IFSCC 2025 full paper (ABSTRACT N° IFSCC2025-1641)

Using Lettuce to Produce Collagen Peptides: A Novel Bio-technological Approach for Enhancing Skin Structure

Stefania Arciello[1], Annalisa Tito [1], Annamaria Cefariello[1], Antonio Colantuono[1], Danila Falanga[1], Maura Angelillo[2], Alessandro Vitale[3] and Vincenzo Fogliano[1,4*]

[1]Arterra Bioscience SpA, Naples, Italy; [2]Vitalab srl, Naples, Italy; [3]IBBA/CNR, Milan, Italy; [4]University of Wageningen, Wageningen, Netherlands

1. Introduction

Collagen is the most abundant structural protein in the extracellular matrix essential for maintaining skin strength, elasticity and structural integrity [1]. Type I collagen, the dominant form in human skin dermis, consists of three polypeptide chains that assemble into a stable triple-helix structure [2]. The correct folding and assembly of these chains are critical for collagen's mechanical properties and biological function [3]. Defective assembly can lead to compromised tissue integrity and contribute to skin aging and connective tissue disorders. Following chronological and extrinsic aging, the balance of collagen homeostasis is disrupted due to a decline in collagen synthesis and a parallel increase in its degradation [4]. One of the primary enzymes involved in collagen breakdown is matrix metalloproteinase 1 (MMP1), upregulated by UV exposure and oxidative stress, playing a major role in photoaging and wrinkle formation [5]. In contrast, Secreted Protein Acidic and Rich in Cysteine (SPARC) contributes positively to collagen homeostasis favoring ECM remodeling, collagen fibrillogenesis and the regulation of cell-matrix interactions. It binds to collagen and integrins, facilitating proper alignment and deposition of collagen fibers. Moreover, SPARC has been shown to upregulate collagen gene expression in dermal fibroblasts, making it a key factor in promoting dermal regeneration and structural integrity [6]. Consequently, collagen and its modulators have become core targets in cosmetic formulations aimed at enhancing skin appearance and texture [7]. In recent years, recombinant technologies have enabled the production of bioactive collagen peptides, offering sustainable alternatives to animal-derived sources. Collagen derived peptides, in particular, have attracted attention for their superior skin penetration, bioavailability and ability to stimulate endogenous collagen production [8]. In this context the present study introduces for the first time a novel plant-based expression platform utilizing *Lactuca sativa* (lettuce) as transient host system for producing human type I collagen-derived peptides. Transient expression was preferred over stable transformation due to its ability to facilitate rapid gene expression, typically within a few days, without integration of the target gene into the host plant genome. This transient nature considerably reduces regulatory concerns commonly associated with genetically modified organisms (GMOs). Moreover, agroinfiltration-based transient expression systems frequently yield higher levels of recombinant protein compared to conventional stable expression systems making them particularly advantageous for rapid and scalable protein production. [9]. To achieve the expression of human type I collagen-derived peptides in plant a specifically designed chimeric peptide incorporated the collagen interhelix region, as well as binding domains for integrins and SPARC-motifs known to stimulate collagen synthesis and assembly in human skin cells. To enhance the efficient accumulation of the recombinant polypeptide, the construct included an endoplasmic reticulum (ER) retention signal at its C-terminus. The ER is regarded as the most favorable subcellular compartment for the accumulation and stabilization of chimeric polypeptides, owing to its relatively low proteolytic activity. To facilitate the release of individual collagen peptides from the precursor polypeptide, the construct was engineered with an interspersed amino acid sequence derived from *P. vulgaris* storage protein β phaseolin a vacuole protein selectively cleaved by cysteine proteases. This cleavage site was intended to enable specific proteolytic processing, particularly in the event that the chimeric polypeptide was partially delivered to the vacuole as a consequence of ER quality control mechanisms [10]. This innovative approach provides a promising foundation for the development of biofunctional, plant-based, cosmetic ingredients tailored for skin rejuvenation.

2. Materials and Methods

Construct engineering and *Agrobacterium tumefaciens* transformation: The nucleotide sequence of the selected construct was synthesized by GenScript, subcloned into the pDHA vector, and the entire expression cassette containing the 35S promoter and nos terminator transferred to the binary vector pCambia 1302, previously deleted of the Hygromycin resistance gene and the reporter GFP gene (Green Fluorescence Protein), to obtain a safer plasmid (pCambia1302ΔHYGΔGFP) expressing the recombinant collagen gene.

