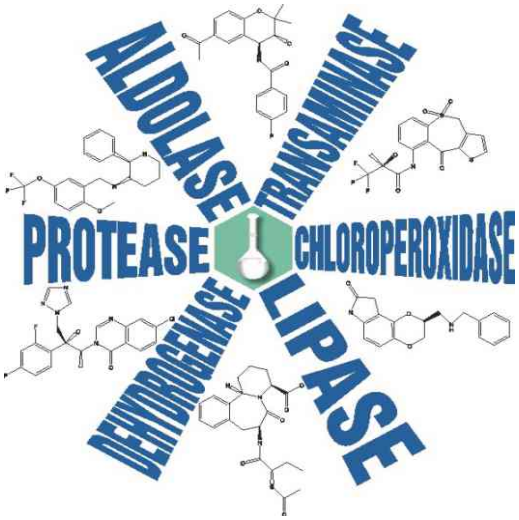


Vth VUFB Conference on

Modern Methods in Synthesis and Analysis of Active Pharmaceutical Substances

Programme

Wednesday, November 23, 2005

08.30 – 10.00 *Registration*
10.00 – 10.15 *Opening Ceremony*
J. Michal
Invitation of participants on behalf of Zentiva company

Session 1
10.15 – 11.00 S. Blecher:
Sequentional formation of C-C bonds using domino metathesis reactions
11.00 – 11.45 A. Minaard:
Asymmetric catalysis in the preparation of biologically active compounds
11.45 – 12.30 I. Starý:
Advanced methods in the synthesis of enantiopure bioactive substances
12.30 – 13.30 *Lunch*

Session 2
13.30 – 14.15 D. Berkeš:
The application of CIAT methodology in the synthesis of active pharmaceutical substances
14.15 – 14.45 L. Bartek, P. Klusoň, L. Červený:
Challenges in using of homogenous and immobilized catalysts in the production of chiral pharmaceutical compounds
14.45 – 15.15 S. Rádl, J. Stach:
Statin hypolipidemics and our experience with their preparation
15.15 – 15.45 *Coffee break*

Session 3
15.45 – 16.30 P. Coufal:
Capillary analytical chemistry in pharmaceutical analysis
16.30 – 17.15 J. Barek, J. Zima:
Modern electroanalytical methods in the analysis of bioactive compounds
17.15 – 17.35 H. Petříčková:
X-ray diffraction applied in pharmaceutical industry
17.35 – 17.55 M. Hušák:
The use of synchrotrone radiation for the structural analysis of substances in powder samples and microcrystals

18.00 – 20.30 *Welcome Reception*

Thursday, November 24, 2005

Session 4
9.00 – 9.15 *Introductory Remarks*
9.15 – 10.00 D. R. Boyd:
The use of both enzymatic and non-enzymatic methods for the preparation of enantiopure molecules
10.00 – 10.45 R. A. Sheldon:
Biocatalytic, chemocatalytic and chemoenzymatic methods for the synthesis of pure enantiomers
10.45 – 11.15 *Coffee break*

Session 5
11.15 – 12.00 L. Martínková, V. Křen:
Preparation of pharmaceutical substances by the use of nitrile- and amide-converting enzymes.
12.00 – 12.15 Z. Prokop:
Enantioselectivity of haloalkane dehalogenases
12.15 – 12.30 J. Damborský:
Computer-assisted engineering of enantioselective dehalogenases
12.30 – 13.30 *Lunch*

Session 6
13.30 – 14.15 Gracza T.:
Novel bicyclisation of unsaturated polyols and aminopolyols in a „magic“ $PdCl_2$ -$CuCl_2$ -AcOH catalytic system
14.15 – 15.00 M. Hocek:
Cross-coupling reactions in the synthesis of pharmaceutical active substances. A focus on bioactive purine bases and nucleosides
15.00 – 15.15 J. Hašek:
Drug design based on X-ray diffraction and steered molecular dynamics
15.15 – 15.30 *Coffee break*

Session 7
15.30 – 15.45 D. Procházková:
New stationary phases for HPLC and their advantages in comparison with RP-C18 phase
15.45 – 16.00 L. Ridvan:
Synthesis of chiral N,N-dialkylaminoethers as intermediates for preparation of oxetines
16.00 – 16.15 P. Krňák:
CEM systems for microwave organic synthesis
16.15 – 16.35 V. Kubelka:
How chemical reactions of ionic species can elucidate the structure of compounds
16.35 – 16.55 A. Popkov:
Evaluation of [^{18}F]fluorobenzaldehyde for preparation of ^{18}F–labelled substance P and an asymmetric approach to the radiosynthesis of both enantiomers of -[^{11}C]methyldopa and -[^{11}C]methyltyrosine for positron emission tomography.

16.55 – 17.00 *Closing Address*

Vth VUFB Conference on

MODERN METHODS IN SYNTHESIS AND ANALYSIS OF ACTIVE PHARMACEUTICAL SUBSTANCES

Praha, November 23 - 24, 2005

SEQUENTIAL FORMATION OF C-C BONDS USING DOMINO METATHESIS REACTIONS

Siegfried Blechert

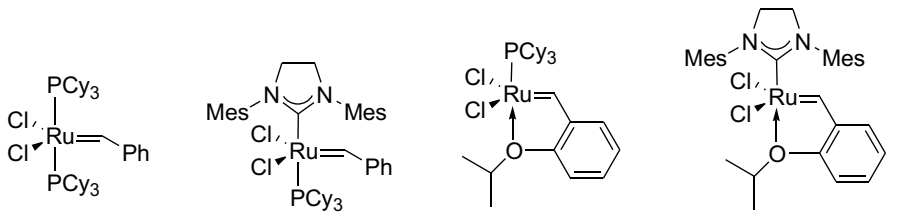
Department of Organic Chemistry, Technical University Berlin, Germany

Olefin metathesis has become widely established as an important tool for the formation of carbon-carbon bonds. Early developments centred on the use of molybdenum complexes, but it was with the advent of the more 'user-friendly' ruthenium precatalysts that the power of the metathesis reaction began to be fully exploited. The majority of metathesis reactions that are conducted at the present time make use of one of the four commercially available precatalysts.

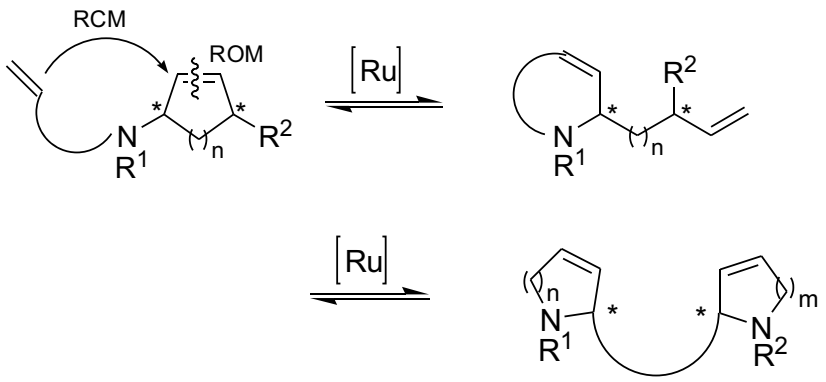
The Blechert group has pioneered the use of ring rearrangement metathesis (RRM) as a methodology through which rapid access to complex alkaloid structures can be achieved. Domino metathesis processes that involve bond cleavage or bond formation are conducted in sequential steps where the functionality formed in the first process is used in the subsequent transformation. The construction of fused carbocycles as well as heterocycles is possible. Stereochemical information is transferred from the starting compound so this methodology allows for the formation of enantiomerically pure heterocycles in a rapid one-pot process.

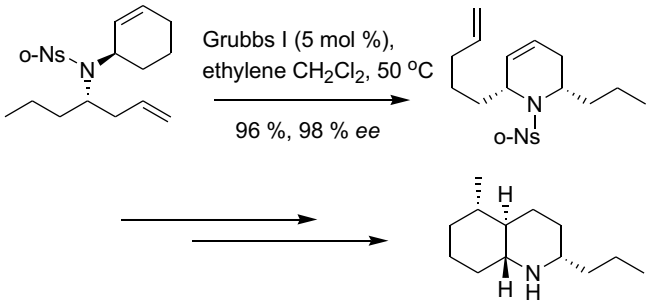
This methodology has been used in the Blechert group in the synthesis of a variety of piperidine and pyrrolizidine alkaloids. A representative example of this strategy can be seen in our recent synthesis of the unnatural enantiomer of (-)-*trans*-195A, a compound isolated from amphibian skin that has been shown to be a noncompetitive blocker of nicotinic receptor channels. The key RRM reaction gave the piperidine in an excellent yield and with no loss of chirality. The synthesis was completed using a zirconium mediated Negishi coupling to give (+)-*trans*-195A.

The synthesis of mono and bicyclic piperidines and pyrrolidines through a sequential cross metathesis (CM)-reductive cyclisation strategy has also been an area of extensive research in the Blechert laboratory. These compounds are some of the most ubiquitous structural motifs seen in natural products and many biologically important compounds contain an *N*-heterocycle at their core. The synthetic strategy that we have chosen makes use of the cross metathesis of allylic and homo allylic amines with electron deficient double bonds. Previous work in our group has demonstrated that such CM reactions proceed with excellent chemo and stereo control to give the enone



Commercially available olefin metathesis precatalysts





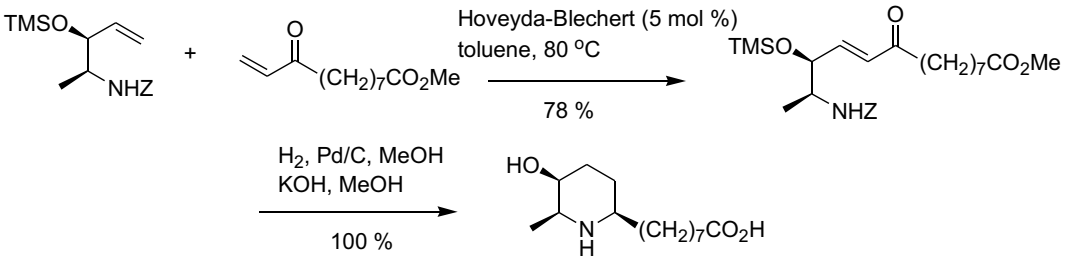
The synthesis of (+)-*trans*-195A

product as the *trans* double bond isomer. We have coupled this reaction with a reductive cyclisation to give a general approach to the synthesis of substituted piperidines and pyrrolidines.

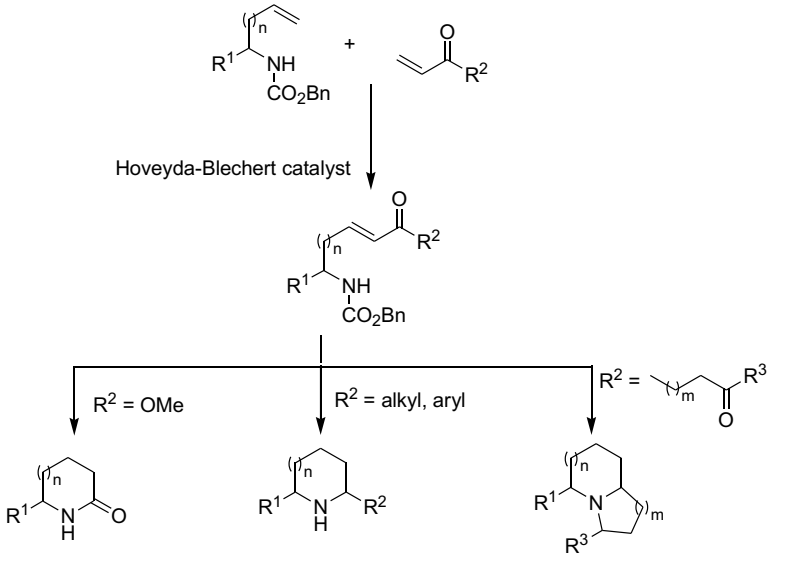
(+)-Carpamic acid, a 2,6-disubstituted 3-piperidinol, belongs to a small sub-group of alkoloids that have been shown to have interesting pharmacological properties. The CM step was investigated using the Hoveyda-Blechert catalyst and it was found necessary to increase the steric bulk of the starting material to suppress formation of the homodimer. This was achieved by protecting the alcohol as the silyl ether and the desired cross product was obtained in excellent yield. Reductive hydrogenation of the enone then gave (+)-carpamic acid.

Double reductive cyclisation of the products obtained from the CM of protected dicarbonyl substrates allows access to bicyclic compounds. This procedure was showcased in the extremely convergent synthesis of (+)-xenoeveine, a pyrrolizidine alkaloid found in the thief ant *Solenopsis xenovenum*. The CM proceeded without incident to give the enone with double reductive cyclisation giving the target compound in 73 % yield.

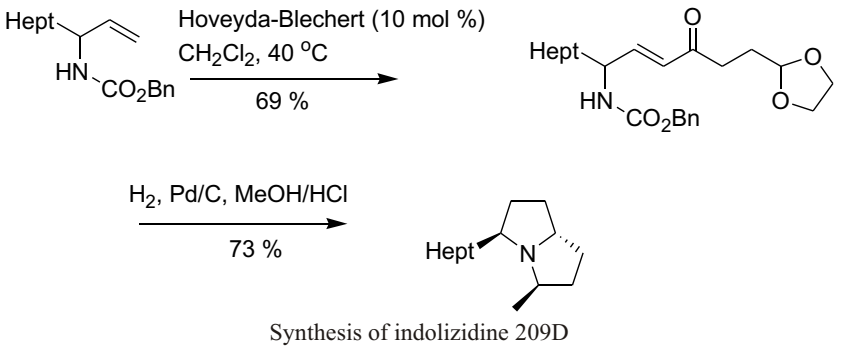
These examples are representative of the recent uses of olefin metathesis in the Blechert laboratory to give access to biologically important natural products. Our general strategy of employing sequential metathesis reactions to deliver complex molecules, illustrated by selected synthetic examples, will be discussed in this lecture.



Synthesis of (+)-carpamic acid



Synthesis of substituted *N*-heterocycles



Synthesis of indolizidine 209D

ASYMMETRIC CATALYSIS IN THE PREPARATION OF BIOLOGICALLY ACTIVE COMPOUNDS

Adriaan J. Minnaard, Ben L. Feringa

Stratingh Institute, University of Groningen, Nijenborgh 4, 9747 AG, Groningen, The Netherlands. A.J. Minnaard@rug.nl

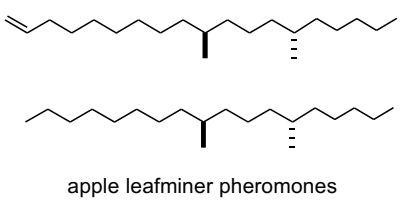
Asymmetric catalysis, and in particular homogeneous transition metal catalysis, has become one of the most important ways of making chiral compounds with high enantioselectivities. In this field, asymmetric conjugate addition reactions of organometallic reagents catalyzed by copper, rhodium and palladium are thoroughly studied as both a carbon-carbon bond and a stereocenter are formed.

In our group we have successfully developed the copper-catalyzed asymmetric conjugate addition of dialkylzinc reagents and Grignard reagents to unsaturated ketones, lactones, alkylidene malonates, and nitro compounds. In our efforts to use this methodology in natural product synthesis we realized that the preparation of linear hydrocarbons containing multiple stereocenters is a challenging field to apply these conjugate addition reactions.

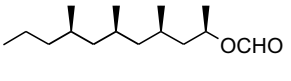
Recently, we have developed a general method to prepare enantiopure saturated isoprenoid building blocks [1]. The method allows the preparation of all four stereoisomers (ee > 99%, de > 98%). To demonstrate the synthetic versatility of this catalytic approach, it was employed in the total synthesis (see Scheme) of two pheromones of the apple leafminer (*Lyonetia prunifoliella*), a pest endemic to the eastern regions of North America [2].

A second application of the methodology is the preparation of enantiopure deoxypropionate building blocks by iterative conjugate addition reactions [3]. This has been used in an efficient synthesis of Lardolure, the aggregation pheromone of the acarid mite, *Lardoglyphus Konoi*.

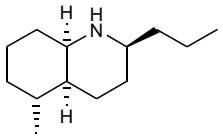
1. Van Summeren, R.P.; Reijmer, S.V.; Feringa, B.L.; Minnaard, A. J. *Chem. Comm.* **2005**, 1387 and references cited therein.
2. For earlier synthesis of these pheromones see: Y. Nakamura and K. Mori, *Eur. J. Org. Chem.*, **2000**, 2745.
3. Des Mazery, R.; Pullez, M.; López, F.; Harutyunyan, S.R.; Minnaard, A.J.; Feringa, B.L. *J. Am. Chem. Soc.* **2005**, *127*, 9966.



apple leafminer pheromones



(-)-Lardolure



(-)-pumiliotoxin C

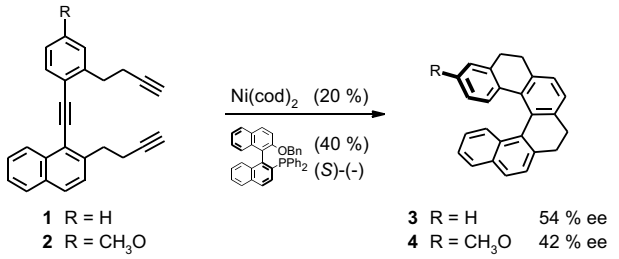
ADVANCED METHODS IN THE SYNTHESIS OF ENANTIOPURE BIOACTIVE SUBSTANCES: THE QUEST FOR NEW TYPES OF CHIRAL LIGANDS APPLICABLE TO ENANTIOSELECTIVE CATALYSIS

Ivo Starý,[*,§] Irena G. Stará,[*,§] Zuzana Alexandrová,[§] Petr Sehnal,[§] Jiří Míšek,[§] Filip Teplý, David Šaman, and Lubomír Rulíšek[§]

[§]Center for Biomolecules and Complex Molecular Systems
Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, 166 10 Prague 6, Czech Republic

Enantioselective catalysis plays an important role in the preparation of optically active compounds. Enzymes, nonracemic metal complexes or organocatalysts are aimed at propagating chirality so that the desired enantiomer of a product may dominate in the reaction vessel. This methodology reflects principles of "green chemistry" by reducing wastes and the energy consumption. It may exhibit high efficiency and, in the case of a low catalyst loading or its recycling, substantially reduce the costs of a process. Enantioselective catalysis is used in academic laboratories as well as in industry and its importance is growing up steadily.

