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Pancreatic lipase and α-amylase inhibitory activities of plants used in Traditional Chinese Medicine (TCM)

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To find new, plant based drugs for the treatment of obesity and/or diabetes mellitus type 2 through the inhibition of essential digestive enzymes, *in vitro* tests were carried out on selected plants or fungi with weight-reducing, blood glucose-reducing or related potential, used in Traditional Chinese Medicine (TCM). Aqueous and methanolic extracts of 32 Chinese herbal medicines were assayed for their *in vitro* inhibitory activity against pancreatic lipase (PL) and α-amylase (PA). PL activity was measured by using an enzymatic *in vitro* assay based on the hydrolysis kinetics of an oleate ester of 4-methylumbelliferone. For the determination of α-amylase activity an enzyme assay based on the hydrolytic cleavage of a modified starch derivative was used. Our findings have shown that the methanolic extract of *Lycopus lucidus* Turcz. var. *hirtus* Regel (Lamiaceae) was a very effective PL inhibitor (IC_{50}: 88.3±4.1 µg/mL). A high anti-amylase activity showed the methanolic extract of *Trichosanthes kirilowii* Maxim. (Curcurbitaceae, IC_{50}: 248.8±67.3 µg/mL). This work provides a priority list of interesting plants for further study with respect to the treatment of obesity and associated metabolic diseases.

1. Introduction

According to the World Health Organization (WHO), Traditional Chinese Medicine (TCM) has existed for more than 3000 years (Nestler 2002). TCM modalities include Chinese herbal medicine, acupuncture, Chinese massage, mind/body exercise, and dietary therapy (Lao et al. 2012). Of these, Chinese herbal medicine is one of the most commonly used modalities. It represents a central part of traditional Chinese culture and medical practice and includes plants, minerals, and animal parts. The herbs are believed to act synergistically to harmonize their effects and to reduce possible toxicity of the individual constituents (Lao et al. 2012).

In the treatment of obesity and associated diseases as diabetes mellitus type 2, TCM plants are of increasing interest. Worldwide, obesity has more than doubled since 1980 and becomes more and more a major challenge for our society. In 2014, more than 1.9 billion adults were registered as overweight, over 600 million of them were obese (WHO 2015). One strategy for the treatment of obesity and its related diseases is the development of inhibitors of nutrient digestion and absorption (Foster-Schubert and Cummings 2006). In particular, the manipulation of essential enzymes of the digestive system is an attractive approach. Inhibition of pancreatic lipase (PL, pancreatic triacylglycerol lipase (EC: 3.1.1.3)) and pancreatic α-amylase (PA, 1,4-alpha-D-glucan glucanohydrolase (EC: 3.2.1.1)) represent useful targets for the discovery of potent agents for patient treatment (Ali et al. 2006; Ros 2000; Slanc et al. 2009; Thomson et al. 1997). Examples of enzyme inhibitors in clinical use are orlistat, acarbose, miglitol and voglibose (Ali et al. 2006; Sergent et al. 2012). However, the use of these inhibitors is compromised by gastrointestinal adverse reactions like oily stools, flatulence and diarrhea (Chaput et al. 2007; Chiasson et al. 2002). The aim of current research is to identify novel inhibitors that lack of some of these unpleasant side effects. In this context, the use of plant based resources as a potential platform for the discovery and development of new drugs is an interesting starting point. Natural products generally have the advantage in being milder in their activity than totally synthetic drugs, in having reduced toxic and systemic adverse effects by low absorption rates in the gut and having a broad spectrum of possible multiple/synergetic effects (Bustanji et al. 2011; Gholamhoseinian 2010). Another advantage is their high presence in the gut after oral administration, so that no further metabolic processes are necessary for an effect. In this context, TCM plants represent an attractive alternative for the discovery and development of new drugs.

In the present study, we have screened a total of 32 TCM plants and fungi using simple, fast, efficient and reliable fluorescence-based *in vitro* enzyme assays in an attempt to provide a preliminary selection of plants with potential PL and α-amylase inhibitory activities for further consideration of more detailed research. Only TCM plants and fungi were tested, which were attributed to a beneficial effect in the treatment of obesity and diabetes mellitus type 2 as described in the literature. The tested TCM plants may be considered as interesting specimens for further work if they have shown potentials for invoking lipase and amylase inhibitor activity.

