A Journey from the Pool of Chiral Synthetic Building Blocks to Cell-Penetrating Peptides, to a Novel Type of Enzyme – and back

Dieter Seebach*

Abstract: The roles of polyhydroxy-butyrates/alkanoates (PHB/PHA) in biology, for the preparation of chiral building blocks, and as a source of inspiration for the discovery of β- and γ-peptides are discussed. The syntheses and structures of β-peptides are outlined. The prerequisites for mimicking peptide/protein interactions with β-peptides and two examples are presented. Single terminal β-amino-acid residues can lead to stabilization of peptides (*cf.* NTS(8-13)) in plasma. Cell-penetrating α-L-, α-D-, mixed α-L/D- and β-oligoarginines (OAs) and -oligoprolines, as well as the mechanism(s) of internalization are compared. Recent studies show that infected erythrocytes, parasitic organisms and mycobacteria are entered by OA-derivatives, which have been employed as transporters of the antibiotic fosmidomycin. While β-peptides are generally enzymatically stable (for days in mammals), a microorganism (*S. xenopeptidilytica*) with an Ntn enzyme (3-2W4 BapA) was discovered that cleaves *only* β-peptides, and that was applied in preparations of (enantiopure) β-amino acids and β-peptides.

Keywords: Cell-penetrating peptides · Fosmidomycin · Oligoarginines · Polyhydroxybutyrate · β-Peptides · Peptidomimetics · Terminal homologation of peptides

1. Introduction

The history of peptide chemistry in our group[1] has its origin in the early work on the pool of chiral building blocks ('chiral pool'),[2] when we used 3-hydroxybutanoates[3] and other readily available enantiopure natural products[4] to prepare macrodiolides,[5] chiral dendrimers,[6] and α-branched amino acids.[7] Key intermediates in these investigations were, among others, lithium enolates of the general *formulae* **1**–**5**[8] (Fig. 1). The most abundant source of (*R*)-3-hydroxybutanoic acid is its polymer (PHB), a microbial storage material and a component of all biological cells, where it is a posttranslational appendix of proteins and a component of complexes with polyphosphate (polyP) and nucleic acids; the results of investigations of PHB over the past decades can be put in a nutshell by the statement "no life on this planet without PHB".[3,9] Fascinated by this role of a simple polyester we searched for structural information to find that the PHB-backbone is extremely flexible in solution, while in the solid state, in phos-

pholipid bilayers and in complexes with polyP it may adopt an (*M*)-2$_1$- or a (*P*)-3$_1$-helical structure[3c] (Fig. 2a). Inspection of the 3$_1$-helix led to the idea to replace the chain-bound oxygen by NH to stabilize the helix by hydrogen bonding. This led to β-peptides, which indeed, fold to helices in solution (Fig. 2b).

2. Synthesis

In between the idea and the discovery of the world of β-, and later of γ-peptides (Fig. 3), lay hard-core organic synthesis with a large group of coworkers for many
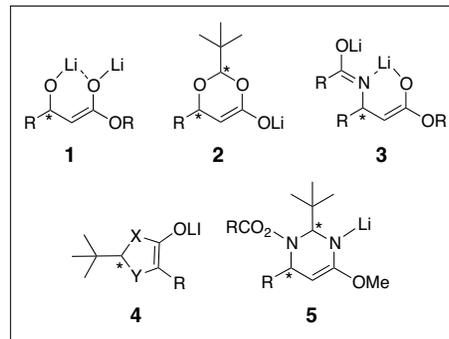

Fig. 1. Chiral Li-enolate derivatives **1**–**5** of β- and α-hydroxy, -amino and -mercapto-carboxylic acids. In **4** X may be O or NR, Y can be O, S, NCOR. * indicates (*R*) or (*S*) configuration.

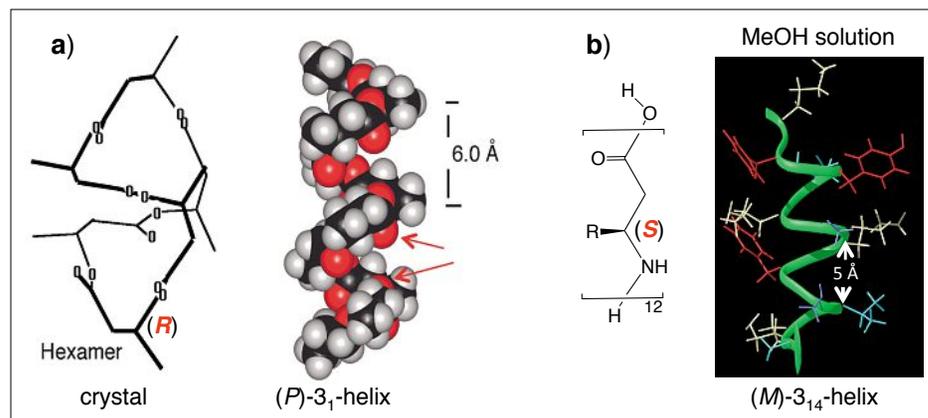Fig. 2. Helices of β-hydroxy- and β-amino-acid oligomers. a) The cyclic HB hexamer has the shape of the number 8, with 2 (*P*)-helical pitches, from which the 3$_1$-helix was constructed in silico.[3] b) NMR Structure of a β^3-dodecapeptide in solution.[10]

