A mechanistic investigation on kokumi-active γ-Glutamyl tripeptides – A computational study to understand molecular basis of their activity and to identify novel potential kokumi-tasting sequences

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Highlights

- Mechanistic and chemical basis of kokumi γ-Glutamyl tripeptides has been depicted
- The stabilization of the closed conformation of Calcium-sensing receptor was modeled
- Specific receptor movements were monitored to assess kokumi activity of molecules
- A virtual library of 400 γ-Glutamyl tripeptides was screened
- γ-Glu-Pro-Ala and γ-Glu-Pro-Ser were described as potential potent kokumi compounds

A mechanistic investigation on kokumi-active γ-Glutamyl tripeptides – a computational study to understand molecular basis of their activity and to identify novel potential kokumi-tasting sequences

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Abstract

Kokumi is an important taste perception whose chemical basis still needs clarifications and for which the development of high-throughput tools of analysis is desirable. The activation of Calcium-sensing receptor (CaSR) was described as the basis of kokumi perception allowing the use of molecular modelling to deepen its chemical rationale and related mechanisms. This study focused on γ-Glutamyl tripeptides, computationally ~~investigated mechanistic basis~~ providing mechanistic insights on ~~of~~ their CaSR-activating properties and extended the comprehension of their structure-activity relationship. A library of 400 γ-Glutamyl tripeptides was also screened. γ-Glu-Pro-Ala and γ-Glu-Pro-Ser were identified for further dedicated investigations based on their promising CaSR-activating potential and their presence should be checked accordingly in food matrices to better profile the kokumi fingerprint. This work provided a meaningful tool for the top-down analysis of kokumi-active molecules that may support either the identification of kokumi molecules concealed in food or the rational design of kokumi-active molecules *de novo*.

Keywords: γ-glutammyl peptides, kokumi, molecular modeling, taste receptors, calcium-sensing receptor

1. Introduction

The term “kokumi” refers to as a complex taste sensation characterized by thickness, mouthfulness and continuity (Toelstede, Dunkel, & Hofmann, 2009; Toelstede & Hofmann, 2008; Zhao, Schieber, & Ganzle, 2016). Such a sensation is important to determine taste, flavour, mouthfeel and aftertaste qualities of a wide variety of foods with critical implications for food acceptance and consequences on consumers behaviour and diet habits (Keast, Costanzo, & Hartley, 2021; Tang, Tan, Teo, & Forde, 2020). For this reason, the identification and characterization of kokumi-active molecules in food is an important piece of information, *inter alia*, to make connections between the chemistry of food and its perception and consumption. At the same time, the design of new kokumi-active molecules meant to be intentionally added in food can be important from a food design standpoint to endow designed food with desired flavour-related properties (e.g. (Ruijschop, Burseg, Lambers, & Overduin, 2009)). However, the kokumi-active fraction is hard to profile with precision in food due to experimental issues, and the *de novo* design of novel kokumi molecules is hampered by the uncomplete understanding of their structure-activity relationship.

From a mechanistic point of view, kokumi perception has been associated with the binding between kokumi-active molecules and the Calcium Sensing Receptor (CaSR), which results in its subsequent allosteric activation (Guha & Majumder, 2022). CaSR is a G-protein coupled receptor (GPCR) consisting in 1078 amino acids and expressed on the surface of diverse cell types. It has been under investigation since its discovery for its involvement in several pathologies (Huang, Zhao, Dong, & Hu, 2021; Sundararaman & van der Vorst, 2021). However, its expression was proved in the gustatory tissues of rats, further demonstrated in the taste buds in lingual epithelia, and the CaSR’s involvement was soundly proposed at the

basis of kokumi perception (Guha & Majumder, 2022; Maruyama, Yasuda, Kuroda, & Eto, 2012; Ohsu et al., 2010). Specifically, the so called CaSR's Venus fly trap (VFT) domain has been described as the binding site of kokumi substances and its capability to close over ligands has been reported as the molecular initiating event underpinning CaSR activation and kokumi perception (Li, Zhang, & Lametsch, 2022; Wellendorph & Brauner-Osborne, 2009).

Concerning the profiling of taste molecules in food, including kokumi-active compounds, the methods commonly used are still complex and time-consuming. They typically require composite multi-step experimental protocols based on the decomposition of a given matrix followed by the precise description of its taste-active chemical components (J. Kim et al., 2022; Vasilaki, Panagiotopoulou, Koupantsis, Katsanidis, & Mourtzinos). In line with the methods used to identify bioactive peptides of food origin, these methods can be defined "bottom-up" in the sense that they ideally require a comprehensive description of molecules in a certain food followed by their characterization in terms of taste activity (Schrader, 2018). Conversely, "top-down" approaches refer to techniques to create organized (bio)active structures either by etching down a bulk material or by engineering them into specific locations (Smith, Tejeda-Montes, Poch, & Mata, 2011). In the context of bioactive peptides of food origin, "top-down" approaches refer to food matrix-independent methods targeting the identification of bioactive sequences with specific features, the occurrence of which in food is searched after their bioactivity has been proved (Lammi et al., 2021). Similarly, taste-active molecules can be identified using "top-down" approaches defining their potential taste activity first, and then searching their presence in food matrices assessing their role in the overall food taste. In the context of kokumi-active molecules analysis, "top-down" approaches may ensure high-throughput investigations

providing ground either to better understand the chemistry underpinning the taste of specific foods *a posteriori*, or to support a knowledge-based design of taste molecules meant to be added in food.

In this context, computational analysis proved to efficiently support the characterization of taste molecules (Bo et al., 2022; J. Yang et al., 2021) with promising applications either for a bulk or precise discovery of novel taste-active compounds (Spaggiari, Di Pizio, & Cozzini, 2020). In this context, the present work aimed at defining a 3D molecular modelling framework useful for a "top-down" identification of potent kokumi γ-Glutamyl tripeptides to: I) ~~decipher~~ get insights on the chemical and mechanistic basis of potent kokumi-active γ-Glutamyl tripeptides; and ii) to screen a combinatory library of 400 γ-Glutamyl tripeptides to identify novel and potentially potent kokumi-active sequences worthy of further dedicated investigations. The study focused on the class of γ-Glutamyl tripeptides as it includes potent kokumi-active compounds with glutathione (GSH, γ-Glu-Cys-Gly) and γ-Glu-Val-Gly among the most potent kokumi-active molecules described so far (Kuroda et al., 2013; Ohsu et al., 2010). The workflow described here focused on the molecular initiating event of kokumi taste and was based on docking simulations and molecular dynamics to study the interaction between γ-Glutamyl tripeptides and CasR, which has been recently described as the target taste receptor of kokumi molecules (Maruyama et al., 2012). Overall, this work presented a rational, useful and straightforward strategy either to better understand the 3D structure-activity relationship of kokumi-active γ-Glutamyl tripeptides or to identify new, potentially potent, and previously uncharacterized sequences for further analysis.

