ibuprofen to other non-phosphorothioate oligomeric constructs, including peptide nucleic acids,[27–29] methyl phosphonates,[30] morpholinos,[31] the methylene(methylimino) backbone modification,[32] and phosphoramidates[33] and locked nucleic acids,[34] may also enhance the pharmacokinetic and tissue distribution properties of these constructs.

[1] S. T. Crooke, *Handb. Exp. Pharmacol.* **1998**, *131*, 1.
[2] R. E. Olson, D. D. Christ in *Annual Reports in Medicinal Chemistry, Vol. 31* (Ed.: J. A. Bristol), Academic Press, San Diego, **1996**, pp. 327.
[3] E. Y. Rykova, L. V. Pautova, L. A. Yakubov, V. N. Karamyshev, V. V. Vlassov, *FEBS Lett.* **1994**, *344*, 96.
[4] S. K. Srinivasan, H. K. Tewary, P. L. Iversen, *Antisense Res. Dev.* **1995**, *5*, 131.
[5] S. T. Crooke, M. J. Graham, J. E. Zuckerman, D. Brooks, B. S. Conklin, L. L. Cummins, M. J. Greig, C. J. Guinosso, D. Kornbrust, M. Manoharan, H. M. Sasmor, T. Schleich, K. L. Tivel, R. H. Griffey, *J. Pharmacol. Exp. Ther.* **1996**, *277*, 923.
[6] S. Agrawal, *J. Drug Targeting* **1998**, *5*, 303.
[7] W. Y. Gao, F. S. Han, C. Storm, W. Egan, Y. C. Cheng, *Mol. Pharmacol.* **1992**, *41*, 223.
[8] A. A. Levin, D. K. Monteith, J. M. Leeds, P. L. Nicklin, R. S. Geary, M. Butler, M. V. Templin, S. P. Henry in *Antisense Research and Applications, Vol. 131* (Ed.: S. T. Crooke), Springer, Berlin, **1998**, pp. 169.
[9] J. S. Cohen, S. T. Crooke, B. Lebleu in *Antisense Research and Applications* (Ed.: C. P. B. Raton), **1993**, pp. 205.
[10] S. M. Freier, K. H. Altmann, *Nucleic Acids Res.* **1997**, *25*, 4429.
[11] L. L. Cummins, S. R. Owens, L. M. Risen, E. A. Lesnik, S. M. Freier, D. McGee, C. J. Guinosso, P. D. Cook, *Nucleic Acids Res.* **1995**, *23*, 2019.
[12] P. Martin, *Helv. Chim. Acta.* **1995**, *78*, 486.
[13] K.-H. Altmann, N. M. Dean, D. Fabbro, S. M. Freier, T. Geiger, R. Häner, D. Hüsken, P. Martin, B. P. Monia, M. Müller, F. Natt, P. Nicklin, J. Phillips, U. Pieles, H. Sasmor, H. E. Moser, *Chimia* **1996**, *50*, 168.
[14] M. Teplova, G. Minasov, V. Tereshko, G. B. Inamati, P. D. Cook, M. Manoharan, M. Egli, *Nat. Struct. Biol.* **1999**, *6*, 535.
[15] R. S. Geary, T. A. Watanabe, L. Truong, S. Freier, E. A. Lesnik, N. B. Sioufi, H. Sasmor, M. Manoharan, A. A. Levin, *J. Pharmacol. Exp. Ther.* **2001**, *296*, 890.
[16] U. Kragh-Hansen, *Pharmacol. Rev.* **1981**, *33*, 17.
[17] T. Peters, Jr., *Adv. Protein Chem.* **1985**, *37*, 161.
[18] T. J. Peters, *All About Albumin, Biochemistry, Genetics and Medical Applications*, Academic Press, San Diego, **1997**.
[19] D. C. Carter, J. X. Ho, *Adv. Protein Chem.* **1994**, *45*, 153.
[20] M. Manoharan, K. L. Tivel, L. K. Andrade, P. D. Cook, *Tetrahedron Lett.* **1995**, *36*, 3647.
[21] M. Manoharan, K. L. Tivel, P. D. Cook, *Tetrahedron Lett.* **1995**, *36*, 3651.
[22] R. Zini, J. Barre, F. Bree, J. P. Tillement, B. Sebille, *J. Chromatogr.* **1981**, *216*, 191.
[23] A. N. Kuznetsov, G. V. Gyul'khandanyan, B. Ebert, *Mol. Biol. (Moscow)* **1977**, *11*, 1057.
[24] S. Manalili, H. Sasmor in *XIII International Round Table Nucleosides, Nucleotides and Their Biological Applications, Proceedings*, Montpellier, France, **1998**, poster 403.
[25] A. A. Levin, S. P. Henry, D. Monteith, M. V. Templin, *Antisense Drug Technol.* **2001**, 201.
[26] M. Tanaka, Y. Asahi, S. Masuda, T. Ota, *Chem. Pharm. Bull.* **1991**, *39*, 1.
[27] M. Egholm, P. E. Nielsen, O. Buchardt, R. H. Berg, *J. Am. Chem. Soc.* **1992**, *114*, 9677.
[28] P. E. Nielsen, *Methods Enzymol.* **2000**, *313*, 156.
[29] P. E. Nielsen, *Biomed. Chem.* **2000**, 371.
[30] P. S. Miller in *Applied Antisense Oligonucleotide Technology* (Ed.: C. A. Stein, A. M. Krieg), Wiley-Liss, New York, **1998**, pp. 3.
[31] J. Summerton, D. Weller, *Antisense Nucleic Acid Drug Dev.* **1997**, *7*, 187.
[32] Y. S. Sanghvi, E. E. Swayze, D. Peoc'h, B. Bhat, S. Dimock, *Nucleosides Nucleotides* **1997**, *16*, 907.
[33] S. M. Gryaznov, *Biochim. Biophys. Acta* **1999**, *1489*, 131.
[34] H. Orum, J. Wengel, *Curr. Opin. Mol. Ther.* **2001**, *3*, 239.
[35] M. Manoharan, *Antisense Nucleic Acid Drug Dev.* **2002**, 12, 103.