2. Investigations, results and discussion

The results of PL and PA inhibition by various aqueous and methanolic plant and fungi extracts have been summarized in Tab. **1**. Depending on their inhibitory activity, the respective extracts could be arranged in five groups. The first group describes minor inhibitory activity (< 20 %). The second group offered an inhibition in a range of 20 % – 49.9 %. The following groups represent an inhibition range of 50 % – 69.9 %, and 70 % to 89.9 %. The extracts in group five show an inhibition higher than 90 %.

A variety of the tested extracts showed a strong inhibitory potential to the digestive enzymes. It is notable, that in most cases the methanolic extracts showed a stronger effect on enzymatic activity than aqueous extracts. The best inhibitory activities to both enzymes (> 90 % inhibition) showed the extracts of *Coptis chinensis* Franch. (Ranunculaceae), *Crataegus pinnatifida* Bge. var. *major* N.E.Br. (Rosaceae), *Dioscorea opposite* Thunb. (Dioscoreaceae), *Rehmannia glutinosa* Libosch (Plantaginaceae), and *Trichosanthes kirilowii* Maxim (Curcurbitaceae). Zhou et al.

(2014) showed in their *in vitro* study an α-glucosidase inhibiting effect of *Coptis chinensis* Franch (Zhou et al. 2014). They could identify five compounds that are able to bind to the α-glucosidase of the plant: coptisine, epiberberine, jatrorrhizine, berberine and palmatine. An inhibitory effect to PL and PA has, to the best of our knowledge, not yet been described. The extract of *Crataegus pinnatifida* Bunge was also analyzed for PL inhibitory activity by Lee et al. (2012). Stem and twig of the plant showed 42.5 % lipase inhibition. For the plant extract of *Dioscorea opposite* Thunb. no data with regard to PL and PA inhibition are given in the litera-

Table 1: Overview of PL and PA inhibitory activity of the tested plant and fungi extracts

Scientific name	Lipase inhibition MeOH extract	Lipase inhibition aqueous extract	α-Amylase inhibition MeOH extract	α-Amylase inhibition aqueous extract
Aconitum carmichaelii Eebx.	++++	-	++++	++++
Agastache rugosa (Fisch. et Mey.) O. Ktze	+++++	-	++++	+++
Alisma orientale (Sam.) Juzep.	++++	++	++++	+++++
Anamarrhena asphodeloides Bge.	+++++	++	++++	++
Angelica sinensis (Oliv.) Diels	++	-	+++	++
Artactylodes macrocephala Koidz.	+++++	++	++++	+++
Asparagus cochinchinensis (Lour.) Merr.	++++	++	++++	+++
Astragalus membranaceus (Fisch.) Bge.	++++	n.d.	+++	+++
Aucklandia lappa Decne.	+++++	++	++++	++
Carthamus tinctorius L.	+++++	++++	++++	++++
Codonopsis pilosula (Franch.) Nannf.	+++++	-	++	++
Coptis chinensis Franch.	+++++	+++++	+++++	++++
Crataegus pinnatifida Bge. var. *major* N.E.Br.	+++++	++++	+++++	+++++
Curcuma wenyujin Chen et Ling	n.d.	+++	++++	+++++
Cyathula officinalis Kuan	+++++	-	++++	+++
Dioscorea opposite Thunb.	+++++	+++	+++++	+++++
Gardenia jasminoides Ellis	+++++	+++++	+++++	++++
Glycyrrhiza uralensis Fisch.	+++++	n.d.	++++	+++
Leomurus japonicas Houtt.	+++++	+++	++++	+++
Ligusticum chuanxiong Hort.	n.d.	++	++++	++++
Lycium barbarum L.	++++	-	+++	++++
Lycopus lucidus Turcz. var. *hirtus* Regel	+++++	+++	++++	++
Ophiopogon japonicas (L.f) Ker-Gawl.	+++	-	++	++
Paeonia lactiflora Pall.	++++	-	++++	++++
Phellodendron chinense Schneid.	+++++	+++	++++	+++
Polygonatum kingianum Coll. et Hemsl.	++	++	+++	++
Poria cocos (Schw.) Wolf	+++++	+++++	++++	+++++
Pueraria lobata (Willd.) Ohwi	+++++	-	+++	+++
Rehmannia glutinosa Libosch	+++++	++++	+++++	++++
Scrophularia ningpoensis Hemsl.	++++	+++	+++	++
Scutellaria baicalensis Georgi	+++++	++++	++++	++++
Trichosanthes kirilowii Maxim.	+++++	++	+++++	+++++

