Journal Pre-proofs

Food-inspired peptides from spinach Rubisco endowed with antioxidant, antinociceptive and anti-inflammatory properties

Lorenza Marinaccio, Gokhan Zengin, Stefano Pieretti, Paola Minosi, Edina Szucs, Sandor Benyhe, Ettore Novellino, Domiziana Masci, Azzurra Stefanucci, Adriano Mollica

PII: S2590-1575(23)00082-2
DOI: https://doi.org/10.1016/j.fochx.2023.100640
Reference: FOCHX 100640

To appear in: *Food Chemistry: X*

Received Date: 1 December 2022
Revised Date: 22 February 2023
Accepted Date: 10 March 2023

Please cite this article as: L. Marinaccio, G. Zengin, S. Pieretti, P. Minosi, E. Szucs, S. Benyhe, E. Novellino, D. Masci, A. Stefanucci, A. Mollica, Food-inspired peptides from spinach Rubisco endowed with antioxidant, antinociceptive and anti-inflammatory properties, *Food Chemistry: X* (2023), doi: https://doi.org/10.1016/j.fochx.2023.100640

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2023 Published by Elsevier Ltd.

Journal Pre-proofs

1 **Food-inspired peptides from spinach Rubisco endowed with antioxidant, antinociceptive and**
2 **anti-inflammatory properties**

3 Lorenza Marinaccio,[a] Gokhan Zengin,[b] Stefano Pieretti,[c] Paola Minosi,[c] Edina Szucs,[d] Sandor
4 Benyhe,[d] Ettore Novellino,[e] Domiziana Masci,[f] Azzurra Stefanucci,[a,*] Adriano Mollica[a]

5 [a]Department of Pharmacy, G. d'Annunzio University of Chieti-Pescara, 66100 Chieti, Italy;

6 [b]Department of Biology, Science Faculty, Selcuk University, Konya 42130, Turkey;

7 [c]National Centre for Drug Research and Evaluation, Istituto Superiore di Sanità, 00161 Rome, Italy;

8 [d]Institute of Biochemistry, Biological Research Centre, 6726 Szeged, Hungary;

9 [e]NGN Healthcare, Via Nazionale Torrette, 207, 83013 Mercogliano, Italy;

10 [f]Department of Basic Biotechnological Sciences, Intensivological and Perioperative Clinics, Catholic
11 University of Sacred Heart, Largo Francesco Vito 1, 00168 Rome, Italy.

12 ***Correspondence:** a.stefanucci@unich.it; phone: +39-08713554904

13

14

15

16

17

18

19

20

21

22 **ABSTRACT:** Rubiscolin-6 (amino acid sequence: YPLDLF) is a selective δ-opioid receptor peptide
23 isolated from spinach Rubisco. Its synthetic analogue, peptide YPMDIV is the most potent described
24 so far for its increased opioid activity, thus in this work it was considered as *lead compound* for the
25 design of twelve new analogues *e.g.* **LMAS1-12**. Firstly all the novel compounds have been tested
26 for their antinociceptive and anti-inflammatory capacity *in vitro* and *in vivo* in order to evaluate their
27 ability to maintain or loss the original activity. Among them peptides **LMAS5-8** gave the best results,
28 thus their antioxidant properties have been investigated along with their enzymatic inhibitory ability.
29 Peptide **LMAS6** shows a strong antioxidant (154.25 mg TE/g CUPRAC) and inhibitor activity on
30 tyrosinase (84.49 mg KAE/g), indicating a potential role in food industry as anti-browning agent,
31 while peptides **LMAS5** and **LMAS7** possess a modest cholinesterase inhibitory activity suggesting
32 a conceivable use for nutraceuticals production.

33 **KEYWORDS:** rubiscolin-6, Rubisco, antioxidants, anti-browning agent, nutraceuticals

Journal Pre-proofs

1. INTRODUCTION

Plant and animal food proteins are considered rich sources of bioactive peptides, which are classified on the basis of their biological effects, *e.g.*, antithrombotic, antimicrobial, antihypertensive, opioid, immunomodulatory and antioxidant (Sánchez & Vázquez, 2017; Udenigwe & Aluko, 2012). Given their small size, sample preparation's process, high potency and selectivity, they are an excellent example of tools to inhibit protein-protein interactions. Food derived bioactive peptides may play an important role in the regulation of various human functions acting directly through food's consumption or after *in vivo* or *in vitro* hydrolysis of the proteins in which they are encrypted (Hartmann & Meisel, 2007). In fact they are generally a mixture of small size-peptides because of the high cost of purification and low yield of extraction; after purification, some of them may lose their additive or synergistic activity with polyphenols or other food components, resulting in inactive compounds that are useless as nutraceuticals. However, the literature reports examples of isolated bioactive compounds with proven physiological effects that can be added to food products (Day, Seymour, Pitts, Konczak, & Lundin, 2009). For instance, antioxidant peptides derived from the digestion process of different proteins may be added to food products to provide them antioxidant benefits (Lafarga & Hayes, 2014). Indeed, bioactive peptides with antioxidant properties have been used as free radical scavengers in meat products (Sohaib et al., 2017; Tkaczewska, 2020). The antioxidant effect of protein hydrolysates is related to their amino acids content (Sánchez et al., 2017).

Rubiscolin-6 (amino acid sequence: YPLDLF) is an interesting natural peptide recently evaluated for its antioxidant activity; it was identified in the spinach Rubisco large subunit (residues 103-108) (Stefanucci et al., 2020; Yang et al., 2001). Rubisco (D-ribulose-1,5-bisphosphate carboxylase/oxygenase; EC 4.1.1.39) represents the 10-30% of the whole amount of leaves proteins. It is composed of two subunits A and B, combined in a type-structure A_8B_8 (Kobbi et al., 2017; Taylor & Andersson, 1997; Yoshikawa, 2015). This enzyme plays an important role in carbon dioxide fixation and photorespiration (Yoshikawa, 2015), and it is involved in food's production for the whole biosphere. A recent study showed that Rubisco hydrolysate from alfa-lfa green juice has significant antioxidant properties preventing linoleic acid oxidation, decreasing ferric ion and inducing stable ABTS^{*+} compared to the unhydrolysate protein (Kobbi et al., 2017).

Rubiscolin-6 is a selective δ-opioid receptor peptide agonist highly conserved in a huge variety of plants (Yang et al., 2001). It exhibits several effects, *e.g.*, antinociceptive activity, anxiolytic-like

Journal Pre-proofs

74 effect, orexigenic action, enhancement of glucose uptake in skeletal cell lines, memory consolidation
75 and reduction of skin inflammation (Chajra, Amstutz, Schweikert, Auriol, Redziniak, & Lefevre,
76 2015; Hirata, Sonoda, Agui, Yoshida, Ohinata, & Yoshikawa, 2007; Kairupan et al., 2019; Kaneko
77 et al., 2012; Miyazaki et al., 2014; Yang, Kawamura, & Yoshikawa, 2003; Yang et al., 2001).
78 Numerous rubiscolin-6 analogues have been already synthesized to evaluate its structure-activity
79 relationships in order to improve its δ-opioid activity (Yang, Sonoda, Chen, & Yoshikawa, 2003).
80 The structural features of rubiscolin-6 analogues required to guarantee a high δ-opioid activity have
81 been also determined through 3D quantitative structure-relationship (QSAR) analysis (Caballero,
82 Saavedra, Fernández, & González-Nilo, 2007). Thus researchers have considered the hypothesis of
83 using rubiscolin-6 and its derivatives for the production of functional foods and as *lead compounds*
84 for the development of new analogues (Stefanucci et al., 2020).

