Induction of Cell Differentiation in Transformed Keratinocytes by Synthetic (Glyco)peptides from the Homophilic Recognition Domain of E-Cadherin**

Jörg Habermann, Kerstin Stüber, Tanja Skripko, Tanja Reipen, Raimund Wieser, and Horst Kunz*

Dedicated to Professor Peter Welzel on the occasion of his 65th birthday

The epithelial cell adhesion glycoprotein E-cadherin plays an essential role in the adhesion of epithelial cells.[1] E-cadherin is also involved in other important processes of cellular organization, including differentiation,[2] growth regulation, and morphogenesis.[3,4] The E-cadherin-mediated cell adhesion is decreased in carcinomas, whereas the CD44-mediated adhesion is increased.[5] The reduced expression of E-cadherin is evidently a significant indication of tumorigenesis.[5–8] E-Cadherin is linked to the actin filament network through the catenins and, therefore, integrated into key pathways of adhesion-dependent morphogenetic processes.[7] The interaction of E-cadherin on epithelial cells is both homotypic (i.e. among the same cell type) and homophilic (i.e. among the same molecules).[9,10] The homophilic recognition between molecules of E-cadherin occurs through the formation of dimers and is mediated by a recognition domain that is embedded in the furthermost (E-CAD1) of five extracellular units (Figure 1). According to NOE NMR spectroscopic analysis, a surface formed by three β sheets (βC, βF, βG) seems to be crucial for the recognition.[9] This surface includes the turn-motif Ser 83–Gly 85 and presents the recognition motif His 79-Ala 80-Val 81 located in βF in a well-defined steric arrangement (Figure 1).

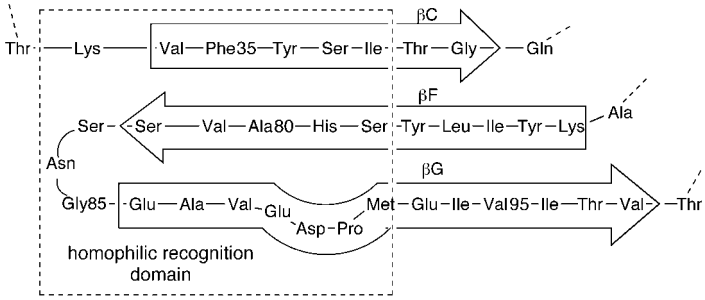


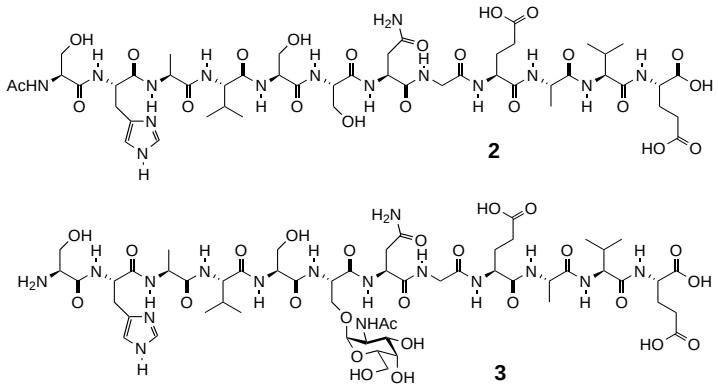
Figure 1. Schematic representation of the homophilic recognition domain of E-cadherin according to ref. [9].

As a result of the importance of E-cadherin to morphogenesis, tumor development, and self-recognizing effects[9,10] it is interesting to investigate the influence of synthetic partial structures of the recognition domain of E-cadherin on the self-recognition phenomenon.

The peptide, glycopeptide, and cycloglycopeptide structures of sequence **1** from the homophilic recognition surface were synthesized by solid-phase methodologies on Tentagel resin[11] as a solid support by using the allylic HYCRON linker.[12] These partial structures begin with Glu 89 at the C-terminus (Figure 1) and contain a portion of β-sheet βG, the turn-motif Ser 83–Gly 85 (partly glycosylated on serine), and a portion of β-sheet βF, including the His 79-Ala 80-Val 81 recognition motif.[1,9]

–Ser 78-His-Ala 80-Val-Ser-Ser-Asn-Gly 85-Glu-Ala-Val-Glu– **1**

The first synthesis of the dodecapeptide Ser 78–Glu 89 (**2**) with tetramethyl-*O*-(1-benzotriazolyl)uronium tetrafluoroborate (TBTU)[13] as coupling reagent suffered from low coupling yields from the eighth coupling step onwards (Val 81), apparently as a result of backfolding effects. Hence *N*,*N*,*N*′,*N*′-bis(tetramethylene)-*O*-pentafluorophenyluronium hexafluorophosphate (PfPyU)[14] was used as the coupling reagent in further solid-phase syntheses.