An ideal chiral metal catalyst or organocatalyst can be characterized by exhibiting both high enantioselectivity and high reactivity. Actually, an enormous effort has been devoted to meet the former criterion and, within the realm of enantioselective transition metal catalysis, so called "privileged ligands" have been identified. Interestingly, the attention has been paid almost exclusively to the ligands possessing the elements of central, axial, or planar chirality. By contrast, the helically chiral ligands have been rather rare and their potential has practically been unexplored so far. A very limited number of examples demonstrating the utilization of helicenes [1] either in stoichiometric asymmetric synthesis [2] or in enanti-



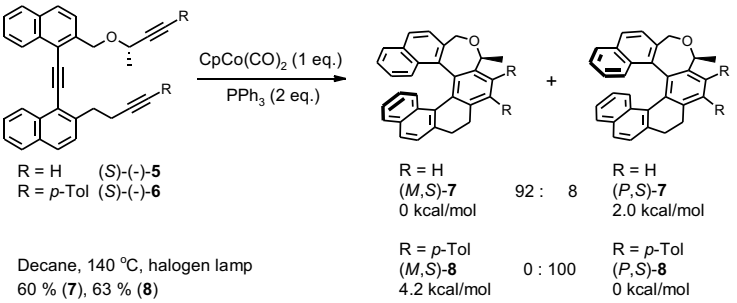
Scheme 1

oselective catalysis [3] can be found in literature. Thus, we have embarked on helicene-based chiral entities taking advantage of our expertise in helicene chemistry and being stimulated also by no patent claims in the field of enantioselective catalysis exploiting helicenes. Accordingly, we have assumed that there is enough room for the future applications of the helicene-based ligands or catalysts to the synthesis of enantiopure bioactive substances.

Helicenes are unique three-dimensional aromatic systems that are inherently chiral, thermally stable, and usually well soluble. However, the difficult preparation and the absence of a more general synthesis methodology to get individual enantiomers on a preparative scale have hampered their broader use. Currently, the renewed interest in helicene chemistry can be seen as various original approaches to racemic or nonracemic helicenes and related compounds have emerged [4-10]. Regardless of such recent progress and intriguing past achievements, the racemate resolution dominates in getting nonracemic helicenes and their derivatives [11].

We have recently demonstrated that the intramolecular [2+2+2] cycloisomerization of aromatic triynes under Co^I or Ni^0 catalysis is a feasible concept exhibiting a high degree of synthetic flexibility [12]. Using this methodology, we synthesized various racemic helicene derivatives ranging from penta- to heptacyclic structures. However, the [2+2+2] cycloisomerization reaction should, in principle, allow for a helicity control in the key helicity forming step. First, we focused on enantioselective catalysis which has never been applied to the construction of helicene skeleton before. The use of chiral ligands for nickel, obviously phosphines, might result in controling helicity. In the presence of Hayashi's MOP ligand or better its benzyl analogue[13] we observed moderate enantioselectivities in [2+2+2] cycloisomerization of aromatic triynes **1** and **2** providing tetrahydro[6]helicene **3** [12d] or the 3-methoxyderivative **4** [12a], respectively (Scheme 1).

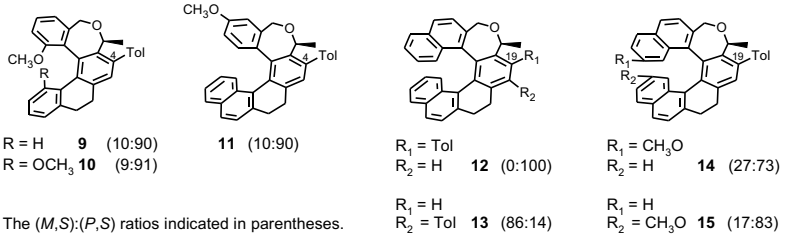
In parallel, the attention has been paid to the diastereoselective synthesis of helicene-like compounds. We assumed that a fully aromatic backbone of helicenes might not be essential for the utilization of these compounds in enantioselective catalysis. Accordingly, we devised nonracemic helicene-like structures whose diastereoselective synthesis relies on a helicity induction by the asymmetric center already present in the triyne molecule (Scheme 2) [14]. We found that [2+2+2] cycloisomerization of chiral triyne (*S*)-**5** mediated by the Co^I/PPh_3 system afforded two possible diastereomers (*M*,*S*)-**7** and (*P*,*S*)-**7** in a 92 : 8 ratio. The analogous triyne (*S*)-**6** with two *p*-tolyl groups at the pendant acetylene units provided only (*P*,*S*)-**8** possessing the opposite helicity. Such a striking stereochemical dichotomy requires explanation. If the reaction proceeds under thermodynamic control its stereochemical outcome should reflect the energy contents of the diastereomeric products. Thus, we calculated the relative energies of both pairs (*M*,*S*)-**7** *versus* (*P*,*S*)-**7** and (*M*,*S*)-**8** *versus* (*P*,*S*)-**8** in decane at 140 °C using the DFT method (B3LYP/TZV+P), and COSMO solvation model. The calculated free energies clearly indicated a more stable diastereomer from the relevant pair being expected to prevail in the reaction mixture, in excellent agreement with the experimental results.



Scheme 2

As it has been clearly shown, the helicity control is possible and diastereoselectivity of the cyclization could be good to excellent, we have decided to explore the scope and limitations of this approach to precursors of nonracemic helicene-like ligands. Accordingly, we studied key [2+2+2] cycloisomerization of a series of chiral triynes in the presence of $CpCo(CO)_2/PPh_3$ in decane at 140 °C to obtain helicene-like compounds **9**-**15** (Scheme 3). The stereochemical outcome of the reaction was found to be practically invariant to the helicene size and the presence of a functional group attached to the terminal benzene rings. In all cases the diastereomeric ratio ranged between 73:27 and 100:0. However, the presence of the *p*-tolyl group at C-4 (compounds **9**-**11**) or C-19 (compounds **12**, **14**, **15**) effectively controlled the cyclization in favor of (*P*) helix while its absence at C-19 (compound **13**) resulted in the predominant formation of (*M*) helix.

The synthesis of nonracemic helicene-based ligands is under progress as well as their utilization in enantioselective catalysis.



Scheme 3

Acknowledgement. *This research was supported by the Ministry of Education (Center for Biomolecules and Complex Molecular Systems, the project LC512), Zentiva and the Ministry of Industry and Trade (the grant FI-IM/073), and the Institute of Organic Chemistry and Biochemistry (this work is a part of the research project Z4 055 0506).*

1. For selected reviews on helicenes, see: (a) A. Urbano. *Angew. Chem. Int. Ed.* **42** (2003) 3986. (b) H. Hopf. *Classics in Hydrocarbon Chemistry: Syntheses, Concepts, Perspectives*, p. 323. VCH, Weinheim (2000). (c) T. J. Katz. *Angew. Chem. Int. Ed.* **39** (2000) 1921. (d) G. Oremek, U. Seiffert, A. Janecka. *Chem.-Ztg*. **111** (1987) 69. (e) F. Vögtle. *Fascinating Molecules in Organic Chemistry*, p. 156. Wiley, New York (1992). (f) K. P. Meurer, F. Vögtle. *Top. Curr. Chem*. **127** (1985) 1. (g) W. H. Laarhoven, W. J. C. Prinsen. *Top. Curr. Chem*. **125** (1984) 63.

2. B. B. Hassine, M. Gorsane, J. Pecher, R. H. Martin. *Bull. Soc. Chim. Belg.* **95** (1986) 547.

3. (a) Nakano, D.; Yamaguchi, M. *Tetrahedron Lett.* 44 (2003)*, 4969.* (b) Sato, I.; Yamashima, R.; Kadowaki, K.; Yamamoto, J.; Shibata, T.; Soai, K. *Angew. Chem. Int. Ed*. **40** (2001), 1096. (c) Dreher, S. D.; Katz, T. J.; Lam, K. C.; Rheingold, A. L. *J. Org. Chem*. **65** (2000) 815. (d) Reetz, M. T.; Sostmann, S. *J. Organomet. Chem*. **603** (2000) 105. (e) Reetz, M. T.; Beuttenmuller, E. W.; Goddard, R. *Tetrahedron Lett*. **38** (1997), *38*, 3211.

4. (a) T. Thongpanchang, K. Paruch, T. J. Katz, A. L. Rheingold, K. C. Lam, L. Liablesands. *J. Org. Chem.* **65** (2000) 1850 . (b) S. D. Dreher, D. J. Weix, T. J. Katz. *J. Org. Chem.* **64** (1999) 3671.

5. (a) M. C. Carreńo, M. Gonzales-Lopez, A. Urbano. *Chem. Commun.* **611** (2005). (b) M. C. Carreńo, S. Garcia-Cerrada, A. Urbano. *Chem. Eur. J*. **9** (2003) 4118. (c) M. C. Carreńo, S. Garcia-Cerrada, A. Urbano. *J. Am. Chem. Soc.* **123** (2001) 7929.

6. Y. Ogawa, M. Toyama, M. Karikomi, K. Seki, K. Haga, T. Uyehara. *Tetrahedron Lett.* **44** (2003) 2167.

7. R. El Abed, B. Ben Hassine, J. P. Genet, M. Gorsane, J. Madec, L. Ricard, A. Marinetti. *Synthesis* 2513 (2004).

8. (a) A. Rajca, M. Miyasaka, M. Pink, H. Wang, S. Rajca. *J. Am. Chem. Soc*. **126**, 15211 (2004). (b) M. Miyasaka, A. Rajca, M. Pink, S. Rajca. *Chem. Eur. J*. **10**, 6531 (2004).

9. K. Tanaka, H. Suzuki, H. Osuga. *J. Org. Chem*. **62**, 4465 (1997).

10. For selected references, see: (a) I. G. Stará, I. Starý, M. Tichý, J. Závada, V. Hanuš. *J. Am. Chem. Soc*. **116**, 5084 (1994). (b) T. J. Katz, A. Sudhakar, M. F. Teasley, A. M. Gilbert, W. E. Geiger, M. P. Robben, M. Wuensch, M. D. Ward. *J. Am. Chem. Soc*. **115**, 3182 (1993). (c) J. Vanest, R. H. Martin. *Recl. Trav. Chim. (Pays-Bas)* **98**, 113 (1979). (d) H. J. Bestmann, W. Both. *Chem. Ber*. **107**, 2923 (1974).

11. For recent examples, see: (a) B. Laleu, P. Mobian, C. Herse, B. W. Laursen, G. Hopfgartner, G. Bernardinelli, J. Lacour. *Angew. Chem. Int. Ed*. **44**, 1879 (2005). (b) M. T. Reetz, S. Sostmann. *Tetrahedron* **57**, 2515 (2001). (c) J. Lacour, A. Londez, C. Goujon-Ginglinger, V. Buß, G. Bernardinelli. *Org. Lett*. **2**, 4185 (2000). (d) K. Tanaka, H. Osuga, H. Suzuki, Y. Shogase, Y. Kitahara. *J. Chem. Soc., Perkin Trans. 1* 935 (1998). (e) H. Okubo, M. Yamaguchi, C. Kabuto. *J. Org. Chem*. **63**, 9500 (1998). (f) H. Osuga, H. Suzuki, K. Tanaka. *Bull. Chem. Soc. Jpn*. **70**, 891 (1997).

12. (a) F. Teplý, I. G. Stará, I. Starý, A. Kollárovič, D. Šaman, P. Fiedler, Š. Vyskočil. *J. Org. Chem.* **68**, 5193 (2003). (b) I. G. Stará, I. Starý, A. Kollárovič, F. Teplý, D. Šaman, P. Fiedler. *Collect. Czech. Chem. Commun.* **68**, 917 (2003). (c) F. Teplý, I. G. Stará, I. Starý, A. Kollárovič, D. Šaman, L. Rulíšek, P. Fiedler. *J. Am. Chem. Soc.* **124**, 9175 (2002). (d) I. G. Stará, I. Starý, A. Kollárovič, F. Teplý, Š. Vyskočil, D. Šaman. *Tetrahedron Lett*. **40**, 1993 (1999). (e) I. G. Stará, I. Starý, A. Kollárovič, F. Teplý, D. Šaman, M. Tichý. *J. Org. Chem*. **63**, 4046 (1998).

13. Y. Uozumi, A. Tanahashi, S.-Y. Lee, T. Hayashi. *J. Org. Chem*. **58**, 1945, (1993).

14. I. G. Stará, Z. Alexandrová, F. Teplý, P. Sehnal, I. Starý, D. Šaman, M. Buděšínský, J. Cvačka. *Organic Lett*. **13**, 2547 (2005).

THE APPLICATION OF CIAT METHODOLOGY IN THE SYNTHESIS OF ACTIVE PHARMACEUTICAL SUBSTANCES

Pavol Jakubec[1], Andrej Kolarovič[2] and Dušan Berkeš[1]

[1]Department of Organic Chemistry, Slovak Technical University, Bratislava, Slovak Republic;
[2]Department of Chemistry and Chemical Biology, Northeastern University, 360 Huntington Ave, 349 Egan, Boston, MA 02115-5000

Worldwide market of chiral substances in single enantiomeric form continues in persistent outstanding growth and represents a stimulus for the development of new efficient methods for the asymmetric synthesis and for the improvement of the known methodologies of the enantiomerically pure compounds preparation, respectively. This tendency is clearly manifested on the worldwide chiral drugs sales. Recent presumption of the enantiomerically pure drugs proportion on pharma-market exceed 40 % continue in growth.

Regardless of tremendous advances have been made in asymmetric synthesis, either substrate driven or catalytically induced, resolution of racemates or covalently bounded diastereoisomers is still the most important approach to the synthesis of enantiomerically pure compounds (EPC´s). Although resolution via diastereoisomeric salt formation is not looked upon as „state of the art" like catalytic asymmetric synthesis, it is still the most widely used method to obtain of EPC´s in industry. The presumptions show that the 30-50 % of enantiomerically pure substances are produced using this methodology [1]. The resolution processes *via* diastereoisomeric salt formation are still intensively studied [2]. Understanding of the principles followed by optimization of the resolution methodology is important not only for industrial applications but also for the academic research.

The principal drawback of the standard racemate resolution is the limitation of the maximum theoretical yield of 50 % and the necessity of subsequent treatment of unwanted isomer in the separate process (for example in the re-racemization). This limitation affects the economical, technical and ecological characteristics of the production.

However, the scenario changes markedly when chiral compound contains labile stereogenic center capable to undergo „in situ“ racemization, which means, when there is a dynamic equilibrium between the isomers in the solution and one of the isomers is separated from the mixture by enantio- or diastereoselective process. It is named crystallization-induced asymmetric transformation (CIAT) when the crystallization is the above mentioned stereoselective process. The same meanings have the terms crystallization-induced dynamic resolution and an older Eliel´s expression – asymmetric transformation of the second kind.[3]

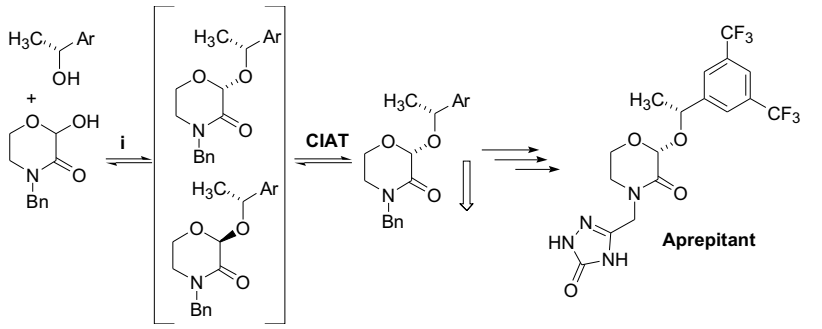


Figure 1. (i) $CF_3CO)_2O$, $BF_3.Et_2O$, (*R*)-3,5-bis(trifluoromethyl)-sec-phenylethyl alcohol 95-98 % (d.r.55:45); **(CIAT)** heptane/0.9 equiv tetrahydrolinalool, 0.3 equiv. of potassium tetrahydrolinaloolate, -10 °C, 5 h, 83 % (> 99 % e.e.).

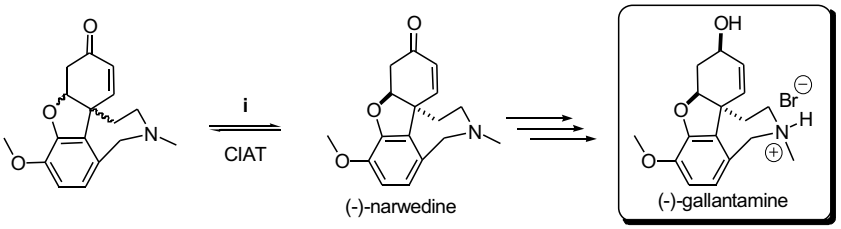


Figure 2. (i) Et_3N/EtOH, seeding with 2,5 % crystalls of (-)- enantiomer, yield 84% (ee = 99%).

CIAT is promising technique for the control of stereochemistry on the chirally labile stereogenic centers. The principal requirement for this type of asymmetric transformation is that one of the isomers must be crystalline at a convenient temperature and mustn't form a solid solutions or quasi-racemates. Because racemization and crystallization take place simultaneously, the relative rates between crystal growth and racemization are likely to be important. However, for the industrial applications mainly it is more convenient when the racemization (epimerization) takes faster than the crystallization. When both these conditions are fulfilled the desired enantiomer (diastereoisomer) can be isolated from the starting mixture by simple filtration and the yields reach theoretical 100 % regardless the equilibrium in the solution.

In spite of exceptional effectiveness of CIAT processes the number of known applications is relatively low. A critical point in the CIAT process development is the compatibility of the racemization conditions or equilibrium between the isomers in the solution with the conditions for the crystal growth and nucleation.

The reaction mixture is heterogeneous and it is not easy to describe such system by the physical model, although some efforts have been published [4]. The prediction, which isomer will be precipitated from the reaction mixture is not a trivial task. However, any discovered example is directly headed to the industrial application. This tendency will be showed on the next examples targeted on the synthesis of the active pharmaceutical substances.

EPA Presidential Green Chemistry Award for the year 2005 honored Merck's chemists for redesign of the synthesis of Aprepitant, the active ingredient in EMEND, a drug used to reduce nausea and vomiting caused by cancer therapy. Using CIAT process as a key step allowed to double the overall yield to 76 % and to reduce significantly operating costs and the production waste (85 % reduction), Figure 1 [5].

An impressive example of successful CIAT process represents resolution of racemic narwedine *via* reversible *oxa*-Michael addition under basic conditions. (-)-Narwedine is the key intermediate for the multikilogram synthesis of (-)-gallantamine used in the treatment of Alzheimer disease, Figure 2 [6].

Another recent example of patented CIAT application based on the thermally induced epimerization is the preparation of a key intermediate of the benazepril synthesis, Figure 3 [7].

