In-silico evaluation of peptide hybrids of di-31 as potential plant defense modulators via activation FLS2

Evaluación *In-sillico* híbridos peptídicos de di-31 como posibles moduladores de defensa de las plantas mediante la activación FLS2

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ABSTRACT

In order to manage stress, plants must balance growth and defense processes at molecular level. Some control over these processes, would allow mankind to develop an efficient and sustainable agriculture. Within this field, the study of defense stimulators' response steps out as possible substituents of agrochemicals. Thus, by hybridizing the steroidal biostimulant DI-31 with the γ-core motif of the antifungal defensin MtDef4 (GRCRGFRRRC), it was intended to potentiate the steroid bioactivity. Molecular docking studies of the designed hybrids against FLS2 receptor, as a potential way for inducing the immune response in plants, were carried out along with DFT calculations including frontier molecular orbitals analysis for the ligands; pKd values for ligand-protein complex were also estimated with the neural network NNScore 2,0. The results displayed the possibility that mono-steroidal hybrid DI31-GMA4, could be recognized by the studied receptor and, subsequently, induce the corresponding biological activity as defense stimulator.

Keywords: FLS2; flagellin; steroid-peptide hybrids; defensin; plant defense.

RESUMEN

Para controlar el estrés, las plantas deben equilibrar los procesos de crecimiento y defensa a nivel molecular, un control sobre estos procesos permitiría a la humanidad desarrollar una agricultura eficiente y sostenible. Para potenciar la bioactividad de los esteroides, se utilizó la hibridación del bioestimulante esteroideo DI-31 con el *γ-core* del antifúngico defensiva MtDef4 (GRCRGFRRRC) y se llevaron a cabo estudios de acoplamiento molecular contra el receptor FLS2, como potencial para inducir una respuesta inmune en las plantas, junto a cálculos DFT que incluyeron un análisis de Orbitales Moleculares para los ligandos. También se estimaron los valores de pKd para el complejo ligando-proteína con la red neuronal NNScore 2,0. Los resultados mostraron la posibilidad de que el híbrido DI31-GMA4, pudiera ser reconocido por el receptor estudiado y posteriormente inducir la actividad biológica correspondiente como estimulador de defensa.

Palabras clave: FLS2; flagelina; híbridos esteroide-péptido; defensina; defensa de plantas.

Recibido: 28/5/2023

Aprobado: 24/7/2023

Introduction

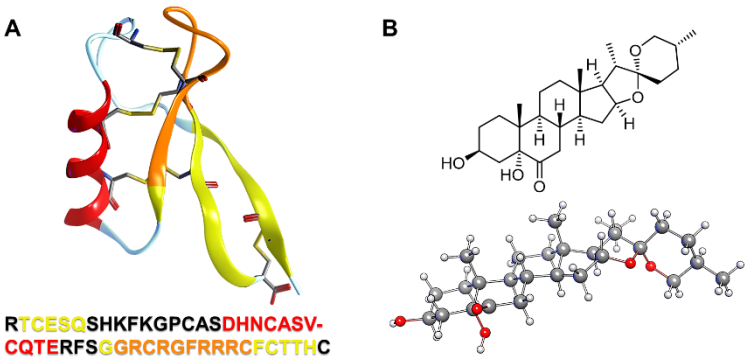
Plants are sessile organisms that live in a forced and permanent interaction with the environment which generally conditions stress states. When pathogens are absent or any environmental changes take place, young tissues must delete immune and adaptative responses to maximize growth, while mature organs are more prepared for defense. Thus, activation of defense mechanisms, at the expense of determined growth processes restrictions, is the result of a delicate balance at the molecular level better-known as growth-defense trade-offs (GDT). GDTs are associated to a variety of signaling opposite routes, regulated by phytohormones, and depending on the availability of nutritional resources in the environment.[1,2]

Plants possess membrane receptors that can recognize common molecular patterns associated to pathogens (PAMPs). Upon activation in the presence of PAMPs, these receptors form complexes with other proteins and trigger signaling cascades which make up plant innate defense response.[3,4] It is important to point out that the constant exposure to the above-mentioned stimuli keep the plant organism in a defense priming state that makes possible to respond in a quicker and more effective way to biotic stresses the plant is familiar with. Moreover, this fact could be a primitive analogue to the immune memory of mammals.[5]

Flagellin Sensitive 2 (FLS2) is a receptor like kinase (RLK) responsible for the recognition of the conserved pattern *flg22* (QRLSTGSRINSAKDDAAGLQIA) at the N-terminus of flagellin the protein of bacteria flagellum.[6] The *flg22* motif couples to

FLS2 and stabilizes the formation of a heterodimer with BAK1, then transduction cascades are activated, regulated by the corresponding phytohormones, acting through the modulation of an array of defense genes which activate proteases, proteins for cell protection, reactive oxygen species (ROS), antimicrobial peptides (AMPs), etc.[7-9] Additionally, BAK1 is the co-receptor for BRI1, also belonging to RLK family, which regulates growth processes upon brassinosteroids (BRs) recognition; therefore, such protein-kinase plays a key role in GDT regulation since it is directly involved in the molecular processes necessary for the aforementioned balance.[10]

In this sense, plant defensins are AMPs expressed ubiquitously all over the plant kingdom, mainly as a result of innate immunity. Also, it has been proven that these molecules can present constitutive and physiological activities such as growth and stress response regulators.[11-13] Despite they are formed up by a huge diversity of aminoacidic residues, it was demonstrated that the biocide activity of defensins mainly resides on a cationic conserved motif known as γ-core. For instance, peptide GMA4 (GRCRGFRRRC), γ-core of antifungal defensin MtDef4 (Figure 1A), was able to inhibit growth of *Fusarium graminearum*, so did hexapeptide RGFRRR, one of the most conserved motifs amongst plant defensin family. [14-16] What it is not assured yet is if such fragment take part in the other activities reported for defensins.



