An Antibody that Reconstitutes the "Base-On" Form of B_{12} Coenzymes**

Renate B. Hannak, Robert Konrat, Wolfgang Schüler, Bernhard Kräutler,* Maria-Teresa M. Auditor, and Donald Hilvert*

The cobalt-coordinating 5,6-dimethylbenzimidazole (DMB) nucleotide is a structural feature of coenzyme B_{12} and of methylcobalamin[1,2] that controls the reactivity of these organometallic cofactors.[3,4] The DMB nucleotide and the functionalized corrin ring are both important for tight and selective binding to B_{12} apoenzymes. Several coenzyme B_{12} dependent enzymes were recently shown to bind the B_{12} cofactor in an unanticipated "base-off/His-on" form,[5–7] in which the DMB nucleotide is displaced from the cobalt center by a histidine residue from the protein. In contrast, diol dehydratase binds coenzyme B_{12} "base-on", as described in a crystal structure at the end of the 1990s.[8] Here we report on a monoclonal antibody raised against coenzyme B_{12}, which binds the "base-on" form of natural coenzyme B_{12} analogues that prefer to be "base-off" in solution. This B_{12} antibody thus causes an unprecedented "reverse" coordinative "base-off" to "base-on" reconstitution, which also results in a significant change in the reactivity of the bound B_{12} coenzyme.

Over the last 15 years, antibodies have proved useful as catalysts for a set of remarkable chemical reactions and as receptors for probing ligand-binding mechanisms.[9–14] Versatile cofactors such as coenzyme B_{12} have considerable potential to extend the chemistry of these systems. To investigate the ability of antibodies to recognize and modulate the chemical reactivity of corrinoid coenzymes, we have raised monoclonal antibodies against coenzyme B_{12} (**1**). Although other antibodies that recognize B_{12} derivatives are known,[15] their binding properties were never scrutinized spectroscopically.

For immunization, coenzyme B_{12} (**1**) was coupled to the carrier protein thyroglobulin (TG) with a succinic acid linker[16] (48 haptens/TG). An immune response was elicited against the TG–hapten conjugate, and a panel of monoclonal antibodies with good antigen recognition was prepared and purified by conventional means.[17] After initially screening 20 antigen binders by competition ELISA (enzyme-linked immunosorbent assay),[18] antibody 2C2 was selected for detailed characterization because it recognized both the DMB nucleotide and the corrin ring.

Antibody 2C2 binds **1** with an estimated dissociation constant (K_d) of $9.8 \pm 2.6\,\mu$M. As summarized in Table 1, vitamin B_{12} (**2**; Scheme 1) and several B_{12} derivatives compete effectively with the antigen. Dicyanocobinamide (**3**), an analogue of **1** and **2** lacking the nucleotide function, is the weakest corrinoid ligand tested, and neither benzimidazole nor 2-amino-isopropylribazole phosphate[19] alone compete with the antigen. These results suggest that the complete cobalt-coordinating nucleotide loop is required for formation of the B_{12}–antibody complex. To test this hypothesis, pseudocoenzyme B_{12} (**4**)[20,21] and Co_β-5′-deoxyadenosyl factor A (**5**)[21,22] were also investigated as competitive inhibitors. These natural coenzyme B_{12} analogues differ from **1** by the replacement of the DMB base by adenine and 2-methyladenine, respectively (Scheme 1). In aqueous solution, **4** and **5** exist mainly in the base-off form, as deduced from UV/Vis, CD, and ^{1}H NMR spectroscopy.[20,21] As expected, these base-off adenosylcobamides bind 3 to 7 times weaker than **1**. Clearly, the constitution of the nucleotide base and its intramolecular interaction with the cobalt center play important roles in the recognition of corrinoids by antibody 2C2.

