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Céline Ollivaux^{a,b*}, Daniel Soyez^{d,e}, Jean-Yves Toullec^{f,g}

Biogenesis of D-amino acid containing peptides/proteins: where, when and how?

Céline Ollivaux: ^aSorbonne Universités, UPMC Univ Paris 06, UMR 8227, Integrative Biology of Marine Models, Station Biologique de Roscoff, CS 90074, F-29688, Roscoff cedex, France. ^bCNRS, UMR 8227, Integrative Biology of Marine Models, Station Biologique de Roscoff, CS 90074, F-29688, Roscoff cedex, France

Daniel Soyez: ^dSorbonne Universités, UPMC Univ Paris 06, ER3, Biogenèse des signaux peptidiques, 7 Quai Saint Bernard, F-75251, Paris cedex 05, France. ^eCNRS, ER3, Biogenèse des signaux peptidiques, 7 Quai Saint Bernard, F-75251, Paris cedex 05, France

Jean-Yves Toullec: ^fSorbonne Universités, UPMC Univ Paris 06, UMR 7144, Adaptation et Diversité en Milieu Marin, Station Biologique de Roscoff, CS 90074, F-29688, Roscoff cedex, France. ^gCNRS, UMR 7144, Adaptation et Diversité en Milieu Marin, Station Biologique de Roscoff, CS 90074, F-29688, Roscoff cedex, France

*Corresponding author: Céline Ollivaux

E-mail: ollivaux@sb-roscoff.fr

Phone: + 33 2 98 29 25 53

Fax: + 33 2 98 29 23 10

Abstract

Peptides and proteins are chiral molecules with their structure determined by the composition and configuration of the amino acids constituting them. Natural amino acids (except glycine) display two chiral types (L- and D-enantiomers). For example, the presence of octopine, a derivative of L-arginine and D-alanine in octopus or peptidyl poly-D-glutamic acid in a bacterial cell wall was demonstrated in the 20ies and 30ies, respectively. Nevertheless, an old dogma in biology was that proteins (in a strict sense) are composed of amino acids in the L-configuration exclusively, until a D-alanyl residue was reported in a frog skin opioid peptide in the early 80ies and since, numerous D-amino acid containing peptides (DAACPs) have been discovered in multicellular organisms. Several hypotheses may be formulated to explain the origin of a D-residue in the peptide/protein chain. It may result from different mechanisms such as incorporation of a D-amino acid, non-enzymatic racemisation associated with aging or diseases

1 and enzymatic post-translational modification. In the last case, the DAACPs are synthesised via a
2 ribosome-dependent manner, and a normal codon for L-amino acid is present in the mRNA at the
3 position where the D-residue is processed in the mature peptide by peptidyl aminoacyl L-D
4 isomerisation, a peculiar and subtle posttranslational modification. In this review, the different
5 pathways of biogenesis of DAACPs not only in bacteria but also in multicellular organisms are
6 discussed, along with the description of the cellular specificity, the enzyme specificity and the
7 substrate specificity of peptidyl aminoacyl L-D isomerisation.
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14 **Keywords:** peptidyl isomerisation - racemisation - D-amino acid containing peptides - biogenesis
15 - review
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18 **Abbreviations:** cDNA, complementary DeoxyriboNucleic Acid; CHH, Crustacean Hyperglycaemic
19 Hormone; D-Phe₃-CHH, Crustacean Hyperglycaemic Hormone with a D-phenylalanine in position
20 3; DAACP, D-amino acid containing peptide; DLP, Defensin Like Peptide; EST Expressed
21 Sequence Tag; IgG-Fc, Immunoglobulin G-fragment crystallisable région; MS, Mass Spectrometry;
22 mRNA, Messenger ribonucleic acid. ORF, open reading frame; OvCNP, C-type natriuretic peptide
23 (ovCNP-39) from platypus (*Ornithorhynchus anatinus*) venom; RACE, Rapid Amplification of cDNA
24 Ends; RP-HPLC Reverse-Phase High Performance Liquid Chromatography; SG, sinus gland; VIH,
25 Vitellogenesis Inhibiting Hormone; D-Trp₄-VIH, Vitellogenesis Inhibiting Hormone with a D-
26 tryptophane in position 4.
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34 Introduction

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36 A central dogma that has stood the test of time in biology was that proteins were
37 composed exclusively of amino acids in the L-configuration and that unusual D- α -amino-acids (D-
38 AAs) found in proteins (in a large sense) of several organisms were considered “special cases”.
39 For example, in the 20ies, the presence of octopine, a derivative of L-arginine and D-alanine was
40 shown in muscle from octopus [1] and scallop [2] in the 30ies. The presence of a peptidyl poly-D-
41 glutamic acid, a virulence factor in the bacterial cell envelope of the virulent *Bacillus anthracis*
42 was also demonstrated in the 30ies [3]. Later, in the 40ies, a rapidly increasing number of
43 microbial antibiotic peptides, containing D-AAAs were discovered (review in [4]). At that time, the
44 natural occurrence of D-AAAs in proteins (in a large sense) was considered as a peculiarity of
45 microorganisms; however, from the early 80ies, proteins (in the strict sense) containing a D-AA
46 were found in animals, including man. For example, D-Asp has been found in several human
47 proteins, such as dentin [5], α A-crystallin from the lens of patients with cataracts [6] and the β -
48 amyloid peptide from the brain of Alzheimer’s patients [7]. In these long-living proteins, the origin
49 of peptidyl D-AAAs may be explained by non-enzymatic racemisation and isomerisation associated
50 with aging or disease [8]. However, independently from these phenomena, the first peptide
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1 containing *de novo* a D-AA was the dermorphin, an opioid peptide with a D-Ala², characterised in
2 skin secretion of the tree frog *Phyllomedusa sauvagei* [9]. Although at the time of publication, the
3 scientific community was quite doubtful about this work, this was a real breakthrough in peptide
4 studies. Since then, D-AA residues of different nature and at different positions have been found
5 in bioactive peptides from the venom or nervous tissue of various species of molluscs, arachnids,
6 crustaceans and vertebrates (platypus and frogs) (review in [10,11]). Only a single D-AA has
7 been found in each peptide chain, close to one end, most frequently near the N-terminus.
8 Moreover, a classical codon has always been found in the mRNA at the position where the D-
9 residue is present in the mature peptide [12,13]. The presence of a D-residue in the peptide chain
10 may result from different mechanisms such as conversion of a free L-AA into its D-counterpart
11 before its incorporation, or enzymatic posttranslational modification of an L-residue after
12 polypeptide chain synthesis. At least, the change of a specific residue from the L- to the D-
13 configuration results often in a modification of the biological activity of the peptide [14].

22 During the studies of these peptides, a number of different analytical methods have been
23 used particularly chromatography because of their differential hydrophobicity and mass
24 spectrometry because of their differential fragmentation [11,15-18]. However, detection of D-AAs
25 in natural peptides has been, and remains a challenging task, as peptidyl aminoacyl L-D
26 isomerisation does not induce any change in physicochemical properties of the molecule such as
27 pI, primary sequence or molecular mass. Therefore, a D-AA residue in a peptide chain is very
28 difficult to detect and its characterization requires specific analytical approaches that will be
29 described below.

35 In this report, along with the description of the diverse analytical techniques used for the
36 detection and separation of DAACPs, the natural occurrence and the functional significance of
37 these particular peptides will be reviewed followed by a description of the various pathways of the
38 biogenesis of D-residues in polypeptide chains. It will focus on spatio-temporal characteristics,
39 cellular and enzyme specificities and known mechanisms of the peptidyl aminoacyl L-D
40 isomerisation.

47 **Incorporation of free D-amino acids before or during peptide elongation**

51 Many compounds, such as amino acids, can be processed via routes not involving mRNAs
52 and ribosomes. These non-ribosomal peptides (NRPs) can contain D-AAs, which are
53 incorporated by different biosynthetic pathways. In general, epimerisation of non-ribosomal
54 peptides either occurs prior to amino acid activation, or during peptide elongation, neither of
55 which is possible for the ribosomal peptides (RPs) based upon the current understanding of their
56 biosynthesis [19]. In numerous bacteria, essential D-AAs have been notably found in the
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1 manufacture of cell walls. For example, D-alanine, D-aspartic and D-glutamic acids are commonly
2 used for the synthesis of the peptidoglycan (i.e. a polysaccharide cross-linked with a short
3 peptide) an essential constituent of the cell wall of Gram-positive and Gram-negative bacteria
4 (Figure 1) [20,21] (review in [22]). The synthesis of the bacterial cell wall is believed to start with
5 UDP-*N*-acetylmuramic acid, to which assorted free amino acids are added (L-Ala, D-Glu and
6 *meso*-diaminopimelic acid) to generate UDP-*N*-acetylmuramyl-L-Ala-D-Glu-*meso*-diaminopimelate
7 ([23] cited in [24]). Then, a D-Ala dipeptide is coupled to this intermediate of synthesis by UDP-*N*-
8 acetylmuramoyl-tripeptide-D-Ala-D-Ala ligase. This dipeptide is produced by two enzymes:
9 alanine racemase, and D-Ala-D-Ala ligase. The three-dimensional structure of the alanine
10 racemase, the first one converting two common L-enantiomers of alanine to the D-counterparts
11 has been elucidated (Figure 2). The D-Ala-D-Ala ligase generates a D-Ala dipeptide, which is then
12 incorporated into the growing peptidoglycan peptide chain by UDP-*N*-acetylmuramoyl-tripeptide-
13 D-Ala-D-Ala ligase [24]. From the point of view of translational research, these products
14 incorporated in the bacterial cell wall are interesting as they provide potential chemical markers to
15 determine the presence of pathogens in animals, such as cows and pigs [25].

26 Other non-ribosomal peptides include antibiotics with a “simple” structure like the penicillins,
27 which contain a D-valine moiety and a cycloserine derived from D-serine, but also more complex
28 peptide antibiotics such as gramicidin, actinomycin, bacitracin, and polymyxin which are
29 assembled in a stepwise fashion by the action of specific enzymes or enzyme complexes (the
30 peptide synthetases) catalysing individual reactions. For example, the first step in the
31 biosynthesis of penicillin G is the condensation of two amino acids, L-amino-adipic acid which is
32 an intermediate in the lysine biosynthetic pathway, and L-cysteine (Figure 3) [26]. Then, a L-valine
33 is added to the two residues and epimerised to the D-form to generate the L-L-D-tripeptide, i.e. δ -
34 (L- α -Aminoadipyl)-L-Cys-D-Val (ACV) by a catalytic multi-enzyme, δ -(L- α -aminoadipyl)-L-Cys-D-
35 Val synthetase that displays an epimerase domain in its C-terminal region [27]. The following
36 steps in the biosynthesis of penicillin G are the oxidative conversion of linear ACV into the
37 bicyclic intermediate isopenicillin N by a specific enzyme, the isopenicillin N synthase and a
38 transamidation by isopenicillin acyltransferase to produce penicillin G (review in [28,29]).