Correspondence: Prof. Dr. D. Seebach
Laboratorium für Organische Chemie der ETH Zürich
Wolfgang-Pauli-Strasse 10
CH-8093 Zürich
Tel.: +41 44 632 29 90
E-mail: seebach@org.chem.ethz.ch

years (Fig. 4).[10] There are only four types of α-amino acids ((L), (D), α-branched such as Aib, and cyclic, such as Pro). In contrast, the β-amino acids can carry the substituent in the 3- or 2-position, and they may be geminally disubstituted in these positions, or can be cyclic (2-amino-cyclopentane or -cyclohexane carboxylic acid[11]), so that a much larger structural variety results (Fig. 4, center). For the syntheses of the most simple β³- and β²-amino acids we used the *Arndt-Eistert* homologation and a modified *Evans*-auxiliary application, respectively. While 18 of the 20 β³-aminoacids with proteinogenic side chains (ready for solid-phase coupling by the Fmoc strategy) are now commercial, most of the β²-analogs and other types of derivatives have to be tediously prepared as shown for β²h-histidine in Fig. 5.[12] The effort was rewarding in many respects.

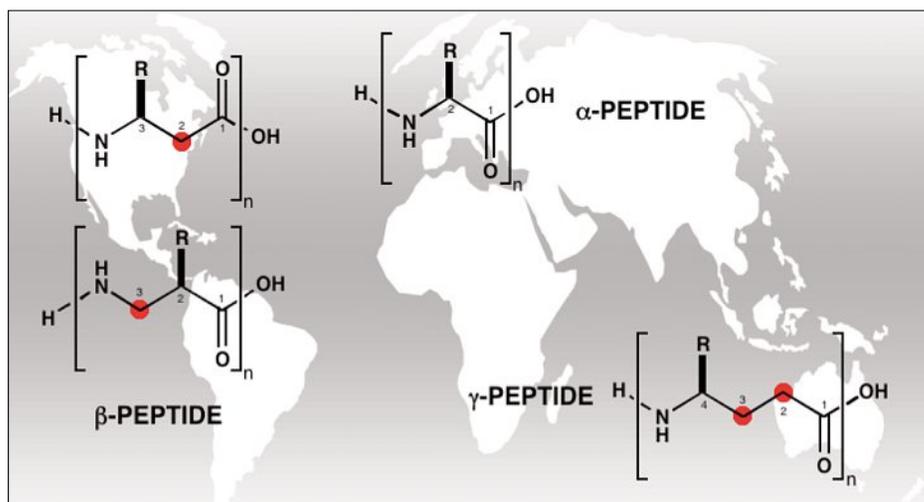Fig. 3. Relationship between α-, β³-, β²- and γ⁴-peptides with the proteinogenic side chains R. For further structural variations see Fig. 4. Our research was restricted to peptides consisting of homologated proteinogenic amino acids[10] lacking the back-bone fortifying effects of cyclic amino-carboxylic acid residues.[11]

3. Structures

Depending upon the substitution patterns (constitution) and upon the (relative and absolute) configurations of the residues a 'brave new world' of peptide structures[13] was uncovered. As with α-peptides and proteins, there are all the structural elements (strand, sheet, turn, helix) but with different dimensions, distances, polarities, stabilities and exclusivities (Fig. 4). Generally, the secondary structures are more stable than those of the α-peptidic counterparts. Also, the sequences can be chosen in such a way that a strand is enforced, which can not aggregate to a sheet, and a sheet structure can be created that can not possibly fold to a turn or helix, while α-peptidic chains with very similar sequences may be found either as part of sheets or of helices. There is no mac-

