



Crystal Structure of Sol i 2: A Major Allergen from Fire Ant Venom

Aline S. Borer¹, Paul Wassmann¹, Margit Schmidt²,
Donald R. Hoffman³, Jing-Jiang Zhou⁴, Christine Wright⁵,
Tilman Schirmer¹ and Zora Marković-Housley^{1*}

¹Department of Structural Biology, Biozentrum, University of Basel, CH-4056 Basel, Switzerland

²Department of Biology, East Carolina University, Greenville, NC 27858, USA

³Department of Pathology and Laboratory Medicine, Brody School of Medicine at East Carolina University, Greenville, NC 27834, USA

⁴Department of Biological Chemistry, Rothamsted Research, Harpenden AL5 2JQ, UK

⁵Department of Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA 22908-0735, USA

Received 11 August 2011;
received in revised form
5 October 2011;
accepted 7 October 2011

Edited by I. Wilson

Keywords:

major fire ant allergen;
Sol i 2;
crystal structure;
hydrophobic cavity;
odorant binding protein and
pheromone binding protein
LUSH

Sol i 2 is a potent allergen from the venom of red imported fire ant, which contains allergens Sol i 1, Sol i 2, Sol i 3, and Sol i 4 that are known to be powerful triggers of anaphylaxis. Sol i 2 causes IgE antibody production in about one-third of individuals stung by fire ants. *Baculovirus* recombinant dimeric Sol i 2 was crystallized as a native and selenomethionyl-derivatized protein, and its structure has been determined by single-wavelength anomalous dispersion at 2.6 Å resolution. The overall fold of each subunit consists of five helices that enclose a central hydrophobic cavity. The structure is stabilized by three intramolecular disulfide bridges and one intermolecular disulfide bridge. The nearest structural homologue is the sequence-unrelated odorant binding protein and pheromone binding protein LUSH of the fruit fly *Drosophila*, which may suggest a similar biological function. To test this hypothesis, we measured the reversible binding of various pheromones, plant odorants, and other ligands to Sol i 2 by the changes in *N*-phenyl-1-naphthylamine fluorescence emission upon binding of ligands that compete with *N*-phenyl-1-naphthylamine. The highest binding affinity was observed for hydrophobic ligands such as aphid alarm pheromone (*E*)- β -farnesene, analogs of ant alarm pheromones, and plant volatiles decane, undecane, and β -caryophyllene. Conceivably, Sol i 2 may play a role in capturing and/or transporting small hydrophobic ligands such as pheromones, odors, fatty acids, or short-living hydrophobic primers. Molecular surface analysis, in combination with sequence alignment, can explain the serological cross-reactivity observed between some ant species.

© 2011 Elsevier Ltd. All rights reserved.

*Corresponding author. E-mail address:
zora.housley@unibas.ch.

Abbreviations used: SeMet, selenomethionine; PDB, Protein Data Bank; OBP, odorant binding protein; PBP, pheromone binding protein; cVA, 11-*cis*-vaccenyl acetate; NPN, *N*-phenyl-1-naphthylamine; CSP, chemosensory protein.

Introduction

Fire ants of the genus *Solenopsis* have spread over large parts of the temperate and tropical world from their original habitats in South and Central America.¹ Today, fire ants are serious pests in the southern United States, Pacific Islands,² Southeast

Asia,^{3,4} and Middle East.⁵ In the southeastern United States, the stings of imported fire ants are the most common cause of insect venom anaphylaxis.^{1,6,7} Fire ant venom consists of a mixture of very basic piperidine alkaloids⁸ and a small aqueous phase containing four major proteins.⁹ A single fire ant sting is sufficient to cause specific IgE antibody production, although it contains only 1–10 ng of protein.¹ In some endemic areas, 30–50% of the population shows evidence of sensitization to fire ant venom, as revealed by the presence of serum-specific IgE antibodies and by skin testing.⁶

Four allergenic proteins have been isolated from *Solenopsis invicta* venom and characterized.⁹ Sol i 1 is a phospholipase A¹B that is similar to those found in wasp venoms and belongs to the lipoprotein lipase family.¹⁰ It contains carbohydrates and exhibits some cross-reactivity with wasp venom proteins. Sol i 3 is a member of the antigen 5/pathogenesis-related protein family.^{11,12} It is not cross-reactive with wasp venom antigen 5, since there is almost no conservation of surface structure.¹³ Sol i 2 and Sol i 4 are members of a unique protein family.^{9,14,15} Sol i 2 is a covalent dimer composed of two identical monomers. Each monomer contains seven cysteines: six cysteines form three intramolecular disulfide bridges that stabilize the structure, whereas the seventh cysteine (Cys22) links two monomers by a disulfide bridge. Sol i 4 shares a 37% sequence identity with Sol i 2, lacks the dimerizing cysteine, and is present in venom as a monomer rather than as a disulfide-linked dimer. Proteins similar to Sol i 2 are found in venoms of other *Solenopsis* species, including *Solenopsis richteri* (Sol r 2), *Solenopsis geminata* (Sol g 2), and *Solenopsis saevissima* (Sol s 2).¹⁶ Sol i 2 has a high level of sequence identity with Sol r 2 (78.1%), as well as with Sol s 2 and Sol g 2 (71.4%). Although all of the Sol 2 allergens show a high degree of immunological cross-reactivity among the species tested, there are some species-specific determinants in the Sol 2 family of allergens.^{16,17} Mouse monoclonal antibodies that are species-specific have been produced, and human IgE cross-reactivity between *S. invicta* and *S. richteri* venoms is not complete.^{16,17} Sol i 2 proteins are less stable than Sol i 3 proteins, and their decomposition is responsible for the decay of allergen activity in vaccine extracts.¹⁸

Only a few three-dimensional structures of insect venom allergens are known to date: the X-ray structure of the major bee venom allergens phospholipase A2¹⁹ and melittin;²⁰ hyaluronidases from bee venom²¹ and wasp venom;²² antigen 5 from *Vespa vulgaris*²³ and red fire ant;¹³ and the complex between bee venom hyaluronidase and its specific IgG Fab.²⁴ To date, no unique structural feature responsible for the onset of allergenicity has been identified. In this work, the crystal structure of a major allergen from red fire ant (Sol i 2) is reported

and compared to its closest structural homologue, that is, the protein LUSH from *Drosophila*, which has specific alcohol and pheromone binding functions *in vivo*.^{25,26} The structures of Sol i 2 and LUSH have similar folds consisting of five and six α -helices that surround a central hydrophobic cavity. To verify whether the structural similarity to LUSH also implies functional similarity, we studied the binding of various insect pheromones, repellent, attractants, and plant volatiles to Sol i 2.

Results and Discussion

Structure determination

The crystal structure of the mature recombinant major allergen from fire ant venom, Sol i 2 (residues

Table 1. Data collection and refinement statistics

	P	Ia	Ib
<i>Data collection</i>			
Protein	Native	SeMet	SeMet
Space group	P2 ₁ 2 ₁ 2 ₁	I222	I222
Number of monomers per asymmetric unit	8	2	2
Cell dimensions a, b, c (Å)	110.2, 139.2, 62.1	68.7, 111.7, 100.9	67.8, 100.9, 148.520
Wavelength (Å)	1.0000	0.97930	1.48520
Number of unique reflections	29,059	3638	3739
Resolution (Å)	70–2.58 (2.72–2.58)	58–3.30 (3.48–3.30)	56–3.10 (3.27–3.10)
R _{sym} (%) ^a	5.5 (28.4)	6.4 (68.3)	5.6 (39.3)
Completeness (%)	95.0 (86.3)	90.7 (37.9)	94.4 (62.8)
Redundancy	3.2 (2.3)	16.6 (3.0)	7.0 (2.8)
I/σ(I)	17.3 (3.8)	24.5 (2.0)	15.6 (1.9)
<i>Refinement statistics</i>			
Resolution range (Å)	15.0–2.6		
Number of unique reflections	26,886		
R _{work} ^b /R _{free} ^c (%)	23.7/27.1		
Number of atoms			
Protein	6872		
Water	79		
Heptane (HP6)	56		
RMSD from ideal values			
Bond lengths (Å)	0.0097		
Bond angles (°)	1.00		
Average B-factors (Å ²)			
Protein	37.9		
Water	36.1		
Heptane	33.1		
Ramachandran plot (%)			
Favored region	98.5		
Allowed region	1.5		
Disallowed region	0.0		

The highest-resolution shell is shown in parentheses.