49 **Conversion of L- to D-amino acids in ribosomally synthesised antibiotics**

51 Unlike non-ribosomal peptides (NRPs), the natural ribosomal peptide products cannot (as
52 far as is known) utilise amino acids beyond the canonical twenty proteinogenic amino acids,
53 which, to a certain extent, limits their structural diversity. However, they can be extensively post-
54 translationally modified, and these modifications can lead to products with many features
55 resembling those of the NRPs. In fact, many of the modifications commonly thought of as
56 “nonribosomal” are also found in ribosomally synthesized peptides. Among these post-
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translational modifications, the conversion from the L- to the D-configuration without residue excision and replacement has been observed in another group of peptide antibiotics called lantibiotics (*lanthionine-containing antibiotic* peptides), produced by Gram-positive bacteria such as *Streptococcus* and *Streptomyces* to attack other Gram-positive bacteria, and as such, they are considered as members of the bacteriocin family. For instance, three examples of D-alanine containing peptides have been reported in these microbial peptides: lactocin S which contains three D-alanines and is produced by *Lactobacillus sakei* [30], and Ltn- α and Ltn- β , the individual components of the two-peptide lantibiotic lactacin 3147 originally produced by *Lactococcus lactis*. Ltn- α and Ltn- β possess one and two D-alanines, respectively (Figure 4) [31,32]. The mechanism by which these D-alanines are generated in lantibiotics is unique and involves the conversion of a ribosomally introduced L-serine to a D-alanine, thus changing both the side group and chirality of the original residue. When this phenomenon was first discovered in lactocin S, it was postulated that a conversion of L-serine to D-alanine occurred via a two-step process of α -carbon stereoinversion [30]. The first step involves the dehydration of serine to dehydroalanine (Dha), a common occurrence during lantibiotic biosynthesis, which, for the majority of lantibiotics, is catalysed by a lantibiotic synthetase. The second step, described for lactacin 3147, involved the enzymatically stereospecific hydrogenation of the Dha to form D-alanine. This is, to date, the only example whereby the enzyme(s) responsible for this modification, a lantibiotic dehydrogenase, has been identified. While lactacin 3147 and lactocin S are the only ribosomally synthesized prokaryotic peptides known to possess the D-AAs, a number of homologues of the dehydrogenase have been identified in the genomes of *Staphylococcus aureus*, *Pediococcus pentosaceus*, and *Nostoc punctiforme* suggesting that others lantibiotics could contain D-AAs [33].

Racemisation in ageing human proteins

Over recent decades, D-AAs have been detected in various human tissues in the free form and as D-amino acyl residues in peptides and proteins. In particular, D-aspartyl residues have been detected in diverse proteins (elastin, myelin, crystallins) from various tissues of elderly individuals such as eye, brain, tooth and skin (for review [34]). D-Serine was also found in β -amyloid protein of patients with Alzheimer's disease [35] and in crystallin [36]. These findings are summarized in Table 1. Importantly, the proteins containing these D-AAs are derived from tissues that are metabolically inert. Thus, D-amino acyl residues arise due to racemisation of AAs in the proteins during the life span of the individual. Crystallin is a major structural protein of the lens, which is composed of α -, β - and γ -forms. α -Crystallin is comprised of two types of subunits α A and α B. Contrary to β - and γ -forms, α A- and α B-crystallins, both display racemisation of aspartic acid and asparagine. In α A-crystallin, the D/L ratio is higher than 1.0 for Asp-151 and Asp-58; this

means that the residues might undergo an inversion of configuration of L- to D-Asp and are not due to a simple racemisation [37]. A potential spontaneous mechanism by which Asp residues in proteins, especially α A-crystallin are isomerised to the D-form with age under particular physiological conditions, has been comprehensively described in [38,39]. Simultaneous formation of D- and β -Asp residues (named D- β -Asp) from aspartyl residues in this protein could be explained as follows (Figure 5) [40]: (1) When the carbonyl group in the side chain of the L-aspartyl residue is attacked by the nitrogen of the amino acid residue following the Asp residue, L-succinimide is formed by intramolecular cyclisation. (2) It is converted to D-succinimide through an intermediate [enol form] whose prochiral α -carbon is in the plane of the ring. (3) Protonation of the intermediate may proceed from the upper or lower side of the plane in an ordinary peptide or protein. The area surrounding Asp-151 and Asp-58 residues in α A-crystallin is thought to form a chiral environment that promotes formation of D-succinimide over L-succinimide. (4) D- and L-succinimide are hydrolyzed at either side of their two carbonyl groups, yielding both β - and α -Asp residues. As shown in Figure 5, D-Asp formation is also accompanied by isomerisation from the natural α -Asp to the biologically rare β -Asp (isoaspartate). Thus, four isomers, L-Asp, L- β -Asp, D-Asp and D- β -Asp, are simultaneously formed in the protein. The formation of these isomers at Asp-151 and Asp-58 of human α A-crystallin begins shortly after birth and thereafter they accumulate gradually during the aging process. As described above, the area surrounding the Asp may form a chiral environment, which allows the inversion of L- to D-configuration. Indeed, if the neighbouring amino acid of the Asp residue has a small side chain, such as alanine or serine, the formation of succinimide occurs easily because there is no steric hindrance [41]. In the primary amino acid sequence of α A-crystallin, Asp-151 and Asp-58 are followed by alanine and serine, respectively. Therefore, formation of succinimide is anticipated to occur readily along with inversion and this depends not only on the primary structure, but also on the higher order structure of the protein.

Isomerisation of peptides in animals

Independently of the age of proteins, it has long been assumed that in animals, natural residues in proteins existed exclusively in the L-form. But, contrary to all predictions, in 1981, a D-Ala residue was reported to be present at the second position in an opioid peptide, dermorphin, from the skin secretion of the tree frog *Phyllomedusa sauvagei* [9]. At the time of publication, the scientific community was quite doubtful about this work, but this subsequently proved to be a real breakthrough in peptide studies. Over the past three decades, more than 40 examples of DAACPs in animals have been reported. D-Amino acyl residues of different types have been found in bioactive peptides from the venom or nervous tissues of various species of molluscs, arachnids, crustaceans and vertebrates (platypus and frogs) (Table 2; reviews in [10,11,42-44]).

Where to find DAACPs? Occurrence in animals and functional significance

To date, only a single D-amino acyl residue has been found in a given peptide sequence. The nature of the D-residue varies according to the peptides, although it has consistently been found near the extremities of the sequences at the second or third position, mostly near the N-terminal end (Crustacean Hyperglycaemic Hormone, dermorphin...). A D-residue has been found near the C-terminus in few cases such as in a toxin isolated from the venom of the funnel web spider *Agelenopsis aperta* (ω -agatoxin 4B) [45,46] and in the R11 conotoxins (a, b and c) from the marine mollusc cone snail (Table 2). The situation is particular for short peptides like contryphans, since these hepta- or octapeptides isolated from the venom of the marine gastropods *Conus* display a D-residue (tryptophan or leucine) at the central positions 3 or 4 [47,48] (Table 2).

From a biological point of view, different cases of figures can be observed. These are summarised in Table 3:

1) Contrary to the D-isomer, the L-form is not naturally produced or has not been found

(a) Isomerisation of one residue in the peptidic chain has been found to be crucial for proper bioactivity. For example, the presence of Ala² in the D-form is the *sine qua non* for the biological activity of the dermorphin from frog skin, binding exclusively to the μ -opioid receptors [9,49]. This was supported by a number of experiments in which synthetic dermorphins containing an L-Ala² (which has never been found in skin extracts) was consistently shown to be inactive, even in the presence of protease inhibitors [50]. This is also the case of the dermenkephalin with D-Met² since L-Met²-dermenkephalin is virtually devoid of opioid activity [51,52].

(b) However, in some cases, even if the L-form is not naturally produced or has not been found, the synthetic peptide with a L-residue displays slight activity. It is the case of the L-counterparts of the cardio-excitatory tripeptide (Asn-D-Trp-Phe) purified from the heart of the gastropod mollusc *Aplysia kurodai* [53] or of the pentapeptide (Phe-D-Asn-Glu-Phe-Val) fulicin extracted from ganglia of the pulmonate mollusc *Achatina fulica* that enhances the contraction of the penis retractor muscle [54]. They are at least 1000 and 3000 times more potent than the L-isomers, respectively.

(c) Another possibility is that the synthetic peptide with a L-residue and the natural D-form display the same activity. An interesting example is the FMRFamide-related decapeptide isolated from the anterior byssus retractor muscle of the bivalve mollusc, *Mytilus edulis* (Mytilus-FRFamide with the sequence H-Ala-D-Leu-Ala-Gly-Asp-His-Phe-Phe-Arg-Phe-NH₂). Indeed, Fujisawa *et al.* [55] showed in 1992 that the synthetic peptides with an L-Leu² and D-Ala¹ have

1 almost equipotent effects as the native peptide containing a D-Leu². Their results suggest that the
2 all-L C-terminal tetrapeptide fragment structure of *Mytilus*-FMRFamide is very important for
3 excitatory action on muscle and that the N-terminal hexapeptide portion containing the D-residue
4 is not essential for activity. Contrary to most examples described above, the D-residue is probably
5 not required for maintaining the correct molecular conformation of the peptide for activation of its
6 receptors.
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11 2) *In other cases, both natural isomers were found in the tissue.*

12 (a) In the African giant snail *Achatina fulica*, achatin I, a tetrapeptide (Gly-D-Phe-Ala-Asp)
13 purified from the suboesophageal and cerebral ganglia was found as both isomers in tissue
14 extracts [56]. The D-form may have a potent neuroexcitatory effect by inducing a stereospecific
15 voltage-dependant inward current, due to Na⁺ ions, on several heart regulatory cerebral neurons
16 of the same snail. Likewise, a synthetic peptide with D-Ala (Gly-D-Phe-D-Ala-Asp), was also
17 proved to be active, but at a level of less than 10⁻³ as potent compared to the natural Gly-D-Phe-
18 Ala-Asp isomer. In contrast, the peptide formed with only L-AAs, also present in the ganglia, is
19 devoid of activity and to date no other function has been attributed.
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23 (b) Like achatin, both natural ω -agatoxin isomers were found in natural extracts, isolated
24 from the web spider venom [57]. However, in this case, the L-form displays slight activity. Indeed,
25 the D-isomer (IVB) was found to be a more potent blocker of the P-type voltage sensitive calcium
26 channel in rats than its L-serine containing counterpart (IVA), which showed 80-90 fold less
27 potent inhibition [46].
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30 (c) In several Astacidae species (lobsters, crayfishes), Crustacean Hyperglycaemic
31 Hormone (CHH) is synthesised in the X organ-sinus gland complex. CHH is a 72-residues
32 neuropeptide and was characterised as two isomers differing in the configuration of the Phe³,
33 either as an L- or a D-form. Both isomers exhibit hormonal activities and regulate energy
34 metabolism but the change in configuration results in modifications to the biological activity of the
35 peptide [58,59]. Indeed, with regard to the physiological significance of this modification, it was
36 established that CHH isomers display differences in the time course of the hyperglycaemic
37 response following injection of the two peptides into crayfish. It was observed that the injection of
38 CHH evoked a rapid hyperglycaemia with a maximal value reached after 2 h, whereas the
39 maximal response was attained only after 3-4 h when using D-Phe³-CHH. Moreover, this isoform
40 increased potency in its hyperglycaemic effect (10 times more potent than the L-counterpart).
41 Other additional functions were reported as it exhibits a potent inhibitory activity upon the molting
42 gland during ecdysteroidogenesis [59,60]. More recently, its role in osmoregulation has also been
43 demonstrated [61].
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56 (d) The presence of both isomers with a difference in biological function has also been
57 observed in the Vitellogenesis Inhibiting Hormone (VIH) a neurohormone found in the major
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neuroendocrine organ of decapod crustaceans, the X organ-sinus gland complex located in eyestalk. The D-form has only been characterised in a few macrouran crustaceans, particularly in lobster [62,63]. It is synthesised with either an L- or a D-tryptophanyl residue (VIH and D-Trp⁴-VIH, respectively) in three Astacidae species (American and European lobster, Norway lobster) and acts on the reproduction of the lobster *Homarus americanus*. When American lobster VIHs were tested in a heterologous *in vivo* assay, only the all-L isomer VIHs displayed significant inhibitory activity with respect to repressing oocyte growth that had been induced by eyestalk removal in grass shrimps [64]. To date, contrary to all the cases described above, no function has been assigned to the counterpart D-Trp⁴-VIH.

(e) A peptide with a D-amino acid residue, C-type natriuretic peptide (OvCNPb with a D-Leu²) has also been described in the venom of a mammal, the platypus (*Ornithorhynchus anatinus*). But the functional significance of the D-amino acid in this peptide is unclear at present. Indeed, experiments performed by de Plater and coworkers [65] did not reveal any difference in activity between the two isomers (OvCNPb and OvCNPa its L-counterpart); they display the same potency in elevating cGMP levels, and producing vasodilatation in cultured vascular smooth muscle. Moreover, OvCNP also causes oedema and mast cell histamine release, and may have other biological activities that are yet to be discovered. A more comprehensive pharmacological study is therefore required in order to compare the spectrum of activities of the two isoforms. The platypus venom gland also produces Defensin Like Peptides (DLP), whose structure resembles those of the mammalian anti-microbial β -defensin. They are present in both configurations (DLP4 and DLP2 with a D-Met²) and their tertiary structures are very similar [66]. The incorporation of the D-AA has minimal effect on the overall structure in solution and to date, the role of both natural isomers in platypus is unknown.

