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Review Article

Mechanisms and Therapeutic Prospects of Peptides in Skin Pigmentation

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Abstract

Clinically modifying skin pigmentation is a valuable approach that can treat pigmentation and photosensitivity disorders and provide a degree of protection from skin cancers. Skin pigmentation is due to the presence of eumelanin, a biological pigment that is generated by the highly regulated melanogenesis pathway. Proteins within this pathway, including the melanocortin 1 receptor (MC1R), microphthalmia-associated transcription factor (MITF), and tyrosinase, can be activated or deactivated in order to bring about the desired change in eumelanin content, and thus pigmentation.

A novel platform to modulate skin pigmentation is the use of oligopeptides designed with affinity towards the active sites in molecules of the melanogenesis pathway. A useful approach developing such peptides is to imitate active sites of existing hormones and signaling molecules with melanogenesis modulating properties. This platform is especially useful since oligopeptides can be designed to increase activity, prolong in vivo stability, promote penetration into cells and minimize side effects when compared to the molecules they mimic, or other drugs.

Abbreviations

α-MSH = α-Melanocyte Stimulating Hormone; ASIP = agouti signaling protein; cAMP = cyclic adenosine monophosphate; DCT = dopachrome tautomerase; EPP = erythropoietic protoporphyria; HQ = hydroquinone; IL= interleukin; MC1R = melanocortin 1 receptor; MITF = microphthalmia-associated transcription factor; PLE = polymorphic light eruption; SU = solar urticaria; TGF-β1 = transforming growth factor β; TRP = tyrosinase related protein