Table 1. Apparent ligand dissociation constants with antibody 2C2.[a]

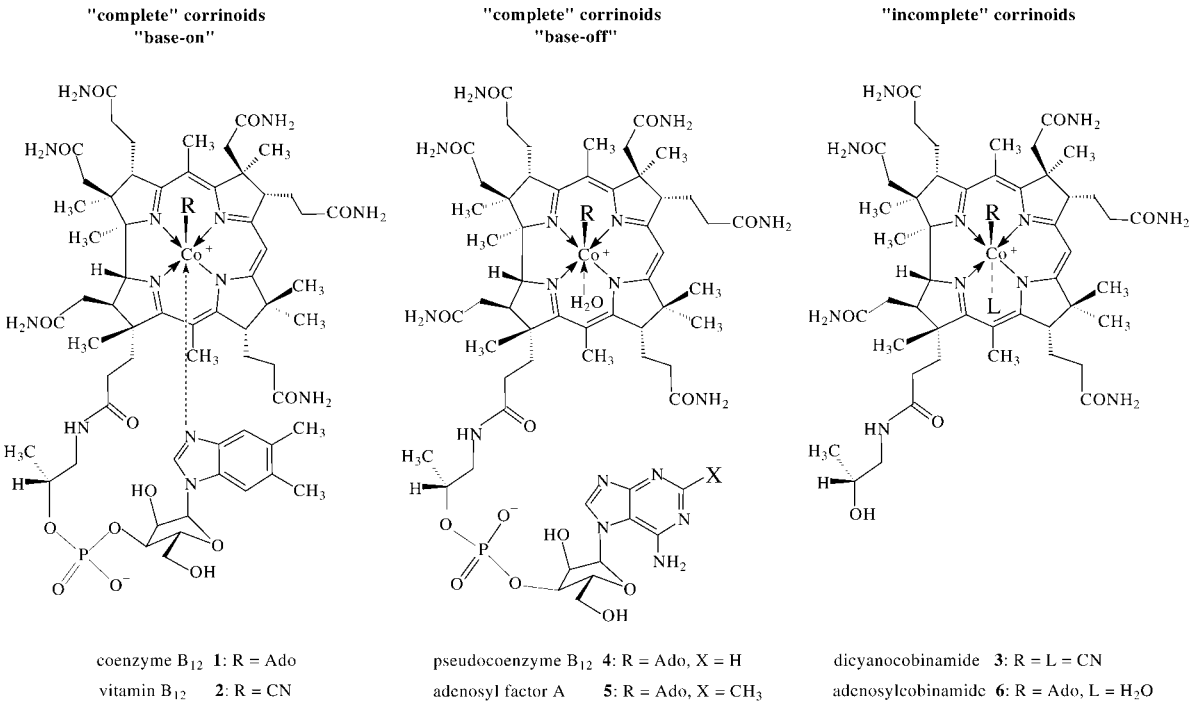
Ligand	K_d [μM]
coenzyme B_{12} (**1**)	10
vitamin B_{12} (**2**)	65
dicyanocobinamide (**3**)	740
pseudocoenzyme B_{12} (**4**)	68
adenosyl factor A (**5**)	29

[a] Determined by competition ELISA.[18] Coenzyme B_{12} conjugated to bovine serum albumin was immobilized to a microtiter plate and bound antibody was detected using glucose oxidase conjugated goat antimouse IgG antibody in the presence and absence of various ligands. Absorbance at 405 nm was determined after developing the plates with 2,2′-azino-di(3-ethylbenzothiazoline-6-sulfonate) (ABTS).

The recognition properties of 2C2 were further examined spectroscopically. Vitamin B_{12} derivatives show diagnostic absorbances at wavelengths longer than 300 nm,[21,22] which are easily monitored in the presence of the colorless antibody. Addition of 2C2 to coenzyme B_{12} (**1**) caused a small shift in the visible part of the UV/Vis spectrum (from 524 to 536 nm) with an 8% decrease of the extinction coefficient. Similar changes have been observed for other B_{12}–protein complexes[23] and indicate that binding of **1** to the antibody does not significantly alter the coordination pattern of the Co^{III}–corrin. In contrast, dramatic changes were detected upon binding of **4** and **5** to the antibody. In these cases, addition of 2C2 caused a marked decrease in the absorbance at 463 nm and an increase at 529 nm (Figure 1a). The spectra of **4** and **5** bound to 2C2 match that of (base-on) coenzyme B_{12} (**1**)