Fig, 1- (A) Secuence and 3D (PDB code: 2LR3) structure of defensin MtDef4 [11], highlighted in orange the γ-core motif (GRCRGFRRRC); (B) Structrure and 3D model of DI-31

On the other hand, it has been demonstrated recently that diosgenin derivative DI-31 (Figure 1B), a synthetic analogue of BRs, can induce plant growth and biotic stress resistance.[17] Additionally, molecular docking studies showed that such activity might be through BRI1-BAK1 complex activation and stabilization.[18] Therefore, it could be hypothesized that DI-31 peptide hybrids with GMA4 fragment could induce defense response and biotic stress resistance in plants. That is why, in this work it is intended to study the interactions of DI-31-GMA4 hybrids with FLS2-BAK1 complex as a possible way to induce defense response, by using molecular docking and frontier molecular orbital (FMO) analysis using DFT calculations.

Materials and methods

Protein FLS2 from A thaliana was downloaded from the Protein Data Bank (www, rcsb, org, PDB code: 4MN8, 3,06 Å) which was crystalized forming the heterocomplex with its natural ligand *flg22* and the co-receptor BAK1, Water, saccharides, anions and other molecules were deleted using PYMOL v1,8,4,0 and protonation states at pH = 7,0 were determined with PROPKA webserver (https://server,poissonboltzman,org/pdb2pqr), The generated structure was then saved in PDB to be later converted into PDBQT format using AutoDockTools v1,5,6 (ADT).

Peptide-protein poses were predicted with HPEPDOCK [(19)] webserver (http://huanglab,phys,hust,edu,cn/hpepdick/). As an input the PDB file with the 3D structure of FLS2-BAK1 complex was submitted along with the FASTA sequence of peptide (>GMA4; GRCRGFRRRC). To specify the active site some residues were listed as 148:A, 272:A, 296:A, 152:A, 294:A, 316:A, 342:A, 52:B, 54:B, 60:B, 61:B, 300 conformations were generated which were ranked increasingly respecting to the returned scoring.

Top 5 conformations generated by HPEPDOCK were used to assemble mono- and bis-steroidal hybrids, using AVOGADRO 1,1,1. Hybrid structures were minimized with the steepest descent algorithm of AVOGADRO, using MMFF94 [(20)] force field, PDBQT structures were obtained with ADT [(21)] making rotatable only side chains bonds of the peptide and succinate linkers of hybrids. The peptide backbone and the steroidal framework were declared rigid.

The top 5 conformations of GMA4, the two hybrids in their respective 10 conformations, and the natural ligand *flg22* underwent molecular docking using Vina [(22)] in order to predict binding modes and their affinity. The simulation box was centered at (29,4, 19,0, -0,5) with a size of 36x48x70 Å3, 10 independent runs were carried out, generating 3 binding modes each (num_modes = 3; exhaustiveness = 32), giving a total 30 poses whice were clustered in 3 clusters (RMSD < 2,5 Å) to determine the representatives binding modes. Each cluster was analyzed by its population, average binding energy, estimated pKd with NNScore 2,0 [(23)] and studied contacts with BINANA.[(24)] All 3D images were generated with PYMOL v1,8,4,0.

DFT calculations were performed with Gaussian 09.[(25)] All structures were optimized using Density Functional Theory at B3LYP density functional [(26)] with the adding the D3(BJ) dispersion correction [(27)] and using the using 6-311G (d,p). Pople's basis set, The frontier orbitals HOMO/LUMO were visualized directly from the optimized structure with DFT/B3LYP/6-311G(d,p) quantum mechanical level of theory by Gaussview 6,0.[(28)]

Results and discussion

The followed workflow involved several stages: (1) design of hybrids based on FLS2 active site characteristics; (2) conformational analysis of peptide GMA4 in its

interaction with FLS2-BAK1 complex; (3) molecular docking analysis of GMA4 and its hybrid with DI-31 against the heterodimer FLS2-BAK1; and (4) DFT calculations and Frontier Molecular Orbital analysis.

A mono- and a bis-steroidal hybrids (Figure 2) were designed taking into account the synthetic efficiency and the possibility of great scale production, along with the structural aspects that ensure the interactions with FLS2 receptor, as a possible way to induce an immune response:

- GMA4 is an arginine-rich decapeptide synthetically scalable. It has the amphipathicity required for the interaction with the receptor and for stabilizing FLS2-BAK1 complex.

- The hybridization with DI-31 at the N-terminus of the peptide GMA4 would favor the interaction with FLS2-LRR, it would confer rigidity to the peptide, making easier the adequate positioning inside the groove that make up the FLS2 active site.

- The functionalization with succinate linker at the C-3 only require one synthetic step, also its flexibility and equatorial stereochemistry facilitates the facial interactions of the steroid with the receptor.