Whereas acid-labile side-chain protection could be employed in the syntheses of linear peptide **2** and glycosylated peptide **3** (*tert*-butyl group for serine and glutamic acid as well as trityl group for asparagine and histidine),[14] the synthesis of the cyclized peptides needed a more differentiating protecting-group strategy at the γ-carboxy group of the glutamic acid units. The formation of the cyclopeptide should favor a turn-type conformation that is in accordance with the secondary structure of the homophilic-binding domain of E-CAD1.

In view of the sequence it appeared to be favorable to carry out the cyclization not through the C-terminal carboxy group in solution[15,16] or on a solid-phase,[17,18] but rather through the γ-carboxy group of the C-terminal glutamic acid. The absence of lysine at the N-terminus[19] required the use of the terminal amino group of Ser 78 in the cyclization. Glycopeptide **5** was synthesized by starting from fluorenylmethoxycarbonyl (Fmoc)-glutamic acid γ-*tert*-butylester **4** loaded on a solid support.[14] Cyclization on the solid-phase to provide **6**,

[*] Prof. Dr. H. Kunz, Dr. J. Habermann, Dr. T. Skripko, Dipl.-Chem. T. Reipen
Institut für Organische Chemie, Universität Mainz
Duesbergweg 10–14, 55128 Mainz (Germany)
Fax: (+49) 6131-392-4786
E-mail: hokunz@mail.uni-mainz.de
K. Stüber, Priv.-Doz. Dr. R. Wieser[+]
Institut für Toxikologie, Universität Mainz
[+] Present address: Innotides GmbH, its 52499 Baesweiler (Germany)

[**] This work was supported by the Volkswagenstiftung and the Deutsche Forschungsgemeinschaft. JH is grateful for a postdoctoral grant from the Fonds der Chemischen Industrie.