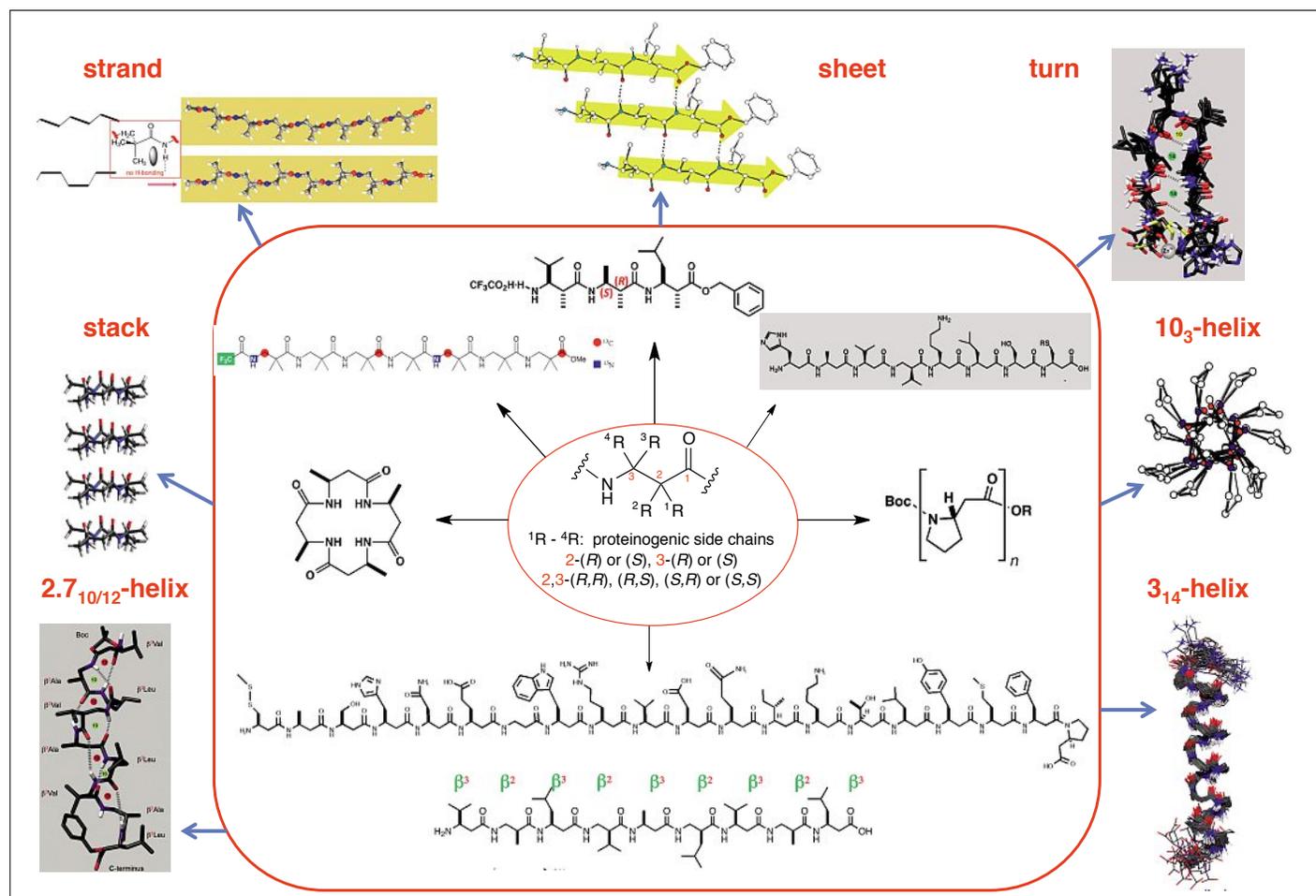Fig. 4. Various types of β-amino acids (center), various residue sequences and the corresponding structures of β-peptides with proteinogenic side chains.[10]
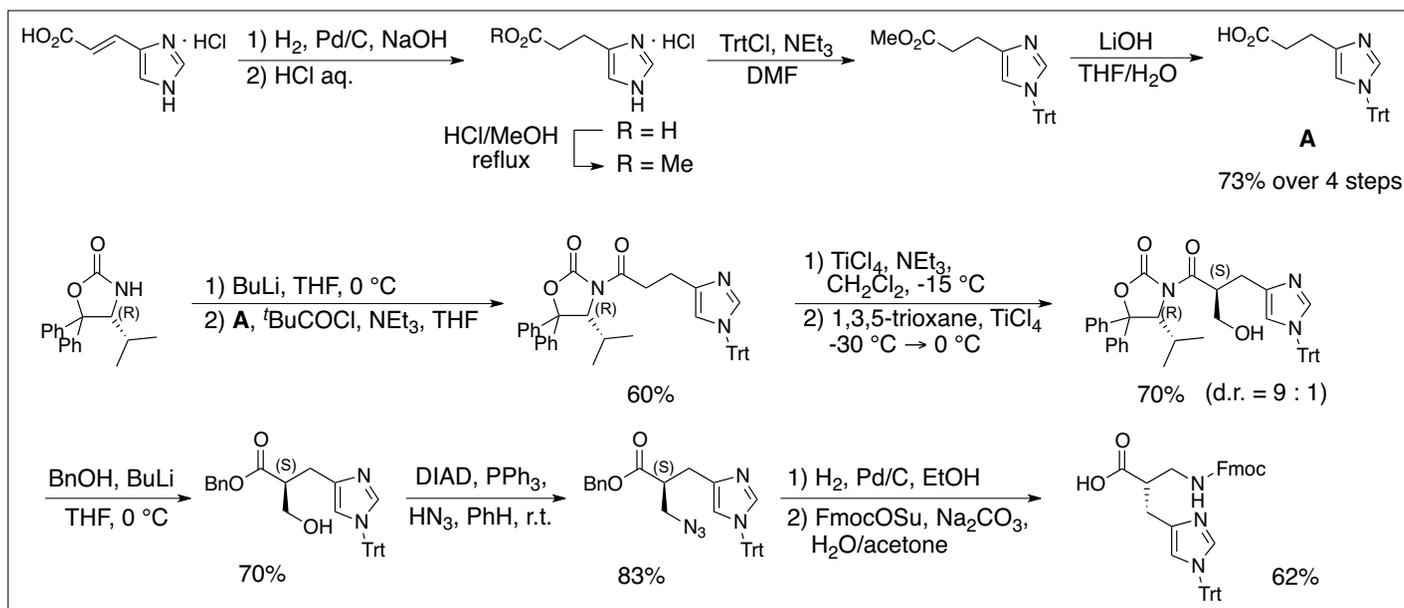
Fig. 5. Preparation of Fmoc-β²h-His(Trit)-OH from des-amino-histidine[12a] in eight steps (overall yield 11%), using the modified Evans auxiliary with 5.5-diphenyl-substitution on the oxazolidinone ring (DIOZ[12b]).