^a $R_{\text{sym}} = \sum |I_i - \langle I_i \rangle| / \sum I_i$, where I_i is the intensity of the i th observation and $\langle I_i \rangle$ is the mean intensity of the reflection.

^b $R_{\text{work}} = \sum (|F_o| - |F_c|) / \sum |F_o|$.

^c $R_{\text{free}} = R$ value for a randomly selected subset (5%) of the data that were not used in the refinement.

1–119 of the mature protein corresponding to residues 20–138 of the full-length protein, which includes the signal sequence), was determined to a resolution of 2.6 Å (Table 1) in space group $P2_12_12_1$ (crystal form P) with eight monomers in the asymmetric unit. Selenomethionine (SeMet)-derivatized Sol i 2 crystallized in space group $I222$, crystal forms Ia and Ib, which each contained two monomers per asymmetric unit. Initial phases were obtained by single-wavelength anomalous dispersion phasing of SeMet-derivatized crystal form Ia and by subsequent 4-fold multicrystal averaging with crystal form Ib. Finally, the electron densities of all 12 monomers of the three crystal forms were cyclicly averaged, yielding an interpretable electron density map.

Crystallographic statistics are shown in Table 1. The eight protomers of the $P2_12_12_1$ crystal with virtually identical structures (RMSD of 0.20–0.26 Å for all C^α positions) are arranged into four nearly identical dimers: A–B, C–D, E–F, and G–H. Refinement with strong noncrystallographic symmetry constraints yielded a final model (Fig. 1) with an R/R_{free} value of 23.7%/27.1%. The residual density remained in the central hydrophobic cavity of each monomer, but its extent and appearance differed from monomer to monomer. In Fig. 1, the residual density of monomers A and B is tentatively modeled with a short hydrocarbon fragment.

The following residue segments are absent in the final model due to missing electron density: residues 118–119 in chains A, C, D, E, and G; residue 21 in chain A; residue 4 in chain B; residue 22 in chain C; residues 1, 4, 14, 19, and 46 in chain D; residues 4 and 21 in chain F; and residues 1–4 in chain H.

The monomers within four nearly identical dimers are held by one disulfide bridge (Cys22–Cys22') and by an average of 22 hydrogen bonds and 16 salt bridges. Most intermonomeric contacts within the dimers are made by residues belonging to the loop between helices $\alpha 1$ and $\alpha 2$ and to helix $\alpha 5$ and the following loop. In the crystal packing, most of the interdimeric interactions are observed between chains A–C and E–G, which are held together by 10 hydrogen bonds and 8 salt bridges formed by residues located in the N-terminal part of the $\alpha 3$ helix and the preceding loop. The final model comprises protein chains A–H (Asp1–Lys119), 79 water molecules, and 7 hydrocarbon groups (*n*-heptane). The following discussion is restricted to the A–B dimer (Fig. 1).

The overall structure of Sol i 2

The overall structure of the Sol i 2 monomer consists of five α -helices and is stabilized by three intramolecular disulfide bridges: Cys15–Cys38,

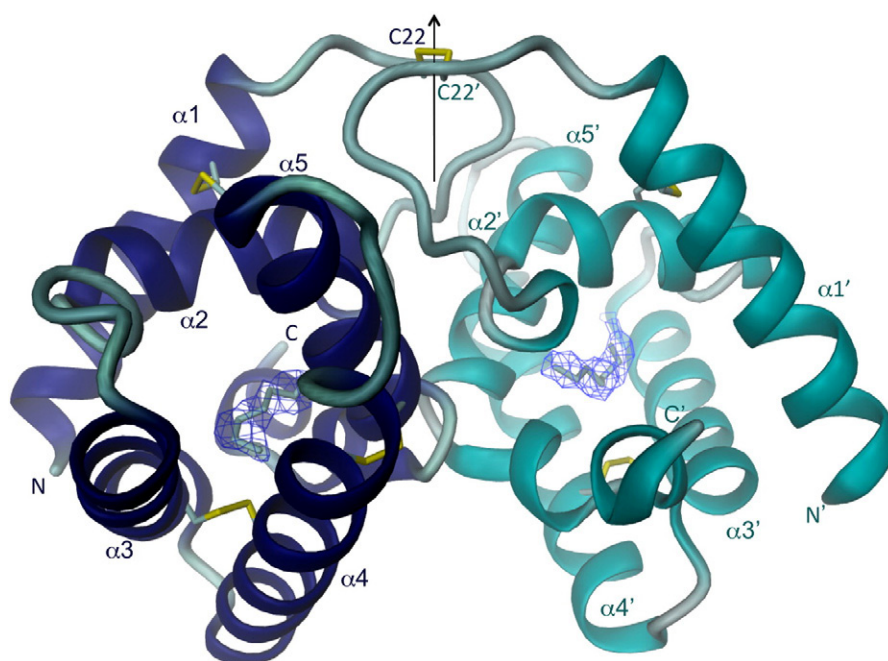


Fig. 1. Ribbon diagram showing the overall crystal structure of Sol i 2 homodimer. The two virtually identical monomers (blue and turquoise) form a disulfide-bonded dimer involving residue Cys22 from both protomers. Each monomer is composed of five α -helices, whereby helices $\alpha 2$ – $\alpha 5$ surround a central hydrophobic cavity. Residual electron density ($F_o - F_c$ map counteracted at 3σ) is observed in the hydrophobic cavity of each monomer and is tentatively modeled with a hydrocarbon chain (*n*-heptane).

Sol i 2 is a globular dimeric protein of 2-fold symmetry with approximate core dimensions of $49 \text{ \AA} \times 41 \text{ \AA} \times 37 \text{ \AA}$. The two chains are covalently linked by a disulfide bridge formed by residue Cys22 and by its symmetry mate Cys22', located in the long loop joining helices $\alpha 1$ and $\alpha 2$ (Fig. 1). The dimer interface was determined to be $1187 \text{ \AA}^2$. The native form of Sol i 2 isolated from fire ant venom is also found as a disulfide-linked dimer, as shown by SDS-PAGE with and without a reducing agent.¹¹

Structural homologues of Sol i 2

A search for proteins with similar structures in the Protein Data Bank (PDB) was performed with the program Dali,²⁷ and superpositioning of molecules was performed with the program Dalilite.²⁸ Sol i 2 is structurally most similar to general odorant binding proteins (OBPs) and pheromone binding proteins (PBPs), with the following general characteristics: usually composed of 130–150 amino acids; with a compact structure mainly composed of α -helices; contain six conserved cysteines paired in three intramolecular disulfide bridges; and the signal sequence of an immature protein. The highest structural similarity is observed with the structure of LUSH from the fruit fly *Drosophila melanogaster*, which has alcohol and pheromone binding functions *in vivo*.^{25,29} In terms of protein sequence, LUSH and Sol i 2 are very different, sharing only an 11.6% identity (18.6% similarity) (Fig. 2b). The top score revealed by Dali is observed with LUSH, as found in a complex with the pheromone 11-*cis*-vaccenyl acetate (cVA) (PDB ID: 2gte).²⁹ After the superpositioning of the C $^{\alpha}$ chains of Sol i 2 and LUSH, the RMSD was found to be $3.4 \text{ \AA}$ for 102 residues (Fig. 2a). The fold of Sol i 2 and LUSH consists of five and six α -helices, respectively, whereby helices $\alpha 2$ – $\alpha 5$ in Sol i 2 and helices $\alpha 2$ – $\alpha 6$ in LUSH surround a hydrophobic cavity that is a suitable binding site for apolar molecules.

Structure-based sequence alignment is shown in Fig. 2b. Two intramolecular disulfide bridges of Sol i 2 (Cys15-Cys38 and Cys82-Cys103) and LUSH (Cys17-Cys50 and Cys92-Cys112) are conserved. Structural alignment shows that the major differences are observed near the N-terminus, where (i) the long loop following helix $\alpha 1$ in Sol i 2 is aligned with helix $\alpha 2$ of LUSH and (ii) the insertion of 14 residues in Sol i 2, at the beginning of helix $\alpha 2$, resulted in the alignment of helices $\alpha 2$ – $\alpha 5$ of Sol i 2 with helices $\alpha 3$ – $\alpha 6$ of LUSH (Fig. 2a and b).