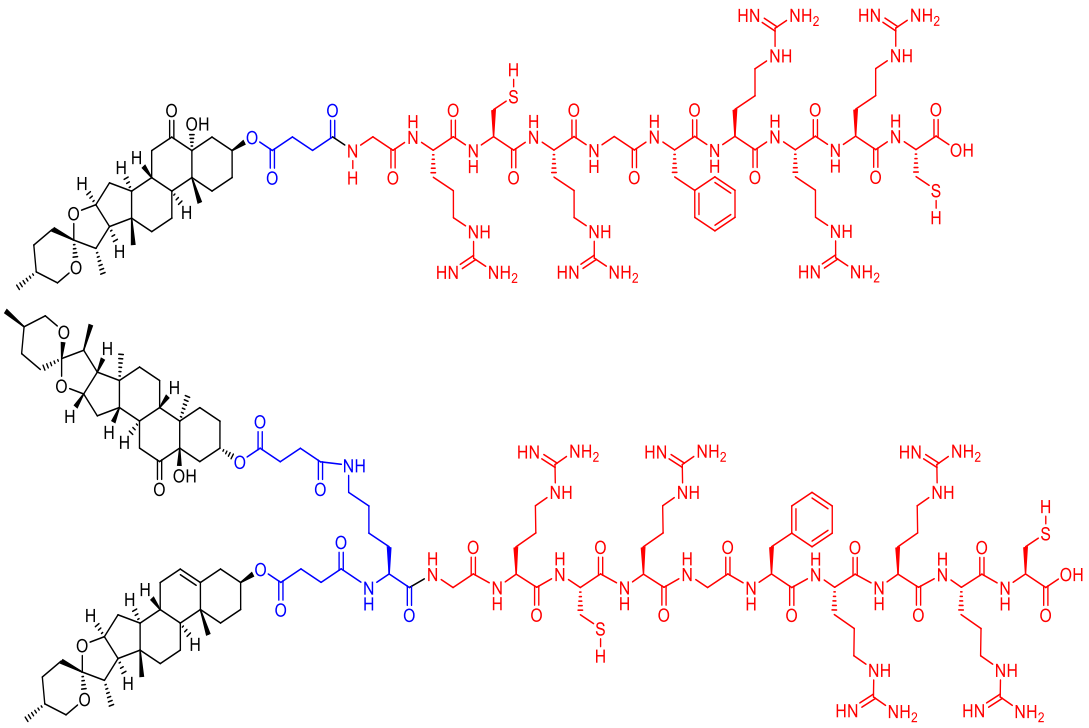


Fig. 2- Structure of designed hybrids, Above mono-steroidal hybrid and below bis-steroidal hybrid

Conformational analysis of peptide

Since fragment GMA4 is naturally occurring in plant defensins it does not have a known 3D structure, thus it was necessary to make a preliminary conformational analysis of the peptide. For that purpose, HPEPDOCK [19] webserver was used, since its hierarchical algorithm returns a set of 300 conformations that the peptide can adopt in its interaction with the active site of FLS2 receptor. In table 1 the scoring for top 10 generated model is shown, as in figure 3 conformations of top-five models are displayed interacting with the active site of FLS2.

Table 1. HPEPDOCK top 10 scoring.

Model	Scoring	Diff,
model_1	-219,119	---
model_2	-213,765	5,354
model_3	-205,209	8,556
model_4	-201,447	3,762
model_5	-200,502	0,945
model_6	-198,589	1,913
model_7	-196,598	1,991
model_8	-196,517	0,081
model_9	-196,319	0,198
model_10	-195,929	0,390

The conformations and the scoring for the natural ligand *flg22* couldn't be obtained because for peptides longer than 20 residues results could be too random, so a reference scoring for comparison wasn't gotten, Nevertheless, top five models were used to evaluate the affinity of GMA4 for the FLS2-BAK1 complex in different conformations, as well as their hybrids with DI-31.

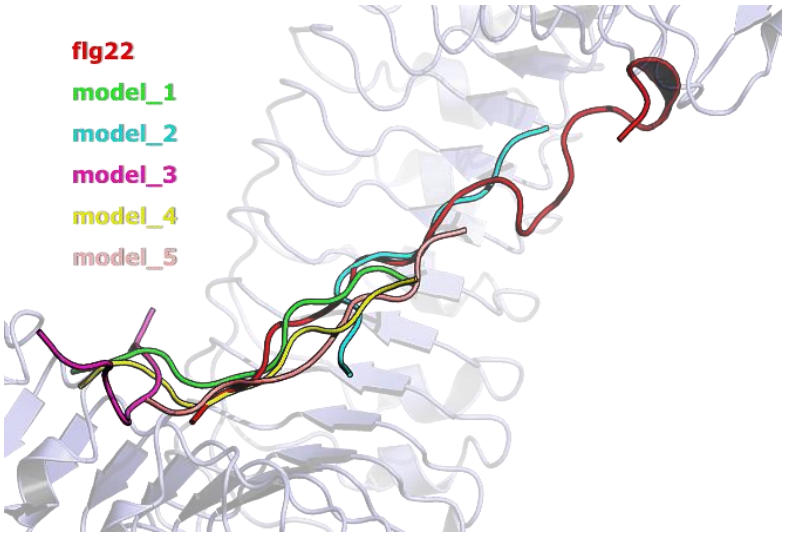


Fig. 1- Top-5 HPEPDOCK models interacting with FLS2 receptor, Red ribbon represents the *flg22* ligand with the crystalized conformation

Molecular docking analysis

In order to determine biding affinity for FLS2-BAK1 complex a total amount of 15 conformations were assessed using software Autodock Vina [(22)], five for each ligand, Also, for each designed ligand and natural ligand *flg22* were generated, by local molecular docking, 30 poses and obtained their respective affinity values, which were grouped into three clusters based on a structural criterion of RMSD < 2,5 Å. In table 2 total average energy and average affinity of each cluster, as well as their population are summarized. Moreover, in figure 4 a comparison of average total energy is presented.