[*] Prof. Dr. B. Kräutler, Dr. R. B. Hannak, Prof. Dr. R. Konrat, Dr. W. Schüler
Institute of Organic Chemistry
University of Innsbruck
Innrain 52a, 6020 Innsbruck (Austria)
Fax: (+43) 512-507-2892
E-mail: bernhard.kraeutler@uibk.ac.at
Prof. Dr. D. Hilvert, Dr. R. B. Hannak
Laboratory of Organic Chemistry
ETH Hönggerberg, 8093 Zürich (Switzerland)
Fax: (+41) 1-632-1486
E-mail: hilvert@org.chem.ethz.ch
M.-T. M. Auditor
Departments of Chemistry and Molecular Biology
The Scripps Research Institute
La Jolla, CA 92037 (USA)

[**] We would like to thank Dr. Wolfgang Fieber for providing graphics, Prof. Paul Renz (University of Stuttgart) for the generous gift of Factor A, and Dr. Frederik Deroose for preparing the protein–hapten conjugates. We are also grateful to the Austrian National Science Foundation (FWF, project no. 13595 to B.K. and a Schrödinger Fellowship to R.H.), the European Commission, the ETH Zürich, and Novartis Pharma for support of this work.



Scheme 1. Structural formulae of vitamin B_{12} derivatives. Left: "Complete corrrinoids" in their base-on form with a DMB base and varying ligand R. Center: The dominant base-off form of the complete corrinoids with an adeninyl base. Right: Cobinamides as "incomplete corrinoids" (Ado = 5′-deoxy-5′-adenosyl).

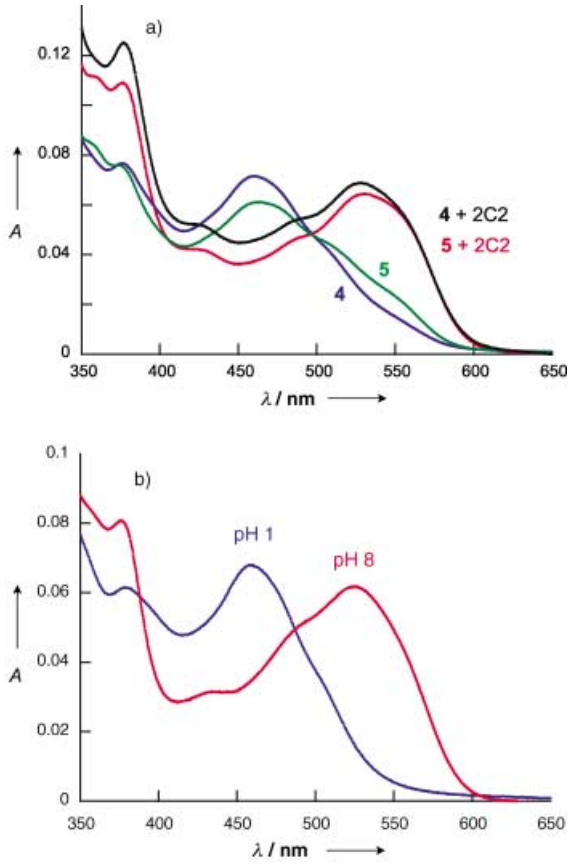


Figure 1. a) UV/Vis spectra of pseudocoenzyme B_{12} (**4**) and of 5′-deoxyadenosyl factor A (**5**) in aqueous buffer (10 mM phosphate, 160 mM NaCl, pH 7.4; blue and green curves, respectively) and in the presence of approximately 20 μM antibody 2C2 (black and red curves, respectively). b) UV/Vis spectra of aqueous solutions of coenzyme B_{12} (**1**) at pH 8 (base-on form of **1**; red curve) and at pH 1 (base-protonated base-off form of **1**; blue curve).

remarkably well (Figure 1b). In contrast, the presence of 2C2 had no effect on the spectrum of 5′-deoxyadenosylcobinamide (**6**), nor did bovine serum albumin alter the spectra of **4** and **5**.