Fig. 6. β-Peptides are stable in essentially all organisms tested so far (for an exception see Fig. 11). The β-amino-acid residues are not recognized by *exo*- or *endo*-peptidases, or by metabolizing enzymes (*cf*. glucuronylation in insects), see section 10 in the review article in ref. [10b], and ref. [16].

rodipole destabilization of the β-peptidic 3_{14}-helix. Furthermore, a peculiar, novel type of $2.7_{10/12}$-helix is formed by sequences of alternating β²- and β³-aminoacid residues, with no resulting macrodipole, consisting of narrow 10-membered and wider 12-membered hydrogen bonded pitches. After a learning process of approximately eight years between discovery[14] and a comprehensive review[10b] we were able to create these secondary structures by design.

4. Biological Investigations

The β- and γ-amino acid residues of the new peptides do not fit into active sites of peptidases or metabolizing enzymes (Fig. 6). Thus, like α-D-peptides,[15] the β-peptides are excreted unchanged after administration to mammals,[10b,16] suggesting that there is no interaction of β-peptides with proteins (PPI), *i.e.* with the active sites of proteases and metabolizing enzymes. In fact, there can be no mixed α/β-peptide sheet or helical zipper[17] (Fig. 7a). On the other hand, structurally not so strictly defined interactions, such as polar binding of anchoring groups in pockets, hydrophobic binding of amphipathic helices in non-polar grooves, or 'face-to-face' interactions between large surface areas of proteins and their ligands can be mimicked with β-peptides (Fig. 7b), see a review article entitled 'β-*Peptidic Peptidomimetics*'.[18] Another biological process, in which no PPI is involved, is the cell penetration of polycationic peptides (CPPs) and other polyelectrolytic compounds. Nature uses this surprising effect for carrying proteins (*cf*. HIV-Tat) into cells, most commonly
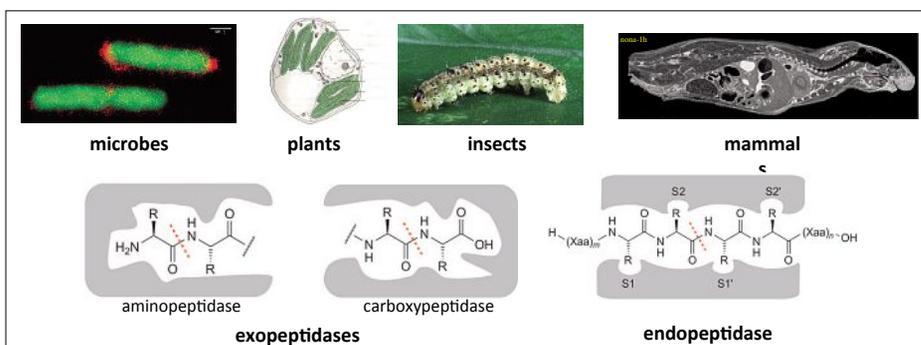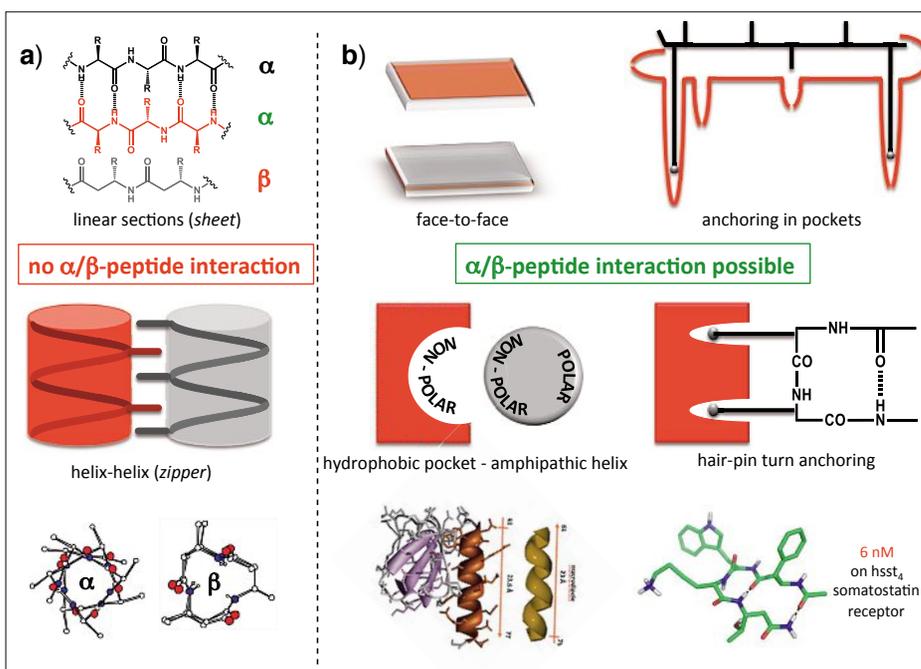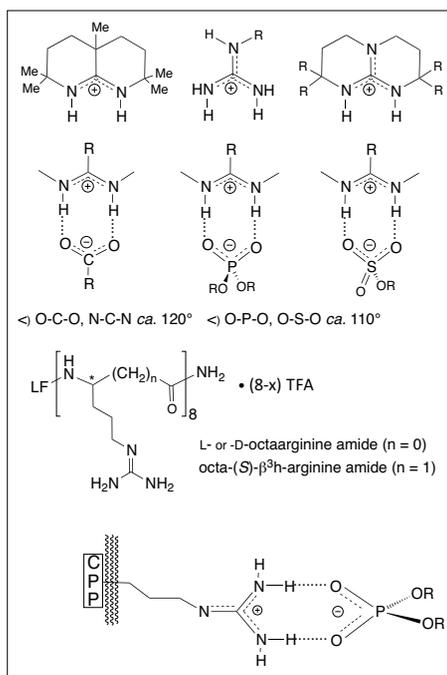