2. Material and methods

2.1. Construction of γ-Glutamyl tripeptides library, tryptophan and glycine residues

The structures of γ-Glutamyl tripeptides analysed in this study were built using an in-house python script interfaced with PyMol (version 2.3.0; www.pymol.org). All possible duplets of the 20 proteogenic amino acids were generated, for a total of 400 combinations. These elements were symbolically represented by arrays of characters. The generated strings will be then divided into the representative characters of the single amino acid and added sequentially to the gamma carbon of the glutamate. The integrity of the carboxy-terminal was ensured adding an oxygen atom will be added to the carboxy terminal. The entire set of compounds generated was stored in the Protein Data Bank format (.pdb) first and then recursively converted in the Tripos Mol2 format (.mol2) for subsequent analysis using Open Babel (version 3.0.0) (O'Boyle et al., 2011) and setting the protonation state at pH 7 (i.e., carboxy- and ammino-terminal set deprotonated and protonated, respectively). The consistency of binding geometries and bond and atom type assignment were visually inspected before further analysis.

The library included 400 different entries grouping all the possible combinations for γ-Glutamyl tripeptides with a γ-Glutamyl residues at the 1st position (Ammino terminus) and all the possible combinations including permutations of 20 proteinogenic amino acids at the 2nd and 3rd position (Carboxy terminus).

The structure of L-tryptophan (L-Trp) and glycine (Gly) were retrieved from PubChem (S. Kim et al., 2021) in the 3D Structure Data File format (.sdf; CID 6305 and 750, respectively) and further converted in the .mol2 format as described above.

2.2. Ligand-based virtual screening of γ-Glutamyl tripeptides library

The library of γ-Glutamyl tripeptides underwent a ligand-based virtual screening to sort entries according to their similarity to γ-Glu-Val-Gly, which is the most potent CaSR-activator γ-Glutamyl tripeptide described *in vitro* so far (Ohsu et al., 2010). The ligand-based virtual screening was done using the LiSiCA (Ligand Similarity using Clique Algorithm) algorithm (Legnik et al., 2015). This algorithm provides a fast ligand-based virtual screening platform to quantify chemical similarities between a reference template (a strong kokumi γ-Glutamyl tripeptide in this case) and a database of target compounds. LiSiCA expresses similarities using the Tanimoto coefficient, a gold standard to quantify chemical analogies. LiSiCA's default parameters were used, with the exception of considering the 3D structures of ligands and setting the maximum allowed atom spatial distance for 3D product graph at 2, in agreement with a previous study (Lammi et al., 2021). This fast virtual screening procedure served to identify a short selection of sequences to carry forth to the slower and more demanding subsequent molecular modelling studies (see below).

2.3. CaSR model construction

The 3D model of human CaSR receptor was derived from the crystallographic structure recorded in the Protein Data Bank (https://www.rcsb.org; Berman et al., 2000) with PDB code 5FBK (chain A) (Zhang et al., 2016). This PDB record reported a high-resolution structure of the wild-type extracellular domain of human CaSR (residues 22-539), called venus flytrap (VFT) domain, which binds kokumi active molecules and is involved in the molecular initiating events of kokumi perception (Goralski & Ram, 2022; Mun et al., 2004). Such a structure was chosen based on its high higher resolution (2.10 Å) and more complete set of resolved coordinates compared to the others structure available at the time of

146 analysis (last database access 14th January 2022). However, it presented unresolved
147 coordinates for residues 361-390, which were not available in any of the structures
148 recorded in PDB at the time of analysis (last database access 14th January 2022). This portion
149 consisted in a small peripheral domain the integrity of which was obtained combining data
150 from homology modelling and expert structure editing. In more detail, the whole 3D
151 structure of VTF domain (residues 22-539) was obtained first using trRosetta
152 (https://yanglab.nankai.edu.cn/trRosetta) (J. Y. Yang et al., 2020) using the FASTA sequence
153 of 5FBK structure, in agreement with a previous study (Louisse, Dorne, & Dellafiora, 2022).
154 In more detail, trRosetta is an algorithm for a fast and accurate protein structure prediction
155 that derive protein structures based on direct energy minimizations with a constrained
156 optimization by Rosetta (Simons, Bonneau, Ruczinski, & Baker, 1999). The restraints include
157 inter-residue distance and orientation distributions, predicted by a deep residual neural
158 network. The model provided by trRosetta showed a high confidence with a TM score of 0.9
159 (scores above 0.5 point to correct topological assignment, in agreement with previous
160 studies (J. Y. Yang et al., 2020)). Such model was superimposed to the crystallographic
161 structure and used to tailor the crystallographic structure by substituting the residues 338-
162 402 ensuring the best backbone overlap between the model obtained from homology
163 modelling and the crystallographic structure.

164

165 **2.4. Docking studies**

166 The docking analysis aimed at providing a plausible binding architecture for L-Trp, Gly and
167 the γ-Glutamyl tripeptides under investigation and was performed using the GOLD software
168 (version 2021). The binding site has been defined within a 10-angstrom radius sphere

centred at the centroid of the substrate binding site of the VTF domain model with the xyz coordinates set in correspondence with the alpha-carbon of the crystallographic ligands of 5FBK (Zhang et al., 2016). Docking protocol was set according to previous studies keeping ligands fully flexible (i.e. all the routable bonds were set free to rotate) and protein semi-flexible allowing polar hydrogens free to rotate (Dellafiora et al., 2017). As a minor modification, 10 poses for each ligand were generated and scored using the internal scoring function GOLDScore as it has been optimized for the prediction of ligand binding positions, according to the manufacturer declaration (https://www.ccdc.cam.ac.uk). Considering that the scoring assignment is proportional to the ligand-pocket fitting (the higher the score, the higher the match to the pocket physico-chemical properties), only the best scored pose for each docked ligand was considered for the analysis, in agreement with previous studies (Dellafiora et al., 2017). Also, the positioning of each ligand was facilitated using the "Similarity constraint" option choosing the crystallographic pose of 5FBK's ligand as template and choosing to avoid the generation of docking poses when the constraint is physically impossible.