About 100 nanograms of plasmid was added to 0.1 ml of Agrobacterium cells (strain LBA4 404) for the electroporation, according to the protocol (MicroPulser electropop, Biorad). The resulting colonies were verified by specific PCR reactions.

Transient expression in lettuce plants: *L. sativa* plants (*cv* D'Estate di Sarno) cultivated in a growth chamber for 6-8 weeks at 25°C under photoperiod (16 hours light/8 hours dark) were vacuum agroinfiltrated with a suspension of *A. tumefaciens* harboring the target gene, previously resuspended in infiltration buffer (10mM $MgCl_2$, 10mM MES pH5.5 and 150μM Acetosyringone). After agroinfiltration, the plants were incubated for a period between 3 and 7 days in the plant growth chamber until the analysis.

Obtainment of Ls-CP extract: Leaves collected from agroinfiltrated plants, expressing the target gene, were homogenized in a Tris-based buffer at pH 7.5 and centrifuged for 15 min at 7000 x g, then the supernatant was recovered and analyzed. The resulting extract was named Ls-CP.

Western blotting: Thirty micrograms of Ls-CP were subject to SDS-PAGE (4-16%) and electroblotted onto PVDF (Polyvinylidene difluoride) membranes (Immobilon; Millipore, Milan, Italy). After blocking, membranes were incubated with the anti-Flag antibody (GenScript) (1:10.000) for 16 h at 4°C and then with horseradish peroxidase-conjugated secondary antibody (1:5000; 1 h at room temperature). The immune complexes were visualized by enhanced chemiluminescence, according to the manufacturer's protocol (Pierce™ ECL Western Blotting Substrate, Thermo Fisher Scientific).

Skin cell cultures and skin explants: Human Dermal Fibroblasts (HDF) (Cell Application Inc., San Diego, CA) were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). Skin explants, obtained from the skin of healthy female donors following breast surgery, were cultured in 24-transwell plates in DMEM/FBS with antibiotics in air-liquid conditions at 37°C in 5% CO_2 humidified air. All donors provided written informed consent for the use of skin tissues, in accordance with the Declaration of Helsinki.

Collagen Production Assay: 8×10^4 HDF were seeded in 96-well plates and treated for 24 h with Ls-CP or with TGFβ 2.5 ng/mL as positive control. The ELISA was performed using the antibody sc-166,572 from Santa Cruz Biotechnology, Dallas, TX, USA.

Gene expression analysis: For MMP1 analysis, HDF were pre-incubated overnight with Ls-CP, stressed with UVA 10J/cm^2 and incubated for further 6 hours before total RNA extraction. For Sparc analysis, HDF were treated for 6 hours before collection. Total RNA was extracted with the GenElute Mammalian Total RNA Purification Kit (SIGMA) following provided instructions. Gene expression analysis was performed using gene specific primers and the Quantum RNA 18S internal standard (Thermo Fisher, Life Technologies, CA).

Staining of insoluble collagen by Sircol assay: Skin explants were used to obtain 4 mm punches which were treated with Ls-CP or a positive control and incubated for 5 days. The

samples were collected, weighed and incubated overnight at 4°C in a solution containing 0.1mg/ml of pepsin dissolved in 0.5 M of acetic acid. The following day, the punches were centrifuged, and both the supernatants and the punches were analyzed according to the manufacturer's instructions for the Sircol Collagen Assay kit (Biocolor). A 200µL aliquot of each sample was measured spectrophotometrically at 556 nm using a Victor Nivo plate reader (PerkinElmer).

Collagen Hybridizing Peptide Cy3 conjugate (R-CHP) staining: Skin explants were used to obtain 8mm punches, incubated in DMEM, 10% FBS with Ls-CP for 24h. They were then irradiated with UVA 22J/cm^2 and treated again for 48h. At the end of the treatments the punches were incubated in PFA 4%, then 15% sucrose and then in 30% sucrose and finally frozen. Cryosections (5µm thick)) were obtained using a Leica CM1520 cryostat. The slides with the sections were washed with PBS and incubated with CHP 20µM overnight at 4°C or 1µg/ml DAPI. After incubation, the slides were mounted, sealed and analyzed by fluorescence microscopy. Images were analyzed using ImageJ software.