Received: July 2, 2002 [Z 446]

A Signal Released by an Endophytic Attacker Acts as a Substrate for a Rapid Defensive Reaction of the Red Alga *Chondrus crispus*

Florian Weinberger,[b] Georg Pohnert,*[a] Bernard Kloareg,[b] and Philippe Potin[b]

KEYWORDS:

amino acids · biosynthesis · chemical defense · hydrogen peroxide · plant–pathogen interaction

Signal reception and regulation of physiological responses by macroalgae are only poorly understood to date. Besides some remarkable exceptions from the pheromone field[1] and the defensive reactions of certain brown algae,[2] only phenomenological descriptions of reactions to environmental cues have been given. The existing evidence nevertheless indicates that brown[3] as well as red[4] algae have developed elaborate strategies to detect the presence of herbivores or pathogens in their aqueous environment. Observed reactions include the immediate release of hydrogen peroxide or halogenated low-molecular-weight hydrocarbons as well as a long-term up-regulation of defensive metabolites. Comparable reactions are well known in higher plants. First evidence indicates that the underlying principles of signal recognition in algae differ significantly from those observed in higher plants, which urges the need for profound investigations in the marine environment.[5]

One of the better understood examples of an algae/pathogen interaction is the system of the red alga *Chondrus crispus* and its pathogen, the green alga *Acrochaete operculata*. The green alga is able to grow within the tissue of the red algal host. This process, called endophytism, can be fended off during certain developmental phases of *C. crispus*.[6] During the resistant gametophytic phase of the life cycle, *C. crispus* can recognise the attacker[6] and kill it by an immediate release of increased levels of H_2O_2 followed by the long-term up-regulation of defensive metabolites. In contrast, during the susceptible sporophytic phase, the green alga can penetrate the tissue of the host and parasitize it.[6]

Here we report the identification of the signal that triggers the early defense reaction of resistant gametophytes of the red alga. In addition, details are given about the biochemical processes that lead to the initial release of micromolar concentrations of

[a] *Dr. G. Pohnert*
Max-Planck-Institut für Chemische Ökologie
Winzerlaer Strasse 10, 07745 Jena (Germany)
Fax: (+49) 3641-571-256
E-mail: pohnert@ice.mpg.de

[b] *Dr. F. Weinberger, Dr. B. Kloareg, Dr. P. Potin*
UMR 1931, CNRS and Laboratoires Goëmar
Station Biologique de Roscoff
Roscoff (France)

H_2O_2 into the surrounding medium, which is sufficient to kill penetrating pathogens.

