release of the other resource required for this defense is triggered by the host upon contact with the pathogen. This new mechanism stands as a unique signal/response reaction and strikingly demonstrates how independent principles have developed for signal reception and response in marine algae.

Experimental Section

General: Unialgal culturing of *A. operculata* (obtained from J. Correa, Santiago, Chile) was performed as previously described.[6] *C. crispus* was collected bimonthly in Pointe St.-Barbe, Roscoff, France and cultivated in tanks with continuous seawater exchange.

Bioassay with *C. crispus*: *C. crispus* was challenged and incubated in sterile seawater for 30 min at a density of 50 g L^{-1}. H_2O_2 in the medium was then quantified as horseradish-peroxidase-catalyzed luminol-dependent luminescence as previously described.[6]

Purification of the active signal from *A. operculata*: Cell-free extracts from *A. operculata* were prepared according to the method described by Bouarab et al.[6] Prior to HPAEC separation, macromolecules were removed from crude extracts by ethanol precipitation (90%), centrifugation, dialysis of the concentrated supernatant (cut-off: 1000 Da) and concentration of the dialysate. A DX500 (Dionex) instrument with CarboPac PA100 (250/4) column was used for HPAEC. Gradient: $NaOH/H_2O$; 1 mL min^{-1}; 0 mM, 5 min at 0 mM then linear to: 15 min, 0.15 M; 20 min, 0.15 M; 20 – 30 min, 1 M. Elution of the active compound was observed at pH 12.

Identification of the active signal from *A. operculata*: HPLC separation was achieved on an HP1100 (Agilent technologies) device equipped with a Macherey Nagel nucleosil 100 – 5 NH_2 aminopropyl column (250/4). Gradient: CH_3CN/H_2O; 1 mL min^{-1}; 90%, 5 min at 90%, then linear to: 25 min, 60%; 30 min, 20% CH_3CN. LC MS^n was performed on a Finnigan LCQ system. LC APCI MS/MS: vaporiser temperature: 470 °C; capillary temperature: 150 °C; discharge current: 4 μA; 133 $[M+H]^+$. The MS^2 spectrum showed fragments with masses of 116(20) and 87(100). The MS^3 spectrum of the ion at $m/z =$ 87 showed an abundant fragment at $m/z = 70$. In gel filtration experiments, the active principle eluted with H_2O after 1.25 void volumes from Sephadex G10.

Identification of 2-oxo succinamic acid: Derivatisation and HPLC separation of *C. crispus* growth medium after administration of different amounts of asparagine was performed according to the procedure described by Lange et al.[11] 2-Oxo octanoic acid was used as a standard. The retention time and MS fragmentation pattern of the derivatised 2-oxo succinamic acid (222 $[M+H]^+$, 178, 161) matched those of a reference compound prepared by treatment of asparagine with amino acid oxidase (Sigma).

Asparagine release after administration of carrageenans: *A. operculata* was incubated in seawater medium at a density of 500 mg mL^{-1} in the presence of the antibiotics Cefotaxim and Gentamycin at 100 μg mL^{-1} and Polymyxin B, Chloramphenicol, Erythromycin and Kanamycin at 20 μg mL^{-1}. After four days, λ- or κ-oligocarrageenan was added to the cultures at a concentration of 250 μg mL^{-1}. Biomass and medium were separated by centrifugation after 12, 24, 36 and 48 h and the asparagine in the medium was quantified as described by Lindroth and Mopper.[12]

We are indebted to Prof. Dr. W. Boland for his support during the preparation of this work.

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A Short Aib/Ala-Based Peptide Helix Is as Stable as an Ala-Based Peptide Helix Double Its Length

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KEYWORDS:

alanine · aminoisobutyric acid · peptides · protein design · protein mimetics

Among the 20 naturally occurring amino acids, alanine (Ala) has the highest helix propensity.[1] Among noncoded α-amino acids, α-aminoisobutyric acid (Aib) is the helicogenic amino acid[2] that has been used the most for designing helices,[3] including those that have biological relevance.[4] In order to establish the advantages and limitations of using Aib to design a peptide helix, that would otherwise be comprised of naturally occurring amino acids like Ala, it is important that a comparative analysis of

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the stabilities of Aib- and Ala-based peptide helices be performed. Although chaotropic-agent-induced unfolding of Ala-based peptide helices has been studied as a function of helix length,[5] to date no such systematic study exists for Aib-based peptide helices. We report here, for the first time, the unfolding of a 14-mer Aib/Ala-based peptide helix, ABGY (Ac-Ala-Aib-Ala-Lys-Ala-Aib-Lys-Ala-Lys-Ala-Aib-Gly-Gly-Tyr-NH_2),[6] with guanidine hydrochloride (GnCl) and urea. Comparison of the unfolding data with similar studies on Ala-based peptide helices[5] shows that the 14-mer Aib/Ala-based helix at 25 °C is as stable as a 26-mer Ala-based helix at 0 °C.