85 Rubiscolin-6 and its *C*-terminal amide analogue have been recently tested *in vitro* for their antioxidant
86 activity, however rubiscolin-6 *C*-amide showed low DPPH radical scavenging activity (2.72 mg
87 TE/g), while rubiscolin-6 gave the best result in ABTS assay (8.86 mg TE/g) (Stefanucci et al., 2020).
88 Among the novel analogues, YPMDIV showed an increased δ-opioid activity on mouse vas deferens
89 (MVD) assay in comparison with rubiscolin-6 (Yang, Sonoda, et al., 2003). In the current study,
90 YPMDIV has been assumed as *lead compound* for the design of twelve new analogues named
91 **LMAS1-12** (Figure 1), with the aim to find bioactive peptides endowed with antioxidant and enzyme
92 inhibitory activities potentially useful as food ingredients or in the development of new nutraceuticals.

93 ## 2. MATERIALS AND METHODS

94 ### *2.1 Design*

95 A rational structure-based design approach has been applied to the development of novel peptide
96 analogues of rubiscolin-6. Considering the multi-residue replacement described by a SAR study in
97 literature (Yang, Sonoda, et al., 2003), we selected the synthetic peptide YPMDIV as the *lead*
98 *compound* for further structural modifications. The following findings have been taken in
99 consideration in order to design the novel bioactive peptides: *i*) the protonated Tyr at the *N*-terminus
100 fits the essential requirement of message domain for opioid receptor binding; *ii*) the Pro^2 residue
101 contributes to the restriction of peptide conformation and stability; *iii*) the presence of Met^3 enhances
102 the opioid activity in MVD about four times, while Ala^3 and Val^3, Phe^3 and Trp^3 decreased the activity
103 in MVD assay; *iv*) Asp^4 is the ideal residue for high activity and selectivity on δ-opioid receptor
104 (DOR); *v*) in the fifth position of Rubiscolin-6, hydrophobic residues increase the potency at DOR,
105 the preferential order is Leu > Ile > Met > Val > Ala; *vi*) the Phe^6 in rubiscolin-6 can be replaced by
106 aliphatic residue without losing δ-opioid activity. In a first attempt we explored the substitution of
107 Met^3 in the *lead compound* with an unnatural residue of (2*S*,4*S*)-4-(methylthio)pyrrolidine-2-
108 carboxylic acid (Proline-Methionine chimeras) prepared in laboratory following the procedure
109 previously described by Mollica *et al*. (Mollica, Paradisi, Varani, Spisani, & Lucente, 2006) linked
110 to both D/L-Pro^2 (compounds **LMAS1-4**), with the aim to improve the metabolic stability of the
111 former peptides as *C*-terminal acids and amides. Since methionine is one of the most documented
112 amino acid responsible of antioxidant activity in several peptides (Lorenzo et al., 2018), the
113 replacement with Cys and the shortening of the Met^3 side chain have been also investigated in
114 peptides of the series **LMAS5-8** and **LMAS9-12** respectively. According to the SAR studies (Yang,
115 Sonoda, et al., 2003), all the other amino acids in position first, fourth, fifth and sixth have been
116 conserved. These modifications are intended to understand the role exerted by this amino acid in
117 varying the biological activity of such modified analogues of rubiscolin-6. The *lead compound* has
118 been prepared as reference for the biological assays (Figure 1).

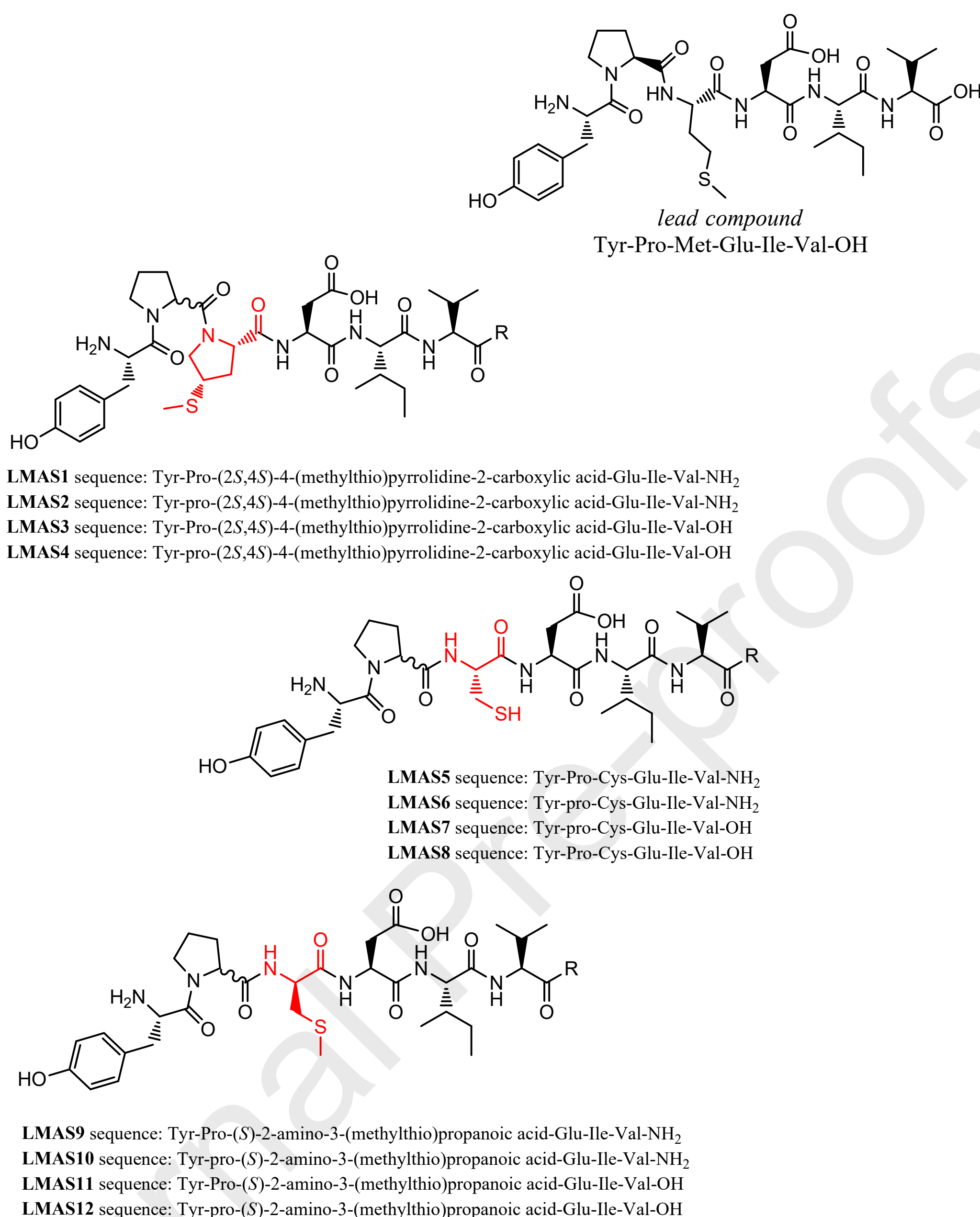


Figure 1. Structure of the *lead compound* (YPMDIV) and peptides **LMAS1-12**.