We used a protocol for our experiments that enabled us to grow the red and green algae separately in aquaria. This allowed the extraction of the active principle from the green alga and its addition to the resistant red alga. The immediate production of H_2O_2 by the red alga after challenge with crude green algal extracts and fractions of these extracts served as the indicative reaction for a bioassay-guided structure elucidation used to establish the nature of the involved signal.

The active principle released by the green algal parasite *A. operculata* can be enriched from cell-free extracts by use of a high pH anion exchange chromatography (HPAEC) cartridge. The compound is insensitive to heat (95 °C), acidic and basic conditions and was not inactivated after treatment with pentafluorobenzyloxime, a derivatising reagent for aldehydes and ketones. In contrast, the activity was strongly reduced or completely suppressed after treatment with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide or diazomethane, which indicates the requirement of an acidic group for full activity. Samples of *A. operculata* growth medium were further characterised by direct submission to liquid chromatography/atmospheric pressure mass spectrometry with an aminopropyl column as the stationary phase. The active fraction could be identified by monitoring the biological response (H_2O_2 production) of *C. crispus* gametophytes challenged with the eluate (Figure 1). Use of different gradients and source parameters allowed the active principle to be identified from the complex matrix as a low molecular weight ($[M+H]^+ = 133$) molecule. This result was in accordance with gel filtration experiments, which showed a molecular weight below 200 Da. High-resolution ESI MS of this compound gave a molecular composition of $C_4H_8N_2O_3$ which, together with the physical behaviour and the results from the derivatisation experiments suggested the free amino acid asparagine as a likely candidate. Indeed, L-asparagine coeluted with the active principle from *A. operculata*, exhibited the same MS and MS^n fragmentation pattern and induced the same biological response as the purified fraction from the extract (Figure 2a,b). This reaction was specific and neither D-asparagine (Figure 2c) nor up to millimolar amounts of L-glutamine, L-aspartic acid, L-α,γ-diamino butyric acid or succinamic acid triggered this defensive response. Kinetic characterisation of the signal/response reaction of *C. crispus* after exposure to

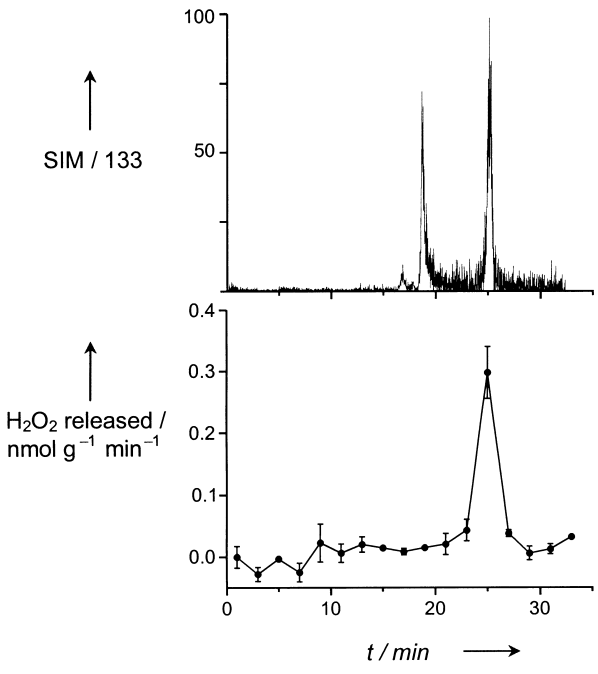

Figure 1. *HPLC separation of extracts from* A. operculata. *Upper trace: LC atmospheric pressure chemical ionisation (APCI) MS (SIM = single ion monitoring, m/z = 133). Lower trace: activity (H_2O_2 release induced in* C. crispus*) of fractions obtained by HPLC. Separation was achieved on an aminopropyl stationary phase; see the Experimental Section for details.*