+++++ inhibition higher than 90%, ++++ inhibition 70-90%, +++ inhibition 50-69.9%, ++ inhibition 20-49.9%, + inhibition lower than 20%,- no inhibitory activity to determine, n.d. not determinable; *Positive control:* Orlistat (PL): 100 % inhibition, acarbose (PA): 100% inhibition

Table 2: IC_{50} values (mg/mL) of the tested methanolic plant extracts needed to inhibit PL and alpha-amylase

Scientific name	Used part	Main constituents	IC_{50} [mg/mL] Lipase	IC_{50} [mg/mL] ⊠-Amylase
Lycopus lucidus Turcz. var. *hirtus* Regel	Stem, leaf	Triterpenes (Fang et al. 2008)	0.088 ± 0.004	1.008 ± 0.009
Carthamus tinctorius L.	Corolla	Pigments (carthamin, precarthamin, safflower yellow A and B), safflower oil (Fang et al. 2008)	0.091 ± 0.001	0.849 ± 0.156
Trichosanthes kirilowii Maxim.	Root	Saponins, organic acids, salts, resins, sugars and pigments (Chinese Herbs Healing 2015)	0.100 ± 0.017	0.249 ± 0.067
Phellodendron chinense Schneid.	Bark	Phenolic acids (ferulic and caffeic acid), isovanillin, berberin (Wang et al. 2009)	0.136 ± 0.001	1.847 ± 0.074
Cyathula officinalis Kuan	Root	Phenolic acids, isoflavones (daidzin, puerarin) (Zhoub et al. 2005)	0.520 ± 0.001	3.178 ± 0.320
Aucklandia lappa Decne.	Root	Amino acids, inulin, aplotaxene, cotuslactone, sterol (Fang et al. 2008)	0.897 ± 0.109	2.003 ± 0.134

Positive control: IC_{50} (Orlistat): 0.19 ± 0.03 ng/mL; IC_{50} (Acarbose): 1.31 ± 0.12 µg/mL

ture. An α-glucosidase inhibitory effect of *Rehmannia glutinosa* Libosch was described by Gao et al. (2008). Furthermore, Kim and Kang (2005) analyzed the lipase inhibitory effect of different Korean medicinal plants. The root of *Rehmannia glutinosa* was investigated and showed a lipase inhibition of 14.5 %. The results of the current study also show a PA inhibitory potential. Moreover, Kim and Kang (2005) analyzed the root of *Trichosanthes kirilowii*, which showed 25.6 % lipase inhibition.

Strong inhibitory effects only to PL showed the extracts of *Agastache rugosa* (Fisch. et Mey.) O. Ktze (Lamiaceae), *Carthamus tinctorius* L. (Asteraceae), and *Lycopus lucidus* Turcz. var. *hirtus* Regel (Lamiaceae). The inhibitory effect of *Agastache rugosa* (Fisch. et Mey.) O. Ktze is confirmed by the results of Sharma et al. (2005). They have measured a lipase inhibition of 40.5 % for an extract of the whole plant. An α-glucosidase inhibitory effect of *Carthamus tinctorius* L. was described by Takahashi and Miyazawa (2012). The compounds active as α-glucosidase inhibitors were serotonin derivatives and showed a strong effect (best IC_{50} value: 47.2 µM). The lipase inhibitory effect was confirmed by Adisakwattana et al. (2012). A flower extract of *Carthamus tinctorius* L. was analyzed and showed PL inhibition (IC_{50}: 0.56±0.04 mg/mL). For the plant extract of *Lycopus lucidus* Turcz. var. *hirtus* Regel no data with regard to PL inhibitory potential were found in the literature.