2.2 Chemistry

Coupling reagents and solvents have been purchased by VWR (Radnor, PN, USA). Fmoc-Tyr(*t*-Bu)-OH, Fmoc-Pro-OH, Fmoc-D-Pro-OH, Fmoc-Met-OH, (*S*)-2-amino-3-(methylthio)propanoic acid, Fmoc-Cys(Trt)-OH, Fmoc-Asp(*t*-Bu)-OH, Fmoc-Ile-OH and Fmoc-Val-OH by Chem-Impex (Wood Dale, IL, USA). The synthetic amino acid residue (2*S*,4*S*)-4-(methylthio)pyrrolidine-2-carboxylic acid has been synthesized as previously reported in literature (Mollica, Paradisi, Varani, Spisani, & Lucente, 2006). Commercial amino acid (*S*)-2-amino-3-(methylthio)propanoic acid has been protected with fluorenylmethoxycarbonyl protecting *group* (Fmoc) before its use (Carpino and Han, 1979). Fmoc-Rink amide and 2-chlorotrityl chloride resins (Loading coefficient: 0.74 mmol/g and 1.60 mmol/g respectively) by IRIS Biotech GmbH (Marktredwitz, DH, Germany). Peptides have been prepared by manual solid-phase synthesis (SPPS) using TBTU/HOBt/DIPEA and TBTU/2,4,6-trimethylpyridine for Fmoc-Cys(Trt)-OH reaction, as coupling mixture; a solution of piperidine 20%/DMF for Fmoc-deprotection. A strong cleavage has been applied to the peptide-resin system

134 using TFA/TIPS/H_2O for **LMAS1-4,9-12** or TFA/TES/H_2O for **LMAS5-8** as cocktails (95:2.5:2.5)
135 (Scheme S1, see SI). The final peptides as TFA salts have been purified by RP-HPLC system
136 equipped with Waters XBridge Prep BEH130 C18, 5.0 μM, 250-10 mm, 7.0 mL/min, Waters Binary
137 1525 pump, H_2O + 0.1% TFA/ACN + 0.1% TFA gradient from 5% to 95% of ACN in 35 minutes
138 (wavelengths: 213 nm, 254 nm and 275 nm). Peptides purity as TFA salts was assessed using ^{1}H-
139 NMR (Varian Inova 300MHz) in DMSOd_6 and RP-HPLC analytic column C-18, 4.6-150 mm, 1
140 mL/min, H_2O + 0.1% TFA/ACN + 0.1% TFA gradient from 5% to 95% of ACN in 30 minutes. Mass
141 spectrometer LCQ (Thermo Finnigan, San Jose, CA, USA) with capillary temperature 300°C,
142 electrospray ion Source (ESI), 4.00 Kv, auxiliary and preservative gas N_2 has been used to confirm
143 the compound's identity. The purity of all the final peptides resulted to be ≥ 95% after purification
144 (see SI).

145 *2.3 Opioid binding assay and GTP stimulation*

146 The opioid receptor binding affinity has been calculated performing displacement assay on μ-opioid
147 receptor (MOR), δ-opioid receptor (DOR) and κ-opioid receptor (KOR) on rat and guinea pig brain
148 membrane homogenates. For the detailed procedures see the supporting information.

149 *2.4 In vitro Antioxidant assay*

150 Methodologies applied in our previous literature have been used (Uysal et al., 2017), considering the
151 following parameters: mg Trolox equivalents (TE)/g extract in 2,2-diphenyl-1-picrylhydrazyl
152 (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging,
153 cupric reducing antioxidant capacity (CUPRAC) and ferric reducing antioxidant power (FRAP) tests,
154 and mmol TE/g extract in phosphomolybdenum assay.

155 *2.5 In vitro Enzymatic inhibitory assay*
156 The enzyme inhibitory assays were performed following the procedure reported in literature (Zengin,
157 2016). The following parameters have been choosen: acetylcholinesterase (AChE) and
158 butyrylcholinesterase (BChE) inhibition in mg galanthamine equivalents (GALAE)/g extract;
159 tyrosinase inhibition in mg kojic acid equivalents KAE/g extract; amylase and glucosidase inhibition
160 in mmol acarbose equivalents (ACAE)/g extract.
161 *2.6 In vivo antinociceptive assays*

162 Tail flick and formalin tests, and zymosan-induced edema formation have been carried out following
163 the procedures previously decribed (Pieretti, Saviano, Mollica, Stefanucci, Aloisi, & Nicoletti, 2022;
164 Della Valle et al., 2021) using groups of 7-8 animals. The experimental protocols performed in the
165 present study were in accordance with Italian Legislative Decree 27/92 and approved by the local
166 ethics committee (approval number: 198/2013-B). Detailed procedures are described in the
167 Supporting Information.

168 2.7 *Data analysis and statistics*

169 Data obtained from *in vivo* experiments are reported as mean ± s.e.m. Data analysis of tail flick and
170 formalin test was carried out using one-way ANOVA followed by Dunnett's multiple comparisons
171 test. Data from zymosan-induced paw edema experiments were analysed using two-way ANOVA
172 followed by Dunnett's multiple comparisons test. GraphPad Prism 9.0 software (San Diego, CA,
173 USA) for data elaboration. Means are considered statistically significant at $P \leq 0.05$.

174 **3. RESULTS AND DISCUSSION**

175 *3.1 Chemical synthesis*

176 The novel peptides as TFA salts were prepared following a straightforward and highly efficient
177 solution phase peptide synthesis protocol, both as *C*-terminal amides and acids (Table S1, Scheme S1
178 see SI). Their amino acid sequences and overall yields are shown in Table S1 (see SI). All peptides
179 contain a L or D-proline in position 2 in combination with (2*S*,4*S*)-4-(methylthio)pyrrolidine-2-
180 carboxylic acid (**LMAS1-4**), cysteine (**LMAS5-8**) or (*S*)-2-amino-3-(methylthio)propanoic acid
181 (**LMAS9-12**) in third position. The synthetic procedure applied in this work allowed us to optimize
182 the reactions time, reagent/solvents costs and the purification steps since the crude final products
183 present less detectable by-products in RP-HPLC chromatographic traces, they are completely soluble
184 in water/methanol medium and they are easier to purify than those obtained by solution phase peptide
185 synthesis.

186 *3.2 Opioid binding assay*

All the final peptides and the *lead compound* have been tested for their ability to bind the MOR, DOR and KOR in presence of the reference ligands [^{3}H]DAMGO, [^{3}H]Ile^{5,6}-delthorphin II and [^{3}H]HS665 respectively (Figure S1, Table S2, see SI). Peptide **LMAS4** seems to be selective for MOR with a moderate binding affinity (Ki: 137.4 $\pm$ 0.15 nM). All the other compounds are not able to bind the three opioid receptors at 10 μM concentration. It's worth to note that none of them exhibits a significant binding affinity for δ-opioid receptor, for which the *lead compound* shows a IC_{50} value of 0.12 μM in [^{3}H]DPDPE binding assay (Yang, S., Sonoda, S., Chen, L., & Yoshikawa, M., 2003).

