So, where do natural products fit in this brave new world? Two words are all that are needed to answer this, quality and utility. We should remember that natural-products chemistries represent the greatest sources of scaffold diversity and that these scaffolds are pharmacophore rich, *i.e.*, the molecules are biologically relevant and have been conserved through an evolutionary process on the basis that they confer competitive advantage on the producing organism. The biological relevance of these molecules is manifest in growth and differentiation modulation, in enzyme inhibition or regulation, in receptor agonism or antagonism – biological events common to human disease processes. The ultimate test of quality and utility is market sales, at the very pinnacle of which is the presence of 8 natural-products drugs in the top 25 drugs of 1994.

However, the '*Achilles* heel' of natural-products drug discovery is the time and cost on a per target basis. *Xenova* has revolutionised its approach to natural products to create chemical libraries that enable natural-products drug discovery to be undertaken quickly and cost-effectively and on a level-playing field with synthetic chemicals. The three most important elements of this are technological innovation (including informatics), process engineering and process management techniques.

Xenova has harnessed natural-products chemistries across many therapeutic targets to create a group of preclinical programmes with its corporate partners and to establish a proprietary pipeline of synthetic and semi-synthetic compounds from preclinical research through to clinical evaluation. The chemical output is over 300 bioactive compounds, one-third of which are novel including numerous new chemical scaffolds.

New techniques, including combinatorial biology and biotransformations, will expand access to novel and challenging chemistries based on natural scaffolds and combinatorial-chemistry technologies will be applied increasing to expand the structural diversity around pharmacophores of natural origins. The author believes that natural products will continue to provide valuable drugs and will continue to represent one-third of the top 25 drugs for decades to come.

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Rational Enzyme Design: Computer Modeling and Site-directed Mutagenesis for the Modification of Catalytic Specificity in Organophosphorus Hydrolase

Kaihua Lai[a]), **Janet K. Grimsley**[a]), **Barbara D. Kuhlmann**[a]), **Leonardo Scapozza**[b]), **Steven P. Harvey**[c]), **Joseph J. DeFrank**[c]), **Jan E. Kolakowski**[c]), and **James R. Wild**[a])*

Organophosphorus neurotoxins are widely used as insecticides in crop production, municipal hygiene, and disease vector control as well as providing the major classes of chemical warfare neurotoxins (V-agents and G-agents). Organophosphorus hydrolase (OPH) is a bacterial metalloenzyme which performs a hydrolytic cleavage of a variety of organophosphorus neurotoxins including common insecticides and chemical nerve agents. The enzyme is capable of hydrolyzing P–O, P–F, P–S, and P–CN bonds of toxic inhibitors of acetyl- and/or butyryl-cholinesterases (AChEs and BChEs) as well as neurotoxic esterases (NTEs). While there are numerous 'OP Anhydrolases' (E.C. 3.1.8.1) in many different organisms, most of them have limited substrate specificities, and there are dramatic differences in the hydrolytic capacity between classes of substrates: phosphotriesters (P–O bonds), fluorophosphonates (P–F bonds), and phosphothioates (P–S bonds).

The enzyme has extremely high efficiency in hydrolysis of many different phosphotriester and phosphothiolester pesticides (P–O bond) such as paraoxon (k_{cat} > 5,000 s^{-1}) and coumaphos (k_{cat} = 800 s^{-1}) or fluorophosphonate (P–F) neurotoxins such as DFP (k_{cat} = 350 s^{-1}) and the chemical warfare agent Sarin (k_{cat} = 350 s^{-1}). In contrast, the enzyme has poor specificities for phosphorothioate insecticides such as acephate (k_{cat} = 5 s^{-1}) and the nerve agent VX (*O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate) (k_{cat} = 0.3 s^{-1}) and its analogues as reflected by the specificity constants (k_{cat}/K_m values for VX ~ 0.75 x 10^3 $M^{-1}s^{-1}$ as compared to 5.5 x 10^7 $M^{-1}s^{-1}$ for paraoxon. Different metal-associated forms of the enzyme with Co or Zn at the binuclear metal-active center demonstrated significantly different hydrolytic capabilities for VX and its analogues; the activity of OPH (Co) was consistently greater than that of OPH (Zn) by five- to ten-fold. Significant improvement of the catalytic activity (k_{cat}) and substrate specificity (k_{cat}/K_m) of this stable, quite flexible enzyme (OPH) has been achieved through site-directed mutagenesis of histidinyl residues affecting the metal content of the enzyme and apparently modifying the boundaries of the active site. Individual mutants have been developed which have demonstrated 20-fold improvement in activity against analogues of VX and 30-fold improvement in activity against Soman. Many of these mutants retain excellent catalytic activity and specificity for the native enzyme's preferred phosphotriester substrates such as paraoxon, despite the loss of one of the two molecules of metal present in each native enzyme. X-Ray crystallographic coordi-

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nates of the native wild-type apoenzyme (from *H. Holden, U. Wisconsin*) and modeling of important features at the active site suggest critical attributes that can be further modified to continue the rational design of this enzyme for bioremediation of pesticide contaminants and CWA stockpiles. OPH is one of the few enzymes which has been shown to be capable of hydrolyzing the P–S bond of various OP pesticides; however, it possesses a wide range of catalytic rates (0.0067–167 s^{-1}). Nonetheless, it has been possible to enhance the unique P–S bond hydrolysis of this enzyme by selecting specific changes in the amino acids bordering the active site. Thus, it appears that the capacity for further improvement is remarkable, and the opportunity for a variety of biotechnical applications from the development of transgenic soil fungi and plants to whole cell hydrolysis in slurry-bed bioreacter systems to air stream purging and development of neurotoxin-specific bioreactors (see references) is quite pronounced.