Binding of coenzyme B_{12} analogues **4** and **5** to 2C2 is thus accompanied by coordination of an N-ligand to cobalt. However, the UV/Vis spectra cannot differentiate clearly between coordination of an internal nucleotide base or an external histidine molecule. Indeed, the insensitivity of these methods to the identity of the axial N-ligand in adenosylcobamides impeded the discovery of the now well-known base-off/His-on form of protein-bound **1** in enzymes such as methylmalonyl CoA mutase[6] and glutamate mutase.[7,24]

On the assumption that 2C2 recognizes the more stable "base-on" form of coenzyme B_{12} (**1**), it seemed possible that the antibody might force the adenine group of pseudocoenzyme B_{12} (**4**) and 2-methyladenine in adenosyl factor A (**5**) to coordinate to the cobalt center. The base-on form of these coenzyme B_{12} analogues, **4b** and **5b**, has not been previously observed, but is known for the corresponding cyano-Co^{III}-corrins pseudovitamin B_{12} and factor A.[25] Utilization of binding energy to counterbalance an equilibrium that favors the base-off form of **4** and **5** in solution would help explain their lower affinities for 2C2, compared to **1**.

Transfer NOE experiments with **5** and 2C2 were performed to establish how the corrinoid binds at the antibody active site. Cross-relaxation measurements on protein–ligand complexes deliver information concerning spin-transfer between two protons of a bound ligand and the free state by fast chemical exchange.[26,27] The NOESY spectrum of **5** in the presence of 2C2 shows an overall increase in NOE intensities and specific increases in the cross-peaks between the heteroaromatic proton H(8N) of the 2-methyladenine base and the corrin e and f side chains of **5** (Figure 2). The latter protons are not