Fig. 7. Peptide–peptide and peptide–protein interactions (PPIs). a) Mixed α/β-peptide sheets or zippers are structurally 'forbidden'. b) Possible PPIs between β-peptides and protein receptors (examples are the binding of β-peptidic ligands to CD40, MHCs, GPCRs, SR-B1, hDM2 receptors and the helix-protein interaction in hIL-8, see refs [10b] and [18] and references cited therein).

with arginine-rich peptide sequences. In spite of numerous investigations into the mechanism, the details are still being discussed. Since the polycationic species, such as oligoarginines, do not pass the phospholipid bilayer of vesicles, which are often used as simple models for cell walls, since no trans-membrane transporter is involved, and since endocytotic entry was excluded in many cases, the potential across the cell wall, generated by proton and ion pumps, was proposed to play a major role.[19] Amidinium and guanidinium ions are known[20] to form thermodynamically stable salts with carboxylates (*cf.* phosphatidylserine), phosphates (*cf.* phospholipids) and sulfates (*cf.* heparan sulfate) (Fig. 8), and formation of polycation–counterion complexes of this type is considered decisive for cell penetration.[21]

β-Oligoarginines (β-OAs) appear to enter eukaryotic cells especially fast (Fig. 9b),[10b] which may be due to the fact that in a strand conformation the positively charged side chains, repelling each other, are all on the same face of the chain, which should lead to a higher affinity for lipophilic counterions (Fig. 9a). Like α-OAs the

◁ O-C-O, N-C-N *ca.* 120°   ◁ O-P-O, O-S-O *ca.* 110°

L- or -D-octaarginine amide (n = 0)
octa-(*S*)-β³h-arginine amide (n = 1)

β-AOs do not enter healthy erythrocytes but infected ones,[22a] and they are able to penetrate through the most elaborate cell walls of mycobacteria (Fig. 9c) and para-

Fig. 8. Carboxylate, phosphate and sulfate salts ('adducts') of amidinium and guanidinium cations[20] are involved in the cell penetration of oligoarginines, consisting of α- or β-arginine residues.[21] The best fit between cation and anion is present with the carboxylate ($r_{H,H} \approx r_{O,O} \approx 2.2$ Å); in the phosphate and sulfate $r_{O,O} \approx 2.4$–2.5 Å. The oligoarginine derivatives employed in CPP investigations are TFA salts, as obtained in standard SPP syntheses.