The structural similarity of Sol i 2 and LUSH also applies to the central cavity, which is lined mainly by hydrophobic residues but also contains three polar residues: Tyr46, Ser58, and Thr113 in Sol i 2 (Fig. 3a), and Thr48, Ser52, and Thr57 in LUSH (Fig. 3b). Two polar groups—Ser52 and Thr57 in LUSH

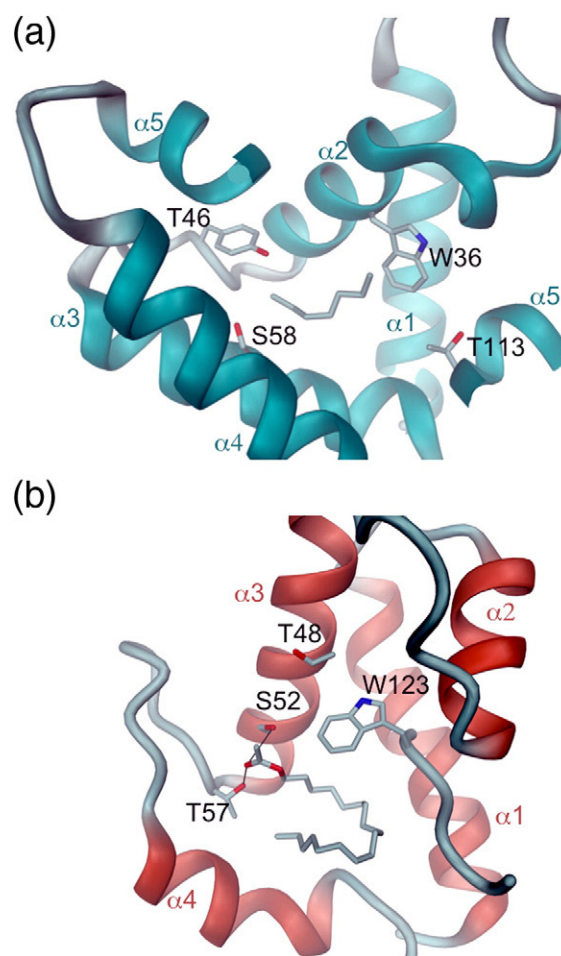


Fig. 3. Close-up view of the hydrophobic cavities of Sol i 2 (a) and LUSH (b) shown as ribbon representation. The same molecular orientation in (a) and (b) is chosen to optimize the viewing of the inner cavity. (a) Stick presentation of *n*-heptane (gray) modeled in the residual electron density of the Sol i 2 inner cavity. Part of helix $\alpha 5$ (residues 100–111) is omitted for clarity. (b) Ligand binding pocket of LUSH with the ligand cVA (gray) filling up the pocket and forming polar interactions with Ser52 and Thr57. For clarity, helices $\alpha 5$ and $\alpha 6$ (residues 81–118) are not shown. No cysteines are shown in (a) and (b).

and Ser58 and Tyr46 in Sol i 2—occupy a similar location in the hydrophobic cavity. In LUSH, Ser52 and Thr57 form a polar edge of the alcohol binding pocket formed by residues from helix $\alpha 3$, helix $\alpha 6$, and the C-terminal end.²⁵ In the structures of LUSH complexes with alcohols (ethanol and butanol)²⁵ and *Drosophila* pheromone cVA,²⁹ residues Ser52 and Thr57 form hydrogen bonds with the OH[−] group of alcohols and with the O atom of cVA (Fig. 3b), with Thr57 being the most critical residue for alcohol binding, as shown by mutagenesis studies.²⁶ In Sol i 2, the polar residues Tyr46 and Ser58 form a polar edge similar to that of LUSH. The study aimed

to verify whether the structural similarity to LUSH also implies the binding of similar ligands, as reported below.

Solution binding of competitive ligands to Sol i 2

The binding method is based on the reduction of the fluorescence emission signals of the probe *N*-phenyl-1-naphthylamine (NPN) bound to Sol i 2 upon competitive binding of various ligands. The fluorescence probe NPN reversibly binds to Sol i 2 ($K_d = 0.447 \mu\text{M}$). In the absence of NPN, the intrinsic fluorescence of a single tryptophan residue of Sol i 2, Trp36 (Fig. 3a), located in the hydrophobic cavity shows a strong blueshift (330 nm) as compared to tryptophan in solution (348 nm). The binding of NPN to Sol i 2 reduces the intrinsic fluorescence emission of the tryptophan at 330 nm and induces a new emission peak at 400 nm. This indicates that the bound NPN lies close to Trp36 and that there is fluorescence resonance energy transfer from the tryptophan, resulting in an increase in NPN fluorescence emission at 400 nm. Upon the addition of an increasing concentration of competitive binding ligands, the intensity of NPN fluorescence at 400 nm decreases due to the displacement of NPN from the binding pocket caused by ligand binding.

The binding of various insect pheromones, repellent, attractants, and plant volatiles to the hydrophobic cavity of Sol i 2 was examined in order to determine if there is a possible functional similarity to the *in vivo* binding of the pheromones and alcohols to protein LUSH. The ability of 24

Table 2. Ligand affinity for Sol i 2 as measured *in vitro* and as estimated by modeling

Ligand	Modeling scores ^a			Affinity constant ^b $1/K_i^c$ (μM^{-1})
	Monomer A	B	Average A+B	
β -Farnesene	1335.7	1231.3	1283.5	29.6
β -Caryophyllene	993.2	992.3	992.8	25.5
Solenopsin ^d	887.1	997.7	942.4	ND
Decane	828.6	829.3	828.9	29.6
Heptane	647.5	733.9	690.7	3.1
1-Octen-3-ol	370.6	795.3	582.9	4.8
Ethanol	-164.5	3.0	-80.8	1.3

ND, not determined.

^a The overall binding score is the sum of the binding values of all interacting atoms. The binding value for a given interacting atom pair is defined in Materials and Methods.

^b As also shown in Fig. 3.

^c K_i is the equilibrium dissociation constant. The competition of fluorescence probe (NPN) binding and the dissociation constant (K_i) of the ligand (0.05–50 μM) are determined by fitting the binding curve (GraphPad Prism 5) using 5 μM NPN and an NPN equilibrium binding constant (K_d) of 0.447 μM .

^d (2*R*)-2-Methyl-6-alkenylpiperidine.

different ligands to displace NPN from the NPN–Sol i 2 complex was measured by following the changes in NPN fluorescence emission, which was used to determine the affinity constant for each ligand (Fig. 4). For LUSH, there are no quantitative binding data for these compounds, except for alcohols (ethanol, butanol, and hexanol) and cVA, which were shown to bind to LUSH by crystallization.^{25,29} The crystal structure of LUSH,

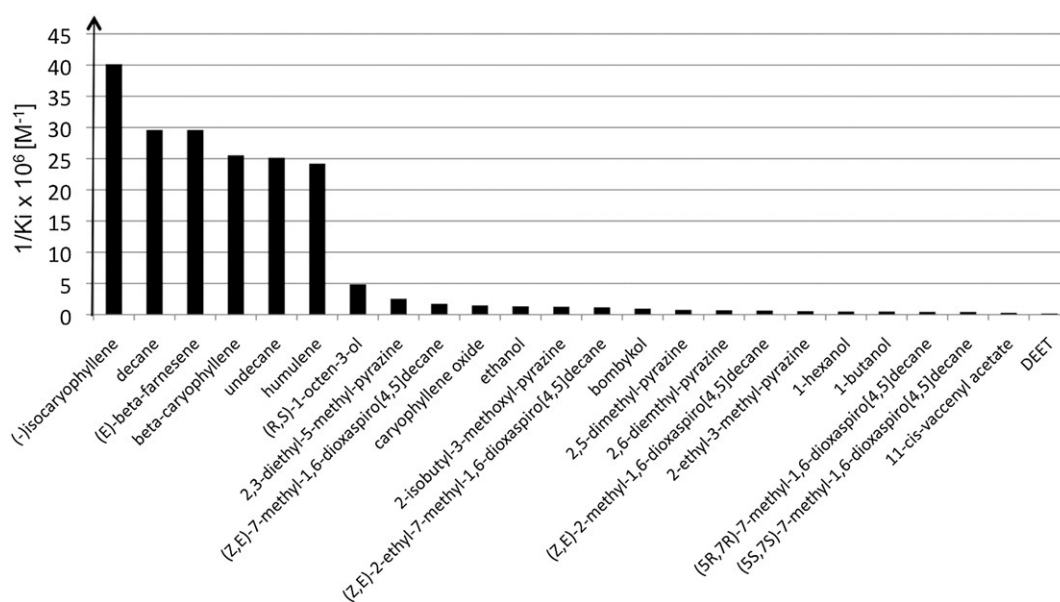


Fig. 4. The affinity constant $1/K_i$ of various ligands for Sol i 2, as measured by competitive ligand binding assay, whereby K_i (M) is the equilibrium dissociation constant.