First we studied the solution conformation of ABGY by ^{1}H NMR and circular dichroism (CD) spectroscopy. Conclusive support for a helical backbone of ABGY came from ^{1}H NMR spectroscopy experiments.[7] This support comes from: 1) sequential NN(i,$i+1$) ROESY crosspeaks, almost across the entire sequence (Figure 1), 2) an upfield shift (from random coil values) of more than 0.14 ppm for all C$^\alpha$H resonances except the two Gly residues,[3b] 4) amide temperature coefficients $(\Delta\delta/\Delta T) < 5$ ppb K^{-1} from Ala3 through to Aib11, and 4) $^3J_{N\alpha}$ values ≈ 5 Hz for all five alanine residues.[8] Collectively, these results all strongly indicate a helical backbone for ABGY.[9]

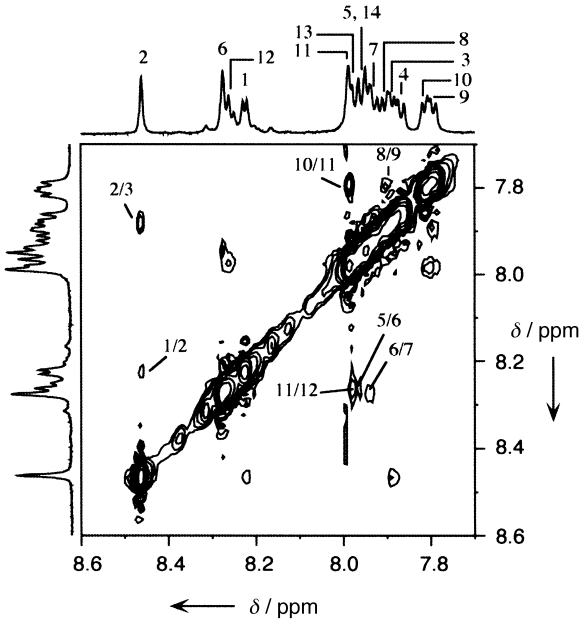

Figure 1. *The NN amide region of the ROESY ($\tau_m = 300$ ms) spectrum of ABGY at 27 °C and pH 3.1. Of the total possible 13 NN(i,i + 1) crosspeaks, 7 were detected (the rest are too close to the diagonal to be observed). Residues are sequentially numbered from the N terminal.*

The CD spectrum of ABGY at 25 °C,[10] shown in Figure 2, also exhibited the conspicuous double-negative-maximum helical signature.[11a] However, the $[\theta]_{222}$ value was low; this is not uncommon with helices containing α,α-dialkyl residues[11b,c] and does not necessarily indicate low helicity.[12]

Next we studied the unfolding of ABGY at 25 °C. Variation of $[\theta]_{222}$ as a function of added GnCl or urea is shown in Figure 3. As with Ala-based peptides,[5] variation of $[\theta]_{222}$ for ABGY reflects a

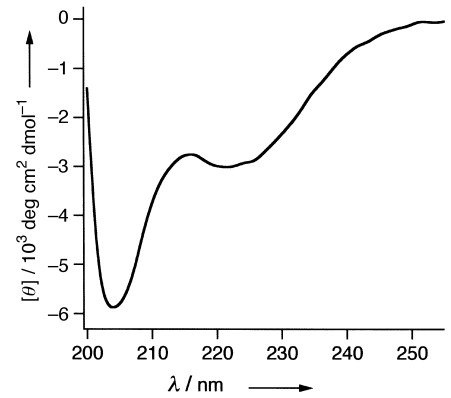

Figure 2. *The far-UV CD spectrum (smoothed) of ABGY at 25 °C and pH 3.1 (10 mM phosphate buffer) with 1 M NaCl.*