sitic species to deliver drug cargoes[19b,22b] (Fig. 9). These microorganisms produce isoprenoids in a non-mevalonate pathway, the DXR enzyme of which is inhibited by fosmidomycin or FR-900098 (natural antibiotics). Structural investigations (X-ray, NMR[23]) of the complexes of DXR with fosmidomycin, a 2-valent cation, and NADPH have shown that the antibiotic is totally embedded by the enzyme, *i.e.* it has to be released from a CPP in order to be active. We have recently shown that a salt formed from an octaarginine and fosmidomycin (1:4) is highly active against the malaria parasite *in vitro* (Fig. 9d), and that even a covalently attached OA conjugate of FR-900098 phosphonate diester (≥5 µM) is able to inhibit *M. bovis* growth (Fig. 9e).[22b]
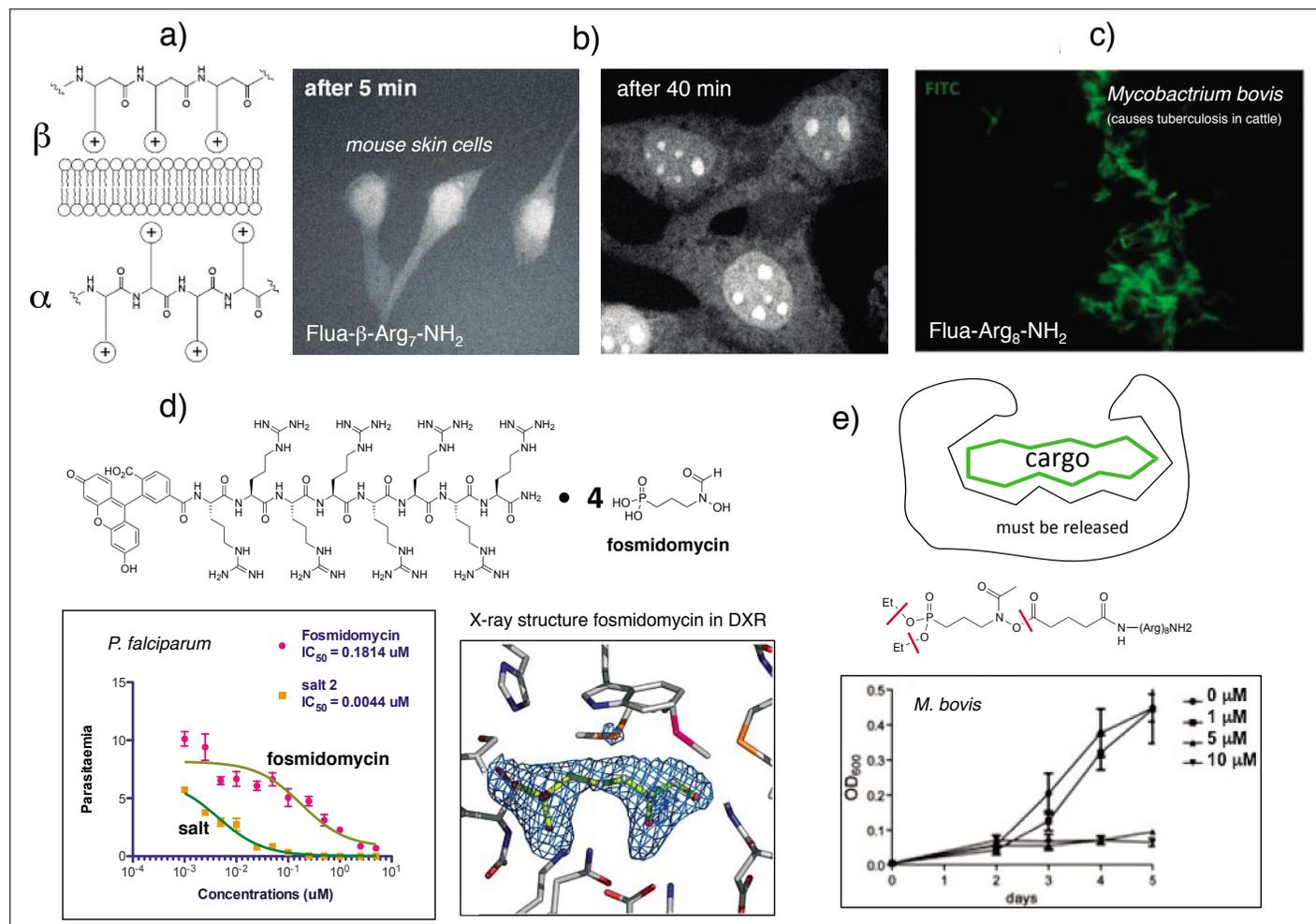
Fig. 9. Cell penetration of α- and β-oligoarginines (OAs) and delivery of antibiotics into *plasmodium* and *mycobacterium* species. a) Structural differences between α- and β-OA. b) Entry of β-OA into the nucleus of skin cells. c) Fluorescence labeled α-OA having entered an *M. tuberculosis* species. d), e) Transport of fosmidomycin or FR-900098 into a parasite and into a mycobacterium with OA-conjugates. The presentations are partially taken from refs [10b], [22b] and [23a].