Moreover, GOLD uses a genetic algorithm that may introduce variability in the score assignment. Therefore, each ligand was run in triplicate to check the respective scoring fluctuations. Score assignment was found reliably stable for active compounds (further details are reported in the Results and Discussion section) with a maximum coefficient of variation below 0.9 % (scores are expressed as mean ± standard deviation).

RMSD values between the calculated and crystallographic poses of L-Trp were calculated using the align command implemented in PyMol disallowing cycles correction and considering the all set of not-hydrogen atoms.

2.5. Pharmacophoric analysis

The pocket of VFT domain was defined using GetCleft (Gaudreault, Morency, & Najmanovich, 2015), while the respective pharmacophoric fingerprint was derived using the IsoMIF algorithm (Chartier & Najmanovich, 2015), as previously described (Lammi et al., 2021).

2.6. Molecular dynamics

Molecular dynamics aimed at studying the stability of VTF domain in complex with the various ligands over the time. They were performed using GROMACS (version 2019.4) (Abraham et al., 2015) with CHARMM27 all-atom force field parameters support (Best et al., 2012) for protein, L-Trp and Gly. γ-Glutamyl tripeptides have been processed and parameterized with CHARMM27 all-atom force field using the SwissParam tool (http://www.swissparam.ch) (Zoete, Cuendet, Grosdidier, & Michielin, 2011). Input structures were solvated with SPCE waters in a cubic periodic boundary condition, and counter ions (Na^+ or Cl^-) were added to neutralize the system. Prior to running simulations, each system was energetically minimized to avoid steric clashes and to correct improper geometries using the steepest descent algorithm with a maximum of 5,000 steps. Afterwards, all the systems underwent isothermal (300 K, coupling time 2 psec) and isobaric (1 bar, coupling time 2 psec) 100 psec simulations before running 25 nsec simulations (300 K with a coupling time of 0.1 psec and 1 bar with a coupling time of 2.0 psec).

3. Results and Discussion

Kokumi perception is a complex phenomenon, and its underpinning early mechanisms depend on the activation of CaSR. CaSR is a class C G protein-coupled receptor (GPCR) working as a homodimer with a large extracellular VFT domain, which is involved in the recognition of kokumi active molecules (Ling et al., 2021). The network of molecular mechanisms underpinning the activation of CaSR and the transduction of signal through the membrane is complex and still need clarifications. However, the capability of VFT domain to close over ligands has been described as the molecular initiating event underpinning CaSR activation and kokumi perception (Wellendorph & Brauner-Osborne, 2009). On this basis, the present work assessed whether a computational workflow could estimate the capability of ligands to elicit a kokumi perception by triggering the very early mechanism at the basis of kokumi taste, i.e. the stabilization of the closed and active structure of CaSR VFT domain. In this respect, computational methods and bioinformatics already succeeded to investigate taste peptides (Iwaniak, Minkiewicz, Darewicz, & Hrynkiewicz, 2016). Specifically, in this work, a 3D molecular modelling approach was used combining docking studies, pharmacophoric analysis and molecular dynamics simulations to: i) ~~understand~~ get insights on the chemical basis of VFT domain-kokumi molecule interaction; and ii) study the early molecular mechanisms of receptor activation over the time. Once assessed the model performances, a virtual library of 400 γ-Glutamyl tripeptides was screened to identify novel and potentially strong kokumi-active sequences never described before.

3.1. Assessment of model performances

Model performances were assessed following a multi-tier approach in agreement with previous studies (e.g., (Louisse et al., 2022)). First, the capability to reproduce the binding architecture of L-Trp, taken as a kokumi reference ligand (Laffitte et al., 2021), was done

comparing its calculated pose with the crystallographic architecture of binding reported in the PDB structure having code 7DTU (Ling et al., 2021). As shown in Figure 1, docking simulation was able to reliably reproduce the binding architecture of L-Trp and its network of polar interactions, with an average RMSD value of 0.6 Å, confirming the geometrical reliability of the model. Then, the capability of model to discriminate between CaSR ligands (i.e., kokumi molecules) and non-ligands (i.e., kokumi-inactive molecules) was assessed combining data from docking studies and molecular dynamic simulations. Specifically, the weak CaSR activator Gly (chosen as negative controls due to its negligible kokumi activity), the moderate CaSR activator and kokumi-active molecules L-Trp, the strong activator and kokumi-active molecule γ-Glu-Val-Gly and the inactive γ-Glu-Val-Trp (chosen as a negative control) were considered in this study (Conigrave, Quinn, & Brown, 2000; Geng et al., 2016; Ohsu et al., 2010; Zhao et al., 2016). As shown in Table 1, the whole set of molecules included in this study recorded relatively high docking scores pointing to their possible capability to match pocket requirements (the higher the score, the higher the theoretical capability of ligands to match the pocket). Conversely, the inspection of docking poses revealed a substantial difference between the weak-to-strong activators (Gly, L-Trp and γ-Glu-Val-Gly) and the inactive sequence γ-Glu-Val-Trp. Specifically, the formers showed an architecture of binding that resembled the crystallographic pose of L-Trp with the alpha carboxy- and amino-group of γ-Glutamyl residue involved in polar contact with Ser147, Ala168 and Ser170 (Figure 2). The comparison between the docking poses and the pharmacophoric analysis of the binding pocket provided a mechanistic rationale for the experimental data reported so far for this set of molecules. In particular, the higher capability of L-Trp to activate CaSR compared to Gly could be explained, at least in part, by the better capability of the former to interact with the VFT domain. Indeed, the indole ring

