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Solid-Phase Synthesis and Biological Evaluation of a Pepticinnamin E Library**

Michael Thutewohl, Lars Kissau, Boriana Popkirova, Ionna-Maria Karaguni, Thorsten Nowak, Michael Bate, Jürgen Kuhlmann, Oliver Müller, and Herbert Waldmann*

Mutations in the genes coding for Ras proteins are found in approximately 30% of all human tumors.[1,2] The proper function of Ras is critically dependent on posttranslational lipidation. Oncogenic Ras only serves as a molecular switch for the transduction of growth signals if the Ras is S-farnesylated at the C-terminus and if located at the plasma membrane. Thus, inhibitors of the enzyme protein farnesyltransferase (PFT) are of particular interest as new antitumor therapeutic agents[1,2] However, some of the most important and fundamental aspects of this application of the signal transduction therapy[3] remain unclear. The crucial substrate of PFT, the farnesylation of which is suppressed by these compounds, is still unknown.[2] In this context, inhibitors which induce apoptosis (that is, programmed cell death), and which are bisubstrate inhibitors of the PFT, are of particular interest.[2,4]

To investigate these biological questions in a focussed but flexible manner, a class of potential PFT inhibitors are required, the structure of which can be varied rapidly and efficiently by combinatorial solid-phase synthesis. This approach should furnish inhibitors which are competitive to the protein substrate, to the farnesylpyrophosphate (FPP), or even to both substrates, as well as substances which induce apoptosis in Ras-transformed cells but not in the untransformed wild-type cells. Pepticinnamin E[5] (**1**; Scheme 1) is a naturally occurring bisubstrate inhibitor of PFT ($IC_{50} = 42\,\mu M$). Based on the total synthesis of this modularly built natural product,[6] we have developed a solid-phase synthesis

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[**] This work was supported by the Deutsche Forschungsgemeinschaft, AstraZeneca (Case Award for M.T.), and the Fonds der chemischen Industrie (Kekulé fellowship for M.T. and L.K.). We are grateful to Helmuth Kipp for the access to the Fluoroscan-Plate-Reader and to Christine Nowak for the isolation of rat PFT.

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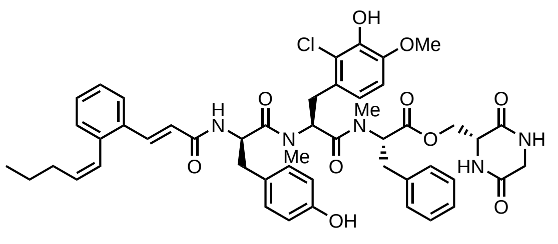


