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Antidiabetic *in vitro* and *in vivo* evaluation of cyclodipeptides isolated from *Pseudomonas fluorescens* IB-MR-66e†

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Three cyclodipeptides [cyclo(L-Pro-L-Leu), **1**; cyclo(L-Pro-L-Val), **2**; and cyclo(L-Pro-L-Phe), **3**] were isolated from *Pseudomonas fluorescens* IB-MR-66e. The structures were established by spectral means and corroborated by synthesis. The antidiabetic potential of compounds **1–3** was explored *in vivo*, *in vitro* and *in silico*. The three peptides showed important inhibitory activity against the α-glucosidase enzyme. Further analysis *in vivo* using a sucrose tolerance test corroborated that compounds **1** and **3** (1–30 mg kg^{-1}) significantly reduced the postprandial state. Peptide **1** (1–30 mg kg^{-1}) also reduced the postprandial peak after a glucose challenge and exhibited significant hypoglycemia during an insulin tolerance test; thus, its antidiabetic action involved also an improvement of insulin utilization not related to Akt phosphorylation nor to an increment in mitochondrial bioenergetics nor insulin secretion.

1 Introduction

Type 2 diabetes (T2DM) is a metabolic disease characterized by insulin deficiency resulting from inadequate insulin secretion or resistance.[1] The global prevalence of T2DM is rapidly increasing; thus, according to the International Diabetes Federation, in 2017 there were 425 million people with this disease worldwide, and 1% of these patients died from associated complications.[2] Therefore, there is an urgent need to discover new therapeutic alternatives to treat this important health problem. In this scenario, herein we have explored the potential of *Pseudomonas fluorescens* Migula (Pseudomonadaceae), isolated from mineral soils in Fresnillo, Zacatecas, Mexico, as a source of antidiabetic compounds.

P. fluorescens is a Gram-negative rod-shaped bacterium found in a wide range of environments, including the rhizosphere, mammalian hosts, and soil, as well as plants and indoor wall surfaces, just to mention the most important.[3] *P. fluorescens* biosynthesizes a few important secondary metabolites comprising 2,4-diacetylphloroglucinols, pseudomonic acid A derivatives, phenazines,[3–6] pyrrole derivatives,[7] indole alkaloids,[3,8] pyochelin type peptides,[9] and cyclodipeptides (CDPs) of the diketopiperazine (DKP) family.[10] Among the last group of compounds *P. fluorescens* biosynthesizes cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Phe) and cyclo(L-Pro-L-Leu). The first two are toxic to cells and seedlings of *Pinus thunbergii*,[11] while the other two are effective antifungal agents.[6]

The CDPs of the DKP family are also found in fungi, chocolate[12] and roasted food.[13,14] It has been suggested that their presence in food is the result of degradation of larger peptides.[12–14] In microorganisms these compounds are assembled by the catalysis of CDP-synthases.[15]

DKPs have recently gained interest due to their wide array of biological activities, including antibacterial, anti-fungal,[16] phytotoxic,[17] antiviral,[18,19] anti-prion[20] and antitumor.[21] Furthermore, a recent Japanese patent claims that some DKPs are α-glucosidase inhibitors (AGIs).[22] However, no *in vivo* test has supported this finding. AGIs are important therapeutic agents for treating the prediabetic condition and modulating post-prandial hyperglycaemia in combination with first line antidiabetic agents.[23]

As a part of a bioprospection program for the search of new antidiabetic agents, herein we describe the activity of a few dipeptides from *P. fluorescens* on yeast α-glucosidase using a well-known enzymatic assay. Furthermore, different *in vivo* and cell-based evaluations were carried out in order to assess the CDPs' effects on glycaemia, insulin secretion and mitochondrial respiration.

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2 Materials and methods

2.1 General experimental procedures

Melting points are uncorrected and were determined on a Fisher-Johns apparatus. IR spectra were recorded using a PerkinElmer 400 FT-IR spectrophotometer (Waltham, MA, USA). NMR spectra, including bidimensional, were recorded in CD_3OD or $CDCl_3$ solutions on a Bruker-Avance III HD spectrometer (Billerica, MA, USA) at either 500 MHz (^1H) or 125 MHz (^{13}C) using tetramethylsilane as an internal standard.