  0044-8249/02/4119-3614 $ 20.00+.50/0

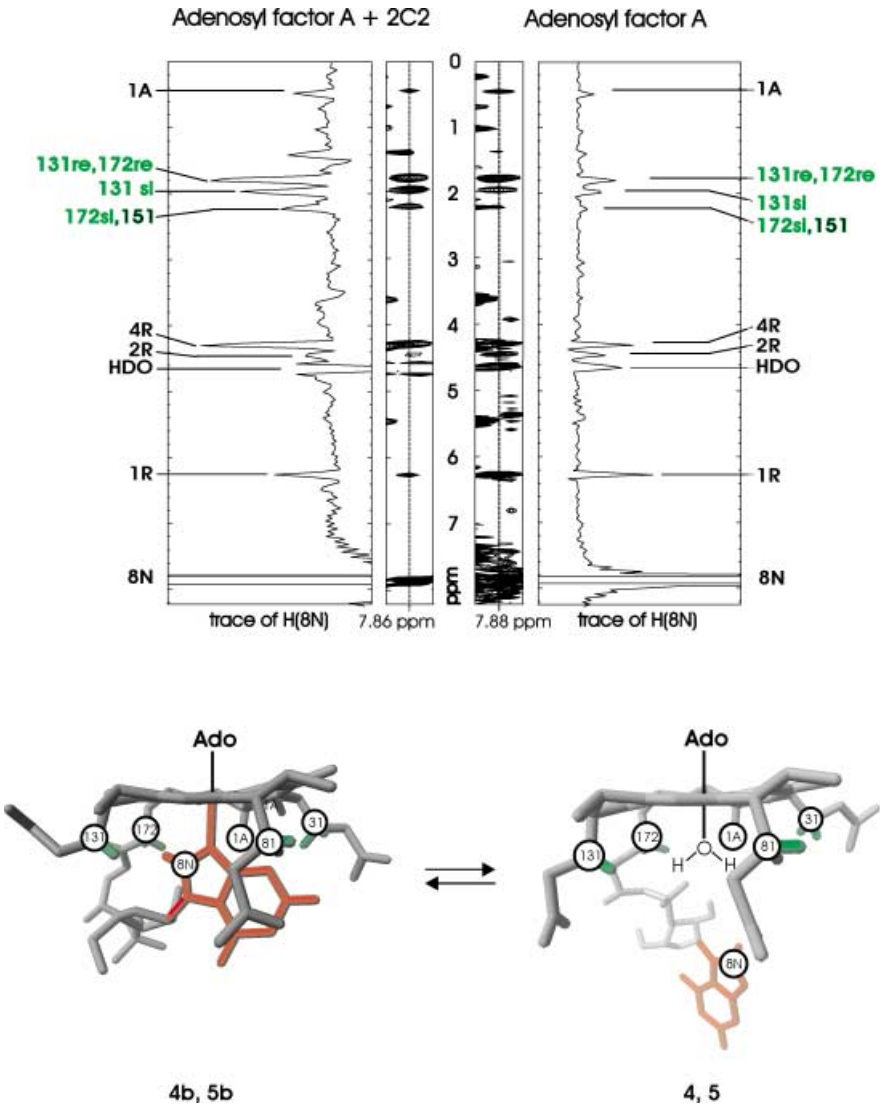


Figure 2. Top: NOE correlations of the signal arising from the H(8N) (at $\delta = 7.86$ and 7.88 ppm) from 500 MHz ^{1}H NMR spectra of 5′-deoxyadenosyl factor A (**5**) in the presence (left, NOESY spectrum) and in the absence (right, ROESY spectrum) of antibody 2C2. The spectra were recorded in 50 mM potassium phosphate buffer, 100 mM NaCl at pH 7.5 and 26 °C; [**5**] = 1.5 mM and [2C2] = 50 μM. The NOESY spectrum of **5** without 2C2 shows only very weak signals because of the similar magnitude of the correlation times of the B_{12} derivative and the critical correlation time at 500 MHz. The H(8N) of the cobalt-coordinated DMB base in **1** is < 3.5 Å (through space) from the protons at 1A, 131(H_{re} and H_{si}), 172(H_{si}), of the corrin ring, and at 4R, of the ribose segment. (Note: the signal at $\delta = 1.5$ ppm is an artefact). Bottom: The base-off/base-on constitutional switch of complete cobamides by (de)-coordination of their nucleotide base at the corrin-bound cobalt center.

proximal to one another in the base-off form of **5** in free solution, but are apparently juxtaposed in antibody-bound **5**, as expected if the corrinoid adopts a base-on mode (Figure 2).

All of the experimental data thus support the conclusion that the B_{12} antibody 2C2 preferentially binds adenosylcorrinoids in a base-on form, as programmed by the hapten. Binding pseudocoenzyme B_{12} (**4**) and its homologue **5** in this way requires formation of a cobalt–nitrogen bond and (presumably[3,4]) replacement of a cobalt-bound water molecule. The base-off to base-on restructuring observed here is opposite to that required for binding of coenzyme B_{12} in a base-off/His-on form in the carbon skeleton mutases.[6,7,24]

The antibody-induced base-off/base-on switch changes the chemical reactivity of the corrinoids. The rate of the conversion of adenosylcobamides (such as **1**, **4**, and **5**) into dicyanocobamides, for example, depends upon the coordination of the nucleotide base.[22] In the presence of a 2.5-fold excess of antibody, the reaction with cyanide was inhibited by over 90% relative to the reaction in the absence of protein (conditions: 15 μM 2C2, 6 μM B_{12}, 4 mM KCN in phosphate buffer, pH 7.5; $T = 25$ °C; spectrophotometric monitoring at 368 nm: relative rates: $k_{\mathbf{4}+2C2}/k_{\mathbf{4}\text{-}2C2} = 0.096$; $k_{\mathbf{5}+2C2}/k_{\mathbf{5}\text{-}2C2} = 0.040$). The base-off/base-on switch of these B_{12} derivatives reduces the tendency of the metal centers to react with the cyanide nucleophile. This observation is in line with the known "trans effect" of the nucleotide coordination on the organometallic reactivity of "complete" corrins.[3,4,22]