An immense material base for the CIAT applications represent racemic chiral drugs, which are finalized in the form of biologically accepted salts. Discovery of the „in situ“ racemization conditions, compatible with the nucleation and crystal growth of such salt, open also the door for the „racemic switch“ distinguished by exceptional technological simplicity and qualitative improvement of the final substance by the ballast isomer disposal [8]. In the case of

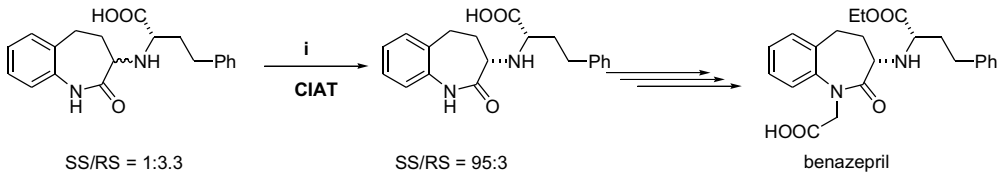


Figure 3. (i) *p*-xylene, reflux 3 h, 99 % (ee = 96 %).

enantiomerically pure derivatives CIAT technology eliminates the necessity of the undesirable isomer treatment mostly in a separate process.

More than one half of the known CIAT examples represent the asymmetric transformations of amino acid derivatives [9]. Resolution *via* diastereoisomeric salt formation is one of the leading synthetic strategies for the preparation of the desired stereoisomer.

Our research interests are devoted to the systematic study of CIAT applications for last five years. We have discovered and optimized several CIAT processes in tandem with *aza*-Michael addition of chiral *N*-nucleophiles [10a,b] or with the addition of achiral amines to the chiral unsaturated substrates precursors [10c], and also the CIAT mechanism of the stereoconvergent lactonization of -hydroxy- -aryl- -aminocarboxylic acids [10d]. We have achieved optimization of CIAT procedure with simultaneous formation of two new stereogenic centers [10e]. We also developed the synthetically useful pathway leading to the substituted homophenylalanine derivatives *via* easy accessible diastereoisomeric salt formation [10f], Figure 4.

The scope and limitation of CIAT processes in *aza*-Michael additions 4-oxo-2-alkenoic acids and the applications of the obtained enantiomerically pure aroyl- or heteroaroylalanines into the synthesis of biologically active substances like kynurenine-3-hydroxylase inhibitors and HPA-12 analogs will be discussed in the second part of the lecture.

Financial support by the Slovak Grant Agency No. 1/2469/05 is gratefully acknowledged.

1. Afraz M.C., Ariaans G.J.A., Broxterman Q.B., Bruggink A., *Arkivoc* (2004), 64-71.
2. a) Kelloggs R.M., Nieuwenhuizen J.W. et al. , *Synthesis* (2003), 1626-1638; b) Fogassy E., Nógradi M., Pálovics E., Schindler, *J. Synthesis* (2005), 1555-1568.
3. Eliel, E. L.; Wilen, S. H.; Mander, L. N. *Stereochemistry of organic compounds*; John Wiley & Sons: New York: 1994; pp. 364-374.
4. Schroer J.W., Ng K.M. *Ind. Eng. Chem. Res.* **42**, (2003) 2230-2244.
5. Ritter S.K., *Chem. Eng. New*s **83(26)**, 2005 40-43.
6. Czollner L. et al.: *Tetrahedron Lett.* **39**, (1998) 2087-2090.
7. Tseng W.-H., Schloemer G.: US Pat. Appl. 2002/0183515 (2002).
8. a) Agranat I., Caner H., Caldwell *J. Nature Rev. Drug Discov*. **1** (2002**)** 753-768.; b) Valentova J., Hutt A.J. Čes.Slov.Farm. **53** (2004) 285-293.
9. a) E. J. Ebbers, G. J. A. Ariaans, J. P. M. Houbiers, A. Bruggink and B. Zwanenburg, *Tetrahedron*, **53** (1997**)** 9417-9476.; b) Strauss U.T., Felfer U., Faber K., *Tetrahedron: Asymmetry* **10** (1999) 107-117.; c) Kolarovič A., Thesis, STU Bratislava, (2003).
10. a) Kolarovič A., Berkeš D., Baran P., Považanec F.: *Tetrahedron Lett.* **42** (2001) 2579-2582.; b) Šafář P., Horváthová H., Koreňová A., Berkeš D., *Annals of the Polish Chemical Society*, **3** (2004) Vol.3, 76-79. c) Jakubec P., Berkeš D., Považanec F. Tetrahedron Lett. **45** (2004) 4755-4758. d) Berkeš D., Kolarovič A., Manduch R., Baran P. , Považanec F. *Tetrahedron: Asymmetry* **16** (2005) 1927-1934; e) Berkeš D., Kolarovič A., Baran P. Považanec F. *Tetrahedron Lett.* **46** (2005) 975-978.; f) Berkeš D., Lopuch J., Proksa B., Považanec F.: *Chem. Papers* **57** (2003**)** 350-354.

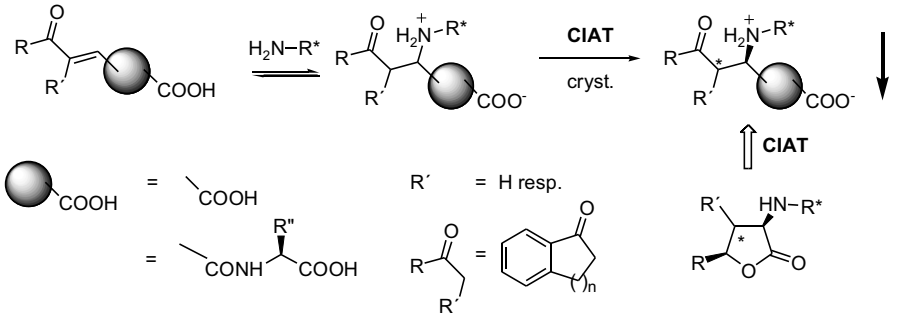


Figure 4.

CHALLENGES IN USING OF HOMOGENOUS AND IMMOBILIZED CATALYSTS IN THE PRODUCTION OF CHIRAL PHARMACEUTICAL COMPOUNDS

Lukáš Bartek, Petr Klusoň and Libor Červený

Department of Organic Technology, Institute of Chemical Technology - Prague Technická 5, 166 28 Prague 6 - Dejvice, Czech Republic

Outline

Asymmetric catalysis has become an essential part of many practical manufacturing processes in the production of chiral fine chemicals. Homogeneous and heterogeneous systems with ability of enantiodifferentiation have been intensively studied in last two decades [1-3]. Association of selective and active homogeneous catalysts with easy separable and reusable heterogeneous systems into one is one of the major tasks of the research in the area of applied catalysis. Immobilization of transition metal complexes on various supports (inorganic materials, polymers) represents a prospective way combining benefits of both. Development of effective immobilized catalysts applicable in the field of fine chemistry is a complex process dependent on many variables (behaviour of the support, leaching of transition metal complex, reusability, etc.). Enantioselective hydrogenations play a key role among other processes of asymmetric catalysis. Performance of the cationic chiral Ru-BINAP catalytic complex (either homogeneous or immobilized) in asymmetric hydrogenation of methyl-3-oxobutyrate (methylacetoacetate) in methanol was studied (scheme 1) [4, 5].

One of the major objectives of the presented work was the immobilization of the cationic complex [(R)-(+)-2,2`-bis(diphenylphosphino)-1,1`-binapthalene]chloro (p-cymene) ruthenium chloride. Immobilization techniques utilizing heteropolyacid [6] as the tethering agent were tested. In comparison the anchoring method aimed at the immobilization via support - cationic complex electronic forces has also been employed. A number of inorganic oxides (alumina, silica, zeolites) was tested as supports. Spectroscopic determination of the leached amount of a catalyst represents the general way to prove the stability of the heterogenized system. The immobilized catalysts and the homogeneous analogues have been employed in asymmetric hydrogenation of methylacetoacetate.

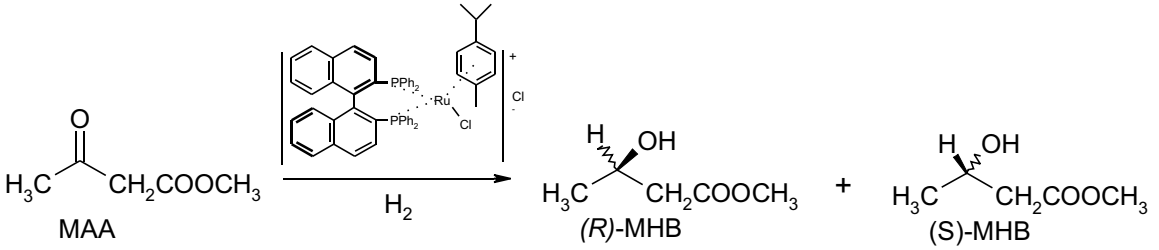
Results and Discussion

Effects of temperature and pressure on activity and enantioselectivity in homogeneous phase were discussed. Catalytic hydrogenations of methylacetoacetate were studied using the laboratory prepared Ru-BINAP homogeneous catalyst in a liquid phase at 303 – 333 K and pressure up to 5 MPa. The optimal values of TOF and the highest enantioselectivity were attained under the conditions of 5 MPa, 333 K and with the addition of 3 wt. % of water to the solvent (17 ml of methanol, S/C ratio 1 500). Presence of water can restrict the acetal formation in the initial stage of hydrogenation, when alcohol is used as a solvent. Immobilization of the Ru-BINAP complex using phosphotungstic acid and silicomolybdic acid as a tethering agents has been tested. Various inorganic materials such as Al_2O_3, SiO_2, TiO_2, zeolites, (montmorillonite, beta, X, Y, mordenite, ZSM-5) were employed as supports. The catalytic behaviour and the stability of the anchored cationic complex have been investigated. Montmorillonite and beta zeolites represented best supports regarding activity, enantioselectivity and leaching of the resulting catalysts. XPS and FTIR-DRIFT spectroscopy represented an effective tool for characterization of anchoring bond between the support, heteropolyacid and the complex and afforded a closer inspection of this rather new and unexplored system.

Acknowledgement

The authors acknowledge the Grant Agency of the Czech Republic, grant No. 104/03/0409 for supporting this research. L.B. thanks also to the Grant Agency of the Czech Republic for funding his fellowship grant No. 203/03/H140 and grant No. 1M4531477201.

1. Osawa T., Harada T., Takayasu O.: *Top. Catal.* **13**, (2000) 155.
2. Noyori R.: *Angew. Chem., Int. Ed.* **41** (2002) 2008.
3. Noyori R., Ohta M., Hsiao Y., Kitamura M., Ohta T., Takaya H.: *J. Am. Chem. Soc.* **108** (1986) 7117.
4. Bartek L., Drobek M., Kuzma M., Kluson P., Cerveny L.: *Catal. Commun.* **6** (2005) 61.
5. Wolfson A., Vankelecom I. F. J., Geresh S., Jacobs P. A.: *J. Mol. Catal. A: Chem.* **198** (2003) 39.
6. Augustine R. L., Tanielyan S. K., Mahata N., Gao Y., Zsigmond A., Yang H.: *Appl. Catal. A*: *General* **256** (2003) 69.



Scheme 1

STATIN HYPOLIPIDEMICS AND OUR EXPERIENCE WITH THEIR PREPARATION

Stanislav Rádl, Jan Stach

Zentiva a.s., U kabelovny 130, 102 01 Prague 10, Dolní Měcholupy, Czech Republic
stanislav.radl@zentiva.cz

Elevated cholesterol, or hypercholesterolemia, is an important risk factor for Coronary Heart Disease (CHD). Cholesterol in the body is either exogenous or formed in the liver. The mode of action of statins is inhibition of the enzyme 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase. This enzyme catalyzes the conversion of HMG-CoA to mevalonic acid (Fig. 1), which is the major rate-limiting step of the sterol pathway [1]. Statins are widely used for the therapeutic reduction of cholesterol-containing atherogenic lipoproteins. This reduction is a result of depletion of intracellular mevalonate leading to reduction of the regulatory sterol pool, which in turn causes upregulation of HMG-CoA reductase and other enzymes of the sterol pathway, most importantly the LDL receptors principally in the liver. However, a range of additional effects of statins on cells that are independent of cholesterol homeostasis has been described.

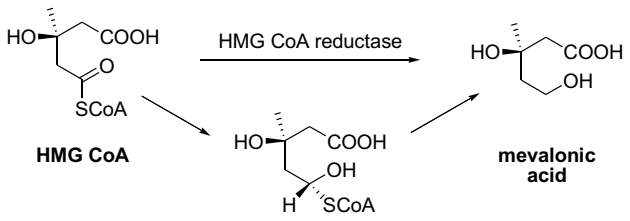


Figure 1.

Sankyo scientists screened more than 6000 wild bacterial and fungal strains isolated from soil samples from all over the globe and found that some microorganisms are able to inhibit cholesterol formation by an unknown mechanism, probably as a defense against cholesterol-dependent bacterial strains. In 1971 they isolated from several broth extracts compounds with cholesterol-lowering activity; the most active compound, later named mevastatin, was isolated from *Penicillium citrinum.* Inspired by this promising research, in 1976 the Merck Company started a project that in fact repeated the Sankyo strategy and as a result isolated from *Asperillus terreus* broth lovastatin [2], which was launched as Mevacor in 1987 (Fig. 2).

Both of these compounds are lactons and serve as prodrugs of the corresponding active 3,5-dihydroxy acids, which have affinity to the active site of (HMG-CoA) reductase higher by about 4 orders of magnitude then the natural ligand shown in Fig. 1.

A Sankyo study of active metabolites of natural statins led to the discovery of pravastatin isolated from the dog urine [3]. The compound was later launched in the form of its sodium salt under several brand names, e.g., Mevastin (Sankyo) or Pravachol (BMS) (Fig. 2). The commercial production is done by microbial oxidation of mevastatin using *Streptomyces carbophylus*.

Extensive research into semisynthetic statins, mainly based on lovastatin molecule modification, led to some SAR. The assumption, that the 3,5-dihydroxy carboxylic region present also in mevalonic acid is essential was confirmed. The most frequent modification was replacement of the acyl group in the tetralin skeleton by various aliphatic and aromatic acyls. As a result, simvastatin (Zocor) was discovered by Merck [4]. This drug is at present the only semisynthetic statin on the market.

Further logic development was research into fully synthetic statins. The tetralin skeleton was substituted by various aromatic and heterocyclic moieties. The possibility of the replacement of the ethylenic bridge was also studied. This modification led to the discovery of a number of active compounds, but only limited number of them had affinity similar or better than lovastatin or simvastatin. For the synthetic statins, the synthesis of the most active stereoisomer by a way useful for the commercial production was a great challenge. Novartis solved this problem by introducing fluvastatin (Lescol), which is used as a sodium salt of *cis*-racemate [5]. However, fluvastatin is the only statin on the market that is not used as the only enantiomer. The currently most important statin atorvastatin (Lipitor, Sortis) is used as the corresponding hemicalcium salt [6] (Fig. 3).

In 1998 Bayer launched very active cerivastatin (Baycol, Lipobay) with daily doses in tenths of mg while other statins are used in doses of two orders of magnitude higher [7]. However, when more than 50 cases of death due to rhabdomyolyse were registered, the drug was withdrawn in 2001. Most of these lethal cases were probably caused by simultaneous use of a fibrate gemfibrozil.

In 2003 rosuvastatin (Crestor) of Astra-Zeneca was launched. The drug is also used as the hemicalcium salt. The most recent statin on the market is itavastatin [9] launched in Japan (Fig. 4).

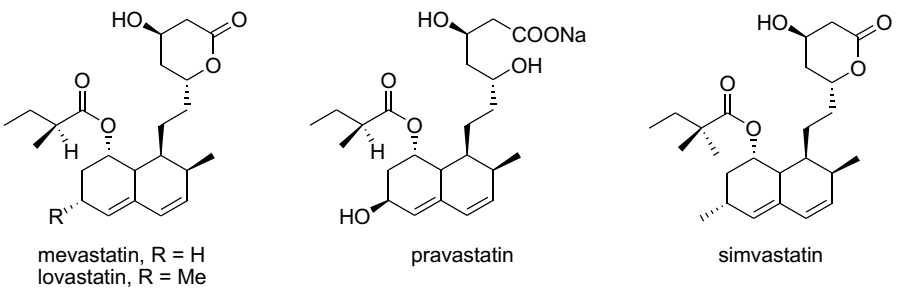


Figure 2.

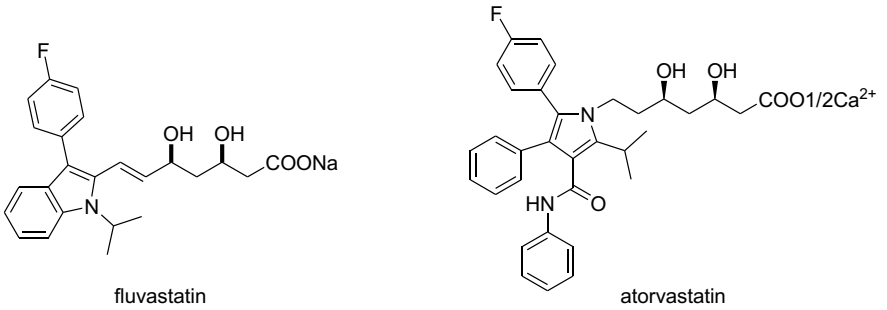


Figure 3.

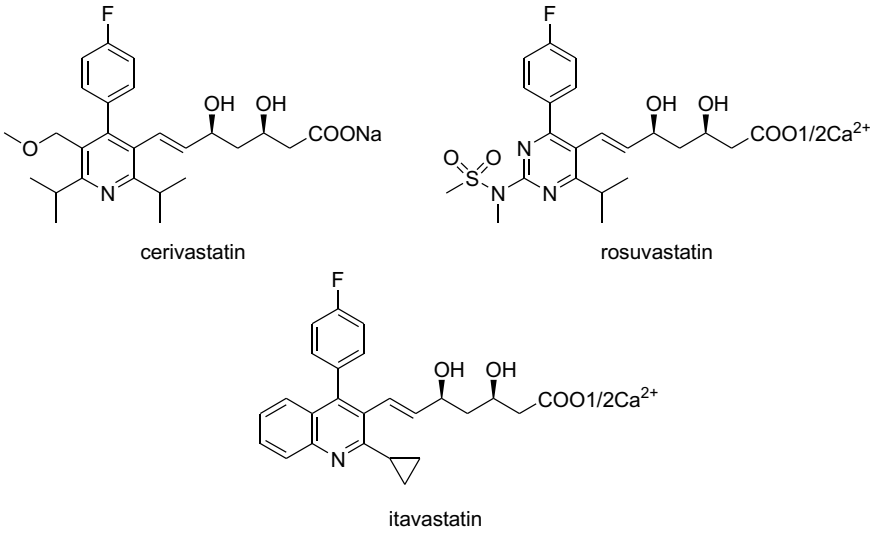


Figure 4.