Among the plant extracts examined, a lot of them showed a high anti-lipase and anti-amylase activity at a concentration of 2.5 mg/mL. A total of six methanolic extracts which were highly active and widely used in TCM, was selected and analyzed in terms of their IC_{50} values. In Table 2 the IC_{50} values of the methanolic plant extracts are reported. All tested extracts inhibited enzyme activity in a dose-dependent manner. The extract of *Lycopus lucidus* Turcz. var. *hirtus* Regel (Lamiaceae) was the most effective PL inhibitor (IC_{50}: 88.3±4.1 µg/mL). The extract of *Trichosanthes kirilowii* Maxim. (Curcurbitaceae) showed the strongest anti-amylase activity (IC_{50}: 248.8±67.3 µg/mL). Furthermore, the methanolic extracts of *Carthamus tinctorius* L. (Asteraceae, IC_{50}: 90.7±0.9 µg/mL), *Trichosanthes kirilowii* Maxim. (IC_{50}: 100.4±17.2 µg/mL) and *Phellodendron chinense* Schneid. (Rutaceae, IC_{50}: 135.7±1.4 µg/mL) showed a high PL inhibitory activity. However, all tested extracts were less potent than the synthetic PL inhibitor orlistat (IC_{50}: 0.19±0.03 ng/mL or 0.38±0.05 nM) and the α-amylase inhibitor acarbose (IC_{50}: 1.31±0.12 µg/mL or 2.03±0.19 µM).

Inhibition of digestive enzymes is one of the most widely studied mechanisms for the treatment of obesity and its associated diseases. In this study, we screened 32 Chinese medicinal plants and fungi using fluorescence-based *in vitro* enzyme assays in an attempt to find extracts with potential pancreatic lipase and α-amylase inhibitory activities. The results show, that a lot of the tested plant extracts have a high anti-lipase and anti-amylase activity and support the view, that herbs of TCM represent a rich source of inhibitory compounds. A total of six methanolic extracts with high activity and widely used in TCM was selected and analyzed in terms of their IC_{50} values. The results pointed out that the methanolic extract of *Lycopus lucidus* Turcz. var. *hirtus* Regel (Lamiaceae) was the most effective PL inhibitor (IC_{50}: 88.3±4.1 µg/mL). The strongest anti-amylase activity showed the methanolic extract of *Trichosanthes kirilowii* Maxim. (Curcurbitaceae, IC_{50}: 248.8±67.3 µg/mL).

Although our results are preliminary and need further confirmation, this work provides a priority list of interesting plants for further study. Fractionation, concentration and isolation steps will be needed to investigate a more precise dosage and specific compound group dependent inhibition.

3. Experimental

3.1. Chemicals

Traditional Chinese drugs were purchased from YI-KANG Pharmacy (Yangling, China) in March, 2014. Orlistat, 4-methylumbelliferyl oleate and acarbose (Sigma-Aldrich Chemie GmbH Steinheim, Germany); porcine pancreas powder (pancreatis pulvis PH. EUR. 6.3, Caesar & Loretz GmbH, Hilden, Germany) and EnzChek® *Ultra Amylase Assay Kit* (Life Technologies, Carlsbad, CA, USA) were used. All the other chemicals were reagent-grade or analytical grade, respectively.

3.2. Plant extraction

The list of TCM plants and fungi used in this study (including the origin province and the used plant organ) and their described effect are given in Table 3. Plants were ground to a fine powder, stored at room temperature, and protected from light until extraction. Extraction of samples was as follows: for the water extraction 5 g of the powdered plant parts were extracted with 100.0 mL of boiling distilled water in a refluxed condenser for 15 min. After filtration, the aqueous solution was lyophilized immediately and stored dry at room temperature and protected from light. In addition to the aqueous extracts, also methanolic extracts were examined to record a broad range of ingrediants. They were prepared by extraction of 5 g powder with 100.0 mL of boiling methanol in a refluxed condenser for 1 h. After filtration, the solution was distilled under reduced pressure at 40 °C, and then lyophilized and stored at 4 °C and protected from light until use. To assess the inhibitory potential of the selected plant and fungi extracts, two fluorescence-based *in vitro* enzyme assays were used.