In the latter case the phosphonate ester *and* the O-acyl bonds must be hydrolyzed before binding to DXR can occur. For *in vivo* investigations L-OA-derivatives are unsuitable because they are quickly degraded, D- and β-OAs are possibly too stable; modulation of enzymatic stability and other properties of OAs is, however, possible with mixed L/D-OAs.[24] In these recent studies we have totally moved away from β-amino acids and β-peptides!

Besides OAs, we also studied L-, D-, β²-, and β³-oligoprolines (OPs) as cell-penetrating *and* phospholipid bilayer passing peptides.[25] They enter HFF cells much more slowly (12 h/37 °C) than the AOs and, being lipophilic, use endocytotic pathways. For measurement of the structure-dependent bilayer membrane translocation of the OPs we used the nanoFAST chip technology, by which the rate of permeation (molecules/(s · µm²)) can be determined simultaneously for hundreds of cavities. Whether oligoprolines can be used to carry cargoes other than fluorescent labels into cells remains to be determined.

The use of single β-amino-acid residues for protection of peptides against exopeptidases was demonstrated with the *C*-terminal, fully active neurotensin segment NTS(8-13). The neurotensin GPC receptor family NT1-3 is present in the brain and in the digestive system, and NTS modulates numerous processes related to dopamine neurotransmission, analgesia, inhibition of food intake, growth of cancer cells *etc*.[26] For diagnostic and therapeutic applications the peptidolytic stability of NTS and other physiologically active peptides has to be increased with as little loss of activity as possible. The degradation by exo-peptidases is commonly inhibited by various structural modifications (Fig. 10a), which cause serious changes of the chemical properties and structures.[27] In contrast, terminal homologation leaves the entire peptide chain from the *N*- to the *C*-terminal stereocenter unscathed and preserves the terminal NH_3^+ and CO_2^- charges, which are just shifted by one C,C-bond distance. In the case of NTS(8-13) the plasma stability increases from minutes to many hours and days after single and double terminal homologation (Fig. 10b), with almost complete retention of receptor affinity.[27,28]

Finally, we have discovered a unique type of enzyme, as a result of concern of youngsters in the group, asking whether β-peptides would ever disappear when put into a natural environment. Samples were collected from flowerpots, forests, fields, compost heaps and water-treatment plants. It took a year until we found a sample (from the sewerage plant of Leutschenbach, a suburb of Zurich), which showed very slow growth of a colony of microbes on the β-tripeptide $^+H_2$-β³hVal-

β³hAla-β²hLeu-O⁻ as the single carbon and energy source. Only one of the organisms in the colony (*Sphingosinicella xenopeptidilytica* sp. 3-2W4) was actually able to cleave the β-peptide, releasing products of degradation for the others to survive in a symbiosis (Fig. 11a).[10b,29a] To make a 12-year-long story short: the β-peptide-cleaving enzyme (BapA) was isolated, its encoding gene cloned in *E. coli* to prepare larger quantities, its activity tested with a variety of substrates, and its X-ray crystal structure determined[29b] (Fig. 11). The
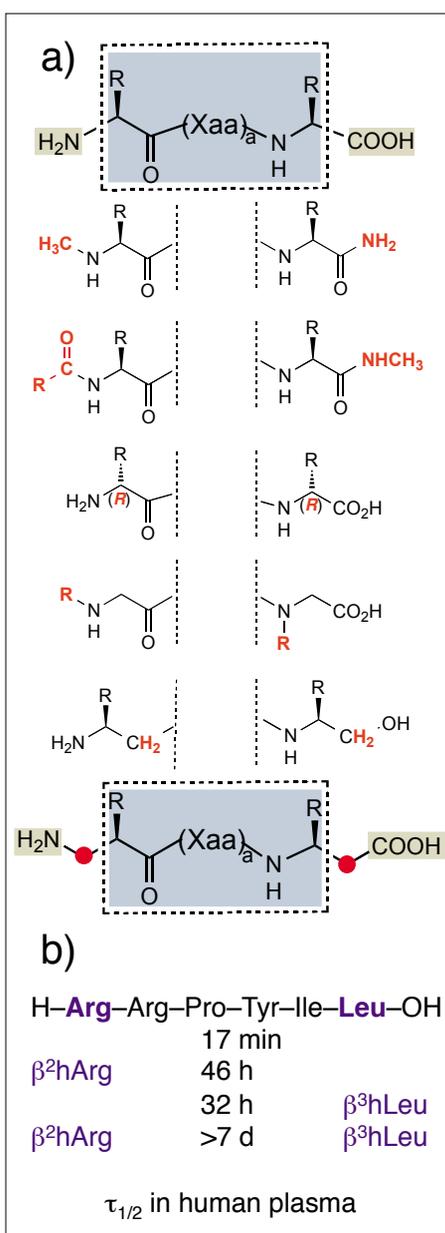