Currently the statins are commercially the most successful therapeutic group and therefore they are targeted by many generic companies. Several leading Indian and Chinese companies mastered the very demanding production of key intermediates and practically all leading generic companies have their own production of some statins. In Zentiva, we have a successful production of API of simvastatin that is marketed as Simvacard. In 2005 generic version of atorvastatin was launched as Torvacard. The API of atorvastatin was developed in the Research Institute of Pharmacy and Biochemistry, a daughter of Zentiva. Of course, we are also interested in rosuvastatin and itavastatin.

The lecture will discuss some aspects of development of these statins in Zentiva. These aspects include situation with polymorphs, some synthetic aspects, as well as stability problems of these drugs. Of course, the intellectual property interests of Zentiva will limit the information discussed. Some perspective combinations of statins with other classes of hypolipidemics will also be briefly mentioned.

1. Endo A.: *J. Lipid. Res*. **33** (1992) 1569.
2. Tolbert J. A.: *Natl. Rev. Drug, Discov.* **2** (2003) 517.
3. Kishida Y, Naito A, Iwado S, Terahara A, Tsujita Y.: *Yakugaku Zasshi* **111** (1991) 469.
4. Castaner J., Prous J.: *Drugs Fut.* **13** (1988) 531.
5. Lawrence J. M., Reckless J. P.: *Expert Opin. Pharmacother.* , **3** (2002) 1631.
6. Castaner J., Graul A.: *Drugs Fut.* **22** (1997) 956.
7. a) McClellan K. J., Wiseman L. R., McTavish D.: *Drugs* , **55**, (1998) 415; b) Plosker G. L., Dunn C. I., Riggitt D. P.: *Drugs* **60** (2000) 1179.
8. Castaner J., Graul A.: *Drugs Fut.* **24** (1999) 511.
9. Flores N. A.: *Curr. Opin. Investig. Drugs*. **3** (2002) 1334.

CAPILLARY ANALYTICAL CHEMISTRY IN PHARMACEUTICAL ANALYSIS

Pavel Coufal

Charles University, Faculty of Science, Dept. of Analytical Chemistry, Albertov 2030, 128 40 Prague

Nowadays, miniaturization is a common trend strongly influencing scientific research, technical and technological progress and each activity of human beings. The miniaturization, in general, helps people to distinguish the smallest particles of matter, manipulate with these particles on nanometer level, read information hidden in them and speed up communication between any two points of our space.

In the past decade, miniaturization started to affect strongly the analytical chemistry as well. This scientific field seems to be under a permanent pressure of analytical chemists form the pharmaceutical and chemical industry to improve significant parameters of all analytical methods developed and under current development. The analytical chemists want to increase sensitivity, improve selectivity, shorten analysis time, decrease sample volume, use expensive agents, reduce analysis uncertainty, guarantee preciseness and accuracy, cut down expenses and minimize impact on environment at each analytical method. Analytical procedures applying methods based on advantages of the miniaturization show many of the above mentioned parameters improved. Capillary separation techniques, like capillary electrophoresis and capillary liquid chromatography, are consequences of the miniaturization influence in the field of analytical separation methods.

Recently, the miniaturization in the analytical separation techniques has reached such a great progress that these techniques were realized in the planar chip format. After construction of first gas chromatograph on the chip, activity of scientists was focused on the capillary electrophoresis since its realization in the planar format on chip is less difficult than in the case of liquid chromatography. However, the capillary electrophoresis does not exhibit such a versatility and robustness in separation mechanisms as the liquid chromatography, whose miniaturization and chip realization are characterized by many technical problems. Despite all these technical difficulties, the capillary liquid chromatography was placed on the chip and this format of liquid chromatography can be used for solving such difficult analytical problems like the identification of proteins, peptides and metabolites of chemotherapeutics.

Capillary separation techniques represent a miniaturization level lying in between the conventional separation methods and the planar ones realized in the chip format. On the one hand, capillary separation methods bring features of the miniaturization, but on the other hand, they do not suffer from the big technical difficulties being typical at the chip format techniques. Moreover, the capillary separation methods are very compatible with mass spectrometry as the detection technique, which enables a reliable identification of analytes. Capillary electrophoresis is generally considered as the separation technique, using which an analytical method for quantification of new analytes can be developed very fast. The developed electrophoretical method mostly produces analytical results in a very short analysis time, however, the results are of a low accuracy and poor reproducibility. Capillary liquid chromatography, on the other hand, generates reproducible and precise analytical results, collection of which is more expensive and time-consuming.

Ropinirol, 4-[2-(dipropylamino)ethyl-1,3-dihydro- 2H -indol-2-one, is anti-Parkinson's disease chemotherapeutic discovered by SmithKline Beecham Pharmaceuticals. Capillary zone electrophoresis and capillary liquid chromatography methods developed and optimized for quantification of ropinirol and its five impurities from synthesis are critically compared. Both capillary analytical techniques enables determination of the ropinirol impurities on the concentration level as low as 0,1 % (w/w). It is demonstrated that the capillary zone electrophoresis results are faster but less reproducible than the capillary liquid chromatography ones. As a matter of fact, capillary zone electrophoresis makes it possible to evaluate dissociation constants of ropinirol and its impurities from the experimental data of electrophoretic mobilities measured during optimization of the electrophoretic method. The low reproducibility of results in capillary zone electrophoresis may be caused by unstable electroosmotic flow, velocity of which is always influenced by many experimental factors. Addition of some agents into the background electrolyte in capillary zone electrophoresis can significantly reduce and stabilize electroosmotic flow and hence, improve its reproducibility including repeatability of the analytical results. This kind of effect of hydroxyethylcellulose on the electroosmotic flow and analytical method reproducibility is shown in capillary zone electrophoretic analysis of amino acids.

Capillary zone electrophoresis and capillary liquid chromatography are powerful miniaturized separation techniques suitable for analyses of various samples. They may replace conventional high performance liquid chromatography in routine analyses in pharmaceutical and chemical industry as they bring all the advantages of miniaturization into the analytical separation techniques. Capillary zone electrophoresis seems to be better for fast quantification of analytes in samples from the production procedure. Capillary liquid chromatography is a perfect analytical tool for precise and reproducible quantification of compounds in final products of pharmaceutical and chemical industry.

1. P. Coufal, K. Štulík, H. A. Claessens, M. J. Hardy, M. Webb, "A determination of the dissociation constants of ropinirole and some impurities and their quantification using capillary zone electrophoresis" *J. Chromatogr. B*, **720** (1998) 197-204.
2. P. Coufal, K. Štulík, H. A. Claessens, M. J. Hardy, M. Webb, "Separation and quantification of ropinirole and some impurities using capillary liquid chromatography", *J. Chromatogr. B*, **732** (1999) 437-444.
3. P. Coufal, J. Zuska, T. van de Goor, V. Smith, B. Gaš, "Separation of twenty underivatized essential amino acids by capillary zone electrophoresis with contactless conductivity detection", *Electrophoresis*, **24** (2003) 671-677.

MODERN ELECTROANALYTICAL METHODS IN THE ANALYSIS OF BIOACTIVE COMPOUNDS

Jiří Barek and Jiří Zima

UNESCO Laboratory of Environmental Electrochemistry, Department of Analytical Chemistry, Charles University, 128 43 Prague 2, Czech Republic, e-mail: Barek@natur.cuni.cz

Many chemical substances can positively or negatively affect human health even in extremely low concentrations. Therefore, the monitoring of their extremely low concentrations in complex environmental and biological matrices is among top priorities of modern analytical chemistry. In many cases modern voltammetric techniques can successfully compete with advanced chromatographic or spectrometric methods because they are inexpensive, extremely sensitive and they present an independent alternative to so far prevalent spectrometric and separation techniques.

The aim of this contribution is to demonstrate the scope and limitations of modern electroanalytical methods in the analysis of biologically active organic compounds. First, the relationship between structure and electrochemical behavior will be briefly discussed to show which types of biologically active organic substances are amenable to voltammetric and/or amperometric determination. Afterwards, recent developments in the field of electroanalytical chemistry of biologically active substances will be described taking into account the fact that the development of modern voltammetric and amperometric techniques proceeds in three parallel lines: Development of electrodes (hanging mercury drop electrode (HMDE), solid amalgam electrodes, carbon paste electrodes, chemically modified electrodes, screen printed electrodes, etc.), development of measuring techniques (DC voltammetry, differential pulse voltammetry, etc.) and development of preconcentration techniques (anodic stripping voltammetry, adsorptive stripping voltammetry, etc.).

The quality of the electrode is probably the most important factor, the electrode material is playing the crucial role. Even 83 years after the invention of polarography by professor Heyrovský, mercury electrodes are still the best available voltammetric sensors. Nevertheless, great attention is being paid to the development, testing and application of new electrode materials with the aim to increase sensitivity, selectivity and available potential window and to decrease noise and problems with electrode passivation. In this contribution, some of the advantages of less usual electrode materials for the determination of nanomolar concentrations of selected biologically active compounds will be shown. The application of the following non-traditional types of electrodes suitable for the determination of biologically active organic compounds either in batch analysis or in flowing systems (especially high performance liquid chromatography (HPLC), flow injection analysis (FIA), and sequential injection analysis (SIA)) will be demonstrated on methods recently developed in our UNESCO laboratory of environmental electrochemistry:

- Solid amalgam electrodes (environmentally friendly alternatives to mercury electrodes suitable both for batch analysis and for HPLC-ED or FIA-ED with limit of determination (LOD) down to 10^{-7} mol/L)
- Bare and cyclodextrin modified carbon paste electrodes (suitable for the determination of oxidizable carcinogens with LOD down to 10^{-7} mol/L).
- Glassy carbon paste electrodes (compatible with a high content of organic solvent and thus applicable for HPLC-ED with a high content of organic modifier in a mobile phase with LOD down to 10^{-7} mol/L).
- Diamond film electrodes (very useful alternative to glassy carbon electrodes especially for flowing systems characterized by very low noise and broad potential window and applicable to HPLC-ED or FIA-ED of electrochemically oxidizable organic substances with LOD down to 10^{-8} mol/L).
- Platinum and copper electrodes (new types of tubular or microcylindrical platinum or copper electrodes suitable for HPLC-ED determination of oxidizable organic compounds with LOD around 10^{-8} mol/L).

Attention will be paid to various possibilities of increasing both the sensitivity and selectivity of these methods using preconcentration of tested substances on the surface of working electrodes and their preliminary separation and preconcentration using liquid-liquid extraction or solid phase extraction.

Moreover, the advantages of combination of HPLC, FIA and SIA with electrochemical detection will be discussed in terms of sensitivity, selectivity and investment and running costs and some recent applications will be shown.

Finally, the possibilities of new approaches based on the combination of nanotechnologies and biotechnologies with electroanalytical methods will be described. Attention will be paid to the application of carbon paste electrodes modified with gold nanoparticles or with multi wall nanotubes for monitoring of electrochemically active substances in immobilized cells, to the application of magnetic field to increase both the sensitivity and the selectivity of various amperometric enzymatic determinations and to the application of magnetic beads for determination of extremely low concentrations of nucleic acids. Some other interesting aspects of the combination of nanotechnologies with electrochemical methods will be discussed as well.

This research was supported by the Grant Agency of the Czech Republic (project 203/03/0182).

X-RAY DIFFRACTION APPLIED IN PHARMACEUTICAL INDUSTRY

Hana Petříčková

R&D Analytical Department Zentiva a.s., U kabelovny 130, 102 37 Prague 10, Czech Republic

Modern Pharmaceutical R D department is toothless without solid-state characterization techniques. XRPD plays an important role at this field; it is a method of first choice when crystalline material is analysed. On the other hand also amorphous materials can be treated using X-rays. This is possible due to the general fact that powder diffraction pattern is the fingerprint of studied material. Even in the mixtures each composites can be identified.

Main pharmaceutical applications of X-ray diffraction such as polymorphic studies, stability testing at non-ambient conditions (prediction possible risks during storage), LOD determination or crystal structure determination will be discussed. Identification of low amount of impurities (undesired polymorphic form) is standard test involved in validation of the XRPD method. Crystal structure determination from powder will be compared with commonly used single crystal structure determination.

THE USE OF SYNCHROTRON RADIATION FOR THE STRUCTURAL ANALYIS OF SUBSTANCES IN POWDER SAMPLES AND MICROCRYSTALS

Hušák M.

Department of Solid State Chemistry, Institute of Chemical Technology Prague, Technická 5, 166 28 Prague 6, Czech Republic

We are often faced with the following situation: it is necessary to solve the structure of a phase for which all attempts failed to prepare single crystal big enough for the standard measurement on laboratory diffractometer. In such situation it is impossible to obtain the structure parameters by standard single-crystal X-ray diffractions methods. There are often some reasons why it is necessary to solve the structure of such phase at any cost. The phase structure is necessary for commercial pharmaceutical product characterization, the phase has some unique physical properties determined by its unknown structure or alternatively, we need to confirm the absolute configuration of the molecules forming the phase.

The help of high intensity X-ray sources, especially synchrotron radiation, could solve such sort of problems. In the lecture we will discuss 2 examples of the synchrotron radiation usage. The first one will be the complete structure solution from powder data only of simvastatin low temperature phases. The second will be structure solution of metergoline phase II from micro crystal. Data measurements were in both cases done on BM01 beam line of ESRF facility in Grenoble.

In the first case we were trying to solve the structure of low temperature phases of simvastain. Preliminary measurement had indicated the phase stable at room temperature shows first phase transition at 260 K and second phase transition at 220 K. High resolution powder data of both low-temperature phases were measured. The structures were solved by FOX software and refined by GSAS system. It was found that the phase existing at 260 K has identical space group ($P2_12_12_1$) as the room temperature phase. It differs only in the molecule side chains orientation. The second phase formed under 220 K differs in space group ($P2_1$), crystal system (monoclinic) and it has 2 independent molecules in the unit cell. The data obtained by the structure solution from powder were independently confirmed by solid state NMR measurement.

The second example of the synchrotron radiation is the solution of metergoline phase II. All efforts to solve the structure from normal single crystals failed, because it was impossible to obtained big enough crystals. We had measured a high-resolution powder diffraction data on BM01. Unfortunately indexing of the powder data had indicated unit cell to big for any existing powder structure solution method. The size of the unit cell indicated presence of 2 independent molecule of metergoline – this gives too much degree of freedom for powder diffraction based structure determination. We had decided to try to obtain enough good monocrystal diffraction data from micro crystal by the help of high intensity radiation and four cycle diffractometer with area detector. After some trial and error experiments enough good micro crystal was finally found and the structure was solved (monoclinic, space group $C2$, two molecules in asymmetric unit cell as expected).

THE USE OF ENZYMATIC AND NON-ENZYMATIC METHODS FOR THE PREPARATION OF ENANTIOPURE MOLECULES

Derek R. Boyd

The Queen's University of Belfast, Belfast, BT9 5AG, UK.

Bacterial redox enzymes have the potential to catalyse a wide range of oxidation reactions in a highly regio- and stereo-selective manner. Examples of the application of bacterial non-heme iron oxygenase (dioxygenase) enzymes to produce enantiopure monohydroxylation (benzylic oxidation), monosulfoxidation (heteroatom oxidation), dihydroxylation (arene and alkene oxidation), trihydroxylation (benzylic/arene oxidation) and tetrahydroxylation (arene *bis*-dihydroxylation) products *via*-asymmetric synthesis and kinetic resolution routes have been found (Fig. 1).

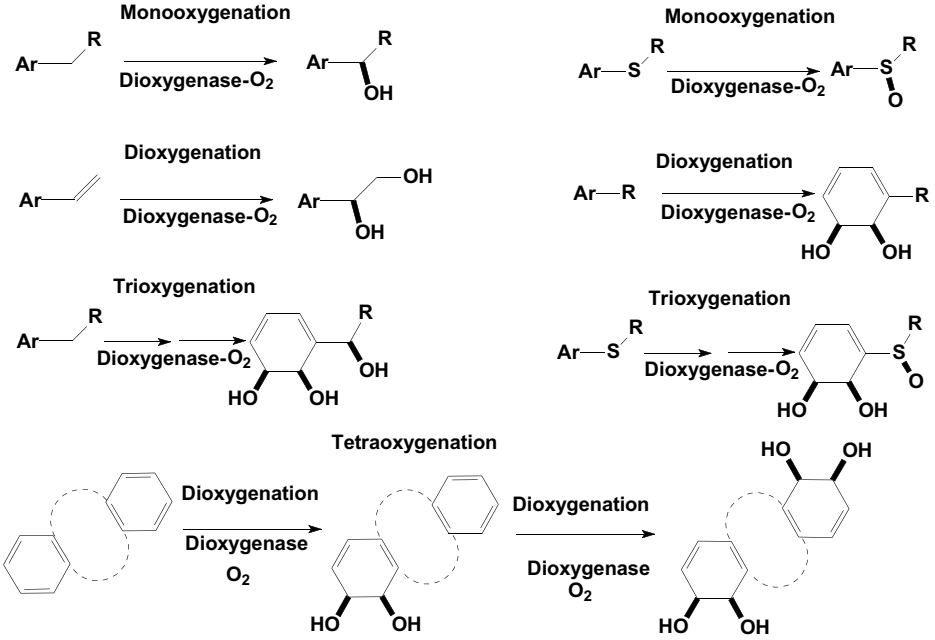


Figure 1.

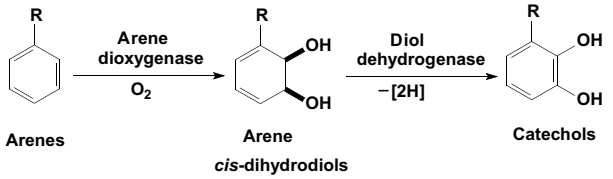


Figure 2.

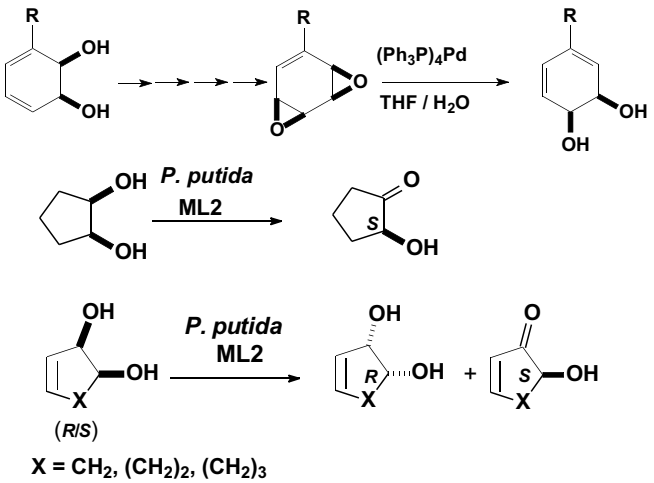


Figure 3.