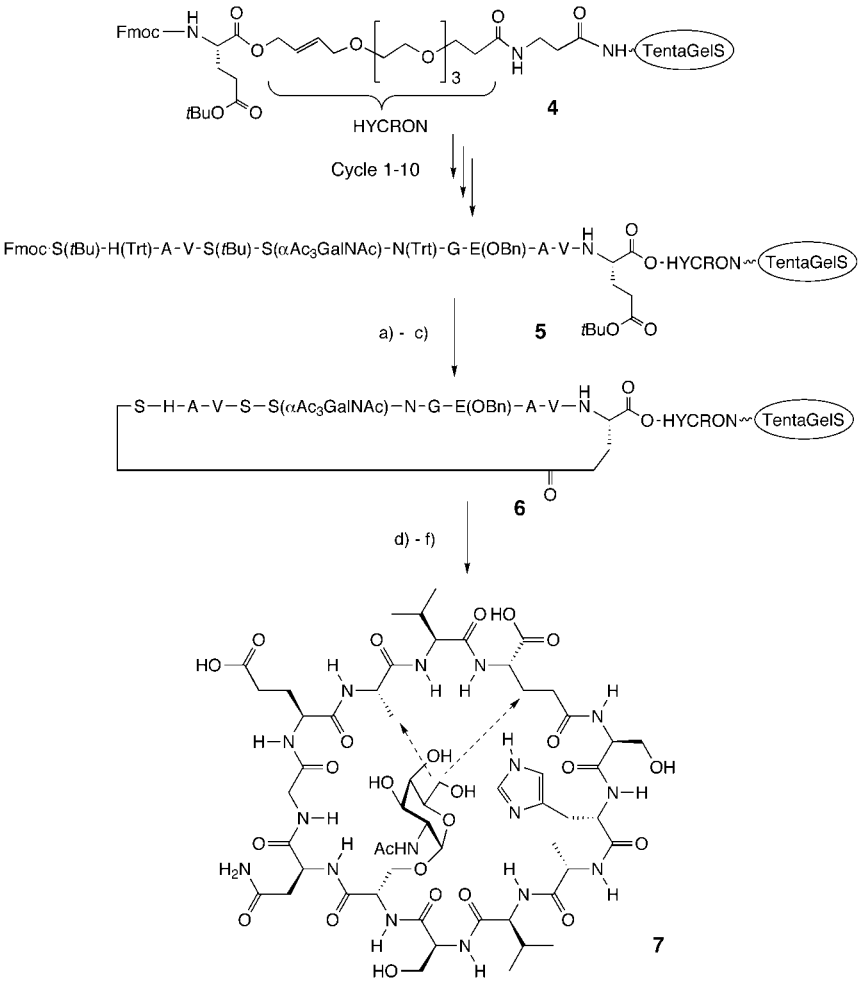
  0044-8249/02/4122-4249 $ 20.00+.50/0

removal from the solid support, and subsequent deprotection yielded cyclopeptide **7** (Scheme 1).

After cleavage of the Fmoc group of **4**, the dipeptide Fmoc-Ala-Val-OH was coupled by the use of PfPyU[14] in the presence of 1-hydroxy-7-aza-benzotriazol (HOAT).[20] The coupling of a dipeptide requires a mild but efficient carboxy activation owing to the risk of epimerization, but in the subsequent Fmoc removal the detachment of the peptide from the resin by intramolecular aminolysis is prevented. The second glutamic acid building block was utilized as γ-benzyl ester (Fmoc-Glu(OBzl)-OH) in a five- to sixfold excess, as was the case with all the carboxy building blocks. As an exception, only 2 equivalents of the α-*O*-galactosamine–serine conjugate were used.

Prior to the on-resin cyclization, the immobilized glycododecapeptide **5** was deprotected by a mixture of trifluoroacetic acid (TFA)/water/triisopropylsilane (95:5:5), and the Fmoc group was cleaved with morpholine/DMF. The allylic linker was not affected under these conditions. Activation of the γ-carboxy group of Glu 89 by PfPyU in the presence of Hünig's base and *sym*-collidine furnished the cyclized structure **6**. The cycloglycopeptide was detached from the resin by using Pd^0-catalyzed allyl transfer to morpholine.[12] Hydrogenation of the Glu-γ-benzyl ester and removal of the *O*-acetyl groups from the carbohydrate moiety by Zemplén transesterification (pH 9) yielded the pure cycloglycopeptide **7**[21] after purification by preparative HPLC in water/acetonitrile (0.1 % TFA). The analytical HPLC showed that **7** exists in several conformations,[16] which can be converted into one another by tempering at 40 °C.

The structure of **7** was confirmed by ROESY NMR spectroscopy experiments in D_2O which showed contacts between 6-H of the *N*-acetylgalactosamine and one methyl group of alanine group (presumably position 3, i.e. Ala 87) and the β-methylene group of a glutamic acid residue (presumably position 1). This orientation was surprising at first, as the nonglycosylated peptide **2** does not adopt any preferred conformation in solution (D_2O) and, in contrast, the glycosylated glycododecapeptide **3** prefers a different turn-type structure in water. In its ROESY NMR spectrum, cross peaks caused by dipolar coupling between the valine γ-methyl protons and 2-H of the imidazole ring on the one hand and 6-H of the GalNAc residue on the other hand are found. In addition, a cross peak between the α-H of Val and 2-H of the imidazole ring and a weaker coupling between the γ-H of Val and 4-H of the imidazole ring is observed (Figure 2).



Scheme 1. Solid-phase synthesis of cycloglycododecapeptide **7** on allylic HYCRON resin (experimental description in ref. [11]). Cycle 1: 1) morpholine/*N*,*N*-dimethylformamide (DMF); 2) Fmoc-Ala-Val-OH, PfPyU, HOAt, *s*-collidine, *N*-methylpyrrolidone (NMP); 3) Ac_2O, pyridine; cycles 2–10: 1) morpholine/DMF; 2) Fmoc-Xaa-OH, PfPyU, HOAt, *s*-collidine, NMP; a) Trifluoroacetic acid (TFA), triisopropylsilane, H_2O; b) morpholine, DMF; c) PfPyU, Et*i*Pr_2N, *s*-collidine, NMP; d) [$Pd(Ph_3P)_4$], morpholine, DMF, dimethyl sulfoxide; e) H_2/Pd in MeOH; f) NaOMe in MeOH, pH 9. In the scheme, the amino acids are represented with their 1-letter codes.

