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TYROSINASE INHIBITORS FROM BOLIVIAN MEDICINAL PLANTS

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ABSTRACT.—Bioassay-guided fractionation monitored by mushroom tyrosinase (EC 1.14.18.1) activity, afforded six inhibitors from three Bolivian medicinal plants, *Buddleia coriacea, Gnaphalium cheiranthifolium,* and *Scheelea princeps*. These inhibitors, which are all known phenolic compounds, inhibited the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) mediated by a mushroom tyrosinase.

As part of our continuing investigation of naturally occurring alternative insect control agents, we have searched for tyrosinase inhibitors from plant sources (1,2). Because tyrosinase is one of the key enzymes in the insect molting process (3), its inhibitors might ultimately provide clues to the control of insect pests. Moreover, tyrosinase inhibitors have become increasingly important for cosmetic (4) and medicinal products, primarily in relation to hyperpigmentation (5–7). Tyrosinase inhibitors may prevent the production of dermal melanin pigmentation since tyrosinase plays an important role in the process of melanin biosynthesis (8,9). Thus, melanin synthesis inhibitors are used topically for treatment of localized hyperpigmentation in humans such as lentigo, nevus, ephelis, post-inflammatory state, and melanoma of pregnancy (10). Although animal tyrosinase differs somewhat from mushroom tyrosinase, the latter was used for this investigation because of its ready availability (11).

In a preliminary screening using mushroom tyrosinase, the MeOH extracts of three medicinal plants, *Buddleia coriacea* Remy (Loganiaceae), *Gnaphalium cheiranthifolium* Lam (Compositae), and *Scheelea princeps* (Mart.) Karst. (Palmae), which are botanically unrelated but endemic to Bolivia, showed significant (<200 μg/ml) inhibitory activity for the oxidation of L-DOPA.

Fractionation guided by tyrosinase inhibitory activity using mushroom tyrosinase led to the isolation of six active principles from the three Bolivian medicinal plants. The identities of these tyrosinase inhibitors were characterized based on spectroscopic studies, in particular by nmr.

The MeOH extract of the aerial parts of *B. coriacea*, a plant locally known as "quishuara," was suspended in H_2O and extracted with EtOAc, a fraction of which in subsequent bioassay was shown to be active. Two flavonol glycosides, buddlenoids A and B, were isolated as tyrosinase inhibitors after the application of repeated chromatographic methods. Their final purification was achieved by recycle-hplc (r-hplc) (12) using an ODS C_{18} column. These two isolates have been deduced from spectroscopic evidence to be kaempferol 7-β-D-(6″-*p*-coumaroyl)-glycoside and isorhamnetin 7-β-D-(6″-*p*-coumaroyl)glycoside (1). Bioassays of the purified compounds indicated that both buddlenoids exhibited potent mushroom tyrosinase inhibitory activity and showed a dose-dependent effect on the oxidation of L-DOPA. The ID_{50} values were 0.39 mM for buddlenoid A and 0.44 mM for buddlenoid B (Table 1). Their limited availability prevented further biological study to determine their mode of inhibition. However, we have recently reported that the partial structure "a" as illustrated with the bold line in the quercetin molecule in Figure 1 is required to inhibit tyrosinase oxidation of L-DOPA by

TABLE 1. ID_{50} Values and Mode of Inhibition of Tyrosinase Inhibitors.

Compound Tested	ID_{50} (mM)	Mode of Inhibition	Reference
Buddlenoid A	0.39	—[a]	1
Buddlenoid B	0.44	—[a]	1
Quercetin	0.07	Competitive	13
Agrimoniin	0.061	—[a]	—[a]
p-Hydroxybenzoic acid	1.9	Competitive	—[a]

[a]Not tested.

competitive inhibition (13). This was based on a comparison study using a series of flavonoids including luteolin 7-β-D-glucoside. Since both buddlenoids possess a partial structure which closely resembles the structure of the substrate, L-DOPA, it is logical to assume their mode of inhibition to be of a competitive nature. Thus, in contrast to quercetin 3-β-D-glucoside, the bulky *p*-coumaroyl–glucose moiety at the 7-position in the buddlenoids does not block the approach of this molecule to the active site of the enzyme.