The capacity of biocatalysts to form only one enantiomer is often perceived as a disadvantage. The use of mutant and recombinant bacterial strains containing different types of dioxygenase enzymes has, in some cases, allowed unnatural regioisomers and enantiomers (enantiocomplementarity) of oxygenated bioproducts to be formed. Alternative chemoenzymatic routes to unnatural arene *cis*-diols have been developed (Fig. 2).

Cis-diol dehydrogenase enzymes have also been used to produce enantiopure molecules (*cis* -diols and ketols) by both asymmetric synthesis and kinetic resolution methods (Fig. 3).

The synthetic versatility of this new chiral pool of enantiopure bioproducts obtained using dioxygenase enzymes, has been demonstrated by the chemoenzymatic synthesis of a wide range of natural and unnatural products. The proof of concept is exemplified by the synthesis of selected target molecules including alkaloids, marine alkaloids, inositols, pseudosugars, and pharmaceuticals (Fig. 4).

A recently developed application of bioproducts obtained using dioxygenase-catalysed asymmetric hydroxylation methods has been in the production of chiral ligands for use in homogenous catalytic asymmetric synthesis. Examples of enantiopure *cis*-1,2-aminoalcohols, 2,2'-bipyridines and the corresponding *bis*-amine N-oxides have been produced by chemoenzymatic synthesis. The utilisation of these chiral ligands in selected asymmetric oxidation, reduction and C-C bond forming reactions is presented (Fig. 5).

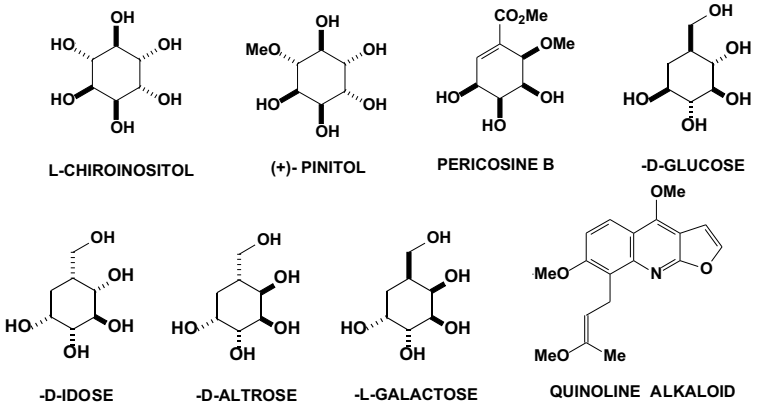
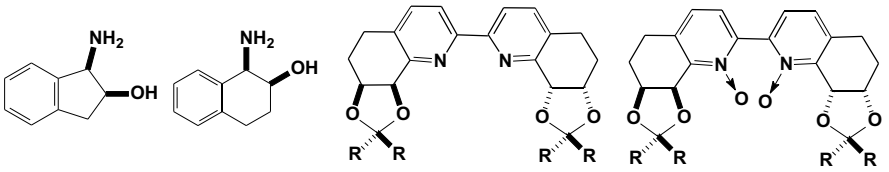


Figure 4.

1. D. R. Boyd, G. N. Sheldrake, *Nat. Prod. Reports*, **15** (1998) 309**.**
2. T. Hudlicky, D. Gonzalez, D. T. Gibson, *Aldrichim. Acta*., **32** (1999) 35.
3. D. T. Gibson, R.E. Parales, *Curr. Opin. Biotechnol*., **11** (2000) 236.
4. D. R. Boyd, N. D Sharma, C. C. R. Allen, *Curr. Opin. Biotechnol.*, 12 (2001) 564.
5. R. A. Johnson, *Org. Reactions*, **63** (2004) 117.
6. D. R. Boyd and T. Bugg, *Org. Biomol. Chem.*, (2005), in the press.



BIOPRODUCT-DERIVED ENANTIOPURE *CIS*-AMINOALCOHOL AND BIPYRIDINE LIGANDS

Figure 5.

BIOCATALYTIC, CHEMOCATALYTIC AND CHEMOENZYMATIC METHODS FOR THE SYNTHESIS OF PURE ENANTIOMERS

Roger A. Sheldon

Biocatalysis and Organic Chemistry, Delft University of Technology, The Netherlands
R.A.Sheldon@tnw.tudelft.nl

The use of biocatalytic transformations for the production of fine chemicals in general, and enantiomerically pure compounds in particular, has undergone an explosive growth in the last decade. Advances in recombinant DNA techniques has made it possible to produce virtually any enzyme for a commercially acceptable price. And advances in protein engineering and *in vitro* evolution have enabled the fine tuning of enzymes such that they exhibit the optimum properties with regard to substrate specificity, activity, (enantio)selectivity, and stability. Furthermore, the development of an ever increasing arsenal of immobilization techniques has provided effective methods for optimizing the operational performance and recovery and recycling of the enzyme.

At the same time, significant advances have been achieved in the application of catalytic asymmetric synthesis with chiral metal complexes, particularly for enantioselective reductions. New developments in the biocatalytic and chemocatalytic synthesis of enantiomerically pure alcohols and amines *via* asymmetric synthesis and (dynamic) kinetic resolution processes will be reviewed. The combination of organometallic catalysis with biocatalysis in chemoenzymatic syntheses of pure enantiomers will also be discussed. Furthermore, a highly effective methodology for the immobilization of enzymes (cross-linked enzyme aggregates CLEAs) will be presented. The use of combi-CLEAs containing more than one enzyme for one-pot, multi-step syntheses will also be discussed.

1. R. A. Sheldon, 'Chirotechnology: the Industrial Synthesis of Optically Active Compounds', Marcel Dekker, New York, 1993.
2. R. A. Sheldon and F. van Rantwijk, *Austr. J. Chem.* **57**, (2004) 281-289.
3. A. J. J. Straathof, S. Panke and A. Schmid, *Curr. Opin. Biotechnol.* **13** (2002) 548-556.
4. F. van Rantwijk and R.A. Sheldon, *Tetrahedron* **60** (2004) 501-519.
5. L. Cao, L.M. van Langen and R.A. Sheldon, *Curr. Opin. Biotechnol.* **14** (2003) 387-394.
6. L.M. van Langen, R.P. Selassa, F. van Rantwijk and R.A. Sheldon, *Org. Lett.* **7** (2005) 327-329.

PREPARATION OF PHARMACEUTICAL SUBSTANCES BY THE USE OF NITRILE- AND AMIDE-CONVERTING ENZYMES

Ludmila Martínková and Vladimír Křen

Laboratory of Biotransformation, Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-142 20 Prague; E-mail:martinko@biomed.cas.cz

Nitriles are fairly widespread in nature, mainly in plants but also in microorganisms and the animal kingdom [1]. Therefore, it is not surprising that enzymes exist that are able to degrade nitriles. The most important pathway in nitrile degradation (Scheme 1) is hydrolysis, which proceeds either directly (pathway I) or *via* an intermediate amide (pathway II).

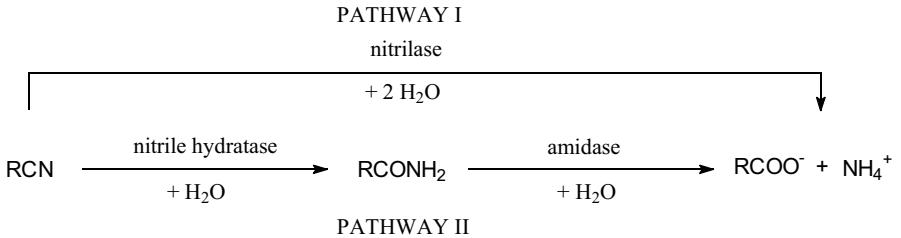
The nitrile- and amide-transforming enzymes are able to accept a vast number of unnatural nitriles as substrates. This property has been widely used in biocatalysis. The benefits of using nitrile-converting enzymes (reduction in energy costs, reduction of waste, pure products, high product yields) have been highly appreciated. New substrates and practical applications of these enzymes are constantly reported. In particular, the potential of nitrile- and amide-converting enzymes to afford optically pure products attracts an increasing attention in the recent years. To date a relatively low number of the processes have been commercialized (see below). Nevertheless, the numbers of processes studied at laboratory scale indicate that the commercial use of nitrile-converting enzymes will increase in near future. In the past several years the nitrile- and amide-converting enzymes have been studied as tools for the production of compounds, which are of interest as building blocks for pharmaceuticals with a broad spectrum of biological activities (antibacterial, -antagonist, hemostatic, antidepressant, antihypertensive, antiflammatory *etc.*) [2, 3]. The aim of this lecture is to provide a survey of these applications (both industrial and laboratory-scale) including our results in the field.

Many companies (e.g., Mitsubishi Rayon Co., Lonza, DuPont, Dow, Diversa, BASF and DSM) [4] became interested in the applications of nitrile- and amide-converting enzymes. Though multiton-scale productions of this type are rare, other processes are in the exploratory phase. Lonza has a long tradition in the use of biocatalysts, among them nitrile hydratases, nitrilases and amidases [5]. Nicotinamide (food and feed additive) is produced at a scale of over 3,500 tons per years by this company under a license from Mitsubishi Rayon Co. who introduced the first industrial use of nitrile hydratase (production of acrylamide) in the 1980s. Furthermore, the use of enzymatic nitrile/amide hydrolysis for the production of several chiral building blocks has been implemented by Lonza at smaller scales. These products, the processes of their manufacture and applications in drug synthesis are summarized in Table 1.

Racemic cyanohydrins have been studied, e.g. by BASF [6] and Diversa [7], as substrates affording valuable optically active -carboxylic acids on enzymatic hydrolysis. The production of (*R*)-mandelic acid and derivatives thereof has been performed by BASF at a scale of several tons per year [6].

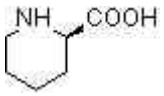
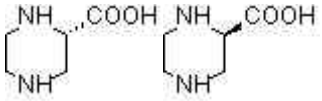
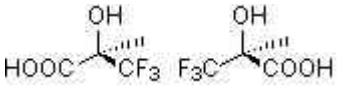
An alternative route to enantiopure -carboxylic acids is the two-step process consisting in;
1) the enzymatic synthesis of optically pure cyanohydrins using the strictly enantioselective (*R*- or *S*-selective) oxynitrilases (hydroxynitrile lyases) and 2) the stereo retentive enzymatic hydrolysis of these intermediates using non-selective nitrile hydratases/amidases. This method was applied to the synthesis of (*R*)-2-chloromandelic acid, the chiral building block of an antithromobotic agent, and (*R*)-2-hydroxy-4-phenylbutyric acid, the key intermediate for the synthesis of ACE-inhibitors [8].

In our Laboratory we concentrate mainly to the development of novel biocatalysts for nitrile and amide biotransformations. The bacterial nitrile-utilizing isolates (*Bacillus subtilis*, *Bacillus licheniformis*, *Rhodococcus equi*, *Corynebacterium* sp.) metabolized nitriles *via* pathway II (Scheme 1). *Rhodococcus equi* A4 (the strain with the highest nitrile hydratase activity) proved to be efficient for the selective biotransformations of more than 60 nitriles of distinct structures (aliphatic, arylaliphatic, aromatic, heterocyclic, alicyclic compounds) [2, 9]. The nitrile hydratase from this strain is a useful tool for processes, which require a mild and selective hydration of a cyano group in sensitive compounds such as cyanohydrins, acyloxynitriles, -methylenenitriles or -ketonitriles. For example, -ketoamides are versatile intermediates for the synthesis of the antibiotic tirandamycin, alkaloids, AMPA receptor antagonists *etc.* The chemical hydration of the corresponding nitriles is difficult due to side reactions (mainly polymerization), while whole-cell catalyzed hydration gives a mixture of amides and acids unless an inhibitor of the amidase is used [10]. On the other hand, the purified



Scheme 1.

Table 1. Chiral building blocks produced by Lonza [5]..

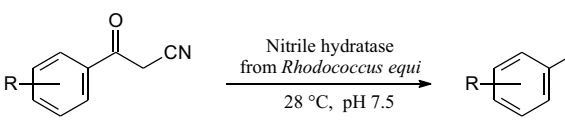
Product	Key enzyme	Product applications
 (*S*)-pipecolic acid	Amidase (*Pseudomonas fluorescens*)	Intermediate for Incel (Vertex treatment of cancer multi-drug resistance) and local anaesthetics Naropin (Astra-Zeneca) and Chirocaine (Chiroscience)
 (*R*)- and (*S*)-piperazine-2-carboxylic acid	(*R*)-Selective amidase (*Burkholderia* sp.) (*S*)-Selective amidase (*Klebsiella terrigena*)	Intermediates of Crixivan (Merck HIV protease inhibitor), *N*-methyl-D-aspartate antagonist, cardioprotective nucleoside transport blocker *etc.*
 (*R*)- and (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionic acid	Amidase (*Klebsiella oxytoca*, expressed in *E. coli*)	Potential intermediates for pharmaceuticals (e.g., for treatment of diabetes)
 (*S*)-2,2-dimethyl-cyclopropanecarboxamide	Amidase (*Comamonas acidivorans*, expressed in *E. coli*)	Intermediate for Cilastatin (Merck inhibitor of degradation of penem and carbapenem antibiotics in kidney)

nitrile hydratase from *R. equi* affords -ketoamides as the only products from -ketonitriles (Scheme 2) [11].

The two-step reaction catalyzed by whole cells of the microorganism was applied to the conversion of sensitive nitriles into carboxylic acids. A remarkable enantioselectivity was achieved by the combination of the nitrile hydratase and amidase for some racemic substrates such as -arylpropionitriles [9], precursors of NSAIDs, and alicylic -aminonitriles which afford optically enriched -amino acids (Scheme 3) useful for the synthesis of -oligopeptides and heterocycles or exhibiting biological activities *per se*, such as the antifungal antibiotic cispentacin (1*R*,2*S*-*cis*-aminocyclopentane carboxylic acid) [12].

An amidase active towards the amido group in lysergamide was also found in *Rhodococcus equi* [13]. This enzymatic activity was exploited in the chemoenzymatic preparation of lysergic acid from peptide alkaloids (Scheme 4).

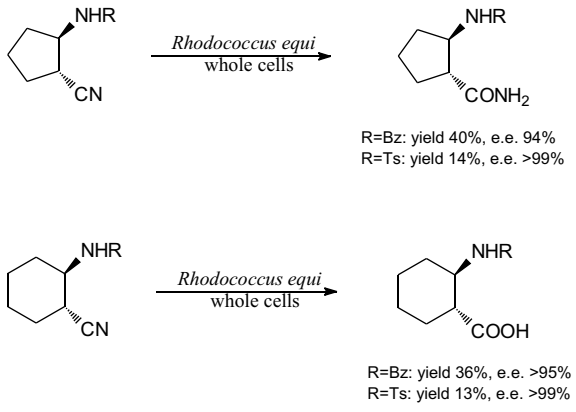
At the second stage of our study we turned our attention to screening of filamentous fungi as a largely unexplored source of nitrile-converting enzymes. This work complemented our library of nitrile- and amide-converting enzymes with "true" nitrilases catalyzing the direct hydrolysis of nitriles through pathway I (Scheme 1) [14]. Several nitrilases differing in substrate specificity, reaction optima, temperature and pH stability became available from strains of *Aspergillus niger*, *Fusarium solani, Fusarium oxysporum* and *Penicillium multicolor*. Most of these enzymes surpass the nitrile hydratases in their stability that makes immobilization and continuous use possible. Superior substrates of the fungal nitrilases are aromatic and heterocyclic nitriles. Therefore, the enzymes can be employed in the production of valuable carboxylic acids, e.g.,



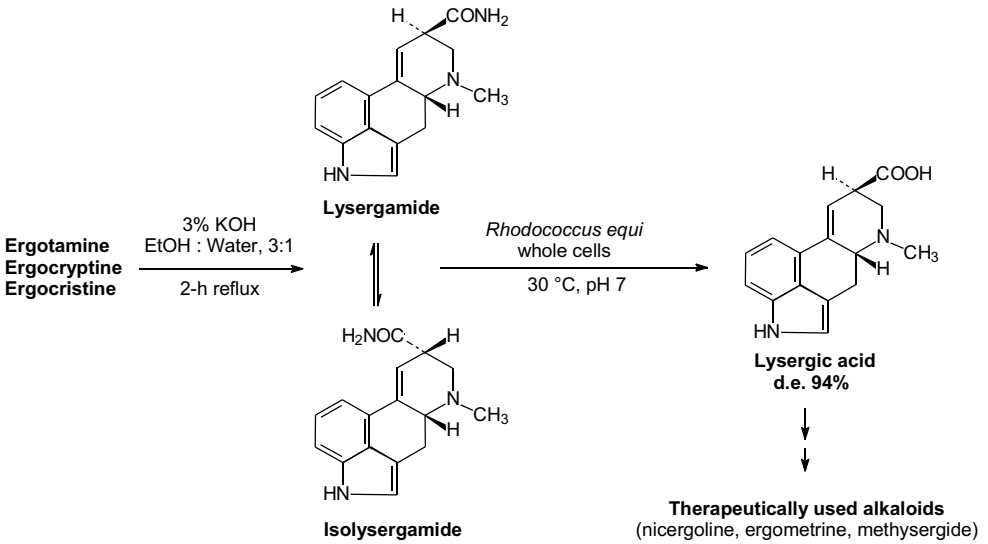
R = *m*-Cl, *p*-Cl, *m*-OMe

isol. yield 86-96%

Scheme 2



Scheme 3



Scheme 4

nicotinic acid (food and feed supplement), isonicotinic acid (active in antitubercular therapy) and picolinic acid (novel antiviral agent [15]) from the corresponding nitriles.

Despite their biocatalytic utility, the nitrile- and amide-converting enzymes have not yet found such a broad application as other hydrolytic enzymes (lipases, esterases, proteases). The reasons seem to be the unsatisfactory choice of these enzymes on the market,their price and sometimes also their low stability. Therefore, organic chemists often prefer to prepare whole-cell biocatalysts on site. Besides, previously nitrilases and nitrile hydratases were considered to be insensitive towards the chirality of the substrate. Though this is true for some reactions, examples of excellent stereoselectivity have been demonstrated for these enzymes. Obviously, more research towards new enzyme resources and improvement of the enzyme operation is needed in order to exploit the potential of this group of enzymes.