in complex with ethanol and butanol, revealed the presence of a single binding site involving residues Ser52 and Thr57, which were hydrogen-bonded to the OH group of alcohols.²⁵ Similarly, in LUSH complexes with cVA, the polar moieties of Ser52 and Thr57 are also hydrogen-bonded to an O atom of cVA (Fig. 3b), whereby LUSH undergoes a pheromone-specific conformational change.²⁹ In Sol i 2, the residues Tyr46 and Ser58 could be expected to form similar hydrogen-bonded interactions with ethanol and cVA. However, the binding affinity for Sol i 2 of ligands containing polar groups such as -OH or oxygen [e.g., 1-octen-3-ol (4.8 μ M), ethanol (1.3 μ M), and cVA (0.3 μ M)] is significantly lower than that of purely hydrophobic ligands such as iso-caryophyllene (40.1 μ M), decane (29.6 μ M), and (*E*)- β -farnesene (29.6 μ M) (Fig. 4 and Table 2). This is in agreement with the high hydrophobicity of the central cavity. Furthermore,

the mutagenesis study of LUSH³⁰ revealed that hydrogen-bonding interactions involving residues Ser52 and Thr57 are crucial mainly for short-chain alcohols such as ethanol, whereas the contribution of hydrophobic interactions becomes dominant for alcohols with longer alkyl chains. Indeed, independent competitive binding studies by Zhou *et al.* revealed that ethanol could not displace NPN in complex with LUSH, even at ethanol concentrations as high as 15 mM.³¹ Since the binding of a purely hydrophobic compound to LUSH was not tested, it is conceivable that, similar to Sol i 2, the hydrophobic ligands may bind to LUSH with a higher affinity than polar ligands.

HINT modeling of ligands

The program HINT (Hydropathic INTERactions)^{32–34} was used to theoretically evaluate the binding

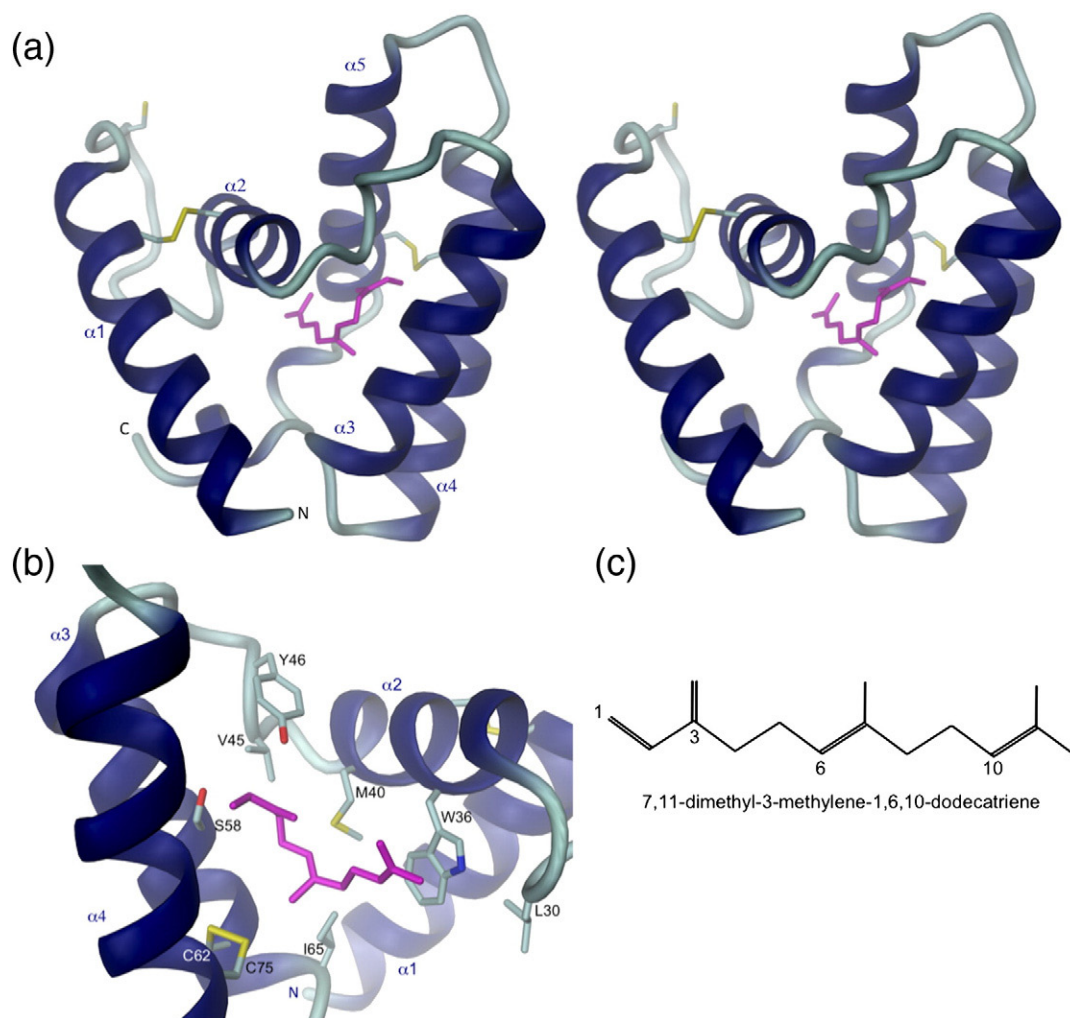


Fig. 5. (a) Stereo view of the ribbon diagram of Sol i 2 with (*E*)- β -farnesene (magenta) modeled in the inner hydrophobic cavity. (b) Close-up view of the ligand binding cavity. Helix $\alpha 5$ is omitted for clarity. (c) Chemical structure of (*E*)- β -farnesene.

strengths of several ligands when complexed with the newly determined structure of Sol i 2. These ligands, identified from fluorescence binding studies, were manually docked into the known hydrophobic binding pocket of Sol i 2, which was followed by energy minimization of ligand–protein complexes. This process allowed the adjustment of the atomic positions of the ligand and protein atoms, contained within a sphere of 6 Å from the ligand, for optimum binding distances. HINT calculations were carried out on these minimized complexes for each monomer (A and B) separately. The overall HINT binding scores, which were subsequently computed, represent net values summed over all positive and negative contributions from each interacting atom pair within the 6-Å sphere. Tables were generated for each complex to examine and compare in detail the nature of those interactions (hydrophobic, hydrophilic, and acid–base) that contribute significantly to the overall binding score.³⁴ It was possible to select the ligand orientations that resulted in optimal binding using these HINT scores as a quantitative tool.

Table 2 lists the HINT binding scores for seven ligands together with their experimentally determined solution binding constants $1/K_i$ (μM^{-1}). With error estimates (± 200) for each of these types of binding constant taken into account, the HINT results correlate reasonably well with the affinity constants obtained from solution binding studies, except for one anomaly. The similar affinity constants for decane and (*E*)- β -farnesene (29.6 μM) do not correlate well with their respective HINT scores (829 and 1283). Perhaps other conformations of decane, besides the one modeled, exist in solution, or its binding mode is different. The binding data shown in Table 2 lead to the conclusion that nonpolar (aliphatic) compounds such as (*E*)- β -farnesene (Fig. 5), β -caryophyllene, decane, and heptane constitute better ligands for Sol i 2 than those containing hydrophilic groups (1-octen-3-ol and ethanol). This is reasonable considering the highly hydrophobic nature of the pocket interior. Data analysis shows that the presence of oxygen atoms in the alcohols causes unfavorable interactions with several hydrophobic protein side chains. Comparing the HINT values for decane and heptane, assuming an estimated error of ± 200 , we observed a reasonable correlation. Heptane was chosen because of its excellent fit to the residual electron density observed in the electron density map (Fig. 1). In the case of (*E*)- β -farnesene and β -caryophyllene, which share the same molecular size ($\text{C}_{15}\text{H}_{24}$), the higher binding affinity for (*E*)- β -farnesene (29.6 μM), as compared to β -caryophyllene (25.5 μM), may be explained by its intrinsic structure, which allows for a higher degree of conformational flexibility and thus greater adaptability to the nonpolar side chains inside the pocket,

compared with the constrained ring structure of β -caryophyllene.