When differences in biological actions and functions are observed between the L- and D-forms, several hypotheses can be proposed to explain this phenomenon. (i) It may be because naturally existing DAACPs, rather than their natural or synthetic L-form counterparts, interact with the endogenous receptors to cause the required physiological responses [67]. For a particular function, the receptor can be specific of the ligand containing a D-residue, whether the L-isomer is present or not. (ii) It is also thought that the modified structure of DAACPs makes it more difficult for the peptides to be degraded by endogenous aminopeptidases, thus increasing their stability [58]. Bansal *et al.* [68] proposed that this “stability bias” is exacerbated by the rate of isomerisation of the D-form of the substrate peptide being slower, than the L-isomer. Hence, in HPLC analysis, the greater abundance of the D-form of the substrate pairs is not only because of the equilibrium of the isomerisation reaction being biased toward the D-isomer, but the all L-peptide is more likely to be hydrolyzed by aminopeptidases in the medium, depleting it further than would otherwise be the case. The medium in which the peptides are immersed may be the

1 internal medium of the animal itself as in case of the hormones CHH and VIH or the tissues of the
2 victims injected with toxins, such as the spider agatoxin, mollusc conotoxins and the platypus
3 DLP; the D-form would be more stable in the victim thus prolonging its effects such as pain. In
4 this biochemical context, a recent concept of mechanism of metabolite separation named “chiral
5 compartmentation” in metabolism, that emerged from the stereo-specificity of enzymes for their
6 substrate(s) has been proposed from these observations [69]. In the case of platypus DLP, chiral
7 compartmentation applies after envenomation: the D-containing peptide DLP4 is resistant to
8 aminopeptidase, which is L-specific. Hence the venom component will reside in the tissue of the
9 victim for longer that would otherwise have been the case because the DAACP is “operationally
10 compartmentalized” from L-stereospecific aminopeptidase(s). Thus, the authors speculated that a
11 selective advantage exists for the platypus to have venom components that persist for longer in
12 the envenomated victim. However, the hypothesis of an increased stability of the D-isomer
13 regarding the putative L-stereospecific aminopeptidase(s) was refuted for CHH since a recent
14 publication [70] has demonstrated that both isoforms of CHH were cleared at a similar rate from
15 the haemolymph of the Crayfish *Astacus leptodactylus*. Rather, the difference in biological
16 activity, for CHHs at least, may be, as proposed above for the hypothesis (i), attributed to binding
17 to different target tissues or, in the same tissue, onto different receptors.

30 ***At which step of peptide biogenesis does isomerisation occur? Timing of the reaction***

31 Since the discovery of the first DAACP, dermorphin in 1981, the origin of the D-AAs in
32 animal peptides has been under debate. The two hypotheses are that, either these peptides are
33 assembled in a stepwise fashion with the incorporation of a D-residue in this process, or the
34 chemical modification of an L- into a D-residue takes place as described above for the bacterial
35 antibiotics. The second hypothesis is that the peptides are biosynthesized by a classical
36 ribosome-associated mechanism. In the latter, the question arises whether the D-residue is
37 incorporated directly into the peptide sequence, or is formed, after peptide synthesis, by a post-
38 translational reaction. Moreover, in this second case, does this modification occur before or after
39 cleavage of the precursor?

40 Using molecular cloning techniques, it has been demonstrated that numerous DAACPs
41 are derived from larger precursor molecules [71]. The classical isolation procedure was PCR
42 amplification of either cDNA libraries or mRNAs prepared from tissues, using degenerate sense
43 and antisense primers from the sequence encoding a similar peptide of another animal species.
44 The normal codon for an L-AA was always described in the position where a D-residue was
45 present in the protein end product. Thus, it is now generally agreed that peptides containing a D-
46 AA are derived from precursor polypeptides comprising only L-residues [72].

47 The next question was to identify if isomerisation is an early reaction that occurs in the
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1 intact precursor polypeptide (as e.g. the hydroxylation of prolines [73]), or a reaction that takes
2 place after partial or complete processing late in the secretory pathway.

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4 Evidence presented by Mor *et al.* [74] suggested that the D-residue is already present in
5 the precursor of frog dermorphin, a heptapeptide with a D-Ala². High molecular weight material
6 was purified by chromatography from extracts of *P. sauvagei* skin and digested with trypsin and
7 carboxypeptidase B. As expected from the precursor sequence, this digestion yielded the
8 decapeptide dermorphin-Gly-Glu-Ala. Using specific antibodies, it could be demonstrated that the
9 isolated fragment already had a D-alanyl residue in the second position. This suggested that the
10 conversion of L- to D-Ala takes place in the intact precursor. However, these results do not
11 exclude the presence of pre-pro-dermorphin with a L-Ala² that is produced ribosomally. In
12 contrast, Auvynet *et al.* [75] did not find a D-residue in pre-pro-deltorphin I, another precursor
13 produced also in the frog skin. Indeed, by using specific antibodies, they showed that deltorphin I
14 (also a heptapeptide with a D-Ala²), and dermaseptin B2 are stored together in secretion granules
15 in the serous glands. Although the pre-pro-dermaseptin B2 and pre-pro-deltorphin I have a
16 common pre-pro-sequence containing signal peptide and acid peptide, and enter identical
17 subcellular compartments where they are processed to produce the mature products (deltorphin
18 I, L-Ala²-deltorphin I and dermaseptin B), only deltorphin I is transformed by isomerisation. These
19 three peptides are all stored together in secretion granules, which accumulate in the cytoplasm of
20 the serous glands. The authors concluded that the isomerisation of deltorphin I occurs in the
21 secretory granules as a late post-translational event. Nevertheless, to our mind, the question
22 whether the D-residue is already present in the precursor or not, remains unanswered, since the
23 presence of the all-L-preprodeltorphin I does not exclude the existence of a putative
24 preprodeltorphin I with a D-Ala².

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39 Study of the dynamics of precursor maturation of CHH isomers (CHH and D-Phe³-CHH)
40 allowed the dissection of the spatio-temporal characteristics of the different steps of isomerisation
41 [76,77]. To our knowledge, this is the only model for which the different steps of the post-
42 translational processing of the precursor (cleavage, C-amidation, isomerisation and N-
43 pyroglutamination) have been comprehensively characterised and the kinetics of isomer
44 maturation estimated in the different parts of the neuroendocrine complex. It has been clearly
45 highlighted using the complementary techniques of radioactive labelling, enzymatic cleavage and
46 immunochemistry with specific antibodies, that the isomerisation of CHH occurs after the pro-
47 peptide cleavage and before N-terminal cyclisation (in a step-wise fashion to avoid steric
48 hindrance). Moreover, results indicate that newly synthesised CHHs are preferentially released,
49 for both isomers. A robust demonstration of the absence of proCHH containing a D-Phe³ has
50 been performed by purification of the only HPLC peak corresponding to the proCHH, and
51 enzymatic cleavage of the material from this peak followed by ELISA with specific antibodies
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1 raised against CHH and D-Phe³-CHH. No immunoreactivity for the D-form was detected by this
2 method.
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4 In conclusion, even if the paucity of information on the isomerisation stage in DAACPs of
5 species, except crustaceans and frogs, prevents any overall generalisations, it is now largely
6 accepted that the DAACP precursor is an all-L sequence. Alternative possibilities, such as the
7 incorporation of D-AAAs into the primary product of translation or excision of certain L-AAAs and
8 reinsertion of the corresponding D-isomer are unlikely.
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13 ***Is there a cellular specificity of isomerisation? Cellular and subcellular distribution of*** 14 ***isomers*** 15 16

17 As described above, over the past 30 years, DAACPs have been regularly discovered in
18 the various tissues of different animal species but rarely described at the cellular level. The frog
19 skin serous glands which are syncytial, produce the D-isomer of deltorphin I (a heptapeptide with
20 a D-Ala²), and isomerisation occurs in all the glands [75]. Staining the serous glands with anti-
21 deltorphin I or anti-L-Ala²-deltorphin I antibodies produced similar overall patterns of fluorescence
22 in all glands, with dense labelling confined to the secretory granules. There was no noticeable
23 difference in the relative intensities and granular localisations of the specific labelling with the
24 anti-deltorphin I and anti-L-Ala²-deltorphin I antibodies, although several serous glands were
25 examined. The absence of glands labelled exclusively by the anti-deltorphin I or the anti-L-Ala²-
26 deltorphin I antibodies suggested that there was no cellular or glandular specificity as the serous
27 glands were producing mixtures of both deltorphin isoforms.
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35 The situation is very different in the X-organ sinus gland (XO-SG) model of crustaceans because
36 D-amino acid containing neurohormones are restricted to discrete, well-characterised
37 neuroendocrine cells located in an easily accessible organ. So, cellular studies in crustaceans are
38 more developed than in other animal models, in which the investigations have been mainly dealing
39 with peptide characterisation and mode of action. Thus, the crustacean XO-SG complex enables
40 the study of the precise cellular and sub-cellular localisations of the isomerisation process. As
41 described earlier, the study of the dynamics of precursor maturation of CHH isomers using the
42 complementary techniques of pulse-chase incubations followed by HPLC/ELISA/MS allowed the
43 dissection of the spatio-temporal characteristics of isomerisation [77]. The conclusion that this
44 post-translational reaction occurs preferentially in the soma of neuroendocrine cells was confirmed
45 by immunofluorescent labelling using specific antibodies against CHH and D-Phe³-CHH followed
46 by analyses using confocal microscopy of the XO-SG complex [76]. In this study, an investigation
47 of the structure of the CHH-producing system was undertaken to elucidate what types of
48 neurosecretory cells were involved in the production of the CHH isomers. Two distinct CHH cell
49 types were observed, either labelled only by the antibody specific for the L-form (called L-cells) or
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1 labelled by antisera recognising both L- or D-isomers, (called D-cells; Figure 6 A, B, C). The
2 number of the different CHH cells per organ was also constant in the studied species, the crayfish
3 *Orconectes limosus*, with a total of 8 D-cells within a total of over 30 CHH cells, as suggested in an
4 earlier immunohistochemical study [78] (Figure 6A) and in the lobster *Homarus americanus*
5 ([62,63]; Figure 6C). Soyez *et al.* [76] have observed that, in the D-cells producing a mixture of
6 both isoforms, secretory granules identified with different antibody labels were not evenly
7 distributed in the cytoplasm (Figure 6B). The clusters of L-labelled granules were generally in the
8 perinuclear region whereas the peripheral part of the cytoplasm was enriched with mixed L- and D-
9 labelled or only D-labelled clusters. A major observation was that in every case, L-labelled
10 fluorescence was no longer detectable as the granules entered the axon of these cells, suggesting
11 that the majority of CHH was already isomerised when the granules left the soma (Figure 6B).
12 Nevertheless, the greater sensitivity of immunogold labelling showed, at the ultrastructural level,
13 that L-immunoreactivity in the axons of D-cells still appeared to be present but in very minute
14 amounts [79]. To our knowledge, this is the only model for which a cellular specificity of this post-
15 translational reaction has been clearly shown. It is almost certainly due to differential expression of
16 the putative isomerase(s) in the two cell types.

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18 In addition to the example of CHH, VIH and D-Trp⁴-VIH are two isomers produced by the same
19 organ, the XO-SG complex but present in distinct cellular locations (Figure 6D) [62]. On the
20 contrary, the use of specific antibodies against D-Trp⁴-VIH and D-Phe³-CHH showed that the D-
21 forms of the two neuropeptides are not only produced in the same cells but, more importantly, are
22 also co-packaged within the same secretory vesicles (Figure 6E). Consequently, with regard to
23 these two hormones, five cell types in the XO-SG complex of lobster could be distinguished: VIH
24 cells, CHH cells, D-Trp⁴-VIH cells, D-Phe³-CHH cells and cells producing both D-Trp⁴-VIH and D-
25 Phe³-CHH (Figure 7). A sixth cell type, producing a mixture of L-epimers of CHH and VIH has been
26 postulated, but, a colocalisation of VIH and CHH has never been observed by confocal or electron
27 microscopy techniques. Similarly, no axon terminal exhibited simultaneous labelling for CHH and
28 D-Trp⁴-VIH or VIH and D-Phe³-CHH.