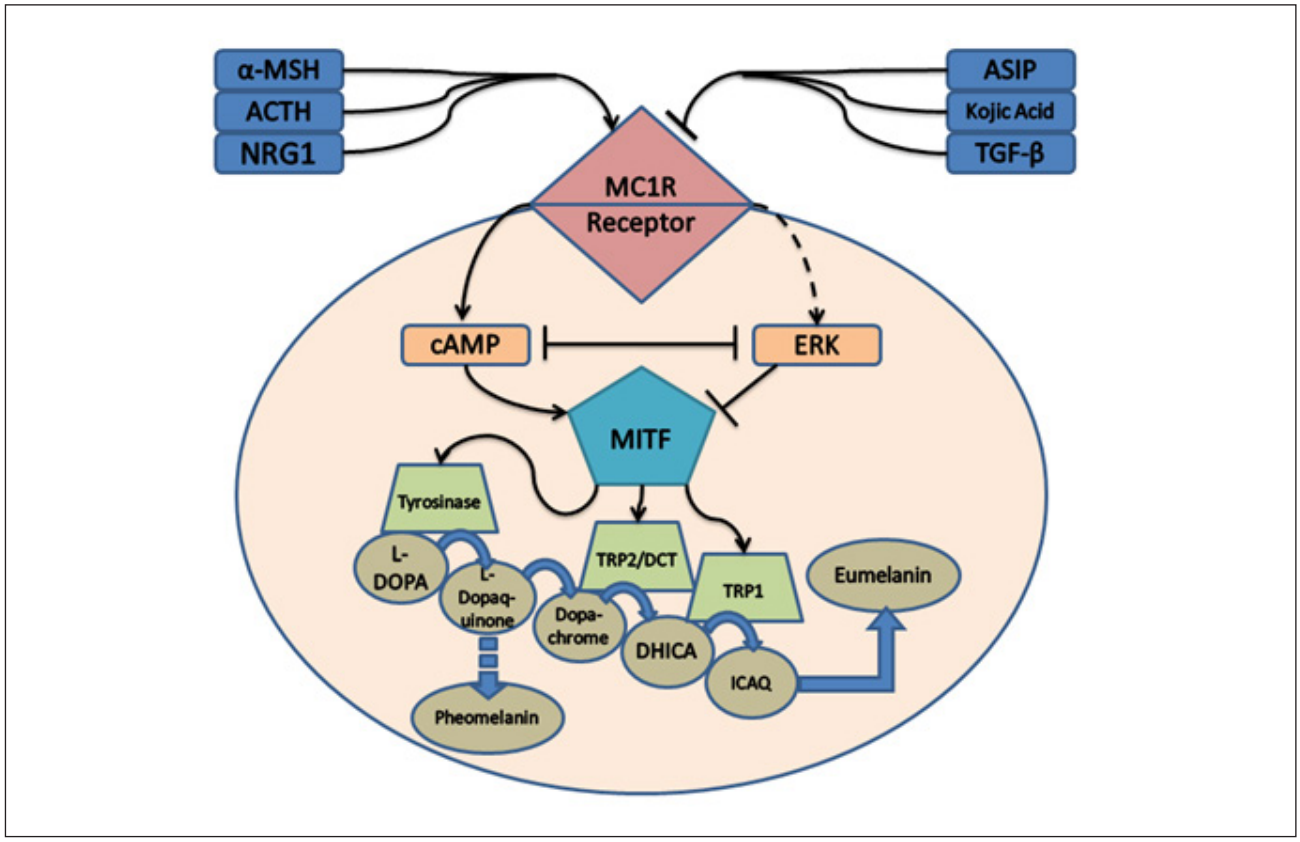
Introduction

Development of novel therapies that modulate skin pigmentation is a fast-growing field that addresses disorders such as melasma, vitiligo, erythropoietic protoporphyria (EPP), solar urticaria (SU), and polymorphic light eruption (PLE) [1-6]. Studies have also suggested that inducing hyperpigmentation in patients may be useful prophylactically in preventing development of skin cancer [7]. Thus, it is evident that treatments that induce or reduce skin pigmentation are clinically valuable.

Melanin is a biological pigment synthesized within melanosomes, then transported into keratinocytes within the surrounding epidermal melanin unit and deposited on the nuclei of cells. There, eumelanin is capable of absorbing UV radiation and neutralizing oxygen radicals, thus protecting genomic integrity [8-11]. The melanogenesis pathway has been exhaustively studied and outlined and is known to consist of a series of enzymatic reactions that convert precursor amino acids into melanin [12-15]. Tyrosinase, a transmembrane oxidase that exists on the surface of melanosomes, is involved in several steps of the melanogenesis pathway, including catalyzing the rate limiting conversion of L-DOPA to L-dopaquinone [16,17]. Our aim was to review mechanism-based approaches in therapy development. Each cell receptor and enzyme involved in the pathway is a potential target for activation or deactivation, which can then effect a downstream increase or decrease in melanogenesis. These targets can be activated or inhibited by short sequence oligopeptides whose remaining amino acid sequences can be designed to increase in vivo stability, promote penetration into cells, and minimize cytotoxicity and side effects.

Mechanisms of the melanogenesis pathway

Mapping out the melanogenesis pathway is useful in the pursuit of targets for modulation. Each of the proteins and enzymes that can be activated or deactivated in order to bring about the desired clinical effect is associated with its own set of upstream or downstream hormones and transcription factors that regulate melanogenesis. These signaling molecules can then be analyzed for their mechanism of action and mimicked with peptides in order to bring about their effect therapeutically. Figure 1 maps out



the melanogenesis pathway and its key regulators and effectors.

Melanocortin 1 receptor (MC1R) is a specialized G protein-coupled cell surface receptor found on melanocytes. A variety of MC1R alleles exist in humans, with particular variants contributing to phenotypic characteristics related to pigmentation (such as red hair, sensitivity to sunlight and UV, or risk of developing skin cancers) [18]. As a G protein-coupled cell surface receptor, MC1R activates adenylyl cyclase mediated conversion of ATP into cyclic adenosine monophosphate (cAMP). cAMP activation and upregulation subsequently, in conjunction with micropthalmia transcription factor (MITF), a dimeric transcription factor with a basic helix-loop-helix leucine zipper, promote the transcription of critical melanogenesis proteins tyrosinase, tyrosinase related protein 1 (TRP1), and tyrosinase related protein 2/dopacrhome tautomerase (TRP2/DCT) [19-23]. The central role played by MC1R, cAMP, and MITF make these molecules valuable targets for therapies seeking to modulate pigmentation. Activating or promoting MC1R, cAMP, or MITF may increase melanocyte proliferation, melanogenesis, and pigmentation, while treatments that seek to block or inhibit this pathway may have depigmentation effects.