Cluster 1 was the most populated for all the studied conformations of the evaluated ligands, and it generally presented the lower energy values, in some cases below the natural ligand *flg22*. Consequently, random representative poses from this cluster were chosen to study contact residues and estimate pKd values, using BINANA [(24)], algorithm and neural network NNScore 2,0 [(23)], respectively. The pKd values are displayed in the bar chart of Figure 5 and the analysis with BINANA of redocked *flg22* showed that the main contacts required to activate the receptor response are the interactions with residues Y272; Y296; H344; D414; H417; F435 of FLS2, and residues L53; V54; T55 from BAK1 are necessary for heterodimer stabilization. The variation in pKd values is associated to the binding modes variation of different conformations and the contact they can stablish with the corresponding receptor FLS2. The representations of all ligand's conformations interacting with FLS2-BAK1 complex are shown in the supplementary information.

Table 2. Vina results of docked models, Total average Vina affinity, cluster energy and cluster population are shown.

Compounds	Avg, E*	%Pop,C1[a]	E,C1*	%Pop,C2[a]	E,C2*	%Pop,C3[a]	E,C3*
flg22[1]	-9,3	85,71	-9,5	14,29	-8,0	—	--
DI_Model_1	-10,7	2,,00	-11,1	10,00	-10,6	—	--
2DI_K_Model_1	-11,1	23,33	-11,2	16,67	-10,9	16,67	-11,6
Model_1	-10,0	73,33	-10,2	20,00	-9,7	—	--
DI_Model_2	-10,0	30,00	-10,5	20,00	-9,9	—	--
2DI_K_Model_2	-10,8	20,00	-11,1	16,67	-10,6	13,33	-10,7
Model_2	-9,1	43,33	-9,2	23,33	-8,8	13,33	-9,1
DI_Model_3	-10,4	23,33	-11,0	20,00	-10,6	13,33	-10,1
2DI_K_Model_3	-10,9	13,33	-11,2	10,00	-10,0	10,00	-10,8
Model_3	-8,7	53,33	-8,6	20,00	-9,3	—	--
DI_Model_4	-11,2	60,00	-11,4	13,33	-11,2	—	--
2DI_K_Model_4	-11,2	53,33	-11,2	16,67	-10,6	10,00	-11,0
Model_4	-9,2	23,33	-8,6	20,00	-8,9	10,00	-9,8
DI_Model_5	-10,1	30,00	-10,3	23,33	-9,8	10,00	-10,0
2DI_K_Model_5	-10,9	30,00	-10,6	10,00	-10,6	10,00	-10,9
Model_5	-9,3	46,67	-9,2	30,00	-9,4	—	--

*All binding energies (E) were expressed in kcal/mol
[a]Cluster population percentage (%Pop,Cx) are relative to the 30 generated poses
[1]Only 28 poses were generated for the ligand