3. Results

Design and obtainment of *Lactuca sativa* expressing Collagen peptides (Ls-CP)

Specific motifs of the collagen type I human sequence were selected as target peptides to be transiently expressed in *Lactuca sativa* plants. Beyond the typical collagen repeats rich in glycine and proline residues, other motifs were strategically chosen for their ability to establish triple helix interactions, integrin and SPARC binding sites.

Four tandem repeats of the selected motifs were separated by cleavage sites recognized by vacuolar cysteine proteases and fused to an efficient signal peptide derived from Arabidopsis basic chitinase to ensure targeting to the secretory pathway. Flag epitope was added at the C-terminus for immunological detection, along with the KDEL tetrapeptide for retention within the endoplasmic reticulum (ER). The construct design is illustrated in Figure 1.

.

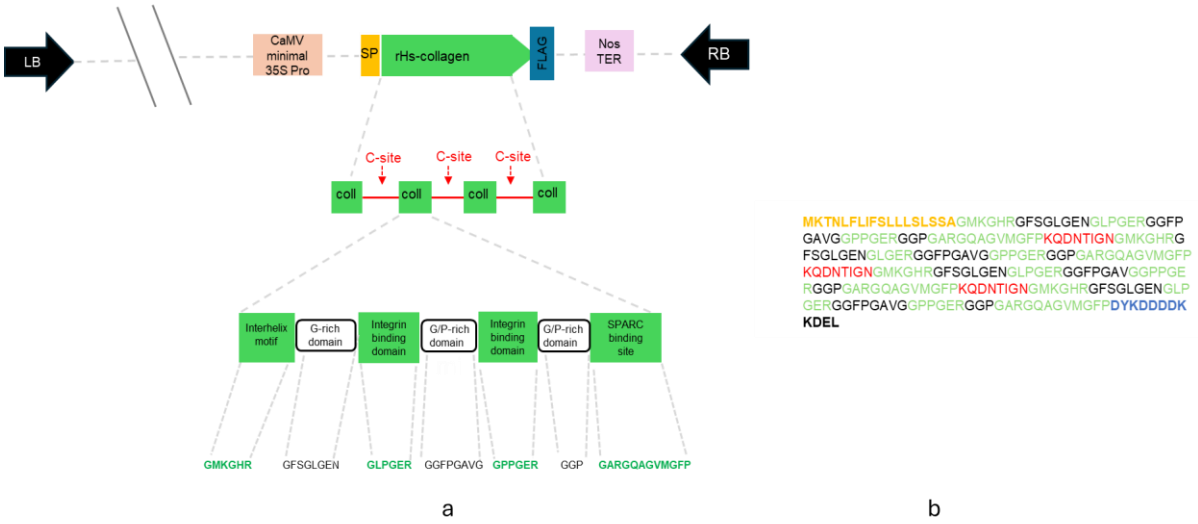

Figure 1 Construct design. a: Schematic representation of the expression cassette cloned in the binary vector pCambia 1302ΔHYGΔGFP. b: amino acid sequence of the recombinant polypeptide.

The expression vector, carrying the exogenous gene, was introduced into *A. tumefaciens* cells, used to transiently transform *Lactuca sativa* plants. Lettuce tissues were collected and processed for total protein extraction over a time course from 3 to 7 Days Post Infection (DPI) in order to establish the best conditions for the highest expression level of the recombinant collagen-derived peptide (Ls-CP). Western blot analysis, using an anti-FLAG antibody (figure 2), revealed a polypeptide of the expected molecular weight (~23KDa) which accumulated at high level in plant tissues. Moreover, the time-course analysis indicated that 3 DPI was the optimal time point for achieving the highest accumulation of the recombinant polypeptide, as well as, peptide fragments generated through partial processing by endogenous plant cysteine proteases.