Column chromatography (CC) was carried out using silica gel. Flash chromatography was carried out with a Teledyne CombiFlashRf + Lumen (Thousand Oaks, CA, USA) chromatograph equipped with a photodiode array detector and an evaporative light scattering detector, using a RediSepRf high-performance GOLD silica gel column and eluting with a gradient of hexane, chloroform, and methanol. Thin layer chromatographic analyses were performed on silica gel 60 F254 plates (Merck, Kenilworth, NJ, USA), and visualization of the plates was carried out using a $Ce_2(SO_4)_3$ (10%) solution in H_2SO_4.

2.2 Bacterial material

Pseudomonas fluorescens strain IB-MR-66 was isolated from mineral soils randomly collected in Fresnillo Zacatecas, Mexico, in August 2014, as previously described.[24] The bacterium was identified based on morphological characteristics and sequence data of the [16S ribosomal RNA] region was deposited in GenBank as accession MH810270. Data available at GenBank aligning with strain IB-MR-66 suggested this bacterium is *P. fluorescens*.

2.3 Fermentation, extraction, and isolation

A single colony of *P. fluorescens* was inoculated into Luria Broth (LB) medium at 25 °C for 2 days. Then, the culture was inoculated into 1.5 litres LB medium in a 2 L flask and cultured under agitation at 25 °C for 7 days. A 1 : 1 mixture of MeOH–CH_2Cl_2 was added to the final culture. A liquid–liquid extraction was carried out (300 mL × 3) and the organic phase was evaporated to dryness under a vacuum. The extract was suspended in 150 mL of a mixture of MeCN–MeOH (1 : 1) and partitioned with *n*-hexane (8 × 150 mL). The combined MeCN–MeOH fractions were dried under a vacuum, and the resulting residue (0.3 g) was dissolved in CH_2Cl_2 and subjected to flash chromatography using a gradient of *n*-hexane–$CHCl_3$ (0.0–10.0 min, 100 : 0 → 0 : 100) and $CHCl_3$–MeOH (10.0–17.5 min, 100 : 0, 17.5–25.0 min, 100 : 0 → 98 : 2; 25.0–32.5 min, 98 : 2 → 95 : 5; 32.5–45.0 min, 95 : 5 → 90 : 10; 45.0–65.0 min, 90 : 10 → 80 : 20, 65.0–90.0 min, 80 : 20 → 0 : 100). The separation process yielded nine primary fractions (F1–F9). Based on their yield F7 and F9 were further purified. F7 (45 mg) was subjected to open silica gel column eluting with CH_2Cl_2–MeOH (80 : 20) to yield **1** (5 mg). F9 (35 mg), was also purified by silica column chromatography eluting with CH_2Cl_2–MeOH (70 : 30) to yield **3** (3 mg).

2.4 Inhibition of α-glucosidases

The bacterial extract, fractions, compounds, and acarbose (positive control) were dissolved in MeOH. Aliquots of 0–40 μL of testing materials (triplicated) were incubated for 10 min with 20 μL of enzyme stock solution (0.4 units mL^{-1} in phosphate buffer solution 100 mM, pH 7). After incubation, 10 μL of substrate (*p*NPG 5 mM) was added and further incubated for 30 min at 37 °C, and the absorbance of each sample was determined. The inhibitory activity was expressed as IC_{50} as previously reported.[25]

2.5 Theory calculation

The minimized structures for docking simulations were prepared using Autodock Tools package v1.5.4 (ADT, http://mgltools.scripps.edu/) as previously described.[25]

2.6 Animals

Experimental procedures were implemented in accordance with internationally accepted principles for laboratory animal use and care and following the Mexican Official Norm for Laboratory Animal Care and Use (NOM-062-ZOO-1999). The Institutional Committee for Care and Use of Laboratory Animals (CICUAL-FQ) of Facultad de Química UNAM approved the protocols (FQ/CICUAL/232-2/17 and FQ/CICUAL/334/18). Evaluations were conducted on 4-week old male ICR mice, obtained from Centro UNAM-Envigo (Envigo RMS S.A. de C.V.). Animals were held under a controlled temperature (22 ± 2 °C) on a light–dark (12 h–12 h) cycle, with free access to a standard diet and purified water before experiments. Six-to-eight normoglycaemic and/or hyperglycaemic mice were grouped for each experiment. Blood samples were obtained by venipuncture on the lateral saphenous vein of the right leg using a 31 G × 5 mm needle. Glycaemia (mg dL^{-1}) was measured using a commercial glucometer (One Touch Ultra 2, Johnson & Johnson, NJ, USA). At the end of the experiments, mice were euthanized by hypoxia in a CO_2 chamber.[26] For organ and tissue recovery, mice were euthanized by porta-exsanguination after isoflurane anaesthesia.