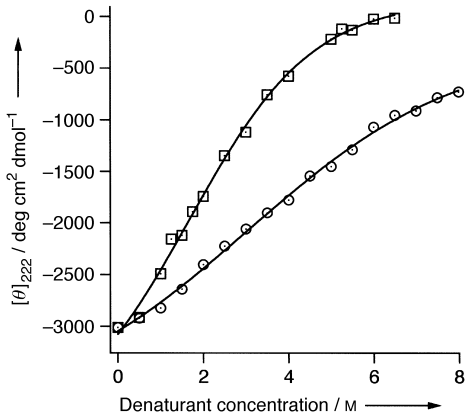

Figure 3. *Variation in $[\theta]_{222}$ values with GnCl (squares) and urea (circles) for ABGY at 25 °C. The curves are the best fits (2S and ZB-LEM models).*

broad transition from a folded to an unfolded state. The unfolding curves were analyzed by: 1) a two-state (2S) model,[13] commonly used for protein unfolding, which assumes the equilibrium existence of a folded and an unfolded state, and 2) the Zimm-Bragg model[14] for helix-coil transition with the linear extrapolation[15] method (ZB-LEM).[16] The best fits to the data, according to both the models are shown in Figure 3 while the best-fit parameters are presented in Table 1.

Analyses of both GnCl and urea unfolding data yield very similar values of s_0 (ZB-LEM model) for ABGY (1.58 and 1.57). The corresponding m values are 51.1 and 28.8 cal mole^{-1} M^{-1} res^{-1}. The higher m value for GnCl compared to urea is consistent since GnCl is known to be a stronger denaturant than urea.[17] For a direct comparison of our data with that for Ala-based peptide helices, we looked at the work by Scholtz et al.,[5] who studied the unfolding of a series of Ala-based peptide[18] helices by urea and GnCl at 0 °C. They had performed a global analysis of their unfolding data (14-, 20-, 26-, 32-, and 50-mer peptides) in terms of the ZB-LEM model to obtain s_0 values of 1.30 and 1.34 for unfolding with GnCl and urea, respectively. The corresponding m values were 50.4 and 23.0 cal mole^{-1} M^{-1} res^{-1}. Although the m values of the Ala-based peptides and ABGY are similar, the s_0 values are conspicuously larger in ABGY. The respective free energies of helix propagation ($-$RTlns_0) are -0.14 and

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Table 1. *Unfolding parameters of ABGY and Ala-based[18] peptides.*[a]

Peptide	Denaturant	s_o[16]	m[16] [cal mole^{-1} M^{-1} res^{-1}]	ΔG_{FU}[13] [kcal mole^{-1}]	m_{FU}[13] [kcal mole^{-1} M^{-1}]
ABGY	GnCl[b]	1.58 ± 0.02	51.5 ± 1.4	0.69 ± 0.16	0.41 ± 0.05
ABGY	urea[b]	1.57 ± 0.05	28.8 ± 4.1	0.72 ± 0.15	0.24 ± 0.03
20-mer	GnCl[c]	1.30[e]	50.4[e]	0.22 ± 0.08	0.41 ± 0.02
20-mer	urea[d]	1.34[e]	23.0[e]	0.64 ± 0.01	0.23 ± 0.01
26-mer	GnCl[c]	1.30[e]	50.4[e]	0.79 ± 0.06	0.52 ± 0.02
26-mer	urea[d]	1.34[e]	23.0[e]	1.12 ± 0.01	0.27 ± 0.01

[a] The value of σ[16] was fixed at 0.003.[5] S_U and S_F (2S and ZB-LEM models) were allowed to float freely during fitting. Reported errors are standard deviations from the curve fit (absolute errors < 0.01 reported as 0.01). [b] 25 °C, 1 M NaCl. [c] 0 °C, 1 M NaCl. [d] 0 °C, 0.1 M NaCl. [e] Global analysis best-fits (S_U, S_F, and their linear variation with denaturant were fixed to predetermined values) of Scholtz et al.[5] Errors in m are 0.4 – 0.7 cal mole^{-1} M^{-1} res^{-1}, errors in $\ln s_0$ are 0.005 – 0.009.

-0.27 kcal mole^{-1} res^{-1} for the Ala-based peptides (0 °C) and ABGY (25 °C).

Assuming identical σ values for Aib/Ala- and Ala-based peptides, this implies that the 14-mer ABGY helix at 25 °C is as stable as a 26-mer Ala-based peptide helix at 0 °C.[19] Analysis of the unfolding data according to the 2S model also leads to a similar conclusion. Using the reported ZB-LEM unfolding parameters of Scholtz et al.,[5] we reconstructed the unfolding profiles for the entire set of Ala-based peptides and refitted them to the 2S model. A comparison of the unfolding parameters showed that ABGY and 26-mer Ala-based peptides were equivalent, as shown in Table 1.[20] Despite the limitations of the 2S model (no intermediates allowed), the qualitative picture that emerges from the analysis is in agreement with the ZB-LEM model.