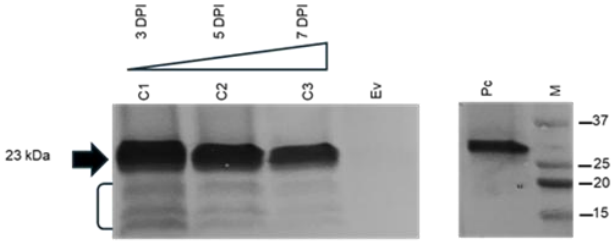

Figure 2: Characterization of Ls-CP. Western blot of total proteins extracted from three different samples (C1, C2, C3) at 3-5 and 7 DPI (Day Post Infection), probed with antisera to flag epitope; Ev: total protein extracted form plant agroinfiltrated with the empty vector; M: protein standard; Pc: multiple Tags Positive Control

Effects of Ls-CP on ProCollagen I in human dermal fibroblasts

The Ls-CP extract was tested in human dermal fibroblasts (HDF) for its ability to increase synthesis of collagen type I. HDF treated with Ls-CP at dose 0.002% and 0.003% or with TGFβ 2.5ng/ml, as positive control, were processed by ELISA using a specific antibody against Procollagen type I. As shown in Figure 3, treatment with Ls-CP led to an increase in ProCollagen type I production by approximately 56% at a concentration of 0.002%, and by 66% at 0.003%, comparable to the effect observed with the positive control.

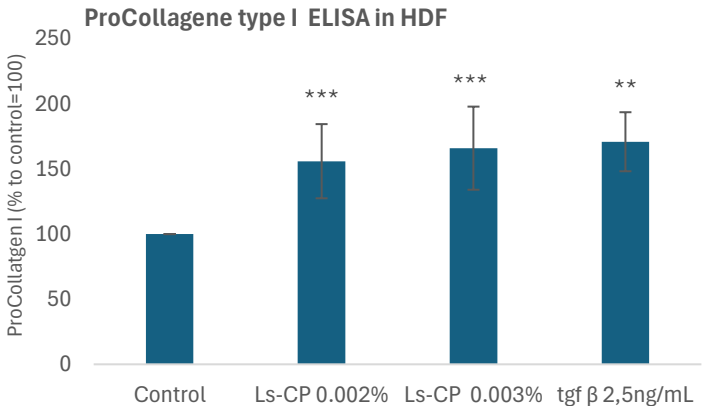

Figure 3: ELISA assay on Procollagen I in HDF cells. Data represent the average of three independent experiments ± standard deviations with statistical significance determined using Student's t-test (**p < 0.01 and *** p < 0.001).

Effect of Ls-CP on MMP1 gene expression.

MMP1 gene expression was evaluated in HDF pretreated with Ls-CP and subsequently irradiated with UVA 10J/cm^2. As shown in figure 4, UVA radiation increased the MMP1 expression by 20% respect untreated cells while pretreatment with Ls-CP prevented this upregulation, leading instead to a reduction in MMP1 expression by approximately 24% at a concentration of 0.002% and 37% at 0.003%, These effects were comparable to those observed with retinoic acid, used as positive control.

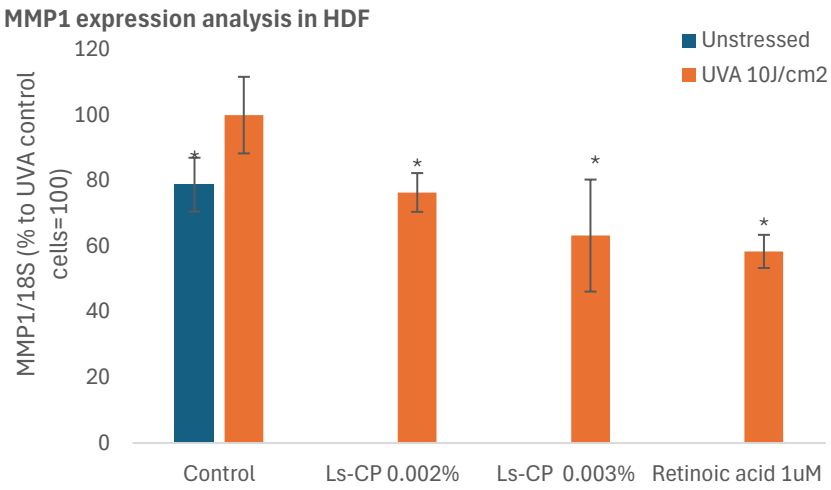

Figure 4: Expression analysis, by semi quantitative RT-PCR, of MMP1 gene in HaCaT cells. Data represent the average of 3 independent experiments ± standard deviations with statistical significance determined using Student's t-test (*p < 0.001).

Effect of Ls-CP on SPARC gene expression.

