molecules, the amidation strategy was chosen. The amide functionality is the most used over the ester group in various drugs, due to its metabolically stablility and it can also act as hydrogen bond donors and/or receptors [24 - 26].

Following the known procedure, where glaucine was preliminary transformed to its 3-aminomethylglaucine analogue [27] and then successfully derivatized with

cinnamic acids [28], the novel boldine modifications were obtained under the similar reaction conditions, as described in Scheme 1.

Initially, the commercially available (+)-boldine (1) was subjected to *a*-amidomethylation with *N*-(hydroxymethyl) acetamide in acetic and sulfuric acid at room temperature (i). Then, the resulting analogue (2) was further hydrolyzed upon heating in dilute hydrochloric acid (4 h at 100°C) to give 3-(aminomethyl) boldine (3) in 73 % isolated yield.

On the next step (iii), the coupling by acylation of intermediate 3 with the corresponding carboxylic acids (ferulic acid, sinapic acid or caffeic acid) via *in situ* activation with carbodiimide coupling reagent (EDC),



Scheme 1. Synthesis of the newly boldine analogues (2, 3) and the target hydroxycinnamoylamides 4 - 6. Reagents and conditions: (*i*) $HOCH_2$-NH-C(O)CH_3, conc. H_2SO_4, glacial CH_3COOH (100 %); (*ii*) aq. HCl, heating; (*iii*) hydroxycinnamic acid (ferulic acid (FA): $R_1 = OCH_3$, $R_2 = H$; sinapic acid (SA): $R_1, R_2 = OCH_3$; or caffeic acid (CafA): $R_1 = OH$, $R_2 = H$), EDC/ HOBt, NMM, CH_2Cl_2, R.T., 20 h.

and in the presence of a catalytic amount of HOBt [29] afforded target compounds 4 - 6. After purification by preparative TLC, the desired boldine-containing hydroxycinnamoyl amides (4 - 6) were provided in moderate yields. The NMR and ESI-MS data were consistent with the assigned structures.

Mushroom tyrosinase activity

Tyrosinase has been reported to possess broad substrate specificities, acting with its both activities (monophenolase and diphenolase activities). Thus, various polyphenols are defined as substrates by tyrosinase, however the inhibitory potential of polyphenols on tyrosinase activity depends on the presence and position of additional substituents [30].

Usually, for estimation of anti-tyrosinase activity, the mushroom tyrosinase is chosen as a frequently used model for *in vitro* investigations [31]. Despite this enzymatic test is easy to perform, providing reproducible results, however, it has the drawback that the results cannot be be utilized in the development of products for human use.

Considering the promising anti-tyrosinase activities of the both structural phenolic units (boldine and hydroxycinnamic acids) comprising our target compounds 4 - 6, herein, we evaluated these hybrids 4 - 6 for diphenolase inhibitory activity of mushroom tyrosinase using *L*-DOPA as a substrate [32].

The percentage tyrosinase activities of tested amides and the standard hydroquinone (HQ) are presented in Fig. 2.

The values are given as the mean ± confidence interval, calculated at level of significance 0.05.

Compared to standard (HQ), the inhibitory activities decrease in the following order: 6 > HQ ≈ 4 > 5.

It is noteworthy that amongst the all tested compounds, caffeoylamide of boldine (**6**; CafA-Boldine**)** exhibited the most potent tyrosinase inhibitory activity. Indeed, this inhibition is expected for this catechol - containing compound 6, due to its structural resemblance to *L*-DOPA, which is a substrate for tyrosinase. Moreover, the feryloylamide (4; FA-Boldine**)** showed comparable tyrosinase inhibitory activity to that of hydroquinone, whereas other amide (5; SA-Boldine)) were less active than used reference.

Considering our preliminary results, we can suggest that the inhibitory effect is dependent on the nature and position of the phenyl ring substitutions.

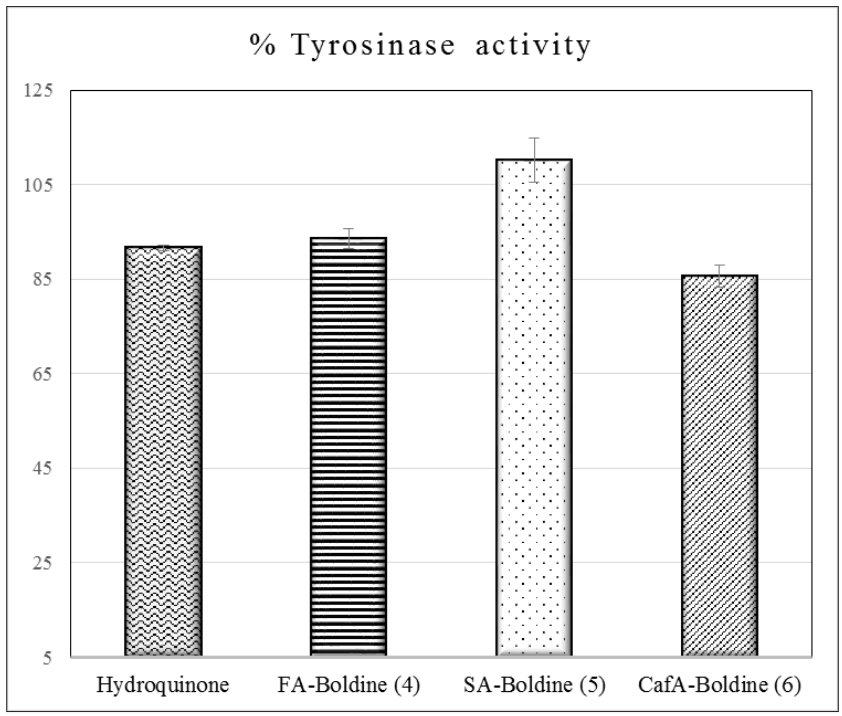


Fig. 2. Effects of the newly hydroxycinnamoyl amide of 3-aminomethylboldine on the diphenolase activity of mushroom tyrosinase. Hydroquinone (HQ) was used as reference compound.

This popular assumption is valid for versatile research work, describing the cinnamoyl derivatives with different substitution patterns as potent tyrosinase inhibitors.

CONCLUSIONS

Herein, two novel boldine analogues (2, 3) and three hydroxycinnamoyl amides (4 - 6) have been synthesized in moderate yields. Moreover, the effects of synthetic amides (4 - 6) on diphenolase activity of mushroom tyrosinase have also been evaluated.

Our preliminary results reveal that among the compounds tested, caffeoylamide (6) having catechol moiety attached to the olefinic side chain, displayed the strongest inhibitory activity, even more potent than the reference compound hydroquinone. However, the feruloylamide (4) also deserves more attention, because it exerted commensurable effect to that of hydroquinone (known as strong, but unstable, cytotoxic to melanocytes, and an easily oxidized tyrosinase inhibitor).

Accordingly, these two amides (4, 6) might be promising candidates as cosmetic or food perserving agents, however further investigations will be needed.

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