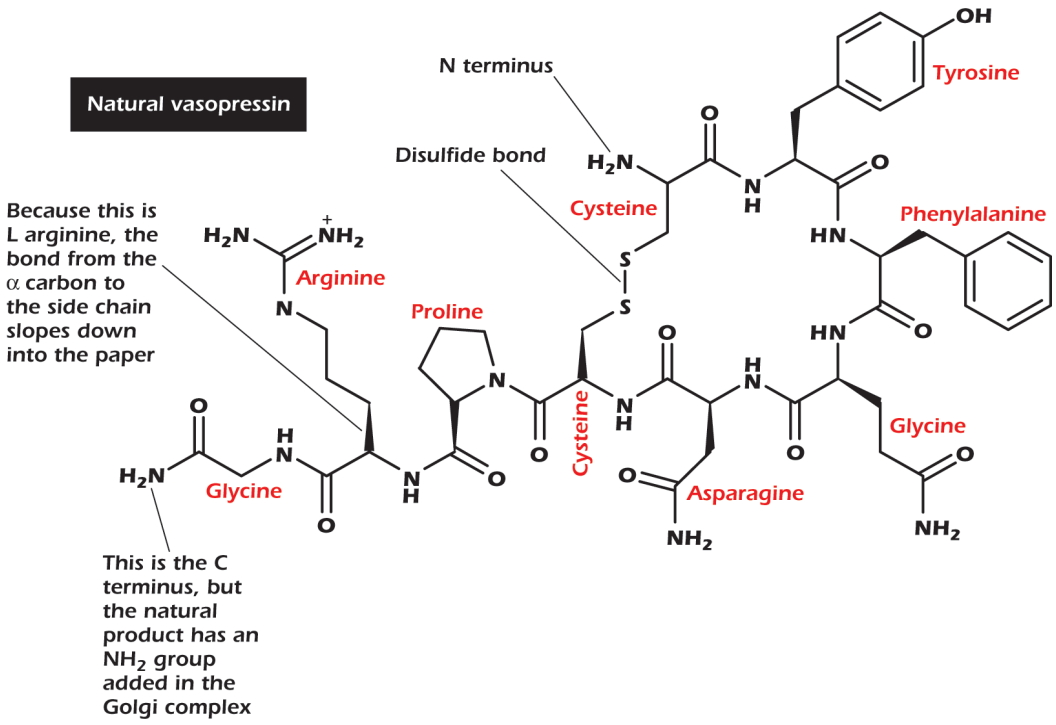
Chapter 8

Web Text Box 2

No ribosome required: making peptides in vitro

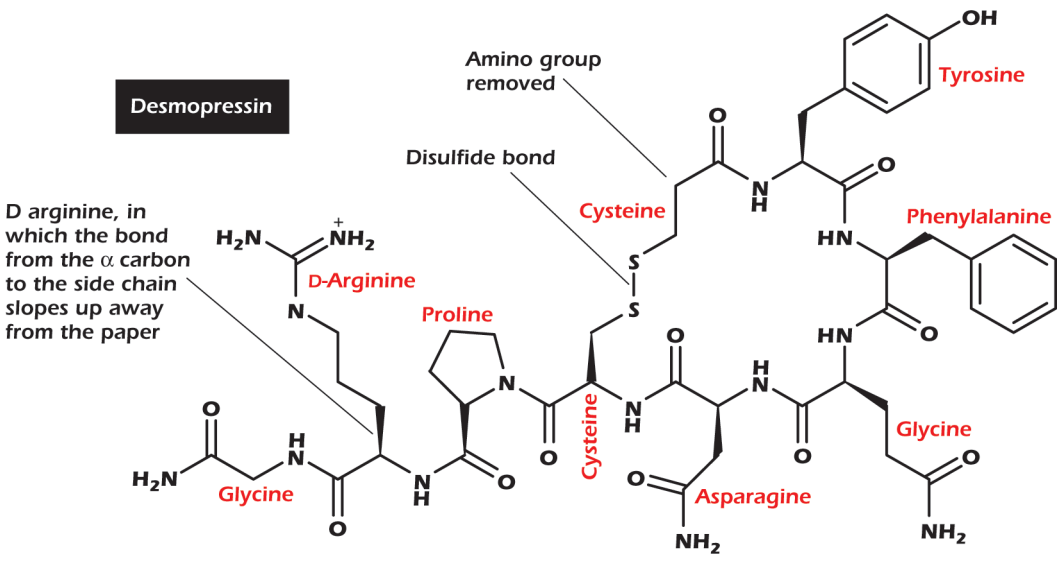
Although the most important polypeptides are proteins, chains of less than fifty amino acids, called peptides, also play diverse and important roles in organisms. Some are antibiotics (such as valinomycin and gramicidin, see In Depth 9.2 on book page 147) while others are hormones. For this reason synthetic peptides can be very useful drugs. Natural vasopressin, released by the pituitary gland in the head, is a 9 amino acid peptide that contains a disulphide bond. Vasopressin targets the kidneys to stimulate them to retain water. Because of its action it is sometimes called antidiuretic hormone or ADH).