To confirm the role of Ls-CP in stabilizing the extracellular matrix and promoting the organization of collagen fibers, SPARC gene expression was evaluated in HDF treated with Ls-CP at the dose 0.002% and 0.003% and TGFβ 2.5ng/mL as positive control. As shown in Figure 5, Ls-CP induced SPARC gene expression by about 24% at the dose 0.002% and 63% at the dose 0.003% in way similar to the positive control.

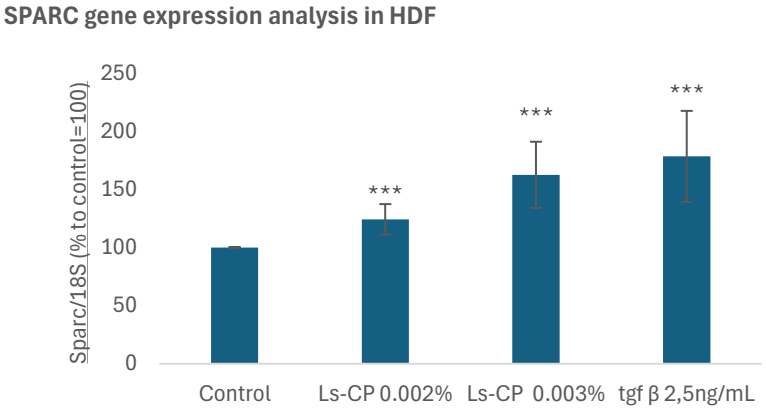

Figure 5: Expression analysis, by semi quantitative RT-PCR, of SPARC gene in HDF. Data represent the average of 3 independent experiments ± standard deviations with statistical significance determined using Student's t-test (***p < 0.05).

Effect of Ls-CP on Insoluble Collagen in ex vivo skin explants

The effect of Ls-CP on the proper assembly of collagen fibers was evaluated in an *ex vivo* study using the Sircol assay, which specifically stains correctly assembled, insoluble collagen fibers that are resistant to pepsin digestion. In particular the skin explants were treated for one week with Ls-CP at the dose 0.003% and TGFβ 2.5ng/ml. As shown in Figure 6, Ls-CP

induced correct collagen deposition in the extracellular matrix by about 63% respect untreated explants.

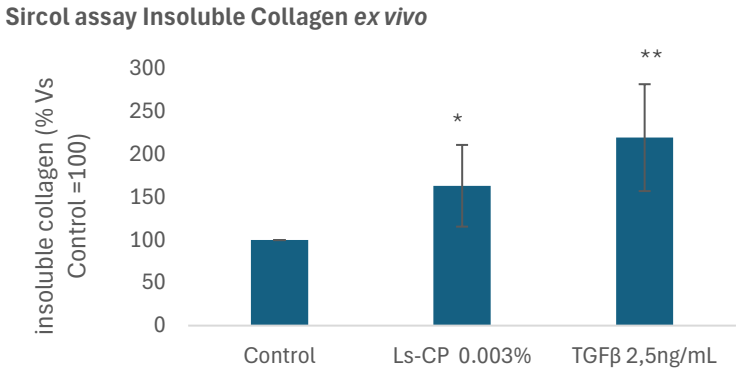

Figure 6: SIRCOL assay on skin explants. Data represent the average of 3 independent experiments ± standard deviations with statistical significance determined using Student's t-test (*p <0.05; **p < 0.01).

Effect of Ls-CP on protection from collagen degradation

The ability of Ls-CP to protect the collagen fibers from degradation induced by UVA was evaluated in skin explants pretreated with it at dose 0.003%, followed by UV irradiation at 22J/cm$^{2.}$ After a second treatment with Ls-CP for 72 hours the explants were processed and stained with Collagen Hybridizing Peptide (CHP), which binds to damaged collagen. As shown in Figure 7 Ls-CP protected and repaired collagen fibers reducing the CHP specifical binding to damaged collagen. Fluorescence intensity analysis (data not shown) revealed a reduction of damaged collagen by Ls-CP of 43% respect the irradiated explants. This effect is comparable to that of ascorbic acid, used as positive control.