Protein helices are typically 10 – 12 residues long.[21] Stabilized by Ala residues, isolated peptides need to be almost double this length to exhibit significant helicity. The central finding of this work, that a 14-mer Ala-/Aib-based helix with about 20% Aib content shows stability at 25 °C similar to an Ala-based peptide helix almost twice its length at 0 °C,[22] implies that it is viable to design short protein-like helices by using the Aib/Ala strategy. These, with suitable side chains, can act as peptidomimetic drugs. In fact, we have already designed Aib-based helical mimics that are stable in isolation and show functional properties similar to the parent protein helix.[23]

The only other study that compared the stabilities of Ala- and Aib-containing peptides (urea unfolding of two Ala $\leftrightarrow$ Aib α-helix-dimer mutants)[24] found the Aib mutant to be more stable (by 1 kcal mole^{-1}). Another study found Ala $\rightarrow$ Aib substitution to increase the thermostability of a protein.[25] Our results complement these studies with important implications for designing helical peptidomimetics.

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[6] ABGY was synthesized by using a standard 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis protocol where Fmoc pentafluorophenyl ester was used for Xxx–Aib couplings and Aib–Xxx peptide bond formation was achieved with benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and *N*,*N*-diisopropylethylamine (DIPEA). All couplings were performed in the presence of 1-hydroxybenzotriazole (HOBT). The peptide was fully characterized by ^{1}H NMR spectroscopy. The Aib/Ala residues in ABGY were incorporated for inducing helicity, while the three Lys residues were incorporated to solubilize the peptide and to reduce the risk of self-association (by reducing amphiphilicity). The two Gly residues were incorporated to keep the Tyr chromophore, used for measuring peptide concentration, away from the helix. In designing ABGY, we used the Aib/Ala ratio of 3:5 to 1) maintain 60% total (Aib + Ala) content in ABGY so that ABGY data can be compared with 60% Ala-containing peptide data[5] and 2) avoid the risk of ABGY being 3_{10}-helical[2] and water insoluble by minimizing the Aib content.

[7] NMR spectroscopy experiments were performed on a Bruker DRX500 spectrometer. Samples were prepared in 10 mM phosphate buffer (pH 3.1) containing 10% D_2O (v/v) and 1 M NaCl with 3-(trimethylsilyl)-propionic-2,2,3,3-$d_4$acid, sodium salt (TSP). TOCSY and ROESY experiments were performed according to standard protocols.[9a]

[8] Due to severe crowding of the amide proton resonances of ABGY, the $(\Delta\delta/\Delta T)$ and the $^3J_{N\alpha}$ data are from NMR spectroscopy studies on a very similar peptide (identical to ABGY without the two Gly residues) which showed very similar CD and NN-ROESY spectra to ABGY.[3b]

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[10] Far-UV CD spectra were recorded at 25 °C in a JASCO J-600 spectropolarimeter in a 1- or 2-mm pathlength cuvette (10 mM phosphate buffer (pH 3.1), 60 μM peptide, 1 M NaCl).

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[12] The low $[\theta]_{222}$ value could be due to the presence of both right- and left-handed helices (Aib is achiral), or due to the fact that the CD spectrum of the unfolded state of Aib-containing peptides is nonstandard. The pronounced minimum at 204 nm compared to 222 nm possibly reflects the presence of some 3_{10}-helical conformation as well.[11c]

[13] $S = \{S_U + S_F \exp(\Delta G_{FU} - m_{FU}[d])/RT\}/\{1 + \exp(\Delta G_{FU} - m_{FU}[d])/RT\}$, where S = observed signal (U and F stand for the unfolded and folded states,