Natural Vasopressin

Patients with the rare genetic condition diabetes insipidus fail to synthesize or secrete vasopressin and therefore lose a great deal of water in the urine. Can the patients be treated with exogenous vasopressin? In the body, peptide hormones such as vasopressin are made as much larger proteins that fold and have their disulphide bonds created in the correct positions. When the short peptide hormone is required it is cleaved off the parent protein by a specific protease. We could therefore consider transfecting cells with plasmids encoding the vasopressin parent protein plus the proteases so that large amounts of vasopressin could be manufactured *in vitro*. However, natural vasopressin has a half-life in the body of 10 to 30 minutes, so that use of natural vasopressin to treat diabetes insipidus would require almost constant administration of the hormone.

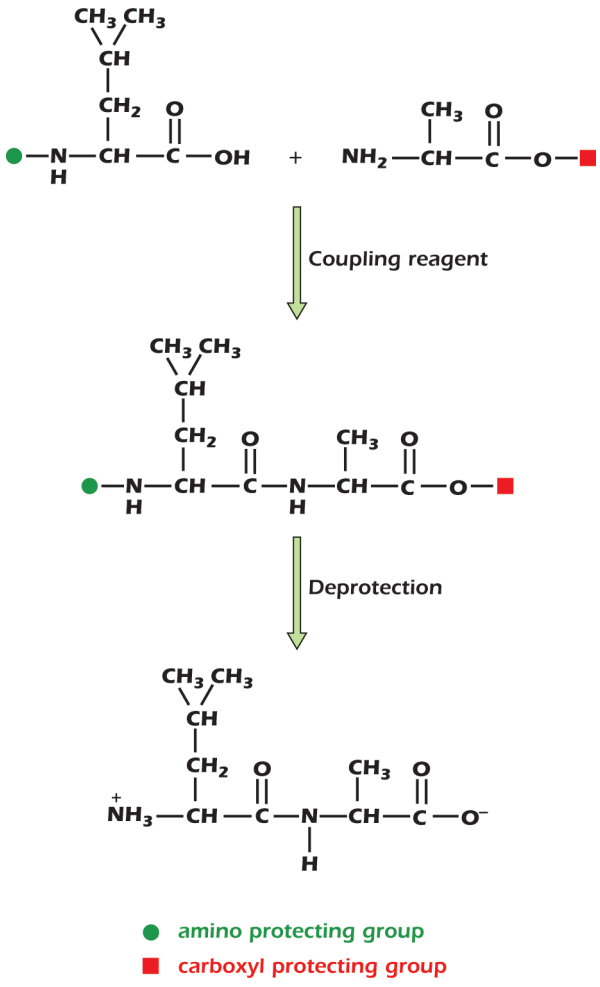
A modified vasopressin called desmopressin proves to be both more effective at the vasopressin receptors and has a longer lifetime in the blood, so that patients need only dose themselves twice a day (usually as a nasal spray). Desmopressin differs from vasopressin in that the N terminal amino group has been removed and D arginine replaces the normal L form.