There are several factors, however, that may complicate the therapeutic utility of MC1R as a target. First, the signal transduction pathway from MC1R is not linearly responsive to activation. Indeed, in vitro attempts to activate MC1R to increase melanogenesis have been shown to work only in short pulses [23]. This is likely due to the fact that overactivation of MC1R results in activation of ERK, which in turn inhibits melanogenesis through blocking cAMP and reducing MITF levels through ubiquitination and subsequent proteolysis [20]. Indeed, ERK/map-kinase (MAPK) mediated inhibition of melanogenesis can be intentionally activated through c-kit, or with agents like caffeoylserotonin [24,25]. Second, modulating MITF has potentially severe side effects, since mutations to the transcription factor causing unusual activity have been seen in many skin cancers, raising the possibility that abnormal MITF signaling may be involved in cancer pathogenesis [21]. Indeed, the transcription factor has been shown to promote survival of some melanoma cancers [26]. Thus, more research into the relationship between modulation of MC1R and MITF and the development of skin cancer is necessary to ensure the safety of treatments that target these molecules.

Further downstream from MC1R in the melanogenesis pathway is the transmembrane copper containing enzyme tyrosinase, which is found in the membrane of melanosomes, and which has a catalytic domain inside the organelle [27,28]. As mentioned above, tyrosinase is involved in various steps in melanogenesis, including the rate limiting conversion of L-DOPA into L-dopaquinone [29-31]. Dysregulation of the enzyme is associated with the pathology of several pigmentary disorders, including vitiligo and melasma [2,3]. The enzyme has also been studied in non-pigmentation contexts, since it has been shown to be involved in certain cancers and neurodegenerative diseases, such as Parkinson's disease [32,33]. There are many potent tyrosinase modulators known today, the most important of which have been extensively discussed in these comprehensive reviews [17,34,35]. This enzyme is the second major target of modulation

for many peptide treatments, a selection of which is presented in this paper.

Activators of melanogenesis

Hyperpigmentation can be effected through building peptide analogs of melanogenesis activators. Major natural activators include α-melanocyte stimulating hormone (α-MSH), which activates MC1R, and neuregulin-1 (NRG1), which is closely linked with the MAPK pathway; the former has been shown to promote melanogenesis, and the latter has been found highly expressed in Fitzpatrick Type VI skin-derived fibroblasts [19,36-38]. Peptides based on these hormones have the potential of becoming an effective therapy not only against hypopigmentation disorders, but also potentially in melanoma prevention and are discussed further below [39].

Activators of MC1R

There exist several peptide treatments that activate MC1R. A vast majority of them are α-MSH analogs. α-MSH is a natural hormone activator of the receptor. However, in addition to mimicking the effects of α-MSH, peptide-based approaches are capable of higher affinity engagement and activation of MC1R versus α-MSH, as evidenced in the study by Abdel-Malek et al. on the tetrapeptide α-MSH analogs n-Pentadecanoyl- and 4-Phenylbutyryl-His-D-Phe-Arg-Trp-NH2, both of which were shown to have greater melanogenesis-stimulating properties than α-MSH [39]. Among these modified α-MSH class of peptides, the linear [Nle4-D-Phe7]-α-MSH, which is clinically known as afamelanotide, melanotan-1, or NDP-MSH, and which has 2 amino acid substitutions that contribute to the peptide's resistance to enzymatic degradation in vivo, is among the most extensively used and studied [40]. Several clinical studies were conducted to determine the safety and efficacy of the peptide in promoting melanogenesis.

In a 2006 study, 65 patients, with varying degrees of skin burns, were injected with [Nle4-D-Phe7]-α-MSH at 0.16 mg/kg for 3, 10-day cycles over 3 months [41]. Reflectance spectroscopy was used before and after treatment to quantitatively measure changes in skin pigmentation. The authors found that melanin content in patients with low constitutive pigmentation increased by 41% ($P<0.0001$), and apoptotic "sunburn" cell count reduced by over 50% ($P<0.001$) in skin exposed to 3 times the minimum erythema dose. These results were consistent with a similarly designed previous study which also found that while patients exhibited an increase in eumelanin levels, their pheomelanin levels remained relatively constant [42]. Figure 1 offers a potential explanation for this phenomenon; NDP-MSH's binding to MC1R leads to activation of MITF, which in turn promotes TRP1 activity. Since TRP1 only provides catalysis for a step in the production of eumelanin but not pheomelanin, it is possible that this increased eumelanin: pheomelanin ratio in patients. However, patients in both studies suffered severe side effects, including nausea, vomiting, and peripheral vasodilation, which the authors attributed to systemic effects of high concentrations of the peptide experienced immediately post-injection. This hypothesis is supported by a 2009 study, which showed use of a slow-release formulation of NDP-MSH decreased incidence of side effects in patients [43,44].