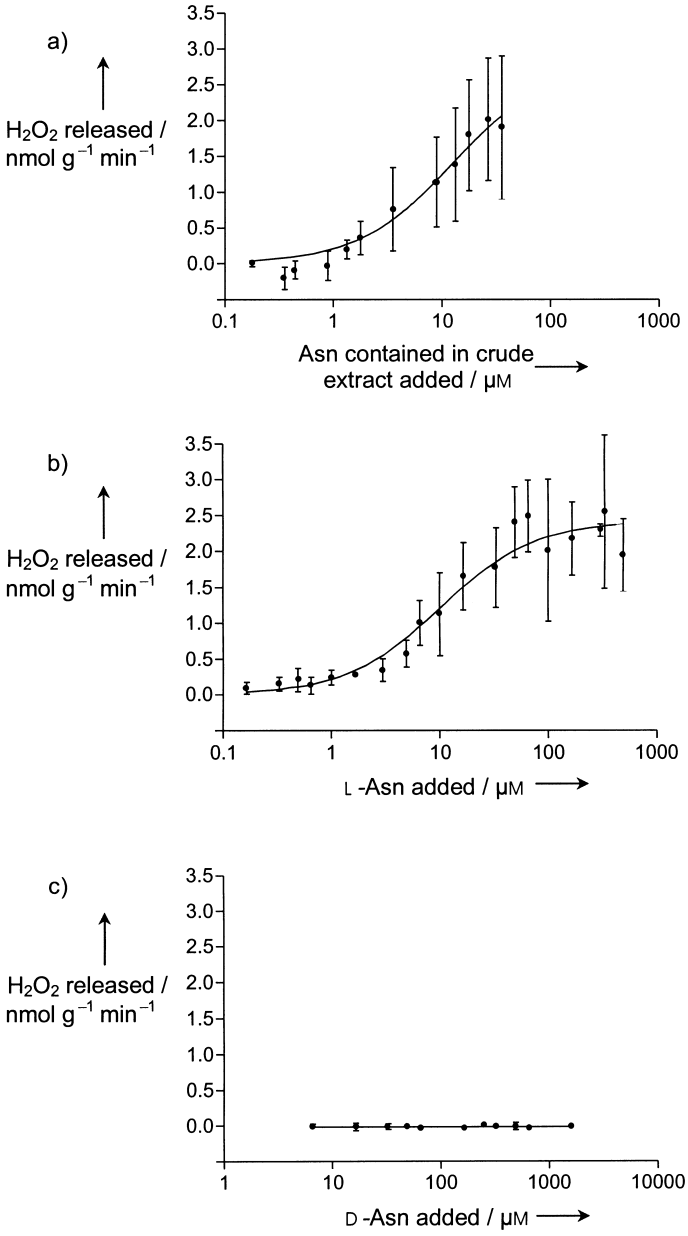

Figure 2. *H_2O_2 release induced in* C. crispus *with a) cell-free extracts (exposure to extracts at high concentrations resulted in interference with the assay); b) L-asparagine; c) D-asparagine. Lines represent the best-fit Michaelis–Menten functions.*

L-asparagine showed that the Michaelis – Menten constant K_m for H_2O_2 release was 13.2 μM (95% confidence interval: 8.0 – 18.4; Figure 2b). This value corresponded with the K_m value for induction of the same response with crude extracts of the pathogen (corresponding to 12.4 μM asparagine; 95% confidence interval: 4.1 – 20.7; Figure 2a).

We elucidated the early biochemical processes of the defensive reaction in order to understand the mechanism involved in this immediate response of the red alga. We found that H_2O_2 release by *C. crispus* after it was challenged with L-asparagine correlated with an accumulation of aldehydes or ketones[7] and ammonium[8] in the medium. This suggests that the defensive reaction is catalysed by an L-amino acid oxidase,[9] which transforms L-asparagine selectively into the corresponding α-keto acid, hydrogen peroxide and ammonium. In accordance with this mechanism, 100 μM concentrations of quinacrine, an inhibitor of flavoproteins, fully prevented the response. In addition, we could show that the amino acid oxidase product 2-oxosuccinamic acid is released by the red alga after application of asparagine. LC MS after derivatisation with phenylhydrazine allowed the characterisation and quantification of this oxo acid. A direct correlation between this product and the released H_2O_2 was observed, which demonstrates that the signal released by the pathogen acts as a substrate for the production of the defensive metabolite from the host.