In addition to these two flavonol glycosides, a more active principle was also purified in large quantities (0.46%, dry wt) from *B. coriacea*. This more potent tyrosinase inhibitor was isolated from the H_2O portion after repeated chromatography using a variety of methods. This polar compound has been identified by spectroscopic studies as the known hydrolyzable tannin, agrimoniin, which was previously isolated from *Agrimonia pilosa* and *Potentilla kleiniana* (14). The ID_{50} value of this tannin was 0.061 mM, which is the highest of the six compounds isolated and bioassayed (Table 1). The purified agrimoniin seems to be rather unstable since it gradually started changing color to grey on isolation from the extract. It appears that agrimoniin itself is auto-oxidized. The inhibition kinetics of this tannin were analyzed by a Lineweaver-Burk plot. The three slopes, obtained from the uninhibited enzyme and from the two different concentrations of agrimoniin, were found to be almost parallel, indicating an uncompetitive inhibition. Because tannins are known to react with proteins by cross-linking, a process known as tanning, agrimoniin is expected to somehow irreversibly inhibit the tyrosinase (a protein). The instability of agrimoniin, coupled with its tanning property, complicated our attempts to characterize the mode of inhibition.

The MeOH extract of the aerial part of *G. cheiranthifolium*, locally known as "huira," was also suspended in H_2O and extracted with EtOAc, a fraction of which in subsequent bioassays was shown to be active. Two active principles were isolated after repeated chromatography and identified by means of spectroscopic methods as gnaphalin (15) and luteolin 4′-β-D-glucoside (16), respectively. Their final purification was achieved by r-hplc (ODS C_{18}). Inasmuch as luteolin 4′-β-D-glucoside possesses the bulky glucose moiety at the 4′-position but still inhibited the enzyme, it was considered of interest to determine its mode of inhibition. How-

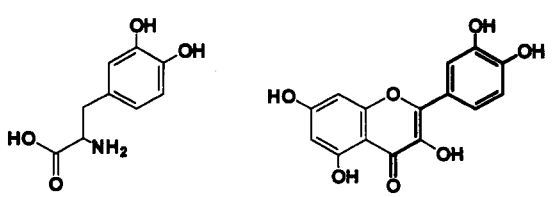

FIGURE 1. L-DOPA and quercetin, and their structural similarity.

ever, these two active compounds exhibited significant (almost 100% at 100 μg/ml) inhibition of the L-DOPA oxidation by tyrosinase. Their limited availability prevented further study so neither ID_{50} values nor their mode of inhibition were investigated.

Similar treatment of the MeOH extract of the root of *S. princeps*, known as "mota cú" in Bolivia, yielded a tyrosinase-inhibitory EtOAc portion. An active principle was isolated from this fraction after separation by a number of chromatographic methods, and identified as the common *p*-hydroxybenzoic acid by comparison of its spectral data with those of an authentic sample. Interestingly, *p*-hydroxybenzoic acid was the only active compound isolated from this source, guided by bioassay, at 200 μg/ml. It showed a concentration-dependent effect on the tyrosinase oxidation of L-DOPA. The ID_{50} value was determined to be 1.9 mM. Although this ID_{50} value represents only moderate potency, because *p*-hydroxybenzoic acid and its derivatives (for example "paraben") have been widely used as preservatives in cosmetic and food products (17), its tyrosinase inhibitory activity was investigated in more detail.