Table 3: List of plants and fungi used in this study, including the origin province and the used plant part

Scientific name	Plant family	Origin province	Used part	Chinese crude drug processing	Described effect	Reference(s)
Aconitum carmichaelii Eebx.	Ranunculaceae	Sichuan	Root	Air dried	Blood glucose ↓	Soumyanath (2006)
Agastache rugosa (Fisch. et Mey.) O. Ktze	Lamiaceae	Hunan	Overground part	Air dried	Plasma cholesterol ↓ TAGs ↓	Jun et al. (2010)
Alisma orientale (Sam.) Juzep.	Alismataceae	Sichuan	Tuber	Air dried	Antidiabetic effect	Jia et al. (2003)
Anamarrhena asphodeloides Bge.	Asparagaceae	Hebei	Rhizome	Air dried	Antidiabetic effect	Jia et al. (2003)
Angelica sinensis (Oliv.) Diels	Apiaceae	Gansu	Root	Air dried	Fasting blood glucose ↓ Body weight ↓ Plasma cholesterol ↓ TAGs ↓	Wang et al. (2015)
Artactylodes macrocephala Koidz.	Compositae	Zhejiang	Rhizome	Fried with wheat bran	Antidiabetic effect	Jia et al. (2003)
Asparagus cochinchinensis (Lour.) Merr.	Asparagaceae	Sichuan	Earthnut	Air dried	Antidiabetic effect	Jia et al. (2003)
Astragalus membranaceus (Fisch.) Bge.	Fabaceae	Neimenggu	Root	Air dried	Antidiabetic effect Fasting blood glucose ↓	Jia et al. (2003) Yin et al. (2008)
Aucklandia lappa Decne.	Compositae	Guangxi	Root	Air dried	Body weight gain ↓ Adipogenesis ↓	Yoon et al. (2010)
Carthamus tinctorius L.	Asteraceae	Xinjiang	Corolla	Air dried	Fasting blood glucose ↓ Plasma cholesterol ↓ LDL ↓, VLDL ↓	Asgary et al. (2012)

Scientific name	Plant family	Origin province	Used part	Chinese crude drug processing	Described effect	Reference(s)
Codonopsis pilosula (Franch.) Nannf.	Campanulaceae	Gansu	Root	Air dried	Blood glucose ↓	He et al. (2011)
Coptis chinensis Franch.	Ranunculaceae	Sichuan	Rhizome	Air dried	Antidiabetic effect	Jia et al. (2003)
Crataegus pinnatifida Bge. var. *major* N.E.Br.	Rosaceae	Shandong	Fruit	Air dried	Antidiabetic potential	Chowdhury et al. (2014)
Curcuma wenyujin Chen et Ling	Zingiberaceae	Shaanxi	Earthnut	Fried or steamed with rice vinegar	Antidiabetic effect	Yanfu (2002)
Cyathula officinalis Kuan	Amaranthaceae	Sichuan	Root	Air dried	Antidiabetic effect	Yanfu (2002)
Dioscorea opposite Thunb.	Dioscoreaceae	Henan	Rhizome	Fried	Antidiabetic effect	Jia et al. (2003)
Gardenia jasminoides Ellis	Rubiaceae	Sichuan	Fruit	Fried	TAGs ↓, Cholesterol ↓ Inhibition PL	Lee et al. (2006)
Glycyrrhiza uralensis Fisch.	Fabaceae	Gansu	Rhizome	Air dried	Fasting blood glucose ↓ Body weight ↓	Yin et al. (2008)
Leonurus japonicus Houtt.	Lamiaceae	Henan	Herb	Air dried	Antidiabetic effect	Yanfu (2002)
Ligusticum chuanxiong Hort.	Apiaceae	Sichuan	Rhizome	Air dried	Antidiabetic effect	He et al. (2011)
Lycium barbarum L.	Solanaceae	Ningxia	Fruit	Air dried	Fasting blood glucose ↓ TAGs ↓, HDL ↑	Yin et al. (2008)
Lycopus lucidus Turcz. var. *hirtus* Regel	Lamiaceae	Sichuan	Stem, leaf	Air dried	Blood glucose ↓ Cholesterol ↓ TAGs ↓	Xiong et al. (2011)
Ophiopogon japonicas (L.f) Ker-Gawl.	Asparagaceae	Shaanxi	Earthnut	Air dried	Antidiabetic effect	Jia et al. (2003)
Paeonia lactiflora Pall.	Paeoniaceae	Gansu	Root	Air dried	Antidiabetic effect	He et al. (2011)
Phellodendron chinense Schneid.	Rutaceae	Hebei	Bark	Air dried	Antidiabetic effect	Yanfu (2002)
Polygonatum kingianum Coll. et Hemsl.	Asparagaceae	Sichuan	Rhizome	Steamed or cooked with rice wine	Antihyperglycaemic activity	Xie and Du (2011)
Poria cocos (Schw.) Wolf	-	Guangxi	Sklerotium	Air dried	Antidiabetic effect	Jia et al. (2003)
Pueraria lobata (Willd.) Ohwi	Fabaceae	Shaanxi	Root	Air dried	Antidiabetic effect	Jia et al. (2003)
Rehmannia glutinosa Libosch	Plantaginaceae	Henan	Earthnut	Air dried	Antidiabetic effect	Jia et al. (2003)
Scrophularia ningpoensis Hemsl.	Scrophulariaceae	Sichuan	Root	Air dried	Antidiabetic effect	Jia et al. (2003)
Scutellaria baicalensis Georgi	Lamiaceae	Gansu	Root	Air dried	Antidiabetic effect	He et al. (2011)
Trichosanthes kirilowii Maxim.	Curcurbitaceae	Hebei	Root	Air dried	Antihyperglycaemic activity	Xie and Du (2011)