2.7 Sample preparation and route of administration

Three logarithmic doses of CDPs **1–3** and single doses of positive controls acarbose and metformin were suspended in a vehicle consisting of isotonic saline solution with 0.05% Tween 80 and given *per os* (p.o., orally) using a gavage feeding needle (18 G, 35 mm; 0.2 mL per 10 g bodyweight). Insulin (Humulin®, Indianapolis IN, USA) was dissolved in sterile isotonic saline solution and intraperitoneally administered (0.1 mL per 10 g bodyweight).

2.8 Oral carbohydrate tolerance tests

Normoglycaemic mice were deprived of food 4 h before the experiment with free access to drinking water. After fasting, basal glycaemia was registered. For the sucrose tolerance test, the mice received the vehicle, or acarbose (5 mg kg^{-1} p.o.), or compounds **1** and **3** (1, 10 and 30 mg kg^{-1} p.o.). Half-an-hour later, all animals received an oral sucrose challenge (2 g kg^{-1}). Blood samples were collected at 10, 20, 30, 60, 90 and 120 min after carbohydrate load. Oral glucose tolerance tests for CDPs **1** and **3** were conducted using a similar experimental design in both normoglycaemic and hyperglycaemic mice, but using metformin (200 mg kg^{-1}) as a positive control, and a glucose challenge of 1 g kg^{-1} p.o.

2.9 Insulin tolerance test and organ recovery

Normoglycaemic mice were deprived of food for 4 h before the experiment. After this time, basal glycaemia was measured, and the mice received either the vehicle, or a single dose of compound **1** (30 mg kg^{-1}) or **3** (10 mg kg^{-1}). Thirty minutes after treatment, the mice received an intraperitoneal dose of 0.05 U kg^{-1} of insulin. Glycaemia was measured at 10, 20 and 30 minutes following insulin administration. In an independent but similar experimental design, liver, adipose tissue and gastrocnemius muscle were obtained from vehicle, insulin and compound **1** + insulin treated mice (n = 3). The organs were removed after 20 min of the administration of insulin. Samples were kept in liquid nitrogen and stored at −70 °C until use.

2.10 Western blot analysis

Frozen liver, adipose tissue and gastrocnemius muscle were homogenized in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 5 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 50 mM NaF and 1% NP-40) with protease inhibitor cocktail (cOmplete, Roche, Merck, Mexico). The protein concentration was determined using the DC protein assay kit (Bio-Rad Laboratories, Richmond, CA). Tissue lysates (20 μg) were combined with Laemmli sample buffer and separated by SDS-PAGE. After electrophoretic separation, the proteins were electrotransferred to a PVDF membrane using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA; USA), blocked and incubated overnight at 4 °C with the anti-AKT (sc-8312, Santa Cruz Biotechnology, Dallas, TX, USA), anti-phospho-AKT (9018, Cell Signaling, Technology, Inc., Danvers, MA, USA) and anti-actin antibody (sc-1615, Santa Cruz Biotechnology, Dallas, TX, USA). Band detection was carried out using a chemiluminescent Western blotting kit (Immobilon Western Chemiluminescent HRP Substrate, Millipore, Burlington, MA, USA). Digital images of the membranes were obtained using a ChemiDoc MP densitometer and processed by Image Lab software (Bio-Rad, Hercules, CA, USA). Results are reported relative to Akt and β-actin. Blots were quantified using NIH Image J software (v. 1.49, National Institutes of Health, USA).

2.11 INS-1E cell culture and insulin secretion assays

Rat insulinoma INS-1E cells (provided by Dr P Maechler, University of Geneva, Switzerland) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. For insulin secretion assays, rat insulinoma INS-1E cells were seeded on 24-well plates and after 48 h treated with 0, 5, 10 or 20 μg μL^{-1} of compound **1** for 2 h. After treatment, rat insulinoma INS-1E cells were washed 3 times with Krebs-Ringer bicarbonate buffer (KRBB: 135 mM NaCl, 3.6 mM KCl, 2 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 10 mM HEPES, pH 7.4, and 0.1% bovine serum albumin) containing 2.5 mM glucose. Rat insulinoma INS-1E cells were incubated for 30 min in KRBB containing 2.5 mM glucose and then maintained on 2.5 mM glucose or switched to 8.3 mM glucose for an additional 1 h. After incubation, the medium was collected and frozen in order to determine the insulin concentration using an ELISA kit. The total insulin content was determined in an aliquot obtained by addition of acidified ethanol (75% ethanol/1.5% HCl) to the attached cells. The insulin secretion was expressed as the percentage of insulin secreted into the media with respect to the sum of the secreted and total insulin content.