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30 NDWamide (named NDWamide with the sequence H-Asn-D-Trp-PheNH₂), a D-tryptophan-
31 containing cardioexcitatory neuropeptide in gastropod molluscs, such as *Aplysia kurodai* and
32 *Lymanea stagnalis* has also been studied at the cellular level [72]. To determine the cellular
33 distribution of this peptide and its transcript (NWF mRNA), abdominal ganglia of *Aplysia kurodai*
34 were subjected to immunohistochemistry (IHC) by sequential double staining with a specific
35 antibody (anti-NDWamide) and by *in situ* hybridisation (ISH) with specific DIG labelled oligo-DNA
36 probes. The results revealed that neurons positively stained with ISH were also positive with IHC,
37 suggesting that the precursor proteins and mature NDWamide peptides co-exist in the same neurons.
38 However, this is not always the case and there were some ISH positive neurons, which did not

show any fluorescence signals by IHC and in contrast some IHC immunopositive neurons that did not show staining positive to ISH probes. Several hypotheses have been formulated to explain this result. For example, in neurons positive with IHC but not with ISH, it may be that plenty of NDWFa peptides are stored, but *de novo* peptide synthesis is blocked at the transcriptional level. Or it may be that another, not yet cloned, transcript of an *NDWFa* gene, not recognised by specific oligo-DNA probes, is used for the synthesis of NDWFa in the neurons. Further investigations are required to understand the difference in distributions between the transcript and the mature peptide [72]. In this framework, to our mind, it would be judicious to use also an antibody against the L-isomer to investigate the cellular specificity of both isomers in the perinuclear regions and in the axons, like for the CHH cells, described above.

Is there an enzyme specificity for each residue converted from the L- to the D-form? Isomerase diversity

All the isomerases studied to date act on intact polypeptides rather than on free AAs and require specific flanking residues to operate efficiently. Therefore, in a strict sense, the enzyme is not a racemase because it catalyses the interchange of diastereoisomers, not of enantiomers. By analogy to peptidyl prolyl cis-trans isomerase, the enzyme could thus be called a peptidyl aminoacyl L-D isomerase [44] or peptide epimerase [45].

The first enzyme catalysing isomerisation was isolated from the venom of the funnel web spider *Agalychnis aperta* [46]. This enzyme was found to interconvert two ω -agatoxin IV, peptide toxins with 48 AAs (IVC and IVB), that differ only by the stereochemistry at serine 46 (L-Ser⁴⁶ and D-Ser⁴⁶, respectively), as described above. The complete primary structure of the isomerase was established by sequence analyses of the polypeptide chain, assignments of disulfide bridges, carbohydrate analyses, and mass spectrometry of sugar chains. The enzyme was found to be a 29 kDa polypeptide with an 18-residue light chain and a 243-residue heavy chain interlinked by a single disulfide bond [80]. The heavy chain contained three intramolecular disulfide bridges and one N-linked oligosaccharide chain with a single trimannosyl core structure [81]. Surprisingly, the heavy chain bears remarkable sequence identity to serine proteases such as thrombin and kallikrein (26% and 35%, respectively), particularly in the region of the conserved catalytic triad. Indeed, these hydrolytic enzymes operate via a catalytic triad, which act as proton acceptors and donors for the residue that will be isomerised in the peptide [82]. However, the residues in this active site of the isomerase have not yet been identified [10] and the gene and the RNA releasing the enzyme are not described, to date.

The first work by Torres *et al.* [66] on the detection of a peptide isomerase in a mammal, i.e. platypus, showed that an *Ornithorhynchus* venom gland extract had enzymatic activity that converted DLP4 to DLP2 (with a D-Met²). In 2006, Torres *et al.* [83] purified the isomerase by size exclusion chromatography followed by SDS-PAGE electrophoresis. Its molecular weight is +/-

1 50–60 kDa and the authors noticed that the platypus isomerase showed similarity in its active site
2 with some amino-peptidases since they both acted on or near the N-terminus and were both
3 inhibited by the peptidase inhibitor amastatin. In another paper [84], isolation of the isomerase
4 was carried out by combined ion-exchange chromatography and reverse-phase high
5 performance liquid chromatography (RP-HPLC) providing a much better molecular separation
6 because both the charge and hydrophobicity properties of proteins were utilized. This enzyme,
7 that was only present in very small quantities in an extract of the venom gland, was thermally
8 stable up to 55 °C, indicating a relatively robust structural fold and it was found by anion-
9 exchange chromatography to be acidic [84]. The authors have also investigated other
10 chromatographic methods such as hydroxylapatite and hydrophobic interaction columns but their
11 separation performance did not give better results. The limited amount of available platypus
12 gland extract and the extent of isomerase activity loss after each chromatographic step restrict
13 more detailed studies of this system. As these techniques cannot be used to further separate and
14 isolate the isomerase, the authors are currently sequencing candidate bands isolated from active
15 fractions on SDS-PAGE gels and are also constructing a cDNA library from the platypus venom
16 gland. This should allow the expression of putative isomerase gene(s) and testing of their
17 enzymatic activities. Recent searches for peptide isomerase activity in other mammals has also
18 shown that echidna venom (another monotreme [85]) and mouse heart contain a putative
19 isomerase that has the same substrate specificity as platypus venom [86]. More surprisingly, a
20 weak isomerase activity has also been reported for the first time in a plant (in papaya extracts).
21 Indeed, the synthetic peptides corresponding to the N-terminus of DLP4 with a L-Met² and DLP2
22 with a D-Met², both produced by and originally identified in the *Ornithorhyncus* venom gland as
23 described above, were found to be substrates of the papaya extract [87]. The phylogenetic
24 distance between these two organisms (platypus and papaya) suggests that every living
25 organism may have some peptide isomerase activity.

26 It was tempting to speculate that the isomerase from frog skin is homologous to that of
27 platypus venom [42] since they both act on the second amino acid residue of peptides (Met₂).
28 Indeed, as described above, opiates (dermorphin with a D-Met² and deltorphin containing a D-
29 Ala²), and antibacterial and haemolytic peptides like bombinins bearing a D-allo-Ileu² have been
30 identified since 1981 in skin secretions of different species of amphibians. Nonetheless, it was
31 only in 2005 that Jilek and co-workers [42] discovered a frog isomerase with an apparent
32 molecular mass of 52 kDa that converted the L-AA at position 2 of its substrate to the D-form. The
33 enzyme was purified from the skin secretions of *Bombina variegata* and the N-terminal sequence
34 of the protein along with some of its cyanogen bromide fragments were determined by automated
35 Edman degradation. Oligonucleotides deduced from these sequences were used for PCR
36 experiments with total mRNA isolated from *Bombina* skin. This amplification yielded a cDNA
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1 fragment representing most of the first half of the isomerase. Northern blot analyses
2 demonstrated that a large mRNA containing 8000 nucleotides encodes the enzyme. All attempts
3 to amplify the rest of the isomerase with 5'- and 3'-RACE failed. In a different approach, Jilek and
4 co-workers in 2005 [42] screened a genomic library prepared from the related species *B.*
5 *orientalis* [88]. With specific primers designed from the previous sequence they isolated two
6 clones containing the sequence of an exon with an ORF for 409 AAs, starting with residue 4 of
7 the mature isomerase and part of an intron. The first 3 AAs of the enzyme and a pro-sequence or
8 spacer sequence are encoded by a separate small exon. These two genomic fragments were
9 assembled to produce a *B. orientalis* cDNA, which was translated into a polypeptide comprising
10 412 AAs. This cDNA coded for a protein with a deduced mass of 45 kDa. The cDNA and
11 genomic fragments containing the genetic information for this protein did not code for a signal
12 peptide and its ORF did not end with a stop codon. Starting with the sequence of the *B. orientalis*
13 isomerase, they screened a cDNA library from skin of *B. variegata* by using PCR and suitable
14 primers. Five different cDNAs, termed va to ve, were amplified and were sequenced fully or in
15 part. All of these cDNAs coded for proteins that were 90% identical to the *B. orientalis* isomerase
16 [42]. These homologous proteins are linked by spacer segments in the following order: -va-(33)-
17 vb- and -vc-(10)-vd-(48)-ve- (numbers in parentheses indicate the length of the spacers). The
18 spacer sequences are relatively rich in proline and charged AAs. Individual isomerase domains
19 are apparently excised from this polyprotein by unknown proteases. A search in the data bases
20 for polypeptides related to the *Bombina* isomerase originally yielded only one major match,
21 namely, the N-terminal H-domain of the human IgG-Fc binding protein [89] and not the platypus
22 isomerase as it was expected. This domain comprises 450 AAs and has no known function.
23 Subsequently, ESTs coding for fragments of related proteins from *X. laevis* and chicken were
24 deposited in the data banks. Finally, in the genome of the *fugu* fish, three genes potentially
25 coding for homologous proteins were characterised (FuguGenscan_26525,
26 FuguGenscan_28866, and FuguGenscan_28655). The authors [42] have also shown that the
27 change of chirality of L-Ile to D-allo-Ile at position 2 of a model heptapeptide with the N-terminal
28 sequence of bombinin H proceeds in both directions via the removal of a proton from the α -
29 carbon and a concomitant addition from the opposite side in an acid-base type of catalysis. As
30 described above, such a mechanism has also been proposed for the isomerases from spider and
31 platypus venoms.

32 To conclude, it seems surprising that structurally unrelated enzymes identified in spider,
33 platypus and frogs can catalyse an identical reaction, the L- to D-isomerisation of AAs in
34 processed peptides. Indeed, as described above, the spider isomerase has significant amino acyl
35 residue sequence similarity with serine proteases, while the frog isomerase has some similarity to
36 the N-terminal H-domain of human IgG-Fc binding protein. However, one must bear in mind that
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1 the AAs subject to the isomerisation reside at very different positions in the substrates of spider
2 (position n-2 near the C-terminal end) and frog/platypus (position 2 near the N-terminal end) and
3 are of different nature (Ser for spider, Ile for frog and Met for platypus). Moreover, there would be
4 no cross specificity since the frog enzyme does not act upon the heptapeptide corresponding to
5 the N-terminus of platypus OvCNP_a [42]. Conversely, platypus peptide isomerase does not
6 convert the tripeptide corresponding to the N-terminus of bombinin H of the frog secretion.
7 Consequently, it can be thought that the enzymes have different active site configurations. This
8 would be consistent with the information obtained on the different fold structures produced from
9 preliminary chromatographic isolation procedures [90]. To date, it seems unconceivable to
10 postulate that the isomerases described above belong to the same family and, as no *consensus*
11 for sequences of the three enzymes has been found except for a pair of histidines, it would be
12 amazing if they process peptides by the same reaction mechanism.
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22 ***How does isomerisation occur? Mechanistic pathway***