Interestingly, in a study conducted on 77 Caucasian individuals with varied MC1R genotypes, including Val60Leu, Asp84Glu, Val92Met, Arg142His, Arg151Cys, and Arg160Trp (which result in reduced MC1R activity and thus may cause constitutively decreased pigmentation, red hair, etc.), authors found that 0.16 mg/kg/day injections of NDP-MSH not only result in melanogenesis and pigmentation in treated patients, but also have greater effectiveness in individuals with the variant genotypes [45]. These findings suggest NDP-MSH has the potential to be used as a prophylactic treatment in individuals who are at greater risk from UV damage due to naturally less-pigmented skin, The effectiveness of NDP-MSH mediated tanning can be enhanced through the combined use of UV-B light and NDP-MSH, which has been shown to be more effective than either UV-B treatment or NDP-MSH treatment alone in promoting melanogenesis, and in treating disorders such as vitiligo [46,47]. This treatment has also been shown to have effectiveness in reducing photosensitivity in patients with EPP, SU, and PLE. [43,44,48-51].

However, it is critical that further clinical trials are performed in order to determine the long-term effects and correct dosage of melanotan. Several case studies have reported development of malignant melanoma several weeks after patients self-injected melanotan [52-55]. While a causative link between melanotan and malignant melanoma remains to be elucidated, (the aforementioned connections between MITF and cancer may be a possible explanation), these case studies highlight the importance of further research into the potential side effects of afamelanotide treatment.

Activators of EGFR

While MC1R is known to involve MAPK pathways, the complexity of the pathway and its prevalence in many other regulatory networks other than melanogenesis makes it difficult to identify a priori targets of the pathway in order to regulate pigmentation. Experimentally, however, by monitoring expression of various paracrine factors in fibroblasts, it is possible to identify potentially useful proteins. One such protein is neuregulin-1 (NRG1). Choi et al. have identified the bioactive peptide sequences PSRYLCKC and LCKCPNEF, which are motifs within NRG1's amino acid sequence; the LCKC motif that both of these peptides share was identified as the minimum fragment of NRG1 necessary to elicit a response [56]. The mechanism of both NRG1 and LCKC motif has been shown to involve ErbB3, a member of the epidermal growth factor receptor (EGFR) family. Interestingly, unlike α-MSH analogs and NRG1 itself, these short peptides are capable of inducing melanogenesis without melanocyte proliferation [38].

However, there remains clinical issues in implementing neuregulin analogs due to the wide range of effects in modulating

Activators of melanogenesis	Mechanism of action	References
α-MSH analogs: [Nle^4,D-Phe^7]α-MSH n-Pentadecanoyl-His-D-Phe-Arg-Trp-NH_2 4-phenylbutyryl-His-D-Phe-Arg-Trp-NH_2	Activators of MC1R	[39,40]
Neuregulin-1 motifs: PSRYLCKC and LCKCPNEF	Activators of EGFR	[56]
Aldehyde dehydrogenase 1A1 (ALDH1A1), human placental proteins and peptide fractions; kappa elastin and the VGVAPG peptide	Activators of tyrosinase	[59-62]
Iinhibitors of melanogenesis		
Agouti signaling protein (ASIP)	Inhibitor of MC1R	[70-74]
TGF-β1 Kojic acid tripeptides Interleukin 6 PKEK tetrapeptide basic FGF (bFGF) Decapeptide-12 Octapeptides P16- P18 K	Inhibitors of tyrosinase	[78-81] [83,84] [87-89] [91,92] [93] [94,95] [84,99,100]

Table 1: Summary of melanogenesis activators and inhibitors.