The dipolar coupling between the side chains of the His-Ala-Val motif and between the side chains and the GalNAc residue indicate a turn conformation **A** of structure **3** that is different from the cycloglycopeptide **7**. Further confirmation for the turn conformation **A** comes from the downfield shift of the signals for 6-H of the carbohydrate residue, presumably caused by the proximity of the aromatic imidazole ring.

It is remarkable that the different conformational properties of (glyco)peptides **2**, **3**, and **7** from the homophilic recognition domain of E-cadherin reflect the dissimilar effects of these compounds on transformed HaCat keratinocytes.

HaCat cells[22] are transformed, immortalized basal keratinocytes that express E-cadherin. However, within one week no differentiation to normal keratinocytes can be detected. These HaCat cells, confluently cultivated in a cell-culture medium (CG medium, Vitromex), supplemented with fetal calf serum (FCS; 0.5 %) were treated with the E-CAD-(glyco)peptide structures **2**, **3**, and **7** (25 μmol mL^{-1}; 18–20 μM) under an atmosphere containing 10 % CO_2 at a humidity of 85 %. They were incubated for 24 h. To investigate

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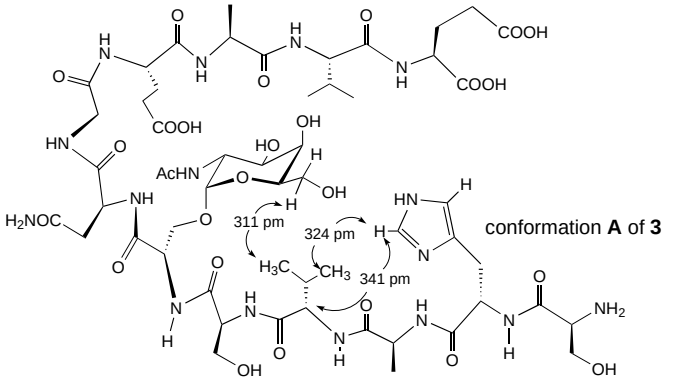

Figure 2. NOE effects (arrows) derived from the ROESY ^{1}H NMR spectrum of E-CAD-glycododecapeptide **3** in D_2O and the deduced conformation **A**.

their cell status, the cells were washed three times with buffer solution (PBS, pH 7.4, 37 °C), fixed, and permeabilized with acetone (5 min). Subsequently, the following antibody solutions containing 5 % BSA (bovine serum albumin) were added to the cells: mouse-anti-involucrin (1:50), mouse-anti-CD44 (1:20), and rabbit-anti-contactinhibin receptor[23] (1:20). To visualize the binding of these antibodies in immunofluorescence assays, the second antibodies attached to the first antibodies were tagged with CY3 dye: anti-mouse-CY3 (1:300) and anti-rabbit-CY3 (1:300) in PBS buffer solution. Typical results of the immunofluorescence assay of fixed and permeabilized HaCat cells are shown in Figure 3.

Involucrin (hINV)[24] is an important marker in the keratinocyte differentiation process. Compared to the control (Figure 3 a,) the amount of involucrin is clearly increased in HaCat cells after treatment with linear glycododecapeptide **3** (Figure 3 b,). In contrast, this is not the case after treatment with linear peptide **2** (Figure 3 c) or cyclopeptide **7**. The interaction of the contactinhibin receptor[23] with the cell-surface glycoprotein contactinhibin is decisively involved in the contact-dependent regulation and inhibition of cell growth. The expression of the contactinhibin receptor is strongly induced by the E-CAD1 glycododecapeptide **3** (Figure 3 e), whereas peptide **2** (Figure 3 d, control is similar) and cyclopeptide **7** (Figure 3 f) do not show any influence on the contactinhibin receptor. In addition, the expression of the CD44 receptor,[25] which plays a role in the adhesion and metastasis of tumors, is distinctly decreased after treatment of the HaCat cells with synthetic glycopeptide **3** (Figure 3 h) in comparison with the control (Figure 3 g). The results of the immunohistochemical experiments are associated with the impressive alteration of the cellular morphology and the three-dimensional architecture of the HaCat cell culture after addition of the biologically active glycopeptide **3**: the HaCat keratinocytes grow into the suprabasal level, form supracellular structures and a differentiated cytoskeleton. Furthermore, the expression of differentiation markers such as involucrin and the contactinhibin receptor is enhanced (Figure 3 b and e). The contactinhibin receptor is transferred from the cytosol into the cell membrane. Cells treated with the inactive peptides **2** and **7** show no alteration compared to the control experiments and do not undergo distinct differentiation within 10 days, even though both structures contain the identical amino acid sequence as the active structure **3**.