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24 Although this clearly depends on the animal model and the peptide involved, one question
25 that arises is whether this particular post-translational reaction is a racemisation yielding a
26 mixture of L- and D-compounds or a quantitative (even complete) inversion of the chirality of the
27 α -carbon with the formation of the D-isomer as either exclusively or as a major proportion of the
28 end product. This is difficult to determine exactly as there is a bias if the initial reaction takes
29 place at the same time as the degradation by exo- or endo-peptidases of the L-isomers, which
30 would then decrease in relative abundance. Moreover, the possibility that the presence of the L-
31 isomer may simply be the result of a slow inversion rate cannot be excluded, especially when it is
32 found in small quantity. For the frog opioid dermorphin, the all L-containing peptide could not be
33 detected [91]. But in the majority of the cases, peptides containing the L-AA are also present,
34 albeit in smaller quantities. For example, the L/D proportions are 1:4 for frog bombinins [92], 1:2
35 for spider agatoxins [57] and 1:3 for lobster VIH [62]. Nevertheless, in this latter species, the
36 CHH, another peptide also produced in the sinus gland, displayed an inversion in the ratio L/D
37 with the majority of the peptide being the L-form (3:1) [62]. These ratios were determined by
38 quantifying the isomers purified from organs by chromatography (RP-HPLC). As previously
39 described, cellular studies in crustaceans have been extensively developed, because the D-
40 amino acid containing neurohormones are produced in discrete, well-identified neuroendocrine
41 cells located in an easily accessible organ, i.e. a tractable model system. This has enabled more
42 detailed studies aimed at determining the reasons behind the ratio of CHH/D-Phe³-CHH observed
43 on the chromatogram (i.e. 3:1). Indeed, observations of whole mounts of the X-organ using
44 confocal microscopy showed that in crayfish a constant number of 8 cells were found to contain a
45 mixture of L- and D-Phe³-CHH with a greater abundance of the D-isomer (called D-cells),
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1 especially in axonal terminals (about 100%), and approximately 25 cells exclusively synthesised
2 the L-isomer (called L-cells with 100% of L-isomer [76]). So, proportions of the different isomers
3 estimated using the analytical approach (3:1) and the cellular observations (3 L-cells for 1 D-cell)
4 correlate exactly. Interestingly, at a sub-cellular level, quantification by image analysis using
5 electronic microscopy after double immunogold labelling of L-cells revealed that D-labelling was
6 significantly increased from the cell body to the axon terminal. This was interpreted as a
7 progression in the Phe³ isomerisation process during granule migration within the neurosecretory
8 cell. Accordingly, the proportion in D-cells of granules containing only CHH was lower in the axon
9 terminals (5%) compared with the cell bodies (40%) [79]. These studies performed at different
10 cellular levels (organ, cell and secretory granule) with various techniques clearly indicate that the
11 conclusions drawn from any ratio measurements should be interpreted with great care depending
12 on the level at which the observation is made.
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21 Different mechanisms for the production of D-AAAs in peptides have been proposed since
22 the discovery of the first DAACP in 1981 by Montecucchi *et al.* [9]. Today, it is generally accepted
23 that this post-translational reaction proceeds by enzymatic action. Free D-AAAs can be formed
24 from the corresponding L-enantiomer by the action of amino acid racemases [93,94]. Most of
25 these enzymes require pyridoxalphosphate as a cofactor and the reaction proceeds via a Schiff
26 base as an intermediate. However, another class of racemases operates without any cofactor (in
27 particular, the addition of the pyridoxalphosphate co-factor does not influence the conversion
28 reaction [95]). Examples are the proline, aspartate, and glutamate racemases from different
29 bacteria [23,96,97]. Isotope-exchange studies indicated that all the pyridoxalphosphate-
30 independent racemases employed a “two-base” mechanism to catalyse epimerisation, and
31 studies using chemical modifications and site directed mutagenesis revealed that these enzymes
32 utilised their two-cysteinylyl residues as the proton abstractor and the proton donor [98]. The
33 reaction catalysed by these enzymes proceeds via deprotonation–protonation at the α -carbon of
34 the substrate (Figure 8) [99]. More precisely, one enzymatic base (i.e. a thiolate group of one
35 cysteinylyl residue) deprotonates the substrate, and the conjugate acid of a second enzymatic
36 base (i.e. the thiol group of the other cysteinylyl residue) protonates the resulting intermediate from
37 the opposite face [100]. However, the spider-venom isomerase apparently did not contain free -
38 SH groups, only disulfide pairings [80], and it was only partly inhibited by N-ethylmaleimide, an
39 alkylating reagent, that covalently modifies nucleophilic thiol residues such as cysteine in proteins
40 [101]. Nevertheless, based on kinetic and isotope exchange experiments, it has been shown that
41 the chiral inversion also proceeds via an exchange of the proton, in this case on the α -carbon of
42 serine-46 of spider ω -agatoxin IVC [101, 102]. But the residues (not cysteinylyl) in the active site of
43 the isomerase, which acted as proton acceptors and donors, respectively, have not yet been
44 identified [10]. As described above, it has been found that the spider-venom isomerase exhibits
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1 significant homology to serine proteases such as trypsin or chymotrypsin. These two hydrolytic
2 enzymes operate via a “catalytic triad” (His, Asp and Ser) whereby, through hydrogen bonding to
3 a histidine and an aspartic acid, the hydroxyl group of the serine present in the active site is
4 deprotonated and thus becomes a strong nucleophile binding to the carbonyl group of the scissile
5 peptide bond. In the case of the spider isomerase, through a similar “triad”, the enzyme serine
6 oxy-anion may act as a strong base abstracting the proton from the substrate (i.e. the α -carbon of
7 an AA, e.g. Ser₄₆) most likely generating a planar anionic intermediate [82].
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12 As described above, a second isomerase has been purified from skin secretions of
13 *Bombina* species [42]. The frog skin enzyme also acts on its substrate via a deprotonation /
14 reprotonation reaction (Figure 8). This was demonstrated by carrying out the reaction in the
15 presence of tritiated water. Radioactivity was incorporated into the second AA of the N-terminal
16 heptapeptide sequence of bombinin H [42]. The peptide side chains, which are present in the
17 active site of the enzyme, are currently not known. As described previously, the comparison of
18 the putative isomerases from different species has shown that a central region, which includes
19 two cysteinyl or two histidyl residues, is highly conserved. The catalytic activity is, however,
20 insensitive to treatment with iodoacetamide; therefore, these cysteinyl residues can be excluded
21 from acting as catalytic bases. In contrast, incubation with diethylpyrocarbonate resulted in loss
22 of activity, which indicates that at least one histidyl residue participates in the catalytic reaction
23 [42].
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32 Similarly, platypus-venom isomerase activity was not inhibited by phenylmethylsulfonyl
33 fluoride or iodoacetamide [68]. These results appear to rule out the presence of a serine-
34 protease-like reactive triad or the involvement of a sulhydryl group (-SH) from a cysteinyl residue
35 in the active site. However, the peptide isomerase was inhibited by diethyl pyrocarbonate. This
36 would suggest the presence of at least one histidyl residue in or near the active site of the
37 enzyme, as has been reported for the peptide isomerase from the frog [42]. Moreover, the
38 candidate gene of platypus isomerase contains two histidyl residues in its central portion
39 (accession number XM_003428816) [68]. On the basis of these studies and similar earlier reports
40 [101], a two-base mechanism has also been proposed for the platypus isomerase with the
41 hypothesis that two histidyl residues are positioned on the opposite sides of the substrate during
42 isomerisation, and they act as a proton abstractor and a proton donor, respectively (Figure 8).
43 During peptide isomerisation, one of the histidyl bases of enzyme deprotonates the α -carbon
44 atom of the second AA (Met) of DLP and forms a planar intermediate [68]. At the same time, the
45 other histidyl base in its conjugated acid form reprotonates the intermediate at the opposite side
46 of the α -carbon atom to form the other isomer. Furthermore, in this study, it was shown that
47 isomerisation of the L- to D-residues in DLP was faster than for the D- to L-reaction. This
48 suggests, that in the two-base mechanism, there is an asymmetry at the active site, in which the
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1 L-isomer is more favourably positioned for proton abstraction than the D-isomer. In relation to
2 understanding the likely mechanism of the enzyme reactions, two fundamental points appear: (i)
3 identification of a target AA by platypus isomerase in a peptide for isomerisation; (ii)
4 deprotonation and reprotonation of a weakly acidic proton from the α -carbon atom of an AA
5 residue. To summarise, only peptides with a particular sequence will act as substrates and the
6 active site is very specific and restricted to accommodate only certain peptide sequences. The
7 high energy process that involves the removal of a weakly acidic proton by weakly basic catalysts
8 may be explained by the involvement of a high energy hydrogen bonds that results in the
9 formation of tight binding between the enzyme and substrate to produce a relatively stable
10 anionic intermediate [103,104] (central peptide in Figure 8). This outcome can be achieved
11 through the formation of a strong hydrogen bond with the carbonyl carbon of the AA thereby
12 lowering the activation energy barrier for the formation of an enolic intermediate of the bound
13 substrate; this will facilitate the removal of the proton. Alternatively, isomerisation may be a
14 concerted process where incipient carbanion formation, deprotonation, and reprotonation take
15 place simultaneously in the transition state, similar to an S_n2 -type mechanism that results in
16 inversion of the configuration [68].

17 To conclude, although it is now clear that an enzymatic activity is involved, the precise
18 mechanism by which the configuration of a specific internal residue is manipulated is still not
19 totally clear. A more accurate understanding of the mechanism should be possible when three-
20 dimensional structural data of the enzyme-substrate complex becomes available. To date, the
21 only putative model is the 3D structure of the spider isomerase derived from its primary sequence
22 by homology modelling using the X-ray structure of trypsin (Brookhaven Protein Data Bank code
23 2PTN, [102]).

40 ***What are the structures recognised by such an enzyme? Substrate specificity***

41 The three isomerases described so far in the literature interconvert in both directions (L->D
42 and D->L) with variations in L/D ratios and in reaction speeds (reaction rates); for further
43 discussion on the dynamic aspect of isomerisation, see [68,101,102,105].

44 Since the discovery of the DAACPs in frog skin secretions, the dermorphin with a D-Ala² [9]
45 and the dermenkephalin with a D-Met², several questions concerning the specificity of the
46 isomerisation, and therefore of the isomerase, remain unanswered with respect to a possible
47 regulatory mechanism that ensures recognition of a particular residue within a peptide sequence.
48 In other words, 1) why does the DAA occur in one position rather than another, e.g. D-Met at
49 position 2 but not at pos. 6 in dermenkephalin and D-Phe in pos. 3 but not in pos. 17 in CHH? 2)
50 is the nature of the "isomerable" residue crucial?, and 3) do the neighbouring residues influence
51 the isomerised one? The first data towards answering these questions came from Jilek *et al.*
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[105] To study the substrate specificity of the frog isomerase, they used different AAs at positions 1, 2 or 3 of the natural substrate bombinin H containing an Ile at position 2 converted to D-allo-Ile². Surprisingly, this enzyme has a rather low selectivity for residues at position 2 where the change of chirality at the α -carbon takes place. Indeed, compared to bombinin H with an Ile², the peptide with Trp² displayed the same reaction rates and consequently, was also a good substrate. Even, five other AAs reacted about ten times faster in the isomerisation reaction, namely methionine, phenylalanine, leucine, norleucine and phenylglycine. Interestingly, the role of the amino terminus for substrate recognition was in contrast crucial one since after replacing the α -amino with a hydroxyl group or an acetylated amino group the isomerisation reaction decreased by several orders of magnitude or even did not occur. Likewise, a hydrophobic amino acid at position 1 (Ile, Phe or Trp) and a small one at position 3 (Gly) of the substrate are essential. To conclude, the frog isomerase acts exclusively on the second amino acid, while residues 1 and 3 largely define the substrate spectrum of the enzyme. At least, the natural L-forms of dermorphins and deltorphins, were not found because they are unstable and apparently degraded by proteases present in the preparation [42,106-108]. However, a chimeric peptide with the N-terminal tripeptide sequence of deltorphin (Tyr-Met-Phe) linked to the C-terminal end of bombinin (Pro-Val-Leu) plus a cysteinyl residue was sufficiently stable during the assay and was observed to be weakly converted at the Met² by the *Bombina* isomerase [105]. Consequently, frog isomerase seems to display a rather low selectivity for the residue at position 2 but an activity influenced by neighbouring residues of the isomerised one.

Furthermore, in view of the rather broad specificity of the frog isomerase, a databank search was made for potential substrates of such an enzyme. Surprisingly, numerous peptides of amphibians and mammals were found which fulfil the requirements determined in this study. For example, alyteserin-2 from the midwife toad *Alytes obstetricans* [109], several ranalexins, temporins 1DYa and PTa and brevenins 1PTa and 1PTb from diverse *Rana* species [110,111], hylin a1 from the spotted treefrog (*Hypsiboas albopunctatus*) [112] as well as kassorin S from the African hyperoliid frog (*Kassina senegalensis*) [113] were predicted as good substrate candidates for the *Bombina* isomerase.