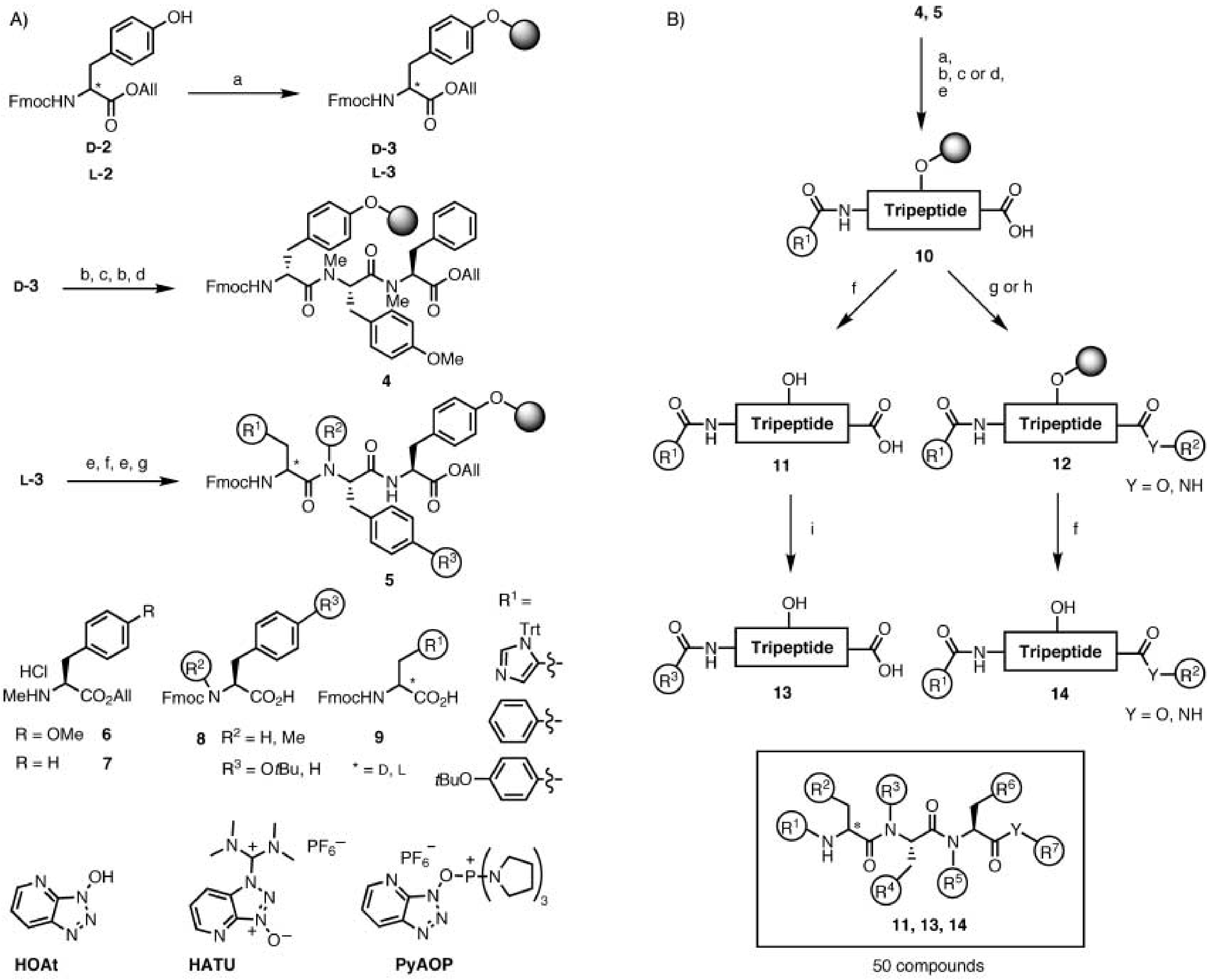
Pepticinnamin E (**1**)
IC_{50} = 42 µM

Scheme 1. The natural PFT inhibitor pepticinnamin E.

of a library of pepticinnamin E analogues, as shown in Schemes 2a and 2b.

To vary all three central amino acid fragments, we developed two independent synthesis sequences starting from different positions of the natural product. The orthogonally protected tyrosines D-**2** und L-**2** were attached to Wang resin by using a Mitsunobu reaction[7] (loading levels 0.5–0.53 mmol g^{-1}, yields between 54–59%; the loading was determined by the Fmoc method[8]). To build up the tripeptide unit **4** by C-terminal chain elongation, starting from D-**3**, the allyl ester groups were cleaved by Pd^0 catalysis, using phenylsilane or *N*-methylaniline as allyl scavengers. The N-methylated amino acid building blocks **6** and **7** were attached by application of the highly active reagents HATU and PyAOP (Scheme 2a).[9]