Acknowledgement. *This work is supported by the grants IAA 4020213 (Grant Agency of the ASCR), 203/05/2267 (Czech Science Foundation), OC D25.001 (Ministry of Education of the CR) and the institutional research concept AV0Z50200510 (Institute of Microbiology ASCR).*

1. Legras J.L., Chuzel G., Arnaud A. and Galzy P. (1990) Natural nitriles and their metabolism: Review. *World J. Microbiol. Bioechnol.* 6, 83-108.
2. Martínková L. and Křen V. (2002) Nitrile- and amide converting microbial enzymes: stereo-, regio- and chemoselectivity. *Biocatal. Biotrans.* 20, 73-93.
3. Martínková L. and Mylerová V. (2003) Synthetic applications of nitrile-converting enzymes. *Curr. Org. Chem.* 7, 1279-1295.
4. Brady D., Beeton A., Zeevart J., Kgaje C., van Rantwijk F. and Sheldon R.A. (2004) Characterization of nitrilase and nitrile hydratase biocatalytic systems. *Appl. Microbiol. Biotechnol.* 64, 76-85.
5. Shaw N.M., Robins K.T. and Kiener A. (2003) Lonza: 20 years of biotransformations. *Adv. Synth. Catal.* 345, 425-435.
6. Schmid A., Hollman F., Park J.B. and Bühler B. (2002) The use of enzymes in the chemical industry in Europe. *Curr. Opin. Biotechnol.* 13, 359-366.
7. DeSantis G., Zhu Z., Greenberg W.A., Wong K., Chaplin J. *et al.* (2002) An enzyme library approach to biocatalysis: development of nitrilases for enantioselective production of carboxylic acid derivatives. *J. Am. Chem.* Soc. 124, 9024-9025.
8. Osprian I., Fechter M.H., Griengl H. (2003) Biocatalytic hydrolysis of cyanohydrins: an efficient approach to enantiopure -hydroxy carboxylic acids. *J. Mol. Catal. B: Enzymatic* 24-25, 89-98.
9. Přepechalová I., Martínková L., Stolz A., Ovesná M., Bezouška K. *et al.* (2001) Purification and characterization of the enantioselective nitrile hydratase from *Rhodococcus equi* A4. *Appl. Microbiol. Biotechnol.* 55, 150-156.
10. Gotor V., Liz R. and Testera A.M. (2004) Preparation of unsubstituted -ketoamides by *Rhodococcus rhodochrous*-catalyzed hydration of -ketonitriles. *Tetrahedron* 60, 60-618.
11. Kubáč D., Vejvoda V., Slámová K., Kaplan O., Přikrylová V. *et al.* (2005) Mild conversion of nitriles by free and immobilized nitrile-converting enzymes. Biotrans 2005 Symposium Delft (NL) 3-8 July 2005, p. 195.
12. Winkler M., Martínková L., Knall A.C., Krahulec S., Klempier N. (2005) Synthesis and microbial transformation of -amino nitriles. *Tetrahedron* 61, 4249-4260.
13. Martínková L., Křen V., Cvak L., Ovesná M. and Přepechalová I. (2001) Hydrolysis of lysergamide to lysergic acid by *Rhodococcus equi* A4. *J. Biotechnol.* 84, 63-66.
14. Kaplan O., Nikolaou K., Pišvejcová A. and Martínková L. (2005) Hydrolysis of nitriles and amides by filamentous fungi. *Enzyme Microb. Technol.*, in print.
15. Romanowski E.G., Yates K.A., Kowalski R.P., Mah F.S., Gordon Y.J. (2005) Novel topical antiviral agent, PCL-016 (picolinic acid), inhibits adenovirus replication in the Ad5/NZW rabbit ocular model. *Invest. Ophth. Vis. Sci.* 46, 1924 Suppl. S.

ENANTIOSELECTIVITY OF HALOALKANE DEHALOGENASES

Zbynek Prokop[1], Yukari Sato[2], Tomas Mozga[1], Petr Jerabek[1], Dick B. Janssen[3], Yuji Nagata[4], Toshiya Senda[2], Jiri Damborsky[1]

[1]Loschmidt Laboratories, Masaryk University, Kamenice 5/A4, 625 00 Brno, Czech Republic;
[2]Advanced Institute for Science and Technology, 2-41-6 Aomi, Koto-ku, Tokyo 135-0064Japan;
[3]University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands;
[4]Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Sendai, 980-8577, Japan

When pharmaceuticals, agrochemicals, food additives and their synthetic intermediates are marketed as single enantiomers, high enantiomeric purities are required. Enzyme-catalyzed reactions have become popular alternatives to classical organic chemistry for its high selectivity and activity under mild reaction conditions. Haloalkane dehalogenases (EC 3.8.1.5) are enzymes able to remove halogen from halogenated aliphatic compounds by a hydrolytic replacement, forming the corresponding alcohols (Figure 1). Any cofactor or metal ion is not required for enzymatic activity of haloalkane dehalogenases [1].

Although, a number of haloalkane dehalogenases from different bacteria have been biochemically characterised, there have been no report that this family of hydrolytic enzymes can have a sufficient enantioselectivity for production of optically active alcohols. In 2001, Pieters and co-workers [2] have investigated chiral recognition of haloalkane dehalogenases DhlA from *Xanthobacter autotrophicus* GJ10 [3] and DhaA from *Rhodococcus rhodochrous* NCIMB 13064 [4]. The magnitude of the chiral recognition was low; a maximum E-value of 9 was reached after some structural optimization of the substrate. Twenty years after discovery of the first haloalkane dehalogenase, the development of enantioselective dehalogenases for use in industrial biocatalysis was defined as one of the major challenges of the field [5]. Hydrolytic dehalogenation of extended series of racemic substrates catalysed by haloalkane dehalogenases DhlA, DhaA, LinB from *Sphingomonas paucimobilis* UT26 [6] and DbjA from *Bradyrhizobium japonicum* USDA 110 [7] was performed in this study. High enantioselectivity was observed for haloalkane dehalogenase DbjA recently isolated from *B. japonicum* exhibiting E-value > 200 in kinetic resolution of several halogenated propionates and butyrates. High enantioselectivity was observed in synthesis of optically pure secondary alcohols, for example (*S*)-2-pentanol (E-value 145) and (*S*)-2-heptanol (E-value 28), which are key intermediates in production of anti-Alzheimer´s drugs. These experiments demonstrated for the first time that a member of haloalkane dehalogenase family possesses sufficient enantioselectivity for synthesis of optically pure compounds.

Further research is focused on study of structure-function relationships and mechanism of haloalkane dehalogenases enantioselectivity heading towards rational design of enzymes with improved selectivity and catalytic efficiency (see abstract Damborsky *et al*.).

1. Janssen, D. B., Pries, F., and Van der Ploeg, J. R. *Annual Review of Microbiology* **48** (1994) 163-191.
2. Pieters, R. J., Spelberg, J. H. L., Kellogg, R. M., and Janssen, D. B. *Tetrahedron Letters* **42** (2001) 469-471.
3. Keuning, S., Janssen, D. B., and Witholt, B. *Journal of Bacteriology* **163** (1985) 635-639.
4. Kulakova, A. N., Stafford, T. M., Larkin, M. J., and Kulakov, L. A. *Plasmid* **33** (1995) 208-217.
5. Janssen, D. B. *Current Opinion in Chemical Biology* **8** (2004) 150-159.
6. Nagata, Y., Miyauchi, K., Damborsky, J., Manova, K., Ansorgova, A., Takagi, M. *Applied and Environmental Microbiology* **63** (1997) 3707-3710.
7. Sato, Y., Monincová, M., Chaloupková, R., Prokop, Z., Ohtsubo, Y., Minamisawa, K., Tsuda, M., Damborský, J., Nagata, Y. *Applied and Environmental Microbiology* **71** (2005) 4372-4379.

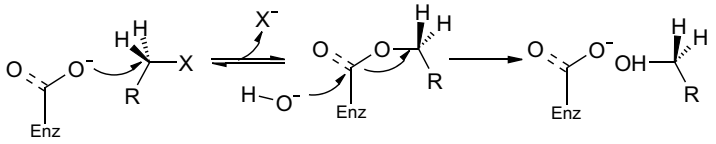


Figure 1 Reaction mechanism of haloalkane dehalogenases (Enz … enzyme)

COMPUTER-ASSISTED ENGINEERING OF ENANTIOSELECTIVE DEHALOGENASES

Jiri Damborsky[1], Petr Jerabek[1], Zbynek Prokop[1], Tomas Mozga[1], Yukari Sato[2], Yuji Nagata[3], Toshiya Senda[2]

[1]Loschmidt Laboratories, Masaryk University, Kamenice 5/A4, 625 00 Brno, Czech Republic;
[2]Advanced Institute for Science and Technology, 2-41-6 Aomi, Koto-ku, Tokyo 135-0064Japan;
[3] Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Sendai, 980-8577, Japan

High enantioselectivity with selected halogenated substrates was recently discovered for the member of haloalkane dehalogenases protein family - enzyme DbjA from *Bradyrhizobium japonicum* USDA 110 (see abstract Prokop *et al.*). Objectives of this research are to: (i) reveal molecular mechanism of enantioselectivity and (ii) engineer haloalkane dehalogenases for enantioselective biocatalysis. To meet these goals, we have solved the structure of DbjA by protein crystallography to atomic resolution and compared it with the structures of two closely related dehalogenases DhaA and LinB [1, 2]. Molecular docking was used to prepare the theoretical models of enzyme-ligand complexes for the substrates showing high enantioselectivity. Molecular dynamic simulation was used to monitor flexibility of ligands in the enzyme active site and quantum mechanic calculations to map reaction co-ordinates for individual enantiomers. The first results from these comparative and modelling analyses provided testable hypotheses on molecular mechanism of enantioselectivity of DbjA and lead to design of protein variants. Experimental construction and characterization of mutant proteins is currently in progress.

1. Newman, J., Peat, T. S., Richard, R., Kan, L., Swanson, P. E., Affholter, J. A., Holmes, I. H., Schindler, J. F., Unkefer, C. J., and Terwilliger, T. C. Haloalkane dehalogenase: structure of a *Rhodococcus* enzyme. *Biochemistry* **38** (1999) 16105-16114.

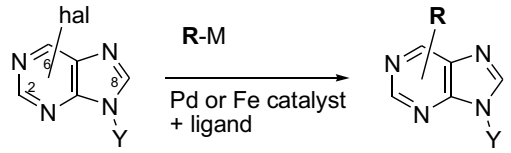
2. Marek, J., Vevodova, J., Kuta-Smatanova, I., Nagata, Y., Svensson, L. A., Newman, J., Takagi, M., and Damborsky, J. Crystal structure of the haloalkane dehalogenase from *Sphingomonas paucimobilis* UT26. *Biochemistry* **39** (2000) 14082-14086.

CROSS-COUPLING REACTIONS IN THE SYNTHESIS OF PHARMACEUTICAL ACTIVE SUBSTANCES. A FOCUS ON BIOACTIVE PURINE BASES AND NUCLEOSIDES

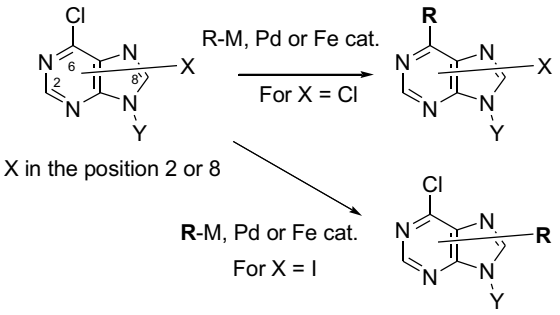
Michal Hocek

Centre for New Antivirals and Antineoplastics, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, CZ-16610 Prague 6, Czech Republic; e-mail: hocek@uochb.cas.cz

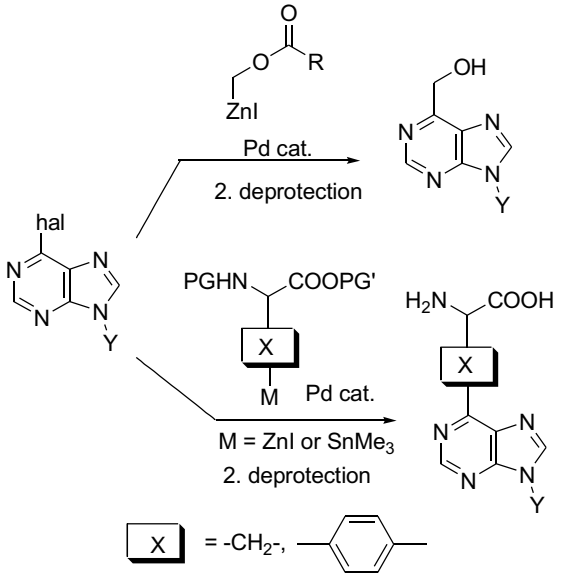
Cross-coupling reactions of aryl or alkyl halides with diverse types of organometallics catalyzed by transition metal complexes (usually Pd) are now one of the most important tools of organic synthesis formation of C-C bonds. In the first part of the talk, general introduction to this type of reactions will be given with selected examples of applications in the synthesis of pharmaceutically active compounds. In the second part, applications of cross-coupling reactions in the synthesis of novel modified nucleobases and nucleosides will be summarized and the biological activity of these compounds will be discussed.



Cross-coupling reactions of halopurines with diverse types of organometallics under Pd, Ni or Fe catalysis generally give access to the C-substituted purines. Each type of organometallic reagents appeared to be superior for an introduction of different substituents and also an effective catalytic system (metal+ligands) was found for each type of the reaction [1]. Thus alkylzinc halides or trialkylaluminum reagents under Pd-catalysis or alkylmagnesium halides under Fe-catalysis are the reagents of choice for an introduction of alkyl groups. The Stille couplings of alkenylstannanes are superior methodology for alkenylations, while the Sonogashira reactions of terminal acetylenes in presence of Cu(I) salts for introduction of alkynyl groups. Aryl groups are easily introduced via the Suzuki-Miyaura reactions of arylboronic acids, while hetaryl groups could be attached by cross-coupling reactions of hetarylzinc, -tin or -boron reagents. Alternative approach to the synthesis of 6-arylpurines by cyclomerizations of 6-alkynylpurines has also been developed [2].



Regioselectivity of the cross-coupling reactions of 2,6- and 6,8-dihalopurines has been studied and established. In dichloropurines, the cross-coupling occurs preferentially in the position 6, while in chloroiodopurines, it occurs in



the position of the iodine. The remaining chlorine atom could be replaced in another cross-coupling or nucleophilic substitution. This methodology has been applied [3] to the regioselective synthesis series of disubstituted purine bases and nucleosides for biological activity screening.

The above mentioned cross-coupling reactions were originally performed with simple unfunctionalized alkyl-, alkenyl- or arylorganometallics. However, for H-bonding interactions with target cellular systems (enzymes or receptors) a presence of polar functional groups (OH, NH_2, COOH etc.) on the C-substituent would be desirable. Therefore, we systematically study reactions of suitably protected functionalized organometallics with halopurines. By applications of such reactions we have developed practical methodologies of synthesis of 6-(hydroxymethyl) purines, (purin-6-yl)alanines and some other related types of compounds [4].

All these cross-coupling reactions were performed with THP-protected purine bases or with acyl- or silyl-protected nucleosides. These protecting groups generally withstand the reaction conditions and reagents and the intermediates are easily deprotected under very mild conditions to free purine bases or nucleosides. This methodology was extensively applied in the synthesis of modified purine bases and nucleosides directed to medicinal chemistry (biological activity screening of new nucleobases and nucleosides) and chemical biology. 6-Aryl-, 6-trifluoromethyl- and 6-(hydroxymethyl)purine ribo-nucleosides, as well as some bis(purin-6-yl)acetylenes displayed significant cytostatic activity [5] while 6-hetarylpurine ribonucleosides exerted high antiviral activity against HCV virus [6].

This work was supported by Centre for New Antivirals and Antineoplastics (1M6138896301); by the Grant Agency of the Czech Republic (grant No. 203/03/0035); and by Sumitomo Chemical Co. Ltd. (Osaka, Japan). The author thanks to all members of his group and to external collaborating colleagues for their contribution.

1. Review: Hocek, M. (2003) *Eur. J. Org. Chem*. **2003**, 245-254; and all references cited therein.
2. (a) Turek, P.; Kotora, M.; Tišlerová, I.; Hocek, M.; Votruba, I.; Císařová, I. *J. Org. Chem.* **2004**, *69*, 9224-9233. (b) Turek, P.; Kotora, M.; Hocek, M.; Votruba, I. *Collect. Czech. Chem. Commun.* **2005**, *70*, 339-349.
3. (a) Hocek, M.; Hocková, D.; Štambaský, J. (2003) *Collect. Czech. Chem. Commun.* **2003**, *68*, 837-848. (b) Hocek, M.; Votruba, I.; Dvořáková, H. *Tetrahedron* **2003**, *59*, 607-611. (c) Hocek, M.; Dvořáková, H. *J. Org. Chem.* **2003**, *68*, 5773-5776. (d) Hocek, M.; Hocková, D.; Dvořáková, H. *Synthesis* **2004**, 889-894. (e) Hocek, M.; Pohl, R. *Synthesis* **2004**, 2869-2876.
4. (a) Šilhár, P.; Pohl, R.; Votruba, I.; Hocek, M. *Org. Lett.* **2004**, *6*, 3225-3228. (b) Šilhár, P.; Pohl, R.; Votruba, I.; Hocek, M. *Org. Biomol. Chem.* **2005**, *3*, 3001-3007. (c) Čapek, P.; Pohl, R.; Hocek, M. *J. Org. Chem.***2004**, *69*, 7985-7988. (d) Čapek, P.; Pohl, R.; Hocek, M. *J. Org. Chem.* **2005**, *70*, 8001-8008.
5. (a) Hocek, M.; Holý, A.; Votruba, I.; Dvořáková, H. *J. Med. Chem*. **2000**, *43*, 1817-1825. (b) Hocková, D.; Hocek, M.; Dvořáková, H.; Votruba, I. *Tetrahedron* **1999**, *55*, 11109-11118. (c) Hocek, M.; Votruba, I. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1055-1057. (d) Nauš, P.; Votruba, I.; Hocek, M. *Collect. Czech. Chem. Commun.* **2004**, *69*, 1955-1970.
6. Hocek, M.; Nauš, P.; Pohl, R.; Votruba, I.; Furman, P. A.; Tharnish, P. M.; Otto, M. J. *J. Med. Chem.* **2005**, *48*, 5869-5873.