2.12 Mitochondrial respiration

A Seahorse Extracellular Flux (XF) 96 Analyzer (Seahorse Bioscience, Inc, North Billerica, MA, USA) was used to measure the oxygen consumption rate (OCR) in mouse myoblast cells C2C12 after sequential addition of oligomycin (2 μM), FCCP (0.5 μM) and a mixture of rotenone plus antimycin A (0.5 μM) to the respiring cells. Basal mitochondrial and ATP-linked respiration, proton leak and the spare capacity were then calculated as previously described.[27] The OCR was measured after the cells were incubated for 18 h with compound **1** (10 μg mL^{-1}).

2.13 Data treatment and statistical analyses

Glycaemia values were used to calculate the percentage of glycaemia variation.[26] Furthermore, areas under the curve (AUC) applying the trapezoidal rule were obtained. The results are expressed as the mean ± SEM of glycaemia variation or AUC. Statistical significance ($p < 0.05$) was assessed with the GraphPad Prism software (version 5.0; GraphPad Inc., La Jolla, CA, USA) using one-way or two-way ANOVA tests followed by an appropriate *post hoc* test.

3 Results and discussion

3.1 Isolation, synthesis and characterization

An organic extract from the culture of *P. fluorescens* inhibited the activity of yeast α-glucosidase in a concentration dependent manner (IC_{50} = 1000 ppm). Fractionation of the extract led to the isolation of the three CPDs (**1–3**) shown in Fig. 1.

The compounds were characterized by spectral and synthetic means as cyclo(L-Pro-L-Leu) (**1**), cyclo(L-Pro-L-Val) (**2**), and cyclo-(L-Pro-L-Phe) (**3**).[28–30] The synthesis of **1–3** was performed as previously described (Scheme 1),[28] but using monowave Anton

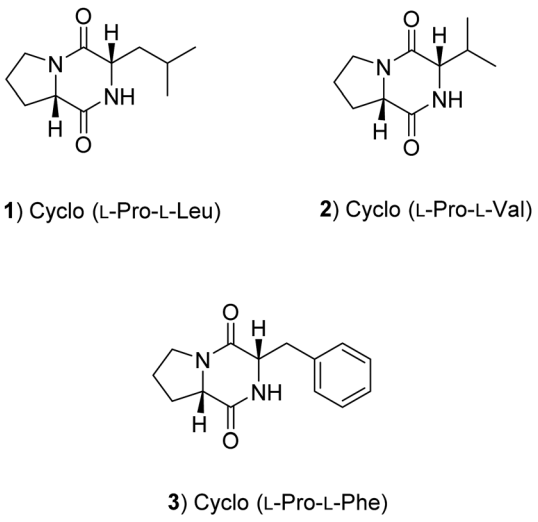
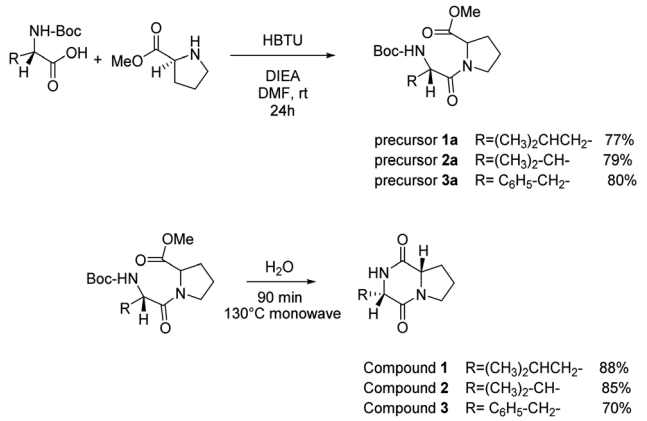


Fig. 1 CPDs isolated from the culture of *P. fluorescens*.