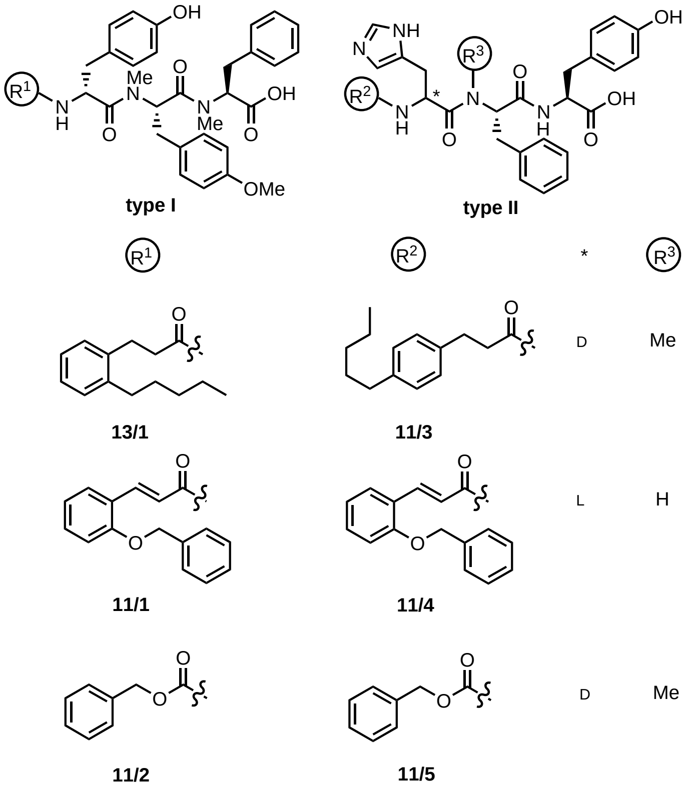
The alternative N-terminal elongation starting from building block L-**3** by sequential basic Fmoc removal and coupling of the fragments **8** and **9** furnished the tripeptide **5** (Scheme 2a). After removing the Fmoc group from the tripeptides **4** and **5**, various cinnamic acid and hydrocinnamic acid derivatives R^1COOH, as well as benzylchloroformates



Scheme 2. A) a) DEAD, PPh_3, THF, 0 °C, 1 h, RT, 12 h, loading: 0.50–0.53 mmol g^{-1}, 54–59%; b) $Pd(PPh_3)_4$, $PhSiH_3$ or NMA, 24 h; c) **6**, HATU, HOAt, *i*$PrNEt_2$, NMP; 2 × 24 h; d) **7**, PyAOP, HOAt, *i*$PrNEt_2$, NMP; 2 × 24 h; e) piperidine/DMF 1:4, 2 × 5 min; f) **8**, HATU, *i*$PrNEt_2$, NMP, 2.5 h; g) **9**, HATU, DIPEA, NMP or DIC, HOAt, DMF, 14 h. B) a) Piperidine/DMF 1:4, 2 × 5 min; b) R^1CO_2H, HATU, HOAt, *i*$PrNEt_2$, NMP, 12 h ($R^1 = ArCH_2CH_2$-); c) R^1CO_2H, PyAOP, DMAP, *i*$PrNEt_2$, CH_2Cl_2/DMF 1:2, 12 h (R^1 = ArCH = CH-); d) R^1COCl, HOAt, DMAP, *i*$PrNEt_2$, CH_2Cl_2/DMF 2:1, 7 h; e) $Pd(PPh_3)_4$, morpholine, THF, 4 h; f) TFA/H_2O 95:5, 5 h; g) R^2NH_2, PyAOP, *i*$PrNEt_2$, NMP, 2 × 8 h (Y = NH); h) Cs_2CO_3, KI, RX, DMF, 24 h, RT (X = Br) or 24–48 h, 50 °C (X = Cl); i) 10% Pd/C, H_2, dioxane or MeOH, 8 h.

R^1COCl, were attached. For the attachment of the cinnamic acids, only a combination of PyAOP and DMAP gave satisfactory results (Scheme 2b). Deprotection of the carboxylic acid group by Pd^0-catalyzed allyl transfer to morpholine und acidic cleavage of the N-acylated tripeptides **10** from the polymeric support furnished the compounds **11**. Hydrogenation of the double bonds resulted in further analogues **13** with saturated residues R^3. Starting from the polymer-bound compounds **10**, esters and amides **12** were synthesized by efficient alkylation of the carboxylic acid functionality and PyAOP-mediated amine-coupling, respectively. Acidic release from the resin furnished the compounds **14**. Following this strategy, we synthesized a library of 50 pepticinnamin E analogues by variation of up to eight structural parameters, in overall yields of between 3–63 %, corresponding to an average yield of between 65–91 % per step (in general, 80 % yield). In most cases purities were higher than 90 %.[10]