Moreover, the substrate spectrum of a recently reported isomerase activity present in the venom of male Platypus is apparently similar to that of the *Bombina* enzyme [68,83]. In delineating the catalytic mechanism of the platypus isomerase and the broader roles in the animal, N-terminal segments of DLP4 and DLP2 (Ile-D-Met-Phe-Phe-....) and natriuretic peptide OvCNP a and b (Leu-D-Leu-His-Asp-...) from the venom were used as substrates. The DLP analogues IMFsrS and IDMFsrS (srS is a solubilising chain) were effective substrates for the isomerase that appeared to recognise the N-terminal tripeptide sequence Ile-X-Phe-. 26 mutants of these hexapeptides were synthesized with the second residue (Met) exchanged with another

1 amino acid, viz. Ala, α -aminobutyric acid, Ile, Leu, Lys, norleucine, Phe, Tyr, and Val. The mutant
2 peptides incorporating norleucine and Phe were effective substrates and exhibited L- or D-
3 isomerisation, but mutant peptides that contained residues with shorter, β -branched or long side
4 chains with polar terminal groups, viz. Ala, α -aminobutyric acid, Ile, Val, Leu, Lys, and Tyr,
5 respectively, were not found to be substrates. It was also demonstrated that at least three N-
6 terminal amino acyl residues are absolutely essential for L- to D-isomerisation and the third
7 residue must be a Phe. Contrary to DLP, none of the hexapeptides based on Leu-Leu-His, the
8 first three residues of OvCNP, were substrates. To conclude, platypus isomerase appeared to
9 recognise the N-terminal tripeptide sequence Ile-X-Phe- with specific characteristics of the
10 residue X.
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12 Moreover, it was established that the spider enzyme catalysing isomerisation, i.e.
13 *Agelenopsis* isomerase had a minimum substrate recognition site, which is a tetrapeptide (-Leu-
14 Ser-Phe-Ala) corresponding to the C-terminus of the spider ω -agatoxin IVC with a L-Ser⁴⁶ (48
15 AAs). The enzyme targeted various peptides with serine methyl ester and Cys instead of Ser⁴⁶,
16 and was able to weakly isomerise Ala but not Thr (e.g. X in Ac-Met-Glu-Gly-Leu-X-Phe-Ala-OH)
17 [101]. No other natural peptide is known in this spider and no other heterologous peptide has
18 been tested for this isomerase.
19

20 At least, the examples of D-Phe³-CHH and D-Trp⁴-VIH in lobster raise the question of the
21 substrate specificity of the putative isomerase(s). Indeed, the same cells and even the same
22 secretory granules, contained both DAACPs but axon terminals never exhibited simultaneous
23 labelling for CHH / D-Trp⁴-VIH or VIH / D-Phe³-CHH [62]. So, a unique enzyme might act on both
24 hormones. Nevertheless, these peptides display a D-residue of different natures (Phe or Trp) and
25 at different positions (3 or 4), hence the isomerisation by two distinct enzymes, one for each
26 peptide type, should also be considered. It is and will remain an enigma until the characterisation
27 and identification of the putative peptide isomerase(s) contained in these specialised cells of the
28 X organ–sinus gland complex of lobster. To date, the occurrence of two distinct enzymes with
29 different substrate specificities and a variable expression pattern appears as the most likely
30 working hypothesis.
31

32 To conclude, even if the neighbouring AAs must have particular characteristics, especially
33 regarding polarity and charge, these enzymes display a relatively broad specificity for the type of
34 isomerised residue. It even seems that, at least for the frog isomerase, that heterologous
35 peptides can be substrates. The expression of isomerases with similar characteristics in other
36 species can therefore be expected to catalyse the formation of DAACPs. Nevertheless, in each
37 species, a single isomerase seems to be insufficient, as the currently isolated and characterised
38 enzymes do not interconvert potentially isomerisable peptides (e.g. dermorphin in frog and
39 natriuretic peptide in platypus). Moreover, even if the position of the isomerised residue has not
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1 been extensively studied, it is notable that only one D-AA is found in each peptide chain, and it is
2 always close to one end, most frequently near the N-terminus (position 2,3 or 4), but also near
3 the C-terminus (n-2) but never at the extremities (1 or n). This is why, in our opinion, the
4 substrate spectrum of the enzyme is largely defined by residues neighbouring the isomerised
5 one. This hypothesis is supported by the fact that dermenkephalin and DLP DLP4 contain a D-
6 Met₂ and not a D-Met₆. In the same way, the octapeptide contryphan from the venom of a fish-
7 hunting snail contains tryptophan residues at positions 4 and 7, of which the first is in the D-
8 configuration, while the second is the 6-bromo-derivative of this amino acid [114,115].

15 Conclusion and future issues

18 In the present paper, we gathered the current knowledge concerning the biogenesis of
19 DAACPs (in a large sense) in the living world, by describing the occurrence of the peptides, the
20 spatio-temporal and mechanistic aspects of isomerisation at the cell level and, the specific
21 enzyme(s) involved in this maturation step, the peptidyl aminoacyl L-D isomerase.

22 Since the discovery in the 30s of a D-AA in bacterial proteins, numerous examples of D-
23 amino acyl residues of different natures and at different positions in the peptide chain were
24 characterised in invertebrates (molluscs, arachnids, crustaceans) and vertebrates (platypus,
25 frogs and humans). More surprisingly, a weak isomerase activity has also been reported for the
26 first time in a plant (papaya), suggesting the presence of a DAACP. Consequently, the
27 phylogenetic distance between these species suggests that every living organism may own some
28 peptide isomerase activity. Moreover, at the tissue level, specialised cells were observed for
29 isomerisation of peptides in several crustaceans and in *Aplysia*, a gasteropod mollusc,
30 suggesting a cellular specificity of isomerase. In this context of specificity, a recent concept of
31 mechanism of metabolite separation named “chiral compartmentation”, that emerged from the
32 stereo-specificity of enzymes for their substrate(s) must also be now taken into consideration
33 [69].

34 Biosynthesis of DAACPs has been shown to occur by several ways, by the incorporation of
35 D-AAAs during translation or isomerisation of the L-AA generated by a normal codon after the
36 building of the peptide or the protein via a non-enzymatic pathway (for e.g. α -crystallin) or by
37 specific isomerase(s) which apparently acts after the cleavage of the precursor. On the basis of
38 current studies on isomerase from frog, spider and platypus, a two-base mechanism has been
39 proposed for L- to D-conversion with the hypothesis that two residues (e.g. histidyl) in the active
40 site (e.g. a catalytic triad) are positioned on the opposite sides of the substrate during
41 isomerisation, and that they act as a proton abstractor and a proton donor, respectively. At least,
42 even if the neighbouring AAAs of the isomerised residue must have particular characteristics,
43 especially for polarity and charge, these enzymes display a relatively broad specificity for the type

1 of isomerised residue, contrary to what was originally thought.

2 Even if the number of DAACPS discovered is growing each year, too little is known about the
3 characteristics of peptidyl aminoacyl L-D isomerisation, one of the most unusual and fascinating
4 posttranslational modifications. This review opens up fascinating perspectives in understanding
5 of isomerisation processes:
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9 1- One of the major challenges remaining for the future is the identification of the receptors of
10 DAACPs and their L-counterpart, when it is present in the tissue, aiming to provide insights on the
11 functional significance of isomerisation.
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14 2- More information about the mechanism of this modification is needed. A more accurate
15 understanding should be possible when three-dimensional structural data on the isomerase(s)-
16 substrate complex will be available.
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19 3- Increasing awareness of the presence of a D-AA in proteins in the otherwise dominant 'L-chiral
20 biosphere' is largely due to recent developments in rapidly-applicable, analytical–chemical
21 methods. Nevertheless, in spite of efforts over the last decade, especially with the development
22 of abundant direct and indirect chromatographic methods for the enantioseparation and
23 stereochemical assignment of D- and L-AAs e.g. in hydrolysates of isolated peptides,
24 identification of DAACPs remains a challenging task. Indeed, isomerisation does not induce any
25 change in the primary sequence or in physicochemical properties of the molecule such as
26 molecular mass or in pI. Moreover, as it has been shown for DLP2 and DLP4 from platypus
27 venom, the overall fold of the full-length protein is not substantially changed, despite of the
28 retention time difference observed in RP-HPLC (Figure 9) [66]. As a consequence, it may be
29 speculated that many DAACPs present in animal tissues remain unknown, though they might
30 well be of great physiological or pathological relevance. Therefore, the development of proper
31 analytical methods for detecting the presence of a D-residue in a peptide/protein is obviously
32 valuable, and especially for a high-throughput screening of complex mixture of peptides by
33 proteomic approach. To date, analysis with synthetic peptides by mass spectrometry gave
34 interesting results. This method seems to be promising to go deeper in the characterisation of
35 natural peptides. Finally, an interesting alternative approach for DAACP characterisation could be
36 identification of isomerase orthologous sequences in genomic and transcriptomic data sets
37 obtained from an increasing number of animals, bacteria and even plants.
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51 **Acknowledgments**

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56 Council, Cambridge, United Kingdom) for critical reading of the manuscript.
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Legends of figures

Table 1. D-amino acid containing proteins observed in various tissues of the living body. Reprinted from Fuji *et al.* [34] with permission.

Table 2. Peptides with a D-amino acid residue isolated from eukaryotic tissues. The list is not exhaustive. The D-residue is in bold and italics. The bombinin with the asterisk is an antimicrobial peptide. Both isomers of CHH display hyperglycaemic activity whereas D-Phe³-CHH also presents osmoregulatory and molt-inhibitory effects. VIH is an inhibitor of vitellogenin synthesis whereas the function of D-Trp⁴-VIH is unknown. Reprinted and adapted from Soyez *et al.* [116] with permission.

Table 3. Consequences of a single amino-acid residue isomerisation on the biological activity of the peptides. Numbers and letters in brackets refers to the text. 0 for no biological activity compared to that of the counterpart; - for a slight biological activity compared to the counterpart; + for biological activity which is as high as that of the counterpart.

Figure 1. Biosynthesis of the terminal peptidoglycan stem peptide of Gram-negative bacteria. The chirality of the α -carbon of two L-alanine (L-Ala) is inverted from the L- to the D-configuration by alanine racemase (Alr). The two D-alanine (D-Ala) are ligated together by D-alanine-D-alanine ligase (Ddl) and the dipeptide D-alanyl-D-alanine (DA-DA) is subsequently added to the stem tripeptide (L-Ala-D-Glu-m-DAP) by a UDP-N-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase (MurF) resulting in a pentapeptide. m-DAP, meso-diaminopimelic acid; MurNAc, N-acetylmuramic acid; UDP, uridine diphosphate. Reprinted and adapted from Liechti *et al.* [117] with permission.

Figure 2. Monomeric structure of the bacteria *Streptomyces lavendulae* alanine racemase dimer. The two subunits that form the monomer structure are shown in different colors (α -helices colored red and β -sheets colored blue). The pyridoxal 5'-phosphate shown in green (PLP) is covalently bound to Lys₃₈ to form a PLP-binding residue (N'-pyridoxyl-lysine-5'-monophosphate) that resides in the active site. The active site is composed of residues from the α/β barrel domain of one monomer and residues from the β -strand domain of the other monomer. Reprinted from Noda *et al.* [118] with permission.