of L-Trp was arranged within a hydrophobic region of the binding site likely adding additional hydrophobic/hydrophobic favourable interactions compared to Gly. In this respect, Gly recorded a docking score significantly lower than the other molecules, in agreement with its very weak capability to activate CaSR. These additional contributions to the binding event of L-Trp could suggest a major effect to stabilize the closed conformation of VTF domain compared to Gly determining a more effective CaSR activation and a more pronounced kokumi perception, in agreement with previous studies (Mun et al., 2004; Goralsky and Ram, 2022). Following this line of interpretation, the stronger activity of γ-Glu-Val-Gly could be explained by the additional contribution to the binding event due to the polar contact between its Gly C-terminus and the side chain of Asn102 (Figure 2C). Conversely, γ-Glu-Val-Trp, which was described as unable to activate CaSR (Ohsu et al., 2010), showed a substantially different architecture of binding which missed all the key contacts described above and reported in crystallographic studies despite the high score recorded. The incapability to mimic the binding pose of γ-Glu-Val-Gly was due to the local lack of a sufficient space to receive the bulky side chain of Trp at the 3rd (C-terminal) position that forced this peptide to find an alternative arrangement into the pocket. The relatively high score was reasonably due to the high number of hydrophobic/hydrophobic contributions. However, an appreciable interaction with VFT domain was not deemed likely based on the lack of the network of key polar contacts well reported by crystallographic records and calculated for γ-Glu-Val-Gly. In addition, γ-Glu-Val-Trp recorded more variable scores compared to the other ligands considered here, with a coefficient of variation (CV) of 3.6 % while the other activators showed CV values below 1%. The higher variability of γ-Glu-Val-Trp scores could indicate the incapability to find a unique and stable binding architecture, which is typically associated with nonoptimal ligands that are unable to

satisfyingly match pocket requirements (Aichinger et al., 2020). Taken together these results pointed to the possible relevance and usability of docking scores and poses to distinguish CaSR activators from inactive molecules.

Keeping in mind that the stabilization of the closed conformation of VFT domain is a key mechanistic event underpinning CaSR activation and kokumi taste perception, the stability of VFT domain in complex with Gly, L-Trp, γ-Glu-Val-Gly and γ-Glu-Val-Trp or in the unbound state was monitored over the time with molecular dynamic simulations. In particular, the protein's α-carbon root mean squared deviation (RMSD) was measured along with specific inter-molecular movements to describe VFT opening/closure (Figure 3). Specifically, three inter-atomic distances were considered to monitor VFT domain's motions and to describe either the stable maintenance of a closed conformation or its opening preluding to ligand detachment and receptor inactivation (Figure 3). –With respect to the RMSD analysis, the complexes considered and the VFT domain in the unbound state showed a similar trend ~~similar~~, although the complex with γ-Glu-Val-Trp showed slightly higher values from 15 nanoseconds onward (Figure 1S; Supporting material). This might point to a slight less stable geometrical organization of the complex with γ-Glu-Val-Trp compared to the other ligands or to the unbound VFT domain. Concerning the intra-molecular distances considered to monitor VFT domain closure/opening, a clearly different trend was observed for moderate and strong activators (i.e., L-Trp and γ-Glu-Val-Gly) compared to the weak activator Gly, the inactive peptide γ-Glu-Val-Trp and the unbound VFT domain. As shown in Figure 3B, the strong activator γ-Glu-Val-Gly and the moderate activator L-Trp showed a similar trend in the three intra-molecular contact regions (C1, C2 and C3) considered, which were kept tightly close along the whole simulation. Conversely, an early detachment was observed for all the contact regions in the unbound VFT domain (more marked for C2 than C1 or C3)

pointing as unfavourable the closed geometry of VFT domain in the unbound state, in agreement with previous studies (Ling et al., 2021). Of note, monitoring such intra-molecular distances could provide a semi-quantitative comparison of ligands in terms of activity: the very weak activator Gly showed for the three contact point regions considered a trend that was intermediate between the inactive sequence γ-Glu-Val-Trp and the two activators L-Trp and γ-Glu-Val-Gly. This was particularly evident for C2 and C3 where the interatomic distances tended to resemble the scenario drawn for the unbound VFT domain. In other words, Gly showed a limited capability to stabilize the closed conformation of VFT domain causing its opening as time went by during the simulation. This outcome could provide a mechanistic basis to better understand the rationale for the very weak activity reported for Gly. Concerning the inactive peptide γ-Glu-Val-Trp, all the interatomic distances considered constantly increased during the simulation pointing to a clear opening of the VFT domain. The close inspection of complex trajectories confirmed such hypothesis as constant opening movements were observed for all the contact regions considered (Figure 3C). As described above for Gly, this evidence provided a mechanistic rationale to understand the molecular basis underpinning the incapability of γ-Glu-Val-Trp to activate CaSR.

Taken together, these results highlighted the usability of the 3D modelling presented to investigate the early molecular event of kokumi perception and described a combination of docking simulations, pharmacophoric analysis and molecular dynamics as a meaningful tool to estimate the CaSR-activating potential of γ-Glutamyl tripeptides. At the same time, although the moderate activator L-Trp was not distinguished from the stronger γ-Glu-Val-Gly peptide preventing the possibility to directly correlate computational results to experimental values (e.g. EC_{50}), the reliability to semi-quantitatively estimate the CaSR-activating potential of γ-Glutamyl peptides was pointed out. Specifically, the procedure

proved efficacy to soundly distinguish moderate-to-strong γ-Glutamyl peptides from weak or inactive molecules.

3.2. Virtual screening of γ-Glutamyl peptides library

Based on the reliability of modelling framework discussed above, a virtual library grouping 400 γ-Glutamyl tripeptides was screened to identify novel sequences with a high theoretical potential as CaSR activators to study in further dedicated investigations. To this end, a ligand-based virtual screening was done to pre-filter the library and identify few promising candidates to investigate with docking simulations and molecular dynamics. Such pre-screening was done using a ligand-based fast search with LiSiCA algorithm and using γ-Glu-Val-Gly as reference template. LiSiCA scores ideally range from 0 (no similarity found) to 1 (high similarity found) and sorted γ-Glutamyl tripeptides according to their chemical similarity to γ-Glu-Val-Gly, which is one of the most potent CaSR activator and kokumi-active molecule described so far. The rationale of this choice was that chemical similarities may ensure a certain degree of conservation in terms of biological activity (CaSR-activating and kokumi properties in this case), as previously demonstrated (Lammi et al., 2021). The virtual screening results for the entire library of 400 γ-Glu-peptides are reported in Table 1S, Supporting material. Of note, 90% of the most potent γ-Glutamyl peptides described by Osho and co-workers (Osho et al., 2010) (i.e., with a reported CaSR activity < 30 μM) were at the top of LiSiCA rank with scores above 0.8 (Table 1S; Supporting material). This evidence validated the use of γ-Glu-Val-Gly as a template to search potent CaSR-activating γ-Glutamyl tripeptides and to enrich the top region of LiSiCA hierarchization with highly potent sequences.