The members of the synthesized library were subsequently evaluated in two assays for their potential inhibitory activity towards PFT. We applied a fluorescence-based assay using rat PFT and a dansyl-labelled Ras peptide[11] which is adapted to the 96 well-plate format.[12] Moreover, in a second assay, tritium-labelled farnesylpyrophosphate, K-Ras protein as substrate, and human placental PFT were applied.[13] The library contained two groups of inhibitors (altogether 28 compounds) with remarkable activity. The inhibitors with the highest activities are shown in Scheme 3. The central tripeptide fragment of the natural product is conserved in the inhibitors of type I; the substituent R^1 was varied. In contrast, inhibitors of type II contain structurally varied tripeptides; in particular, they contain a histidine residue. Furthermore, the structure of the lipophilic substituent R^1 is varied. In both types, the C-terminal diketopiperazine is missing. While compounds **11/1**–**11/5** inhibit rat PFT in the low, mainly single-digit micromolar range, **13/1** is only weakly active against this enzyme (Table 1). However, the human PFT is inhibited efficiently by **13/1**, as well as by **11/1**, **11/2**, **11/4**, and **11/5**.



Scheme 3. Selected members of the pepticinnamin E library.

Table 1. Inhibition of PFT by selected compounds.

Entry	Compound	PFT (IC_{50}) [μM][a]	PFT (IC_{50}) [μM][b]	PFT[a] w.r.t. CAAX[c]	PFT[a] w.r.t. FPP[d]
1	**13/1**	> 50	6.4	n.m.	n.m.
2	**11/1**	9.3	57 %[e]	n. comp.	comp.
3	**11/2**	5.3	6.7	n. comp.	comp.
4	**11/3**	10.5	n.m.	comp.	n. comp.
5	**11/4**	6.4	19.2	comp.	comp.
6	**11/5**	8.0	6.4	comp.	n. comp.

[a] Fluorescence test against rat PFT.[12] [b] Radioactive test against human PFT.[13] [c] Variation of the dansyl peptide concentration in the presence of the inhibitor with constant FPP concentration. [d] Variation of the FPP concentration in presence of the inhibitor with constant dansyl peptide concentration. [e] 57 % inhibition at 30 μM concentration of inhibitor. comp. = competitive; n. comp. = not competitive; n.m. = not investigated; w.r.t. = with respect to.

Compounds **11/1**–**11/5** were investigated with regard to their mechanism of inhibition (Table 1, see also the Supporting Information). As a result, **11/1** and **11/2** show competitive inhibition with respect to FPP, while **11/3** and **11/5** compete only with the peptide substrate. Moreover, as hoped, we were able to identify compound **11/4** as a bisubstrate inhibitor. These results of the enzymatic assays demonstrate that different types of inhibitors can be developed efficiently and flexibly on the basis of the natural product pepticinnamin E. The diverse modes of inhibition were examined by molecular modelling experiments.[14] Figure 1a illustrates the calculated binding mode for the bisubstrate inhibitor **11/4**. It is obvious that both binding pockets are occupied (the substituted cinnamic acid substituent points backwards into the FPP binding pocket) and that the histidine residue is coordinated to the zinc atom in the active center of PFT. Figure 1b shows that the FPP-competitive inhibitor **11/2** blocks the appropriate binding pocket, but not the binding site of the peptide. Figure 1c shows that the peptide-competitive inhibitor **11/3** blocks the peptide binding site, but not the FPP binding site.

Finally we examined, with the help of an assay developed by us,[15] whether or not the pepticinnamin E library contains compounds which induce apoptosis in Ras-transformed cells but not in the corresponding wild-type cells. In this assay, MDCK-f3 tumor cells from dog kidney were used, which were transfected with oncogenic H-Ras.[16] A cell-division-inhibiting, morphology-changing, or apoptosis-inducing effect of a substance on MDCK-f3 cells indicates an influence on the Ras signal-transduction cascade.