activation of the EGFR family. NRG1 is also involved in stimulation of connective tissue growth factor, and thus has been implicated in promoting hypertrophic scarring [57]. Should NRG1 increase fibrous scar tissue in conjunction with pigmentation, its utility may be limited as a treatment. More concerning than the adverse risks that may be associated with clinical use of NRG1 is its potential to induce genomic instability via ErbB3 activation, and thus the additional more serious risk of promoting progression of benign nevi to malignant melanoma [58]. Thus, while NRG1 has been shown to be capable of promoting pigmentation, its implication in both hypertrophic scarring and melanoma suggests a need for further clinical studies to establish its therapeutic index. As indicated above, however, it is possible that short peptides that utilize the melanogenesis activating regions of NRG1 may lack the undesirable effects, such as melanocyte proliferation, of the full protein. Thus, it may be possible to synthesize specialized peptides that are able to bring about the desired clinical effect without side effects.

Activators of tyrosinase

There are several agents that promote tyrosinase activity, and thus melanogenesis. Paterson et al. have identified the enzyme aldehyde dehydrogenase 1A1 (ALDH1A1) and Sarkar et al. have identified human placental proteins and peptide fractions as tyrosinase activating [59,60]. Elastin-derived peptides have also been recently shown to have tyrosinase stimulatory effects [61]. Different tropoelastin fragments, such as kappa elastin and the VGVAPG peptide have been shown to stimulate melanogenesis [61,62]. However, there are no in vivo studies that determine the efficacy of these proteins and peptides in promoting tyrosinase activity. Analysis on the active sites of these proteins may yield specific peptide sequences that have a capacity for tyrosinase activation, thus providing a series of potential peptides that can be used to stimulate melanogenesis.

Inhibitors of melanogenesis

Currently, 2-4% hydroquinone (HQ) is one of the most widely used treatments used to reduce melanogenesis [63]. However, the molecule has been shown to have a variety of deleterious side effects, including inducing apoptosis in melanocytes, promoting oxidative damage to membrane lipids and proteins, and leading to a host of clinical contraindications, including contact dermatitis, erythema, leukoderma, hypochromia, and ochronosis [64-69]. Thus, it is important to develop novel therapies to effect reduced pigmentation without these deleterious effects.

Inhibitors of MC1R

Just as α-MSH and its analogs activate MC1R to promote melanogenesis, inhibitors of MC1R can be employed to effect depigmentation. In humans, agouti signaling protein (ASIP) regulates pigmentation by antagonizing binding of α-MSH to MC1R [70-73]. A study using a murine model showed that ASP (the murine analog of the human ASIP) was capable of downregulating the MC1R pigmentation pathway, resulting in a decrease in melanin synthesis within 1 day of treatment [74]. Interestingly, the study indicates that melanocyte turnover makes cessation of melanogenesis sufficient to effect visual depigmentation, rather than requiring degradation of existing melanin. The importance of ASIP in limiting MC1R activation makes this protein a valuable target. Analogs to the agouti protein have the potential for reducing pigment production, similar to how α-MSH analogs have the potential to promote melanogenesis. For example, human β-defensin 3 (HBD3) has been shown to bind MC1R and block α-MSH, similar to ASIP [75]. Alternatively, agents such as caffeoylserotonin and fucoxanthin can attenuate downstream effects of MC1R via modification of cAMP levels and MAPK pathway-induced MITF synthesis [24,76].

A recent summary of hypopigmenting agents working through MAPK pathway modification can be found in the following review [77]. With regards to peptide based approaches to altering pigmentation, identifying active regions of ASIP that block MC1R may be fruitful in yielding peptides that can be used for depigmentation, without involving modification of the MAPK signal cascade and cAMP levels, both of which have a high potential for side effects.

Inhibitors of tyrosinase

Several natural proteins, such as transforming growth factor (TGF-β1), have been shown to inhibit tyrosinase, with TGF-β1 additionally inhibiting MITF and interfering with melanosome maturation and migration [78-81]. The active sites of these proteins can be mimicked with peptides. Agents that have been studied include kojic acid, interleukin (IL)-6, and bFGF; these molecules provide templates for novel peptides that can bring about hypopigmentation through tyrosinase inhibition.

Kojic acid, found in fungal species such as Aspergillus, is a molecule that can form a chelate complex with copper atoms in tyrosinase and suppress its activity [82]. A peptide-kojic acid conjugated molecule has been shown to be significantly more stable and bioactive, repressing tyrosinase ~100 times more effectively than kojic acid alone [83,84]. However, kojic acid alone has been associated with several side effects, including dermatitis and erythema, and has been shown to be carcinogenic in rats. Further clinical studies are required to establish ipeptide-kojic acid's potential safety in humans [85,86].