[1] B. Xu, J.R. Wild, C.M. Kenerley, *J. Fermentation and Bioengineering* **1996**, *81*, 473.
[2] J.K. Grimsley, V.K. Ratogi, J.R. Wild, 'Biological Systems for the Detoxification of Organophosphorus Neurotoxins: Potential for Environmental Remediation Applications', in 'Bioremediation: Principles and Practice', Eds. S.K. Sikdar and R.L. Irvine, Technomic Publishing Co. Inc., 1996, in press.
[3] E.I. Rainina, A.L. Simonian, E.N. Elremenco, S.D. Varfolomeyev, J.R. Wild, *Biosensors and Bioelectronics* **1996**, in press.
[4] K. Lai, N.J. Stolowich, *Arch. Biochem. Biophys.* **1995**, *318*, 59.
[5] F. Yang, J.R. Wild, A.L. Russell, *Biotechnol. Prog.* **1995**, *11*, 71.
[6] F.C.G. Hoskin, J.E. Walker, W-D. Dettbarn, J.R. Wild, *Biochem. Pharmacol.* **1995**, *49*, 711.
[7] J.E. Kolakowski, J.J. DeFrank, K. Lai, J.R. Wild, 1996, submitted for publication.
[8] K.I. Dave, L. Phillips, V.A. Luckow, J.R. Wild, *Biotech. and Appl. Biochem.* **1994**, *19*, 271.
[9] K. Lai, K.I. Dave, J.R. Wild, *J. Biol. Chem.* **1994**, *269*, 16579.
[10] L. Pei, G. Omburo, W.D. McGuinn,. I. Petrikovics, K.I. Dave, F.M. Raushel, J.R. Wild, J.R. DeLoach, J.L. Way, *Toxicol. Appl. Pharmacol.* **1994**, *124*, 296.
[11] K.I. Dave, C.E. Miller, J.R. Wild, *Chem.-Biol. Interact.* **1993**, *87*, 55.

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Biocatalysis and Process Integration in the Synthesis of Semi-synthetic Antibiotics

Alle Bruggink*

Introduction

The fine chemical industry is one of the industry segments where the impact of biocatalysis is felt most profoundly. Possible explanations are [1][2]:
- the need to replace traditional, stoichiometric processes in order to improve the product to waste ratio,
- the failure to translate chemocatalytic processes from petrochemicals to fine chemicals,
- the ready acceptance of enzymes by the organic chemist as part of his toolbox, whereas organic chemistry is still the dominating discipline in fine chemical industry [3],
- the low entry barrier, *i.e.* low investments, for new technologies in this small scale industry, facilitated by the fact that fine chemical companies become increasingly part of larger industrial conglomerates.

Synthesis of Semi-synthetic Antibiotics

The industrial production of semi-synthetic antibiotics, with a history of some 30 years, is an outstanding example of the development of biocatalysis. Cefalexin, with an annual consumption of almost 2000 t, the largest cephalosporin on the world market, serves as a useful illustration. The original synthesis (see *Fig.*) starting from benzaldehyde and fermentation of penicillin G was a ten-step process employing stoichiometric chemistry only and causing a waste stream of 30–40 kg per kg of end product. Often 4–6 different companies were involved to serve the chain from basic raw materials to bulk drug; nowadays one or two companies cover the full production column.

The *Chemferm* Process

In the *Chemferm* process (*Fig.*) only six steps are needed, whereby biocatalysis is involved in three of them [4]; a major improvement through the eyes of the organic chemist [5]. However, when it comes to the design of the production plant for the final coupling step, the engineers are faced with an equilibrium process requiring recycle of starting materials and handling of many solids:
- crystallization and isolation of the desired cefalexin,
- crystallization and recovery of excess 7-ADCA (= 7-aminodeacetoxycephalosporanic acid),
- crystallization and isolation of phenylglycine from undesired hydrolysis of both end product and side-chain precursor.

So far, in our developments at *Chemferm*, the environment has been the main winner. Only aqueous waste streams containing some simple inorganic salts are produced, whereas the traditional process releases methylene chloride and other solvents and needs stoichiometric amounts of silylating agents, *Dane*-salt-protected side chains and acylating promotors (such as pivaloyl chloride) which all end up as waste.

The NOVO Process

An alternative process was developed by NOVO, acquired and further improved by *Chemferm*. Using β-naphthol as complexing agent, which surprisingly is compatible with the enzymatic condensation conditions, an almost quantitative yield of

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