The crystal structure of an aptamer[28] for vitamin B_{12} revealed that the RNA moiety binds (only) the corrin ring of the cobalamin.[29] Herein, coenzyme B_{12} (**1**) was used to induce the formation of an antibody that selectively recognizes the base-on form of **1** and of other B_{12} coenzymes. The nucleotide loop, which is a unique structural motif of the base-on forms of complete cobamides, steers the reactivity of the cobalt corrins[3,4] and also serves as a still poorly understood recognition element for selective interactions with B_{12}-binding proteins.[8] Antibody 2C2 recognizes the intact DMB nucleotide loop and reconstitutes the base-on form of coenzyme B_{12} analogues,whose solution structures are mainly base-off. Antibody C2C may represent a functional model for natural B_{12}-binding proteins, such as (human) intrinsic factor and transcobalamin.[23] These important B_{12} receptors preferentially bind cobamides with a DMB nucleotide[23,30] and are selective for structurally related B_{12} derivatives that also prefer a base-on conformation in solution.

Received: April 2, 2002 [Z19024]

[1] P. G. Lenhert, D. C. Hodgkin, *Nature* **1961**, *192*, 937 – 938.
[2] J. Pickworth-Glusker in *B_{12}, Vol. I* (Ed.: D. Dolphin), Wiley, New York, **1982**, pp. 24 – 106.
[3] B. Kräutler in *Vitamin B_{12} and B_{12} Proteins* (Eds.: B. Kräutler, B. T. Golding, D. Arigoni), Wiley-VCH, Weinheim, **1998**, pp. 3 – 43.
[4] J. M. Pratt in *B_{12}, Vol. I* (Ed.: D. Dolphin), Wiley, New York, **1982**, pp. 325 – 392.
[5] a) C. L. Drennan, S. Huang, J. T. Drummond, R. G. Matthews, M. L. Ludwig, *Science* **1994**, *266*, 1669 – 1674; b) M. L. Ludwig, P. R. Evans