Desmopressin

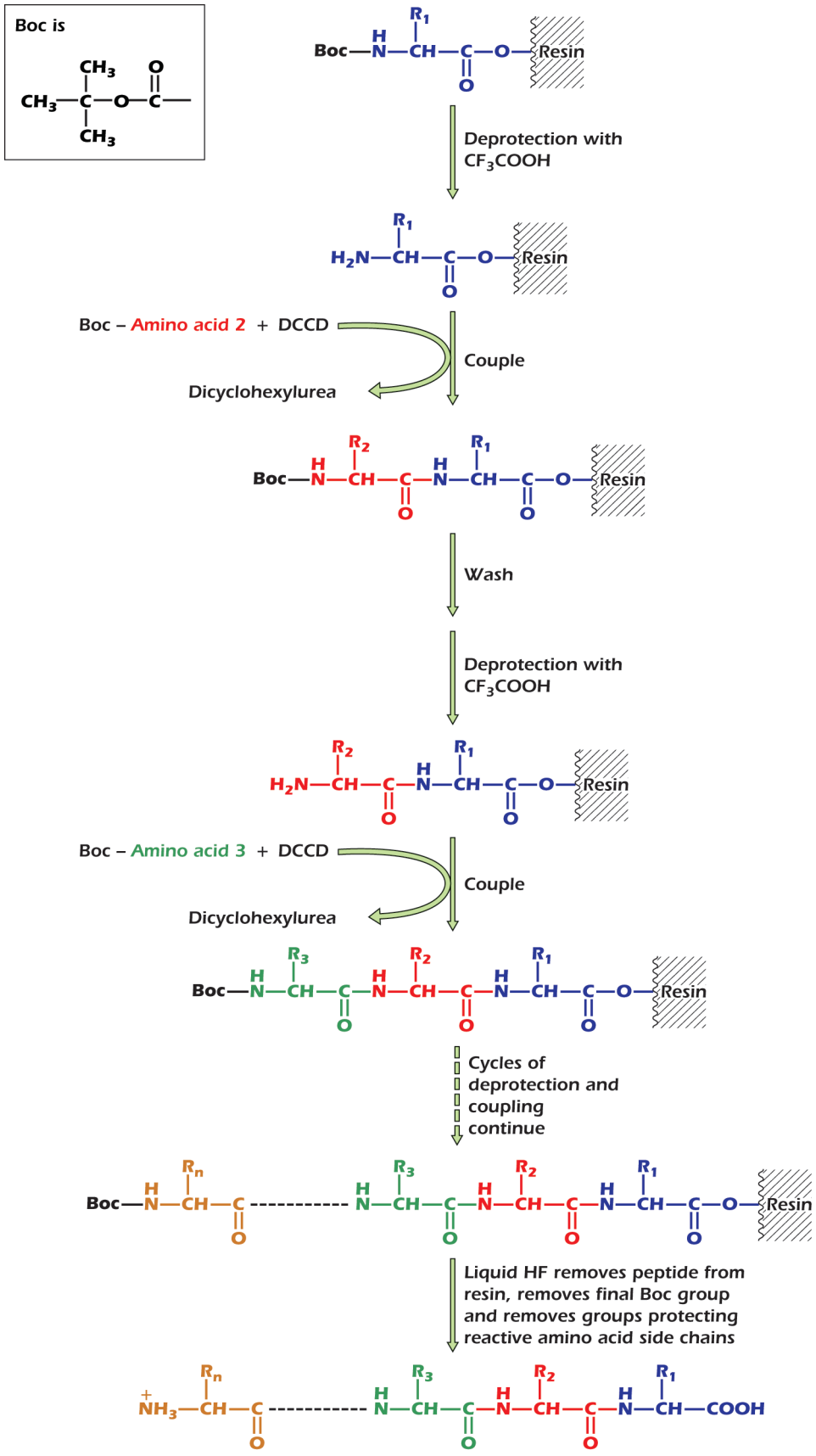
Since desmopressin contains a D amino acid it cannot be synthesized on ribosomes and rather is generated completely artificially, in a chemical synthesis.

Clearly the chemical synthesis of peptides is desirable; unfortunately it is not easy. Suppose you wish to make the dipeptide leucylalanine. If you simply mix leucine and alanine with reagents capable of causing peptide bond formation you will end up with a mixture of the product you want, leucylalanine, but you will also have alanylleucine, alanylalanine, leucylleucine, plus a soup of tri- and longer peptides. Even in this simple case purifying the product would not be a trivial task.



The answer, outlined in the diagram above, is to block alanine's amino group and leucine's carboxyl group so that only the correct peptide can form. The blocking groups (often called protecting groups) are then removed. If instead of the dipeptide leucylalanine you needed to make a longer peptide you would use conditions that removed the carboxyl protecting group leaving the amino group still blocked. The dipeptide could then have another amino acid added, and so on as required. Finally the amino protecting group would be removed.