Then, a selection of top-ranked sequences was considered for further analysis with docking studies and molecular dynamics. Among the uncharacterized sequences in terms of CaSR activity at the time of analysis and to the best of our knowledge, γ-Glu-Pro-Ala, γ-Glu-Ile-Ala, γ-Glu-Pro-Ser, γ-Glu-Ile-Cys and γ-Glu-Ile-Pro were chosen based on their sequence analogies to highly potent sequences already described (Table 1S; Supporting material) to maximize the chance to identify novel potent CaSR-activating γ-Glutamyl tripeptides. As shown in Figure 2, and despite the relatively high and stable scores recorded (Table 1), γ-Glu-Ile-Cys and γ-Glu-Ile-Pro showed a pose similar to the inactive peptide γ-Glu-Val-Trp. Therefore, their pose was considered not promising to stabilize a closed conformation of VFT domain and were not considered for further analysis. Conversely, γ-Glu-Pro-Ala, γ-Glu-Ile-Ala and γ-Glu-Pro-Se showed a binding pose similar to γ-Glu-Val-Gly and drawn a comparable network of polar interactions with the pocket suggesting that they could efficiently stabilize the closed conformation of VFT domain. This hypothesis was further assessed with molecular dynamic simulations. As shown in Figure 3D, γ-Glu-Ile-Ala caused an opening of the VFT domain as suggested by the increase of interatomic distances at the contact regions C1, C2 and C3. On this basis, it was not deemed to be a potent CaSR-activating peptide. Conversely, the interaction of γ-Glu-Pro-Ala and γ-Glu-Pro-Ser determined the stable closure of the receptor over the time, and they were both considered theoretically potent CaSR-activating and kokumi-tasting γ-Glutamyl peptides worthy of further dedicated analysis. Of note, γ-Glu-Pro-Ala showed a closure tighter than both γ-Glu-Pro-Ser and γ-Glu-Val-Gly at the contact region C3 possibly pointing to a marked capability to stabilize the closed conformation of VTF domain with possible consequences on its kokumi perception.

From a general point of view, the lack of appreciable activity calculated for γ-Glu-Ile-Ala, γ-Glu-Ile-Cys and γ-Glu-Ile-Pro agreed with the data available so far in terms of potent CaSR-activating γ-Glutamyl tripeptides. Indeed, among the most potent sequences reported so far, the 2nd position of γ-Glutamyl tripeptides is rarely occupied by bulky residues and Ile is reported at the 2nd position in the reference set of already characterized γ-Glutamyl tripeptides (Table 1S; Supporting material) only in one moderately active sequence (γ-Glu-Ile-Gly). This evidence is in line with the observed limited space the pocket had to receive the side chain of residues at the 2nd position (Figure 4A). This structural requirement was reasonably at the basis of the VFT domain opening to allow the arrangement of the bulky side chain at the 2nd position of γ-Glu-Ile-Ala and might generally determine either a low or no activation of CaSR by γ-Glutamyl tripeptide with bulky side chains at the 2nd position. Conversely, less bulky side chains, like Val, ~~or~~ Pro or Cys, could be better tolerated at the 2nd position, and they may efficiently stabilize the closed and active conformation of VFT domain, in line with the evidence reported for the reference set of γ-Glutamyl tripeptides (Table 1S; Supporting material). Specifically, although the pharmacophoric analysis revealed regions able to receive polar groups (Figure 3S; Supporting material), steric constraints at the 2nd position seemed important to determine the capability of γ-Glutamyl tripeptide to properly bind the VFT, in line with the lack of potent kokumi γ-Glutamyl tripeptide with a bulky polar residue at the 2nd position (Table 1S; Supporting material). In this respect, the reference set of γ-Glutamyl tripeptides (Table 1S; Supporting material) included only one kokumi active peptide with a polar (and small) residue at the 2nd position, i.e. γ-Glutamyl-Thr-Gly, further supporting this hypothesis. Concerning the space receiving the side chains in 3rd position (C-terminus), the analysis of VFT domain binding site revealed an opening to a solvent exposed region (Figure 4B) that could explain the higher tolerance for bulky side

chains as observed for some CaSR-activating γ-Glutamyl tripeptides (e.g., γ-Glu-Val-Phe and γ-Glu-Val-Arg, Table 1S; Supporting material). Nonetheless, the existence of a certain specificity could be inferred considering the incapability of γ-Glu-Val-Trp to properly dock the binding site and to active CaSR.

4. Conclusion

The identification of kokumi molecules, although still challenging, is important to understand the chemistry of food and related consequences on diet habits and consumer behaviour. The recent advances in the comprehension of mechanisms underpinning kokumi perception, and in particular the identification of CaSR as the receptor responsible for kokumi molecules recognition, paved the way to use molecular modelling approaches as a meaningful tool of analysis. In this context, the present work showed that modelling the interaction with the CaSR's VFT domain could efficiently estimate the activating potential of γ-Glutamyl tripeptides. In particular, the study identified relevant early mechanistic features at the basis of CaSR activation, such as the way γ-Glutamyl tripeptides arranged into the binding site and the related effects on VFT domain opening, that could be efficiently modelled to extend knowledge on ~~understand~~ the chemistry of γ-Glutamyl tripeptides and used to estimate the potential of still uncharacterized sequences. This may be used in future bulk analysis for a systematic and high throughput analysis supporting the characterization of taste molecules in food. In addition, this study described precisely structural features affecting CaSR-activating potential of γ-Glutamyl tripeptides and the 3D structure-activity relationship analysis could serve as a useful blueprint to improve the understanding of chemical basis for kokumi molecules. Also assessing the interaction between the γ-Glutamyl

tripeptide under analysis and other GPCRs might be considered for further analysis to better characterize the biological activity beyond their taste properties, in line with the various activity they may exert in living organisms (Lu et al., 2021).

Overall, this work provided a useful, reliable and high-throughput tool for a top-down screening of γ-Glutamyl tripeptides which may be integrated into the conventional characterization and sensory tests of taste molecules facilitating the fingerprinting of kokumi compounds in food. Similarly, the model could help the rational design of libraries of compounds in the context of the *de novo* synthesis of kokumi molecules.