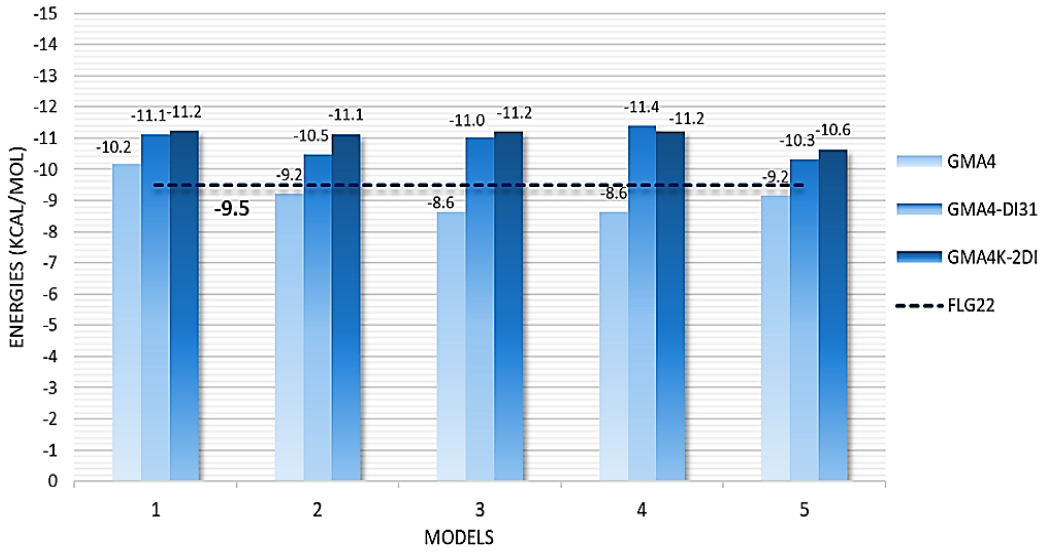


Fig. 4- Vina average total binding energies for FLS2-ligand complex

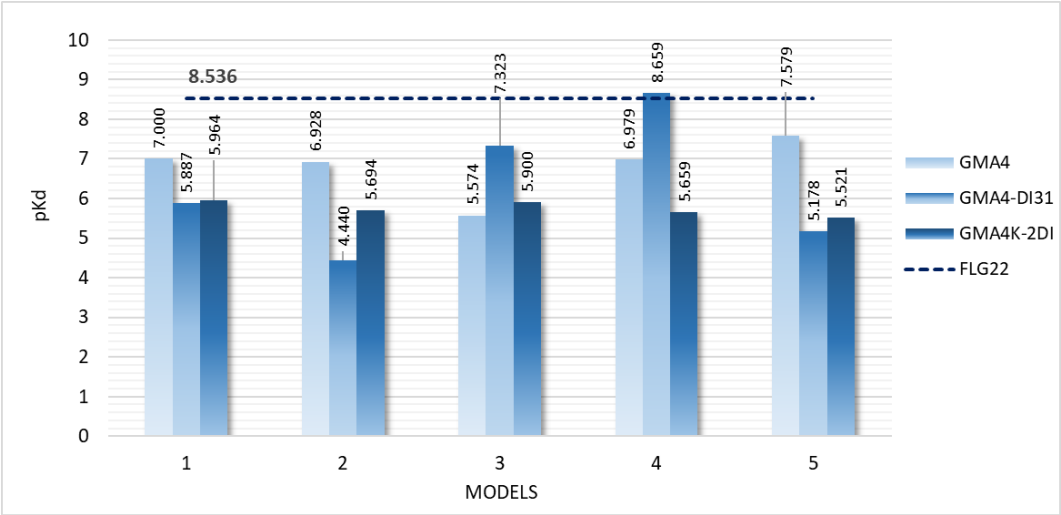


Fig. 5-pKd values estimated by NNScore 2,0

DFT calculations and FMO analysis

In this paper, theoretical DFT calculations were performed in gas phase at B3LYP [26], combined with Grimme´s dispersion correction D3(BJ) and 6-311G (d,p) basis set [27], to predict the structure for the compounds, and to determine the factors that control the geometry of these molecules. All optimized geometries were characterized by harmonic vibrational analysis to ensure that they represent minima on the potential energy surface and were found to have only positive eigenvalues. The calculated minimum energy structures of DI-31, 2DI-31_K and GMA4 compounds are shown in figure 6.

As a result of DFT calculations. Table 3 shows low dipole moments for steroids, a measure of the net molecular polarity and the charge distribution in a molecule. The

highest dipole moment was observed for the 2DI-31_K (3,63 Debye) whereas the smallest one was observed for the DI-31 (1,98 Debye). In the case of GMA4, the adding of polar groups promotes the formation of hydrogen bonds and increases significantly this magnitude.

Table 3. Calculated energies, dipole moments and quantum chemical parameters at DFT- B3LYP-D3 (BJ) / 6-311G (d,p) level.

Estimated Parameters	DI-31	2DI-31_K	GMA4
ENERGY (hartree)	-1 811,523 22	-3 967,423 40	-4 914,083 672
E_HOMO (eV)	-6,58	-6,38	-5,86
E_LUMO (eV)	-0,80	-0,65	-1,05
ΔE (HOMO-LUMO) (eV)	5,79	5,73	4,81
Electronegativity - χ (eV)	3,69	3,51	3,45
Global hardness - η (eV)	2,89	2,86	2,41
Softness - δ (eV)	0,35	0,35	0,42
Electrophilicity - ω (eV)	19,71	17,66	14,36
Ionization potential - I (eV)	6,58	6,38	5,86
Electron affinity - A (eV)	0,80	0,65	1,05
Dipole moment (Debye)	1,98	3,63	12,55

Results show that the presence of hydrogen bonds has a major role in the final geometry of 2DI-31_K, It was calculated that -OH groups interact actively forming H bonding, Calculations predicted a tendency to form this kind of interaction between the two steroids units, Interaction of H of the corresponding ring A of the steroid moieties with the carbonyl group of DI-31 was found. The carbonyls s-trans conformation in the amino acid moieties were also reported.

A detailed analysis of the HOMOs and LUMOs orbitals is listed in table1, where orbital energies, energy band gap and reactivity descriptors (like electron affinity, chemical softness, ionization potential, chemical softness) are reported. A higher energy gap indicates the harder and more stable molecule while a lower the energy gap indicates the soft and more polarizable nature of the molecule; this is the case of peptide GMA4. The FMO energy gap of the target molecules are found to be 5,79 eV and 5,73 eV for the steroidal compounds. The isodensity surface plots of HOMO and LUMO for investigated compounds are shown in figure 6.