To our delight, inhibitor **13/1** induced apoptosis in the tumor cell line (Figure 2). After 6 h at 100 μM inhibitor concentration, the final stage of apoptosis was observed, after 6 h at 50 μM concentration, an early stage of apoptosis had occurred. In a comparison experiment under identical con-

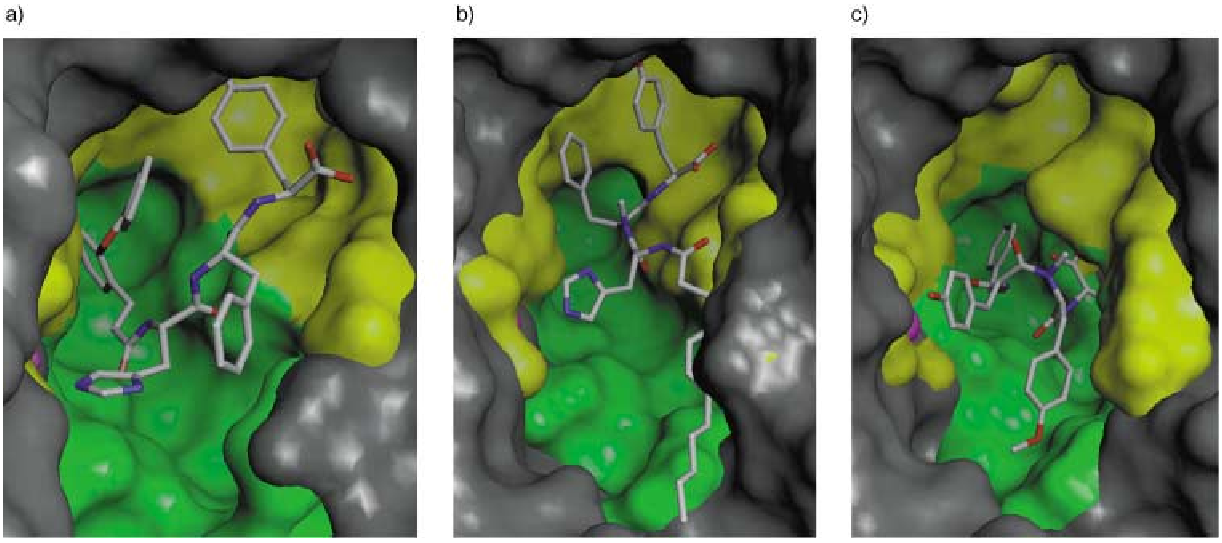


Figure 1. Results of the molecular modeling experiments. The binding site of FPP is shown in green, the binding site of the peptide substrate in yellow. The Zn^{2+} ion in the active center is shown in purple. a) Compound **11/4** in the active site of PFT; b) compound **11/3** in the active site of PFT; c) compound **11/2** in the active site of PFT.

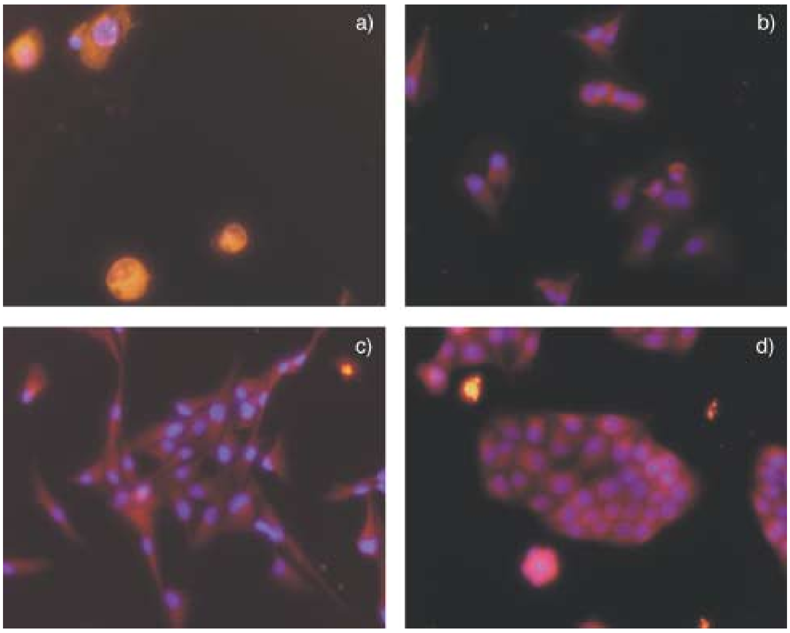


Figure 2. Effect of inhibitor **13/1** on MDCK-f3 cells: a) 100 μM, 6 h (apoptosis); b) 50 μM MDCK-f3 (apoptosis early stage); c) negative control, MDCK-f3 without inhibitor (no apoptosis); d) effect of **13/1** (100 μM, 6 h) on wild-type MDCK cells (no apoptosis).

ditions the wild-type MDCK cells showed no apoptotic effect.[17] Thus compound **13/1** is not generally cytotoxic and its action is directly involved in influencing the Ras signal pathway.