Fig. 10. Terminal protection of peptides against *exo*-peptidases by chemical modifications. a) Commonly applied methods and the new terminal homologation by replacing the *N*- and/or the *C*-terminal residue by a β²- and a β³-amino-acid residue, respectively. b) Effect on plasma stability of terminal homologation of NTS(8-13); the doubly homologated derivative has full affinity as agonists of the NT1 receptor in human cancer tissues.[27]

results are published in *ca*. 20 papers.[29c] The β-peptidyl aminopeptidase BapA belongs to the well-known group of so-called *N*-terminal nucleophile (Ntn) hydrolases, only five of which have now been identified as β-amino-peptidases. Our BapA enzyme has the peculiar property of accepting all *N*-terminal β-amino-acid residues (small, large, non-polar, polar, except for β-Asp and β-Glu), with a large pocket for the side chains in the active site, as if it were constructed for non-selective degradation of β-peptides (which have not been detected in nature!). BapA stops cleaving when it arrives at an α-amino-acid residue in a mixed β/α-peptide, opposite to all 'normal' amino peptidases, which stop with the first β-amino-acid residue in an α/β-peptide.[30]

5. Back to Organic Synthesis

With the BapA enzyme available on larger scale we have tested it for synthetic purposes: BapA is capable of coupling a β-amino acid to an α-amino acid, it cleaves (*S*)-β²hPhe-OH from a *rac*-β-peptide,[29c] and it can be used for preparative kinetic resolutions, for instance of *rac*-β-amino-acid amides to give the enantiopure (*S*)-acid and (*R*)-amide (Fig. 11d).[29c,31] Thus our journey has taken us far into biological, pharmacological and biomedical research areas and back to – enzymatic – organic synthesis.

6. Conclusion

The story I have told herein is a demonstration of the statement about curiosity-driven research: "One can certainly plan research, but not the result".[32] For me and my collaborators this trip was a lot of fun and a constant process of learning about the miraculous chemistry of life.
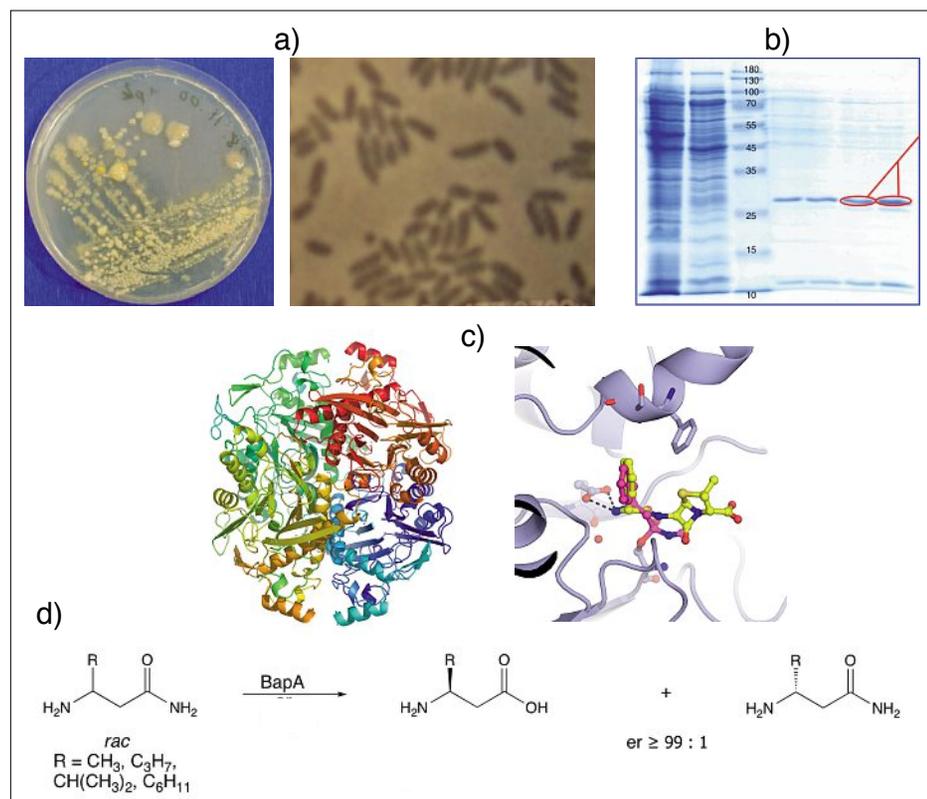
Fig. 11. Discovery of the microorganism *Sphingosinicella xenopeptidilytica* 3-2W4 and its β-peptide-cleaving enzyme BapA.[29] a) In a symbiotic colony of microorganisms the species containing the Ntn hydrolase BapA was identified. b) Isolation of the enzyme and preparation by *E. coli* was followed by c) crystallization, by co-crystallization (*cf.* with antibiotic β-lactams, which inhibit BapA, but are not cleaved by it), and X-ray structural analyses (also of precursors and mutants of the enzyme). d) BapA can be used for the preparation of β-amino acids by enzymatic kinetic resolution.

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