The inhibition kinetics of *p*-hydroxybenzoic acid were analyzed by a Lineweaver-Burk plot as shown in Figure 2. The slopes, obtained from the uninhibited enzyme and from the two different concentrations of *p*-hydroxybenzoic acid, intercepted on the original axis. The results indicated that *p*-hydroxybenzoic acid was a characteristic competitive inhibitor. However, preincubation in the presence of 1.9 mM *p*-hydroxybenzoic acid and in the absence of the substrate (L-DOPA) did not indicate that *p*-hydroxybenzoic acid is a direct inhibitor of the enzyme since it did not significantly decrease enzymatic activity. The inhibition was increased only from 50% to 55%. Thus, *p*-hydroxybenzoic acid

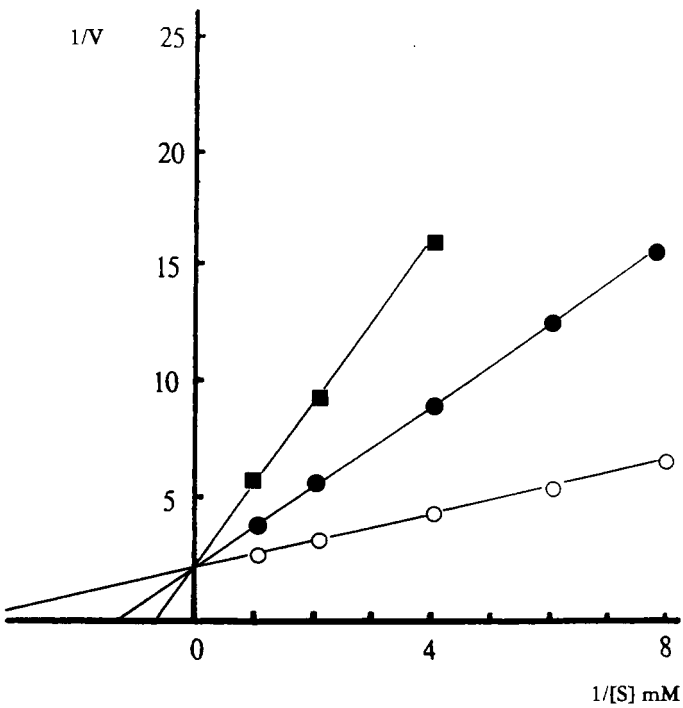

FIGURE 2. Lineweaver-Burk plots of mushroom tyrosinase and L-DOPA without (-○-, control) and with [(-●-, 133 μg/ml (1.0 mM); -■-, 233 μg/ml (1.7 mM)] *p*-hydroxybenzoic acid. 1/V:1/Δ475 nm/min.

appears to competitively displace L-DOPA from the active site.

Tyrosinase contains a strongly coupled binuclear copper active site and functions both as a monooxygenase (monophenol+O_2→*o*-diphenol+H_2O) and as an *o*-dihydroxyphenolase (2 *o*-diphenol+O_2→2 *o*-quinone+2 H_2O) (18–20). The competitive inhibitor *p*-hydroxybenzoic acid binds preferentially to the binuclear copper center with the more acidic carboxylic group and competes with the substrate, L-DOPA (18). More importantly, this enzyme apparently has separate catalytic sites for these two oxidations, and yet another independent binding site for L-DOPA as a co-factor (21–25). Presumably, the above-mentioned quercetin and probably the buddlenoids displace at the active site of the co-factor as competitive inhibitors, since these flavonoids have partial structures that closely resemble the structure of the substrate. Thus, these flavonoids fit loosely into the active site of the co-factor and prevent its entry. *p*-Hydroxybenzoic acid is, on the other hand, an inhibitor rather than an inactivator of the enzyme (26). As a result, it seems that the modes of inhibition of *p*-hydroxybenzoic acid and quercetin are different, even though both were found to be competitive inhibitors (27).

Interestingly, all the six tyrosinase (polyphenol oxidase) inhibitors isolated by bioassay-guided fractionation from the three plants of Bolivian origin were found to be common phenolic compounds.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Fabms spectra were obtained on a JEOL DX-303HF instrument using a glycerol matrix. Nmr spectra were recorded using a JEOL GSX-500 instrument (500 MHz for ^1H and 125 MHz for ^{13}C). Recycle-hplc (r-hplc) was performed by a JAI LC-09 instrument. Dimethylsulfoxide (DMSO) and *p*-hydroxybenzoic acid were purchased from Sigma Chemical Co. (St. Louis, MO). L-DOPA was obtained from Aldrich Chemical Co. (Milwaukee, WI).