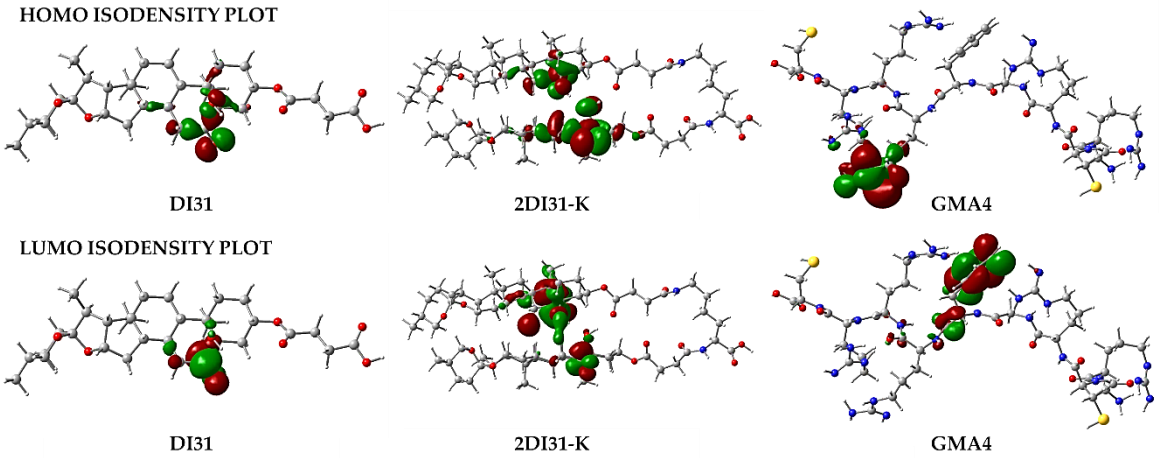


Fig. 6- The isodensity surface plots of HOMO and LUMO obtained at the DFT- B3LYP-D3 (BJ) / 6-311G (d,p) level

Structural studies have shown that *flg22* fragment binds to FLS2 concave surface, whist its C-terminus interact with BAK1 residues, stabilizing the protein complex. The peptide *flg22* could be divided in two segments at residues N10 and S11. The former, oriented to the N-terminus, interacts with leucine-rich repeat (LRR) strands of FLS2, specifically LRR 2-6, where hydrophobic contacts predominate. The residues at such section does not play a crucial role in FLS2 signaling nor in BAK1 interaction, since shorter analogues of *flg22*, such as *flg15* (RINSAKDDAAGLQIA), have been able to start a defense response upon FLS2 activation. Even in mutants of *A, thaliana* where LRR 2-6 were not expressed, a defense response was triggered in the presence of *flg22*.[9.28,29]

On the other hand, the 15 residues segment (flg15: RINSAKDDAAGLQIA) towards the C-terminus can establish mainly polar interactions with FLS2 residues Y272, Y296, D414 and other greasy contacts that set up the ligand in the correct position to interact with residues T52 and V54 of BAK1.[29,30] All these aspects were taken into account when designing the DI31-GMA4 hybrids; where not only two plant defense inducers were selected, but combined in a specific way that resembles the size, amphipathic character, and the interaction modes of the natural ligand *flg22*.

Local docking with HPEPDOCK server allowed to evaluate several conformations that peptide GMA4 might present inside the active site of FLS2. This preliminary step was very important since it showed that GMA4 could bind to the receptor in a similar way as the natural ligand does; thus, top five conformations were used to obtain binding affinity values by redocking the peptide and its hybrid using Vina. Nonetheless returned scoring was only useful to select the best conformations because we couldn't get a comparative criterion for *flg22* and hybrids couldn't be assessed since the server only admits peptide residues as input.

For the analysis with Vina two factors were taken into account: biding mode population and binding affinity. That's why all 30 generated conformers of each ligand were

clustered (RMSD < 2,5 Å), getting for all cases that the most populated cluster was the first one (C,1), so it was taken as reference for ulterior analyses. Moreover, it was demonstrated that all ligands presented biding affinities around the value of -9,5 kcal/mol, corresponding with the natural ligand. The higher energy (-8,6 kcal/mol) was obtained for conformations model_3 and model_4 of GMA4 alone, while the lowest energy for the peptide (-10,2 kcal/mol) belonged to the first conformation (model_1), which also presented a high cluster population (73,33 %). Therefore, as previously shown by HPEPDOCK server, peptide might be recognized by FLS2 receptor preferably in the conformation model_1.

Molecular docking studies additionally showed that hybridization with DI-31 lowered the binding energy in all cases; the cluster population were affected though. These results must be associated with the fact that the steroid could interact better with LRRs of FLS2, and that the flexibility of succinate linker used in hybrids' design might have increased the randomness of poses generation, attempting against cluster population not greater than 30 % in the majority of cases, thus not considered representative, An interesting exception was observed for model_4 hybrids. The peptide alone presented one of the lowest affinity and cluster population (23,33 %), but the hybridization increased significantly the population and the affinity, getting for the mono- and bis-steroidal hybrids a biding energy of -11,4 kcal/mol (60,00 %) and -11,2 kcal/mol (53,33 %), respectively. Such result might be in correspondence with the statement that steroid-peptide joint favor the linear configuration and rigidity of peptide, and the facial amphipathicity, making the molecule to bind to the receptor in a more suitable fashion.

Due to the better results of energy and population were obtained for conformations model_1, DI_model_4, and 2DI_K_model_4, it could be said a priori that the biding of such molecules to FLS2 is thermodynamically favored, Additionally, BINANA analysis showed that the ligands might be able to interact with the active site's residues in a similar way to *flg22*, so it might make possible FLS2-BAK1 complex stabilization.

More specifically, model_1 enters in the active site by positioning its C-terminus in a positive hole given by residue R294, It's also stabilized by polar interactions with residues Y296; E321; H344; D414 and Q485 and hydrophobic interactions with V340; H344; F369; L412 and F435. This pose is able to interact with BAK1 residues T52 y V54 through hydrogen bond and greasy interactions, respectively (Figure 7).