In summary, we have developed a flexible and efficient solid-phase synthesis of a pepticinnamin E library furnishing different types of PFT inhibitors and apoptosis-inducing compounds. These effects could be rationalized well by molecular modelling studies. This class of compounds should open up new opportunities in the chemical biology of Ras proteins.[18]

Received: April 24, 2002 [Z19161]

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[9] Under standard conditions, considerable racemization occurred during the attachment of building block **7**. This effect could be suppressed by coupling with PyAOP.

[10] For a detailed presentation of the synthesis results see the Supporting Information (Table S1 and Figure S1).

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[12] Modification of the procedure in [11]: A Fluoroscan-FL Fluorometer of Ascent Labsystems was used; filter: $\lambda_{ex} = 355$ nm, $\lambda_{em} = 460$ nm. 96 well-plate: Per well 20 μL of a solution of rat PFT (2.6 μM in the buffer medium indicated below) was added by the dispenser function of the apparatus to 180 μL of a preincubated solution (at 30 °C) of FPP (10 μM), dansyl-GCVLS-peptide (10 μM), varied concentrations of the inhibitor in 20 μL of methanol and 160 μL of buffer solution (50 mM Tris/HCl pH 7.5, 5 mM DTE, 10 μM $ZnCl_2$, 5 mM $MgCl_2$, 0.2 % (w,v) *n*-octyl-*β*-D-glucopyranoside) and then the fluorescence development was recorded for 5 min at 30 °C.

[13] Modification of a procedure according to D. L. Pompliano, E. Rands, M. D. Schaber, S. D. Mosser, N. J. Anthony, J. B. Gibbs, *Biochemistry* **1992**, *31*, 3800 – 3807. To a solution of 100 μL of K-Ras (0.27 mg L^{-1}),

human PFT in the buffer medium (8.6 mM $MgCl_2$, 17.1 μM $ZnCl_2$, 1.32 mg mL^{-1} DTT, 86 mM Tris/HCl pH 8.0) were pipetted 10 μL of a [^{3}H]FPP-solution (3.3 mM, 15–30 Ci mmol^{-1}, New England Nuclear) and incubated for 30 min at 37 °C. Then the reaction was stopped by addition of 100 μL of a solution of concentrated HCl in ethanol (15 %), the precipitated Ras-protein was filtered through filter pads (type B) in a Tomtec-Harvester and the transferred radioactivity was measured by using a Wallac 1024 Betaplate scintillation counter.

[14] For details of the molecular modelling see the Supporting Information. For molecular modelling studies of the PFT see a) J. Sakowski, M. Bohm, I. Sattler, H. M. Dahse, M. Schlitzer, *J. Med. Chem.* **2001**, *44*, 2886–2899; b) M. Schlitzer, M. Bohm, I. Sattler, *Bioorg. Med. Chem.* **2002**, *10*, 615–620; c) A. Perdetti, L. Villa, G. Vistoli, *J. Med. Chem.* **2002**, *45*, 1460–1465.

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[17] At 100 μM and higher inhibitor concentrations up to 1 mM no visible effect was observed for MDCK cells.

[18] Abbreviations: DEAD = Diethylazodicarboxylate, HATU = 1-(bis-(dimethylamino)methylene)-1*H*-1,2,3-triazolo-[4,5b]pyridinium hexafluorophosphate 3-oxide, PyAOP = 7-aza-1-hydroxybenzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate, NMP = 1-methyl-2-pyrrolidone, DIC = *N,N'*-diisopropylcarbodiimide, HOAt = 7-aza-1-hydroxybenzotriazole, DTT = 1,4-dithio-DL-erythritol, DTE = 1,4-dithioerythritol, DIPEA = diisopropylethylamine, NMA = *N*-methylaniline, Fmoc = 9-fluorenylmethoxycarbonyl, DMAP = 4-dimethylaminopyridine, TFA = trifluoroacetic acid.