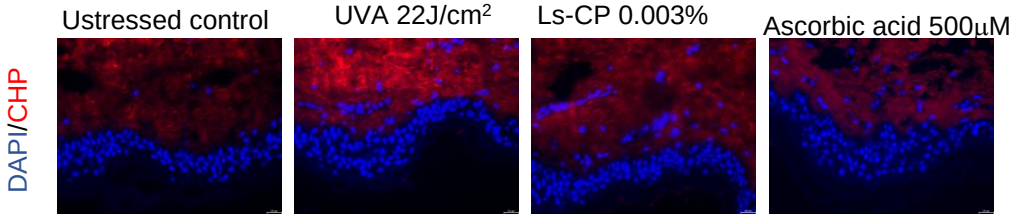

Figure 7. **Collagen Hybridizing Peptide (CHP) staining**. Representative pictures of CHP-stained slices.

3. Discussion

In this study, we investigated the feasibility of producing specific peptides derived from human collagen type I alpha, containing key functional motifs, using a lettuce-based plant transient expression system. The selected peptide sequences, located within the coding region of the alpha chain of type I collagen, were chosen for their roles in regulating collagen synthesis and for their potential interactions with integrins or SPARC. Considerable effort was devoted to engineering the peptide for expression in plant systems. Our strategy, which involved expressing multiple repeats of collagen peptides as polypeptide targeted to the endoplasmic reticulum (ER), proved to be highly effective. By employing the Arabidopsis basic chitinase signal peptide, renowned for its efficiency in directing proteins into the plant secretory pathway [11;12], and the KDEL, an ER retention signal, we successfully achieved accurate targeting of the chimeric polypeptide which facilitated its high accumulation by limiting its exposure to the proteolytic activities present in the vacuole or apoplast. Lettuce, utilized as a transient expression system, offered a rapid, efficient, and reproducible alternative to the traditional use of tobacco tissues. Unlike tobacco, which contains nicotine and other toxic alkaloids, that may restrict its applicability [13], lettuce presents a safer option for large-scale production. Furthermore, this system obviated the need for extensive polypeptide purification, enabling the direct recovery of a collagen-enriched recombinant extract with improved suitability for biotechnological applications. Although direct evidence is currently lacking, the detection of both full-length expressed collagen polypeptide and peptide fragments of the expected size, indicative of partial processing of the full-length polypeptide, suggests that the phaseolin sequence included in the construct functioned as intended. The release of individual peptide units may have resulted from partial trafficking of the polypeptide to the vacuole through ER quality control pathways. The biological activity of the peptide-enriched plant extracts was assessed on human skin cells. Treatment of human dermal fibroblasts (HDFs) with Collagen peptides enriched-lettuce extract (Ls-CP) resulted in a significant increase in procollagen production but also in preventing its degradation reducing MMP1 expression. Furthermore, the extract showed specific activity in enhancing extracellular matrix stability and promoting collagen fiber organization, as evidenced by the upregulation of the SPARC gene. The effect on collagen stabilization was further demonstrated by *ex vivo* test, in which it was found that Ls-CP was able to reduce the quantity of damaged collagen, as revealed by CHP staining, but also to improve collagen stabilization acting on its correct assembly. Indeed, staining with a dye specific for insoluble and thus correctly assembled-collagen revealed a significant increase in collagen levels.

5. Conclusion

Ls-CP, a novel extract derived from *Lactuca sativa* plants genetically engineered to express human collagen-derived peptides, was revealed as an agent able to boost synthesis of new collagen but also to favor its correct assembly and its protection against degradation. To our knowledge, this is the first report highlighting the potential of lettuce as a viable platform for the production of recombinant human peptides and as a suitable environment for generating active ingredients.

6. References

[1] https://doi.org/10.1146/annurev.biochem.77.032207.120833
[2] https://doi.org/10.3390/biomedicines10092307
[3] https://doi.org/10.3390/cells10030662
[4] https://doi.org/10.3390/ijms23126655
[5] https://doi.org/10.1016/j.bbamcr.2017.04.015
[6] https://doi.org/10.1074/jbc.M700167200
[7] https://doi.org/10.3390/metabo11080527
[8] https://doi.org/10.1038/s41598-018-28492-w
[9] https://doi.org/10.4161/hv.7.3.14103
[10] https://doi.org/10.1093/mp/ssn066
[11] https://doi.org/10.1124/mi.5.4.5
[12] https://doi.org/10.1007/s12033-021-00381-0
[13] https://doi.org/10.1016/S0031-9422(00)80751-7