Finally, two molecules with a high potential in terms of CaSR activating properties and kokumi taste were identified (i.e., γ-Glu-Pro-Ala and γ-Glu-Pro-Ser). Although such molecules have been prioritized for further dedicated investigations and sensory tests to ultimately verify their actual kokumi properties, it is advised their inclusion in the list of molecules to check in food matrices to characterize their possible kokumi fingerprint at a molecular level.

Declaration of interest

The authors have nothing to disclose.

Acknowledgment

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Figures

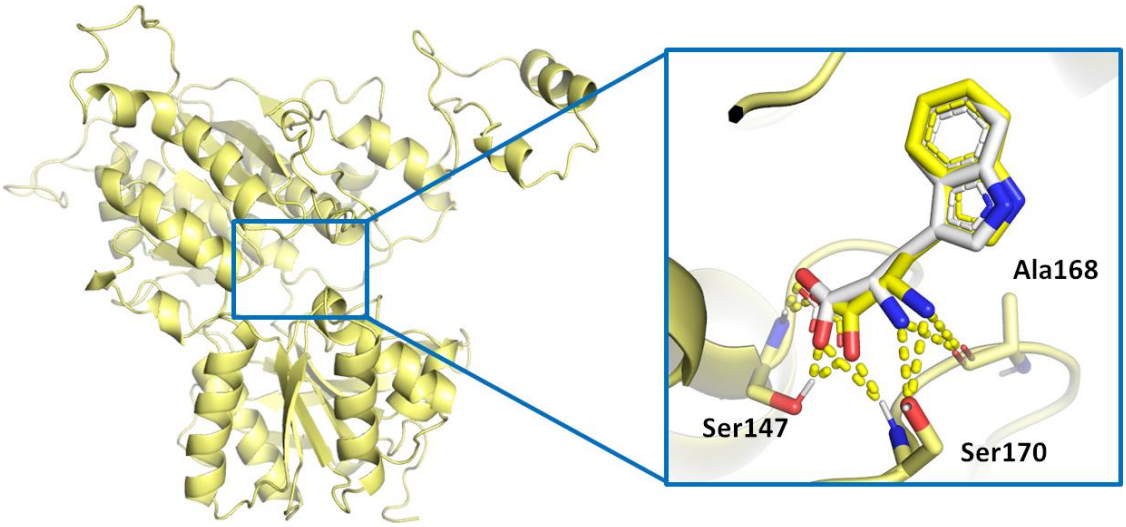


Figure 1. Graphical representation of CaSR VTF domain. The protein is represented in cartoon while ligands are represented in stick. The close-up on the right reports the comparison between the calculated (yellow) and crystallographic (white) pose of L-Trp. Yellow dashed lined indicate to formation of polar contacts.

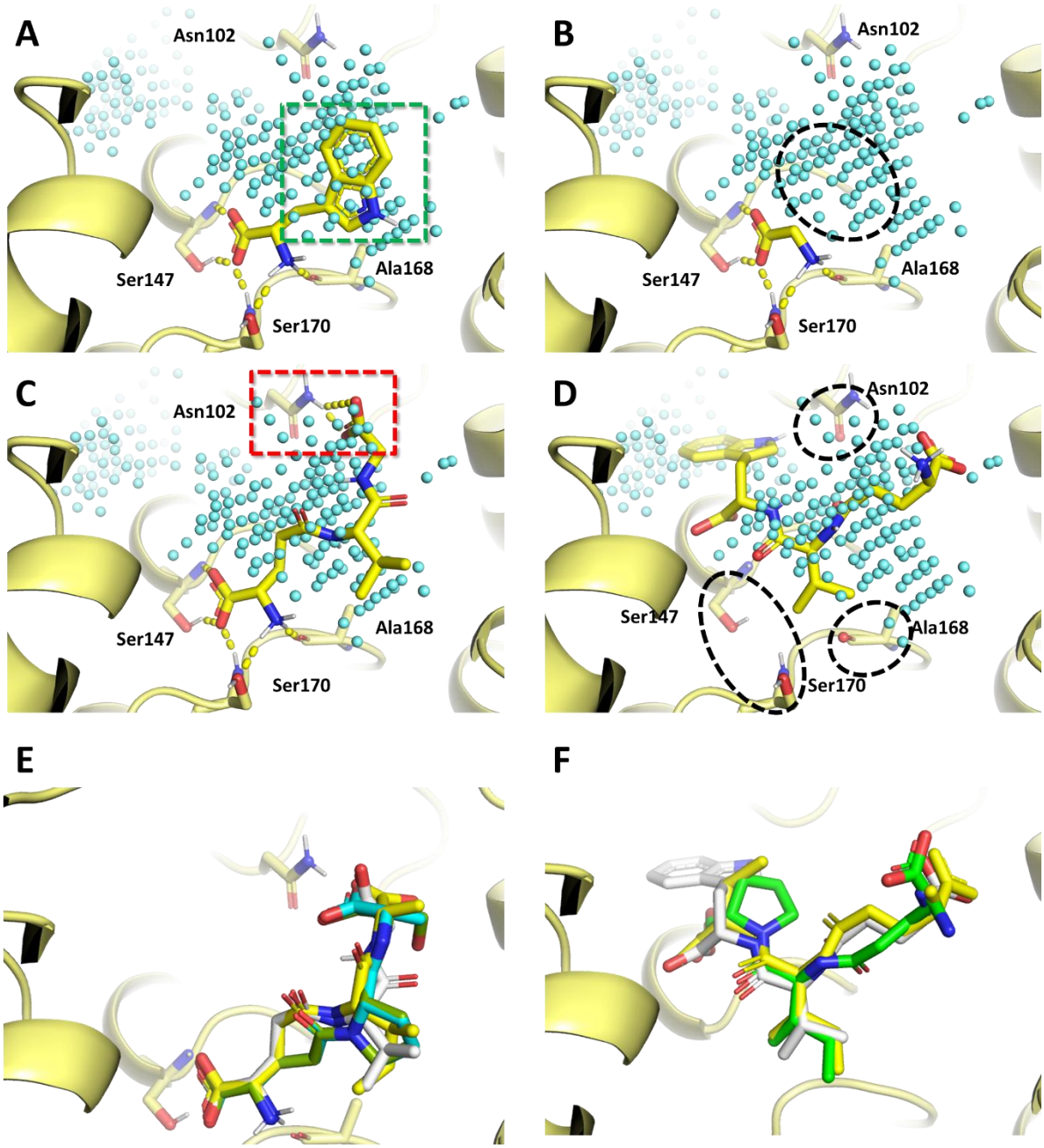


Figure 2. Docking pose of molecules under investigation. The protein is represented in cartoon while ligands and protein's amino acids involved in polar contacts are represented in sticks. Yellow dashed lines indicate polar contacts while cyan spheres represent the region of the binding pocket energetically suitable to receive hydrophobic groups. **A.** L-Trp. The green dashed box indicates the proper arrangement of the hydrophobic indole ring within a region of the pocket suitable to receive hydrophobic groups. **B.** Gly. The dashed black ring indicates the lack of hydrophobic-hydrophobic interactions. **C.** γ-Glu-Val-Gly. The