187 This result is not surprising since the sequence D-Pro-(2*S*,4*S*)-4-(methylthio)pyrrolidine-2-carboxylic
188 acid (proline-methionine chimera) contained in **LMAS4** has been already reported to confer an
189 increased metabolic stability to plasma and enzyme's degradation (Mollica et al., 2012), as well as to
190 be responsible of an improved μ-opioid receptor selectivity, thanks to the ability of such sequence to
191 assume a well-defined 3D structure (Stefanucci et al., 2011).

192 *3.3 GTP stimulation assay*

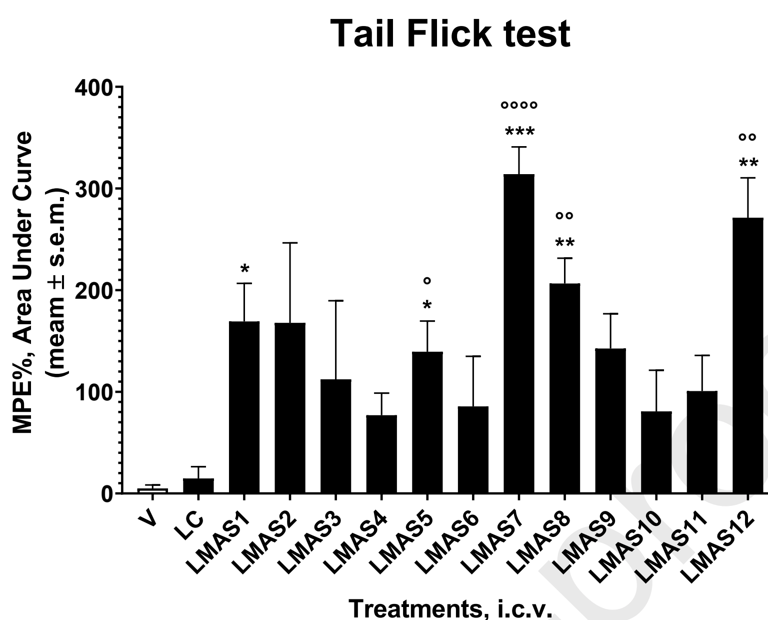
193 In the GTP stimulation assay, all the peptides were tested to evaluate their capacity to stimulate the
194 G-protein coupled receptor (Figure S2, Table S2 see SI). Their efficacy almost stayed around the
195 basal activity, with the only exception of compound **LMAS4** (E_{max}: 139.8 $\pm$ 2.8%). In agreement with
196 the binding data, this analogue of rubiscolin-6 is more efficacious and potent in GTP stimulation
197 assay than the *lead compound*, which is in turn about 20 times more potent than rubiscolin-6 in MVD
198 assay (EC_{50} value of 5.65 μM) (Yang, S., Sonoda, S., Chen, L., & Yoshikawa, M., 2003). The novel
199 analogue shows a diverse affinity/selectivity profile *in vitro* and a more potent antinociceptive activity
200 than rubiscolin-6 and its analogue previously described by Yang et al.

201 *3.4 In vivo experiments*

202 *3.4.1 Tail flick test*

203 In the first series of experiments, the effects of the *lead compound* and **LMAS1-12** peptides were
204 investigated in an animal model of acute nociception induced by thermal stimuli as the tail flick tests.
205 Peptides were injected via intracerebroventricular route (i.c.v.) at the dose of 10 μg/mouse (Figure
206 2). Peptides **LMAS1-12** induce a greater antinociceptive effect than vehicle-treated animals, which
207 reaches statistical significance for **LMAS1,5,7,8,12**. All of them also increased antinociceptive
208 response times in comparison with *lead compound*-treated animals. Statistical analysis reveals that
209 this effect is significant in **LMAS5,7,8,12** treatment groups. All of them exert an antinociceptive

210 effect in this assay higher than that of rubiscolin-6 and its *C*-terminal amide derivative previously
211 described by us (Stefanucci et al., 2020), which could be due to an improved enzymatic stability at a
212 central level.

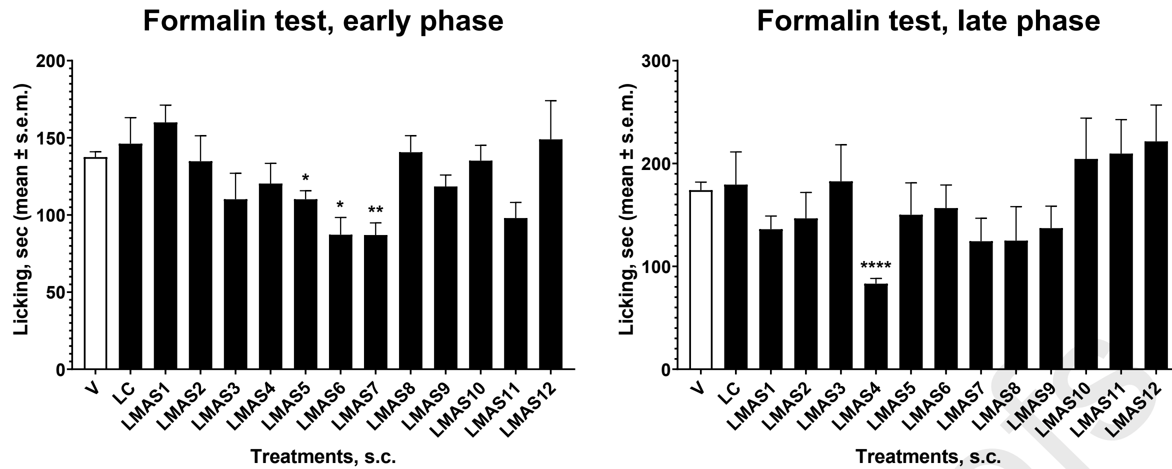


213

214 **Figure 2**. Antinociceptive effects induced by the *lead compound* (LC) and **LMAS1-12** peptides in the tail flick test.
215 Peptides were administered i.c.v. at the dose of 10 µg/mouse. Tail flick data were reported as area under %MPE curve
216 (AUC). * is for P<0.05, ** is for P<0.01 and *** is for P<0.001 *vs* vehicle-treated animals (V, DMSO 0.1% in saline); °
217 is for P<0.05, °° is for P<0.01 and °°°° is for P<0.0001 *vs* LC-treated animals. N=7.

218 *3.4.2 Formalin test*

219 In the formalin test, the *lead compound* and **LMAS1-12** peptides were administered subcutaneously
220 (s.c.) into the mice hind paw at a dose of 100 µg/mouse, 15 min before formalin (Figure 3). In the
221 early phase of the formalin test, **LMAS3,4,5,6,7,9,11** reduced the licking behaviour induced by
222 formalin, although only the antinociceptive effects of **LMAS5-7** peptides reach statistical
223 significance in comparison with vehicle treated animals (Figure 3, left panel). In the late phase of the
224 formalin test, **LMAS1,2,4-9** reduced formalin-induced nociceptive effect, even if only the **LMAS4**
225 peptide is able to significantly reduce the licking activity induced by the aldehyde (Figure 3, right
226 panel). These results partially support our findings described in paragraph 3.2, where peptide **LMAS4**
227 exerted the best binding affinity for µ-opioid receptor probably due to an improved plasma or
228 metabolic stability. On the contrary, the antinociceptive effect of this peptide and analogues **LMAS5-**
229 **7** is lower than that of rubiscolin-6 *C*-terminal amide, previously described by us (Stefanucci et al.,
230 2020) in the early phase of the formalin test, while it appears to be stronger than the parent compound
231 in the late phase.