Scheme 1 General synthesis of dipeptides **1–3**.

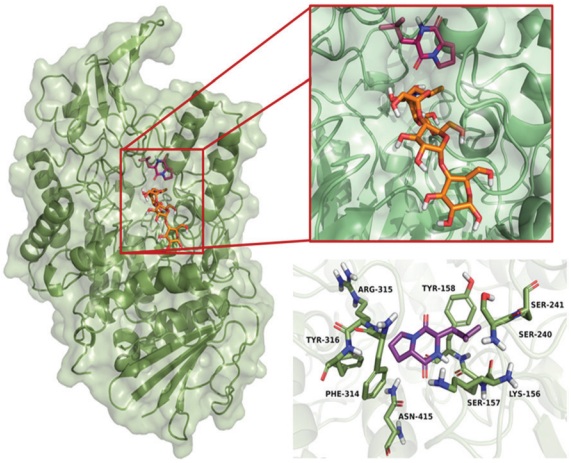


Fig. 3 Model of binding for compound **1** and acarbose (purple and orange sticks respectively) with αGHY (green cartoon, PDB 3A4A).

Paar equipment for heating at 120 °C for 1 h 30 min. This heating method allowed faster results than that of an autoclave at 180 °C for 24 h previously used.[28]

3.2 Inhibition of α-glucosidases

Compounds **1–3** were tested for their ability to inhibit yeast α-glucosidase activity. The IC_{50}s calculated were 4.6 ± 0.2, 3.2 ± 0.5 mM and 3.4 ± 0.6, respectively *versus* 1.6 ± 0.1 mM for acarbose. Interestingly when the synthetic lineal peptides (**1a–3a**) used as precursors of the natural products were tested against the enzyme, no inhibitory activity was observed, revealing that the diketopiperazine subunit had an important impact in the inhibitory action.

3.3 Oral sucrose tolerance test

The inhibitory activity of compounds **1–3** on yeast α-glucosidase prompted us to evaluate their *in vivo* effect on mammal enzymes during a sucrose tolerance test (OSTT) in normal ICR mice. Compound **1** was able to reduce glycaemia levels during the first 30 minutes of the OSTT; this effect was not dose dependent and the highest activity was observed at doses 10 and 30 mg kg^{-1} (Fig. 2, panel A). Compound **3** decreased the postprandial peak only at a dose of 30 mg kg^{-1} (Fig. 2, panel B). Compound **2** was inactive. The *in vivo* effects of **1** and **3** are consistent with the inhibitory action of CPDs on α-glucosidases.

3.4 Docking

In order to envisage the putative binding mode of the most active compound **1**, a docking analysis was carried out using the crystallized structure of α-glucoside hydrolase from *Saccharomyces cerevisiae* (αGHY, PDB code 3A4A).[31] The results revealed that compound **1** bound to the catalytic site of αGHY: the binding energy was −6.1 kcal mol^{-1} while that for acarbose was −8.6 kcal mol^{-1}. The interactions in this site included hydrophobic contacts with Lys156, Tyr158, Asp242, Val232, Asp233, Phe314, Arg315, Tyr316 and Asn415, and hydrophilic interactions with Ser157, Ser240 and Ser241 (Fig. 3). Hydrogen bonding between compound **1** and Ser157 was also observed.

3.5 Oral glucose tolerance test

Compounds **1** and **3** were tested in an oral glucose tolerance test (OGTT), in order to determine their antidiabetic potential