NOVEL BICYCLISATION OF UNSATURATED POLYOLS AND AMINOPOLYOLS IN A 'MAGIC' $PDCL_2$-$CUCL_2$-ACOH CATALYTIC SYSTEM

Matej Babjak[1], Ľuboš Remeň[1], Peter Szolcsányi[1], Oľga Karlubíková[1], Peter Zálupský[1], Dušan Miklóš[2] and Tibor Gracza[1]

[1]Department of Organic Chemistry, Slovak University of Technology, Radlinského 9, SK-812 37 Bratislava, Slovak Republic;
[2]Department of Inorganic Chemistry, Slovak University of Technology, Radlinského 9, SK-812 37 Bratislava, Slovak Republic

Since development of the Wacker process, many various transformations mediated by palladium(II) compounds have been described. Intramolecular versions of these processes are very useful in the synthesis of oxygen and nitrogen heterocycles [1-7]. We have developed a general strategy for homologation of aldoses to optically active anhydroaldonolactones, [8, 9] a class of compounds that have proven most versatile intermediates for C-glycosides/ C-nucleosides syntheses (Scheme 1). Our entry into this field features is the stereocontrolled Pd(II)-catalysed oxy-/aminocarbonylation of unsaturated enitols and amino derived enitols as a key step. The performance of this methods was illustrated by flexible approaches to some natural products (anti-tumor-active lactones, notably (+)-goniofufurone [10, 11] (+)-7-*epi*-goniofufurone [12] 7-deoxygoniofufurone [12] and (+)-goniothalesdiol [13-15], erythroskyrine [16], plakorto-lactones [17-19] A, B, C, D, kumausynes [20, 21], Hagen gland's lactones [17, 22] enzyme inhibitors; homo-DLX [23] homo-DMDP [23] homo-DNJ, homo-L-*ido*-DNJ [24, 25].

A 'magic' catalytic system that is mostly efficient for oxidations, carbonylations, cycloacetali-zations and the other type of such reactions contains both palladium(II) chloride and cooper(II) chloride in acetic acid with sodium acetate as a buffer [3, 4].

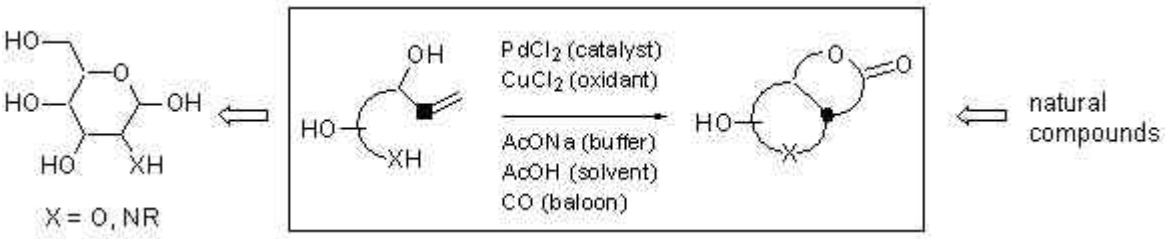
In this account, we will discuss the new type of bicyclization of unsaturated polyols and aminopolyols in "magic" $PdCl_2$-$CuCl_2$-AcOH catalytic system (Scheme 2). The reaction shows exceptional demand for substrate configuration, when substrates bearing *xylo*- alignment at , , - carbons afford desired bicyclic dianhydroalditols, whereas non-*xylo* substrates undergo Wacker oxidation instead [26].

Mechanistic considerations of the problem, based on semi-empirical calculations and 3D modelling, will be discussed (Scheme 3).

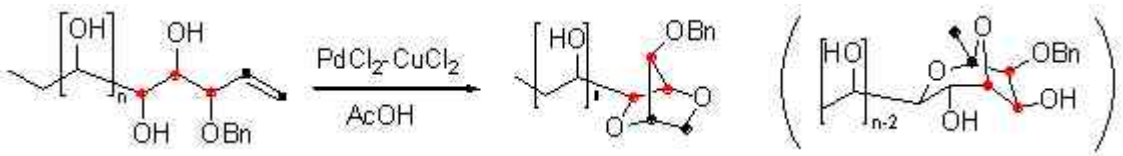
Concerning the aspect utility in organic synthesis, tandem of Pd(II)-catalysed bicyclisation of unsaturated polyols and ring-opening of the dianhydroalditols represents a new synthetic access to 2,3-*trans*-tetrahydrofurans and is complementary to oxycarbonylation methodology producing 2,3-*cis*-configured tetrahydrofuran derivatives. The stereochemistry of the product is controlled by hydroxyl in -position or -position with *cis* arrangement according to newly formed stereogenic centre (Scheme 4).

A simple stereoconvergent synthesis of C-2 symmetrical bicyclic product from the equimolar diastereomeric mixture of D-*erythro*-/D-*threo*-1-pentenitols, followed with directed diastereoselective ring opening [27], will be presented as a smart route to polysubstituted tetrahydrofurans with rare D-*lyxo* configuration (Scheme 5), which is complementary to D-*arabino*, routinely accessible from conventional iodocyclisation [28].

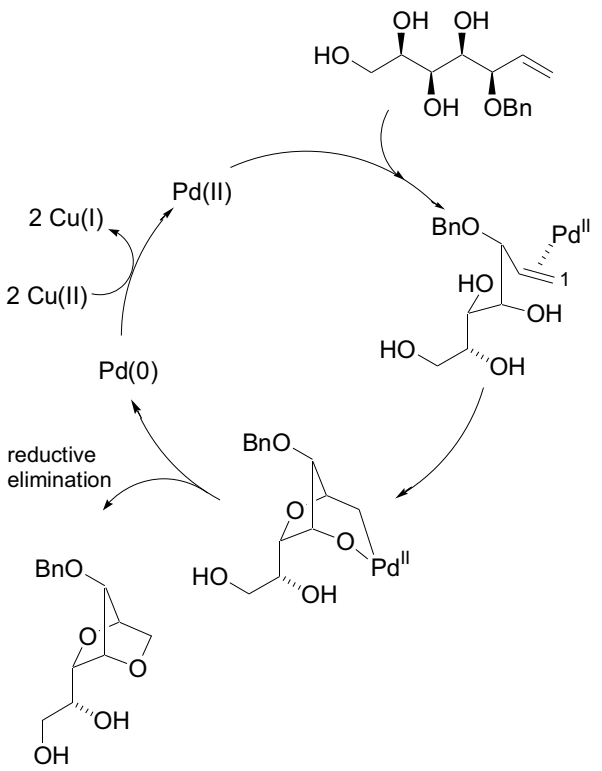
In conclusion, we have found a novel type of $PdCl_2$/$CuCl_2$-catalysed bicyclisation of sugar-derived unsaturated polyols that leads to 1,4:2,5-dianhydroalditols in good yields [29]. This useful synthetic method is highly substrate-selective and displays a strong stereochemical preference for alkenitols with C3, C4, C5-all-*syn* (*xylo*) relative configuration. Moreover, the transformation is diastereospecific due to the formation of new C-2 stereogenic centre with *threo*-relationship exclusively (Scheme 6).



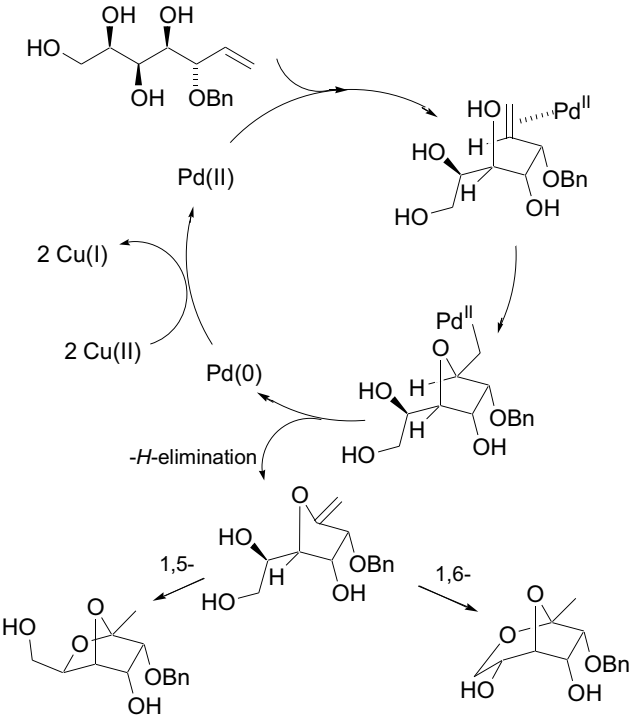
Scheme 1.



Scheme 2.

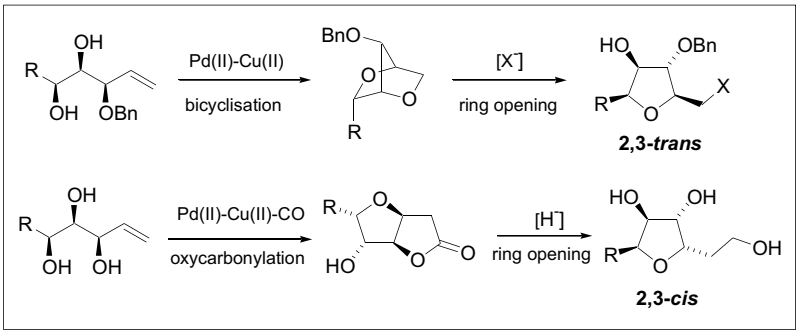


Bicyclisation

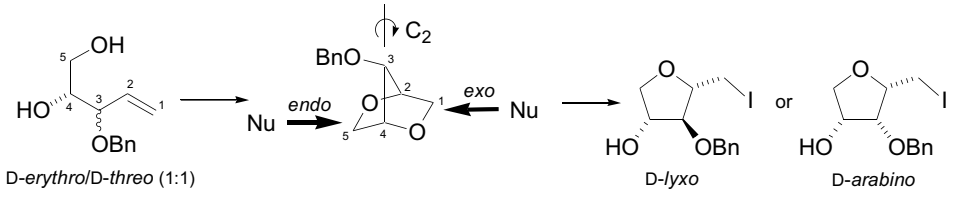


Wacker-type reaction

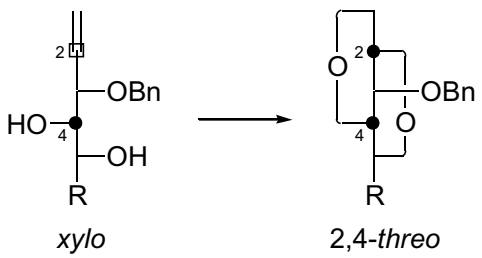
Scheme 3.



Scheme 4.



Scheme 5.



Scheme 6.

1. Collman, J. P.; Hegedus, L. S.; Norton, J. R.; Finke, R.G. in: *Principles and Applications of Organotransition Metal Chemistry*, 2nd Edition, University Science Books 1987.
2. Hegedus, L. S. in: *Transition Metals in the Synthesis of Complex Organic Molecules*, University Science Books 1994.
3. Malleron, J.-L.; Fiaud, J.-C.; Legros, J.-Y. in: *Handbook of Palladium Catalysed Organic Reactions* 1997.

4. Orena, M. in: G. Helmchen, R. Hoffmann, J. Mulzer (Eds.), *Cycloetherification*, Houben Weyl, 4th edition, Georg Thieme Verlag Stuttgart - New York, 1995, Vol. E21c, 4698.
5. Elbracht, D. in: G. Helmchen, R. Hoffmann, J. Mulzer (Eds.), *Addition to Olefinic Double Bonds Catalysed by Transition Metals*, Houben Weyl, 4th edition, Georg Thieme Verlag Stuttgart - New York, 1995, Vol. E21c, 2488.
6. Schmalz, H. G. in: Ei-ichi Negishi (Ed.), *Handbook of Organopalladium Chemistry for Organic Syntheses*, John Wiley&Sons, Inc., New York, 2002, Vol. 2, 2377.
7. Farina, V.; Eriksson, M. in: Ei-ichi Negishi (Ed.), *Handbook of Organopalladium Chemistry for Organic Syntheses*, John Wiley&Sons, Inc., New York, 2002, Vol. 2, 2351.
8. Jäger. V.; Gracza, T.; Dubois, E.; Hasenohrl, T.; Hummer, W.; Kautz, U.; Kirschbaum, B.; Lieberknecht, A.; Remeň, Ľ.; Stahl, U.; Stephan, O. *OSM 5,* Springer Verlag, Heidelberg, G. Helmchen, J. Dibo, D. Flubacher, B. Wiese (Eds.), **1997**, 331.
9. Gracza, T.; Hasenöhrl, T.; Stahl, U.; Jäger, V. *Synthesis* **1991**, 1108.
10. Gracza, T.; Jäger, V. *Synlett*. **1992**, 191.
11. Gracza, T.; Jäger V. *Synthesis* **1994**, 1359.
12. Gracza, T.; Szolcsányi P. *Molecules* **2000**, 5, 1386.
13. Babjak, M.ô Kapitán, P.; Gracza, T. *Tetrahedron Lett*. **2002**, *43*, 6983.
14. Langer, V.; Gyepesova, D.; Koman, M.; Kapitán, P.; Babjak, M.; Gracza, T.; Koóš, M. *Molecules* **2003**, *8*, 599-606.
15. Babjak, M.; Kapitán, P.; Gracza, T. *Tetrahedron* **2005**, *61*, 2471.
16. Dixon D. J.; Ley S. V.; Gracza T.; Szolcsányi P. *J. Chem. Soc., Perkin Trans 1*, **1999**, *8*, 839.
17. Mereyala, H. B.; Gadikota, R. R.; Joe, M.; Arora, S. K.; Dastidar, S. G.; Agarwal, S. *Bioorg. Med. Chem*. **1999**, *7*, 2095.
18. Paddon-Jones G. C., Hungerfod N. L., Hayes P., Kitching W. *Org. Lett*. **1999**, *1*, 1905.
19. Semmelhack M. F., Shanmugan P. *Tetrahedron Lett*. **2000**, *41*, 3567.
20. Boukouvalas, J.; Fortier, G.; Radu, I.-I. *J. Org. Chem*. **1998**, *63*, 916.
21. Mereyala H. B., Gadikota R. R. *Indian. J. Chem. Sec. B* **2000**, *39*, 166.
22. Paddon-Jones, G. C.; Moore, Ch. J.; Brecknell, D. J.; Konig, W. A.; Kitching, W.; *Tetrahedron Lett*. **1997**, *38*, 3479.
23. Hümmer W., Dubois E., Gracza T., Jäger V., *Synthesis* **1997**, 634-642.
24. Szolcsányi P., Gracza T., Koman M., Prónayová N., Liptaj T. *Chem.Commun*. **2000**, 471.
25. Szolcsányi P., Gracza T., Koman M., Prónayová N., Liptaj T. *Tetrahedron Asymmetry*. **2000**, 2579.
26. Babjak, M.; Remeň, L.; Szolcsányi, P.; Zálupský, P.; Gracza, T. *J. Organometall. Chem*. **2005** (in press).
27. Babjak, M.; Remeň, L.; Karlubíková, P.; Gracza, T. *Synlett* **2005**, 1609.
28. Bravo, F.; Castillon, S. *Eur. J. Org. Chem*. **2001**, 507. For an analogous Pd(II)/$CuCl_2$-catalysed *N,O*-bicyclisation of aminoalkenitols leading to corresponding 6-oxa-2-azabicyclo[3.2.1]octanes see: Szolcsányi, P.; Gracza, T. *Chem. Commun*. **2005**, 3948.
29. For an analogous Pd(II)/$CuCl_2$-catalysed *N,O*-bicyclisation of aminoalkenitols leading to corresponding 6-oxa-2-azabicyclo[3.2.1]octanes see: Szolcsányi, P.; Gracza, T. *Chem. Commun*. **2005**, 3948.

DRUG DESIGN BASED ON X-RAY DIFFRACTION AND STEERED MOLECULAR DYNAMICS

Hašek J., Skálová T., Dohnálek J., Dušková J., Petroková H., Vondráčková E., Zimmermann K.

Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Heyrovskeho nam.2, 162 056 Praha 6, hasek@imc.cas.cz

Introduction

HIV, human immunodeficiency virus is a causal agent of AIDS - acquired immunodeficiency syndrome. HIV-1 protease is an enzyme which cleaves primary polyprotein of a virion into mature viral proteins. Thus, inhibition of HIV-1 protease aborts the life cycle of the virus. However, gradual development of new generations of the virus with mutated forms of HIV-1 proteases resistant to inhibitors applied in medical treatment of AIDS remains a crucial problem. The paper shows some recent advances in searching for mutation-resistant drugs.

X-ray diffraction

In the recent five years we determined structures of 12 complexes formed by three HIV-1 protease mutants and 10 chemically similar ligands [1, 3-7]. The ligands belong to 4 different inhibitor classes (Table 1) and form a chemically systematic sequence varying chemical composition and absolute configuration of isostere [-***(R/S)*** **COH-**] and hydrophobicity of the P_2' site ($\mathbf{R}^{2'}$= Glu, Gln, Ile). Inhibitors were selected with a large scale of inhibition constants K_i (from 0.02 to 1000 nmol/l) to demonstrate clearly the differences between the binding mode of high- and low-affinity inhibitors. One of these studies - the structure of HIV-1 protease inhibited by the product of autoproteolytic process (denoted here as the YDQIL peptide), showing a

Table 1. Experimental material.

HIV-1 proteases: native (BRU isolate), mutants (L63P, A71V, V82T, I84V) , (A71V, V82T, I84V)
Inhibitors with ethyleneamine,hydroxyethylamine,hydroxyethylene and hydroxymethyl isosteres :
YDQIL peptide = fragment of HIV-1 protease cleft at Leu63 Ile64.
PTPI-EA = R^2 - CONH - CHR^1- **CH_2 CH_2NH** - $CHR^{1'}$ - CONH - $CHR^{2'}$ - CONH - $CHR^{3'}$- $CONH_2$
PTPI-HEA = R^2 - CONH - CHR^1- **COH CH_2NH** -$CHR^{1'}$ - CONH - $CHR^{2'}$ - CONH - $CHR^{3'}$- $CONH_2$
SNPI = R^2 - CONH - CHR^1 - **COH CH_2** - $CHR^{1'}$ - NHCO - $R^{2'}$
SNPI = R^2 - CONH - CHR^1 - **COH** - $CHR^{1'}$ - NHCO - $CHCH(CH_3)^2$ -NHCO-$R^{3'}$

snapshot of the substrate cleavage process, deserves a special attention [2].

Inhibitors:

Preparation and characterization of the experimental material were described elsewhere. The HIV-1 protease mutants were prepared at the Institute of Organic Chemistry and Biochemistry (IOCB) and Institute of Molecular Genetics (IMG) in Prague. The series of the pseudotetrapeptide inhibitors (PTPI) with the hydroxyethylamine isostere (HEA) and the ethylenamine isostere (EA) with inhibition constatnts K_i in the range 0.2 - 1000 nmol/l were prepared in the IOCB. The series of symmetrical non-peptidic inhibitors (SNPI) with K_i in the range 0.02 - 5 nmol/l was prepared at the TU Lodz. Diffraction measurements were performed at the source of synchrotron radiation ESRF in Grenoble and at ELETTRA in Trieste.

Molecular dynamics under externally applied forces

Static and dynamic simulations with externally applied forces showed that the protease without inhibitor is in a relaxed state with its flaps having a large spectrum of conformations of similar energy. On complexation, the protease flaps always close over the inhibitor and bind strongly to the ligand carbonyls at P_1 and P_1' positions. However, forced flap opening, flaps flipping and a forced extraction of inhibitors showed that HIV-1 protease can accommodate under its flaps much larger highly hydrophobic ligands than expected. Molecular simulations of inhibitor extraction and flaps opening were carried out by AMBER and ORAL [7, 8].