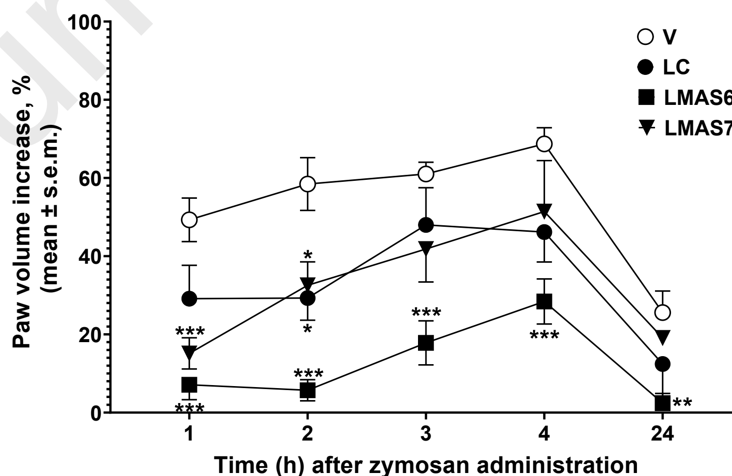


232

233 **Figure 3**. Antinociceptive effects induced by the *lead compound* (LC) and **LMAS1-12** peptides in the formalin test. In
234 the left panel, the result obtained in the early phase of the test are reported. The right panel reports the licking activity
235 recorded in the late phase of the formalin test. Peptides were administered s.c. into the hind paw at the dose of 100
236 μg/mouse. * is for P<0.05, ** is for P<0.01 and **** is for P<0.0001 *vs* vehicle-treated animals (V, DMSO:saline, ratio
237 1:3 *v/v*). N=7.

238 *3.4.3 Zymosan-induced edema formation*

239 Since some of the tested peptides show the best *in vivo* antinociceptive profile, we decided to study
240 the possible anti-inflammatory effects of **LMAS6,7** and *lead compound* (Figure 4). All of them
241 induced an anti-inflammatory effect, although a significant reduction of edema volume for the *lead*
242 *compound* was observed after 2 hours and for the **LMAS7** peptide, 1 and 2 hours after zymosan
243 administration. The **LMAS6** peptide appears to be the most active, as it was able to significantly
244 reduce the formation of edema for the entire duration of the observation period. This result pairs with
245 the data obtained by the formalin test in the early phase after subcutaneous administration, leading us
246 to suppose a possible analgesic activity at the periphery, however further investigation is required to
247 support such hypothesis raising by an embryonic stage of the work.



248

249 **Figure 4**. Anti-inflammatory effects induced by the *lead compound* (LC) and **LMAS6,7** peptides. Inflammatory paw
250 edema was induced by zymosan, and peptides were administered s.c. at the dose of 100 μg/mouse 30 min before. The
251 increase in paw volume was evaluated as the percentage difference between the paw volume at each time point and the

252 basal paw volume. * is for P<0.05 and **** is for P<0.0001 *vs* vehicle-treated animals (V, DMSO:saline, ratio 1:3 *v/v*).
253 N=8.

254 Overall, the cluster of peptides **LMAS5-8** exhibits the best *in vivo* antinociceptive results.
255 Surprisingly they are not able to bind opioid receptors neither to stimulate G protein coupled to them,
256 thus their activity *in vivo* should be related to the activation of other systems involved in nociceptive
257 stimuli control. It is well known that several non-opioid peptides, some of them recently marketed,
258 are powerful antinociceptive agents as evidenced in pre-clinical studies. Non-opioid peptides acting
259 directly or indirectly at different ion channels or non-opioid G-protein coupled receptors (GPCRs)
260 localized in the nociceptive pathways include peptides targeting Ca^{2+}, Na^{+} and K^{+} voltage-gated ion
261 channels, the neuronal nicotinic receptors (nAChR), transient receptor potential channels (TRP), the
262 calcitonin gen-related peptide (CGRP), cannabinoid, bradykinin and neurotensin receptors. (Pérez de
263 Vega MJ, Ferrer-Montiel A, González-Muñiz R. 2018; Dimmito et al., 2021; Mollica et al., 2017;
264 Mollica et al. 2015). These are all targets involved in the mechanism of pain through interaction with
265 peptidic endogenous ligands, also showing a certain overlapping positioning with opioid system in
266 the central nervous system (CNS) (Dvoracsko et al. 2015). Thus we cannot exclude the possibility of
267 a single or multitarget interaction with some of them located at central level or periphery. These
268 peptides are characterized by the presence of a cysteine residue in position 3 in place of methionine
269 contained in the *lead compound*. In light of these data, we decided to test the most active compounds,
270 *e.g*. **LMAS5-8** in a battery of antioxidant and enzyme inhibition assays.

271 *3.5 Antioxidant activity*

272 Peptides **LMAS5-8** have been evaluated for their antioxidant properties using radical scavenging
273 assays (DPPH and ABTS), reducing power assays (CUPRAC and FRAP) and Phosphomolybdenum
274 assay (Table 1).

275

276 **Table 1.** Antioxidant activities of *lead compound* and **LMAS5-8** in DPPH, ABTS, FRAP, CUPRAC and
277 Phosphomolybdenum assays*.

Compounds	**DPPH** mg TE/g	**ABTS** mg TE/g	**FRAP** mg TE/g	**CUPRAC** mg TE/g	**Phosphomolybdenum** mg TE/g
Lead compound	na	8.69 ± 0.64	14.21 ± 0.64	19.35 ± 0.64	0.02 ± 0.00
LMAS5	88.84 ± 2.25	136.03 ± 0.61	24.71 ± 0.37	96.79 ± 0.98	1.67 ± 0.03
LMAS6	91.02 ± 0.74	154.25 ± 0.21	27.01 ± 0.11	156.91 ± 2.16	2.18 ± 0.01
LMAS7	62.43 ± 0.46	139.45 ± 1.58	18.53 ± 0.86	99.67 ± 0.97	1.72 ± 0.15
LMAS8	75.75 ± 2.65	131.28 ± 1.78	19.71 ± 0.17	99.64 ± 1.02	1.61 ± 0.03

278 *Values are reported as mean ± SD of three parallel experiments. TE: Trolox Equivalent; na: not active.

279 Peptide **LMAS6** was the most remarkable antioxidant compound showing the best activity in DPPH
280 and ABTS assays, among the other analogues. Furthermore, it shows a good activity in FRAP,
281 CUPRAC and phosphomolybdenum assays. Interestingly its antioxidant potential is higher than that
282 of the *lead compound*, which seems to be only slightly active. The improved antioxidant activity of
283 **LMAS6** could be due to its amino acid sequence, in particular the presence of cysteine in position 3.
284 Indeed, sulfur-containing amino acids already showed a paramount effect in the reduction of Fe^{3+}-
285 ferricyanide complex (Nwachukwu & Aluko, 2019; Udenigwe & Aluko, 2011). Sulphur group
286 contained in cysteine may neutralize free radicals forming cysteine sulfoxide, a stable oxidation
287 compound (He, Ju, Yuan, Wang, Girgih, & Aluko, 2012). Furthermore, proline and tyrosine could be
288 involved in direct electrons transfer causing the enhancement of free radical scavenging activity
289 (Ketnawa, Wickramathilaka, & Liceaga, 2018; Nwachukwu et al., 2019).