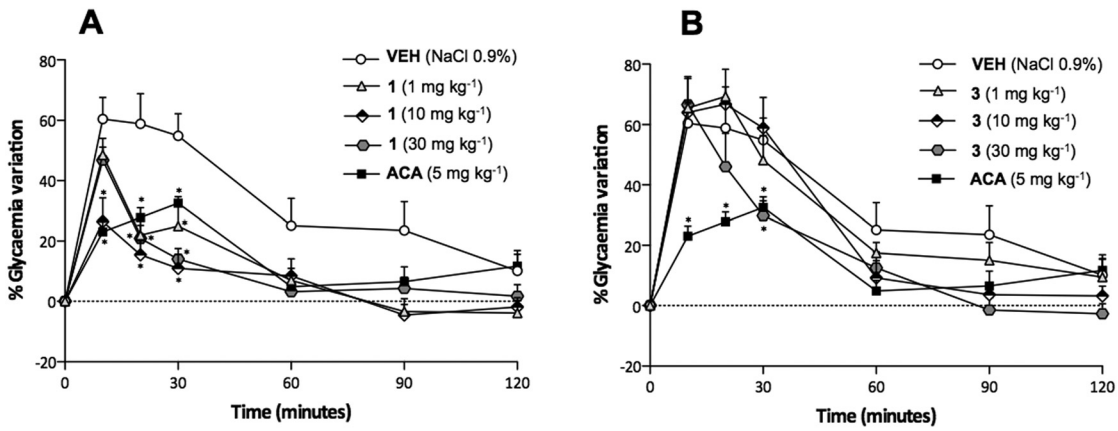


Fig. 2 Effect of cyclodipeptides **1** (panel A) and **3** (panel B) in normoglycaemic ICR mice during an oral sucrose tolerance test (OSTT). VEH: vehicle; ACA: acarbose. Each point represents the mean ± SEM of 6–8 mice in each group. *$p < 0.01$ significantly different from the vehicle group (two-way ANOVA followed by a Bonferroni *post hoc* test).