Conclusions

The X-ray experiments and molecular simulations lead to the following conclusions:

- An efficient inhibitor should have a low-energy conformation that fits well the protease binding tunnel and satisfies all the hydrogen bond donors.
- In spite of the fact that the protease possesses two-fold symmetry, a good inhibitor need not be symmetrical.
- There is also no need to imitate the transition state of substrate as declared earlier.
- Hydrophilic void cavities between ligand and protein are possible if their size allows inclusion of 1-2 bridging water molecules.
- X-ray studies showed that even a small change in the chemistry of the inhibitor molecule can lead to a significant change of the dense network of multiple hydrogen bonds required for good affinity of the inhibitor to protein.
- Large conformational differences between the inhibitor bound in the protease and that in the crystal of neat inhibitor were found. But surprisingly, these differences were only in a few torsion angles responsible for proper re-orientation of side chains to fit in the clefts in protease surface.
- Hydrophilic and hydrophobic groups form a special array on the surface of binding tunnel (Fig.1). Some ligands can adopt multiple conformations in the binding tunnel formed under protease flaps satisfying all the hydrogen-bond-forming groups.
- Inhibitors, very flexible in solution, adopt fixed conformations on complexation. The corresponding entropy changes and deformation energies of both inhibitor and protease are highly competitive with the enthalpy of newly formed weak bonds between inhibitor and protease.
- The energy loss is almost in all cases reduced by water molecules built in between protease and inhibitor.
- The ability of the inhibitor to form large hydrophobic surface areas oriented to the bottom parts of flaps is very important. A large conformational freedom of these hydrophobic groups with respect to the inhibitor skeleton is desirable.
- In the case of pseudopeptide inhibitors, the protease flaps always close over the inhibitor and bind strongly to the ligand carbonyls at P_1 and P_1' positions during the inhibition process.

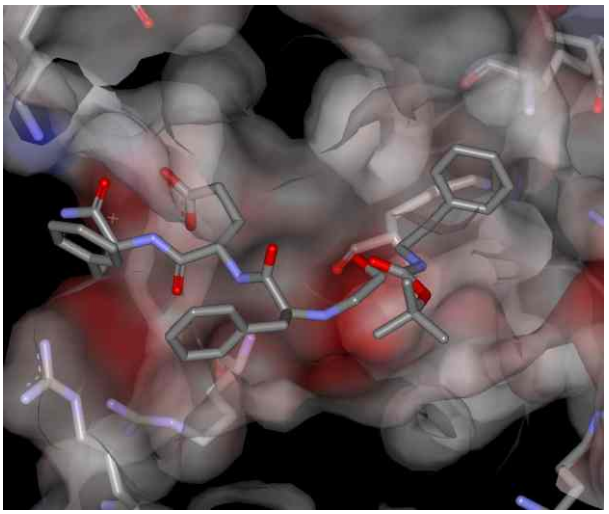


Figure 1. Top view of the complex of the RE inhibitor and HIV-1 protease mutant (A71V, V82T, I84V). The protease flaps were removed to show the bottom of the protease binding tunnel with stripes of hydrophilic and hydrophobic surfaces.

- Static and dynamic simulations with externally applied forces showed that the protease without inhibitor is in a relaxed state with its flaps having a large spectrum of conformations of similar energy. In particular, the flaps are able to accommodate much larger ligands than it has been expected so far.

All these results strongly indicate that in spite of the ten-year development of HIV protease inhibitors, we can expect a boom in designing more universal inhibitors of new chemical composition. These could hopefully overcome the long-standing problem of adaptability of the virus to drug treatment.

Project was supported by GAAV KJB 4050312.

References

1. Dohnálek J., Hašek J., Dušková J., Petroková H., Hradílek M., Souček M., Konvalinka J., Brynda J., Sedláček J., Fábry J. *Distinct Binding Mode of a Hydroxyethylamine Isostere Inhibitor of HIV-1 Protease. Acta Crystallogr. D* **57** (2001) 472-476**.**
2. Buchtelová, E., Hašek, J., Dohnálek, J., Petroková, H., Dušková, J., Skálová, T., Brynda, J., Sedláček, J., Hradilek, M., Konvalinka, J., Tykarska, E., Jaskolski, M, Olivi, L.: *Product of autodigestion at Leu63-Ile64 site binds to HIV-1 protease instead of inhibitor Boc-Phe- [(S)-CH(OH)CH2NH]Phe-Ile-Phe-NH2,* Mater. Struct., **8** (2001) 31-32.
3. Dohnálek J., Hašek J., Dušková J., Petroková H., Hradílek M., Souček M., Konvalinka J., Brynda J., Sedláček J., Fábry J.: *Hydroxyethylamine Isostere of an HIV-1 Protease Inhibitor Prefers Its Amine to the Hydroxy Group in Binding to Catalytic Aspartates. J. Med. Chem.* **45** (2002) 1432-1438**.**
4. Skálová T., Hašek J., Dohnálek J., Petroková H., Buchtelová E. *Mutant HIV-1 protease complexed with tetrapeptide inhibitor. Acta Phys. Pol. A* vol. **101** (2002) 659-663.
5. Skálová T., Hašek J., Dohnálek J., Petroková H., Buchtelová E., Dušková J., Souček M., Majer P., Uhlíková T., Konvalinka J.: *An ethylenamine inhibitor binds tightly to both wild type and mutant HIV-1 proteases. Structure and energy study. J. Med. Chem.* **46** (2003) 1636-1644.
6. Skálová T., Dohnálek J., Dušková J., Petroková H., Vondráčková E., Hašek J.: Series of *HIV Protease Nanomolar Inhibitor; Binding to WT and Mutant Protease. Acta Crystallogr* **A 61**, (2005) C243.
7. Petroková H., Dušková J., Dohnálek J., Skálová T., Vondráčková-Buchtelová E., Souček M., Konvalinka J., Brynda J., Fábry J., Sedláček J., Hašek J.: *Role of Hydroxyl Group and R/S Configuration of Isostere in Binding Properties of HIV-1 Protease Inhibitors. Eur. J. Biochem.* **271** (2004) 4451-4461.
8. Hašek J., Dohnálek J., Skálová T., Dušková J., Petroková H., Vondráčková E., Kolenko P., Zimmermann K.: *HIV Protease Inhibition Seen by X-Ray Diffraction and Molecular Dynamics. Protein Sci.* **14**, (2005) S1, 276.

NEW STATIONARY PHASES FOR HPLC AND THEIR ADVANTAGES IN COMPARISON WITH RP-C18 PHASE

D. Procházková

Sigma Aldrich, s.r.o., Pobřežní 46, 186 00 Prague 6

The most common approach to HPLC method development is often begun with alkyl-bonded stationary phases like C18 and C8. Too often, however, unknown impurities in a sample are unretained, too retained or unresolved. These unknown impurities may go unnoticed until much later in the life cycle of the method demanding costly redevelopment, revalidation and reanalysis [1].

Several new LC stationary phases have recently become popular because they exhibit improved retention and selectivity over standard C18 phases and extend the usefulness of reversed phase LC. Alkyl chains with embedded polar groups, fluorinated alkyl and aryl groups, and various forms of carbon have all found wide acceptance as alternatives to alkyl chains. These alternative stationary phase interactions often provide retention and selectivity not obtained on traditional alkyl phases. Due to the differences in the interactions possible with the various stationary phase chemistries, the probability of separating components of a sample substantially increases. The end result is greater confidence that the final method conditions will allow detection of potentially critical sample components and degradation products.

Polar reversed phases can provide dramatically different separations compared to C18. The PEG phase produced excellent separations of phenolic solutes and should be considered for applications such as drug metabolite analysis. The F5 phase exhibits unique, interesting separations of amine-containing solutes and should be considered for applications such as basic pharmaceuticals. In particular, the Discovery HS F5 phase exhibit reversed-phase and normal-phase retention for polar analytes as well as ion-exchange behavior.

Both phases offer valuable alternatives to C18 when confronted with poor resolution on C18. Retention and selectivity of polar reversed phases is a continuing research and development focus at Supelco. Our aim is to develop unique, valuable phases and applications for HPLC [1].

Although silica-based stationary phases remain the widely used for high-performance liquid chromatography (HPLC) analyses, separations based on modified zirconia phases are fast becoming a popular alternative. More recently, zirconia based phases have also been studied as a selective alternative to popular silica-based phases. The interest in zirconia columns stems from their ability to withstand extreme pH and temperature conditions as well as their offering of unique selectivity and retention for various classes of compounds [2].

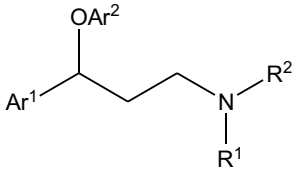
1. *Discovery HPLC Columns Developed for Pharmaceutical Analysis and Purification* Sigma-Aldrich, Co. 2004.
2. *Discovery Zr: High pH and High Temperature Method Development*, Sigma-Aldrich, Co. 2002.

SYNTHESIS OF CHIRAL *N,N*-DIALKYLAMINOETHERS AS INTERMEDIATES FOR PREPARATION OF OXETINES

Luděk Ridvan, Petr Hrubý, Lukáš Plaček, Hana Petříčková

Zentiva a.s., U kabelovny 130, 102 01, Praha 10, Czech Republic,
Tel: + 420 602 558 414; Fax: + 420 267 244 088; ludek.ridvan@zentiva.cz

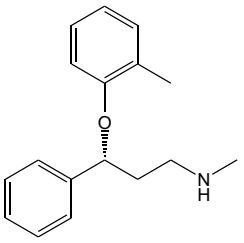
N,N-Dialkylaminoethers of a general formula **I** can be used as intermediates for synthesis of pharmacologically important substances, e.g. group of CNS drugs called "oxetines".



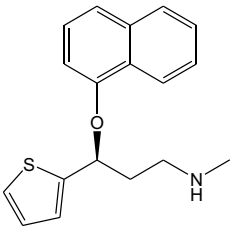
I

Most of newly developed chiral drugs are used as single enantiomers. In spite of several different approaches to the synthesis of pure enantiomers (asymmetric synthesis, chiral pool, biochemical methods, etc.), the classical approach based on resolution *via* salt formation with a chiral base or an acid is still often the method of choice in many industrially used processes. This methodology using various chiral acids has been described for preparation of optically pure *N*-methyl-3-aryl-3-aryloxyamines **I** [1, 2].

We would like to present our recent results obtained during our study aimed at preparation of optically pure *N,N*-dimethyl-3-aryl-3-aryloxyamines **I** as intermediates for preparation of atomoxetine and duloxetine [1, 3].



Atomoxetine



Duloxetine

1. US 0052492 (B. J. Foster, E. R. Lavagnino, *Eli Lilly*, 1982).
2. WO 2004/056795 A1 (D.R. Rao, R.N. Kankan, C.P. Wain, *CIPLA*, 2004).
3. EP 0273658 (D.V. Robertson, D. T. Wong, J. H. Krushinski, *Eli Lilly*, 1990).

HOW THE CHEMICAL REACTIONS OF IONIC SPECIES CAN ELUCIDATE THE STRUCTURE OF COMPOUNDS

Vladislav Kubelka

Zentiva a.s., U kabelovny 130, 102 01, Praha 10, Czech Republic

To understand thoroughly the capabilities, and especially the limitations, of a mass spectrum for structure elucidation, it is necessary to be familiar with the basic theoretical aspect of unimolecular ion decompositions. The basic process for getting a mass spectrum of an organic compound is an ionisation of the molecule. The ionisation of the sample molecules with electrons, photons or charged and excited molecules produces molecular ions (M^{+*} or M^{-}) whose internal energy values cover a broad range (0-20 eV). This content of energy of the initially formed M^{+*} ions is responsible for their decomposition behaviour.

The other ionisation techniques like the chemical ionisation, the fast atom bombardment, the field ionisation, the lasser matrix assisted ionisation produce mainly pseudomolecular ions [MH^{+}, $(M\text{-}H)^{-}$, MNa^{+}, $(MNH_4)^{+}$] with a low excess of internal energy in the molecular ions.

Only unimolecular reactions are possible for the gaseous ions under usual MS operating conditions. The nature and extent of these reactions depend only on the ion's structure and the method of ionisation. Without collisions it is the energy originally deposited in the ion that causes the ion to decompose or isomerize. The unimolar decompositions of the molecular ions yield the different ion's products through kinetic and thermodynamic control– the enthalpy and the entropy requirements for decomposition of ions. These offsetting enthalpy and enthropy effects in general lead to a substantial number of competing primary reactions as well as the consecutive secondary and further reactions, thus the mass spectrum of a large molecule can have hundreds of peaks.

For this reason, in interpreting an unknown spectrum it is helpful not only to study the spectra of closely related compounds but also to take into account the result reactions of ionic and radical species which are well known in organic chemistry – liquid face reactions.

Some examples of how to interpret the structure of an unknown compound from its mass spectra will be shown during the presentation.

EVALUATION OF [^{18}F]FLUOROBENZALDEHYDE FOR PREPARATION OF ^{18}F -LABELLED SUBSTANCE P AND AN ASYMMETRIC APPROACH TO THE RADIOSYNTHESIS OF BOTH ENANTIOMERS OF -[^{11}C]METHYLDOPA AND -[^{11}C]METHYLTYROSINE FOR POSITRON EMISSION TOMOGRAPHY

Alexander Popkov[1,7,8], Milan Nádvorník[2], Josef Jirman[3], Pavla Kružberská[4], Antonín Lyčka[5], Tomáš Weidlich[5] , Jozef Kožíšek[6], Martin Breza[6], Szabolcs Lehel[7], Nicholas Gillings[7], Philip Elsinga[8]

[1]Faculty of Health and Social Studies, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic;
[2]Department of General and Inorganic Chemistry, Faculty of Chemical Technology, Pardubice University, Czech Republic;
[3]Zentiva a.s., U kabelovny 130, 102 01 Praha 10, Czech Republic;
[4]Department of Analytical Biochemistry, Institute of Entomology, Branišovská 31, 37005 České Budejovice, Czech Republic;
[5]Research Institute for Organic Syntheses, Rybitví 296, 532 18 Pardubice 20; CzechRepublic;
[6]Department of Phys. Chemistry, Faculty of Chemical and Food Technology, Slovak University of Technology, 812 37 Bratislava, Slovak Republic;
[7]PET & Cyclotron Unit, Copenhagen University Hospital, Blegdamsvej 9, 2100 Copenhagen, Denmark;
[8]Nuclear Medicine and Molecular Imaging, University Medical Center Groningen, Groningen, Netherlands.

Substance P (SP) is an agonist for the NK_1-receptor and is considered to be a neuromodulator or neurotransmitter. Changes in SP have been implicated in Parkinson's disease, arthritis, inflammatory bowel disease and asthma.

The reaction of an oxoamino derivative of SP [1] with [^{18}F]fluorobenzaldehyde to form an oxime, was investigated as described by Poethko *et al.* [2]. In the production of [^{18}F]fluorobenzaldehyde, DMSO was replaced by 1,3-dimethylimidazolidin-2-one (DMI). The reaction in DMSO led to the formation of substantial amounts of [^{18}F]fluorobenzoic acid due to oxidation of aldehyde group by the solvent. The amount of radioactive by-products was reduced significantly using DMI. The radiochemical yield of purified aldehyde was up to 40%.

After dilution of the reaction mixture with water, [^{18}F]fluorobenzaldehyde was purified by solid-phase extraction on Oasis HLB cartridge. Radioactive impurities were removed by elution with mixtures of water and organic solvent. Finally, [^{18}F]fluorobenzaldehyde was eluted with methanol. This solution was added to the oxoamino derivative of SP dissolved in 0.5 ml of phosphate buffer pH 2.7 (the oxoamino group was attached to the N-terminus of peptide) and heated at 60 C for 20 min. Based on HPLC data, the radiochemical yield of the Schiff base formation was 35%. The whole procedure was fully automated with a Zymark robotic system. Biological studies involving *in vivo* metabolic stability and pharmacokinetics of ^{18}F-labelled SP are underway.

In positron emission tomography (PET) -methyl amino acids can play a dual role:

1. precursors of non-metabolised neurotransmitters (analogues of serotonin, dopamine, tyramine etc) for the study of neurodegenerative diseases;

2. non-metabolised analogues of proteinogenic amino acids for the study of amino acids uptake into normal and cancer cells. The difference in the uptake rates during a PET scan could visualise cancer cells in a human body.

Clinical applications of such amino acids are strongly limited due to their poor availability. For the synthesis of the only enantiomerically pure ^{11}C-labelled -methyl amino acid, -[^{11}C]methyltryptophan, an industrial procedure was adopted [3, 4]. All attempts to prepare enantiomerically pure -[^{11}C]methylated tyrosine or phenylalanine failed [5, 6].

Our approach to enantiomerically pure [^{11}C]methyl amino acids is based on chiral metalocomplex synthons of amino acids. We carried out [^{11}C]methylation of metalocomplex synthons derived from protected DOPA or tyrosine. For [^{11}C]methylation, sodium hydroxide (5 mg of fine dry powder) was sealed in a vial, which was flushed with dry nitrogen before addition of a solution of the complex (10 mg) and $^{11}CH_3I$ in DMI (300 l). After 10 min at 25 C, a 9% radiochemical yield (decay corrected) of a mixture of the diastereomeric -[^{11}C]methylDOPA complexes or a 7% radiochemical yield of a mixture of the diastereomeric -[^{11}C]methyltyrosine complexes was achieved. Individual diastereomers were successfully separated by preparative HPLC, diluted with excess of water and extracted on C_{18} cartridges. Optimisation of the procedure followed by hydrolysis of the complexes and purification of the enantiomers of -[^{11}C]methylDOPA and -[^{11}C]methyltyrosine is currently underway.

1. Kindly donated by Dr M. Schottelius and Dr H. J. Wester, Technical University Munich.

2. Poethko T.; Schottelius M.; Thumshim G.; Hersel U.; Herz M.; Henriksen G.; Kessler H.; Schwaiger M.; Wester H. J. *J. Nucl. Med.* **2004**, *45*, 892.

3. Bourne G. T., Crich D., Davies J. W., Horwell. D. C.: *J. Chem. Soc., Perkin Trans.1* **1991**, 1693.

4. Plenevaux A., Lemaire C., Delfiore G., Comar D.: *Appl. Radiat. Isot.* **1994**, *45*, 651.

5. Fasth K. J.; Långström B.: *Acta Chem. Scand.* **1990**, *44*, 720.

6. Gee A. D., Långström B.: *J. Chem. Soc., Perkin Trans. 1* **1991**, 215.