Concentrations of H_2O_2 of around 100 μM are required for efficient induction of cell death of the pathogen.[10] In order to establish whether asparagine concentrations that would lead to the release of this amount of H_2O_2 may be available for *C. crispus* during infections, we monitored the content of extracellular asparagine in cultures of *A. operculata*. The degree of susceptibility of *C. crispus* tissues to *A. operculata* is correlated with the type of carrageenan that is present in the tissue of the host,[6] therefore we determined the asparagine release of the pathogen after administration of different carrageenan oligosaccharides. It was observed that λ-carrageenans from the susceptible life stage of *C. crispus* lead to an increased virulence of *A. operculata*, while pretreatment of the pathogen with κ-carrageenans from the resistant life stage of *C. crispus* significantly reduces its virulence.[6] In accordance with these findings, the asparagine release of *A. operculata* was strongly dependent on the type of carrageenan produced by the host (Figure 3). λ-oligocarrageenans from the susceptible life stage of the red alga do not trigger an increased release of asparagine. In contrast, a pronounced release of asparagine takes places 24 – 36 h after contact of the pathogen with κ-oligocarrageenans. The concentration of asparagine reaches levels required for a maximum production of about 66 μmol per kg algal wet weight H_2O_2 over a period of 30 min by the resistant host (calculated from Figure 2b), which is within the range of the lethal concentration for *A. operculata*. Since the intercellular space between host and pathogen is limited, both asparagine and H_2O_2 will accumulate to even higher local concentrations than found in the surrounding medium.

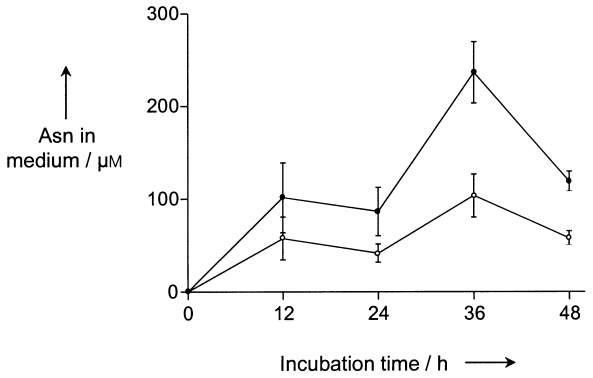

Figure 3. *Quantification of asparagine released by* A. operculata *after treatment with oligocarrageenans from* C. crispus. *(●) Treatment with κ-oligocarrageenans; (○) treatment with λ-oligocarrageenans.*

The interaction of the host and pathogen involves a complex communication between these organisms (Figure 4). After successful penetration of the outer cell layers of resistant, gametophytic *C. crispus*, filaments of the pathogen reach the inner tissue of the host and macerate its κ-carrageenan cell wall matrix, which leads to the release of κ-oligocarrageenans.[6] These molecules trigger the release of the signal asparagine from the pathogen, which finally serves as a substrate for the production of the defensive principle H_2O_2 (Figure 4).

This is to our knowledge the first example in chemical ecology where the induced signal of an attacker serves directly as a substrate for the production of a chemical defensive metabolite. Only the presence of an amino acid oxidase that can produce elevated levels of H_2O_2 is required to fend off the pathogen. The