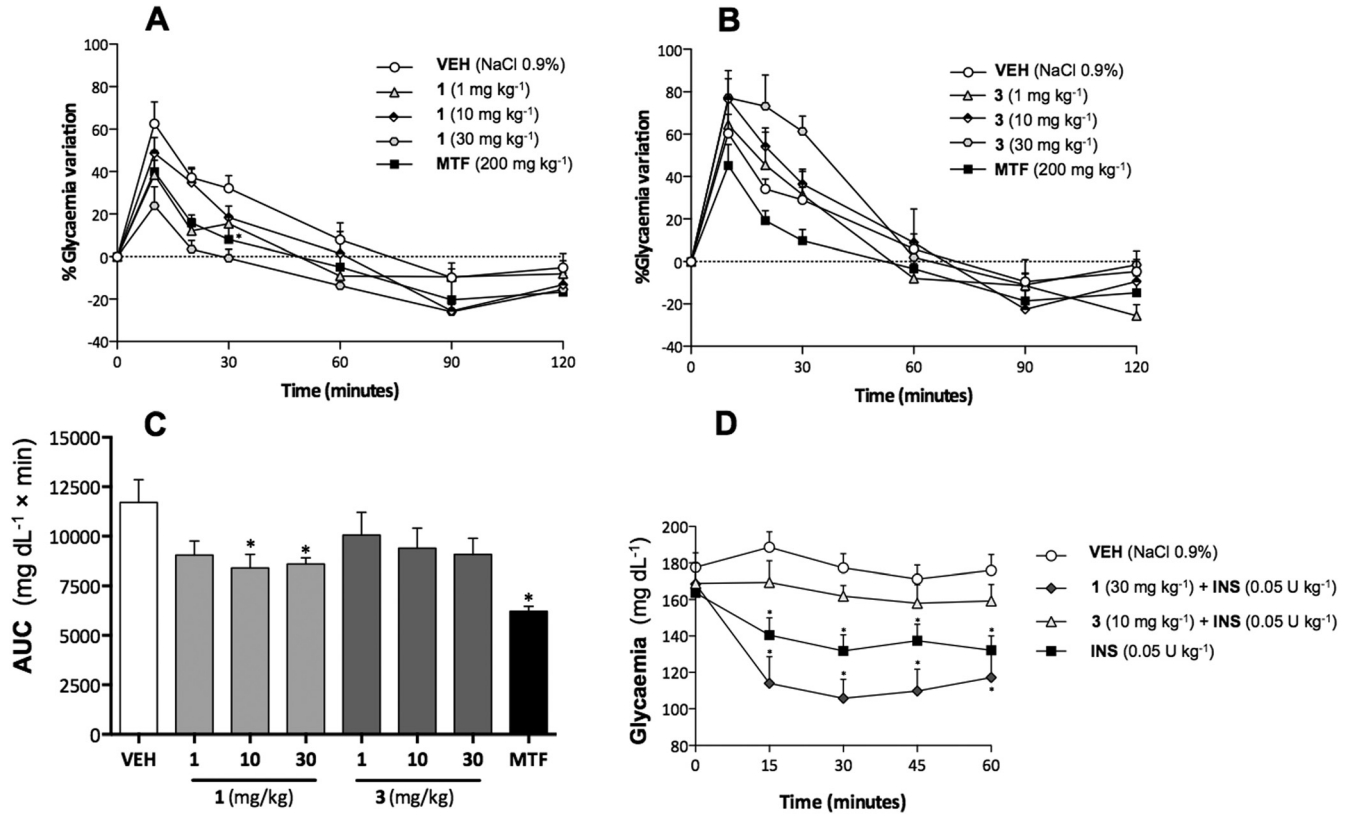


Fig. 4 Effect of cyclodipeptides **1** and **3** during an oral glucose tolerance test (OGTT) and intraperitoneal insulin tests. Panels A and B, OGTT in normoglycaemic mice treated with CDPs **1** and **3**, respectively; panel C, OGTT in hyperglycaemic mice treated with CDPs **1** and **3**; panel D, insulin tolerance test in normoglycaemic mice treated with CDPs **1** and **3**. VEH: vehicle, MTF: metformin, INS: insulin. Each point or bar represents the mean ± SEM of 6–8 mice per group. $*p < 0.05$ significantly different from vehicle group (ANOVA followed by Bonferroni or Dunnet's *post hoc* tests).

independent of the α-glucosidase inhibition. As shown in Fig. 4, in normoglycaemic mice, cyclodipeptide **1** was able to reduce the hyperglycaemia provoked by the oral glucose challenge at a dose of 30 mg kg^{-1} (panel A); on the other hand, cyclopeptide **3** did not have any effect at the doses tested (panel B). In hyperglycaemic mice only **1** (10 and 30 mg kg^{-1}) had antihyperglycaemic action (panel C) suggesting that compound **1** might increase insulin sensitivity. Indeed, further testing during an insulin tolerance test revealed that **1** elicited a significant hypoglycaemic effect. In contrast, peptide **3** reverted the hypoglycaemic action of insulin (Fig. 4, panel D). The hypoglycaemic effect of compound **1** could be attributed to an improvement in insulin signalling, modification of insulin secretion and/or an increase in mitochondrial bioenergetics, among other mechanisms.

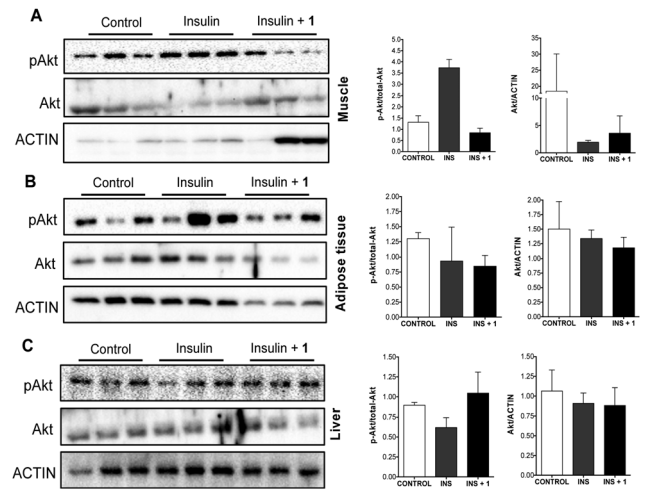


Fig. 5 Akt phosphorylation in gastrocnemius muscle (A), adipose tissue (B) and liver (C) of mice treated with compound **1** during an insulin tolerance test (0.05 U kg^{-1}). Representative blots are shown. The bar graphs represent the mean ± SEM of quantitative densitometry of 3 mice.

3.6 Effect of compound 1 on insulin signalling

To evaluate whether the hypoglycaemic effect of compound **1** involved an improvement in insulin sensitivity due to enhanced

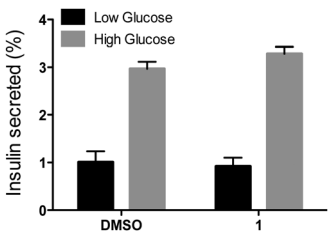


Fig. 6 Effect of compound **1** on insulin secretion in rat insulinoma INS-1E cells incubated with compound **1** (10 μg mL^{-1}) for two hours before the insulin secretion assay. Each bar is the mean ± SEM of two independent experiments.