respectively), ΔG_{FU} = free energy of unfolding in absence of denaturant, and m_{FU} = rate of change of ΔG_{FU}(d) with denaturant concentration [d].
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[16] $S = f_H(S_F - S_U) + S_U$, with $f_H = [\sigma \times s/(s-1)^3][n \times s^{n+2} - (n+2)s^{n+1} + (n+2)s - n]/[n\{1 + [\sigma \times s/(s-1)^2][s^{n+1} + n - (n+1)s]\}]$ and $s = \ln s_0 - m$[d]/RT, where s = propagation parameter and σ = nucleation parameter.
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[18] The peptides studied had the general sequence Ac-Tyr-(Ala-Glu-Ala-Ala-Lys-Ala)$_n$-Phe-NH$_2$, where $n = 2$, 3, 4, 5, and 8.
[19] This follows from the fact that $14 \times RT\ln s_0$(ABGY) $\approx 27 \times RT\ln s_0$(Scholtz peptides). ABGY and the 26-mer Ala-based peptide share similar (Aib + Ala) content (~60%). It should be pointed out that the difference in s_o for the two sets may also originate from a higher value of the helix nucleation parameter σ in Aib-based helices (held constant at 0.003 by us and Scholtz et al.[5]).
[20] The presence of NaCl can alter the denaturant effect in peptides.[5] However, identical concentrations of NaCl used in GnCl experiments with ABGY and the Ala-based peptides cancel any salt effect in the two sets, which are compared.
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[22] Apart from the fact that ABGY data corresponds to 25 °C, ABGY, unlike the Ala-based peptides, contains two Gly residues that are known to disfavor the helical conformation. So, without the Gly residues and at 0 °C, the stability of ABGY is expected to increase even more.
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Fluorinated Protective Groups for On-Resin Quantification of Solid-Phase Oligosaccharide Synthesis with ^{19}F NMR Spectroscopy

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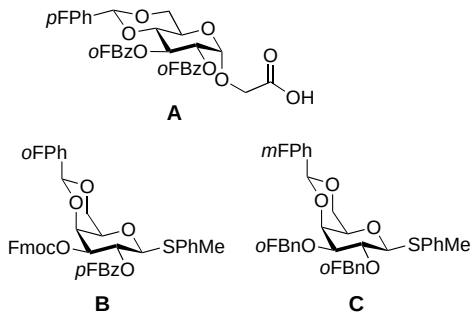
KEYWORDS:

carbohydrates · fluorine · glycosylation · NMR spectroscopy · solid-phase synthesis

Solid-phase synthesis is an attractive approach for the generation of large numbers of compounds for biological studies, for instance in the pharmaceutical industry.[1] In addition, automated procedures for solid-phase synthesis of oligopeptides and oligonucleotides have been of major importance in developing an understanding of the functions of proteins and nucleic acids in the processes of life. As compared to knowledge about these two biopolymers, the roles played by carbohydrates in different glycoconjugates in nature are less well understood.[2] Unfortunately, the difficulties associated with the synthesis of oligosaccharides constitute a major impediment for the development of glycobiology.[3] For instance, the assembly of complex oligosaccharides on solid support or in solution is still a considerable challenge for all but a few laboratories.

Although major advances in the solid-phase synthesis of oligo- and polysaccharides have been made during the past few years,[4] the lack of simple and powerful analytical techniques for on-resin validation of the chemistry involved is a significant limitation.[5] In general, the use of nondestructive methods, such as IR and NMR spectroscopy, for elucidating reactions directly on the solid phase constitutes the most attractive approach.[6, 7] However, applications of these techniques in solid-phase oligosaccharide synthesis are rare and include the use of high-resolution magic-angle-spinning NMR spectroscopy,[8] as well as employment of ^{13}C-enriched acetyl protective groups.[9, 10] These methods are somewhat restricted by costs and the requirement for specialized NMR spectroscopy equipment. On the other hand, fluorinated reagents corresponding to the most common protective groups used in oligosaccharide synthesis are commercially available and are usually cheap. Use of saccharide building blocks that carry fluorinated protective groups should therefore allow optimization of solid-phase oligosaccharide synthesis by using gel-phase ^{19}F NMR spectroscopy. Gel-phase ^{19}F NMR spectroscopy has several favorable properties including high sensitivity (the natural abundance of ^{19}F is 100%) and a wide dispersion of the ^{19}F chemical shifts. Hence, magic-angle spinning is not required and high-quality spectra can be obtained in a couple of minutes with a conventional NMR spectrometer.[11–16]

To investigate the potential of ^{19}F NMR spectroscopy for monitoring solid-phase oligosaccharide synthesis we have undertaken the synthesis of the α-Gal epitope (Gal(α1-3)-Gal(β1-4)Glc), which is responsible for hyperacute rejection in xenotransplantation of porcine organs.[17] The synthetic sequence started with immobilization of glycoside **A** (Scheme 1) on the linker-loaded ArgoGel resin **1**[15] through an ester linkage[18] to afford resin **2** (Scheme 2). The outcome of the



Scheme 1. *Monosaccharide building blocks used for solid-phase synthesis. Bn = benzyl, Bz = benzoyl, Fmoc = 9-fluorenylmethoxycarbonyl.*

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