TAGs: triacylglycerides; VLDL: very low density lipoprotein; HDL: high density lipoprotein

3.3. Pancreatic lipase inhibition

PL activity was measured by using an enzymatic *in vitro* assay based on the hydrolysis kinetics of an oleate ester of 4-methylumbelliferone. The assay was adopted from Sergent et al. (2012) and performed as follows: A volume of 25 µL of the sample solution (aqueous/methanolic plant extract, final concentration in the reaction mixture: 2.5 mg/mL) dissolved in dimethylsulfoxide (DMSO) and 25 µL of pancreatic lipase solution were mixed in the well of a 96-well microtiter plate. The enzyme and extract solutions were prepared immediately before use. Porcine pancreas powder was suspended in TRIS-HCl buffer (13 mM TRIS-HCl, 150 mM NaCl, 1.3 mM $CaCl_2$, pH 8.0) to give a concentration of 0.5 mg/mL. The mixture was pre-incubated for 10 min. Afterwards, 50 µL 4-methylumbelliferyl oleate (0.5 mM) were added to each well to initiate the enzyme reaction. The amount of 4-methylumbelliferone (4-MU) was measured at 37 °C over 30 min using a fluorescence reader (Tecan Group Ltd., Maennedorf, Switzerland) at an excitation and emission wavelength of 360 nm and 465 nm. For all measurements a 100 %-activity control, where the extract was replaced with the same volume of DMSO, was examined. Orlistat was used as positive control.

3.4. α-Amylase inhibition

The α-amylase inhibition was determined by using the fluorescence-based EnzChek® *Ultra Amylase Assay Kit* (Life Technologies, Carlsbad, CA, USA). This *in vitro* enzyme assay is based on the hydrolytic cleavage of a modified starch derivative. 25 µL of the sample solution (aqueous/methanolic plant extract dissolved in DMSO, final concentration in the reaction mixture: 2.5 mg/mL) and 25 µL α-amylase solution (porcine pancreas powder prepared at 1.25 µg/mL in the same buffer as used in pancreatic lipase activity assay) were mixed in a 96-well microtiter plate and pre-incubated for 10 min. The enzyme and extract solutions were prepared immediately before use. Then, 50 µL of substrate solution (DQ starch from corn, BODIPY FL conjugate, 200 µg/mL) were added to start the reaction. The accompanying increase in fluorescence is proportional to amylase activity and was monitored over 30 min at 37 °C using a fluorescence microplate reader (Em/Ex = 535/485). A 100 %-activity control was applied. Acarbose was used as positive control in this study.

3.5. Calculation of results

The measured fluorescence of each sample was corrected by subtracting the fluorescence of the respective blank. The enzyme activity was defined as increase of relative fluorescence units (RFU) per minute. The inhibitory activity of the extracts was defined as the difference between the enzyme activity in the 100 %-activity control (no inhibitor added) and the enzyme activity in the reaction mixture containing the aqueous/methanolic extract, expressed as a percentage of the enzyme activity of the positive control. The inhibitory activity was tested for each extract in quadruplicate.

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