Figure 3. Biosynthesis of penicillin G by the fungus *Penicillium chrysogenum*. The first step in the biosynthesis of penicillin G is the condensation of two amino acids, L-amino-adipic acid and L-cysteine. Then, a, L-valine is added to the two residues and epimerised to the D-form to generate the L-L-D-tripeptide, i.e. δ -(L- α -Amino adipyl)-L-Cys-D-Val (ACV-tripeptide) by a catalytic multi-enzyme, δ -(L- α -amino adipyl)-L-Cys-D-Val synthetase (AVCS) that displays an epimerase domain in its C-terminal region. The following steps are the oxidative conversion of linear ACV-tripeptide

1 into the bicyclic intermediate isopenicillin N by isopenicillin N synthase (INPS) and a
2 transamidation by isopenicillin acyltransferase (IAT) to produce penicillin G.
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5 Figure 4. A. Structure of Ltn- α and Ltn- β of the lantibiotic lactacin 3147, with the 23
6 posttranslationally modified residues shaded gray. The locations of the three D-alanine residues
7 are indicated by black circles. B. The proposed pathway for the conversion of L-serine to D-
8 alanine via dehydroalanine (Dha) is also depicted. LtnM is a lantibiotic synthetase required to
9 dehydrate serines. LtnJ is a lantibiotic dehydrogenase to generate D-alanines. Reprinted and
10 adapted from Cotter *et al.* [31] with permission.
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17 Figure 5: Reaction pathways for spontaneous inversion and isomerisation of aspartyl residues in
18 peptides/proteins. The formation of D- and β -aspartyl residues (named D- β -Asp) from aspartyl
19 residues in this protein begins (1) with the attack of the carbonyl group in the side chain of the L-
20 aspartyl residue by the nitrogen of the amino acid residue following the Asp residue. L-
21 succinimide is formed by intramolecular cyclisation and (2) is converted to D-succinimide through
22 an intermediate [enol form] whose prochiral α -carbon is in the plane of the ring. (3) Protonation of
23 the intermediate may proceed from the upper or lower side of the plane in an ordinary peptide or
24 protein. The area surrounding Asp-151 and Asp-58 residues in α A-crystallin is thought to form a
25 chiral environment that promotes formation of D-succinimide over L-succinimide. (4) D- and L-
26 succinimide are hydrolysed at either side of their two carbonyl groups, yielding both β - and α -Asp
27 residues. As shown in Figure 5, D-Asp formation is also accompanied by isomerisation from the
28 natural α -Asp to the biologically rare β -Asp (isoaspartate). Thus, four isomers, L-Asp, L- β -Asp, D-
29 Asp and D- β -Asp, are simultaneously formed in the protein. Reprinted and adapted from
30 Takahashi *et al.* [40] with permission.
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43 Figure 6. Confocal micrographs of double immunolabelled whole mounts of crustacean X organ–
44 sinus gland. Images were collected as a focal series and processed to create 2D projections
45 (single composite images). Central drawing: schematic representation of the eyestalk nervous
46 structures and the neuroendocrine complex (X organ–sinus gland). OX, X organ; SG, sinus
47 gland; R, retina; LG, lamina ganglionaris; ME, medulla externa; MI, medulla interna; MT, medulla
48 terminalis. (A) Distribution of CHH cells in X organ of the crayfish *Orconectes limosus*, showing
49 green cell bodies (L-CHH cells) labelled only with gp-anti-pQL, synthesising CHH, specific of
50 CHH and orange somata (D-CHH cells) corresponding to labelling with gp-anti-pQL and rb-anti-
51 pQD antisera, producing both isomers of CHH, CHH and D-Phe³-CHH, specific of D-Phe³-CHH.
52 (B) Enlarged view of L-CHH cells (green) and D-CHH cells showing the different granule cluster
53 colorations (green, orange and red) and the axon appearing in red (arrow). (C) Distribution of
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1 CHH cells in X organ of the lobster *Homarus americanus* showing green cell bodies (L-CHH cells)
 2 labelled only with gp-anti-pQL and orange somata (D-CHH cells) corresponding to labelling with
 3 gp-anti-pQL and rb-anti-pQD antisera. (D) General view of X organ labelled with r-anti-L, specific
 4 of VIH (red) and gp-anti-DW4, specific of D-Trp⁴-VIH (green) to visualise small L-VIH cells (red
 5 producing VIH) and larger D-VIH cells (green or yellow) synthesising both isomers of VIH, VIH
 6 and D-Trp⁴-VIH. (E) Immunolocalisation of D-Trp⁴-VIH and D-Phe³-CHH in the X organ where
 7 three cell types were observed: D-CHH cells producing only D-Phe³-CHH (red, thin arrow), D-VIH
 8 cells containing only D-Trp⁴-VIH (green, short arrow) and D-cells producing both D-isomers
 9 (orange, long arrows). Reprinted and adapted from Ollivaux *et al.* [62] and Soyez *et al.* [76] with
 10 permission.
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 19 Figure 7. General diagram of precursor processing of VIH and CHH isomers in relation to the
 20 different cell types in X organ–sinus gland complex. CPRP, CHH precursor related peptide; CHH
 21 VIH, L-CHH and L-VIH cells D-CHH and D-VIH cells.

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 24 ^aAmidation can be pre-, co- or post-cleavage of CPRP.

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 26 ^bCyclisation of CHH N-terminus is optional (N-terminus unblocked CHH can be released) and,
 27 similar to isomerisation, it occurs after CPRP cleavage.

28
 29 ^cBy contrast to CHH, VIH is not N-terminal cyclised. L-CHH and L-VIH cells secrete exclusively
 30 CHH and VIH, respectively, whereas D-CHH and D-VIH cells release mainly the D-isomer of the
 31 respective hormone, in addition to a variable amount of L-isomer. D-cells secrete mainly the D-
 32 form of both CHH and VIH. Besides isomerisation, the same post-translational modifications
 33 occur in every type of CHH or VIH cell. L-CHH cells, cells synthesising CHH; D-CHH cells, cells
 34 producing both isomers of CHH, CHH and D-Phe³-CHH; L-VIH cells, cells synthesising VIH; D-
 35 VIH cells, cells producing both isomers of VIH, VIH and D-Trp⁴-VIH; D-cells, cells producing both
 36 D-isomers (D-Phe³-CHH and D-Trp⁴-VIH). Reprinted from Ollivaux *et al.* [62] with permission.
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 45 Figure 8. A two-base mechanism is proposed for the peptide isomerase. Abstraction of a proton
 46 from one face of an activated α -carbon atom by one enzymatic base (B:) is concomitant with
 47 delivery from the opposite face by the conjugate acid of a second enzymatic base (BH). aa:
 48 amino acid. Reprinted and adapted from Bansal *et al.* [68] with permission.
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 53 Figure 9. Structure of Defensin Like Peptide isomers (DLP2 and DLP 4). (A) Ribbon diagram of
 54 DLP2 structure showing the secondary-structural elements and disulfide connectivities. (B)
 55 Ribbon diagram of DLP4 showing the secondary-structural elements and disulfide connectivities.
 56 (C) Superposition of DLP2 (grey) and DLP4 (red) structures over the backbone of residues 7–42.
 57 Only the polypeptide backbone chains are shown. Reprinted and adapted from Torres *et al.* [66]
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Table 1

Tissue	Protein	Amino acid	Disease
Teeth	Phosphophoryn	D-Asp	?
Bone	Osteocalcin	D -Asp	?
Aorta	Elastin	D -Asp	Arteriosclerosis
Brain	β -Amyloid	D-Asp, D-Ser	Alzheimer
Brain	α -synuclein	D-Asp	Parkinson
Lens	α A, AB-crystallin	D-Asp	Cataract
Retina	α	D-Asp	AMD ^a
Conjunctivae	?	D-Asp	Pingueculae
Cornea	?	D-Asp	CDK ^b
Skin	Elastin	D-Asp	Elastosis
	Keratin	D-Asp	Sun damage
Lung	Elastin	D-Asp	?
Intestine	?	D-Asp	?

^a AMD: age-related macular degeneration

^b CDK: climatic droplet keratopathy

Table 2

Peptide [reference]	Sequence	Organism
Frog skin opioids and *antibacterial peptides		
Dermorphin [9]	Tyr- Ala -Phe-Gly-Tyr-Pro-Ser-NH ₂	<i>Phyllomedusa sauvagei</i>
Met-Deltorphin [119] or Dermenkephalin [51]	Tyr- Met -Phe-His-Leu-Met-Asp-NH ₂	<i>Phyllomedusa bicolor</i>
Deltorphins I/II [13]	Tyr- Ala -Phe-Asp/Glu-Val-Val-Gly-NH ₂	<i>Phyllomedusa bicolor</i>
Leu-Deltorphin [120]	Tyr- Leu -Phe-Ala-Asp-Val-Ser-....	<i>Phyllomedusa burmeisteri</i>
*Bombinin H [92]	Ile- alle -Gly-Pro-Val-Leu-Gly-...	<i>Bombina variegata</i>
Spider venom toxins		
ω-Agatoxin [45, 121]	...-Gly-Leu- Ser ₄₆ -Phe-Ala	<i>Agelenopsis aperta</i>
Mollusc venom toxins		
Conotoxin R11a [122]	...-Ser-Phe- Phe ₄₄ -Lys-Ile	<i>Conus radiatus</i>
Conotoxin R11c [123]	...-Ser-Phe- Leu ₄₂ -Thr	<i>Conus radiatus</i>
Bromocontryphan-R [115]	Gly-Cys-Hyp- Trp -Glu-Pro-Trp	<i>Conus radiatus</i>
Contryphan [47]	Gly-Cys-Hyp- Trp -Glu-Pro-Trp-Cys-NH ₂	<i>Conus radiatus</i>
Contryphan-P [124]	Gly-Cys-Pro- Trp -Asp-Pro-Trp-Cys-NH ₂	<i>Conus radiatus</i>
Contryphan-R [124]	Gly-Cys-Hyp- Trp -Glu-Pro-Trp-Cys-NH ₂	<i>Conus radiatus</i>
Contryphan-Sm ([124])	Gly-Cys-Hyp- Trp -Gln-Pro-Trp-Cys-NH ₂	<i>Conus stercusmuscarum</i>
Contryphan-Tx [48]	Gly-Cys-Hyp- Trp -Gln-Pro-Tyr-Cys-NH ₂	<i>Conus textile</i>
Leu-Contryphan-P [125]	Gly-Cys-Val- Leu -Leu-Pro-Trp-Cys	<i>Conus purpurascens</i>
Leu-Contryphan-Tx [48]	Cys-Val- Leu -Tyr-Pro-Trp-Cys-NH ₂	<i>Conus textile</i>
Des-(Gly ¹)Contryphan-R [47]	Cys-Hyp- Trp -Glu-Pro-Trp-Cys-NH ₂	<i>Conus radiatus</i>
Conomap-Vt [126]	Ala- Phe -Val-Lys-Gly-Ser-Ala-Gln-Arg-Val-Ala-His-Gly-Tyr	<i>Conus vitulinus</i>
Conomarphin [127]	Asp-Trp-Glu-Tyr-His-Ala-His-Pro-Lys-Hyp-Asn-Ser- Phe -Trp-Thr	<i>Conus marmoreus</i>
Conophan [128]	Ala-Hyp-Ala-Asn-Ser- Val -Trp-Ser	<i>Conus vitulinus</i>
Mammal venom toxins		
OvCNP [129]	Leu- Leu -His-Asp-His-Pro-Asn-....	<i>Ornithorhynchus anatinus</i>
DLP2 [66]	Ile- Met -Phe-Phe-Glu-Met-....	<i>Ornithorhynchus anatinus</i>
Mollusc neuroexcitatory peptides		
Achatin [56]	Gly- Phe -Ala-Asp	<i>Achatina fulica</i>
Fulicin [130]	Phe- Asn -Glu-Phe-Val-NH ₂	<i>Achatina fulica</i>
Fulyal [131]	Tyr- Ala -Glu-Phe-Leu-NH ₂	<i>Achatina fulica</i>
FFRFa [55]	Ala- Leu -Ala-Gly-Asp-His-Phe-Phe-Arg-Phe-NH ₂	<i>Mytilus edulis</i>
NdWFa [53]	Asp- Trp -Phe-NH ₂	<i>Aplysia kurodai</i>
Ocp-1/4 [132]	Gly- Phe -Gly-Asp/Gly- Ser -Trp-Asp	<i>Octopus minor</i>
Crustacean neurohormones		
CHH [58]	pGln-Val- Phe -Asp-Glu-Ala-Cys-....	Astacidea
VIH [62]	Ala-Ser-Ala- Trp -Phe-Thr-Asn-....	Homaridea