Hydrophobic cavity: Biological implications

Chemical communication in insects is mediated by two types of small soluble proteins that have distinct structures: OBPs (130–150 amino acids), which are specific for pheromones and general odorants, and chemosensory proteins (CSPs), which are slightly smaller (100–120 residues).³¹ Although the structures of OBPs and CSPs differ, both contain an inner hydrophobic cavity and perform a similar function (i.e., to reversibly bind small ligands with dissociation constants in the micromolar range). OBPs and CSPs are essential components of insect olfactory systems that mediate neuronal responses to airborne stimuli of pheromones and food odors. Both OBPs and CSPs are secreted into the lymph of chemosensilla, where their concentration can reach millimolar levels. The striking self-organization of social insect colonies is due to well-developed communication, which is mainly performed by sociochemicals, including both releaser and primer pheromones. Pheromones are very volatile chemicals that are released by one member of a particular species into the environment, where they affect the behavior or the physiology of another member of the same species. Insects belonging to Hymenoptera have several pheromone-producing glands. It has been reported that the venom gland of *S. invicta* is the storage site for the attractive pheromone.³⁵ The trail pheromones of *S. invicta*, produced by Dufour's gland, are essential for the recruitment and orientation of ant workers to food sources and foraging areas.^{36,37}

In terms of protein sequences, Sol i 2 is very different from any OBP sequence, including PBPs, CSPs, and antennal-specific proteins. In contrast, the structure of Sol i 2 is very similar to that of the OBP and PBP LUSH of the fruit fly *D. melanogaster*, which may suggest a similar function. Although the biological function of Sol i 2, produced by the venom gland and released through the stinger, is not yet known, the following analysis of the ant pheromone-producing machinery may allow some insights. Dufour's gland is one of the pheromone-producing exocrine glands and is located near the venom gland, at the base of the sting apparatus,^{36,37} through which its content and the venom gland content are released. The most prominent trail pheromone produced in Dufour's gland of *Solenopsis* species is *Z,E*- α -farnesene.³⁸ It is more effective in combination with homofarnesene synergists from Dufour's gland, but even more effective in combination with incompletely characterized primer pheromones.³⁹ In *S. invicta*, Dufour's gland contains trail primer pheromones, which are small hydrophobic compounds required to trigger orientation

response upon release through the stinger. This was shown by the observation that addition of Dufour's gland extract at the beginning of a trail increased the recruitment activity of *Z,E*- α -farnesene by more than 4-fold.⁴⁰ In one study, the short-living hydrophobic primer stored in Dufour's gland was shown to be replaceable with heptadecane.³⁹ Modeling of (*E*)- β -farnesene, the isomer of *Z,E*- α -farnesene that differs by the location of one double bond, showed that it is one of the ligands with the strongest binding to Sol i 2 (Table 2, Fig. 5). Thus, the role of Sol i 2 may be to bind and protect the hydrophobic moiety of pheromones, fatty acids, or a short-living hydrophobic primer needed for, for example, mass recruitment or transport to their receptor analogous to an OBP.

Another possible role of Sol i 2 is related to the chemical composition of fire ant venom, which is very rich in piperidine alkaloids (Chen and Fadamiro⁴¹ and references therein). The venom of *S. invicta* is composed mainly of water-insoluble 2-methyl-6-alkyl or 2-methyl-6-alkenyl piperidines, known as solenopsins.⁴¹ Figure 4 shows that two alkanes (decane and undecane) and one alkene [(*E*)- β -farnesene] (Fig. 5) bind to the hydrophobic cavity of Sol i 2. These compounds are similar to alkane and alkene chains attached at the sixth position of piperidine alkaloids. It is thus conceivable that Sol i 2 could also be involved either in the transport of an alkaloid ingredient from the site of synthesis to the venom reservoir or in the formation of a protective complex with the alkaloid in the venom duct.

Allergenicity

Sol i 2 is a potent major allergen of red fire ant venom that can induce serious, occasionally fatal, systemic IgE-mediated anaphylactic reactions in humans.^{1,6,7} The structure of the Sol i 2 surface contains an unusually high number of charged residues that are evenly distributed on the surface (Fig. 6). Of all surface residues, 38% are charged: Asp, Glu, Lys, or Arg. This is considerably higher than the average value (27%) for proteins.⁴² Charged amino acids often contribute significantly to the free energy of binding in protein-protein interactions, including antibody-antigen binding. Charged residues have been found in epitopes identified from the crystal structure of the bee venom hyaluronidase in complex with its specific IgG antibody,²⁴ as well as in the structure of the grass pollen allergen Phl p 2 in complex with monoclonal human IgE.⁴³ The importance of surface-charged residues in IgE binding and in the allergenicity of the dust mite allergen Der f 13 and other major allergens has been demonstrated by mutagenesis studies.⁴⁴ Likewise, site-directed mu-

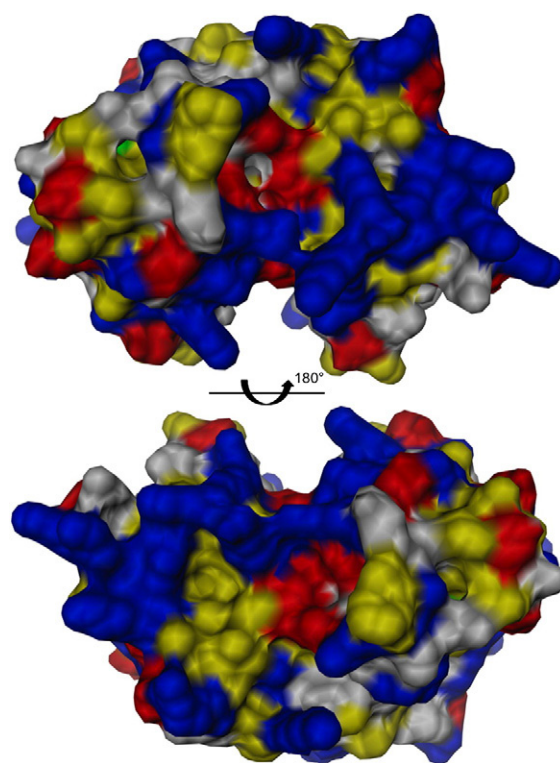


Fig. 6. Molecular surface of Sol i 2. The surface is color-coded according to residue property: red, acidic residues; blue, basic residues; yellow, apolar residues; green, aromatic residues; gray, polar residues. The molecular view at the top row is as shown in Fig. 1. In the bottom row, the molecule has been rotated by 180° along the horizontal axis with respect to the view in the top row.

tagenesis of Sol i 2 surface-exposed charged residues may lead to the identification of the Sol i 2 epitope(s) and the production of mutant variants with low IgE binding activity (hypoallergens), which may be used in immunotherapy without the risk of side effects. The full characterization of antigen-antibody recognition sites will require the elucidation of the structure of complexes between Sol i 2 and its specific antibodies, since allergens' epitopes are generally composed of a discontinuous array of amino acids.⁴⁵

Sensitization of patients to a given insect venom may lead to sensitivity to multiple insect venoms. The IgE antibodies from patients sensitized to fire ant stings are usually cross-reactive among the species, including *S. invicta*, *S. richteri*, *S. geminata*, *S. xyloni*, *S. aurea*, and *S. saevissima*.^{16,17} This cross-reactivity is variable, and some sera exhibit species-specific reactivity with large differences in inhibition of IgE antibody binding.⁴⁷ The IgE cross-reactivity between the venoms of *S. invicta* and *S. richteri* was studied with sera from four patients living in northern Mississippi and sensitized to Sol i

2. The sera from three patients reacted to both Sol r 2 and Sol i 2, whereas one patient exhibited species-specific IgE antibody binding to Sol r 2 but was unreactive to Sol i 2, as shown by RAST and RAST inhibition studies.¹⁷ Cross-reactivity between *S. invicta* and *S. richteri* occurs mainly due to a high sequence similarity between major allergens Sol i 1 and Sol r 1 (phospholipase AB) and Sol i 3 and Sol r 3 (antigen 5), which share a 95.8% sequence identity. Sol i 2 and Sol r 2 share a 78.1% sequence identity and have some species-specific reactivity, whereas an allergen comparable to Sol i 4 is not found in *S. richteri*. The sequence alignment of allergens homologous to Sol i 2 is shown in [Supplementary Fig. 1](#). It has been suggested that IgE epitopes that are responsible for cross-reactivity are preferentially located in the surface area conserved among the species.^{23,46} In [Fig. 7](#), the surface-exposed residues conserved between Sol i 2 and Sol r 2 are shown in green and have highly similar surface properties and thus may account for the observed IgE cross-reactivity, whereas those in yellow may account for species-specific reactivity. The yellow surface area

is composed of the remaining 24 nonconserved residues, of which 19 are surface-exposed and 5 are buried in the protein interior. Although the yellow area is relatively small, it is probably large enough to accommodate at least one epitope specific for either Sol i 2 or Sol r 2 and thus may account for the lack of IgE cross-reactivity observed in some patients.