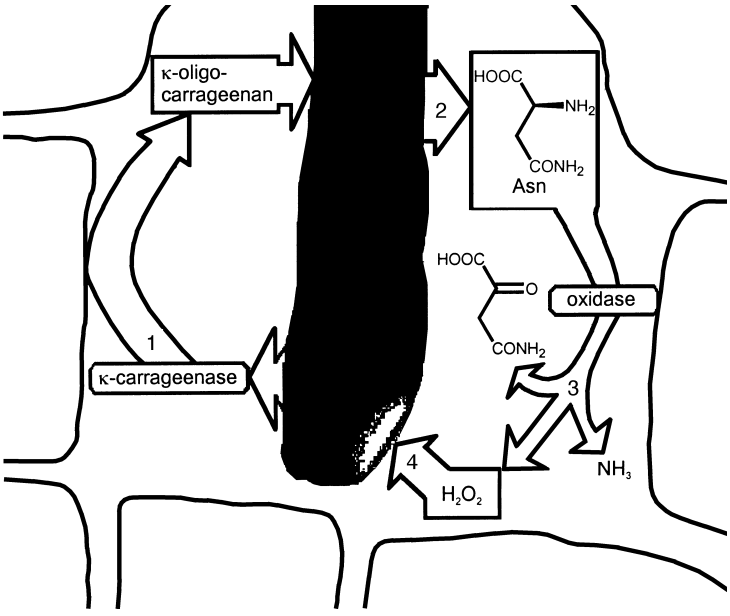

Figure 4. *Model for the involvement of* κ*-carrageenan and asparagine in the interaction of* C. crispus *and* A. operculata. *After penetration of the green algal endophyte (center), the carrageenans of the red algal tissue are broken down (1).[6] The resulting oligocarrageenans trigger the release of asparagine from the pathogen (2). This amino acid is then converted into* H_2O_2 *and* α*-ketosuccinamide by an amino acid oxidase from the red algal host (3). The oxidant causes cell death of the pathogen (4).*

  1439-4227/02/03/12 $ 20.00+.50/0 ChemBioChem **2002**, No. 12

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.

[1] a) G. Pohnert, W. Boland, *Nat. Prod. Rep.* **2002**, *19*, 108 – 122; b) F. Ender, A. Hallmann, P. Amon, M. Sumper, *J. Biol. Chem.* **1999**, *274*, 35 023 – 35 028.
[2] F. C. Küpper, B. Kloareg, J. Guern, P. Potin, *Plant Physiol.* **2001**, *125*, 278 – 291.
[3] G. B. Toth, H. Pavia, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 14 418 – 14 420.
[4] F. Weinberger, C. Richard, B. Kloareg, Y. Kashman, H. G. Hoppe, M. Friedlander, *J. Phycol.* **2001**, *37*, 418 – 426.
[5] P. Potin, K. Bouarab, J.-P. Salaun, G. Pohnert, B. Kloareg, *Curr. Opin. Plant Biol.* **2002**, *5*, 308 – 317.
[6] K. Bouarab, P. Potin, J. Correa, B. Kloareg, *Plant Cell* **1999**, *11*, 1635 – 1650.
[7] Quantification was carried out spectrophotometrically according to: K. Soda, *Agric. Biol. Chem.* **1967**, *31*, 1054 – 1060.
[8] Quantification was carried out spectrophotometrically according to: F. Koroleff in *Methods of Seawater Analysis* (Eds.: K. Grasshoff, K. Kremling, M. Ehrhardt), Wiley-VCH, Weinheim, **1983**, 2nd ed., pp. 159 – 228.
[9] P. Piedras, M. Pineda, J. Muñoz, J. Cárdenas, *Planta* **1992**, 188, 13 – 18.
[10] K. Bouarab, unpublished results.
[11] M. Lange, M. Mályusz, *J. Chromatogr., B: Biomed. Sci. Appl.* **1994**, *662*, 97 – 102.
[12] P. Lindroth, K. Mopper, *Anal. Chem.* **1979**, *51*, 1667 – 1674.

Received: August 27, 2002 [Z 480]

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

Raja Banerjee[a, c] and Gautam Basu*[a, b]

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

[a] *Dr. G. Basu, R. Banerjee*
Department of Biophysics, Bose Institute
P-1/12 CIT Scheme VIIM, Calcutta 700054 (India)

[b] *Dr. G. Basu*
Current address:
Graduate School of Information Science
Nara Institute of Science and Technology
Takayama 8916-5, Ikoma, Nara 601 (Japan)
Fax: (+81) 743-72-5391
E-mail: gautam@is.aist-nara.ac.jp

[c] *R. Banerjee*
Permanent address:
Department of Chemistry, St. Xavier's College
Calcutta 700016 (India)