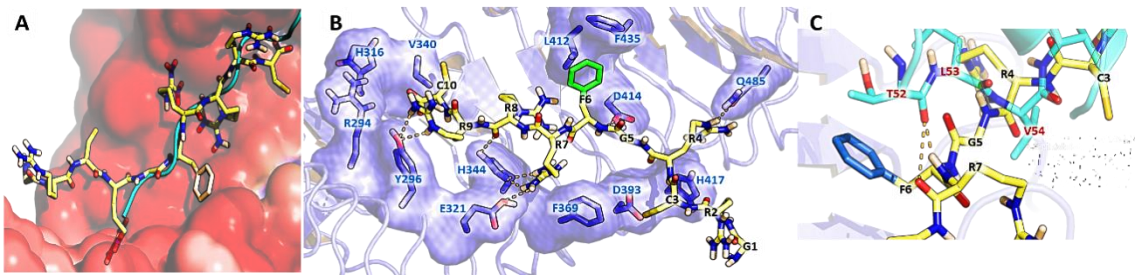


Fig. 7-Peptide interactions of GMA4 with FLS2-BAK1, polar interactions (hydrogen bonds and salt bridges) are represented by dashed lines: A) peptide GMA4 (yellow sticks) on the FLS2 electrostatic potential surface (negative potentials in red, positive potential in blue), cyan ribbon represents the *flg22* biding mode; B) GMA4 contacts with FLS2; C) peptide-BAK1 interactions

In a similar way mono-steroidal hybrid conformation DI_model_4 can establish some contacts within the active site's residues electrostatic interactions are observed with residues E249; Y272; D393; D414 and T342. Hydrophobic interactions were observed with residues H296; H344; F369; L412 and F435, a cation-π interaction was observed between residues R8 and H417. Potentially, contacts with V54 and T55 might suggest FLS2-BAK1 complex stabilization. The steroid moiety did not settle over the active site groove, It did though, within a hydrophobic pocket made up by residues E249; G225; G201 and Y177 of FLS2 (Figure 8). Such interactions could be useful for inducing a more suitable positioning of GMA4 fragment inside the active site and subsequently stabilize heterodimer with BAK1, so it might be possible for such molecule to induce a FLS2-like defense response.

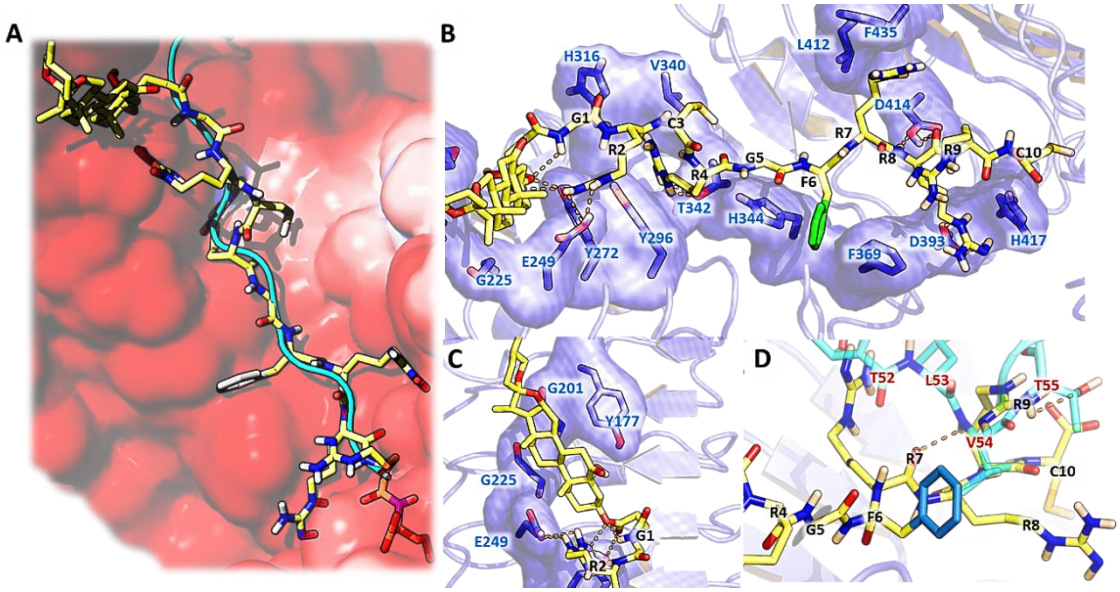


Fig. 8- GMA4-DI interactions with FLS2-BAK1, polar interactions (hydrogen bonds and salt bridges) are represented by dashed lines: A) ligand (yellow sticks) on the FLS2 electrostatic potential surface (negative potentials in red, positive potential in blue), cyan ribbon represents the *flg22* biding mode; B) hybrid contacts with FLS2; C) hydrophobic pocket where steroid moiety is located; D) peptide fragment interactions with BAK1.

For bis-steroidal hybrid it was shown that steroidal moiety bends under the N-terminus of peptide making difficult to correctly interact with both FLS2 and BAK1 residues, Despite some contacts were similar to that obtained for natural ligand *flg22* as well as model_1 and DI_model_4, this compound was unable to properly stabilize complex with BAK1, so potentially not a defense inductor.