The binding of human polyclonal IgE antibodies to Sol i 2 was shown to be conformation-dependent.¹⁵ Mouse monoclonal antibodies A4 and C6 raised against natural Sol i 2 were species-specific but did not inhibit human IgE binding to Sol i 2.^{15,18}

Concluding Remarks

The structure of the fire ant allergen Sol i 2 was solved at 2.6 Å resolution, revealing a homodimer with each subunit consisting of five α -helices that surround a central hydrophobic cavity. This is the first structure reported for Sol i 2 and the family of homologous insect allergens. The highest structural similarity, including the central hydrophobic cavity, is observed with the nonhomologous PBP and alcohol binding protein LUSH. To infer the role of the central hydrophobic cavity, we studied the binding of ant alarm pheromone analogues and other insect attractants of various sizes and hydrophobicities using fluorescence emission measurements. The highest binding affinity was observed with hydrophobic ligands such as aphid alarm pheromone (*E*)- β -farnesene and with plant volatiles such as decane, β -caryophyllene, and iso-caryophyllene. In contrast, the binding affinity of ligands containing polar groups such as ethanol and 1-octen-3-ol was significantly lower, in agreement with the highly hydrophobic character of the Sol i 2 cavity. The affinity of ligand binding correlated well with the highest-fitting scores obtained from ligand modeling, except for decane. Conceivably, the role of Sol i 2 may be similar to other transport proteins, that is, to sequester water-insoluble hydrophobic ligands such as pheromones, fatty acids, or short-living hydrophobic primers and to transport them to their receptor and/or to act as a cofactor in the activation of an odorant receptor. Further studies are required to validate this hypothesis. An analysis of the molecular surface, in combination with sequence alignment, revealed that a large part of the molecular surface is conserved between the two species, thus explaining the observed serological cross-reactivity between *S. invicta* and *S. richteri*.^{16,17} However, a small part of the nonconserved molecular surface may accommodate at least one epitope that is unique for Sol i 2 and thus may account for the lack of IgE cross-reactivity observed in certain patients who are allergic to Sol i 2.¹⁷

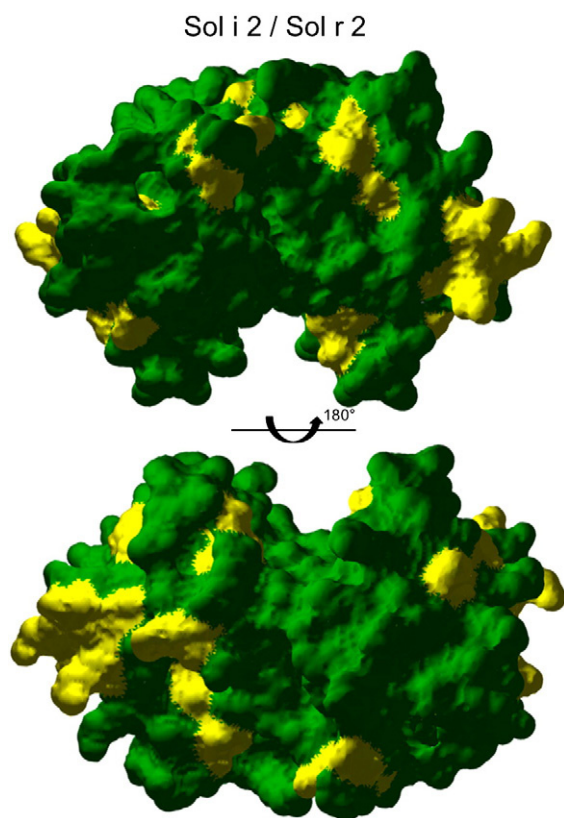


Fig. 7. The molecular surface of Sol i 2. Green denotes amino acids identical with Sol r 2, whereas nonidentical residues are shown in yellow. The molecular view in the bottom row has been rotated by 180° along the horizontal axis with respect to the view in the top row.

Materials and Methods

Expression and purification of recombinant Sol i 2

Mature recombinant Sol i 2, composed of residues 20–138 (UniProt ID: P35775) and a C-terminal 6× His tag, was produced by *Baculovirus*-infected insect cells (Sf9), as described previously.¹⁵ The recombinant wild-type Sol i 2 was produced by infecting 1 L of Sf9 insect cell culture (2×10^6 cells/ml) with a viral high-titer stock at a multiplicity of infection of 1. The supernatant harvested 2 days postinfection was dialyzed and purified by Ni^{2+} -chelate chromatography. The protein purity, tested by SDS/PAGE and Western blot analysis, was higher than 95%, and the yield was 10–11 mg/1 cell culture.

The production of SeMet-derivatized Sol i 2 was performed by infecting Sf9 insect cells (1.8×10^6 cells/ml) with high-titer stock (multiplicity of infection=1) using a modified procedure based on the method of Cronin *et al.*⁴⁷ After 7 h, the insect cells were separated by centrifugation at 600 rpm and resuspended in methionine-free medium ESF-21 (Expression Systems LLC). At 11 h postinfection, DL-SeMet (Calbiochem) was added to a final concentration of 100 mM. The supernatant was harvested 4 days after infection and purified by Ni^{2+} -chelate chromatography. The final yield was 7–8 mg/ml, and the total substitution of methionine by SeMet was between 28% and 40%. The presence of SeMet was verified by liquid chromatography–mass spectrometry of the purified protein digested with Glu-C and by the X-ray fluorescence spectrum of a crystal. Three intramolecular cysteine bridges and the dimerizing Cys22 were verified, using liquid chromatography–mass spectrometry, by the absence of cysteine alkylation with iodoacetamide, which is characteristic of free cysteines.

For crystallization experiments, Sol i 2 was dialyzed against 50 mM Hepes buffer (pH 7.5) and concentrated to 12 mg/ml.

Crystallization, data collection, and structure determination

Two crystal forms suitable for X-ray analysis were obtained by hanging-drop vapor diffusion at 20 °C. The best-diffracting crystals of native Sol i 2 grew in space group $P2_12_12_1$ and were obtained by mixing 1.0 μL of Sol i 2 solution (2.8 mg/ml) with 1.0 μL of 35% polyethylene glycol 8000, 0.1 M glycine (pH 9.2), and 0.1 M Na-acetate, and equilibrated over the latter solution. Two crystal forms (Ia and Ib) of SeMet-derivatized Sol i 2 grew within 2 months in space group $I222$ and were obtained by mixing 1 μL of protein solution (12 mg/ml) with 1 μL of 35% polyethylene glycol 8000, 0.2 M glycine (pH 9.2), and 0.2 M Na-acetate, giving rise to needle-like crystals up to 4 mm in length and 50 μm in thickness.

For crystal structure determination, diffraction data were collected from single crystals (100 K) at the Swiss Light Source (Villigen, Switzerland) using a MAR CCD detector. The images were indexed and integrated using the program MOSFLM,⁴⁸ and the data were reduced and scaled using programs of the CCP4 package⁴⁹ (Table 1).

Three different crystal forms (P, Ia, and Ib) (Table 1) were used for structure determination. Initial phases were determined by single-wavelength anomalous dispersion on a SeMet-derivatized crystal of $I222$ symmetry (Ia) diffracting only to low resolution (3.3 Å). The program SHELXD⁵⁰ identified all six selenium atoms of the 2-fold symmetric dimer of the asymmetric unit. Phases were improved by SHARP,⁵¹ and the cut-out map of the dimer was used for molecular replacement (program PHASER⁵²) to solve crystal form Ib that also belonged to space group $I222$, with a dimer in the asymmetric unit but with significantly distinct cell axes. The map was improved by 4-fold multicrystal averaging over the two dimers using the program DMM.⁴⁹ Then, the map of the averaged dimer was used to solve crystal form P with four dimers in the asymmetric unit by molecular replacement. Finally, multicrystal averaging over all 12 monomers of the three crystal forms and phase extension to the resolution limit of form P (2.6 Å) drastically improved the map.