So far so good but problems remain. The reagents used to remove the protecting groups must not break the peptide bonds. None of the reactions used for peptide bond synthesis and deprotection must be capable of causing racemization – this is conversion of an L amino acid to a mixture of D and L amino acids (or vice versa). The laboring chemist now faces further problems - the amino group of lysine, the carboxyl groups of aspartic acid and glutamic acid and most of the other side chains (except the aliphatic amino acids and phenylalanine) will react either to make peptide bonds or side reactions. These amino acids too must be protected with groups that will remain when the alpha carboxyl group is deprotected at each cycle. These groups must be removable at the end. Each elongation cycle requires purification of the intermediate product.


Boc is
Deprotection with CF_3COOH
Boc – Amino acid 2 + DCCD
Couple
Dicyclohexylurea
Wash
Deprotection with CF_3COOH
Boc – Amino acid 3 + DCCD
Couple
Dicyclohexylurea
Cycles of deprotection and coupling continue
Liquid HF removes peptide from resin, removes final Boc group and removes groups protecting reactive amino acid side chains
Resin

Bruce Merrifield (1921-2006) won the Nobel Prize for chemistry in 1984 for a very bright idea. In the 1960s he developed solid phase peptide synthesis (SPPS), illustrated in the diagram above. The first amino acid was attached through its carboxyl group to an inert porous resin (polystyrene was used initially). The resin – in the form of beads- was held in a reactor tube that allowed fluids to flow through. The first amino-protected amino acid was added with coupling reagents, after the peptide bond between the amino group of the immobilized amino acid and the carboxyl group of the added amino acid was complete, reagent for deblocking the dipeptide now immobilized on the resin was added. After deblocking and washing with solvent the third amino acid was added and so on. The growing chain is immobilized on the resin and the reagents flow past it so there is no need to purify intermediate products. At the end the peptide is cleaved from the resin and the side chain protecting groups removed. Note that chemical synthesis is from the carboxy end to the amino end while the cell does it from the amino end to the carboxyl end.

There are limitations as such an approach requires extremely high efficiency at each step. Suppose you were making a 50 amino acid peptide and that the yield at each individual reaction step was 95%. Since addition of each amino acid requires two steps, coupling and deprotection, there are 100 reactions to perform. The overall percentage yield – the amount of substrate that ends up as the final desired product - is therefore 0.95^{100} x 100 = 0.6%. Clearly to be useful the chemistry must give as close as possible to 100% yield at each individual step. Individual step yields of 99.5% and better are now common, and yields of 99.99% have been achieved.

The actual chemistry is complex. Commonly used protecting groups for the alpha amino group are either the tertiary butyloxycarbonyl group (BOC), as shown in the diagram, or the 9-fluorenylmethoxycarbonyl group (FMOC). Different groups are used to protect the various side chains. Nonaqueous solvents are used. Coupling is carried out with dicyclohexylcarbodiimide. BOC groups are removed with mild acid (trifluoroacetic acid

dissolved in methylene chloride) while FMOC groups removed with an organic base dissolved in dimethyl formamide. Peptides are removed from the resin with anhydrous hydrogen fluoride. A synthesis cycle is shown in the figure. The couple – wash – deprotect – wash cycle is easily automated and most synthesis is now done on automated synthesizers. Some allow synthesis of up to 96 peptides at once. One simply types in the desired sequence and pushes “start” and comes back when it is done. (The fanciest machines will detect problems, stop, and send the operator an email or text message!). Despite the sophistication of the chemistry it may be necessary to purify the product. This is readily accomplished by automated chromatography devices while purity can be monitored by mass spectroscopy.

Routine synthesis is limited to peptides in the 60 to 100 amino acid range. Larger molecules can be divided into fragments and these coupled together in solution. Shumpei Sakakibara and his colleagues synthesized green fluorescent protein (Figure 9.10 on book page 149) chemically in 1998. The synthetic protein, with 238 amino acids, folded correctly and was normal in its fluorescence. This remains the record for a chemical synthesis. As GFP can easily be made in cells this heroic effort really only served to show that it could be done – rather like sailing around the world single-handed or climbing Everest the first time.

For more information see Voet & Voet. 2001. Biochemistry 4th edition. pp206-209, and Jones. 2002. Amino Acid and Peptide Synthesis, 2nd edition.