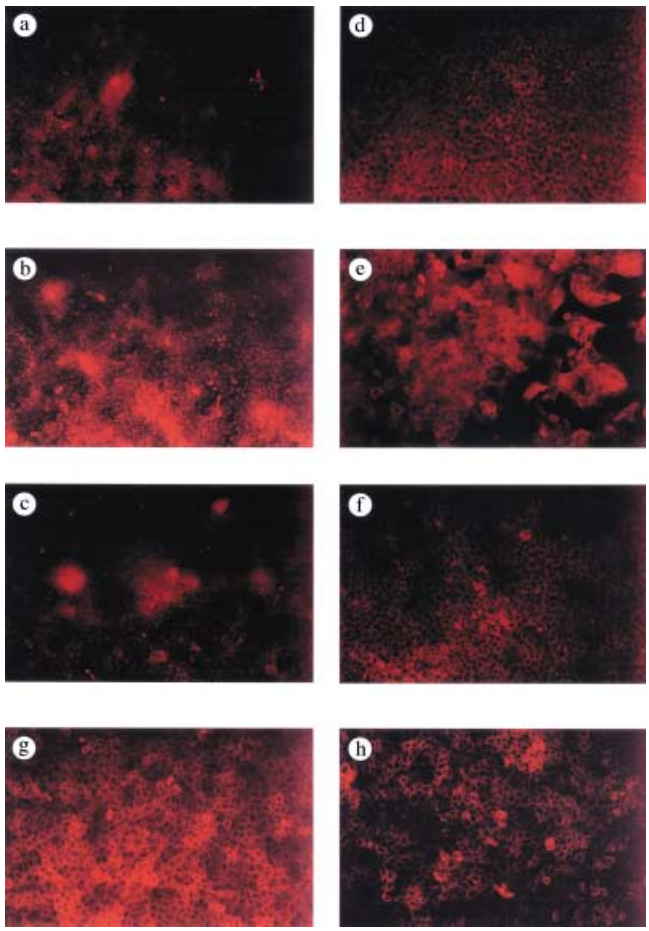

Figure 3. Biological effects of the synthetic (glyco)peptides **2**, **3**, and **7** on transformed basal keratinocytes of the HaCat cell line;[22] immunofluorescence microscopy assays: a) control, 1. mouse-anti-involucrin (Sigma, 1:50 in blocking solution), 2. anti-mouse-CY3 (Jackson, 1:1 in glycerol, 1:300 in PBS; b) E-CAD-glycododecapeptide **3**, 24 h, then immunostaining identical to (a); c) E-CAD-dodecapeptide **2**, 24 h, then immunoassay, for involucrin as in a); d) E-CAD-dodecapeptide **2**, 24 h, rabbit-anti-contactinhibin receptor (ref. [15], 1:2 in blocking solution), 2. anti-rabbit-CY3 (Jackson, 1:1 with glycerol, 1:300 in PBS); e) E-CAD-glycododecapeptide **3**, 24 h, then immunoassay for contactinhibin receptor as in (d); f) E-CAD-cycloglycododecapeptide **7**, 24 h, then immunoassay for contactinhibin receptor as in (d); g) control, 1. mouse-anti-CD44, PE conjugated (Natu-Tec, 1:20 in blocking solution), 2. anti-mouse-CY3 (Jackson, 1:1 with glycerol, 1:300 in PBS); h) E-CAD-glycododecapeptide **3**, then immunoassay identical to (g).