Model building was performed with the programs Coot^{53–55} and O,⁵⁶ starting with a homology model identified by HHpred.⁵⁷ After modeling the side chains, we refined the model with noncrystallographic symmetry restraints and TLS parameters using REFMAC.⁵⁸ The progress of the refinement was monitored with a decrease in free R -factor, while the divergence between R and R_{free} was minimized. The final model includes four dimers of Sol i 2 and has an R/R_{free} value of 23.7%/27.1%. Data statistics are given in Table 1. The stereochemistry of the refined structure was validated with the program Procheck,⁵⁹ which showed that 98.5% of residues were found within the favored region and 1.5% of residues were found within the allowed region of the Ramachandran plot.

All figures were produced with the program DINO†, except for Fig. 7, which was produced with the program SPDBV.⁶⁰

Fluorescence competitive binding assay

The binding affinities of various compounds for Sol i 2 were determined by assays based on the reduction of the fluorescence emission of the probe NPN, which is caused by ligand competition with NPN. To measure the binding of the fluorescent probe NPN to Sol i 2, we titrated a 2 μM protein solution (1 ml) in 20 mM Tris-HCl (pH 7.4) with aliquots of 1 mM NPN dissolved in methanol to final concentrations of 0.05–40 μM . The protein/NPN complex was excited at 280 nm, and emission spectra were recorded between 350 nm and 450 nm. The competitive binding of ligands was measured by using NPN (5 μM) as fluorescent reporter and 0.05–50 μM concentrations of each ligand dissolved in methanol. The total volume of methanol in the protein solution was maintained at 21 μL (2.1%) by adding methanol without ligands. Bound ligand affinity for Sol i 2 was evaluated from the values of fluorescence intensity with a protein/ligand stoichiometry of 1:1 at saturation. The reductions in NPN fluorescence emission at 400 nm were normalized to the NPN fluorescence before titration.

† <http://www.dino3d.org>

Modeling of ligands

Modeling of selected ligands identified by fluorescence binding studies into the hydrophobic cavity was performed manually using the program Coot.^{53,55} This was followed by energy minimization of the protein–ligand complex within a 6-Å sphere of the ligand using the program SYBYL (version 8.1; Tripose Associates, St. Louis, MO).⁶¹ Subsequently, the program HINT,^{32–34} which was integrated into the SYBYL modeling environment, was used to compute an overall HINT binding score (HINT, version 3.1) that analyzes ligand–receptor interactions. This overall binding score is generally useful as a scoring method in molecular docking, as it represents the sum of all binding values from each interacting atom pair. The binding value B_{ij} for a given interacting atom pair ij , where i is an atom on the ligand and j is an atom on the protein, is defined as:

$$B_{ij} = \sum_i \sum_j [s_i a_i s_j a_j R_{ij} + r_{ij}]$$

where s is the solvent-accessible surface area, a is the hydrophobic atom constant, and R_{ij} and r_{ij} are functions of distances between interacting atoms.⁶² HINT is based on the prediction of the hydrophobic atom constants for each atom of the ligand and the receptor and, together with proximity factors, enables the evaluation of the interactions between ligand and biomolecules. HINT has been created and utilized to identify the major noncovalent hydrophobic and polar interactions (so-called hydrophathy) between small molecules and macromolecules.

PDB accession numbers

Coordinates and structure factors have been deposited in the PDB with accession numbers 2ygu and r2ygu, respectively.

Acknowledgements

We would like to thank the staff at synchrotron beamline PX of the Swiss Light Source. This study was supported by Swiss National Science Foundation grant 31-116804 (to Z.M.-H.), the North Carolina Biotechnology Center (D.R.H.), and Rothamsted Research grant-aided support from the Biotechnology and Biological Science Research Council of United Kingdom. We are also indebted to the Institute of Structural Biology and Drug Discovery, Virginia Commonwealth University (Richmond, VA), for the use of their excellent modeling facilities.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jmb.2011.10.009](https://doi.org/10.1016/j.jmb.2011.10.009)

References

- Hoffman, D. R. (1995). Fire ant venom allergy. *Allergy*, **50**, 535–544.
- Lai, L. C., Hua, K. H., Yang, C. C., Huang, R. N. & Wu, W. J. (2009). Secretion profiles of venom alkaloids in *Solenopsis geminata* (Hymenoptera: Formicidae) in Taiwan. *Environ. Entomol.* **38**, 879–884.
- Wu, B. Q., Lu, Y. Y., Zeng, L. & Liang, G. W. (2008). Influences of *Solenopsis invicta* Buren invasion on the native ant communities in different habitats in Guangdong. *Ying Yong Sheng Tai Xue Bao*, **19**, 151–156.
- Knight, D. & Bangs, M. J. (2007). Cutaneous allergic vasculitis due to *Solenopsis geminata* (Hymenoptera: Formicidae) envenomation in Indonesia. *Southeast Asian J. Trop. Med. Public Health*, **38**, 808–813.
- Khan, S., Shelleh, H., Khan, L. & Shah, H. (1999). Black fire ant (*Solenopsis richteri*) sting producing anaphylaxis: a report of 10 cases from Najran. *Ann. Saudi Med.* **19**, 462–464.
- Partridge, M. E., Blackwood, W., Hamilton, R. G., Ford, J., Young, P. & Ownby, D. R. (2008). Prevalence of allergic sensitization to imported fire ants in children living in an endemic region of the southeastern United States. *Ann. Allergy Asthma Immunol.* **100**, 54–58.
- Freeman, T. M. (1997). Hymenoptera hypersensitivity in an imported fire ant endemic area. *Ann. Allergy Asthma Immunol.* **78**, 369–372.
- MacConnell, J. G., Williams, R. N., Brand, J. M. & Blum, M. S. (1974). New alkaloids in the venoms of fire ants. *Ann. Entomol. Soc. Am.* **67**, 131–135.
- Hoffman, D. R., Dove, D. E. & Jacobson, R. S. (1988). Allergens in Hymenoptera venom: XX. Isolation of four allergens from imported fire ant (*Solenopsis invicta*) venom. *J. Allergy Clin. Immunol.* **82**, 818–827.
- Hoffman, D. R., Sakell, R. H. & Schmidt, M. (2005). Sol i 1, the phospholipase allergen of imported fire ant venom. *J. Allergy Clin. Immunol.* **115**, 611–616.
- Hoffman, D. R. (1993). Allergens in Hymenoptera venom: XXIV. The amino acid sequences of imported fire ant venom allergens Sol i II, Sol i III, and Sol i IV. *J. Allergy Clin. Immunol.* **91**, 71–78.
- Schmidt, M., McConnell, T. J. & Hoffman, D. R. (2003). Immunologic characterization of the recombinant fire ant venom allergen Sol i 3. *Allergy*, **58**, 342–349.
- Padavattan, S., Schmidt, M., Hoffman, D. R. & Marković-Housley, Z. (2008). Crystal structure of the major allergen from fire ant venom, Sol i 3. *J. Mol. Biol.* **383**, 178–185.
- Schmidt, M., Walker, R. B., Hoffman, D. R. & McConnell, T. J. (1993). Nucleotide sequence of cDNA encoding the fire ant venom protein Sol i II. *FEBS Lett.* **319**, 138–140.
- Schmidt, M., McConnell, T. J. & Hoffman, D. R. (1996). Production of a recombinant imported fire ant venom allergen, Sol i 2, in native and immunoreactive form. *J. Allergy Clin. Immunol.* **98**, 82–88.
- Hoffman, D. R. (1997). Reactions to less common species of fire ants. *J. Allergy Clin. Immunol.* **100**, 679–683.
- Hoffman, D. R., Smith, A. M., Schmidt, M., Moffitt, J. E. & Guralnick, M. (1990). Allergens in Hymenoptera venom: XXII. Comparison of venoms from two