290 *3.6 Enzyme inhibitory activity*

291 Oxidative stress is the principle cause of a huge number of medical diseases like neurological
292 disorders, inflammatory processes, ischemic diseases, hypertension etc (Lobo, Patil, Phatak, &
293 Chandra, 2010). Bioactive agents able to reduce the oxidative damages simultaneously inhibiting the
294 main enzymes involved in this kind of diseases could be useful for the production of functional foods
295 or nutraceuticals in combination with commercial drugs. For example, *J. acutus, J. maritimus* and *J.*
296 *inflexus* leaves and roots extracts have been tested as acetylcholinesterase and butyrylcholinesterase
297 inhibitors and for their antioxidant activity; the results suggest their potential role as sources of
298 bioactive compounds useful for the production of nutraceuticals with cognitive improvement
299 properties or food additives (Rodrigues et al., 2017).

300 For this reason, peptides **LMAS5-8** have been studied to investigate their *in vitro* inhibitory activity
301 against acetylcholinesterase, butyrylcholinesterase, amylase, glucosidase and tyrosinase (Table 2).
302 Acetylcholinesterase (AChe) and butyrylcholinesterase (BChE) inhibition causes the increase of
303 acetylcholine levels enhancing cognitive functions (Greig et al., 2005; Rodrigues et al., 2017),
304 representing a promising approach for the management of Alzheimer disease. Peptides **LMAS5** and
305 **LMAS7** exhibit an increased inhibitory activity against acetylcholinesterase compared to the *lead*
306 *compound.* Peptide **LMAS5** is more effective against butirylcholinesterase than the reference
307 compound which is not active. Hyperglycaemia-induced reactive oxygen species has widely
308 described in literature (Brownlee, 2001; Vanessa Fiorentino, Prioletta, Zuo, & Folli, 2013). The
309 inhibition of carbohydrate hydrolysing enzymes α-amylase and α-glucosidase, is important in the
310 management of hyperglycaemia (Cardullo et al., 2020; Hakamata, Kurihara, Okuda, Nishio, & Oku,
311 2009). For instance, C-glucosidic ellagitannins and some galloylated glucopyranosis have been
312 recently evaluated for their potential use as food ingredients with anti-diabetic effect due to their
313 inhibitor action on amylase and glucosidase (Cardullo et al., 2020). We tested the *lead compound* and
314 **LMAS5-8** for their amylase and glucosidase inhibitory activities (Table 2). Surprisingly the *lead*
315 *compound* resulted to be the only active glycosidase inhibitor, all the other analogues show low
316 inhibitory activity against amylase. Tyrosinase inhibitory activity has been checked for **LMAS5-8**
317 (Table 2). Tyrosinase is involved in browning reactions in food causing the variation of aspect and
318 organoleptic properties of food products reducing their shelf-life and market value (Chazarra,
319 Escribano, & Cabanes, 2001). The use of tyrosinase inhibitors is a promising strategy to prevent
320 browning phenomenon in food industry (Zheng, Cheng, To, Li, & Wang, 2008). In contrast to the
321 *lead compound*, peptides **LMAS5-8** are active against tyrosinase; among them **LMAS6** gives the best
322 result, suggesting a possible use as anti-browning agent in food industry.

323 **Table 2.** Cholinesterase, tyrosinase, amylase and glucosidase inhibitory activity of *lead compound* and **LMAS5-8**[*].

Compounds	AChE mg GALAE/g	BChE mg GALAE/g	Tyrosinase mg KAE/g	Glucosidase mmol ACAE/g	Amylase mmol ACAE/g
Lead compound	3.17 ± 0.09	na	na	1.57 ± 0.03	0.08 ± 0.00
LMAS5	6.24 ± 0.03	9.57 ± 0.08	59.84 ± 2.28	na	0.09 ± 0.01
LMAS6	2.73 ± 0.05	na	84.49 ± 1.27	na	0.09 ± 0.02
LMAS7	5.87 ± 0.04	na	39.49 ± 2.22	na	0.09 ± 0.00
LMAS8	2.94 ± 0.03	0.48 ± 0.02	49.94 ± 2.28	na	0.11 ± 0.01

324 325 326 *Values are reported as mean±SD of three parallel experiments. AChE: Acetylcholinesterase; BChE: butyrylcholinesterase; GALAE: galantamine equivalents; KAE: kojic acid equivents; ACAE: acarbose equivalents; na: not active.

327 Overall the combined *in vitro* and *in vivo* data confirm the group of synthetic peptides **LMAS5-8** as
328 the most active antinociceptive and anti-inflammatory agents, antioxidants and enzyme inhibitors.
329 These peptides possess a molecular weight very close each other's and a chemical structure
330 characterized by common amino acids in several positions of the primary sequence. The *C*-terminal
331 acid and amide functions don't seem to be responsible of any changes in biological activity, while
332 the presence of a D-Pro^2 is crucial to guarantee efficient antinociceptive and anti-inflammatory
333 activity *in vivo,* as well as the best antioxidant power and anti-tyrosinase activity (*e.g.* **LMAS6**). This
334 could be due to a stronger metabolic or enzymatic stability *in vivo* or *in vitro,* as also recently reported
335 by us for their parent compounds (Stefanucci et al., 2020). Furthermore Cys^3 is responsible of a strong
336 effect encountered by CUPRAC, DPPH, and ABTS assays, being also involved in several
337 neuroprotective roles at the CNS (Su et al., 2020). It's interesting to note that all these described
338 features are not present in the *lead compound*, which resulted to be less active than **LMAS6** in all the
339 performed assays.

4. CONCLUSION

Isolation and identification of bioactive peptides require high-cost equipment to elucidate their secondary and tertiary structures. This aspect is fundamental to correlate the specific 3D-structure with their biological effects. Regarding the antioxidant peptides, their application as prophylactic agents should be emphasized in order to improve the quality of life. In this work, three series of rubiscolin-6 analogues have been designed following modifications in position 2 and 3 of a previously described rubiscolin-6 analogue YPLDLF. The synthetic procedure is easy and straightforward allowing to obtain high purity compounds through a simple isolation technique. The peptides **LMAS5-8** give the best results *in vivo* for their antinociceptive and anti-inflammatory effect. Among them, peptide **LMAS6** shows the best antioxidant and tyrosinase inhibitory activities suggesting a possible use in food industry as preservatives or/and anti-browning agents. In addition, data obtained for the anti-cholinesterase peptides **LMAS5** and **LMAS7** indicate their potential development in new nutraceuticals with cognitive-enhancing properties.

CONFLICT OF INTEREST: Declared none.

Journal Pre-proofs

ACKNOWLEDGMENT: We are grateful to all our collaborators for their efforts. We thank PON Ricerca e innovazione 2014-2020 for supporting PhD. program of L.M.

SUPPORTING INFORMATION DESCRIPTION: Peptides sequences and overall yields, RP-HPLC traces, LRMS spectra, opioid binding and GTP stimulation assays, *in vivo* procedure.