red dashed box indicates the additional polar contact between the peptide C-terminus (Gly at the 3rd position) and the side chain of Asn102. **D.** γ-Glu-Val-Trp. The dashed black rings indicate the lack of interaction with the residues engaged by the other molecules under investigation. **E.** Docking pose superimposition of γ-Glu-Val-Gly (white), γ-Glu-Ile-Ala (yellow), γ-Glu-Pro-Ser (green) and γ-Glu-Pro-Ala (cyan). **F.** Docking pose superimposition of γ-Glu-Val-Trp (white), γ-Glu-Ile-Cys (yellow) and γ-Glu-Ile-Pro (green).

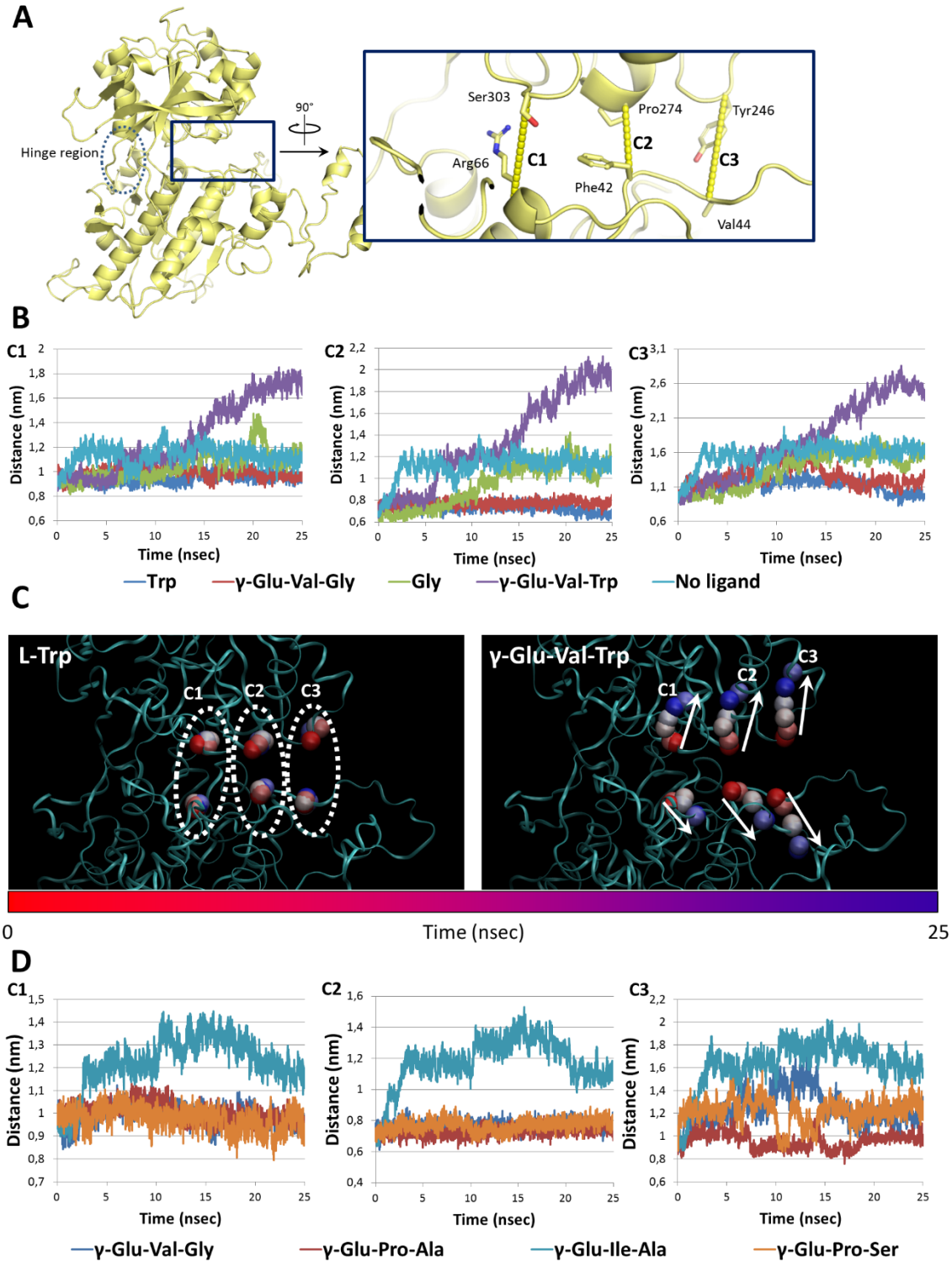


Figure 3. Molecular dynamics results. **A.** Graphical representation of CaSR in cartoon. The dashed ring indicates the hinge region while the three intra-molecular contact region distances considered to measure the opening of VFT domain are indicated with yellow dashed lines in the close-up on the right. Specifically, three contact regions (C1, C2 and C3) were defined between the α-carbon of Arg66-Ser303, Pro274-Phe42 and Tyr246-Val44,

606 respectively. **B.** Distance plots of C1, C2 and C3 contact regions for VTF domain in complex
607 with Gly, L-Trp, γ-Glu-Val-Gly, γ-Glu-Val-Trp or in the unbound state. **C.** Trajectory analysis of
608 contact region C1, C2 and C3 for VTF domain in complex with L-Trp or γ-Glu-Val-Trp. The
609 from-red-to-blue colour switch indicates the stepwise changes of coordinates during the
610 simulation. The dashed white rings indicate the stable fluctuation of interatomic distances
611 for C1, C2 and C3 contact regions when VTF domain was in complex with L-Trp. Conversely,
612 white arrows indicate the progressive detachment of C1, C2 and C3 contact regions for VTF
613 domain in complex with γ-Glu-Val-Trp leading to the structure opening. **D.** Distance plots of
614 C1, C2 and C3 contact regions for VTF domain in complex with γ-Glu-Val-Gly (taken as
615 reference), γ-Glu-Pro-Ala, γ-Glu-Ile-Ala or γ-Glu-Pro-Ser.