PLANT MATERIAL.—The aerial parts of *B. coriacea* and of *G. cheiranthifolium* were provided by PROMENAT (Proyecto de Medicina Native), La Paz, Bolivia. The roots of *S. princeps* were collected in Beni, Bolivia, air-dried immediately after collection, and identified by Ms. I. Hinojosa, Herbario Nacionale de Bolivia, where a voucher specimen has been deposited.

EXTRACTION AND ISOLATION.—The dried pulverized plant materials were each extracted with MeOH at room temperature. The solvent was removed under reduced pressure to give the crude extracts which were submitted for bioassay. The extracts were first dissolved in DMSO and used at suitable concentrations, with the highest one being 0.17% because of solubility limitations.

The dried pulverized aerial parts (1.0 kg) of *B. coriacea*, extracted with MeOH as described above, yielded 150 g of a crude extract. A portion (50 g) of this extract was suspended in H_2O and extracted successively with *n*-hexane and EtOAc. Subsequent bioassay showed both the EtOAc and the H_2O portions to be active. The purification of two of the active flavonoids, buddlenoids A and B, from the EtOAc fraction was as previously reported (1). A portion of the H_2O fraction (4.5 g from the total of 30.6 g) was chromatographed over ODS C_{18} with gradient elution (H_2O-MeOH, initially 1:0, then 0:1). The active fraction (1.307 g) obtained was further chromatographed over Sephadex LH-20. This process was repeated three times to give a hydrolyzable tannin-rich active fraction (800 mg). A part of this fraction (160 mg) was subjected to further separation by r-hplc (12) using an ODS C_{18} column eluted with CH_3CN-H_2O-AcOH (200:800:2) to yield pure agrimoniin (45 mg, 0.459%). The uv, ir, and nmr were identical with the data previously reported (14).

The MeOH extract (54 g) of *G. cheiranthifolium* was treated similarly to that of *B. coriacea* and two active principles were isolated. They were identified, based on spectroscopic studies, as gnaphalin (0.005%) (15) and luteolin 4′-β-D-glucoside (0.004%) (16), respectively.

The MeOH extract (65 g) of *S. princeps* was suspended in H_2O and partitioned with EtOAc. The bioactive EtOAc portion (6.0 g) was chromatographed on Si gel and ODS C_{18} to yield the active principle, which was identified as *p*-hydroxybenzoic acid (0.012%) by a comparison of its spectroscopic data with those of an authentic sample.

ENZYME ASSAY.—The mushroom tyrosinase (EC 1.14.18.1) used for the bioassay was purchased from Sigma Chemical Co. (St. Louis, MO). All the samples were first dissolved in DMSO and used for the experiment at 30 times dilution. The assay was performed as previously described (9,28). An aliquot (1 ml) of 2.5 mM L-DOPA was mixed

with 0.1 ml of the sample solution and 1.8 ml of 0.1 M phosphate buffer (pH 6.8), and incubated at 25° for 10 min. Then, 0.1 ml of the aqueous solution of the mushroom tyrosinase (138 units) was added to the mixture to immediately measure the initial rate of linear increase in optical density at 475 nm, based on the formation of dopachrome. The extent of inhibition by the addition of samples is expressed as the percentage necessary for 50% inhibition (ID_{50}).

The pre-incubation mixture consisted of 1.8 ml of 0.1 M phosphate buffer (pH 6.8), 0.6 ml of H_2O, 0.1 ml of the sample solution (equivalent amount of ID_{50}), and 0.1 ml of the aqueous solution of the mushroom tyrosinase (138 units). The mixture was pre-incubated at 25° for 5 min. Then, 0.4 ml of 6.3 mM L-DOPA was added and the reaction was monitored at 475 nm for 2 min.

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