A possible explanation for these results could be found in DFT results and FMO analysis. It is clear from the figure 2 of the molecules that the HOMO and LUMO orbitals are localized essentially on the -OH group. In DI-31, the negative regions attract proton from the amino acids or protein. These active sites are evidence of the biological activity of the molecules, as previously reported.[17,18,31] In the case of GMA4 the HOMO orbitals are located at Arginine part and illustrate the capacity for electron

donation while LUMO illustrates the capacity for accepting electrons at phenylalanine amino acid, localized mainly on the aromatic group. A small energy gap of HOMO-LUMO means more chemical activity, favoring the biological potential of the compound.

All these results were congruent with pKd estimation using the neural network NNScore 2,0, which not only take into account Vina scoring function, but additionally it considers BINANA contact analysis. Therefore, it was obtained that pKd for conformation DI_model_4 the greatest, equals 8,659, slightly greater compared to the estimated for *flg22* (pKd = 8,536). For model_1 conformation (pKd = 7,000) its pKd evidence that GMA4 alone is not a great defense inductor even though it might present certain affinity for FLS2-BAK1 complex. Besides it proves that hybridization with DI-31 potentially improves receptor affinity and the activity as defense response inductor.

The decrement of pKd estimated for 2DI_model_4, could be associated to the fact that double steroid hybridization increases degrees of freedom of the molecule, making more difficult the interaction with the studied receptor. There's also evidence for this in the fact that bis-steroidal conformations gave the less populated cluster in Vina analysis, Besides most stable conformation obtained by DFT calculations display both steroids in a stacked position which favors intramolecular rather than intermolecular interactions, along with the observation that FMO are oriented towards the inside of the molecule, not the protein surface.

Moreover, it is known that some bacteria have evolved to *flg22* polymorphic forms mainly in fragment 18GLQI21 in order to avoid plant perception. So, it is needed for *flg22*-I21 to stablish non-polar interactions with BAK1-T58 and I483 and I507 from FLS2 receptor. These contacts favor the stabilization of heterodimer by interactions with residues 52TLV54 from BAK1. Non-polar interactions of these residues could make difficult the solvent penetration inside this receptor region inhibiting the competition for polar interactions with BAK1 residues.[29] For peptide GMA4 alone, the residues interacting with the co-receptor (1GRCRG5) didn't quite stablish the reported contacts, although it was gotten peptide could interact with residues C57 from BAK1 and Q485 from FLS2; the latter was through a hydrogen bond. Even though such contacts are close to those identified above, they're not a prove for FLS2-BAK1 stabilization, also could be another explanation for the obtained pKd value, smaller than that of *flg22*.

In the studied system for mono-steroidal hybrid, despite sequence divergence, the residues of the ligand (6FRRRC10) interacting with the co-receptor were potentially able to form up polar contacts with residues 52-54 and 4 Å hydrophobic interactions with T58 from BAK1 and I483 from FLS2. Thus, this is another hint that hybridization with DI-31 enhance the capacity of peptide for being recognized by FLS2 in a similar way to *flg22*, as previously predicted by Vina studies, contact analysis and pKd estimated value, similar to the natural ligand, Hence it could be assumed that DI-31-GMA4 hybrid it's a potential plant immune-stimulator via FLS2 recognition.

Generally, it is reported that FLS2 is practically exclusive for *flg22*. In 2011 Lee *et al*, [32] suggested peptide CLV3p, related to regulation of stem cell elongation, was capable

to stimulate plant defense via FLS2, as detected by reciprocal co-immunoprecipitation, monitored by the activation of mitogen-activated protein kinases (MAPK). This discovery has been questioned by several research groups that study plant innate immune response based on studies of events associated to FLS2 signaling.[33,34] On the other hand, CLV3p peptide was in silico positively docked to FLS2, however BAK1 complex stabilization wasn't assessed, so its activity as plant defense inductor could not be concluded.[35,36] That's why, in the present work several thermodynamic parameters were obtained using complementary methods, additionally contacts that stabilize FLS2-BAK1 were exhaustively studied, taking *flg22* parameters as a reference. So, despite these results are a primary theoretical approximation, they have shown that GMA4 and its hybrid with DI-31 might be able to be recognized by FLS2, stabilize complex with BAK1 and subsequently induce plant defense.

Conclusions

The possibility of the peptide GMA4 joining the FLS2 receptor, along with its mono- and bis-steroidal hybrids, was assessed via molecular docking, the determination of the pKd values of the ligand-receptor complexes and DFT studies, Overall, the results showed that GMA4 could interact with FLS2 in a favorable way, capable of stabilize the FLS2-BAK1 complex similar to *flg22*. Moreover, the mono-steroidal hybrid displayed an improved affinity for the receptor in opposition to the bis-steroidal one, since the steroid moiety helps the molecule to position appropriately inside the active site of the receptor; these observations were supported by the analysis of contacts and DFT studies.

Acknowledgements

YPB acknowledges the Department of Applied Physical Chemistry of the Universidad Autónoma de Madrid for providing computational resources used for calculations.

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Conflict of interests

Authors declare no conflict of interests.

Authors contribution

Juan Pablo Figueroa-Macías: contributed to the conception of the idea, carried out the corresponding review and the molecular docking calculations, and the writing of the paper.

Fidel E-Morales: contributed to the conception of the idea and the reviewing of the written paper;

Yoana Perez-Badell: carried out the DFT calculations, contributed to the writing, and reviewing of the paper.

Yamilet-Coll: contributed to the conception of the idea and the reviewing of the written paper.