REFERENCES

Brownlee, M. (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature, 414*(6865), 813-820.

Caballero, J., Saavedra, M., Fernández, M., & González-Nilo, F. D. (2007). Quantitative structure–activity relationship of rubiscolin analogues as δ opioid peptides using Comparative Molecular Field Analysis (CoMFA) and Comparative Molecular Similarity Indices Analysis (CoMSIA). *Journal of agricultural and food chemistry, 55*(20), 8101-8104.

Cardullo, N., Muccilli, V., Pulvirenti, L., Cornu, A., Pouységu, L., Deffieux, D., . . . Tringali, C. (2020). C-glucosidic ellagitannins and galloylated glucoses as potential functional food ingredients with anti-diabetic properties: A study of α-glucosidase and α-amylase inhibition. *Food chemistry, 313*, 126099.

Carpino, L.; Han, G. The 9-Fluorenylmethoxycarbonyl Amino-Protecting Group. *J. Org. Chem.* **1979**, *44* (21), 3739–3739. https://doi.org/10.1021/jo01335a600.

Chajra, H., Amstutz, B., Schweikert, K., Auriol, D., Redziniak, G., & Lefevre, F. (2015). Opioid receptor delta as a global modulator of skin differentiation and barrier function repair. *International journal of cosmetic science, 37*(4), 386-394.

Chazarra, S., Escribano, J., & Cabanes, J. (2001). F. Garc? ia-Carmona,"Competitive Inhibition of Mushroom Tyrosinase by 4-Substituted Benzaldehydes,". *Journal of agricultural and food chemistry, 49*, 4060-4063.

Day, L., Seymour, R. B., Pitts, K. F., Konczak, I., & Lundin, L. (2009). Incorporation of functional ingredients into foods. *Trends in Food Science & Technology, 20*(9), 388-395.

Della Valle, A., Stefanucci, A., Scioli, G., Szűcs, E., Benyhe, S., Pieretti, S., . . . Zengin, G. (2021). Selective MOR activity of DAPEA and Endomorphin-2 analogues containing a (R)-γ-Freidinger lactam in position two. *Bioorganic Chemistry, 115*, 105219.

Dimmito, M.P., Stefanucci, A., Della Valle, A., Scioli, G., Cichelli, A., Mollica, A. (2021). An overview on plants cannabinoids endorsed with cardiovascular effects. *Biomed Pharmacother.* 142:111963. doi: 10.1016/j.biopha.2021.111963.

Dvoracsko, S., Stefanucci, A., Novellino, E., Mollica, A. (2015). The design of multitarget ligands for chronic and neuropathic pain. *Future Medicinal Chemistry* 7, 2469-2483.

Greig, N. H., Utsuki, T., Ingram, D. K., Wang, Y., Pepeu, G., Scali, C., . . . Giordano, T. (2005). Selective butyrylcholinesterase inhibition elevates brain acetylcholine, augments learning and lowers Alzheimer β-amyloid peptide in rodent. *Proceedings of the National Academy of Sciences, 102*(47), 17213-17218.

Hakamata, W., Kurihara, M., Okuda, H., Nishio, T., & Oku, T. (2009). Design and screening strategies for α-glucosidase inhibitors based on enzymological information. *Current Topics in Medicinal Chemistry, 9*(1), 3-12.

Hartmann, R., & Meisel, H. (2007). Food-derived peptides with biological activity: from research to food applications. *Current opinion in biotechnology, 18*(2), 163-169.

Journal Pre-proofs

He, R., Ju, X., Yuan, J., Wang, L., Girgih, A. T., & Aluko, R. E. (2012). Antioxidant activities of rapeseed peptides produced by solid state fermentation. *Food Research International, 49*(1), 432-438.

Hirata, H., Sonoda, S., Agui, S., Yoshida, M., Ohinata, K., & Yoshikawa, M. (2007). Rubiscolin-6, a δ opioid peptide derived from spinach Rubisco, has anxiolytic effect via activating σ1 and dopamine D1 receptors. *Peptides, 28*(10), 1998-2003.

Kairupan, T. S., Cheng, K.-C., Asakawa, A., Amitani, H., Yagi, T., Ataka, K., . . . Inui, A. (2019). Rubiscolin-6 activates opioid receptors to enhance glucose uptake in skeletal muscle. *journal of food and drug analysis, 27*(1), 266-274.

Kaneko, K., Lazarus, M., Miyamoto, C., Oishi, Y., Nagata, N., Yang, S., . . . Narumiya, S. (2012). Orally administered rubiscolin-6, a δ opioid peptide derived from Rubisco, stimulates food intake via leptomeningeal lipocallin-type prostaglandin D synthase in mice. *Molecular nutrition & food research, 56*(8), 1315-1323.

Ketnawa, S., Wickramathilaka, M., & Liceaga, A. M. (2018). Changes on antioxidant activity of microwave-treated protein hydrolysates after simulated gastrointestinal digestion: Purification and identification. *Food chemistry, 254*, 36-46.

Kobbi, S., Bougatef, A., Balti, R., Mickael, C., Fertin, B., Chaabouni, S., . . . Nedjar, N. (2017). Purification and recovery of RuBisCO protein from alfalfa green juice: antioxidative properties of generated protein hydrolysate. *Waste and biomass valorization, 8*(2), 493-504.

Lafarga, T., & Hayes, M. (2014). Bioactive peptides from meat muscle and by-products: generation, functionality and application as functional ingredients. *Meat science, 98*(2), 227-239.

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy reviews, 4*(8), 118.

Lorenzo, J. M., Munekata, P. E., Gomez, B., Barba, F. J., Mora, L., Perez-Santaescolastica, C., & Toldra, F. (2018). Bioactive peptides as natural antioxidants in food products–A review. *Trends in Food Science & Technology, 79*, 136-147.

Miyazaki, Y., Kaneko, K., Iguchi, S., Mizushige, T., Kanamoto, R., Yoshikawa, M., . . . Ohinata, K. (2014). Orally administered δ opioid agonist peptide rubiscolin-6 stimulates food intake in aged mice with ghrelin resistance. *Molecular nutrition & food research, 58*(10), 2046-2052.

Mollica, A., Paradisi, M. P., Varani, K., Spisani, S., & Lucente, G. (2006). Chemotactic peptides: fMLF-OMe analogues incorporating proline–methionine chimeras as N-terminal residue. *Bioorganic & medicinal chemistry, 14*(7), 2253-2265.

Mollica, A., Costante, R., Novellino, E., Stefanucci, A., Pieretti, S., Zador, F., Samavati, R., Borsodi, A., Benyhe, S., Vetter, I. and Lewis, R.J. (2015), Design, Synthesis and Biological Evaluation of Two Opioid Agonist and Ca_v2.2 Blocker Multitarget Ligands. *Chem Biol Drug Des, 86*, 156-162.

Mollica, A., Pelliccia, S., Famiglini, V., Stefanucci, A., Macedonio, G., Chiavaroli, A., Orlando, G., Brunetti, L., Ferrante, C., Pieretti, S., Novellino, E., Benyhe, S., Zador, F., Erdei, A., Szucs, E., Samavati, R., Dvrorasko, S., Tomboly, C., Ragno, R., Patsilinakos, A., Silvestri, R. (2017). Exploring the first Rimonabant analog-opioid peptide hybrid compound, as bivalent ligand for CB1 and opioid receptors. *J Enzyme Inhib Med Chem.*, 32(1), 444-451.