In conclusion, the significant induction of differentiation seems to be connected to the preferred conformation of the E-CAD-glycopeptide in water in which the recognition motif His-Ala-Val is presented in a well-defined steric arrangement. The mechanism of the induction of differentiation in HaCat cells[22] has to be investigated further, in particular whether the synthetic E-CAD-glycopeptides **3** bind to E-cadherin itself as is presumed by the homophilic recognition mechanism.[9,10] The expression and activation of E-cadherin seems to be crucial for tumor suppression.[6,7,25] Hence the conformation-dependent effects of the E-CAD-glycopeptide **3** that induce differentiation, as well as adhesion and integration

  0044-8249/02/4122-4251 $ 20.00+.50/0

abilities in transformed HaCaT keratinocytes within 24 h, is of interest to the development of tumor suppressors.

Received: May 21, 2002 [Z19348]

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[21] **7:** $[\alpha]_D^{23} = -48.2$ ($c = 1.0$, H_2O); MALDI TOF (2,5-dihydroxybenzoic acid, positive-ion mode): m/z: 1371.9 $[M+H]^+$; 1393.8 $[M+Na]^+$; ^{1}H NMR (D_2O, 600 MHz, ^{1}H,^{1}H-ROESY): $\delta = 8.60$ (s, 1H; Im 2-H), 7.29 (s, 1H; Im 4-H), 4.88 (br s, 1H; Gal 2-H), 3.57–3.54 (m, 2H; Gal 6-H), 2.20–1.96 ppm (m, 11H; Sβ, 2 × Eβ, 2 × Vβ, CH_3CO_2).
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pH-Responsive Molecular Nanocarriers Based on Dendritic Core-Shell Architectures**

Michael Krämer, Jean-François Stumbé, Holger Türk, Simon Krause, Ansgar Komp, Lydie Delineau, Svetlana Prokhorova, Holger Kautz, and Rainer Haag*

Physical aggregates of amphiphilic molecules, such as micellar structures, are frequently proposed as drug-delivery systems.[1] These aggregates can be unstable under shear force and other kinds of environmental effects as a result of their weak assembly. They are also not very suitable for the active release of the encapsulated species through the application of an external trigger, such as pH change. For drug delivery in biological systems, in particular, the release of the encapsulated species must occur as a result of a weak external signal, for example, a pH drop in tumor and infected tissues (pH 5–6).[2,3] Furthermore, it has been demonstrated that nanoparticles larger than 5 nm, such as liposomes and macromolecular carriers, can pass through biological membranes by different mechanisms than small molecules, and thereby enhance the specificity of drugs for certain tissues (for example, tumor).[4–6]

In contrast to physical aggregates of amphiphilic molecules, the covalent modification of dendritic macromolecules[7] with an appropriate shell results in stable micelle-type structures, which are suitable for the noncovalent encapsulation of guest molecules.[8] While the encapsulation and the transport of guest molecules into these dendritic architectures have been studied by several research groups,[9–16] relatively little is known about the active release of the encapsulated guest molecules by the pH-triggered cleavage of the shell under physiological conditions. So far, a pH-dependent release from dendritic architectures has only been reported under drastic conditions[17] or by protonation of poly(propyleneimine) dendrimers[18] and their derivatives.[19,20]

Herein we describe a simple and general synthetic concept for the generation of pH-responsive molecular nanocarriers based on the selective and reversible shell functionalization of dendritic polymers, such as polyglycerol (PG, **1**) and polyethyleneimine (PEI, **2**, Scheme 1). Polyglycerol (**1**) and polyethyleneimine (**2**) are randomly branched, but well-defined dendritic structures with a degree of branching of 60

[*] Dr. R. Haag, Dipl.-Chem. M. Krämer, Dr. J.-F. Stumbé, Dipl.-Chem. H. Türk, S. Krause, A. Komp, Dr. L. Delineau, Dr. S. Prokhorova, Dipl.-Chem. H. Kautz
Freiburger Materialforschungszentrum und
Institut für Makromolekulare Chemie
Universität Freiburg
Stefan-Meier-Strasse 21, 79104 Freiburg (Germany)
Fax: (+49) 761-203-4709
E-mail: rainer.haag@fmf.uni-freiburg.de

[**] The authors would like to thank Prof. Rolf Mülhaupt for his support, and Katrin Armbruster and Bernhard Siegel for the preparation of some intermediates. BASF AG is kindly acknowledged for the donation of chemicals. R.H. is indebted to the Deutsche Forschungsgemeinschaft Fonds der chemischen Industrie and the Dr. Otto-Röhm Gedächtnisstiftung for financial support.

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  0044-8249/02/4122-4252 $ 20.00+.50/0