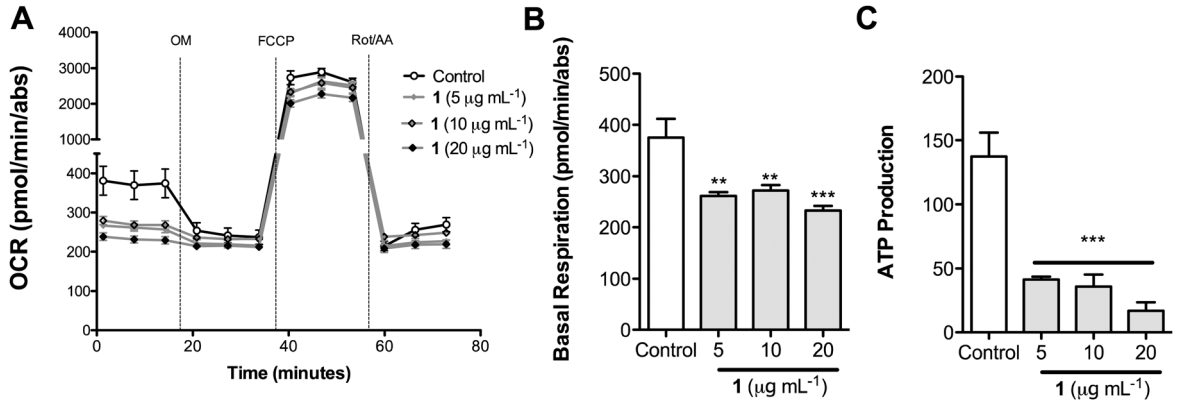


Fig. 7 Oxygen consumption rate, basal respiration and ATP production in mouse myoblast cells C2C12 for **1** (18 h incubation). Each bar is the mean ± SEM of two or three independent experiments. One-way ANOVA followed by Dunnett's test (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

insulin signalling, phosphorylation of Akt was assessed in gastrocnemius muscle, adipose tissue and liver of mice treated with the vehicle or compound **1** during an insulin tolerance test. As expected, insulin increased Akt phosphorylation in gastrocnemius muscle; however, in compound **1**-treated mice there was an important reduction of the phosphorylation of the protein (Fig. 5, panel A). In the adipose and liver tissues, the Akt phosphorylation level did not change in either treated mouse group (Fig. 5, panels B and C), probably due to the fact that the mice used were not obese. Our findings were consistent with the inhibition of Akt phosphorylation in HeLa cells exerted by a mixture of cyclodipeptides obtained from *P. aeruginosa* PAO1.[21] Our results ruled out implication of Akt-phosphorylation in the hypoglycaemic effect of **1**.

3.7 Insulin secretion

Next, to find out if the antihyperglycaemic action of compound **1** implicated modulation of insulin secretion, an *in vitro* cell based test using rat insulinoma INS-1E was performed.[32] As observed in Fig. 6, **1** was not able to promote insulin secretion *in vitro*. Our results are in agreement with a previous report in which cyclo(L-His-L-Pro), a metabolite of the thyrotropin releasing hormone, did not affect insulin secretion in mouse pancreatic islets.[33]

3.8 Effect of compound 1 on mitochondrial bioenergetics

Hyperglycaemia has also been linked to alterations of mitochondrial bioenergetics,[34,35] therefore the effect of **1** on mouse myoblast cell C2C12 mitochondrial energetics was evaluated over 18 hours. The results indicated that the basal oxygen consumption rate (OCR) in mouse myoblast cells C2C12 treated with **1** was significantly reduced. This reduction was primarily due to a decrease in the OCR associated with ATP production in a concentration dependent fashion, thus reducing the mitochondrial oxidative capacity (Fig. 7). Previous reports showed that cyclo(L-His-L-Pro) decreased the mitochondrial capacity due to a reduction in cytochrome *C* reductase and malic enzymes.[36] The results obtained for muscle tissue and mouse myoblast cells C2C12 suggest that compound **1** might have important implications in cell proliferation.

4 Conclusions

These results suggest that compound **1** exerts its antidiabetic action throughout a mechanism involving α-glucosidase inhibition and other mechanisms yet to be established. For instance, an incretin-based mechanism or modulation of other upstream kinases such as phosphorylation of PTP1B cannot be ruled out at the present time. Since compound **1** does not affect insulin secretion but improves its utilization, it might be a good candidate for further investigation for drug development. Moreover, the presence of DKPs in food products might be useful for preventing diabetes or its progression upon their consumption. The fact that compound **1** decreased Akt phosphorylation in muscle and mitochondrial respiration linked to ATP production highlights also its potential as an antitumor agent.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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