Table 3

Peptide	L-isomer	D-isomer
<i>(1) Only D-isomer has been described:</i>		
(a) Dermorphin/dermenkephalin	0	+
(b) Fulicin	-	+
(c) Mytilus-FFRFamide	+	+
<i>(2) Both isomers are present:</i>		
(a) Achatin	0	+
(b) ω -agatoxin	-	+
(c) CHH hyperglycaemic activity	-	+
molt inhibition	0	+
osmoregulation	0	+
(d) VIH	+	0
(e) OvCNP	+	+

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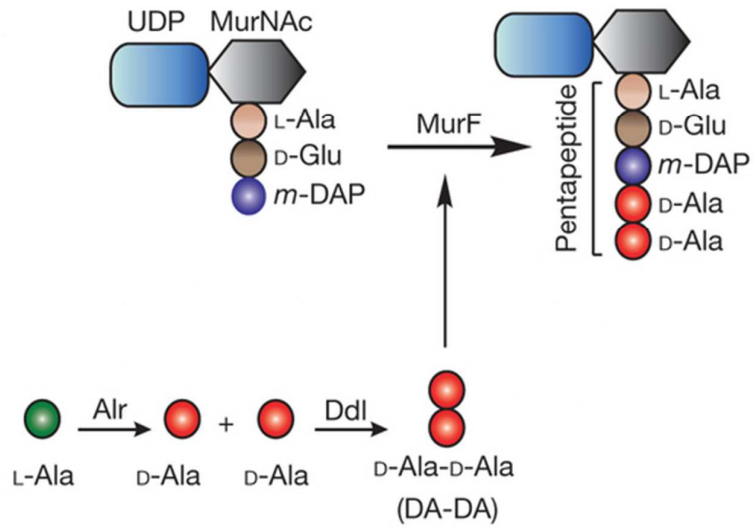
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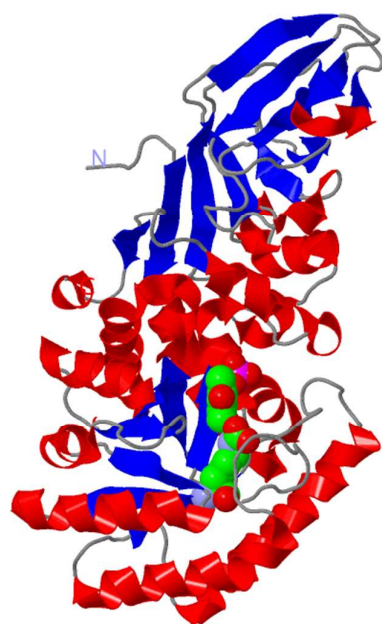
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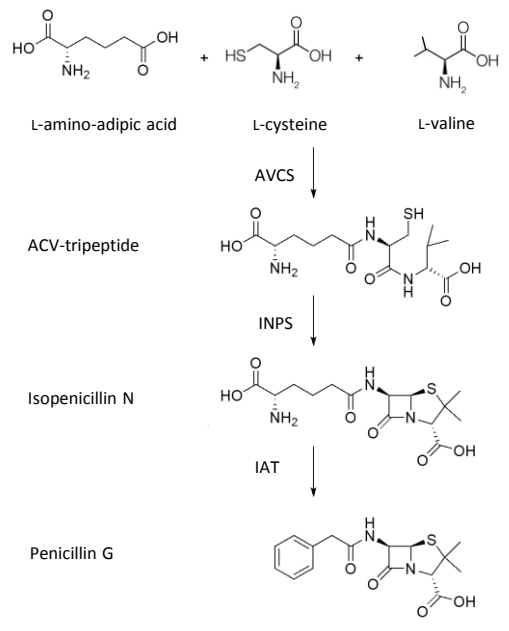
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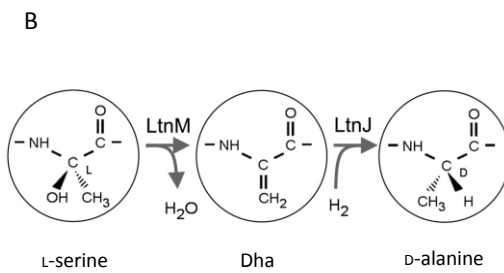
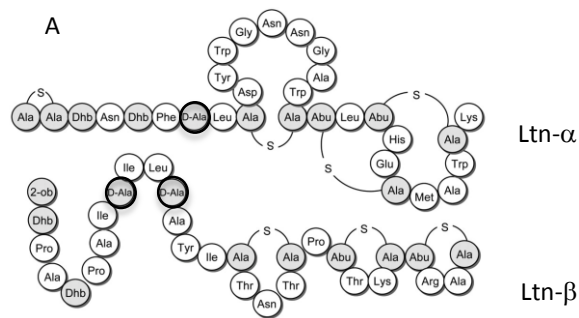
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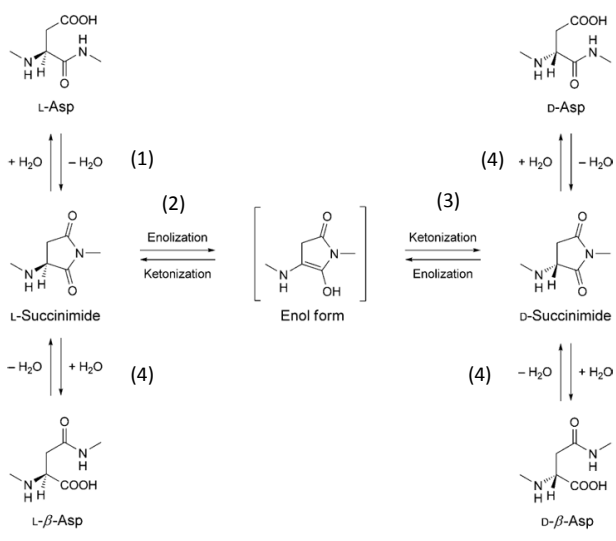
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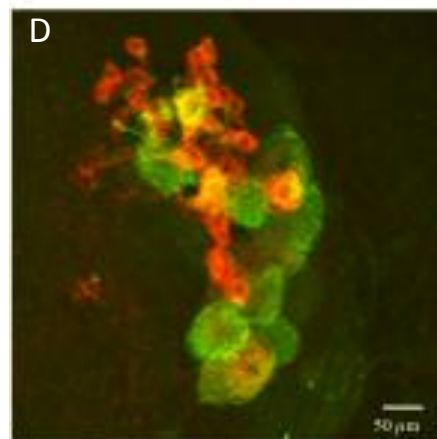
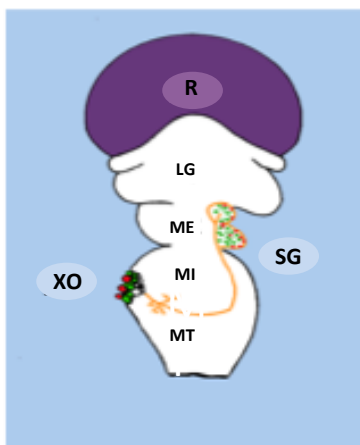
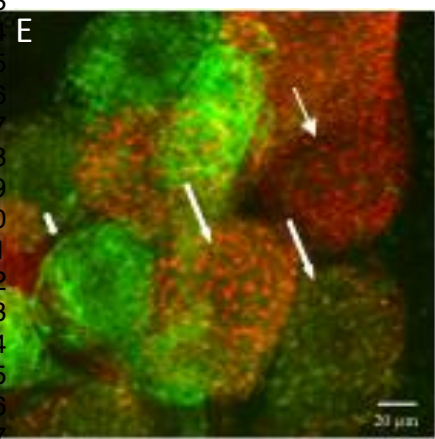
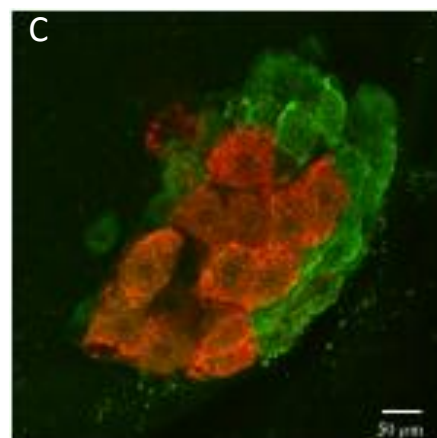
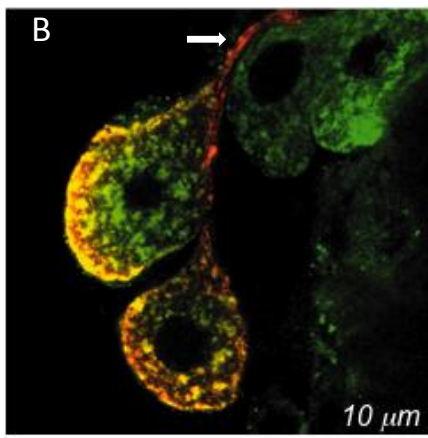
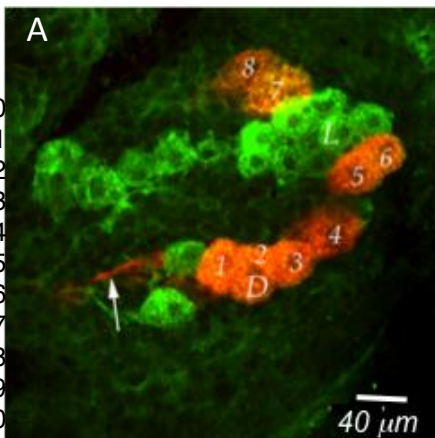
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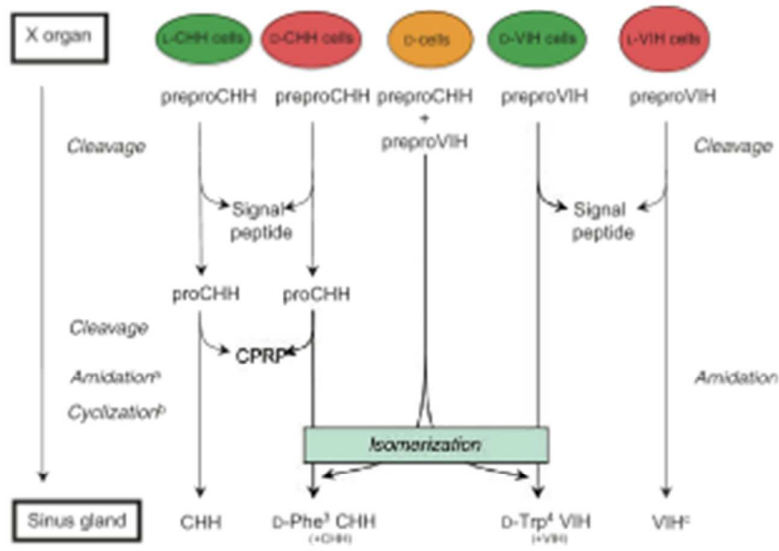


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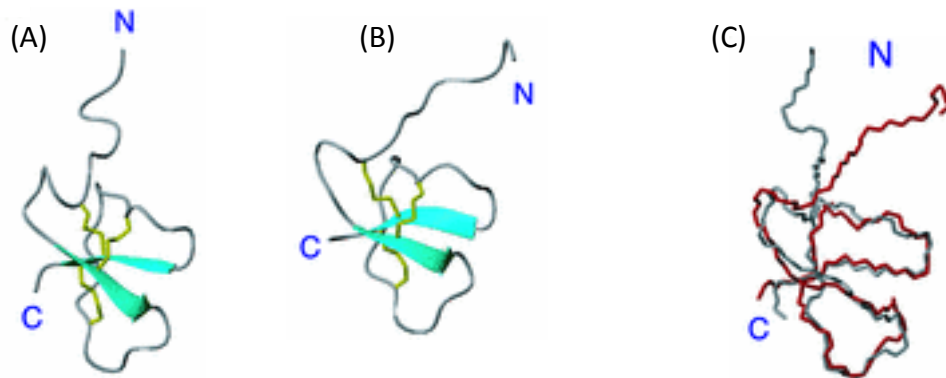
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