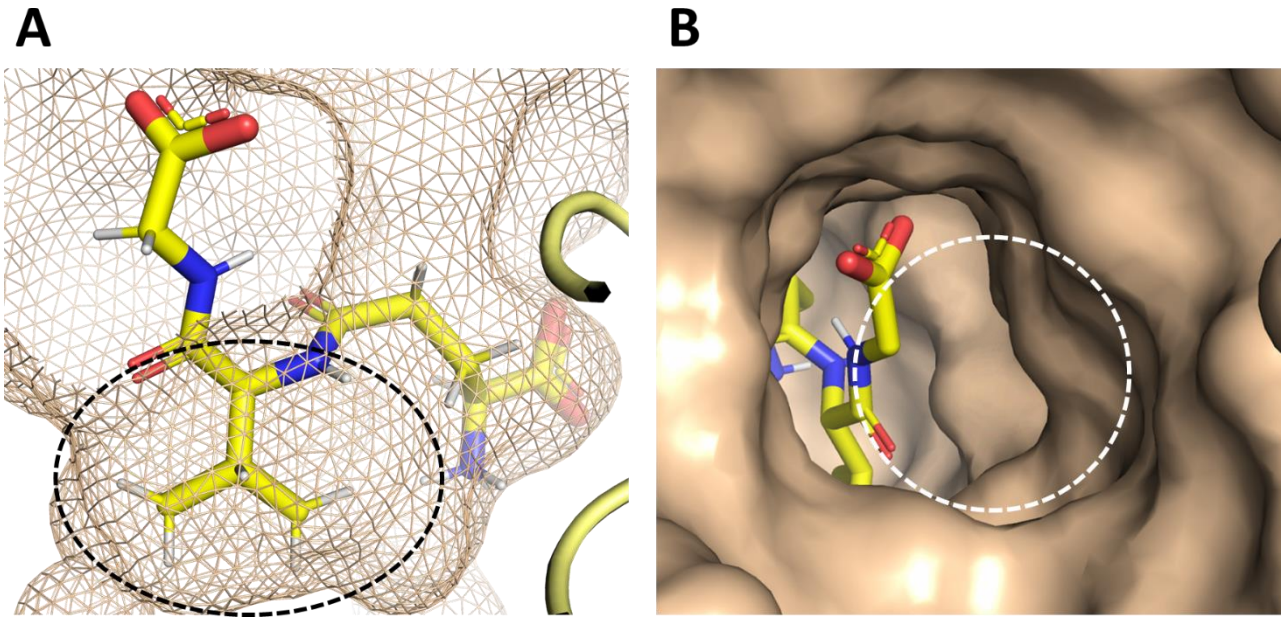


Figure 4. Close-ups of the pocket environment surrounding γ-Glu-Val-Gly. **A.** Focus on the space surrounding the side chain of Val at the 2nd position. The pocket is represented in mesh while γ-Glu-Val-Gly is represented in sticks. The black dashed ring indicates the almost full sub-site occupancy by Val side chain. **B.** Focus on the space surrounding Gly at the 3rd position. The pocket is represented in solid surface while γ-Glu-Val-Gly is represented in sticks. The white dashed ring indicates the presence of a solvent exposed region of the VTF domain binding site that could be occupied by the side chain of certain amino acids at the 3rd position of γ-Glutamyl tripeptides.

625 **Tables**

Table 1. Docking scores and reported activity of molecules under analysis

Peptide	**Docking score (mean ± SD)[1]**	**Experimental activity**
Gly	58.65 ± 0.04	Very weak activator[2]
L-Trp	103.37 ± 0.16	Moderate activator [3]
γ-Glu-Val-Gly	116.70 ± 0.90	Strong activator [4]
γ-Glu-Val-Trp	115.65 ± 4.14	Inactive [4]
γ-Glu-Pro-Ala	123.26 ± 0.86	Unknown
γ-Glu-Ile-Ala	121.86 ± 0.36	Unknown
γ-Glu-Pro-Ser	125.54 ± 1.16	Unknown
γ-Glu-Ile-Cys	124.41 ± 0.82	Unknown
γ-Glu-Ile-Pro	115.42 ± 0.39	Unknown

Note: [1] Docking scores are expressed as mean ± SD of three replicates. [2] According to (Geng et al., 2016). [3] According to (Conigrave et al., 2000). [4] According to (Ohsu et al., 2010).

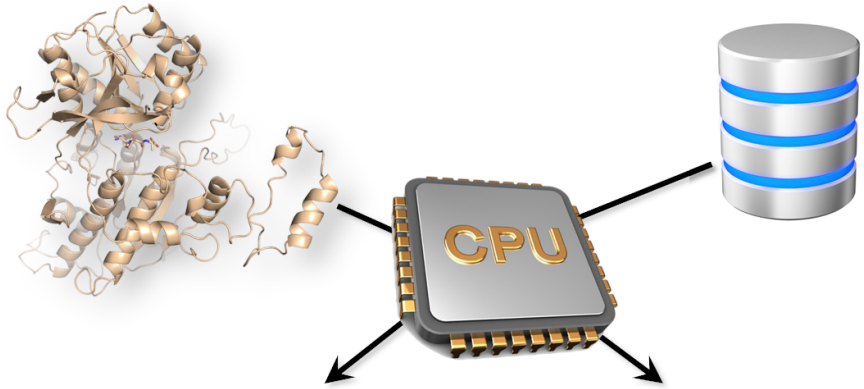
626

Graphical abstract

A mechanistic investigation on kokumi-active γ-Glutamyl tripeptides

3D model of CaSR's venus flytrap domain

Virtual library of 400 γ-Glutamyl tripeptides



3D structure-activity relationship analysis of kokumi-active γ-Glutamyl tripeptides

Tool for the top-down identification of kokumi-active molecules

CRediT author statement

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