A Molecular Beacon for Quantitative Monitoring of the DNA Polymerase Reaction in Real-Time**

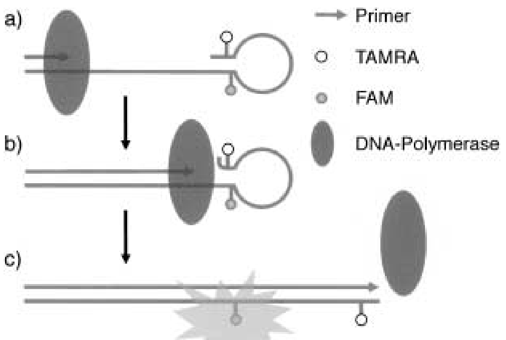
Daniel Summerer and Andreas Marx*

The design of new assay formats that allow fast parallel screening of enzyme function plays a pivotal role in the discovery of new products and reagents ranging from industrial processes to diagnostics.[1] Furthermore, drug discovery and protein engineering are increasingly performed by combinatorial approaches, in which progress is tightly linked to the development of suitable screening systems capable of measuring desired enzyme properties in a high-throughput parallel fashion.[1]

DNA polymerases are involved in all DNA syntheses occurring in nature. This decisive role in biological key processes has made these enzymes to attractive drug targets.[2] Furthermore, DNA polymerases are the workhorses in numerous important molecular-biological core technologies such as the ubiquitous polymerase chain reaction (PCR), cDNA cloning, genome sequencing, and detection of nucleotide variations within genes.[3,4] Nevertheless, emerging drug resistances and the need to tailor DNA polymerases for new technologies, for example, faithful genome-wide nucleotide variation detection, fuel the search for further developments.[4,5] Several approaches to screen DNA polymerases have been reported, all of which have considerable drawbacks,[6] these include the use of laborious gel-based methods and/or rely on the employment of radioactive isotopes for product detection.[6a,b] Several fluorescent-based assays are described, which are nevertheless restricted to specific DNA polymerases (such as thermostable enzymes used in PCR)[7] or endpoint measurements requiring several cost-intensive auxiliary reagents and proteins for signal generation.[6c–f]

Herein we report a new assay format that translates the proceeding DNA synthesis into a fluorescent signal in real-time. The method uses commercially available reagents and allows quantitative monitoring of enzymatic DNA synthesis in a multiwell plate-reader format even in crude lysates of DNA-polymerase overexpressing cells. Thus, this assay supersedes known methods to screen these important enzymes.

We designed a DNA reporter molecule in such a way that the template strand forms a stable hairpin structure (Scheme 1 a). The stem is equipped with a dye (carboxyfluorescein, FAM) the fluorescence of which is quenched through resonance energy transfer (RET) by a quencher (*N,N'*-tetramethylrhodamine, TAMRA) brought in close proximity by the hairpin formation.[8,9] Both, fluorophor and quencher are attached to the C5-position of 2'-deoxyuridines, which can be bypassed by DNA polymerases.[10] An extended 3'-end of our template hairpin construct allows binding of a primer strand to its complementary site (Scheme 1 b). A DNA polymerase triggers opening of the hairpin stem as primer extension proceeds, which results in spatial separation of fluorescent and quencher dyes accompanied by restoration of FAM-emission (Scheme 1).



Scheme 1. Assay design for real-time observation of the DNA polymerase reaction. a) The template probe labeled with fluorophor (carboxyfluorescein, FAM) and acceptor (*N,N'*-tetramethyl-rhodamine, TAMRA) has a hairpin extension in closed conformation before start of reaction. b) While extension proceeds, the DNA polymerase opens the stem and prevents reannealing by DNA duplex formation. c) The increase in the distance between the two labels is reported by restoration of FAM emission.

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[**] Financial support of this project by the DFG is gratefully acknowledged. We thank Dr. S. Brakmann for providing the plasmid pQKlenowExo$^-$ and Professor Dr. M. Famulok for his continuing support.

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