Immunoregulatory proteins are also linked with melanogenesis. IL- 6 is reported to decrease pigmentation via direct effects on tyrosinase and its related proteins, TRP1 and TRP2 [87-89]. The peptide sequences Gly-Gly-His and Gly-His-Lys have both been shown to promote human fibroblast secretion of IL-6, and thus can be used as a means of promoting the depigmenting effects of IL-6 [90]. Conversely, recent studies involving the PKEK tetrapeptide indicate that inhibiting certain inflammatory and immune factors may also lead to reduced pigmentation. In vitro studies with this peptide have demonstrated decreased expression of IL-8 and TNF-α, with a downstream reduction in melanogenesis in vivo studies have demonstrated anti-pigmentation effects in 39 patients treated with a topical therapy of combined PKEK and sodium ascorbyl phosphate [91,92]. The authors did note that proopiomelanocorticotropin, which codes for α-MSH, was downregulated as well by this treatment, which may also account for the reduction in pigmentation.

A previous study found increased levels of basic FGF (bFGF) in serum and blister fluid of vitiligo patients, suggesting that there may be a link between the growth factor and pigmentation levels [93]. In vitro studies confirm this link, with 8 and 10 amino acid length peptide fragments of bFGF causing a reduction in melanin content by 27% and 43%, respectively, in cultured melanoctytes, without cytotoxic effects [94,95]. Kinetic studies also showed that the mechanism of action for this effect is through inhibition of tyrosinase. The 10-amino acid length peptide (called decapeptide-12) was recently tested in vivo in a split-face, double-blind, placebo-controlled pilot study conducted on 5 patients with recalcitrant melasma and who had failed to demonstrate improvement with 6 months of Tri-Luma™ (Galderma, Lausanne, Switzerland) (fluocinolone acetonide, 0.01%; HQ, 4%; tretinoin, 0.05%) or HQ therapy [96]. All 5 patients, who were treated with a topical cream formulated with 0.01% peptide (w/w) over 16 weeks, tolerated the treatment without visible signs of erythema or irritation, and demonstrated significant improvement in their condition. Another clinical study with 4 patients with mild to moderate facial melasma treated with decapeptide-12 showed similar success in all 4 patients, with 1 patient demonstrating complete clearance after 6 weeks of treatment [97].

In vitro studies testing for binding affinities of particular amino acids with the tyrosinase enzyme have identified peptide sequences that contain arginine and phenylalanine combined with leucine, valine, or alanine residues, as well as cysteine and serine tend to have greater tyrosinase binding and inhibitory activity [98]. Additionally, the structure of tyrosinase can be modeled in

silico in order to both identify target sites for peptide interaction, and test for affinity towards any particular novel peptide. Such an approach was used in order to establish that octapeptides P16-18 dock into the tyrosinase catalytic site, with tryptophan residues binding and inhibiting the catalytic ability of tyrosinase through. F hydrophobic-aromatic interactions, thus effecting a reduction in skin pigmentation [84,99,100]. At 200 μM, the 3 oligopeptides were shown to decrease melanin content by 45%, 38%, and 41% respectively, without displaying the cytotoxic effects seen with HQ required to achieve the same extent of melanin reduction. Thus, oligopeptides P16-P18 offer a novel, potent means of inhibiting tyrosinase and effecting depigmentation further in vivo studies (in progress) are required in order to confirm the safety and efficacy of these peptides.

Conclusion

Peptides are becoming more important development options for modification of melanin content clinically. They offer the potential advantages of site-specific design via mimicry of catalytic sites of interest. This approach has been successfully applied to mimic α-MSH, ASIP, and bFGF. As specificity is increased, risk of efficacy limiting toxicity is diminished, potentially accelerating availability of novel therapies to address difficult to treat conditions such as EPP, SU and melasma.

Conflict of Interest

BMH is the inventor of decapeptide-12, a patent held by Stanford University. BMH and AAU are consultants and/or stockholders of Escape Therapeutics, Inc. which holds patents pending for P16-18 and may pursue further commercialization of these 3 oligopeptides.

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