  0044-8249/02/4119-3615 $ 20.00+.50/0

in *Chemistry and Biochemistry of B_{12}* (Ed.: R. Banerjee), Wiley, New York, **1999**, pp. 217 – 226.
[6] F. Mancia, N. H. Keep, A. Nakagawa, P. F. Leadlay, S. McSweeney, B. Rasmussen, P. Bösecke, O. Diat, P. R. Evans, *Structure* **1996**, *4*, 339 – 350.
[7] R. Reitzker, K. Gruber, G. Jogl, U. G. Wagner, H. Bothe, W. Buckel, C. Kratky, *Structure* **1999**, *7*, 891 – 920.
[8] N. Shibata, J. Masuda, T. Tobimatsu, T. Toraya, K. Suto, Y. Morimoto, N. Yasuoka, *Structure* **1999**, *7*, 997 – 1008.
[9] R. A. Lerner, S. J. Benkovic, P. G. Schultz, *Science* **1991**, *252*, 659 – 667.
[10] P. G. Schultz, R. A. Learner, *Science* **1995**, *269*, 1835 – 1842.
[11] D. Hilvert, *Top. Stereochem.* **1999**, *22*, 83 – 135.
[12] D. Hilvert, *Ann. Rev. Biochem.* **2000**, *69*, 751 – 793.
[13] *Catalytic Antibodies* (Eds.: D. J. Chadwick, J. Marsh), Wiley, Chichester, **1991** (Ciba Foundation Symposium 159).
[14] P. Wentworth, Jr., L. H. Jones, A. D. Wentworth, X. Zhu, N. A. Larsen, I. A. Wilson, X. Xu, W. A. Goddard III, K. D. Janda, A. Eschenmoser, R. A. Lerner, *Science* **2001**, *293*, 1806 – 1811.
[15] a) H. Gershman, N. Nathanson, R. Abeles, L. Levine, *Arch. Biochem. Biophys.* **1972**, *153*, 407 – 409; b) D. F. M. Van De Weil, W. T. Goedemans, M. G. Woldring, *Clin. Chim. Acta* **1974**, *56*, 143 – 149; c) S. S. Ahrenstedt, J. I. Thorell, *Clin. Chim. Acta* **1979**, *95*, 417 – 423; d) J. J. O'Sullivan, R. J. Leeming, S. S. Lynch, A. Pollock, *J. Clin. Pathol.* **1992**, *45*, 328 – 331; e) S. C. J. Meskers, P. J. M. Dekkers, *J. Am. Chem. Soc.* **1998**, *120*, 6413 – 6414.
[16] T. Toraya, K. Ohashi, H. Ueno, S. Fukui, *Bioinorg. Chem.* **1975**, *4*, 245 – 255.
[17] E. Harlow, D. Lane, *A Laboratory Manual*, Cold Springs Laboratories, New York, **1988**.
[18] B. R. Clark, E. Engvall, *Enzyme-Immunoassay*, CRC, Boca Raton, FL, **1980**, pp. 167 – 179.
[19] A. Eschenmoser, *Angew. Chem.* **1988**, *100*, 5 – 40; *Angew. Chem. Int. Ed. Engl.* **1988**, *27*, 5—40, and references therein.
[20] H. A. Barker, H. Weissbach, R. D. Smyth, *Proc. Natl. Acad. Sci. USA* **1958**, *44*, 1093 – 1097.
[21] W. Fieber, B. Hoffmann, W. Schmidt, E. Stupperich, R. Konrat, B. Kräutler, *Helv. Chim. Acta* **2002**, *85*, 927 – 944.
[22] W. Friedrich in *Fermente, Hormone, Vitamine, Vol. III/2* (Eds.: R. Ammon, W. Dirscherl), Thieme, Stuttgart, **1975**, pp. 10 – 152.
[23] E. Nexø in *Vitamin B_{12} and B_{12}-Proteins* (Eds.: B. Kräutler, D. Arigoni, B. T. Golding), Wiley-VCH, Weinheim, **1998**, pp. 461 – 471.
[24] E. N. G. Marsh, D. E. Holloway, H.-P. Chen in *Vitamin B_{12} and B_{12} Proteins* (Eds.: B. Kräutler, D. Arigoni, B. T. Golding), Wiley-VCH, Weinheim, **1998**, pp. 253 – 264.
[25] B. Hoffmann, M. Oberhuber, E. Stupperich, H. Bothe, W. Buckel, R. Konrat, B. Kräutler, *J. Bacteriology* **2000**, *182*, 4773 – 4782.
[26] A. P. Campbell, T. M. Tarasow, W. Massefski, P. E. Wright, D. Hilvert, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 8663 – 8667.
[27] A. Bax, G. W. Vuister, S. Grezsiek, F. Delaglio, R. Tschudin, G. Zhu, *Methods Enzymol.* **1994**, *239*, 79 – 105.
[28] J. R. Lorsch, J. W. Szostak, *Biochemistry* **1994**, *33*, 973 – 982.
[29] D. Sussman, J. C. Nix, C. Wilson, *Nat. Struct. Biol.* **2000**, *7*, 53 – 57.
[30] B. Elsenhans, I. H. Rosenberg, *Biochemistry* **1984**, *23*, 805 – 808.

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 μM). Based on the total synthesis of this modularly built natural product,[6] we have developed a solid-phase synthesis

[*] Prof. Dr. H. Waldmann, Dipl.-Chem. M. Thutewohl, Dipl.-Chem. L. Kissau
Max-Planck-Institut für molekulare Physiologie
Abt. Chemische Biologie
Otto-Hahn-Str. 11, 44227 Dortmund (Germany)
und
Fachbereich 3, Organische Chemie
Universität Dortmund (Germany)
Fax: (+49) 231-133-2499
E-mail: herbert.waldmann@mpi-dortmund.mpg.de
Dipl.-Biochem. B. Popkirova, Dipl.-Biol. I.-M. Karaguni, Dr. J. Kuhlmann, Priv.-Doz. Dr. O. Müller
Max-Planck-Institut für molekulare Physiologie
Abt. Strukturelle Biologie
Otto-Hahn-Str. 11, 44227 Dortmund (Germany)
Dr. T. Nowak, M. Bate
AstraZeneca, Mereside, Alderley Park, Macclesfield, Cheshire, SK10 4TG (UK)

[**] 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.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

  0044-8249/02/4119-3616 $ 20.00+.50/0