- species of imported fire ants, *Solenopsis invicta* and *richteri*. *J. Allergy Clin. Immunol.* **85**, 988–996.
18. Hoffman, D. R., Jacobson, R. S., Schmidt, M. & Smith, A. M. (1991). Allergens in Hymenoptera venoms: XXIII. Venom content of imported fire ant whole body extracts. *Ann. Allergy*, **66**, 29–31.
 19. Scott, D. L., Otwinowski, Z., Gelb, M. H. & Sigler, P. B. (1990). Crystal structure of bee-venom phospholipase A2 in a complex with a transition-state analogue. *Science*, **250**, 1563–1566.
 20. Terwilliger, T. C. & Eisenberg, D. (1982). The structure of melittin: II. Interpretation of the structure. *J. Biol. Chem.* **257**, 6016–6022.
 21. Marković-Housley, Z., Miglierini, G., Soldatova, L., Rizkallah, P. J., Müller, U. & Schirmer, T. (2000). Crystal structure of hyaluronidase, a major allergen of bee venom. *Structure*, **8**, 1025–1035.
 22. Skov, L. K., Seppälä, U., Coen, J. J. F., Crickmore, N., King, T. P., Monsalve, R. *et al.* (2006). Structure of recombinant Ves v 2 at 2.0 angstrom resolution: structural analysis of an allergenic hyaluronidase from wasp venom. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **62**, 595–604.
 23. Henriksen, A., King, T. P., Mirza, O., Monsalve, R. I., Meno, K., Ipsen, H. *et al.* (2001). Major venom allergen of yellow jackets, Ves v 5: structural characterization of a pathogenesis-related protein superfamily. *Proteins*, **45**, 438–448.
 24. Padavattan, S., Schirmer, T., Schmidt, M., Akdis, C., Valenta, R., Mittermann, I. *et al.* (2007). Identification of a B-cell epitope of hyaluronidase, a major bee venom allergen, from its crystal structure in complex with a specific Fab. *J. Mol. Biol.* **368**, 742–752.
 25. Kruse, S. W., Zhao, R., Smith, D. P. & Jones, D. N. M. (2003). Structure of a specific alcohol-binding site defined by the odorant binding protein LUSH from *Drosophila melanogaster*. *Nat. Struct. Biol.* **10**, 694–700.
 26. Thode, A. B., Kruse, S. W., Nix, J. C. & Jones, D. N. M. (2008). The role of multiple hydrogen-bonding groups in specific alcohol binding sites in proteins: insights from structural studies of LUSH. *J. Mol. Biol.* **376**, 1360–1376.
 27. Holm, L. & Sander, C. (1995). Dali: a network tool for protein structure comparison. *Trends Biochem. Sci.* **20**, 478–480.
 28. Holm, L. & Park, J. (2000). Dalilite workbench for protein structure comparison. *Bioinformatics*, **16**, 566–567.
 29. Laughlin, J. D., Ha, T. S., Jones, D. N. M. & Smith, D. P. (2008). Activation of pheromone-sensitive neurons is mediated by conformational activation of pheromone-binding protein. *Cell*, **133**, 1255–1265.
 30. Lauren, A., Jones, D. N. M. & Lin, H. (2010). Alcohol binding to the odorant binding protein LUSH: multiple factors affecting binding affinities. *Biochemistry*, **49**, 6136–6142.
 31. Zhou, J. J., Zhang, G. A., Huang, W., Birkett, M. A., Field, L. M., Pickett, J. A. & Pelosi, P. (2004). Revisiting the odorant-binding protein LUSH of *Drosophila melanogaster*: evidence for odour recognition and discrimination. *FEBS Lett.* **558**, 23–26.
 32. Abraham, D. J. & Kellogg, G. E. (1994). The effect of physical organic properties on hydrophobic fields. *J. Comput.-Aided Mol. Des.* **8**, 41–49.
 33. Meng, E. C., Kuntz, I. D., Abraham, D. J. & Kellogg, G. E. (1994). Evaluating docked complexes with the HINT exponential function and empirical atomic hydrophobicities. *J. Comput.-Aided Mol. Des.* **8**, 299–306.
 34. Wright, C. S. & Kellogg, G. E. (1996). Differences in hydrophobic properties of ligand binding at four independent sites in wheat germ agglutinin-oligosaccharide crystal complexes. *Protein Sci.* **5**, 1466–1476.
 35. Vander Meer, R. K., Glancey, B. M., Lofgren, C. S., Glover, A., Tumlinson, J. H. & Rocca, J. R. (1980). The poison sac of red imported fire ant queens: source of a pheromone attractant. *Ann. Entomol. Soc. Am.* **73**, 609–612.
 36. Wilson, E. O. (1959). Source and possible nature of the odor trail of fire ants. *Science*, **129**, 643–644.
 37. Billen, J. P. J. (1987). New structural aspects of the Dufour's and venom glands in social insects. *Naturwissenschaften*, **74**, 340–341.
 38. Vander Meer, R. K., Alvarez, F. & Lofgren, C. S. (1988). Isolation of the trail recruitment pheromone of *Solenopsis invicta*. *J. Chem. Ecol.* **14**, 825–838.
 39. Vander Meer, R. K. (1983). Semiochemicals and the red imported fire ant (*Solenopsis invicta* Buren) (Hymenoptera: Formicidae). *Fla. Entomol.* **66**, 139–161.
 40. Vander Meer, R. K., Alvarez, F. M. & Lofgren, C. S. (1984). Fire ant recruitment and orientation trail pheromones: a unique species isolation mechanism. National Institutes of Health, Bethesda, MD.
 41. Chen, L. & Fadamiro, H. Y. (2009). Re-investigation of venom chemistry of *Solenopsis* fire ants: II. Identification of novel alkaloids in *S. invicta*. *Toxicon*, **53**, 479–486.
 42. Miller, S., Janin, J., Lesk, A. M. & Chothia, C. (1987). Interior and surface of monomeric proteins. *J. Mol. Biol.* **196**, 641–656.
 43. Padavattan, S., Flicker, S., Schirmer, T., Madritsch, C., Randow, S., Reese, G. *et al.* (2009). High-affinity IgE recognition of a conformational epitope of the major respiratory allergen Phl p 2 as revealed by X-ray crystallography. *J. Immunol.* **182**, 2141–2151.
 44. Chan, S. L., Ong, S. T., Ong, S. Y., Chew, F. T. & Mok, Y. K. (2006). Nuclear magnetic resonance structure-based epitope mapping and modulation of dust mite group 13 allergen as a hypoallergen. *J. Immunol.* **176**, 4852–4860.
 45. Laver, W. G., Air, G. M., Webster, R. G. & Smith-Gill, S. J. (1990). Epitopes on protein antigens: misconceptions and realities. *Cell*, **61**, 553–556.
 46. Gajhede, M., Osmark, P., Poulsen, F. M., Ipsen, H., Larsen, J. N., Joost van Neerven, R. *et al.* (1996). X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. *Nat. Struct. Biol.* **3**, 1040–1050.
 47. Cronin, C. N., Lim, K. B. & Rogers, J. (2007). Production of selenomethionyl-derivatized proteins in baculovirus-infected insect cells. *Protein Sci.* **16**, 2023–2029.
 48. Leslie, A. G. W. (1992). Technical Report No. 26. Joint CCP4 and ESF-EACBM Newsletter on Protein Crystallography SERC Daresbury Laboratory, Warrington, UK.
 49. Collaborative Computational Project, Number 4 (CCP4). (1994). The CCP4 suite: programs for protein

- crystallography. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **50**, 760–763.
50. Sheldrick, G. M. (2008). A short history of SHELX. *Acta Crystallogr., Sect. A*, **64**, 112–122.
 51. La Fortelle, E. & Bricogne, G. (1997). Maximum-likelihood heavy-atom parameter refinement for multiple isomorphous replacement and multiwavelength anomalous diffraction methods. *Methods Enzymol.* **276**, 472–494.
 52. Storoni, L. C., McCoy, A. J. & Read, R. J. (2004). Likelihood-enhanced fast rotation functions. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **60**, 432–438.
 53. Emsley, P. & Cowtan, K. (2004). Coot: model-building tools for molecular graphics. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **60**, 2126–2132.
 54. Krissinel, E. & Henrick, K. (2004). Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **60**, 2256–2268.
 55. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). Features and development of Coot. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **66**, 486–501.
 56. Jones, T. A., Zou, J., Cowan, S. & Kjeldgaard, M. (1991). Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr. Sect. A*, **47**, 110–119.
 57. Söding, J., Biegert, A. & Lupas, A. N. (2005). The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Res.* **33**, W244–W248; (Web Server Issue).
 58. Winn, M. D., Isupov, M. N. & Murshudov, G. N. (2001). Use of TLS parameters to model anisotropic displacements in macromolecular refinement. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **57**, 122–133.
 59. Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993). Procheck: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* **26**, 283–291.
 60. Guex, N. & Peitsch, M. C. (1997). Protein modelling for all. *Electrophoresis*, **18**, 2714–2723.
 61. Ghose, A. K., Jaeger, E. P., Kowalczyk, P. J., Peterson, M. L. & Treasurywala, A. M. (1993). Conformational searching methods for small molecules: I. Study of the SYBYL search method. *J. Comput. Chem.* **14**, 1050–1065.
 62. Kellogg, G., Joshi, G. S. & Abraham, D. (1992). New tools for modeling and understanding hydrophobicity and hydrophobic interactions. *Med. Chem. Res.* **1**, 444–453.