Mollica, A., Pinnen, F., Stefanucci, A., Mannina, L., Sobolev, A.P., Lucente, G., Davis, P., Lai, J., Ma, S.W., Porreca, F., Hruby, V.J. (2012). cis-4-amino-L-proline residue as a scaffold for the synthesis of cyclic and linear endomorphin-2 analogues: part 2. *Journal of Medicinal Chemistry,* 55(19), 8477-8482.

Nwachukwu, I. D., & Aluko, R. E. (2019). Structural and functional properties of food protein-derived antioxidant peptides. *Journal of Food Biochemistry, 43*(1), e12761.

Pérez de Vega, M.J., Ferrer-Montiel, A., González-Muñiz, R. (2018). *Arch Biochem Biophys*. 660, 36-52.

Pieretti, S., Saviano, A., Mollica, A., Stefanucci, A., Aloisi, A. M., & Nicoletti, M. (2022). Calceolarioside A, a Phenylpropanoid Glycoside from Calceolaria spp., Displays Antinociceptive and Anti-Inflammatory Properties. *Molecules, 27*(7), 2183.

Rodrigues, M. J., Gangadhar, K. N., Zengin, G., Mollica, A., Varela, J., Barreira, L., & Custódio, L. (2017). Juncaceae species as sources of innovative bioactive compounds for the food industry: In vitro antioxidant activity, neuroprotective properties and in silico studies. *Food and Chemical Toxicology, 107*, 590-596.

Sánchez, A., & Vázquez, A. (2017). Bioactive peptides: A review. *Food Quality and Safety, 1*(1), 29-46.

Sohaib, M., Anjum, F. M., Sahar, A., Arshad, M. S., Rahman, U. U., Imran, A., & Hussain, S. (2017). Antioxidant proteins and peptides to enhance the oxidative stability of meat and meat products: A comprehensive review. *International Journal of Food Properties, 20*(11), 2581-2593.

Stefanucci, A., Dimmito, M. P., Tenore, G., Pieretti, S., Minosi, P., Zengin, G., . . . Cichelli, A. (2020). Plant-derived peptides rubiscolin-6, soymorphin-6 and their c-terminal amide derivatives: Pharmacokinetic properties and biological activity. *Journal of Functional Foods, 73*, 104154.

Stefanucci, A., Pinnen, F., Feliciani, F., Cacciatore, I., Lucente, G., Mollica, A. (2011). Conformationally Constrained Histidines in the Design of Peptidomimetics: Strategies for the χ-Space Control. *International Journal of Molecular Science, 12*, 2853-2890.

Su, T.; Han, M.; Cao, D.; Xu, M. (2020). Molecular and Biological Properties of Snakins: The Foremost Cysteine-Rich Plant Host Defense Peptides. *J. Fungi*, *6*, 220.

Taylor, T. C., & Andersson, I. (1997). Structure of a product complex of spinach ribulose-1, 5-bisphosphate carboxylase/oxygenase. *Biochemistry, 36*(13), 4041-4046.

Tkaczewska, J. (2020). Peptides and protein hydrolysates as food preservatives and bioactive components of edible films and coatings-A review. *Trends in Food Science & Technology, 106*, 298-311.

Udenigwe, C. C., & Aluko, R. E. (2011). Chemometric analysis of the amino acid requirements of antioxidant food protein hydrolysates. *International Journal of Molecular Sciences, 12*(5), 3148-3161.

Udenigwe, C. C., & Aluko, R. E. (2012). Food protein-derived bioactive peptides: production, processing, and potential health benefits. *Journal of food science, 77*(1), R11-R24.

Uysal, S., Zengin, G., Locatelli, M., Bahadori, M. B., Mocan, A., Bellagamba, G., . . . Aktumsek, A. (2017). Cytotoxic and enzyme inhibitory potential of two Potentilla species (P. speciosa L. and P. reptans Willd.) and their chemical composition. *Frontiers in pharmacology, 8*, 290.

Vanessa Fiorentino, T., Prioletta, A., Zuo, P., & Folli, F. (2013). Hyperglycemia-induced oxidative stress and its role in diabetes mellitus related cardiovascular diseases. *Current pharmaceutical design, 19*(32), 5695-5703.

Yang, S., Kawamura, Y., & Yoshikawa, M. (2003). Effect of rubiscolin, a δ opioid peptide derived from Rubisco, on memory consolidation. *Peptides, 24*(2), 325-328.

Yang, S., Sonoda, S., Chen, L., & Yoshikawa, M. (2003). Structure–activity relationship of rubiscolins as δ opioid peptides. *Peptides, 24*(4), 503-508.

Yang, S., Yunden, J., Sonoda, S., Doyama, N., Lipkowski, A. W., Kawamura, Y., & Yoshikawa, M. (2001). Rubiscolin, a δ selective opioid peptide derived from plant Rubisco. *FEBS letters, 509*(2), 213-217.

Yoshikawa, M. (2015). Bioactive peptides derived from natural proteins with respect to diversity of their receptors and physiological effects. *Peptides, 72*, 208-225.

Zengin, G. (2016). A study on in vitro enzyme inhibitory properties of Asphodeline anatolica: New sources of natural inhibitors for public health problems. *Industrial Crops and Products, 83*, 39-43.

Zheng, Z. P., Cheng, K. W., To, J. T. K., Li, H., & Wang, M. (2008). Isolation of tyrosinase inhibitors from Artocarpus heterophyllus and use of its extract as antibrowning agent. *Molecular nutrition & food research, 52*(12), 1530-1538.

Food-inspired peptides from spinach Rubisco endowed with antioxidant, antinociceptive and anti-inflammatory properties

Lorenza Marinaccio,[a] Gokhan Zengin,[b] Stefano Pieretti,[c] Paola Minosi,[c] Edina Szucs,[d] Sandor Benyhe,[d] Ettore Novellino,[e] Domiziana Masci,[f] Azzurra Stefanucci,[a,*] Adriano Mollica[a]

[a]Department of Pharmacy, G. d'Annunzio University of Chieti-Pescara, 66100 Chieti, Italy; [b]Department of Biology, Science Faculty, Selcuk University, Konya 42130, Turkey;

[c]National Centre for Drug Research and Evaluation, Istituto Superiore di Sanità, 00161 Rome, Italy;

[d]Institute of Biochemistry, Biological Research Centre, 6726 Szeged, Hungary;

[e]NGN Healthcare, Via Nazionale Torrette, 207, 83013 Mercogliano, Italy;

[f]Department of Basic Biotechnological Sciences, Intensivological and Perioperative Clinics, Catholic University of Sacred Heart, Largo Francesco Vito 1, 00168 Rome, Italy.

*Correspondence: a.stefanucci@unich.it

HIGHLIGHTS

- Rubiscolin-6 is a linear peptide isolated by the spinach Rubisco.
- New analogues have been prepared via SPPS with high purity and good overall yields.
- Their activity on opioid receptors has been detected *in vitro* and *in vivo*.
- Some of them possess a strong antioxidant and tyrosinase inhibitor activity.
- Peptide **LMAS6** exerts also a significant anti-inflammatory *in vivo*.

Journal Pre-proofs